SYNTHESIS AND CHARACTERIZATION OF TIME-RESOLVED LANTHANIDE (III) LUMINESCENT PROBES FOR POTENTIAL DETECTION OF MELANOMA SKIN CANCER

A thesis presented to the faculty of the Graduate School of Western Carolina University in partial fulfillment of the requirements for the degree of Master of Science in Chemistry.

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November 2017

ACKNOWLEDGEMENTS

I would like to express my gratitude to several people for the completion of this thesis. First and foremost, I would like to thank my parents Randy and Denise Love for their support throughout my academic career and my entire life. I would like to thank Dr. Brian Dinkelmeyer for his knowledge and guidance in helping me become an organic chemist. I would also like to thank my committee members, Dr. Channa De Silva and Dr. William Kwochka, for their assistance throughout this project. Special thanks to Dr. Krista Wilson of Wingate University in Wingate, NC for her resourceful knowledge in solid phase peptide synthesis. Also, a very special thanks to Dr. Matthew Vergne of Lipscomb University in Nashville, TN for obtaining the HPLC-MS data.

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LIST OF ABBREVIATIONS

SPPS	solid phase peptide synthesis
MSH	melanocyte stimulating hormone
FMOC	fluorenylmethyloxycarbonyl chloride
DMF	dimethylformamide
DIC	N,N'-Diisopropylcarbodiimide
DCM	dichloromethane
HOBT	hydroxybenzotriazole
TFA	trifluoroacetic acid
NMR	nuclear magnetic resonance
GC-MS	gas chromatography-mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
HPLC	high performance liquid chromatography
FT/IR	fourier transform/infrared spectroscopy
THF	tetrahydrofuran
PEGO	polyethylene glycol

ABSTRACT

SYNTHESIS AND CHARACTERIZATION OF TIME-RESOLVED LANTHANIDE (III) LUMINESCENT PROBES FOR POTENTIAL DETECTION OF MELANOMA SKIN CANCER

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Western Carolina University, November 2017

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The focal point of this research is to continue the development of a time-resolved lanthanide (III) luminescent probe that may aid in early detection of melanoma skin cancer. If melanoma cancer is found at an early stage, it can successfully be taken out by invasive surgery before spreading to other tissues and organs throughout the body. This timely detection could help decrease the mortality rate of melanoma victims. By attaching a lanthanide (III) luminescent tag to a MSH-4 peptide substrate, the detection of melanoma skin cancer could potentially be measured. The design of our target molecule consists of three components. They include: a Eu^{3+} luminescent complex, a melanocyte stimulating hormone (MSH-4) peptide and a polyethylene glycol spacer molecule. Each component will be independently synthesized and then coupled onto an insoluble support resin via solid phase peptide synthesis. The sequential coupling will allow the indirect attachment of the MSH-4 substrate to the luminescent probe by utilizing a PEGO linker. This separation will ensure that the Eu^{3+} luminescent tag will not hinder the binding interactions between the MSH-4 substrate and its respective G-protein coupled receptor (GPCR). All components of the probe were characterized by using a combination of ^{1}H NMR, ^{13}C NMR, FT/IR, GC-MS, and HPLC-MS.

CHAPTER 1: INTRODUCTION

1.1 Problem Statement

The goal of this research is to develop a bioconjugate that can act as a biosensor for the early detection of melanoma. The ability to detect interactions between an analyte of interest and its target receptor is critical for understanding cell signaling pathways and important for disease diagnosis. Current evaluation methods rely on bioconjugates that covalently attach radioisotopes, fluorophores or enzymes to biological analytes that bond with receptors present in cells.[1] Each of these screening techniques has its own limitations. Radioisotopes are expensive and have negative side effects. Also, the radioactive waste must be handled properly to prevent exposure to public health. Techniques based on fluorescence are limited by their sensitivity due to the background signal that is observed from the analyte's environment. In recent studies, luminescent lanthanide (III) complexes have shown to possess photochemical properties that overcome background fluorescence and deliver greater assay sensitivity. The superior sensitivity of luminescent lanthanide(III) probes can be largely attributed to their narrow emission bands, large Stokes shifts and long luminescent lifetimes.[1] Eu^{3+} labels have exclusively been studied and evidence suggest them to be viable candidates for biological imaging probes.[1,2]

1.2 Background

Skin cancer is one of the most common forms of cancer in the United States. Each year there are over 3.3 million cases of people needing treatment for some form of skin cancer.[3] Melanoma skin cancer is the most dangerous of all forms. It has been estimated that over 76,000 new cases of malignant melanoma skin cancer will be diagnosed in the U.S. in 2017.[3] The major factor that contributes to skin cancer is the exposure of skin to UV light. The sun's rays emit ultraviolet radiation and is the primary source of human exposure. UV light is harmful to the skin because

it can damage the DNA contained within its cells. When DNA is damaged, repair mechanisms within the DNA are activated so homeostasis is maintained. One way DNA protects itself from UV exposure is by producing signals that alert melanocytes to produce melanin. Melanin is a compound within the skin that actively absorbs harmful UV rays and transfers the energy in the form of heat. Skin cancer stems from the overproduction of melanocytes between the epidermal and dermal layers of the skin. If the melanocytes continue to produce at an uncontrollable rate, they can eventually grow and reach the blood vessels deeper within the skin. When the cancer has reached the blood vessels it can travel through the blood and metastasize. The cancer is then able to reach other tissues and organs in the body. Hence, early detection of melanoma skin cancer is essential. It is important that the clinical techniques used for the detection of melanoma be accurate and expeditious as possible. If the melanoma is found at an early stage, it can successfully be taken out by minimally invasive surgery before spreading throughout the body.

The production of melanocytes is regulated by a class of receptors called G-protein coupled receptors (GPCR's). All GPCR's share a major role in governing physiological functions within the cell. These functions include facilitating neurotransmission, releasing hormones and enzymes from endocrine and exocrine glands, and controlling immune responses.[4] The receptor is composed of seven-transmembrane α-helices that are embedded within the cell membrane. The seven α-helices weave in and out of the cell membrane, positioning the receptor on the exterior as well as the interior of the cell.[4] GPCR's will elicit a physiological response when a signaling molecule binds to the receptor on the cell's exterior surface. Consequentially, this induces a conformational change that activates the G-protein found on the interior of the cell.

The signaling molecule that facilitates the production of melanin is a group of peptides entitled melanocyte stimulating hormones (MSH). There are three classes of MSH peptides that

are produced in the brain. The pituitary gland secretes an alpha, beta and gamma MSH peptide that binds to the GPCR located on the surface of the melanocyte.[4] The primary peptide that influences melanin production is the α-MSH. It is of interest to study the effects of the α-MSH because of its high binding affinity to the GPCR. By synthesizing a proper imaging probe onto the α -MSH substrate it could possibly provide a clinical tool to detect melanoma cancer.

Ideal characteristics of fluorophores include: (1) high extinction coefficients; (2) high quantum yields; (3) emit at long wavelengths so that they are easily seen over biological signal background; (4) photo stable; (5) relatively small so as not to disrupt the normal activity of the biological analyte; (6) water soluble; (7) easily conjugated to a biomolecule of interest.[1,5,6] Lanthanide luminescent probes are advantageous over commonly used organic fluorophores in imaging methods due to their long luminescent lifetimes and narrow emission bands.[1,5] This is critical when detecting the presence of an analyte in a biological system. When imaging for an analyte in a biochemical assay there tends to be other signals or noise coming from background emission that influence the signal of interest. To overcome this issue, lanthanide fluorescent tags are being studied due to their ability to detect after signals from other analytes have decayed. One drawback is that lanthanides often have low luminescent quantum yields. This shortcoming is circumvented by the use of a chromophore that will chelate to the europium metal and absorb energy from UV irradiation. This energy can be transmitted to the europium complex in what is known as the antenna effect.[1,5] When the energy from the UV radiation is absorbed by the chromophore it can transfer the energy to the europium metal ion which can then show luminescence. The luminescence light that the complex gives off is in the red visible region that can be distinguished apart from other background radiation in the green/blue visible region.[6] This

luminescent property of the europium metal complex is what may potentially help with the detection of melanoma skin cancer.

Linker molecules are often used when it is necessary to covalently link but spatially separate the biological analyte from other components of the bioconjugate system. Important characteristics for these spacer molecules is that they are biocompatible, water soluble and easily attached to the analyte and do not effect normal activity of the biological analyte. Alkyl chains, aromatics, oligopeptides, oligosaccharides, oligonucleotides and various polymers have all been employed in bioconjugate chemistry. Polyethylene glycol is commonly used because it is biocompatible and is known to improve the water solubility of compounds it which it is covalently bound.

1.3 Design

There are three phases to this project that must be completed for the proposed research to work. The most significant part of the project is the attachment of the luminescent probe to the MSH-4 substrate. The probe will be indirectly attached to the MSH-4 substrate by employing a polyethylene glycol linker molecule. By incorporating a linker molecule into the design, it can help assure that the luminescent tag will not hinder the MSH-4 substrate from binding to its G-protein coupled receptor. Figure 1 shows the molecular structure of the final target molecule.

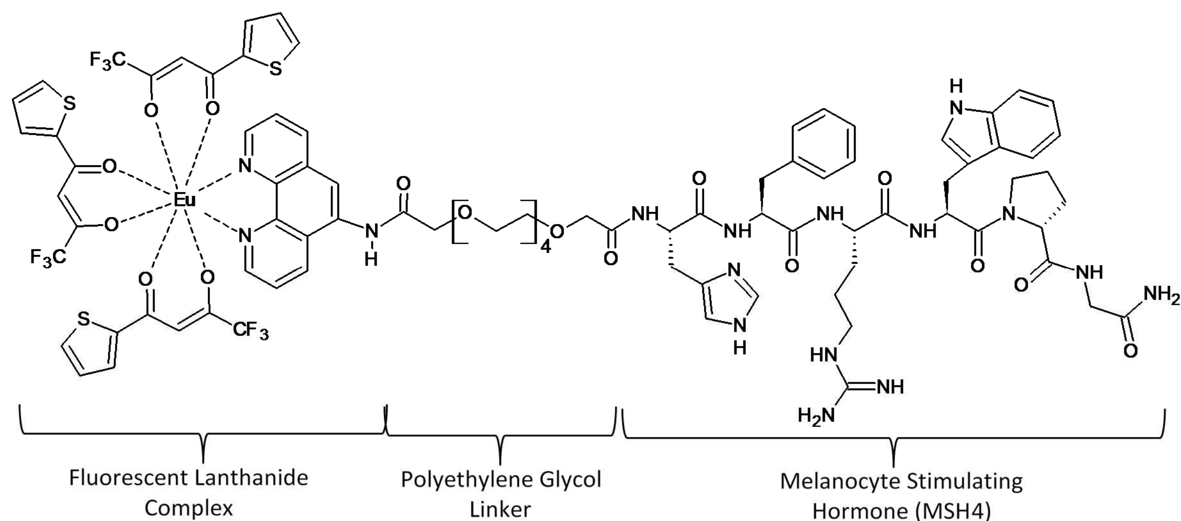
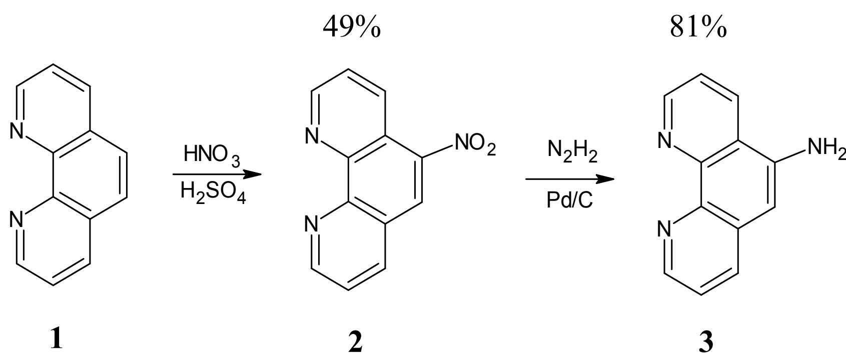


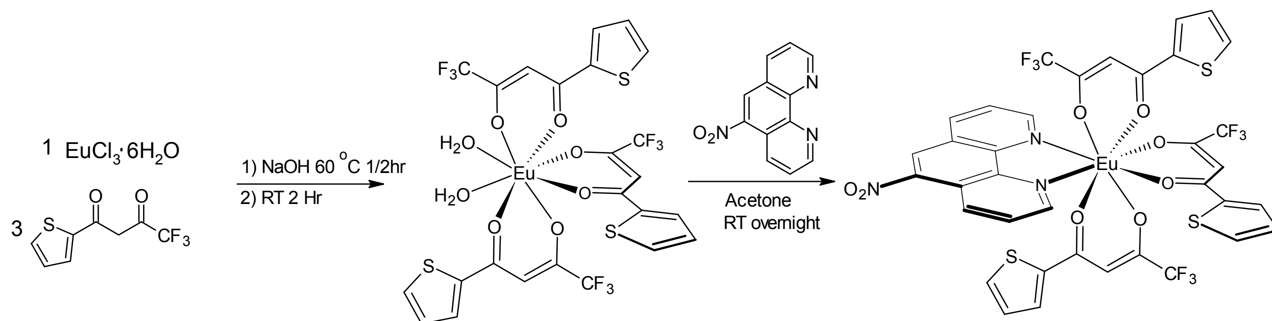
Figure 1. Molecular structure of the final target molecule.

The first part of the project includes the synthesis of the lanthanide fluorescent tag. The fluorescent tag consists of an organic chromophore that coordinates with the Eu^{3+} lanthanide complex. The chromophore selected for this project will be based on 5-amine-1, 10-phenanthroline (**3**) since it was found to produce satisfactory absorption and emission characteristics when chelated to the Eu^{3+} metal.[9] Synthesis of 5-amine-1, 10-phenanthroline (**3**) begins with the nitration of 1, 10-phenanthroline (**1**) (Scheme 1).[7]



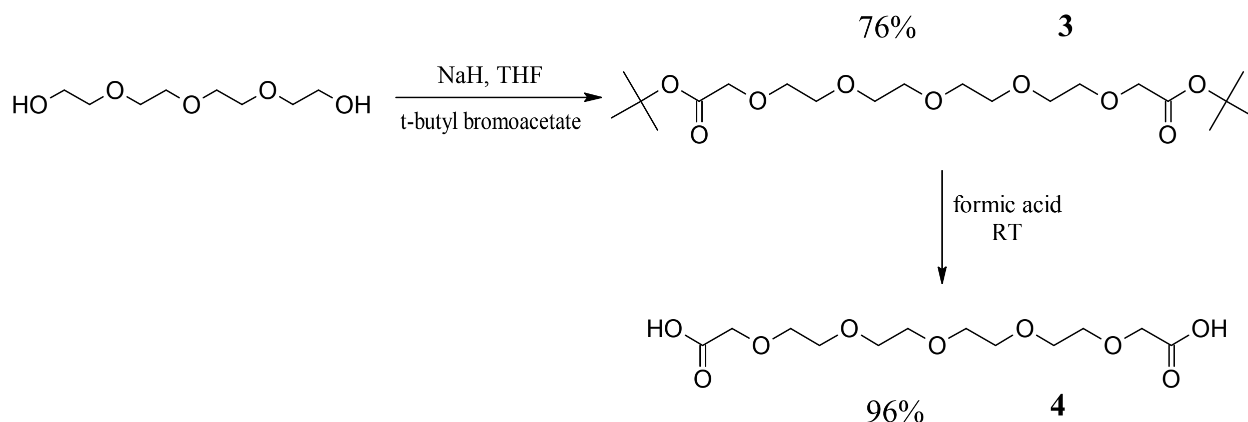
Scheme 1. Synthesis of 5-nitro-1, 10-phenanthroline (**2**) and 5-amine-1, 10-phenanthroline (**3**).

After successful addition of the nitro group, a reduction reaction is performed using hydrazine and a palladium catalyst.[8] This will reduce the nitro group to an amine group. Chelation of 5-amine-1, 10-phenanthroline (**3**) onto the europium metal is the final part in the completion of the fluorescent tag. Coordination of this complex involves the reaction of $Eu(TTA)_3(H_2O)_2$ with the 5-amine-1, 10-phenanthroline ligand (Scheme 2).[9]



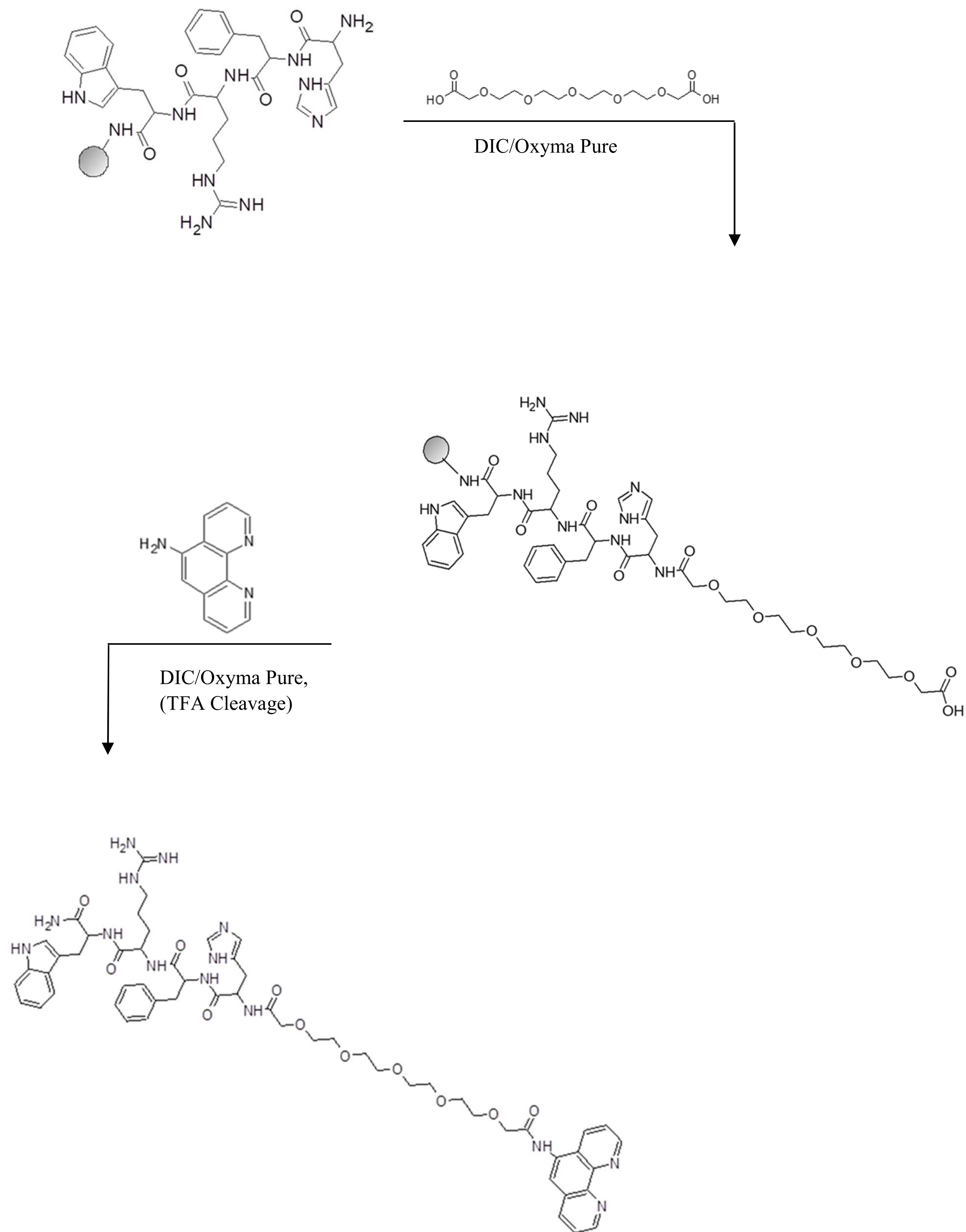
Scheme 2. Synthesis of $Eu(TTA)_3$(amino-phen).

The next objective is the synthesis of an ethylene glycol linker molecule that separates the lanthanide fluorescence tag from the peptide substrate. It is essential that the linker molecule remain soluble in aqueous environments when binding to both the lanthanide fluorescence tag and the MSH-4 peptide. A modified tetra ethylene glycol linker was synthesized because of its solubility features and for its ability to couple with an amine functional group. The first step in synthesizing the modified PEGO linker is to combine tetra ethylene glycol and tert-butyl bromoacetate in a solution of sodium hydride and TFH.[10] This reaction will place a t-butyl protecting group on both ends of the linker molecule. The t-butyl protecting groups are then removed under acidic conditions to afford two carboxylic acid groups that are capable of coupling in solid phase peptide synthesis (Scheme 3).[10]



Scheme 3. Synthesis of modified PEGO linker (di-acid).

The final component of the probe is the MSH-4 peptide which serves as the analyte that binds to G-protein receptors found on the surface of melanoma cancer cells. The peptide will be synthesized on a solid support resin using microwave-assisted SPPS. Under Fmoc protocol, amino acids will be coupled on a polymer supported resin by formation of a peptide bond.[11] After the assembly of the peptide on resin, coupling of the PEGO linker and phenanthroline chromophore onto the peptide can proceed. The coupling of the PEGO linker and phenanthroline chromophore will be accomplished under the same reaction conditions as the amino acid coupling (Scheme 4).[12] Finally, the target molecule can be cleaved from the solid support resin and then coordinated to the Eu^{3+} metal ion.



Scheme 4. Proposed synthetic strategy for coupling the PEGO linker (di-acid) and 5-amine-1, 10-phenanthroline chromophore onto the MSH-4 substrate.

CHAPTER 2: INTRODUCTION TO SOLID PHASE PEPTIDE SYNTHESIS

2.1. General

Solid phase peptide synthesis (SPPS) is a highly efficient organic synthesis technique that enables the production of large biomolecules such as proteins and peptides. The process involves a stepwise assembly of amino acids onto an insoluble polymer support resin by coupling the amine group of one amino acid with the carboxylic acid group of another amino acid to form an amid bond (Figure 2).

