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Ferrocene Constrained Helical Peptides via On-Resin Cyclization

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Ferrocene Constrained Helical Peptides via On-Resin Cyclization

By

Thomas Andrew McTeague

Thesis Submitted to the

Faculty of the Department of Chemistry

Of Trinity College

In Partial Fulfillment of the Requirements for the Degree Of

Bachelor of Science

In

Chemistry

Signatures of Committee

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2012

ABSTRACT

Previous research within the Curran group has demonstrated that ferrocene may be used as an organometallic constraint to induce the formation of α -helices in short peptides which traditionally possess undefined conformations. Through strategic placement of lysine residues at the i and i+3 positions within the peptide, such a constraint was accomplished via the crosslinking of the lysine side chains to ferrocene dicarboxylic acid chloride in solution phase synthesis. The aim of this work was to develop a method for solid phase peptide synthesis (SPPS) for the synthesis of these ferrocene-constrained helices. In particular, we seek to develop a method in which the nascent peptide can be cyclized while it is still anchored to the resin. For the synthesis, Fmoc-protected amino acids were utilized to afford the pentapeptide Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂. For the synthesis, Fmoc-protected amino acids and Aloc side chain protecting groups were utilized due to the mild conditions under which they are removed. This allowed the use of a Rink Amide AM resin, which is cleaved under acidic conditions. In this study it was found that on resin Pd⁰ catalyzed Aloc removal required very specific conditions and work up for efficient deprotection. Additionally, Fmoc-protected amino acids were utilized in an analogous manner to afford the pentapeptide Ac-Lys(Z)-Ala-Val-Lys(Z)-Gly-NH₂. The Z-protected pentapeptide was then removed from the resin, hydrogenated using Pd on C and subsequently cyclized with 1,1'-ferrocenedicarboxylic acid chloride. Results from on-resin cyclization indicate that a new approach must be developed in order to accomplish on-resin cyclization. Future work includes the use of different resins, different protecting groups, and different removal conditions for optimization. In solution phase, the ferrocene coupling, the data indicates that a peptide dimer with a single ferrocene was formed due to incomplete Z protecting group removal. Future work in solution phase includes hydrogenation and cyclization optimization.

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INTRODUCTION

1.1 Protein Chemistry

Within the body, proteins are responsible for a large number of the reactions essential to life. When describing the particular conformation of a protein, scientists describe protein structure in terms of four levels of organization: primary, secondary, tertiary, and quaternary. The primary structure of a protein is simply the linear arrangement of amino acids in a protein. The next level of organization is the secondary structure, which describes areas of coiling or folding which are formed via hydrogen within the protein backbone. The tertiary structure is the full three dimensional structure of a given protein. Finally, if multiple polypeptides make up a given protein complex, the protein is said to have a quaternary structure which describes the overall shape of the associated subunits.

1.2 Alpha-Helices

Among this hierarchy which describes the organization of a protein, secondary structures, in particular, are known to play a key role in many important biological activities of proteins. The most common of these structures, the α -helix, is adopted by 30% of all amino acids in proteins¹. The α -helix is a structural motif involved in an array of vital functions such as protein-DNA interactions², protein-protein interactions³, and membrane-bound receptors⁴. In the ideal α -helix hydrogen bonds form between amide protons and carbonyl groups of amino acids at the i and $i+4$ positions⁵ (see Figure 1.1).

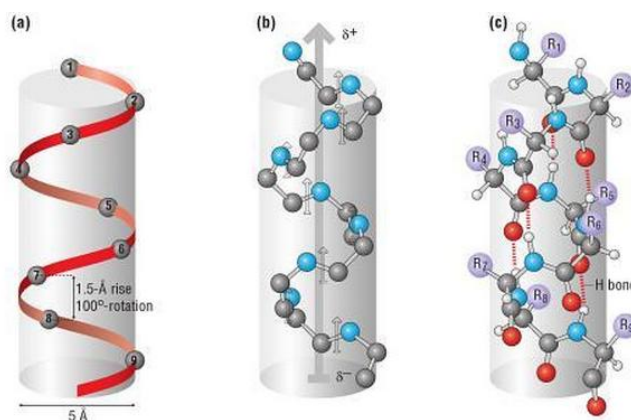


Figure 1.1: An α -helix with (a) C_{α} labeled, (b) peptide backbone labeled, (c) full structure and hydrogen bonds shown⁶.

In this particular conformation there are 3.6 amino acids per complete turn with C_{α} -N bond angles (Φ) of $-64 \pm 7^{\circ}$ and C_{α} - C_{β} bond angles (ψ) of $-41 \pm 7^{\circ}$.⁷

Due to the biological prevalence of α -helices, it is crucial that researchers have accessible model systems in order to further probe the intricacies of helix formation and its bioactivity. One of the largest complications of this need arises from the energetic difficulty which short peptide strands face in forming well-defined conformations due to their inherent flexibility⁸. Currently researchers have devised several reasonably successful methods to induce well defined helices using various conformational constraints^{9,10,11} which can be mainly categorized into three major categories: template, covalent and metal systems.

1.3 Alpha-Helix Prediction

Although researchers have several synthetic methods by which to nucleate alpha-helices, several statistical models can help predict whether a given peptide will form an alpha-helix. Previous analysis has shown that certain amino acids exhibit higher frequencies for different secondary structures. In particular, methionine, alanine, leucine, lysine, and uncharged glutamate have particularly high propensities to form alpha-helices¹². Since the 1960s, researchers have also developed several computational methods to better predict alpha-helix formation, each of which has a varying degree of accuracy depending on the system being analyzed. The Chou-Fasman method relies heavily on the frequency of appearance of each amino acid in alpha-helices and has been shown to be roughly 50-60% accurate at predicting secondary structures¹³. A similar method developed by Garnier, Osguthorpe, and Robson (GOR) uses a similar method that not only takes into account the probability of each amino acid adopting a particular secondary structure but also the contributions of each amino acid's neighbors *a priori* to comparison with proteins of known structure¹⁴. Due to this difference, the GOR method is more sensitive and more accurate (around 65%) than the Chou-Fasman method.

A separate computational approach to predicting secondary structures is to analyze the sequences of

proteins with known structures and compare them to the desired system. Neural network methods machines use this approach by grouping amino acid sequences into sets to identify common sequence motifs of various secondary structures. Similarly based support vector machines use an analogous processing algorithm to achieve the same goal. These methods are typically greater than 70% accurate¹³.

In more recent history, many of these prediction systems have been optimized and modified to include a number of other factors which have also been found to affect secondary structure formation¹⁴, including solvent systems, accessible residue surface area¹⁶, and protein structural class¹⁷.

1.4 The Template Approach

In template systems, the constraint is placed at one end of the peptide chain, which then serves to nucleate the formation of an α -helix. Typically, templates consist of semi-flexible cyclic systems containing multiple carbonyl groups which can participate in hydrogen bonds with the amide protons of the peptide backbone or within their cyclic framework⁹. Using this approach, several laboratories have been relatively successful at synthesizing a number of compounds which tend to adopt α -helical structures.^{18,19,20} (See Figure 1.2).

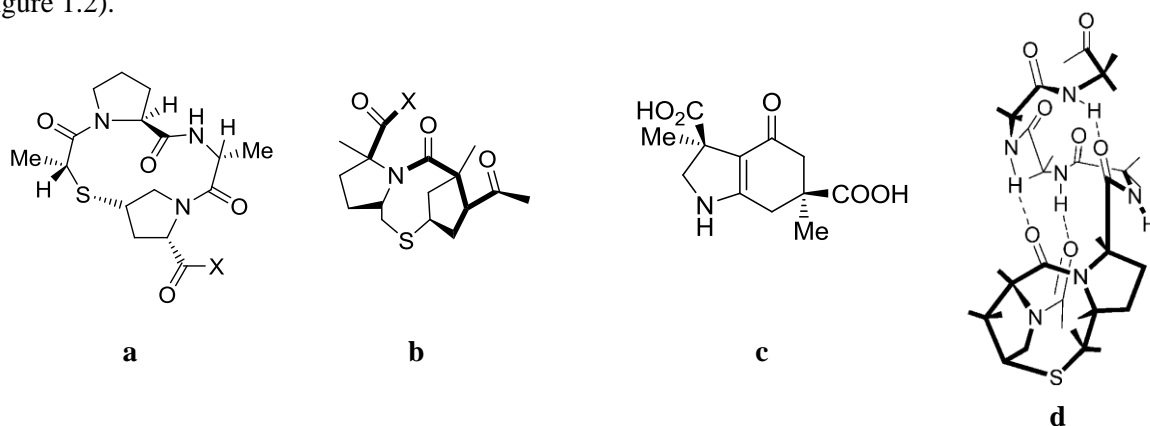


Figure 1.2: Various templates for α -helix nucleation (a) Bisproline-based template developed by Gani, (b) Kemp & Curran's bisproline-based template, (c) 4-oxo-hexahydroindole-3,6-dicarboxylic acid helix cap developed by Bartlett & Hutton, (d) Line angle representation of template mechanism of α -helix nucleation.⁷

Templates may also be successfully constructed by replacing the hydrogen bond promoting carbonyl group with sterically hindered covalent systems²¹ (See Figure 1.3).

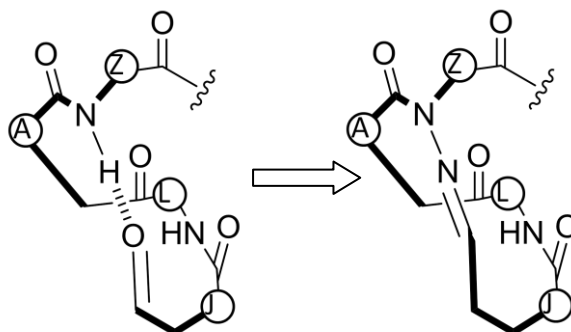


Figure 1.3: Satterwat's hydrazone based hydrogen bond mimic, L = Leu, A = Ala, J = CH₂, Z = CH₂.⁷

While the template systems have been shown to successfully induce helix formation, these α -helices tend to form less than 50% in aqueous solution or were found to be highly length dependent with decreasing helicity as the peptide strand lengthened¹⁰.

1.5 Covalent Constrained Systems

In an alternative system, a covalent constraint is introduced by crosslinking amino acids placed at specific locations within the peptide. There are a variety of available methods for such systems which vary in both the positioning of the linkers and the crosslinking reaction type. Successful techniques include constraint via salt bridges²², lactam formation^{23,24}, disulfide bonds²⁵, thioether formation²⁶, amine formation²⁷, carbon-carbon bond formation (via ring olefin closing metathesis (RCM) reaction)²⁸ and triazole formation via “click” type reactions^{29,30}.

In a relatively simple approach using salt bridges, α -helix formation is promoted by placing oppositely charged amino acids, such as glutamic acid and lysine at the *i* and *i*+4 positions which form ion pairs at desired positions, enhancing helix nucleation.²² Via a condensation reaction a lactam may also be formed to induce helix formation^{23,24}. Additionally, this natural peptide based method has been accomplished by crosslinking two glutamine residues at the *i* and *i*+7 position³¹ (see Figure 1.4).

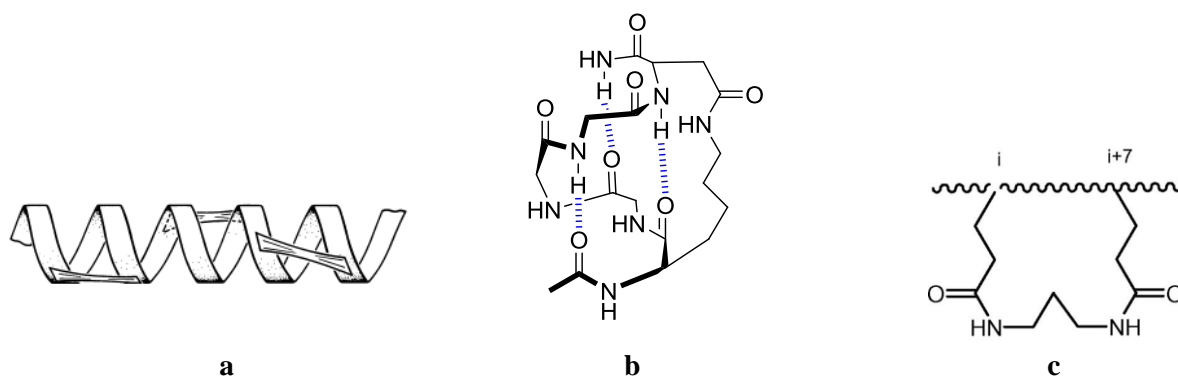


Figure 1.4: (a) Cartoon depicting Glu-Lys salt bridges in stabilized α -helix accomplished by Baldwin²², (b) Glu-Lys lactam-based constraint system developed by Fairlie, (c) Glutamine based lactam constraint system developed by Phelan.⁷

Disulfide bridges, a common structure found in proteins, are also used to form helices from short peptide strands. This process is accomplished by crosslinking modified amino acids at the i and $i+7$ positions of opposite stereochemistry²⁵ via oxidation (see Figure 1.5).

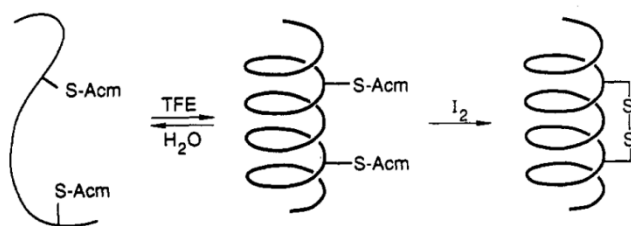


Figure 1.5: Shultz's α -helix nucleation disulfide constraint²⁵

Similarly, two modified amino acids may be joined using bridging units of different lengths to aid in helix nucleation depending on the length of the desired cross linking. By using semi-rigid organic compounds as bridging units, this method has been applied to systems with modified amino acids at the i and $i+7$, and i and $i+11$ positions^{26,27} (see Figure 1.6).

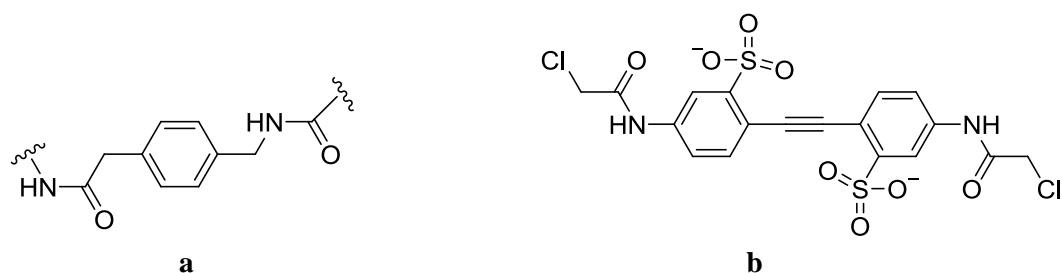


Figure 1.6: Examples of spacer units utilized for α -helix formation, (a) 4-(aminomethyl)phenylacetic acid used by Taylor, (b) 3,3'-ethyne-1,2-diylbis[6-[(chloroacetyl)amino]benzenesulfonic acid] used as a rigid crosslinker by Wooley.⁷

Ring closing metathesis (RCM) has also been applied to the formation of α -helices. In the most successful of these systems modified amino acids with terminal olefins at the i and $i+7$ positions with 10 total methylene spacing groups are crosslinked using Grubbs catalyst²⁸ (see Figure 1.7).

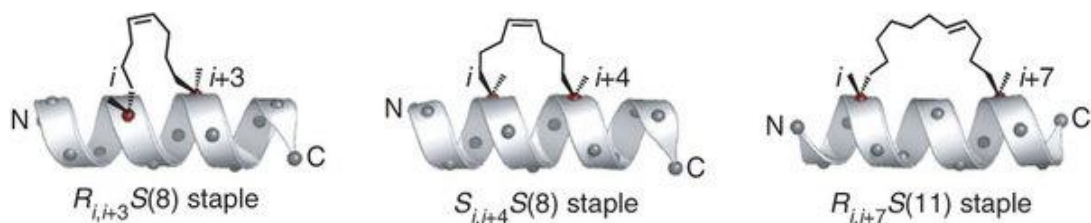


Figure 1.7: Various stapled peptides with different linker lengths and amino acid positions. R and S represent the stereochemistry of the adjacent amino acid position. The number in parentheses corresponds to the linker length.²⁸

More recently, with the discovery of “click” type reactions²⁹, on resin crosscoupling has been achieved using copper catalysts. “Click” mediated systems utilize modified amino acids at the i and $i+3$ positions one of which contains an azide and the other which contains an alkyne^{30,31} (see Figure 1.8).

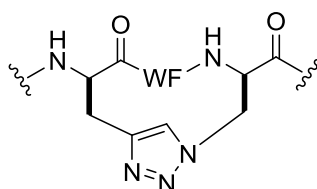


Figure 1.8: Dawson's Triazole based tethering system formed through copper-assisted azide-alkyne cycloaddition (CuAAC).³¹

Despite the success of many covalent systems, such methodologies often require non-native amino acids

such as $\alpha\alpha$ -disubstituted amino acids and dehydroaminoacids. Additionally, a number of these reactions are run with harsh conditions, or expensive cross-coupling reagents such as Pd or Ni based catalysts.

1.6 Metal Constrained Systems

In the metal-ligand mediated system, metal coordinating amino acids such as histidine and cysteine placed within the peptide chain during synthesis are subsequently coordinated with the desired metal, constraining the peptide in such a way to induce formation of an α -helix. Metal constrained systems have been synthesized using a number of modified and native amino acids as well as a range of metals such as Pd^{2+} , Co^{2+} , Cu^{2+} , Rh, and Cd^{2+} 33-36 (see Figure 1.9).

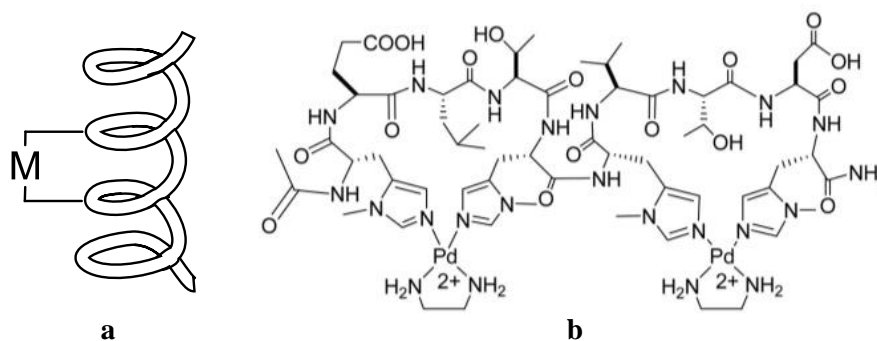


Figure 1.9: (a) Simplified cartoon depiction of metal constrained α -helix, (b) Pd constrained system developed by Fairlie.⁷

Although the chemistry involved with metals is often more simple than incorporation of a specific template, metal-induced α -helices are often very metal specific and typically, as with covalent linking, require unnatural amino acids⁹.

Among these methods, metal-ligand constraint, however, does provide several key advantages. In contrast to template and covalent systems, creation of the α -helix constrained organometallic compound can be achieved in either one or a very few number of simple steps. In comparison, the synthesis of the template alone takes many synthetic steps, and then the template must be incorporated into the peptide. RCM methods contain similar levels of complexity in the synthesis of unnatural amino acids. An organometallic compound containing a transition metal also provides a simple spectroscopic tool for

assessing the reactivity and behavior of the target in biochemical and biological settings. Additionally, the presence of a transition metal can help facilitate X-ray crystal structure elucidation due to its ability to restrict the peptides conformational fluxionality. Furthermore, the transition metal has the ability to undergo oxidation and reduction, enabling an investigation of how electronic transitions might affect the conformation of the complexed peptide.

For the synthesis of peptides, although there are several currently available methods, solid phase peptide synthesis (SPPS) is the preferred method due to its robustness and efficiency³⁷. For a majority of systems utilizing the range of previously mentioned constraints, the constraint was introduced after SPPS was complete and the peptide had been cleaved from the resin. Recently, however, researchers have developed methodologies for introducing the RCM and click reaction triazole covalent constraints while the peptide remains on the solid support^{28,29,30}. As previously mentioned, however, both systems require the use of synthetic amino acids, limiting their potential in fully mimicking biological systems.

Designing an analogous system which would utilize not only the advantages of metal-ligand based constraints but also the ease of on-resin complexation that would work with only the 20 common amino acids would be a significant advancement within the field.

1.7 Thesis Work

Previous research within the Curran lab³⁸ has been successful at inducing a 3_{10} -helix, a helical structure that is slightly tighter than that of the α -helix, by crosslinking lysines at the i and $i+3$ position with 1,1'-ferrocenedicarboxylic acid. By extending this previous research and redesigning its methodologies to work with solid phase peptide synthesis (SPPS) this thesis aims to develop and accomplish the synthesis of compound **1** (see Figure 1.10), an α -helix constrained organometallic.

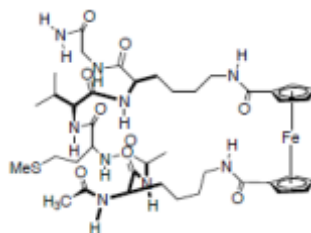
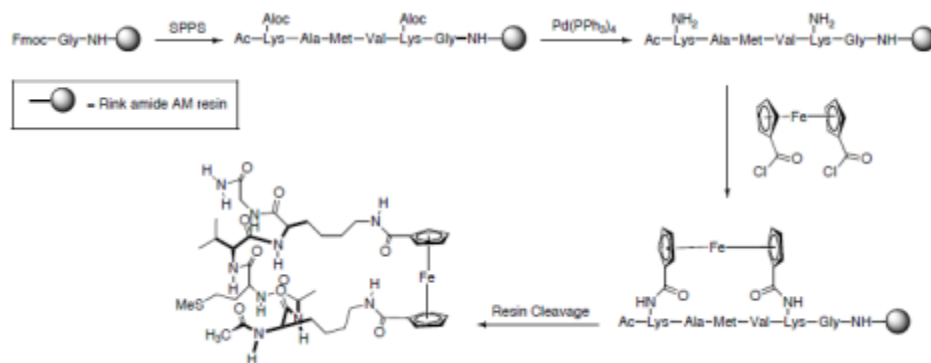


Figure 1.10: Target organometallic compound.

First, an attempt will be made to develop a method of synthesis for the compound compatible with SPPS. Once successful, coupling with ferrocene will be attempted while the peptide remains on its solid support in an effort to minimize polymerization and possibly improve yields (see Scheme 1.1). In the following chapters of this thesis is described the progress towards developing a methodology for the on-resin cyclization of a pentapeptide. Additionally discussed is a second methodology which utilizes SPPS to first synthesize a pentapeptid, which can then be cleaved and cyclized with ferrocene in solution phase.

Scheme 1.1:

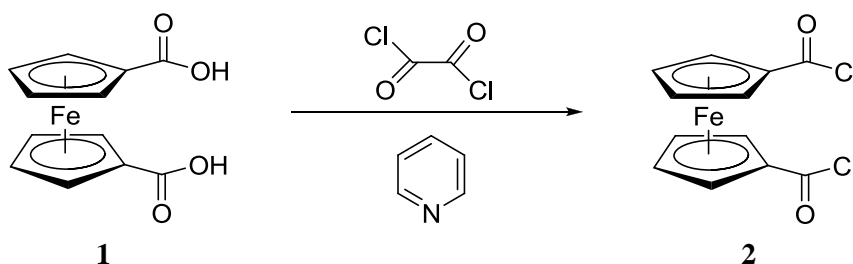


MATERIALS AND METHODS

2.1 Materials & Instrumentation

Commercially available reagents and solvents were used for all purification and synthesis, unless otherwise noted. 1,1'-ferrocenedicarboxylic acid and tetrakis(triphenylphosphine)palladium(0) were obtained from Strem Chemicals. Methanol, acetic anhydride, trifluoroacetic acid, tetrahydrofuran, diisopropylethylamine, dimethylformamide, chloroform, and ether were obtained from Fisher Chemicals. Anisole, oxalyl chloride and 1,8-diazabicycloundec-7-ene (DBU) were purchased from Sigma-Aldrich. All peptide reagents and peptide coupling reagents (HOBt, HATU) were from Chem-Impex International. For the ferrocene diacid chloride synthesis, dry CH_2Cl_2 was obtained from an anhydrous solvent purification apparatus. Prior to Aloc group removal, the scavenger agent, N-methylaniline was freshly distilled. Flash chromatography was performed using 60Å pore Ultra Pure Silica Gel from Silicycle. Thin layer chromatography was performed using EMD chemicals TLC Silica gel 60 F₂₅₄ glass backed plates. All NMR spectra were obtained with a 400 MHz Bruker Avance.

