Structural studies and biological applications of α,γ-hybrid peptide helices composed of γ^4- and β-hydroxy-γ-amino acids(statins)

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

By

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Under the guidance of

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CERTIFICATE

This is to certify that this dissertation entitled "**Structural studies and biological applications of α,γ-hybrid peptide helices composed of γ- and β-hydroxy-γ-amino acids(statins)** " towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by "**Mona Manoj Katariya** at IISER Pune " under the supervision of "**Dr. Hosahudya N. Gopi**, Associate professor, Department of Chemistry, IISER Pune" during the academic year 2015-2016.

28th March 2016

Dr. Hosahudya N. Gopi

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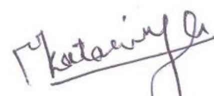
DECLARATION

I hereby declare that the matter embodied in the report entitled "**Structural studies and biological applications of α,γ-hybrid peptide helices composed of γ- and β-hydroxy-γ-amino acids(statins)** " are the results of the investigations carried out by me at the Department of Chemistry, Indian Institute of Science Education And Research, Pune, under the supervision of **Dr. Hosahudya N. Gopi** and the same has not been submitted elsewhere for any other degree.



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ABBREVIATION

ACN = Acetonitrile

Aib/U = 2-Aminoisobutyric acid

Boc = *tert*-Butoxycarbonyl

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

DCM = Dichloromethane

DIEA/DiPEA = Diisoproylethyl amine

DMF = Dimethyl formamide

EtOAc = Ethyl Acetate

Fmoc = 9-Fluorenylmethoxycarbonyl

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

HBTU = *O*-Benzotriazole-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate

HOBt = 1-Hydroxybenzotriazole

HPLC = High performance liquid chromatography

IBX = 2-iodoxybenzoic acid

NMP = *N*-methyl pyrrolidone

$SnCl_2$ = Tin chloride

TFA = Trifluoro acetic acid

THF = Tetrahydrofuran

Val/V = Valine

ABSTRACT

Recent advances in foldamers composed of non-natural β- and γ- amino acids suggested the greater structural diversity in hybrid peptides constituted with mixed αβ, αγ and βγ sequences compared to their homooligomers. The remarkable structural diversity in αβ-hybrid peptides has been exploited to design specific inhibitors for protein-protein interactions, antimicrobials and biomaterials. In comparison with αβ-hybrid peptides, αγ-hybrid peptides have not been extensively explored to design either biologically active molecules or biomaterials. In this context, we sought to investigate the folding behaviour of ααγ-hybrid peptides composed of γ- and β-hydroxy γ-amino acids (statins) and their potential antimicrobial properties. Herein, we are reporting the design, synthesis, single crystal conformations of various novel α,α,γ-peptides incorporated with γ- and statins and their potential antimicrobial properties. The single crystal conformational analysis suggests that ααγ-hybrid peptides adopt 10/12 helical conformations with or without stereochemically constrained amino acids. The helical structures of ααγ-hybrid peptides are stabilized by 4→1 intramolecular H-bonds. Instructively, 10/12-helices showed similarities in the H-bonding pattern (residue to residue) and side-chain projection with 3_{10}-helix of α-peptides and β-peptide 12-helix. Further, our studies also infer that biologically active, naturally occurring β-hydroxy γ-amino acids can be accommodated into the helix without deviating overall helical fold. In addition to the conformational analysis, we have also designed and synthesized water soluble hybrid peptides incorporated with γ^4-amino acids and investigated their potential anti-microbial properties. Results of these studies suggests that peptides incorporated with statin *anti* diastereoisomer shows better potency against various bacteria as compared to that of peptides incorporated with *syn* diastereoisomer and γ^4-amino acids.

INTRODUCTION

Proteins carry out different important functions in biological systems. They fold into complex three dimensional structures to perform these functions. Hydrogen bonding plays a crucial role in stabilizing these structures, Relationship between specific and complex higher order structure with sophisticated function has inspired researchers to ask the question that whether it is possible to design well-defined folded architectures from non-biological oligomers with specific functions. This endeavour has been elegantly demonstrated over the last two decades using non-natural oligomer constructed from β-amino acids and γ-amino acids. These β- and γ-amino acids are backbone homologated α-amino acids. In their pioneering work Dieter Seebach[1] and Samuel Gellman[2] demonstrated definite folding behaviour of β- and γ-peptides. Further, β-amino acids are classified in two β^2 and β^3 depending on position of side chain. In contrast to α-peptides, β-peptides form different types of helices. Based on the H-bonding periodicity, they classified as C_8-, C_{10}-, C_{12}-, $C_{10/12}$- and C14-helices. The subscripts refer to the number of atoms including hydrogen in closed ring formed by the hydrogen bond. In addition to the helices, these peptides also showed β-sheet like conformations.[3] Generally, β-peptide forms more stable helix than α-peptide. In the case of C_{14}-helix, the hydrogen bonds exits between the amide protons of *i* and carbonyls at *i+2* positions and the periodicity repeats approximately every 3 residues forming 14 membered rings. The C_{12}-helix of β-peptide is stabilized by hydrogen bonding between amide carbonyl group at i^{th} position and amide proton at *i+3* position. In C_{10}- and C_{14}-helices, the amide carbonyl and NH group project toward the N- and C-terminus, respectively, resulting in a net dipole reversed when compared to α-helix, whereas in C_8-, C_{12}-helices projection of the amide carbonyl and NH groups is same as that of the α-helix. Incorporation of β-amino acids into α-helices, β-sheets, β-hairpins lead to the generation of hybrid peptides with different hydrogen bonding patterns. Enormous efforts have been in the literature to design various hybrid peptides with different H-bonding periodicity. Some of the secondary structure obtained from β-peptide and α/β hybrid peptides[5] are shown in **Figure 1**.

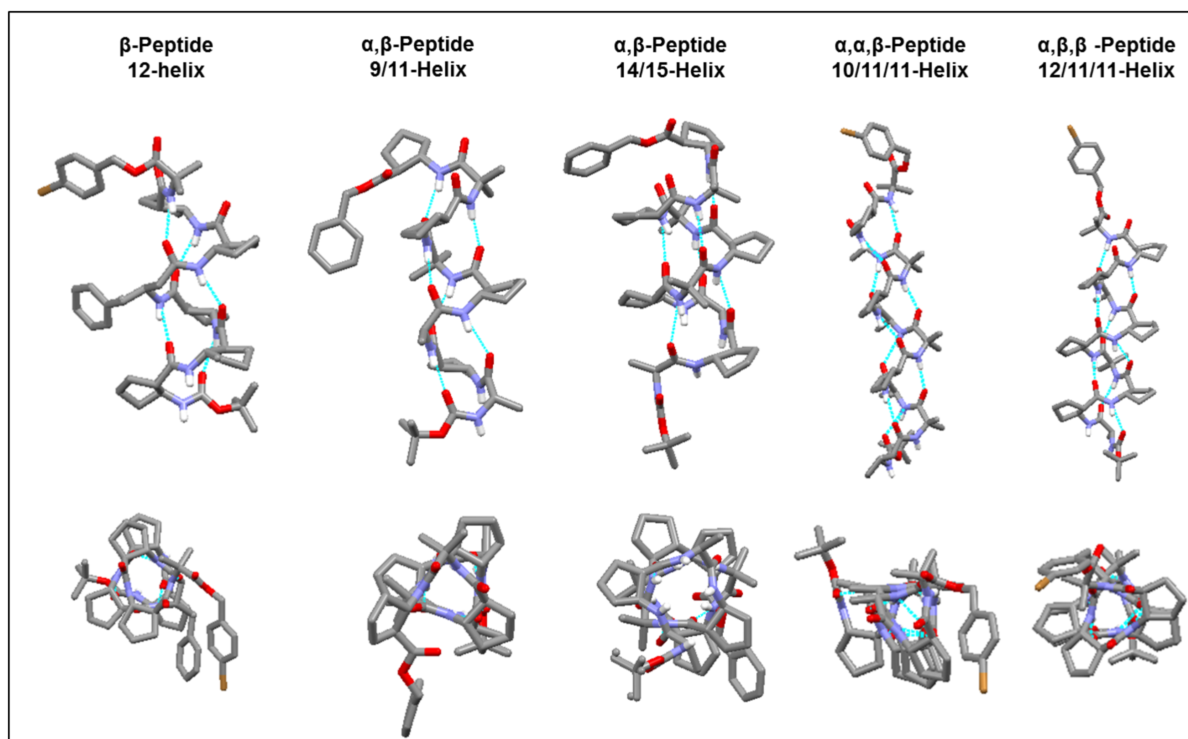


Figure 1: Secondary structures obtained from β-peptides and α,β-hybrid peptide.

Double homologation of α-amino acids are known as γ-amino acids and they are classified as γ^2, γ^3 and γ^4 depending on the position of their side chains. γ-Peptides form C_{14} helices with 2.6 residues per turn whereas α/γ-peptides show either C_{12} or combination of C_{12} and C_{10} hydrogen bonding pattern in helical peptides. α,γ-Hybrid peptide 12-helices showed the projection of side-chains at four corners of the helical cylinder. Previous studies on conformational analysis of short α,α,γ-peptides and α,γ,α-peptides composed of unnatural γ^4-amino acids revealed that side chains are projected at three faces of helical cylinder similar to 3_{10}-helix and β-peptide 12-helix suggesting that they can be used to mimic 3_{10}-Helix and β-peptide 12-helix.[6] Some of the secondary structure obtained from γ-peptides[7] and hybrid α,γ-peptides[6,8] are shown in **Figure 2**.

In contrast to α-peptides, β- and γ-peptides as well as αβ- and αγ-hybrid peptide have showed the higher proteolytic and metabolic stability.[9] These remarkable properties make hybrid peptides composed of γ-amino acids very attractive from chemical biology and medicinal chemistry. However, the progress in field of γ- and hybrid γ-peptides composed of γ^4-amino acids peptide is lagging behind as compared to the β- and hybrid β-peptides. Recently, our group strucural properties of α,γ-hybrid peptides. These 1:1 alternating hydrid peptides

spontaneously fold into 12-helical conforamtions without having any stereochemcial constraints. Inspired from the structural features of α,γ-hybrid peptides, we sought to investigate the structural features of α,α,γ-hybrid peptides composed of both γ- and as well as β-hydroxy-γ-amino acids. It is also worth investigating the hybrid peptide containing statins, since they have been widely studied for their biological properties however very little is known regarding their conformational properties.

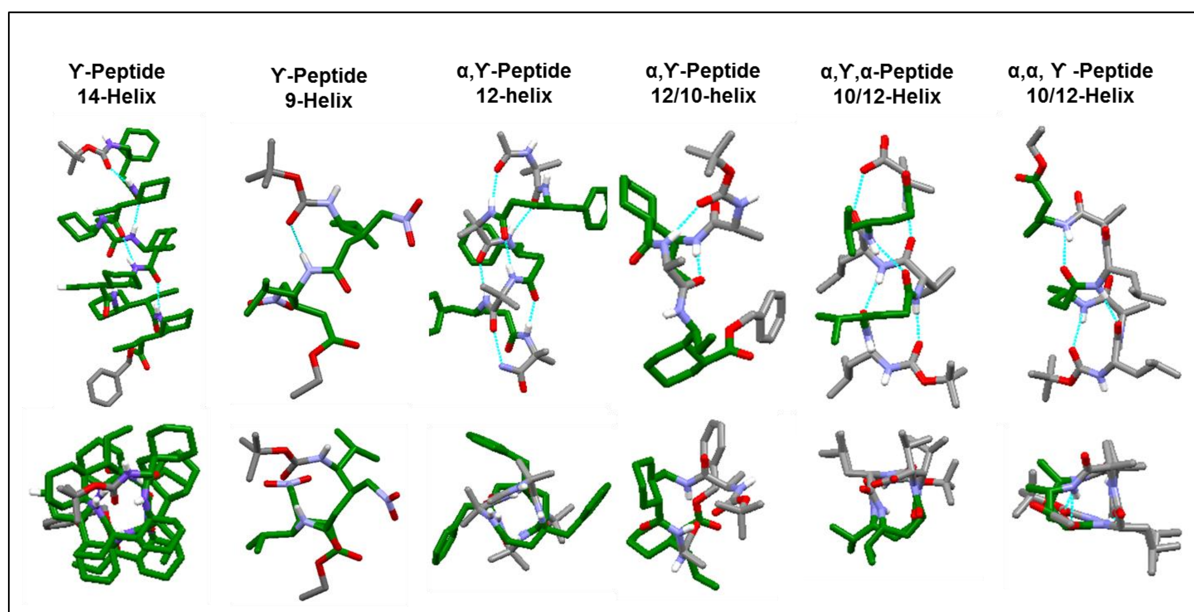


Figure 2: Secondary structures obtained from γ-peptide and hybrid α,γ-peptide. γ-Amino acids are shown in green color.

β-Hydroxy-γ-amino acids also known as statins are non-ribosomal naturally occuring gamma amino acids. Natural peptides containing statins or modified statins such as dolastatins[10] showed excellent anticancer properties showing potency against breast and liver cancers, solid tumours and leukaemia. Other statin containing peptides such as hapolosin,[11] Tamandarins,[12] didemnins[13] etc., also displayed promising anticancer properties They are widely present in saveral other natural products such as peptides used for inhibition of aspartic acid proteases. Naturally occuring pepstatin[14] has shown inhibitory action against pepsin,cathespin D and E, HIV-1 protease, Plasmepsin I and II of malarial parasite *Plasmodium falciparum*, renin etc. Some of the biologically active peptide natural products containing β-hydroxy-γ- amino acids are shown in **Figure 3**.

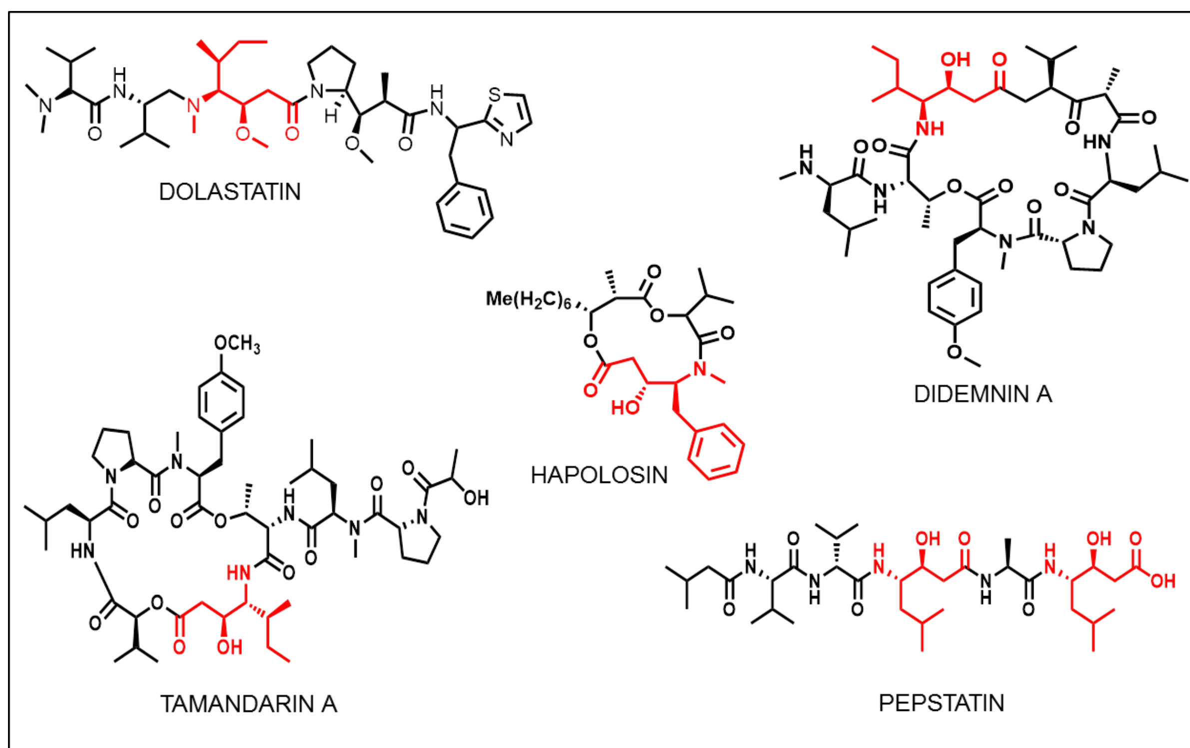


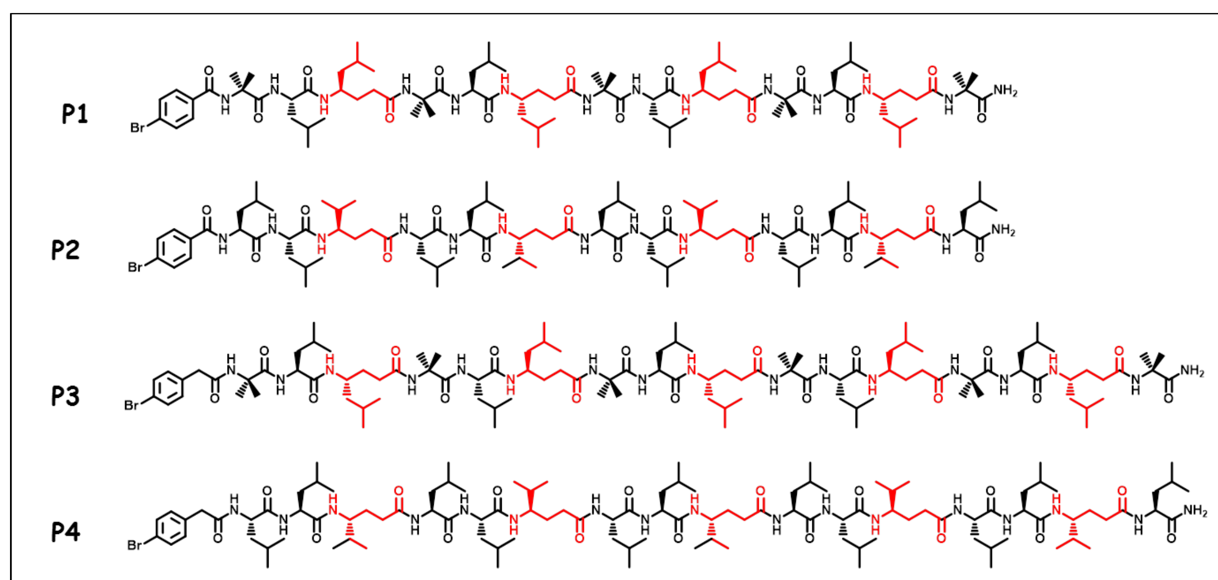
Figure 3: Naturally occuring peptides containing statins.

Encouraged by the structural features of α,γ-hybrid peptides and biological properties of statin containing peptides we also sought to tinvestigate antimicrobial properties of water soluble α,α,γ-peptides composed of non-ribosomal β-hydroxy γ-amino acids and compare them to that of water soluble α,α,γ-peptides composed of γ^4-amino acids and investigate effect of accomodation of hydroxy group at β position in γ^4-amino acids on their antimicrobial activity.