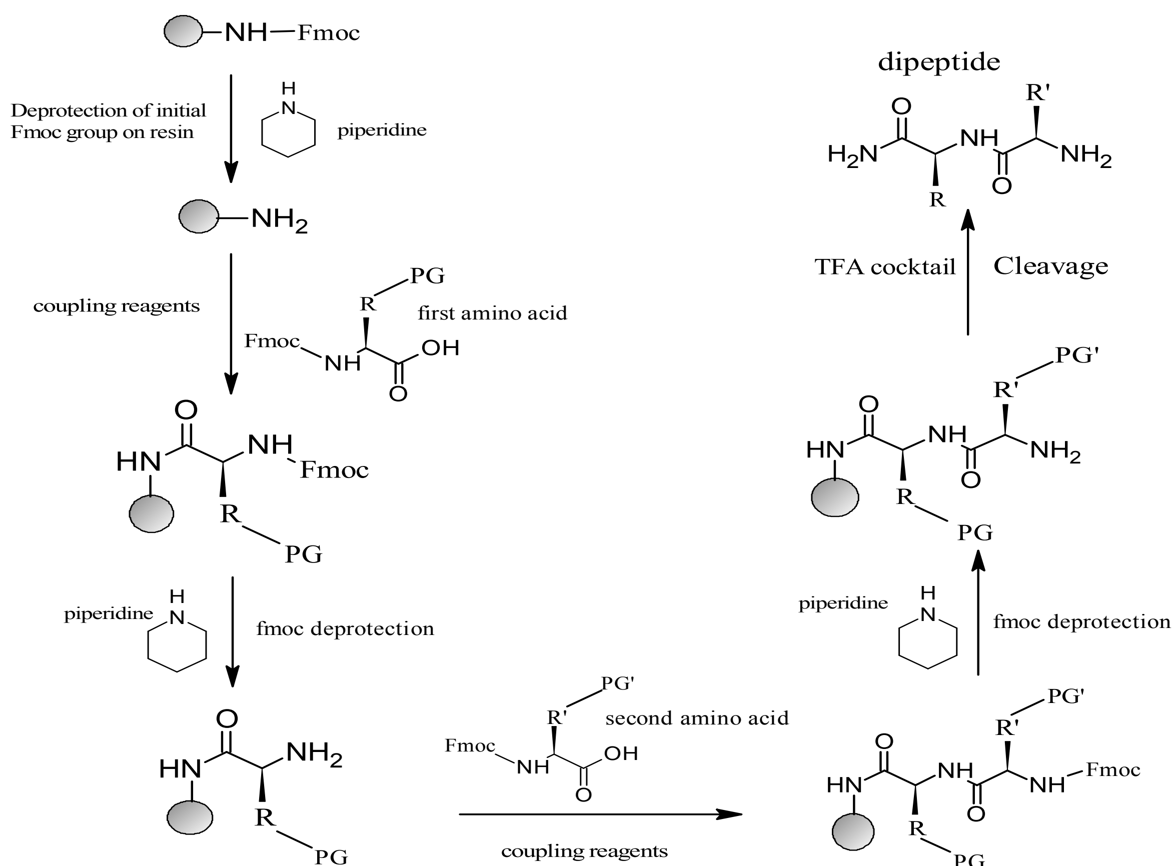


Figure 2. Overview of SPPS under Fmoc protocol.

For this project, SPPS will be employed for the synthesis of the MSH-4 peptide substrate and used for the coupling of the PEGO linker molecule and the phenanthroline chromophore onto the peptide. The benefits of this technique as opposed to older synthetic methods include: speed of reaction, high yields and ease of purification.[13] The use of microwave heating has greatly increased the speed of SPPS. Reactions occur in high yields since coupling reagents and amino acids can be used in excess. Purification and removal of excess/unreacted reagents are easily accomplished by filtration.

In recent years, the pharmaceutical industry has increased production of drug related peptides due to the influence of SPPS. Clinical studies have also increased due to the expansion of drug related peptides, specifically in areas such as cancer research.[14] The speed of amino acid coupling can be largely attributed to the use of microwaves instead of conventional heating methods. For this project, the coupling of each amino acid will be accomplished by using a Discover SP-X microwave synthesizer equipped with a fiber optic temperature probe manufactured by CEM Corporation. Microwave irradiation can rapidly heat reaction solutions and help break up ionic and polar interactions within the growing peptide chain. The disruption of peptide chain aggregation can allow for easier coupling of each amino acid and thus increase the speed of reaction. Faster reaction rates also limit the amount of racemization and side reactions that can occur during coupling.[15]

2.2 Resins

The basis of solid phase peptide synthesis is centered around the insoluble support resin in which the growing peptide chain is built. Performing the synthesis on an insoluble polymer support allows the use of excess reagents which can be easily vacuum filtered and washed away after the reaction is complete. The use of excess reagents ensures very high yields while the

ability to remove unreacted reagents and side products by vacuum filtration makes purification very easy. Older synthetic methods that do not use an insoluble support resin must require a recrystallization step to separate the desired peptide from excess reagent impurities.[13] There are many resins available that can be used for SPPS. Resins are chosen based upon their loading and which synthetic strategy is used to produce the peptide. The features of the peptide will determine which synthetic strategy is most useful. The synthetic strategy used to produce the MSH-4 peptide in this thesis is Fmoc based.

The most common resin used for the Fmoc synthetic route is rink amide resins. Generally, the insoluble resin matrix is made of 1% divinylbenzene crosslinked polystyrene.[13, 14] Rink amide resins usually come with a linker (or handle) that attach directly to the polystyrene beads. All resins come with a specific loading constant. Typically, these loading constants range from 0.10-0.80 mmol/g. The loading constant signifies how many millimol of peptide can be produced per gram of resin. Low loading resins (0.10-0.30 mmol/g) allow for longer length peptides. Higher loading resins (0.60-0.80 mmol/g) are used only for shorter length peptides. The lower loading resins have fewer moieties available for initial amino acid attachment, which is beneficial when synthesizing a peptide with over 30 amino acids in its sequence. Higher loading resins have many more moieties available for initial amino acid attachment. The high loading resin becomes a problem when trying to synthesize a peptide with a sequence of 30 amino acids or greater. Secondary structure formation and interaction between neighboring peptide chains can interfere with coupling steps which can significantly decrease purity.

2.3 Preparing the resin for peptide synthesis

The linker has an amino functional group protected by an initial Fmoc group. This is where the first amino acid will be coupled to create the growing peptide chain.

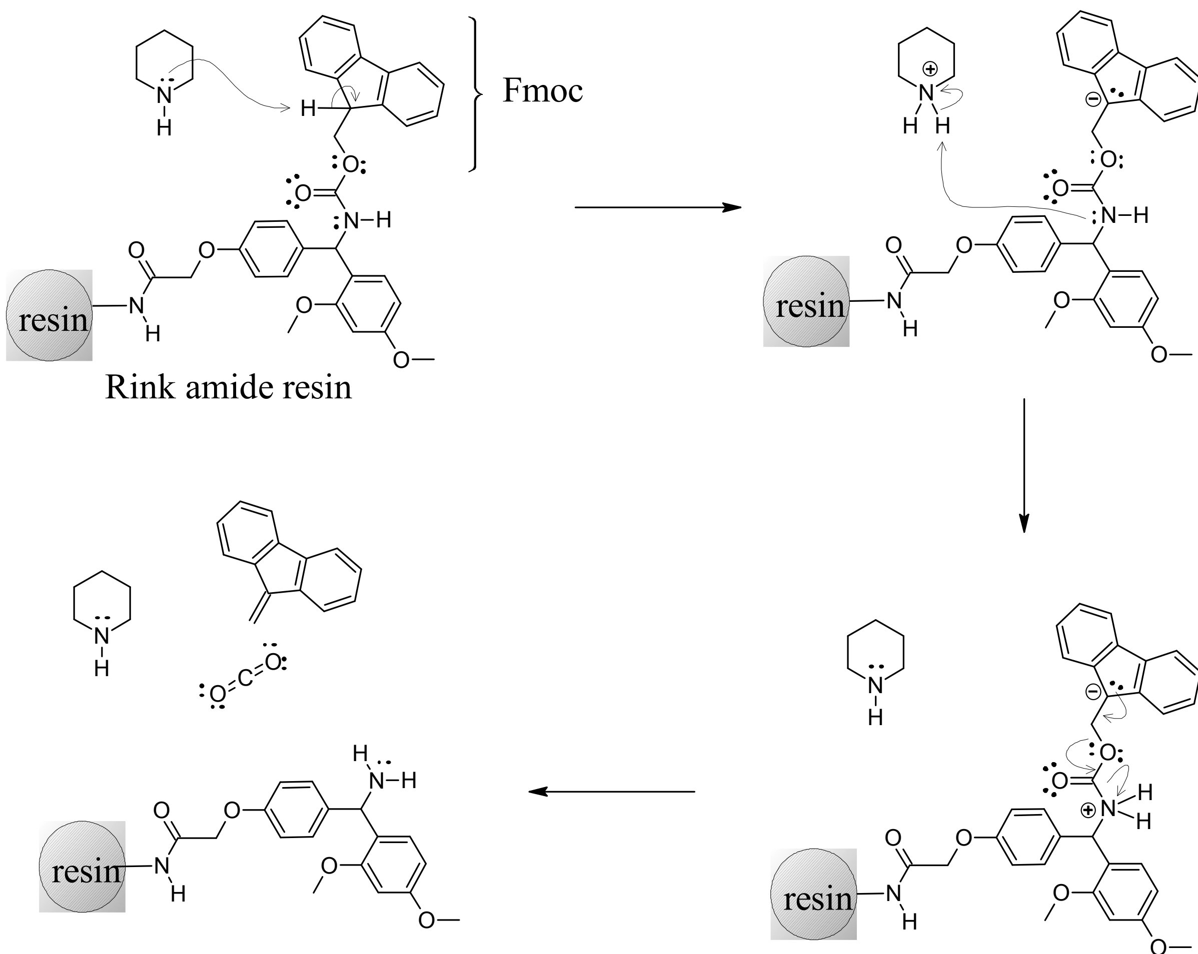


Figure 3. Rink Amid ProTide resin produced by CEM Corporation. This resin is made of insoluble polystyrene beads that come protected with initial Fmoc group. The Fmoc group is removed with piperidine before attaching the first amino acid.

Before amino acid coupling, the resin must be swollen in a suitable solvent to make the active sites accessible for coupling and the initial Fmoc protecting group removed before coupling of the first amino acid.[15] This step is responsible for creating a free N-terminus amine that is reactive with the C-terminus carboxylic acid of an amino acid. The Fmoc group is base labile and is deprotected using a solution of 20% piperidine in DMF. The key step is initial deprotonation of the fluorene ring to generate the aromatic cyclopentadiene intermediate, which rapidly eliminates to form dibenzofulvene (Figure 3).[15, 16] After the deprotection step is

complete, DMF and by-products are vacuum filtered from the fritted vessel leaving the resin dry. The resin is then washed and filtered to remove impurities. A Kaiser (ninhydrin) test is then used to test the effectiveness of the N-Fmoc deprotection. The Kaiser test will qualitatively indicate whether the Fmoc protecting group has successfully been removed. The test requires that a few resin beads be placed into a test tube and a few drops of each of the Kaiser reagent be added. The Kaiser reagents will interact with the free amine and stain the resin beads a dark blue color if deprotection is successful. However, if the resin beads are yellow after the Kaiser test then deprotection was unsuccessful and another deprotection step should be performed. The resin is now ready for peptide synthesis.

2.4 Amino Acid Coupling

Once the Kaiser test is positive for a free amine the first amino acid can be coupled onto the resin. Amino acids and coupling reagents are used in excess to help push the reaction forward. The coupling reagents that are used in this protocol are diisopropylcarbodiimide (DIC) and oxyma pure. Another coupling reagent mixture that is commonly used is DIC/HOBt. Each of these reagents along with the amino acids are used in a 5-fold excess. These reagents activate the ester for nucleophilic attack by the resin supported amino acid (Figure 4).[15, 16] A stable urea containing molecule is produced as a side product which makes the process thermodynamically favorable.

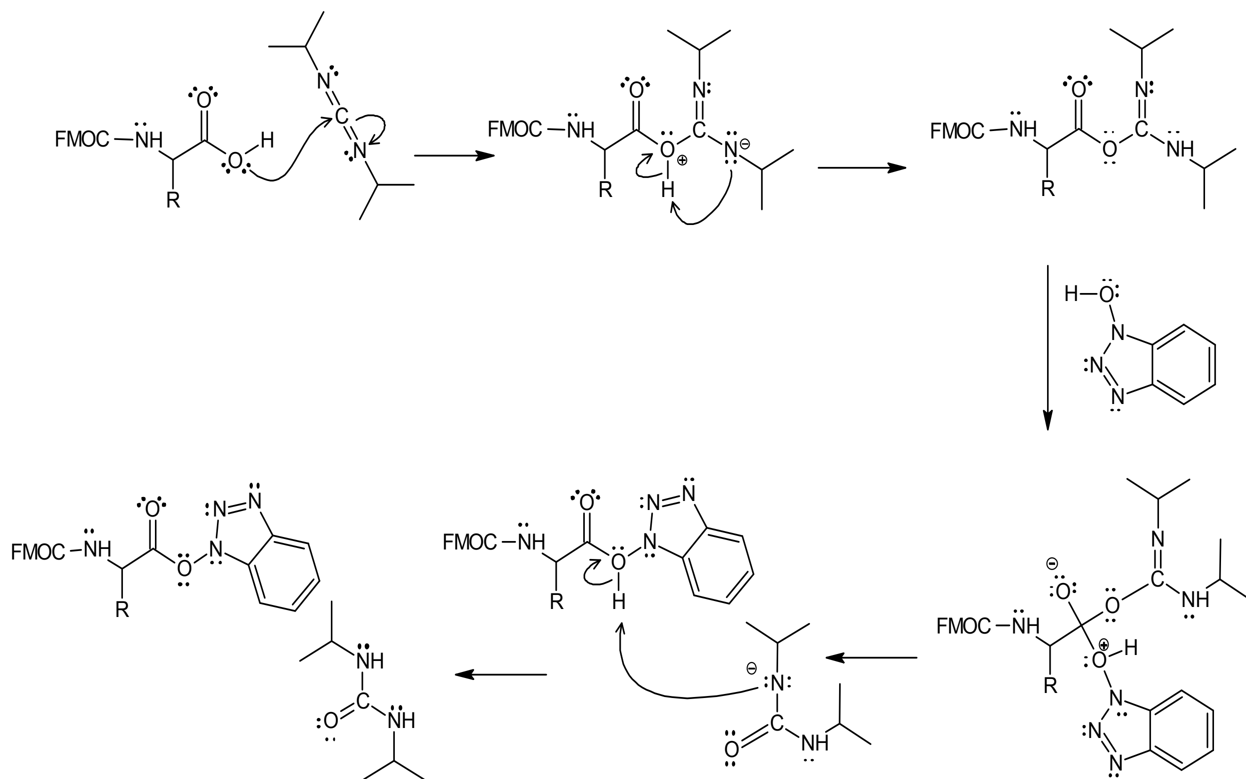


Figure 4. Reaction mechanism of a pre-activated amino acid ester for peptide coupling.

The peptide bond is formed when the active ester of the amino acid is reacted with the free amine on the resin (Figure 5).[16] After the reaction is complete, the solvent and excess reagents are filtered off leaving behind the newly coupled amino acid on resin. Another Kaiser test is performed to provide proof that the peptide bond has formed. The Kaiser test should stain the resin beads yellow if the peptide bond has formed. This indicates that a free amine is no longer present and ensures that further coupling can begin. This process of ester activation, nucleophilic attack by the resin bound amino acid and deprotection is repeated for the addition of each amino acid until the desired peptide is complete.

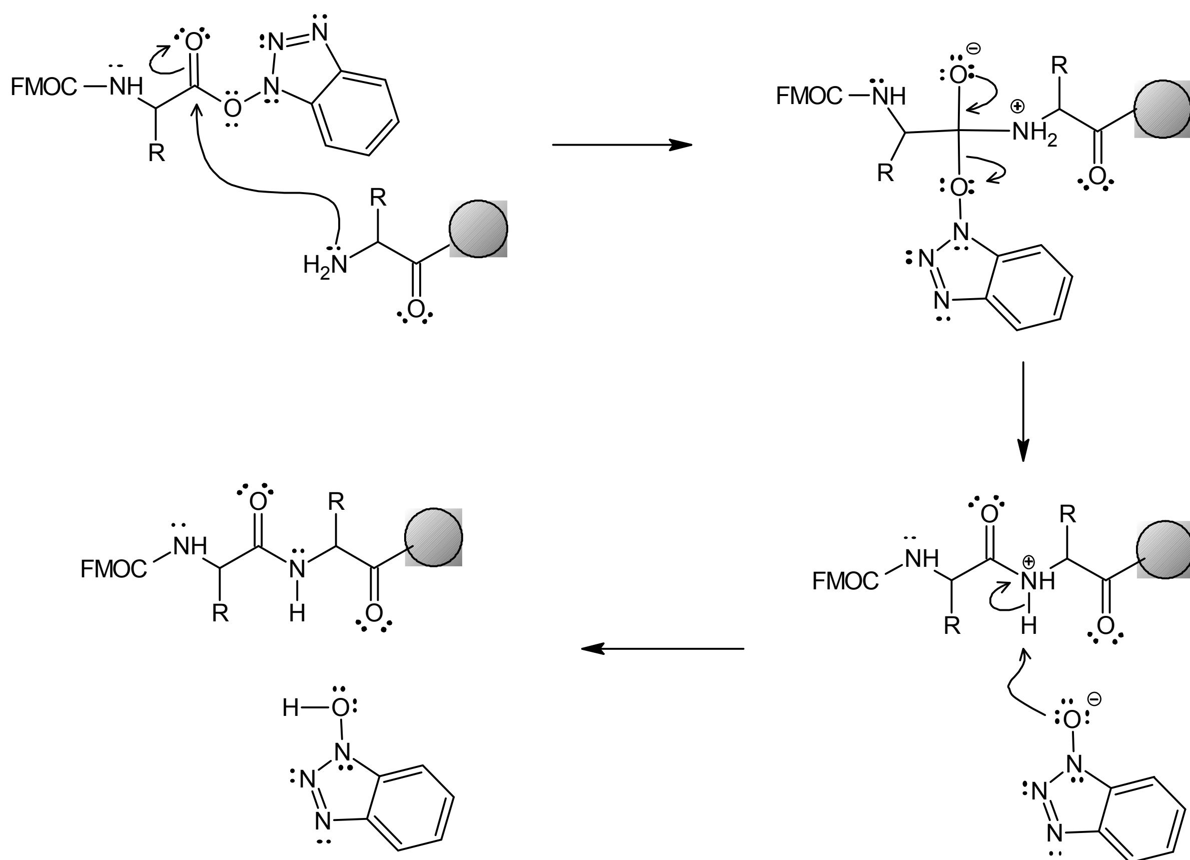


Figure 5. Amino acid coupling mechanism involving the pre-activated amino acid ester and the free amine on resin.

After an amino acid has been coupled, its Fmoc protecting group must be removed from its amino group before another amino acid can be coupled to the growing peptide chain. This is accomplished by the addition of piperidine followed by vacuum filtration and rinsing (Figure 6). The presence of a free amine group is then tested for by performing a Kaiser test. The appearance of a blue color indicates that removal of the Fmoc group was successful and the peptide is ready to be coupled to the next amino acid. This process of ester activation, nucleophilic attack by the resin bound amino acid and deprotection is repeated for the addition of each amino acid until the desired peptide is complete.

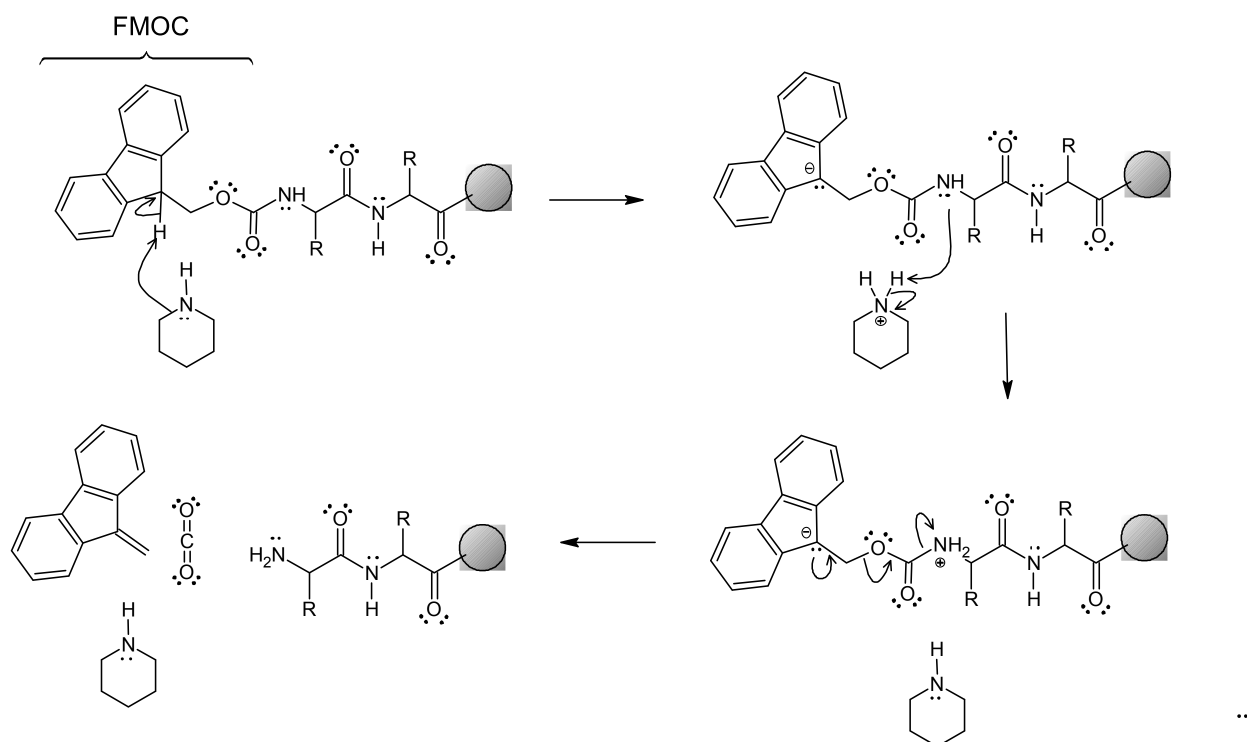


Figure 6. Removal of the N-Fmoc group with piperidine is necessary before coupling the next amino acid.

2.5 Cleavage

The final step in peptide synthesis is the cleavage of the peptide from resin and removal of protecting groups on amino acid R groups. This step is accomplished under acidic conditions. Trifluoroacetic acid (TFA) is the most popular cleavage reagent used in SPPS (Figure 7). Under these conditions, highly reactive cationic species are generated when protecting groups are released from their amino acids.[13, 14] These species can react with electron-rich functional groups on the amino acids and therefore modify the desired peptide. To prevent this from happening, nucleophilic reagents are mixed in with TFA. These reagents act like scavengers and quench the ions that are formed. A typical cleavage cocktail consists of (82.5% TFA, 5% thioanisole, 5% water, 5% phenol, 2.5% 1, 2-ethanedithiol v/v).

