Probing the stability of designed coiled-coil motifs using small synthetic fluorescent amino acids

A thesis submitted towards partial fulfillment of

BS-MS dual degree programme

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By

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CERTIFICATE

This is to certify that this dissertation entitled "**Probing the stability of designed coiled-coil motifs using small synthetic fluorescent amino acids**" towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune submitted by Sumeet Kumar Singh carried out by the candidate at the Indian Institute of Science Education of Research (IISER), Pune, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.

02nd April, 2013

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Declaration

I hereby declare that the thesis entitled "**Probing the stability of designed coiled-coil motifs using small synthetic fluorescent amino acids**" submitted for the partial fulfillment of the BS-MS dual degree programme at Indian Institute of Science Education of Research (IISER), Pune has not been submitted by me to any other University or Institution. This work was carried out at the Indian Institute of Science Education of Research (IISER), Pune, India under supervision of Dr. Hosahudya N. Gopi.

2nd April, 2013

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Sumeet Kumar Singh

CONTENT

ABBREVIATIONS

Ac = Acyl

Ac_2O = Acetic anhydride

AcOEt = Ethyl acetate

ACN = Acetonitrile

Bn = Benzyl

Boc = tert-Butoxycarbonyl

$(Boc)_2O$ = Boc anhydride

Bu^t = Tertiary butyl

Cbz = Benzyloxycarbonyl

CD = Circular Dichroism

DCM = Dichloromethane

DiPEA = Diisopropylethyl Amine

DMF = Dimethylformamide

DNA = Deoxyribonucleic acid

EtOH = Ethanol

EtOAc = Ethyl acetate

Fmoc = 9-Fluorenylmethoxycarbonyl

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

g = gram

hrs = hours

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

HCl = Hydrochloric acid

HOBt = Hydroxybenzotriazol

HPLC = High Performance Liquid Chromatography

IBX = 2-Iodoxybenzoicacid

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

Me = Methyl

MeOH = Methanol

mg = miligram

min = Minutes

µL = Microliter

µM = Micromolar

mL = milliliter

mM = millimolar

mmol = millimoles

MSA = Methanesulfonic acid

NMP = N-methyl pyrrolidone

NMR = Nuclear Magnetic Resonance

PG = Protecting Group

ppm = Parts per million

Py = Pyridine

RT = Room Temperature

TFA = Trifluoroacetic acid

THF = Tetrahydrofuran

HRMS = High resolution mass spectrometry

Abstract

Coiled coils are very important super secondary structural motifs widely present in many protein structures. The conformations of coiled-coils are prescribed primarily by a seven-residue ('heptad') sequence repeat, denoted as ***a-b-c-d-e-f-g***. Positions ***a*** and ***d*** are predominantly occupied by the hydrophobic side-chains and the polar residues generally occupied other positions. Here we are reporting effective incorporation of synthetic fluorescent amino acids as molecular fluorescent probes without much deviation from the overall folding of the coiled-coil peptides. Thermodynamic parameters of the coiled-coil peptides were calculated from the temperature dependent CD spectroscopy. Coumarin amino acids were found to be insensitive towards changes in the polarity of the solvents, while NBD fluorescent amino acid showed huge change in the fluorescent properties with the change in polarity of the solvents. Ironically, peptide P5 was found to be a unique example which changes its conformations with change in the pH of the solution. These results show great promise for the creation of ''smart'' biomaterials whose assembly could be regulated by environmental factors, such as solvents and pH of the solution.

INTRODUCTION

Our understanding of protein structure and function has advanced rapidly over the past few years, providing a mechanistic insight into wide variety of biological events. Examination of three-dimensional protein structures suggests complex tertiary folds and quaternary associations. The disintegration of the protein tertiary structures leads to a limited number of secondary structural elements, such as strands, helices, and turns, which are assembled using loosely structured loops. The secondary structures are locally defined, and there can be many different secondary motifs present in one single protein molecule. Association of two or more secondary structures connected by loops leads to a super secondary structural motifs. Helices associate to form super secondary structures such as α-helical hairpins, coiled-coils, or helical bundles.

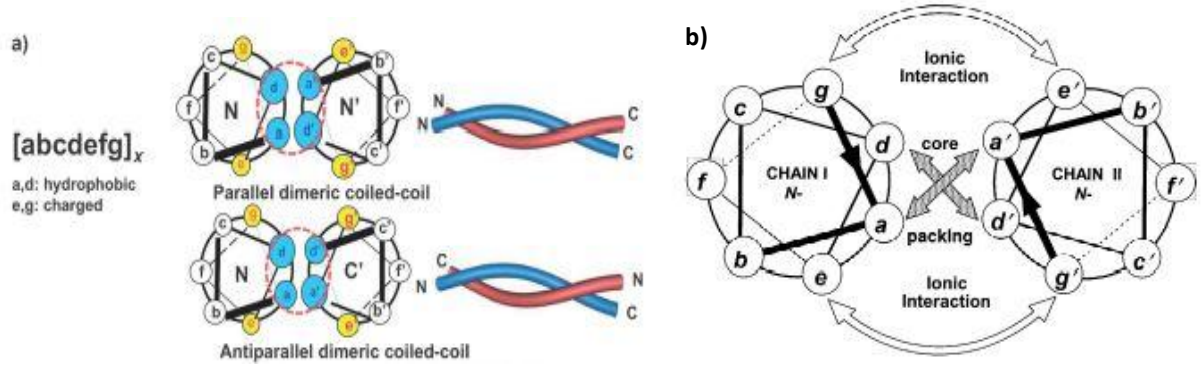


Figure 1. a) Helical-wheel diagram of a dimeric coiled-coil, showing possible structure of coiled-coil motifs. b) Core packing and different interactions.

The Coiled-coils are very important super secondary structural motifs widely used by nature in many protein structures [1,2]. The existence of such domains was first predicted by Crick in 1953 based on the X-ray diffraction pattern of α-keratin [3]. He suggested that a coiled-coil is a structural domain where two or more right-handed amphipathic α-helices wrap around each other to adopt a slightly left-handed super coil [4,5]. The amino acid sequence of the

first coiled-coil was identified initially in 1972 in tropomyosin protein [6,7]. This protein was the smallest and simplest protein postulated to contain a coiled-coil structure at that time. Analysis of the primary sequence of the same protein revealed a seven-amino acid repeating motif denoted as $(\boldsymbol{a}\text{-}\boldsymbol{b}\text{-}\boldsymbol{c}\text{-}\boldsymbol{d}\text{-}\boldsymbol{e}\text{-}\boldsymbol{f}\text{-}\boldsymbol{g})_n$ where the ***a*** and ***d*** positions are predominantly occupied by amino acids containing hydrophobic residues, while the other positions are generally occupied by the polar residues. The schematic representation of the coiled-coil motif is shown in Figure 1. Two helices associate together through a hydrophobic interface between **a** and **d**, making **b**, **c**, and **f** face outward. The coiled-coiled structures are further stabilized by the interhelical salt bridges originate from the side chains of the residue ***g*** of one helix and the residue ***e'*** of the other helix. The prime indicate the residues in the opposite helix. Because of the 2-fold symmetry of the dimeric coiled coil, there are two ion pairs per heptad: *g*-*e*' and *g*'-*e.* These ionic interactions are shown in the **Figure 1b**. Depending on their sequences, α-helices may associate as homodimers, heterodimers in parallel or antiparallel alignments, or form higher order (for example tetramer) aggregates. [8-10]

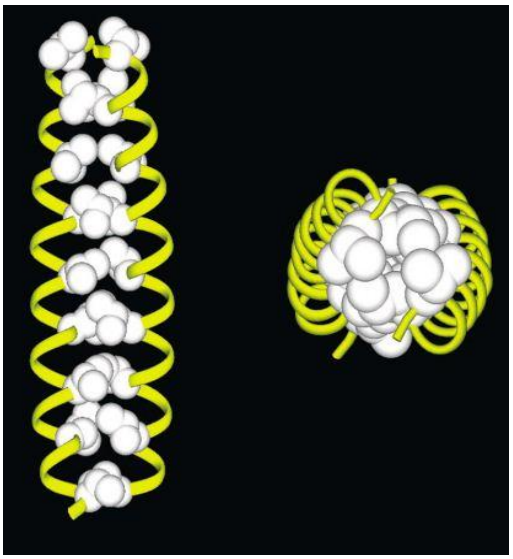


Figure 2: Side (left) and top (right) views of a computer generated dimeric coiled-coil. The yellow ribbons represent the helical backbone while the white balls represent side chains at the hydrophobic core. Notice that while each individual helix is right-handed, the supercoil is left-handed. This is a direct consequence of the heptad sequence repeat.

The abundance of coiled-coil domains can also be seen in many structural and functional proteins including muscle proteins, cytoskeleton proteins, transcription factors, cell and viral surface proteins, tumor suppressors, molecular motors, and many disease- and organ-specific auto-antigens. [11] The coiled-coil domains play very significant role in the specific functions of these proteins. A unique feature of coiled-coils is the specific spatial recognition, association, and dissociation of helices, making it an ideal model for the generation of protein biomaterials in which the higher order structures may be predicted based on the primary sequence. Specific intermolecular interactions of coiled-coil peptides can be recognized through the exact position of the various functional groups in the coiled-coil structures [12] (**Figure 2**).

Typically α-helix is a right-handed helix and constitutes 3.6 amino acid residues per turn and the structure is stabilized by the canonical 13-membered intramolecular H-bonds. The α-helices in the coiled-coils are slightly distorted so that the helical repeat is 3.5 residues rather than 3.6, as in a regular α-helix. One heptad repeat in coiled-coils forms exactly two helical turns. In nature, coiled-coils with different periodicities, for example, 11-residues periodicities, or with insertions of one or more residues into the heptad pattern can also be found in many protein structures. [4,13] The dimers, trimers, and tetramers are most frequently observed in nature, however, the formation of multimers and more complex assemblies are also well-known. [14]

The functional and the structural diversity of coiled-coil domains lead to the extensive investigations of these structures from outside protein context. Degrado and colleagues designed the coiled-coil tertra helical bundles using protein engineering [15]. Kim and colleagues showed crystal conformations of the fragments of GCN4 coiled-coils.[16] Further, persubstitution of *e*/*g* positions with either Glu or Lys, and inclusion of a buried core asparagine (Asn) has also been established to favor the heterodimer formation from equimolar mixtures of the resultant peptides. However, there is some controversy ensued about stability gains from surface salt bridges in the heterodimer, destabilization of the competing homodimers (presumably through electrostatic repulsion) was clearly demonstrated. The utility of the acid/base heterodimer in the programmed assembly of desired components has subsequently been established in

numerous contexts. Recently, Fairman and colleagues reinvestigated the coiled-coil heterotetramers initially investigated by the Kim et al. In this new design, Fairman et. al introduced the glutamate residues at the **c'** instead of **g'** which leads to the stable coiled-coils structural motifs through a better electrostatic interactions between the acids and bases.[17] We anticipate that introducing the fluorescent probes as molecular reporters may provide addition evidence to understand the coiled-coil structural properties. In addition, these studies also provide tolerance of coiled-coil peptides for guest fluorescent molecules. Herein, we are reporting the synthesis, incorporation and structural investigation of coiled-coil hereotetramers containing small fluorescent amino acids. The stability of the coiled-coil peptides against the pH change and temperature also investigated.

