Synthesis and utilisation of β –hydroxy γ-amino acids (Statines) in the design of hybrid peptide foldamers and their biological applications

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

By

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20091070

Under the guidance of

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CERTIFICATE

This is to certify that this dissertation entitled "***Synthesis and utilisation of β-hydroxy γ-amino acids in the design of hybrid peptide foldamers and their biological applications***" 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 "***Ankita Malik*** at IISER Pune " under the supervision of "***Dr. Hosahudya N. Gopi***, Associate professor, Department of Chemistry, IISER Pune" during the academic year 2013-2014.

1st April 2014

Dr. Hosahudya N. Gopi

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DECLARATION

I hereby declare that the matter embodied in the report entitled "***Synthesis and utilisation of β-hydroxy γ-amino acids in the design of hybrid peptide foldamers and their biological applications***" 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.

1st April 2014

Ankita Malik

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CONTENTS

ABBREVIATION

Ac = Acyl

Ac_2O = Acetic anhydride

Aib/U = 2-Aminoisobutyric acid

Ala/A = Alainine

Arg/R = Arginine

Boc = tert-Butoxycarbonyl

$(Boc)_2O$ = Boc anhydride

DiPEA = Diisoproylethyl amine

DMF = Dimethyl formamide

EtOAc = Ethyl Acetate

Fmoc = 9-Fluorenylmethoxycarbonyl

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

GABA = γ-Aminobutyric acid

Glu = Glutamic acid

Gly/G = Glycine

HBTU = O-Benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate

HCl = Hydrochloric acid

HOBt = Hydroxybenzotriazol

HPLC = High performance liquid chromatography

IBC-CL = Isobutyl chloroformate

IBX = 2-iodoxybenzoic acid

Leu/L = Leucine

LS(R,S) = *syn (3R, 4S)* β-hydroxy γ-leucine

MeOH = Methanol

Met = Methionine

$NaBH_4$ = Sodium Borohydride

NaOH = Sodium hydroxide

NMP = N-methyl pyrrolidone

NMR = Nuclear Magnetic Resonance

PhAc = Phenyl acetyl

Phesta(R,S)/FS(R,S) = *syn (3R, 4S)* β-hydroxy γ-phenylalanine

Phesta(S,S)/FS(S,S) = *anti (3S, 4S)* β-hydroxy γ-phenylalanine

^{D}Pro/P = ^{D}Proline

Ser = Serine

$SnCl_2$ = Tin chloride

TFA = Trifluoro acetic acid

THF = Tetrahydrofuran

Val/V = Valine

ABSTRACT

Gamma-amino-β-hydroxy acids (statines) have been extensively investigated for their biological activities, however, very little is known regarding their conformational behaviour. The natural occurrence and their excellent biological activities inspired us to investigate the conformational behavior of these amino acids in peptides. In this regard, various hybrid peptide helices and hybrid β-hairpins were designed and synthesized. The conformational properties of designed hybrid peptides were studied in both solution and in single crystals. Results of these preliminary investigations reveal that statines can be incorporated into the helices irrespective of the stereochemistry of the β-hydroxyl group; however, the hydroxyl group with *anti* stereochemistry with respect to the side-chain may facilitate the helical folds through additional H-bond between the β-hydroxyl group and amide carbonyl groups. Further, statine residues at the facing position of anti-parallel β-strands also stabilize the overall β-hairpin conformation without much deviation from the overall β-hairpin conformation in solution. In addition to understanding the conformational properties of statines in the helix and β-hairpin structure mimetics, we have also designed a water soluble analogue of universal aspartic acid protease inhibitor pepstatin and studied its inhibitory activity against protease pepsin. Preliminary results suggest that soluble pepstatin inhibits the proteolytic activity of pepsin.

INTRODUCTION

Designing protein secondary structure mimetic from the unnatural monomers is a very attractive field of interest. The folding properties of unnatural amino acid oligomers do not only give insight into the protein folding but also provide proteolytically stable protein structure mimetics. These properties of unnatural foldamers have made them very attractive from the perspective of medical and biological chemistry. Understanding of peptide secondary structures through *de novo* design has drastically expanded our knowledge towards the folding and functions of proteins.[1] The success of this endeavor has been elegantly demonstrated in the synthetically derived oligomers of β- and γ-amino acids as well as hybrid peptides composed of α/β, α/γ and β/γ-amino acids.[2] The β-amino acids are homologated species of α-amino acids. Depending on the position of the side chain, they are classified as β^3- and β^2-amino acids. Similarly, the double homologated γ-amino acids are classified as γ^2, γ^3 and γ^4-amino acids. The secondary structures displayed by the oligomers of β-amino acids can be further classified as helices, sheets and turns. The helices produced by the β-peptides are recognized as C_{14}-, C_{12}-, C_{10}- and C_8-helices. The helices from β-peptides have displayed different polarities with respect to their C and N-termini. The C_8- and C_{12}-helices have a hydrogen bond direction (C ←N), which is the same as that observed in α-peptides, whereas in the C_{10}- and C_{14}- structures the hydrogen bond directions (N ←C) are reversed. The stability of the helices increases upon progressing from α- to β- to γ-peptides. The γ- peptides form a right handed C_{14}-helix with 2.6 residues per turn. Comparatively, γ- and hybrid γ-peptides composed of γ^4-amino acids are less studied than the β- and other γ-peptide counterparts. Various ordered secondary structure mimetics have also been derived from the hybrid peptides composed of mixed α/β, α/γ and β/γ sequences. The advantage of hybrid peptides (heterooligomer) over the homooligomers is that various ordered helical structures can be designed by varying the position and sequence patterns of constituent amino acids. These fascinating structural features of hybrid peptide foldamers with expanded H-bond psuedocycles have greatly

expanded the repertoire of the folded polypeptide structures. Some of the secondary structure mimetics obtained from the α/β hybrid peptides[3] are shown in **Figure 1**.

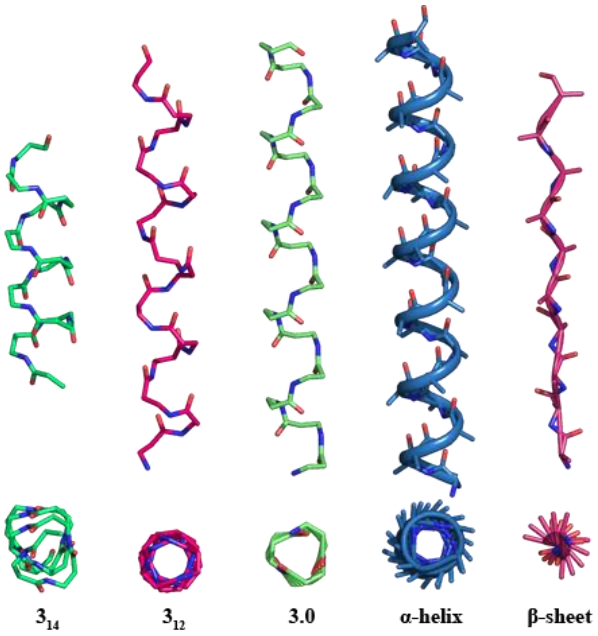


Figure 1: strucutres based on β2 and β3 amino aicds

The conformational analysis of γ- and hybrid γ-peptides with various types of γ-amino acids, γ^4-amino acids,[4] 3, 3-dialkyl γ-amino acids,[5] cyclic γ-amino acids,[6] and 2, 3, 4-alkyl substituted γ-amino acids[7] revealed their preference towards helical organization. However, peptides with α, β-unsaturated γ-amino acids[8] and 4,4-dimethyl substituted γ-amino acids[9] preferred to adopt extended sheet type of structures. We have been interested in the conformational analysis of various γ- and α,γ-hybrid peptides and sought to investigate the conformational behaviour of naturally occurring biologically important non-ribosomal β-hydroxy γ-amino acids (statines) and hybrid peptides composed of these statine amino acids.

Gamma-amino-β-hydroxy acids (statines) are naturally occurring non-ribosomal γ-amino acids widely present in many peptide natural products. Peptides constituted with statines have been used as inhibitors for aspartic acid proteases. For example, pepstatin,[10] a naturally occurring peptide has shown broad inhibitory activities against

various aspartic acid proteases[11] such as, pepsin, cathespin D and E, rennin, HIV-1 protease, β-secretase, plasmepsin I and II of malarial parasite *Plasmodium falciparum* etc,. In addition, several natural peptides containing statines or modified statines, such as didemnins,[12] dolastatins,[13] hapolosin,[14] Tamandarins,[15] etc displayed promising anti-cancer properties. Some of the representative examples of biologically active peptide natural products containing statine amino acids are shown in **Figure 2**.

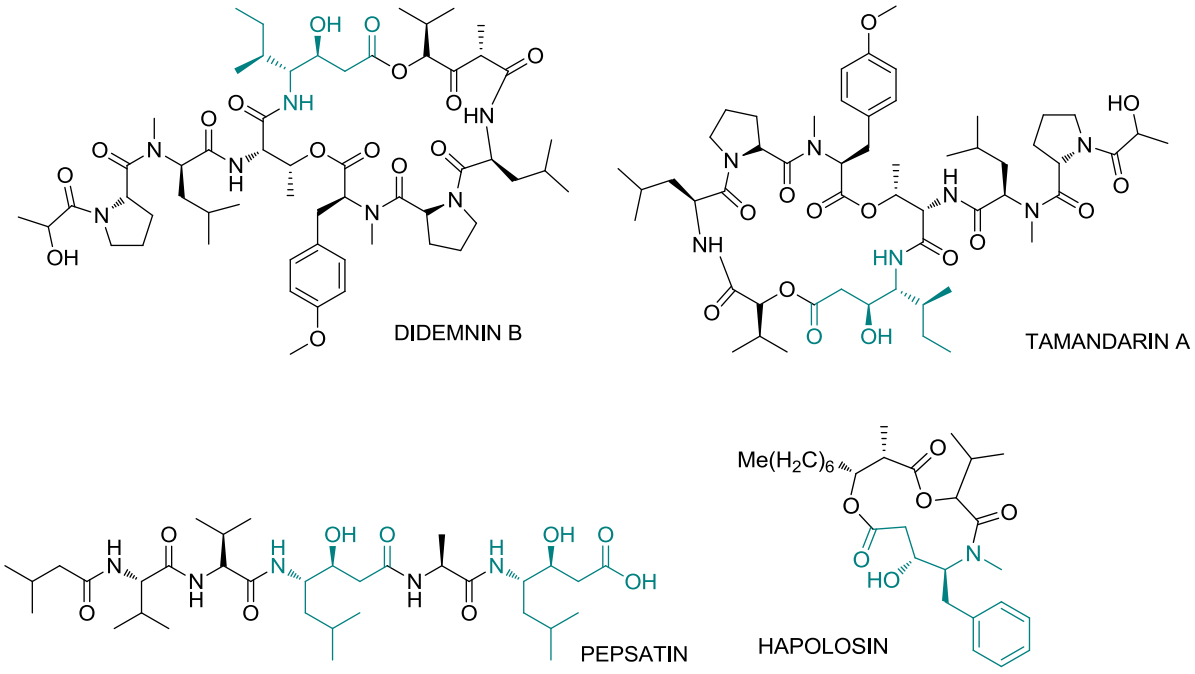


Figure 2: structure of naturally occurring statine based peptides

Inspired by the remarkable biological applications of these peptide natural products, various synthetic strategies have also been developed for the synthesis of statines.[16] Though the peptides containing statines have been extensively studied for their biological properties, however, very little is known regarding their conformational properties. The natural occurrence and their excellent biological activities inspired us to investigate the conformational behavior of these amino acids in designed hybrid helices and β-hairpins. In addtion, we also sought to investigate whether the highly insoluble

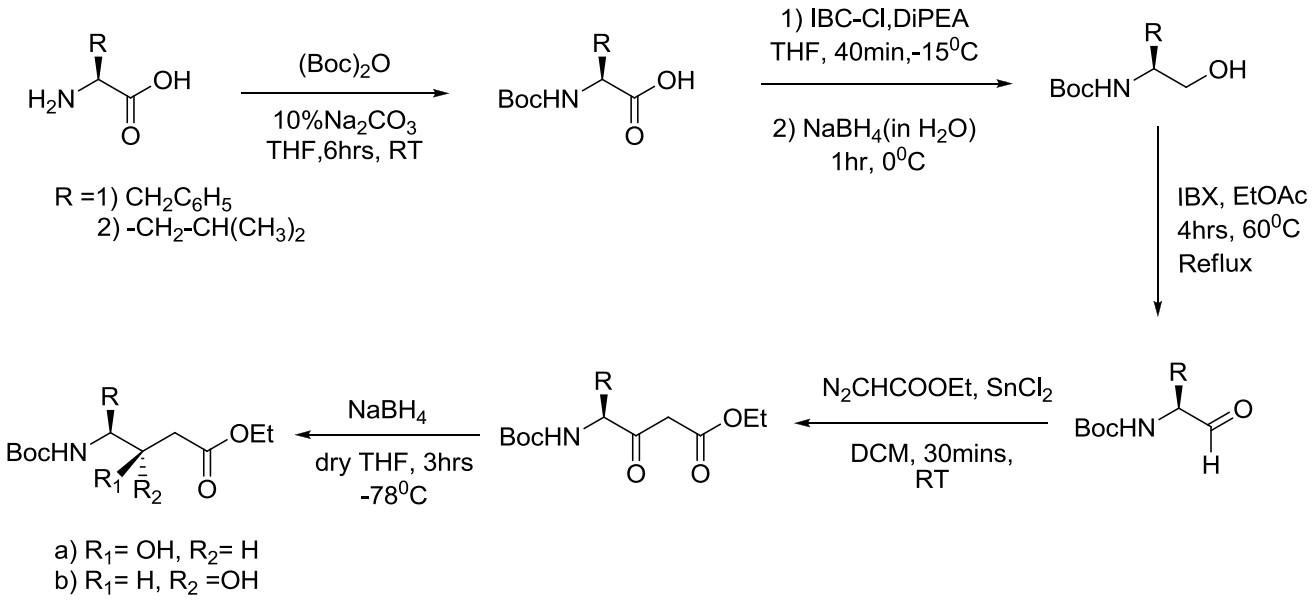
universal aspartic acid protease inhibitor pepstatin can be converted to water soluble analogue without affecting its biological activity.

RESULTS AND DISCUSSIONS

Synthesis of β-hydroxy γ-amino acids:

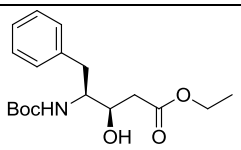
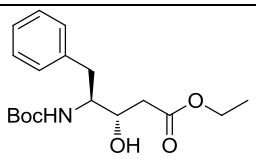
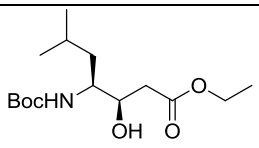
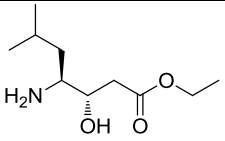
We have chosen to use β-keto-γ-amino esters as starting materials to synthesize β-hydroxy-γ-amino esters. The main reason is that the mild reduction of β-keto-esters gives both the diastereoisomers of statine residues and both the statine diastereoisomers can be exploited in the design of hybrid peptide foldamers. We recently reported the tin(II)chloride mediated synthesis of β-keto-γ-amino esters starting from α-amino aldehyde and ethyl diazoacetate. We utilize the same strategy for the synthesis of β-keto-γ-amino esters. The amino aldehydes were synthesized 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 IBC-Cl in the presence of a weak base DIEA. After obtaining β-keto-γ-amino esters through the modified Roskamp protocol[17], we subjected them to mild reduction using NaBH4 reported by Rich et al.[18] The schematic representation of the reaction is shown in **Scheme 1**. To understand the compatibility and the stereochemical output, we initially subjected β-keto-γ-amino esters to the $NaBH_4$ reduction in dry THF at -78 °C. The mild reduction of β-keto-γ-amino esters gave anti (the β-hydroxyl group is anti with respect to the amino acid side-chain) is a major product and *syn* (β-hydroxyl group is *syn* with respect to amino acid side-chain) product as minor isomer.[19] The *syn* and *anti* diastereoisomers were separated using column chromatography. The list of statine amino acid diastereoisomers synthesized using this protocol is given in the **Table 1**. These were further used for the solution phase synthesis of peptides after the selective deprotection Boc- and ester groups. Solid phase compatible Fmoc-amino acids were synthesized from the N- and C-terminal free statine amino acids obtained from the ester hydrolysis and subsequent

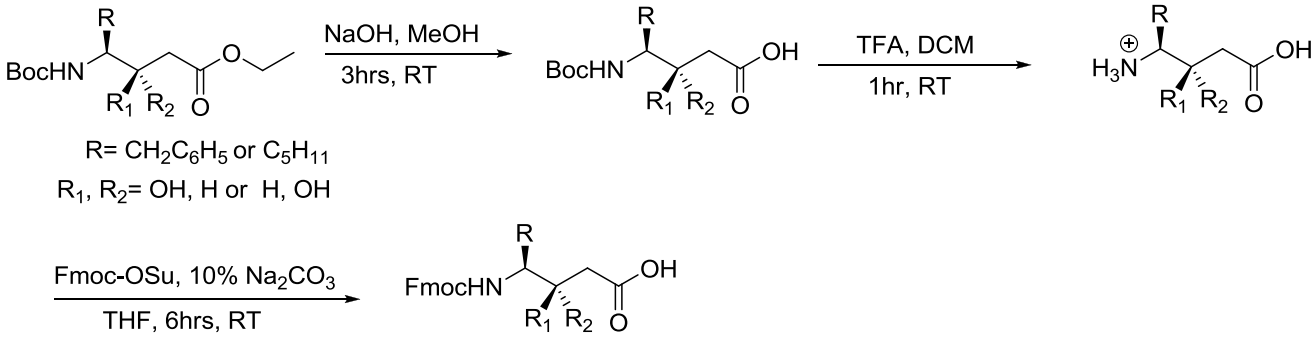
Boc-deprotection. The Fmoc-group was introduced using Fmoc-OSu in the mild basic environment. The schematic representation of the reaction is shown in **Scheme 2.**



Scheme 1: Synthesis of Boc -protected β hydroxyl γ amino esters from α amino acid.

