Selective Nitroalkane-alkene 1,3-Dipolar Cycloaddition: A New Strategy for Peptide Conjugation

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

By

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Under the guidance of

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Certificate

This is to certify that this dissertation entitled "***Selective Nitroalkane-alkene 1,3-Dipolar Cycloaddition: A New Strategy for Peptide Conjugation***" towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents work carried out by Rajat Patel at IISER Pune under the supervision of **Prof. Hosahudya N. Gopi**, Department of Chemistry, IISER Pune during the academic year 2018-2019.

20th March 2019

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Professor
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Declaration

I hereby declare that the matter embodied in the report entitled ***"Selective Nitroalkane-alkene 1,3-Dipolar Cycloaddition: A New Strategy for Peptide Conjugation"*** are the results of the work carried out by me at the Department of Chemistry, IISER PUNE under the supervision of **Dr. Hosahudya N. Gopi** and the same has not been submitted elsewhere for any other degree.

20th March 2019

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Table of Contents

LIST OF FIGURES

LIST OF SCHEMES

Abbreviations

ACN	=	Acetonitrile
AcOH	=	Acetic acid
AIBN	=	Azobisisobutyronitrile
Boc	=	*tert*-Butoxycarbonyl
$(Boc)_2O$	=	Di-*tert*-butyl-dicarbonate (Boc anhydride)
DCM	=	Dichloromethane
DIPEA	=	Diisoproylethyl amine
DMF	=	Dimethyl formamide
DMSO	=	Dimethyl sulfoxide
EDC.HCl	=	*N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride
EtOAc	=	Ethyl Acetate
Et_3N	=	Triethylamine
Fmoc	=	9-Fluorenylmethoxycarbonyl
Fmoc-OSu	=	*N*-(9-Fluorenylmethoxycarbonyloxy) succinimide
$HClO_4$	=	Perchloric acid

HOBt	=	1-Hydroxybenzotriazole
RP-HPLC	=	Reverse Phase High Performance Liquid Chromatography
IBC-Cl	=	Isobutyl chloroformate
KOH	=	Potassium hydroxide
Leu	=	Leucine
LiOH	=	Lithium hydroxide
$NaBH_4$	=	Sodium borohydride
Na_2CO_3	=	Sodium carbonate
$NaNO_2$	=	Sodium nitrite
NMP	=	*N*-methyl pyrrolidone
NMR	=	Nuclear Magnetic Resonance
PPh_3	=	Triphenyl phosphine
TFA	=	Trifluoro acetic acid
THF	=	Tetrahydrofuran

1. Abstract

Chemoselective 1,3-dipolar cycloaddition reaction between the nitroalkane functionalized peptides and alkenes is reported. The reactive nitrile oxide can be generated *in situ* through dehydration reaction using phenyl isocyanate. The *in situ* generated nitrile oxide was trapped with alkenes to give isoxazolines on peptides. The nitrile oxide-alkene 1,3-dipolar cycloaddition reaction was found to be compatible with solution as well as solid phase peptide synthesis. In a sharp contrast to the copper catalyzed 1,3-dipolar cycloaddition of azides, the nitrile oxides can undergo cycloaddition with both alkynes and alkenes. More importantly, alkenes are easy to synthesize compared to alkynes and also a variety of alkenes are commercially available. The selective and mild nitro-alkene cycloaddition reaction reported in this project can be utilized to functionalize peptides and other small molecules. Though the reaction is mild and compatible for broad substrates, however still there is a need to improve the efficiency of the reaction and also finding alternative to phenyl isocyanate to activate the nitro group is important.

2. Introduction

Since past few decades, peptides serve as an excellent tool for bio-conjugation, leading to various types of applications in diagnostics,[1] tissue engineering,[2] and drug delivery.[3] Since peptides are consisting of a variety of amino acids arranged in several different ways, this gives it unique properties which can be utilized to design varieties of biomaterials, probes and drug molecules, also we can exploit the peptide properties like biodegradability and functional diversity. Since in recent years, bio-conjugation has an excellent tool for the synthesis of a variety of different compounds via one of the most celebrated reaction named “Click Chemistry”, because these reactions are selective, fast, efficient, orthogonal and compatible at physiological pH. The term click was introduced by Sharpless in 2001.[4] In addition to click reactions various types other conjugation reactions such as Thiol-ene,[5] Oxime ligation,[6] Rh catalysed 1,3 dipolar cycloaddition reaction,[7] Diels-Alder,[8] strain promoted azide-alkyne cycloaddition[9] have been developed and introduced to peptides for facile conjugation. 1,3 Dipolar cycloaddition reactions were performed by Huisgen in presence of heat via a concerted mechanism, involving 3-atom 4 electron but it gave 1,4 and 1,5 disubstituted1,2,3-triazole with poor regioselectivity along with the requirement of high temperature[10]. However, the copper catalysed click reaction give exclusive 1,4-disubstituted triazoles.[4,5] Fokin and co-workers showed by taking Ruthenium complex to get particular 1,5 disubstituted triazole product in high yield.[7] To avoid the use of Cu(I) in organic reactions, Bertozzi designed copper free strain promoted azide-alkyne cycloaddition reaction.[9] The schematic representation of different types azide-alkyne 1,3-dipolar cycloaddition reactions are shown in the **Figure 1.**

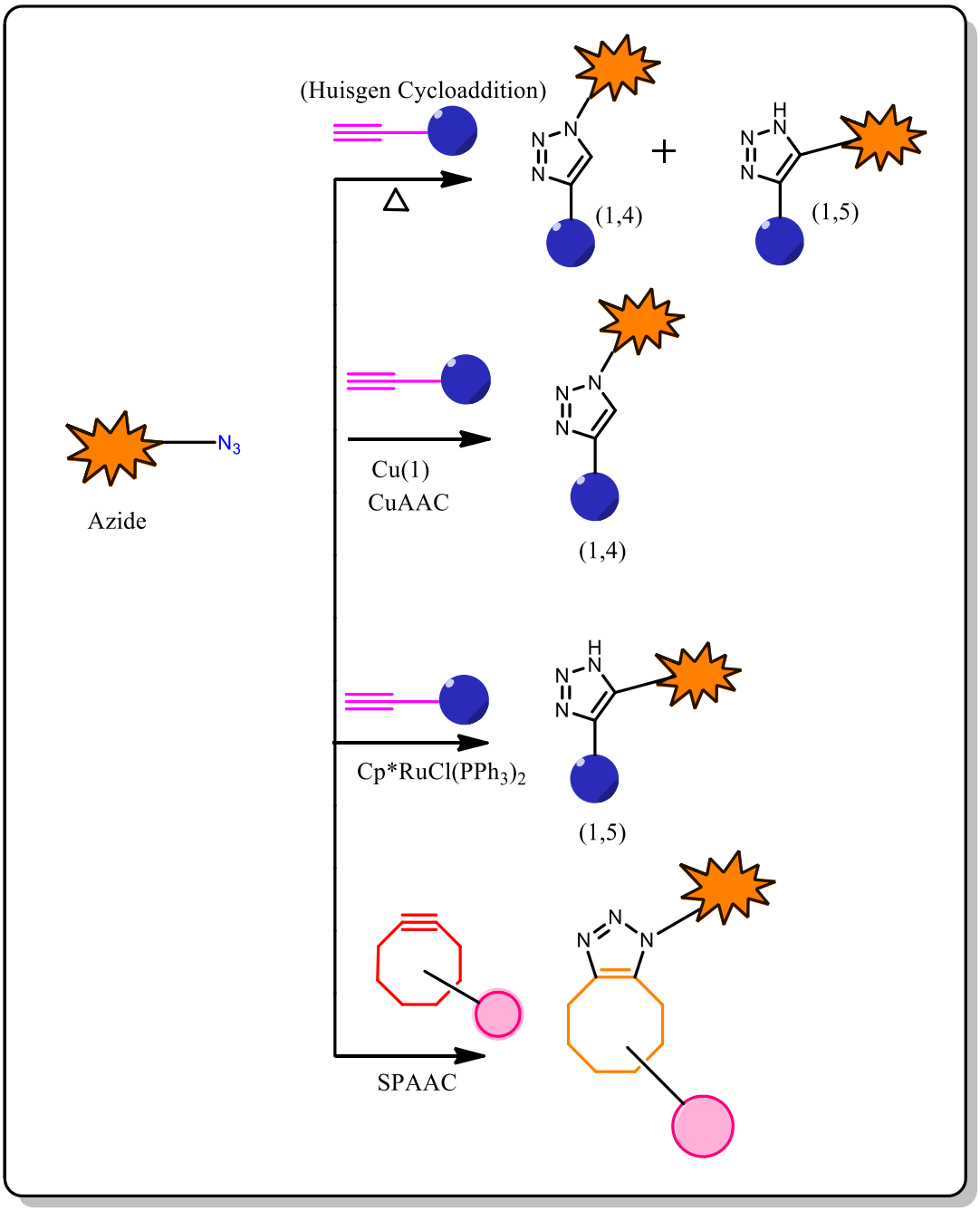


Figure 1: Click reactions used for bioconjugation

The alkyl nitro group is one of the most versatile functional group in organic chemistry. It can be transformed into many different functional groups such as aldehydes, amines, nitriles oxides, acids etc.[11] In addition to these

transformations, alkyl nitro group can also be converted into reactive nitrile oxide which can used for 1,3-dipolar cycloaddition reaction.[12,13] In the process, we can replace azide by nitro group functionality which can be easily converted into nitrile oxide through dehydration. There several protocols reported for the synthesis of nitrile oxide as shown in **Figure 2**. Recently, we reported the synthesis new amino acid consisting of nitroalkane functionality and it's utility in the orthogonal 1,3-dipolar cycloaddition reaction on peptides.[14] Our group had showed the compatibility of the 1,3 dipolar cycloaddition reaction between nitroalkane and alkyne in both solid and solution phase peptide synthesis.[14] In this project, we sought to investigate whether 1,3 dipolar cycloaddition reaction between the new nitroalkane amino acid in the peptide sequence and the alkenes are possible or not. Here we report for the first time that nitroalkane appended peptides can undergo 1,3-dipolar cycloaddition reaction with alkenes in both solid and solution phase methods. As azide cannot react with alkenes, this reaction can also be used as orthogonal to azide-alkyne click reaction.

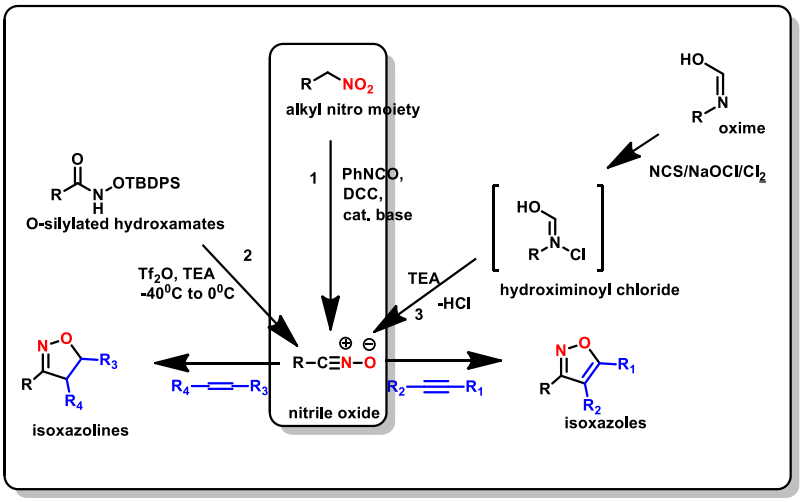


Figure 2: Different ways of preparation of nitrile-oxide.

3. Methods

3.1. Chemicals

All amino acids and chemicals were purchased from the commercial sources. The rink amide resin was bought from Novabiochem. Solvents were brought locally and distilled before use. Column chromatography was performed on silica gel as a stationary phase (120-200 mesh).

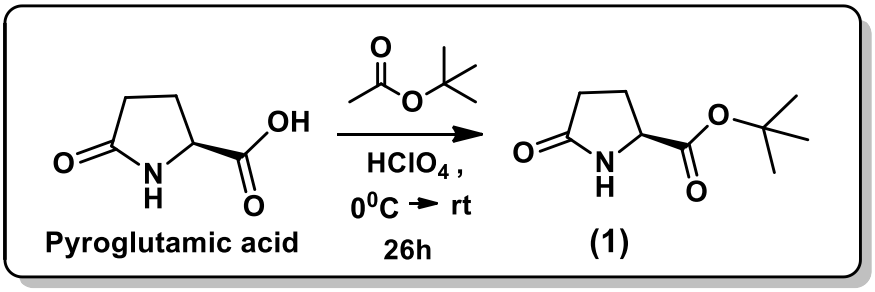
3.2. Instrumentation

Peptides were purified on C_{18} column in RP-HPLC ($MeOH/H_2O$ 50:50-95:5 as a gradient with flow rate 2.0 mL/min). ^{1}H and ^{13}C NMR spectra were recorded on 400 MHz, 100MHz in BRUKER NMR spectrophotometer respectively using the $CDCl_3$ solvent having tetramethylsilane (TMS) as internal standard. Chemical shifts (δ) were reported in ppm. Mass of pure peptides was confirmed by HRMS and MALDI-TOF/TOF.

3.3. General Procedures:

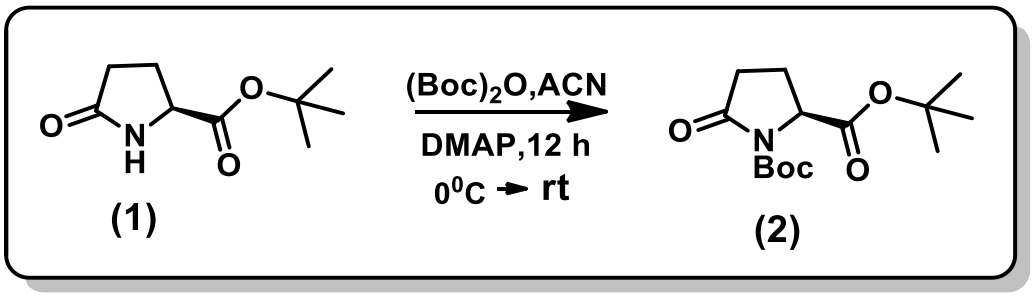
3.3.1. Synthesis of Compound1:

L-Pyroglutamic acid (3.225 g, 25 mmol) was dissolved in 50 mL of *tert*-butyl acetate and reaction mixture was cooled under ice-cold condition. To this reaction mixture 3 mL of perchloric acid ($HClO_4$) was added in dropwise manner which results in clear transparent solution. The reaction was allowed to continue for about 26 hr and completion of reaction was monitored by thin layer chromatography (TLC). After the completion of reaction, unreacted perchloric acid was neutralized by 10% Na_2CO_3, reaction mixture was extracted by DCM (50 mL x 3) and followed by washing with brine (30 mL x 3) and dried over anhydrous $MgSO_4$ and organicsolvent was evaporated under reduced pressure to obtain white coloured solid compound **1**. Yield: 4.16 g (89.99%).