2.0 Design of coiled-coil peptides

In order to understand the coiled-coil structures, we adopted similar strategy reported by the Fairman and colleagues to design the coiled-coil peptides. The coiled-coil sequences were redesigned by replacing the N-terminal Met residue with Leu. The sequence of the coiled-coil peptides are shown in Scheme 1. Both the peptides were synthesized by solid phase method

P1: LKEIEDK LEEIESK LYEIENE LAEIEKL

P3: LKKIKDK LEKIKSK LYKIKNE LAKIKKL

Scheme 1: Sequence coiled-coil heptad repeats

using standard Fmoc chemistry on Rink amide resin. Coupling reactions were carried out using HBTU/ HOBt coupling reagents. After completion of the synthesis the N-terminal acetylated peptides were released from the resin using the cocktail mixture of TFA, thioanisole and water. The peptides were purified by reverse phase HPLC on C18 column using acetonitrile water gradient system. Homogeneity of the peptides was confirmed using analytical HPLC and MALDI-TOF/TOF. The circular dichroism (CD) spectra of **P1**, **P3** and **P1+P3** are shown in **Figure 3**. As

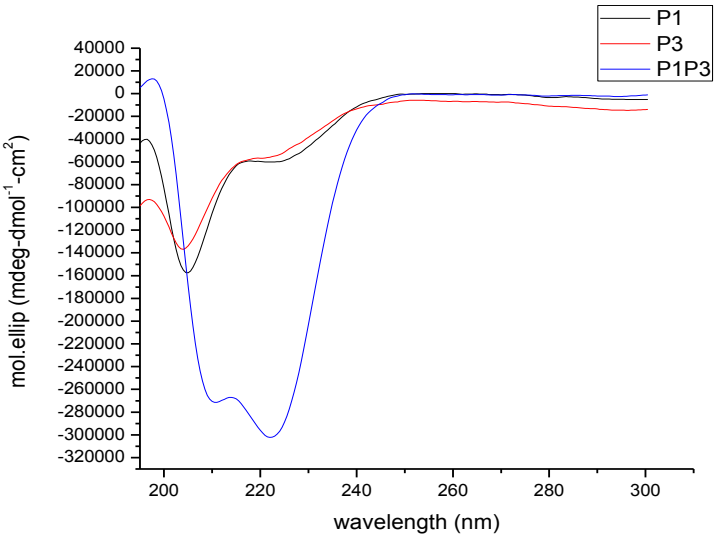
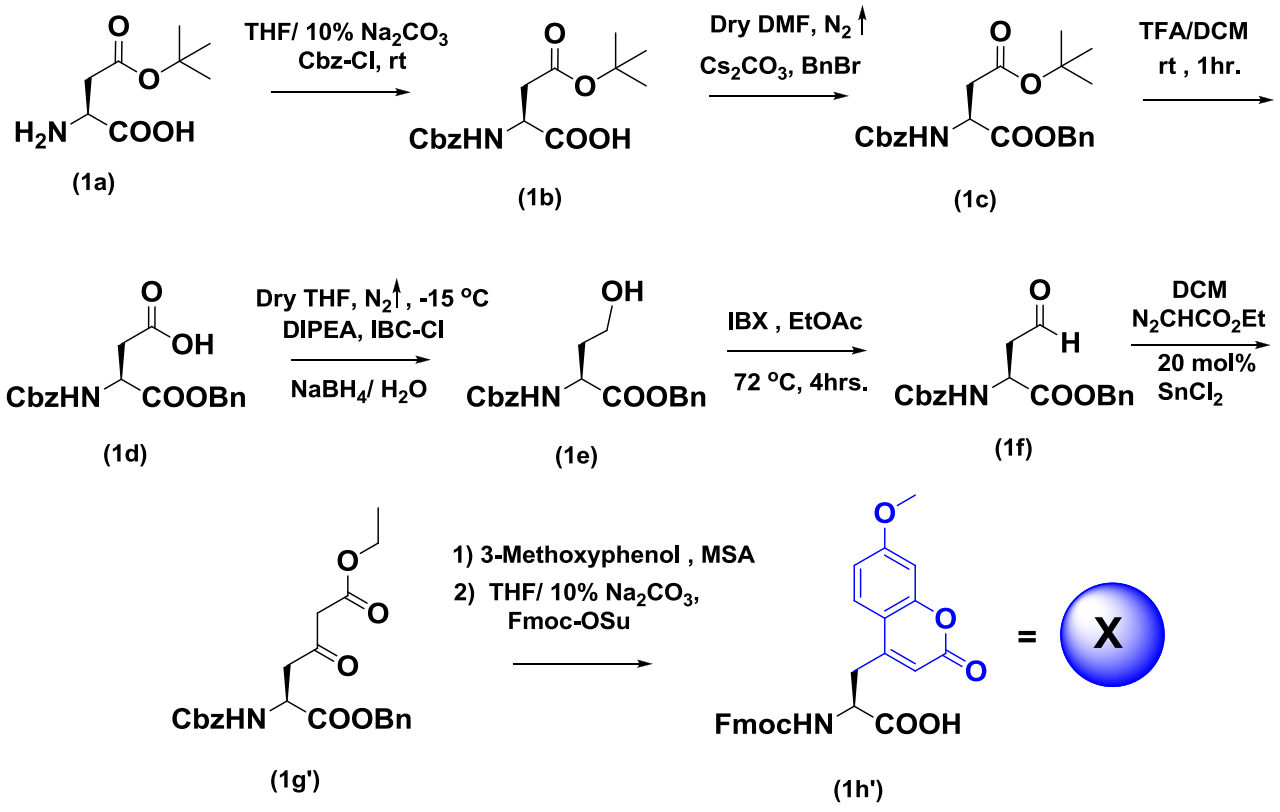


Figure 3: CD spectra of P1, P3, and P1+P3.

anticipated, **P1** and **P3** remain unfolded in solution due to the electrostatic repulsions between the like-charged residues at the positions **e** and **c'** and 1:1 mixture of **P1** and **P3** showed a stable coiled-coil conformation. Calculation of the stability of coiled-coil conformation of **P1+P3** using the Young's model suggest the 100% helicity of the peptides. With this encouraging result, we move forward to introduce the fluorescent amino acids at d11 hydrophobic positions of the **P1** and **P3**. We recently reported the synthesis of coumarin amino acids starting from the β-keto γ-amino esters.[18] We adopted the same methodology for the synthesis of amino acid containing coumarin side-chain. The schematic representation of the synthesis of solid phase compatible N-Fmoc-protected coumarin amino acid **1h'** is shown in Scheme 2. Briefly, the coumarin amino

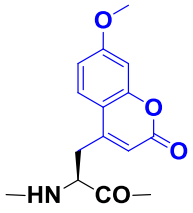


Scheme 2: Synthesis of side chain coumarin amino acid.

acid (1h') was synthesized starting from the commercially available NH_2-Asp(O^tBu)-OH. The amine and carboxylic acids were protected with Cbz- and Bzl groups, respectively (1c). The orthogonally protected t-butyl group was removed by the treatment of 50% TFA in DCM and the free side chain carboxylic acid was converted to aldehyde [Cbz-Asp(CHO)-OBzl] through the oxidation of corresponding alcohol using IBX. The aldehyde was transformed to β-keto ester (1g') by reacting with ethyl diazoacetate in the presence of $SnCl_2$ catalyst. The Pechmann condensation of 1g' with 3-methoxyphenol in the presence of MSA led to the

P2: LKEIEDK LEEXESK LYEIENE LAEIEKL

P4: LKKIKDK LEKXKSK LYKIKNE LAKIKKL

Where X= 

Scheme 3: Sequence of coiled-coil peptides containing coumarin amino acids

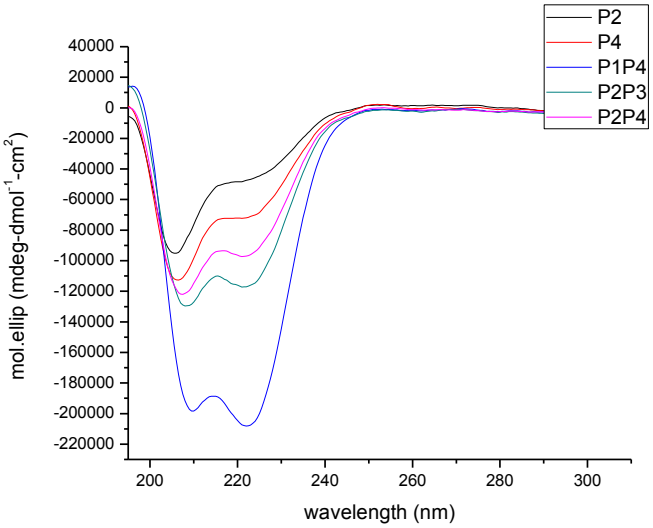


Figure 4: CD spectra of P2, P4, P2P3, P2P4, and P1P4.

formation of methanesulfonate salt of amino acid coumarin. The free amino group was again protected with Fmoc- group in the presence of Na_2CO_3. The pure amino acid 1h was used in the solid phase synthesis of coiled-coil peptides P2 and P4. The sequences of P2 and P4 are shown in Scheme 3. Similar to the P1 and P3, peptides P2 and P4 were synthesized using solid phase method. Pure peptides were subjected to the CD analysis and the results are shown in Figure 4. Results reveal that similar to the P1 and P3, P2 and P4 are unstructured in solution, while the 1:1 combination of P2 and P4 displayed the helical conformation. In addition, 1:1 combination of P1 and P4, P2 and P3 also displayed the helical conformations (Figure 4). These results reveal that coumarin amino acids can be incorporated into the coiled-coil sequence without much deviation in their helical conformations. We further subjected these peptides for fluorescence studies. Results of the fluorescence properties of the peptide are summarized in the **Figure 5**.

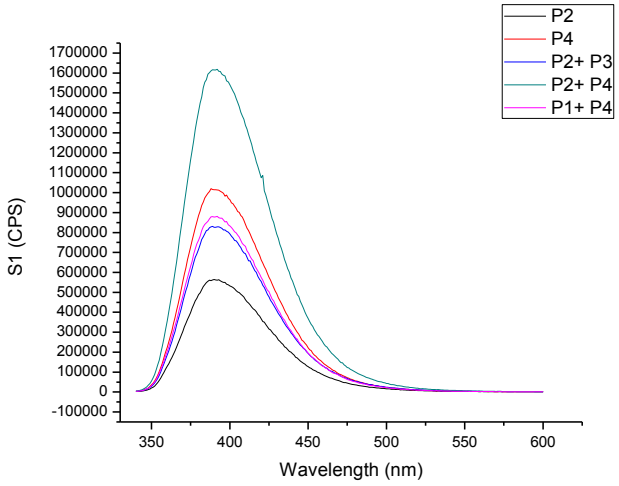
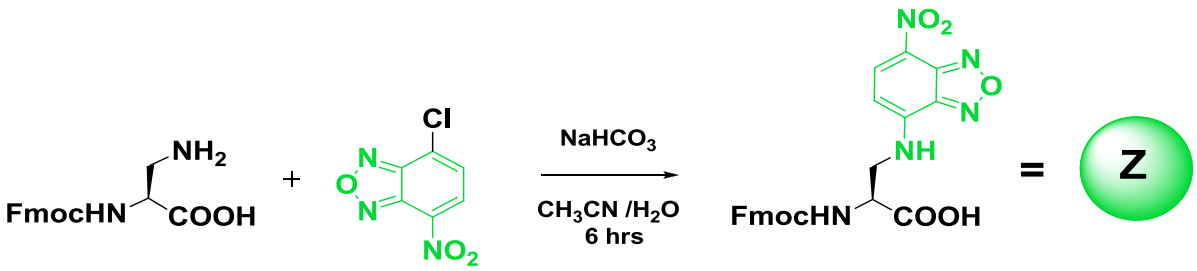


Figure 5: Fluorescence spectra of P2, P4, P2P3, P2P4, and P1P4.

The huge fluorescence intensity observed at 420 nm after exciting the peptides at 320 nm is unfortunately not environment sensitive. In addition, the CD spectral analysis suggests that that the combination of P1and P2, P3 and P4 displayed better helical conformation than the P2 and P4. These results promoted us look for another small and environment sensitive fluorescent amino acid.

