OPTIMIZATION OF LINKER SYSTEM OF POTENTIAL MELANOMA SKIN CANCER PROBE

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 Chemistry.

By

Sarah Elizabeth Lee Ballard

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

Committee Members: Dr. Channa De Silva, Chemistry and Physics
Dr. William Kwochka, Chemistry and Physics

April 2019

ACKNOWLEDGMENTS

I would like to thank my committee members and director for their assistance and encouragement. In particular, Dr. Brian Dinkelmeyer for his guidance, patience, and expertise. Dr. Channa De Silva, for his expertise in the field of inorganic Chemistry and words of encouragement. Dr. William Kwochka for his help with synthesis methods and advice on trouble shooting. Without the help and support of my committee I would not have been able to expand my knowledge of chemistry so greatly and this project would have been much more difficult.

I also extend sincere thanks to the following people, without whom this thesis would not have been possible: My mother and father, Beth and Perry Ballard, for their continued support and love, without which I would not be here. My High school band director, Neil Underwood, who supported my dream to become a chemist by encouraging me to do what I love no matter what. My college adviser and head of the department of chemistry at Wingate University, Dr. Christopher Dahm, for his support and encouragement when times were hard during my undergraduate studies at Wingate University. My research adviser from Wingate University, Dr. Krista Wilson, for her knowledge and expertise in peptide synthesis as well as her support. My Hazardous Materials professor, Dr. James Hall, for his exciting classes and for helping prepare me for industrial chemistry. To the late Dr. Michael Gibson, my Organic professor from Wingate, who taught me several useful lab skills and how to live life to the fullest. To my adviser at Western Carolina University, Carmen Huffman, for her advice and patience.

To my friends and colleagues who have struggled with me and helped me along the way, thank you, you will never know how much your friendship has helped me.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABREVIATIONS

Bz-4EG	Monobenzyl tetraethylene glycol
Bz-4EG-CO_2H	1-Phenyl-2,5,8,11-tetraoxatridecan-13-oic acid
DCM	Dichloromethane
FT-IR	Fourier-Transform Infrared
GC-MS	Gas Chromatography mass spectrometry
GPCR	G-Protein Coupling receptor
PEGO	Polyethylene Glycol
HPLC	High performance Liquid chromatography
MSH-4	Melanocyte stimulating hormone
TTA	2-Thenoyltrifluoroacetone
IR(ATR)	Infra-red (attenuated total reflection)

ABSTRACT

OPTIMIZATION OF MELANOMA SKIN CANCER PROBE

Sarah Elizabeth Lee Ballard M.S.

Western Carolina University (April 2019)

Director: Dr. Brian Dinkelmeyer

The aim of our proposed research is to develop clinical tools for detecting cancer cells and to further expand methods for the early detection of cancer. The focus of this research is to optimize the synthesis of a probe for detecting melanocyte stimulating hormone(MSH) G-proteins that are present in melanocyte cancer cells. An europium complex was chosen since it is an excellent luminescent tag with a narrow emission band and has a long luminescent lifetime. MSH-4 peptide was selected as the analyte since it binds specifically with MSH G proteins in melanoma cells. The melanocyte stimulating hormone (MSH-4) and its analogs were synthesized via a solid phase peptide synthesis method using F-moc protocol. The lanthanide tag was synthesized by chelating 5-amine-1,10- phenanthroline to $Eu(TTA)_3(H_2O)$ complex. A tetraethylene linker was successfully synthesized and attached to the phenanthroline ligand. This was done by oxidizing the monobenzyl tetraethylene glycol to a carboxylic acid and adding it to the 5-amino-1,10-phenathroline amine using a standard peptide coupling procedure. The linker synthesis was found to be reproducible. The chelation of the ligand to the europium TTA complex was also completed. All compounds synthesized were characterized using the following instrumentation: GC-MS, UV-Vis, HPLC, FT-IR, NMR and fluorescence spectroscopy.

CHAPTER ONE: INTRODUCTION

The US Cancer Statistics Working Group states that, in 2014, 76,665 people were diagnosed with melanoma and of those who were diagnosed, 9,324 people died from melanoma.[1] Melanoma skin cancer is caused by the skin's or eye's over exposure to UV light which damages the DNA in the cell.[2] When melanocytes in the body become cancerous the usual result is a "mole or lesion"-like tumor on the surface which can spread out all over the body and lead to cancers forming elsewhere.[2] During the early stages of melanoma, the growth stays within 1mm in thickness and has not reached the blood vessels and can be removed fairly easily with a simple surgical procedure.[2] Studying melanocytes and their channeling pathways could lead to early detection of and fewer deaths from melanoma in the future.

Bioconjugation is a set of techniques which allows for site specific creation of a covalent bond between a biomolecule and a chemical that has the desired properties.[3] This hybrid having properties of both components can serve as a more useful tool for site specific conjugation.[3] This bioconjugation method for this project combines a melanocyte stimulating hormone (MSH) with a luminescent label that is separated by a polyethylene glycol linker. MSH (melanocyte stimulating hormone) corresponds to the melanocytes in the body which is part of the family of G-protein coupled receptors.[4] MSH has the potential to be a diagnostic tool for melanoma because it has been used in conjugation with cytotoxin and cytotoxic T-cells for killing melanoma cells by recognizing their MSH receptors.[4] G-protein coupling receptors or GPCR's are present in cells throughout most of the organs in the body.[5] This receptor is a globular polypeptide

embedded in a cell's surface which binds to a signaling molecule, causing a conformational change and activating a G-Protein on the interior of the cell acting like a switch, turning off or on through signal-receptor interactions on the cell's surface as seen in Figure 1.[5]

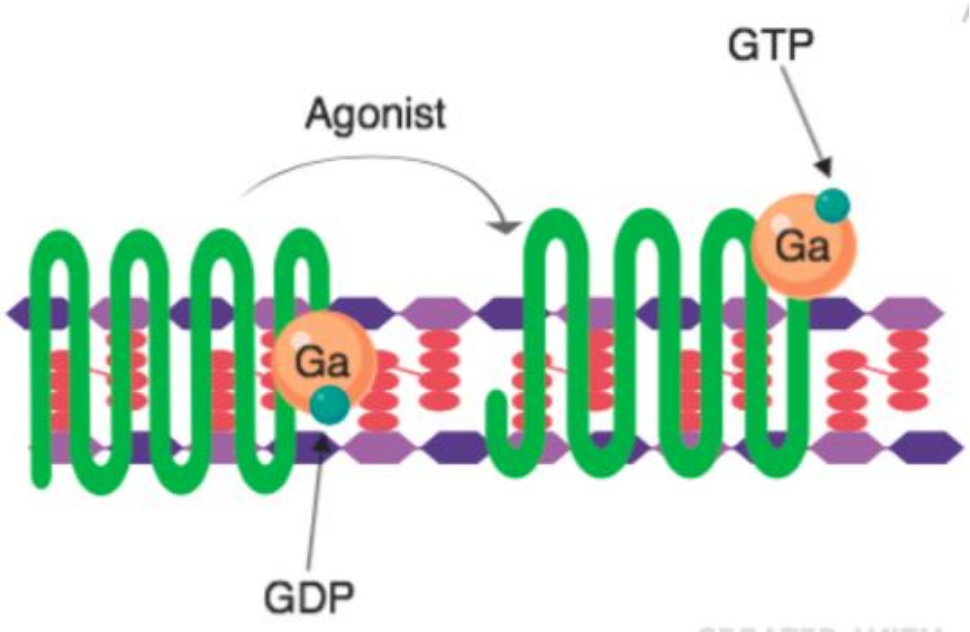


Figure 1: A G-Protein Coupled Receptor activating a cell's signaling pathway through the presence of an agonist (signaling molecule).[5]

The MSH- peptide was made using Solid phase peptide synthesis. Solid phase peptide synthesis is a method of synthesizing peptides on a solid support or resin by a C-terminus. The peptide is the built backwards from C-terminus to N-terminus which is opposite of how are bodies make peptides.[6] The amino acids(AA) are added on individually by first removing the protecting group on the current AA on the resin, a washing step to remove any soluble by-products, then coupling a protected AA, and followed by another wash step.[6] These steps are repeated until the desired amount of peptide chain length has been reached. The peptide remains attached to the resin

throughout the wash steps.[6] The peptide will then be removed with a final cleaving step which will be strong enough to separate the peptide from the solid support. [6]All of the reactions mentioned take place inside of a microwave that aids in speeding up the reaction.[6]

Lanthanides can be used for biological imaging due to their long lifetime and narrow emission bands.[7] Lanthanides exhibit these properties due to the shielding of the 4f orbitals by the filled 5s and 5p sub-shells.[7] This makes excitation of f-orbital electrons difficult and prevents an emissive state and makes f to f* transitions forbidden. Due to this low likelihood of electric dipole f-f* transition, direct excitation of the europium(III) ion is difficult, an organic chromophore ligand is used as an antenna to transfer energy directly to a europium(III) complex as represented in Figure 2 and Figure 3.[7]

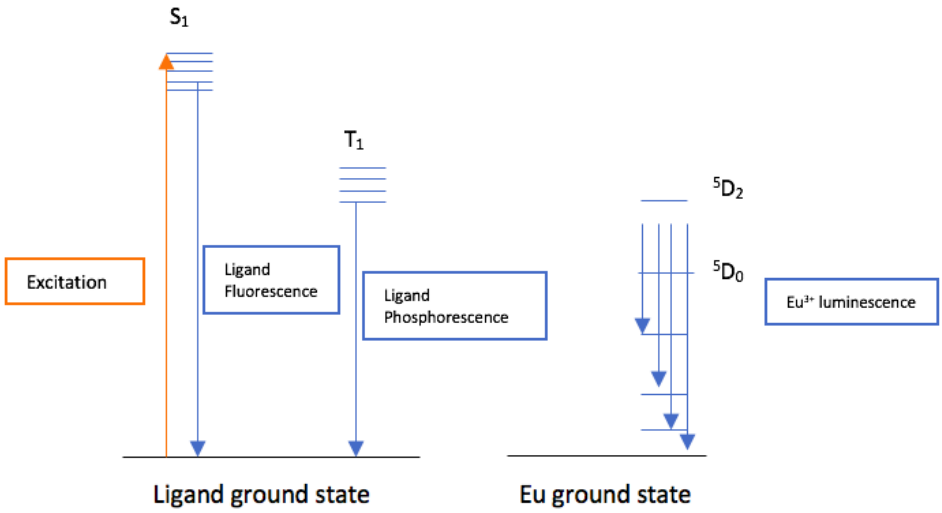


Figure 2: Jablonski diagram showing the transfer of energy from the ligand to the europium ion and to the ground state via luminescence.[7]

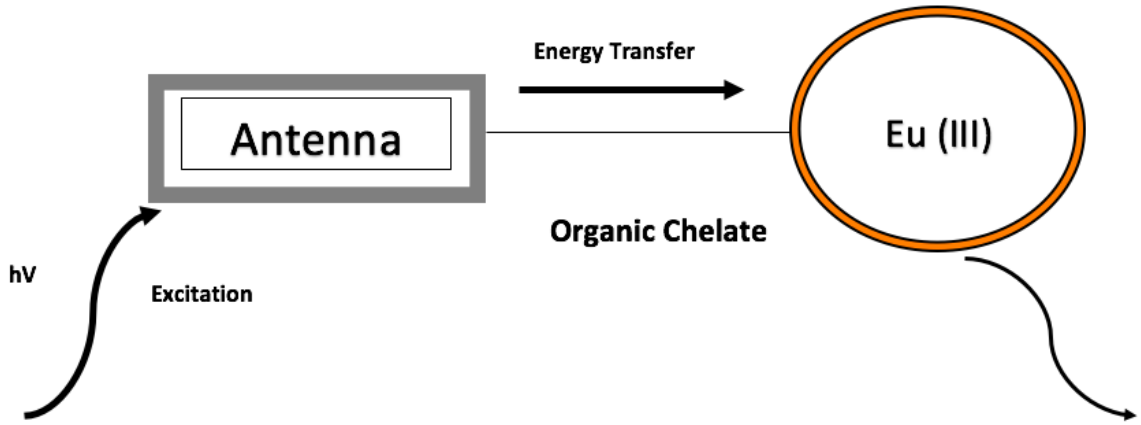


Figure 3: Illustration of the antenna effect where energy is absorbed by the organic chelate and transferred to europium metal.

Linkers are needed to separate the bioactive site from the luminescent tag. Tetra-ethylene glycol is commercially available and is commonly used in PEGylation synthesis, this will be used in the synthesis of this probe. Polyethylene glycol is a nontoxic and non-immunogenetic polymer that is used strategically to overcome disadvantages associated with some biopharmaceuticals.[8] Some of these disadvantages include conformation, electrostatic binding, and hydrophobicity.[8] Using PEGylation can improve pharmacokinetic behavior of drugs by changing the physical and chemical properties of the biomedical molecules.[8] By changing the way the cell interprets the molecule the cell will not begin to break down the bioconjugate and the bioactive portion of the molecule can function without being hindered sterically by the luminescent label.[8] The linker for this bioconjugate will be made with a benzyl protected polyethylene glycol (PEG) linkers through a modification of biological molecules by covalent bonding called PEGylation.[8]

The current probes used by dermatologist has short life only fluorescing for a short period of time. By having a longer life time and increasing the visibility of the probe, as compared to dyes that are currently being used today, this probe could aid in the further studies of melanoma skin cancer and could be used in the identification and removal of affected melanocytes. The goal of my part of the project is to optimize the synthesis of the linker system of the melanoma skin cancer probe in order to further separate the peptide and the tag. Without proper separation of the two active sites of this probe the two ends could hinder their effectiveness due to steric hinderance.

CHAPTER TWO: PROJECT DESIGN

The aim of our proposed research is to develop clinical tools for detecting and studying G-proteins in cancer cells and to further expand methods for the early detection of cancer. The essential feature of our design is attaching an easily detectable fluorescent probe molecule to a peptide substrate which specifically binds to melanocytes. A successful design must meet two criteria; First, the fluorescent probe must be easily detectable over the background fluorescence of biological samples. Second, the florescent probe must be attached to the substrate peptide such that it does not interfere with its normal binding to the G-protein binding site. This configuration is only possible if the label and peptide are separated by the linker enough to prevent steric interference. Our proposed structure is shown in Figure 4.

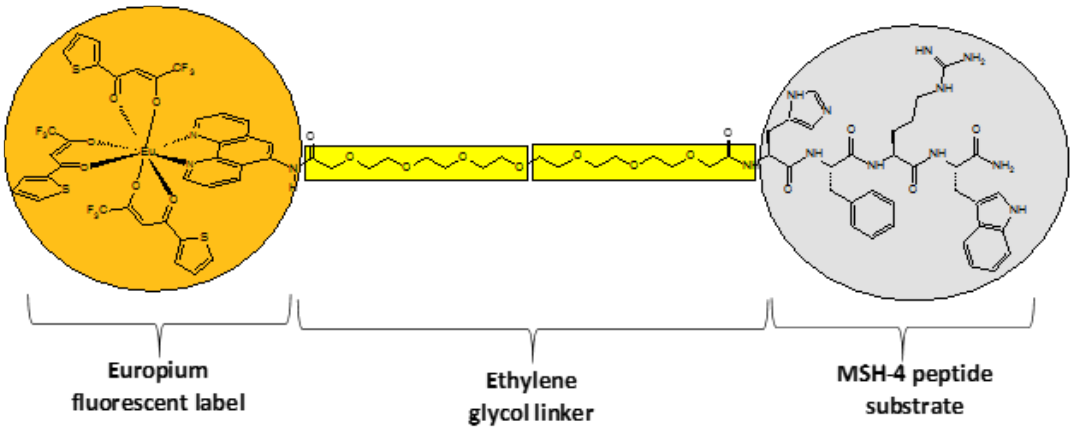
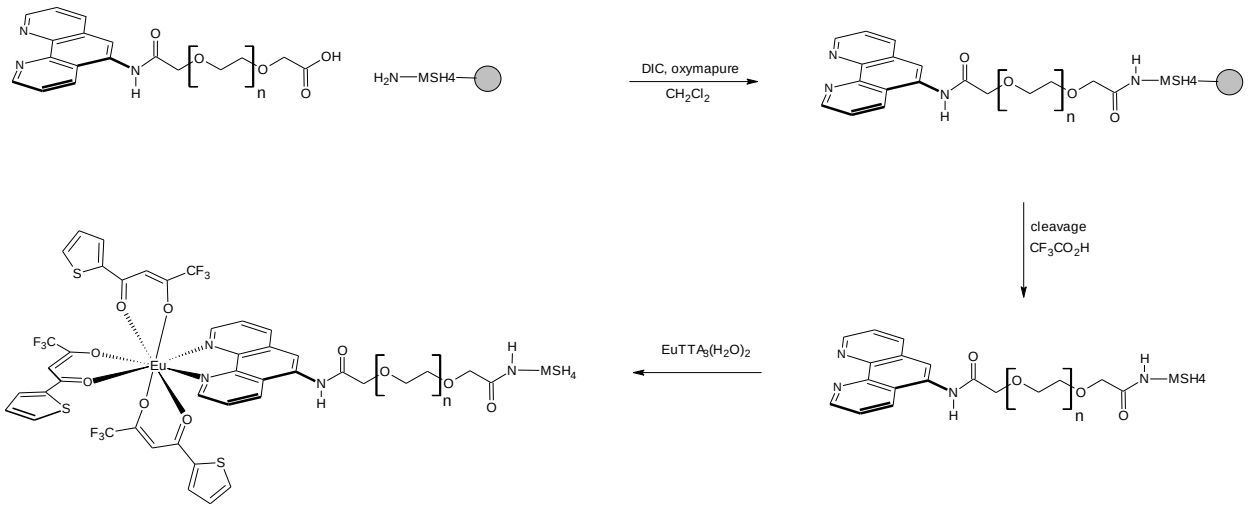


Figure 4: Generalized structure of the proposed probe.