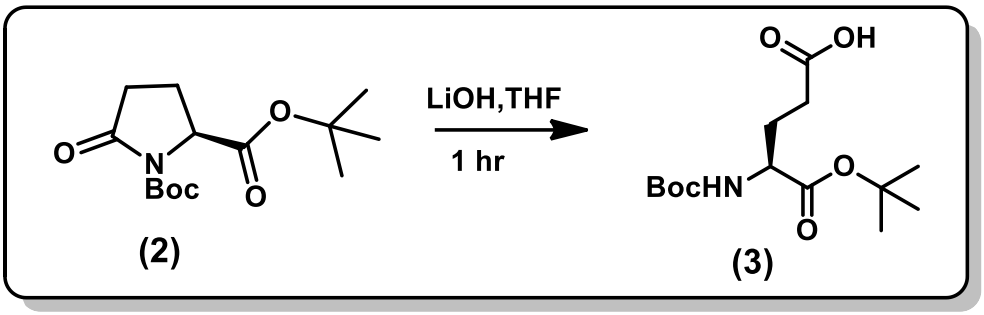
3.3.2. Synthesis of Compound 2:

Compound **1** (4 g, 21.62 mmol) was dissolved in 60 mL of acetonitrile (ACN) followed by addition of DMAP (261 mg, 2.162 mmol). Then reaction mixture was cooled under ice-cold condition. After that $(Boc)_2O$ (7.44 mL, 32.43 mmol) in 30 mL of acetonitrile was added. Then the reaction was allowed to continue for 12 hr. The progress of reaction was monitored by TLC. After completion of reaction, acetonitrile was evaporated and the aqueous layer was extracted with ethyl acetate (50 mL x 3) and followed by washing with brine (30 mL x 3) and dried over anhydrous $MgSO_4$ and evaporated under reduced pressure to obtain orange coloured viscous product. Yield: 4.7 g (76.31%).



3.3.3. Synthesis of Compound 3:

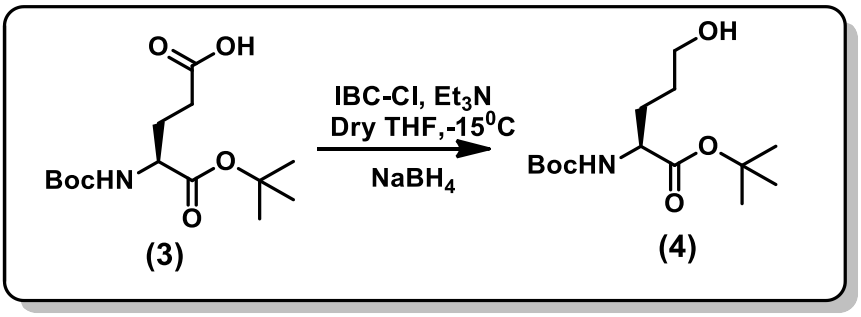
Compound **2** (4.7 g, 16.5 mmol) was dissolved in 50 mL of THF. To this solution, 30 mL of 1*N* LiOH was added in dropwise manner. The progress of reaction was monitored by TLC. After completion of reaction, THF solvent was evaporated under reduced pressure and reaction mixture was acidified with 10% HCl solution and then the aqueous layer was extracted with ethyl acetate (50 mL x 3) and followed by washing with brine (50 mL x 3) and dried over anhydrous $MgSO_4$. Organic solvent was evaporated under reduced pressure on rota evaporator to obtain orange coloured viscous compound **3**. Yield: 4.3 g (86%).



3.3.4. Synthesis of Compound4:

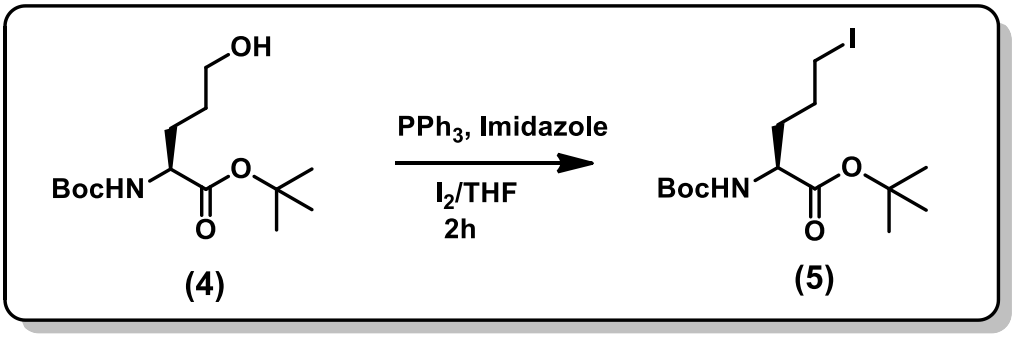
Compound **3** (4.3 g, 14.19 mmol) was dissolved in 50 mL dry THF under nitrogen atmosphere and reaction mixture was cooled to -15°C by using salt-ice mixture. To the reaction mixture, triethylamine (2.36 mL, 17 mmol) was added followed by drop wise addition isobutyl chloroformate (2 mL, 14.19 mmol). Then the reaction was allowed to continue for about 40 min at -15°C. After that the active ester was reduced to alcohol using $NaBH_4$ (2.6 g, 70.95 mmol). Progress of reaction was monitored by TLC, after completion of reaction, THF solvent was evaporated under reduced pressure and excess $NaBH_4$ was quenched by 10% HCl solution and then the aqueous layer was extracted with ethyl acetate (50 mL x 3) and then followed by washing with with 10% HCl solution (50 mL x3), 10% Na_2CO_3 solution (50 mL x 3) and then brine solution (50 mL x 3) and dried over anhydrous Na_2SO_4. Organic solvent was evaporated and obtained product was

purified by column chromatography using ethyl acetate and pet ether as eluent to obtain pure compound **4**. Yield: 3.5 g (85%).



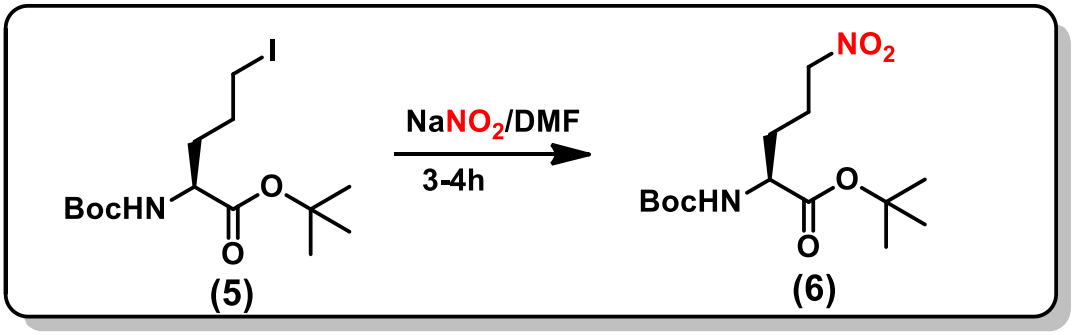
3.3.5. Synthesis of Compound 5:

Compound **4** (3.5 g, 12.1mmol) was dissolved in 50 mL dry THF under nitrogen atmosphere and cooled under ice-cold condition. PPh_3 (4.75 g, 18.16 mmol), imidazole (1.23 g, 18.17 mmol) and iodine (4.6 g, 18.17 mmol) were added to this solution respectively. Then the reaction was allowed to continue for 40 min. Progress of reaction was monitored by TLC, after completion of reaction, THF solvent was evaporated under reduced pressure and then the aqueous layer was extracted with ethyl acetate (50 mL x 3) and followed by washing with 10% $Na_2S_2O_3$ solution (50 mL x 3), brine solution (50 mL x 3) and dried over anhydrous Na_2SO_4. Then the organic solvent was evaporated under reduced pressure and purified though silica gel column chromatography using ethyl acetate and pet ether as eluent to obtain pure compound **5**. Yield: 3.0 g (61.48%).



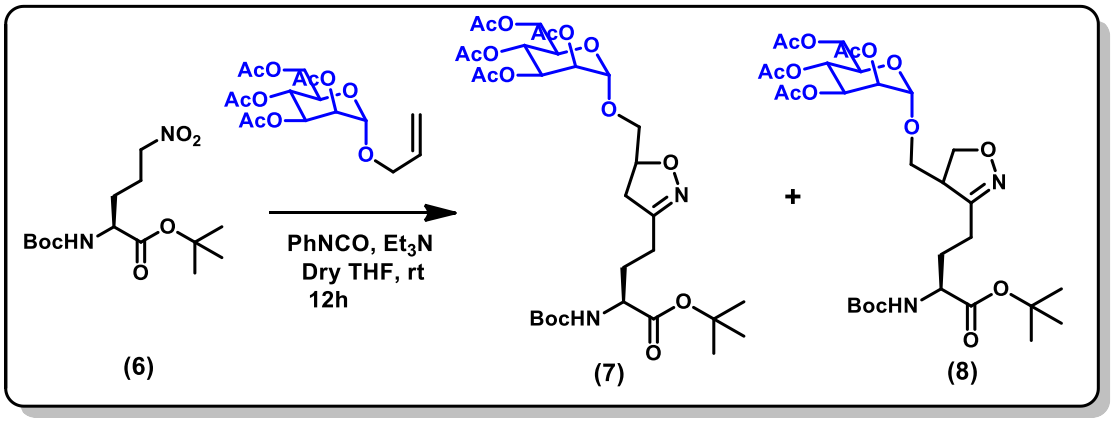
3.3.6. Synthesis of Compound 6:

Compound **5** (3 g, 7.44 mmol) was dissolved in 10 mL of DMF under nitrogen atmosphere and cooled under ice-cold condition. To this solution, sodium nitrite (1.28 g, 18.16 mmol) was added. After that the reaction was allowed to continue for 3 hr. Progress of reaction monitored by TLC, after completion of reaction, 10% $Na_2S_2O_3$ solution (50 mL) was added to reaction mixture and then the aqueous layer was extracted with ethyl acetate (50 mL x 3) and then followed by washing with 10% $Na_2S_2O_3$ solution (50 mL x 3), brine solution (50 mL x 3) and dried over anhydrous Na_2SO_4. The organic solvent was evaporated under reduced pressure and purified via silica gel column chromatography using ethyl acetate and pet ether as eluent to obtain pure compound **7**. Yield: 1.0 g (42.26%).



3.3.7. Synthesis of Compound 7:

Compound **6** (100 mg, 0.31 mmol) was dissolved in 3 mL of dry THF under nitrogen atmosphere under ice-cold condition. To this solution, propargyl glucose pentaacetate (610 mg, 1.57 mmol) was added. After that, phenylisocynate (170 μL, 1.55 mmol) and triethylamine (215 μL, 1.55 mmol) was added in dropwise manner to reaction mixture at 0°C. The progress of the reaction was monitored by TLC. After completion of the reaction, the THF was evaporated and the crude compound was purified using ethyl acetate and pet ether system through silica gel column chromatography to obtain mixture of compound **7** and **8**.



3.3.8. Synthesis of nitroalkane tethered tetra peptide P1:

The peptide **P1** was synthesised by solution phase condensation strategy. *N*-Boc protected leucine was dissolve in DMF under nitrogen atmosphere under ice-cold condition. To this solution, coupling reagents EDC.HCl and HOBt were added and followed by the addition of DIPEA and stirred for about 15 min. After that methyl ester of L-valine was added. Progress of reaction was monitored by TLC. After the completion of reaction, 10% HCl and brine was added and the organic compound was extracted with EtOAc (30 mL x 3), then followed by washing with with 10% HCl solution (50 mL x 3),10% Na_2CO_3 solution (50 mL x 3) and then brine solution (50 mL x 3) and dried over anhydrous Na_2SO_4. The organic solvent was evaporated under reduced pressure to obtain compound **9**.

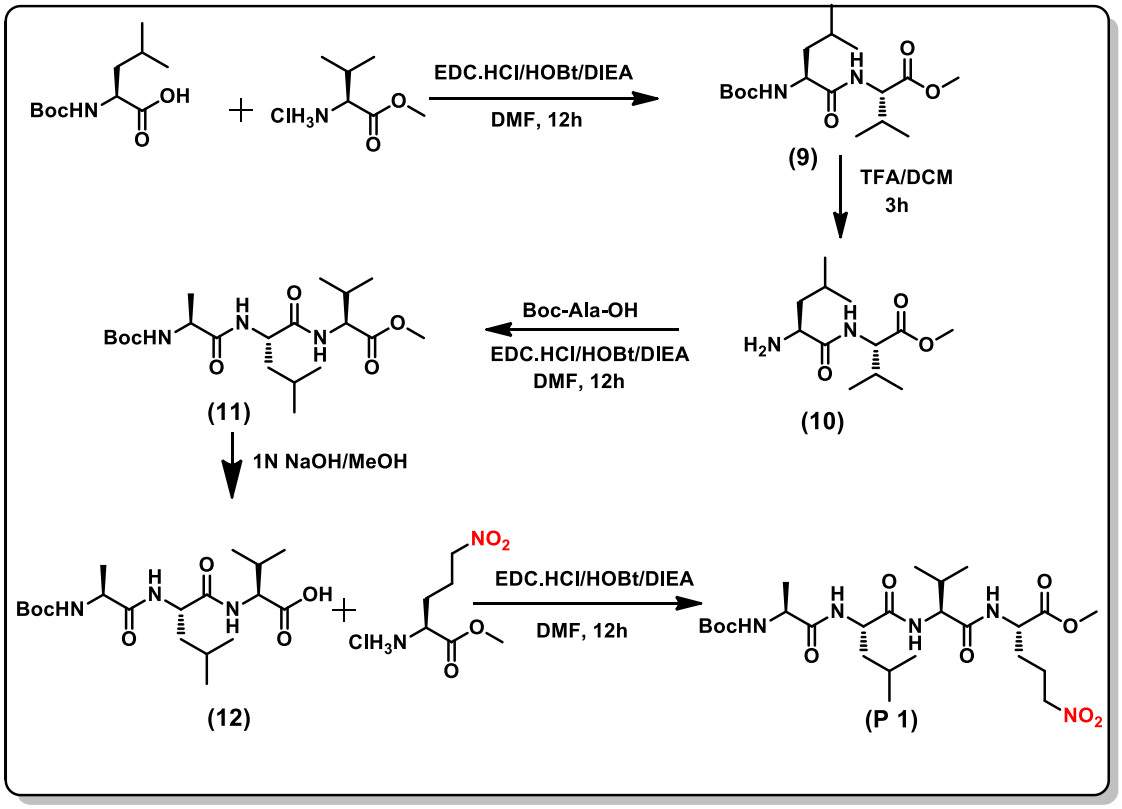
Compound **9** was subjected to a solution of trifluoroacetic acid and dichloromethane respectively (1:1). Progress of reaction was checked by TLC and after completion of reaction, solvent was evaporated several times with DCM to remove excess TFA to obtain compound **10**.