Based on the literature survey,[19,20] we anticipate that 7-nitrobenzo-2-oxa-1,3-diazole (NBD) derivatives of amines can be used as environment sensitive fluorescent reporters. We speculate that NBD can be introduced to the amino acids through amidation via S_NAr mechanism. We utilize free amine side-chain of the commercially available Nα-Fmoc, L-diamino propionic acid (Fmoc-Dap-OH) to introduce NBD derivative. The schematic representation of the synthesis of fluorescent NBD amino acid is shown Scheme 4. The choice of the solvent was crucial for compounds solubility and integrity. The apparent value of the pH had to be maintained between 8.1 and 9 by $NaHCO_3$ addition. During the reaction, the complete disappearance of Fmoc-Dap-OH necessitated the addition of a second aliquot (0.5 equiv.) of NBD-Cl despite equimolar stoichiometry. This synthesis procedure leads to a crude product with 85±90% purity. The pure amino acid was subjected to the solid phase synthesis.

a)



b) P5: LKKIKDK LEKZKSK LYKIKNE LAKIKKL

Scheme 4: a) Synthesis of amino acid NBD derivative. b) Peptide sequence P5 analogous to the sequence of P1 except the NBD derivative at the position d11.

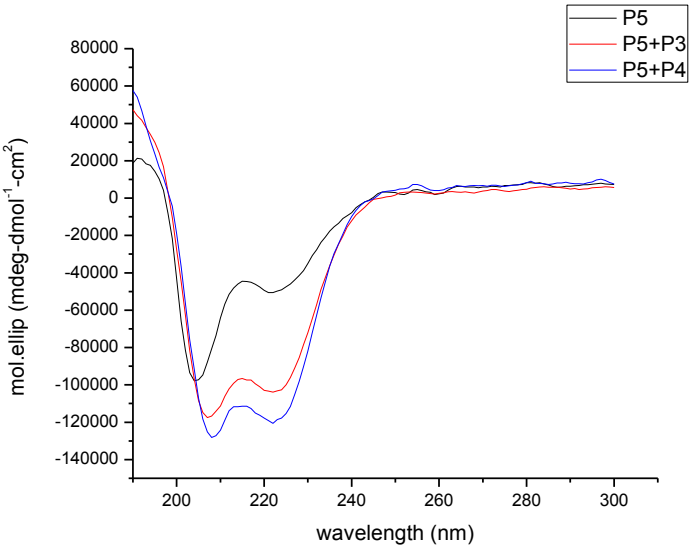


Figure 6: CD spectra of P5, P5P3, and P5P4.

The CD spectra of spectra of P3 and P5, P4 and P5 and is shown in the **Figure 6.** Results of CD spectral analysis suggest that P4+ P5 and the P3 +P5 both showed the coiled-coil conformations.

2.1 Investigation of the stability of coiled-coil peptides using temperature dependent CD spectroscopy.

We used circular dichroism (CD) to test for helical content as a measure of coiled-coil formation. CD spectra were acquired for P1, P2, P3, P4 and P5 or an equimolar P1/P3, P1/P4, P2/P3, P2/P4, P5/P3 and P5/P4 mixture (all at 30 µM total peptide concentration) in 10 mM sodium phosphate (pH 7.0) and 150 mM NaCl (Figures 3-6). The spectra of P1, P2, P3, P4 and P5 showed that all peptides were largely unfolded, with strong minima at 202 nm and very weak minima at 222 nm. The equimolar P1/P3, P1/P4, P2/P3, P2/P4, P5/P3 and P5/P4 mixture, on the other hand, was judged to be fully helical, with the 202 nm minimum shifted to 208 nm and the minimum at 222 nm of approximately equal intensity to the 208 nm minimum.

Temperature dependant circular dichroism spectroscopy was used to experimentally probe the thermodynamic stability of the dimers. All of the peptide combinations show cooperative thermal unfolding transitions upon heating from 20 to 80 ^{o}C (**figure 7 & 8**). Upon heating the coiled-coil peptides started unfolding from helical conformation to random coils. We calculated the thermodynamic parameters by following the decrease in the CD minima at 222 nm using the equation $\theta = (\theta_M - \theta_D)P_M + \theta_D$, where θ is normalized ellipticity, θ_M and θ_D represent the ellipticity values for the fully unfolded monomer and fully folded dimer and P_m is fraction of unfolding. P_m was calculated from the $P_M = [(8KC + 1)^{1/2}-1]/4KC$, where C is the total peptide concentration and K is equilibrium constant.[21,22] The Gibbs–Helmholtz equation can be used to express the temperature dependence of ΔG in terms of ΔH_m and T_m as given by Equation:

$$\Delta G = \Delta H_m \,.\, (1 - T/T_m) + \Delta C_p \,.\, (T - T_m - T \,.\, ln(T/T_m))$$

The thermodynamic parameters of unfolding are summarized in Table 1. Analysis reveal that the coiled-coil structure of P1 and P3 showed the greater stability followed by the peptides with single substitution of either coumarin or NBD at the 11d or 11d'. Coiled-coiled structure of peptides P4 and P5 where the isoleucine substituted by the Asp(Cum)-OH and Dap(NBD)-OH respectively, showed the considerable decrease the in the thermodynamic stability. The melting temperature of P1P3 was found to be 63 °C while P4P5 was found to be 41 °C. We further calculated the Gibbs free energy of the coiled-coil structures at 37 °C and the results are shown in the Figure 9. Results reveal that coiled-coil structure of P1P3 is approximately 2 kcal/mol more stable than the coiled-coil structure of P4P5 at 37 °C.

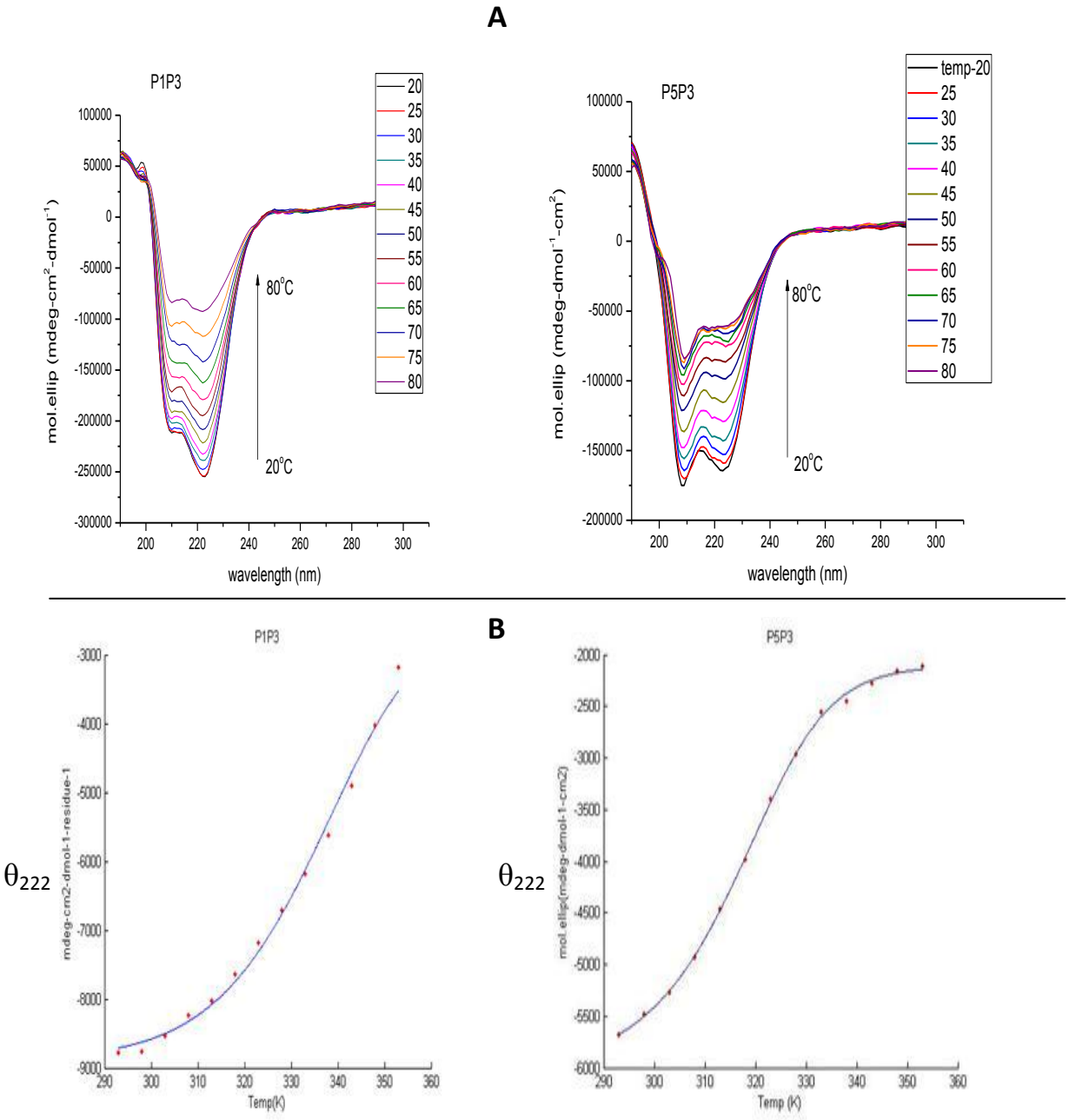


Figure 7: A) Temperature dependent CD spectra of P1P3 and P5P3 showing thermal unfolding of coiled-coil peptide from 20 – 80 ^{o}C . **B)** Mol. Ellip at λ = 222nm (θ_{222}) vs temp (K) best fit curve.

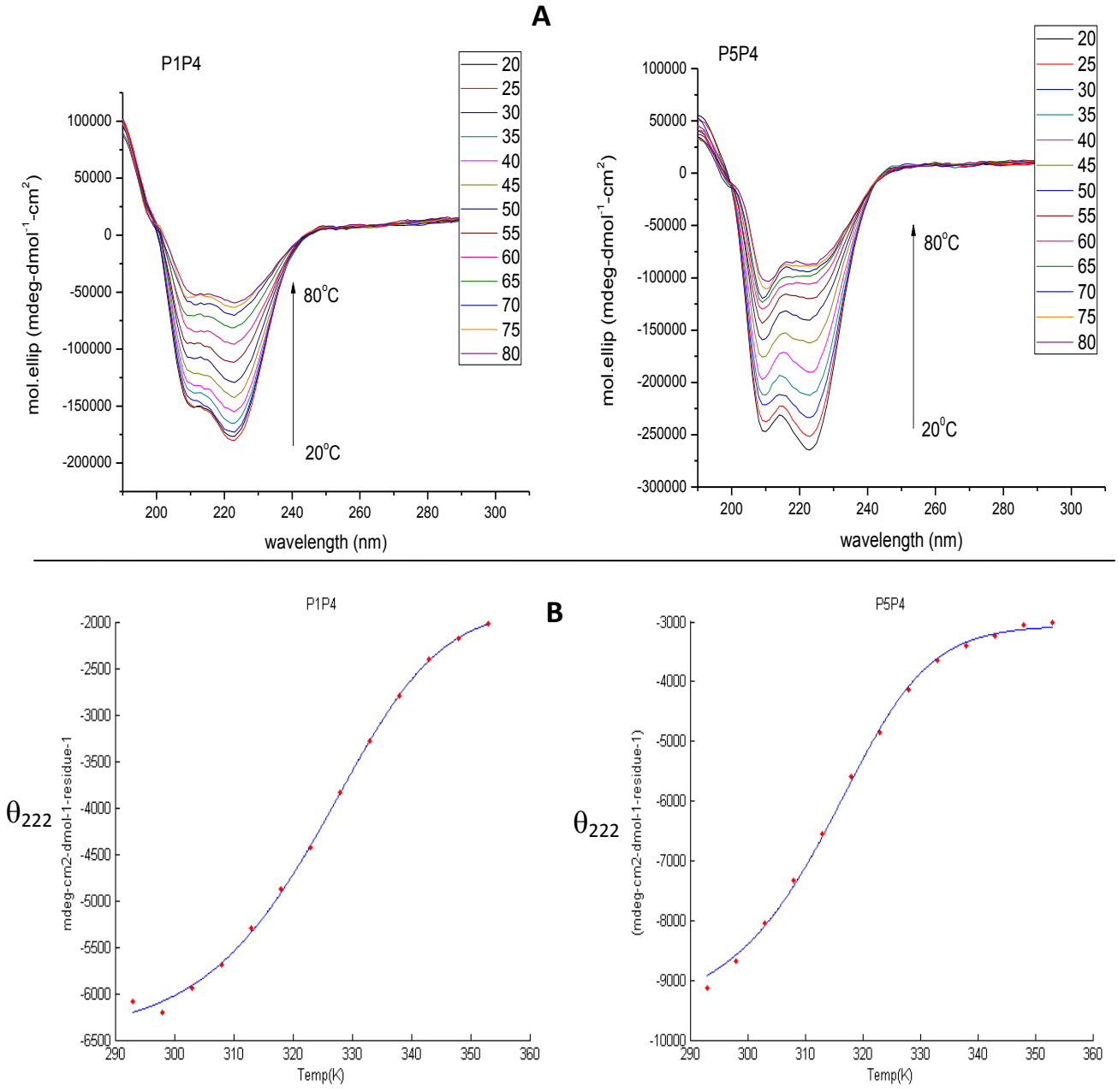


Figure 8: A) Temperature dependent CD spectra of P1P4 and P5P4 showing thermal unfolding of coiled-coil peptide from 20 – 80 ^{o}C . **B)** mol. Ellip at λ = 222nm (θ_{222}) vs temp (K) best fit curve.