Table 1: List of β hydroxyl γ amino acids synthesised from α amino acids

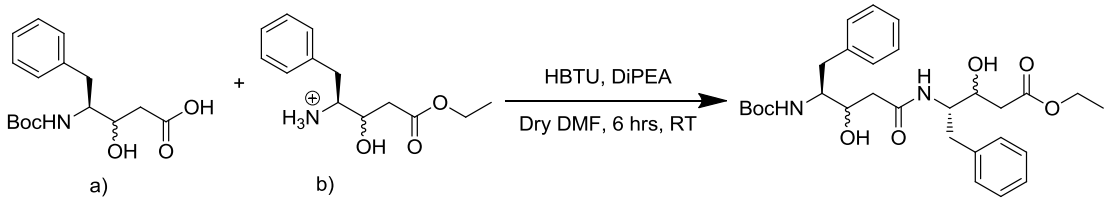
S.No.	R	R_1	R_2	Statine	Yield %	Overall yield%
1a	$-CH_2C_6H_5$	-OH	-H		65	95
1b	$-CH_2C_6H_5$	-H	-OH		35	
2a	$-CH_2CH(CH_3)_2$	-OH	-H		64	92
2b	$-CH_2CH(CH_3)_2$	-H	-OH		36	



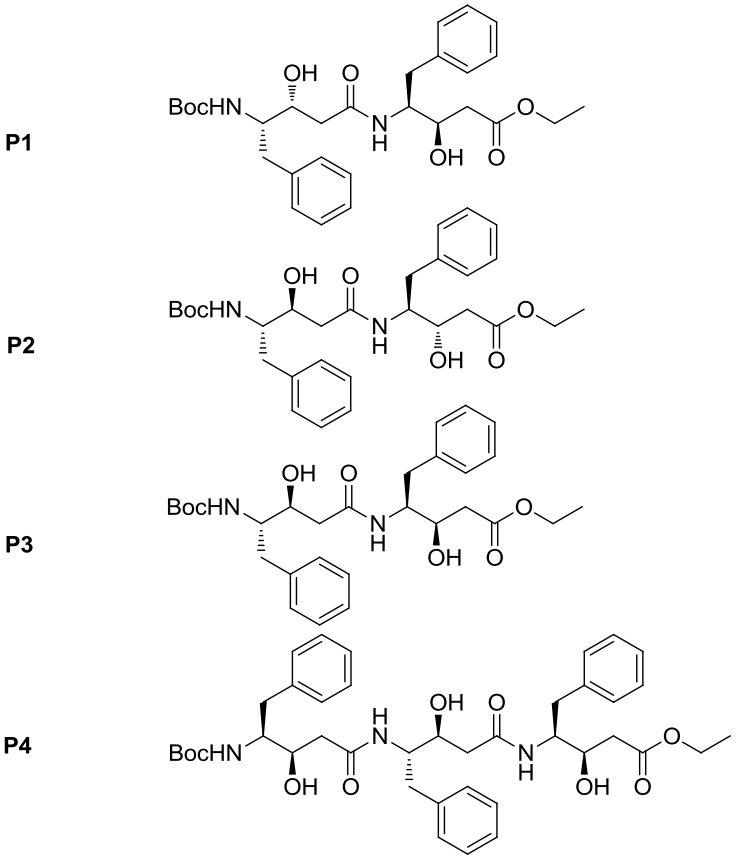
Scheme 2: Synthesis of Fmoc-protected statine amino acids from the ethyl esters of Boc-protected statines.

Synthesis of statine homooligomers:

To understand whether the statine peptides can be synthesized without protection of the β-hybroxyl group, we subjected both the diastereoisomers of 1a and 1b to the dipeptide synthesis. The dipeptides **P1**, **P2** and **P3** were synthesized in solution phase using Boc chemistry. In the case of **P1**, the diastereoisomer 1a was used and in the case of **P2** the diastereoisomer 1b was used. Both 1a and 1b were used in the case of **P3**. The schematic representation of the synthesis of statine dipeptides is shown in **Scheme 3**. The Boc-group deprotected N-terminal free statine was coupled with free carboxylic acid of the other statine. The coupling reactions were performed using HBTU/HOBt standard coupling conditions. All three dipeptides were isolated in good yield after the column purification. These results suggest that statines can be used in the peptide synthesis without protection of the β-hydroxyl group. Inspired by these results, we further extended same strategy to the synthesis of tripeptide **P4**. The N-free dipeptide **P3** was coupled to the free acid of amino acid 1a to give tripeptide **P4**. The tripeptide **P4** was purified by reverse phase on C18 column using MeOH/H_2O gradient system. The sequence of all four statine peptides are shown in **Scheme 4.**



Scheme 3: Synthesis of dipeptide in solution phase



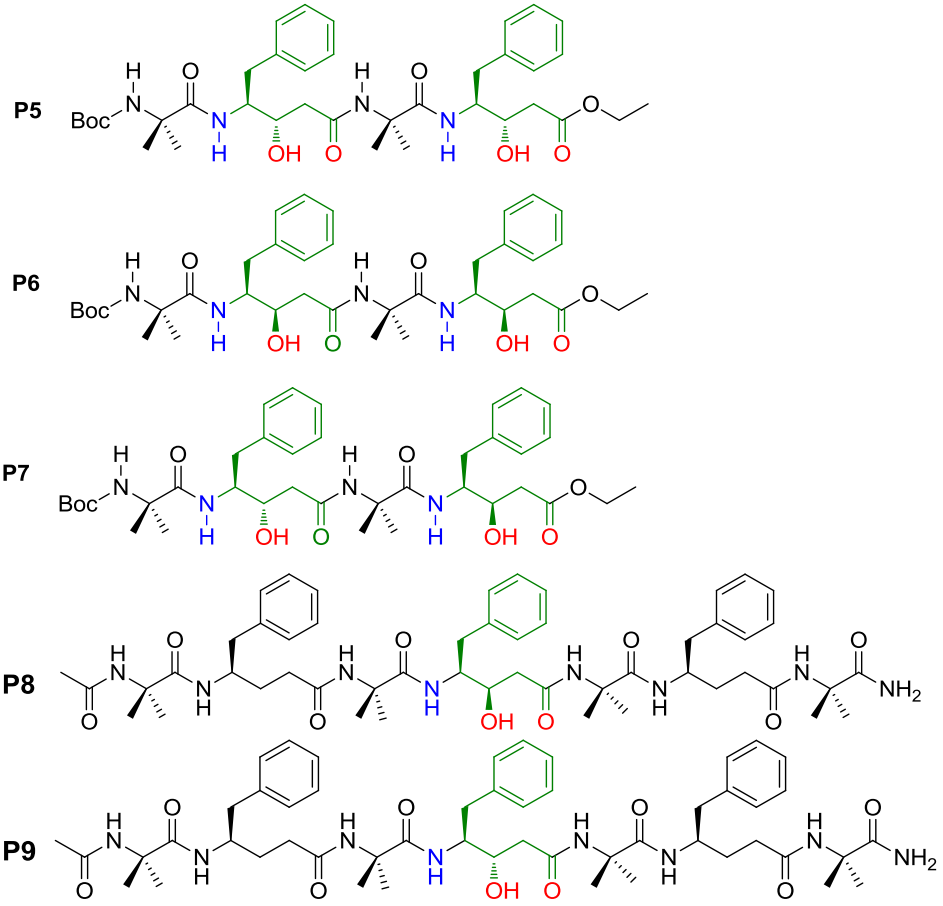
Scheme 4: Sequences of dipeptides and tripeptides generated from the β-hydroxy-γ-phenylalanine

The conformational analysis of these statine oligomers is under progress.

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

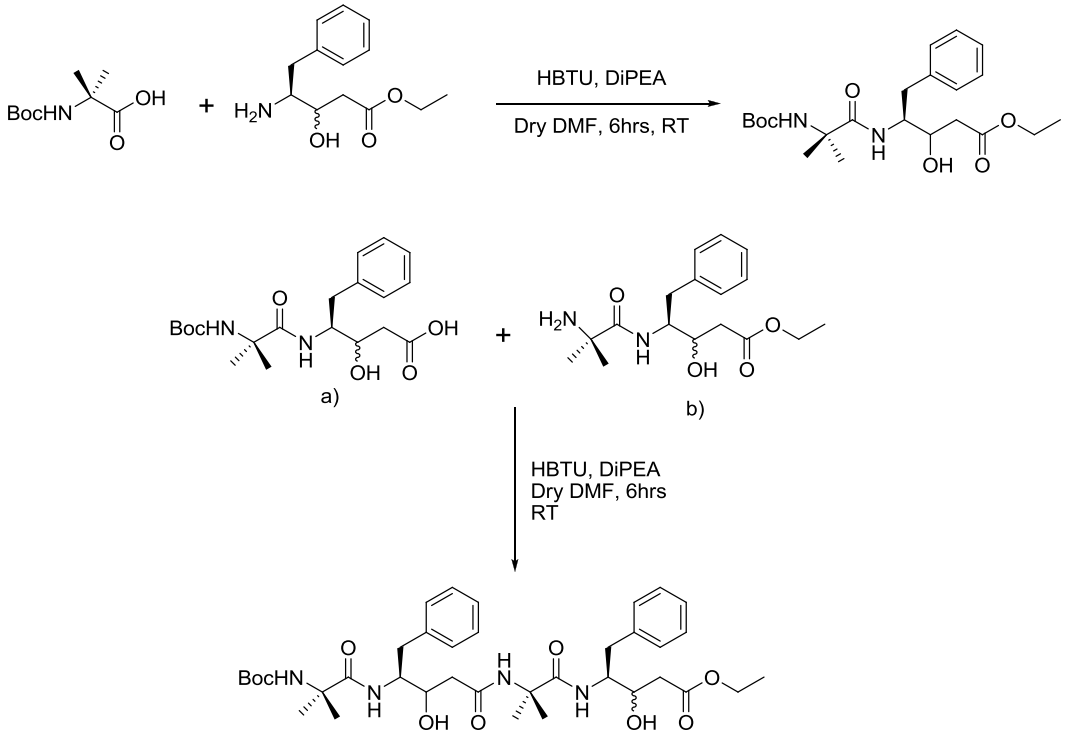
Recently, we reported the stable 12-helical conformations of α,γ-hybrid peptides composed 1:1 alternating α- and γ^4-amino acids. In order to understand the conformational behaviour of statine residues in the hybrid peptide sequences, we

designed four hybrid peptides. The sequences of these hybrid peptides along with recently reported α/γ-statine hybrid peptide **P5** are shown in the **Scheme 5**. The hybrid peptides **P6** and **P7** are composed of 1:1 alternating α- and statine residues, while **P8** and **P9** composed of single statine residues along with saturated γ^4-amino acids in 1:1 alternating α, γ- hybrid peptides. Synthesis and crystal conformation of **P5** is recently reported.[20] In order to get clear understanding about the conformational properties of statine residues, **P6** and **P7** were synthesised through solution phase chemistry using 2+2 convergent strategy and purified by column chromatography. The schematic representation of the tetrapeptides synthesis is shown in the **Scheme 6**. The heptapeptides, **P8** and **P9,** were synthesized by solid phase method on Knorr amide resin using standard Fmoc- chemistry. The coupling reactions



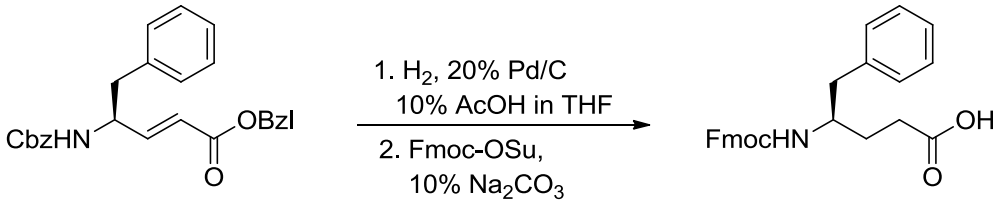
Scheme 5: Sequences of hybrid peptides composed of 1:1 alternating Aib and Phestatine residues.

were performed using HBTU/HOBt coupling reagents. The γ^4-Phe was synthesized through Wittig reaction as reported earlier by our group.[21] The α, β-



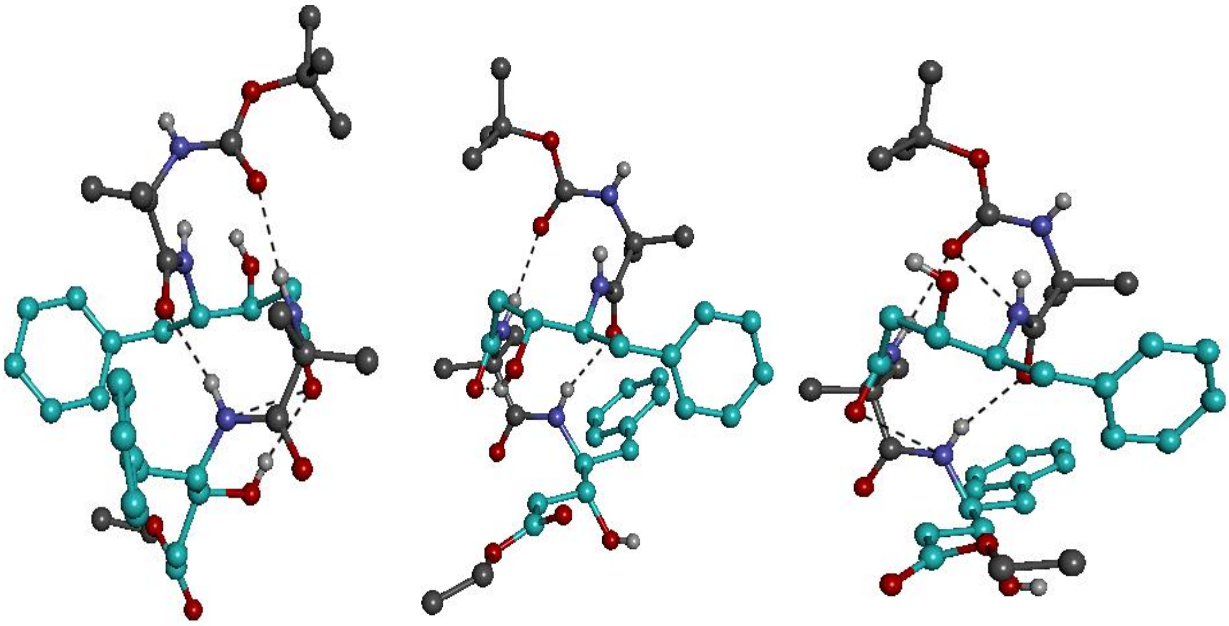
Scheme 6: synthesis of α/γ hybrid tetrapeptide

unstaturated γ-amino acid obtained after the Witting reaction was reduced to saturated γ-amino acid through catalytic hydrogenation. The schematic representation of the synthesis γ^4-Phe is shown in **Scheme 7**.



Scheme 7: Synthetic scheme for γ phenyl alanine[22]

To understand the conformations of **P6** and **P7** we subjected both the peptides for crystallization in various solvent combinations. The X-ray quality single crystals were obtained from the slow evaporation of solution of peptides in aqueous methanol. The X-ray structures of **P6** and **P7** peptides are shown in **Figure 3** along with **P5**.



P5 **P6** **P7**

Figure 3: X-ray structures of **P6** and **P7** along with **P5**.

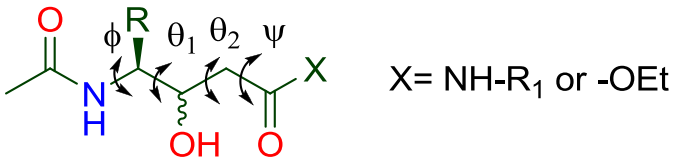


Figure 4: Local torsional variables of statine residues

As reported earlier, the helical conformation of **P5** is stabilized by two intramolecular 12-membered H-bonds.[20] The analysis of the crystal structure of **P6** and **P7** with (*3R, 4S*) *β*-hydroxy-*γ*-phenylalanine (*syn*) suggests the presence of a single molecule in their asymmetric unit. Similar to **P5**, **P6** and **P7** also adopted 12-helical conformations in the single crystals and the structure is stabilized by two intramolecular H-bonds between

the residues *i* and *i+3*. The torsional angles of statine residues were measured by introducing two additional variables θ_1 (N-C^{γ}-C^{β}-C^{α}) and θ_2 (C^{γ}-C^{β}-C^{α}-C') along with ϕ (C'-N-C^{γ}-C^{β}) and ψ (C^{β}-C^{α}-C'-N/O) (**Figure 4**). The torsion values of statines residues in all three peptides along with statine amino acid diastereoisomers 1a and 1b are given the **Table 2**.

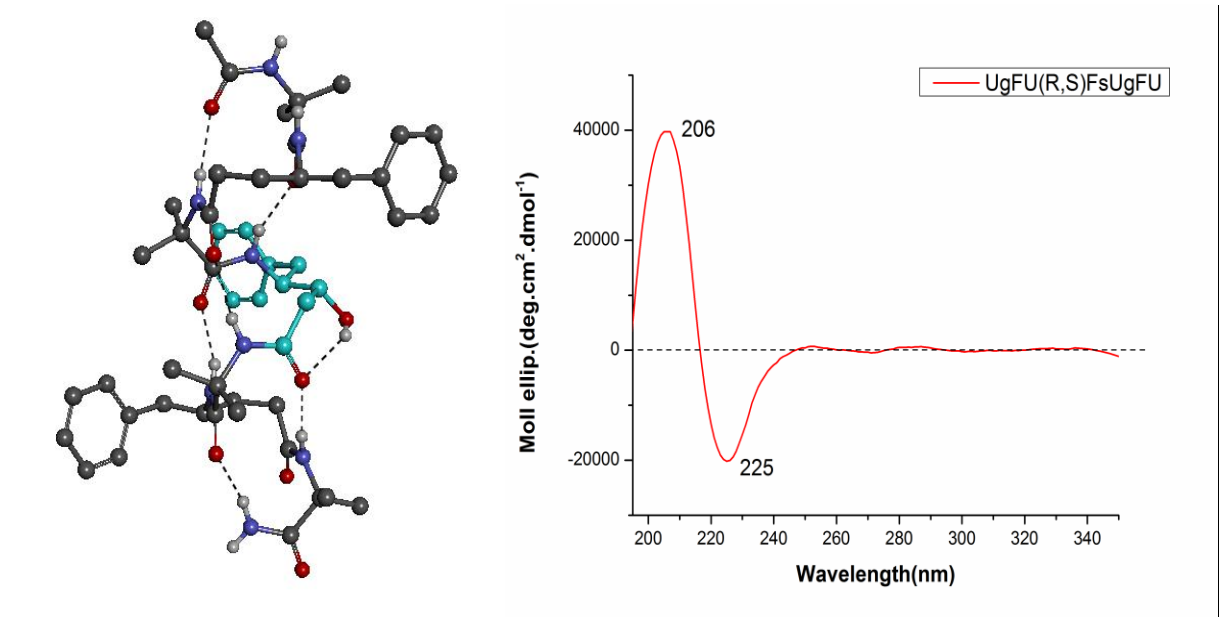
Table 2: Tortional values for statine in tetrapeptides

No.	Residue	ϕ	θ1	θ2	ψ
AAs	1a	-135	35	162	94
	1b	-131	59	-169	-172
P5	Phesta2	-123±3	53±3	60±3	-122±1
	Phesta4	-105±3	173±3	168±9	154 (-57)
P6	Phesta2	-126	51	63	-121
	Phesta4	-105	56	-170	-158
P7	Phesta2	-128	54	56	-121
	Phesta4	-110	57	-179	27

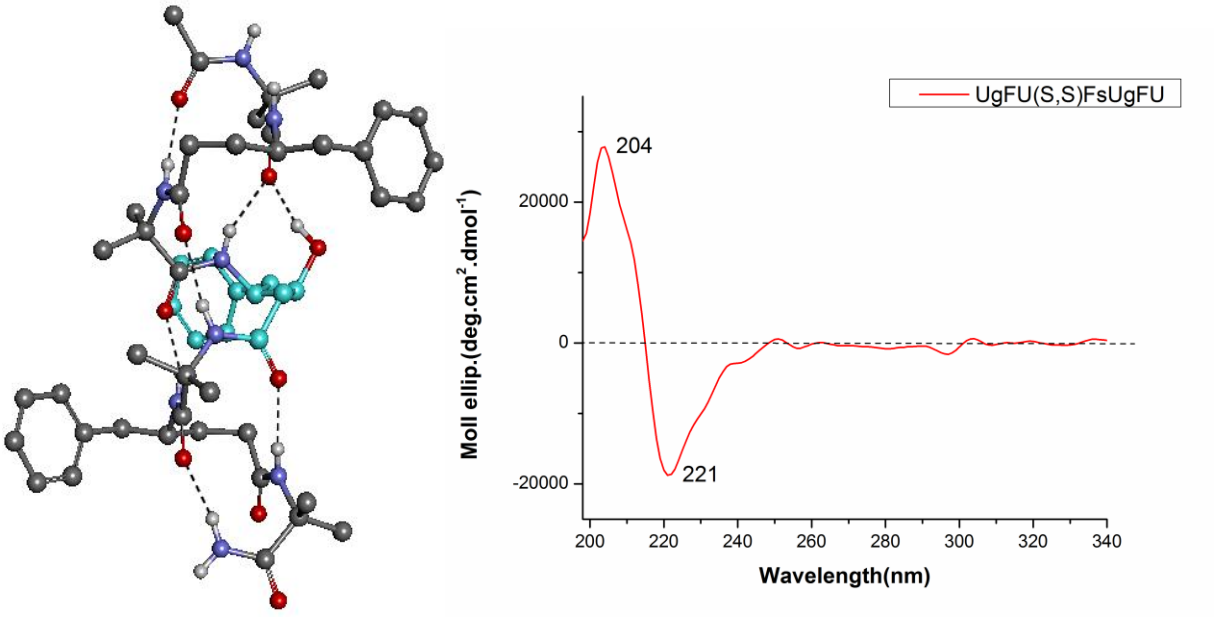
Similar to the other α,γ-hybrid peptide 12-helices, the α-residues displayed ϕ and ψ values around -60 and -40, respectively. Similar to **P5**, the Phe-sta2 in both **P6** and **P7** accommodated nicely into the 12-helical conformation by adopting g^+, g^+ conformation along θ_1 and θ_2. However, as observed in **P5**, Phe-sta4 in both **P6** adopted extended conformation by having the torisonal values θ_2 and ψ -170° and -158°, however, ψ displayed 27° in the case of **P7**. Due to the stereochemical requirement, the *β*-OH group of (*3R, 4S*) *β*-hydroxy-*γ*-phenylalanine in **P6** pointed towards C-terminus of the helix similar to the amide CO groups. In contrast to the *β*-hydroxyl group of *anti* (*3S,4S*)Phe-sta2 in **P5**, the *β*-OH group of the Phe-sta2 in **P6** is involved in the six membered intramolecular H-bonding with CO of the same residue with a C=O---H distance of 2.09 Å and C=O---H-O angle 142°. Interestingly, Phe-sta2 **P7** is not involved in the intramolecular H-bonding. The intraresidue H-bonding is not observed in the terminal Phe-sta4 in all the peptides as it is not participating in the helix due to the lack

of C-terminal amide NH, however, it is involved in the strong intermolecular H-bonding with the solvent methanol. The structural analysis of **P5**, **P6** and **P7** reveal that irrespective of the stereochemistry at the ***β***-position, these peptides adopted right handed helical conformation, except the projection of the β-hydroxyl groups and their pattern of H-bonding. Overall, these results suggest that short hybrid peptides constituted with *syn* and *anti* diastereoisomers of ***β***-hydroxy γ-amino acids adopted 12-helical conformations without much deviation from the hybrid peptides composed of α- and γ^4-amino acids. Further, statine hybrid peptides displayed additional inter and intramolecular H-bonds due to the presence of ***β***-hydroxyl groups.