RESULTS AND DISCUSSION

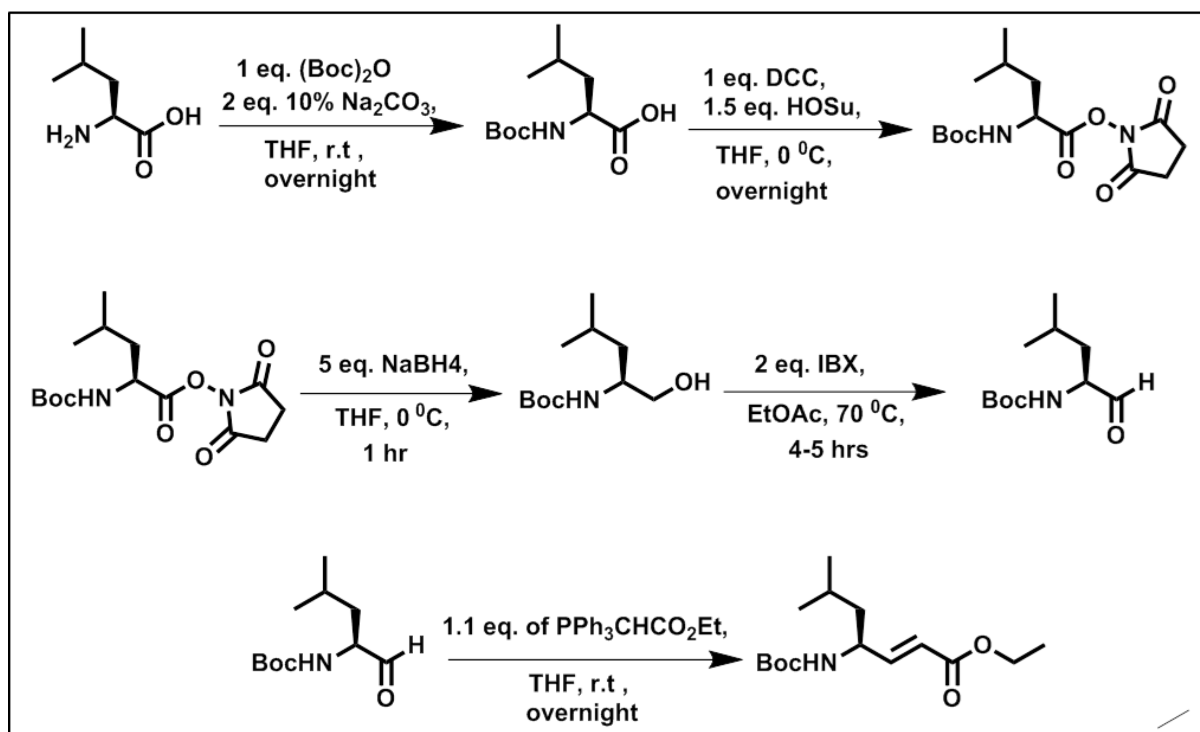
Design, synthesis and conformational analysis of α,α,γ- peptide helices

In order to understand the conformational behaviour of higher oligomers of α, α,γ- peptides, we designed four hybrid peptides composed of γ^4-amino acids. The sequences of these hybrid peptides are shown in the **Scheme 1**.



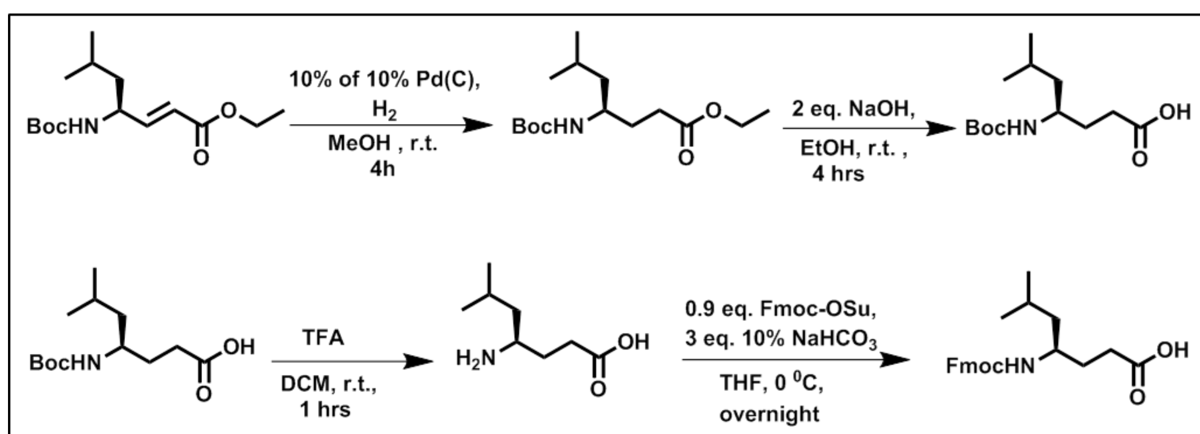
Scheme 1: Sequences of α,α,γ-peptides.

The hybrid peptides **P1** and **P3** are composed of repeated tripeptide composed of Aib, Leu and γ4-Leu residues, while **P2** and **P4** composed of tripeptide Leu, Leu and γ4-Val repeats. The **P2** and **P4** were designed to understand the whether α,α,γ- hybrid peptides require stereochemically constrained amino acids to induce the helical fold in the peptides. Required γ-amino acids were synthesized through Wittig reaction reported earlier by our group [15] using α-amino aldehydes. The synthesis of intermediate *E*-vinylogus residues is in the **Scheme 2**.



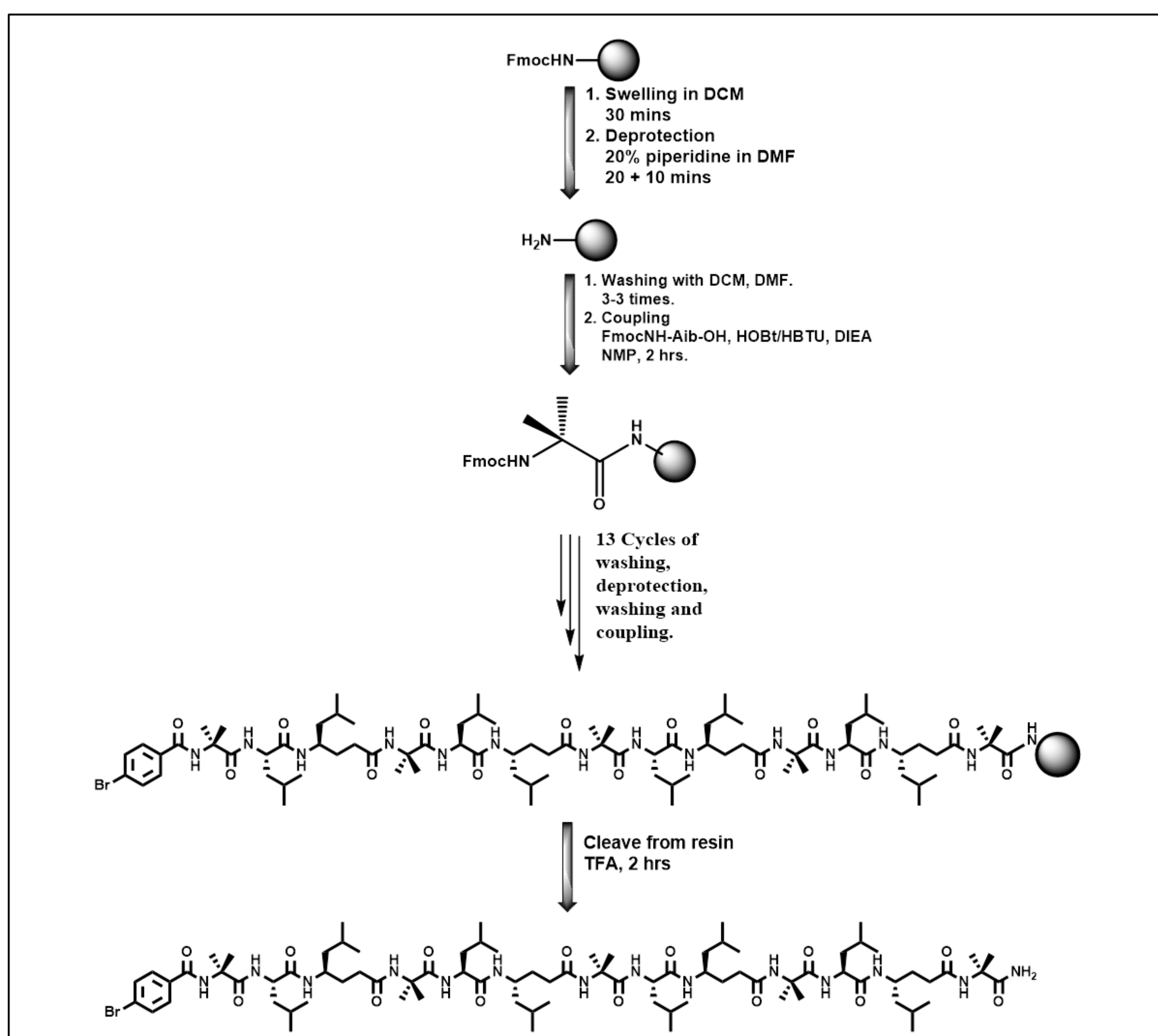
Scheme 2: synthesis of α,β-unsaturated γ^4-Lue.

The α, β- unstaturated γ-amino acid obtained after the Witting reaction was reduced to saturated γ-amino acid through catalytic hydrogenation. Solid phase compatible Fmoc-amino acids were synthesized from the C- and N-terminal free γ-amino acids obtained from the ester hydrolysis followed by Boc-deprotection. The Fmoc-group was introduced using Fmoc-OSu in the mild basic environment.[16] The schematic representation of the reaction is shown in **Scheme 3.** Similar procedure was followed for synthesis of Fmoc-protected γ^4-Val.



Scheme 3: Synthesis of Fmoc-protected γ^4-Lue.

The pure Fmoc-protected γ-amino acids obtained from **Scheme 3** are directly used to for the synthesis of peptides. All four peptides **P1, P2, P3** and **P4** were synthesized by solid phase method using HBTU/HOBt as coupling agents on Rink amide as resin using standard Fmoc- chemistry. After completion of synthesis, the N-terminal was protected with 4-bromobenzoic acid or 4-bromo phenyl acetic acid. The peptide was then cleaved from resin using pure TFA. The schematic representation of the solid phase synthesis of **P1** is shown in **Scheme 4**.



Scheme 4: Schematic representation of solid phase synthesis of **P1.**

All four peptides were insoluble in methanol, ACN and water. In order to purify peptides we centrifuged them using methanol, ACN, EtOAc and DCM as solvent followed by removal of supernatant in each case to get pure precipitated peptide. To understand the conformations of **P1, P2, P3** and **P4,** we subjected these peptides for

crystallization in various solvent combinations. The X-ray quality single crystals were obtained for **P1** and **P2** from the slow evaporation of solution of peptides in aqueous methanol/DCM (1/1) and methanol/DCM/trifluroethanol (1/1/2). The X-ray structures of **P1** peptide is shown in **Figure 4**.

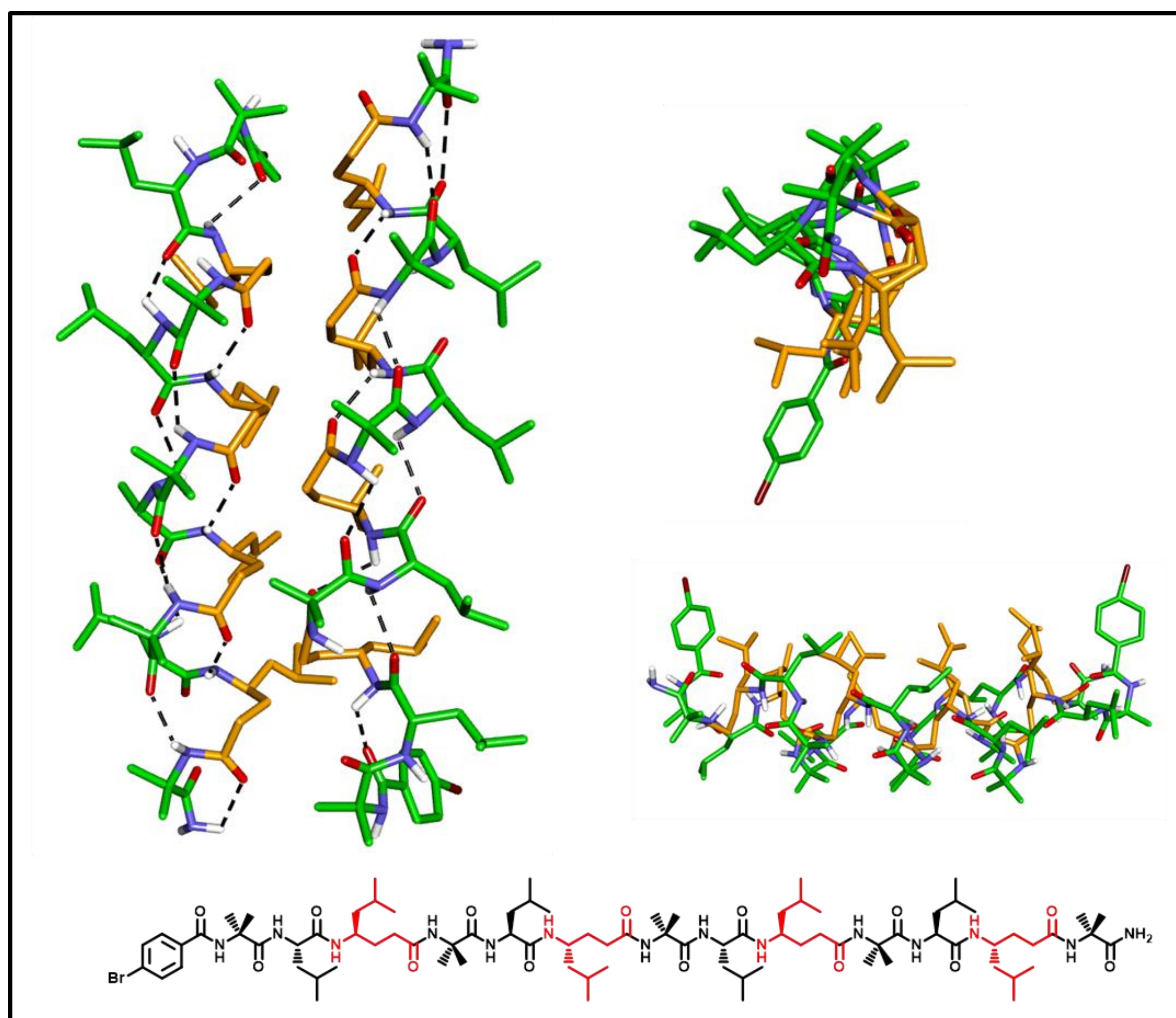


Figure 4: A. X-ray structure of **P1 B.** top view of single molecule showing distinct projection of side chains **C.** side view of **P1** showing bending of peptide. All γ^4-amino acids are shown in yellow color.

The analysis of the crystal structure of **P1** showed the presence of a dimer in which molecules are antiparallel in their asymmetric unit. As anticipated, **P1** favoured formation of 10/12-hybrid helical conformations in the single crystals. The structure is stabilized by four C_{10} and seven C_{12} intramolecular H-bonds. The top view of helix revealed the projection of side-chains along the three corners of the helical cylinders, which is very similar to 3_{10}-helix[6] and β-peptide 12-helix.[17] These results suggested that α,α,γ-hybrid peptides can be used to mimic 3_{10}-helix and β-peptide 12-helix.

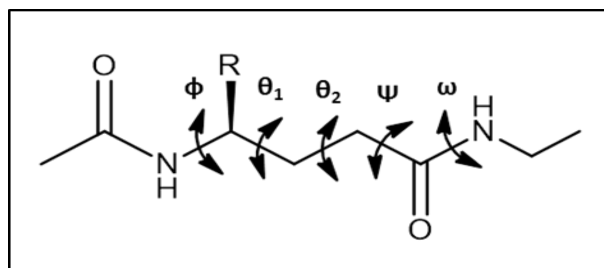


Figure 5: Torsional variables of γ^4-amino acids

Torsional angles of α-amino acids and γ^4-amino acids were measured by introducing three additional variables θ_1 (N-C_γ-C_β-C_α), θ_2 (C_γ-C_β-C_α-C') and ω(C_α-C'-N-C) along with ϕ (C'-N-C_γ-C_β) and ψ (C_β-C_α-C'-N/O) as shown in **Figure 5**. The torsion values of all residues in **P1** are given in **Table 1**.

			Molecule 1					Molecule 2		
Residue	**φ**	**θ_1**	**θ_2**	**Ψ**	**ω**	**φ**	**θ1**	**θ2**	**Ψ**	**ω**
Aib 1	**-49**	**--**	**--**	**-44**	**-179**	**-60**	**--**	**--**	**-26**	**-169**
Leu 2	**-91**	**--**	**--**	**8**	**-178**	**-72**	**--**	**--**	**-15**	**167**
Υ^4 Leu 3	**-109**	**54**	**63**	**-134**	**-169**	**-101**	**56**	**64**	**-150**	**-175**
Aib 4	**-48**	**--**	**--**	**-42**	**180**	**-58**	**--**	**--**	**-29**	**-176**
Leu 5	**-67**	**--**	**--**	**-23**	**180**	**-69**	**--**	**--**	**-20**	**-175**
Υ^4 Leu 6	**-127**	**46**	**60**	**-127**	**-164**	**-123**	**47**	**58**	**-122**	**-167**
Aib 7	**-53**	**--**	**--**	**-38**	**-175**	**-58**	**--**	**--**	**-34**	**-173**
Leu 8	**-74**	**--**	**--**	**-20**	**-174**	**-65**	**--**	**--**	**-27**	**177**
Υ^4 Leu 9	**-129**	**52**	**57**	**-122**	**-171**	**-135**	**55**	**53**	**-109**	**-168**
Aib 10	**-55**	**--**	**--**	**-41**	**-177**	**-52**	**--**	**--**	**-39**	**-173**
Leu 11	**-79**	**--**	**--**	**-7**	**179**	**-66**	**--**	**--**	**-22**	**175**
Υ^4 Leu 12	**-140**	**53**	**53**	**-114**	**-167**	**-131**	**60**	**58**	**-124**	**-177**
Aib 13	**-47**	**--**	**--**	**-50**	**--**	**-66**	**--**	**--**	**-41**	**--**

Table 1: Torsion angle values of all amino acids in **P1**.

Stereochemical analysis of all eight crystallographically characterized γ^4-Leu residues showed that they adopt the g^+-g^+ conformation about the backbone C^γ-C^β (θ_1) and C^β-C^α (θ_2) bonds with average values of torsion angles: θ_1= 53±4.74^0 and θ_2 = 58±3.98^0. The structure also showed the unusual torsional values for α-residue Leu.

In addition to distinct projection of side chains, P1 showed unusual curvature of helix probably due to the unusual torsion angles adopted by the Leu residues. In order to check whether this unusual distinct structural feature remains consistent for other α,α,γ-peptides composed of different γ4-amino acids or not, we designed **P2**

by replacing Aib and γ4-Leu with Leu and γ4-Val respectively. We designed **P3** and **P4** to check whether this unusual structural feature remains constant with length or not.

The structural analysis **P2** in single crystals showed the presence of a dimer in which molecules are parallel in their asymmetric unit. The X-ray structure of **P2** is shown in Figure 6. Similar to **P1**, **P2** also adopted 10/12-hybrid helical conformations in their single crystals. The structure is stabilized by four C_{10} and seven C_{12} intramolecular H-bonds. Similar **P1, P2** also show the curvature in the helix suggesting unique structural feature of α,α,γ-helix.