Table 1: Thermodynamic parameters of coiled-coil peptides.

peptide	a	$\theta_m(0)$	$\theta_D(0)$	$\Delta H(T_m)$ (Kcal/mole)	T_m (K)	ΔG_o (37°C) (Kcal/mole)	R^2
P1P3	0.01999	-2500.000	-8874.856	-30.183	336.16 (63.16°C)	-8.7505	0.99368
P1P4	-0.01999	-1827.718	-6411.937	-31.2353	325.53 (52.53°C)	-7.8886	0.99887
P5P3	-0.01999	-2104.2632	-5970.87463	-32.82953	317.26 (44.26°C)	-7.1460	0. 9994
P5P4	-0.0200	-3054.9366	-9500.0000	-33.6369936	314.73 (41.73°C)	-6.8996	0.9984

Free energies of coiled-coil peptides at 37 °C

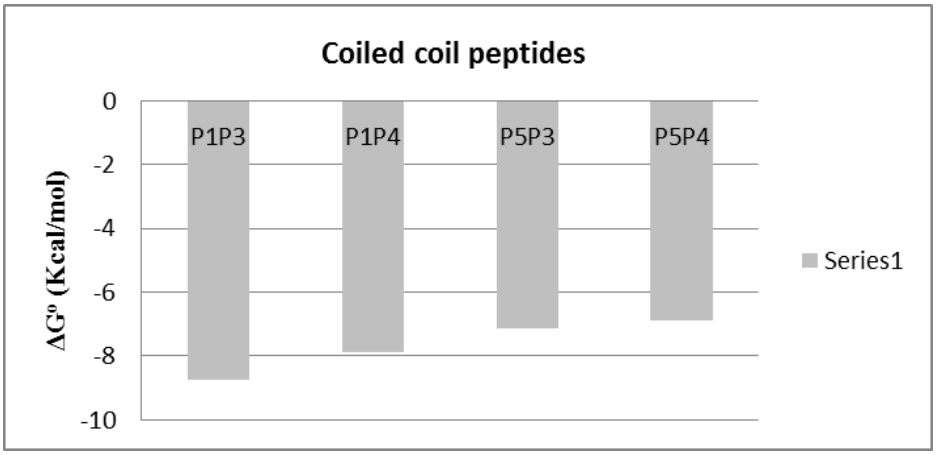


Figure 9: Relative stability (ΔG^o at 37 °C) of coiled-coil peptides.

2.5 pH dependent conformational analysis of coiled-coil peptides using CD and Fluorescence spectroscopy.

We further investigated the influence of pH change on the coiled coil conformations using circular dichorism (CD) and fluorescence spectroscopy. For CD studies all peptide sample were prepared in sodium phosphate buffer at pH 7.0 similarly as described earlier. The CD spectra were recorded for all coiled-coil peptides, P1P3, P3P5, P4P5 and individual unstructured peptides P1, P2, P3, P4 and P5. To understand the any influence of the pH change on the coiled-coil conformation, we subjected all peptides to pH studies. Acidic pH was achieved by adding diluted acetic acid and basic pH was achieved using potassium carbonate (K_2CO_3) to the peptides in phosphate buffer. The CD spectra were recorded at different pHs and the results are shown in **Figure 10**. Analysis of CD spectra reveals very interesting results. In the case of P1P3, decreasing pH resulted in no change in the coiled-coil conformation, while increase in the pH resulted in destabilization of the coiled-coil conformation. Intriguing results were observed in the case of P5P3 and P4P5. Either increase or decrease in the pH leads to the transition from coiled-coil to β-sheet structure. This transition from the coiled-coil to β-sheet structures have rarely observed in the literature. This type of transition was not observed in the other coiled-coil peptides, P1P3, P2P3, P2P4 and P1P4. Further to understand the response of individual peptides to the pH change, we subjected all peptides P1, P2, P3, P4 (data shown) and P5 for the CD studies. In contrast to the other peptides, P5 showed very interesting behavior. The random coil behavior of P5 at neutral pH changed to β-sheet signature at lower pH while at higher pH it displayed α-helical character (**Figure 11**). Ironically, peptide P5 was found to be a unique example which changes its conformations with change in the pH of the solution.

We further investigated the fluorescence properties of the coiled-coil peptides in response to the change in the pH. Results are compiled in the **Figure 12A**. Analysis of the fluorescence spectra reveals the influence of the environmental change on the properties NBD derivatives. The maximum fluorescence intensity was observed at neutral pH and the fluorescence intensity deceases either increasing or decreasing the pH of the solution. Similar fluorescence properties are also observed in the P5, however with lower intensities. The plot of fluorescence intensities

verses pH is shown in **Figure 12b**. In contrast to the P3P5, P4P5 showed the lower fluorescence intensities the solvent exposed NBD derivative due to the steric class of NBD and coumarin derivatives at the hydrophobic pocket.