Inspired by these results, we designed two heptapeptide **P8** and **P9** by incorporating single statine residue at the fourth position of the known α,γ-hybrid peptide composed of Aib and γ-Phe to understand the influence of β-hybroxyl group on the stability of α,γ-hybrid peptide 12-helix. Both peptides were synthesized using solid phase method and purified by HPLC. The CD analysis of the both the hybrid peptides displayed the characteristic signature of the parent 12-helix (**Figure 5**), suggesting that the accommodation of the statine residues in higher ordered helices. The 2D NMR and X-ray analysis of these peptides is currently under progress. The model structures of **P8** and **P9** generated based on the X-ray structures of the parent peptides using Discovery studio are shown in **Figure 5**.



P8



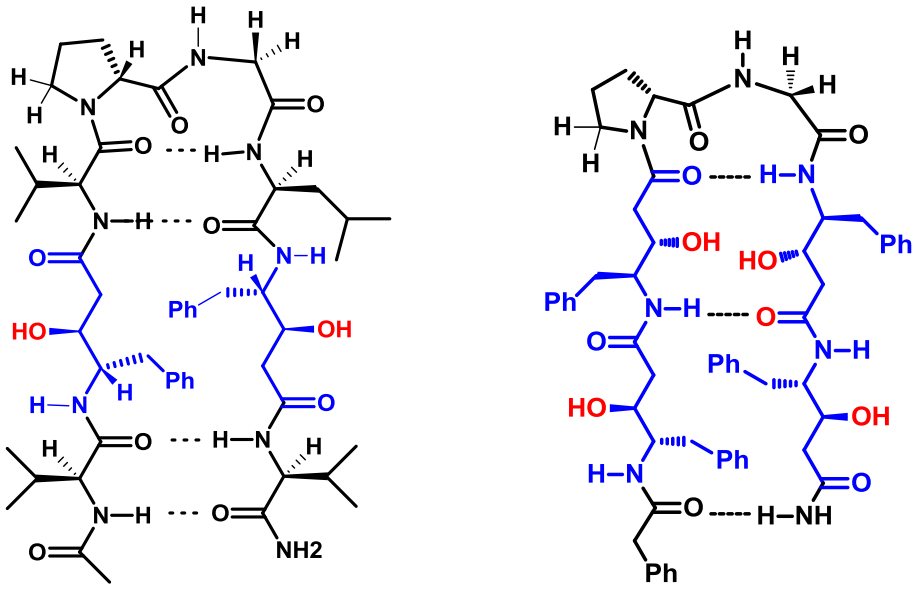
P9

Figure 5: Model structures and CD spectra of **P8** and **P9** depicting 12-helical conformations. The models were generated through Discovery studio using crystal structure information of the parent α,γ-hybrid peptide.

Overall these studies reveal that the statine residues can be incorporated into hybrid peptide 12-helices without much deviation from the overall folding of the 12-helix. The statine amino acids with anti stereochemistry may influence the stability of the helical folds through additional H-bonding.

Design, synthesis and conformational analysis of hybrid β-hairpins

Beta-hairpins are another very important class of secondary structures widely present in protein structures. A β-hairpin is a simplest structural motif consists of anti-parallel β-sheets and a reverse turn. Many proteins often constitute a β-hairpin scaffold for biomolecular recognition. In contrast to the helical secondary structures, β-hairpins have not been extensively studied. Unlike α-helical peptides, where structure is stabilized by local H-bonds, β-strand requires a structural context for the stabilization. It has been shown that β-hairpin structures from outside the protein context can be stabilized using the sequences ^{D}Pro-Xxx (Xxx is Gly, Ala, Pro, Aib etc.,), Asn-Gly, Aib-Gly at the turn segment[23]. Further, the β-strand residues that modulate the β-hairpin stability depending upon their intrinsic β-sheet propensities through both cross strand and diagonal side chain-side chain interaction. Recently, our group reported the stable β-hairpin structure by exploiting the geometrical rigidity of α, β-unsaturated γ amino acids. Further it has been shown that saturated analogue of the unsaturated hairpin (hairpin with γ-amino acids) obtained after the catalytic hydrogenation destabilises the hairpin conformation through poor registry of the β-strands[24]. To understand whether the statine residues can stabilize the β-hairpin conformation through additional H-bonding along the anti-parallel β-strands, we designed two hybrid β-hairpins **P10** (Ac-Val-Phesta(S,S)-Val-^{D}Pro-Gly-Leu-Phesta(S,S)-Val-NH_2) and **P11** **(**Ac-Phesta(S,S)-Phesta(S,S)-^{D}Pro-Gly-Phesta(S,S)-Phesta(S,S)-NH_2) incorporated with statine residues at the facing position of the anti-parallel β-strands. The schematic representation of the **P10** and **P11** is shown in **Scheme 8**.



P10 **P11**

Scheme 8: Schematic representation of designed β-hairpins **P10** and **P11**. Statines are highlighted in blue colour.

Both β-hairpins **P10** and **P11,** were synthesised on solid phase using HBTU/HOBt as coupling agents on Knorr amide as resin using standard Fmoc- chemistry. After completion of synthesis, the N-terminal was protected with acyl group using acetic anhydride and pyridine. The peptide was then cleaved from resin using pure TFA. In addition to the N-acetylation, we also observed O-acetylation of the peptides in the MALDI TOF mass spectra. To remove O-acetylation, we further subjected crude peptides for the selective mild $NaBH_4$ reduction in methanol. After obtaining the O-deacetylated products, the peptides were subjected to the purification by reverse phase HPLC on C18 column using MeOH/H_2O solvent system. The pure peptides were further subjected to the 2D NMR and CD analysis to understand their solution state conformations.

The CD spectrum of **P10** recorded in methanol is shown in **Figure 6**. Peptide displayed CD minima at 230 nm suggesting that well folded conformation of the peptide in solution. In addition, the well dispersed amide proton signals (^{1}H NMR) of NH, CαH and CγH in CD_3OH indicate the well folded signature of the peptide in solution.

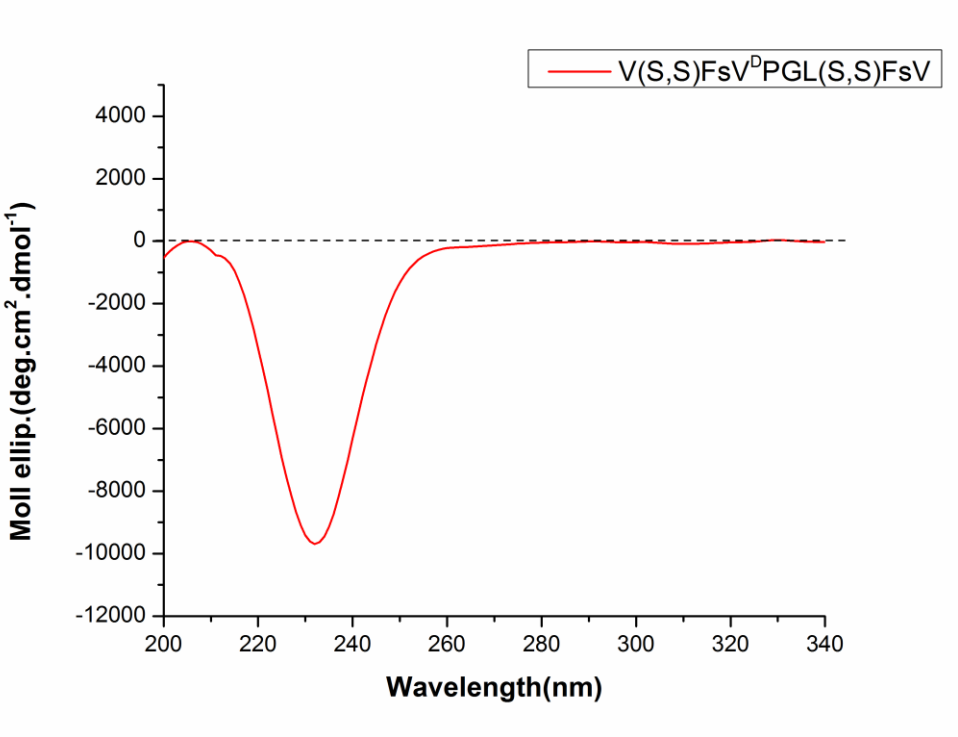


Figure 6: CD Spectra of the β hairpin **P10**

Further, we subjected the peptide **P10** to the 2D NMR analysis. As anticipated, the 2D NMR (TOCSY and ROESY) analysis showed the characteristic turn and cross-strand NOEs of antiparallel *β*-strands confirming a *β*-hairpin structure in solution. The fully assigned TOCSY spectrum of the peptide **P10** is shown in **Figure 7**. The fully assigned characteristic sequential NH↔C_αH, cross-strand NH↔NH and C_αH↔C_γH NOEs of ROESY spectrum are shown in the **Figures 8, 9** and **10**, respectively. The critical NOEs observed in the ROESY spectrum are highlighted in the model shown in **Figure 11**. The strong C_αH↔C_γH NOEs between the statine residues in **P10** indicating the stable antiparallel *β*-sheet character in solution. Further, we perform the temperature dependent ^{1}H NMR to understand the involvement of amide NHs in the intermolecular H-bonding. The ^{1}H NMR with increasing temperature is shown in **Figure 12**. The linear up field chemical shifts of *β*-strand amide NHs in *vt* NMR (temperature ranging from -10 to 40 ^{0}C), indicating the strong registry of the antiparallel *β*-sheets

(**Figure 13**). Further the higher (-ve scale) temperature co-efficient values (dδ/dt) of all amide NHs obtained from the slope suggesting grater sheet character of the peptide.

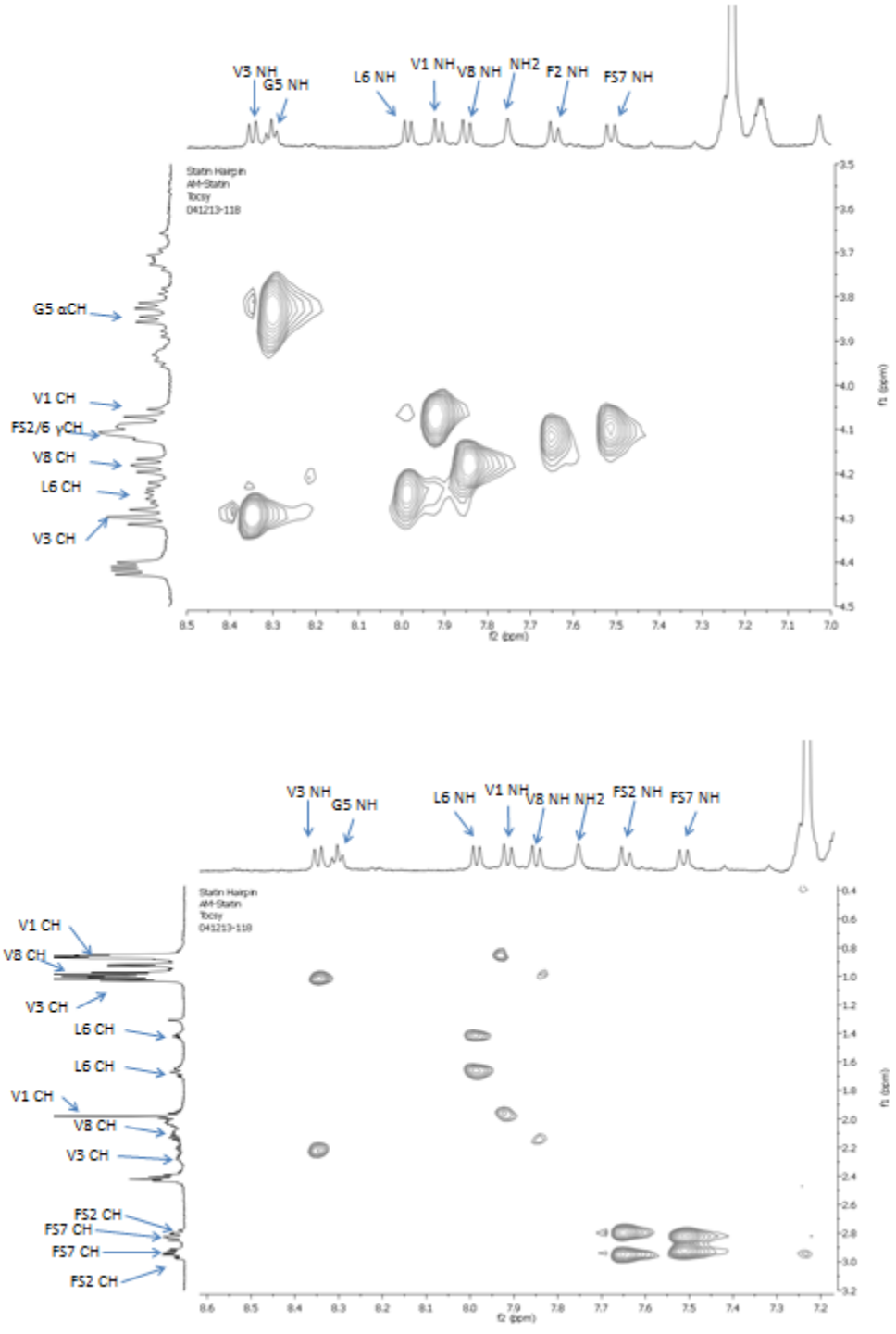
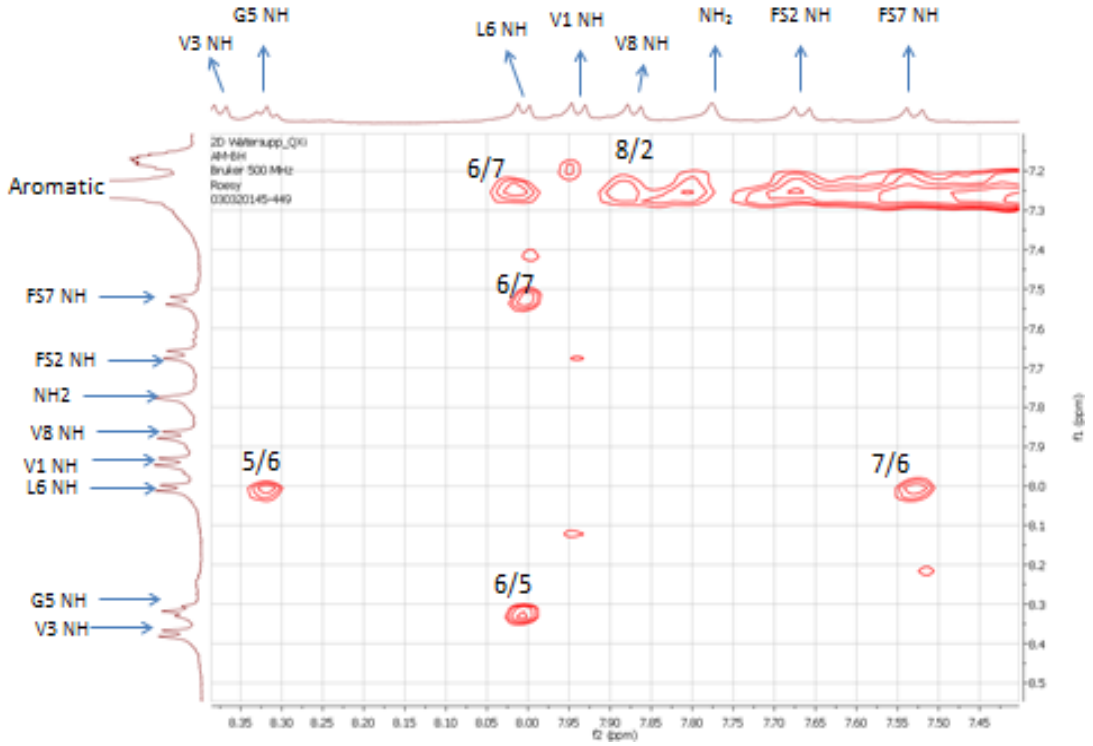
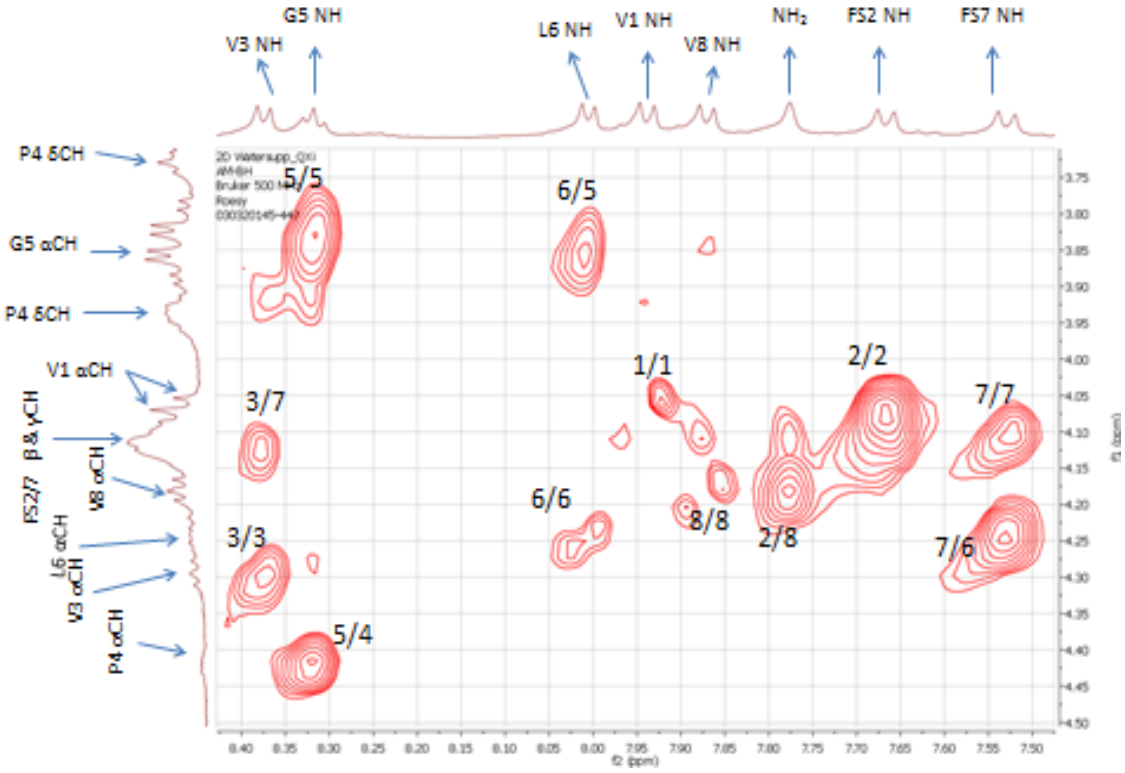