Then *N*-Boc protected alanine was dissolve in DMF under N_2 atmosphere at 0 ºC. To this solution, coupling reagents EDC.HCl and HOBt were added and followed by the addition of DIPEA and stirred for about 15 min. After that

compound **10** was added. Progress of reaction was monitored by TLC. After the completion of reaction, 10% HCl and brine was added and the organic compound was extracted with EtOAc (30 mL x 3), then followed by washing with 10% HCl solution (50 mL x 3), 10% Na_2CO_3 solution (50 mL x 3) and then brine solution (50 mL x 3) and dried over anhydrous Na_2SO_4. The combined organic layer was evaporated under reduced pressure to obtain compound **11**.

Compound **11** was hydrolyzed using 1*N* NaOH in MeOH. Progress of the reaction is checked by TLC and after completion of the reaction the solvent MeOH was evaporated under reduced pressure. Then mixture was neutralized with10% HCl and extracted with EtOAc (30 mL x 3), then followed by washing with brine solution (50 ml x 3) and dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to obtain compound **12**.

Compound **12** was dissolve in DMF under N_2 atmosphere at 0 ºC. To this solution, coupling reagents EDC.HCl and HOBt were added and followed by the addition of DIPEA and stirred for about 15 min. After that compound **12** was added. Progress of reaction was monitored by TLC. After the completion of reaction, 10% HCl and brine was added and the organic compound was extracted with EtOAc (30 mL x 3), then followed by washing with 10% HCl solution (50 mL x 3) followed by 10% Na_2CO_3 solution (50 mL x 3) and then brine solution (50 mL x 3) and dried over anhydrous $MgSO_4$. The organic solvent was evaporated under reduced pressure to obtain tetrapeptide **P1**.



Scheme 1: Synthetic scheme of nitro-alkane tetrapeptide **P1**

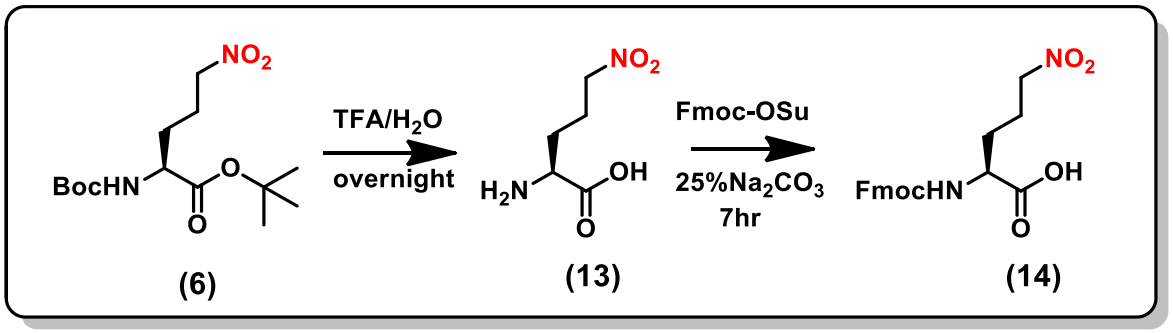
3.3.9. 1,3 Dipolar cycloaddition reaction on nitroalkane tethered tetrapeptide P1:

Tetra peptide (100mg, 0.178mmol) was dissolve in 5 mL of dry THF at 0°C under nitrogen atmosphere. To this solution, styrene (5 eq.), phenylisocynate (5 eq.) and trimethylamine (5eq.) were added respectively. The progress of the reaction was monitored by TLC. After completion of reaction, solvent THF was evaporated and by-product urea was filtered out using filter paper and evaporated under reduced pressure. The crude compound was purified by Reverse Phase High Pressure Liquid Chromatography (HPLC) using C_{18} column and MeOH/H_2O as a solvent system to obtain pure peptide **P2**. Same protocol has been utilized for the synthesis of the other peptides (**P3-P6**)

.3.3.10. Synthesis of nitro alkane tethered hepta peptide on solid phase:

Synthesis of Fmoc-Nitro-OH:

Compound **6** (1 g, 3.14 mmol) was dissolved in 10 mL of DCM. To this solution, 10 mL of TFA and 1 mL of water were added. After that the reaction mixture was stirred for overnight. The progress of reaction was monitored by TLC. After completion of reaction, reaction mixture was several times evaporated with DCM to get gummy compound **13**. Then compound **13** was dissolved in 15 mL of THF and 25% Na_2CO_3 (15 mL) solution under ice-cold condition and stirred for another 10 min. After that, Fmoc-OSu (953 mg, 2.82 mmol) dissolve in 10 mL of THF was added and reaction was allowed to stirred for 12 hr. After completion of reaction, solvent THF was evaporated and aqueous solution was acidified with 10% HCl and extracted with EtOAc (30 mL x 3), then followed by washing with brine solution (50 mL x 3) and dried over anhydrous $MgSO_4$ and evaporated under reduced pressure to give gummy compound **14**, which is purified by column chromatography using ethyl acetate and pet ether as eluent to obtain pure compound to get brown coloured solid compound which can be further utilized to use in solid phase peptide synthesis.

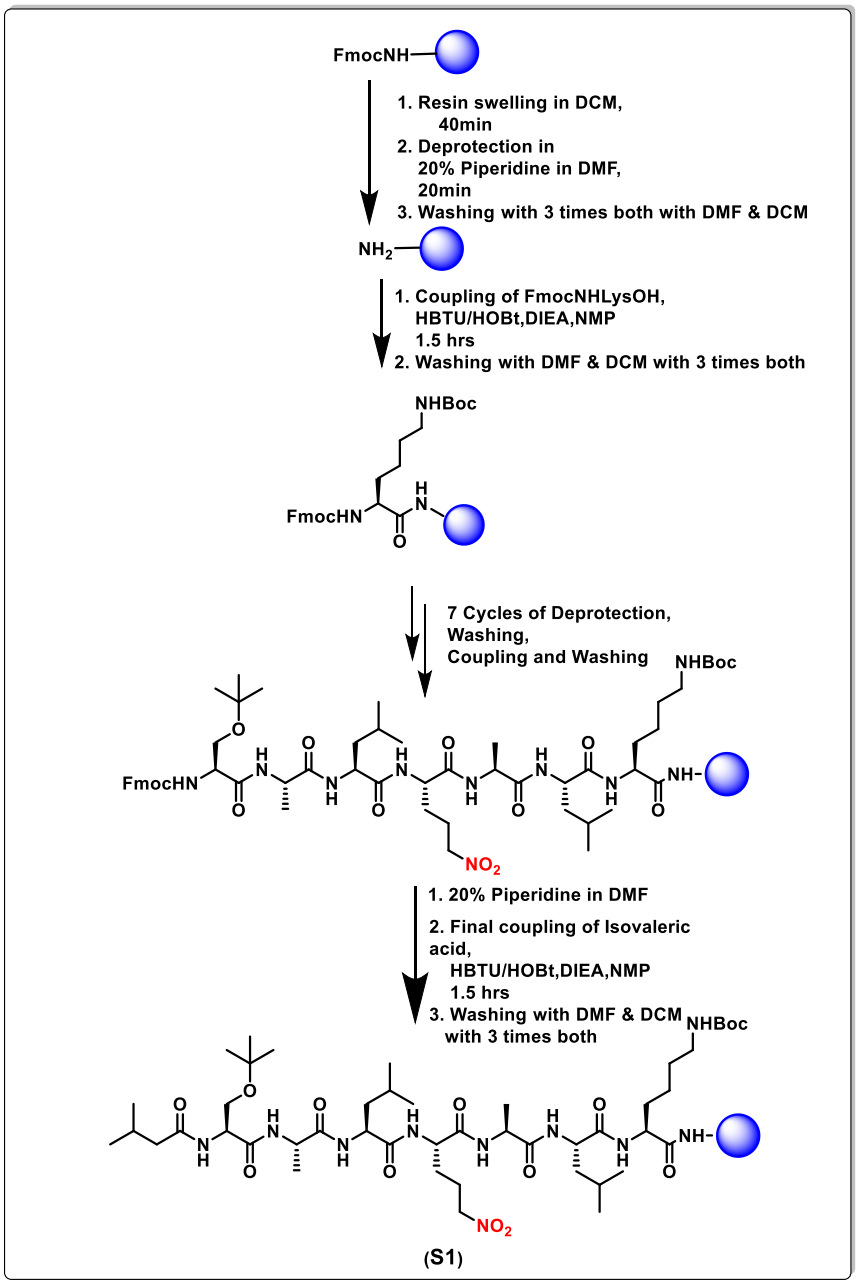


(6) (13) (14)

3.3.11 Synthesis of nitroalkane peptide S1 on solid support:

Synthesis of peptide was done by solid phase peptide synthesis protocol on rink amide resin (0.2 mmol scale) using Fmoc-chemistry. Coupling reactions were performed in NMP solvent and HBTU/HOBt as a coupling reagents and DIPEA

as a base. All Fmoc deprotections were performed in 20 % piperidine in DMF. After performing all couplings, *N*-terminous of hepta peptide was deprotected using 20 % piperidine in DMF and was coupled to isovaleric acid by using HBTU/HOBt as a coupling reagent and DIPEA as a base to get peptide**S1**.

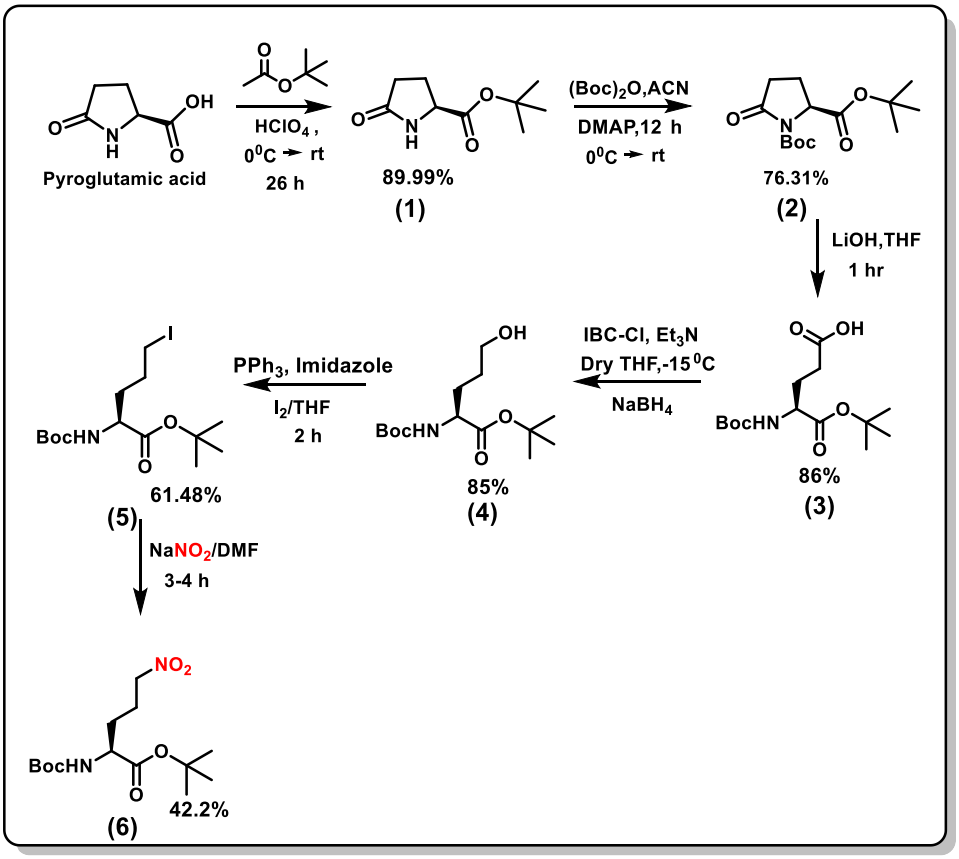


Scheme 2: Solid phase peptide synthesis protocol of peptide **S1.**

4. Results and Discussion:

4.1. Synthesis of nitroalkane amino acid:

Synthesis of nitroalkane amino acid was done via protection of amine and carboxylic acid group of pyroglutamic acid with *tert*-butoxycarbonyl and *tert*-butyl ester respectively and then *N*-Boc protected pyroglutamic ester was subjected to ester hydrolysis in THF using lithium hydroxide which led to opening of five membered ring introducing carboxylic group in side chain. Then carboxylic acid was converted to active ester using isobutyl chloroformate and then reduced to alcohol using sodium borohydride. The alcohol was converted to iodo using modified apple reaction protocol. Finally, the iodo compound was converted to nitro alkane using sodium nitrite via S_N2 mechanism and purified via silica gel column chromatography.

