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Synthesis of a Fluorescently Tagged Bioactive Probe Specific to Neutrophils in a Zebrafish Model

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SYNTHESIS OF A FLUORESCENTLY TAGGED BIOACTIVE PROBE SPECIFIC TO NEUTROPHILS IN A ZEBRAFISH MODEL

by

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ABSTRACT

Fluorescent bioimaging has proven to be a powerful tool in non-invasively studying biological processes in living systems. One application of this technique is being used in a transgenic zebrafish model to study the innate immune response to infection of *Candida Albicans*. Methods utilizing genetic engineering, however, are limited by time constraints that arise when having to depend upon the ability to modify genetics of the model. To bypass these constraints, this project seeks to produce a modified version of a previously developed biofluorescent probe, which was validated in a mouse model. The probe consists of three components, a hexapeptide, a fluorophore, and a polyethylene glycol (PEG) polymer. The peptide was found to bind to the formyl peptide receptors (FPRs) of the neutrophils in mice. Synthesis of this peptide was accomplished using solid phase peptide synthesis and standard Fmoc-chemistry. The peptide was purified via high performance liquid chromatography (HPLC), and characterized via mass spectrometry and ^{1}H NMR and ^{13}C NMR. The product had a yield of 9 mg. The PEG polymer serves to increase bioavailability and was conjugated to the peptide in sodium borate acetonitrile buffer system. This molecule was characterized via MALDI mass spectrometry and resulted in a yield of 4.9 mg. Further studies will verify the successful conjugation of the fluorophore to the PEG polymer. Once characterized, the probe will be validated through application to an unmodified zebrafish model.

ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Dr. Matthew Brichacek, for welcoming me into his lab. Before joining the Brichacek lab, research as a whole seemed to be incredibly daunting. However, through his mentorship, Dr. Brichacek showed me how incredibly beautiful scientific research truly is. My experience was enriched through his guidance, and I have gained invaluable skills that have taught me to be a better chemist. I would like to also thank my committee members for their time, patience, and support throughout this process, as well as their mentorship throughout my undergraduate career at the University of Maine. I would also like to thank my fellow labmates in the Brichacek lab, and especially Billy. Each of them has taken time at some point in their busy days to put what they were doing on hold to answer a question, troubleshoot an instrument, or help locate a missing piece of equipment. Their daily support and willingness to teach me, in combination with their passion for chemistry and research, has impacted me greatly. I would also like to thank all of the past teaching assistants that have taught me in all of my laboratory courses. Though the content was different from class to class, I gained skills that are universal to the research setting, and they too contributed to making me the researcher I am. I must thank my friends and family, and especially my parents, for their ceaseless support through my academic career. They have supported and encouraged me through countless achievements and trepidations. Without them, I would not be the person I am today. Finally, I would like to thank the Chemistry department at the University of Maine. Since I decided to become a chemistry major, the department has been nothing but welcoming, and has provided me with endless opportunities.

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CHAPTER 1: INTRODUCTION

The role of the innate immune system in the presence of infection has been of great interest to researchers in recent years. Bioimaging is a technique that involves molecular-guided imaging of living systems at the cellular level.[1] It is unique in that it enables the analysis of cellular interactions at the molecular level and is being developed to be utilized in research and clinical settings for diagnostics and treatment of disease.[2] Bioimaging is powerful due to the nature in how it enables researchers to essentially "see" and visually observe cellular events and interactions. Furthermore, this technique may be performed on biological systems in vitr o as well as *in vivo*, allowing for visualization in real-time. It also provides information concerning the localization and quantity of the molecules of interest. As a result, bioimaging has further expanded possibilities in biological research by the level of information it can provide researchers.

The hallmark feature of bioimaging is the probe that is used for visualization. The probe is a molecule that is designed specifically for the cellular interaction of interest and is modified to include a molecular component that will enable visualization, usually a fluorescent dye or a radiolabel.[1] Reflective of the vast diversity of cellular interactions that occur in a living system as complex as the human body, a number of different probes have been developed to investigate these interactions, showing the versatility of this technique.

One application of the bioimaging technique has been to investigate the role of the innate immune system in the presence of infection.[3] Currently, there are studies to understand the mechanisms of the innate immune response in a host infected with the microbe *Candida Albicans* (*C. albicans*).[4] Currently, researchers are using a method of

fluorescent imaging that involves use of a fluorescent transgenic zebrafish model to visualize the cellular interactions of the zebrafish neutrophil. However, a bioactive probe is of interest to provide an alternative synthetic approach to bioimaging these models, and further understanding the role of the innate immune system in the presence of infection in human systems.

1.1. General characteristics of the innate immune system and response in humans

Humans contain and carry a plethora of different microbes that reside on all surfaces of the body. The body contains at least 10 times more bacterial cells than it does human cells.[5] Many of these microbes thrive in colonies throughout the body and maintain a commensal relationship with the host, and some others even share a mutualistic-type of relationship. Infection occurs when a microbe enters the host organism and multiplies to a point of overgrowth, and the number of organisms is no longer in a state of equilibrium with the host.[6] Disease differs from infection in that it affects a small percentage of infected people and causes damage to cells of the body.[5]

In general, the immune system is comprised of many different cells that work alone and together in different combinations against different types of attacks on the body. Historically, the immune system was regarded as two systems, the innate response and the adaptive response.[7] Another subcategory is comprised of anatomical and physiological barriers against foreign substances, pathogens, and any other cells that are not compatible with the host organism. After the anatomical and physiological barriers were breached, it was concluded that the innate immune response was the host organism's first line of defense against infection. The innate system was considered to be a non-specific, but immediate response, and was overlooked by the next line of defense, the adaptive immune

response, which was considered to be a specific mode of defense and much more sophisticated. However, in recent years, research has proven the importance of the innate immune system through a greater understanding of its role in infection and disease.

When infection occurs, the innate immune response is activated by the recognition of the microbes and danger signals that are released from host cells that have been damaged.[8] A hallmark of the innate immune system is its mediation via phagocytic cells, such as macrophages and neutrophils. Understanding the differences, roles, and mechanisms of these different cells are important to fully understanding the process of innate immunity. Conveniently, the nature of these cells can be investigated through the use of fluorescence bioimaging.

Neutrophils are of specific interest in studying host defense and innate immunity response. These cells are major pathogen-fighting agents in the body.[9] The ability to be quickly recruited to sites of infection and inflammation, to recognize and phagocytose microbes, and kill pathogens through phagocytic and cytotoxic mechanisms are hallmarks of the neutrophils, and are characteristics that make the cell so remarkable. Additionally, recent research has suggested that there is a line of communication between neutrophils, macrophages, and adaptive immune system. Originally, it was thought that neutrophils were only present during the acute stage of infection, but evidence shows that neutrophils play a larger role in influencing the immune system.

1.2. General characteristics of bioactive peptides and their role in bioimaging

Bioactive peptides have been broadly defined as protein fragments that have a positive or negative effect on body functions or conditions and may have an influence on health.[11] These molecules have been found to exist in all living systems, and play critical

roles in a variety of functions such as the immune response in the presence of infection and the regulation of blood pressure and glucose levels.[10] They are derived from a variety of sources, such as food and bacteria, and a number of biological mechanisms have been proposed in regards to these peptides' roles in the human body systems (Figure 1.1).

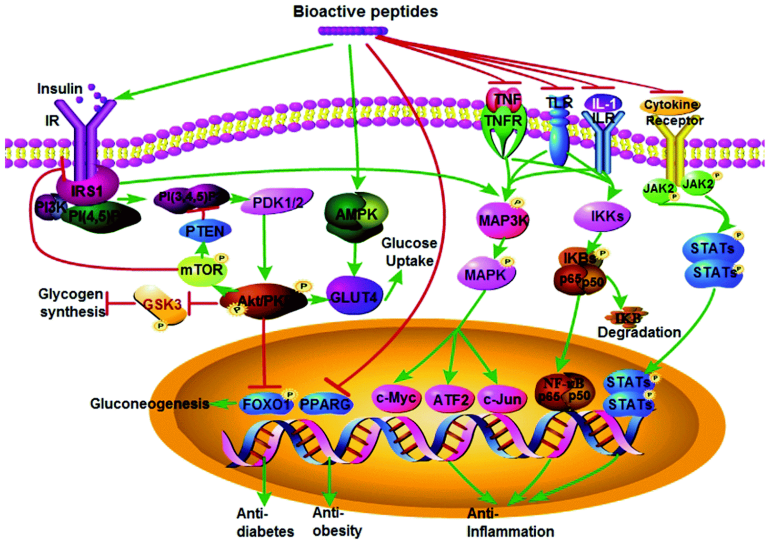


Figure 1. 1 Several proposed biological mechanisms for different bioactive peptides, including anti-diabetes effects, anti-obesity effects, and anti-inflammation effects [Adapted from Li, et al., 2017].[10]

These molecules are of interest for therapeutic targets, based on their binding characteristics to specific protein receptors. Drug molecules are being developed which utilize bioactive moieties to selectively deliver encapsulated drugs, by targeting the protein receptors that may be found on the surface of the cell of interest.[12] This same principle is being adapted for molecular-guided imaging technique.

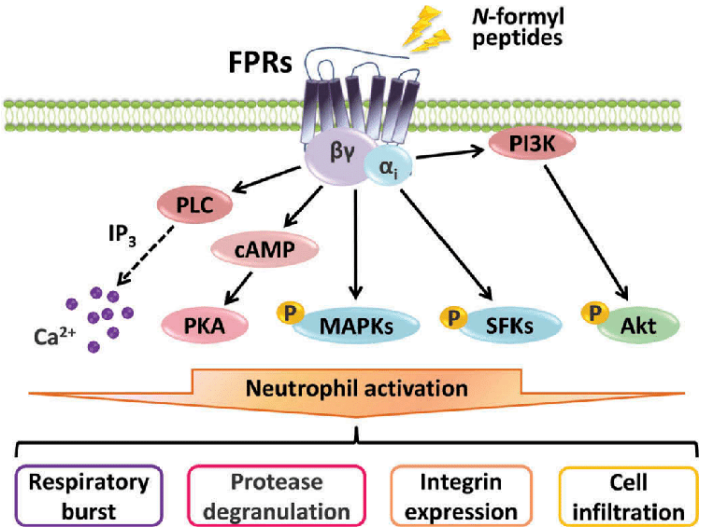


Figure 1. 2 A cartoon depiction magnifying the surface of a neutrophil and showing the transmembrane formyl peptide receptors [Adapted from Tsai, et al., 2016].[13]

For this project, the specific binding function of bioactive peptides will be utilized for targeting the neutrophil cells of the zebrafish model for imaging, but will be modified to have molecules useful for fluorescent imaging techniques. On the surface of the neutrophil cells are protein receptors called formyl peptide receptors (FPRs) (Figure 1.2).[14] There are three known types of these receptors, FPR1, FPR2, and FPR3. These receptors are part of a larger class of receptors called G-protein coupled receptors (GPCRs), which are involved in the recruitment of neutrophils in inflammatory events. However, they can also be repurposed as receptors to bind a bioactive peptide modified with a fluorescent tag to image neutrophil interactions with high selectivity. Studies have shown that these three receptors do not always bind the same ligands.[15] This aspect could be useful in developing a peptide designed to target a specific FPR.

1.3. The transgenic model and its application to study a host-pathogen interaction

Candida Albicans (*C. albicans*) is the most abundant type of fungal species in the human microbiota.[6] These organisms typically colonize different areas of the body, commonly the gastrointestinal and genitourinary tracts. In healthy individuals, the species falls into the commensal-type relationship with its host. However, in some cases when alteration has occurred to the host microbiota or when alteration has occurred in the host immunity, overgrowth of *C. albicans* can occur. It is the overgrowth of the fungi that results in a wide range of infections within the host, such as commonly known yeast infections and diaper rash. *C. albicans* is of the few fungal species that is disease-causing in humans. It is a microorganism of great interest due to its notoriety for forming biofilms on various medical devices, which presents a challenge in clinical settings to prevent patient infection. This organism alone accounts for 15% of nosocomial sepsis cases.[16]

The impact *C. albicans* has on human health has created a push to further understanding the role of the innate immune system response in the event of infection. Having knowledge of this response mechanism could provide insight for therapeutics and drug development. The zebrafish model is one tool researchers have used to study the mechanisms of a wide range of infections in a preclinical setting, including that of *C. albicans*.[8,4]

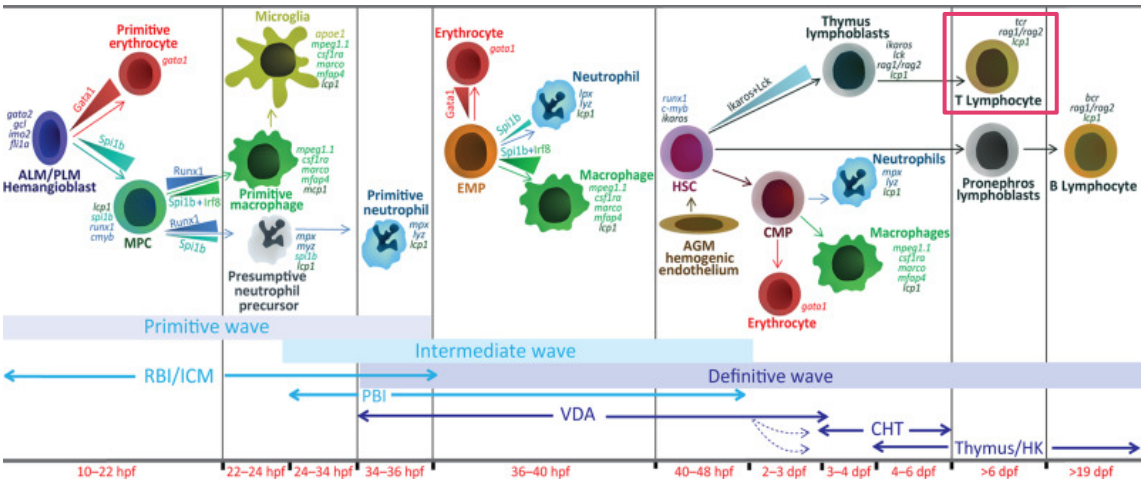


Figure 1. 3 A timeline showing the three hallmark stages of zebrafish development [Adapted from Masud, et al., 2017][8]

The zebrafish is an efficient model for a number of reasons. Similar to humans, the immune response in zebrafish is a cooperative function between the innate and adaptive immune systems. Further, the adaptive immune response in zebrafish matures much later than the innate, thus zebrafish larvae are a prime candidate for studying innate immunity in separation from the adaptive immune system (Figure 1.3). There is a window in which the organism has not developed an adaptive immune response. Development of the adaptive immune system is indicative by the presence of T cells.

Another major benefit of using zebrafish is the optimized optical accessibility the specimen provides as a result of its transparent tissues. Finally, zebrafish embryos are readily and easily colonized by commensal flora that are commonly found on surfaces of the human body. Over the past decade, embryonic, larval, and adult zebrafish have been used in a range of models to study the complexities of immune response.[8]

Another important aspect of the zebrafish model is the availability of genetically engineered specimen which have been altered to express fluorescently actively proteins.

The genome of the model of interest in this research project has been transformed to include either the mpx:GFP or mpx:mCherry transgene. [4] In essence, the myeloperoxidase promoter is included in the organism's genome, which promotes the neutrophil-specific expression of either the proteins green fluorescent protein (GFP) or the mCherry protein. These proteins absorb and emit light at different wavelengths, but both within the visible range, allowing for fluorescent imaging studies to be performed to visualize the cellular interactions of the neutrophils. Furthermore, the wavelengths at which these proteins fluoresce differ enough from the wavelengths that cause macrophages to fluoresce, which ensures the ability to selectively visualize neutrophil cells.

While a useful method for imaging cellular interactions of neutrophils, this method is tedious in its reliance on genetics. In the event that a fishline lacks the promoter necessary to expresses fluorescently active proteins, the issue may be remedied by crossing fishlines. However, the amount of time necessary to produce a functional model is costly to the flow of research and is a disadvantage to this means of imaging. A synthetic alternative that can be introduced to the zebrafish model endogenously is of interest in order to remove the time hindrance associated with current methods while maintaining imaging functionality.

1.4 Current bioactive probes designed to target study of the innate immune system

As previously discussed, a number of bioactive probes have been developed for use in bioimaging techniques. One such probe was developed by a research group at the University of Virginia that targets the FPRs on the surface of neutrophil cells in mice.[17] This probe consisted of three components, a bioactive peptide, a polyethylene glycol (PEG) polymer, and a Cyanine 7 fluorophore (Figure 1.4).

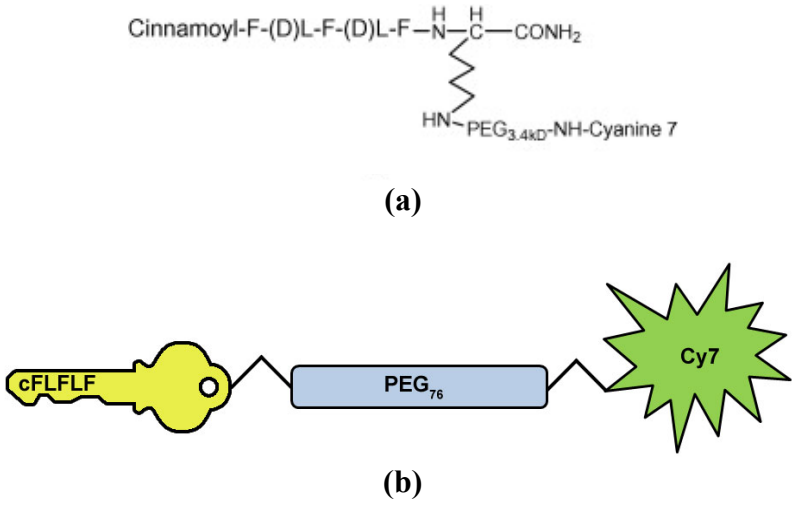
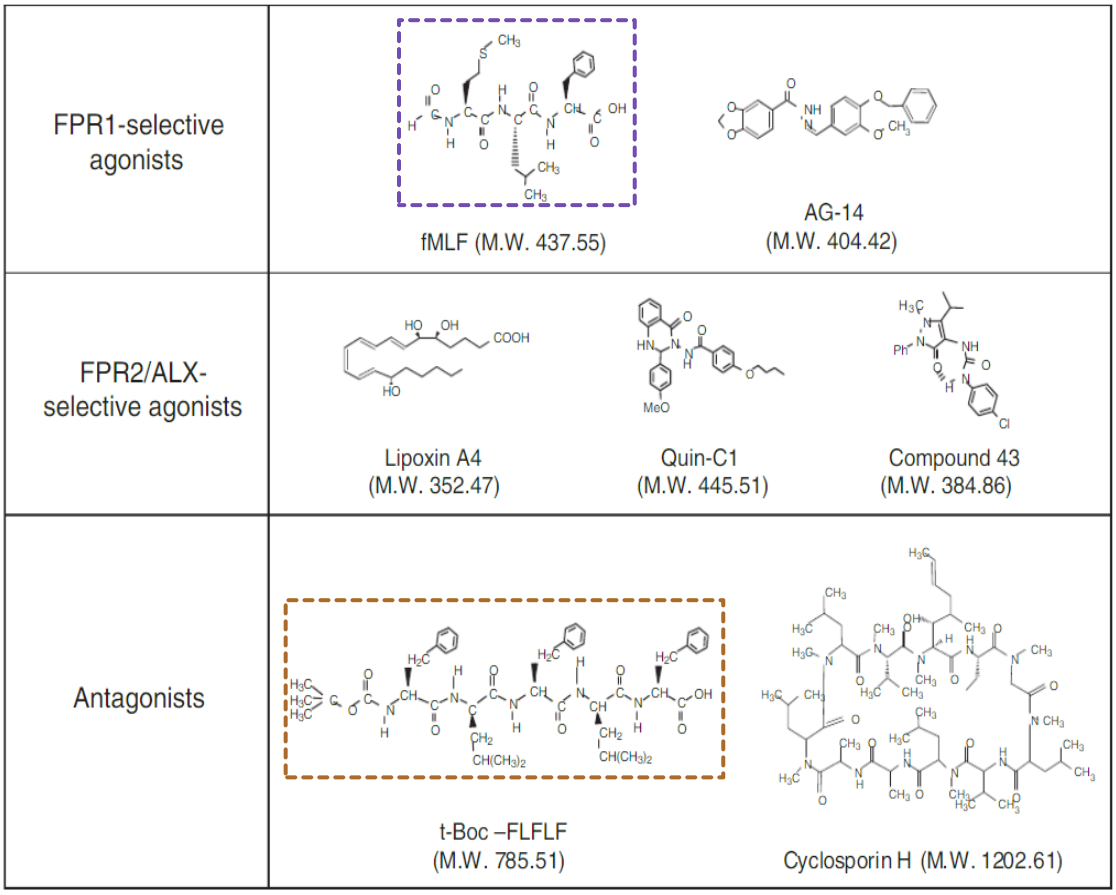


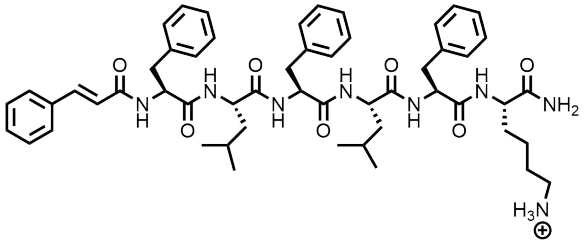
Figure 1. 4 (a) Hybrid structure of the bioactive probe developed by Xiao, et al [Adapted from Xiao, et al., 2010].[17] The complex includes a short hexapeptide capped with a cinnamoyl, cFLFLFK, a polyethylene glycol (PEG) polymer and a Cyanine 7 fluorophore. (b) A graphical depiction of a bioactive probe developed to selectively target the FPRs on the neutrophils in mice [Adapted from Riedel, 2013].[18]

Because the functionality of the bioactive probe depends solely on the bioactive peptide that serves as the foundation, design of the bioactive peptide is important when developing imaging probes. Figure 1.5 compares a few known ligands that have binding

affinities FPRs (a) to the structure of the peptide designed for use as a bioactive probe specific to mouse neutrophils.



(a)



(b)

Figure 1. 5 (a) Some known N-formylated ligands with binding affinities for FPRs [Adapted from Ye, et al., 2017].[15] (b) Structure of the target bioactive peptide, cFLFLFK.

One similarity between some of the known ligands and the target molecule is the presence of phenylalanine residues and leucine residues (Figure 1.5a in purple and yellow, respectively). Another element in peptide ligands for FPRs are the presence of N-formylated methionine residues.[19] The mechanism of this binding event is not clear, but studies have shown that N-formylated peptides with a methionine residue at the the N-terminus commonly act as ligands to FPRs. The peptide highlighted in purple has a methionine residue at the N-terminus and has been formylated (Figure 1.5a). The target peptide (Figure 1.5b) is not formylated, nor does it include a methionine residue. However, the cinnamoyl cap on the target peptide may mimic the effects of the N-formylated methionine residue, in that it promotes binding to the FPR. It may also increase antagonistic characteristics of the peptide, so that it binds to the FPR but does not result in signal transduction. In essence, the peptide would have similar binding affinities, but the neutrophil would not recognize the peptide as a recruiting molecule to elicit an immune response against the peptide.