Figure 7: Tocsy spectra of peptide **P10**


G5 NH
V3 NH
L6 NH
V1 NH
V8 NH
NH2
FS2 NH
FS7 NH
Aromatic
FS7 NH
FS2 NH
NH2
V8 NH
V1 NH
L6 NH
G5 NH
V3 NH
6/7
8/2
6/7
5/6
7/6
6/5
f2 (ppm)
f1 (ppm)


G5 NH
V3 NH
L6 NH
V1 NH
V8 NH
NH2
FS2 NH
FS7 NH
P4 δCH
G5 αCH
P4 δCH
V1 αCH
FS2/7 β & γCH
V8 αCH
L6 αCH
V3 αCH
P4 αCH
5/5
6/5
1/1
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8/8
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3/3
7/6
5/4
f2 (ppm)
f1 (ppm)

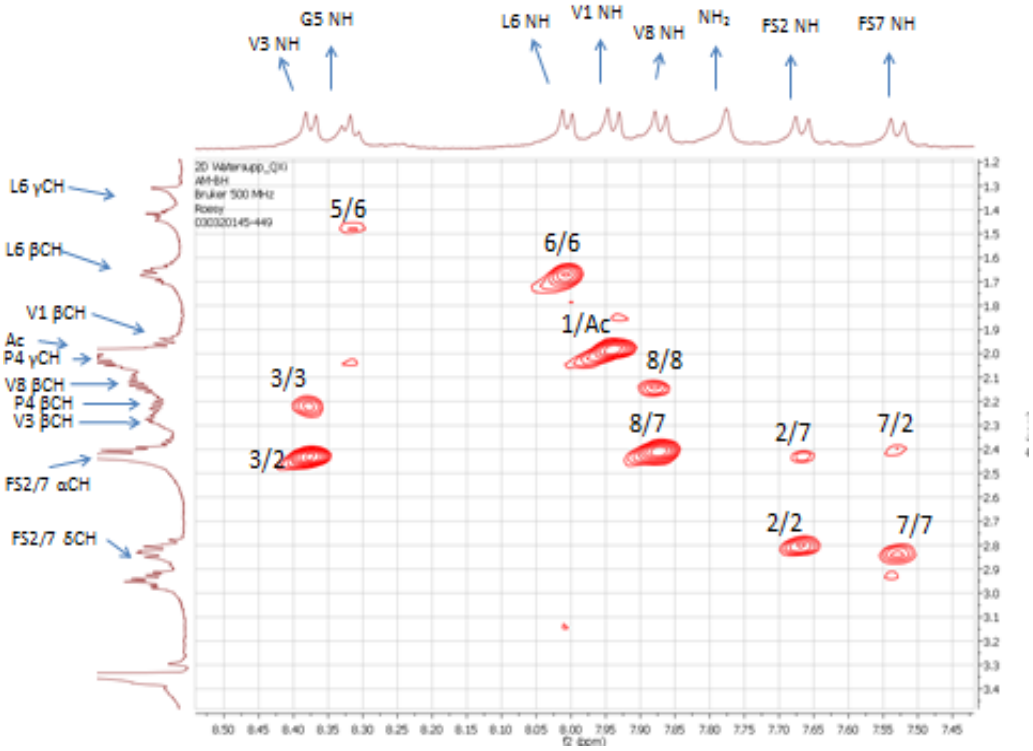


Figure 8-10: Roesy spectra for β hairpin 8) correlation between NH-NH region 9) correlation between NH-C_{α}H 10) correlation between NH-side chain protons

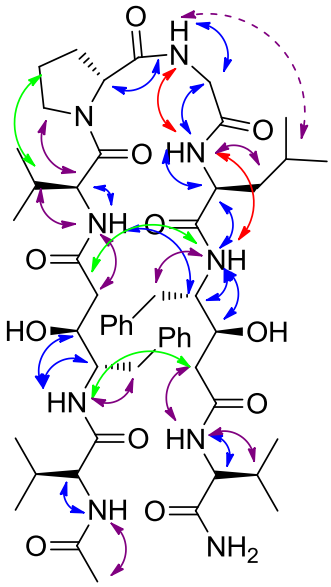


Figure 11: The NOEs observed in the ROESY is shown in double headed arrows.

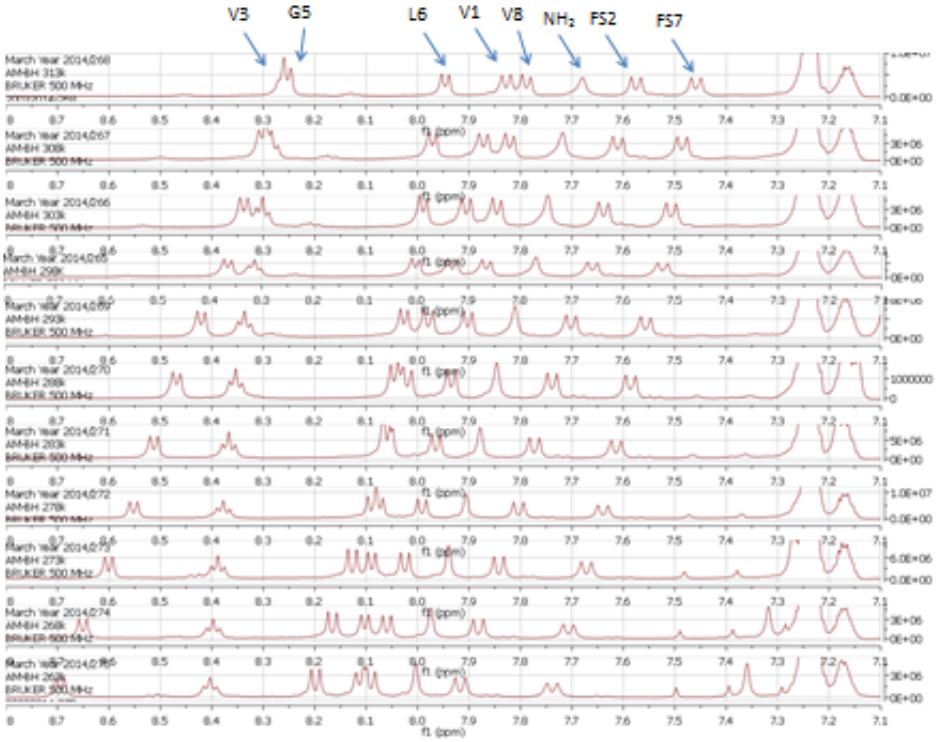


Figure 12: ^{1}H NMR spectra with increasing temperture.

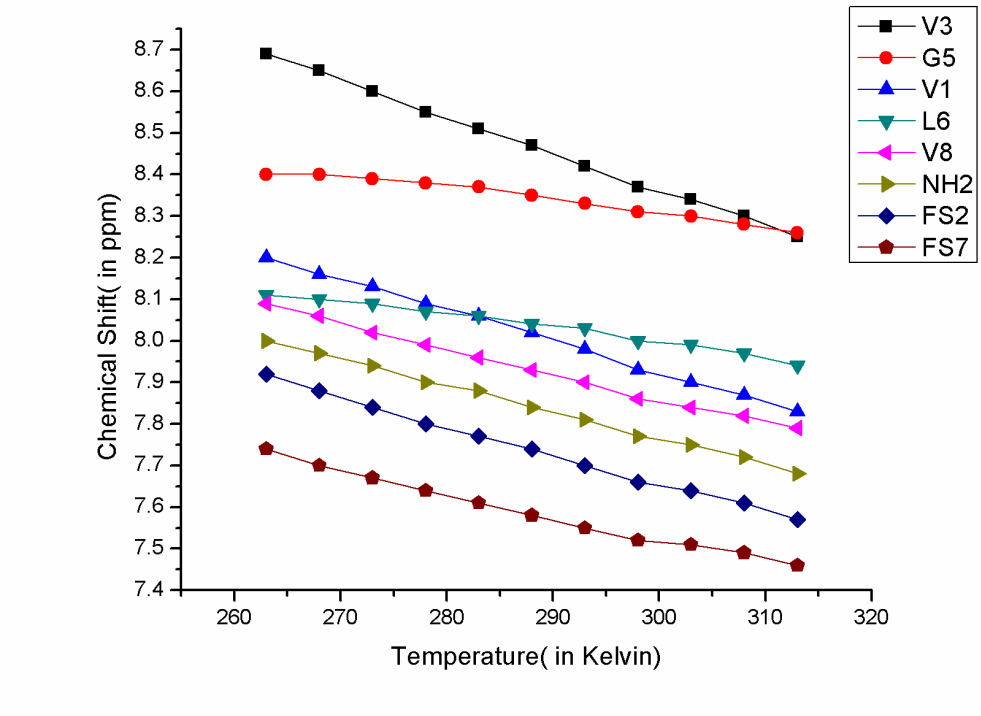


Figure 13: A plot dicting the amide NH chemical shift versus temperture.

Table 3: Chemical shifts of the amide NHs with increasing temperature.

Residue	Chemical Shift of NH with temperature(ppm)											dδ/dT (ppb/K)
	263	268	273	278	283	288	293	298	303	308	313	
Val3	8.69	8.65	8.6	8.55	8.51	8.47	8.42	8.37	8.34	8.3	8.35	-8.78
Gly5	8.4	8.4	8.39	8.38	8.37	8.35	8.33	8.31	8.3	8.28	8.26	-2.96
Val1	8.2	8.16	8.13	8.09	8.06	8.02	7.98	7.93	7.9	7.87	7.83	-7.45
Leu6	8.11	8.1	8.09	8.07	8.06	8.04	8.03	8	7.99	7.97	7.94	-3.35
Val8	8.09	8.06	8.02	7.99	7.96	7.93	7.9	7.86	7.84	7.82	7.79	-6.04
NH_2	8	7.97	7.94	7.9	7.88	7.84	7.81	7.77	7.75	7.72	7.68	-6.36
FS2	7.92	7.88	7.84	7.80	7.77	7.74	7.70	7.66	7.64	7.61	7.57	-6.87
FS7	7.74	7.7	7.67	7.64	7.61	7.58	7.55	7.52	7.51	7.49	7.46	-5.49

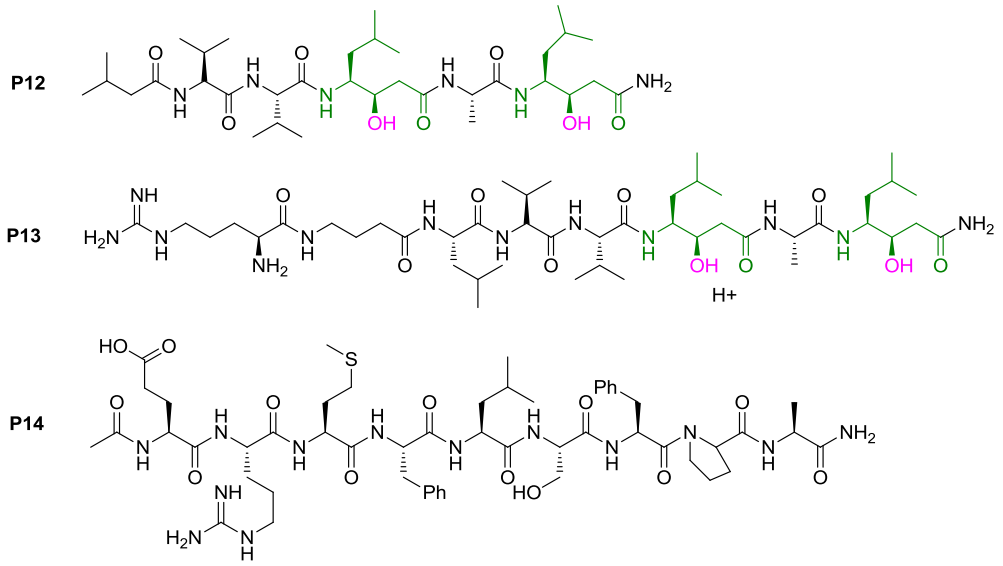
We are presently investigating the solution state conformational properties of the other hybrid β-hairpin **P11**.

Overall, these studies suggest that statine residues can be incorporated into the canonical β-hairpin structures without much deviation from the overall fold of the β-hairpin conformation. Results of these investigations can be further explored in the design of biologically active β-hairpin structures as protease inhibitors as well as inhibitors for protein-protein interactions.

Design synthesis and biological evaluation of water soluble pepstatin analogues

The peptide natural product, pepstatin showed broad spectrum inhibitory activities against various aspartic acid proteases, including pepsin, cathespin D and E, rennin, HIV-1 protease, β-secretase, plasmepsin I and II of malarial parasite *Plasmodium falciparum* etc. The major disadvantage of the pepstatin is its insolubility in aqueous solvents and buffers. Because of its insolubility, pepstatin has not been well exploited

as an inhibitor against various proteases *in vivo*. Due to the difficulty in the synthesis and isolation of stereochemically pure statine amino acids, very few attempts have been made in the literature to design the water soluble pepstatin analogues. As we have been standardized the protocol for the synthesis and the isolation stereochemically pure of statines, and their utility in both solution and solid phase peptide synthesis, we sought to further explore the design of water soluble pepstatin analogues. The sequence of the pepstatin (**P12**, $(CH_3)_2CHCH_2CO$-Val-Val-LS(R,S)-Ala-LS(R,S)NH_2) is shown in the **Scheme 9**. The natural peptide composed of two leucine statines at the positions 3 and position 5 (C-terminal), along with α-Val residues at the position 1 and 2 and α-Ala at the position 4. The N-terminal of the peptide is protected with isovaleric acid. Based on the pepstatin sequence, we designed peptide **P13** (Arg-GABA-Leu-Val-Val-LS(R,S)-Ala-LS(R,S)-NH_2) without disturbing the overall

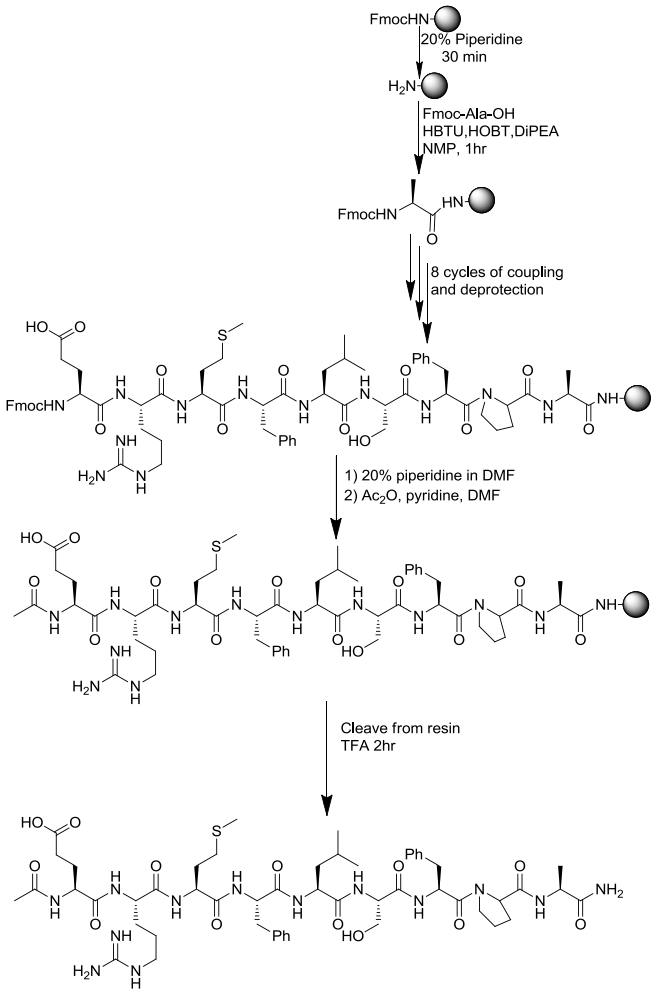


Scheme 9: Sequences of pepstatine, designed water soluble pepstatin and protease substrate.

sequence order of the peptide (**Scheme 9**). We introduced Leu residue at position 3 to compensate the structure of the isovaleric acid. The polar residue Arg was introduced at the N-terminus to induce the solubility of the peptide and GABA was used as a linker to separate polar residue Arg with the hydrophobic patch of pepstatin residues. Both

the peptides, **P12** and **P13** were synthesized using solid phase method on Knorr amide resin as described earlier. The schematic representation of the solid phase synthesis of **P13** is shown in **Scheme 10**. Due to the insolubility of **P12,** we were unable to purify

SCHEMATIC REPRESENTATION OF SOLID PHASE PAPTIDE SYNTHESIS



Scheme10: Solid phase synthesis of water soluble pepstatin analogue **P13.**

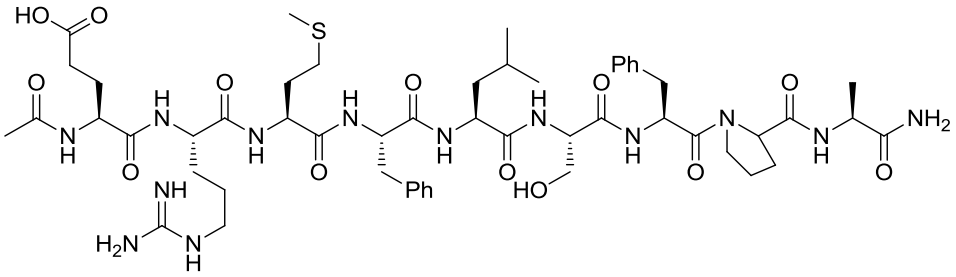
this peptide however, the mass of peptide was confirmed using MALDI-TOF. The designed peptide **P13** turned out to be highly soluble in water and other aqueous buffers. The peptide was purified by reverse phase HPLC using MeOH/H_2O gradient

system with 0.1%TFA. The mass spectrum of the peptide is given below. To understand the inhibitory activity of the water soluble pepstatin analogue **P13** against the aspartic acid proteases, we selected pepsin as a model protease. The protease substrate was derived from the plasmepsin cleavage site of the haemoglobin. The sequence of the protease substrate (**P14**) is shown in the **Scheme 9**. We used MALDI-TOF as a tool to understand the proteolytic activity of pepsin and the inhibitory effect of **P13**. The literature suggests that pepsin cuts the substrate at 4^{th} or 5^{th} residue that is between leucine and phenylalanine or phenylalanine and methionine. The preliminary investigations reveal the cleavage of the substrate P14 at position 4 as well as at position 5 by the pepsin. The cleavage products were identified using MALD-TOF analysis. To understand whether the water soluble pepstatin analogue **P13** can inhibit the activity of pepsin, we incubated **P13** with pepsin prior to the treatment of protease substrate **P14**. The mass spectral analysis suggests that there is no cleavage of **P14** and the intact substrate was observed in the mass spectrum. These preliminary results suggest that water soluble pepstatin inhibits the activity of pepsin protease. We are currently investigating the kinetics of protease activity in the presence and absence of inhibitor **P13**. In addition to the pepsin, we are also investigating the inhibitory activity of the **P13** with various other aspartic acid proteases and binding thermodynamic parameters.

