

2012

# Synthesis and purification of potential antimicrobial peptides

Christopher Ryan Snyder

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Synthesis and Purification of Potential Antimicrobial Peptides

By

Christopher Ryan Snyder

Thesis

Submitted to the Department of Chemistry

Eastern Michigan University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

in

Chemistry

Deborah Heyl-Clegg, PhD, Chair

March 15, 2012

Ypsilanti, Michigan

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Synthesis and Purification of Potential Antimicrobial Peptides

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## **Abstract**

The increasingly growing resistance to antibiotics has geared research efforts towards understanding the structure and function of antimicrobial peptides to gradually replace or reinforce the roles of antibiotics in therapeutic applications. Cathelicidins are a family of structurally diverse antimicrobial peptides that have only a single representative in humans, an antimicrobial peptide called LL37. LL37 is believed to play an important role in the first line of defense against local infection and systemic invasion of pathogens at sites of inflammation and wounds. LL37 kills bacteria by disrupting their membranes through non-specific peptide-lipid interactions, making it far more difficult for bacteria to develop resistance against than current antibacterial agents. Examining analogs of LL37 could provide insight into its structure, function, and antimicrobial properties. Three of these variations were synthesized and purified: two truncated forms of LL37 (SK29 and FF33) as well as N-terminally extended LL37 (FALL39) for comparative studies.

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## 1. Introduction

### 1.1 Amino Acids, Peptides, and Proteins

There are twenty amino acids that occur regularly in nature and make up the building blocks for peptides and proteins. All amino acids have a general formula illustrated in Figure 1.

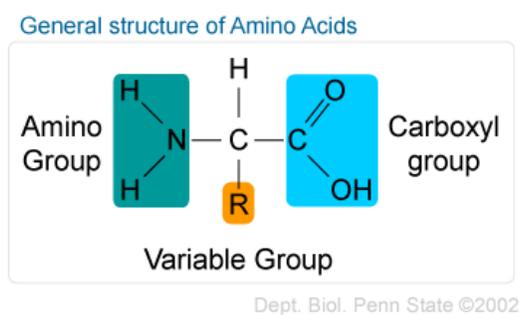


Figure 1: General structure of amino acids.<sup>1</sup>

Amino acids possess an acidic carboxyl group and a basic amino group, in addition to a unique side chain bound to the same chiral carbon atom. All amino acids produced *in vivo* occur in the L-conformation with respect to the central chiral carbon atom. Amino acids are commonly classified based on the nature of their side chain (R-group). These classes include non-polar, polar, basic, and acidic amino acids. The twenty naturally occurring amino acids are shown in Figure 2, grouped into their respective classes.

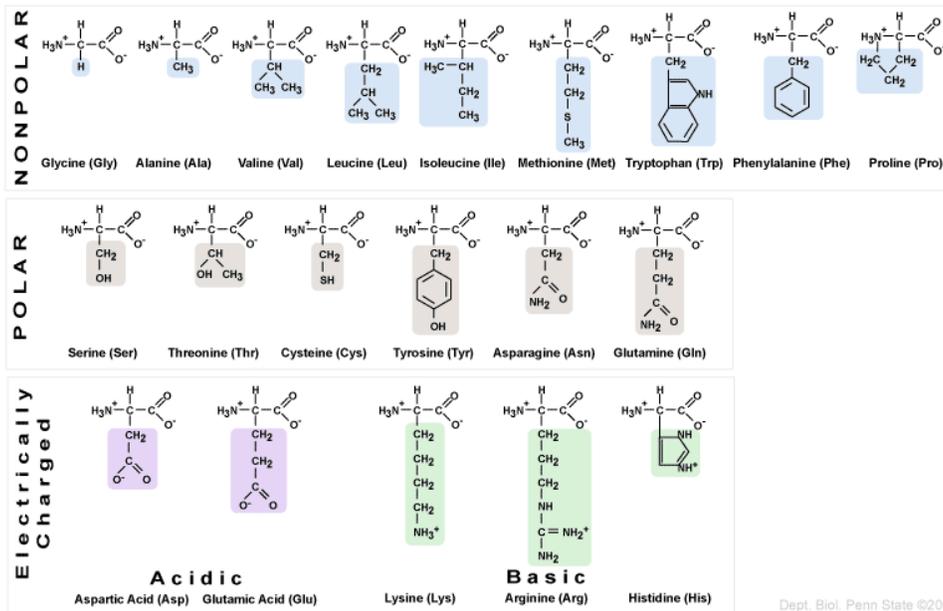


Figure 2: The twenty naturally occurring amino acids.<sup>1</sup>

Amino acids are commonly joined through a condensation reaction in which the carboxy-terminus of one amino acid reacts with the amino-terminus of another to form a peptide bond and water. This reaction is illustrated below in Figure 3.