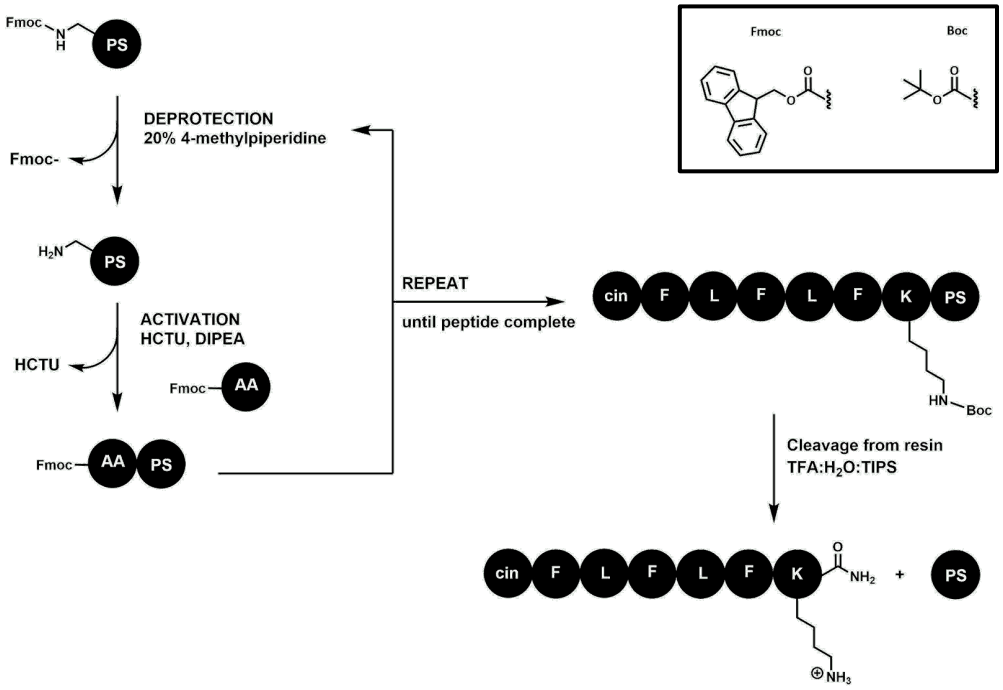


Figure 1. 6 Overview of solid phase peptide synthesis using standard Fmoc-chemistry to produce the peptide cFLFLFK.

For synthesis of the bioactive probe, first the peptide was produced via automated solid phase peptide synthesis using standard Fmoc-chemistry.[20] In solid phase synthesis, there are two main steps that make up one coupling cycle of an amino acid fragment (Figure 1.6). The first step involves the removal of a protecting group, fluorenylmethyloxycarbonyl (Fmoc), from the terminal amine of the peptide chain. In the Figure above the N^{α} that is deprotected is the amine on the terminal end of the solid support (denoted as PS). In subsequent coupling cycles, the amine to undergo deprotection will be a part of the peptide backbone. This functional group is used to control the reactivity of the terminal amine throughout reactions and reduces the likelihood of a doubling coupling event to occur throughout the synthesis.[21] Another protecting group, tert-butyloxycarbonyl (Boc), is used to control the reactivity of the terminal amine of the lysine residue side chain, so to prevent side chain reactions from occurring.[22]

The use of protecting groups for N^{α}-amino protection is an important feature in solid phase peptide synthesis techniques.[21] The Fmoc group replaced the previously preferred Boc group due to the milder conditions necessary for removal of the Fmoc group. Deprotection of Fmoc requires a mild base, such as 4-methylpiperidine, whereas the Boc group becomes labile in acidic conditions. The milder conditions allowed for the elimination of repetitive acidolysis steps and the final strong acidolysis step which were hallmarks of solid phase synthesis that used Boc group for N^{α} protection. This characteristic is also more compatible with peptides that are susceptible to acid-catalyzed side reactions, making the Fmoc compound a more efficient protecting group. However, the compatibility between these two molecules based on their stabilities in different conditions (basic/acidic) allows for selective deprotection of N^{α}-amino groups and selective protection of reactive side chains throughout the synthesis of the peptide.

After deprotection, the first amino acid residue may be loaded onto the resin in the next step. A coupling agent is used, 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), to activate the carboxylic acid of the incoming amino acid fragment. Then the carboxylic acid donates the acyl group, which forms the peptide bond. The peptide bond presents a nucleophilic attack on the α-amino group of another amino acid.[22] The incoming amino acid residue is also protected with an Fmoc group, so to load the next amino acid the cycle begins again with removal of the Fmoc group. Upon completion, the peptide can be released from the solid support under strong acidic conditions. This step also globally deprotects the Boc-protected sidechains. Stepwise, peptides can be synthesized from the C to the N terminus using N^{α}-protected amino acids.[23]

The modification to the peptide to include a PEG polymer was found to be necessary to increase the bioavailability of the peptide.[17] The peptide is composed of largely non-polar amino acid residues (phenylalanine and leucine), further it is capped with the cinnamoyl group at the N-terminus and the C-terminus has been functionalized with an amide, rather than the typical carboxylate group. As a result, the peptide is highly hydrophobic and its ability to remain in biological systems proved itself a challenge. A PEG polymer of 3.4 KDa (76 repeat units) was determined to be a sufficient length to increase the bioavailability and ensure the peptide could exist in aqueous environments (i.e. when injected into the bloodstream if a mouse model). This molecule was attached to the terminal amine of the lysine residue side chain through a conjugative addition reaction in a 0.1 M acetonitrile sodium borate buffer system (pH 8.5).

The selection of the Cyanine 7 (Cy7) fluorophore was based on its emission wavelength, 800 nm, which is in the near-infrared region (NIR). This was necessary due to the application of the probe to a mouse model. At the NIR region, mammalian tissue has low absorbance, thus the signal could better penetrate through the tissue. Fluorophores chosen in the visible range would be difficult to visualize. This compound was conjugated to the terminal amine of the PEG_{76} molecule using similar conditions to attach the PEG polymer to the peptide.

The completed probe was synthesized using the methods previously described. In addition to the target molecule, a series of derivatives were produced as well, with slight modifications made. These modifications included varying lengths of the PEG polymer, complete removal of the PEG polymer, and conjugation of the fluorophore to different functional groups on the peptide and peptide-PEG molecule. The set of these bioactive

probes were validated using a mouse model, and it was determined that the most effective probe included the peptide, PEG_{76} polymer, and fluorophore conjugated to the PEG_{76} polymer.

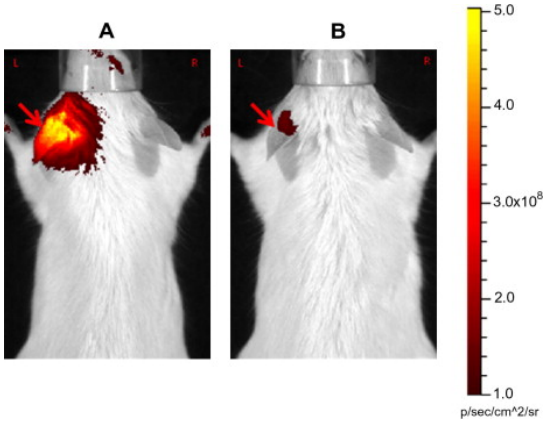


Figure 1. 7 Representative image taken using NIR fluorescence imaging of mouse models 3 hours post-injection with the bioactive probe. Normal group (a); blocking group (b) pre-injected with a non-fluorescently active bioactive probe before injection with bioactive probe [Adapted from Xiao, et al., 2010].

Specificity tests of the probe were performed on a mouse model. Acute inflammation was induced by a topical application of phorbol myristate acetate (PMA) to the left earlobes of the mice. This caused ear dermatitis, which ultimately promoted the migration of neutrophils to the afflicted area. The fluorescent bioactive probe was injected into the mouse model via a tail vein, and in vivo NIR fluorescence imaging was performed. In models injected with the probe, fluorescence intensities were recorded at higher levels than the control, suggesting high binding affinity for the FPRs on neutrophil cells. Specificity experiments were conducted as well by pre-injecting mice with a non-fluorescent peptide one hour before injecting the specimen with the fluorescent probe. It was expected that the models pre-injected with blocking agents would not show high neutrophilic activation or accumulation under fluorescence studies if the bioactive peptide was selective for FPRs. Comparative analysis showed lower fluorescence intensities in the

blocking group than in the normal group (Figure 1.7), suggesting that the probe has high specificity for FPRs. The group concluded that a potential probe was synthesized for NIR fluorescence bioimaging specifically targeted towards neutrophilic activation and accumulation.[17] This probe is available for commercial purchase.[16] However, the attachment of only the Cy7 fluorophore limits the range of use for the tool. Bioactive probes such as the molecule developed for use in a mouse model that could be modified with a range of fluorophores would increase the versatility of the probe and widen the possibilities for bioimaging techniques.

1.5. Synthetic strategies to reproduce bioactive probe cFLFLFK-PEG$_{76}$-fluorophore

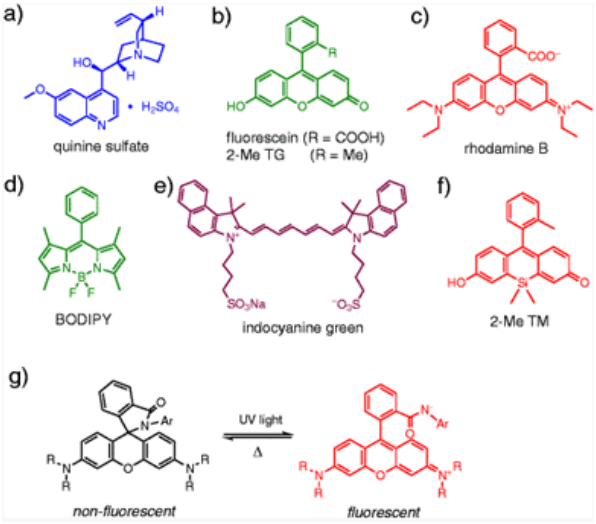


Figure 1. 8 Chemical structures of various platforms of organic fluorescing molecules. The color of each molecules loosely represents the color it emits.[2]

Due to the established superiority of the zebrafish model and the heightened interest and importance of fluorescence bioimaging, there is a demand for the development of bioactive probes that can be utilized for bioimaging studies. These probes are attractive

based on the modes in which they are endogenously introduced to the model. There is a movement towards developing new, novel fluorescent probes and biosensors that are a hybrid between organic fluorescent molecules and synthetic molecules, reflective of the probe developed by Xiao, et al.[17] A host of different organic fluorescent molecules have been discovered and reported to emit a range of different colors and have become commonly used organic molecules in fluorescent imaging (Figure 1.8).[2] The bright and photostable dyes with red to near-infrared (NIR) fluorescence are highly attractive choices for in vivo imaging in living systems because tissue is transparent in this wavelength region. Cyanine dyes (Figure 1.8e) have been modified and adapted to accommodate this demand. Fluorescein (Figure 1.8b) and rhodamine (Figure 1.8c) are the mostly commonly used molecules for labelling or as probes for bioimaging.

The fluorescein and rhodamine dyes are also analogous to the GFP and mCherry proteins previously discussed. These small molecules have potential to be incorporated into a synthetic peptide targeting FPRs and replace the need for transgenic zebrafish models used for studies of host-pathogen interactions in the presence of infection. Furthermore, as there is a wide variety of known fluorophore molecules, there is potential to create a library of probes that would increase the versatility of bioimaging of zebrafish models for infection.

1.6 Thesis Project Overview

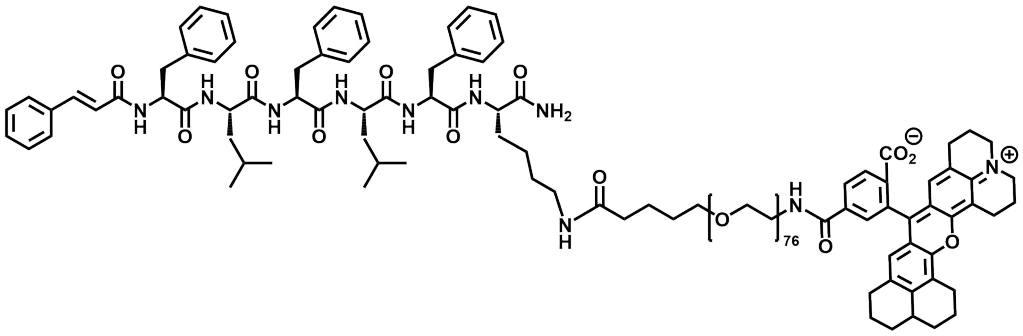


Figure 1. 9 Structure of the target molecule of interest, cFLFLFK-PEG_{76}-5(6)-ROX. The attached fluorophore is a 5(6)-ROX, a derivative of the rhodamine class. This particular derivative is analogous to that of the mCherry protein.

Synthesis of a bioactive probe for use in a zebrafish model to study host-pathogen interactions was conducted using similar methods previously discussed. The target molecule consisted of a bioactive peptide, PEG polymer, and small molecule fluorophore (Figure 1.9). The short hexapeptide capped with a cinnamoyl, cFLFLFK, was produced using solid phase peptide synthesis procedures. Both manual and automated methods were investigated for production of the peptide. The addition of a PEG_{76} polymer was done through bioconjuation in a buffer system. The fluorophores of interest were chosen based on their similarities in characteristics to the current proteins encoded in transgenic zebrafish models. A 5(6)-ROX fluorophore was chosen as the synthetic alternative to the mCherry protein. It will be attached to the peptide-PEG_{76} complex through bioconjugation. After the completion of each component (peptide, peptide-PEG_{76}, peptide-PEG_{76}-fluorophore), purification was performed via high performance liquid chromatography (HPLC) and size exclusion chromatography. Characterization studies were performed for each molecule

with mass spectrometry, ^{1}H NMR, ^{1}C NMR, and UV-vis analyses. Once the target molecule is completed, validation of the probe will be confirmed through application to an unmodified zebrafish model.

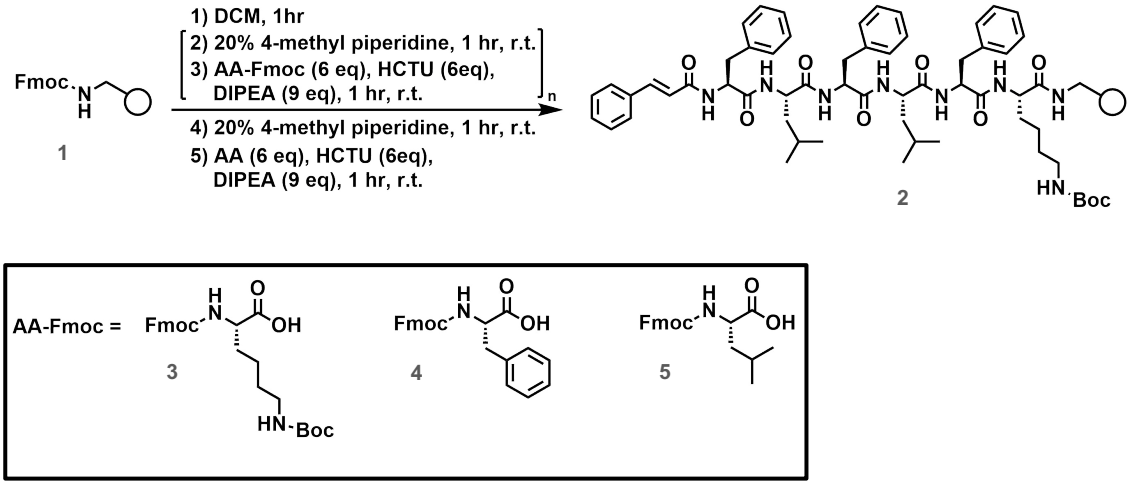
CHAPTER 2: EXPERIMENTAL

2.1. Materials and general considerations

All chemicals were available from commercial suppliers and did not undergo any purification or altering unless specified. For automated peptide synthesis an AAPPtec automated peptide synthesizer was used, and all reactions were run at room temperature under N_2. Analytical to semi-preparative high-performance liquid chromatography (HPLC) was performed with a Gilson GX-271 liquid handler and a Gilson 322 pump. A Kintex 5 μm EVO C18 100Å 4.6 x 150 mm column and Kinetex 5 μm EVO

C18 100Å 21.1 x 150 mm column were used for analytical and preparative purposes, respectively. Data was rendered in Trilution LC software. Products were characterized by ^{1}H and ^{13}C NMR, liquid chromatography/mass spectrometry (LC/MS), matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and UV-vis analysis. A Varian UNITY INOVA 400 MHz instrument was used to obtain ^{1}H and ^{13}C NMR data. An Agilent 1100 series HPLC coupled with a diode array detector (DAD, model G1315A) and a mass selective detector (MSD, model G2445A) operating in electrospray ionization (ESI) was used to collect LC/MS data. A Bruker Daltonics UltrafleExtreme MALDI TOFTOF instrument was utilized to collect MALDI-TOF MS data. A Beckman DU 7500, Diode Array UV-vis spectrophotometer was used for UV-vis analysis. A SpectraMax i3x UV-vis spectrophotometer was also used for UV-vis analysis.

2.2. Synthesis of hexapeptide, cFLFLFK-NH_2, via solid phase peptide synthesis using manual coupling techniques



Scheme 2. 1 Overview of synthesis of hexapeptide, cFLFLFK, with solid phase peptide synthesis using standard Fmoc chemistry.

2.2.1. Preparation of Resin

The target peptide, a hexapeptide, cFLFLFK-NH_2, was synthesized using a modified procedure published by Xiao, et al[13]. To a polyprep column (BioRad), 0.1 g of Fmoc-PAL-AM resin (0.5-0.8 mmol/g, EMD Milipore) was added. To prepare the solid support, the resin was swelled by adding 2 mL of dry dichloromethane to the polyprep column and then the column was agitated in an incubator at 190 rpm and room temperature. After 1 hour, the dichloromethane was eluted, and the resin was washed with 2 mL of DMF three times.

2.2.2. Removal of Fmoc protecting group

For removal of the Fmoc group, a solution of 20% 4-methyl piperidine was prepared in an inert environment and stored in a flame-dried 40-mL vial. Then 8 mL of the prepared solution was added to the polyprep column containing the swelled resin and the column was agitated in an incubator at 190 rpm, at room temperature, for 1 hour. The 20% 4-methyl piperidine solution was then eluted, and the resin was washed with 10 mL of DMF, three times.

2.2.3. Elongation of peptide

For coupling of the first amino acid, an Fmoc-protected lysine, a coupling cocktail was prepared by dissolving 0.281 g of Fmoc- Lysine (0.6 mmol), 0.248 g of O-(6-Chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HCTU; 0.6 mmol), and 0.157 mL of diisopropylethylamine (DIPEA; 0.9 mmol) in 1 mL of DMF. The solution was immediately transferred to the polyprep column, and the reaction was agitated in an incubator at 190 rpm, at room temperature, for 1 hour. Once complete, the coupling cocktail was eluted, and the resin washed with 10 mL of DMF three times.

2.2.4. Monitoring of reaction progress via UV-vis spectroscopy

To monitor the progress of the coupling reaction, 8 mL of DMF was added to the column. The column was briefly shaken, to evenly disperse resin, and then 20 μL aliquot of the solution was reserved and transferred to a 7 mL vial. The resin was dried under vacuum to remove DMF. Then 3 mL of the 20% 4-methylpiperidine was added to the vial, and the resin was agitated in an incubator at 190 rpm for about 20 minutes. Then a UV-vis reading was taken at 290 nm against a blank of 20% 4-methyl piperidine.

For the coupling of the subsequent amino acids, the previously described procedure for deprotection of the Fmoc group and the coupling of the activated amino acid was followed (Sections 2.2.2 and 2.2.3.). After each coupling reaction (described in section 2.2.3), loading was monitored via UV-vis.

2.2.5. Coupling of cinnamoyl cap

To cap the peptide with a cinnamoyl group, the Fmoc group was first removed from the terminal amino acid (phenylalanine) following the previously described methodology. A coupling cocktail was similarly prepared by dissolving 0.089 g cinnamic acid (0.6 mmol), 0.248 g of HCTU (0.6 mmol), and 0.157 mL of DIPEA (0.9 mmol) in 3 mL of DMF. The solution was immediately transferred to the polyprep column and the reaction was agitated in an incubator at 190 rpm, at room temperature, for 20 hours. At this point, the plastic polyprep column, originally clear and colorless, was stained a dark brown, and the eluent also a dark brown color. A pale brown colored sediment formed in the bottom of the column. The resin was separated from the layer of sediment by adding DCM to the column and collecting the top layer that formed, which contained only resin. The resin was then washed with 5 mL of DCM three times.

2.3. Synthesis of peptide cFLFLK via automated synthesis

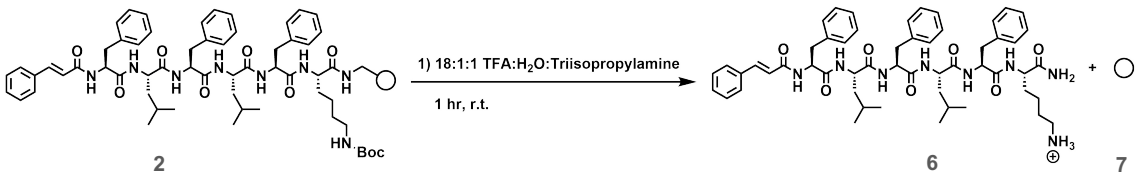
This procedure followed for manual coupling was adapted for synthesis of the peptide using an automated peptide synthesizer. In set-up for this reaction, a solution of 20% piperidine in DMF was prepared, 0.5 M solutions of the amino acids were prepared, 0.5 M solution of HCTU was prepared, and a 0.75 M solution of DIPEA was prepared. To the synthesizer 0.050 g of PAL-AM resin was added, and swelled in the peptide

synthesizer, in DCM for 1 hour then washed with DMF three times before deprotection and coupling proceeded.

For deprotection, 2.0 mL of 20% 4-methylpiperidine was transferred to the resin, and the resin was incubated and shaken for 1 hour. Then the resin was washed with DMF 3 times. Then the instrument delivered 0.7 mL of 0.5 M Fmoc-protected amino acid solution, 1 mL of 0.5 M HCTU, and 1 mL of 0.75 mL of DIPEA to the resin. The resin was incubated and shaken for 1 hour, then washed with DMF 3 times, and the deprotection and coupling cycles would begin for the next amino acid.

After the final coupling of the last phenylalanine residue, before removing the final Fmoc group, the resin was washed a final time and then dried under N_2 on the instrument. The dried resin was transferred to a polyprep column, and the final deprotection and coupling with the cinnamic acid was carried out following manual coupling procedures.

2.4. Cleavage of peptide from PAL-AM resin



Scheme 2. 2 Cleavage of synthesized peptide, cFLFLFK, from solid support using an 18:1:1 solution of TFA, water, and triisopropylsilane.

To remove the synthesized peptide from the solid support after both manual coupling and automated synthesis methods, the resin was first dried under a stream of N_2 gas for 1 hour. Then a cleavage cocktail was prepared by combining 4.5 mL of TFA, 0.25

mL of water, and 0.25 mL of triisopropylsilane (TIPS). The solution was immediately transferred to the polyprep column containing the loaded resin. The reaction was agitated in an incubator at 190 rpm, room temperature, for 1 hour. Then the solution was eluted and collected in a 20 mL vial. The TFA, water, and TIPS solution was removed via a rotary evaporator and dried under vacuum.

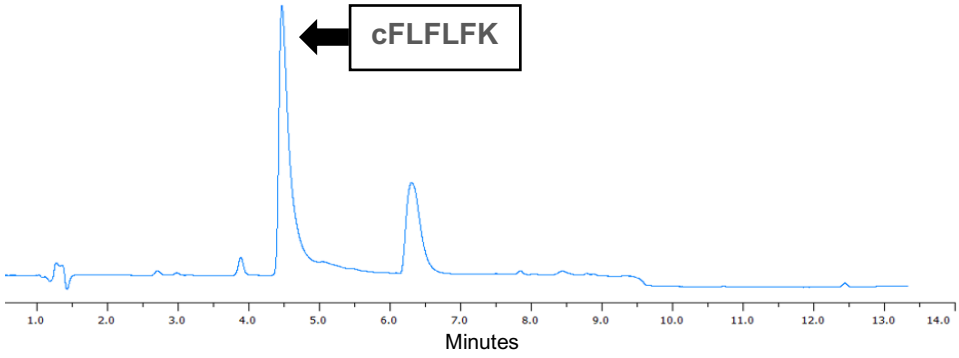


Figure 2. 1 Analytical HPLC trace for purification of peptide cFLFLFK. Stationary phase: C18; mobile phase water:acetonitrile, 30-70%, over 10 minutes. UV-vis detected at 254 nm and 280 nm. The peak retained at 4.5 minutes was collected characterization.

The product was purified via high-performance liquid chromatography (HPLC). First analytical HPLC was performed, and UV-vis measurements were recorded at 254 nm and 280 nm to optimize conditions. It was determined that at 30-70% water to acetonitrile, over 10 minutes, the product was retained on the column for 4.5 minutes (Figure 2.1). The product was then purified with preparative HPLC.