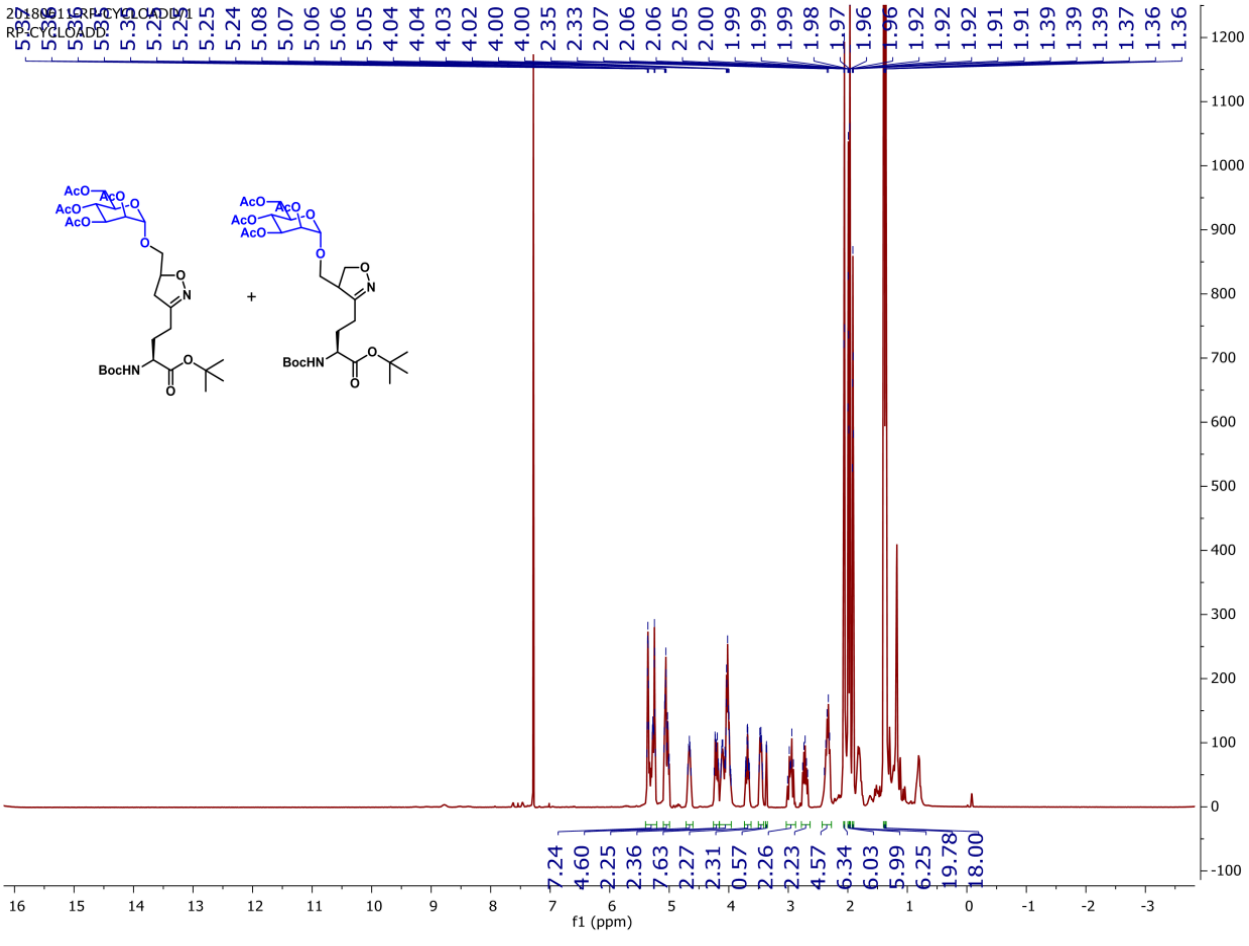


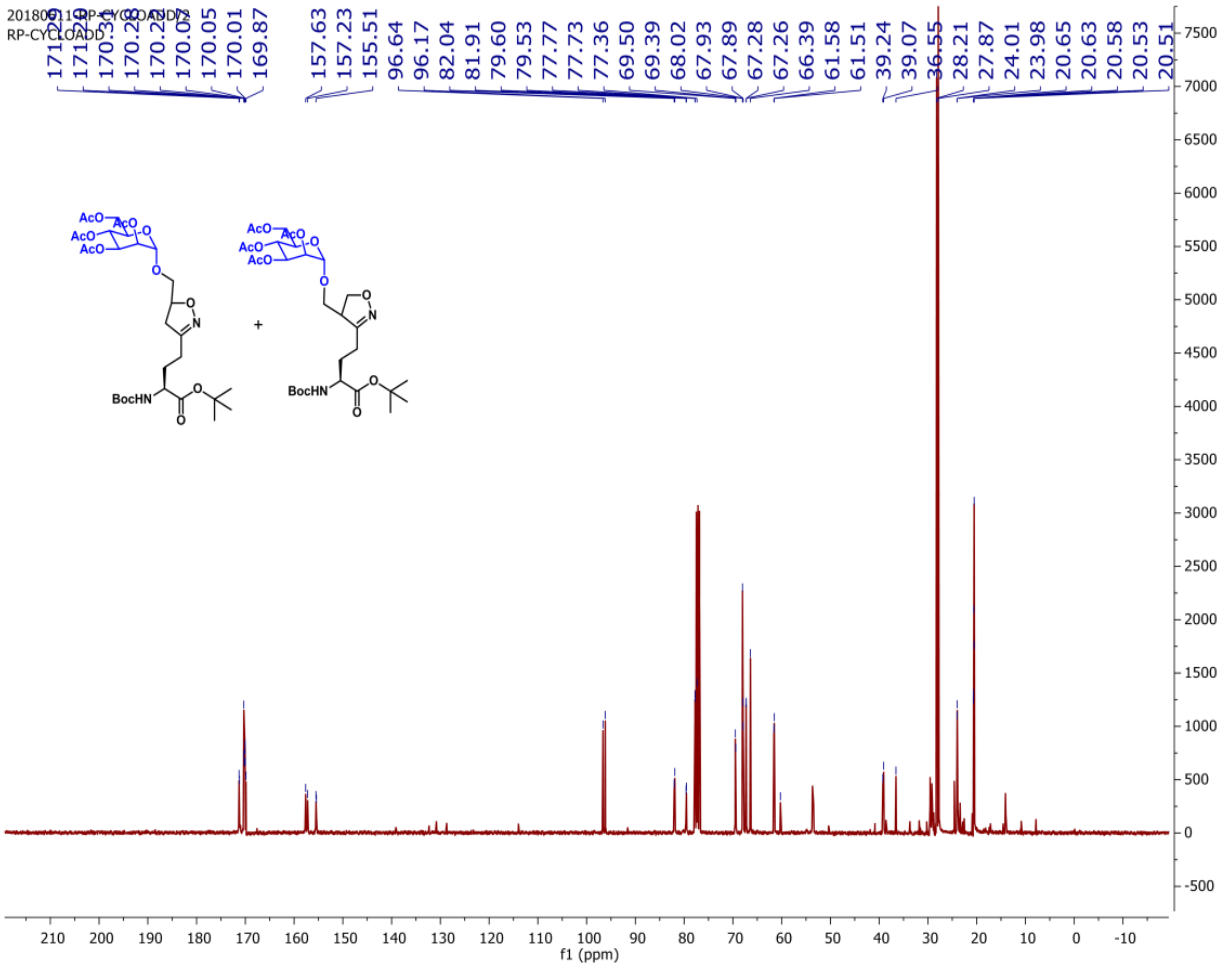
Scheme 3: Synthetic scheme of nitro alkane monomer.

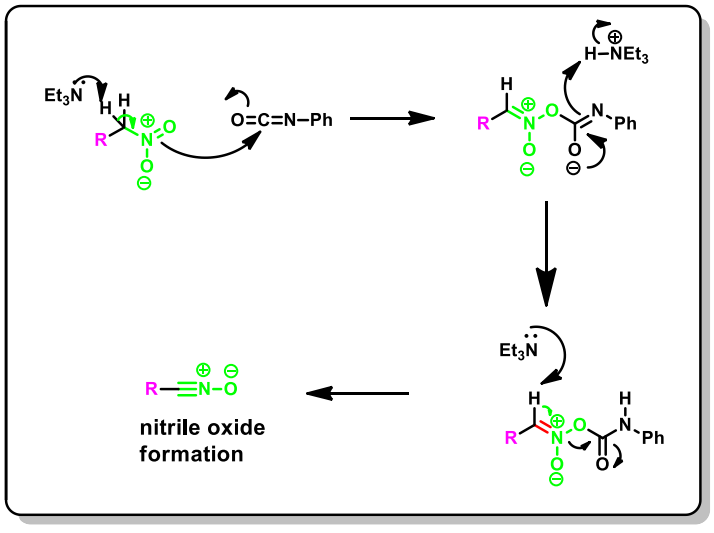
Formation of nitro amino acid was done by ^{1}H NMR, ^{13}C NMR and MALDI-TOF shown in supplementary data.

4.2. Cycloaddition reaction on Nitro monomer:

Nitroalkane amino acid was subjected to cycloaddition with alkene having sugar moiety using phenylisocynate, triethylamine as a reagent in ice cold condition under N_2 atmosphere.Triethylamine act as a base which abstracts the acidic proton present next to nitro group lead to formation of urea and corresponding isoxazoline product and purified via silica gel column chromatography. The corresponding cycloaddition product had two isomers which were differentiated by ^{1}H and ^{13}C NMR techniques.



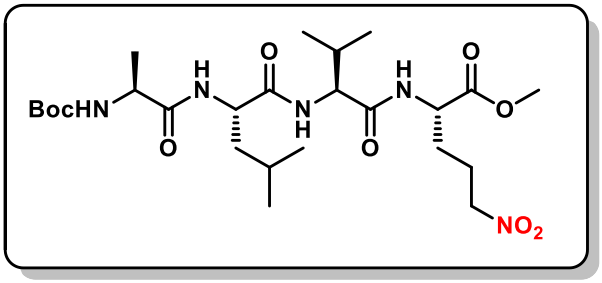




Scheme 4: Mechanism of nitrile oxide formation from nitroalkane.

4.3. Cycloaddition reaction on tetra peptide Boc-Ala-Leu-Val-Nitro-OMe on solution phase:

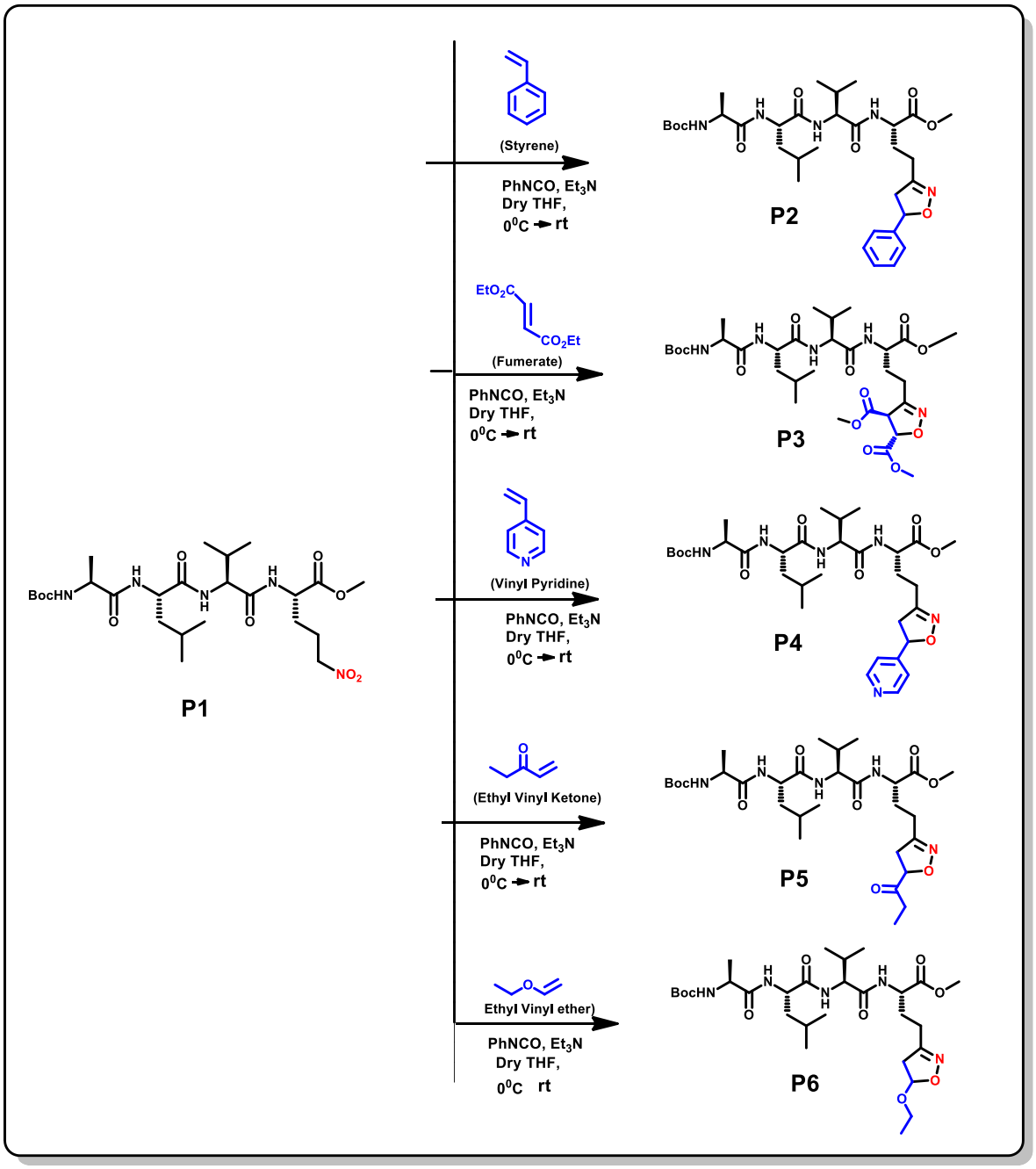
Synthesis of tetrapeptide was done by 1+2+1 methodology in solution phase using EDC.HCl and HOBt as a coupling reagent and DIPEA as a base. Conformation of tetra peptide was characterized by NMR and MALDI-TOF techniques.



Then this tetrapeptide was subjected to cycloaddition with different types of alkenes (disubstituted and mono substituted) using phenylisocynate, triethylamine as a reagent in ice condition under N_2 atmosphere. Side product Urea was eliminated via silica gel column chromatography to remove urea and crude was further purified via reverse phase High Pressure Liquid Chromatography to get isoxazoline product. The corresponding cycloaddition product was characterized by ^{1}H NMR and MALDI-TOF/TOF techniques.

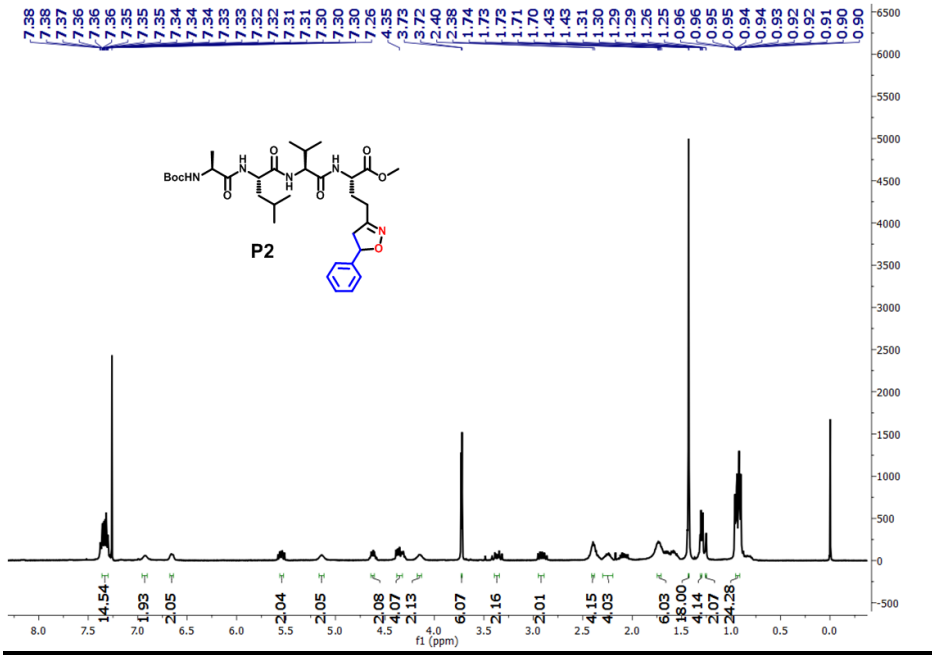
We tried the cycloaddition reaction several different types of alkenes, for example in case of cyclohexene, the yield of the reaction was almost negligible. Since the reactivity of alkene are low compare to alkyne so the yield of these reactions are low. As we know that attaching electron withdrawing group near to alkene improves the reactivity of electrophile and improves the yield of reaction. So we had taken the disubstituted and terminal alkenes having electron withdrawing substituents like carbonyl, pyridine and performed the reaction which results in yield in range of 20% to 30%. Then we tried the 1,3 dipolar cycloaddition reaction with 4 different vinyl alkenes (styrene, vinyl pyridine, ethyl vinyl ketone and ethyl vinyl ether) and one trans disubstituted alkene (fumerate), in all cases reaction went with yield around 25% - 30%. There is a necessity to improve the

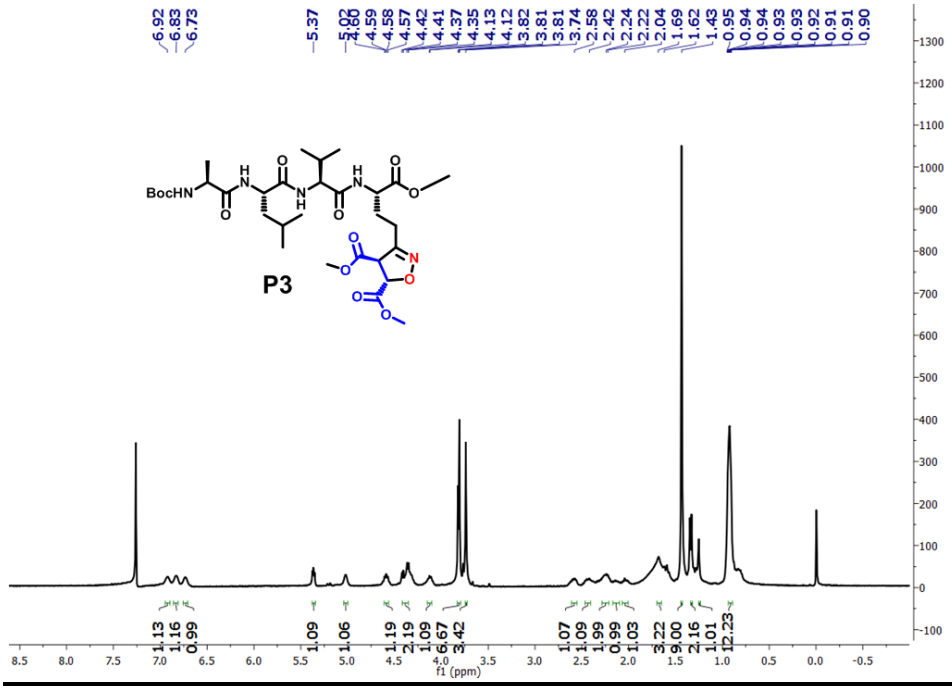
reaction condition for nitrile oxide-alkene cycloaddition reaction in solution phase. By the above protocol peptides (**P1 – P6**) were synthesized **Scheme 5** and characterized by ^{1}H NMR and MALDI-TOF shown below.