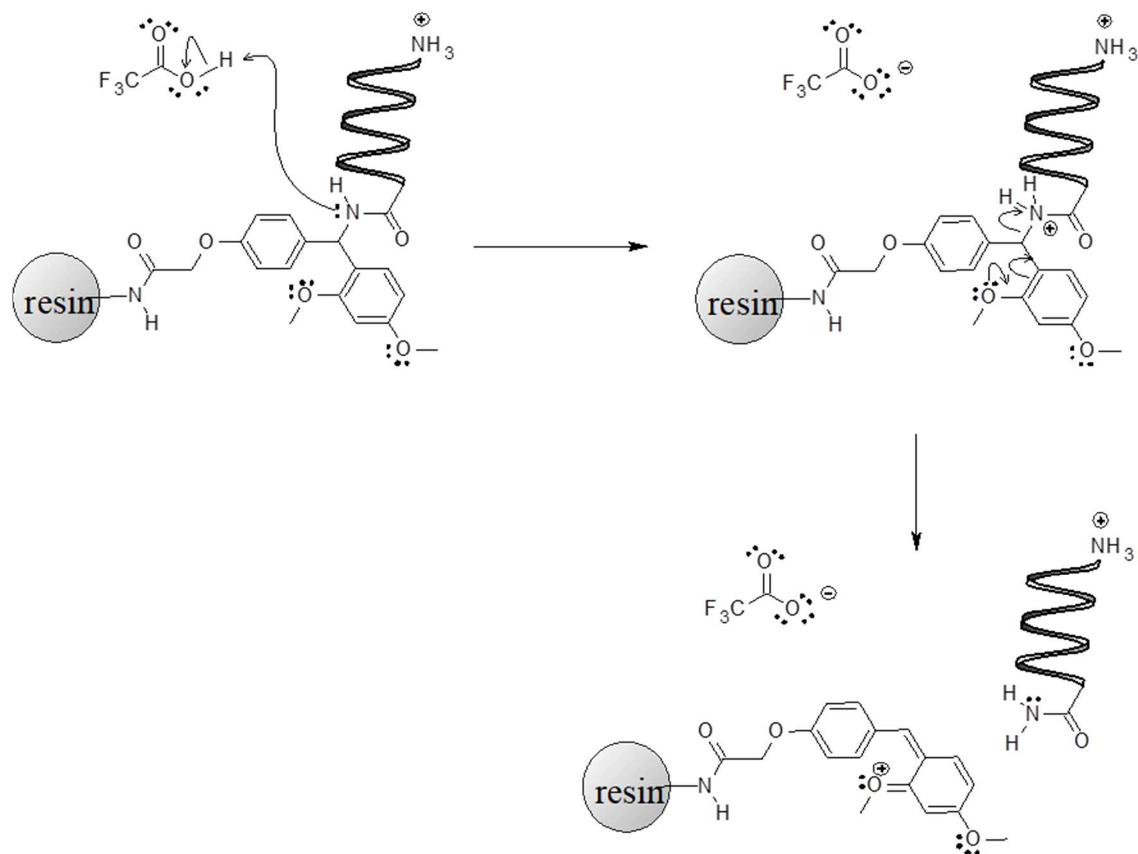


Figure 7. Cleavage of the peptide is accomplished by the addition of trifluoroacetic acid.

2.6 Purification and characterization

Cleaving the peptide from the resin releases the peptide into the TFA solution. The peptide can be precipitated by the addition of cold diethyl ether and then isolated by repeated centrifugation. After purification, peptides should be lyophilized to help preserve them. The peptide is then analyzed by HPLC and mass spectroscopy (LCMS). The shelf-life of each peptide will be unique and dependent upon which amino acids make up its sequence. For example, peptides that contain Cys, Met, Trp residues are susceptible to oxidation and should be stored in an anaerobic environment. Peptides can also absorb moisture from the air if not properly stored, which can cause degradation over time. To prevent this from happening it is recommended to store the peptide in a desiccator at 4˚ C or colder.[11, 15]

CHAPTER 3: RESULTS AND DISCUSSION

The aim of this thesis was to continue the development of a fluorescent probe with the potential to detect melanoma cancer. In a previous thesis, all three components of the proposed molecule were synthesized and characterized, but attachment of all three parts had not yet been completed. By using the previous thesis as a guide, all three parts were successfully remade and characterized. The plan for this project was to utilize a different synthetic route that would afford our designed luminescent probe. Currently, attachment of the polyethylene glycol linker onto the MSH-4 peptide has been synthesized and characterized. In the sections below, further detail about the synthetic routes employed are given. Ongoing research is still being conducted to attach the phenanthroline chromophore onto the peptide-linker, as it is the last step before completion of the entire molecule.

3.1 Tert-Butyl 15-hydroxy-3, 6, 9, 12-tetraoxa-tetradecanoate (**5**)

Tert-butyl 15-hydroxy-3, 6, 9, 12-tetraoxa-tetradecanoate (**5**) was synthesized from tetraethylene glycol and t-butyl bromoacetate (Scheme 5).[17] Tetraethylene glycol was used in excess so that only one end of the molecule would become protected with a t-butyl group. First, tetraethylene glycol was added to a basic solution containing potassium t-butoxide and deprotonation of the hydroxyl group occurred. The nucleophile was able to undergo a substitution reaction with t-butyl bromoacetate by displacing the bromide leaving group. This step formed tert-butyl 15-hydroxy-3, 6, 9, 12-tetraoxa-tetradecanoate (**5**) with a 77% yield. Removal of excess tetraethylene glycol was accomplished by washing with a saturated sodium chloride solution. ^{1}H-NMR revealed the indicative peaks of the product but also displayed t-butyl bromoacetate in the spectrum. Starting material was removed under reduced pressure and GC-MS showed the product to have a purity greater than 88%.

Potassium t-butoxide; THF, t-butyl bromoacetate

5 77%

Scheme 5. Synthesis of tert-butyl 15-hydroxy-3, 6, 9, 12-tetraoxa-tetradecanoate (**5**).

3.2 Synthesis of Allyl bromoacetate (**6**)

The synthesis of allyl bromoacetate (**6**) was carried out using bromoacetic acid and allyl alcohol in the presence of an acid catalyst (Scheme 6).[18] A Dean and Stark apparatus was employed to help remove the water that was formed throughout the course of the reaction. After the reaction, the acid catalyst was neutralized by washing with a dilute sodium bicarbonate solution. Removal of solvent left a colorless liquid whose vapors were very irritating to the eyes. The yield for allyl bromoacetate (**6**) was 80%. ^{1}H-NMR displayed the correct signals, suggesting that the compound had been synthesized. GC-MS showed the sample to have a purity of 98%.

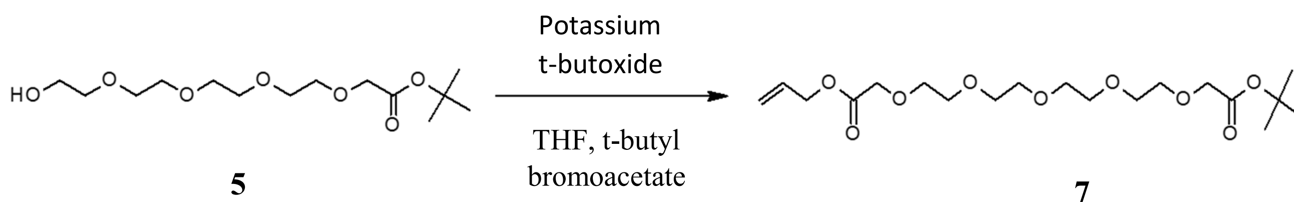
80%

pTsOH; cyclohexane

6

Scheme 6. Synthesis of allyl bromoacetate (**6**).

The next route chosen was to react allyl bromoacetate (**6**) with tert-butyl 15-hydroxy-3, 6, 9, 12-tetraoxa-tetradecanoate (**5**) under the same reactions used in Scheme 5. This proposed synthesis would modify the hydroxyl group on tert-butyl 15-hydroxy-3, 6, 9, 12-tetraoxa-tetradecanoate (**5**) by attaching an allyl protecting group (Scheme 7).



Scheme 7. Proposed synthetic route for tert-butyl 11-oxo-3, 6, 9, 12-tetraoxatetradecane-14-en-1-oate (**7**).

Unfortunately, this reaction did not produce the intended product as the ^{1}H-NMR spectra was cluttered with additional peaks and the signal from the t-butyl group seemed to disappear. After GC-MS analysis, it was believed that tert-butyl 15-hydroxy-3, 6, 9, 12-tetraoxa-tetradecanoate (**5**) was undergoing an intramolecular reaction by producing a cyclic lactone (Figure 8). This outcome was deemed as a possibility before synthesis, but due to the length of the molecule it was thought that maybe this would not happen. The reaction was attempted multiple times, and each try produced similar results.

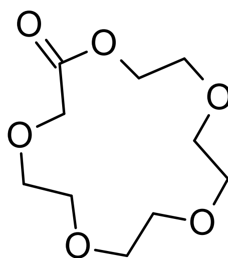
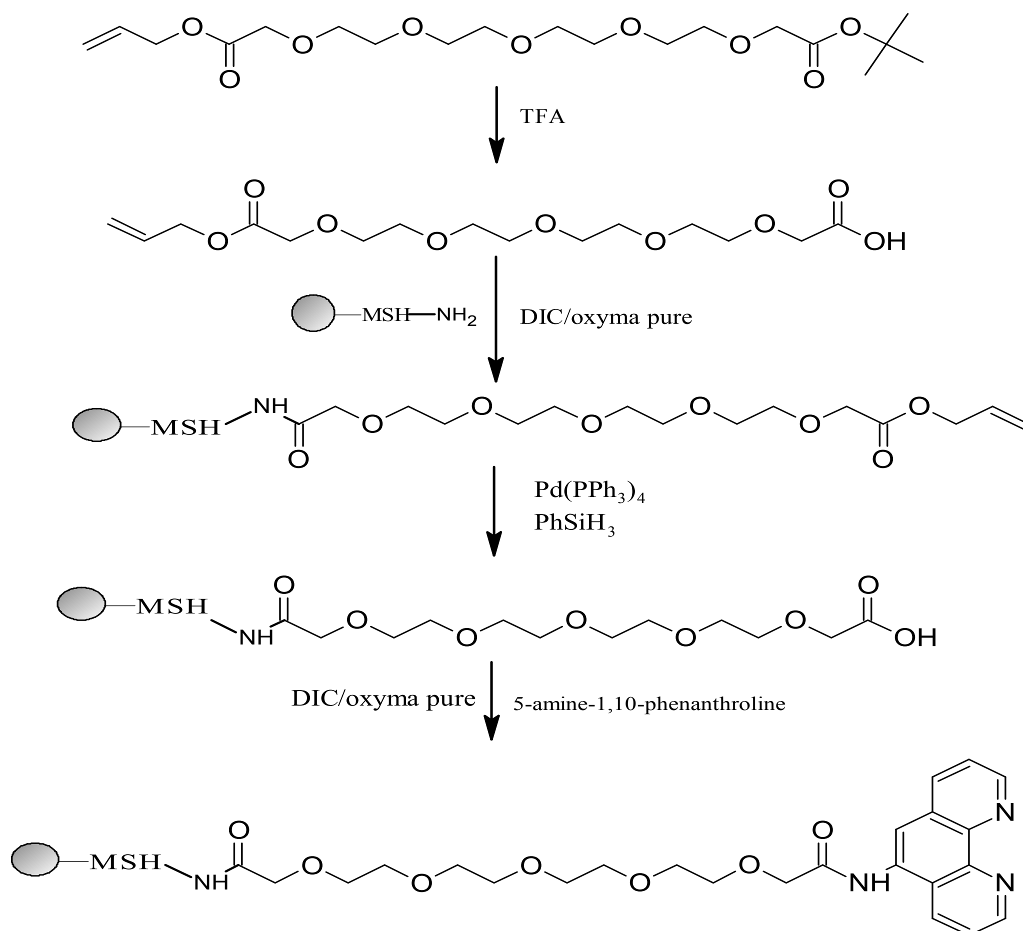


Figure 8. Structure of 1, 4, 7, 10, 13-pentaoxacyclopentadecan-2-one, a cyclic lactone.

The rationale behind adding tert-butyl and allyl protecting groups was to systematically couple the linker to the MSH-4 peptide on resin, and lastly couple the phenanthroline chromophore onto the opposite end of the linker (Scheme 8).



Scheme 8. Proposed synthetic route for the coupling of the PEGO linker and phenanthroline chromophore onto the MSH-4 peptide.

The reaction scheme was to first deprotect the t-butyl group in a solution of TFA.[10] This step would release the t-butyl group and generate a carboxylic acid. The C-terminus could then couple onto the free amine on the MSH-4 peptide substrate by forming an amide bond. After confirmation of the peptide-linker amide bond, deprotection of the allyl group from the opposite

end of the linker could be performed. Deprotection of the allyl group by a palladium catalyst would once again generate a carboxylic acid.[19] The free C-terminus could then couple with the amine group on the phenanthroline chromophore by forming an amide bond.

The next approach taken was to synthesize the di-acid form of the PEGO linker molecule, and then react it with the free amine group on the peptide substrate using Fmoc coupling reagents. There was speculation that both carboxylic acid groups would react with a free amine moiety from two different MSH-4 peptide molecules projecting off the resin. This would generate an undesired product, leaving two MSH peptides coupled on each side of the linker (Figure 9). To prevent this from happening a low loading resin was used. With fewer moieties available there would hopefully be less of a chance for cross coupling.

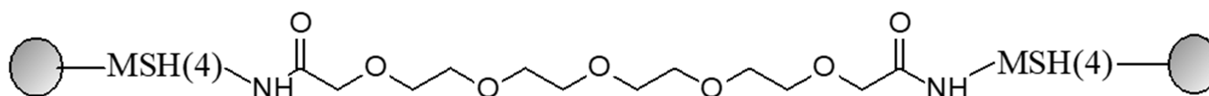
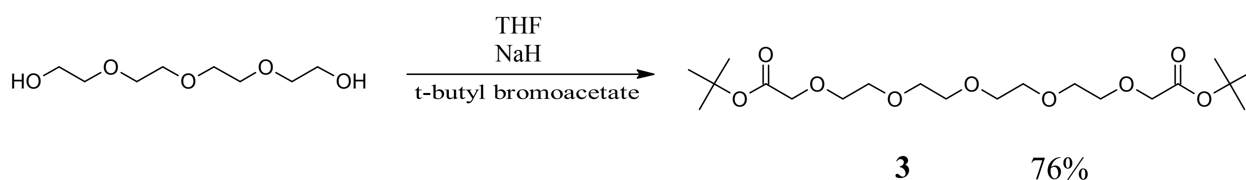


Figure 9. Cross coupling of PEGO linker, leaving two MSH peptides attached on each end.

3.3 Synthesis of tert-butyl 17-hydroxy-19, 19-dimethyl-3, 6, 9, 12, 15, 18-hexaoxaicosanoate (**3**)



Scheme 9. Synthesis of tert-butyl 17-hydroxy-19, 19-dimethyl-3, 6, 9, 12, 15, 18-hexaoxaicosanoate (**3**).

The synthesis of tert-butyl 17-hydroxy-19, 19-dimethyl-3, 6, 9, 12, 15, 18-hexaoxaicosanoate (**3**) was carried out using tetraethylene glycol and t-butyl bromoacetate under basic conditions

(Scheme 9).[10] This reaction was very challenging as it often produced half reacted side product. Increasing the amount of sodium hydride proved to generate more of the di-tert-butyl than mono-tert-butyl product. Excess t-butyl bromoacetate was also difficult to remove. Removal of the starting material was accomplished by using reduced-pressure distillation. After many efforts of experimenting with different amounts of starting material and reaction times, the synthesis would always result in half reacted side product. To achieve the desired purity column chromatography was utilized. A solvent system composed of a 1:4 ratio of ethyl acetate to hexanes was found to help separate not only the mono-tert-butyl product but also t-butyl bromoacetate from the desired product. Post column chromatography showed the di-tert-butyl product to be close to 85% pure after GC-MS analysis. ^{1}H-NMR displayed a 18H singlet at δ 1.5, representing the hydrogen nuclei from the two t-butyl groups. This confirmed that a majority of the starting material and half reacted product had been removed.

3.4 Synthesis of 2□hydroxy□1λ^{3}, 4, 7, 10, 13, 16□hexaoxaoctadec□1□yn□18□oic acid (**4**)

formic acid

RT

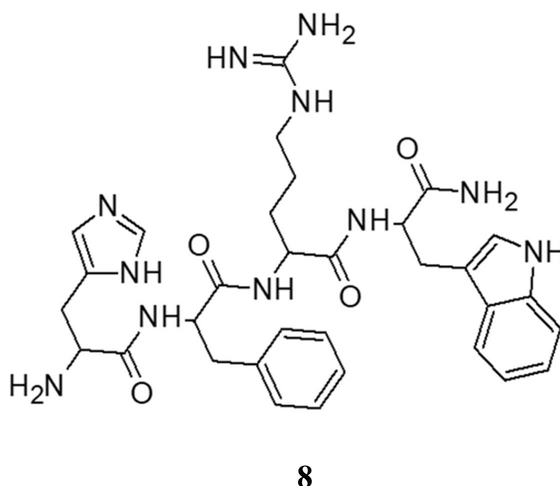
3 **4** 96%

Scheme 10. Synthesis of 2□hydroxy□1λ^{3}, 4, 7, 10, 13, 16□hexaoxaoctadec□1□yn□18□oic acid (**4**).

The synthesis of 2□hydroxy□1λ^{3}, 4, 7, 10, 13, 16□hexaoxaoctadec□1□yn□18□oic acid (**4**) involved stirring tert-butyl 17-hydroxy-19, 19-dimethyl-3, 6, 9, 12, 15, 18-hexaoxaicosanoate (**3**) in a solution of excess formic acid (Scheme 10).[10] This reaction was responsible for removing the terminal t-butyl groups and generating t-butyl alcohol as a side product. Removal of t-butyl

alcohol and excess formic acid were accomplished by reduced-pressure distillation. HPLC showed the product to have a purity of 71%. ^{1}H-NMR spectra revealed the disappearance of the t-butyl groups and showed the emergence of a broad 2H singlet, representing the two carboxylic acid functional groups generated from the reaction. LC-MS displayed a M^{+1} peak of 311 indicating the correct mass for the intended product.

3.5 Synthesis of Melanocyte Stimulating Hormone (4) peptide (**8**)



8

Scheme 11. Synthesis of Melanocyte Stimulating Hormone (4) peptide using Fmoc protocol (**8**)

Synthesis of Melanocyte Stimulating Hormone (4) peptide (**8**) was performed under Fmoc protocol using a Discover SP-X microwave synthesizer equipped with a fiber optic temperature probe manufactured by CEM Corporation (Scheme 11).[12] A low loading (0.10-0.30 mmol/g) rink amide resin was chosen as the solid support to help prevent cross coupling with the ensuing synthesis attachment of the PEGO linker. Approximately 500 mg of resin was weighed and synthesis was performed using Fmoc protocol on a 0.1 mmol scale. The resin beads were swelled for 40 minutes at room temperature in a 50:50 (by volume) mixture of DMF and DCM. After swelling was complete the solvent mixture was filtered and coupling proceeded. The

resin's initial Fmoc protecting group was removed by adding approximately 3 mL of the piperidine reagent to the fritted vessel. A fiber optic temperature probe was inserted into the reaction vessel to alert the microwave to turn off once the preferred temperature was attained. The microwave settings for the deprotection method included adjusting the power to 50 W, while heating the reaction at 70˚ C for a period of 30 seconds. Following deprotection, the resin beads were washed and filtered three times with approximately 2 mL of DMF to remove all excess reagents and by-products. A Kaiser test was performed to indicate the presence of a free amine group. A few resin beads were placed in a test tube with the Kaiser reagent and heated in the microwave at 120˚ C for a period of 30 seconds. The interaction between the amine group and Kaiser reagents stained the resin beads blue, which indicated that deprotection was successful.

The next step was to couple the first amino acid residue. The coupling reagents used were diisopropylcarbodiimide (DIC) and ethyl (hydroxyamino)cyanoacetate. Each of these reagents along with the amino acids were used in a 5-fold excess. A solution of coupling reagents along with the intended amino acid were dissolved in DMF beforehand to help pre-activate the amino acid. The reaction conditions for coupling were set at 75˚ C for a period of 7 minutes. After the reaction, the resin was washed and filtered three times with approximately 3 mL of DMF. This process was repeated for each additional amino acid. The amino acid sequence consisted of tryptophan, arginine, phenylalanine and histidine. Cleavage of the peptide from resin and releasement of protecting groups from amino acid residues was not performed in the microwave, but rather at room temperature using a rocker device. Ideally, cleavage should be performed in the microwave but due to lacking the necessary equipment the reaction was done using benchtop methods. The cleavage cocktail consisted of (82.5% TFA, 5% thioanisole, 5% water, 5% phenol, 2.5% 1, 2-ethanedithiol v/v). Reaction times for cleavage can vary

depending upon what amino acids and how many are present in the peptide sequence. Generally, a reaction time between 3-6 hours is sufficient for most peptides. To begin cleavage, the peptide-resin was placed into a plastic syringe and capped. The cleavage cocktail was poured over the resin and the syringe plunger was inserted. The syringe was placed on the rocker device and the contents were mixed. Afterwards, the resin beads were filtered off from the cleavage solution containing the peptide. Precipitation of the peptide from the cleavage solution was accomplished by adding cold diethyl ether. It is recommended that the TFA and other volatile scavengers be evaporated by at least half their volume before adding ether to increase peptide yields. The solution was then placed in a freezer for 1 hour to allow for further precipitation. Afterwards, the peptide-ether suspension was centrifuged for 20 minutes at 0˚ C. The ether was carefully decanted making sure not to disturb the peptide film at the bottom of the centrifuge tube. The precipitation process was performed two additional times to optimize purity. The sample was sonicated after each addition of fresh ether to break the peptide into smaller granules to help remove any trapped impurities. The peptide was air dried, dissolved in acetic acid/water mixture and then lyophilized.