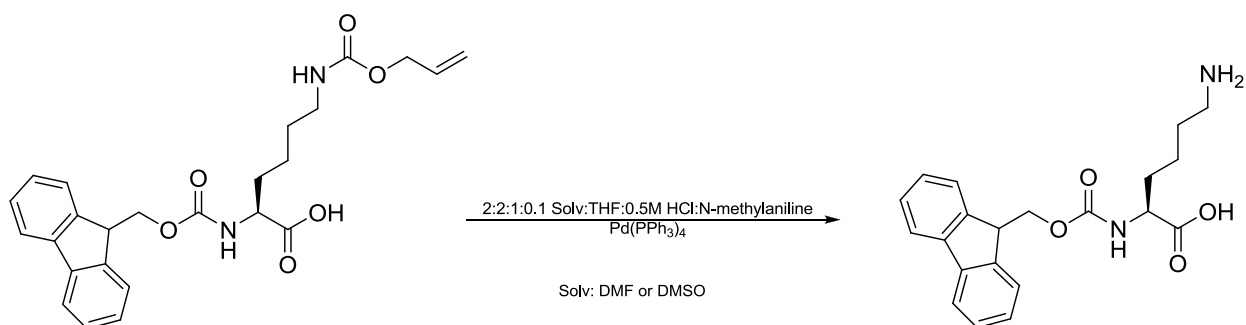
2.2 Preparation of 1,1'-Ferrocenedicarboxylic Acid Chloride³⁹



Scheme 2.1: Reaction scheme for the preparation of 1,1'-ferrocenedicarboxylic acid chloride

First, 2.011 g (7.34mmol) of ferrocenedicarboxylic acid was reacted with 3.1 mL (4.9 equiv.) of oxalyl chloride and several drops of pyridine in 35mL of CH_2Cl_2 . A cardboard box and aluminum foil were used to cover the reaction vessel to protect it from light. The reaction was stirred at room temperature for 12 hours, followed by 6 hours at reflux. The CH_2Cl_2 was removed by rotary evaporation, and the product was left to dry overnight under vacuum. The desired product was extracted using approximately 5 x 100 mL of boiling ligroin ($\approx 80^\circ\text{C}$) and subsequently decanting the ligroin into a second round bottom. The ligroin was subsequently removed using rotary evaporation, yielding 1.781g of red crystals (78% yield). The obtained crystals had a melting point of $91\text{-}95^\circ\text{C}$, in close agreement with literature values (98°C - 100°C)³⁹.

2.3 Investigation of Solvent Effects on Allyloxycarbonyl (Aloc) Group Removal⁴⁰



Scheme 2.2: Solution phase Aloc removal

First, 1.008g of Fmoc-Lys(Aloc)-OH was massed and transferred into two separate reaction flasks. The reaction vessels were then sealed with a rubber septums and put under inert N₂ atmosphere. For the first flask, labeled A, 0.0638g of tetrakis(triphenylphosphine)palladium(0) was dissolved into a solution of 0.1:2:2:1 N-methylaniline:THF:DMF:0.5M HCl, quickly agitated, and added to the reaction flask using a syringe. For the second reaction flask, labeled B, 0.0638g of (triphenylphosphine)palladium(0) was dissolved into a solution of 0.1:2:2:1 N-methylaniline:THF:DMSO:0.5M HCl and added to the reaction flask using a syringe. The reactions were then stirred and monitored by TLC with samples taken every 60 minutes for analysis. A 1:1 MeOH:CHCl₃ was used as the eluent. To aid in the analysis, the reaction mixtures A and B were run in comparison to the unreacted Fmoc-Lys(Aloc)-OH starting material, labeled U on the TLC plates (Figure 2.1).

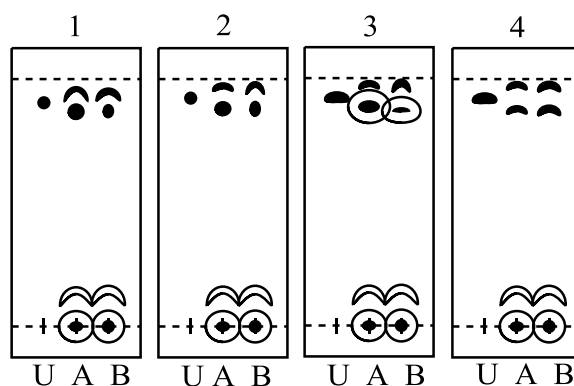


Figure 2.1: TLC plates of Aloc removal reaction mixture with an eluent of 1:1 MeOH:CHCl₃

After 20 hours, both reactions were removed from stirring and the solvent solution was evaporated with a rotary evaporator. The crude product mixtures were then analyzed and compared using ¹H NMR (Figures 2.2, 2.3, and 2.4).

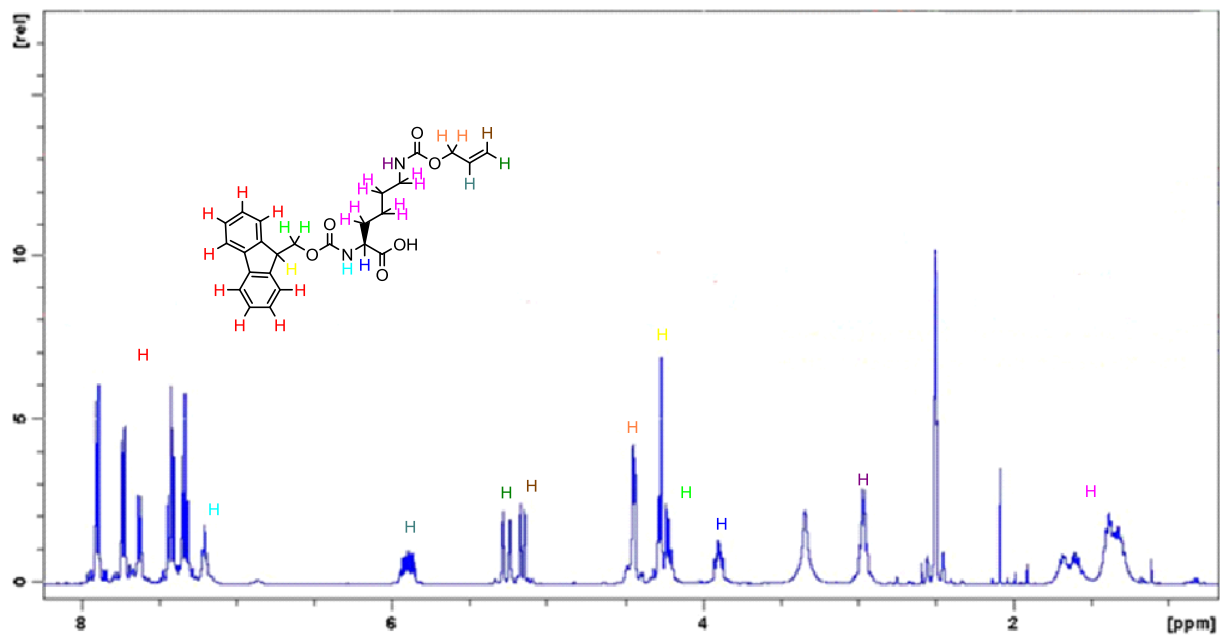


Figure 2.2: ^1H NMR of Fmoc-Lys(Aloc) OH in DMSO- d_6

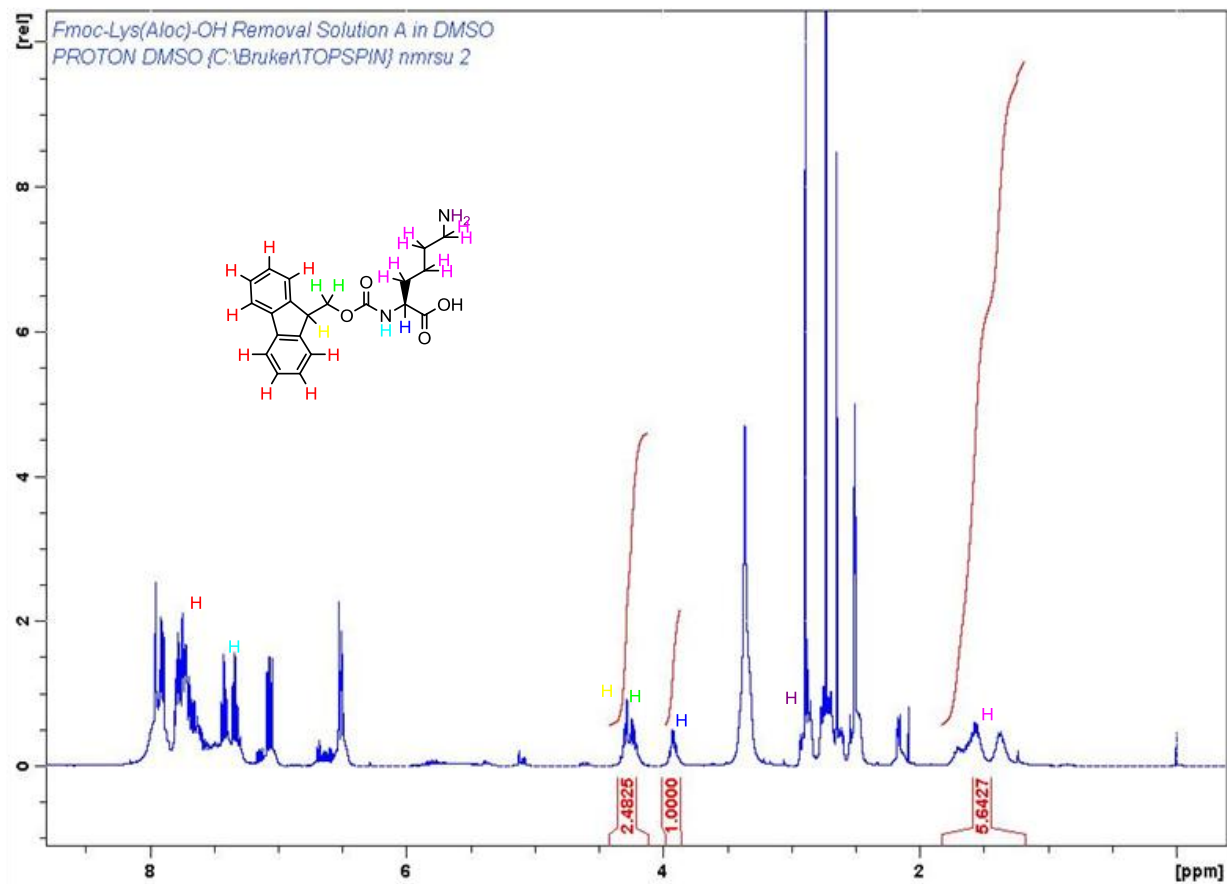


Figure 2.3: ^1H NMR of crude product of Aloc removal attempted with DMF

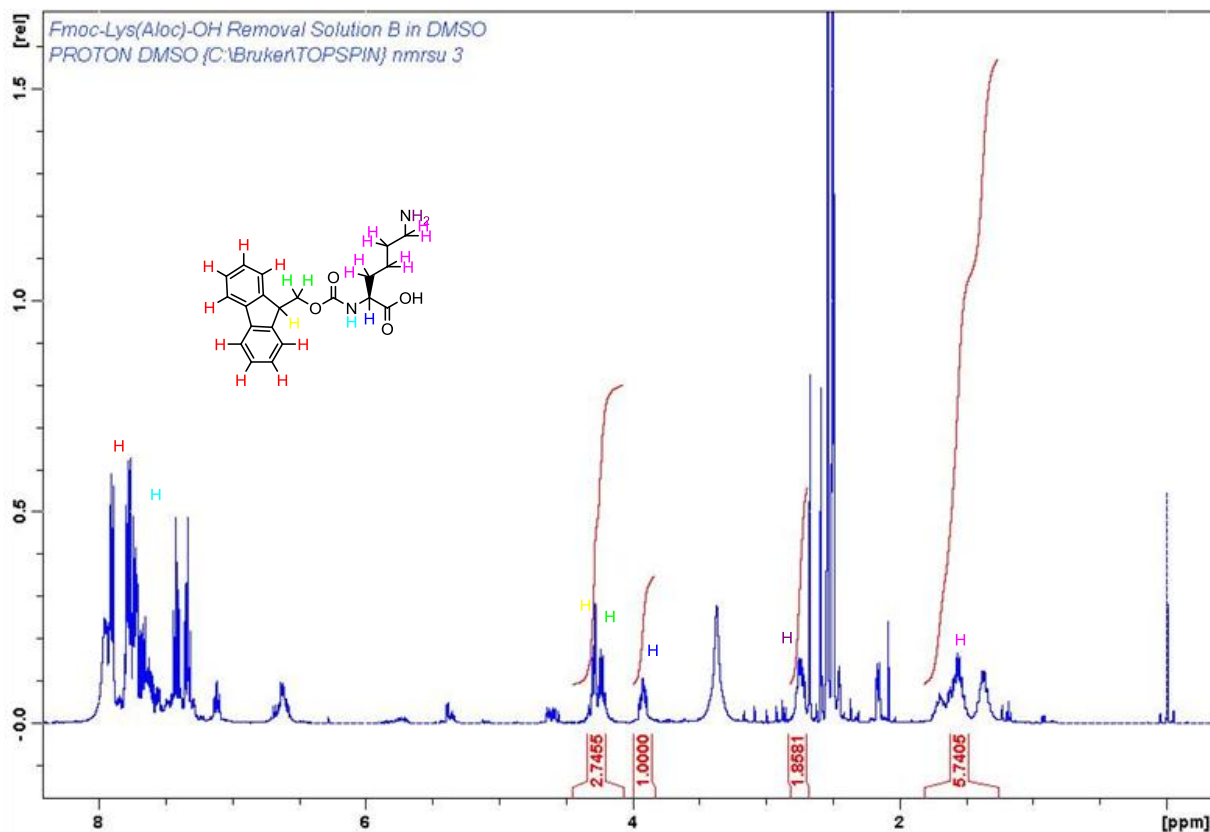
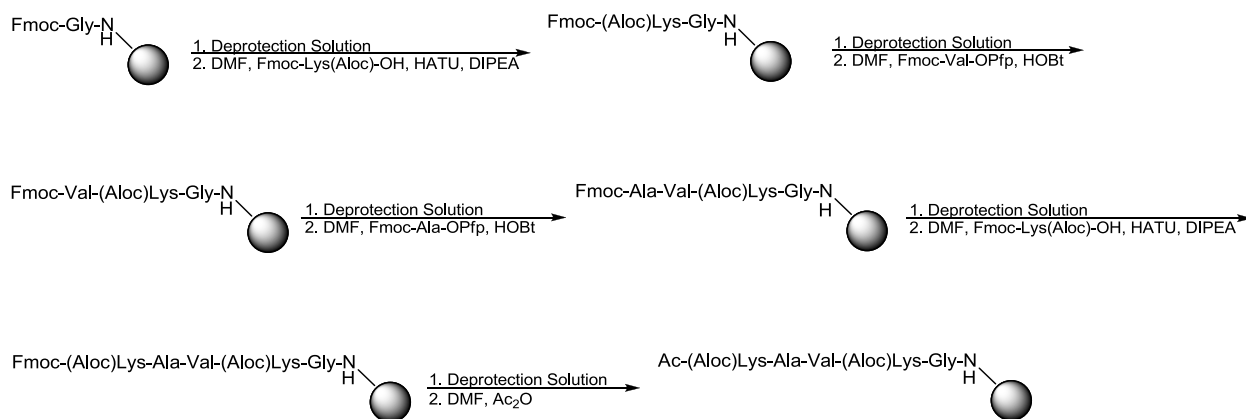


Figure 2.4: ^1H NMR of crude product of Aloc removal attempted with DMSO

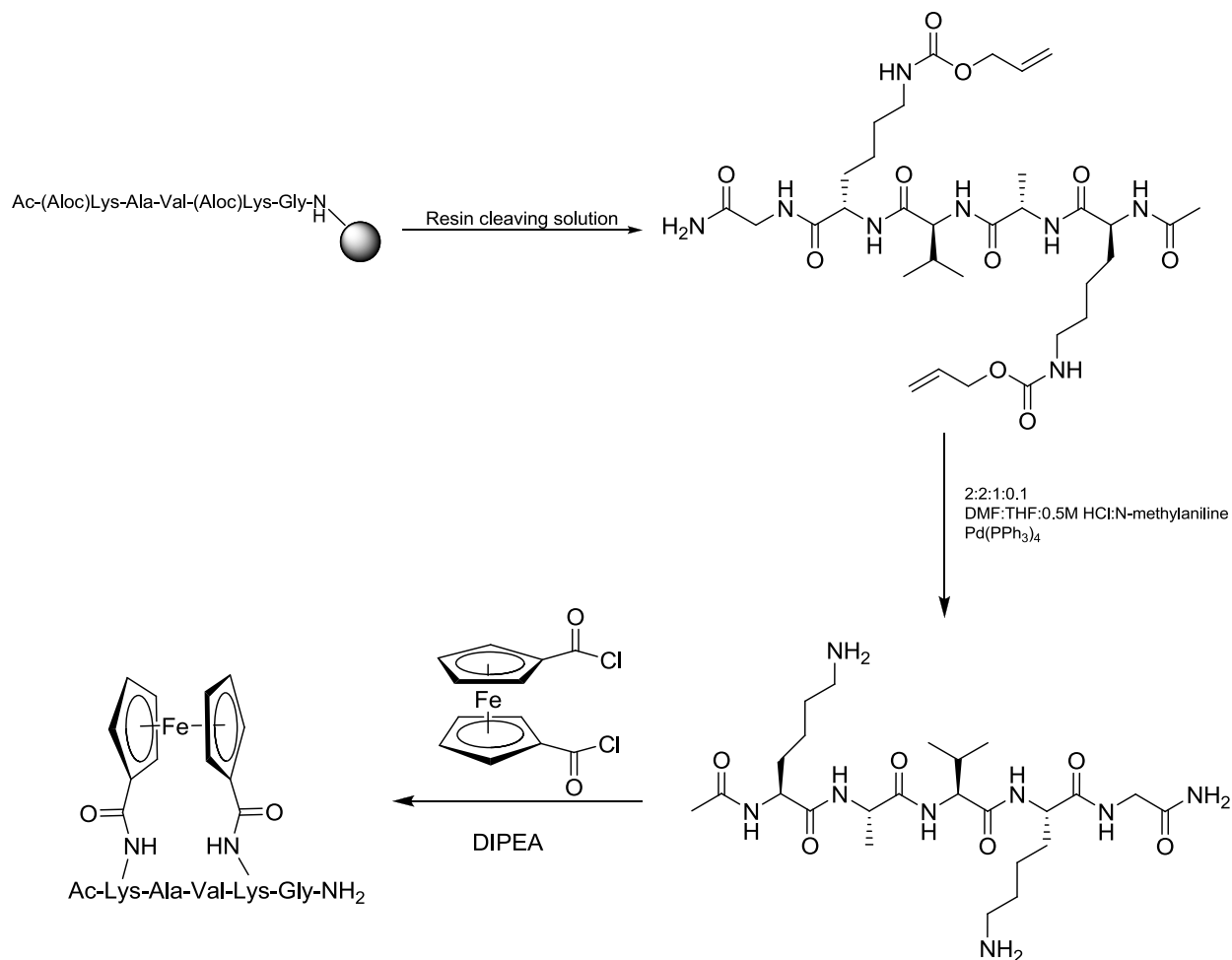
2.4 Preparation of Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂



Scheme 2.3: Solid phase preparation of Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂

First 0.500g (0.365 mequiv) of Fmoc-Gly-Rink amide AM resin (Chem-Impex International) was added to a solid phase reaction vessel. Approximately 4.5mL (3x the resin volume) of CH₂Cl₂ was then added to the vessel and the resin was allowed to swell for 30 minutes with gentle agitation. The CH₂Cl₂ was subsequently drained and the resin was washed with 4.5mL of DMF with gentle agitation for 2 minutes, followed by draining. The DMF wash was performed 5 times. The Fmoc group was then removed using 1.5mL (1x the resin volume) of a deprotecting solution (1:1:48 DBU:piperidine:DMF). The resin was washed 5 times with DMF in 2 minute intervals with gentle agitation and draining in between each rinse. A second round of DMF washes was then performed. To add the next amino acid, 0.826g (5 equiv) of Fmoc-Lys(Aloc)-OH and 0.682g of HATU (4.9 equiv) were first dissolved in a minimal amount (≈12mL) of DMF. Immediately prior to adding the peptide solution to the resin, 0.640mL of DIPEA were added to the peptide solution, the mixture was stirred, and subsequently added to the resin. After 1 hour of gentle agitation the remaining solution was drained off and the resin was once again washed with DMF as described above. The deprotection and DMF washes were then repeated. Next, 0.922g (5equiv) of Fmoc-Val-OPfp and 0.253g (5 equiv) of HOBt were dissolved in a minimal amount of DMF and added to the reaction vessel. After agitation for 1 hour, the solution was then drained and the wash/deprotect/wash procedure was followed once again. Using this method 0.875g (5equiv) of Fmoc-Ala-OPfp and 0.829g (5 equiv) Fmoc-Lys(Aloc)-OH were then added to the solid phase vessel and reacted. For the activated ester amino acids derivatives (those with a OPfp group) HOBt was utilized, whereas those with the unactivated amino acids (those with an OH) were coupled using the combination of HATU and DIPEA. After the resin was washed 5 times with DMF, 5 washes of deprotection solution, and 5 times with DMF, 0.345 mL (10 equiv.) of acetic anhydride in a minimal amount of DMF was added to the resin and allowed to react for 1 hour with gentle agitation.

2.5 Solution phase Ferrocene Coupling with Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂



Scheme 2.4: Solution phase crosslinking of pentapeptide with ferrocene

In addition to the attempted on-resin coupling, a solution phase methodology for crosslinking the synthesized pentapeptide with ferrocene was also attempted. First 0.0946 g (1 equiv) of dried resin was transferred to a second reaction vessel. The peptide was cleaved from the resin via the addition of 3mL (3x the resin volume) of 95:2.5:2.5 TFA:anisole:H₂O v/v solution with gentle agitation for 3 hours. The solution within the reaction vessel was then drained into a round bottom flask. The resin was subsequently washed 2 times with 3mL of TFA and combined with the previous removal solution fractions. After the solvent was evaporated using a high vacuum rotary evaporator, the resulting product was then triturated with ether several times to remove impurities. The ¹H NMR of the product was consistent with the desired pentapeptide. COSY and ¹H NMR were utilized to make peak assignments for each hydrogen found within the product (Figure 2.5, Table 2.1, Figure 2.6, Table 2.2).

2.93	2 H	m		Lys-N-CH ₂
2.93	2 H	m		Lys-N-CH ₂
1.97	1 H	m		Val-CβH
1.83	3 H	s		Ac-CH ₃
1.69-1.21	13 H	m		Lys-CH ₂
1.19	3 H	m		Ala-CH ₃
0.82	6 H	d	³ J: 10.90 Hz	Val-CH ₃

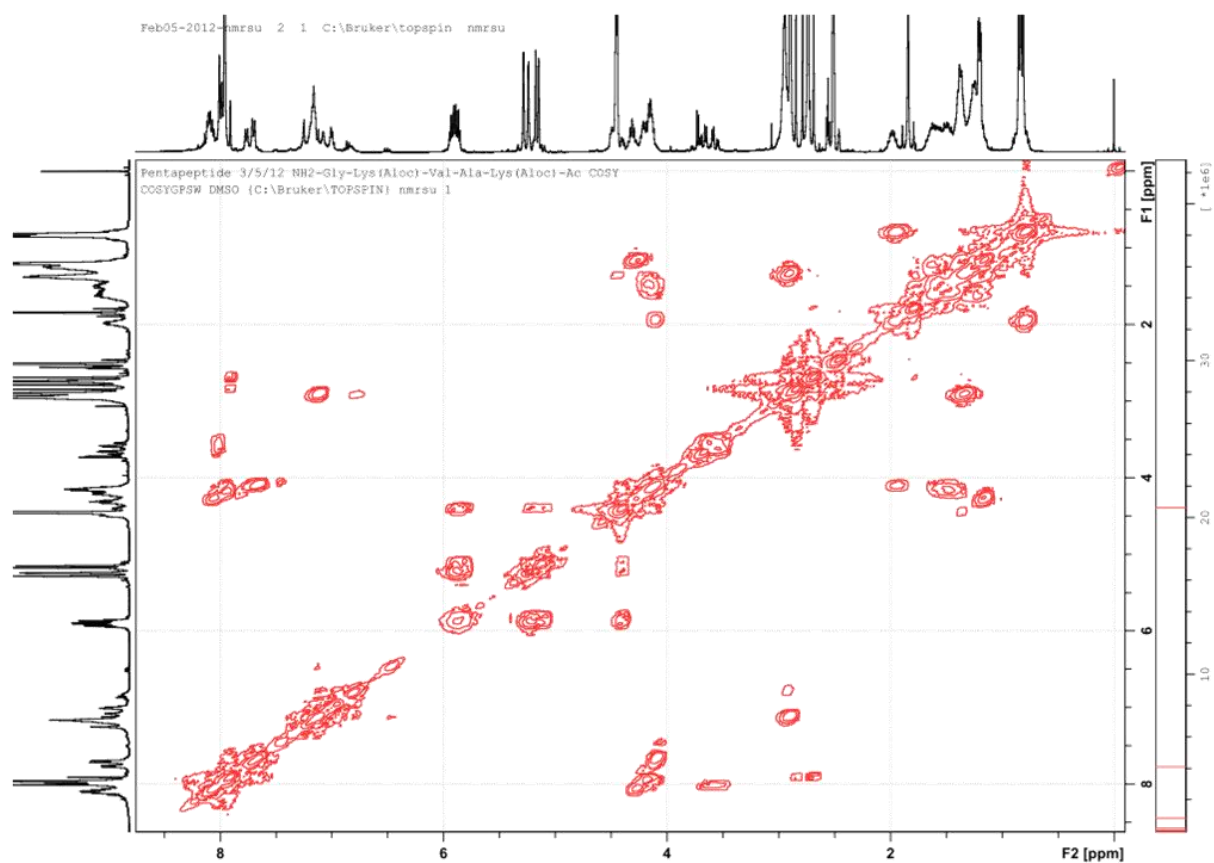


Figure 2.6: 2D COSY NMR of Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂ in DMSO-d₆.

Table 2.2: Cross Coupling Peaks for COSY of Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂ in DMSO-d₆.