The synthetic strategy that was settled upon involved first synthesizing the ethylene glycol linker-phenanthroline unit. This would then be attached to the MSH-4

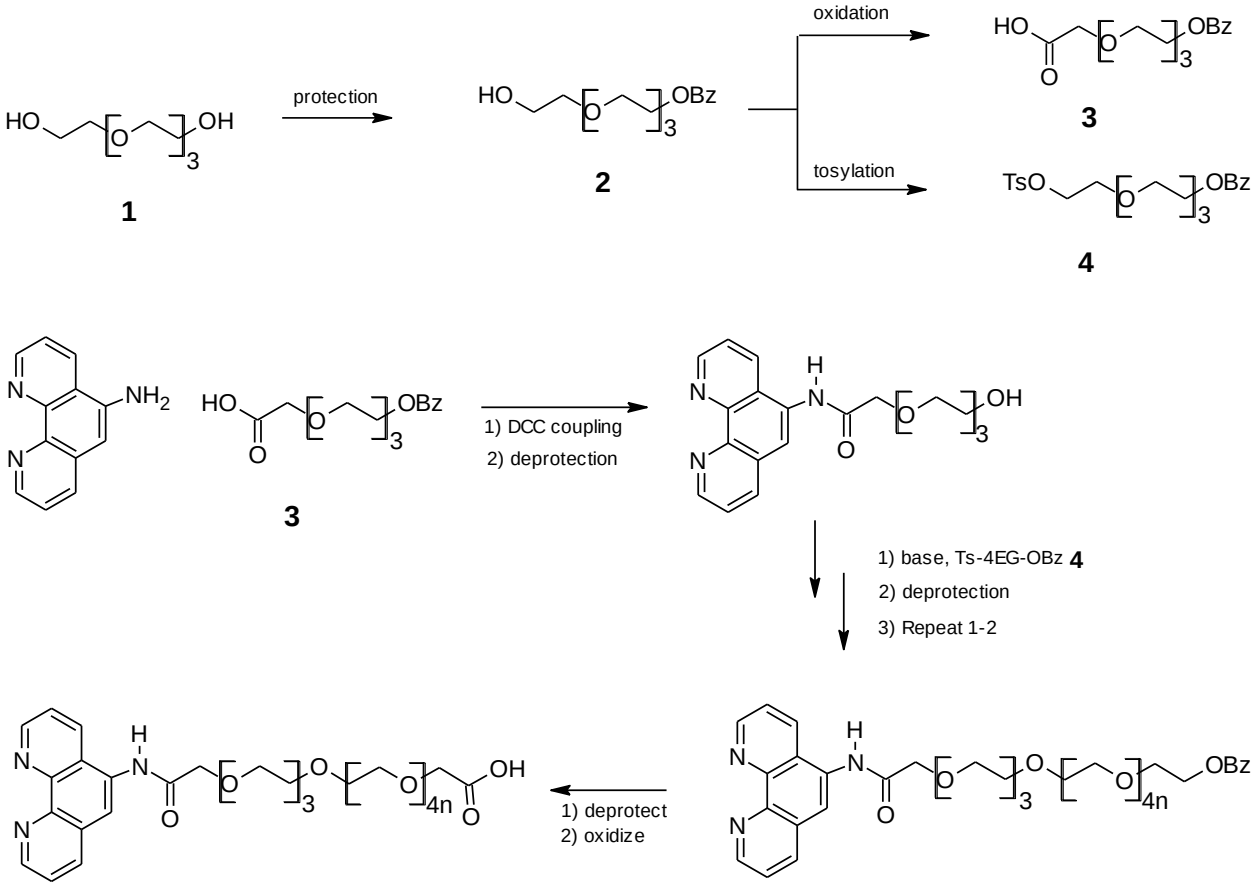
peptide using solid-phase peptide techniques (Scheme 1). The europium TTA complex phenanthroline will be formed last after the MSH4-linker-ligand compound is cleaved from the resin.



Scheme 1: Synthesis of MSH-4 (1) and attachment of the linker and ligand (2).

The proposed synthesis of the linker-phenanthroline unit is shown in Scheme 2. The plan entails synthesizing mono-benzyl protected tetra-ethylene glycol (**2)** which can be oxidized to a carboxylic acid (**3**) or tosylated to form compound **4**. The carboxylic acid terminated **3** is then coupled to the 5-amine-1,10-phenanthroline ligand. The length of the ethylene glycol unit can be progressively made longer by first removing the benzyl group followed by reaction with the tosylated compound **4**. Repetition of these steps will result in phenanthroline ligands with longer and longer ethylene glycol tails. Finally, the PEGylated phenanthroline ligands can be oxidized to create a carboxylic acid that can be coupled to the MSH-4 peptide on the solid support.

The first part of the probe synthesis, involves the synthesis of 5-amino-1,10-phenanthroline(**5**) ligand which will be used as to create the antenna effect will be made from commercially available 1,10-phanathroline. The linker system will be attached to this ligand in order to separate the two active ends of the probe. The linker system will be synthesized from benzyl protected tetra-ethylene glycol which has either been tosylated or oxidized to a carboxylic acid. The carboxylic acid linker can be attached to the 5-amine-1,10-phenanthroline and sequentially lengthened by deprotecting followed by reaction with the tosylated linker (Scheme 2).



Scheme 2: Proposed synthesis of ethylene glycol linker-phenanthroline probe.

The synthesis of the monobenzyl tetraethylene glycol (**2**) and tosylated glycol monobenzyl (**4**) compounds will be performed on a large scale to produce large quantities since these compounds will be used multiple times during the chain lengthening phase of the synthetic strategy. The 5-amine-1,10-phenanthroline (**5**), synthesized previously, will then be added to the 1-Phenyl-2,5,8,11-tetraoxatridecan-13-oic acid (**3**). The benzyl protecting group is removed and more of the linkers can be added on sequentially.

The MSH-4 peptide is made using solid phase peptide synthesis with a standard F-moc deprotection protocol on a solid support. This involves the deprotection of an F-moc-protected N-terminus and its subsequent coupling which is all done in a microwave synthesizer. The coupling is confirmed via Kaiser test and the process is repeated to yield highly pure peptides. The beta varieties of the MSH-4 has an additional Proline-Glycine on the end to provide more distance between the binding site and the luminescent tag. These peptides will be characterized using HPLC and LCMS.

After the synthesis of the MSH-4 peptide, the benzyl protected end of the linker attached to the ligand is deprotected and converted to a carboxylic acid. This is then coupled to the peptide which is on solid support. The ligand-peptide complex is then cleaved from solid support. After cleavage the Europium TTA (**7**) complex is added to the ligand completing the probe as shown in Scheme 2. The probe will be complete when the luminescent label is added as shown in Scheme 2.

CHAPTER THREE: EXPERIMENTAL

3.1 General information

Unless otherwise stated, all solvents and other reagents are commercially available and were used without further purification. All reagents were weighed and handled in air at room temperature. All thin-layer chromatography (TLC) was done with Sorbtech silica gel plated w/UV254. Silica used for all column chromatography was Silicycle R030B 40-63µm. The NMR used is a JEOL 300MHz Eclipse. Chemical shifts(δ) are quoted in ppm. Multiplicity is reported with the following abbreviations: s= singlet, d = doublet, t = triplet, q = quartet, dt = doublet of triplets, td = triplet of doublets, tt = triplet of triplets. The GC-MS used was an Agilent 7890A paired with a 5975C mass spectrometer capable of both electron ionization (EI). The IR used was a Perkin Elmer SpectrumONE FTIR with diamond ATR. The UV-Vis used was an Agilent Cary 5000 UV-VIS-NIR system. The Fluorometer used was a Perkin Elmer LS-55 spectrometer.

3.2 Synthesis and characterization of monobenzyl tetraethylene glycol (2)[9]

This synthesis was modified from the synthesis found in the Niederer, K. et. al. paper.[9] Tetra-ethylene glycol (60.0 mL, 60.54g, 0.312mol, 3.95eqv.) was added to 165 mL of aqueous NaOH solution (50% m/m) and was stirred at room temperature for 20 min.[9]The solution formed a gel that was difficult to stir.[9] Benzyl bromide (10.8mL, 15.552g, 0.091mol, 1.0 eqv.) was then added to the mixture solution. [9]The mixture was refluxed and stirred for 24 h and then cooled to room temperature.[9] The mixture was separated retaining the dark top layer.[9] The top layer was diluted with water (50 mL) and its pH adjusted to 4 with 2M HCl.[9] The acidified reaction mixture was extracted three times

with 50 ml diethyl ether. [9] The combined ether layers were washed with brine and dried over $MgSO_4$.[9] The solvent was removed by rotary evaporation providing 17.5457g of crude reaction mixture containing the mono and dibenzyl derivatives. The residue was purified by dry column chromatography eluting with 1:1 ethyl acetate/n-hexane while gradually increasing the proportion of ethyl acetate (Rf =0.5) to afford 6.6576g (6.1mL, 74%) of the desired product as a light yellow oil. H^1 NMR (300MHz, $CDCl_3$, TMS) :3.66(m,16H), 4.56(s, 2H), 7.33ppm(d,5H). ^{13}C NMR (300MHZ,CDCl3): 61.72, 69.39, 70.31, 70.58, 70.60, 72.90, 73.23, 127.59, 127.76, 128.34, 138.16ppm. IR(ATR): 3443.48, 2866.56, 1719.23, 1453.94, 1350.19, 1093.77, 939.42, 885.20, 738.56, 698.33 cm^{-1}. MS(ESI)10.479 minutes: 281.1 m/z, 207.0, 135.0, 117.0, 91.1, 58.1, 32.0. The structure is shown in Figure 5.

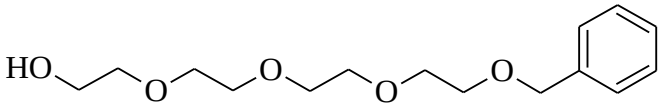


*Figure 5: Structure of Monobenzyl tetraethylene glycol (**2**).*

3.3 Synthesis and characterization of tosylated tetraethylene glycol monobenzyl (4)[10]

This synthesis was modified from the synthesis found in the Yoshimoto, M. et. al. paper.[10] In a dry round bottom flask, mono-benzyl tetra-ethylene glycol (0.519g, 0.00175mol, 1eqv.) and tosylchloride (0.8150g, 0.00427mol, 4eqv.) was dissolved in dry methylene chloride(28.5mL) and KOH (0.2407g, 0.00439mol, 2.5eqv.) was added.[10] This mixture was then cooled to -10°C with an ice/salt bath and slowly over 5 minutes. The mixture was allowed to stir and reach room temperature overnight becoming less

translucent as the reaction progressed.[10] Then a saturated aqueous ammonium chloride solution was added followed by extraction with methylene chloride.[10] The organic layers were then washed with saturate ammonium chloride and sodium bicarbonate and then with ammonium chloride again. The mixture was then dried with brine and magnesium sulfate. The solvent was removed by rotary evaporation to produce clear almost colorless oil. Flash column chromatography was performed using 1:1 hexanes to ethyl acetate.(100:0 v/v, Rf = 0.25). the yield was 0.586g (39% yield). H^1NMR (300MHz, $CDCl_3$, TMS): 2.43(s,3H), 3.67(m,14H), 4.12(t,3H), 4.56(s,2H), 7.34(m,5H), 7.82(d,2H). ^{13}C NMR (300 MHZ, $CDCl_3$): 68.75, 69.33, 69.49, 70.69, 73.32, 127.15, 127.85, 128.07, 128.45, 129.90, 130.32, 133.07, 138.29 ppm. IR (ATR): 2869.95, 1701.06 , 1597.17, 1495.81, 1453.59, 1292.67, 1354.45, 1249.67, 1189.15, 1017.66 , 915.86, 814.22, 774.13, 747.79 , 699.05, 662.55, 654.93,571.16, 528.59 cm^{-1}. MS(EIS): 355.0 m/z, 327.1, 281.1, 243.1, 199.0, 155.0,119.0, 91.0, 45.0. The structure is shown in Figure 6.

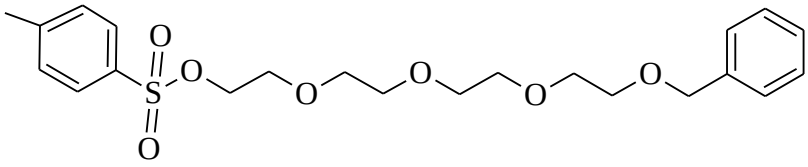


*Figure 6: Structure of Tosylated Tetraethylene glycol monobenzyl (**4**).*

3.4 Synthesis and characterization of 5-amino-1,10-phenathroline(5)[11]

This synthesis was modified from the synthesis found in the Binnemans, K. et. al. paper.[11] 5-nitro-1,10-phenanthroline (2.0196g, 8.975mmol, 1.0 eqv.) was added to a 250 round bottom flask and dissolved in ethanol(90.2mL) and sonicated for 1 hour at room temperature.[11] After dissolution the solvent was purged with argon and a 10%

Pd/C catalyst (0.09g) was added along with a stir bar.[11] The reaction was capped with a septum and purged with argon, hydrazine monohydrate (7.6mL, 0.1569mol, 17.5 eqv.) was added in slowly.[11] The reaction was heated to 65°C and stirred over-night. After reaction was complete the catalyst was removed via vacuum filtration. After filtration the solution was dissolved in chloroform and washed thrice with water to remove the excess hydrazine monohydrate.[11] The organic layer was then placed under reduced pressure and the product was a yellow/green color 0.8121g(53.6% yield). H^1 NMR (300MHz, CDCl3, TMS): 1.237ppm (m,1 H)1.689ppm (s, 5H) 4.903ppm(s,4H), 6.941ppm (s,1H), 7.500ppm (m,1H), 7.652 (m,1H), 7.962 (m, 1H), 8.263 (m,1H), 8.936(m,1H), 9.194 (m,1H). ^{13}C NMR (300MHZ,$CDCl_3$): 106.59, 126.64, 126.89, 135.39, 135.65, 137.54, 137.54, 137.54, 145.30, 147.51, 149.63, 150.98, 154.16, 177.74 ppm. IR(ATR):3335.53 cm^{-1}, 2866.56 cm^{-1}, 1722.49 , 1633.90, 1611.04, 1488.83 , 1406.97, 1270.98, 1133.96 , 802.28, 736.85, 707.52 , 531.87 cm^{-1}. The structure is shown in Figure 7.

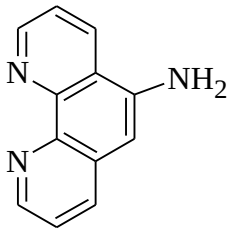


*Figure 7: Structure of 1,10-phenathroline-5-amine (**5**).*

3.5.a Synthesis and characterization of 1-Phenyl-2,5,8,11-tetraoxatridecan-13-oic acid (3)[12]

This synthesis was modified from the synthesis found in the Kichler, A et. al. paper.[12] Monobenzyl tetraethylene glycol (2.0151g, 0.00696mol,)was dissolved in acetone (92

mL) in a 250mL round bottom flask. Jones reagent (4.0 ml), consisting of 25g CrO_3 in 25mL of concentrated H_2SO_4 and 75mL H_2O was added to the round bottom slowly over 20min with stirring.[12] The mixture was stirred at ambient temperature for 1 hour. To this mixture was then added 3mL of 2-propanol and allowed to stir or twenty minutes.[12] Water (61.2 mL)was then added and the mixture vacuum filtered to remove excess chromium oxide.[12] The acetone was removed by rotary evaporation. An extraction was preformed using dichloromethane(DCM) in 20 mL increments totaling up to 12 times.[12] The organic layers were then dried with magnesium sulfate and the DCM was removed under vacuum pressure. The resulting oil was then placed under vacuum to remove solvent and the oil was placed in the freezer. Yield was 2.176g (38%).

3.5.b Purification of 1-Phenyl-2,5,8,11-tetraoxatridecan-13-oic acid (3) via ion exchange chromatography.[13]

This purification step was done via ion exchange resin modified from the procedure developed by Bookser, B. C. et. al.[13] The ion exchange resin used to purify was Dowex1X2-400(5.053g), which was swelled in methanol(25mL) overnight then drained off the methanol.[13] Then some of the carboxylic acid(1.0076g,0.0034mol,1eqv.) and DIEA(0.8799g,0.068mol, 2eqv.) were mixed with 25ml of methanol and then added to the resin.[13] This mixture was allowed to sit for 1 hour.[13] Then the resin was washed with methanol and the wash was collected.[13] To the resin was added a solution of 95:5 Methanol: TFA (100mL) and allowed to sit for 30 minutes then the solvent was collected and the resin was washed with more of the 95:5 Methanol: TFA(100mL) this was collected and the solvent was removed via vacuum.[13] The light yellow oil was collected and determined to be two separate carboxylic acids according to the GC-MS. H^1

NMR(300MHz,CDCl3,TMS): 3.62-3.74 (m,13H), 4.13-4.15 (d,2H), 4.58(s, 2H), 7.25-7.33(m,5H). ^{13}C NMR (300MHZ,CDCl3): 0.073, 51.883, 68.710, 69.482, 70.726, 71.395, 73.331, 74.202, 127.853, 138.291, 171.015ppm. IR(ATR):3348.08 cm^{-1}, 2875.57, 1734.82 , 1453.91, 1351,19, 1218.46, 1086.34, 1017.67, 943.83, 848.11, 740.84, 699.05 cm^{-1}. MS(ESI) 9.785 minutes : 268.1 m/z, 209.2, 191.0, 162.1, 135.1, 116.1, 91.1, 59.1, 39.1. MS(ESI) 10.864 minutes: 340.9 m/z, 312.2, 269.1, 237.3, 206.1, 179.1, 148.1, 117.1, 91.1, 59.1, 31.1. The structure is shown in Figure 8.

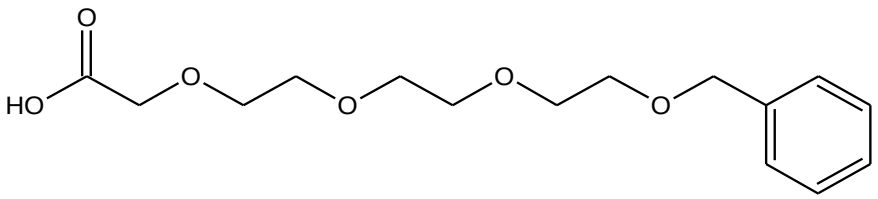


Figure 8: Structure of 1-Phenyl-2,5,8,11-tetraoxatridecan-13-oic acid (3).

