Exploration of Naturally Occurring γ-amino acids in the Design of Foldamers and Biologically Active Peptidomimetics

A thesis
Submitted in partial fulfilment of the requirements
Of the degree of
Doctor of Philosophy

By

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Indian Institute of Science Education and Research, Pune

Dedicated to My Lovely Sister

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**Exploration of Naturally Occurring γ-amino acids in the Design of Foldamers and Biologically Active Peptidomimetics**" submitted by **Anindita Adak** carried out by the candidate at the Indian Institute of Science Education and Research (IISER), Pune, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or Institution.

Date: December 15, 2021

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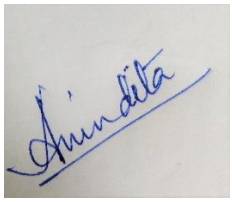
Declaration

I hereby declare that the thesis entitled "**Exploration of Naturally Occurring γ-amino acids in the Design of Foldamers and Biologically Active Peptidomimetics**" submitted for the degree of Doctor of Philosophy in Chemistry at Indian Institute of Science Education and Research (IISER), Pune has not been submitted by me to any other University or Institution. This work was carried out at Indian Institute of Science Education and Research (IISER), Pune, India under the supervision of Dr. Hosahudya N. Gopi.

Date: December 15, 2021



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Acknowledgement

First of all, I would like to thank my supervisor Dr. H. N Gopi, for his immense support and belief in me. Not only during my research work but his support to me personally was always there due to which today I am in a position to write my thesis. Sir, thank you for always being there on my side and inspiring me to be optimistic. I will always remember the day I joined him and the kind of freedom for research he has provided being my inclination more towards biology than chemistry. I will be always grateful to my sir from the bottom of my heart.

My sincere thanks to the Director and all faculty members of IISER-Pune for their time to time encouragement and support towards me, from the day I joined as Integrated PhD. I would like to thank Dr. Thulasiram, Dr. Amrita and Dr. Mrinalini Puranik, for their valuable comments and suggestions for my research projects as my RAC members.

I am very thankful to my lab. Members Dr. Anupam, Dr, Mali, Dr. Sandeep, Dr. Rajkumar, Dr. Veersh, Dr. Rahi, Dr. Ganesh, Dr. Susheel, Puneeth, Sajit, Sachin, Mona, Rupal, Manjeet, Pahan, Panda, Roy, Manish, Nithun, Serena, Varsha, Ankita, Anagha and Lipi. They are like the second family to me.

All the chemistry department technical staff who have helped me during my IISER- journey from MS to PhD I will be always grateful to them. Special thanks to Swati (Di) for being available always for me in my time of need and doing n-number of samples for MALDI analysis. Hemlata di and Tejaswani di for always providing tasty Maharashtrian food during my low time.

Dr. Aamod and Dr. Santosh my batchmates, owe a great thanks from me for being there in my life from the day I joined IISER. May God bless both of you. My special thanks to all my IISER friends, seniors and juniors, Neelam di, Kavita Di, Viraj Di, Ajay, Sahaji, Prabhakar, Bappa, Yashwant ji, Brijesh ji, Pragati, Vishnu, Akansha, Sneha and Prachi for a joyful and memorable IISER life and wonderful Hostel life as well.

In the end, I would like to thank my father, My Mother, my beloved Sister and My Brother-in-law for their faith, support and patience.

I will pray may you all have good health and joyful life.

Thank You Sai Baba

Anindita Adak

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Abbreviations

Ac_2O = Acetic anhydride

ACN = Acetonitrile

Aib = α-Amino isobutyric acid

aq. = Aqueous

Bn = Benzyl

Boc = tert-Butoxycarbonyl

$(Boc)_2O$ = Boc anhydride

^tBu = tertiary Butyl

Calcd. = Calculated

Cbz-Cl = Benzyl chloroformate

CCDC no. = Cambridge Crystallographic Data Centre number

CD = Circular Dichroism

CIF = Crystallographic Information File

dγ and dg = dehydro gamma

DBU = 1,8-Diazabicyclo[5.4.0]undec-7-ene

DCC = N, N' –Dicyclohexylcarbodiimide

DCM = Dichloromethane

DiPEA = Diisopropylethyl Amine

DMF = Dimethylformamide

DMSO = Dimethylsulfoxide

EtOH = Ethanol

Et = Ethyl

EtOAc = Ethyl acetate

Fmoc = 9-Fluorenylmethoxycarbonyl

Fmoc-OSu = N-(9-Fluorenylmethoxycarbonyloxy) succinimide

g = gram

h = hours

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

H-bond = Hydrogen bond

HOBt = Hydroxybenzotriazole

HCl = Hydrochloric acid

IBX = 2-Idoxybenzoic acid

LAH = Lithium Aluminium Hydride

MALDI-TOF/TOF = Matrix-Assisted Laser Desorption/Ionization-Time of Flight

Me = Methyl

MeOH = Methanol

mg = Milligram

min = Minutes

µL = Micro liter

mL = Milliliter

mM = Millimolar

mmol = millimoles

m.p = Melting Point

MS = Mass Spectroscopy

N = Normal

NHS = N-hydroxy succinimide

NMP = N-methyl pyrrolidone

NMR = Nuclear Magnetic Resonance

ORTEP = Oak Ridge Thermal-Ellipsoid Plot Program

PG = Protecting Group

ppm = Parts per million

Py = Pyridine

t_R = Retention time

RP- HPLC = Reversed Phase-High Performance Liquid Chromatography

RT = Room Temperature

TFA = Trifluoroacetic acid

THF = Tetrahydrofuran

UV = Ultraviolet–Visible Spectroscopy

*Z*dγ = Z-dehydro gamma

ITC = Isothermal Titration Calorimetry

Abstract

Extensive efforts have been made over the years to design protein structure mimetics using a variety of unnatural amino acid building blocks. The advancement in the field of peptide foldamers not only facilitated understanding the mechanism of folding but also proved to be relevant in the field of drug discovery, molecular recognition and bio-catalysis. Perhaps, the oligomers of the β and γ amino acids are among the most extensively studied unnatural oligomers. Our previous studies have shown the utility of γ-amino acids in the design of helices, β-hairpins, β-double helices, and multi-stranded β-sheets. In the present study, we have reported a cost-effective, efficient and racemize free protocol for the synthesis of β-amino alcohols using 2-MBT and further utilized them to synthesize γ^4-amino acids, β-hydroxy γ-amino acids, β-keto γ-amino esters and *E*-vinylogous amino acids. Later, we have explored naturally occurring β-hydroxy γ-amino acids (statine) in the design of peptide foldamers. Further, we have examined the inhibitory potency of α, γ-hybrid peptides against the γ-secretase, a multi-subunit protease complex known to be responsible for the pathogenesis of Alzheimer's disease. We have also utilized β-hydroxy γ-amino acid to design proteolytically stable water-soluble analogues of pepstatin (a universal aspartic acid protease inhibitor) and studied their inhibitory activity against proteases. We have also studied antimicrobial properties of short hybrid cationic lipopeptides containing β-hydroxy γ-amino acids and water-soluble cationic β-hairpins composed of *E*-vinylogous amino acids with lipid chain at the *N*-terminal. These peptides have displayed broad-spectrum antimicrobial activities against various microbial strains. Finally, we have shown the transformation of amino acids β-keto esters, precursors for the synthesis statines, into 1,4-dihydropyridines. Overall, the synthesis,

conformational properties, inhibitory activity against the aspartic acid proteases, antimicrobial activity of γ^4 and functionalized γ^4-amino acids have been reported in this thesis that open wide opportunities to further, explore them as building blocks for the design of biologically active peptidomimetics.

Publications

1. **Adak A**, Koppal P. A Facile Transformation of Amino acids into 1,4-dihydropyridines and their Crystallographic Analysis. ChemRxiv. Cambridge: Cambridge Open Engage; 2021; This content is a preprint and has not been peer-reviewed.(https://doi.org/10.33774/chemrxiv-2021-vpvqq)

2. Ganesh Kumar, M., Thombare, V.J., Bhaisare, R.D., **Adak, A**. and Gopi, H.N. (2015), Synthesis of Tetrasubstituted Symmetrical Pyrazines from β-Keto γ-Amino Esters: A Mild Strategy for Self-Dimerization of Peptides. Eur. J. Org. Chem., 2015: 135-141. https://doi.org/10.1002/ejoc.201403237

Chapter 1

General Introduction

1.1 Introduction

Proteins are biomacromolecules that carry a vast array of biological functions in organisms. Folding into a well-defined three-dimensional structure plays a very important role in its functionality. The structure of Protein can be well explained at four levels where the primary structure of proteins consists of sequence and number of amino acids in a polypeptide chain. Local arrangement of these polypeptide sequences due to hydrogen-bonding between the atoms and the backbone results in helices, beta-hairpin and reverse-turn, held together with loosely structured loops represents the secondary structure of a protein in nature.[1] Various covalent and non-covalent interactions of amino acid side chains result in the overall three-dimensional structure of a single polypeptide chain representing the tertiary structure of a protein. When two or more polypeptide chains called subunits come together to form a complex it represents the quaternary structure of a protein. Other than these, super secondary structures of protein also exist which bridges the gap between the local arrangement of amino acid as in secondary structure to that of the highly organized tertiary structure of proteins. Beta meander, Greek key motif, Beta hairpin, helix turn helix, helix hairpin, beta alpha beta motifs, staircase-like motifs etc. are few among them. Various structural elements present in protein structures are shown below in Figure 1.

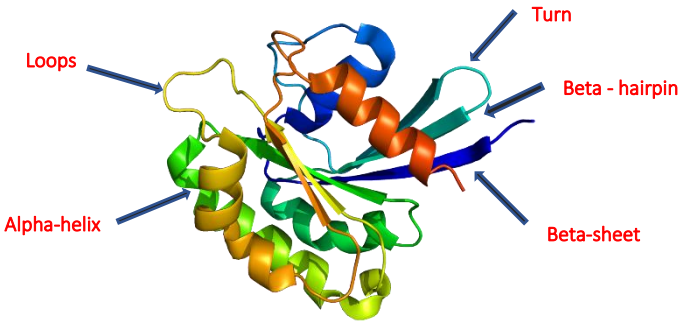


Figure 1: Structural elements in protein structure (PDB code-1ZD9).

Many covalent interactions including hydrogen bonding are the governing factor involved in the three-dimensional structure of proteins which is directly related to their sophisticated well-defined functions. The relation of complex structures to their functions inspired many researchers to discover structural based molecular scaffolds using various alpha-amino acids present in proteins.[2] The major drawback of using alpha-amino acid was their low serum stability thus reducing their half lifetime inside the cell (*in-vivo*). Mimicking the biologically relevant secondary structure of proteins using artificial molecules and non-ribosomal amino acids not only facilitated in understanding the mechanism of folding but also proved to be relevant in the area such as drug chemistry, molecular recognition, bio-catalysis, etc because

of their high proteolytic and metabolic stability.[3] The role of using these artificial moieties has also been proved to be recognizable in designing more efficient peptidomimetics for designing protease inhibitors, antimicrobial agents, biomaterials, understanding protein-protein interactions, etc.[4] This endeavour has been gracefully recognized using non-ribosomal or non-proteinogenic homologated alpha-amino acid derivatives such as β-amino acids, γ-amino acids and ω-amino acids by various researchers over the last two decades. Also, backbone functionalized modified naturally present γ-amino acid such as α, β-unsaturated γ-amino acid, β-keto γ-amino acid and β-hydroxy γ-amino acid *etc*. came into the picture recently for utilizing them as a building block for peptidomimetics.[5] It can be well said that frontiers of today's molecular science lie in between chemistry, drug chemistry and material science etc.

1.2 Foldamer

In 1998, Samuel Gellman first coined the term Foldamer- "**any polymer with a strong tendency to adopt a specific compact conformation**" or "**polymer backbone with a discrete and predictable folding tendency"**.[6] Over these past three decades several templates have been utilized in designing foldamers of various conformations and also with various biological and medicinal applications.

1.3 Peptide foldamers from homologated α-amino acid

β-amino acids are single homologated α-amino acid (one extra methylene group present in the backbone). Considering the position of the side chain, they are termed as β^2, β^3, $\beta^{2,3}$. γ-amino acids are double homologated α-amino acid (two methylene groups present extra in the backbone). Considering the position of the side chain, they are termed as γ^2, γ^3 and γ^4. Varieties of β and γ-amino acid scaffold of cyclic and acyclic structures have been utilized in designing secondary protein structures such as helix, beta-hairpin and beta-turn etc. are shown in Figure 2.

1.3.1 β and γ- peptide Helices

β-oligomers helices obtained from β-amino acid residues mostly adapt stable C_8, C_{10}, C_{12}, C_{14} and $C_{12/10}$ configurations where subscript represent the number of atoms involved in hydrogen bonding.[7] These helices have shown different polarity to that of alpha-amino acid helices. The C_8 and C_{12} helices have shown similar macrodipole as of alpha-helices (C←N) whereas, C_{10} and C_{14} helices have shown reverse macrodipole (N←C). Gellman and co-workers provided the first crystallographic analysis of helices with cyclic β-amino acid residues such as *trans*-2

amino cyclopentane carboxylic acid (ACPC) and *trans*-2-amino cyclohexane carboxylic acid (ACHC).[8]

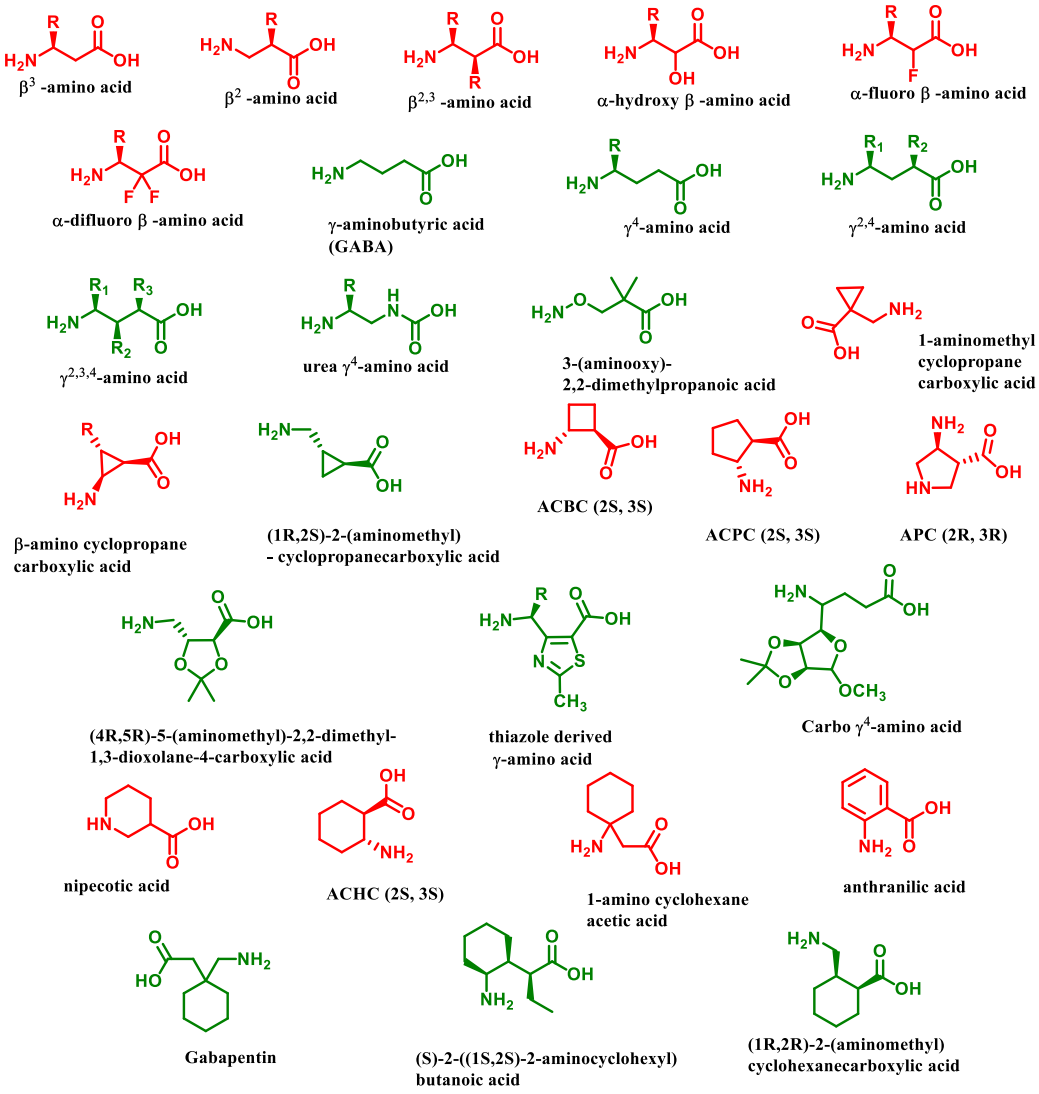


Figure 2: Examples of β and γ- amino acid building blocks. (β showed in red and γ in green)

The β-peptide from *trans*-ACPC adopted C_{12} helix with similar polarity to that of the alpha-helix (C←N) but surprisingly *trans*-ACHC showed C_{14} helix with opposite polarity (N←C). During the same time, Seebach and co-workers have shown β peptide helices using acyclic chiral β-amino acid residues. The β^2 peptide helix and β^3 peptide helix have shown C_{14} configuration but with opposite polarity, left-handed (C ←N) and right-handed (N ←C) respectively. They have also shown helices containing alternate β^3 and β^2-residues having right-handed $C_{12/10}$ helicity containing alternate C_{12} and C_{10} hydrogen bonding patterns.[9] Aitken and co-workers obtained C_{12} helices both in solution and solid phase using cyclo butane β-amino acid residues in β-peptide foldamers.[10] Later, Fulop and co-workers have also utilized cyclic

β-amino acid residue *cis*-2-Aminocyclohex-3-ene carboxylic acid (*cis*-ACHEC) in designing α, β-hybrid peptides and obtained $C_{10/12}$ helices.[11] Sharma and co-workers have incorporated carbohydrate base β-amino acid residues in helices.[12]

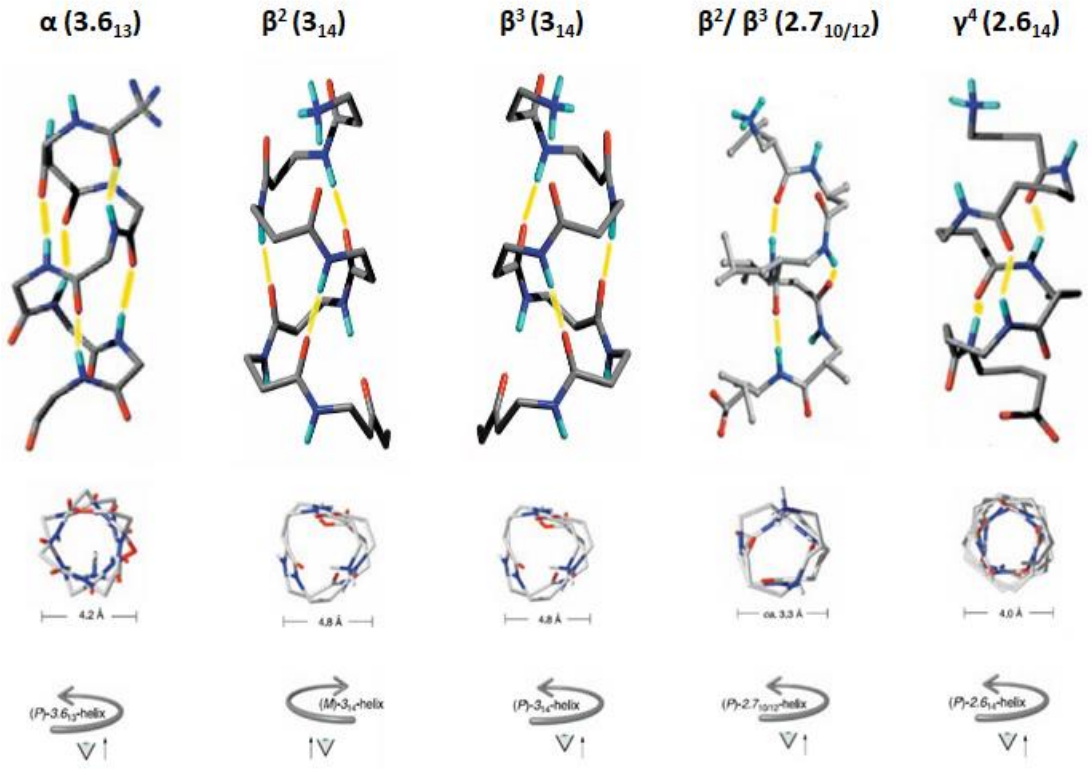
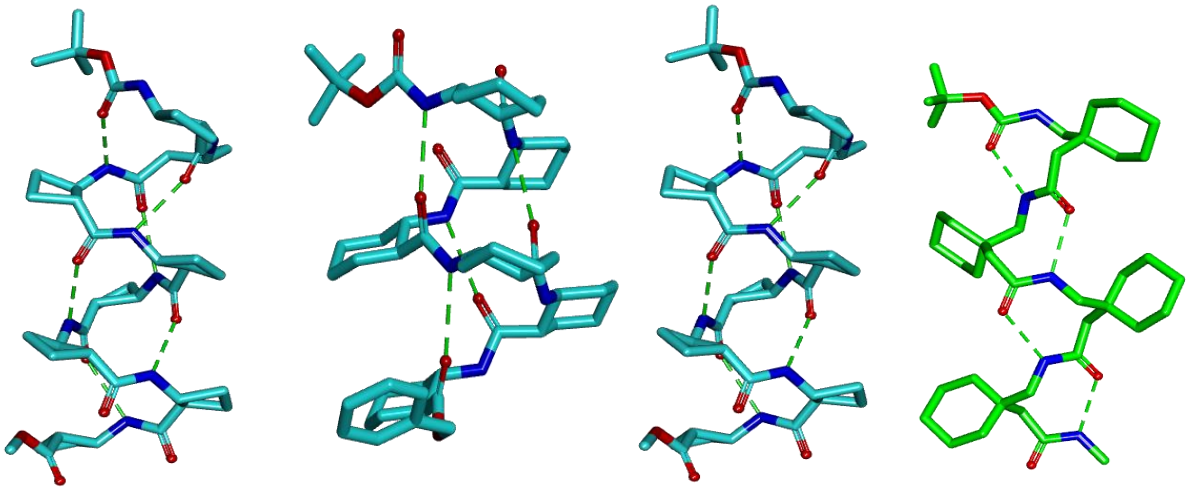


Figure 3: Comparison of acyclic β^2, β^3, β^2/ β^3 and γ^4 homo-oligomers of various helicity and directional pattern with α-helix.[9,13]

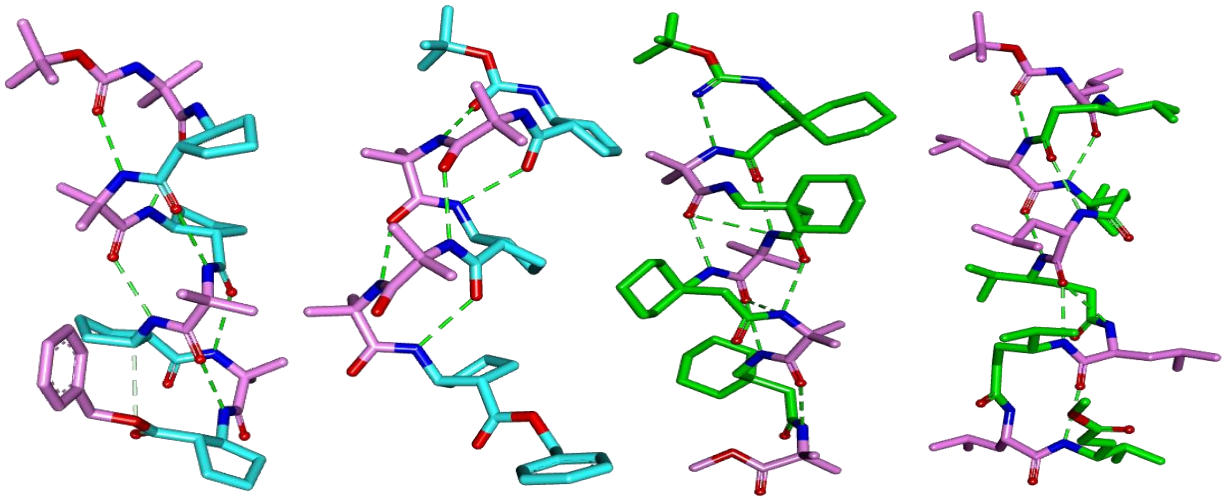
In comparison with the study of the folding pattern of β or hybrid β peptides still very little is known about γ-peptides due to less feasible synthetic procedure for obtaining stereo-chemically pure γ^4 amino acid. In the beginning, Seebach and co-workers[13] and Hanessain and co-workers[14] came up with C_{14} helices obtained using homo-oligomer of γ^4-amino acid. Surprisingly the polarity of γ^4 C_{14} peptide is similar to that of alpha C_{14} peptide except 2.6 residues involved per turn to that of 3.6 in alpha peptide and 3 residues per turn in C_{14} β-peptide. Using carbo-γ^4 amino acid and γ-aminobutyric acid dipeptide as repetitive units, Sharma and co-workers have observed left-handed C_9 helix and helix-turn-helix .[15] Later stable C_9 helix was also investigated by Balaram and co-workers of 3,3-dialkyl γ-amino acid residue (gabapentin) tetrapeptide.[16] Further, Gellman and co-workers have shown a right-handed C_{14} helix of cyclic γ-amino acids homo-oligomers.[17] Further, Smith and co-workers showed C_7

helical pattern with homo-oligomer of $\gamma^{2,3}$ *trans*-dioxolane in solution.[18] Hoffmann and co-workers with *ab initio* theoretical study provided an overview of a wide range of helical combinations with γ^4 -amino acid and α,γ-(1:1) hybrid peptide.[19] Nonetheless, Balaram and co-workers[20] and Gellman and co-workers[21] obtained right-handed C_{12} helix of α,γ-hybrid peptides (1:1) with gabapentin and cyclic $\gamma^{2,3,4}$ amino acid residues, respectively.



(a) (b) (c) (d)

Figure 4: Examples of homo-oligomers of cyclic β and γ- amino acid. (a) C_{12} of trans-ACPC (β)[8(c)] ,(b) C_{14} of trans-ACHC[8(a)] (β), (c) C_{12} of cyclobutane (β)[10] and (d) C_9 of gabapentin(γ).[16(a)]



(a) (b) (c) (d)

Figure 5: Examples of hybrid peptides with various β and γ- amino acids. (a) α,β with C_{11} helix, Boc-Aib-ACPC-Aib-ACPC-Aib-ACPC-Aib-ACPC-OBn,[21(b)] b) ααβ with 10/11/11 helix, Boc-ACPC-Aib-Aib-ACPC-Aib-Aib-ACPC-OBn,[21(c)](c) α/γ with C_{12} helix, Boc-(Gpn-Aib)4,[20] (d) α,γ with C_{12} helix, Boc-(Leu- γVal)$_5$-OMe.

In addition to the use of cyclic and acyclic γ-amino acids for designing γ-peptide helices, reports are also present on utilizing heteroatoms (oxygen and nitrogen) in the backbone of γ-amino acids. Yang and co-workers investigated the conformational arrangement of α-aminooxy peptides and β-aminooxy peptides and observed C_8 and C_9 helical conformations respectively.[22] Further, Le Grel and co-workers showed helices using aza-amino acids with similar properties related to β-aminooxy peptides.[23] Guichard and co-workers reported urea-peptides adopted to C_{14} helical conformation.[24] Further, thiazole derivative of γ-amino acids was also been used to design γ-peptide foldamers.[25] Recently, our group structural properties of α, γ-hybrid peptides. These 1:1 alternating hybrid peptides spontaneously fold into 12-helical conformations without having any stereochemical constraints.[26] Few of the homo-oligomeric and heteromeric helices are shown in **Figures 3, 4** and **5** with cyclic and acyclic homologated amino acids.

1.3.2 β and γ-peptide hairpins and turns

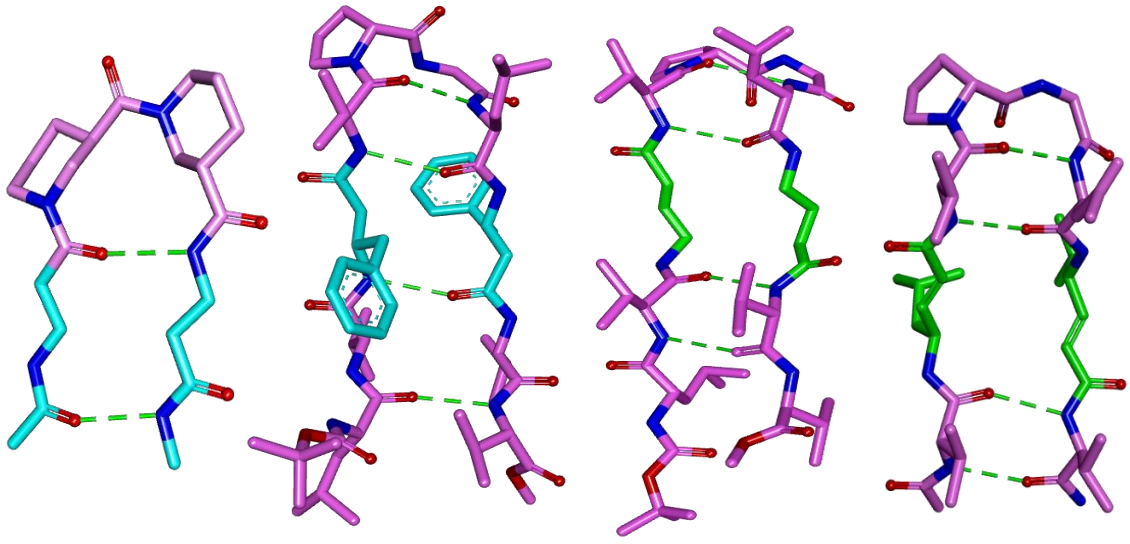


Figure 6: Examples of β-hairpins using β and γ-amino acids. (a) Ac-βGly-Nip-Nip-βGly-NHMe,[30] (b) Boc-Leu-Val-β^{3}Phe-Val-DPro-Gly-Leu-β^{3}Phe-Val-Val-OMe,[28] (c) Boc-Leu-

Val-γAbu-Val-DPro-Gly-Leu-γAbu-Val-Val-OMe[29] and (d) Ac-Val-dgLeu-Val-DPro-Gly-Leu-dgVal-Val-$CONH_2$.[33]

Another super secondary structure of proteins called beta-hairpins are most widely found in globular proteins. In a beta-hairpin architecture, two strands are connected by a loop segment and the extended structure of beta-hairpins occurs as parallel and antiparallel sheet structures in proteins. Remarkable progress has been observed in the field of designing helix mimetics using β and γ amino acids but also Gellman and co-workers and Balaram and co-workers have explored using β and γ-amino acids in designing β-hairpin structures. Seebach has shown the solution structure of $\beta^{2,3}$ amino acid in inducing turn in methanol.[27]

Balaram and co-workers have shown that β^3 amino acids[28] and γ-amino acid residue γAbu (4-amino butyric acid)[29] can be well- incorporated into a recognized β-hairpin structure. Gellman and co-workers have shown utilization of *R*-Nipecotic acid (nip)[30] as a turn inducer necessary for hairpin folding as well as the crystal structure of γ-peptides containing acyclic alpha, β-disubstituted β-amino acid[31] and cyclic *trans*-3-ACPC[32] as the γ-amino acid well accommodated in parallel sheet secondary structure differently. Recently our group have shown incorporation of *(E)* vinylogous γ-amino acids in hairpin strands as well as using them for developing a three-stranded antiparallel β-sheet structure.[33] Few examples of β-hairpins using β and γ-amino acids are shown above in Figure 6.

1.4 Naturally occurring non-ribosomal γ-amino acids and their biological importance

Discussion of the utilization of β and γ-amino acids will be incomplete without putting light on naturally occurring non-ribosomal γ-amino acids such as γ amino β-keto acids and γ- amino β-hydroxy acids, α, β-Unsaturated γ-amino acids for designing various biological moieties and new building blocks.

α, β-Unsaturated γ-amino acids contains –(CH=CH)– between $C_\alpha H$ and CO of α-amino

acids. These moieties are widely present in peptide-based natural products, such as Gallinamide A, Cyclotheonamide A, Miraziridine A, *etc*. Gallinamide A is known for its anti-malarial potency since it was isolated in 2009 from marine cyanobacterial metabolites. Later, they have also shown potent and selective anti-protease activity towards human Cathepsin L, a cysteine protease.[34] Cyclotheonamide A, a macrocyclic marine natural product isolated from a marine sponge and shows strong inhibition towards thrombin, a serine like protease. The vinylogous

tyrosine amino acid residue is found to be responsible for binding to thrombin.[35] Miraziridine A, is also a peptide-based natural product first isolated from a marine sponge. Miraziridine A is known as a unique class spanning inhibitors for serine, cysteine and aspartic acid proteases due to the presence of aziridine, statine and vinylogous amino acid moiety, respectively.[36] Few examples of α, β-Unsaturated γ-amino acids containing natural products are shown in Figure **7.**

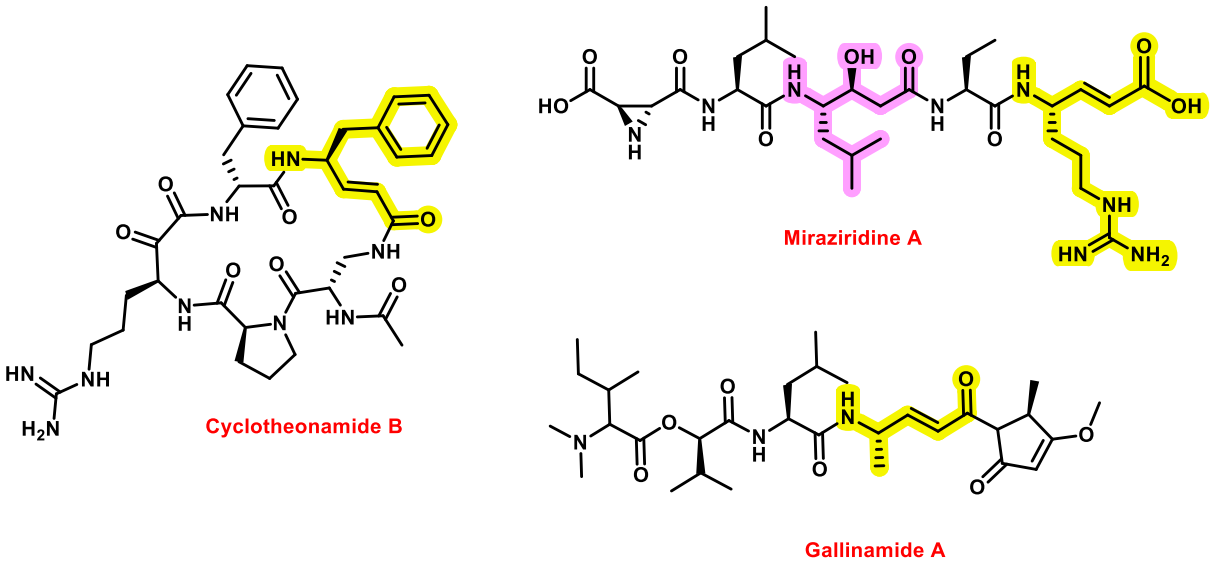


Figure 7: Examples of α, β-Unsaturated γ-amino acids in natural products.

γ-amino β-keto acids contains –C(O)CH_2– between $C_\alpha H$ and CO of α-amino acids. These non-ribosomal γ-amino β-keto acids are widely present in many biologically active molecules such as Majusculamide C, Desmethoxymajusculamide C (DMMC), Nostopeptolide etc. Majusculamide C, a cyclic depsipeptide that was isolated from a blue-green alga, *Lyngbya majuscula* have shown antifungal properties against various fungal plant pathogens.[37(a-d)] Desmethoxymajusculamide C (DMMC), another natural product isolated from *Lyngbya majuscula*, which is active in both cyclic as well as linear form have shown potent and selective anti-solid tumour activity through depolarization of actin protein.[37(e)] Nostopeptolide, play a role in cell differential governing factor for the symbiotic cyanobacteria *Nostoc punctiform.*[37(f)] Few examples of **γ-amino β-keto acids** containing natural products are shown in Figure 8.

γ amino β-hydroxy acids (statins) contains –C(OH)CH_2– between $C_\alpha H$ and CO of α -amino acids and they exist in two conformers *Anti* (R, S) and *Syn* (S, S) concerning the position of OH with amino acid side chain. Several peptide natural products have been reported containing statins or modified statins moiety such as Dolastatin, Tamandarin, Didemnins, Hapalosin,

Stictamides, *etc*. having several biological importance. Statines came into the limelight since their discovery by Umezawa and Co-workers in 1970 as aspartic acid protease (pepsin, HIV-1 protease, cathepsin D and E, Plasmepsin I and II, renin, *etc*.) inhibitor universally known as Pepstatin A.[38] Hapalosin, a 12-membered cyclic depsipeptide with multiple drug reversal (MDR) agent, is thus widely used in chemotherapy undergoing cancer patients.[39(a-b)] Stictamides A-C, extracted from *Sticta sp*. Of lichen shows inhibitory activity against MMP-12 (Matrix metalloproteinase) hence, shows anti-tumour activity.[39(c)]

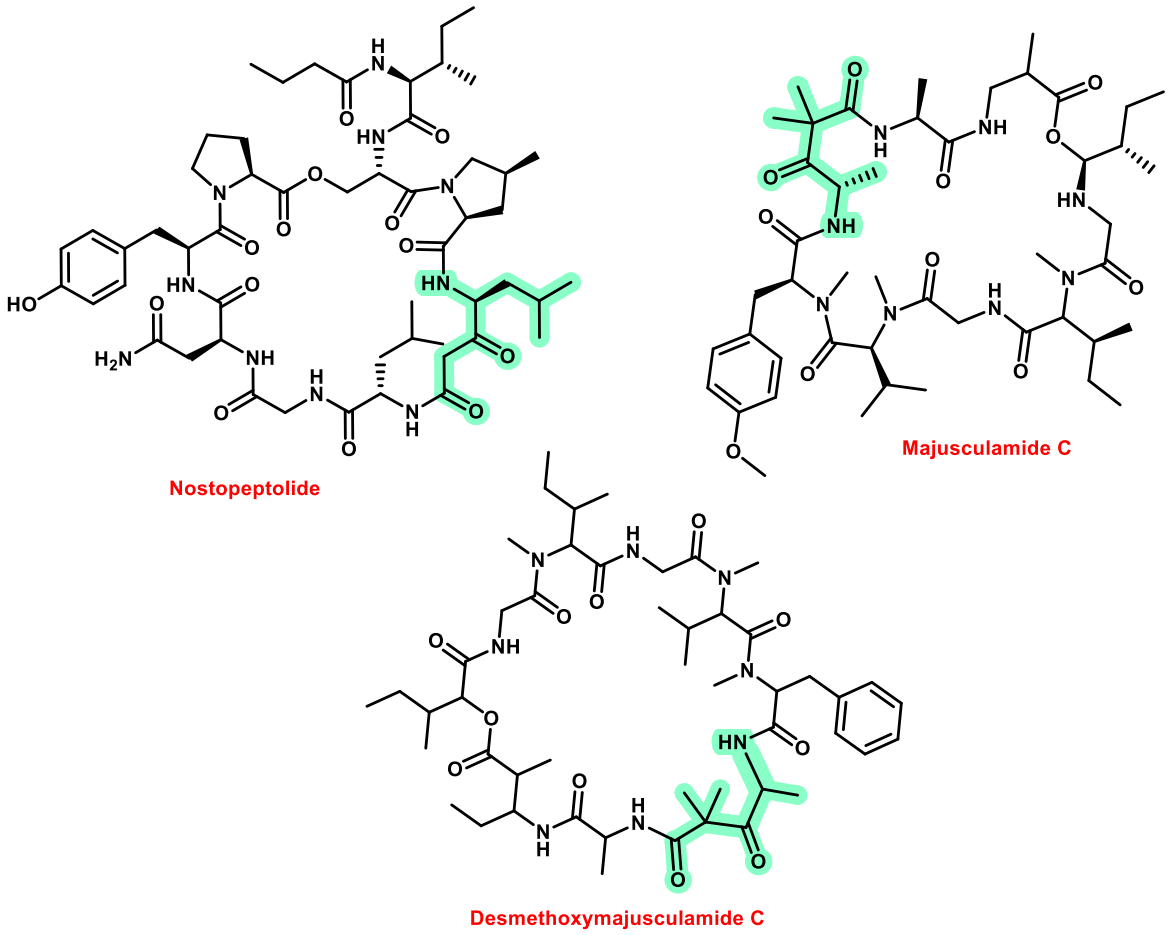


Figure 8: Examples of γ-amino β-keto acids in natural products.

Didemnin B, a class of cyclic depsipeptide which was first isolated from a tunicate and known to have potent anti-viral against herpes simplex virus type 1, anti-tumour activity against murine leukaemia cell lines and strong immunosuppressive properties.[39(d-f)] Didemnin being the first marine peptide-based drug that went into clinical trials and was tried over humans. Tamandarins A and B, a cytotoxic depsipeptide which was first isolated from marine ascidians. They have shown more potent antitumour activity than Didemnins for human cancer cell lines.[39(g,h)] Polymyxin B, a γ-amino acid containing natural product has shown antibacterial

activity against various multi-drug resistant gram-negative bacteria. It is bactericidal by disrupting their cell membrane and then inhibit the respiration of bacteria.[40] Few examples of γ-amino β-hydroxy acids containing natural products are shown in Figure 9**.**

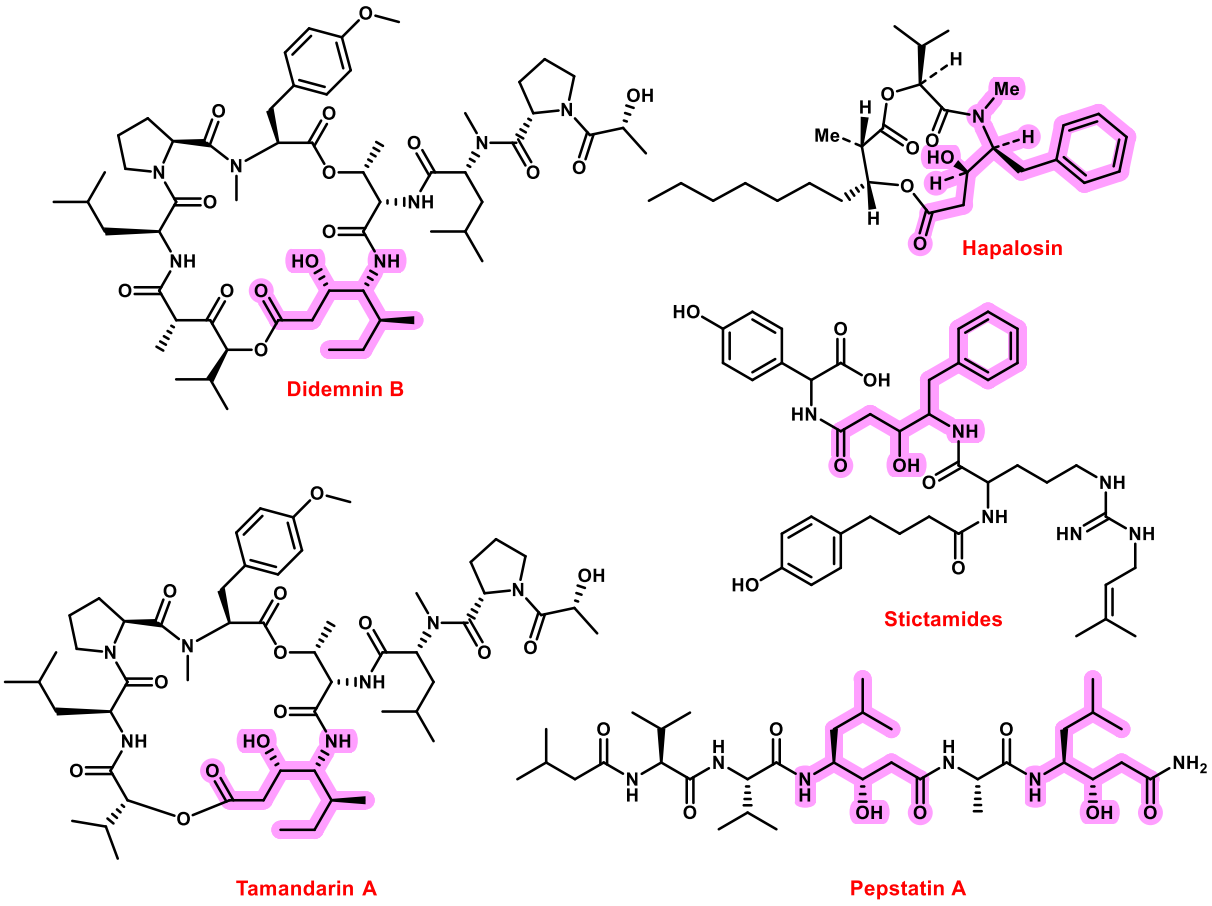


Figure 9: Examples of γ amino β-hydroxy acids (statine) in natural products.

1.5 Peptidomimetics

Modified homologated oligopeptide with anticipated conformational properties has shifted the research interests of many in the direction of studying their biochemistry, drug chemistry and medicinal chemistry**.** The rich diversity of the antimicrobial peptides presents in nature adopt different secondary structures and can be classified into different classes such as linear (Indolicidin), helical (Magainin), β-sheet (Human β-defensin-3), cyclic (Circulin A and B) or mixed peptides. They can be cationic (e.g. Polymyxin) as well as anionic (Daptomycin).[41] The remarkable protein secondary structure mimetics displayed by β and γ-peptide oligomers have

fascinated researchers to exploit them in designing inhibitors for various protein-protein interactions, antimicrobials and biomaterials etc. The major drive towards this field of peptidomimetics is because of protease stability of β and γ-peptide oligomers are much higher than that of α-peptides oligomers thus increasing their serum life *in-vivo*.[42]

DeGrado and co-workers and Seebach and co-workers came with an idea of amphiphilic antimicrobial peptide helices arranged in such a manner that the polar and apolar sides will be well segregated from each other on the opposite side of the helix. For designing such β-peptide helices, a series of repeated tri-peptides were synthesized where β-hLys was chosen as a positively charged polar and β-hLeu and/or β-hVal was chosen as a hydrophobic residue. The β-peptides have shown antimicrobial effect but they were not selective between pathogenic cell lines and human RBC. Later less hydrophobic β-hAla was inserted in the place of β-hLeu/ β-hVal. These peptides shown potencies comparable to that of magainin, the naturally occurring antimicrobial peptide and also shown more selectivity.[43] These amphiphilic β-peptides have shown C_{14} helicity. Further, Gellman and co-workers have designed C_{12} antimicrobial peptides using cyclic β-amino acid residues such as ACPC as hydrophobic residue and APC as positively charged hydrophilic residue of 14 amino acid residues named as β-17.[44] This peptide have also shown segregation of polar and apolar residues on the opposite side of C_{12} helix. These helices are more rigid than C_{14} helices and have also shown excellent activity and less haemolytic values. Later during the year, they came up with helical analogues (with similar helical pattern to that of alpha peptides) of antifungal peptide Aurein 1.2 composed of various combinations of α and β amino acid residues as repetitive units which have shown improved activity and selectivity over Aurein on *Candida albicans* species.[45]

Seebach and co-workers for the first time demonstrated protein-protein interaction (PPI) peptidomimetics by the mimicking of Lipid transport protein (SR-B1) with amphipathic β-nonapeptide of 3_{14} helical conformations and thus suppressing the absorption of cholesterol and fat molecules by small intestine cell line.[46] The same approach was later used by them to mimic interleukin(hIL8) 3.6_{13} alpha-helical portion with 3_{14}-amphipathic β^3-peptide helix.[47] Schepartz and co-workers came up with various 3_{14}-helical β-decapeptide ligands as peptidomimetics of P53 and oncoprotein-hDM2 interactions as well as inhibitors of HIV fusion peptides.[48]

Seebach and co-workers introduced cyclic β-peptides which mimic the peptide hormone somatostatin and shown a strong affinity towards various somatostatin receptors.[49a] They also

studied similar cyclic β-peptides against enterobactin receptors and shows antiproliferative activities towards various human cancer cell lines. Studies have also been done in mimicking CD-40L with cyclic tri-Arg β-peptide macrocycle (due to similarity in structure) which showed more affinity with its receptors CD40, a human immune response glycoprotein causing apoptosis in leukaemia and lymphoma cells.[49b-c] Ghadiri and co-workers designed cyclic β-peptides which have shown properties similar to ion channels in lipid bilayers.[50] Studies have also been done on interactions of β-petadecapeptide (βArg, βGlu and βlys) with DNA duplexes by Seebach and co-workers[51] Gellman and co-workers. have shown selective binding of β-undecapeptide derived from TAT-peptide sequence to TAR-RNA thus disturbing the PPI of TAT protein with TAR-RNA(transcription activator responsive element).[52]

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Chapter 2(a)

Efficient synthesis of β-amino alcohol using 2-MBT

2a.1 Introduction

The **β-amino alcohols** are found in nature at the *C*-terminal end of a biologically significant family of antibiotic peptides called "**Peptaibols**" such as Alamethicin and lipovelutibol A-D.[1] They are also present in several other biologically active peptides such as octreotide, an inhibitor for growth hormone,[2] various analogues of growth hormone secretase,[3] several insecticidal agents,[4] various cholecystokinin antagonists[5] *etc*. Few examples are shown in Figure 1.

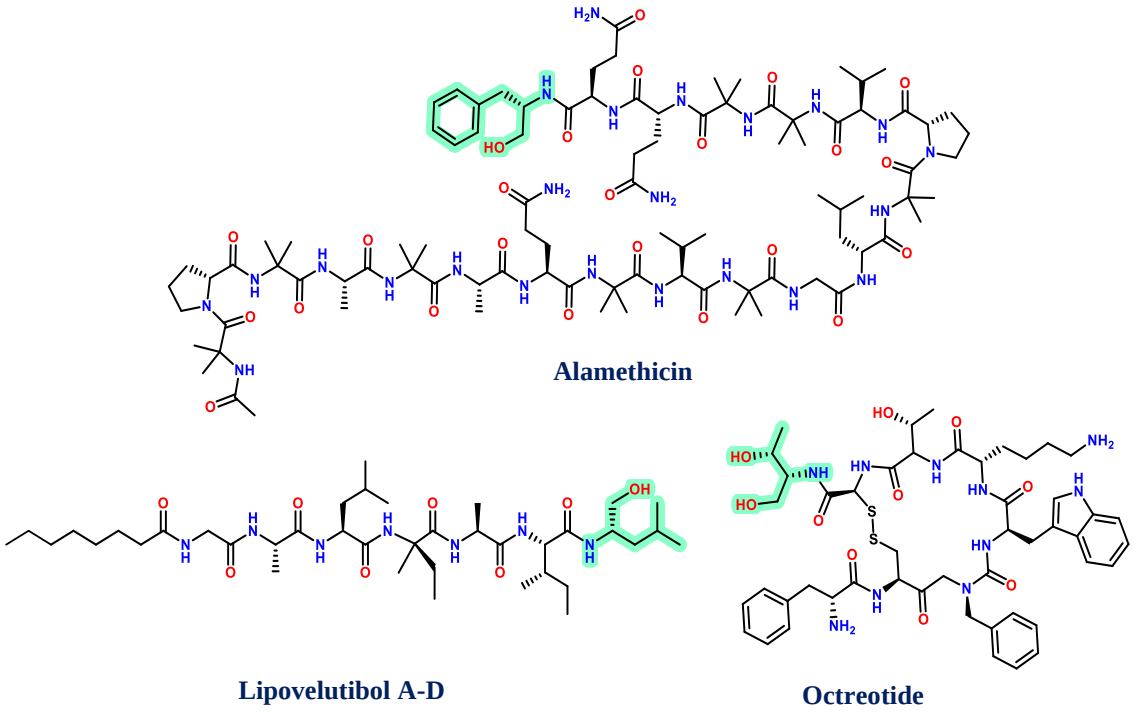


Figure 1: Few Examples of Peptaibols.

They are found to be useful as a chiral auxiliary in the synthesis of 4-substituted oxazolidine-2-ones,[6] asymmetric C-C bond formation,[7] in primary amine separation[8] *etc*. β-amino alcohols are also involved in the synthesis of β-amino acid,[9] α-haloβ-amines,[10] diamines, triamines,[11] optically active α-amino aldehydes,[12] γ-amino acids, *etc.* a starting material.

Earlier, aminolysis of epoxides was used to synthesize β amino alcohols in the presence of an excess amine at heating conditions. Though this method had many drawbacks, the use of various catalysts has improved this method such as $ZnCl_2$,[13] $InBr_3$,[14] $Bi(OTf)_3$,[15] $CeCl_3.7H_2O$,[16] $Cu(BF_4)_4.xH_2O$,[17] Alumina,[18] $COCl_2$,[19] *etc*. Another way of β-amino alcohols synthesis reported in the literature is reduction of activated *N*-protected amino acid. Various activated

carboxylic acid such as acid chlorides,[20] mixed anhydrides,[21] 1-hydroxybezotriazole esters,[22] acid fluorides,[23] 1-succinimidyl esters,[24] pentafluorophenyl esters,[25] *etc*. on reduction with various reducing agents $NaBH_4$,[26] $ZnBH_4$,[27] $(EtO)_3SiH$,[28] I_2 ,[29] *etc.* gives β-amino alcohols.

Over the last few decades, there has been significant progress in the use of 2-mercaptobenzothiazole(2-MBT) or its derivatives in organic synthesis. 2-mercaptobenzothiazole(2-MBT) is known to be associated with the vulcanization process in the rubber industry as well as involved in the extraction of gold in the mining industry.[30] Recently, several applications of 2-mercaptobenzothiazole(2-MBT) and its derivatives have been reported to possess biological activities such as antibacterial, antifungal, insecticidal, *etc*.[31]

In recent years, our lab has reported various foldamers of γ and functionalized γ-amino acids. β-amino alcohols are used as the starting material for the synthesis of γ-amino acids. We have followed the mixed anhydride method and HOSu method for the above till date.[32] In sought of developing some mild, low cost and faster methodology we proposed the use of 2-MBT as an ideal component by looking into cost-effectiveness and easy accessibility.

2a.2 Aim and rationale of present work

Here we are reporting a method that is rapid, efficient, cost-effective and racemization-free for the synthesis of β-amino alcohols through mild reduction of 2-MBT esters of *N*-protected α-amino acids. These β-amino alcohols can be utilized later for the synthesis of γ^4-amino acids, α-β, unsaturated γ-amino acids, β-keto γ-amino acids and β-hydroxy γ-amino acids *etc*.

2a.3 Results and Discussion

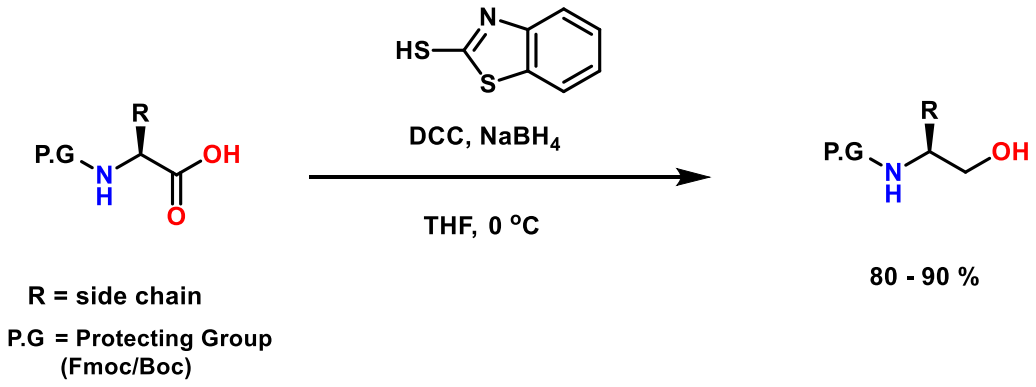
2a.3.1 Synthesis of *N*-protected β-amino alcohols:

Initially, a systematic study was done with various equivalents of 2-MBT to undergo the MBT-ester formation of *N*-Fmoc α-amino acids. We found that 1.5 equivalent of 2-MBT is sufficient to undergo the reaction with *N*-Fmoc α-amino acids with DCC as a coupling reagent in THF at ice-cold conditions to form MBT esters of amino acid in 1hour. These esters were taken directly without purification to undergo mild reduction using dissolved $NaBH_4$ in THF at ice-cold conditions to get *N*-Fmoc β-amino alcohols in 5-15 mins time with visible effervescence. The schematic representation of the synthesis of *N*-protected β-amino alcohols is shown in Scheme 1. We have synthesized various *N*-Fmoc β -amino alcohols from their respective *N*-

Fmoc α-amino acids using this methodology with good yields, shown in Table 1. The melting points and optical rotations were also recorded for each for showing similarity with the literature reported values. The yields of various synthesized alcohols show almost similar values to alcohols synthesized using the HOSu method but much better than the IBC-Cl method, comparison of these three methods is shown in Table 2. But the major advantage of using this method is less time of reaction and much cheaper price of 2-MBT reagent proving the reported protocol to be cost-effective. We obtain a single crystal for compound 3, Fmoc-Leu-ol by slow evaporation in ethyl acetate, shown in Figure 2.

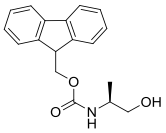
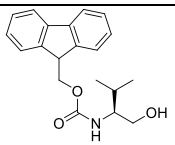
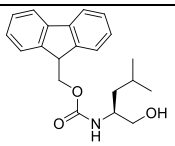
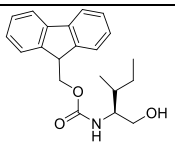
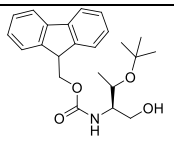
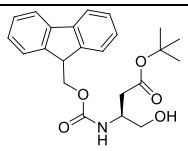
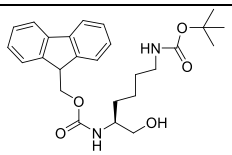
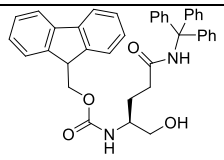
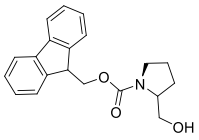
To investigate the protocol to be racemization free, we subjected Fmoc-Ileu-ol to pass through HPLC on chiralpack AD column, in isocratic mode with 2mL/min flow rate with a solvent system of 90% Isopropanol in n-hexane. We observed a single peak at 5.44 min which indicates the presence of a single enantiomer indicating the result to be racemized free, HPLC-chromatogram has shown in Figure 3.

Inspired by the above results and to investigate the compatibility of this reaction with other amine protecting groups we subjected this method to various *N*-Boc α-amino acids. We have synthesized various *N*-Boc β-amino alcohols using this methodology, shown in Table 3. The results showed this protocol is compatible with other amine protecting groups and other functionalities present on side chains such as alcohol, carboxylic acid, guanidine and thiols protecting groups. This proves, this methodology of synthesizing β -amino alcohols to be efficient, cost-effective, selective, compatible and racemize free.



Scheme 1: Schematic representation of the synthesis of *N*(Fmoc/Boc) β -amino alcohols from their respective *N*(Fmoc/Boc) α-amino acids using the 2-MBT method.

Table 1: List of *N*-Fmoc β-amino alcohols using the 2-MBT method.

Entry	Fmoc-AA-ol	% Yield	m.p.	$[\alpha]_D^{25}$ (c =1, MeOH)
1		90	150- 153	-2.0
2		85	121-122	-13.2
3		80	133-135	-20.0
4		70	114-116	-12.0
5		83	oil	+6.2
6		90	93-96	-7.4
7		78	136-138	-6.8
8		83	74-77	-6.0
9		78	90-92	-29.1

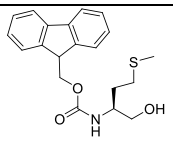
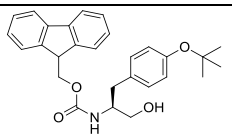
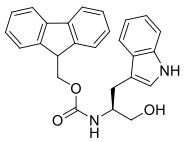
10		90	136-137	-17.3
11		79	113-116	-18.8
12		85	88-90	-26.8

Table 2: Comparison of 2-MBT, HOSu and IBC-Cl methods in terms of percentage yields.

Entry	**Fmoc-AA alcohol**	**Yield (%)**		
		MBT esters	**HOSu**	**IBC-Cl**
1	Fmoc-Ala-ol	90	90	70
2	Fmoc-Val-ol	85	90	76
3	Fmoc-Leu-ol	80	81	68
4	Fmoc-Ileol	70	65	50
5	Fmoc-Thr (OtBu)-ol	83	85	55
6	Fmoc-Asp (OtBu)-ol	90	92	47
7	Fmoc-Lys (Boc)-ol	93	75	64
8	Fmoc-Asn (Trt)-ol	92	90	65

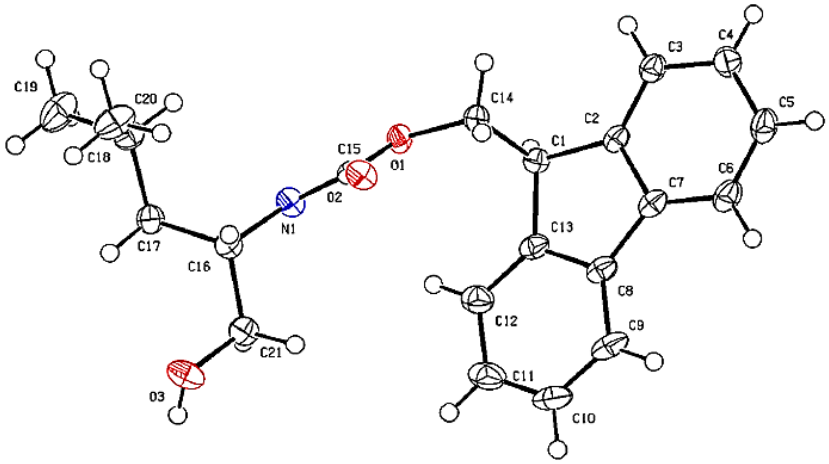


Figure 2: Single crystal of compound 3, (*N*)Fmoc-Leucine-ol.

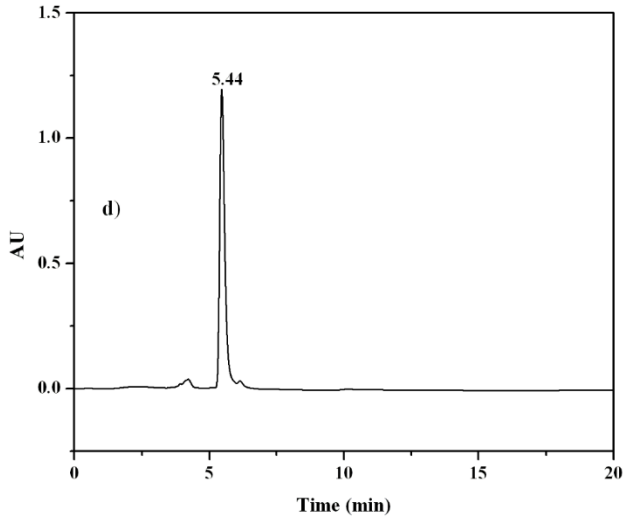
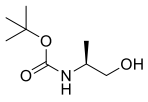
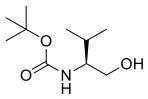
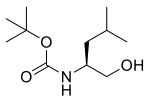
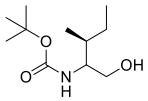


Figure 3: Chiral HPLC chromatogram of compound 4, (*N*)Fmoc-Isoleucine-ol.

Table 3: List of *N*-Boc β -amino alcohols using the 2-MBT method.

Entry	**Boc-AA-ol**	**% Yield**	**m.p.**	**$[\alpha]_D^{25}$ (c =1, $CHCl_3$)**
12		75	oil	-9

13		75	oil	-16.5
14		80	oil	-22.5
15		73	oil	-23.2

2a.4 Conclusion

All the *N*-protected β-amino alcohol have been synthesized in 80-90% yield and characterized well with NMR and Mass analysis. We have proved the reaction to be efficient, versatile, cost-effective, selective, compatible and racemize free. Further, β-amino alcohols synthesized with this methodology can be used for the synthesis of other building blocks like β-keto- γ-amino acid, β-hydroxy γ-amino acids and peptaibols.

2a.5 Experimental section

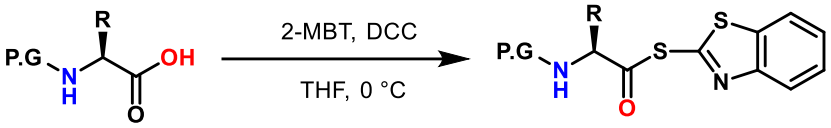
2a.5.1 General experimental details:

All α-amino acids and Di-t*ert*-butyl dicarbonate were obtained from Merck. 2-Mercaptobenzothiazole (2-MBT), $NaBH_4$ and dry THF were purchased from Spectrochem. Column chromatography was achieved on silica gel of 120-200 mesh from Merck. Thin-layer chromatography (TLC) was used to monitor reaction using Merck 60 F_{254} precoated silica gel plates. For visualization UV light and ninhydrin or phosphomolybdic acid (PMA) stains were used then charring on a hot plate. Melting points were recorded on Veego VMP-DS hot stage apparatus. Specific rotations were recorded at ambient temperature on the Rudolph Analytical Research instrument using $CHCl_3$ and MeOH as solvents. Mass was analysed on Applied Biosciences with MALDI TOF/TOF on positive ion mode.

NMR spectroscopy

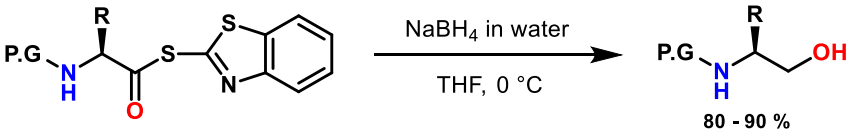
Jeol 400 MHz (or 100 MHz for ^{13}C) spectrometer were used for recording ^{1}H spectra and ^{13}C NMR. The chemical shifts (δ) and coupling constants (J) were reported in ppm and Hz, respectively. The residual solvents signals were used as internal reference ($CDCl_3$ δ_H, 7.24 ppm, δ_c 77.0 ppm).

2a.5.2 General procedure for the synthesis of MBT ester from *N*-protected α-amino acid:



N-protected α-amino acid (10 mmol) and 2-Mercaptobenzothiazole (2.505 g, 15 mmol) were mixed in THF (20 mL). The reaction mixture was allowed to cool to 0 °C. After cooling, DCC (2.06 g, 10 mmol) was added to the reaction and stirred for the next 1 h. TLC was performed for monitoring the reaction. On the completion of the reaction, precipitated DCU was filtered. The filtrate was concentrated beneath the *vacuum* to attain a gummy mixture. Further, EtOAc/Hexane was used for precipitating the mixture to give MBT ester of *N* (protected) amino acid. This precipitated product was used without purification directly for the alcohol synthesis.

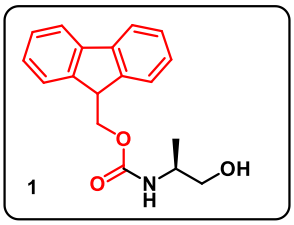
2a.5.3 General procedure for the synthesis of *N*-protected β-amino alcohol from 2-Mercaptobenzothiazole esters:



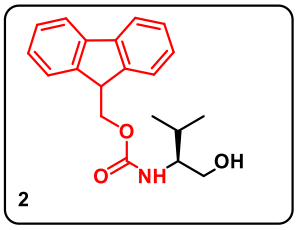
THF (30ml) was used to dissolve the above obtained *N*-protected MBT ester and was allowed to cool to 0 °C using an ice bath. $NaBH_4$ in water (50 mmol in 15 ml of water) was added to the reaction mixture step by step to observe effervescence and kept for the next 30 mins. Once the reaction is completed it was neutralized using 10% HCl. Later, the reaction mixture was kept beneath a vacuum to evaporate the THF used for the reaction. The aqueous layer was extracted thrice using ethyl acetate. The combined organic extract was washed further with 10% Na_2CO_3 and later with brine followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure *N*-protected β-amino alcohol with 80-90% yield.

Characterization of compounds

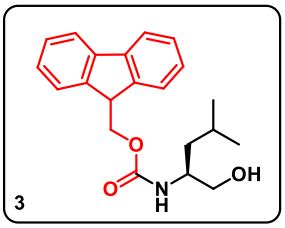
Spectroscopic Data for *N*-Fmoc β-amino alcohols obtained from 2-Mercaptobenzothiazole (2-MBT) method:



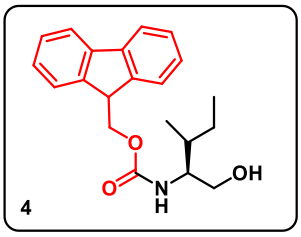
(*S*)-(9H-fluoren-9-yl)methyl 1-hydroxypropan-2-ylcarbamate (1) : white solid (0.534g, 90%); mp 150-153°C; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.77 (d, *J* =7.36 Hz, 2H), 7.60 (d, *J* = 7.32 Hz, 2H), 7.47 (t, *J* = 7.52 Hz, 2H), 7.32 (td, *J* = 0.9 Hz, *J* = 7.62 Hz, 2H), 4.86 (bs, 1H), 4.43 (d, *J* = 6.44 Hz, 2H), 4.22 (t, 6.44 Hz, 1H), 3.83 (bs, 1H), 1.18 (d, *J* = 6.44 Hz, 3H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 156.60, 143.86, 141.32, 127.69, 127.04, 125.01, 119.97, 66.92, 66.62, 48.94, 47.23, 17.34; **MALDI TOF/TOF**- *m/z* calcd. for $C_{18}H_{19}NO_3$ $[M+K]^+$ 336.1002, obsrvd. 335.9779, **$[\alpha]_D^{25}$** = -2.0 (c = 1, MeOH).



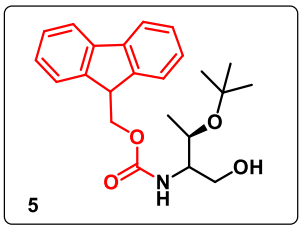
(*S*)-(9H-fluoren-9-yl)methyl 1-hydroxy-3-methylbutan-2-ylcarbamate (2) : white solid (0.520 g, 80%), mp 121-122 °C, **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.33 Hz, 2H), 7.59 (d, *J* = 7.30 Hz, 2H), 7.45 (t, *J* = 7.51 Hz, 2H), 7.32 (td, *J* = 0.9 Hz, *J*=7.62 Hz, 2H), 4.90 (bs, 1H), 4.45 (d, *J* = 6.44 Hz, 2H), 4.21 (t, *J* = 6.44 Hz, 1H), 3.66 (m, 2H), 3.46 (m, 1H), 2.04 (bs, 1H), 1.83 (m, 1H), 0.94 (m, 6H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 157.05, 143.86, 141.32, 127.65, 127.02, 124.97, 119.94, 66.54, 63.72, 58.53, 47.29, 29.15, 19.47, 18.61; **MALDI TOF/TOF**- *m/z* calcd. for $C_{20}H_{23}NO_3$ $[M+Na]^+$ 348.1576, obsrvd. 348.0117, **$[\alpha]_D^{25}$** = -13.0 (c = 1, MeOH).



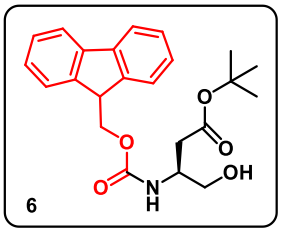
(*S*)-(9H-fluoren-9-yl)methyl 1-hydroxy-4-methylpentan-2-ylcarbamate (3): white solid (0.549 g, 81%), mp 133-135°C, **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.77 (d, J = 7.76 Hz, 2H), 7.59 (d, J = 7.32 Hz, 2H), 7.40 (t, J = 7.32 Hz, 2H), 7.32 (t, J = 7.32 Hz, 2H), 4.78 (bs, 1H), 4.45 (d, J = 6.88 Hz, 2H), 4.21 (t, J = 6.88 Hz, 1H), 3.85 (m, 1H), 3.58 (m, 1H), 1.63 (m, 3H), 1.32 (m,1H), 0.92 (dd, J = 2.28 Hz, J = 6.2 Hz, 6H) **;** **^{13}C NMR** (100 MHz, $CDCl_3$) δ 156.76, 143.85, 141.33, 127.67, 127.03, 125.0, 119.95, 66.47, 66.05, 51.30, 47.30, 40.34, 24.72, 23.04, 22.10; **MALDI TOF/TOF**- *m/z* calcd. for $C_{21}H_{25}NO_3$ $[M+K]^+$ 378.1472, obsrvd. 377.9655, **$[\alpha]_D^{25}$** = -20.1 (c = 1, MeOH).



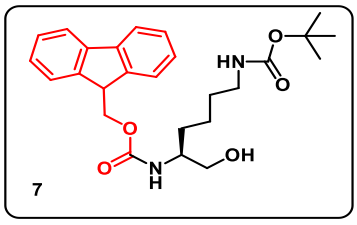
(9H-fluoren-9-yl)methyl *(2S, 3R)*-1-hydroxy-3-methylpentan-2-ylcarbamate (4): white solid (0.423 g, 65%), mp 114-116 °C; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.77 (d, J = 7.8 Hz, 2H), 7.60 (d, J = 7.32 Hz, 2H), 7.41 (t, J = 7.36Hz,2H), 7.32 (dt, J = 1.36 Hz, J=7.32 Hz, 2H), 4.88 (bd, J = 8.24 Hz,1H) , 4.44 (d, J = 6.4 Hz, 2H), 4.22 (t, J = 6.4 Hz, 1H), 4.12 (bs,1H), 3.68 (m,2H), 3.54 (m, 1H), 1.93 (m, 1H), 1.13 (m,2H), 0.92 (m,6H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 157.00, 143.85, 141.32, 141.29, 127.65, 127.02, 124.98, 119.94, 66.51, 63.49, 57.36, 47.31, 35.83, 25.40, 15.48, 11.35; **MALDI TOF/TOF**- *m/z* calcd. for $C_{21}H_{25}NO_3$ $[M+K]^+$ 378.1472, obsrvd. 377.9658, **$[\alpha]_D^{25}$** = -11.7 (c = 1, MeOH).



(9H-fluoren-9-yl) methyl (*2R, 3S*)-3-tert-butoxy-1-hydroxybutan-2-ylcarbamate (5): colorless oil (0.651 g, 85%); **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.76 (d, J = 7.76 Hz, 2H), 7.61 (d, J = 7.6 Hz, 2H), 7.40 (t, J = 7.32 Hz, 2H), 7.32 (t, J = 7.32 Hz, 2H), 5.28 (bd, J = 7.76 Hz,1H) , 4.41 (m,2H), 4.23 (t, J = 6.88 Hz, 1H), 3.96 (m, 1H), 3.66 (m, 3H), 2.86 (bs,1H), 1.21 (s,9H), 1.16 (d, J = 5.96 Hz, 3H) ; **^{13}C NMR** (100 MHz, $CDCl_3$) δ 157.28, 144.16, 141.56, 127.94, 127.30, 125.34, 120.23, 74.59, 67.45, 67.06, 63.96, 57.39, 47.53, 28.91, 20.36 ; **MALDI TOF/TOF**- *m/z* calcd. for $C_{23}H_{29}NO_4$ $[M+Na]^+$ 406.1994, obsrvd. 406.0422; **$[\alpha]_D^{25}$** = +6.1 (c = 1, MeOH).

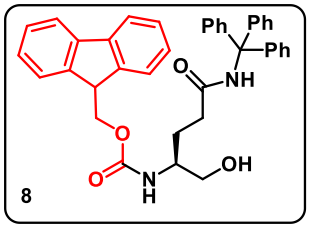


(*S*)-tert-butyl 3-(((9H-fluoren-9-yl)methoxy)carbonylamino)-4-hydroxybutanoate (6): white solid (0.730 g, 92 %), mp 93-96 ^{o}C; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.77 (d, J = 7.8 Hz, 2H), 7.59 (d, J = 7.32 Hz, 2H), 7.40 (t, J = 7.32 Hz, 2H), 7.31 (t, J =7.32 Hz, 2H), 5.54 (bs, 1H) , 4.40 (d, J =6.92,2H), 4.22 (t, J = 6.92 Hz, 1H), 4.03 (m,1H), 3.74 (m,2H), 2.57-2.52 (m,2H), 1.46 (s,9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 171.10, 156.30, 143.79, 141.28, 127.69, 127.03, 125.02, 119.96, 82.00, 67.95, 66.84, 64.53, 49.92, 47.15, 37.26, 28.00; **MALDI TOF/TOF**- *m/z* calcd. for $C_{23}H_{27}NO_5$ $[M+K]^+$ 436.1526, obsrvd. 435.9662; **$[\alpha]_D^{25}$** = -7.4 (c = 1, MeOH).

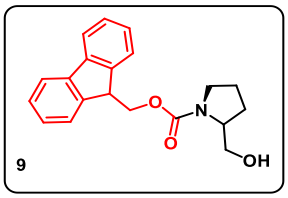


(*S*)-(9H-fluoren-9-yl)methyl6-((tert-butoxycarbonyl)amino)-1-hydroxyhexan-2-ylcarbamate (7): white solid (0.681 g, 75%), mp 136-138 ^{o}C; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.75 (d, J = 7.77 Hz, 2H), 7.60 (d, J = 7.31 Hz, 2H), 7.41 (t, J = 7.32 Hz, 2H), 7.32 (t, J = 7.32 Hz, 2H), 5.10 (bs, 1H), 4.60 (bs, 1H), 4.40 (d, J = 6.90 Hz,2H), 4.21 (t, J = 6.90 Hz, 1H), 3.62 (bs,3H), 3.18-3.07 (m, 2H), 2.56 (bs, 1H), 1.67 (m,2H), 1.47 (m,2H), 1.43 (s,9H), 1.36 (m, 2H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 156.78, 156.53, 144.00, 141.42, 127.77, 127.15, 125.16, 120.05, 80.00, 66.69, 64.78, 53.03, 47.38, 39.61, 30.43, 30.09, 28.50, 22.60; **MALDI**

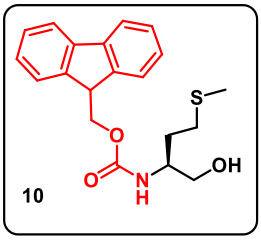
TOF/TOF- *m/z* calcd. for $C_{26}H_{34}N_2O_5$ $[M+Na]^+$ 477.2399, obsrvd. 477.2375 and m/*z* calcd. for $C_{21}H_{26}N_2O_3$ $[M\text{-}Boc+H]^+$ 355.2022, obsrvd. 355.1945, $[\alpha]_D^{25}$ = -6.9 (c = 1, MeOH).



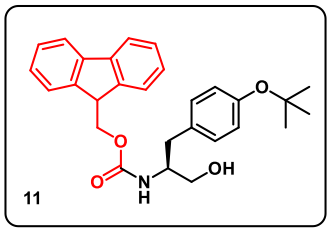
(*S*)-(9H-fluoren-9-yl)methyl 1-hydroxy-5-oxo-5-(tritylamino)pentan-2-ylcarbamate (9) : white solid (1.013 g, 85%), mp 74-77 ^oC ; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.72 (d, *J* = 7.36 Hz, 2H), 7.56 (d, *J* = 7.36 Hz,2H), 7.39-7.16 (m,19H), 6.93 (s, 1H), 5.36 (d, *J* = 8.72 Hz, 1H), 4.50 (bs,1H), 4.37 (m, 2H), 4.17 (t, *J* = 6.88 Hz, 1H) , 5.53 (m,1H), 3.39 (m, 2H), 2.28 (m,2H), 1.79 (m, 2H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 172.40, 156.83, 144.54, 143.94, 141.41, 128.73, 128.06, 127.80, 127.18, 125.19, 120.08, 70.75, 66.59, 64.09, 52.76, 47.36, 33.57, 26.40; **MALDI TOF/TOF**- *m/z* calcd. for $C_{39}H_{36}N_2O_4$ $[M+Na]^+$ 619.2573, obsrvd. 619.2513; $[\alpha]_D^{25}$ = -5.8 (c = 1, MeOH).



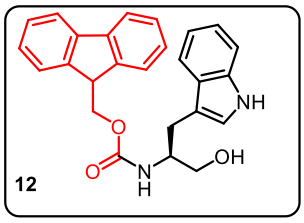
(9H-fluoren-9-yl)methyl 2-(hydroxymethyl)pyrrolidine-1-carboxylate(12) : White solid (0.504 g, 78%), mp 90-92 ^oC; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.77 Hz, 2H), 7.58 (d, *J* = 7.31 Hz, 2H), 7.39 (t, *J* = 7.32 Hz, 2H), 7.31 (t, *J* = 7.32 Hz, 2H), 4.57 (bs, 1H,-OH), 4.42 (m, 2H), 4.24 (t, *J* = 6.88 Hz, 1H), 3.99(m, 1H), 3.63-3.37 (m, 4H), 2.04-1.09 (m, 4H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 157.11, 143.82, 141.29, 127.68, 126.99, 125.01, 119.95, 67.51, 66.96, 60.71, 49.01, 47.28, 33.88, 28.55, 25.56, 24.90, 24.06; **MALDI TOF/TOF**- *m/z* calcd. for $C_{20}H_{21}N_1O_3$ $[M+Na]^+$ 346.1419, obsrvd. 346.1453; $[\alpha]_D^{25}$ = -29.1 (c = 1, $CHCl_3$).



(*S*)-(9H-fluoren-9-yl)methyl 1-hydroxy-4-(methylthio)butan-2-ylcarbamate (15) : White solid (0.642g, 90%), mp 136-137 ^{o}C; **^{1}H NMR** (400 MHz, DMSO d_6) δ 7.87-7.33 (m, 8 H,), 6.51 (d, *J* = 7.76 Hz, 1H), 6.27 (m, 2H), 4.02 (m, 1H), 3.44 (bs,1H), 3.37-3.21 (m, 2H), 2.4 (m,2H), 2.02 (s, 3H), 1.74-1.51 (m,2H); **^{13}C NMR** (100 MHz, DMSO d_6) δ 157.73, 142.57, 139.43, 137.43, 128.95, 127.31, 121.41, 120.05, 109.82, 63.74, 59.76, 51.76, 30.83, 30.10, 14.69; **MALDI TOF/TOF**- *m/z* calcd. for $C_{20}H_{23}N_1O_3S_1$ $[M+Na]^+$ 380.1296, obsrvd. 380.0779; **$[\alpha]_D^{25}$** = -17.3 (c = 1, $CHCl_3$).

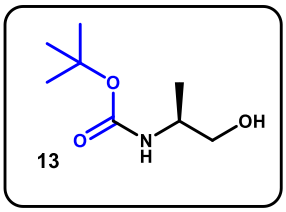


(*S*)-(9H-fluoren-9-yl)methyl1-(4-tert-butoxyphenyl)-3-hydroxypropan-2ylcarbamate(11) : White Solid (0.703g, 79%), mp 113-116 ^{o}C; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.77 Hz, 2H), 7.55 (d, *J* = 7.31 Hz, 2H), 7.37 (t, *J* = 7.32 Hz, 2H), 7.24 (t, *J* = 7.32 Hz, 2H), 7.10-6.88 (m, 4H), 5.00 (d, *J* = 7.76 Hz, 1H), 4.38 (m, 2H), 4.19 (t, *J* = 6.88 Hz, 1H), 3.88 (bs, 1H), 3.66-3.53 (m,2H), 2.79 (d, *J* = 6.88 Hz, 2H), 1.30 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 156.42, 153.92, 143.81, 141.28, 132.30, 129.59, 127.65, 127.01, 124.97, 124.28, 119.94, 80.80, 66.56, 63.87, 54.07, 47.20, 36.57, 28.76; **MALDI TOF/TOF**- *m/z* calcd. for $C_{28}H_{31}N_1O_4$ $[M+Na]^+$ 468.2151, obsrvd. 468.2289; **$[\alpha]_D^{25}$** = -18.8 (c = 1, $CHCl_3$).

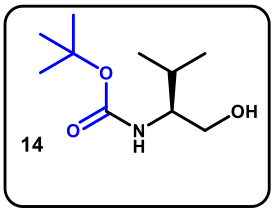


(*S*)-(9H-fluoren-9-yl)methyl 1-hydroxy-3-(1H-indol-3-yl)propan-2-ylcarbamate(13) : White Solid (0.700g, 85%); mp 88-90 ^{o}C; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.39 (bs, 1H), 7.76 -7.00 (m, 13 H), 5.31 (bs, 1H), 4.41 (m, 2H), 4.18 (t, *J* = 6.87 Hz, 1H),4.05 (bs,1H), 3.65-3.60 (m,2H), 3.02 (d, *J* = 3.66 Hz, 2H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 156.55, 143.84, 141.21, 136.19, 127.59, 126.98, 125.03, 122.78, 121.08, 119.88, 119.45, 118.98, 66.45, 63.91, 53.30, 47.17, 33.85, 26.72; **MALDI TOF/TOF**- *m/z* calcd. for $C_{26}H_{24}N_2O_3$ $[M+K]^+$ 412.1787, obsrvd. 412.1424; **$[\alpha]_D^{25}$** = -26.8 (c = 1, $CHCl_3$).

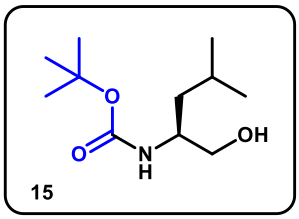
Spectroscopic Data for *N*-Boc β-amino alcohols obtained from 2-Mercaptobenzothiazole(2-MBT) method:



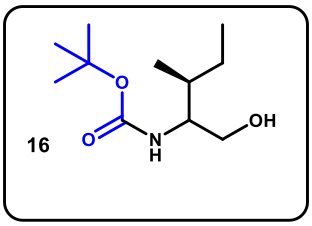
***Tert*-butyl (*S*)-(1-hydroxypropan-2-yl)carbamate:** colorless oil (0.312 g, 75 %);**^{1}H NMR** (400 MHz, Chloroform-*d*) δ 5.16 (d, *J* = 7.8, 1H), 3.95 (s, 1H), 3.76 -3.54 (m, 2H), 3.49 (d, *J* = 11.0, 1H), 1.44 (s, 9H), 1.15 (d, *J* = 6.8, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 156.12, 126.54, 125.29, 122.45, 121.25, 79.13, 77.54, 77.22, 76.91, 65.94, 48.10, 28.38, 17.17. **LCMS/MS** - *m/z* calcd. for $C_8H_{17}NO_3$ [M+Na]+ 198.1106, obsrvd. 198.1172; **$[\alpha]_D^{25}$** = -9 (c = 1, MeOH).



***Tert*-butyl (*S*)-(1-hydroxy-3-methylbutan-2-yl)carbamate**: colorless oil (0.312 g, 75 %); **^{1}H NMR** (400 MHz, CDCl3) *δ* 4.83 (bs, 1H), 3.52 (m, 2H), 3.43 (m,1H), 3.10 (bs, 1H), 1.82 (m.1H), 0.94 (m, 6H); **^{13}C NMR** (100 MHz, CDCl3) *δ* 156.97, 79.57, 64.05, 58.04, 29.33, 28.34, 19.58, 18.53; **LCMS/MS** - *m/z* calcd. for $C_{10}H_{21}NO_3$ [M+Na]+ 226.1419, obsrvd.226.1481; **$[\alpha]_D^{25}$** = -16.5 (c = 1, MeOH).



***Tert*-butyl (*S*)-(1-hydroxy-4-methylpentan-2-yl)carbamate:** colorless oil (0.312 g, 80 %);**^{1}H NMR** (400 MHz, Chloroform-*d*) δ 4.81 (s, 1H), 3.70 – 3.61 (m, 2H), 3.51 (d, *J* = 5.4, 1H), 3.25 (s, 1H), 1.70 – 1.65 (m, 1H), 1.45 (s, 9H), 1.33 (d, *J* = 7.9, 2H), 0.94 (s, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 156.41, 79.22, 77.45, 76.81, 65.57, 50.65, 40.50, 28.62, 24.86, 24.70, 22.99, 22.15. **LCMS/MS** - *m/z* calcd. For $C_{11}H_{23}NO_3$ [M+Na]+ 240.1576, obsrvd. 240.1544; **$[\alpha]_D^{25}$** = -22.5 (c = 1, MeOH).



***Tert*-butyl ((*2R,3R*)-1-hydroxy-3-methylpentan-2-yl)carbamate:** colorless oil (0.312 g, 73 %); **^{1}H NMR** (400 MHz, Chloroform-*d*) δ 4.74 (d, *J* = 8.1, 1H), 3.69 (d, *J* = 10.3, 1H), 3.53 (dd, *J* = 39.4, 6.5, 2H), 2.69 (s, 1H), 1.59 – 1.54 (m, 1H), 1.43 (s, 9H), 1.27 – 1.05 (m, 2H), 0.91 – 0.88 (m, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 156.78, 79.42, 63.54, 56.90, 36.00, 28.43, 28.37, 25.35, 15.49, 11.44. **LCMS/MS** - *m/z* calcd. For $C_{11}H_{23}NO_3$ [M+Na]+ 240.1576, obsrvd. 240.1544; **$[\alpha]_D^{25}$** = -23.2 (c = 1, MeOH).

2a.5.4 Crystallographic Information

General procedure for crystallization

Glass sample vials (2 mL) were washed with acetone and dried under a nitrogen gas stream before use and PARAFILM were used to close the vials. Purified compounds were dissolved in a minimum amount of HPLC-grade EtOAc and kept for crystallization at room temperature. Data for X-ray structure determination were obtained from Bruker APEXII DUO diffractometer using Mo-Kα graphite monochromatic radiation (λ = 0.71073 Å) at room temperature.

Crystal structure data for Fmoc-Leu-ol :

A colourless crystal with approximate dimensions 0.90 x 0.25 x 0.10 mm was obtained with chemical formula $C_{21}H_{25}N_1O_3$ and monoclinic space group *P*2(1)*; a* = 5.211(4), *b* =12.337(10), *c* = 14.187(12) Å, *α* =90, *β*=97.308(16), *γ* =90°; *V* = 904.7(13)Å^3; *T* = 296 (2) K; *Z* = 2; ρ_{calcd} =1.246 Mgm^{-3}; *2θmax* = 57.28°; *MoKαλ*= 0.71073 Å. Graphite monochromator in Fine-focus sealed tube source was used. *R* = 0.0547 (for 3460 reflections with *I>2σ (I)*; *wR* = 0.1362 which was refined against $|F^2|$ and *S* = 0.818 for 229 parameters and 4239 unique reflections. SHELXS-97 [33] was used to obtain the structure by direct methods using with μ = 0.083mm^{-1} and minimum/maximum residual electron density –0.247/0.292eÅ^{-3}.

2a.6 References

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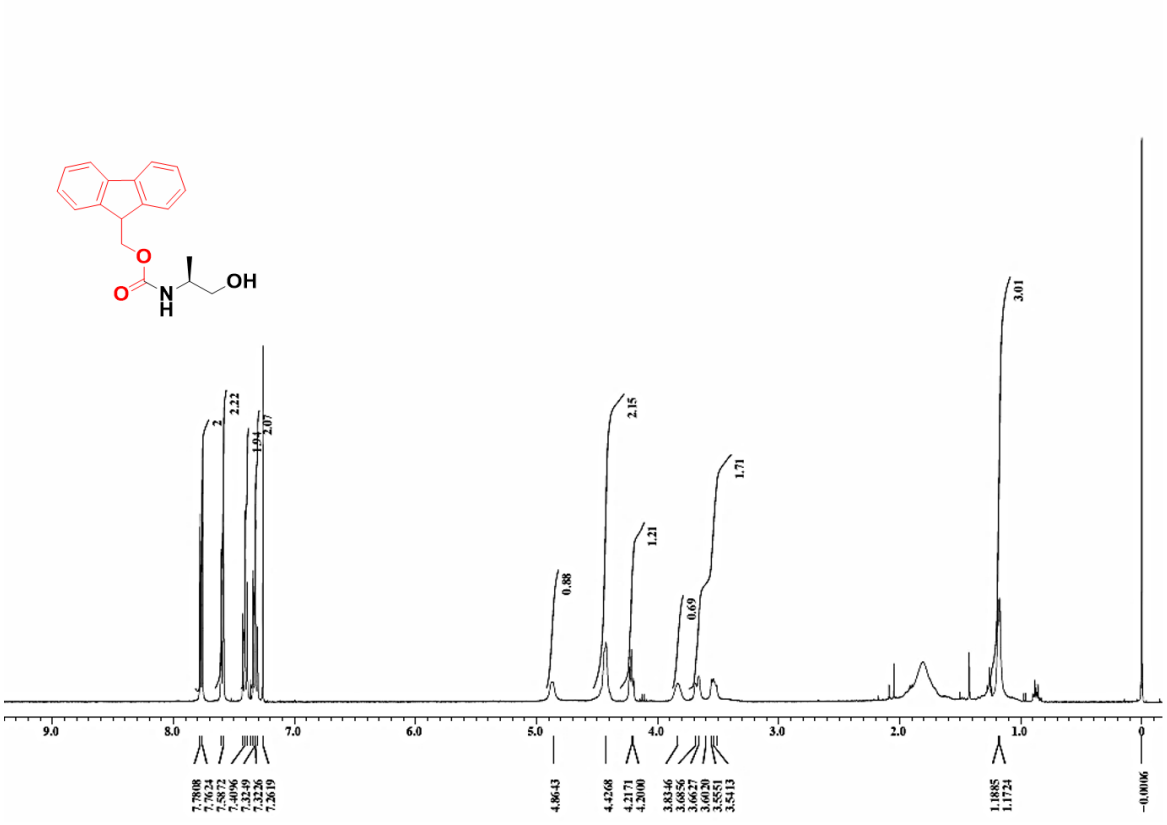
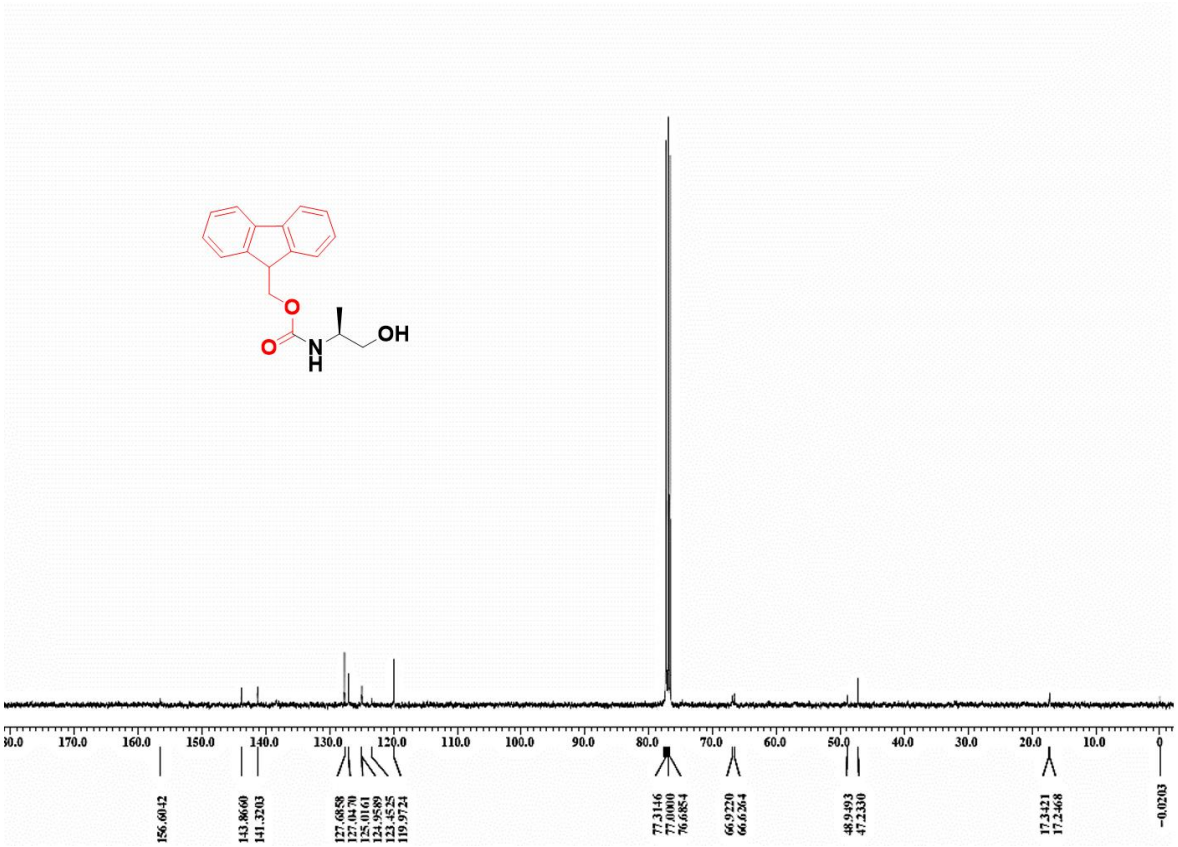
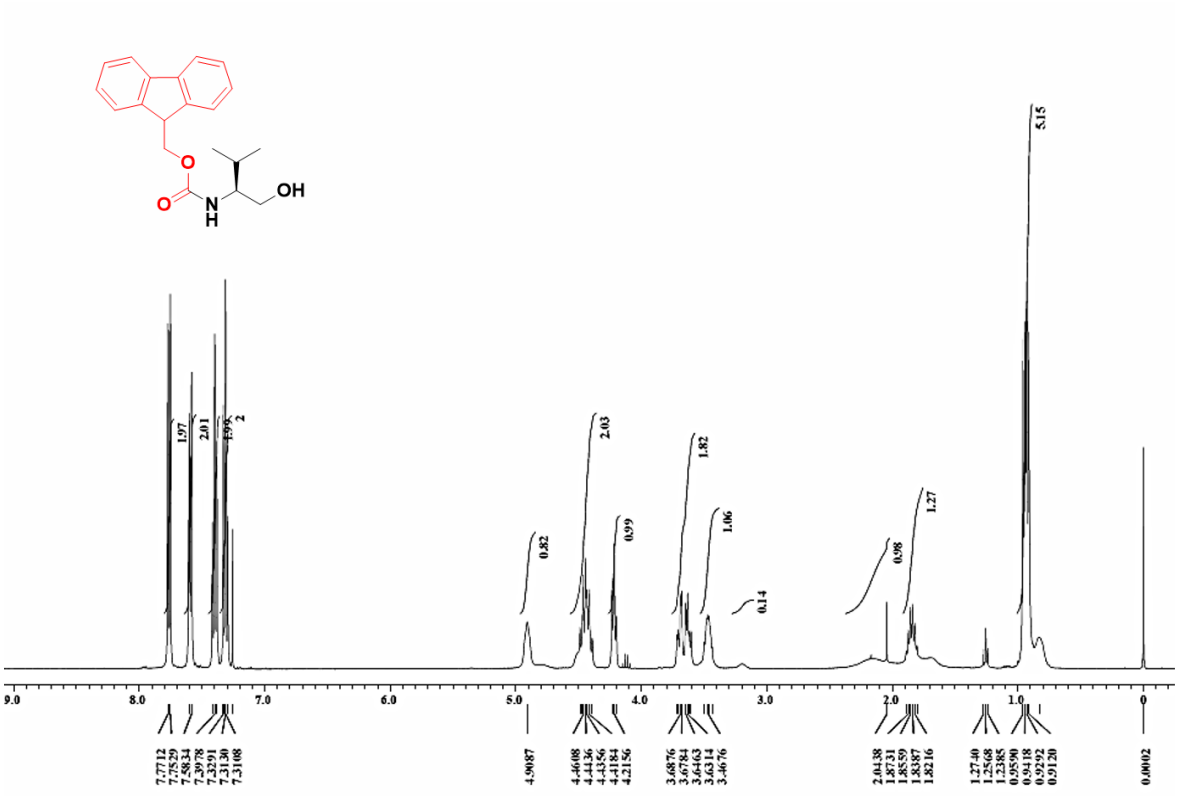
2a.7 Appendix I: ^{1}H NMR spectra,^{13}C NMR and Mass spectra of compound 1-16.



