SYNTHESIS OF TIME-RESOLVED FLUORESCENCE PROBES FOR THE FUTURE DETECTION AND IMPROVED STUDY OF 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.

By

Alyssa Marie Frediani

Director: Dr. Brian Dinkelmeyer
Associate Professor of Chemistry
Department of Chemistry and Physics

Committee Members: Dr. Channa De Silva, Dr. William Kwochka, Dr. Rangika Hikkaduwa Koralege

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ABBREVIATIONS

PEG	polyethylene glycol
TTA	thenoyltrifluoroacetone
NMR	nuclear magnetic resonance
GPCR	G-protein coupling receptor
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
FT-IR	Fourier-Transform Infrared
QY	Quantum Yield
BPAH	2-bromo-N-(1,10-phenanthroline-5-yl) acetamide hydrobromide
Bz-4EG	monobenzyl protected polyethylene glycol linker
THF	tetrahydrofuran
DCC	dicyclohexylcarbodiimide
TFA	trifluoroacetic acid
MSH	melanocyte stimulating hormone
FMOC	fluorenylmethyloxycarbonyl chloride
DMF	dimethylformamide
DIC	N,N'-Diisopropylcarbodiimide
DCM	dichloromethane
LC/MS	liquid chromatography coupled mass spectrometry
HNMR	proton NMR
CNMR	carbon NMR
IR	infrared

ABSTRACT

SYNTHESIS OF TIME-RESOLVED FLUORESCENCE PROBES FOR THE FUTURE DETECTION AND IMPROVED STUDY OF CANCER

Alyssa M. Frediani, M.S.
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Director: Dr. Brian Dinkelmeyer

The goal of this project is to develop a diagnostic tool for detecting melanoma cells. The design entails using a polyethylene glycol (PEG) linker to attach a luminescent lanthanide complex to a peptide (MSH-4) that specifically targets G-protein coupling receptors on melanocytes. Lanthanide chelate labels, such as europium thenoyltrifluoroacetone ($Eu(TTA)_3$), have great potential as imaging and detection agents since their luminescent properties allow them to be visualized with high efficiency over the fluorescence of a biological background. The synthesis of each component; linker, ligand, complex and peptide was attempted. The MSH-4 protein was successfully synthesized on a Rink-amide resin using an FMOC protection/deprotecting as evidenced by positive ninhydrin tests after each coupled amino acid. The phenanthroline amine ligand was successfully synthesized as well as its europium complex made. The quantum yield for the $Eu(TTA)_3$-5-amine-1,10-phenanthroline complex was measured and was determined to be sufficient for our purposes. However, difficulties arose in attaching the PEG linker to the phenanthroline amine ligand. The amine functional group was too poor of a nucleophile to react with an O-tosylated PEG linker. This unforeseen challenge necessitated a change in the synthetic strategy. The idea came to add a functional group with a better leaving group. This led to using bromo acetic anhydride to convert the amine to a bromo acetamide. The reaction to couple this anhydride to the phenanthroline amine ligand was deemed successful by using proton and carbon NMR. The quantum yield of the new acetamide-phenanthroline ligand had reduced quantum yield as compared to the

originally proposed ligand. This was investigated and concluded that the weaker electron-donating ability of the amide in this compound that is correlated to the carbonyl, negatively impacted the fluorescence of the europium complex. Future work will investigate coupling the monosubstituted PEG linker with the ligand by nucleophilic substitution of the bromine.

CHAPTER 1: INTRODUCTION

1.1 Background

Melanoma cancer rates have seen an overall increase over the past 20 years; however, melanoma cancer has the one of the lowest mortality rates amongst all forms of cancer, being only 1.5%. For the year of 2019, it is projected that 99,000 new cases of melanoma will affect the Unite States population. With that projection, 13,000 cases will assume to be fatal. [1] Melanoma cancer can mutate anywhere on the body that there are melanocytes. This includes skin, eyes, and even fingernails. Melanoma tumors can appear as moles, abrasions, or sores on the external organs of the body. If untreated, melanoma tumors will grow large enough to penetrate beneath the dermis, approximately 1mm into the skin, and to the blood vessels at which point it is called metastatic melanoma.[2] Metastatic melanoma releases cells into the body that can spread into lymph nodes and various organs causing the start and spread of more fatal cancer cells throughout the person affected. If caught early, melanoma, or suspected lesions, can be removed by minor surgical procedures for biopsy.[3] Early detection is critical in lowering the fatality rate for patients with melanoma cancers. A common method of biopsy is to observe the cells under a fluorescent microscope to further inspect abnormalities microscopically. In this technique, the sample is first stained with a fluorescent dye. This detection of diseased cells can be difficult because current dyes and biological cell samples, both fluoresce at short wavelengths making it difficult to differentiate between abnormal and normal cells. An improved detection method would use a dye that fluoresces at a vastly different wavelength from the cell background and would specifically bind to melanoma cells.

The most commonly targeted signaling pathway in eukaryotic cells involves G protein coupled receptors (Figure 1). In fast growing melanocytes, MSH-G protein coupled receptors (GPCR) are overexpressed on their cell surfaces. A high concentration of GPCR can be linked to the rapid expansion of tumor growth.[4] Easy detection of these protein coupled receptors would improve the chances for early detection of melanoma cancer. This receptor acts as the inbox to the cell allowing it to translate and send

messages. The GPCR can be stimulated by the introduction of an agonist which can be in the form of light, proteins, sugars, lipids and peptides.

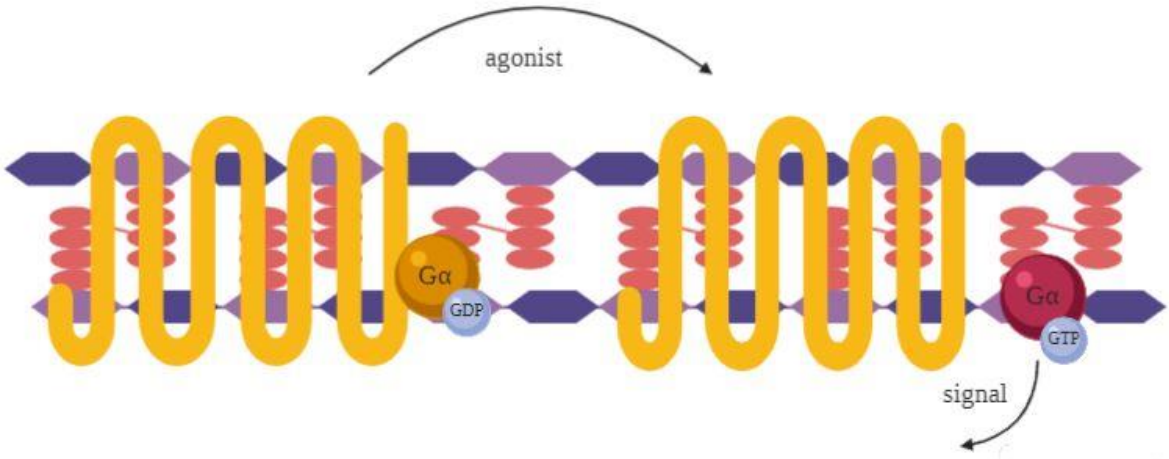


Figure 1: G-protein coupling receptor stimulated by a signaling molecule (agonist).

The prospective probe includes linking a GPCR targeting peptide, melanocyte stimulating hormone (MSH-4), to a chelated lanthanide ligand. This is expected to enhance the fluorescence of melanocytes against normal biological sample background fluorescence by fluorescing at much longer wavelengths while absorbing at shorter wavelengths. The attachment of the highly luminescent lanthanide to MSH-4 will produce a fluorescent dye with high specificity for melanoma cancer cells.

1.2 Project Goal

The overall goal of this project is to develop a diagnostic tool for detecting melanoma cells. The design from left to right, displayed in Figure 2, entails attaching a luminescent lanthanide chelate to a polyethylene glycol (PEG) linker attached to a peptide, MSH-4, that targets and binds to a G-protein coupling receptor found on the surface of and excreted by melanoma cancer cells. The success of this proposed structure relies heavily on making sure the chelated lanthanide ligand does not interfere with the MSH-4 peptide. The interference could hinder the probe's ability to attach properly to the GPCR. This would then interfere with the lanthanides ability to fluoresce properly. Since the peptide is specifically

designed for the GPCR of melanocytes, the probe will not be able to attach to any other GPCRs in any residual biological cells.

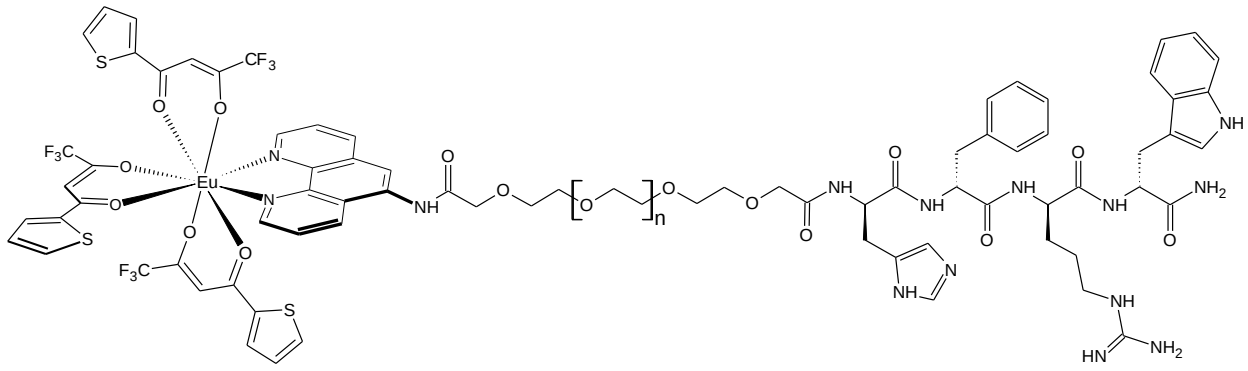


Figure 2: Design of Fluorescent Melanoma Probe

The design of this compound is related to a series of techniques called bioconjugation. Bioconjugation allows bonds between a biological molecule and a synthesized molecule to form.[6] Considering that the molecule being proposed needs an amalgamation of synthetic and biologic properties, this will make it a more useful and successful system for site specified conjugation. The method for bioconjugation of the desired compound includes MSH-4 that is conjugated to a lanthanide chelated ligand, by separation of a PEG linker.

PEGylation has been making large advancements in the field of anti-cancer therapy.[7] The PEG units are used to separate bioactive sites from a cell targeting drug, or in relation to this project design, a luminescent lanthanide chelate label that would be used for imaging. Polyethylene glycol has some of the most desired properties for pharmaceuticals. These properties include being nontoxic, non-immunogenic, and hydroscopic.[7] These properties associated with PEGylation allow for biopharmaceuticals to change their physical and chemical properties. This aids in cell interpretation of the molecule, so that the PEG linker can enhance the retention time that the drug remains in the targeted cell as well as protects them from rapid degradation.[8] The PEG linker also acts as a separation unit between the MSH-4 peptide and

the lanthanide chelated ligand to prevent steric hinderance. For this project a monosubstituted benzyl-PEG will be used for the bioconjugation of the proposed molecule.

Lanthanide complexes are used in a wide array of bioimaging applications. These complexes have desirable properties that aid in their success and reliance. The properties include narrow emission bands and long luminescence lifetimes[9]. The lanthanide being used for this project is Eu^{3+}. The narrow emission bands stem from the 4f orbitals of the lanthanide (III) ions being shielded by the full 5s and 5p sub-shells. The 4f-4f electronic transitions of the europium ions are forbidden by the electric dipole selection rules.[9] These rules state that the transition is forbidden because the initial and final state of the orbital symmetries are the same. These forbidden transitions of direct excitation of f-f electrons are an inefficient luminescent characteristic of europium ions.[9] Because of this difficulty of direct excitation of the lanthanide (III) ion and performing an f-f transition is rare, an organic chromophore can be utilized to aid in the transfer of energy to the lanthanide (III) ion, Figure 4. To enable the transfer of energy, light energy is passed onto lanthanide (III) ion from the organic ligand. The ligands are able to absorb light and transfer the energy from the light to the lanthanide. This is known as the antenna effect shown in Figure 3.

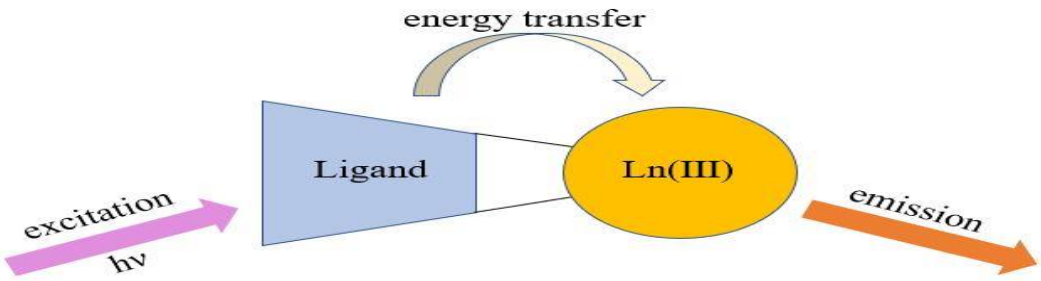


Figure 3: Illustration of the antenna effect of an organic ligand chelated to a lanthanide (III) ion.

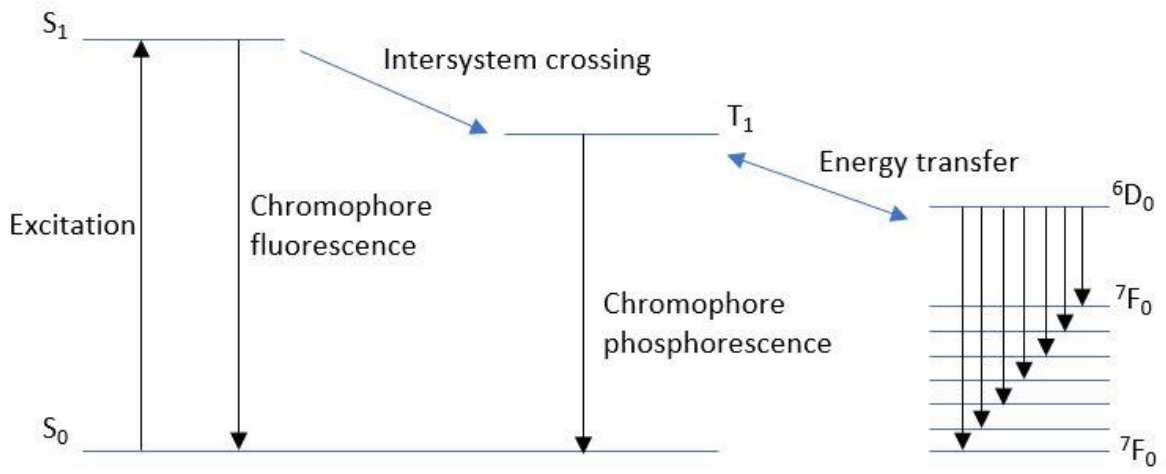


Figure 4: Jablonski diagram showing the energy transfer from the excited state of the ligand to the excited state of the europium ion and to the ground state via luminescence.

Europium (III) complexes that are paired with beneficial organic ligands have several advantages over other lanthanide (III) complexes as bio-imaging agents. One of the advantageous characteristics of Eu^{3+} complexes is that they are sensitive to longer wavelengths of light (visible range) which has fewer negative effects on biological analytes such as cells and tissues, than UV light excitation. Also, Eu^{3+} complexes have narrow emission bands that emit reddish orange light which has minimal interference with the luminescence emission of biological samples.[10] In addition, Eu^{3+}complexes have large Stokes shifts, long luminescent lifetimes, and high luminescence quantum yields.[9] These characteristics make Eu^{3+} complexes extremely useful agents to utilize organic chromophores for biomedical imaging applications.