<u>Cross Peak Coupling Hydrogens</u>
Ala-NH (8.09ppm) with Ala-Cα-H (4.30ppm)
Gly-NH (8.00ppm) with Gly-Cα-H (3.60ppm)
Lys-NH (7.97ppm) with Lys-Cα-H (4.20ppm)
Lys-NH (7.75ppm) with Lys-Cα-H (4.15ppm)
Val-NH (7.69ppm) with Val-Cα-H (4.11ppm)
Lys-Amine (7.15ppm) with Lys-CH ₂ -N (2.93ppm)
Lys-Amine (7.07ppm) with Lys-CH ₂ -N (2.93ppm)
Aloc-CH (5.89ppm) with Aloc-CH ₂ (4.44ppm)
Aloc-CH (5.89ppm) with Aloc Terminal CH (5.25ppm)

Aloc-CH (5.89ppm) with Aloc Terminal CH (5.15ppm)
Aloc Terminal CH (5.25ppm) with Aloc-CH ₂ (4.44ppm)
Aloc Terminal CH (5.15ppm) with Aloc-CH ₂ (4.44ppm)
Ala-C α -H (4.30ppm) with Ala-CH ₃ (1.19ppm)
Lys-C α -H (4.20ppm) with Lys-CH ₂ (1.69-1.21ppm)
Lys-C α -H (4.15ppm) with Lys-CH ₂ (1.69-1.21ppm)
Val-C α -H (4.11ppm) with Val-C β -H (1.97ppm)
Lys-CH ₂ -N (2.93ppm) with Lys-CH ₂ (1.69-1.21ppm)
Val-C β -H (1.97ppm) with Val-CH ₃ (1.69-1.21ppm)
Lys-CH ₂ (1.69-1.21ppm) with Lys-CH ₂ (1.69-1.21ppm)

The allyloxycarbonyl (Aloc) groups on the two lysine residues at *i* and *i*+3 positions within the peptide were then removed by reaction with a solution of 2:2:1:0.1 DMF:THF:0.5M HCl: freshly distilled *N*-methylaniline v/v solution (\approx 5.1mL/100mg resin). In this reaction Pd(PPh₃)₄ was used as a catalyst with a 0.25 equivalent load (0.01995g). To protect the catalyst, the reaction was run under inert N₂ atmosphere. The reaction was left stirring for 24 hours and the solvent was subsequently removed under high vacuum. Upon heating to remove the solvent, the solution transitioned from a light yellow color to a dark purple. The color change was attributed to the poisoning of the Pd catalyst.

Ferrocene crosslinking was then attempted with the crude product. The unpurified mixture was first dissolved in approximately 12.5mL of DMF. A solution of 0.0215g (1 equiv) of 1,1'-ferrocenedicarboxylic acid chloride in 5 mL of DMF was first added to a stirring solution of the crude unprotected peptide followed by the addition of 115 μ L of DIPEA. The coupling reaction was conducted under inert N₂ atmosphere. After 24 hours, the solvent was then removed using a high vacuum rotary evaporator. Due to the known impurity of the product, no accurate mass of the desired product could be obtained, but the mass of the crude sample was approximately 150mg.

The ¹H NMR of the product indicates that complete removal of the Aloc group was incomplete (Figure 2.7).

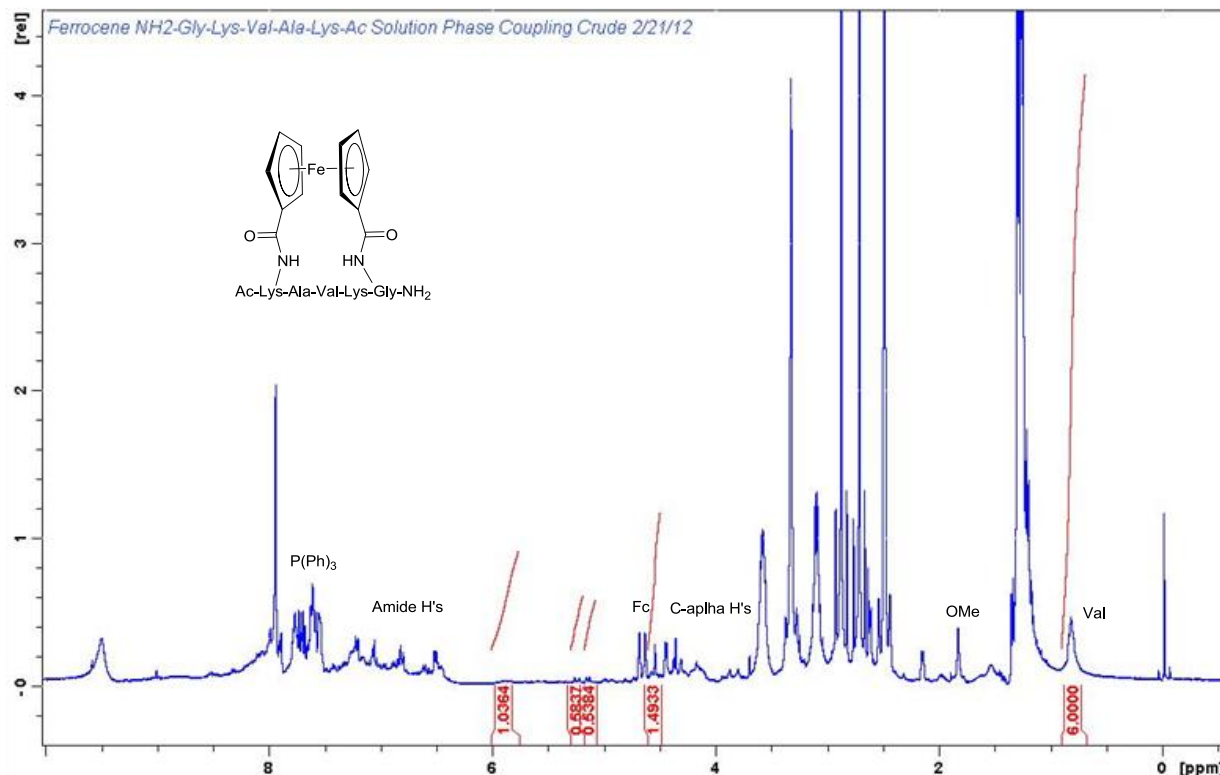
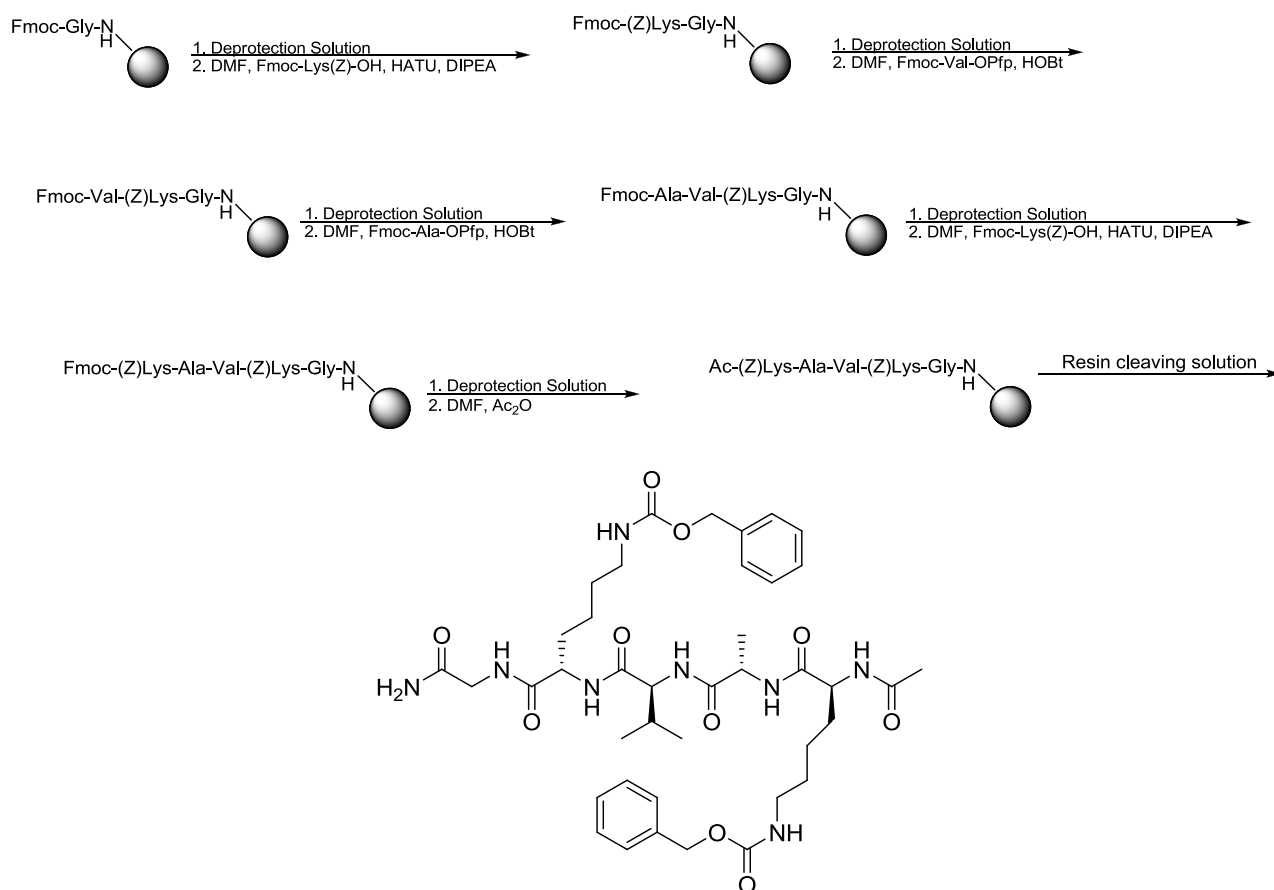


Figure 2.7: ^1H NMR of solution phase cyclization attempt with unpurified peptide

Based on integration values, about 50% of the Alloc groups were removed. Despite this, the ^1H NMR also indicates the presence of peaks in the 4.5-5ppm region, characteristic of ferrocene being present, so flash chromatography was attempted. Flash chromatography, with an eluent of 10:1 chloroform:methanol, was used to separate the product mixture. The crude product was then dissolved in a minimal amount of HFIP and added to the packed 1.5cm column. A total of 36 2mL fractions from the column were collected and analyzed using thin layer chromatography (TLC). Due to the difficulty of separation to obtain a pure crosslinked product, purification was halted.

2.6 Preparation of Ac-Lys(Z)-Ala-Val-Lys(Z)-Gly-NH₂



Scheme 2.5: Solid phase preparation of Ac-Lys(Z)-Ala-Val-Lys(Z)-Gly-NH₂

First 0.1028g (0.07504 mequiv) of Fmoc-Gly-Rink amide AM resin (Chem-Impex International) was added to a solid phase reaction vessel. Approximately 3.0mL (3x the resin volume) of CH₂Cl₂ was then added to the vessel and the resin was allowed to swell for 30 minutes with gentle agitation. The CH₂Cl₂ was subsequently drained and the resin was washed with 3mL of DMF with gentle agitation for 2 minutes, followed by draining. The DMF wash was performed 5 times. The Fmoc group was then removed using a 1.0mL (1x the resin volume) a deprotecting solution (1:1:48 DBU:piperidine:DMF). The resin was washed 5 times with DMF in 2 minute intervals with gentle agitation and draining in between each rinse. A second round of DMF washes was then performed. To add the next amino acid, 0.1920g (5 equiv) of Fmoc-Lys(Z)-OH and 0.1399 of HATU (4.9 equiv) were first dissolved in a minimal amount (\approx 4mL) of DMF. Immediately prior to adding the peptide solution to the resin, 0.130mL (10 equiv) of DIPEA were added to the peptide solution, the mixture was stirred, and subsequently added to the resin. After 1 hour of gentle agitation the remaining solution was drained off and the resin was once again

washed with DMF as described above. The deprotection and DMF washes were then repeated. Next, 0.1911g (5equiv) of Fmoc-Val-OPfp and 0.0517g (5 equiv) of HOBt were dissolved in a minimal amount of DMF and added to the reaction vessel. After agitation for 1 hour, the solution was then drained and the wash/deprotect/wash procedure was followed once again. Using this method 0.1808g (5equiv) of Fmoc-Ala-OPfp and 0.1691g (5 equiv) Fmoc-Lys(Z)-OH were then added to the solid phase vessel and reacted. For the activated amino acids (those with a OPfp group) HOBt was utilized, whereas with the unactivated amino acids (those with an OH) were coupled using the combination of HATU and DIPEA. After the resin was washed 5 times with DMF, 5 washes of deprotection solution, and 5 times with DMF, 0.345 mL (10 equiv.) of acetic anhydride in a minimal amount of DMF ($\approx 1.5\text{mL}$) was added to the resin and allowing to react for 1 hour with gentle agitation. After draining and washing with DMF ($5 \times 3\text{mL}$) The resin was subsequently dried under N_2 . The peptide was then cleaved from the resin using 3mL of a removal solution of 95:2.5:2.5 TFA:anisole: H_2O v/v solution with gentle agitation for 3 hours. Subsequently, the resin was washed with 2mL of TFA for 2 minutes and all of the filtrates were collected in a single round bottom flask. The solvent was then evaporated off yielding a crude mass of 0.64mg. The ^1H NMR of the product was consistent with the desired pentapeptide. COSY and ^1H NMR were utilized to make peak assignments for each hydrogen found within the product (Figure 2.8, Table 2.3, Figure 2.9, Table 2.4).

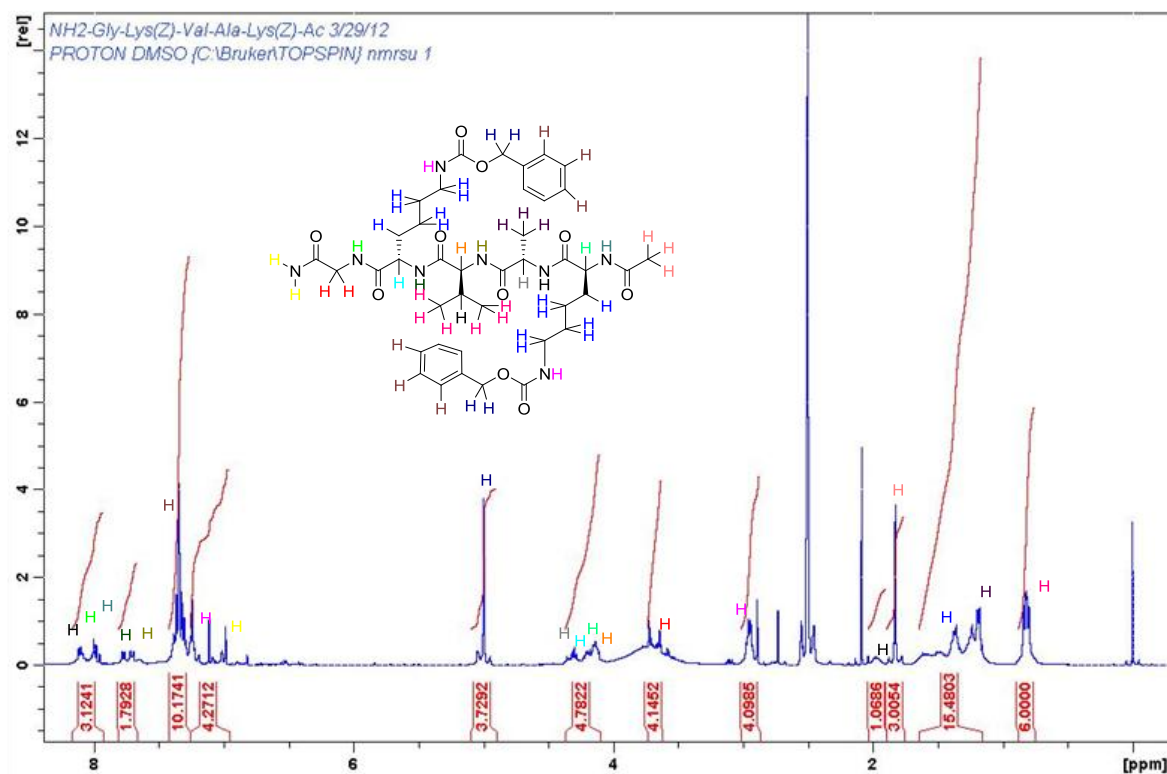


Figure 2.8: ^1H NMR of $\text{Ac-Lys(Z)-Ala-Val-Lys(Z)-Gly-NH}_2$ in $\text{DMSO-}d_6$

Table 2.3: Peak data for the ^1H NMR of Ac-Lys(Z)-Ala-Val-Lys(Z)-Gly-NH₂ in DMSO-*d*₆

δ (ppm)	Integral	Splitting	J-Coupling	Identity
8.11	1 H	m		Ala-NH
8.05	1 H	m		Gly-NH
8	1 H	m		Lys-NH
7.77	1 H	d	^3J : 7.89 Hz	Lys-NH
7.71	1 H	d	^3J : 8.97 Hz	Val-NH
7.34	10 H	m		Cbz-Benzyl
7.23	1 H	m		Lys-Amine
7.11	1 H	m		Lys-Amine
6.89	2 H	s		Terminal Amide
5	4 H	s		Cbz-CH ₂
4.3	1 H	m		Ala-C α H
4.2	1 H	m		Lys-C α H
4.15	1 H	m		Lys-C α H
4.11	1 H	m		Val-C α H
3.67	2 H	m		Gly-C α H
2.95	2 H	m		Lys-N-CH ₂
2.95	2 H	m		Lys-N-CH ₂
1.97	1 H	m		Val-C β H
1.83	3 H	s		Ac-CH ₃
1.69-1.21	13 H	m		Lys-CH ₂
1.19	3 H	m		Ala-CH ₃
0.82	6 H	d	^3J : 10.90 Hz	Val-CH ₃

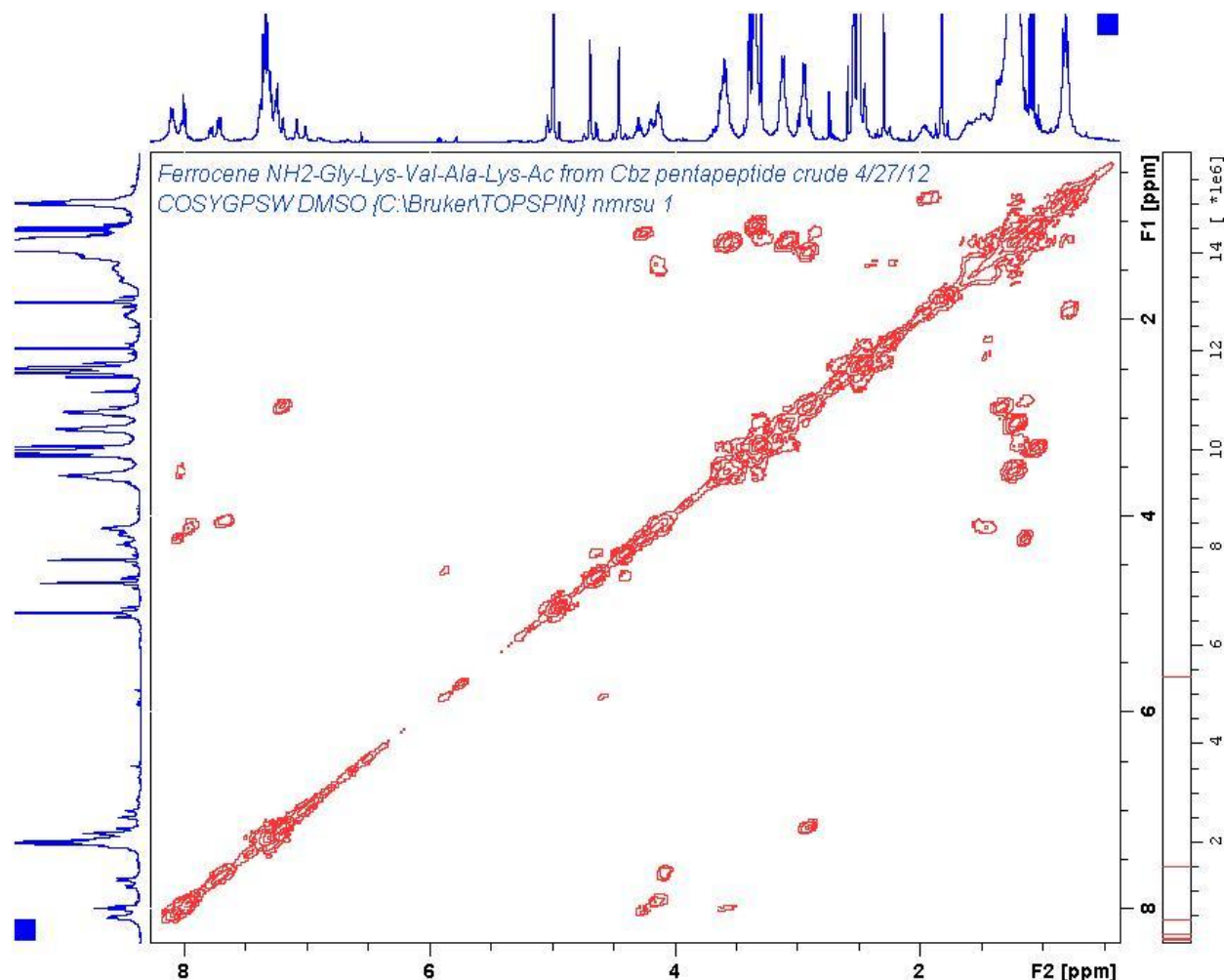


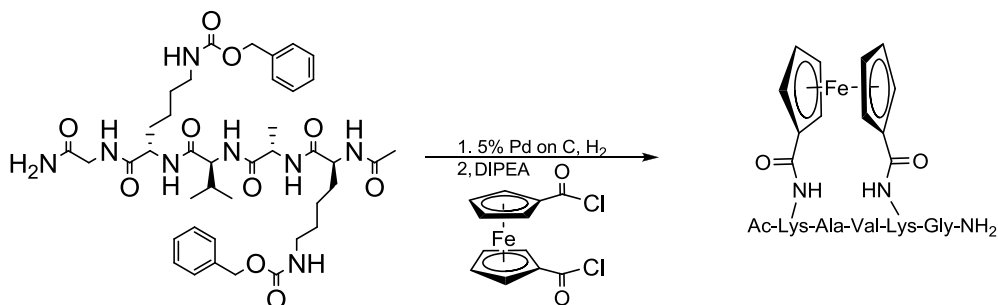
Figure 2.9: 2D COSY NMR of Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂ in DMSO-d₆.

Table 2.4: Cross Coupling Peak data for the ¹H NMR of Ac-Lys(Z)-Ala-Val-Lys(Z)-Gly-NH₂ in DMSO-d₆

<u>Cross Peak Coupling Hydrogens</u>
Ala-NH (8.11ppm) with Ala-C α -H (4.30ppm)
Gly-NH (8.05ppm) with Gly-C α -H (3.67ppm)
Lys-NH (8.00ppm) with Lys-C α -H (4.20ppm)
Lys-NH (7.77ppm) with Lys-C α -H (4.15ppm)
Val-NH (7.71ppm) with Val-C α -H (4.11ppm)
Cbz-Benzyl (7.34ppm) with Cbz-Benzyl (7.34ppm)
Lys-Amine (7.23ppm) with Lys-CH ₂ -N (2.95ppm)
Lys-Amine (7.11ppm) with Lys-CH ₂ -N (2.95ppm)
Ala-C α -H (4.30ppm) with Ala-CH ₃ (1.19ppm)
Lys-C α -H (4.20ppm) with Lys-CH ₂ (1.69-1.21ppm)
Lys-C α -H (4.15ppm) with Lys-CH ₂ (1.69-1.21ppm)
Val-C α -H (4.11ppm) with Val-C β -H (1.97ppm)

Lys-CH ₂ -N (2.95ppm) with Lys-CH ₂ (1.69-1.21ppm)
Val-Cβ-H (1.97ppm) with Val-CH ₃ (1.69-1.21ppm)
Lys-CH ₂ (1.69-1.21ppm) with Lys-CH ₂ (1.69-1.21ppm)

2.7 Solution Phase synthesis of Ferrocene Crosslinked Ac-Lys-Ala-Val-Lys-Gly-NH₂



Scheme 2.6: Solution phase cyclization using Z protected peptide

To begin, all of the resin cleaved pentapeptide, Ac-Lys(Z)-Ala-Val-Lys(Z)-Gly-NH₂, product from the previous step (≈ 50 mg) was dissolved in 12.5mL of DMF and transferred to a Parr hydrogenation flask. Under a stream of running N₂, 0.0267g of 5% Pd on C was then added to the solution. The flask was then placed in a Parr hydrogenation apparatus and hydrogenated for 24 hours at 40psi. The catalyst was subsequently removed via vacuum filtration through celite into a three neck flask. The solution was then brought to a total volume of 70mL of DMF. An addition funnel was added and the system was put under N₂. 0.191g of ferrocene dicarboxylic acid chloride was dissolved in 10mL of DMF and added to the addition funnel. To the solution was then added 0.250mL of DIPEA via a syringe. The ferrocene dicarboxylic acid chloride solution was then added to the reaction flask over the course of an hour and the reaction was allowed to stir for 24 hours. The bronze colored solution was then transferred to a second flask and the DMF was evaporated using a rotary evaporator. The crude mixture was then washed with MeOH and the MeOH was decanted into a second round bottom flask. The MeOH was then evaporated to yield a crude black film with a mass of 50mg. The ¹H NMR indicates that there is ferrocene in the product mixture (Figure 2.10).