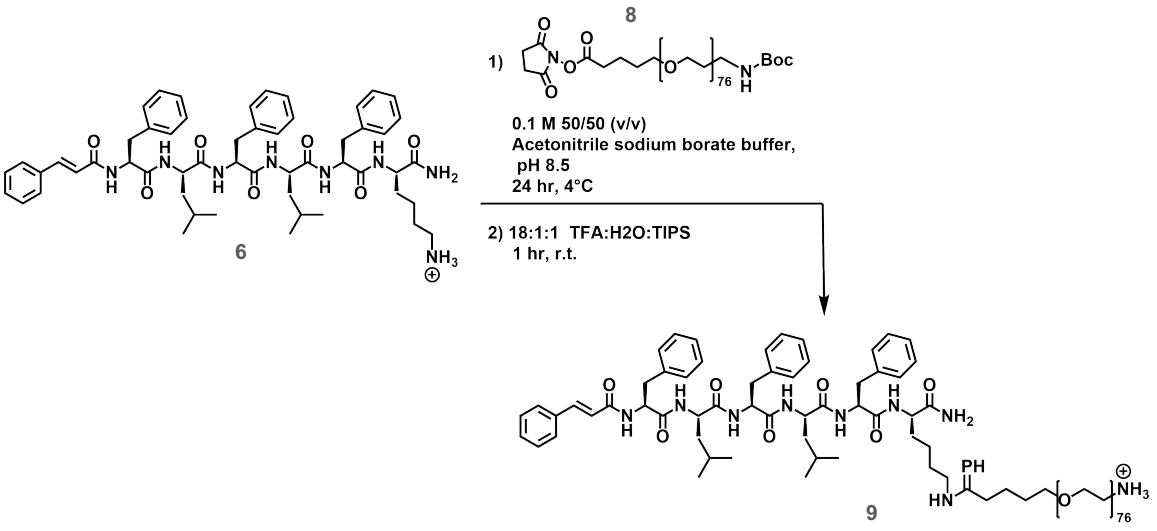
For manual coupling methods, the yield was 9 mg (9.52 µmol, 38%) of product. For automated synthesis methods, the yield was 5.9 mg (6.25 µmol, 25%) of product. Using HPLC, the retention time of cFLFLFK-NH_2 was 4.5 minutes. The compound produced using manual coupling procedures was characterized with LC/MS and ^{1}H NMR and ^{13}C

NMR. The solvent used was methanol-D. The mass of the product was calculated to be 944.5 g/mol. Results from the LC/MS showed that the product was 944.3 g/mol.

^{13}C NMR (101 MHz, CD_3OD) δ 175.33 (s), 174.42 (s), 174.08 (s), 173.91 (s), 173.20 (s), 172.27 (s), 168.22 (s), 141.74 (s), 137.31 (d, *J* = 4.7 Hz), 136.62 (s), 134.75 (s), 130.24 – 129.66 (m), 128.32 (t, *J* = 55.2 Hz), 126.70 (s), 119.81 (s), 57.20 (s), 55.77 (d, *J* = 28.0 Hz), 53.84 (s), 53.26 (s), 52.81 (s), 40.02 (s), 39.50 (d, *J* = 16.9 Hz), 36.98 (s), 36.52 (s), 30.68 (s), 26.50 (s), 24.51 (d, *J* = 15.0 Hz), 22.20 (d, *J* = 23.5 Hz), 20.63 (d, *J* = 24.9 Hz).

^{1}H NMR (400 MHz, CD_3OD) δ 8.64 – 8.55 (m, 8H), 8.64 – 8.31 (m, 36H), 8.10 (s, 5H), 7.95 – 7.77 (m, 41H), 7.66 – 7.46 (m, 40H), 7.46 – 7.32 (m, 36H), 7.32 – 6.73 (m, 158H), 6.72 (s, 9H), 4.87 (s, 307H), 4.44 (dd, *J* = 46.6, 39.2 Hz, 50H), 4.15 (dd, *J* = 15.3, 10.3 Hz, 23H), 3.37 – 3.20 (m, 107H), 3.19 – 3.00 (m, 44H), 3.00 – 2.21 (m, 92H), 2.02 (s, 5H), 1.88 (s, 9H), 1.71 (d, *J* = 9.6 Hz, 14H), 1.64 – 1.33 (m, 93H), 1.27 (d, *J* = 7.4 Hz, 13H), 1.11 (s, 6H), 0.96 – 0.74 (m, 104H).

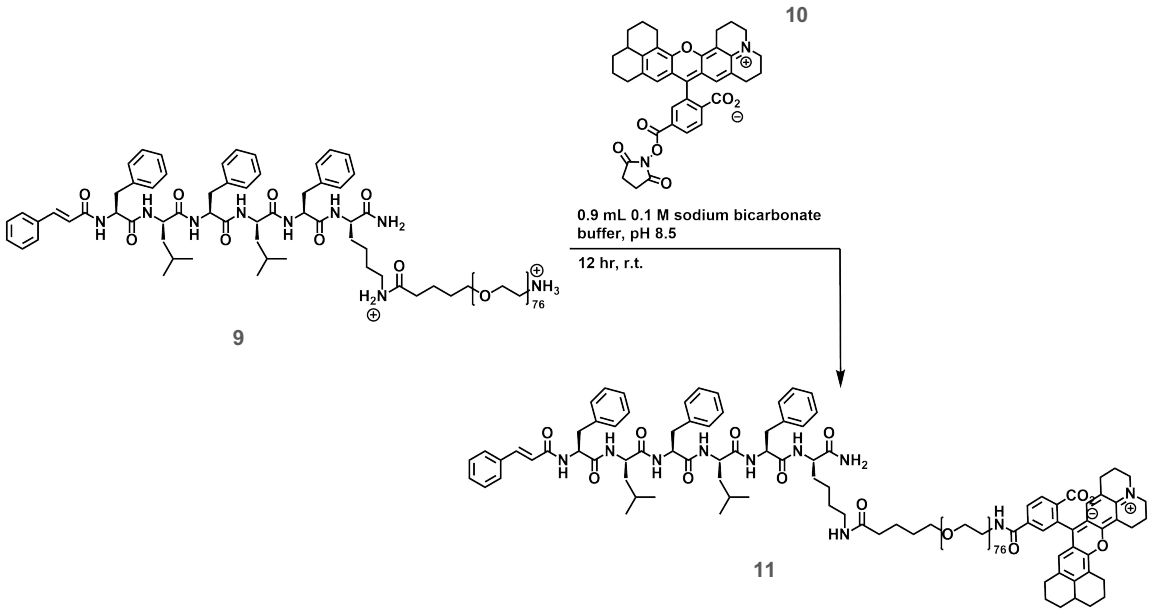
2.5. Synthesis of peptide cFLFLFK-PEG$_{76}$-NH$_2$



Scheme 2. 3 Conjugation of polyethylene glycol polymer (PEG$_{76}$) in a 0.1 M acetonitrile sodium borate buffer of pH 8.5.

For the conjugation of the polyethylene glycol molecule to the ω-NH_2 of the lysine residue, a procedure previously published by Zhang, et al. was followed[20]. In a 7 mL vial, 3.2 mg of peptide cFLFLFK-NH_2 and 12.6 mg of *tert*-Boc-PEG-NHS ($PEG_{76,}$ MW-3400 Da; Laysan Bio Inc.) was dissolved in a 1:1 mixture of acetonitrile and sodium borate buffer (0.1 M, pH 8.5), at 4°C or 24 hours. The reaction was monitored via analytical HPLC (Figure A1). The stationary phase was a C18 column and the mobile phase was water:acetonitrile (50-80%). Absorbance was measured at 215 nm and 254 nm. The product was then purified via preparative HPLC using the conditions optimized in analytical experiments and characterized via mass spectrometry (MALDI-TOF). The calculated mass of the compound was 4489.6 g/mol. The experimental mass of the compound was found to be 4511.9 g/mol.

2.6. Conjugation of fluorophore, 5(6)-ROX NHS ester



Scheme 2. 4 Conjugation of 5(6)-ROX NHS ester fluorophore in a 0.1 M sodium bicarbonate buffer of pH 8.5.

2.6.1. Conjugate addition of 5(6)-ROX NHS ester

For the conjugation of the 5(6)-ROX NHS ester fluorophore, an adapted protocol was used previously published by the manufacturer.[24] In a 7-mL vial, covered completely in aluminum foil, 2.8 mg of 5(6)-ROX NHS ester (3.48 μmol, 8 equivalents) were dissolved in 1/10th of the reaction volume of DMSO (0.1 mL). In separate 7-mL vial, 1.9 mg of cFLFLFK-PEG_{76}-NH_3^+ compound was dissolved in 9/10th of the reaction volume of 0.1 M sodium bicarbonate buffer (pH 8.5; 0.9 mL). The reaction was run for 12 hours at room temperature. The reaction was monitored via HPLC. The stationary phase was a C18 column, and the mobile phase was water:acetonitrile (30-70%).

2.6.2. Purification of cFLFLFK-PEG_{76}-5(6)-ROX via size exclusion chromatography

Before purification, the product was condensed using rotary evaporation until about 0.1 mL of solvent remained. Solid precipitate formed, and 0.8 mL of distilled water was added to the reaction vessel to re-dissolve product. Then the product was purified using size exclusion chromatography on a Sephadex G-25 column. The Sephadex G-25 media was pre-swelled in distilled water and then packed into a 4.5 cm tall column with diameter 2 cm. The column was equilibrated with distilled water. Then half of the solution containing product (about 0.5 mL) was loaded onto the column. Over and hour and a half, 19 1-mL fractions were collected in 1.5-mL microcentrifuge tubes. The first 10 fractions that eluted from the column were clear and colorless. The subsequent fractions were still clear but were a pink hue that gradually intensified and then weakened again.

The fractions were analyzed via UV-vis spectroscopy. A plate was prepared with 100 μL of each fraction collected. A full absorbance spectrum was measured for each fraction against a blank of distilled water. Based on this analysis, fractions 4-10 and then fractions 11-17 were combined and condensed for further study. Instead of using a plate reader, the samples scanned in a quartz cuvette against a blank of distilled water. Then the sample containing fractions 11-17 was analyzed via mass spectrometry (MALDI-TOF MS).

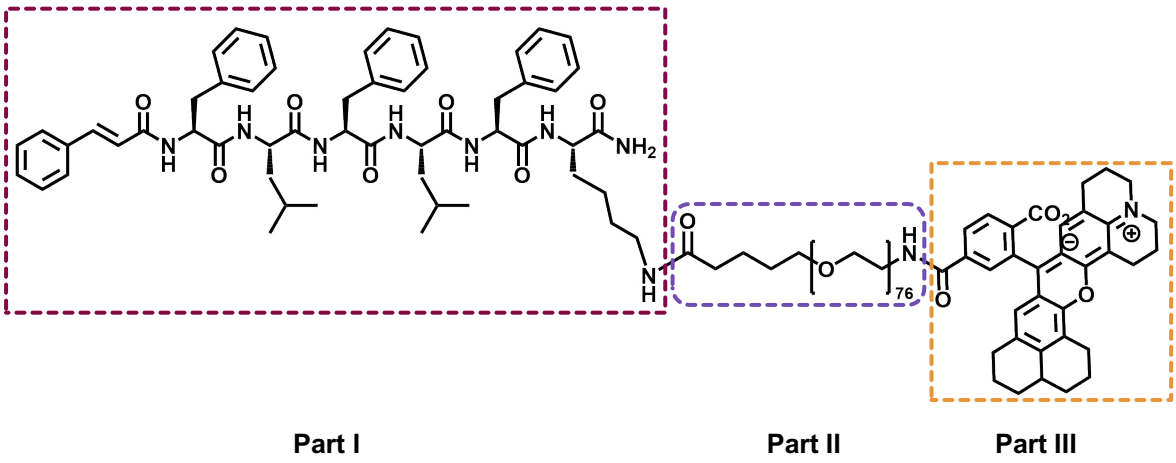


Figure 3. 1 Structure of overall target molecule, cFLFLFK-PEG$_{76}$-5(6)-ROX. This is a bioactive, fluorescently tagged peptide modified with a polyethylene glycol (PEG$_{76}$) polymer. Synthesis of the molecule was divided into three parts.

The target bioactive probe is of interest due to previous studies determining that this peptide has a specific binding affinity to the formyl peptide receptors found the surface of neutrophils in mice.[17] This binding affinity provides researchers with a method to attach small molecules, such as fluorophores, to the neutrophils. As a result, researchers are enabled to visualize cellular interactions of the neutrophils at the molecular level. Furthermore, these fluorescent imaging studies utilizing the bioactive probe may be performed *in vivo*, as it is biologically relevant to living systems.

The target molecule in this study was a short hexapeptide, FLFLFK, capped with a cinnamoyl, that was modified with the addition of a PEG$_{76}$ polymer and tagged with a fluorophore (Figure 3.1). The synthetic route was divided into three parts. First the peptide capped with the cinnamoyl was to be synthesized. This molecule was targeted based on its binding affinity to the formyl peptide receptors on the neutrophils in a mouse model. Then

the PEG_{76} polymer could be conjugated to the peptide. The addition of the PEG_{76} molecule was determined to be important in increasing the bioavailability of the peptide.[17] Finally, a fluorophore could be conjugated to the PEG_{76} polymer to produce the target molecule. The fluorophores were chosen based on their similarities in absorbance and emission characteristics to the fluorescent proteins currently genetically encoded in zebrafish models.

3.1. Synthesis of peptide cFLFLFK

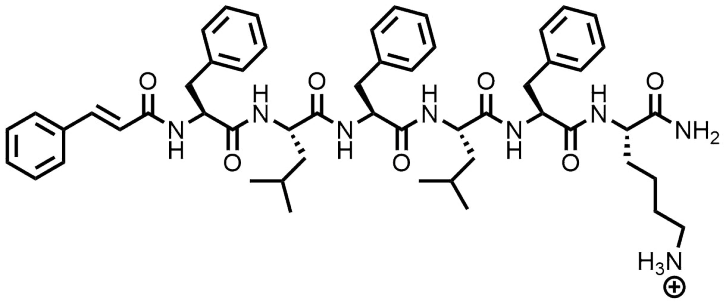


Figure 3. 2 Structure of target molecule, peptide FLFLFK, capped with a cinnamoyl group.

A procedure modified from Zhang, et al., was followed to synthesize the short hexapeptide, FLFLFK (Figure 3.2). This peptide was synthesized via manual and automated coupling procedures. Similar conditions were used to cap the peptide with a cinnamoyl group.

3.1.1 Selection of Resin Fmoc-PAL AM

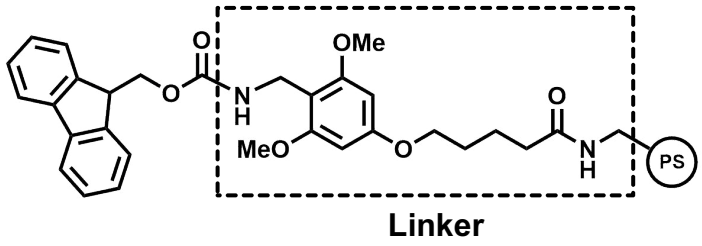


Figure 3. 3 Structure Fmoc-PAL AM resin, consisting of an Fmoc-protected linker attached to a polystyrene bead.

For the synthesis of the peptide cFLFLFK, an Fmoc-protected PAL AM resin was selected (Figure 3.3). This solid support is composed of a polystyrene bead and a linker that attached to and extends from the bead. The use of polystyrene as the anchor for peptide synthesis allows for a more uniform reaction mixture because this material is porous when swelled. When swelled, reagents and solvents are able to pass through the pores of the material.

This resin was also selected based on its linker, as it would result in a carboxamide at the C-terminus after cleavage from the solid support, rather than the typical carboxylate. The carboxamide serves as a pseudo protecting group since the amide is less likely to be deprotonated and form the conjugate base of the amide. The formation of the amide allows for selective reactivity of the terminal primary amine on the Lysine residue in later reactions when the polyethylene glycol polymer is attached through a conjugation reaction.

Another important aspect in selecting a resin is its loading value. For the Fmoc-PAL AM resin, the loading value ranges between 0.50-0.80 mmol/g. This means that for every gram of resin that participates in a synthesis, between 0.50 and 0.80 mmol of product is expected to be produced. This value ranges because, while each polystyrene bead has

multiple linkers attached, not every linker will load an amino acid. Furthermore, in subsequent coupling reactions, amino acid residues may not be loaded onto every peptide on the solid support. The combination of the three aspects discussed above contributed to the final selection of the Fmoc-PAL AM resin for this synthesis.

3.1.2 Protecting Groups

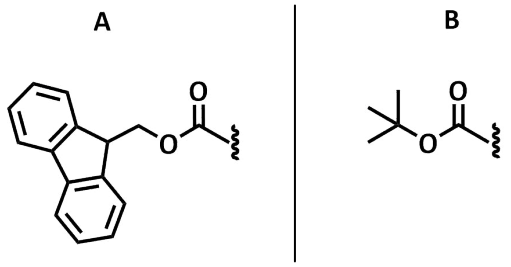


Figure 3. 4 Structures of the (a) fluorenylmethyloxycarbonyl (Fmoc) protecting group and (b) the tert-butyloxycarbonyl (Boc) protecting group

A key aspect in solid phase synthesis is the use of protecting groups. In this study, the two that are utilized are the fluorenylmethyloxycarbonyl (Fmoc) protecting group (Fig. 3.4a) and the tert-butyloxycarbonyl (Boc) protecting group (Fig 3.4b), which are considered to be orthogonal to one another. The Fmoc group is stable in acid conditions but becomes labile under basic conditions, while the Boc group is stable in basic conditions and labile in acidic conditions. The inverse relationship of the stabilities and reactivities of these two groups allows for control of specific functional groups throughout a reaction.

In this study, the Fmoc group was used to control the reactivity of the primary amine at the terminal end of the linker of the resin, as well as the amines of the amino acid residues that participate in the peptide backbone. For removal of the Fmoc group, a 20% 4-methylpiperidine solution was used.

The primary amine at the terminal end of the lysine residue side chain was of concern due to its reactivity and the possibility of forming impurities through side chain involved reactions. It could not be protected with an Fmoc group, as it was the first amino acid residue to be loaded onto the solid support, and the reactivity would be recovered in the next deprotection step. Thus, a lysine compound was selected that had an Fmoc group protecting the terminal amine that would participate in the peptide backbone and a Boc group protecting the terminal amine in the side chain of the lysine residue. Because of the orthogonal properties of the two protecting groups, the amine of the lysine side chain would remain unfunctionalized throughout synthesis of the peptide. After completion of the synthesis the functionality of the amine in the lysine side chain could be recovered by removing the Boc protecting group in a trifluoracetic acid cocktail.

The selection of the PAL-AM resin was also related to this idea of orthogonal protecting group schemes. The linker attached to the polystyrene bead is stable in basic conditions and becomes labile only in acidic conditions, which makes it a good support choice for Fmoc-chemistry synthetic routes. This provides a selective route for cleavage of the peptide from the resin after the completion of the synthesis.

3.1.3. Fmoc deprotection conditions

Because the Fmoc protecting group was used to reduce nucleophilicity of the amine groups in the amino acid residues, a solution of 4-methylpiperidine was used for removal of this protecting group. The 20% 4-methyl piperidine solution was prepared in dry dimethylformamide (DMF). The dry DMF used was purchased, but it was also stored with molecular sieves to maintain dryness. This polar, aprotic solvent was chosen in order to

reduce the likelihood of hydrolysis of the peptide backbone occurring throughout the synthesis of the peptide. A lower water content in the solvent was desired for this purpose as well.

A challenge with the 4-methylpiperidine solution was the rate at which the 4-methylpiperidine compound underwent oxidation. For this reason, small quantities of solution were prepared at a time. For manual coupling procedures, the solution was prepared several times through the course of the synthesis. For automatic synthesis, the solution was able to be prepared for one synthesis and the remaining solution was discarded before the next synthesis.

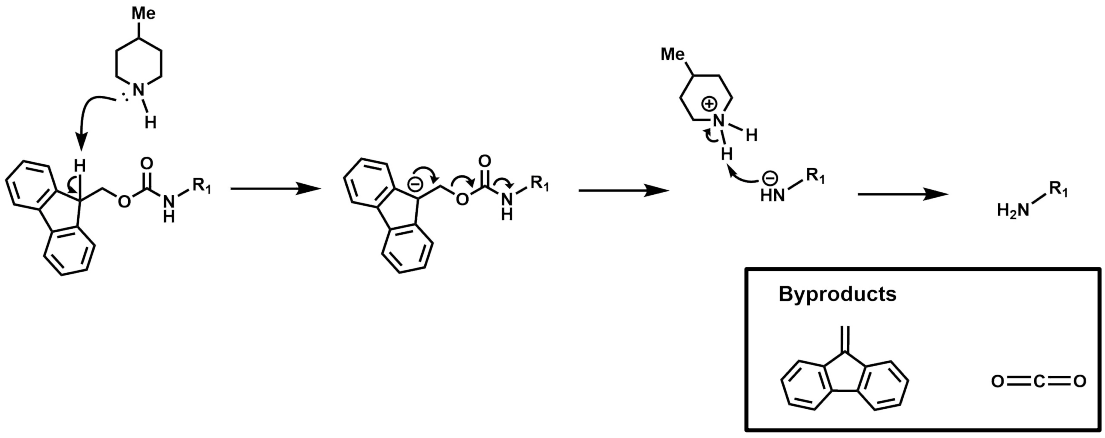


Figure 3. 5 Mechanism of removal of the Fmoc protecting group by 4-methylpiperidne to recover functionality of terminal amine of the peptide on solid support.

In the deprotection reaction the 4-methylpiperidine deprotonates the alpha proton, transforming the aromatic system in the Fmoc molecule to become a good leaving group (Figure 3.5). The reaction proceeds an E2 type elimination reaction, producing a byproduct molecule. Decarboxylation proceeds and produces CO_2 gas as another byproduct. The

aromatic byproduct remains in the 20% 4-methylpiperidine solution and is removed when the deprotection solution is removed.

In order to maintain a uniform reaction solution, the reaction vessel was incubated and agitated with each deprotection. Reaction times never exceeded more than one hour, as the possibility of the resin being degraded by the 4-methylpiperidine compound increased over time. The resin and reaction column were thoroughly washed after each deprotection treatment, in order to fully remove all 4-methylpiperidine. Even trace amounts of the deprotection solution could result in removal of the Fmoc group protecting subsequent amino acid resides. This could result in double coupling events and would ultimately change the peptide sequence. Consequentially, with the thorough washings, the Fmoc byproduct was removed after each deprotection.

3.1.4. Reaction monitoring via UV-vis spectroscopy

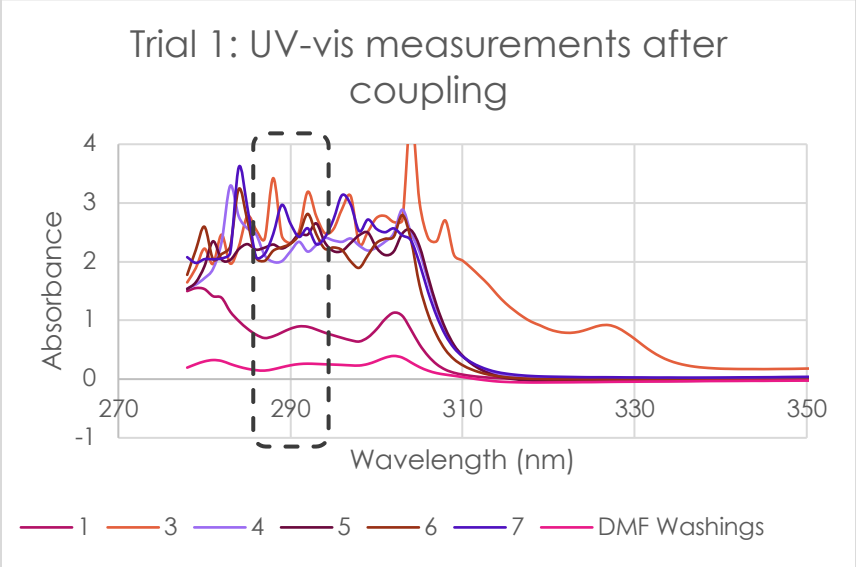


Figure 3. 6 UV-vis measurements at 290 nm of Fmoc washings after coupling cycles.

During synthesis via manual coupling, the loading of each amino acid fragment was monitored via UV-vis spectroscopy. The deprotection solution, containing the aromatic Fmoc byproduct formed in the deprotection scheme (Figure 3.6) was analyzed after each coupling reaction, as well as the deprotection to remove the Fmoc group from the resin. After several washing cycles, DMF washings were analyzed to determine that no Fmoc byproducts remained and confirm that the resin and reaction volume could be cleaned efficiently with DMF rinses.