3.6.a Synthesis and characterization of N-(5-phenanthryl)-12-benzyl-3,6,9,12-tetraoxa dodecamide (Phen-4EG-BZ) (6)[14]

This synthesis was modified from the synthesis found in the Elmes, R. B. P. et. al. paper.[14] To a 250mL round bottom flask was added 5-amine phenanthroline(0.5056g, 2.59mol, 1eqv.) dissolved in 100mL of DCM and cooled to 0°C via ice bath.[14] The 1-Phenyl-2,5,8,11-tetraoxatridecan-13-oic acid (0.653g, 2.19mmol, 1eqv.) was added to the flask.[14] DMAP(0.31g, 2.537mmol) and EDCI(1.23g, 7.91mmol) are added and the flask and the mixture is allowed to stir at 0°C for an hour and allowed to reach room temperature over-night.[14] The solvent was removed from the mixture and then water(50mL) was added to the reaction.[14] The water mixture was then extracted with DCM. The organic layers were collected and the solvent was removed from the product to afford a brownish-

green solid(0.4073g).[14] This solid was then dissolved in DCM and then ran through a silica plug using 1:25 methanol to chloroform as a eluent to remove unreacted 5-amine phenanthroline.[14] The sample was then dissolved in methanol (25mL) and purified via ion exchange resin.[13]

3.6.b Purification of N-(5-phenanthryl)-12-benzyl-3,6,9,12-tetraoxa dodecamide (Phen-4EG-BZ) (6)[13]

This purification step was modified from the Bookser, B. C. et. al.[13] The ion exchange resin used to purify was Dowex1X2-400(5.278g), which was swelled in methanol(25mL) overnight then drained off the methanol.[13] Then the dissolved solid was added to the resin.[13] This mixture was allowed to sit for 1 hour. Then the resin was washed with methanol and the wash was collected.[13] To the resin was added a solution of 95:5 methanol: TFA (100mL) and allowed to sit for 30 minutes then the solvent was collected and the resin was washed with more of the 95:5 methanol: TFA (100mL)this was collected and the solvent was removed via vacuum.[13] The first wash of methanol was collected and the solvent was removed under vacuum yielding a dark orange solid(0.476g, %). The methanol: THF wash was found to contain unreacted carbocyclic acid. DIEA was found to still be in the final product. H^1 NMR(300MHz,DMSO D-6,TMS):1.24-1.29 (m,1H),3.51-3.62(m,15H), 4.12(s, 2H), 4.47(s, 2H), 6.91(s, 1H), 7.26-7.34(m, 5H), 7.61-7.65(m,1H), 7.80-7.84(m, 1H), 8.22-8.25(m,1H), 8.69-8.71(m,1H), 8.76-8.79(m,1H), 9.09-9.10(m,1H). IR(ATR): 3416.51 cm^{-1}, 3322.44, 3208.13, 2863.63, 2083.69, 1754.28, 1635.6, 1610.32, 1592.67, 1552.67, 1543.56, 1504.88, 1489.03 , 1455.50, 1428.02, 1406.73, 1302.57, 1271.70 , 1254.34 , 1215.84, 1107.28,

1032.94, 885.85, 841.79, 825.85, 812.54, 792.22, 738.51, 707.05, 650.88, 620.62 cm^{-1}. The structure is shown in Figure 9

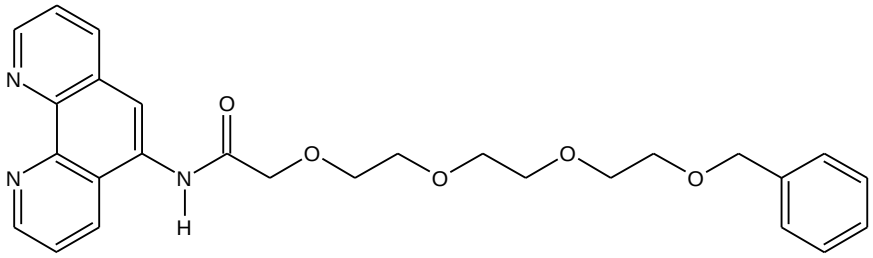


Figure 9: Structure of Phen-4EG-BZ (6).

3.7 Synthesis and characterization of monotetrahydropyran-protected tetraethylene glycol (THP-4EG)[15] (7)

This synthesis was modified from the synthesis found in the paper by Loiseau, F. A. et. al.[15] To a 1000mL flask was added Tetraethylene glycol (100mL, 0.579mol, 4.9eqv.) and (0.0360g,0.209mmol,1.8eqv.)p-toluene sulfonic acid.[15] This was dissolved in dichloromethane (300mL) and allowed to stir at room temperature.[15] To this mixture was added dropwise 3,4 dihydro-2,4-pyran 99% (9.9657g, 0.1185mol, 1eqv.).[15] This mixture was allowed to react over-night while stirring.[15] Half of the solvent was removed via rotary evaporation.[15] Water(300mL) was then used to wash the mixture, the organic layer was collected then washed with a brine mixture (100mL) six times.[15] The organic layer was then dried using sodium sulfate.[15] The remaining solvent was removed from the organic layer resulting in a clear oil (12.8846g). The oil was then proven to be a 8 to 1 ratio of mono substituted to di substituted linker via GC-MS. H^1NMR (300MHz, CDCl3, TMS): 1.50-1.60(m,6H), 2.76-2.78(m,1H), 3.50-3.70(m,15H), 3.85-3.87(m, 2H), 4.61-4.63(m,1H). C13 NMR (300MHZ,CDCl3):19.39, 25.36, 30.46, 62.69, 70.30, 70.49,

70.53, 70.56, 70.59, 72.50, 98.88ppm. IR(ATR): 3450.21 cm^{-1}, 2939.24, 2868.64, 1454.79,1349.99, 1284.86,1258.79,1201.55,1184.01 ,1120.70 , 986.70 , 929.61, 871.68 , 813.71, 792.27 cm^{-1}. MS(ESI)13.141 minutes: 195.0 m/z, 165.0, 150.9, 133.0, 119.0, 101.0, 85.0, 72.0, 58.1, 45.0, 32.0. MS(ESI)16.019 minutes: 280.8 m/z, 259.0, 206.9, 188.8, 164.9, 133.0, 114.9, 85.0, 55.0, 32.0. The structure is shown in Figure 10.

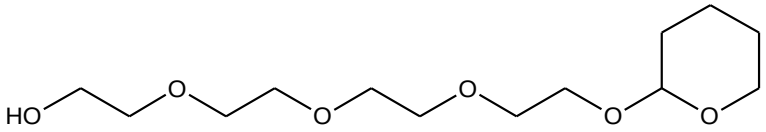


Figure 10: Structure of Monotetrahydropyran-protected tetraethylene glycol (THP-4EG).

3.8 Europium(III)$(TTA)_3$ $(H_2O)_2$(8)[16]

This synthesis was modified from the synthesis found in the Leonard, J. P. et. al. paper.[16] 2-Thenoyltrifluoroacetone (0.67 g; 3 mmol, 3eqv.) was dissolved in 1M NaOH (3 mL) and water (20mL).[16] A solution of $EuCl_3 \bullet 6H_2O$ (0.37 g; 1 mmol,1eqv.) in 10 mL of water was made.[16] The two solutions were stirred separately until completely clear the TTA solution was added dropwise to the europium solution.[16] A precipitant formed and was removed via vacuum filtration and washed with water (200 mL). the precipitant was then washed with diethyl ether to dry the solid. Yield was 0.689g (81% yield) of solid europium(III)$(TTA)H_2O$. IR(ATR): 3356.28 cm^{-1},1600.19, 1580.90, 1538,1509, 1459, 1406.84, 1356.77, 1285.55,1247.95, 1230.82, 1131.99, 1080.82, 1051.10, 1037.14, 929.00, 850.11, 786.77, 751.11, 734.02 , 711.07, 698.81, 680.58 , 604.08. UV/Vis λ_{max}: 343 nm. Fluoresce λ_{max}: 615.5 nm. The structure is shown in Figure 11.

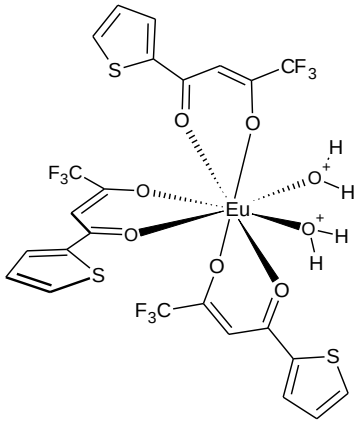


Figure 11:Structure of Europium(III)(TTA)3 (H2O)2 Complex.

3.9 Europium(TTA) 5-amine phenanthroline(9)[11]

This synthesis was modified from the synthesis found in the Binnemans K, et. al. paper.[11] To a 100ml Round bottom flask was added Europium(TTA)3 H_2O (0.1090g, 0.1303mmol, 1eqv.) and dissolved in DCM (30mL).[11] In a separate flask 5-amine phenanthroline(0.0236g, 0.1303mmol, 1eqv.) was dissolved in DCM(30mL).[11] The 5-amine phenanthroline mixture was added to the europium TTA mixture and stirred at 60°C for 30 minutes.[11] After heating, the mixture was allowed to stir overnight.[11] The mixture was placed in a scintillation vile and the solvent was allowed to evaporate out leaving a very small amount of solvent that will be removed via vacuum filtration.[11] The crystal yield was 0.0365g (30% yield). IR (ATR):1598.14 cm^{-1}, 1588.94, 1505.95, 1450.58, 1412.26, 1355.88, 1305.95,1248.92, 1231.71, 1192.50, 1132.55, 1062.85, 933.34, 859.32, 787.07, 734.93, 714.76, 683.05, 640.85. UV/Vis λ_{max}: 340 nm. Fluorescence λ_{max}: 615 nm. The structure is shown in Figure 12.

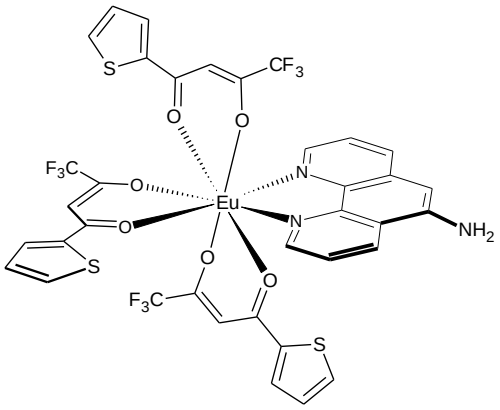


Figure 12: Structure of Europium(TTA) 5-amine phenanthroline Complex.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Statement of previous plans and redirection

The project is not complete but significant progress has been made. Linker synthesis has been optimized. The coupling of the linker to the ligand has been achieved. The synthesis of the europium TTA water was achieved. The chelating of **8** to the ligand (**5**) has been achieved. Our initial synthetic strategy has changed due to time constraints and technical difficulties. Originally, ethylene glycol linkers of varying length were to be synthesized by systematically combining monobenzyl mono-tosylated tetraethylene glycol (**4**) with THP protected tetra ethylene glycol (**7**). This would produce a longer ethylene chain with different protecting groups at each end. Selective removal of the THP protecting group followed by addition of **4** would lengthen the chain by four ethylene glycol units. Repeating the process would lengthen the chain further as seen in Figure 13.

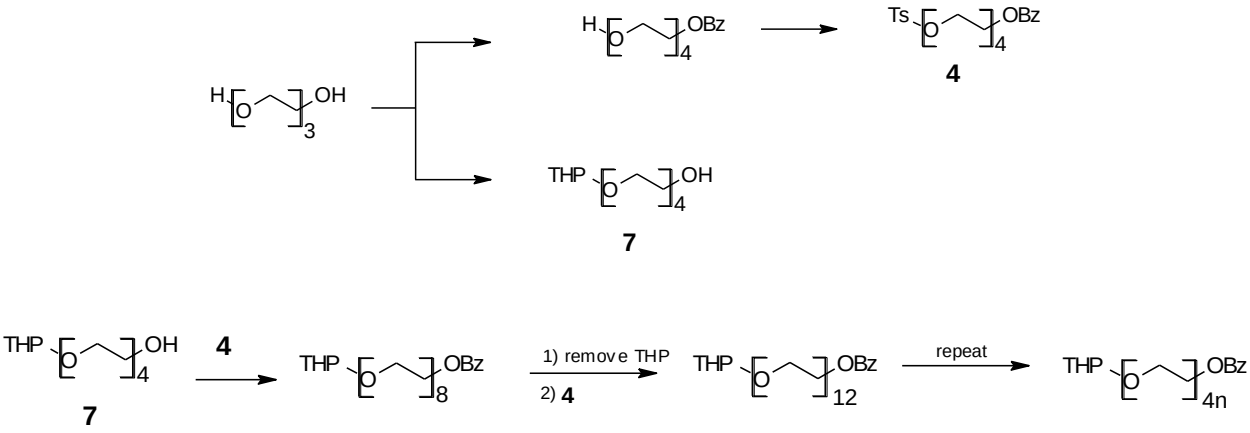


Figure 13: Initial Synthetic Strategy.

The Benzyl/THP protected chains would be deprotect and tosylate followed by attachment to 5-aminophenathroline by an SN2 reaction (Fig.14). This synthetic plan ultimately failed and required us to reevaluate the strategy. The amine group on the 5-amine 1,10-phenathroline was found to be a poor nucleophile because of the nitrogen lone pair electrons are stabilized by resonance to an electron withdrawing aromatic ring. Additionally, the reaction was moisture sensitive and drying reactants and reagents was problematic. Both the ethylene glycol compounds and the 5-aminophenathroline **5** were extremely hygroscopic.

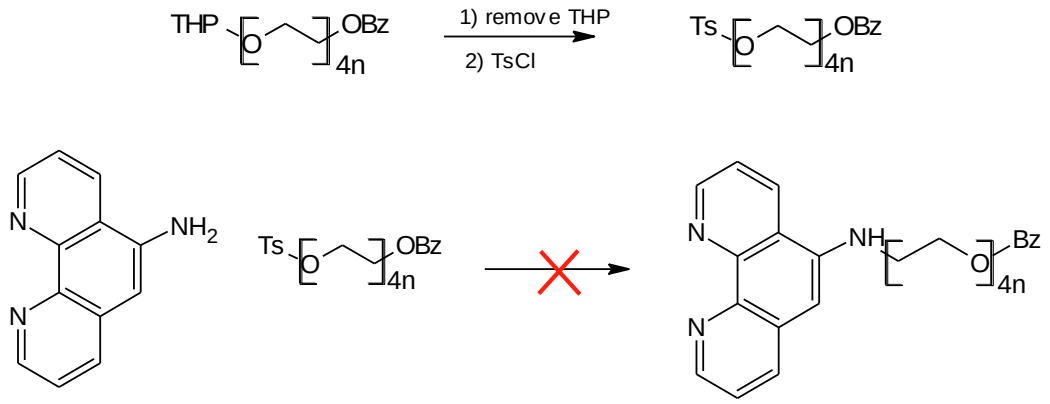


Figure 14: Unsuccessful coupling attempt of tosylated monobenzyl tetraethylene glycol to 5-amine 1,10-phenathroline.

The original reaction was preformed using toluene as a solvent with triethylamine to deprotonate the amine group, this was found to be unsuccessful. Great care was taken to dry all of the solvents and reagents used. Solvent stills were used to dry both the toluene and triethylamine. The 5-aminophenathroline was dried by removing water as an azeotrope from toluene. Despite these careful precautions the reaction was still unsuccessful. Other literature procedures with different solvents and bases were then tried. The toluene/triethylamine was replaced with dry THF/ K_2CO_3 which this was also

unsuccessful. The reaction was attempted with microwave heating which was also unsuccessful. After multiple attempts and months of research the synthetic plan was shortened and changed.

The new synthetic plan relied on oxidizing the monobenzyl tetraethylene glycol to a carboxylic acid. This would then be added to the 5-amine1,10-phenanthroline(5) using techniques generally used in peptide synthesis (Figure 15). This was determined to be plausible and the synthesis was carried out using DMAP and EDIC to couple the carboxylic acid(3) to the amine group(5), this was successful. The plan for addition of the linkages will now be to add the tosylated monobenzyl tetraethylene glycol(4) on to the coupled reaction(6) after removal of the benzyl group. The following sections detail the progress made in synthesizing the phenanthroline linker system.

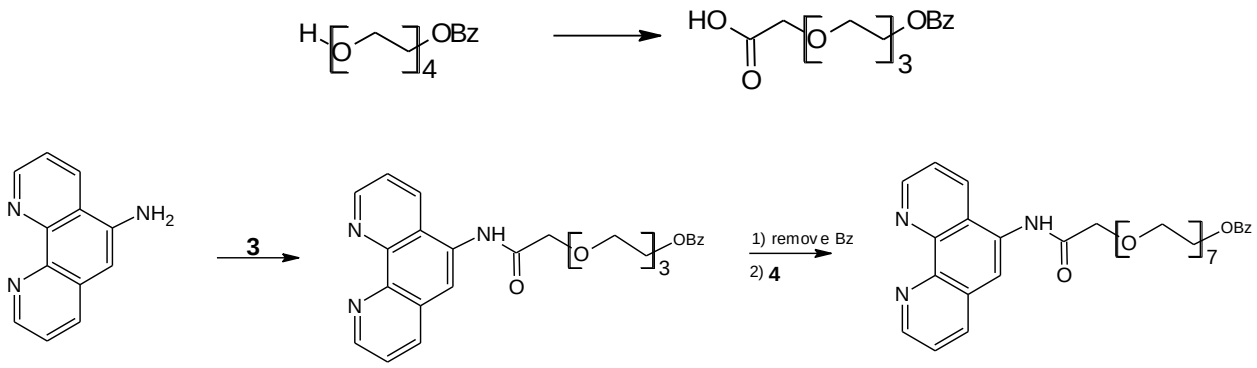


Figure 15: New coupling synthesis.

4.2 Synthesis and characterization monobenzyl tetraethylene glycol (2)[9]

This synthesis was modified from the synthesis found in the Niederer, K. et. al. paper.[9] Tetra-ethylene glycol was added to aqueous NaOH solution and was stirred at room temperature for 20 min this mixture was very thick and was a yellowish color. After

adding the benzyl bromide the solution formed an orange top layer with a cream colored liquid at the bottom. After refluxing for 24 h and then cooled to room temperature the top layer as now thick and burgundy, while the bottom layer was still a cream color. The mixture was separated retaining the dark top layer. The top layer was diluted with water and acidified dark solids were observed. The acidified reaction mixture was extracted three times with diethyl ether and the clear combined ether layers were washed with brine and dried over $MgSO_4$. The solvent was removed yielding a dark transparent orange oil containing a mixture of mono and dibenzyl derivatives. The residue was purified by dry column chromatography yielding a light yellow oil.

The H^1 NMR spectrum confirmed that the reaction was a success. The singlet at 4.56ppm corresponds to the CH_2 benzyl hydrogens. The multiplet at 7.33ppm corresponds to the five hydrogens of the benzyl group. The ^{13}C NMR showed the 11 expected carbon peaks. GC-MS was used to determine purity and the presence of functional groups. A molecular ion peak could not be found because ethylene glycol chains fragment easily in ESI MS. The mass spectra of that peak indicated presence of the benzyl group giving a large peak at 91.1m/z. The product was analyzed via IR and confirms the presence of the benzyl group at approximately 3100 cm^{-1} (aromatic peak). The tetraethylene glycol at 1350.19 cm^{-1} (C-O stretch) and a strong 1093.77 cm^{-1} (C-O stretch). Also the primary alcohol at 3443.48cm^{-1} (OH stretch). (H^1 NMR , C^{13} NMR, IR, and GC-MS spectra found in supplemental material section.)