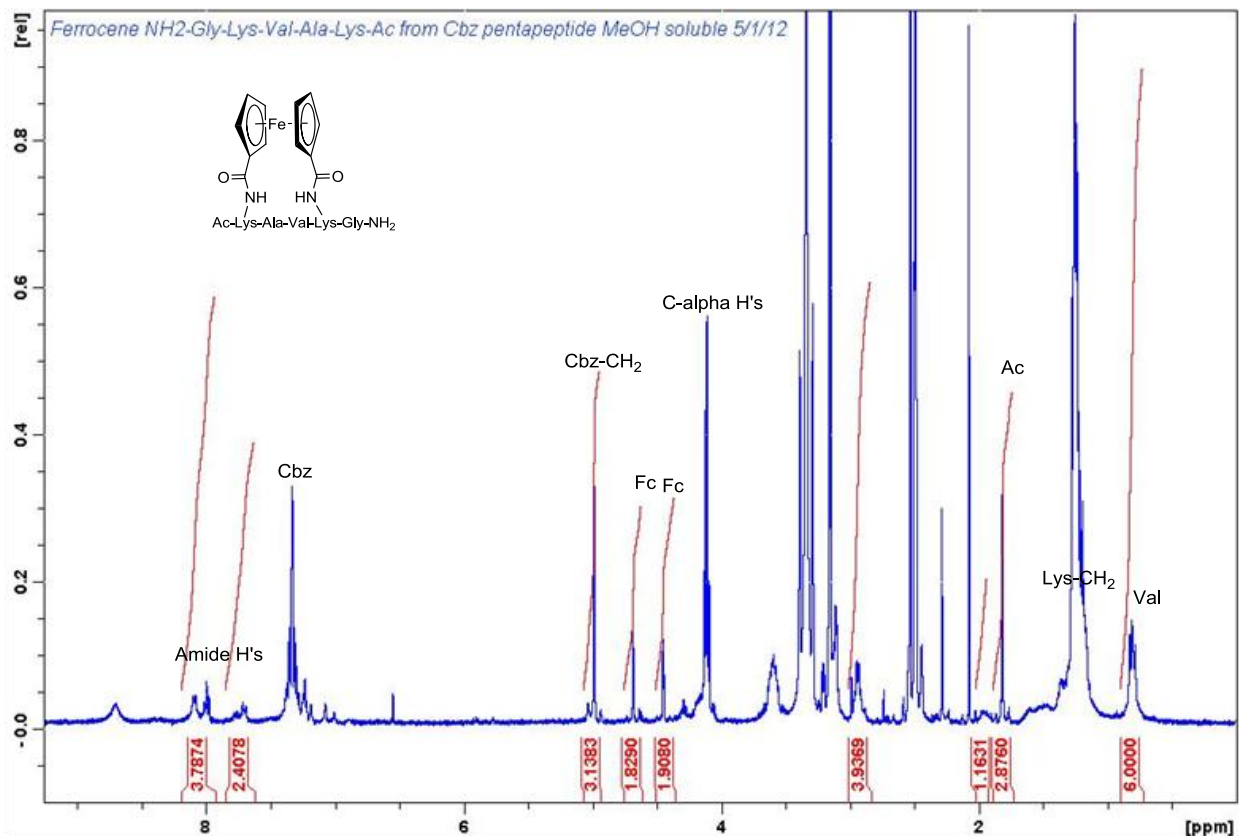
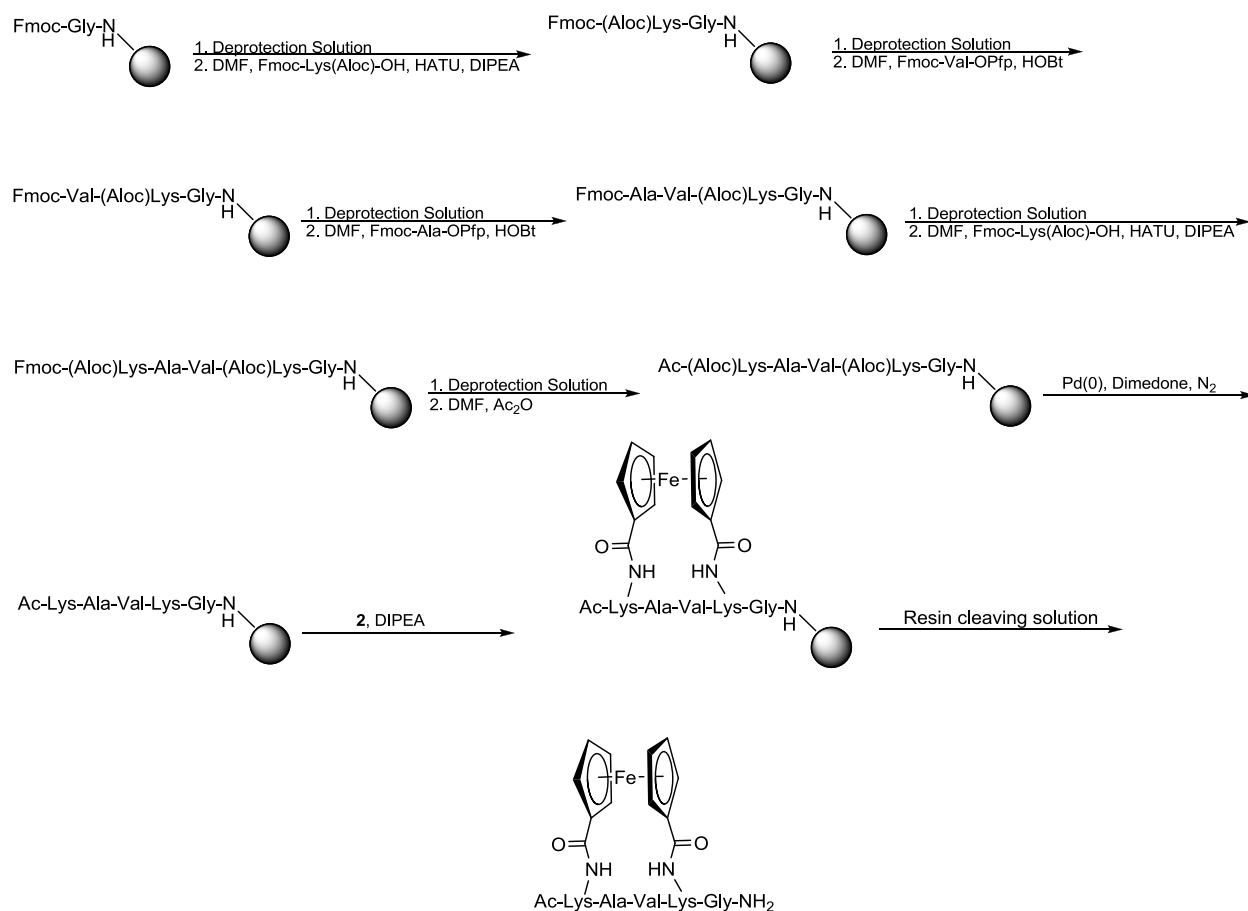


Figure 2.10: Crude solution phase ferrocene cyclized product using Z protected peptide

Flash chromatography was attempted on the crude mixture, but after evaporation, the obtained black film was no longer soluble in MeOH. Due to time constraints, flash chromatography could not be fully completed, although initial TLC analysis indicated that pure MeOH was not a sufficient eluent and that acetic acid must be added for adequate separation. It is also noted that upon dissolution in DMF, the black film had a redish tint, indicative of the presence of ferrocene.

2.8 Preparation of Ferrocene Crosslinked Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂ On-resin



Scheme 2.7: On resin preparation of Ferrocene crosslinked Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂

To begin 0.1019g (0.07439 mequiv) of Fmoc-Gly-Rink amide AM resin (Chem-Impex International) was added to a solid phase reaction vessel. Approximately 3.0mL (3x the resin volume) of CH₂Cl₂ was then added to the vessel and the resin was allowed to swell for 30 minutes with gentle agitation. The CH₂Cl₂ was subsequently drained and the resin was washed with 3.0mL of DMF with gentle agitation for 2 minutes, followed by draining. The DMF wash was performed 5 times. The Fmoc group was then removed using 1.0mL of a deprotecting solution (1:1:48 DBU:piperidine:DMF). The resin was washed 5 times with DMF in 2 minute intervals with gentle agitation and draining in between each rinse. A second round of DMF washes was then performed. To add the next amino acid, 0.1695g (5 equiv) of Fmoc-Lys(Aloc)-OH and 0.1383g of HATU (4.9 equiv) were first dissolved in a minimal amount (\approx 4mL) of DMF. Immediately prior to adding the peptide solution to the resin, 0.1296mL of DIPEA were added to the peptide solution, the mixture was stirred, and subsequently added to the resin. After 1 hour of gentle agitation the remaining solution was drained off and the resin was once again washed with DMF as described above. The deprotection and DMF washes were then repeated. Next, 0.1923g (5equiv) of

Fmoc-Val-OPfp and 0.0514g (5 equiv) of HOBt were dissolved in a minimal amount of DMF (\approx 4mL) and added to the reaction vessel. After agitation for 1 hour, the solution was then drained and the wash/deprotect/wash procedure was followed once again. Using this method 0.1529g (5equiv) of Fmoc-Ala-OPfp and 0.1691g (5 equiv) Fmoc-Lys(Aloc)-OH were then added to the solid phase vessel and reacted. For the activated ester amino acid derivatives (those with a OPfp group) HOBt was utilized, whereas with the unactivated amino acids (those with an OH) were coupled using the combination of HATU and DIPEA. After the resin was washed 5 times with DMF, 5 washes of deprotection solution, and 5 times with DMF, 0.070 mL (10 equiv.) of acetic anhydride in a minimal amount of DMF was added to the resin and allowing to react for 1 hour with gentle agitation. The resin was then dried under N_2 and subsequently swelled for 30 minutes using CH_2Cl_2 . After draining off the CH_2Cl_2 , the reaction vessel was covered with aluminum foil and flushed with N_2 . Next, 0.0872g (1 equiv) of tris(triphenylphosphine)palladium(0) and 0.1055g (10 equiv) of dimedone were then dissolved in a solution of dry, degassed CH_2Cl_2 and THF (0.5mL:0.5mL). Using a syringe this solution was then added to the reaction vessel and agitated using a shaker for 6 hours. The solution was then drained and the resin was washed with 0.5% v/v DIPEA in DMF (5×10 mL), 0.5% w/v dimethyldithiocarbamic acid sodium salt solution (10×10 mL), dry CH_2Cl_2 (5×10 mL) and dry diethylether (5×5 mL). The resin was then dried under vacuum for several minutes. In order to crosslink the deprotected peptide, after flushing the reaction vessel with N_2 , 0.0261g (1.13equiv) of 1,1'-ferrocene dicarboxylic acid chloride in 1mL of DMF was added to the resin in addition to 0.130mL of DIPEA and the reaction vessel was shaken. After 96 hours, the reaction vessel was drained and subsequently washed with DMF (5×4 mL) in two minute intervals. In order to acylate any unreacted free amines, 0.070mL (10 equiv) of acetic anhydride was dissolved in 1mL of DMF and subsequently added to the resin. After 1 hour of shaking, the solution was drained and washed with DMF once again. The resin was subsequently dried under N_2 and the peptide was cleaved from the resin using a removal solution of 95:2.5:2.5 TFA:anisole: H_2O v/v solution with gentle agitation for 3 hours. The resin was then finally washed with two volumes of TFA for 2 minutes and all of the filtrates were collected in a single round bottom flask. The solvent was then evaporated off using a rotary evaporator yielding a crude mass of 0.1052g. The 1H NMR of the crude product does not contain any substantial peaks within the 4.5-5.0ppm region, characteristic of ferrocene Cp rings (Figure 2.11). Due to absence of ferrocene peaks, no purification attempt was made of the crude product.

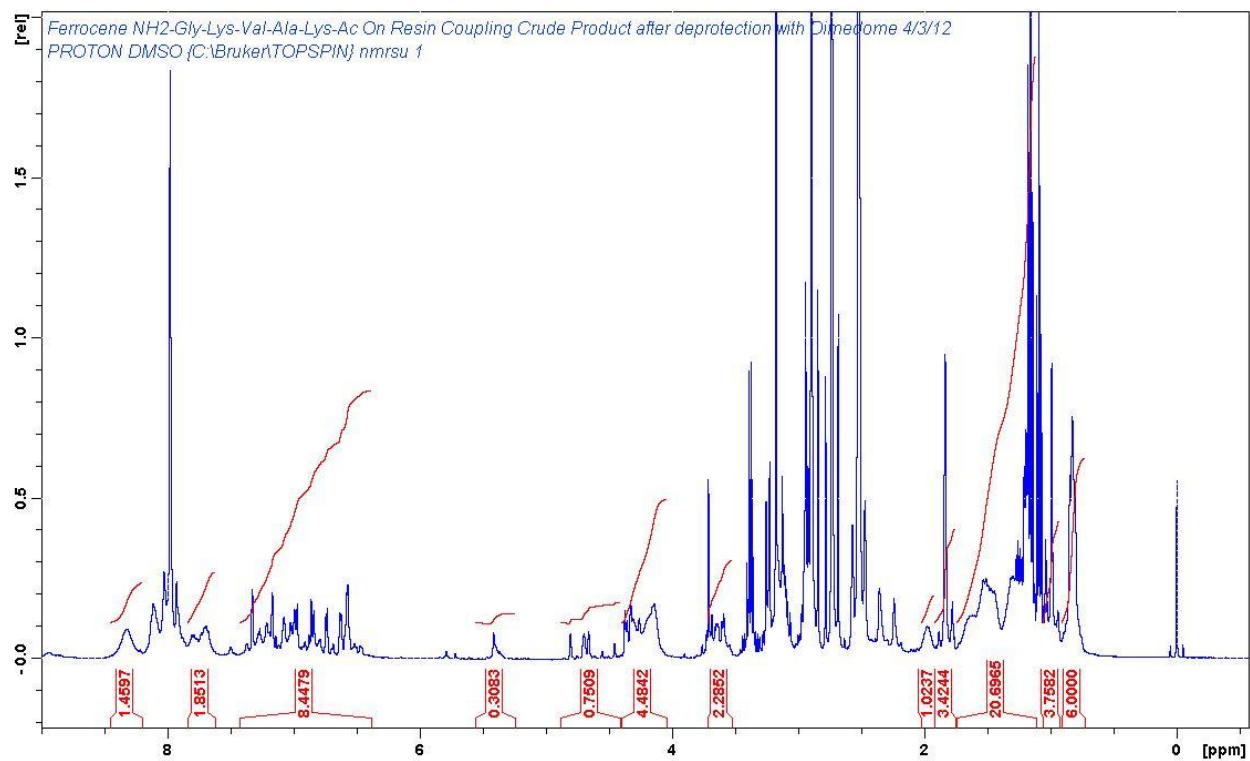
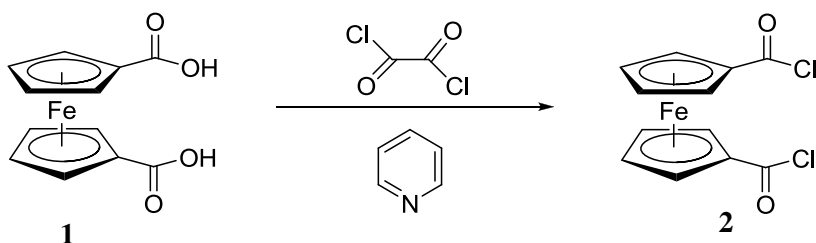


Figure 2.11: Crude on resin ferrocene cyclization with Aloc protected peptide

RESULTS AND DISCUSSION

The goal of this study was to successfully synthesize a pentapeptide with lysine residues at the i and i+3 positions which could be crosslinked with 1,1'-ferrocenedicarboxylic acid chloride while still on-resin. For the on resin synthesis, Fmoc based solid phase peptide synthesis (SPPS) was utilized. SPPS is advantageous due to the high purity and efficiency it affords in comparison to solution phase peptide synthesis. In SPPS, the amino acid is immobilized on the surface of an insoluble polymer. This methodology allows for sequential addition of amino acids to the N-terminus of the immobilized amino acid, enabling precise control of peptide assembly. During synthesis, the terminal amino acid is deprotected and the next amino acid is added. This process is repeated until the desired peptide is synthesized. Subsequently, the peptide chain is cleaved from the resin and desired product is obtained. Currently, there are two major protecting groups compatible with SPPS: Fmoc and Boc. Fmoc based SPPS was chosen over Boc based systems due to the milder reaction conditions and lack of special equipment required for the synthesis. Additionally, the highly acidic conditions for Boc removal would lead to premature cleavage of the peptide from the resin compatible with the possible on resin cyclization. To begin, 1,1'-ferrocenedicarboxylic acid chloride was prepared using previously published syntheses¹ (Scheme 3.1).

3.1 Preparation of 1,1'-Ferrocenedicarboxylic Acid Chloride¹



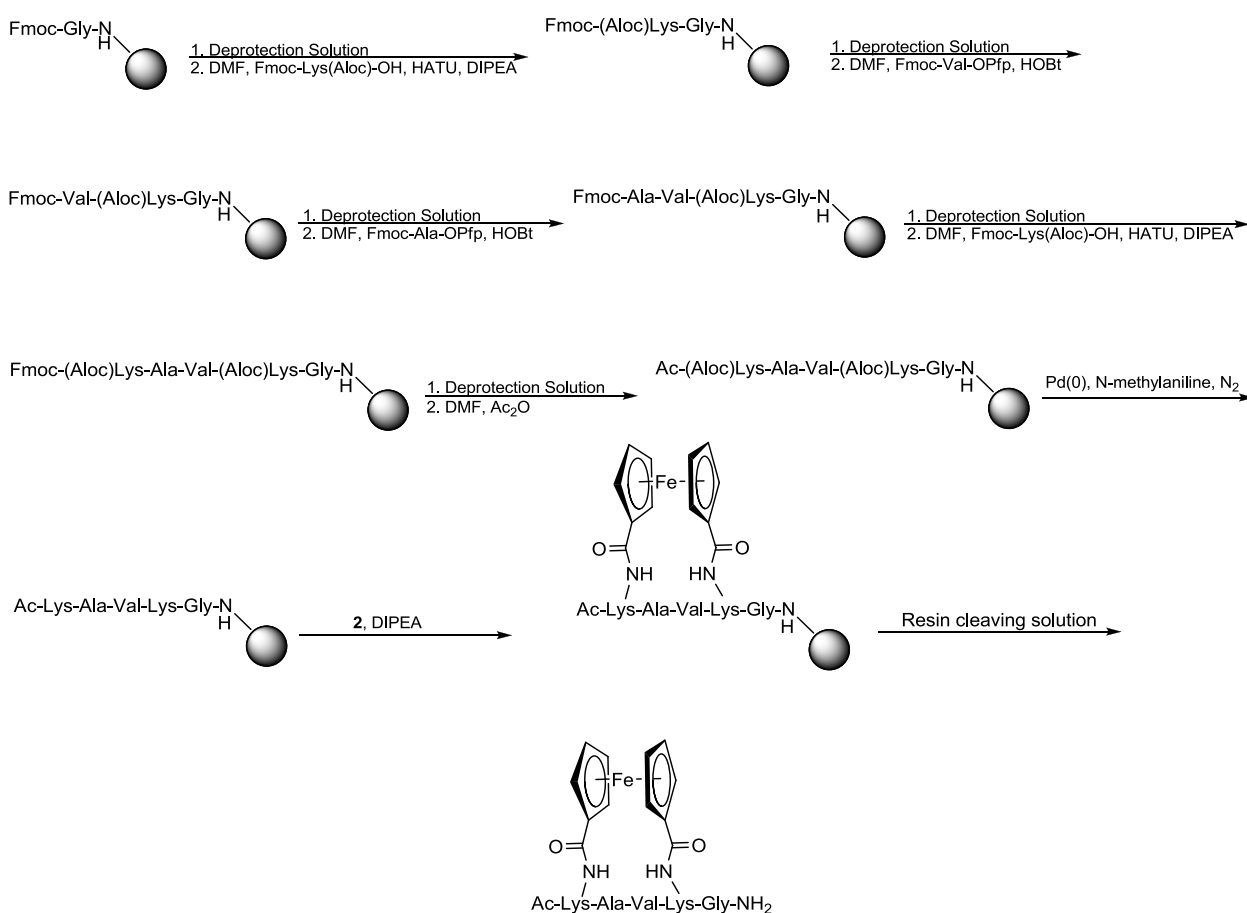
Scheme 3.1: Reaction scheme for the preparation of 1,1'-ferrocenedicarboxylic acid chloride

This methodology requires that the reaction be kept in the dark for 12 hours. Aluminum foil covering the flask was found to be sufficient. To limit exposure to water vapor, the reaction was also run with a rubber septum pierced with a syringe tip for ventilation. In order to confirm the product, melting point analysis was performed on the resulting red/crimson solid. The melting point was found to be 87°C-95°C, slightly lower than the reported literature value of 98°C-100°C. It is assumed that trace impurities are responsible for the slightly lower experimental value determined. The main source of impurities for the formed diacid chloride is hydrolysis to the starting dicarboxylic acid product, which has a melting point of 210°C-215°C. Given that the experimental value was lower than reported, it was assumed that this impurity was present only in trace amounts. In order to prolong the life of the 1,1'-ferrocenedicarboxylic acid chloride

product, the solid was kept under either N_2 or vacuum in a refrigerator. Additionally, prior to each use, the crude product was washed redissolved with dry ethyl ether, filtered to remove impurities, and the solvent was evaporated to ensure purity as the diacid chloride is soluble in ether, whereas, the diacid is not. The experimental yield of 78% was comparable to the published results, validating this as an effective methodology for the preparation of 1,1'-ferrocenedicarboxylic acid chloride.

3.2 Preparation of Ferrocene Crosslinked Ac-Lys-Ala-Val-Lys-Gly-NH₂

In the first attempt, the entire synthesis of Ac-Lys-Ala-Val-Lys-Gly-NH₂ cyclized on resin with ferrocene was carried out in its entirety (Scheme 3.2)



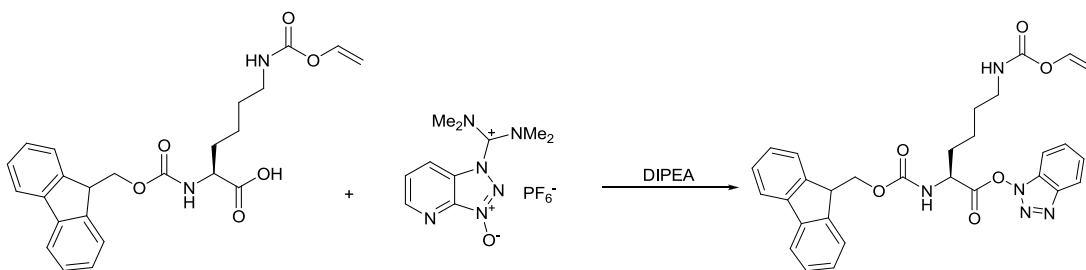
Scheme 3.2: On-resin cyclization of Ac-Lys-Ala-Val-Lys-Gly-NH₂

To begin, the pentapeptide Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂ was prepared on resin. This was accomplished using Fmoc solid phase peptide synthesis (SPPS).

For the synthesis, Fmoc-protected amino acids and Aloc-protected lysines residues were utilized due to

the mild conditions under which they are removed. This allowed for the use of a Rink Amide AM resin, which is cleaved under acidic conditions. In order to place lysine residues at the *i* and *i*+3 positions, only a tetrapeptide is required. The only commercially available lysine bound Rink Amide AM resin, however, has a Boc side chain protecting group. Boc groups are easily cleaved under the same conditions as the Rink Amide AM resin, making it unusable for our purposes. Due to the fact that the lysine residue could not be placed on the C-terminus, a glycine bound Rink Amide AM resin was utilized. A glycine residue was chosen due to its minimal steric bulk. Therefore a pentapeptide, rather than a tetrapeptide was synthesized. In addition all amino acids were used in their carboxylic acid or OPfp ester forms and activated using coupling reagents during synthesis. In particular the use of OSu derivatives was avoided due to previous studies which have shown that even in the presence of HOBt, reactions with OSu esters tend to be slow and form unwanted side products⁴¹.

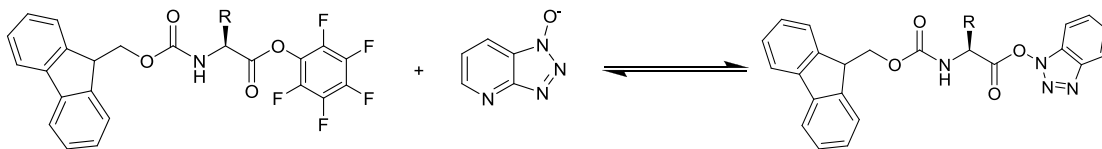
The first steps of SPPS are relatively simple. In order for efficient reaction, the dried resin must first be swollen to allow adequate access to the resin bound amino acid. This is done by adding the dried resin to the solid phase vessel and swelling with CH₂Cl₂ for 30 minutes with agitation. The Fmoc protecting group is then removed via a series of washes, followed by addition of the next amino acid dissolved in a minimal amount of DMF. The reaction was then gently agitated by shaking for one hour. The resin was then washed and the Fmoc group was once again removed, followed by subsequent addition of the next amino acid. This process could then be repeated as many times as necessary to complete synthesis of the desired peptide. In this manner, alloxycarbonyl protected lysine, valine, alanine, and alloxycarbonyl protected lysine were added to form the desired pentapeptide Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂. The lysine derivatives were purchased in their carboxylic acid form, and as such required activation for effective addition. This was accomplished using *in situ* generation of the activated ester derivative by reaction with HATU and DIPEA (Scheme 3.3).



Scheme 3.3: *In situ* activation of carboxylic acid amino acids with HATU

All other amino acids were used as OPfp activated esters. Although not required, HOBt was used to activate these amino acids *in situ* in order to increase yields. The generation of the activated ester is very

similar to that of HATU (Scheme 3.4).



Scheme 3.4: In situ activation of the OPfp amino acids

Finally, the Fmoc group was removed from the terminal residue and an acetyl group was added via the addition of acetic anhydride in DMF to the resin, in the same manner as the previous amino acids. It is important to note that these groups were removed due to the lability of Fmoc protecting groups under basic conditions and their tendency to make reaction mixtures more difficult to separate.