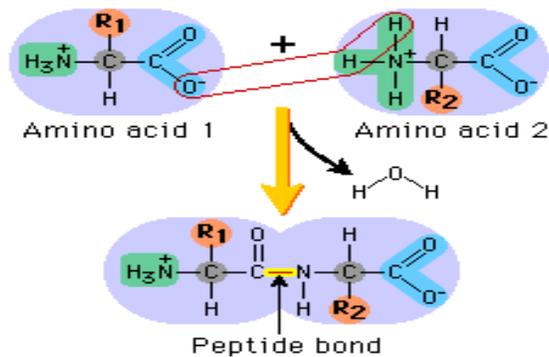


Figure 3: Formation of the peptide bond.<sup>2</sup>

When fifty amino acids or less are joined by peptide bonds, the molecule is typically called a peptide. Once the chain lengthens past fifty residues, it is generally referred to as

a polypeptide. Proteins are formed when multiple polypeptides are joined together in a biologically functional way. Slight modifications in the sequence of amino acids can result in drastic changes in the structure and function of proteins.

## 1.2 Protein Structure

Protein structure is classified into four distinct levels: primary, secondary, tertiary, and quaternary. The primary structure is simply the actual amino acid sequence that makes up a protein prior to any folding. As the chain of amino acids lengthens, some rotation occurs around the peptide bonds, and hydrogen bonding (H-bonds) typically results in the formation of a secondary structure. The hydrogen bonds may be within one chain or between different chains. This interaction of the amino acids leads to formation of either an  $\alpha$ -helix,  $\beta$ -sheet, or random coil, which are synonymous with protein secondary structure. In the  $\alpha$ -helix, hydrogen bonds are formed within the same chain, between amino acids four residues apart, as in the  $\alpha$ -helix of hemoglobin. In  $\beta$ -sheets, hydrogen bonding links adjacent chains, or can be intrachain, but the backbone is more extended. Tertiary structure results from the interaction of multiple secondary structures as the protein folds and coils to attain a more compact three dimensional structure. These structures are held together by several different forces, including hydrogen bonds, disulfide bonds, van der Waals forces, and electrostatic and hydrophobic interactions. These different levels of protein structure are shown in Figure 4.