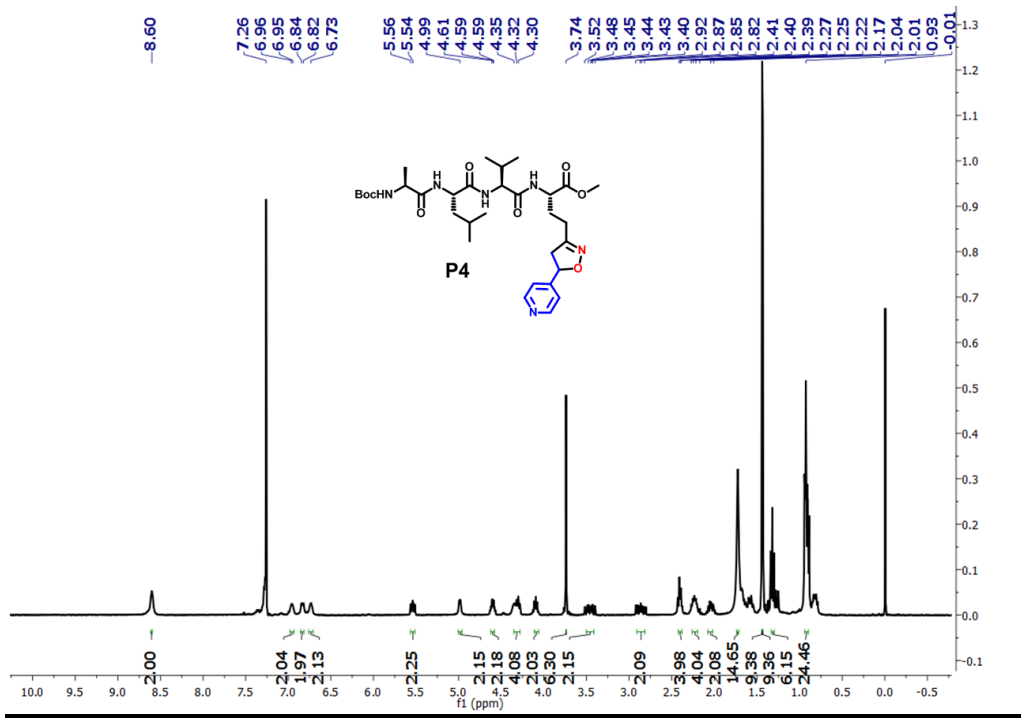


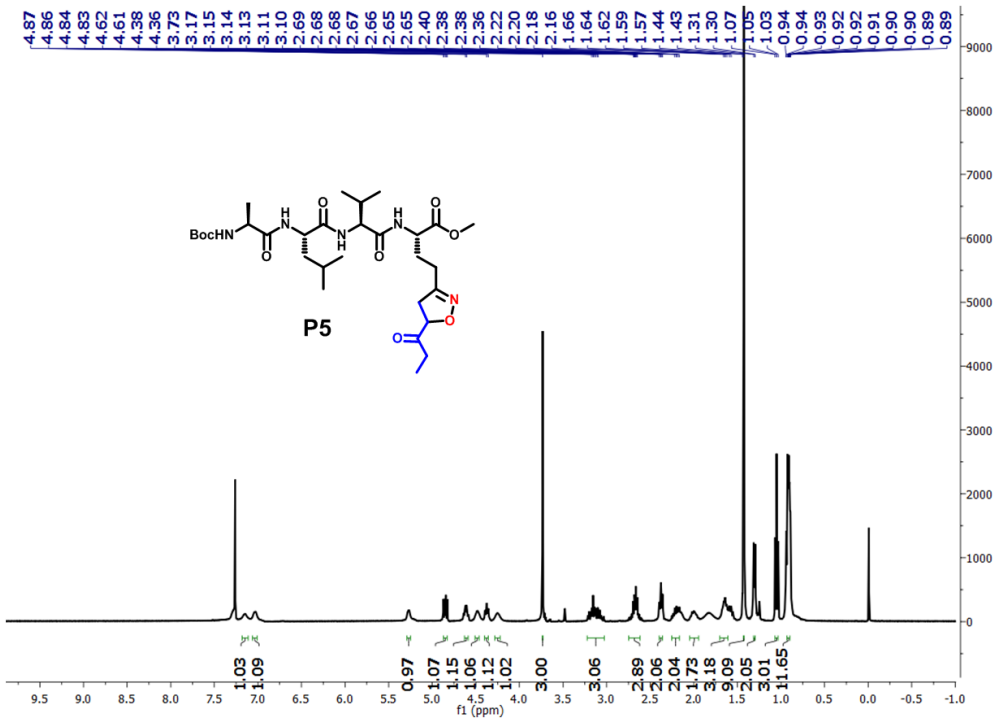
Scheme 5: Cycloaddition reaction of peptide **P 1** with different alkene substrates on solution phase.

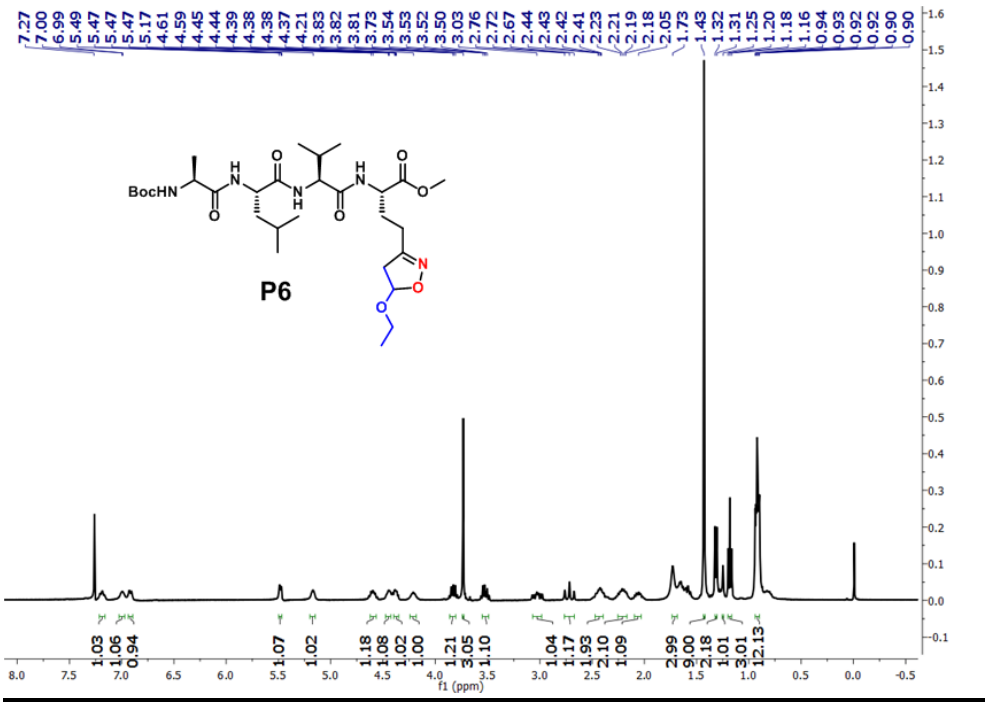
^{1}H NMR spectra of peptides P2-P6






P4
BocHN
f1 (ppm)


P5
BocHN
f1 (ppm)



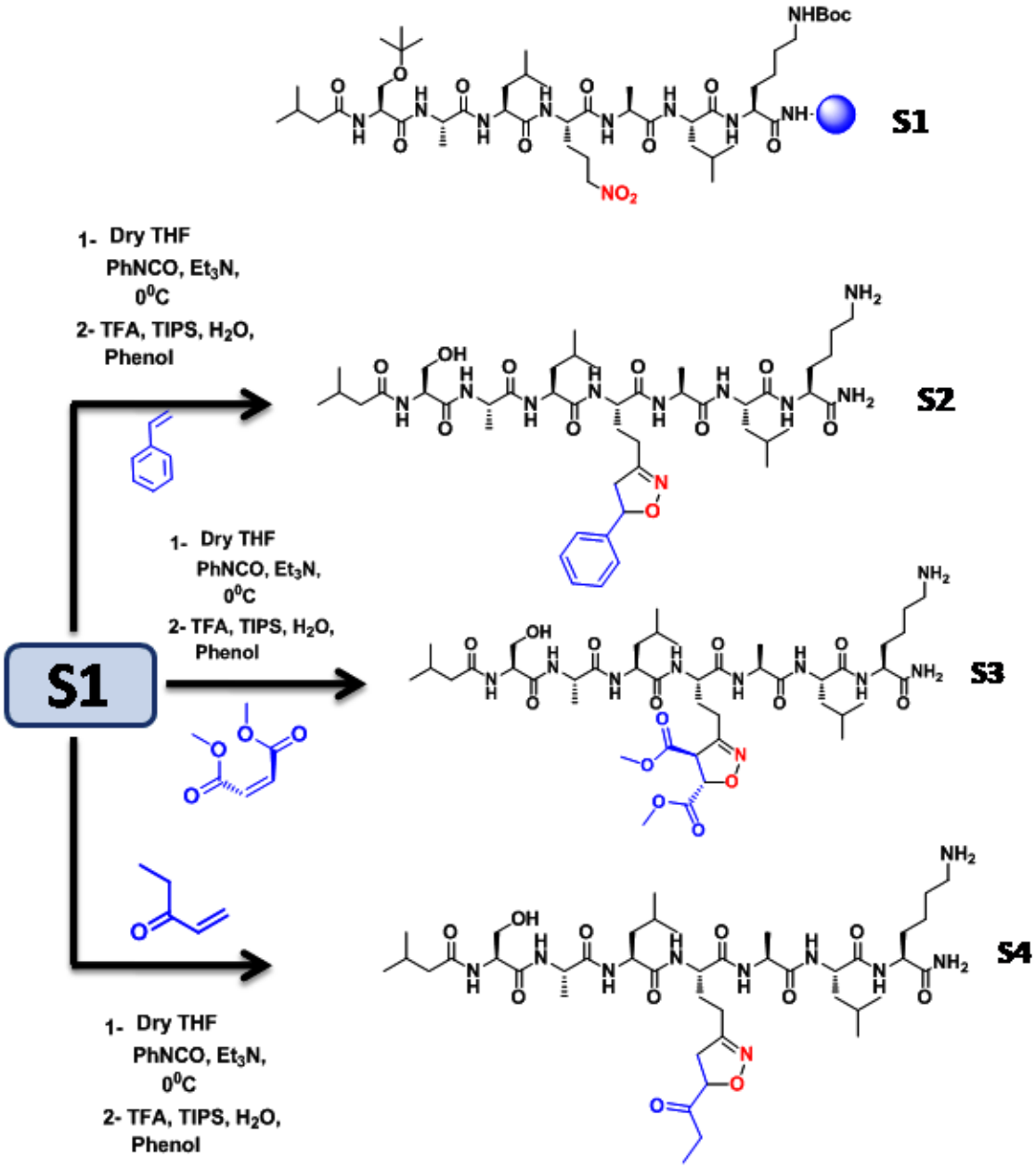
4.4. Cycloaddition reaction of hepta peptide S1 on solid support:

Synthesis of hepta peptide was done in solid phase peptide synthesizer using rink amide resin. Couplings were performed using HBTU/HOBt as coupling reagents in NMP solvent. 20% piperidine in DMF was used to deprotect Fmoc group and washing were done in DCM and DMF solvents.

Resin bound peptide **S1**was suspended in dry THF at 0 ºC under N_2 atmosphere. To this suspension solution styrene (5eq), phenylisocynate (5eq) and triethylamine were added. After completion of reaction urea was removed by washing with MeOH in sintered funnel and resin was collected and resin cleavage was performed under cocktail mixture consisting of 95 % TFA, 2.5 % H_2O and 2.5 % triisopropylsilane and cycloaddition product **S2** was purified on C_{18} column in RP-HPLC and characterized by MALDI-TOF/TOF.

Peptides **S3** and **S4** were synthesized by using similar protocol using and purified using fumerate and ethyl vinyl ketone as alkene. In contrast to solution phase

significant improvement in the yields were observed in 1,3-dipolar cycloaddition on solid support.



Scheme 6: Cycloaddition reaction of peptide **S1** with different alkenes on solid support.

5. Conclusion

We had demonstrated 1,3-dipolar cycloaddition reactions between alkenes and *in situ* generated nitrile oxide from nitro-alkane amino acid and peptides. We successfully showed that incorporating this nitro-alkane amino acid in peptide sequence and then performing cycloaddition reaction leads to cyclized isoxazoline product in both solution and solid support. We showed that terminal alkene (vinyl) can easily undergo cycloaddition reactions with nitroalkanes tethered peptides compared to the disubstituted alkenes. Since alkenes are not as reactive as alkynes so yields of the cycloaddition reactions were found to be less and there is a necessity to improve reaction conditions to improve the yields. Nevertheless, it is important to note that 1,3-dipolar cycloaddition reaction between azide and alkene is not feasible and it is possible in nitrile oxide case. In comparison, better yields were obtained on solid support reactions than solution method. The main problem in solution method is the separation of urea byproduct. We are making efforts to improve the yields of this important nitrile oxide-alkene cycloaddition and its orthogonality with other 1,3-dipolar cycloaddition reactions. Overall, there is a great scope for this nitroalkane mediated nitrile oxide –alkene cycloaddition reactions for peptide conjugations.