Aloc group removal was then attempted. Prior to reaction, the tetrakis(triphenylphosphine)palladium(0) catalyst was washed with methanol to remove impurities. The solution was then decanted and discarded while the remaining solid was placed under vacuum to remove any remain MeOH. The reaction vessel was then drained and purged with flowing N₂. Tetrakis(triphenylphosphine)palladium(0), dissolved in 5mL of 2:2:1:0.5 solution of DMF:THF:0.5M HCl: N-methylaniline was then added to the reaction flask. The resin was subsequently washed and repurged with N₂. Cyclization was then attempted via the addition of 1,1'-ferrocenedicarboxylic acid chloride and DIPEA as a base. In order to gauge and potentially quantify the amount of free amine present (if any) in the resulting product, the resin was treated with acetic anhydride in DMF for 1 hour prior to resin cleavage. In order to remove the cyclized peptide from the solid support, the resin was treated with a removal solution and agitated by shaking for 3 hours. The resulting solution was then drained into a flask and freeze dried overnight. The pale pink solid was then characterized via 1D ¹H NMR, 2D ¹H NMR COSY and ROESY, as well as circular dichroism. Initial ¹H NMR data indicated that the Aloc groups had not been successfully removed (Figure 3.1).

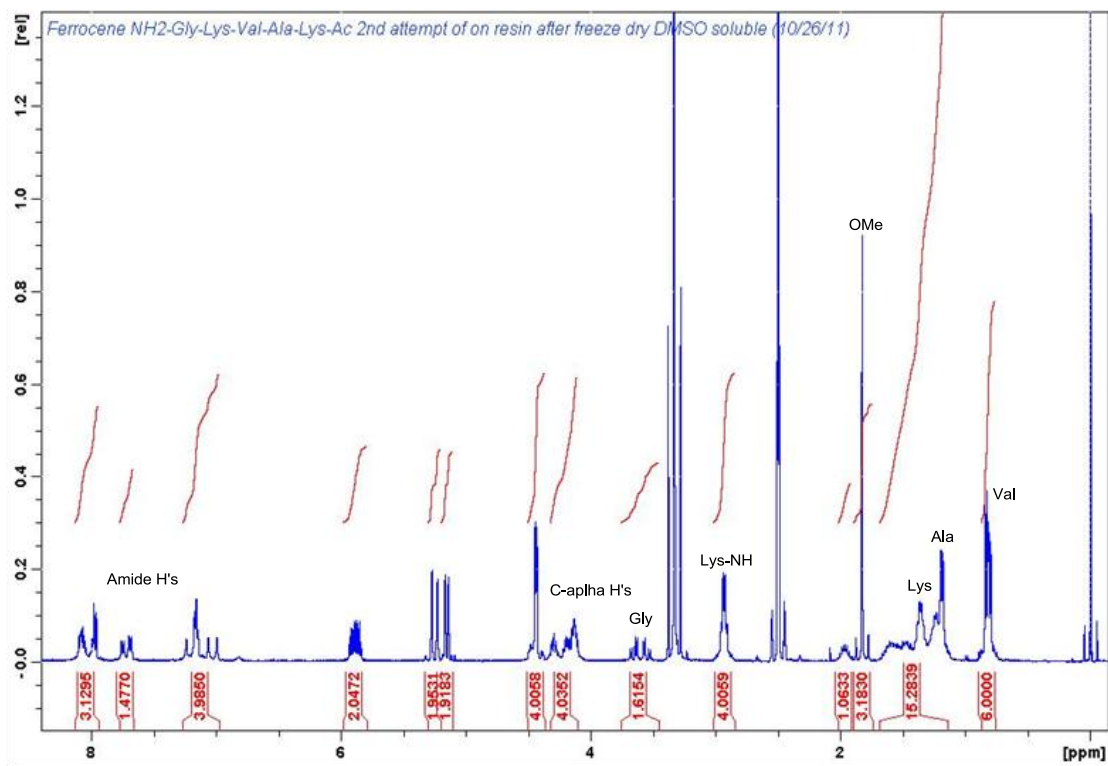
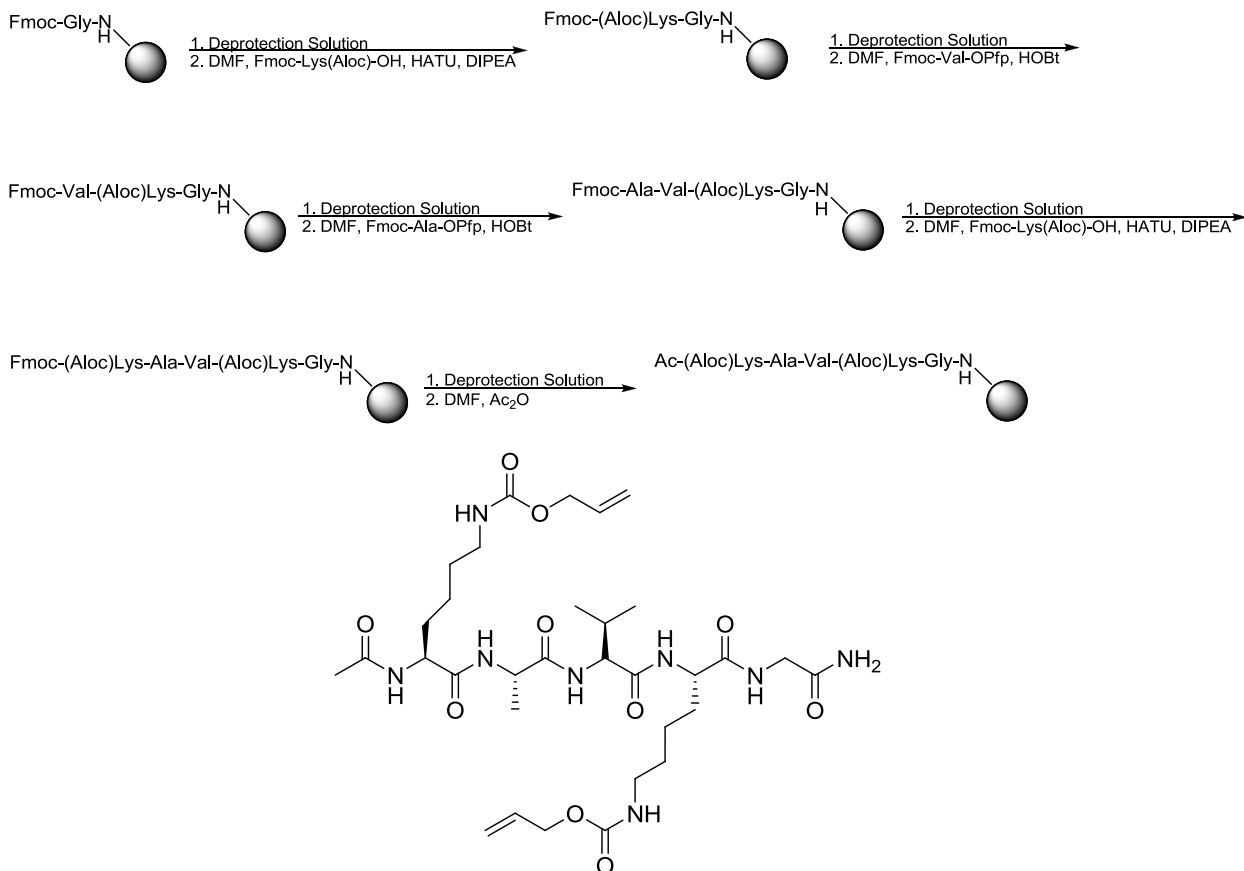


Figure 3.1: Crude product from on-resin Cyclization of Ac-Lys-Ala-Val-Lys-Gly-NH₂ with Ferrocene

3.3 Solid Phase Preparation of Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂



Scheme 3.4: Solid phase preparation of Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂

As stated, during the characterization of the on-resin cyclization product, the reaction was also attempted off-resin. First, the dried resin was added to the solid phase vessel and the resin was swelled with CH₂Cl₂ for 30 minutes with agitation. The Fmoc protecting group was then removed via a series of washes, followed by addition of the next amino acid dissolved in a minimal amount of DMF. The reaction was then gently agitated by shaking for one hour. The resin was then washed and the Fmoc group was once again removed, followed by subsequent addition of the next amino acid. This process could then be repeated as many times as necessary to complete synthesis of the desired peptide. In this manner, alloxycarbonyl protected lysine, valine, alanine, and alloxycarbonyl protected lysine were added to form the desired pentapeptide Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂. In order to remove the peptide from the solid support, the resin was treated with a removal solution and agitated by shaking for 3 hours. After rotary evaporation, the resulting peptide was triturated with diethyl ether to yield a beige solid.

This solid was characterized by 1D ¹H NMR, which indicated that the desired product was present with little to no impurities. To confirm the peak assignments a 2D ¹H COSY, which exhibits crosspeaks for

hydrogens one bond apart, was obtained. The exhibited cross peaks were consistent with the initial assignments from 1D ^1H NMR. An MS of the resulting product was not obtained because the instrument was not working at the time, but based on consistency of the 1D and 2D ^1H NMR data, it was assumed that the peptide was successfully synthesized.

3.4 Solution Phase Aloc Deprotection of Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂

Using the off-resin pentapeptide, a solution phase removal of the protecting Aloc groups was attempted. The previously prepared pentapeptide was first placed under an inert atmosphere of N_2 in a round bottom flask. To the flask was then added tetrakis(triphenylphosphine)palladium(0) (purified as previously discussed) in 5mL of a 2:2:1 solution of DMF:THF:0.5M HCl with N-methylaniline as the organic scavenger. After addition of the removal solution via a syringe, the reaction was monitored via TLC. Samples of the product mixture were then taken every 45 minutes for 7 hours. Within this time period, no noticeable difference in the R_f was observed, with all plates exhibiting 3 major spots with R_f values of 0.96, 0.91, and 0 respectively.

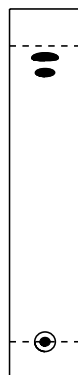


Figure 3.2: TLC of Aloc removal reaction mixture run with 1:1 CHCl_3 :MeOH mixture.

The reaction was run overnight and a final sample was analyzed via TLC, but no measurable differences in R_f values were observed. A 1D ^1H NMR was then taken of the crude product mixture to determine the efficiency of the Aloc removal.

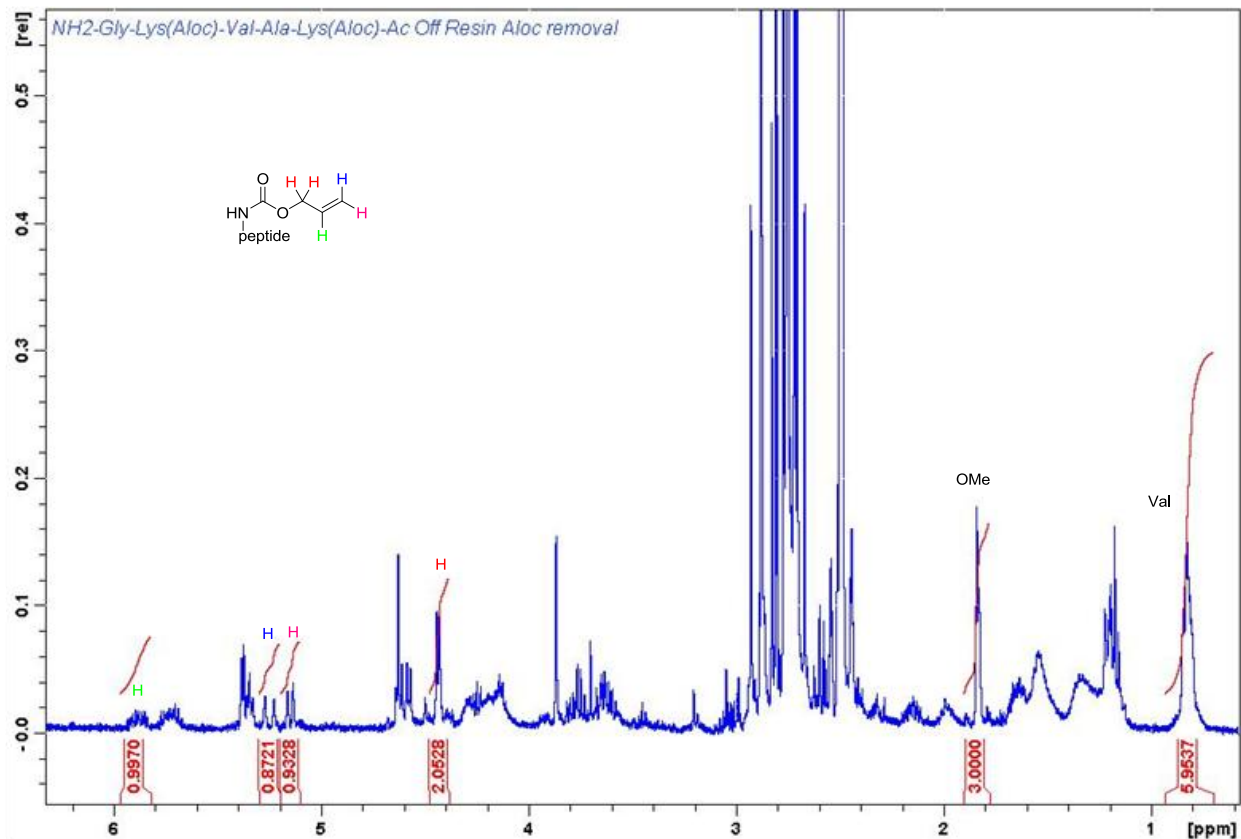


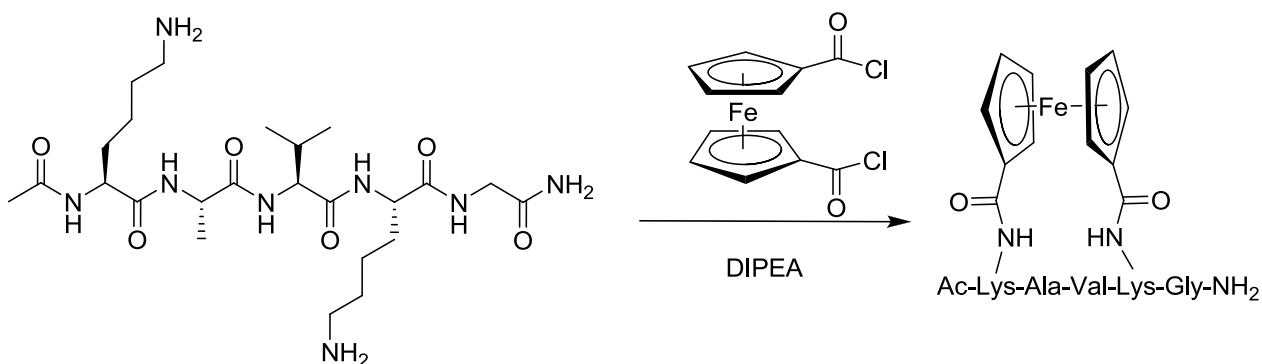
Figure 3.3: ¹H NMR of crude solution phase Aloc removal product in DMSO-*d*₆

Within the ¹H NMR spectrum, the Aloc peaks can still be observed, indicating that once again, the Aloc group removal was incomplete. Using the integration values of the Aloc peaks, the efficiency of the reaction was then be estimated. The complete peptide contains two Aloc groups, meaning that the integration values should be 2:2:2:4 for the assigned peaks in Figure 3.3. The experimental values, however, are half of the expected values, indicating that only about ½ of the Aloc groups are being removed from the peptide. There are several factors which could have contributed to the lack of success/efficiency. Due to the sensitivity of the Pd(0) catalyst, it was hypothesized that the catalyst precursor was either no longer active or that the active catalyst was being poisoned during the reaction by impurities in the N-methylaniline scavenger. In order to avoid this complication, the reaction was then run in the same manner but using freshly distilled N-methylaniline. It is of note that the freshly distilled N-methylaniline was significantly paler after distillation and noticeably more viscous. Once again, TLC was used to monitor the reaction, but no observable differences in R_f values were observed across the reaction duration. The observed spots had R_f values of 0.95, 0.88, 0.77, and 0 with an eluent of 1:1 CHCl₃:MeOH (Figure 3.4).

Aloc protecting groups. With the relative success of the Aloc removal, it was assumed that impurities in the undistilled N-methylaniline were mostly responsible for poisoning the catalyst in previous attempts. Due to the reactivity of the free amines in the unprotected pentapeptide, synthesis was continued without purification of the crude product mixture.

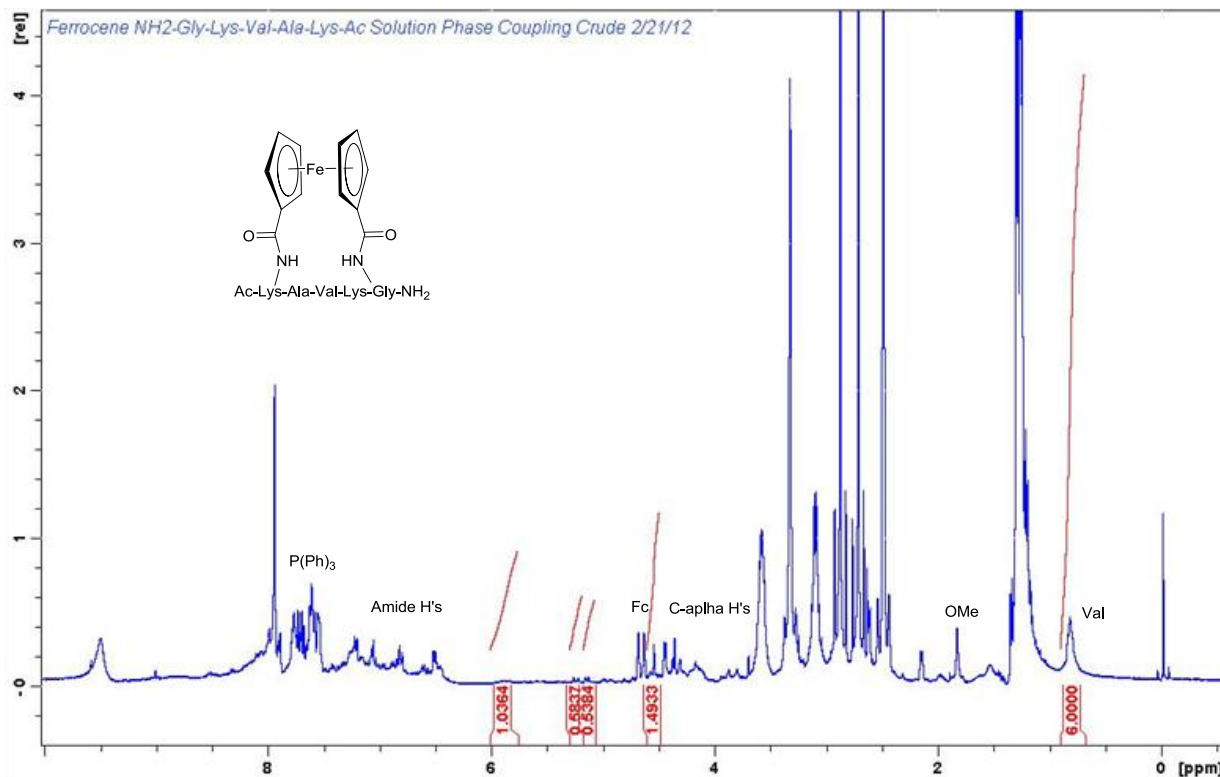
3.5 Solution Phase Cyclization of Ac-Lys-Ala-Val-Lys-Gly-NH₂

With a majority of the Aloc protecting groups successfully removed, cyclization was attempted in solution phase (Scheme 3.5).



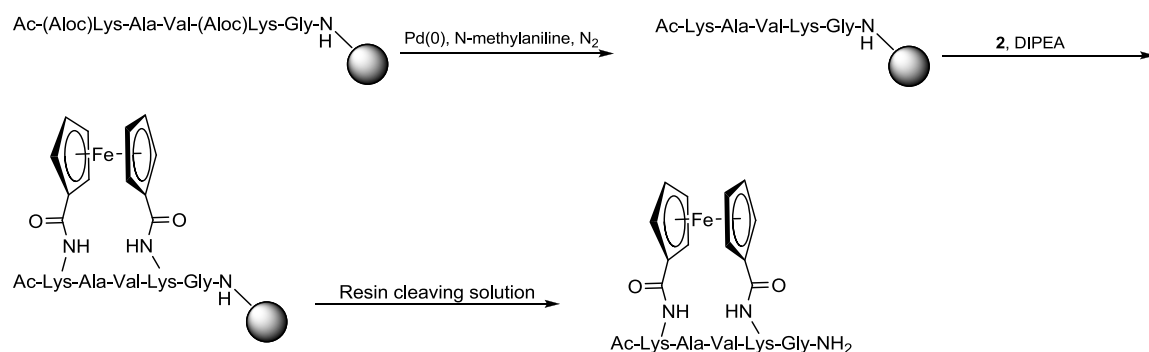
Scheme 3.5: Solution Phase Cyclization of Ac-Lys-Ala-Val-Lys-Gly-NH₂

Before the reaction could be conducted, the small volume of DMSO-d₆ used as the NMR solvent was evaporated off. During the process, it was observed that the product mixture turned from yellow to purple upon heating. It is believed that the observed color change is mostly due to degradation of the Pd(0) catalyst. For the synthesis, the crude product mixture was first placed under an inert N₂ gas atmosphere. 1,1'-ferrocenedicarboxylic acid chloride, dissolved in DMF and an excess of DIPEA were then added via a syringe and the reaction mixture was stirred. The cyclization was allowed to run for 94 hours. The solvent was then evaporated using a rotary evaporator and a 1D ¹H NMR of the crude product mixture was taken.



was recorded. The ^1H NMR spectrum, however, did not provide sufficient resolution to confirm the product. The spots shown in Figure 3.7 which remained on the origin, were purple, and therefore, it was assumed that they were primarily Pd catalyst. Other fractions were not isolated due to the prevalence of the palladium catalysts throughout the column, which after loading, was entirely purple in color due to the lack of separation. It is believed that most of the crude mixture, which was insoluble in most organic solvents, preferentially stayed dissolved in the HFIP. As an extremely polar molecule, HFIP moves fairly quickly through the column, and as a result, most of the product ran too quickly, resulting in minimal separation. Given that the product mixture had so many impurities, purification techniques were halted. Instead, having proven successful Aloc group removal using freshly distilled N-methylaniline, the on-resin cyclization was once again reattempted.

3.6 On-resin cyclization of Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂



Scheme 3.6: On-resin cyclization of Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂ with distilled N-methylaniline

Having successfully removed a majority of the Aloc groups in a solution phase reaction with freshly distilled N-methylaniline, the on-resin deprotection and cyclization of Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂ was then reattempted. SPPS should also make the isolation of the final product more efficient. First, the pentapeptide, Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂, was prepared using SPPS, as previously described. To the resin, under inert N₂ atmosphere, was then added tetrakis(triphenylphosphine)palladium(0) (purified as previously discussed) dissolved in 5mL of a 2:2:1 solution of DMF:THF:0.5M HCl with freshly distilled N-methylaniline. The reaction was then agitated for 13 hours under flowing N₂. Upon returning to the laboratory, it was noted that all of the solvent had evaporated in the elapsed time. As a result, 5mL of the reaction solution were then added once again and the reaction was agitated for an additional 13 hours without flowing N₂. The resin was then washed and repurged with N₂. A solution of 1,1'-ferrocenedicarboxylic acid chloride in DMF was then added to the

resin and the reaction mixture was agitated for 74 hours. The resin was then washed and treated with acetic anhydride in DMF. The peptide was then removed from the resin and the solvent was evaporated. 1D ^1H NMR and 2D ^1H COSY spectra were then taken for the crude product.