4.3 Synthesis and characterization of tosylated tetraethylene glycol monobenzyl (4)[10]

This synthesis was modified from the synthesis found in the Yoshimoto, M. et. al. paper.[10] Benzylbromide was allowed to react with an excess of tetrethyleneglycol and excess of concentrated sodium hydroxide.[10] Excess tetrethylene glycol was removed by extyraction yielding a mixture of mono- and di-protected product. Column chromatography was used to isolate the monobenzyl tetraethylene glycol.[10] The product was identified using H^1 NMR. The singlet at 2.43ppm corresponds to the methyl on the tosylate group. The multiplet at 3.67ppm corresponds to the methylenes on the tetraethylene glycol . The triplet at 4.12ppm corresponds to the terminal methylene adjacent to the tosyl group. The singlet at 4.56ppm is from the benzyl methylene group. The multiplet at 7.34ppm corresponds to the aromatic hydrogens of the benzyl protecting group. The doublets in the aromatic region correspond to the tosylate group hydrogens. The C^{13} NMR showing 13 carbon peaks as expected. The IR spectrum confirms the presence of the tosyl group at 1354.45 cm^{-1} (S=O asymmetric stretch), and 1017.66 cm^{-1} (S=O symmetric stretch). The benzyl group at approximately 3100 cm^{-1} (aromatic peak). The GC-MS of the purified product shows the presence of tosylate group because of the large peaks at 155m/z (C_6O_2S) and 91m/z (C_7H_7). The benzyl group can also be confirmed due to the large size of the 91m/z peak. (H^1 NMR , C^{13} NMR, IR, and GC-MS spectra found in supplemental material section.)

4.4 Synthesis and characterization 1,10-Phenathroline-5-amine (5)[11]

This synthesis was modified from the synthesis found in the Binnemans, K. et. al. paper.[17] 5-nitro-1,10-phenanthroline was reduced to the amine with 10% Pd/C and hydrazine.[17] After reaction, the catalyst was removed filtration and the filtrate washed with water to remove the excess hydrazine monohydrate.[17] The mostly pure product

was recrystallized from absolute ethanol and cyclohexane.[17] The H^1 NMR of the product had the expected peaks. The singlet at 6.941 ppm corresponds to the amine group. The peaks at 7.652, 7.962, 8.263, 8.936, and 9.194 correspond to the aromatic hydrogens on the 1,10-phenathroline. The C13 NMR showed fourteen peaks in the aromatic region as expected. The IR spectrum had bands at 3335.53 and 3222.81 cm^{-1} indicating that an amine had formed (H-N-H asymmetric/symmetric). The absence of N=O nitro stretch also confirms the formation of the amine. (H^1 NMR , C^{13} NMR, IR, and GC-MS spectra found in supplemental material section).

4.5 Synthesis and characterization of 1-phenyl-2,5,8,11-tetraoxatridecan-13-oic acid (3)[12,13]

This synthesis was modified from the synthesis found in the Kichler, A et. al. paper. Monobenzyl tetraethylene glycol was oxidized to a carboxylic acid using Jones reagent. The reaction mixture turns orange when the jones reagent is added and then as the jones reagent oxidizes the mixture turns teal with dark teal solid. The color change is a good indication that the chromium agent is being consumed and that the carboxylic acid is forming. The crude product was a clear oil that contained many side products. The acidic reaction conditions were harsh and resulted in cleavage of the ethylene glycol chain. Some of these ethylene glycol fragments were further oxidized to mono and dicarboxylic acids. Most of these side products were successfully removed by sending the reaction mixture through a silica plug. Further purification by column chromatography was unsuccessful.

Further purification required using an ion exchange resin. This purification step was modified from the Bookser, B. C. et. al. paper.[13] Dowex 1x2 (Cl) ion exchange resin

was swelled overnight with methanol.[13] The swelled resin was fluffy in texture and had a yellowish white appearance.[13] The product mixture was combined with DIEA in methanol and added to the swelled ion exchange resin and allowed to sit for one hour.[13] The addition of DIEA is believed to improve the effectiveness of the ion exchange resin in adsorbing carboxylic acids.[13] The resin was filtered and the filtrate analyzed to determine if the carboxylic acid (**3**) had been adsorbed. The carboxylic acid (**3**) was displaced from the resin by washing it with a CH_3OHl/TFA solution. After removal of the CH_3OHl/TFA, the resulting oil was then characterized by GC/MS. The oil consisted primarily of the desired product **3** with a small amount of impurity present. The mass spectrum of the impurity was extremely similar to the product. Carboxylic acid (**3**) was used without further purification.

The H^1 NMR confirmed the identity of the product. The peak at 3.62-3.74 ppm (m,13H) correlates to the ethylene glycol CH_2 units. Peak at 4.13-4.15 ppm (d, 2H) and 4.58 ppm (s,2H) correlates to the methylenes adjacent to the carboxylic acid. The IR spectrum confirmed the presence of a carboxylic acid. The appearance acid of a peak at 170.9 ppm in the C^{13} NMR also indicated that a carboxylic had been made. The MS data of the larger peak shows peaks indicating loss of a carboxylic acid at 59.1m/z ($C_2H_3O_2$). The MS data also confirms the presence of a carboxylic acid and benzyl group with peaks at 59.1 m/z (CH_2CO2H) and 91.1 m/z (C_7H_7). (H^1 NMR , C^{13} NMR, IR, and GC-MS spectra found in supplemental material section.)

4.6 Synthesis and characterization of N-(5-phenanthryl)-12-benzyl-3,6,9,12-tetraoxa dodecamide (Phen-4EG-BZ) (6)[13,14]

This synthesis was modified from the synthesis found in the Elmes, R. B. P. et. al. paper.[14] Standard peptide coupling procedures (EDCI/DMAP) were used to attach 1-phenyl-2,5,8,11-tetraoxatridecan-13-oic acid (**3**) to 5-amino-1,10-phenathgroline (**5**) to afford a light orange solid. The sample did not move on silica gel TLC plates so column chromatography could not be used to purify. Instead, ion exchange resin was used to remove any unreacted carboxylic acid (**3**). The unreacted carboxylic acid (**3)** was recovered by rinsing the resin with 95:5 methanol: TFA. The H^1 NMR confirmed the presence of the desired product. The multiplet at 3.51-3.62 ppm corresponds to the fifteen hydrogens on the tetraethylene glycol portion of the product. The singlet at 4.12 ppm corresponds to the O=C-CH_2-O hydrogens adjacent to the amide. The singlet at 4.47 ppm corresponds to the benzyl methylene group. The singlet at 6.91ppm corresponds to the aromatic methine on C6 of the phenanthroline. The multiplet at 7.26-7.34 ppm corresponds to the hydrogens on the lone benzyl group on the end of the linker. The peaks at 7.61-7.65 ppm, 7.80-7.84 ppm, 8.22-8.25 ppm, 8.69-8.71 ppm, 8.76-8.79 ppm, 9.09-9.10 ppm all correspond to aromatic hydrogens attached to the phenanthroline portion of the ligand. The FTIR spectrum confirmed the formation of an amide bond with peaks at 3415.51 cm^{-1} (amide N-H stretch) and 1635.64 cm^{-1} (amide N-C=O stretch). The appearance of a strong peak at 1107.28 cm^{-1} (C-O) indicates the presence of the tetraethylene glycol ether. (H^1 NMR , C^{13} NMR, IR, and GC-MS spectra found in supplemental material section.)

4.7 Synthesis and characterization of monotetrahydropyran-protected tetraethylene glycol (THP-4EG) (7)[15]

This synthesis was modified from the synthesis found in the paper by Loiseau, F. A. et. al.[15] Tetraethylene glycol was reacted with 3,4 dihydro-2,4-pyran using p-toluene sulfonic acid as a catalyst to produce a clear colorless oil. The oil contained the mono and di-protected product in a 8:1 ratio. Further use of compound **7** was not considered unnecessary since the synthetic strategy had changed (Fig. 15-16). (H^1 NMR , C^{13} NMR, IR, and GC-MS spectra found in supplemental material section).

4.8 Synthesis and characterization of Europium(III)$(TTA)_3(H_2O)_2$ (8)[16]

This synthesis was modified from the synthesis found in the Leonard, J. P.et. al. paper. 2-Thenoyltrifluoroacetone (TTA) was dissolved in water and NaOH and an aqueous $EuCl_3$ solution was added dropwise. The solution was filtered to produce a white solid that was fluoresced orange under UV light and had the desired luminescent properties. The complex had an excitation wavelength of 340 nm and emission wavelength of 615.5 nm. The product was analyzed via IR and had peaks at 3356.28 cm^{-1} (O-H) indicating the presence of water in the complex. Peaks were also found in 1131.99 cm^{-1} (C-F stretch) and indicating the presence of TTA. (IR, UV-VIS, and Florescence spectra found in supplemental material section.)

4.9 Europium(TTA) 5-amine phenanthroline(9)[11]

This synthesis was modified from the synthesis found in the Binnemans K, et. al. paper.[17] Europium complex **8** was combined with 5-amine phenanthroline in CH_2Cl_2 and stirred at 60 °C for 30 min and then at room temperature overnight. Small crystals formed upon evaporation of the solvent.[17] The 5-aminophenthroline-europium complex **9** had a much more intense fluorescent band 615.5 nm then starting complex **8**. This is expected since the phenanthroline ligand is supposed to act as an antennae for

absorbing light and transferring the energy to the europium. (IR, UV-VIS, and Florescence spectra found in supplemental material section.)

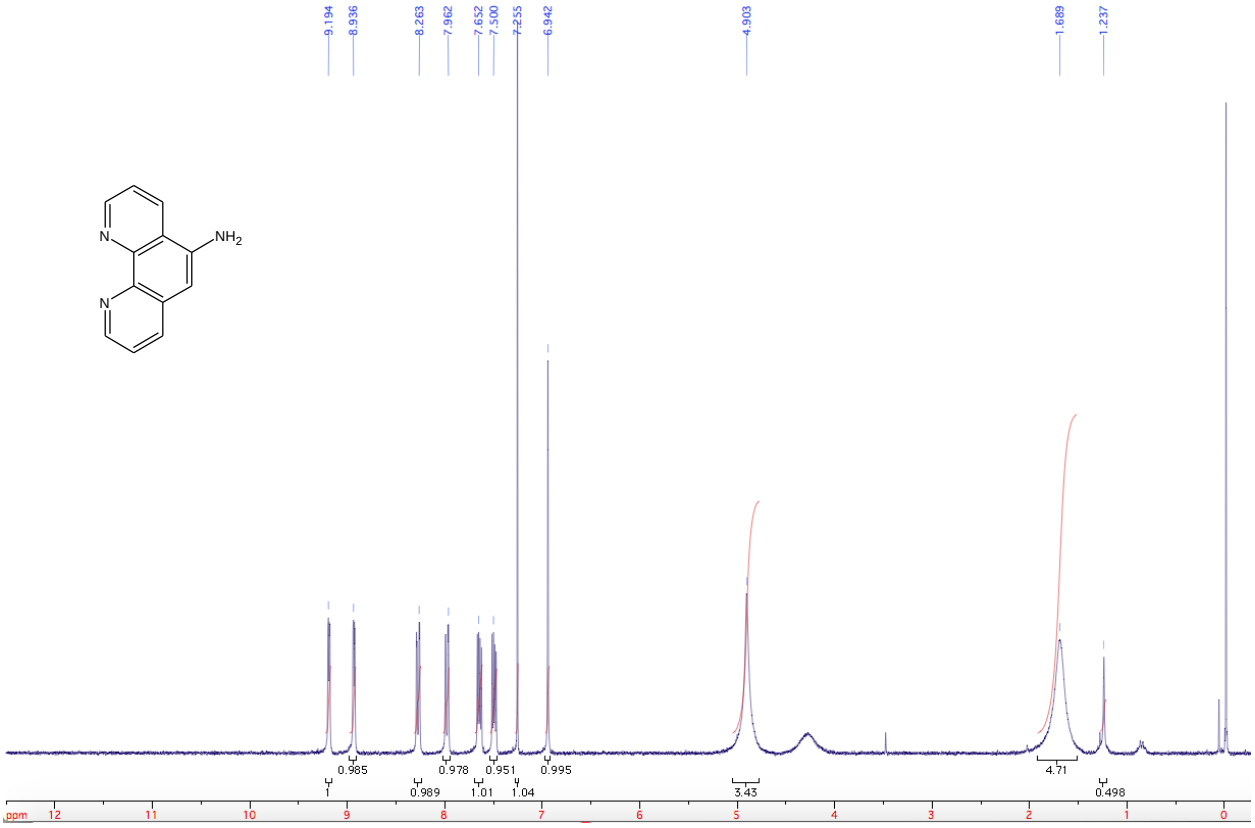
4.10 Conclusion

In this project, the linker synthesis was found to be reproducible and the purification of the products can now be assured upon the improvement of the purification methods. This will allow for the ease of synthesis of the probe. The coupling of the linker was found to be successful, the purification is not complete until further purification can be completed. The purification steps isolated the unreacted products, those of which can be reused. Europium label synthesis has been accomplished and can now be used for further analysis. All components of this project were made, purified, and analyzed to insure optimal usage for future assembly of the probe. The linker synthesis has now been optimized allowing for extension of the N-(5-phenanthryl)-12-benzyl-3,6,9,12-tetraoxa dodecamide (**6**).

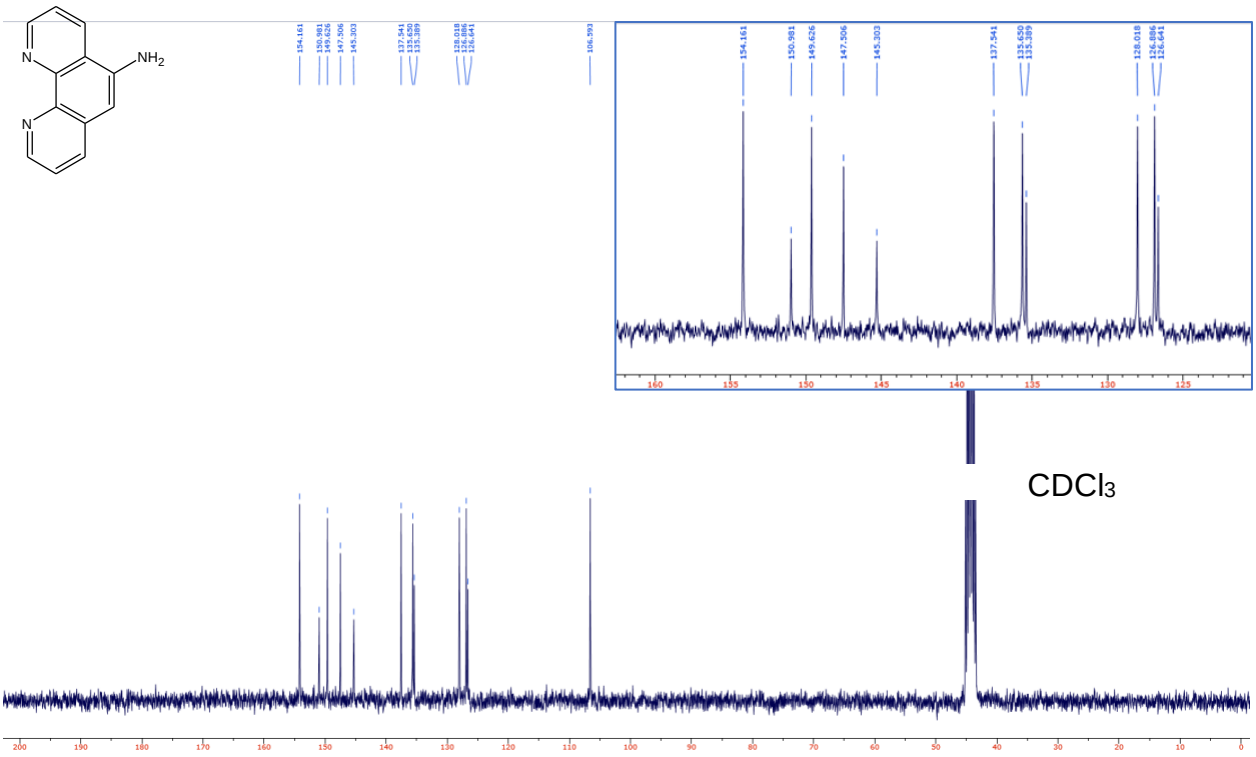
4.11 Future Work

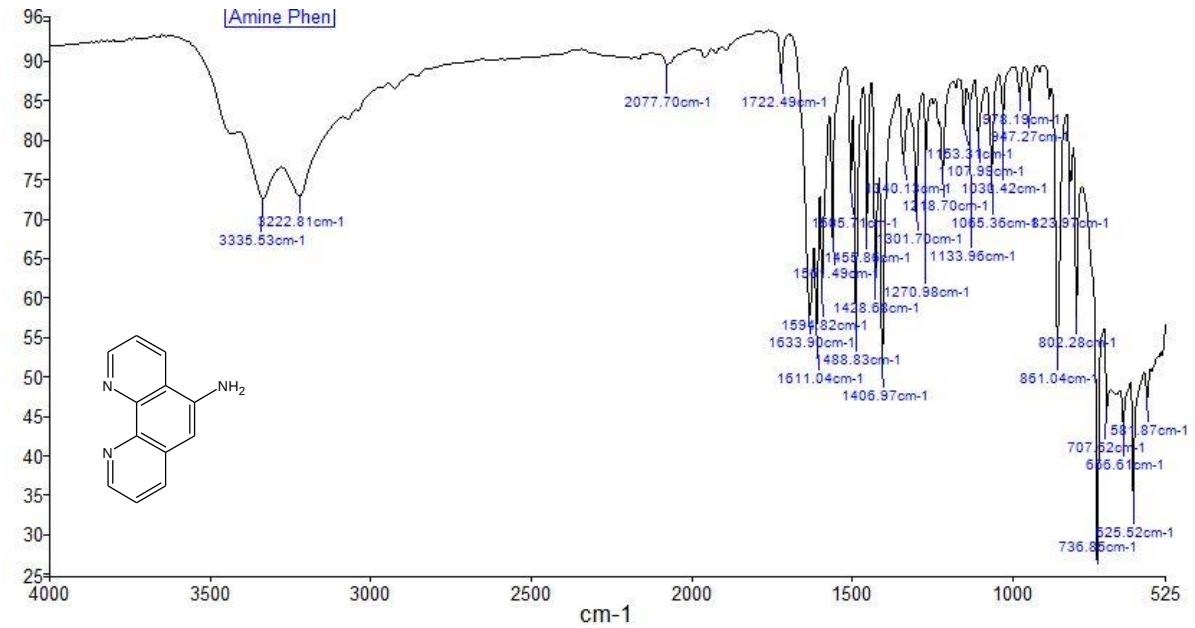
Future members of this research group can now work on adding more linkers to the ligand after purification and begin attempting to attach the linker to the MSH-4 peptide. The europium complexes will be added to the ligand attached with the inker to the MSH-4. After the successful synthesis of the probe cell testing can be done.