A full absorption spectrum was measured for analysis. It was expected for the highest absorbance to occur at 290 nm. It was also predicted that this method of monitoring reaction progress would provide insight into the percent of each coupling. As previously discussed, the loading percentage decreases after each coupling cycle. As a result, it was expected for intensity of absorbance to decrease at 290 nm after each coupling cycle. This trend, however, was not observed.

The challenge with this method of monitoring the reaction was removing a consistent amount of resin for analysis after each coupling of the next amino acid. Due to the small amount of resin collected each time (about 1-2 mg), it was difficult to weigh the amount analyzed accurately and the loading percent could not be concluded from this method.

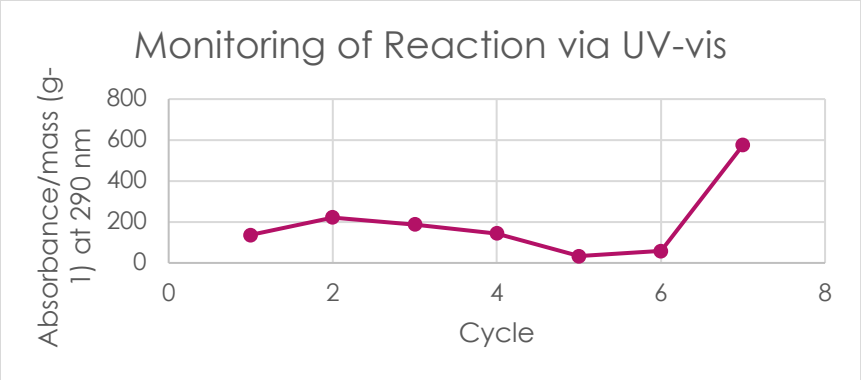


Figure 3. 7 Standardized curve measuring presence of Fmoc byproduct.

In later trials, rather than drying the resin and attempting to record a weight, 20 µL aliquot of solution containing resin was removed and then dried and weighed. Then this sample was treated with the deprotection solution, which was collected for analysis. The challenge with this method was ensuring that the resin was evenly suspended in the solution when the sample was taken, and a consistent amount of resin still was unable to be collected for analysis. The absorbance values were standardized to correct for the varying amounts of resin that were analyzed (Figure 3.7). From the UV-vis measurements, it could be determined that the Fmoc deprotection was occurring at each step, however, the percent of coupling after each cycle could not be determined throughout the synthesis.

3.1.5. Coupling conditions -

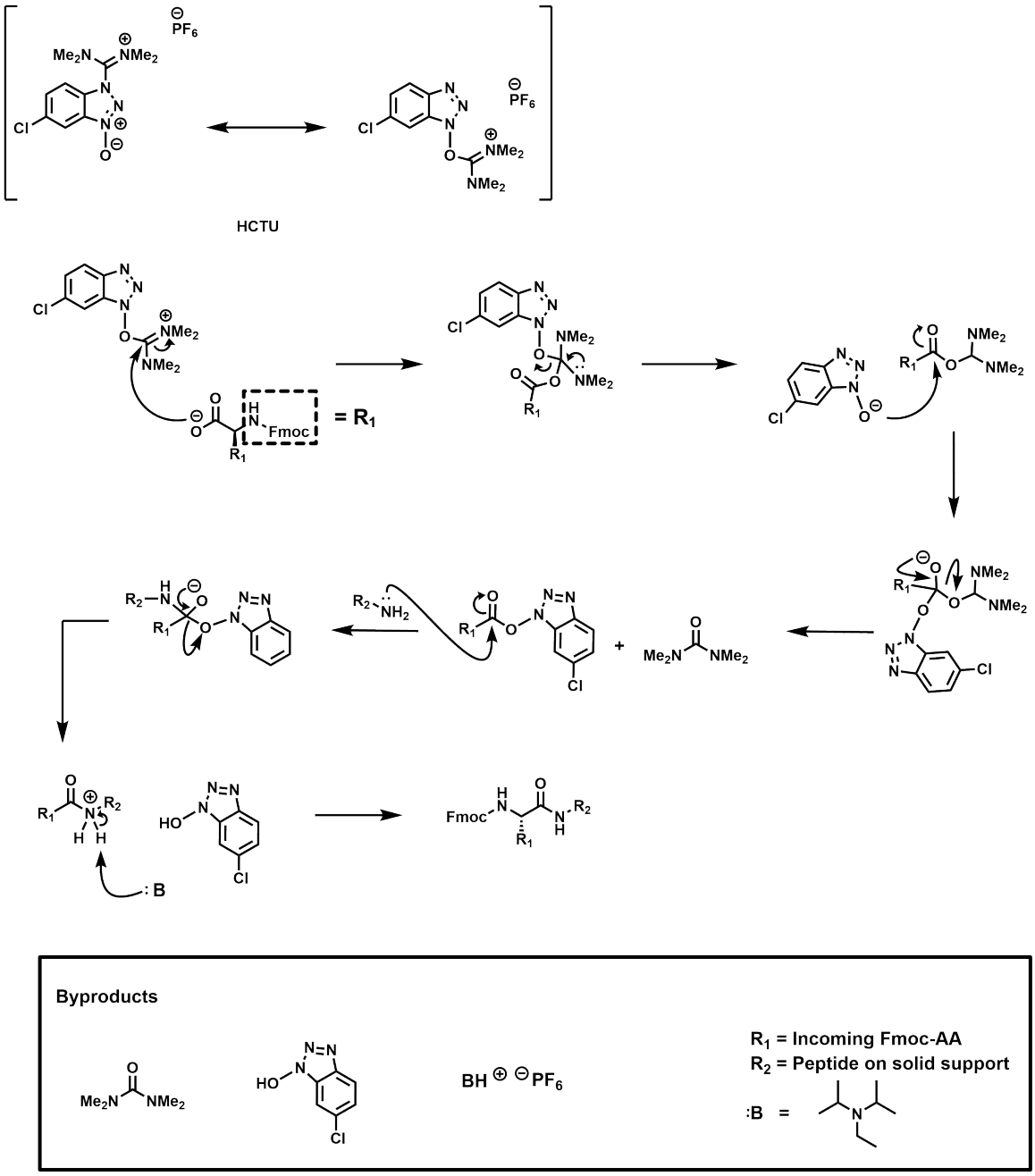


Figure 3. 8 Coupling mechanism using HCTU as coupling reagent. Hunig's base (N,N-diisopropylethylamine) was used as the base.

For the loading of subsequent amino acid residues, there were three major components in the coupling cocktail, which were the coupling reagent, a base, and the amino acid residues. The coupling reagent chosen for this synthesis was HCTU (O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate). This compound was developed to have two functionalities in coupling reactions (Figure 3.8). First, a base is used to form the carboxylate of the amino acid residue. Hunig's base (diisopropylethylamine) in excess was utilized in this synthesis for this purpose. Then the carboxylate performs a nucleophilic attack on the carbon of the amide bond. An ester intermediate forms containing the amino acid residue and the HCTU compound is transformed so that it contains a deprotonated hydroxyl. The coupling reagent now serves as the nucleophile and the carbon within the carbonyl of the ester intermediate acts at the electrophile. The amino acid is transferred back to the coupling reagent to form the fully activated amino acid residue, and tetramethyl urea is produced as a byproduct. The terminal amine of the peptide on solid support is able to attack the activated amino acid residue at the electrophile carbon of the carbonyl. A tetrahedral intermediate is formed, and the product that forms results in the new amino acid residue finally being attached to the peptide chain on the solid support. The coupling reagent fragment is produced as byproduct. The excess base is used to deprotonate the nitrogen in the newly formed amide bond, and the elongation of the peptide is complete.

As previously discussed, in manual coupling procedures, the amino acid residue was activated in a coupling cocktail prepared externally from the reaction vessel containing the resin. For automated synthesis, the coupling cocktail was prepared within the reaction vessel already containing resin. In both procedures, the amino acid residues to be coupled

were selectively activated. During manual coupling procedures, the coupling cocktail was prepared immediately before the reaction was to begin, as it was learned that the activated amino acid has limited stability before degrading. But, because the amino acid compound and the coupling reagent needed to be dissolved in solvent, the solution was prepared externally before being transferred to the reaction vessel.

3.1.6. Cleavage and Boc deprotection conditions -

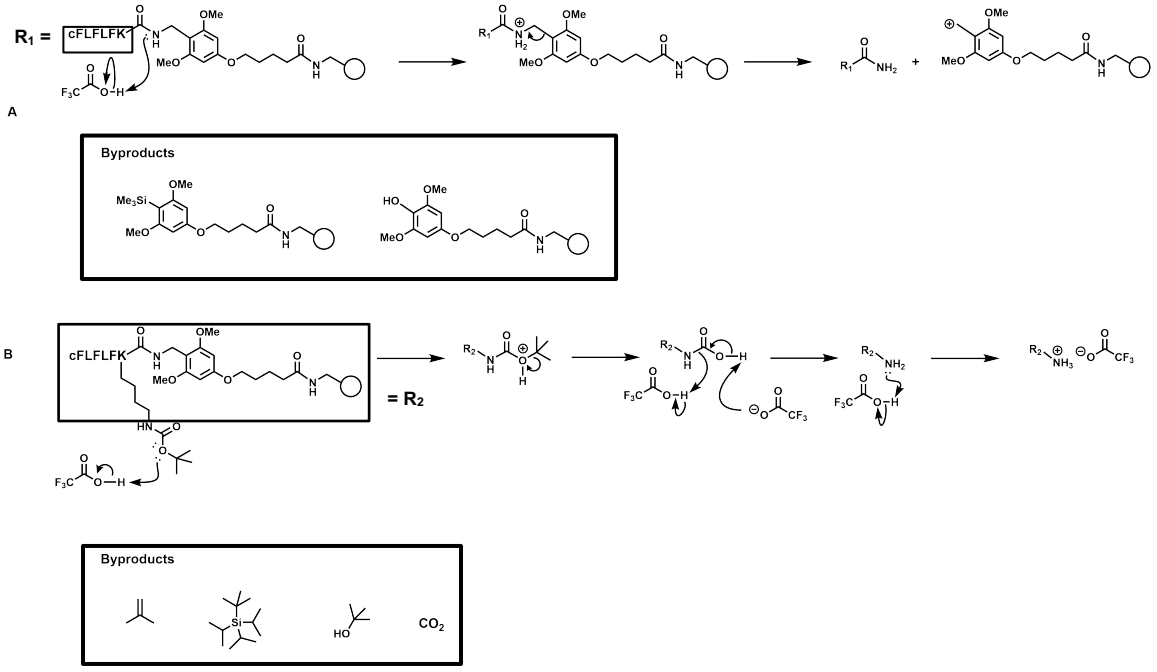


Figure 3. 9 (a) Mechanism for cleavage of peptide and (b) global deprotection of Boc protecting group attached to the lysine residue sidechain.

After the completion of the synthesis and capping of the peptide, it was removed from the solid support. A cleavage cocktail of trifluoroacetic acid (TFA), water, and triisopropylsilane (TIPS) was used (Figure 3.9). This cleavage cocktail also resulted in the global deprotection of the Boc side chain on the lysine residue. The water and TIPS

compound were used as nucleophilic scavengers for the carbocation produced in each reaction. Carbon dioxide gas was also produced as a byproduct in each of these reactions. The reaction solution and byproducts were removed by evaporation.

3.1.7. Isolation and purification techniques of cFLFLFK -

3.1.7.1. Purification of cFLFLFK via high performance liquid chromatography (HPLC)

For purification, the crude product was dissolved in methanol for HPLC analysis and purification. In several trials, the product was attempted to be dissolved in water, however, a gel-like substance formed. Methanol was added, and the gel partially dissolved and solid precipitates formed. Centrifugation and syringe filtration were used to remove the solid particles, and the methanol solution was injected on the analytical HPLC. It was hypothesized that the formation of the gel was an effect of residual TFA and base forming a salt.

3.1.7.2. Isolation via cold ether precipitation

In one trial, after the completion of peptide synthesis, isolation of product was attempted using cold diethylether. It was expected for the product to crash out of solution. This precipitation event did not occur immediately, and the solution was left overnight at 4°C, however no precipitates formed. It was later determined that this isolation method was not possible due to the nonpolar characteristics of the molecule. The modifications to yield C-terminus amide and the N-terminus capped with the cinnamoyl group, the product was highly nonpolar. The compound would not form a precipitate in the cold ether solution.

3.1.9. Characterization of peptide cFLFLFK

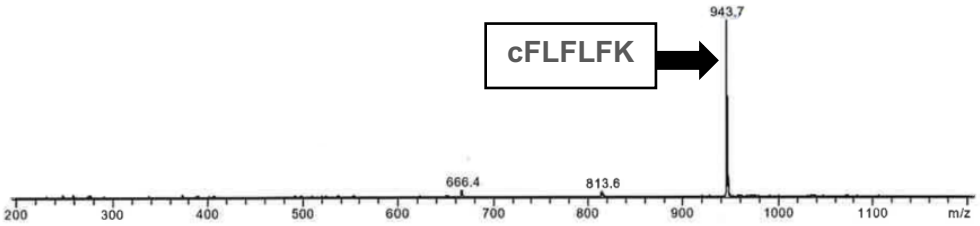
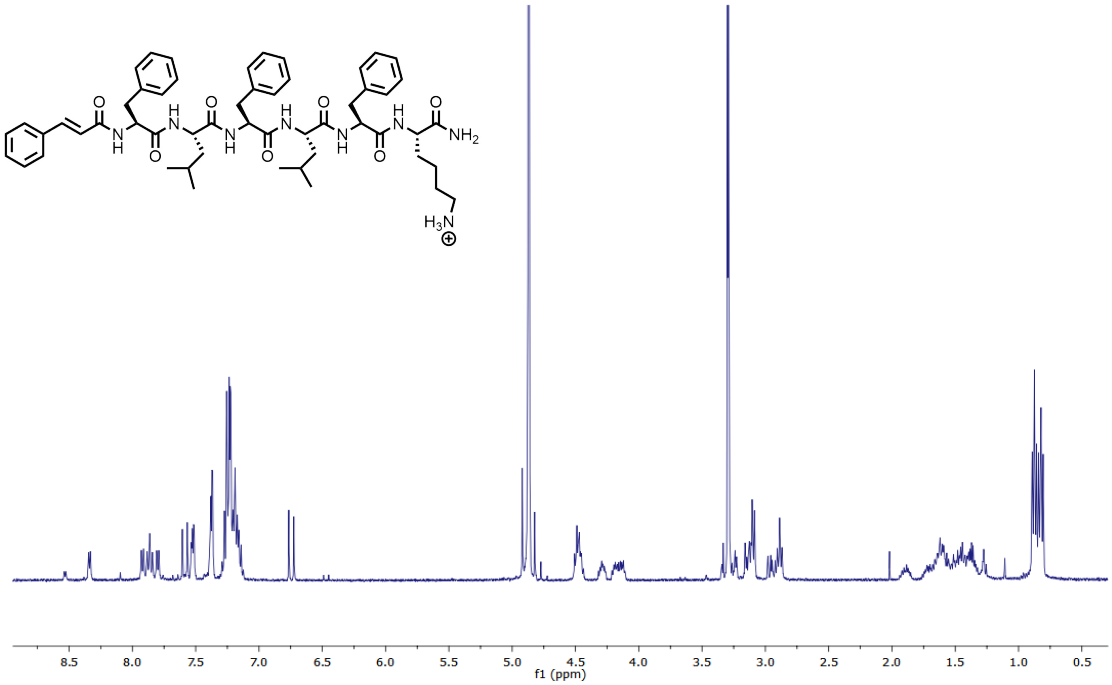


Figure 3. 10 Chromatogram of LC/MS analysis of peptide cFLFLFK

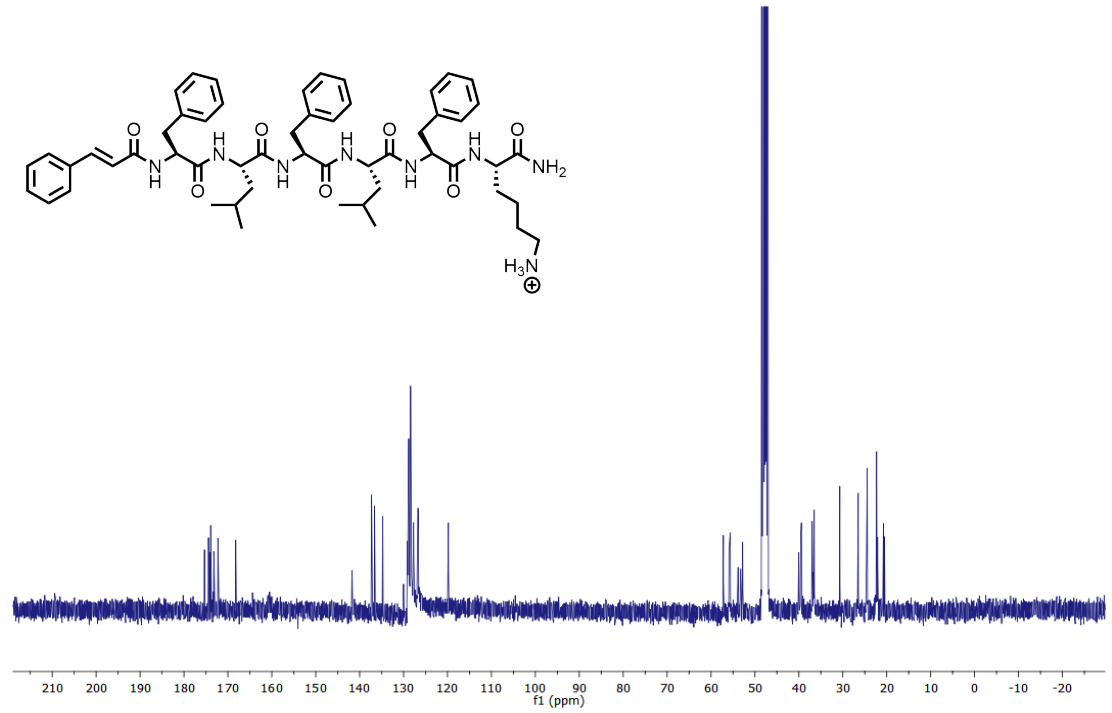
After purification, the product was characterized via liquid chromatography/mass spectrometry. The product was expected to be protonated at the terminal amine of the lysine residue sidechain, and had an expected mass of 944.5 g/mol. Mass spectrometry results measured a mass of 943.7 g/mol. The slight difference (0.9 g/mol) was attributed to error of the instrument, and it was concluded that the product was most likely the expected peptide.

The product was further analyzed via ^{1}H NMR and ^{13}C NMR (Figure 3.11). In both spectra, various regions showed chemical shifts expected for the different characteristics present in the target molecule. Based on these spectra, it was further concluded that the desired product had been synthesized.

Neither of these techniques provide insight to the order of the connectivity of the amino acid fragments in the peptide chain. However, based on the reaction conditions and the low likelihood of contamination occurring among amino acid compounds, and the thorough washings that occurred in between each reaction step, it was concluded that the peptide had been produced with the desired sequence.



(a)



(b)

Figure 3. 11 (a) ^{1}H NMR spectrum of peptide cFLFLFK (solvent: methanol-D) and (b) ^{13}C NMR spectrum of peptide cFLFLFK (solvent: methanol-D)

3.1.10. Summary of synthesis of peptide cFLFLFK

Several trials were performed to attempt to optimize reaction conditions and isolation and purification methods. In the case of manual coupling, the largest hurdle to overcome was isolating and purifying product. It was determined that purification via HPLC was the most efficient tool for this purpose. Smaller challenges that presented involved the stability window of activated amino acid fragments and the ability to monitor the progress of the reaction after each coupling cycle.

In the case of automated peptide synthesis, the largest issue had to do with the system software and creating reaction files to run the synthesis. Smaller technicalities related to the instrument also needed to be addressed before the instrument was run properly. Optimization of reagent concentrations was another obstacle that was troubleshooted for these reactions. Once these issues were resolved, the automated synthesis protocol proved to be the superior route for synthesizing this product. The time taken to produce the peptide was reduced from two weeks to about ten hours. One downside to this method of synthesis is the inability to monitor the reaction with each coupling cycle. Another downside in this synthetic route is the need to perform the final coupling with the cinnamoyl cap manually. However, these disadvantages are mitigated by the efficiency of this instrument, and the sizeable reduction of time needed to produce product.

3.2. Conjugation of polyethylene glycol (PEG) polymer

Relative to most biomacromolecules, the peptide cFLFLFK is very hydrophobic. This causes the bioavailability of the molecule to decrease in living systems. The low bioavailability is usually due to the compound low solubility or segregation to fatty tissues,

rather than the intended target cells, which usually reside in relatively aqueous environments. To increase bioavailability, a polyethylene glycol molecule was conjugated to the terminal amine of the lysine residue in the peptide chain.

3.2.1 Selection of biheterofunctionalized PEG_{76} moiety, tBOC-PEG_{76}-NHS ester -

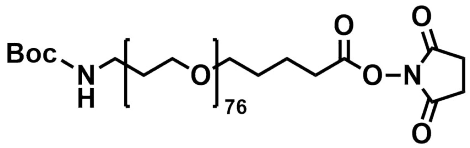


Figure 3. 12 Structure of biheterofunctionalized tBoc-PEG_{76}-NHS ester

A PEG polymer with an average length of 76 repeat units was selected that was biheterofuntionalized in order for the conjugate addition to occur selectively at the terminal amine of the lysine residue size chain (Figure 3.12). One end of the polymer was functionalized with an N-hydroxysuccinimide ester. The other end of the polymer was functionalized with a primary amine. The reactivity of this terminal amine was masked with use of a Boc protecting group. The reactivity could be later recovered after removal of the Boc group using a TFA cleavage cocktail. The cleavage of the Boc group proceeded as the mechanism described in section 3.1.6 and shown in Figure 3.9.

3.2.2. Reaction conditions for conjugation of tBoc-PEG$_{76}$-NHS ester to cFLFLFK

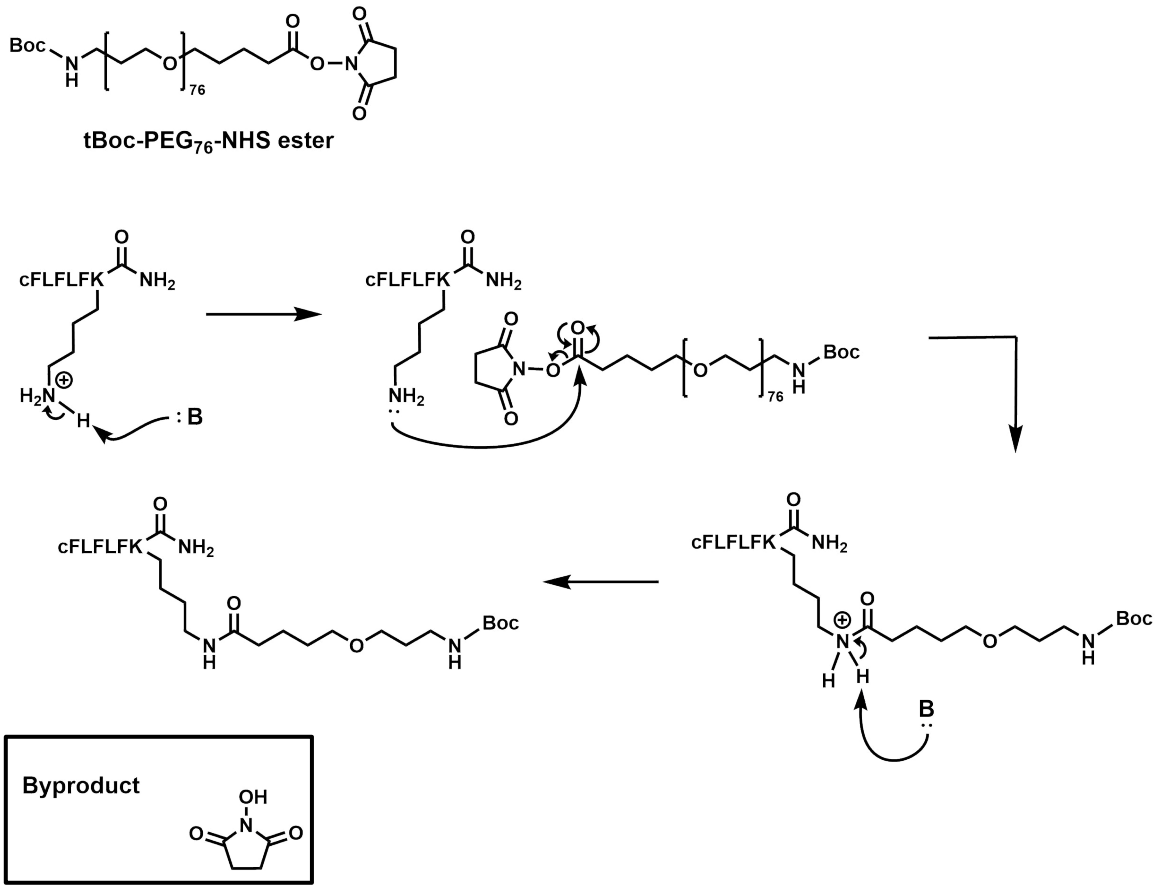


Figure 3. 13 Reaction mechanism for the conjugate addition of the tBoc-PEG$_{76}$-NHS ester to the terminal amine of the lysine residue side chain.