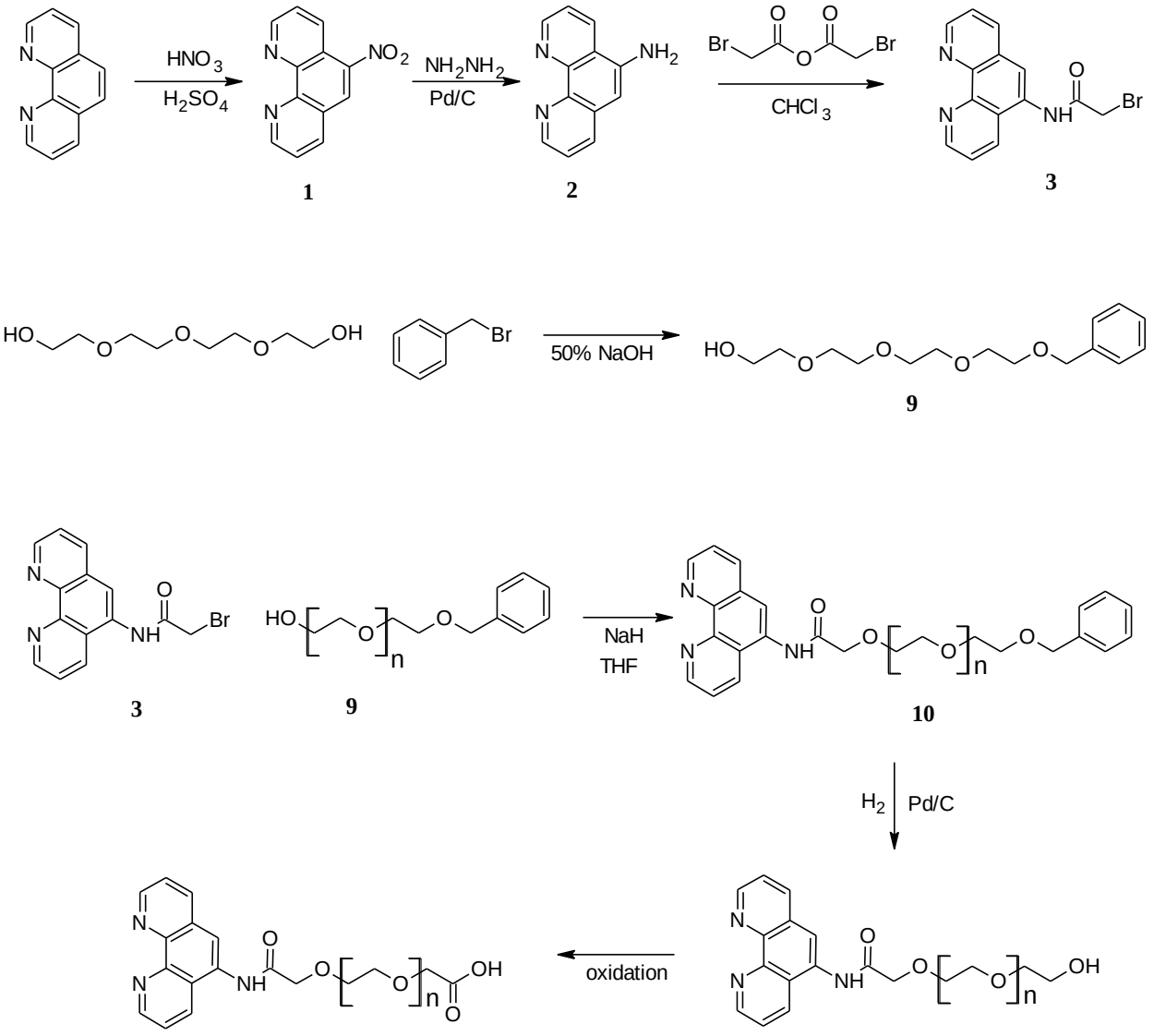
The MSH-4 peptide is a specially designed chain of amino acids to target the GPCR of melanocytes. Therapeutic peptides have made several advancements in cancer detection and treatment. The advantages of using therapeutic peptides include low toxicity, higher target specificity and selectivity, and they are

easy to synthesize[12]. For the synthesis of the MSH-4 peptide, solid phase peptide synthesis will be utilized.

Solid phase peptide synthesis is a highly successful technique which is used for large biomolecules that include peptides and proteins. Some of the advantages to this technique include, higher yields, faster and more efficient reactions, and minimal purification[12]. The higher yields are common because the amino acids and coupling reagents can be used in extreme excess. Excess and unreacted reagents are easily removed by filtration, allowing for purification of the peptide to be easy and efficient. The overall process of solid phase peptide synthesis comprises a step by step construction of amino acids on an insoluble polymer support resin. This is done by coupling the amine group of one amino acid to the carboxylic acid functional group of the next amino acid to form an amid bond.

1.3 Synthetic plan

The project requires synthesizing a PEGylated-ligand that can be easily coupled to an MSH-4 peptide. The MSH-4 peptide will be synthesized using microwave assisted solid-state peptide synthesis. 1,10-phenthroline ligand was selected since it is known to make stable highly fluorescent complexes with europium (III) thenoyltrifluoroacetone. The synthetic strategy is shown in Scheme 1. Attaching the ethylene glycol to the ligand will require adding an amine group by first nitration of phenanthroline followed by reduction to an amine. This resulting 5-amino-1,10-phenthroline **2** can then be converted to a Bromo acetamide derivative **3** which can then be reacted with a benzyl protected tetra ethylene glycol **9** to produce compound **10**. Deprotection and oxidation of **10** will produce a PEGylated-ligand that can be attached directly to the MSH-4 peptide while it is still attached to the resin.



Scheme 1: Synthetic plan of overall project

CHAPTER 2: EXPERIMENTALS

All starting materials were purchased by Western Carolina University Department of Chemistry and Physics from Fisher Scientific, Acros Organics, and Alfa Aesar and used without further purification. Chloroform and dichloromethane were dried before use by sitting for at least 48 hours over 3Å sieves. Spectral Data were collected by means of nuclear magnetic resonance (NMR), fourier-transform infrared spectroscopy (FT-IR), UV-Vis, and fluorescence. All instruments used to obtain data were located at Western Carolina University.

2.1 Instrumentation

2.1.1 Nuclear Magnetic Resonance Instrumentation

The instrument used for NMR spectroscopy is a JEOL 300 MHz Eclipse NMR. This instrument utilizes a 5mm tunable probe to detect ^{1}H, ^{13}C, ^{19}F, and ^{31}P. For the premise of this project, only ^{1}H and ^{13}C were measured using this instrument. The measured samples for ^{1}H NMR were prepared by dissolving approximately 10-20 mg of analyte in deuterated chloroform or deuterated dimethyl sulfoxide and set for a minimum of 8 scans to a maximum of 24 scans to gain more precise measurements. For ^{13}C NMR the samples were dissolved in deuterated solvents until a readable saturation level was achieved and set for a maximum of 2400 scans.

2.1.2 Fourier-Transform Infrared Instrumentation

The instrument used for FT-IR spectroscopy is a Perkin-Elmer Spectrum One. This instrument analyzes materials using single bounce attenuated total reflectance (ATR) infrared technology, with measurements collected in the range of 4000 cm^{-1} to 600 cm^{-1}. All analytes were measured at room temperature with a background measurement collected before each measurement.

2.1.3 Ultraviolet-Visible Instrumentation

The instrument used for UV-Vis spectroscopy is an Agilent 8453 Diode Array Spectrometer. This instrument utilized both tungsten and deuterium lamps to be able to take measurements from 190 nm to 1100 nm. This spectrometer has a slit width of 1 nm to ensure that a 1nm gradient of light is shown at the sample for each measurement. The samples were prepared by dissolving the sample in dichloromethane or methanol and transferred to a quartz cuvette with a 1.0 cm path length. The samples were diluted accordingly until the desired absorption level was achieved. The measurements were made at room temperature with a blank cuvette filled with the respective solvent measured first to subtract background noise from the spectra.

2.1.4 Fluorescence Instrumentation

The instrument used for fluorescence spectroscopy was a Perkin-Elmer LS-55 Fluorometer. This instrument uses a xenon pulse lamp and highly sensitive, holographic gratings to ensure the spectra gathered is accurate and reproducible. This spectrometer has a slit width of 0.1 nm to keep stray light interference as minimal as possible. The sample is prepared in a quartz cuvette with a 1.0 cm path length using the same sample preparation in section 2.1.3. The previously measured UV-Vis absorption wavelength of each sample was used to excite the sample to achieve its fluorescence emission. All measurements were made at room temperature.

2.1.5 Quantum Yield Measurements

The UV-Vis and fluorescent measurements were used to calculate the luminescent quantum yield according to equation 1. The quantum yield is used to measure the fluorescence efficiency of the europium complexes synthesized in this study. Quantum yield is simply the ratio of the number photons emitted to the number of photons absorbed.

Equation 1

$$\phi_S = \phi_R \frac{Abs_R}{Abs_S} \frac{A_S}{A_R} \frac{n_S^2}{n_R^2}$$

Where Abs is the absorbance, A is the area under the luminescence curve, and n is the refractive index of the solvent. The subscripts R and S are representative of reference sample and sample respectively. Phi (φ) is representative of quantum yield itself. The reference sample that was used was Cresyl violet, with a known quantum yield of 0.54, or 54% using methanol as the solvent.

2.2 Synthetic procedures

2.2.1 Europium thenoyltrifluoroacetone water complex, $(Eu(TTA)_3(H_2O)_2)$ [4].[17]

NaOH pellets (3.36 mmol, 0.1467 g, 3 eq) was dissolved in 20 mL of water and a magnetic stir bar was added. Thenoyltrifluoroacetone (tta) (3.41 mmol, 0.7622 g, 3 eq) was added to the NaOH solution and allowed to stir at room temperature for 10 minutes. A second solution consisting of $EuCl_3 \cdot 6H_2O$ (1.13, 0.4193 g, 1 eq) dissolved in 12 mL of water and allowed to a stir at room temperature. The NaOH/tta solution was then added dropwise to the europium solution. A white/yellow precipitate immediately formed. Once all of the solution had been added, a septum was placed on the round bottom flask and the solution stirred under argon for 3 hours at 60˚C. The cream-colored $Eu(TTA)_3(H_2O)_2$ precipitate was isolated by vacuum filtration and with rinsed with 100 mL of water twice and with 3 mL of hexanes once. The complex as characterized using UV-Vis and Fluorescence spectroscopy in methylene chloride and was used without further purification. Yield: 0.7846 g (83%) UV (CH_2Cl_2) λ_{max}, 340 nm. Fluorescence (CH_2Cl_2) $\lambda_{excitation}$, 340 nm, $\lambda_{emission}$, 615 nm

2.2.2 5-nitro-1,10-phenanthroline ligand, (nitro-phenanthroline) [1].[14]

1,10-phenanthroline (5.65 mmol, 1.0185 g, 1 eq) was added to a 25 mL round bottom flask with a magnetic stir bar. Concentrated sulfuric acid (126.03 mmol, 6.0 mL, 3 eq) was added slowly to the round

bottom flask. The reaction vessel was fitted with a reflux condenser and fuming nitric acid (112.56 mmol, 3.0 mL, 6 eq) was carefully added dropwise into the round bottom flask. A reddish-brown gas was produced while the nitric acid was being added. The solution stirred at room temperature for 15 minutes. The round bottom flask was then placed into a paraffin oil bath and refluxed at 160˚C for 3 hours. The reaction mixture was allowed to cool to room temperature and poured over ice in a 150 mL Erlenmeyer flask. Once the ice had melted, the pH of the solution was carefully adjusted to a pH of 3 to induce precipitation using saturated NaOH solution. Product yield was negatively affected if the pH was adjusted higher than 3. The yellow solid was collected by vacuum filtration and was washed with water and allowed to air dry overnight. Yield= 0.9670 g (76%). ^{1}H-NMR 300MHz ($CDCl_3$): δ 9.3 (2H mult.), δ 8.99 (1H, dd), δ 8.64 (1H, s), δ 8.41 (1H, dd), δ 7.79 (2H, mult.). C^{13} NMR($CDCl_3$): 156.432, 153.655, 151.563, 147.654, 146.207, 144.270, 137.994, 132.558, 125.634, 124.521, 121.049 ppm. IR (ATR): 3023, 1505, 1349, 1144, 805, 731 cm^{-1}.

2.2.3 5-amine-1,10-phenanthroline ligand, (amine-phenanthroline) [2].[15]

Previously synthesized nitro-phenanthroline (1.107 mmol, 0.2573 g, 1 eq) was dissolved in 20 mL of absolute ethanol in a 50 mL round bottom flask. Dissolution of the nitro-phenanthroline required sonication for 5-60 minutes. Once dissolved, argon was bubbled through the solution to displace any dissolved oxygen and then 5% palladium on carbon (25.0mg) was added. A stir bar and a rubber septum were added and the reaction vessel was purged with argon gas. Hydrazine (19.375 mmol, 0.7 mL, 19 eq) was added dropwise via syringe over a period of 15 minutes. The round bottom flask was placed in an oil bath and heated to 70˚C. The solution was allowed to stir at 70˚C overnight. The Pd/C catalyst was removed by vacuum filtering the solution through a celite plug. The filtrate was diluted with chloroform and washed 3 times with water. The solvent was removed under reduced pressure using a rotary evaporator to produce a brick red solid. Yield: 0.1376 g (63%). H^{1} NMR ($CDCl_3$): δ 9.208 (1H, dd), δ 8.95 (1H, dd), δ 8.29 (1H, dd), δ 8.00 (1H, dd), δ 7.67 (1H, qt), δ 7.51 (1H, qt), δ 6.96 (1H, s), C^{13}

NMR($CDCl_3$) 149.935, 146.899, 146.599, 142.180, 139.858, 133.434, 129.782, 129.411, 123.224, 122.194, 122.088, 105.486 ppm. IR (ATR): 3338, 3220, 1609, 1491, 1408, 862, 738, 628 cm^{-1}.

2.2.4 2-bromo-N-(1,10-phenanthroline-5-yl) acetamide hydrobromide (BPAH) [3].[16]

5-amine-1,10-phenanthroline was dried before use by dissolving it in toluene followed by rotary evaporation. The dried 5-amine-1,10-phenanthroline (0.4018 g, 2.06 mmol, 1 eqv) was added to an oven dried 100ml round bottom flask and dissolved in 40 mL of anhydrous chloroform. The round bottom flask was fitted with a reflux condenser and a septum and purged with argon. A solution consisting of Bromo acetyl bromide (0.31 mL,4.18 mmol, 1.2 eqv) in 40 mL of dry chloroform was added slowly to the 100 mL round bottom flask over 3 to 5 minutes. The reaction mixture was refluxed overnight. The resulting solid was isolated by vacuum filtration and recrystallized by hot filtration form methanol. Yield: 0.4078 g (63%) Characterized by carbon and proton NMR and IR. ^{1}H-NMR 300MHz (DMSO-D6): δ 10.92 (1H, s), δ 9.35 (1H, dd), δ 9.23 (1H, dd), δ 9.06 (1H, dd), δ 8.59 (1H, s) C^{13}-NMR 300MHz (DMSO-D6): δ 172.387, 166489, 149.191, 145.311, 143.347, 143.042, 136.199, 135.625, 134.957, 133.067, 129.452, 125.705, 125.213, 118.668, 61.962, 30.042 cm^{-1}. IR (ATR): 3028, 2909, 1711, 1540, 1496, 1413, 1202, 878, 799, 724 cm^{-1}.