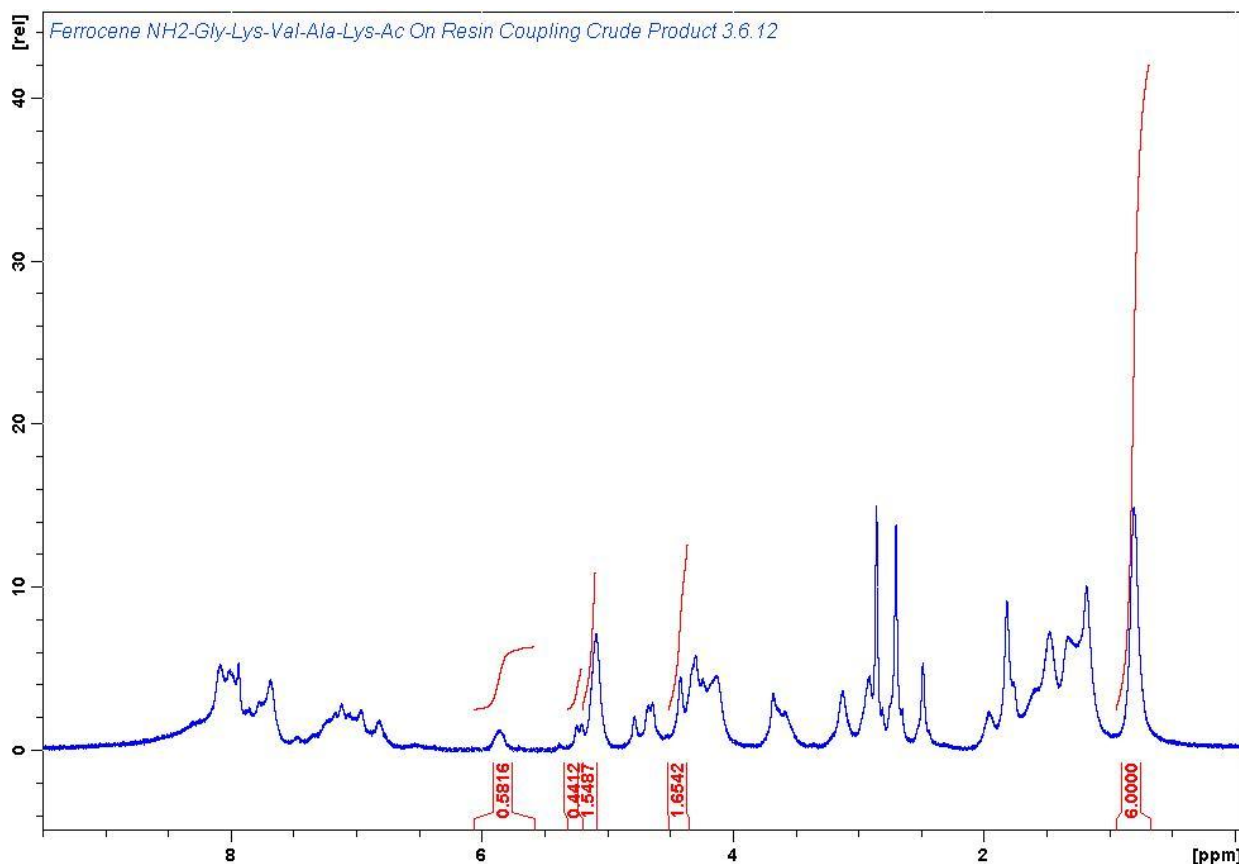
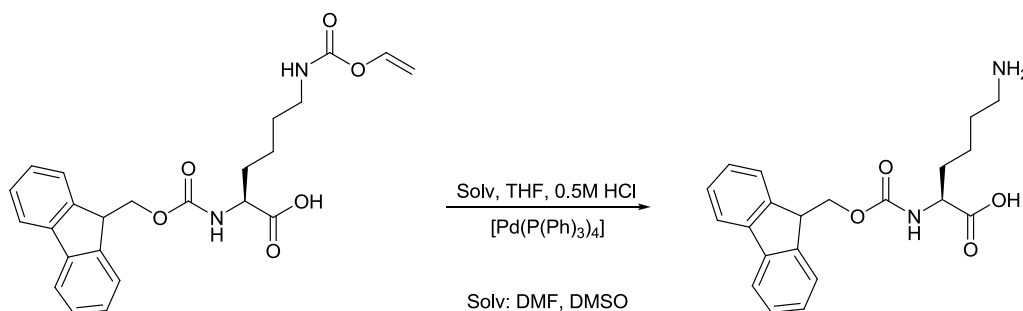


Figure 3.8: On resin cyclization attempt with distilled *N*-methylaniline

Similar to the solution phase deprotection, based on integration values, the Aloc deprotection step was found to only be about 75% effective. Before full synthesis could be carried out again, it was decided that a more effective method must first be developed for the peptide deprotection in order to maximize product purity and yields.

3.7 Solvent effects on Aloc deprotection efficiencies

It is widely known that catalysis can be highly dependent on the solvent within which the reaction is run. In all of the attempted synthesis, a mixture of DMF, THF, and 0.5M HCl had been utilized in contrast to literature reports which used DMSO instead of DMF. While the two are both polar aprotic solvents, side by side reactions were run in order to detect any differences in the purity/efficiency. For ease, the Aloc removal was performed on Fmoc-Lys(Aloc)-OH in solution phase.



Scheme 3.7: Aloc deprotection efficiencies using DMSO or DMF

To simplify the comparison, the two reactions were run using equal amounts of each reagent for identical durations. First, the protected amino acid was placed in a round bottom flask and placed under an inert N_2 atmosphere. To the reaction flask was then added 5mL of a 2:2:1 solution of Solv:THF:0.5M HCl where the solvent was either DMF or DMSO. It is noted that the Pd(0) catalyst precursor was significantly more soluble in DMSO than in DMF initially. The solutions were then added via a syringe to the flasks and the reactions were stirred for 24 hours. The reaction mixture was monitored via TLC with samples taken every hour. Across the reaction time, as was found with previous Aloc removal studies, there was no significant difference observed in the R_f values of the spots. After the allotted reaction time, the solvents were evaporated from each sample and 1D ^1H NMR's of each were taken (Figure 3.9)

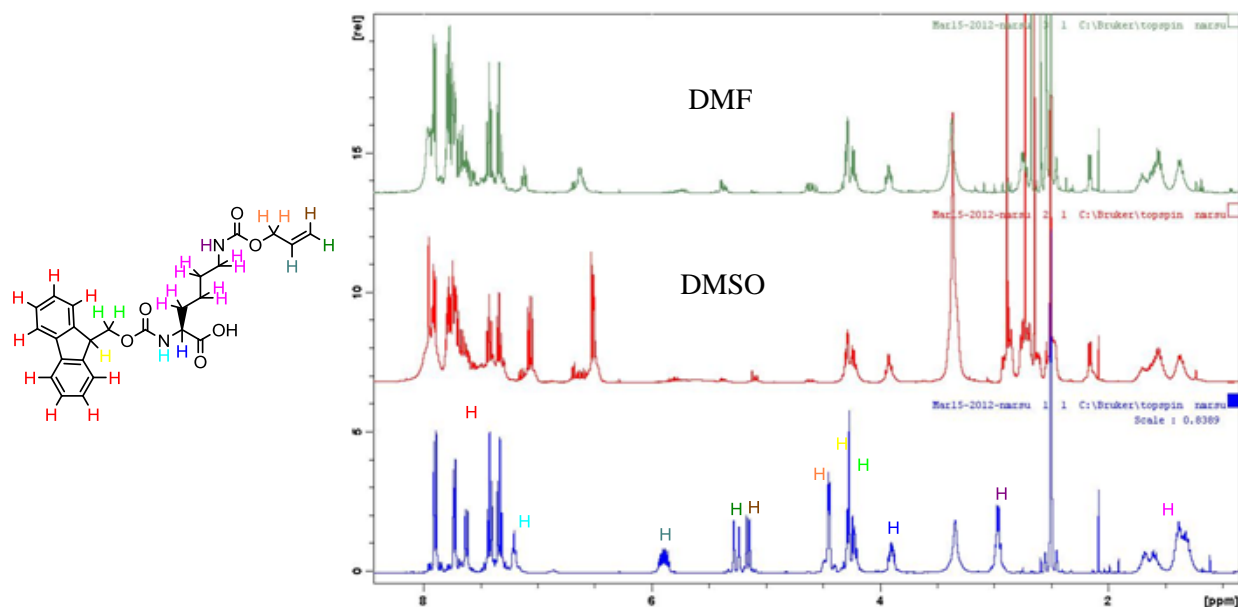


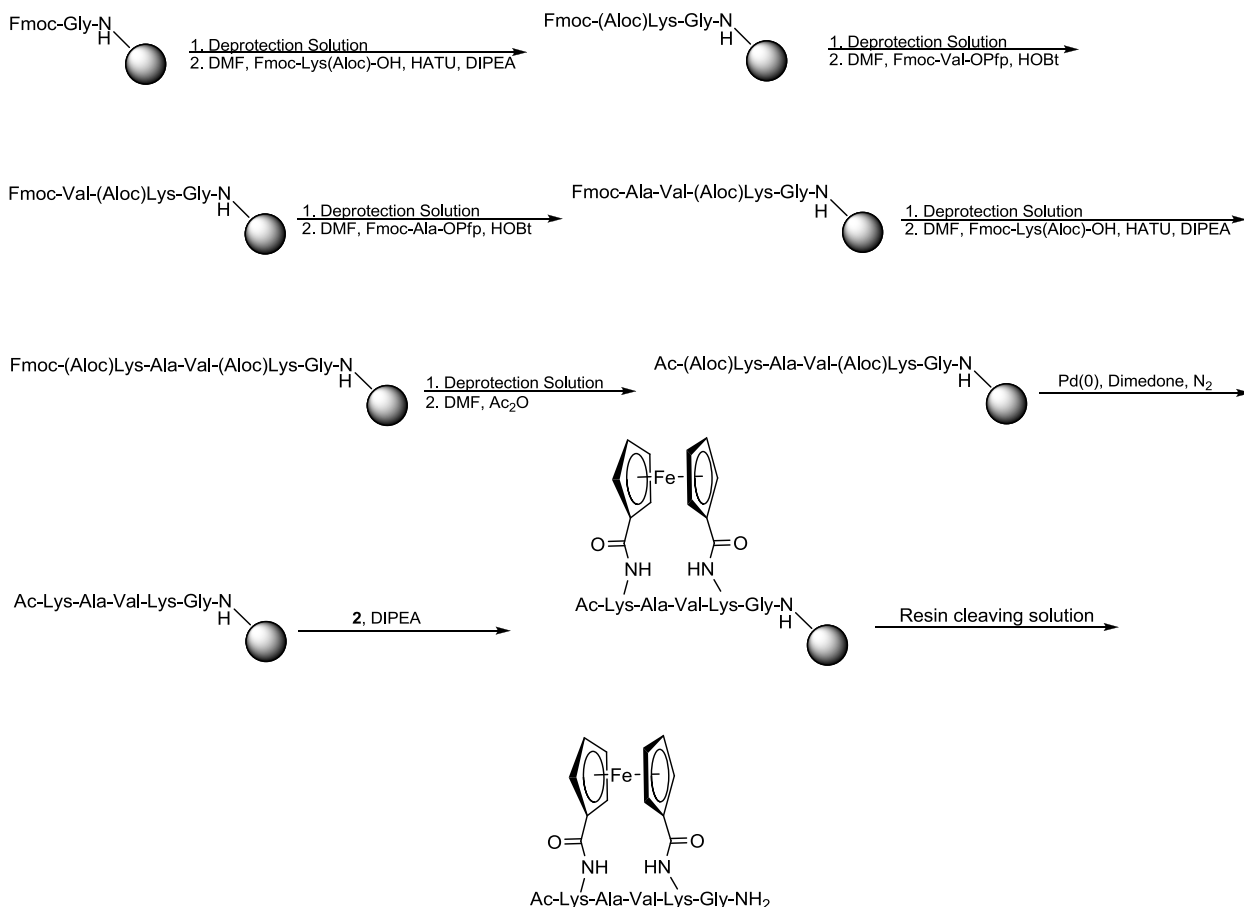
Figure 3.9: Aloc deprotection of Fmoc-Lys(Aloc)-OH with different solvents

From the ^1H NMR, both methods were shown to effectively remove the protecting Aloc groups. It is noted that the conversion efficiency within this simple system was almost 25% more effective than that of on-resin Aloc removal. It is therefore proposed that on-resin deprotection is slightly less effective

possibly due to accessibility issues. Given the limited success on-resin, alternative methods of Aloc deprotection were then investigated.

3.8 On-resin Aloc Deprotection of Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂ with Dimedone⁴²

After performing a literature search, an alternative method for Aloc removal utilizing dimedone as the scavenger was found. This previously published method² was then applied to the system at hand.



Scheme 3.8: On-resin cyclization of pentapeptide using Dimedone as scavenger

First, the pentapeptide was synthesized using SPPS as was accomplished earlier. Prior to catalyst addition, the reaction vessel was purged with N₂ and covered with aluminum foil to exclude light. To the swollen resin, was then added 1 equivalent of tetrakis(triphenylphosphine)palladium (0) (purified as previously discussed) and 10 equivalents of dimedone in 2mL of a 1:1 solution of CH₂Cl₂:THF. The solution was then agitated for 6 hours and subsequently washed with a 0.5% DIPEA/DMF (v/v) solution and a 0.5% dimethyldithiocarbamic acid sodium salt solution in DMF (w/v) to remove any palladium residues. Next, 1 equivalent of 1,1'-ferrocenedicarboxylic acid chloride and 10 equivalents of DIPEA in 10mL of DMF were added to the resin under N₂. The reaction was agitated for 24 hours to allow for

cyclization to occur. The resin was then treated with acetic anhydride in DMF to acylate any free amines which still may be present. The peptide was subsequently cleaved from the resin, and the solvent evaporated. The ^1H NMR of the crude product (Figure 3.10) does not appear to contain peaks within the ferrocene region (4.50ppm-5.00ppm) which integrate proportionally.

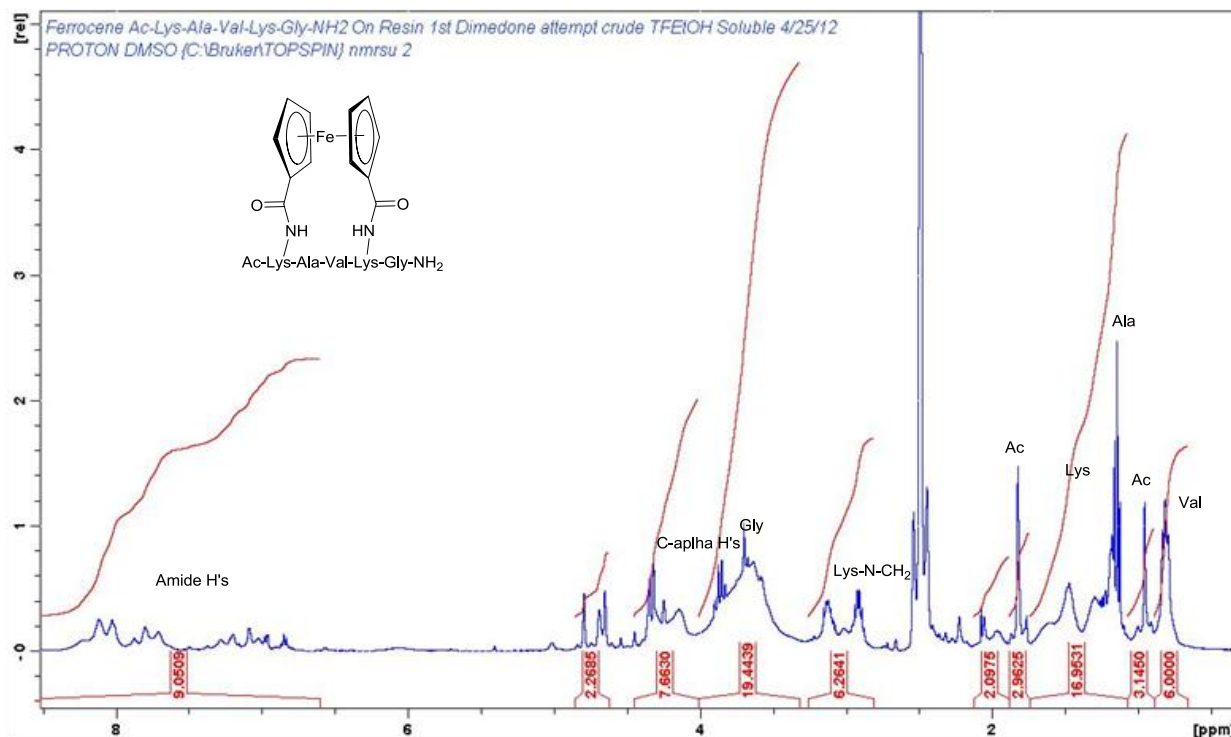
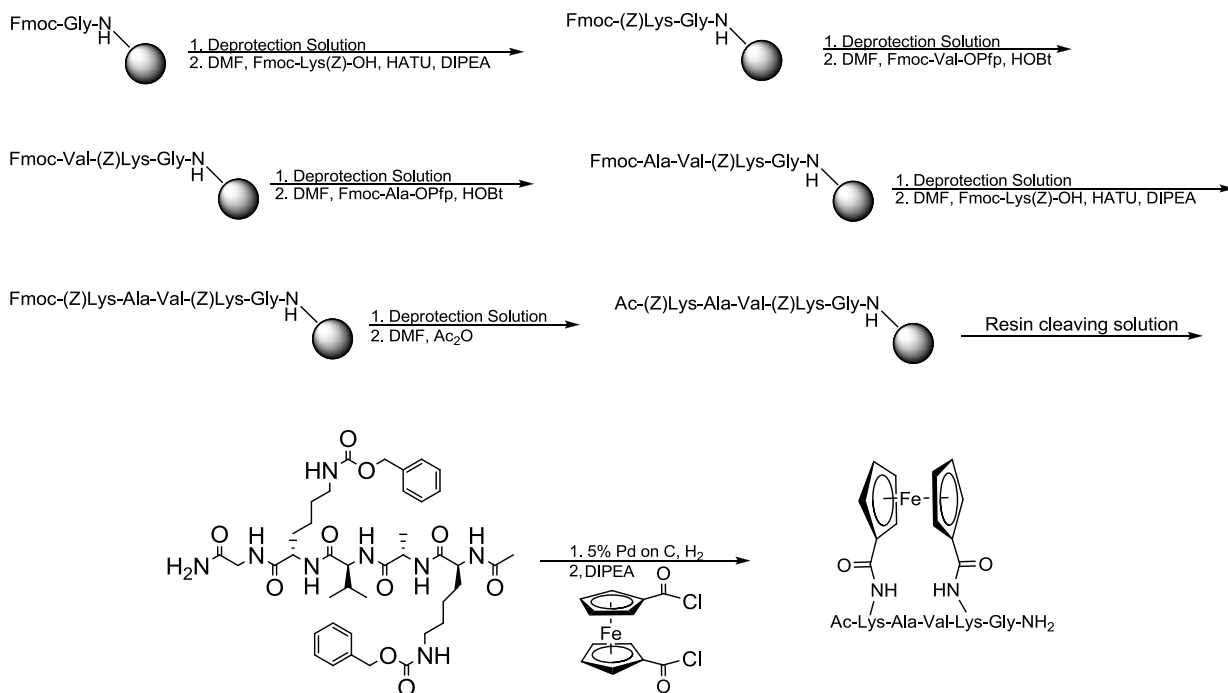


Figure 3.10: On resin cyclization of pentapeptide using dimedone as the scavenger

Based on the rough integrations, it was assumed that no appreciable quantity of the desired cyclized product was synthesized and no purification attempts were made.

It is of note that the source for this methodology was the first to mention the necessity of washing the resin with specific solutions post deprotection in order to remove any remaining palladium residues. Neither of the previous literature papers provided such details. Upon deeper investigation, it was discovered that all methodologies require special washes post deprotection. Given this, it is believed that the lack of removal of Pd(0) in previous attempts may have interfered with the cyclization process. Pd(0) is known to add to acid chlorides, and as a result, excess catalyst may have been reacting with the 1,1'-ferrocenedicarboxylic acid chloride during the subsequent step in synthesis.

3.9 Solution Phase Cyclization of Ac-Lys(Z)-Ala-Val-Lys(Z)-Gly-NH₂



Due to the unsuccessful on-resin cyclization, a solution phase variant was attempted using Z protected lysine residues. First, the dried resin was added to the solid phase vessel and the resin was swelled with CH₂Cl₂ for 30 minutes with agitation. The Fmoc protecting group was then removed via a series of washes, followed by addition of the next amino acid dissolved in a minimal amount of DMF. The reaction was then gently agitated by shaking for one hour. The resin was then washed and the Fmoc group was once again removed, followed by subsequent addition of the next amino acid. This process could then be repeated as many times as necessary to complete synthesis of the desired peptide. In this manner, carboxybenzyl protected lysine, valine, alanine, and carboxybenzyl protected lysine were added to form the desired pentapeptide Ac-Lys(Z)-Ala-Val-Lys(Z)-Gly-NH₂. In order to remove the peptide from the solid support, the resin was treated with a removal solution and agitated by shaking for 3 hours. After rotary evaporation, the resulting peptide was triturated with diethyl ether to yield an off white solid.

1D ¹H NMR indicated that the desired product was present with little to no impurities. To confirm the peak assignments, a 2D ¹H COSY was also taken and the exhibited cross peaks were consistent with the initial assignments from 1D ¹H NMR.

The product was then hydrogenated with 5% Pd on C under 40psi of H₂ for 24 hours. The palladium catalyst was then filtered off through celite and cyclization with ferrocene was attempted. To a stirring

solution of the peptide under N_2 , 1 equivalent of 1,1'-ferrocenedicarboxylic acid chloride in 10mL of DMF was added dropwise over an hour. The final concentration of 1,1'-ferrocenedicarboxylic acid chloride in the solution was 0.75mM. The solution was allowed to stir for 24 hours and the solvent was subsequently removed using a rotary evaporator. The 1H NMR of the product indicates that ferrocene is present in the crude product mixture (Figure 3.11).

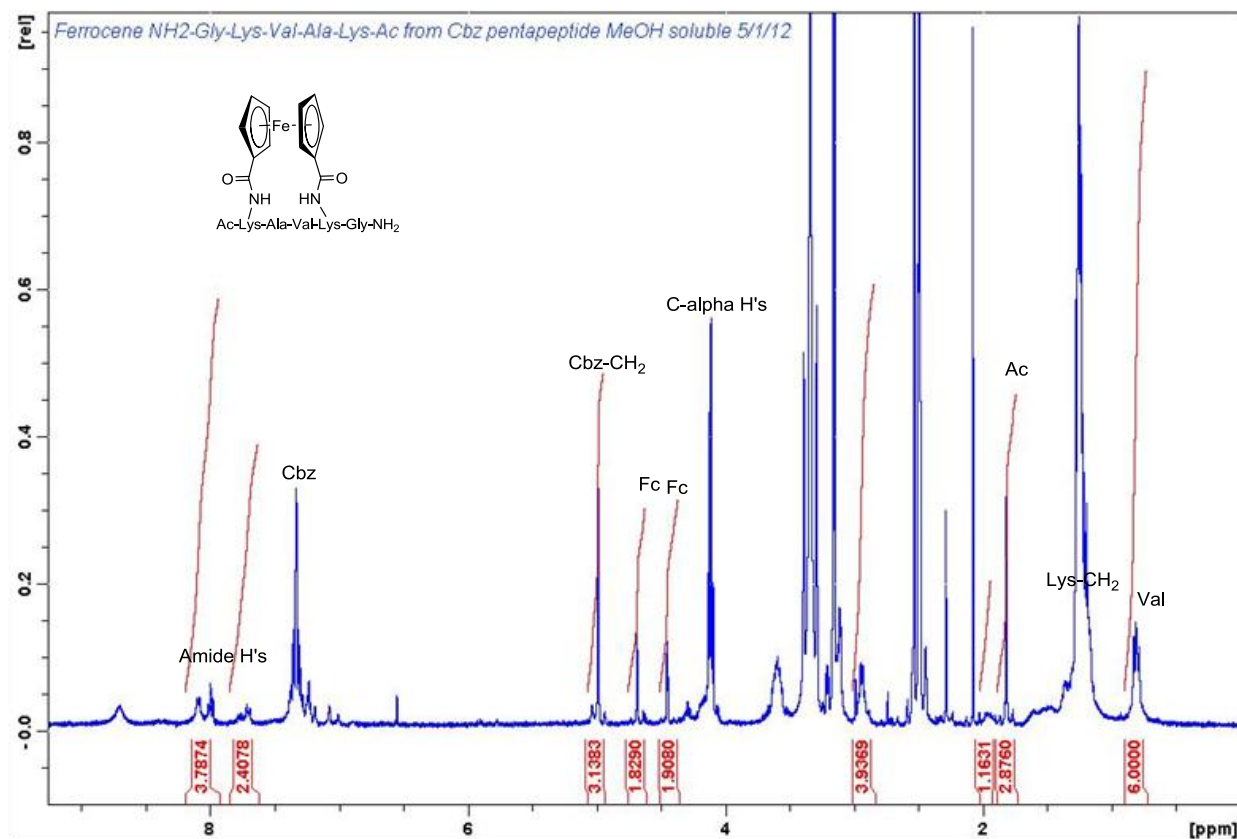


Figure 3.11: Solution Phase Cyclization of Ac-Lys-Ala-Val-Lys-Gly-NH₂ with ferrocene

After evaporation the obtained black film was no longer soluble in MeOH. Due to time constraints, flash chromatography could not be fully completed, although initial TLC analysis indicated that pure MeOH was not a sufficient eluent. It is also noted that upon dissolution in DMF, the black film had a redish tint, possibly indicative of the presence of ferrocene.

3.10 Summary and Conclusions

The pentapeptides Ac-Lys(Aloc)-Ala-Val-Lys(Aloc)-Gly-NH₂ was successfully synthesized using standard solid phase peptide synthesis techniques. While both solution and solid phase cyclization with 1,1'-ferrocenedicarboxylic acid chloride was attempted, neither yielded the desired products in any recoverable quantity. Additionally, neither Aloc removal technique utilized for solution or solid phase

synthesis was fully successful. As such, it is evident that an alternative technique must be utilized for Alloc removal, or the current conditions must be refined for maximum efficiency.

In addition, the pentapeptide Ac-Lys(Z)-Ala-Val-Lys(Z)-Gly-NH₂ was successfully synthesized. Based on the ¹H NMR spectrum, hydrogenation with 5% Pd on C under H₂ only partially removed the Z protecting groups. It is believed, however, that the catalyst loading and reaction time can be optimized to ensure complete removal of the Z protecting groups. While full characterization of the product could not be completed due to time constraints, the ¹H NMR spectrum appears to indicate the presence of ferrocene in the product. The best hypothesis based on the 1D ¹H NMR integral values of the crude product, is that the product is mostly comprised of a single ferrocene coupled to two pentapeptides.