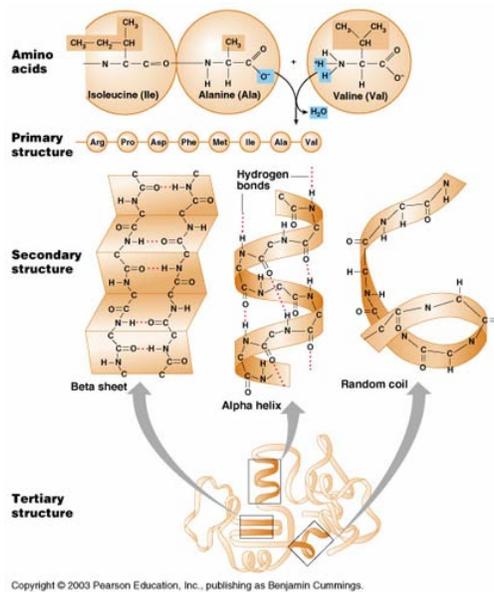


Figure 4: Primary, secondary, and tertiary protein structure.<sup>1</sup>

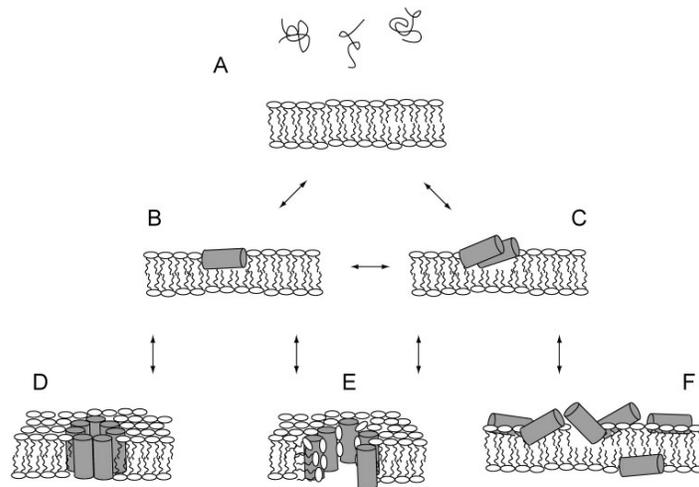
The fourth level of protein structure is quaternary structure. Quaternary structure is composed of multiple tertiary structures formed into compact units called sub-units, which combine to form the overall protein. Once proteins are fully formed, they typically have at least one location where they interact with a specific biochemical. These locations are called active sites and are designed so that only certain molecules with distinct structures or characteristics can bind to them.<sup>3</sup> Any changes to the protein's structure in that region could inactivate the site, thus preventing binding and/or causing loss of function.

### 1.3 Antimicrobial Peptides

Antimicrobial peptides show a broad spectrum of antimicrobial activity and are found among all classes of life.<sup>4</sup> These peptides seem to be representative of an ancient

defense strategy of eukaryotes against bacteria due to their high conservation and large diversity, which is observed even in closely related species.<sup>5</sup> Since antimicrobial peptides are so widespread, they are thought to be conserved throughout evolution as part of the innate immune response. The increasingly growing resistance to antibiotics has geared research efforts towards understanding the mechanistic and potential roles of antimicrobial peptides with development for replacing or reinforcing the roles of antibiotics in therapeutic applications.<sup>6</sup> In addition, antimicrobial peptides have also been reported to modulate various disease curbing processes in the body such as influencing inflammation, chemotaxis, the release of cytokines, and maintaining the balance of proteases and protease inhibitors.<sup>7</sup> Thus, they also have a role as potential drugs in the treatment of wounds and inflammation.

The exact mechanism by which antimicrobial peptides kill microorganisms has yet to be fully understood. They are known to actively bind to microbial membranes, although the exact conformation they are in during binding is still being researched. Antimicrobial peptides tend to be rich in the positively charged amino acids: lysine, arginine, and histidine. This allows them to bind the typically negatively charged cell membranes of bacterial pathogens while leaving neutral charged eukaryotic membranes intact.<sup>8</sup> Once bound to the membrane, they seem to make it permeable to ions and other cellular content, which causes great harm or even death to the cells. There are currently three different models to explain how antimicrobial peptides permeate bacterial cell membranes, as shown in Figure 5.



**Figure 5: An overview of AMP membrane permeability models.<sup>9</sup>**

Figure 5 (A) shows the antimicrobial peptides in solution not yet bound to the lipid bilayer bacterial membrane. The antimicrobial peptide is bound to the membrane as a cylindrical monomer in (B), and multiple peptides have self-assembled and bound to the membrane in (C). The three models of membrane permeability are shown in (D), (E), and (F). In the first model, (D), the peptides form a pore across the membrane. This is known as the “barrel-stave” model. The toroidal wormhole model shown in (E) appears very similar to (D), with a pore through the membrane, but closer examination shows negatively charged lipid head groups lining the pore that compensate for the positive charged peptides. The final model shown in (F) is known as the carpet model, where the membrane is simply disrupted by a high local concentration of peptides in a non-structured manner.

## 1.4 The Human Cathelicidin, LL37

Cathelicidins are a family of structurally diverse antimicrobial peptides that are located at the carboxyl terminus of a 15-18 kDa highly conserved cathepsin-L-inhibitor (cathelin)-like domain.<sup>10</sup> All cathelicidin family members are synthesized and stored in cells as two-domain proteins. These are split on demand to produce a cathelin protein and an antimicrobial peptide.<sup>11</sup> Cathelicidins have only a single representative in humans, an antimicrobial peptide called LL37. It derives its name from the first two residues at the N-terminus (**L**eucine, **L**eucine) and its length of amino acids (37). It is synthesized as an 18 kDa propeptide (hCAP18) consisting of a 13.5 kDa N-terminal cathelin-like domain and the 4.5 kDa active peptide, LL37.<sup>10</sup> A diagram of the structure of hCAP18 is shown in Figure 6.