The conjugate addition was completed in an acetonitrile sodium borate buffer solution of pH 8.5 over a period of 24 hours (Figure 3.13). The reaction progress was monitored via analytical HPLC (Figure A1). The PEG$_{76}$ molecule is does not absorb light, which posed as a challenged in this experiment. The absorbance was measured at 254 nm to monitor the peptide compound. To selectively monitor the PEG$_{76}$ molecule, absorbance was measured at 215 nm. At this wavelength, the Boc protecting group could be traced on the PEG$_{76}$ molecule, and as the polymer was the only molecule with this protecting group, absorbance at this wavelength would be indicative of PEG$_{76}$ only.

The reaction was run in basic conditions, to control the deprotonation of the terminal amine of the lysine residue side chain (Figure 3.10). This transformed this amine into a good nucleophile, which attacked at the electrophilic carbon of the N-hydroxysuccinimide ester. A tetrahedral intermediate formed and when it degraded, the N-hydroxysuccinimide acted as the leaving group produced the conjugated product.

3.2.3. Purification of cFLFLFK-PEG_{76}-Boc via HPLC

The product cFLFLFK-PEG_{76}-Boc was purified via HPLC. After the conjugate addition of the PEG polymer, the product was able to be dissolved water. Purification was performed using the same methods optimized in the analytical experiments. In two trials, the peak collected for characterization did not have the expected retention time. In one trial, the peak eluted at 13.5 minutes. In another, the peak eluted at 2.5 minutes. The varying retention times was attributed to the loading difference between the analytical column and the preparatory columns. In both trials, the UV-vis data were a strong indicator for selecting which peaks to collect and perform characterization analyses on.

3.2.4. Characterization via mass spectrometry (MALDI-TOF)

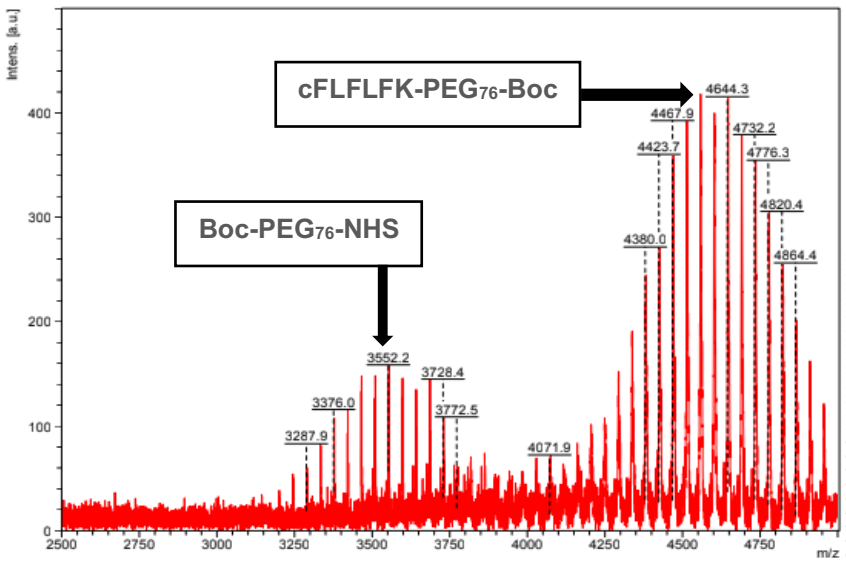


Figure 3. 14 Mass spectrometry spectrum of cFLFLFK-PEG$_{76}$-Boc compound (method: MALDI-TOF)

As previously discussed, a PEG$_{76}$ moeity was selected, which has an average molecular weight of 3.4 kDa. The length of this polymer was determined in previous studies to increase the bioavailability of the peptide when attached. After conjugation, the calculated mass of the product was 4489.6 g/mol. Due to the mass of this product, mass spectrometry was performed using a matrix assisted laser desorption ionization-time of flight (MALDI-TOF) method (Figure 3.14).

The resulting spectrum for this analysis appeared as a bimodal distribution curve. The distribution nature of the curve was attributed to the factor of the PEG polymer that was purchased exists in a range of lengths, with an average of 76 repeat units. The average molecular weight of the compound (3.4 kDa) reflects this characteristic of the polymer. This was also confirmed by calculating the mass difference between two neighboring peaks on the curves. The PEG monomer has a molecular weight of 44.053 g/mol, and the mass

difference between neighboring peaks was calculated to be 44.05 g/mol, confirming that the distribution curve was a result of the varying lengths of PEG polymer.

It was also confirmed that the bimodal distribution was a result of unconjugated PEG_{76} compounding remaining in solution with the product. The first distribution curve correlated to masses expected for the tBoc-PEG_{76}-NHS ester compound. The second correlated to masses expected for the product. The mass resulting from the analysis was 4511.9 g/mol, and it was found that the extra unexpected mass was caused by the addition of a sodium ion (MW = 23.0 g/mol). These ions could have been contributed from matrix or from the glassware the peptide was stored in. When the mass of the product was recalculated to consider the addition of a sodium ion, the expected mass was 4512.6 g/mol. The 0.7 g/mol difference was attributed to instrument error, and based on this analysis, it was concluded that the conjugate addition of the PEG_{76} polymer was successful.

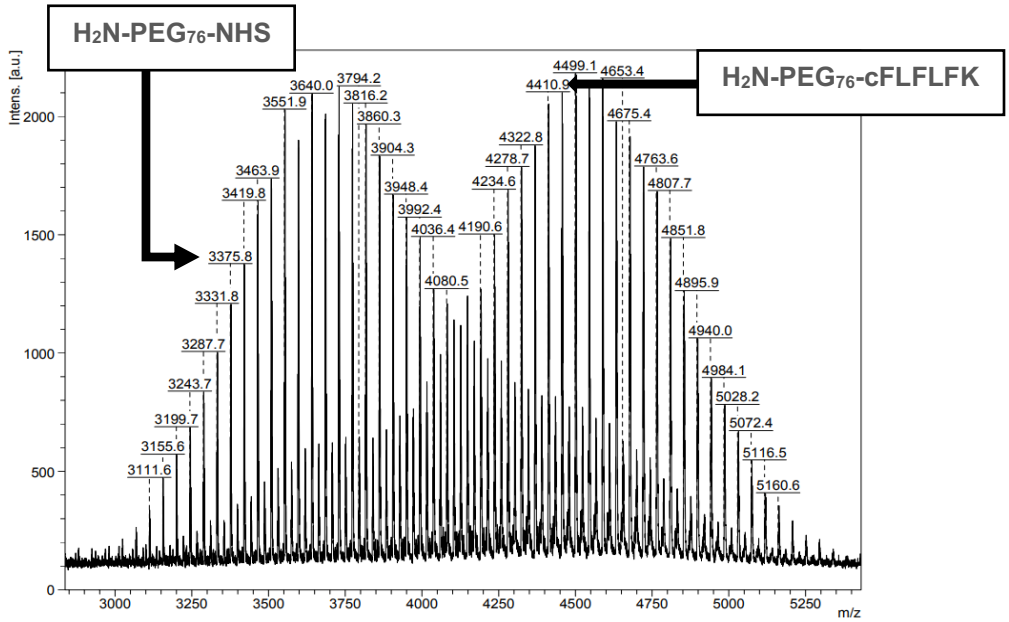


Figure 3. 15 Mass spectrometry spectrum of cFLFLFK-PEG_{76}-NH_2 compound (method: MALDI-TOF)

In a second study, cleavage of the Boc protecting group was performed before analyzing product (Figure 3.15). A similar spectrum resulted, showing two distribution curves. The distribution resulting from the varying length of peptide, and the two curves resulting from the presence of unconjugated PEG_{76} compound as well as the product was confirmed following similar calculations. After removal of the Boc group, the expected mass of the compound was 4412.5 g/mol (considering the addition of a Na^+ ion). The observed mass was 4410.9 g/mol, resulting in a 1.7 g/mol difference in this study. This difference was again attributed to instrument error, and it was concluded that product had formed and the Boc deprotection was successful.

3.3. Conjugation of fluorophore 5(6)-ROX NHS ester

The final component of the overall target molecule involves the attachment of a fluorophore. The fluorophore chosen was analogous to one of the proteins that are expressed in the transgenic zebrafish model. The fluorophore, 5(6)-ROX is of the rhodamine family, and has been modified to absorb at 580 nm and emit at 601 nm. It was functionalized with an NHS ester, and coupling to the terminal amine of the PEG_{76} molecule proceeded similarly to the conjugate addition of the PEG_{76} polymer to the terminal amine of the lysine residue sidechain.

3.3.1. Conjugation conditions

A procedure published by the manufacturers of the fluorophore was adapted for this reaction.[24] Due to the narrow window of solvents that the fluorophore is soluble in, dimethyl sulfoxide (DMSO) was chosen for this reaction, as it is a polar aprotic solvent.

Because the selected fluorophore was functionalized with an NHS ester, a buffer with a pH of 8.5 was selected. NHS esters are strongly reactive with primary amines in environments of pH 7.0-9.0. The mechanism of this reaction was similar to that of the reaction mechanism detailed in section 3.2.2. and shown in Figure 3.13. The reaction was monitored via HPLC to determine whether the starting material was being consumed (Figure A2). After optimization of the mobile phase so that the fluorophore had a reasonable retention time on the stationary phase, the retention time for both the fluorophore and the cFLFLFK-PEG_{76}-NH_2 compound coincided at around 6 minutes. This however, was not problematic due to the different wavelengths the two compounds were detected at by the UV-vis detector. The peptide-PEG_{76} compound was detected at 215 nm and the fluorophore was detected at 575 nm. The selectivity of these two wavelengths was determined by analyzing two standard solutions of each compound separately. Through this study, the conjugation reaction could be visualized and was confirmed by retention times that were detected at both wavelengths.

3.3.2. Purification of cFLFLFK-PEG_{76}-5(6)-ROX-NHS ester

Purification of this product was performed via size exclusion chromatography. The calculated mass for this product was 4928.96 g/mol. Due to the size of the compound, a Sephadex G-25 media was selected for the column. Before loading the compound on the column, the sample was first condensed via rotary evaporation. However, the low solubility of the fluorophore proved to be a challenge and a solid precipitate fell out of the small amount of solution. The sample was re-dissolved in 0.8 mL of water to total about 1 mL of sample. For loading, the sample was divided in half, and only 0.5 mL of sample was loaded on the column at once.

The sample was a vibrant purple color, a result from the 5(6)-ROX fluorophore. This allowed for visualization of the bands that formed as the sample moved through the Sephadex gel. Three apparent bands formed on the column. The first was very thin and a pale purple color. The second was thicker in height, about 4 mm, and a dark magenta color. The third band was much thicker, about 10 mm, and was a less vibrant magenta color. As the bands separated and become more defined, the loss of uniformity of the bands was observed. They became more wavy-looking than straight, as they neared the bottom of the column, it was apparent that different sections of the bands were eluting at a varying rates. It was hypothesized that this lack of uniformity in the appearance of the bands may have been caused because such a small sample was loaded making it difficult to load it evenly over the entire surface of the gel.

Based on the principles of size exclusion chromatography, however, it was still expected for the largest molecules to elute from the column first, while the smaller particles would retain on the column longer respective to their size. A total of 19 fractions were collected in 1-mL aliquots and reserved for analysis to confirm the order of elution.

3.3.3. Characterization via UV-vis spectroscopy -

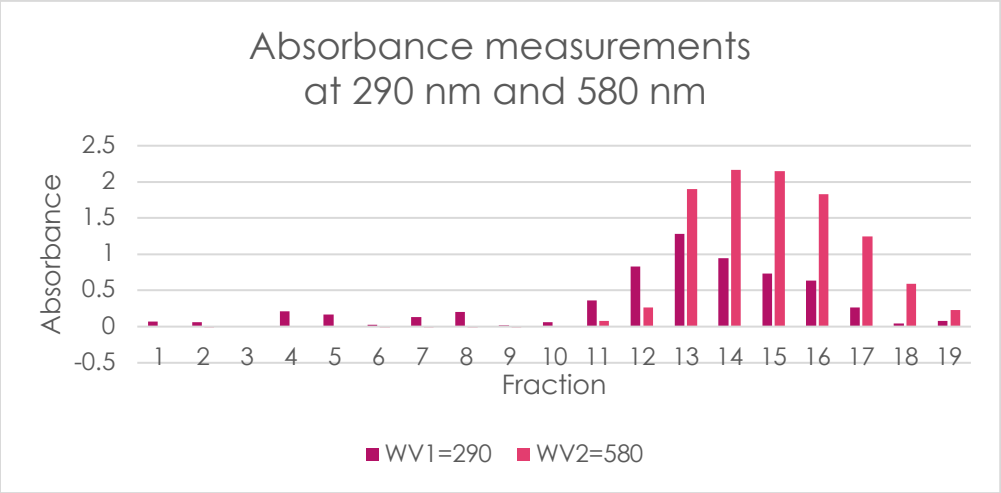


Figure 3. 16 UV-vis analysis of fractions from purification of cFLFLFK-PEG_{76}-5(6)-ROX compound via size exclusion chromatography. Figure (a) shows the full spectrum absorbance scan per fraction and Figure (b) shows comparison of fractions that absorbed at 290 nm and fractions that absorbed at 580 nm.

The 19 fractions were all analyzed using UV-vis spectroscopy. First, specific wavelength scans taken of the samples. Absorbance was measured at 254 nm to determine whether the peptide containing compound was present. This wavelength was chosen for the UV-vis active aromatic side chain in the phenylalanine residues. To detect the presence of the fluorophore, absorbance was measured at 580 nm. Results of these scans were inconclusive and did not reflect the presence of either compound.

Full spectrum absorbance scans measured from 230 nm to 1000 nm were then taken of all samples. The results of these scans showed high absorbance at 290 nm and 580 nm in fractions 11-17 (Figure 3.16). It was determined that the absorbance at 580 was comparable to the absorbance first predicted for the fluorophore (575 nm) and was most likely reflective of the presence of fluorophore. It was hypothesized that the higher absorbance at 290 nm was an effect of the material the samples were scanned in. The multi-

well plate was composed of polystyrene, which absorbs in the range of 230 nm to 280 nm. This hypothesis was supported by the data from the UV-vis scans, which had consistently recorded a zero value for all fractions in the 230 nm to 280 nm range.

Despite the lack of absorbance between 230 nm and 280 nm, these UV-vis analyses still provided information on the composition of the fractions. The wavelengths 290 nm and 580 nm were selected for the comparison of the nineteen fractions, which is illustrated in Figure 3.13b. Fractions 1-10 showed absorbance at 290 nm. The intensity was low, but was expected as a very low amount of starting material was used in this conjugation reaction. Furthermore, only half of the sample was loaded on the column for purification. Fractions 11-17 showed absorbance at both wavelengths, which suggested the presence of both peptide-PEG_{76} compound and the fluorophore. Based on this comparison, fractions 4-10 and then fractions 11-17 were condensed for further analysis.

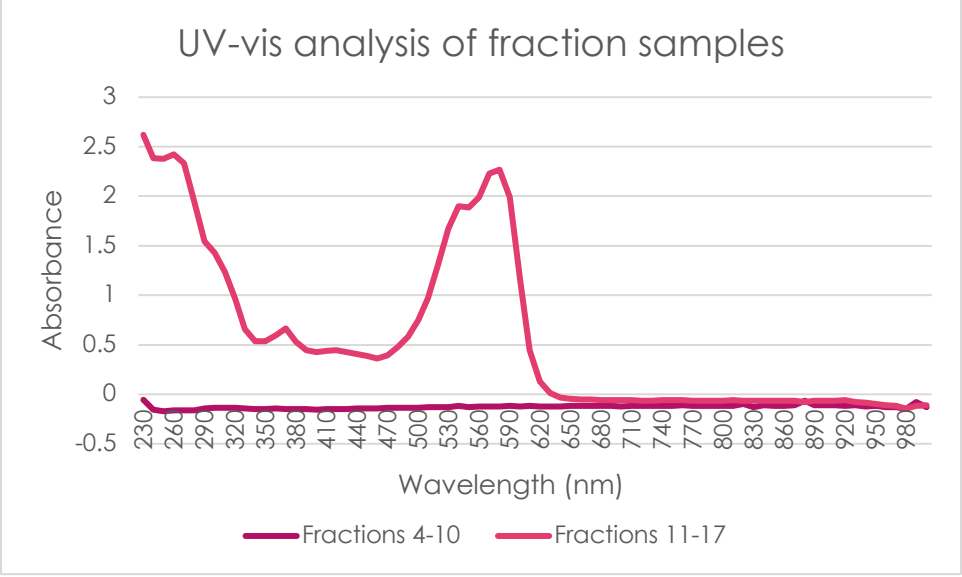


Figure 3. 17 UV-vis analysis of (a) sample containing fractions 4-10 and (b) sample containing fractions 11-17.

To avoid the absorbance issues at wavelength 254 nm caused by the material of the plate used in the first UV-vis study, the two samples containing fractions 4-10 and 11-17 were measured in a quartz cuvette. The sample containing fractions 4-10 showed no absorbance at neither 254 nm nor 580 nm (Figure 3.a) However, the sample containing fractions 11-17 showed absorbance at both the target wavelengths. This was suggestive of the presence of both compound as well as the fluorophore. The disadvantage to this analysis however, is its limitation in providing detail in regard to connectivity of the two molecule. The presence of absorbance at both wavelengths cannot confirm that the conjugation reaction was successful. As a result, this sample will need further analysis to determine whether the two molecules underwent reaction and product was produced.

CHAPTER 4: CONCLUSION AND FUTURE WORK

The goal of this project was to synthesize a bioactive fluorescently active probe that would serve as a synthetic replacement for the use of a transgenic zebrafish model in host-pathogen interaction fluorescence studies. In this study, the production of the bioactive peptide, cFLFLFK, and production of the peptide-PEG_{76} compound was completed successfully. The peptide was synthesized using manual and automated coupling procedures. The PEG_{76} polymer was attached to the terminal amine of the lysine residue side chain via a conjugative addition in a buffer system. The synthesis of these two molecules was confirmed through ^{1}H NMR and ^{13}C NMR and mass spectrometry analyses.

A third molecule, the peptide-PEG_{76}-fluorophore compound was synthesized using a 5(6)-ROX fluorophore through a conjugative addition in a buffer system. This third molecule was purified via size exclusion chromatography, and characterization experiments were performed with UV-vis analysis. However, further characterizations tests must be conducted to confirm product, such as mass spectrometry. If product is not confirmed, another goal will be to explore other reaction conditions for this final conjugation.

Once final product is confirmed, future work should include the validation of the product in a zebrafish model. Upon validation, if the proposed synthetic route in this project is approved, other small molecule fluorophores may begin to be considered to serve as modifications to the probe.

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APPENDICES

Appendix A

HPLC experiments to monitor coupling of peptide and tBoc-PEG$_{76}$ compound

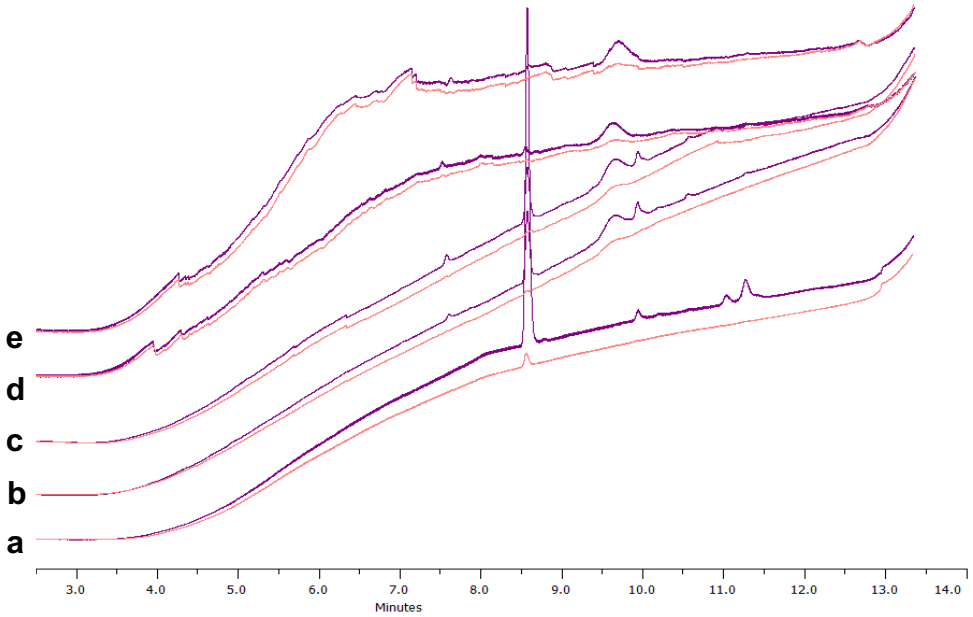

Figure A 1 HPLC analysis of conjugate addition reaction of tBoc-PEG$_{76}$-NHS ester. (stationary phase: C18; mobile phase: water:acetonitrile, 50-80%, 10 minutes, UV-vis detection at 215 nm [red] and 254 nm [purple])

Table A. 1 HPLC experiments monitoring PEG$_{76}$ conjugation

Time (hr)	**Trace**
0	a
1	b
3	c
4	d
24	e

Appendix B

HPLC experiments monitoring coupling of cFLFLFK-PEG_{76} and 5(6)-ROX NHS ester

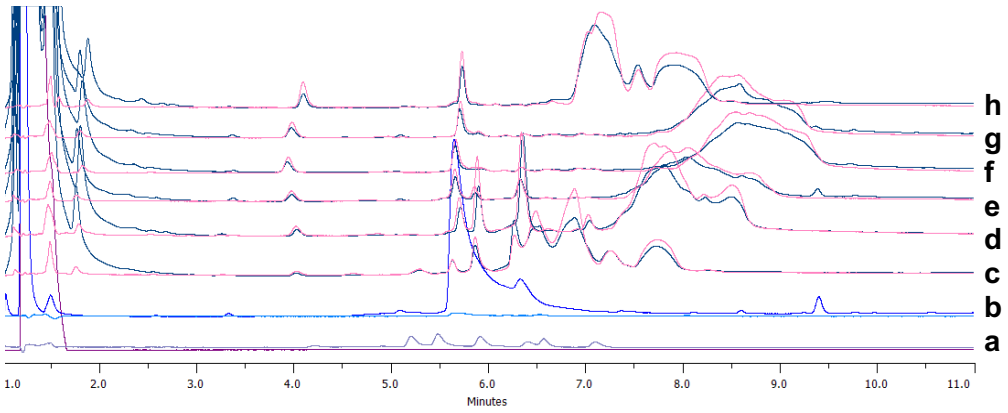

Figure A 2 HPLC analysis of conjugate addition reaction of the fluorophore 5(6)-ROX-NHS ester

Table A. 2 HPLC experiments monitoring fluorophore conjugation

Time	**Sample**	**254 nm**	**575 nm**
5(6)-ROX NHS ester in water	a	Purple	Blue
cFLFLFK-PEG-NH_2 in water	b	Dark blue	Light blue
0	c	Blue	Pink
0.5	d	Blue	Pink
1	e	Blue	Pink
3	f	Blue	Pink
4	g	Blue	Pink
20	h	Blue	Pink