2.2.5 $Eu(tta)_3$(1,10-phenanthroline) [5][18]

$Eu(tta)_3(H_2O)_2$ (0.1195 mmol, 0.1097 g, 1 eq) was added it to a 100 mL round bottom flask with 30 mL of methylene chloride and a magnetic stir bar. In a 50 mL Erlenmeyer 1,10-phenanthroline (0.1195 mmol, 0.0274g, 1 eq) and 30 mL of methylene chloride were added and dissolved with a magnetic stir bar. The phenanthroline solution was then added the europium solution dropwise. The round bottom flask was then added to an oil bath to 60˚C and allowed to stir overnight. The solution was gravity filtered into a 100 mL Erlenmeyer flask and then allowed the solvent to evaporate by air. Characterized by UV-Vis and Fluorometer using methylene chloride as the solvent. UV (CH_2Cl_2) λ_{max}, 344 nm. Fluorescence (CH_2Cl_2) $\lambda_{excitation}$, 340 nm, $\lambda_{emission}$, 615 nm. QY: 4.83 ± 0.815%

2.2.6 Synthesis of $Eu(tta)_3$(5-nitro-1,10-phenanthroline) [6]

This synthesis was preformed using identical synthetic procedures as section 2.2.5; although 5-nitro-1,10-phenanthroline (0.1195 mmol, 0.0273 g, 1 eq) was used. Characterized by UV-Vis and Fluorometer using methylene chloride as the solvent. UV (CH_2Cl_2) λ_{max}, 344 nm. Fluorescence (CH_2Cl_2) $\lambda_{excitation}$, 340 nm, $\lambda_{emission}$, 615 nm. QY: 2.78 ± 0.523%

2.2.7 Synthesis of $Eu(tta)_3$(5-amine-1,10-phenanthroline) [7]

This synthesis was preformed using identical synthetic procedures as section 2.2.5; although 5-amine-1,10-phenanthroline (0.1195 mmol, 0.0243 g, 1 eq) was used. Characterized by UV-Vis and Fluorometer using methylene chloride as the solvent. UV (CH_2Cl_2) λ_{max}, 342 nm. Fluorescence (CH_2Cl_2) $\lambda_{emission}$, 615 nm. QY: 5.02 ± 1.14%

2.2.8 Synthesis of $Eu(tta)_3$(BPAH) [8]

This synthesis was preformed using identical synthetic procedures as section 2.2.5; although BPAH (0.1195 mmol, 0.0438 g, 1 eq) was used. Characterized by UV-Vis and Fluorometer using methylene chloride as the solvent. UV (CH_2Cl_2) λ_{max}, 346 nm. Fluorescence (CH_2Cl_2) $\lambda_{emission}$, 615 nm. QY: 0.417 ± 0.107%

2.2.9 Synthesis of monobenzyl protected polyethylene glycol linker (Bz-4EG) [9][19]

Tetra-ethylene glycol (60.0 mL, 60.54 g, 0.312 mol, 3.95eqv.) was added to 165 mL of 50% v/v NaOH aqueous solution and stirred at room temperature for 20 min. Benzyl bromide (10.8 mL, 15.552 g, 0.091 mol, 1.0 eqv.) was then added to the solution. The solution refluxed and stirred for 24 hours and cooled at room temperature. The mixture formed two layers; the top a dark, syrup layer and the bottom a transparent orange layer. The top layer was removed and diluted with water (50 mL). The pH was adjusted to 4 using 2.0 M HCl. The solution was then extracted with diethyl ether (50 mL) three times, washed with a brine solution, and then dried over magnesium sulfate. The solvent was removed by rotary

evaporation. The product was a mixture containing both mono and di benzyl substituted PEG. The separation of this mixture was done by using column chromatography in a 1:1 ethyl acetate/hexane solution, while the solution increased in concentration of ethyl acetate. The product was characterized using carbon and proton NMR and FT-IR. Yield: 6.66 g (74%) H^1 NMR 300MHz, ($CDCl_3$) : δ 3.66(16H, m), δ 4.56(2H, s), δ 7.33 (5H, d). C^{13} NMR 300MHz, (CDCl3): 61.72, 69.39, 70.31, 70.58, 70.60, 72.90, 73.23, 127.59, 127.76, 128.34, 138.16 ppm. IR(ATR): 3443, 2866, 1719, 1453, 1350, 1093, 939, 885, 738, 698 cm^{-1}.

2.2.10 Synthesis of MSH-4 peptide [10][20-21]

The peptide was synthesized using traditional microwave assisted FMOC procedures and solid phase peptide synthesis. The microwave used was a Discover SP-X microwave synthesizer equipped with a fiber optic temperature probe manufactured by CEM Corporation. A low loading (0.10-0.30 mmol/g) rink amide resin was the solid support for binding. 500 mg of resin was weighed and swelled for 40 minutes at room temperature in a 50:50 (by volume) mixture of DMF and DCM. After swelling was complete the solvent was filtered from the resin. The beginning FMOC protecting group was removed by adding 3 mL piperidine to the fritted microwave vessel. To ensure that the microwave temperature was consistent, a fiber optic temperature probe was inserted into the reaction vessel to direct the microwave to stop once the preferred temperature was reached. The microwave settings for the deprotection method adjusted the power to 50 W and heated the reaction to a maximum of 70˚ C for 30 seconds. Following deprotection, the resin beads were washed and filtered three times with 2 mL of DMF to elute excess reagents. A Kaiser test was performed to indicate a free amine group. This was done by removing a few resin beads which were added to a test tube with the Kaiser reagent and heated in the microwave at 120˚ C for 30 seconds. A positive Kaiser test exuded a blue color for successful deprotection. Next, the first amino acid was coupled to the resin. For this, DIC and ethyl (hydroxyamino)cyanoacetate were used in a 5x molar excess as coupling reagents. The reagents were combined with a 5x excess of the amino acid and dissolved in DMF. This solution was added to the reaction vessel with the unprotected resin. The reaction

conditions included a maximum temperature of 75˚ C for 7 minutes. The resin was then washed and filtered three times with 3 mL of DMF. A positive Kaiser test was needed between each coupling to move to the next step. This process was repeated for each amino acid in the order of tryptophan, arginine, phenylalanine and histidine. The peptide was cleaved from the resin at room temperature using a rocker. The cleavage solution included 82.5% TFA, 5% thioanisole, 5% water, 5% phenol, and 2.5% 1, 2-ethanedithiol (percentages are v/v-based values). The peptide was cleaved for a minimum of 3 to a maximum of 6 hours. The resulting solution included the peptide whilst the resin beads remained to be filtered. The peptide solution was allowed to evaporate at room temperature until the volume was reduced by half. The peptide was then precipitated from solution using cold diethyl ether and being placed in the freezer for a minimum of 1 hour and a maximum overnight. The peptide precipitate in solution was then centrifuged at 0 ˚C for 20 minutes. Diethyl ether remained as the supernatant and was decanted from the solid peptide at the bottom of the centrifuge tube. The peptide was sonicated into diethyl ether and centrifuged twice to purify. The remaining solid was dried overnight by air, dissolved in a 50:50 mixture of acetic acid and water and lyophilized for 8 hours. The sample was characterized and verified by Dr. Matthew Vergne of Lipscomb University in Nashville, TN using LC/MS.

CHAPTER 3: RESULTS AND DISCUSSION

The ultimate goal of this project is to develop a diagnostic tool for detecting melanoma cells. The design entails attaching a luminescent lanthanide chelate to a polyethylene glycol (PEG) linker attached to a melanocyte stimulating hormone (MSH-4) that targets and binds to a G-coupling protein receptor found on the surface of and excreted by melanoma cancer cells. The success of the synthesis of this proposed structure relies heavily on making sure the chelated lanthanide ligand does not interfere with the MSH-4 peptide. The interference could hinder the probe's ability to attach properly to the GPCR. This would then interfere with the lanthanides ability to fluoresce properly. Since the peptide is specifically designed for the GPCR of melanocytes, the probe will not be able to attach to any other GPCRs in any residual biological cells.

3.1 Synthesis of the MSH-4 peptide

The MSH-4 peptide was synthesized using a microwave assisted FMOC synthesis procedure. Each peptide was added in series following standard deprotection/protection synthesis. There were few difficulties when synthesizing the peptide. One being that the deprotection step was needed to be repeated to ensure that the vast majority of the amino acids were deprotected and available to have the next amino acid attach to the sequence. If this step wasn't repeated and checked then that would have resulted in lower yield of complete and correct peptide sequences. To ensure proper assembly and reduce error, a checklist was created, see supplemental materials for compound **10**. This allows for other members to follow proper FMOC protocol, as well as help troubleshoot common errors if necessary.

For the characterization methods of the MSH-4 peptide, Western Carolina University does not have a LC/MS, therefore the sample peptides that were created and purified in the lab, had to be shipped to and analyzed by Dr. Matthew Vergne of Lipscomb University in Nashville, TN. They were verified by Dr. Vergne to be the MSH-4 peptide that was being designed for this project. The spectra is referenced in supplemental materials for compound **10**.

3.2 Synthesis of 1,10-N,N-phenanthroline ligands

The synthesis of each phenanthroline ligand began by using commercially synthesized N,N-1,10-phenanthroline and then nitrating it at the 5th carbon using concentrated sulfuric acid and then adding fuming nitric acid dropwise. This allows for control of the reaction and ensuring that the gas production is under control. The resulting reaction mixture was added to ice to allow for rapid cooling and preparation for pH adjustment. The pH was neutralized to a pH of 3 using NaOH. The importance of this step was to allow for any remaining acid to be protonated and dissolvable in water, whilst allowing the resulting product to precipitate from solution. The solid was washed with water and allowed to dry. The 5-nitro 1,10phenathroline (**1**) was sufficiently pure for use in the next reaction.

The reduction of the nitro group of **1** to an amine was done by adding an excess of hydrazine dropwise to a solution of **1** in absolute ethanol containing a catalytic amount of palladium on activated charcoal. Argon was bubbled through ethanol/**1** solution to remove any dissolved oxygen before the hydrazine was added. The reaction was heated to 70^{o} C and stirred overnight under an inert atmosphere overnight. This allows for the nitro group of 1 to be reduced to form 5-amino 1,10phenathroline (**2**).

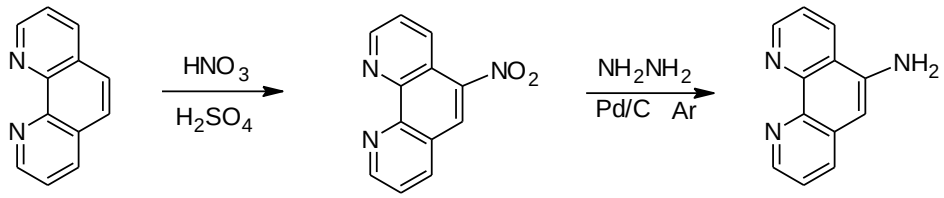


Figure 5. Synthetic scheme for nitration and reductive amination of 1,10-phenanthroline.

The original project plan was to attach the peg linker to the phenanthroline linker by performing a SN2 reaction between aminephenanthroline (**2**) with a mono-tosylated mono-benzylated tetra ethylene glycol (Fig 6). Previous attempts by another researcher were unsuccessful. Multiple attempts varying

solvent and base failed in producing the product.[13] It was determined that the amino group attached to the aromatic ring was too poor of a nucleophile. Next, DCC peptide coupling methods were attempted using a ethylene glycol containing a carboxylic acid. After several attempts using traditional peptide coupling methods the product could only be produced in low yields (Fig. 6). One possible reason for failure may have been the difficulty in drying the starting materials. Both phenanthroline and polyethylene glycol are difficult to keep dry and any excess water can react with DCC. The synthetic plan was then rerouted to add another functional group to the phenanthroline that might be more practical (Fig 7).

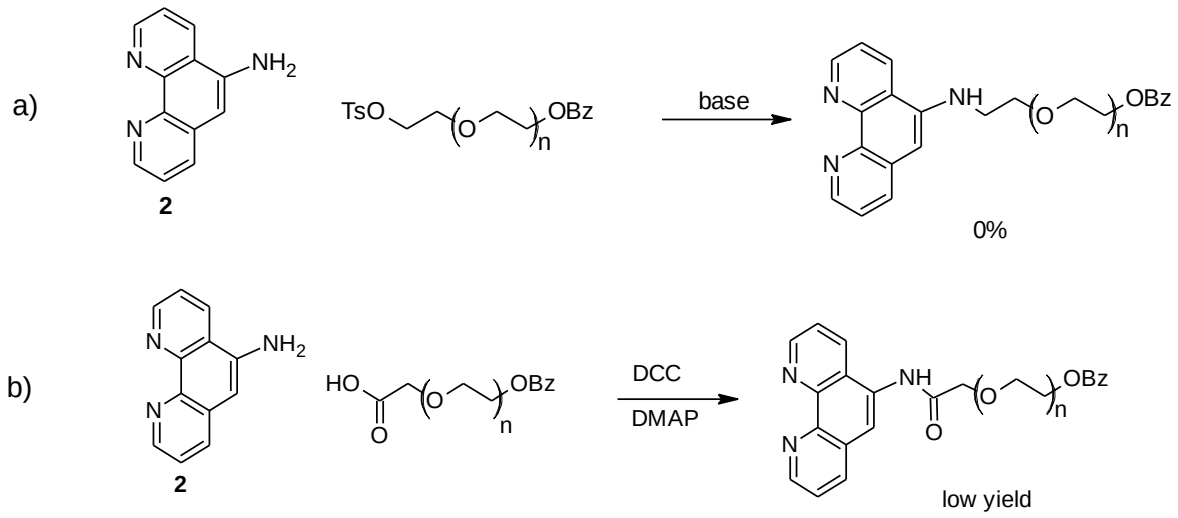


Figure 6. Previous attempts to attach PEG to phenanthroline ligands. a) Nucleophile attack of phenanthroline amine on a O-tosylated tetra ethylene glycol. b) peptide coupling methods were attempted to attach the PEG to the ligand.

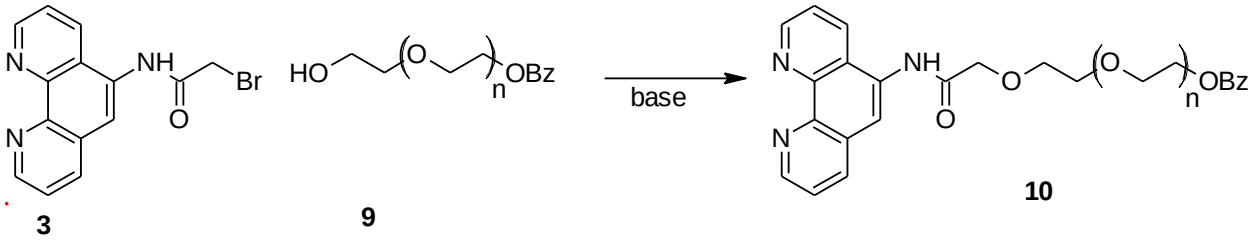


Figure 7. Adding a bromo acetamide group to the phenanthroline ligand may allow attaching PEG using an SN2 reaction where the PEG acts as the nucleophile.

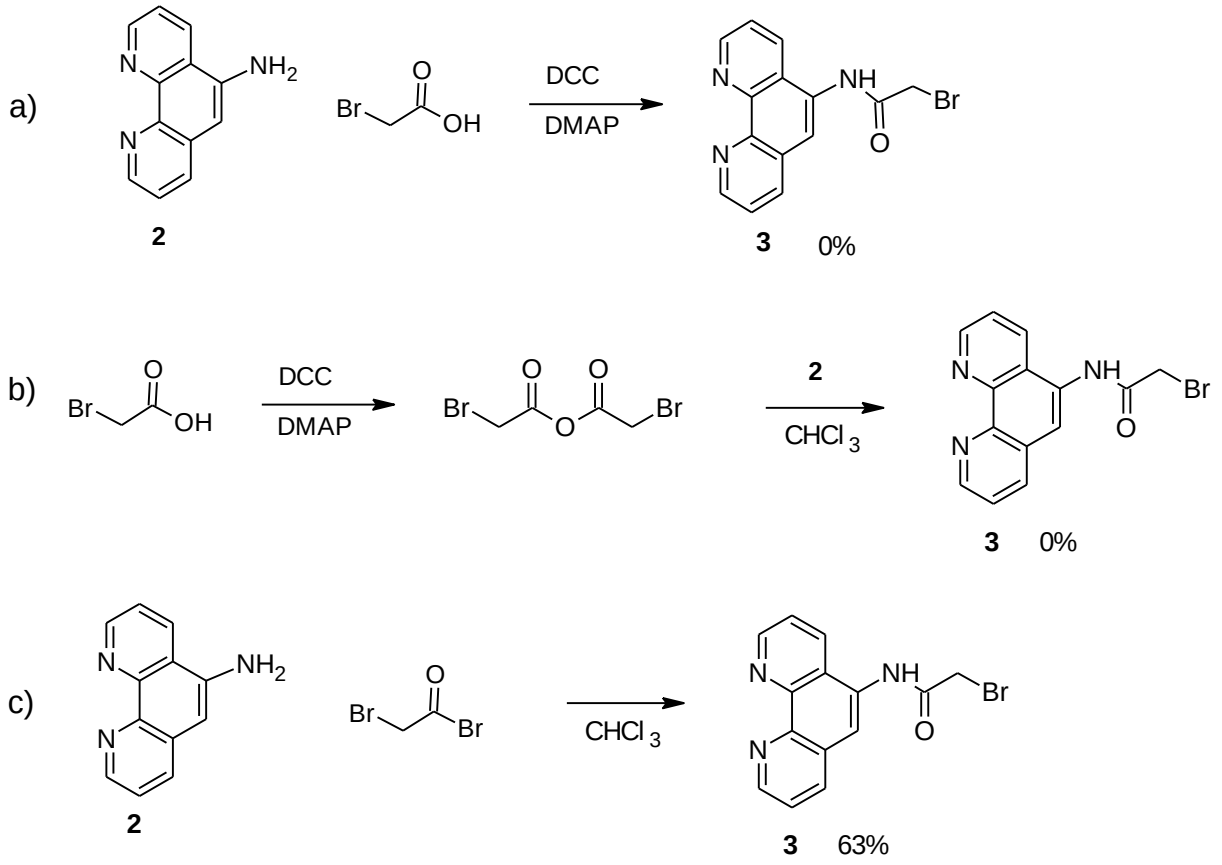


Figure 8. Synthetic strategies to form the bromo acetamide attached to the amine.