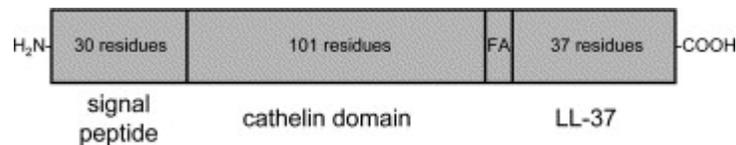


Figure 6: Diagram illustrating the structure of human cathelicidin – hCAP18.<sup>12</sup>

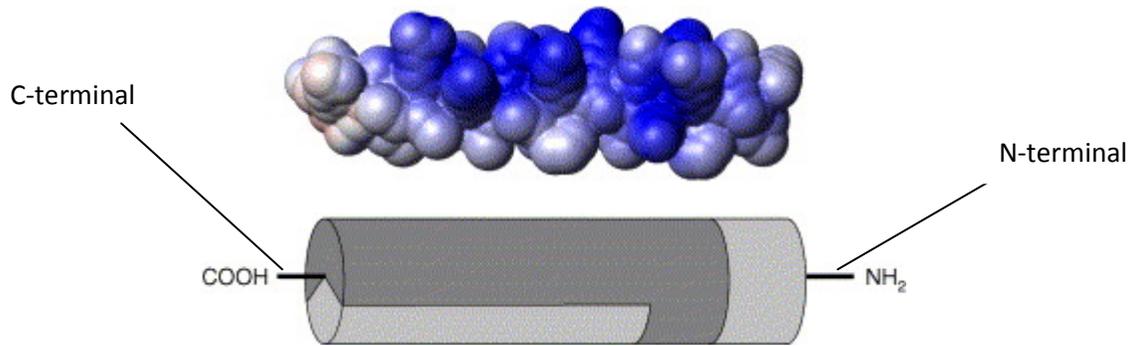
The mature active peptide was originally isolated from degranulated granulocytes.<sup>5</sup> LL37 showed moderate antibacterial activity in LB (Luria-Bertani) medium but a pronounced increase in antibacterial activity on E (a salt medium used for culturing *E. coli*) medium.<sup>13</sup> Since LL37 still retains broad-spectrum activity in high salt medium, this represents typical physiological conditions and could be advantageous for potential therapeutic applications. LL37 is a positively charged molecule with the charge

being conferred by the high content of basic amino acids, arginine and lysine. At physiological pH, LL37 has 16 out of its 37 residues charged, including two aspartic acid and three glutamic acid residues carrying negative charge, and six lysine and five arginine residues bearing positive charge. The resultant net charge at physiological pH is therefore positive (+6). LL37 contains no cysteine residues and is thus absent of disulfide bridges. This makes synthesizing it much easier and less expensive. The exact sequence is shown below in Figure 7.

LLGDF–FRKSK–EKIGK–EFKRI–VQRIK–DFLRN–LVPRT–ES

Figure 7: Primary structure of LL37

The secondary structure of LL37 is typically an  $\alpha$ -helix, with its specific structure being anion-, pH-, and concentration dependent.<sup>13</sup> It adopts an  $\alpha$ -helix structure in hydrophilic environments and a coiled structure in hydrophobic environments.<sup>14</sup> The amphipathic nature of LL37 enables it to bind bacteria with both hydrophobic and electrostatic interactions. Figure 8 shows an electrostatic surface plot of LL37 in an assumed perfect  $\alpha$ -helical conformation. You can clearly see the hydrophilic surface on the top (blue) and a hydrophobic surface on the bottom (white), which is a common feature of all antimicrobial peptides. In the bottom part of Figure 8 is a cartoon representation showing that the N-terminal region of LL37 shows a lack of amphipathic topology. Instead, this region consists of stretches of hydrophilic and hydrophobic residues that may be responsible for the complex signaling functions of LL37, rather than its antimicrobial properties.<sup>11</sup>



**Figure 8: Hypothetical amphipathic  $\alpha$ -helix structure of LL37.<sup>11</sup>**

Upon accumulation of a threshold concentration of LL37 on bacterial cell membranes, the membrane is altered through an unknown mechanism, resulting in the formation of ion channels. These aqueous pores typically lead to cell death via hypoosmotic lysis. The entry of the peptide into the cell membrane also leads to leakage of the cell cytoplasm and its contents into the extracellular space. The electron micrographs in Figure 9 show how a bacterium is affected by LL37. The bacterium dies if a threshold called “minimum inhibitory concentration” (MIC) is reached.<sup>15</sup> Substantial damage to the cell is still observed, even if LL37 is present at a lower concentration than the MIC.