Appendix C

ChemFile for synthesis of peptide FLFLFK via automated coupling procedures

16:52:01.56 Starting ChemFile - C:\AAPPTEC\SNI144.CHM
16:52:01.94 1 REM ** This is the data for cycle 1. ** SynthPos=1
16:52:02.27 2 <Set Active> SynthPos=2
16:52:03.75 3 Goto ChemFile swell2.cht, line 1 SynthPos=3
16:52:05.07 1 REM SWELL THE RESIN SynthPos=4
16:52:05.40 2 Mix "RV48" for 60.00 minutes at 600 rpm(s) and wait. SynthPos=5
16:52:08.75 Mixer 0 on at 600 rpm
17:52:12.03 Mixer 0 off
17:52:12.36 3 Empty RV48 for 2.000 minute(s) SynthPos=6
17:54:10.45 4 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=7
17:54:41.48 Washing with DMF (A) 3.00ml per vessel to RV48
17:54:51.42 5 Mix "RV48" for 5.00 minutes at 450 rpm(s) and wait. SynthPos=8
17:54:53.18 Mixer 0 on at 450 rpm
17:59:55.32 Mixer 0 off
17:59:55.71 6 Empty RV48 for 1.000 minute(s) SynthPos=9
18:00:53.44 7 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=10
18:00:53.60 Washing with DMF (A) 3.00ml per vessel to RV48
18:01:03.49 8 Mix "RV48" for 20.00 minutes at 450 rpm(s) and wait. SynthPos=11
18:01:05.30 Mixer 0 on at 450 rpm
18:21:07.18 Mixer 0 off
18:21:07.56 9 Empty RV48 for 1.000 minute(s) SynthPos=12
18:22:05.46 10 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=13
18:22:05.62 Washing with DMF (A) 3.00ml per vessel to RV48
18:22:15.51 11 Return SynthPos=14
18:22:15.89 4 Goto ChemFile depro48.cht, line 1 SynthPos=15
18:22:17.21 1 REM FMOC DEPROTECTION SynthPos=16
18:22:17.54 2 Transfer 2.00ml from Pip-350mL[1](PIP) to RV48[1-48] using DMF (1) SynthPos=17
18:22:17.59 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
18:22:17.59 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
18:22:19.52 Pick up 9000 microL and 0 steps
18:22:28.69 A2700R
18:22:38.69 Pick up 9000 microL and 0 steps
18:22:47.91 A2700R
18:23:07.25 Pick up 110 microL and 0 steps
18:23:07.96 A33R
18:23:08.35 Dispense 99 microL and 0 steps
18:23:08.35 A30R
18:23:11.20 --Cleaned Probe with 18000µl of DMF (1)

18:23:11.97 Pick up 110 microL and 0 steps
18:23:12.41 A33R
18:23:12.74 Dispense 99 microL and 0 steps
18:23:12.74 A30R
18:23:18.73 Pick up 2225 microL and 0 steps
18:23:25.43 A668R
18:23:29.22 Dispense 2126 microL and 0 steps
18:23:29.22 A638R
18:23:35.53 --Aspirated 2001µl from Pip-350mL[1](PIP)
18:23:37.73 Dispense 126 microL and 0 steps
18:23:37.73 A38R
18:23:44.93 --Dispensed 2000µl to RV48[11]
18:23:45.31 Dispense 1 microL and 0 steps
18:23:45.31 A0R
18:23:49.65 --Dispensed 126µl to RV48[11]
18:23:52.94 3 Mix "RV48" for 3.00 minutes at 450 rpm(s) and wait. SynthPos=18
18:23:54.70 Mixer 0 on at 450 rpm
18:26:55.68 Mixer 0 off
18:26:56.07 4 Empty RV48 for 1.500 minute(s) SynthPos=19
18:28:24.44 5 Transfer 2.00ml from Pip-350mL[1](PIP) to RV48[1-48] using DMF (1) SynthPos=20
18:28:24.44 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
18:28:24.44 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
18:28:25.16 Pick up 110 microL and 0 steps
18:28:25.92 A33R
18:28:26.25 Dispense 99 microL and 0 steps
18:28:26.25 A30R
18:28:32.24 Pick up 2225 microL and 0 steps
18:28:38.94 A668R
18:28:42.73 Dispense 2126 microL and 0 steps
18:28:42.73 A638R
18:28:48.83 --Aspirated 2001µl from Pip-350mL[1](PIP)
18:28:51.08 Dispense 126 microL and 0 steps
18:28:51.08 A38R
18:28:58.22 --Dispensed 2000µl to RV48[11]
18:28:58.66 Dispense 1 microL and 0 steps
18:28:58.66 A0R
18:29:02.94 --Dispensed 126µl to RV48[11]
18:29:06.24 6 Mix "RV48" for 8.00 minutes at 450 rpm(s) and wait. SynthPos=21
18:29:08.05 Mixer 0 on at 450 rpm
18:37:10.74 Mixer 0 off
18:37:11.07 7 Empty RV48 for 1.500 minute(s) SynthPos=22
18:38:39.44 8 Return SynthPos=23
18:38:39.77 5 Goto ChemFile washdp48.cht, line 1 SynthPos=24
18:38:41.09 1 REM Wash after Deprotection SynthPos=25

18:38:41.47 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=26
18:39:12.51 Washing with DMF (B) 3.00ml per vessel to RV48
18:39:22.45 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=27
18:39:24.21 Mixer 0 on at 450 rpm
18:40:26.16 Mixer 0 off
18:40:26.49 4 Empty RV48 for 1.500 minute(s) SynthPos=28
18:41:54.48 5 Repeat from step 2, 3 times SynthPos=29
18:41:55.80 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=30
18:41:55.97 Washing with DMF (B) 3.00ml per vessel to RV48
18:42:05.91 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=31
18:42:07.66 Mixer 0 on at 450 rpm
18:43:09.24 Mixer 0 off
18:43:09.57 4 Empty RV48 for 1.500 minute(s) SynthPos=32
18:44:37.45 5 Repeat from step 2, 3 times SynthPos=33
18:44:38.76 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=34
18:44:38.93 Washing with DMF (B) 3.00ml per vessel to RV48
18:44:48.87 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=35
18:44:50.63 Mixer 0 on at 450 rpm
18:45:53.24 Mixer 0 off
18:45:53.57 4 Empty RV48 for 1.500 minute(s) SynthPos=36
18:47:21.45 5 Repeat from step 2, 3 times SynthPos=37
18:47:22.77 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=38
18:47:22.94 Washing with DMF (B) 3.00ml per vessel to RV48
18:47:32.82 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=39
18:47:34.64 Mixer 0 on at 450 rpm
18:48:36.70 Mixer 0 off
18:48:37.03 4 Empty RV48 for 1.500 minute(s) SynthPos=40
18:50:05.46 5 Repeat from step 2, 3 times SynthPos=41
18:50:06.78 6 Return SynthPos=42
18:50:07.16 6 REM HBTU48 SynthPos=43
18:50:07.49 7 Dispense Sequence SNI141.dsp with 0.699ml to RV48 rack using DMF (1) SynthPos=44
18:50:07.49 Dispensing from Rack 4x7 vessel 11 to Rack RV48 vessel 11
18:50:09.42 Pick up 4000 microL and 0 steps
18:50:13.65 A1200R
18:50:27.93 Pick up 110 microL and 0 steps
18:50:28.69 A33R
18:50:29.02 Dispense 99 microL and 0 steps
18:50:29.02 A30R
18:50:31.94 --Cleaned Probe with 4000µl of DMF (1)
18:50:32.65 Pick up 110 microL and 0 steps
18:50:33.03 A33R
18:50:33.36 Dispense 99 microL and 0 steps
18:50:33.36 A30R
18:50:38.91 Pick up 924 microL and 0 steps
18:50:41.66 A277R

18:50:45.34 Dispense 823 microL and 0 steps
18:50:45.34 A247R
18:50:51.43 --Aspirated 698µl from 4x7[11](Lys(Boc))
18:50:53.47 Dispense 0 microL and 0 steps
18:50:53.47 A0R
18:50:58.85 --Dispensed 823µl to RV48[11]
18:51:02.09 8 Transfer 0.70ml from HBTU-350mL[1](HCTU) to RV48[1-48] using DMF (1) SynthPos=45
18:51:02.09 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
18:51:02.09 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
18:51:04.01 Pick up 2250 microL and 0 steps
18:51:06.43 A675R
18:51:19.01 Pick up 110 microL and 0 steps
18:51:19.78 A33R
18:51:20.11 Dispense 99 microL and 0 steps
18:51:20.11 A30R
18:51:23.02 --Cleaned Probe with 2250µl of DMF (1)
18:51:23.73 Pick up 110 microL and 0 steps
18:51:24.11 A33R
18:51:24.44 Dispense 99 microL and 0 steps
18:51:24.44 A30R
18:51:30.93 Pick up 924 microL and 0 steps
18:51:33.67 A277R
18:51:37.52 Dispense 823 microL and 0 steps
18:51:37.52 A247R
18:51:43.83 --Aspirated 698µl from HBTU-350mL[1](HCTU)
18:51:46.03 Dispense 124 microL and 0 steps
18:51:46.03 A37R
18:51:51.25 --Dispensed 700µl to RV48[11]
18:51:51.69 Dispense 0 microL and 0 steps
18:51:51.69 A0R
18:51:56.03 --Dispensed 123µl to RV48[11]
18:51:59.32 9 Transfer 0.70ml from DIEA-350mL[1](DIPEA) to RV48[1-48] using DMF (1) SynthPos=46
18:51:59.32 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
18:51:59.32 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
18:52:01.19 Pick up 2250 microL and 0 steps
18:52:03.66 A675R
18:52:16.18 Pick up 110 microL and 0 steps
18:52:16.95 A33R
18:52:17.28 Dispense 99 microL and 0 steps
18:52:17.28 A30R
18:52:20.19 --Cleaned Probe with 2250µl of DMF (1)
18:52:20.85 Pick up 110 microL and 0 steps

18:52:21.29 A33R
18:52:21.62 Dispense 99 microL and 0 steps
18:52:21.62 A30R
18:52:27.83 Pick up 924 microL and 0 steps
18:52:30.63 A277R
18:52:34.47 Dispense 823 microL and 0 steps
18:52:34.47 A247R
18:52:40.79 --Aspirated 698µl from DIEA-350mL[1](DIPEA)
18:52:43.04 Dispense 124 microL and 0 steps
18:52:43.04 A37R
18:52:48.21 --Dispensed 700µl to RV48[11]
18:52:48.65 Dispense 0 microL and 0 steps
18:52:48.65 A0R
18:52:52.93 --Dispensed 123µl to RV48[11]
18:52:56.22 10 Mix "RV48" for 45.00 minutes at 450 rpm(s) and wait. SynthPos=47
18:52:58.04 Mixer 0 on at 450 rpm
19:38:00.43 Mixer 0 off
19:38:00.76 11 Empty RV48 for 1.500 minute(s) SynthPos=48
19:39:28.47 12 Goto ChemFile washcp48.cht, line 1 SynthPos=49
19:39:29.79 1 REM Wash after Coupling SynthPos=50
19:39:30.17 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=51
19:40:01.21 Washing with DMF (A) 3.00ml per vessel to RV48
19:40:11.09 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=52
19:40:12.91 Mixer 0 on at 450 rpm
19:41:14.26 Mixer 0 off
19:41:14.64 4 Empty RV48 for 1.500 minute(s) SynthPos=53
19:42:42.47 5 Repeat from step 2, 3 times SynthPos=54
19:42:43.79 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=55
19:42:43.95 Washing with DMF (A) 3.00ml per vessel to RV48
19:42:53.89 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=56
19:42:55.65 Mixer 0 on at 450 rpm
19:43:58.10 Mixer 0 off
19:43:58.43 4 Empty RV48 for 1.500 minute(s) SynthPos=57
19:45:26.48 5 Repeat from step 2, 3 times SynthPos=58
19:45:27.79 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=59
19:45:27.96 Washing with DMF (A) 3.00ml per vessel to RV48
19:45:37.90 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=60
19:45:39.66 Mixer 0 on at 450 rpm
19:46:41.07 Mixer 0 off
19:46:41.39 4 Empty RV48 for 1.500 minute(s) SynthPos=61
19:48:09.44 5 Repeat from step 2, 3 times SynthPos=62
19:48:10.76 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=63
19:48:10.92 Washing with DMF (A) 3.00ml per vessel to RV48
19:48:20.86 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=64
19:48:22.62 Mixer 0 on at 450 rpm
19:49:24.58 Mixer 0 off

19:49:24.91 4 Empty RV48 for 1.500 minute(s) SynthPos=65
19:50:53.45 5 Repeat from step 2, 3 times SynthPos=66
19:50:54.77 6 Return SynthPos=67
19:50:55.15 13 REM ** This is the data for cycle 2. ** SynthPos=68
19:50:55.86 14 <Set Active> SynthPos=69
19:50:57.35 15 Goto ChemFile depro48.cht, line 1 SynthPos=70
19:50:58.67 1 REM FMOC DEPROTECTION SynthPos=71
19:50:59.05 2 Transfer 2.00ml from Pip-350mL[1](PIP) to RV48[1-48] using DMF (1) SynthPos=72
19:50:59.05 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
19:50:59.05 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
19:51:00.97 Pick up 2250 microL and 0 steps
19:51:03.39 A675R
19:51:15.91 Pick up 110 microL and 0 steps
19:51:16.68 A33R
19:51:17.07 Dispense 99 microL and 0 steps
19:51:17.07 A30R
19:51:19.92 --Cleaned Probe with 2250µl of DMF (1)
19:51:20.64 Pick up 110 microL and 0 steps
19:51:21.02 A33R
19:51:21.41 Dispense 99 microL and 0 steps
19:51:21.41 A30R
19:51:27.34 Pick up 2225 microL and 0 steps
19:51:34.09 A668R
19:51:37.88 Dispense 2126 microL and 0 steps
19:51:37.88 A638R
19:51:44.20 --Aspirated 2001µl from Pip-350mL[1](PIP)
19:51:46.40 Dispense 126 microL and 0 steps
19:51:46.40 A38R
19:51:53.59 --Dispensed 2000µl to RV48[11]
19:51:53.98 Dispense 1 microL and 0 steps
19:51:53.98 A0R
19:51:58.31 --Dispensed 126µl to RV48[11]
19:52:01.61 3 Mix "RV48" for 3.00 minutes at 450 rpm(s) and wait. SynthPos=73
19:52:03.37 Mixer 0 on at 450 rpm
19:55:05.01 Mixer 0 off
19:55:05.34 4 Empty RV48 for 1.500 minute(s) SynthPos=74
19:56:33.44 5 Transfer 2.00ml from Pip-350mL[1](PIP) to RV48[1-48] using DMF (1) SynthPos=75
19:56:33.44 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
19:56:33.44 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
19:56:34.15 Pick up 110 microL and 0 steps
19:56:34.86 A33R
19:56:35.25 Dispense 99 microL and 0 steps

19:56:35.25 A30R
19:56:41.24 Pick up 2225 microL and 0 steps
19:56:47.94 A668R
19:56:51.78 Dispense 2126 microL and 0 steps
19:56:51.78 A638R
19:56:57.88 --Aspirated 2001µl from Pip-350mL[1](PIP)
19:57:00.13 Dispense 126 microL and 0 steps
19:57:00.13 A38R
19:57:07.27 --Dispensed 2000µl to RV48[11]
19:57:07.66 Dispense 1 microL and 0 steps
19:57:07.66 A0R
19:57:11.99 --Dispensed 126µl to RV48[11]
19:57:15.29 6 Mix "RV48" for 8.00 minutes at 450 rpm(s) and wait. SynthPos=76
19:57:17.05 Mixer 0 on at 450 rpm
20:05:19.62 Mixer 0 off
20:05:19.95 7 Empty RV48 for 1.500 minute(s) SynthPos=77
20:06:48.44 8 Return SynthPos=78
20:06:48.77 16 Goto ChemFile washdp48.cht, line 1 SynthPos=79
20:06:50.14 1 REM Wash after Deprotection SynthPos=80
20:06:50.47 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=81
20:07:21.50 Washing with DMF (B) 3.00ml per vessel to RV48
20:07:31.44 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=82
20:07:33.20 Mixer 0 on at 450 rpm
20:08:35.32 Mixer 0 off
20:08:35.65 4 Empty RV48 for 1.500 minute(s) SynthPos=83
20:10:03.48 5 Repeat from step 2, 3 times SynthPos=84
20:10:04.80 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=85
20:10:04.96 Washing with DMF (B) 3.00ml per vessel to RV48
20:10:14.90 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=86
20:10:16.66 Mixer 0 on at 450 rpm
20:11:18.73 Mixer 0 off
20:11:19.11 4 Empty RV48 for 1.500 minute(s) SynthPos=87
20:12:47.49 5 Repeat from step 2, 3 times SynthPos=88
20:12:48.80 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=89
20:12:48.97 Washing with DMF (B) 3.00ml per vessel to RV48
20:12:58.91 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=90
20:13:00.67 Mixer 0 on at 450 rpm
20:14:02.07 Mixer 0 off
20:14:02.46 4 Empty RV48 for 1.500 minute(s) SynthPos=91
20:15:30.45 5 Repeat from step 2, 3 times SynthPos=92
20:15:31.77 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=93
20:15:31.93 Washing with DMF (B) 3.00ml per vessel to RV48
20:15:41.87 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=94
20:15:43.63 Mixer 0 on at 450 rpm
20:16:46.08 Mixer 0 off
20:16:46.47 4 Empty RV48 for 1.500 minute(s) SynthPos=95

20:18:14.46 5 Repeat from step 2, 3 times SynthPos=96
20:18:15.78 6 Return SynthPos=97
20:18:16.16 17 REM HBTU48 SynthPos=98
20:18:16.49 18 Dispense Sequence SNI142.dsp with 0.699ml to RV48 rack using DMF (1) SynthPos=99
20:18:16.49 Dispensing from Rack 4x7 vessel 13 to Rack RV48 vessel 11
20:18:18.36 Pick up 4000 microL and 0 steps
20:18:22.59 A1200R
20:18:36.92 Pick up 110 microL and 0 steps
20:18:37.64 A33R
20:18:38.02 Dispense 99 microL and 0 steps
20:18:38.02 A30R
20:18:40.88 --Cleaned Probe with 4000µl of DMF (1)
20:18:41.59 Pick up 110 microL and 0 steps
20:18:41.97 A33R
20:18:42.30 Dispense 99 microL and 0 steps
20:18:42.30 A30R
20:18:47.91 Pick up 924 microL and 0 steps
20:18:50.71 A277R
20:18:54.33 Dispense 823 microL and 0 steps
20:18:54.33 A247R
20:19:00.48 --Aspirated 698µl from 4x7[13](Phe)
20:19:02.35 Dispense 0 microL and 0 steps
20:19:02.35 A0R
20:19:07.73 --Dispensed 823µl to RV48[11]
20:19:11.03 19 Transfer 0.70ml from HBTU-350mL[1](HCTU) to RV48[1-48] using DMF (1) SynthPos=100
20:19:11.03 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
20:19:11.03 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
20:19:12.95 Pick up 2250 microL and 0 steps
20:19:15.37 A675R
20:19:27.95 Pick up 110 microL and 0 steps
20:19:28.66 A33R
20:19:29.05 Dispense 99 microL and 0 steps
20:19:29.05 A30R
20:19:31.90 --Cleaned Probe with 2250µl of DMF (1)
20:19:32.62 Pick up 110 microL and 0 steps
20:19:33.00 A33R
20:19:33.38 Dispense 99 microL and 0 steps
20:19:33.38 A30R
20:19:39.81 Pick up 924 microL and 0 steps
20:19:42.61 A277R
20:19:46.40 Dispense 823 microL and 0 steps
20:19:46.40 A247R
20:19:52.72 --Aspirated 698µl from HBTU-350mL[1](HCTU)

20:19:54.97 Dispense 124 microL and 0 steps
20:19:54.97 A37R
20:20:00.19 --Dispensed 700µl to RV48[11]
20:20:00.57 Dispense 0 microL and 0 steps
20:20:00.57 A0R
20:20:04.91 --Dispensed 123µl to RV48[11]
20:20:08.21 20 Transfer 0.70ml from DIEA-350mL[1](DIPEA) to RV48[1-48] using DMF (1) SynthPos=101
20:20:08.21 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
20:20:08.21 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
20:20:10.13 Pick up 2250 microL and 0 steps
20:20:12.55 A675R
20:20:25.12 Pick up 110 microL and 0 steps
20:20:25.89 A33R
20:20:26.22 Dispense 99 microL and 0 steps
20:20:26.22 A30R
20:20:29.24 --Cleaned Probe with 2250µl of DMF (1)
20:20:29.96 Pick up 110 microL and 0 steps
20:20:30.40 A33R
20:20:30.73 Dispense 99 microL and 0 steps
20:20:30.73 A30R
20:20:36.93 Pick up 924 microL and 0 steps
20:20:39.73 A277R
20:20:43.52 Dispense 823 microL and 0 steps
20:20:43.52 A247R
20:20:49.84 --Aspirated 698µl from DIEA-350mL[1](DIPEA)
20:20:52.09 Dispense 124 microL and 0 steps
20:20:52.09 A37R
20:20:57.31 --Dispensed 700µl to RV48[11]
20:20:57.70 Dispense 0 microL and 0 steps
20:20:57.70 A0R
20:21:02.03 --Dispensed 123µl to RV48[11]
20:21:05.28 21 Mix "RV48" for 45.00 minutes at 450 rpm(s) and wait. SynthPos=102
20:21:07.09 Mixer 0 on at 450 rpm
21:06:08.98 Mixer 0 off
21:06:09.31 22 Empty RV48 for 1.500 minute(s) SynthPos=103
21:07:37.47 23 Goto ChemFile washcp48.cht, line 1 SynthPos=104
21:07:38.79 1 REM Wash after Coupling SynthPos=105
21:07:39.17 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=106
21:08:10.20 Washing with DMF (A) 3.00ml per vessel to RV48
21:08:20.09 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=107
21:08:21.90 Mixer 0 on at 450 rpm
21:09:23.69 Mixer 0 off
21:09:24.02 4 Empty RV48 for 1.500 minute(s) SynthPos=108
21:10:52.45 5 Repeat from step 2, 3 times SynthPos=109

21:10:53.77 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=110
21:10:53.94 Washing with DMF (A) 3.00ml per vessel to RV48
21:11:03.88 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=111
21:11:05.64 Mixer 0 on at 450 rpm
21:12:07.43 Mixer 0 off
21:12:07.76 4 Empty RV48 for 1.500 minute(s) SynthPos=112
21:13:35.47 5 Repeat from step 2, 3 times SynthPos=113
21:13:36.79 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=114
21:13:36.95 Washing with DMF (A) 3.00ml per vessel to RV48
21:13:46.90 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=115
21:13:48.65 Mixer 0 on at 450 rpm
21:14:51.27 Mixer 0 off
21:14:51.60 4 Empty RV48 for 1.500 minute(s) SynthPos=116
21:16:19.48 5 Repeat from step 2, 3 times SynthPos=117
21:16:20.80 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=118
21:16:20.96 Washing with DMF (A) 3.00ml per vessel to RV48
21:16:30.85 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=119
21:16:32.66 Mixer 0 on at 450 rpm
21:17:34.84 Mixer 0 off
21:17:35.22 4 Empty RV48 for 1.500 minute(s) SynthPos=120
21:19:03.49 5 Repeat from step 2, 3 times SynthPos=121
21:19:04.81 6 Return SynthPos=122
21:19:05.19 24 REM ** This is the data for cycle 3. ** SynthPos=123
21:19:05.52 25 <Set Active> SynthPos=124
21:19:07.00 26 Goto ChemFile depro48.cht, line 1 SynthPos=125
21:19:08.32 1 REM FMOC DEPROTECTION SynthPos=126
21:19:08.70 2 Transfer 2.00ml from Pip-350mL[1](PIP) to RV48[1-48] using DMF (1) SynthPos=127
21:19:08.70 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
21:19:08.70 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
21:19:10.57 Pick up 2250 microL and 0 steps
21:19:13.04 A675R
21:19:25.57 Pick up 110 microL and 0 steps
21:19:26.34 A33R
21:19:26.72 Dispense 99 microL and 0 steps
21:19:26.72 A30R
21:19:29.58 --Cleaned Probe with 2250µl of DMF (1)
21:19:30.29 Pick up 110 microL and 0 steps
21:19:30.68 A33R
21:19:31.06 Dispense 99 microL and 0 steps
21:19:31.06 A30R
21:19:36.99 Pick up 2225 microL and 0 steps
21:19:43.69 A668R
21:19:47.54 Dispense 2126 microL and 0 steps
21:19:47.54 A638R

