Selective Orthogonal nitroalkane-alkyne 1, 3-dipolar Cycloadditions on peptides: A versatile approach for Biomolecular 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 Orthogonal nitroalkane-alkyne 1,3-dipolar Cycloadditions on peptides: A versatile approach for Biomolecular Conjugation***" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by "**Sereena Sunny** at IISER Pune" under the supervision of "**Dr. Hosahudya N. Gopi**, Associate professor, Department of Chemistry, IISER Pune" during the academic year 2016-2017.

20th March 2017

Dr. Hosahudya N. Gopi

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DECLARATION

I hereby declare that the matter embodied in the report entitled "***Selective Orthogonal nitroalkane-alkyne 1,3-dipolar Cycloadditions on peptides: A versatile approach for Biomolecular Conjugation***" are the results of the work 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.

20th March 2017

Sereena Sunny

5th Year BS-MS

IISER Pune

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I extend my gratitude towards people who helped for doing MALDI/TOF, X-ray diffraction, and NMR studies.

I am deeply grateful to IISER Pune for providing me excellent facilities and beautiful atmosphere to carry out research.

I owe my deepest gratitude to my whole family especially my parents and lovely brothers for their unconditional love, caring and unfailing support throughout my years of study. This accomplishment would not have been possible without them.

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LIST OF SCHEMES

ABBREVATIONS

ACN = Acetonitrile

AcOH = Acetic acid

Aib/U = 2-Aminoisobutyric acid

AIBN = Azobisisobutyronitrile

Boc = tert-Butoxycarbonyl

Bu_3SnH = Tributyltin hydride

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

Cbz-Cl = Benzyl chloroformate

$CuSO_4$ = Copper (II) sulphate

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

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

$HClO_4$ = Perchloric acid

HOBt = 1-Hydroxybenzotriazole

RP-HPLC = Reverse Phase High Performance Liquid Chromatography

IBC-Cl = Isobutyl chloroformate

K_2CO_3 = Potassium carbonate

K_2HPO_4 = Dipotassium phosphate

$KMnO_4$ = Potassium permanganate

KOH = Potassium hydroxide

Leu = Leucine

LiOH = Lithium hydroxide

$NaBH_4$ = Sodium borohydride

Na_2CO_3 = Sodium carbonate

NaH = Sodium hydride

$NaNO_2$ = Sodium nitrite

NMP = N-methyl pyrrolidone

NMR = Nuclear Magnetic Resonance

N = Normal

Ph-NCO = Phenyl isocyanate

PPh_3 = Triphenyl phosphine

t-BuOH = tert-Butyl alcohol

TFA = Trifluoro acetic acid

THF = Tetrahydrofuran

Val/V = Valine

1. ABSTRACT

The mild and substrate compatible copper catalyzed 1, 3-dipolar cylcoaddition reaction between azide and alkyne (click chemistry) has been extensively used in the conjugation chemistry. Though the click chemistry has proven its wide applications in bioconjugations, however suffers with toxicity of copper in the biological systems. In addition, performing more than one cycloaddition reactions on a substrate containing two or more azide functionalities is proved to be difficult. In this context, we sought to investigate 1, 3-dipolar cycloaddition reactions orthogonal to the azide-alkyne cycloadditions. Nitroalknes have been serving as versatile intermediates in various organic transformations including 1, 3-dipolar cycloadditions with alkenes and alkynes. We hypothesized that nitroalkanes can serve attractive alternatives to azides in 1, 3-dipolar cycloadditions. Herein, we are reporting the mild 1, 3-dipolar cycloaddition reaction between alkyl nitro and alkynes. The new nitro amino acid was synthesized and introduced into the peptide sequence along with azidolysine. The reaction was found to be orthogonal to the azide-alkyne chemistry. In addition, the nitroalkane-alkyne cycloaddition reaction was compatible with both solid and solution phase chemistry. Using mild and orthogonal cycloaddition reactions, various nitroalkane-alkyne and azide-alkyne cycloaddition reactions were performed step by step in both solution as well as on solid phase. Besides the cycloaddition, the nitro group was further transformed into various other functional groups on peptides. Overall, we have demonstrated the mild functional group transformations of nitro amino acid on the peptides including orthogonal 1, 3-dipolar cycloaddition reactions.

2. INTRODUCTION

Peptides are attracting widespread interest in the field of bioorthogonal chemistry due to its structural complexity, functional diversity, biodegradability and ease of synthesis. Each peptide sequence have its own unique properties which can be exploited to design wide variety of biomaterials by combing these with carbohydrates, nucleic acids, drug molecules, polymers, Nanoparticles and diagnostic probes[1]. The conjugated peptides have been finding much application in therapeutic[2], diagnostic[3], tissue engineering[4] and drug delivery[5]. For the efficient synthesis of peptide-conjugates, one need to develop methodologies that are cost effective, selective, bioorthogonal as well as compatible with physiological conditions. The reactions which can satisfy the above mentioned criteria are termed as "Click reactions" by the famous scientist Barry Sharpless in 2001[6]. During last few years, researchers have developed several click reactions to synthesize peptide-conjugates including Cu(I)-catalyzed azide–alkyne cycloaddition, Strain-promoted azide–alkyne cycloaddition, Thiol–ene reaction[7], Oxime ligation[8], Diels–Alder reaction[9], Staudinger ligation[10] and native chemical ligation[11] (**Figure 2.1**). Among these, Copper (I) catalysed azide-alkyne cycloaddition reaction is one of the most celebrated chemical reaction in recent days[12]. The pioneering work of Hüisgen established a large range of 3 atom-4 electron cycloaddition reaction with dipolarophile[13]. The copper catalysed azide-alkyne reactions satisfied all criteria required for click reaction. Therefore, it has become the quintessential click reaction''. The cycloaddition between the azide and terminal alkyne can also be catalysed by Ru(II) complexes to achieve 1,5 disubstituted 1,2,3 triazole products [14]. Copper catalysed click reaction has been extensively utilized to labeled the biomolecules in vitro and ex vivo. To avoid the toxicity of copper (I) in biological systems, Bertozzi et al developed copper free strain promoted azide-alkyne cycloaddition reaction (SPPAC) [15]. Besides the biological applications, click reaction has been extensively explored to conjugate peptide and small molecules.

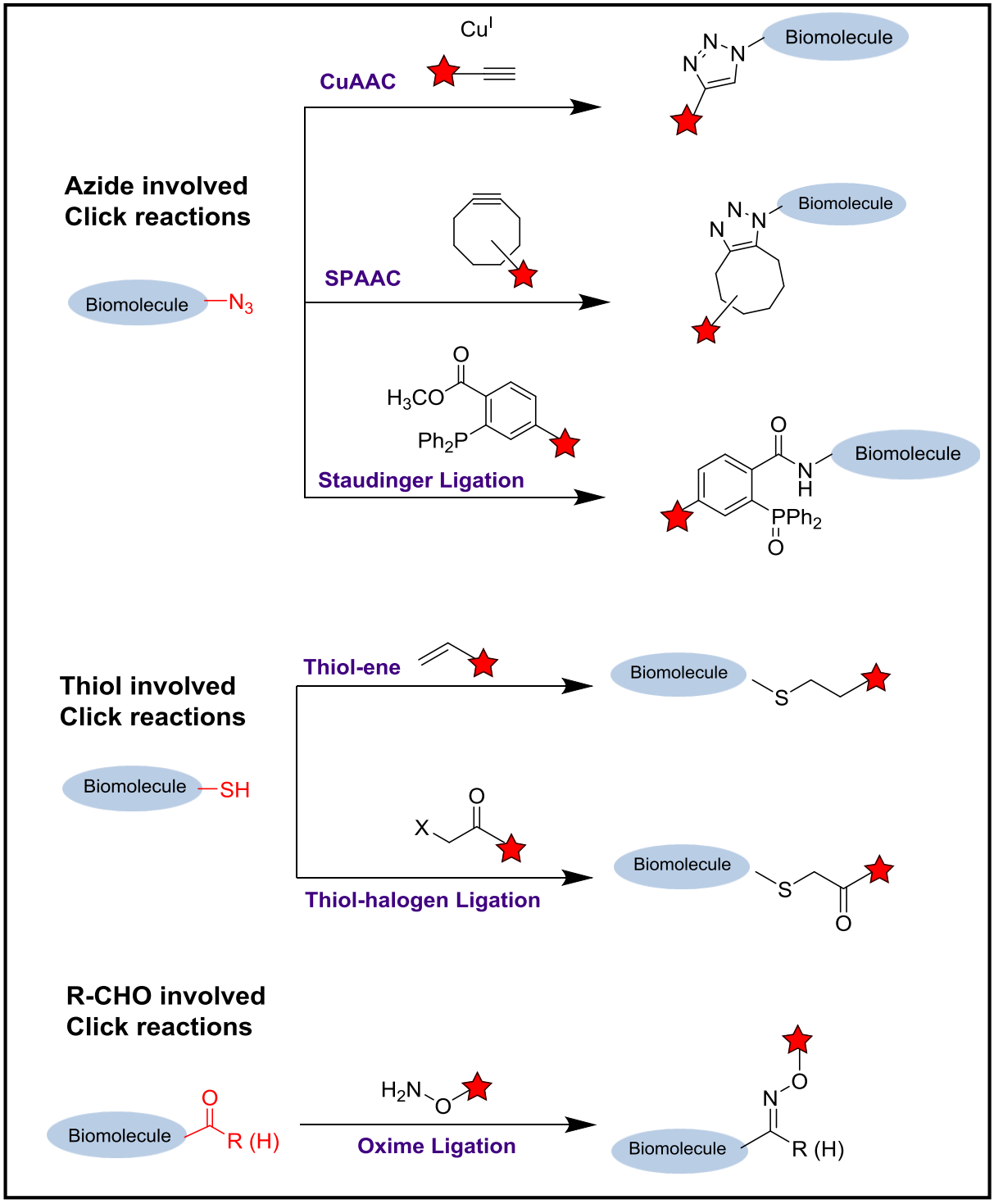


Figure 2.1: Click reactions that used to synthesis peptide-conjugates

In order to use click conjugated peptides more efficiently for biomedical applications, researchers have to develop simple and effective strategies for synthesising multifunctional biomacromolecules by using orthogonal click reactions. For this purpose, we need to design a scaffold with a set of functional groups which should not react with each other and the conjugation reaction between chosen functional groups and the added molecules has to be orthogonal to each other [16]. The

major issue related with the azide-alkyne click conjugation is that it cannot be used for more than one cycloaddition in a single peptide. Nevertheless, Wolfeis and colleagues have demonstrated the dual labelling of biomolecules with and without copper using alkynes and strain alkynes in a peptide respectively[17]. In addition, Carell and colleagues demonstrated the triple modification of DNA by incorporating suitable protected alkynes on the DNA backbone [18]. Boturyn and colleagues demonstrated a novel strategy of biomolecular assemblies using orthogonal oxime bond formation and copper (I) mediated azide-alkyne cycloaddition reaction on peptide backbone[19]. Besides the alkyne and the azides, a variety of 1, 3 dipoles (3 atom system) has been explored in the biomolecular conjugation[20]. Recently, Boturyn et. al showed the bioorthogonal conjugation reaction using azide-alkyne and thioacid-amine coupling reaction on a peptide template by Cu (I) catalyst[21]. We recently demonstrated facile peptide coupling reactions using thioacids and its chemistry on peptides [22].

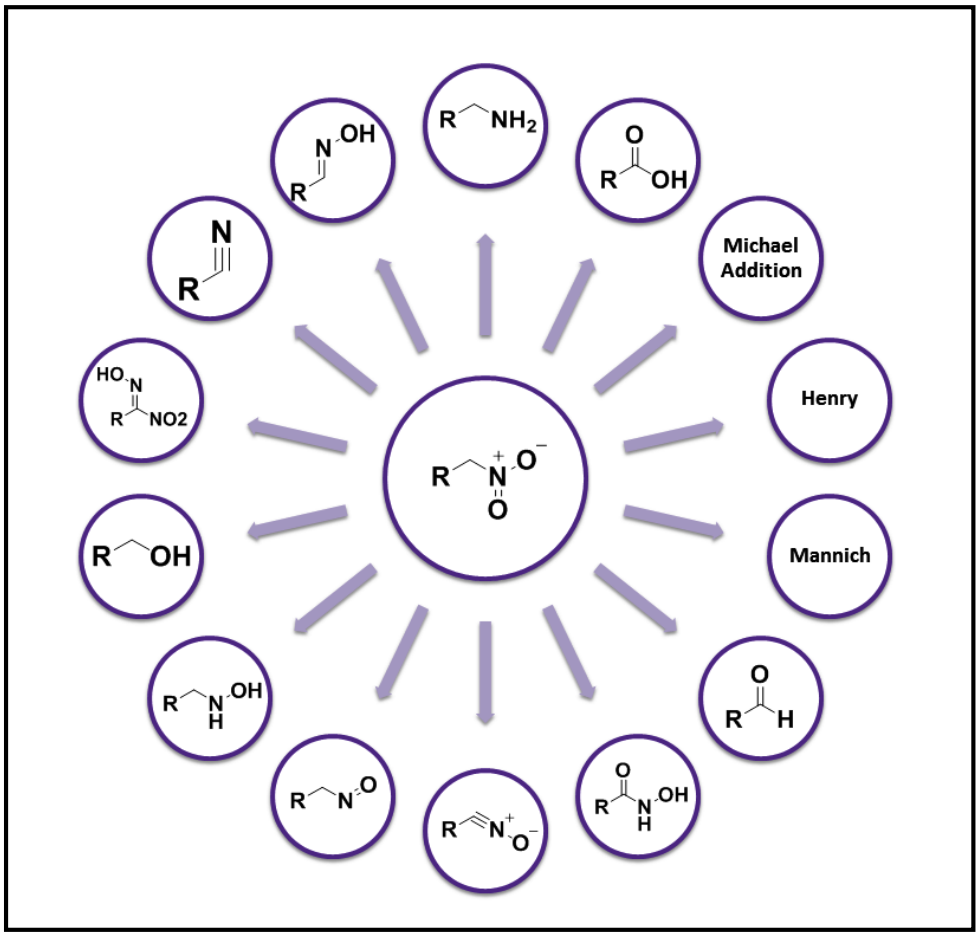


Figure 2.2: Diverse functional group transformations of nitroalkanes

Recently, we demonstrated Michael addition of CH_3NO_2 to unsaturated amino acid to derive γ-peptide foldamers and their reactivity[23]. Nitroalkanes are served as versatile

intermediates for the transformation of diverse functional groups such as amines, carboxylic acids, aldehydes, ketones and cyclised products[24] (**Figure 2.2**). In spite by the versatile properties of nitroalkanes, we sought to investigate whether the nitroalkanes can be explored as orthogonal to the azide-alkyne click reactions on peptides. Herein we are reporting an efficient strategy of 1, 3 dipolar nitroalkane-alkyne cycloaddition reaction in the presence of azide and alkynes in solution as well as solid phase.

3. METHODS

3.1 CHEMICALS

All the amino acids, phenyl acetylene, AIBN, Bu_3SnH, activated Pd/C, Triphenylphosphine were purchased from Sigma-Aldrich. The rink amide resin was bought from Novabiochem. HBTU, HOBt, EDC.HCl, TFA, DIPEA, IBC-Cl, Cbz-Cl, Triethylamine, Sodium nitrite was obtained from Spectrochem. DCM, DMF, NMP, DMSO, toluene, THF were purchased from Spectrochem and used without purification. MeOH was distilled before use. Column chromatography was performed on silica gel (120-200 mesh).

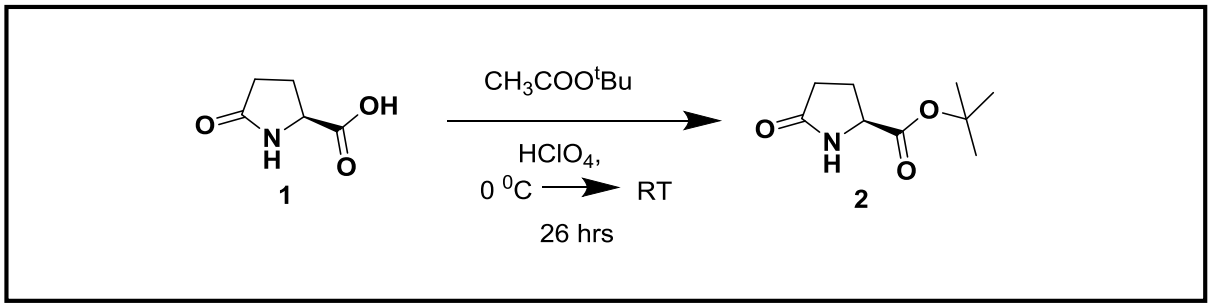
3.2 INSTRUMENTATION

Final peptides were purified by reverse phase HPLC (C_{18} column, MeOH/H_2O 70:30-95:5 as a gradient with flow rate 2.0 mL/min).). ^1H NMR and ^{13}C NMR spectra were recorded on JEOL 400 MHz, 100MHz respectively using the residual solvent signal as internal standards ($CDCl_3$ and DMSO). Chemical shifts (δ) reported in parts per million (ppm) and coupling constants (J) reported in Hz. Mass of pure peptides was confirmed by MALDI/TOF (4800 Plus from Applied Biosystems).

3.3 GENERAL PROCEDURES

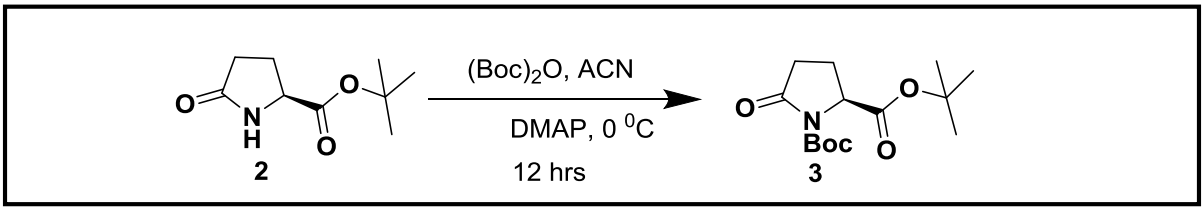
3.3.1 Synthesis of tert-butyl (S)-5-oxopyrrolidine-2-carboxylate: L-Pyroglutamic acid (3.25 g, 25 mmol) was taken in a round bottom (RB) flask. To this 50 ml, tertiary butyl acetate was added. Then it was cooled to 0 ºC. After that 2.5 ml, perchloric acid was added dropwise to the reaction mixture. The reaction was kept for 26 hrs at room

temperature. After the completion of the reaction, $HClO_4$ was neutralized with 10 % $NaHCO_3$. Then the compound was extracted from the aqueous layer with DCM (3 X 30 ml). Finally, the DCM layer was washed with brine solution and dried over anhydrous $MgSO_4$. Then the combined DCM layer was evaporated on rota evaporator to give solid white colour compound 2. Yield: 4.16 g (90%).



3.3.2 Synthesis of di-tert-butyl (S)-5-oxopyrrolidine-1,2-dicarboxylate

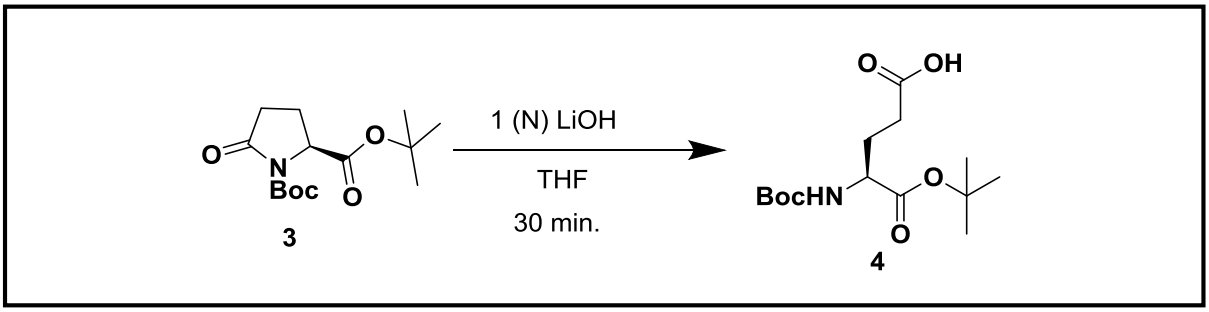
After dissolving 4.16 g (22.5 mmol) compound 2 in 35 ml acetonitrile, DMAP (274 mg, 2.25 mmol) was added to the solution. Then the reaction mixture was cooled to 0 ^{0}C. Boc anhydride (7.74 ml, 33.75 mmol) was dissolved in 35 ml acetonitrile and added dropwise to the reaction mixture. Then the reaction was kept for 12 hrs. After completion of the reaction, the acetonitrile solvent was evaporated on rota evaporator and the compound was extracted with ethyl acetate 3 times (50 ml X 3). Then the organic layer was washed with brine solution (35 ml X 3) and dried over anhydrous Na_2SO_4. Then the combined organic layer was evaporated in rota evaporator to give viscous compound 3. Yield: 5.78 g (90%).



3.3.3 Synthesis of (S)-5-(tert-butoxy)-4-((tert-butoxycarbonyl)amino)-5-oxopentanoic acid

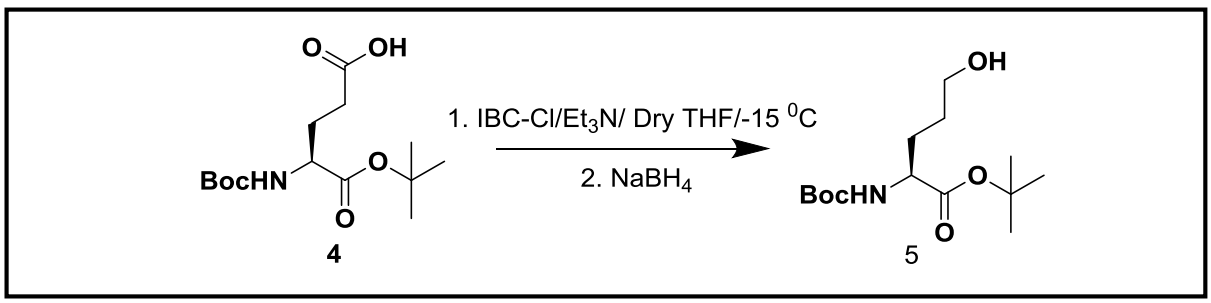
Compound 3 (5.78 g, 20.25 mmol) was dissolved in THF. Then 35 ml 1(N) lithium hydroxide solution was added dropwise to the reaction mixture and kept for 30 min. After the completion of the reaction, THF was evaporated on rota evaporator and the aqueous layer was neutralized with 10% HCl solution. Then the compound was

extracted with ethyl acetate (3 X 50 ml). After that, the organic layer was washed with brine solution (3 X 35 ml) and dried over Na_2SO_4. Then the combined organic layer was evaporated in rota evaporator to give solid compound 4.Yield: 5.41 g (88%).



3.3.4 Synthesis of tert-butyl (S)-2-((tert-butoxycarbonyl)amino)-5-hydroxypentanoate

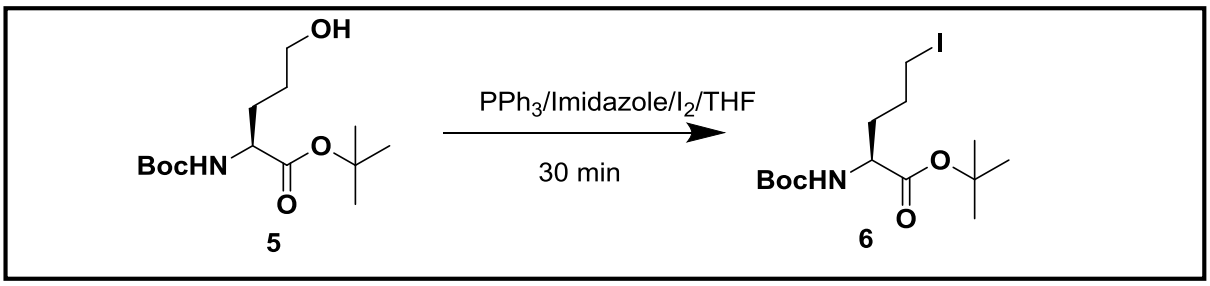
Compound 4 (5.41 g, 17.82 mmol) was dissolved in 30 ml Dry THF. Then the reaction mixture was cooled to -15 ^{0}C by salt ice combination and triethylamine (1 eq.) was added to this. After 5 min, isobutyl chloroformate (1.5 eq.) was added dropwise under nitrogen atmosphere and kept it for 30 min. Then $NaBH_4$ (3.38g, 89.1 mmol) was added to the reaction mixture and kept it for another 30 min. After 30 min, the completion of the reaction was monitored by the TLC. Then the THF was evaporated on rota evaporator and the excess $NaBH_4$ was quenched by 10% HCl solution. After that, it was extracted with ethyl acetate (3 X 50 ml). Then the organic layer washed with 10% HCl solution (3 X 50 ml), 10% Na_2CO_3 (3 X 50 ml) and brine solution (3 X 50 ml). Then dried over anhydrous Na_2SO_4. The impure compound was purified by column chromatography in hexane/EA system to get the pure compound. Yield: 4.13g (80%).



^{1}H NMR ($CDCl_3$, 400 MHz) δ_{ppm} 5.20 (d, J = 8 Hz, 1H), 4.15 (m, 1H), 3.63 (m, 2H), 2.49 (bs, 1H), 1.82 (m, 2H), 1.59 (m, 2H), 1.43 (s, 9H), 1.40 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ_{ppm} 172.06, 155.67, 82.03, 79.84, 62.12, 53.72, 29.71, 28.41, 28.07 HR-MS m/z calculated value for $C_{14}H_{27}NO_5$ is $[M+Na]^+$ 312.1786 and observed 312.1793.

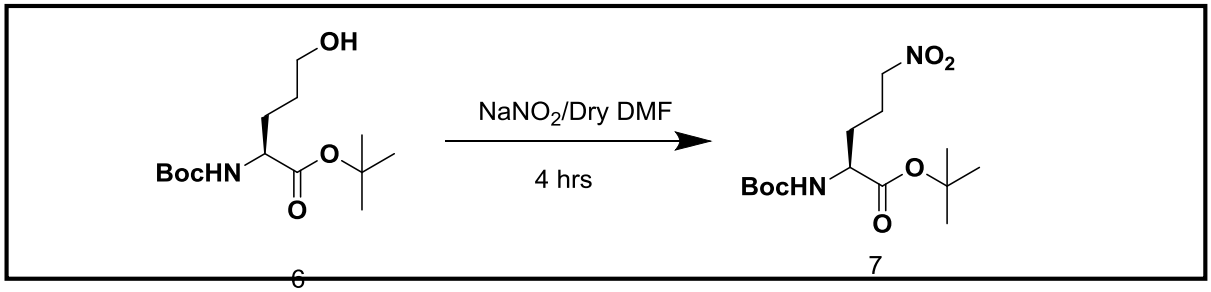
3.3.5 Synthesis of tert-butyl (S)-2-((tert-butoxycarbonyl)amino)-5-iodopentanoate

Compound 5 (4.13 g, 14.25 mmol) was dissolved in 30 ml Dry THF under a nitrogen atmosphere. Then (1.5 eq., 5.6 g, 21.38 mmol) triphenyl phosphine, imidazole (1.5 eq., 1.4 g, 21.38mmol) and Solid iodine (1.5 eq, 5.4 g, 21.38 mmol) were added respectively. After 30 min, the completion of the reaction was monitored by TLC. After that, the THF was evaporated under reduced pressure and extracted with ethyl acetate (3 X 50 ml). The combined organic layer was washed with 10% $Na_2S_2O_3$ solution (3 X 50 ml) and brine solution (3 X 50 ml). Then the organic layer was dried over anhydrous Na_2SO_4. After that, the organic layer was evaporated under reduced pressure and purified with column chromatography by hexane /EA system. Yield: 3.98 g (70%).



3.3.6 Synthesis of tert-butyl (S)-2-((tert-butoxycarbonyl)amino)-5-nitropentanoate

Compound 6 (3.98 g, 9.98 mmol) was dissolved in 5 ml Dry DMF. To the solution, $NaNO_2$ (2.5 eq, 1.6 g, 24.95 mmol) was added and kept for 4 hrs. The completion of the reaction was monitored by TLC. Then DMF was evaporated, and the compound was dissolved in ethyl acetate. The organic layer was washed with 10% $Na_2S_2O_3$ solution, brine solution and dried over anhydrous Na_2SO_4. Then the combined organic layer was evaporated and the compound the purified by column chromatography using hexane/EA solvent system. Yield: 1.27 g (40%).

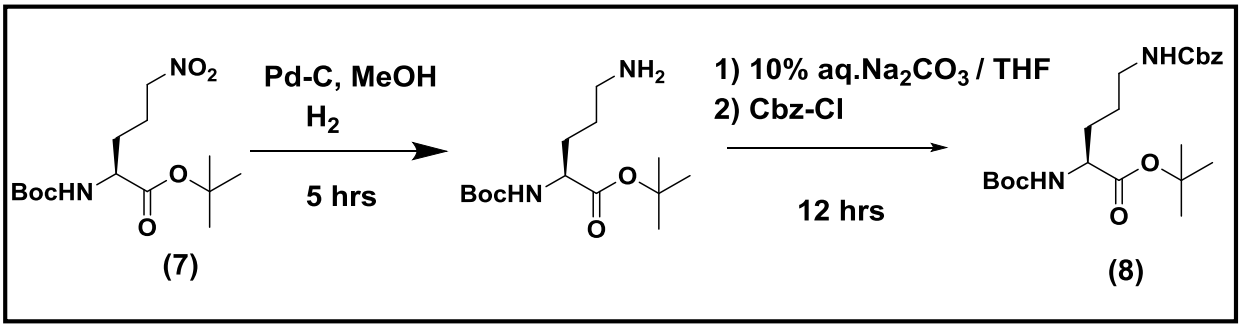


^{1}H NMR ($CDCl_3$, 400 MHz) δ_{ppm} 5.13(d, J = 8 Hz, 1H), 4.42 (t, J = 8 Hz, 3H), 4.42 (m, 1H), 2.40 (m, 2H), 1.90 (m, 1H), 1.63 (m, 1H), 1.47 (s, 9H), 1.44 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ_{ppm} 171.18, 155.52, 82.72, 80.17, 75.05, 53.17, 29.97, 28.44, 28.10, 23.29. HR-MS m/z calculated value for $C_{14}H_{26}N_2O_6$ is $[M+Na]^+$ 341.1688 and observed 341.1690.

3.3.7 Synthesis of tert-butyl (S)-5-(((benzyloxy)carbonyl)amino)-2-((tert-butoxycarbonyl)amino)pentanoate

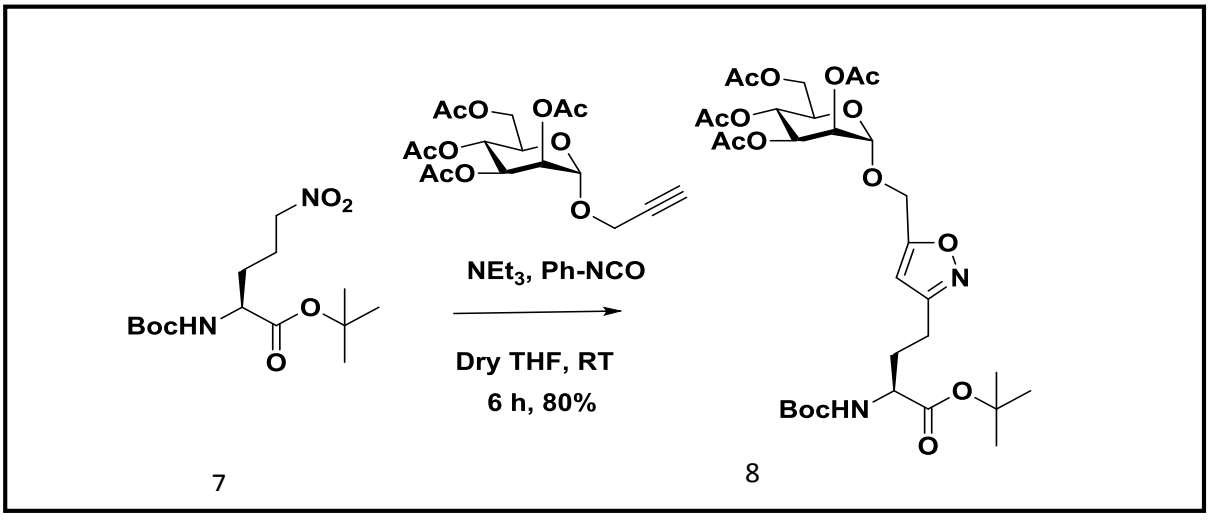
Compound 7 (0.478 g, 1.5 mmol) and 10% Pd-C was dissolved in 30 ml EtOH in an RB contained magnetic bar and stirred for 8 hrs. under H_2 atmosphere provided by the balloon. The progress of the reaction was monitored by TLC. After completion of the reaction, the Pd-C was removed by sintered funnel, and the EtOH was evaporated under reduced pressure to get the free amine. Then the compound was dissolved in 10 ml THF and the pH was adjusted around ~10 by slow addition of 10% Na_2CO_3 solution. Then Cbz-Cl in 10 ml THF was added slowly at 0 ^{0}C and the reaction mixture was kept for 12 hrs. After completion of the reaction, reaction mixture acidified with 2N HCl and the aqueous layer extracted with EtOAc (3x 30 ml). The combined organic layer was washed with brine and dried over anhydrous Na_2SO_4. The crude product was purified by silica gel chromatography to get 65% (0.98 mmol) pure product.

^{1}H NMR (400 MHz, Chloroform-*d*) δ*ppm* 7.35 – 7.30 (m, 5H), 5.09 (s, 3H), 4.90 (s,1H), 4.24 – 4.11 (m, 1H), 3.21 (dd, *J* = 12.4, 6.2 Hz, 1H), 1.68 (s, 2H), 1.58 (ddt, *J* = 13.9, 10.5, 7.1 Hz, 2H), 1.45 (s, 9H), 1.43 (s, 9H). **^{13}C NMR** (100 MHz, Chloroform-*d*) δ*ppm* 171.67 , 156.39 , 155.41 , 136.57 , 82.08 , 79.78 , 66.65 , 53.53 , 40.61 , 30.32 , 29.68 (d, *J* = 3.9 Hz), 28.33 , 28.00 , 25.75 .



3.3.8 Synthesis of (2R,3R,4S,5S,6S)-2-(acetoxymethyl)-6-((3-((S)-4-(tert-butoxy)-3-((tert-butoxycarbonyl)amino)-4-oxobutyl)isoxazol-5-yl)methoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate

To the dry THF (2 ml) solution of compound 7 (0.478 g, 1.5 mmol), propargylated penta acetate sugar (2.8 g, 7.5 mmol) and phenyl isocyanate (0.815 ml, 7.5 mmol) were added. Triethylamine (1.04 ml, 7.5 mmol) was added to the above solution dropwise at room temperature and stir the reaction for 6 hrs. After completion of the reaction, urea (by-product) was filtered out through celite using EtOAc (50 ml). The filtrate was washed with 5% HCl (3 × 20 ml), 10% Na_2CO_3 (3 × 20 ml), brine solution (3 × 20 ml) and dried over anhydrous Na_2SO_4. The crude product was purified by silica gel chromatography to get 80% (1.2 mmol) pure product.

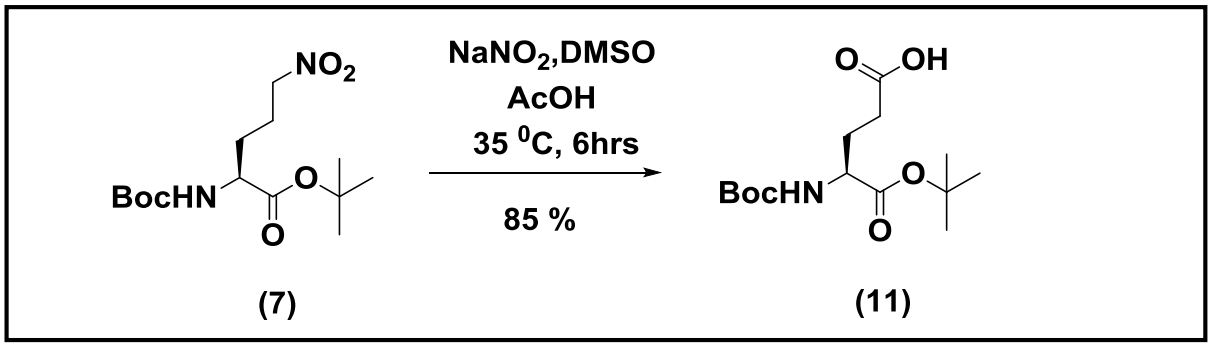


^{1}H NMR ($CDCl_3$, 400 MHz) δ_{ppm} 6.16 (s, 1H), 5.27 (m, 2H), 5.23 (m, 1H), 5.17 (d, J = 8 Hz, 1H), 4.90 (s, 1H), 4.65 (q, J = 12 Hz, 2H), 4.23-4.27 (m, 2H), 3.99-4.09 (m, 3H), 2.64-2.77 (m, 2H), 2.12 (s, 3H), 2.07 (s, 3H), 2.00 (s, 3H), 1.95 (s, 3H), 1.44 (s, 9H), 1.41 (s, 9H) ; **^{13}C NMR** (100 MHz, $CDCl_3$) δ_{ppm} 171.33, 170.65, 169.99, 169.88,

169.74, 167.23, 163.10, 103.71, 97.33, 82.73, 79.89, 69.28, 69.08, 68.89, 65.94, 62.31, 60.11, 53.66, 31.47, 28.37, 28.05, 22.38, 20.88, 20.78, 20.73, 20.69**. HR-MS** m/z calculated value for $C_{31}H_{46}N_2O_{15}$ is $[M+H]^+$ 687.2976 and observed 687.2991.

3.3.9 Synthesis of (S)-5-(tert-butoxy)-4-((tert-butoxycarbonyl)amino)-5-oxopentanoic acid

Compound 7 (0.478 g, 1.5 mmol) was dissolved in DMSO (3 ml). To this solution $NaNO_2$ (206.7 mg, 3 mmol) and 0.5 ml acetic acid was added. Then the reaction mixture was kept at 35 ^{0}C for 6 hrs. After completion of the reaction, the reaction mixture was extracted with EtOAc (3x 25 ml) and washed with brine solution (3 X 25 ml). Then the combined organic layer dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to yield 85 % (1.27 mmol) of the acid.



(7) **(11)**

^{1}H NMR ($CDCl_3$, 400 MHz) δ_{ppm} 12.12 (s, 1H), 7.13 (d, J = 8 Hz, 1H), 3.79-3.84 (m, 1H), 2.24-2.29 (m, 2H), 1.83-1.91 (m, 1H), 1.66-1.76 (m, 1H), 1.39 (s, 9H), 1.38 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ_{ppm} 173.74, 171.52, 155.53, 80.33, 78.08, 53.55, 30.03, 28.18, 27.63, 25.91. **HR-MS** m/z calculated value for $C_{14}H_{25}NO_6$ is $[M+Na]^+$ 326.1579 and observed 326.1580.

3.3.10 Nitro to acid transformation (P1 to P4): 25 mg resin of peptide **P2** was taken in 25 ml round bottom (RB) flask. To this 100 mg of $NaNO_2$, 0.5 ml DMSO and 0.1 ml acetic acid were added. Then the reaction mixture was kept 110 ^{0}C for 10 hrs. After the completion of reaction the resin was filtered by sintered funnel and the peptide was cleaved from the resin by cocktail mixture TFA/TIPS/H_2O. After 2 hrs, the cleaved peptide solution was evaporated under reduced pressure and precipitated out by cold diethyl ether. Then the crude product was purified by reverse phase HPLC on C_{18}

column by MeOH/H_2O system. MALDI TOF/TOF- *m/z* calculated for $C_{33}H_{58}N_8O_{10}$ $[M+Na]^+$ 749.41 , observed 749.33.

3.3.11 Transformation of P1 to P5: resin (25 mg, 0.015 mmol) was taken in 10 ml RB flask. To this 3 ml Dry THF was added under N_2 atmosphere. Then it was cooled to 0 ^{0}C. After phenyl acetylene (110 µl, 1mmol) was added to it, followed by triethylamine (139 µl, 1mmol), and phenyl isocyanate (112 µl, 1 mmol) at 0 ^{0}C. The reaction mixture was continued to stir for about 12 hrs. After completion of the reaction, the resin was filtered through sintered funnel. Then peptide was cleaved from the resin by cocktail mixture of TFA/TIPS/Phenol. After 2 hrs. the resin was filtered through sintered funnel and the filtrate was evaporated under reduced pressure and precipitated by adding cold diethyl ether. The precipitate was dissolved in MeOH and purified by reverse phase HPLC on C_{18} column by MeOH/H_2O system. MALDI TOF/TOF- *m/z* calculated for $C_{41}H_{63}N_9O_9$ $[M+Na]^+$ 848.46 and observed 848.95.

3.3.12 Cycloaddition reaction with Nitro functionality (P2 to P6): Resin (25 mg, 0.015 mmol) was taken in 10 ml RB flask. To this 3 ml, Dry THF was added under N_2 atmosphere. Then it was cooled to 0 ^{0}C. After that Cbz protected propargylamine (189 mg, 1 mmol) was added, followed by triethylamine (139 µl, 1 mmol), and phenyl isocyanate (112 µl, 1 mmol) at 0 ^{0}C. Then the reaction mixture was kept for 12 hrs. After completion of the reaction, the resin was filtered through sintered funnel.

Then peptide was cleaved from the resin by a cocktail mixture of TFA/TIPS/Phenol. After 2 hrs the resin was filtered through sintered funnel and the filtrate was evaporated on rota evaporator and precipitated by adding cold diethyl ether. The precipitate was dissolved in MeOH and purified by reverse phase HPLC on a C_{18} column by MeOH/H_2O system.). MALDI TOF/TOF- *m/z* calculated for $C_{52}H_{83}F_5N_8O_{11}$ $[M+Na]^+$ 1232.71 and observed 1237.35.

3.3.13 Click reaction with azide functionality (P2 to P7): The resin of protected heptapeptide (25 mg, 0.015 mmol) was suspended in 2 mL of acetonitrile/ water/ DIEA/ pyridine (4:4:1:0.5) mixture. To this solution, phenyl acetylene (109.8 µl, 1 mmol) was added, followed by a catalytic amount of CuI. The reaction was stirred overnight at room temperature; the solution was filtered and washed with 5% HCl, an excess of DMF and dichloromethane. The peptide was cleaved from the resin by using a cocktail mixture of 95:2:2:1 TFA/ ethylene dithiol/ water/ thioanisole and purified by reverse

HPLC using a C_{18} column by MeOH/H_2O system. MALDI TOF/TOF- *m/z* calculated for $C_{52}H_{86}N_{12}O_{10}$ $[M+Na]^+$ 1061.65 and observed 1061.40.

3.3.14 Cycloaddition reaction with Nitro functionality in the presence of click adduct product (P7 to P8): Resin (25 mg, 0.015 mmol) was taken in 10 ml RB flask. To this 3 ml, Dry THF was added under N_2 atmosphere. Then it was cooled to 0 ^{0}C. After that Cbz protected propargylamine (189 mg, 1 mmol) was added to it. After that triethylamine (139 µl, 1mmol), and phenyl isocyanate (112 µl, 1mmol) at 0 ^{0}C. Then the reaction mixture was kept for 12 hrs. After completion of the reaction the resin was filtered through sintered funnel. Then peptide was cleaved from the resin by cocktail mixture of TFA/TIPS/Phenol. After 2 hrs, the resin was filtered through sintered funnel and the filtrate was evaporated on rota evaporator and precipitated by adding cold diethyl ether. The precipitate was dissolved in MeOH and purified by reverse phase HPLC on C_{18} column by MeOH/H_2O system. MALDI TOF/TOF- *m/z* calculated for $C_{55}H_{89}N_{13}O_{11}$ $[M+K]^+$ 1146.6442 and observed 1144.25.

3.3.15 Synthesis of Fmoc-Lys (N_3)-OH: Fmoc-Lys (N_3)-OH was synthesised by using the protocol already available in the literature [25]. Initially, Boc-protected L-Lysine (2.5 g, 10.15 mmol) was dissolved in MeOH (50 ml) .To this solution K_2CO_3 (2.23 g, 18.09 mmol, 1.8 eq.), copper(II) sulfate pentahydrate (253 mg, 1.01 mmol, 0.1eq.) and imidazole-1-sulfonyl azide HCl salt (3.18 g, 15.17 mmol, 1.5 eq.) were added. After stirring for 16 h at r.t, half of the solvent was evaporated under vacuum before the solution was acidified with2M HCl. The mixture was extracted with DCM (3×100 ml), and the organic layer was dried over Na_2SO_4 and concentrated in vacuum. The crude product was then dissolved in DCM (100 ml) and extracted with 5% aq. $NaHCO_3$ (3×100 ml). The combined aqueous layers were washed twice with DCM (100 ml), acidified with 1M HCl and extracted with DCM (3×100 ml). The organic layer was dried over Na_2SO_4 and concentrated in vacuum to afford the product as a colorless oil. The Boc protected L-Lys (N_3)-OH was used for the solution phase peptide synthesis. For the solid phase peptide synthesis, the Boc group was deprotected by TFA/DCM, and it was further protected with Fmoc and purified by column chromatography to get the final Fmoc protected L-Lys(N_3)-OH.

3.3.16 Synthesis of the tetrapeptide P3: The tetrapeptide **P3** was synthesized by the 1+2+1 strategy. First Boc-protected valine was coupled with the methyl ester of

leucine with EDC.HCl/HOBt as a coupling reagent and DIPEA as a base. Then the Boc group of dipeptide BocNH-Val-Leu-OMe was deprotected by TFA/DCM in 0 ^{0}C. And coupled with BocNH-Lys (N_3)-OH by EDC.HCl/HOBt as a coupling reagent and DIPEA as a base in dry DMF. After that, the tripeptide BocNH-Lys (N_3)-Val-Leu-OMe was coupled with the methyl ester of alpha nitro amino acid by EDC.HCl/HOBt as a coupling reagent and DIPEA as a base in dry DMF to get the **P3**.The tetrapeptide **P3** was further purified by silica gel column chromatography by EA/Hexane.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.16 (d, *J* = 8.1 Hz, 1H), 6.97 (d, *J* = 8.4 Hz, 1H), 6.62 (d, *J* = 6.8 Hz, 1H), 5.01 (d, *J* = 4.2 Hz, 1H), 4.60 (tt, *J* = 8.4, 4.4 Hz, 2H), 4.43 (t, *J* = 6.9 Hz, 2H), 4.21 (dd, *J* = 6.8, 4.5 Hz, 1H), 3.94 (dt, *J* = 7.8, 4.9 Hz, 1H), 3.77 (s, 3H), 3.33 (t, *J* = 6.6 Hz, 2H), 2.40 (dd, *J* = 12.3, 6.4 Hz, 1H), 2.18 – 2.04 (m, 2H), 1.85 (m, *J* = 12.4, 9.6, 5.4 Hz, 3H), 1.69 (s, 9H), 1.48 (s, 9H), 1.01 (d, *J* = 6.9 Hz, 3H), 0.94 (dt, *J* = 6.4, 4.8 Hz, 10H). MALDI TOF/TOF- *m/z* calculated for $C_{28}H_{50}N_8O_9$ $[M+K]^+$ 681.33 and observed 681.10.

3.3.17 Cycloaddition reaction with Nitro functionality (P3 to P9): (160 mg, 0.25 mmol) Tetrapeptide **P3** was dissolved in Dry THF under N_2 atmosphere and cooled to 0 ^{0}C. To this solution (236 mg, 1.25 mmol) of Cbz protected propargylamine was added. Then to this solution (135 µl, 1.25 mmol) phenyl isocyanate and (174 µl, 1.25 mmol), triethylamine (173 µl, 1.25 mmol) was added. The reaction mixture was kept for 12 hrs at room temperature. After completion of the reaction the urea was filtered through filter paper, and the THF was evaporated. The crude product was dissolved in MeOH, and the compound was purified by Reverse Phase HPLC on MeOH/H_2O system.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.34 (s, 4H), 7.02 (d, *J* = 8 Hz, 1H), 6.87 (d, *J* = 8.5 Hz, 1H), 6.60 (d, *J* = 6.8 Hz, 1H), 6.06 (s, 1H), 5.53 (s, 1H), 5.12 (s, 2H), 4.98 (d, *J* = 8 Hz, 1H), 4.52 (dd, *J* = 54.2, 5.4 Hz, 1H), 4.22 – 4.06 (m, 1H), 3.96-3.92 (m, 1H), 3.72 (s, 1H), 3.25 (t, *J* = 6.6 Hz, 1H), 2.70 (dt, *J* = 15.1, 8.3 Hz, 1H), 1.84 – 1.70 (m, 1H), 1.59 (s, 15H), 1.43 (s, 3H), 0.96 (d, *J* = 6.9 Hz, 1H), 0.92 – 0.84 (m, 4H). MALDI TOF/TOF- *m/z* calculated for $C_{39}H_{59}N_9O_{10}$ $[M+Na]^+$ 836.42 and observed 836.24.

3.3.18 Click reaction with azide functionality (P3 to P10): (160 mg, 0.25 mmol) Tetrapeptide **P3** was dissolved in 3 ml THF/H_2O (1:1) mixture. To this solution (53 µl, 0.50 mmol) phenyl acetylene was added. After that, to this solution, $CuSO_4$, $5H_2O$ (125

mg, 0.5 mmol) and Sodium ascorbate (50 mg, 0.25 mmol) was added. Then the reaction mixture was kept for 12 hrs at room temperature. After completion of the reaction mixture, the THF was evaporated and the compound was extracted with ethyl acetate (3 X 25 ml).Then the organic layer was washed with brine solution (3 X 25ml) and dried over Na_2SO_4. After that, the combined organic layer was evaporated by rota evaporator and dissolved in MeOH. Then the crude compound was purified by reverse phase HPLC on a C_{18} column by MeOH/H_2O system. MALDI TOF/TOF- *m/z* calculated for $C_{36}H_{56}N_8O_9$ $[M+Na]^+$ 767.40 and observed 767.53.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.05 – 7.65 (m, 1H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.37 – 7.28 (m, 1H), 7.17 (d, *J* = 8.0 Hz, 1H), 6.58 (d, *J* = 8 Hz, 1H), 5.13 (d, *J* = 3.6 Hz, 1H), 4.55 (dt, *J* = 8.4, 4.1 Hz, 1H), 4.41 (dt, *J* = 19.3, 6.8 Hz, 1H), 4.14 (dd, *J* = 4 Hz, 4 Hz, 1H), 3.89-3.85 (m, *J* = 1H), 3.72 (s, 3H), 2.41 – 2.27 (m, 1H), 2.11 – 1.95 (m, 4H), 1.87 – 1.80 (m, 2H), 1.70-1.62 (m, 8H), 1.42 (s, 9H), 0.93 (d, *J* = 4.0 Hz, 2H), 0.91 – 0.85 (m, 10H).

3.3.19 Transformation of P10 to P11: Click adduct tetrapeptide **P7** (111 mg, 0.15 mmol) was dissolved in Dry THF under N_2 atmosphere and cooled to 0 ^{0}C. To this solution (141 mg, 0.75 mmol) of Cbz protected propargylamine was added. Then to this solution (81 µl, 0.75 mmol) phenyl isocyanate and (104 µl, 0.75 mmol), triethylamine was added. The reaction mixture was kept for 12 hrs, at room temperature. After completion of the reaction the urea was filtered through filter paper and the THF was evaporated. The crude product was dissolved in MeOH and the compound was purified by Reverse Phase HPLC on MeOH/H_2O system.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.84 (dd, *J* = 9.6, 2.7 Hz, 1H), 7.43 (t, *J* = 7.5 Hz, 1H), 7.39 – 7.32 (m, 2H), 6.84 (d, *J* = 4 Hz, 1H), 6.67 (d, *J* = 8 Hz ,1H), 6.03 (d, *J* = 26.7 Hz, 1H), 5.31 (d, *J* = 7.4 Hz, 1H), 5.15 (s, 1H), 4.66 – 4.24 (m, 2H), 3.71 (s, 1H), 2.72-2.64 (m, 2H), 1.96-1.88 (m, 2H), 1.82-1.78 (m, 2H), 1.70-1.59 (m, 10 H), 1.42 (s, 9 H), 1.01 – 0.88 (m, 12H). MALDI TOF/TOF- m/z calculated for $C_{47}H_{65}N_9O_{10}$ $[M+Na]^+$ 938.47 and observed 938.26.

4. RESULTS and DISCUSSION

4.1 Synthesis of α-nitro amino acid

To introduce nitroalkane functionality on the peptide backbone, we synthesized a new nitroalkane functionalized amino acid shown in **Figure 4.1.1**. The schematic representation of synthetic procedure is shown in **Scheme 4.1.1.**

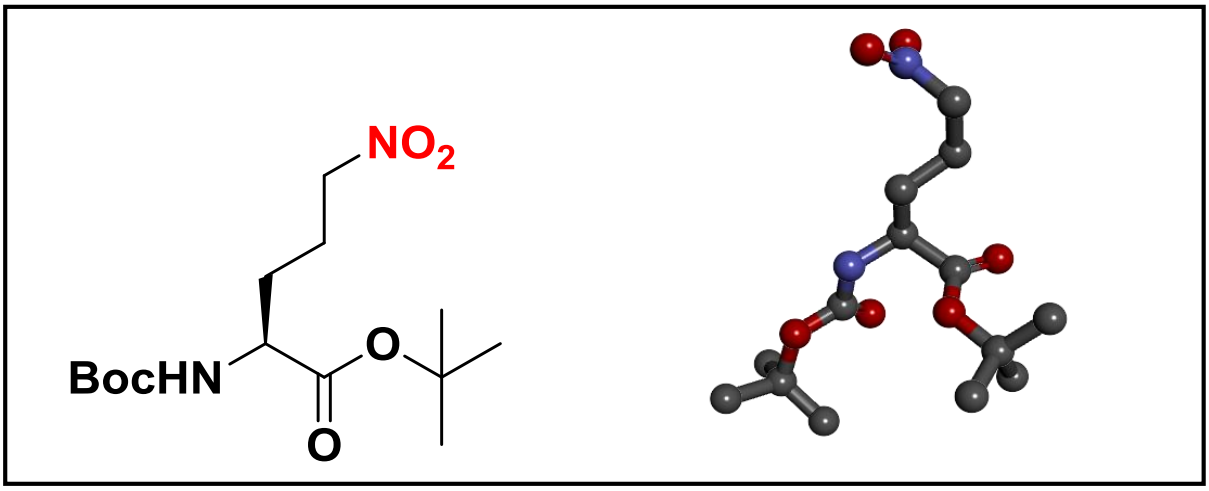
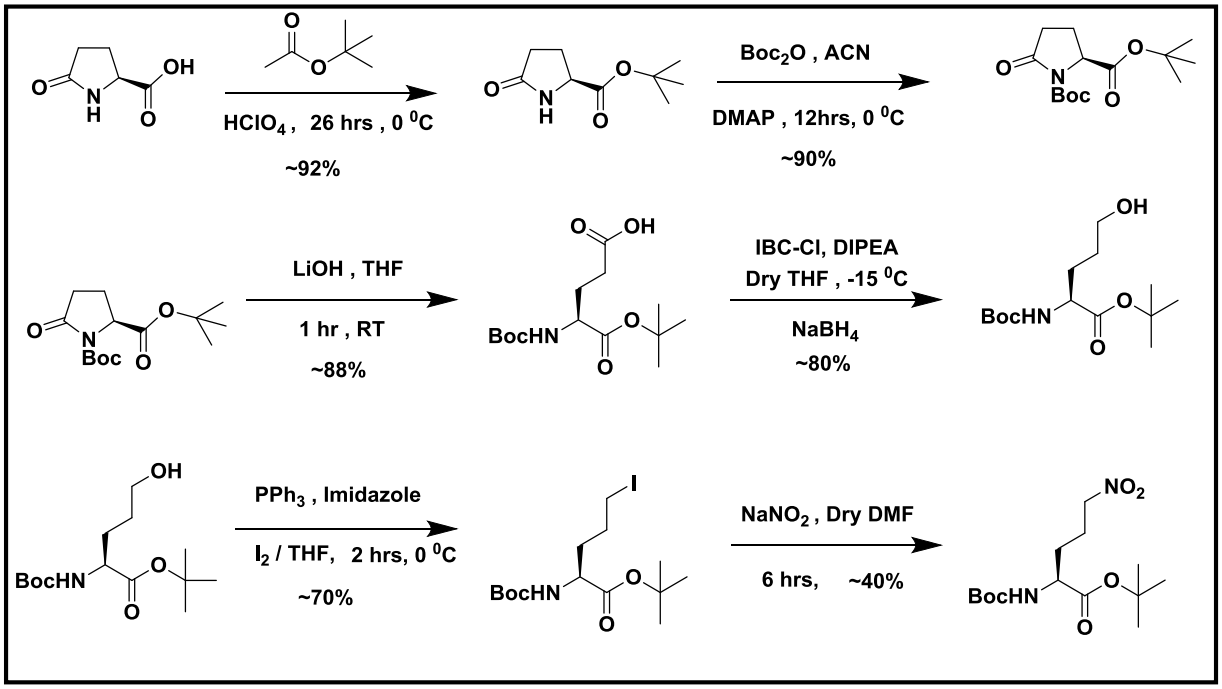


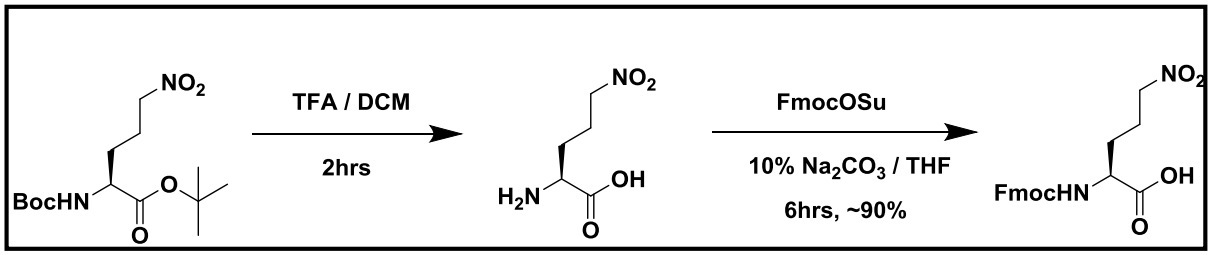
Figure 4.1.1: Boc-protected α-nitro amino acid and its X-ray crystal structure

For this synthesis, L-pyroglutamic acid was chosen as starting material since it is a commercially available natural amino acid and the conversion of this to corresponding alcohol is feasible by series of reactions. After the appropriate protection of carboxyl and amino group of L-pyroglutamic acid with tertiary butyl ester and tert-Butoxycarbonyl (Boc), the compound was subjected to hydrolysis with LiOH to obtain corresponding carboxylic acid. The conversion of carboxylic acid to alcohol was successfully done after the activation of acid with IBC-Cl in the presence of weak base DIPEA at -15 ^{0}C followed by mild $NaBH_4$ reduction. The formation of product was confirmed by using both ^{1}H NMR and X-ray crystallography. In order to convert alcohol to nitro functionality, we transformed –OH functionality to Iodo with the help of modified Appel reaction. Since this compound was unstable, we converted iodo to nitro by using $NaNO_2$ in dry DMF immediately after purification by column chromatography. The formation of desired product was confirmed with the help of ^{1}H NMR and X-ray crystallography after purification by column chromatography. The ^{1}H NMR spectra and ^{13}C NMR spectra of **AA 1** are shown in supplementary data.



Scheme 4.1.1: Synthesis of Boc protected α-nitro amino acid 1 (**AA 1**)

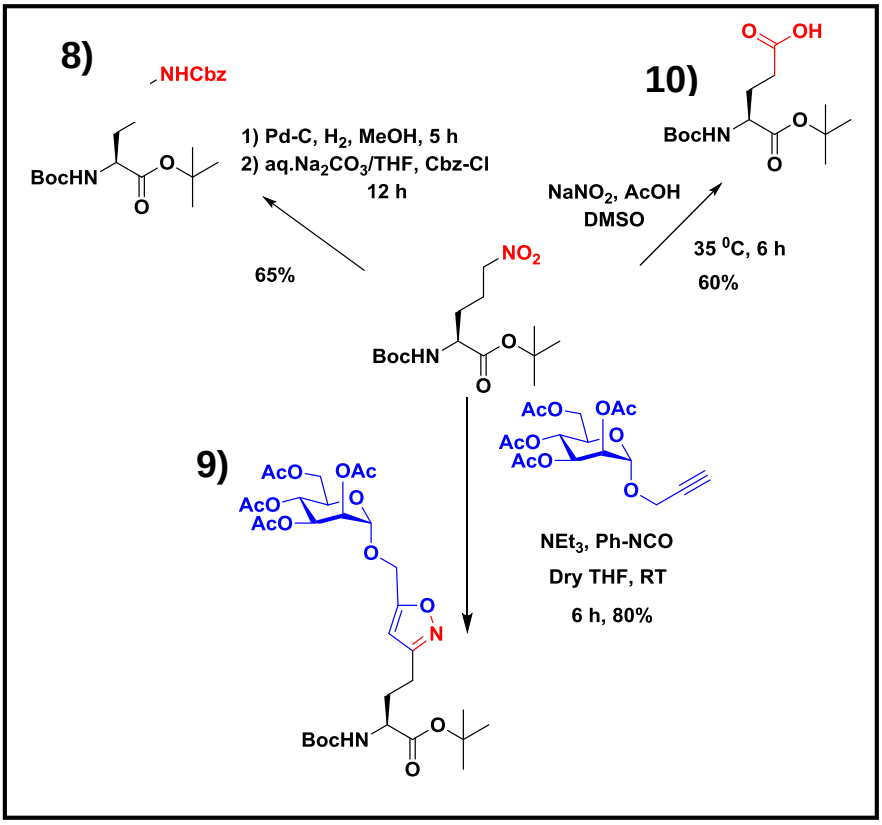
In order to make the new amino acid AA 1 compatible with Fmoc solid phase peptide synthesis, we have synthesized corresponding Fmoc protected derivative. The reactions are shown in **Scheme 4.1.2**.



Scheme 4.1.2: Synthesis of Fmoc protected α-nitro amino acid.

4.2 Organic transformations of α-nitro amino acid to different functionalities

To understand whether the nitroalkane amino acid can undergo a transformation similar to that of the other nitroalkanes[24], we subjected the amino acids to various chemical transformations such as amines[26], acids[27] and cycloaddition with olefins[28]. The schematic representation of all of these conversion reactions is shown in **Scheme 4.2.1**.



Scheme 4.2.1: Organic transformation of amino acid AA1 to compound **8**, **9** & **10**.

Initially, the Boc-protected nitro amino acid **AA 1** was subjected to Pd/C catalyzed hydrogenation reaction in methanol resulted in the formation of the corresponding amine. The free amine was then protected with Cbz-Cl and purified by column chromatography to obtain the pure product. The reactions are shown in **Scheme 4.2.2a**. The ^{1}H NMR and ^{13}C NMR spectra of the final product were shown in **Figure 4.2.2a & b**. Further, the conversion of a nitro group to carboxylic acid was successfully done through simple oxidation reaction with $NaNO_2$/AcOH mixture in DMSO (**Scheme 4.2.2b**). After purification, the pure product was subjected to ^{1}H NMR and ^{13}C NMR analysis. The NMR spectra are shown in **Figure 4.2.2c & d.** The 1, 3 dipolar cycloaddition reaction of **AA 1** with propargylated mannose resulted in the formation of the corresponding isoxazole with an excellent yield (**Scheme 4.2.1c**). The reaction was carried out in the presence of phenyl isocyanate and triethylamine to facilitate the conversion of the nitro group to nitrile oxide so that it can react with an olefin to give corresponding cyclised product under mild conditions [29]. The mechanism of formation of nitrile oxides from primary nitroalkane is shown in **Figure 4.2.1a**. The product was

isolated and analyzed by ^{1}H NMR and ^{13}C NMR spectroscopy. The NMR spectra were presented in **Figure 4.2.2 e & f**.

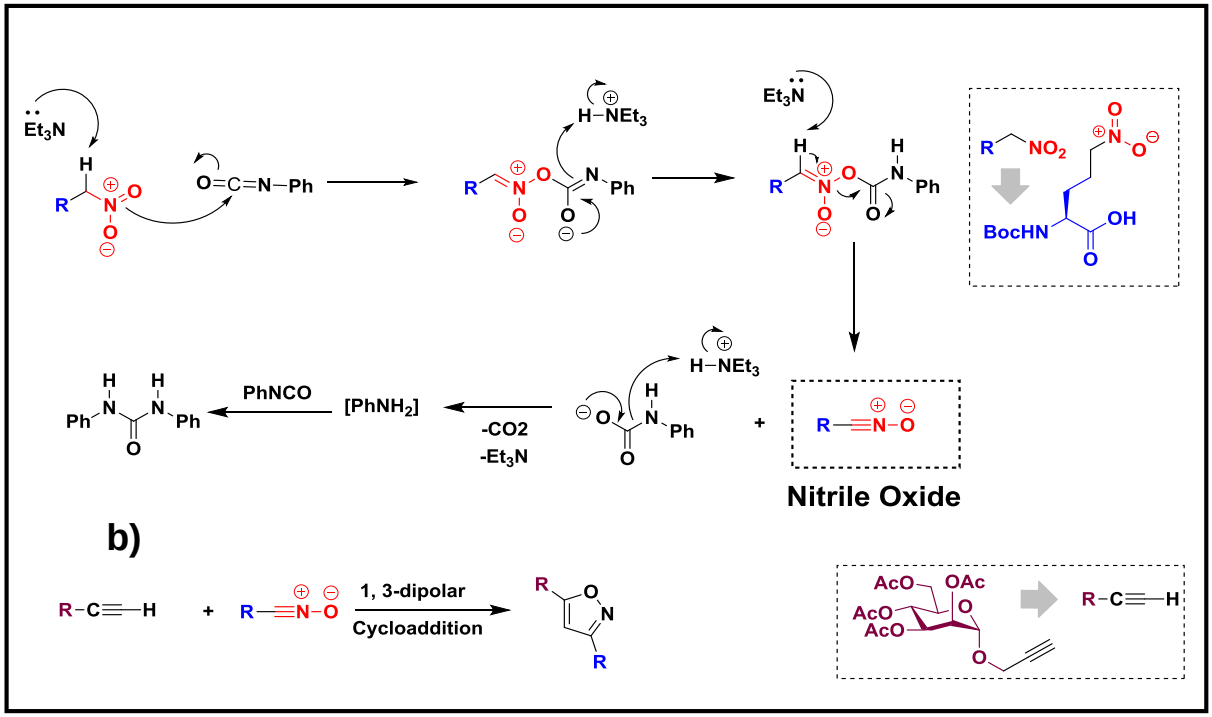
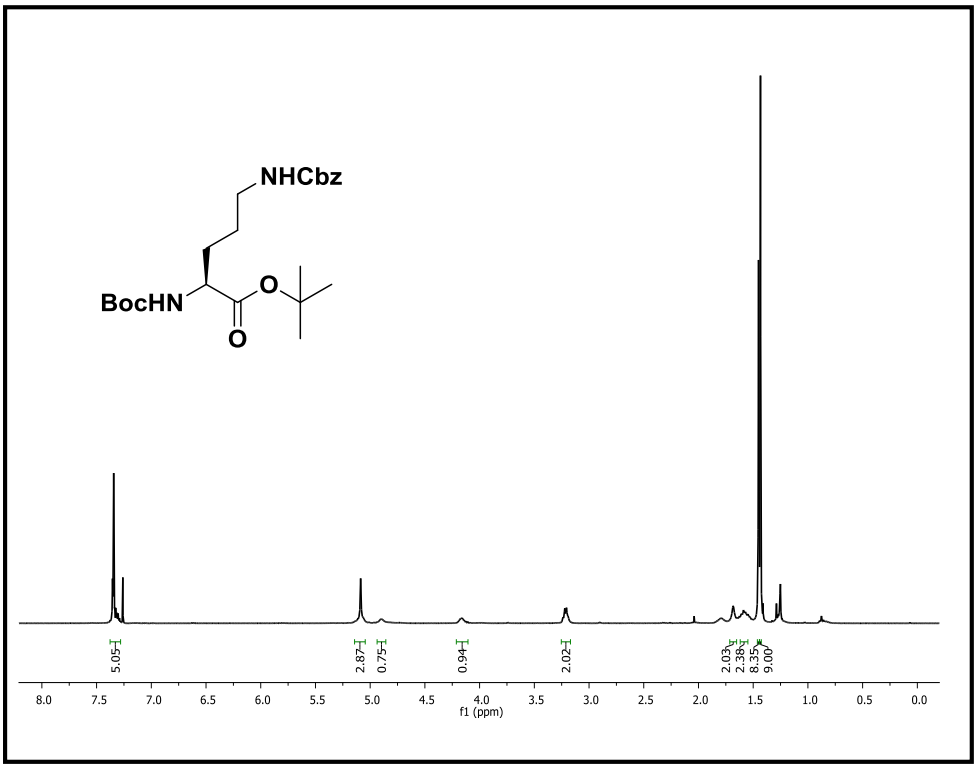
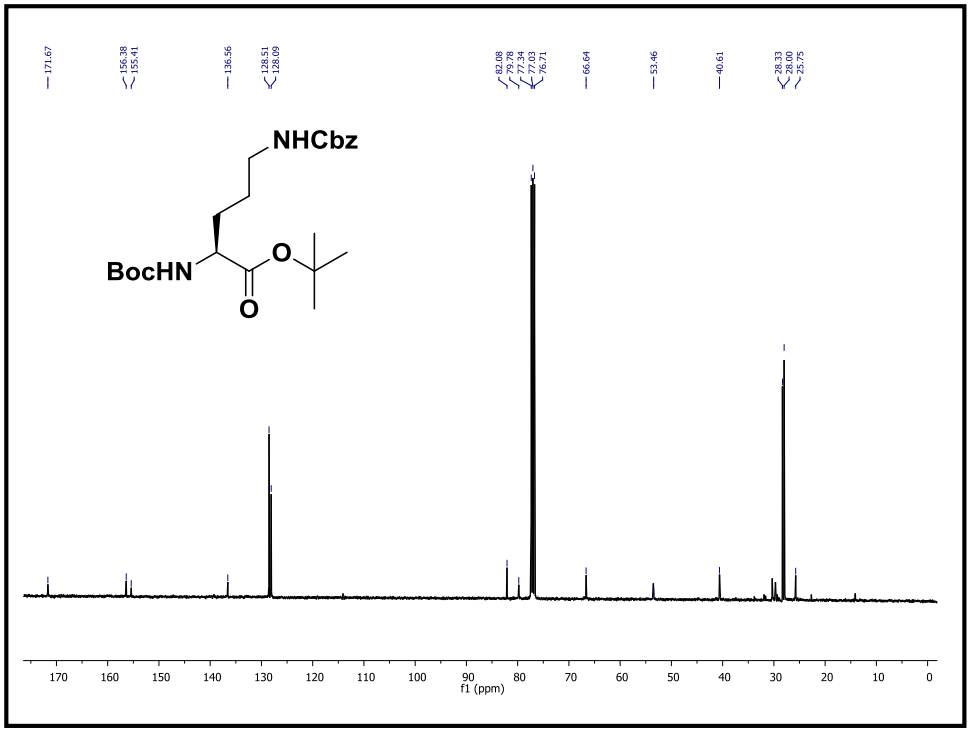


Figure 4.2.1: a) Mechanism of formation of nitrile oxides from nitroalkanes. b) Formation of isoxazole by nitroalkane-alkyne 1,3-dipolar cycloaddition reaction

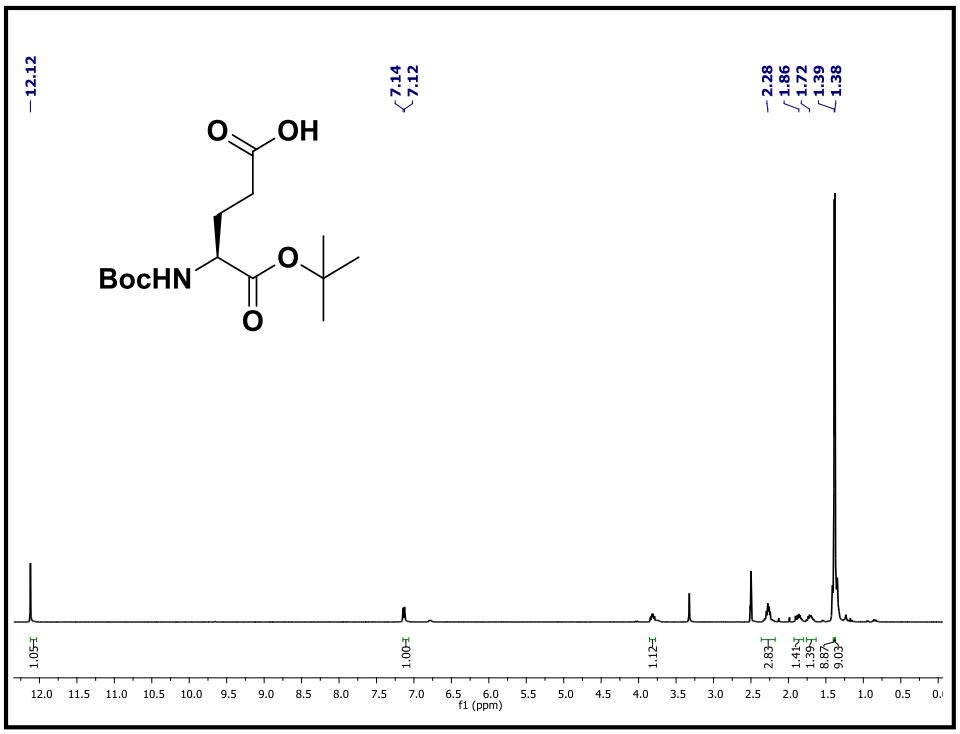
a)



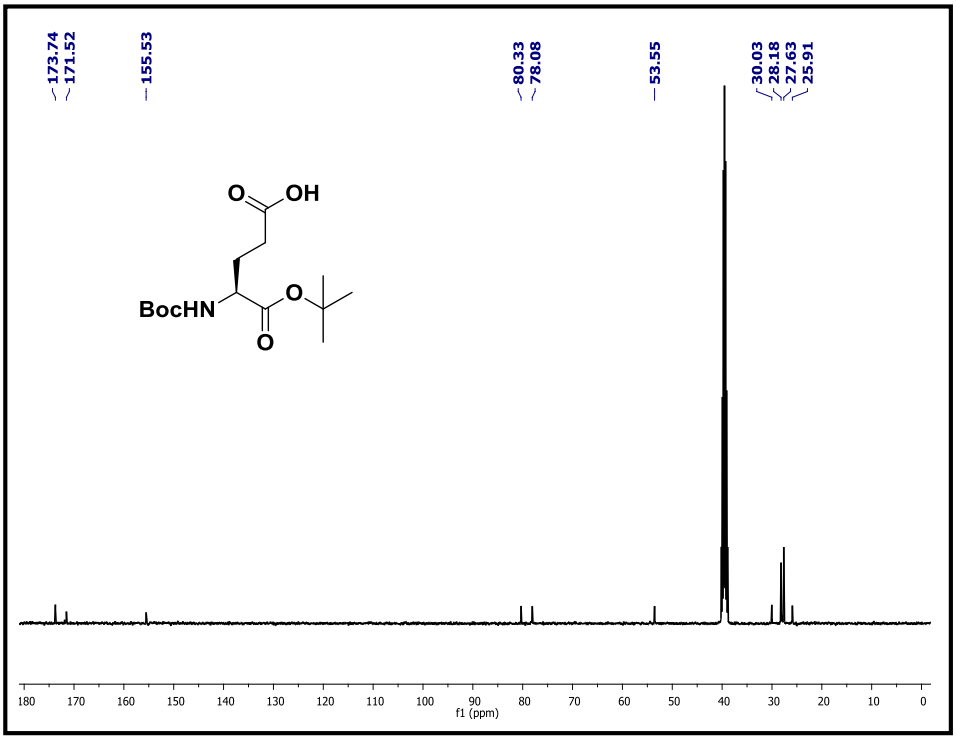
b)


171.67
156.38
155.41
136.56
128.51
128.09
82.08
79.78
77.34
77.03
76.71
66.64
53.46
40.61
28.33
28.00
25.75
NHCbz
BocHN
O
O
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

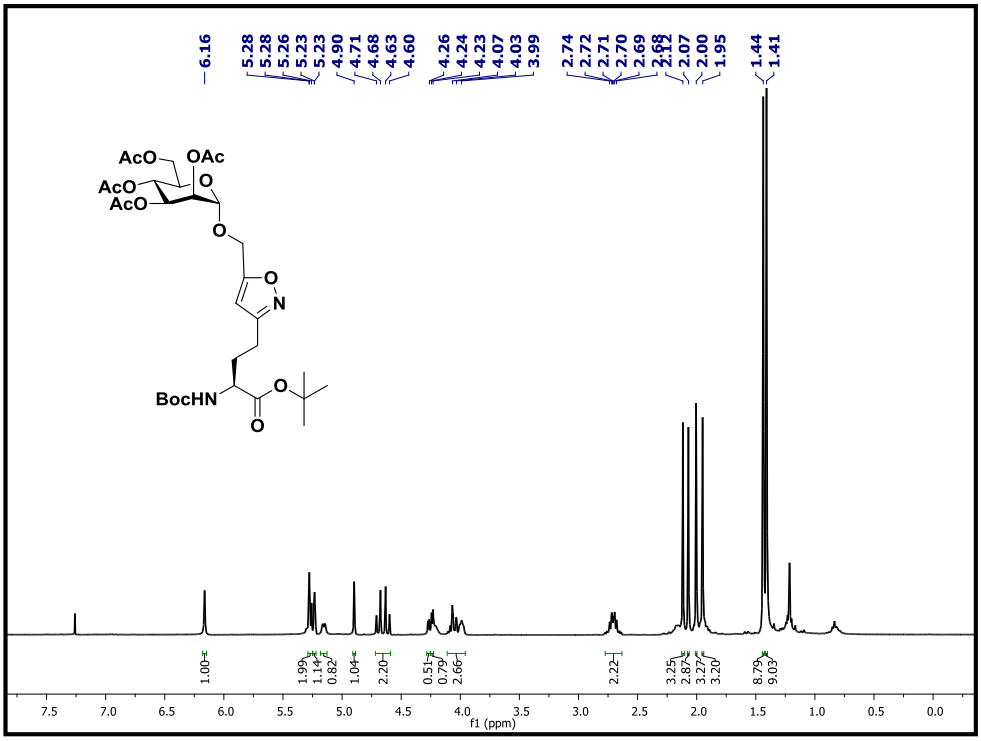
c)


12.12
7.14
7.12
2.28
1.86
1.72
1.39
1.38
O
OH
BocHN
O
O
1.05
1.00
1.12
2.83
1.41
1.39
8.87
9.03
12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)

d)


173.74
171.52
155.53
80.33
78.08
53.55
30.03
28.18
27.63
25.91
O
OH
BocHN
O
O
f1 (ppm)

e)


6.16
5.28
5.28
5.26
5.23
5.23
4.90
4.71
4.68
4.63
4.60
4.26
4.24
4.23
4.07
4.03
3.99
2.74
2.72
2.71
2.70
2.69
2.68
2.07
2.00
1.95
1.44
1.41
AcO
OAc
AcO
AcO
O
O
N
BocHN
O
O
1.00
1.99
1.14
0.82
1.04
2.20
0.51
0.79
2.66
2.22
3.25
2.87
3.27
3.20
8.79
9.03
f1 (ppm)

f)

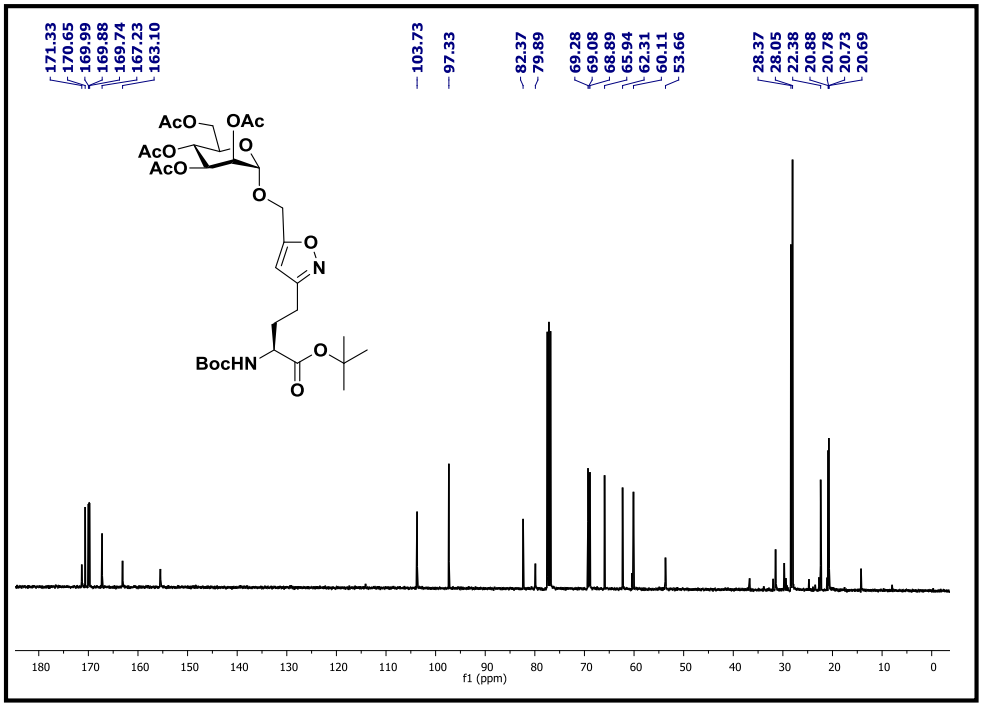
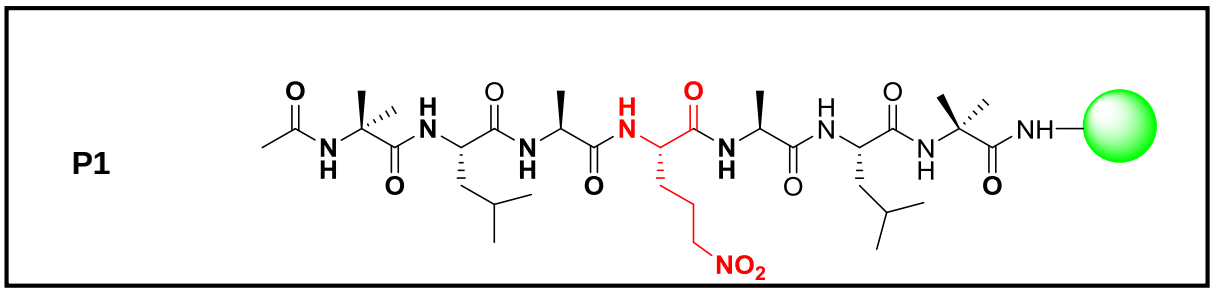


Figure 4.2.2: ^{1}H NMR & ^{13}C NMR spectra of Organic transformations of amino acid **AA 1** to compound **8** (a & b), **9** (c & d) and **10** (e & f) respectively.

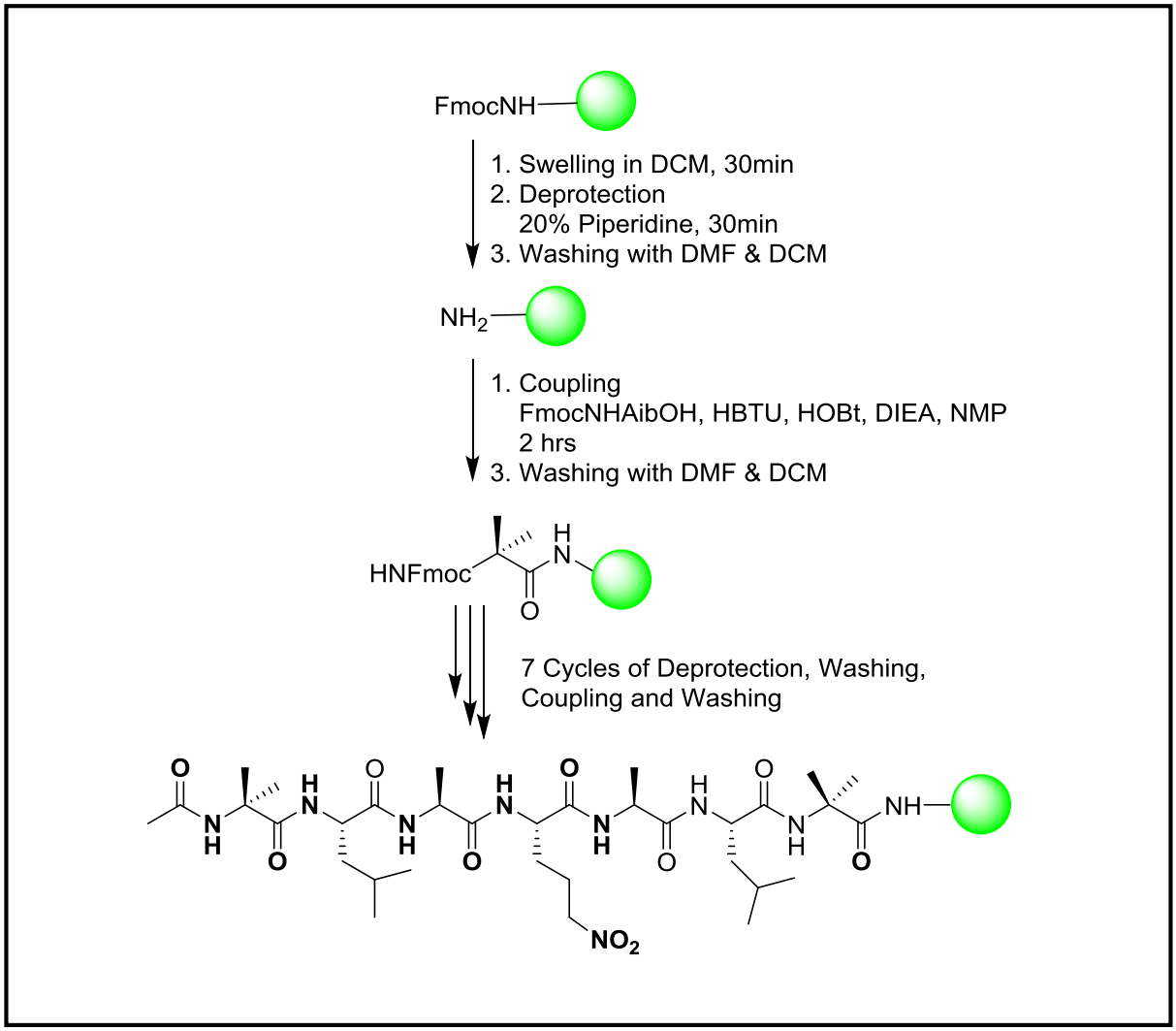
4.3 Organic transformation of peptides on solid phase

To understand whether the transformation of the nitro group to various functionalities can be compatible with the solid phase, we designed peptide **P1** composed of Aib, Leu, Ala and α-nitro amino acid (**Scheme 4.3.1**).



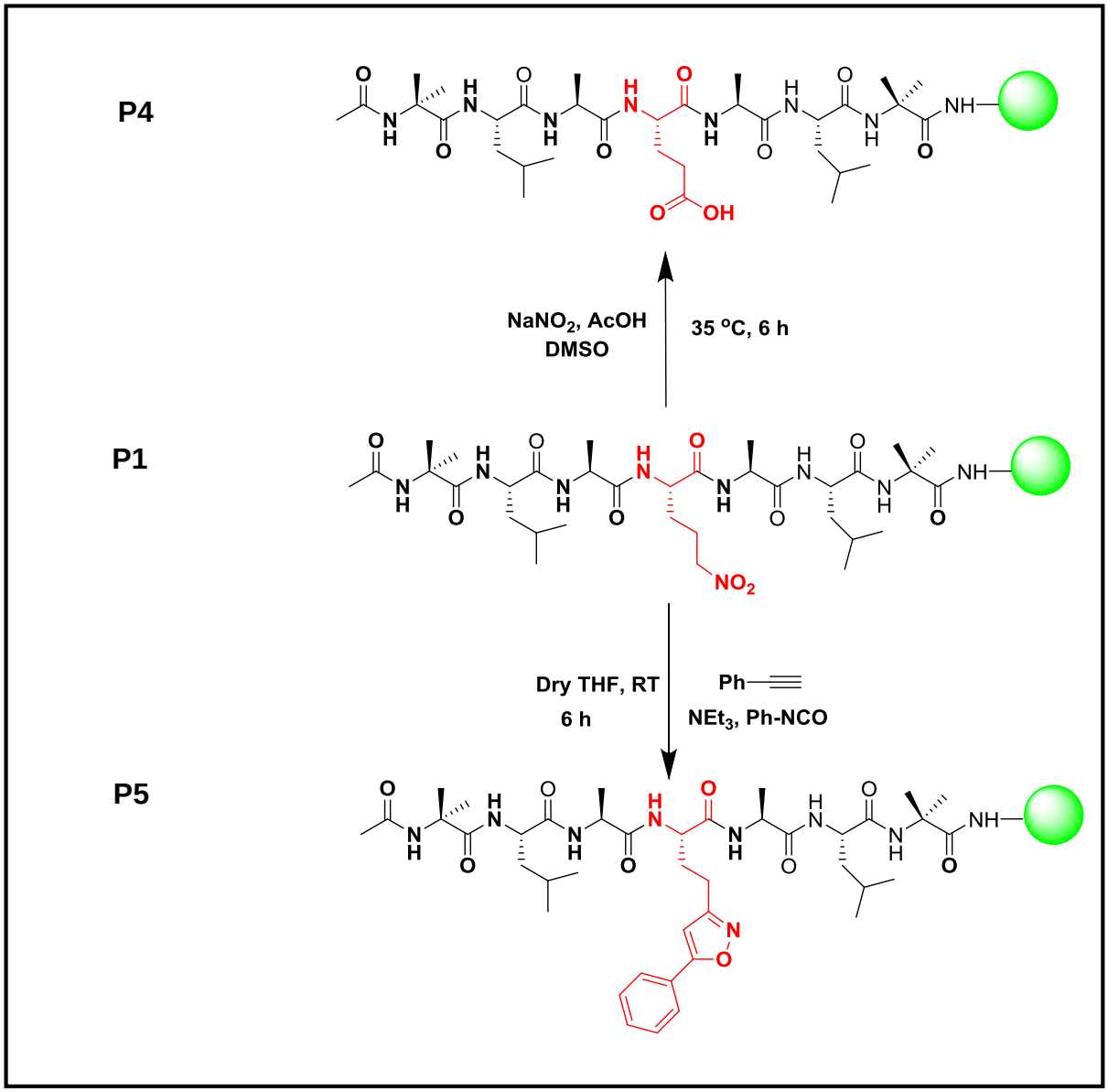
Scheme 4.3.1: Sequence of Peptide **P1**

The peptide **P1** was synthesized by using standard Fmoc solid phase peptide synthetic method on rink amide resin.



Scheme 4.3.2: Fmoc solid phase synthesis of peptide **P1**

The amino acid coupling reactions were carried out in the presence of HOBt /HBTU coupling reagents and DIPEA. At the end of the synthesis, N-terminal of the peptide was protected with the acyl group by the treatment of acetic anhydride in the presence of pyridine. The schematic representation of solid phase peptide synthesis of peptide **P1** was shown in **Scheme 4.3.2**.These resin bound peptides were further used for various transformation reactions of nitro functionality. Initially, we subjected the resin bound peptide **P1** for the conversion of nitro to carboxylic acid (**P4**) by using $NaNO_2$/AcOH in DMSO. The schematic representation of reaction is shown in **Scheme 4.3.3**.



Scheme 4.3.3: Organic transformation of peptide **P1** into **P4** and **P5**

After the reaction completes, the resin was washed thoroughly with DMF and DCM to remove unreacted reagents and side products. Then the peptide was cleaved from the resin by using TFA. Further, **P4** was purified by using reverse phase HPLC on a C_{18} column in MeOH/H_2O system. The transformation from **P1** to **P4** was confirmed by MALDI-TOF. The HPLC trace of the peptide is given in **Figure 4.3.1**. To check whether the 1, 3 dipolar cycloaddition of the nitro group with olefin is possible on a solid phase, the resin bound peptide **P1** was subjected to cycloaddition reaction with phenyl acetylene in the presence of PhNCO and triethylamine under mild conditions (**Scheme 4.3.3**). After the reaction completes, the resin was thoroughly washed to remove

unreacted reagents. Subsequently, the resulting peptide **P5** was cleaved from the resin and purified by using reverse phase HPLC in MeOH/H_2O system. The formation of isoxazole was confirmed by using MALDI-TOF. The HPLC trace of the peptide **P4** is given in **Figure 4.3.1**. The HPLC analysis suggested that both reactions are neat and achieved a quantitative transformation of **P1** to **P4** and **P1** to **P5.** The mass spectra of both **P4** & **P5** are given in the supplementary data session.

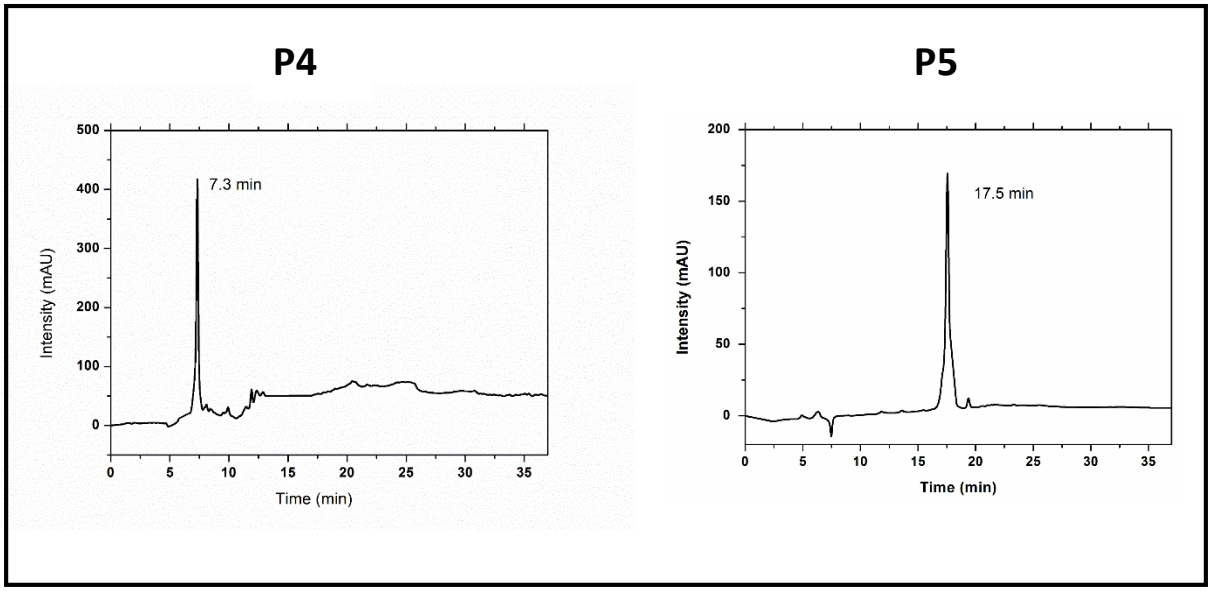
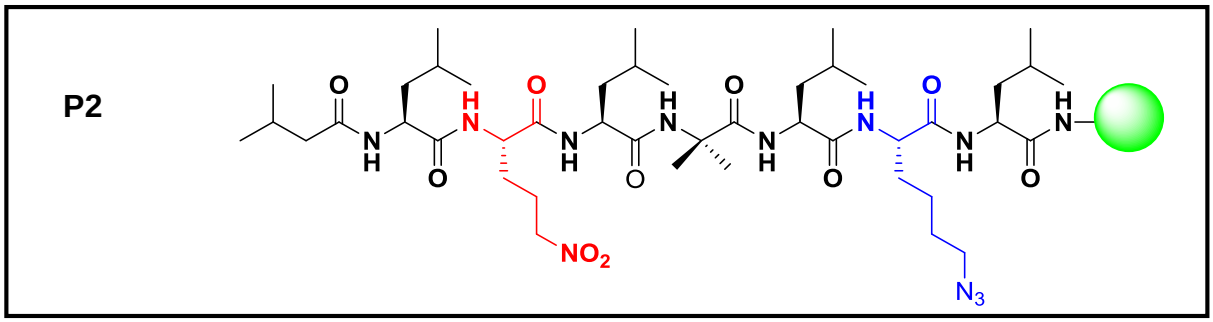


Figure 4.3.1: HPLC Traces of peptides **P4** and **P8**.

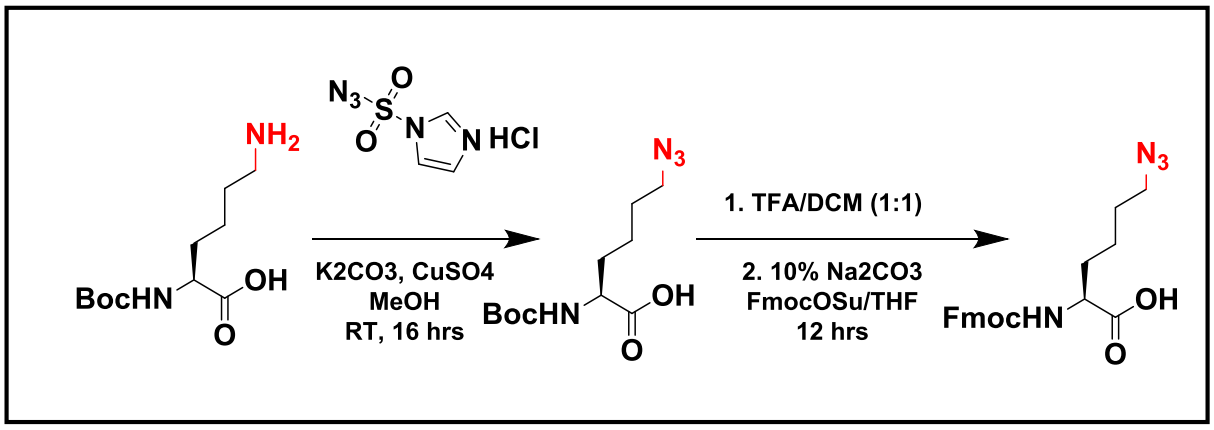
4.4 Orthogonal nitroalkane-alkyne and azide-alkyne 1, 3-dipolar cycloaddition on peptides – Solid phase

Inspite by the multifaceted nature of amino acid **AA1**, we hypothesized that it could serve as an orthogonal functionality for both azides and alkynes. Motivated by the quantitative transformation of **P4** and **P5** from **P1** we designed peptide **P2** containing Leu, AA 1, Aib and Azidolysine (**Scheme 4.4.1**).



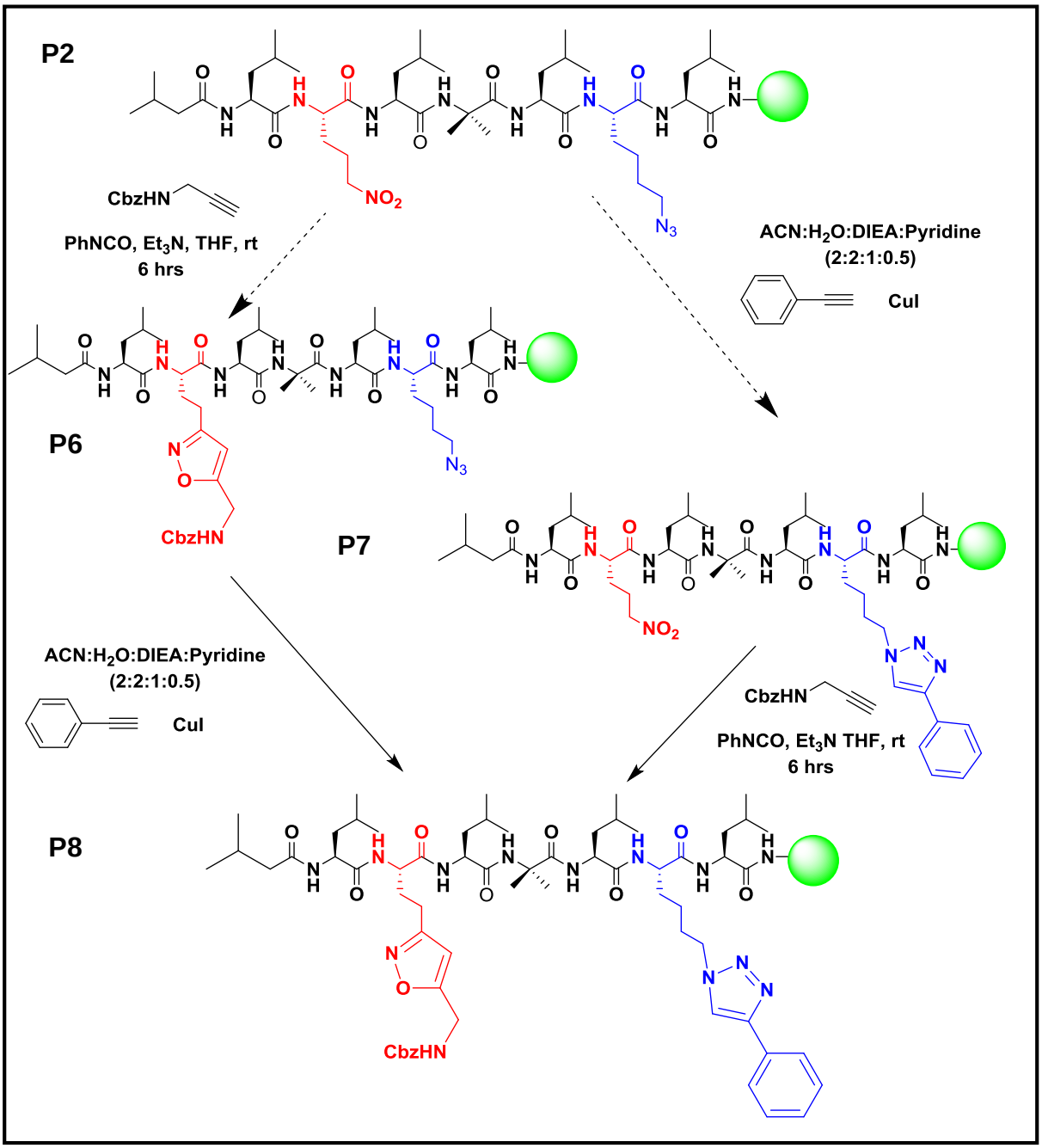
Scheme 4.4.1: Sequence of peptide **P2**

Initially, Fmoc-Lys (N_3)-OH was synthesized from L-Lysine by a method which is already reported in the literature. The schematic representation of synthetic procedure is given in **Scheme 4.4.2**. Boc-protected Lysine was treated with Imidazole-1-sulfonyl azide hydrochloride and Copper pentahydrate ($CuSO_4.5H_2O$) in the presence of the base to obtain Boc-Lys (N_3)-OH. Then the deprotection of Boc followed by protection of amine with FmocOSu gave Fmoc-Lys (N_3)-OH. The peptide **P2** was synthesized similar to **P1** by Fmoc solid phase peptide synthesis. In peptide **P2** N-terminal was protected with isovaleric acid instead of the acyl group. The incorporation of both **AA 1** and azidolysine was confirmed by MALDI/TOF mass spectra.



Scheme 4.4.2: Synthesis of Fmoc-Lys (N3)-OH

The resin bound peptide **P2** was then subjected to cycloaddition reaction separately with alkynes to achieved **P6** and **P7** (**Scheme 4.4.3**). In the case of **P6**, the cycloaddition reaction with Cbz protected propargylamine was carried out in the presence of both azide and nitro group. The reaction was done by using phenyl isocyanate and triethylamine as reagents in THF at room temperature in the absence of Cu (I).



Scheme 4.4.3: Orthogonal cycloaddition reaction with azide on the solid support.

Interestingly, we have observed only the formation of isoxazole and no cycloaddition product with azide was formed in the reaction mixture. In the case of **P7** cycloaddition was carried out with phenyl acetylene in the presence of Cu (I) catalyst. In this case, we have witnessed only the formation of 1, 2, 3-triazole due to the 1, 3 dipolar reaction between both phenyl acetylene and azide. No cycloaddition product was observed with nitro amino acid. Further, Cu (I) catalyzed click as well as nitro-alkyne cycloaddition was performed on both **P6** and **P7** respectively to yield **P8** (**Scheme 4.4.3**). The quantitative transformation of nitro and azide cycloaddition products are achieved. The peptide **P8** was cleaved from the resin and purified by HPLC. HPLC traces of peptides **P6, P7,** and **P8** is shown in **Figure 4.4.1**. The MALDI/TOF spectra of peptides **P3**, **P6**, **P7** & **P8** are given in supplementary data session.

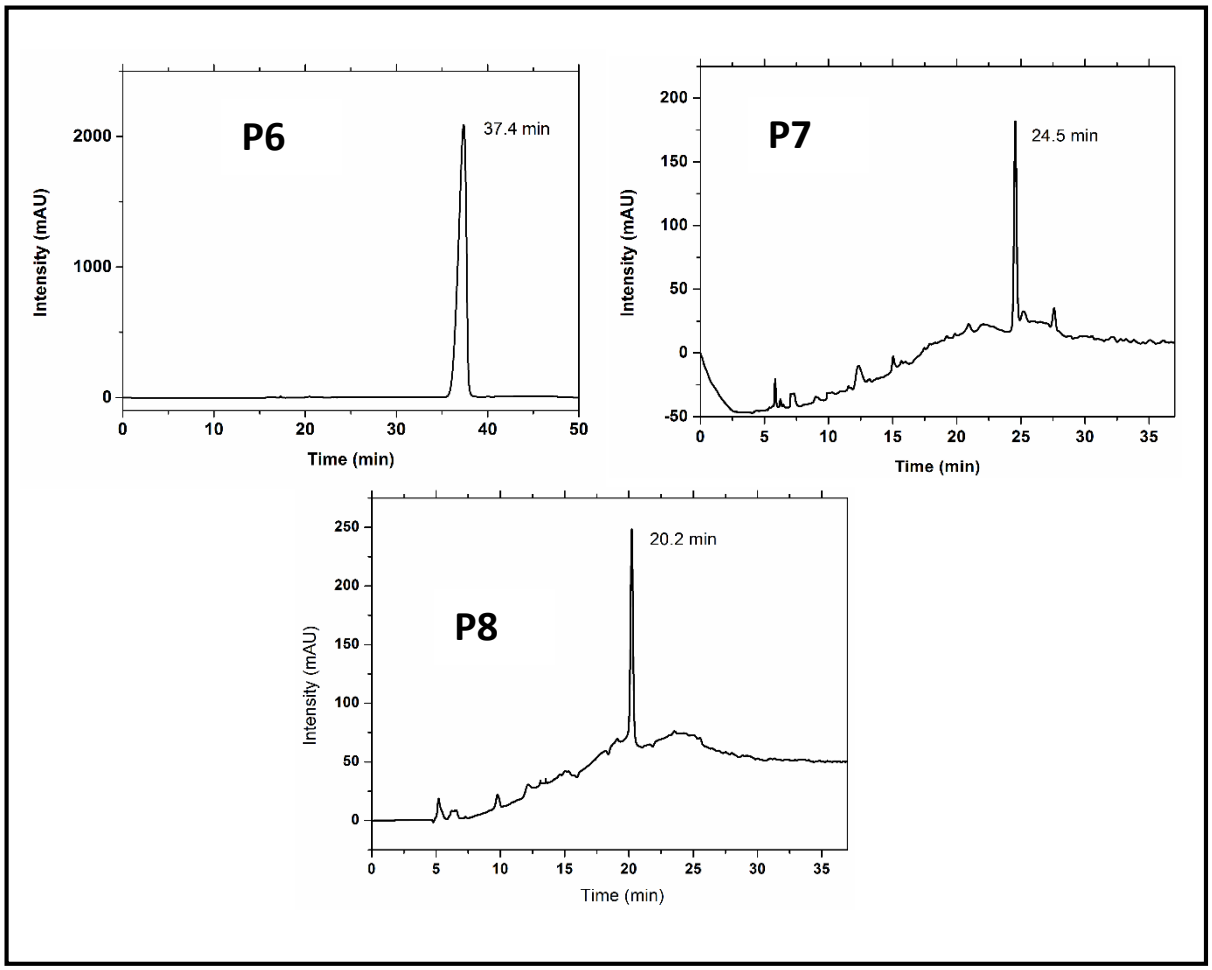
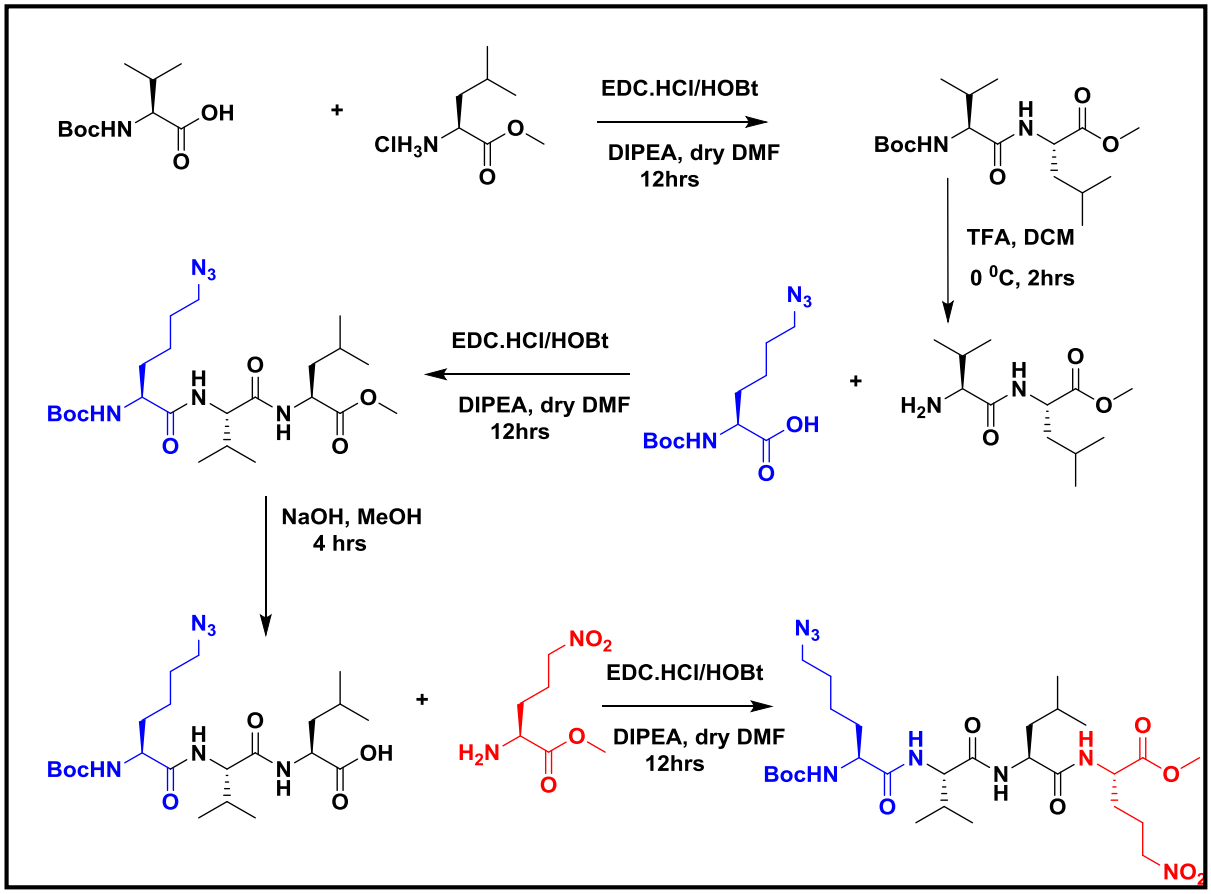


Figure 4.4.1: HPLC Traces of peptides **P6**, **P7** and **P8**.

4.5 Orthogonal nitroalkane-alkyne and azide-alkyne 1, 3-dipolar cycloaddition on peptides – Solution phase

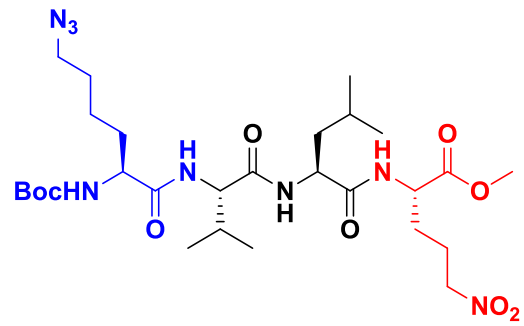
In spite by the orthogonal cycloaddition of nitro and azide on a solid phase, we sought to investigate whether the same reaction can be performed in solution. In this context, we synthesized peptide **P3** by incorporating azidolysine and nitro amino acid (**Scheme 4.5.2**). The tetrapeptide **P3** was synthesized by the 1+2+1 strategy in solution phase. First Boc-protected valine was coupled with the methyl ester of leucine with EDC.HCl/HOBt as a coupling reagent and DIPEA as the base in dry DMF. Then the Boc group of dipeptide BocNH-Val-Leu-OMe was deprotected by TFA/DCM in 0 ^{0}C and coupled with BocNH-Lys (N3)-OH by EDC.HCl/HOBt as a coupling reagent and DIPEA as the base in dry DMF. After hydrolysis, the tripeptide BocNH-Lys(N3)-Val-Leu-OH was coupled with the methyl ester of alpha nitro amino acid by EDC.HCl/HOBt as a coupling reagent and DIPEA as a base in dry DMF to get the peptide **P3**.The tetrapeptide **P3** was further purified by silica gel column chromatography by EA/Hexane. Synthesis procedure is shown in **Scheme 4.5.1**.



Scheme 4.5.1: Synthesis scheme of tetrapeptide **P3**

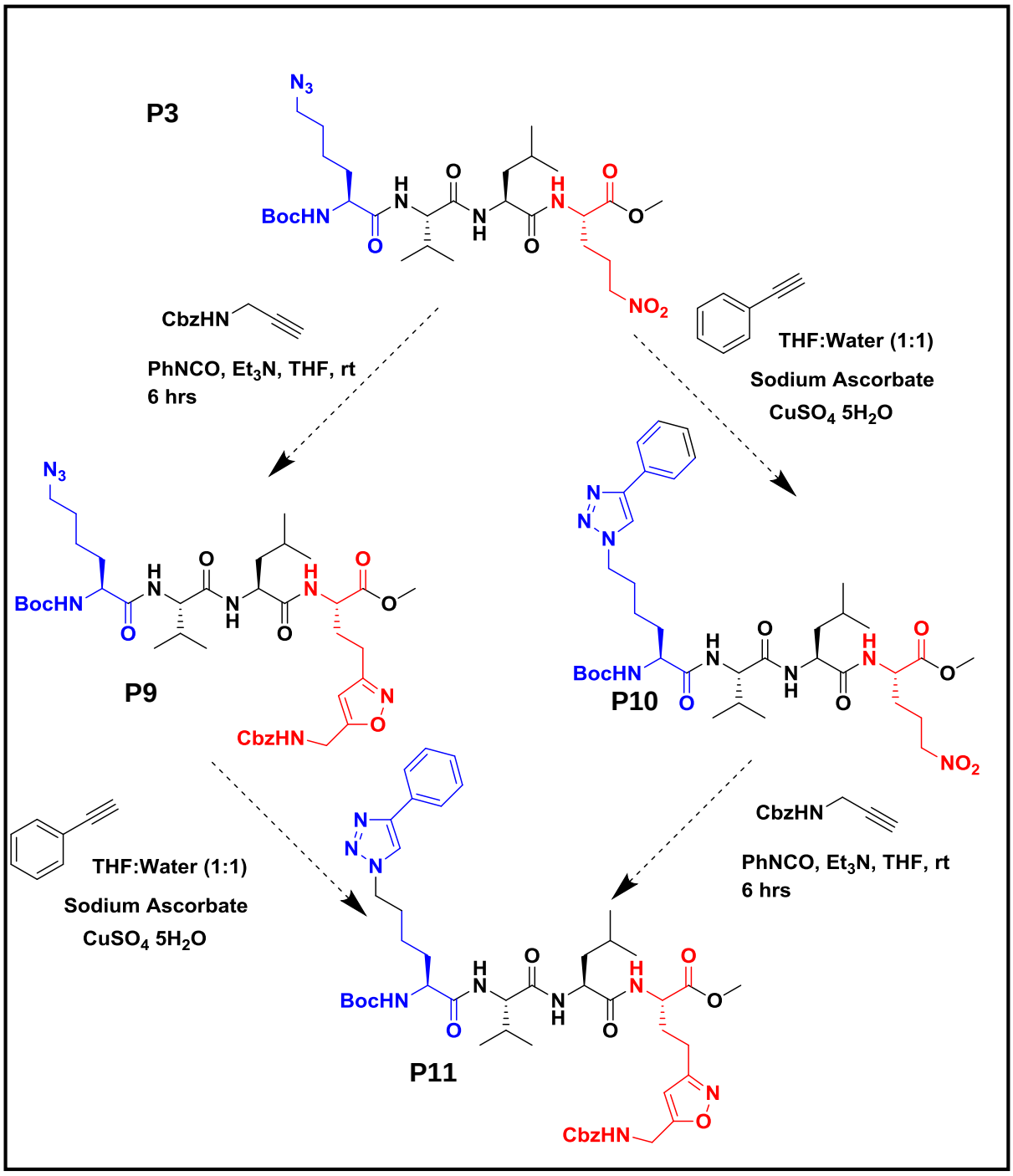
The purified peptide **P3** was subjected to ^{1}H NMR analysis to confirm the incorporation of amino acids. The NMR spectra of peptide **P3** is shown in **Figure 4.5.1a.**

P3



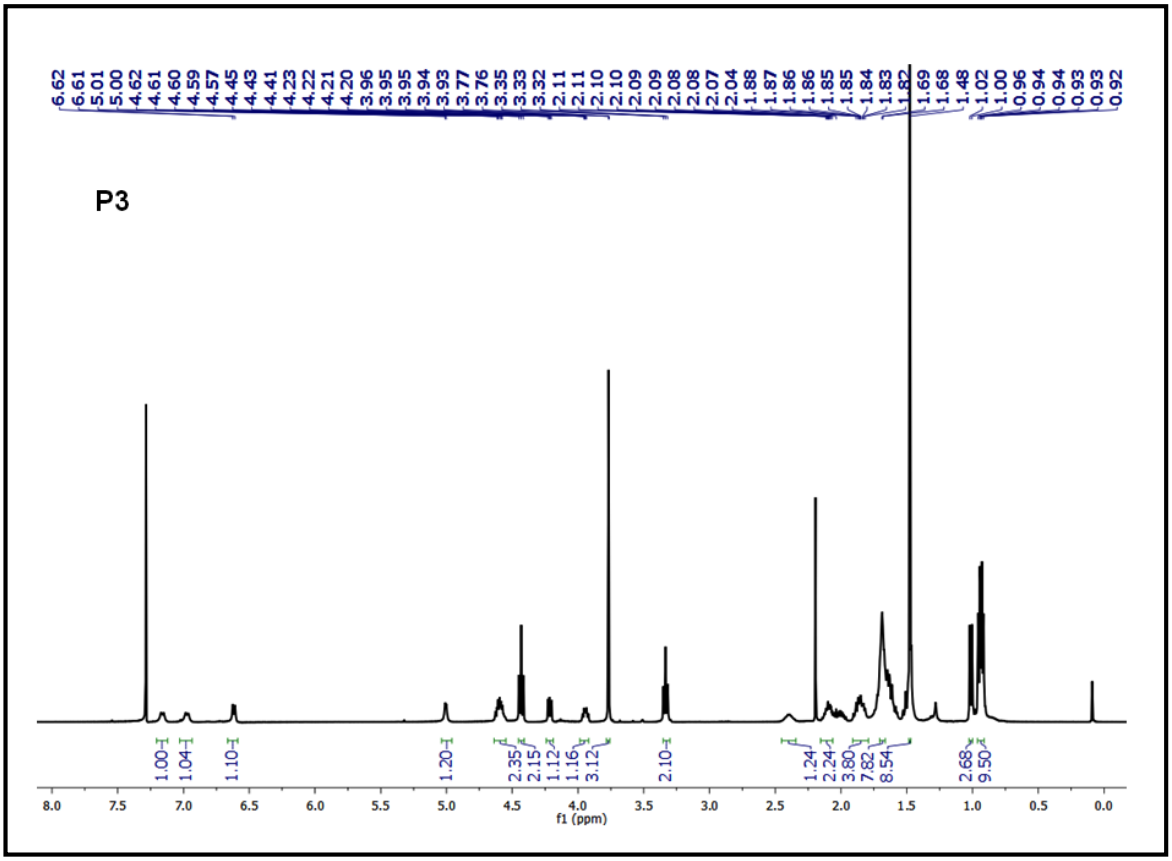
Scheme 4.5.2: Sequence of tetrapeptide **P3**