1,10-phenathroline-5-amine (5)

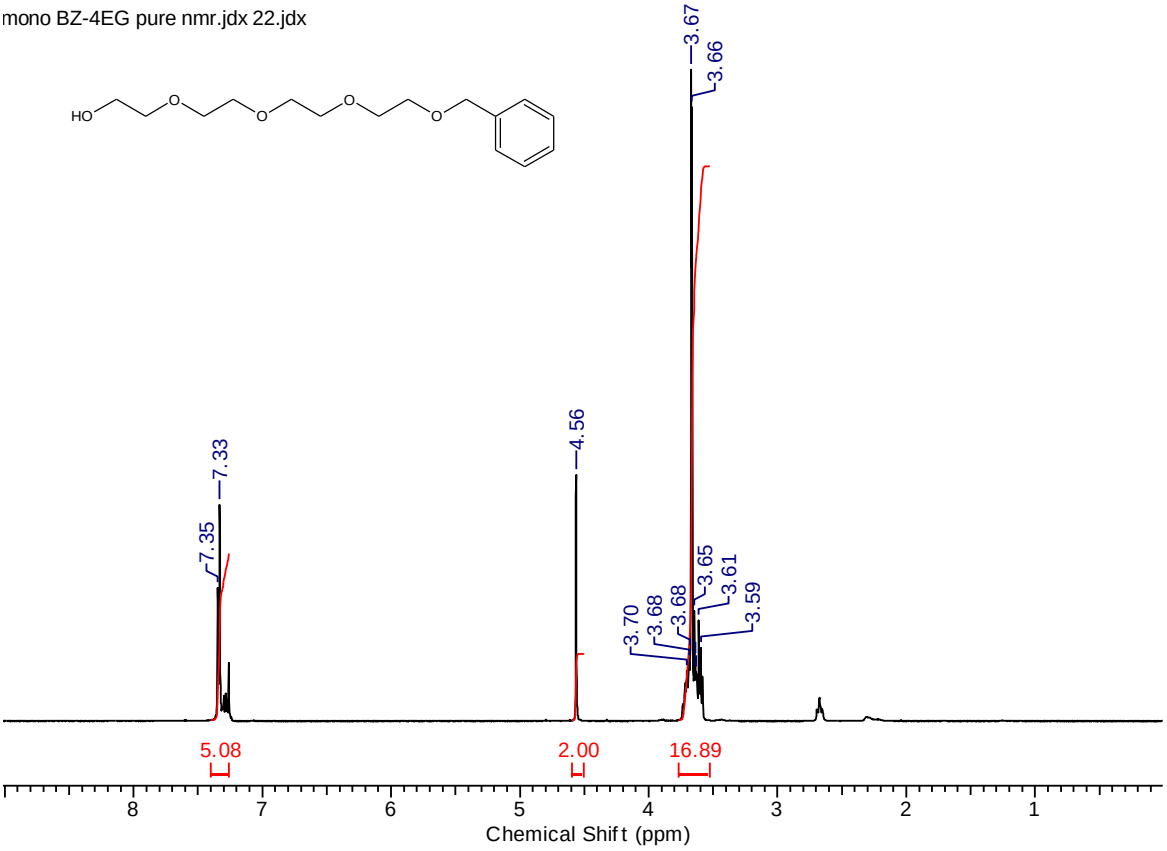


1,10-phenathroline-5-amine (5)

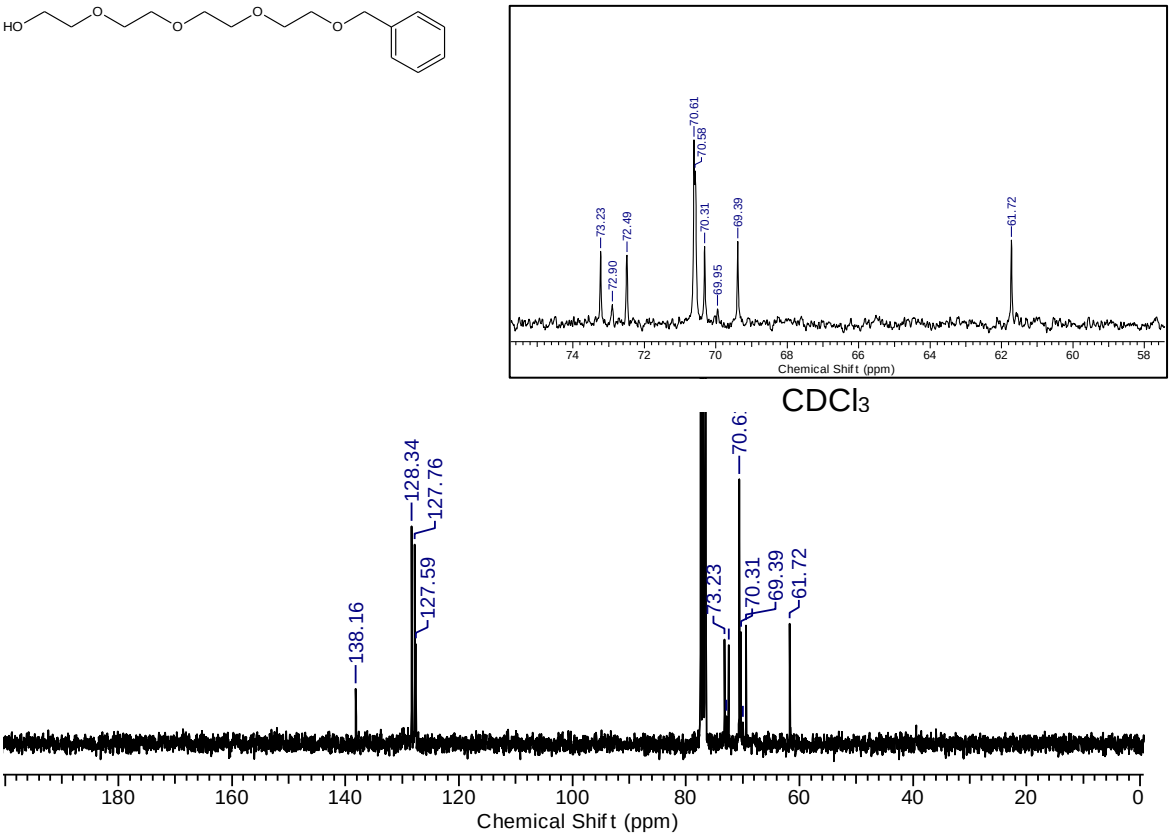




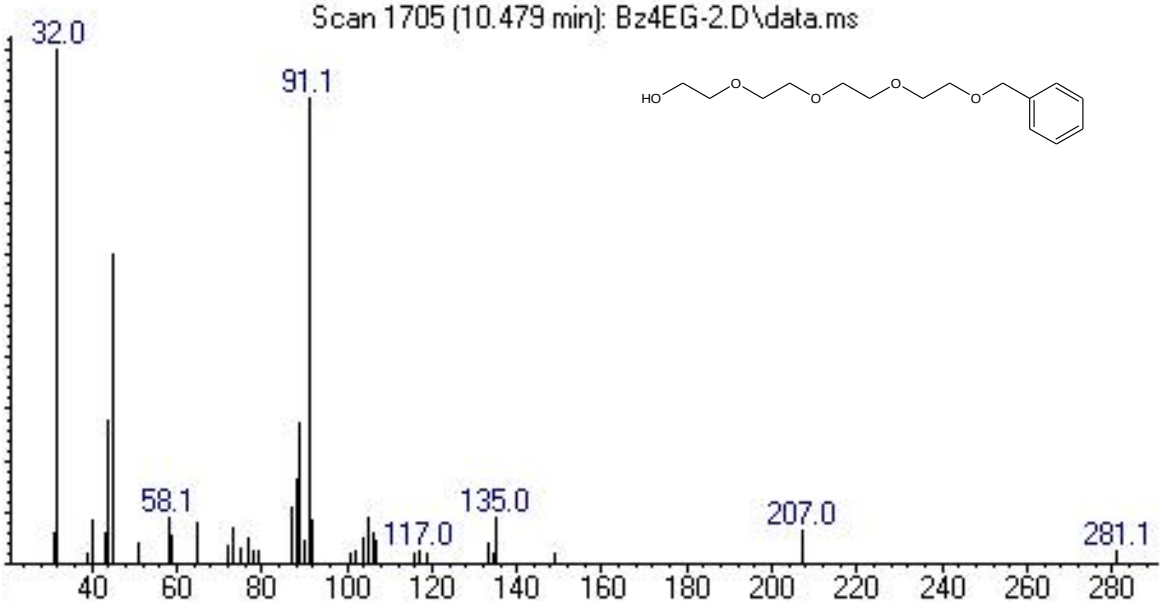
benzyl-tetraethylene glycol (2): 4-EG-Bz