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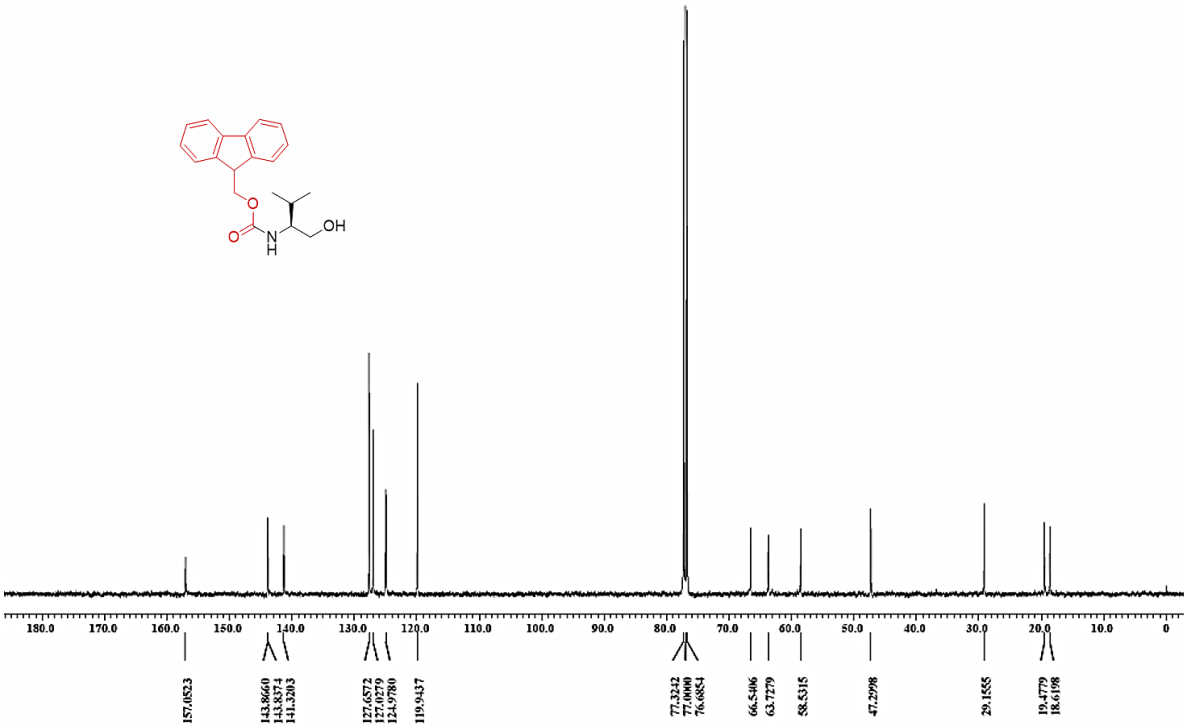
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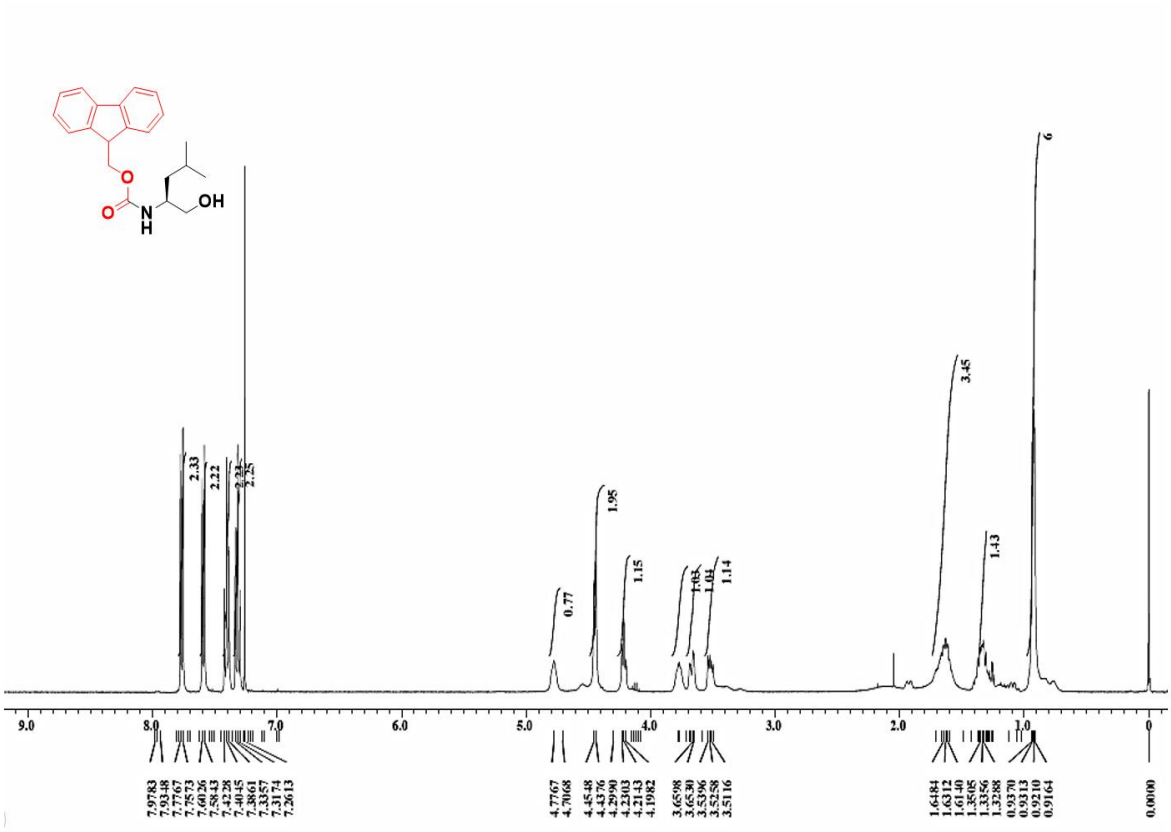
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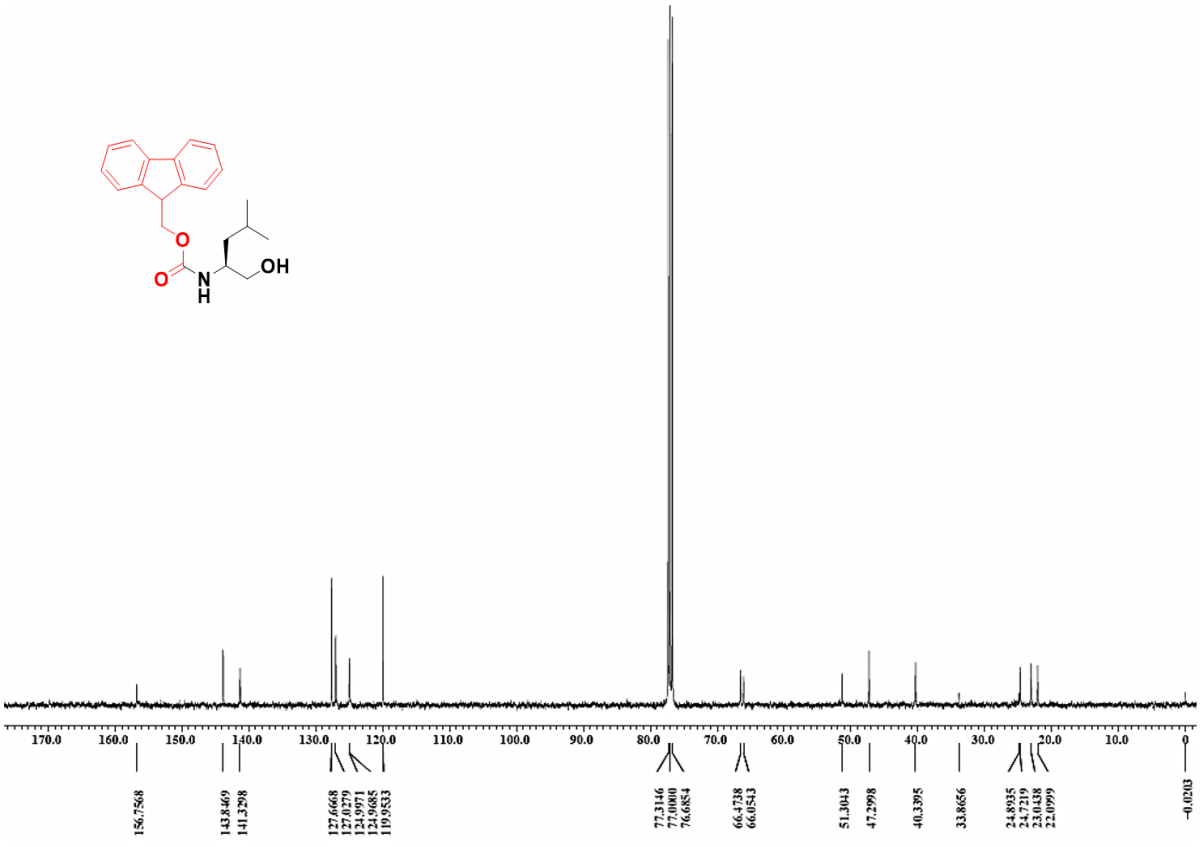
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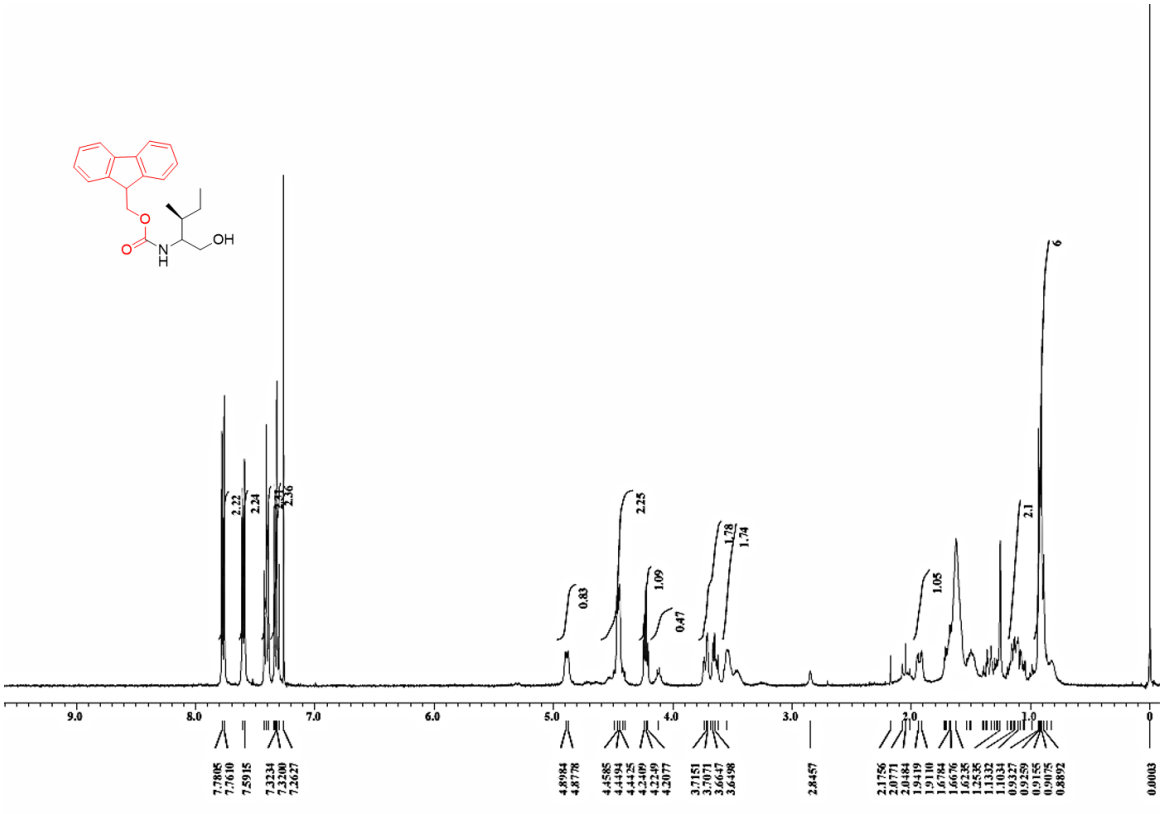
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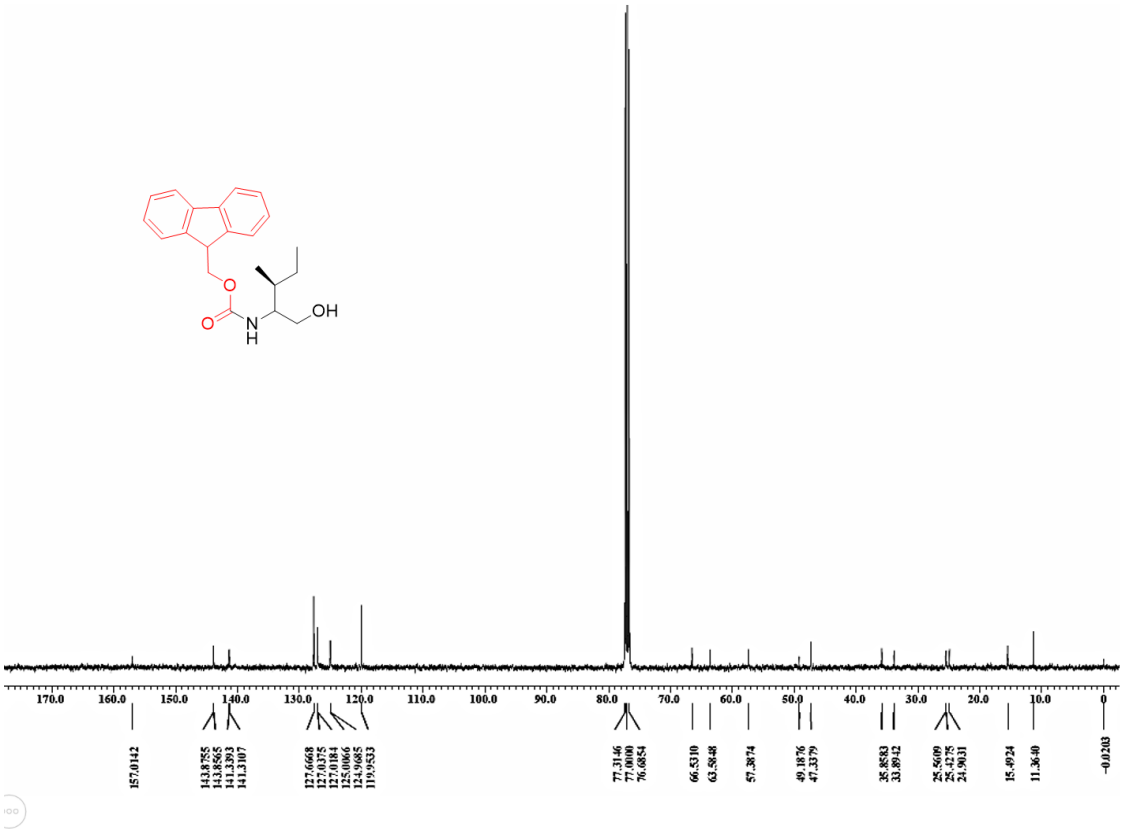
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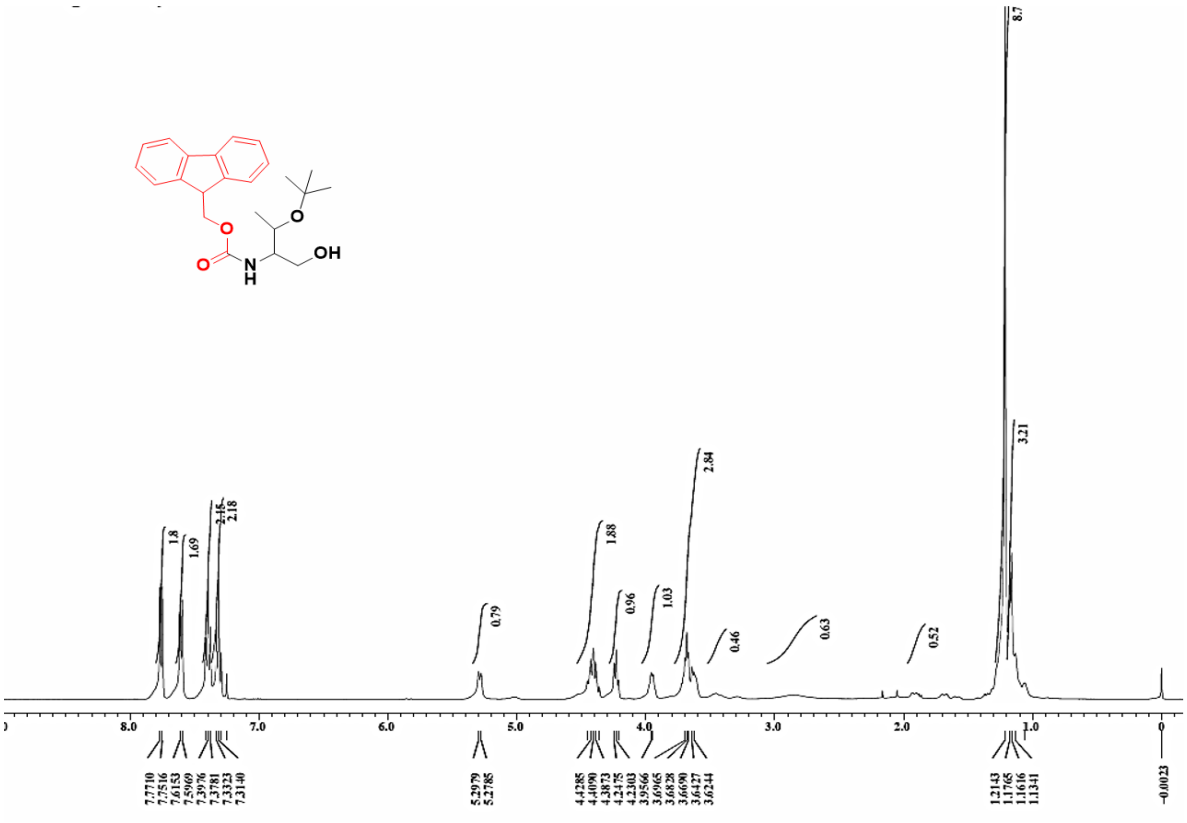
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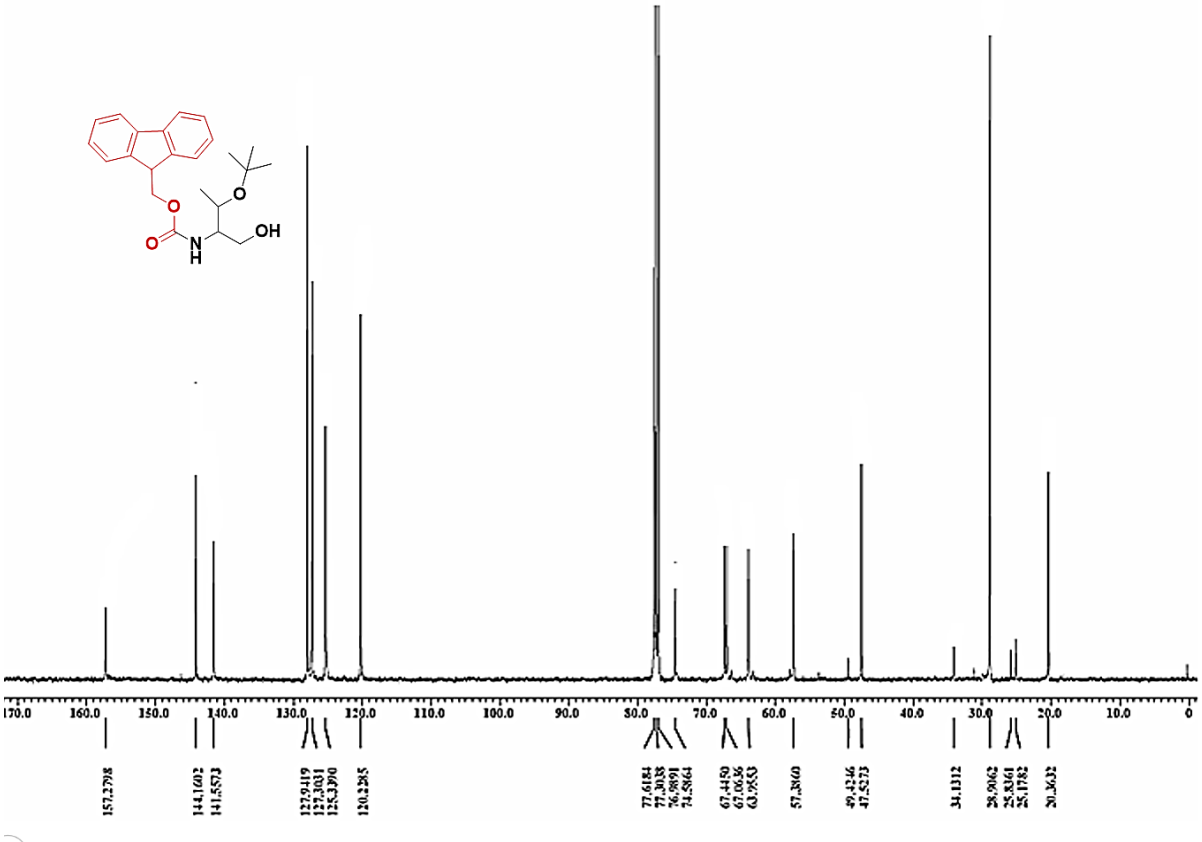
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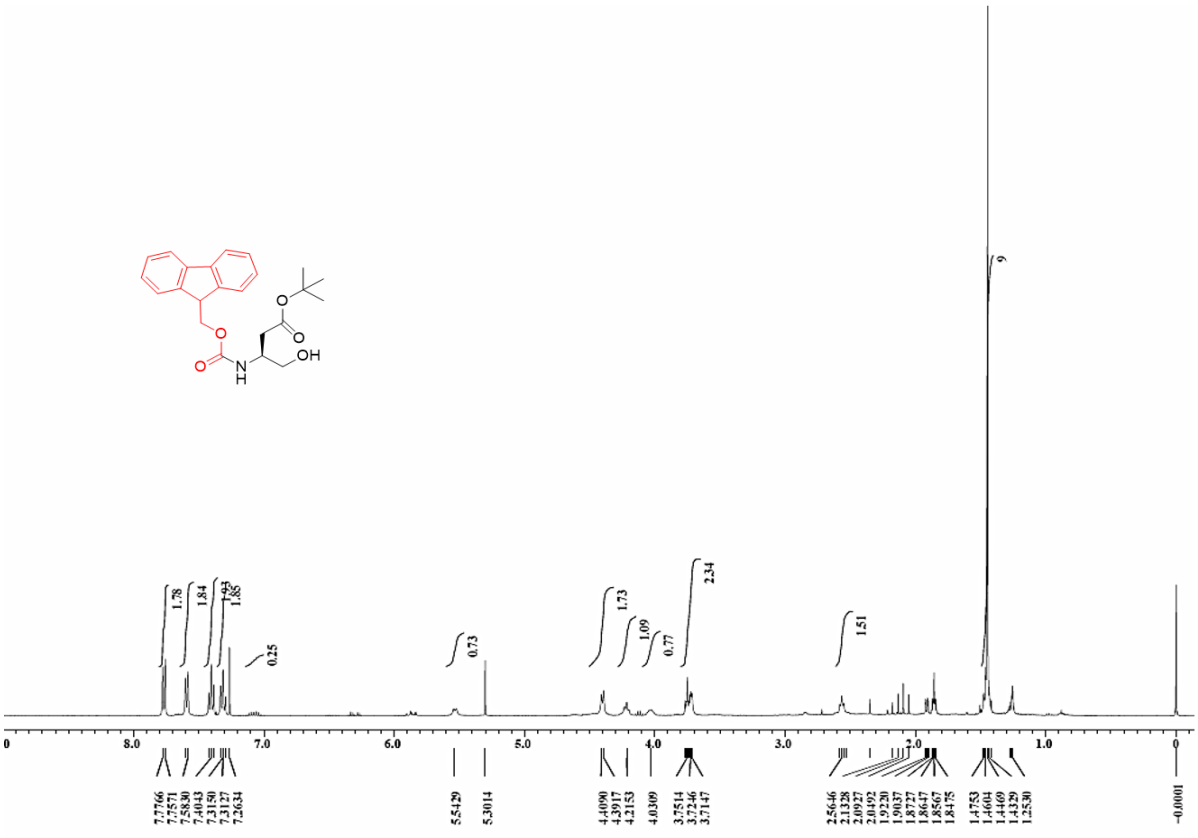
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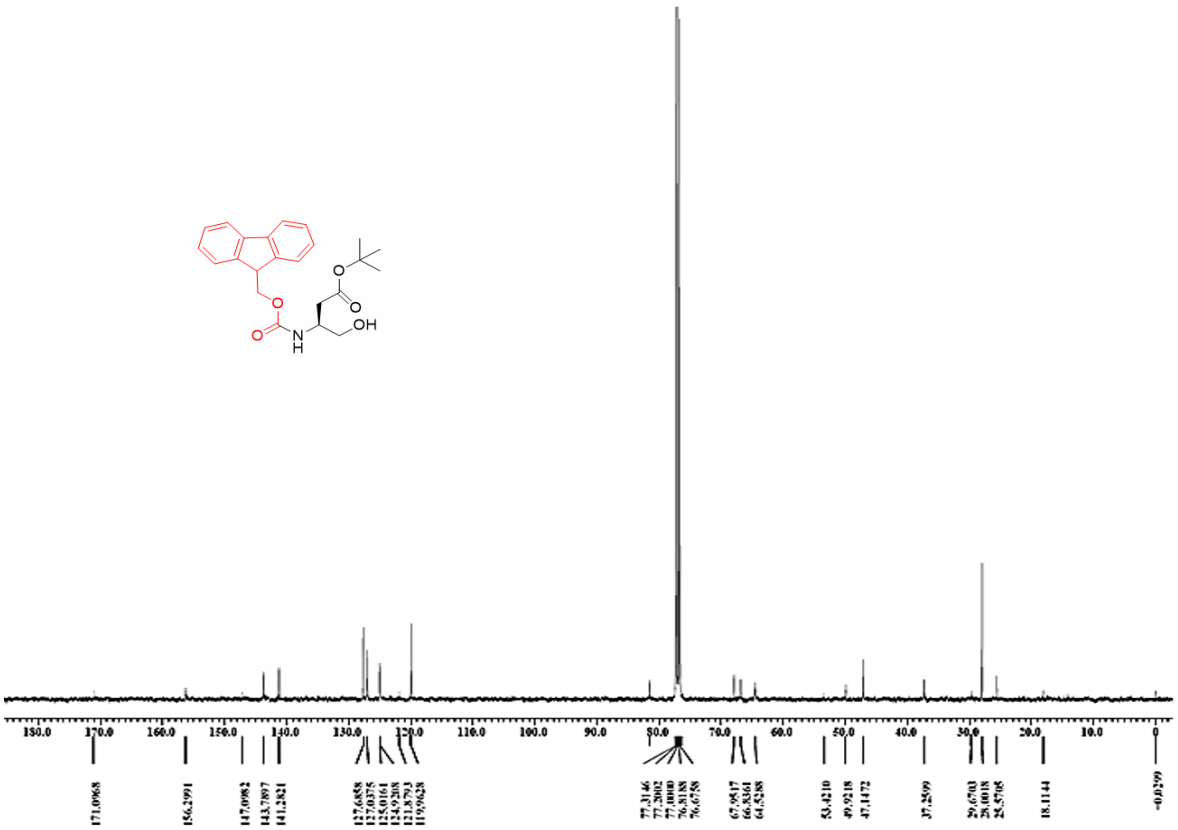
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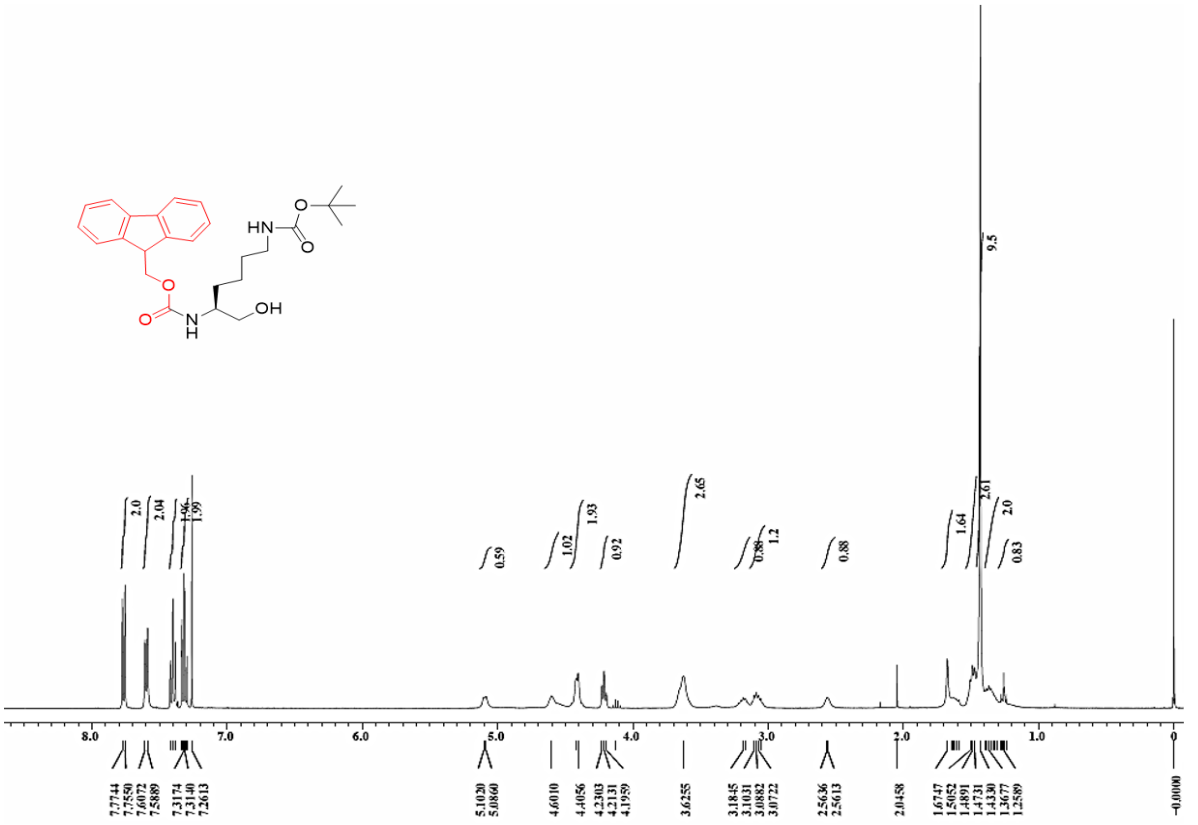
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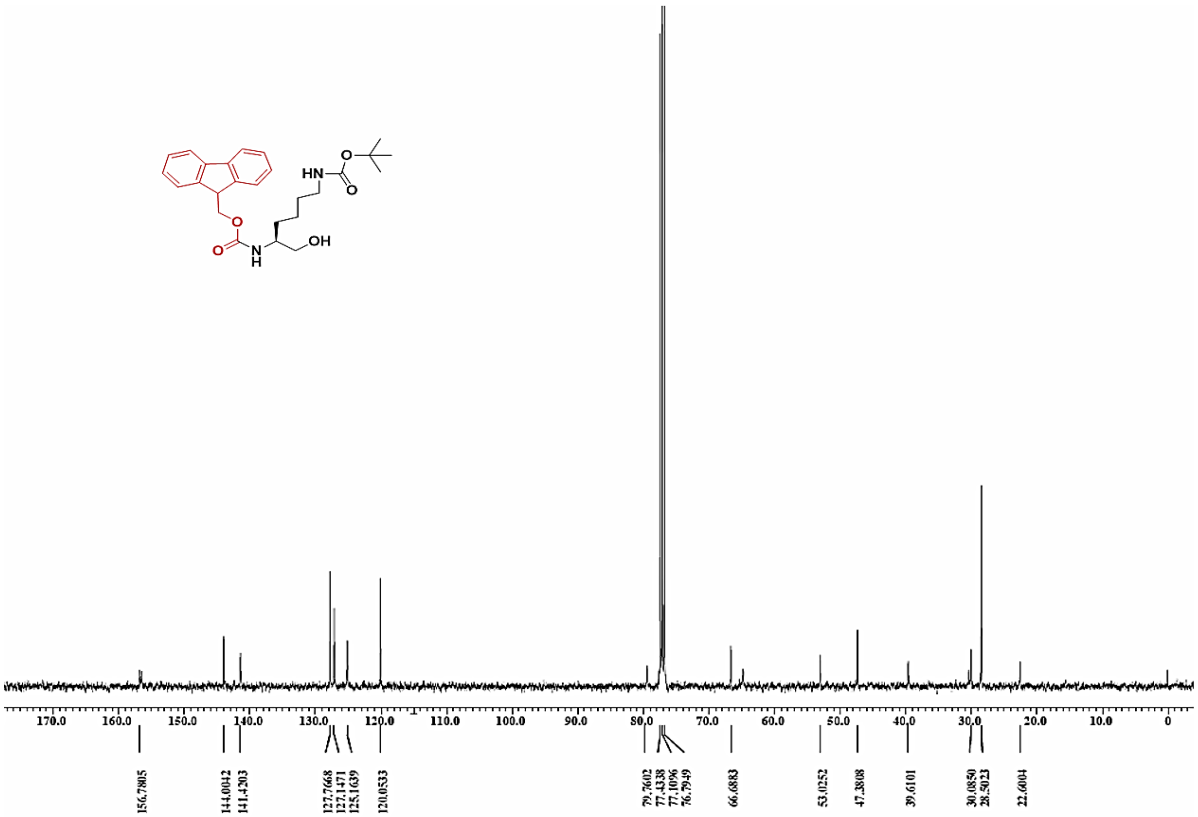
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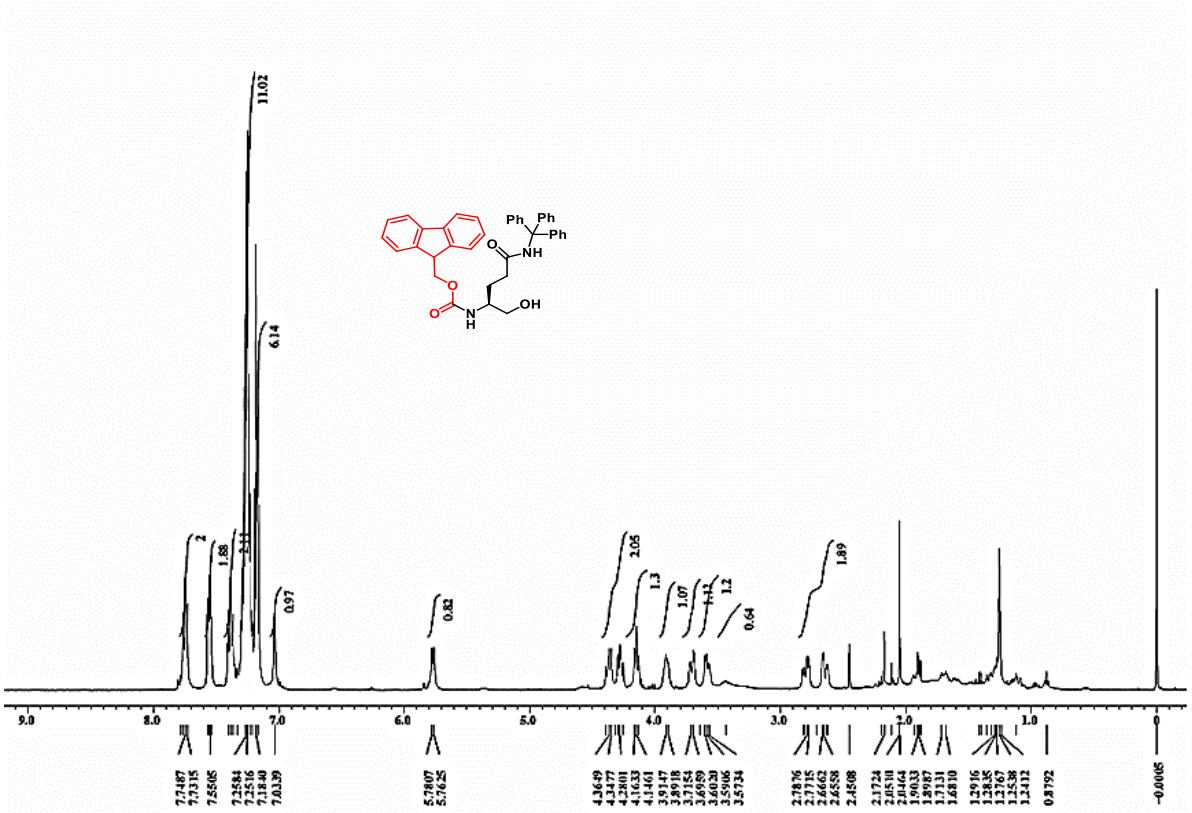

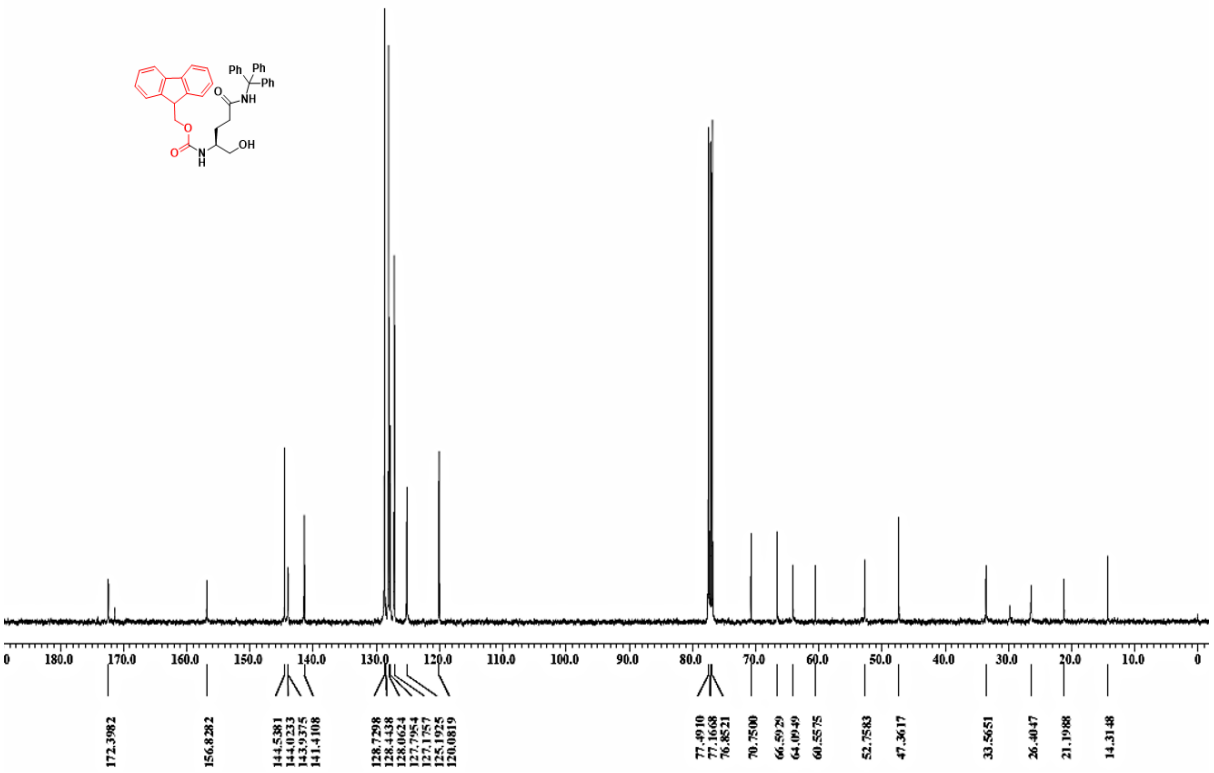

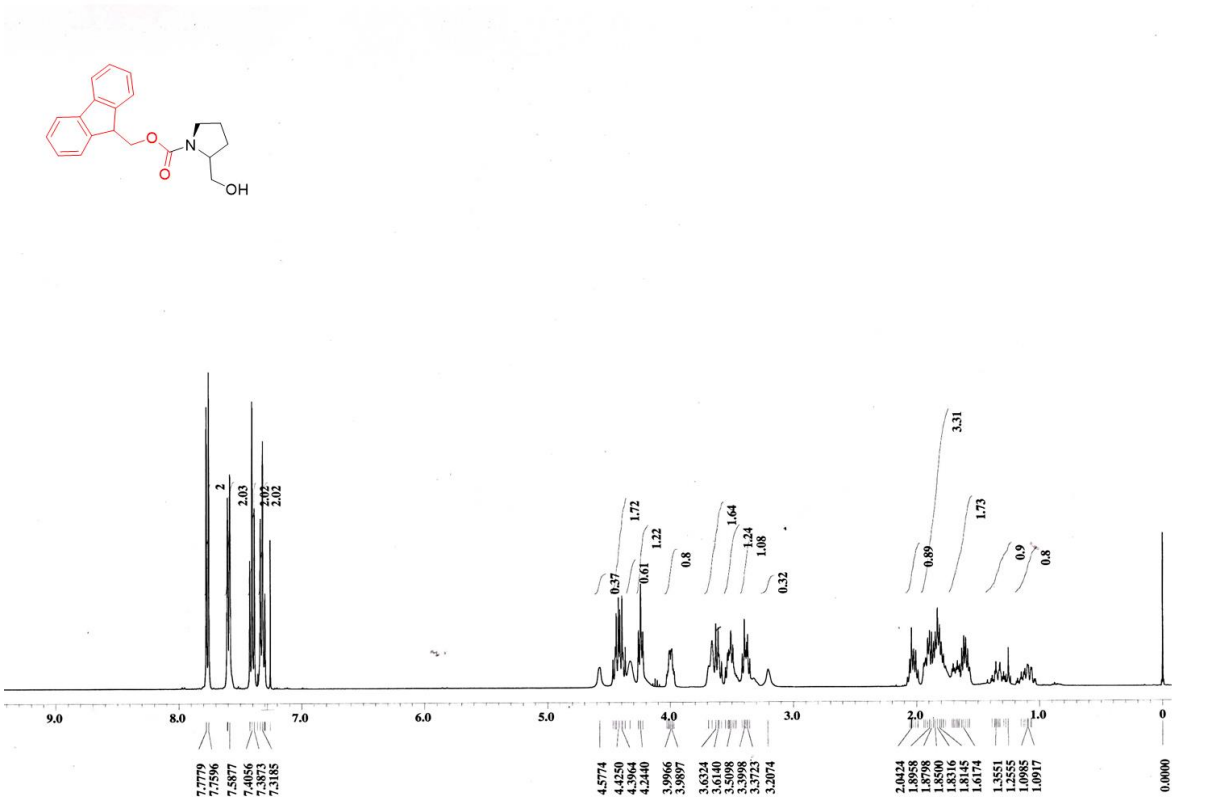
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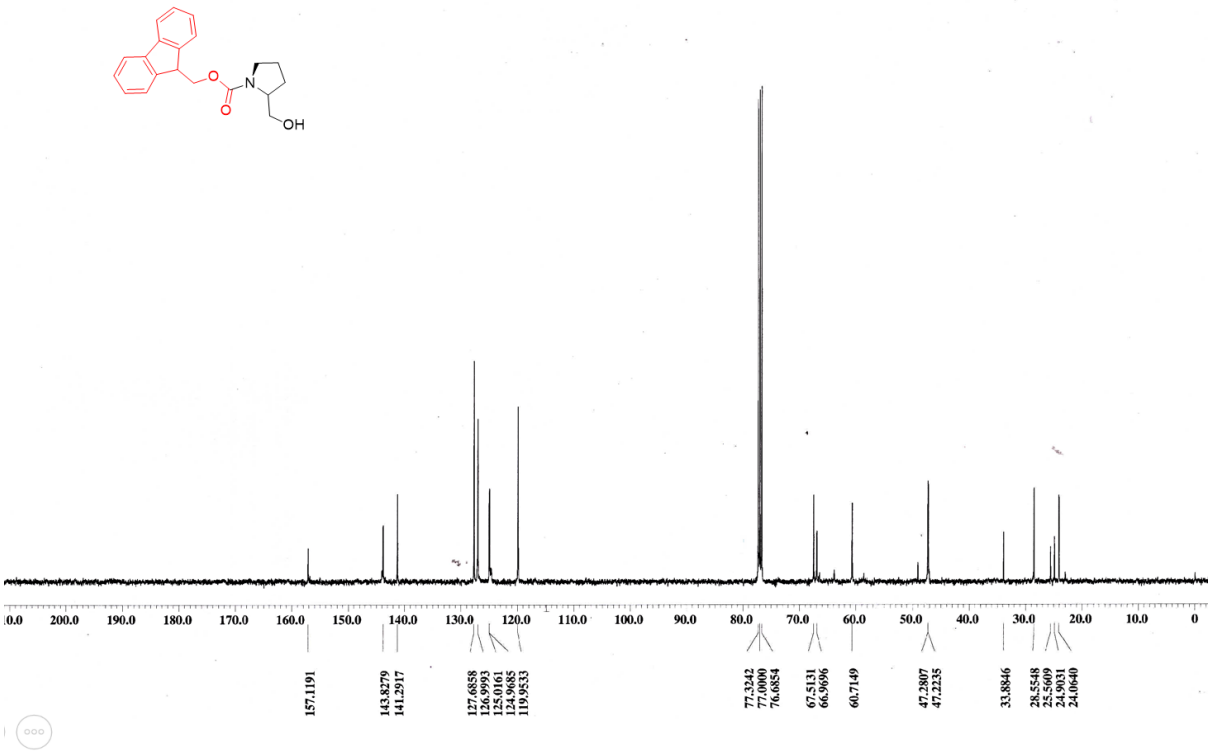
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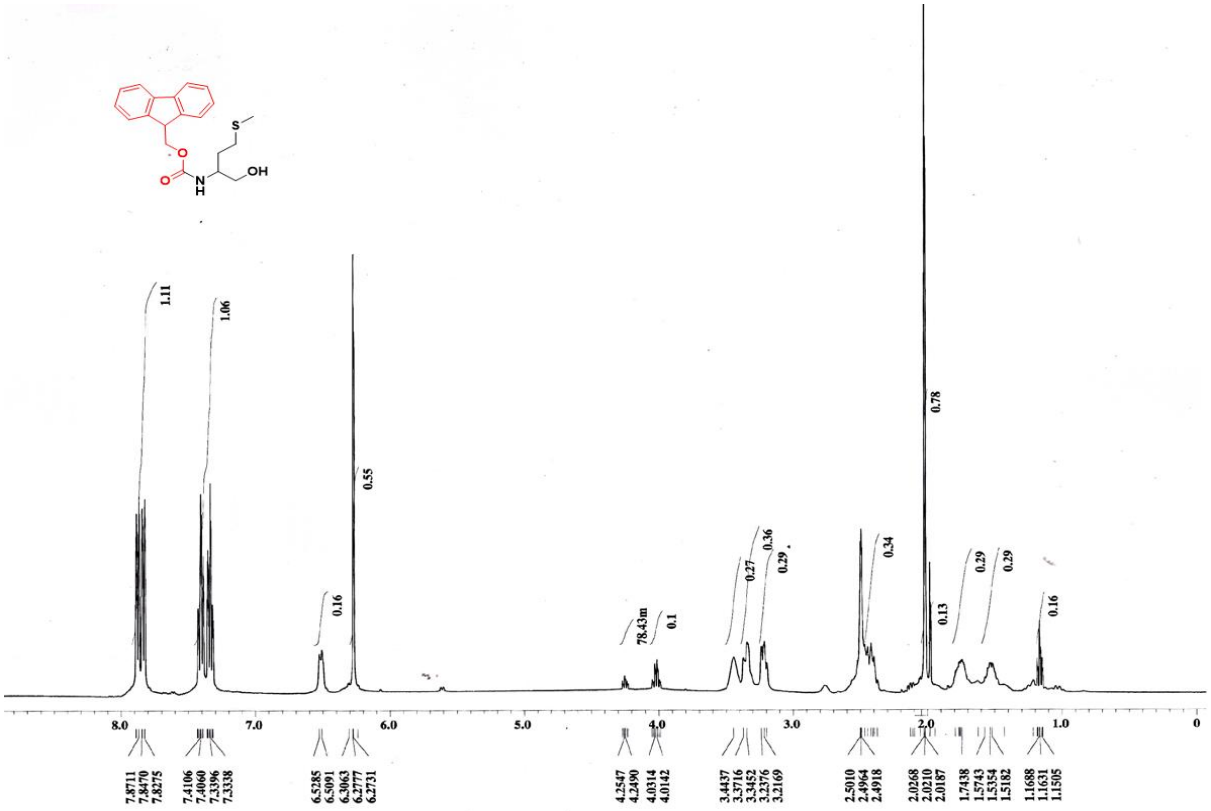
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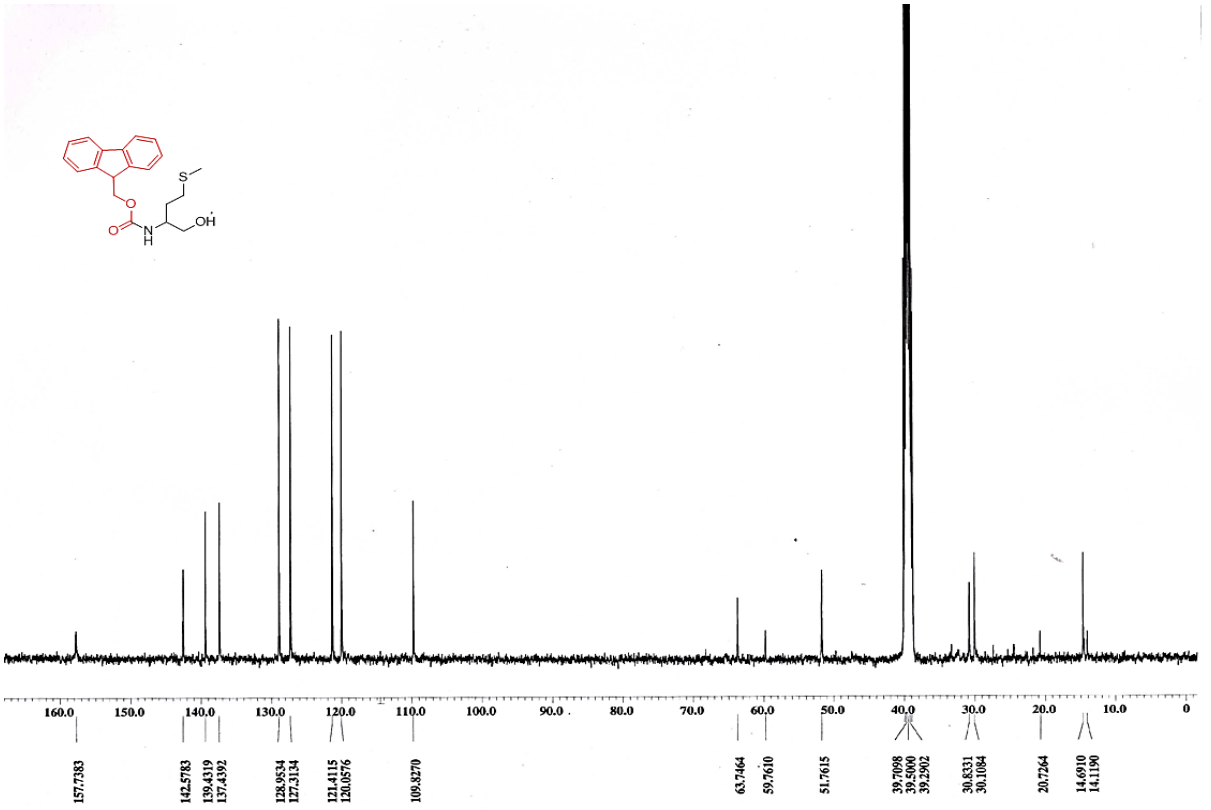
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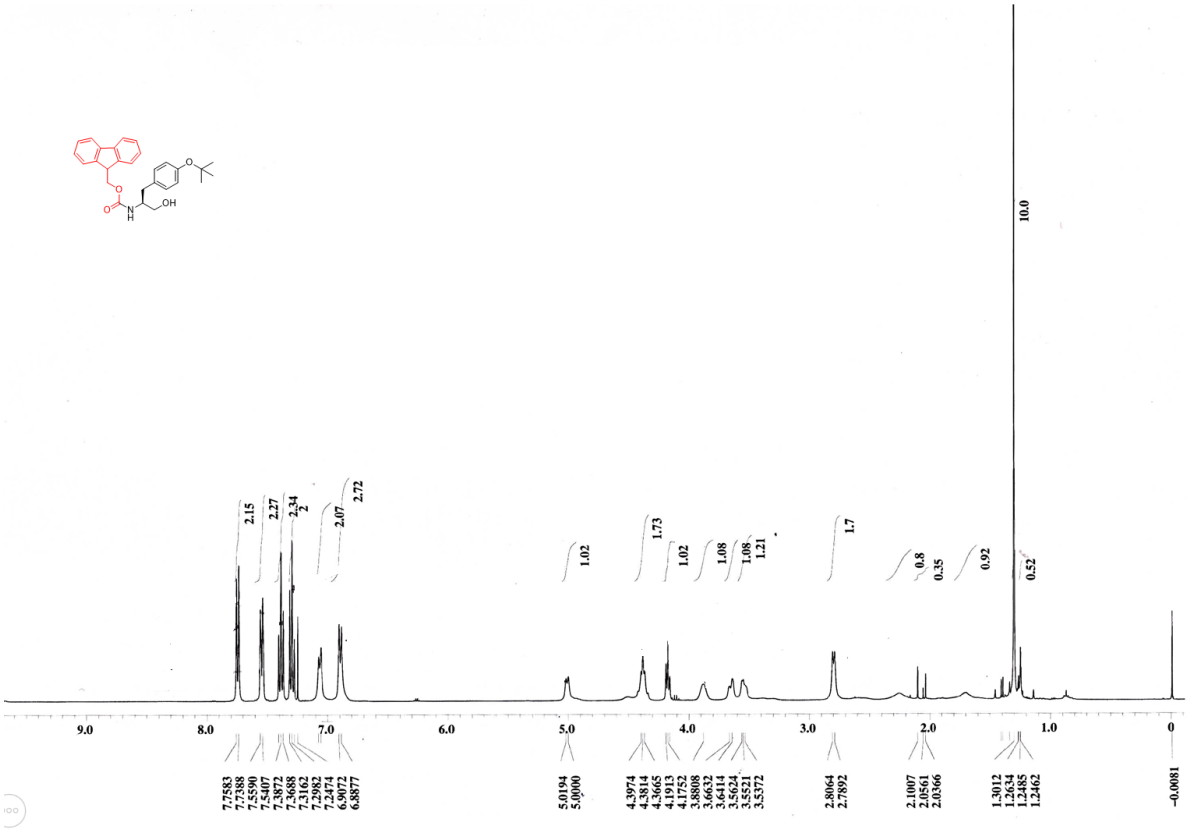
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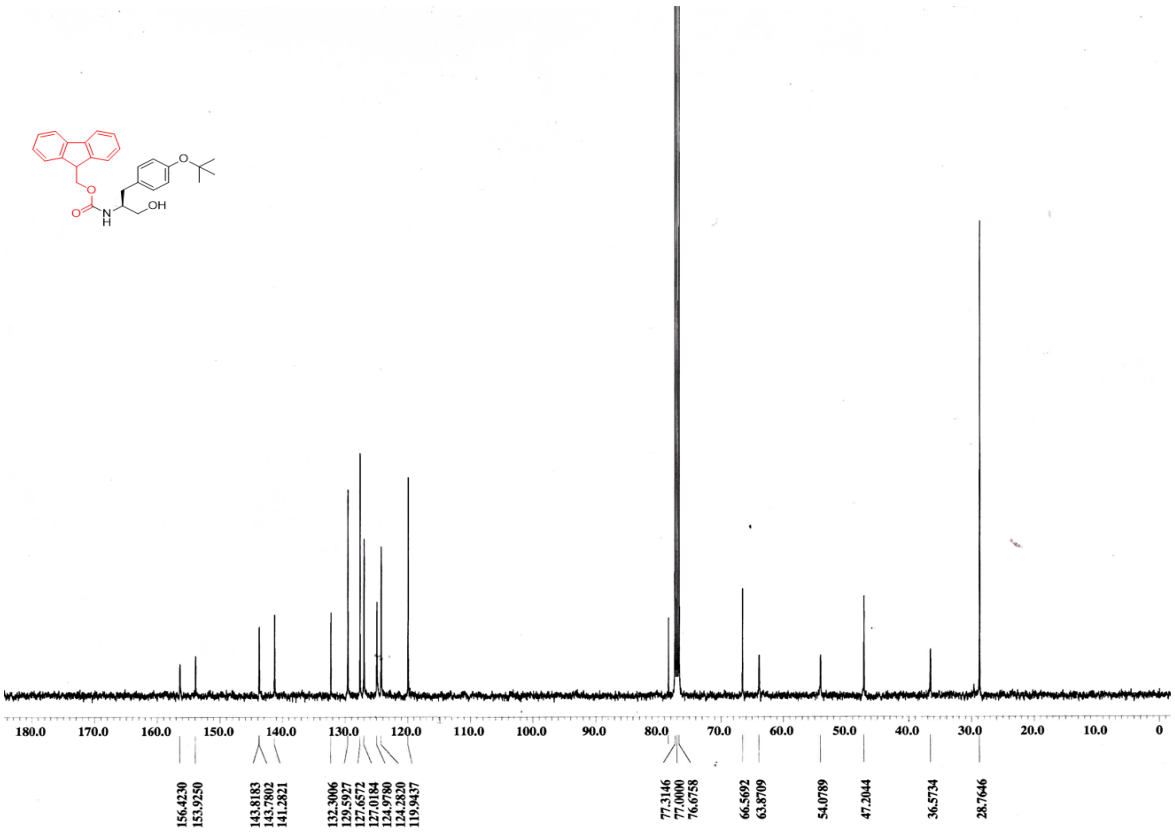
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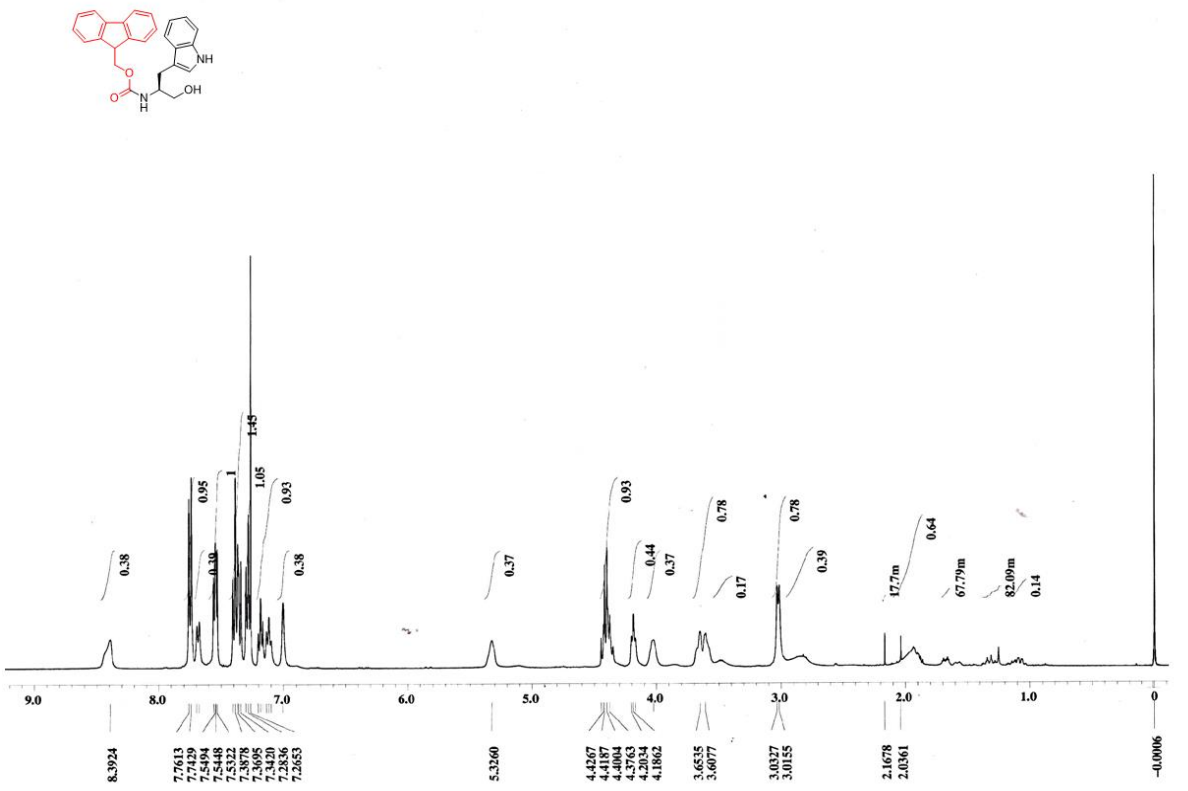