Overall, we designed and synthesized water soluble analogue of universal aspartic acid protease inhibitor pepstatin and studied its inhibitory activity against the pepsin protease using MALDI-TOF. Presently, we are investigating the kinetic and thermodynamic binding parameters of the pepsin and inhibitor **P13** interactions.

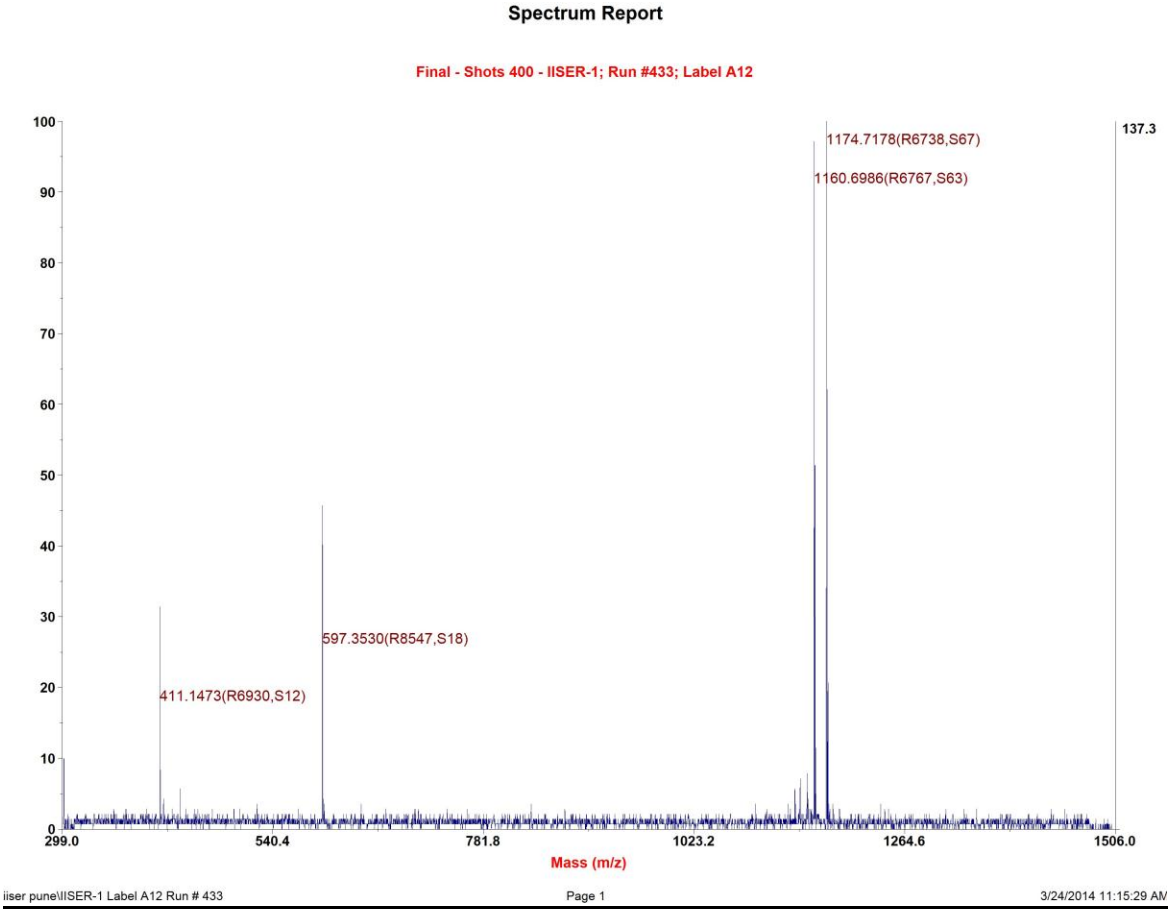
MALDI-TOF based experiment for the inhibition study of pepstatin

Substrate(P14):

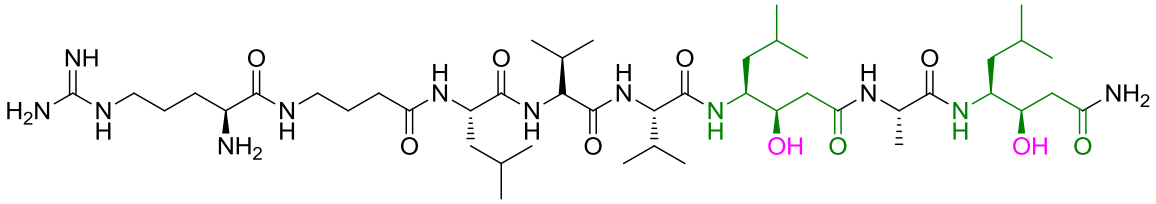


MALDI-TOF

m/z calcd for $C_{53}H_{79}N_{13}O_{13}S$ ($M+Na^+$) 1160.5539. Observed($M+Na^+$) 1160.6986

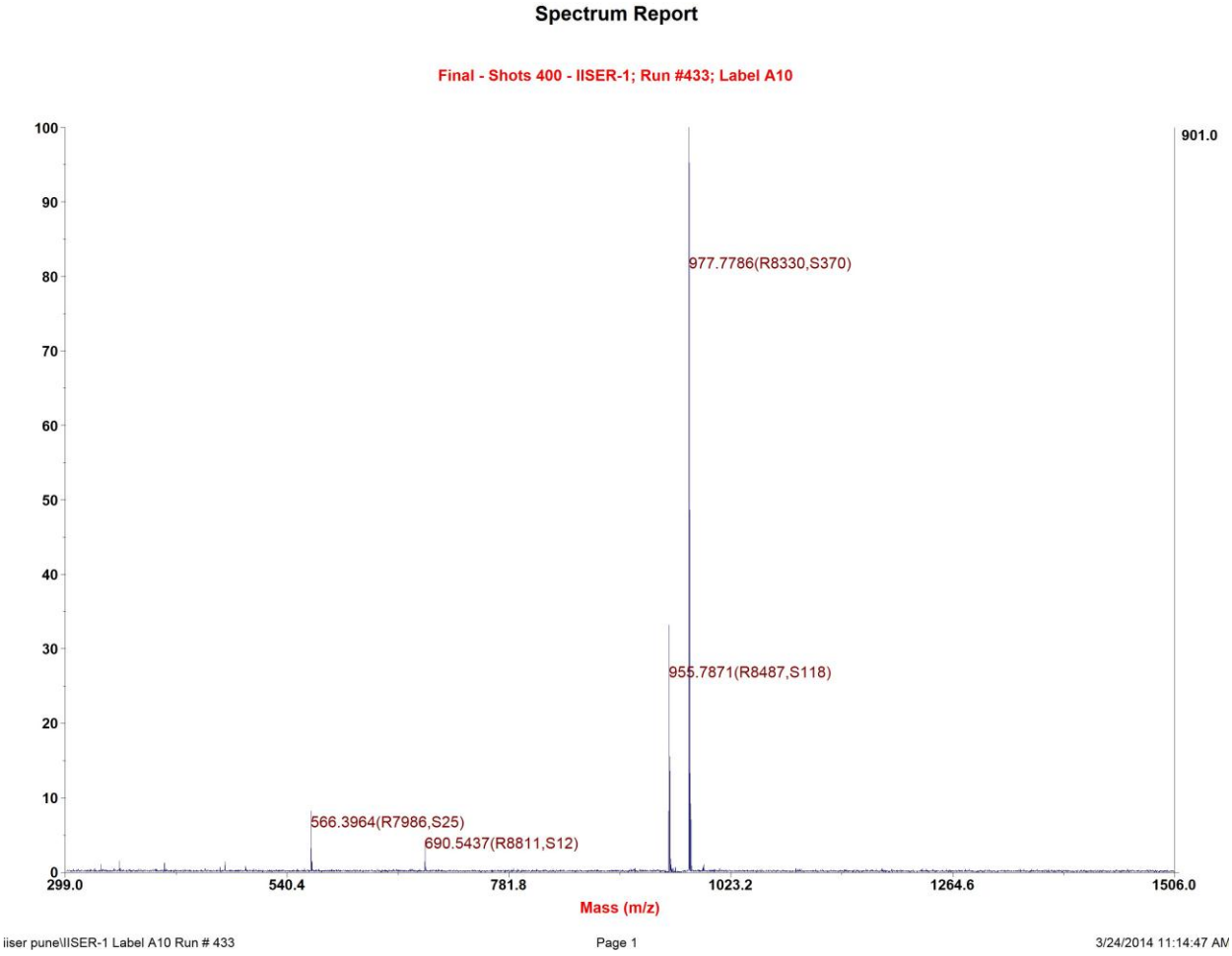


Inhibitor(P13):



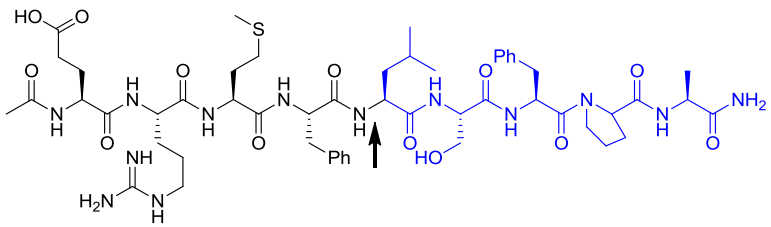
MALDI-TOF

m/z calcd for $C_{45}H_{86}N_{12}O_{10}$ $(M+H^+)$955.6668, $(M+Na^+)$977.6482. Observed $(M+H^+)$955.7871, $(M+Na^+)$ 977.7786

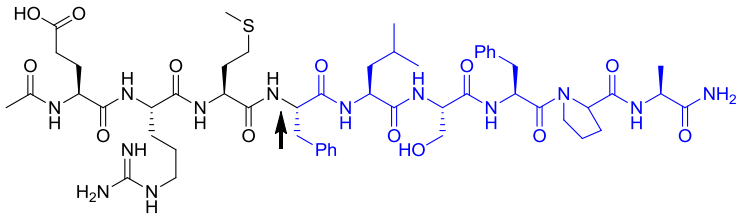


Substrate+ Enzyme(Pepsin): Proteolysis of the control was observed

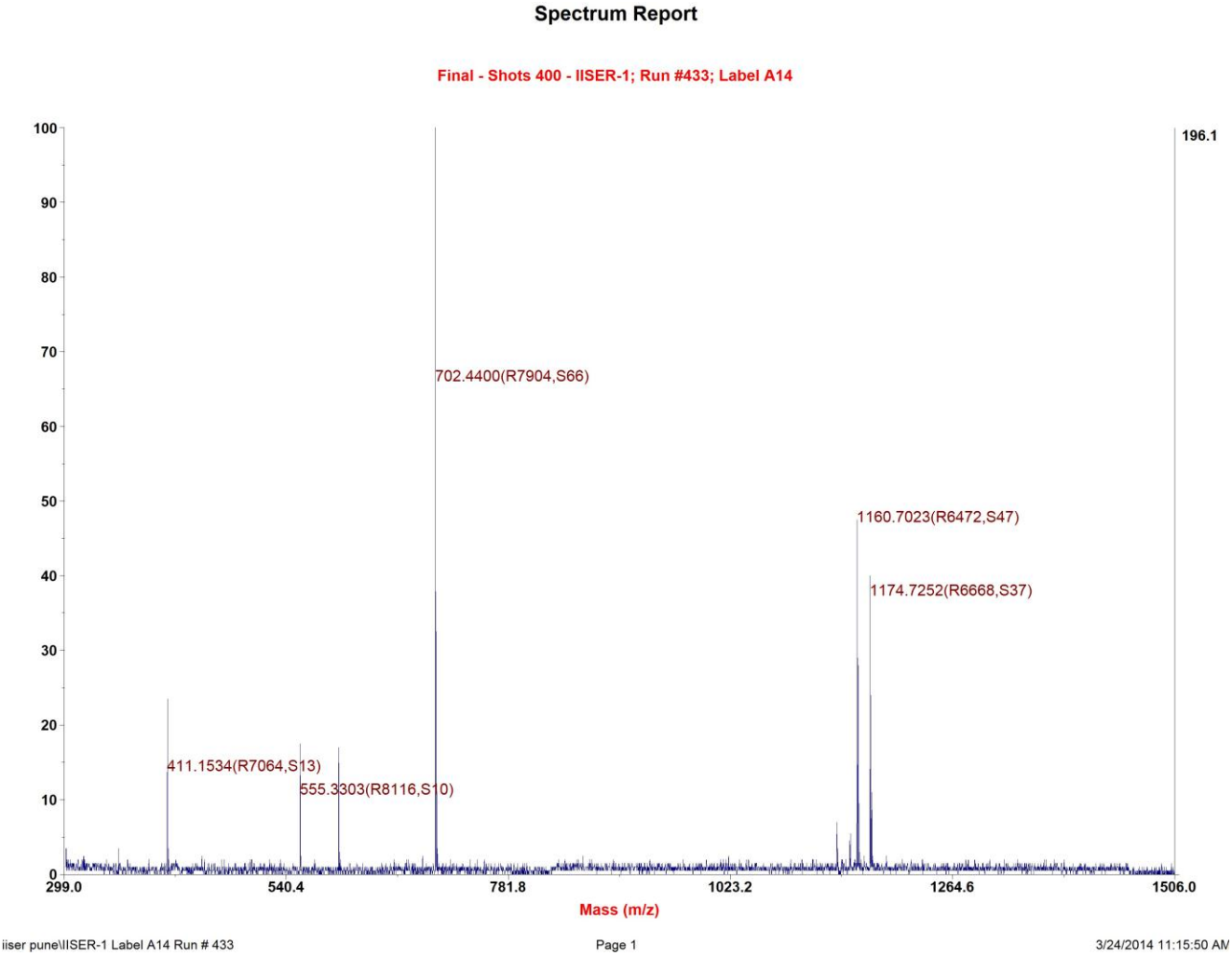
Analysis: 1160.7023 corresponds to the (P13+Na^+)



m/z calcd for the (fragment+K^+)555.2454. Observed(fragment+K^+) 555.3303



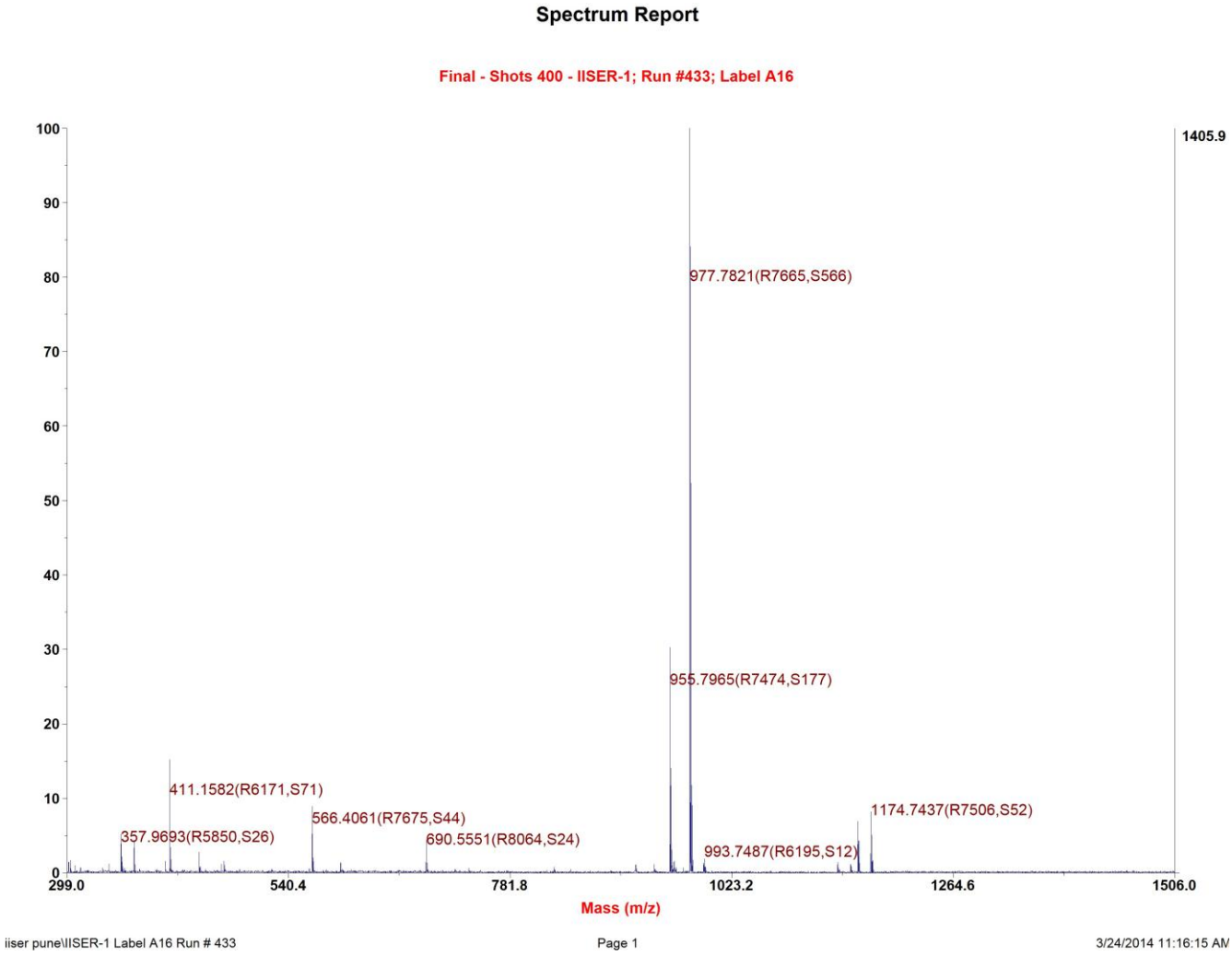
m/z calcd for the (fragment+K^+)702.3143. Observed(fragment+K^+) 702.4400



Addition of control in mixture of inhibitor and enzyme

No proteolysis was observed

Inhibitor inhibits the activity of the enzyme. So, 702 and 555 mass peak were not observed.



CONCLUSION

In conclusion, we have demonstrated the facile synthesis of β-hydroxy γ-amino acids through the mild reduction of β-keto-γ-amino esters and their utility in the design of various hybrid peptides and homooligomers. The conformational properties of hybrid helices incorporated with stereochemically pure *anti (3S, 4S)* and *syn (3R, 4S)* β-hydroxyγ-phenylalanine reveal that irrespective of the stereochemistry β-carbon center, both diastereoimers can be accommodated into 12-helices without much deviation of the overall 12-helical conformation. However, the β-hydroxyl groups in *syn* diastereoisomer projected upwards similar to the amide NHs in the helix, while in *anti* diastereoisomers β-hydroxyl group projected downwards similar to the amide carbonyl. These sterochemical requirements enable the β-hydroxyl groups to involve in the six membered intraresidue H-bonds in the *syn* diastereoisomers and ten membered interresidue H-bonds in the *anti* diastereoisomers, respectively. The influence of these additional -OH group interactions with backbone carbonyl groups in higher ordered helical peptides is currently under investigation. Further, we investigated the influence of statine residues in the structural stability of β-hairpins. The designed octapeptide β hairpin incorporated with *(3R, 4S)* β-hydroxy γ-phenylalanine at the facing positions of the anti-parallel strands displayed a well folded conformation in solution, suggesting that these statines can be readily accommodated into the canonical β-hairpin structures without much deviation from the overall folding of the β-hairpin conformation. In addition, we have also demonstrated the design of water soluble analogue of universal aspartic acid protease inhibitor pepstatin and studied their inhibitory activity against model protease pepsin using α-peptide substrate. The kinetic and thermodynamic parameters substrate protease as well as the inhibitor protease interactions is currently under progress. Over all we demonstrated the facile synthesis and isolation of statine amino acids and their utility in the design of hybrid peptide foldamers as well as their utility in the design of biologically active water soluble pepstatin.