Before lyophilizing, the peptide is dissolved in a suitable solvent or solvent mixture. There is not a single solvent that will dissolve all peptides, so a trial-and-error approach must be taken. Solubility will depend upon the amino acid composition of the peptide. If the peptide at hand has many ionic charges in its structure, then chances are it will dissolve in water. A common solvent mixture that will dissolve most charged peptides is water and acetic acid. Peptides that contain a lot of hydrophobic residues may have a hard time dissolving in an aqueous solution. Organic solvents such as acetonitrile will help dissolve peptides that contain more than 50% hydrophobic residues. Once the peptide is dissolved it should be frozen in a

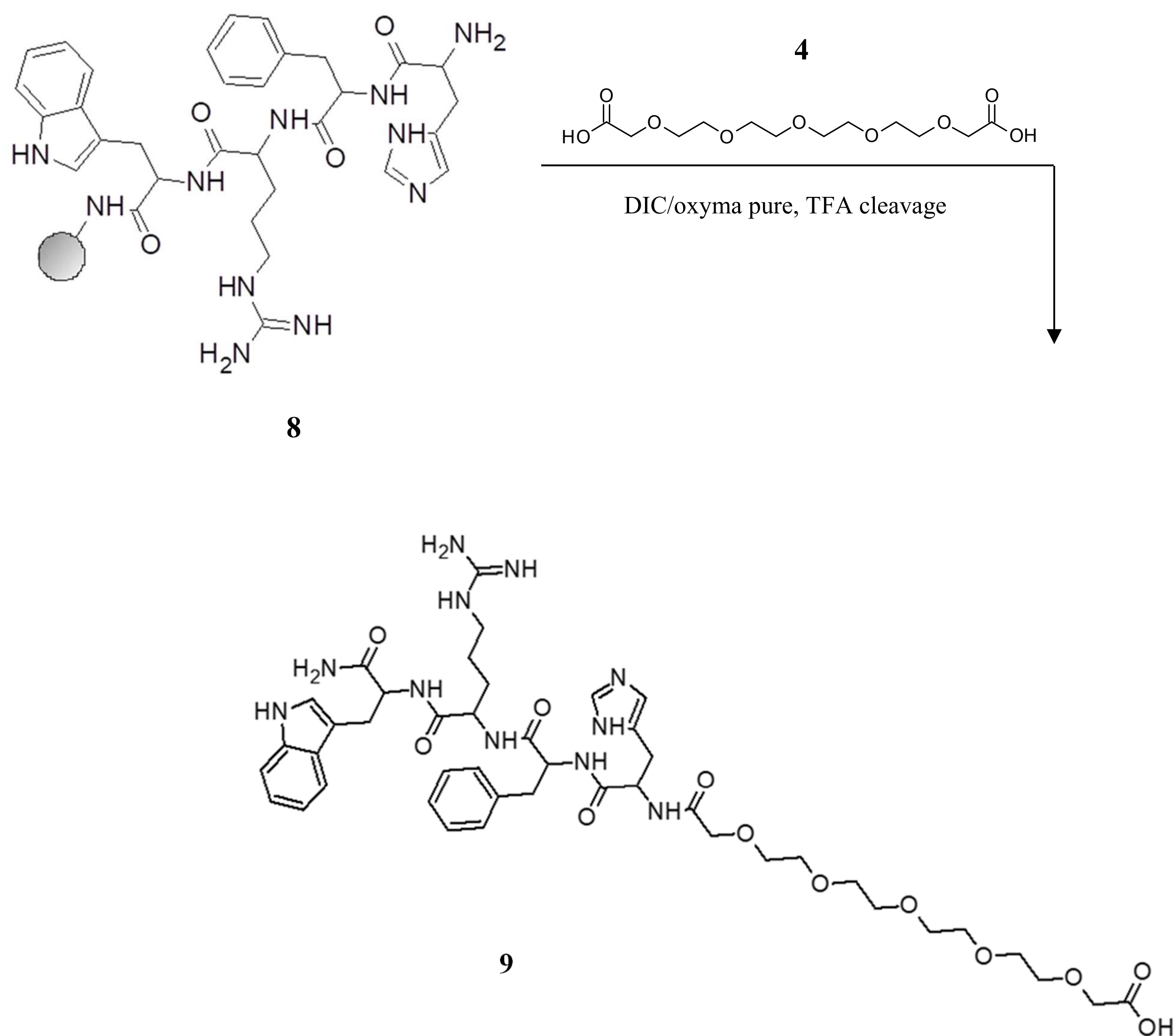
cryogen such as liquid nitrogen and then placed inside the lyophilizer to sublimate for a period of at least 14 hours.

The standard analytical tool used for purity assessment of proteins and peptides is reverse-phase HPLC. Reverse-phase HPLC uses a polar mobile phase and a hydrophobic stationary phase. A C_{18} silica column with a porosity between 100-300 Å is preferable. Due to the length of most peptides it is recommended that the porosity size be on the larger side. Samples are eluted from the column using a solvent gradient mixture of water and acetonitrile. A general starting gradient of 5% acetonitrile with a linear increase in acetonitrile to 90% is used to establish a chromatographic behavior of the peptide. After initial analysis the gradient system can be tailored to help obtain optimal separation between peaks. The ultra violet lamp is generally set between 210-250 nm to monitor the peptide after elution from the column. To confirm the structure of the peptide a mass spectrometer is coupled to the HPLC. After the peptide sample leaves the HPLC column it is received by the mass spectrometer. The mass spectrometer ionizes the peptide and subjects it to a magnetic field where the mass to charge ratio is determined. When analyzing peptides, it is often common to find not only M^{+1} peaks in the mass spectrum but also M^{+2} and M^{+3} peaks.

A peptide sample was sent off site to Lipscomb University and analyzed by Dr. Vergne's research group. The LC-MS was set on positive ion mode and showed a M^{+1} peak of 644, and a M^{+2} peak of 323. HPLC analysis showed the peptide to have a purity greater than 80%. The HPLC chromatogram displayed the peptide eluting first at 0.1 minutes and two impurities eluting at 6.0 and 8.9 minutes, respectively. The two impurities were thought to be the peptide with protecting groups still attached on. Since the column is reverse phase it would make sense for

the impurities to elute much later than the peptide because of the nonpolar nature of the protecting groups.

3.6 Synthesis of MSH-4 Peptide/Polyethylene Glycol Linker (di-acid) (**9**)

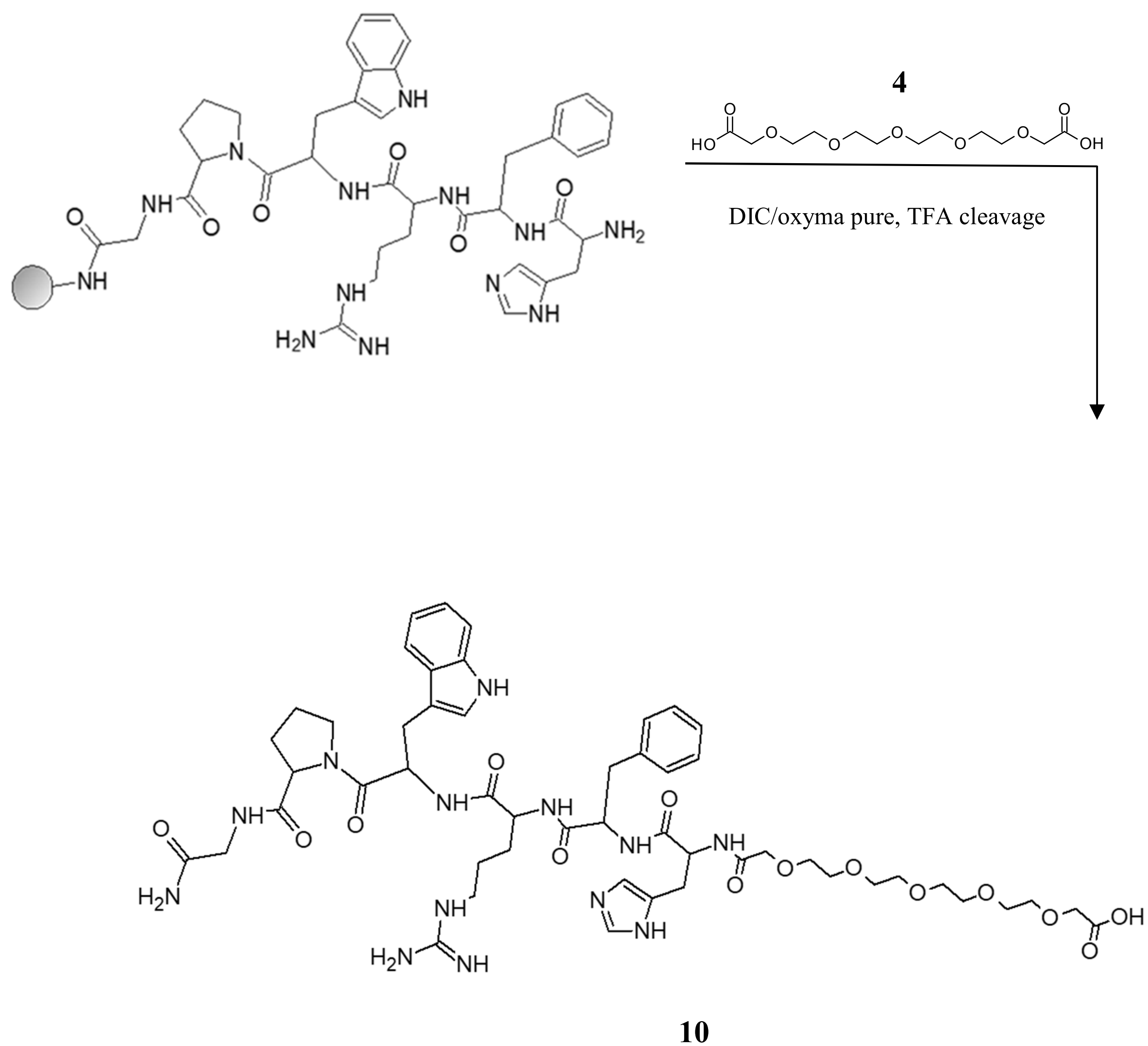


Scheme 12. Synthesis of MSH-4 Peptide/Polyethylene Glycol Linker (di-acid) (**9**).

The coupling of the PEGO linker (di-acid) onto the MSH-4 peptide was performed under the same reaction conditions as Fmoc amino acid coupling (Scheme 12). Before coupling 2□ hydroxy-1λ³, 4, 7, 10, 13, 16-hexaoxaoctadec-1-yn-18-oic acid (**4**) to the peptide-resin it was first allowed to pre-activate in a solution containing DMF, DIC and oxyma pure. After microwave heating, a Kaiser test was performed to reveal if the primary amine on the peptide had reacted. The Kaiser test revealed that the free amine was no longer present due to the yellow stained resin beads. A small sample of the peptide-resin was cleaved from the resin and lyophilized. The sample was injected into the HPLC and the major product MSH-4 Peptide/Polyethylene Glycol Linker (di-acid) (**9**) eluted at approximately 0.5 minutes. The area under the curve demonstrated the product to be greater than 85% pure. Following HPLC analysis, the sample was injected into the LC-MS and set to positive ion mode. The mass spectrum displayed an intense M^{+2} peak of 469 indicating the correct mass for our product.

To confirm that the PEGO linker (di-acid) was in fact coupling to the MSH-4 peptide-resin a second peptide was incorporated into the project. The second peptide was synthesized from a previous thesis and was kept stored in the freezer with the peptide bound to resin.[9] The peptide identity was the MSH-4-(PG) analog that consists of two additional amino acid residues, proline and glycine.

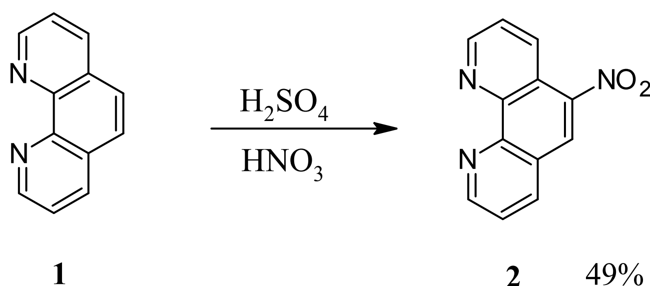
3.7 Synthesis of MSH-4-(PG) Peptide/Polyethylene Glycol Linker (di-acid) (**10**)



Scheme 13. Synthesis of MSH-4-(PG)/Polyethylene Glycol Linker (di-acid) (**10**).

The MSH-4-(PG) analog was removed from the freezer and swelled for an extra hour due to its prolonged storage time. Before coupling the linker, 2-hydroxy-1λ^3, 4, 7, 10, 13, 16□ hexaoxaoctadec-1-yn-18-oic acid (**4**) was pre-activated in a solution containing DMF, DIC and oxyma pure (Scheme 13). Following microwave heating, a Kaiser test was able to stain the resin beads yellow indicating that the free amine on the peptide was no longer present. After cleaving a small portion of the peptide-resin the sample was injected into the HPLC. The chromatogram displayed a nice single peak with the UV detector set at 215 nm. The elution of the major product MSH-4-(PG) Peptide/Polyethylene Glycol Linker (di-acid) (**10**) at approximately 0.5 minutes was almost identical as the retention time for the MSH-4 Peptide/Polyethylene Glycol Linker (di-acid) (**9**). The purity of the product was greater than 95% as demonstrated by the area under the curve. The LC-MS displayed two molecular ion peaks representing the correct mass of our product. The mass spectrum showed an intense M^{+2} peak of 546 and a M^{+1} peak of 1090, confirming that the reaction scheme for the PEGO linker coupling was successful.

3.8 Synthesis of 5-nitro-1, 10-phenanthroline (**2**)

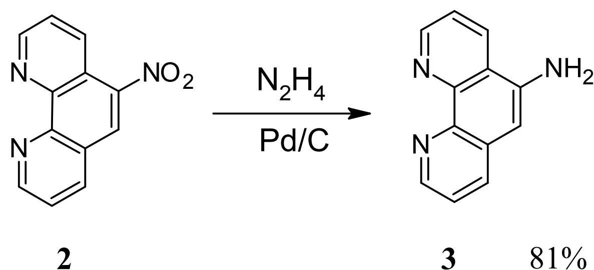


Scheme 14. Synthesis of 5-nitro-1, 10-phenanthroline (**2**).

The next phase of the project was to synthesize the 5-amine-1, 10-phenanthroline (**3**) chromophore that could potentially couple with the free C-terminus on the MSH-4 Peptide/Polyethylene Glycol Linker (di-acid) (**9**). To begin, 1, 10-phenanthroline (1) was

nitrated by combining nitric and sulfuric acid in excess (Scheme 14).[7] After allowing the reaction mixture to reflux for 3 hours, the solution was poured over ice water. A 6M NaOH solution was titrated into the reaction mixture to help adjust the pH to 7, so that 5-nitro-1, 10-phenathroline (**2**) would precipitate out of solution. The reaction mixture would oftentimes generate solid $NaSO_4$ as a by-product with the addition of sodium hydroxide. This presented a problem when filtering due to 5-nitro-1, 10-phenathroline (**2**) mixing in with the sulfate impurities. To remove the $NaSO_4$ impurities 5-nitro-1, 10-phenanthroline (**2**) was dissolved in chloroform and then filtered. The yield for this reaction was quite low at times because of the product being trapped within the sodium sulfate impurities. A yield of 49% was the best yield obtained from this reaction. The product was characterized by ^{1}H-NMR and FT/IR. The ^{1}H-NMR spectra revealed the indicative signals that suggested the compound was synthesized. The ^{1}H-NMR spectra also showed a water signal due to the hygroscopic nature of the two nitrogen atoms. No further drying methods were employed to remove the water from the sample since it was not thought to effect the coupling reaction with the MSH-4 Peptide/Polyethylene Glycol Linker (di-acid) (**9**). FT/IR displayed a stretch (N-O stretch) at 1517 cm^{-1} and at 1330 cm^{-1} that confirmed the presence of the nitro group.

3.9 Synthesis of 5-amine-1, 10-phenanthroline (**3**)



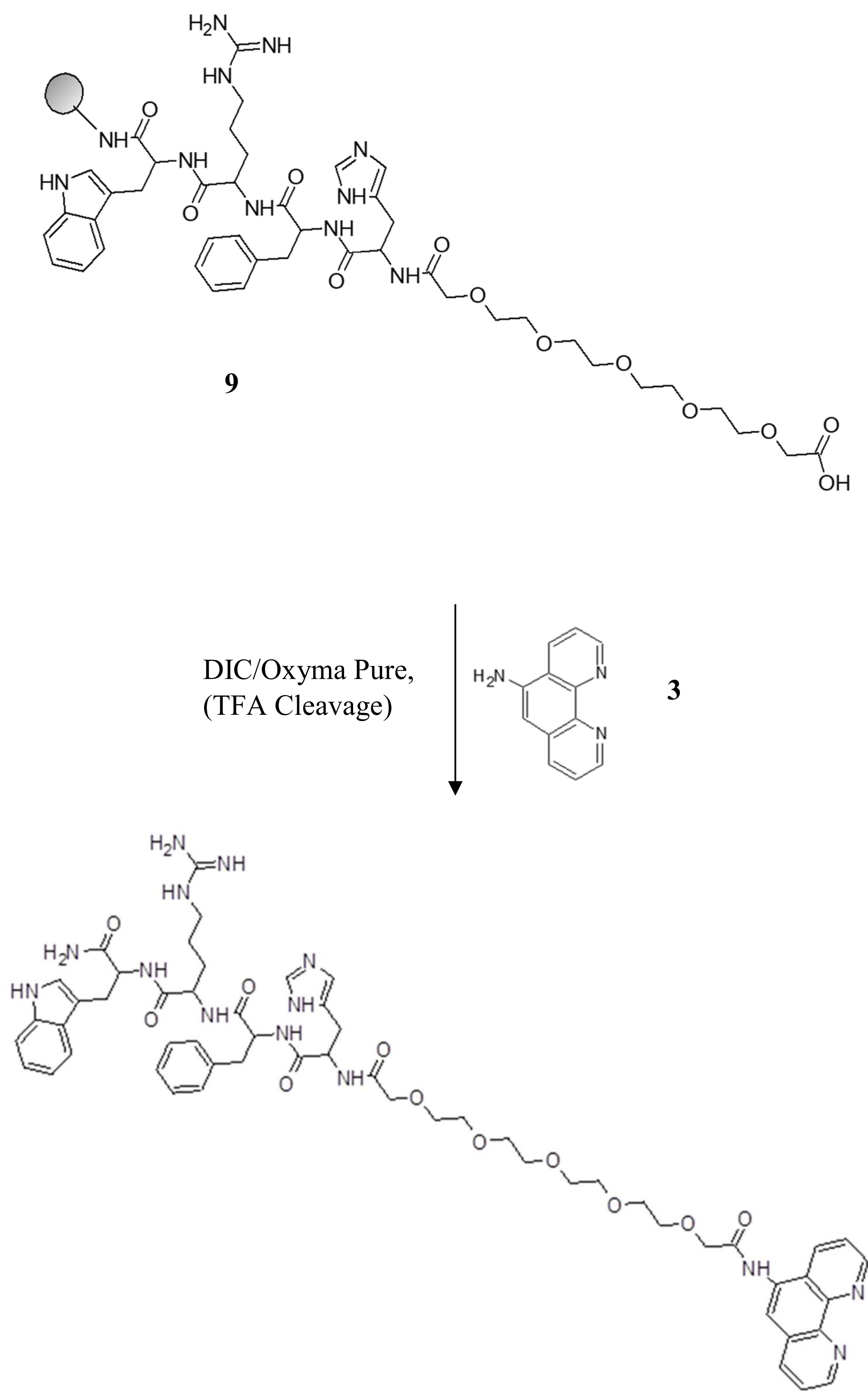
Scheme 15. Synthesis of 5-amine-1, 10-phenanthroline (**3**).

The reduction of the nitro group was performed using hydrazine monohydrate in the presence of a Pd/C catalyst to generate 5-amine-1, 10-phenanthroline (**3**) (scheme 15).[8] First, 5-nitro-1, 10-phenanthroline (**2**) was dissolved in absolute ethanol. The solid starting material did not readily dissolve in absolute ethanol, so the mixture was placed in a sonicator bath until dissolution was complete. Before the addition of hydrazine monohydrate, argon gas was bubbled through the solvent to help provide an inert reaction environment. The reduction reaction seemed to go smoothly by producing an 81% yield. The ^{1}H-NMR spectra revealed a 2H singlet at δ 6.16 indicating the presence of the newly formed amine group. In some cases, crude product showed there to be excess hydrazine monohydrate in the ^{1}H-NMR spectra. To remove the unwanted starting material, the product was re-dissolved in chloroform and washed three times with H_2O. This step was able to remove all the hydrazine monohydrate. FT/IR spectra showed the presence of an amine stretch (N-H stretch) at 3415 cm^{-1} and at 3319 cm^{-1}. The FT/IR spectra also revealed the disappearance of the nitro bands (N-O stretch) at 1517 cm^{-1} and 1330 cm^{-1}.

3.10 Synthesis of MSH-4 Peptide/Polyethylene Glycol (di-acid)/5-amine-1, 10-phenanthroline

After synthesis and characterization of the 5-amine-1, 10-phenanthroline (**3**) chromophore, the next step was to attach it onto the peptide-linker using Fmoc coupling reagents (Scheme 16). In this case, the C-terminus used to form the amide bond would be from the linker projecting off the peptide on resin. To activate the linker C-terminus, Fmoc coupling reagents (DIC, oxyma pure) were added to the peptide-linker on resin. The reagents were stirred briefly to allow the ester intermediate to form. Next, a solution of 5-amine-1, 10-phenanthroline (**3**) in DMF was poured over the resin and the mixture was heated for 14 minutes at 75 ˚C. For this specific reaction a qualitative Kaiser test would not work since there was no free amine to test

for. A sample of the resin was cleaved and lyophilized. The LC-MS showed there to be numerous peaks in the chromatogram and did not display a correct m/z charge signal that would suggest the phenanthroline had successfully been coupled. The reason for this outcome could be due to the amino group on the phenanthroline not being nucleophilic enough to react and form the amide bond. The lone pair on the nitrogen atom could be stabilized due to resonance of the conjugated pi-electron ring structure.



Scheme 16. Proposed synthesis strategy for the coupling of the 5-amine-1, 10-phenathroline chromophore onto the peptide-linker.

CHAPTER 4: EXPERIMENTALS

Materials and Instrumentation

All reagents were purchased from Sigma Aldrich, Acros Organics, or CEM corporation and used without further purification unless otherwise stated. Synthesized ligands and complexes were characterized using Fourier-transform infrared spectroscopy (FT-IR), nuclear magnetic resonance (NMR), high pressure liquid chromatography (HPLC), gas chromatography (GC), and mass spectroscopy (GCMS, LCMS). The methods used for characterization will be discussed in this section along with all instrument specification.

FT-IR spectra were obtained using a Perkin Elmer Spectrum One. All measurements were performed at room temperature with a scanning range of 4000 cm^{-1} – 600 cm^{-1}, using single-bounce attenuated total reflectance with a diamond crystal. For all of the materials that were measured as a solid powder (all solvents had been removed), the background was performed on the instrument room environment. The ATR plate was cleaned with a Kimwipe and acetone between each measurement.