APPENDIX

A.1 Synthesis of Boc-Cys(SCH₂CCH)-OH

Under an inert atmosphere of N₂, 1.030g (0.044mol, 4.0 equiv) of sodium metal was dissolved in ~60mL of absolute ethanol. Once fully dissolved, 1.956g (0.0111mol, 1.0 equiv.) of L-cysteine hydrochloride hydrate and 4.90mL (0.044mol, 4.0 equiv.) of propargyl bromide was added to the solution and allowed to react for 2 hours. The ethanol was then evaporated off using a rotary-evaporator and the remaining product was redissolved in a minimal amount of THF. To the solution was then added 4.865g (0.0223mol, 2.0 equiv.) of Boc₂O that was previously dissolved in approximately 12.5mL of THF. Additionally, 5.0mL (0.055mol, 5.0 equiv) of triethylamine and 25.0mL of deionized H₂O was added to the flask and allowed to stir covered overnight with a syringe inserted into the rubber septum for gas change. The THF was then evaporated off and the solution was made basic through the addition of 1M NaOH. The aqueous layer was then washed with 3× ~25mL of ethyl acetate, acidified to a pH of 2 using concentrated HCl, and extracted using 3× ~25mL ethyl acetate. The remaining ethyl acetate layer was dried using MgSO₄ and filtered into a round bottom flask (tare mass: 99.647g) and evaporated off using the rotary evaporator to yield a dark brown oil. The final mass of the flask was 101.606g (1.959g, 0.0133mol product). The product was then confirmed using ¹H NMR and its purity was determined by TLC (Figure A1). The product had an R_f value of 0.67 with an eluent of 1:1 ethyl acetate:methanol.

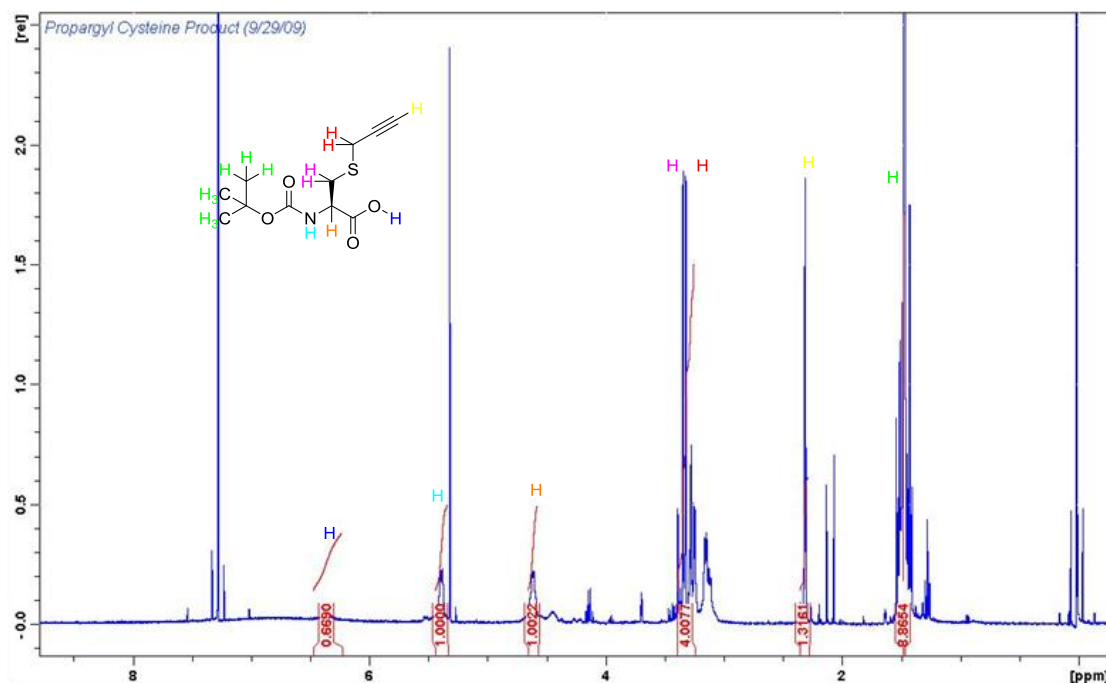


Figure A1: ¹H NMR of Boc-Cys(Prg)-OH in CDCl₃

^1H NMR: (CDCl_3) δ 6.356ppm (1H, bs, OH), 5.392ppm (1H, d, $^3\text{J}=7.66$ Hz, NH), 4.617ppm (1 H, q, $^3\text{J}=5.86$, C α -H), 3.3357ppm (2H, dd (qd), $^2\text{J}=8.80\text{Hz}$ $^3\text{J}=2.70\text{Hz}$, CH_2), 3.2687ppm (2 H, dd, $^2\text{J}=13.27\text{Hz}$ $^3\text{J}=4.84$, CH_2), 2.3155ppm (1 H, s, alkyne), 1.498ppm (9H, s, Boc- CH_3).

A.2 Synthesis of Boc-Cys(SCH₂CCH)-Anisidine

0.500g (0.00193mol) of previously synthesized Boc-Cys(SCH₂CCH)-OH was dissolved in 2.07mL of CH_2Cl_2 to obtain a concentration of 0.9M. To the flask was then added 0.261g (0.00212mol, 1.1 equiv.) anisidine and the solution was allowed to stir in an ice water bath. Once the solution had reached 0°C (approximately 5 minutes) 0.443g (0.00231mol, 1.2 equiv.) of EDC was added and the solution was allowed to slowly warm to room temperature over a 4 hour period. The CH_2Cl_2 was evaporated off using a rotary evaporator and the remaining oil was redissolved in ethyl acetate and washed with 3 \times 25mL of 0.1M HCl, 3 \times 25mL of saturated NaHCO_3 solution, and 1 \times 25mL brine, after which the ethyl acetate layer was dried with MgSO_4 and the ethyl acetate was rotovapped off. The product was purified via flash chromatography (3cm diameter column, 2:1ethyl acetate/hexanes eluent) and massed at 80.116g (tare: 79.858g). The structure was confirmed by ^1H NMR and its purity was determined by TLC (Figure A2). The product had an R_f value of 0.57 with an eluent of 2:1 hexanes:ethyl acetate. ESMS was also performed on the given sample, and the molecular ion peak, in addition several fragments were confirmed to be present.

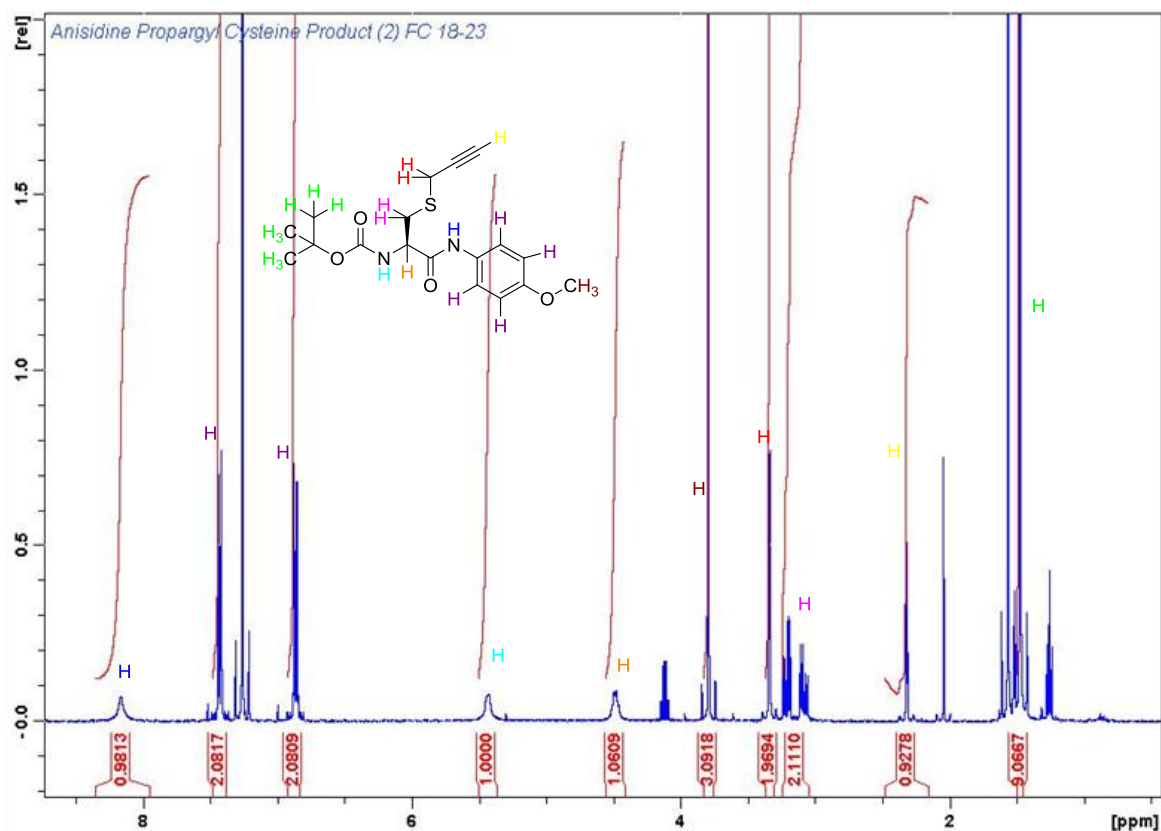


Figure A2: ^1H NMR of Boc-Cys(Prg)-Anisidine in CDCl_3

^1H NMR: (CDCl_3) δ 8.1661ppm (1H, s, Ani-NH), 7.4289ppm (2 H, d, $^3\text{J}=8.93\text{Hz}$, Ani-CH), 6.8674ppm (2H, d, $^3\text{J}=9.05$, Ani-CH), 5.4372 (1H, d, $^3\text{J}=6.64\text{Hz}$, Cys-NH), 4.4887ppm (1 H, q, $^1\text{J}=6.76$, CaH), 3.7954ppm (3H, s, OMe), 3.343ppm (2 H, d, $^3\text{J}=2.60$, Prg- CH_2), 3.1499ppm (2H, s (qd), $^2\text{J}=52.21\text{Hz}$, $^3\text{J}=14.02\text{Hz}$, Cys- CH_2), 2.3223ppm, 1H, s, Alkyne-H), 1.4785ppm, 9H, s, Boc-(CH_3) $_3$).

A.3 Synthesis of Boc-Cys(SCH_2CCH)-OSu

0.500g (0.00193mol) of Boc-Cys(SCH_2CCH)-OH was dissolved in 4.00mL of CH_2Cl_2 and to this solution was added 1.56g (0.0145mol, 7.5 equiv.) of N-hydroxysuccinimide. The solution was then placed in an ice bath and allowed to cool to 0°C . Once at the desired temperature, 0.444g (0.00232mol, 1.2 equiv.) of EDC was added to the flask and the reaction was left for 4 hours while the solution slowly warmed to room temperature. An additional 15mL of CH_2Cl_2 was then added to the reaction flask and the solution was washed with $3 \times 25\text{mL}$ of 0.1HCl, $3 \times 25\text{mL}$ of saturated NaHCO_3 solution, and $1 \times 25\text{mL}$ brine. The CH_2Cl_2 layer was then dried using MgSO_4 , filtered, and evaporated using a rotary evaporator. The crude product was then purified using flash chromatography (3cm diameter column, 7:3 hexanes/ethyl acetate (350mL) and pure ethyl acetate eluents). The product was then massed at 45.198g (tare: 45.086g) and its

structure was confirmed by ^1H NMR and its purity was determined by TLC (Figure A3). The desired product had an R_f value of 0.65 with an eluent of 2:1 ethyl acetate:hexanes.

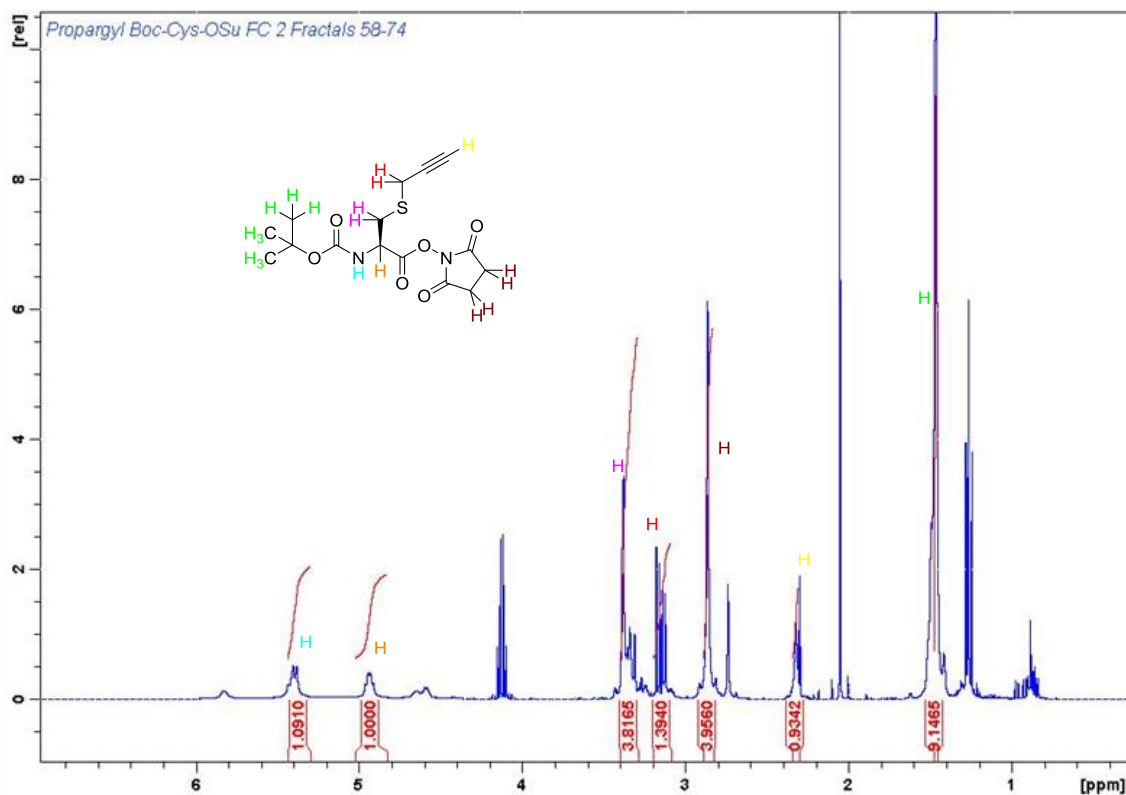


Figure A3: ^1H NMR of Boc-Cys(Prg)-OSu in CDCl_3

^1H NMR: (CDCl_3) δ 5.396ppm (1H, d, $^3\text{J}=8.74\text{Hz}$, Cys-NH), 4.9367ppm (1H, q, $^2\text{J}=5.46\text{Hz}$, $^3\text{J}=6.92\text{Hz}$, $\text{C}\alpha\text{H}$), 3.3824ppm (2H, d, $^3\text{J}=2.37\text{Hz}$, Prg- CH_2), 3.2248ppm (2H, s (qd), $^2\text{J}=40.17\text{ Hz}$, 14.40Hz , $^3\text{J}=7.48$, Cys- CH_2), 2.8634ppm (4H, s, OSu), 2.3214ppm (1H, s, Alkyne-H), 1.4663ppm (9 H, s, Boc CH_3)₃.

A.4 Synthesis of $\text{CF}_3\text{CO}_2[\text{Cys}(\text{SCH}_2\text{CCH})\text{-Anisidine}]$

0.100g(0.000274mol) of Boc-Cys(SCH_2CCH)-Anisidine was dissolved in ~1ml of CH_2Cl_2 and a single drop of anisole was added to the stirring solution. The reaction flask was placed in an ice bath and allowed to cool to 0°C . Once at the target temperature, 1mL of TFA was additionally added to the solution. The flask was slowly warmed to room temperature by the ambient room temperature (~4-5hours) and the CH_2Cl_2 and TFA were stripped off using a high vacuum rotary evaporator. The grey-yellow colored oil was left on the vacuum line overnight and massed the next day 37.832g (tare: 37.709g).

A.5 Synthesis of Boc-Cys(SCH₂CCH)-Cys(SCH₂CCH)-Anisidine

0.0726g(0.000274mol) of CF₃CO₂[Cys(SCH₂CCH)-Anisidine] was first dissolved in a minimal amount of CH₂Cl₂ and a stir bar was added to the flask. To the stirring solution were then added 0.98g(1 equiv.) of Boc-Cys(SCH₂CCH)-OSu and 0.29mL(7.6 equiv) of triethylamine in quick succession. The solution was allowed to react for 4 hours at room temperature and the CH₂Cl₂ was stripped off using a rotary evaporator. The golden yellow brown residue was then redissolved in ethyl acetate and transferred to a separatory funnel. The solution was washed with three times with 10mL of 0.1M HCl, three times with 10mL of saturated NaHCO₃ solution, and once with 10mL of brine. The ethyl acetate layer was then transferred into a round bottom flask and the ethyl acetate was stripped from the solution and the flask was massed at 55.672g (tare: 55.630g), yielding a product mass of 0.042g. The product was then confirmed by both TLC and ¹H NMR (Figure A4). The product was shown to be pure by TLC with an R_f of 0.60 in 3:2 ethyl acetate:hexanes. ESMS was also performed on the given sample, and the molecular ion peak, in addition several fragments were confirmed to be present.

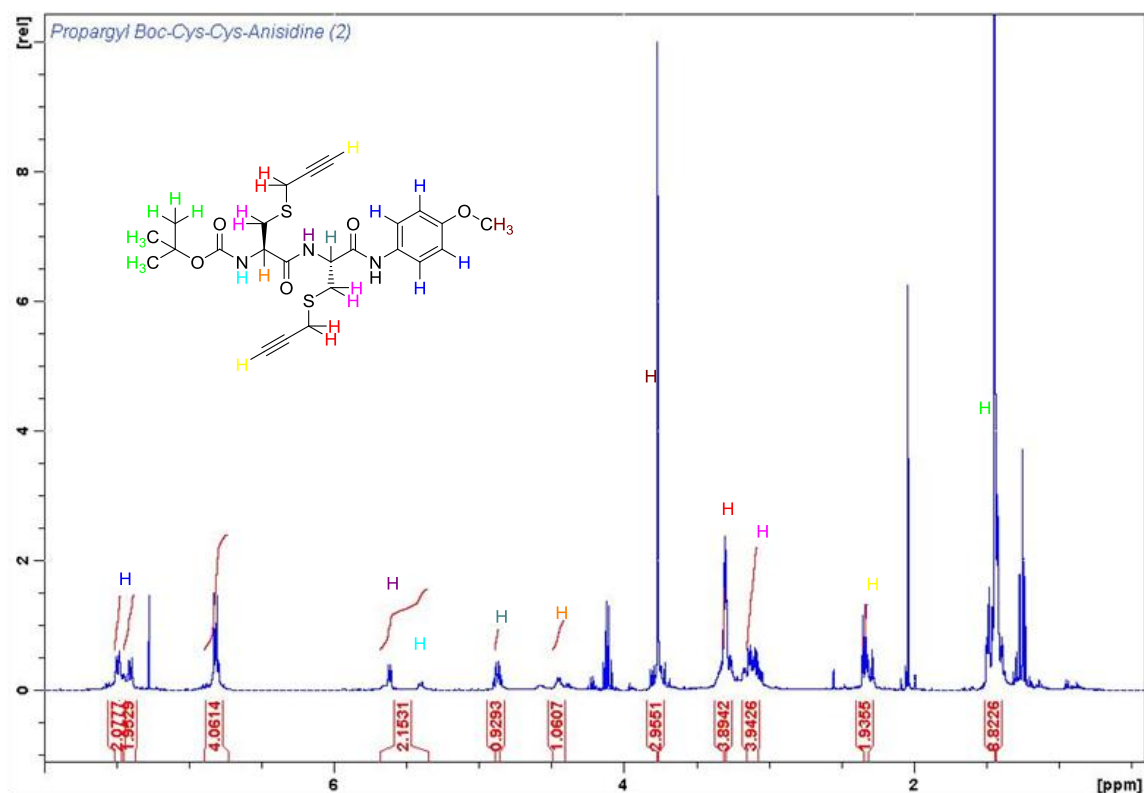


Figure A4: ¹H NMR of Boc-Cys(Prg)-Anisidine in CDCl₃

^1H NMR: (CDCl_3) δ 8.707ppm (1H, s, Ani-NH), 7.4502ppm (2H, d, $^2\text{J}=35.1\text{Hz}$, $^3\text{J}=9.13$, Ani-CH), 6.1893ppm (2H, d, $^3\text{J}=9.02\text{Hz}$, Ani-H), 5.6185ppm (1H, d, $^3\text{J}=6.37$, Cys-NH), 5.523ppm (1H, d, $^2\text{J}=99.54\text{Hz}$, $^3\text{J}=7.64$, Cys-NH), 4.8748ppm (1H, q, $^2\text{J}=5.94$, $^2\text{J}=5.94$, C α), 4.4526ppm (1H, q, $^2\text{J}=5.94$, $^2\text{J}=5.94$, C α), 3.7697ppm (3H, s, OMe), 3.306ppm (4H, m, Cys- CH_2), 3.119ppm (4H, m, Cys- CH_2), 2.332ppm (2H, m, Alkyne-H), 1.4418ppm (1H, s, Boc-(CH_3) $_3$).

A.6 Synthesis of $W(\text{dmtc})_2(\text{Boc-Cys}(\text{SCH}_2\text{CCH})\text{-Cys}(\text{SCH}_2\text{CCH})\text{-Anisidine})$

Approximately 1500mL of methanol was first degassed using a partial vacuum and filtration flask. In the hood a 2L 3-neck Schlenk flask was set up within which 0.504g of $W(\text{dmtc})_2(\text{CO})_3$, which had been previously synthesized, was transferred. About 1.1L of the degassed methanol was then added to the 3-neck reaction flask in addition to a stir bar. The stirring solution was then put under an inert atmosphere of N_2 and stirred until most of the solid had dissolved. The solution was then brought to a gentle reflux using a heating mantle. Once at the moderate reflux, 0.504g of Boc-Cys(SCH_2CCH)-Cys(SCH_2CCH)-Anisidine, which had been dissolved in a 7mL of the degassed methanol, was added to the stirring solution using a 10mL syringe. Upon addition of the peptide, the solution immediately became a dark forest green color, indicating formation of the mono-alkyne complex. Reflux of the solution was continued until the solution had become a lemon yellow color, indicating that both alkynes had been coordinated, which took approximately 45 minutes to accomplish. The methanol was then stripped from solution. The product was then purified using flash chromatography and an eluent of 1:1 ethyl acetate:hexanes. Fractions 115-140 were then combined and transferred to a round bottom flask which was massed at 63.329g (tare: 62.291g), yielding a product mass of 0.408g. The desired product was then confirmed via ^1H NMR, TLC, and ESI-MS (Figure A5, Figure A7). The desired product had an R_f value of 0.20 with an eluent of 1:1 ethyl acetate:hexanes. The purity of the product was also tested using HPLC (Figure A6). For HPLC analysis the gradient is provided in Table A1. Absorbance readings were all recorded at 310nm.

Table A1: HPLC Gradient programming for Purity Analysis of $W(\text{dmtc})_2(\text{Boc-Cys}(\text{Prg})\text{-Cys}(\text{Prg})\text{-Ani})$

Time	0.1% TFA	Acetonitrile	Flow
0	80	20	1 ml/min
6	50	50	1
12	0	100	1
20	0	100	1
22	80	20	1

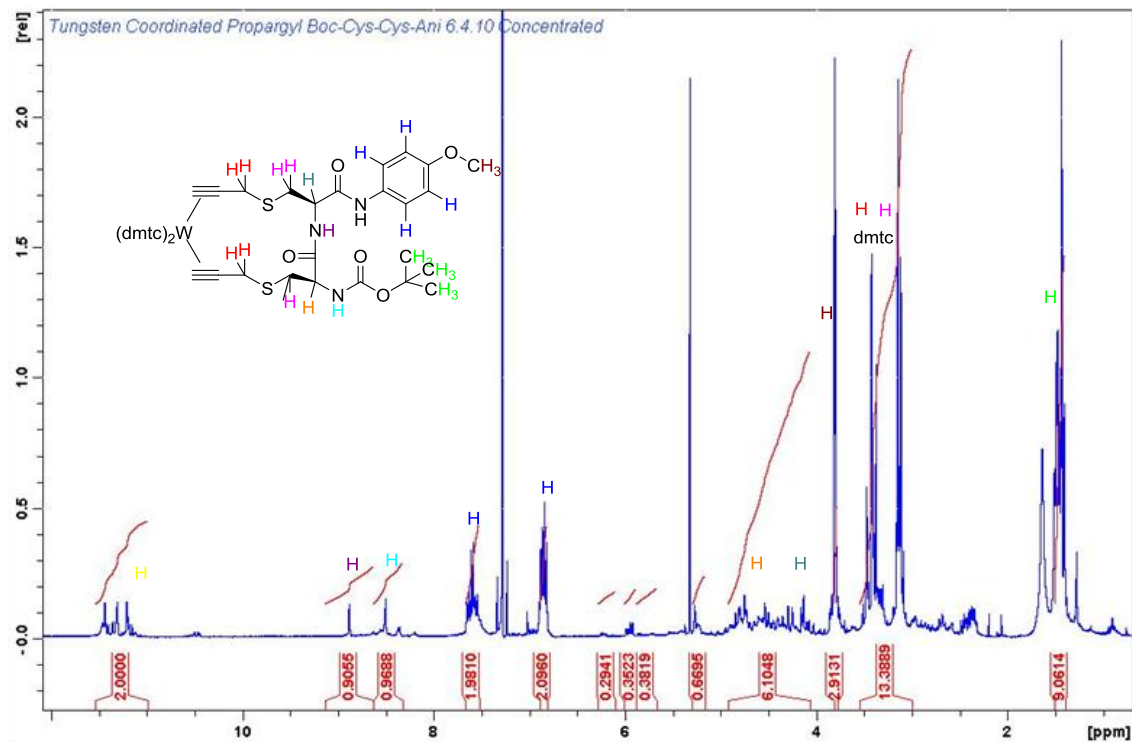
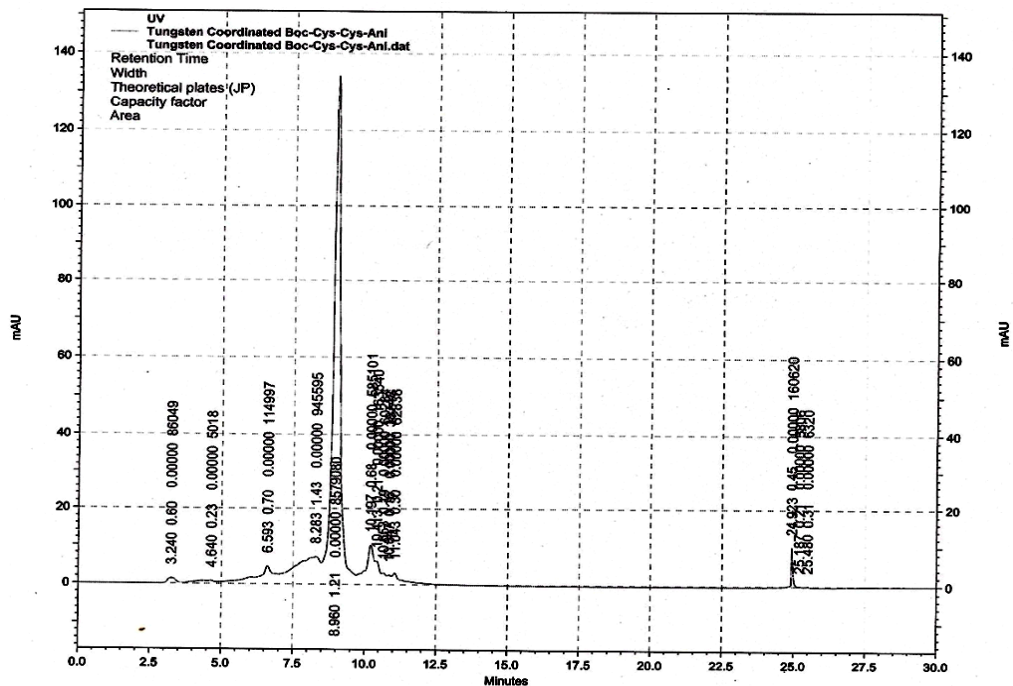


Figure A5: ^1H NMR of $\text{W}(\text{dmtc})_2(\text{Boc-Cys}(\text{Prg})\text{-Cys}(\text{Prg})\text{-Ani})$ in CDCl_3

^1H NMR: (CDCl_3) δ 11.3ppm (2H, s (m), Alkyne-H), 8.707ppm (1H, s, Ani-NH), 8.529ppm (1H, s, Gly-NH), 7.5935ppm (2H, m, Ani-CH), 6.8515ppm (2H, m, Ani-CH), 5.324ppm (1H, m, Gly-NH), 3.7997ppm (3H, m, OMe), 3.3137ppm (10H, m, DMTC + CH_2), 3.127ppm (10H, m, DMTC + CH_2), 1.461ppm (9H, m, Boc- $(\text{CH}_3)_3$).