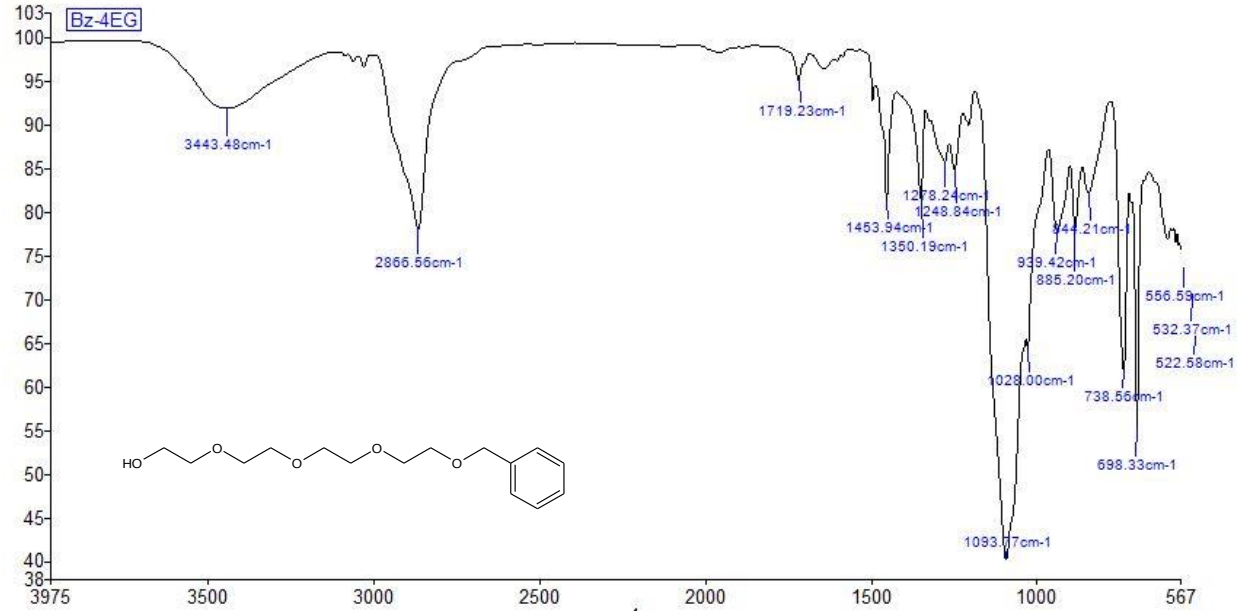


benzyl-tetraethylene glycol (2): 4-EG-Bz

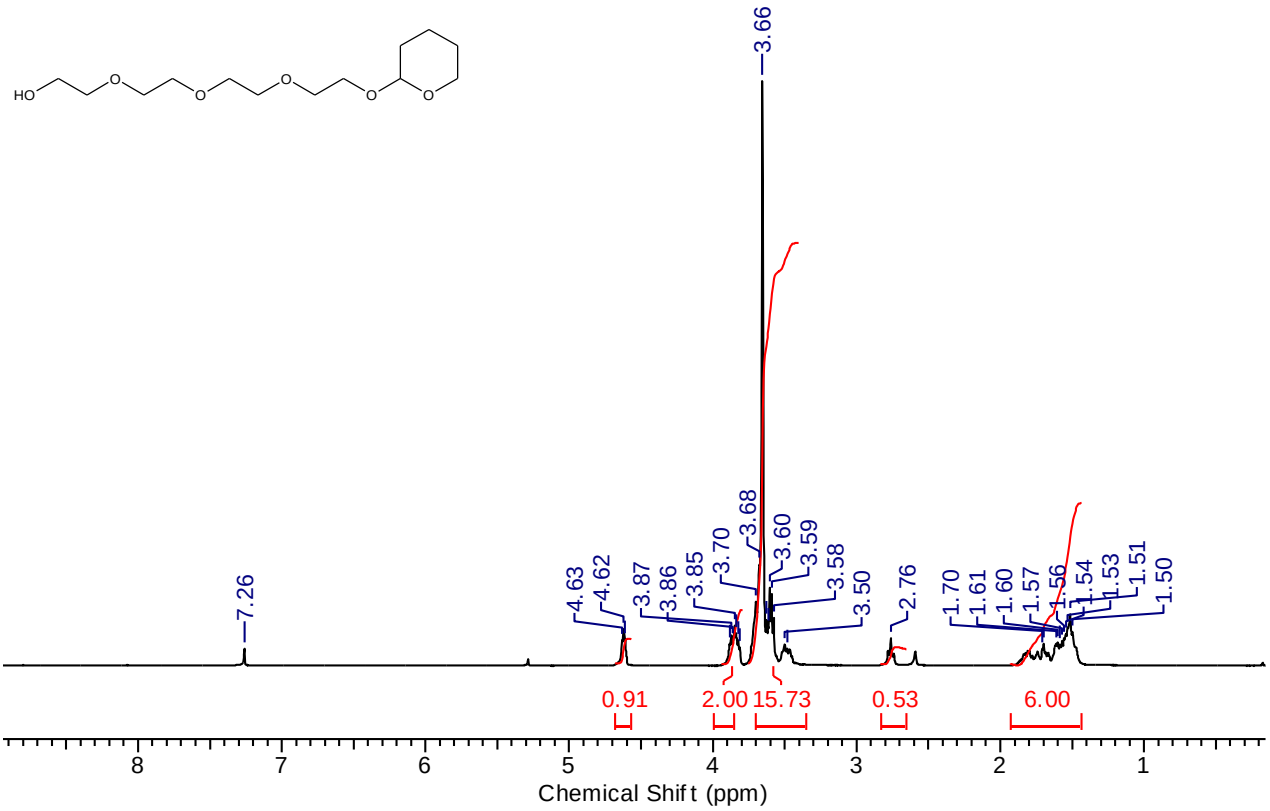


benzyl-tetraethylene glycol (2): 4-EG-Bz

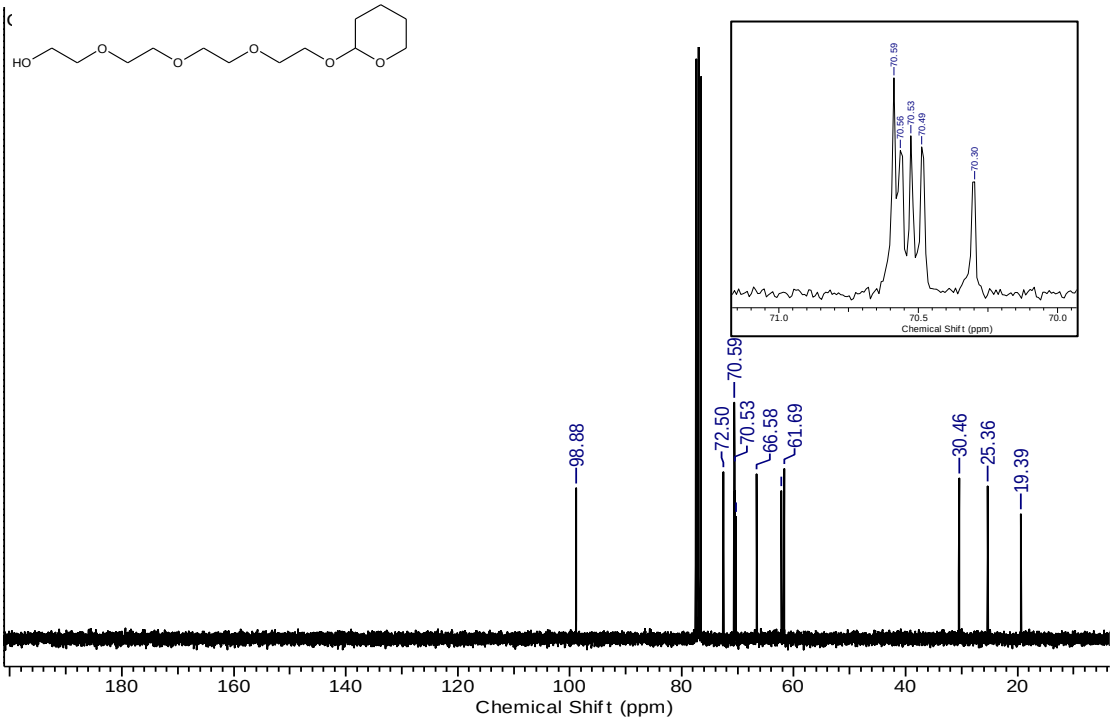


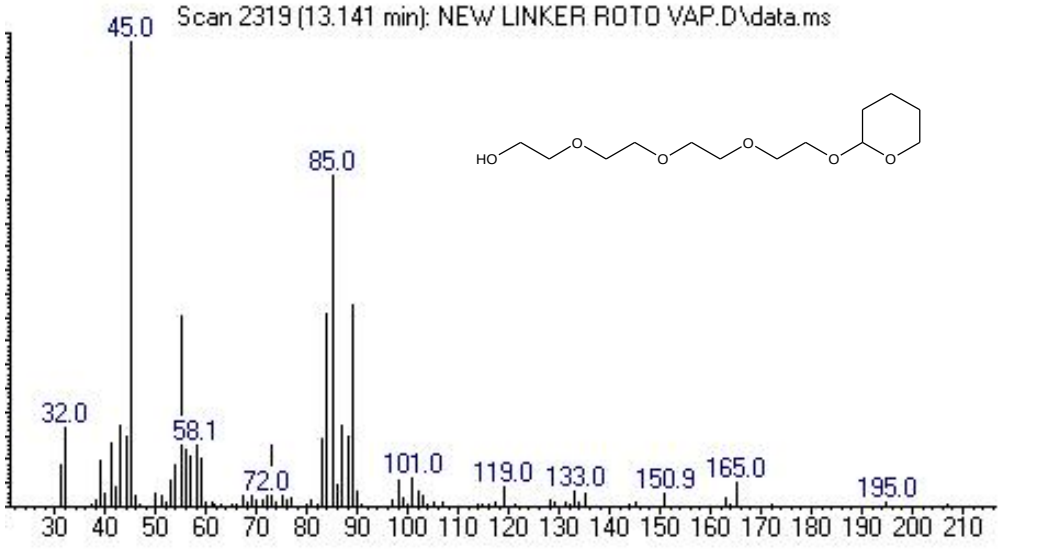


tetrahydropyranyl tetraethylene glycol *(7)*

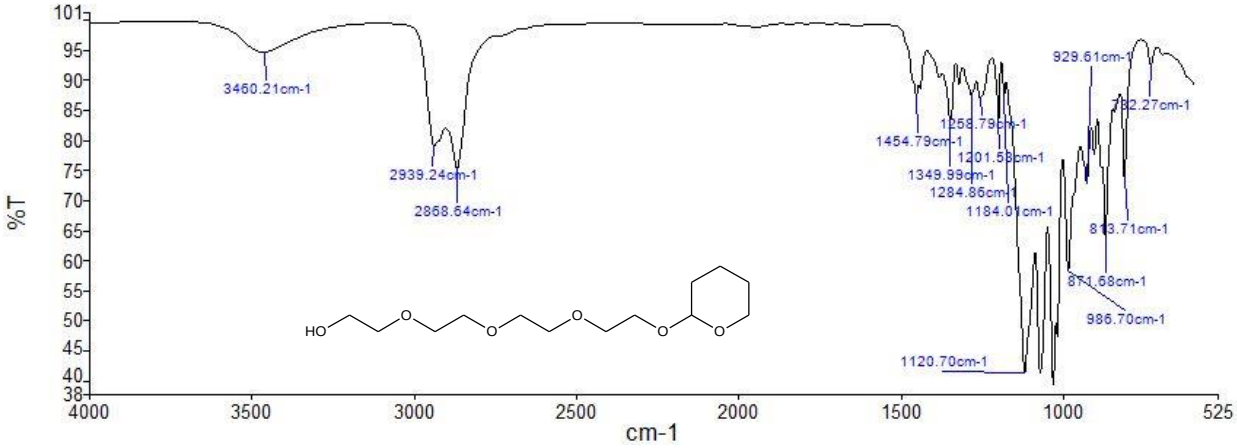


tetrahydropyranyl tetraethylene glycol *(7)*

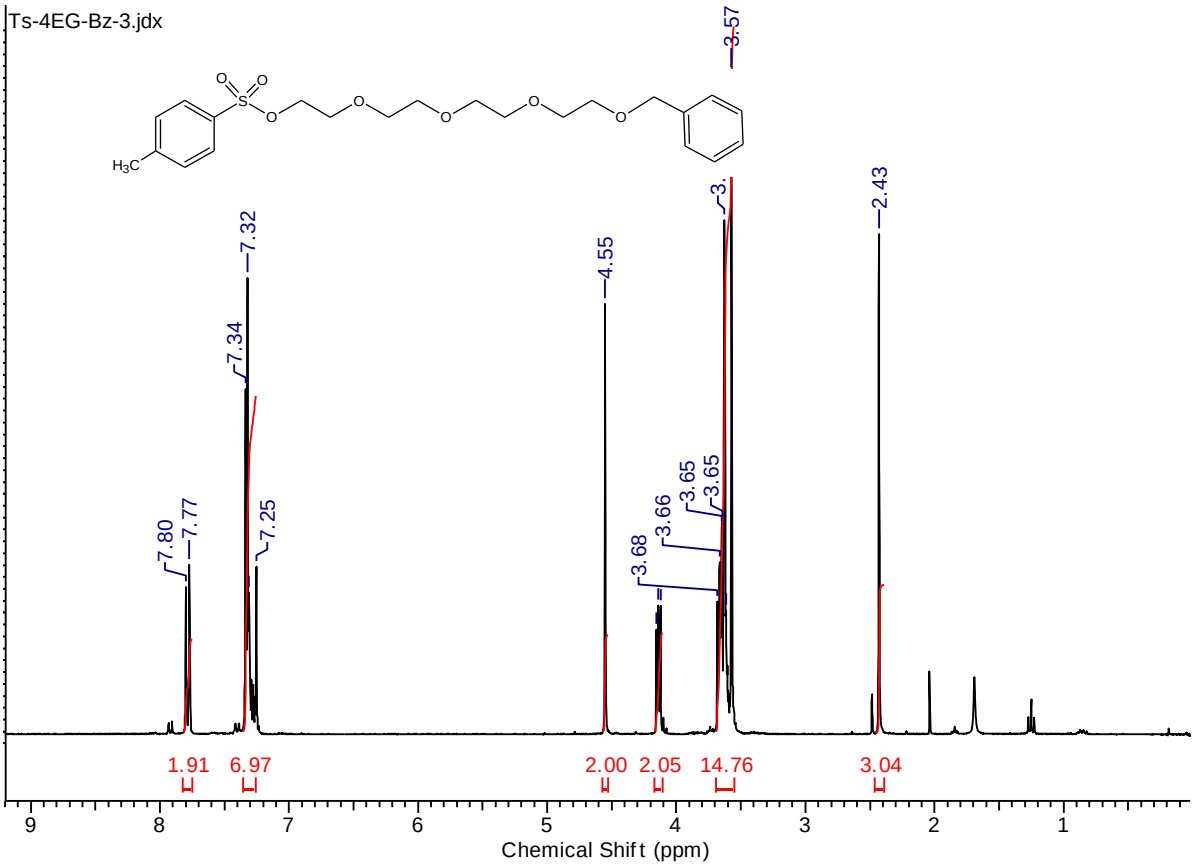




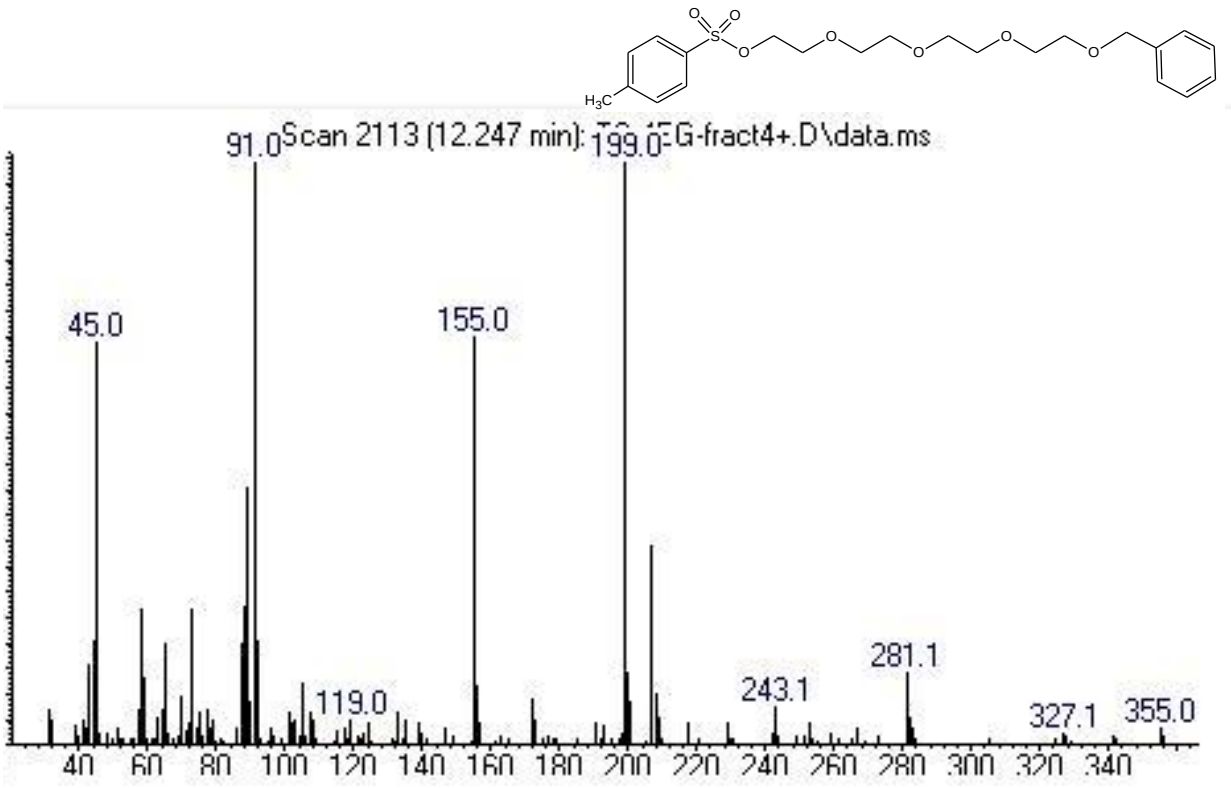
tetrahydropyranyl tetraethylene glycol *(7)*



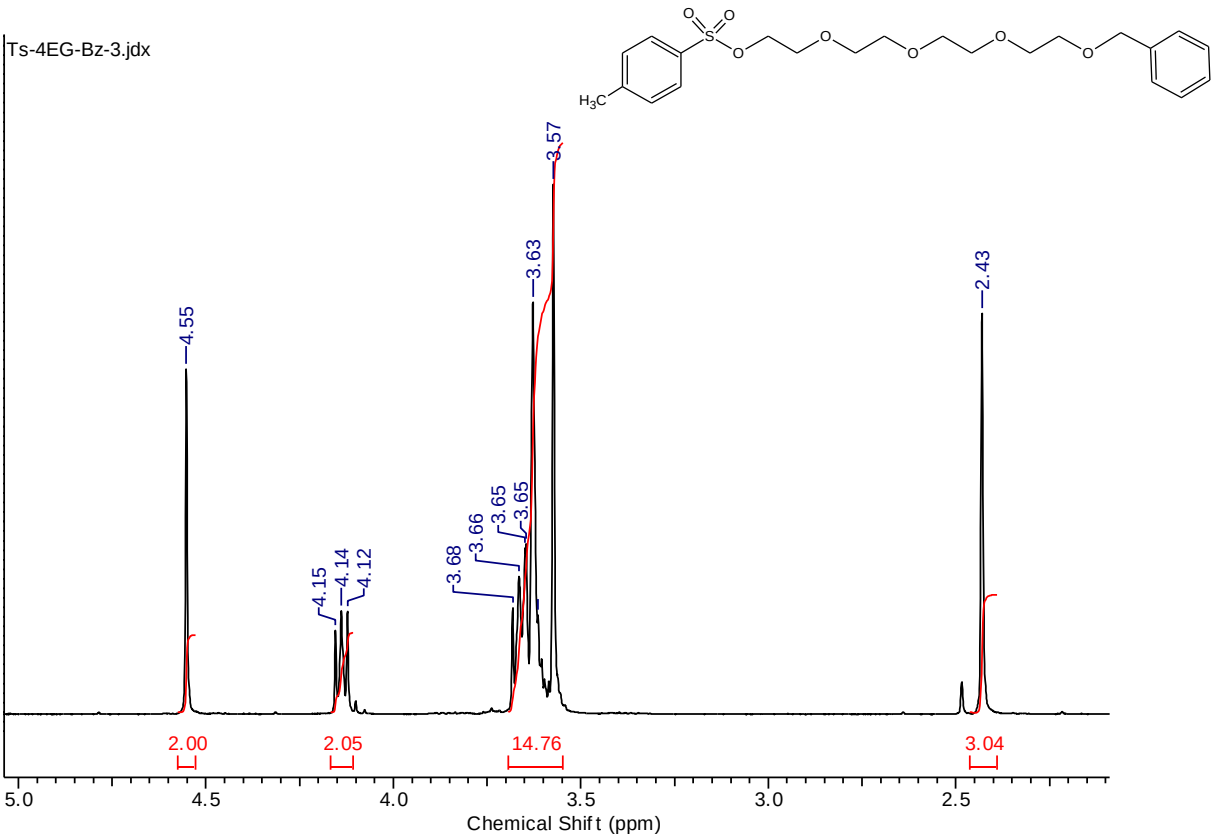
Mono-tosyl-mono-benzyl tetraethylene glycol (4)



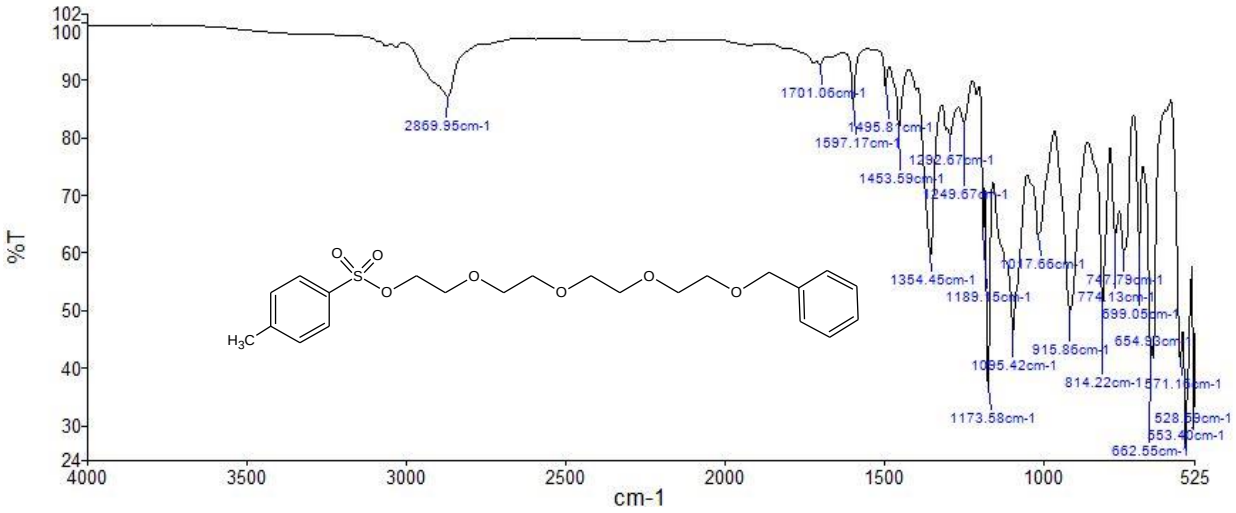
Mono-tosyl-mono-benzyl tetraethylene glycol (4)



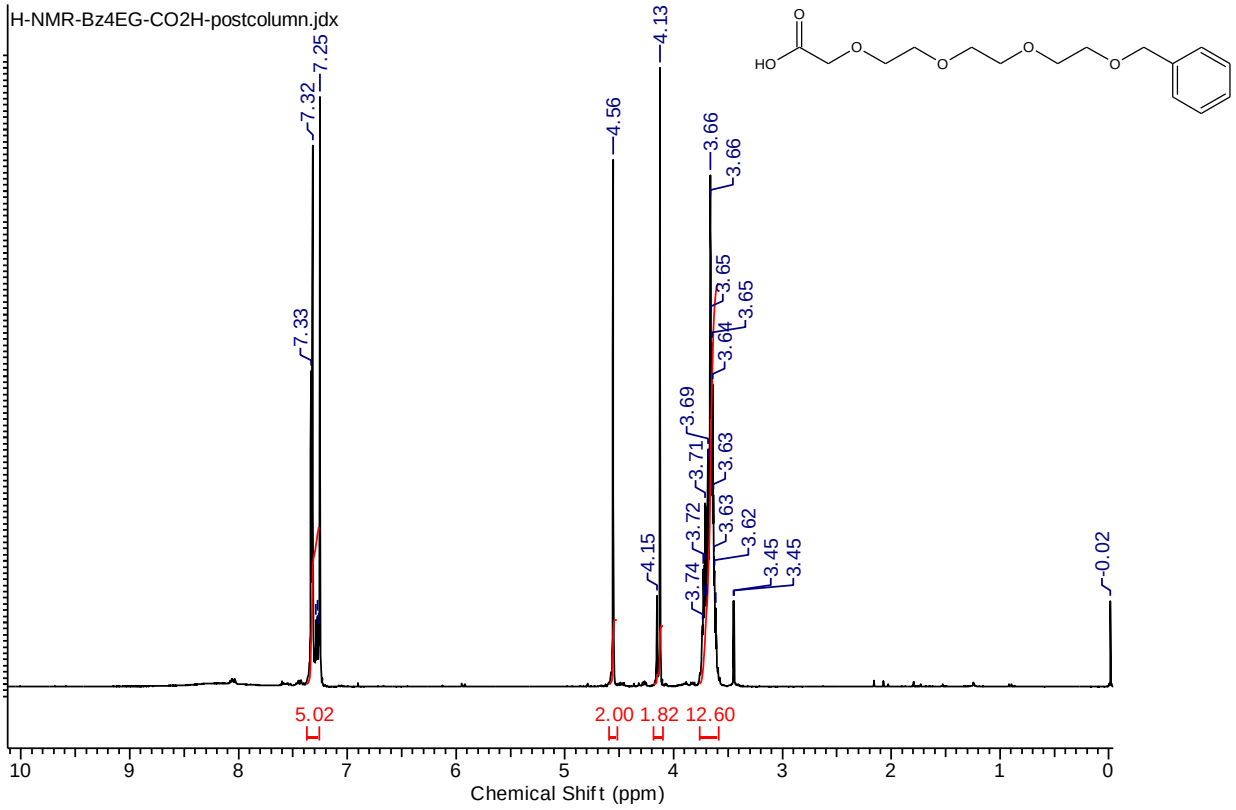
Mono-tosyl-mono-benzyl tetraethylene glycol (4)



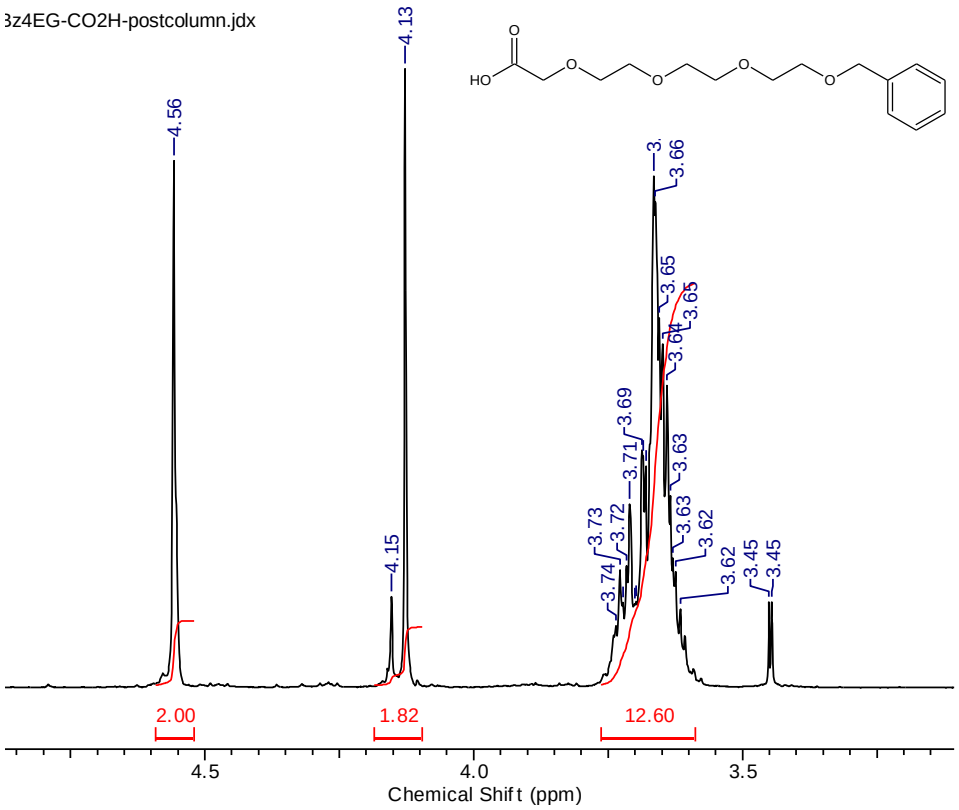
Mono-tosyl-mono-benzyl tetraethyleneglycol (4)