METHODS

CHEMICALS

All the amino acids, Ethyl diazoacetate, TFA, DiPEA were bought from Aldrich. IBCCl, DCM, THF, DMF were obtained from spectrochem and used without further purification unless specified otherwise. Silica gel for column chromatography was 120-200 mesh obtained from Merck.

INSTRUMENTATION

The ^{1}H spectra were recorded on BRUKER 500 MHz (or 125 MHz for ^{13}C) and JEOL 400 MHz (or 100 MHz for ^{13}C) using residual solvents signals as an internal reference [($CDCl_3$δH, 7.26 ppm, δC 77.0 ppm) and CD_3OH δC 49.3 ppm]. The chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. MALDI-TOF/TOF mass spectra were obtained on Model 4800 (Applied Biosystems) instrument. WATERS HPLCwas used for the purification of peptide using distilled Methanol-Water system with preparatory column. ZEISS EVO series Scanning Electron Microscope Model EVO 50 was used to obtain SEM images of the peptides after coating them with a gold layer.

EXPERIMENTAL

General procedure for the Synthesis of N-Boc-protected β-keto γ-amino esters: N-protected amino aldehyde (10 mmol) was dissolved in 15 ml of DCM at room temperature. To the reaction mixture, tin (II) chloride (20 mol%) was added followed by 1.05eq. of 15% ethyl diazoacetate in toluene (7.5 ml). spontaneous evolution of N_2 gas is observed and reaction is allowed to stir for 1hr. reaction progress is monitored with TLC. After completion of reaction, the reaction mixture is acidified using 10% HCl and extracted with DCM. The organic layer is washed with brine solution, dried over anhydrous sodium sulphate and concentrated over reduced pressure to obtain β-keto γ-amino ester. Pure compound were obtained after purification by column chromatography.

General procedure for Synthesis of N-Boc-protected β-hydroxy γ-amino esters: The β-keto-γ amino ester was dissolved in 30 ml of dry THF under nitrogen atmosphere. The solution is brought to -78^{0}C using dry ice and acetone. 1.4 eq. of NaBH4 was added to the reaction mixture and reaction was allowed to stir vigorously for 3 hrs. After completion of reaction, the reaction mixture was quenched using 10% 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 β-hydroxyl γ-amino ester. Diastereomers were separated using silica gel based column chromatography. These diastereomers were further used for solution phase peptide synthesis.

General procedure for Synthesis of N-Boc-protected β-hydroxy γ-amino acids: The β-hydroxy γ-amino ester (4.5 mmol) was dissolved MeOH (54 ml) and allowed to stir to obtain a clear solution. To the reaction mixture 3eq of 1NaOH (13.5 ml, 13.5 mmol) was added drop wise and reaction was allowed to stir for 3hrs at room temperature. After the completion of the reaction, the reaction mixture was quenched with 10% 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 N-protected β-hydroxyl γ-amino acid. It was used without any further purification.

General procedure for the synthesis of β-hydroxy γ-amino acids: The N-protected β-hydroxy γ-amino acid was dissolved in 3 ml of DCM and cooled to 0 ^{0}C. To the solution 5ml of TFA was added slowly and reaction was brought to room temperature. The reaction mixture was allowed to stir for about 1hr. After completion of the reaction, TFA was completely evaporated and the free amine was for the next step without any further purification.

General procedure synthesis of N-Fmoc protected β-hydroxy γ-amino acids: The N-Boc- deprotected β-hyroxy γ-amino acid (4 mmol) was dissolved in 10% sodium carbonate (21.2 ml) and reaction mixture was maintained at basic pH. To the reaction mixture, THF (15.8 ml) was added and cooled to 0 ^{0}C. The Fmoc-OSu was dissolved in 15 ml THF and added drop wise to the reaction mixture. After the addition, the solution was brought to room temperature and allowed to stir for 5hrs. After completion of the reaction, it was

quenched with 10% HCl solution and extracted with ethyl acetate. The organic layer was washed with brine solution, dried over anhydrous sodium sulphate and concentrated under reduced pressure to obtain N-Fmoc protected β-hydroxyl γ-amino acid. The gummy product was precipitated using ethyl acetate and pet ether and directly used for the solid phase peptide synthesis.

General procedure synthesis of statine dipeptides (P1-P3): The trifluoroacetate salt of the statine ester (0.24 gm, 1 mmol) was dissolved in 0.5 ml of DMF. The solution was cooled to 0 °C, followed by the addition of 3 eq. of DiPEA (0.52 ml, 3 mmol) drop wise. After that reaction was allowed to attain room temperature, Boc protected statine acid (0.340 gm, 1 mmol), HBTU (0.38 gm, 1 mmol) and HOBt (0.135 gm, 1 mmol) dissolved in 1ml of DMF were added and reaction was allowed to stir overnight. Reaction progress was monitored by TLC. After completion, the reaction was quenched with 10% HCl and extracted with ethyl acetate. The organic layer was washed with sodium carbonate and brine solution and dried over anhydrous sodium sulphate. The pure dipeptide was obtained after silica gel column chromatography.

Synthesis of tripeptide (P4): The trifluoroacetate salt of the dipeptide ester was dissolved in dissolved in 0.5 ml of DMF. The solution was cooled to 0 °C, followed by the addition of 3 eq. of DiPEA (0.52 ml, 3 mmol) drop wise. After that reaction was allowed to attain room temperature, Boc protected statine acid (0.34 gm, 1 mmol), HBTU (0.38 gm, 1 mmol) and HOBt (0.135 gm, 1 mmol) dissolved in 1ml of DMF were added and reaction was allowed to stir overnight. Reaction progress was monitored by TLC. After completion of the reaction, reaction was quenched with 10% HCl and extracted with ethyl acetate. The organic layer was washed with sodium carbonate and brine solution and dried over anhydrous sodium sulphate. The pure dipeptide was obtained after HPLC purification using MeOH/H_2O solvent system.

General procedure synthesis of tetrapeptides (P6 and P7): The two dipeptides were prepared as mentioned above. One dipeptide was deprotected other was hydrolysed and the same procedure as mentioned for the dipeptide was followed in order to obtain the tetrapeptide. Which was then purified using HPLC with MeOH/H_2O as solvent system.

Deprotection of the O-acetyl groups obtained from the solid phase synthesis: The O-acylation of the peptide was the major problem faced while using statines in peptides. To deprotect the O-acetylation, the crude peptide was dissolved in MeOH and treated with 5eq. of $NaBH_4$ in one batch. The reaction mixture was allowed to stir at room temperature for 5 to 6 hrs in order to obtain complete deacetylation of the peptide. Unreacted sodium borohydride was neutralised using 10% HCl. And peptide was extracted in ethyl acetate and purified using reverse phase HPLC using methanol and water as solvent system.

Cleavage of peptides from solid phase resin: The dried resin after the synthesis of peptide was treated with cocktail mixture TFA/water (99/1) and allowed to stir for 2 hrs at room temperature. After completion of the reaction, reaction mixture was filtered and washed with DCM to separate resin from peptide. TFA was completely evaporated and peptide was precipitated either ethyl acetate/ pet. ether (hydrophobic sequence) or diethyl ether (hydrophilic sequence). The peptides were purified using reverse phase HPLC on C_{18} column using methanol/water gradient system.

NMR details for the monomers

(R,S)-N-Boc protected β-hydroxyl γ-phenyl alanine ester:**^{1}H NMR** (400 MHz, $CDCl_3$ **:** δ_H 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, $CDCl_3$): δ_C 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; **ESI** m/z **:** Calcd. $[M+Na]^+$ 360.1786, observed 360.1789.

(S,S)-N-Boc protected β-hydroxyl γ-phenyl alanine ester: **^{1}H NMR** (400 MHz, $CDCl_3$ **:** δ_H 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, $CDCl_3$) **:** δ_C 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; **ESI** m/z : Calcd. $[M+Na]^+$ 360.1786, observed 360.1792.

(R,S)-N-Boc protected β-hydroxyl γ-leucine ester: **^{1}H NMR** (400 MHz, $CDCl_3$ **:** δ_H 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, $CDCl_3$) **:** 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 **:** Calcd. $[M+Na]^+$ 326.1943, observed 326.1949.

(R,S)-N-Boc protected β-hydroxyl γ-leucine ester: **^{1}H NMR** (400 MHz, $CDCl_3$ **:** δ_H 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, $CDCl_3$) **:** 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 **:** Calcd. $[M+Na]^+$ 326.1943, observed 326.1946.

NMR details for dipeptides

N-Boc protected (S,S)phesta-(S,S)phesta ethyl ester:**1H NMR** (400 MHz, DMSO-d6) δ_H 7.64 (d, *J* = 8.6 Hz, 1H), 7.25 – 7.07 (m, 10H), 6.45 (d, *J* = 9.2 Hz, 1H), 5.10 (d, *J* = 5.8 Hz, 1H), 4.81 (d, *J* = 5.8 Hz, 1H), 3.97 (q, *J* = 7 Hz, 2H), 3.93 – 3.83 (m, 2H), 3.81 – 3.70 (m, 1H), 3.58 (dd, *J* = 6.6, 3.8 Hz, 1H), 2.76 (ddt, *J* = 12, 8.8, 4.8 Hz, 2H), 2.63 – 2.48 (m, 2H), 2.34 – 2.20 (m, 2H), 2.19 – 2.05 (m, 2H), 1.26 (d, *J* = 2.6 Hz, 9H), 1.10 (t, *J* = 7 Hz, 3H);**^{13}C NMR** (100 MHz, DMSO-D6) δ_C 171.85, 171.36, 155.91, 139.78, 129.58, 128.52, 126.43, 78.11, 69.16, 67.88, 60.20, 56.45, 54.59, 36.55, 28.72, 14.60.

N-Boc protected (S,S)phesta-(R,S)phesta ethyl ester: **^{1}H NMR** (400 MHz, DMSO-d6) δ_H 7.67 (d, *J* = 8.2 Hz, 1H), 7.21 – 7.10 (m, 10H), 6.40 (d, *J* = 9.4 Hz, 1H), 5.07 (d, *J* = 6.6 Hz, 1H), 4.70 (d, *J* = 5.6 Hz, 1H), 3.99 (q, *J* = 7 Hz, 2H), 3.81 – 3.71 (m, 2H), 3.71(s, 1H) 3.56 (s, 1H), 2.84 (dd, *J* = 60.4, 12.6 Hz, 2H), 2.55 – 2.49 (m, 2H), 2.31 – 2.11 (m, 2H), 2.11 – 1.90 (m, 2H), 1.25 (s, 9H), 1.12 (t, *J* = 7.0 Hz, 3H). **^{13}C NMR** (100 MHz, DMSO-D6) δ_C 172.16, 171.10, 157.81, 155.91, 139.78, 129.72, 128.67, 126.29, 78.08, 70.19, 68.89, 60.21, 56.16, 55.25, 36.88, 36.04, 28.71, 14.65.

N-Boc protected (S,S)phesta-(R,S)phesta ethyl ester: **^{1}H NMR** (400 MHz, DMSO-d6) δ_H 7.72 (d, *J* = 9 Hz, 1H), 7.27 – 7.05 (m, 10H), 6.56 (d, *J* = 9.4 Hz, 1H), 5.10 (d, *J* = 7.0 Hz, 1H), 4.94 (d, *J* = 5.8 Hz, 1H), 4.02 (q, *J* = 7 Hz, 2H), 3.86 – 3.71 (m, 2H), 3.63 (s, 1H), 3.40 (s, 1H), 2.96 (dd, *J* = 41.4, 13.8 Hz, 2H), 2.75 – 2.55 (m, 2H), 2.40 (dd, *J* =

14.0, 10.8 Hz, 1H), 2.18 (dd, *J* = 15.2, 10 Hz, 1H), 2.02 (d, *J* = 6.4 Hz, 2H), 1.21 (s, 9H), 1.17 (s, 3H). **^{13}C NMR** (100 MHz, DMSO-D6) δ_C 172.39, 171.35, 155.81, 139.94, 129.59, 128.38, 126.26, 77.84, 71.03, 67.61, 60.22, 57.04, 55.22, 36.44, 28.70, 14.67.

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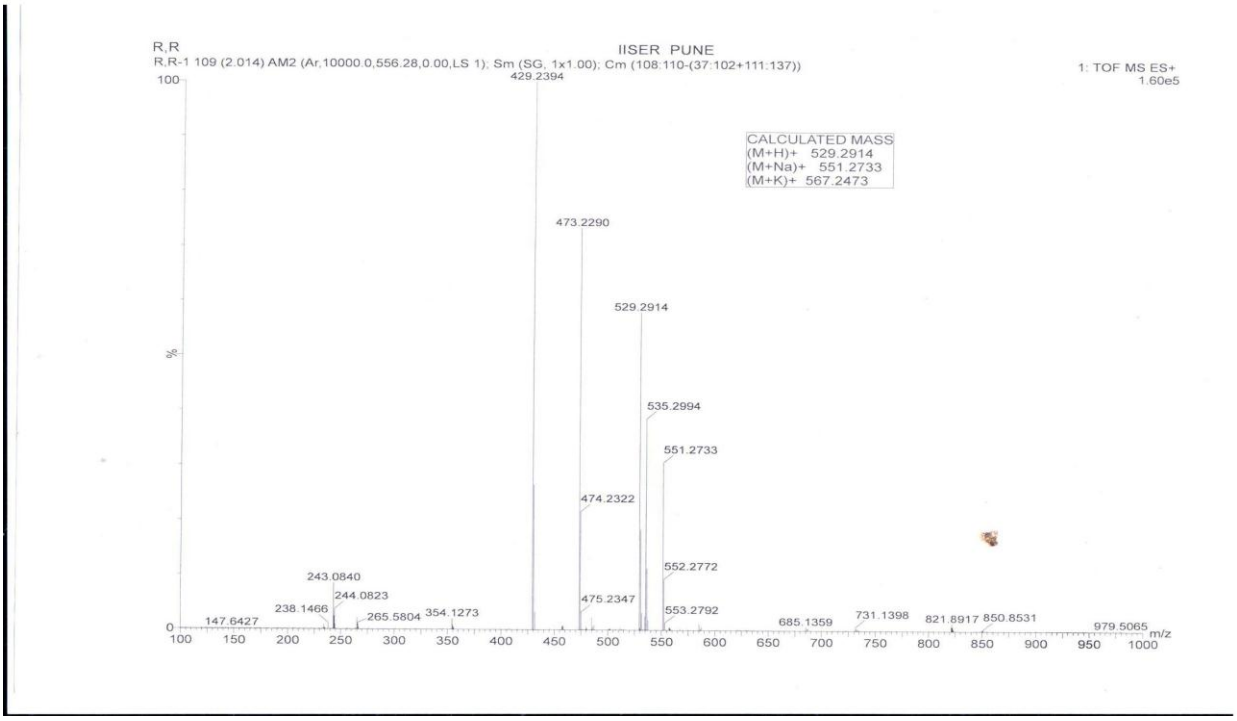
SUPPLEMENTRY DATA

HRMS Spectras **P1** mass calcd. ($M+H^+$) 529.2908 Observed 529.2914



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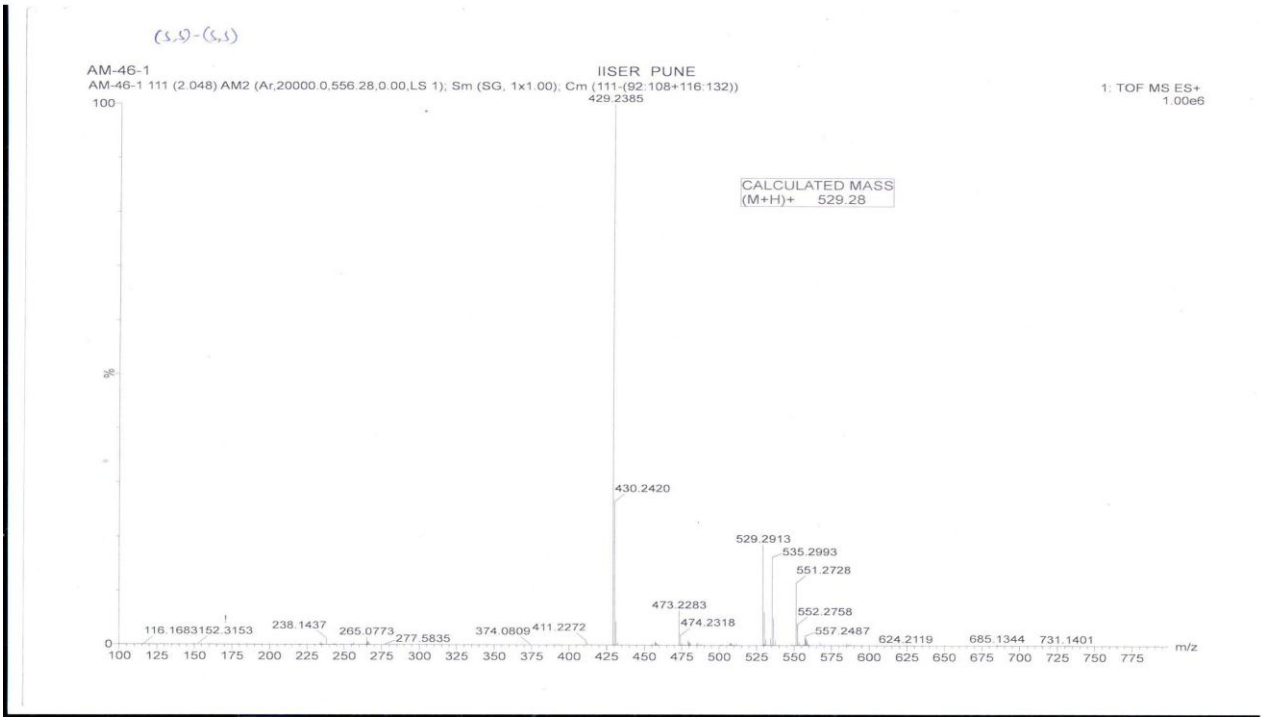