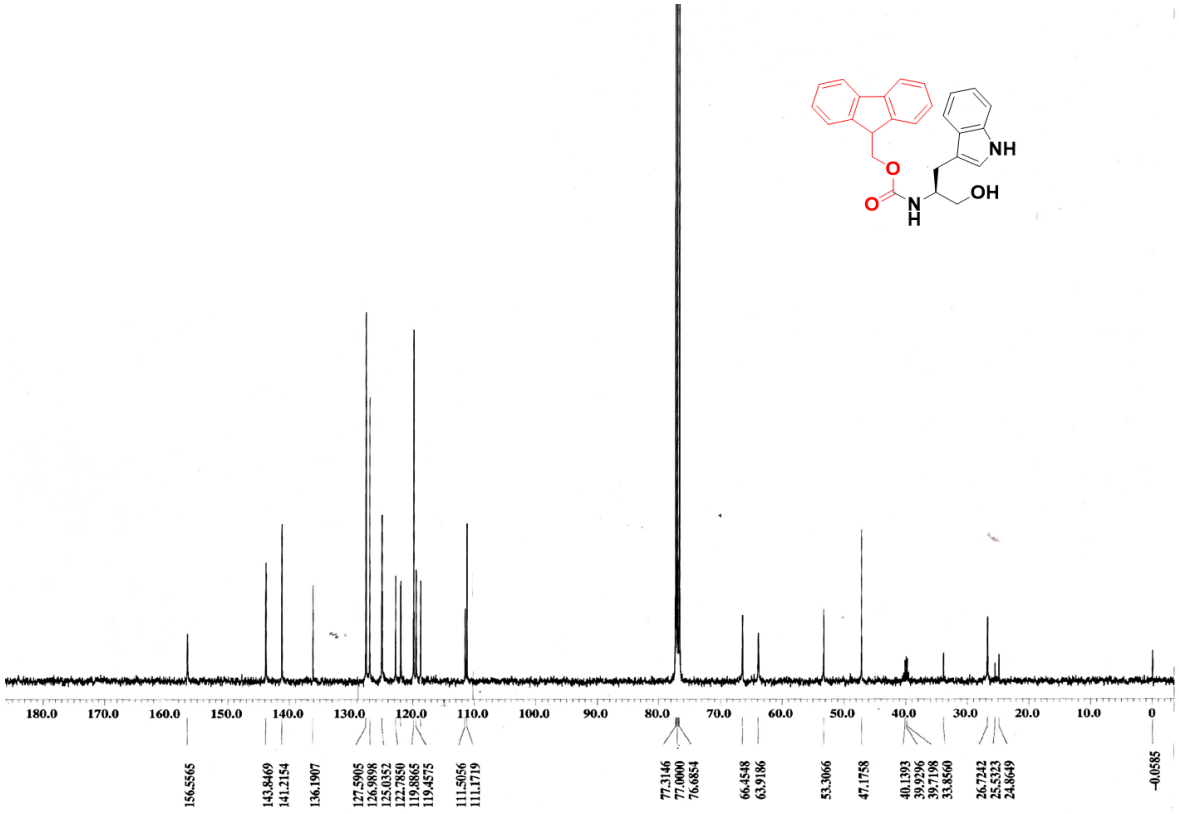

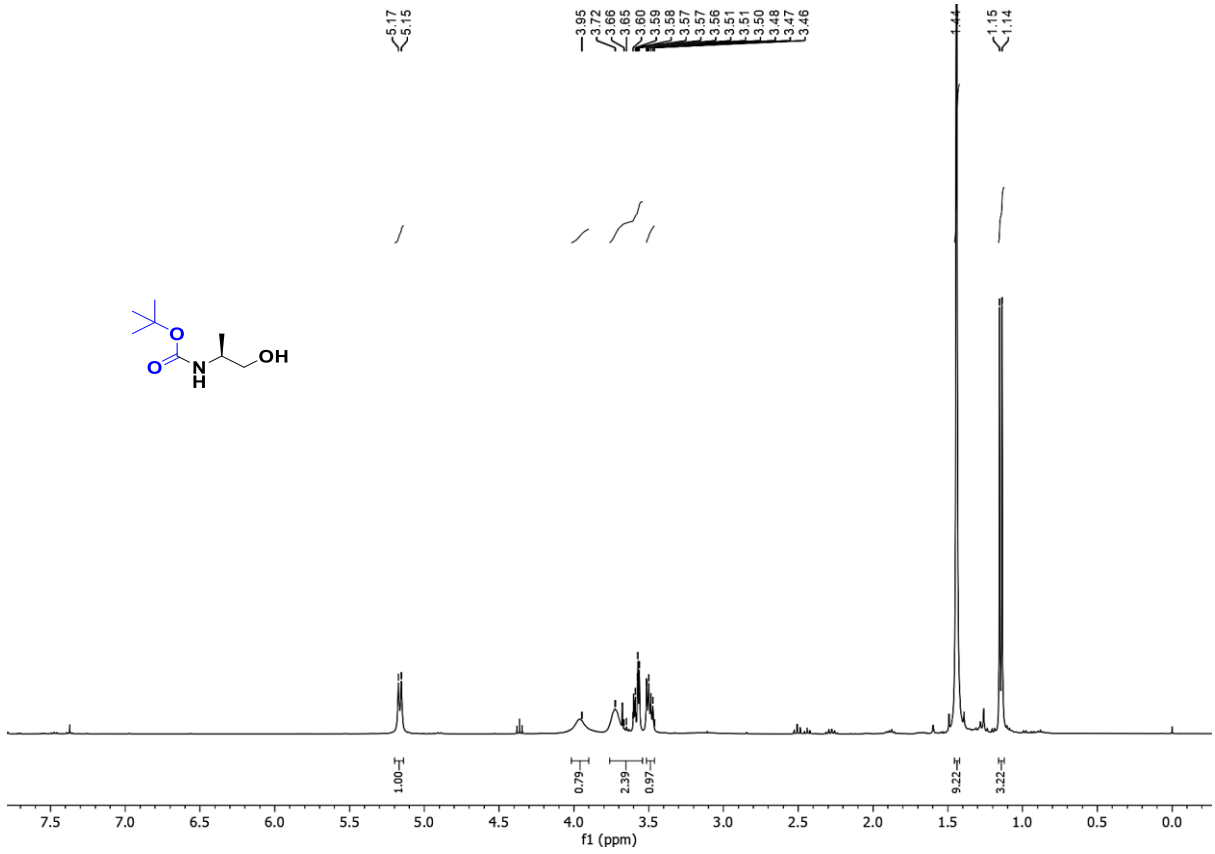
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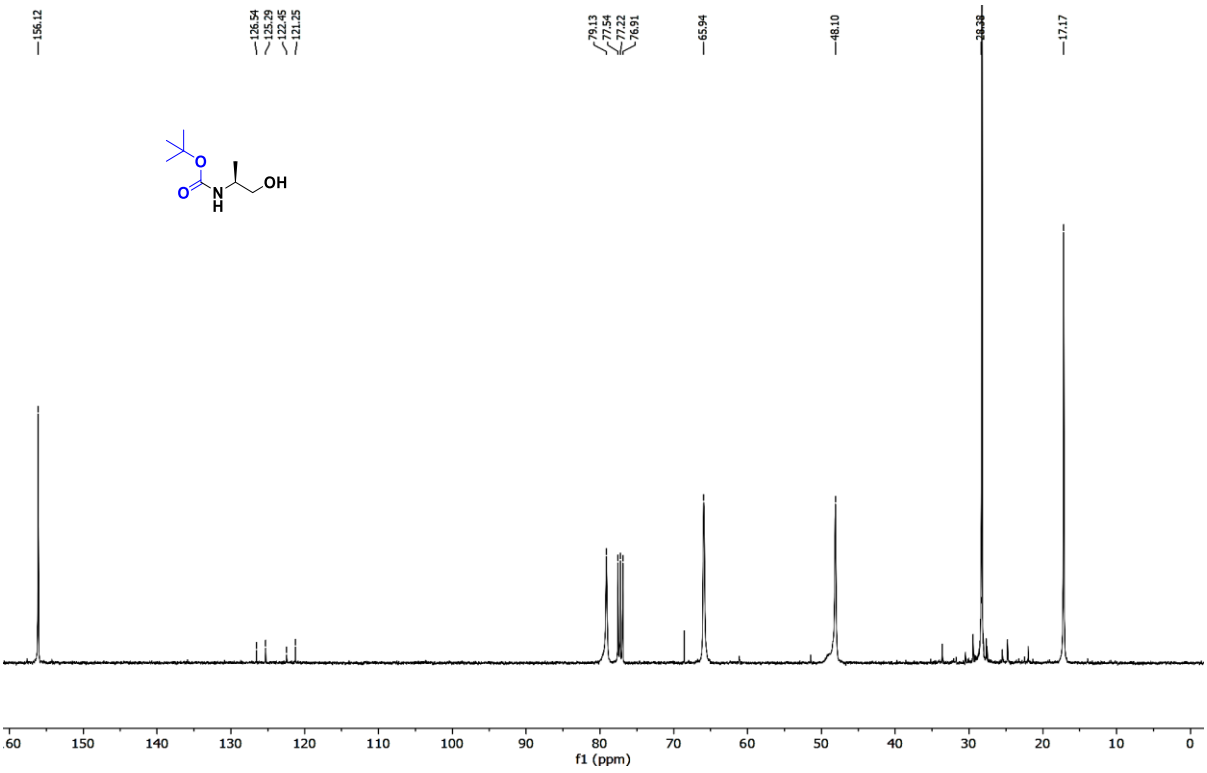
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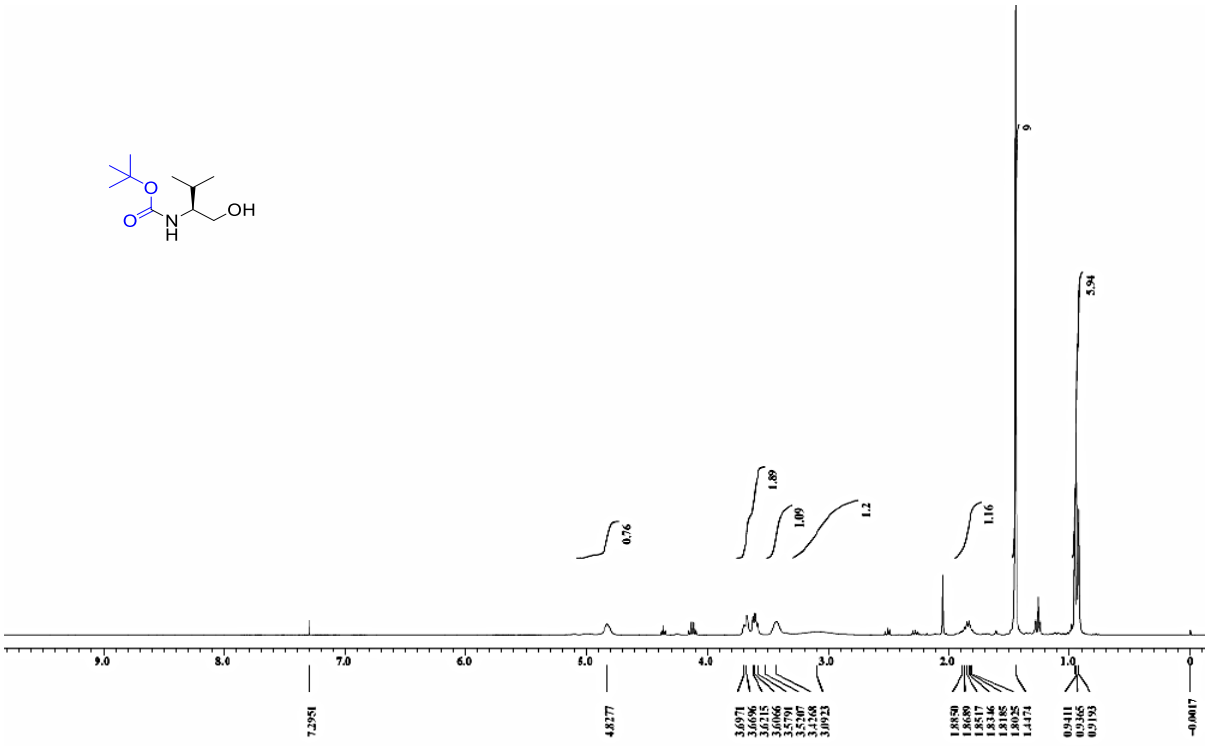
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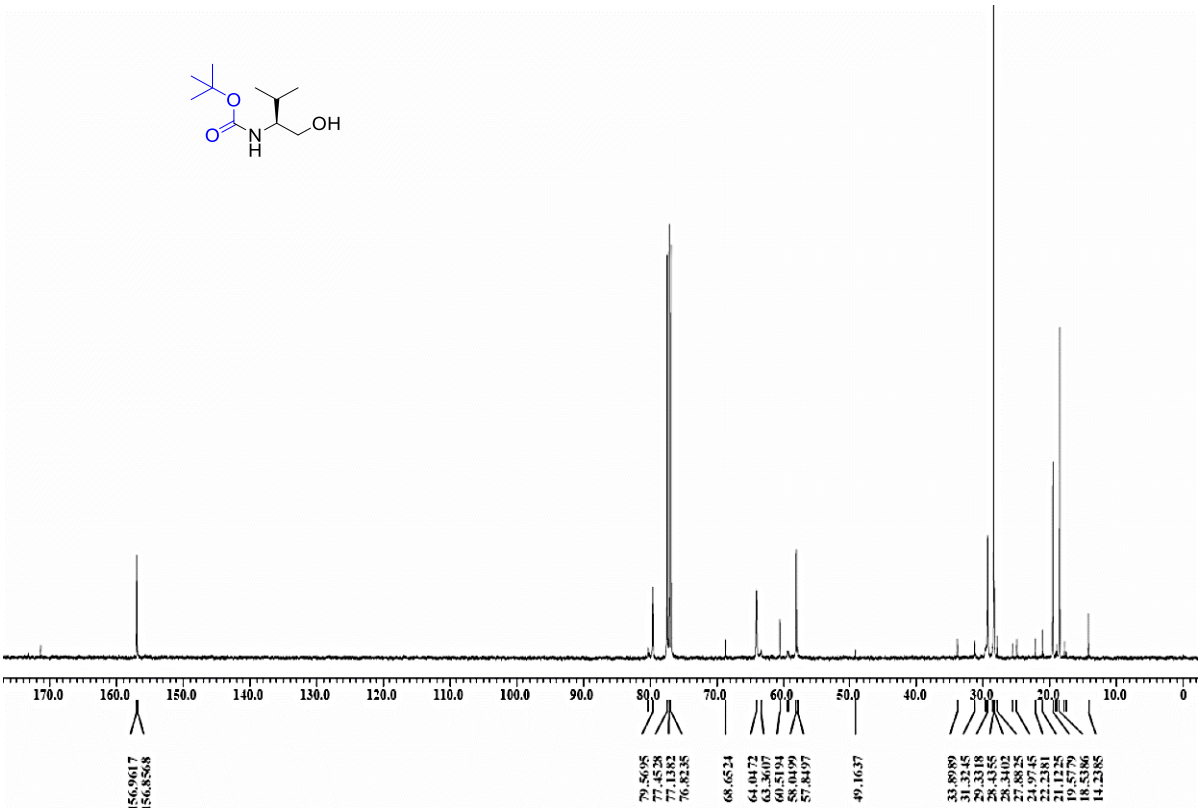
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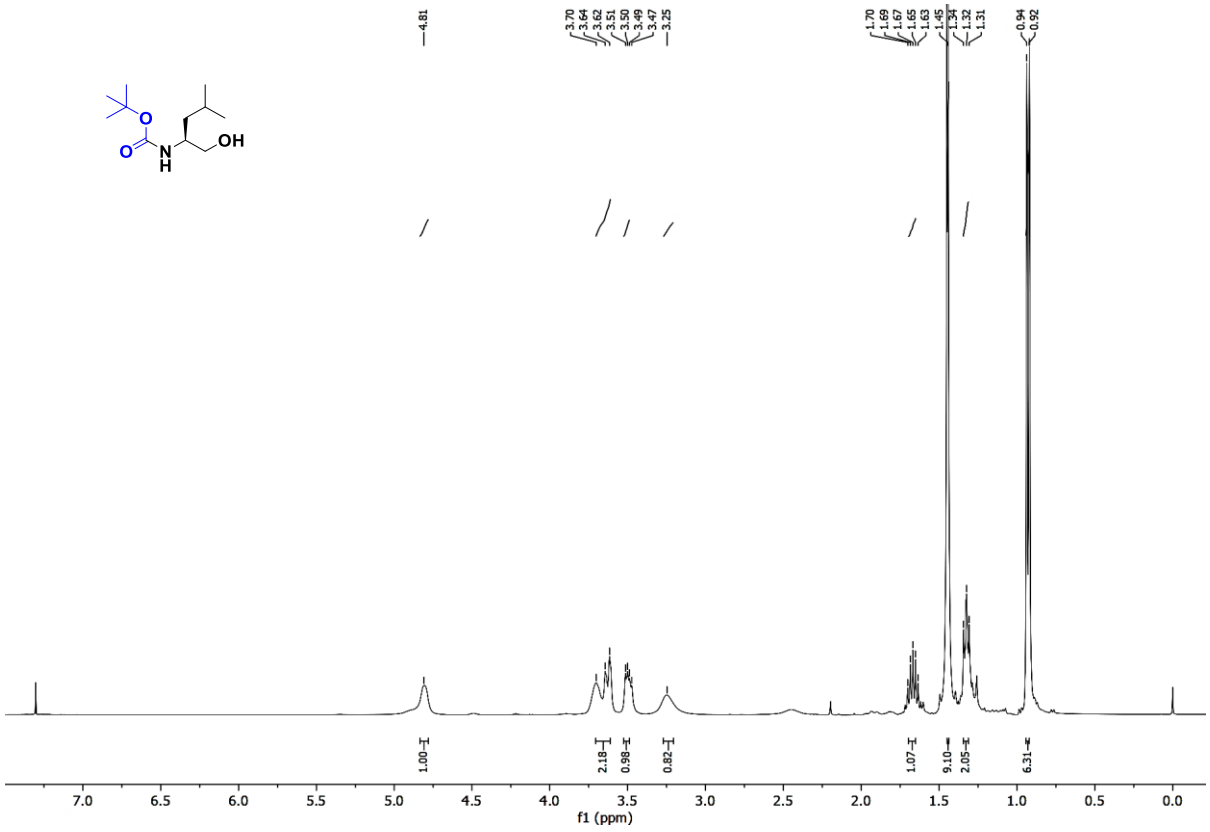
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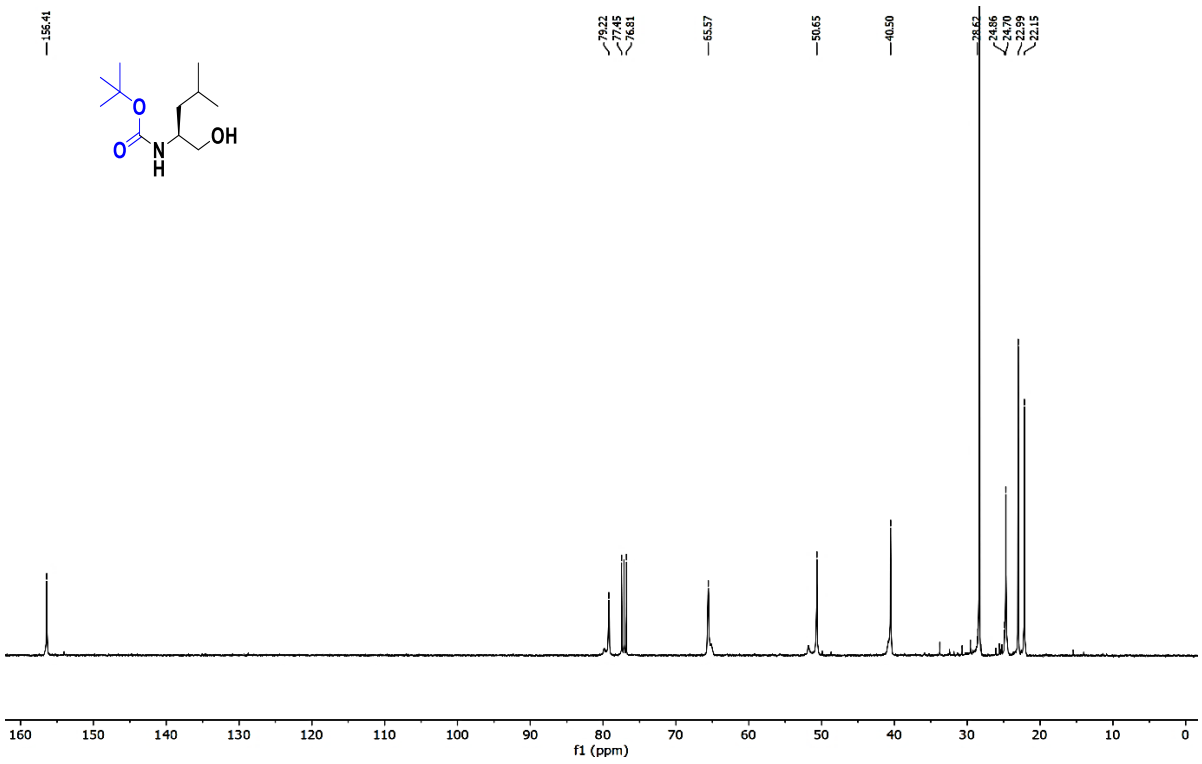
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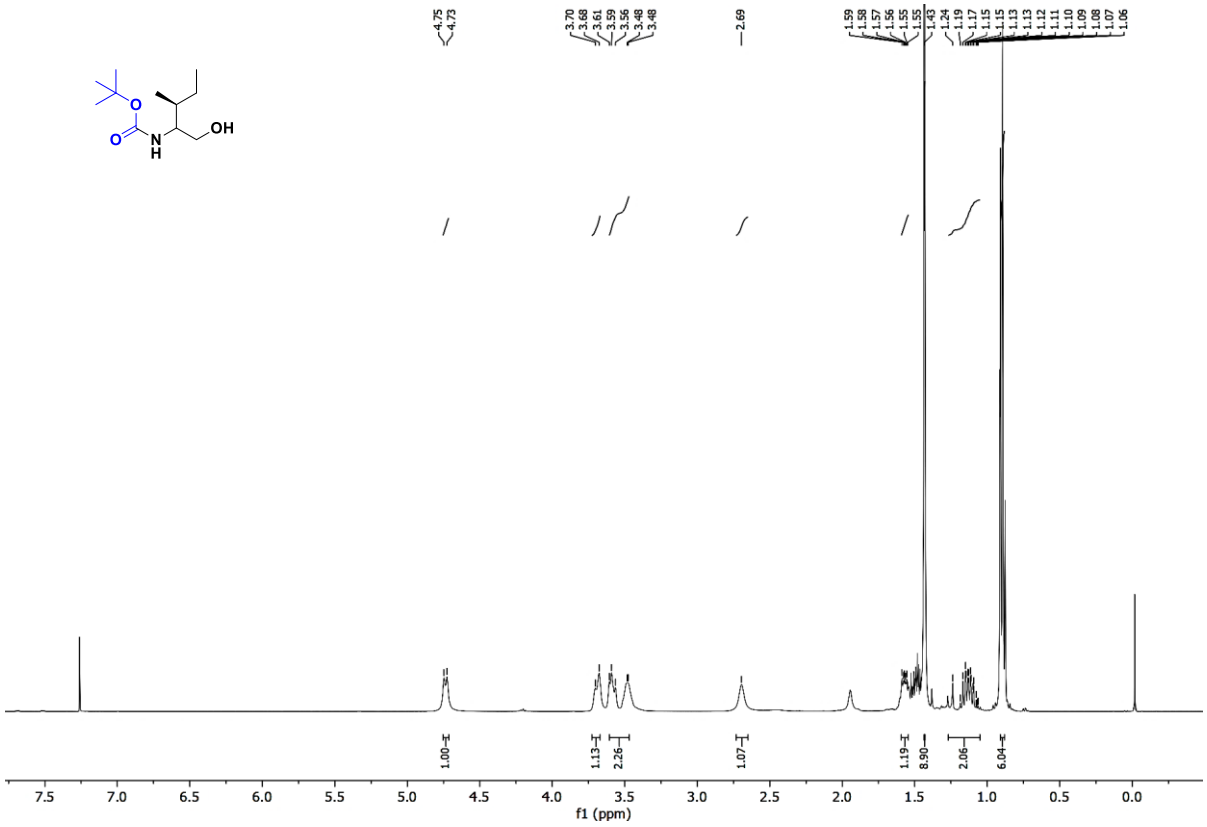
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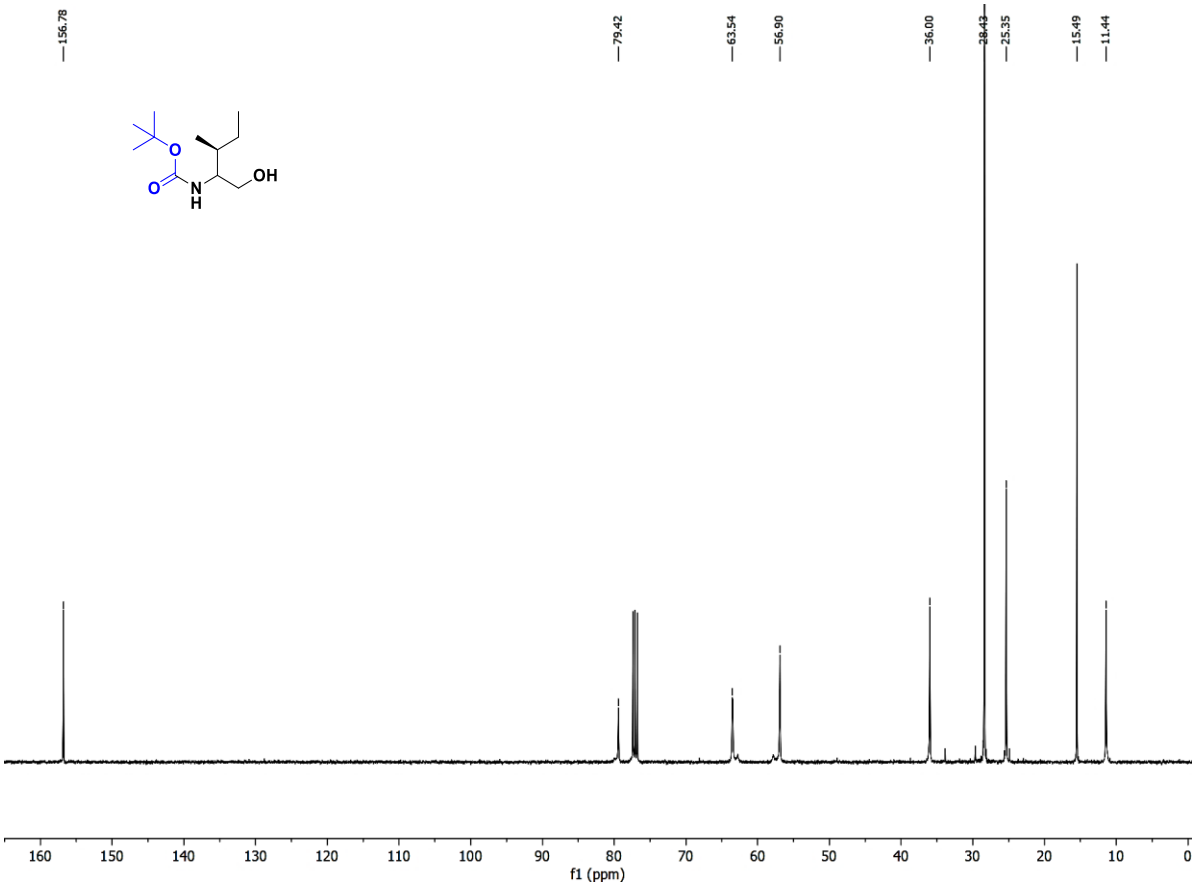
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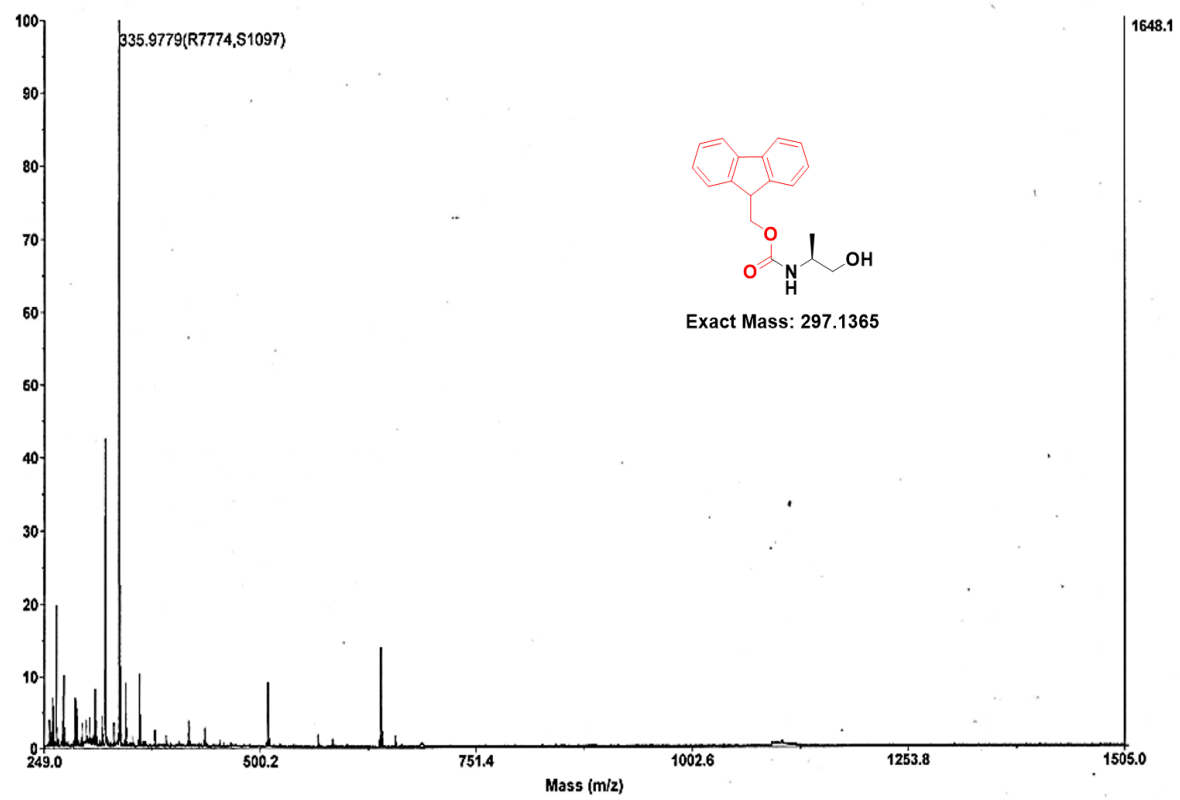
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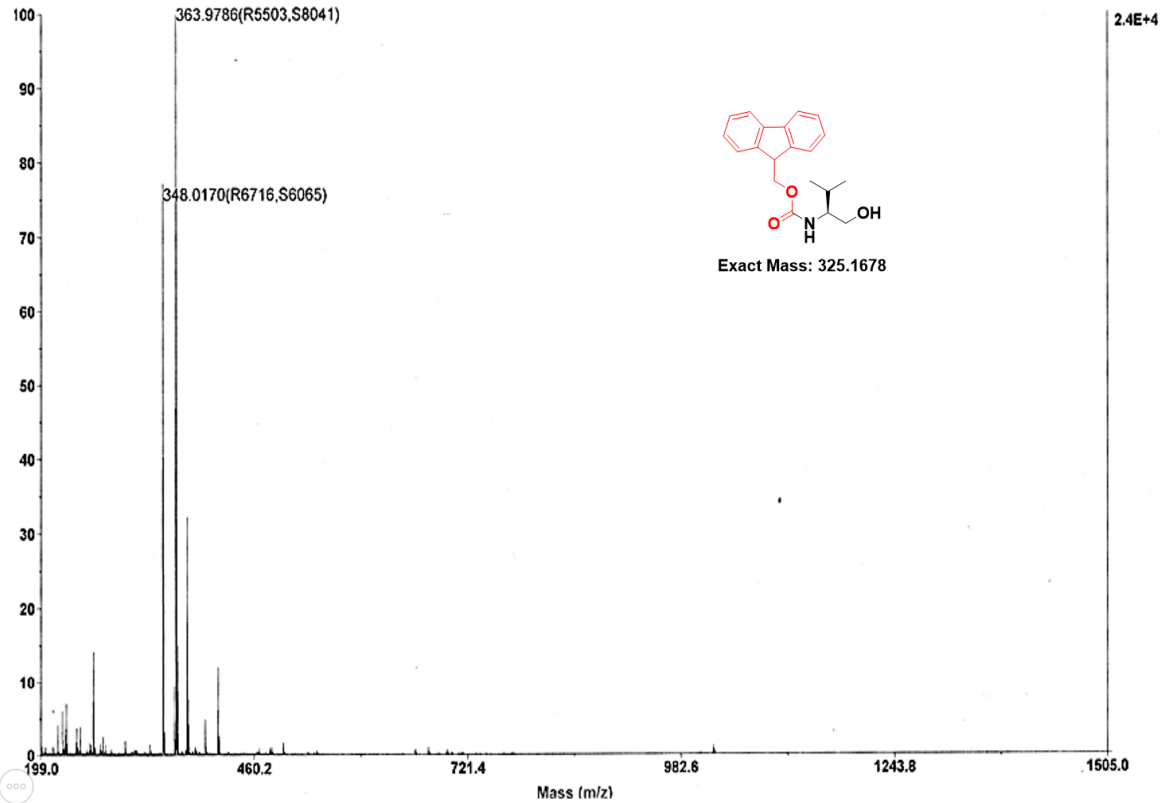
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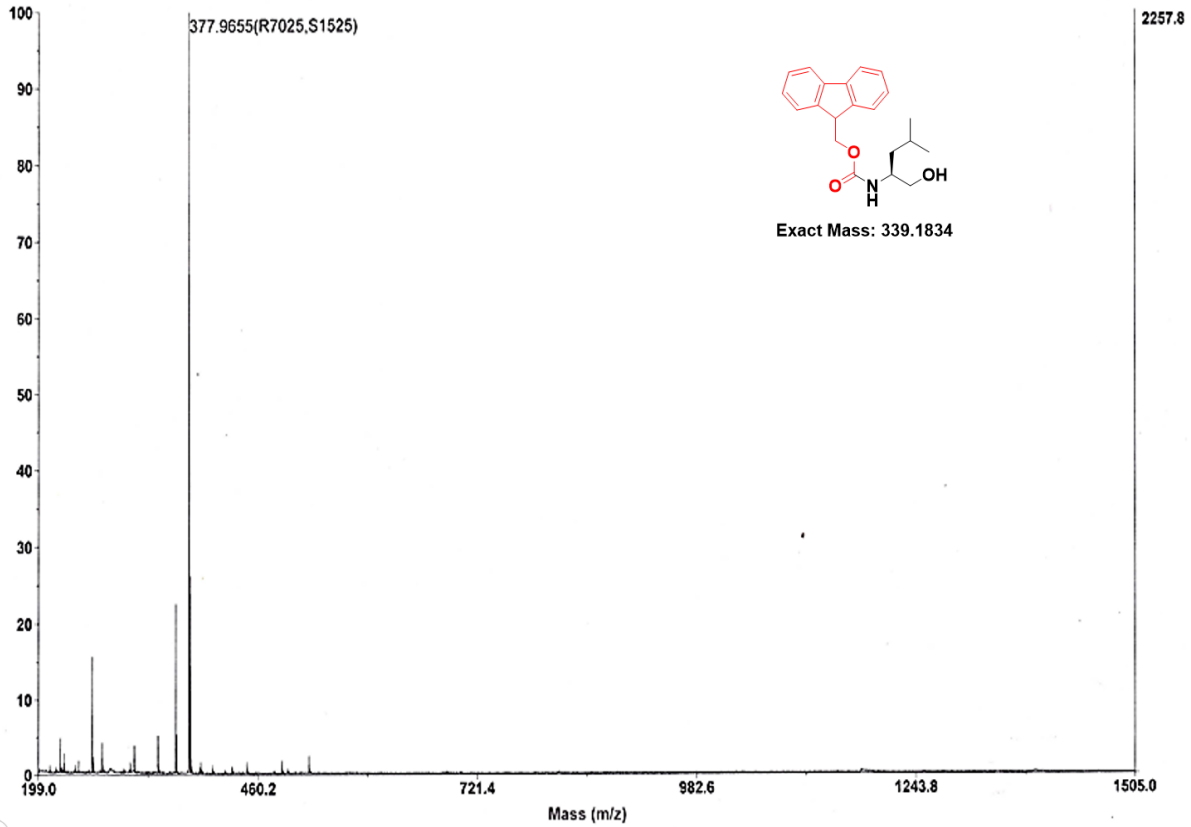
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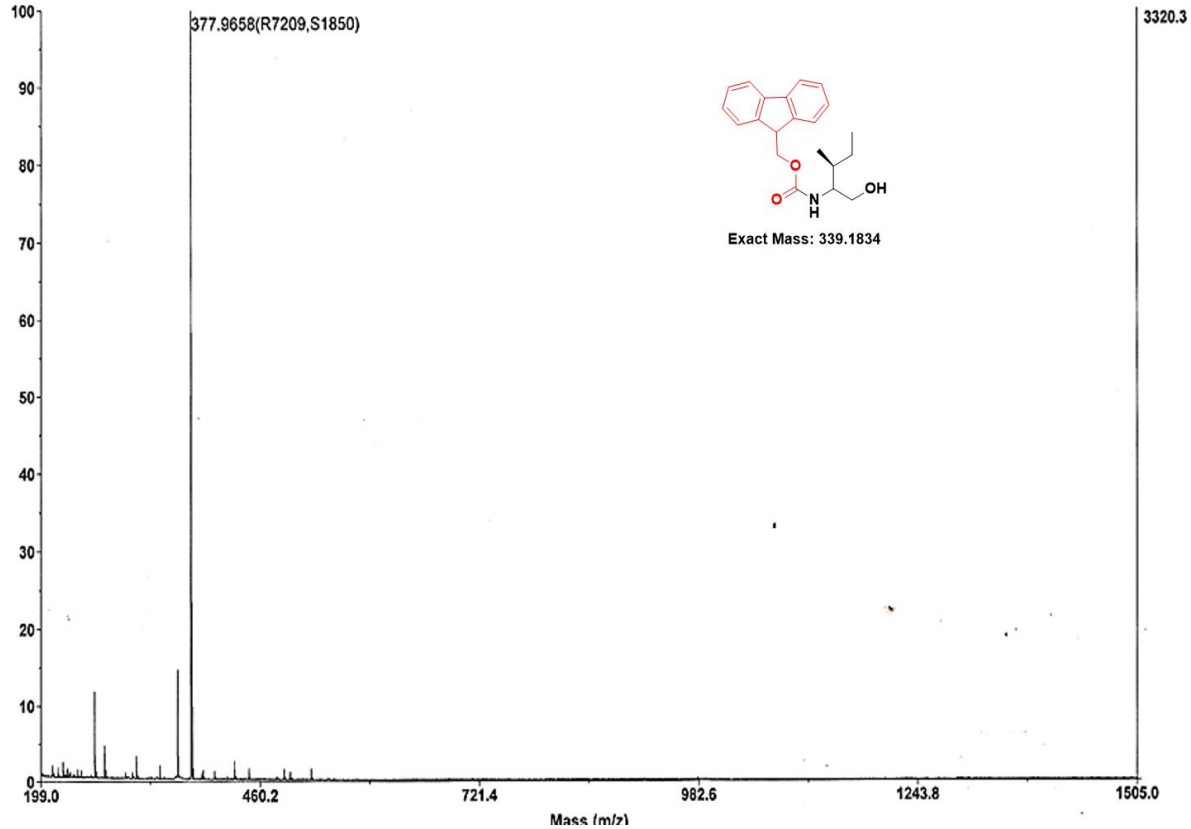
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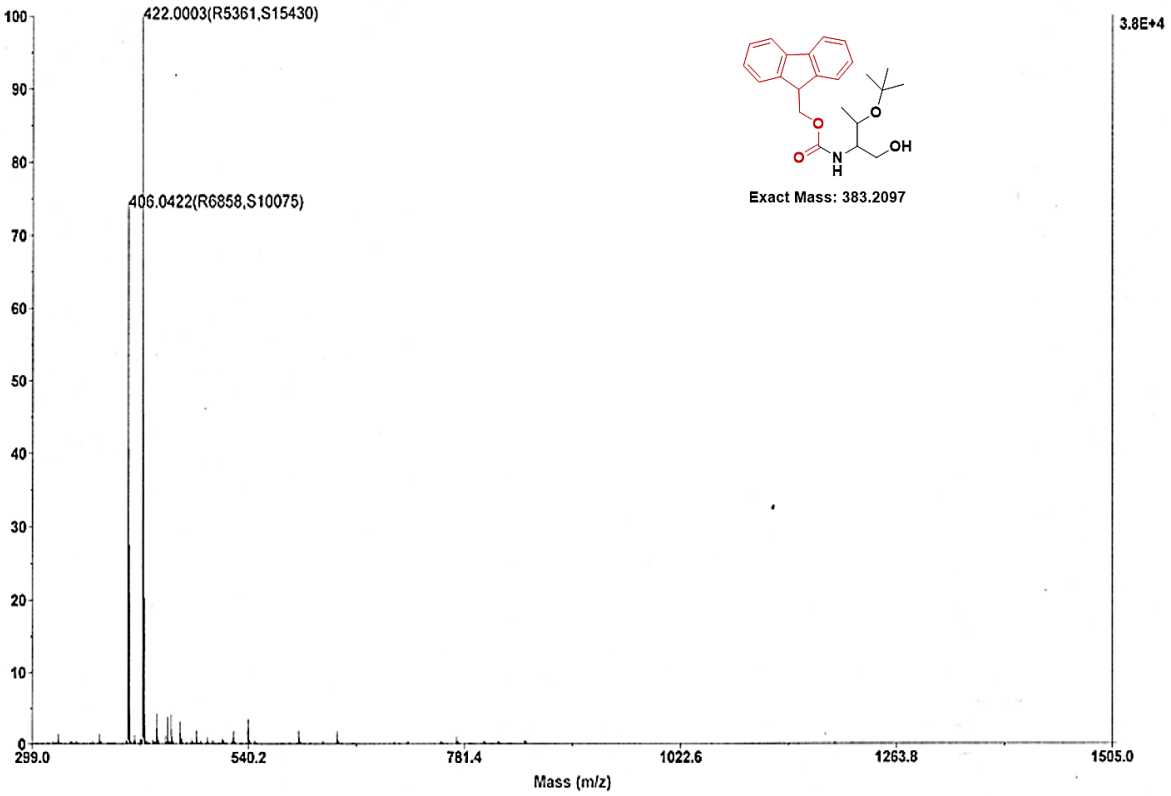
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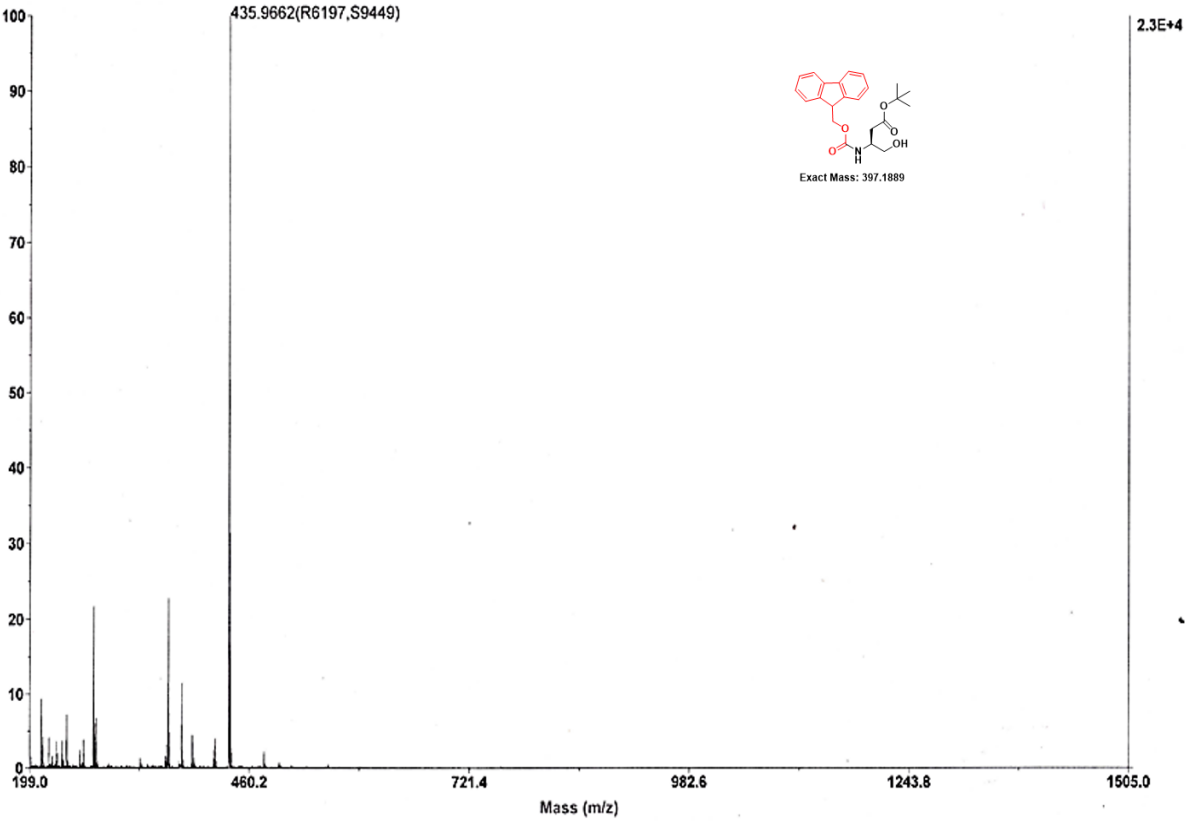
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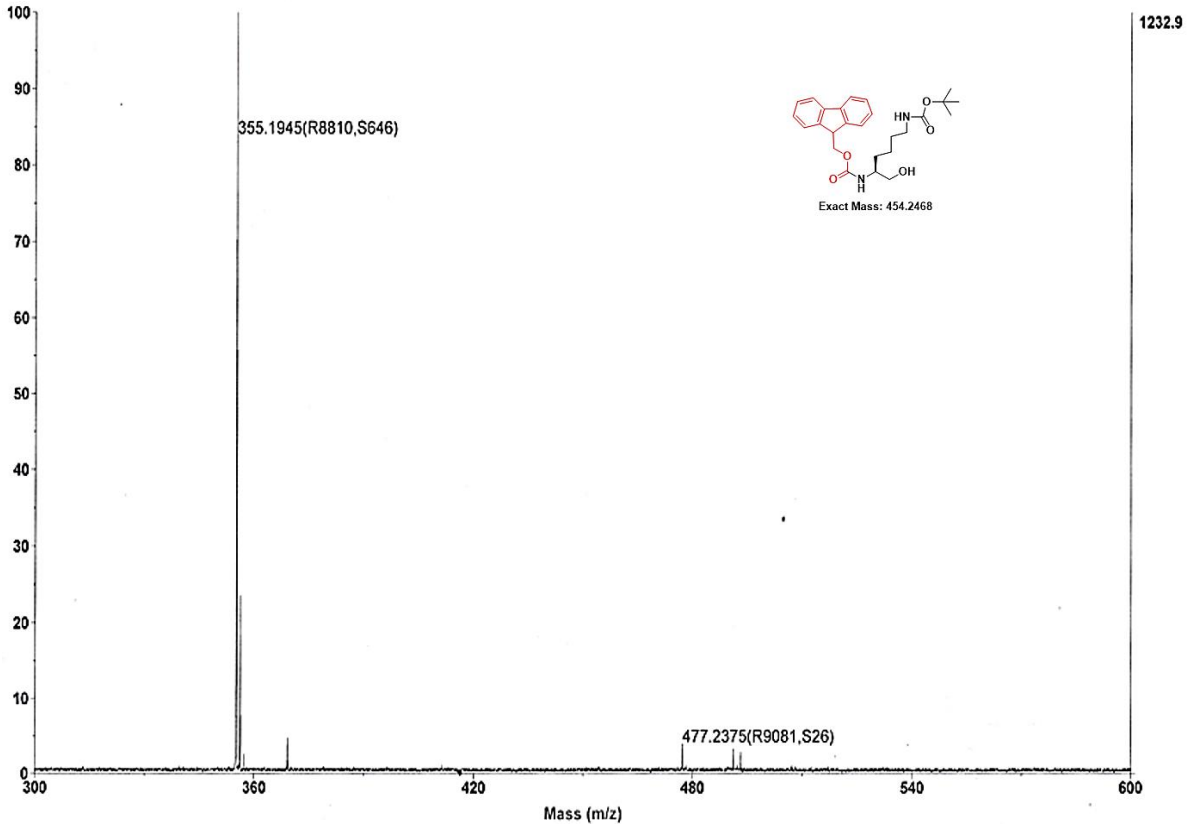
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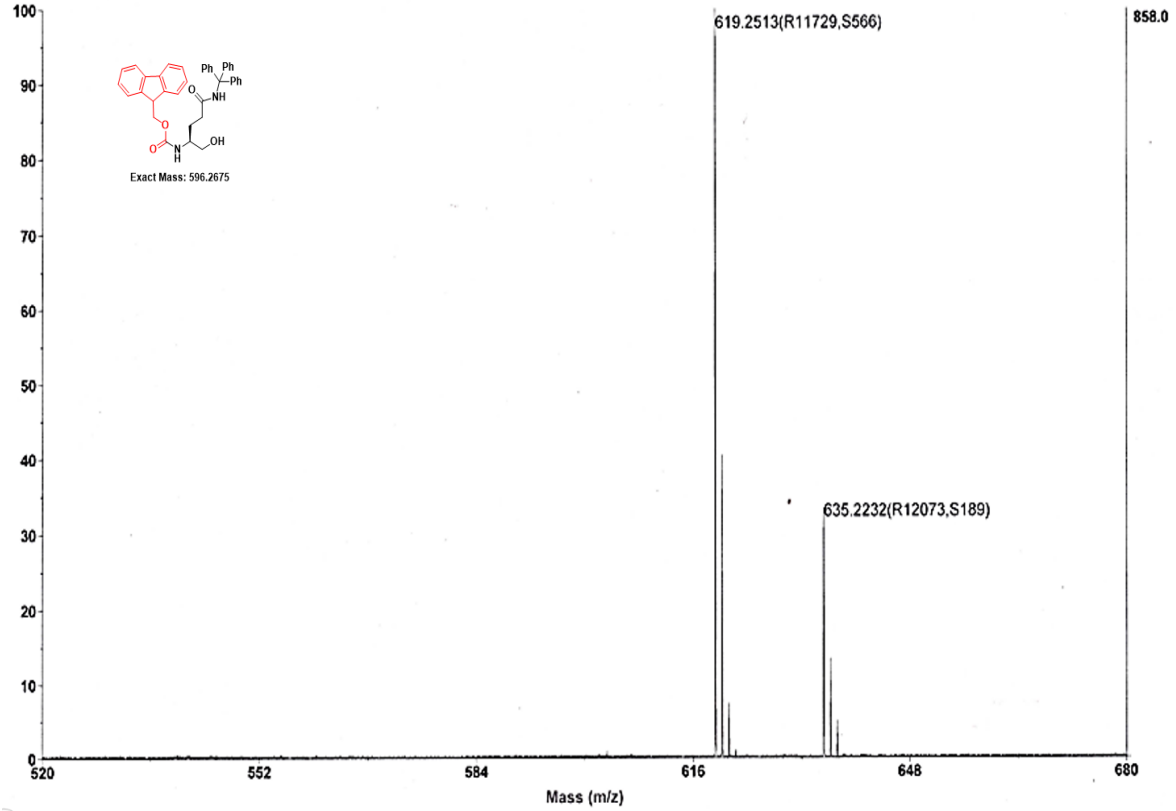
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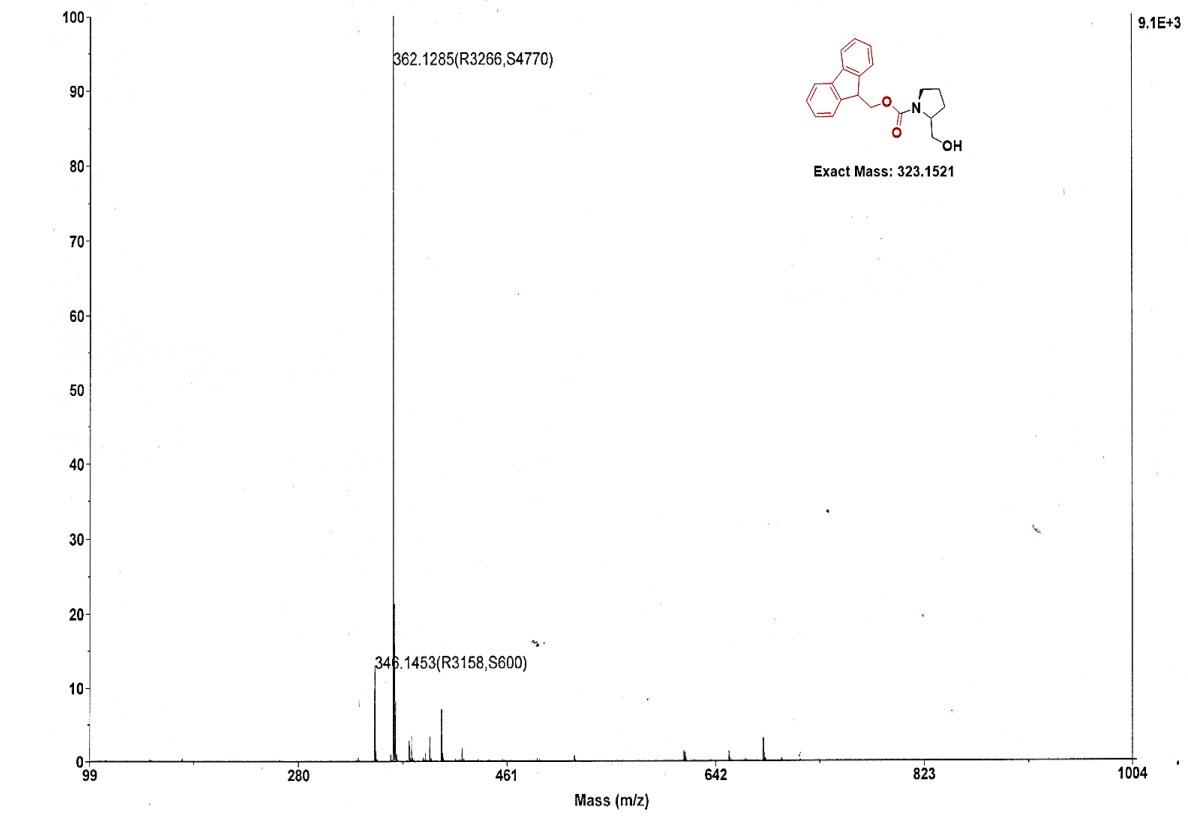
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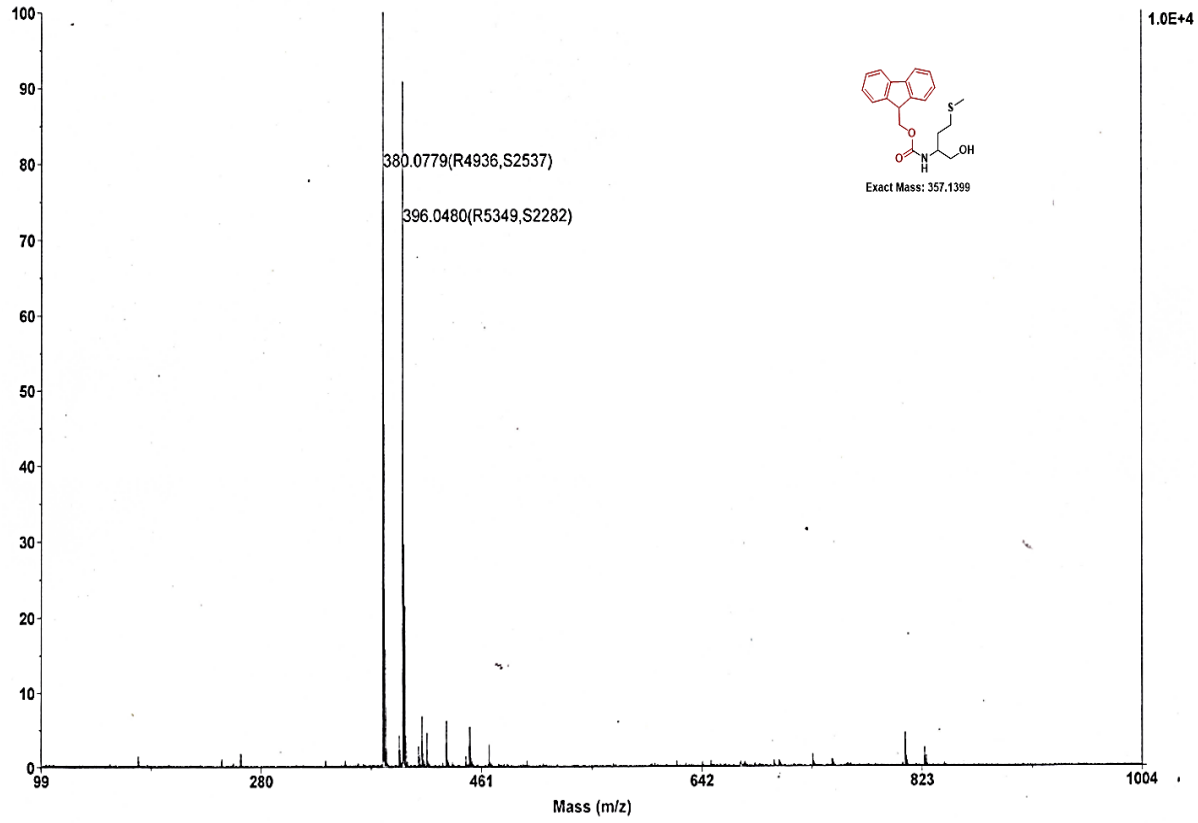
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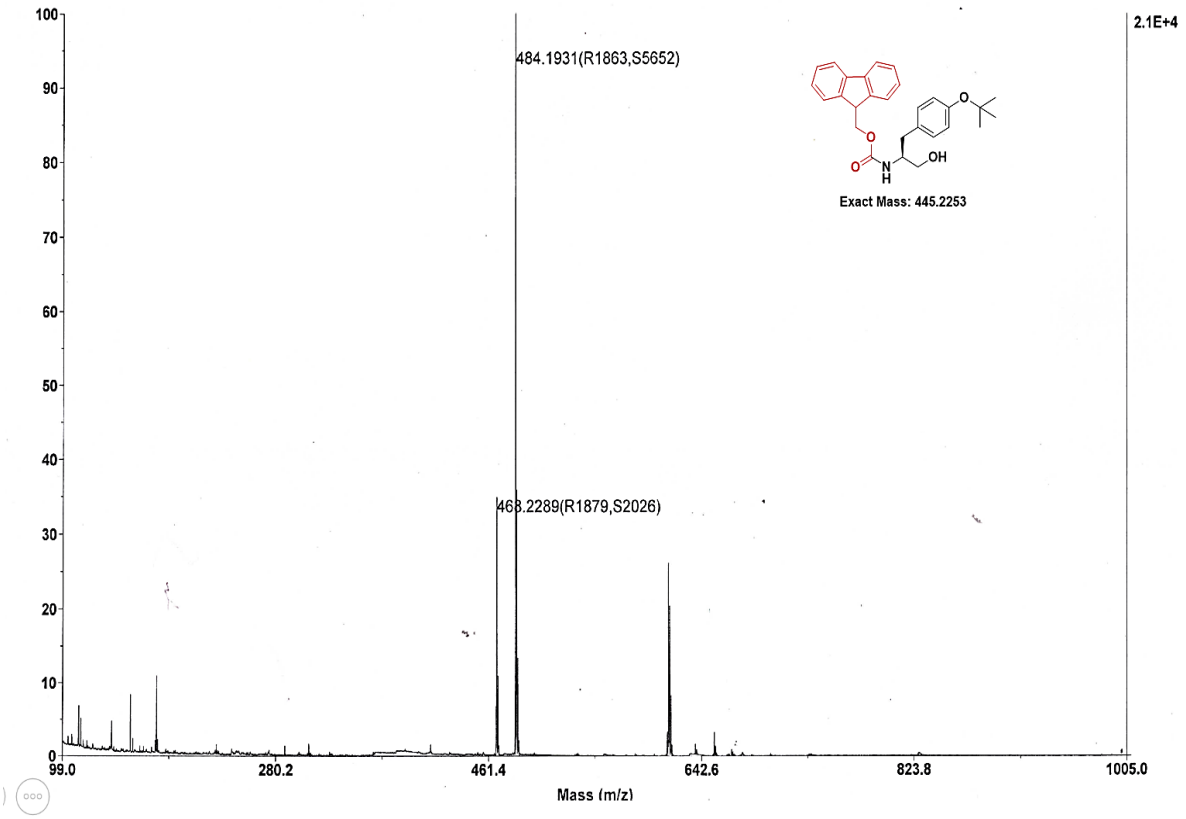
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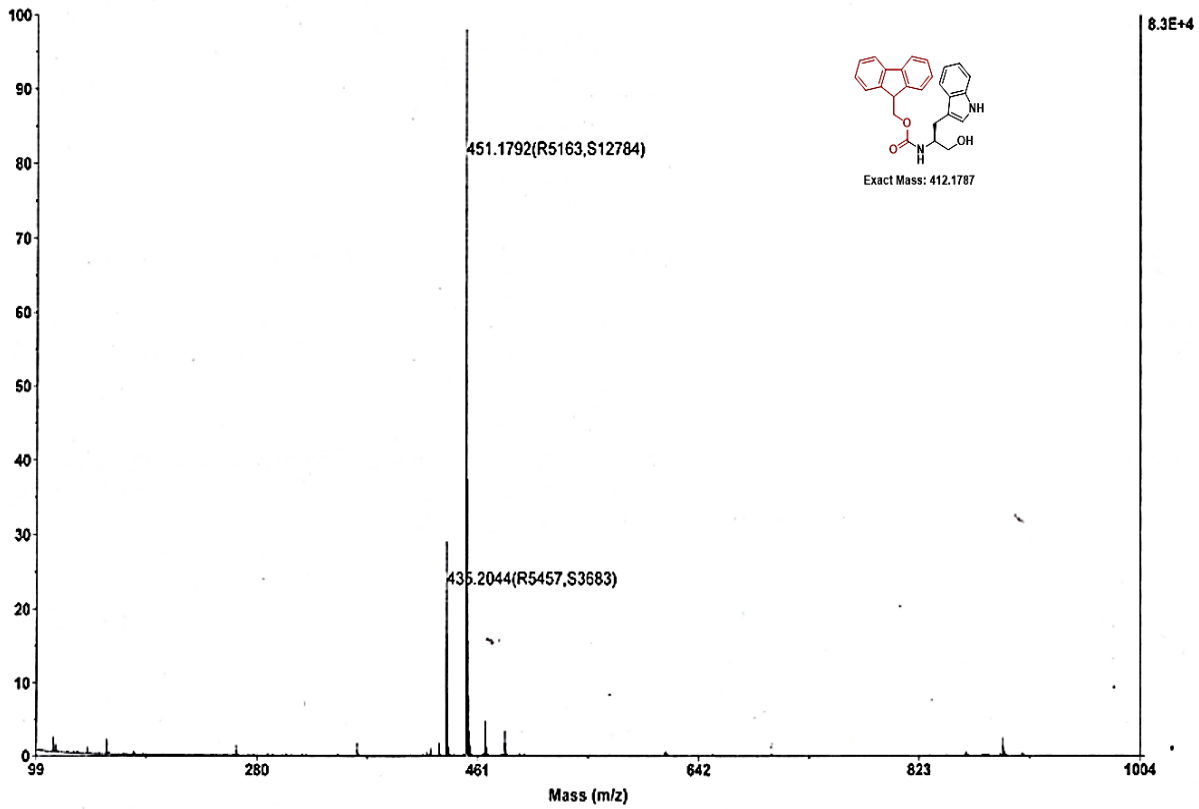
Mass (m/z)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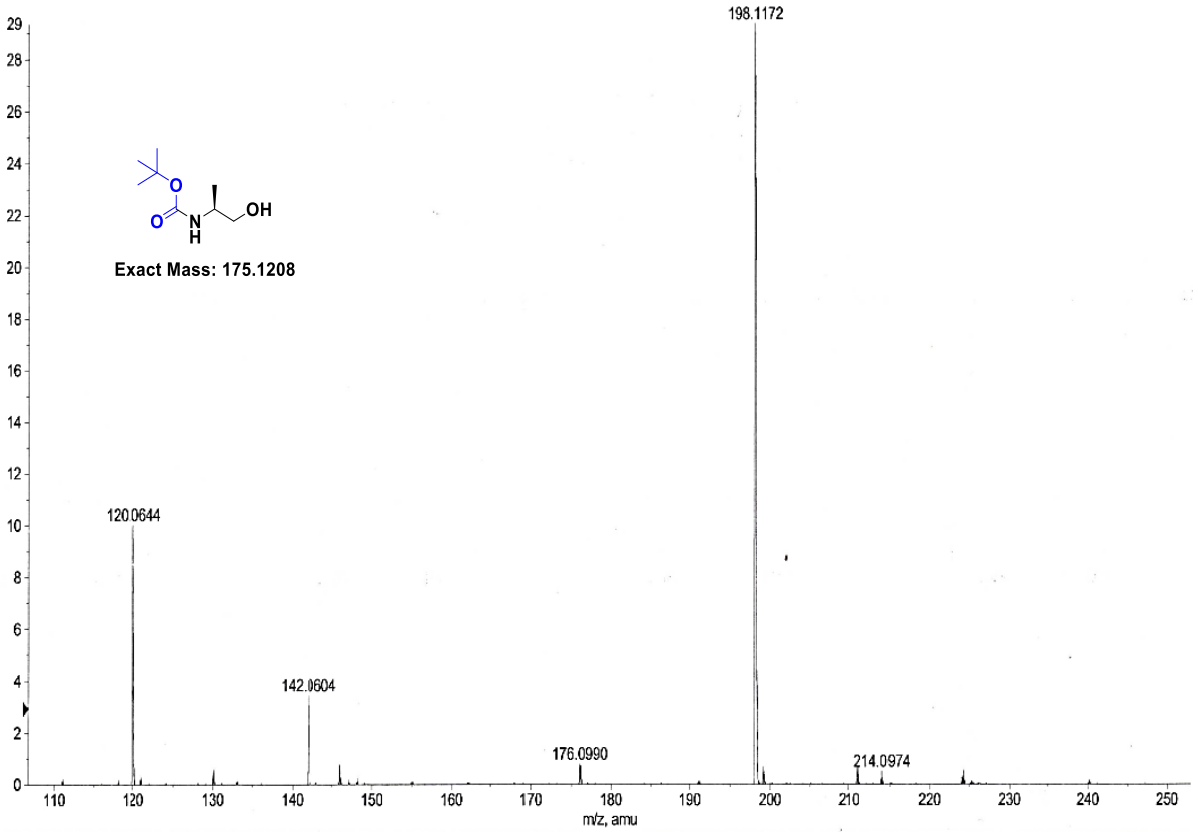
Mass (m/z)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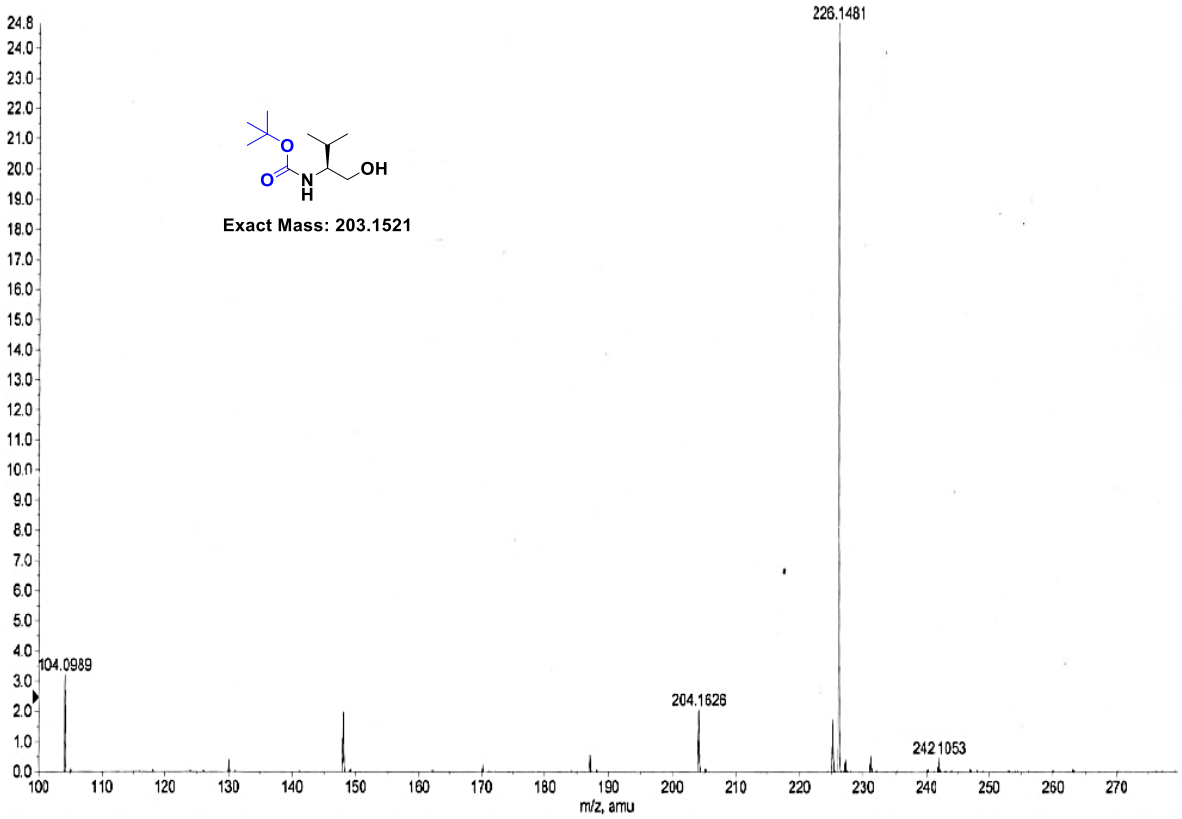
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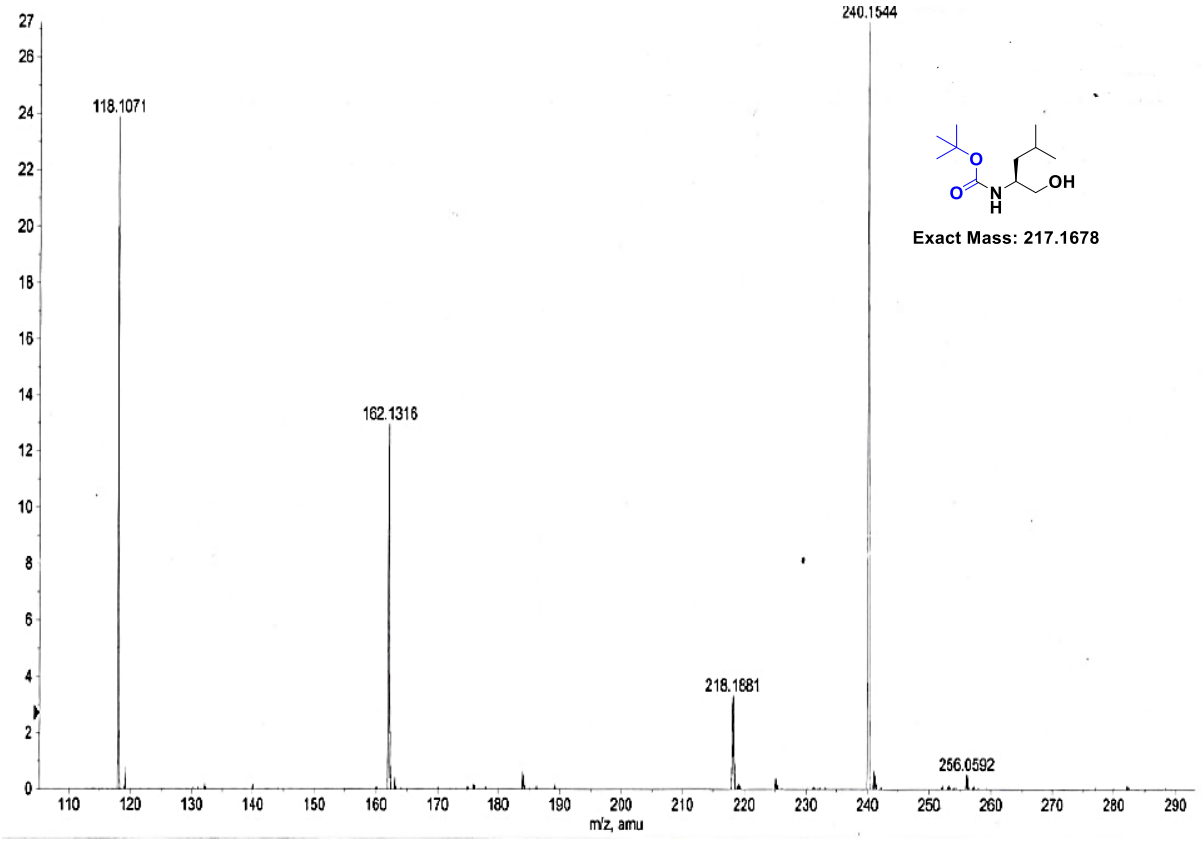
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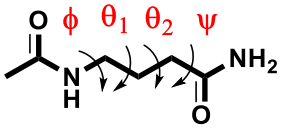
Chapter 3(b)

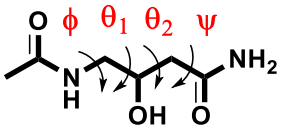
Exploration of naturally occurring β-hydroxy γ-amino acids in the design of foldamers

2b.1 Introduction

β-Hydroxy γ-amino acids also called statines are naturally occurring non-coded or non-ribosomal amino acids. A variety of natural cyclic and acyclic peptides composed of statine residues have been isolated and studied for their biological activities.[1] The presence of hydroxyl functional group at the beta-position is the major difference between the β-hydroxy γ-amino acids to other γ-amino acids.



γ-amino acid



β-hydroxy γ-amino acid

Over the years, it has been demonstrated that γ-amino acids can be utilized to design protein secondary structure mimetics. The natural α-helix can be represented as C_{13}-helix. The homo-oligomers of γ-amino acids are shown to adopt C_{14} and C_9-helical conformations, where C represents the pseudo cycles of H-bonds and number represents the total atoms involved in the hydrogen bonding pseudo cycles. In their pioneering work, Seebach and coworkers[2] and Hanessian and colleagues[3] demonstrated that backbone flexible oligomers of γ^4-amino acids tend to adopt C_{14}-helical structures. In addition, computation studies carried out by Hofmann and colleagues suggested that homo-oligomers of unsubstituted γ-residues will be able to form two types of helical structures such as C_9 and C_{14}.[4] Balaram and colleagues demonstrated C_9 and C_{14} helical conformations in single crystals from the oligomers of 3,3-disubstituted γ-amino acids ($\gamma^{3,3}$) and 4-substituted γ-amino acids (γ^4), respectively.[5] Polypeptides composed of complete γ^4-amino acids preferred to adopt C_{14}-helix, while the γ-peptides composed complete 3,3-dialkyl substituted γ-amino acids preferred to adopt C_9-helical conformation. Further, Smith and colleagues reported the parallel beta-sheet sort of structures in the single-crystal using short oligomers of cyclopropane γ-amino acids.[6] The short parallel β-sheet structures are stabilized by inter-strand H-bonds. Besides the regular H-bonds, these β-sheet structures are also involved in CH---OH bonds.

In continuation, the oligomers of α,β-unsaturated 4,4-disubstituted γ-amino acids ($\gamma^{4,4}$) have shown to adopt β-double helical conformations in single crystals.[7] In the case of β-double helical structures, the two strands are intertwined and the structure is stabilized by inter-strand

H-bonds. The structure of γ-peptides homo-oligomeric foldamers C_9, C_{14} and β-double helices are shown below in Figure 1.

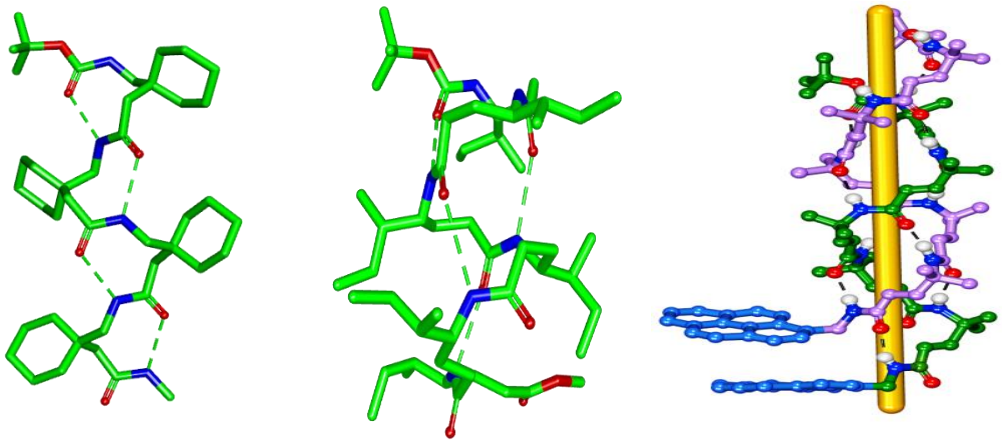


Figure 1: Gamma peptides homo-oligomeric foldamers (a) C_9 helix,[5] (b) C_{12} helix[5] and (c) β-double helix.[7]

In addition to the homo-oligomers, incredible efforts have been made in the literature to know about the conformational properties of γ-amino acids in the hybrid peptide sequences composed of α, β and γ-amino acids. Using theoretical calculations Hofmann and co-workers have predicted the various helical types available to the oligopeptides composed of alternating α and γ-amino acids in 1:1 ratio.[8] Among these helical types, they suggested 12-helix is the most stable conformation for the hybrid peptides composed of 1:1 alternating α and γ- amino acids. The 12-helix structure is stabilized by the 4→1 H-bonds between the residues *i* and *i+3*. In addition to 12-helix, these α,γ-hybrid peptides have also been reported to adopt 12/10 or 18/20 helical conformations. Interestingly, the 12/10 helices are mixed helices consisting of opposite directionality of the alternating intramolecular H-bonds along the helix.

In their exceptional work, Balaram and colleagues demonstrated 12-helical conformation from the α,γ-hybrid tetrapeptide composed of alternating Aib and gabapentin ($\gamma^{3,3}$-amino acids).[9] Further, Sharma and co-workers have reported the 12/10-helical conformation in solution state of α,γ-peptides with alternating L-Ala and γ-Caa residues (C-linked carbo-γ-amino acid from D-mannose).[10] Further, the helix with 12/10-mixed hydrogen-bonding pattern was also observed in the single crystals. Gellman and colleagues have demonstrated the 12-helix and

12/10 mixed helices from the hybrid peptides of alternating α and sterically constrained cyclic γ-amino acids.[11] The helical structure of α,γ-hybrid peptides are shown in Figure 2.

In addition to the α,γ-hybrid peptides, efforts have also been made in the literature to understand the conformations of β,γ-hybrid peptides. These peptides have been shown to adopt 11-, 13-, mixed 11/13-helices. Gellman and colleagues demonstrated the 13-helical conformations from hybrid peptides with cyclic β and cyclic γ-amino acids.[12] Sharma and colleagues and others have reported 11/13-helices from β, γ-hybrid peptides.[13] Our group has shown the 11-helices from the peptides composed of β^3 and $\gamma^{4,4}$ or $\gamma^{3,3}$-amino acids.[14]

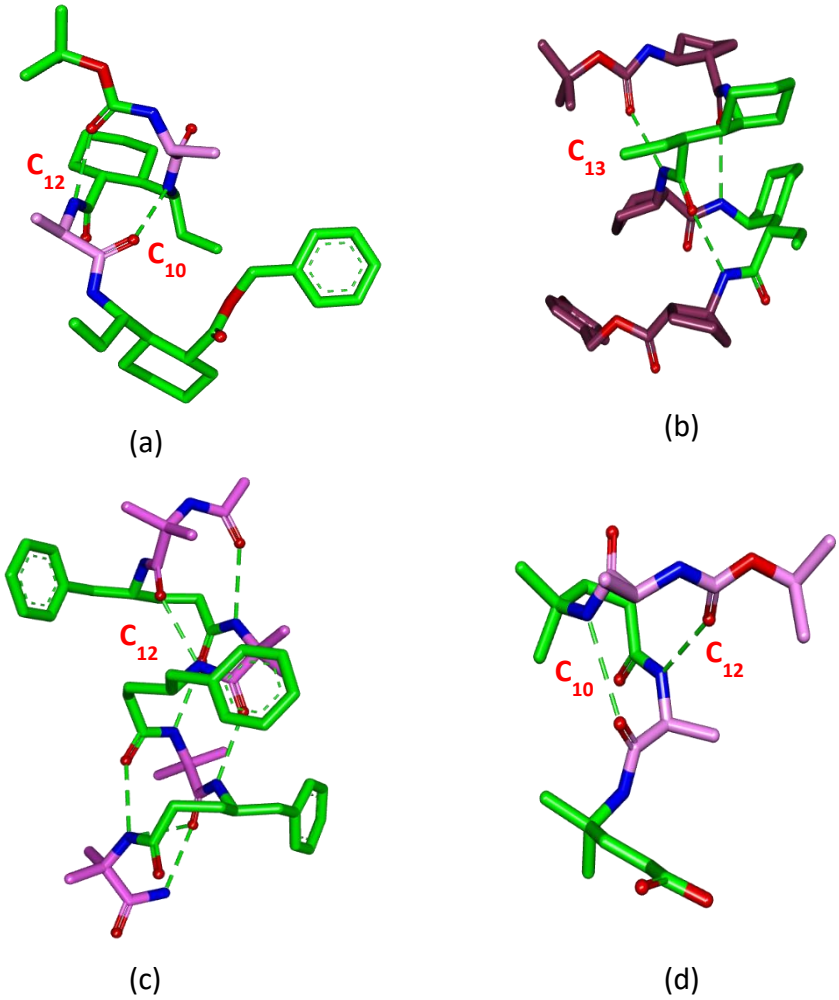


Figure 2: Hybrid peptide foldamers (a) α/(cyclic)γ - peptides 12/10 helix,[11a] (b) β/γ - peptides 13 helix,[12] (c) α/γ - peptides 12 helix[15b] and (d) α/γ - peptides 12/10 helix.[16]

Earlier work by our group

Our lab has been working in the area of peptide foldamers with the main focus on understanding the conformational properties of γ-amino acids. Our group has reported various hybrid peptides with 12-helical conformations with α and γ^4-amino acids in 1:1 ratio,[15] mixed 12/10-helical conformations with alternating α-amino acids and $\gamma^{4,4}$-amino acids[16] and 12 and 15/17-helices from sterically constrained dialkyl amino acids Aib and $\gamma^{4,4}$-amino acids.[17]

2b.2 Aim and rationale of the present work

Natural and synthetic peptides consisting of statines residues have shown a variety of biological activities, especially as inhibitors against various proteases, discussed in detail in chapter 3(b). An extension of our efforts to study the conformational behaviour of various types of γ^4 and modified γ^4-amino acids and inspired by the wide range of biological applications of statine containing peptides, we were now interested in exploring the conformational behaviour of β-hydroxy γ-amino acids containing γ-peptides and the role of an extra hydroxyl group on the peptide H-bonding pattern.

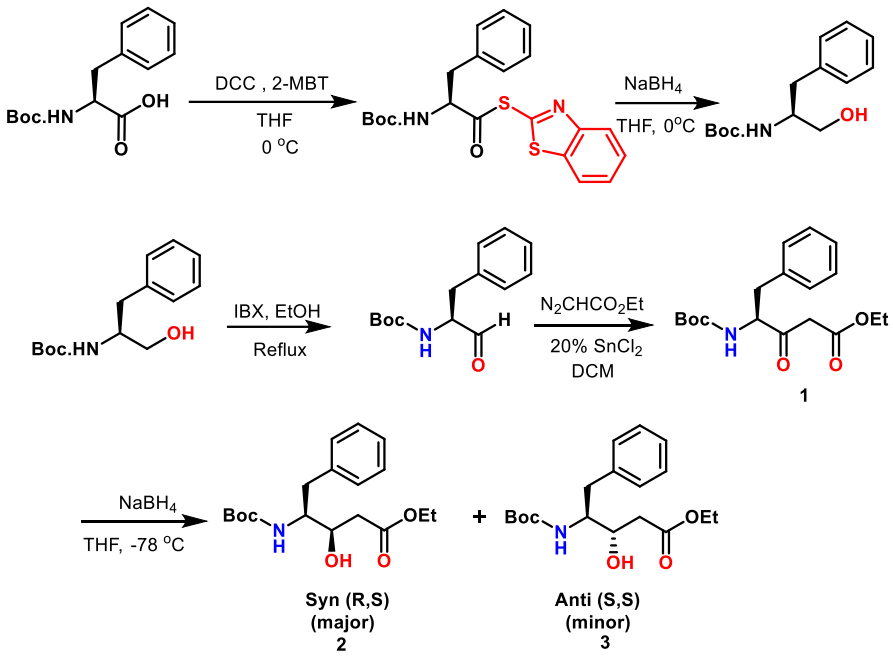
2b.3 Results and Discussion

2b.3.1 Design and synthesis of monomers

*(N)*Boc β-amino alcohol synthesised by the protocol reported in previous chapter 2(a) from *L*-phenylalanine, which on oxidation by using IBX produce (*N*)Boc amino aldehydes. Later, (*N*)Boc amino aldehydes in the presence of ethyl diazoacetate and tin chloride (20 mol %) as a catalyst in DCM gives ethyl esters of γ-amino β-keto amino acids, **1**. The (*N*)Boc γ-amino β-keto esters were purified through silica column chromatography in good yields before using it for the next step. Further, (*N*)Boc β-hydroxy γ-amino esters were synthesized by mild reduction of (*N*)Boc γ-amino β-keto esters with $NaBH_4$ as per the procedure reported earlier by Rich *et al*., shown in Scheme 1. Just after the reduction both the conformers were isolated in good yields through column purification, where *Syn (S, S)*, **3** was the minor and *Anti(R, S)*, **2** was the major product, shown in Table 1.

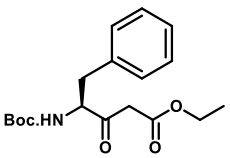
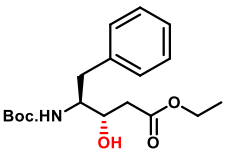
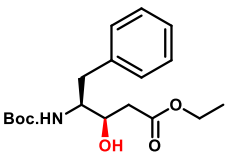
2b.3.2 Design, synthesis and purification of peptides

Herein, we thought of studying the peptides composed only with *anti* (*3R, 4S*) because it is the major conformer obtained during the synthesis. We thought of utilizing this major formed product for synthesizing a homo dipeptide, α,γ-hybrid tetrapeptide and α,γ-hybrid pentapeptide.



Scheme 1: Schematic representation of the synthesis of (*N*)Boc β-hydroxy γ-amino ester from *L*-Phenylalanine.

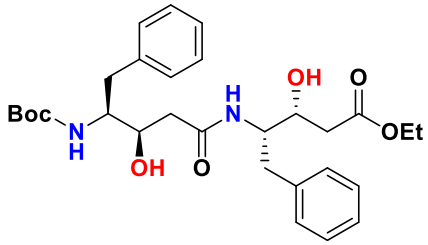
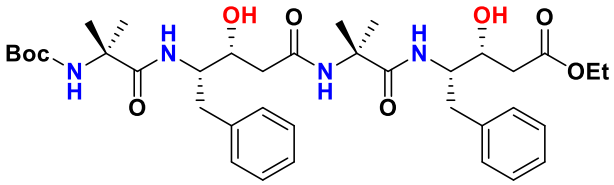
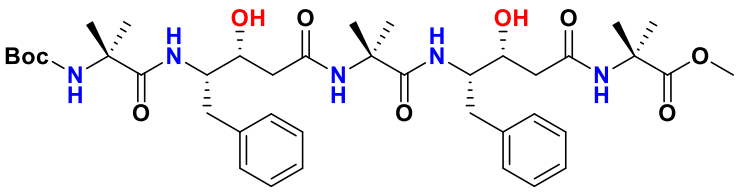
Table 1: List of (*N*)Boc β-keto γ-amino ester and their respective (*N*)Boc β-hydroxy γ-amino ester.

Entry	β-keto γ-amino ester	β-hydroxy γ-amino acid *(S, S)*	β-hydroxy γ-amino acid *(R, S)*	Overall yield %
1.		 *Anti* (S,S) (Minor) **35%**	 *Syn* (R,S) (Major) **65%**	**95%**

We anticipated that the extra hydroxyl group on the backbone of amino acid will participate in intramolecular hydrogen bonding other than the usual amide and carbonyl H-bonding, which may provide extra stability to the structural pattern. The sequence of these designed peptides is shown in Table 2. All three peptides **P1**, **P2** and **P3** were synthesized using standard solution-phase peptide synthesis. The statine amino acid esters were hydrolyzed using 1*N* NaOH in methanol. As described earlier, the hydroxyl groups were not protected and the

peptides were synthesized standard coupling conditions. The HBTU was used as a coupling agent and HOBt as an additive. The Dipeptide **P1** was synthesized using 1+1 fragment coupling strategy. The tetrapeptide **P2** was synthesized using a 2+2 fragment coupling strategy, while the pentapeptide **P3** was synthesized using a 4+1 fragment coupling strategy. The schematic representation of the synthesis of **P1, P2** and **P3** are shown in Scheme 2. All three peptides were subjected to crystallization after purification. We were unable to get good quality single crystals for the dipeptide. The structural properties of peptide **P2** were reported earlier and were resynthesized to have a comparative analysis with **P3** concerning the change in their structural properties.

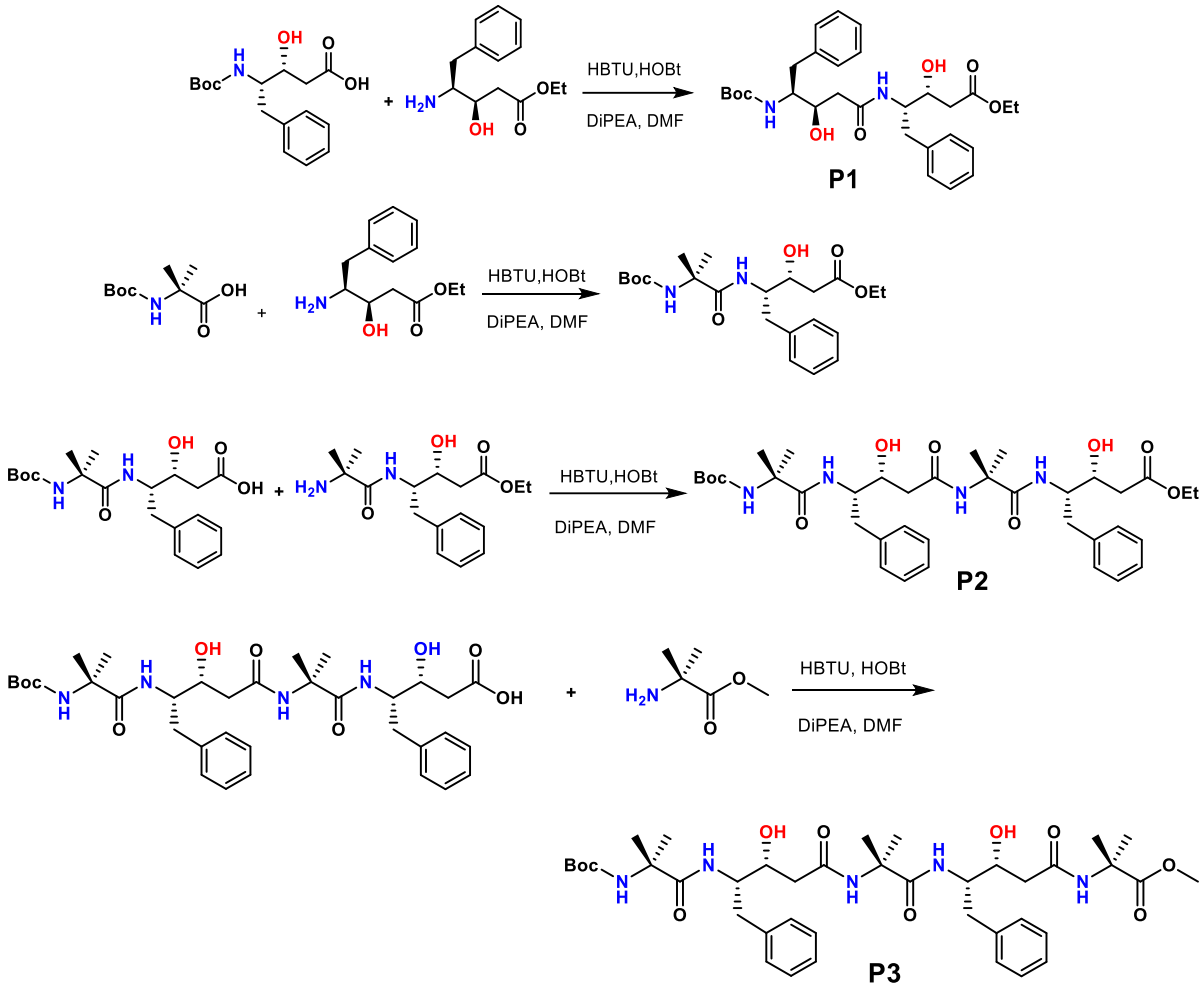
Table 2: Sequence of hybrid peptides **P1-P3** composed of (*3S,4R*) β-hydroxy γ-amino acids.

P1	 **Boc-γ(R,S)Phe-γ(R,S)Phe-OEt**	**Homo-oligomer (dimer)** γγ
P2	 **Boc-Aib-γ(R,S)PheAib-γ(R,S)Phe-OEt**	**Hetro-oligomer (tetramer)** αγαγ
P3	 **Boc-Aib-γ(R,S)PheAib-γ(R,S)Phe-Aib-OMe**	**Hetro-oligomer (pentamer)** αγαγα

2b.3.3 Crystal structure analysis of peptide P2

Peptide **P2** adopted a regular 12-helix conformation, shown in Figure 3. The structure is stabilized by the two independent consecutive 12-membered H-bonds. The torsion angles of and β-hydroxy γ-amino acids can be measured using two additional variables θ_1 (N-C$^{\gamma}$-C$^{\beta}$-C$^{\alpha}$) and θ_2 (C$^{\gamma}$-C$^{\beta}$-C$^{\alpha}$-C') along with ϕ (C'-N-C$^{\gamma}$-C$^{\beta}$) and ψ (C$^{\beta}$-C$^{\alpha}$-C'-N/O) similar to the other γ-

amino acids. The crystal structure analysis revealed that the γ-residue StaPhe2 adopted *gauche*$^+$(g^+), *gauche*$^+$(g^+) conformation along with C^{γ}-C^{β} and C^{β}-C^{α} bonds similar to other γ-residues. Both torsion angles θ_1 and θ_2 attain the torsion angles 51° and 63°, respectively. The torsion angles ϕ and ψ attain the values -126 and -121, respectively.



Scheme 2: Schematic representation of the synthesis of the dipeptide **P1**, tetrapeptide **P2** and the pentapeptide **P3**.

In contrast to StaPhe2, the C-terminal StaPhe4 adopted a different conformation. It adopted g^+ conformation along C^{γ}-C^{β} bond (θ_1 = 55), while anti (*t*) conformation along C^{β}-C^{α} bond (θ_2 = -170). The torsion angles ϕ and ψ attain the values -105 and -158, respectively. The structural analysis reveals that the terminal statin adopted extended conformation. Both Aib residues attain regular helical conformations. The torsion angles of the peptide **P2** are tabulated in Table 3. In addition to the intramolecular H-bonds, the *N*-terminal amide two NH groups and C-terminal two carbonyl groups are exposed to intermolecular H-bonds with solvent molecules

and helical peptides. The β-hydroxyl group in the Staphe2 involved in the intramolecular H-bond (C=O---H-O) with the CO of the same residue. In contrast, the β-OH group of the terminal Staphe4 is not involved in the intramolecular H-bonds. The H-bond parameters of peptide **P2** are tabulated in Table 4.

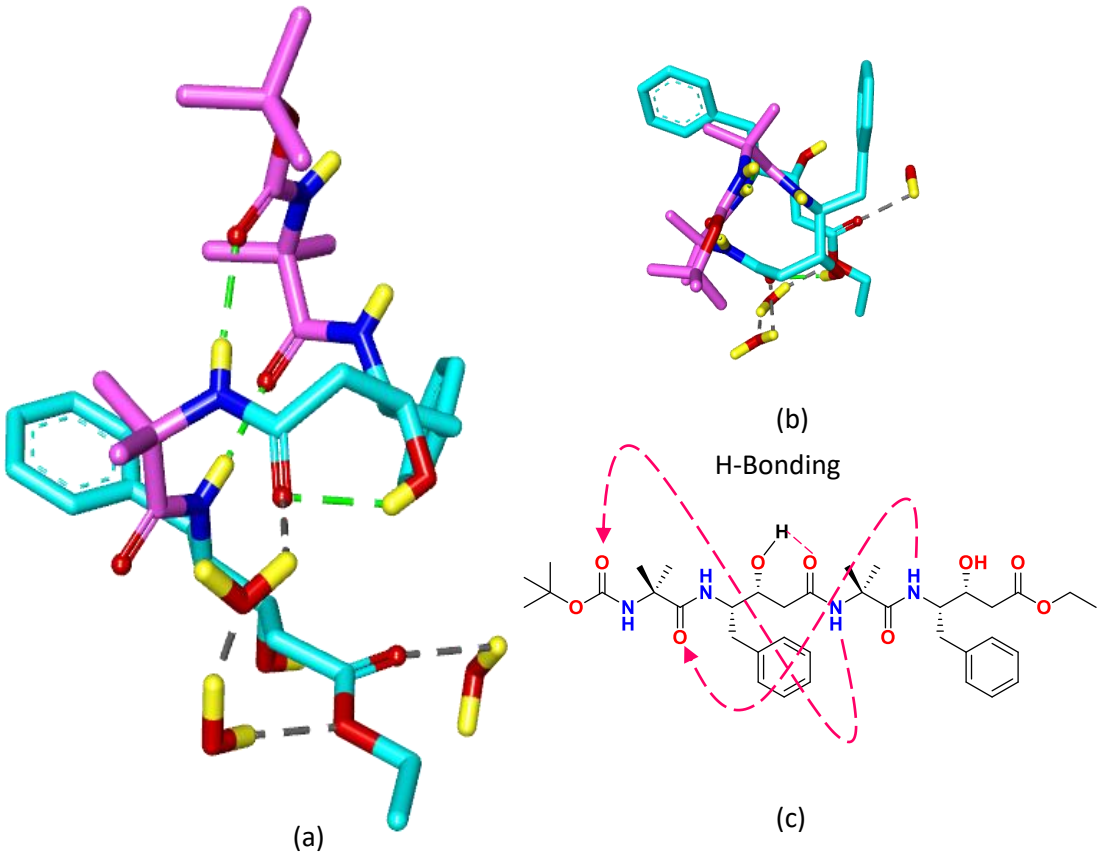


Figure 3: X-ray diffracted structure of peptide **P2 (**Boc-Aib-(*R, S*)Phe(β-OH)-Aib-(*R, S*)Phe(β-OH)-COOEt) (a) side view, (b) top view (c) Backbone H-bond directionality shown. H-atoms of polar atoms and β, γ C-atoms of statine residue [for (*R, S*) stereochemistry] are shown for clarity. H-bonds are represented by dotted lines.

Table 3: The torsion angles of peptide **P2**.

Pept.	Resd.	ϕ	θ_1	θ_2	ψ
P1	Aib1	-61	-	-	-39
	(*3R, 4S*)γPhe2	-126	51	63	-121
	Aib3	-58	-	-	-39

	(*3R*, *4S*)γPhe4	-105	55	-170	-158

Table 4: Intramolecular Hydrogen bonding parameters observed in the crystal structures of peptide **P2**.

Type of H-bonds	Donar (D)	Acceptor (A)	D⋯⋯A (Å)	D-H⋯⋯A (Å)	∠N-H⋯O (deg)	∠N⋯O=C (deg)
Boc-Aib-(3*R*, *4S*)-γPhe-Aib-(3*R*, *4S*)-γPhe-OEt (P2)						
1←4	N3	O2(Boc)	2.92	2.07	172.3	149.6
1←4	N4	O3	2.83	2.04	157.1	141.5
Intra residue	O4	O5	2.79	2.09	142.8	84.8 (C=O⋯O)

2b.3.4 Crystal structure analysis of peptide P3

The peptide **P3** crystallized with the symmetry group P 2_1, shown in Figure 4. The peptide adopted distorted helix type conformation. The *N*-terminal part of the peptide adopted helix conformation and the *C*-terminal part adopted extended conformation. The *N*-terminal helix part is stabilized by two independent H-bonds between the residues i and i+3 (1←4). Similar to the peptide **P2**, the StaPhe2 adopted g^+, g^+ conformation along C^{γ}-C^{β} and C^{β}-C^{α} bonds, respectively. Both torsion angles θ_1 and θ_2 attain the torsion angles 52° and 62°, respectively. The torsion angles ϕ and ψ attain the values -128 and -121, respectively. In contrast to StaPhe2, the C-terminal StaPhe4 adopted a different conformation. The conformation of StaPhe4 observed in **P3** is a little different than that of StaPhe4 in peptide **P2**. It adopted g^+ conformation along C^{γ}-C^{β} bond (θ_1 = 57), while anti (*t*) conformation along C^{β}-C^{α} bond (θ_2 = 177). The torsion angles ϕ and ψ attain the values -105 and 139, respectively. The StaPhe4 adopted extended conformation. Interestingly, the *C*-terminal Aib residue adopted left-handed conformation with ϕ and ψ values 40°, 56° respectively. The torsion angles are tabulated in

Table 5. Along with the two intramolecular H-bonds, the *N*-terminal two amide NH groups are involved in the intermolecular H-bonds with other helices and solvent molecules. The StaPhe4 adopted extended conformation and Aib5 adopted left-handed helix conformation. The CO group of the Aib3 is involved in the intermolecular H-bond with other helix molecules. The β-OH group of StaPhe2 is involved in the intramolecular H-bond with CO of the same residue. The β-OH group of StaPhe4 is not involved in the intramolecular H-bonds. The H-bond parameters are tabulated in Table 6.

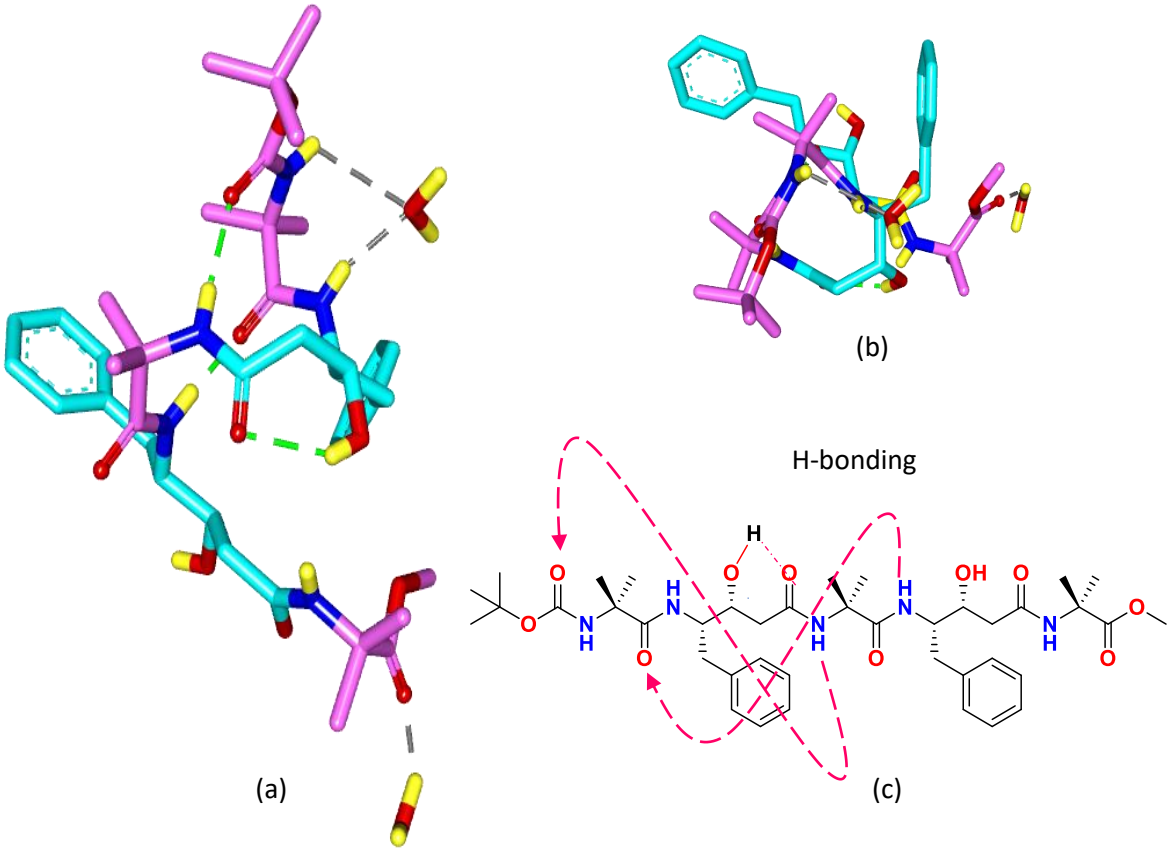


Figure 4: X-ray diffracted structure of peptide **P3 (**Boc-Aib-(*R*, *S*)Phe(β-OH)-Aib-(*R*,*S*)Phe(β-OH)-Aib-COOMe) (a) side view, (b) top view (c) Backbone H-bond directionality shown. H-atoms of polar atoms and β, γ C-atoms of statine residue [for (*R*, *S*) stereochemistry] are shown for clarity. H-bonds are represented by dotted lines.

Table 5: The torsional angles of peptide **P3.**

Pept.	Resd.	ϕ	θ_1	θ_2	ψ
	Aib1	-76	-	-	-39

P3	(*3R*, *4S*)γPhe2	-128	52	62	-121
	Aib3	-57	-	-	-44
	(*3R*, *4S*)γPhe4	-109	57	177	139
	Aib5	40	-	-	56

Table 5: Intramolecular Hydrogen bonding parameters observed in the crystal structures of peptide **P3**.

Type of H-bonds	Donar (D)	Acceptor (A)	D···A (Å)	D-H···A (Å)	∠N-H···O (deg)	∠N···O=C (deg)
Boc-Aib-(3*R*, *4S*)-γPhe-Aib-(3*R*, *4S*)-γPhe-Aib-OMe (P3)						
1←4	N3	O2(Boc)	2.93	2.07	176.9	150.7
1←4	N4	O3	2.79	1.97	160.0	143.2
Intra residue	O4	O5	2.92	2.23	142.0	84.0 (C=O···O)

The structural analysis of the peptides **P1** and **P2** reveal that (*3R*, *4S*) β-hydroxy γ-amino acids can be used to design helical conformation, however, incorporation of more than one residue in the α,γ-hybrid peptide leads to distortion in the helical conformation. In comparison to the previous results on peptides containing statine residue with *anti*-stereochemistry, it may be difficult to accommodate *syn* statins in 1:1 α,γ-hybrid peptide helices. As *C*-terminal statine in both peptides displayed extended conformation, indicating that these can be useful to design β-sheet type extended structures.

2b.4 Conclusion

In summary, we have demonstrated the conformational behaviour of γ-peptides containing β-hydroxy γ-amino acid residues. Single crystal analysis suggests that the tetrapeptide adopted

12-helix conformation as observed in the α,γ-hybrid peptides. Interestingly, the addition of helix promoting another Aib residue at the *C*-terminus of the tetrapeptide leads to a distorted 12-helical structure. The possible explanation of the distorted helix is the steric clash between the Aib side-chains and the β-hydroxyl group at the statin residues. Overall, it may be difficult to design longer 12-helices composed of 1:1 alternating α and (*3S*, *4R*) β-hydroxy γ-amino acid residues. Though *anti* residues are involved in the intramolecular H-bonds, the conformation properties of longer sequences containing these *anti*-residues is yet to investigate. These reported notable structural behaviour of statines containing γ-peptides have given direction to utilize them further in the design of biologically active foldameric peptidomimetics in the later chapters.

2b.5 Experimental section

2b.5.1 General Experimental Details:

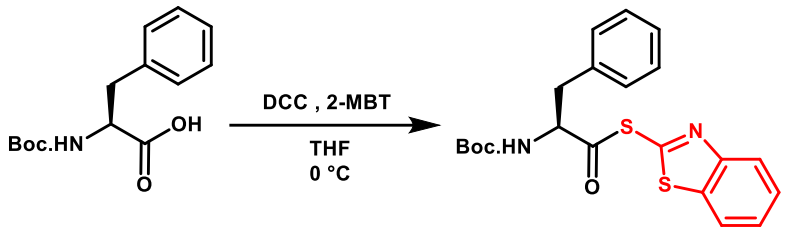
All α-amino acids, Ethyl diazoacetate, Tin chloride and Di-t*ert*-butyl dicarbonate were obtained from Merck. 2-Mercaptobenzothiazole (2-MBT), Fmoc-Osu, DCC, $NaBH_4$, HPLC grade acetonitrile, dry DCM and dry THF were purchased from Spectrochem. Column chromatography was achieved on silica gel of 120-200 mesh from Merck. Thin-layer chromatography (TLC) was used to monitor reaction using Merck 60 F_{254} precoated silica gel plates. For visualization UV light and ninhydrin or phosphomolybdic acid (PMA) stains were used then charring on a hot plate. Melting points were recorded on Veego VMP-DS hot stage apparatus. Specific rotations were recorded at ambient temperature on the Rudolph Analytical Research instrument using $CHCl_3$ and MeOH as solvents. Reverse Phase-HPLC of Waters was used to purify peptides using Acetonitrile/H_2O gradient with 0.1% TFA from C-18 column. Mass was analysed on Applied Biosciences with MALDI TOF/TOF on positive ion mode.

NMR spectroscopy

Jeol 400 MHz (or 100 MHz for ^{13}C) spectrometer were used for recording ^{1}H spectra and ^{13}C NMR. The chemical shifts (δ) and coupling constants (J) were reported in ppm and Hz, respectively. The residual solvents signals were used as internal reference ($CDCl_3$ δ_H, 7.24 ppm, δ_c 77.0 ppm).

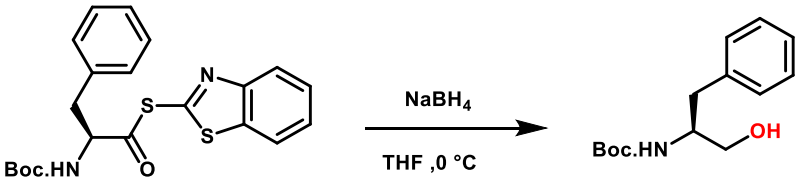
2b.5.2 General procedure for the synthesis of monomers

General procedure for the synthesis of MBT ester from *N*-Boc phenylalanine amino acid:



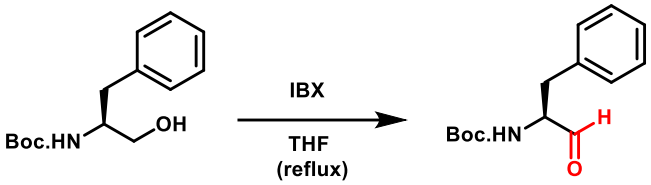
N-Boc phenylalanine amino acid (10 mmol) and 2-Mercaptobenzothiazole (2.505 g, 15 mmol) were mixed in THF (20 mL). The reaction mixture was allowed to cool to 0 ˚C. After cooling, DCC (2.06 g, 10 mmol) was added to the reaction and stirred for the next 1 h. TLC was performed for monitoring the reaction. On the completion of the reaction, precipitated DCU was filtered. The filtrate was concentrated beneath the *vacuum* to attain a gummy mixture. Further, EtOAc/Hexane was used for precipitating the mixture to give MBT ester of *N*-Boc amino acid. This precipitated product was used without purification directly for the alcohol synthesis.

General procedure for the synthesis of (*N*)Boc β-amino alcohol from 2-Mercaptobenzothiazole esters:



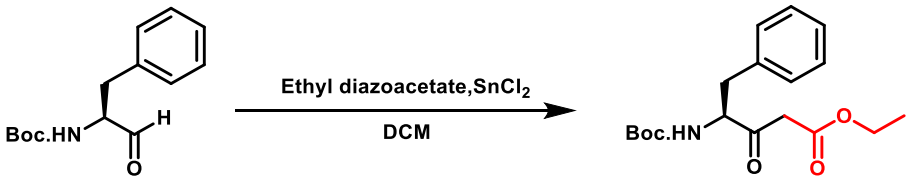
THF (30ml) was used to dissolve the above obtained *N*-protected MBT ester of phenylalanine and was allowed to cool to 0 ˚C using ice-bath. $NaBH_4$ in water (50 mmol in 15 ml of water) was added to the reaction mixture step by step to observe effervescence and kept for the next 30 mins. Once the reaction is completed it was neutralized using 10% HCl. Later, the reaction mixture was kept beneath a vacuum to evaporate the THF used for the reaction. The aqueous layer was extracted thrice using ethyl acetate. The combined organic extract was washed further with 10% Na_2CO_3 and later with brine followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure *N*-protected β-amino alcohol with 80-90% yield.

General procedure for the Synthesis of *N*-Boc amino aldehydes:



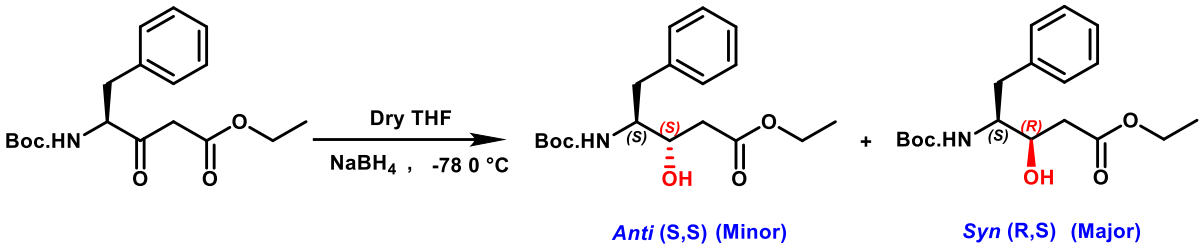
Ethyl acetate (50 ml) was used to dissolve *N*-Boc β-amino alcohol (10 mmol) and to that IBX (30 mmol) was added. The reaction was kept for stirring at a refluxing temperature over oil-bath for the next 4 hours. TLC was performed for monitoring the reaction. On the completion of the reaction, the reaction mixture was filtered while washing with ethyl acetate. The filtrate was concentrated beneath the *vacuum* to attain an oily mixture. This oily product was used without purification directly for the β-keto synthesis.

General Procedure for the Synthesis of *N*-Boc β-keto γ-amino esters:



DCM (15 ml) was used to dissolve the above formed *N*-Boc phenylalanine β-amino aldehyde and to that Tin (II) Chloride (20 mol%) was added at room temperature. After 5 mins, 15% Ethyl diazoacetate in toluene (10.05 mmol, 7.5 ml) was added slowly drop by drop and instant evolution of nitrogen can be seen till the reaction completes. TLC was performed for monitoring the reaction. Once the reaction is completed it was neutralized using 5 % HCl and the aqueous layer was extracted thrice using DCM. The combined organic extract was washed with brine followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure *N*-Boc β-keto ester.

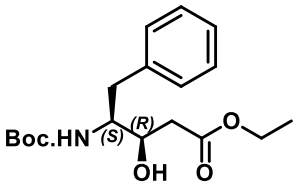
General Procedure for Synthesis of *N*-Boc β-hydroxy γ-amino esters:



The purified phenylalanine β-keto γ-amino ester (10 mmol) was dissolved in 30 ml of dry THF under a nitrogen atmosphere. The reaction is brought to -78 0 ˚C using dry ice and acetone. $NaBH_4$ (0.529 g, 14 mmol) was added to the reaction mixture and allowed to stir for 3-4 hours. The reaction was well monitored with TLC. After completion of the reaction, the reaction mixture was quenched using a 5% HCl solution and extracted with ethyl acetate. The organic layer was washed with brine solution, dried over anhydrous sodium sulphate and concentrated at reduced pressure to obtain the diastereomers of *N*-Boc phenylalanine β-hydroxyl γ-amino ester. The *syn* and *anti* diastereoisomers were separated using silica (200-400 mesh) column chromatography using ethyl acetate and hexane.

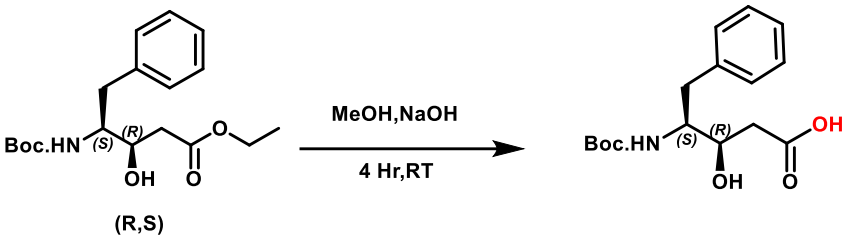
Characterisation of N-Boc β-hydroxy γ-amino acids:

(3*S*, 4*R*)-ethyl 4-((tert-butoxycarbonyl)amino)-3-hydroxy-5-phenylpentanoate:



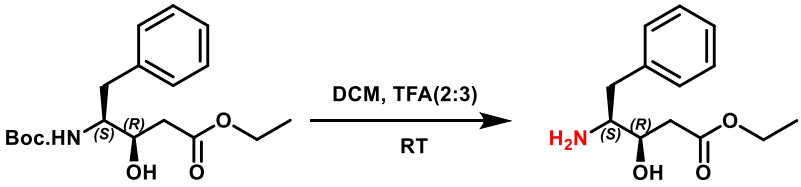
White powder **^{1}H NMR** (400 MHz, $CDCl_3$) **:** 7.32-7.22 (m, 5H), 4.56-4.54 (d, *J* = 9.8, 1H), 4.21-4.16 (q, *J* = 7.2, 2H), 4.00-3.99 (d, *J* = 6.5, 1H), 3.90-3.84 (m,1H), 3.61 (b, 1H), 3.01-2.82 (m, 2H), 2.61-2.47 (m, 2H), 1.36 (s, 9H), 1.30-1.26 (t, *J* = 7.2, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) **:** 173.01, 155.71,137.60, 129.47, 128.45, 126.43,79.60, 70.06, 60.89, 55.11, 38.12, 35.79, 28.24, 14.13. **MALDI-TOF/TOF** m/z value Calcd. [M+Na]+ 360.1787, observed 360.1732.

General Procedure for Synthesis of (*3S,4R*) *N*-Boc β-hydroxy γ-amino acid:



MeOH (50 ml) was used to dissolve pure *(R, S) N*-Boc β-hydroxy γ-amino ester (5 mmol) and was allowed to stir to get a clear solution. 3eq. of 1NaOH (15 ml, 15 mmol) was added to the reaction mixture dropwise and kept for the next 2 hours at room temperature. TLC was performed from time to time for monitoring the reaction. Once the reaction is completed it was neutralized using 10% HCl. Later, the reaction mixture was kept beneath a vacuum to evaporate the MeOH used in the reaction. The aqueous layer was extracted thrice using ethyl acetate. The combined organic extract was washed with brine solution followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate to obtain pure *N*-Boc β-hydroxy γ amino acid, no further purification was required.

General procedure for the synthesis of NH_2-β-hydroxy γ-amino-COOEt:



DCM (10 ml) was used to dissolve *N*-Boc β-hydroxy γ amino ester (10 mmol) and was allowed to cool to 0 °C using ice-bath. 10 ml of TFA was added to the reaction mixture slowly and kept for next 30 mins. Once the reaction is completed TFA was co-evaporated using DCM (15ml five times) and recrystallized using DCM and hexane to obtain solid triflate salt of NH_2-β-hydroxy γ-amino acid which can be used without purification directly for the next step.

2b.5.3 Solution-phase peptide synthesis and purification

General procedure for the synthesis of Boc-(*R, S*) Phe(β-OH)-(*R, S*) Phe(β-OH)-COOEt (P1):

Dipeptide was synthesized by a conventional way of solution-phase peptide synthesis with fragment condensation strategy. Deprotection of Boc(*N*)-termini was performed by using

trifluoracetic acid (TFA) and C-terminal ester by saponification method. HBTU and HOBt were used as coupling agents in the presence of DIEA as a base in N, N-Dimethylformamide (DMF) as a solvent for overnight. Dipeptide was synthesized using a 1+1 condensation strategy involving Boc-(*R, S*) Phe(β-OH)-COOH and NH_2-(*R, S*) Phe(β-OH)-OEt. After the completion of the reaction, the reaction mixture was extracted in ethyl acetate, later acid wash and base wash was given thrice each, with a brine wash at the end. The organic layer was evaporated under a vacuum to obtain a gummy mixture. The peptide was purified using RP-HPLC, C_{18} - column using Methanol/water solvent system.

General procedure for the synthesis of Boc-Aib-(*R, S*) Phe(β-OH)-Aib-(*R, S*) Phe(β-OH)-COOEt (P2):

Tetrapeptide was synthesized by a conventional way of solution-phase peptide synthesis with fragment condensation strategy. Deprotection of Boc(N)-termini was performed by using trifluoracetic acid (TFA) and C-terminal ester by saponification method. HBTU and HOBt were used as coupling agents in the presence of DIEA as a base in N, N-Dimethylformamide (DMF) as a solvent for overnight. Tetrapeptide was synthesized using a 2+2 condensation strategy involving Boc-Aib-(*R, S*) Phe(β-OH)-COOH and NH_2-Aib-(*R, S*) Phe(β-OH)-OEt. After the completion of the reaction, the reaction mixture was extracted in ethyl acetate, later acid wash and base wash was given thrice each, with a brine wash at the end. The organic layer was evaporated under a vacuum to obtain a gummy mixture. The peptide was purified using RP-HPLC, C_{18} - column using Methanol/water solvent system.

Boc-Aib-(*R, S*)Phe(β-OH)-Aib-(*R, S*)Phe(β-OH)-COOEt (P2): White crystalline powder . 1H NMR (500 MHz, $CDCl_3$) : δH 7.78-7.76 (d, J = 8.78, 1H), 7.33-7.10 (m, 11H), 5.60-5.98 (d, J = 10), 4.90 (s, 1H), 4.33-4.28 (m, 1H), 4.21-4.14 (m, 5H), 3.69-3.67 (d, J = 9.81, 1H), 3.23-2.79 (m, 5H), 2.64-2.34 (m, 3H), 1.44 (s, 3H), 1.41 (s, 9H), 1.36 (s, 3H), 1.29-1.27 (t, J = 7, 3H), 1.13 (s, S20 3H), 0.93 (s, 3H). ESI m/z : Calcd. for $C_{37}H_{54}N_4O_9$ [M+H]+ 699.3969, observed 699.3976.

General procedure for the synthesis of P3

Boc-Aib-(*R, S*)Phe(β-OH)-Aib-(*R, S*)Phe(β-OH)-Aib-COOMe (P3):

Pentapeptide was synthesized by a conventional way of solution-phase peptide synthesis with fragment condensation strategy. Deprotection of Boc(N)-termini was performed by using trifluoracetic acid (TFA) and C-terminal ester by saponification method. HBTU and HOBt

were used as coupling agents in the presence of DIEA as a base in N, N-Dimethylformamide (DMF) as a solvent for overnight. Pentapeptide was synthesized using a 4+1 condensation strategy involving Boc-Aib-(*R, S*) Phe(β-OH)- Aib-(*R, S*) Phe(β-OH)-COOH and NH_2-Aib-*(R, S*)-OMe. After the completion of the reaction, the reaction mixture was extracted in ethyl acetate, later acid wash and base wash was given thrice each, with a brine wash at the end. The organic layer was evaporated under a vacuum to obtain a gummy mixture. The peptide was purified using RP-HPLC, C_{18}-column using Methanol/water solvent system.

Boc-Aib-(*R, S*)Phe(β-OH)-Aib-(*R, S*) Phe(β-OH)-Aib-COOMe (P3): White crystalline powder. 1H NMR (500 MHz, $CDCl_3$) : δH 7.70-7.68 (d, J=8.5, 1H), 7.31-7.09 (m, 11H), 5.89-5.87 (d, J=9.1, 1H), 5.08 (s, 1H), 4.94 (s, 1H), 4.61 (s, 1H), 4.29-4.27 (d, 1H), 4.11 (s, 1H), 3.88 (s, 1H), 3.72(s, 1H), 3.63 (s, 1H), 3.45-3.42 (d, J=9.7, 1H), 3.19-3.16(d, J=8.7, 1H), 2.88-2.0 (m, 4H), 2.61-2.33 (m, 2H), 1.60 (s, 1H), 1.55 (s, 1H), 1.48 (s, 1H), 1.40 (s, 1H), 1.37 (s, 1H), 1.21(s, 1H), 0.088 (s, 1H). ESI m/z: Calcd. for $C_{40}H_{59}N_5O_{10}$ [M+Na]+ 792.4172 , [M+K]+ 808.3900 observed 792.4276 and 808.3900 respectively.

2b.5.4 Crystallographic Information

Crystal structure analysis of Boc-Aib-(*R, S*) Phe(β-OH)-Aib-(*R, S*) Phe(β-OH)-COOEt (P2):

Crystals of peptide were grown by slow evaporation from a solution of methanol/water. A single crystal (0.15 × 0.13 × 0.07 mm^3) was mounted on loop with a small amount of the paraffin oil. The X-ray data were collected at 100K temperature on a Bruker APEX(II) DUO CCD diffractometer using Mo K_α radiation (λ = 0.71073 Å), ω-scans (2θ = 52.52), for a total of 20402 independent reflections. Space group P 21, a =10.5440(8), b = 9.6062(8), c = 21.5794(16), a= 90°, b = 100.859(2), g = 90°, V = 2146.6(3) $Å^3$, Monoclinic C, Z = 2 for chemical formula C38 H60 N4 O13, with one molecule in asymmetric unit; ρcalcd = 1.208 gcm^{-3}, μ = 0.091 mm^{-1}, F (000) = 840, R_{int}= 0.0347. The structure was obtained by direct methods using SHELXS-97.[62] The final R value was 0.0548, (wR2 = 0.1503) 8413 observed reflections ($F_0 \geq 4\sigma$ ($|F_0|$)) and 516 variables, S = 1.069. The largest difference peak and hole were 0.692 and -0.467 $eÅ^{-3}$, respectively.

Crystal structure analysis of Boc-Aib-(*R, S*) Phe(β-OH)-Aib-(*R, S*) Phe(β-OH)-Aib-COOMe (P3):

Crystals of peptide were grown by slow evaporation from a solution of Methanol/Water. A single crystal (0.13 × 0.12 × 0.02 mm^3) was mounted on loop with a small amount of the paraffin oil. The X-ray data were collected at 100K temperature on a Bruker APEX(II) DUO CCD diffractometer using Mo K_α radiation (λ = 0.71073 Å), ω-scans (2θ = 52.52), for a total of 4055 independent reflections. Space group P21, a = 10.481(5), b = 9.750(5), c = 22.545(10), β = 93.178(8), V = 2300.3(19) $Å^3$, Monoclinic, Z = 2 for chemical formula C40 H59 N5 O13, with three molecule in asymmetric unit; ρ calcd = 1.181 gcm^{-3}, μ = 0.088 mm^{-1}, F(000) = 876, R_{int}= 0.1064. The structure was obtained by direct methods using SHELXS-97. The final R value 0.0968 (wR2 = 0.2092) 4055 observed reflections ($F_0 \geq 4\sigma$ ($|F_0|$)) and 535 variables, S = 1.231. The largest difference peak and hole were 0.399 and -0.330 $e.Å^{-3}$, respectively.