21:19:53.85 --Aspirated 2001µl from Pip-350mL[1](PIP)
21:19:56.05 Dispense 126 microL and 0 steps
21:19:56.05 A38R
21:20:03.19 --Dispensed 2000µl to RV48[11]
21:20:03.63 Dispense 1 microL and 0 steps
21:20:03.63 A0R
21:20:07.91 --Dispensed 126µl to RV48[11]
21:20:11.21 3 Mix "RV48" for 3.00 minutes at 450 rpm(s) and wait. SynthPos=128
21:20:13.02 Mixer 0 on at 450 rpm
21:23:14.88 Mixer 0 off
21:23:15.21 4 Empty RV48 for 1.500 minute(s) SynthPos=129
21:24:43.48 5 Transfer 2.00ml from Pip-350mL[1](PIP) to RV48[1-48] using DMF (1) SynthPos=130
21:24:43.48 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
21:24:43.48 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
21:24:44.19 Pick up 110 microL and 0 steps
21:24:44.96 A33R
21:24:45.29 Dispense 99 microL and 0 steps
21:24:45.29 A30R
21:24:51.28 Pick up 2225 microL and 0 steps
21:24:58.03 A668R
21:25:01.82 Dispense 2126 microL and 0 steps
21:25:01.82 A638R
21:25:07.92 --Aspirated 2001µl from Pip-350mL[1](PIP)
21:25:10.17 Dispense 126 microL and 0 steps
21:25:10.17 A38R
21:25:17.31 --Dispensed 2000µl to RV48[11]
21:25:17.69 Dispense 1 microL and 0 steps
21:25:17.69 A0R
21:25:22.03 --Dispensed 126µl to RV48[11]
21:25:25.33 6 Mix "RV48" for 8.00 minutes at 450 rpm(s) and wait. SynthPos=131
21:25:27.09 Mixer 0 on at 450 rpm
21:33:29.50 Mixer 0 off
21:33:29.83 7 Empty RV48 for 1.500 minute(s) SynthPos=132
21:34:57.49 8 Return SynthPos=133
21:34:57.82 27 Goto ChemFile washdp48.cht, line 1 SynthPos=134
21:34:59.19 1 REM Wash after Deprotection SynthPos=135
21:34:59.52 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=136
21:35:30.55 Washing with DMF (B) 3.00ml per vessel to RV48
21:35:40.44 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=137
21:35:42.25 Mixer 0 on at 450 rpm
21:36:43.93 Mixer 0 off
21:36:44.26 4 Empty RV48 for 1.500 minute(s) SynthPos=138
21:38:12.47 5 Repeat from step 2, 3 times SynthPos=139
21:38:13.79 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=140

21:38:13.96 Washing with DMF (B) 3.00ml per vessel to RV48
21:38:23.90 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=141
21:38:25.66 Mixer 0 on at 450 rpm
21:39:27.72 Mixer 0 off
21:39:28.05 4 Empty RV48 for 1.500 minute(s) SynthPos=142
21:40:56.48 5 Repeat from step 2, 3 times SynthPos=143
21:40:57.80 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=144
21:40:57.96 Washing with DMF (B) 3.00ml per vessel to RV48
21:41:07.91 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=145
21:41:09.66 Mixer 0 on at 450 rpm
21:42:11.18 Mixer 0 off
21:42:11.56 4 Empty RV48 for 1.500 minute(s) SynthPos=146
21:43:39.44 5 Repeat from step 2, 3 times SynthPos=147
21:43:40.76 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=148
21:43:40.93 Washing with DMF (B) 3.00ml per vessel to RV48
21:43:50.87 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=149
21:43:52.63 Mixer 0 on at 450 rpm
21:44:54.03 Mixer 0 off
21:44:54.36 4 Empty RV48 for 1.500 minute(s) SynthPos=150
21:46:22.46 5 Repeat from step 2, 3 times SynthPos=151
21:46:23.78 6 Return SynthPos=152
21:46:24.17 28 REM HBTU48 SynthPos=153
21:46:24.50 29 Dispense Sequence SNI143.dsp with 0.699ml to RV48 rack using DMF (1) SynthPos=154
21:46:24.50 Dispensing from Rack 4x7 vessel 10 to Rack RV48 vessel 11
21:46:26.42 Pick up 4000 microL and 0 steps
21:46:30.59 A1200R
21:46:44.93 Pick up 110 microL and 0 steps
21:46:45.64 A33R
21:46:46.03 Dispense 99 microL and 0 steps
21:46:46.03 A30R
21:46:48.88 --Cleaned Probe with 4000µl of DMF (1)
21:46:49.60 Pick up 110 microL and 0 steps
21:46:49.98 A33R
21:46:50.37 Dispense 99 microL and 0 steps
21:46:50.37 A30R
21:46:55.86 Pick up 924 microL and 0 steps
21:46:58.66 A277R
21:47:02.28 Dispense 823 microL and 0 steps
21:47:02.28 A247R
21:47:08.38 --Aspirated 698µl from 4x7[10](Leu)
21:47:10.41 Dispense 0 microL and 0 steps
21:47:10.41 A0R
21:47:15.80 --Dispensed 823µl to RV48[11]
21:47:19.09 30 Transfer 0.70ml from HBTU-350mL[1](HCTU) to RV48[1-48] using DMF (1) SynthPos=155

21:47:19.09 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
21:47:19.09 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
21:47:21.01 Pick up 2250 microL and 0 steps
21:47:23.43 A675R
21:47:36.01 Pick up 110 microL and 0 steps
21:47:36.72 A33R
21:47:37.11 Dispense 99 microL and 0 steps
21:47:37.11 A30R
21:47:40.02 --Cleaned Probe with 2250µl of DMF (1)
21:47:40.68 Pick up 110 microL and 0 steps
21:47:41.06 A33R
21:47:41.45 Dispense 99 microL and 0 steps
21:47:41.45 A30R
21:47:47.87 Pick up 924 microL and 0 steps
21:47:50.67 A277R
21:47:54.52 Dispense 823 microL and 0 steps
21:47:54.52 A247R
21:48:00.84 --Aspirated 698µl from HBTU-350mL[1](HCTU)
21:48:03.09 Dispense 124 microL and 0 steps
21:48:03.09 A37R
21:48:08.25 --Dispensed 700µl to RV48[11]
21:48:08.69 Dispense 0 microL and 0 steps
21:48:08.69 A0R
21:48:12.97 --Dispensed 123µl to RV48[11]
21:48:16.27 31 Transfer 0.70ml from DIEA-350mL[1](DIPEA) to RV48[1-48] using DMF (1) SynthPos=156
21:48:16.27 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
21:48:16.32 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
21:48:18.19 Pick up 2250 microL and 0 steps
21:48:20.61 A675R
21:48:33.19 Pick up 110 microL and 0 steps
21:48:33.96 A33R
21:48:34.28 Dispense 99 microL and 0 steps
21:48:34.28 A30R
21:48:37.20 --Cleaned Probe with 2250µl of DMF (1)
21:48:37.86 Pick up 110 microL and 0 steps
21:48:38.29 A33R
21:48:38.62 Dispense 99 microL and 0 steps
21:48:38.62 A30R
21:48:44.83 Pick up 924 microL and 0 steps
21:48:47.63 A277R
21:48:51.42 Dispense 823 microL and 0 steps
21:48:51.42 A247R
21:48:57.74 --Aspirated 698µl from DIEA-350mL[1](DIPEA)

21:48:59.99 Dispense 124 microL and 0 steps
21:48:59.99 A37R
21:49:05.21 --Dispensed 700µl to RV48[11]
21:49:05.65 Dispense 0 microL and 0 steps
21:49:05.65 A0R
21:49:09.99 --Dispensed 123µl to RV48[11]
21:49:13.28 32 Mix "RV48" for 45.00 minutes at 450 rpm(s) and wait. SynthPos=157
21:49:15.04 Mixer 0 on at 450 rpm
22:34:18.47 Mixer 0 off
22:34:18.80 33 Empty RV48 for 1.500 minute(s) SynthPos=158
22:35:46.46 34 Goto ChemFile washcp48.cht, line 1 SynthPos=159
22:35:47.78 1 REM Wash after Coupling SynthPos=160
22:35:48.17 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=161
22:36:19.20 Washing with DMF (A) 3.00ml per vessel to RV48
22:36:29.14 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=162
22:36:30.90 Mixer 0 on at 450 rpm
22:37:32.30 Mixer 0 off
22:37:32.63 4 Empty RV48 for 1.500 minute(s) SynthPos=163
22:39:00.46 5 Repeat from step 2, 3 times SynthPos=164
22:39:01.78 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=165
22:39:01.94 Washing with DMF (A) 3.00ml per vessel to RV48
22:39:11.88 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=166
22:39:13.64 Mixer 0 on at 450 rpm
22:40:16.48 Mixer 0 off
22:40:16.81 4 Empty RV48 for 1.500 minute(s) SynthPos=167
22:41:44.47 5 Repeat from step 2, 3 times SynthPos=168
22:41:45.79 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=169
22:41:45.95 Washing with DMF (A) 3.00ml per vessel to RV48
22:41:55.89 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=170
22:41:57.65 Mixer 0 on at 450 rpm
22:42:59.17 Mixer 0 off
22:42:59.50 4 Empty RV48 for 1.500 minute(s) SynthPos=171
22:44:27.49 5 Repeat from step 2, 3 times SynthPos=172
22:44:28.80 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=173
22:44:28.97 Washing with DMF (A) 3.00ml per vessel to RV48
22:44:38.91 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=174
22:44:40.67 Mixer 0 on at 450 rpm
22:45:43.01 Mixer 0 off
22:45:43.34 4 Empty RV48 for 1.500 minute(s) SynthPos=175
22:47:11.44 5 Repeat from step 2, 3 times SynthPos=176
22:47:12.76 6 Return SynthPos=177
22:47:13.14 35 REM ** This is the data for cycle 4. ** SynthPos=178
22:47:13.47 36 <Set Active> SynthPos=179
22:47:14.95 37 Goto ChemFile depro48.cht, line 1 SynthPos=180
22:47:16.27 1 REM FMOC DEPROTECTION SynthPos=181

22:47:16.60 2 Transfer 2.00ml from Pip-350mL[1](PIP) to RV48[1-48] using DMF (1) SynthPos=182
22:47:16.60 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
22:47:16.66 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
22:47:18.52 Pick up 2250 microL and 0 steps
22:47:21.00 A675R
22:47:33.52 Pick up 110 microL and 0 steps
22:47:34.29 A33R
22:47:34.67 Dispense 99 microL and 0 steps
22:47:34.67 A30R
22:47:37.53 --Cleaned Probe with 2250µl of DMF (1)
22:47:38.24 Pick up 110 microL and 0 steps
22:47:38.63 A33R
22:47:39.01 Dispense 99 microL and 0 steps
22:47:39.01 A30R
22:47:45.00 Pick up 2225 microL and 0 steps
22:47:51.70 A668R
22:47:55.49 Dispense 2126 microL and 0 steps
22:47:55.49 A638R
22:48:01.81 --Aspirated 2001µl from Pip-350mL[1](PIP)
22:48:04.06 Dispense 126 microL and 0 steps
22:48:04.06 A38R
22:48:11.20 --Dispensed 2000µl to RV48[11]
22:48:11.58 Dispense 1 microL and 0 steps
22:48:11.58 A0R
22:48:15.92 --Dispensed 126µl to RV48[11]
22:48:19.22 3 Mix "RV48" for 3.00 minutes at 450 rpm(s) and wait. SynthPos=183
22:48:20.97 Mixer 0 on at 450 rpm
22:51:22.28 Mixer 0 off
22:51:22.67 4 Empty RV48 for 1.500 minute(s) SynthPos=184
22:52:50.44 5 Transfer 2.00ml from Pip-350mL[1](PIP) to RV48[1-48] using DMF (1) SynthPos=185
22:52:50.44 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
22:52:50.44 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
22:52:51.15 Pick up 110 microL and 0 steps
22:52:51.87 A33R
22:52:52.25 Dispense 99 microL and 0 steps
22:52:52.25 A30R
22:52:58.24 Pick up 2225 microL and 0 steps
22:53:04.94 A668R
22:53:08.73 Dispense 2126 microL and 0 steps
22:53:08.73 A638R
22:53:14.88 --Aspirated 2001µl from Pip-350mL[1](PIP)
22:53:17.08 Dispense 126 microL and 0 steps

22:53:17.08 A38R
22:53:24.22 --Dispensed 2000µl to RV48[11]
22:53:24.66 Dispense 1 microL and 0 steps
22:53:24.66 A0R
22:53:28.94 --Dispensed 126µl to RV48[11]
22:53:32.24 6 Mix "RV48" for 8.00 minutes at 450 rpm(s) and wait. SynthPos=186
22:53:34.05 Mixer 0 on at 450 rpm
23:01:36.57 Mixer 0 off
23:01:36.90 7 Empty RV48 for 1.500 minute(s) SynthPos=187
23:03:05.44 8 Return SynthPos=188
23:03:05.77 38 Goto ChemFile washdp48.cht, line 1 SynthPos=189
23:03:07.14 1 REM Wash after Deprotection SynthPos=190
23:03:07.47 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=191
23:03:38.50 Washing with DMF (B) 3.00ml per vessel to RV48
23:03:48.45 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=192
23:03:50.20 Mixer 0 on at 450 rpm
23:04:52.43 Mixer 0 off
23:04:52.76 4 Empty RV48 for 1.500 minute(s) SynthPos=193
23:06:20.48 5 Repeat from step 2, 3 times SynthPos=194
23:06:21.80 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=195
23:06:21.96 Washing with DMF (B) 3.00ml per vessel to RV48
23:06:31.85 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=196
23:06:33.66 Mixer 0 on at 450 rpm
23:07:35.29 Mixer 0 off
23:07:35.67 4 Empty RV48 for 1.500 minute(s) SynthPos=197
23:09:03.44 5 Repeat from step 2, 3 times SynthPos=198
23:09:04.76 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=199
23:09:04.93 Washing with DMF (B) 3.00ml per vessel to RV48
23:09:14.87 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=200
23:09:16.63 Mixer 0 on at 450 rpm
23:10:18.25 Mixer 0 off
23:10:18.58 4 Empty RV48 for 1.500 minute(s) SynthPos=201
23:11:46.46 5 Repeat from step 2, 3 times SynthPos=202
23:11:47.78 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=203
23:11:47.95 Washing with DMF (B) 3.00ml per vessel to RV48
23:11:57.83 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=204
23:11:59.65 Mixer 0 on at 450 rpm
23:13:01.44 Mixer 0 off
23:13:01.77 4 Empty RV48 for 1.500 minute(s) SynthPos=205
23:14:29.48 5 Repeat from step 2, 3 times SynthPos=206
23:14:30.80 6 Return SynthPos=207
23:14:31.18 39 REM HBTU48 SynthPos=208
23:14:31.51 40 Dispense Sequence SNI144.dsp with 0.699ml to RV48 rack using DMF (1) SynthPos=209
23:14:31.51 Dispensing from Rack 4x7 vessel 13 to Rack RV48 vessel 11
23:14:33.44 Pick up 4000 microL and 0 steps

23:14:37.61 A1200R
23:14:51.95 Pick up 110 microL and 0 steps
23:14:52.72 A33R
23:14:53.04 Dispense 99 microL and 0 steps
23:14:53.04 A30R
23:14:55.96 --Cleaned Probe with 4000µl of DMF (1)
23:14:56.67 Pick up 110 microL and 0 steps
23:14:57.05 A33R
23:14:57.38 Dispense 99 microL and 0 steps
23:14:57.38 A30R
23:15:02.99 Pick up 924 microL and 0 steps
23:15:05.79 A277R
23:15:09.47 Dispense 823 microL and 0 steps
23:15:09.47 A247R
23:15:15.56 --Aspirated 698µl from 4x7[13](Phe)
23:15:17.49 Dispense 0 microL and 0 steps
23:15:17.49 A0R
23:15:22.81 --Dispensed 823µl to RV48[11]
23:15:26.11 41 Transfer 0.70ml from HBTU-350mL[1](HCTU) to RV48[1-48] using DMF (1) SynthPos=210
23:15:26.11 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
23:15:26.11 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
23:15:28.03 Pick up 2250 microL and 0 steps
23:15:30.45 A675R
23:15:42.97 Pick up 110 microL and 0 steps
23:15:43.74 A33R
23:15:44.13 Dispense 99 microL and 0 steps
23:15:44.13 A30R
23:15:46.98 --Cleaned Probe with 2250µl of DMF (1)
23:15:47.70 Pick up 110 microL and 0 steps
23:15:48.08 A33R
23:15:48.46 Dispense 99 microL and 0 steps
23:15:48.46 A30R
23:15:54.89 Pick up 924 microL and 0 steps
23:15:57.64 A277R
23:16:01.48 Dispense 823 microL and 0 steps
23:16:01.48 A247R
23:16:07.80 --Aspirated 698µl from HBTU-350mL[1](HCTU)
23:16:10.05 Dispense 124 microL and 0 steps
23:16:10.05 A37R
23:16:15.27 --Dispensed 700µl to RV48[11]
23:16:15.65 Dispense 0 microL and 0 steps
23:16:15.65 A0R
23:16:19.99 --Dispensed 123µl to RV48[11]

23:16:23.23 42 Transfer 0.70ml from DIEA-350mL[1](DIPEA) to RV48[1-48] using DMF (1) SynthPos=211
23:16:23.23 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
23:16:23.29 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
23:16:25.15 Pick up 2250 microL and 0 steps
23:16:27.63 A675R
23:16:40.15 Pick up 110 microL and 0 steps
23:16:40.92 A33R
23:16:41.25 Dispense 99 microL and 0 steps
23:16:41.25 A30R
23:16:44.16 --Cleaned Probe with 2250µl of DMF (1)
23:16:44.82 Pick up 110 microL and 0 steps
23:16:45.26 A33R
23:16:45.59 Dispense 99 microL and 0 steps
23:16:45.59 A30R
23:16:51.79 Pick up 924 microL and 0 steps
23:16:54.59 A277R
23:16:58.38 Dispense 823 microL and 0 steps
23:16:58.38 A247R
23:17:04.76 --Aspirated 698µl from DIEA-350mL[1](DIPEA)
23:17:06.95 Dispense 124 microL and 0 steps
23:17:06.95 A37R
23:17:12.17 --Dispensed 700µl to RV48[11]
23:17:12.56 Dispense 0 microL and 0 steps
23:17:12.56 A0R
23:17:16.89 --Dispensed 123µl to RV48[11]
23:17:20.19 43 Mix "RV48" for 45.00 minutes at 450 rpm(s) and wait. SynthPos=212
23:17:21.95 Mixer 0 on at 450 rpm
00:02:23.30 Mixer 0 off
00:02:23.63 44 Empty RV48 for 1.500 minute(s) SynthPos=213
00:03:51.45 45 Goto ChemFile washcp48.cht, line 1 SynthPos=214
00:03:52.77 1 REM Wash after Coupling SynthPos=215
00:03:53.15 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=216
00:04:24.19 Washing with DMF (A) 3.00ml per vessel to RV48
00:04:34.13 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=217
00:04:35.89 Mixer 0 on at 450 rpm
00:05:37.40 Mixer 0 off
00:05:37.73 4 Empty RV48 for 1.500 minute(s) SynthPos=218
00:07:05.45 5 Repeat from step 2, 3 times SynthPos=219
00:07:06.77 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=220
00:07:06.93 Washing with DMF (A) 3.00ml per vessel to RV48
00:07:16.87 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=221
00:07:18.63 Mixer 0 on at 450 rpm
00:08:19.98 Mixer 0 off
00:08:20.31 4 Empty RV48 for 1.500 minute(s) SynthPos=222

00:09:48.47 5 Repeat from step 2, 3 times SynthPos=223
00:09:49.78 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=224
00:09:49.95 Washing with DMF (A) 3.00ml per vessel to RV48
00:09:59.89 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=225
00:10:01.65 Mixer 0 on at 450 rpm
00:11:03.55 Mixer 0 off
00:11:03.88 4 Empty RV48 for 1.500 minute(s) SynthPos=226
00:12:31.49 5 Repeat from step 2, 3 times SynthPos=227
00:12:32.80 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=228
00:12:32.97 Washing with DMF (A) 3.00ml per vessel to RV48
00:12:42.91 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=229
00:12:44.67 Mixer 0 on at 450 rpm
00:13:46.35 Mixer 0 off
00:13:46.68 4 Empty RV48 for 1.500 minute(s) SynthPos=230
00:15:14.45 5 Repeat from step 2, 3 times SynthPos=231
00:15:15.77 6 Return SynthPos=232
00:15:16.15 46 REM ** This is the data for cycle 5. ** SynthPos=233
00:15:16.48 47 <Set Active> SynthPos=234
00:15:17.96 48 Goto ChemFile depro48.cht, line 1 SynthPos=235
00:15:19.28 1 REM FMOC DEPROTECTION SynthPos=236
00:15:19.67 2 Transfer 2.00ml from Pip-350mL[1](PIP) to RV48[1-48] using DMF (1) SynthPos=237
00:15:19.67 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
00:15:19.67 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
00:15:21.59 Pick up 2250 microL and 0 steps
00:15:24.01 A675R
00:15:36.53 Pick up 110 microL and 0 steps
00:15:37.30 A33R
00:15:37.63 Dispense 99 microL and 0 steps
00:15:37.63 A30R
00:15:40.54 --Cleaned Probe with 2250µl of DMF (1)
00:15:41.25 Pick up 110 microL and 0 steps
00:15:41.64 A33R
00:15:42.02 Dispense 99 microL and 0 steps
00:15:42.02 A30R
00:15:48.01 Pick up 2225 microL and 0 steps
00:15:54.71 A668R
00:15:58.50 Dispense 2126 microL and 0 steps
00:15:58.50 A638R
00:16:04.87 --Aspirated 2001µl from Pip-350mL[1](PIP)
00:16:07.07 Dispense 126 microL and 0 steps
00:16:07.07 A38R
00:16:14.21 --Dispensed 2000µl to RV48[11]
00:16:14.65 Dispense 1 microL and 0 steps
00:16:14.65 A0R

00:16:18.93 --Dispensed 126µl to RV48[11]
00:16:22.23 3 Mix "RV48" for 3.00 minutes at 450 rpm(s) and wait. SynthPos=238
00:16:24.04 Mixer 0 on at 450 rpm
00:19:26.28 Mixer 0 off
00:19:26.61 4 Empty RV48 for 1.500 minute(s) SynthPos=239
00:20:54.44 5 Transfer 2.00ml from Pip-350mL[1](PIP) to RV48[1-48] using DMF (1) SynthPos=240
00:20:54.44 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
00:20:54.44 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
00:20:55.10 Pick up 110 microL and 0 steps
00:20:55.87 A33R
00:20:56.25 Dispense 99 microL and 0 steps
00:20:56.25 A30R
00:21:02.24 Pick up 2225 microL and 0 steps
00:21:08.94 A668R
00:21:12.73 Dispense 2126 microL and 0 steps
00:21:12.73 A638R
00:21:18.88 --Aspirated 2001µl from Pip-350mL[1](PIP)
00:21:21.08 Dispense 126 microL and 0 steps
00:21:21.08 A38R
00:21:28.22 --Dispensed 2000µl to RV48[11]
00:21:28.66 Dispense 1 microL and 0 steps
00:21:28.66 A0R
00:21:32.94 --Dispensed 126µl to RV48[11]
00:21:36.24 6 Mix "RV48" for 8.00 minutes at 450 rpm(s) and wait. SynthPos=241
00:21:38.05 Mixer 0 on at 450 rpm
00:29:41.01 Mixer 0 off
00:29:41.34 7 Empty RV48 for 1.500 minute(s) SynthPos=242
00:31:09.44 8 Return SynthPos=243
00:31:09.77 49 Goto ChemFile washdp48.cht, line 1 SynthPos=244
00:31:11.09 1 REM Wash after Deprotection SynthPos=245
00:31:11.47 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=246
00:31:42.50 Washing with DMF (B) 3.00ml per vessel to RV48
00:31:52.45 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=247
00:31:54.20 Mixer 0 on at 450 rpm
00:32:56.65 Mixer 0 off
00:32:57.04 4 Empty RV48 for 1.500 minute(s) SynthPos=248
00:34:25.47 5 Repeat from step 2, 3 times SynthPos=249
00:34:26.79 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=250
00:34:26.95 Washing with DMF (B) 3.00ml per vessel to RV48
00:34:36.84 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=251
00:34:38.65 Mixer 0 on at 450 rpm
00:35:40.28 Mixer 0 off
00:35:40.61 4 Empty RV48 for 1.500 minute(s) SynthPos=252
00:37:08.49 5 Repeat from step 2, 3 times SynthPos=253