NMR spectra were obtained using a JEOL 300 MHz Eclipse NMR with a 5mm probe capable of detecting ^{1}H and ^{13}C nuclei. Proton NMR samples were prepared using ~10 mg of material, in either deuterated chloroform ($CDCl_3$) or deuterated dimethyl sulfoxide (d6-DMSO) unless otherwise stated, and spectra were obtained using a varying number of scans ranging from 16-128 as to ensure an adequate signal-to-noise ratio was acquired. ^{13}C NMR samples were prepared similarly, except material was added until the deuterated solution became saturated.

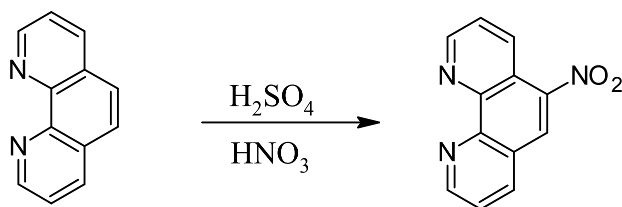
Gas chromatograms were obtained using an Agilent Technologies 7890 A GC System with a 7693 Autosampler, a 5975 C Inert XL EI/CI MSD (with Triple Axis Detector), and an Agilent J&W GC Column-HP-5MS (30 m × 0.25 mm × 0.25 μm) using a split inlet. Flow rate: 1.2mL/min,

APC-3 pressure: 1.0, Inlet Temperature: 250°C, Oven Temperature: 80°C, MS Quad Temperature: 150°C, MS Source Temperature: 230°C.

High pressure liquid chromatography was performed using an Agilent Technologies 1220 Infinity LC with a 99301 Prevail Select C18 column (L: 250 mm, ID: 4.6 mm) and a UV-Vis detector. Mobile phase: A: 10% CH_3CN B: 90% H_2O, Flow Rate: 1.5mL/min, Column Temperature: 25°C, Detection: 214nm/280nm. For peptide purity assessment, samples were sent off site to Lipscomb University and analyzed by Dr. Matthew Vergne's research group. Each assessment was performed using a Shimadzu Prominence XR Ultra HPLC system (Shidmadzu Scientific Instruments, Columbia, MD) which included two Shimadzu LC-20ADXR pumps, a SIL-10AVP UV-Vis detector and a Shimadzu LCMS 8030 mass spectrometer. Chromatographic separation was achieved on with a Phenomenex Kinetex C18 coloumn (50 x 2.1 mm, 1.7 µm) maintained at temperature of 40˚ C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was 0.25 mL/min. Initially, solvent B concentration was 5% and held at 5% for 0.5 minutes. Solvent B concentration was increased linearly to 60% form 0.5 min to 7 minutes, B was increased to 95% from 7 to 8 minutes, and B was held at 95% from 8.0 min. to 8.2 min. Solvent B was returned to 5% at 8.22 min. and remained at 5% until the send of the time program at 3.0 minutes to equilibrate the column for the next run. The injection volume was 1 µL. The UV wavelength of 254 nm was analyzed. For mass spectrometry, an electrospray ionization source was utilized with the following parameters: DL temperature, 250˚ C; nebulizing gas flow, 3.00 L/min; heat block, 450˚ C; drying gas flow, 20 L/min. The MS program consisted of a quadrupole scan (Q3) event form m/z 200 to m/z 1100 with a 0.100 sec. event time in the positive ion mode.

All peptide samples were centrifuged using a Sorvall RC-5C Plus Superspeed Centrifuge. The samples were placed into the centrifuge at 0˚ C for 20 minutes. Each sample was centrifuged at a speed of 3200 RPM. All peptide samples were lyophilized into a dry powder using a Labconco Freeze Dry System (7740021). The samples were first dissolved in a suitable aqueous buffer and then frozen in liquid nitrogen. The frozen samples were placed inside the freeze dryer under reduced pressure (~.250 mbar) and allowed to sublimate for a period of 14 hours.

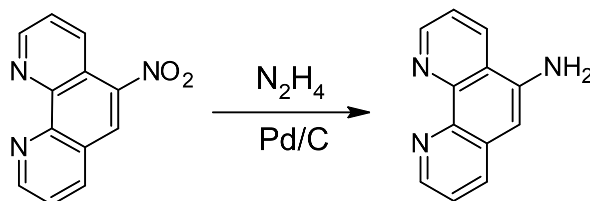
Synthesis of 5-nitro-1, 10-phenanthroline (2)



1, 10-phenanthroline (1.0 g, 5.55 mmol, 1.0 eqv) was added to a 25-ml round bottom flask. Sulfuric acid (6.0 ml, 0.113 mol, 20.5 eqv) was added and the mixture was stirred until the 1, 10-phenanthroline was dissolved. After heating the solution to reflux, concentrated nitric acid (3 ml, .071 mol, 14.2 eqv) was added dropwise. The reaction was refluxed for three hours at 160 ˚C. The reaction mixture was taken off reflux and poured into ice water. The ice water solution was then titrated with 6M NaOH (38 ml) until a yellow solid precipitate formed. The precipitate of 5-nitro-1, 10-phenanthroline was collected by vacuum filtration. To remove Na_2SO_4 impurities the solid 5-nitro-1, 10-phenanthroline was re-dissolved in chloroform. The sodium sulfate impurities were filtered off leaving a clear solution. Chloroform was removed by reduced pressure leaving behind a yellow solid. The yellow solid was air dried to yield 5-nitro-1, 10-phenanthroline (**2**) (0.560 g, 2.49 mmol, 49.2% yield). ^{1}H-NMR 300MHz ($CDCl_3$): δ 9.35 (2H mult.), δ 9.06 (1H, dd), δ 8.73 (1H, s), δ 8.45 (1H, dd), δ 7.83 (2H, mult.). ^{13}C NMR 300 MHz ($CDCl_3$): δ 120.83, 124.20, 124.29, 125.27, 125.38, 132.31, 137.74, 144.07, 146.02, 147.47,

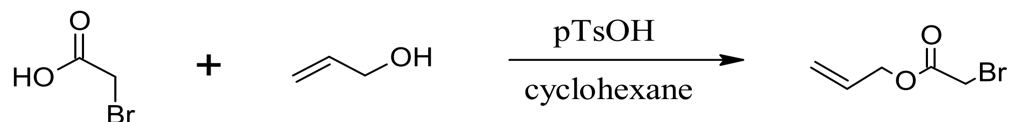
151.35, 153.43. FT/IR (ATR): 1517, 1502, 1344, 1330 (N-O stretch), 806, 733 (=C-H bending) cm^{-1}.

Synthesis of 5-amine-1, 10-phenanthroline (3)



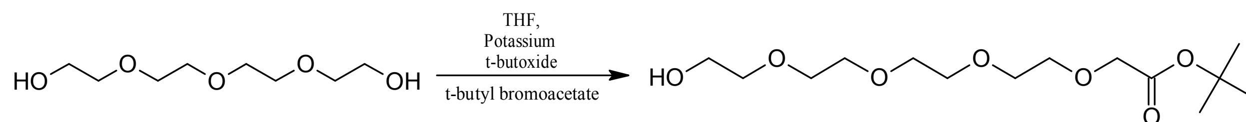
5-nitro-1, 10 phenanthroline (**2**) (0.560 g, 2.48 mmol, 1.0 eqv) was added to a 50-ml round bottom flask and dissolved in absolute ethanol (25 ml). To help guarantee all the 5-nitro-1, 10-phenanthroline was dissolved the reaction mixture was placed in a sonicator for 1 hour at room temperature. After complete dissolution of 5-nitro-1, 10-phenanthroline, the solvent was purged with argon and a Pd/C catalyst (5% Pd/C, 50 mg) was added to the flask along with a stir bar. The reaction flask was capped with a septum and hydrazine monohydrate (2.13 ml, 43.5 mmol, 17.5 eqv) was added dropwise via syringe for a period of 30 minutes. The reaction mixture was heated to 70 ˚C and stirred for 10 hr. After the reaction was complete, the solution was filtered to remove the Pd/C. To remove excess hydrazine monohydrate, the crude product was re-dissolved in chloroform and washed three times with H_2O. The filtrate was placed under reduced pressure and a brick red 5-amine-1, 10-phenanthroline (**3**) (0.395 g, 81.4% yield) precipitate appeared. ^{1}H-NMR 300MHz (DMSO): δ 9.06 (1H, d), δ 8.68 (2H, dd), δ 8.04 (1H, d), δ 7.73 (1H, mult.), δ 7.50 (1H, mult.), δ 6.86 (1H, s), δ 6.16 (2H, s). ^{13}C-NMR 300MHz (DMSO): δ 101.94, 122.51, 123.16, 124.09, 131.45, 131.90, 134.69, 139.34, 143.79, 144.35, 145.54, 150.02. FT/IR (ATR): 3415, 3319 (N-H stretch), 1610 (N-H bending), 841, 739 (=C-H bending) cm^{-1}.

Synthesis of allyl bromoacetate (6)



Bromoacetic acid (48.25 g, 0.34 mol, 1.0 eqv) and allyl alcohol (24 mL, 20.54 g, 0.34 mol, 1.0 eqv) were added to cyclohexane (200 mL). An acid catalyst pTsOH (20 mg) was added to the mixture and the solution was heated to reflux. A Dean and Stark apparatus was attached to the reflux condenser to ensure the removal of water over the course of the reaction. After refluxing for 3 hours, the reaction mixture was added to a separatory funnel and neutralized by washing with a dilute sodium bicarbonate solution. The mixture was then washed with brine and dried using $MgSO_4$. Cyclohexane was removed by reduced pressure and an irritating colorless liquid remained (48.25 g, 80.4% yield). ^{1}H NMR 300 MHz ($CDCl_3$): δ 5.92 (1H, mult.), δ 5.31 (2H, mult.), δ 4.68 (2H, d), δ 3.87 (2H, s).

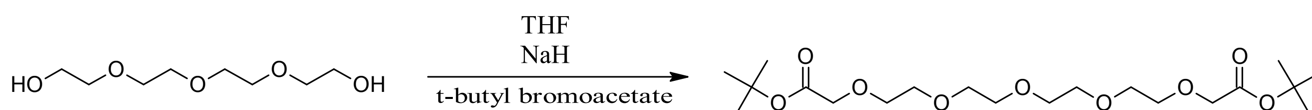
Synthesis of tert-butyl 15-hydroxy-3, 6, 9, 12-tetraoxatetradecan-1-oate (5)



Potassium t-butoxide (4.2 g, 37.43 mmol, 1.0 eqv) was added to a 250-ml round bottom flask and the flask was capped with a septum. The flask was purged with argon to remove atmospheric oxygen. Tetraethelyne glycol (60 ml, 67.5 g, excess) along with dry THF (25.0 ml) were added to the flask via syringe. The solution was heated between 40-50˚C until the potassium t-butoxide was completely dissolved. The mixture was then placed into an ice bath and allowed to cool to 0˚ C. One portion of t-butyl bromoacetate (6.0 ml, 7.93 g, 40.63 mmol, 1.0 eqv) was added to the flask via syringe. The solution was taken from the ice bath and

allowed to stir at room temperature for 24 hours. After stirring for 24 hours, the solution had turned yellow-orange in color. The solution was placed into a separatory funnel and diluted with ethyl acetate. The mixture was washed with brine three times and all organic layers were collected. Solvents were removed under vacuum to yield tert-butyl 14 hydroxy-3, 6, 9, 12-tetraoxatetradecan-1-oate (**5**) (8.89 g, 77.1% yield) as a viscous yellow-orange liquid. ^{1}H NMR 300 MHz ($CDCl_3$): δ 4.01 (2H, s), δ (16 H, mult.), δ 3.66 (1H, s), δ 1.46 (9H, s).

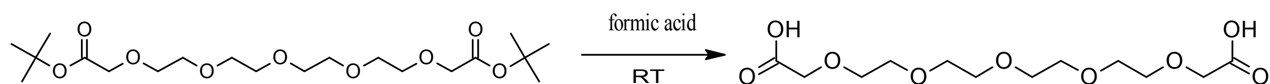
Synthesis of tert-butyl 17-hydroxy-19, 19-dimethyl-3, 6, 9, 12, 15, 18-hexaoxaicosanoate (3)



Sodium hydride (0.979 g, 40.8 mmol, 4.0 eqv) was placed into a 100-mL round bottom flask and capped with a septum. The flask was purged with argon to ensure an inert environment. Dry THF (20.0 ml) was added to the flask and the solution was stirred. Tetraethylene glycol (1.78 ml, 2.0 g, 10.2 mmol, 1.0 eqv) in 5.0 ml of dry THF was slowly added to the solution via syringe. The solution started to bubble as the evolution of hydrogen gas was given off. After complete addition of tetraethylene glycol, the solution was stirred at room temperature for a period of 2 hours. A separate solution of tert-butyl bromoacetate (6.02 ml, 7.96 g, 40.8 mmol, 4.0 eqv) in 10 ml THF was placed in an ice bath and cooled to 0˚ C. The tetraethylene glycol solution was then added dropwise to the chilled tert-butyl bromoacetate solution. The reaction was stirred at 0˚ C for 2 hours and then at room temperature for 14 hours. The solution was then placed under reduced pressure and solvent was removed. Removal of solvent created a solid white residue that was re-dissolved in water and extracted using diethyl ether. The extract was

dried using magnesium sulfate and solvent was removed by rotary evaporation. This left an impure, yellow viscous liquid. Dry column chromatography was utilized to remove unwanted starting material and other by-products. The column apparatus consisted of a porosity sinter funnel connected to a parallel-sided vacuum filter funnel that was attached to a receiving flask. The dimensions of the column were 50 mm by length and 40 mm by diameter. TLC analysis revealed a solvent mixture of ethyl acetate and hexanes (1:4) to give a R_f value of 0.5 for the least polar constituent in the sample. The column was packed with approximately 30 g of silica and pre-eluted using a 100-mL solution of ethyl acetate and hexanes (1:4). There was a total of seven, 50-mL fractions collected. The first fraction consisted of 25% ethyl acetate /75% hexanes by volume. Each successive fraction contained a 5% increase in ethyl acetate. The last fraction consisted of a 100% ethyl acetate flush. Solvent was removed under reduced pressure for each fraction leaving behind good product for fractions 3 through 6. Fractions 3 through 6 were combined to yield tert-butyl 17-hydroxy-19, 19-dimethyl-3, 6, 9, 12, 15, 18-hexaoxaicosanoate (**3**) (2.78 g, 6.37 mmol, 75.9 % yield). ^{1}H NMR 300 MHz ($CDCl_3$): δ 4.02 (4H, s), δ 3.68 (16H, mult.), δ 1.47 (18H, s). ^{13}C NMR 300 MHz ($CDCl_3$): δ 169.78, 81.62, 77.43, 70.80, 70.69, 70.65, 69.12, 28.19.

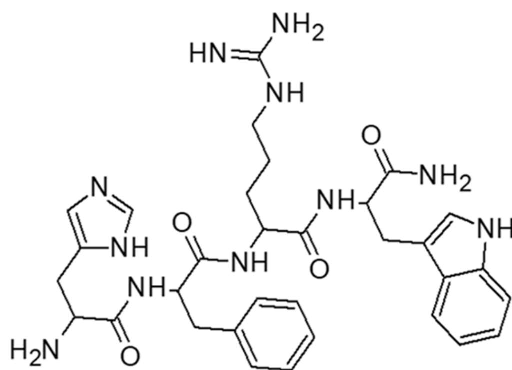
Synthesis of 2-hydroxy-1λ³, 4, 7, 10, 13, 16-hexaoxaoctadec-1-yn-18-oic acid (4)



Tert-butyl 17-hydroxy-19, 19-dimethyl-3, 6, 9, 12, 15, 18-hexaoxaicosanoate (**3**) (1.0 g, 2.36 mmol, 1.0 eqv) was added 25-mL round bottom flask. Formic acid (6.0 ml, 0.159 mol, 67.4 eqv) was added to the flask and the solution was stirred at room temperature for 3 hours. Excess formic acid and by-products were removed under vacuum to yield 2□hydroxy□1λ³, 4, 7, 10, 13,

16hexaoxaoctadec1yn18oic acid (**4**) as a viscous yellow oil (0.701 g, 2.26 mmol, 95.6 % yield). HPLC analysis revealed a purity of 71% as demonstrated by the area under the curve. ^{1}H NMR 300 MHz ($CDCl_3$): δ 5.35 (2H, s), δ 4.16 (4H, s), δ 3.68 (16H, mult.). ^{13}C NMR 300 MHz ($CDCl_3$): δ 172.54, 71.26, 70.62, 70.40, 70.21, 69.17. LC-MS: $[(M+H)^+]$ calculated, *m/z* = 310.2; found *m/z* = 311.2.

Synthesis of Melanocyte Stimulating Hormone (4) peptide (8)

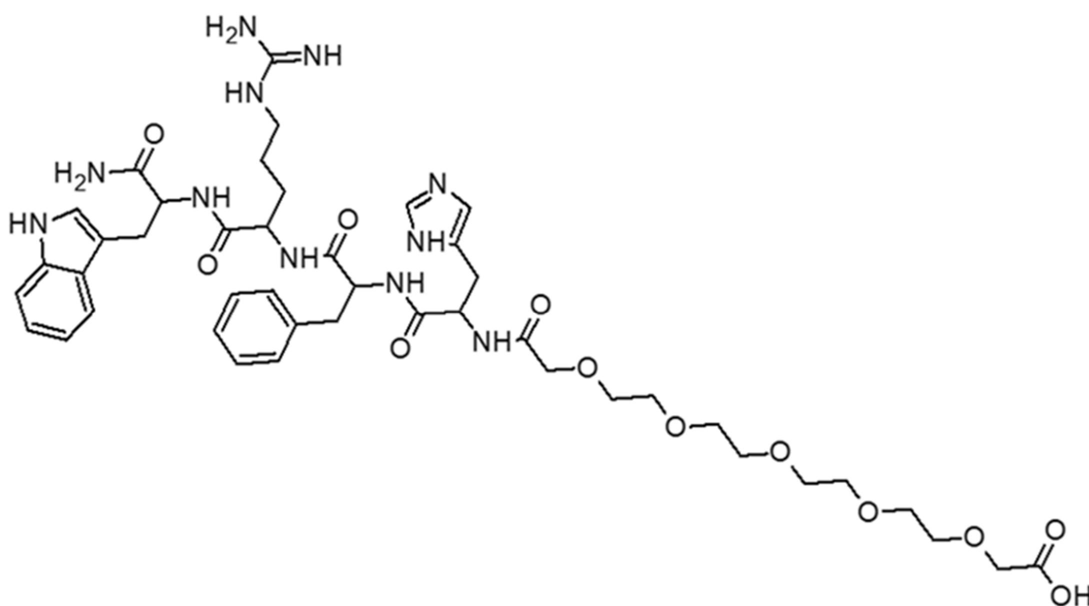


Synthesis of Melanocyte Stimulating Hormone (4) **(8)** was performed using solid phase peptide synthesis. The coupling of each amino acid was carried out on an insoluble support resin using a Discover SP-X microwave, equipped with a fiber optic temperature probe that was developed by CEM Corporation. ProTide Rink Amide Resin (555 mg, 0.1 mmol) was weighed out and placed into a fritted reaction vessel. The resin was first swelled in a 50:50 mixture of DMF and DCM for a period of 40 minutes. Removal of solvent by vacuum filtration left a dry resin that was fit for coupling. Before the addition of the first amino acid a deprotection step was performed to remove the initial FMOC group. A deprotection solution of 20 % piperidine in DMF (3 mL) was added to the reaction vessel. The vessel was placed inside the microwave and reacted for 30 seconds at 70˚ C, and then again for 30 seconds at 75˚ C. The deprotection solution was filtered off and a Kaiser test was used to test for a free amine. A few resin beads were placed into a test tube and 2 drops of each of the Kaiser reagents were added. The test tube was placed into the

microwave and reacted for 30 seconds at 120˚ C. The resin beads appeared blue in color indicating the presence of a free amine. Before coupling of the first amino acid the resin was washed three times with approximately 3 mL of DMF. A solution of the first amino acid in DMF along with coupling reagents were prepared in a scintillation vile. Fmoc-Trp(Boc)-OH (0.158 g, 0.3 mmol, 3.0 eqv) was added to 3 mL of DMF. Also added to the mixture was DIC (78.0 μL, 0.5 mmol, 5.0 eqv) and oxyma pure (0.071 g, 0.5 mmol, 5.0 eqv). This solution was vortexed to ensure uniform mixing and then poured over the resin beads. The mixture was heated in the microwave for 7 minutes at 75˚ C. Another Kaiser test was performed to determine if an amide bond had formed. The Kaiser test turned resin beads yellow, indicating that the reaction was successful. Resin beads were washed with DMF (3mL) three times before each coupling. The assembly of the remaining amino acids onto the resin were done using the previous steps. The remaining amino acids included: Fmoc-Arg(Pbf)-OH, Fmoc-D-Phe-OH, and Fmoc-His(Trt). After coupling the last amino acid, a final deprotection was conducted and the final Fmoc group was removed. The resin was finally washed two times with DCM (4 mL) and once with a 5-mL portion of DCM. Approximately 200 mg portion of peptide on resin was used for cleavage. A 5-mL cleavage mixture, composed of (82.5% TFA, 5% phenol, 5% water, 5% thioanisole, 2.5% 1, 2-ethandithiole) was added to the resin in a syringe. The syringe was placed on a rocker device for 3.5 hours at room temperature. Afterwards, resin beads were filtered off leaving behind cleaved peptide in solution. Argon gas was bubbled through the solution to evaporate most of the TFA. Cold ether (10-mL) was added and the peptide precipitated from solution. The peptide was then centrifuged at 3200 rpm for 20 minutes at 0˚ C causing it to separate from the cleavage solution. The cleavage solution was decanted off leaving behind the solid peptide. Fresh cold ether was poured over the peptide and was sonicated for 10 minutes.

The peptide was centrifuged and sonicated for a second and third time to ensure purity. Lastly, the peptide was air dried, dissolved in nano pure water, and lyophilized for 12 hours. Lyophilization rendered a white fluffy, powder-like substance. HPLC analysis revealed an MSH-4 peptide with a purity greater than 80% as demonstrated by the area under the curve. LC-MS detected two molecular ion peaks confirming the synthesis of the MSH-4 peptide. The mass spectra displayed a M^{+1} peak of 644, and a M^{+2} peak of 323.