This led to trying to form a bromo acetic anhydride using bromo acetic acid and coupling it to the previously synthesized **2**. This was attempted done using dry ethyl acetate and acetonitrile as solvents and dicyclohexylcarbodiimide as the coupling reagent. After several trials this reaction proved ineffective. It was believed that the water associated with the phenanthroline amine **2** was interfering with the reaction. The next plan of action was to perform the same reaction, but separate it by first forming the anhydride in a separate reaction vessel. The formation of the anhydride was confirmed by FTIR. The next step was to try react the anhydride with **2**. This was done in dichloromethane and was deemed unsuccessful by H^1 NMR. It seems that the amine of **2** is too poor of a nucleophile to react with an anhydride. The final attempt to attach a bromo acetyl group to **2** was by reacting the amine with bromo acetyl bromide. The acid bromide is a stronger is a stronger electrophile than an anhydride and should react even with a poor

nucleophile. This reaction was carried out using dry chloroform as the solvent and under inert atmosphere. The resulting precipitate was filtered and purified using hot recrystallization in methanol. 5-N-bromoacetylphenathroline **3** was successfully synthesized as determined by H^1 and C^{13} NMR as well as IR. However, in the C NMR there is a peak of interest at 172 ppm. This peak correlates to a carbon that is part of a carboxylic acid functional group; therefore, more investigation needed to be done to eliminate whether or not there is a bromine on the targeted compound. This was done by preforming the flame test. The flame test can help determine whether or not a compound has a halogen within the system. A positive test for bromine would show a green flame when the compound is heated over a Bunsen burner. To carry-out this test a copper wire was burnt over flame until an orange flame was at glow. Copper ions turn flame green, so this was done to eliminate the possibility of a false positive. The wire was then cooled and dipped into bromo acetyl bromide and then held over flame to verify that the color that should be displayed is green. Then wire was then held over flame until the flame turned orange, again to make sure that all excess would be burnt off to prevent false positive. Compound **3** was then tested and showed a positive result for bromine in the sample. Therefore, the peak at 172 can be ruled as an impurity and that the synthesis deemed successful.

3.3 Synthesis of the PEG linker O-benzyl tetra ethylene glycol.

In the original plan for this project, a tosylated tetra ethylene glycol chain was synthesized. This was done by adding p-toluene sulfonyl chloride, tetra ethylene glycol and 16M KOH in THF. After the solution was refluxed for 1 hour, the reaction stirred at room temperature overnight. The product was extracted using diethyl ether and washed with a brine solution. The resulting product was dried using magnesium sulfate and condensed using rotary evaporation. The reaction yielded both a monosubstituted tosyl-PEG as well as a disubstituted tosyl-PEG. This created a problem when it came time to separate the products. Where both had extremely similar polarities, a TLC plate with various combinations of solvent systems did not allow it to move from the baseline. Therein the plan was diverged.

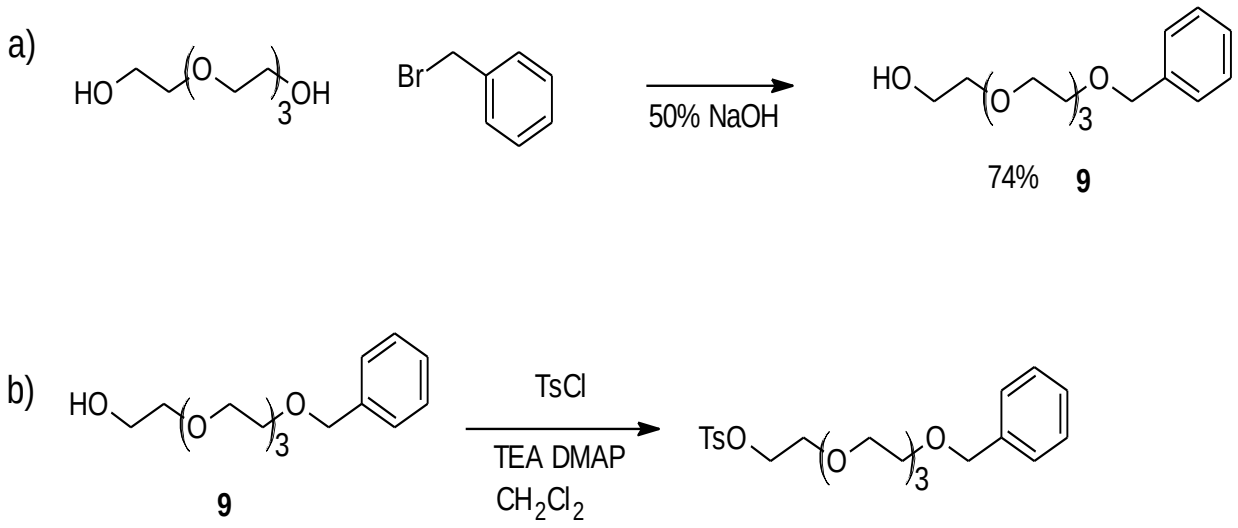


Figure 9: Synthetic plan revisions for the PEG linker

The next idea was to add a benzyl group to the tetra ethylene glycol. This was done as shown in experimental 2.2.1 **9**. Once the monosubstituted benzyl-PEG was isolated, a tosyl group was added using p-toluene sulfonyl chloride, DMAP, and triethyl amine in DCM. This reaction was done under argon and allowed to stir overnight at room temperature. The resulting product was checked against starting materials by using a 1:1(v/v) hexanes: ethyl acetate solvent system. To purify, a separatory funnel was used by extracting with ethyl acetate and washing with a 5% sodium bicarbonate wash, twice, and a brine wash, twice. The resulting solution was concentrated using rotary evaporation.

3.4 Coupling of the PEG linker

After the succession of the targeted phenanthroline ligand and the PEG linker, the next step was to couple this ligand to the PEG linker. Another member of the research group had tried to couple the amine phenanthroline to the tosylated monosubstituted benzyl-PEG linker. This reaction was deemed unsuccessful; therefore, there was another deviation in the project's plan. Stated in the previous section 3.2, the aminephen **2** was unsuccessful in the coupling reaction, so a better leaving group was needed to be able to perform the needed SN2 reaction to be able to couple the two compounds.

3.5 Synthesis of $Eu(tta)_3$ complexes

Three different 5-substituted ligands were synthesized in this project containing a nitro (**2**), amine (**3**) and a Bromo acetamide (**4**) group. Each of these groups are expected to influence the luminescent properties of their europium complexes differently. The nitro group is a powerful electron with-drawing group, the amine is a powerful electron donating group and the acetamide is a moderate electron donating-group. Europium-TTA complexes of all three ligands and phenanthroline (R = H) were synthesized and their quantum yields measured to determine their ability as fluorescent dyes.

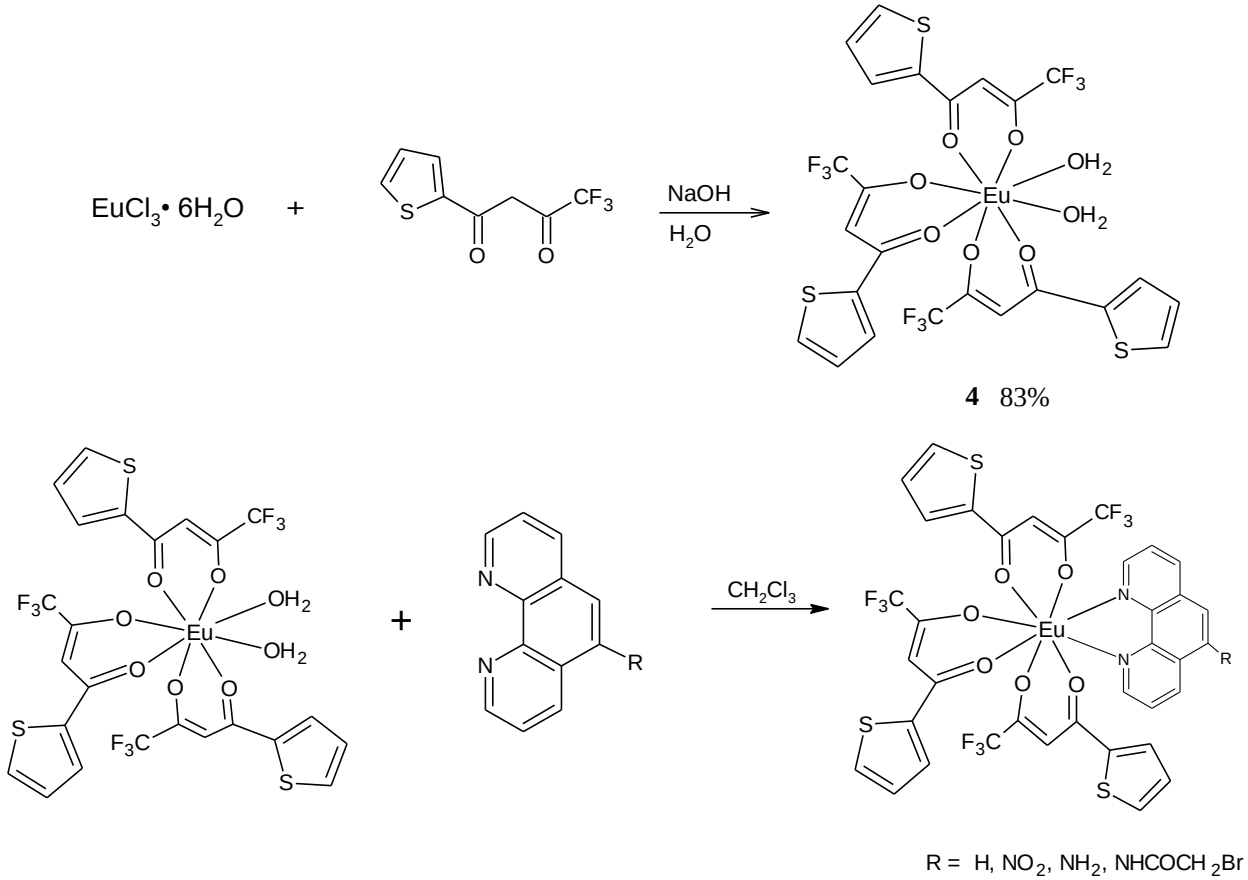


Figure 10: Synthetic strategy for chelation of the phenanthroline ligands to $Eu(tta)_3$.

The synthesis of $Eu(tta)_3(H_2O)_2$ began by combining NaOH solution with thenoyltrifluoroacetone (TTA). This was done so that the basic solution can deprotonate the TTA by removing a hydrogen between the two carbonyls. This allows for the enolate to form. Once this solution is added to the europium chloride,

most of the hydroxide from the NaOH solution has turned into water and allows the sodium and chloride to form a salt whilst the europium forms six bonds with three of the deprotonated TTA as shown in figure 10. The washing of the resulting product allows for the remaining salt to be removed from the precipitate. The success of this reaction was determined by shining the complex under a UV lamp to display its luminescent properties.

The chelation of this complex to the organic ligands involves separately preparing solutions of $Eu(tta)_3$ and the phenanthroline ligand in dichloromethane. By keeping them separated and adding them dropwise this allows for there to be control over the selectivity of the chelation between the $Eu(tta)_3$ and the nitrogen locations of the phenanthroline ligands. The resulting complexes were characterized by UV-Vis and Fluorescence spectroscopy.

3.6 UV-Vis and Fluorescence Spectra of $Eu(tta)_3$(n-phenanthroline) complexes

Qualitative spectra were taken for each ligand, complex, and reference sample. In figure 11, phenanthroline was shown to absorb around 265 nm. This proves that the absorbance around 340 in figure 12 is TTA, which matches known absorbance values for TTA. This trend follows with the phenanthroline ligands with different functional groups show in figures 11 and 12. Upon chelation of the phenanthroline ligands, the absorption spectra are shown to have two peaks, one peak between 240 and 300 and one peak between 330 and 360. These peaks are the phenanthroline ligands and the TTA respectively. The TTA peak is displayed to have shifted once the organic ligands were chelated to the europium. The fluorescence spectra display appropriate peaks for the fluorescence of TTA in figure 13.

Although there are noticeable differences of the UV-Vis spectra for each organic ligand and complex, the fluorescence spectra for all four complexes look almost identical in that their emission wavelength is the same (615nm). This is expected since the fluorescence of europium results from electronic transitions in f-orbitals whose energies are not involved in binding to ligands. However, the different ligands do affect the intensities of emission and are all different. The two complexes with the

lower quantum yields, nitrophenanthroline **(2)** and BPAH **(3)**, also have lower intensities. This is extremely important when looking at the absorbance spectra for BPAH in figure 11. In order to get a spectrum with a smooth curve and noticeable peaks, the concentration had to be significantly higher for this ligand than the others. This is also true for the $Eu(tta)_3$-BPAH complex in figure 12, as well. However, the fluorescence spectra for the larger concentration has a lower intensity of the main 615 nm peak. This is directly correspondent to the low quantum yield of the complex being less than 1%, (table 1.)