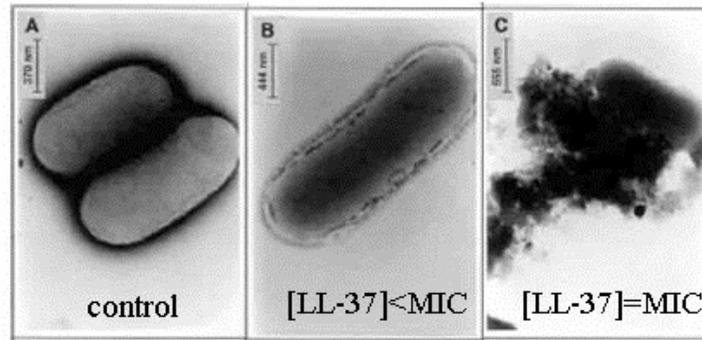


Figure 9: A series of electron micrographs of LL37 affected bacterium.<sup>15</sup>

*In vivo*, LL37 is bound by plasma proteins, thereby inhibiting its activity toward human cell membranes. Studies have demonstrated that truncating the peptide further reduces its cytotoxic activity towards eukaryotic cells, making it amenable as a possible therapeutic drug.<sup>16</sup> Research efforts are therefore geared towards developing truncated LL37 peptides that selectively kill microbes and avoid inflicting any damage to human cells.

LL37 is encoded by a gene located on chromosome three in humans and is expressed in the squamous epithelium of airways, mouth, and intestines.<sup>17</sup> This peptide is constitutively expressed in the spleen, liver, stomach, and intestines. In addition, various body secretions such as semen, wound fluid, saliva, and sweat contain LL37.<sup>18</sup>

Studies by Oren et al. have identified LL37 as having several properties setting it apart from other antimicrobial peptides.<sup>16</sup> Most native antimicrobial peptides are highly susceptible to enzymatic degradation in solution, unlike LL37, which oligomerizes in solution, thus protecting it from proteolytic degradation. The fact that LL37 is able to

oligomerize in solution is especially surprising given the fact that it is positively charged. Given that the N-terminal of LL37 is hydrophobic and contains 30% of the hydrophobic residues in the peptide, they may serve as a hydrophobic core of the oligomer. LL37 also has five negatively charged residues spread throughout the chain, which may form salt bridges with the positively charged residues, thus stabilizing the oligomer. The peptide also resists degradation when bound to both zwitterionic (mimicking mammalian membranes) and negatively charged membranes (mimicking bacterial membranes). Most other antimicrobial peptides are protected from degradation only when bound to negatively charged membranes. LL37 is also able to bind and permeate both zwitterionic membranes and negatively charged membranes, while other native antimicrobial peptides only permeate negatively charged membranes efficiently. Last, LL37 is self-associated when bound to zwitterionic phospholipid vesicles but dissociates into monomers upon binding negatively charged vesicles. Antimicrobial peptides typically are monomeric when bound to zwitterionic and negatively charged membranes.

Aside from its important antimicrobial properties, LL37 also has been reported to serve other roles such as a chemo-attractant of mast cells, neutrophils, monocytes, NK cells, and T-cells to the sites of infection. This is achieved through interactions with the formyl peptide receptor like-1 that also promotes angiogenesis.<sup>19,20</sup> Also, elevated levels of LL37 are seen in areas of inflammation, such as an infected tissue.<sup>21</sup> LL37 induces mast cells to degranulate and releases pro-inflammatory mediators such as histamines, which promote infiltration of inflammatory cells.<sup>22</sup> LL37/hCAP18 was found to bind lipopolysaccharide, inhibit its multiple biological activities in vitro, and reduce its

lethality in murine models of endotoxaemia.<sup>23</sup> LL37 has been associated with tissue repair and wound healing.<sup>24</sup>

## 2. Research Goals and Objectives

This project involves the synthesis, purification, and analysis of three peptides based on LL37, a human cathelin-associated antimicrobial peptide. LL37 is believed to play an important role in the first line of defense against local infection and systemic invasion of pathogens at sites of inflammation and wounds.<sup>16</sup> Understanding its mode of action may assist in the development of new antimicrobial agents that mimic the human immune system rather than conventional antibiotics.