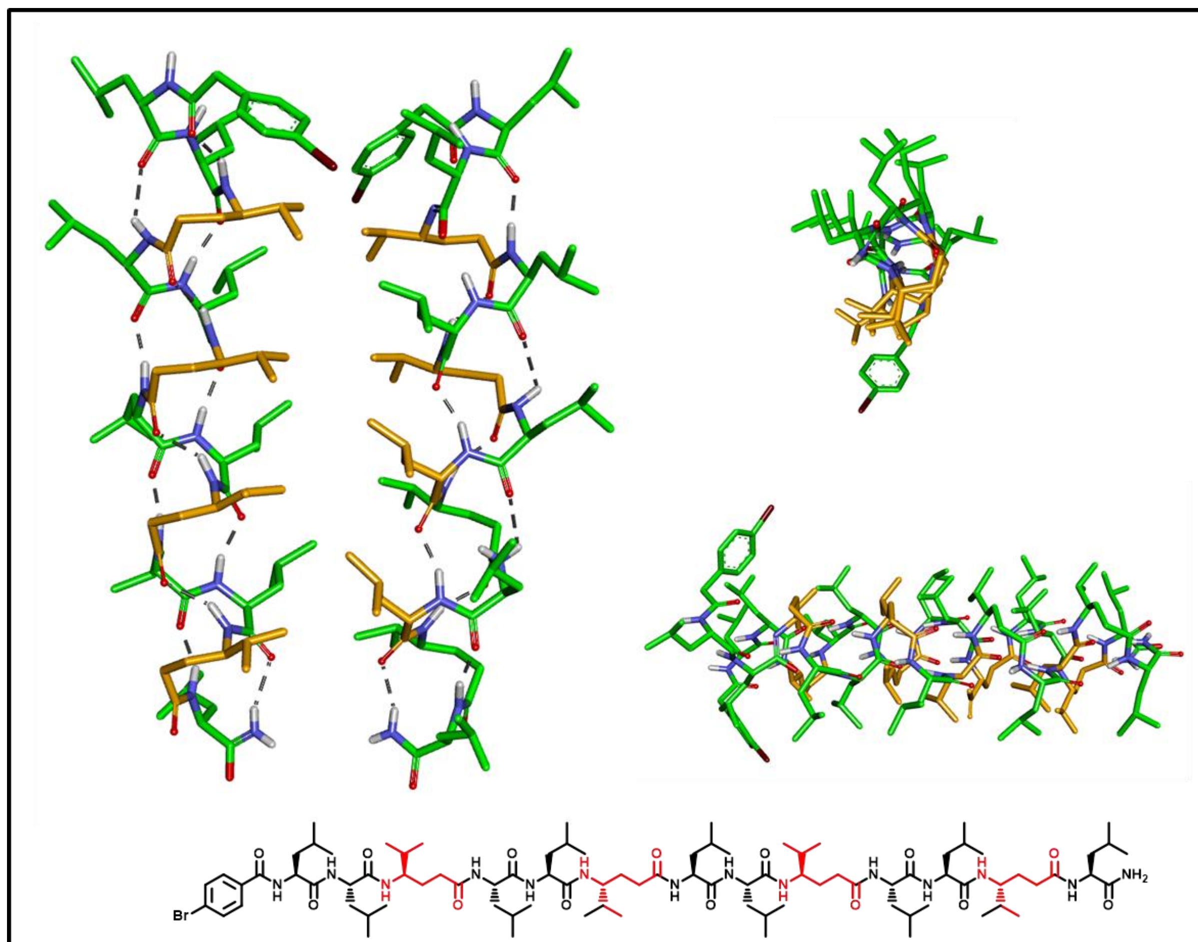


Figure 6: A. X-ray structure of **P2** **B.** top view of single molecule showing distinct projection of side chains **C.** side view of **P2** showing no bending in peptide. All γ^4-amino acids are shown in yellow color.

The torsion angle values of all residues in **P2** are given in **Table 2**. Similar to **P1**, all eight crystallographically characterized γ^4-Val residues adopt the *g*$^+$-*g*$^+$ conformation about the backbone C^γ-C^β (θ_1) and C^β-C^α (θ_2) bonds with the average torsion angle values : θ_1= 47±3.51^0 and θ_2 = 59±3.64^0. Similar to **P1**, α-Leu residues showed distinct torsional values.

			Molecule 1					Molecule 2		
Residue	**φ**	**θ_1**	**θ_2**	**Ψ**	**ω**	**φ**	**θ1**	**θ2**	**Ψ**	**ω**
Lue 1	**-61**	**--**	**--**	**-24**	**177**	**-51**	**--**	**--**	**-42**	**-175**
Leu 2	**-73**	**--**	**--**	**-18**	**178**	**-73**	**--**	**--**	**-19**	**-178**
γ^4 Val 3	**-125**	**45**	**60**	**-123**	**-173**	**-130**	**50**	**56**	**-122**	**-163**
Leu 4	**-50**	**--**	**--**	**-44**	**-176**	**-69**	**--**	**--**	**-23**	**177**
Leu 5	**-69**	**--**	**--**	**-23**	**-179**	**-75**	**--**	**--**	**-18**	**180**
γ^4 Val 6	**-123**	**44**	**64**	**-131**	**-167**	**-130**	**50**	**57**	**-115**	**-174**
Leu 7	**-62**	**--**	**--**	**-29**	**177**	**-50**	**--**	**--**	**-46**	**-173**
Leu 8	**-71**	**--**	**--**	**-21**	**-178**	**-68**	**--**	**--**	**-24**	**-177**
γ^4 Val 9	**-120**	**42**	**66**	**-132**	**-163**	**-124**	**45**	**58**	**-121**	**-165**
Leu 10	**-67**	**--**	**--**	**-24**	**179**	**-59**	**--**	**--**	**-37**	**-175**
Leu 11	**-76**	**--**	**--**	**-13**	**178**	**-76**	**--**	**--**	**-9**	**174**
γ^4 Val 12	**-135**	**51**	**56**	**-111**	**-175**	**-136**	**50**	**58**	**-106**	**-175**
Leu 13	**-61**	**--**	**--**	**-42**	**--**	**-65**	**--**	**--**	**-38**	**--**

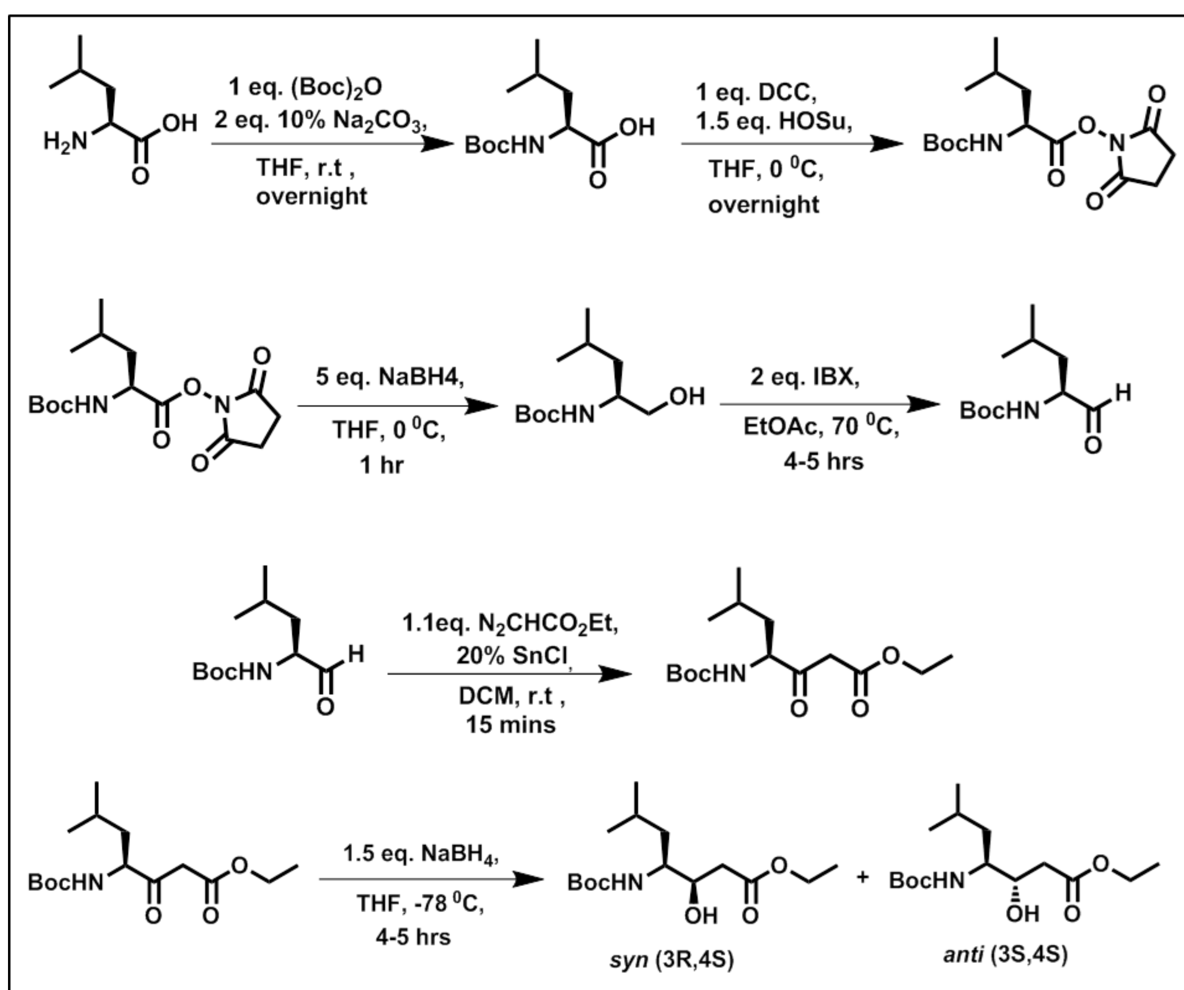
Table 2 : Torsion angle values of all amino acids in **P2.**

These helical structures of α,α,γ -hybrid peptides showed 10/12 H-bonding pattern which is different than H-bonding pattern for helices with $-(\alpha,\gamma,\alpha)_n-$ sequences reported by Balaram and colleagues.[8] However, H-bonding pattern can be tuned by varying the length of peptide.

Synthesis of β-hydroxy γ-amino acids

Further, we investigated the structural properties of α,α,γ-hybrid peptides composed of β-hydroxy α-amino acids. We recently reported synthesis of β-hydroxy-γ-amino esters starting from β-keto-γ-amino esters using $NaBH_4$ as reducing agent. We utilize the same strategy for the synthesis of β-hydroxy-γ-amino esters. The β-keto-γ-amino esters were synthesized from α-amino aldehyde and ethyl diazoacetate using tin(II)chloride as catalyst.[18] The amino aldehydes were obtained from the oxidation of corresponding amino alcohols using IBX. The amino alcohols

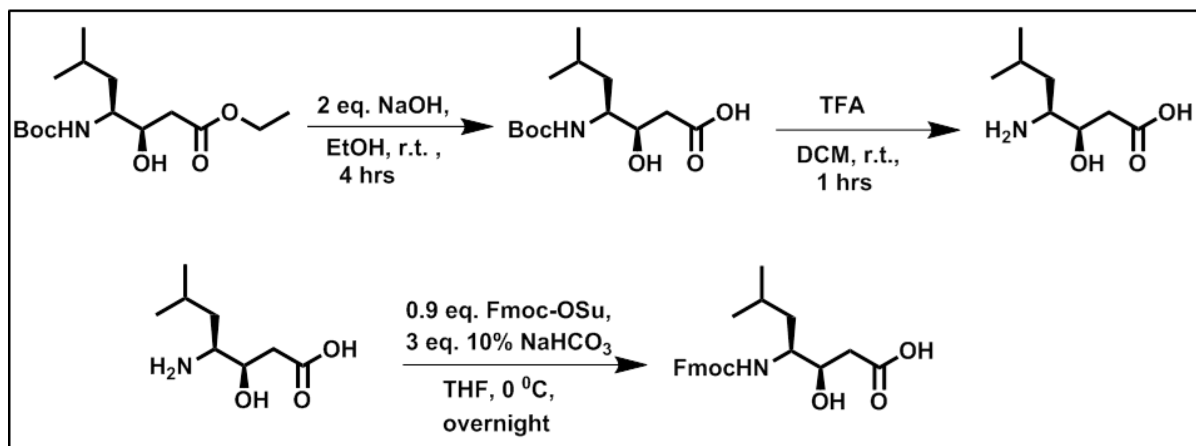
were synthesized through the mild $NaBH_4$ reduction of mixed anhydrides generated from the reaction between protected amino acids and HOSu/DCC. After obtaining β-keto-γ-amino esters, we subjected them to mild reduction using $NaBH_4$ reported by Gopi et al.[19] The schematic representation of the reaction is shown in **Scheme 5**. The mild reduction of β-keto-γ-amino esters gives the *syn* (β-hydroxyl group is *syn* with respect to amino acid side chain) stereoisomer as a major product and *anti* (the β-hydroxyl group is anti with respect to the amino acid side-chain) stereoisomer as a minor product.[19] The *anti* and *syn* diastereoisomers were separated using column chromatography.



Scheme 5: synthesis of Boc-protected-β-hydroxy-γ-luecine ethyl ester.

Solid phase compatible Fmoc-amino acids were synthesized from the N- and C-terminal free β-hydroxy-γ-amino acids obtained from the ester hydrolysis followed by Boc-deprotection. The Fmoc-group was introduced using Fmoc-OSu in the mild

basic environment. The schematic representation of the reaction is shown in **Scheme 6.** Similar procedure was followed for *anti*-isomer.

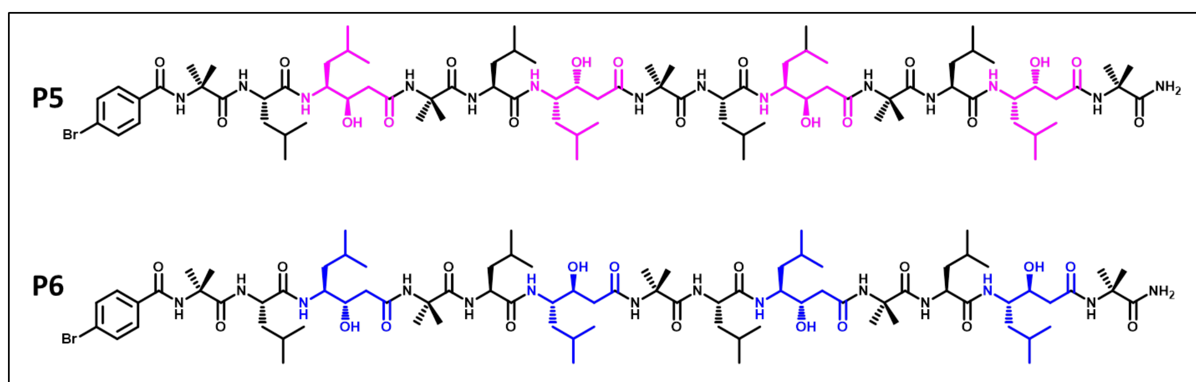


Scheme 6: Synthesis of Fmoc- protected β-hydroxy-γ-Leucine

Design, synthesis and conformational analysis of α,α,γ- peptide helices

Synthesis of hydrophobic sequences for structural analysis:

In order to understand the conformational behaviour of β-hydroxy-γ-amino acids in α,α,γ- peptide sequences. We designed **P5** and **P6** hybrid peptides composed of alternating α- and β-hydroxy-γ-amino acids in 2:1 ratio. The sequences of these hybrid peptides are shown in the **Scheme 7.**



Scheme 7: Design of hydrophobic α,α,γ-peptides composed of alternating Aib, Leu and β-hydroxy-γ-leucine.

Hybrid peptides **P5** and **P6** are composed of alternating Aib, Leu and β-hydroxy-γ-Leu residues (*anti* and *syn* isomer respectively). Both **P5** and **P6** were synthesized by solid phase method using HBTU/HOBt as coupling agents on Rink amide as resin using standard Fmoc- chemistry. After completion of synthesis, the *N*-terminal was protected with 4-bromobenzoic acid and 4-bromo phenyl acetic acid respectively. The peptide was then cleaved from resin using pure TFA. Both the peptides were insoluble in methanol, ACN and water. In order to purify peptides we centrifuged them using methanol, ACN, EtOAc and DCM as solvent followed by removal of supernatant in each case to get pure precipitated peptide. To understand the conformations of **P5** and **P6,** we subjected these peptides for crystallization in various solvent combinations. The X-ray quality single crystals were obtained for **P5** from the slow evaporation peptide in aqueous methanol: DCM (1:1) solution. The X-ray structures of **P5** peptide is shown in **Figure 7**.

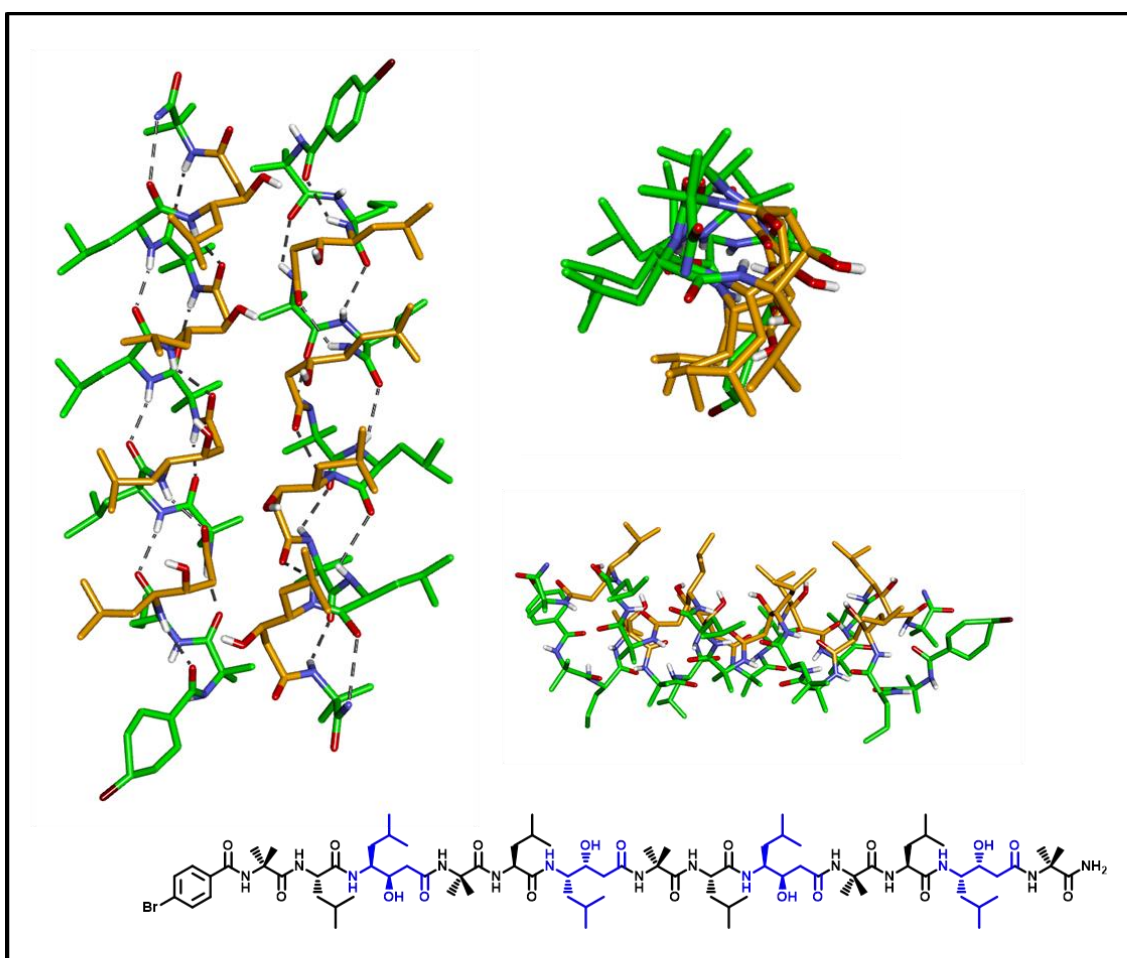


Figure 7: A. X-ray structure of **P5** **B.** top view of single molecule showing distinct projection of side chains **C.** side view of **P5** showing bending in peptide. All β-hydroxy-γ-amino acids are shown in yellow color.

Peptide **P5** adopted 10/12-helix conformation similar to **P1** and **P3**. Another interesting feature is that the peptide favoured formation of intramolecular H-bonding between hydroxyl group of statin residue and carbonyl of the same statin residue giving six-membered ring. The insertion of *syn* hydroxyl group (with respect to the side-chain) did not affect the overall folding of the peptide, suggesting that small functional groups can be accommodated at the β-position of γ-amino acids without deviating the overall helical fold of the molecule. The *β*-hydroxyl group of *syn*-(*3R, 4S*) *β*-hydroxy-γ-Leu is pointed towards C-terminus of the helix similar to the amide CO groups. Side-view of **P5** showed backbone bending similar to **P1**. The torsion angle values of all residues in **P5** are given in **Table 3**.