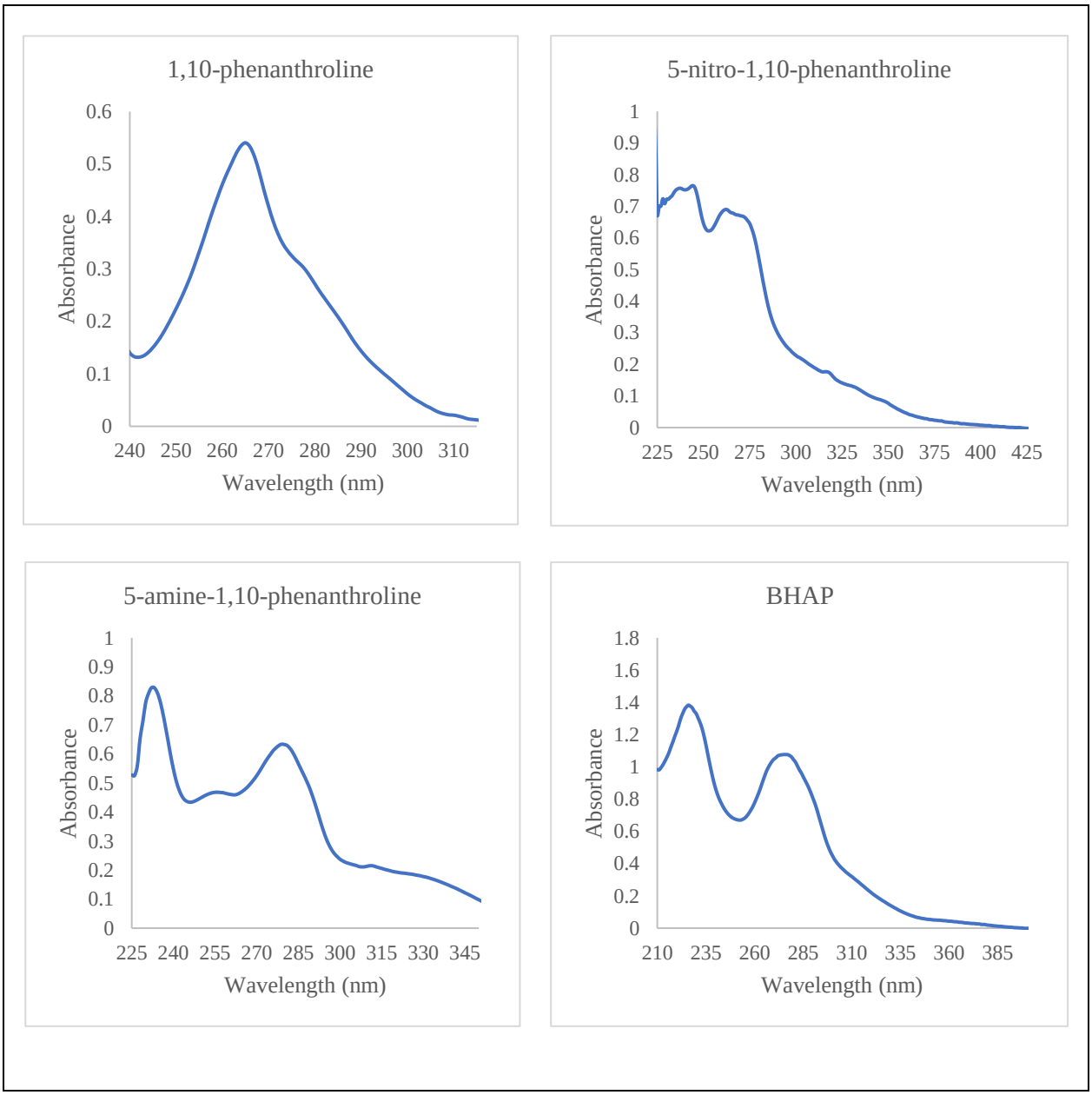


Figure 11: UV-Vis spectra of phenanthroline ligands

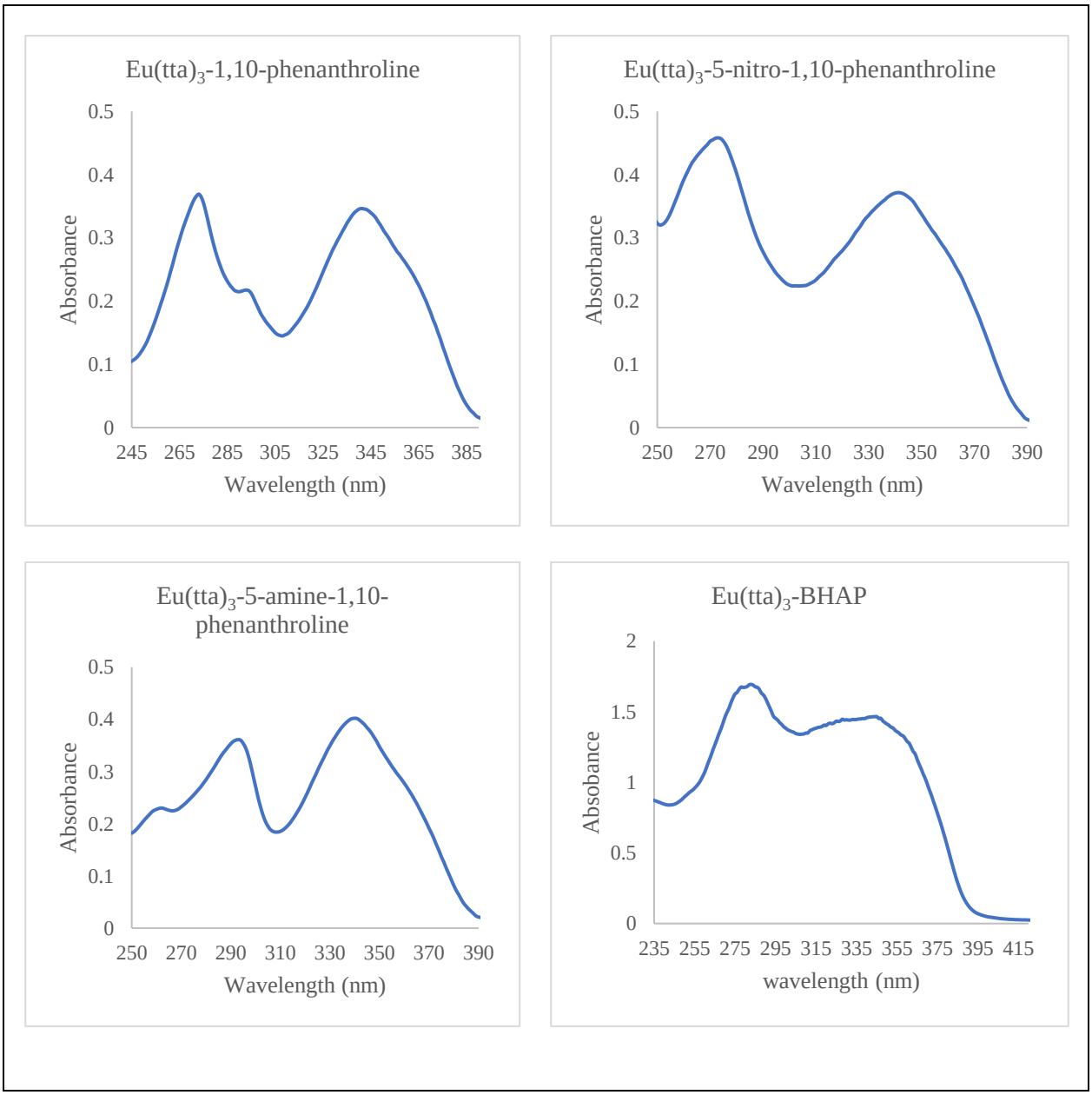


Figure 12: UV-Vis spectra of $Eu(tta)_3$-phenanthroline complexes

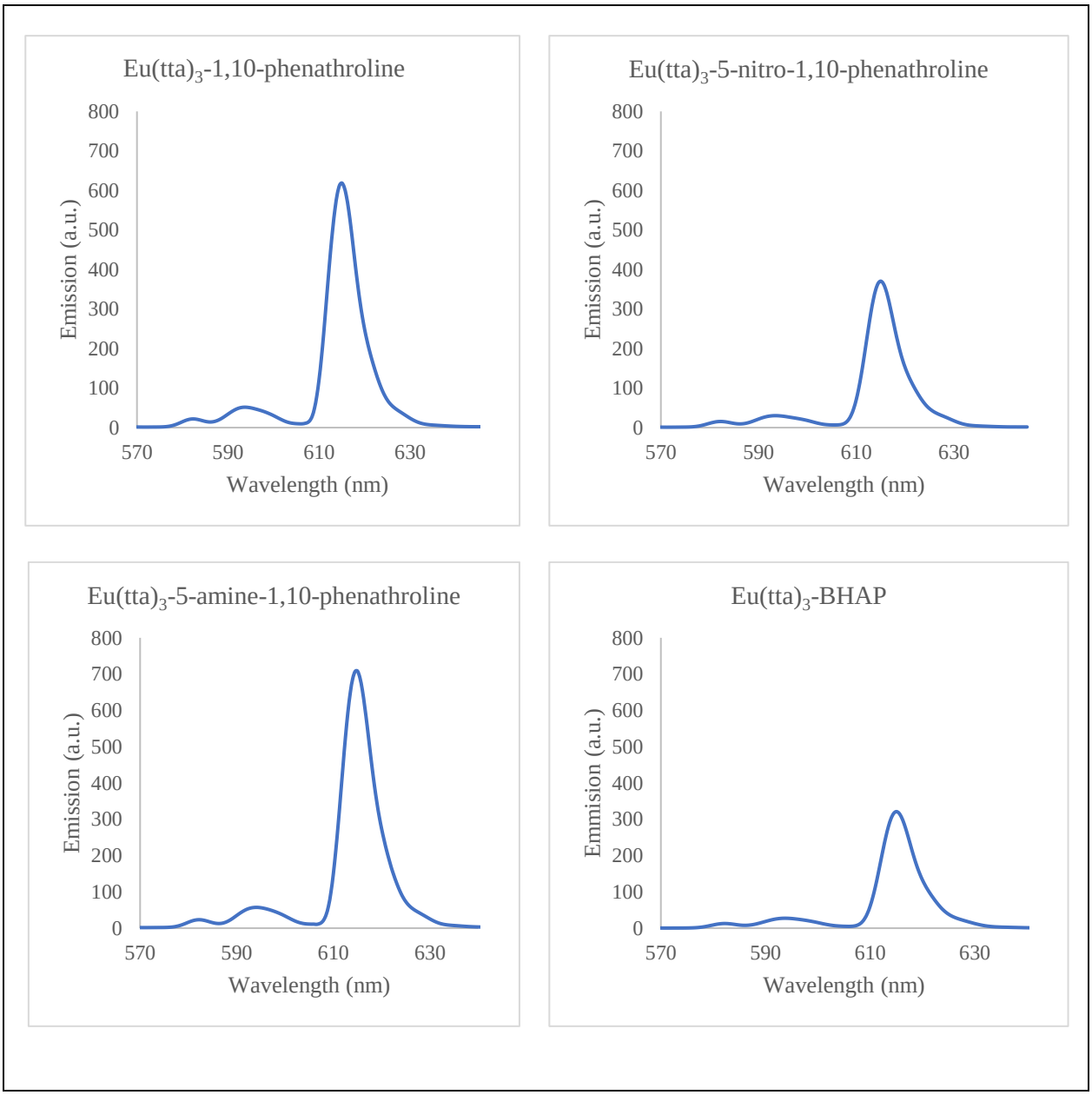


Figure 13: Fluorescence spectra of $Eu(tta)_3$-phenanthroline complexes excited at 340 nm

3.7 Quantum Yield

Quantum yield is a ratio of the number of photons absorb in comparison to the number of photons that are emitted by a fluorophore. Quantum yield is determined by measuring the UV-Vis absorbance and fluorescence spectra of each sample thrice, over a period of three day. The data that is collected is then input into Equation 1 displayed in Chapter 2 section 2.1.5. The quantum yields of each complex are

shown in table 1. The quantum yield is used to measure the fluorescence efficiency of the europium complexes synthesized in this study. Quantum yield is simply the ratio of the number photons emitted to the number of photons absorbed.

Equation 1

$$\phi_S = \phi_R \frac{Abs_R}{Abs_S} \frac{A_S}{A_R} \frac{n_S^2}{n_R^2}$$

Where Abs is the absorbance, A is the area under the luminescence curve, and n is the refractive index of the solvent. The subscripts R and S are representative of reference sample and sample respectively. Phi (φ) is representative of quantum yield itself. The reference sample that was used was Cresyl violet, with a known quantum yield of 0.54, or 54%.

Within this table, and discussed in prior sections, the quantum yield of the $Eu(tta)_3$BPAH complex is significantly lower than that of the other chelated ligand complexes. This is unfortunate; however, we still feel that these systems can work as biological imaging reagents. Ideally the complex would have a high quantum yield which would allow lower concentration of the dye to be used in imaging. With some investigation as to why this has happened, the absorption and fluorescence spectra of his complex has been taken into accord. The absorption spectra had to be at a greater concentration to obtain a readable spectrum, as well as the fluorescence of the complex is at a lesser intensity as well. With the objective of the fluorophore to be able to luminesce at greater intensities than that of others, this had presented a problem. The reason in using BPAH was to have a better SN2 reaction to attach to the PEG linker, however if the quantum yield is low then the problem arises as to why and how to fix this problem, or to try another method for coupling to the PEG linker. The first idea was to see if the BPAH had a significant fluorescence itself, as opposed to other organic ligands. This proved to be ineffective as it has no considerable difference to other organic ligands. Next, the structure itself was taken into account. The $Eu(tta)_3$aminephen has the highest quantum yield out of all complexes because it is such a good electron

donator to the system. When looking at the structure of BPAH [3], one would assume that the attachment of the bromo acetamide wouldn't have made a significant difference, however the carbonyl oxygen acts as an electron magnet. This restricts the electrons on the amine from conjugating with the phenanthroline to aid in the antenna effect. This concludes that there needs to be future work to be able to reduce the carbonyl oxygen or to remove it altogether.

Table 1: Quantum Yield Values

Complex	Quantum yield (%)	Std Dev
$Eu(tta)_3$phen	4.83	0.815
$Eu(tta)_3$nitrophen	2.78	0.523
$Eu(tta)_3$aminephen	5.02	1.14
$Eu(tta)_3$BPAH	0.417	0.107

3.8 Future work

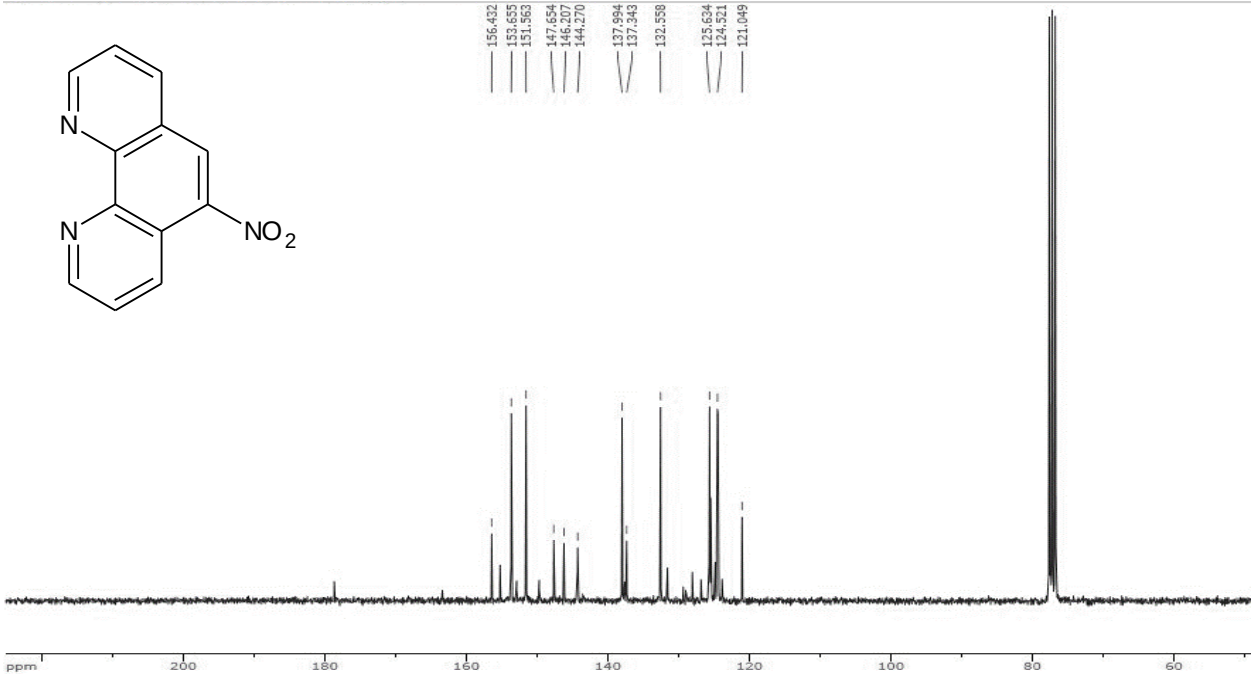
Future work for this project would entail designing a better ligand to attach to the linker without hindering the quantum yield of the lanthanide (III) complex. This could also necessitate having a better functional group on the PEG linker to be able to utilize the aminephenanthroline. This would ensure that the quantum yield would not be hindered by any groups on the ligand. Future work would also include attaching the MSH-4 peptide to the PEG linker with the ligand already coupled. Having the linker, ligand, and peptide attached would presumably be unchallenging to chelate the lanthanide (III) complex.