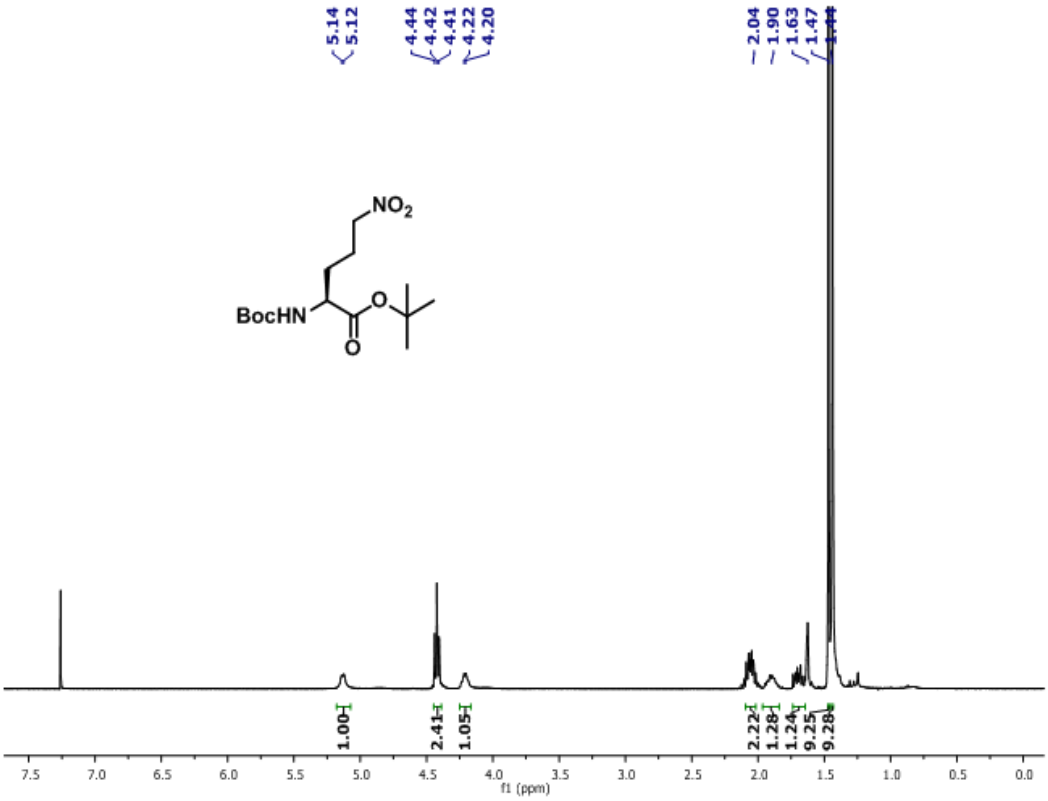
6. References

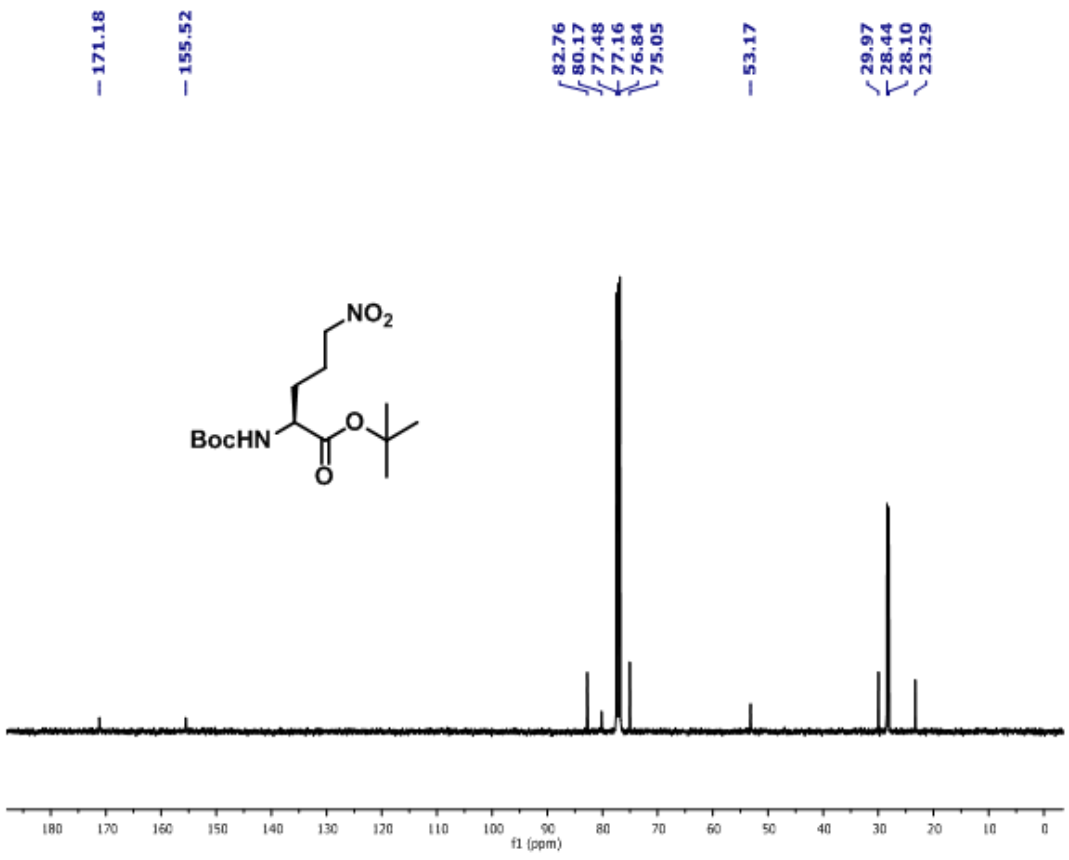
1. (a) Shokeen, M.; Pressly, E. D.; Hagooly, A.; Zheleznyak, A.; Ramos, N.; Fiamengo, A. L.; Welch, M. J.; Hawker, C. J.; Anderson, C. J. *ACS Nano*. **2011**, *5*, 738. (b)Sachin, K.; Jadhav, V. H.; Kim, E.-M.; Kim, H. L.; Lee, S. B.;Jeong, H.-J.; Lim, S. T.; Sohn, M.-H.; Kim, D. W. *BioconjugateChem.* **2012**, *23*, 1680. (c)Wanglerl, C.; Maschauer, S.; Prante, O.; Schafer, M.;Schirrmacher, R.; Bartenstein, P.; Eisenhut, M.; Wangler, B.*ChemBioChem*. **2010**, *11*, 2168
2. (a) DeForest, C. A.; Polizzotti, B. D.; Anseth, K. S. *Nat. Mater.***2009**, *8*, 659. (b) Zheng, J.; Liu, K.; Reneker, D. H.; Becker, M. L. *J. Am. Chem. Soc.***2012**, *134*, 17274. (c) Lin, F.; Yu, J.; Tang, W.; Zheng, J.; Xie, S.; Becker, M. L. *Macromolecules* **2013**, *46*, 9515.
3. (a) Kamphuis, M. M. J.; Johnston, A. P. R.; Such, G. K.; Dam, H. H.; Evans, R. A.; Scott, A. M.; Nice, E. C.; Heath, J. K.; Caruso, F. *J. Am. Chem. Soc.***2010**, *132*, 15881. (b) von Maltzahn, G.; Ren, Y.; Park, J.-H.; Min, D.-H.; Kotamraju, V. R.; Jayakumar, J.; Fogal, V.; Sailor, M. J.; Ruoslahti, E.; Bhatia, S. N. *Bioconjugate Chem*. **2008**, *19*, 1570. (c) Krishnamurthy, V. R.; Wilson, J. T.; Cui, W.; Song, X.; Lasanajak, Y.; Cummings, R. D.; Chaikof, E. L. *Langmuir*, **2010**, *26*, 7675.
4. Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem*. **2001**, *40*, 2004.
5. Hoyle, C. E.; Bowman, C. N. *Angew. Chem., Int. Ed*. **2010**, *49*, 1540.
6. (a) Saxon, E.; Bertozzi, C. R. *Science*, **2000**, *287*, 2007. (b) Lin, F. L.; Hoyt, H. M.; van Halbeek, H.; Bergman, R. G.; Bertozzi, C. R. *J. Am. Chem. Soc*. **2005**, *127*, 2686.
7. Gramlich, P. M. E.; Wirges, C. T.; Manetto, A.; Carell, T. *Angew. Chem. Int. Ed.***2008**, *47*, 8350**.**
8. Blackman, M. L.; Royzen, M.; Fox, J. M. *J. Am. Chem. Soc.***2008**, *130*, 13518.

9. (a) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc*. **2004**, *126*, 15046. (b) Baskin, J. M.; Prescher, J. A.; Laughlin, S. T.; Agard, N. J.; Chang, P. V;
10. Miller, I. A.; Lo, A.; Codelli, J. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci.***2007**, *104*, 16793. (c) Agard, N. J.; Baskin, J. M.; Prescher, J. A.; Lo, A.; Bertozzi, C. R. *ACS Chem. Biol*., **2006**, *1*, 644.
11. R. Huisgen, *Angew. Chem.***1963**, *75*, 604.
12. Mukaiyama, T; Hoshino, T.*J. Am. Chem. Soc*. **1960**, *82*, 5339.
13. Liu K. C., Shelton B. R., Howe R. K ; *J. Org. Chem*. 1980, 45, 3916 – 3918.
14. Reja, R. M.; Sunny, S.; Gopi, H. N. *Org. Lett.*2017, 19, 3572.

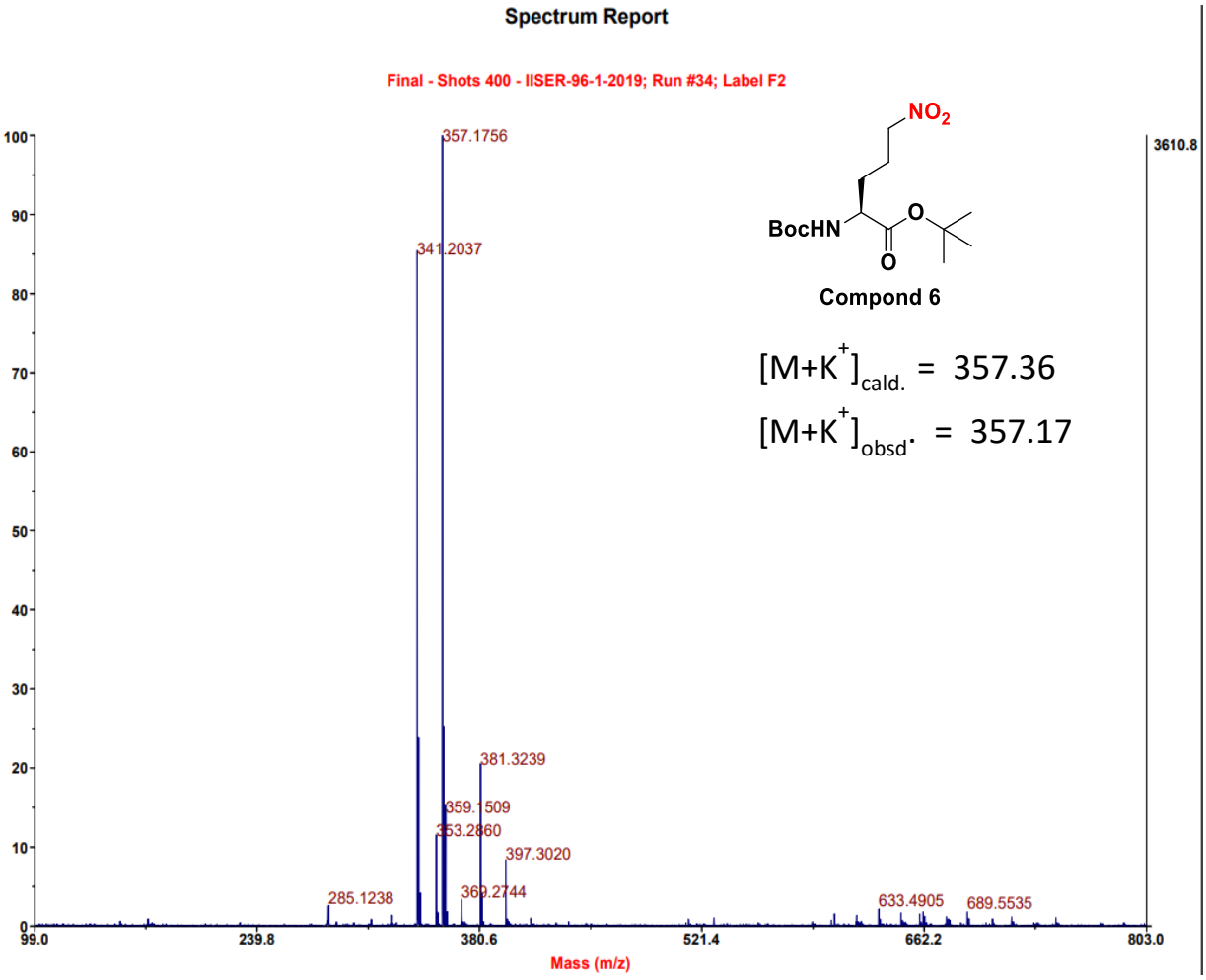
7. Supplementary Data

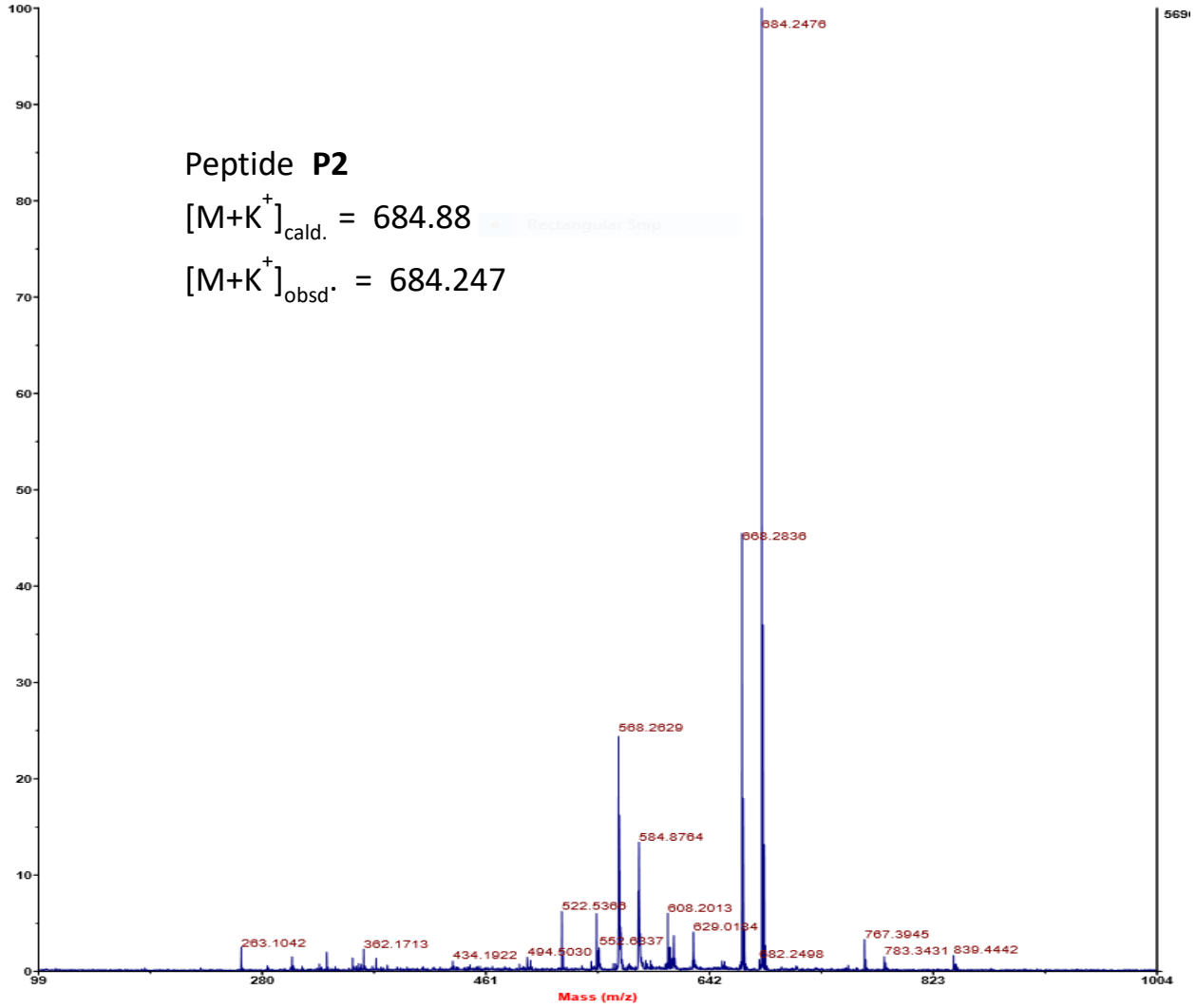
^{1}H NMR and ^{13}Cof Spectra of Compound 6