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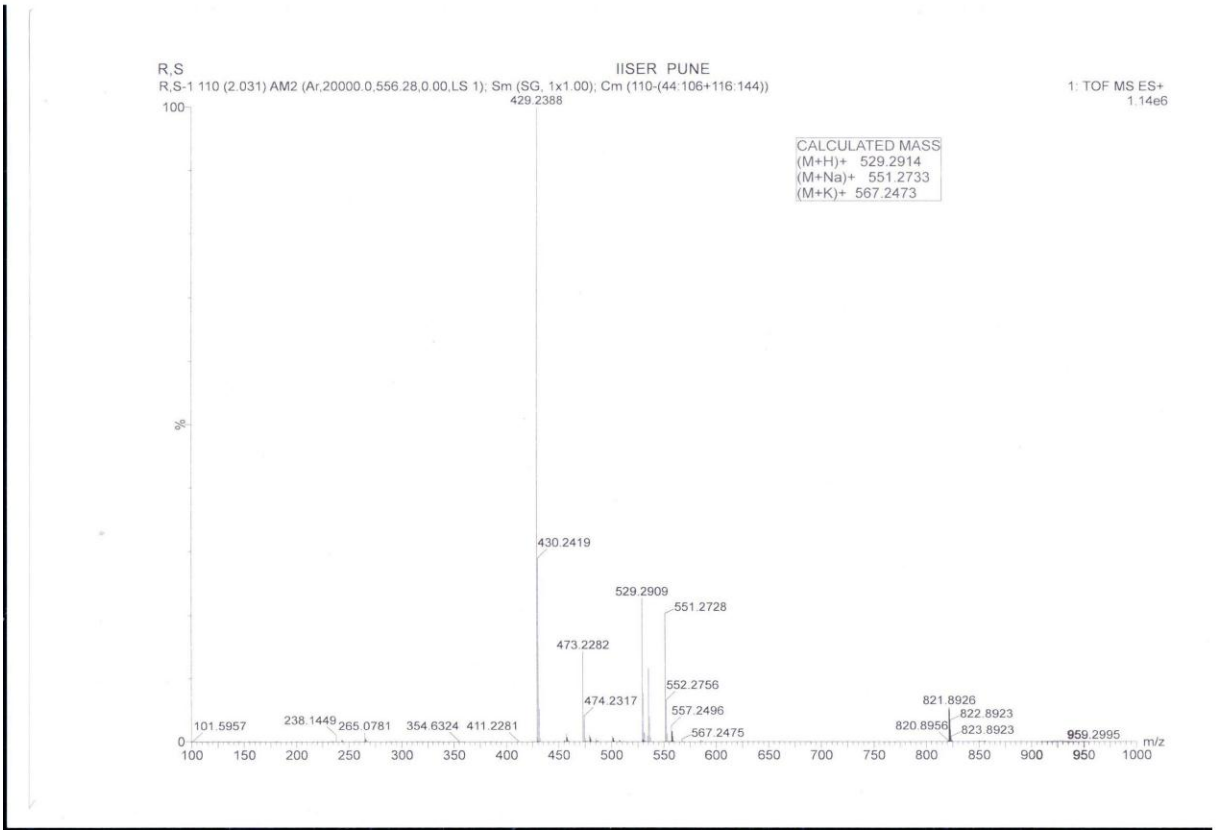

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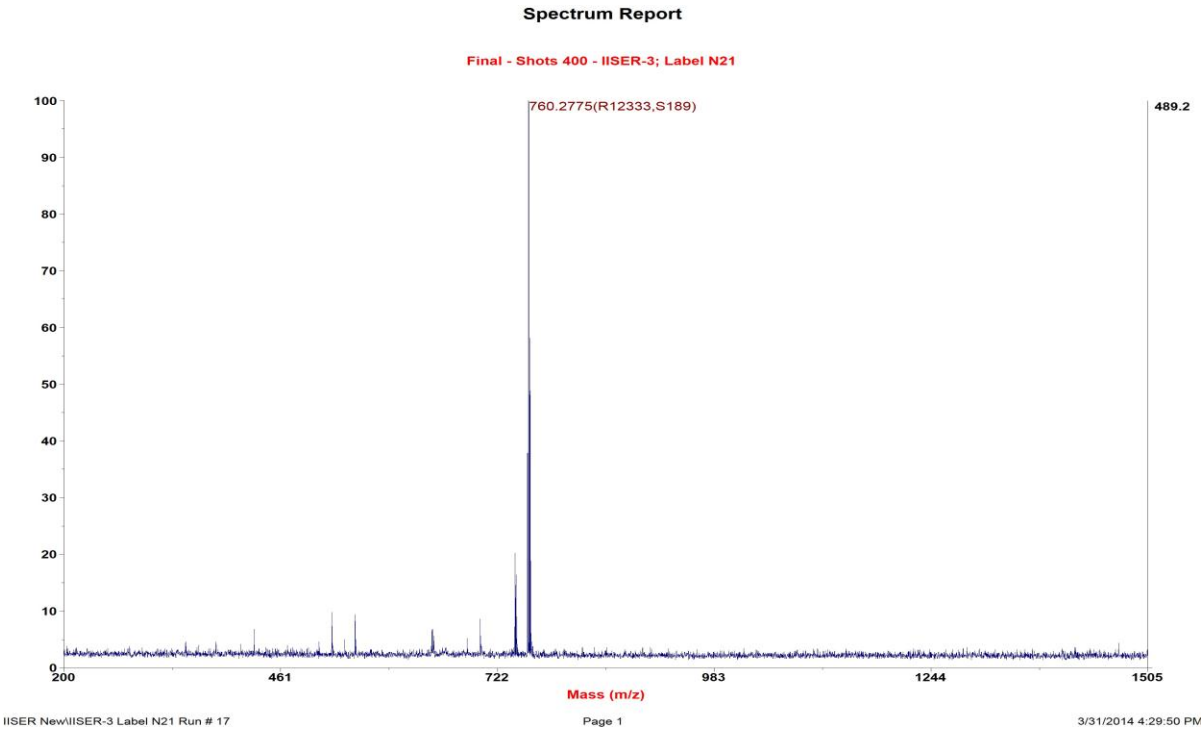

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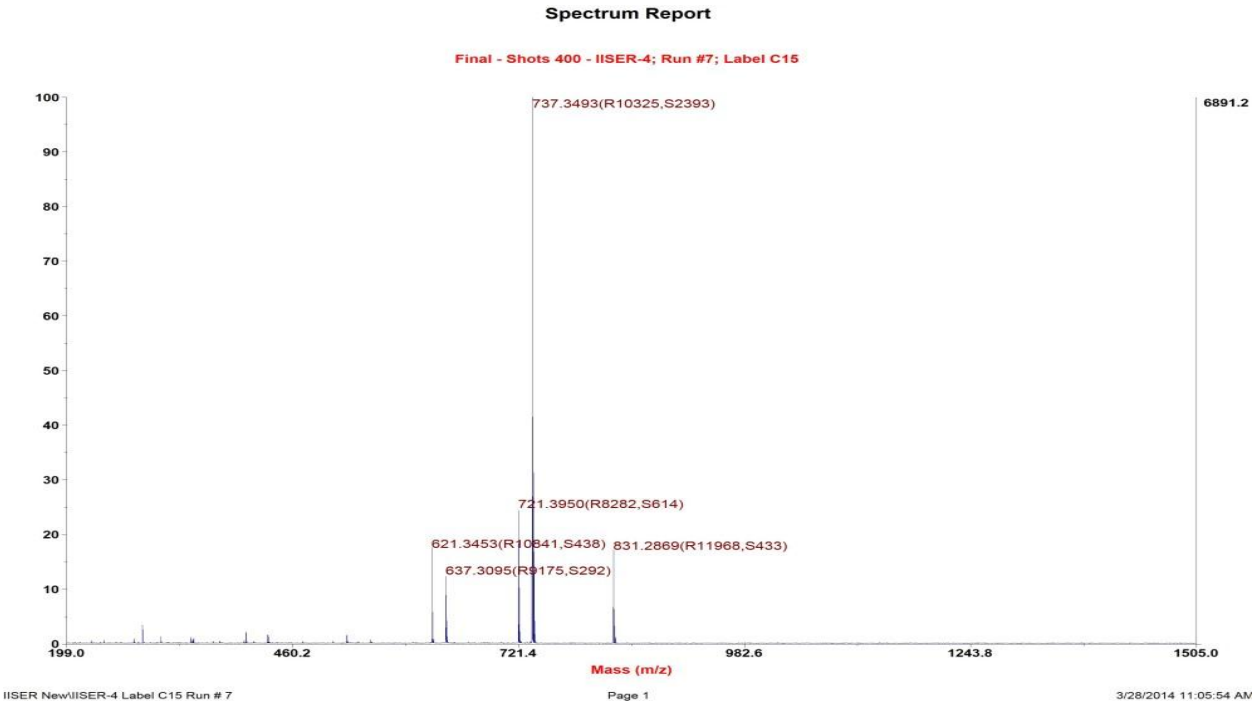

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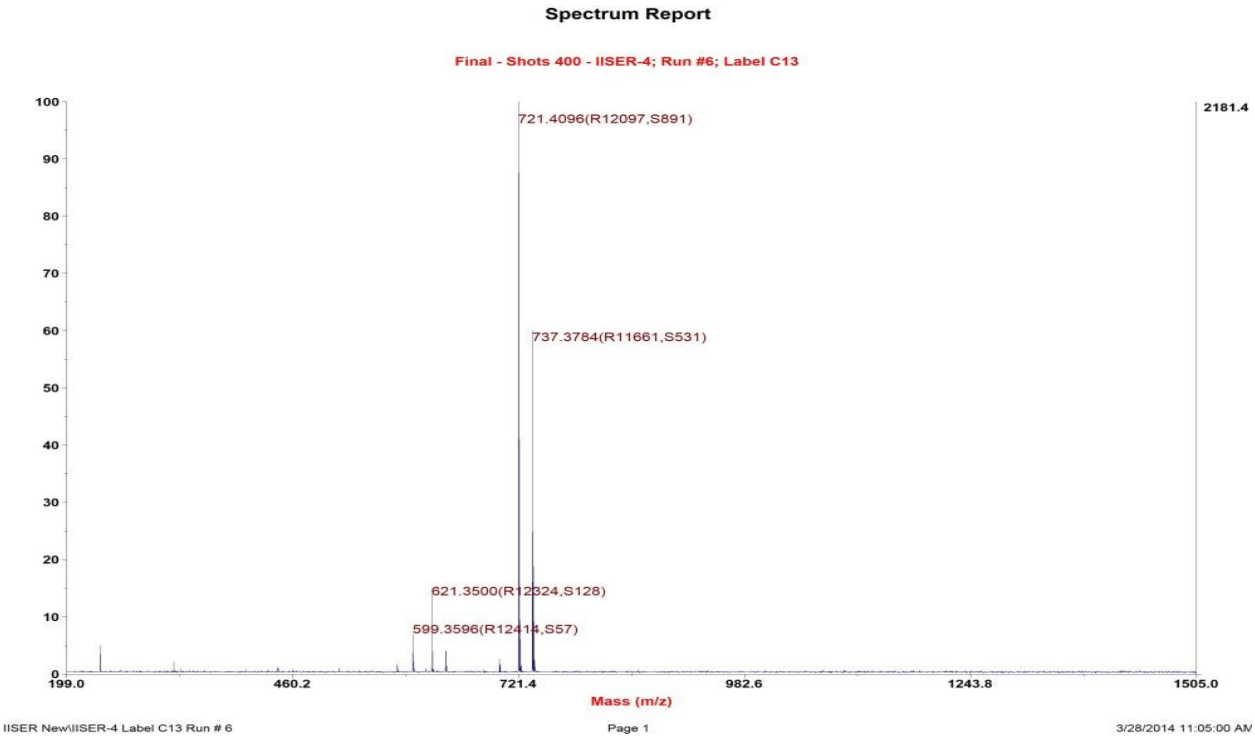

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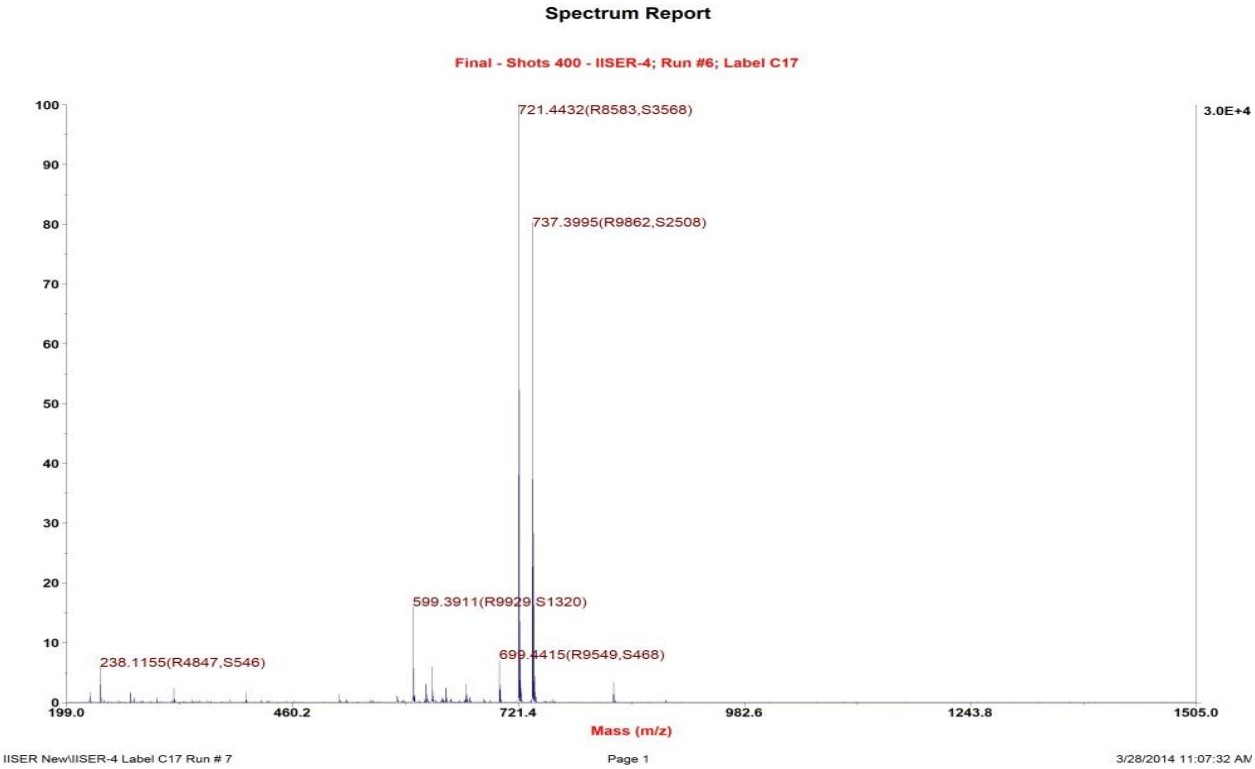

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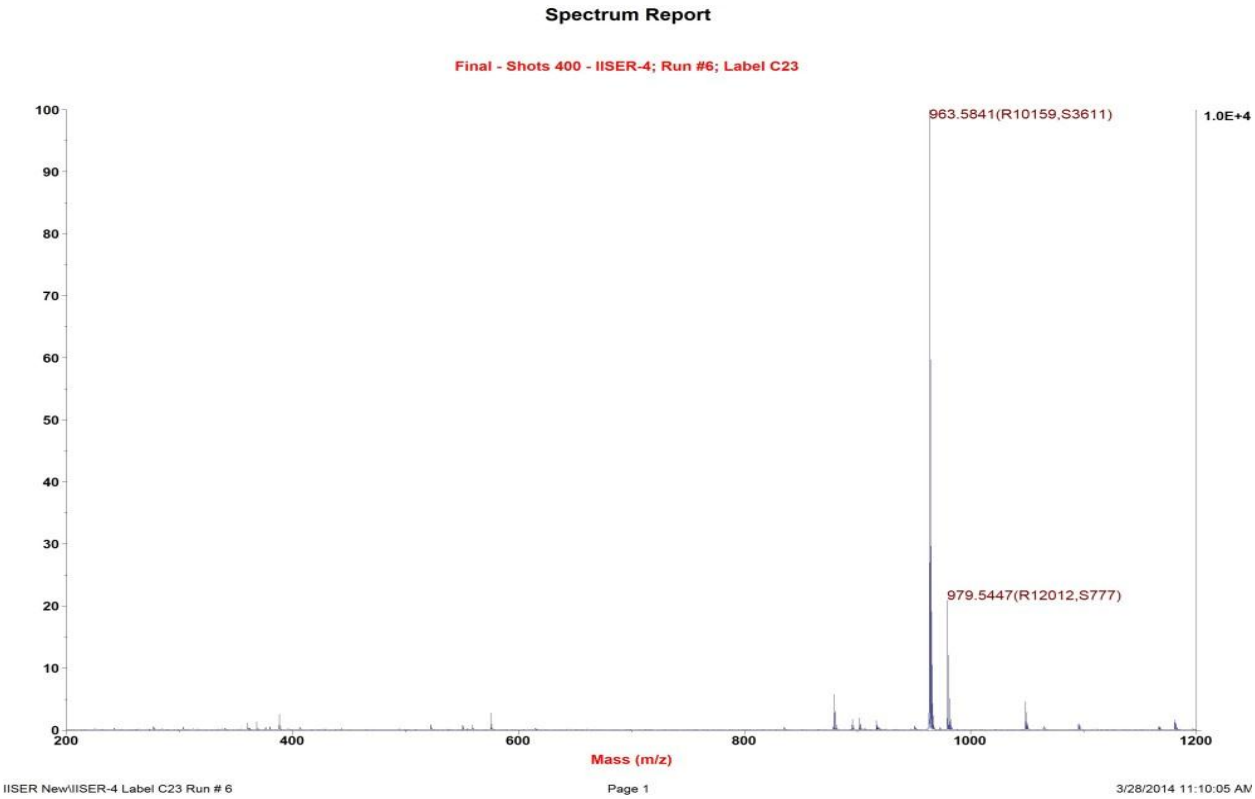

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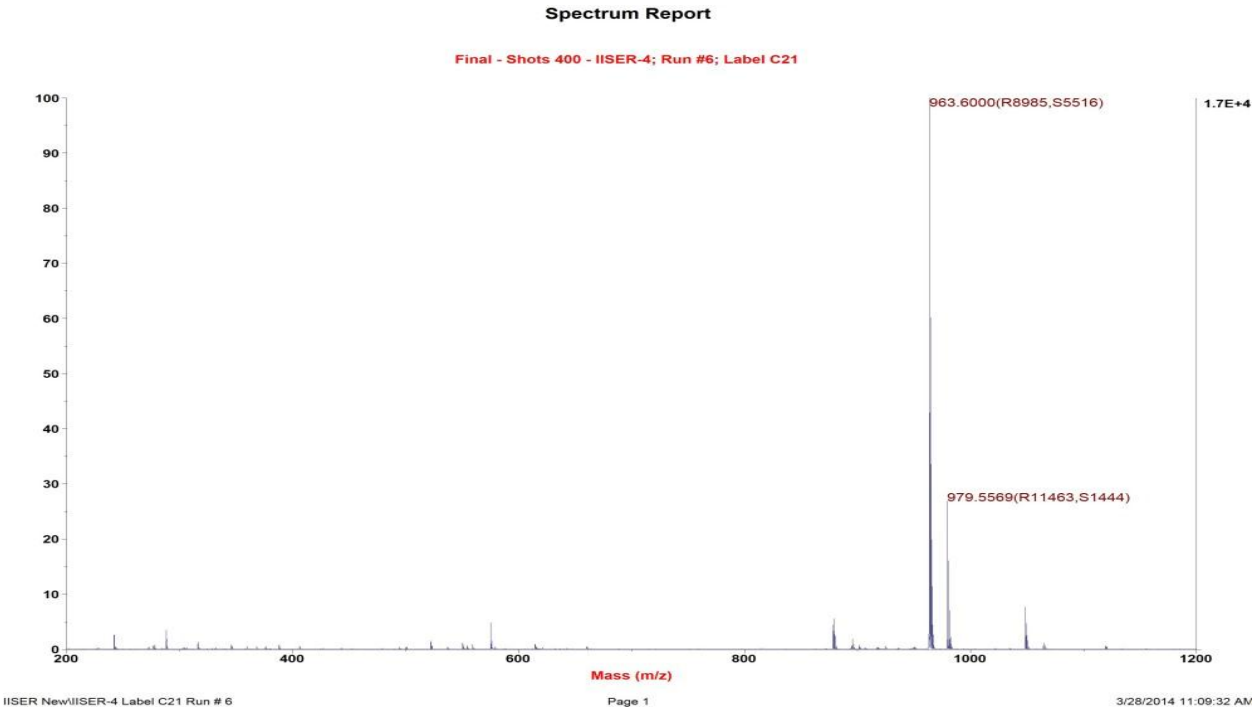