CHAPTER 4: SUPPLEMENTAL DATA

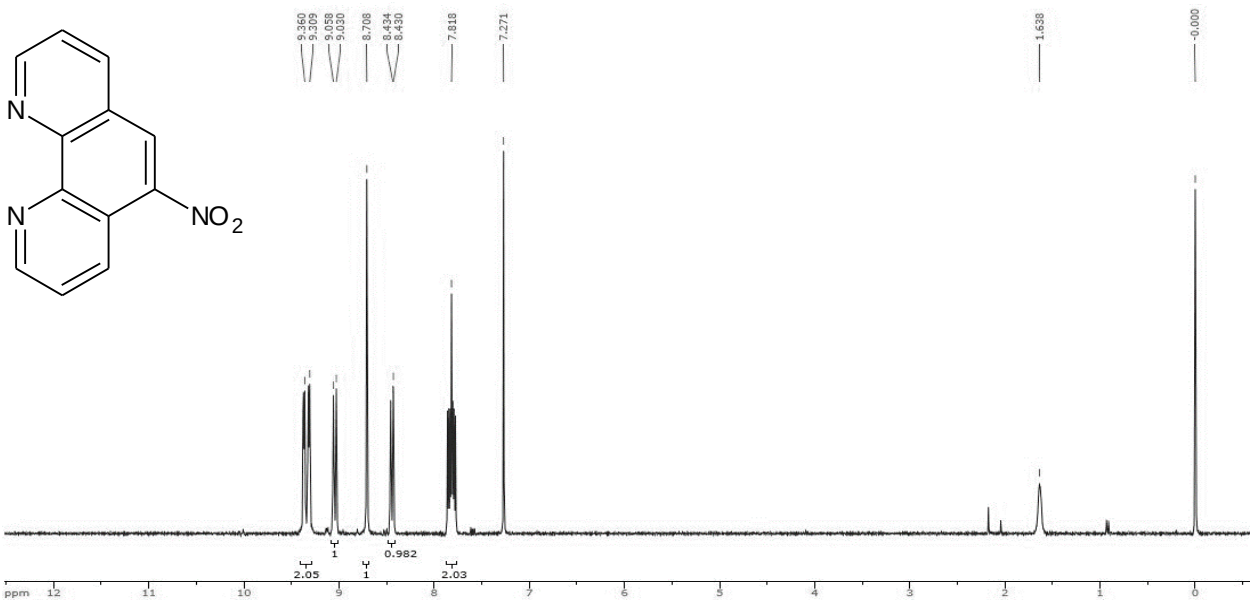
This chapter includes all spectra referenced in previous chapters. The solvents used are listed within the experimental chapter for each compound respectively. The spectra included in this chapter are of compounds that were synthesized within the lab, with the exception of one UV spectra for commercially purchased 1,10-phenanthroline.

4.1 NMR and IR spectra

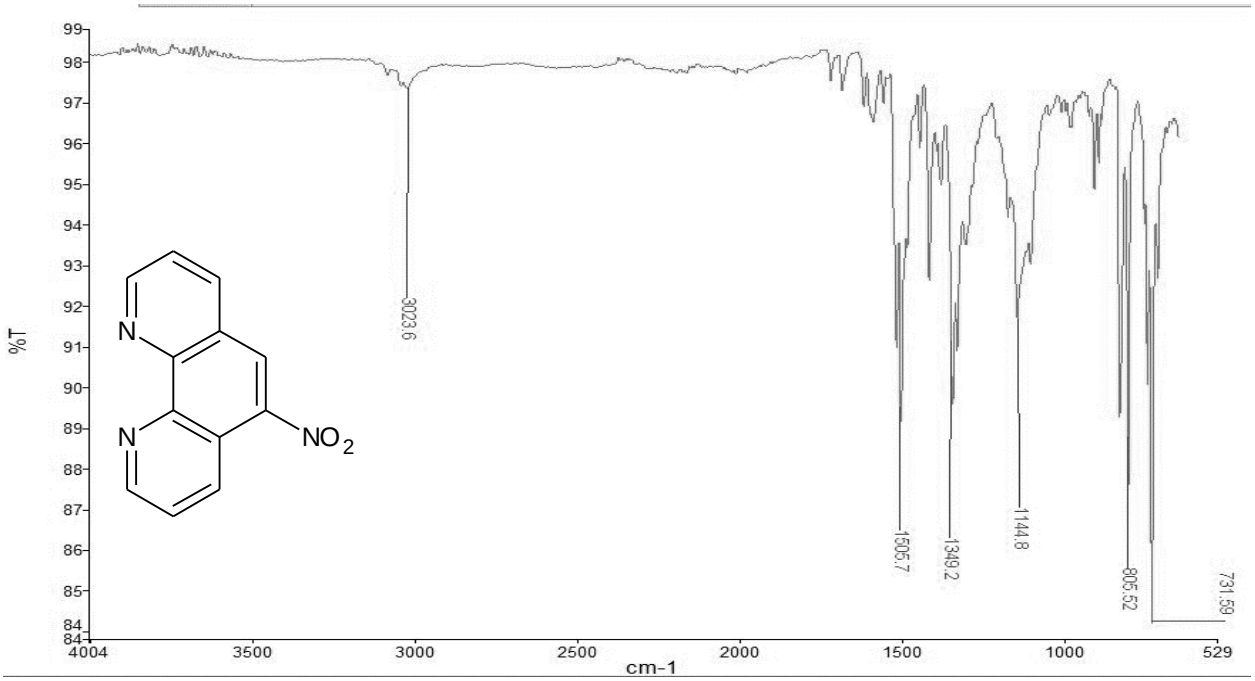
4.1.1 5-nitro-1,10-phenanthroline, (nitrophenanthroline) [1]



^{13}C NMR nitrophenanthroline

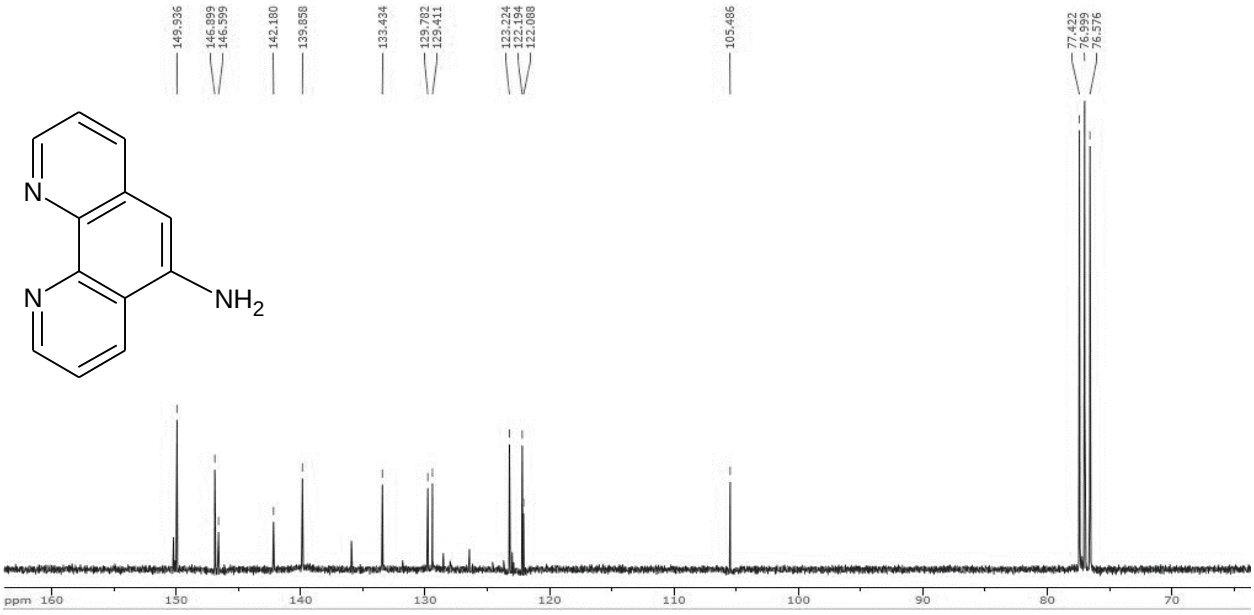


^{1}H NMR nitrophenanthroline

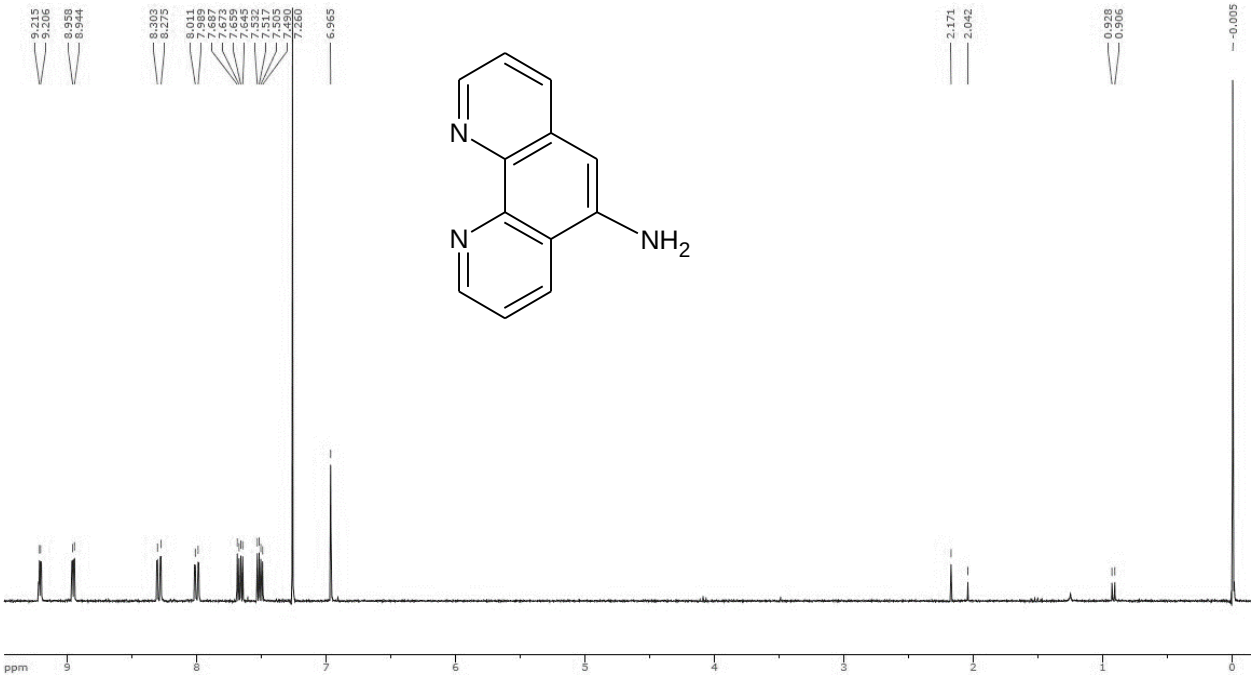


IR- nitrophenanthroline

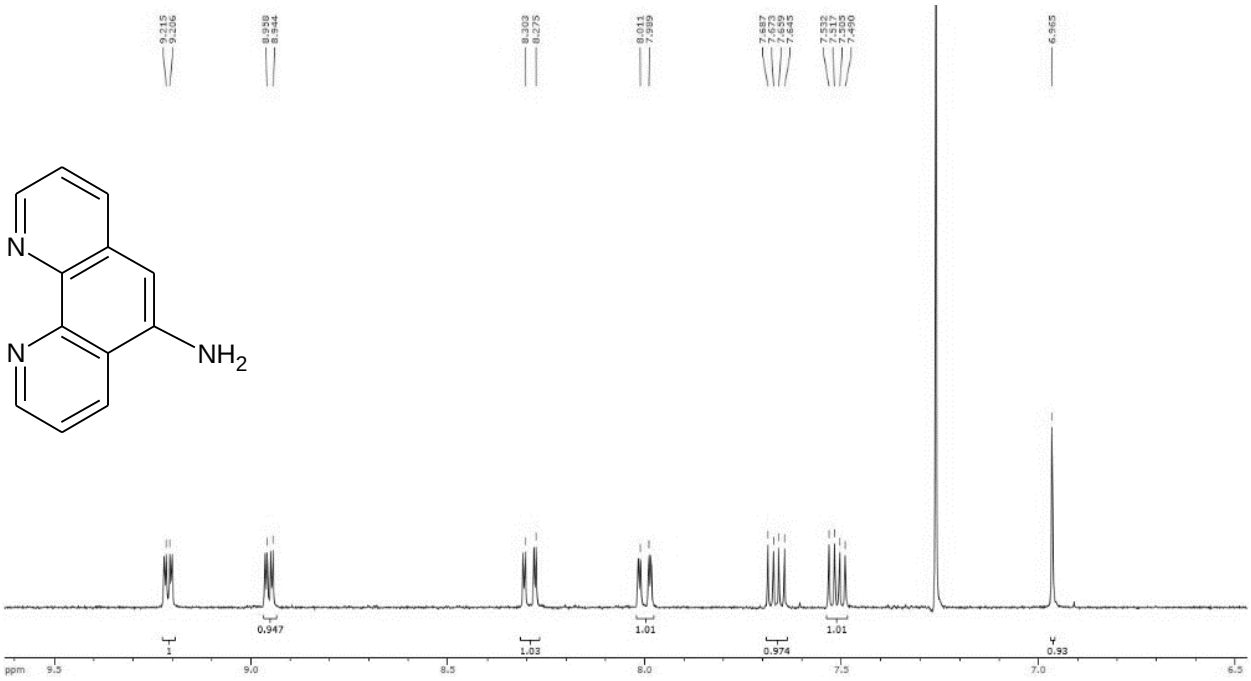
4.1.2 5-amine-1,10-phenanthroline,(aminephenanthroline) [2]



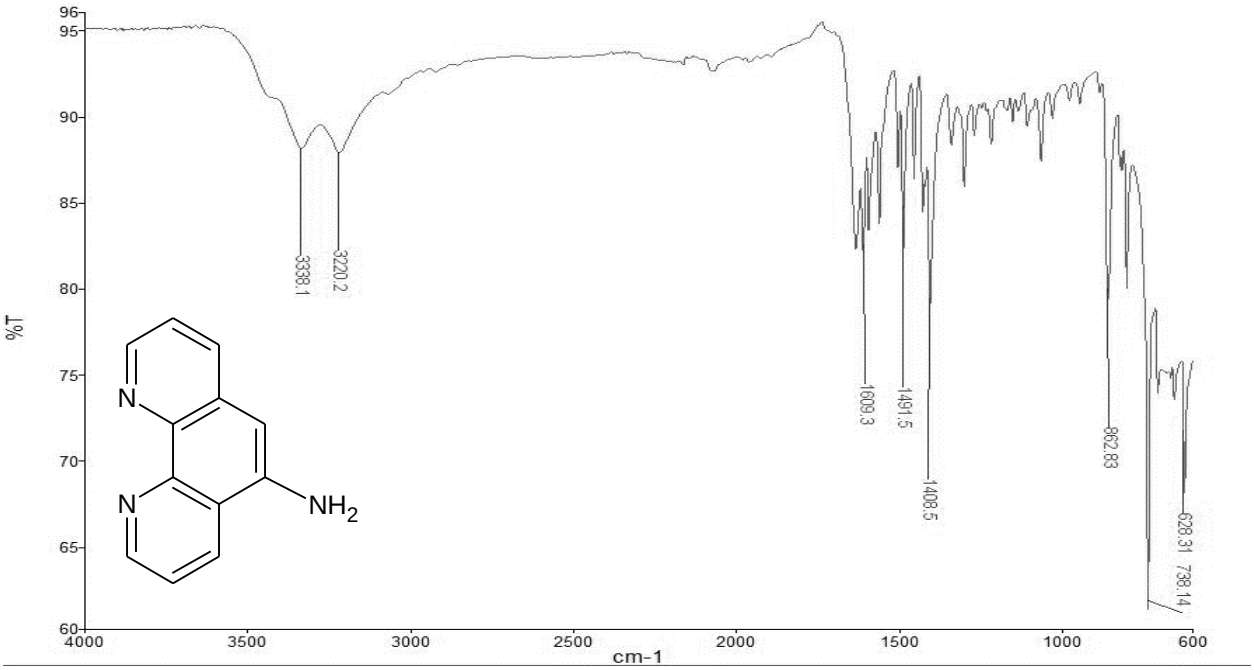
^{13}C NMR-animephenanthroline



^{1}H NMR aminephenanthroline

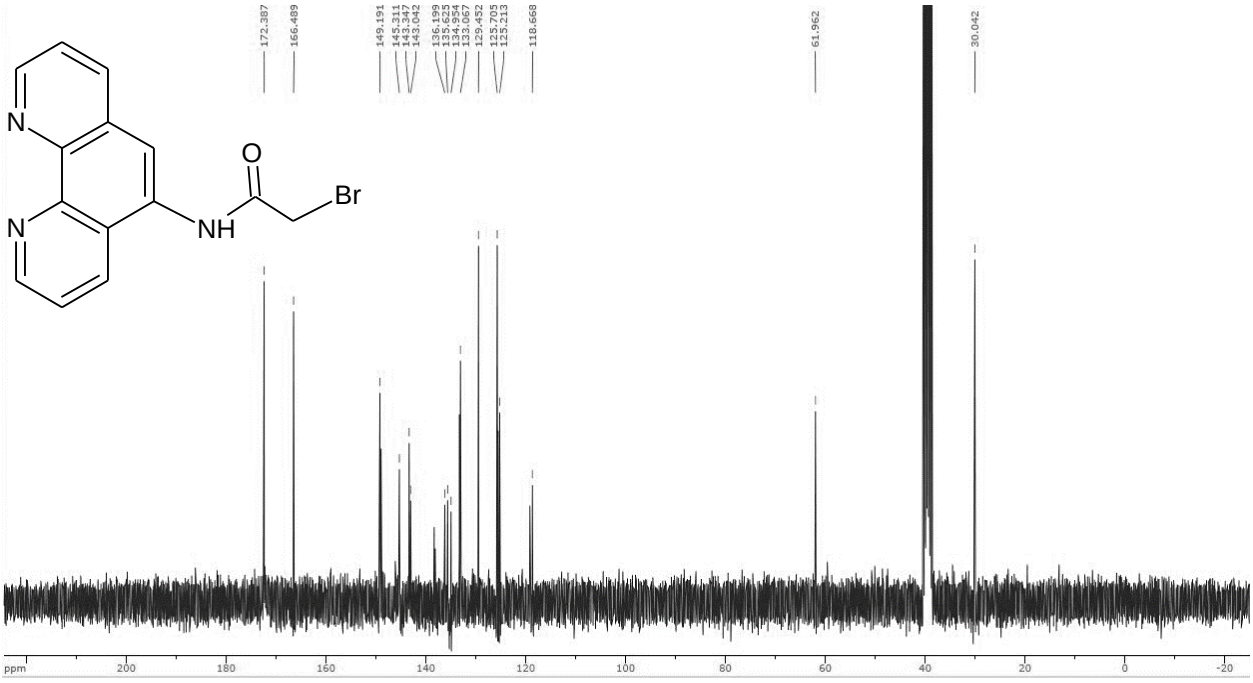


^{1}H NMR- aminephenanthroline (zoomed in)