The emergence of antibiotic-resistant strains of several different bacteria has necessitated the development of new ways to fight resistant bacterial infections. LL37 kills bacteria by disrupting their membranes through non-specific peptide-lipid interactions, making it more difficult for bacteria to develop resistance against than some current antibacterial agents. Although the general mechanism of action is known (permeabilization of the cell membrane), the exact mechanism is still under debate. LL37's specific membrane selectivity targets bacteria while leaving other cells intact. Additionally, LL37 has been shown to have roles in wound healing, attraction of white blood cells, and inflammation regulation. LL37 has been examined extensively, and several variations of its sequence have also been studied due to its possible pharmaceutical properties. Examining its analogs could provide more potent potential drugs or information on its function. My goal was to synthesize three of these variations, two truncated forms of LL37 (SK29 and FF33) and an N-terminally extended LL37

(FALL39) for comparative studies. The amino acid sequences of these peptides are shown in Figure 10.

	1	5	10	15	20	25	30	35																															
LL37	L	L	G	D	F	F	R	K	S	K	E	K	I	G	K	E	F	K	R	I	V	Q	R	I	K	D	F	L	R	N	L	V	P	R	T	E	S		
FF33					F	F	R	K	S	K	E	K	I	G	K	E	F	K	R	I	V	Q	R	I	K	D	F	L	R	N	L	V	P	R	T	E	S		
SK29									S	K	E	K	I	G	K	E	F	K	R	I	V	Q	R	I	K	D	F	L	R	N	L	V	P	R	T	E	S		
FALL39	F	A	L	L	G	D	F	F	R	K	S	K	E	K	I	G	K	E	F	K	R	I	V	Q	R	I	K	D	F	L	R	N	L	V	P	R	T	E	S

**Figure 10: Amino acid sequences of proposed antimicrobial peptides.**

These peptides were prepared using standard Fmoc solid phase peptide synthesis on a Symphony automated peptide synthesizer from Protein Technologies, Inc. and purified by reverse phase HPLC. Major challenges included modification of the synthesis in the event that the correct peptide could not be isolated and obtained with a final purity >90%.

### 3. Peptide Synthesis: A Brief History

The current most common method of peptide synthesis uses solid phase techniques initially developed by R. B. Merrifield and published in the *Journal of the American Chemical Society* in 1963.<sup>25</sup> Merrifield used a chloromethylated polystyrene polymer as a support for repeated step-wise carbodiimide coupling reactions, which lengthened the peptide from its amino-end. His initial synthesis yielded a four-amino acid sequence with many by-products from incomplete coupling reactions, but his idea would eventually become the industry standard that is still the preferred method today.

Merrifield's solid phase technique for peptide synthesis ignored some of the basic principles of organic synthesis at the time. His method did not isolate and characterize the product at each step, instead waiting until the completion of the synthesis to cleave the peptide from the solid support and purify it to the desired level. This resulted in a method that was fast, simple, and efficient. He later improved upon his original conditions through the use of N-terminal t-butoxycarbonyl protecting groups and benzyl derivatives for side chain and carboxy terminal protection. He was able to synthesize and purify the nonapeptide sequence of bradykinin in a mere nine days, with an astounding yield for the time at 68%.<sup>26</sup>

Although his method was certainly groundbreaking, it was not without faults. The temporary N-terminal protecting groups, the more permanent side chain protecting groups, and the peptide-resin linkage were all acid labile. This meant that some of the side chain protecting groups would be removed at each step and resulted in undesired

products at the conclusion of the synthesis. In addition, small amounts of the peptide would be cleaved from the resin and washed away at each step, lowering the overall yield. These effects were compounded throughout the synthesis, making the possibility of synthesizing longer peptides quite difficult. In addition, the technique required the use of strong acids that are very hazardous and dangerous to handle. Trifluoroacetic acid (TFA) was typically used for the N-terminal deprotections, and hydrogen fluoride (HF) was used for the peptide cleavage and removal of side chain protecting groups.

In 1972, Carpino suggested the use of a base labile N-terminal protecting group, 9-fluorenylmethoxycarbonyl (Fmoc).<sup>27</sup> Although its adoption was initially slow, Fmoc solid phase peptide synthesis quickly took off with the arrival of commercially available Fmoc-amino acids. Now the resin-bound peptide could be deprotected prior to each coupling using a 20% solution of piperidine in dimethyl formamide (DMF). The acid labile peptide-resin linkage as well as the amino acid side chain protecting groups would remain intact as the chain was lengthened.<sup>28,29</sup> Once the chain was completed, TFA could be used to cleave the peptide from the resin and remove the amino acid side chain protecting groups, thus avoiding HF altogether. The general scheme of solid phase peptide synthesis is shown below in Figure 11.