Synthesis of MSH-4 Peptide/Polyethylene Glycol Linker (di-acid) (9)



MSH-4 peptide on resin was swelled in a 50:50 mixture of DCM and DMF for a period of 40 minutes. The solvent mixture was removed by vacuum filtration and the resin was allowed to air dry. A solution of polyethylene glycol linker (diacid) and coupling reagents were prepared in a scintillation vile. Exactly (0.3 mmol, 0.093 g, 3 eqv) of 2□hydroxy□1λ^3, 4, 7, 10, 13, 16□ hexaoxaoctadec□1□yn□18□oic acid, (0.5 mmol, 0.071 g, 5 eqv) of oxyma pure, and (0.5 mmol, 78 µL, 5 eqv) of DIC were added together in a 3-mL solution of DMF and vortexed. The

contents were poured over the peptide on resin. The reaction mixture was placed inside the microwave and reacted at 75˚ C for a period of 14 minutes. Afterwards, the reaction solution was filtered off and the resin was washed three times with 3-mL of DMF. A Kaiser test stained the resin beads yellow, indicating that the free amine was no longer present. The resin beads were finally washed with 10-mL of DCM. Cleavage of the MSH-4 peptide/polyethylene glycol linker (diacid) from the resin was performed. A sample of approximately 200 mg of MSH-4 peptide/polyethylene glycol linker (di-acid) (**9**) on resin was used for the cleavage. A 5-mL cleavage cocktail, consisting of (82.5% TFA, 5% phenol, 5% water, 5% thioanisole, 2.5% 1, 2-ethandithiole) was added to the resin in a syringe. The contents were then mixed on a rocker device for a period of 3.5 hours. After the reaction was complete, the resin beads were filtered off leaving behind the product in solution. Argon gas was bubbled through the solution and most of the TFA was evaporated. Cold ether (10 mL) was added to the solution and the product precipitated out. The product/solution mixture was left in the freezer for 30 minutes and further precipitation occurred. Removal of reagent impurities and by-products were accomplished by centrifugation. The product was centrifuged three separate times at 3200 rpm for a period of 20 minutes at 0˚ C. After each centrifuge, the ether was decanted off and fresh ether was added. The product was sonicated for 10 minutes after the addition of fresh ether. Following centrifugation, the product was air dried and later dissolved in nano pure water. Lastly, the product was lyophilized for 14 hours. This yielded a white powder-like solid. HPLC analysis revealed the major product having a purity greater than 85% as demonstrated by the area under the curve. LC-MS detected a M^{+2} peak of 469, indicating the presence of the desired product.

Synthesis of Melanocyte Stimulating Hormone 4-(PG)/Polyethylene Glycol Linker (di-acid) (10)

Synthesis of MSH 4-(PG) peptide was synthesized in a previous thesis and had been stored at 4˚ C. MSH 4-(PG) peptide on resin was swelled in a 50:50 mixture of DCM and DMF for 1 hour. The solvent mixture was removed by vacuum filtration and the resin was air dried. A solution of polyethylene glycol linker (diacid) and coupling reagents were prepared in a scintillation vile. Exactly (0.3 mmol, 0.093 g, 3 eqv) of 2□hydroxy□1λ³, 4, 7, 10, 13, 16□hexaoxaoctadec□1□yn□18□oic acid, (0.5 mmol, 0.071 g, 5 eqv) of oxyma pure, and (0.5 mmol, 78 µL, 5 eqv) of DIC were added together in a 3-mL solution of DMF and vortexed. The contents were poured over the peptide on resin. The reaction mixture was placed inside the microwave and reacted at 75˚ C for a period of 14 minutes. Afterwards, the reaction solution was filtered off and the resin was washed three times with 3-mL of DMF. A Kaiser test stained the resin beads yellow, indicating that the free amine was no longer present. The resin beads were finally washed with 10-mL of DCM. Cleavage of the MSH-4 peptide/polyethylene glycol linker (di-acid) (**10**) from the resin was performed. A sample of approximately 200 mg of MSH 4-(PG) peptide/polyethylene glycol linker (diacid) on resin was used for the cleavage. A 5-mL cleavage

cocktail, consisting of (82.5% TFA, 5% phenol, 5% water, 5% thioanisole, 2.5% 1, 2-ethandithiole) was added to the resin in a syringe. The contents were then mixed on a rocker device for a period of 3.5 hours. After the reaction was complete, the resin beads were filtered off leaving behind the product in solution. Argon gas was bubbled through the solution and most of the TFA was evaporated. Cold ether (10 mL) was added to the solution and the product precipitated out. The product/solution mixture was left in the freezer for 30 minutes and further precipitation occurred. Removal of reagent impurities and by-products were accomplished by centrifugation. The product was centrifuged three separate times at 3200 rpm for a period of 20 minutes at 0˚ C. After each centrifuge, the ether was decanted off and fresh ether was added. The product was sonicated for 10 minutes after the addition of fresh ether. Following centrifugation, the product was air dried and later dissolved in nano pure water. Lastly, the product was lyophilized for 14 hours. This yielded a white powder-like solid. HPLC analysis revealed the major product having a purity greater than 95% as demonstrated by the area under the curve. LC-MS detected two molecular ion peaks that confirmed the synthesis of the desired product. The mass spectra displayed a M^{+1} peak of 1090, and a M^{+2} peak of 546.

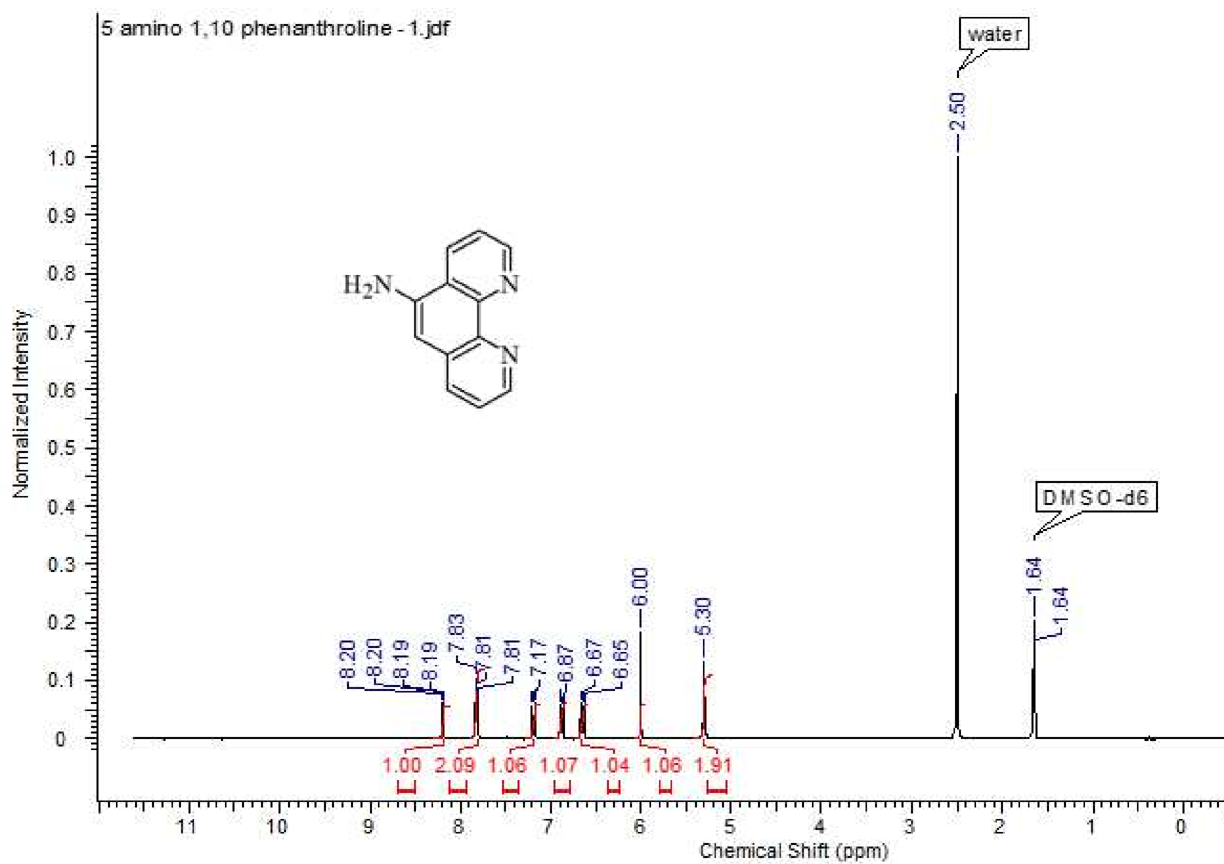


Figure 10. ^{1}H NMR spectra of 5-amino-1, 10-phenanthroline in $CDCl_3$.

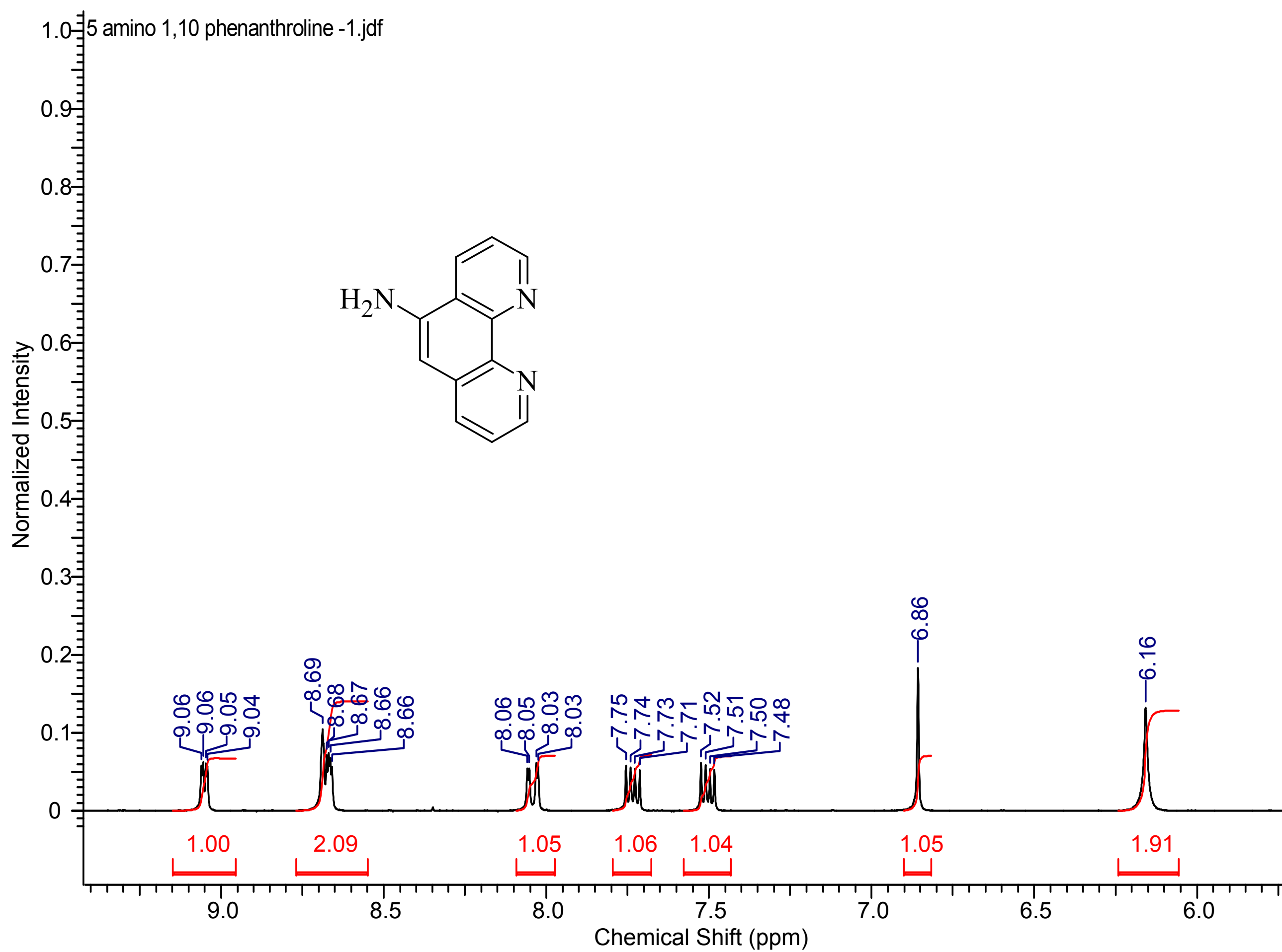


Figure 11. ^{1}H NMR spectra of 5-amino-1, 10 phenanthroline in $CDCl_3$ (zoomed in).

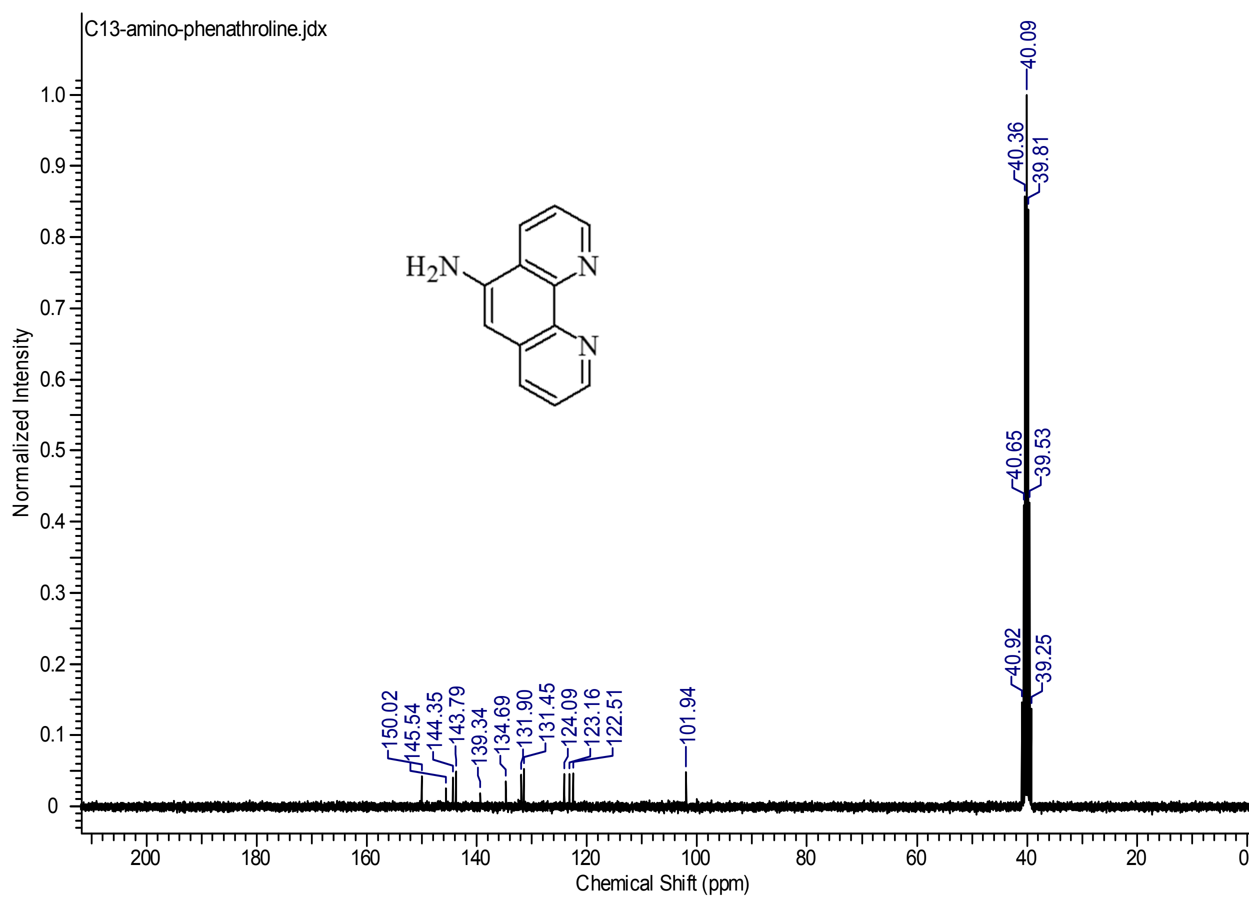


Figure 12. ^{13}C NMR spectra of 5-amino-1, 10-phenanthroline in $CDCl_3$.

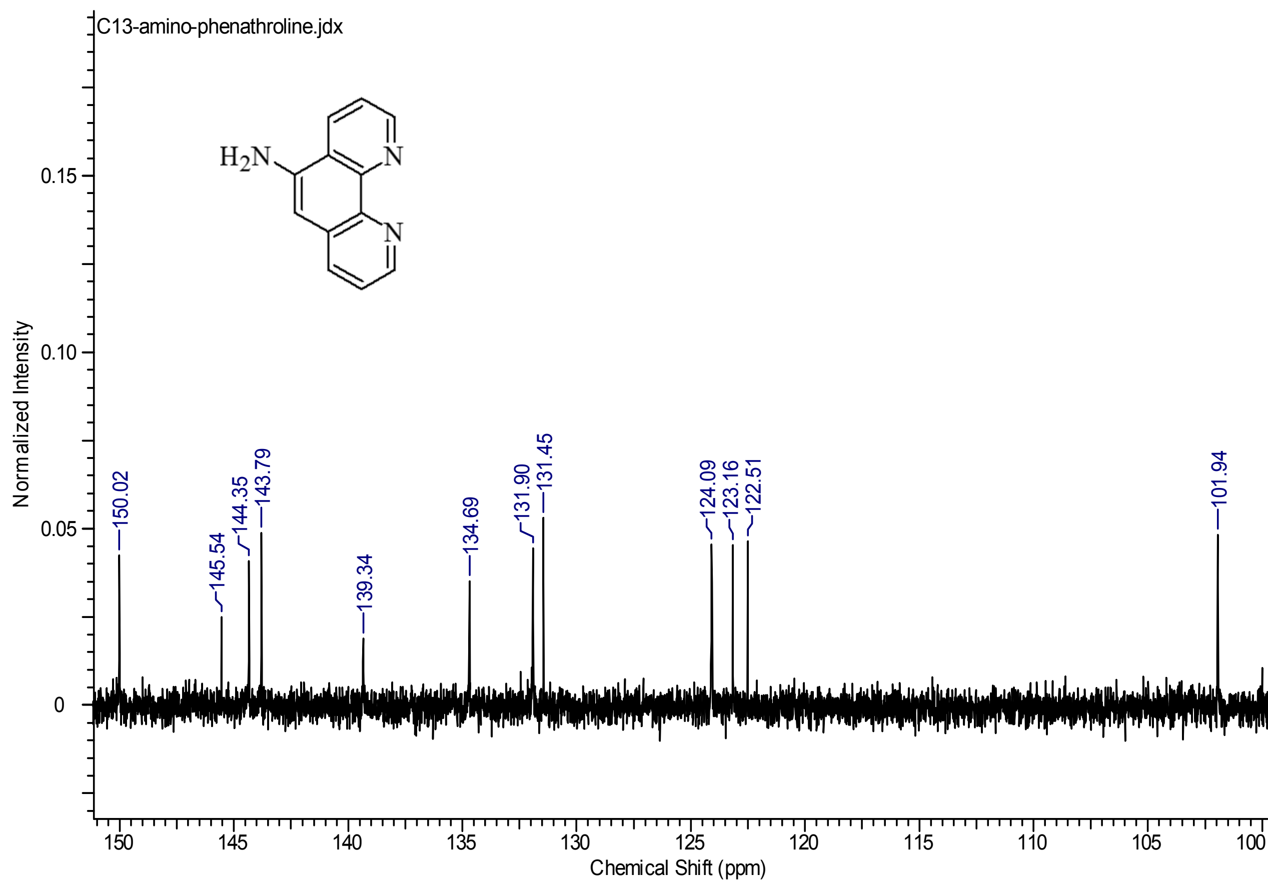


Figure 13. ^{13}C NMR spectra of 5-amino-1,10-phenanthroline in $CDCl_3$ (zoomed in).

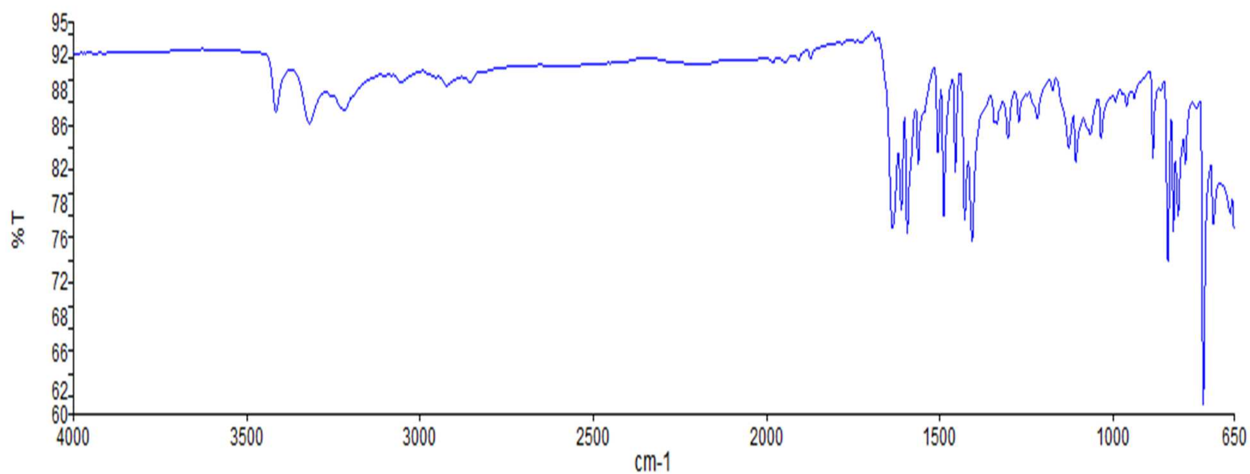


Figure 14. FT-IR spectra of 5 amino-1,10-phenanthroline.