Influence of pH on the conformational properties of coiled-coils

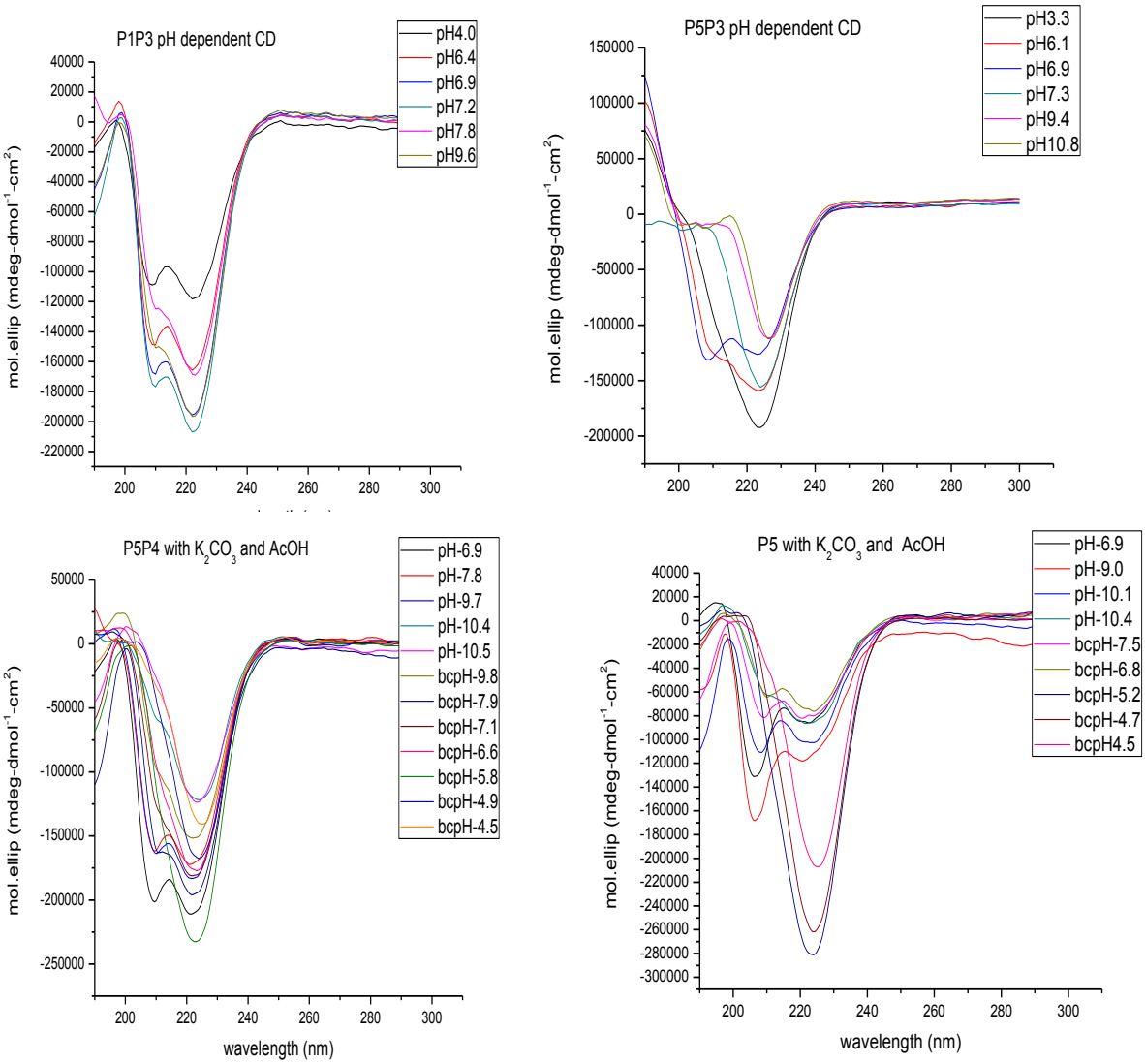


Figure 10: Influence of pH on the coiled-coil conformations**.**

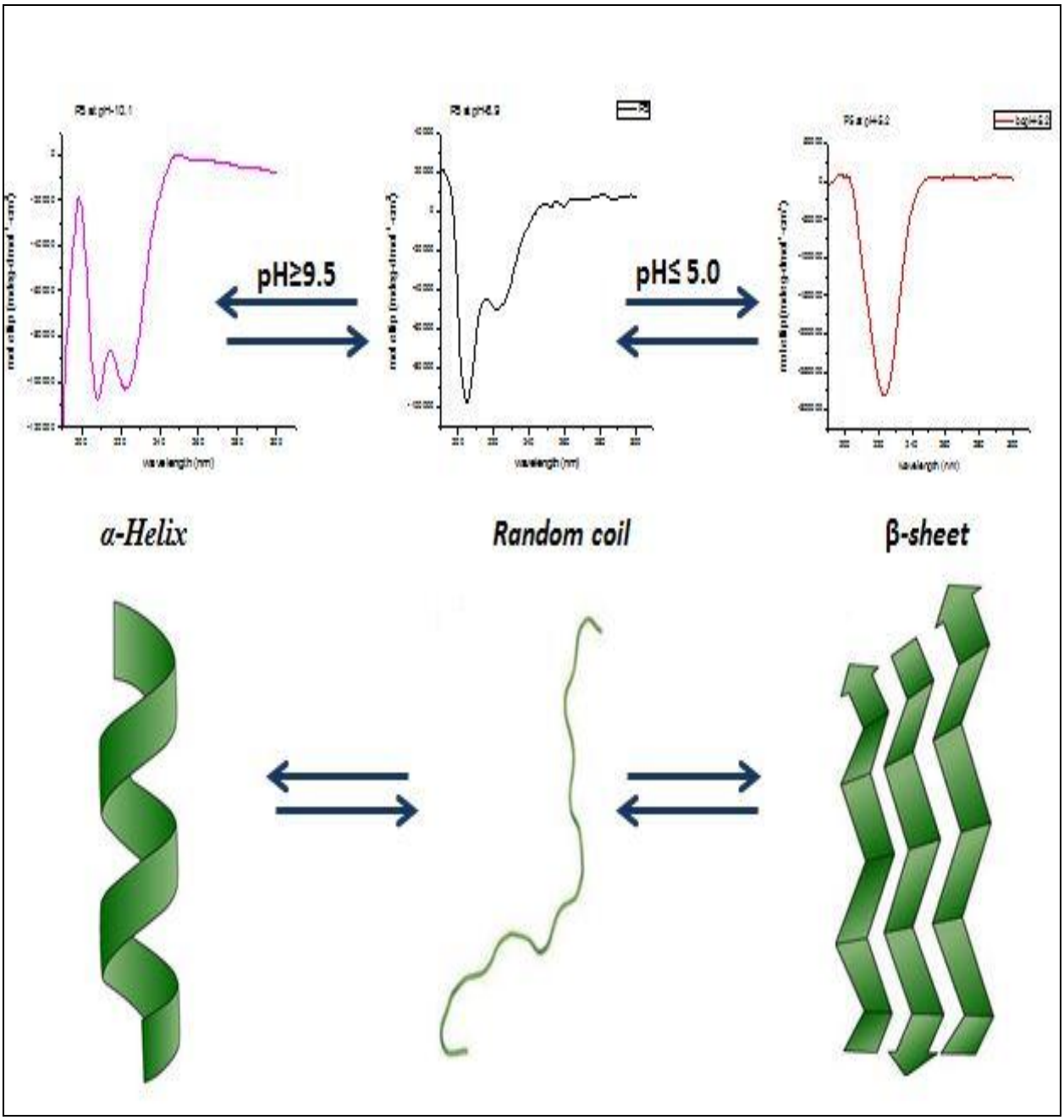


Figure 11: Conformational change of **P5** with pH change monitored by CD spectra.

Influence of pH on the fluorescent properties of coiled-coils

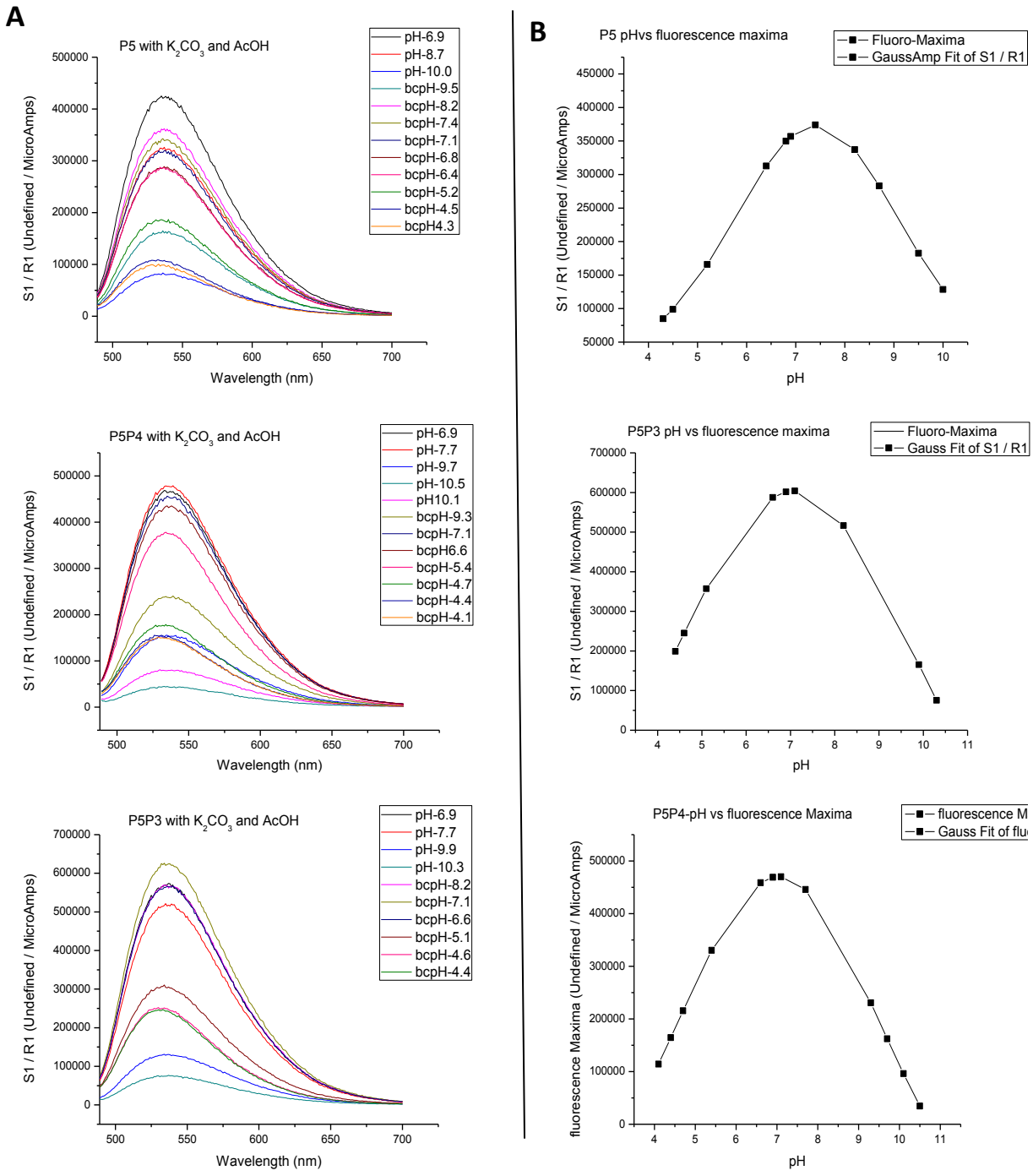


Figure 12: A) Fluorescence spectra of colied-coils with change in the pH. **B)** pH vs fluorescence maxima for the same peptide combination.

Conclusion

We have demonstrated the incorporation of small fluorescent amino acids as guest molecules into the host coiled-coil peptides without much deviation from the coiled-coil conformation of the peptides. Thermodynamic parameters of the coiled-coil peptides calculated from the temperature dependent CD, suggests that single substitution at hydrophobic sites decreases the stability of the peptides. The peptides substituted with coumarin and NBD derivatized amino acids at d_{11} and d'_{11} melt at 41 °C. In contrast to the NBD derivatives, coumarin derivatives are not sensitive to the environmental change. The transition of coiled-coil to β-sheets is unique example with respect to the coiled-coil design. In addition, the peptide P5 adopted three different conformations in response to the change in the pH of the solution. The low Tm values and their transition from coiled-coil to β-sheets structures in the response to the pH change can be further utilized in the design of smart biomaterials as well as promoters for the drug release from lipid vesicle drug delivery systems.

METHODS

3.0 Experimental Section

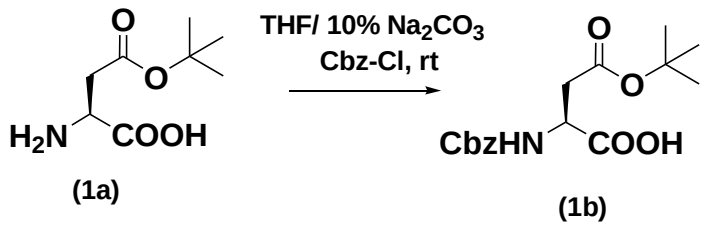
3.1 Materials

All amino acids, DiPEA, TFA, triphenylphosphine were purchased from Aldrich. THF, DCM, DMF, NaOH were purchased from Merck. Ethyl bromoacetate, HBTU, HOBt, EtOAc, NMP, Pet-ether (60-80 ^{o}C) were obtained from Spectrochem and used without further purification. THF and DiPEA were dried over sodium and distilled immediately prior to use. Column chromatographies were performed on Merck silica gel (120-200 mesh). 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 [($CDCl_3$ δ_H, 7.26 ppm, δ_C 77.0 ppm) and CD_3OH δ_C 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.

3.1 <u>Synthesis of side chain coumarin amino acid.</u>

a) Synthesis of N-Cbz-protected Asp(OBu^{t})-COOH

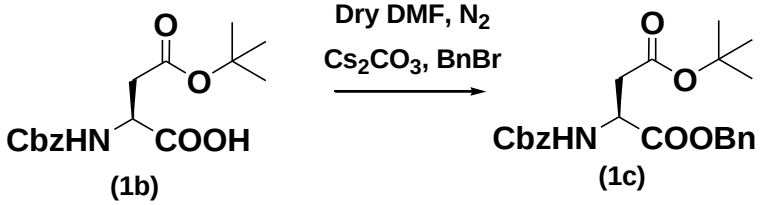
NH_2-Asp(O^{t}Bu)-OH (10 mmol) was dissolved in 20mL of 10% Na_2CO_3 solution and 10 mL of THF was added in same mixture. Cbz-Cl (1.43mL, 10 mmol) was added in reaction mixture and stirred for 12 hrs. After completion of reaction THF was evaporated under reduced pressure, the residue was dissolved in ethyl acetate (50ml) ,acidified with 10% HCl , extracted by 3x100mL of EtOAc, washed with Brine, dried over Na_2SO_4 , and concentrated over reduced pressure to get Cbz- Asp(O^{t}Bu)-OH as a white solid (**100%**)



(1a) (1b)

1b) <u>Benzyl- protection of N-Cbzprotected Asp (OBu^t)-COOH</u> :-

Cbz-Asp(O^{t}Bu)-OH (1 eq.) was dissolved in dry DMF under N_2 atmosphere, 1.1 eq. of Cs_2CO_3 and 1.2 eq. of BnBr (Benzyl bromide) was added in reaction mixture and it was stirred for 6-7 hrs. After reaction completion (checked by TLC) it was filtered and filtrate was concentrated over reduced pressure to get Cbz- Asp(O^{t}Bu)-OBzl.



(1b) (1c)

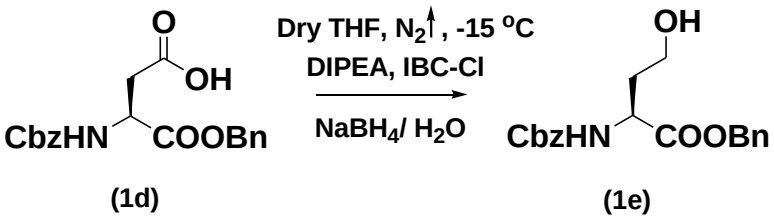
1c) <u>Procedure for tertiary butyl (^{t}Bu) deprotection</u>:-

Compound 1c was dissolved in 5mL of DCM and 6mL TFA was added in that reaction mixture under ice cooled condition and reaction was stirred for 1 hrs. After that DCM and TFA was evaporated out over reduced pressure to get 1d.

1d) <u>Synthesis of Cbz-protected amino alcohol:-</u>

1 eq. of Cbz-Asp(OH)-OBzl (1d) was dissolved in dry THF (10 mL) under nitrogen atmosphere, and cooled to -15 ^oC, and then treated with DiPEA (1.2 eq.) followed by isobutyl chloroformate (1eq.). White hydrochloride salt of DiPEA was precipitated out immediately after the addition of isobutyl chloroformate. The reaction was continued to stir for another 10 mins. A solution of $NaBH_4$ (2 eq.) in 5 mL of water was added with vigorous stiring under ice cold condition. Immediate evolution of gas was observed after the addition. THF was evaporated from the reaction mixture and diluted with EtOAc (150 mL). The organic layer was washed with 5 % HCl

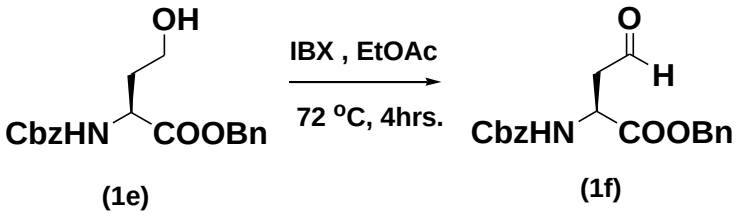
(5 % by volume in water, 2 X 50 mL), 5 % Na_2CO_3 solution in water (2 X 50 mL), followed by brine (50 mL). Organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The alcohol used were used for next step without purification.