			Molecule 1					Molecule 2		
Residue	**φ**	**θ1**	**θ2**	**Ψ**	**ω**	**φ**	**θ1**	**θ2**	**Ψ**	**ω**
Aib 1	**-58**	**--**	**--**	**-40**	**-168**	**-52**	**--**	**--**	**-37**	**-177**
Leu 2	**-83**	**--**	**--**	**-18**	**-174**	**-71**	**--**	**--**	**-19**	**-178**
Leusta 3	**-124**	**46**	**57**	**-123**	**-168**	**-96**	**38**	**55**	**-122**	**-164**
Aib 4	**-51**	**--**	**--**	**-43**	**-170**	**-44**	**--**	**--**	**-39**	**-170**
Leu 5	**-80**	**--**	**--**	**-12**	**-178**	**-81**	**--**	**--**	**-14**	**-176**
Leusta 6	**-126**	**38**	**57**	**-114**	**-169**	**-120**	**40**	**60**	**-119**	**-168**
Aib 7	**-43**	**--**	**--**	**-39**	**-173**	**-50**	**--**	**--**	**-41**	**-172**
Leu 8	**-57**	**--**	**--**	**-36**	**-162**	**-66**	**--**	**--**	**-33**	**-178**
Leusta 9	**-133**	**51**	**55**	**-122**	**-168**	**-117**	**53**	**55**	**-126**	**-164**
Aib 10	**-49**	**--**	**--**	**-41**	**-173**	**-62**	**--**	**--**	**-32**	**-178**
Leu 11	**-76**	**--**	**--**	**-11**	**179**	**-80**	**--**	**--**	**-7**	**180**
Leusta 12	**-125**	**43**	**60**	**-107**	**-170**	**-126**	**47**	**55**	**-119**	**-173**
Aib 13	**-52**	**--**	**--**	**-63**	**--**	**-55**	**--**	**--**	**-55**	**--**

Table 3: Torsion angle values of all amino acids in **P5.**

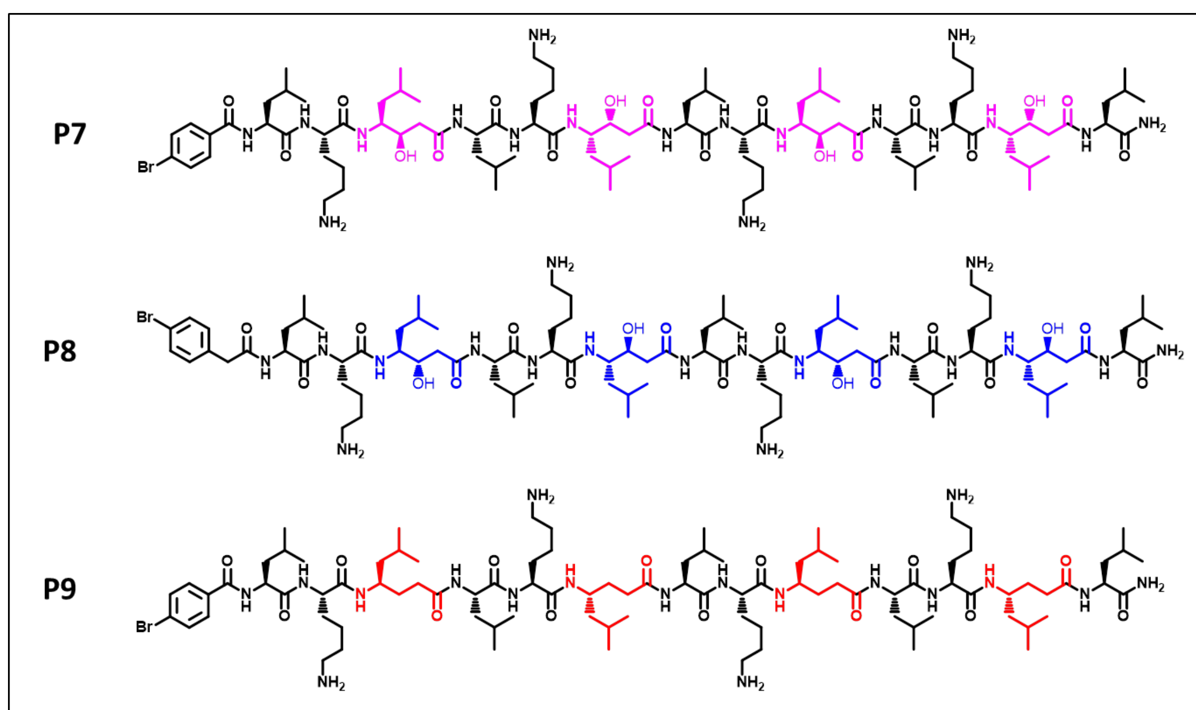
All eight crystallographically characterized β-hydroxy-γ-Leu residues adopt the g^+-g^+ conformation about the backbone C^γ-C^β (θ_1) and C^β-C^α (θ_2) bonds with the average torsion angle values : $\theta_1 = 45\pm5.73^0$ and $\theta_2 = 57\pm2.18^0$. In addition, we observe similar curvature in the helix.

Synthesis of hydrophilic sequences for investigation of antibacterial property

The rapid increase in multi-drug resistance bacteria has created an acute problem to antibacterial therapies demanding an urgent requirement for new antibiotics with no toxicity and different mode of actions defeating known mode of action of resistance. Due to this increased resistance against known antibiotics,

discovery and development of new antimicrobial agents is becoming very important field of interest. AMPs are one of the promising candidates for antimicrobial agents. AMPs are also known as host defence peptides and part of host innate immune system present an activity against broad spectrum of microbes. Synthetic AMP mimics have become novel strategy to offset the several problems including selectivity, toxicity and bioavailability.

We sought to investigate whether the α,α,γ-peptides composed of β-hydroxy-γ-gamma amino acids as well as γ-amino acids can be used as antimicrobials and also compare the activity hybrid peptides. The sequences of three water soluble hybrid peptides are shown in the **Scheme 8.**



Scheme 8: Schematic representation of hydrophilic peptides.

The hybrid peptide **P7** and **P8** are composed of alternating Leu, Lys and β-hydroxy-γ-Leu residues, whereas **P9** is composed of alternating Leu, Lys and γ^4-Leu residues. All three peptides **P7, P8** and **P9** were synthesized by solid phase method using HBTU/HOBt as coupling agents on Rink amide resin using standard Fmoc-chemistry. After completion of synthesis, the *N*-terminal was protected with 4-bromobenzoic acid or 4-bromo phenyl acetic acid. Peptides were then cleaved from

the resin using pure TFA. All three peptides **P7, P8** and **P9** were purified by reverse phase HPLC on C18 column using ACN/H2O with 0.1% TFA gradient system. Depending on the reverse phase HPLC retention time, the order of hydrophobicity of the β-hydroxy-γ-amino acid peptides **P7** and **P8** and γ^4-amino acid peptide **P9** was found to be **P9**>**P7**>**P8**. Reverse phase HPLC gradient system used for the purification of **P7**, **P8** and **P9** and retention time (t_R) for all three peptides are given in **Figure 8**.

1.

	Solvent A H_2O:ACN (95:5)	Solvent B ACN:H_2O (95:5)
Time (mins)	%A	%B
0	70	30
2	70	30
10	50	50
15	40	60
18	50	50
20	70	30

2.

Peptide	t_R
P7	14.07
P8	12.34
P9	15.71

Figure 8: **1.** Reverse phase HPLC gradient system used for purification of peptides. **2**. Retention time (t_R) for peptides.

The pure peptides were subjected to CD analysis to understand their conformations. CD spectra for **P7**, **P8** and **P9** in water and SDS are given in **Figure 9**. Solution structural analysis of these peptides using NMR is under investigation.

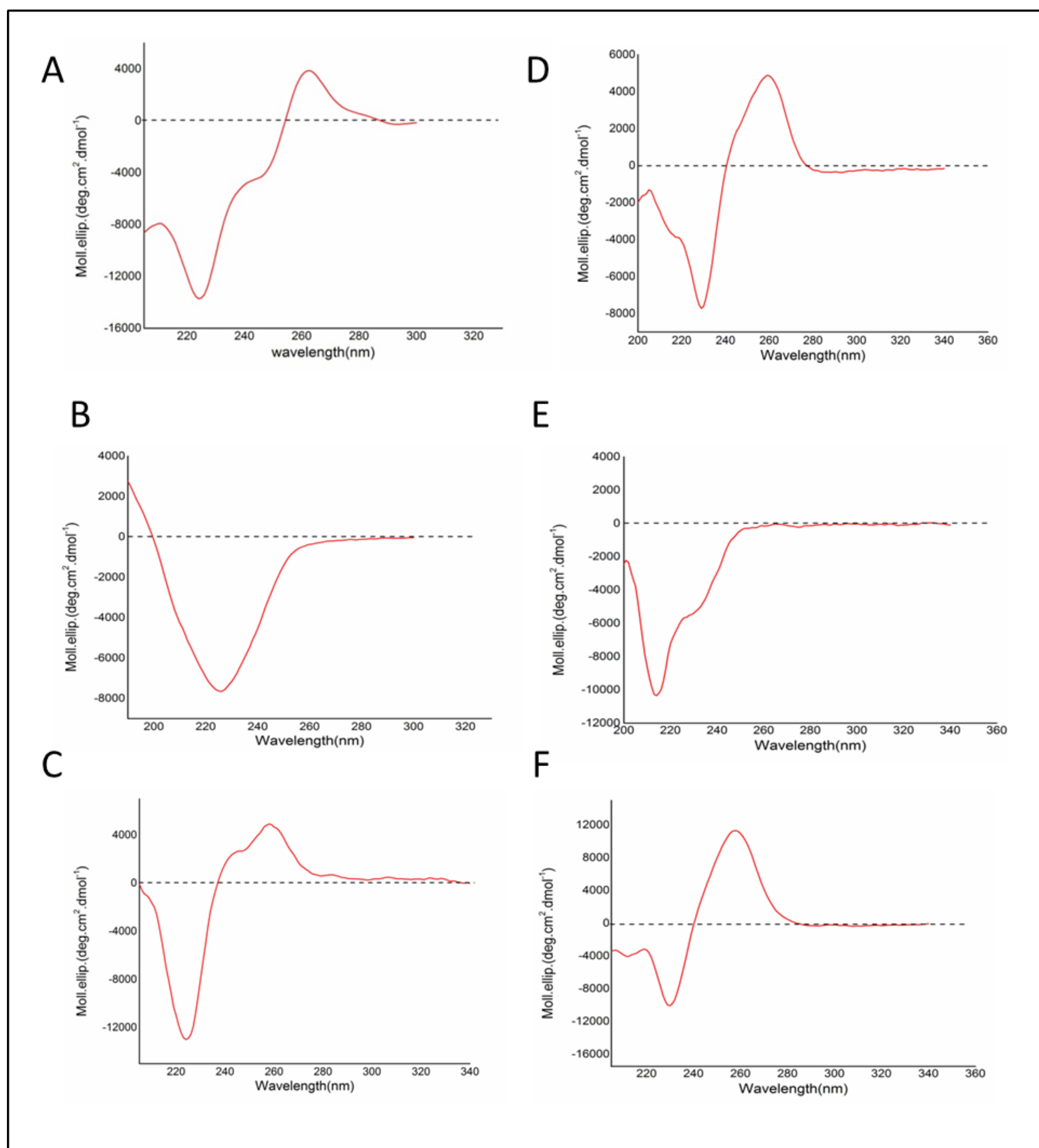


Figure 9: CD spectra **A. P7** in water **B. P8** in water **C. P9** in water **D. P7** in SDS **E. P8** in SDS **F. P9** in SDS.

CD spectra analysis showed increased helicity for **P8** when we change media from water to SDS compared to **P7** and **P9**.

Antibacterial activity

Both β-hydroxy-γ-amino acids containing peptides **P7** and **P8** were subjected to antibacterial studies along with control peptide **P9** composed of γ^4-amino acids

against *Escherichia coli, Pneumonia, Aureus, Typhimurium* and *Pseudomonas*. **P7** and **P8** showed better potency against all gram-positive and gram-negative bacteria than control **P9** peptide suggesting that β-hydroxy-γ-amino acids containing α,α,γ-peptides are better antibacterial agents than γ^4-amino acid containing α,α,γ-peptide except against *Escherichia coli*. Both **P7** and **P8** showed same potency against *Typhimurium* and *Pseudomonas.* Among the statins containing peptides, (3S,4S)-β-hydroxy-γ-leucine containing peptide **P8** showed 2-fold more activity against against *Escherichia coli, Pneumonia* and *Aureus* than (3R, 4S)-β-hydroxy-γ-leucine containing peptide **P7** suggesting that both presence of additional H-bond donor and its orientation are important for the enhanced antibacterial activity. The MIC values of all the peptides are given in the **Table 4**.

Table 4: MICs of α,α,γ-peptides (μg/ml).

Micro-organisms	P7	P8	P9
Escherichia coli	64	32	32
Pneumonia	64	32	>250
Aureus	64	32	>250
Typhimurium	32	32	64
Pseudomonas	64	64	250

The percentage of bacterial growth studies with respect to the concentration of peptides (μg/ml). Finding of above studies are shown in **Figure 10**.

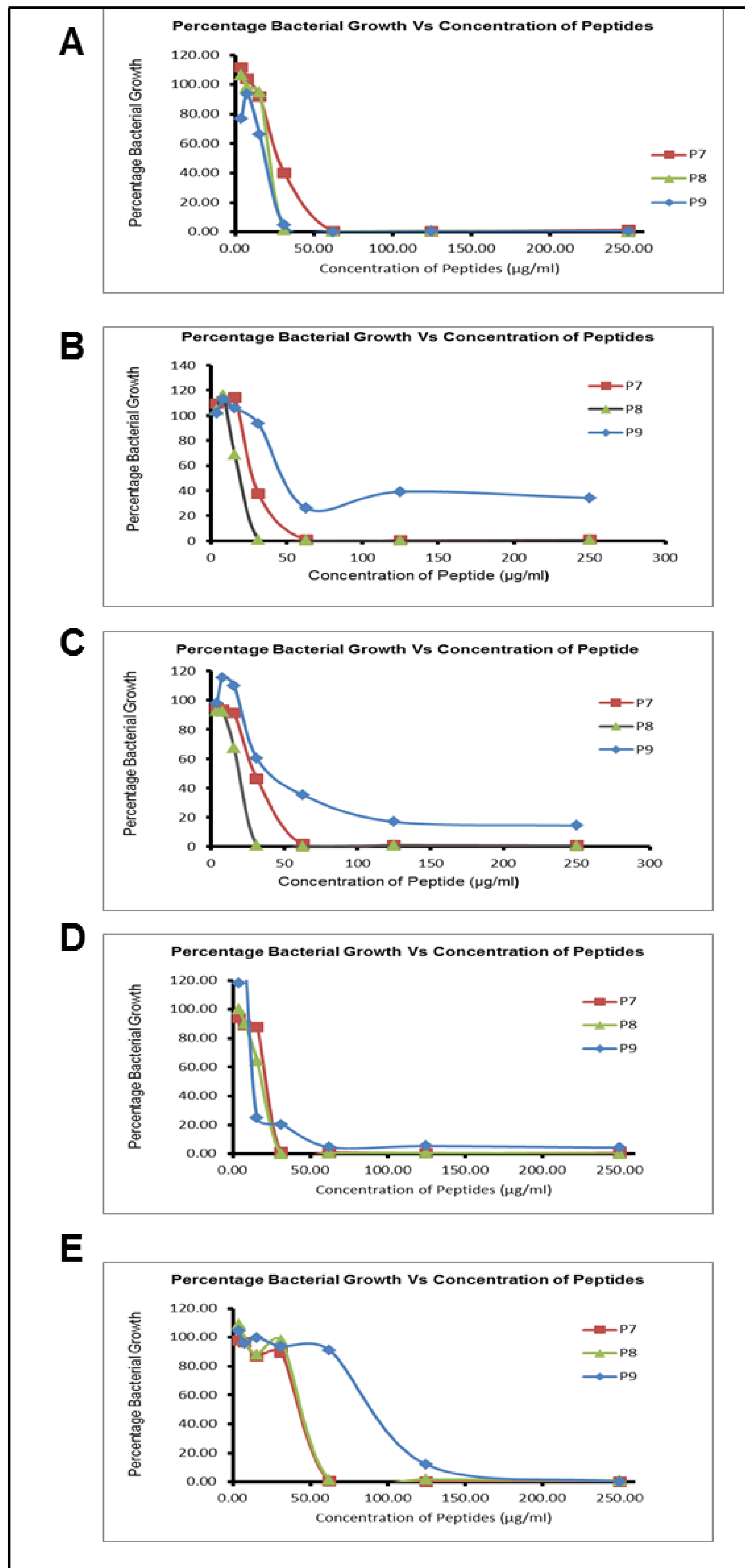


Figure 10: Percentage bacterial growth as a function of the concentration of peptides P7, P8 and P9 against **A.** *Escherichia coli* **B.** *Pneumonia* **C.** *Aureus* **D.** *Typhimurium* and **E.** *Pseudomonas* .

CONCLUSIONS

Conformational properties of higher oligomers of α,α,γ-peptides incorporated with γ^4-amino acids showed similar H-bonding pattern as that of the short α,α,γ-peptides containing γ^4-amino acids. In contrast to short peptides, higher oligomers showed curvature in the helix, suggesting the unique feature of higher order α,α,γ-hybrid helices. Analysis of the crystal structure revealed the projection of the side-chians along three corners of helical cylinder similar to 3_{10}-helix, β-peptide 12-helix. Thus they can be used as 3_{10}-helix, β-peptide 12-helix mimetic.

The conformational analysis of α,α,γ-helices containing *syn*-β-hydroxy-γ-leucine reveal that additional hydroxyl group at β-position can be accommodated into 10/12-helices without deviating overall helical folding. Similar to ααγ-peptides containing γ^4-leucine, ααγ-peptides composed of *syn*-β-hydroxy-γ-leucine also showed the curvature in the helix. The β-hydroxyl group in *syn* diastereoisomer is projected downwards similar to amide carbonyl and involves itself in formation of six membered intraresidue H-bond. Influence of hydroxyl group with *anti*-stereochemistry is currently under investigation.

In addition to conformational analysis of ααγ-peptides, we have demonstrated the design of water soluble peptides and studied their potency against the bacteria. Our investigation showed that peptides containing β-hydroxy-γ-leucine are more active against bacteria as compared to peptide containing γ^4-leucine. Peptide incorporated with *anti* diastereoisomer showed 2-fold more potency compared to the peptide containing *syn* diastereoisomer. NMR studies, haemolysis assay and beta-galactosidase assay are currently under investigation.

METHODS

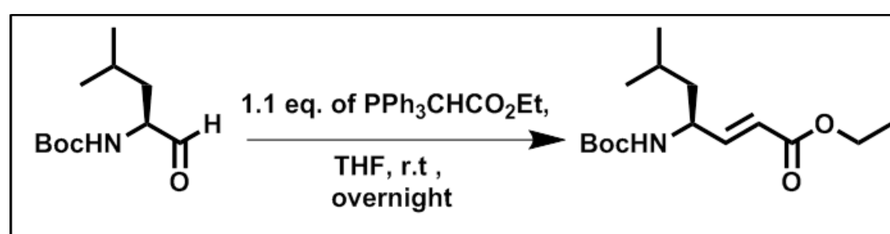
CHEMICALS

All amino acids, activated palladium charcoal, triphenyl phosphine, ethyl bromoacetate, ethyl diazoacetate, TFA, DIPEA and tin(II) chloride were obtained from Aldrich. HOBt, HBTU were purchased from spectrochem. THF, DMF and DCM were bought from Merck used without further purification. Sillica gel used for column chromatography was purchased from Merck (120-200 mesh).

EXPERIMENTAL

General Procedure for the Synthesis of N-Boc-protected E-vinylogous amino ester:

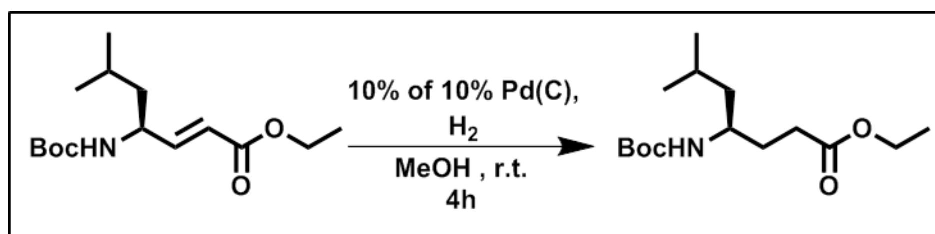
N-Boc-Amino aldehyde (15 mmol) was dissolved in 50 mL of dry THF followed by Wittig ylide (16.5 mmol) was added at RT. Reaction mixture was stirred for about 5h at RT. Completion of the reaction was monitored by TLC. After completion, solvent was evaporated and the crude product was purified by column chromatography using EtOAc/pet ether.