00:37:09.81 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=254
00:37:09.97 Washing with DMF (B) 3.00ml per vessel to RV48
00:37:19.91 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=255
00:37:21.67 Mixer 0 on at 450 rpm
00:38:23.24 Mixer 0 off
00:38:23.57 4 Empty RV48 for 1.500 minute(s) SynthPos=256
00:39:51.45 5 Repeat from step 2, 3 times SynthPos=257
00:39:52.77 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=258
00:39:52.93 Washing with DMF (B) 3.00ml per vessel to RV48
00:40:02.88 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=259
00:40:04.63 Mixer 0 on at 450 rpm
00:41:06.15 Mixer 0 off
00:41:06.48 4 Empty RV48 for 1.500 minute(s) SynthPos=260
00:42:34.47 5 Repeat from step 2, 3 times SynthPos=261
00:42:35.79 6 Return SynthPos=262
00:42:36.17 50 REM HBTU48 SynthPos=263
00:42:36.50 51 Dispense Sequence SNI145.dsp with 0.699ml to RV48 rack using DMF (1) SynthPos=264
00:42:36.50 Dispensing from Rack 4x7 vessel 10 to Rack RV48 vessel 11
00:42:38.42 Pick up 4000 microL and 0 steps
00:42:42.60 A1200R
00:42:56.93 Pick up 110 microL and 0 steps
00:42:57.70 A33R
00:42:58.03 Dispense 99 microL and 0 steps
00:42:58.03 A30R
00:43:00.94 --Cleaned Probe with 4000µl of DMF (1)
00:43:01.60 Pick up 110 microL and 0 steps
00:43:02.04 A33R
00:43:02.37 Dispense 99 microL and 0 steps
00:43:02.37 A30R
00:43:07.86 Pick up 924 microL and 0 steps
00:43:10.67 A277R
00:43:14.29 Dispense 823 microL and 0 steps
00:43:14.29 A247R
00:43:20.44 --Aspirated 698µl from 4x7[10](Leu)
00:43:22.47 Dispense 0 microL and 0 steps
00:43:22.47 A0R
00:43:27.86 --Dispensed 823µl to RV48[11]
00:43:31.10 52 Transfer 0.70ml from HBTU-350mL[1](HCTU) to RV48[1-48] using DMF (1) SynthPos=265
00:43:31.10 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
00:43:31.10 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
00:43:33.02 Pick up 2250 microL and 0 steps
00:43:35.44 A675R
00:43:48.02 Pick up 110 microL and 0 steps

00:43:48.78 A33R
00:43:49.11 Dispense 99 microL and 0 steps
00:43:49.11 A30R
00:43:52.02 --Cleaned Probe with 2250µl of DMF (1)
00:43:52.68 Pick up 110 microL and 0 steps
00:43:53.12 A33R
00:43:53.45 Dispense 99 microL and 0 steps
00:43:53.45 A30R
00:43:59.88 Pick up 924 microL and 0 steps
00:44:02.68 A277R
00:44:06.47 Dispense 823 microL and 0 steps
00:44:06.47 A247R
00:44:12.79 --Aspirated 698µl from HBTU-350mL[1](HCTU)
00:44:15.04 Dispense 124 microL and 0 steps
00:44:15.04 A37R
00:44:20.26 --Dispensed 700µl to RV48[11]
00:44:20.64 Dispense 0 microL and 0 steps
00:44:20.64 A0R
00:44:24.98 --Dispensed 123µl to RV48[11]
00:44:28.28 53 Transfer 0.70ml from DIEA-350mL[1](DIPEA) to RV48[1-48] using DMF (1) SynthPos=266
00:44:28.28 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
00:44:28.28 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
00:44:30.14 Pick up 2250 microL and 0 steps
00:44:32.61 A675R
00:44:45.14 Pick up 110 microL and 0 steps
00:44:45.91 A33R
00:44:46.24 Dispense 99 microL and 0 steps
00:44:46.24 A30R
00:44:49.15 --Cleaned Probe with 2250µl of DMF (1)
00:44:49.81 Pick up 110 microL and 0 steps
00:44:50.25 A33R
00:44:50.58 Dispense 99 microL and 0 steps
00:44:50.58 A30R
00:44:56.78 Pick up 924 microL and 0 steps
00:44:59.58 A277R
00:45:03.43 Dispense 823 microL and 0 steps
00:45:03.43 A247R
00:45:09.74 --Aspirated 698µl from DIEA-350mL[1](DIPEA)
00:45:12.00 Dispense 124 microL and 0 steps
00:45:12.00 A37R
00:45:17.16 --Dispensed 700µl to RV48[11]
00:45:17.60 Dispense 0 microL and 0 steps
00:45:17.60 A0R
00:45:21.88 --Dispensed 123µl to RV48[11]

00:45:25.18 54 Mix "RV48" for 45.00 minutes at 450 rpm(s) and wait. SynthPos=267
00:45:26.99 Mixer 0 on at 450 rpm
01:30:28.28 Mixer 0 off
01:30:28.61 55 Empty RV48 for 1.500 minute(s) SynthPos=268
01:31:56.44 56 Goto ChemFile washcp48.cht, line 1 SynthPos=269
01:31:57.76 1 REM Wash after Coupling SynthPos=270
01:31:58.14 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=271
01:32:29.17 Washing with DMF (A) 3.00ml per vessel to RV48
01:32:39.11 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=272
01:32:40.87 Mixer 0 on at 450 rpm
01:33:42.22 Mixer 0 off
01:33:42.55 4 Empty RV48 for 1.500 minute(s) SynthPos=273
01:35:10.49 5 Repeat from step 2, 3 times SynthPos=274
01:35:11.81 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=275
01:35:11.97 Washing with DMF (A) 3.00ml per vessel to RV48
01:35:21.91 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=276
01:35:23.67 Mixer 0 on at 450 rpm
01:36:25.08 Mixer 0 off
01:36:25.41 4 Empty RV48 for 1.500 minute(s) SynthPos=277
01:37:53.45 5 Repeat from step 2, 3 times SynthPos=278
01:37:54.77 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=279
01:37:54.94 Washing with DMF (A) 3.00ml per vessel to RV48
01:38:04.88 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=280
01:38:06.63 Mixer 0 on at 450 rpm
01:39:07.77 Mixer 0 off
01:39:08.10 4 Empty RV48 for 1.500 minute(s) SynthPos=281
01:40:36.47 5 Repeat from step 2, 3 times SynthPos=282
01:40:37.79 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=283
01:40:37.95 Washing with DMF (A) 3.00ml per vessel to RV48
01:40:47.90 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=284
01:40:49.65 Mixer 0 on at 450 rpm
01:41:51.44 Mixer 0 off
01:41:51.77 4 Empty RV48 for 1.500 minute(s) SynthPos=285
01:43:19.44 5 Repeat from step 2, 3 times SynthPos=286
01:43:20.75 6 Return SynthPos=287
01:43:21.14 57 REM ** This is the data for cycle 6. ** SynthPos=288
01:43:21.47 58 <Set Active> SynthPos=289
01:43:22.95 59 Goto ChemFile depro48.cht, line 1 SynthPos=290
01:43:24.27 1 REM FMOC DEPROTECTION SynthPos=291
01:43:24.65 2 Transfer 2.00ml from Pip-350mL[1](PIP) to RV48[1-48] using DMF (1) SynthPos=292
01:43:24.65 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
01:43:24.65 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
01:43:26.52 Pick up 2250 microL and 0 steps
01:43:28.99 A675R

01:43:41.52 Pick up 110 microL and 0 steps
01:43:42.28 A33R
01:43:42.67 Dispense 99 microL and 0 steps
01:43:42.67 A30R
01:43:45.53 --Cleaned Probe with 2250µl of DMF (1)
01:43:46.24 Pick up 110 microL and 0 steps
01:43:46.68 A33R
01:43:47.01 Dispense 99 microL and 0 steps
01:43:47.01 A30R
01:43:52.99 Pick up 2225 microL and 0 steps
01:43:59.70 A668R
01:44:03.49 Dispense 2126 microL and 0 steps
01:44:03.49 A638R
01:44:09.86 --Aspirated 2001µl from Pip-350mL[1](PIP)
01:44:12.05 Dispense 126 microL and 0 steps
01:44:12.05 A38R
01:44:19.19 --Dispensed 2000µl to RV48[11]
01:44:19.58 Dispense 1 microL and 0 steps
01:44:19.58 A0R
01:44:23.92 --Dispensed 126µl to RV48[11]
01:44:27.21 3 Mix "RV48" for 3.00 minutes at 450 rpm(s) and wait. SynthPos=293
01:44:28.97 Mixer 0 on at 450 rpm
01:47:30.28 Mixer 0 off
01:47:30.66 4 Empty RV48 for 1.500 minute(s) SynthPos=294
01:48:58.44 5 Transfer 2.00ml from Pip-350mL[1](PIP) to RV48[1-48] using DMF (1) SynthPos=295
01:48:58.44 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
01:48:58.44 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
01:48:59.09 Pick up 110 microL and 0 steps
01:48:59.86 A33R
01:49:00.25 Dispense 99 microL and 0 steps
01:49:00.25 A30R
01:49:06.23 Pick up 2225 microL and 0 steps
01:49:12.94 A668R
01:49:16.73 Dispense 2126 microL and 0 steps
01:49:16.73 A638R
01:49:22.88 --Aspirated 2001µl from Pip-350mL[1](PIP)
01:49:25.07 Dispense 126 microL and 0 steps
01:49:25.07 A38R
01:49:32.21 --Dispensed 2000µl to RV48[11]
01:49:32.65 Dispense 1 microL and 0 steps
01:49:32.65 A0R
01:49:36.94 --Dispensed 126µl to RV48[11]
01:49:40.23 6 Mix "RV48" for 8.00 minutes at 450 rpm(s) and wait. SynthPos=296
01:49:42.05 Mixer 0 on at 450 rpm

01:57:44.62 Mixer 0 off
01:57:45.01 7 Empty RV48 for 1.500 minute(s) SynthPos=297
01:59:13.44 8 Return SynthPos=298
01:59:13.77 60 Goto ChemFile washdp48.cht, line 1 SynthPos=299
01:59:15.14 1 REM Wash after Deprotection SynthPos=300
01:59:15.47 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=301
01:59:46.50 Washing with DMF (B) 3.00ml per vessel to RV48
01:59:56.44 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=302
01:59:58.20 Mixer 0 on at 450 rpm
02:01:00.32 Mixer 0 off
02:01:00.65 4 Empty RV48 for 1.500 minute(s) SynthPos=303
02:02:28.48 5 Repeat from step 2, 3 times SynthPos=304
02:02:29.79 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=305
02:02:29.96 Washing with DMF (B) 3.00ml per vessel to RV48
02:02:39.90 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=306
02:02:41.66 Mixer 0 on at 450 rpm
02:03:43.18 Mixer 0 off
02:03:43.50 4 Empty RV48 for 1.500 minute(s) SynthPos=307
02:05:11.44 5 Repeat from step 2, 3 times SynthPos=308
02:05:12.76 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=309
02:05:12.92 Washing with DMF (B) 3.00ml per vessel to RV48
02:05:22.87 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=310
02:05:24.62 Mixer 0 on at 450 rpm
02:06:26.14 Mixer 0 off
02:06:26.52 4 Empty RV48 for 1.500 minute(s) SynthPos=311
02:07:54.46 5 Repeat from step 2, 3 times SynthPos=312
02:07:55.78 2 Fast Wash using System Fluid DMF (B) 3.00ml to RV48 SynthPos=313
02:07:55.94 Washing with DMF (B) 3.00ml per vessel to RV48
02:08:05.83 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=314
02:08:07.64 Mixer 0 on at 450 rpm
02:09:09.82 Mixer 0 off
02:09:10.15 4 Empty RV48 for 1.500 minute(s) SynthPos=315
02:10:38.47 5 Repeat from step 2, 3 times SynthPos=316
02:10:39.79 6 Return SynthPos=317
02:10:40.17 61 REM HBTU48 SynthPos=318
02:10:40.50 62 Dispense Sequence SNI146.dsp with 0.699ml to RV48 rack using DMF (1) SynthPos=319
02:10:40.50 Dispensing from Rack 4x7 vessel 13 to Rack RV48 vessel 11
02:10:42.42 Pick up 4000 microL and 0 steps
02:10:46.60 A1200R
02:11:00.93 Pick up 110 microL and 0 steps
02:11:01.70 A33R
02:11:02.03 Dispense 99 microL and 0 steps
02:11:02.03 A30R
02:11:04.94 --Cleaned Probe with 4000µl of DMF (1)
02:11:05.66 Pick up 110 microL and 0 steps

02:11:06.04 A33R
02:11:06.37 Dispense 99 microL and 0 steps
02:11:06.37 A30R
02:11:12.03 Pick up 924 microL and 0 steps
02:11:14.77 A277R
02:11:18.45 Dispense 823 microL and 0 steps
02:11:18.45 A247R
02:11:24.55 --Aspirated 698µl from 4x7[13](Phe)
02:11:26.47 Dispense 0 microL and 0 steps
02:11:26.47 A0R
02:11:31.80 --Dispensed 823µl to RV48[11]
02:11:35.10 63 Transfer 0.70ml from HBTU-350mL[1](HCTU) to RV48[1-48] using DMF (1) SynthPos=320
02:11:35.10 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
…
02:11:35.10 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
02:11:37.02 Pick up 2250 microL and 0 steps
02:11:39.43 A675R
02:11:51.96 Pick up 110 microL and 0 steps
02:11:52.73 A33R
02:11:53.11 Dispense 99 microL and 0 steps
02:11:53.11 A30R
02:11:55.97 --Cleaned Probe with 2250µl of DMF (1)
02:11:56.68 Pick up 110 microL and 0 steps
02:11:57.07 A33R
02:11:57.45 Dispense 99 microL and 0 steps
02:11:57.45 A30R
02:12:03.88 Pick up 924 microL and 0 steps
02:12:06.68 A277R
02:12:10.52 Dispense 823 microL and 0 steps
02:12:10.52 A247R
02:12:16.84 --Aspirated 698µl from HBTU-350mL[1](HCTU)
02:12:19.04 Dispense 124 microL and 0 steps
02:12:19.04 A37R
02:12:24.25 --Dispensed 700µl to RV48[11]
02:12:24.69 Dispense 0 microL and 0 steps
02:12:24.69 A0R
02:12:28.98 --Dispensed 123µl to RV48[11]
02:12:32.27 64 Transfer 0.70ml from DIEA-350mL[1](DIPEA) to RV48[1-48] using DMF (1) SynthPos=321
02:12:32.27 RV48 : Vessel 0 DEACTIVE -- VesselsLeft 47
… 7
02:12:32.27 RV48 : Vessel 47 DEACTIVE -- VesselsLeft 1
02:12:34.20 Pick up 2250 microL and 0 steps
02:12:36.61 A675R
02:12:49.19 Pick up 110 microL and 0 steps

02:12:49.90 A33R
02:12:50.29 Dispense 99 microL and 0 steps
02:12:50.29 A30R
02:12:53.14 --Cleaned Probe with 2250µl of DMF (1)
02:12:53.86 Pick up 110 microL and 0 steps
02:12:54.24 A33R
02:12:54.63 Dispense 99 microL and 0 steps
02:12:54.63 A30R
02:13:00.83 Pick up 924 microL and 0 steps
02:13:03.64 A277R
02:13:07.43 Dispense 823 microL and 0 steps
02:13:07.43 A247R
02:13:13.80 --Aspirated 698µl from DIEA-350mL[1](DIPEA)
02:13:15.99 Dispense 124 microL and 0 steps
02:13:15.99 A37R
02:13:21.21 --Dispensed 700µl to RV48[11]
02:13:21.65 Dispense 0 microL and 0 steps
02:13:21.65 A0R
02:13:25.93 --Dispensed 123µl to RV48[11]
02:13:29.23 65 Mix "RV48" for 45.00 minutes at 450 rpm(s) and wait. SynthPos=322
02:13:30.99 Mixer 0 on at 450 rpm
02:58:32.44 Mixer 0 off
02:58:32.77 66 Empty RV48 for 1.500 minute(s) SynthPos=323
03:00:00.49 67 Goto ChemFile washcp48.cht, line 1 SynthPos=324
03:00:01.81 1 REM Wash after Coupling SynthPos=325
03:00:02.19 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=326
03:00:33.22 Washing with DMF (A) 3.00ml per vessel to RV48
03:00:43.17 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=327
03:00:44.92 Mixer 0 on at 450 rpm
03:01:46.71 Mixer 0 off
03:01:47.04 4 Empty RV48 for 1.500 minute(s) SynthPos=328
03:03:15.47 5 Repeat from step 2, 3 times SynthPos=329
03:03:16.79 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=330
03:03:16.96 Washing with DMF (A) 3.00ml per vessel to RV48
03:03:26.90 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=331
03:03:28.66 Mixer 0 on at 450 rpm
03:04:29.90 Mixer 0 off
03:04:30.28 4 Empty RV48 for 1.500 minute(s) SynthPos=332
03:05:58.44 5 Repeat from step 2, 3 times SynthPos=333
03:05:59.76 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=334
03:05:59.92 Washing with DMF (A) 3.00ml per vessel to RV48
03:06:09.86 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=335
03:06:11.62 Mixer 0 on at 450 rpm
03:07:13.52 Mixer 0 off
03:07:13.91 4 Empty RV48 for 1.500 minute(s) SynthPos=336
03:08:42.45 5 Repeat from step 2, 3 times SynthPos=337

03:08:43.76 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=338
03:08:43.93 Washing with DMF (A) 3.00ml per vessel to RV48
03:08:53.87 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=339
03:08:55.63 Mixer 0 on at 450 rpm
03:09:57.64 Mixer 0 off
03:09:58.02 4 Empty RV48 for 1.500 minute(s) SynthPos=340
03:11:26.45 5 Repeat from step 2, 3 times SynthPos=341
03:11:27.77 6 Return SynthPos=342
03:11:28.16 68 Goto ChemFile washfn48.cht, line 1 SynthPos=343
03:11:29.47 1 Goto ChemFile C:\AAPPTEC\WASHCP48.CHM, line 1 SynthPos=344
03:11:30.79 1 REM Wash after Coupling SynthPos=345
03:11:31.18 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=346
03:11:31.34 Washing with DMF (A) 3.00ml per vessel to RV48
03:11:41.23 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=347
03:11:43.04 Mixer 0 on at 450 rpm
03:12:45.93 Mixer 0 off
03:12:46.26 4 Empty RV48 for 1.500 minute(s) SynthPos=348
03:14:14.47 5 Repeat from step 2, 3 times SynthPos=349
03:14:15.79 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=350
03:14:15.95 Washing with DMF (A) 3.00ml per vessel to RV48
03:14:25.90 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=351
03:14:27.65 Mixer 0 on at 450 rpm
03:15:29.83 Mixer 0 off
03:15:30.16 4 Empty RV48 for 1.500 minute(s) SynthPos=352
03:16:58.48 5 Repeat from step 2, 3 times SynthPos=353
03:16:59.80 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=354
03:16:59.96 Washing with DMF (A) 3.00ml per vessel to RV48
03:17:09.90 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=355
03:17:11.66 Mixer 0 on at 450 rpm
03:18:13.62 Mixer 0 off
03:18:13.95 4 Empty RV48 for 1.500 minute(s) SynthPos=356
03:19:42.49 5 Repeat from step 2, 3 times SynthPos=357
03:19:43.80 2 Fast Wash using System Fluid DMF (A) 3.00ml to RV48 SynthPos=358
03:19:43.97 Washing with DMF (A) 3.00ml per vessel to RV48
03:19:53.91 3 Mix "RV48" for 1.00 minutes at 450 rpm(s) and wait. SynthPos=359
03:19:55.67 Mixer 0 on at 450 rpm
03:20:57.18 Mixer 0 off
03:20:57.57 4 Empty RV48 for 1.500 minute(s) SynthPos=360
03:22:25.45 5 Repeat from step 2, 3 times SynthPos=361
03:22:26.77 6 Return SynthPos=362
03:22:27.15 2 Dispense System Fluid MEOH (2) 3.00ml to RV48[1-48] SynthPos=363
03:22:29.07 Pick up 9000 microL and 0 steps
03:22:38.30 A2700R
03:22:49.18 Pick up 9000 microL and 0 steps
03:22:58.35 A2700R
03:23:19.11 Pick up 40 microL and 0 steps

03:23:19.61 A12R
03:23:20.81 Dispense 29 microL and 0 steps
03:23:20.81 A9R
03:23:23.73 --Cleaned Probe with 18000µl of MEOH (2)
03:23:24.06 Pick up 3000 microL and 0 steps
03:23:27.19 A900R
03:23:27.84 --Aspirated 2999µl of System Fluid MEOH (2)
03:23:31.31 Dispense 0 microL and 0 steps
03:23:31.31 A0R
03:23:40.37 --Dispensed 2999µl to RV48[11]
03:23:43.22 3 Mix "RV48" for 2.00 minutes at 475 rpm(s) and wait. SynthPos=364
03:23:45.26 Mixer 0 on at 475 rpm
03:25:47.25 Mixer 0 off
03:25:47.58 4 Empty RV48 for 3.000 minute(s) SynthPos=365
03:28:45.48 5 Repeat from step 2, 2 times SynthPos=366
03:28:46.80 2 Dispense System Fluid MEOH (2) 3.00ml to RV48[1-48] SynthPos=367
03:28:47.18 Pick up 3000 microL and 0 steps
03:28:50.37 A900R
03:28:50.97 --Aspirated 2999µl of System Fluid MEOH (2)
03:28:54.49 Dispense 0 microL and 0 steps
03:28:54.49 A0R
03:29:03.49 --Dispensed 2999µl to RV48[11]
03:29:06.41 3 Mix "RV48" for 2.00 minutes at 475 rpm(s) and wait. SynthPos=368
03:29:08.44 Mixer 0 on at 475 rpm
03:31:11.69 Mixer 0 off
03:31:12.02 4 Empty RV48 for 3.000 minute(s) SynthPos=369
03:34:10.47 5 Repeat from step 2, 2 times SynthPos=370
03:34:11.79 2 Dispense System Fluid MEOH (2) 3.00ml to RV48[1-48] SynthPos=371
03:34:12.18 Pick up 3000 microL and 0 steps
03:34:15.36 A900R
03:34:15.97 --Aspirated 2999µl of System Fluid MEOH (2)
03:34:19.48 Dispense 0 microL and 0 steps
03:34:19.48 A0R
03:34:28.54 --Dispensed 2999µl to RV48[11]
03:34:31.40 3 Mix "RV48" for 2.00 minutes at 475 rpm(s) and wait. SynthPos=372
03:34:33.43 Mixer 0 on at 475 rpm
03:36:35.37 Mixer 0 off
03:36:35.75 4 Empty RV48 for 3.000 minute(s) SynthPos=373
03:39:33.44 5 Repeat from step 2, 2 times SynthPos=374
03:39:34.75 6 Empty RV48 for 5.000 minute(s) SynthPos=375
03:44:32.45 7 Return SynthPos=376
03:44:39.81 Ending ChemFile - C:\AAPPTEC\SNI144.CHM

AUTHOR'S BIOGRAPHY

Sadie Novak was born in Kunming, China on February 22, 1997. She was adopted and raised in Akron, Ohio and Chautauqua, New York before moving to the beautiful state of Maine in 2014. In 2015, she graduated from Hampden Academy and began her undergraduate career at the University of Maine. Through her studies in organic chemistry, she discovered her love for the field and chose to pursue a degree in Chemistry. She has received a Center for Undergraduate Research grant and an Honors College Thesis Fellowship.

While at the University of Maine, Sadie maintained an active role in the campus community through her involvement with the University of Maine College Democrats and Sophomore Eagles, as well as by serving as an officer for the Student Heritage Alliance Council. She also contributed to her community through the time she spent volunteering and interning for political campaigns.

Upon graduation, Sadie will return to New York to attend Syracuse University and earn her doctorate in biological organic chemistry.