171.18
155.52
82.76
80.17
77.48
77.16
76.84
75.05
53.17
29.97
28.44
28.10
23.29
NO2
BocHN
O
O
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

MALDI/TOF Spectra of Compond 6 and Peptides P1-P6 and S2-S4




Peptide P2
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[M+K+]obsd. = 684.247
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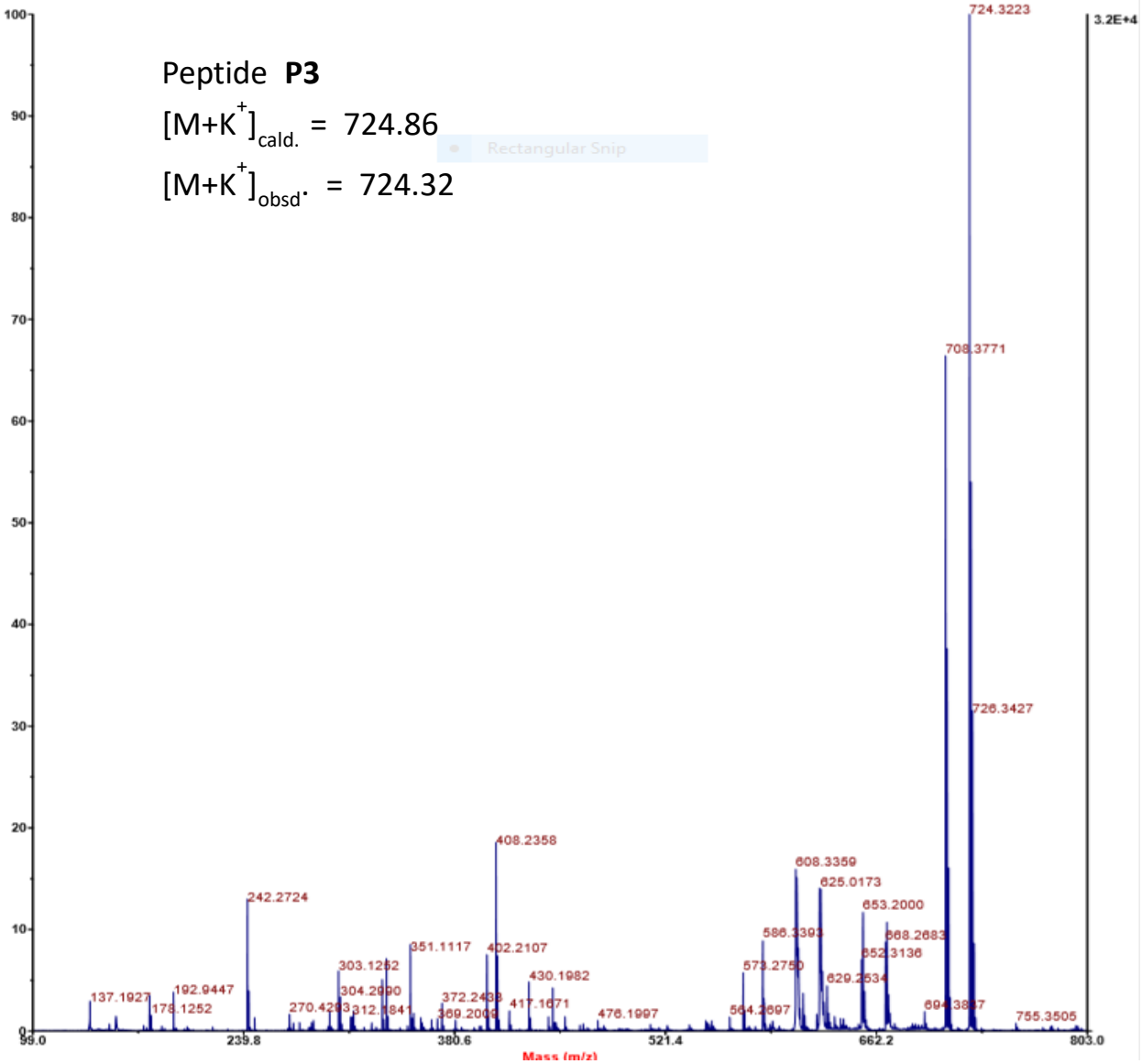
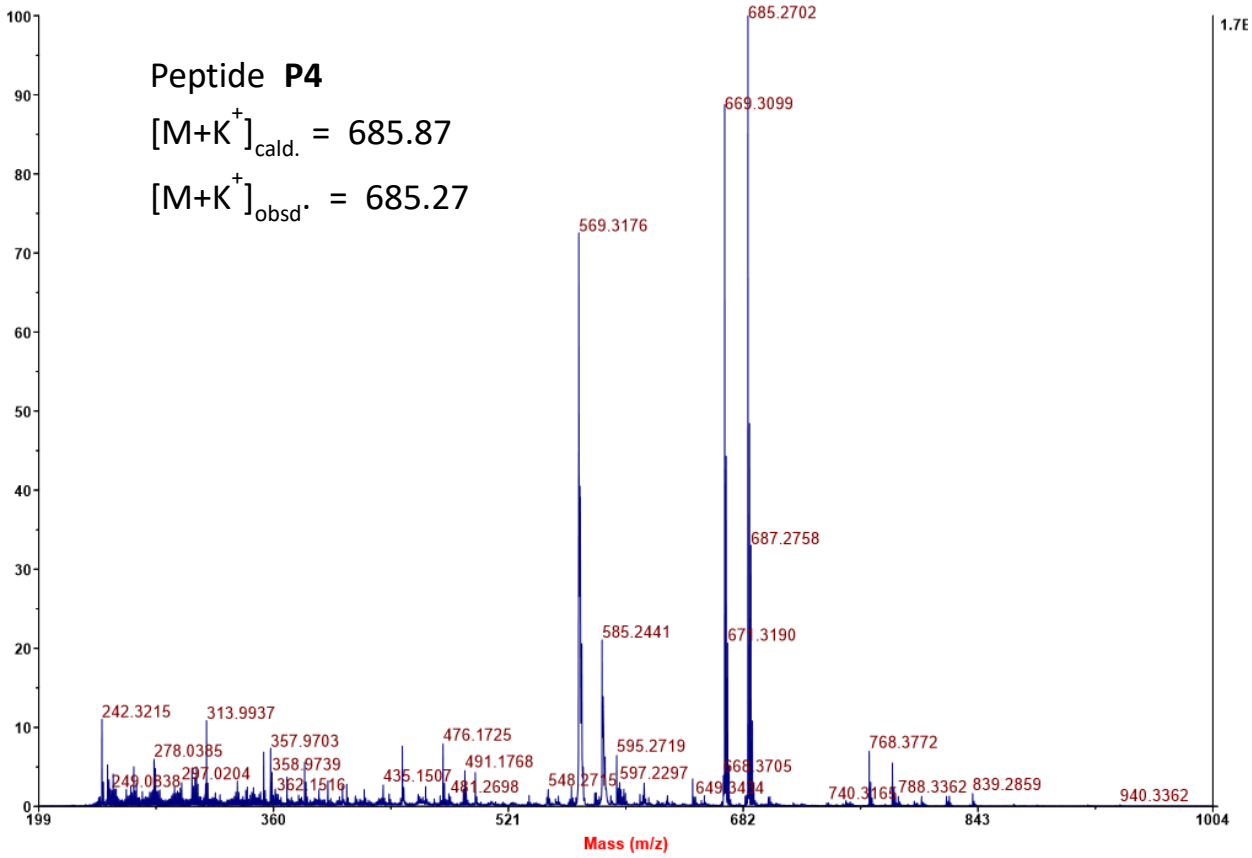
522.5368
608.2013
629.0184
263.1042
362.1713
434.1922
494.5030
552.6837
682.2498
767.3945
783.3431
839.4442
Mass (m/z)

Peptide **P3**

$[M+K^+]_{cald.}$ = 724.86

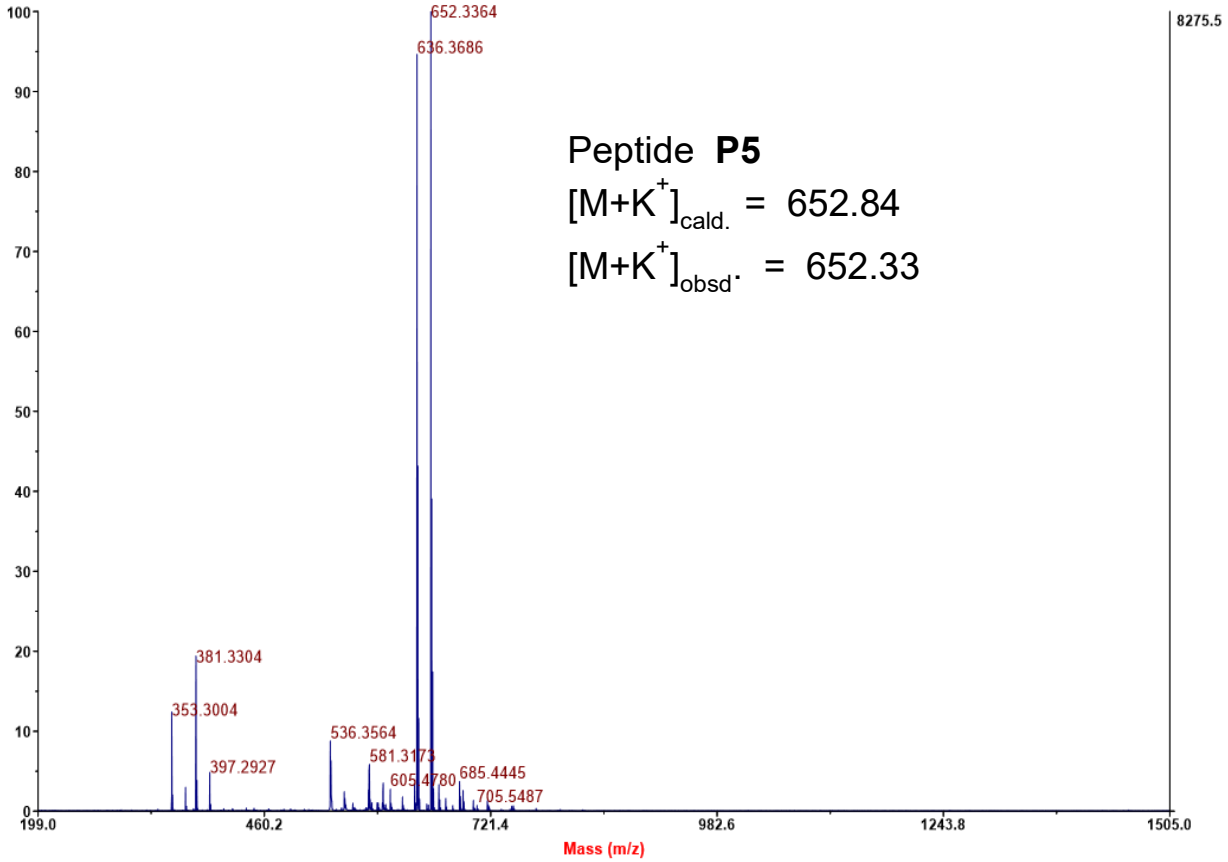
$[M+K^+]_{obsd.}$ = 724.32




Peptide **P4**
$[M+K^+]_{cald.}$ = 685.87
$[M+K^+]_{obsd.}$ = 685.27
685.2702
669.3099
569.3176
687.2758
585.2441
671.3190
242.3215
313.9937
476.1725
278.0385
357.9703
491.1768
595.2719
768.3772
297.0204
358.9739
597.2297
435.1507
548.2715
839.2859
940.3362
Mass (m/z)