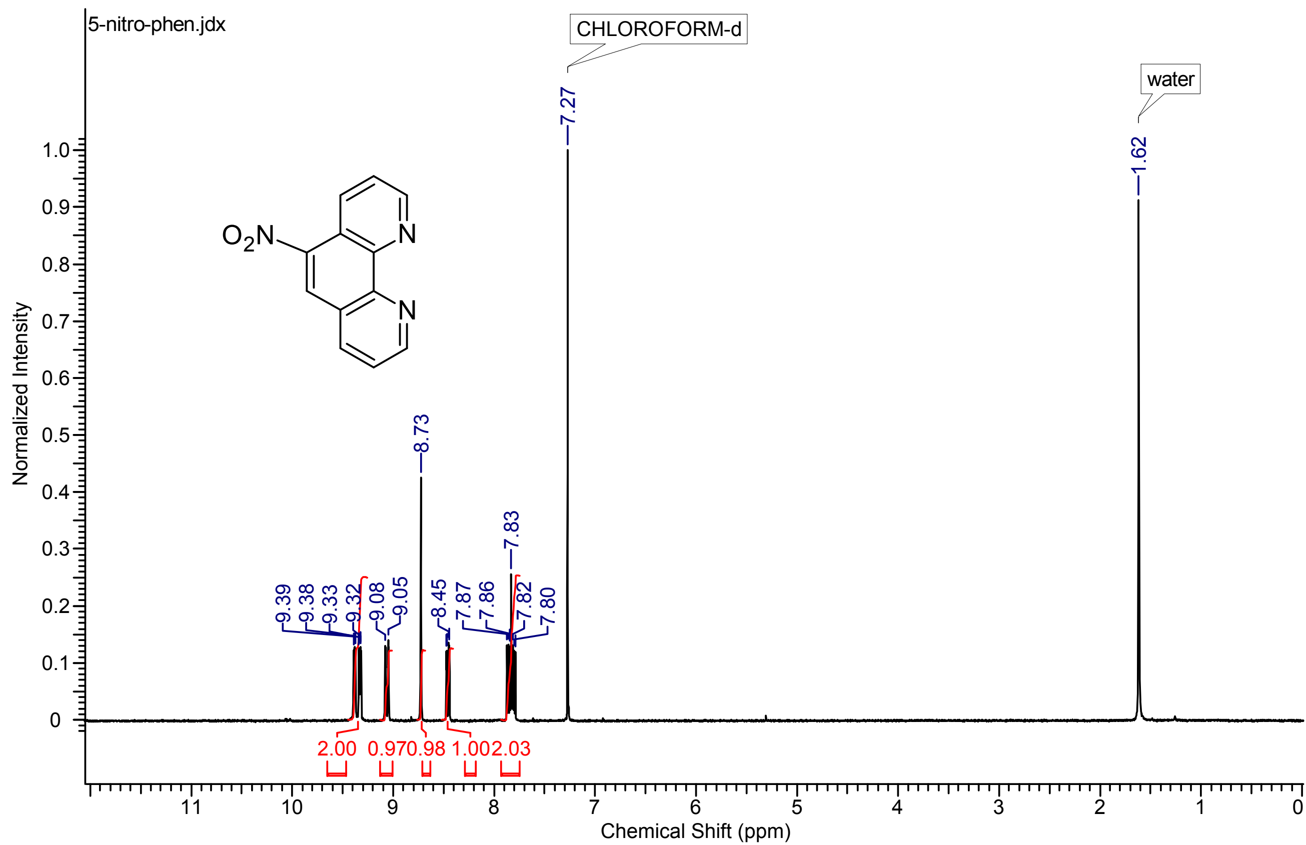


Figure 15. ^{1}H NMR spectra of 5-nitro-1,10-phenanthroline in $CDCl_3$.

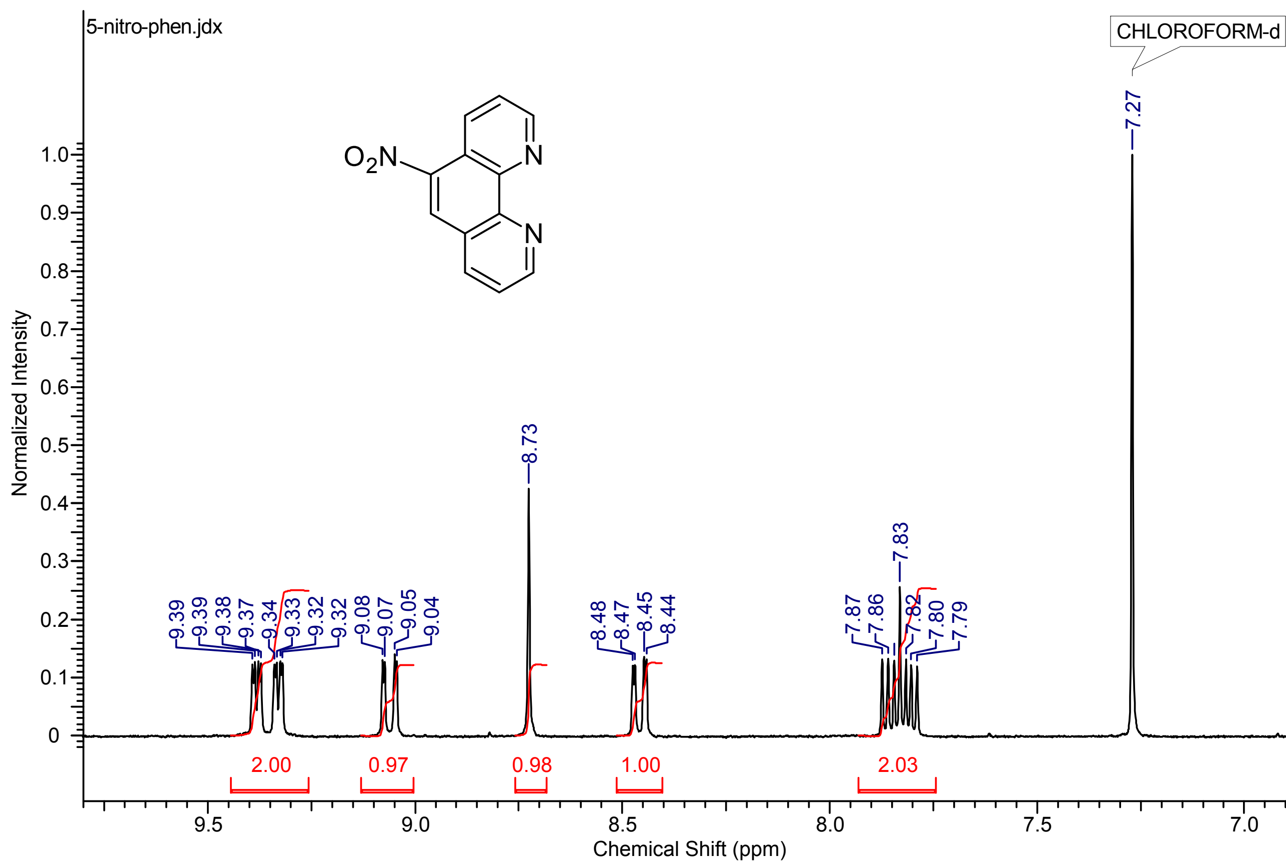


Figure 16. ^{1}H NMR spectra of 5-nitro-1,10-phenanthroline in $CDCl_3$ (zoomed in).

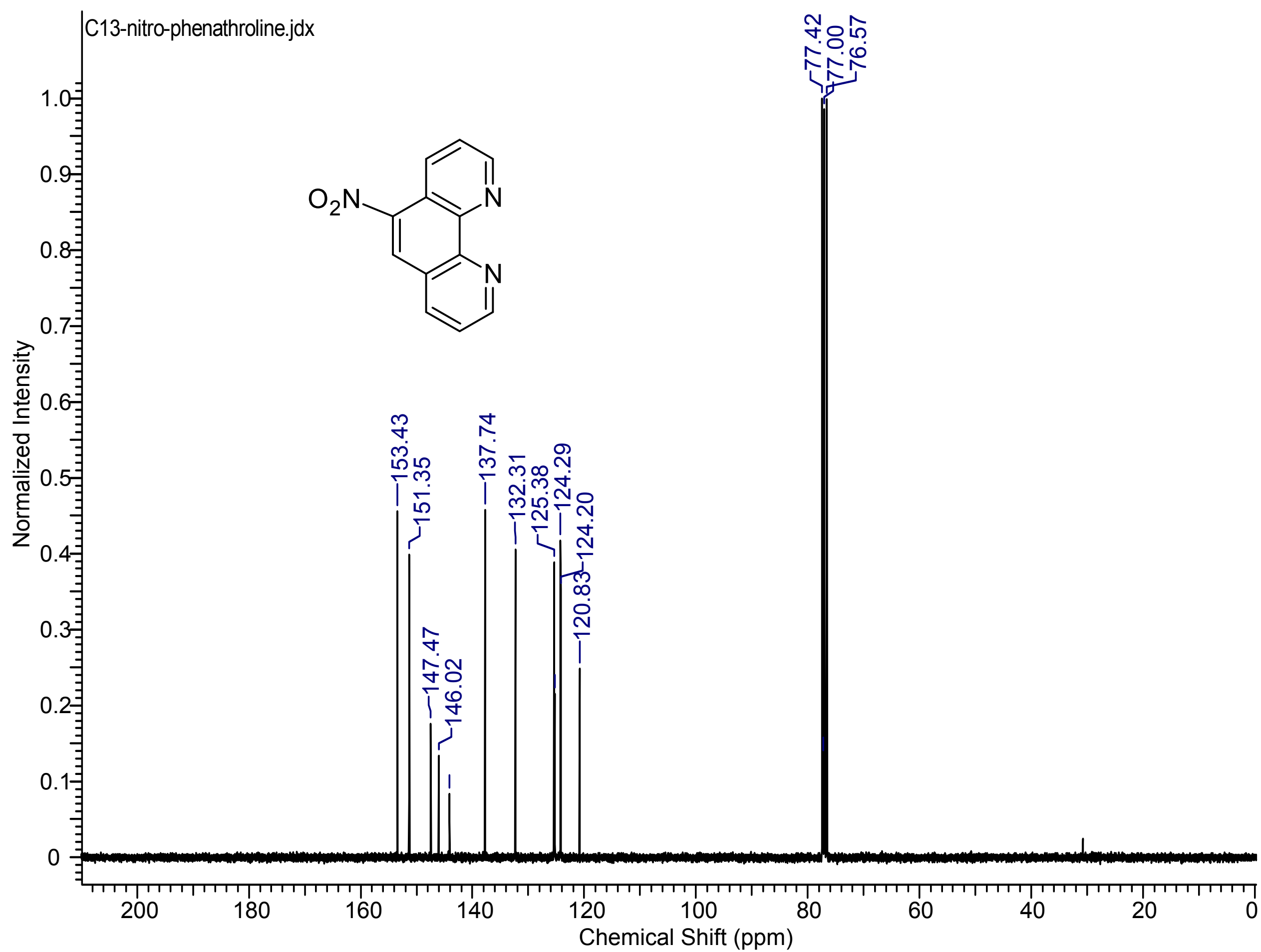


Figure 17. ^{13}C NMR spectra of 5-nitro-1, 10-phenanthroline in $CDCl_3$.

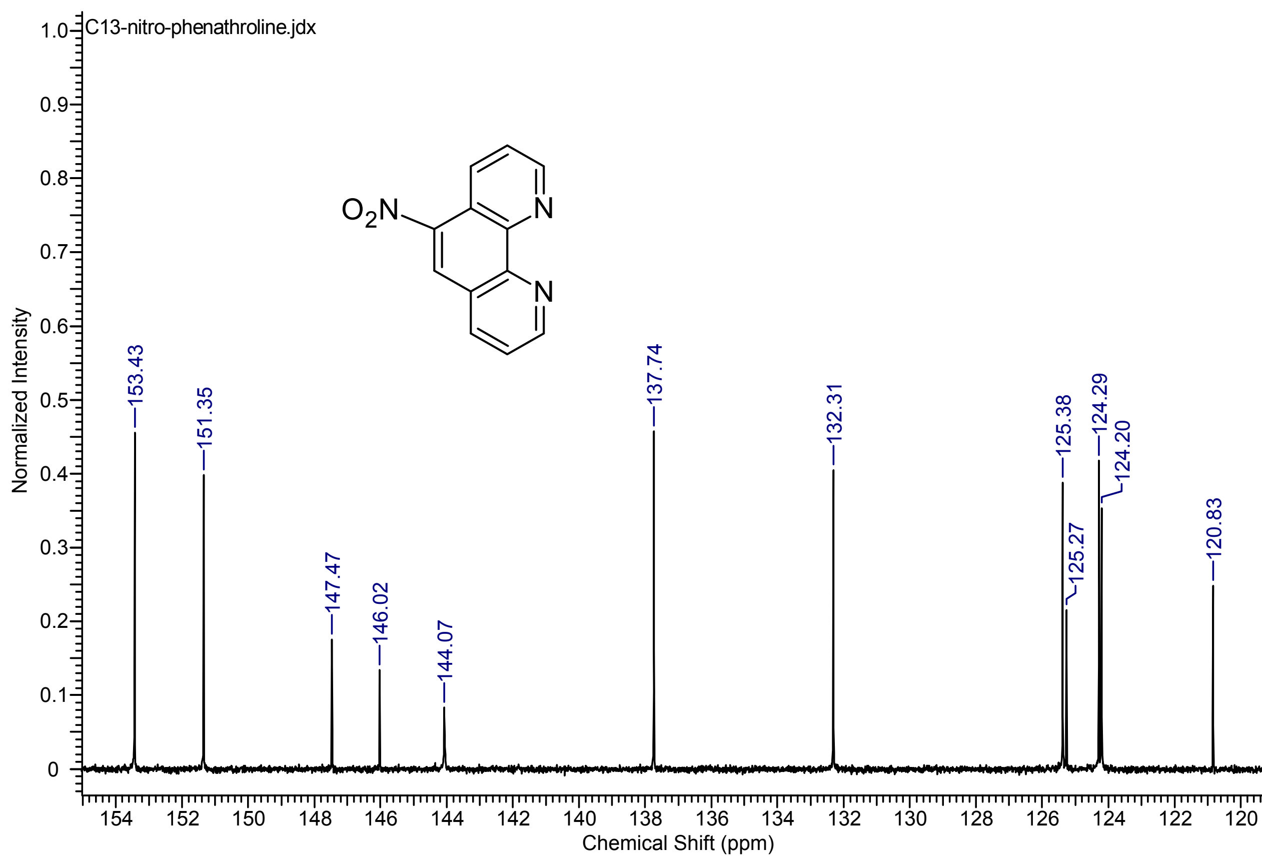


Figure 18. ^{13}C NMR spectra of 5-nitro-1,10-phenanthroline in $CDCl_3$ (zoomed in).

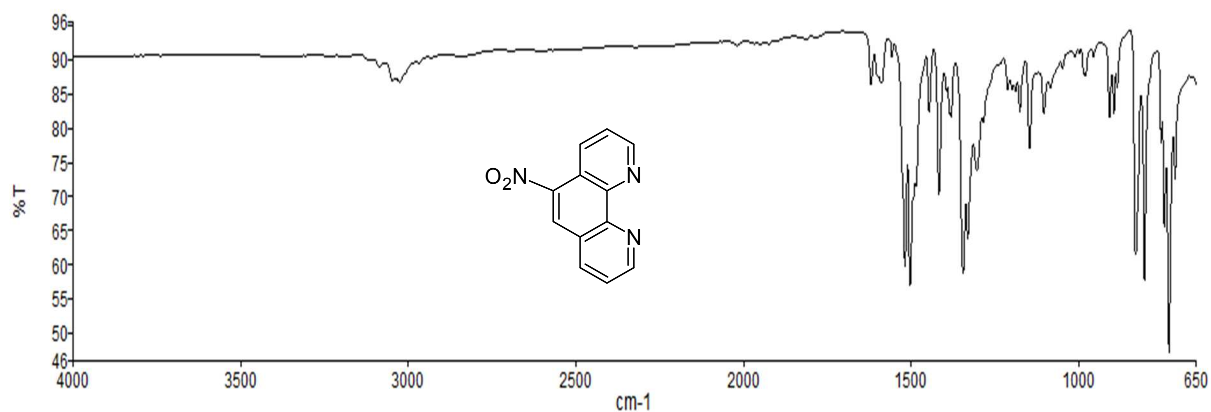


Figure 19. FT-IR spectra of 5-nitro-1,10-phenanthroline.

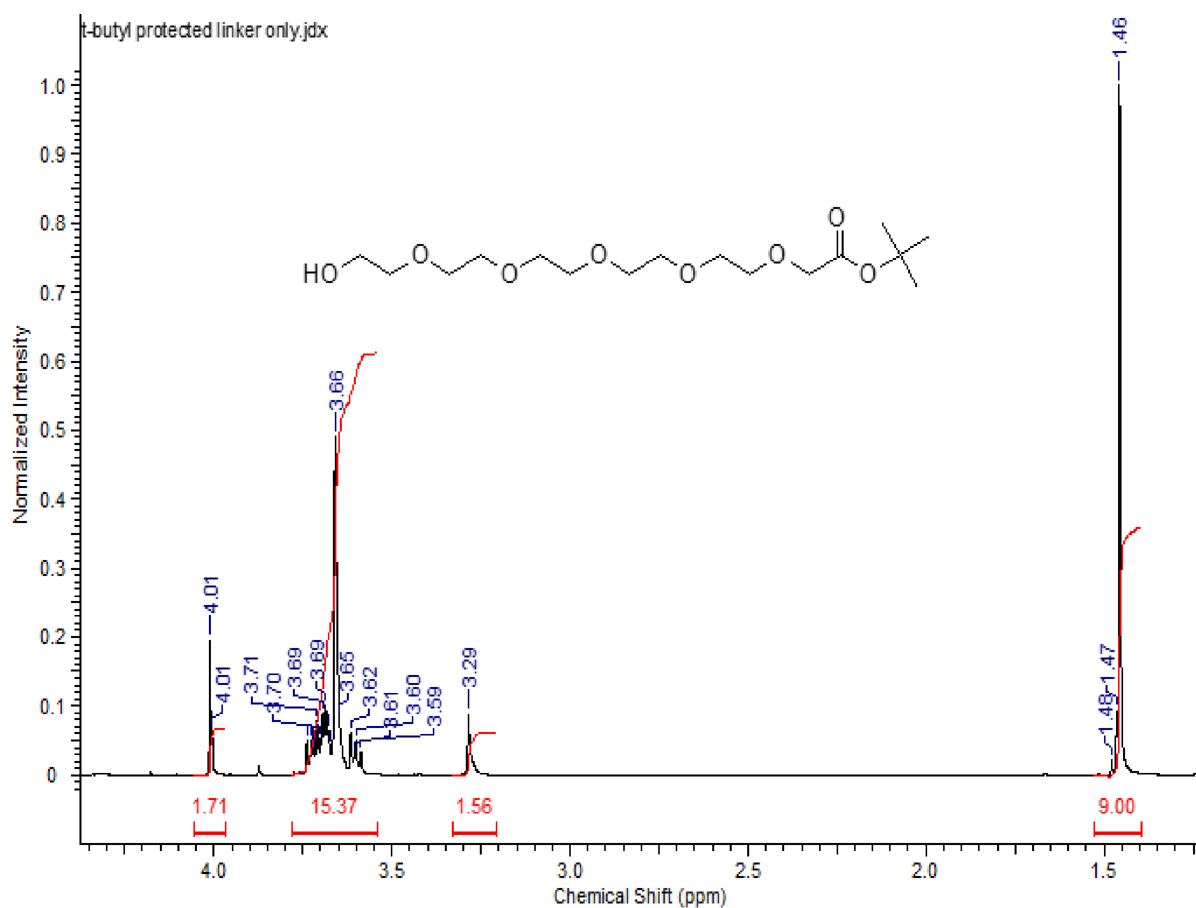


Figure 20. ^{1}H NMR spectra of tert-butyl 15-hydroxy-3, 6, 9, 12-tetraoxa-tetradecanoate in $CDCl_3$.

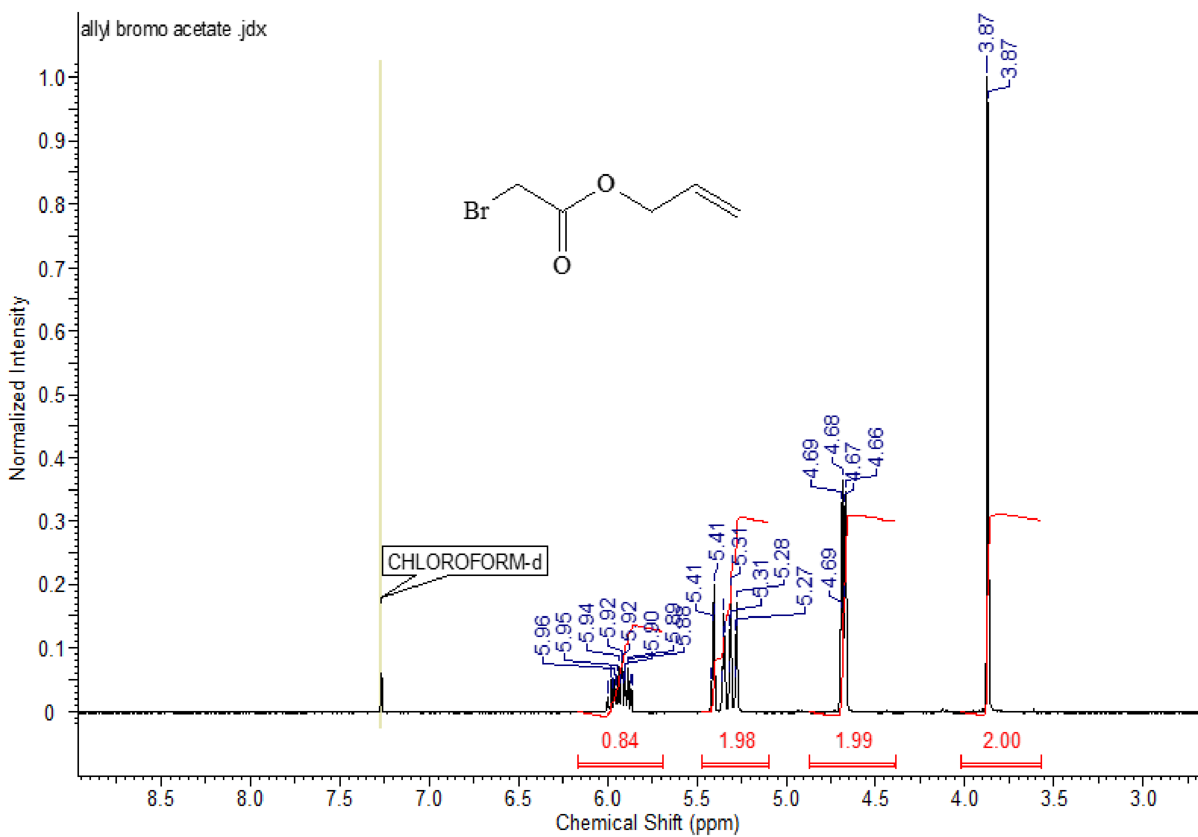


Figure 21. ^{1}H NMR spectra of allyl bromoacetate in $CDCl_3$.