General procedure for the Synthesis of N-Boc-Protected γ^4-amino ester

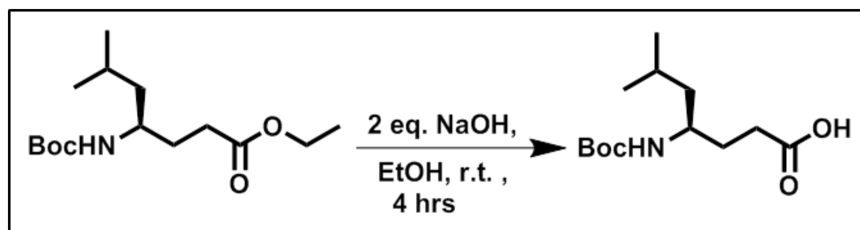
The suspension of activated Pd/C (20% by weight) and benzyl esters of N-Boc-protected vinylogous amino esters (5 mmol), which was synthesized using the

reported method, in 10% acetic acid in EtOH (40 mL) was stirred overnight at room temperature in the presence of hydrogen. After completion of the reaction, Pd/C was filtered through the bed of Celite and the filtrate was evaporated to dryness under vacuum to obtain the corresponding free γ^4- amino ester.



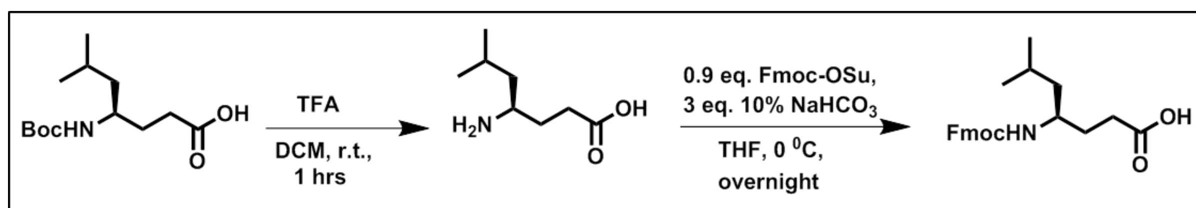
General procedure for the Synthesis of N-Boc-Protected γ^4-amino acid

The N-Boc-protected γ^4- amino ester (3.5 mmol) was dissolved in 15 mL ethanol followed by slow addition of 1N NaOH (2 eq.). The reaction mixture was stirred for about 4h. The progress of the reaction was monitored by TLC. After completion of the reaction, methanol was evaporated under reduced pressure. Then the residue was diluted with water (60 mL), acidified (pH~ 4) with 0.5 N HCl and extracted the compound with EtOAc (3 × 60 mL). The combined organic layer was washed with 50 mL brine solution, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to get free carboxylic acid.



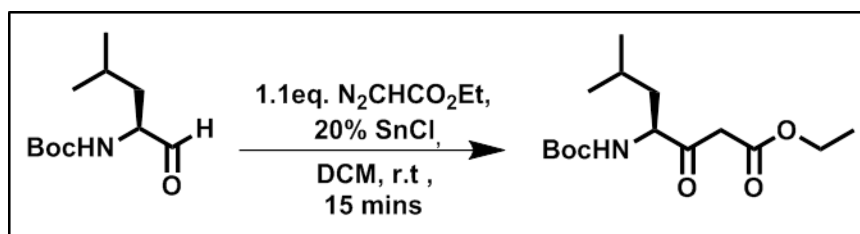
General procedure for the synthesis of Fmoc-protected γ^4-amino acids:

The solution of N-Boc-protected γ^4- amino acids (3 mmol) in 3 mL of DCM was cooled to 0 °C in ice bath followed by addition of neat 5 mL of TFA. After completion of the reaction (~ 30 min), solvent was evaporated under reduced pressure. Residue was dissolved in 10 mL of water and adjusts pH to ~8 by addition of solid Na_2CO_3. The solution of Fmoc-OSu (2.7 mmol) in 10 mL of THF was added slowly to the reaction mixture and stirred overnight at RT. After completion of the reaction, the reaction mixture was acidified with 1N HCl under cold condition and extracted with ethyl acetate (3× 50 mL). The combined organic layer was washed with brine solution, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give white solid product of Fmoc-γ^4-amino acids in 72-80% yields. They were directly used for solid phase peptide synthesis without further purifications.



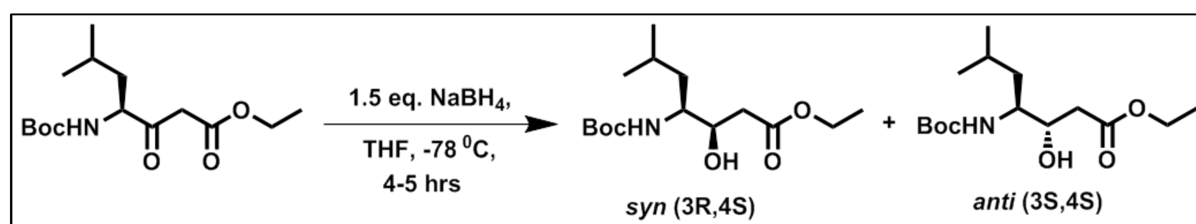
General procedure for the Synthesis of N-Boc-protected β-keto γ-amino esters

In general, the *N*-protected amino aldehyde (10 mmol) was dissolved in 15 mL of DCM at room temperature (20–25 °C) and then anhydrous tin(II) chloride (0.796 g, 20 mol%) was added to the reaction followed by ethyl diazoacetate (1.19 g, 10.5 mmol). Immediate gas evolution was observed and it ended within 30 min. The progress of the reaction was monitored by TLC. After completion (~30 min) of the reaction, it was quenched with 60 mL of 0.5 N HCl and immediately extracted with DCM (80 mL × 3). The combined organic layer was washed with brine (100 mL), dried over anhydrous sodium sulfate and concentrated under reduced pressure to give a greenish oily crude product which was further purified by silica gel column chromatography to get pure β-keto γ-amino ester.



General procedure for Synthesis of N-Boc-protected β-hydroxy ϒ-amino ester

The *N*-protected β-keto γ-amino ester (10 mmol) was dissolved in 10 mL of dry THF under N_2 atmosphere, cooled to -78 ^{o}C, and then $NaBH_4$ (15 mmol) was added in one portion. The reaction mixture was stirred for further 3h to complete the reaction. The progress of the reaction was monitored by TLC. After completion of the reaction, it was quenched by pouring the mixture into ice-cold 1 *N* hydrochloric acid (10 mL). The aqueous phase was extracted with ethyl acetate (3 × 50 mL). Then the combined organic layers was washed with brine (60mL) and dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The diastereoisomer mixtures were separated passing through normal silica gel column chromatography using pet-ether (60-80 ^{o}C)–ethyl acetate solvent system.

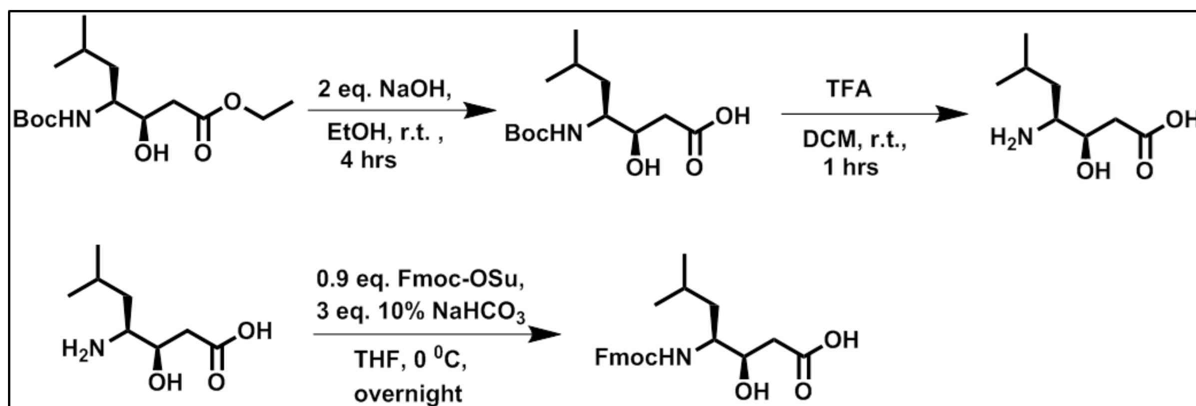


General procedure synthesis of N-Fmoc protected β-hydroxy ϒ-amino acids

The N-Boc-protected β-hydroxy γ-amino ester (3.5 mmol) was dissolved in 15 mL ethanol followed by slow addition of 1N NaOH (2 eq.). The reaction mixture was stirred for about 4h. The progress of the reaction was monitored by TLC. After completion of the reaction, methanol was evaporated under reduced pressure. Then

the residue was diluted with water (60 mL), acidified (pH~ 4) with 0.5 N HCl and extracted the compound with EtOAc (3 × 60 mL). The combined organic layer was washed with 50 mL brine solution, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to get free carboxylic acid. The solution of N-Boc-β-hydroxy γ-amino acids (3.5 mmol) in 3 mL of DCM was cooled to 0 °C in ice bath followed by addition of neat 5 mL of TFA. After completion of the reaction (~ 30 min), solvent was evaporated under reduced pressure.

Residue was dissolved in 10 mL of water and adjusts pH to ~8 by addition of solid Na_2CO_3. The solution of Fmoc-OSu (3.15 mmol, 0.9 eq.) in 10 mL of THF was added slowly to the reaction mixture and stirred overnight at RT. After completion of the reaction, the reaction mixture was acidified with 1N HCl under cold condition and extracted with ethyl acetate (3× 50 mL). The combined organic layer was washed with brine solution, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give white solid product of Fmoc-γ^4-amino acids in good yields. They were directly used for solid phase peptide synthesis without further purifications.



Solid Phase Peptide Synthesis:

All peptides were synthesized at 0.2 mmol scales on Rink Amide resin using standard Fmoc-chemistry. HBTU/HOBT was used as coupling agents. Fmoc deprotections were facilitated using 20% piperidine in DMF. The coupling reactions

were monitored by Kaiser Test. Fmoc-γ- amino acids were prepared using reported method. Each statine (2 eq and re-coupling with 1 eq) and Aib (5 eq) were used for couplings. Final couplings were done using *p*-Bromobezoic acid and *p*-Bromophenyl acetic acid. After completion of the synthesis, peptides were cleaved from resin with 15 mL of TFA for 3 h. After cleavage, the resin was filtered and washed with TFA. The cleavage mixture was evaporated under reduced pressure to give gummy product. Peptide was further precipitate using cold diethyl ether and followed by lyophilization gave crude peptide. Crude peptide further purified using RP-HPLC and mass was confirmed by MALDI-TOF/TOF.

Antibacterial activity

The bacteria used in these experiments were collected from National Collection of Industrial Microorganisms (NCIM) *Escherichia coli* (NCIM 2065), *Klebsiella pneumoniae* (NCIM 2957), *Staphylococcus aureus* (NCIM 5021), *Salmonella typhimurium* (NCIM 2501), *Pseudomonas aeruginosa* (NCIM 5029). The minimal inhibitory concentration (MIC) of the active compounds was determined by following the previously published protocol.[20]

The antibacterial activities of ααγ-peptides were performed in 96-well microtiter plate using broth serial dilution method. The bacterial cultures were grown over night at 37 °C in autoclaved sterile MHB (Mueller-Hinton broth) media. Peptides to be tested were dissolved in autoclaved sterile MHB. Two-fold serial dilutions with MHB medium were carried out for each peptide in a sterile 96-well microtiter plate to a final volume of 50 μl in each well. 3-4 single babcterial colonies were suspended in 2ml of MHB media then optical density of 1ml of the solution was adjusted to 0.1($1*10^8$ colony forming units/ml) at 600nm. 200 μl of resulting solution was added to 19.8ml of sterile MHB medium to achieve a concentration of $5*10^5$ colony forming units/mL . By adding an aliquot of 50 μl bacterial solution to each well containing serially diluted 50μl of peptides total volume was made 100μl. Three different controls were prepared **1.** Peptide control: 50μl of peptide solution in 50μl of MHB media **2.** Media control: 100μl of MHB **3.** Growth control: 50μl of bacterial solution in 50μl of sterile MHB media. Plates were incubated at 37 °C for 18-20 hrs and the MIC

of peptide was obtained by measuring the absorbance at 492 nm. Lowest concentration of peptide which inhibited complete bacterial growth is recorded as MIC for that sample peptide. All the experiments were performed in triplicates and repeated for three different times. Scematic representation of procedure is shown in **Figure 11**.

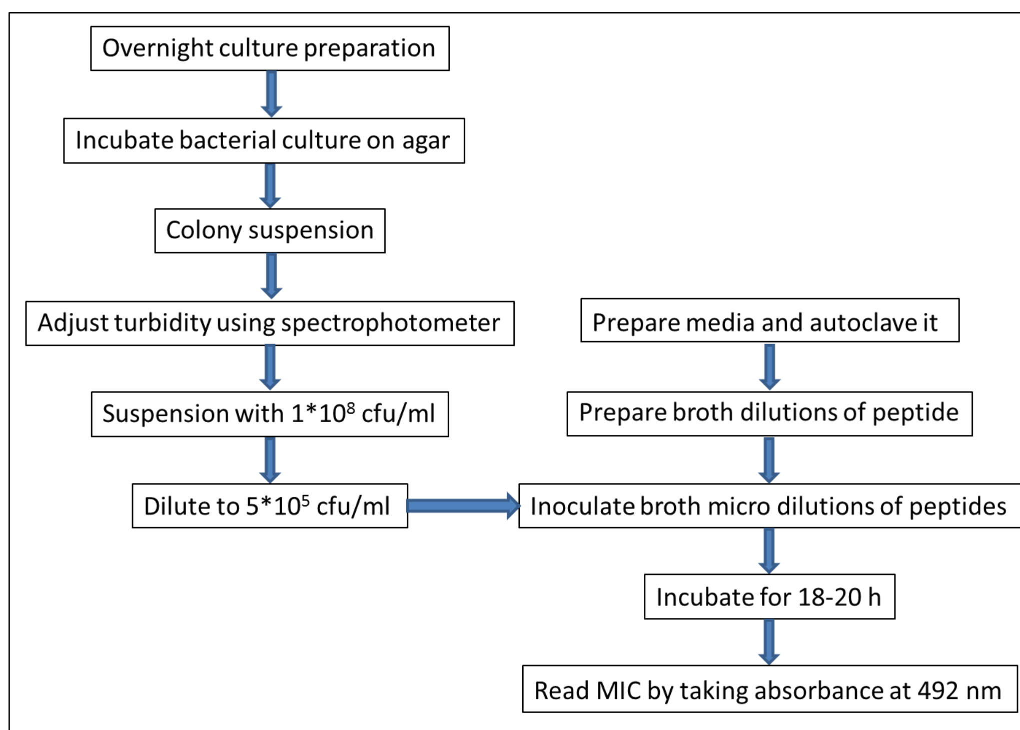
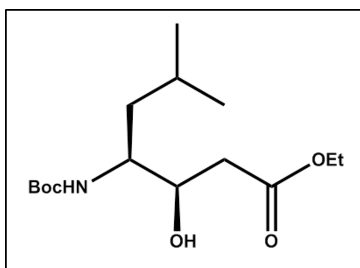


Figure 11: Schematic representation of MIC protocol.

NMR details for the monomers

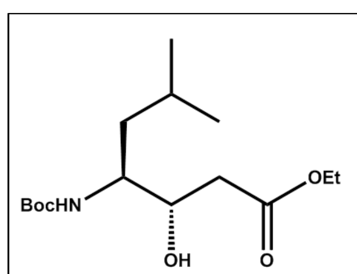
(R,S)-N-Boc protected β-hydroxyl γ-leucine ester:

Yellow colour solid (0.98g, 60.0%); ^{1}**H NMR** (400 MHz, $CDCl_3$) **:** δ_H 4.59-4.57 (d, *J* = 8.8, 1H, NH), 4.20-4.15 (q, *J* = 7, 2H, -OC*H*2), 4.05-4.01 (m,1H, C*H*OH), 3.70-3.63 (m, 1H, -C*H*), 3.45 (b, 1H, -OH), 2.48-2.42 (m, 2H, -C*H*2CO-), 1.66-1.57 (m,2H, -δC*H*2-), 1.44 (s, 9H, Boc -(C*H*3)3), 1.34-1.31 (t, *J* = 6.8, -ωC*H*-) 1.29-1.26 (t, *J* = 7.2,3H, -C*H*3), 0.95-0.91 (m,6H, -(C*H*3)2); ^{13}**C NMR** (100 MHz, $CDCl_3$) **:** δ_C 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; **ESI** *m/z* value Calcd. $[M+Na]^+$ 326.1943, observed 326.1949.



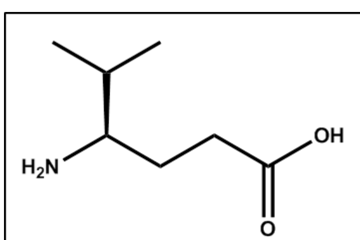
(S,S)-N-Boc protected β-hydroxyl γ-leucine ester:

Light yellow color liquid (0.48g, 30.1%); **^{1}H NMR** (400 MHz,$CDCl_3$) δ_H 4.73-4.71 (d, *J* = 10, 1H, NH), 4.20-4.15 (q, *J* = 7.2, 2H, -OC*H*2), 4.03-4.01 (d, *J*= 8, 1H,-C*H*-OH), 3.63-3.60 (m, 1H, -C*H*-), 3. 30 (b, 1H, -OH), 2.59-2.46 (m, 2H, -C*H*2CO),1.69-1.58 (m, 3H, -C*H*2C*H*-), 1.44 (s, 9H, Boc -(C*H*3)3) ,1.30-1.26 (t, *J* = 7.2, 3H, -C*H*3), 0.94-0.92 (m,6H, -(C*H*3)2); **^{13}C NMR** (100 MHz, $CDCl_3$)**:** δ**C** 173.55, 156.00, 79.18, 69.65,51.93, 41.70, 38.67, 28.35, 24.73, 23.01, 22.24, 14.13; **ESI** *m/z* value Calcd. for [M+Na]+ 326.1943,



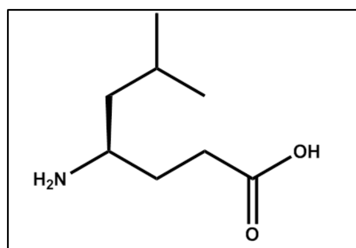
(*R*)-4-amino-5-methylhexanoic acid:

White solid (0.62 g, 80 %); **^{1}H NMR** (400 MHz, D_2O) *δ* 3.06 (m, 1H), 2.35 (m, 3H), 1.76 (m, 2H), 0.92 (m, 6H); **^{13}C NMR** (100 MHz, D_2O) *δ* 174.81, 57.33, 33.70, 29.66, 25.97, 17.46, 16.71; **MALDI TOF/TOF**- *m/z* calcd. for $C_7H_{15}NO_2$ $[M+Na]^+$ 168.1000, obsrvd. 168.1035.



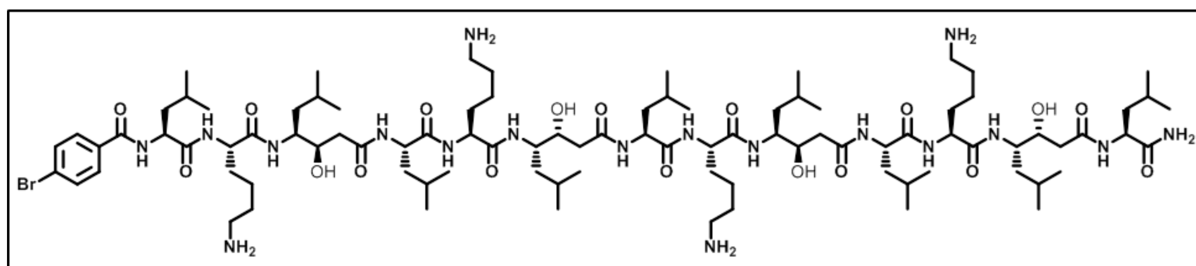
(*R*)-4-amino-6-methylheptanoic acid

White solid (0.70 g, 75 %), **^{1}H NMR** (400 MHz, D_2O) *δ* 3.25 (m, 1H), 2.25 (m, 2H), 1.81 (m, 2H), 1.62 (m, 1H), 1.42 (m, 2H), 0.84 (d, *J* = 4 Hz, 6H) ; **^{13}C NMR** (100 MHz, D_2O) *δ* 181.55, 50.02, 41.18, 33.30, 29.00, 23.80, 21.77, 21.24; **MALDI TOF/TOF**- *m/z* calcd. for $C_8H_{17}NO_2$ $[M+Na]^+$ 182.1157, obsrvd. 182.0886.