2b.6 References

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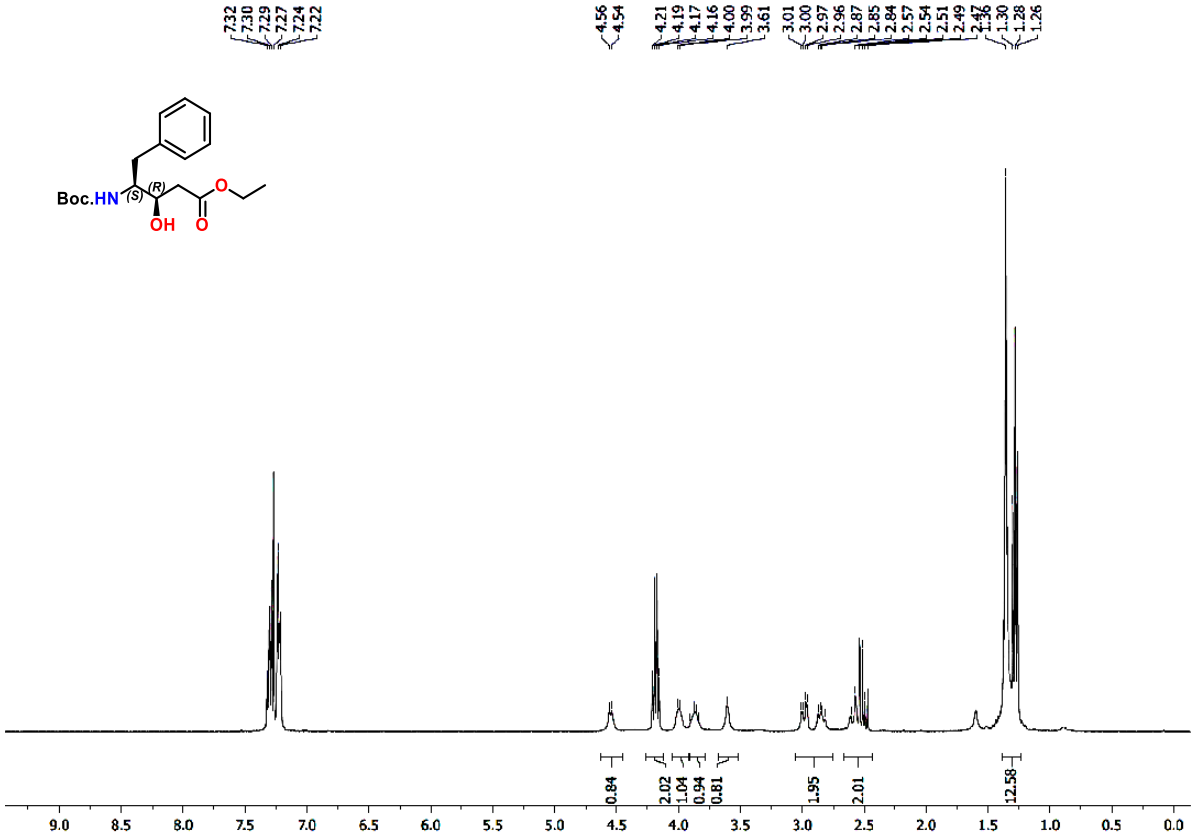
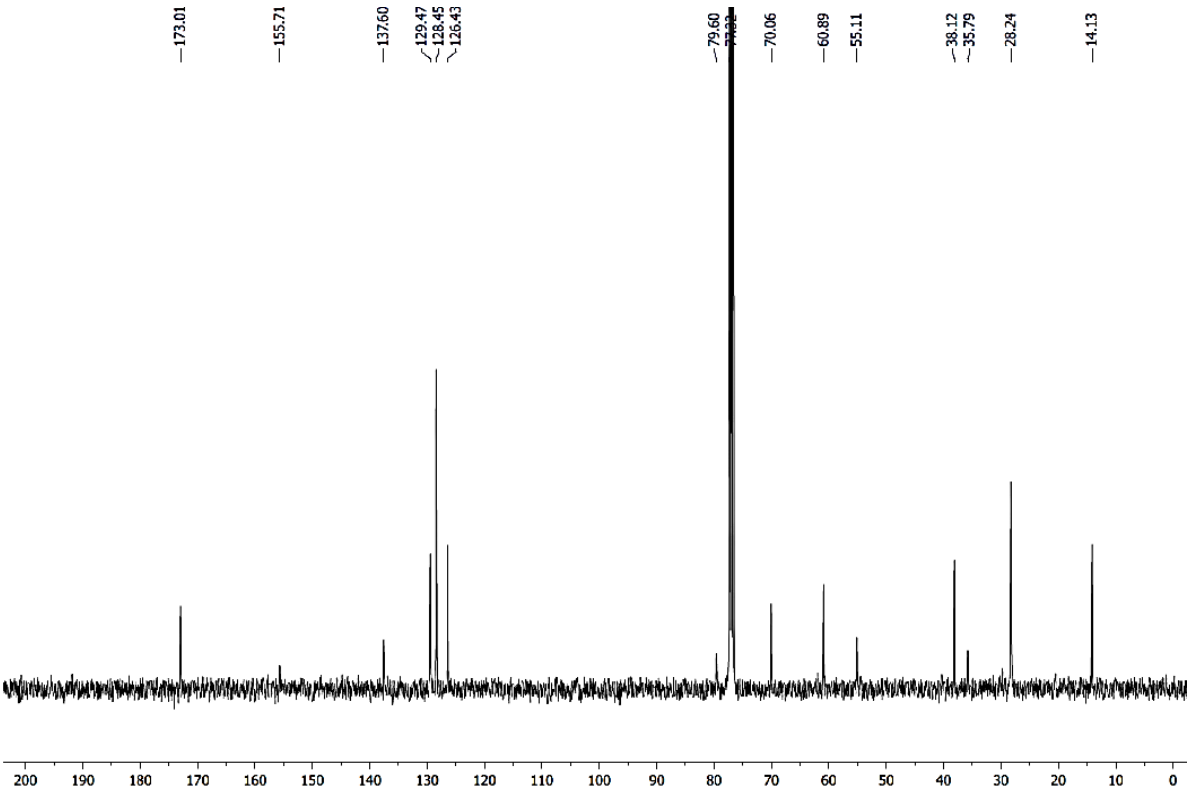
2b.7 Appendix I: ^{1}H NMR spectra,^{13}C NMR and Mass spectra

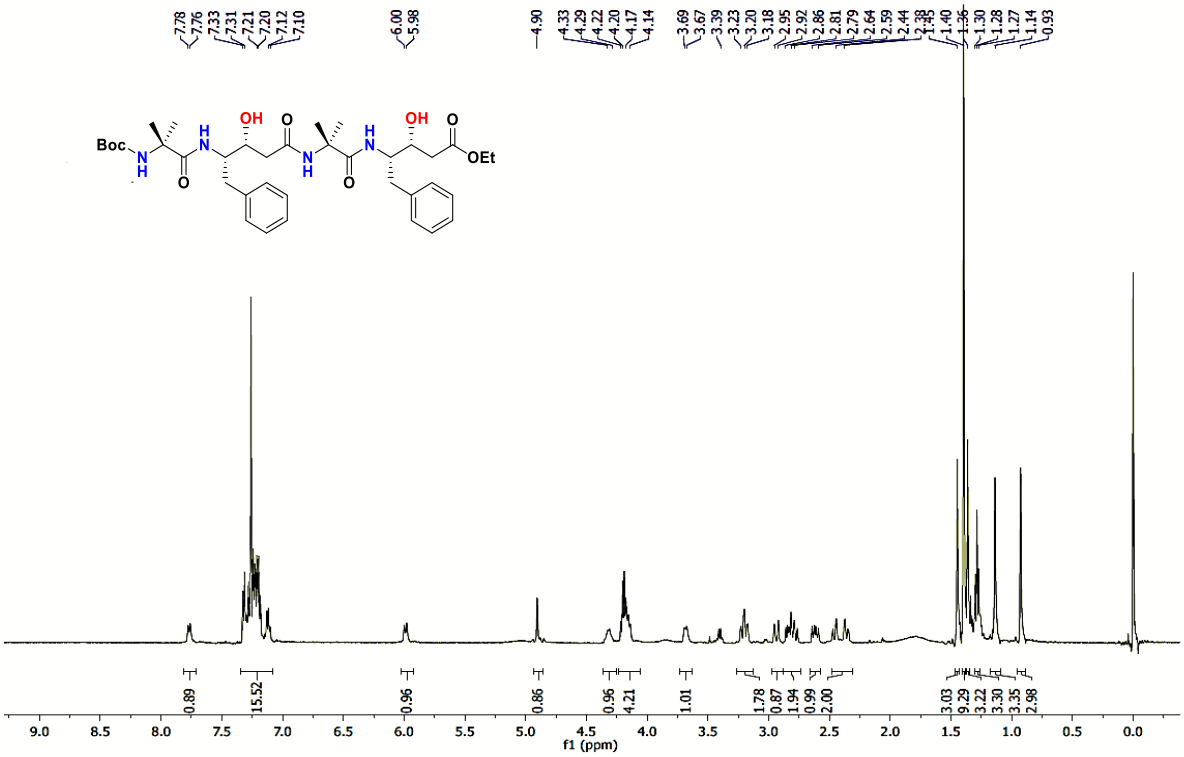
^{1}H NMR *N*-Boc (*R, S*)Phe-(β-OH)-COOEt



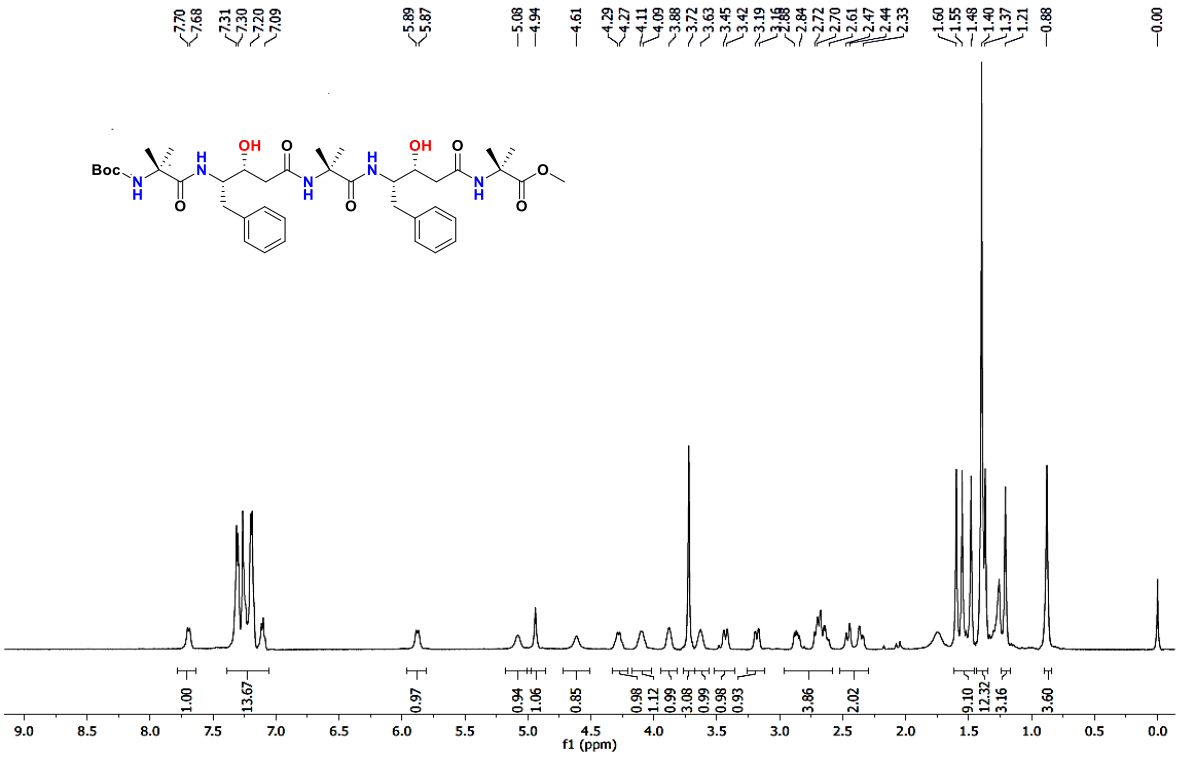
^{13}C NMR of *N*-Boc (*R, S*)Phe-(β-OH)-COOEt



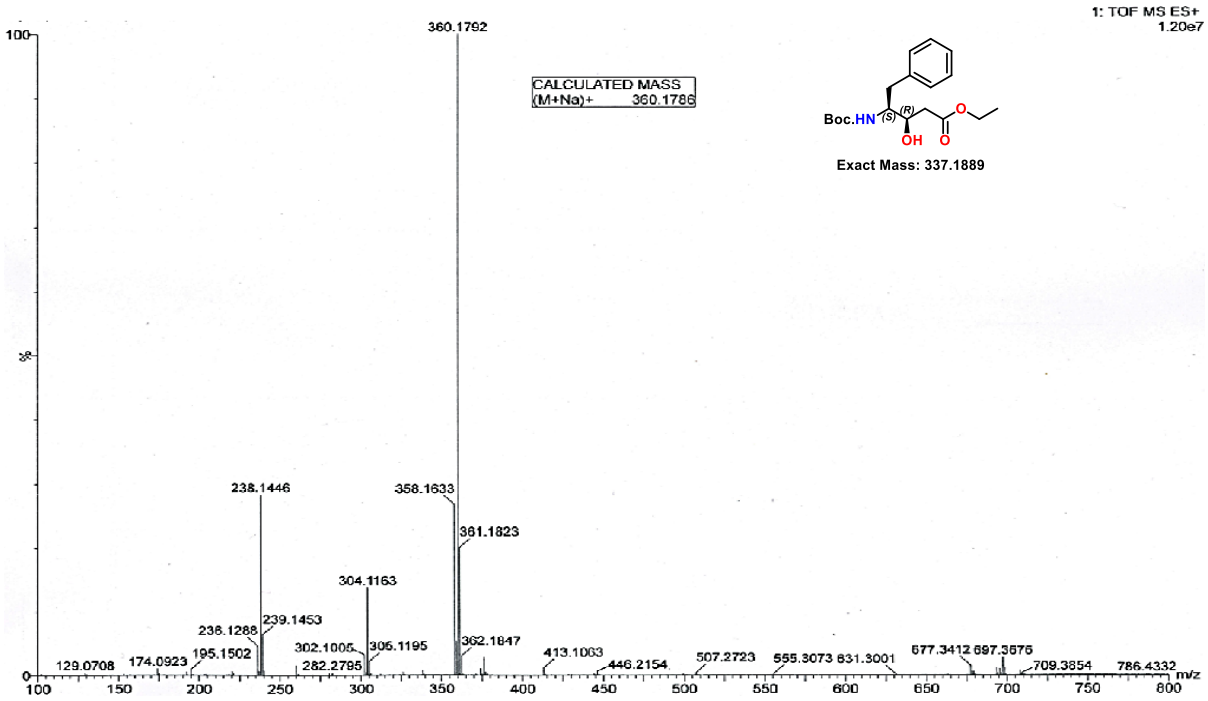
^{1}H NMR Peptide P2



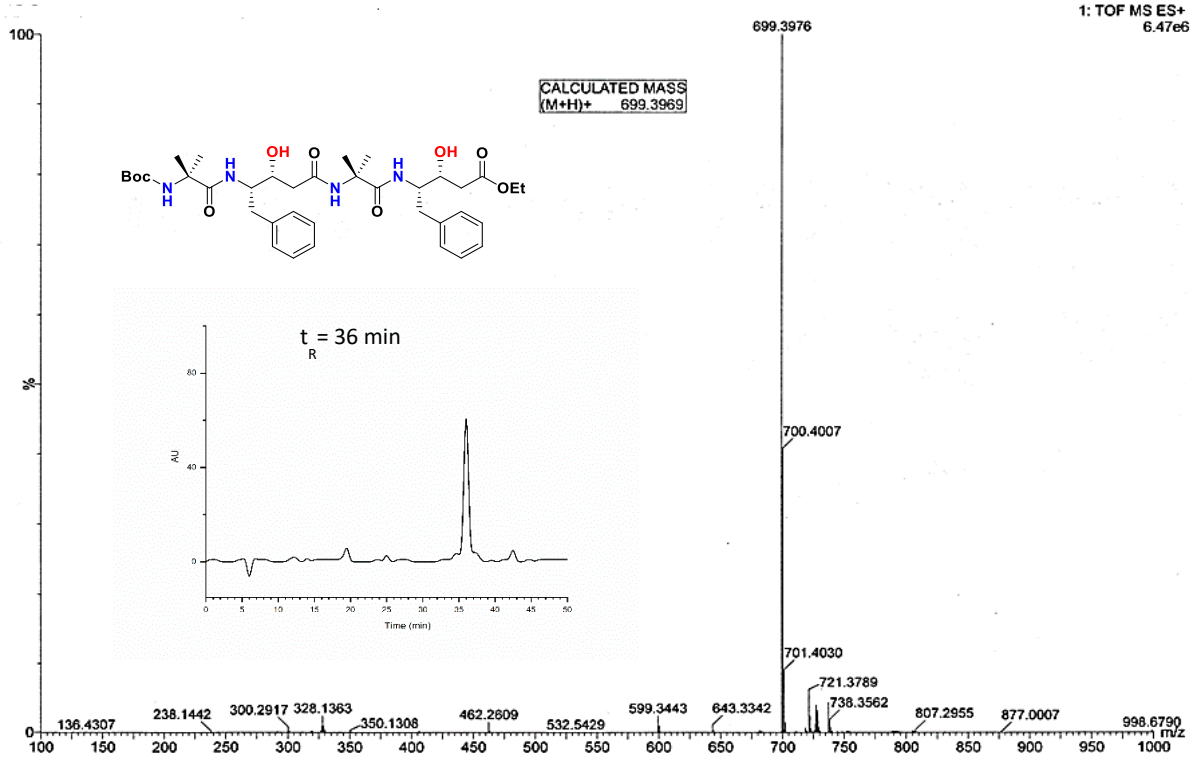
^{1}H NMR Peptide P3



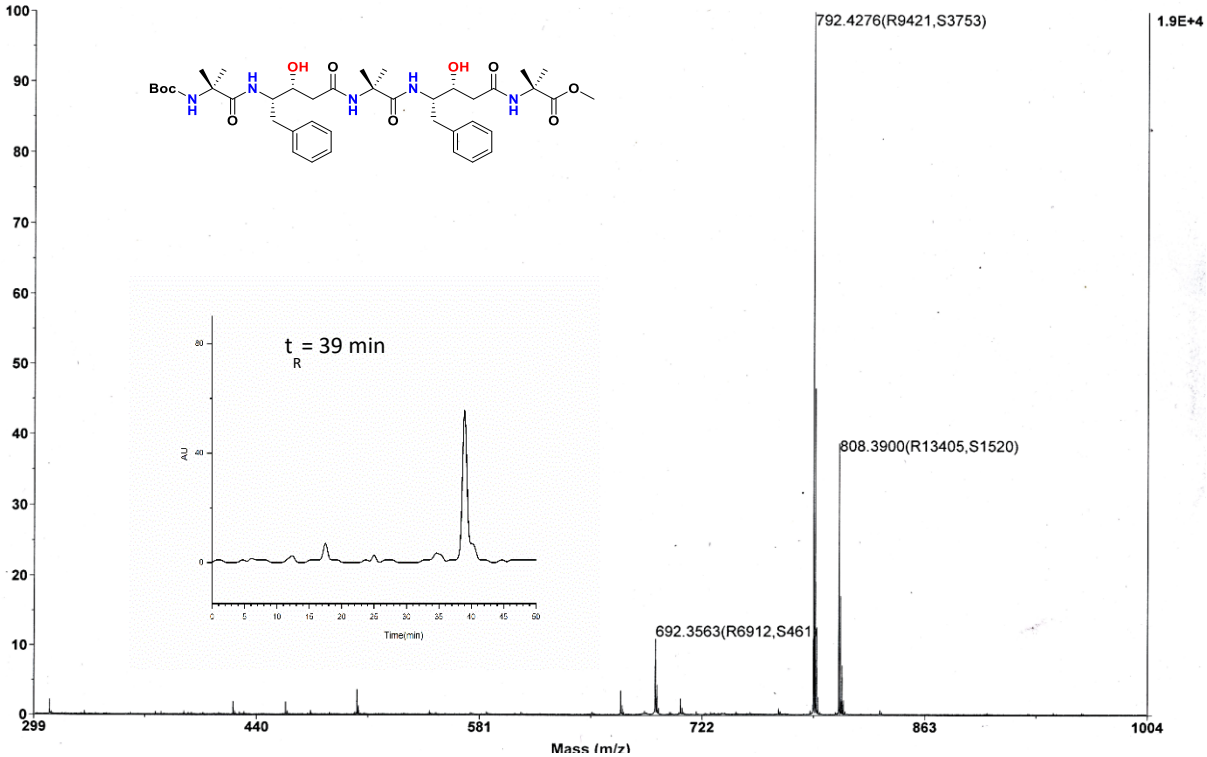
Mass spectra of *N*-Boc (*R, S*)Phe-(β-OH)-COOEt



Mass spectra and HPLC trace of Peptide P2



Mass spectra and HPLC trace of Peptide P3



Chapter 3(a)

Inhibition of γ-Secretase Activity by α,γ-hybrid Helical Peptides

3a.1 Introduction

Alzheimer's Disease (AD) is known as a neurological disorder or neurodegenerative disorder causing shrinkage of the brain and destruction of nerve cells.[1] The Alzheimer's brain is different from the normal brain due to the accumulation of amyloid β- peptides (Aβ) as amyloid plaques in between the neurons. Dementia, loss of memory and thinking ability is the early sign of AD.[2] Over the decades, huge research has been done to support the fact that disorder in the ratio of $Aβ_{40/42}$, few peptide fragments of Amyloid β-peptides are majorly associated with the pathogenesis of Alzheimer Disease.[3] The sequential proteolysis of Amyloid Precursor Protein (APP, a transmembrane protein) by β and γ-secretase (an aspartic acid proteases), generates Amyloid β-peptides (Aβ-peptides). According to the Amyloidogenic pathway of APP proteolysis firstly, APP is proteolyzed by β-secretase at C-terminal to generate APP-C99 or β-CTF fragment.[4] Later, which was cleaved by γ-secretase at γ-site of β-CTF to generate Aβ and at ε-site to release AICD (APP Intracellular Domain). This fragmented protein, Aβ consist of various lengths but $Aβ_{1-40}$ and $Aβ_{1-42}$ are majorly responsible to form non-soluble fibrils or senile plaque formation and hence associated with the pathogenesis of Alzheimer disease.[5] The potential target for treating AD pathogenesis is developing drugs to inhibit γ-secretase (GSI-Gamma Secretase Inhibitors), hence inhibiting the release of Aβ fragments in brain.[6]

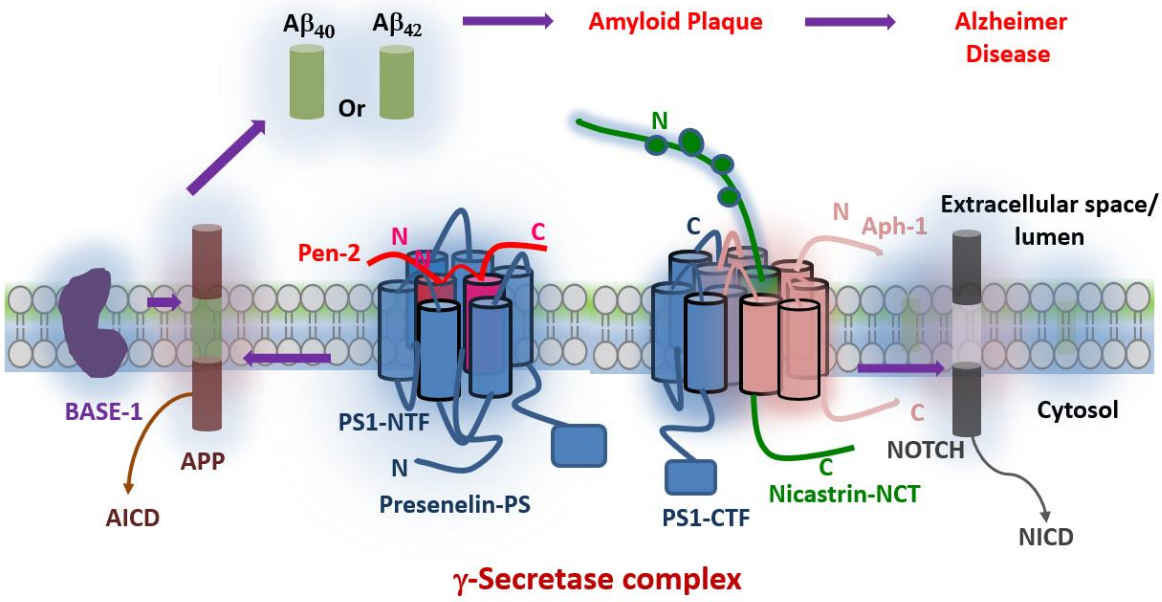


Figure 1: Gamma secretase complex and the proteolysis of transmembrane proteins APP and Notch.

γ-secretase structure is a complex assembly of heterogenous membrane protein subunits which include four components: Presenilin (PS) heterodimer, Presenilin enhancer-2 (PEN-2), Nicastrin (NCT) and Anterior pharynx-defective 1 (APH-1) shown in Figure 1.[7]

Presenilin (PS) is a heterodimer complex (PS1 and PS2) with nine transmembrane domain units and contains N-terminal fragment (NTF) and C terminal fragment (CTF) as subunits which offer two aspartate molecules required for the catalytic activity of the active GS complex.[8] Hence, PS can be said as the catalytic component of GS.[9] PEN-2 consists of three transmembrane domain units, which participates in the enzymatic hydrolysis of the PS domain to produce active GS.[10] Nicastrin consists of glycosylated large ectodomain which binds to NTF and CTF of PS complex during maturation of GS and also serves as receptors for GS substrates.[11]APH-1 is a membrane protein that consists of seven transmembrane domain units and is involved in the selectivity and stability of GS- complex.[12]

γ-secretase being a potential pharmacological target to treat ADs there are several drawbacks associated also. γ-secretase is not only involved in the proteolysis of APP but also involved in the processing of other transmembrane proteins which are involved in several biological processes, one such example is Notch protein.[13] Hence, inhibiting γ-secretase non-selectively can cause inhibition of notch processing resulting in disturbance of homeostasis in small intestine goblet cells causing gastrointestinal toxicity.[14] This becomes the major challenge for inhibitors that can selectively inhibit the processing of APP and sparing Notch processing. Several pharmacologically potent inhibitors have been developed during the past few decades for effective and selective inhibition of γ-secretase. These γ-secretase inhibitors can be broadly classified into two classes based on their chemical structure as nonpeptide inhibitors and peptides-based inhibitors.

Generally, non-peptide inhibitors reported to date are small molecules either benzodiazepine derivatives[15] or Sulfonamides derivatives.[16] Several peptide-based GSIs have been reported with various functionalities such as peptide aldehyde derivatives,[17] Hydroxyethylene dipeptide isostere derivatives,[18] Difluoroketones derivatives,[19] dipeptide analogues[20] and alpha-helical peptide analogues.[21] DAPT, a dipeptide type GSI was the first to be reported as orally active *in-vivo* to treat Aβ-42 deposition in the brain. This has also shown the property of inhibiting breast cancer cell lines and treating tumour angiogenesis.[22] Another potent inhibitor of dipeptide nature, LY-411,575 have shown better potency than DAPT *in-vivo*.[23] Koenig *et al.* have reported a derivative of the former compound named LY-450139, which becomes the

first to went for clinical trial Phase III but stopped for further use due to its side effects.[24] These all are transition state analogues of peptide hydrolysis and thus inhibits γ-secretase by blocking its active sites.[25] The transmembrane protein, APP is known to exist in helical form and mimicking this protein structure by various helical peptides can selectively inhibit the processing of APP by γ-secretase and sparing the processing of Notch by substrate docking. Considering the above-discussed factor several alpha-helical peptidomimetics have been reported as γ-secretase inhibitors with Aib residue,[26] and D and L-amino acid residues.[27] Since, "foldamer"[28] came into the picture in recent decades various beta-peptides synthetic foldamers, which have shown to exist in various helical conformations have been reported as potent GSI by Imamura *et al*.[29] The advantage of these synthetic peptidomimetics over alpha helix is their proteolytic stability or increased serum half-life making them more potent inhibitors for *in-vivo* studies.[30] Few examples of both types of potent GSIs are shown in Figure 2.

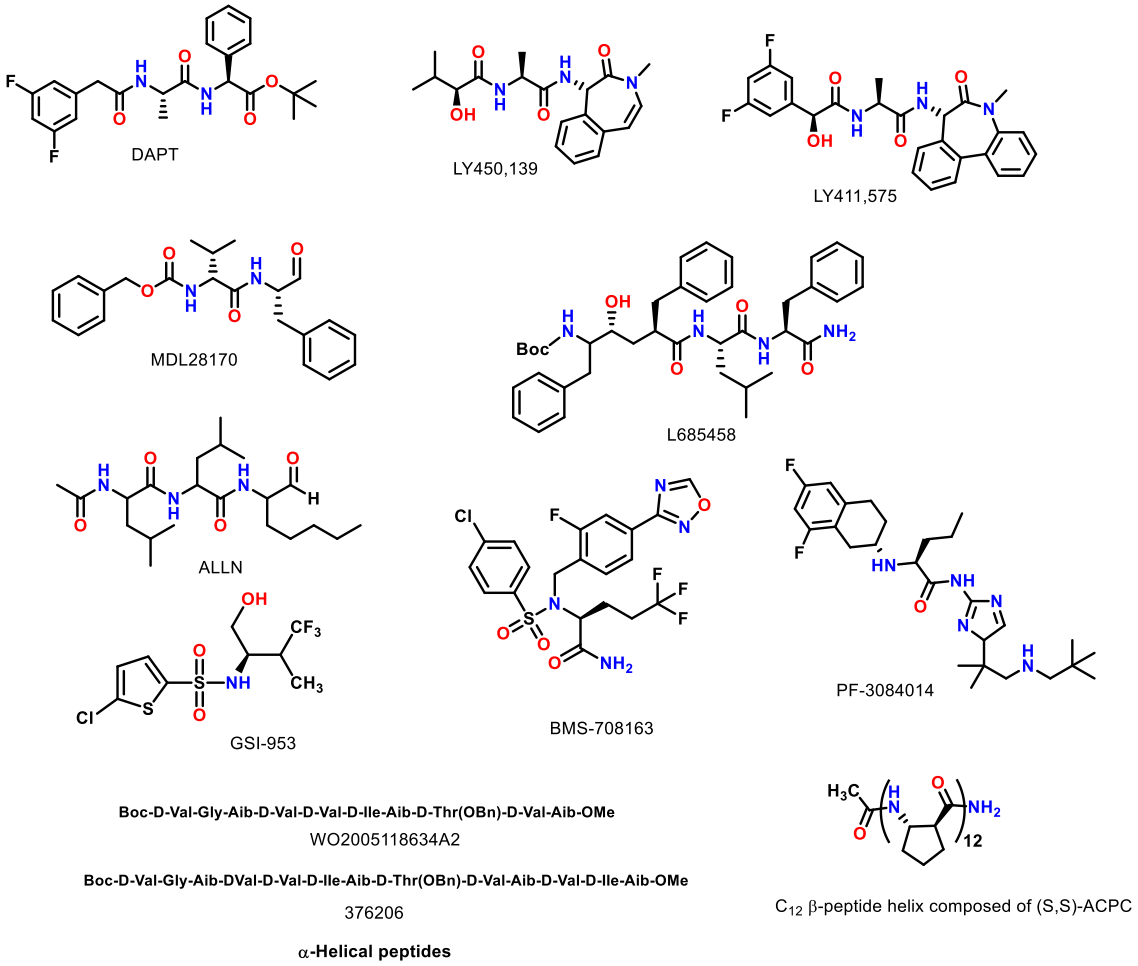


Figure 2: Few examples of potent γ-secretase inhibitors reported in the literature.

3a.2 Aim and Rationale of the present work

Our group have reported various γ^4 and functionalized γ amino acids such as β-hydroxy γ-amino acid and α,β-unsaturated γ amino acids well accommodated in C_{12} helices composed of alternate α and γ amino acids (briefly described and reported in chapter 2b).[31] Inspired by the immense biological importance of γ^4 and functionalized γ amino acid as inhibitors against various types of proteases and their predictable folding patterns, we thought of investigating inhibitory potency of various designed α, γ hybrid helices against γ-secretase. We thought of studying behavioural patterns of designed hybrid helices both *ex-vivo* and *in-vitro* to reduce $A\beta_{40}$ and $A\beta_{42}$ productions and also against notch proteolysis.

3a.3 Results and Discussion

3a.3.1 Synthesis of various *(N)*Fmoc γ- amino acids:

We have synthesized *(N)*Boc β-hydroxy γ-amino ester of phenylalanine and leucine amino acid (as per the reported procedure)[32] by mild reduction of their respective β-keto γ-amino esters using $NaBH_4$ as per the procedure described in chapter 2b section 2b.3.1. Later by saponification to obtain free acid and subsequently by Boc-deprotection and lastly by protecting the free amine using Fmoc-OSu to obtain ***(N)*Fmoc β-hydroxy γ- amino acid.**

We have synthesized *(N)*Boc α,β-unsaturated γ-amino ester by Wittig reaction on *(N)*Boc protected amino acid aldehyde, as per the procedure reported by our group.[33] Later by saponification to obtain free acid and subsequently by Boc-deprotection and lastly by protecting the free amine using Fmoc-OSu to obtain ***(N)*Fmoc α,β-unsaturated γ- amino acid.**

The above obtained *(N)*Boc α,β-unsaturated γ- amino ester was reduced to get *(N)*Boc γ^4-amino ester by using Pd/C as a catalyst. Later by saponification to obtain free acid and subsequently by Boc-deprotection and lastly by protecting the free amine using Fmoc-Osu to obtain ***(N)*Fmoc γ^4 - amino acid.** The list of synthesized *(N)*Fmoc γ-amino acids and α- amino acids to design various hybrid α and γ hybrid helix are shown in table 1. with their percentage yields are shown in Table 1.

3a.3.2 Design and synthesis of α and γ hybrid peptides

To evaluate the inhibitory potency of alternate α and various types of γ- amino acids containing hybrid peptides, we have designed six peptides (**GSI1-GSI6**). **GSI-1** and **GSI-2** are the peptides sequences that were reported in chapter 2b to show C_{12} conformation and resynthesized here investigate the role of a hydroxyl group in the comparative analysis with

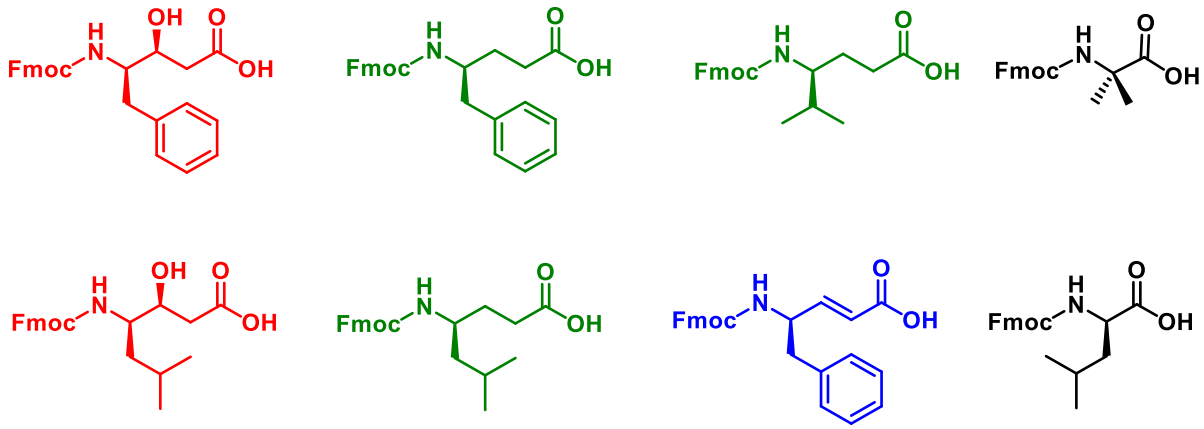


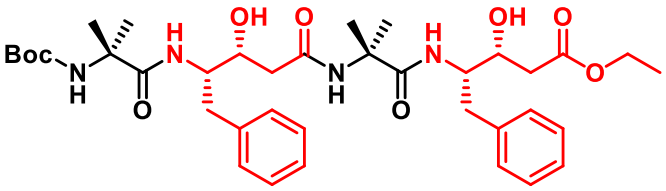
Table 1: The list of synthesized *(N)*Fmoc γ-amino acids and α- amino acids to design hybrid α and γ hybrid peptide where γ^4- amino acid residues, α,β-unsaturated γ-amino acid residues, β-hydroxy γ-amino acid residues and α- amino acid residues are shown in green, blue, red and black respectively.

other γ- amino acids for inhibiting $A\beta_{40}$ and $A\beta_{42}$ productions. The C_{12} - conformational properties of peptides **GSI-3** and **GSI-4** were already been reported by our group earlier.[34] These peptides were resynthesized here using solution-phase peptides synthesis by using a 4+2 coupling strategy, for studying their biological properties. Peptide **GSI-5** and **GSI-6** were synthesized with mixed γ^4 and β-hydroxy γ-amino acids using solid-phase peptide synthesis on Knorr amide resin and HOBt and HBTU as a coupling reagent. All the peptides were well purified through Reverse Phase HPLC on the C-18 column and purity was checked by taking HPLC traces on the analytical C-18 column. Mass of the peptides was confirmed using MALDI-TOF/TOF. Later the hybrid peptides were examined for biological activity. The list of the synthesized hybrid peptides is shown in Table 2, where γ^4- amino acid residues, α,β-unsaturated γ-amino acid residues, β-hydroxy γ-amino acid residues and α- amino acid residues are shown in green, blue, red and black respectively.

3a.3.3 *In-vitro* gamma-secretase assay

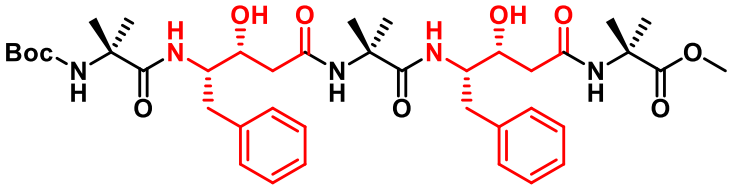
For evaluating the inhibitory potency of the designed hybrid helical peptides, *in-vitro* gamma-secretase assay has been carried out. Using recombinant C100FmH as a substrate (as described by Takahashi et al., JBC 2003),[16a] we have detected the activity of γ-secretase in CHAPSO-solubilized membrane. The substrate was incubated with 1 and 10 μM concentration of each peptide for 24 hours and then de novo Abeta was quantified by sandwiched ELISA.

GSI-1



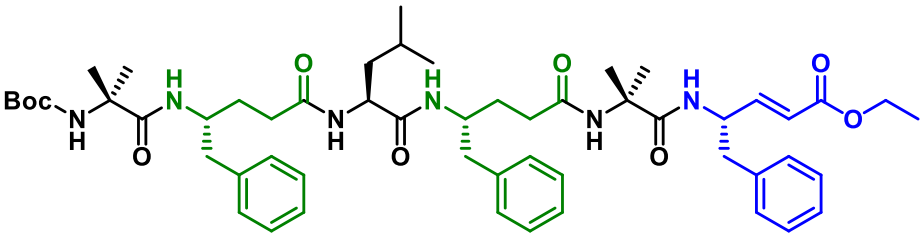
Boc-Aib-γ(R,S)Phe-Aib-γ(R,S)Phe-OEt

GSI-2



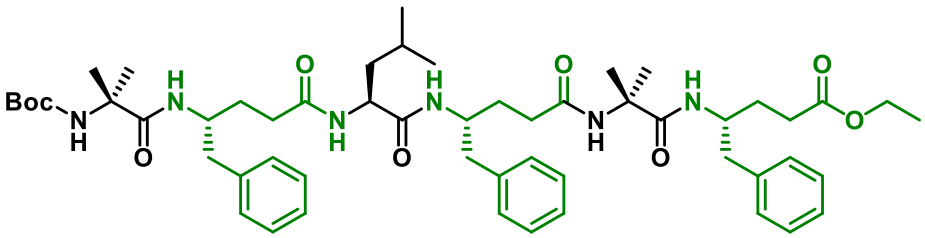
Boc-Aib-γ(R,S)Phe-Aib-γ(R,S)Phe-Aib-OMe

GSI-3



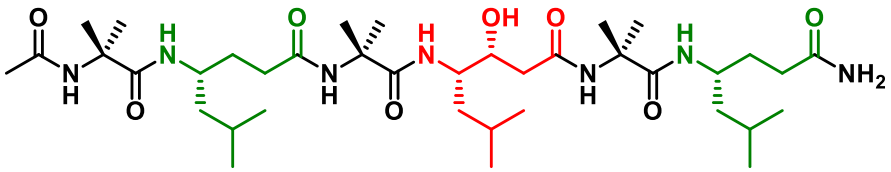
Boc-Aib-γ^4 Phe-Leu-γ^4 Phe-Aib-*dg*Phe-OEt

GSI-4



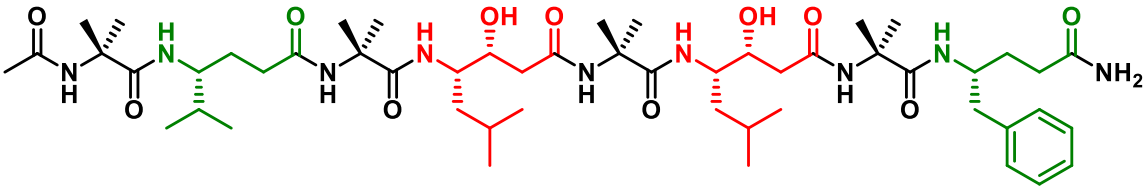
Boc-Aib-γ^4 Phe-Leu-γ^4 Phe-Aib-γ^4 Phe-OEt

GSI-5



Ac-Aib-γ^4 Leu-Aib-γ(R,S) Leu-Aib-γ^4 Leu-NH_2

GSI-6



Ac-Aib-γ^4 Val-Aib-γ(R,S) Leu-Aib-γ(R,S) Leu-Aib-γ^4 Phe-NH_2

Table 2: The sequence of the synthesized α,γ-hybrid peptides.

All the experiments are performed in triplicates (n=3). DMSO was taken as a control and S12 peptide (1μM) reported by Imamura et al., (peptide 6, JACS 2009)[29a] was taken as reference peptide inhibitor or positive control peptide.

By the initial screening of all hybrid helical peptides GSI-1 to GSI-6 *in-vitro* (shown in **Figure 3**), we observed that GSI-1 and GSI-2 which contains statines as gamma residues are slightly decreasing the production of preferentially Abeta42 but not in a concentration-dependent manner. Similar results were observed with GSI-5 and GSI-6 which contains Statine and γ^4 residues. On examining GSI-3 and GSI-4 we have observed inhibition of gamma-secretase activity. Both GSI-3 and GSI-4 inhibited Abeta40 and Abeta42 (preferentially (not much) inhibited Abeta40 production). This suggests that GSI-3 and GSI-4 can be gamma-secretase inhibitors. These results and directly relate to the perfect C_{12} helical pattern observed with alpha and gamma 4 hybrid peptides reported earlier by our group and slight distortion in case of incorporating more than one statine residue in alpha gamma hybrid helical peptides as described in detail in chapter 2b.

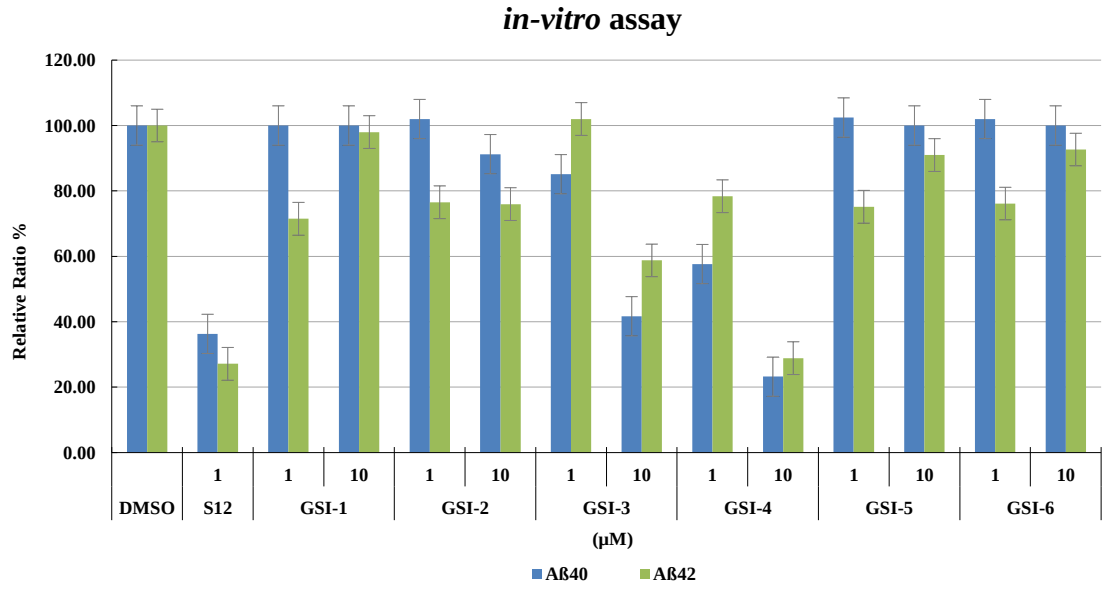


Figure 3: *In-vitro* relative γ-secretase activities at 1 and 10 μM concentration of each peptide (GSI-1 to GSI-6) are shown as the ratio (%) to the control DMSO (left), taken as 100% and S12 peptide at 1μM concentration as positive control peptide.

3a.3.4 Cell-based assay using NLNTK cell line

For investigating the inhibitory potency and selectivity of the designed peptides GSI-1 to GSI-6 for gamma-secretase sparing the notch activity, a cell-based assay was performed using HEK293-based reporter cell lines called NLNTK. These NLNTK cell lines overexpress Swedish mutant APP, truncated Notch, Notch-responsible luciferase reporter and CMV driven EGFP. Using this cell line, we are simultaneously able to analyse Abeta40, Abeta42, Notch activity (as luciferase) and cell viability (as EGFP). NLNTK cell lines were cultured in the presence of 1μM and 10μM concentration of each peptide inhibitor (GSI-1 to GSI-6). Abeta40 and Abeta 42 were quantified using Sandwich ELISA, notch activity via luminescence and EGFP via fluorescence measurement. Each experiment was performed in triplicates (n=3). DMSO is taken as control and S12 peptide as a positive control at 1μM concentration.

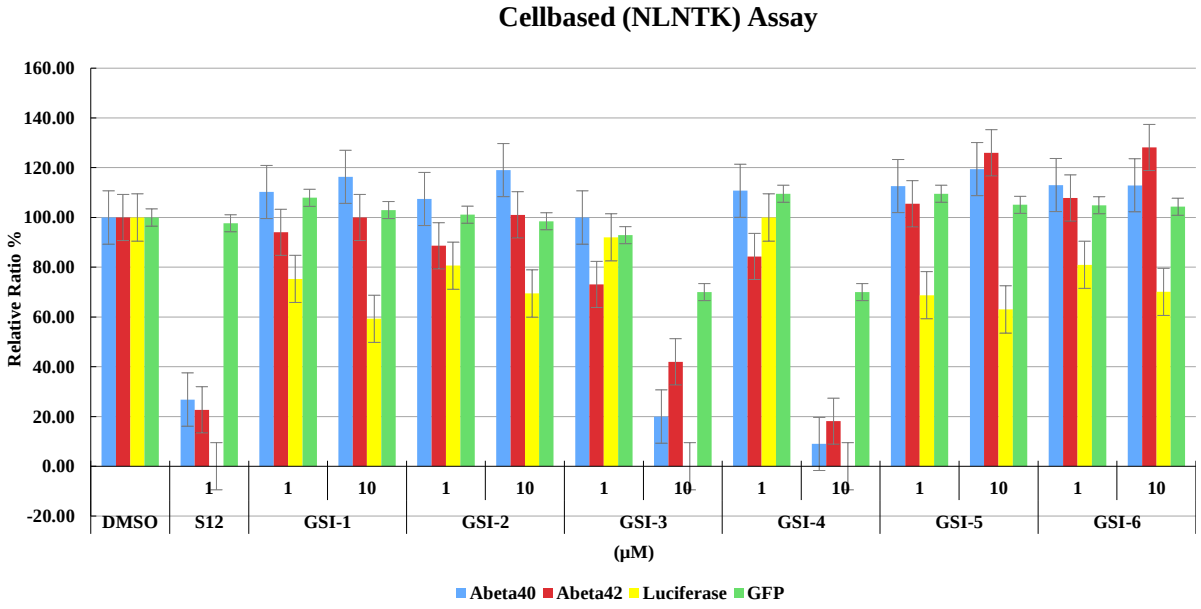


Figure 4: *Ex-vivo* relative γ-secretase activities, notch activity and cell viability using NLNTK cell lines at 1 and 10 μM concentration of each peptide (GSI-1 to GSI-6) are shown as the ratio (%) to the control DMSO (left), taken as 100% and S12 peptide at 1μM concentration as positive control peptide. (In the case of notch activity S12 peptide notch activity at 1μM concentration is taken as 100%).

We have observed similar results as observed in the *in-vitro* assay that only GSI-3 and GSI-4 are showing inhibition against gamma-secretase. Both GSI-3 and GSI-4 inhibited Abeta40 and Abeta42 (preferentially (not much) inhibited Abeta40 production). But rest of the peptides have

shown weak inhibition on notch activity but not on Abeta40/42 production, shown above in Figure 4.

For that reason, GSI-3 and GSI-4 were screened with various concentrations of 0.1, 0.3, 1, 3, 10 μM concentrations on NLNTK cell lines. We observed that inhibition of Abeta formation preferentially Abeta40 but not in a concentration-dependent manner. GSI-3 and GSI-4 are significantly inhibiting at 10μM concentration but showing cytotoxicity too at this active concentration. The peptides showed weak inhibition on Notch signalling, hence suggesting they are not notch sparing, shown below in Figure 5.

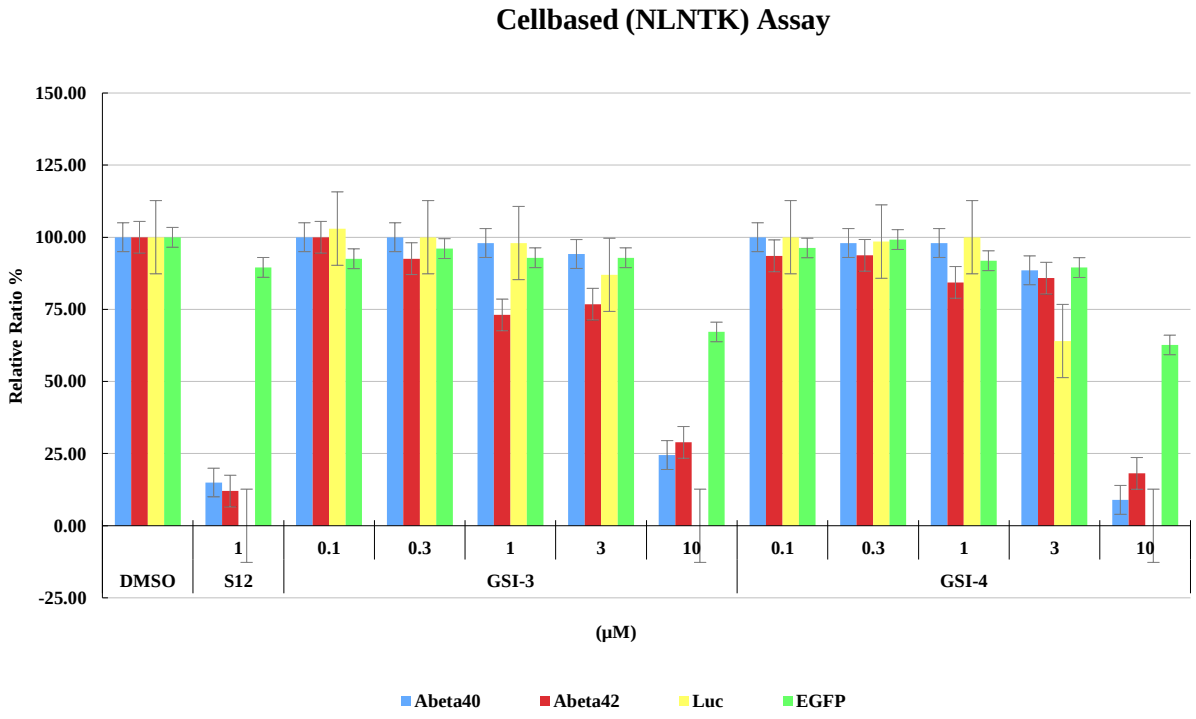


Figure 5*: Ex-vivo* relative γ-secretase activities, notch activity and cell viability using NLNTK cell lines at 0.1, 0.3,1, 3 and 10 μM concentration of active peptide GSI-3 to GSI-4 are shown as the ratio (%) to the control DMSO (left), taken as 100% and S12 peptide at 1μM concentration as positive control peptide. (In the case of notch activity S12 peptide notch activity at 1μM concentration is taken as 100%).

3a.3.5 Cell-based assay using Neuro2a cell line

For evaluating the inhibitory potency of active inhibitor GSI-3 and GSI-4, an *ex-vivo* assay was carried out using Neuro2a cell lines. Neuro2a cell line is derived from mouse neuroblastoma

and expresses APP, BACE and BACE and gamma-secretase. Thus, we can measure endogenous Abeta from endogenous APP. On incubating the peptides GSI3 and GSI-4 with Neuro2a cells at 1 or 10 μM concentration for 24 hours and measured secreted Abeta in the conditioned medium, we have observed similar results that decrease in $A\beta_{40}$ and $A\beta_{42}$ productions more in case of $A\beta_{40}$, suggesting that these peptides are showing inhibition *ex-vivo* at 10 μM concentration. All the experiments were performed in triplicates (n=3). Data is shown in Figure 6.

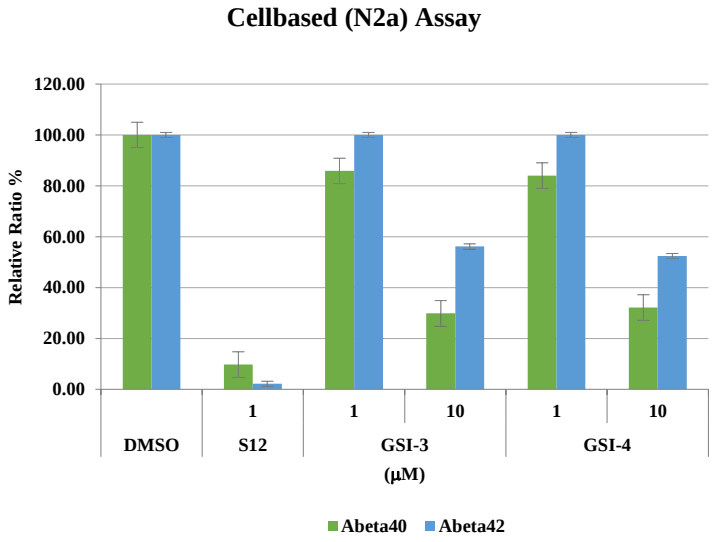


Figure 6: Relative γ-secretase activities *ex-vivo* with N2a cell lines at 1 and 10 μM concentration of active peptide GSI-3 to GSI-4 are shown as the ratio (%) to the control DMSO (left), taken as 100% and S12 peptide at 1μM concentration as positive control peptide.

3a.3.6 Western blot analysis of Neuro2a cell lysate

We analysed the cell lysates of the N2a cell line at 10 μM concentration by western blotting. We observed that protein amounts of cells treated with GSI-3 and GSI-4 were lesser than that of mock-treated cells. Thus, suggesting that GSI-3 and GSI-4, harboured significant cell toxicity at their inhibitory concentration of 10 μM. Supporting this notion, the band pattern of Nicastrin and APP holoprotein was different from the control peptide S12 and intensities were low, hence showing cytotoxicity. No accumulation of C-terminal fragment of APP was observed with GSI-3 and GSI-4 and intense with S12 peptide, shown in Figure 7. As S12 was not toxic to Neuro2a cells, the toxicity of GSI-3 and GSI-4 is independent of the inhibition of the gamma-secretase activity.

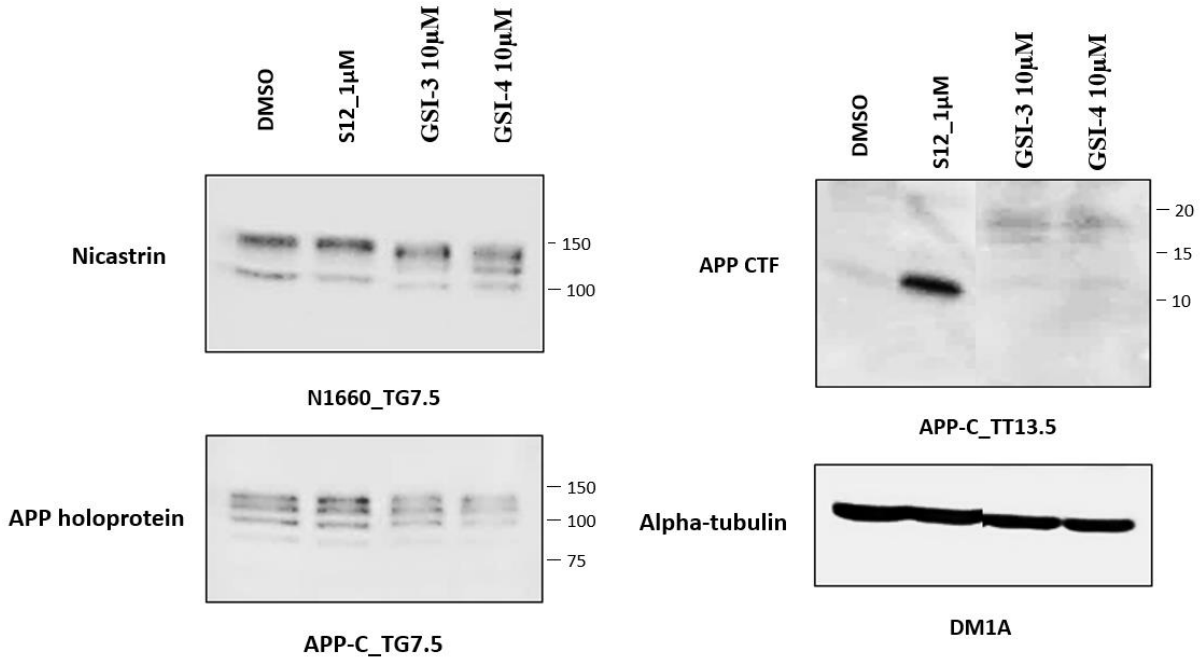


Figure 7: Effect of GSI-3 and GSI-4 at 10 μM concentration on the levels of Nicastrin, APP holoprotein and APP CTF secreted from N2a cell lines. These proteins were detected by immunoblotting using various antibodies (mention below in each case).

3a.4 Conclusion

We are reporting here, the design, synthesis and inhibitory potencies of α,γ-hybrid helices composed of γ^4 and functionalized γ-amino acids. The hybrid peptide containing more of γ^4 residues or with perfect C_{12} helical pattern have shown better inhibition suggesting the relationship of structure to their functions. Also, GSI-3 and GSI-4 have shown inhibition of $A\beta_{40}$ and $A\beta_{42}$ both *in-vitro* and *ex-vivo* assay. From these data, we concluded that GSI-3 and GSI-4 can inhibit the gamma-secretase activity directly, but have also shown cell toxicity at working concentration. This may be because of more aromatic hydrophobic amino acid residue γ^4Phe. In future, relacing γ^4Phe with other γ^4 amino acid residues for designing active gamma-secretase inhibitors can be looked upon in detail.

3a.5 Experimental section

3a.5.1 General experimental details:

All α-amino acids, Ethyl diazoacetate, Tin chloride and Di-t*ert*-butyl dicarbonate were obtained from Merck. 2-Mercaptobenzothiazole (2-MBT), Fmoc-Osu, DCC, $NaBH_4$, HPLC

grade acetonitrile, dry DCM and dry THF were purchased from Spectrochem. Column chromatography was achieved on silica gel of 120-200 mesh from Merck. Thin-layer chromatography (TLC) was used to monitor reaction using Merck 60 F_{254} precoated silica gel plates. For visualization UV light and ninhydrin or phosphomolybdic acid (PMA) stains were used then charring on a hot plate. Melting points were recorded on Veego VMP-DS hot stage apparatus. Specific rotations were recorded at ambient temperature on the Rudolph Analytical Research instrument using $CHCl_3$ and MeOH as solvents. Reverse Phase-HPLC of Waters was used to purify peptides using Acetonitrile/H_2O gradient with 0.1% TFA from the C-18 column. Mass was analysed on Applied Biosciences with MALDI TOF/TOF on positive ion mode.

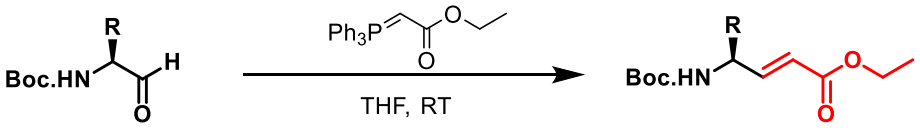
NMR spectroscopy

Jeol 400 MHz (or 100 MHz for ^{13}C) spectrometer were used for recording ^{1}H spectra and ^{13}C NMR. The chemical shifts (δ) and coupling constants (J) were reported in ppm and Hz, respectively. The residual solvents signals were used as internal reference ($CDCl_3$ δ_H, 7.24 ppm, δ_c 77.0 ppm).

All α-amino acids, Ethyl diazoacetate, Tin chloride, HBTU, HOBt, Knorr-Amide resin and Di-t*ert*-butyl dicarbonate were obtained from Merck. 2-Mercaptobenzothiazole (2-MBT), Fmoc-Osu, DCC, $NaBH_4$, HPLC grade acetonitrile, dry DCM and dry THF were purchased from Spectrochem. Column chromatography was achieved on silica gel of 120-200 mesh from Merck. Thin-layer chromatography (TLC) was used to monitor reaction using Merck 60 F_{254} precoated silica gel plates. For visualization UV light and ninhydrin or phosphomolybdic acid (PMA) stains were used then charring on a hot plate. Mass was analysed on Applied Biosciences with MALDI TOF/TOF on positive ion mode.

3a.5.2. General procedure for the synthesis of monomers:

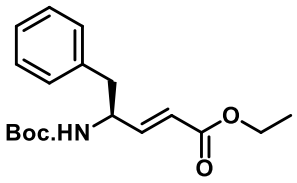
General Procedure for the Synthesis of *N*-Boc α,β-unsaturated γ-amino esters:



Distilled THF (15 ml) was used to dissolve *N*-Boc β-amino aldehyde (10 mmol) and to that Wittig ylide (10 mmol) was added at room temperature and kept on stirring for 6 hours. TLC

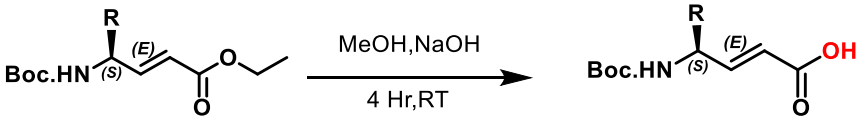
was performed for monitoring the reaction. Once the reaction is completed, the crude mixture was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure *N*-Boc α,β-unsaturated γ-amino ester.

Characterization of *(N)*Boc α,β-unsaturated γ- amino ester of phenylalanine (Boc-*dg*F):



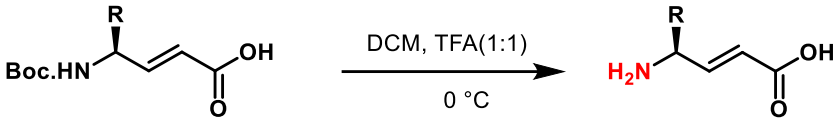
(S, E)-ethyl 4-(tert-butoxycarbonylamino)-5-phenylpent-2-enoate: White powder. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.3006-7.1423(m, 5H), 6.9188-6.8670(dd, *J* = 5.04, *J* = 11, 1H), 5.8586-5.8195 (d, *J* = 17.4, 1H), 4.5956 (b, 1H), 4.5244 (b, 1H), 4.1893-4.1358 (q, *J* = 6.88, 2H), 2.922-2.8555 (m, 2H), 1.3763 (s, 9H), 1.2739-1.2381 (t, *J* = 7.3, 3H) ; **^{13}C NMR** (100MHz, $CDCl_3$) δ 166.1454, 154.9128, 147.5676, 136.3376, 129.3685, 128.5495, 126.8338, 121.0495, 79.8302, 60.4434, 52.1647, 40.8203, 28.2629, 14.1884; **MALDI TOF/TOF** m/z Calcd. For $C_{18}H_{25}NO_4$ [M+Na]+ 342.1681, observed 342.1657.

General Procedure for Synthesis of *N*-Boc α,β-unsaturated γ-amino acids:



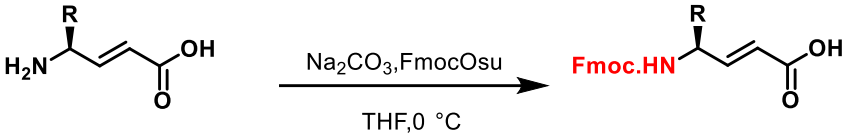
MeOH (50 ml) was used to dissolve pure *N*-Boc α,β-unsaturated γ-amino ester (5 mmol) and was allowed to stir to get a clear solution. 3eq. of 1NaOH (15 ml, 15 mmol) was added to the reaction mixture dropwise and kept for the next 2 hours at room temperature. TLC was performed from time to time for monitoring the reaction. Once the reaction is completed it was neutralized using 10% HCl. Later, the reaction mixture was kept beneath a vacuum to evaporate the MeOH used in the reaction. The aqueous layer was extracted thrice using ethyl acetate. The combined organic extract was washed with brine solution followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate to obtain pure *N*-Boc α,β-unsaturated γ-amino acid, no further purification required.

General procedure for the synthesis of NH_2-α,β-unsaturated γ-amino acids:



DCM (10 ml) was used to dissolve *N*-Boc α,β-unsaturated γ-amino acid (10 mmol) and was allowed to cool to 0 °C using an ice bath. 10 ml of TFA was added to the reaction mixture slowly and kept for the next 30 mins. Once the reaction is completed TFA was co-evaporated using DCM (15ml five times) and recrystallized using DCM and hexane to obtain solid triflate salt of NH_2-α,β-unsaturated γ-amino acid which can be used without purification directly for the next step.

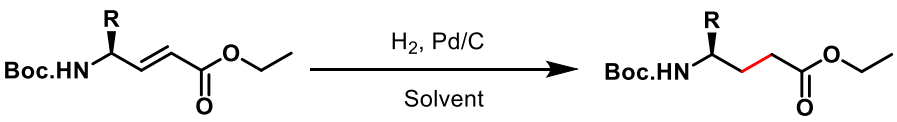
General procedure synthesis of *N*-Fmoc α,β-unsaturated γ-amino acids:



THF (15 ml) was used to dissolve the above obtained NH_2-α,β-unsaturated γ-amino acid(5mmol) and was allowed to cool to 0 °C using an ice bath. 10% sodium carbonate (≅ 25 ml) was added to the reaction mixture step by step to obtain a basic pH of approximately 9. Later, Fmoc-Osu (1.68 g, 5 mmol dissolved in 15 ml THF) was added to the reaction mixture step by step and kept for 6 hours.

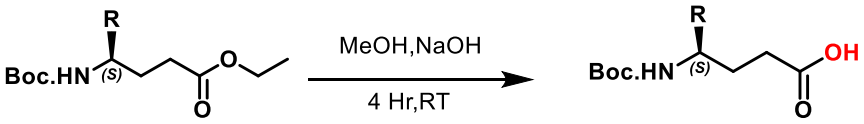
Once the reaction is completed it was neutralized using 5% HCl. Later, the reaction mixture was kept beneath a vacuum to evaporate the THF used for the reaction. The aqueous layer was extracted thrice using ethyl acetate. The combined organic extract was washed further with 10% Na_2CO_3 and later with brine followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure solid *N*-Fmoc α,β-unsaturated γ-amino acids.

General Procedure for the Synthesis of *N*-Boc γ^4-amino esters:



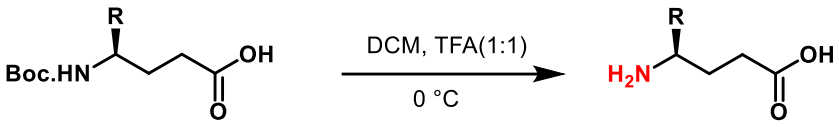
Distilled THF (15 ml) was used to dissolve *N*-Boc α,β-unsaturated γ-amino ester (10 mmol) and activated Pd/C (20% by weight) under nitrogen atmosphere. Later, kept under hydrogen gas kept in a hydrogen balloon at room temperature and kept on stirring for 6 hours. TLC was performed for monitoring the reaction. Once the reaction is completed, the balloon was removed and the crude mixture was filtered through a celite bed and the filtrate was kept beneath the *vacuum* to concentrate to obtain a gummy mixture. This organic mixture was recrystallized using cold diethyl ether and used for further steps.

General Procedure for Synthesis of *N*-Boc γ^4-amino acids:



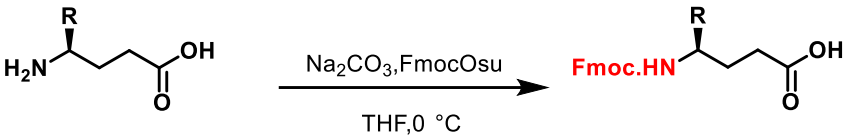
MeOH (50 ml) was used to dissolve pure *N*-Boc γ^4-amino ester (5 mmol) and was allowed to stir to get a clear solution. 3eq. of 1NaOH (15 ml, 15 mmol) was added to the reaction mixture dropwise and kept for the next 2 hours at room temperature. TLC was performed from time to time for monitoring the reaction. Once the reaction is completed it was neutralized using 10% HCl. Later, the reaction mixture was kept beneath a vacuum to evaporate the MeOH used in the reaction. The aqueous layer was extracted thrice using ethyl acetate. The combined organic extract was washed with brine solution followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate to obtain pure *N*-Boc γ^4-amino acid, no further purification was required.

General procedure for the synthesis of NH_2- γ^4-amino acids:



DCM (10 ml) was used to dissolve *N*-Boc γ^4-amino acid (10 mmol) and was allowed to cool to 0 $^{\circ}$C using an ice bath. 10 ml of TFA was added to the reaction mixture slowly and kept for the next 30 mins. Once the reaction is completed TFA was co-evaporated using DCM (15ml five times) and recrystallized using DCM and hexane to obtain solid triflate salt of NH_2 γ^4-amino acid which can be used without purification directly for the next step.

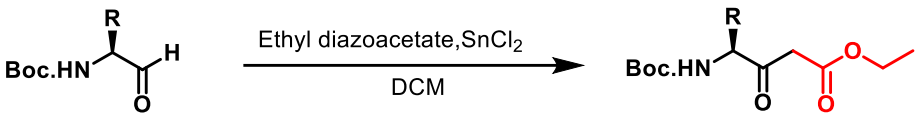
General procedure synthesis of *N*-Fmoc γ^4-amino acids:



THF (15 ml) was used to dissolve the above obtained NH_2 γ^4-amino acid (5mmol) and was allowed to cool to 0 ˚C using an ice bath. 10% sodium carbonate (≅ 25 ml) was added to the reaction mixture step by step to obtain a basic pH of approximately 9. Later, Fmoc-Osu (1.68 g, 5 mmol dissolved in 15 ml THF) was added to the reaction mixture step by step and kept for 6 hours.

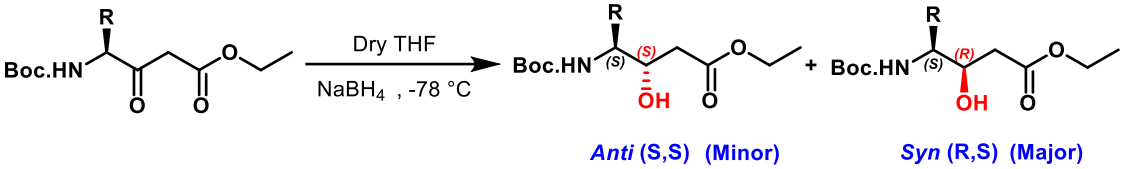
Once the reaction is completed it was neutralized using 5% HCl. Later, the reaction mixture was kept beneath a vacuum to evaporate the THF used for the reaction. The aqueous layer was extracted thrice using ethyl acetate. The combined organic extract was washed further with 10% Na_2CO_3 and later with brine followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure solid *N*-Fmoc γ^4-amino acids.

General Procedure for the Synthesis of *N*-Boc β-keto γ-amino esters:



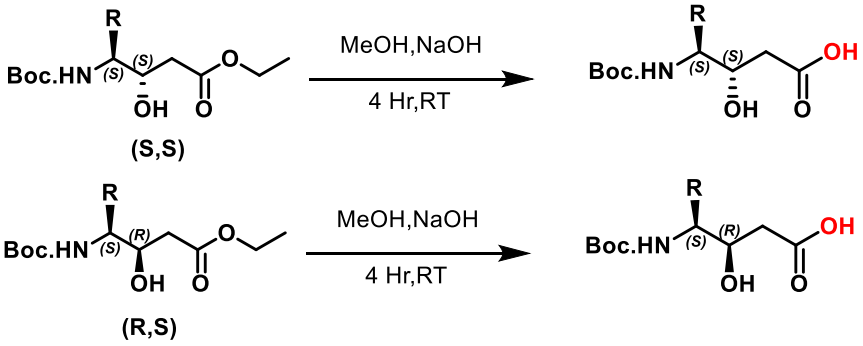
DCM (15 ml) was used to dissolve the above formed *N*-Boc β-amino aldehyde and to that Tin (II) Chloride (20 mol%) was added at room temperature. After 5 mins, 15% Ethyl diazoacetate in toluene (10.05 mmol, 7.5 ml) was added slowly drop by drop and instant evolution of nitrogen can be seen till the reaction completes. TLC was performed for monitoring the reaction. Once the reaction is completed it was neutralized using 5 % HCl and the aqueous layer was extracted thrice using DCM. The combined organic extract was washed with brine followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure *N*-Boc β-keto ester.

General Procedure for Synthesis of *N*-Boc β-hydroxy γ-amino esters:



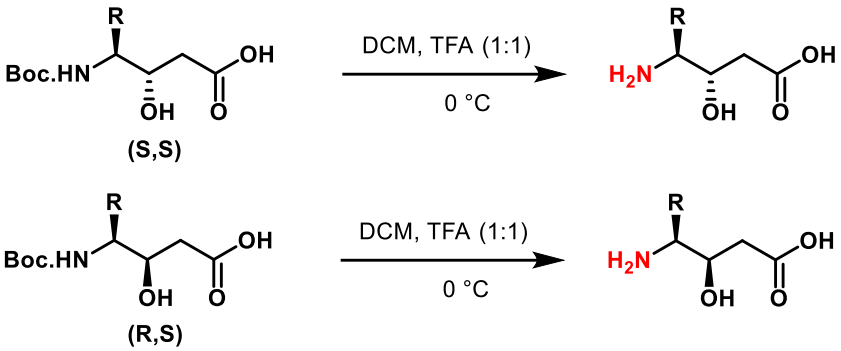
Dry THF (30ml) was used to dissolve the above obtained pure *N*-Boc β-keto γ ester (10 mmol) at inert conditions. By using dry ice and acetone the reaction temperature was brought to -78 ˚C. Solid $NaBH_4$ (0.529 g,14 mmol) was added to the reaction mixture at one step and kept for the next 4 hours. TLC was performed from time to time for monitoring the reaction. Once the reaction is completed it was neutralized using 5 % HCl. Later, the reaction mixture was kept beneath a vacuum to evaporate the THF used for the reaction. The aqueous layer was extracted thrice using ethyl acetate. The combined organic extract was washed further with brine solution followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure both *syn* and *anti N*-Boc β-hydroxyl γ-amino ester (statine) conformers separately.

General Procedure for Synthesis of *N*-Boc β-hydroxy γ-amino acids:



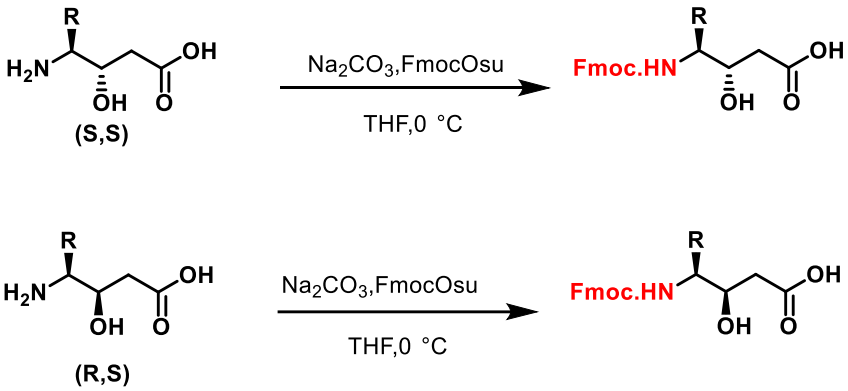
MeOH (50 ml) was used to dissolve pure *N*-Boc β-hydroxy γ amino ester (5 mmol) and was allowed to stir to get a clear solution. 3eq. of 1NaOH (15 ml, 15 mmol) was added to the reaction mixture dropwise and kept for the next 2 hours at room temperature. TLC was performed from time to time for monitoring the reaction. Once the reaction is completed it was neutralized using 10% HCl. Later, the reaction mixture was kept beneath a vacuum to evaporate the MeOH used in the reaction. The aqueous layer was extracted thrice using ethyl acetate. The combined organic extract was washed with brine solution followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate to obtain pure *N*-Boc β-hydroxy γ amino acid, no further purification was required.

General procedure for the synthesis of NH_2-β-hydroxy γ-amino acids:



DCM (10 ml) was used to dissolve *N*-Boc β-hydroxy γ amino acid (10 mmol) and was allowed to cool to 0 °C using an ice bath. 10 ml of TFA was added to the reaction mixture slowly and kept for the next 30 mins. Once the reaction is completed TFA was co-evaporated using DCM (15ml five times) and recrystallized using DCM and hexane to obtain solid triflate salt of NH_2-β-hydroxy γ-amino acid which can be used without purification directly for the next step.

General procedure synthesis of *N*-Fmoc β-hydroxy γ-amino acids:



THF (15 ml) was used to dissolve the above obtained NH_2-β-hydroxy γ-amino acid and was allowed to cool to 0 °C using an ice bath. 10% sodium carbonate (25 ml) was added to the reaction mixture step by step to obtain a basic pH of approximately 9. Later, Fmoc-Osu (1.68 g, 5 mmol dissolved in 15 ml THF) was added to the reaction mixture step by step and kept for 6 hours. Once the reaction is completed it was neutralized using 5% HCl. Later, the reaction mixture was kept beneath a vacuum to evaporate the THF used for the reaction. The aqueous layer was extracted thrice using ethyl acetate. The combined organic extract was washed further with 10% Na_2CO_3 and later with brine followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture

was further purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure solid *N*-Fmoc β-hydroxy γ-amino acid.

3a.5.3 Peptide synthesis and purification

General procedure for the synthesis of GSI-1 and GSI-2

(Procedure for synthesis and characterization of peptides are reported in chapter 2b, section 2b.5.2.)

General procedure for the synthesis of Boc-Aib-γ^{4}Phe-Leu- γ^{4}Phe-Aib-dgPhe-OEt (GSI-3):

Hexapeptide was synthesized by a conventional way of solution-phase peptide synthesis with fragment condensation strategy. Deprotection of Boc(N)-termini was performed by using trifluoracetic acid (TFA) and C-terminal ester by saponification method. Hexafluorophosphate Benzotriazole Tetramethyl Uronium (HBTU) and 1-hydroxy benzotriazole (HOBt) were used as coupling agents in the presence of N-Methyl-2-Pyrrolidone (NMP) as a base in N, N-Dimethylformamide (DMF) as a solvent for overnight. Hexapeptide was synthesized using a 4+2 condensation strategy involving Boc-Aib-γ^{4}Phe-Leu-γ^{4}Phe-COOH and NH_2-Aib-dgPhe-OEt. After the completion of the reaction, the reaction mixture was extracted in ethyl acetate, later acid wash and base wash was given thrice each, with a brine wash at the end. The organic layer was evaporated under a vacuum to obtain a gummy mixture. The peptide was purified using RP-HPLC, C_{18} - column using Methanol/water solvent system.

Characterization of Boc-Aib-γ^{4}Phe-Leu- γ^{4}Phe-Aib-dgPhe-OEt (GSI-3):

White crystalline powder. **^{1}H NMR** (500 MHz, $CDCl_3$) δ 8.59-8.57 (d, *J* = 8.3, 1H), 7.70-7.68 (d, *J* = 10.2, 1H), 7.48-7.47 (d, *J* = 3.85,1H), 7.48-7.07 (m, 17H), 6.53-6.49 (dd, *J* = 17.7, *J* = 2.1, 1H), 6.07-6.05 (d, *J* = 10.3, 1H), 5.23 (s, 1H), 5.13-4.97 (m, 1H), 4.26-4.13 (m, 4H), 3.80-3.76 (m, 1H), 2.98-2.60 (m, 6H), 2.29-2.10 (m, 4H), 1.76 (s, 3H),1.71-1.65 (m, 2H), 1.59 (s, 3H), 1.55-1.48 (m, 2H), 1.45 (s, 3H), 1.43 (s, 9H), 1.30 (s, 3H), 1.29-1.26 (t, *J* = 7, 3H), 1.20-1.14(m, 2H), 0.99 (s, 3H), 0.94-0.88 (m, 2H), 0.83-0.82 (d, *J* = 6.6, 3H), 0.78-0.77 (d, *J* = 6.7, 3H); **MALDI TOF/TOF** m/z Calcd. for $C_{54}H_{76}N_6O_9$ [M+Na]+ 975.5571, observed 975.9615.

General procedure for the synthesis of Boc-Aib-γ⁴Phe-Leu-γ⁴Phe-Aib- γ⁴Phe-OEt (GSI-4):

Hexapeptide was synthesized by a conventional way of solution-phase peptide synthesis with fragment condensation strategy. Deprotection of Boc(N)-termini was performed by using trifluoracetic acid (TFA) and C-terminal ester by saponification method. Hexafluorophosphate Benzotriazole Tetramethyl Uronium (HBTU) and 1-hydroxy benzotriazole (HOBt) were used as coupling agents in the presence of N-Methyl-2-Pyrrolidone (NMP) as a base in N, N-Dimethylformamide (DMF) as a solvent for overnight. Hexapeptide was synthesized using a 4+2 condensation strategy involving Boc-Aib-γ⁴Phe-Leu-γ⁴Phe-COOH and NH_2-Aib-γ⁴Phe-OEt. After the completion of the reaction, the reaction mixture was extracted in ethyl acetate, later acid wash and base wash was given thrice each, with a brine wash at the end. The organic layer was evaporated under a vacuum to obtain a gummy mixture. The peptide was purified using RP-HPLC, C_{18} - column using Methanol/water solvent system.

Characterization of Boc-Aib-γ⁴Phe-Leu- γ⁴Phe-Aib- γ⁴Phe-OEt (GSI-4):

White crystalline powder. **^{1}H NMR** (500 MHz, $CDCl_3$) δ 8.08-8.06 (d, J = 9.25, 1H), 7.66-7.64 (d, J = 10.2, 1H), 7.39-7.38 (d, J = 4,1H), 7.39-7.09 (m, 15H), 7.01 (s, 1H), 5.96-5.94 (d, J = 10.3, 1H), 5.05 (s, 1H), 4.26-4.13 (m, 5H), 3.85-3.82 (m, 1H,), 2.99-2.59 (m, 6H), 2.49-2.02 (m, 6H), 1.99-1.83(m, 6H), 1.72-1.58(m, 6H), 1.56-1.48(m, 6H),1.60 (s, 3H), 1.44 (s, 9H), 1.43 (s, 3H), 1.29 (s, 3H), 1.27-1.22 (m, 5H), 1.13-1.05 (m, 1H), 1.00 (s, 3H), 0.86-0.85 (d, J = 6.6, 3H), 0.81-0.80 (d, J = 6.7, 3H); **MALDI TOF/TOF** m/z Calcd. For $C_{54}H_{78}N_6O_9$ [M+Na]+ 977.5728, observed 977.8813.

3a.5.3 Solid-phase peptide synthesis and purification

General procedure for the synthesis of peptides GSI-5 and GSI-6:

The peptides **GSI-5** and **GSI-6** were synthesized on Knorr amide resin (0.2 mmol scales) using standard Fmoc-chemistry. As coupling reagents, HBTU/HOBt were used. Kaiser test was performed to monitor coupling reactions. After completion of the desired couplings, TFA: water: phenol: TIPS (95:2:2:1) was used as a scavenger cocktails mixture to cleave the peptides from resins. Later, the resin was filtered and the cleavage mixture or the filtrate was co-evaporated using DCM beneath the vacuum to get a gummy product. The product was recrystallized using diethyl ether to obtain a white solid. The obtained white solid was dissolved in water and purified through reverse phase HPLC on a C18 column using an ACN/H_2O with

a 0.1% TFA solvent system with 2mL/min flow rate. The purity of the peptides was further investigated by passing from an analytical C18 column with the same gradient system with 0.75ml/min flow rate. The mass of the peptide was recorded using **MALDI TOF/TOF.**

4b.5.4 Procedure for *in-vitro* gamma-secretase activity

Recombinant C100FmH (C100-FLAG-myc-6xHis) as substrate was prepared as per the reported protocol by Takahashi et al., JBC 2003.[16a] Using this substrate, the inhibitory potencies of the synthesized hybrid peptides GSI-1 to GSI-6 on the activity of gamma-secretase were detected *in-vitro*. CHAPSO-solubilized membrane fraction (250 μg/mL), purified substrate recombinant C100FmH and protease and inhibitor mixture were incubated together in γ- buffer (HEPES buffer with 5 mM EDTA, 5 mM 1,10-phenanthroline, 0.25% CHAPSO, 10 mg/mL phosphor Amidon, 0.01% phosphatidylcholine) for 24 hours at 37 °C. After 24 hours of incubation time, each sample was centrifuged and supernatants were analysed by sandwiched BNT77/BA27 or BNT77/BC05 ELISA. This quantifies the *de novo* generated Abeta40 and Abeta42 peptide fragments. The concentration of 1μM and 10 μM of each helical hybrid peptide Inhibitor from GSI-1 to GSI-6 were examined for inhibiting gamma-secretase activity and each experiment were performed in triplicates (n=3).

4b.5.4 Procedure for *ex-vivo* gamma-secretase activity

For *ex-vivo* assay, the protocol reported by Kurosumi *et al.* (BMCL 2010) was followed.[17a] NLNTK cell lines and N2a cell lines were allowed to culture for 24-30 hours in the presence of various concentrations of peptides. After the incubation time, culture media were centrifuged and subjected to BNT77/BA27 or BNT77/BC05 sandwich ELISA to quantify $A\beta_{40}$ and $A\beta_{42}$ generated. In the case of NLNTK cell lines, Notch activity was detected by luminescence was measured and for checking cell viability fluorescence was measured. All the experiments were performed in triplicates (n=3). The cell lysate of N2a cell lines with 10 μM of GSI-3 and GSI-4 were subjected for Immunoblotting against various antibodies (shown in brackets) to check the levels of Nicastrin (N1660_TG7.5), APP- holoprotein (APP-C_TG7.5), C-terminal factor of APP (APP-C_TT13.5) and α- tubulin (DM1A) proteins.

3a.6 References

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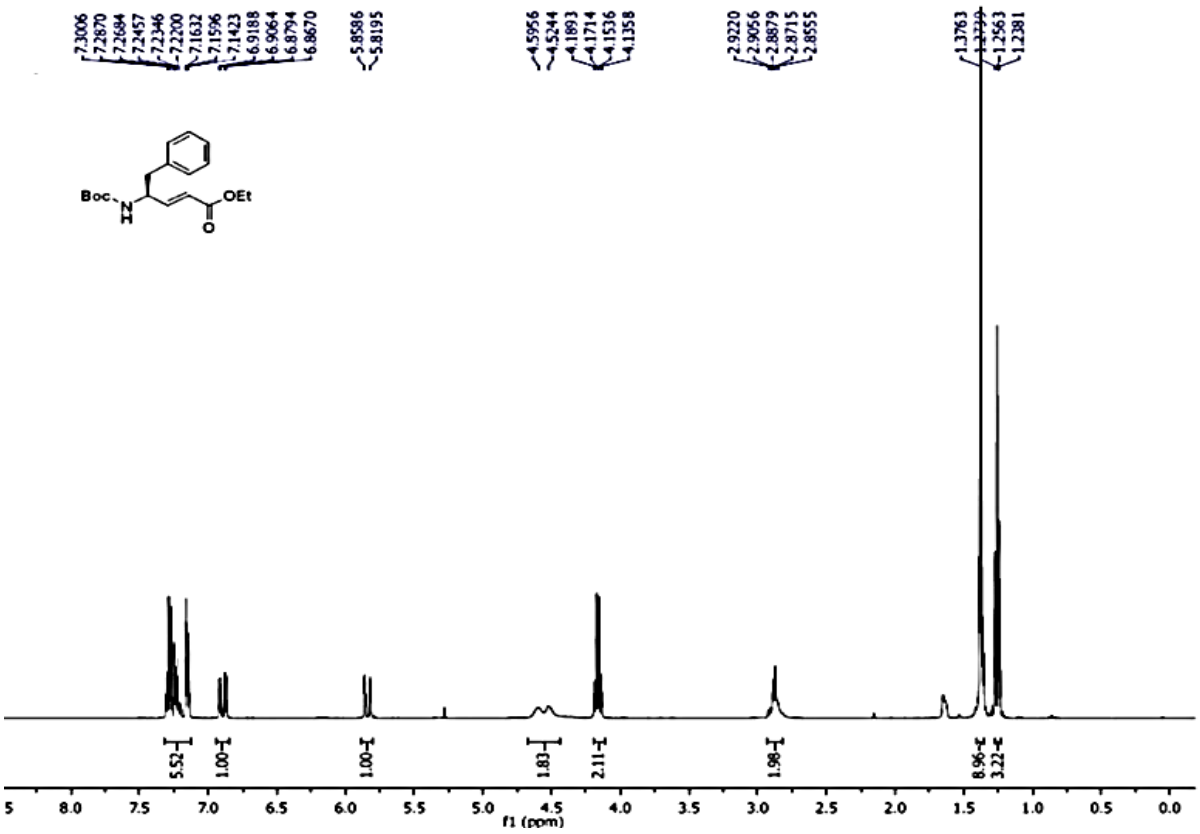
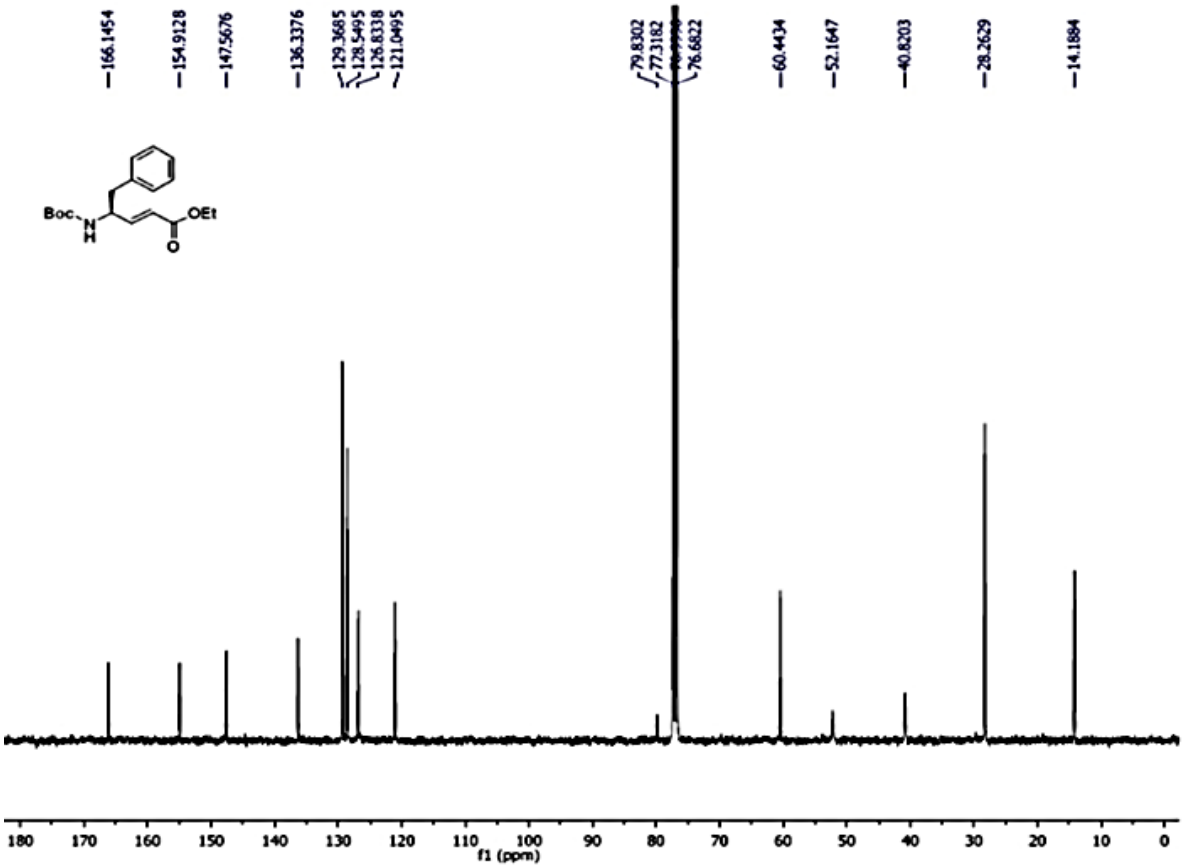
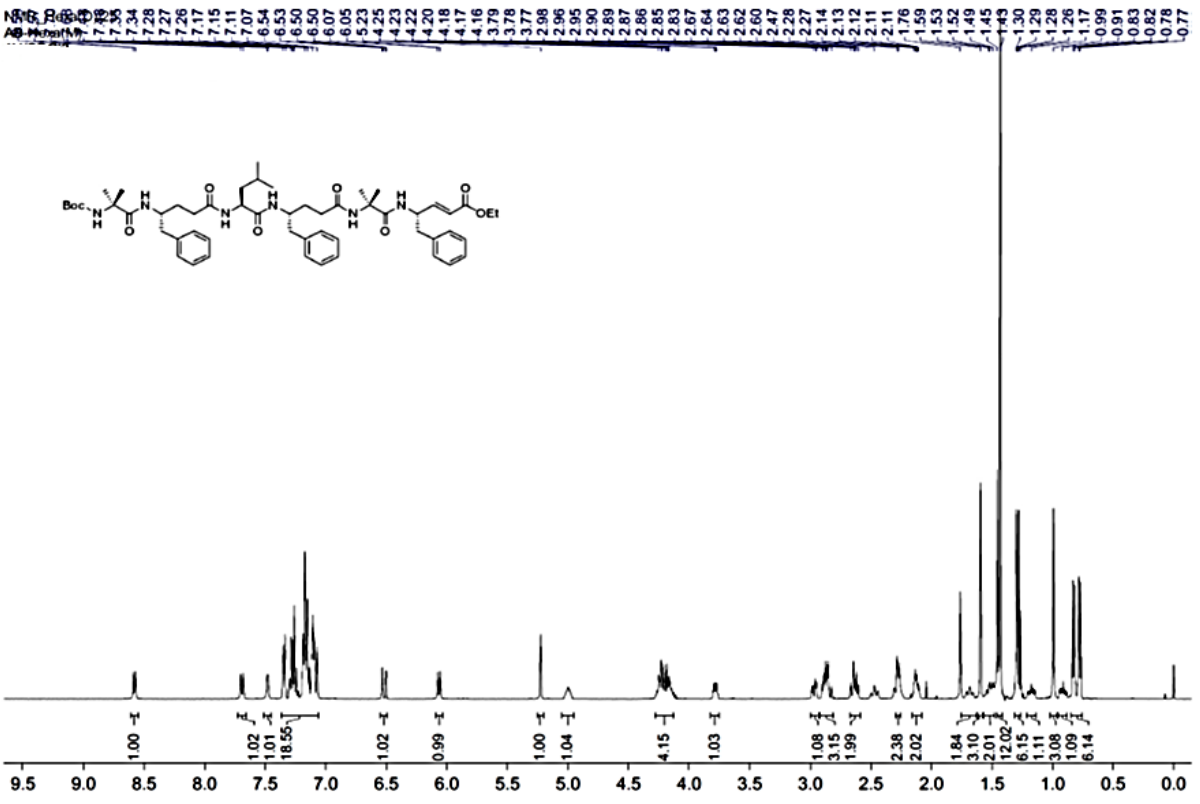
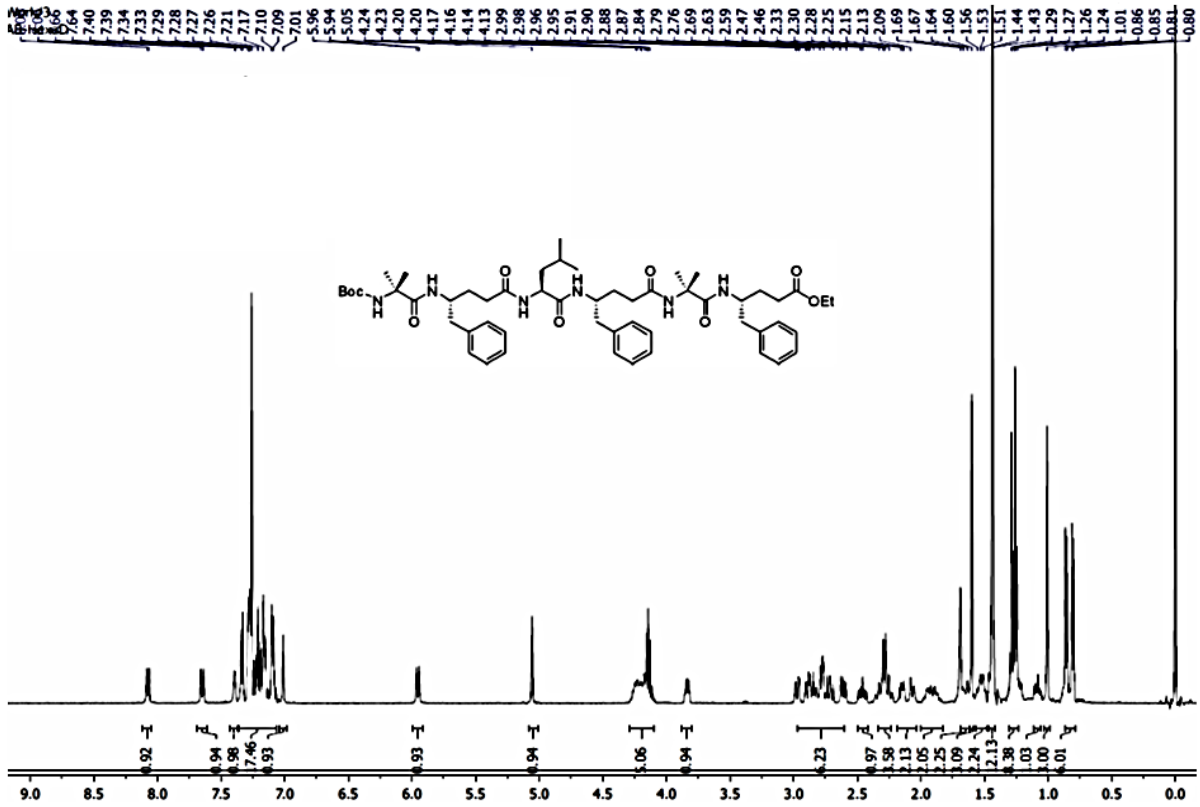
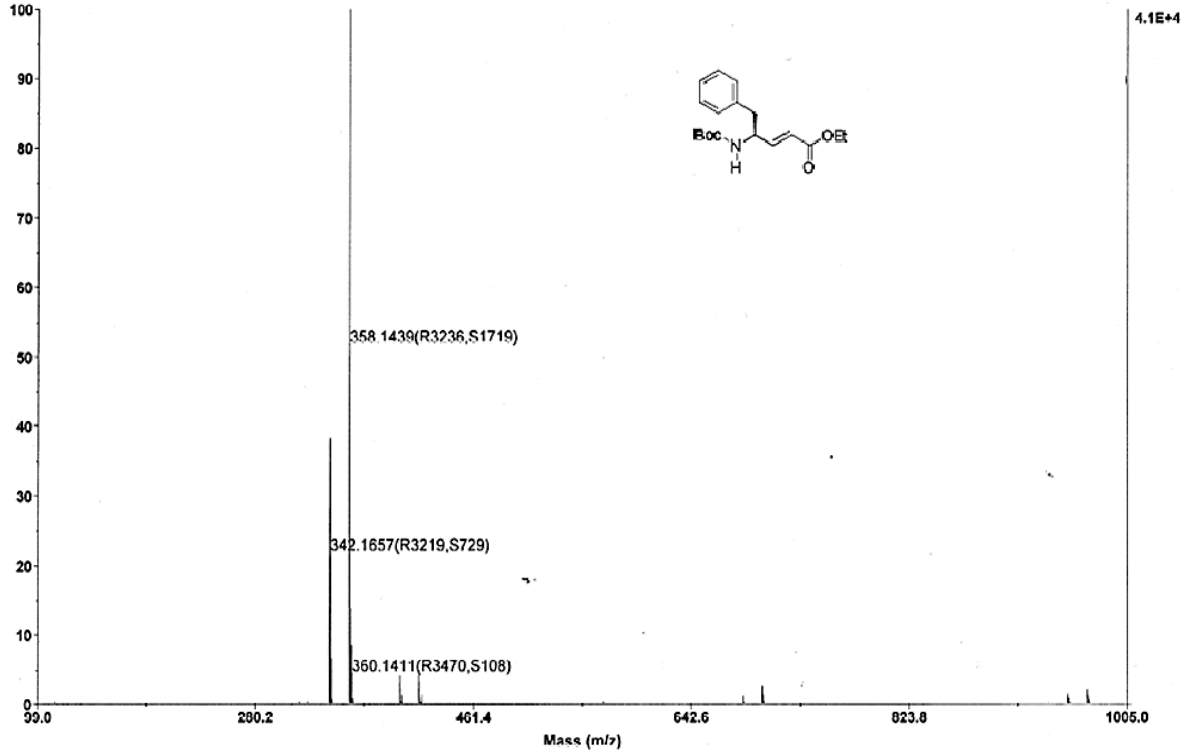
3a.7 Appendix: ^{1}H, ^{13}C, mass spectra with HPLC traces of peptides

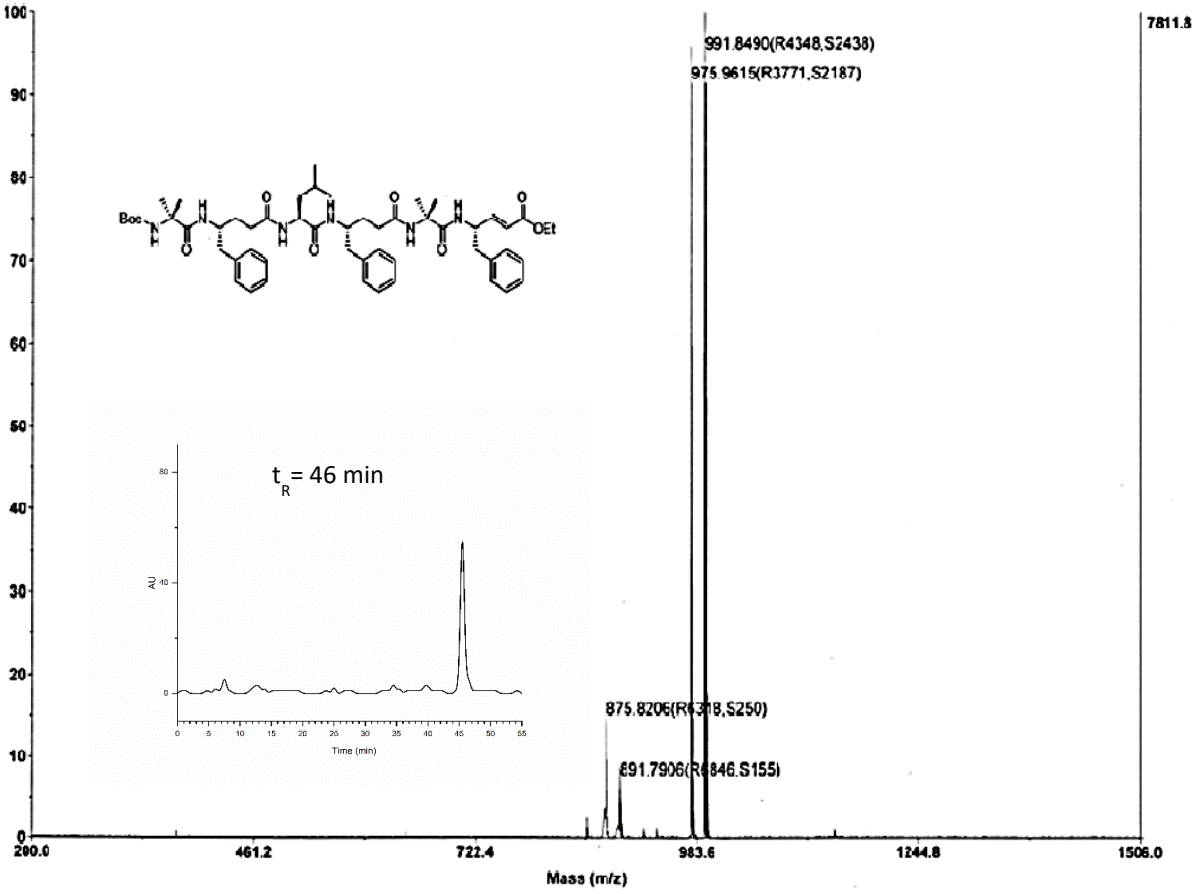


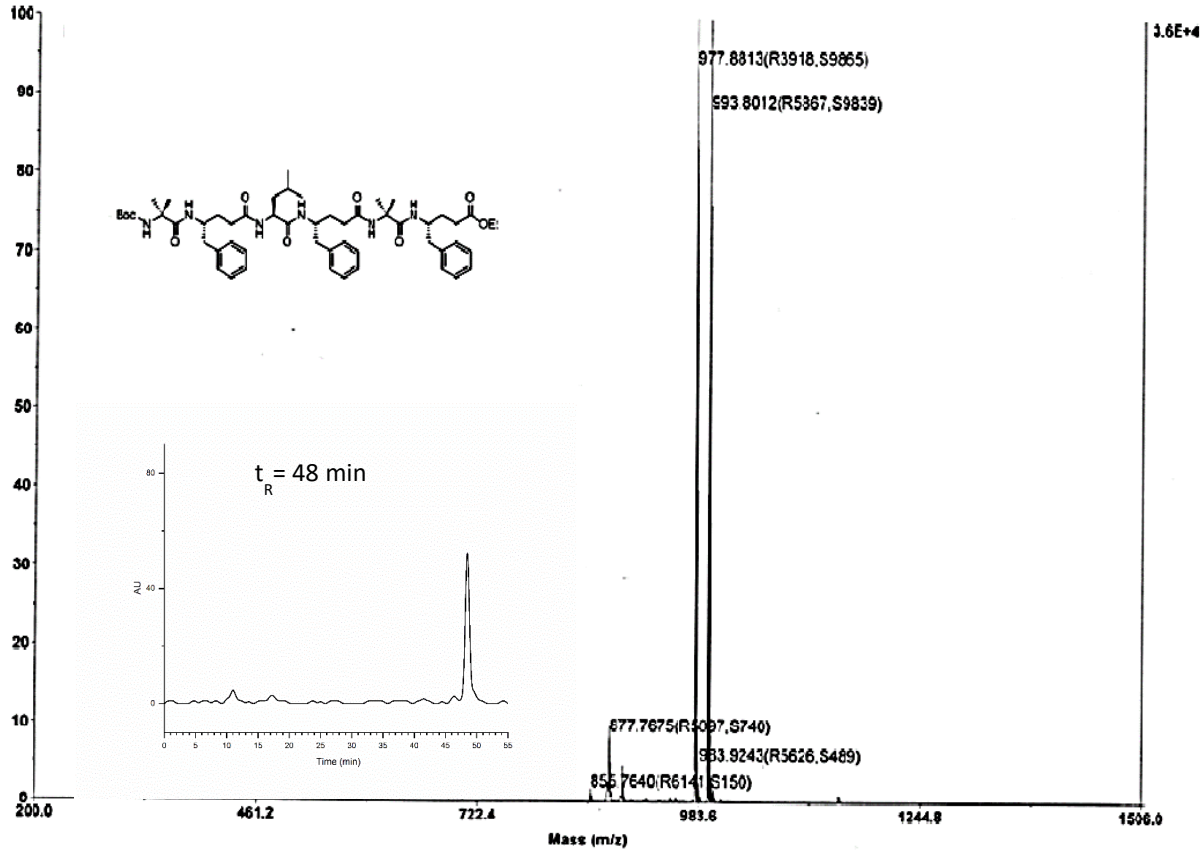


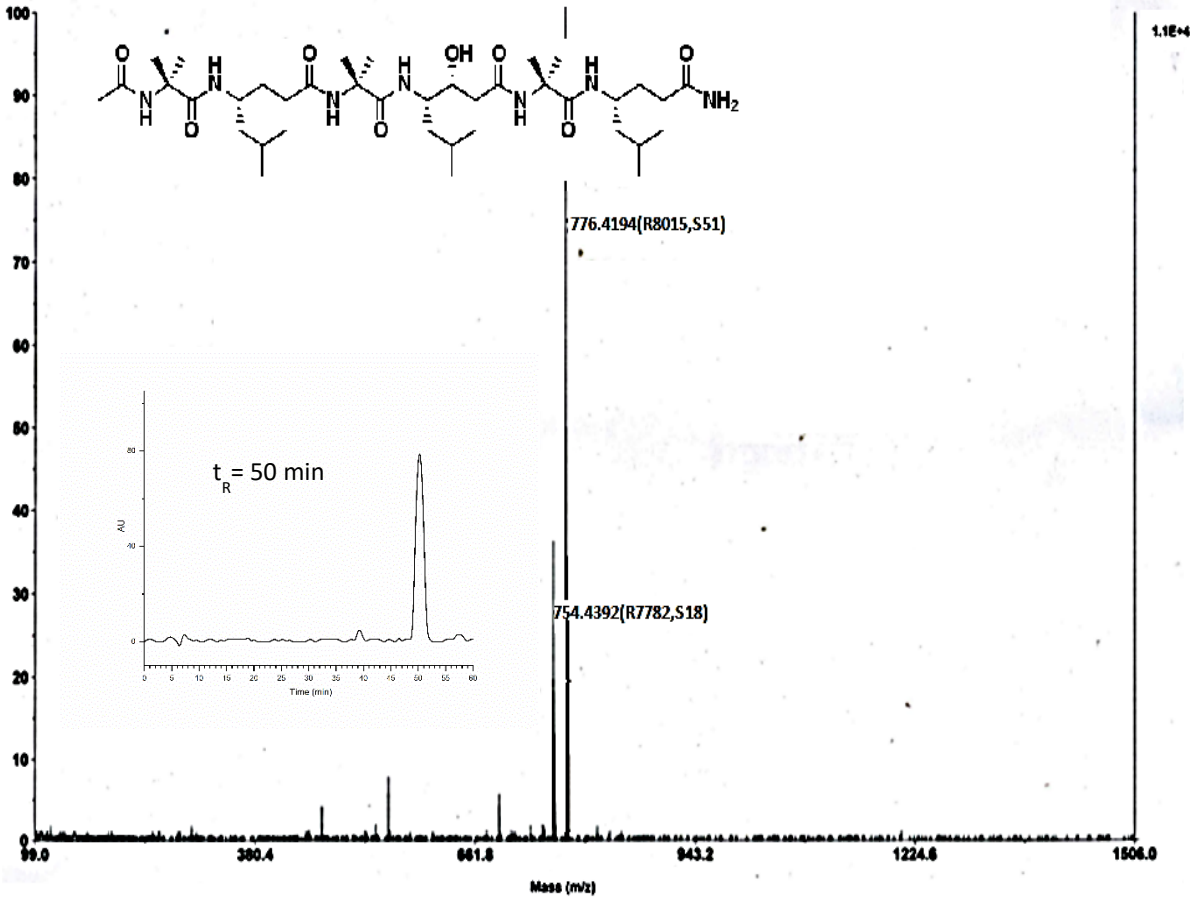


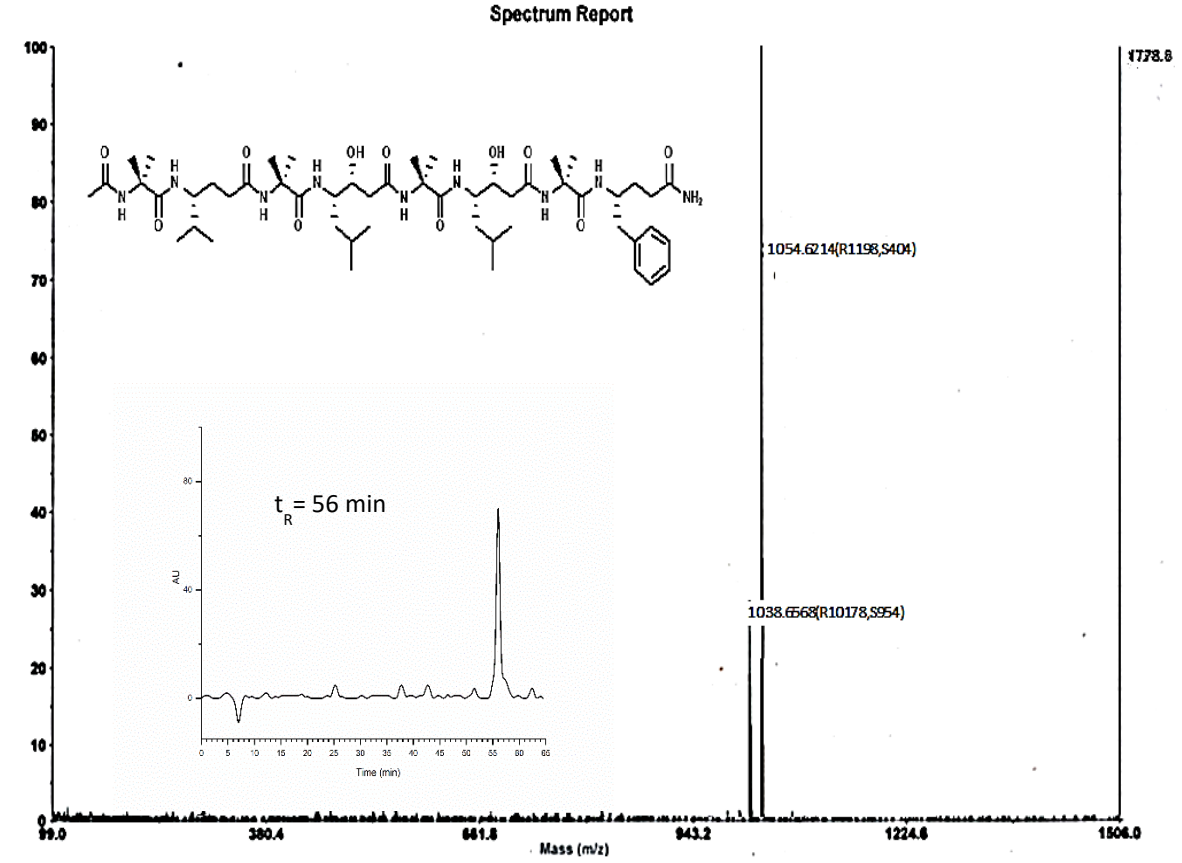



4.1E+4
358.1439(R3236,S1719)
342.1657(R3219,S729)
360.1411(R3470,S108)
Boc
N
H
OEt
O
Mass (m/z)


7811.8
991.8490(R4348,S2438)
975.9615(R3771,S2187)
875.8206(R6318,S250)
691.7906(R6846,S155)
Boc
OEt
t_R = 46 min
AU
Time (min)
Mass (m/z)


977.8813(R3918,S9865)
993.8012(R5367,S9839)
3.6E+4
t R = 48 min
AU
Time (min)
877.7675(R5097,S740)
983.9243(R5626,S489)
855.7640(R6141,S150)
100
90
80
70
60
50
40
30
20
10
0
200.0
461.2
722.4
983.6
1244.8
1506.0
Mass (m/z)


1.1E+4
776.4194(R8015,S51)
754.4392(R7782,S18)
t R = 50 min
AU
Time (min)
100
90
80
70
60
50
40
30
20
10
0
99.0
380.4
661.8
943.2
1224.6
1506.0
Mass (m/z)


Spectrum Report
1778.8
1054.6214(R1198,S404)
1038.6568(R10178,S954)
t R = 56 min
AU
Time (min)
Mass (m/z)
99.0
380.4
661.8
943.2
1224.6
1506.0
2018\IISER-96-2-2018 Label G2 Run # 301
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Chapter 3(b)

Exploration of naturally occurring β-hydroxy γ-amino acids (Statines) in the design of water-soluble Pepstatin analogues.