(1d) **(1e)**

1e) <u>Synthesis of Cbz-protected amino aldehyde:-</u>

The N-protected amino alcohol (5 mmol) was dissolved in 50 mL of EtOAc and then IBX (10 mmol, 2.8 g) was added to it and refluxed. The reaction was monitored by TLC. After completion of the reaction, the reaction mixture was filtered and the filtrate concentrated over reduced pressure to yield N-protected amino aldehyde. It was immediately used for next step without purification.

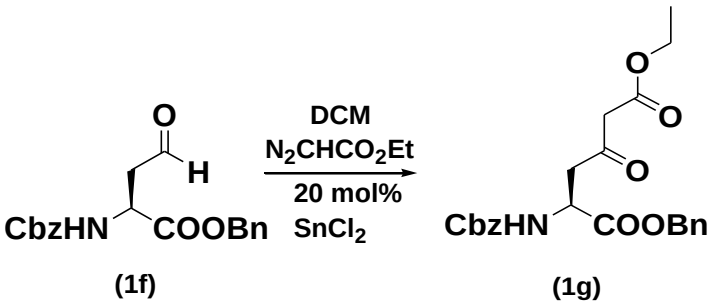


(1e) **(1f)**

1f) <u>Synthesis of N-protected γ-amino β-keto-ester</u>

The N-protected amino aldehyde **1(f)** (2.0 mmol) was dissolved in 15 mL of DCM at room temperature (20–25 ^{o}C) and then 0.0756g (20 mol%) of tin(II) chloride was added followed by 0.239 g (2.1 mmol) of ethyl diazoacetate. Immediate gas evolution was observed. The reaction mixture was stirred and the progress of the reaction was monitored by TLC. After completion of the reaction, it was quenched with 10 mL of 0.5 N HCl and the reaction mixture was extracted with DCM (30 mL x 3). The combined organic layer was washed with 20 mL of brine, dried over

anhydrous sodium sulfate and concentrated under reduced pressure to get a yellowish oily crude product which was purified on silica gel column chromatography.

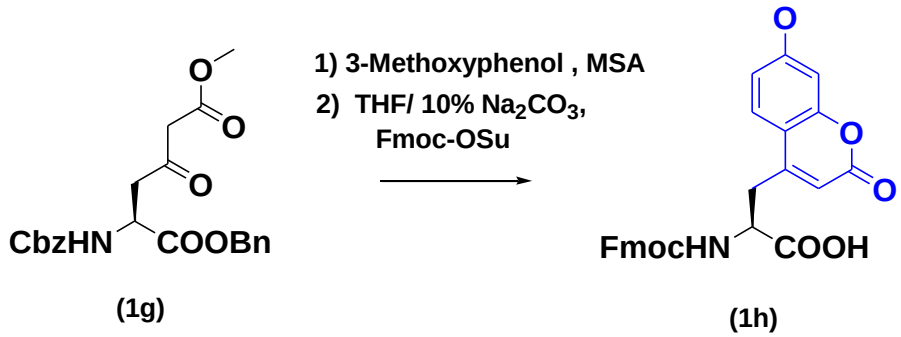


(1f) (1g)

1g) <u>Synthesis of Fmoc-protected coumarin functionalized with proteinogenic amino acid side chain.</u>

Compound 1g (0.427 g, 1 mmol) was treated with 3-methoxyphenol (0.615 g, 5 mmol) and the mixture was cooled to 0^{o}C under N 2 atmosphere. After stirring for 5 min, MSA (2.4 g, 25 mmol) was added slowly under constant stirring. The reaction mixture was allowed to warm to room temperature and the stirring was continued for another 2 h. The progress of the reaction was monitored by TLC. After completion of the reaction (~2 h), it was diluted with diethyl ether (100 mL) and cooled to -15 ◦C. The ether layer was centrifuged (4000 rpm at 4 ◦C) and the red precipitate (methanesulfonate salt of coumarin) was separated. The red precipitate was dissolved in a solution of 30% Na_2CO_3 (20 mL) and THF (5 mL) and cooled to 0 ◦C. A solution of Fmoc-OSu (0.370 g, 1.1 mmol) in THF (5 mL) was added to the reaction mixture and allowed to stir overnight to complete the reaction. After completion (monitored by TLC), it was washed with ether (20 mL x 2) and the aqueous layer was acidified with 3 N HCl (up to pH 3) under ice cold conditions. The combined organic layer was washed with 5% HCl (50 mL x 2), brine (30 mL), dried over anhydrous sodium sulfate and concentrated under reduced pressure to give reddish gummy crude product which was further purified on silica gel column chromatography to give pure white powder. Yield: 0.315 g (62%). UV absorption λ_{max} : 323 nm, fluorescence emission λ_{max} : 383 nm; ^{1}H NMR (400 MHz, DMSO): d 13.09 (s, b, 1H, -COOH), 7.88–7.86 (d, J = 7.32, 2H, aromatic Fmoc), 7.74–7.63 (d, J = 9.16, 1H, aromatic ring of coumarin), 7.63–7.59 (dd, J = 7.36, J = 3.64 2H, aromatic Fmoc), 7.41–7.37 (t, J = 7.56, 2H, aromatic Fmoc), 7.31–7.25 (dd, J = 7.32, J

= 6.88, 2H, aromatic Fmoc) 7.02–6.979 (m, 2H, aromatic ring of coumarin), 6.24 (s, 1H, CCH), 4.33-4.27 (m, 1H, Fmoc-CH), 4.23–4.14 (m, 3H, Fmoc-CH_2), 3.84 (s, 3H, -OCH_3), 3.34– 3.04 (m, 2H, CH-CH_2);

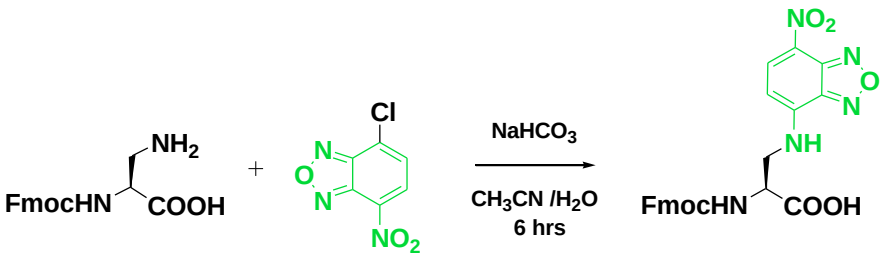


(1g) (1h)

Scheme 2

2a) <u>The synthesis of Dap(NBD)-OH fluorescent compound</u>.

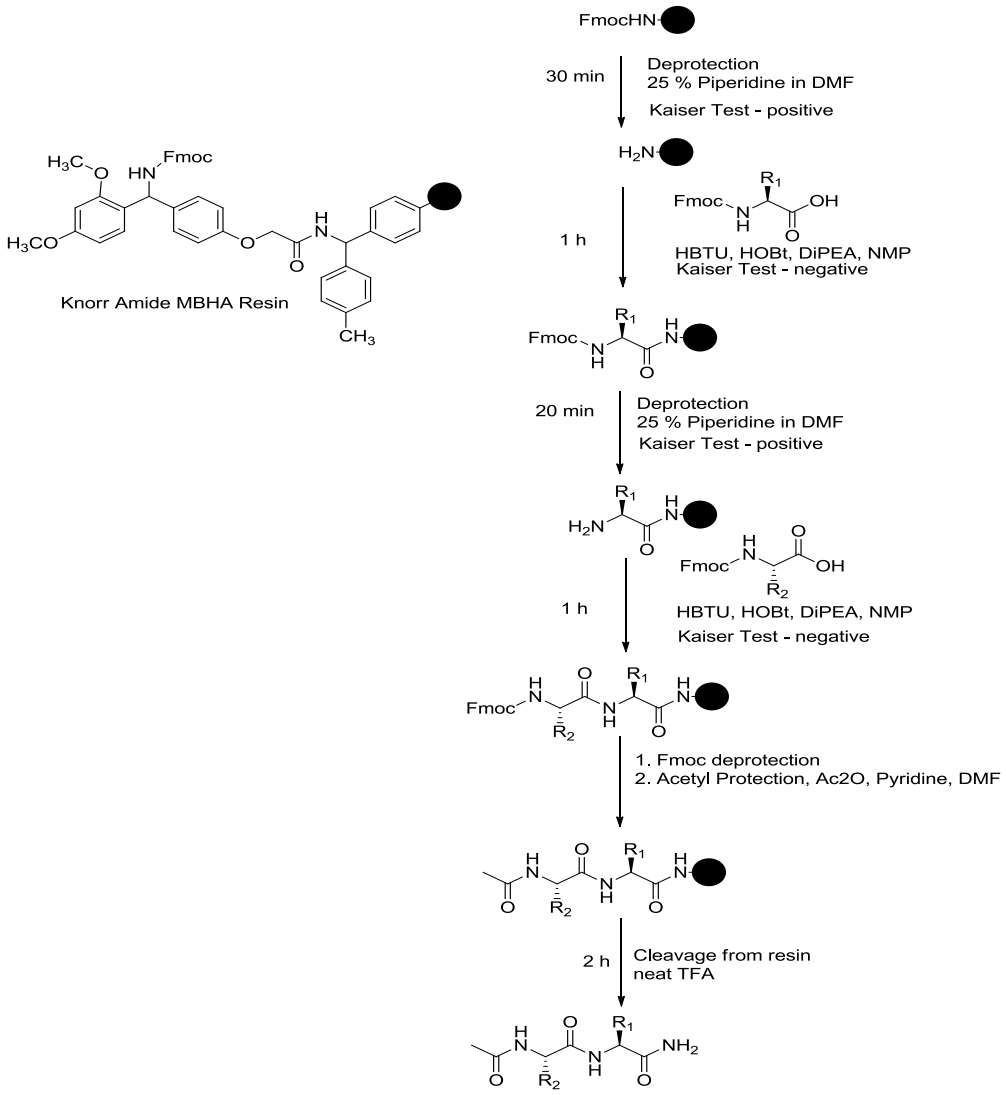
To a stirred solution of Na-Fmoc- L -diaminopropionic acid (1, 0.500 g, 1.53 mmol) in water/acetonitrile (20 mL of a 1 : 1 mixture) was added 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl, 0.367 g, 1.836 mmol) and sodium bicarbonate (0.154 g, 1.83 mmol) and pH was adjusted between 8-9. The reaction mixture was stirred for 6 h at RT. Then, water (10 mL) was added, the mixture was washed with ethyl acetate/hexanes (1 : 5) twice. The aqueous phase was acidified by 1 M HCl until a pH of 5–6 was achieved. The product was extracted from the aqueous solution with ethyl acetate. The combined organic phases were dried over anhydrous sodium sulfate, filtered, and the solvent was removed by rotary evaporation. R_f = 0.37 (CH_2Cl_2 : MeOH = 10 : 2). HRMS (ESI^+): m/z calcd for $C_{24}H_{19}N_5O_7$ $[M + H]^+$ 490.1363, found 490.1345.



3.2 Coiled coil peptide synthesis and purification.

The synthesis of peptides P1, P2 and P5 was carried out by manual solid phase peptide synthesis on Rink amid (for P1 and P2) and Knor amid (for P5) resins using standard Fmoc-chemistry (Schematic representation of solid phase peptide synthesis is shown below). Peptides P3 and P4 were synthesized by microwave peptide synthesizer using Novasyn resin using standard Fmoc- chemistry. HBTU and HOBt were used as coupling agents. Fmoc-group deprotection was performed using 20% piperidine in DMF.

Schematic representation of the solid phase peptide



Cleavage of peptide from resin.