P7:

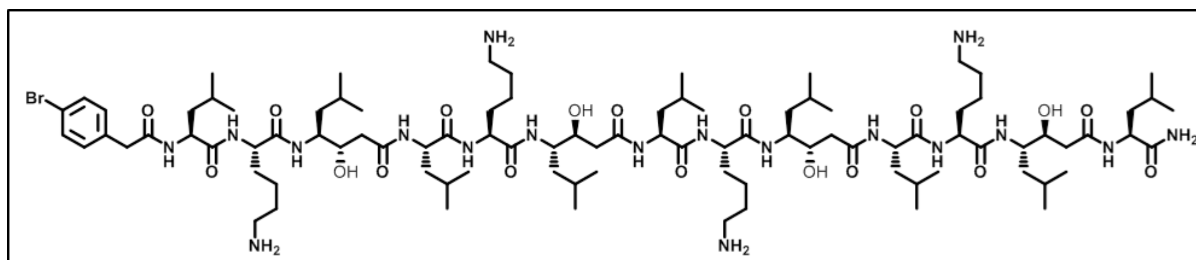
^{1}H NMR (400MHz, 5% D_2O in water): $δ_H$ 8.53 (d, *J* = 4 Hz, 1H), 8.36 (d, *J* = 8 Hz, 1H), 8.24-8.17 (m, 4H), 8.12-8.08 (m, 3H), 7.73(d, *J* = 12, 1H), 7.64-7.59 (m, 7H), 7.51-7.44 (m, 7H), 6.99 (s, 1H), 4.26-4.13 (m, 2H), 3.90-3.76 (m, 7H), 2.94-2.84 (m, 7H), 2.46-2.23 (m, 9H), 1.79-1.23 (m, 54H), 0.89 (d, *J* = 4, 4H), O.86-0.69 (m,58H); **MALDI TOF/TOF**- *m/z* calcd. for $C_{93}H_{169}BrN_{18}O_{18}$$[M+Na]^+$ 1928.1938, obsrvd. 1928.25.



P8:

^{1}H NMR (400MHz, 5% D_2O in water): $δ_H$ 8.40 (d, *J* = 4 Hz, 1H), 8.30-8.27 (m, 3H), 8.17 (d, *J* = 8 Hz, 4H), 8.13-8.03 (m, 4H), 7.65 (s, 1H), 7.57-7.33 (m, 13H), 7.13 (d, *J*

= 8 Hz, 2H), 6.99 (s, 1H), 4.22-4.17 (m, 3H), 3.97-3.88 (m, 8H), 2.93-2.81 (m, 7H), 2.32-2.23 (m, 8H), 1.80-1.20 (m, 55H),O.85-0.73 (m,64H); **MALDI TOF/TOF**- *m/z* calcd. for $C_{94}H_{171}BrN_{18}O_{18}$ $[M+Na]^+$ 1942.2095, obsrvd. 1942.29.



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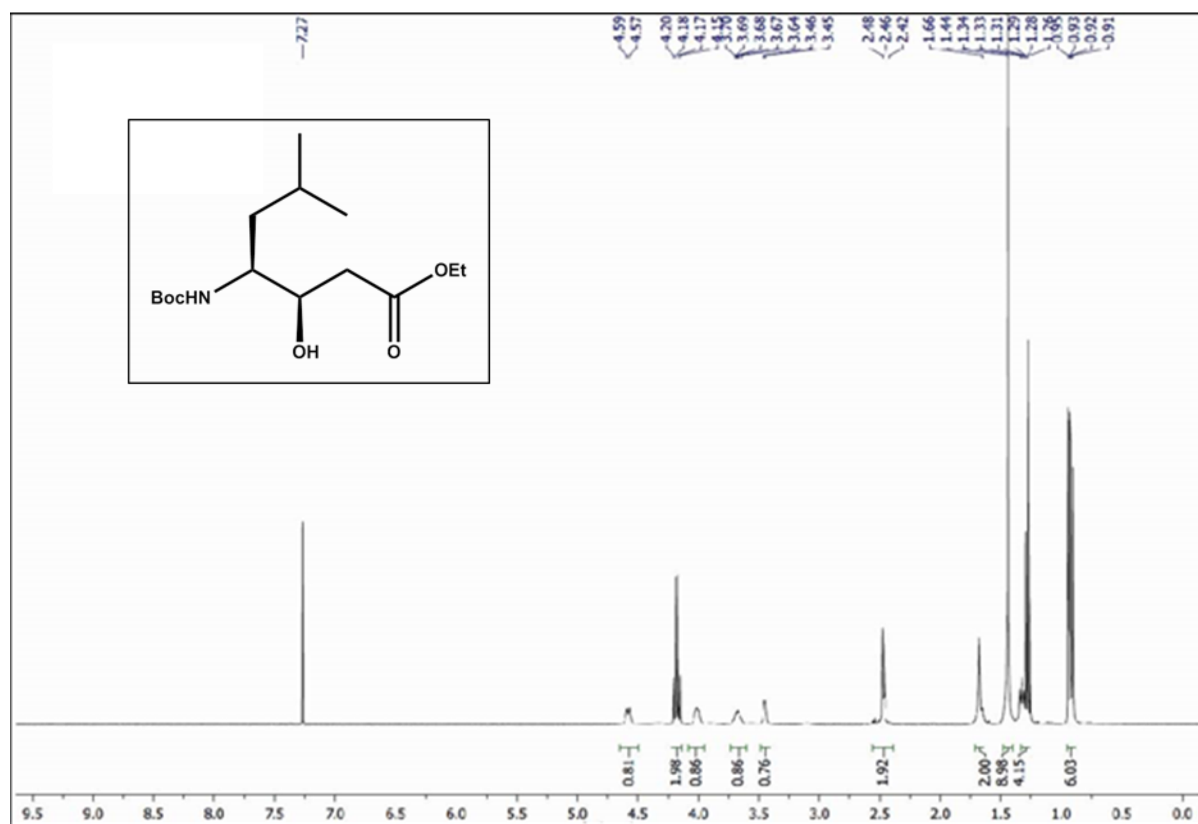
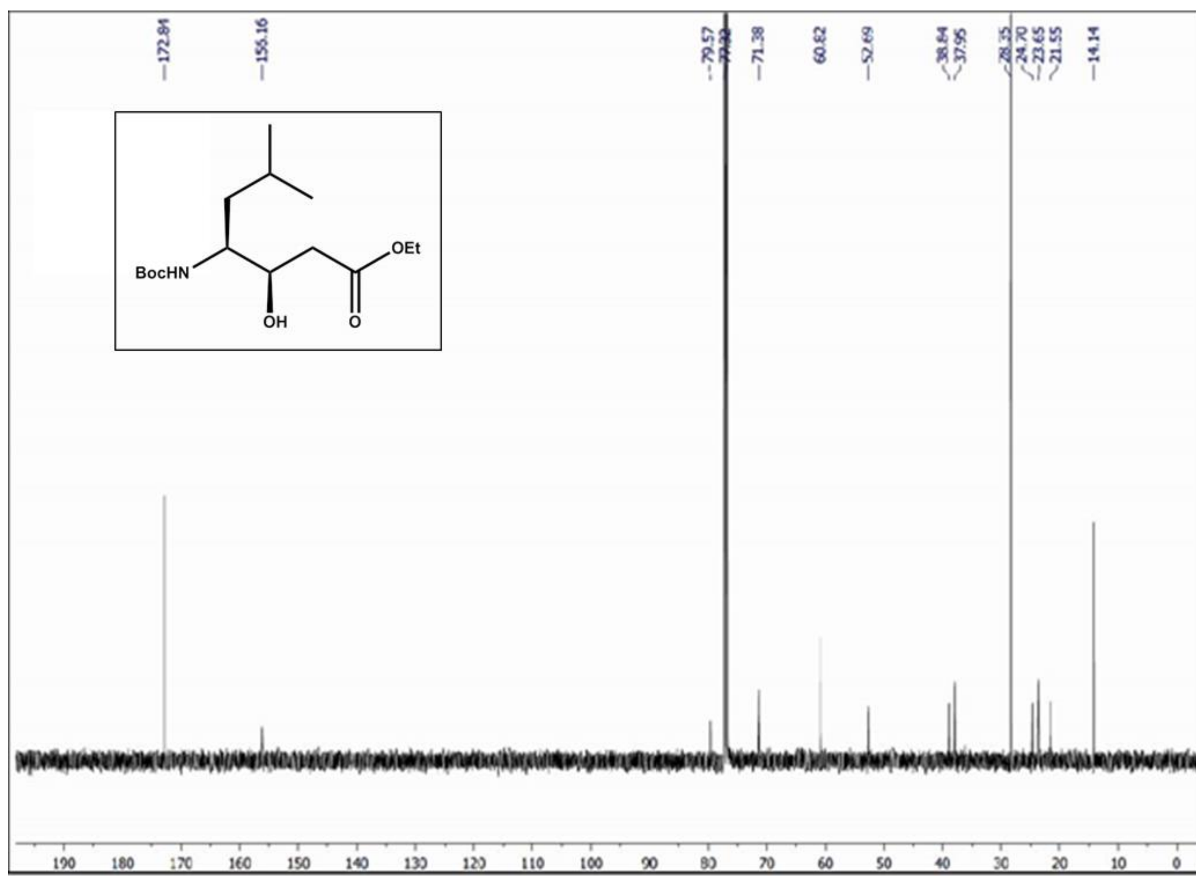
SUPLLEMENTRY DATA

^{1}H NMR for (R,S)-N-Boc protected β-hydroxyl γ-leucine ester



^{13}C NMR for (R,S)-N-Boc protected β-hydroxyl γ-leucine ester



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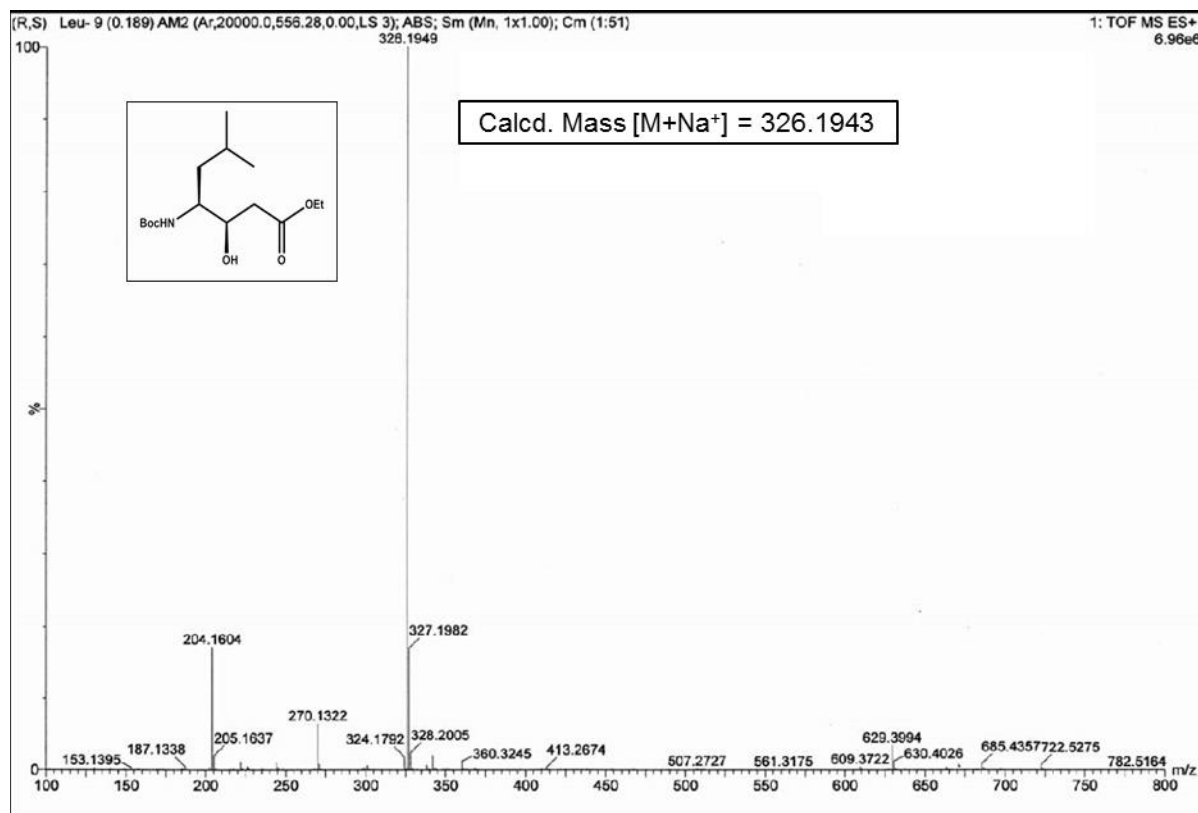

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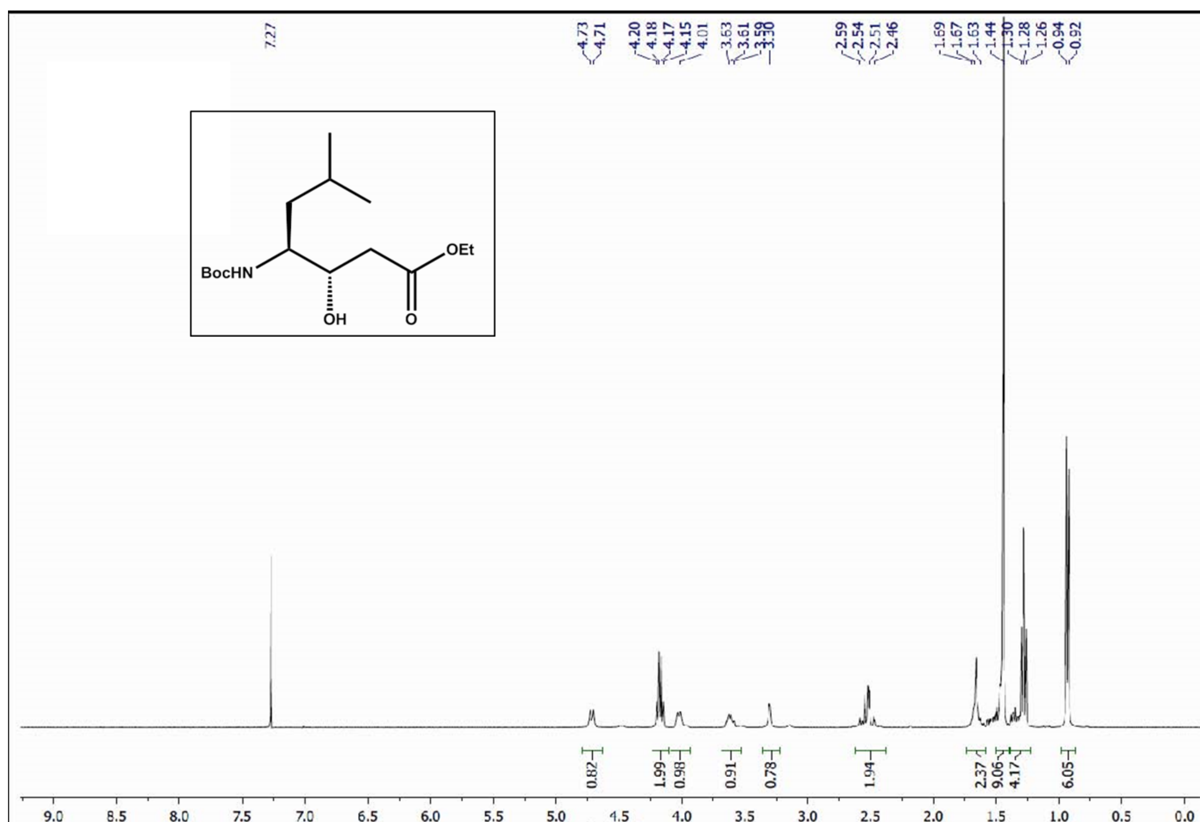

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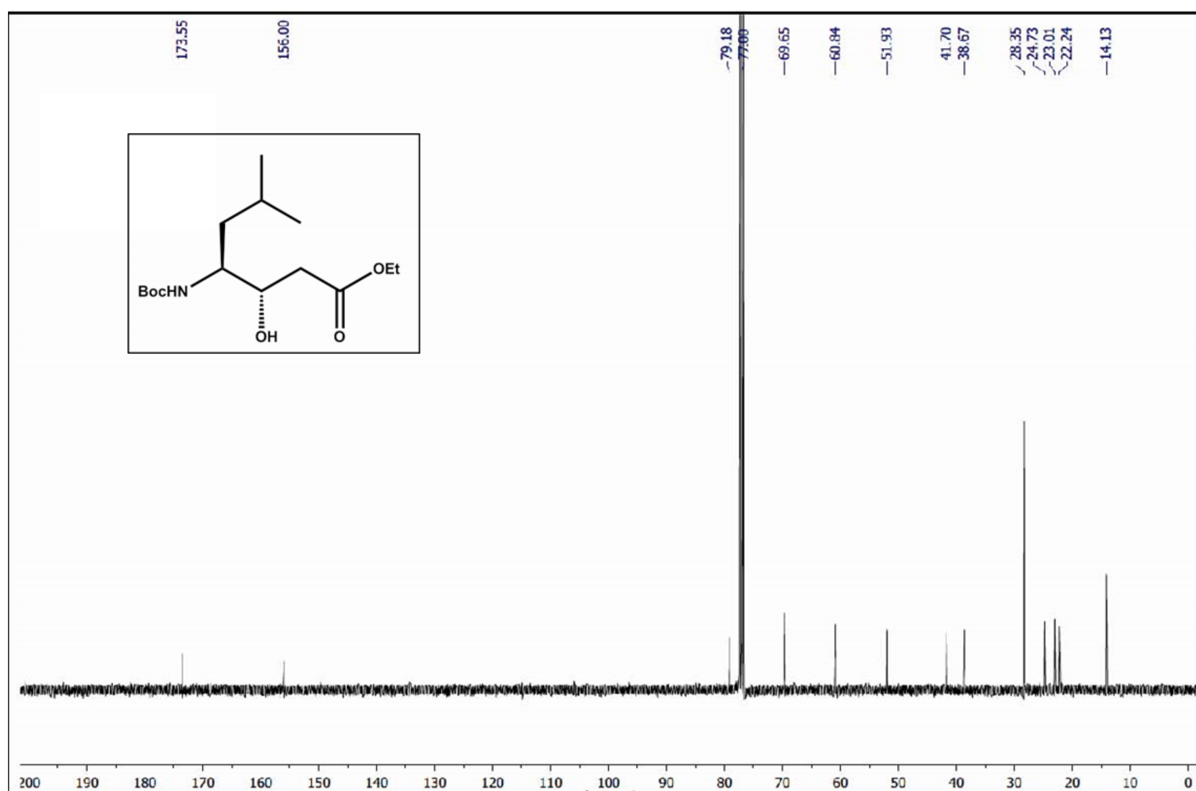