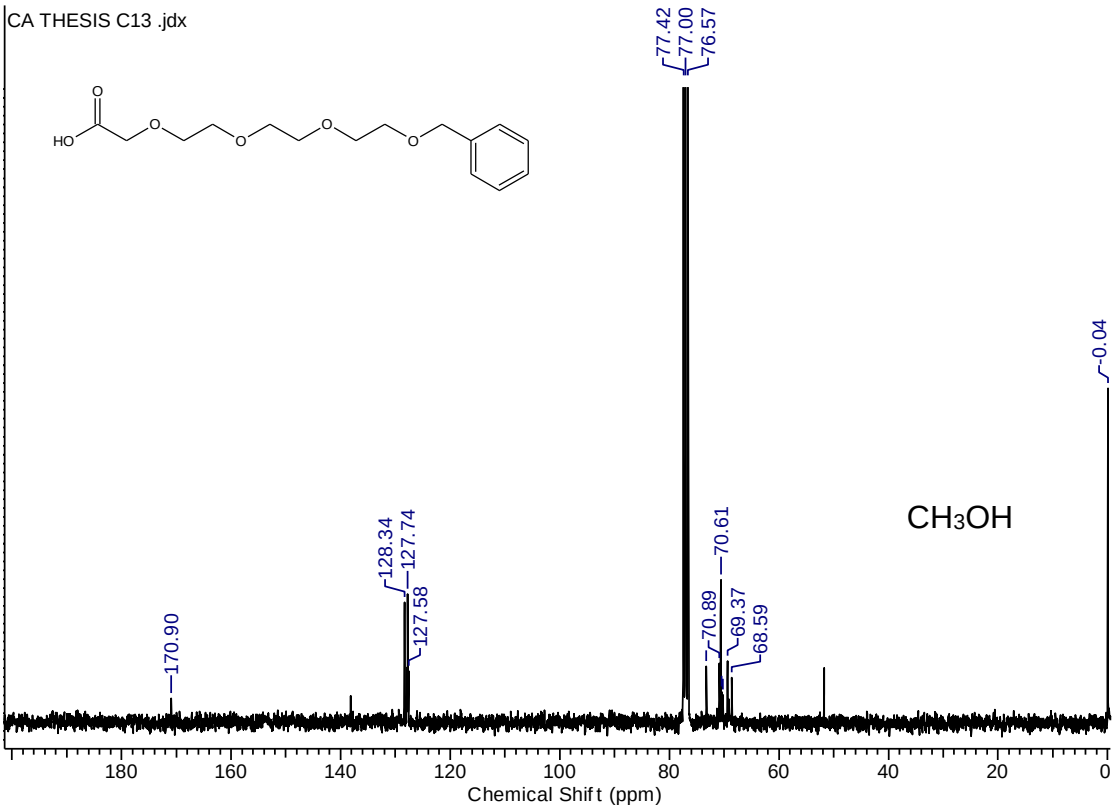
1-phenyl-2,5,8,11-tetraoxatridecane-13-oic acid (3)



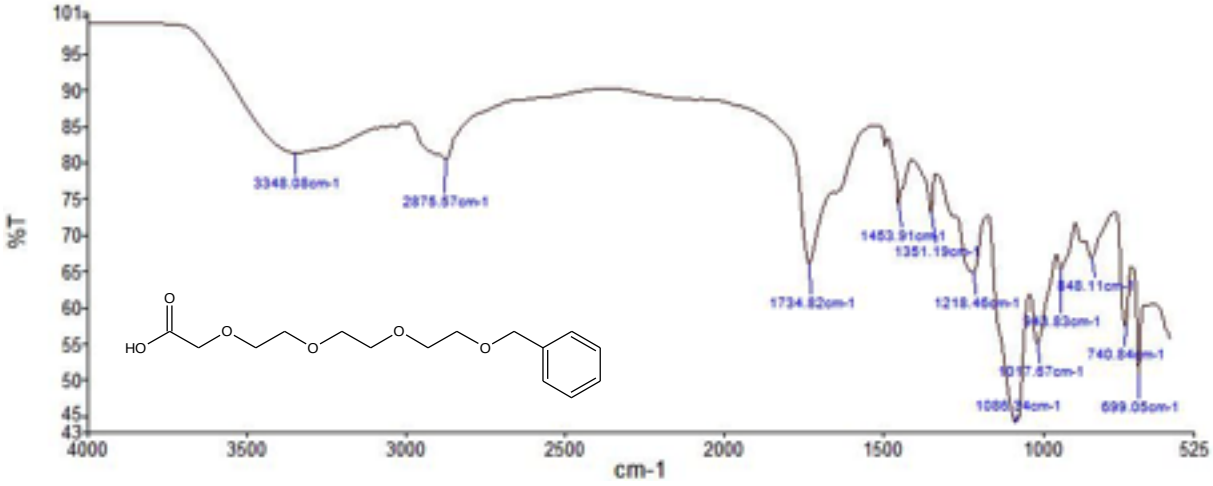
1-phenyl-2,5,8,11-tetraoxatridecane-13-oic acid (3)



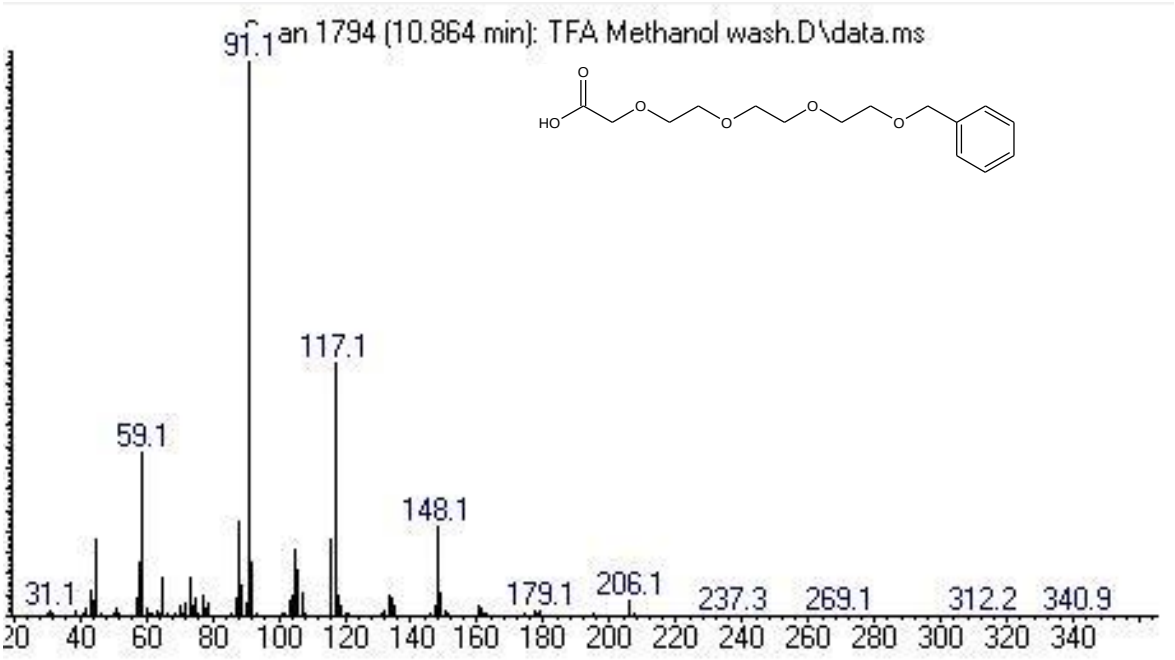
1-phenyl-2,5,8,11-tetraoxatridecane-13-oic acid (3)



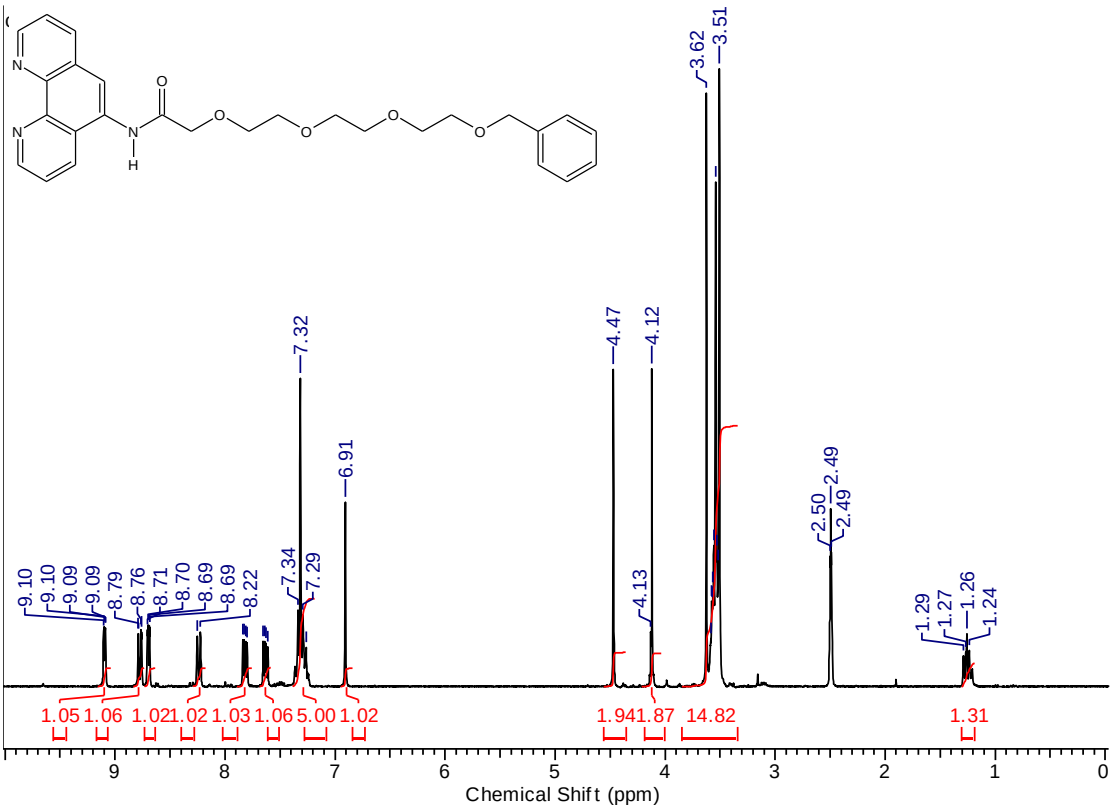
1-phenyl-2,5,8,11-tetraoxatridecane-13-oic acid (3)



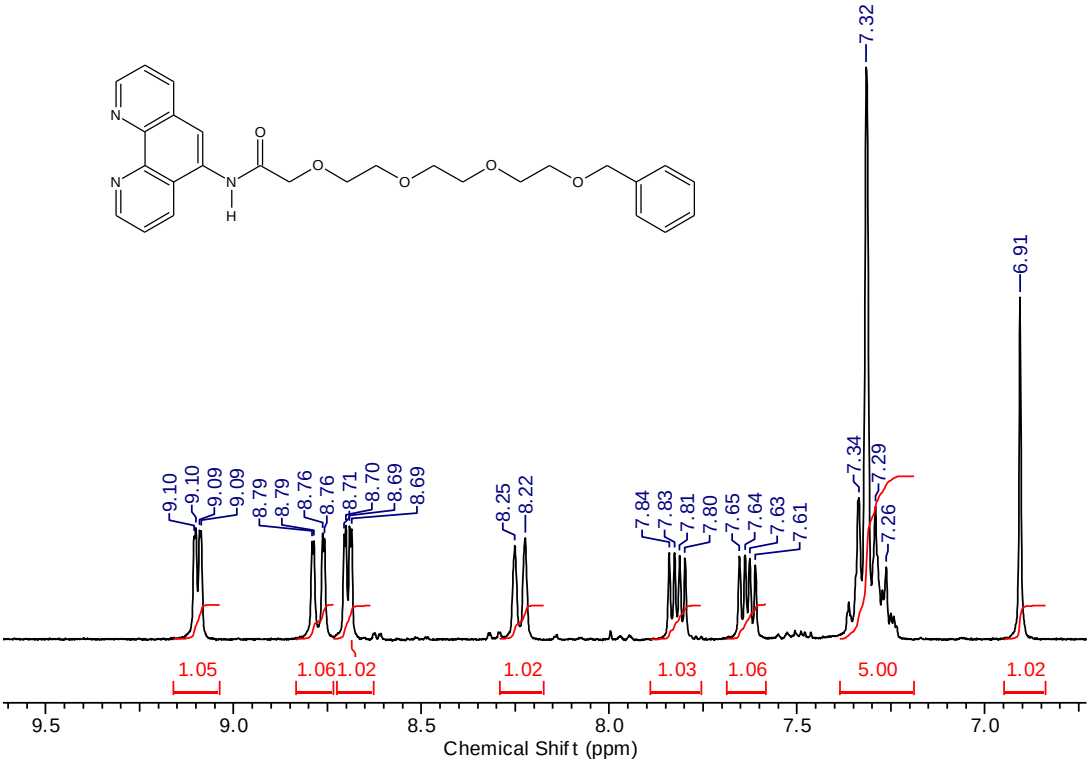
1-phenyl-2,5,8,11-tetraoxatridecane-13-oic acid (3)



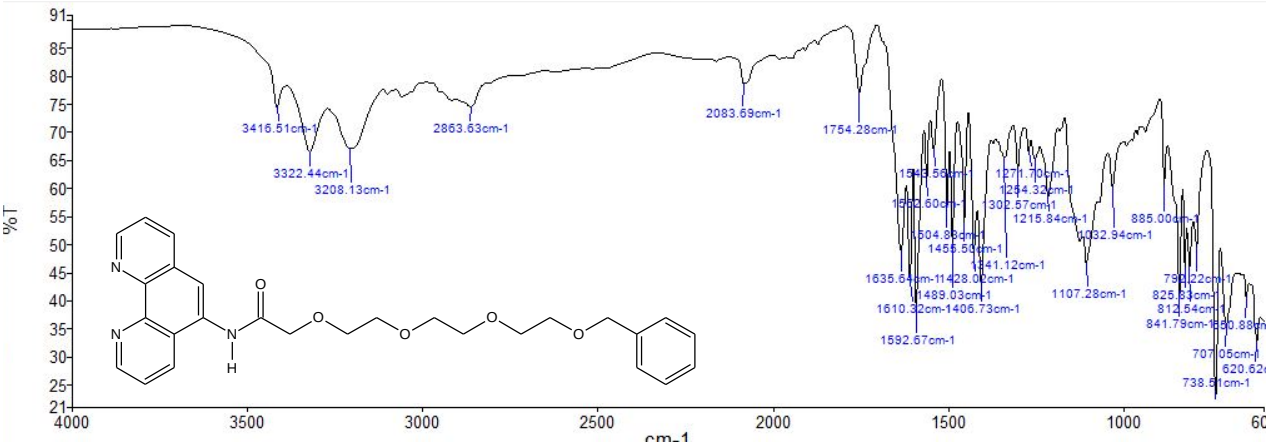
N-(5-phenanthryl)-12-benzyl-3,6,9,12-tetraoxa dodecamide *(6): Phen-4EG-BZ*



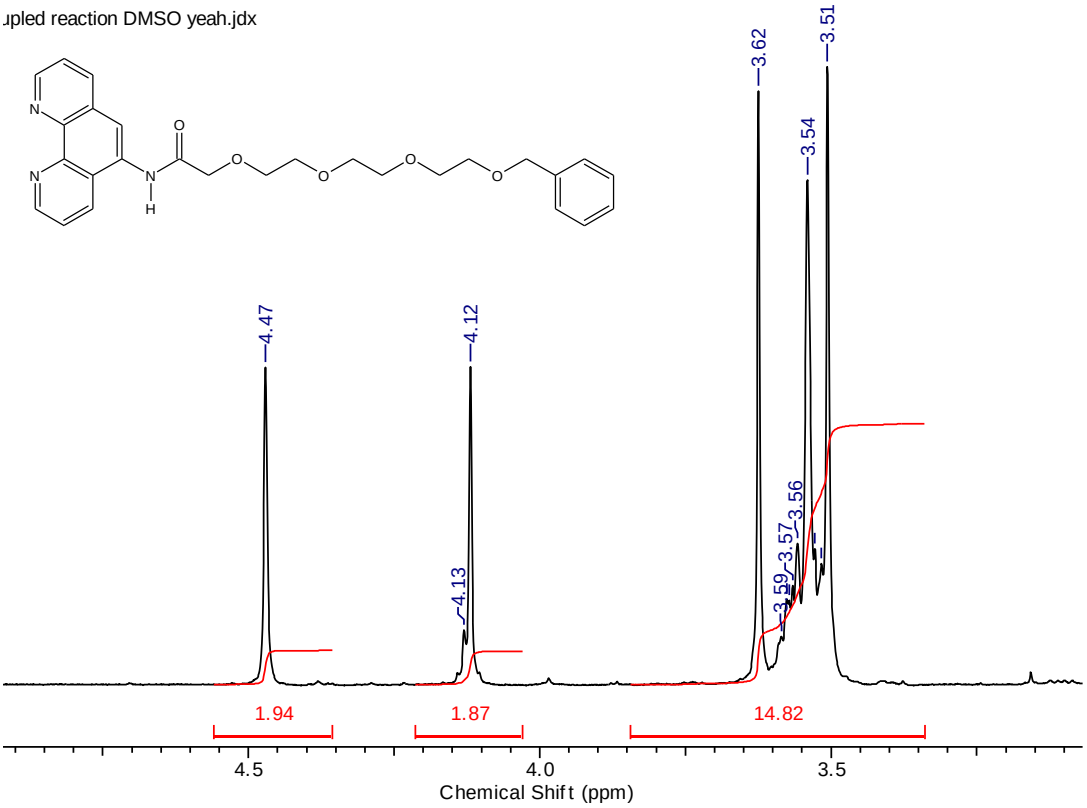
N-(5-phenanthryl)-12-benzyl-3,6,9,12-tetraoxa dodecamide *(6): Phen-4EG-BZ*