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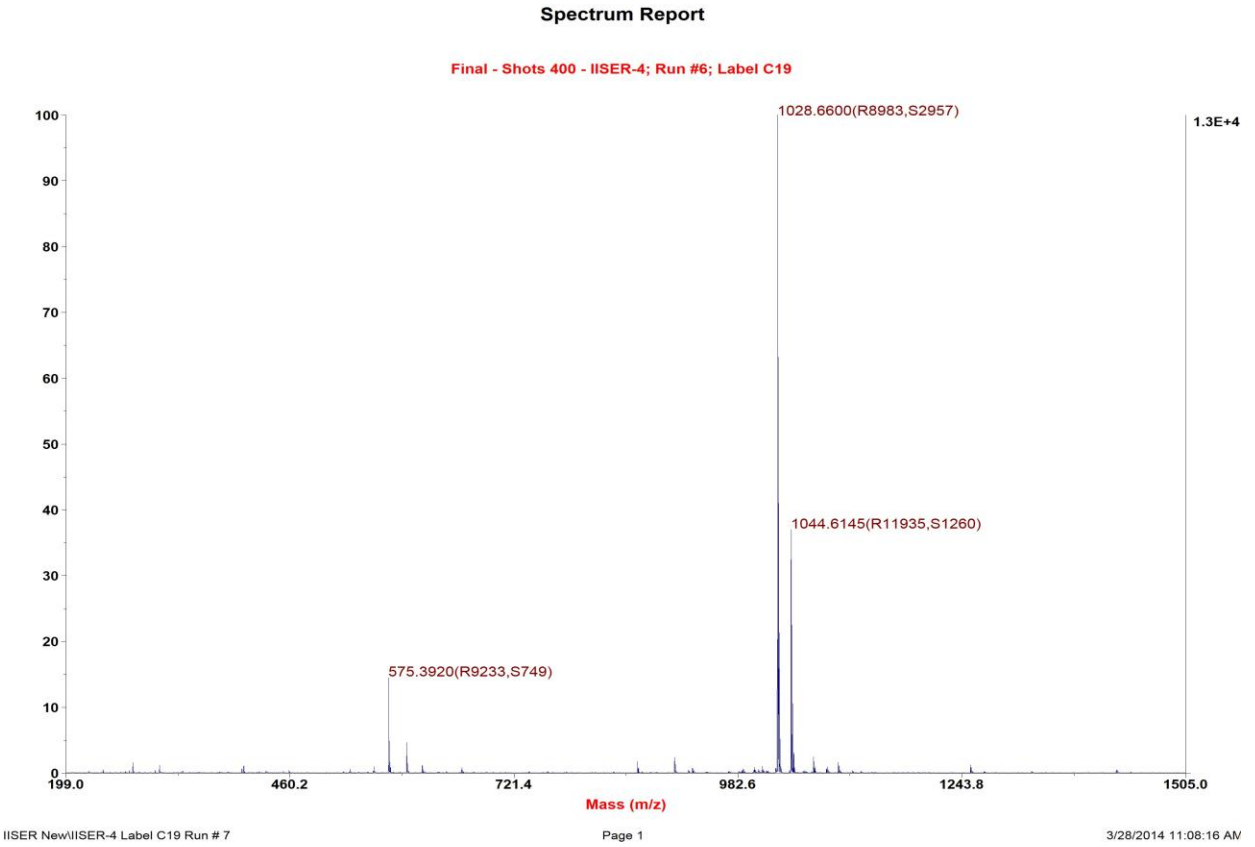

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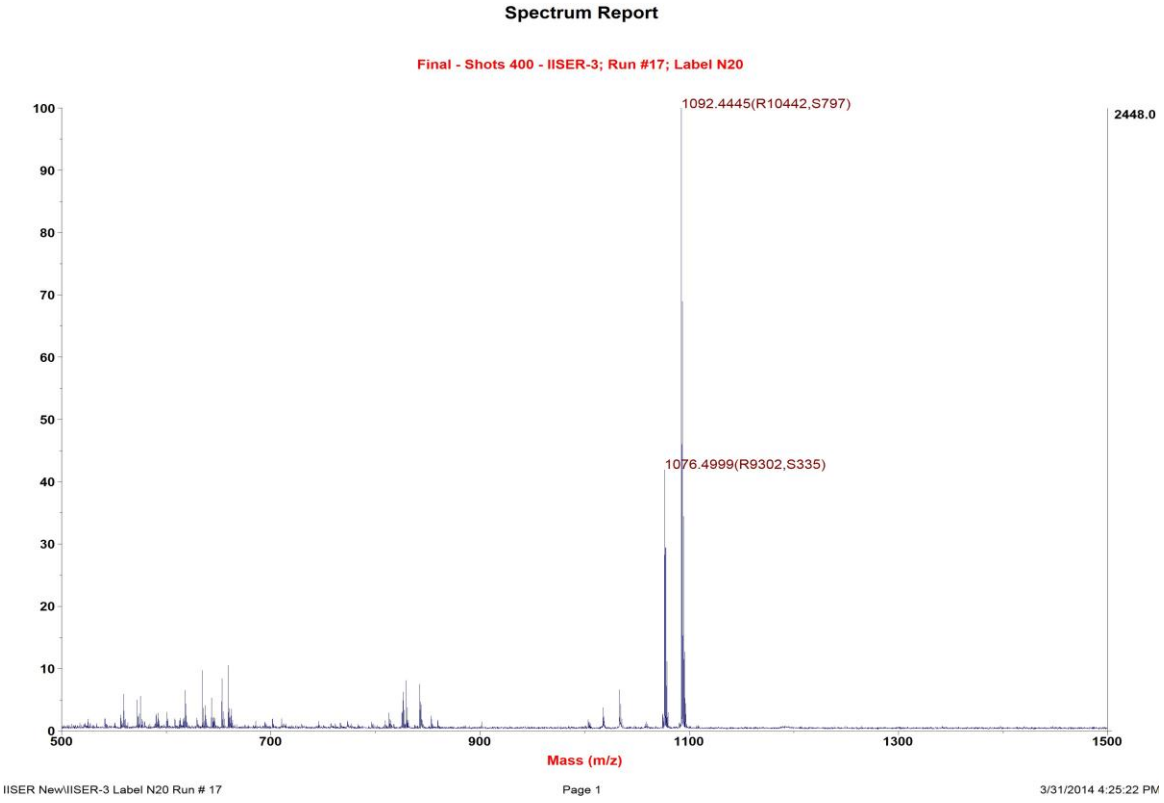

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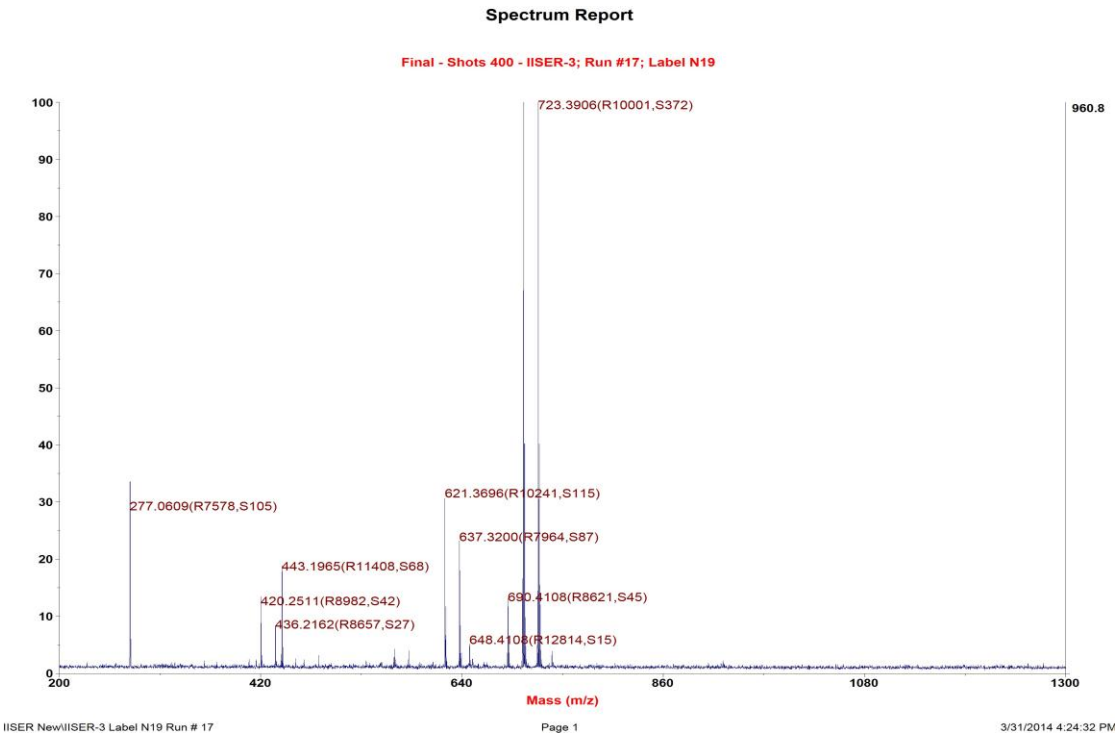
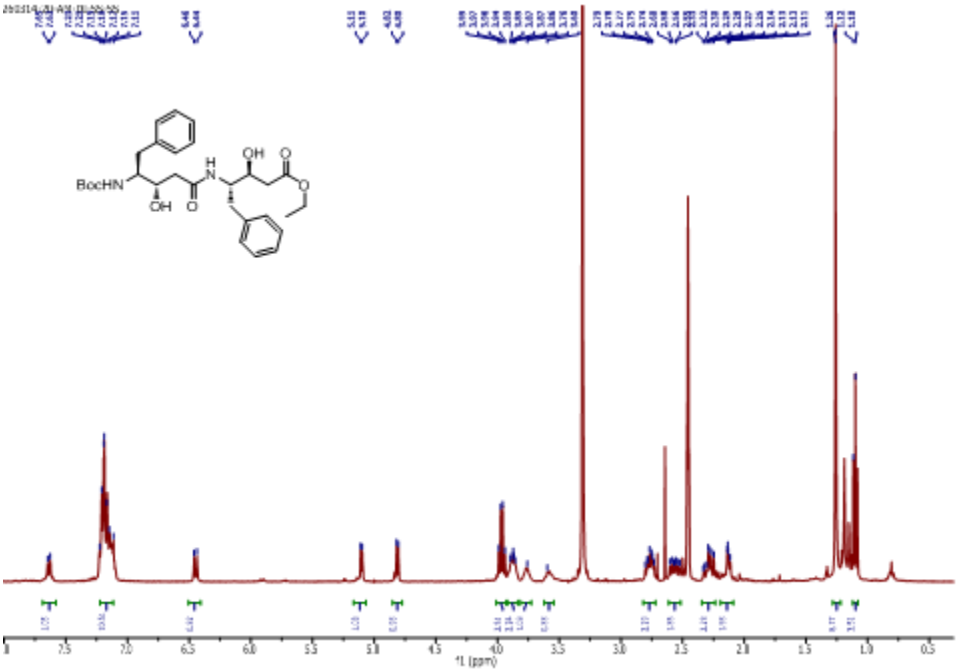


NMR FOR DIPEPTIDES ^{1}H NMR



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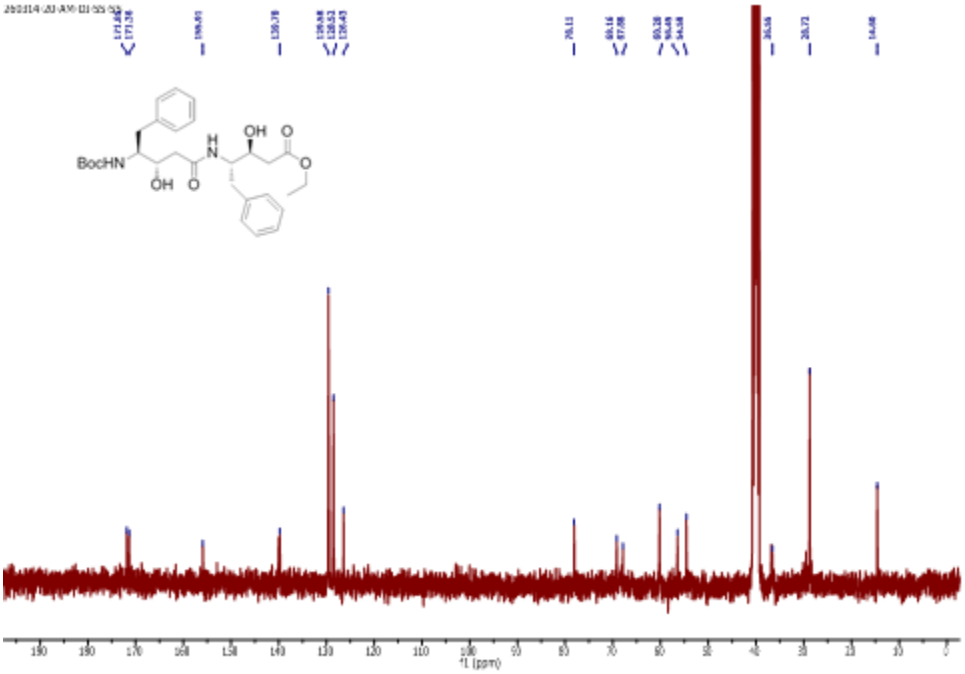

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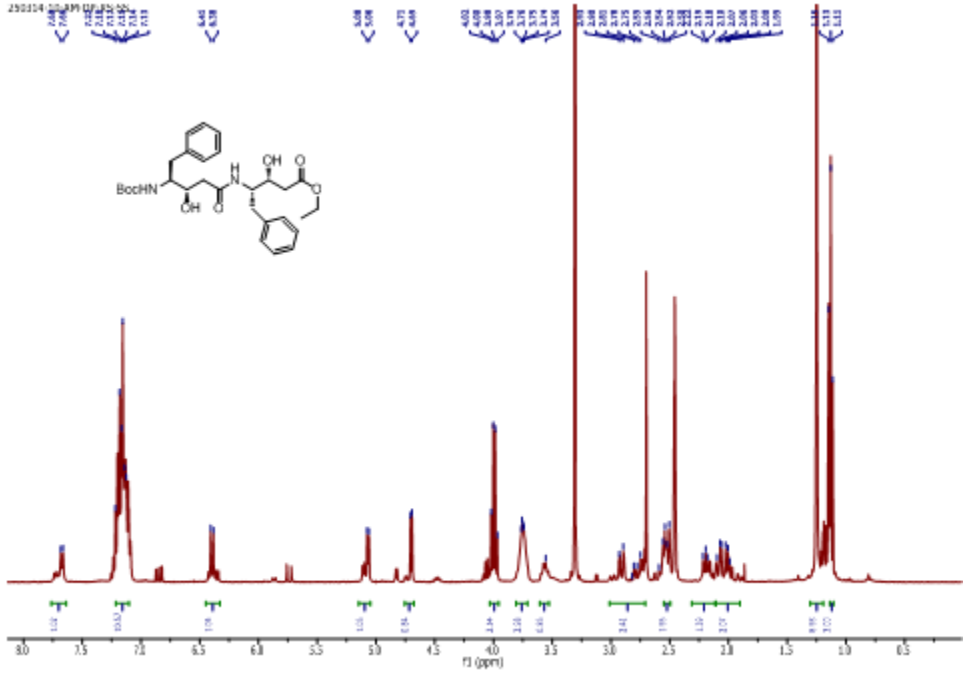

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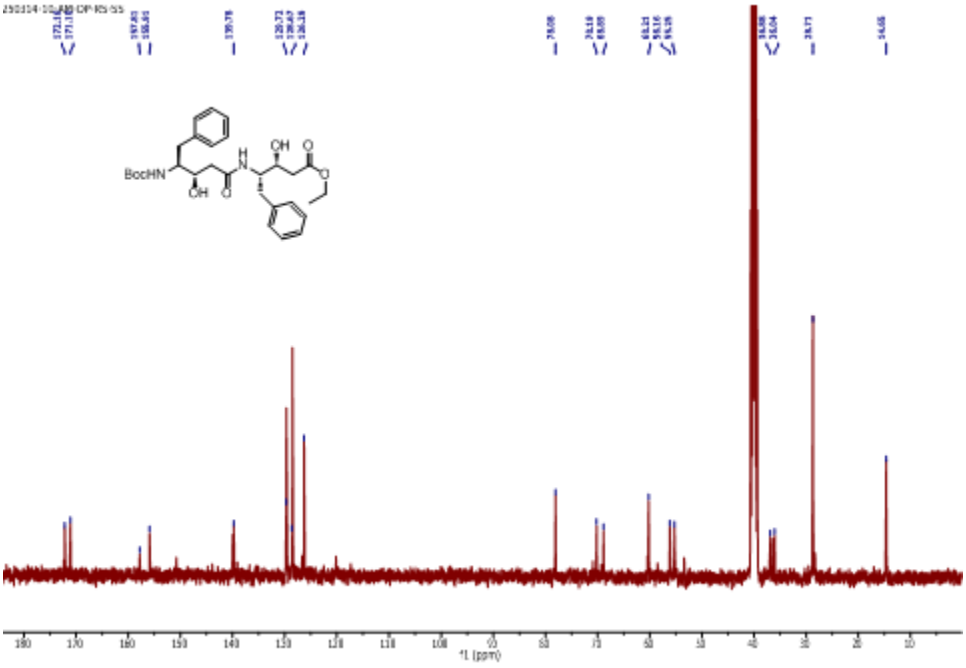
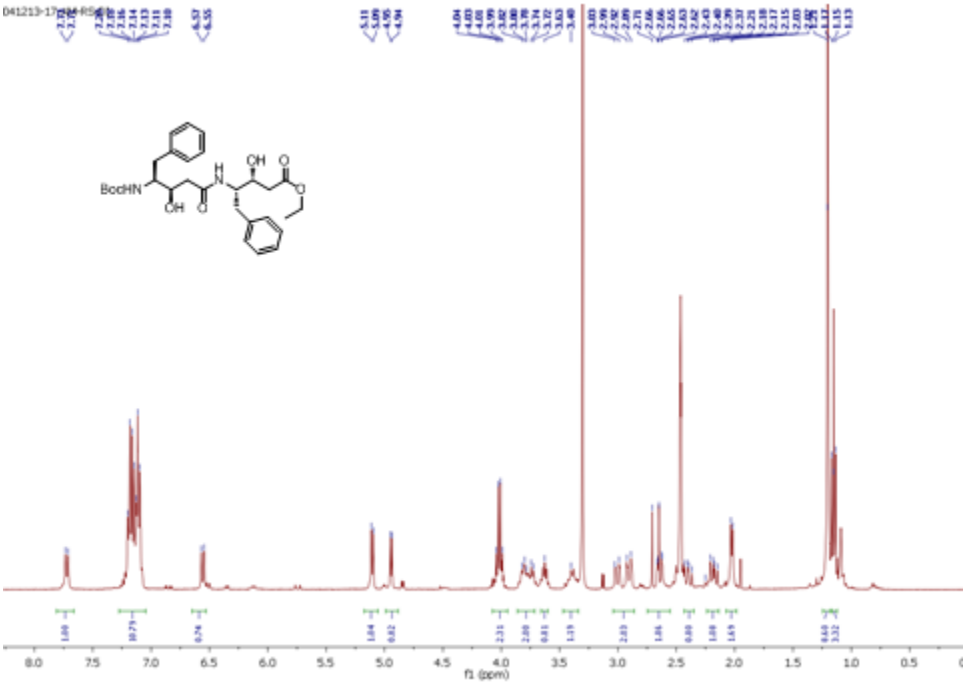


^{1}H NMR



^{13}C NMR