1. The peptide was cleaved from resin using TFA/water/thioanisole cocktail mixture and isolated as solid white crude after precipitation with chilled Diethyl ether. Then the peptide was purified using reverse phase HPLC using ACN/H_2O as solvent systems.

3.2.2 Peptide purification by HPLC.

The crude peptides were purified to >98% purity using reverse phase HPLC C_{18} column with a gradient from 5 to 95% acetonitrile (0.1% TFA) over 50 min at a rate of 2mL/min. Correct masses were verified by MALDI TOF/TOF mass spectrometry.

3.3 Circular dichroism studies.

CD spectra were recorded on a Jasco J-815 spectropolarimeter at 20^{o}C (Jasco PTC-348WI peltier thermostat). Overall peptide concentrations were 30mM (15mM ecE and 15mM ecK) at pH 7.0 (10mM phosphate buffer and 150mM NaCl). CD-spectra were obtained in the far-UV range (190–300 nm) using 0.2 cm Quartz Suprasil cuvettes (Hellma) equipped with a stopper. The nitrogen flow rate was set to 3 L min^{-1}. Ellipticity was normalized to concentration (c [mol L^{-1}]), number of residues (n= 29, including the N-terminal label) and path length (l = 0.2 cm) using Equation (1):

$$[\theta] = \theta_{obs}/(10{,}000 \cdot l \cdot c \cdot n) \quad (1)$$

where θ_{obs} is the measured ellipticity in millidegrees and [θ] the normalized ellipticity in 10^3 deg cm^2 dmol^{-1} residue^{-1}. Melting curves was recorded using the signal at 222nm applying a heating rate of 3 Kmin^{-1} from 20 to 80 ^{o}C. Each sample was prepared three times and both the baseline corrected spectra and the melting curves were averaged.

3.4 Fluorescence studies.

All fluorescent peptides were studied in 10mM potassium phosphate buffer (pH 7.0) and 150mM NaCl solution individually or with their coiled coil partners. Fluorescence was measured using a Fluoromax-4 spectrofluorometer using excitation wavelength of particular peptide.

Spectra were taken at room temperature using a 1-cm pathlength cuvette, an increment of 1 nm, and a 15sec averaging time. pH dependent fluorescence study was done in 3-10 pH range and pH was changed by adding acetic acid or K_2CO_3 diluted solution in peptide-buffer solution and pH was maintained by the help of S2K712ISFECOM, Japan pH meter.

3.5 Calculation of thermodynamic parameters

Thermodynamic parameters were determined by curve fitting of the denaturation curves to the following equations using a non-linear least-squares fitting using Matlab 7.8.0 (R2009a) software. Ellipticity was normalized to fraction monomer using the equation:

$$\theta = (\theta_M - \theta_D)P_M + \theta_D \quad (2)$$

where θ_M and θ_D represent the ellipticity values for the fully unfolded monomer and fully folded dimer species at each temperature. θ_M was found to be constant at the temperatures higher than the melting region for all the pepetides studied. θ_D was approximated by a linear function of temperature $\theta_D = \theta_D[0] + aT$. The fraction monomer ($P_M$) was expressed in terms of the equilibrium constant after solving the equation for a bimolecular reaction 2M → D:

$$P_M = [(8KC + 1)^{1/2} - 1]/4KC \quad (3)$$

where K is the equilibrium constant and C is the total peptide concentration. K was assumed to be temperature dependent according to the equation (4):

$$K = e^{-\Delta G/RT} \quad (4)$$

The Gibbs–Helmholtz equation can be used to express the temperature dependence of DG in terms of ΔH_m and T_m as given by Equation (5):

$$\Delta G = \Delta H_m \,.\, (1 - T/T_m) + \Delta C_p \,.\, (T - T_m - T \,.\, \ln(T/T_m)) \quad (5)$$

where ΔH_m is the enthalpy change at the melting temperature T_m , that is defined as the temperature at which $P_m = 0.5$. ΔC_p is the change in heat capacity that was initially assumed to be zero for the purpose of fitting because due to the high interdependence of ΔH_m and ΔC_p

these parameters cannot be fitted simultaneously. Equations (2) through (5) were combined and the data fitted directly. ΔC_p was calculated afterwards from the dependence of ΔH_m from T_m and the standard free energy of unfolding ΔG_o (1 M standard state) was then calculated at T_o= 37 ^oC according to Equation (6):

$$\Delta G_o = \Delta H_m \,.\, (1 - T_o/T_m) + \Delta C_p \,.\, \{T_o - T_m - T_o \,.\, ln\,(\,T_o/T_m)\} - RT_o ln(C) \qquad (6)$$

Errors were determined by a statistical analysis of the fitted parameters. The error for the free energy of unfolding was calculated using Equation (6) applying the minimum and maximum values for ΔH_m , ΔC_p , and T_m according to their individual errors. To prove the validity of the fit, ΔH_m and T_m were also determined manually using the Van't Hoff equation.

Matlab program file for curve fitting:-

```
function [] = givey1(y)

x = ((20:5:80)+273)';


ftyp = fittype('(m-a*x-d) * ((sqrt(8*exp((h/1.98)*(1/t - 1/x)) + 1) -
1)/(4*exp((h/1.98)*(1/t - 1/x)))) + (a*x+d)');

[fd, gof] = fit(x,y,ftyp,'StartPoint',[-0.01,-5.5e3,-3e4,-2e3,323],'Lower',[-
0.02,-6.5e3,-5e4,-2.5e3,283],'Upper',[0.02,-4.8e3,-2e4,-1e3,343]);

ce = coeffvalues(fd);
disp(ce);
fe = struct2cell(gof);
fe = cell2mat(fe)

xal = (20:0.1:80)';
xal = xal+273;

a = ce(1);
d = ce(2);
h = ce(3);
m = ce(4);
t = ce(5);
yal = (m-d-a*xal) .* ((sqrt(8*exp((h/1.98)*(1/t - 1./xal)) + 1) -
1)./(4*exp((h/1.98)*(1/t - 1./xal)))) + (a*xal+d);

hold on;
plot(xal,yal);
plot(x,y,'.r');

end
```

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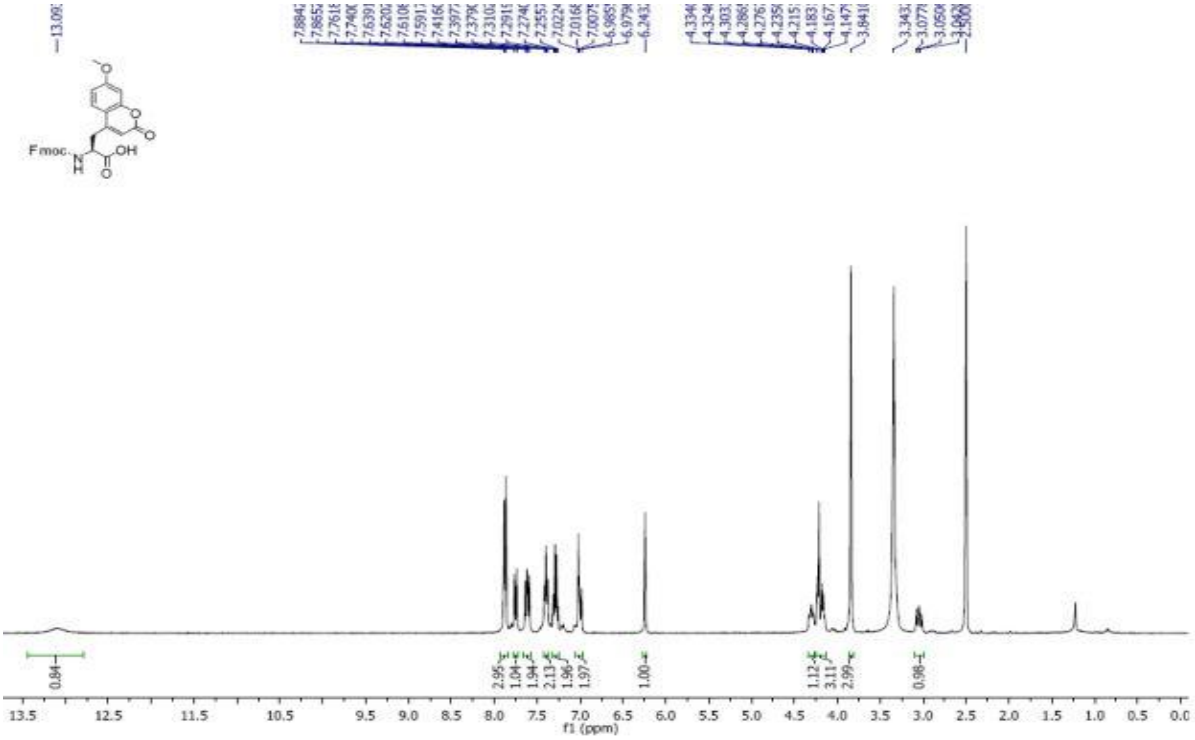
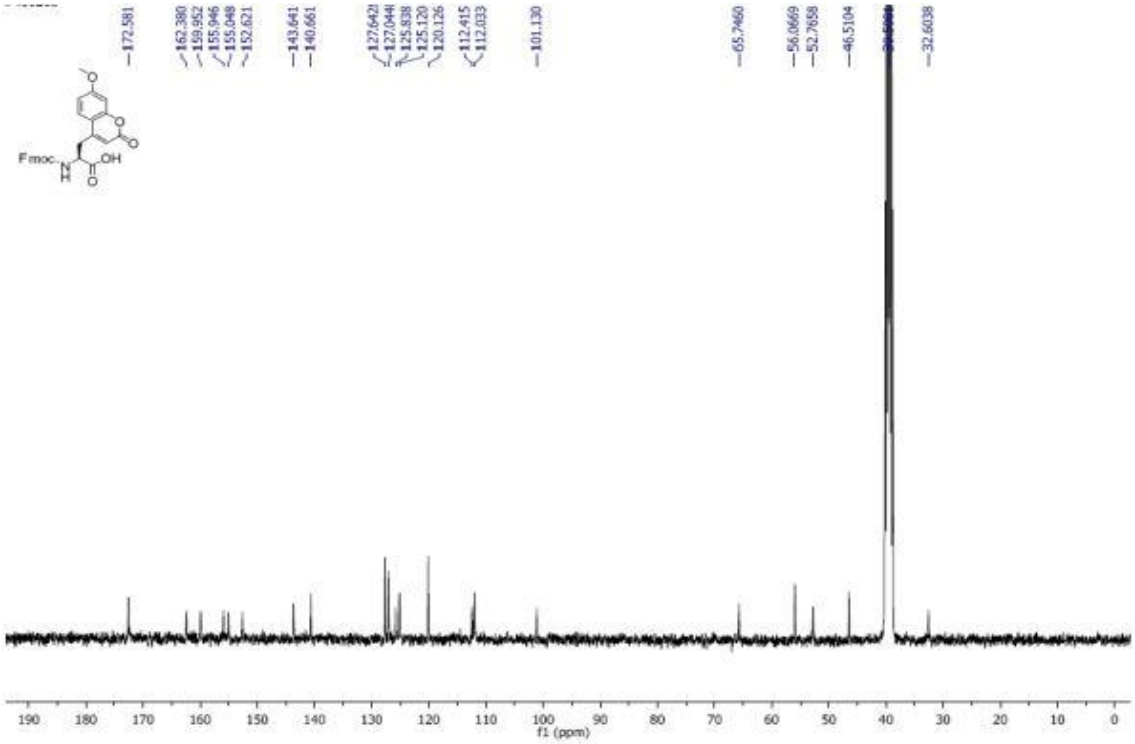
^{1}H-NMR