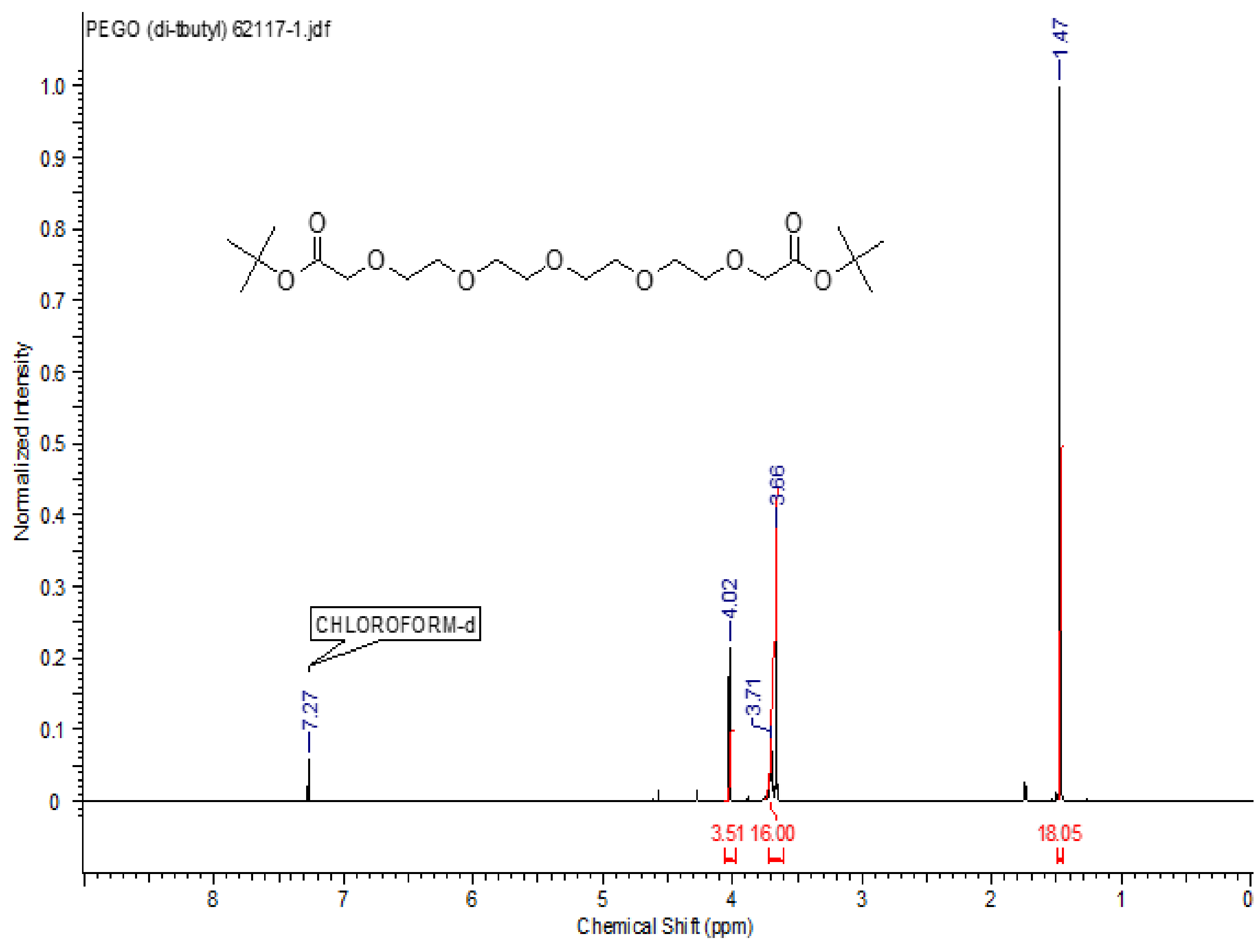


Figure 22. ^{1}H NMR spectra of tert-butyl 17-hydroxy-19,19-dimethyl-3,6,9,12,15,18-hexaoxaicosanoate in $CDCl_3$.

Figure 23. ^{1}H NMR spectra of ^{1}H NMR spectra of tert-butyl 17-hydroxy-19,19-dimethyl-3,6,9,12,15,18-hexaoxaicosanoate in $CDCl_3$ (zoomed in).

Figure 24. ^{13}C NMR spectra of tert-butyl 17-hydroxy-19,19-dimethyl-3,6,9,12,15,18-hexaoxaicosanoate in $CDCl_3$.

Figure 25. ^{13}C NMR spectra of tert-butyl 17-hydroxy-19,19-dimethyl-3,6,9,12,15,18-hexaoxaicosanoate in $CDCl_3$ (zoomed in).

Figure 26. ^{1}H NMR spectra of 2□hydroxy□1λ^{3},4,7,10,13,16□hexaoxaoctadec□1□yn□18□oic acid in $CDCl_3$.

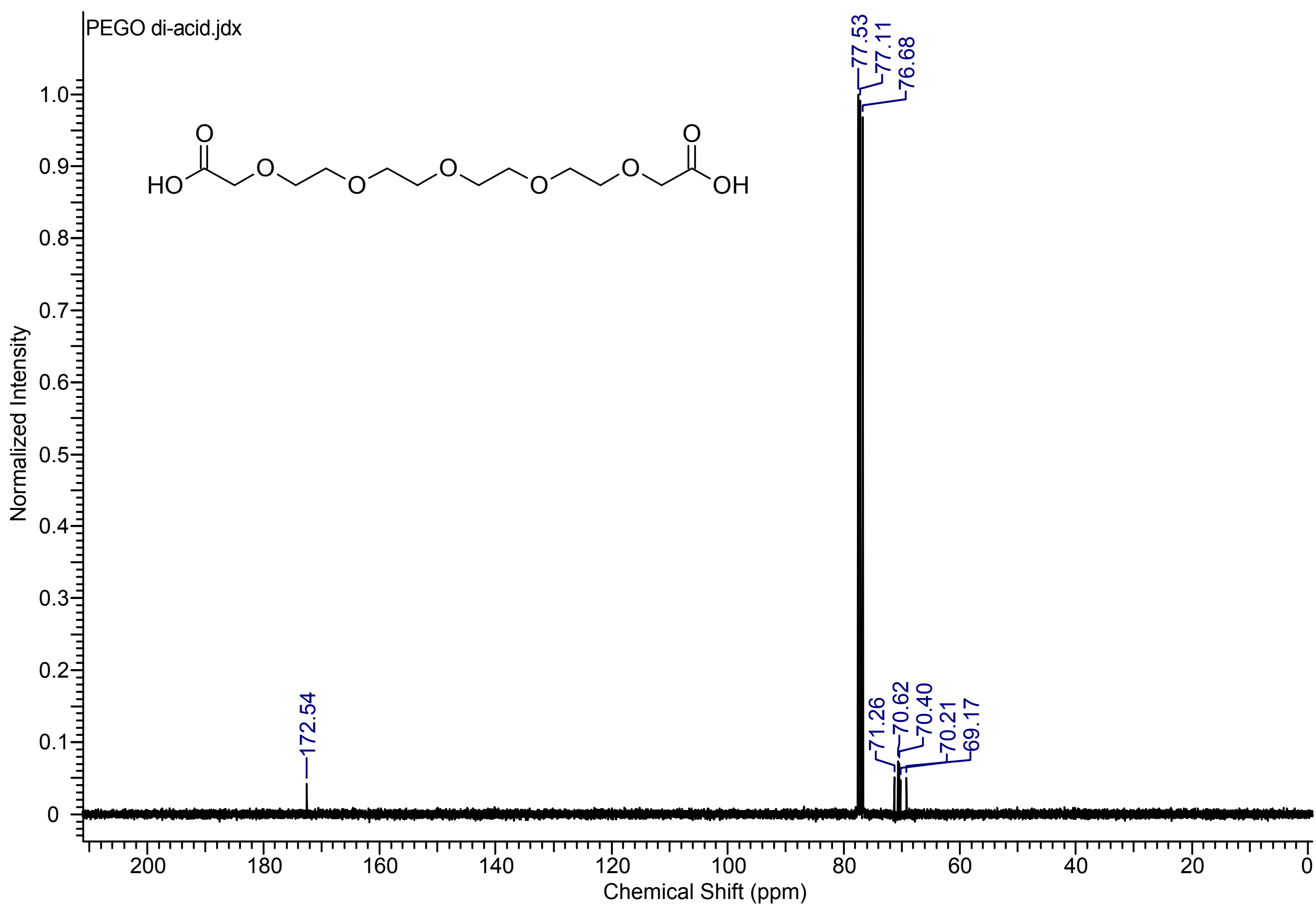


Figure 27. ^{13}C NMR spectra of 2□hydroxy□1λ³,4,7,10,13,16□hexaoxaoctadec□1□yn□18□oic acid in $CDCl_3$.

Figure 28. ^{13}C NMR spectra of 2□hydroxy□1λ³,4,7,10,13,16□hexaoxaoctadec□1□yn□18□oic acid in $CDCl_3$ (zoomed in).

Figure 29. Mass spectra of 2□hydroxy□1λ³,4,7,10,13,16□hexaoxaoctadec□1□yn□18□oic acid.

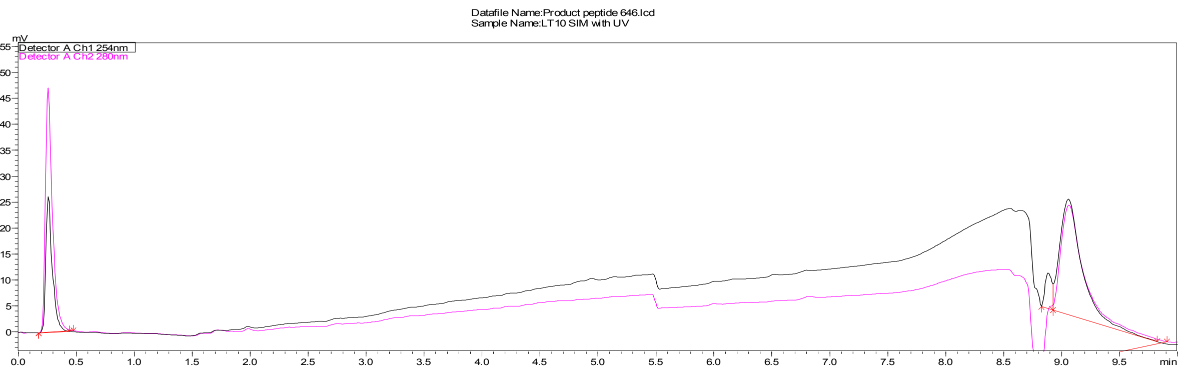


Figure 30. HPLC-UV chromotogram of MSH-4 peptide.

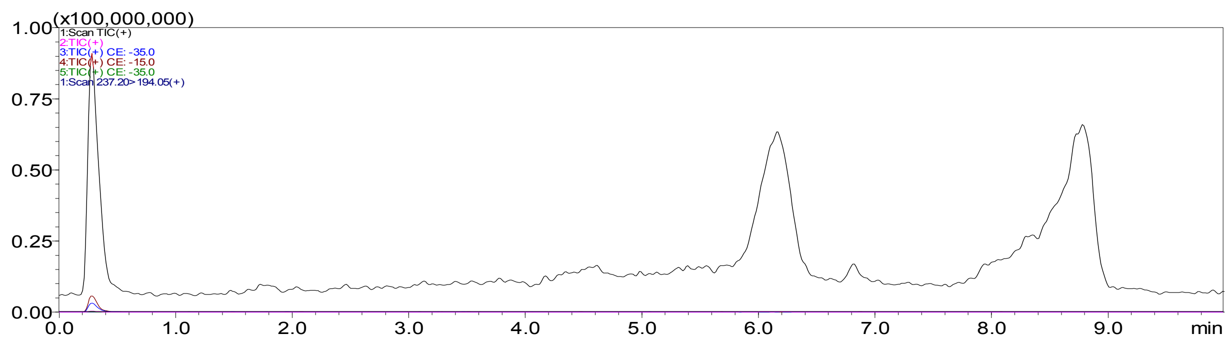


Figure 31. LC-MS chromotogram of MSH-4 peptide.

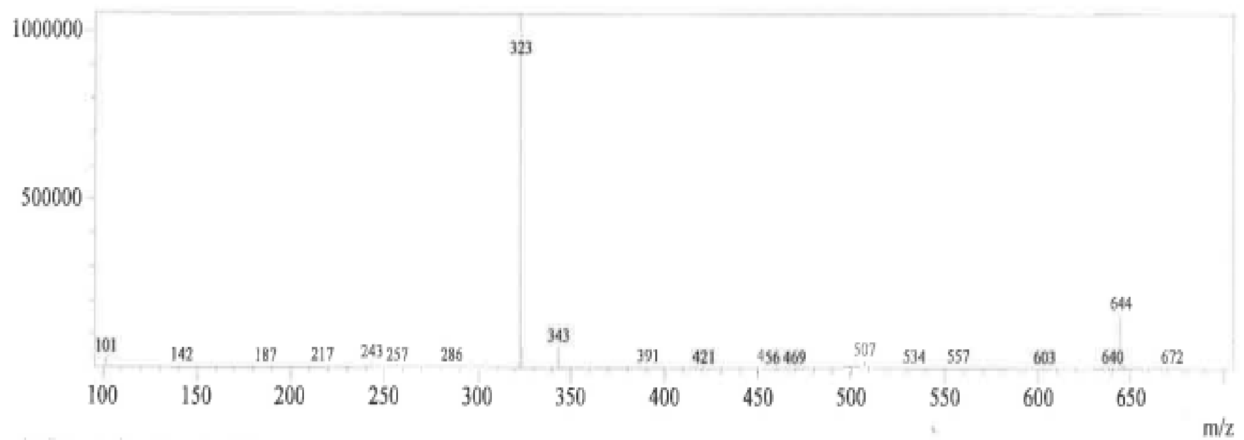


Figure 32. Mass spectra of MSH-4 peptide.

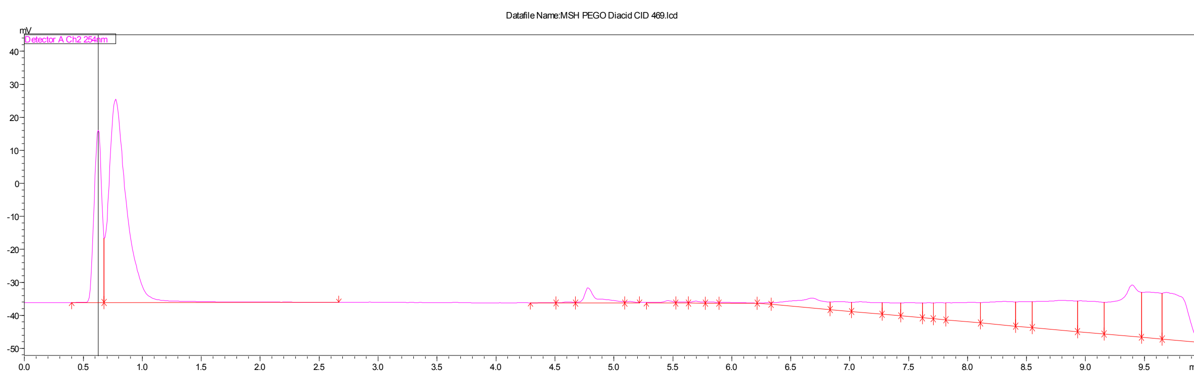


Figure 33. HPLC-UV chromotgram of MSH-4 peptide/PEGO (diacid).

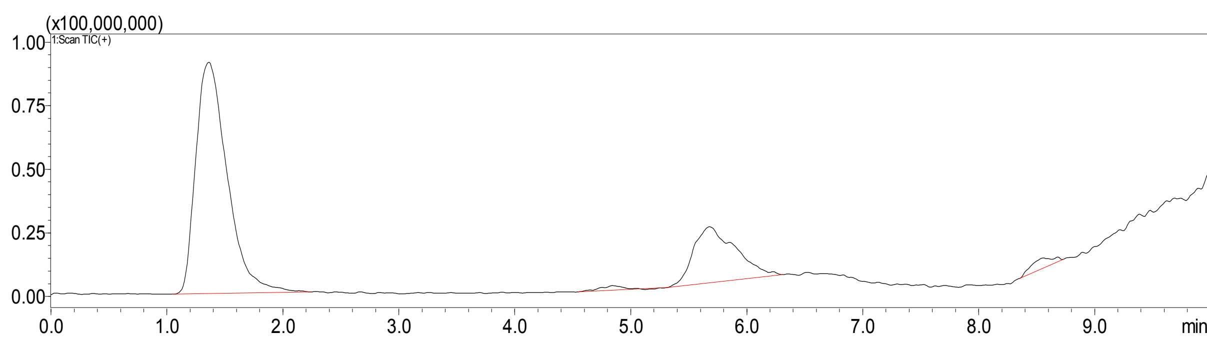


Figure 34. LC-MS chromatogram of MSH-4 peptide/PEGO (diacid).

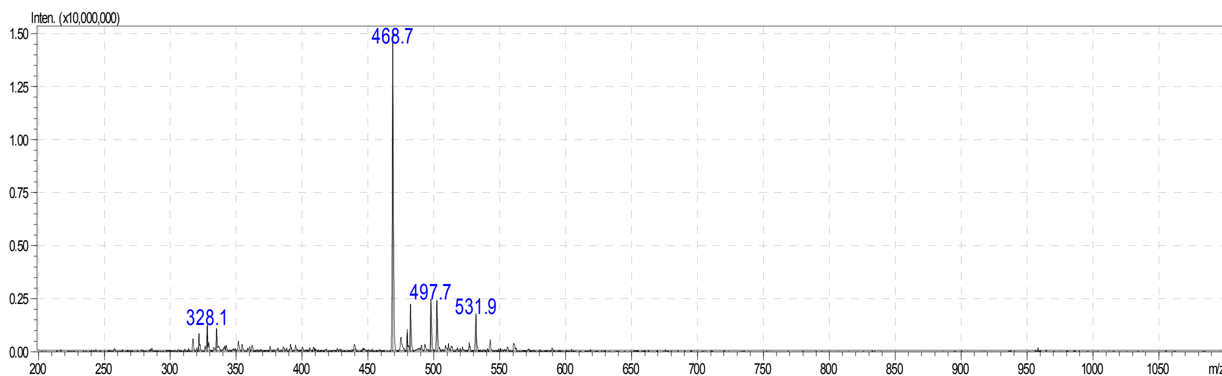


Figure 35. Mass spectra of MSH-4 peptide/PEGO (diacid).

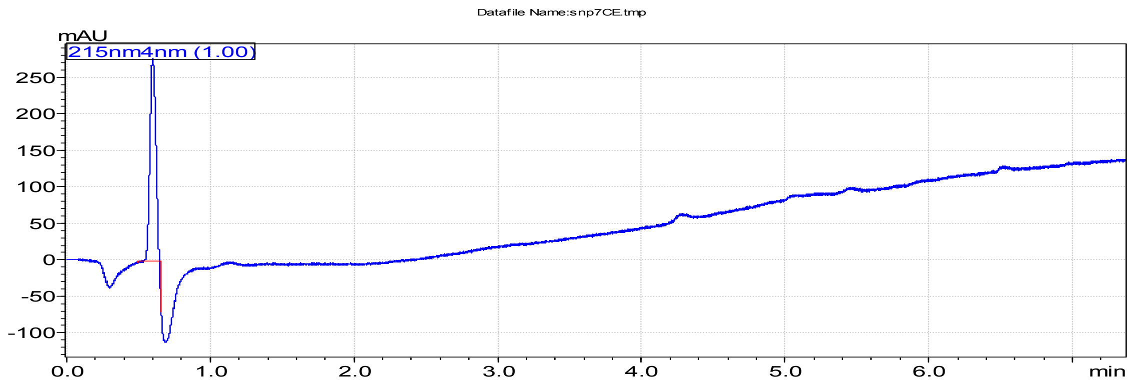


Figure 36. HPLC-UV chromatogram of MSH 4-(PG) peptide/PEGO (diacid).

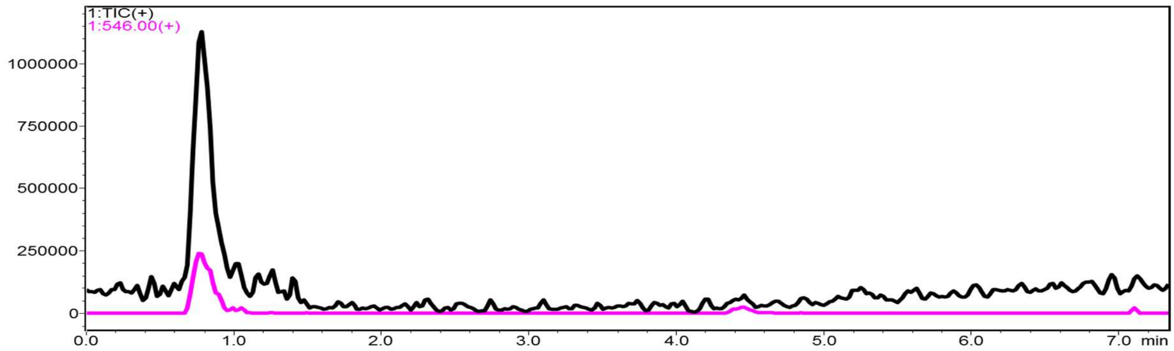


Figure 37. LC-MS chromatogram of MSH 4-(PG) peptide/PEGO (diacid).

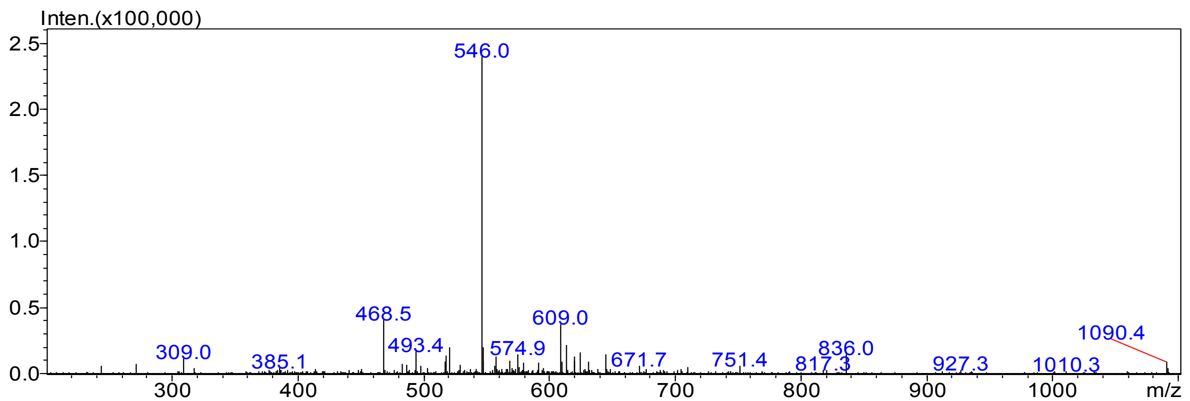


Figure 38. Mass spectra of MSH 4-(PG) peptide/PEGO (diacid).

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