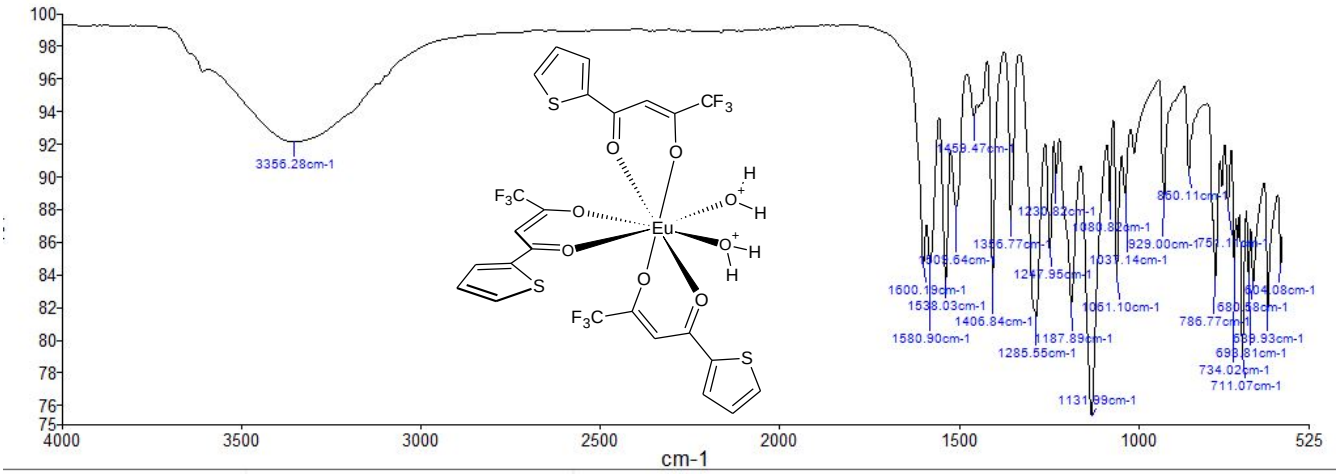
N-(5-phenanthryl)-12-benzyl-3,6,9,12-tetraoxa dodecamide *(6): Phen-4EG-BZ*



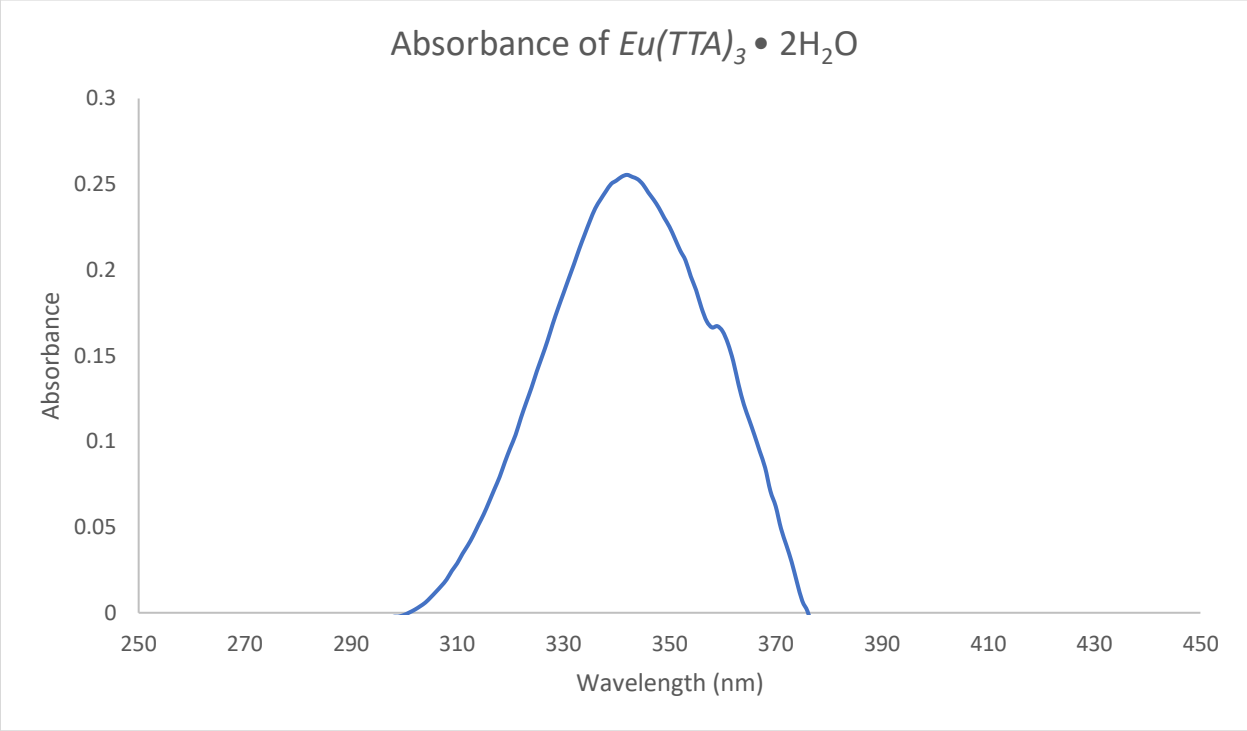
N-(5-phenanthryl)-12-benzyl-3,6,9,12-tetraoxa dodecamide *(6): Phen-4EG-BZ*

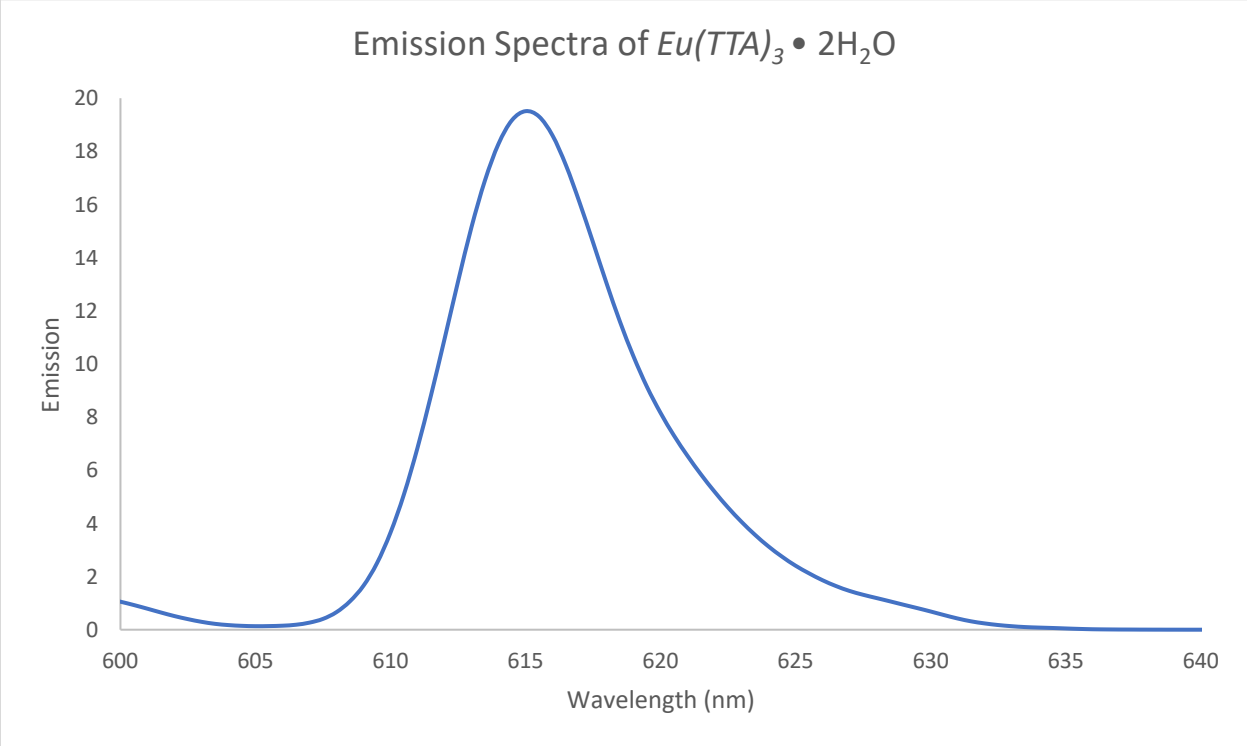


$Eu(TTA)_3(H_2O)_2$ (8)

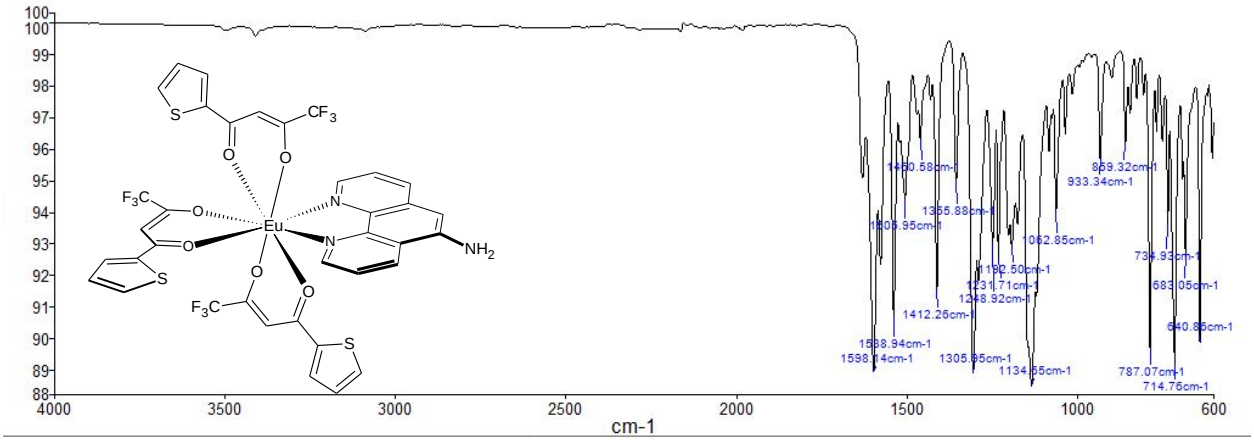


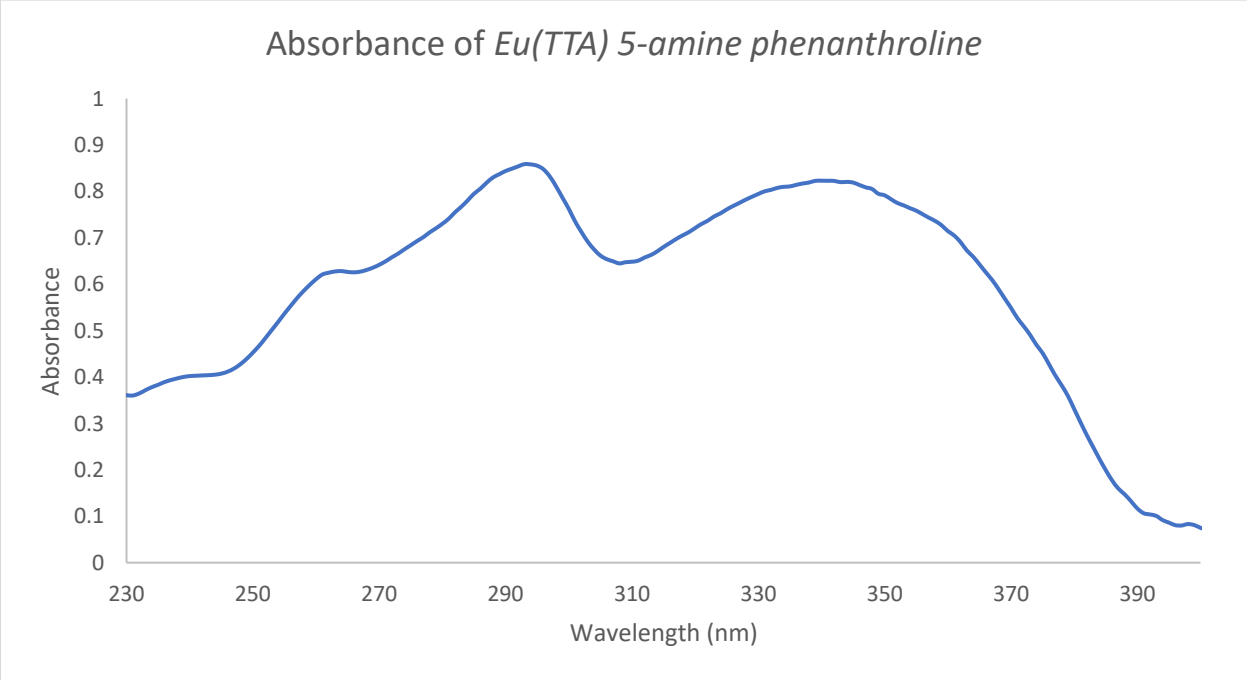
Eu(TTA)$_3$ (H$_2$O)$_2$ (8)



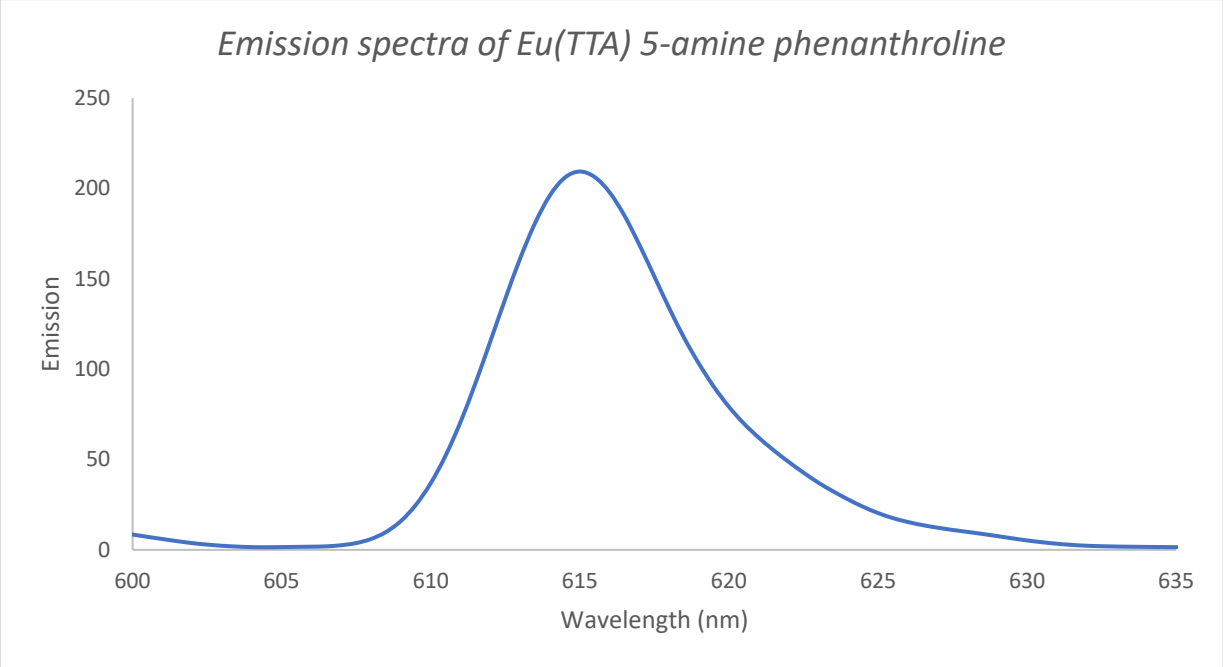


Eu(TTA)3 5-amino-1,10-phenathroline (9)





Eu(TTA)3 5-amino-1,10-phenathroline (9)



REFERENCES

(1) Description, T. United States Cancer Statistics (USCS). *Sites J. 20Th Century Contemp. French Stud.* **2011**, 8–9.

(2) Facts, C. CONCEPTS Cancer-Facts-and-Figures-2018. **2018**.

(3) Koniev, O.; Wagner, A. Developments and Recent Advancements in the Field of Endogenous Amino Acid Selective Bond Forming Reactions for Bioconjugation. *Chem. Soc. Rev.* **2015**, *44* (15), 5495–5551.

(4) Chhajlani, V.; Wikberg, J. E. S. Molecular Cloning and Expression of the Human Melanocyte Stimulating Hormone Receptor CDNA. *FEBS Letters*. 1992, pp 417–420.

(5) Rosenbaum, D. M.; Rasmussen, S. G. F.; Kobilka, B. K. The Structure and Function of G-Protein-Coupled Receptors. *Nature* **2009**, *459* (7245), 356–363.

(6) Wilson, K. R.; Sedberry, S.; Pescatore, R.; Vinton, D.; Love, B.; Ballard, S.; Wham, B. C.; Hutchison, S. K.; Williamson, E. J. Microwave-Assisted Cleavage of Alloc and Allyl Ester Protecting Groups in Solid Phase Peptide Synthesis. *J. Pept. Sci.* **2016**, *22* (10), 622–627.

(7) SYNTHESIS , CHARACTERIZATION , AND LUMINESCENT PROPERTIES OF E U 3 + DIPYRIDOPHENAZINE FUNCTIONALIZED COMPLEXES FOR POTENTIAL BIO- IMAGING APPLICATIONS A Thesis Presented to the Faculty of the Graduate School of Western Carolina University in Partial Fulf. **2014**, No. October.

(8) Veronese, F. M.; Mero, A. The Impact of PEGylation on Biological Therapies. *BioDrugs* **2008**, *22* (5), 315–329.

(9) Niederer, K.; Seiwert, J.; Worm, M.; Wurm, F. R.; Herzberger, J.; Pohlit, H.; Frey, H. Polymerization of Ethylene Oxide, Propylene Oxide, and Other Alkylene Oxides: Synthesis, Novel Polymer Architectures, and Bioconjugation. *Chem. Rev.* **2015**, *116* (4), 2170–2243.

(10) Yoshimoto, M.; Honda, K.; Kurosawa, S.; Tanaka, M. Dynamic Properties of Self-Assembled Monolayers of Mercapto Oligo(Ethylene Oxide) Methyl Ether on an Oscillating Solid-Liquid Interface. *J. Phys. Chem. C* **2014**.

(11) Binnemans, K.; Lenaerts, P.; Driesen, K.; Go, C. A Luminescent Tris (2-Thenoyltrifluoroacetonato) Europium (III) Complex Covalently Linked to a 1 , 10-Phenanthroline- Functionalised Sol – Gel Glass. **2004**, 191–195.

(12) Kichler, A.; Schuber, F. Versatile Synthesis of Bi- and Tri-Antennary Galactose Ligands: Interaction with the Gal/GalNAc Receptor of Human Hepatoma Cells. *Glycoconj. J.* **1995**, *12* (3), 275–281.

(13) Bookser, B. C.; Zhu, S. Solid Phase Extraction Purification of Carboxylic Acid Products from 96-Well Format Solution Phase Synthesis with DOWEX 1 x 8-400 Formate Anion Exchange Resin. *J. Comb. Chem.* **2001**, *3* (2), 205–215.

(14) Elmes, R. B. P.; Orange, K. N.; Cloonan, S. M.; Williams, D. C.; Gunnlaugsson, T. Luminescent Ruthenium(II) Polypyridyl Functionalized Gold Nanoparticles; Their DNA Binding Abilities and Application as Cellular Imaging Agents. *J. Am. Chem. Soc.* **2011**.

(15) Loiseau, F. A.; Hii, K. K.; Hill, A. M. Multigram Synthesis of Well-Defined Extended Bifunctional Polyethylene Glycol (PEG) Chains. *J. Org. Chem.* **2004**, *69* (3), 639–647.

(16) Leonard, J. P.; Gunnlaugsson, T. Luminescent Eu(III) and Tb(III) Complexes: Developing Lanthanide Luminescent-Based Devices. *J. Fluoresc.* **2005**, *15* (4), 585–595.

(17) Binnemans, K.; Lenaerts, P.; Driesen, K.; Go, C. A Luminescent Tris(2-Thenoyltrifluoroacetonato)Europium(. *Synthesis (Stuttg).* **2004**, 191–195.