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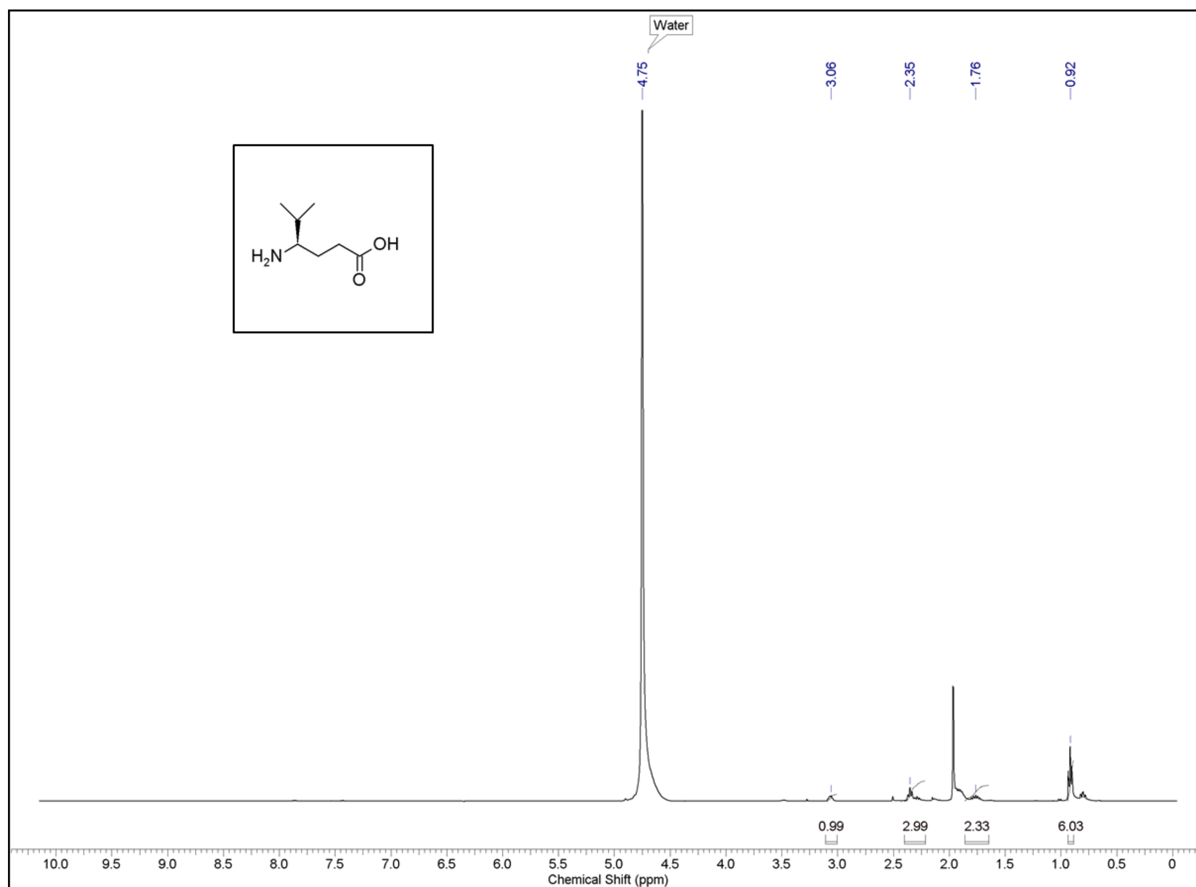

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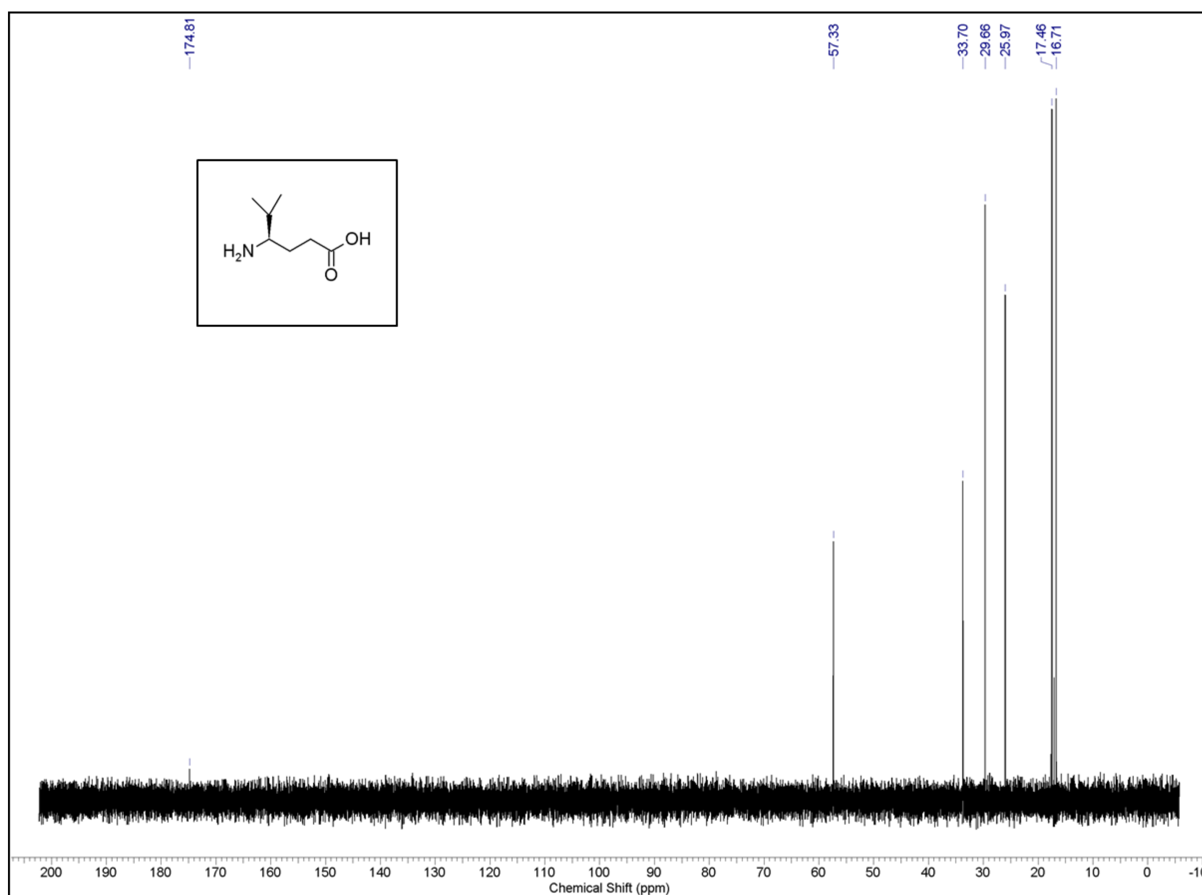

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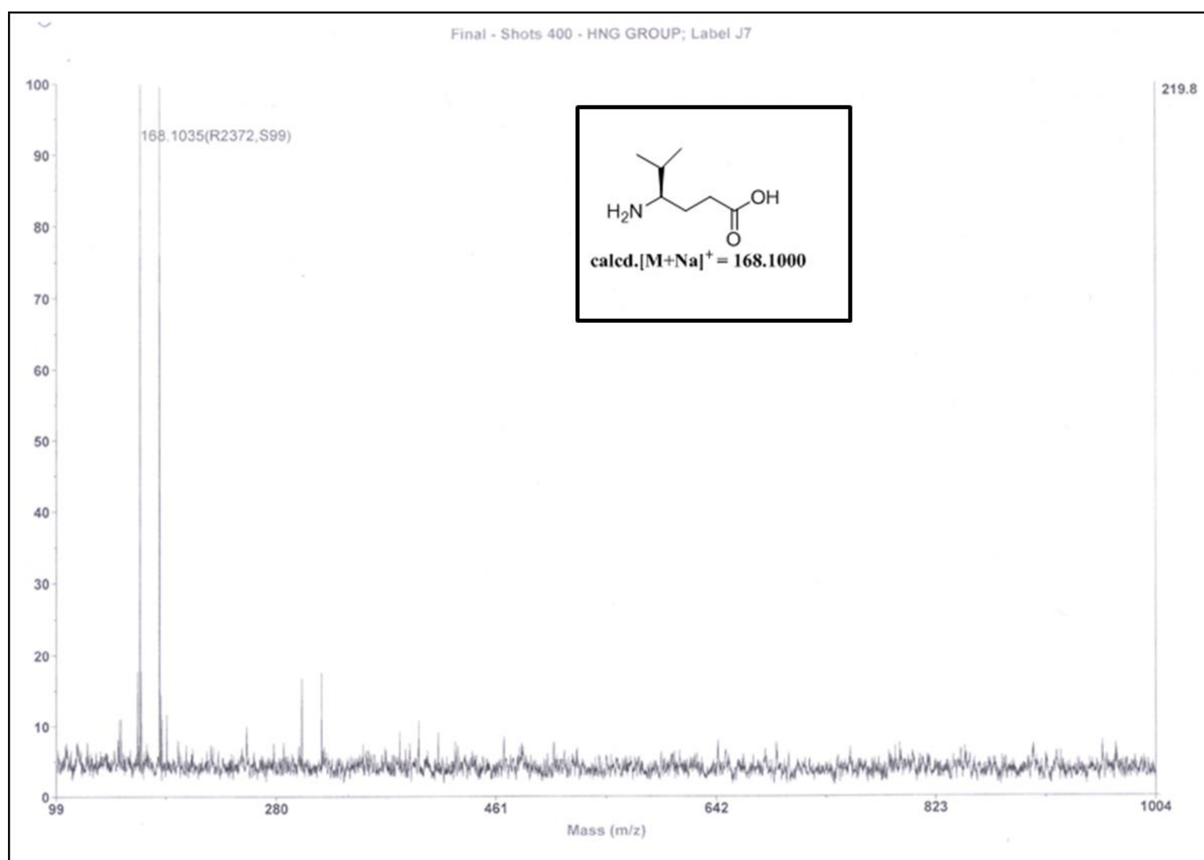
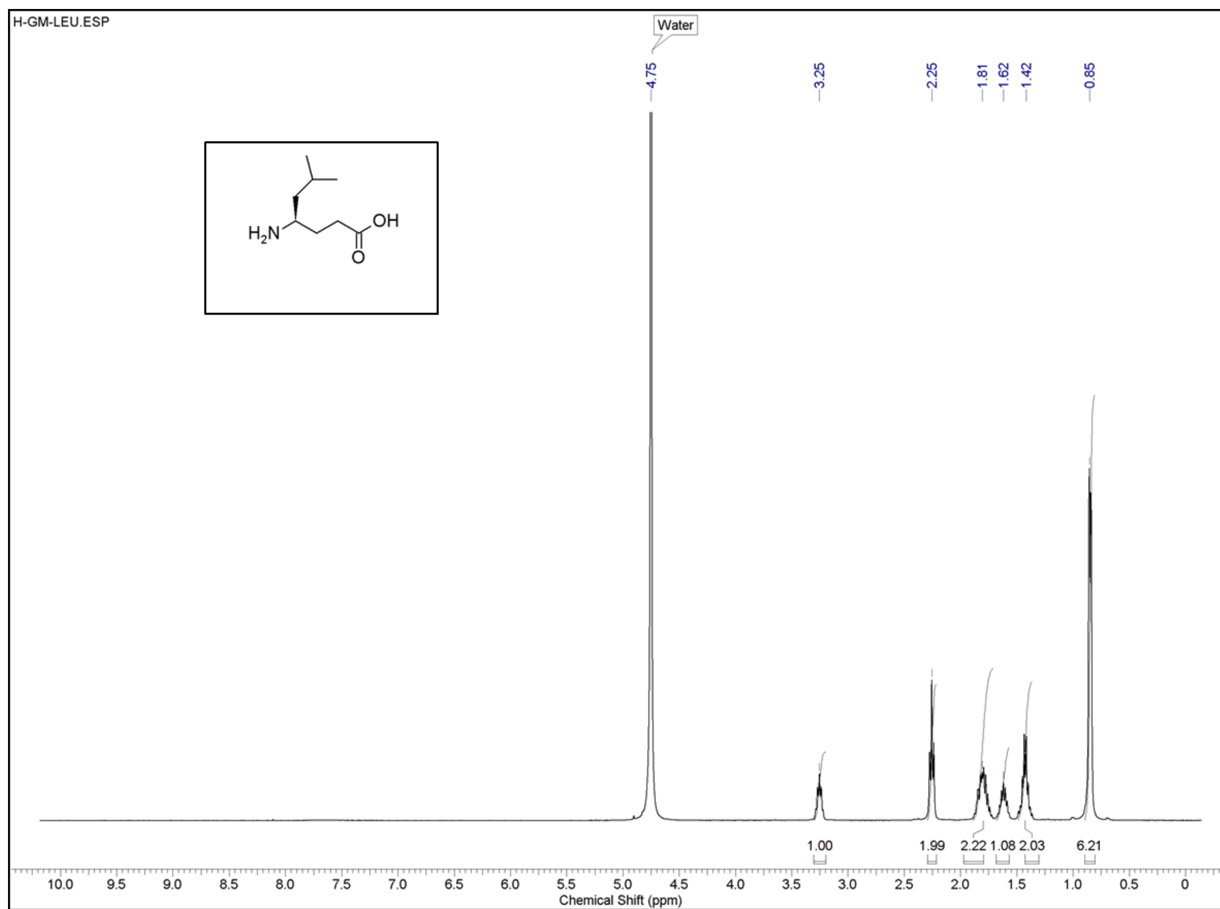


^{1}H NMR for (*R*)-4-amino-6-methylheptanoic acid



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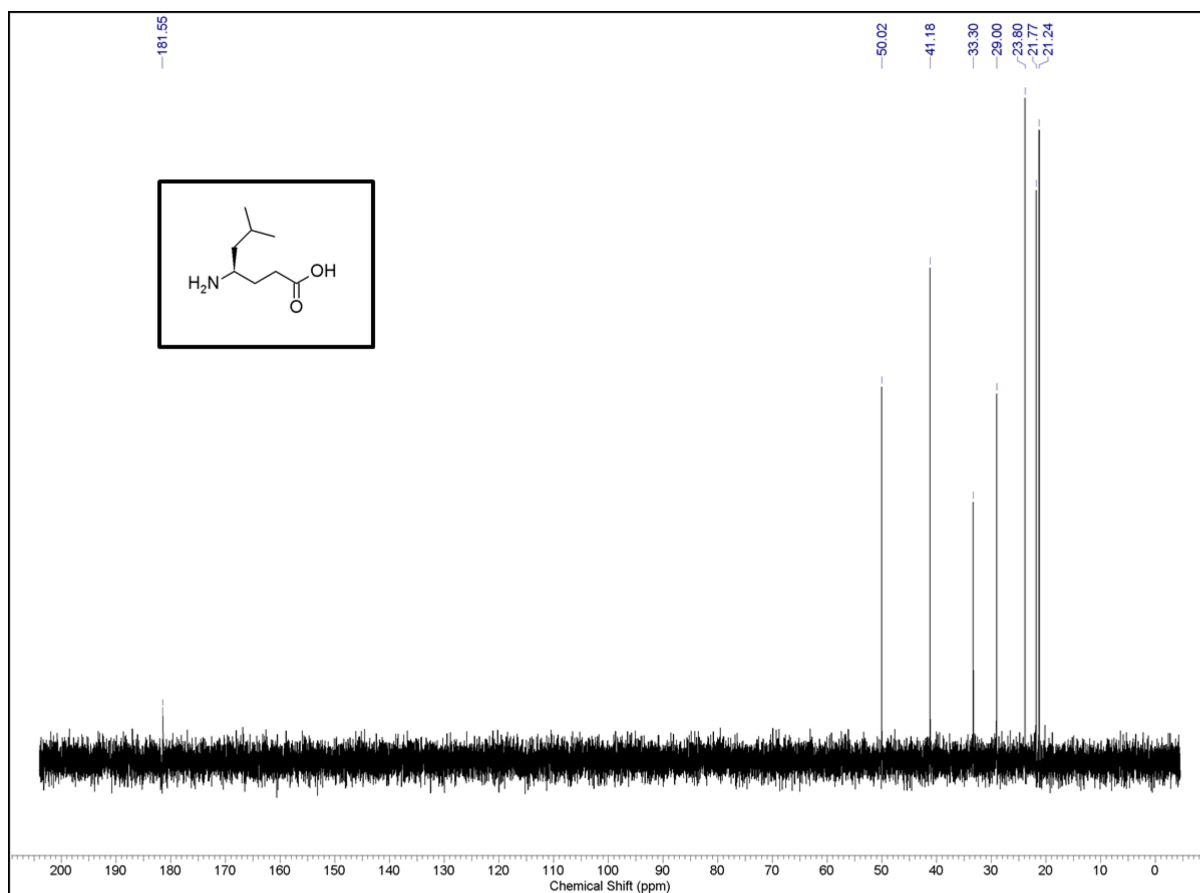