3b.1 Introduction

β-Hydroxy γ-amino acids commonly known as **"statine"** motifs are "**naturally occurring non-ribosomal gamma-amino acid**" residues found as a key element in many natural products. In recent years statine motifs have attracted attention due to their presence in many natural products with various pharmacokinetic and biological properties. **Didemnins** have an antiviral, immunosuppressant and anticancer property and **Didemnins-B** is among the first marine natural product to go into clinical trials against cancer.[1] **Tamandarin**, another cyclic marine natural product also shows anticancer properties with less cytotoxicity.[2] **Hapalosin** having multidrug-resistant reversing properties for tumour cells.[3] **Dolastatin** containing methylated hydroxyl statine moiety is well known to have an antimitotic effect.[4]

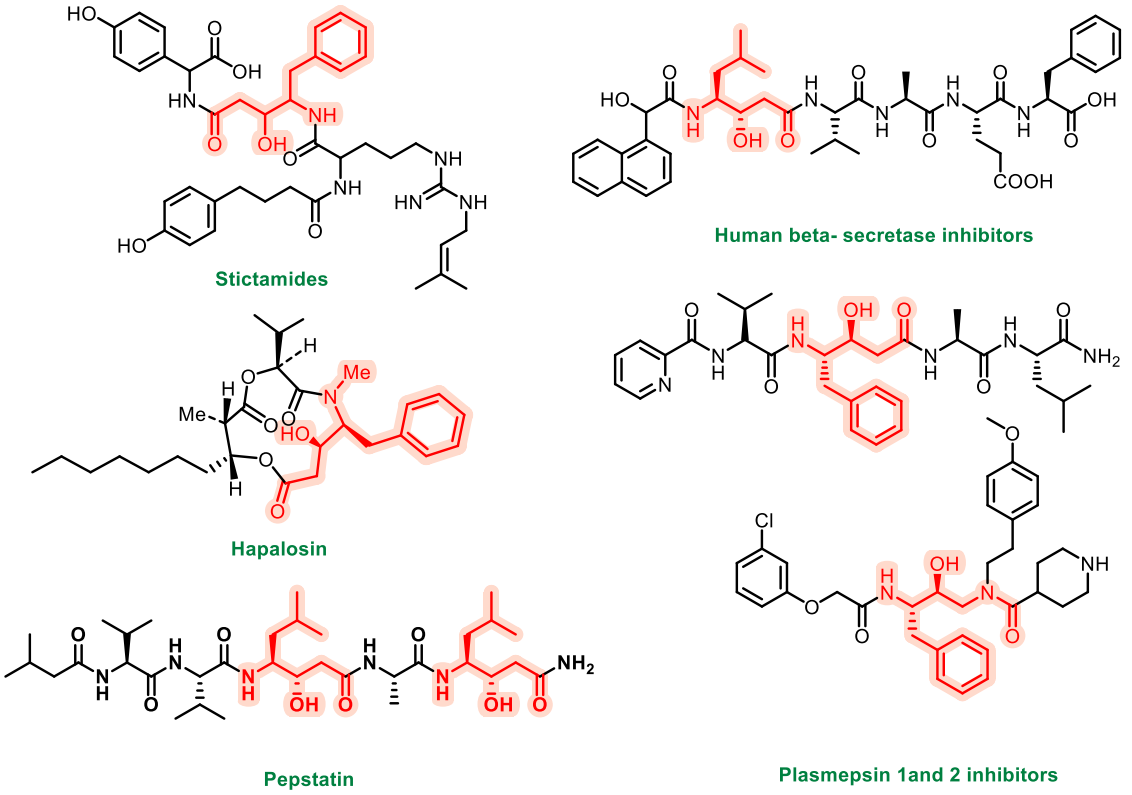


Figure 1: β-Hydroxy γ-amino acid motif-containing natural and synthetic products.

Stictamides serve as MMP-12 inhibitors.[5] Most importantly this residue caught much consideration on **Pepstatin-A**, a carboxyl protease inhibitor discovery by Umezawa *et al*. in 1970.[6] Some of these natural and synthetic products containing β-hydroxy-γ- amino acids are shown in Figure 1.

During peptide hydrolysis tetrahedral transition state plays a very important role in the bioactivity of any protease and mimicking this state by β-Hydroxy γ- amino acids makes these

moieties very important as a design element for peptidomimetic protease inhibitors making them fascinating as protease inhibitors.[7] Naturally occurring pepstatin A has shown potent inhibitory action against several acid proteases such as pepsin, cathepsin D and E, HIV-1 protease, plasmepsin I and II, renin *etc*.[8] Although, having excellent potent protease inhibitor properties pepstatin shows less therapeutic value because of its **lipophilicity** thus, low cell penetration ability and less specificity. Because of which there is always a need for developing potent analogues of pepstatin to increase its specificity and hydrophilicity. By looking into its structure modifications are possible at three positions either *N*-terminal, *C*-terminal or statine moiety site.

Umezawa and co-workers have developed acylated pepstatin analogues by replacing the isovaleryl group at *N*-terminal which has not only increased solubility but also showed enhancement in inhibitory properties too. Several other studies have been done by changing various capping at *N*- terminal flanking region of pepstatin with acylation to Boc and Cbz protection of terminal amino acid.[9] Tabor and co-worker, have demonstrated the utilization of mannose moiety at *C*- terminal of parent pepstatin molecule separated by a linker for making mannose–pepstatin bioconjugates inhibitors. These analogues have enhanced waster solubility and specificity towards dendritic cells for the inhibition of cathepsin D and E proteases involved in the antigen processing pathway.[10] Researchers have also done studies on coupling the *C*-terminal with 4-aminomethyl benzoic acid and their inhibitory potency against human β-secretase (BACE 1).[11] Pepstatin-A analogues were also developed by isoamyl amide coupling at *C*- terminal instead of free acid to much shorter peptide ranges. Few studies have also been done on using various amino acid statine by Rich and co-workers. They have also developed analogues by replacing the statine moieties with keto (pepstatone).[12] Studies have also been done on replacing the isobutyl chain of leucine statine with trifluoromethyl group to make pepstatin analogues which have shown specificity towards plasmepsin protease (PMII) and maintained its inhibitory potency level at nanomolar range.[13] Various researchers have worked on developing fluorinated pepstatin analogues and studied their inhibitory potency against various acid proteases.[14]

In recent years *candida species* have become a major threat to global health due to their wide pathogenesis and spread across various healthcare facilities. They cause several fungal infections *(candidiasis*) especially to the immunodeficient patient under the treatment process and hence affecting morbidity and mortality worldwide.[15] These species secrete various aspartic acid protease (*Sap*) which plays a major role in their pathogenesis. Pichova and *et.al*

have studied various pepstatin based analogues to inhibit *sap* of various pathogenic *candida species* and also studied their effect *in vivo* on a mouse model for treating candidiasis.[16] Due to the difficult multistep procedure for pepstatin synthesis because of difficulty in obtaining stereo chemically pure statine isomer, still detailed works has to be done in the field of making analogues of pepstatin to overcome pepstatin drawbacks.

In recent years, our lab has reported a methodology for the synthesis of β-keto γ-amino esters from an amino aldehyde by Roskamp's procedure.[17] Further statines were obtained by mild reduction using $NaBH_4$ reported by Rich et al.[18] As we have standardized the protocol for the synthesis and isolation of stereochemically pure statine moieties and their utility in both solution and solid-phase peptide synthesis (discussed in section 2b), we thought to further utilize statine in designing water-soluble pepstatin analogues and explore their inhibitory properties against aspartic acid proteases.

3b.2 Aim and rationale of present work

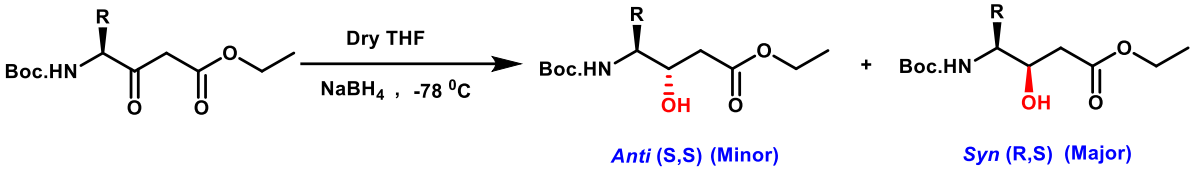
Inspired by the immense biological importance of pepstatin as an inhibitor especially as HIV-1 protease inhibitors and effective methodology for statine synthesis, we thought of utilizing these statine moieties for designing water-soluble pepstatin based analogues and to study their inhibitory potency against Pepsin (an aspartic acid protease). We have utilized statine moieties obtained from phenylalanine, leucine and valine amino acid for the design using both the conformers (***anti*** (*R, S)* and ***syn*** (*S, S*)) to have a comparative study to understand the effect of side-chain and the position of the hydroxyl group for its activity, respectively. We also want to study the proteolytic stability, specificity and anti-fungal activity of the designed analogues.

3b.3 Results and Discussion

3b.3.1 Synthesis of monomers

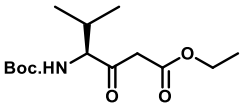
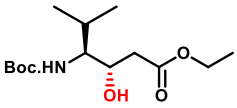
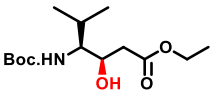
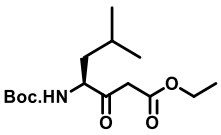
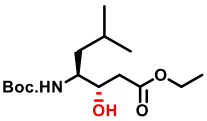
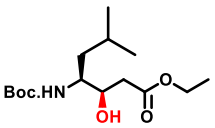
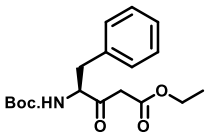
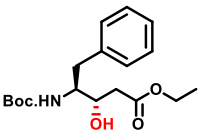
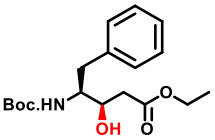
Synthesis of *N*- Fmoc-protected β-hydroxy γ- amino acids

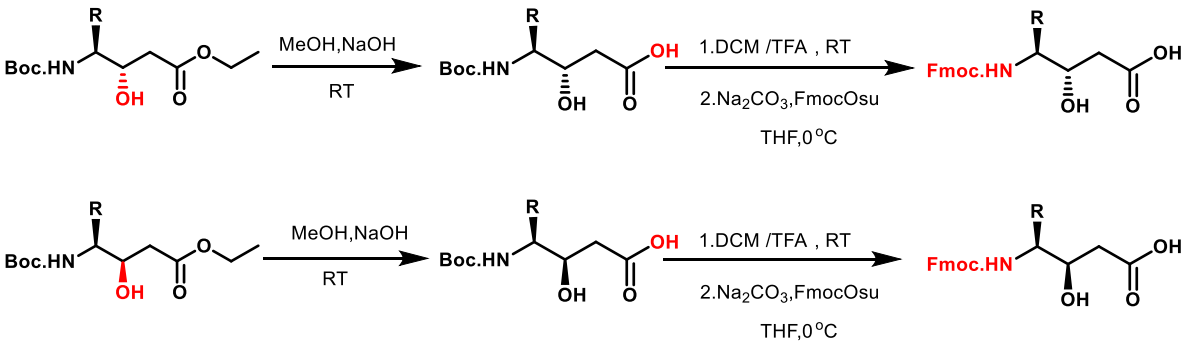
We have synthesized *N*-Boc- β-hydroxy γ-amino esters by mild reduction of β-keto gamma-amino esters with $NaBH_4$ as per the procedure reported earlier by Rich *et al*. as shown in Scheme 3.1. Just after the reduction both the conformers were isolated in good yields through column purification and well-characterized by NMR.



Scheme 1: Conversion of (*N*)Boc β-keto γ-amino ester to (*N*)Boc β-hydroxy γ-amino ester.

Table 3.1: List of *N*-Boc β-keto γ- amino esters and their respective N-Boc β-hydroxy γ-amino esters.

Entry	β-keto γ- amino ester	β-hydroxy γ- amino acid (S.S)	β-hydroxy γ- amino acid (R,S)	Overall yield %
1.		 *Anti* (S,S) (Minor) 32%	 *Syn* (R,S) (Major) 68%	93%
2.		 *Anti* (S,S) (Minor) 36%	 *Syn* (R,S) (Major) 64%	92%
3.		 *Anti* (S,S) (Minor) 35%	 *Syn* (R,S) (Major) 65%	95%



Scheme 2: Synthesis of *N*-Fmoc, β-hydroxy γ- amino acid from *N*-Boc, β-hydroxy γ- amino ester.

Later *N*-Boc-β-hydroxy γ- amino esters were subjected for hydrolysis and Boc was deprotected using TFA/DCM. Further, free amine was again protected with Fmoc-Osu to obtain solid-phase compatible *N*-Fmoc- β-hydroxy γ- amino acid shown in Scheme 2. After the reaction, the product was purified through column purification and used for peptide synthesis.

3b.3.2 Design, synthesis and purification of peptides

The sequence of natural Pepstatin **$(CH_3)_2CHCH_2CO$-Val-Val-*Sta*Leu*(S, S)*-Ala-*Sta*Leu*(S, S)*-COOH** is shown in Figure 1, composed of two leucine statine at position 3 and 5 with free acid at *C*-terminal and isovaleric acid at *N*-terminal with one α-Ala residue at 4 and two α-Val residue at 1 and 2. Keeping the parent sequence of pepstatin in consideration, we have synthesized Analogue 1 (**A1**) as **NH_2-Arg-GABA-Leu-Val-Val-*Sta*Leu*(R, S)*-Ala-*Sta*Leu(*R, S*)-$CONH_2$**. Basically, we did modifications on all the possible sites i.e., *N* and *C*- terminal and at statine moiety. For increasing hydrophilicity, we have introduced Arg-residue with free amine at *N*-terminal and gamma-aminobutyric acid (GABA) as a linker to separate the hydrophilic part with Arg to that of the rest of the sequence of pepstatin and *C*- terminal is kept as an amide, instead of free acid. To mimic the isovaleryl moiety of pepstatin A, we have introduced α- Leu residue. We have incorporated *anti (R, S)* conformer of statine in place of *syn (S, S)* which is present in parent pepstatin A. As already been reported in the literature that isovaleric acid residue doesn't play any role in its activity so we have synthesized analogues **A2-A7** with the basic sequence as **Arg-GABA- Val-Val-Sta-Ala-Sta-NH_2** using Phe, Leu and Val statine motifs both the conformers, a basic design is shown in Figure 2. The schematic representation of the sequence of designed pepstatin analogues is shown in Table 2.

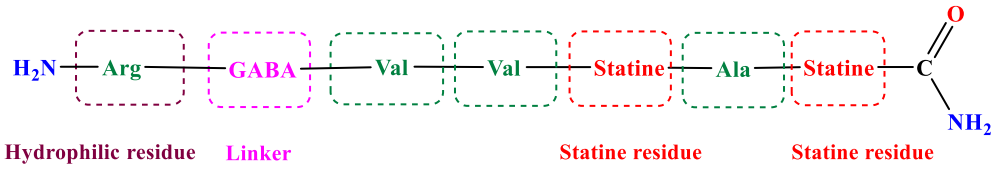
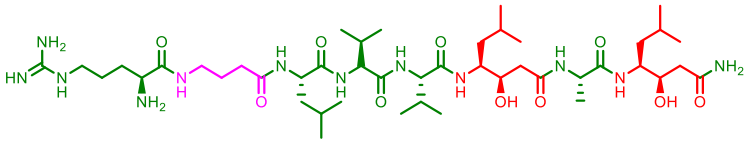
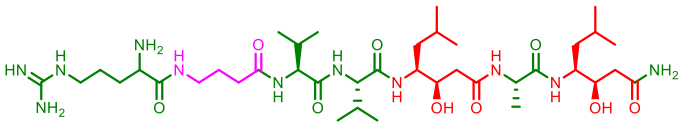
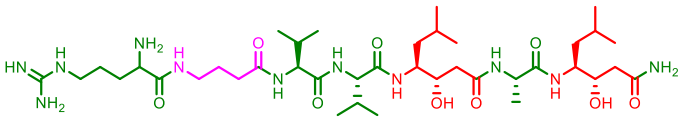
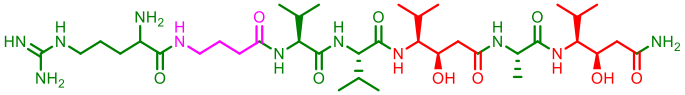
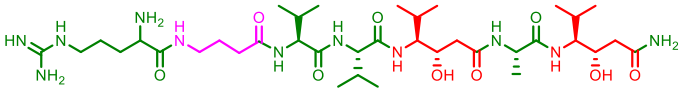
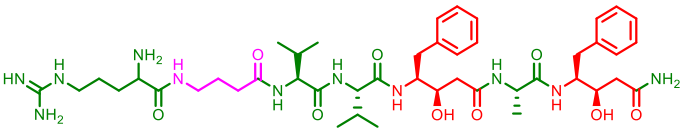
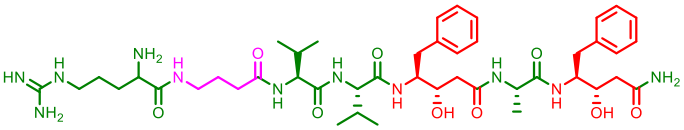


Figure 2: Basic design of water-soluble pepstatin analogues.

The protease substrate sequence chosen for the proteolytic study was derived from the plasmepsin 1 and 2 labile sites of the haemoglobin α -chain, residues 32-37. We have designed two protease substrates **SP1** and **SP2** for studying the inhibitory potency of the designed pepstatin analogues (A1-A7). SP1 contains a total of 9 residues and the cleavage site for pepsin as per literature is in between Met-Phe and Phe-Leu and we can detect the fragments mass using MALDI - TOF/TOF.

Table 2: Design, sequence and molecular weight of pepstatin peptide analogues (**A1**-**A7**) containing β-hydroxy γ- amino acid moiety.

Entry	Pepstatin Analogues	MW
A1		954
A2		841
A3		841
A4		813
A5		813
A6		909
A7		909

To study the enzyme kinetics of protease with and without inhibitor we have designed an internally-quenched fluorescent substrate SP2 having 4-(4-dimethylaminophenylazo)-benzoyl (DABCYL) as quencher moiety and [N(-acetamidoethyl)]-1-naphthylamine-5-sulfonic acid (I-AEDANS) as chromophore moiety, as the intramolecular FRET pair. SP2 on proteolysis will show increased emission of fluorescence at 490 nm on 330 nm excitation wavelength. The schematic representation of the sequence of designed substrates SP1 and SP2 are shown in Figure 3. All the pepstatin analogues **A1**-**A7** and substrate **SP1**-**SP2** were synthesized using standardised solid-phase peptide synthesis using Fmoc-chemistry on Knorr amide resin as

described earlier. All the peptides were purified by reverse phase HPLC using an acetonitrile /water gradient system with 0.1%TFA on the C18 column. All the peptides are well characterized by mass using MALDI TOF/ TOF.

To understand the inhibitory activity of the designed pepstatin analogue **A1**-**A7**, we designated pepsin (aspartic acid protease) as a model protease. To investigate the specificity of these designed analogues for aspartic acid proteases, we have chosen chymotrypsin and trypsin as other classes of proteases.

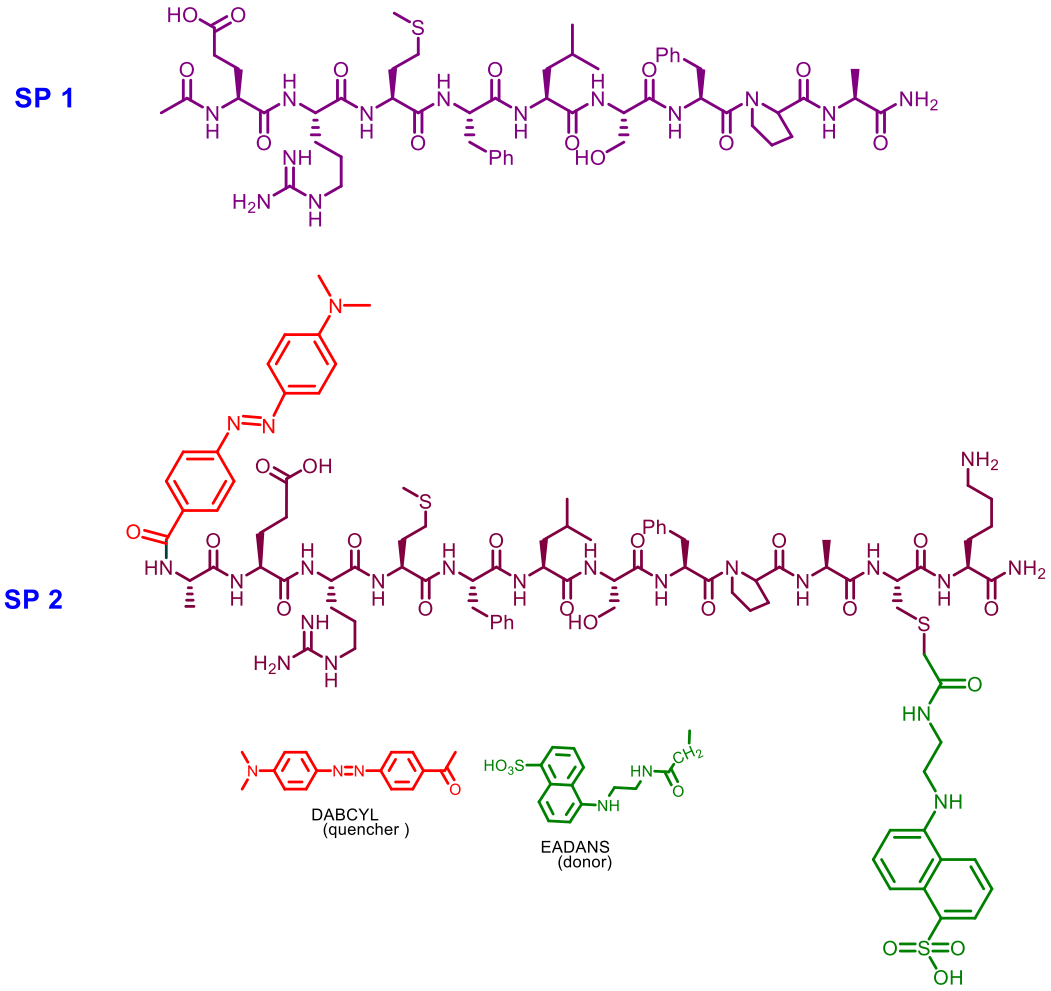
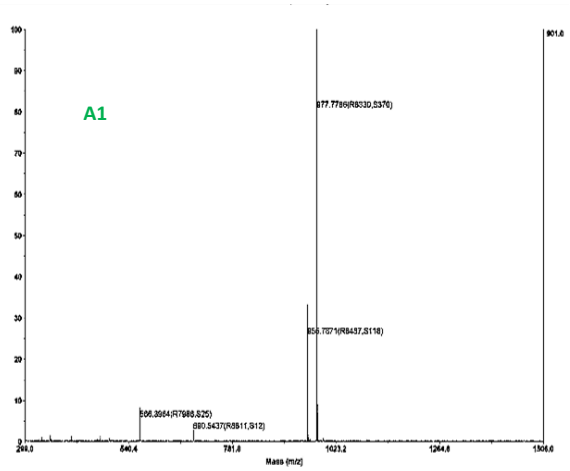


Figure 3: Design and sequence of non-fluorogenic (S1) and fluorogenic (S2) protease substrate.

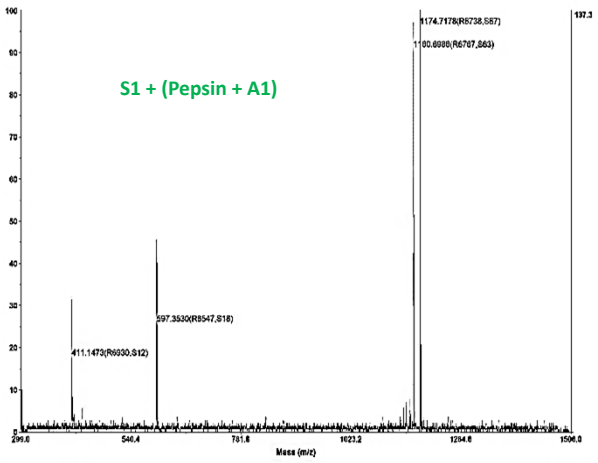
3b.3.3 Inhibition study using Mass Spectrophotometry

We have used mass analysis as a tool to understand the inhibitory effect of **A1**-**A7** on the proteolytic activity of pepsin on the substrate, S1. As mentioned earlier, that pepsin does the proteolysis of the peptide bond between leucine and phenylalanine residue or phenylalanine

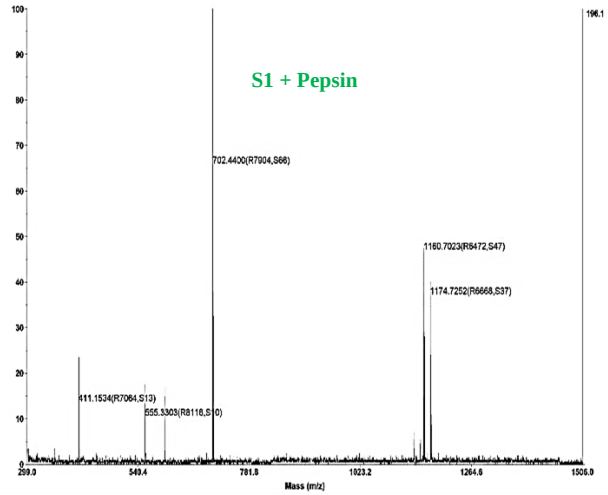
and methionine residues of the substrate, **S1**. So, on proteolysis of substrate **S1** we will get the fragmented mass of **S1** on positive ion mode of MALDI- TOF/TOF. Mass spectra of proteolytic assay of **A1** and **A6** are shown in Figures 4 and 5, respectively.



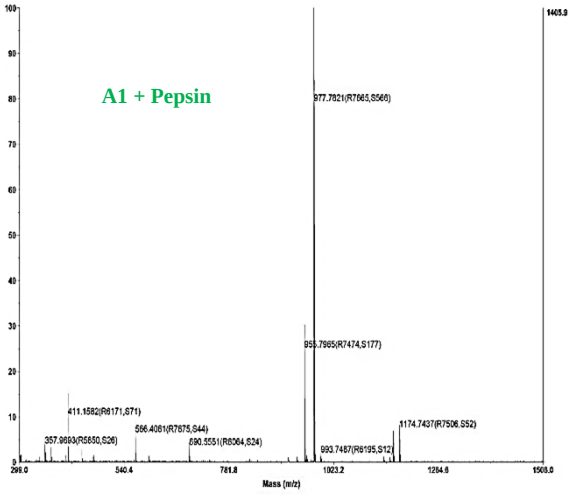
(a) Mass spectra of A1.



(c) Mass spectra of S1 on proteolysis with pepsin pre-treated with A1.



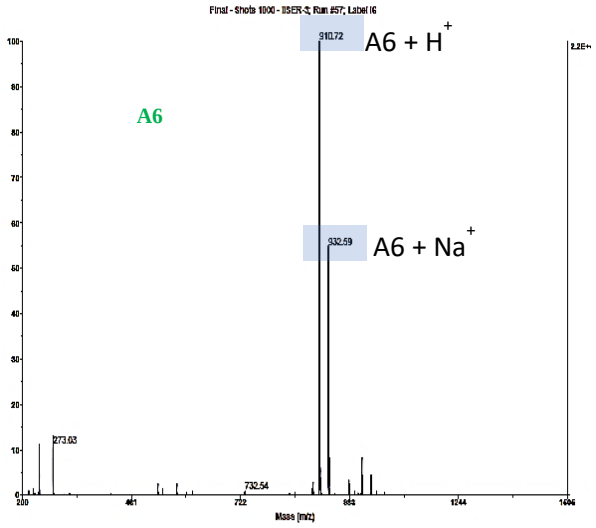
(b) Mass spectra of S1 on proteolysis with pepsin.



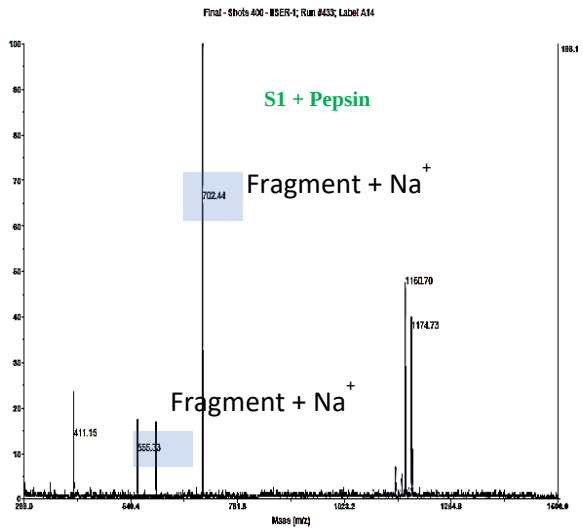
(d) Mass spectra of A1 on proteolysis with pepsin.

Figure 4: Mass spectra of proteolytic assay of pepstatin peptide inhibitor **P1**, pepsin a protease and **S1** as a substrate.

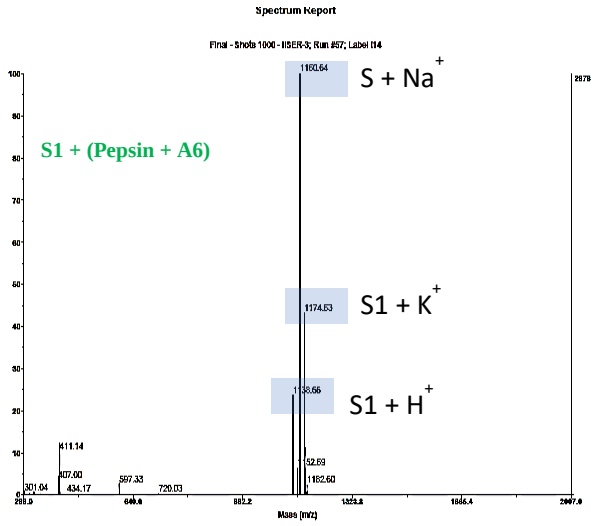
Also, if the pepsin pre-treated with analogues **A1**-**A7** shows fragmented mass of S1 suggesting that particular analogue is not showing inhibition but if no fragmented mass is observed suggesting that particular analogue can be considered to be inhibiting pepsin.



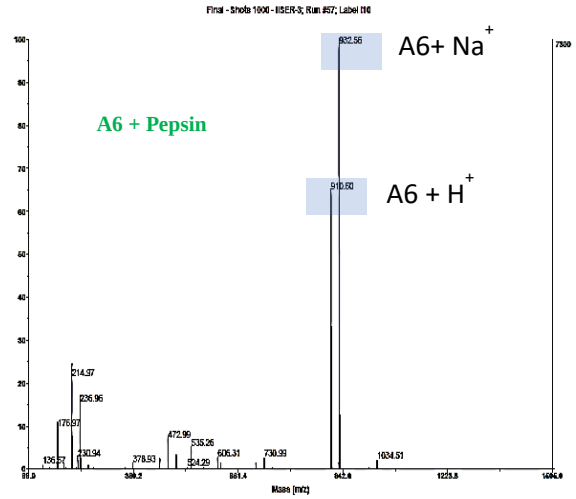
(a) Mass spectra of A6.



(b) Mass spectra of S1 on proteolysis with pepsin.



(c) Mass spectra of S1 on proteolysis with pepsin pre-treated with A6.



(d) Mass spectra of A6 on proteolysis with pepsin.

Figure 5: Mass spectra of proteolytic assay analogue **A6**, pepsin a protease and **S1** as a substrate.

In each case, the first spectra belong to **A1** / **A6** respectively and the second spectra showing the mass of S1 and fragments on proteolysis by pepsin. Third spectra showing the mass of S1 on proteolysis by pepsin preincubated for 5-10 mins with **A1** / **A6** and no S1 fragments mass were observed suggesting inhibition of pepsin by these analogues. As we can see that our designed analogues are completely peptide-based so there is a possibility of proteolysis of these

analogues with proteases so, in fourth spectra, we have shown mass of **A1** / **A6** on proteolysis with pepsin and indicated through mass analysis that these analogues are proteolytically stable. These processes were repeated with each analogue thrice and only mass spectra of peptides **A1** and **A6** are shown. The preliminary results suggest that except **A4** and **A5** which contains Val statine residue is not showing any inhibitory activity but analogues containing Leu and Phe statine can be considered as inhibitory for pepsin activity.

3.3.4 Pepstatin analogues Inhibition study using Fluorescence

We used fluorescence emission measurement as a tool to understand the inhibitory effect of Analogues except for **A**4 and **A**5 on the proteolytic activity of pepsin on a substrate, **S2**. As mentioned earlier, **S2** is an internally quenched substrate having a FRET pair DABCYL and AEDANS at two ends of the peptide such that when it is intact fluorescence is quenched but upon proteolysis, the FRET pair goes apart and shows enhanced fluorescence at an emission wavelength of 490 nm on excitation at 335 nm.

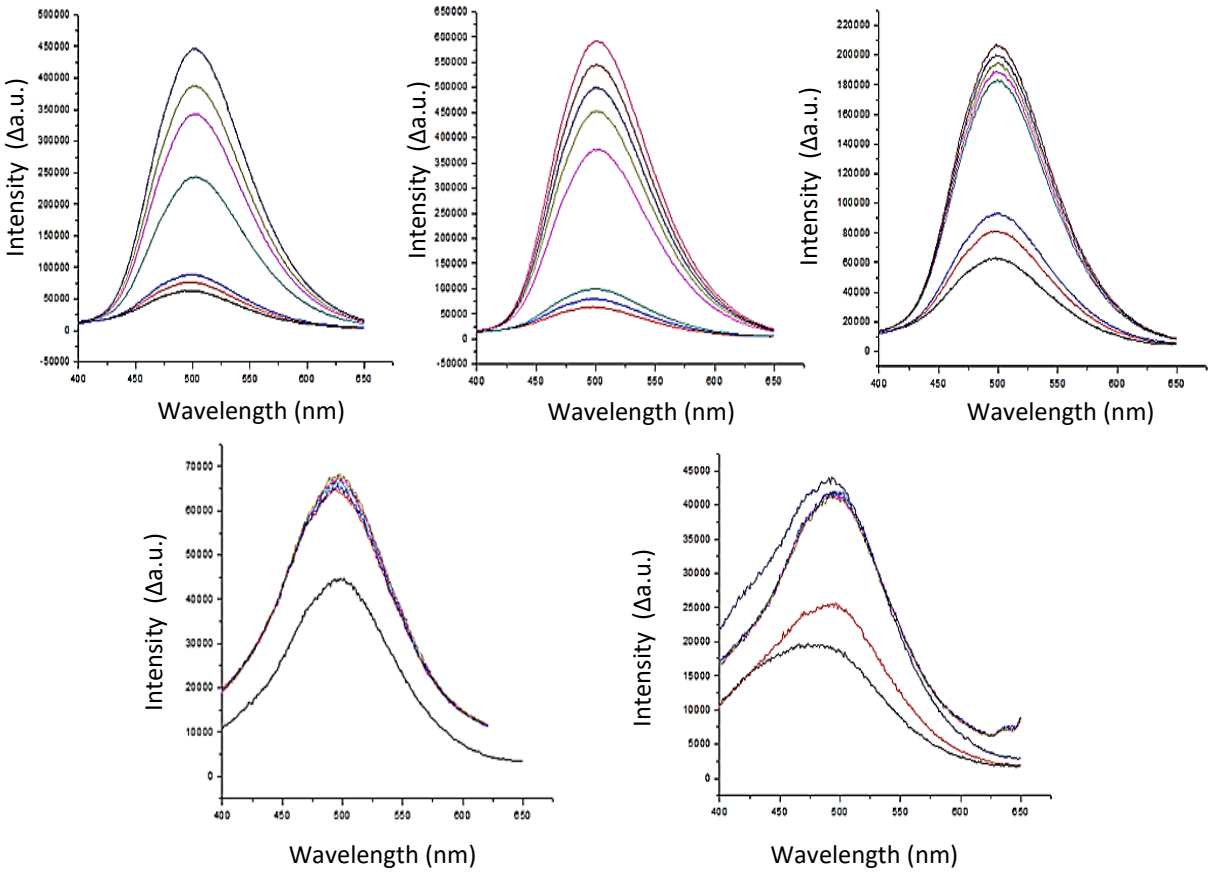


Figure 6: Fluorescence emission spectra of pepstatin peptide analogues **P1** against pepsin as protease and **S2** as a substrate.

So, if any of the pepstatin analogues is active to show inhibition of protease then there will be less fluorescence emission observed when pepsin pre-incubated with analogue does proteolysis on **S2**. We have screened all the analogues except **A4** and **A5** (which showed no inhibition in the previous assay), inhibitory activity against pepsin proteolysis on **S2**, shown in Figure 6. All the analogues were pre-incubated with proteases for 10 mins at the ice-cold condition and later used to check its proteolytic activity on **S2**. In the case of **A1** and **A2** very high fluorescence intensity was observed indicating no inhibiting activity by these analogues at this concentration. In the case of **A3**, a moderate increase in fluorescence intensity with time indicating moderate inhibition by this **A3** analogue. In the case of **A6** and **A7**, we observed a very less increase in intensity, indicating tight inhibition by these two analogues. These results indicate that Phe containing analogues are showing the best results and Leu (ss) statine containing analogue **A3** is also moderately active

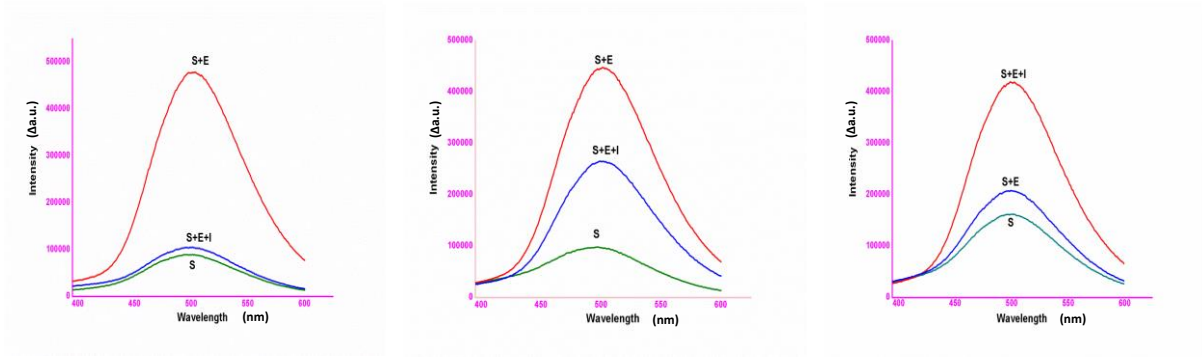


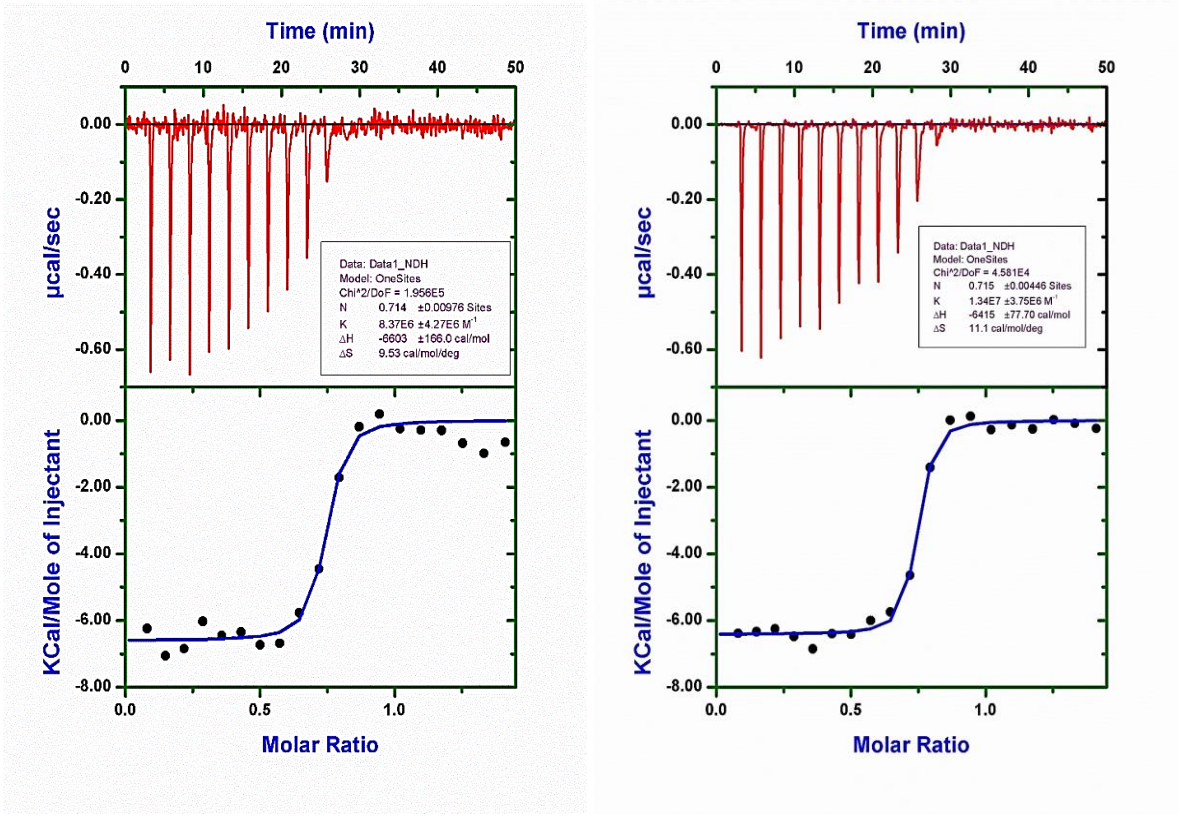
Figure 7: Fluorescence emission spectra of pepstatin peptide analogues **P7** against (a) pepsin, (b) chymotrypsin and (c) trypsin as protease and **S2** as a substrate.

We have also screened the active analogue **A7** against other proteases chymotrypsin and trypsin, to check its specificity towards this class of protease. The emission spectra reveal that this analogue showed more affinity towards pepsin (aspartic acid protease) as showing increased emission in the case of chymotrypsin and trypsin (serine protease). Comparative emission spectra are shown in Figure 7.

3b.3.4 Thermodynamic study using Isothermal Calorimetry

To understand the thermodynamic binding energetics, we have used isothermal titration calorimetry. The binding of the peptide to protease pepsin is shown in Figure 8. The protease was taken in the cell and peptide was titrated against the protease in citrate buffer (pH-3.4) at room temperature. The modified pepstatin bind to the peptide with an affinity of 135 nM. The

stoichiometry was found to be 1:1. These results suggested that modified pepstatin is directly binding to the pepsin with 135 nM affinity. As natural pepstatin is highly insoluble in aqueous buffers, we could not able to measure its binding constants with pepsin.



(a) (b)

Figure 8. Binding isotherms of modified pepstatin analogues (a) **A6** and (b) **A7** with pepsin protease.

3.4 Conclusion

We have successfully designed analogues of naturally occurring pepstatin A. Firstly, all the designed pepstatin analogues have solved the major drawback associated with pepstatin that is its lipophilicity and all the analogues are highly soluble in water as well in any of the aqueous buffer. Secondly, Leu and Phe statine containing analogues have shown inhibition towards pepsin. Also, Phe containing analogues have shown better inhibition against pepsin. These analogues are proteolytically stable and have showed more affinity towards pepsin than chymotrypsin and Trypsin. These pepstatin analogues can be used as inhibitors for aspartic acid proteases.

3b.5 Experimental section

3b.5.1 General experimental details

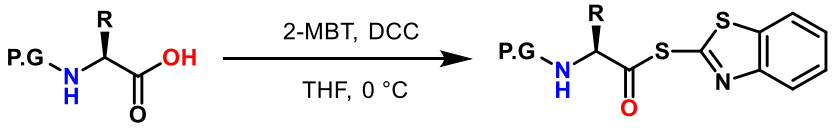
All α-amino acids, Ethyl diazoacetate, Tin chloride and Di-*tert*-butyl dicarbonate were obtained from Merck. 2-Mercaptobenzothiazole (2-MBT), Fmoc-Osu, DCC, $NaBH_4$, HPLC grade acetonitrile, dry DCM and dry THF were purchased from Spectrochem. Column chromatography was achieved on silica gel of 120-200 mesh from Merck. Thin-layer chromatography (TLC) was used to monitor reaction using Merck 60 F_{254} precoated silica gel plates. For visualization UV light and ninhydrin or phosphomolybdic acid (PMA) stains were used then charring on a hot plate. Melting points were recorded on Veego VMP-DS hot stage apparatus. Specific rotations were recorded at ambient temperature on the Rudolph Analytical Research instrument using $CHCl_3$ and MeOH as solvents. Reverse Phase-HPLC of Waters was used to purify peptides using Acetonitrile/H_2O gradient with 0.1% TFA from C-18 column. Mass was analysed on Applied Biosciences with MALDI TOF/TOF on positive ion mode.

NMR spectroscopy

Jeol 400 MHz (or 100 MHz for ^{13}C) spectrometer were used for recording ^{1}H spectra and ^{13}C NMR. The chemical shifts (δ) and coupling constants (J) were reported in ppm and Hz, respectively. The residual solvents signals were used as internal reference ($CDCl_3$ δ_H, 7.24 ppm, δ_c 77.0 ppm).

3b.5.2 General procedure for the synthesis of *(N)*Fmoc β-hydroxy γ-amino acids

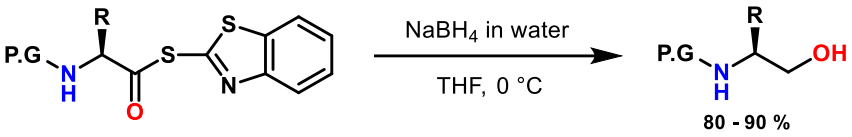
General procedure for the synthesis of MBT ester from *N*-Boc α-amino acid:



N-Boc α-amino acid (10 mmol) and 2-Mercaptobenzothiazole (2.505 g, 15 mmol) were mixed in THF (20 mL). The reaction mixture was allowed to cool to 0 °C. After cooling, DCC (2.06 g, 10 mmol) was added to the reaction and stirred for the next 1 h. TLC was performed for monitoring the reaction. On the completion of the reaction, precipitated DCU was filtered. The

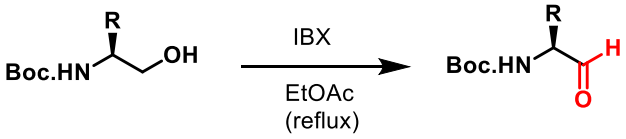
filtrate was concentrated beneath the *vacuum* to attain a gummy mixture. Further, EtOAc/Hexane was used for precipitating the mixture to give MBT ester of *N*-Boc amino acid. This precipitated product was used without purification directly for the alcohol synthesis.

General procedure for the synthesis of *N*-Boc β-amino alcohol from 2-Mercaptobenzothiazole esters:



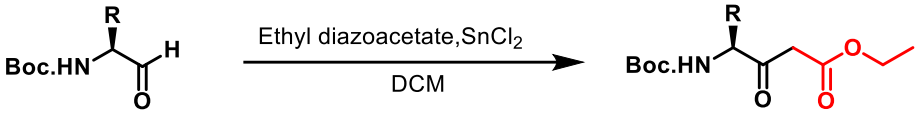
THF (30ml) was used to dissolve the above obtained *N*-protected MBT ester and was allowed to cool to 0 ˚C using an ice bath. $NaBH_4$ in water (50 mmol in 15 ml of water) was added to the reaction mixture step by step to observe effervescence and kept for the next 30 mins. Once the reaction is completed it was neutralized using 10% HCl. Later, the reaction mixture was kept beneath a vacuum to evaporate the THF used for the reaction. The aqueous layer was extracted thrice using ethyl acetate. The combined organic extract was washed further with 10% Na_2CO_3 and later with brine followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure *N*-protected β-amino alcohol with 80-90% yield.

General Procedure for the Synthesis of *N*-Boc amino aldehydes:



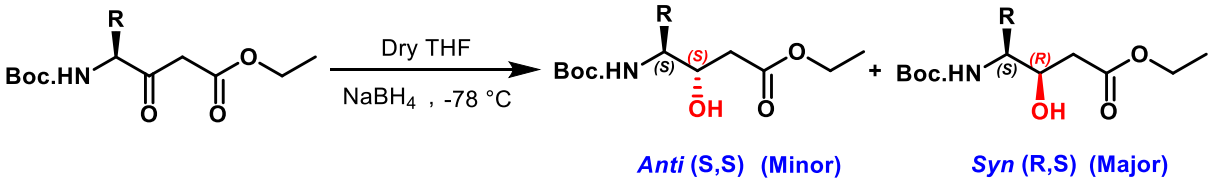
Ethyl acetate (50 ml) was used to dissolve *N*-Boc β-amino alcohol (10 mmol) and to that IBX (30 mmol) was added. The reaction was kept for stirring at refluxing temperature over oil- bath for next 4 hours. TLC was performed for monitoring the reaction. On the completion of the reaction, reaction mixture was filtered while washing with ethyl acetate. The filtrate was concentrated beneath the *vacuum* to attain oily mixture. This oily product was used without purification directly for the β-keto synthesis.

General Procedure for the Synthesis of *N*-Boc β-keto γ-amino esters:



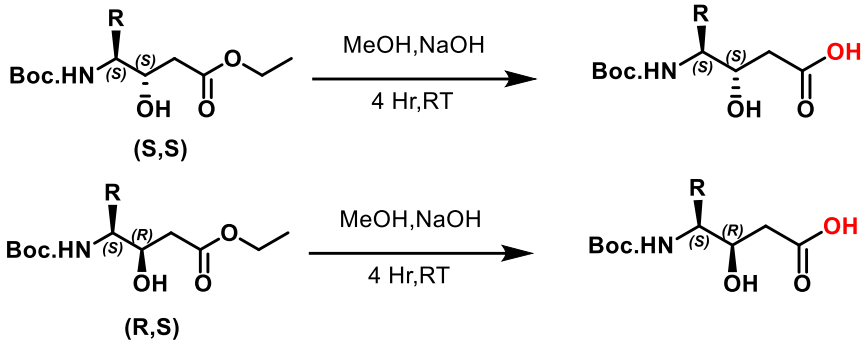
DCM (15 ml) was used to dissolve the above formed *N*-Boc β-amino aldehyde and to that Tin (II) Chloride (20 mol%) was added at room temperature. After 5 mins, 15% Ethyl diazoacetate in toluene (10.05 mmol, 7.5 ml) was added slowly drop by drop and instant evolution of nitrogen can be seen till the reaction completes. TLC was performed for monitoring the reaction. Once the reaction is completed it was neutralized using 5 % HCl and the aqueous layer was extracted thrice using DCM. The combined organic extract was washed with brine followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure *N*-Boc β-keto ester.

General Procedure for Synthesis of *N*-Boc β-hydroxy γ-amino esters:



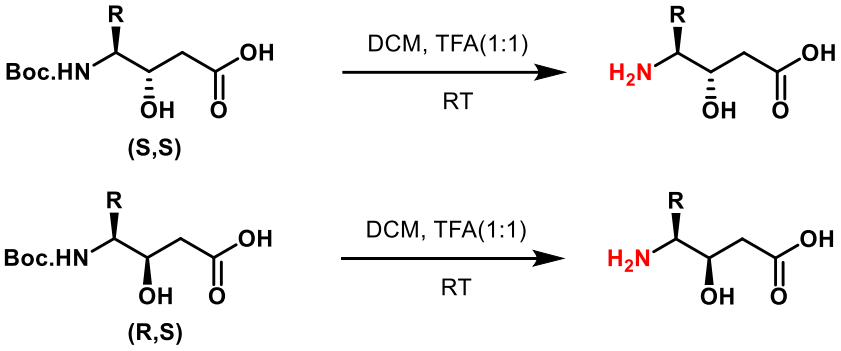
Dry THF (30ml) was used to dissolve the above obtained pure *N*-Boc β-keto γ ester (10 mmol) at inert conditions. By using dry ice and acetone the reaction temperature was brought to -78 °C. Solid $NaBH_4$ (0.529 g,14 mmol) was added to the reaction mixture at one step and kept for the next 4 hours. TLC was performed from time to time for monitoring the reaction. Once the reaction is completed it was neutralized using 5 % HCl. Later, the reaction mixture was kept beneath a vacuum to evaporate the THF used for the reaction. The aqueous layer was extracted thrice using ethyl acetate. The combined organic extract was washed further with brine solution followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure both *syn* and *anti N*-Boc β-hydroxyl γ-amino ester (statine) conformers separately.

General procedure for Synthesis of *N*-Boc β-hydroxy γ-amino acids:



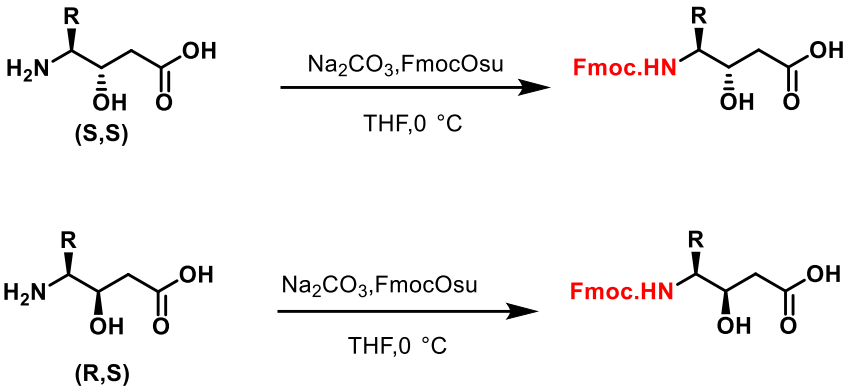
MeOH (50 ml) was used to dissolve pure *N*-Boc β-hydroxy γ amino ester (5 mmol) and was allowed to stir to get a clear solution. 3eq. of 1NaOH (15 ml, 15 mmol) was added to the reaction mixture dropwise and kept for next 2 hours at room temperature. TLC was performed time to time for monitoring the reaction. Once the reaction is completed it was neutralized using 10% HCl. Later, reaction mixture was kept beneath vacuum to evaporate the MeOH used in the reaction. The aqueous layer was extracted thrice using ethyl acetate. The combined organic extract was washed with brine solution followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate to obtain pure *N*-Boc β-hydroxy γ amino acid, no further purification required.

General procedure for the synthesis of NH_2-β-hydroxy γ-amino acids:



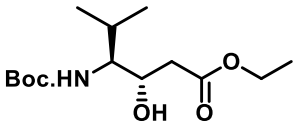
DCM (10 ml) was used to dissolve *N*-Boc β-hydroxy γ amino acid (10 mmol) and was allowed to cool to 0 °C using an ice bath. 10 ml of TFA was added to the reaction mixture slowly and kept for the next 30 mins. Once the reaction is completed TFA was co-evaporated using DCM (15ml five times) and recrystallized using DCM and hexane to obtain solid triflate salt of NH_2-β-hydroxy γ-amino acid which can be used without purification directly for the next step.

General procedure synthesis of *N*-Fmoc β-hydroxy γ-amino acids:

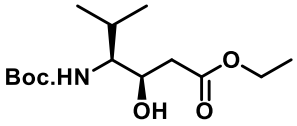


THF (15 ml) was used to dissolve the above obtained NH_2-β-hydroxy γ-amino acid and was allowed to cool to 0 ˚C using an ice bath. 10% sodium carbonate (25 ml) was added to the reaction mixture step by step to obtain a basic pH of approximately 9. Later, Fmoc-Osu (1.68 g, 5 mmol dissolved in 15 ml THF) was added to the reaction mixture step by step and kept for 6 hours. Once the reaction is completed it was neutralized using 5% HCl. Later, the reaction mixture was kept beneath a vacuum to evaporate the THF used for the reaction. The aqueous layer was extracted thrice using ethyl acetate. The combined organic extract was washed further with 10% Na_2CO_3 and later with brine followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure solid *N*-Fmoc β-hydroxy γ-amino acid.

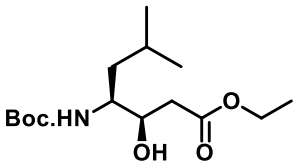
Characterisation of (*N*)Boc β-hydroxy γ-amino acids:



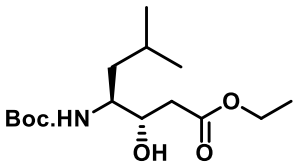
(3*S*, 4*S*)-ethyl 4-(tert-butoxycarbonylamino)-3-hydroxy-5-methyl hexanoate: Colourless liquid. **^{1}H NMR** (400 MHz, $CDCl_3$) **:** 4.87-4.85 (d, *J* = 9.7, 1H), 4.19-4.14 (q, *J* =7.2, 2H), 4.11-4.09 (d, *J* = 8, 1H), 3.67-3.65 (m, 1H), 3.26 (b, 1H), 2.50-2.44 (m, 2H), 1.84-1.79 (m, 1H), 1.41 (s, 9H,) ,1.28-1.24 (t, *J* = 7.2, 3H), 0.92-0.88 (m,6H,). **^{13}C NMR** (100 MHz, $CDCl_3$) **:** 173.63, 155.35, 79.87, 68.37, 64.28, 61.33, 39.26, 28.35, 26.89, 20.03, 14.15. **MALDI-TOF/TOF** m/z value**:** Calcd. [M+Na]+ 312.1787, observed 312.1739.



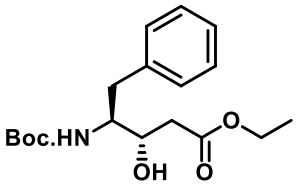
(3*R*, 4*S*)-ethyl 4-(tert-butoxycarbonylamino)-3-hydroxy-5-methylhexanoate: Colourless liquid. **^{1}H NMR** (400 MHz, $CDCl_3$) **:** 4.64-4.62 (d, *J* = 9.8, 1H), 4.18-4.13 (q, *J* =7.1, 2H), 4.09-4.07 (d, *J* = 8, 1H), 3.62-3.58 (m, 1H), 3.24 (b, 1H), 2.52-2.40 (m, 2H), 1.83-1.74 (m, 1H), 1.42 (s, 9H), 1.29-1.25 (t, *J* = 7.2, 3H), 0.93-0.86 (m, 6H). **^{13}C NMR** (100 MHz, $CDCl_3$) **:** 173.13, 155.21, 79.82, 68.11, 63.93, 62.45, 38.79,28.03, 26.32, 19.45, 14.07. **MALDI-TOF/TOF** m/z value**:** Calcd. [M+Na]+ 312.1787, observed 312.1754.



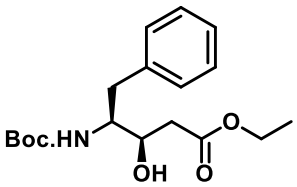
(3R,4S) ethyl 4-((tert-butoxycarbonyl)amino)-3-hydroxy-6-methylheptanoate: Yellow colour solid. **^{1}H NMR** (400 MHz, $CDCl_3$) **:** 4.59-4.57 (d, *J* = 8.8, 1H), 4.20-4.15 (q, *J* = 7, 2H), 4.05-4.01 (m,1H), 3.70-3.63 (m, 1H), 3.45 (b, 1H), 2.48-2.42 (m, 2H), 1.66-1.57 (m,2H), 1.44 (s, 9H), 1.34-1.31 (t, *J* = 6.8) 1.29-1.26 (t, *J* = 7.2,3H), 0.95-0.91 (m,6H,); **^{13}C NMR** (100 MHz, $CDCl_3$) **:** 172.84, 156.16, 79.57, 71.38, 60.82, 52.69, 38.84, 37.95, 28.35, 24.70, 23.65, 21.55, 14.14. **MALDI-TOF/TOF** *m/z* value Calcd. [M+Na]+ 326.1943, observed 326.1949.



(3S,4S)ethyl 4-((tert-butoxycarbonyl)amino)-3-hydroxy-6-methylheptanoate: Light yellow colour liquid . **^{1}H NMR** (400 MHz,$CDCl_3$) : 4.73-4.71 (d, *J* = 10, 1H), 4.20-4.15 (q, *J* = 7.2, 2H), 4.03-4.01 (d, *J*= 8, 1H), 3.63-3.60 (m, 1H), 3. 30 (b, 1H), 2.59-2.46 (m, 2H),1.69-1.58 (m, 3H), 1.44 (s, 9H) ,1.30-1.26 (t, *J* = 7.2, 3H), 0.94-0.92 (m,6H); **^{13}C NMR** (100 MHz, $CDCl_3$) **:** 173.55, 156.00, 79.18, 69.65,51.93, 41.70, 38.67, 28.35, 24.73, 23.01, 22.24, 14.13. **MALDI-TOF/TOF** *m/z* value Calcd. for [M+Na]+ 326.1943, observed 360.1747.



(3*S*, 4*S*)-ethyl 4-((tert-butoxycarbonyl)amino)-3-hydroxy-5-phenylpentanoate: White powder. **^{1}H NMR** (400 MHz, $CDCl_3$) **:** 7.31-7.22 (m, 5H), 4.97-4.95 (d, *J* = 9.8, 1H), 4.16-4.11 (q, *J* = 7.2, 2H), 4.00-3.97 (d, *J* = 8, 1H), 3.76-3.70 (m, 1H), 3.52 (b, 1H), 2.93-2.91 (m, 2H), 2.63-2.35 (m, 2H), 1.42 (s,9H) ,1.27-1.25 (t, *J* = 7.0, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) **:** 173.61, 155.81, 138.14, 129.41, 128.45, 126.36, 79.40, 66.94, 60.85, 55.34, 38.53, 29.68, 28.33, 14.09. **MALDI-TOF/TOF** *m/z* value Calcd. [M+Na]+ 360.1787, observed 360.1747.



(3*S*, 4*R*)-ethyl 4-((tert-butoxycarbonyl)amino)-3-hydroxy-5-phenylpentanoate: White powder.**^{1}H NMR** (400 MHz, $CDCl_3$) **:** 7.32-7.22 (m, 5H), 4.56-4.54 (d, *J* = 9.8, 1H), 4.21-4.16 (q, *J* = 7.2, 2H), 4.00-3.99 (d, *J* = 6.5, 1H), 3.90-3.84 (m,1H), 3.61 (b, 1H), 3.01-2.82 (m, 2H), 2.61-2.47 (m, 2H), 1.36 (s, 9H) ,1.30-1.26 (t, *J* = 7.2, 3H).^{13}**C NMR** (100 MHz, $CDCl_3$) **:** 173.01, 155.71,137.60, 129.47, 128.45, 126.43,79.60, 70.06, 60.89, 55.11, 38.12, 35.79, 28.24, 14.13.**MALDI-TOF/TOF** m/z value Calcd. [M+Na]+ 360.1787, observed 360.1732.

3b.5.3 Solid-phase peptide synthesis and purification

All the designed peptides analogues **A1**-**A7** were synthesized with a 0.2 mmol scale on Knorr amide resin using standard Fmoc-chemistry. HBTU/HOBt were used as coupling reagents. After completion of the synthesis, peptides were cleaved from the resin using a cocktail mixture of TFA: water: thioanisole (98:1:1). The cleavage mixture was then co-evaporated using DCM under reduced pressure to give a gummy product and recrystallized using diethyl ether to give a white solid. The white solid was dissolved in water and purified through reverse phase HPLC on a C18 column using an ACN/H_2O with 0.1% TFA gradient with 2ml/min flow rate. The purity of the peptides was further confirmed by an analytical C18 column with the same gradient system, with 1ml/min flow rate. The mass of the peptide was confirmed using **MALDI TOF/TOF.**

The synthesis of S1 and S2 were performed with 600mg of Knorr amide resin with a bead capacity of 0.265 mmol/g. After the first coupling (exactly with 1 equivalent of the amino acid at 0.25 mmol scale) the resin was treated with acetic anhydride / DMF and pyridine for capping off the unreacted free amine of resin. Later all the couplings including DABCYL (in the case of S2) were done using basic Fmoc chemistry and HBTU as a coupling reagent. The peptide was cleaved from the resin using TFA/ phenol /water/TIPS as a cocktail mixture as cation scavengers. The isolated crude peptide was recrystallized using diethyl ether to obtain a red colour powder. The crude peptide was dissolved in THF / water in a 4:1 ratio (8 ml) at room temperature. I-AEDANS (5 mg) was added to the reaction mixture and pH was maintained to 8 using Triethylamine. The reaction was kept undisturbed for 4 hours. After completion, the reaction mixture was kept under reduced pressure and the crude peptide was dissolved in water and methanol mixture and purified further using reverse phase -HPLC on C18 column and acetonitrile/water with 0.1% TFA as gradient system with 2ml/min flow rate. Later, the peptide was well characterized by mass using MALDI-TOF/TOF. The purity of the peptides was further confirmed by an analytical C18 column with the same gradient system with 1ml/min flow rate.

3b.5.4 Procedure for inhibitory studies using Mass Analysis

The stock solution of 1 mg/ml each of pepsin from the porcine stomach (sigma) and **S1** substrate was made using 66 mM citrate-phosphate buffer, pH 3.0. Inhibitor analogues **A1-A7** stock solution was prepared each with 1mM concentration in buffer solution. Mass of S1 and analogues were already recorded using MALDI-TOF. For the analysis firstly, 5 µl of protease stock solution and 5 µl of **S1** from stock solution was mixed in 500 µl aliquot and buffer solution is taken to make a total volume of 400 µl. The reaction mixture was kept on a shaker for 10-15 mins for the proteolysis to occur. After 10-15 mins, the mass was recorded for the reaction mixture. In this case, we observed the mass of proteolytically cleaved **S1** and its fragments. Mass was recorded at cationic mode so we were able to get mass of positive ions only i.e., 532 and 679.

Now for checking the inhibitory activity of the synthesized analogues **A1**-**A7**, 5 µl of pepsin and 5 µl of **A1** -analogues was taken from its stock solutions to a 500 µl aliquot, mixed well and incubated for 30 mins at 37 ˚C. After incubation time, 5 µl of this mixture (**S1** and **A1**) was picked out and put in an aliquot which contains 5 µl of S1 already in it and buffers solution was put into this to make a final volume of 400 µl and kept in a shaker for 10-15 mins to

undergo proteolysis. After 10-15 mins, the mass was recorded again for this reaction mixture. We observed no fragmented mass of S1. So preliminary results show that **A1** can be an inhibitor for pepsin. This same procedure was repeated with the rest of the analogues (**A2**-**A7**) and surprisingly we observed no fragmented mass of **S1** in each case except with **A4** and **A5**. Suggesting that analogue **A4** and **A5**, which contains Valine statine residue are not showing any inhibition towards pepsin.

For checking proteolytic stability of analogues **A1**-**A7** against pepsin protease, 5 µl remaining mixture of analogues with pepsin which was pre-incubated was also checked for mass analysis. We have observed no proteolysis as an intact mass of **A1**-**A7** were seen in mass spectral analysis. All the studies are carried out at room temperature and stock solutions were kept at 4 $^{\circ}$C, in 66 mM citrate-phosphate buffer, pH 3.0. Using these preliminary results showing all the analogues except **A4**-**A5** are actively inhibiting pepsin we went ahead with the remaining five active analogues for further studies.

3b.5.5 Procedure for inhibitory studies using fluorescence

Stock solution 1 mg/ml of pepsin from the porcine stomach (sigma) and 1 mM substrate **S2** was prepared for each assay. Later with dilution, the final concentration of 0.03 mM of pepsin and 0.5 mM of substrate stock solution was prepared. Fluorescence was recorded using a spectrophotometer (Perkin Elmer). All spectra were recorded at emission wavelength 490 nm with 335 nm excitation wavelength with a slit width adjusted to 10 nm in each case. The enzyme proteolytic assay of pepsin was carried out using citrate phosphate buffer, 66 mM and pH adjusted to 3.0. For checking the proteolytic activity of pepsin, 1 µl of pepsin (0.03 mM) from their stock solution was added to substrate S2 (0.5 mM) present in fluorescence cuvette and the total volume was adjusted to 500 µl using the buffer. An increase in fluorescence emission intensity of **S2** was recorded with a 1min interval time till no further change in intensity was observed.

For checking the inhibitory activity of designed pepstatin analogues which showed active inhibition in the previous assay, a stock solution of each analogue of 1mM concentration was prepared using a buffer solution and then diluted to adjust the final concentration to be 0.3 mM. Before going for proteolysis 5 µl of pepsin stock solution (0.03 mM) and 5 µl of **A1** analogue (0.3mM) were mixed well and preincubated for 30 mins at 37 $^{\circ}$C in 500 µl aliquot and kept on the shaker. After 30 mins, 1 µl from this mixture is added to the S2 substrate (0.5 mM) present in fluorescence cuvette and the total volume was adjusted to 500 µl using the buffer. The

increase in fluorescence intensity was recorded till the saturation point is reached. This same procedure is repeated with the remaining four analogues. We observed **A6** and **A7** which contains phenylalanine statine motifs showed very little increase in intensity indicating strong binding with pepsin and inhibiting its proteolytic activity. Analogue **A3**, containing leucine statine motif with *syn (S, S)* conformer is showing a moderate increase in intensity indicating moderate inhibition of pepsin by **A3**.The **A1** and **A2** analogues which contain leucine statine motif with *anti (R, S)* conformer is showing an enhanced increase in fluorescence intensity of **S2** suggesting that they are not inhibiting the protease at this concentration. All the assays are carried out at room temperature where the concentration of the protease to inhibitor analogue was kept to be 1:10. All the assay was conducted thrice before concluding.

For checking specificity of these analogues, **A7** was checked for the inhibition of chymotrypsin and trypsin, a serine protease with 100 mM triethanolamine buffer with basic pH of 7.5 and the same procedure is repeated.

3b.5.6 Procedure for thermodynamic study using ITC

The thermodynamic study was performed on MicroCal1000 -iTC200 calorimeter at a constant temperature of 25 °C of the cell. Before the experiment A6, A7 and pepsin which was dissolved in Citrate phosphate buffer, 66 mM and pH adjusted to 3.0 were degassed and the syringe, sample cell and reference cell were cleaned, degassed and dried. The experimental parameters were adjusted to 20 injections of 2μl each with a spacing time of 150 s and an initial delay of 60 s. The reference cell was filled with citrate phosphate buffer. The concentration of pepsin in the sample cell was 0.03 μM and analogues A6 and A7 concentration of 0.3 μM stock solution by keeping the ratio of protease to inhibitor in 1:10 molar ratio concentration. All the experiments were performed thrice with each analogue. The data was generated by the instrument installed software, MicroCal origin with: “one binding site model and sigmoidal curve fitting”.

3b.6 References

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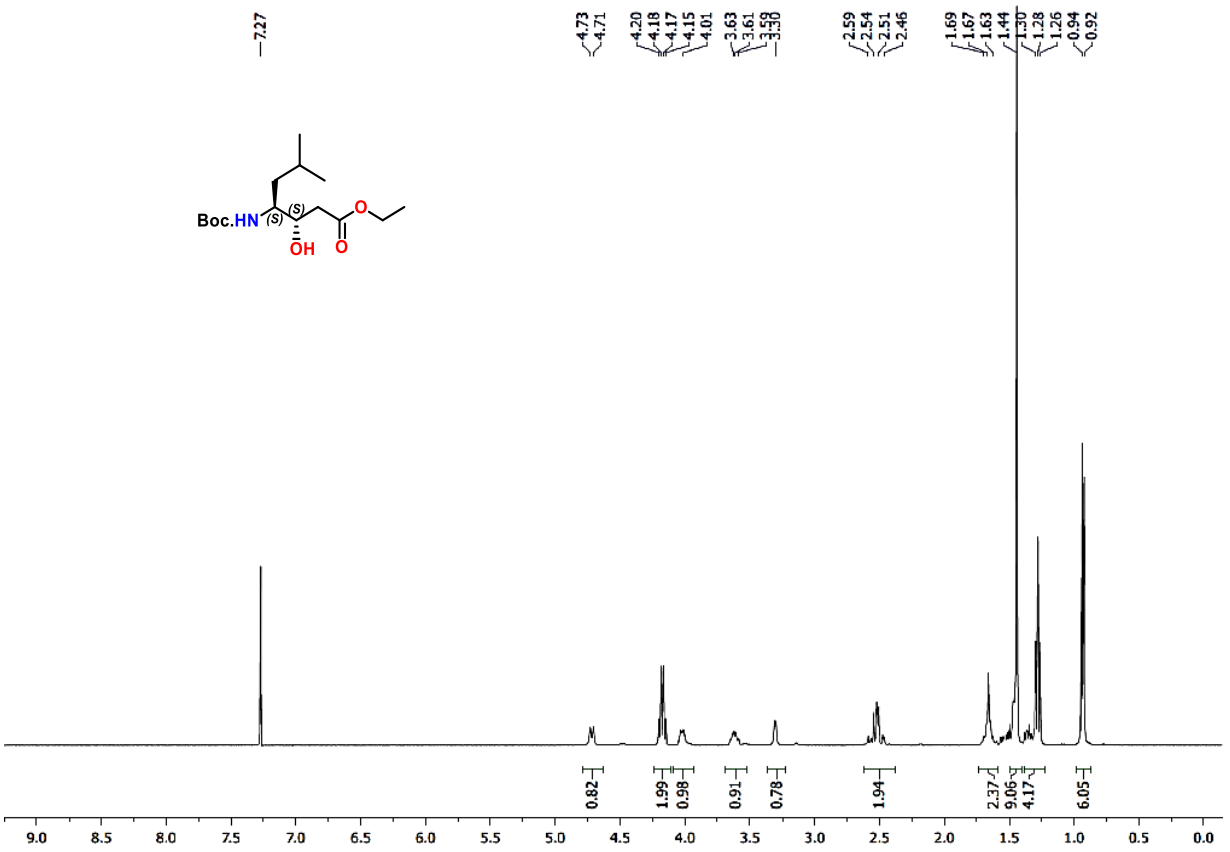
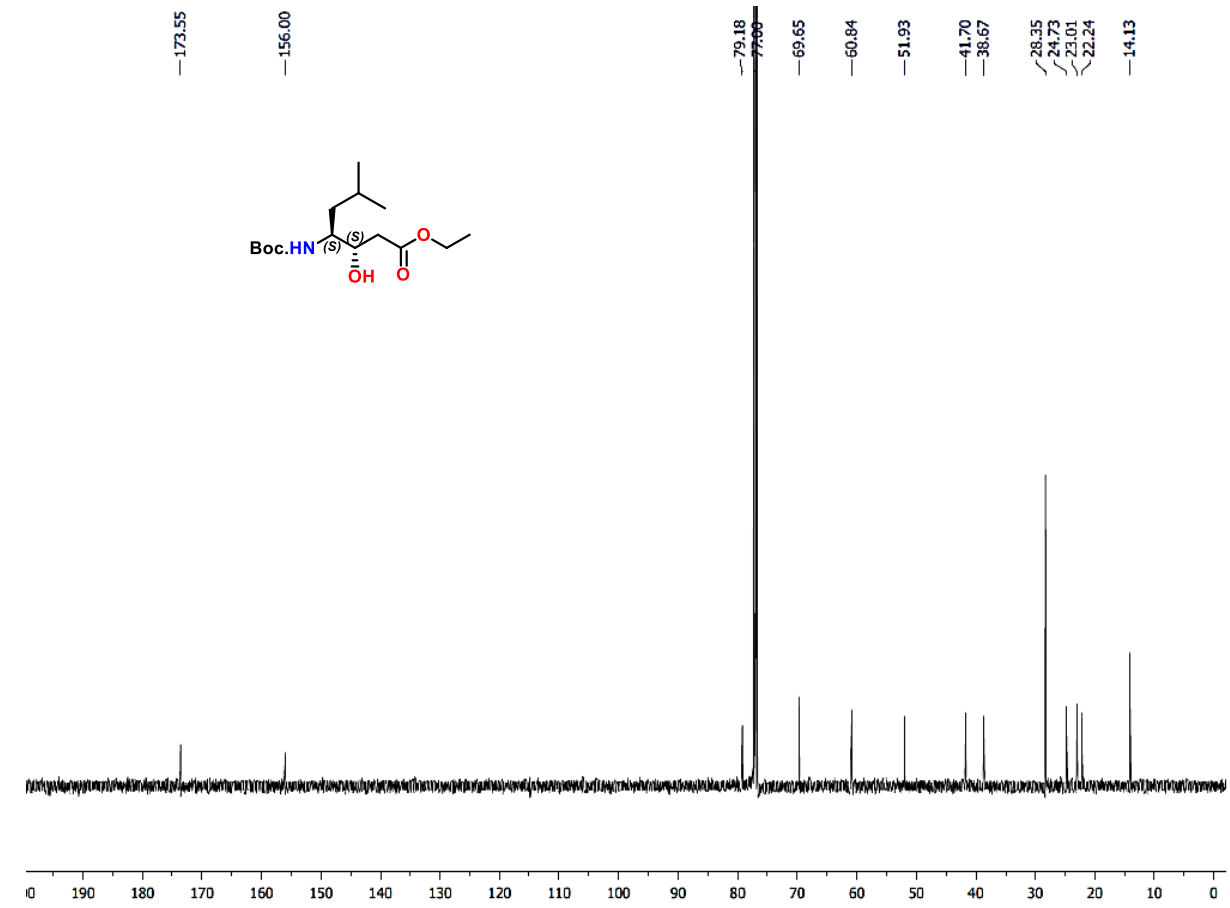
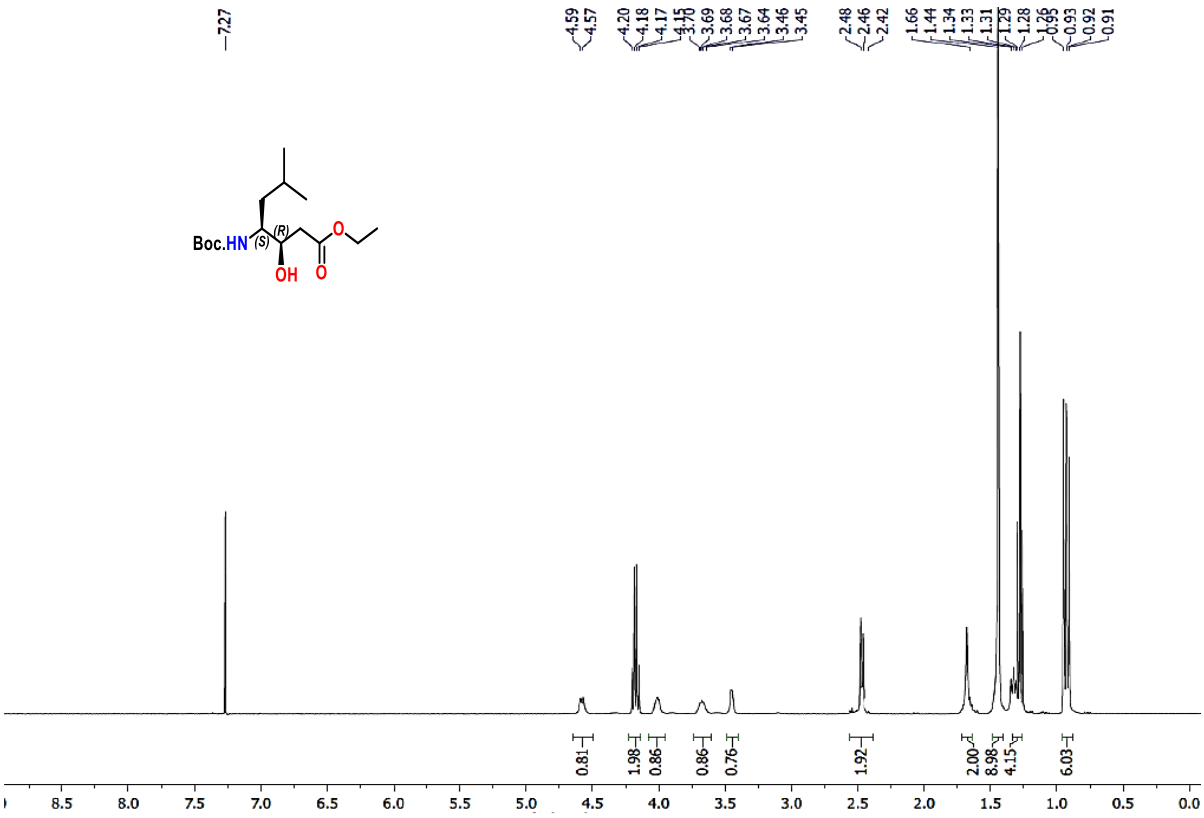
3b.7 Appendix: ^{1}H and ^{13}C NMR, mass spectra and HPLC traces



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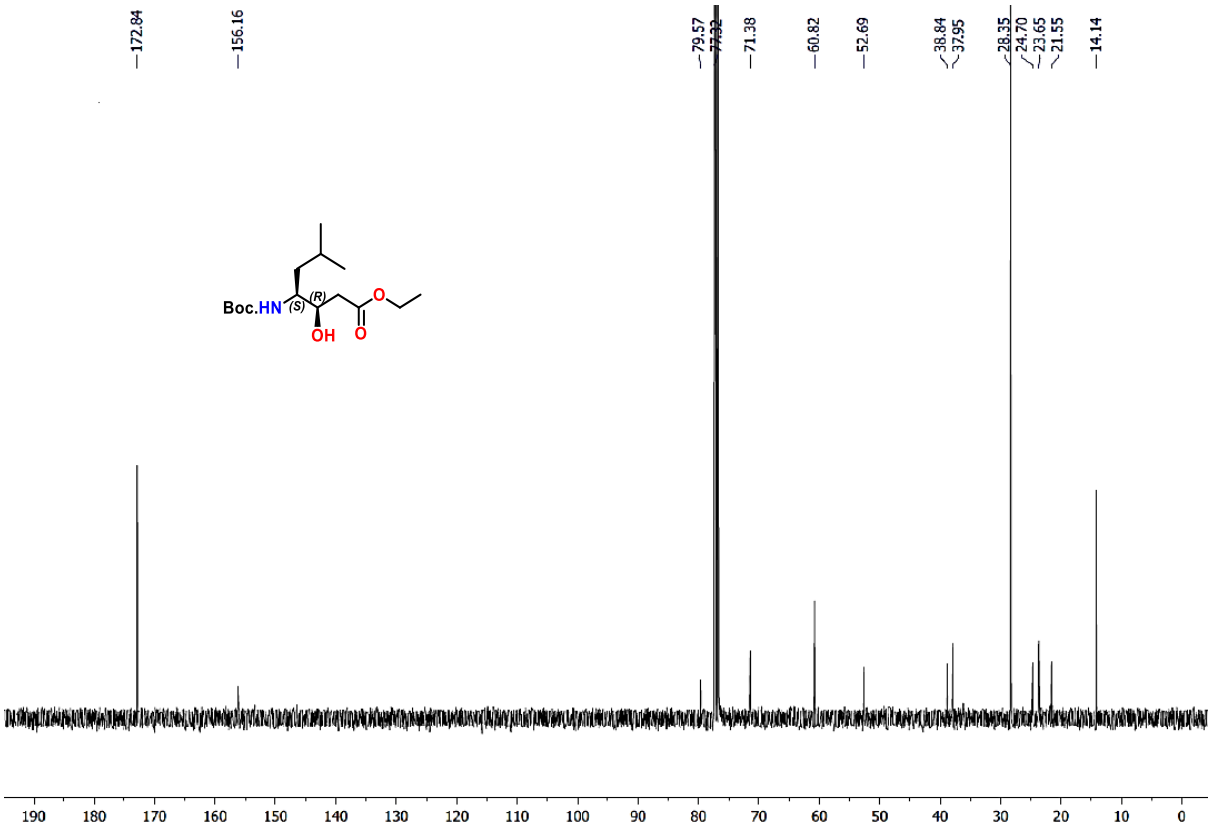
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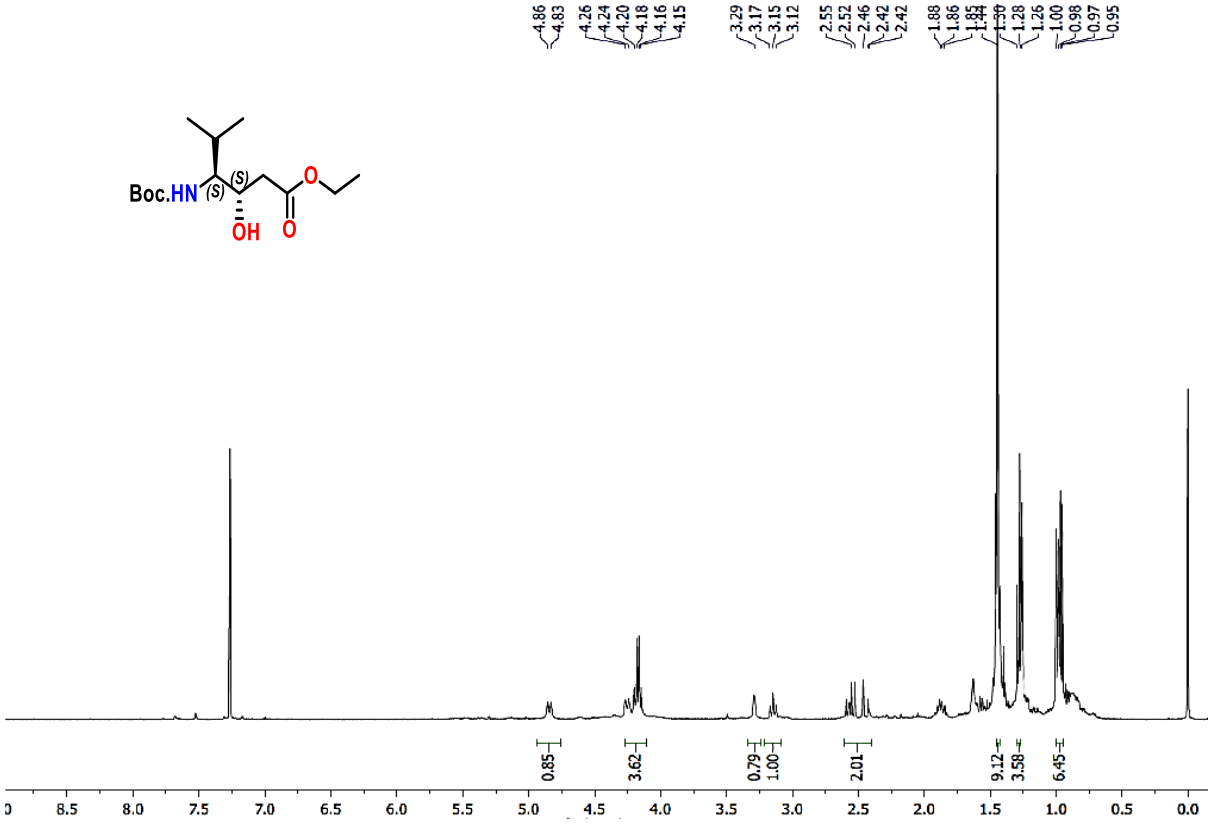
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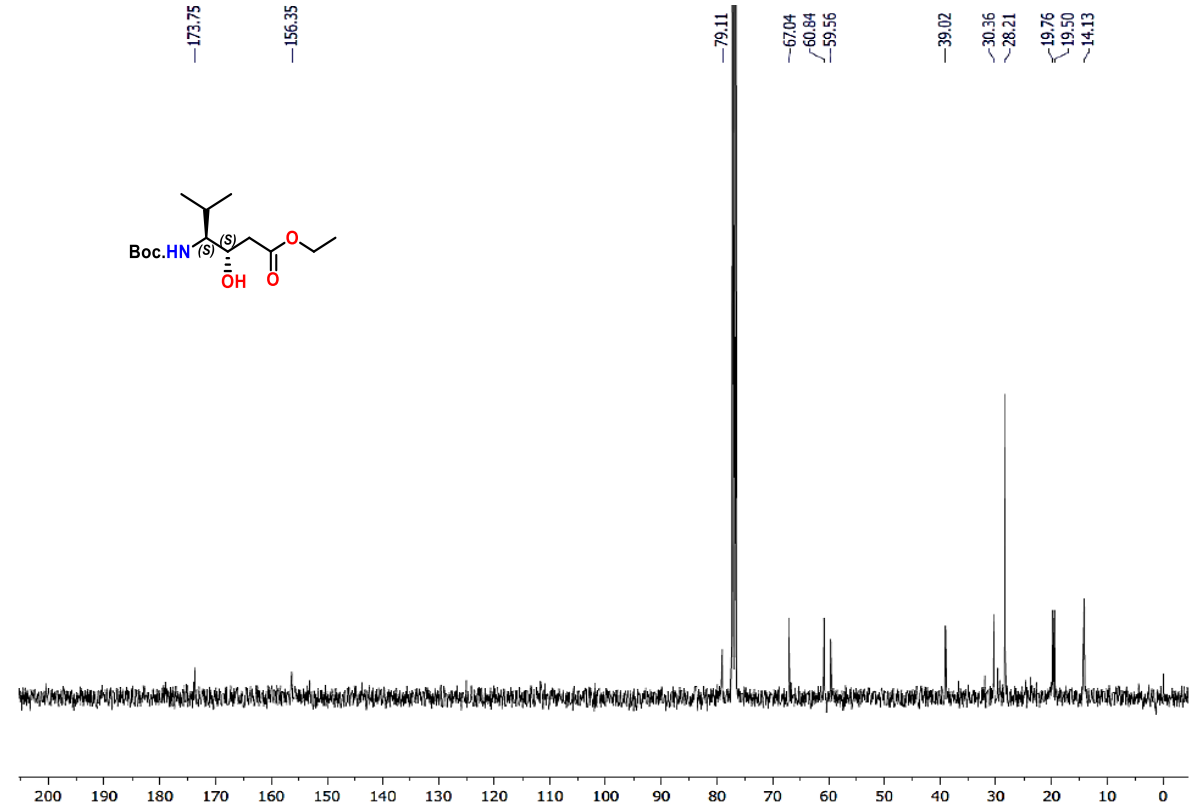
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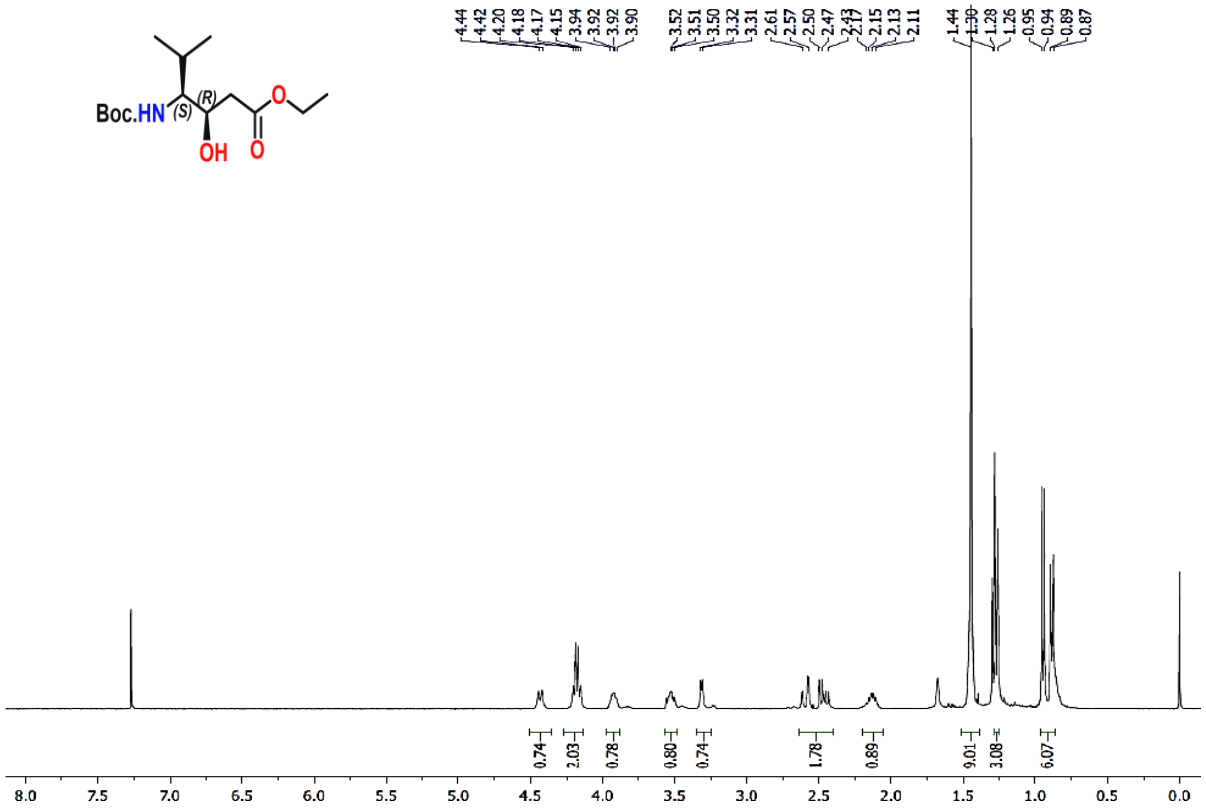
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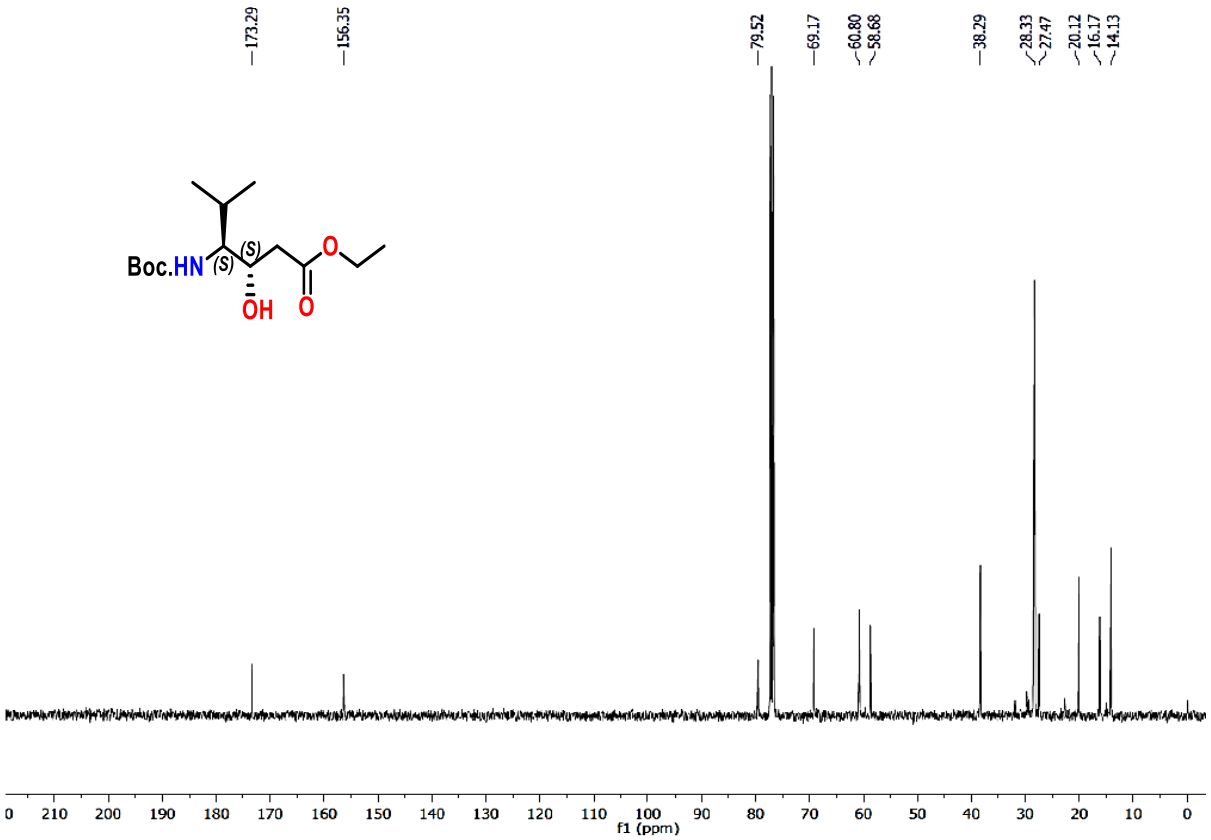
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69.17
60.80
58.68
38.29
28.33
27.47
20.12
16.17
14.13
Boc.HN
(S)
(S)
OH
O
O
0
210
200
190
180
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100
90
80
70
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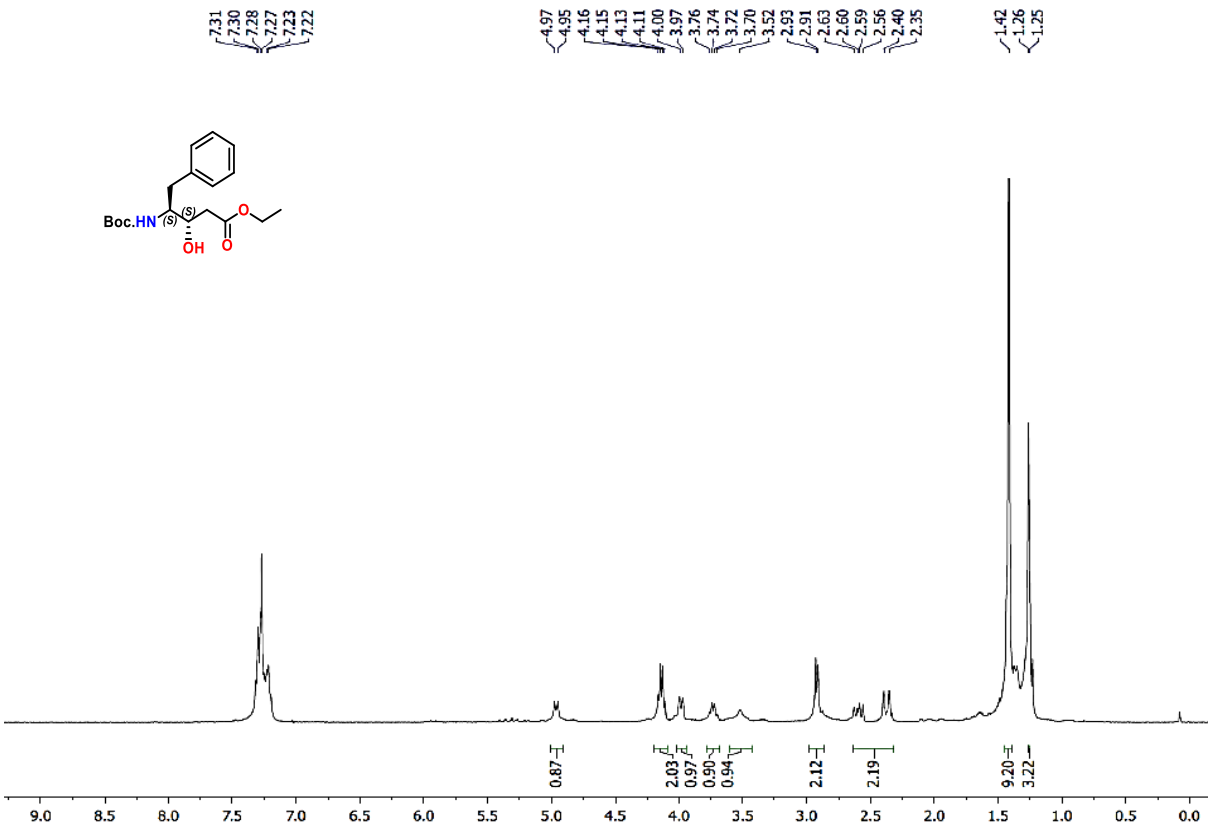
50
40
30
20
10
0
f1 (ppm)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4.00
3.97
3.76
3.74
3.72
3.70
3.52
2.93
2.91
2.63
2.60
2.59
2.56
2.40
2.35
1.42
1.26
1.25
Boc.HN
(S)
(S)
OH
O
O
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2.03
0.97
0.90
0.94
2.12
2.19
9.20
3.22
9.0
8.5
8.0
7.5
7.0
6.5
6.0
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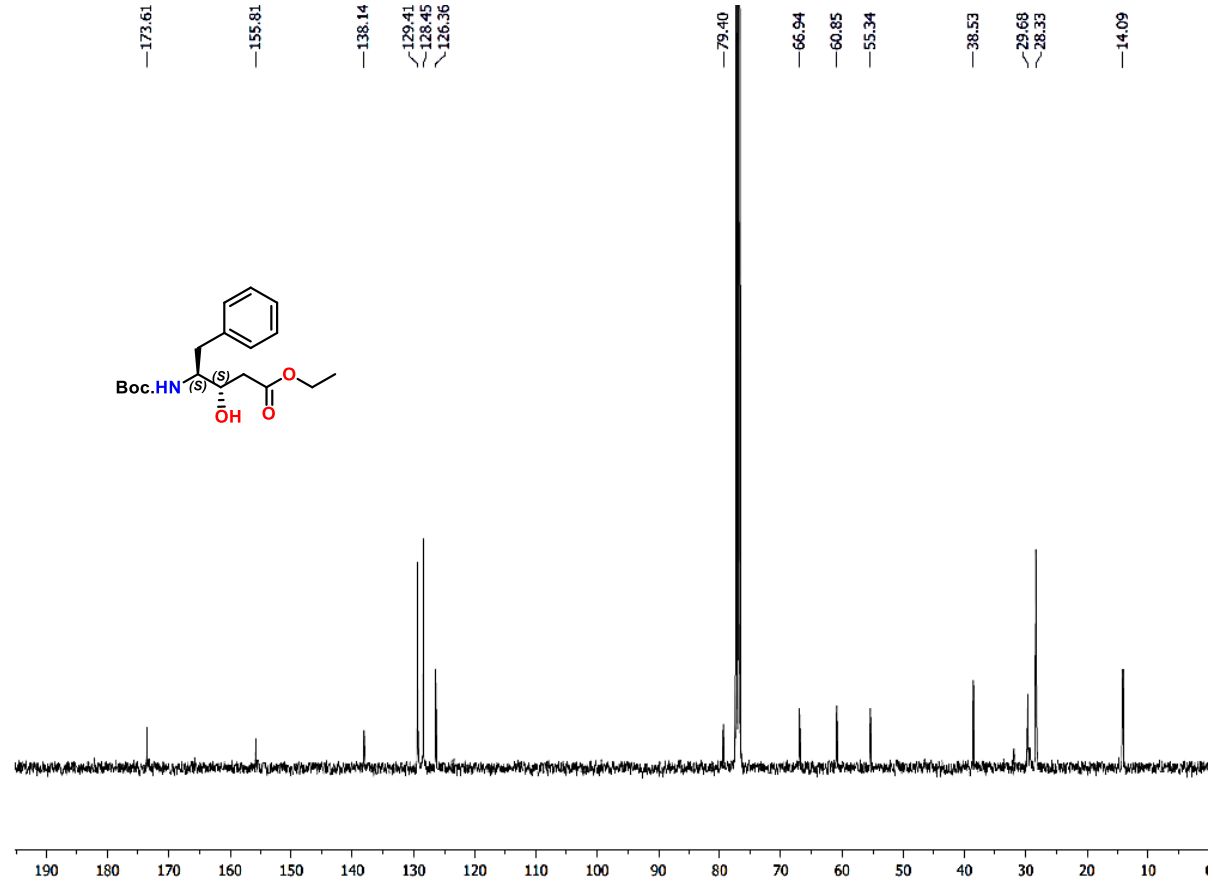
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79.40
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14.09
Boc.HN
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(S)
OH
O
O
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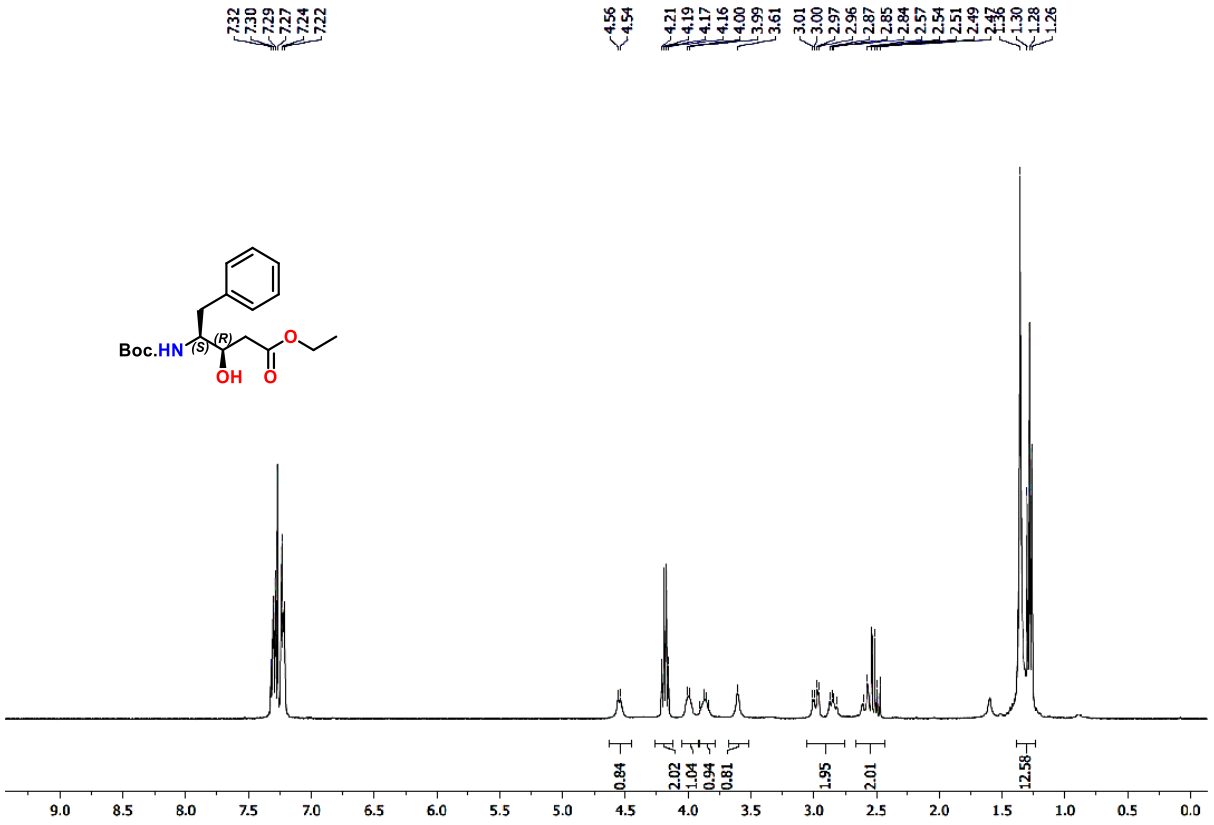
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4.54
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4.19
4.17
4.16
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3.99
3.61
3.01
3.00
2.97
2.96
2.87
2.85
2.84
2.57
2.54
2.51
2.49
1.30
1.28
1.26
Boc.HN
(S)
(R)
OH
O
O
0.84
2.02
1.04
0.94
0.81
1.95
2.01
12.58
9.0
8.5
8.0
7.5
7.0
6.5
6.0
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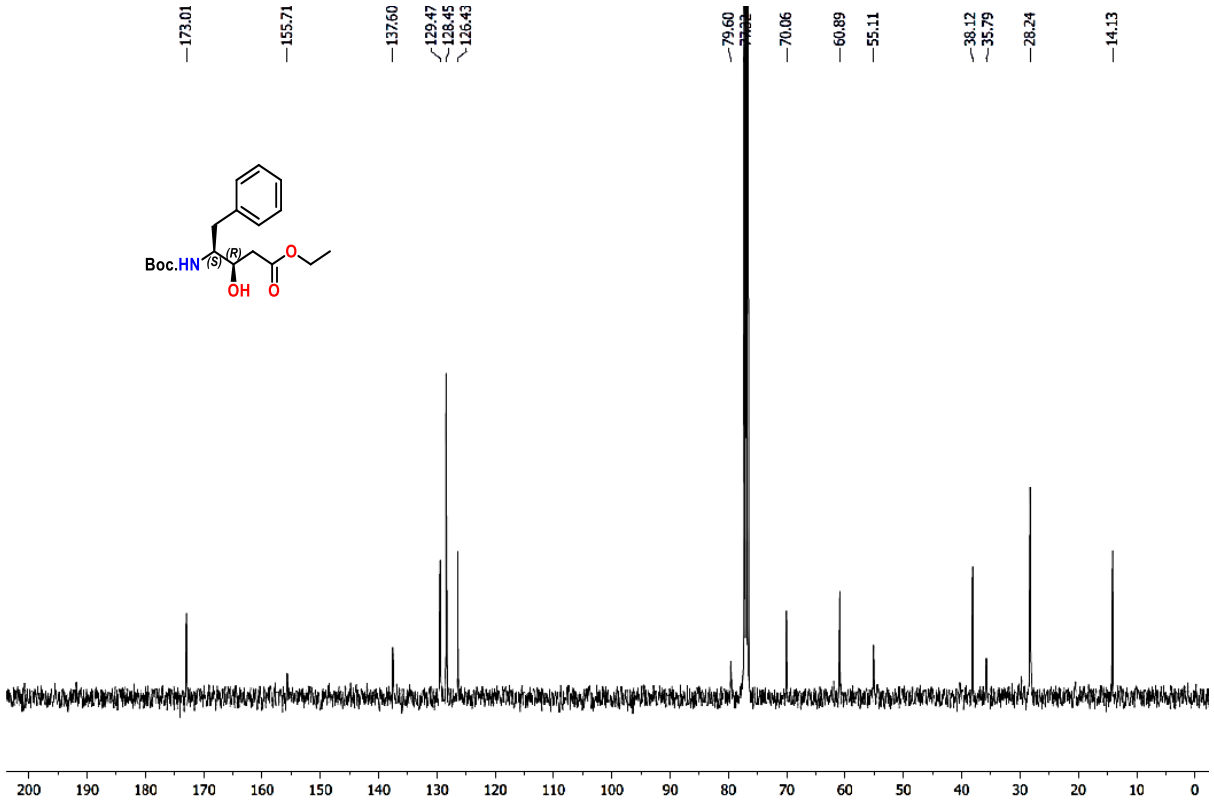
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129.47
128.45
126.43
79.60
70.06
60.89
55.11
38.12
35.79
28.24
14.13
Boc.HN
(S)
(R)
OH
O
O
200
190
180
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150
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110
100
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80
70
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10
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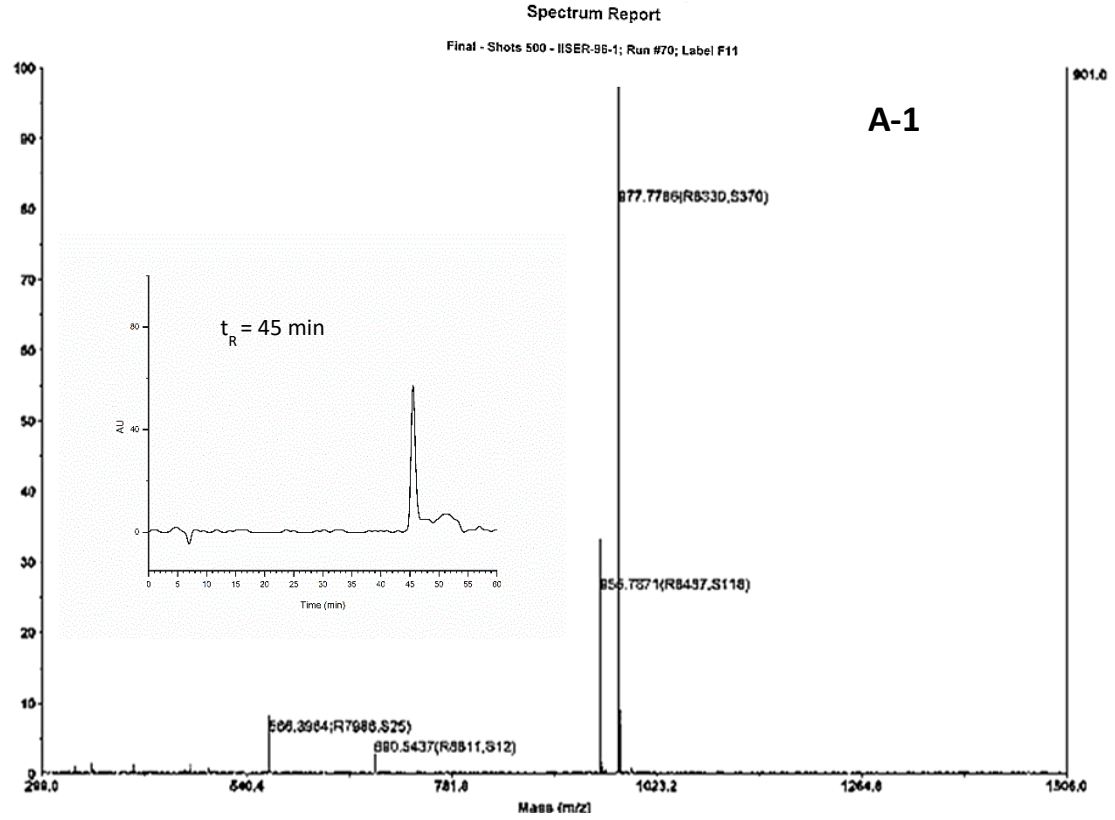
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A-1
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AU
Time (min)
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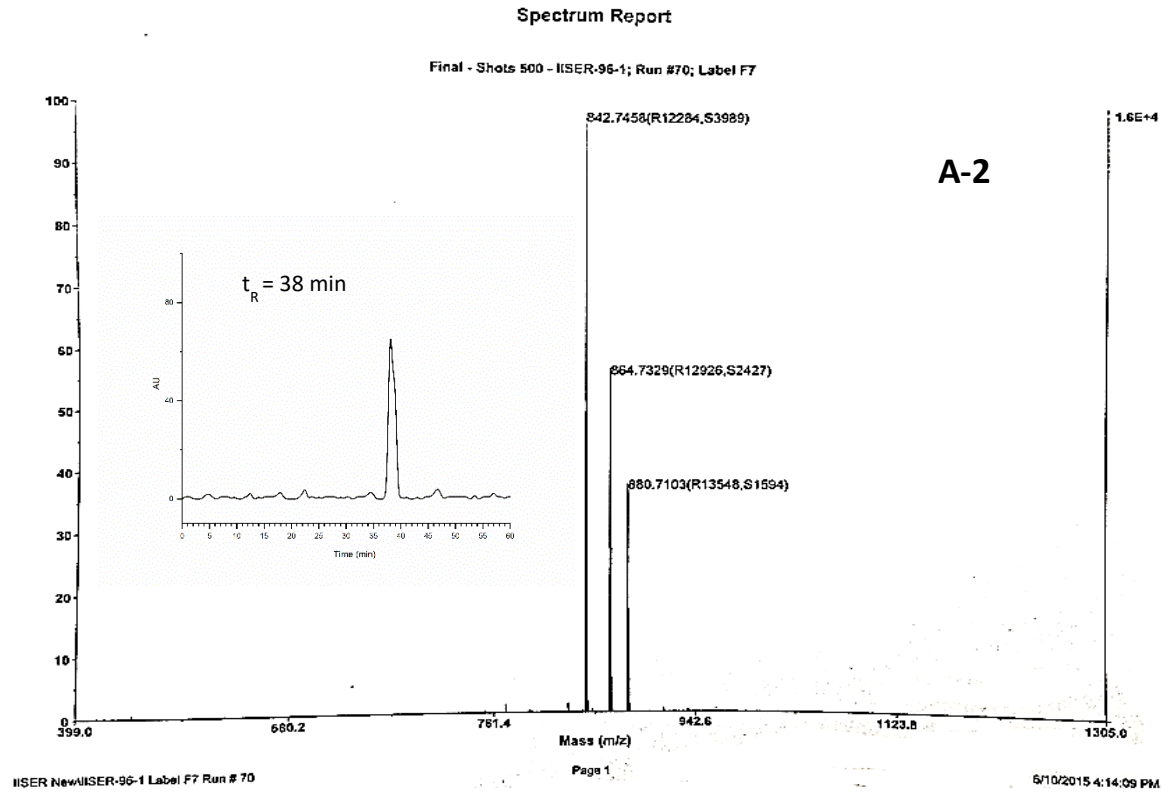
856.7871(R8437,S118)
666.3964;R7986,S25)
680.5437(R8511,S12)
901.0
Mass (m/z)


Spectrum Report
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Time (min)
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1.6E+4
864.7329(R12926,S2427)
880.7103(R13548,S1594)
Mass (m/z)
IISER New\IISER-96-1 Label F7 Run # 70
Page 1
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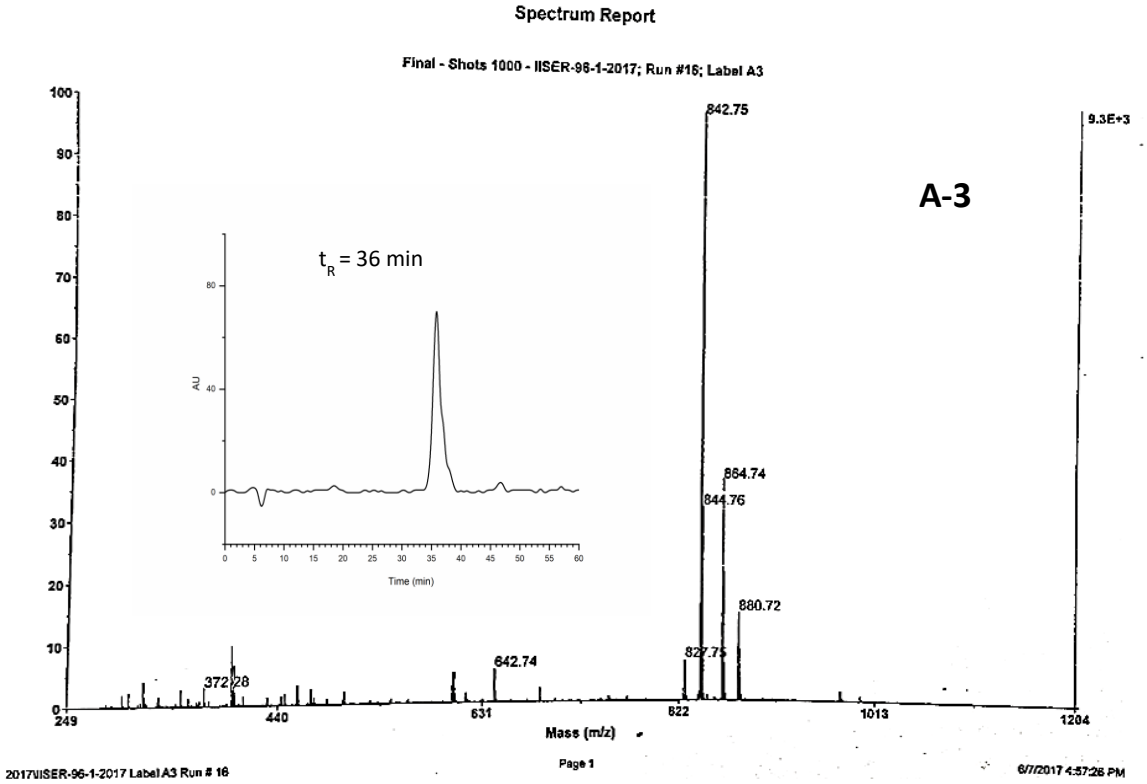
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A-3
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AU
Time (min)
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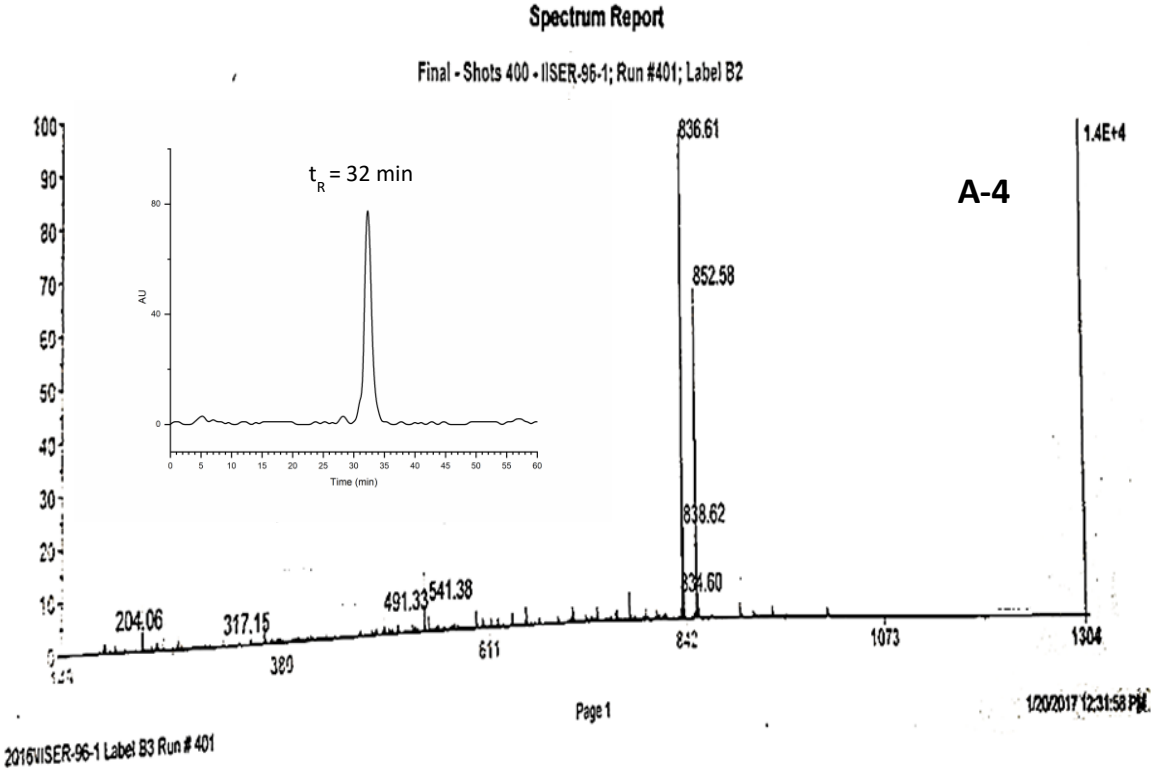
9.3E+3
864.74
844.76
880.72
827.75
642.74
372.28
249
440
631
822
1013
1204
Mass (m/z)
Page 1
2017\IISER-96-1-2017 Label A3 Run # 16
6/7/2017 4:57:26 PM


Spectrum Report
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A-4
t_R = 32 min
AU
Time (min)
836.61
1.4E+4
852.58
838.62
834.60
491.33
541.38
204.06
317.15
389
611
842
1073
1304
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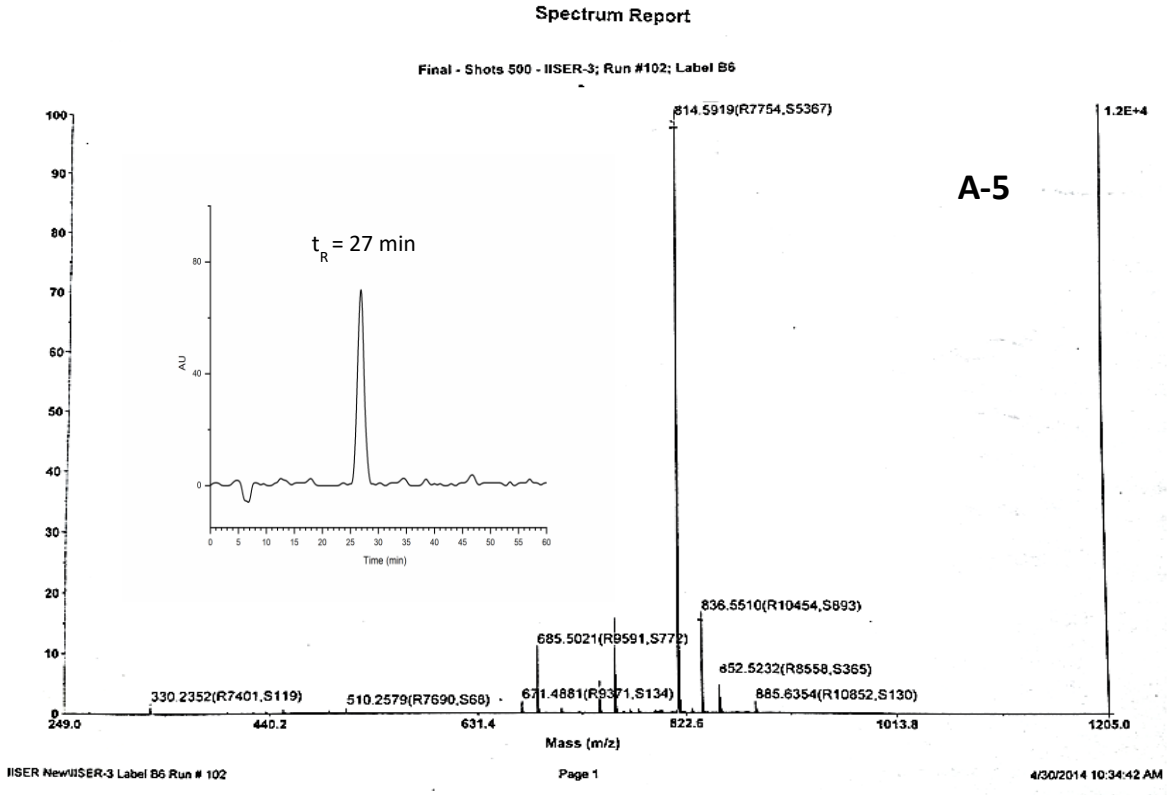
2016\IISER-96-1 Label B3 Run # 401


Spectrum Report
Final - Shots 500 - IISER-3; Run #102; Label B6
814.5919(R7754,S5367)
1.2E+4
A-5
t_R = 27 min
AU
Time (min)
836.5510(R10454,S893)
685.5021(R9591,S772)
852.5232(R8558,S365)
330.2352(R7401,S119)
510.2579(R7690,S68)
671.4881(R9371,S134)
885.6354(R10852,S130)
Mass (m/z)
IISER New\IISER-3 Label B6 Run # 102
Page 1
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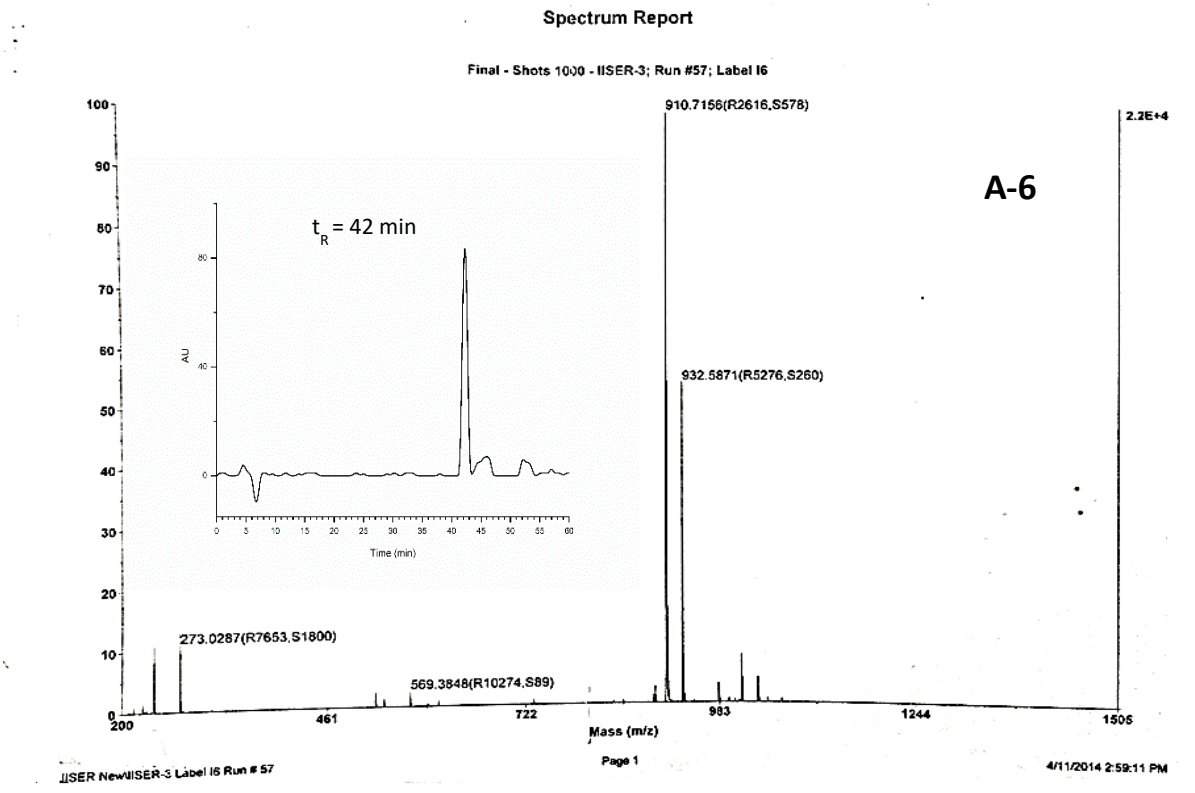
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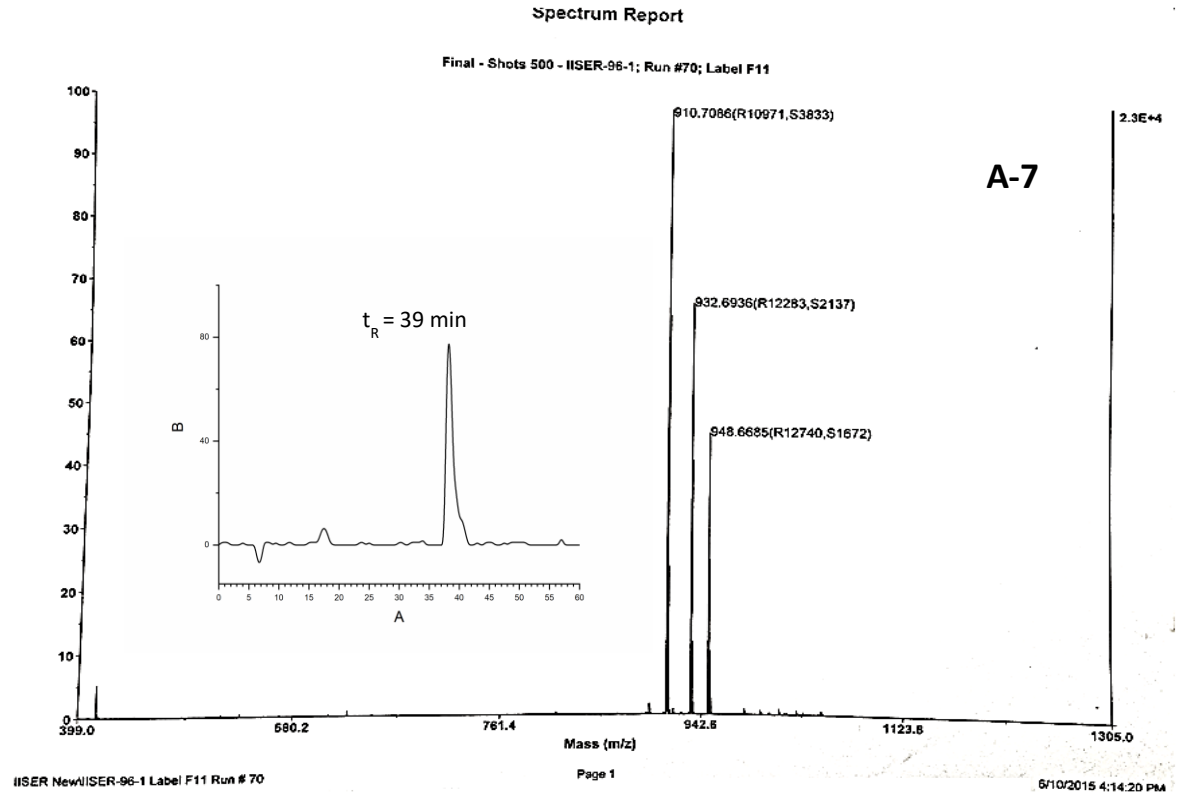
2.2E+4
A-6
t_R = 42 min
AU
Time (min)
932.5871(R5276,S260)
273.0287(R7653,S1800)
569.3848(R10274,S89)
Mass (m/z)
IISER New\IISER-3 Label I6 Run # 57
Page 1
4/11/2014 2:59:11 PM


Spectrum Report
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2.3E+4
A-7
932.6936(R12283,S2137)
948.6685(R12740,S1672)
t_R = 39 min
B
A
399.0
680.2
761.4
942.6
1123.8
1305.0
Mass (m/z)
IISER New\IISER-96-1 Label F11 Run # 70
Page 1
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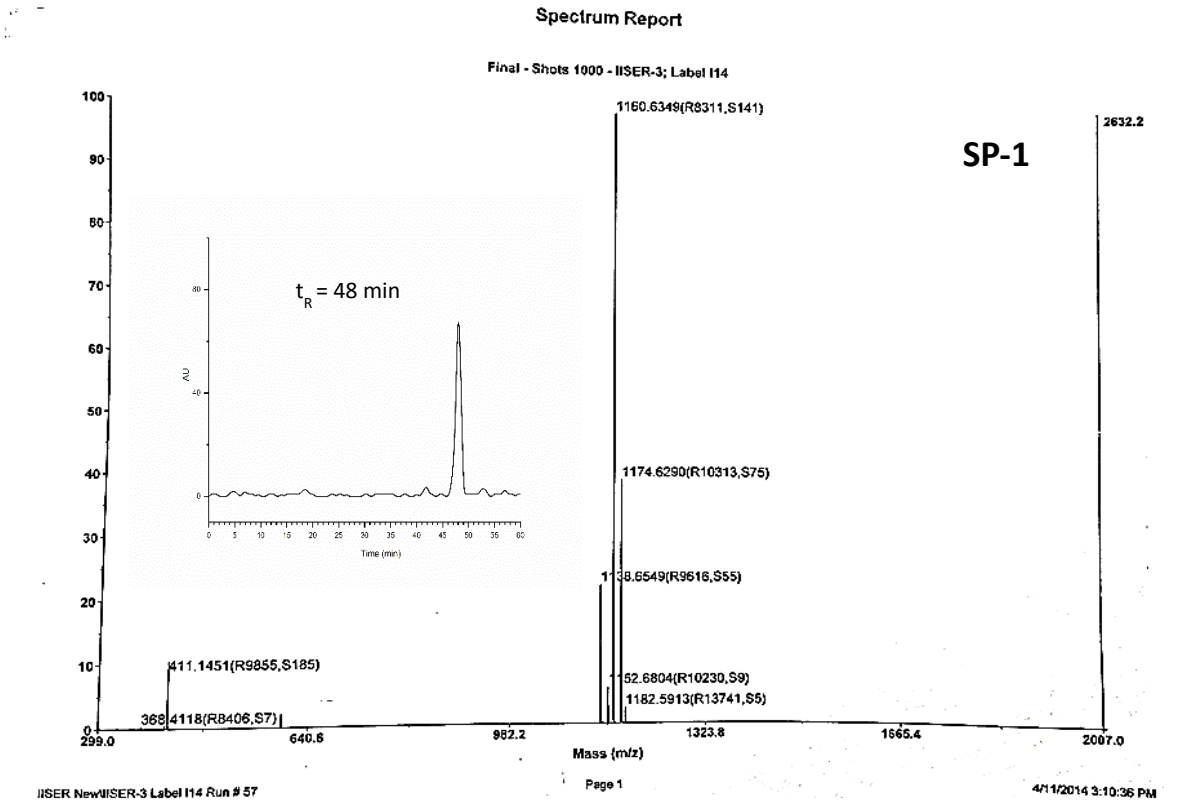
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Final - Shots 1000 - IISER-3; Label I14
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2632.2
SP-1
1174.6290(R10313,S75)
1138.6549(R9616,S55)
411.1451(R9855,S185)
1152.6804(R10230,S9)
1182.5913(R13741,S5)
368.4118(R8406,S7)
t_R = 48 min
AU
Time (min)
299.0
640.6
982.2
1323.8
1665.4
2007.0
Mass (m/z)
IISER New\IISER-3 Label I14 Run # 57
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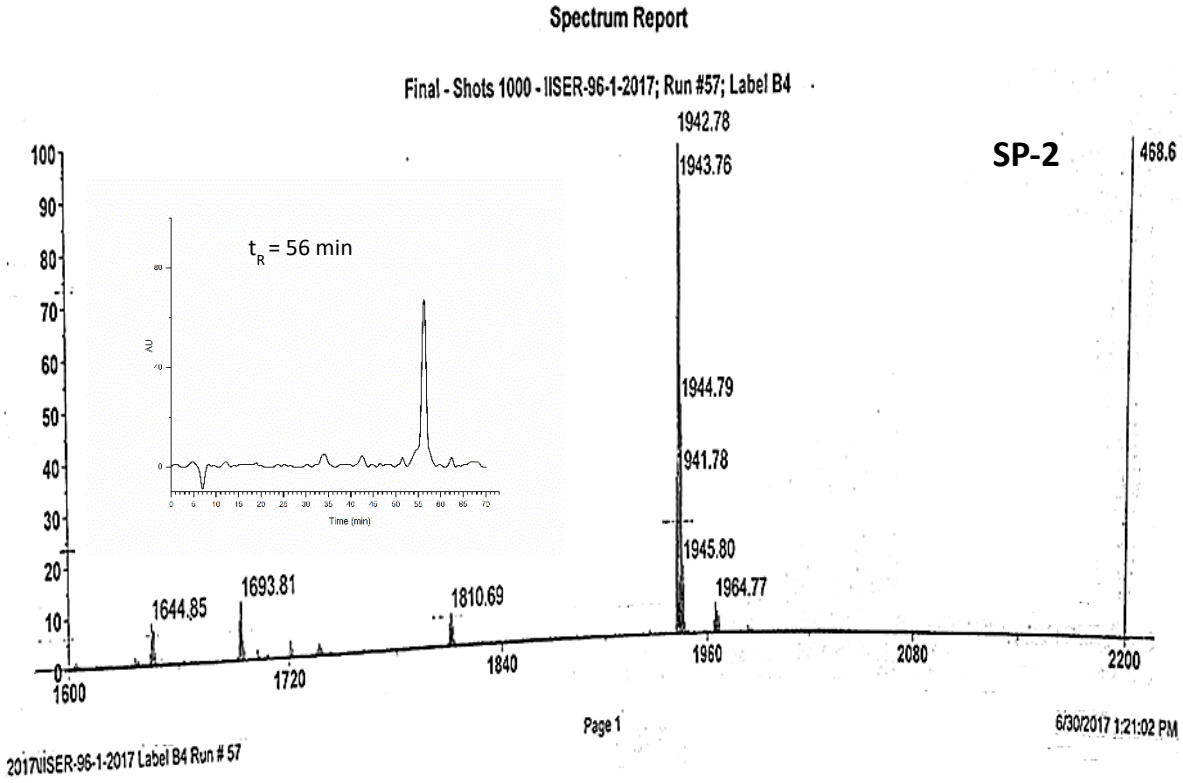
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AU
Time (min)
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468.6
1944.79
941.78
1945.80
1964.77
1644.85
1693.81
1810.69
100
90
80
70
60
50
40
30
20
10
0
1600
1720
1840
1960
2080
2200
Page 1
6/30/2017 1:21:02 PM
2017\IISER-96-1-2017 Label B4 Run # 57

Chapter 4(a)

Design, Synthesis and Antimicrobial Properties of Short Heterogenous Cationic Lipopeptides containing β-hydroxy γ-amino acids.

4a.1 Introduction

Antibiotics have proved to be a major breakthrough in contemporary medicinal science and have shown their vast importance for an era since their discovery in 1928 with the discovery of *Penicillin* by Alexander Fleming.[1] The evolving of new species of pathogens across the globe and their resistance towards multiple drugs (superbugs or multi-drug resistant strains, MDRS) are making antibiotics lose their productivity and efficacy.[2] The overuse and misuse of antibiotics over few decades have causes bacterial strains to evolve and mutate to stand against antibiotics and leads to resistant varieties. Antimicrobial resistance is standing as a severe threat to global health and the clinal use of antibiotics.[3] The exclusive demand today is the need for a novel class of anti-microbial agents. To overcome this problem various Host Defence Peptides (HDP) or specifically called antimicrobial peptides (AMP) have emerged as a new class of potential alternatives to antibiotics in recent decades.[4]

Antimicrobial peptides (AMPs) are widely present in living organisms as a significant part of the innate immune system that provides "defence to the host" by preventing an attack from broad-spectrum microbes including bacteria, protozoa, fungi and viruses. AMPs have also shown immunomodulatory properties in encouraging wound healing, suppressing inflammation, stimulating the production of cytokine and also showed killing effect on few cancerous cells lines.[5] That is the reason, they are often called host defence peptides (HDPs) in place of AMPs. Extensively studied, cathelicidin (LL-37) and beta-defensins family are among such host defence AMPs present in humans.

AMPs are usually short peptides of various lengths with 10 to 50 amino acid residues. They are mostly cationic containing +2 to +9 net positive charge due to the occurrence of lysine or arginine amino acid residues. Although few negatively charged anionic AMPs have also been reported such as dermcidin, a human anionic antimicrobial peptide secreted by sweat glands.[6]

In these peptides, exist a perfect balance of hydrophilicity and hydrophobicity making them amphipathic, which is essential for their activity.[7] The cationic part involves in the process of explicit interaction to negatively charged head groups of lipid chain present on microbial lipid bilayer cell membrane apart from the zwitterionic eukaryotic cell membrane and the hydrophobic residues are engaged to interact with lipid acyl chains and encourage in the insertion process to the membrane.[8]

AMPs are more potent because of their generalised target including disruption of the cell membrane (bactericidal) and not a specific target (bacteriostatic) as in the case of antibiotics, making them less prone to developing resistance by microbes.[9] Even though, few conducts of developing resistance against cell wall disruption by AMPs have been observed.[10]

A literature study reveals that huge research is going on making synthetic mimics of naturally occurring AMPs, called as SMAMPs because of poor bioavailability, poor selectivity and high haemotoxicity of the former.[11] An excellent approach over the years to overcome the protease instability problem is backbone modification. Modification at *N*- terminal by attaching lipid chain (lipopeptides),[12] cyclizations of linear peptides (cyclic peptides),[13] using D-amino acid, β and γ-amino acid instead of L-amino acid (synthetic hybrid peptides from non-ribosomal amino acid),[14] shorter derivatives of naturally occurring AMPs (peptidomimetics)[15] are few among them.

Shai and co-workers have demonstrated the utilization of fatty acid chains to make *N*-terminally modified natural and synthetic AMPs and studied their antimicrobial activities on various strains.[16] They have also reported short lipopeptides of different combinations of D and L-amino acids.[17] They did detailed studies on the mechanism of binding and disruption of the microbial membrane by these antimicrobial peptides.[18] Later, Schweizer and co-workers have also reported various potent antimicrobial lipopeptides.[19] DeGrado and co-workers have reported amphiphilic β^3-peptide with C_{14} helical pattern to exhibit efficient and selective antimicrobial activity.[20] Later, the incorporation of β-amino acids in designing AMPs was extensively studied by Gellman and co-workers[21] and others.[22] Various amphipathic homo-oligomers of cyclic β-amino acid of C_{12} conformations have shown antimicrobial activity similar to magainin but less of haemolytic value. Also, potent antibacterial and antifungal cationic peptides composed of β-amino acid with C_{14} helical pattern have also been reported. They have also reported antimicrobial peptides with a heterogeneous backbone of α,β-amino acids. At the same time, Guichard and co-workers have reported various antimicrobial foldamers containing heterogenous urea backbone with less cytotoxicity and more potency.[23] Gellman and group have reported various antimicrobial and antifungal peptides with nylon-3 copolymers.[24] Few synthetic antimicrobial peptides reported in the literature are shown in Figure 1. The major advantage of β-AMPs over α-AMPs is their high proteolytic stability making them the target for enhancing potency and bio-stability of native AMPs.[25] Over the years various γ-peptide foldamers and natural products containing γ-amino acids with various

biological importance have been reported in the literature (described in chapter 1) but γ-peptides antimicrobial activities were still to be explored.

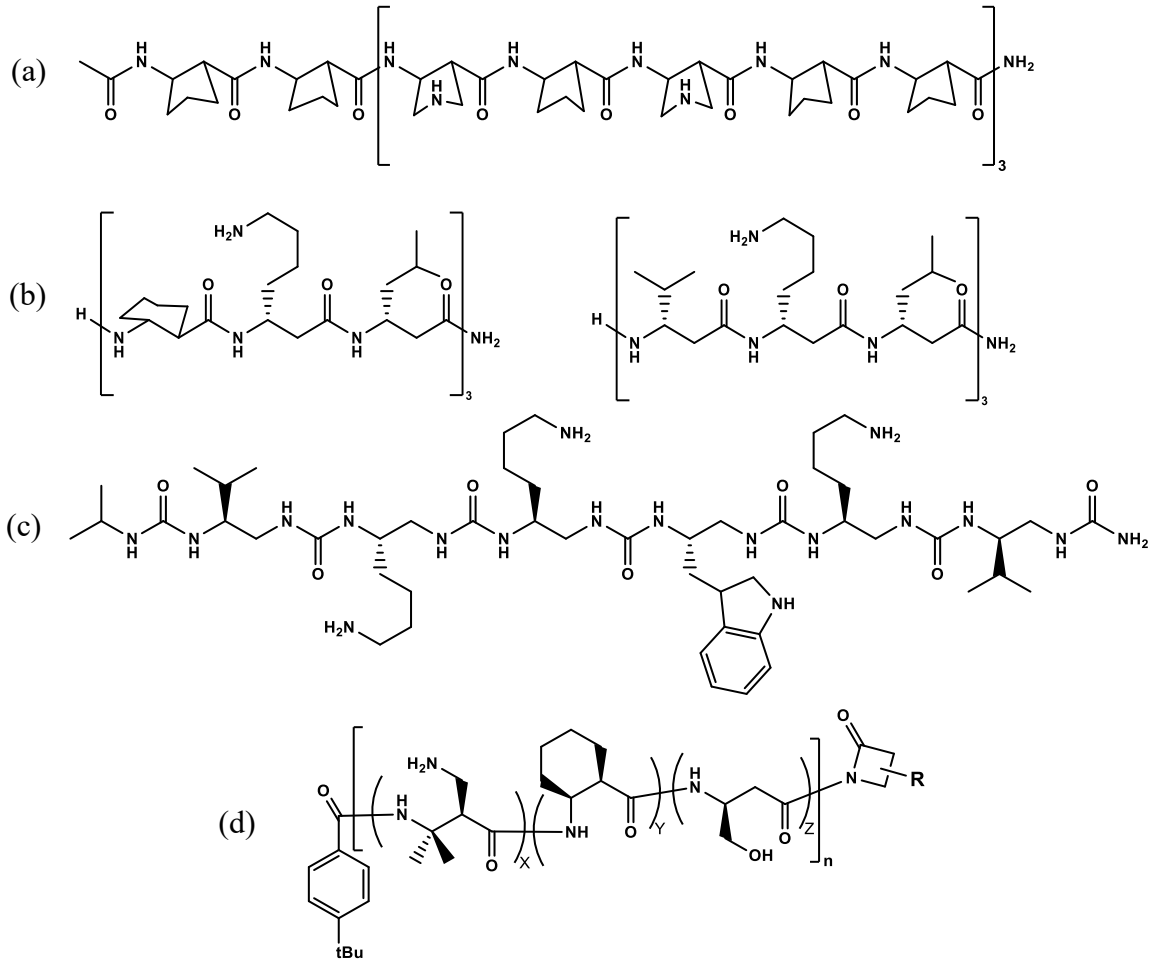


Figure 1: Various synthetic antimicrobial peptides. (a) 12-helical β-peptide,[21a] (b) 14-helical β-peptide,[21b] (c) amphiphilic oligourea[23b] and (d) ternary Nylon-3 copolymer.[24a]

Earlier Work Reported by Our Group

Recently, our lab has reported the utilization of non-proteinogenic α, β unsaturated γ-amino acids[26] and γ^4-amino acid[27] in designing short hybrid lipopeptides which have shown potent antimicrobial activities against a broad spectrum of gram-positive and gram-negative bacteria. Antifungal activity of hybrid α, β unsaturated γ-amino acid containing lipopeptides were also been reported.[26] Their mode of action and self-assembly behaviours were also shown. These lipopeptides have shown specificity towards bacterial cells over RBCs and proved to be non-haemolytic at their MIC concentration. The few effective lipopeptides reported by our group are shown in Figure 2.

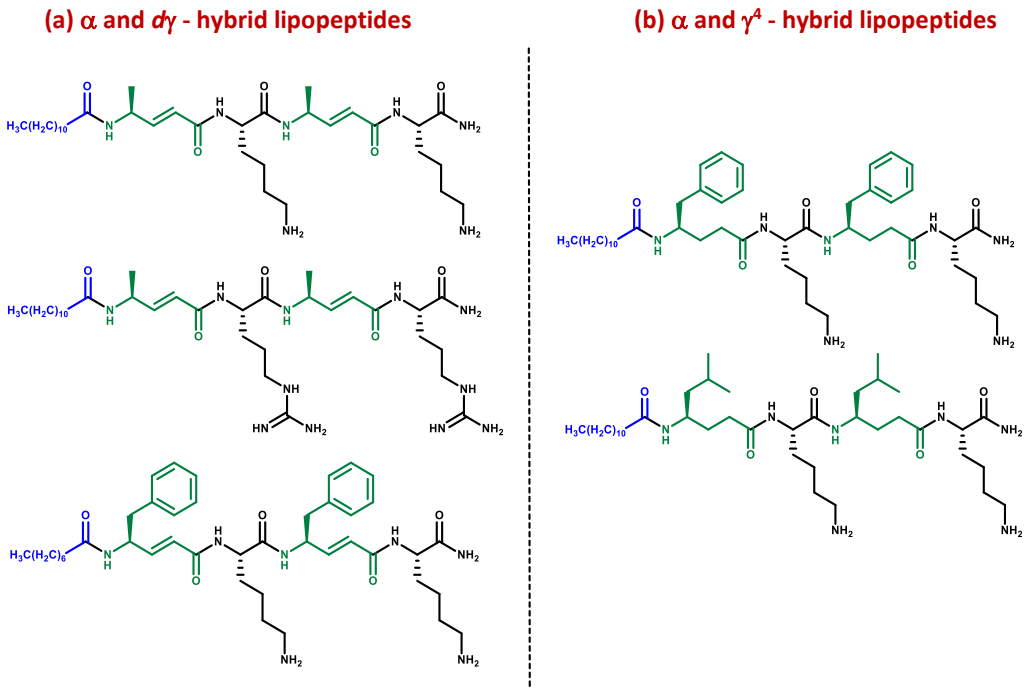


Figure 2: Antimicrobial lipopeptides reported by our group (a) α and α, β unsaturated γ-amino acid - hybrid lipopeptides[26] and (b) α and γ^4 - hybrid lipopeptides.[27]

4a.2 Aim and Rationale of the Present Work

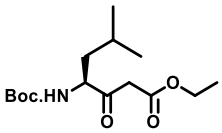
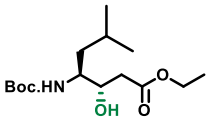
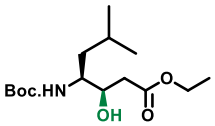
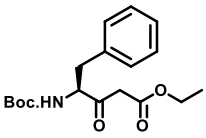
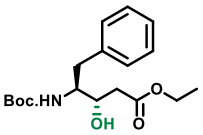
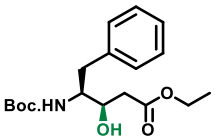
β-hydroxy γ-amino acid moieties are the key components in many of the biologically important natural products, discussed in detail in chapter 3, but their antimicrobial behaviour was never been discussed in the literature. Though few pepstatin analogues have shown inhibitory activity against *Sap* of *Candida species* during its virulence,[28] their effect on inhibiting the growth of bacteria and fungi are still to be explored. In previous sections, we have discussed the utility of β-hydroxy γ-amino acids (statines) moieties in designing peptidomimetics such as C_{12}-helices, gamma-secretase inhibitors and pepstatin analogues. Inspired by this, we sought to design lipopeptides by incorporating statine moieties and study their potency against various strains of gram-positive, gram-negative bacterial strains. We are also interested in investigating their antifungal activities. We have designed these lipopeptides using both the conformers *syn* and *anti* to investigate the role of conformation on the potency of these peptides. We are also attaching various lengths of the fatty acid chain on the *N*-terminal of peptides for studying threshold hydrophobicity and investigating the effect on their antimicrobial behaviour. In this Chapter, we are reporting the design, synthesis and antimicrobial activities of short heterogeneous cationic lipopeptides containing β-hydroxy γ- amino acid or statine.

4a.3 Results and Discussion

4a.3.1 Synthesis of *(N)*Fmoc β-hydroxy γ- amino acid:

We have synthesized *(N)*Boc β-hydroxy γ-amino ester of Phenylalanine and Leucine amino acid by mild reduction of their respective β-keto γ-amino esters using $NaBH_4$ as per the procedure described in chapter 2b section 2b.3.1. The list of synthesized *(N)*Boc β-hydroxy γ-amino esters with their percentage yields is shown in Table 1. Later, on saponification, deprotection of Boc and again protecting the free amine using Fmoc-Osu we have obtained *(N)*Fmoc β-hydroxy γ-amino acid, as described in chapter 3b section 3b.3.1.

Table 1: List of *N*-Boc β-keto γ- amino esters and their respective *N*-Boc β-hydroxy γ- amino esters.

Entry	β-keto γ- amino ester	β-hydroxy γ- amino acid (*S, S*)	β-hydroxy γ- amino acid (*R, S*)	Overall yield %
1.		 *Anti* (S,S) (Minor) 36%	 *Syn* (R,S) (Major) 64%	92%
2.		 *Anti* (S,S) (Minor) 35%	 *Syn* (R,S) (Major) 65%	95%

4a.3.2 Design, synthesis and purification of lipopeptides

To examine the antimicrobial activity of alternate α and β-hydroxy γ- amino acids containing hybrid lipopeptides, we have designed ten lipopeptides (**LP1-LP10**). It has been reported in the literature that though incorporation of arginine and lysine as cationic hydrophilic residues for making potent AMP's but Arg peptides have shown more haemolytic activity,[26] the same is observed with aromatic amino acid residue such as phenylalanine.[26,27] Considering the above observations, we have chosen only Lysine as a hydrophilic, cationic α -amino acid residue.

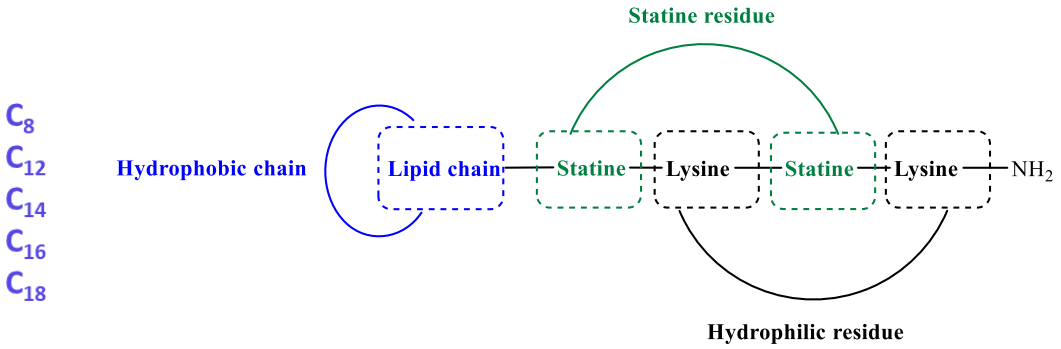


Figure 3: Basic design of β-hydroxy γ- amino acid containing hybrid cationic lipopeptides.

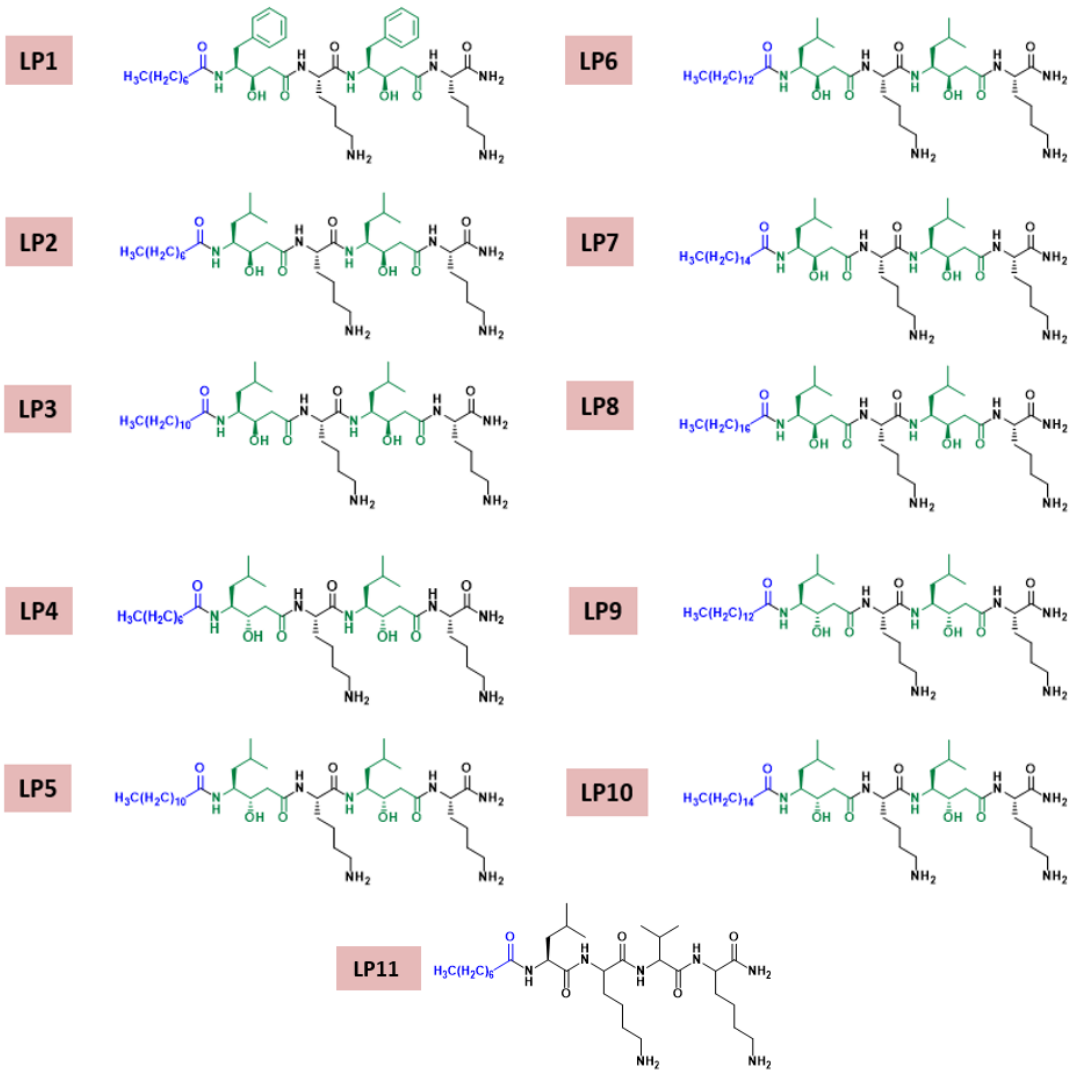


Figure 4: Sequence of hybrid cationic lipopeptides containing α and β-hydroxy γ- amino acid.

We have incorporated $C_{8(caprylic\ acid)}$, $C_{12(lauric\ acid)}$, $C_{14(myristic\ acid)}$, $C_{16(palmitic\ acid)}$ and $C_{18(stearic\ acid)}$ fatty acids chains in the *N*-terminal to understand the role of lipid chain length and calculating the threshold hydrophobicity for the antimicrobial properties of the designed hybrid lipopeptides. The basic design of short hybrid lipopeptides containing statine moiety is shown in Figure 3. We have utilized both the statine conformers *anti* and *syn* to investigate the role of statine conformation on antimicrobial properties. We have also synthesized peptide **LP11** with all alpha-amino acid and C_8-fatty acid chain as control peptide. The sequence of the lipopeptides is shown in Figure 4.

We have synthesized all the hybrid lipopeptides, **LP1-LP10** and the control peptide **LP11** on Knorr-amide resin by manual solid-phase peptide synthesis using solid-phase compatible Fmoc protected amino acids. All the lipopeptides were well purified through reverse phase HPLC on the C-18 column and purity was checked by taking HPLC traces on the analytical C-18 column. Mass of lipopeptides was confirmed using MALDI-TOF/TOF. Later the lipopeptides were examined for antibacterial and antifungal activity.

4a.3.3 Antimicrobial activity

All β-hydroxy γ-amino acid containing hybrid lipopeptides analogues with *N*-terminal fatty acid (**LP1-LP10**) and control peptide (**LP11**) were subjected to antibacterial activity. Exclusively, all C_8 containing lipopeptides **LP1$_{(R,S)}$**, **LP2$_{(R,S)}$**, **LP4$_{(S,S)}$** and control peptide **LP11** (with only α-amino acid), have not shown any antibacterial activity with MIC value (μg/ml) >128, in each case, showing C_8 fatty acid is not appropriate for the threshold hydrophobicity required for the activity.

Lipopeptides with C_{12} fatty acids, **LP3$_{(R,S)}$** have shown MIC value range (μg/ml) of 16-64 and 16 with gram-negative and gram-positive bacterial strains, respectively. Lipopeptides with C_{12} fatty acids, **LP5$_{(S,S)}$** have shown MIC value range (μg/ml) of 32-128 and 64 with gram-negative and gram-positive bacterial strains, respectively. The results showed that the lipopeptide containing statine *anti* conformation, **LP3$_{(R,S)}$** is showing more potent activity than its *syn* conformation containing analogue, **LP5$_{(S,S)}$**.

Lipopeptides with C_{14} fatty acid, **LP6$_{(R,S)}$** have shown MIC value range (μg/ml) of 8-64 and 32 with gram-negative and gram-positive bacterial strains, respectively. Lipopeptides with C_{14} fatty, **LP9$_{(S,S)}$** have shown MIC value range (μg/ml) of 64-128 and 64 with gram-negative and gram-positive bacterial strains, respectively. This result also shows that the lipopeptide containing statine *anti* conformation, **LP6$_{(R,S)}$** is showing more potent activity than its *syn*

conformation containing analogue, **LP9$_{(S,S)}$**. Also, lipopeptides with C_{14} lipid chain, have displayed better antibacterial activity than lipopeptides with C_{12} lipid chain, especially for gram-negative strains.

Lipopeptides with C_{16} fatty acid, **LP7$_{(R,S)}$** have shown MIC value range (µg/ml) of 32-128 and 64 with gram-negative and gram-positive bacterial strains, respectively. Lipopeptides with C_{14} fatty, **LP10$_{(S,S)}$** have shown MIC value (µg/ml) of 128 for all gram-negative and gram-positive bacterial strains. These results also demonstrate more potent activity for the lipopeptides containing statine *anti* conformation compared to the peptide containing statins with *syn* conformation **(LP10$_{(S,S)}$)**. Also, lipopeptides with C_{16} fatty, have displayed less potency than lipopeptides with C_{12} and C_{14} lipid chain.

Lipopeptides with C_{18} lipid chain, **LP8$_{(R,S)}$** have shown MIC value (µg/ml) of 128 for all gram-negative and gram-positive bacterial strains. The above results suggest that lipopeptides **LP6$_{(R,S)}$** is the most potent among all lipopeptides with the least MIC obtained 8µg/ml. Also, lipopeptides with C_{12} fatty C_{14} lipid chain are more potent than lipopeptides with C_8, C_{16} and C_{18} fatty acid chains. In each case, we have observed that lipopeptides with statine *anti* conformation are showing more potent activity than its *syn* conformation containing analogue. MIC values (µg/ml) of all lipopeptides on various bacterial strains are shown in Table 2.

We also subjected hybrid lipopeptides analogues (**LP1**-**LP5**) and control peptide (**LP11**) for antifungal activity. Exclusively, all C_8 containing lipopeptides **LP1$_{(R,S)}$**, **LP2$_{(R,S)}$, LP4$_{(S,S)}$** and control peptide **LP11** (with only α-amino acid), have not shown any antifungal activity with MIC value (µg/ml) >128, in each case, showing C_8 fatty acid is not appropriate for the threshold hydrophobicity required for the antifungal activity.

Lipopeptides with C_{12} fatty acids **LP3$_{(R,S)}$** and **LP5$_{(S,S)}$** displayed good antifungal activity. **LP3$_{(R,S)}$** have shown MIC value range (µg/ml) of 16-32 with various fungal strains. **LP5$_{(S,S)}$** have shown MIC value range (µg/ml) of 32-128 with various fungal strains. In the case of fungal activity also result shows that the lipopeptide containing statine *anti* conformation, **LP3$_{(R,S)}$** is showing more potent activity than its *syn* conformation containing analogue, **LP5$_{(S,S)}$**. Also, these lipopeptides have shown a similar kind of activity for bacterial as well as fungal strains suggesting their broad-spectrum activity as anti-bacterial as well as anti-fungal antimicrobial cationic lipopeptides. MIC values (µg/ml) of lipopeptides on various fungal strains are shown in Table 3.

Table 2: MIC value in µg/ml of lipopeptides on various Bacterial Strains.

			E. coli	*P. aeruginosa*	*S. typhi*	*S. aureus*	*K. pneumoniae*
1	**LP1**	C_8-(Phe(R,S)-Lys)$_2$-$CONH_2$	>128	>128	>128	>128	>128
2	**LP2**	C_8-(Leu(R,S)-Lys)$_2$-$CONH_2$	>128	>128	>128	>128	>128
3	**LP3**	C_{12}-(Leu(R,S)-Lys)$_2$-$CONH_2$	64	32	64	16	16
4	**LP4**	C_8-(Leu(S,S)-Lys)$_2$-$CONH_2$	>128	>128	>128	>128	>128
5	**LP5**	C_{12}-(Leu(S,S)-Lys)$_2$-$CONH_2$	64	32	128	64	64
6	**LP6**	C_{14}-(Leu(R,S)-Lys)$_2$-$CONH_2$	**64**	**32**	**64**	**32**	**8**
7	**LP7**	C_{16}-(Leu(R,S)-Lys)$_2$-$CONH_2$	64	32	128	64	64
8	**LP8**	C_{18}-(Leu(R,S)-Lys)$_2$-$CONH_2$	>128	>128	>128	>128	>128
9	**LP9**	C_{14}-(Leu(S,S)-Lys)$_2$-$CONH_2$	128	64	128	64	64
10	**LP10**	C_{16}-(Leu(S,S)-Lys)$_2$-$CONH_2$	128	128	128	128	128
11	**LP11**	C_8-Leu-Lys-Val-Lys-$CONH_2$	>128	>128	>128	>128	>128

Table 3: MIC value in µg/ml of lipopeptides on various Fungal Strains.

			Candida glabrata	*Candida tropicalis*	*Cryptococcus neoformans*
1	**LP1**	C_8-(Phe(R,S)-Lys)$_2$-$CONH_2$	>128	>128	>128
2	**LP2**	C_8-(Leu(R,S)-Lys)$_2$-$CONH_2$	>128	>128	>128
3	**LP3**	C_{12}-(Leu(R,S)-Lys)$_2$-$CONH_2$	32	16	16
4	**LP4**	C_8-(Leu(S,S)-Lys)$_2$-$CONH_2$	>128	>128	>128
5	**LP5**	C_{12}-(Leu(S,S)-Lys)$_2$-$CONH_2$	128	32	32
6	**LP11**	C_8-Leu-Lys-Val-Lys-$CONH_2$	>128	>128	>128

4a.4 Conclusion

We are reporting the antimicrobial properties of short cationic heterogenous lipopeptides containing β-hydroxy γ- amino acids. These lipopeptides have shown moderate antimicrobial activity than their alpha-amino acid-containing lipopeptides analogues. Lipopeptide **LP3$_{(R,S)}$** and **LP6$_{(R,S)}$** have shown good antibacterial and antifungal activity especially for *Candida* species which can be further looked upon to identify haemolytic activity and their mode of action to make the new class of potent antimicrobial lipopeptide analogues.

4a.5 Experimental section

4a.5.1 General experimental details

All α-amino acids, Ethyl diazoacetate, Tin chloride and Di-t*ert*-butyl dicarbonate were obtained from Merck. 2-Mercaptobenzothiazole (2-MBT), Fmoc-Osu, DCC, $NaBH_4$, HPLC grade acetonitrile, dry DCM and dry THF were purchased from Spectrochem. Column chromatography was achieved on silica gel of 120-200 mesh from Merck. Thin-layer chromatography (TLC) was used to monitor reaction using Merck 60 F_{254} precoated silica gel plates. For visualization UV light and ninhydrin or phosphomolybdic acid (PMA) stains were used then charring on a hot plate. Melting points were recorded on Veego VMP-DS hot stage apparatus. Specific rotations were recorded at ambient temperature on the Rudolph Analytical Research instrument using $CHCl_3$ and MeOH as solvents. Reverse Phase-HPLC of Waters was used to purify peptides using Acetonitrile/H_2O gradient with 0.1% TFA from C-18 column. Mass was analysed on Applied Biosciences with MALDI TOF/TOF on positive ion mode.

4a.5.2 Solid-phase peptide synthesis and purification

All the designed Peptides (**LP1-LP11**) were synthesized on Knorr amide resin (0.2 mmol scale) using standard Fmoc-chemistry. As coupling reagents, HBTU/HOBt were used. Kaiser test was performed to monitor coupling reactions. After completion of the desired couplings, TFA: water: phenol: TIPS (88:5:5:2) was used as a scavenger cocktail mixture to cleave the peptides from resins. Later, the resin was filtered and the cleavage mixture or the filtrate was co-evaporated using DCM beneath the vacuum to get a gummy product. The product was recrystallized using diethyl ether to obtain a white powder. The obtained white powder was dissolved in water and purified through reverse phase HPLC on a C18 column using an ACN/H_2O with a 0.1% TFA solvent system with 2mL/min flow rate. The purity of the peptides was further investigated by passing from an analytical C18 column with the same gradient

system with 0.75ml/min flow rate. The mass of the peptide was recorded using **MALDI TOF/TOF.**

4a.5.3 Procedure for antibacterial activity

The bacteria culture, *Escherichia coli K12* (NCIM2563), *Pseudomonas aeruginosa* (NCIM 5029), *Salmonella typhimurium* (NCIM 2501), *Staphylococcus aureus* (NCIM 5021) and *Klebsiella pneumoniae* (NCIM 2957) used for the MIC investigation were obtained from the National Collection of Industrial Microorganisms (NCIM).

The antibacterial activities of lipopeptides were performed according to the standard protocol reported by Clinical Laboratories Standards Institute (CLSI): M07-A10 in 2015.[29] The antibacterial activities are reported in MIC (minimum inhibition concentration) i.e., the least concentration where no bacterial growth was observed. The standard broth microdilution method was followed to obtain MIC values on a sterile 96-well microtiter plate (F96, NUNC, 200 µL well capacity). The bacterial cultures were grown for 24 hours at 37 °C in MHB media (Muller-Hinton broth). After 24 hours OD was measured for each bacterial culture and then serially diluted to obtain a final concentration of 10^6 colony-forming units/ml using MHB media. All the lipopeptides were dissolved in sterile water and added in a sterile 96-well plate to a final volume of 50 µL in each well with two-fold serial dilution from 128µM-8µM concentration range. Later, bacterial suspensions of 50 µL were added to each well to make a final volume of 100 µL. The plate was kept in an incubator at 37 °C for 18-20 hours. For control, a culture without peptide and only media were also grown parallelly. The MIC values of the lipopeptides were obtained by monitoring the growth inhibition at an absorbance of 492 nm by the Biotek microplate reader. The MIC values obtained here were reproducible for three independent triplicates.

4a.5.4 Procedure for antifungal activity

The fungal culture, *Candida glabrata* (NCIM 3237), *Candida tropicalis* (NCIM 3471) and *Cryptococcus neoformans* (NCIM 3542) used for the MIC investigation were obtained from the National Collection of Industrial Microorganisms (NCIM).

The antibacterial activities of lipopeptides were performed according to the standard protocol reported by Clinical Laboratories Standards Institute (CLSI): M07-A10 in 2015.[29] The antibacterial activities are reported in MIC (minimum inhibition concentration) i.e., the least concentration where no fungal growth was observed. The standard broth microdilution method was followed to obtain MIC values on a sterile 96-well microtiter plate (F96, NUNC, 200 µL

well capacity). The fungal cultures were grown for 48-72 hours at 37 °C in PDB media (Potato dextrose broth). After 48-72 hours OD was measured for each fungal culture and then serially diluted to obtain the final concentration of 2×10^3 colony-forming units/ml using PDB media. All the lipopeptides were dissolved in sterile water and added in a sterile 96-well plate to a final volume of 50 µL in each well with two-fold serial dilution from 128µM-8µM concentration range. Later, fungal suspensions of 50 µL were added to each well to make *a* final volume of 100 µL. The plate was kept in an incubator at 37 °C for 48-2 hours. For control, a culture without peptide and only media were also grown parallelly. The MIC values of the lipopeptides were obtained by monitoring the growth inhibition at an absorbance of 620 nm by the Biotek microplate reader. The MIC values obtained here were reproducible for three independent triplicates.

4a.6 References

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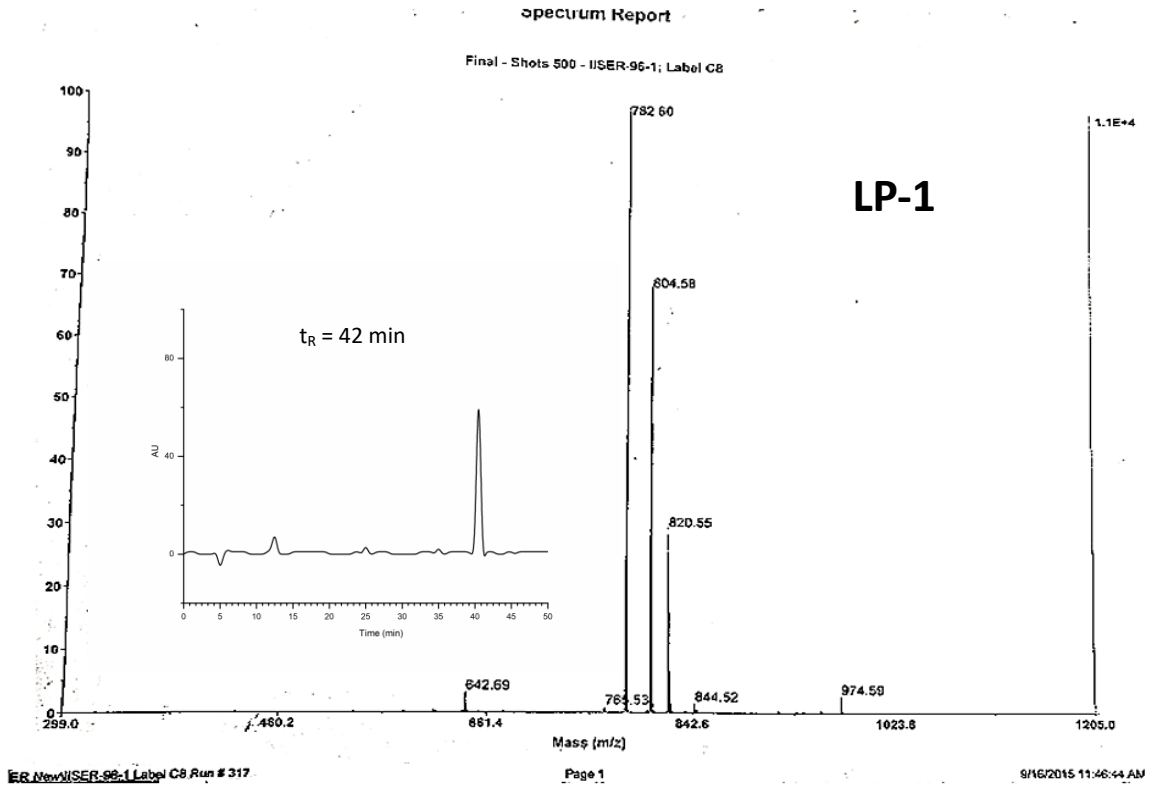
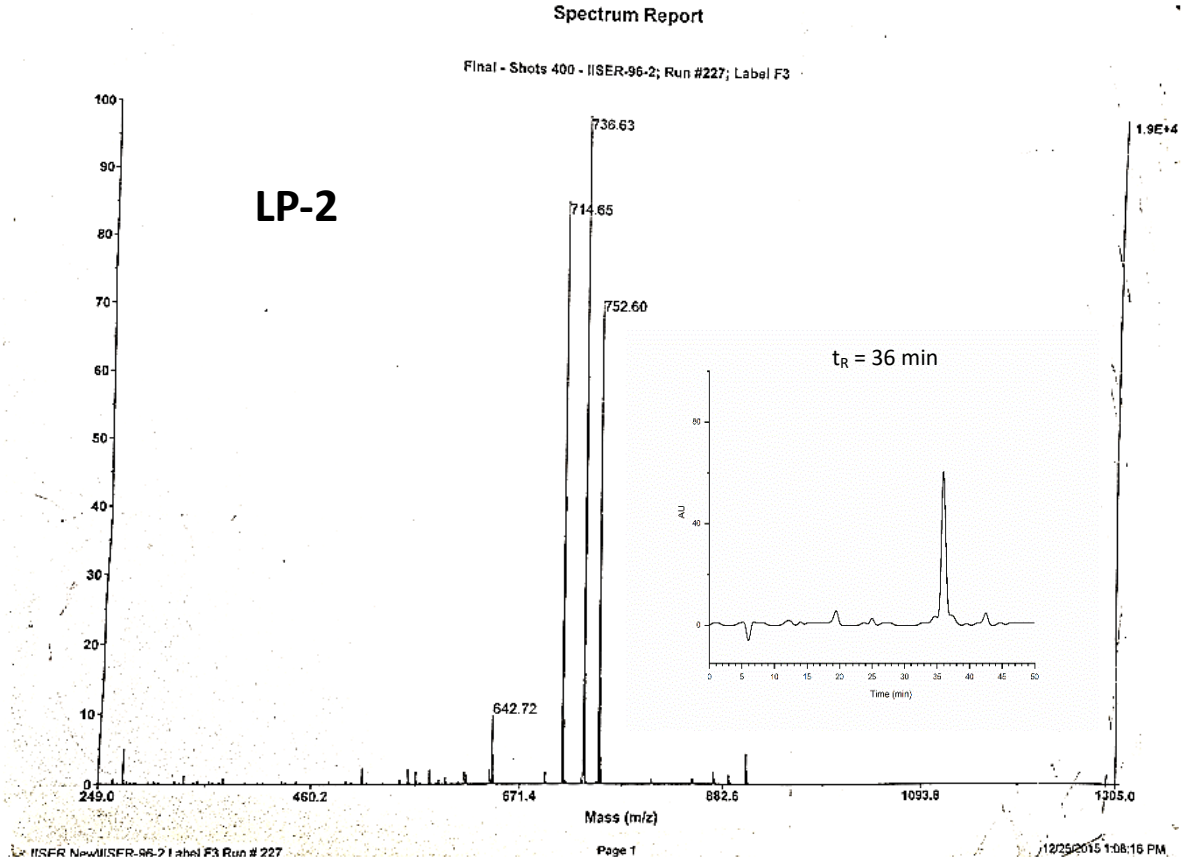
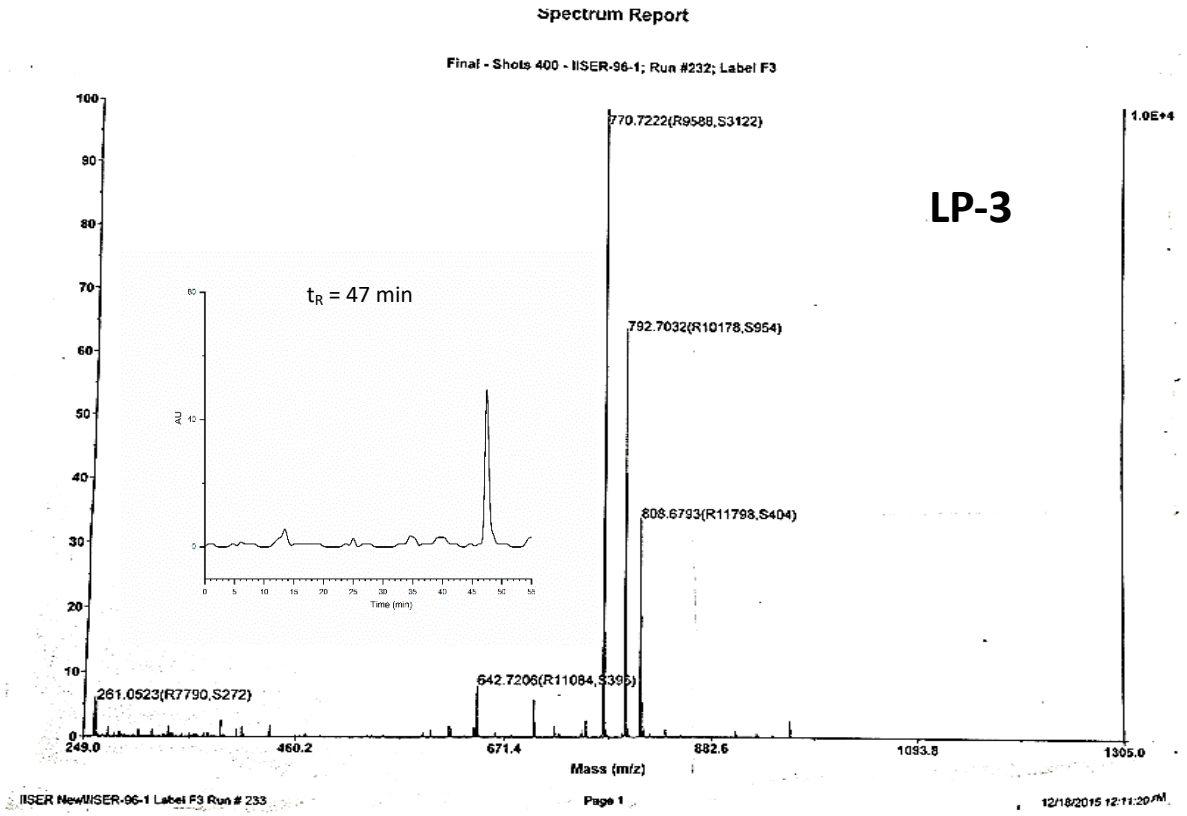
4a.7 Appendix: HPLC traces and Mass spectra of lipopeptides



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642.7206(R11084,S395)
261.0523(R7790,S272)
1.0E+4
249.0
460.2
671.4
882.6
1093.8
1305.0
Mass (m/z)
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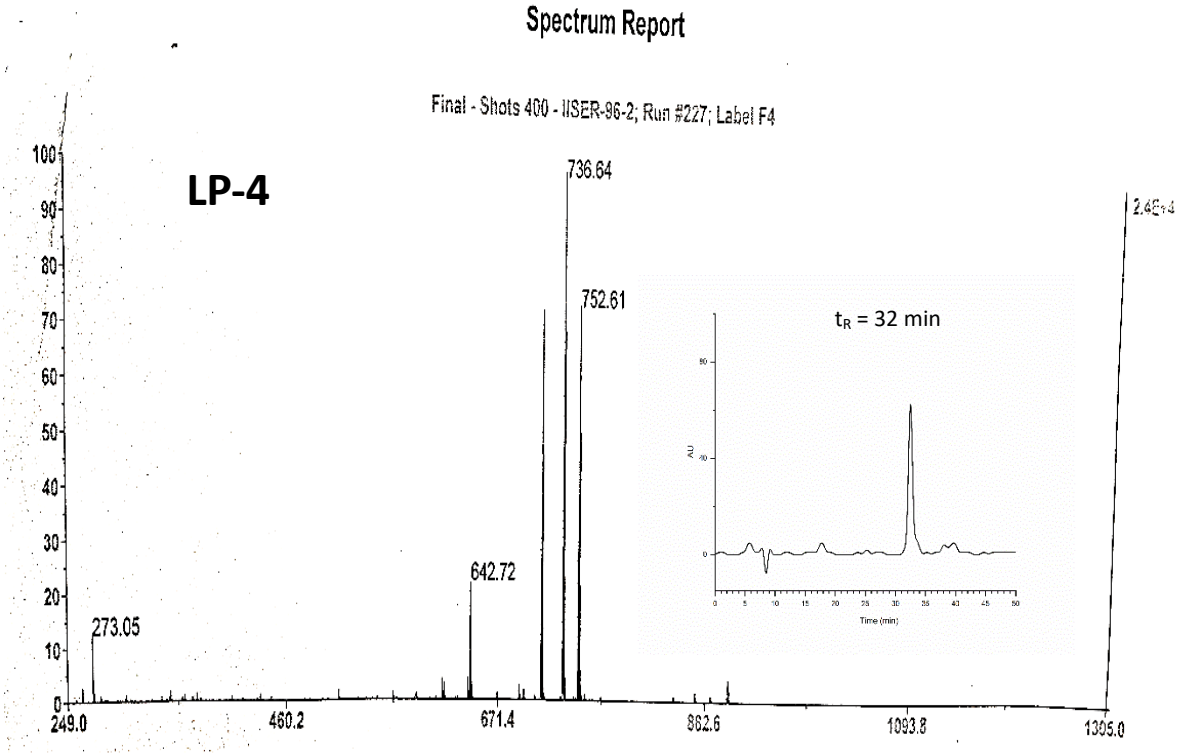
Page 1
12/18/2015 12:11:20 PM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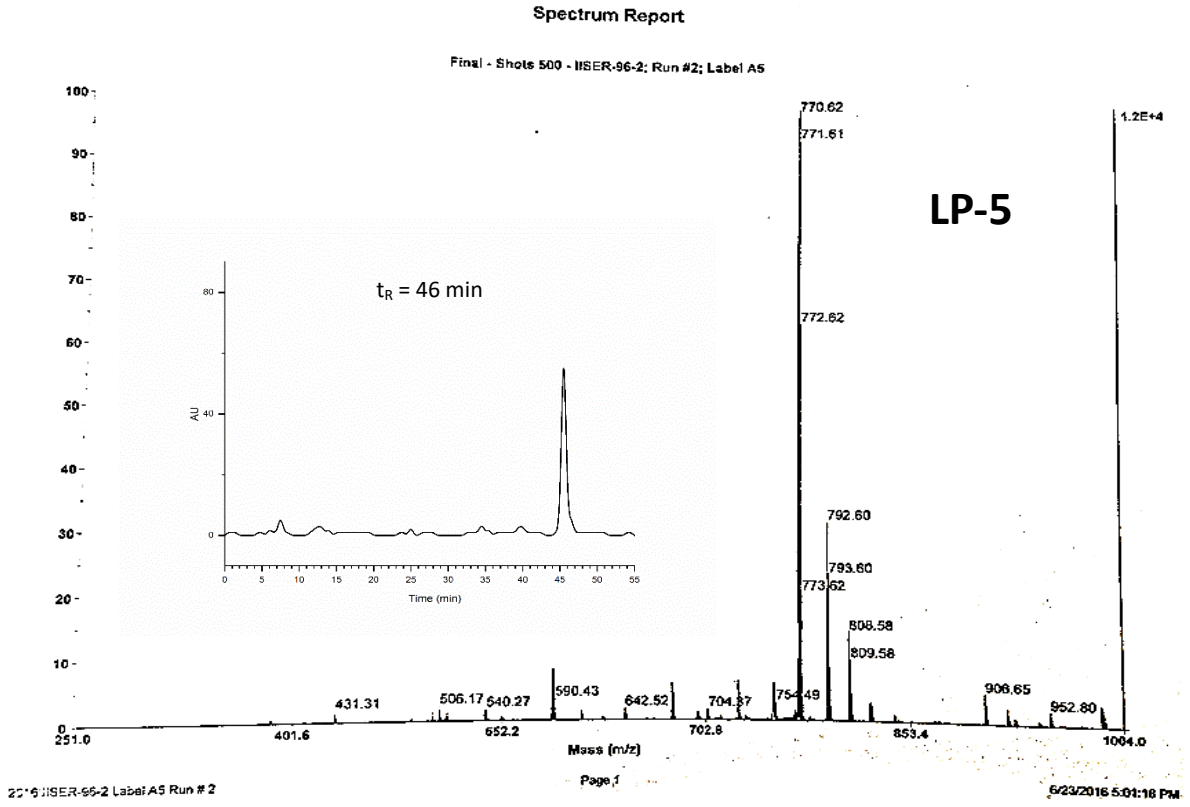
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771.61
772.62
773.62
792.60
793.60
808.58
809.58
431.31
506.17
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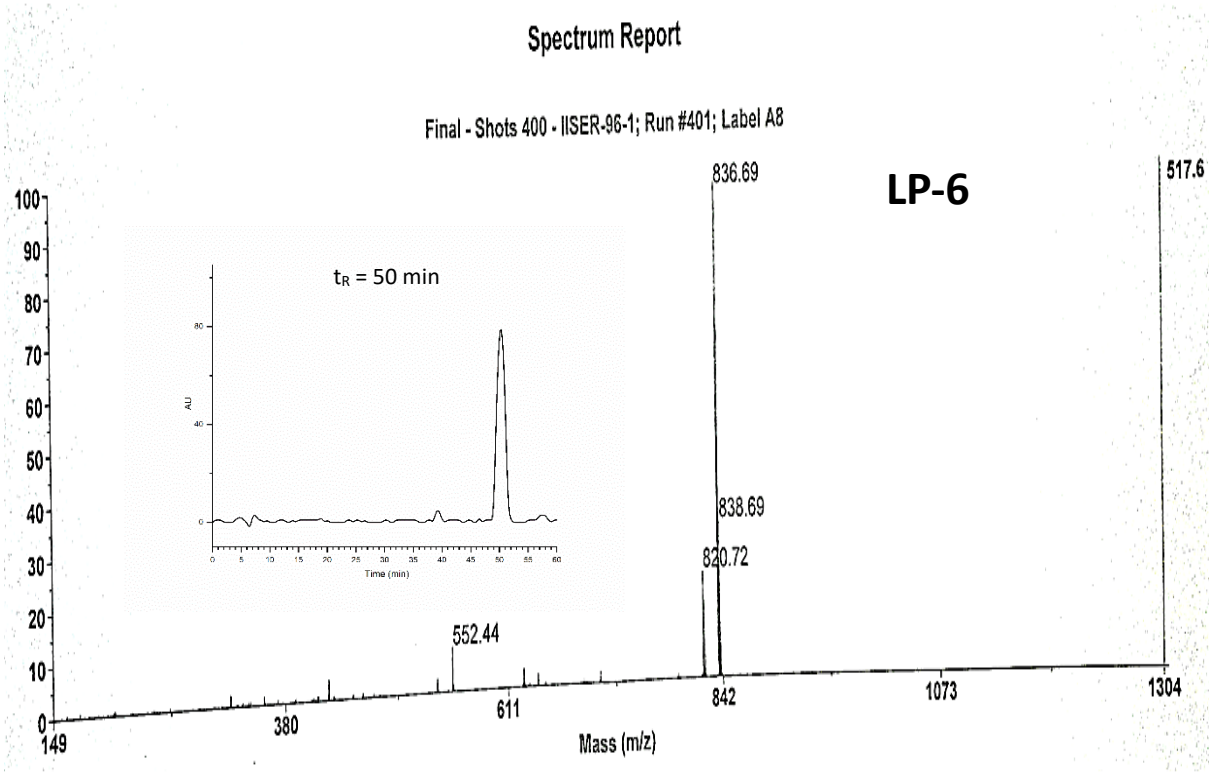
Spectrum Report
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Mass (m/z)
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820.72
552.44
517.6
149
380
611
842
1073
1304

Spectrum Report

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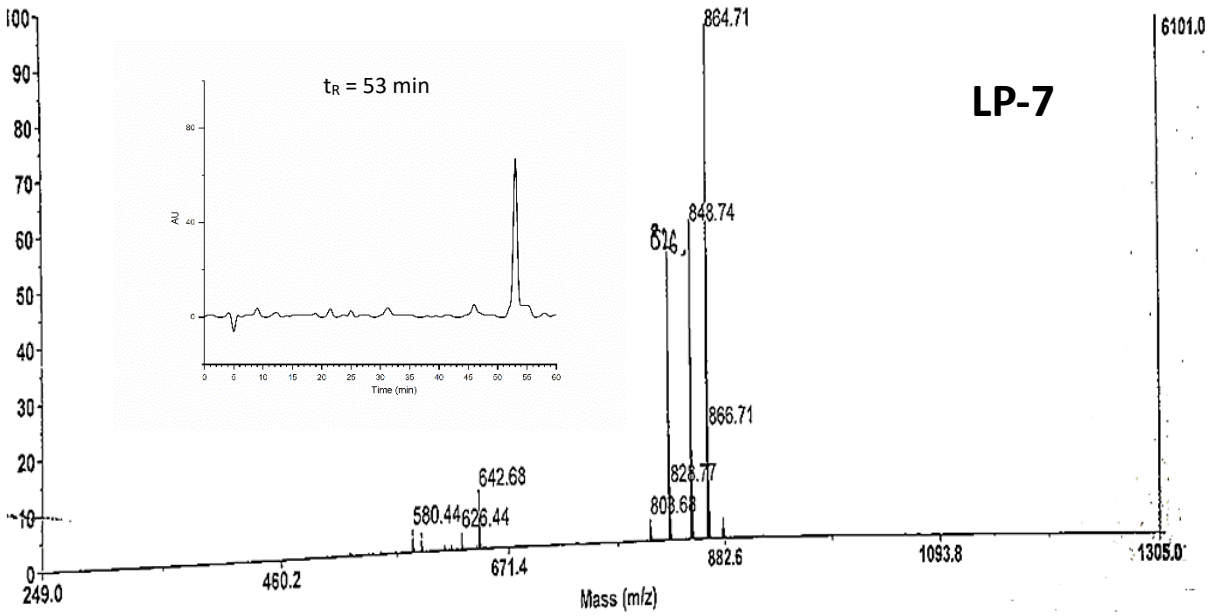

Spectrum Report

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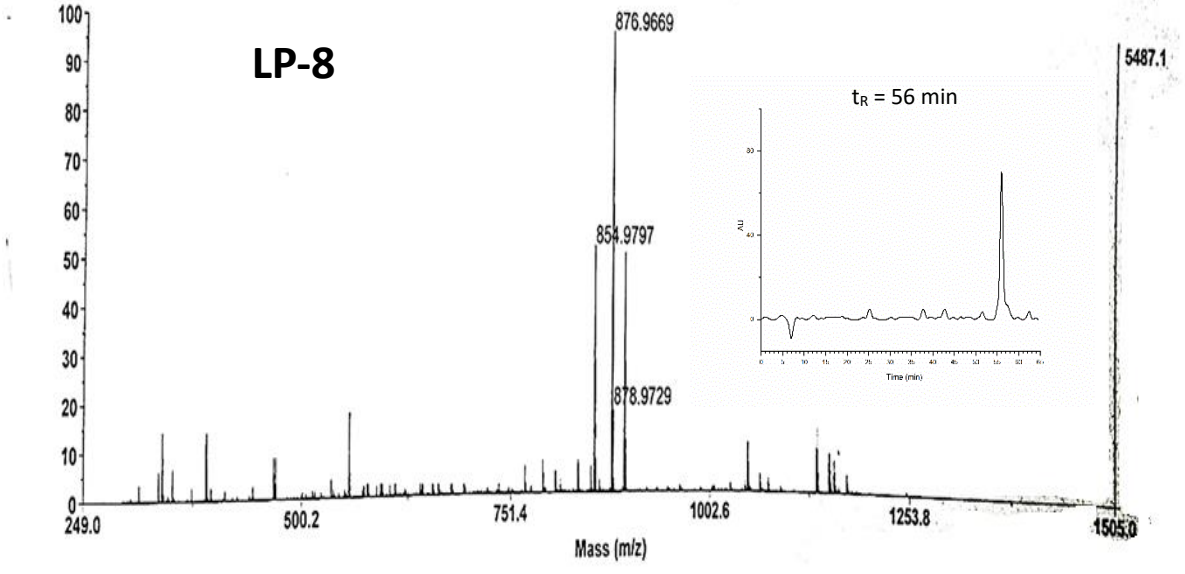
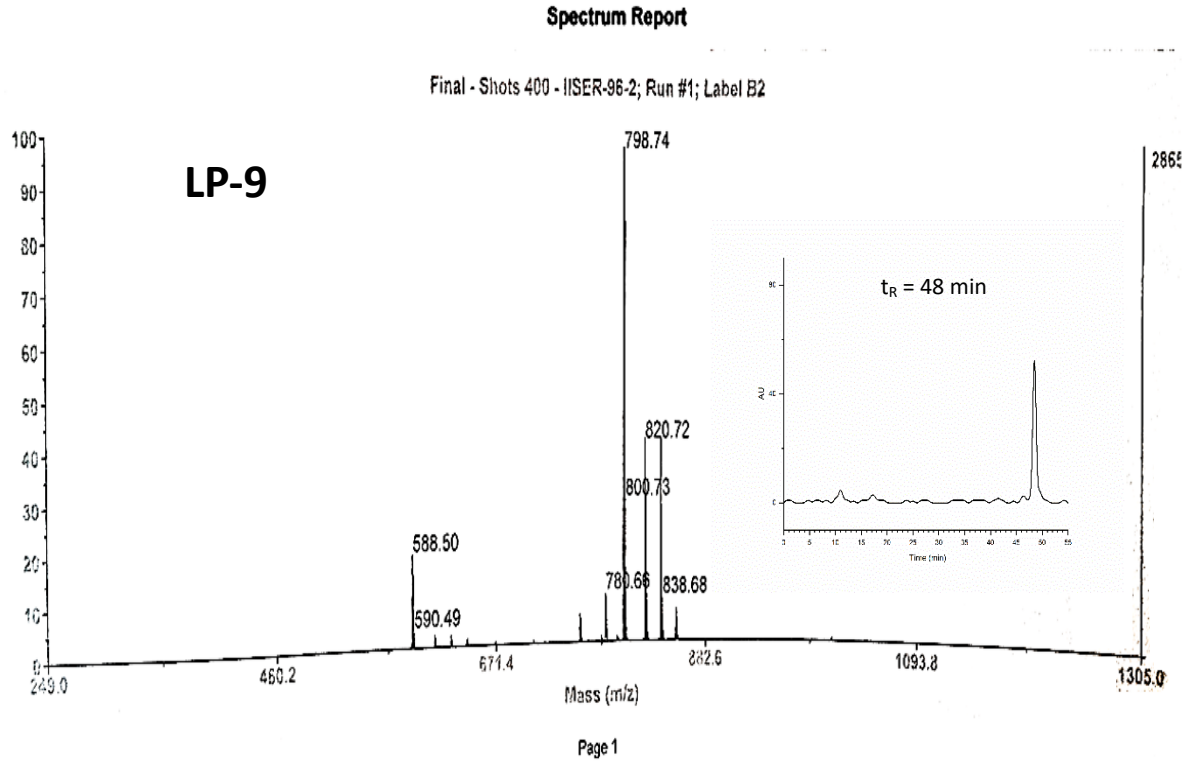


Page 1

2017\IISER-96-1-2017 Label A5 Run # 176


Spectrum Report
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LP-9
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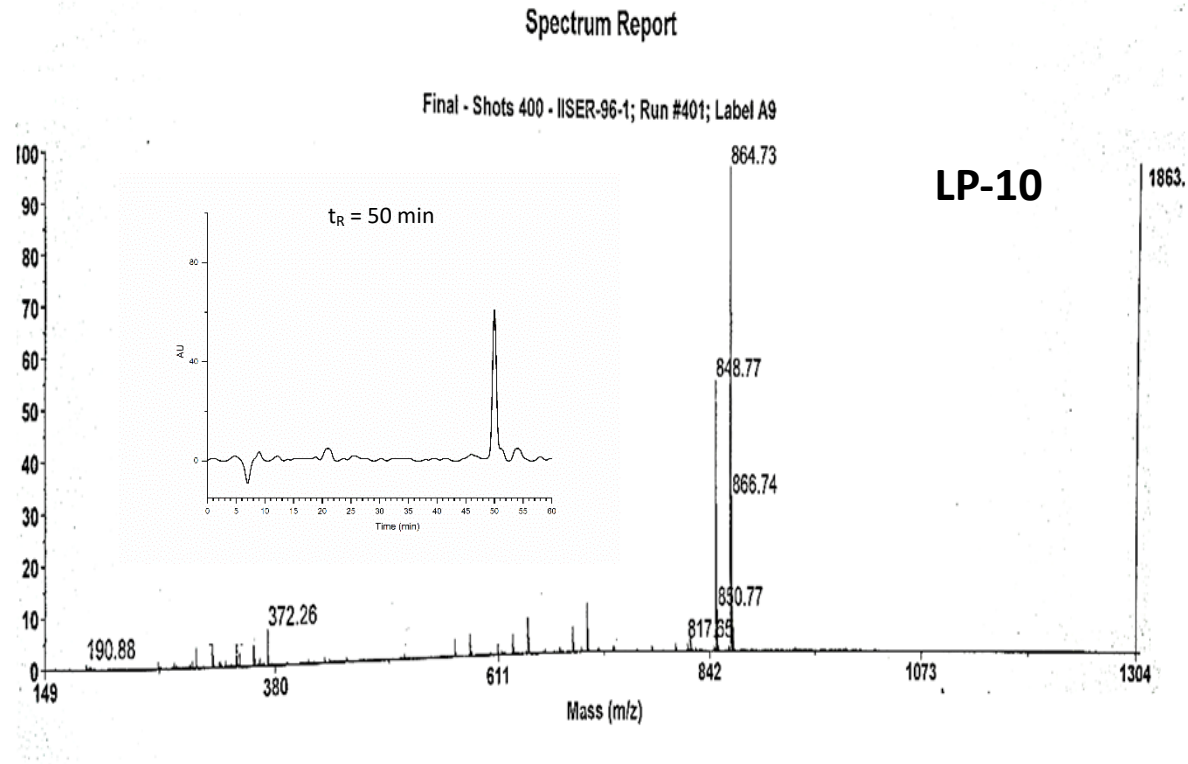
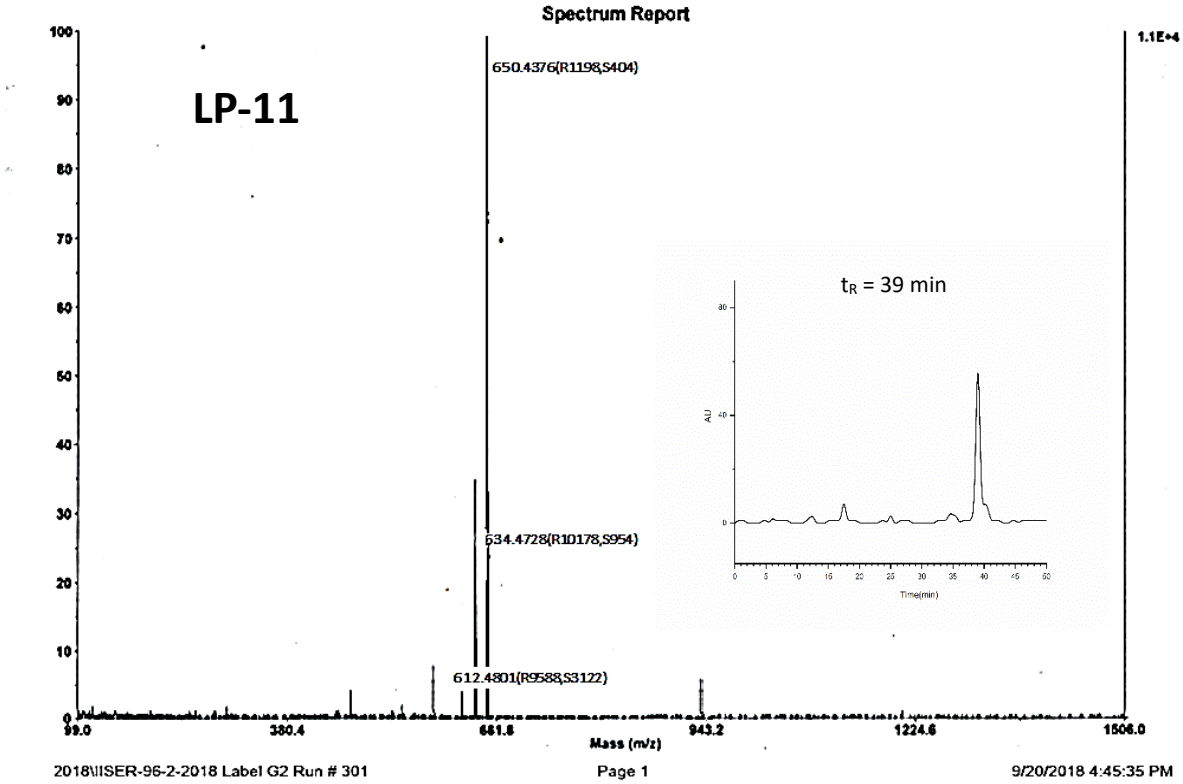
820.72
800.73
588.50
590.49
780.66
838.68
t_R = 48 min
249.0
460.2
671.4
882.6
1093.8
Mass (m/z)
Page 1


Spectrum Report
Final - Shots 400 - IISER-96-1; Run #401; Label A9
LP-10
864.73
848.77
866.74
830.77
372.26
190.88
t_R = 50 min
149
380
611
842
1073
1304
Mass (m/z)


Spectrum Report
LP-11
650.4376(R1198,S404)
634.4728(R10178,S954)
612.4801(R9588,S3122)
t$_R$ = 39 min
AU
Time(min)
Mass (m/z)
2018\IISER-96-2-2018 Label G2 Run # 301
Page 1
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Chapter 4(b)

Design, synthesis and antimicrobial activities of structure-based cationic hybrid beta-hairpin lipopeptides

4b.1 Introduction

We have discussed antimicrobial peptides in the previous section-4a, here we will be discussing the classes of antimicrobial peptides based on their structure. AMPs can be broadly classified in terms of their structure as (a) α-helical such as Magainin[1] and LL-37[2] (b) β-sheets such as Human β-defensin[3] and Protegrin[4] and (c) extended peptides such as Indolicidin[5] and Tritrpticin[6], structures are shown in Figure 1.

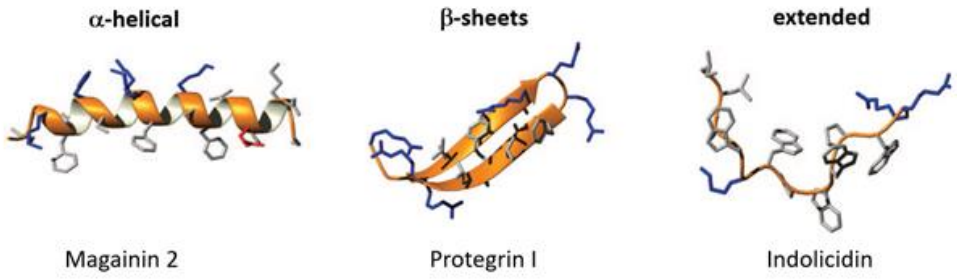


Figure 1: Few examples of antimicrobial peptides based on structure. (Figure is taken from ref[7] with few modifications.

In this section, we will discuss briefly a few examples of β-sheets class of antimicrobial peptides, shown in Figure 2**.**

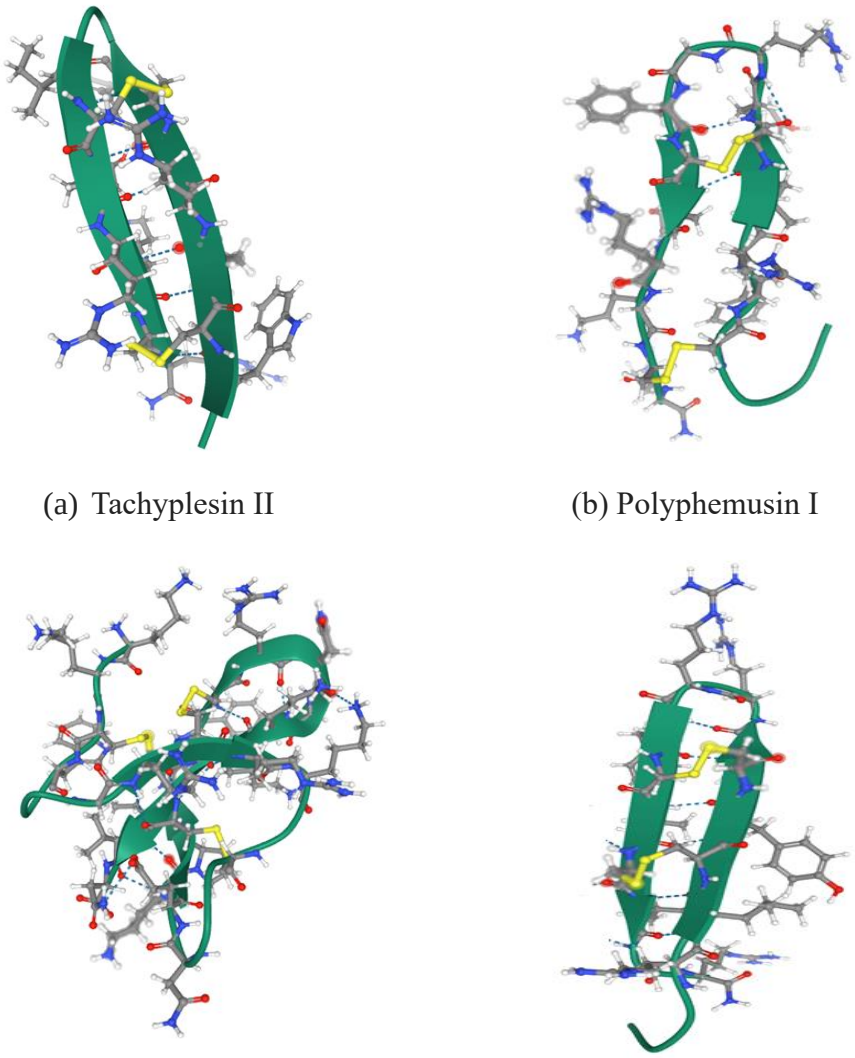
1. Tachyplesins - A cationic host defence antimicrobial peptide isolated from *Tachyplesus tridentatus* (Japanese horseshoe crab), from their haemocytes.[8] This peptide has shown broad-spectrum antibacterial and antifungal activity, by causing bacterial cell membrane disruption hence causing cell death.[9] Wild type Tachyplesin I is composed of 17 residues with four cysteine residues to form two disulphide bonds making them exist as beta-hairpin conformation in the solution state.[10] It's been reported in the literature that replacing these cysteine moieties could lead to an increase in MIC values, hence lowering the antimicrobial activity.[11] Despite its tremendous antimicrobial property, it has low therapeutic potential because of its high haemolytic value, low membrane permeability and poor serum stability.[12]
2. Polyphemusins: A class of cationic host defence antimicrobial peptides isolated from *Limulus polyphemus* (American horseshoe crab) shows great similarity to Tachyplesin in their structure.[13] They are also composed of 17-18 residues with four cysteine residues to form two disulphide bonds to make them exist as antiparallel beta-hairpin conformation in their solution state.[14] They follow a different mechanism called lipid flip-flop mechanism

for permeabilization through lipid membranes of bacteria following carpet and toroidal pore mechanism.[15] They have shown excellent antimicrobial activity against various bacterial strains of MIC as low as > 0.2 μM. But they have shown failed results during the study in animal models showing poor serum stability.[16]

3. Protegrins: Another class of beta-sheet antimicrobial cationic peptide but the mammalian origin and first isolated from porcine leukocyte.[17] They contain 16-18 amino acid residue with 4 cysteine residues similar to Tachyplesin and Polyphemusin but are rich in arginine like beta-defensins, hence known to have combine features of both.[18] They have shown excellent antimicrobial activity against bacteria and fungus especially against sexually transmitted pathogens.[19] Protegrin family have shown activity against few enveloped viruses such as HIV-1 and HSV-2.[20] One of its synthetic derivatives IB-367 have gone into clinical trials for their topical use for oral mucositis induced during radiations and chemotherapy and ventilator-associated pneumonia.[21]
4. Defensins – They are the most prominent host-defence antimicrobial peptides present in animals and plants.[22] Defensins contain approximately 25-45 amino acids and rich in arginine. They can be broadly divided into two alpha-defensins (29-35 residues) and beta-defensins (38-42 residues), with a common structural characteristic of three-stranded anti-parallel β-sheet with three intramolecular disulfide bonds.[23] Another type of defensin called theta defensin of avian origin has a cyclic structure with 18 amino acid residues that have also been reported.[24] They have shown excellent antimicrobial activity against gram-positive and gram-negative bacterial strains and also against various enveloped fungal strains.[25] Defensins do the disintegration of the bacterial cell membrane by pore formation, by changing the electric potential of the membrane surface.[26] They are also known to have immunomodulating properties.[27]

In recent years, antimicrobial peptides containing lipid chains at their *N*-terminal called **lipopeptides** have appeared as a new class of AMPs, shown in Figure 3.[28] Several lipopeptides such as Daptomycin and Polymyxin B have been accepted as a drug and proved to have significant biological activity against various multidrug-resistant bacterial strains.[29] Daptomycin sold with the name - **Cubicin** is known as an alternative for Vancomycin and has shown antimicrobial activity against gram-positive vancomycin-resistant *E. coli* and *Methicillin-resistant Staphylococcus aureus (MRSA*) species.[30] On the other hand, Polymyxin B sold with the name - **Poly Rx**, have shown selective potency against gram-negative bacterial pathogens causing pneumonia, sepsis, urinary tract infections, etc.[31] Lipopeptides are known

to disrupt morphology of bacterial cell membrane hence causing cell lysis.[32] Most of the naturally isolated lipopeptides have shown non-specificity and high toxicity against eukaryotic cells so several pieces of research have been done on making synthetics analogues of these naturally occurring lipopeptides for reducing their toxicity and increasing their potency and specificity.[33] (Described in section 4a.1)



(a) Tachyplesin II (b) Polyphemusin I

(c) Human beta defensin (d) Protegrin 1

Figure 2: Few examples of Beta-sheets peptides (modified from their PDB structure). (a) Tachyplesin II (PDB code – 6PI2),[10b] (b) Polyphemusin I (PDB code -1RKK),[14] (c) Human β-defensin (PDB code – 2LWL)[23e] and (d) Protegrin I (PDB code – 1ZY6)[18c].

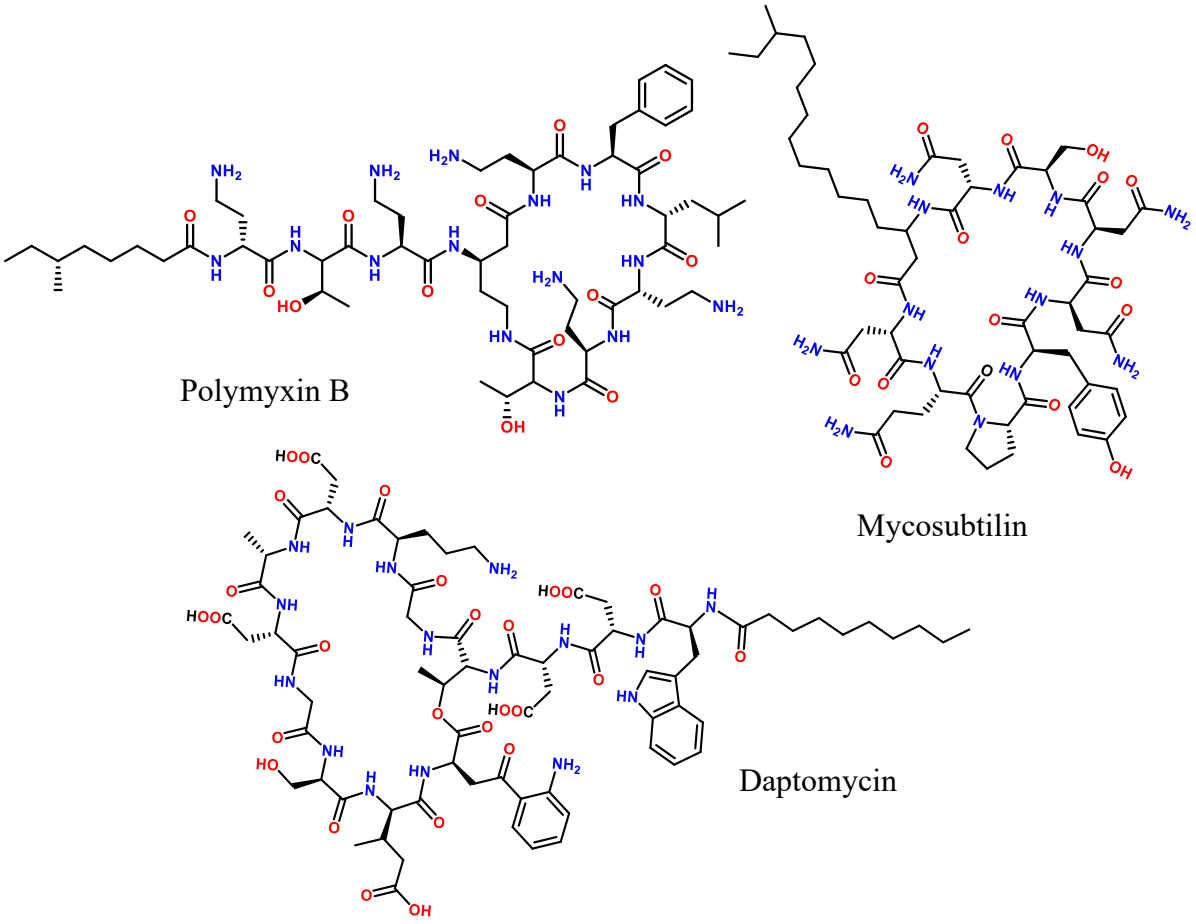


Figure 3: Few examples of naturally occurring antimicrobial Lipopeptides.

Earlier work reported by our group

α, β-Unsaturated γ-amino acids (*E*-vinylogous amino acids or dehydro γ-amino acids) are widely present in many natural products, known to have inhibitory potency against several serine proteases such as Miraziridine A[34] (briefly described in Chapter 1, section 1.7). They contain geometrically constrained double bond that shows their preference for adopting extended sheet-type structures. Since the β-sheet type foldameric pattern was reported by Schreiber and group in dipeptide sequence, several other secondary structures were also been reported, composed of α, β-unsaturated γ-amino acids.[35] Since we have reported the synthesis of *E*-vinylogous amino esters by the witting reaction on amino aldehyde we were interested to study conformational analysis of these moieties in peptide sequences.[36] Previously, our lab has reported conformational analysis of naturally occurring α, β-Unsaturated γ-amino acids (*E*-vinylogous amino acids or dehydro γ- amino acids), few examples shown in Figure 4. Hybrid peptide sequences composed of these constrained non-ribosomal moieties are well accommodated in antiparallel beta-hairpins [37] and three-stranded beta-sheets.[38] We have also reported a Beta meander kind of structure from their hybrid peptide sequence.[39] On the other

way, a homooligomeric sequence with this moiety have been shown to adopt parallel β-sheets and β-double helix type of structure.[40]

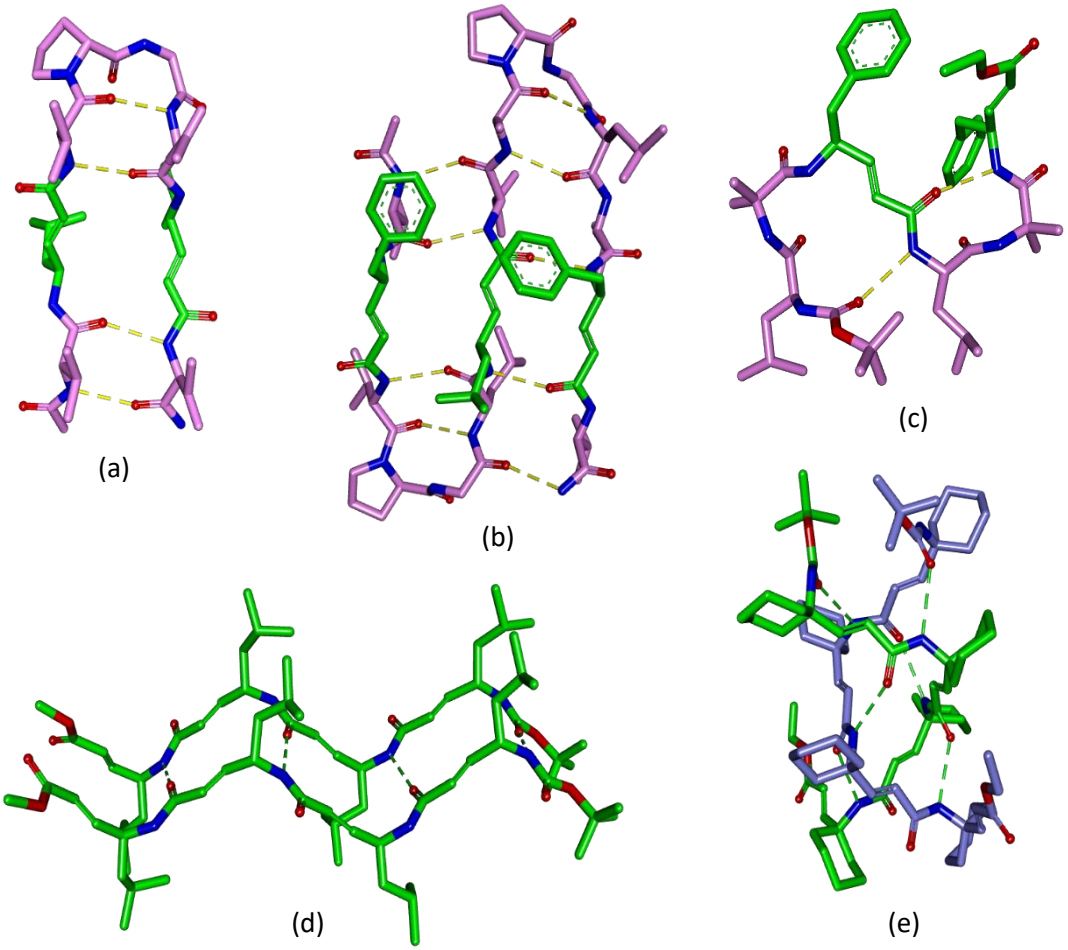


Figure 4: Foldamers reported by our group containing α, β-Unsaturated γ-amino acids in hybrid peptides (a) antiparallel beta-hairpins,[37] (b) three-stranded beta-sheets,[38] (c) Beta-meander[39] and in homooligomeric peptides (d) parallel β-sheets and (e) β-double helix type of structure.[40]

4b.2 Aim and Rationale of Present Work

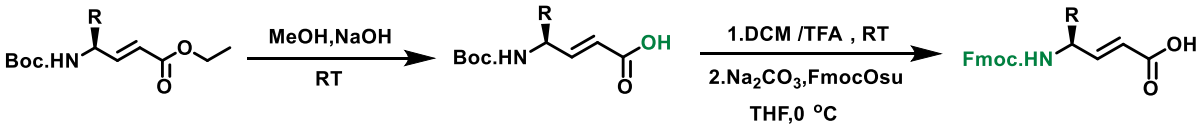
Our group have reported several short hybrid lipopeptides composed of α, β-unsaturated γ-amino acids and their antimicrobial properties.[41] Inspired by the various conformational pattern and biological properties (discussed above) shown by the peptides with α, β-unsaturated γ-amino acids we sought to design cationic beta hairpins with α, β-unsaturated γ-amino acids moieties. In the previous section, we have discussed the potent antimicrobial peptides with lipid chains, lipopeptides. We thought of mimicking protein secondary structure and combining

the biological significance of lipopeptides in a single peptide. For this purpose, we have designed hybrid peptides containing α, β-unsaturated γ-amino acids in parallel beta-hairpin sequence with lysine amino acid as cationic α-residue and fatty acid at *N*-terminus. Later, we want to study their antimicrobial properties against various bacterial strains.

4b.3 Results and Discussion

4b.3.1 Synthesis of (*N*)Fmoc α, β-Unsaturated γ- amino acid:

We have synthesized (*N*)Boc α, β-Unsaturated γ- amino esters by doing a witting reaction on amino aldehyde as described in chapter 3a, section 3a. Later, we did the hydrolysis on (*N*)Boc α, β-Unsaturated γ- amino esters and then deprotecting the Boc using TFA/DCM. Further, amine triflate salt was protected with Fmoc-Osu to produce (*N*)Fmoc α, β-Unsaturated γ- amino acids (schematic representation shown in **scheme 4.1**) to utilize them for solid-phase peptide synthesis, using Knorr Amide resin. We have synthesized (*N*)Fmoc α, β-Unsaturated γ- amino acids of *L*-Leucine, *L*-Valine and *L*-Alanine amino acid.

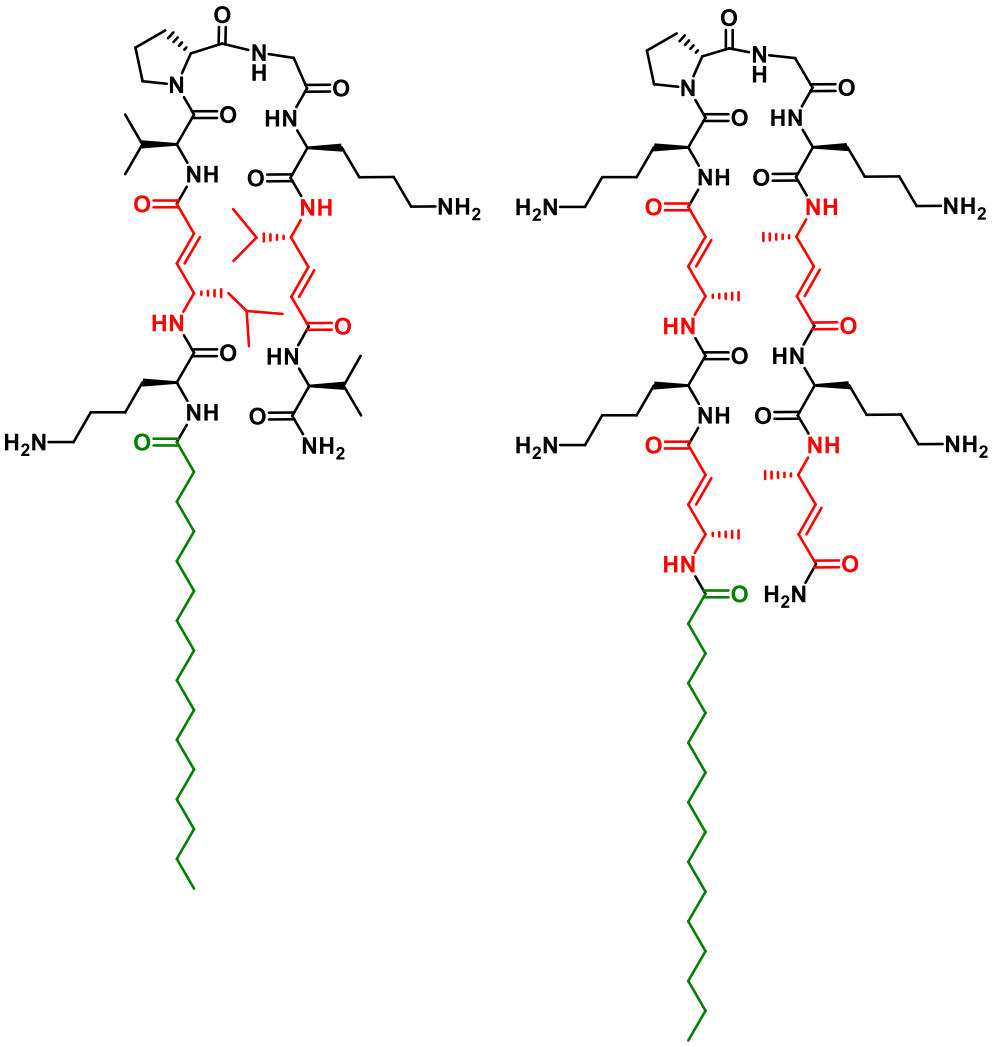


Scheme 4.1: Synthesis of (*N*)Fmoc α,β-unsaturated γ-amino acids from (*N*)Boc α,β-unsaturated γ-amino esters.

4b.3.2 Design, synthesis and purification of lipopeptides

To examine the antimicrobial activity of α, β-unsaturated γ- amino acid containing cationic hybrid beta-hairpin lipopeptides, we have designed two anti-parallel beta-hairpin peptide sequences, **HPL1 and HPL2.** We have chosen lysine as a hydrophilic, cationic α -amino acid residue. For the fatty acids chains in the *N*-terminal, we have incorporated $C_{16(\text{palmitic acid})}$ at the end during peptide synthesis. **HPL1** consists of 8-amino acid residues, with alternate α and α, β-unsaturated γ- amino acid motifs having *D*-proline and glycine as the turn inducer. In **HPL1** we have attached α, β-unsaturated γ- amino acid of valine and leucine amino acid, facing opposite to each other in the two antiparallel strands. For α-amino acids we have incorporated Val and Leu residue and two lysine residues at two opposite strands away from each other. On the other way around, HPL2 consists of 10-amino acid residues, with alternate α and α, β-unsaturated γ- amino acid motifs having *D*-proline and glycine as the turn inducer. In **HPL2** we have attached four α, β-unsaturated γ- amino acid of only Alanine amino acid, facing

opposite to each other in the two antiparallel strands. For α-amino acid we have incorporated four cationic lysine residues. The idea was to increase charge and hydrophilicity. The design of peptides is shown in **Scheme 2**.



Scheme 2: Design of hybrid lipopeptides containing α and β-hydroxy γ-amino acid.

We have synthesized both the hybrid hairpin lipopeptides, **HPL1** and **HPL2** on Knorr-amide resin by manual solid-phase peptide synthesis using solid-phase compatible Fmoc protected amino acids. HBTU and HOBt were used as coupling agents. Both the lipopeptides were purified through Reverse Phase HPLC on the C-18 column and purity was checked by taking HPLC traces on the analytical C-18 column. Mass of lipopeptides was confirmed using MALDI-TOF/TOF. Later the lipopeptides were examined for antibacterial activity.

Table 1: The IC_{50} values of **HPL1** and **HPL2** on various bacterial strains (ampicillin as control).

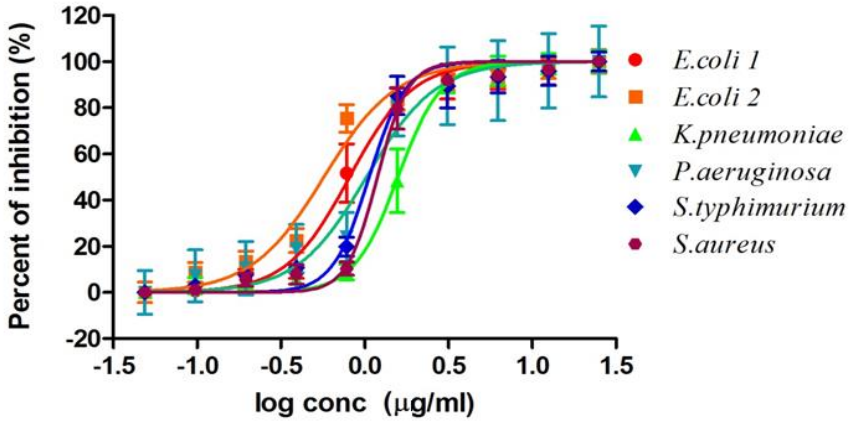
Organisms	HPL-1 (µg/ml)	HPL-2 (µg/ml)	Ampicillin (µg/ml)
Escherichia coli 1	4.052	0.8128	0.183
Escherichia coli 2	2.189	0.5713	0.399
Klebsiella pneumoniae	2.062	1.581	1.208
Pseudomonas aeruginosa	**0.9081**	**1.026**	**16.73**
Salmonella typhimurium	**1.757**	**1.064**	**3.267**
Staphylococcus aureus	0.6255	1.192	0.176

4b.3.3 Antimicrobial activity

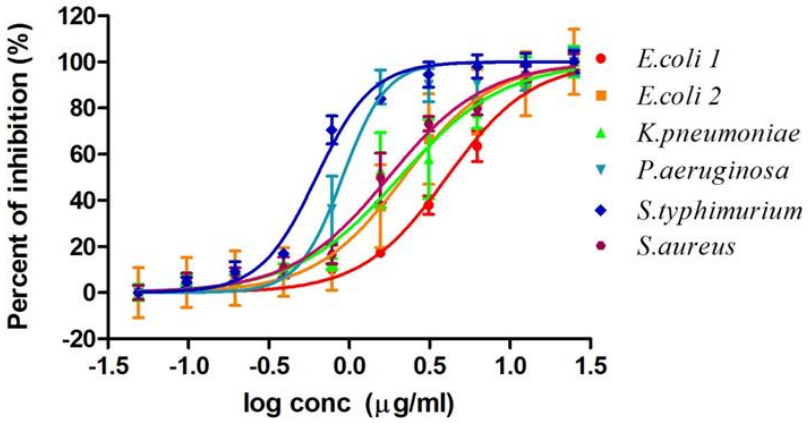
The hybrid hairpin lipopeptides **HPL1** and **HPL2** were subjected for antibacterial activity against various bacterial strains. The antibacterial activities are represented in terms of IC_{50} (the concentration of peptide for killing 50% of bacterial culture), to represent the reponse of various bacterial strains as a function of exposure to different concentrations of lipopeptides. Graphs of % of inhibition of various bacterial strains over various lipopeptide concentrations are shown in figure 5. **HPL1** with two lysine motifs have shown IC_{50} value 4.05- 0.90 µg/ml for gram-negative and 0.6 µg/ml for gram-positive bacterial strains.

HPL2 with four lysine motifs have shown IC_{50} value 0.57- 1.50 µg/ml for gram-negative and 1.19 µg/ml for gram-positive bacterial strains. The inhibition curves of various bacterial strains over ten different lipopeptide concentrations are shown above in Figure 5. The IC_{50} values of **HPL1** and **HPL2** on various bacterial strains are reported in Table 1.

The above results indicate an increase in the activity on increasing hydrophilicity or net positive charge. Also, **HPL1** and **HPL2** are showing better activity than control **Ampicillin** (commercially available antibiotic) against *Pseudomonas aeruginosa* and *Salmonella typhimurium* species, indicating the higher potency of these structure-based hybrids β-hairpin lipopeptides.



(a)



(b)

Figure 5: The inhibition curves of various bacterial strains over different lipopeptide (a) HPL1 and (b) HPL2 concentrations.

Inspired by the above excellent results, we have also subjected these two peptides **HPL1** and **HPL2** against *Mycobacterium smegmatis*, the fastest-growing non-pathogenic gram-positive bacteria which serve as a model for other pathogenic mycobacterium strains such as *M. tuberculosis* and *M. leprae*. **HPL1 and HPL2** have shown IC_{50} values 1.33 and 1.29 µg/ml, respectively, approximately similar activity by both the peptides. The inhibition curves of *Mycobacterium smegmatis* over ten different lipopeptide concentrations are shown above in Figure 6. The IC_{50} values of **HPL1** and **HPL2** on *Mycobacterium smegmatis* are reported in Table 2.

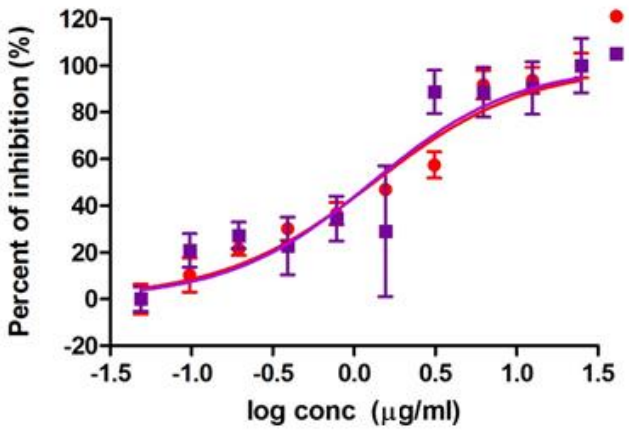


Figure 6: The inhibition curves of *Mycobacterium smegmatis* with different hairpin lipopeptide concentrations. (red for **HPL1** and purple for **HPL2**)

Table 4: The IC_{50} values (µg/ml) of **HPL1** and **HPL2** on *Mycobacterium smegmatis*.

organism	HPL1 (µg/ml)	HPL2 (µg/ml)
Mycobacterium smegmatis	1.330	1.291

4b.4 Conclusion

We are reporting the design, synthesis and antimicrobial activities of structure-based cationic hybrid beta-hairpin lipopeptides containing naturally occurring non-ribosomal α,β-unsaturated γ-amino acid motifs. These peptides have shown high potent antimicrobial activity with IC_{50} as low as 0.5 µg/ml. They have shown better activity than commercially available ampicillin for two bacterial strains. Also, these peptides shave shown excellent activity against *Mycobacterium* strains, showing the efficacy of these hairpin lipopeptides as potent antimicrobial agents.

4b.5 Experimental section

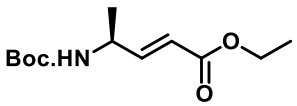
4b.5.1 General experimental details

All α-amino acids, Ethyl diazoacetate, Tin chloride and Di-t*ert*-butyl dicarbonate were obtained from Merck. 2-Mercaptobenzothiazole (2-MBT), Fmoc-Osu, DCC, $NaBH_4$, HPLC grade acetonitrile, dry DCM and dry THF were purchased from Spectrochem. Column chromatography was achieved on silica gel of 120-200 mesh from Merck. Thin-layer chromatography (TLC) was used to monitor reaction using Merck 60 F_{254} precoated silica gel plates. For visualization UV light and ninhydrin or phosphomolybdic acid (PMA) stains were used then charring on a hot plate. Melting points were recorded on Veego VMP-DS hot stage apparatus. Specific rotations were recorded at ambient temperature on the Rudolph Analytical Research instrument using $CHCl_3$ and MeOH as solvents. Reverse Phase-HPLC of Waters was used to purify peptides using Acetonitrile/H_2O gradient with 0.1% TFA from C-18 column. Mass was analysed on Applied Biosciences with MALDI TOF/TOF on positive ion mode.

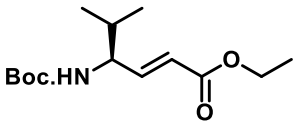
4b.5.2 General procedure for the synthesis of *(N)*Fmoc α,β-unsaturated γ- amino acid

(refer chapter 3a,section 3b)

Characterization of *(N)*Fmoc α,β-unsaturated γ- amino acids

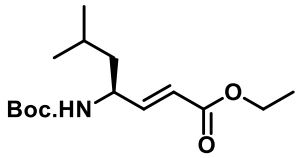


(*S, E*)-ethyl 4-(tert-butoxycarbonylamino)pent-2-enoate: Colourless Oil. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.876-6.827 (dd, 1H), 5.898-5.859 (d, 1H) , 4.5 (br, 1H), 4.38 (br, 1H), 4.198-4.144(q, *J*= 7.3 Hz, 2H), 1.432 (s, 9H), 1.265-1.247 (m, 6H); **^{13}C NMR** (100 MHz, $CDCl_3$) 166.472, 154.974, 120.201, 79.851, 60.534, 47.080, 28.431, 20.422, 14.301. **MALDI.TOF/TOF** m/z Calcd. for $C_{12}H_{21}NO_4$ (M+Na) 266.1368 Observed.266.1365.



(*S, E*)-ethyl 4-(tert-butoxycarbonylamino) 5-methylhex-2-enoate: Colourless solid. **^{1}HNMR** (400 MHz, $CDCl_3$) δ 6.855-6.816 (dd, 1H) 5.924-5.880 (d, *J*=15.6 Hz, 1H), 4.55 (d, 1H), 4.187-4.168 (q, *J* =6.88 Hz, 2H), 1.862-1.84 (m, 1H), 1.429 (s, 9H),1.292-1.256 (t, *J* =7.32 Hz, 3H), 0.932-0.885 (q, *J*=6.4 Hz, 6H); **^{13}C NMR** (100 MHz, $CDCl_3$); 166.2437, 155.3170,

147.3461, 121.4025, 79.6125, 60.3812, 56.6151, 32.1875, 28.2974, 18.8105, 17.9428, 14.1862. **MALDI. TOF/TOF** Calcd. for $C_{14}H_{25}NO_4$ 294.1681 Observed 294.1686.



(*S, E*)-ethyl 4-(tert-butoxycarbonylamino) 6-methylhept-2-enoate: Colorless crystalline soild .**^{1}H NMR** (500 MHz,$CDCl_3$) δ 6.854-6.811(dd, *J*=16 Hz, *J*=5.5 Hz, 1H), 5.936-5.904 (d, *J*=16 Hz, 1H), 4.451 (br, 1H), 4.334 (br, 1H), 4.215-4.152 (q, *J*=7 Hz, 2H),1.706-1.681 (m, 1H), 1.436 (s, 9H), 1.399-1.372 (t, *J*=7 Hz, 2H), 1.334-1.266 (t, *J*=7 Hz, 3H), 0.935-0.922 (d, *J*=6.5 Hz, 6H); **^{13}C NMR** (100MHz, $CDCl_3$) δ 166.4153, 155.0501, 148.8907, 120.3537, 79.6506, 60.4098, 49.7597, 43.7815,28.3164, 24.6742, 22.6815, 22.1476, 14.2053. **MALDI.TOF/TOF** m/z Calcd. For $C_{15}H_{27}NO_4$ [M+Na+] 308.1838, Observed. 308.1840.

4b.5.3 Solid-phase peptide synthesis and purification

The cationic hairpin lipopeptides **HPL1** and **HPL2** were synthesized on Knorr amide resin (0.2 mmol scales) using standard Fmoc-chemistry. As coupling reagents, HBTU/HOBt were used. Kaiser test was performed to monitor coupling reactions. After completion of the desired couplings, TFA: water: phenol: TIPS (95:2:2:1) was used as a scavenger cocktails mixture to cleave the peptides from resins. Later, the resin was filtered and the cleavage mixture or the filtrate was co-evaporated using DCM beneath the vacuum to get a gummy product. The product was recrystallized using diethyl ether to obtain a white solid. The obtained white solid was dissolved in water and purified through reverse phase HPLC on a C18 column using an ACN/H_2O with a 0.1% TFA solvent system with 2mL/min flow rate. The purity of the peptides was further investigated by passing from an analytical C-18 column with the same gradient system with 0.75ml/min flow rate. The mass of the peptide was recorded using **MALDI TOF/TOF.**

4b.5.4 Procedure for testing antibacterial activity

The bacteria culture, *Escherichia coli* (NCIM 2065), *Escherichia coli K12* (NCIM 2563), *Klebsiella pneumoniae* (NCIM 2957), *Pseudomonas aeruginosa* (NCIM 5029), *Salmonella typhimurium* (NCIM 2501), *Staphylococcus aureus* (NCIM 5021), and *Mycobacterium smegmatis* (NCIM 5138) used for the bacterial investigation were obtained from the National Collection of Industrial Microorganisms (NCIM).

The antibacterial activities of hairpin lipopeptides (HPL1 and HPL2) were performed according to the standard protocol reported by Clinical Laboratories Standards Institute (CLSI): M07-A10 in 2015.[42] The antibacterial activities are reported in IC_{50} (half maximal inhibitory concentration) and the standard broth microdilution method was followed on a sterile 96-well microtiter plate (F96, NUNC, 200 μL well capacity). The bacterial cultures were grown for 24 hours at 37 °C in MHB media (Muller-Hinton broth). After 24 hours OD was measured for each bacterial culture and then serially diluted to obtain a final concentration of 10^6 colony-forming units/ml using MHB media. The lipopeptides **HPL1** and **HPL2** were dissolved in sterile water and added in a sterile 96-well plate to a final volume of 50 μL in each well with two-fold serial dilution from 100μg/ml-0.1μg/ml concentration range. Later, bacterial suspensions of 50 μL were added to each well to make a final volume of 100 μL. The plate was kept in an incubator at 37 °C for 18-20 hours. The same procedure is followed for Ampicillin as a reference drug. For control, a culture without peptide and only media were also grown parallelly. The percentage of inhibition was obtained by monitoring the growth inhibition at an absorbance of 492 nm by Biotek microplate reader. Later, plotted using MS-Excel with various *log* concentrations of each sample concerning their percentage of inhibition for each strain, IC_{50} representing the concentration value of lipopeptide where 50% of inhibition of bacterial culture is seen. The IC_{50} values obtained here were reproducible for three independent triplicates.

4b.6 References

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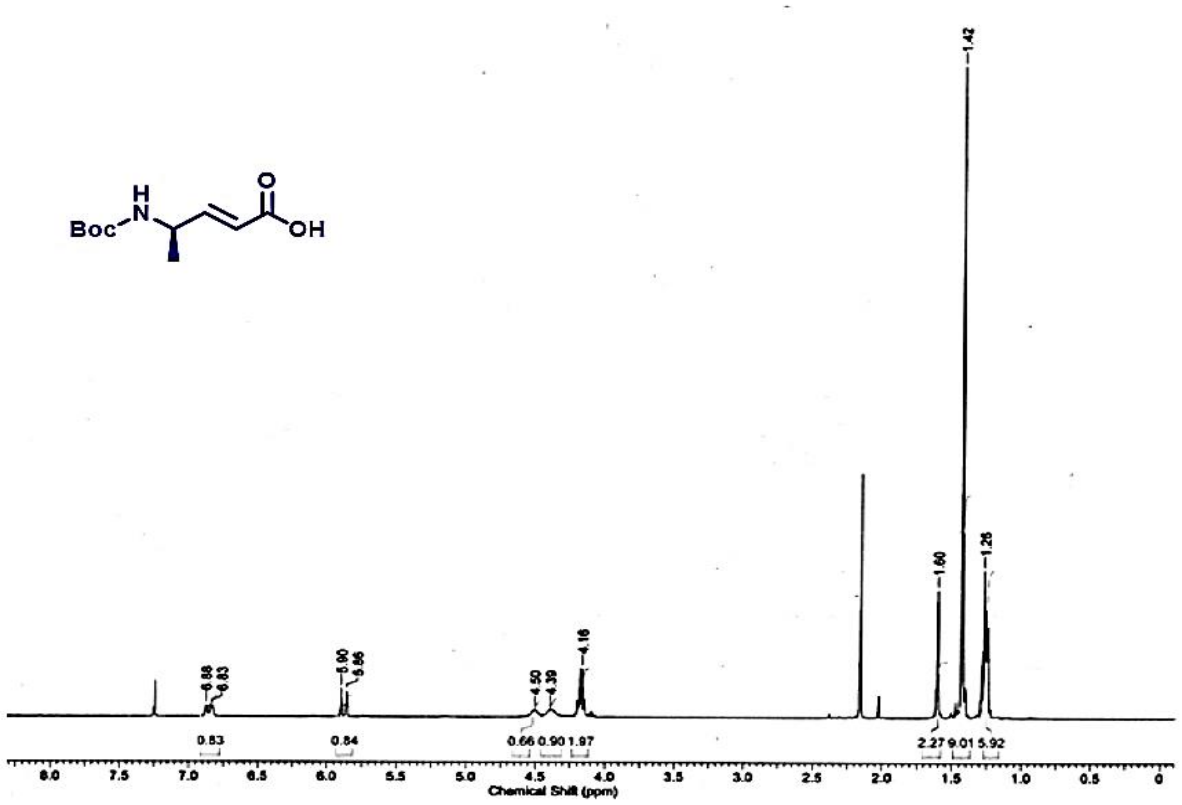
4b.7 Appendix: ^{1}H, ^{13}C, Mass spectra and HPLC traces of monomers and peptides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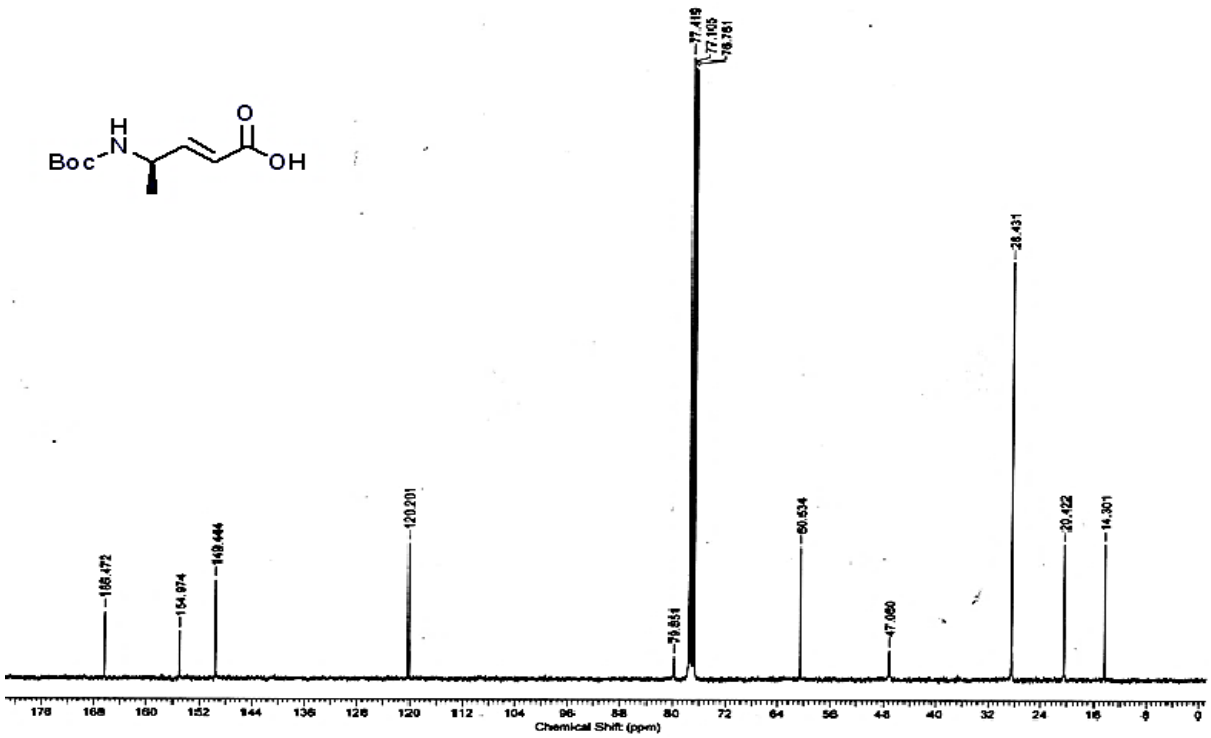
0.90
1.97
2.27
9.01
5.92
Chemical Shift (ppm)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OH
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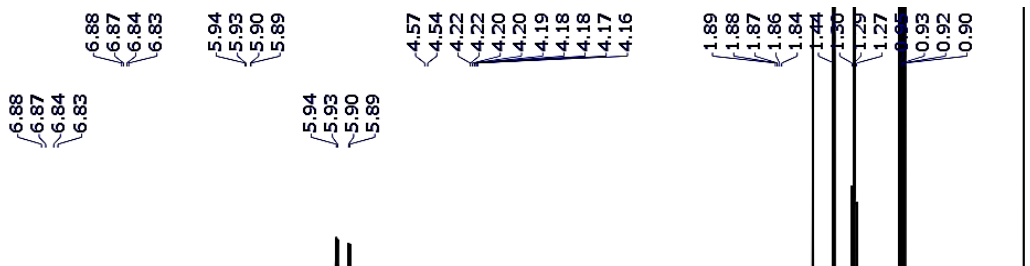
Chemical Shift (ppm)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4.18
4.17
4.16
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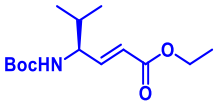
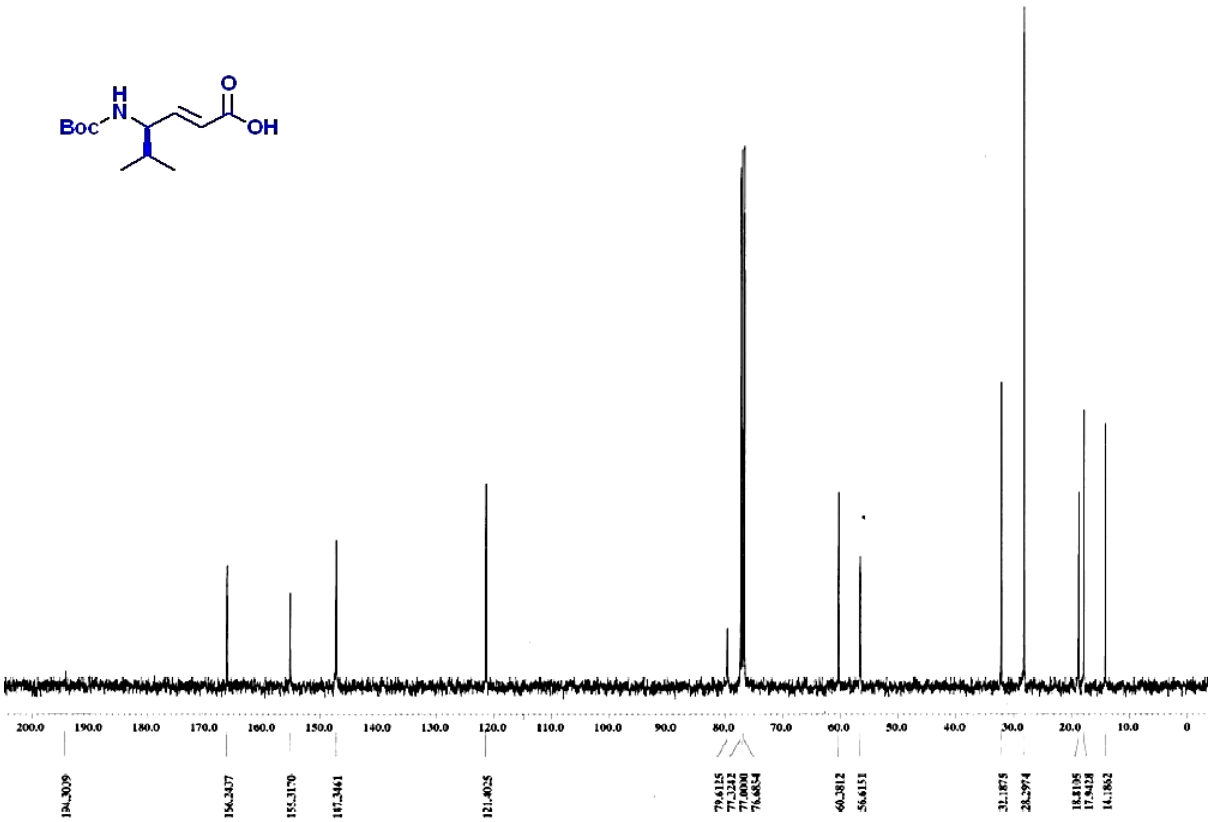
1.29
1.27
0.93
0.92
0.90


BocHN
O
O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OH
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160.0
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140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0
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153.3170
147.3441
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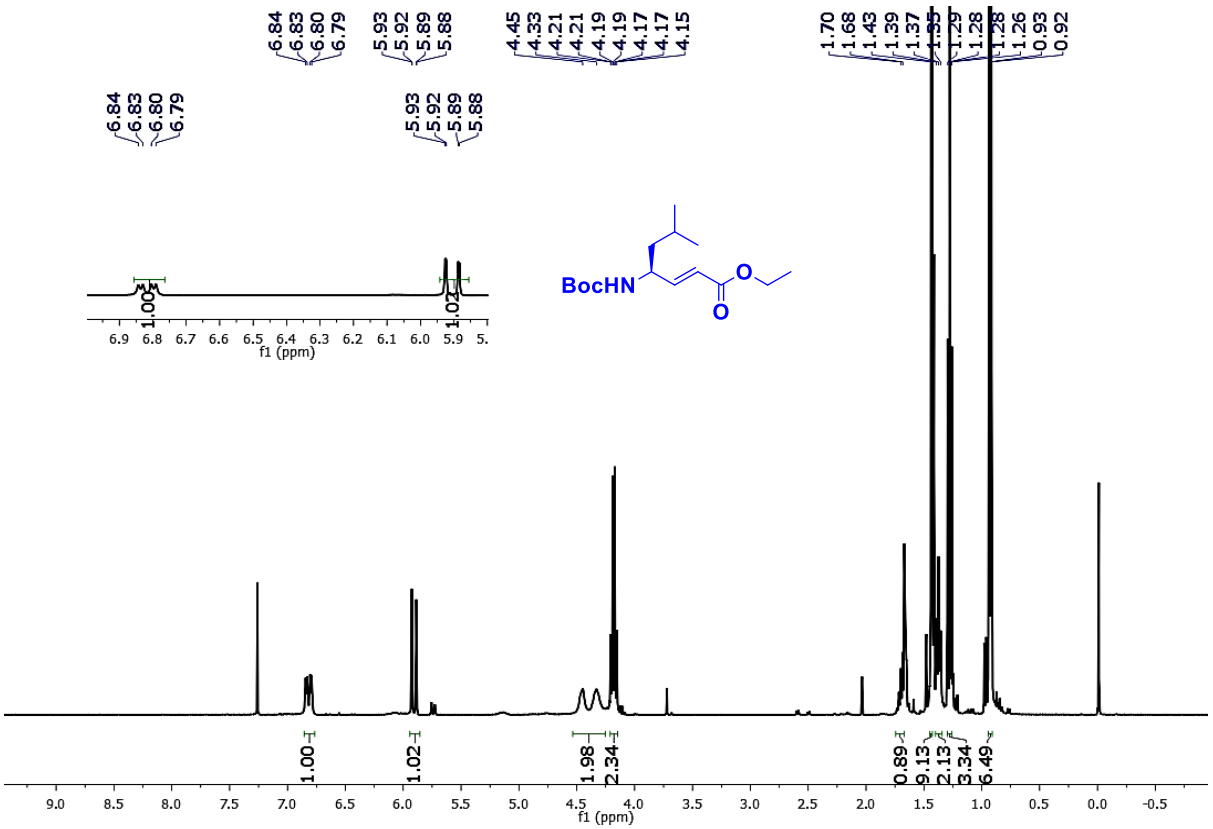
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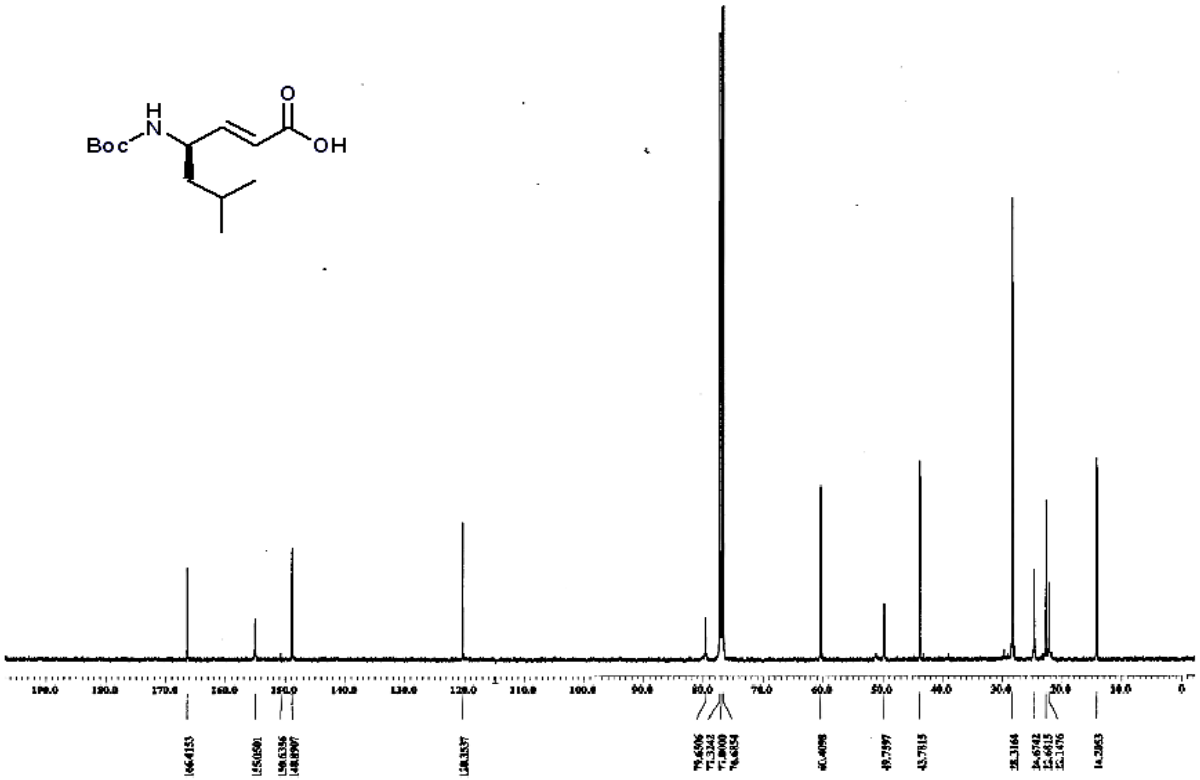
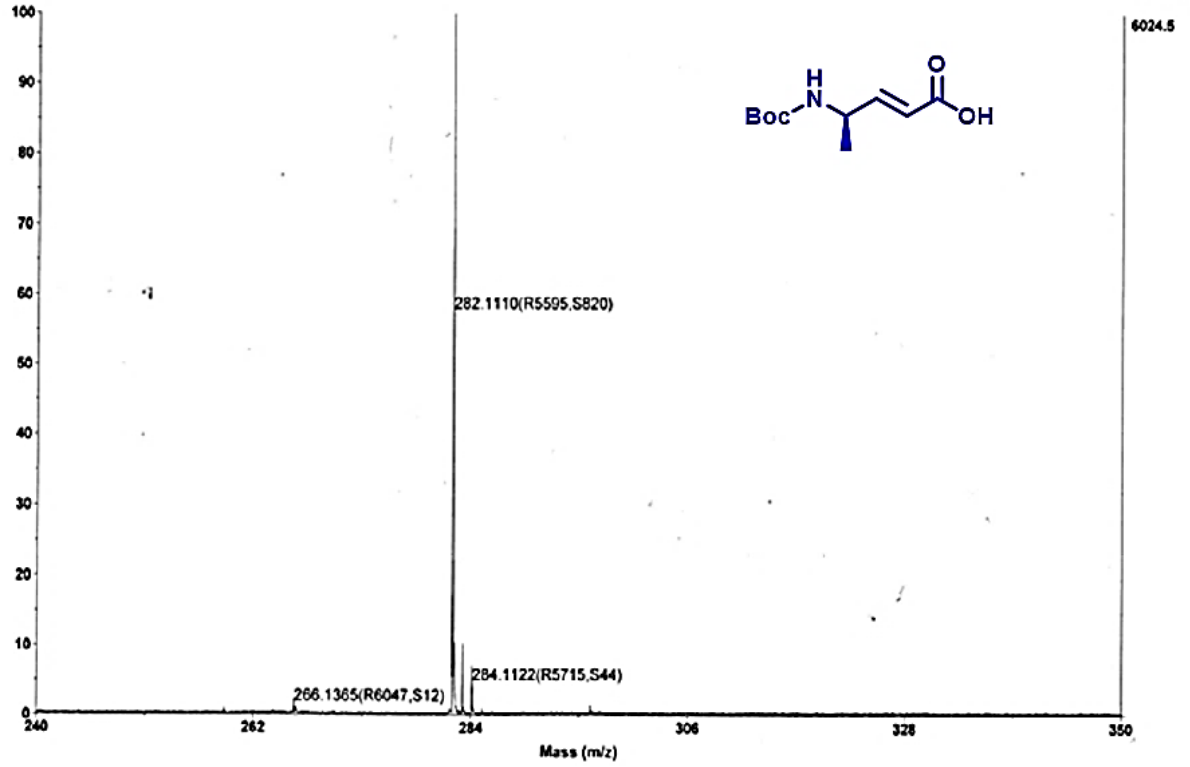
4.19
4.17
4.17
4.15
1.70
1.68
1.43
1.39
1.37
1.35
1.29
1.28
1.28
1.26
0.93
0.92
BocHN
O
O
1.00
1.02
1.98
2.34
0.89
9.13
2.13
3.34
6.49
f1 (ppm)


Boc
N
H
O
OH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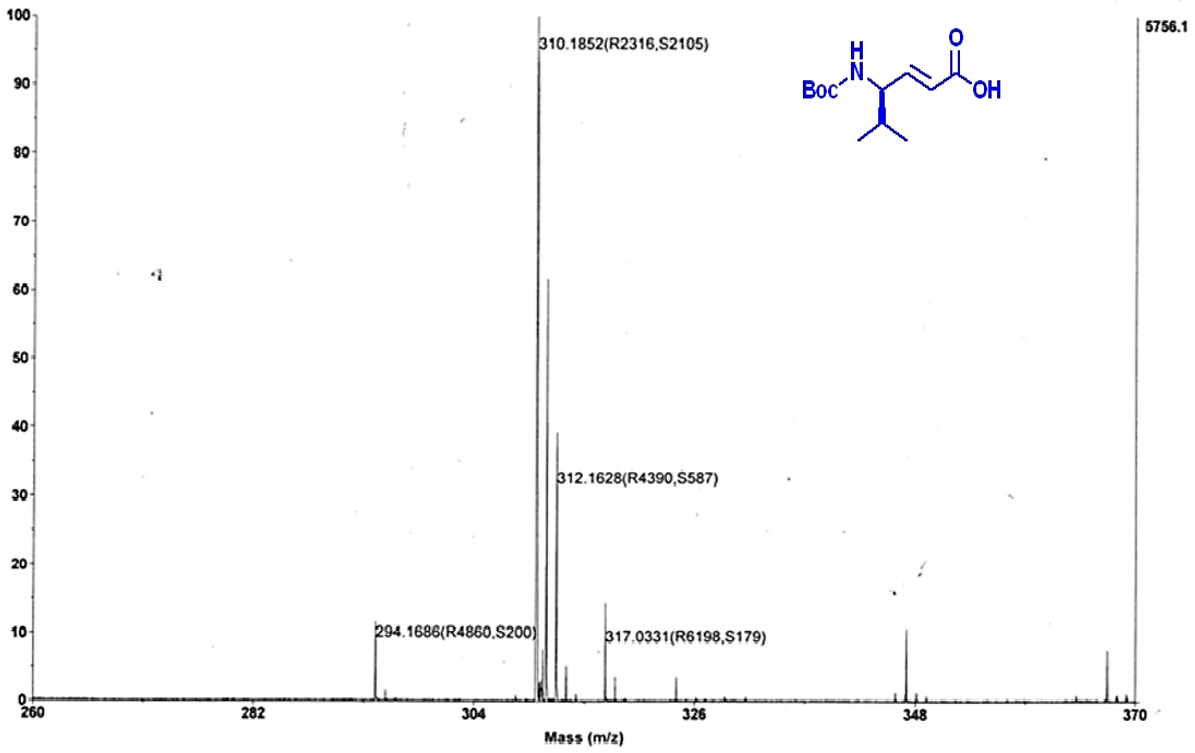
266.1365(R6047,S12)
284.1122(R5715,S44)
Mass (m/z)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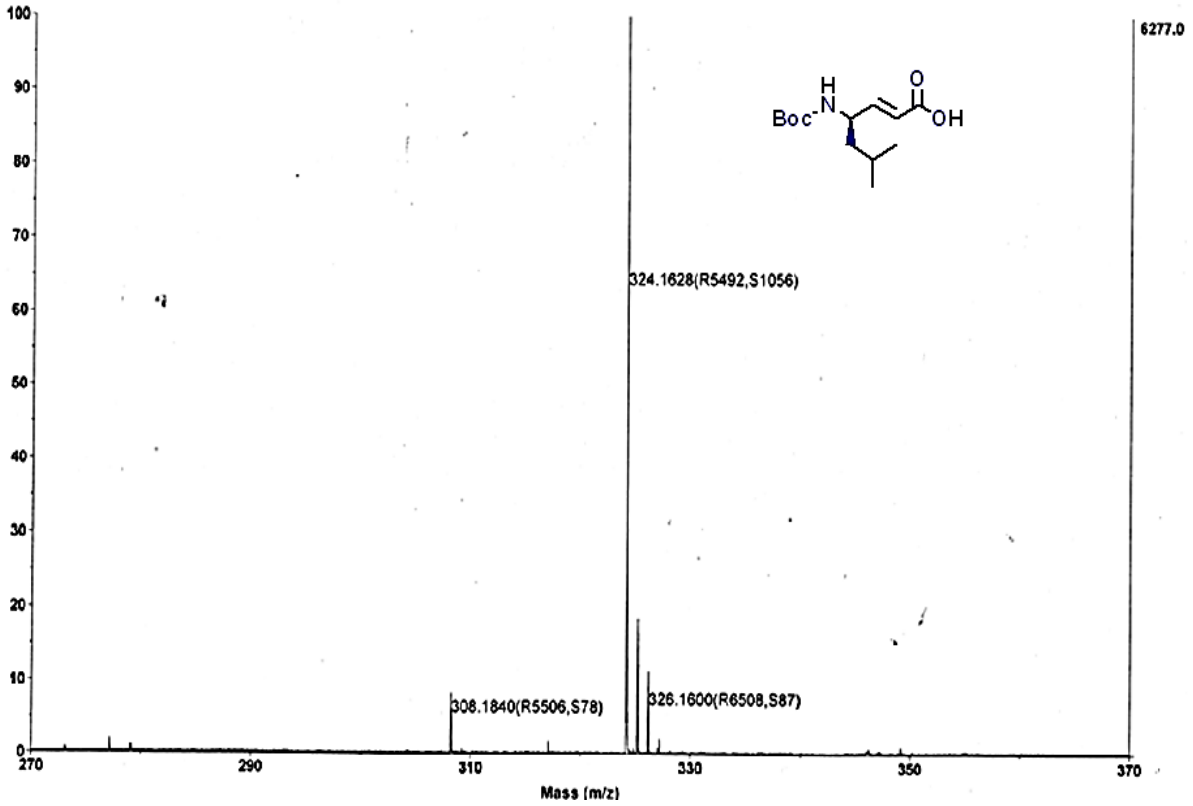
294.1686(R4860,S200)
317.0331(R6198,S179)
Mass (m/z)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100
90
80
70
60
50
40
30
20
10
0
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290
310
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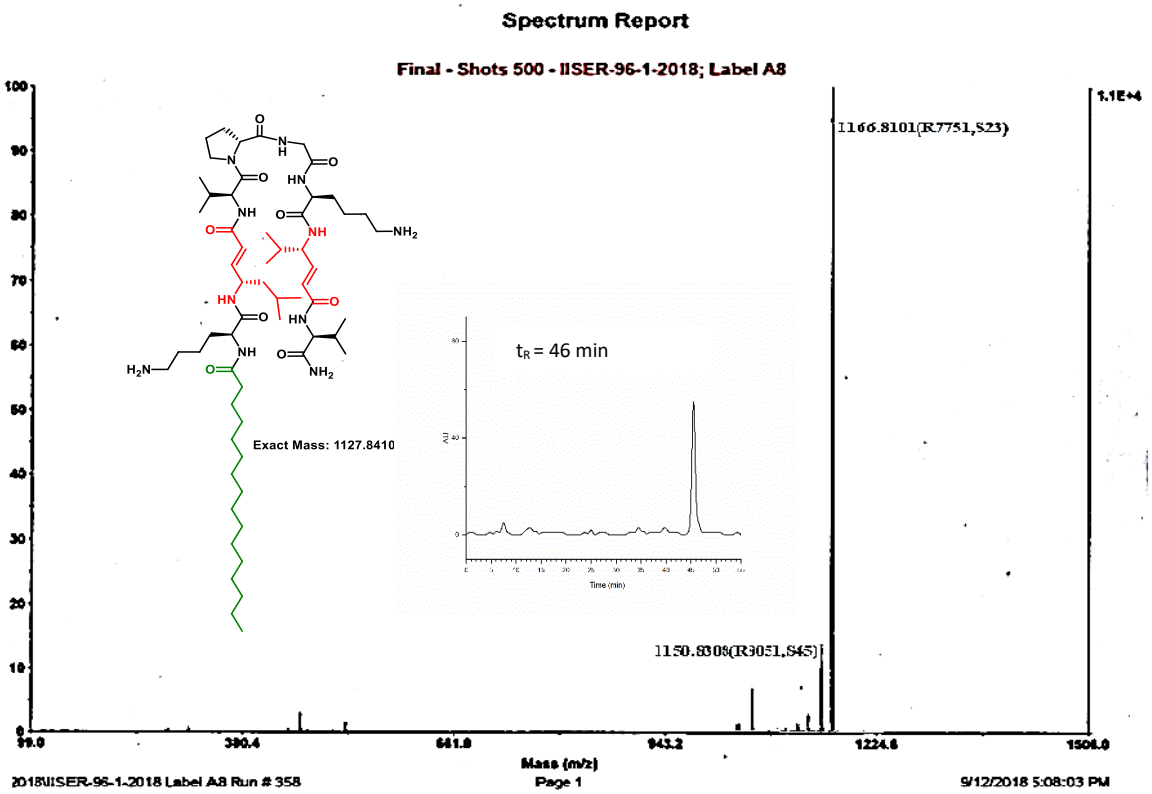
Mass (m/z)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tR = 46 min
Time (min)
AU
100
90
80
70
60
50
40
30
20
10
0
99.0
390.4
681.8
943.2
1224.6
1506.0
Mass (m/z)
2018\IISER-96-1-2018 Label A8 Run # 358
Page 1
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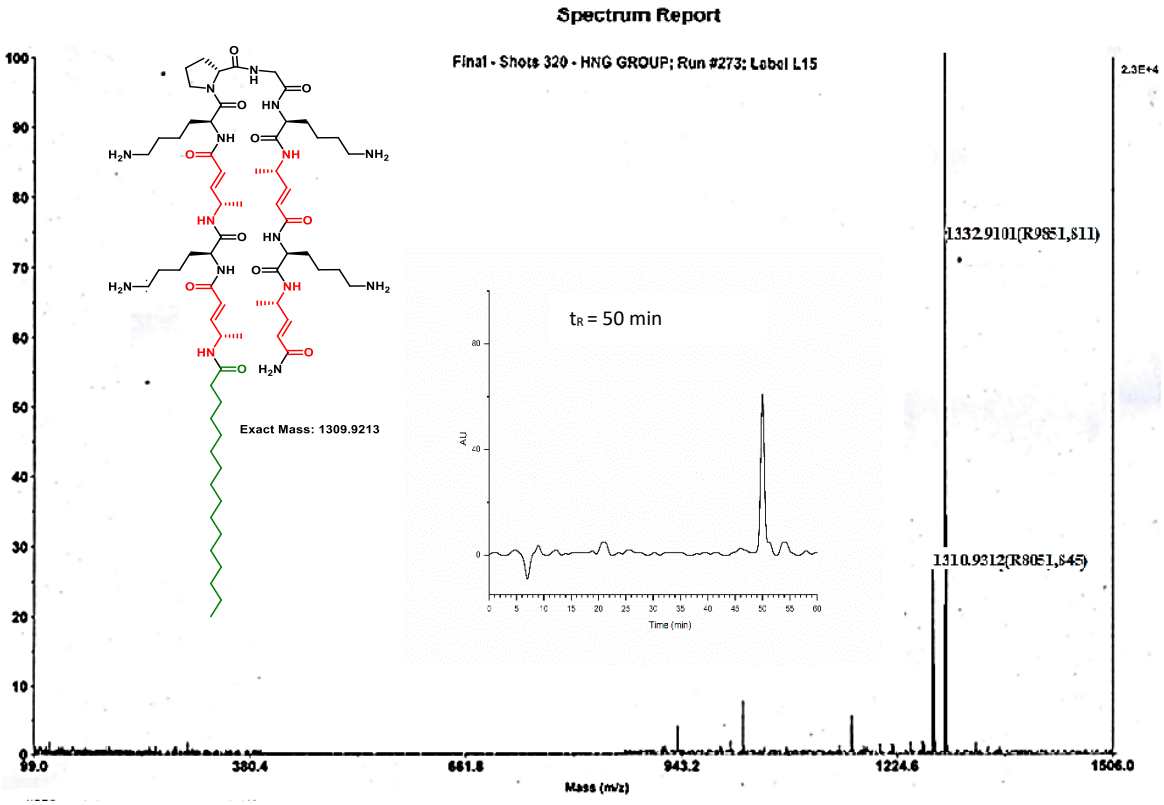
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1310.9312(R8051,845)
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Mass (m/z)
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661.8
943.2
1224.6
1506.0

Chapter 5

A Facile Transformation of Amino acids into 1,4-dihydropyridines and their Crystallographic Analysis

5.1 Introduction

Amino acids are essential components for the existence of life on earth and they are responsible for the structure and function of proteins. Besides their biological role, they have been serving as starting materials for the synthesis of various drugs intermediates,[1] chiral catalyst,[2] heterocyclic compounds,[3] statins,[4] β-amino acids,[5] γ-amino acids,[6] and biodegradable polymers.[7]

1,4-Didropyridine(1,4-DHP) is a semi-saturated nitrogen-containing heterocyclic compound derived from pyridine as a parent molecule. In a few decades, 1,4-Dihydropyridine has emerged as a prolific class of molecule in the field of important biologically active and pharmaceutical compounds. 1,4-dihydropyridine scaffold containing molecules are among the most predominant present in the list of top 20 small molecule-based drugs in the 2010 survey.

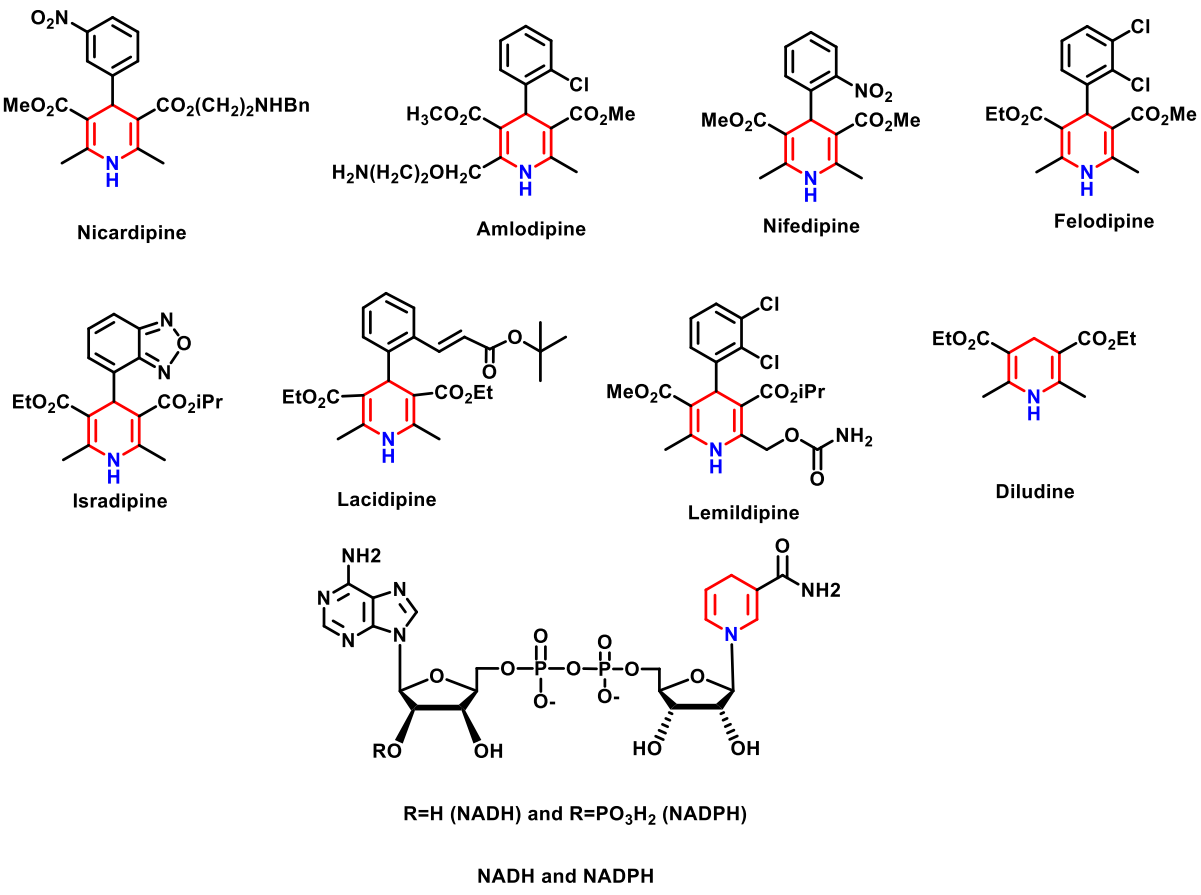


Figure 1. Examples of a clinically valuable 1,4-Dihydropyridine scaffold containing drugs and NADH molecule.

Mainly 1,4-DHP families have found commercial efficacy as a calcium channel blocker.[8] It can be well-illustrated with several examples of 1,4-DHP scaffolds containing drugs clinically accepted as cardiovascular agents and are given for treating hypertension and heart failures such as nicardipine, amlodipine, nifedipine, felodipine, isradipine, etc., examples are shown in Figure 1.[9] Lacidipine, the second-generation calcium channel blocker widely comes in a market with name Lacipil have more of selective tissue bioavailability and biostability and less of side effects.[10]

Moreover, 1,4-DHPs have also been reported in other biologically active compounds and have shown splendid results such as antioxidant,[11] antimalarial,[12] antibacterial,[13] antifungal,[14] antiplasmodial,[15] anti-inflammatory[16] and antidiabetic.[17] Besides, they have also shown promising potential as HIV protease inhibitor,[18] anti-ischemic agents for treating Alzheimer's disease,[19] chemo sensitizers in tumor therapy,[20] platelet antiaggregatory agents,[21] bronchodilators for treating tuberculosis, [22] cystic fibrosis,[23] asthma,[24] neuro protecting agents in neuronal diseases[25] and drugs widely used for treating other various diseases.
1,4-DHP is well known as NADH mimic. In recent years 1,4-DHP came into the picture as biomimetic reducing agents and provides many examples as hydride donors in various chemical reactions such as reduction of alpha-keto electron-withdrawing conjugated olefins, cyclic enones, ketones to alcohols and hydrogenation of alpha-beta unsaturated aldehydes and imines, etc. with extensive application in the field of synthetic chemistry.[26]

By looking into 1,4-DHPs and their great biological importance, developing a new methodology for its synthesis opens a new area of significant research interest. 1,4-DHPs are generally synthesized by the classical Hantzsch condensation method first reported in 1882, which involves cyclo condensation of two equivalents of β-keto esters, aldehyde and ammonia or amines as a nitrogen source by refluxing in water and has been extensively used to synthesize 1,4-DHP derivatives over the years.[27] But due to low product yields obtained by this method which can be because of the solubility of the reagents in water, several modifications have been done to improve the reaction yields. Moreover, this method is better for symmetrical 1,4-DHP synthesis and in literature, it’s been shown that chiral, unsymmetrical 1,4-DHPs present in many drugs have shown higher pharmacological activity.

While in a recent era several routes to synthesize 1,4-DHPs have been described in the literature. Methodologies have recently undergone much progress by using various catalysts such as Lewis acid (Ajavakom, *et al.* and Li, *et al.*),[28] Bronsted acid (Renaud, et al. and

Takemoto *et al*),[29] $PhB(OH)_2$ and PPH_3 (Debache, *et al*.),[30] metal triflates (Donelson *et al.*, Adibi *et al* and Fukazawa, *et al*.),[31] molecular iodine(Yao, *et al*.),[32] organocatalysts,[33] TMS iodide (Sabitha *et al*.),[34] ionic liquids as catalyst (Ji, *et al*.),[35] hetero-polyacid (Heravi, *et al*.),[36] biocatalyst such as baker's yeast (Lee *et al*.) *etc*.[37] In recent years, heterogeneous solid catalysts such as $SiO_2/NaHSO_4$ (Adharvana Chari & Syamasundar),[38] $SiO_2/HClO_4$ (Sridhar, *et al*.)[39] and sulphonic acid on silica gel (Gupta *et al*.)[40] ceric ammonium nitrate, CAN (Perumal, *et al*.)[41] montmorillonite K-10 (Song *et al*.),[42] ionic HY zeolite (Das *et al*),[43] *etc*. have been utilized extensively to improve the yields of the reaction. Catalyst free reactions have received considerable interest in organic synthesis. Various methodology such as the use of microwave radiations (Bazureaua *et al*. and Li *et al*.),[44] ultrasound radiations (Kumar & Maurya),[45] *etc*. came into the picture. Using the above-discussed methods, numerous relevant derivatives of 1,4-DHPs have been synthesized and studied. During few decades wide studies have been done looking to more on economically superior, catalyst-free, environment-friendly and one-pot synthesis rather than a multistep classical method for synthesis of complex classes of a compound having 1,4-DHP moiety. The development of an efficient and versatile method for the preparation 1,4-DHPs is an active ongoing research area and there is scope for further improvements towards mild reaction conditions and improved yields.

However, no method is reporting in the literature for the direct introduction of amino acid functional groups on the 1,4-DHP. This is possibly due to the lack of the commercial availability of starting material i.e. amino β-keto esters. The amino acid functional groups may improve the biological activities of these DHP derivatives.

Earlier work

We have been working in the area of foldamers composed of various types of non-ribosomal γ-amino acids. Besides their utility in the design of foldamers, these naturally occurring non-ribosomal amino acids such as α,β-unsaturated γ-amino acids and γ-amino β-keto esters have served as starting materials for the construction of various small molecules. Recently, we demonstrated the synthesis of γ-lactams from α,β-unsaturated γ-amino amides through a base mediated molecular rearrangement.[46] The γ-amino β-keto esters have been used to synthesize functionalized curcumins and fluorescent amino acids,[47] γ-amino β-hydroxy acids,[48] and symmetrical tetra-substituted pyrazines through air oxidation.[49] These recent examples highlight the level of constant curiosity in the direction of utilizing these modified non-ribosomal γ-amino acids for the synthesis of various molecular scaffolds and this inspired us

to explore γ-amino β-keto esters for the synthesis of pharmacophoric scaffold 1,4-DHP derivatives with amino acid functionalization, which may find applications as a source of bioactive molecules.

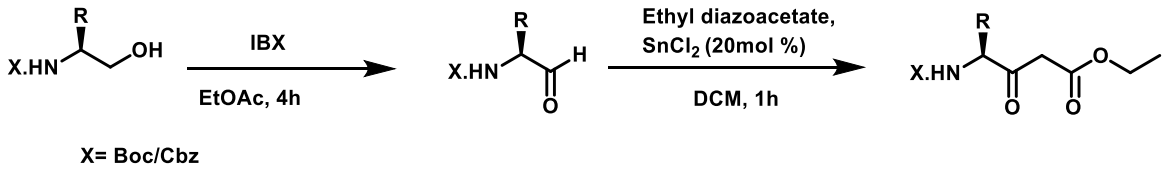
5.2 Aim and rationale of the present work

We envisioned that the amino acid-functionalized 1,4-DHP derivatives can be synthesized using γ-amino β-keto esters thus integrating amino acid functionalities in the 1,4-DHP scaffold. In this Chapter, we are describing the design, synthesis and crystal conformations of 1,4-DHP derivatives from commercially available amino acids.

5.3 Results and Discussion

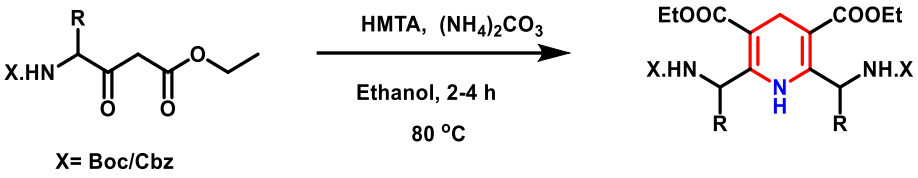
5.3.1 Design and synthesis

In order to investigate whether γ-amino β-keto esters can undergo Hantzsch type reaction to give desired 1,4-dihydropyridine derivatives, we have synthesized various ethyl esters of γ-amino β-keto amino acids from of *N*-protected amino aldehydes in the presence of ethyl diazoacetate and tin chloride (20 mol %) as a catalyst in DCM, shown in **Scheme 1**, as per the protocol reported by our group earlier.[47] All the *N*-protected γ-amino β-keto esters were purified through silica column chromatography in good yields before using it for the next step.



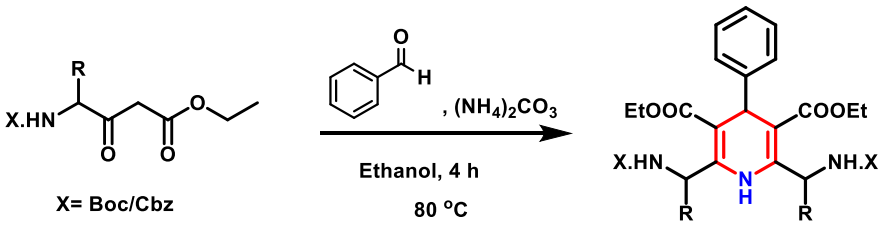
Scheme 1: Synthesis of *N*-protected β-keto γ-amino esters.

Further to go for Hantzsch type reaction we need an aldehyde and a nitrogen source. For the aldehyde source, we have chosen hexamethylenetetramine (HMTA) as it is known for *in-situ* generation of formaldehyde and for the nitrogen source we went ahead with ammonium carbonate and ammonium acetate for investigating. We have also investigated the reaction yields with various solvents such as water, ethanol and toluene. During the investigation, we have observed that the optimum reaction conditions for best yield were obtained using ethanol as solvent and HMTA and ammonium carbonate combinations at reflux temperature for 2 hours for the complete conversion of *N*-protected γ-amino β-keto ester (1a) to obtain 1,4-DHP (2a), shown in **Scheme 2**.



Scheme 2: Synthesis of 1,4-dihydropyridine synthesis from *N*-protected γ-amino β-keto esters.

Encouraged by the above result for the synthesis of **2a**, various hydrophobic *N*-protected γ-amino β-keto esters were synthesized (**1b-1g**) and kept for the reaction in ethanol to obtain desired 1,4-DHPs, shown in Table 1. Later to investigate the interference of side-chain functionalities and different amino acid protecting groups in this reaction, (*N*)Cbz-protected β-keto γ-amino ester (**1g**) were synthesized and subjected under the reaction condition to obtain product **2g.** It is worth noting that the reaction is compatible with various hydrophobic side chains and amine protecting groups for the condensation of β-keto esters of various amino acids to synthesize 1,4 DHPs. All 1,4-DHPs products were isolated in good yields after purification from silica gel column chromatography using hexane and ethyl acetate as solvent systems. Later we kept them for crystallization in Ethyl acetate and x-ray quality single crystal was obtained for **2(b)**, **2(e)** and **2(f)**. The crystal structure of **2(f)** is shown in Figure 2. The crystal structure shows the pyridine ring exists in planer conformation in all of the above. As per the reports in the literature, if no substitution is there at the para position the pyridine ring shows planer structure, which exactly matches our observations in the synthesized amino acid-functionalized 1,4-DHPs.

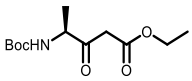
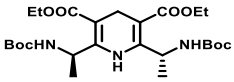
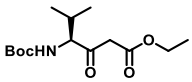
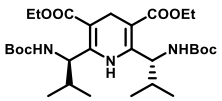
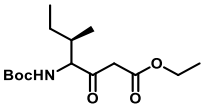
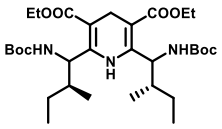
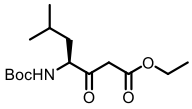
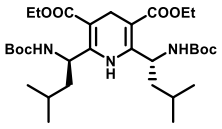
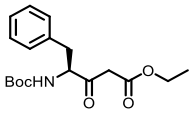
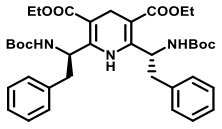
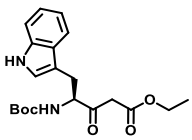
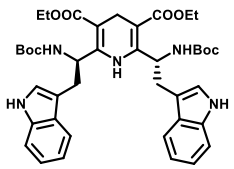


Scheme 3: Synthesis of para-substituted 1,4-dihydropyridine from *N*-protected γ-amino β-keto esters.

Inspired by this result, we thought of investigating the role of substitution at the para position in a conformational change of the pyridine ring we have synthesized para-substituted 1,4-DHP **2(h)**. For the above purpose, we have chosen **1(h)** as γ-amino β-keto ester and for the para

substitution, we have chosen benzaldehyde despite using HMTA as aldehyde source keeping the rest of the reaction condition unchanged, shown in **Scheme 3**. We have observed the complete conversion in 4 hours. Later we purified the product **2(h)** through column chromatography characterized by mass and kept it for crystallization in ethyl acetate. We have observed x-ray quality single crystal for **2(h)** shown in **Figure 3**. From the crystal structure, we wonderfully observed the puckered conformation of 1,4-DHP with substitution at the para position hence matches with the literature observations. As we just want the product **2(h)** for studying the role of substitution at the para position in conformation and thus, synthesizing and characterization other para-substituted DHP's of this series is beyond the scope of this chapter**.**

Table 1: List of γ-amino β-keto esters and their respective symmetrical 1,4-dihydropyridine.

Entry	β-keto esters (1)	1,4-DHPs (2)	% yield
(a)			87
(b)			78
(c)			85
(d)			75
(e)			82
(f)			78

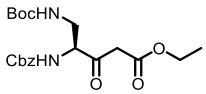
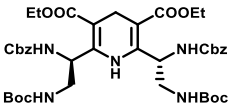
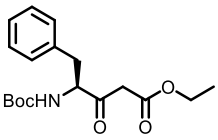
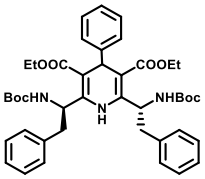
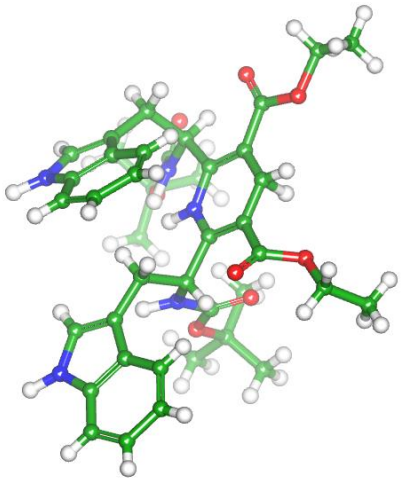
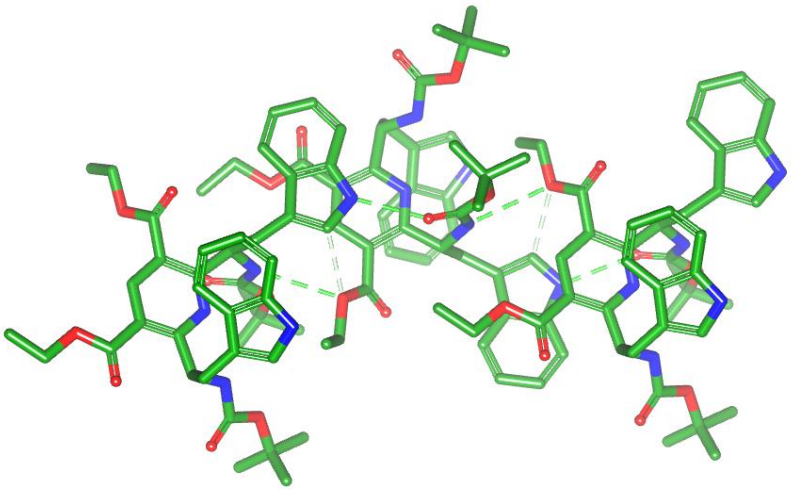
(g)			85

Table 2: List of γ-amino β-keto esters and their respective para-substituted 1,4-dihydropyridine.

Entry	β-keto-esters (1)	1,4-DHP (2)	% yield
(h)			82

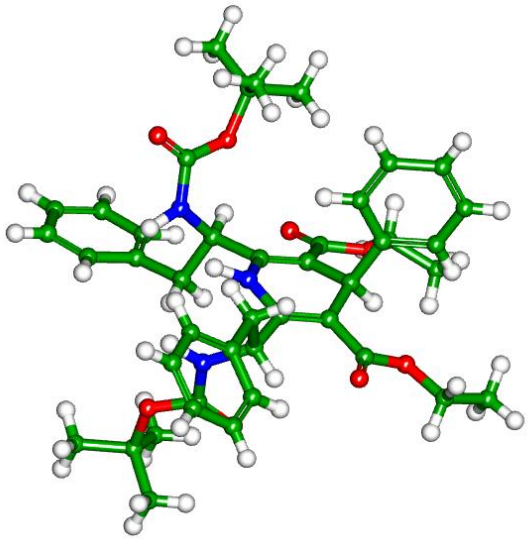


(a)

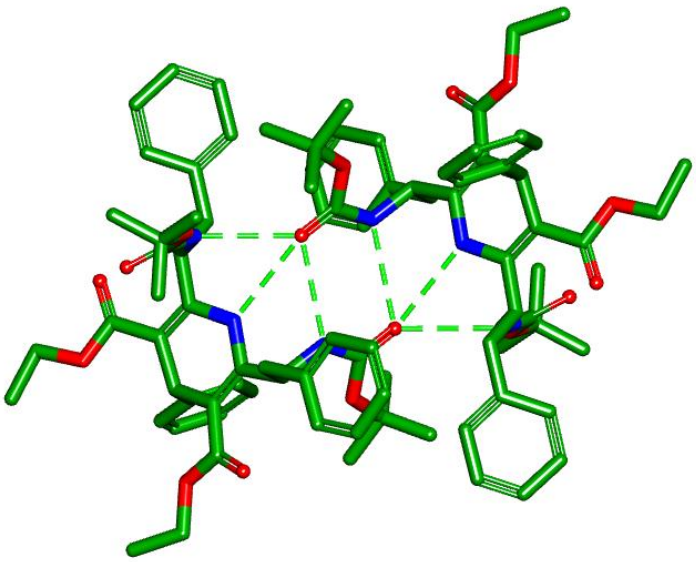


(b)

Figure 2: (a) X-ray structure of compound 2(h) and (b) H-bonding patterns of compound 2(h).



(a)



(b)

Figure 3: (a) X-ray structure of compound 2(f) and (b) H-bonding patterns of compound 2(f).

5.4 Conclusion

In summary, we have proposed an efficient catalyst-free procedure for the synthesis of 1,4-dihydropyridines from various amino acids with many functionalities through a one-pot Hantzsch reaction with satisfactory yields of 70-80 %. The protocol is neat and clean. Through this protocol, it is possible to introduce amino acid side-chains in 1,4-dihydropyridines. The

hydrogen transferability of these new 1,4-dihydropyridines and medicinal properties will be investigated in due course of time.

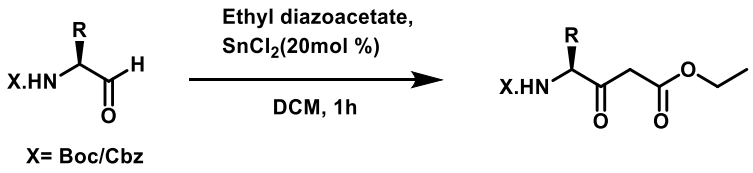
5.5 Experimental Section

5.5.1 General Experimental Details: All α-amino acids, Ethyl diazoacetate, Tin chloride, HMTA and Di-t*ert*-butyl dicarbonate were obtained from Merck. Ethanol, ammonium carbonate, dry DCM and benzaldehyde were purchased from Spectrochem. Column chromatography was achieved on silica gel of 120-200 mesh from Merck. Thin-layer chromatography (TLC) was used to monitor reaction using Merck 60 F_{254} precoated silica gel plates. For visualization UV light and ninhydrin or phosphomolybdic acid (PMA) stains were used then charring on a hot plate. Specific rotations were recorded at ambient temperature on the Rudolph Analytical Research instrument using $CHCl_3$ and MeOH as solvents. Mass was analysed on Applied Biosciences with MALDI TOF/TOF on positive ion mode and Electron Spray Ionization (ESI).

NMR spectroscopy

Jeol 400 MHz (or 100 MHz for ^{13}C) spectrometer were used for recording ^{1}H spectra and ^{13}C NMR. The chemical shifts (δ) and coupling constants (J) were reported in ppm and Hz, respectively. The residual solvents signals were used as internal reference ($CDCl_3$ δ_H, 7.24 ppm, δ_c 77.0 ppm).

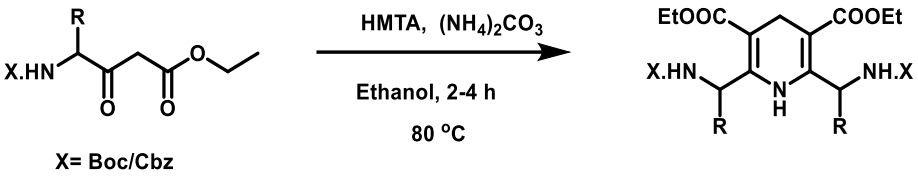
5.5.2 Synthesis of *N*-protected β-keto γ-amino ester



DCM (15 ml) was used to dissolve the above formed *N*-Boc β-amino aldehyde and to that Tin (II) Chloride (20 mol%) was added at room temperature. After 5 mins, 15% Ethyl diazoacetate in toluene (10.05 mmol, 7.5 ml) was added slowly drop by drop and instant evolution of nitrogen can be seen till the reaction completes. TLC was performed for monitoring the reaction. Once the reaction is completed it was neutralized using 5 % HCl and the aqueous layer was extracted thrice using DCM. The combined organic extract was washed with brine followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further purified through column

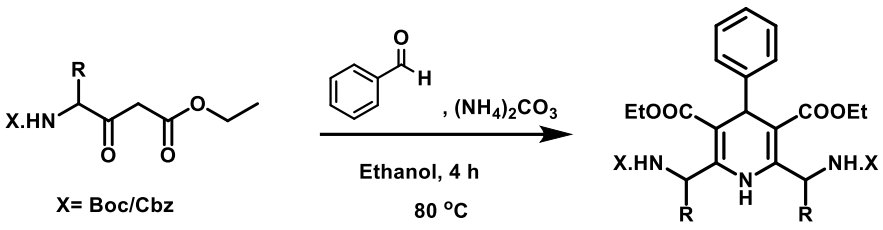
chromatography over silica using EtOAc / Hexane solvent system to obtain pure *N*-Boc β-keto ester. Characterization of the compound was done using NMR and mass analysis.

5.5.3 Synthesis of 1,4-dihydropyridine



Ethanol (10ml) was used to dissolve *N*-protected γ-amino β-keto ester (5 mmol) and kept for stirring at a refluxing temperature under N_2 atmosphere. Later, HMTA (7.5 mmol, 1.05 g) and ammonium carbonate (5.5 mmol, 0.52 g) was added to the reaction mixture and stirred for the next 2 to 4 hours. TLC was performed for monitoring the reaction. Once the reaction is completed it was neutralized using 5 % HCl and Ethanol was evaporated under reduced pressure. The aqueous layer was extracted thrice using Ethyl acetate. The combined organic extract was washed with brine followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure 1,4-DHP. Characterization of the compound was done using NMR and mass analysis.

5.5.4 Synthesis of para-substituted 1, 4-DHP



Ethanol (10ml) was used to dissolve *N*-protected γ-amino β-keto ester (5 mmol) and kept for stirring at a refluxing temperature under N_2 atmosphere. Later, Benzaldehyde (5 mmol, 0.5 ml) and ammonium carbonate (5.5 mmol, 0.52 g) was added to the reaction mixture and stirred for the next 2 to 4 hours. TLC was performed for monitoring the reaction. Once the reaction is completed it was neutralized using 5 % HCl and Ethanol was evaporated under reduced pressure. The aqueous layer was extracted thrice using Ethyl acetate. The combined organic extract was washed with brine followed by drying over anhydrous Na_2SO_4. The organic layer was kept beneath the *vacuum* to concentrate. This concentrated organic mixture was further

purified through column chromatography over silica using EtOAc / Hexane solvent system to obtain pure para-substituted 1,4-DHP. Characterization of the compound was done using NMR and mass analysis.

5.6 Crystal Structure Information

5.6.1 General procedure for crystallization of 1,4-dihydropyridine.

All crystallization attempts were conducted at room temperature. All compounds were purified carefully before keeping for crystallization. Glass sample vials (2 mL) were washed with acetone and dried under a nitrogen gas stream before use. PARAFILM was used to close the vials. HPLC-grade solvents were used for crystallization.

5.6.2 Crystal structure analysis of compound 2(f)

Crystals were grown from ethyl acetate and n-hexane solution by slow evaporation. A single crystal (0.12 x 0.1 x 0.08 mm^3) was mounted on loop with a small amount of the paraffin oil. The X-ray data were collected at 100 K temperature using Mo K_α radiation (λ =0.71073 Å), ω-scans (2θ = 57.028), for a total of 10604 independent reflections. Space group P21/n,a = a = 12.281(8) Å , b = 15.095(10) Å , c = = 22.849(14) Å , α = 90, β = 91.813(10), γ =90, V = 4234(5) $Å^3$, monoclinic, Z= 4 for chemical formula C_{41} H_{45} N_5 O_8 with one molecule in asymmetric unit; ρcalcd = 1.154 Mg/m^3, μ =0.081 mm^{-1} , F (000) = 1560, The structure was obtained by direct methods using SHELXS-97.[1] The final R value was 0.1062, (wR2 = 0.2403), 69439 observed reflections ($F_0 \geq 4\sigma$ ($|F_0|$)) and 495 variables, S = 0.99.The largest difference peak and hole were 0.800 and -0.261 e.$Å^{-3}$ respectively.

5.6.3 Crystal structure analysis of compound 2(h)

Crystals were grown from ethyl acetate and n-hexane solution by slow evaporation. A single crystal (0.10 x 0.08 x 0.06 mm^3) was mounted on loop with a small amount of the paraffin oil. The X-ray data were collected at 100 K temperature using Mo K_α radiation (λ = 1.54178 Å), ω-scans (2θ = 57.028), for a total of 21460 independent reflections. Space group P-1, a = a = 11.5155(9), b = 16.4327(13) , c = 22.5154(17) , α = 90, β = 103.30(4), γ =90(7) V = 4138.9(6) $Å^3$, monoclinic, Z = 4 for chemical formula C_{43} H_{50} N_3 O_8 with one molecule in asymmetric unit; ρcalcd = 1.183 Mg/m^3, μ =0.662 mm^{-1} , F (000) = 1572, The structure was o btained by direct methods using SHELXS-97.[1] The final R value was 0.0701,(WR2 = 0.1893) 29094 observed reflections ($F_0 \geq 4\sigma$ ($|F_0|$)) and 558 variables, S = 1.316.The largest difference peak

and hole were0.672 and -0.257 e.Å^{-3} respectively.

Numerous datasets were collected on single crystals from different batches and one of the highest qualities is reported herein.

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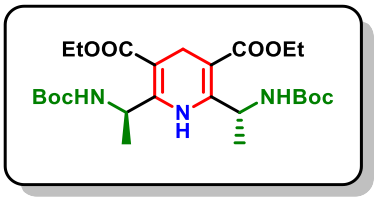
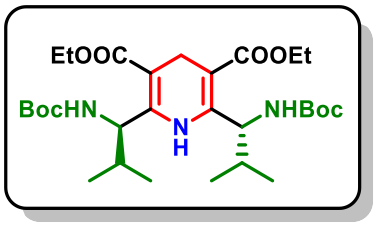
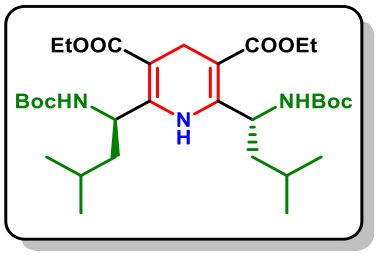
Characterization of Compounds:



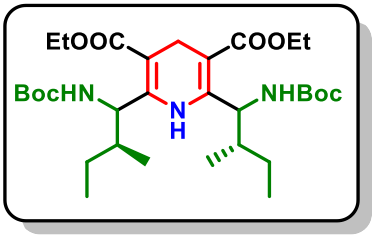
2(a) diethyl 2,6-bis((S)-1-((tert-butoxycarbonyl)amino)ethyl)-1,4-dihydropyridine-3,5-dicarboxylate : pale yellow solid (87%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.67 (d, *J* = 2.8 Hz, 1H), 5.73 (s, 2H), 5.39 – 5.26 (m, 2H), 4.41 (q, *J* = 7.1 Hz, 4H), 4.17 – 4.14 (m, 2H), 1.41 (s, 18H), 1.30 – 1.24 (m, 12H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 165.04, 155.85, 151.30, 122.74, 79.52, 62.03, 59.85, 49.08, 28.50, 18.96, 14. HRMS m/z calculated value for $C_{22}H_{41}N_3O_6$ is [M+H$^+$] 512.30 and observed 512.2972.



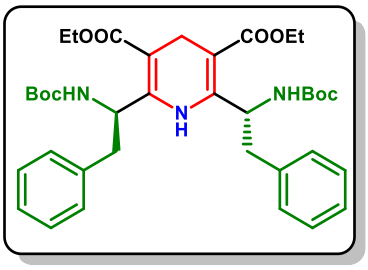
2(b) diethyl 2,6-bis((S)-1-((tert-butoxycarbonyl)amino)-2-methylpropyl)-1,4-dihydropyridine-3,5-dicarboxylate: pale yellow solid (78 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.72 (d, *J* = 13.4 Hz, 1H), 5.67 – 5.59 (m, 2H), 5.28 – 4.84 (m, 2H), 4.46 – 4.37 (m, 4H), 4.18 – 4.13 (m, 2H), 2.12 – 2.02 (m, 2H), 1.42 (s, 18H), 1.27 (td, *J* = 7.2, 2.6 Hz, 6H), 0.94 – 0.85 (m, 12H).^{13}C NMR (101 MHz, Chloroform-*d*) δ 165.21, 155.95, 141.82, 123.36, 79.33, 62.01, 56.80, 44.97, 34.38, 28.36, 19.97, 14.32. HRMS m/z calculated value for $C_{22}H_{41}N_3O_6$ is [M+H$^+$] 568.36 and observed 568.3670.



2(c) diethyl 2,6-bis((S)-1-((tert-butoxycarbonyl)amino)-3-methylbutyl)-1,4-dihydropyridine-3,5-dicarboxylate: pale yellow solid (85 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.64 (d, *J* = 7.7 Hz, 1H), 5.75 (d, *J* = 14.2 Hz, 2H), 5.49 (d, *J* = 36.6 Hz, 2H), 4.41 (q, *J* = 7.3, 6.8 Hz, 4H), 4.13 – 4.08 (m, 2H), 1.39 (d, *J* = 7.0 Hz, 18H), 1.24 (t, *J* = 7.1 Hz, 6H), 1.03 (t, *J* = 6.2 Hz, 6H), 0.91 (q, *J* = 8.7, 7.9 Hz, 12H).^{13}C NMR (101 MHz, Chloroform-*d*) δ 171.28, 165.19, 155.60, 141.69, 79.29, 61.93, 51.28, 46.14, 45.81, 28.49, 25.30, 23.61, 14.28. HRMS m/z calculated value for $C_{22}H_{41}N_3O_6$ is [M+H$^+$] 596.39 and observed 596.3911.

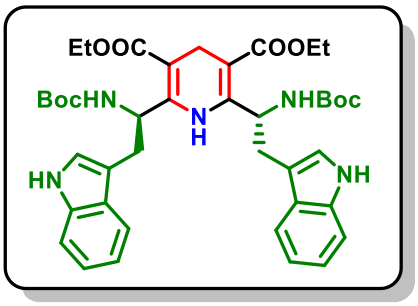


2(d) diethyl 2,6-bis((S)-1-((tert-butoxycarbonyl)amino)-2-methylbutyl)-1,4-dihydropyridine-3,5-dicarboxylate. pale yellow solid (75 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.64 (d, *J* = 7.7 Hz, 1H), 5.75 (d, *J* = 14.2 Hz, 2H), 5.49 (d, *J* = 36.6 Hz, 2H), 4.41 (q, *J* = 7.3, 6.8 Hz, 4H),δ 4.41 (q, *J* = 6.7 Hz, 1H), 1.40 (tt, *J* = 7.6, 3.7 Hz, 8H), 1.31 – 1.17 (m, 1H), 0.92 (m, *J* = 18.4, 7.7 Hz, 2H), 0.86 – 0.74 (m, 2H).^{13}C NMR (101 MHz, Chloroform-*d*) δ 165.18, 155.80, 142.08, 123.33, 79.27, 62.01, 55.73, 40.92, 29.79, 28.48, 27.21, 15.89, 14.34, 11.95. HRMS m/z calculated value for $C_{22}H_{41}N_3O_6$ is [M+H$^+$] 596.39 and observed 596.3911.

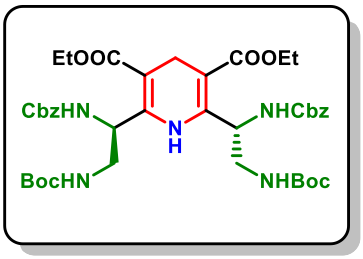


2(e) diethyl 2,6-bis((S)-1-((tert-butoxycarbonyl)amino)-2-phenylethyl)-1,4-dihydropyridine-3,5-dicarboxylate: pale yellow solid (82 %). ^{1}H NMR (400 MHz,

Chloroform-*d*) δ 8.62 (d, *J* = 30.5 Hz, 1H), 7.30 – 7.14 (m, 10H), 6.10 – 5.89 (m, 2H), 5.65 – 5.22 (m, 2H), 4.42 – 4.31 (m, 4H), 4.13 (dq, *J* = 21.5, 7.2 Hz, 2H), 2.97 (dd, *J* = 21.3, 7.1 Hz, 4H), 1.38 (s, 18H), 1.28 – 1.21 (m, 6H).^{13}C NMR (101 MHz, Chloroform-*d*) δ 164.86, 155.30, 141.65, 137.10, 129.79, 129.68, 128.31, 126.61, 79.54, 62.08, 53.27, 47.00, 42.45, 28.51, 14.32. HRMS m/z calculated value for $C_{22}H_{41}N_3O_6$ is [M+H$^+$] 664.36 and observed 664.3598.

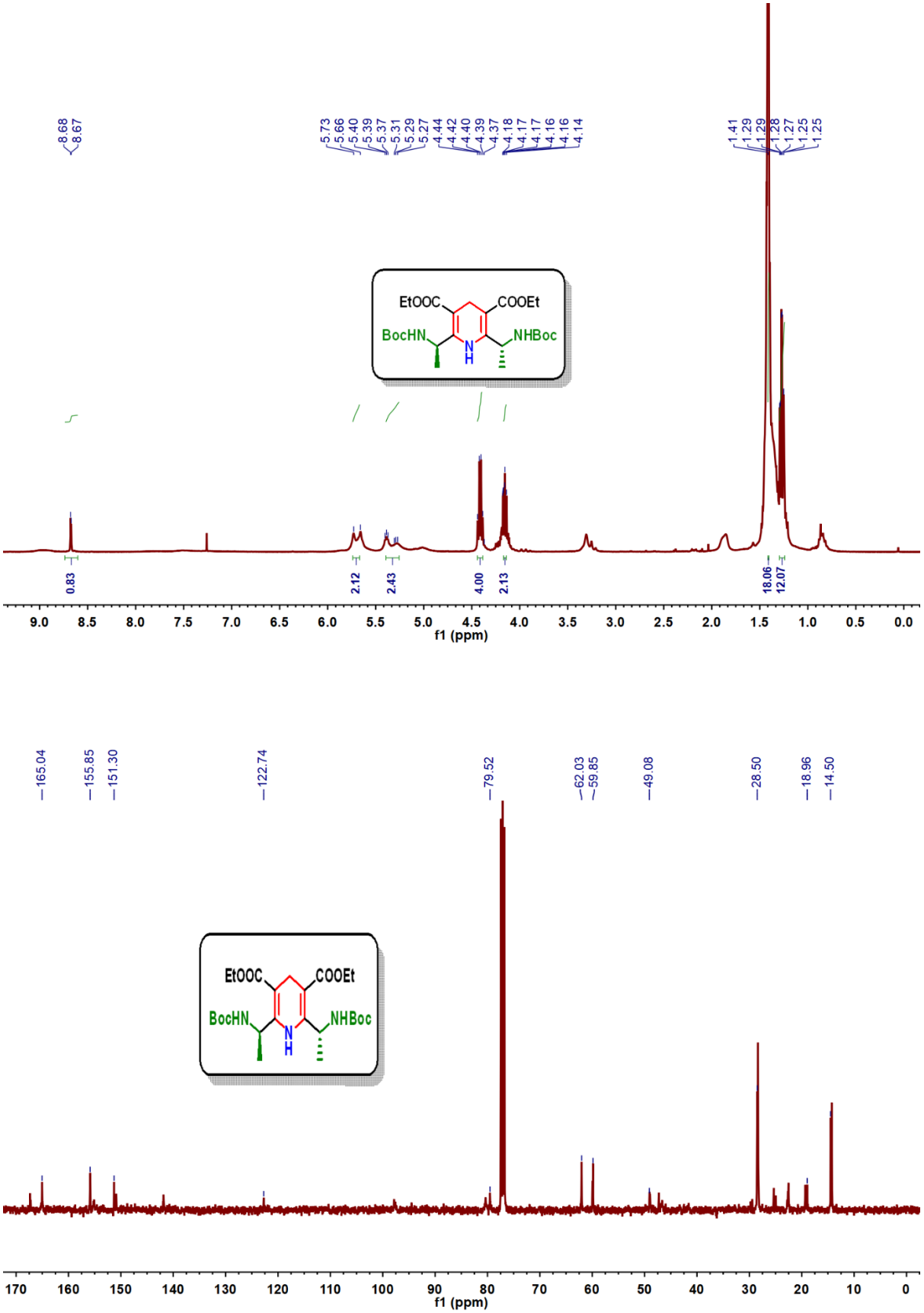


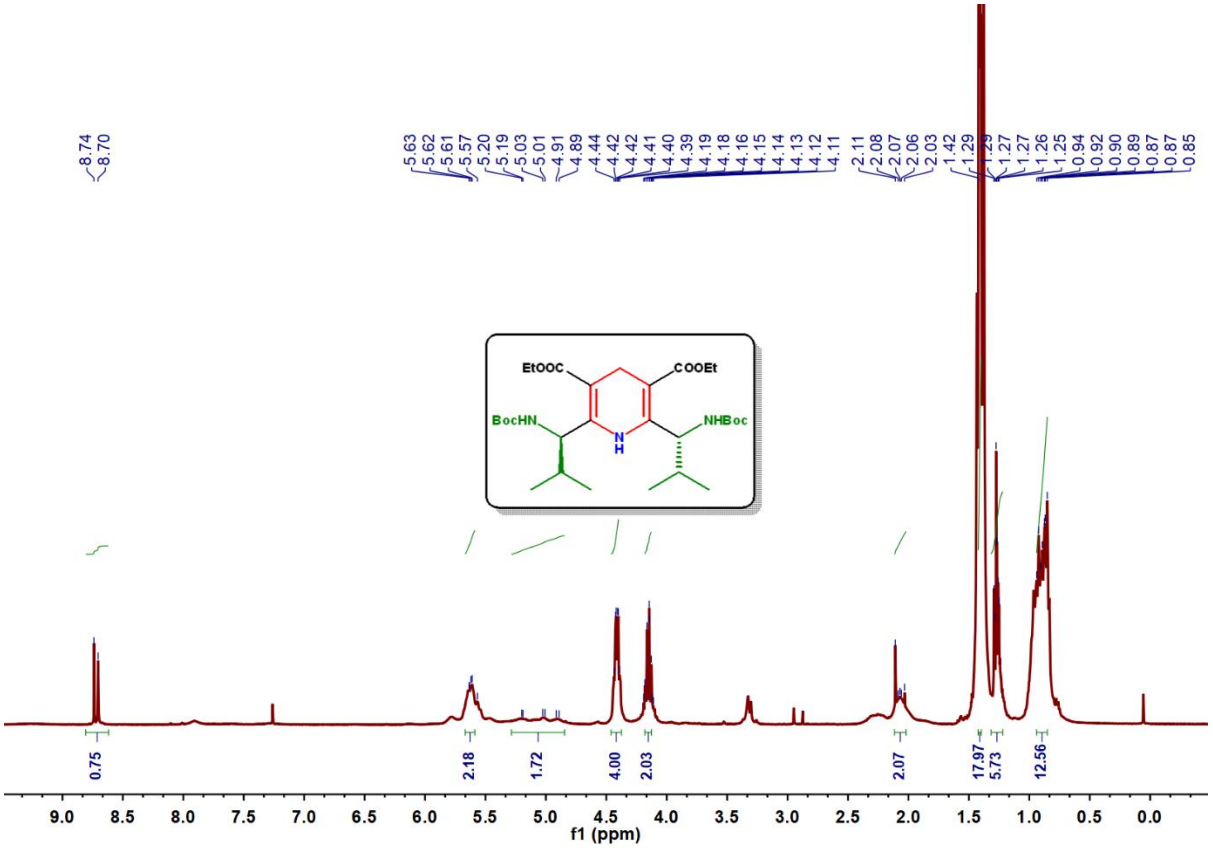
2(f) diethyl 2,6-bis((S)-1-((tert-butoxycarbonyl)amino)-2-(1H-indol-3-yl)ethyl)-1,4-dihydropyridine-3,5-dicarboxylate: pale yellow solid (78 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.21 (s, 2H), 7.80 (s, 1H), 7.34 – 7.08 (m, 10H), 5.65 (s, 2H), 4.99 (s, 2H), 4.22 (dd, *J* = 12.7, 6.9 Hz, 4H), 4.12 (q, *J* = 7.1 Hz, 2H), 3.22 – 2.99 (m, 4H), 1.31 (s, 18H), 1.26 (t, *J* = 7.2 Hz, 6H).^{13}C NMR (101 MHz, Chloroform-*d*) δ 167.38, 156.47, 150.17, 136.42, 127.69, 125.69, 123.20, 122.25, 119.74, 119.27, 111.23, 98.21, 80.31, 60.51, 60.00, 51.34, 28.33, 25.60, 14.58. HRMS m/z calculated value for $C_{22}H_{41}N_3O_6$ is [M+H$^+$] 742.38 and observed 742.3816.



2(g) diethyl 2,6-bis((S)-10,10-dimethyl-3,8-dioxo-1-phenyl-2,9-dioxa-4,7-diazaundecan-5-yl)-1,4-dihydropyridine-3,5-dicarboxylate: pale yellow solid (85 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.61 (s, 1H), 7.38 – 7.28 (m, 10H), 6.65 (s, 1H), 6.19 (s, 1H), 5.55 – 5.41 (m, 2H), 5.28 – 4.92 (m, 6H), 4.17 (q, *J* = 7.0 Hz, 4H), 3.57 (s, 2H), 3.42 – 3.29 (m, 4H), 1.40 (s, 18H), 1.28 (d, *J* = 7.0 Hz, 6H).^{13}C NMR (101 MHz, Chloroform-*d*) δ 170.32, 157.73, 154.91, 136.18, 128.62, 128.56, 127.46, 99.49, 80.54, 66.96, 61.92, 60.03, 42.33, 39.56, 28.22, 14.01. HRMS m/z calculated value for $C_{22}H_{41}N_3O_6$ is [M+H$^+$] 810.38 and observed 810.3867.

5.8 Appendix I: ^{1}H NMR spectra,^{13}C NMR and Mass spectra of compound 2a-h.

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4.42
4.41
4.40
4.39
4.19
4.18
4.16
4.15
4.14
4.13
4.12
4.11
2.11
2.08
2.07
2.06
2.03
1.42
1.29
1.27
1.26
1.25
0.94
0.92
0.90
0.89
0.87
0.87
0.85
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COOEt
BocHN
NHBoc
N
H
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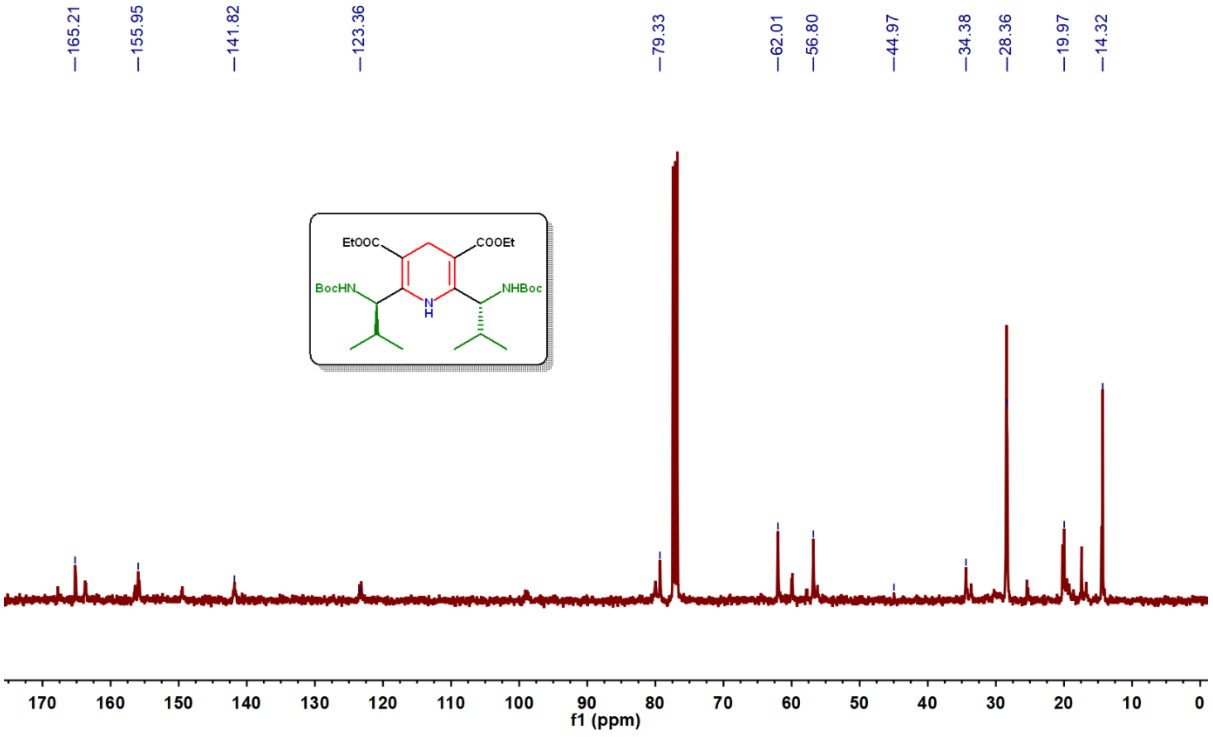
2.18
1.72
4.00
2.03
2.07
17.97
5.73
12.56
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


165.21
155.95
141.82
123.36
79.33
62.01
56.80
44.97
34.38
28.36
19.97
14.32
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COOEt
BocHN
NHBoc
N
H
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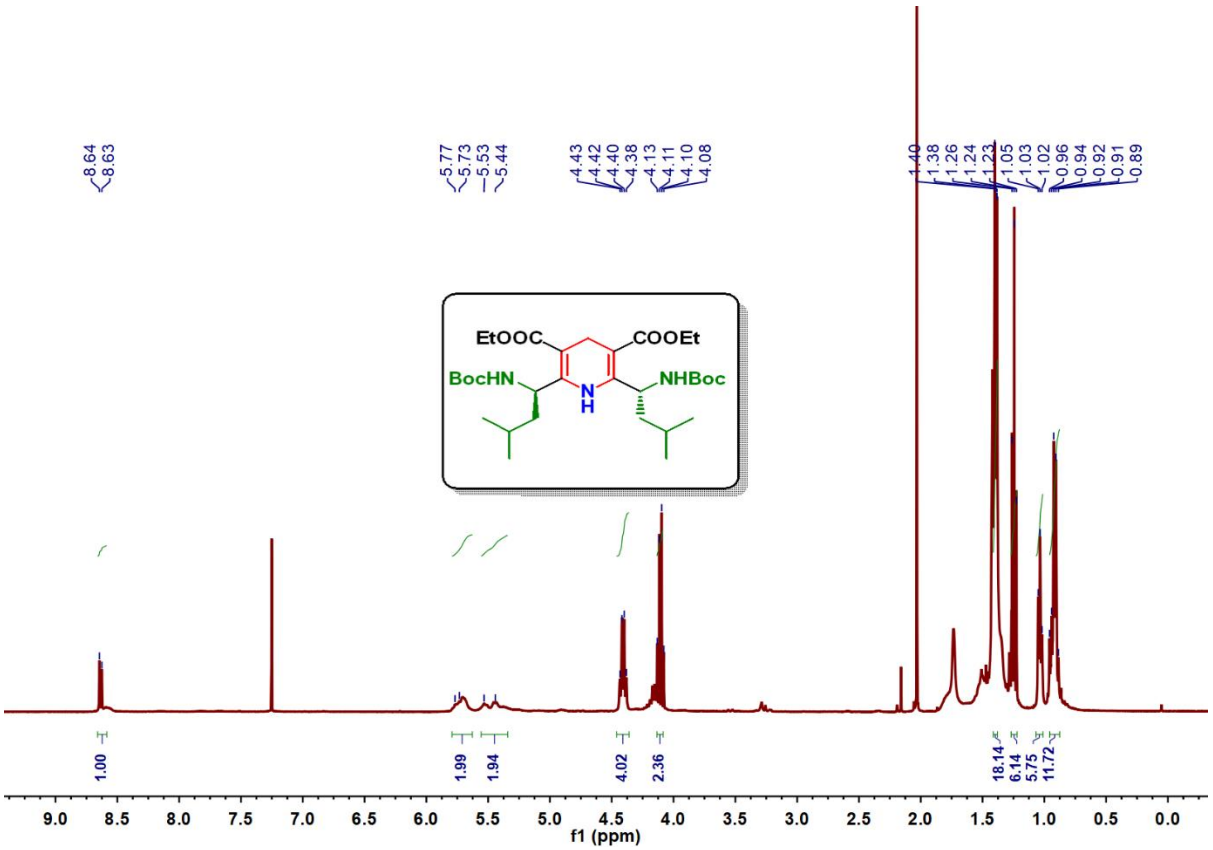
f1 (ppm)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4.42
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4.38
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4.08
1.40
1.38
1.26
1.24
1.23
1.05
1.03
1.02
0.96
0.94
0.92
0.91
0.89
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COOEt
BocHN
NHBoc
N
H
1.00
1.99
1.94
4.02
2.36
18.14
6.14
5.75
11.72
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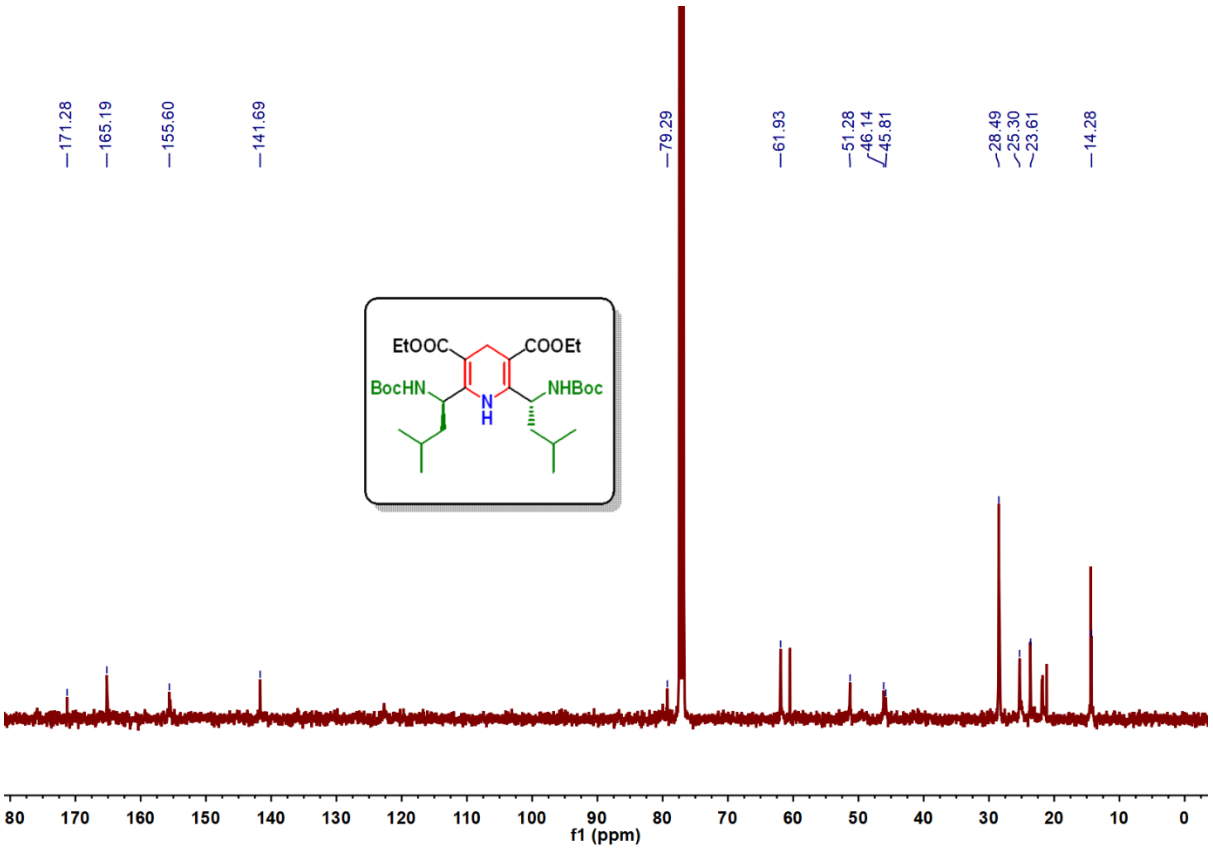
f1 (ppm)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141.69
79.29
61.93
51.28
46.14
45.81
28.49
25.30
23.61
14.28
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COOEt
BocHN
NHBoc
N
H
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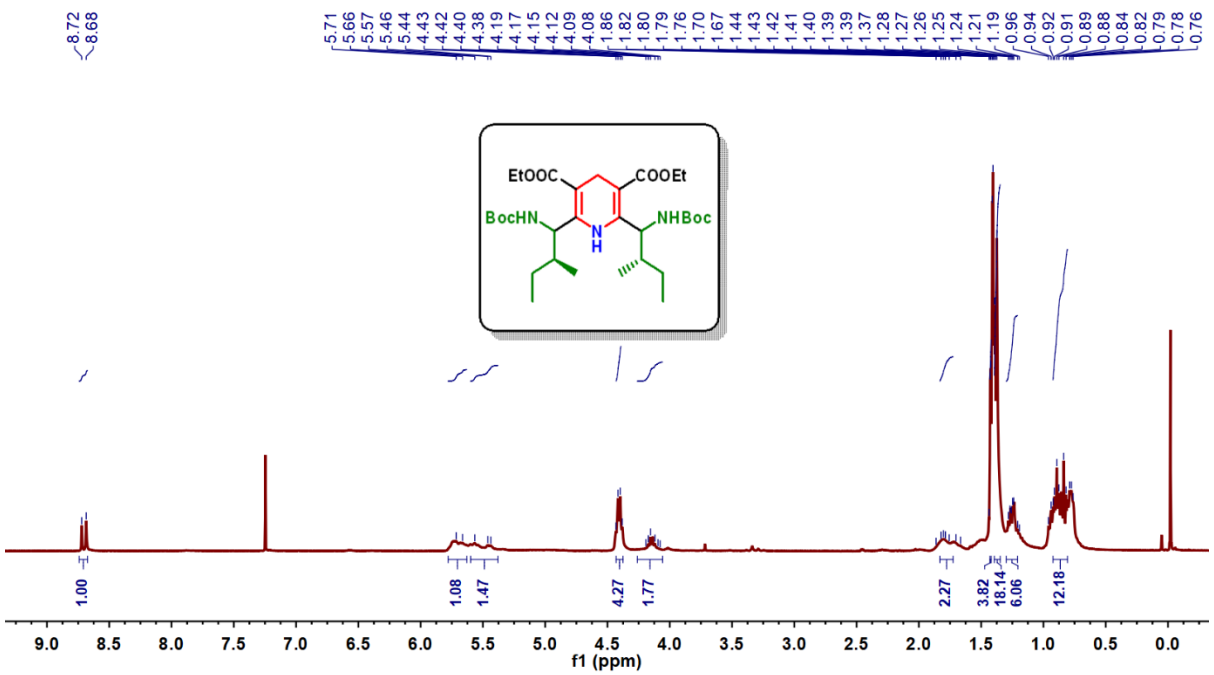
f1 (ppm)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5.44
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4.42
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4.19
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4.15
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4.09
4.08
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1.43
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1.37
1.28
1.27
1.26
1.25
1.24
1.21
1.19
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0.94
0.92
0.91
0.89
0.88
0.84
0.82
0.79
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0.76
EtOOC
COOEt
BocHN
NHBoc
N
H
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1.08
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4.27
1.77
2.27
3.82
18.14
6.06
12.18
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
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3.5
3.0
2.5
2.0
1.5
1.0
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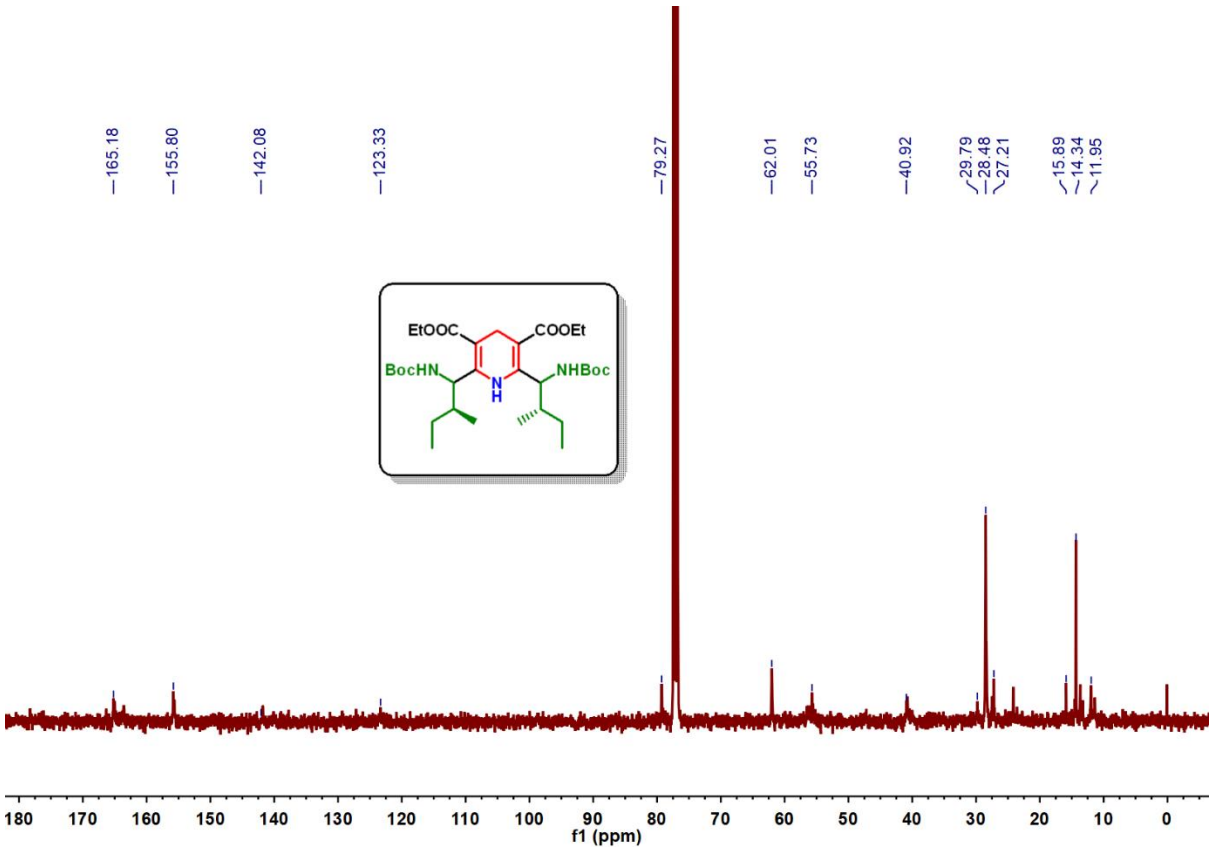
0.0
f1 (ppm)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55.73
40.92
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28.48
27.21
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14.34
11.95
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COOEt
BocHN
NHBoc
N
H
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100
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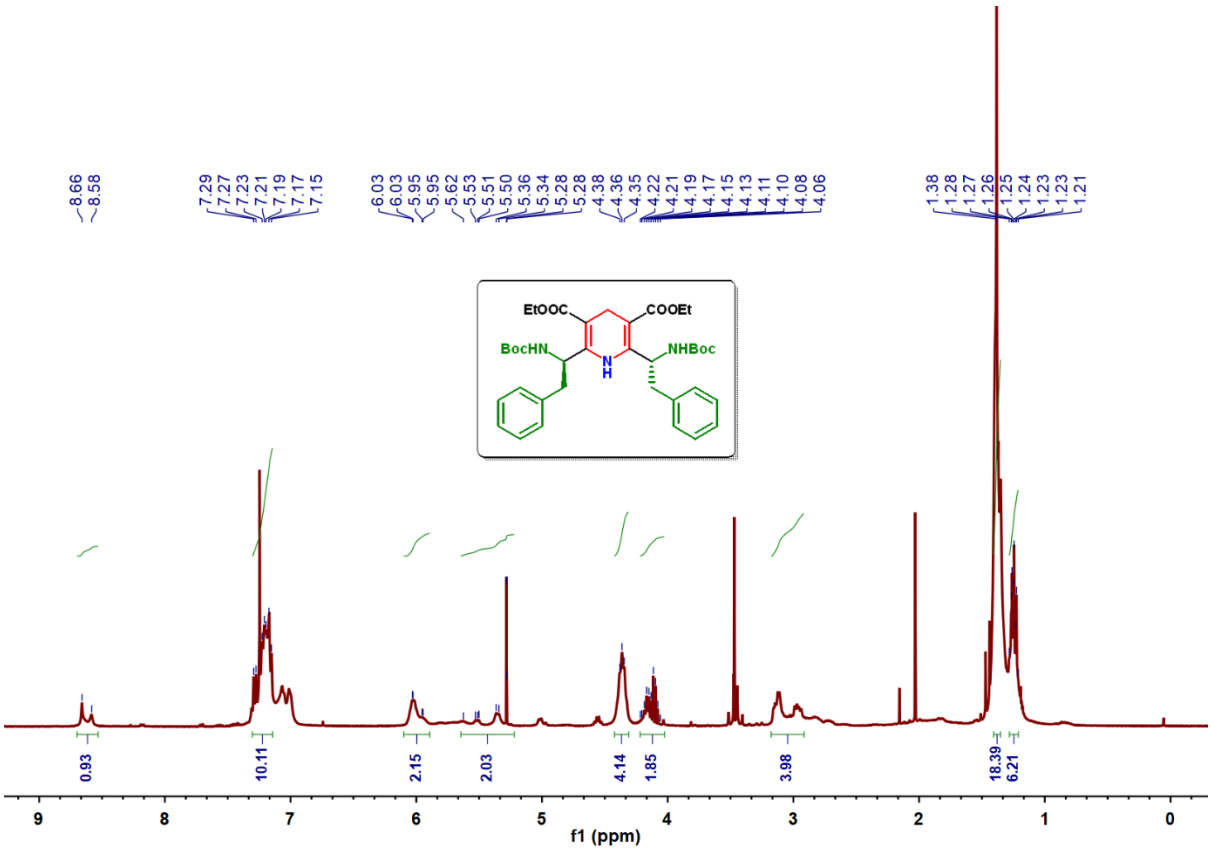
f1 (ppm)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6.03
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5.95
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5.53
5.51
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5.36
5.34
5.28
5.28
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4.21
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4.17
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1.27
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1.25
1.24
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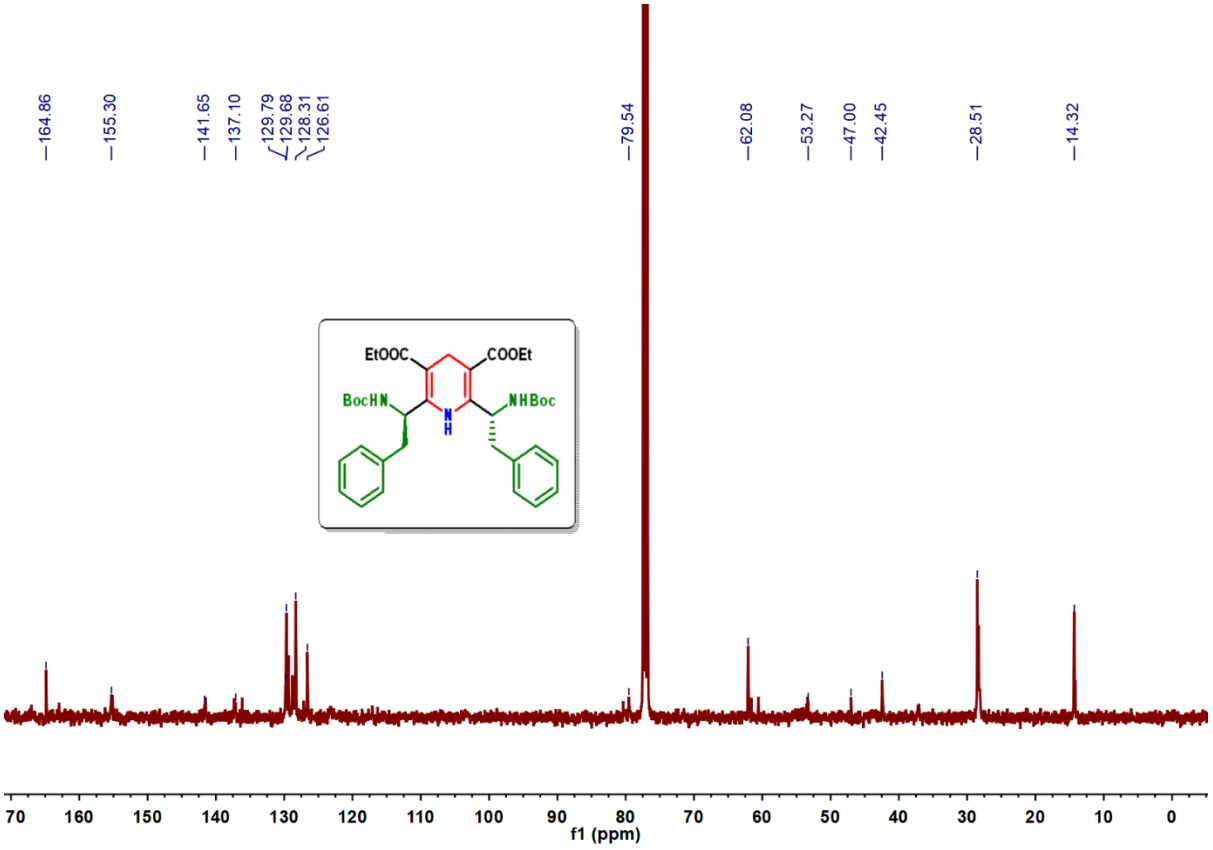
f1 (ppm)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129.79
129.68
128.31
126.61
79.54
62.08
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28.51
14.32
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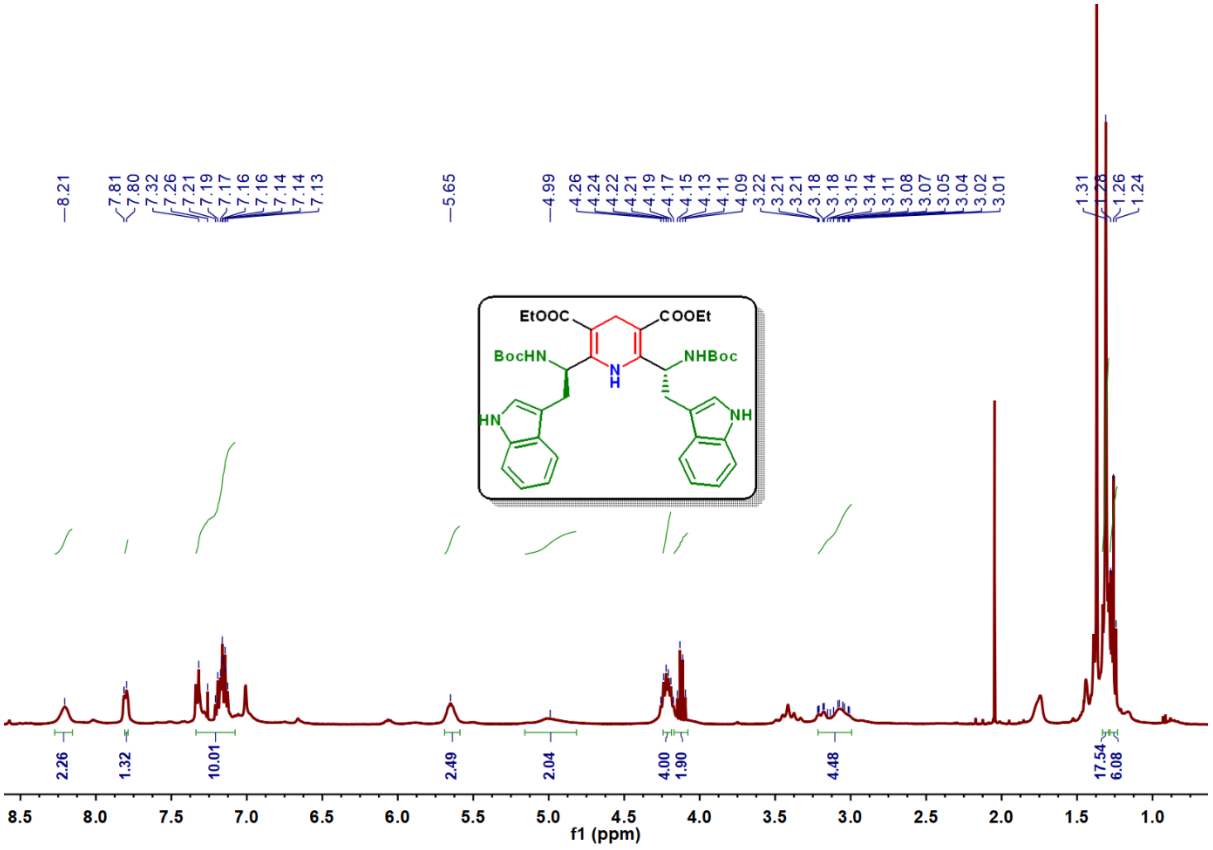
COOEt
BocHN
NHBoc
N
H
f1 (ppm)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3.14
3.11
3.08
3.07
3.05
3.04
3.02
3.01
1.31
1.28
1.26
1.24
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BocHN
NHBoc
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2.04
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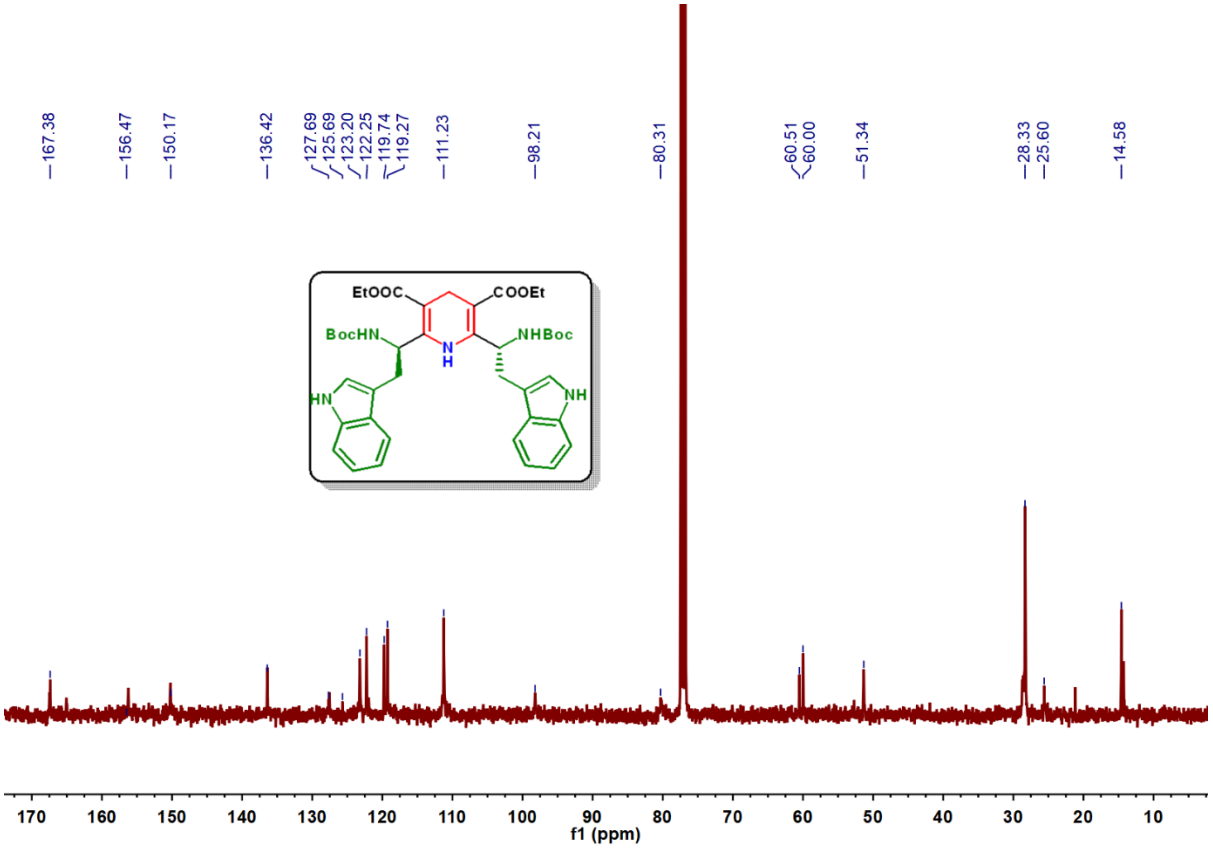
6.08
f1 (ppm)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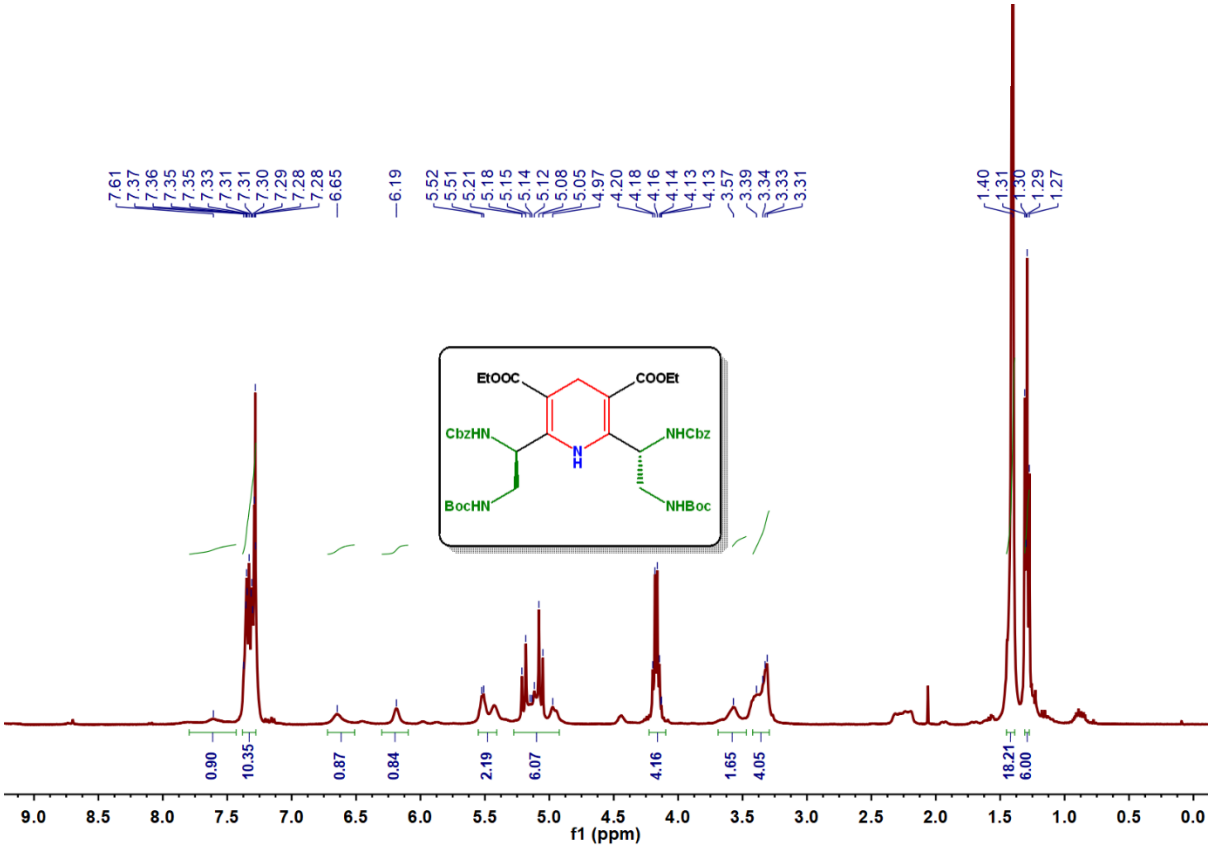
f1 (ppm)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NHBoc
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6.07
4.16
1.65
4.05
18.21
6.00
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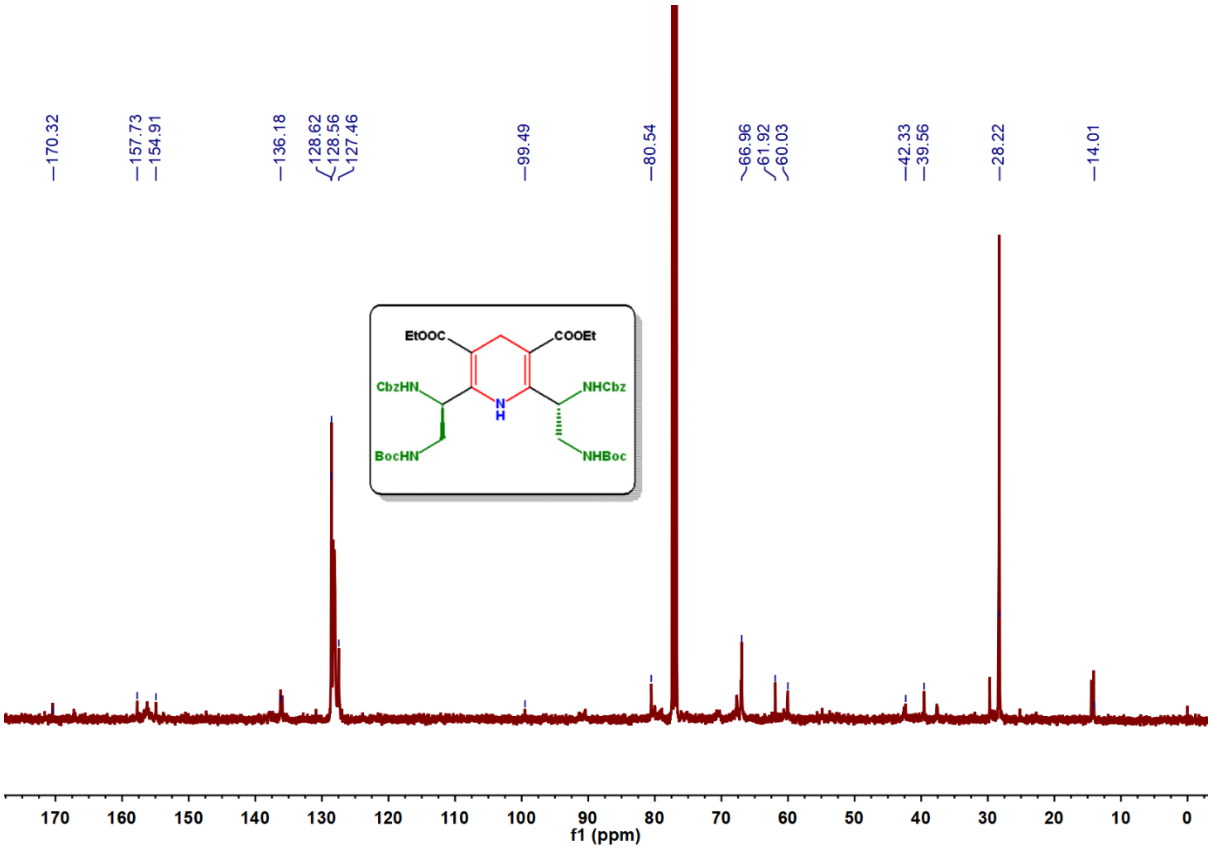
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39.56
28.22
14.01
EtOOC
COOEt
CbzHN
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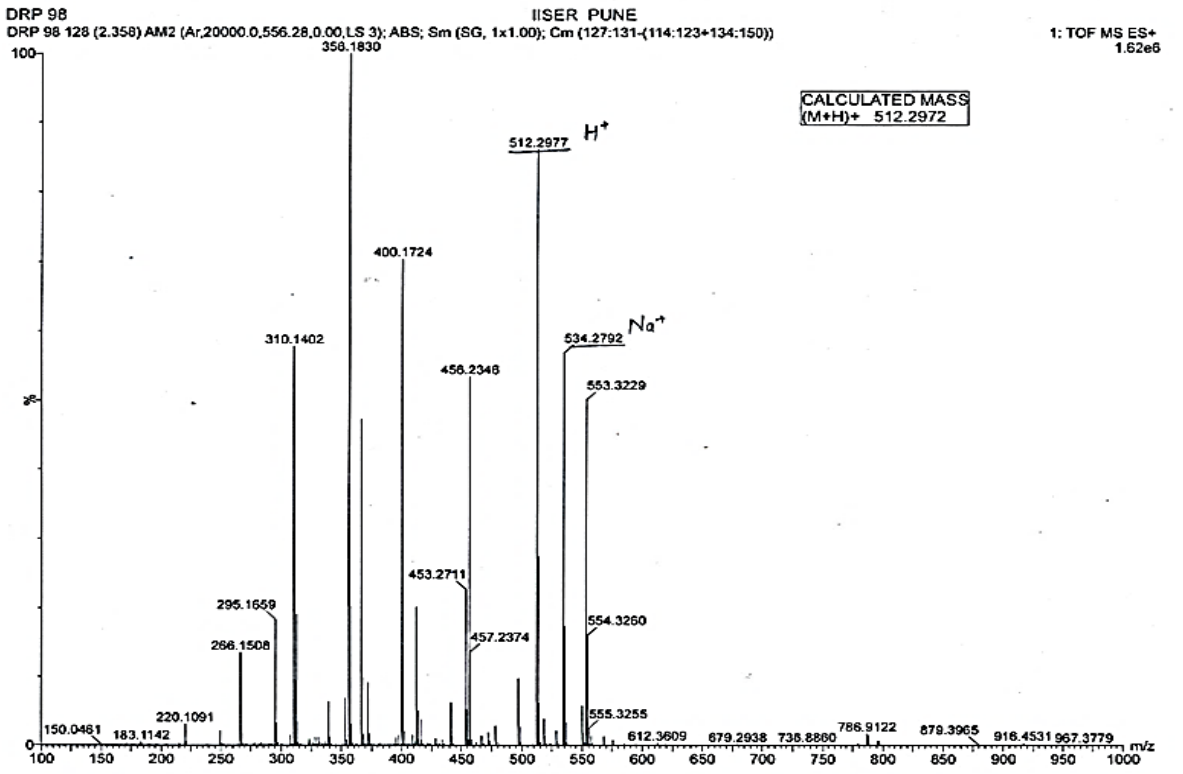
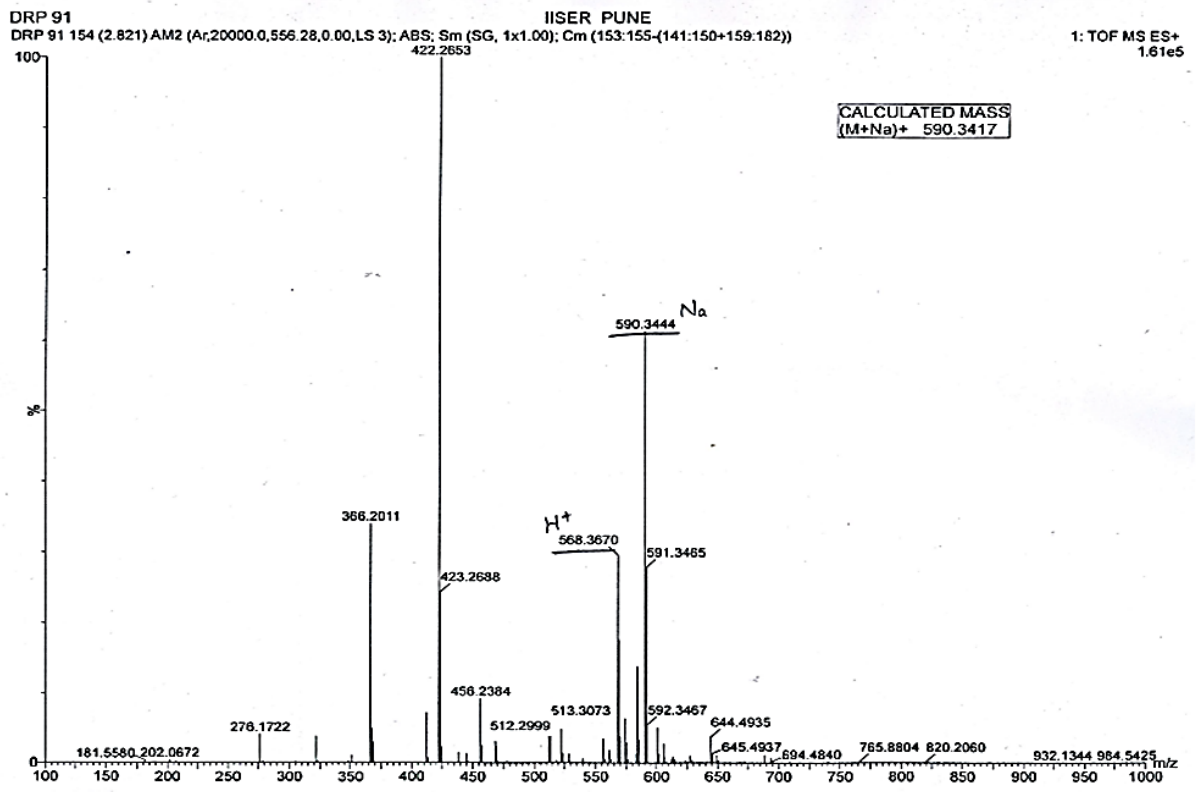
BocHN
NHBoc
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

MALDI TOF/TOF mass of compounds:

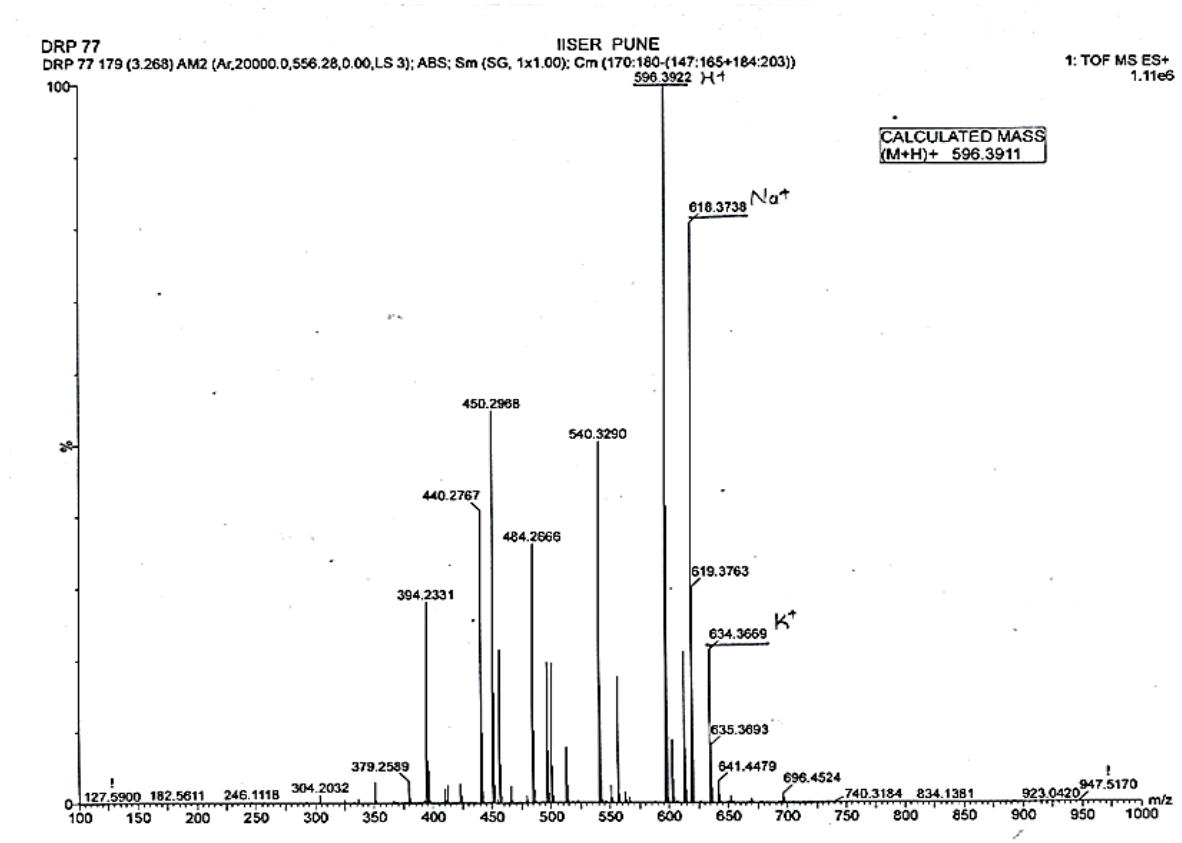
2(a)



2(b)



2(c)


DRP 77
IISER PUNE
DRP 77 179 (3.268) AM2 (Ar,20000.0,556.28,0.00,LS 3); ABS; Sm (SG, 1x1.00); Cm (170:180-(147:165+184:203))
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CALCULATED MASS
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540.3290
440.2767
484.2666
619.3763
394.2331
634.3669 K+
635.3693
379.2589
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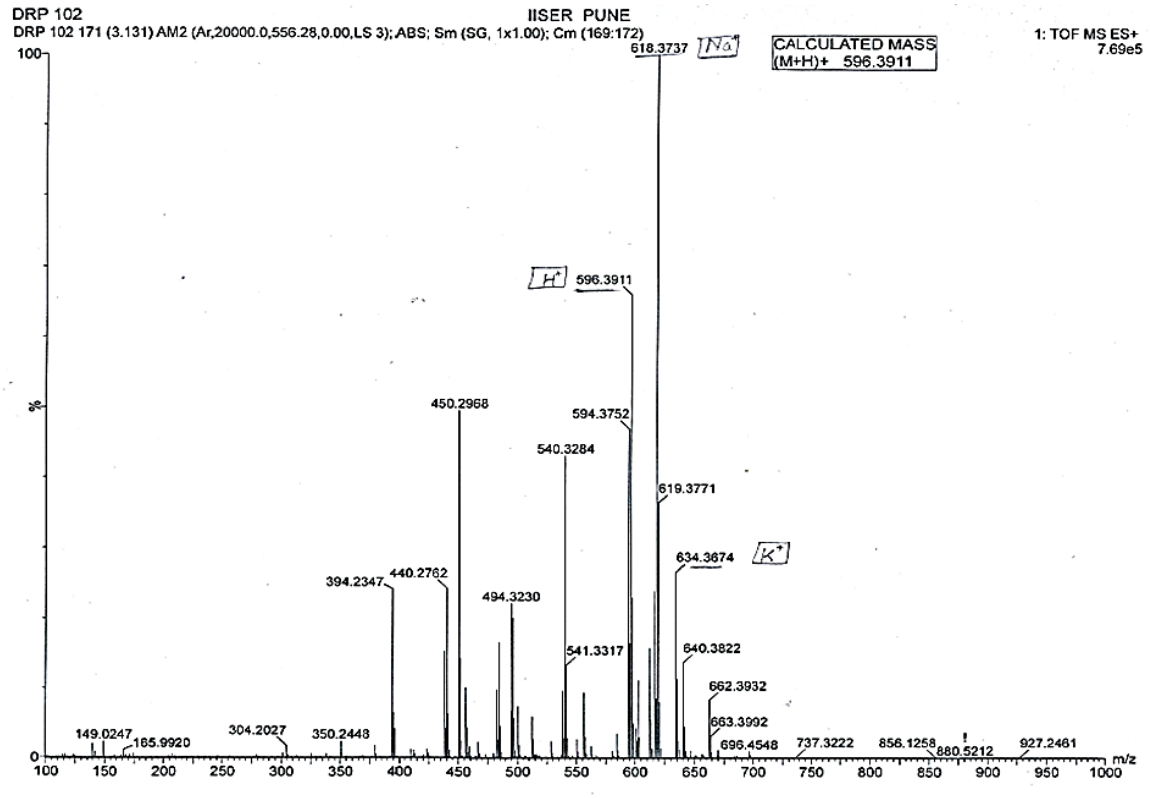
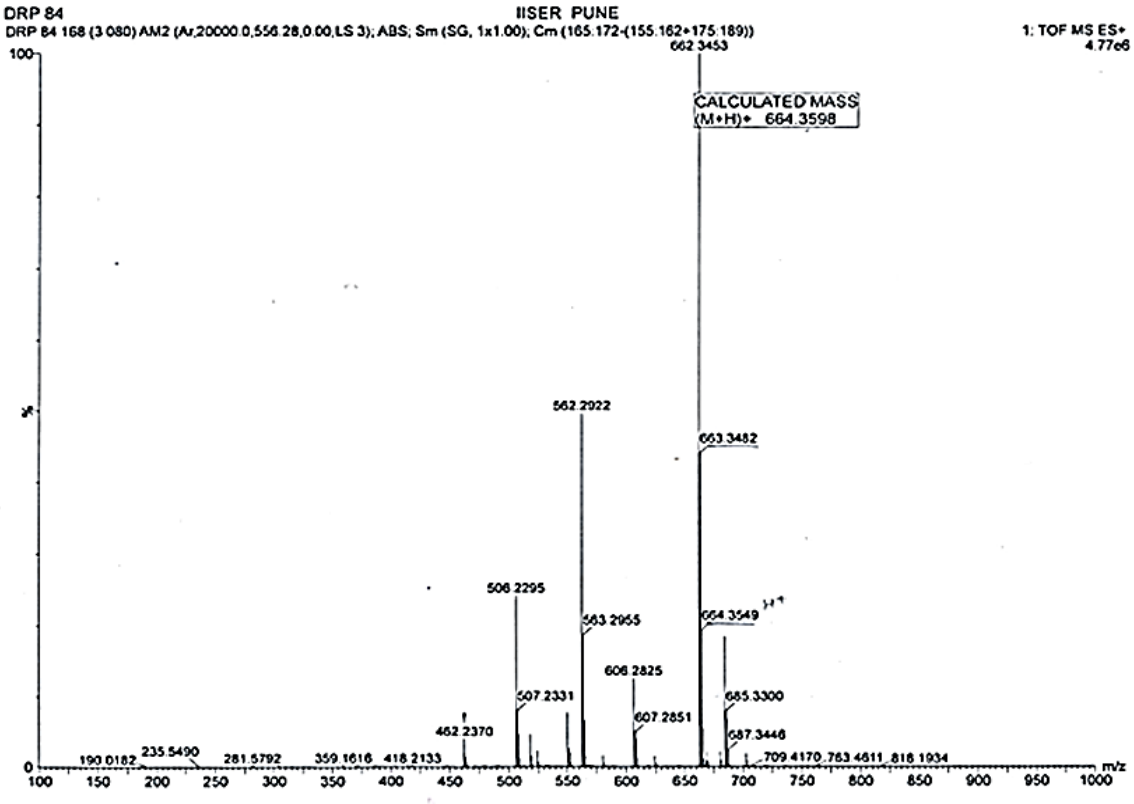
m/z

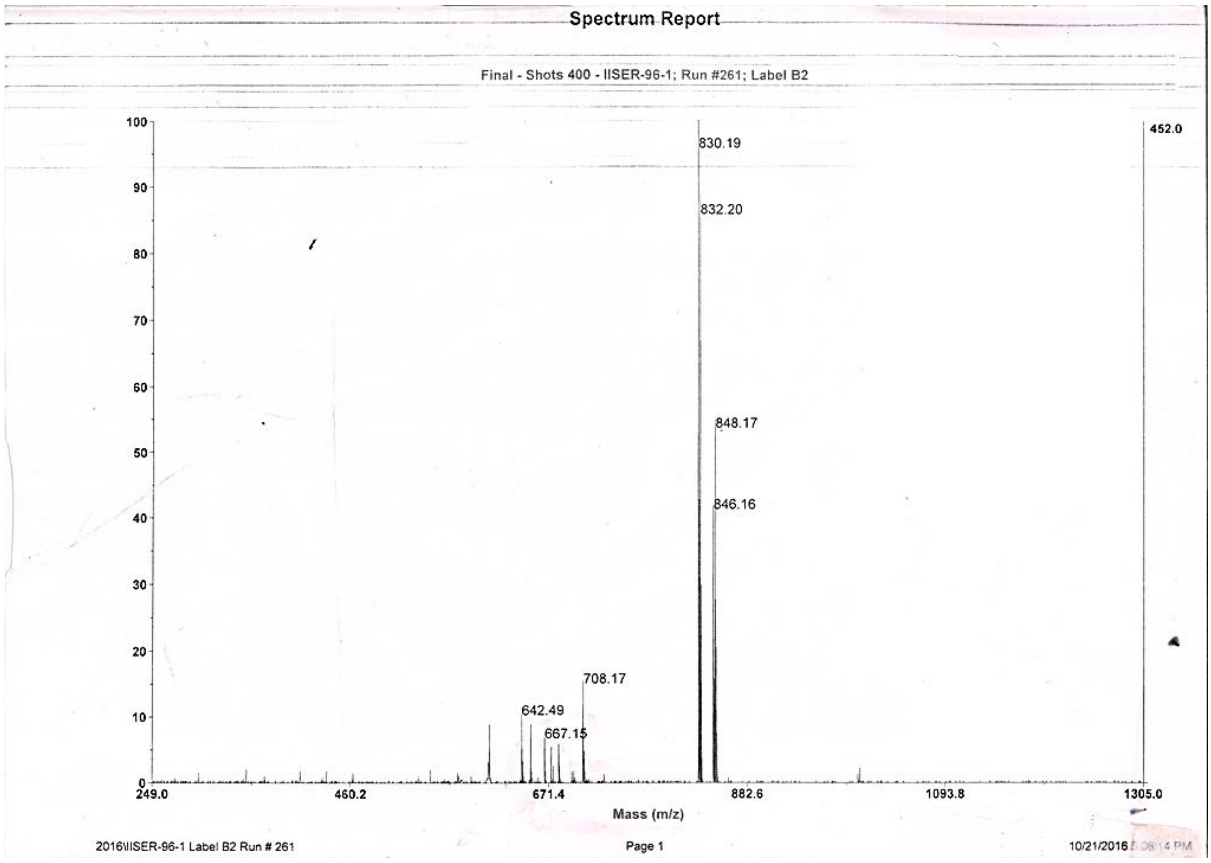
2(d)


DRP 102
IISER PUNE
DRP 102 171 (3.131) AM2 (Ar,20000.0,556.28,0.00,LS 3); ABS; Sm (SG, 1x1.00); Cm (169:172)
1: TOF MS ES+
7.69e5
618.3737 Na+
CALCULATED MASS
(M+H)+ 596.3911
H+ 596.3911
450.2968
594.3752
540.3284
619.3771
634.3674 K+
394.2347
440.2762
494.3230
541.3317
640.3822
662.3932
663.3992
149.0247
165.9920
304.2027
350.2448
696.4548
737.3222
856.1258
880.5212
927.2461
m/z

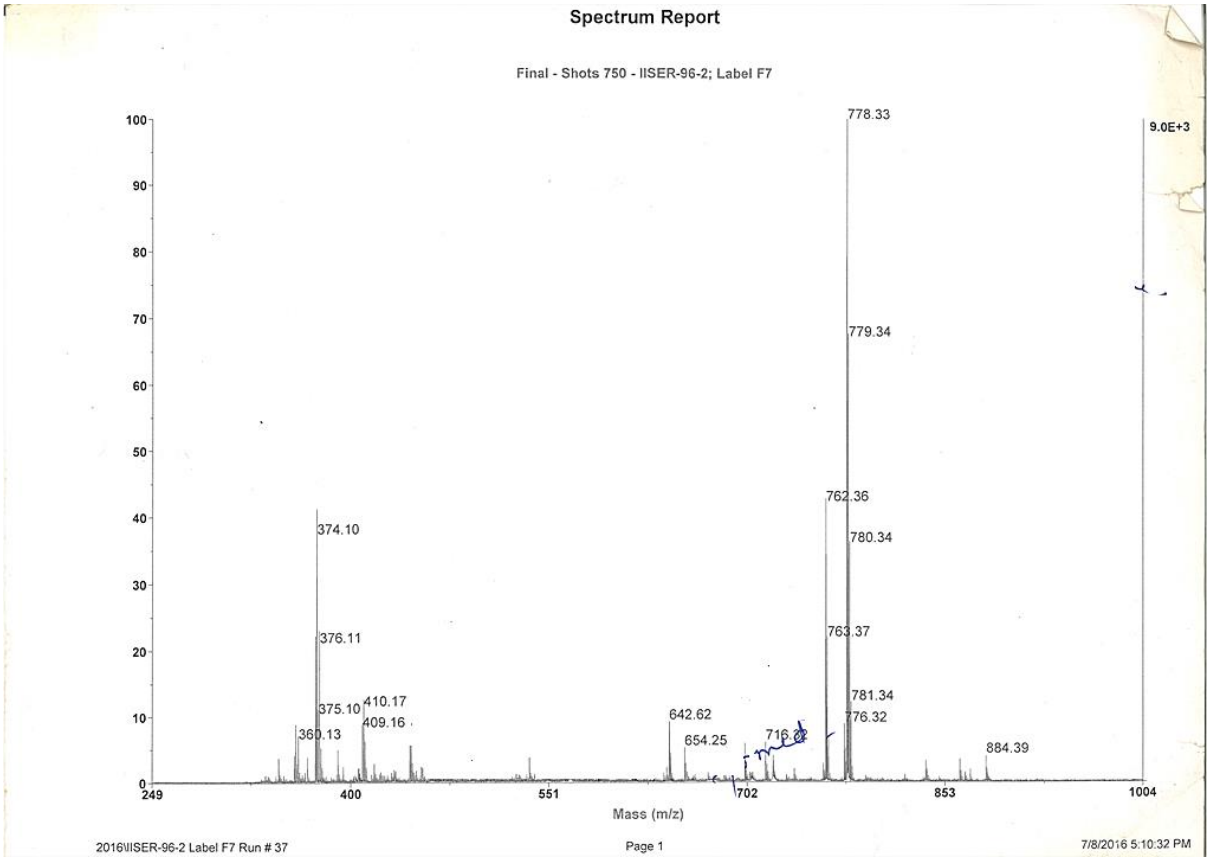
2(e)


DRP 84
IISER PUNE
DRP 84 168 (3.080) AM2 (Ar,20000.0,556.28,0.00,LS 3); ABS; Sm (SG, 1x1.00); Cm (165:172-(155:162+175:189))
1: TOF MS ES+
4.77e6
662.3453
CALCULATED MASS
(M+H)+ 664.3598
562.2922
663.3482
506.2295
563.2955
664.3549
606.2825
507.2331
607.2851
665.3300
462.2370
687.3446
190.0182
235.5490
281.5792
359.1616
418.2133
709.4170
763.4611
818.1934
m/z

2(f)


Spectrum Report
Final - Shots 400 - IISER-96-1; Run #261; Label B2
830.19
832.20
452.0
848.17
846.16
708.17
642.49
667.15
Mass (m/z)
2016\IISER-96-1 Label B2 Run # 261
Page 1
10/21/2016

2(g)


Spectrum Report
Final - Shots 750 - IISER-96-2; Label F7
100
90
80
70
60
50
40
30
20
10
0
778.33
9.0E+3
779.34
762.36
780.34
374.10
763.37
376.11
781.34
410.17
375.10
642.62
776.32
409.16
360.13
654.25
884.39
249
400
551
702
853
1004
Mass (m/z)
2016\IISER-96-2 Label F7 Run # 37
Page 1
7/8/2016 5:10:32 PM

Summary and Perspectives

In a nutshell, the work reported in this thesis demonstrates the feasibility of various types of γ-amino acids as a building block for the synthesis of peptide-foldamers and peptidomimetics. Mimicking these biologically important peptides by utilizing these modified amino acids can be beneficial due to their high proteolytic stability and hence longer serum life.

In **chapter 2a**, the synthesis of highly important β-amino alcohols has been discussed which have been shown to serve as a precursor for the synthesis of γ-amino acids discussed in further chapters. In future, β-amino alcohols can also be utilized further as a building block for the synthesis of peptaibols.

In **chapter 2b**, we have reported the helical properties of α,γ-hybrid peptides by utilizing β-hydroxy γ-amino acid (statine) with *anti* (*R,S*) conformation. These peptides have shown propensity towards β-sheet kind of structure. Based on this observation, these statine moieties can be utilized further for the synthesis of β-hairpins and sheets like structure. In future, they can be utilized to mimic biologically important β-hairpins present in nature such as Tachyplesin, Polyphemusin, *etc*.

In **chapter 3a**, we have reported the inhibitory properties of various α,γ-hybrid peptides with helical conformations containing different types of γ-amino acids against γ- secretase which is primarily responsible for Alzheimer disease. Primary studies reported in this chapter, suggest that more stable helical conformation more is the inhibition properties. In future, helical peptides can be designed using γ^4-amino acids and can be studied further for their inhibitory actions.

In **chapter 3b**, we have reported various water-soluble analogues of pepstatin A, an aspartic acid protease and studied their inhibitory actions against pepsin using MALDI-TOF/TOF, Fluorescence spectrophotometer and Isothermal Titration Calorimetry. We have also reported the relative affinity of synthesized active analogues more towards aspartic acid protease than towards other types of proteases. These analogues have not only shown inhibition against pepsin but also shown high proteolytic stability as well as solubility in water and buffers which was the major drawback associated with naturally occurring Pepstatin A. These analogues must be further analyzed for calculating kinetic parameters such as K_{cat}, K_m and K_i.

In **chapter 4a**, we have reported various α,γ- hybrid lipopeptides containing lipid chains at *N*-terminal by utilizing both the conformations of statine (*anti* and *syn*) as γ-amino acids. We have beautifully shown the effect of hydrophobicity on the antimicrobial properties of these

lipopeptides by varying the length of the fatty acids chain. We have also shown the effect of conformation of statine moiety in their antimicrobial behaviours as in each case we have seen *anti* (*R*,*S*) conformation to be more potent antibacterial and antifungal agent as compared to *syn* (*S*,*S*) conformation.

In **chapter 4b**, we have shown the design, synthesis and antimicrobial activities of structure-based cationic hybrid β-hairpin lipopeptides by utilizing α,β-unsaturated γ-amino acids as building blocks. These peptides have shown highly potent antimicrobial activity with IC_{50} as low as 0.5 µg/ml. Also, these peptides shave shown excellent activity against *Mycobacterium* strains. Hence, showing the efficacy of these hairpin lipopeptides as potent antimicrobial agents. In chapters 4a and 4b, all the reported peptides have to be studied further for their haemolytic activity with human RBCs.

In **chapter 5**, we have reported the catalyst-free synthesis and crystal structure analysis of substituted 1,4-Dihydropyridines synthesized from α-amino acids. The reported method has shown high yield percentage and compatibility with various side chains protecting groups of amino acids thus proving the efficiency of this method. In future, this method can be utilized to synthesize highly versatile heterocyclic 1,4-dihydropyridine derivatives and studied further for their biological importance and reducing properties for various organic reactions.

Thus, the simultaneous study of various γ-peptides for their conformational and biological properties can be utilized for developing biologically significant peptides, is expected to be a line of research over the next few years.