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Figure A6: HPLC analysis of $W(dmtc)_2(Boc-Cys(Prg)-Cys(Prg)-Ani)$

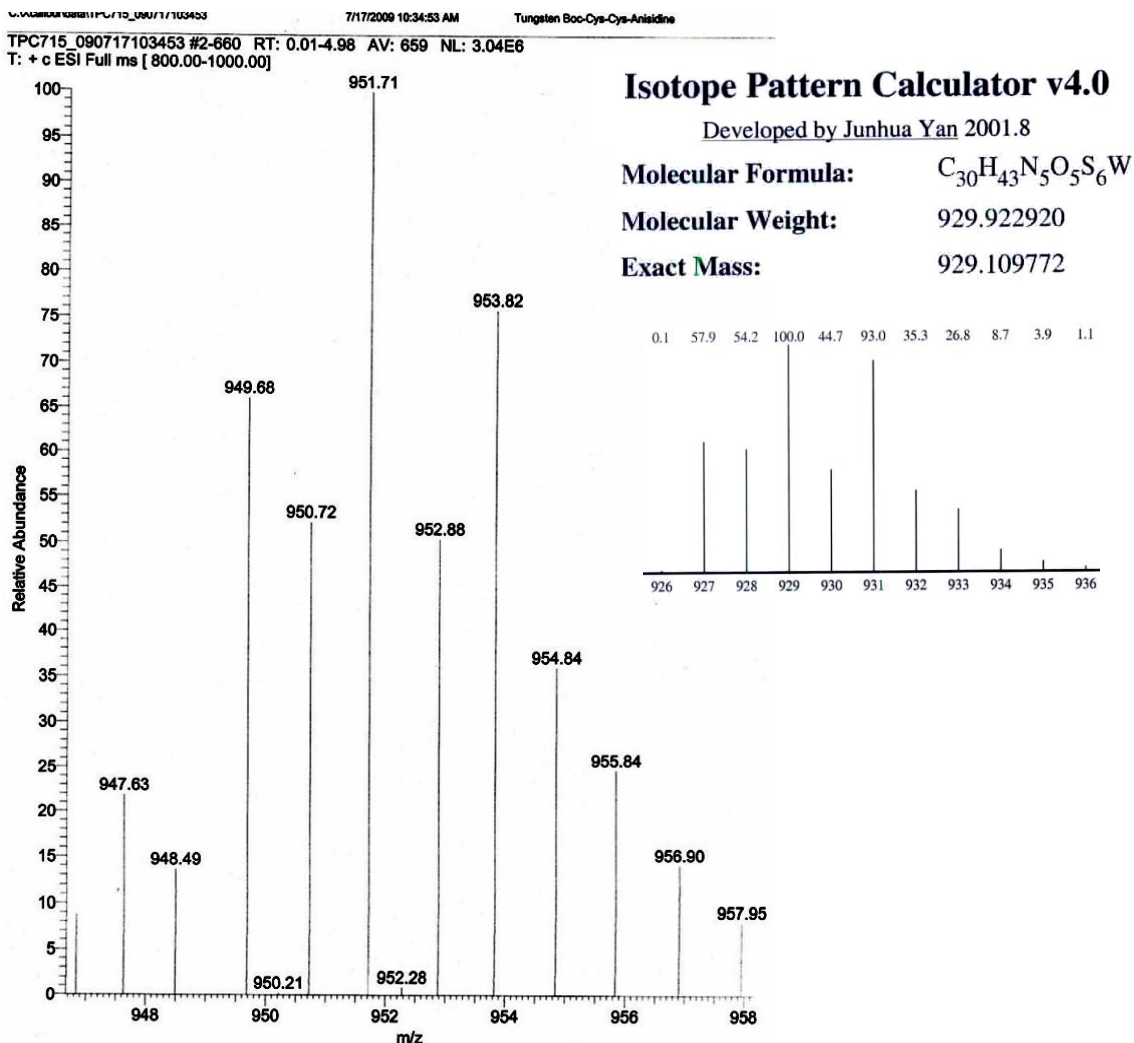


Figure A7: ESI-MS of $W(dmtc)_2(Boc-Cys(Prg)-Cys(Prg)-Ani)$ with theoretical mass and isotope pattern

A.7 Variable Temperature studies of $W(dmtc)_2(Boc-Cys(SCH_2CCH)-Cys(SCH_2CCH)-Anisidine)$

A series of 1D 1H NMR spectrums with increasing sample temperatures was recorded. First, in order to avoid solvent evaporation effects during the heating process, the sample was dissolved in DMSO- d_6 . Next, an initial NMR was recorded at 293K, the normal temperature of the instrument. The probe was then heated to 300K and a second 1H NMR was recorded. 1D 1H NMR's were then taken at 320K, 330K, 340K, 350K, 360K, and 364K, the maximum achievable temperature of the instrument. The sample was then allowed to cool down to its normal temperature (293K) and a final 1H NMR spectrum was recorded (Figure A8).

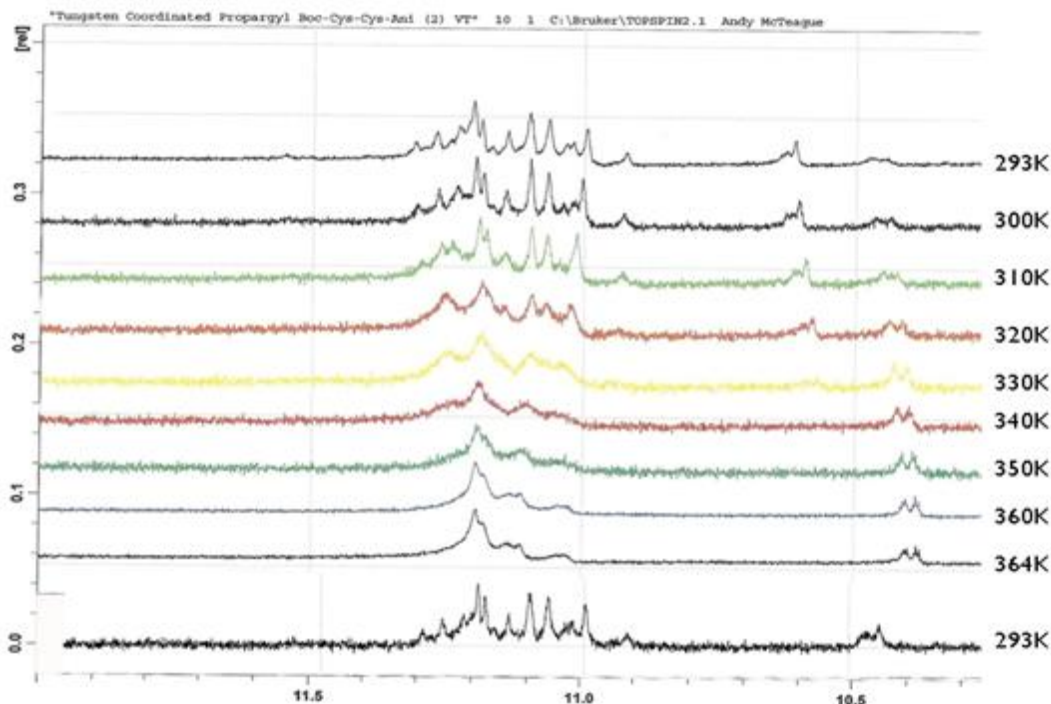


Figure A8: Variable Temperature ¹H NMR of W(dmtc)₂(Boc-Cys(Prg)-Cys(Prg)-Ani) in DMSO-d₆

The results of the VT-¹H-NMR show near coalescence of the sample at 363K.

A.8 DMSO titration of W(dmtc)₂(Boc-Cys(SCH₂CCH)-Cys(SCH₂CCH)-Anisidine)

In addition, a DMSO titration was performed in which 10mg of sample was dissolved in 75μL of CH₂Cl₂ and the concentration of DMSO in the sample was increased in specific intervals. First, a ¹H NMR was recorded in pure CDCl₃. Next, 8μL of DMSO-d₆ was added to the sample and a ¹H NMR was recorded. This was repeated 3 more times. This process was then repeated for two 16μL DMSO-d₆ additions, one 32μL addition and three 50μL additions. The chemical shifts of the two amide protons within the compound were then recorded for each of the ¹H NMR spectrums taken (Figure A9, Table A2). The chemical shift was then plotted as a function of the DMSO-d₆ concentration in the sample. When graphed the results of the DMSO titration clearly indicate that both amide protons in the coordinated peptide are involved in hydrogen bonds.

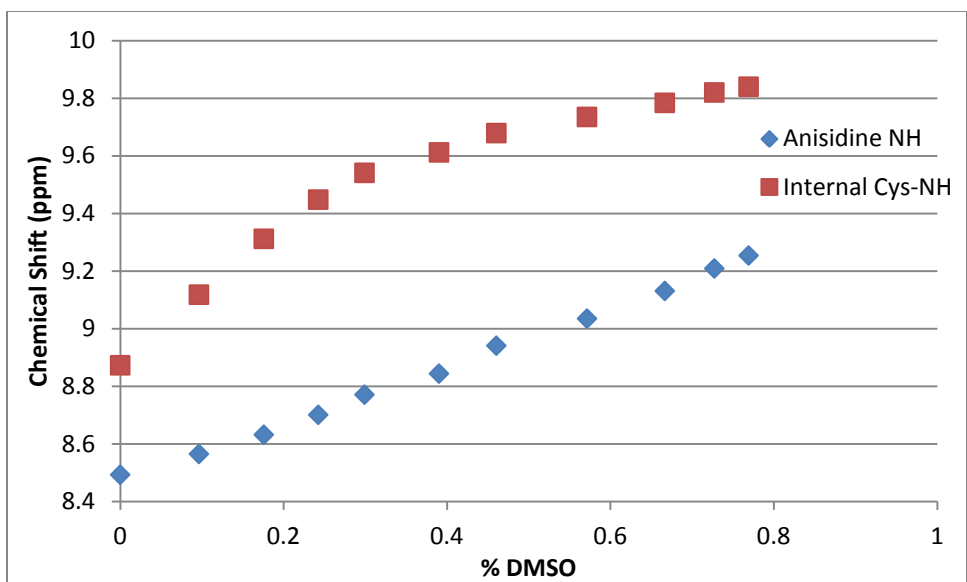


Figure A9: Chemical shift of selected NH peaks as a function of %DMSO of solvent

Table A2: DMSO Titration data

Solvent % DMSO	Location of 1 st NH peak	Location of 2 nd NH peak
0	8.493	8.873
0.096386	8.565	9.118
0.175824	8.632	9.312
0.242424	8.701	9.448
0.299065	8.771	9.541
0.390244	8.844	9.612
0.460432	8.941	9.679
0.571429	9.035	9.735
0.666667	9.131	9.784
0.727273	9.209	9.82
0.769231	9.254	9.84

A.9 Synthesis of $W(dmtc)_2(Boc-Prg-Prg-OMe)$

A sample of Boc-Prg-Prg-OMe was first obtained from previous research and the structure of the sample was confirmed using 1H NMR (Figure A10).

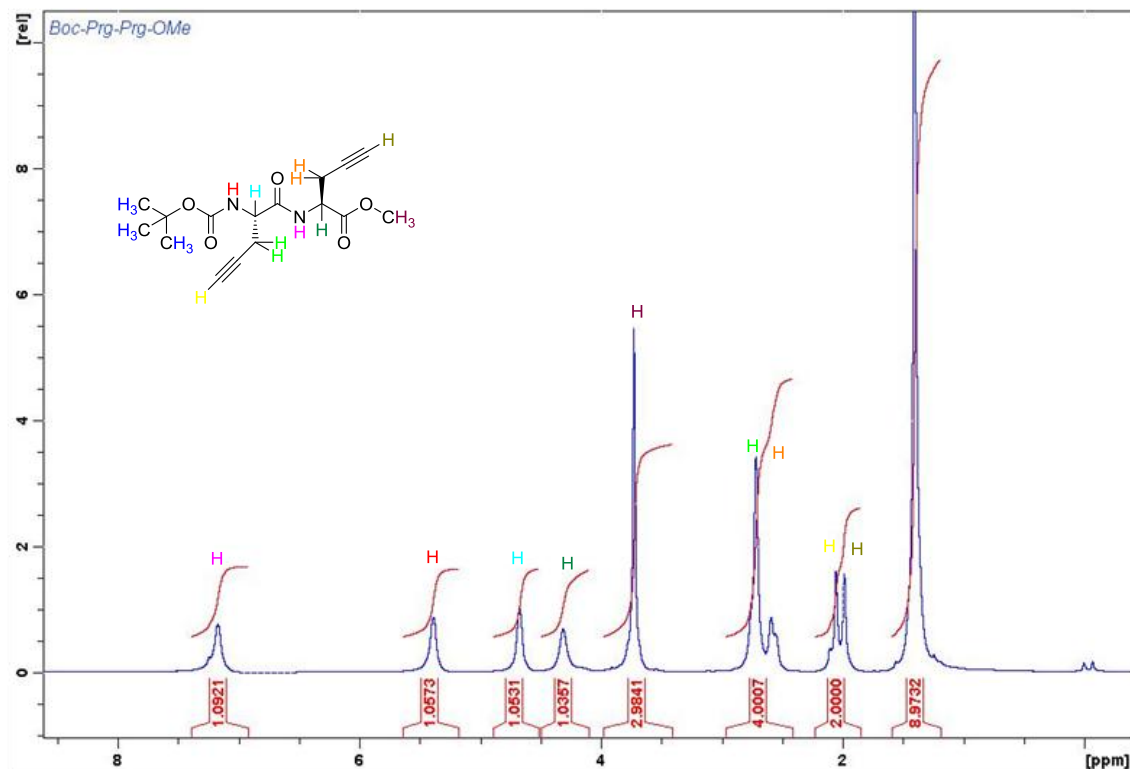


Figure A10: 1H NMR of Boc-Gly(Prg)-Gly(Prg)-OMe in $CDCl_3$

1H NMR: ($CDCl_3$) δ 7.169ppm (1H, s, Boc-Gly-NH), 5.386ppm (1H, s, Gly-NH), 4.673ppm (1H, s, Gly-C α), 4.311ppm (1H, s, Gly-C α), 3.7234ppm (3H, s, OMe), 2.7144pp, -2.5665ppm (4H, s, Gly-Prg CH $_2$), 2.0523ppm (1H, s, Alkyne), 1.983ppm (1H, s, Alkyne), 1.3999 (9H, s, Boc-CH $_3$)

0.0915g (1 equiv) of the sample was then placed within a 3 neck 500mL round bottom flask, while 300mL of methanol was degassed using a partial vacuum and a filter flask. Approximately 200mL of the degassed methanol and a stir bar were then transferred to the reaction vessel and the solution was put under an inert atmosphere of N_2 . The solution was then heated to a moderate reflux until most of the solid had dissolved. 0.055g (0.180mmol) of the Boc-Prg-Prg-OMe was then dissolved in a minimal amount of methanol and added into the stirring solution using a syringe. Upon addition of the peptide, the solution immediately became a dark forest green color, indicating formation of the mono-alkyne complex. Reflux of the solution was continued until the solution had become a lemon yellow color, indicating that both alkynes had been coordinated, which took approximately 2 hours to accomplish. The methanol was then

stripped from solution using a high vacuum rotary evaporator and the flask was massed at 131.260g (tare: 131.121). The product was then redissolved in methylene chloride and methanol and flash chromatography was performed using a 2cm column and ethyl acetate as the eluent. Fractions 9-12 were pooled and confirmed to be the desired product through ^1H NMR (Figure A11). The flask was massed at 64.633g (tare: 64.553g). In addition a variable temperature NMR was conducted starting at 294K and increased at intervals of 10K to 344K, but no evidence of coalescence was noted.

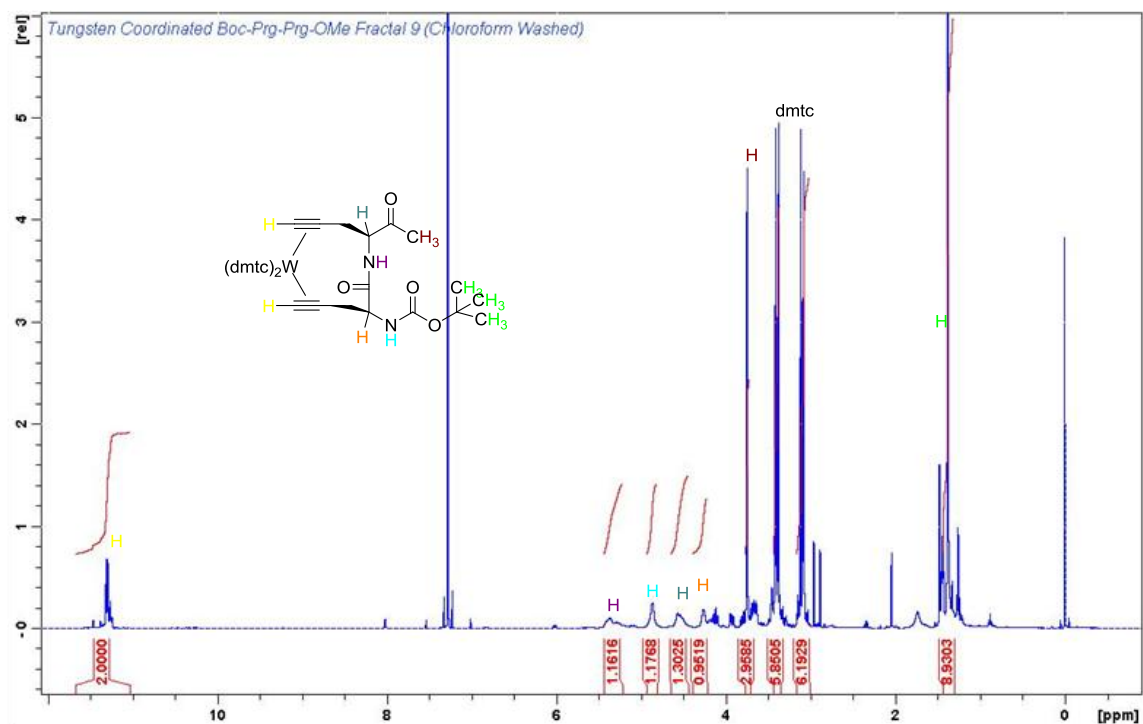


Figure A11: ^1H NMR of $W(\text{dmtc})_2\text{Boc-Gly(Prg)-Gly(Prg)-OMe}$ in CDCl_3

^1H NMR: (CDCl_3) δ 11.302ppm (2H, m, Alkyne-H), 5.369ppm (1H, s (d), $^3\text{J}= 3.64\text{Hz}$, Gly-NH), 4.871ppm (1H, d, $^3\text{J}=3.64\text{Hz}$, Gly-NH), 4.55ppm (1H, s (q), Gly-C α), 4.27ppm (1H, s (q), Gly-C α), 3.753ppm (3H, d (s), $^3\text{J}=4.48\text{Hz}$, OMe), 3.4017ppm (6H, s (dq), $^2\text{J}=10.55$, $^3\text{J}=4.16\text{Hz}$, dmtc), 3.0993ppm (6H, s (dq), $^2\text{J}=10.55$, $^3\text{J}=6.80$, dmtc), 1.3842ppm (9H, s, Boc-(CH_3) $_3$).

A.10 Variable Temperature studies of $W(\text{dmtc})_2\text{Boc-Gly(Prg)-Gly(Prg)-OMe}$

A series of 1D ^1H NMR spectrums with increasing sample temperatures was recorded. First, in order to avoid solvent evaporation effects during sample heating, the compound was dissolved in DMSO-d_6 . Next, an initial NMR was recorded at 293K, the normal temperature of the instrument. The probe was then

heated to 303K and a second ^1H NMR was recorded. 1D ^1H NMR spectrums were then taken at 313K, 323K, 333K, and 343K. The sample was then allowed to cool down to its normal temperature (293K) and a final ^1H NMR spectrum was recorded (Figure A12).

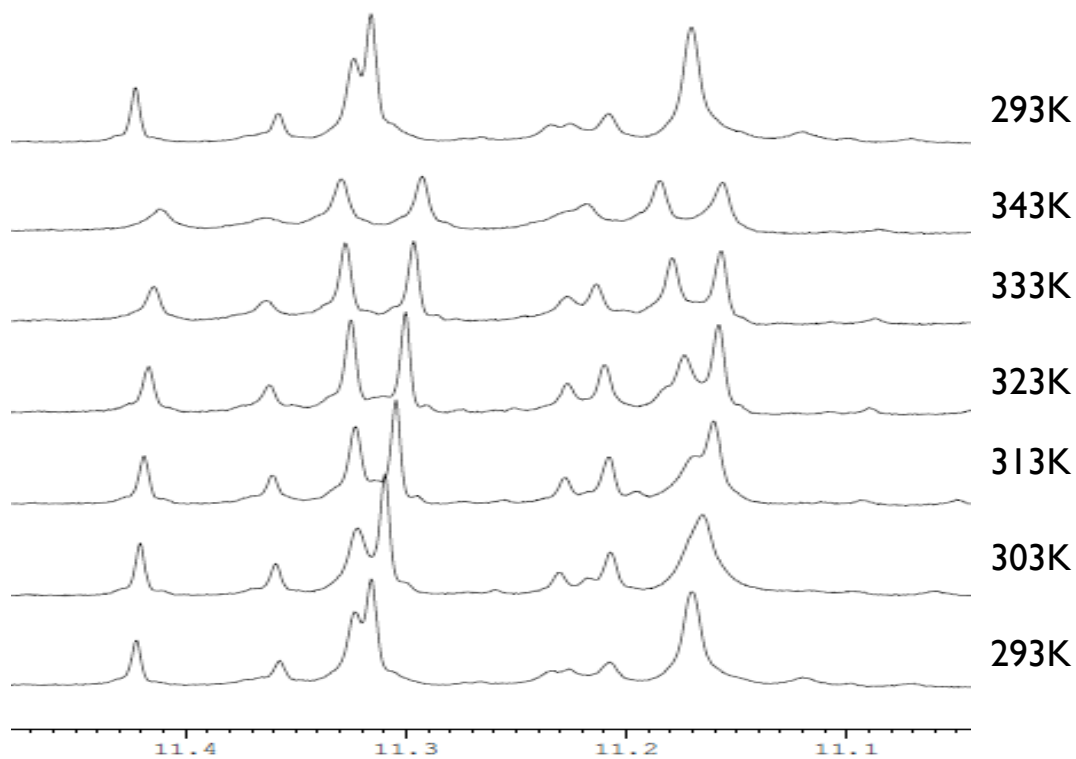


Figure A12: Variable Temperature studies of $W(\text{dmtc})_2\text{Boc-Gly(Prg)-Gly(Prg)-OMe}$ in $\text{DMSO-}d_6$

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