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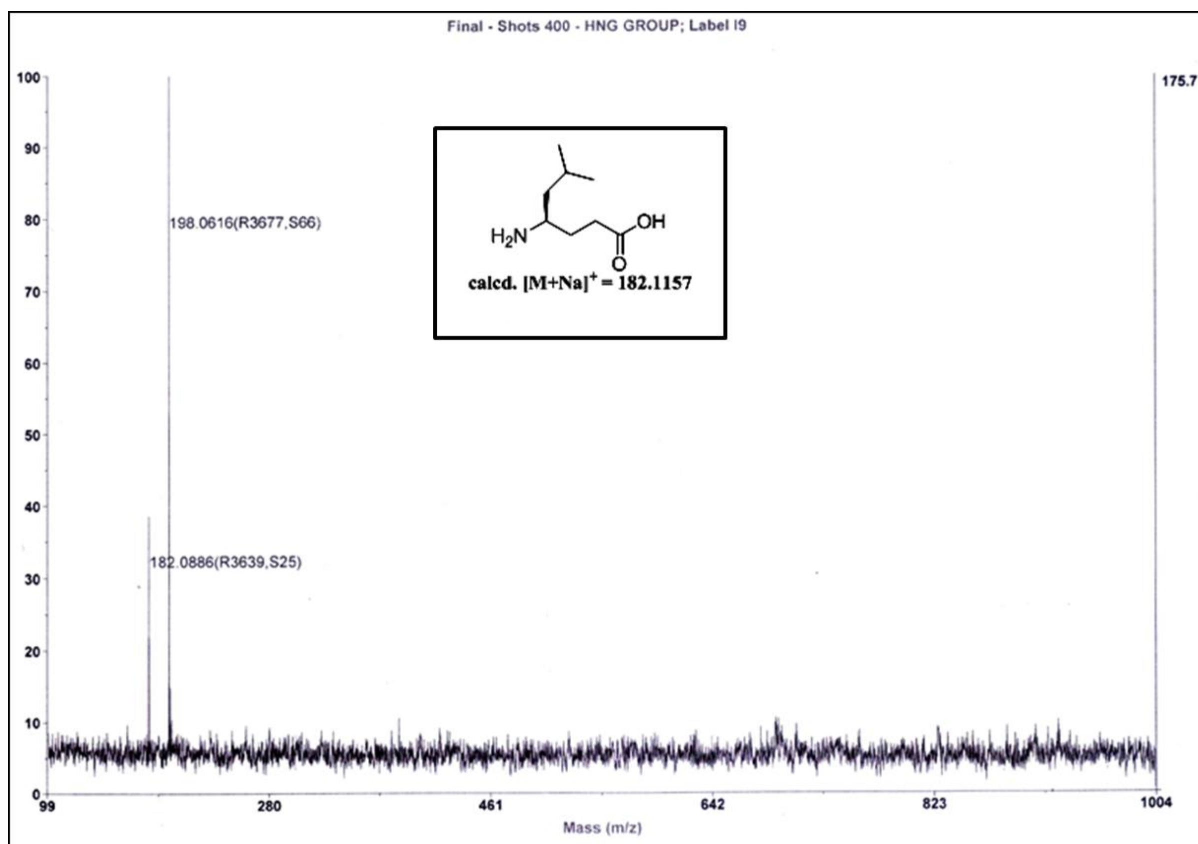
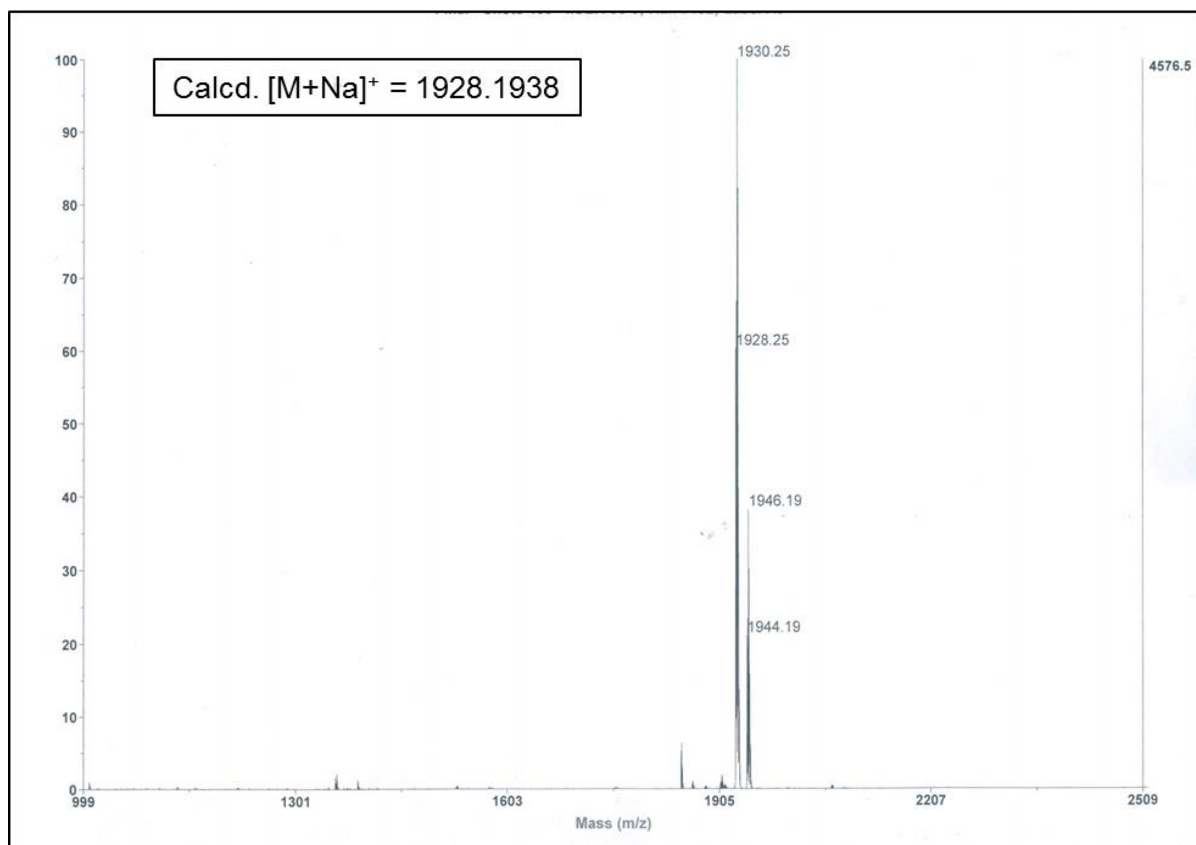
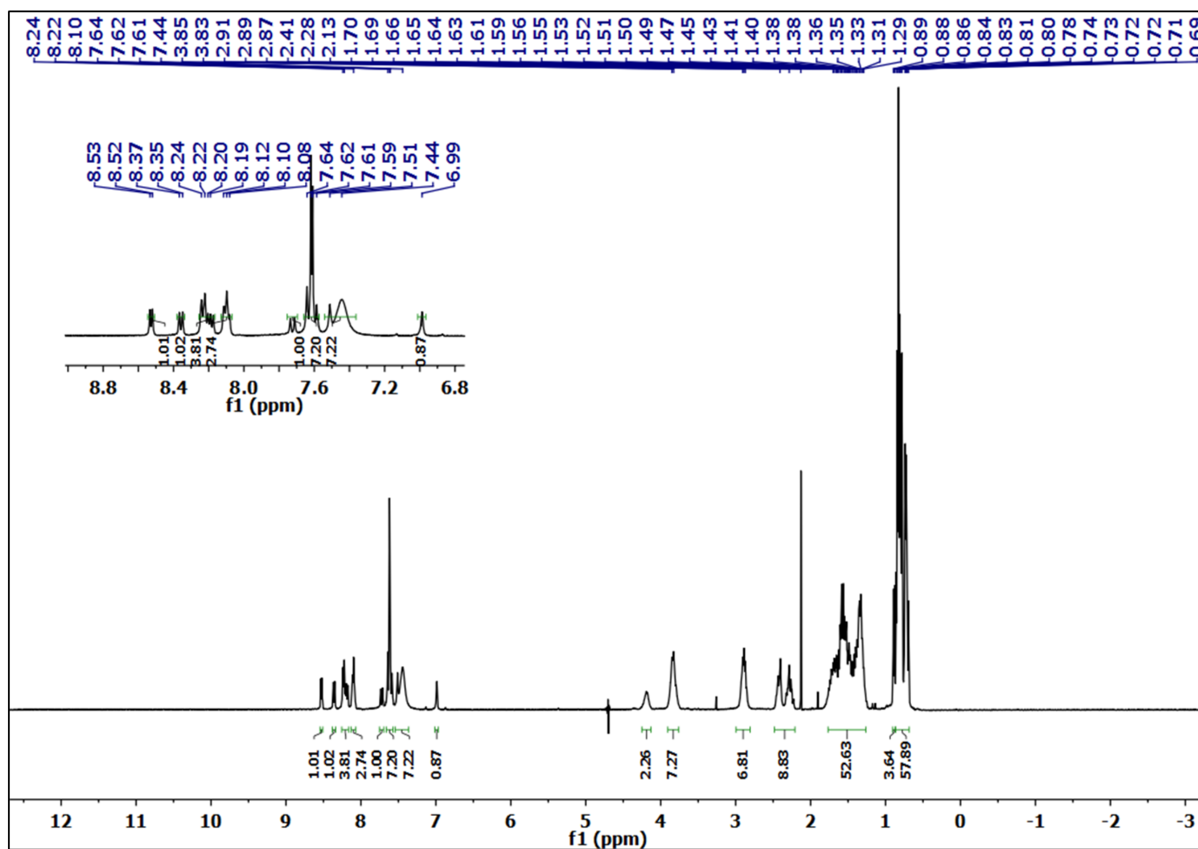


MALDI-TOF P7: mass calcd.[M+Na$^+$]= 1928.1938, mass observed = 1928.25



¹H NMR OF P7:



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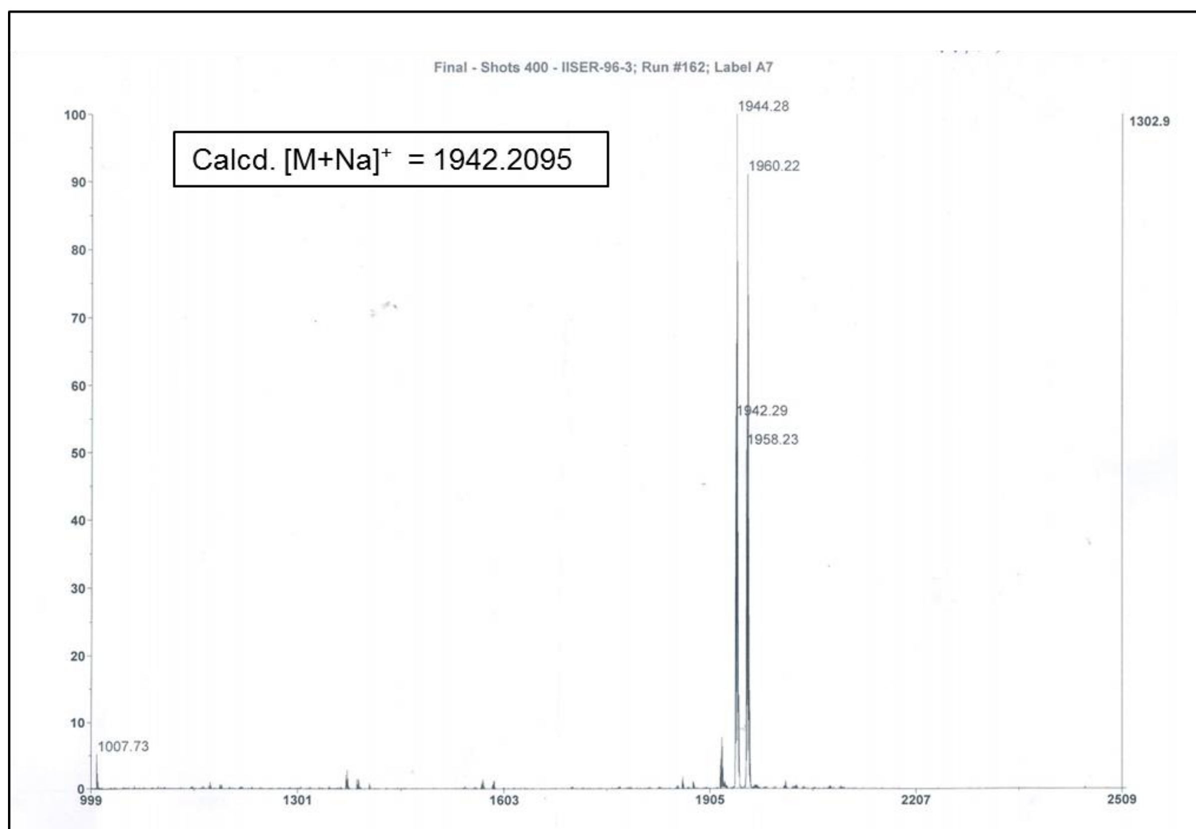
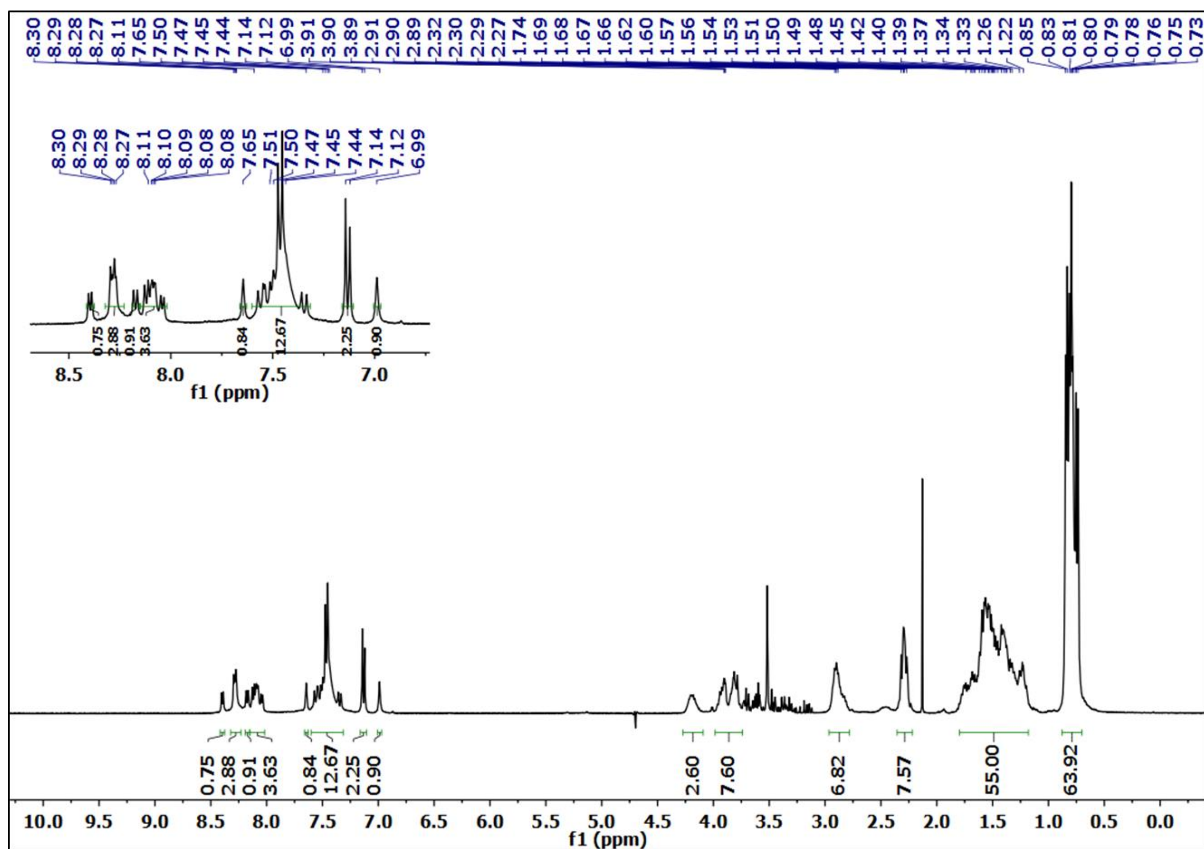


^{1}H NMR FOR P8



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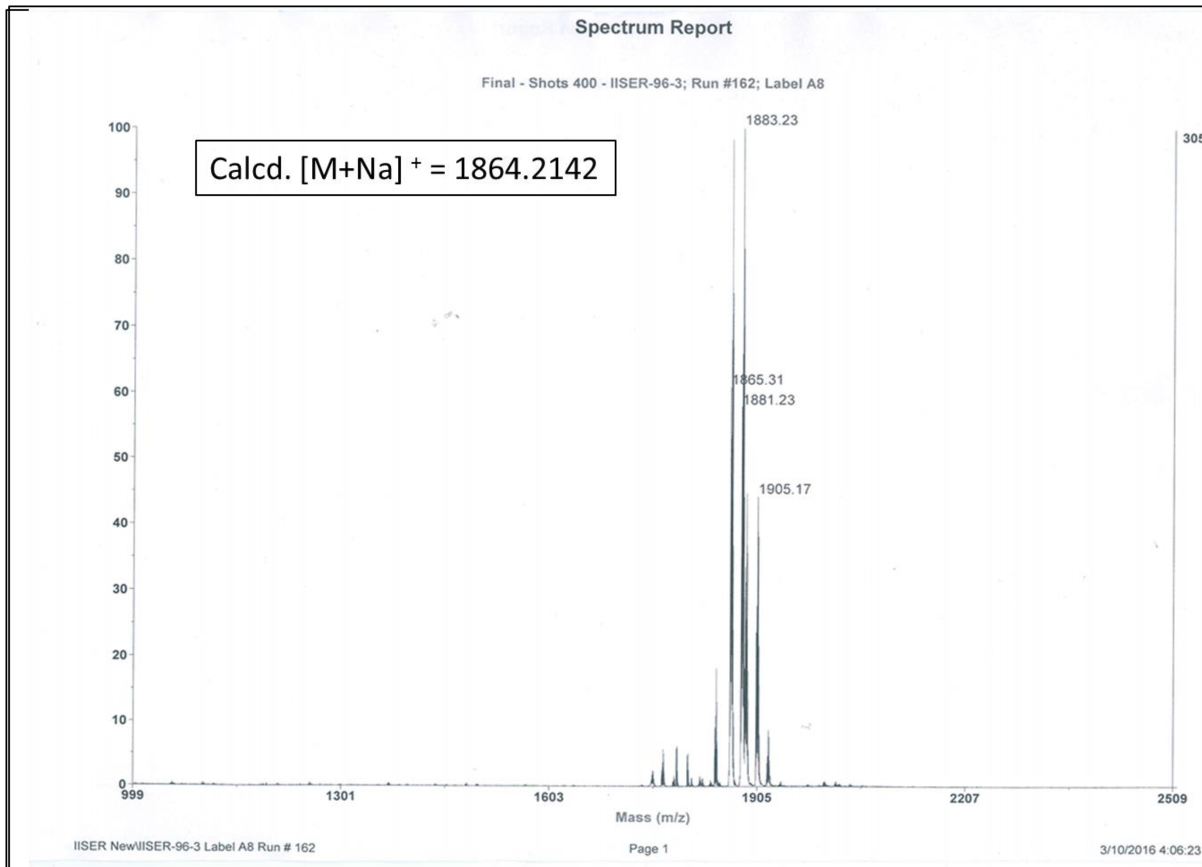
Mass calcd.[M+K$^+$]= 1880.1881, mass observed= 1881.23



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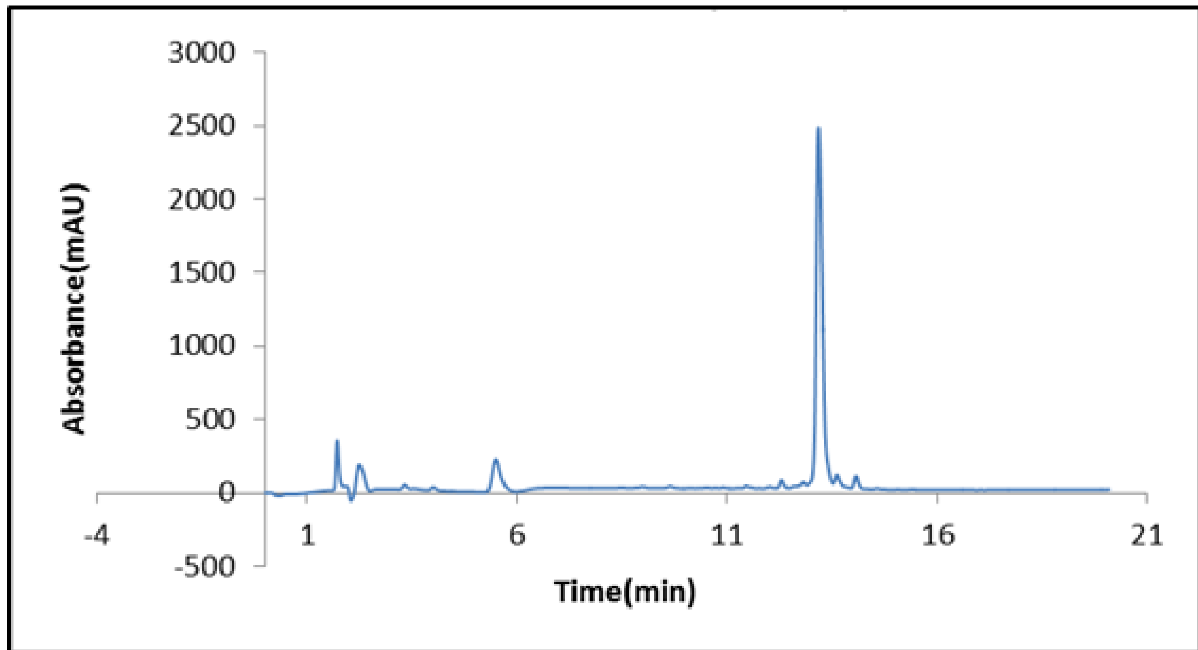
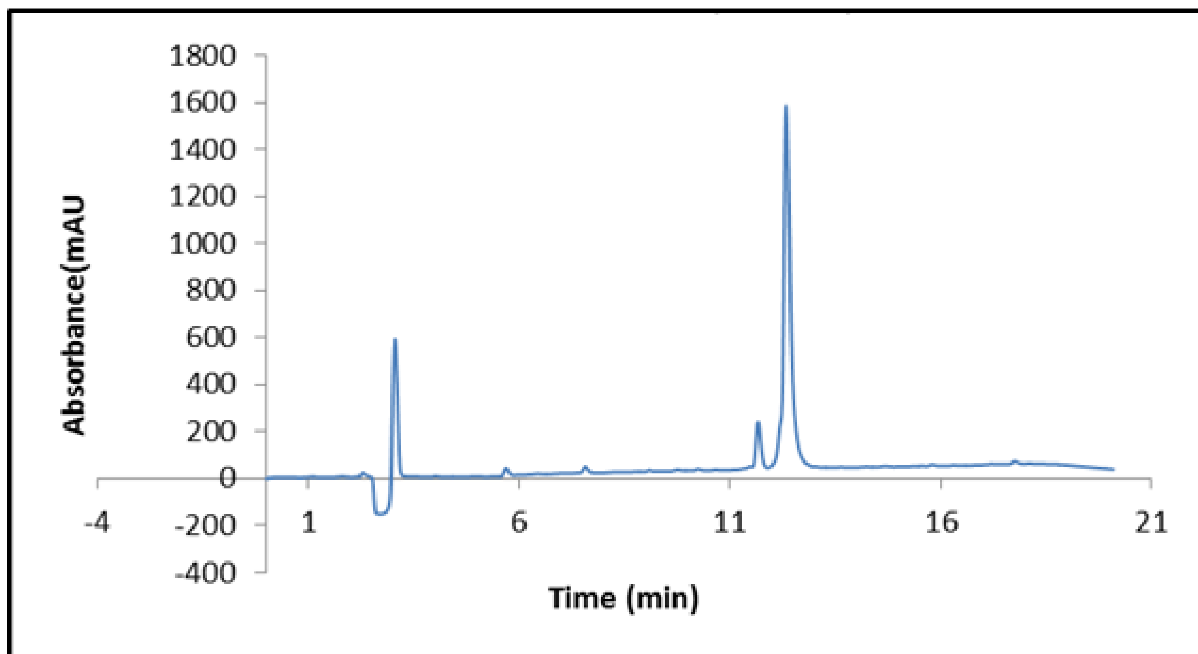


HPLCE TRACE FOR P8:



HPLC TRACE FOR P9: