Synthesis of Peptides with C-terminal *Z*-γ-Lactam via *E*→*Z* isomerization

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the M.Sc. Degree Programme

by

Keerthivason S

Indian Institute of Science Education and Research Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

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Supervisor: Prof. Hosahudya N. Gopi

Certificate

This is to certify that this dissertation entitled "***Synthesis of Peptides with C-terminal Z-γ-Lactam via E→Z isomerization***" towards the partial fulfilment of the M. Sc degree programme at the Indian Institute of Science Education and Research, Pune represents work carried out by S. Keerthivason at Indian Institute of Science Education and Research, Pune under the supervision of Dr. Hosahudya N. Gopi, Professor, Department of Chemistry, during the academic year 2018-2019.

12/04/2024
IISER Pune

Dr. Hosahudya N. Gopi
Professor,
Department of Chemistry
IISER Pune

Declaration

I hereby declare that the matter embodied in the report entitled "***Synthesis of Peptides with C-terminal Z-γ-Lactam via E→Z isomerization***" are the results of the work carried out by me at the Department of Chemistry, Indian Institute of Science Education & Research (IISER) Pune, under the Supervision of Prof. Hosahudya N. Gopi and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

12/04/2024

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Keerthivason S
20226204
M. Sc.

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Keerthivason S

Table of Contents

Abbreviation

RB - Round Bottom

Na_2CO_3 – Sodium Carbonate

Boc - tert-Butoxycarbonyl

Boc_2O - Di-*tert*-butyl dicarbonate

pH - Potential of Hydrogen

HCl - Hydrochloric acid

TFA - Trifluoroacetic acid

Gly - Glycine

DCC - Dicyclohexyl carbodiimide

DCU – Dicyclohexyl urea

DMF – Dimethyl formamide

$NaBH_4$ – Sodium borohydride

Na_2SO_4 – Sodium sulphate

HBTU - Hexafluorophosphate Benzotriazole Tetramethyl Uronium

HATU - Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium

HOBt - Hydroxybenzotriazole

NaOH – Sodium Hydroxide

DIEA – N,N'-Diisopropylethylamine

DIC – N,N'-Diisopropyl carbodiimide

EtOH - Ethanol

MeOH - Methanol

H_2O - Water

TIPS - Triisopropylsilane

HFIP - Hexafluoro-2-propanol

Ac_2O – acetic anhydride

N_2 - Nitrogen

CTC – 2-Chorotrityl chloride

IBx – 2-Iodoxybenzoic acid

THF - Tetrahydrofuran

DCM - dichloromethane

NMR – Nuclear Magnetic Resonance

Abstract

The *Z*-α,β-unsaturated γ-lactams have been found in many biologically active peptide natural products. Due to their biological activities, extensive efforts have been made in the literature to synthesize the *Z*-γ-lactams. Generally, *Z*-γ-lactams have been found at the C-terminus of peptides, which restricts the solid-phase synthesis of these peptides. In addition, *Z*-γ-lactam synthesis involves very strong reagents, which are generally not suitable for use during or after peptide synthesis. Recently, we reported the spontaneous transformation of (*E*)-α,β-unsaturated γ-amino acids into *Z*-α,β-unsaturated γ-lactam through *in-situ* activation of the free carboxylic acid using the peptide coupling reagent HBTU and the base DIPEA at room temperature. The transformation also involves the (*E*)→ (*Z*) isomerization of α,β-unsaturated γ-amino acids. We sought to examine the *Z*-γ-lactam synthesis on peptides consisting of (*E*)-α,β-unsaturated γ-amino acids at the C-terminus. In this work, we are reporting the synthesis of peptides with C-terminal *Z*-γ-lactam at room temperature using the peptide coupling agents HATU and the base DIPEA. These peptides were synthesized through solid-phase on CTC resin and cyclized after releasing from the solid support. This methodology can provide an opportunity to synthesize peptides with C-terminal γ-lactams without affecting other functional groups on the peptide backbone.

Introduction

Proteins play a crucial role in maintaining life in all living organisms through their diverse structures and functions. Proteins are made up of twenty ribosomal amino acids. Using these 20 amino acids, nature has synthesized a myriad of proteins to perform countless functions in biology.[1] The three-dimensional structure of proteins is crucial, as it dictates their functionality. Besides 20 amino acids a variety of non-ribosomal amino acids.[2] Some of these non-ribosomal amino acids are shown in Figure 1.

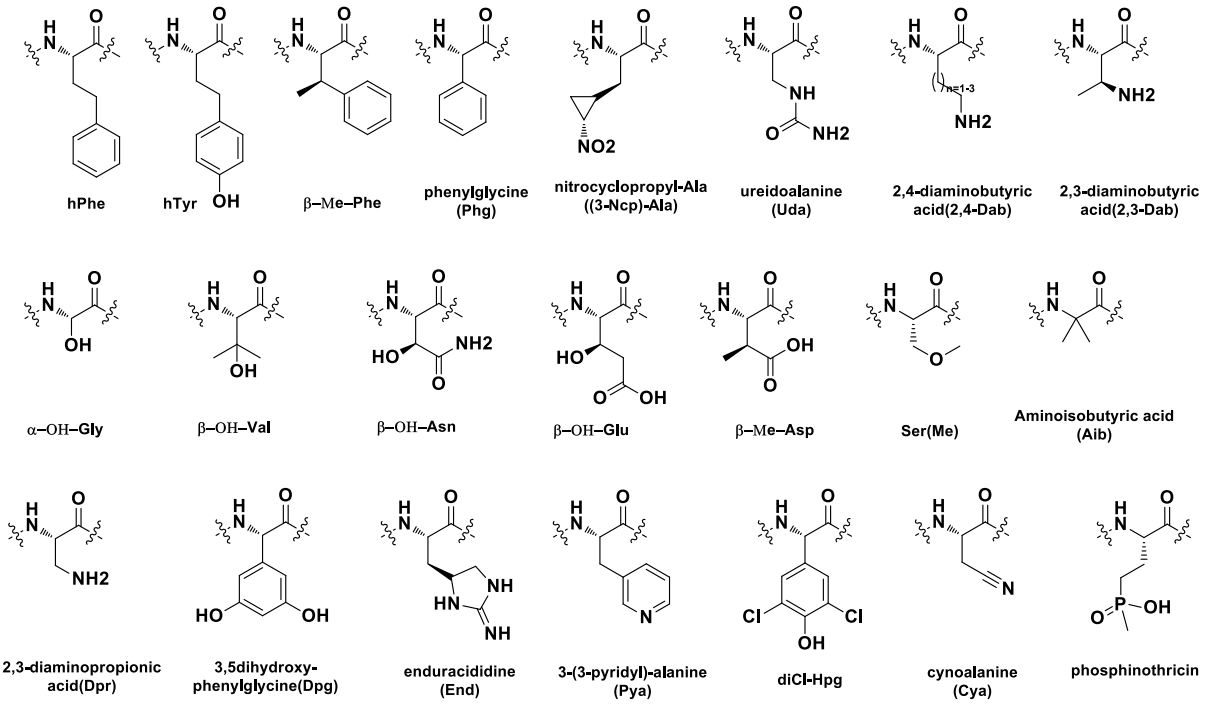


Figure 1. *Some of the non-ribosomal α-amino acids present the peptide natural products.*

Apart from non-ribosomal α-amino acids, a variety of backbone homologated β-, γ- and higher homologue amino acids have been extensively used in the design of protein secondary structure mimetics.[3-10] Some of the synthetic β- and γ-amino acids are shown in Figure 2.

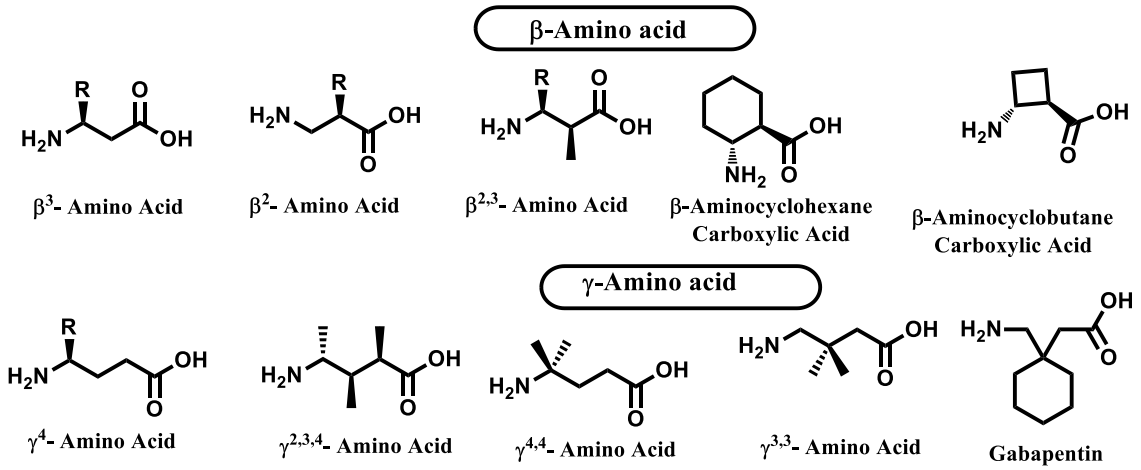


Figure 2. *Representative examples of synthetic β- and γ-amino acids.*

These non-natural β- and γ- amino acids have been extensively used to design protein secondary structure mimetics. A representative examples of protein type secondary structures displayed by the oligomers of β- and γ- amino acids and the hybrid peptide sequences composed of αβ and αγ-amino acids are shown in the Figure 3.

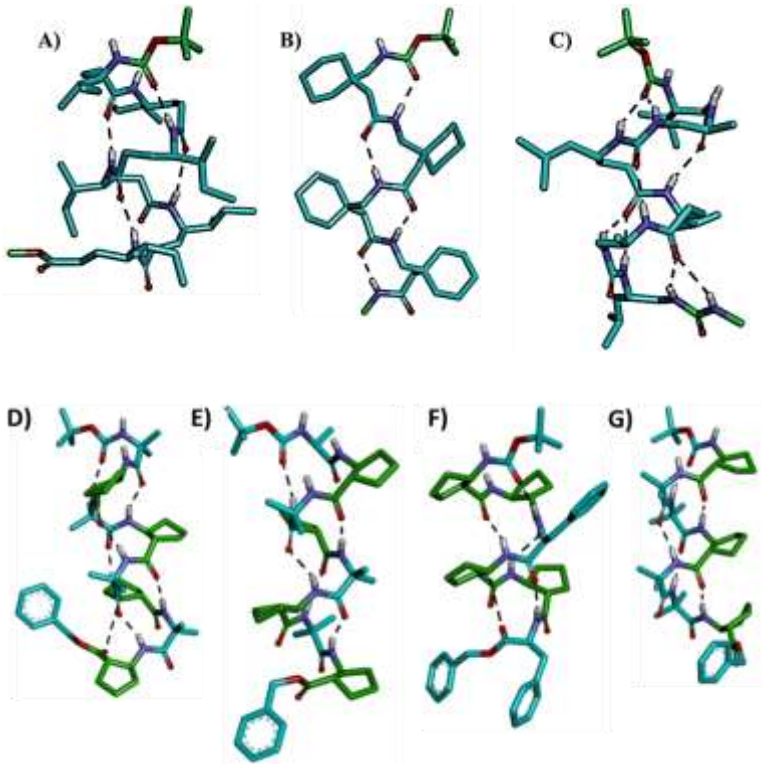


Figure 3. *A) C_{14}-helix of γ^4-peptide[11] B) C_9-helix of gabapentin oligomers,[12] C) C_{14}-helical conformation of oligoureas[13] D) 11-helix from α,β-hybrid peptide[14] E) 14/15-helix from α,β-hybrid peptide[15] F) 12/11/11-helix from β,β,α-hybrid peptide[16] G) 10/11/11-helix from α,α,β-hybrid peptide.[17]*

In addition to the non-ribosomal α-amino acids, a variety γ-amino acids have been found in the peptide natural products.[18-24] Some of the natural products showing the γ- amino acids are shown in Figure 4.

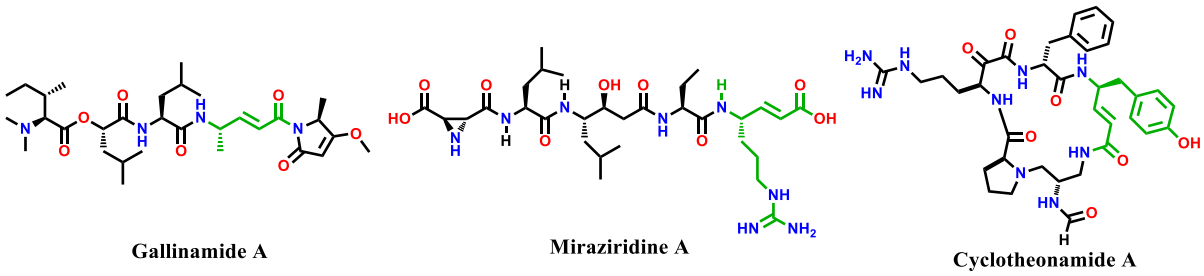


Figure 4: *Representative examples of peptide natural products consisting of non-ribosomal γ-amino acids.*

Conformations of the oligomers of (E)-α,β-unsaturated γ-amino acids.

Our group is interested in understanding the conformational properties and chemical reactivity of naturally occurring non-ribosomal γ-amino acids, particularly (*E*)-α,β-unsaturated γ-amino acids. The structural analysis suggested that (*E*)-α,β-unsaturated γ-amino acids and the oligomers of these amino acids prefer to adopt β-sheet type conformations.[25] The β-sheet promoting signature of these (E)-α,β-unsaturated γ-amino acids has been further utilized to design β-hairpins and multi-stranded β-sheets.[26] The β-sheet promoting nature of these amino acids has further explored to design antimicrobial hybrid lipopeptides.[27] In a sharp contrast to β-sheet type structures of (*E*)-α,β-unsaturated γ-amino acid oligomers, the oligomers consisting of (Z)-α,β-unsaturated γ-amino acids adopted helical conformations.[28] Further, our group showed that β-double helix structure from oligomers of (*E*)-α,β-unsaturated 4,4-dialkyl substituted γ-amino acids.[29] The analysis of the oligomers of (*E*)-vinylogous amino acids suggested that single substitution at γ-position leads to a β-sheet structure while dialkyl substitution leads to β-double helix conformation. In the β-double helix structures, the two peptide strands intertwined together and the structure is stabilized through interstrand H-bonds. In addition, our group recently demonstrated chiral double helices consisting chiral (*E*)-α,β-unsaturated-γ-amino acids along with 4,4-dimethyl α,β-unsaturated γ-amino acids.[30] The representative examples of peptides consisting of E/Z-α,β-unsaturated γ-amino acids are shown in Figure 5, Figure 6 and Figure 7.

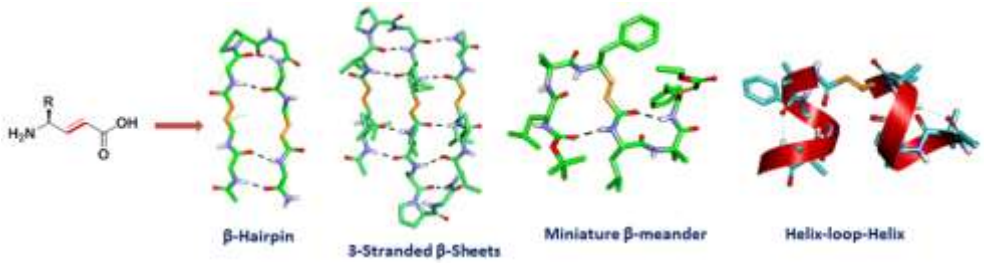


Figure 5: *A variety of structures derived from the hybrid peptides consisting of (E)-α,β-unsaturated γ-amino acids.*

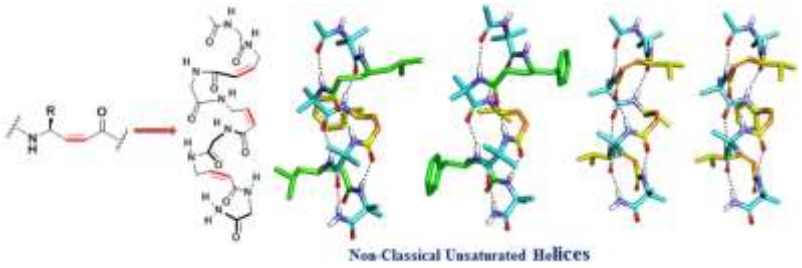


Figure 6: *Hybrid peptide 12-helices consisting of (Z)-α,β-unsaturated γ-amino acids.*

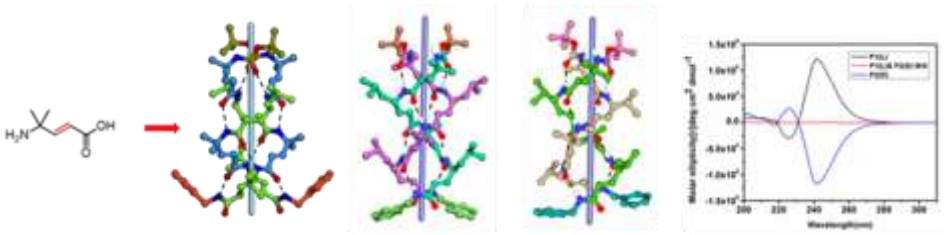
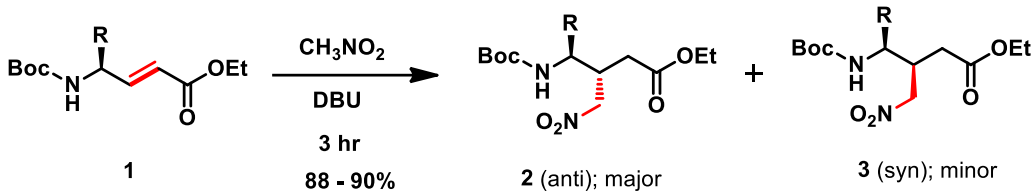


Figure 7: *Chiral and achiral γ-double helices derived from the oligomers of (E)-α,β-unsaturated 4,4-dialkyl-γ-amino acids.*

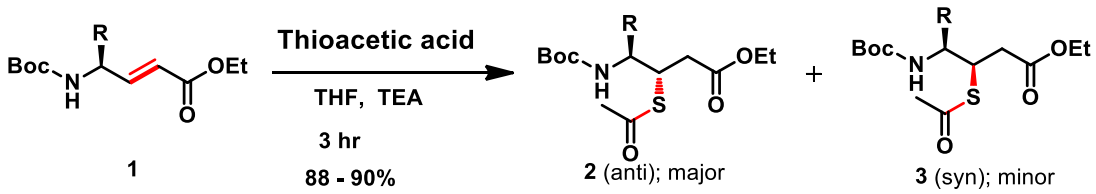
Chemistry of α,β-unsaturated γ-amino acids

The (*E*)-α,β-unsaturated γ-amino acids have been used as starting materials for the synthesis of γ-amino acids through catalytic hydrogenation.[31] Plummer et al examined the Michael addition of nitromethane to N-protected *E*-α,β-unsaturated γ-amino esters.[32] In continuation, our group further demonstrated the diastereoselectivity and the mechanism of nitromethane conjugate addition.[33] Similarly, thioacetic acid conjugate addition was also examined for its high diastereoselectivity.[34] The Schematic representation of the reactions of nitromethane conjugate addition and thioacetic conjugate addition reactions are shown in Scheme 1 and Scheme 2, respectively. Further, our group also demonstrated the base mediated single step transformation of N-protected (*E*)-α,β-unsaturated γ-amino esters into N-protected 5,5-

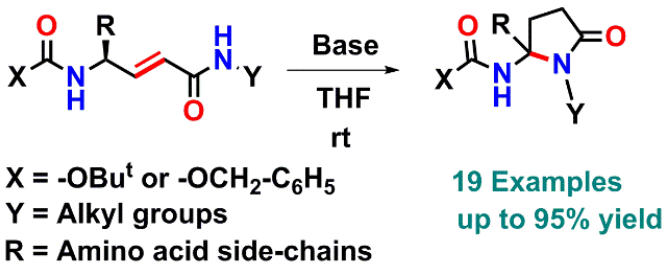
disubstituded γ-lactams through multiple double bond migrations.[35] The Schematic representation of the reaction is shown in Scheme 3.



Scheme 1: *Synthesis of N-Boc-β-nitromethyl substituted γ-amino esters.*

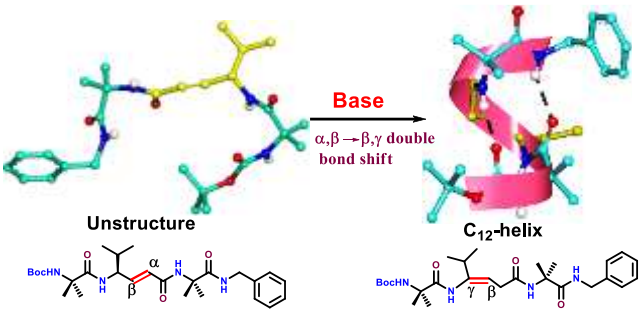


Scheme 2: *Synthesis of N-Boc-β-thioacetic acid substituted γ-amino esters.*



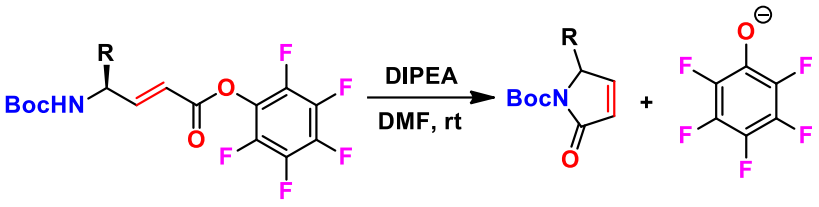
Scheme 3: *Single step transformation of (S, E) N-Boc-α, β-unsaturated γ-amino acid N-benzyl amides into 5,5-disubstituted γ-lactams.*

In addition, our group also demonstrated the base mediated unusual double bond migration in peptides composed of (*E*)-α,β-unsaturated γ-amino acids.[36] The Schematic representation of the reaction is shown in Scheme 4.

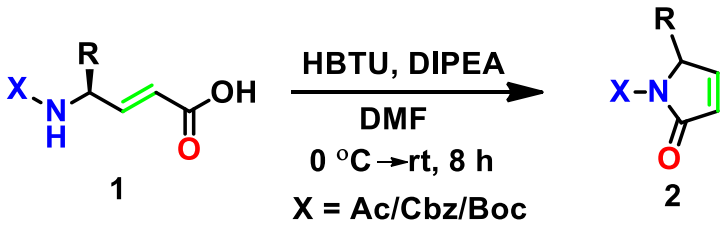


Scheme 4: *Base mediated double bond migration in the peptide composed of (E)-vinylogous amino acid. The unusual planar structure displayed by the starting peptide transformed into 12-helix after double bond migration.*

This transformation involves the unusual *E*→*Z* isomerization of double bond in the absence of catalysts and light. Similar transformation of (*E*)-vinylogous amino acids into corresponding Z-γ-lactams was also observed in the *in situ* carboxylic acid activation using HBTU in the presence of mild base.[38] The schematic representation of these reactions are shown Schemes 5 and 6, respectively. Both transformations involved complete racemization of chiral centres at the γ-position of amino acids.



Scheme 5*: Synthesis of N-Boc-(Z)-α,β-unsaturated γ-lactams from the pentafluorophenyl (OPfp) esters of N-Boc-(E)-α,β-unsaturated γ-amino acids at room temperature.*



Scheme 6. *Synthesis of Z-α,β-unsaturated γ-lactams from E-α,β-unsaturated γ-amino acids via in situ activation of carboxylic acid using HBTU in the presence of mild base. The transformation involves the unusual E→Z isomerization of double bond.*

The Z-γ-lactams have been found in many biologically active peptide natural products such as Majusculamide D,[39] Ypaoamide etc.[40] In addition, Z-γ-lactams are also very

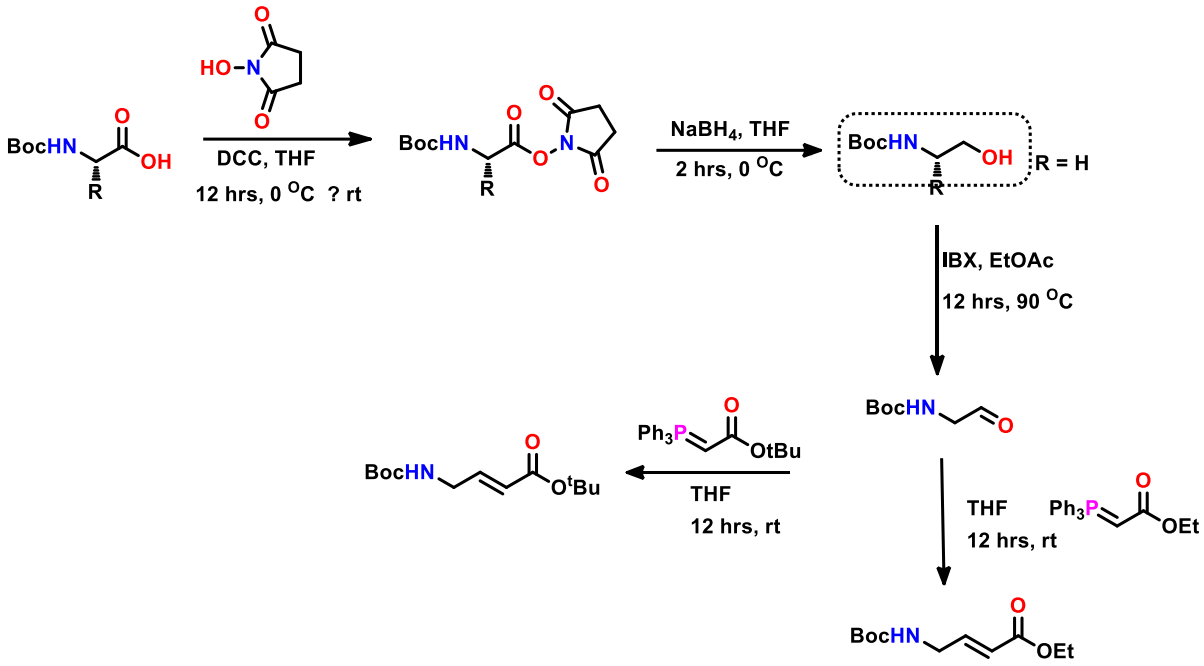
important structural constituents of a variety of natural products including oligopyrroles, heme metabolites and terpenoids.[41] Inspired the mild transformation of *E*-α, β-unsaturated γ-amino acids into Z-α,β-unsaturated γ-lactams, we sought to investigate whether the Z-γ-lactams can be introduced at the C-terminus of the peptides after the solid phase peptide synthesis. Various reports in the literature suggested it is very difficult to introduce the Z-γ-lactams at the C-terminus of peptides. So far, the Z-lactam have been introduced through solution phase synthesis.

Aim of the thesis

In this thesis, we aimed to investigate whether Z-γ-lactams can be introduced at the C-terminus of peptides synthesized via solid-phase methods involving E-vinylogous amino acids. The in-situ activation of the peptide carboxylic acid after release from the solid support may lead to the formation of Z-γ-lactam. This method may offer a mild approach for synthesizing a variety of biologically active peptide natural products. Additionally, Z-γ-lactams at the C-terminus of peptides can be explored as alternatives to maleimides for bioconjugation reactions, facilitating the attachment of different drugs and peptide conjugates containing thiol nucleophiles.

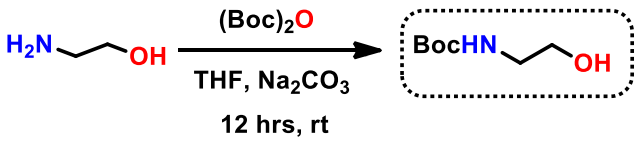
Results and Discussion

As chiral amino acids are prone for the racemization during transformation of (*E*)-α,β-unsaturated γ-amino acids into Z-γ-lactams, we chose to use achiral Gly as a starting material. The Boc-protected Gly was transformed into alkyl ester (ethyl or tert-butyl) of Boc-protected α,β-unsaturated γ-amino acid as reported earlier.[37,38] Briefly, the Boc-Gly was converted into N-hydroxysuccinimide (NHS) active ester using DCC and NHS. The active ester was converted into alcohol through the reduction using $NaBH_4$ in THF. The Boc-amino alcohol was oxidized to aldehyde using IBX. The aldehyde was subjected Wittig reaction using either ethyl 2-(triphenylphosphoranylidene)acetate or tert-butyl-2-(triphenylphosphoranylidene)acetate. The Witting product was isolated in very good yields. The reaction gave exclusively *trans* double bonds. The schematic representation of the reaction is shown in Scheme 7.



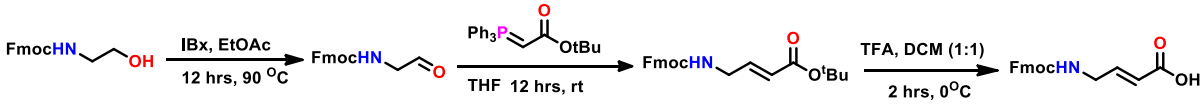
***Scheme 7**. Synthesis of alkyl esters of (E)-α,β-unsaturated γ-amino acid.*

Further, we have slightly modify the procedure for the synthesis of Boc-amino alcohol. Instead of using Gly, we start the synthesis with the commercially available ethanolamine. The schematic representation of the reaction is shown in Scheme 8. The Boc protected amino ethanol was subjected to oxidation using IBX. This route avoids the two steps along with reagents and time.

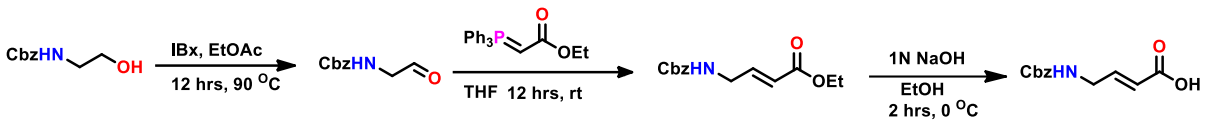


***Scheme 8**: Synthesis of Boc-amino ethanol from ethanolamine.*

Similar to the synthesis of Boc protected (E)-α,β-unsaturated γ-amino acid, we have also synthesized Fmoc protected and Cbz-protected E-α,β-unsaturated γ-amino acids starting from either ehtnaolamine or Gly. The schematic representation of the reactions are shown in the schemes 9 and 10, respectively.



Scheme 9: *Synthesis of Fmoc-protected (E)-α,β-unsaturated γ-amino acid.*



Scheme 10. *Synthesis of Cbz-protected (E)-α,β-unsaturated γ-amino acid.*

In all three methods, we obtained exclusively *E*-double bonds with comparable yields. The N-protected (*E*)-α,β-unsaturated γ-amino acids were characterized using ^{1}H and ^{13}C NMR. In addition, we also obtained the single crystals of *tert*-butyl-ester of N-Boc-protected (*E*)-α,β-unsaturated γ-amino acid and its structure is shown in Figure 8. The carbon-carbon double bond is highlighted in green colour. The double bond adopted *trans* geometry in the crystal structure.

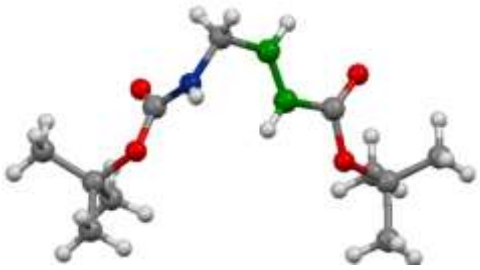
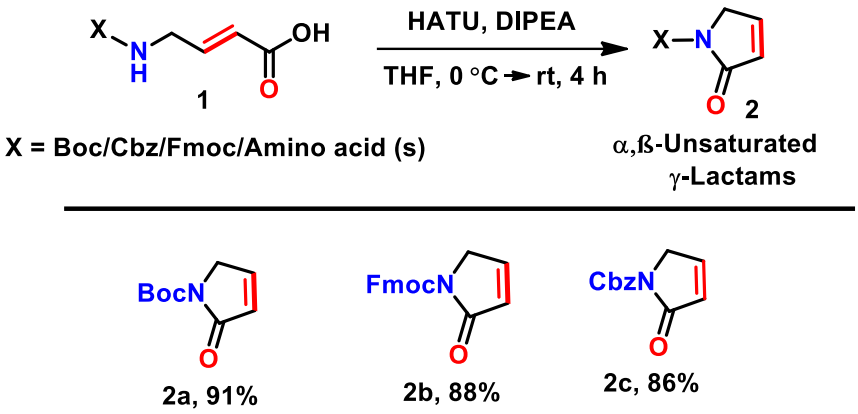


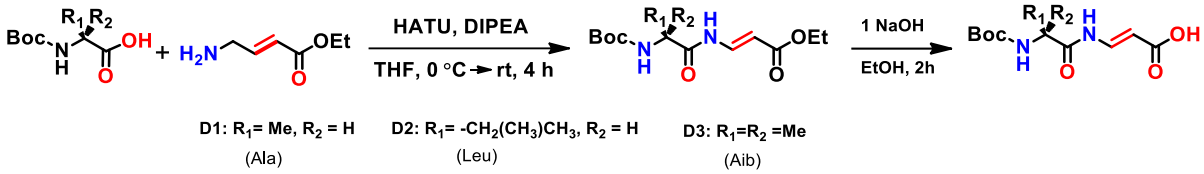
Figure 8. *X-ray structure of tert-butyl-ester of N-Boc-protected (E)-α,β-unsaturated γ-amino acid.*

In order to understand whether or not the N-protected (E)-α,β-unsaturated amino acids will undergo lactamization upon carboxylic acid activation, we subjected them for the intramolecular cyclization in the presence of HATU and DIEA. The schematic representation of the reaction is shown in the Scheme 11. Irrespective of the protection all N-protected (*E*)-α,β-unsaturated γ-amino acids were transformed into N-protected (*Z*)-α,β-unsaturated γ-lactam.

Inspired by the transformation of *E*-vinylogous amino acids into correcting *Z*-γ-lactams, motivated us to examine the transformation on peptides. In this context, we have synthesized three dipeptides using different Boc-protected amino acids to understand whether the same transformation is possible on the peptides. The schematic representation of the synthesis of Boc protected dipeptides is shown in Scheme 12. All dipeptides were characterized using ^{1}H and ^{13}C NMR.



Scheme 11: *Transformation of N-protected (E)-α,β-unsaturated amino acids into corresponding (Z)-α,β-unsaturated γ-lactams.*



Scheme 12: *Synthesis of dipeptide acids.*

Among all the dipeptides, we obtained the single crystals for the ethyl ester of dipeptide D3 and its X-ray structure is shown in Figure 10. The dipeptide adopted a β-turn type structure.

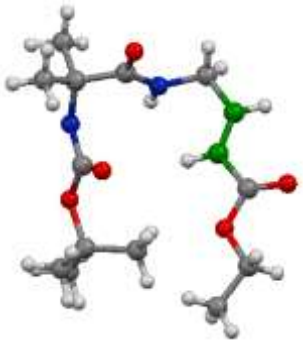
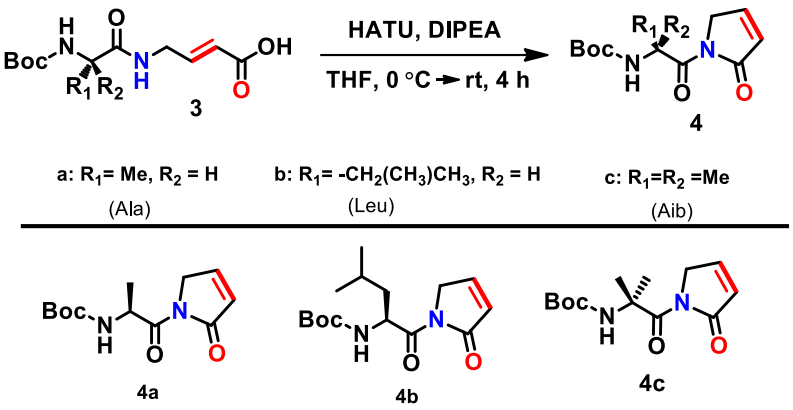


Figure 10. *X-ray structure of dipeptide D3 ethyl ester.*

The dipeptide acids were subjected to lactamization using the coupling agent HATU and the base DIPEA. The schematic representation of the reaction is shown in the Scheme 13. All dipeptides were transformed into dipeptide γ-lactams with very good yields. All lactams were characterized using ^{1}H and ^{13}C NMR.



***Scheme 13**: Synthesis of dipeptide γ-lactams*

Solid phase peptide synthesis

After the synthesis of dipeptide γ-lactams, we turned our attention to the solid phase synthesis peptides consisting of E-vinylogous amino acids at the C-terminus. To obtain free carboxylic acid at the C-terminus we chose the 2-chlorotrityl resin (CTC-resin). To understand the C-terminus lactamization on peptides, we have designed a series of peptides and synthesized using solid phase peptide synthesis. The Schematic representation of the solid phase peptide synthesis is shown in Figure 11.

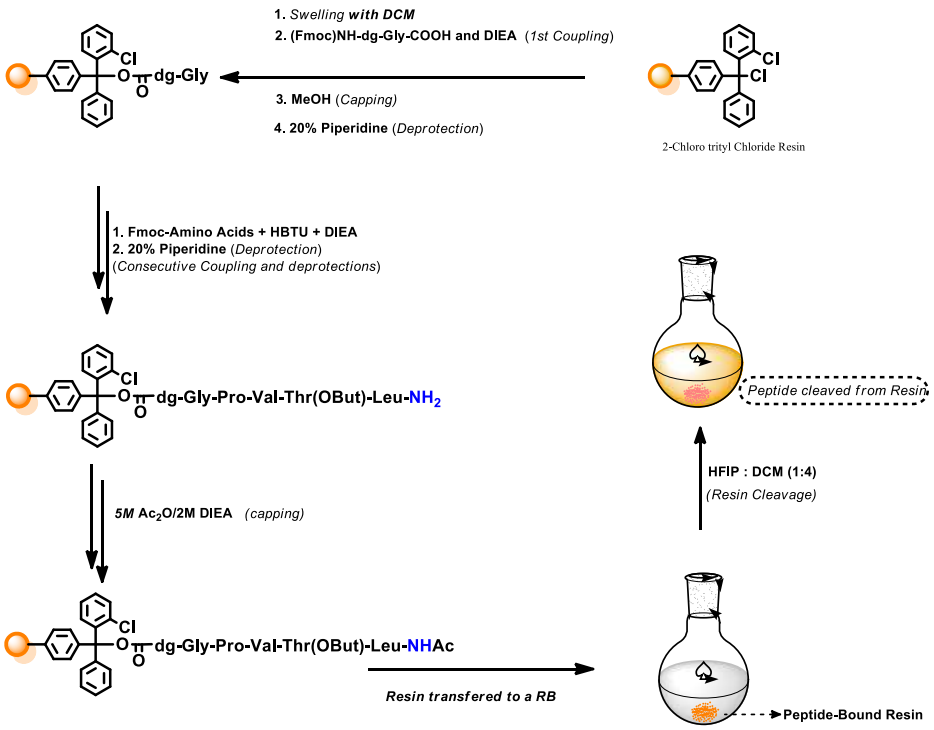
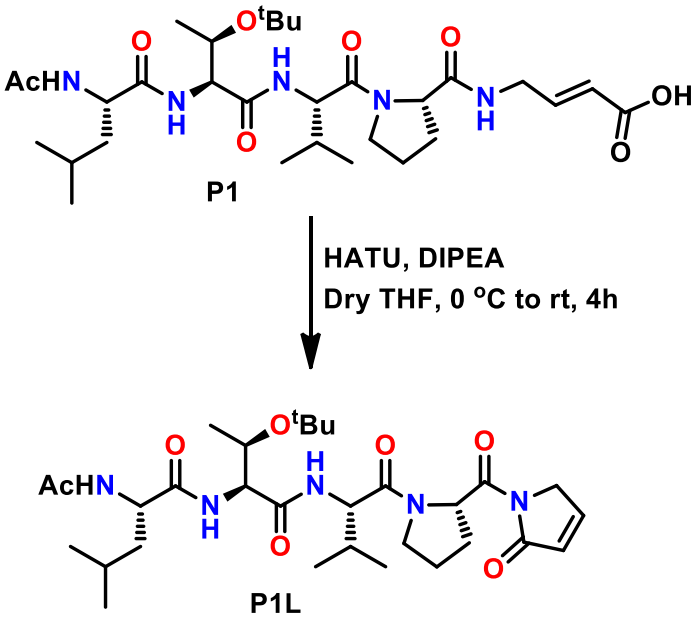


Figure 11. *The schematic representation of solid phase peptide synthesis on CTC-resin.*

To begin with, we designed and synthesized peptide **P1** using solid phase method as shown above. Peptide **P1** was released from the solid support using 1:4 mixture of hexafluoroisopropanol (HFIP) and DCM. The mixture was stirred for about 3 hrs and the resin was filtered and wash again with DCM. The filtrate was evaporated under reduced pressure and the crude peptide was purified using reverse phase HPLC. The pure peptide was subjected to lactamization using HATU and DIPEA in dry THF. The mixture was stirred for about 4 h and reaction progress was monitored using MALDI-TOF. The schematic representation is shown in Scheme 14. The peptide **P1** was completely transformed to lactam **P1L**.



Scheme 14: *Introducing Z-γ-lactams at the C-terminus of peptides synthesized from solid phase synthesis.*

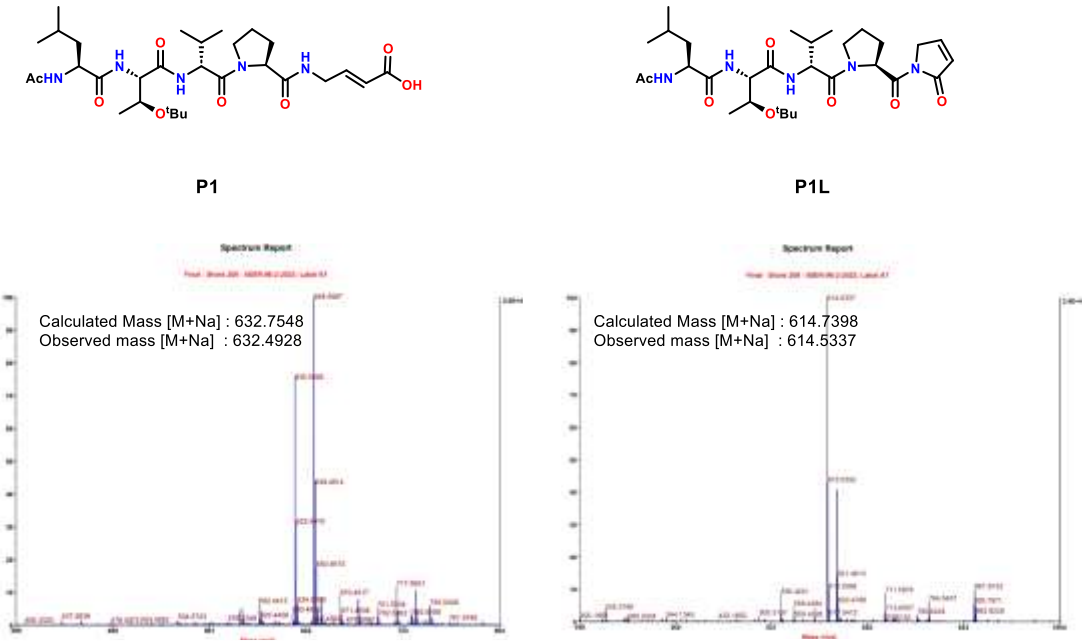
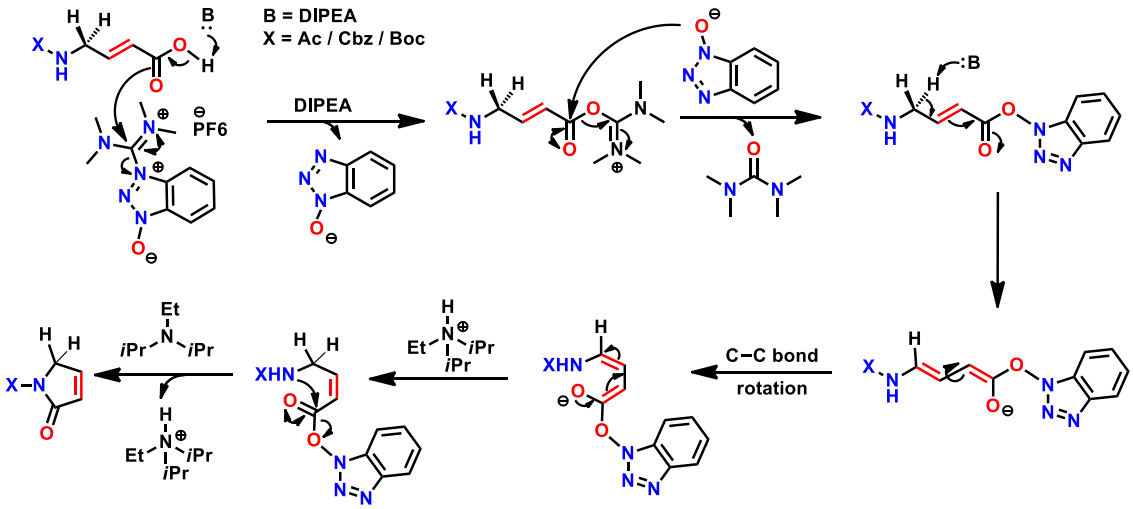
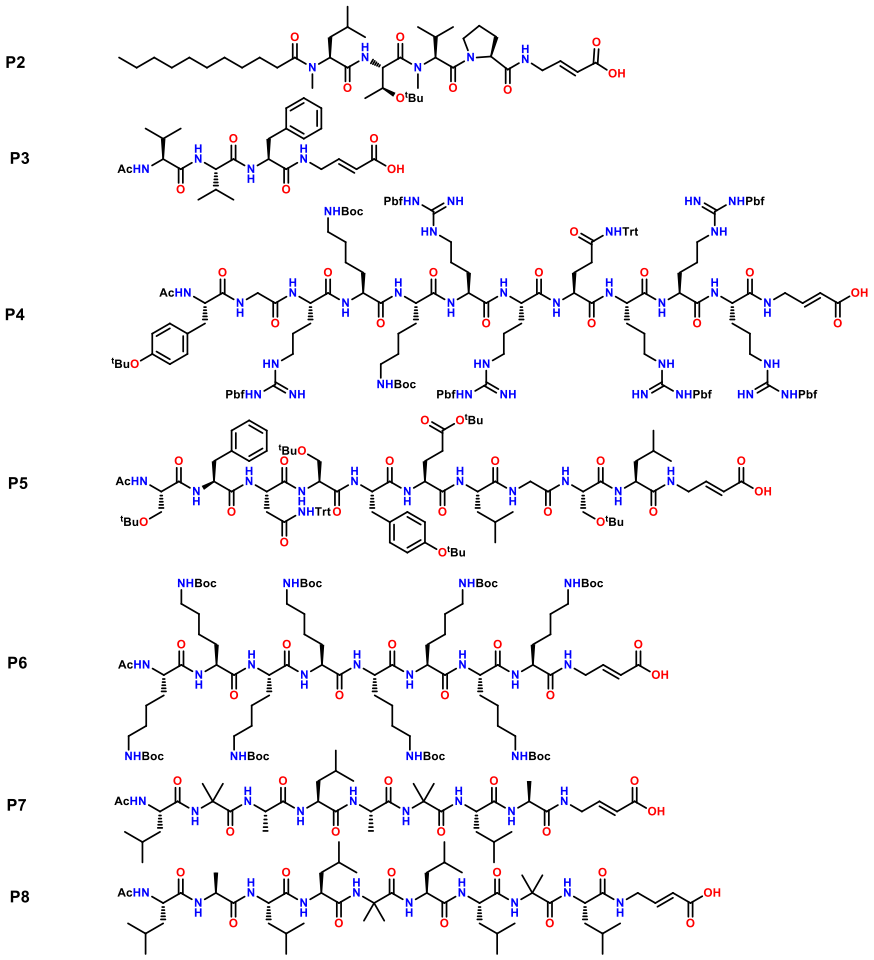


Figure 12. *Mass spectra depicting the starting peptide carboxylic acid **P1** and product **P1L** with C-terminal Z-γ-lactam.*

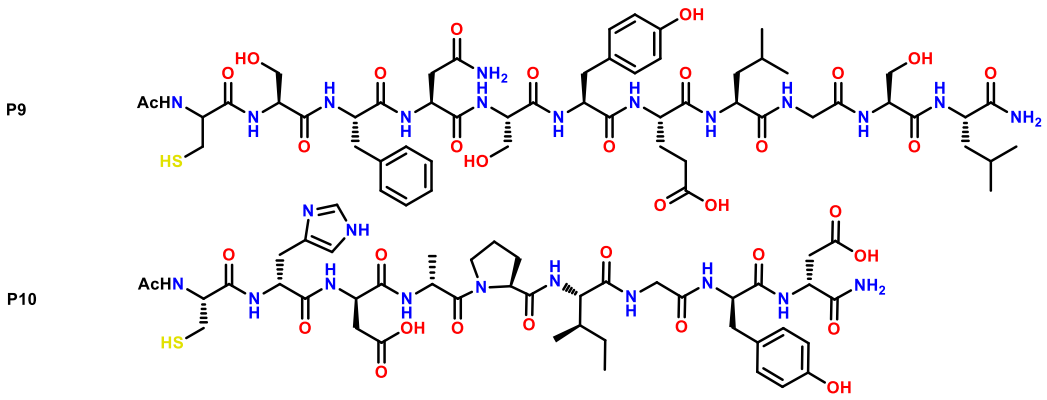
Based on the cyclization of (*E*)-vinylogous amino acids in monomers and also in peptides, we propose the possible mechanism for the transformation of (*E*)-vinylogous amino acids into γ-lactams as shown in Scheme 15 using coupling agent HBTU and DIEA.



Scheme 15. *Possible mechanism of the cyclization of E-vinylogous amino acids into Z-γ-lactams*



Scheme 16: *Peptide sequences consisting of C-terminal carboxylic acids synthesized from solid phase peptide synthesis using CTC resin.*



Scheme 17. *Peptide sequences consisting of N-terminal Cys synthesized through Rink amide resin.*

Inspired by the complete transformation of **P1** into **P1L**, motivated us to design various other peptides with different functional groups. The list of these peptides are shown in

Scheme 16. These were synthesized and their mass was confirmed by the MALDI-TOF mass spectrometry. As the lactam can be used for the bioconjugation reactions, we have also synthesized various peptides consisting of Cys residues at the N-terminus to conjugate cell permeable peptides consisting of Z-γ-lactam at the C-terminus. The list of these peptides are shown in **Scheme 17**. This work is under progress.

Conclusions

In this thesis work we have demonstrated the spontaneous cyclization of (E)-α,β-unsaturated γ-glycine into corresponding (Z)-α,β-unsaturated γ-lactam in monomers, dipeptides and peptides consisting of (E)-α,β-unsaturated amino acid at the C-terminus. The peptides with C-terminus carboxylic acids were easily synthesized from solid phase method on 2-chlorotrityl chloride resin with excellent yields. The cyclization of peptides through in situ activation using HATU or HBTU in the presence of mild base also gave excellent yield. The procedure described here can be extended to any other peptides. We are investigating the cyclization of cell permeable peptides consisting (E)-vinylogous amino acid at the C-terminus and exploring them as delivery agents after conjugating with drugs, antibodies and bioactive molecules. The easily accessible (Z)-α,β-unsaturated γ-lactams described here may serve as valuable alternatives to maleimide based bioconjugation reactions.

Experimental Section

General Method: The lactamization reactions were performed under inert conditions of nitrogen. All the amino acids, Benzyl chloroformate, Ethyl bromoacetate. Boc anhydride, N-(9-Fluorenylmethoxycarbonyloxy)succinimide, $LiAlH_4$, HATU, HBTU, Triphenyl Phosphine (PPh_3), Trifluoroacetic acid (TFA), N, N-Diisopropylethylamine (DIPEA), DMF, THF, EtOAc, Hexane, were purchased from different commercial sources. Ethyl acetate, Hexane, and THF (over sodium) were distilled before use. Column chromatography was performed on silica gel (60-120). Reactions were monitored by analytical thin layer chromatography (TLC) and confirmed by UV light chamber and $KMnO_4$ or ninhydrin staining. ^1H NMR and ^{13}C NMR spectra were

recorded in deuterated solvents on 400 MHz and 101 MHz NMR machines respectively using the residual solvent signal as the internal standard. Chemical shifts are reported in parts per million (ppm, δ) relative to tetramethyl silane (δ 0.00). ^{1}H NMR splitting patterns are assigned as singlet (s), doublet (d), broad singlet (bs), triplet (t), doublet of doublets (dd), and quintet (qn). Coupling constants (*J*) are reported in Hertz (Hz). Mass spectra were recorded using HRMS Electron Spray Ionization (ESI). The mass of the purified peptides was finally confirmed by MALDI-TOF/TOF analysis. The X-ray data were collected at low temperature (150 K) on a Bruker APEX (II) DUO CCD diffractometer using Mo K_α (λ = 0.71073 Å) graphite monochromated radiation.

Synthesis of N-Hydroxy succinimide active esters of Boc/Fmoc-/Gly

In a 250 mL RB Flask, Boc/Fmoc-Cbz-2-aminoisobutyric acid was dissolved in THF under an ice bath. N-Hydroxy Succinimide was added to the solution and left to react for 10 minutes. Afterward, DCC was added, and the reaction mixture was left to react for 12 hours. A white precipitate of DCU was formed as a byproduct. The reaction mixture was then filtered on a cealite bed with THF, and the filtrate was evaporated in a rota-evaporator to obtain crude active esters.

Reduction of the NHS active ester into primary alcohol

Following the previous reaction, the resulting product was dissolved in THF and maintained at a low temperature. The gradual addition of $NaBH_4$ was made to the reaction mixture, which was then allowed to react for an hour. To neutralize the excess $NaBH_4$, 10% HCl was used. The solvent was evaporated, and the product was extracted three times with EtOAc. The organic layer was then carefully washed with 10% Na_2CO_3 and brine solution before it was evaporated and used for the subsequent stage. All protected alcohols were isolated in good yields.

Oxidation of N-protected amino alcohols into aldehyde

The N-protected alcohol obtained was then dissolved in EtOAc. Subsequently, IBx (2-Iodoxybenzoic acid) was added into the reaction vessel, and the reaction mixture was heated under reflux condition for 12 h. After completion of the reaction, the reaction mixture was filtered and washed with EtOAc. The filtrate was then evaporated and used for the next step of the reaction. All aldehydes were isolated in quantitative yields and immediately used for the next reaction.

Synthesis of alkyl esters of E-α,β-unsaturated amino acids using Wittig reaction

The process started by dissolving the N-protected aldehyde in THF. Ethylester-Ylide was added to the reaction vessel and stir the reaction for about 12-14 hours. After completion of the reaction, THF evaporated and the remaining mixture was extracted using ethyl acetate. The product was then purified using silica column chromatography with an 8% ethyl acetate/hexane solvent system. The purified product was used for the next step.

Synthetic procedures for *N*-protected (*E*)-vinylogous amino acids

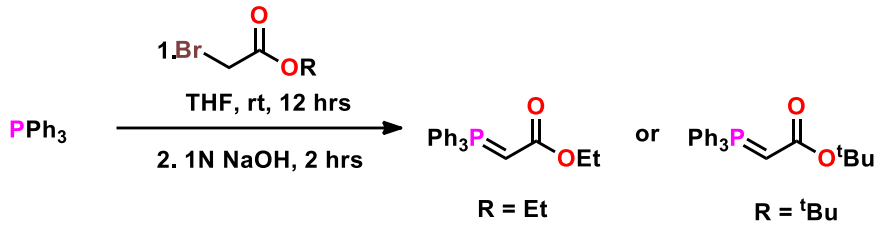
The *N*-Fmoc/Boc- or Cb-α,β-unsaturated γ-amino esters were synthesized by the corresponding N-Fmoc-/Boc or Cbz-Gly are reported earlier. Boc/Fmoc/Cbz and ethyl ester protecting groups were used to protect the N-terminus and C-terminus of the (*E*)-vinylogous glycine amino acid, respectively. The ethyl ester group of Boc/Cbz-(*E*)-$d^{2,3}$γGly-OEt at 2 mmol scale was deprotected by base hydrolysis using 1N NaOH, and Fmoc-(*E*)-$d^{2,3}$γGly-OBu^t using 1:1 TFA in DCM to obtain **1a**-**1c**.

Deprotection of Boc group.

The Boc protected amino ester was dissolved in DCM, and TFA was introduced into the reaction mixture. The reaction was allowed to proceed for a period of 3 hours. Once the reaction was complete, the solvent was removed through evaporation and the reaction mixture was rinsed with DCM around 5 to 6 times for complete evaporation of TFA.

Synthesis of Wittig Ylide

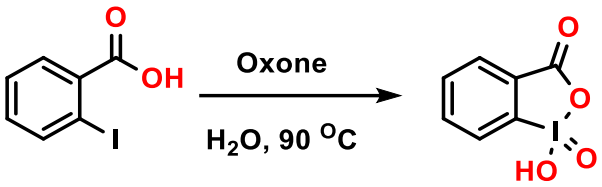
In a 500 ml round bottom flask, 50 mmol of triphenyl phosphine was dissolved in THF. Then, ethyl bromo acetate or tert-butyl bromo acetate (depending on the required product) was added in 1.2 equivalents and stirred for 12 hours. The resulting phosphorous salt was filtered and dissolved in distilled water. 1N NaOH was added to the reaction mixture which caused the ylide to precipitate out in an aqueous medium. The solid was filtered, dried and used for the Wittig reaction.



Scheme 18: *Synthesis of Wittig Ylide*

Synthesis of IBX :

2-Iodobenzoic acid was employed as the starting material for synthesizing the mild oxidizing agent. To a 1000 ml RB flask 450 ml of Distilled water was added along with Oxone (1.5 eq.) to dissolve under oil bath and waited to reach a temperature of 90 ^{O}C.



Scheme 19: *Synthesis of IBx*

(*E*)-4-((*tert*-butoxycarbonyl) amino) but-2-enoic acid (1a)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 6.99 (d, *J* = 15.6 Hz, 1H), 5.93 (d, *J* = 15.6 Hz, 1H), 4.92–4.74 (m, 1H), 3.94 (s, 2H), 1.44 (s, 9H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ_C 170.81, 155.84, 147.25, 120.87, 80.19, 41.46, 28.43. **HRMS**: *m/z* for $C_9H_{15}NO_4Na$ $(M + Na)^+$, cal. 224.0898, found 224.0892. Viscous liquid, yield = 360 mg (90%).

(*E*)-4-((((9H-fluoren-9-yl) methoxy) carbonyl) amino) but-2-enoic acid (1b)

^{1}H NMR (400 MHz, CD_3CN) δ_H 7.83 (d, J = 7.5 Hz, 2H), 7.66 (d, *J* = 7.5 Hz, 2H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.34 (t, *J* = 7.3 Hz, 2H), 6.85 (dt, *J* = 15.7, 4.7 Hz, 1H), 5.93 (s, 1H), 5.84 (d, *J* = 15.7 Hz, 1H), 4.36 (d, *J* = 6.9 Hz, 2H), 4.24 (t, *J* = 6.9 Hz, 1H), 3.86 (t, *J* = 4.1 Hz, 2H); **^{13}C NMR** (101 MHz, CD_3CN) δ_C 167.35, 147.02, 145.17, 142.16, 128.71, 128.11, 126.14, 121.24, 121.00, 67.15, 48.12, 42.16, 1.94, 1.73. **HRMS**: *m/z* for $C_{19}H_{17}NO_4Na$ $(M + Na)^+$, cal. 346.1055, found 346.1056. White solid, yield = 594 mg (92%); **m.p.** 158-161 °C; $[\alpha]_D^{25}$ = -24.0 (*c* 0.1, MeOH).

(*E*)-4-(((benzyloxy) carbonyl) amino) but-2-enoic acid (1c)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.37 (d, *J* = 3.3 Hz, 5H), 6.96 (dt, *J* = 15.7, 4.8 Hz, 1H), 5.99 (dt, *J* = 15.7, 1.9 Hz, 1H), 5.18 (s, 1H), 5.12 (s, 1H), 4.92 (d, *J* = 4.8 Hz, 1H), 4.03–3.96 (m, 2H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ_C 144.90, 128.73, 128.43, 128.34, 121.47, 76.84, 66.53, 41.92. HRMS: *m/z* for $C_{12}H_{12}NO_4$ $(M - H)^-$, cal. 234.0768, found 234.0769. Viscous liquid, yield = 418 mg (89%);

General procedure for the synthesis of achiral α,β-unsaturated γ-lactams (2a-2c).

Dissolve Boc/Fmoc/Cbz-(E)-d2,3γGly-OH (2 mmol) in 50 mL THF, then add DIPEA (6 mmol) to the mixture. Add HATU (2 mmol) at ice-cold conditions and stir the reaction mixture for 4 hours at room temperature under inert conditions. Monitor the reaction by TLC. After completion, evaporate THF using a rotary evaporator, dilute the mixture with ethyl acetate (150 mL), and wash with 10% HCl (3 × 80 mL), 10% Na_2CO_3 (3 × 80 mL), and brine solution (3 × 80 mL). Dry the organic layer with anhydrous Na_2SO_4, concentrate under reduced pressure, and purify the crude product using silica gel column chromatography with an ethyl acetate/hexane solvent system to obtain pure α,β-unsaturated γ-lactams (2a–2c).

***tert*-butyl 2-oxo-2,5-dihydro-1*H*-pyrrole-1-carboxylate (2a)**

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.18 (d, *J* = 6.1 Hz, 1H), 6.15 (dt, *J* = 6.1, 1.9 Hz, 1H), 4.34 (t, *J* = 2.0 Hz, 2H), 1.55 (s, 9H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ_C 169.32, 149.71, 145.27, 128.06, 83.16, 51.77, 28.21. HRMS: *m/z* for $C_9H_{13}NO_3Na$ $(M + Na)^+$, cal. 206.0786, found: 206.0791. Viscous liquid, yield = 311 mg (85%), R_f = 0.50 (40% EtOAc in hexane);

(9*H*-fluoren-9-yl) methyl 2-oxo-2,5-dihydro-1*H*-pyrrole-1-carboxylate (2b)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.77 (t, *J* = 6.8 Hz, 4H), 7.44–7.39 (m, 2H), 7.34 (td, *J* = 7.4, 1.0 Hz, 2H), 7.27–7.24 (m, 1H), 6.22 (dt, *J* = 6.1, 1.9 Hz, 1H), 4.55 (d, *J* = 7.3 Hz, 2H), 4.37 (t, *J* = 2.0 Hz, 2H), 4.34 (d, *J* = 7.3 Hz, 1H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ_C 168.67, 151.23, 146.02, 143.54, 141.38, 127.98, 127.76, 127.32, 125.44, 120.07, 68.66, 51.59, 46.76. **HRMS**: *m/z* for $C_{19}H_{15}NO_3Na$ $(M + Na)^+$, cal. 328.0949, found:

328.0949. White solid, yield = 536 mg (88%), **m.p.** 147-149 °C, R_f = 0.48 (40% EtOAc in hexane).

Benzyl 2-oxo-2,5-dihydro-1*H*-pyrrole-1-carboxylate (2c)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.47–7.42 (m, 2H), 7.39–7.33 (m, 3H), 7.22 (dt, *J* = 6.2, 2.1 Hz, 1H), 6.18 (dt, *J* = 6.2, 2.0 Hz, 1H), 5.32 (s, 2H), 4.41 (t, *J* = 2.0 Hz, 2H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ_C 168.87, 151.01, 145.87, 135.41, 128.74, 128.59, 128.40, 127.81, 68.17, 51.66. **HRMS**: *m/z* for $C_{12}H_{11}NO_3Na$ $(M + Na)^+$, cal. 240.0636, found 240.0632. Viscous liquid, yield = 373 mg (86%), R_f = 0.45 (40% EtOAc in hexane).

Boc-Leu-dgGly-OEt :

^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.86 (dt, *J* = 15.7, 4.9 Hz, 1H), 5.88 (dt, *J* = 15.7, 1.9 Hz, 1H), 5.10 (d, *J* = 7.8 Hz, 1H), 4.15 (q, *J* = 7.1 Hz, 3H), 4.04 – 3.96 (m, 2H), 1.71 – 1.58 (m, 2H), 1.53 – 1.44 (m, 1H), 1.40 (s, 9H), 1.24 (d, *J* = 7.1 Hz, 3H), 0.91 (dd, *J* = 7.4, 6.2 Hz, 6H).
13C : ^{13}C NMR (101 MHz, Chloroform-*d*) δ 172.99, 166.12, 156.10, 143.92, 121.69, 60.51, 40.03, 28.41, 24.84, 23.02, 14.30.

Boc-Aib-dgGly-OEt

^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.88 (dt, *J* = 15.7, 4.8 Hz, 2H), 5.94 (d, *J* = 15.7 Hz, 1H), 5.04 – 4.99 (m, 1H), 4.18 – 4.10 (m, 2H), 4.04 – 3.99 (m, 2H), 1.48 (s, 6H), 1.42 (s, 9H), 1.27 – 1.20 (m, 3H).
13C NMR (101 MHz, Chloroform-d) δ 174.84, 166.13, 154.96, 144.24, 121.41, 60.33, 56.82, 40.17, 28.29, 14.18.

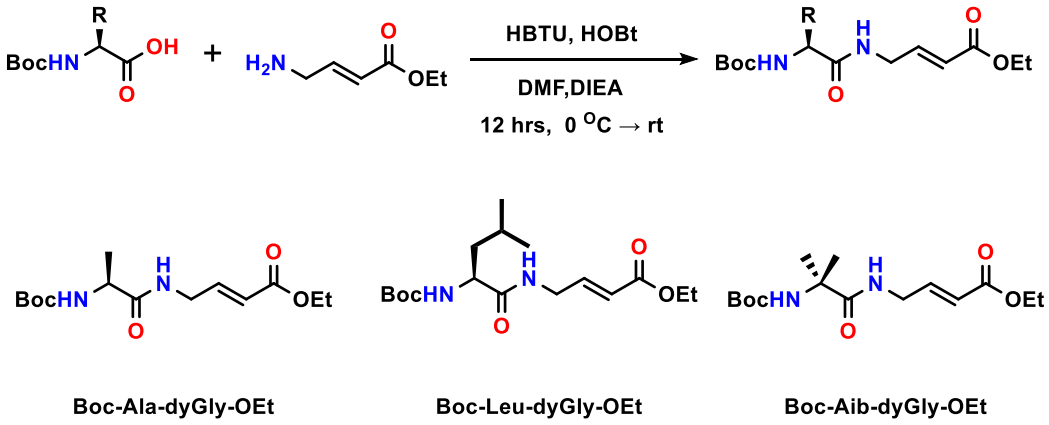
Boc-Ala-DyGly-OEt

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.22 (s, 1H), 6.89 (dt, *J* = 15.7, 4.8 Hz, 1H), 5.91 (dt, *J* = 15.7, 1.9 Hz, 1H), 5.52 (s, 1H), 4.18 (q, *J* = 7.1 Hz, 2H), 4.04 (s, 2H), 2.81 (s, 1H), 1.43 (s, 9H), 1.38 (d, *J* = 7.0 Hz, 3H), 1.27 (t, *J* = 7.1 Hz, 3H).

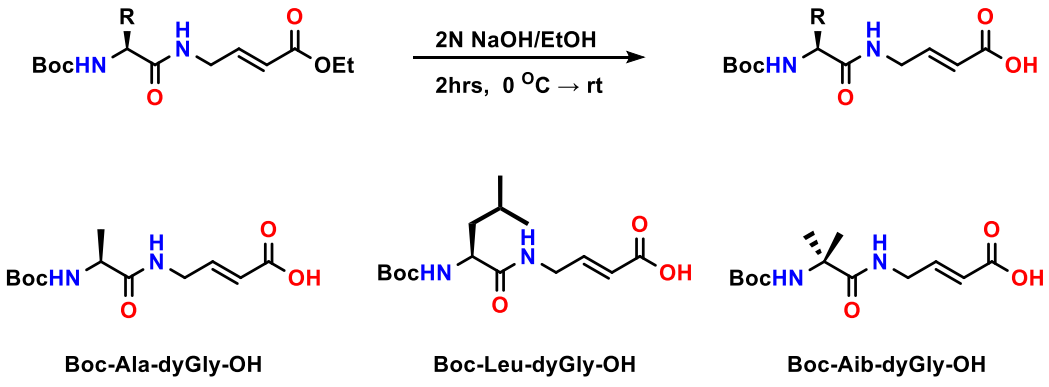
General procedure for the synthesis of dipeptide acids.

To synthesize dipeptide BocHN-Ala/Leu/Aib-d^{2,3}γGly-OEt, initially, BocHN-d^{2,3}γGly-OEt (2.2 mmol) was deprotected by treating it with a DCM-TFA mixture (1:1, 10 mL) in a 25 mL round bottom flask (RB) at room temperature for 30 minutes. The reaction progress was monitored by TLC. After completion, the solution was evaporated under

reduced pressure using a rotary evaporator. Excess TFA was removed by co-evaporating the mixture with DCM four to five times at low temperatures. The resulting TFA.H_2N-d^{2,3}γGly-OEt salt was basified with a small amount of DIPEA. In another 25 mL RB, BocHN-Ala/Leu/Aib-OH (2 mmol) and HBTU (2 mmol) were dissolved in 8 mL DMF. After some time, DIPEA (6 mmol) was added, followed by H_2N-d^{2,3}γGly-OEt (2 mmol). The reaction mixture was stirred at ice-cooled temperature for 5 minutes, then at room temperature for 6 hours. After TLC confirmed completion, the reaction was worked up by diluting with ethyl acetate (150 mL) and washing successively with 10% HCl (3 × 80 mL), 10% Na_2CO_3 (3× 80 mL), and brine solution (2 × 80 mL). The organic layer was dried over anhydrous Na_2SO_4, concentrated under reduced pressure, and the crude compound purified by silica gel column chromatography using ethyl acetate/pet-ether as eluent. The resulting dipeptide were hydrolyzed to get the desired dipeptide acids.



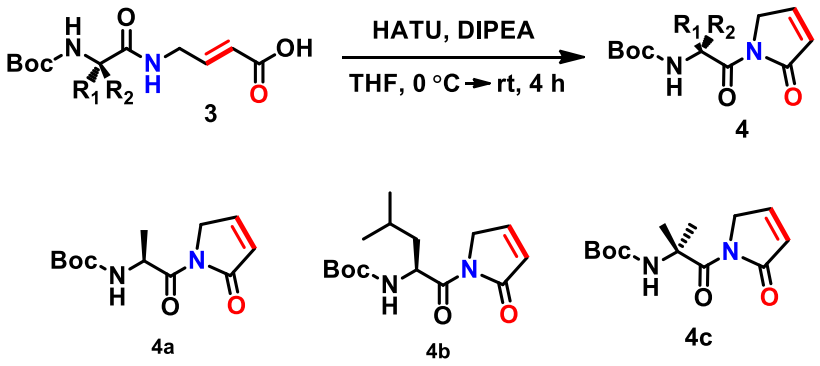
Scheme 20: *Synthesis of Dipeptides*



Scheme 21: *Hydrolysis of the ethyl esters of dipeptides*

General procedure for the lactamization of dipeptide acids (3a-3c).

Dissolve Boc-Ala/Leu/Aib-d^{2,3}γGly-OH (2 mmol) in 50 mL THF, then add DIPEA (6 mmol) to the mixture. Add HATU (2 mmol) at ice-cold conditions and stir the reaction mixture for 4 hours at room temperature under inert conditions. Monitor the reaction by TLC. After completion, evaporate THF using a rotary evaporator, dilute the mixture with ethyl acetate (150 mL), and wash with 10% HCl (3 × 80 mL), 10% Na_2CO_3 (3 × 80 mL), and brine solution (3 × 80 mL). Dry the organic layer with anhydrous Na_2SO_4, concentrate under reduced pressure, and purify the crude product using silica gel column chromatography with an ethyl acetate/hexane solvent system to obtain pure α,β-unsaturated γ-lactams (4a–4c).



General procedure for the solid phase synthesis

To synthesize peptides with C-terminal carboxylic acids, solid-phase synthesis was conducted on 2-Chlorotrityl chloride (CTC) resin at a 0.2 mmol scale using Fmoc chemistry. Initially, the 2-CTC resin was pre-swelled in dichloromethane (DCM) for approximately 60 minutes, followed by treatment with a mixture of Fmoc-d^{2,3}gGly-OH and DIPEA in DCM for 1 hour. Subsequently, all amino acids with Fmoc protection were dissolved in NMP solvent, and then HBTU and DIPEA were added to the reaction mixture. The resulting mixture was transferred to the solid-phase peptide synthesizer, and coupling was performed according to the solid-phase peptide synthesis (SPPS) protocol. Each coupling was conducted at room temperature for 45 minutes. Upon completion of peptide synthesis, the peptide-bound resin was washed sequentially with DMF (5 mL x 3), DCM (5 mL x 3), and DMF (5 mL x 3), and then dried under vacuum. To release the peptides from the resin, a mixture of HFIP:DCM (3:7, 10 mL) was added to the resin, which was stirred for 2 hours at room temperature in a 25 mL round-bottom flask. The resin was removed by filtration, and purification was

performed before further synthesis. The crude peptide was dissolved again in a small amount (approximately 5 mL) of methanol and purified using reversed-phase HPLC equipped with a C18 column as the stationary phase. Methanol and water gradient systems at a flow rate of 2 mL/min were employed for peptide purification. The mass of the purified peptides was further confirmed by MALDI-TOF analysis.

Finally, peptide acids were cyclized into the corresponding lactams with high yields using HATU and DIPEA.

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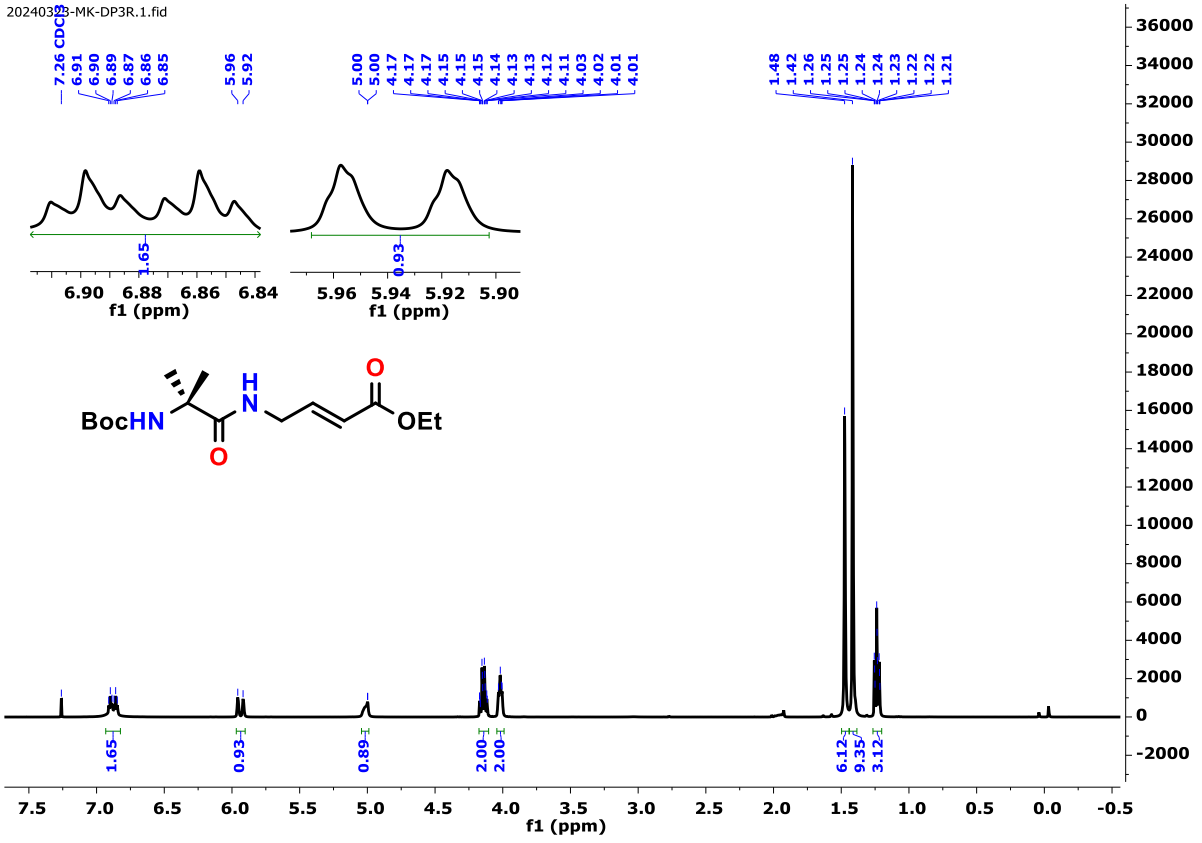
Spectra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4.15
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4.13
4.13
4.12
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4.02
4.01
4.01
1.48
1.42
1.26
1.25
1.25
1.24
1.24
1.23
1.22
1.22
1.21
1.65
0.93
6.90 6.88 6.86 6.84
f1 (ppm)
5.96 5.94 5.92 5.90
f1 (ppm)
BocHN
H
N
O
O
OEt
1.65
0.93
0.89
2.00
2.00
6.12
9.35
3.12
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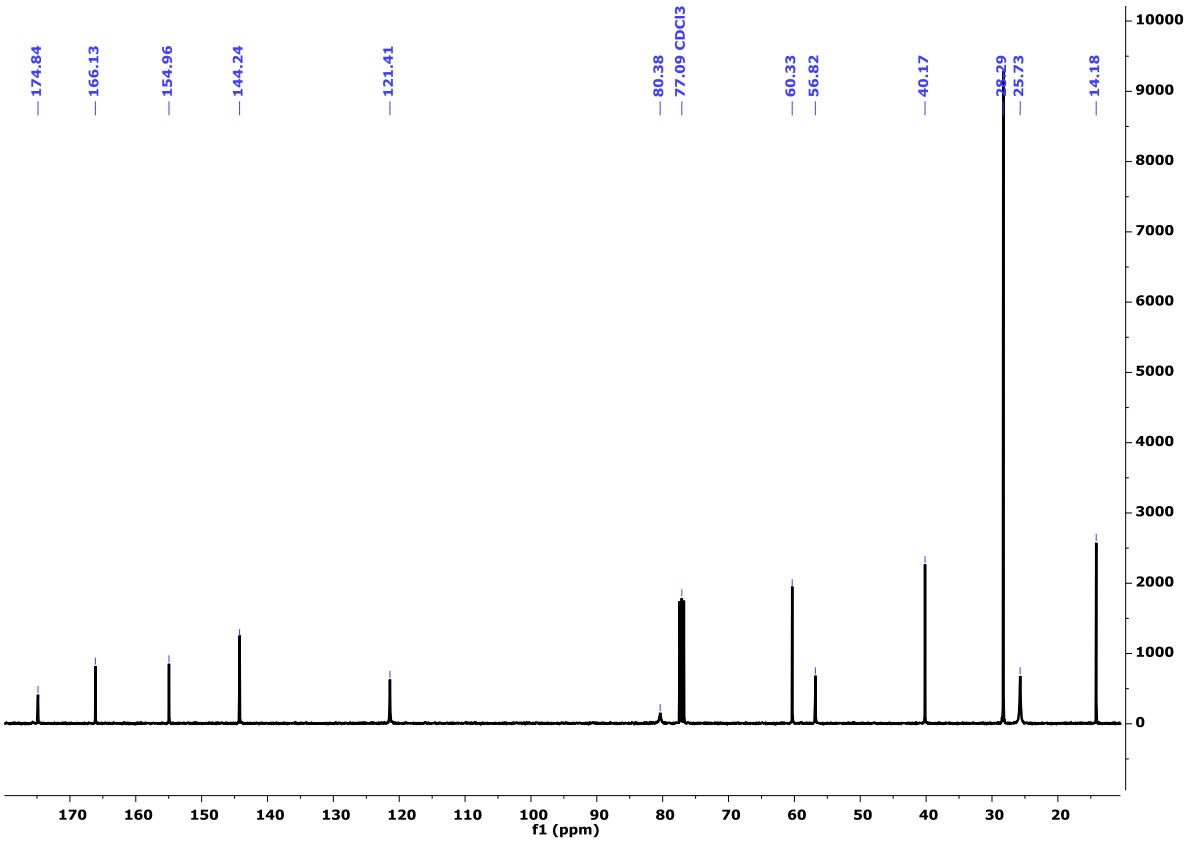
f1 (ppm)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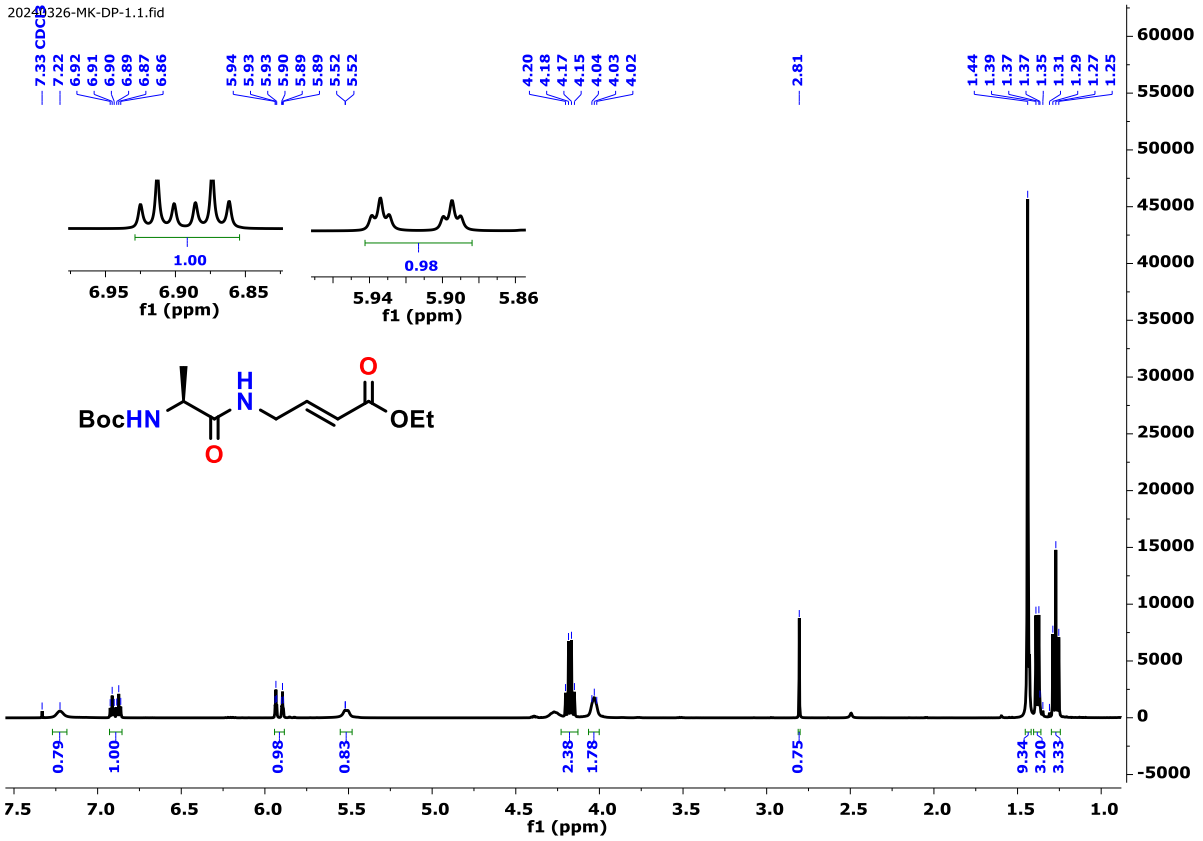
f1 (ppm)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5.93
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5.90
5.89
5.89
5.52
5.52
4.20
4.18
4.17
4.15
4.04
4.03
4.02
2.81
1.44
1.39
1.37
1.37
1.35
1.31
1.29
1.27
1.25
1.00
6.95 6.90 6.85
f1 (ppm)
0.98
5.94 5.90 5.86
f1 (ppm)
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H
N
O
O
OEt
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1.00
0.98
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2.38
1.78
0.75
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3.20
3.33
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
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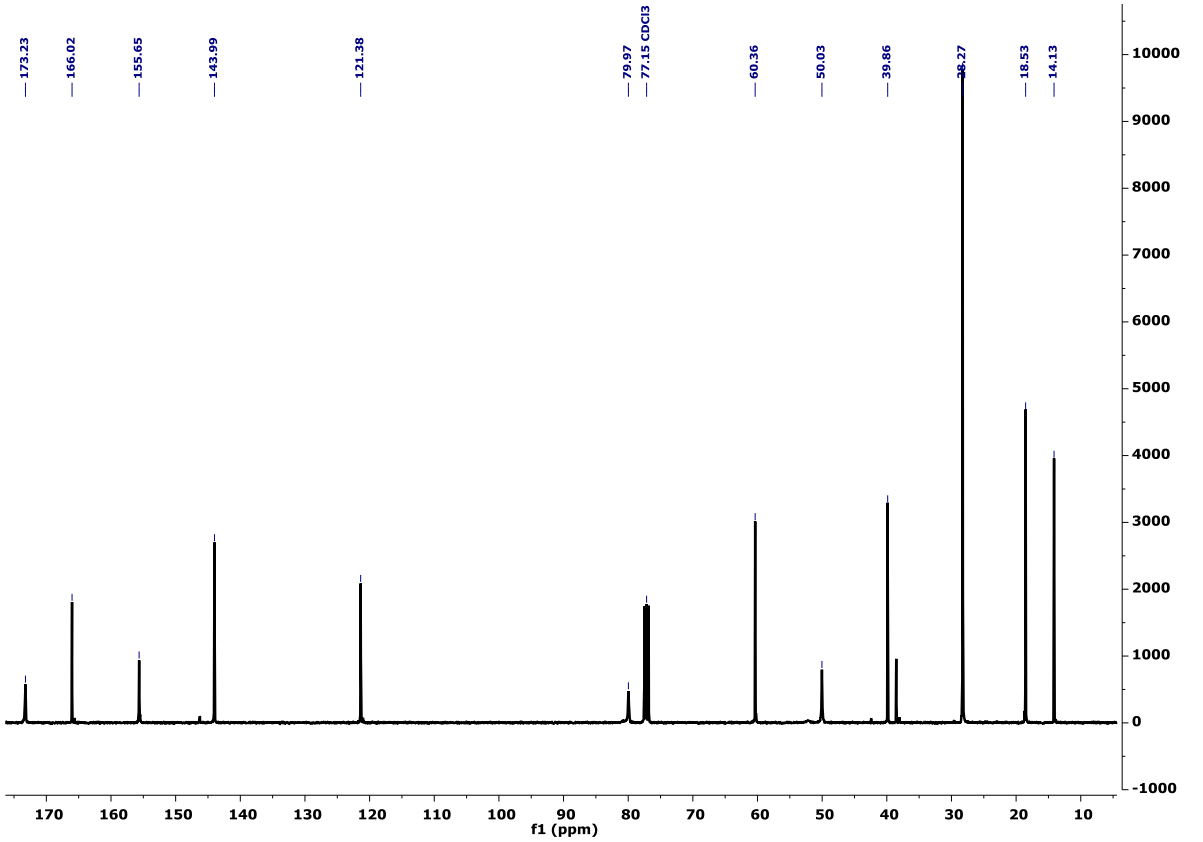
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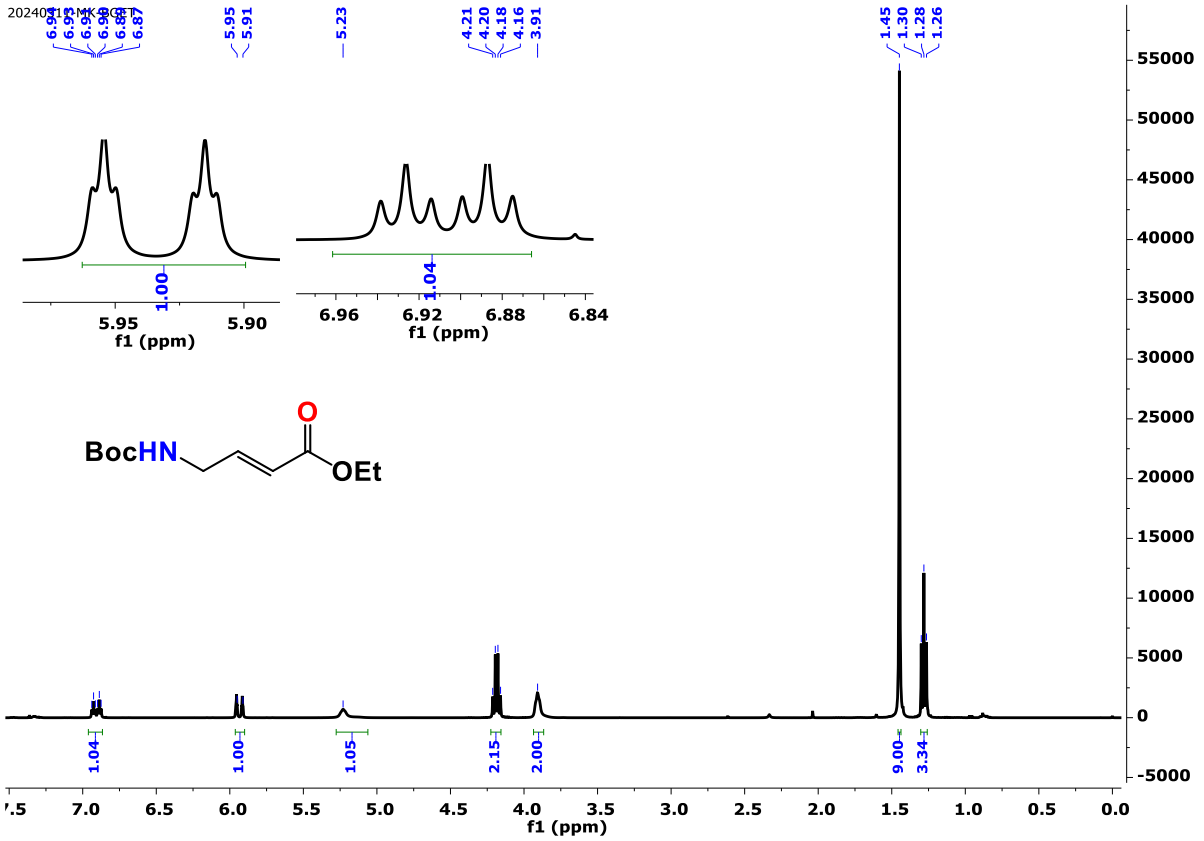
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4.16
3.91
1.45
1.30
1.28
1.26
1.00
1.04
5.95
5.90
f1 (ppm)
6.96
6.92
6.88
6.84
f1 (ppm)
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O
OEt
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1.00
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2.15
2.00
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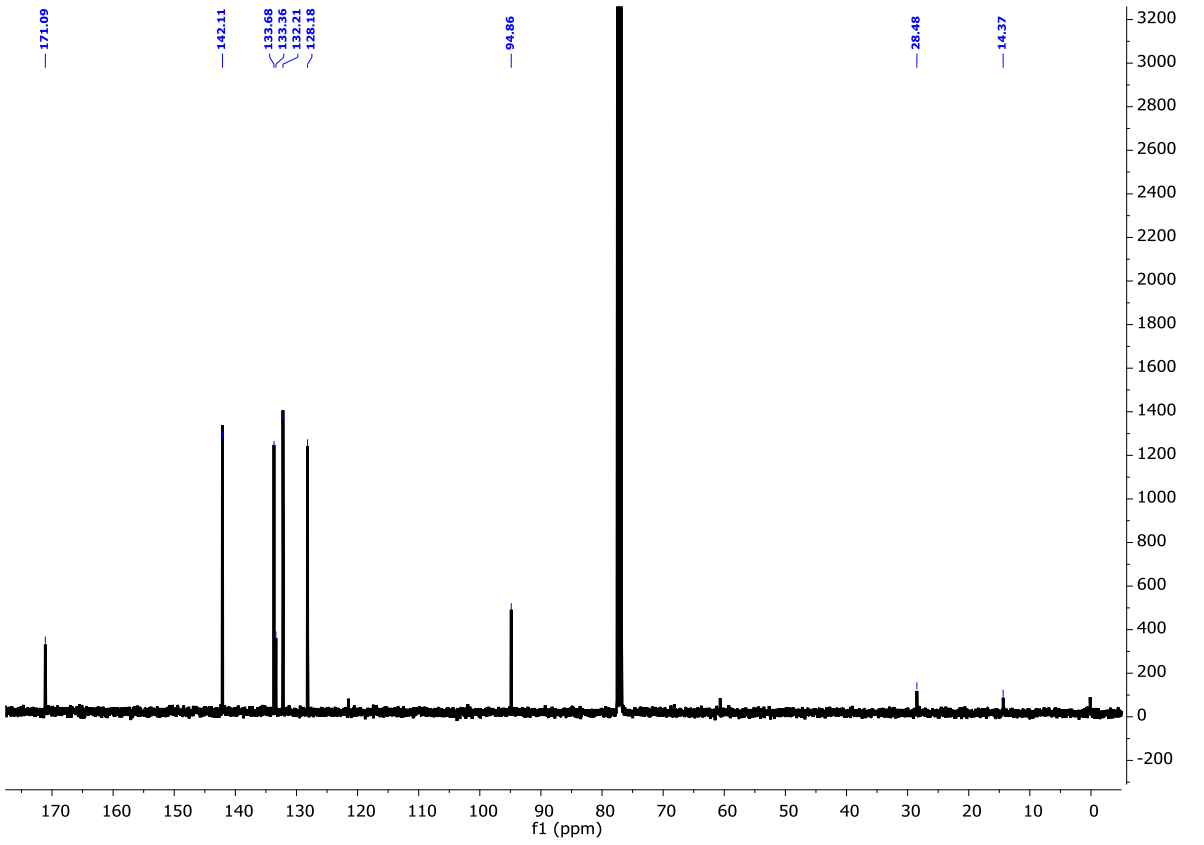
3.34
f1 (ppm)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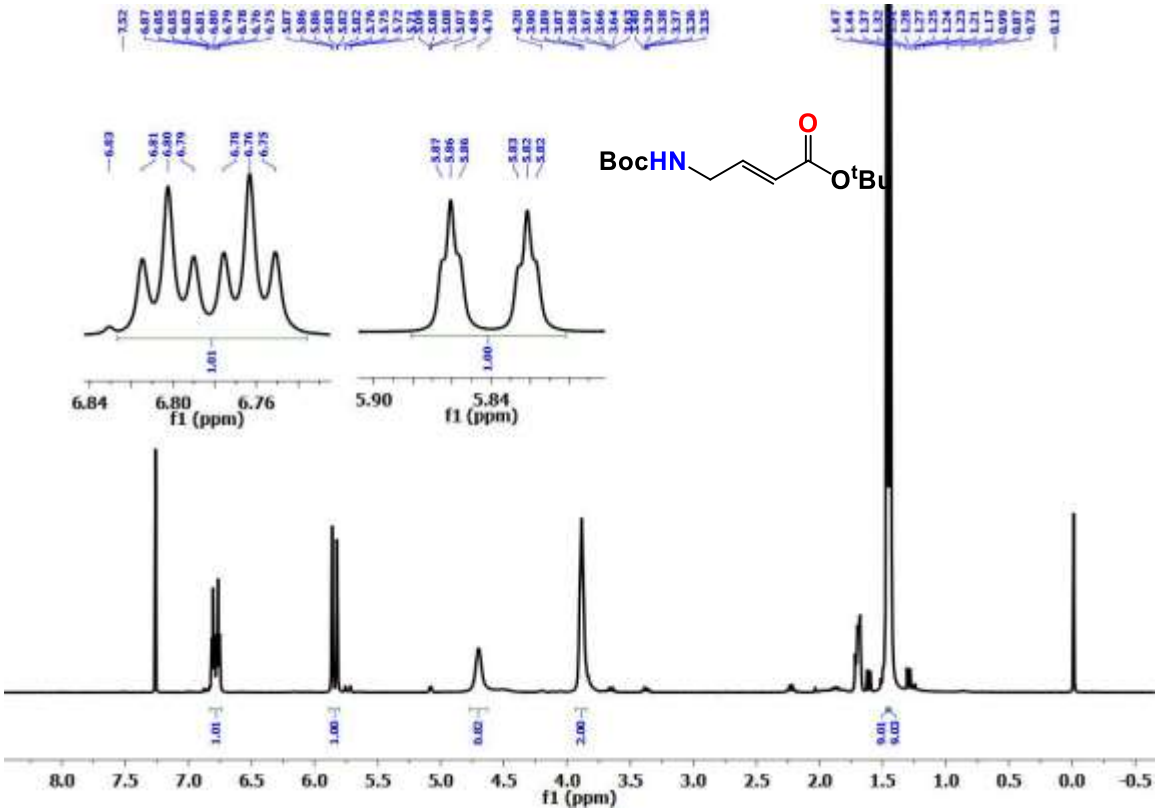
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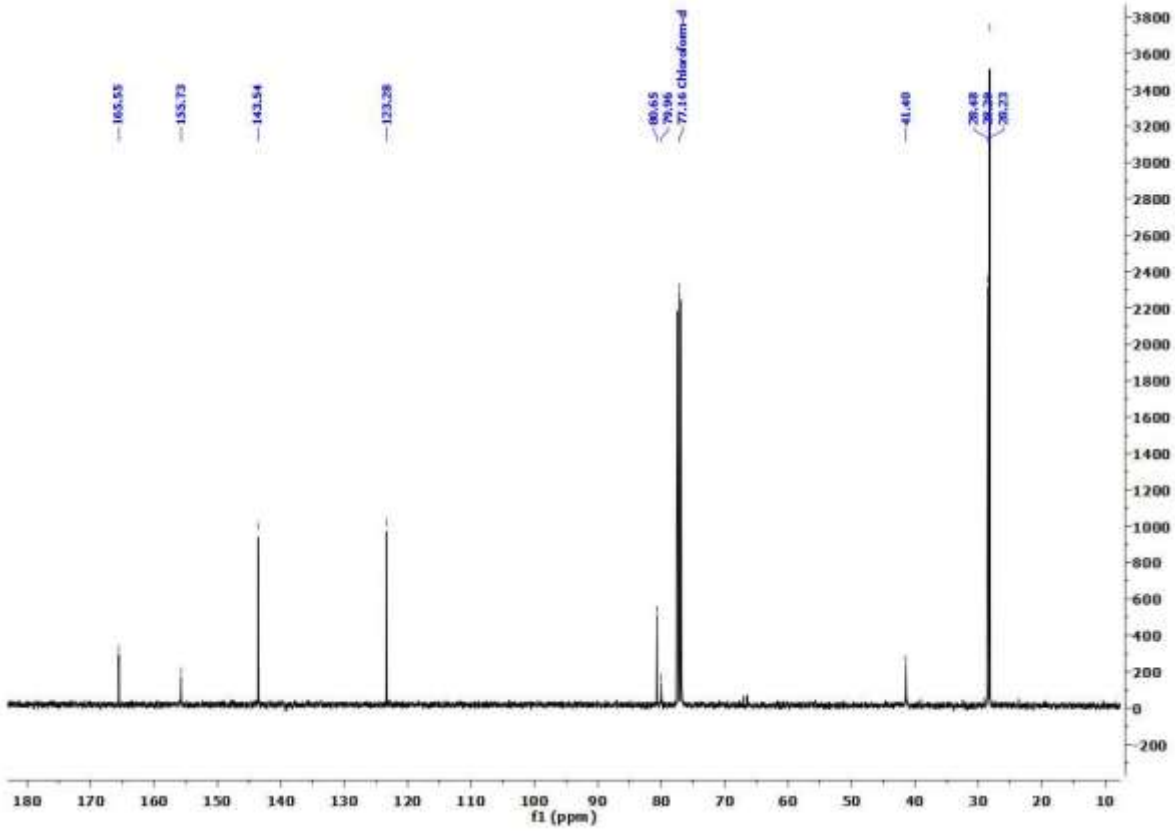
f1 (ppm)
f1 (ppm)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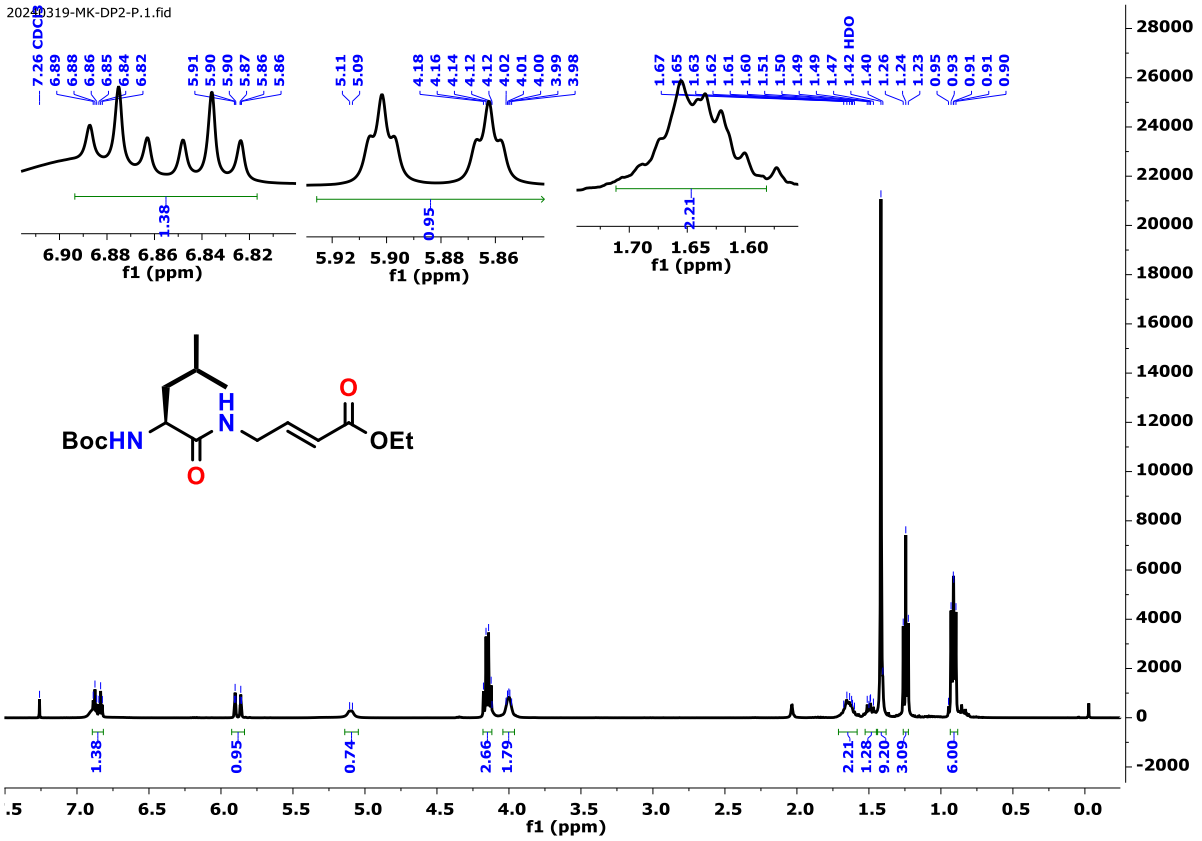
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0.95
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f1 (ppm)
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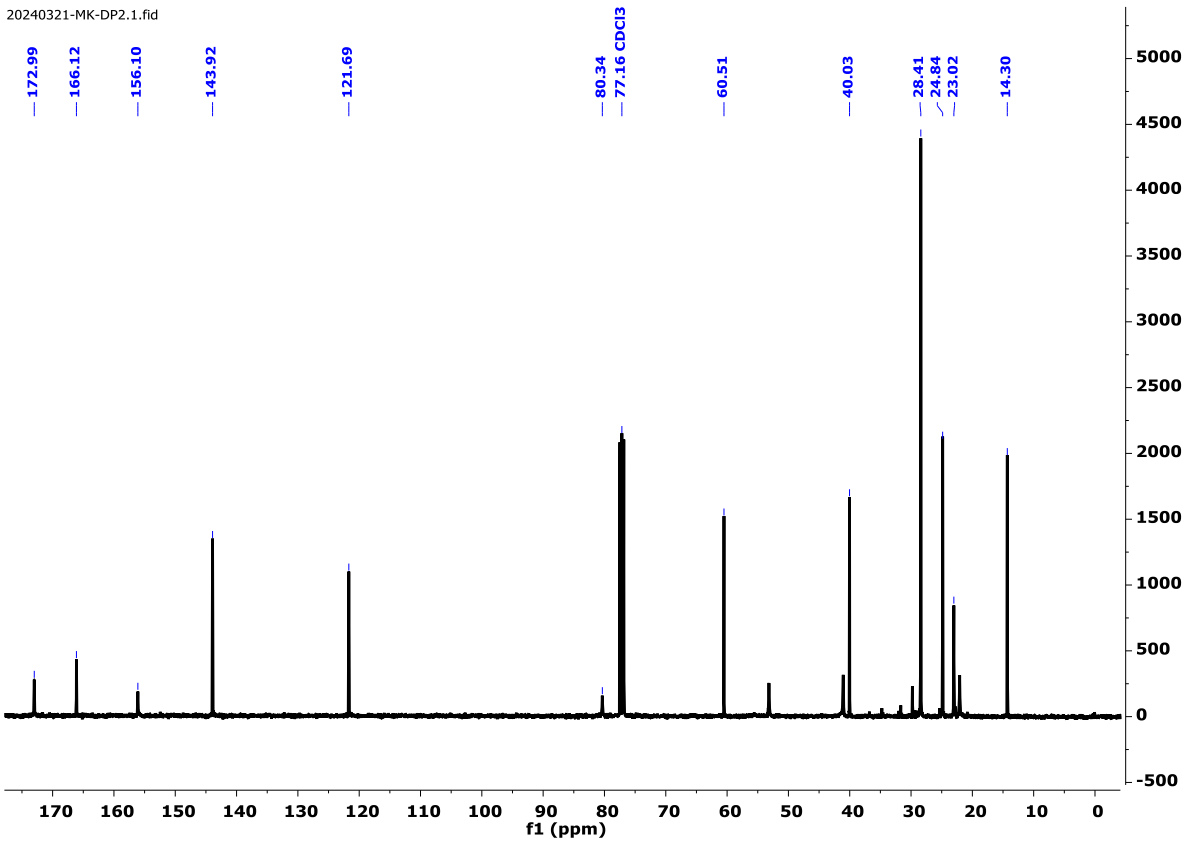
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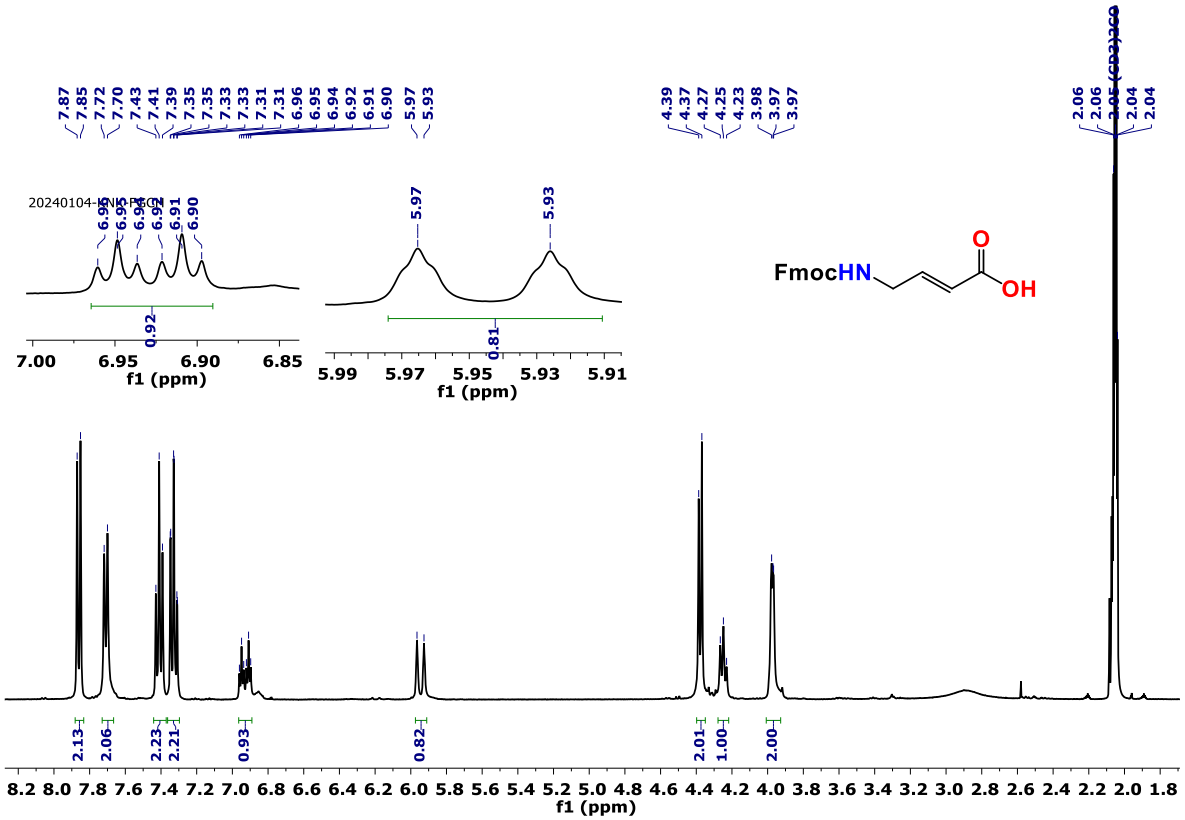
14.30
f1 (ppm)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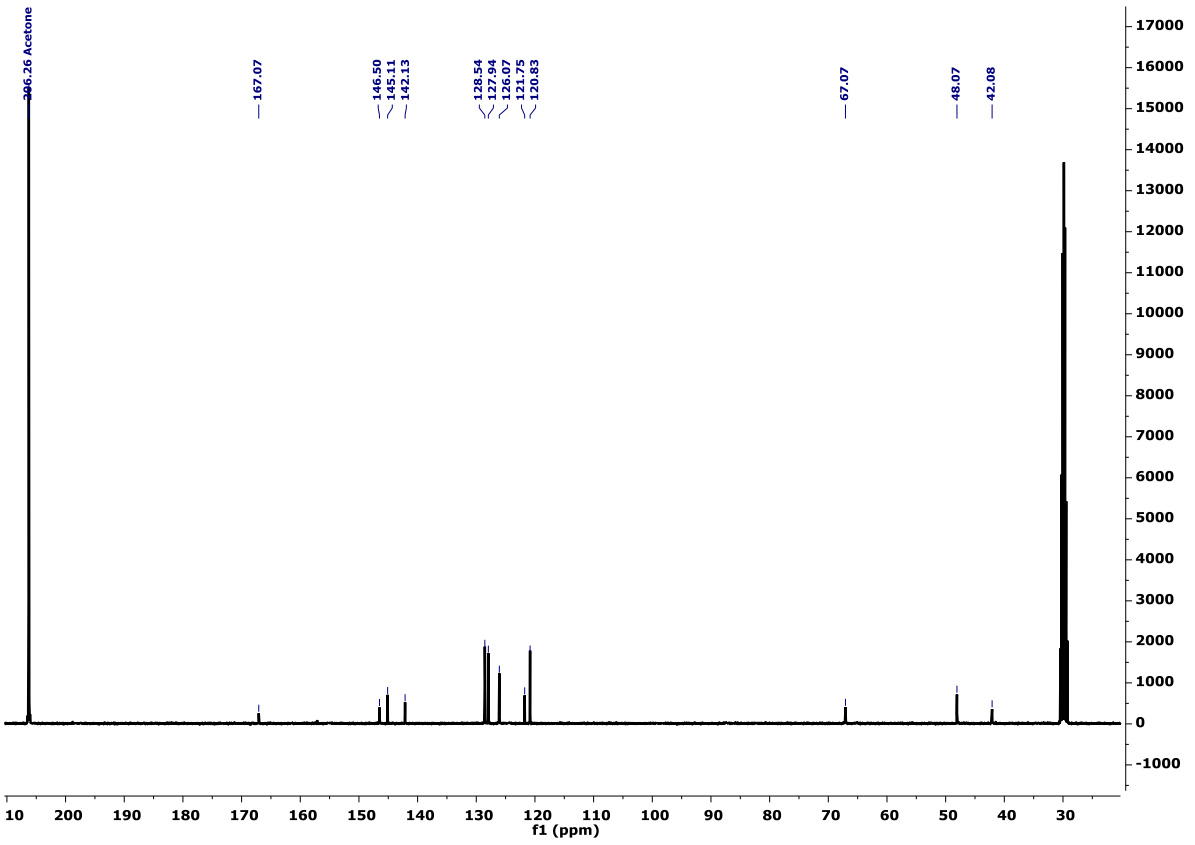
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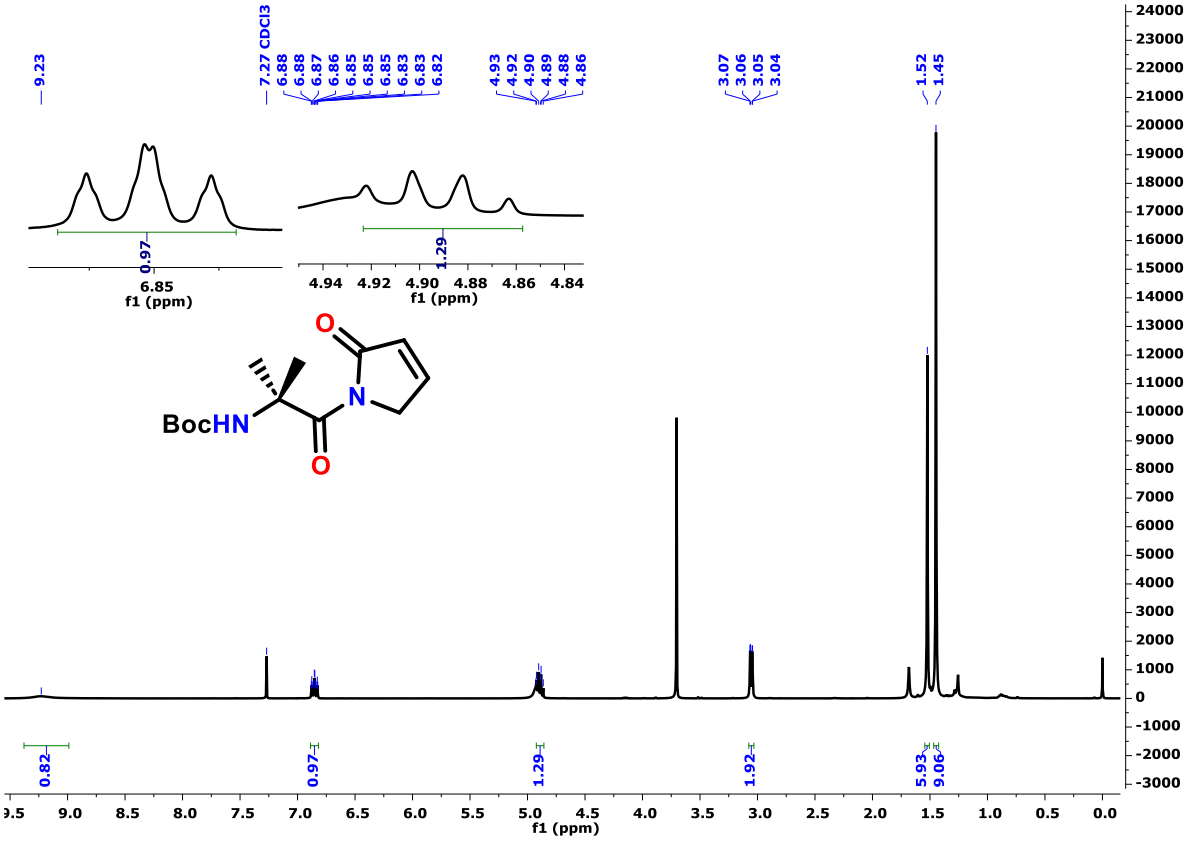
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4.89
4.88
4.86
3.07
3.06
3.05
3.04
1.52
1.45
0.97
1.29
6.85
f1 (ppm)
4.94 4.92 4.90 4.88 4.86 4.84
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1.92
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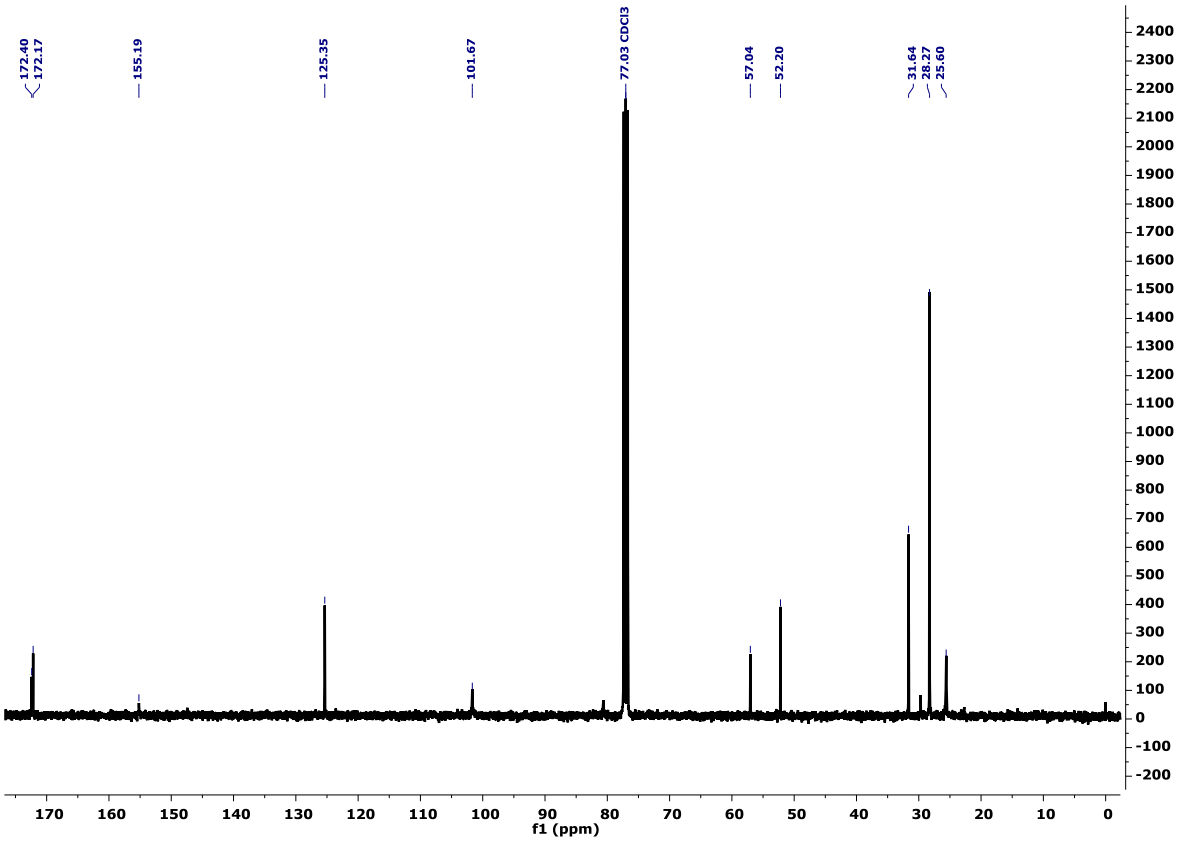
9.06
f1 (ppm)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f1 (ppm)

Spectrum Report

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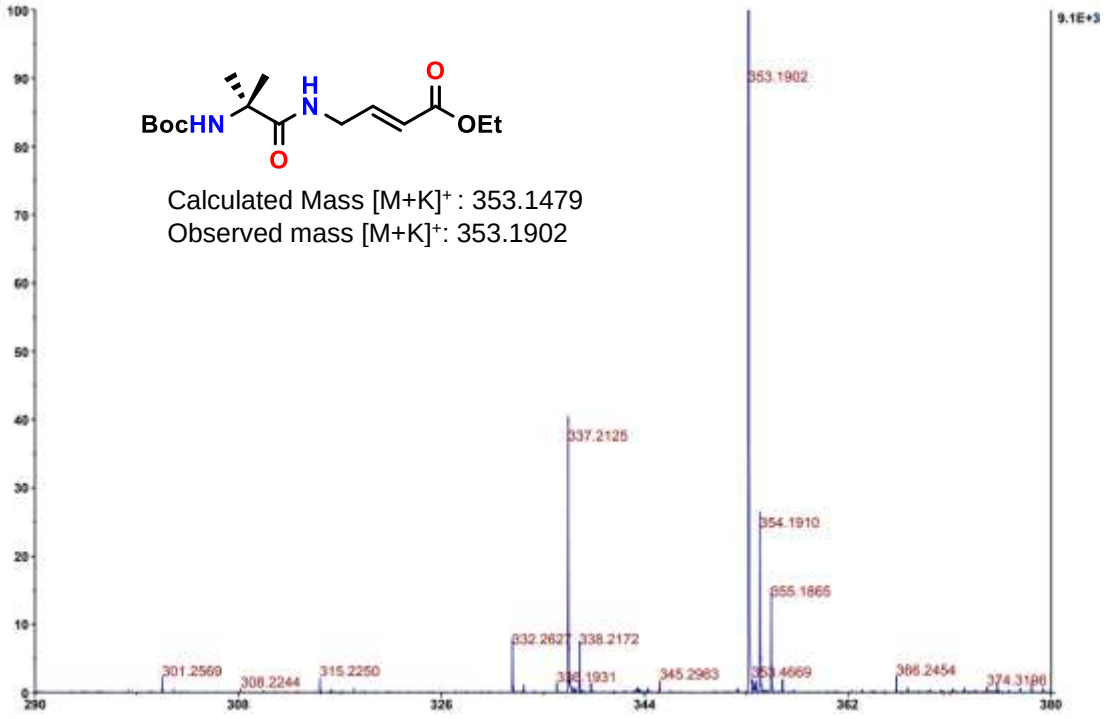

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Spectrum Report

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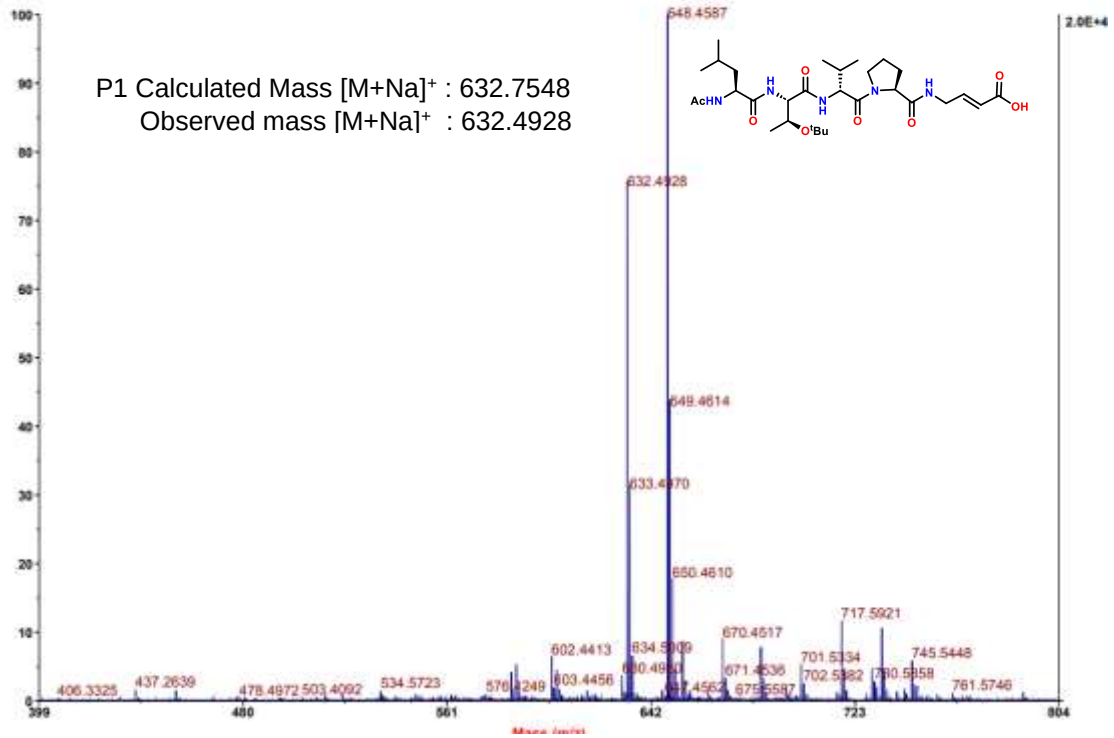