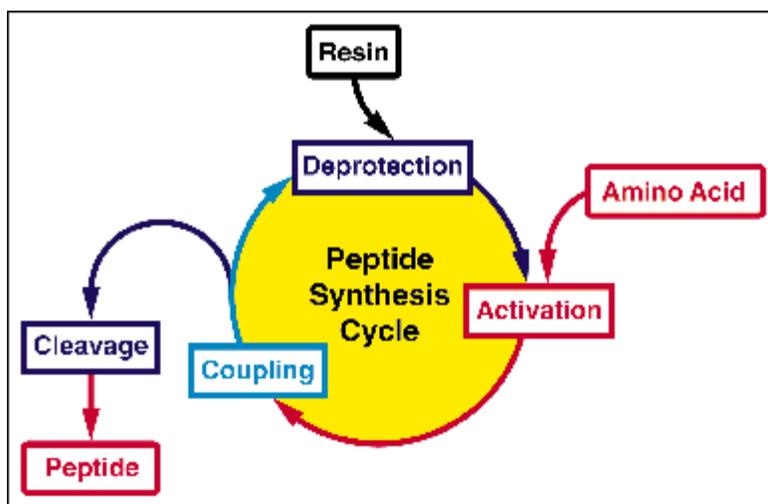


Figure 11: The general scheme of SPPS.<sup>30</sup>

Another advantage was that fluorine derivatives have strong ultraviolet absorption that could be monitored for the completion of each deprotection step.<sup>31</sup> This led to the development of fully automated peptide synthesizers, which could monitor the progression of the synthesis from one step to the next and even make decisions based on the ultraviolet absorption data. Although Fmoc solid phase peptide synthesis is now the preferred method, many complex reactions are still performed using the original Boc chemistry. The original Boc method is performed when unnatural amino acids or other derivatives that are base sensitive are incorporated into the sequence.

## 4. Synthesis and Analysis of Peptides

### 4.1 Fmoc Solid Phase Peptide Synthesis

The first step in solid-phase peptide synthesis is to determine which resin to build the peptide on. Resin selection greatly affects the outcome of the synthesis, and there are many options commercially available.<sup>32</sup> With relatively long, difficult sequences being synthesized, there is a need to consider the swelling properties of the resin as well as its ability to deter aggregation. Typically, SPPS is carried out on cross-linked polystyrene, and excellent results have previously been obtained using polystyrene (PS) resins that employed polyethylene glycol linkers (PEG-PS). PEG-PS resins are typically quite expensive. A literature search showed that a cheaper alternative was developed by Peptides International: Cross-Linked Ethoxylate Acrylate (CLEAR) resins. These resins offer the benefits of polyethylene glycol linkers for disruption of aggregation and also have better swelling properties than polystyrene resins in a wider variety of solvents.<sup>33</sup> In addition, they were available with the first residue of all the proposed sequences, serine, preloaded. Ultimately, the shortest sequence of the LL37 related peptides, SK29, was attempted on the Fmoc-Ser(tBu)-CLEAR-Acid Resin (Product Code CFS-1243-PI), and the resin selection for the remaining, longer peptides was based on the analytical results of the SK29 synthesis.

## 4.2 Synthesis of SK29

100  $\mu$ mol of Fmoc-Ser(tBu)-CLEAR-Acid Resin was weighed to a disposable fritted reaction vessel and placed on the Symphony peptide synthesizer. Each amino acid reservoir was filled with a 100 mM concentration of amino acid in DMF. For a 100  $\mu$ mol synthesis, this will represent a 2.5x molar excess with a 2 volume (2.5 ml) delivery. Some of the amino acids require side chain protecting groups, and the ones used are listed below in Figure 12.

Fmoc-Asp(OtBu)-OH  
Fmoc-Asn(Trt)-OH  
Fmoc-Gln(Trt)-OH  
Fmoc-Glu(OtBu)-OH  
Fmoc-Lys(Boc)-OH  
Fmoc-Ser(tBu)-OH  
Fmoc-Thr(tBu)-OH  
Fmoc-Arg(Pbf)-OH

**Figure 12: Amino acid side chain protecting groups.**

In addition to the 20 pressurized amino acid reservoirs on the front of the instrument, the Symphony has 6 available pressurized solvent bottles in the lower cabinet. Their contents are shown in Figure 13.