Similar to **P2,** the peptide **P3** was treated separately with propargylamine and phenyl acetylene to obtain selective cycloaddition product **P9** and **P10**. To obtain **P9, P3** was reacted with propargylamine in the presence of mild base triethylamine in dry THF and no cycloaddition product with azide was observed. For **P10**, **P3** was treated with phenyl acetylene in the presence of Cu catalyst in THF and no cycloaddition product with a nitro group was seen (**Scheme 4.5.3**). Finally, **P11** has achieved by the cycloaddition of corresponding azide and nitro with respective alkynes. The peptides **P9**, **P10** were purified by HPLC and subjected to the ^{1}H NMR analysis. This suggests the formation of **P9** and **P10** in quantitative yield (**Scheme 4.5.3**). The structure of **P11** was confirmed by ^{1}H NMR and mass spectra. The ^{1}H NMR spectra of **P9** is shown in **Figure 4.5.1b**. The ^{1}H NMR spectra of **P10** & **P11** are shown in **Figure 4.5.2a & 4.5.2b.**



Scheme 4.5.3: Orthogonal cycloaddition reaction with azide in solution phase

a)



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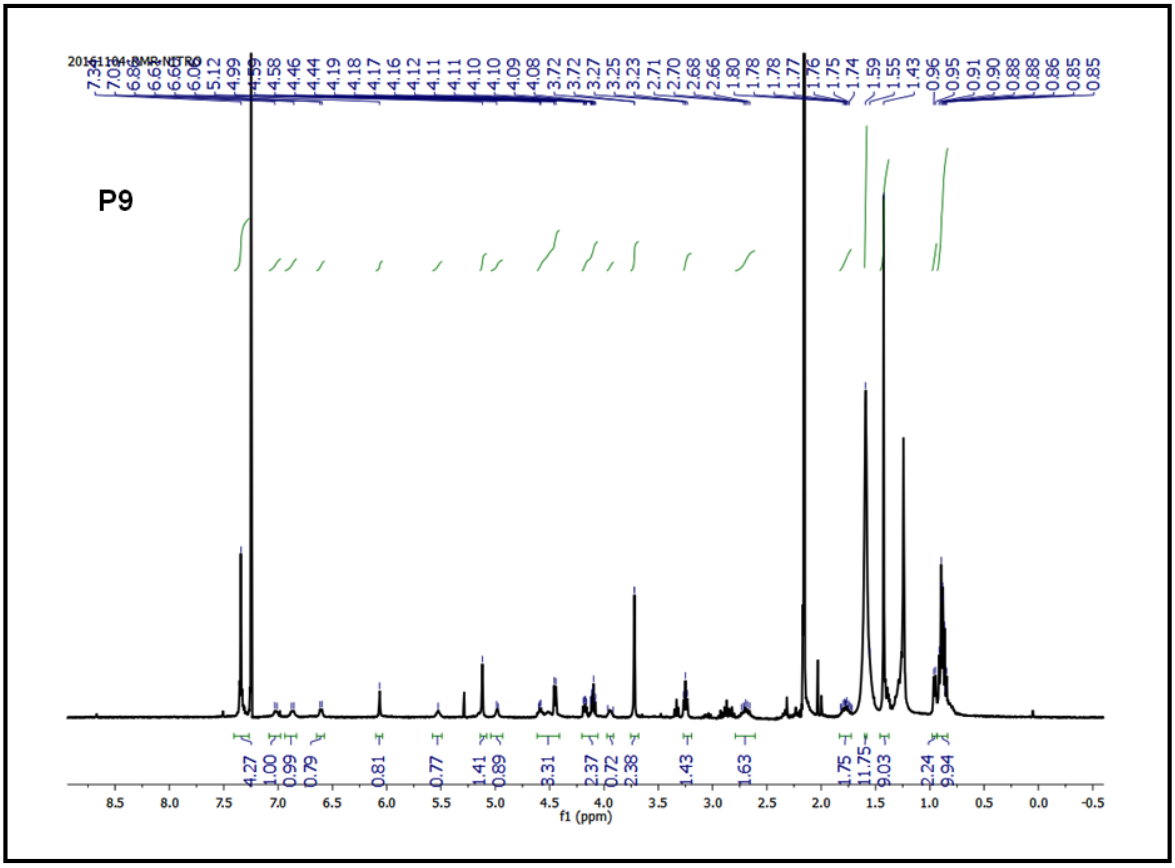
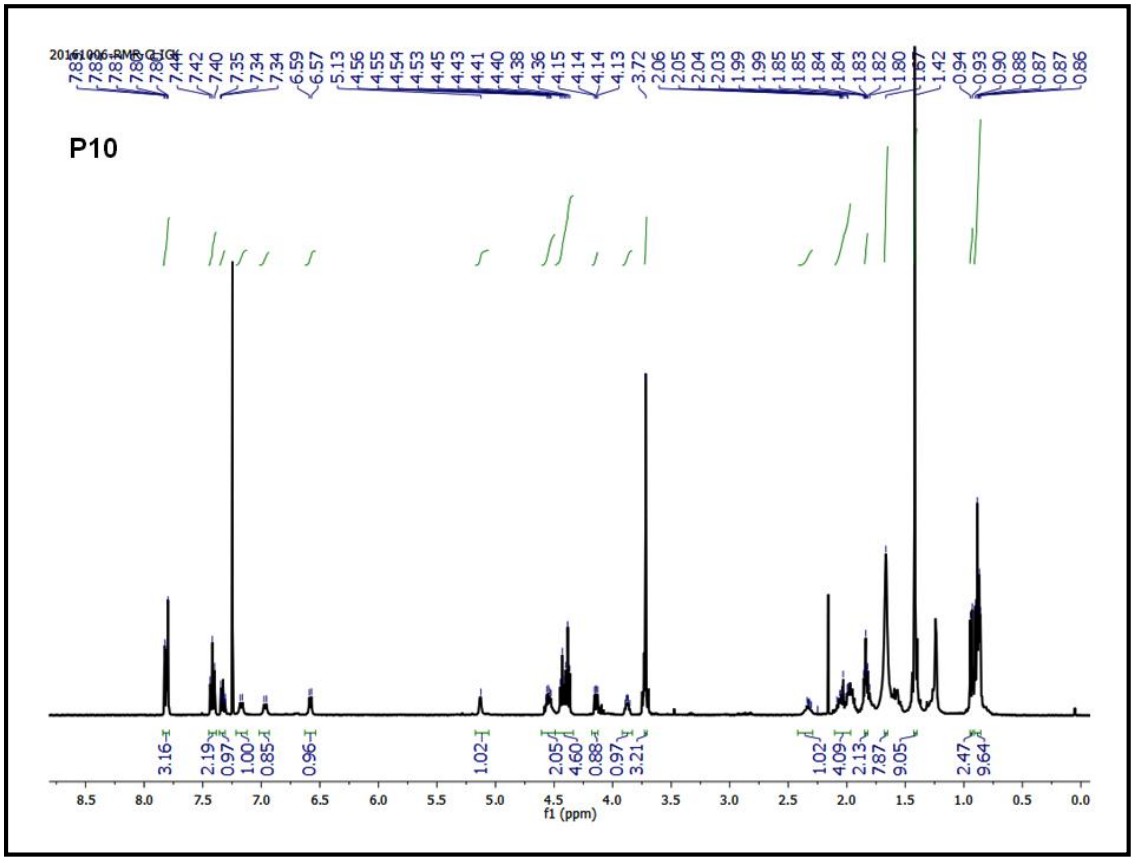


Figure 4.5.1: a) The ^{1}H NMR spectra of **P3**. b) The ^{1}H NMR spectra of **P9**

a)



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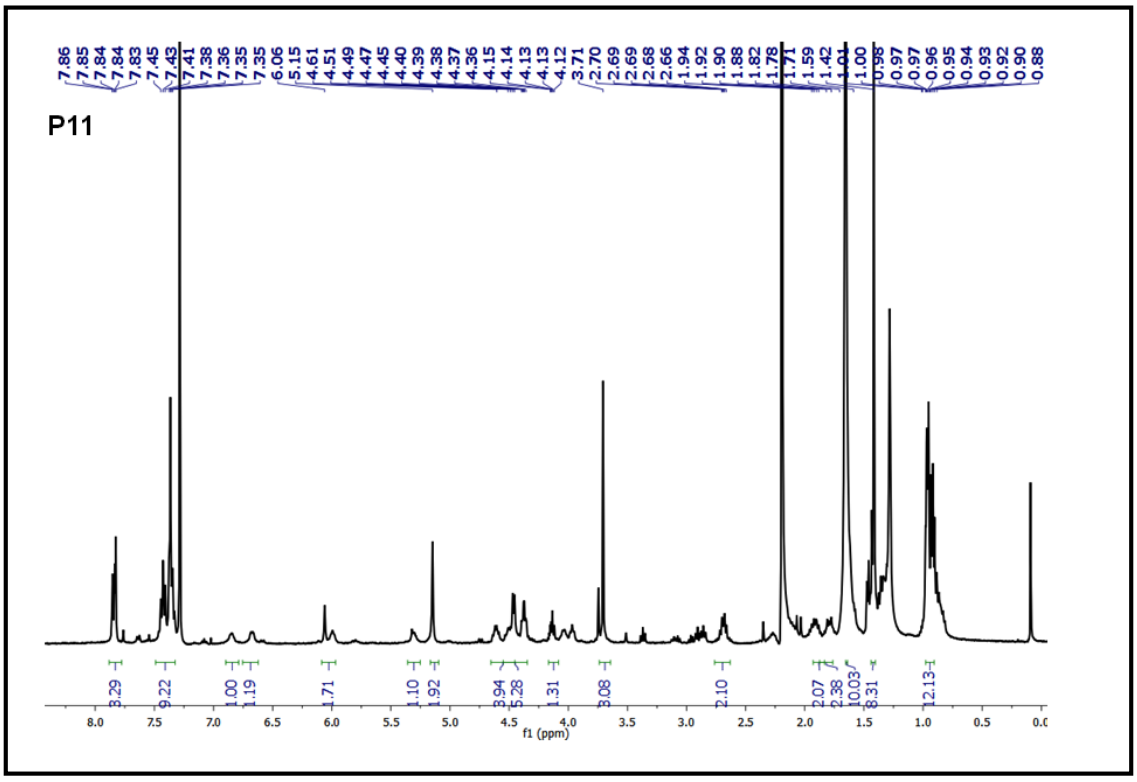

Figure 4.5.2: a) The ^{1}H NMR spectra of **P10**. b) The ^{1}H NMR spectra of **P11.**

CONCLUSION

In conclusion, we have demonstrated the orthogonal nitroalkane-alkyne 1, 3-dipolar cycloaddition reactions mediated by the phenylisocyanate at room temperature. As shown in both solution as well as solid phase methods, the selective nitroalkane-alkyne cycloadditions can be performed in the presence of azido group. In addition, azide-alkyne click reaction can be performed without affecting the nitroalkane functionality. Both azide-alkyne and nitroalkane-alkyne reaction can be performed selectively step-by- step on peptides. The nitro group is simply acts as spectator while performing click reaction. Similarly, azide as a spectator while performing nitroalkane-alkyne cycloaddition reaction. The side-chain functional groups particularly amine functionalities must be protected while performing nitroalkane-alkyne cycloaddition reaction. In addition, the reaction is compatible only in organic solvents. These are the two shortcomings of the nitroalkane-alkyne cycloaddition reaction. Besides the cycloaddition reactions, we further demonstrated the versatile nature of nitro amino acid by transforming it into various other functionalities. Overall, the mild orthogonal nitroalkane-alkyne cycloaddition reactions and the manifold nature of the nitro amino acid reported here can be further explored in the bioconjugations on peptides as well as other organic molecules.

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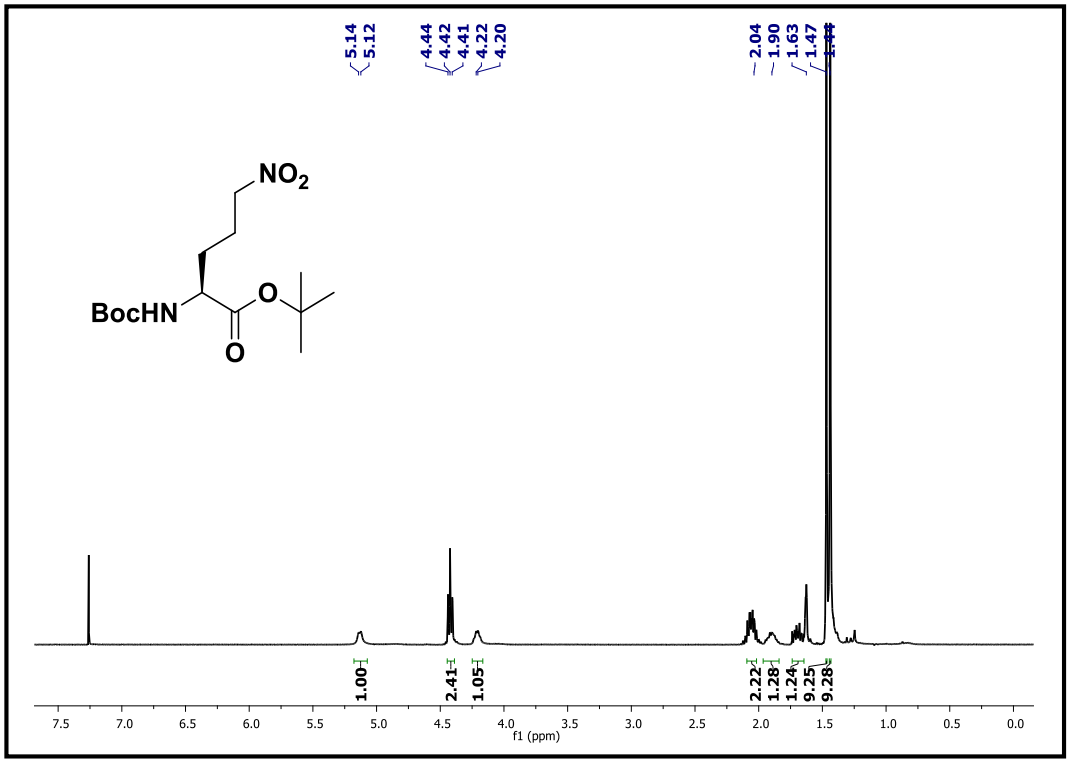
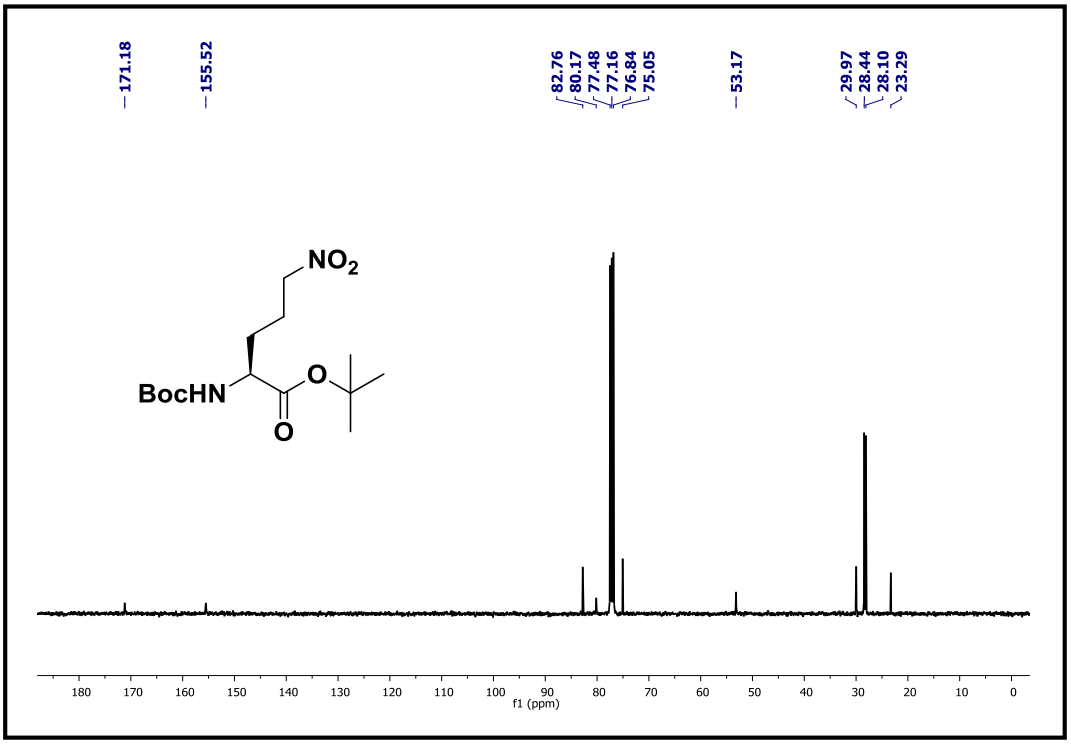
SUPPLEMENTARY DATA

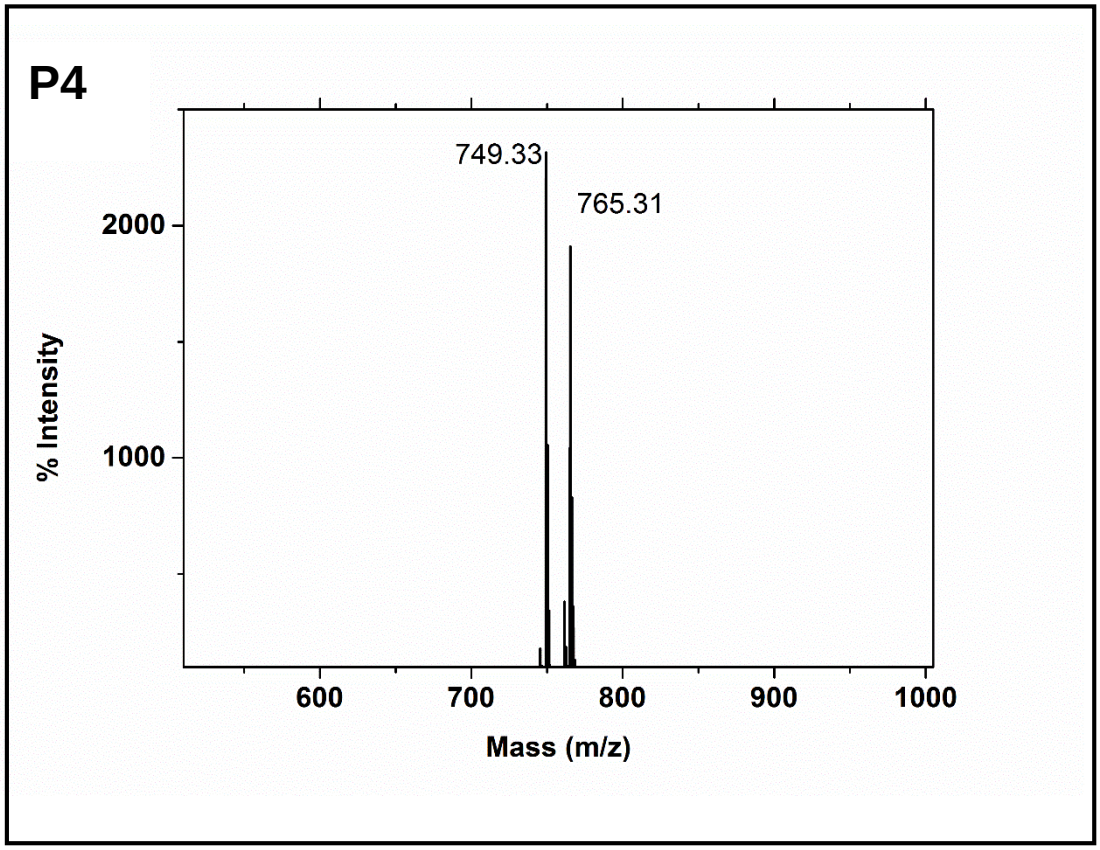
^{1}H NMR spectra of α-nitro amino acid AA 1 & ^{13}C NMR spectra of α-nitro amino acid AA 1