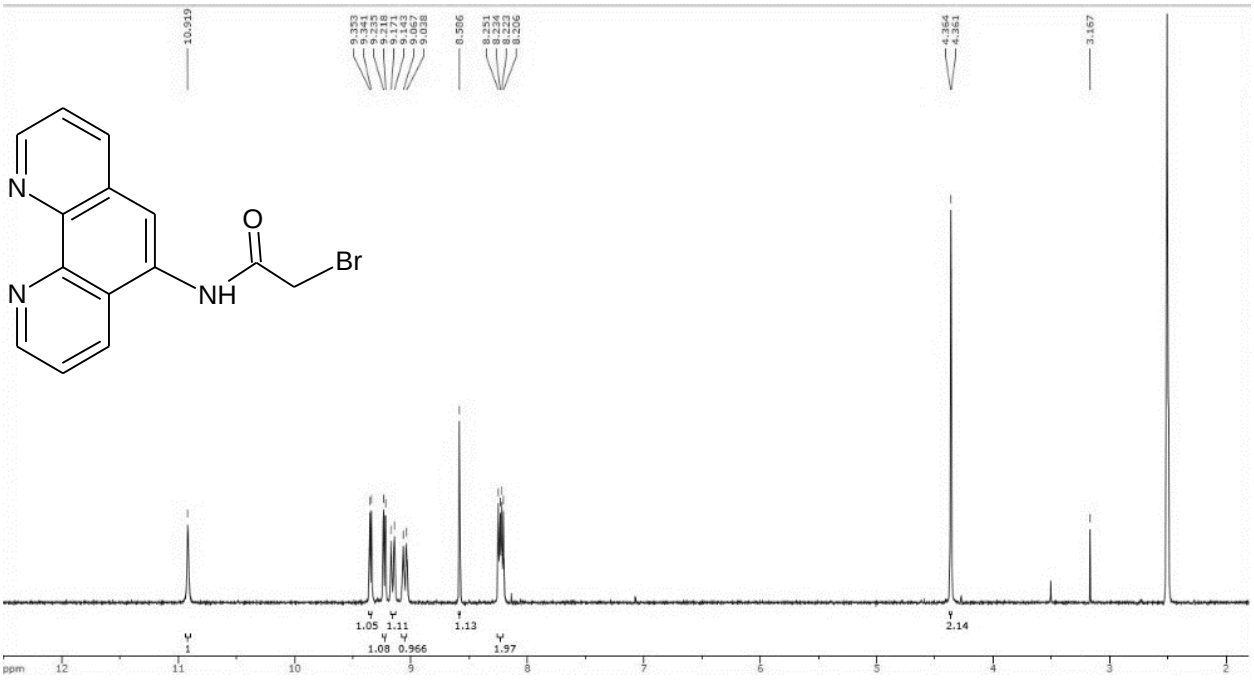


IR-aminephenanthroline

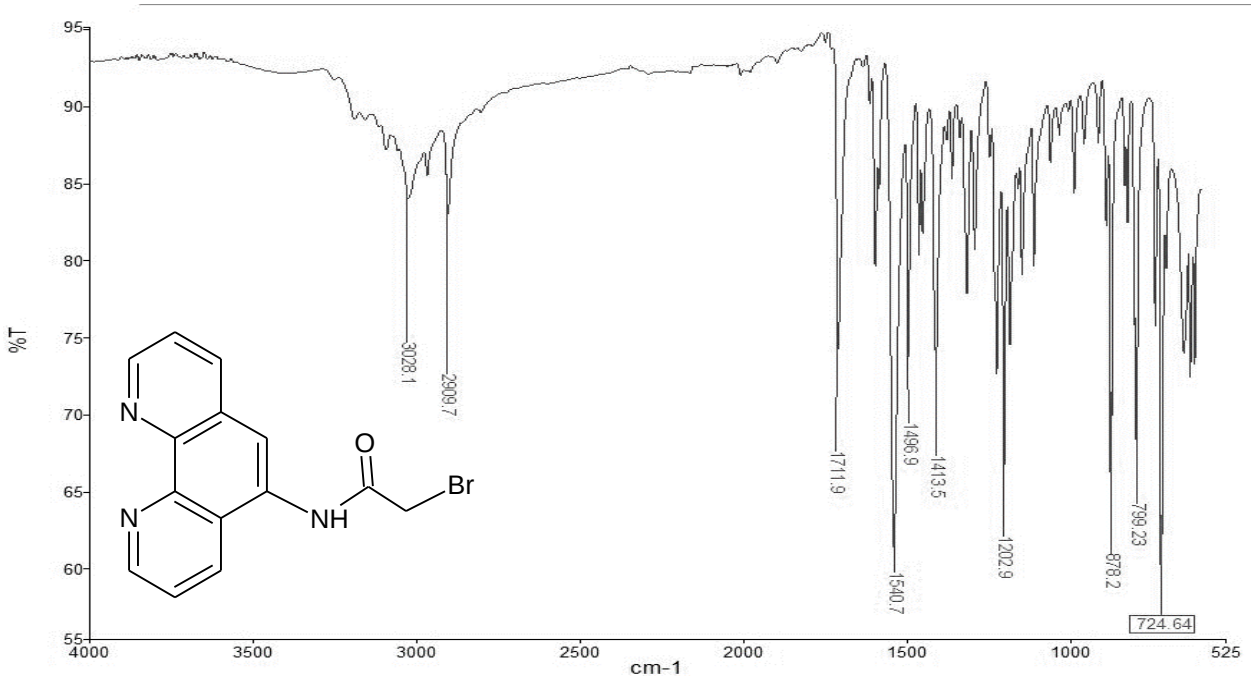
4.1.3 2-bromo-N-(1,10-phenanthroline-5-yl) acetamide hydrobromide, (BPAH) [3]



^{13}C NMR- BPAH

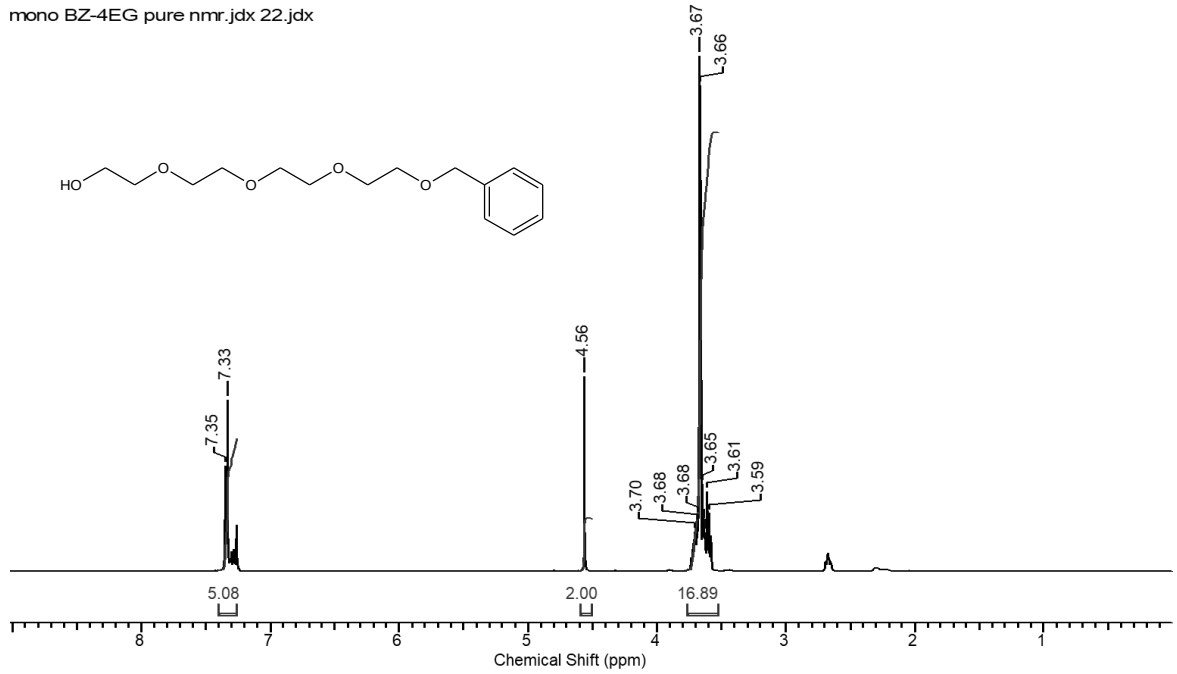


^{1}H NMR-BPAH

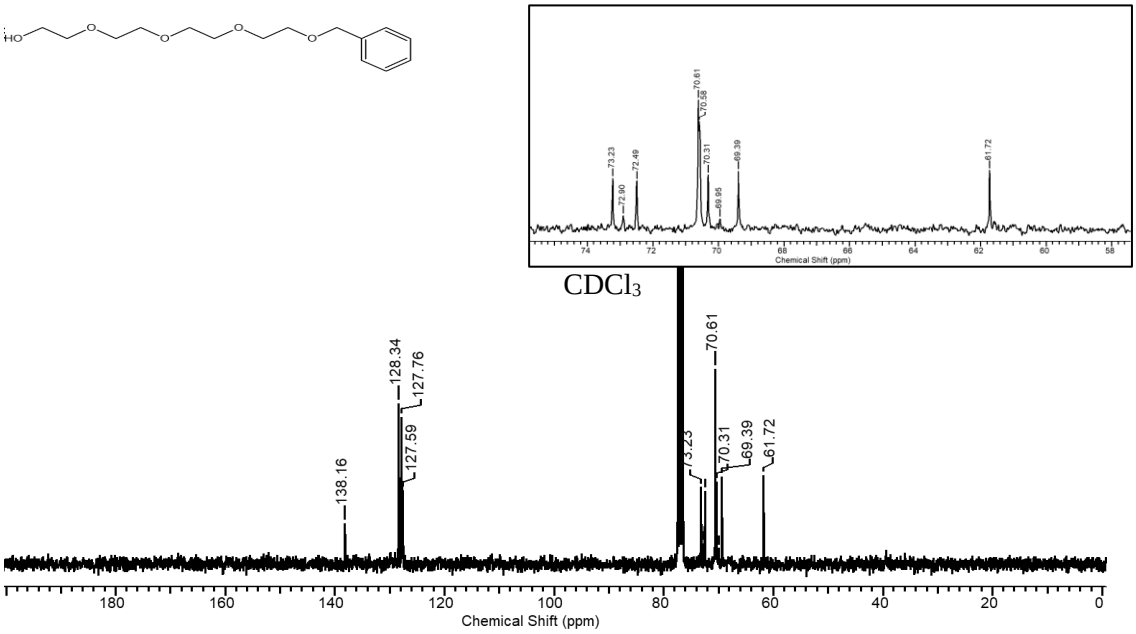


IR-BPAH

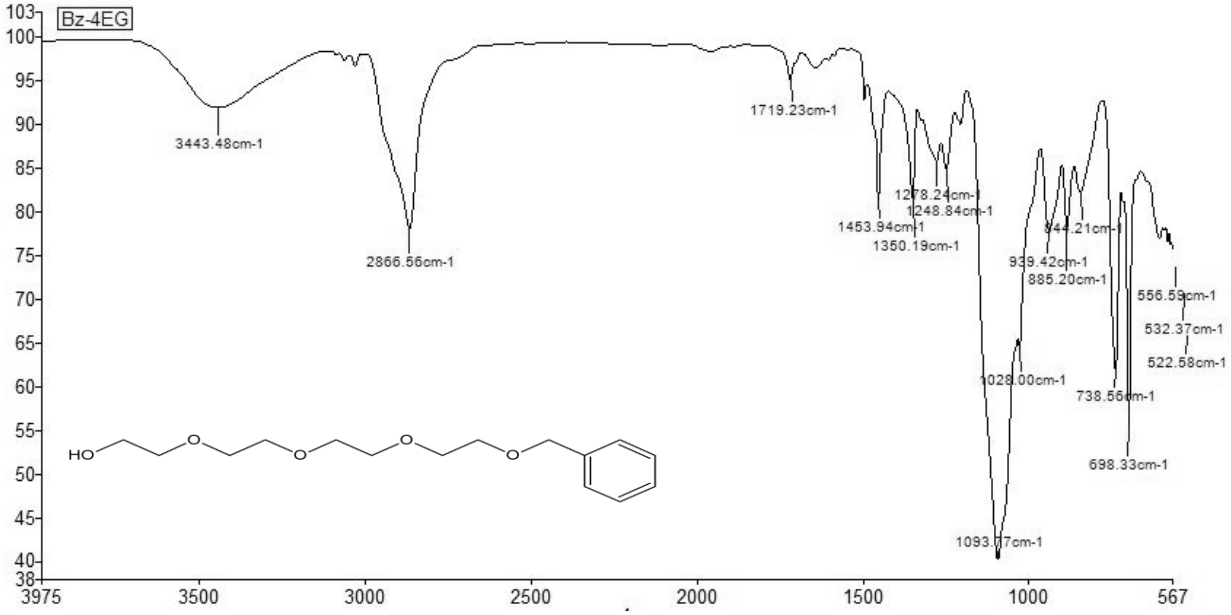
4.1.4 Monobenzyl protected polyethylene glycol linker [9][19,13]