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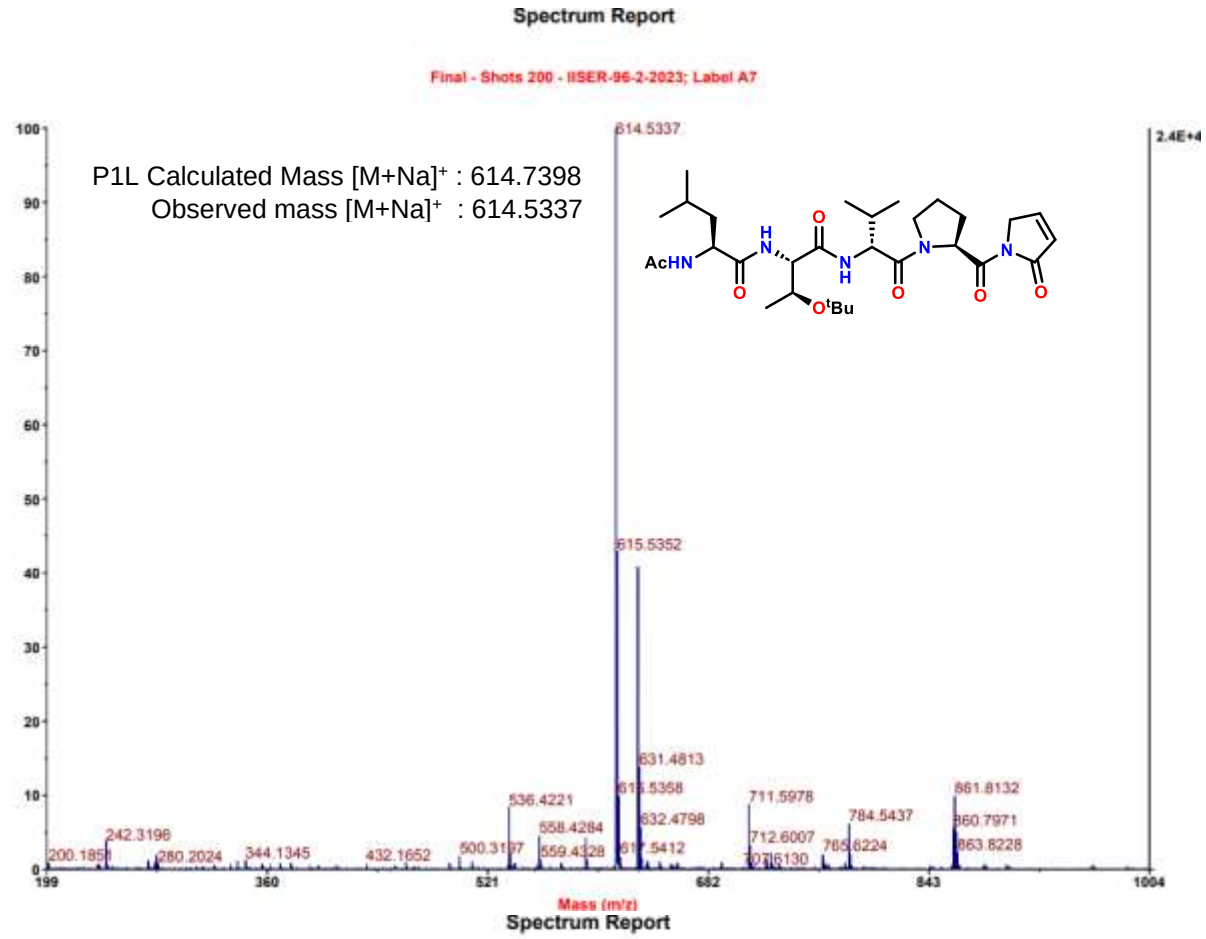
Spectrum Report
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536.4221
632.4798
711.5978
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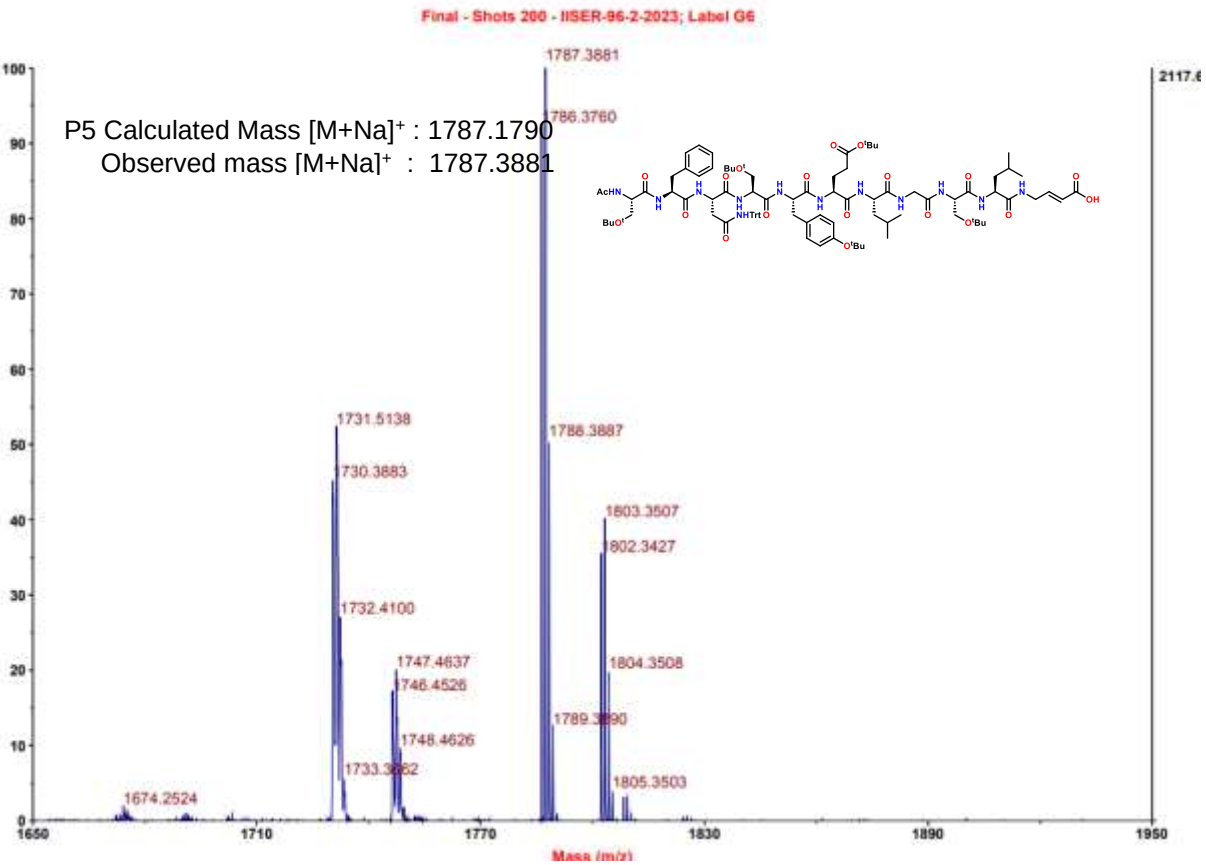
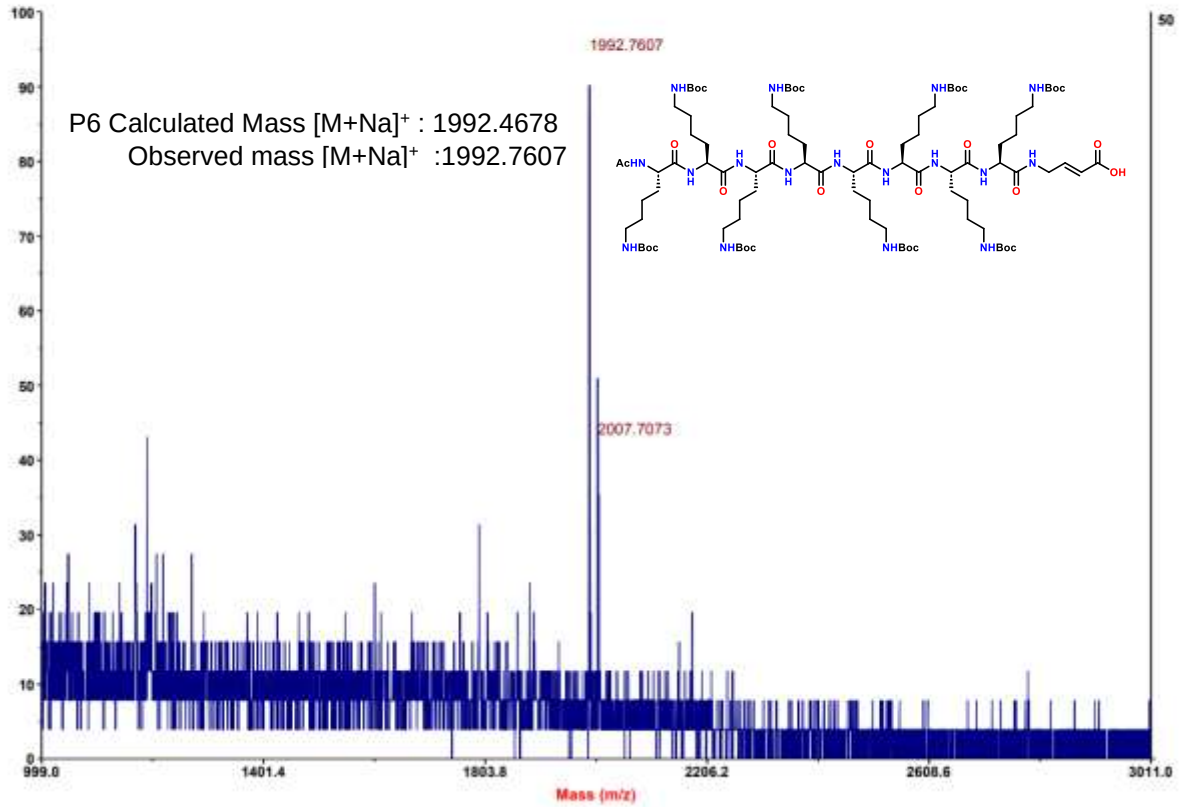
785.6224
863.8228
Mass (m/z)


Spectrum Report
Final - Shots 200 - IISER-96-2-2023; Label G6
P5 Calculated Mass [M+Na]+ : 1787.1790
Observed mass [M+Na]+ : 1787.3881
1787.3881
1786.3760
1731.5138
1788.3887
1730.3883
1803.3507
1802.3427
1732.4100
1747.4637
1804.3508
1746.4526
1748.4626
1805.3503
1674.2524
Mass (m/z)

Spectrum Report

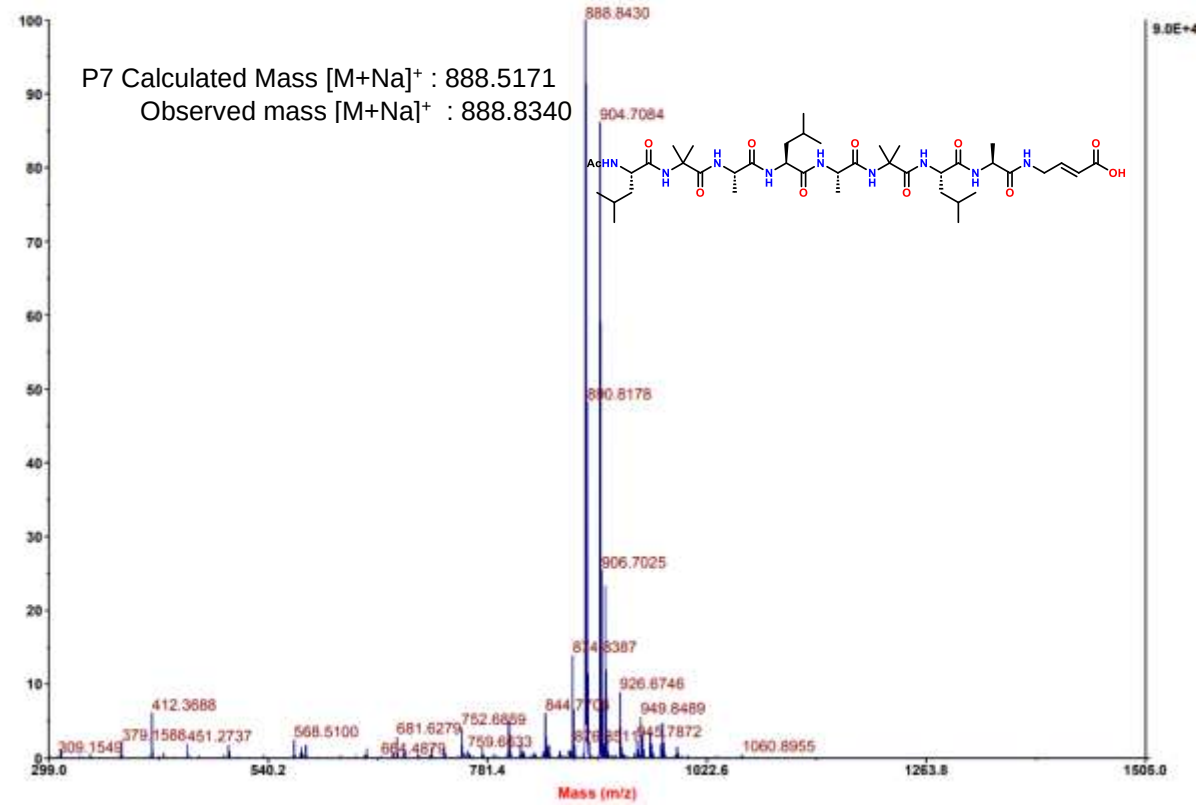
Final - Shots 200 - IISER-96-2-2023; Label C5



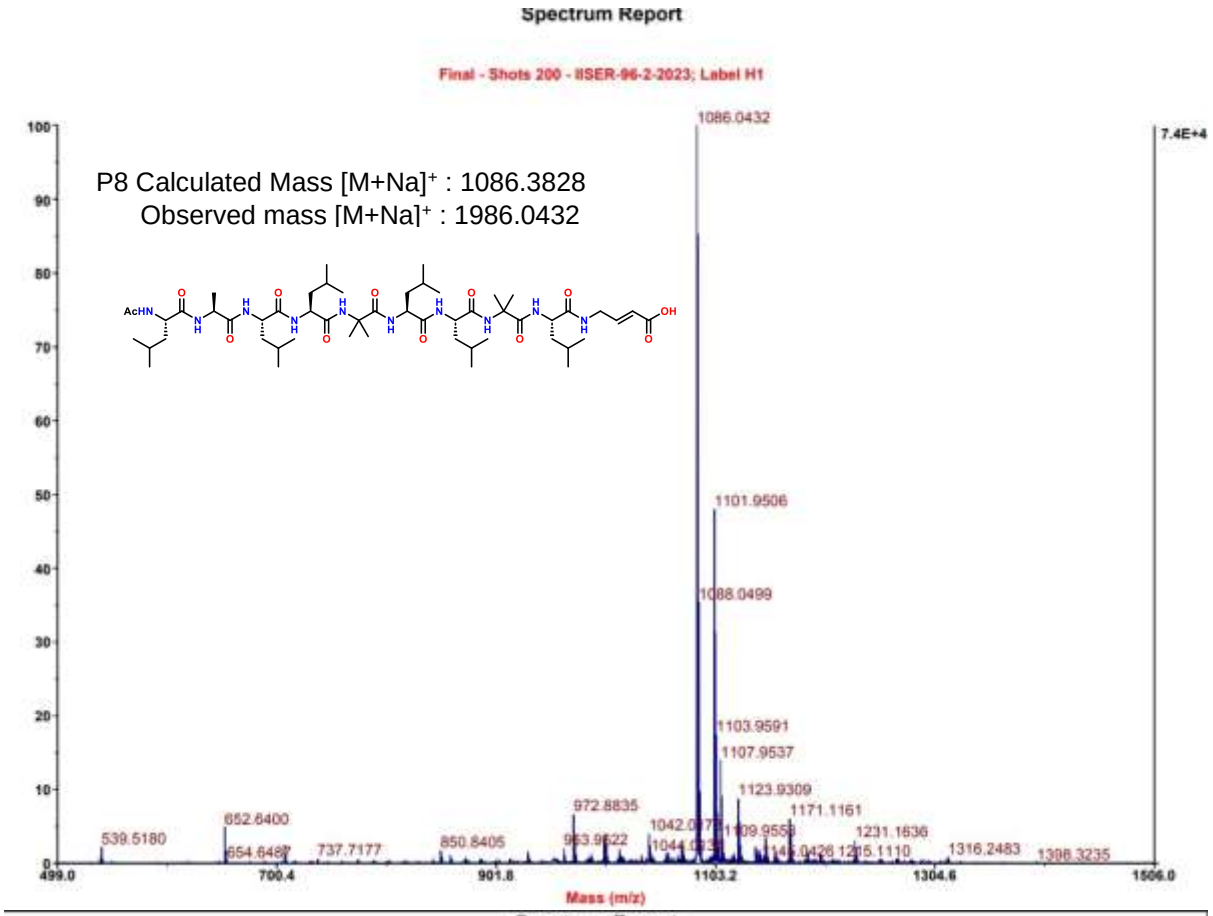
P6 Calculated Mass $[M+Na]^+$: 1992.4678
Observed mass $[M+Na]^+$:1992.7607

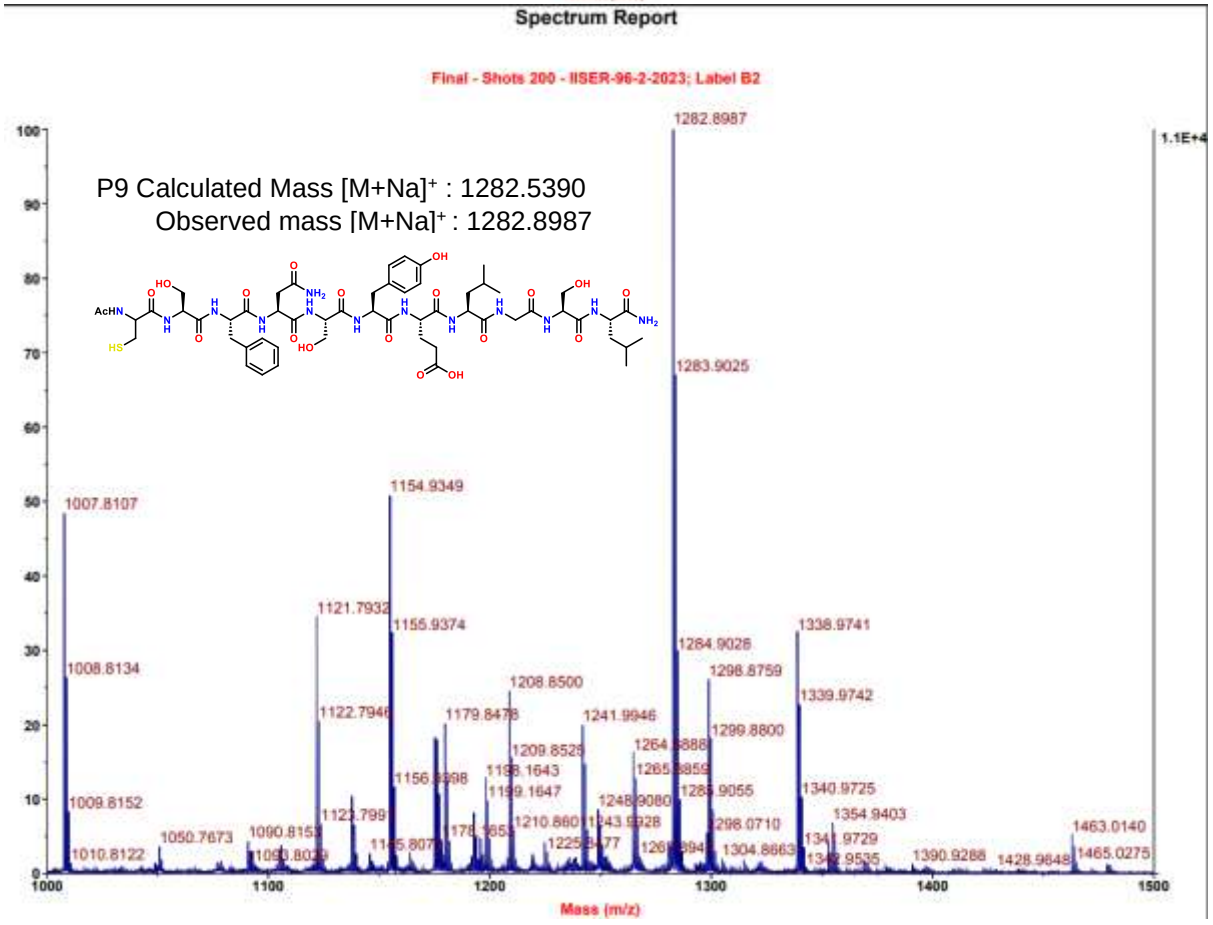
Spectrum Report

Final - Shots 200 - IISER-96-2-2023; Label G7



P7 Calculated Mass $[M+Na]^+$: 888.5171
Observed mass $[M+Na]^+$: 888.8340


Spectrum Report
Final - Shots 200 - IISER-96-2-2023; Label H1
P8 Calculated Mass [M+Na]+ : 1086.3828
Observed mass [M+Na]+ : 1986.0432
1086.0432
1101.9506
1088.0499
1103.9591
1107.9537
1123.9309
1171.1161
1231.1636
1316.2483
1398.3235
972.8835
652.6400
539.5180
654.6487
737.7177
850.8405
953.9622
Mass (m/z)


Spectrum Report
Final - Shots 200 - IISER-96-2-2023; Label B2
P9 Calculated Mass [M+Na]+ : 1282.5390
Observed mass [M+Na]+ : 1282.8987
1282.8987
1283.9025
1154.9349
1007.8107
1121.7932
1155.9374
1008.8134
1284.9028
1338.9741
1298.8759
1208.8500
1339.9742
1122.7946
1179.8478
1241.9946
1299.8800
1209.8525
1156.8998
1198.1643
1199.1647
1009.8152
1248.9080
1340.9725
1354.9403
1122.7997
1210.8601
1243.9928
1298.0710
1050.7673
1090.8151
1010.8122
1304.8663
1390.9288
1428.9848
1463.0140
1465.0275
Mass (m/z)

Spectrum Report

Final - Shots 200 - IISER-96-2-2023; Label B1

P10 Calculated Mass $[M+H]^+$: 1031.4257
Observed mass $[M+H]^+$: 1031.6731