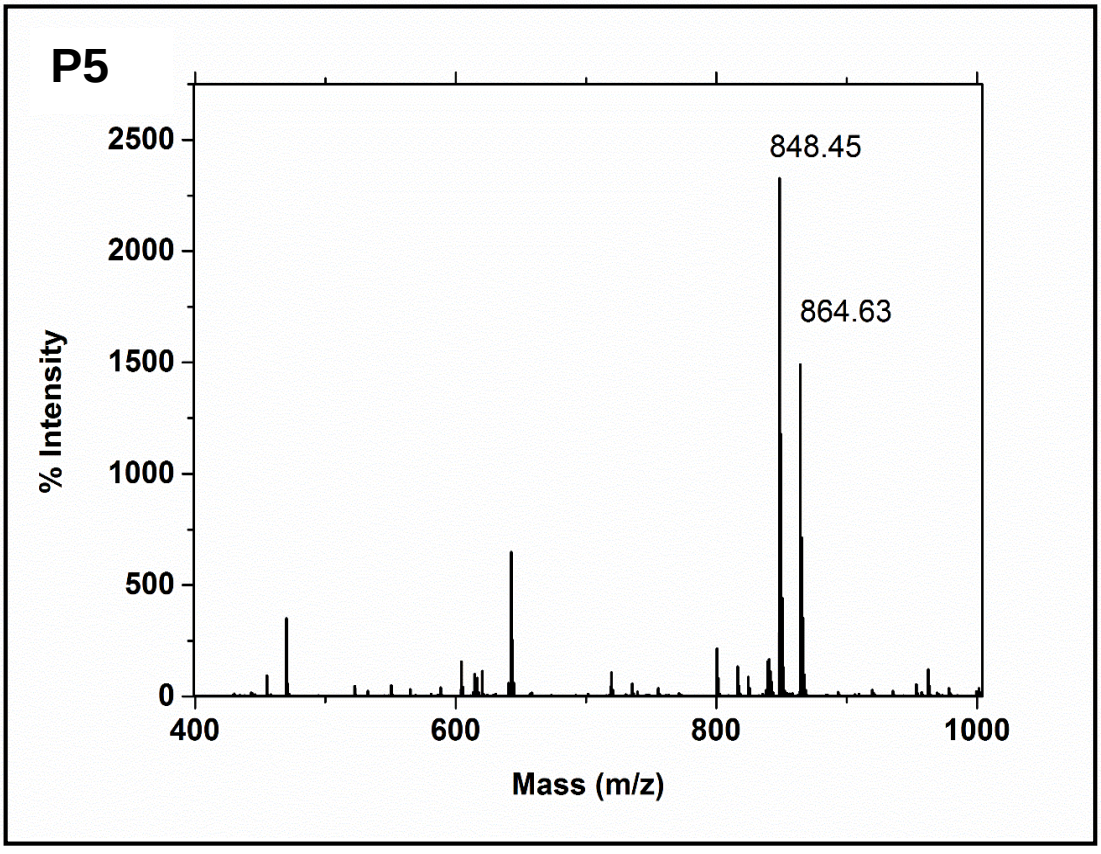
1



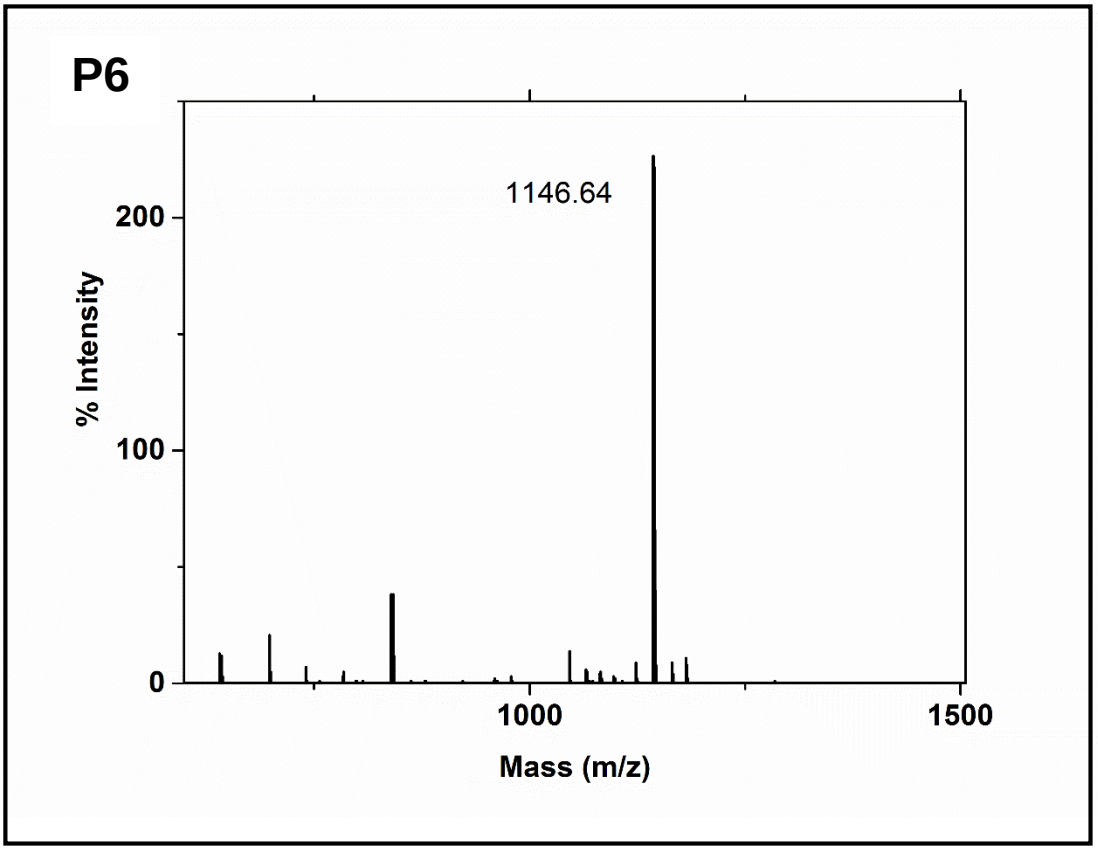


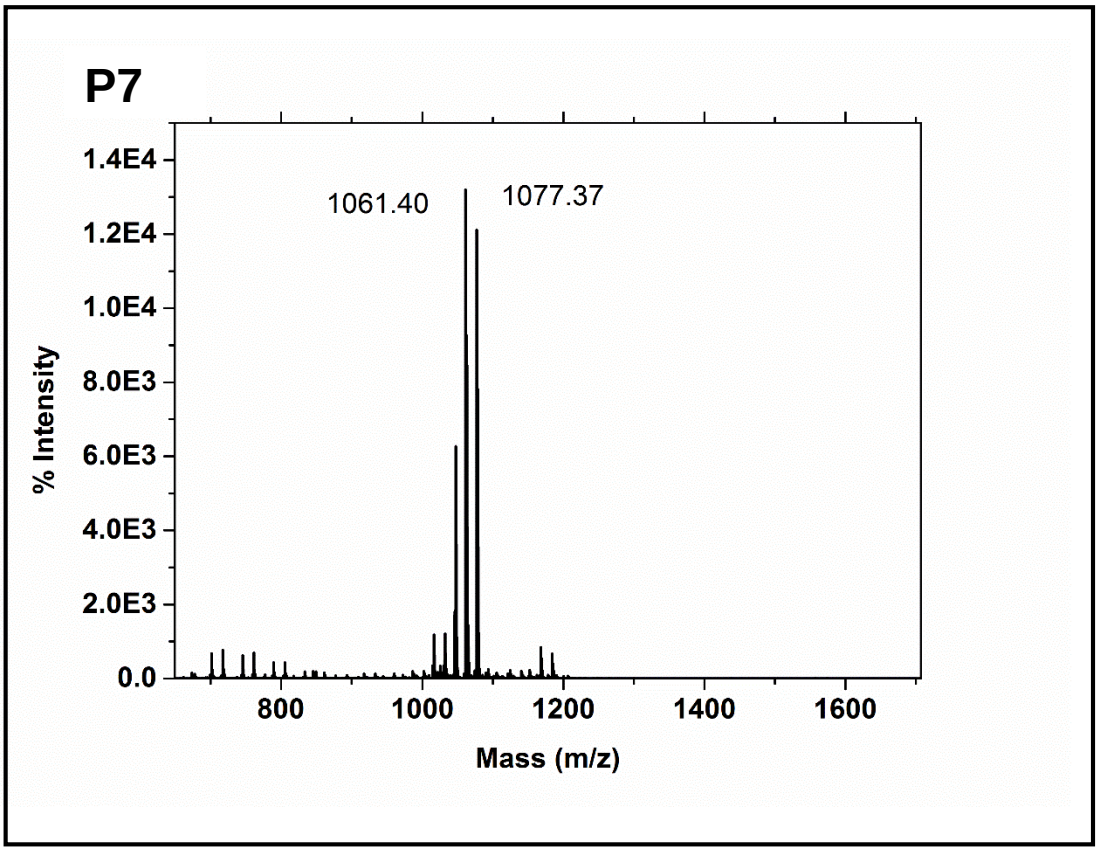
MALDI/TOF Spectra of peptides P4 & P5

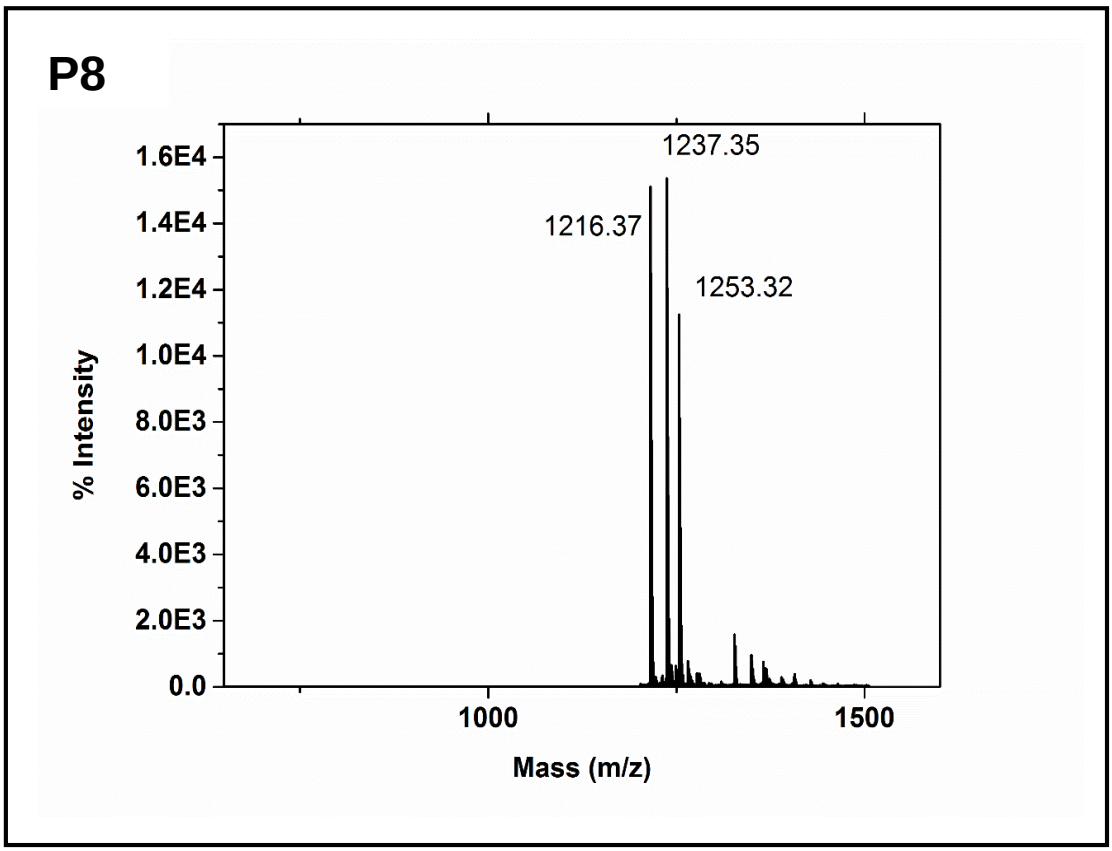




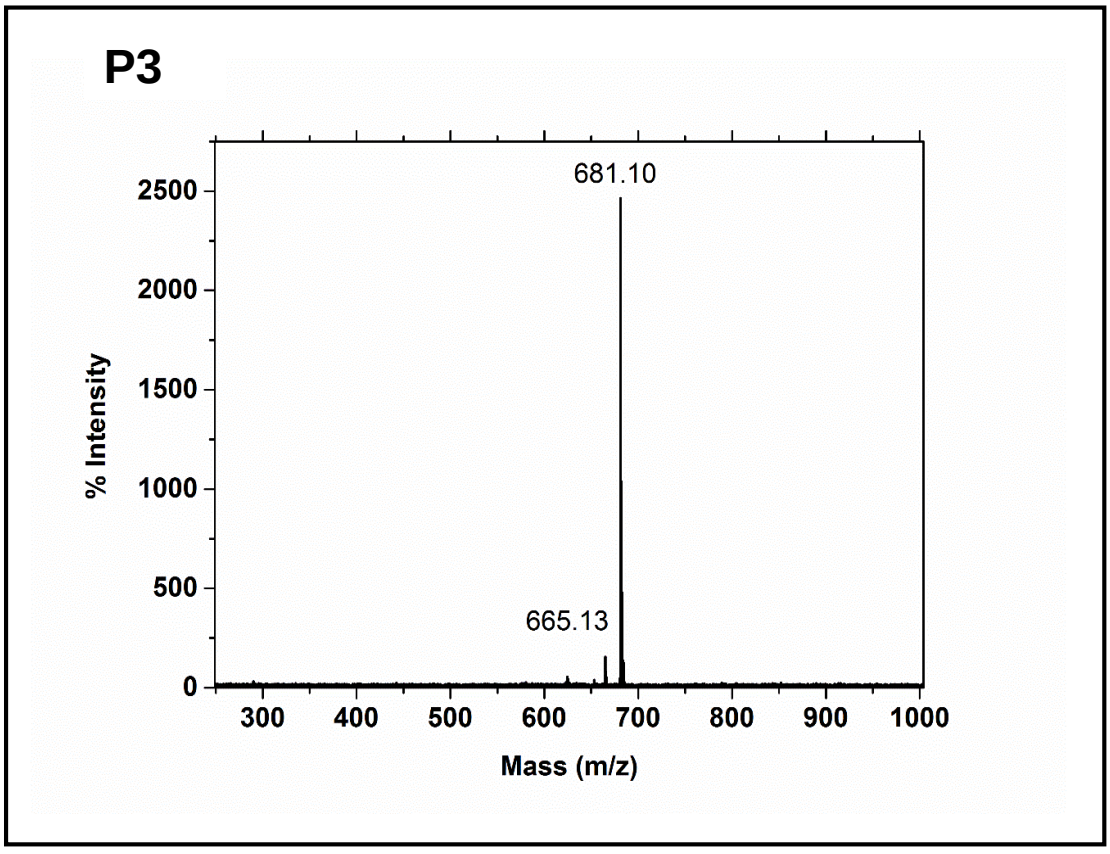
MALDI/TOF Spectra of peptides P6, P7 & P8



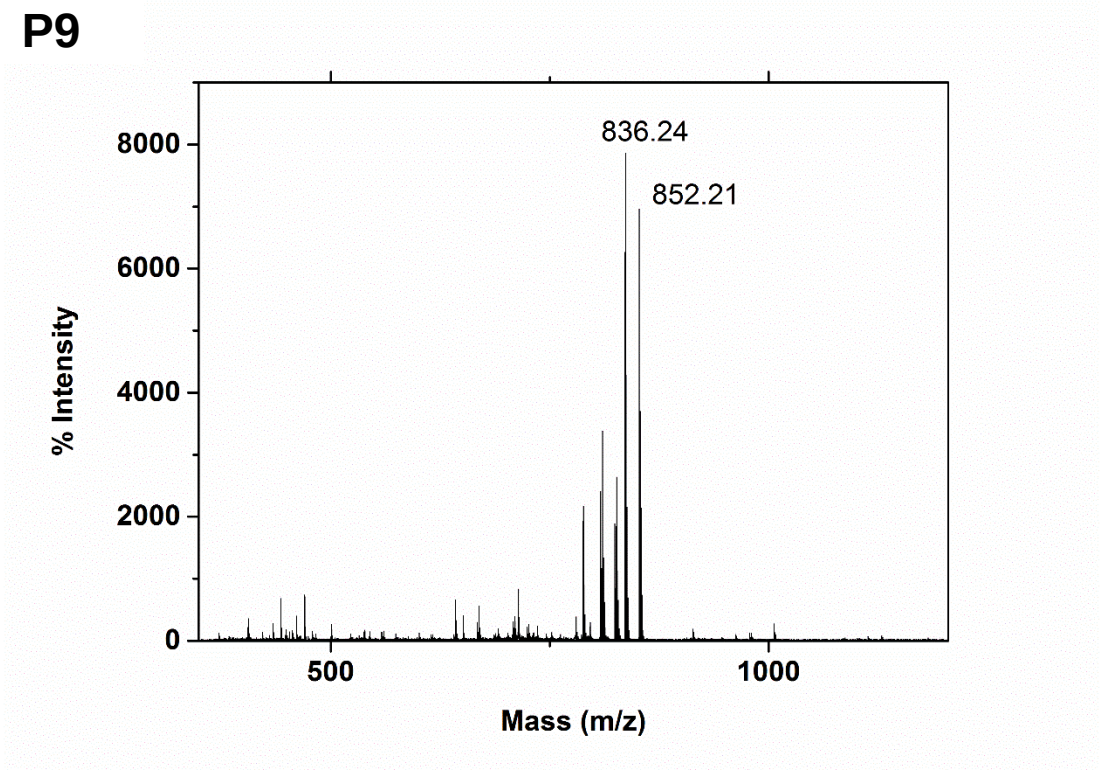




MALDI/TOF Spectra of peptides P3, P9 & P11



P9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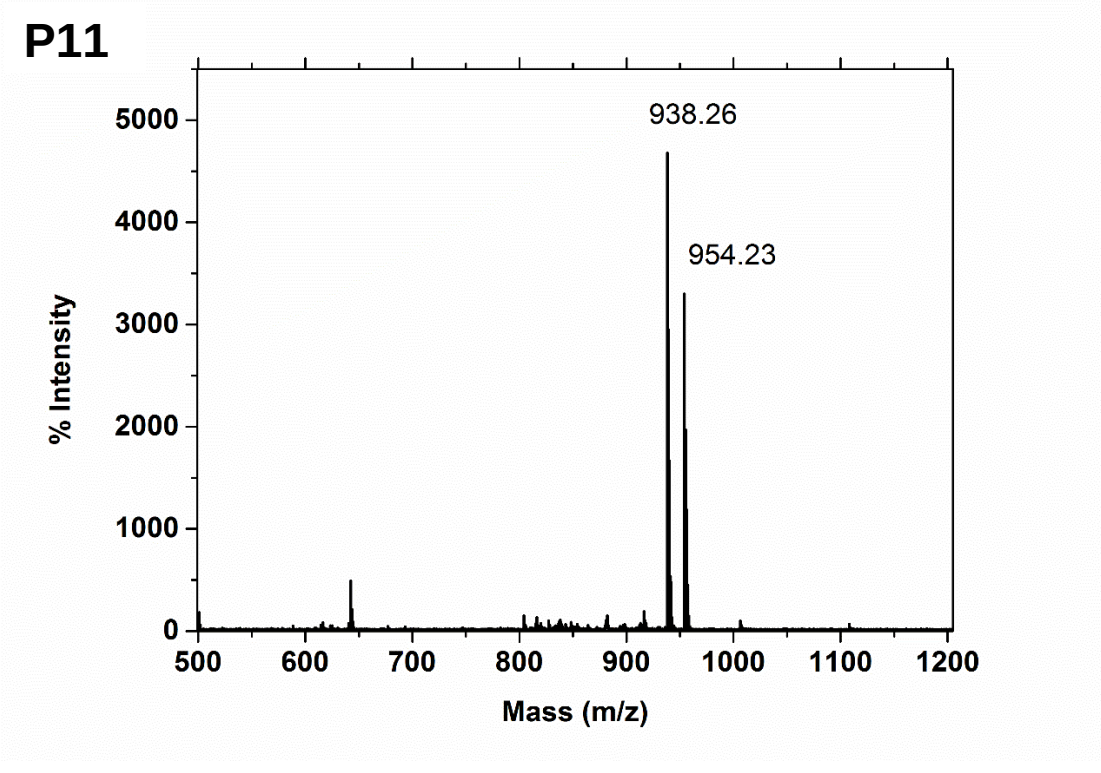
6000
4000
2000
0
% Intensity
500
1000
Mass (m/z)

P11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