Spectrum Report

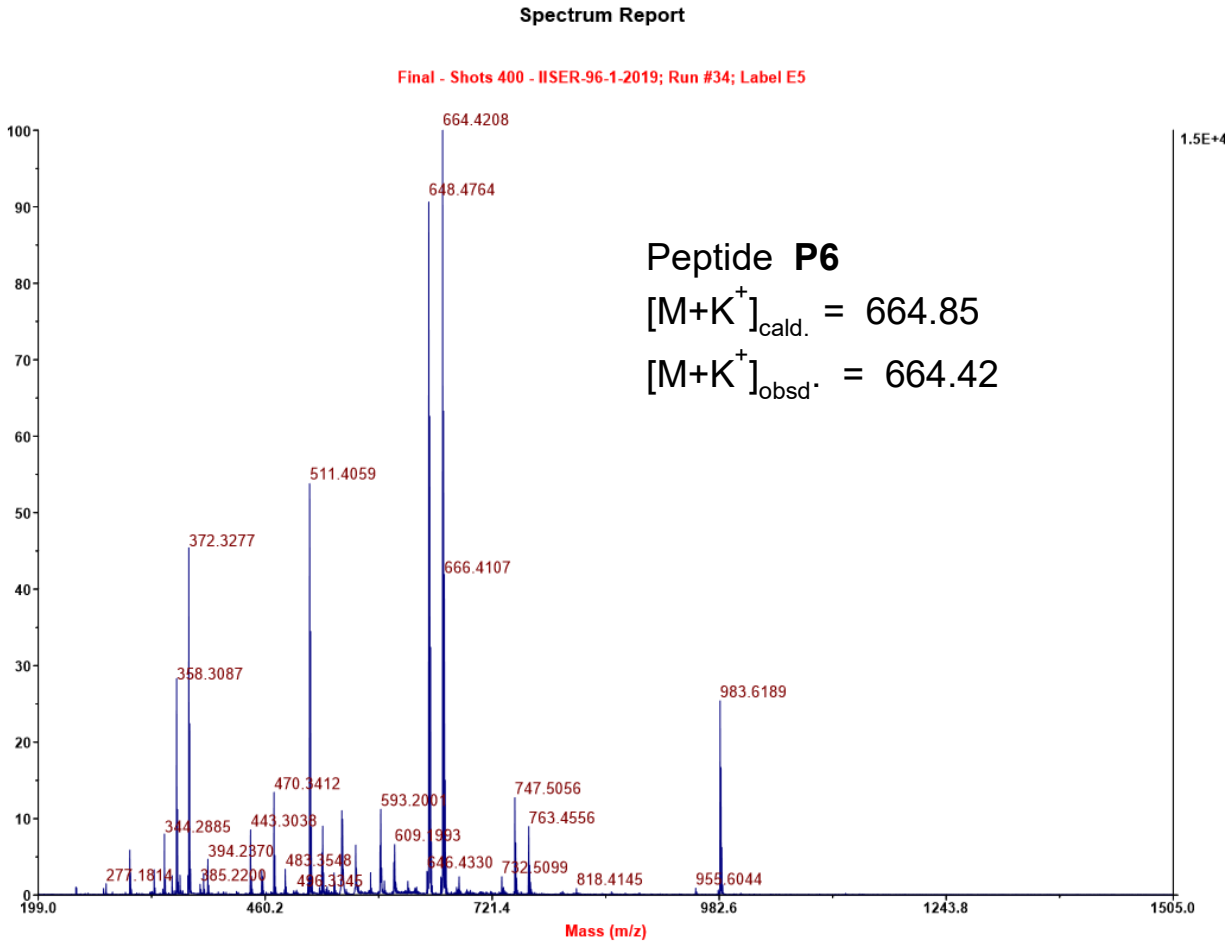
Final - Shots 400 - IISER-96-1-2019; Run #34; Label F4



Peptide **P5**

$[M+K^+]_{cald.}$ = 652.84

$[M+K^+]_{obsd.}$ = 652.33


Spectrum Report
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358.3087
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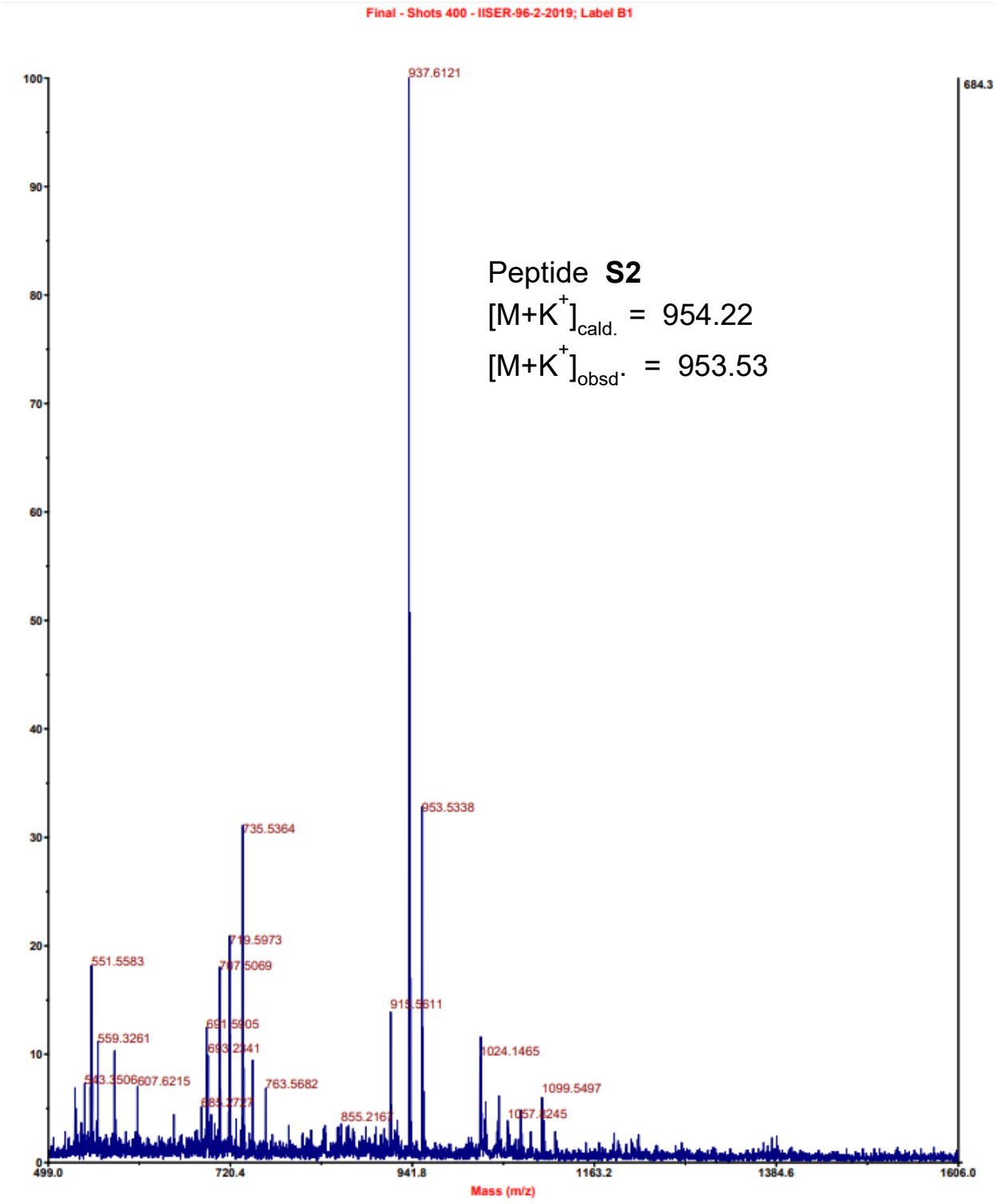
470.3412
593.2001
747.5056
763.4556
344.2885
443.3038
609.1993
394.2370
483.3548
646.4330
732.5099
818.4145
955.6044
277.1814
385.2200
496.3345
Peptide P6
[M+K+]cald. = 664.85
[M+K+]obsd. = 664.42
1.5E+4
100
90
80
70
60
50
40
30
20
10
0
199.0
460.2
721.4
982.6
1243.8
1505.0
Mass (m/z)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691.5605
559.3261
693.2941
1024.1465
543.3506
607.6215
763.5682
1099.5497
685.2727
855.2167
1057.5245
Peptide S2
[M+K+]cald. = 954.22
[M+K+]obsd. = 953.53
100
90
80
70
60
50
40
30
20
10
0
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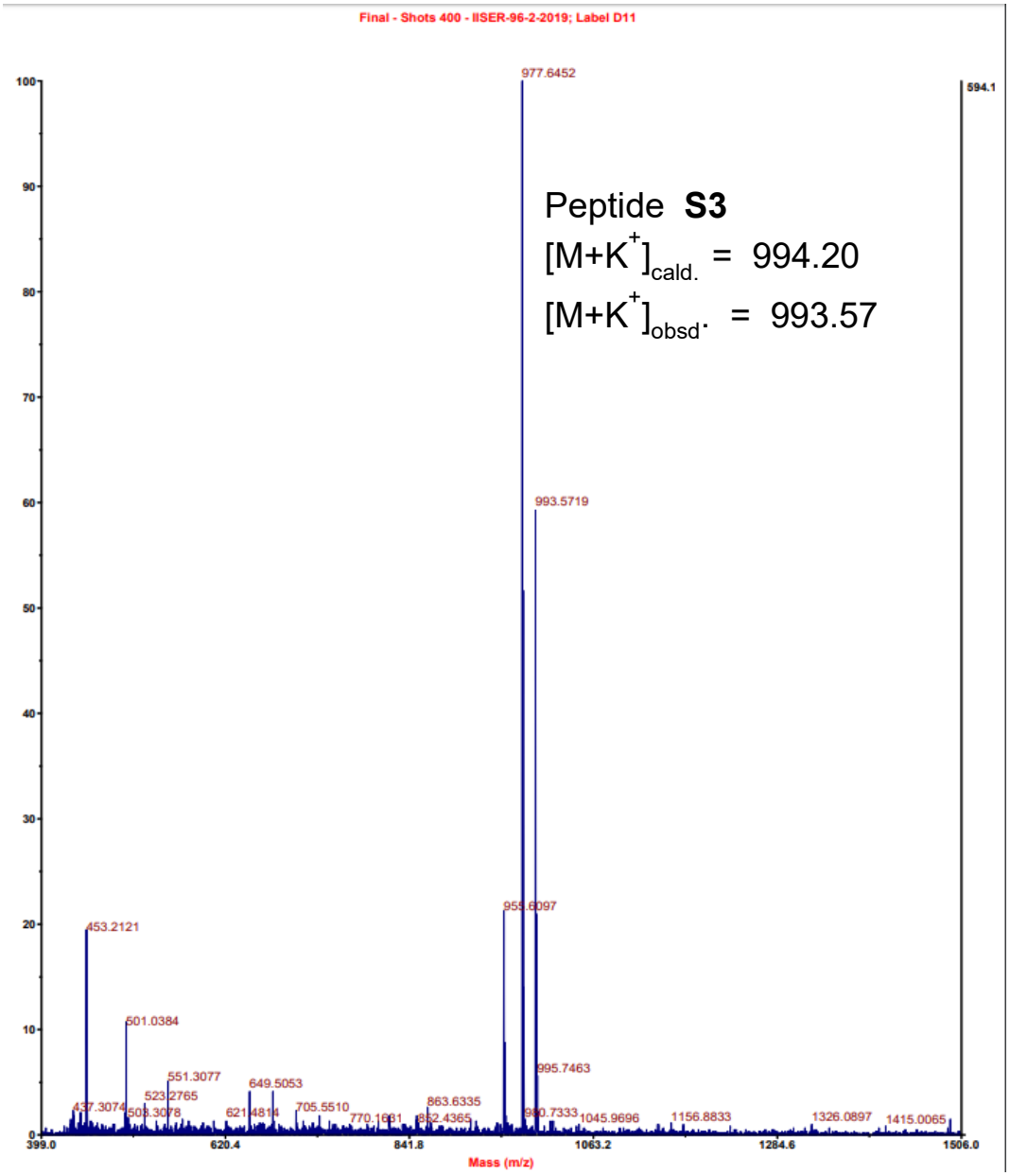
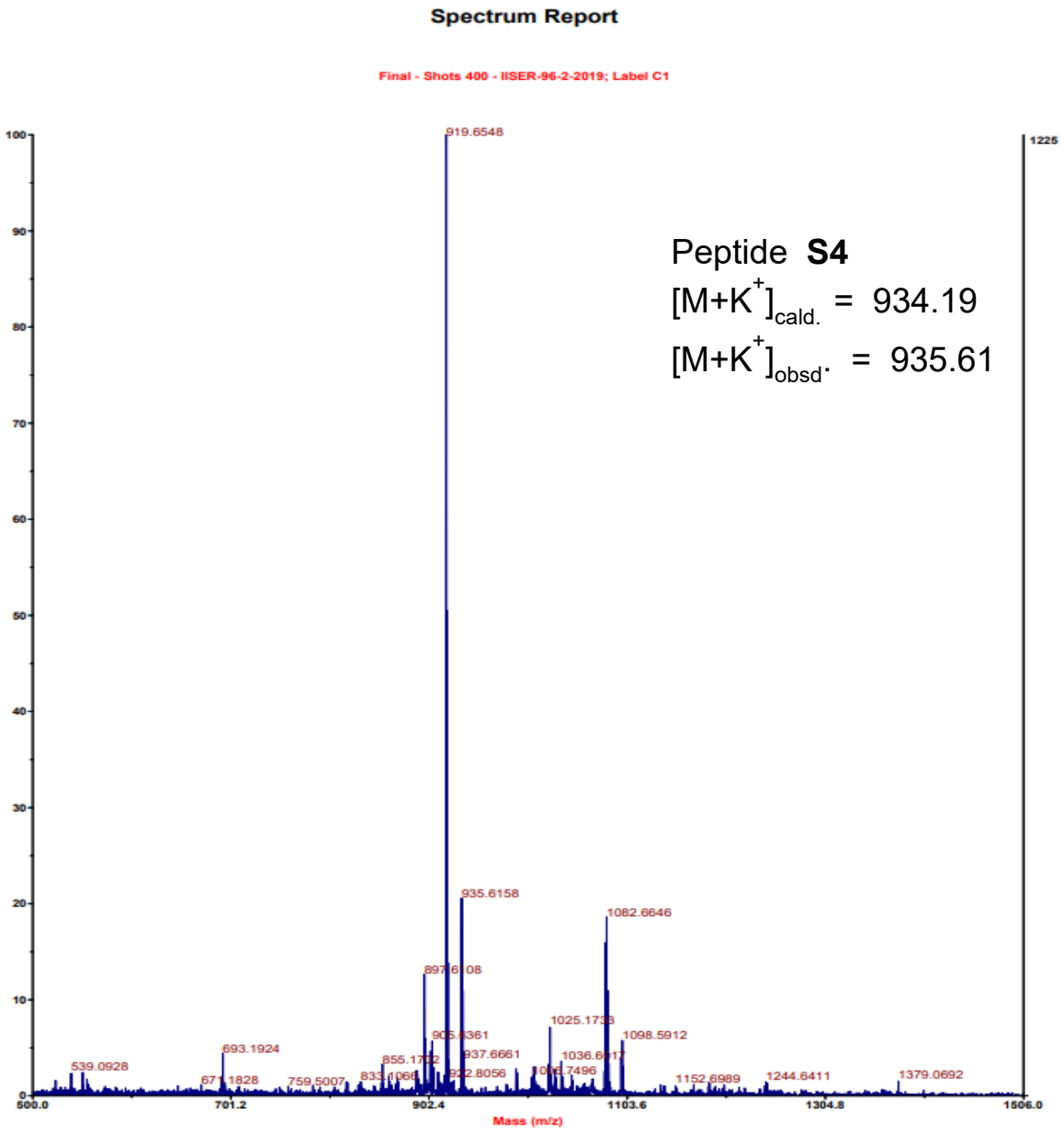
720.4
941.8
1163.2
1384.6
1606.0
Mass (m/z)



Peptide **S3**

$[M+K^+]_{cald.}$ = 994.20

$[M+K^+]_{obsd.}$ = 993.57


Spectrum Report
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937.6661
1025.1733
1244.6411
1379.0692
1152.6989
759.5007
Mass (m/z)
Peptide S4
[M+K+]cald. = 934.19
[M+K+]obsd. = 935.61