^{1}H NMR Monobenzyl protected polyethylene glycol linker

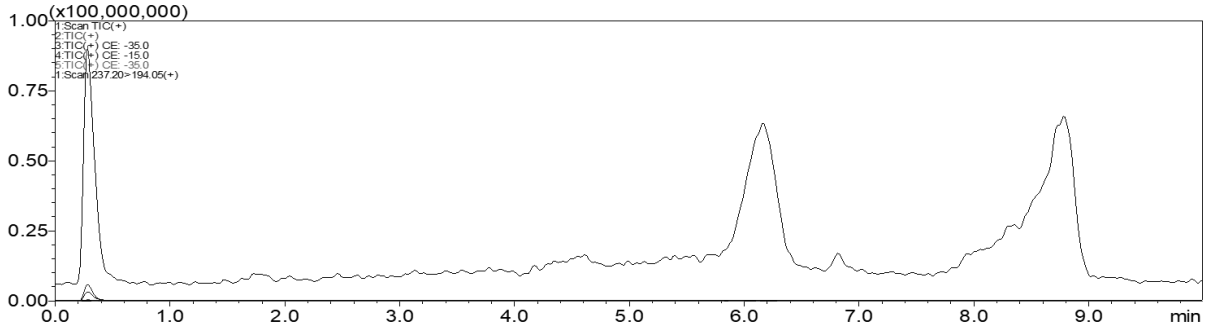


^{13}C NMR Monobenzyl protected polyethylene glycol linker

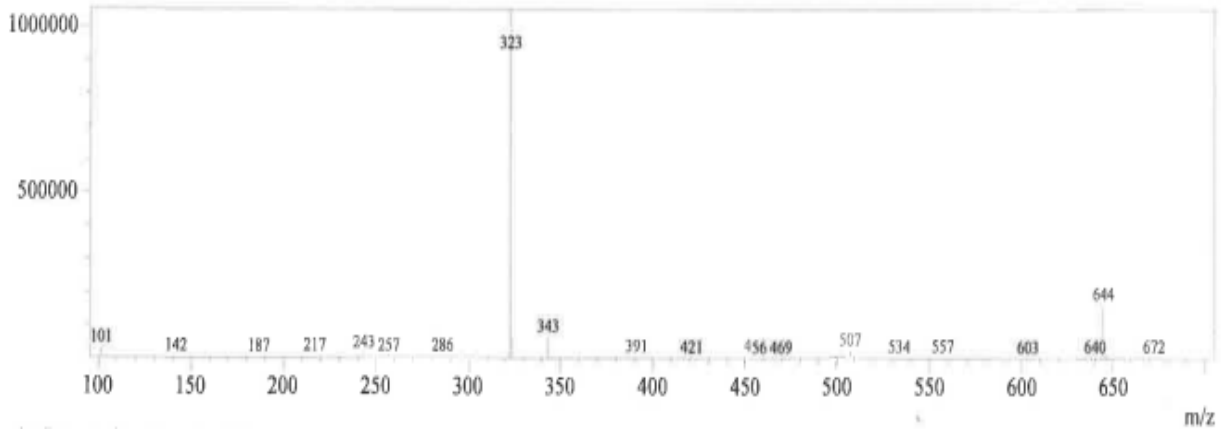


IR Monobenzyl protected polyethylene glycol linker

4.2 LCMS and MS spectra[22]



LC-MS chromotogram of MSH-4 peptide



Mass spectra of MSH-4 peptide

Fmoc Synthetic Strategy

Peptide ID: tetrapeptide
Resin Specs : ProTide Rink Amide Low Loading Resin (0.18 mmol/g)
N to C terminal: His-DPhe-Arg-Trp-NH_2

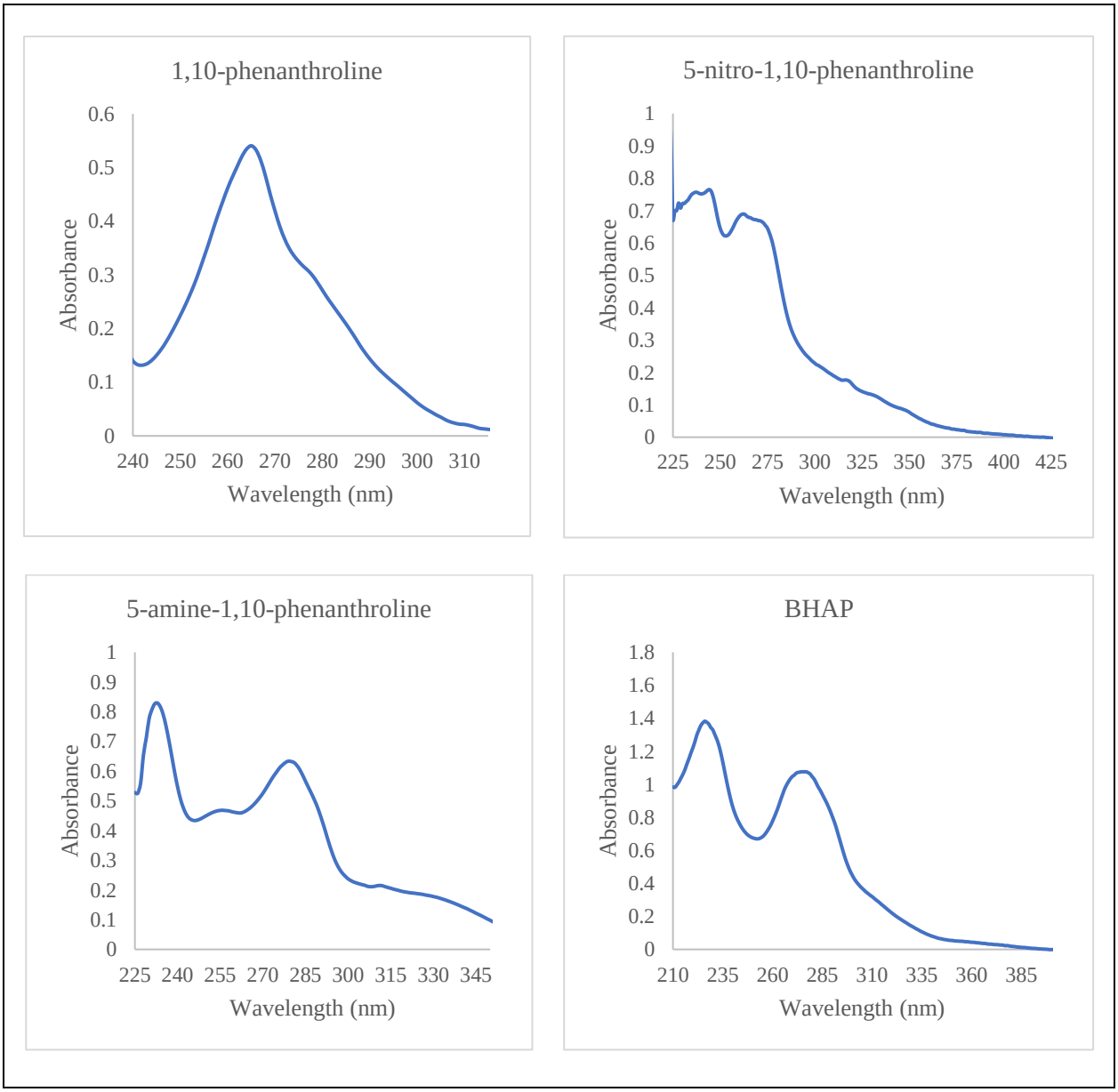
Date					
Amino Acid C to N terminal	**Trp**	**Arg**	**Dphe**	**His**	**Deprotect**
DMF rinse	◊◊◊	◊◊◊	◊◊◊	◊◊◊	◊◊◊
20% piperidine, "Rapid Dep" (30s @70˚C, 30s @75˚C)	◊	◊	◊	◊	◊
DMF rinse	◊◊◊	◊◊◊	◊◊◊	◊◊◊	
Ninhydrin (30s @120˚C)					Blue/Purple=go Yellow=repeat
Fmoc AA (3x excess)	◊	◊	◊	◊	
Oxyma Pure (5x excess)	◊	◊	◊	◊	
DIC (5x excess)	◊	◊	◊	◊	
Coupling (7 min @75˚C)					
DMF rinse	◊◊◊	◊◊◊	◊◊◊	◊◊◊	
Ninhydrin (30s @120˚C)					Yellow=go Blue/Purple=repeat
Recoupling (5 min @75˚C)					
DMF rinse	◊◊◊	◊◊◊	◊◊◊	◊◊◊	
Ninhydrin (30s @120˚)					Yellow=go Blue/Purple=repeat

Swell Resin by saturating in 50/50 DMF/DCM 20 min in microwave before starting
If left overnight before whole chain is finished, swell again before adding another AA

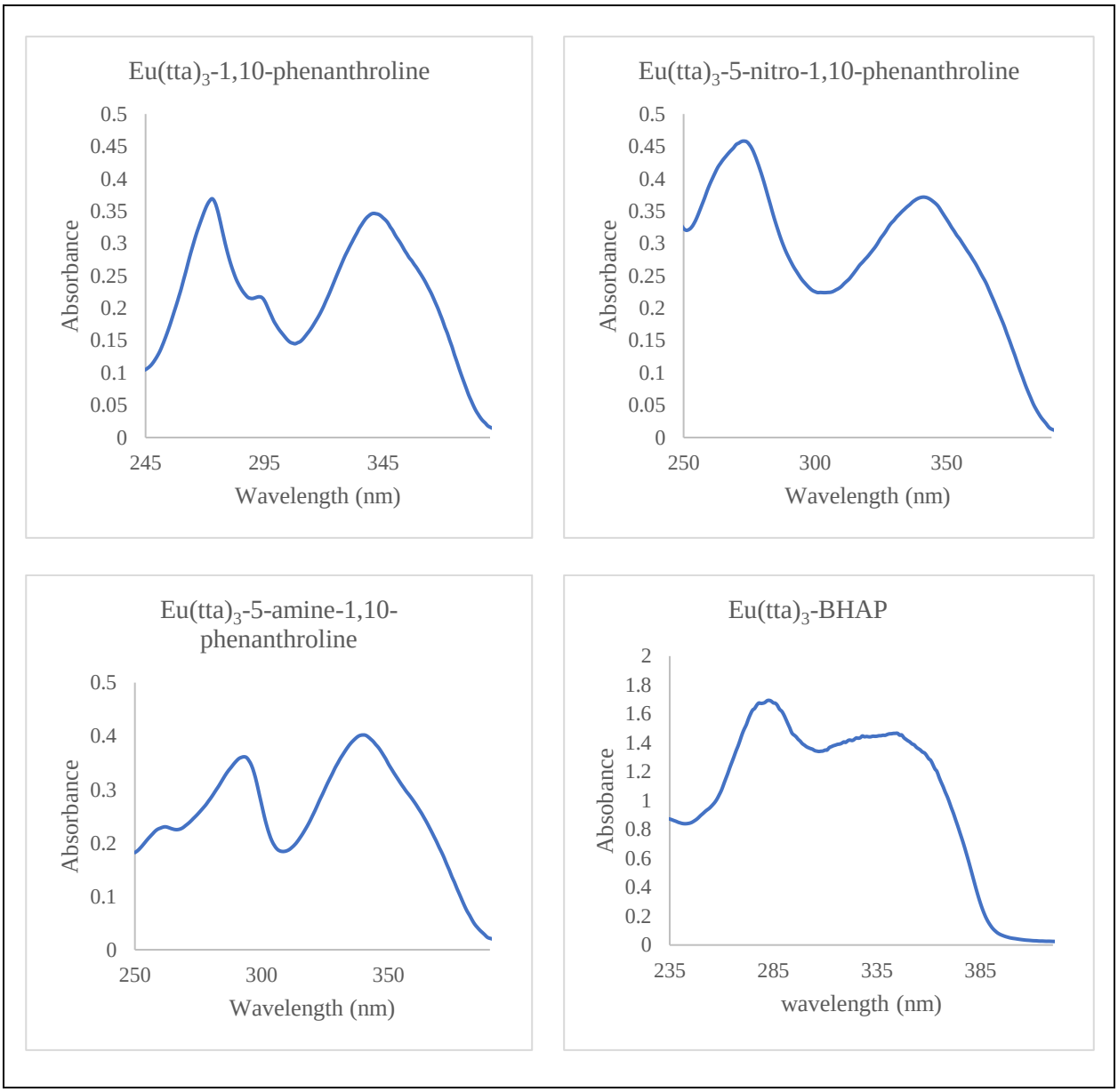
Combine separately in a synth vile before adding to microwave vessel

Peptide synthesis checklist

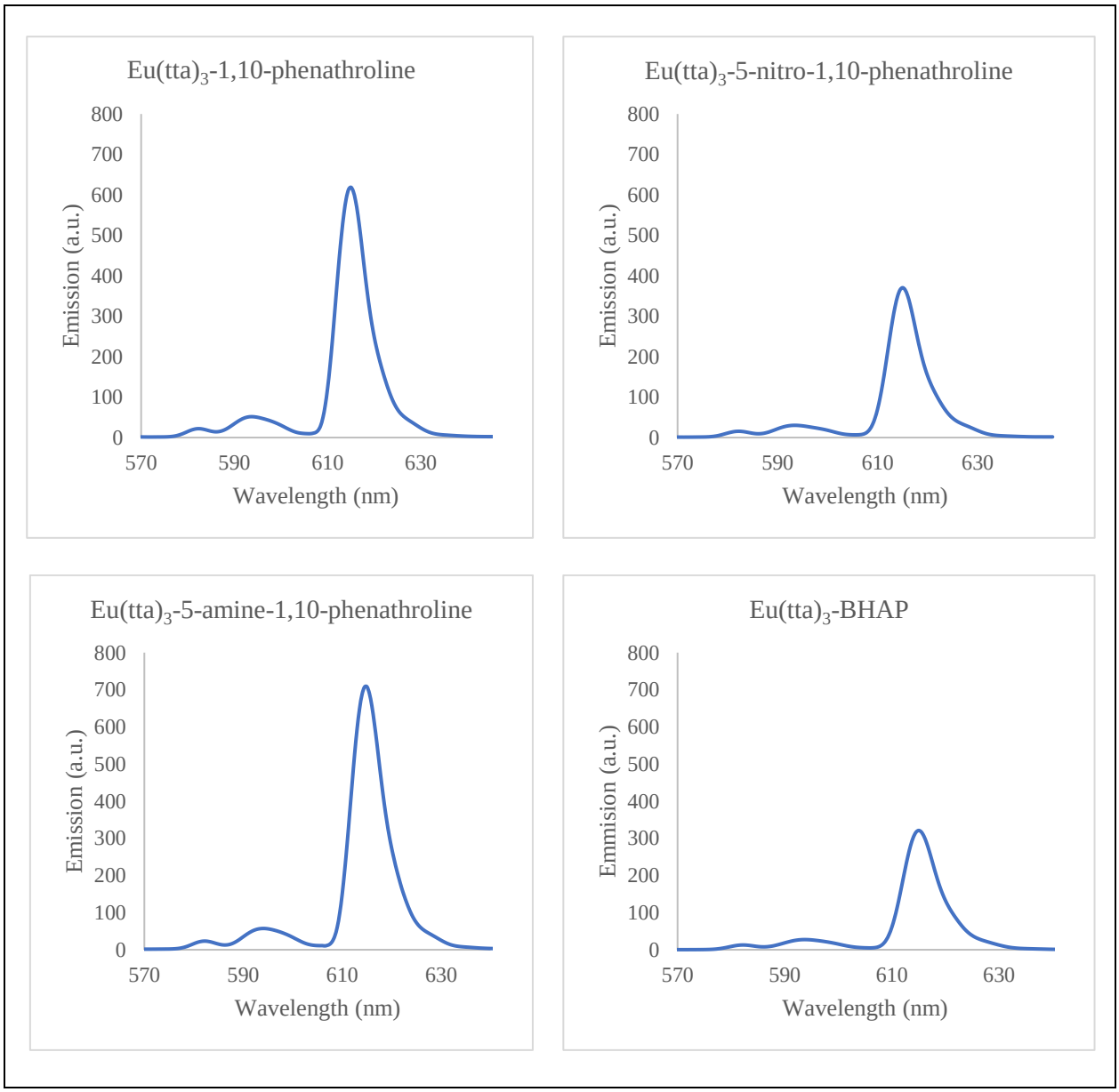
4.3 UV-Vis and Fluorescence spectra



UV-Vis spectra of phenanthroline ligands



UV-Vis spectra of $Eu(tta)_3$-phenanthroline complexes



Fluorescence spectra of $Eu(tta)_3$-phenanthroline complexes excited at 340 nm

4.3.1 Quantum Yield Table

Table 2: Quantum Yield Values

Complex	Quantum yield (%)	Std Dev
$Eu(tta)_3$phen	4.83	0.815
$Eu(tta)_3$nitrophen	2.78	0.523
$Eu(tta)_3$aminephen	5.02	1.14
$Eu(tta)_3$BPAH	0.417	0.107

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