^{13}C-NMR



51 - D

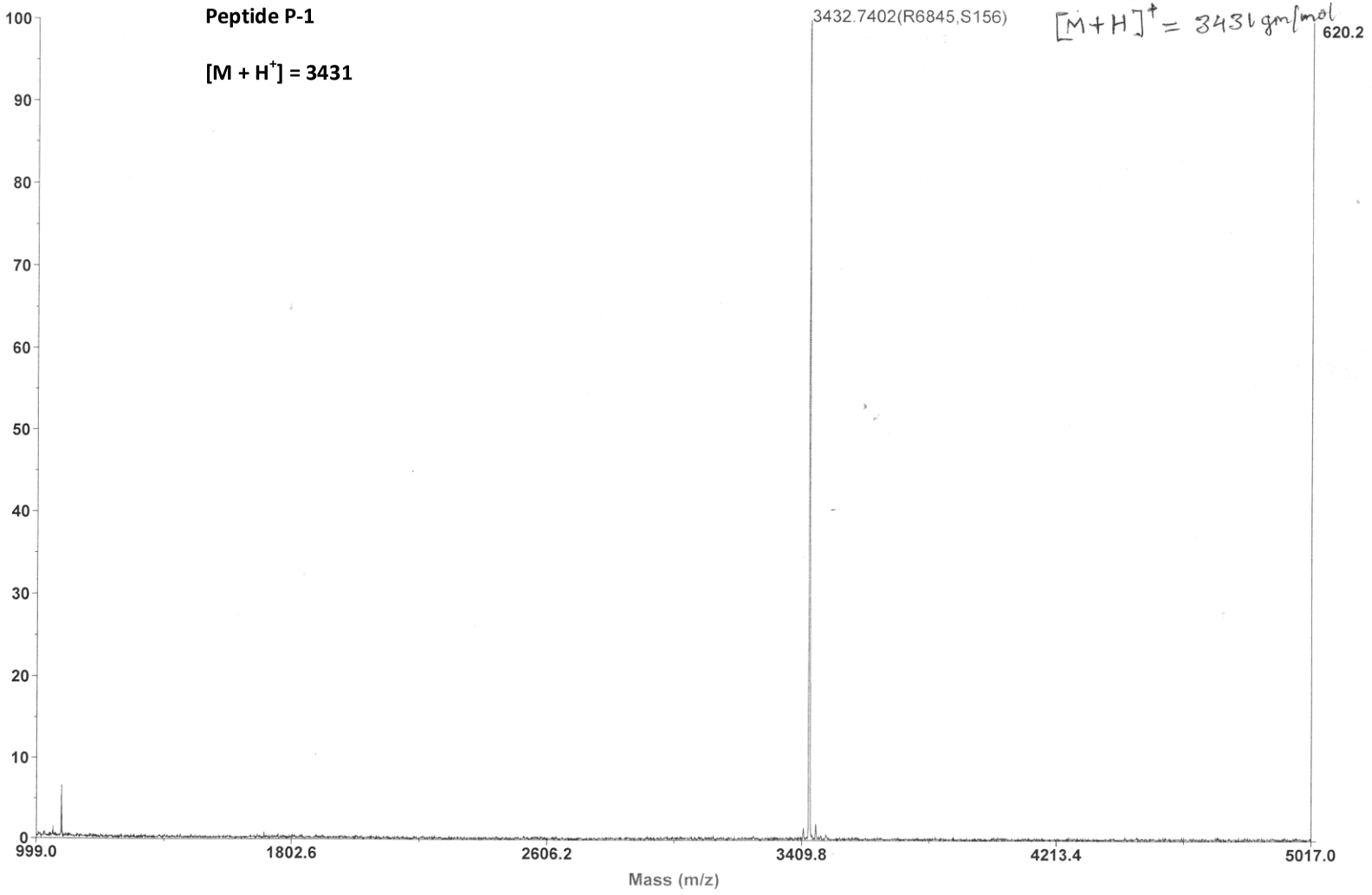
P-1

$[M+H]^+ = 3431\ gm/mol$

Final - Shots 400 - IISER-; Label B7

Peptide P-1

$[M + H^+] = 3431$



Spectrum Report

28-mer Cu peptide

P-2

Final - Shots 500 - IISER-; Run #406; Label B9

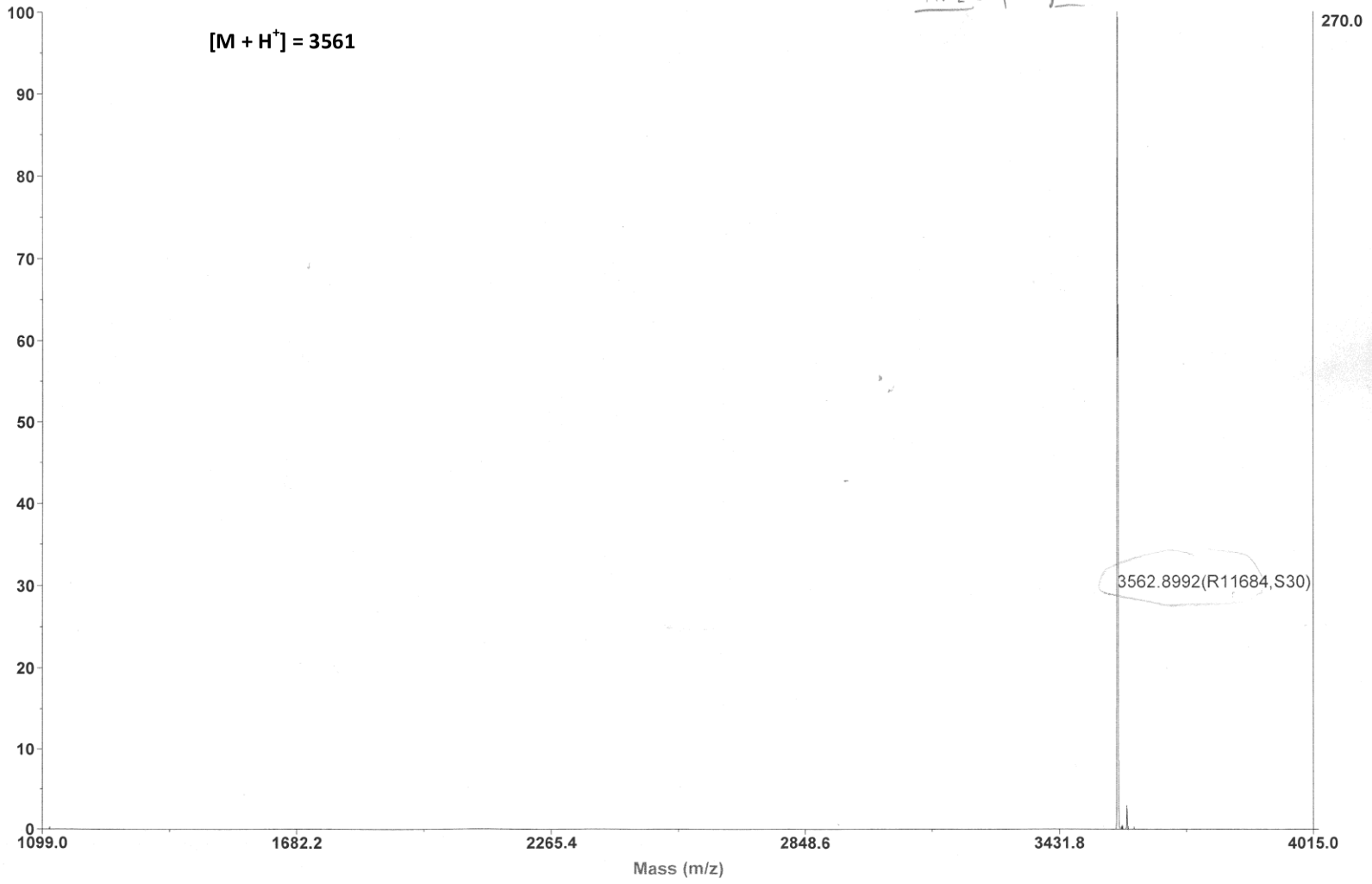
~~M+H~~ =

HPLC purified

$[M+H]^+ = 3562$

Peptide P-2

$[M + H^+] = 3561$



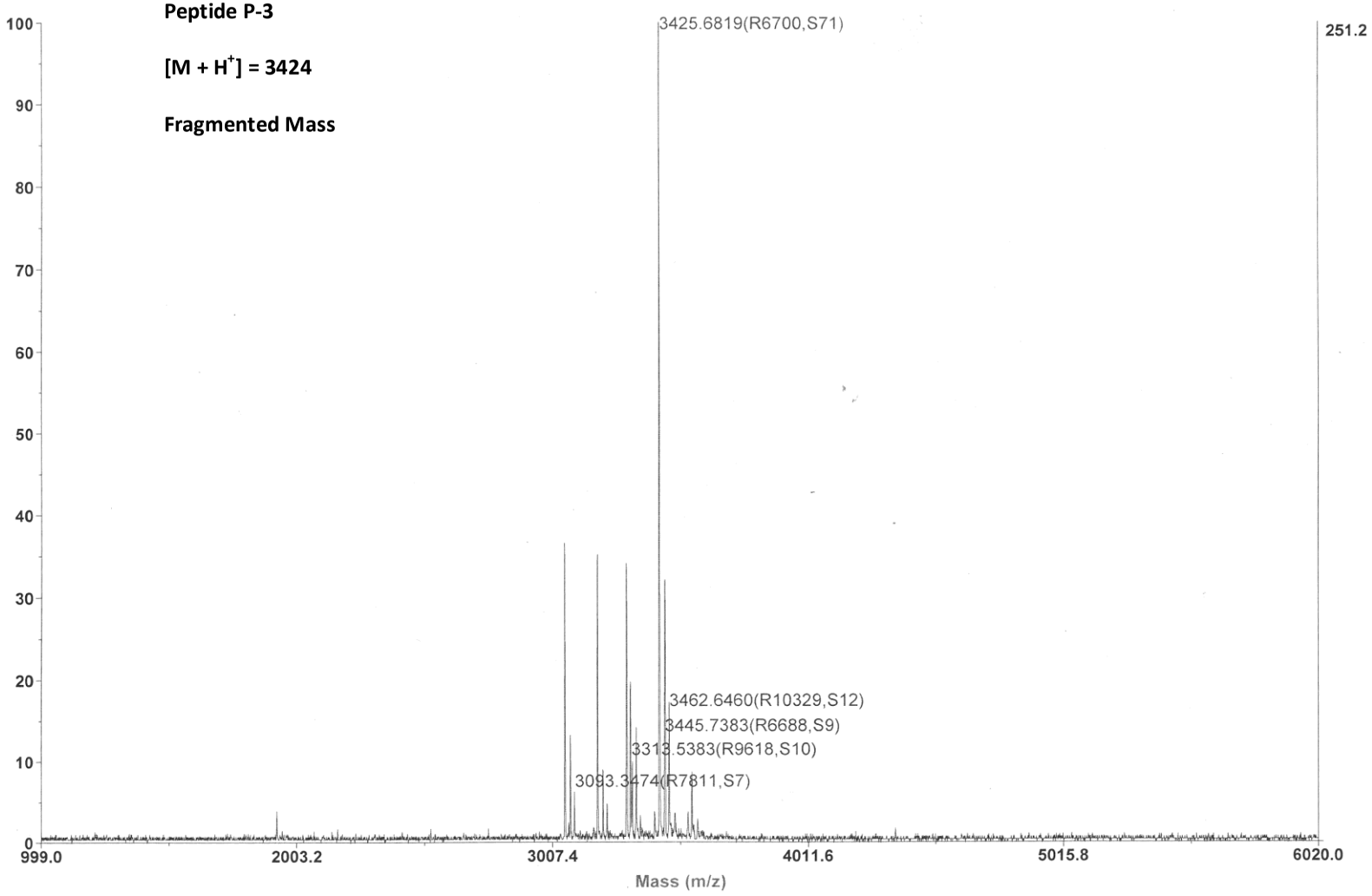
Spectrum Report

Final - Shots 400 - IISER; Run #1013; Label O19

Peptide P-3

[M + H^+] = 3424

Fragmented Mass



Spectrum Report

Final - Shots 400 - IISER; Run #1013; Label O20

Peptide P-4

[M + Na^+] = 3578