Solvent 1 bottle: DMF solvent (wash)  
Solvent 2 bottle: 20% piperidine in DMF (deprotect)  
Solvent 3 bottle: 100 mM O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU) and N-Hydroxybenzotriazole (HOBT)/400 mM N-Methylmorpholine (NMM) in DMF  
Solvent 4 bottle: empty  
Solvent 5 bottle: Dichloromethane (DCM)  
Solvent 6 bottle: Cleavage cocktail (Reagent B)

**Figure 13: Symphony reagent list.**

The Symphony works by following a series of programs detailing what to deliver to the reaction vessel and also various other functions such as mixing and draining. All of the bottles on the instrument are pressurized with nitrogen, and the reaction vessel is mixed by bubbling nitrogen from the bottom inlet of the reaction vessel. Solvents are drained from the bottom inlet of the reaction vessel by vacuum. The program for the first amino acid added is slightly different from the remaining residues to be coupled because the resin requires swelling and there is only a single coupling. Table 1 shows the program used for the coupling of the first amino acid residue.

<b>Step</b>	<b>Solvent Bottle</b>	<b>Volumes</b>	<b>Mix Time</b>	<b>Drain</b>	<b>Rep</b>	<b>Comments</b>
1	1	2	00:30	ON	1	Resin wash
2	1	2	15:00	ON	1	Resin swelling
3	1	2	00:30	ON	2	Resin wash
4	2	2	10:00	ON	1	1x 10 min. deprotection
5	1	2	00:30	ON	3	3x wash with DMF
6	AA	2	00:00	OFF	1	Addition of amino acid
7	3	2	45:00	ON	1	1x 45 min. coupling
8	1	2	00:30	ON	3	3x wash with DMF

**Table 1: The program for the coupling of the first residue.**

Once the first amino acid residue was coupled, the remaining residues were all added by double coupling, using the program provided in Table 2.

<b>Step</b>	<b>Solvent Bottle</b>	<b>Volumes</b>	<b>Mix Time</b>	<b>Drain</b>	<b>Rep</b>	<b>Comments</b>
1	1	2	00:30	ON	3	3x wash with DMF
2	2	2	05:00	ON	1	1x 5 min. deprotection
3	2	2	10:00	ON	1	1x 10 min. deprotection
4	1	2	00:30	ON	6	6x wash with DMF
5	AA	2	00:00	OFF	1	Addition of amino acid
6	3	2	45:00	ON	1	1x 45 min. coupling
7	AA	2	00:00	OFF	1	Addition of amino acid
8	3	2	30:00	ON	1	1x 30 min. coupling
9	1	2	00:30	ON	3	3x wash with DMF

**Table 2: Standard program for double coupling.**

### 4.3 Peptide Cleavage

The Protein Technologies Symphony peptide synthesizer allows for automated cleavage of the peptide from the resin. Once the cleavage is completed, the instrument delivers the product from the reaction vessel to a 10 mL centrifuge tube in a vented compartment. There is a separate pressurized bottle for the cleavage cocktail of your choice. For the cleavage of the LL37 related peptides, fresh Reagent B was prepared. Reagent B consists of 88% TFA/5% phenol/5% water/2% triisopropylsilane.<sup>34</sup> Reagent B can result in the alkylation of unprotected tryptophan residues, but since none of the sequences contain tryptophan, this was not an issue.

Following the synthesis, each peptide was bound to the resin within the reaction vessel, with N-terminal Fmoc protection still intact. The resin was then deprotected once

for five minutes followed by a second, ten-minute deprotection. It was then washed six times with DMF, followed by three washes of DCM. It was then dried with a stream of nitrogen for 30 minutes. At this point, 5 mL of reagent B was delivered to the reaction vessels and allowed to mix using nitrogen bubbling for two hours. The cleavage cocktail was then transferred to the collection tube. An additional 2.5 mL of reagent B was delivered to the reaction vessel and bubbled with nitrogen for five minutes as a wash step. This additional cleavage cocktail was then also transferred to the collection tube. The collection tube was then removed from the Symphony and added dropwise to 40 mL of cold (-20°C) methyl tert-butyl ether (MTBE) in a 50 mL centrifuge tube. The 10 mL cleavage collection tube was then rinsed with excess cold MTBE and transferred to the 50 mL centrifuge tube. The ether solution was then stored overnight in a freezer (-20°C). The following day, the ether solution was centrifuged, resulting in the precipitated peptide collecting in the bottom of the 50 mL centrifuge tube. The ether solution was poured off, and fresh cold ether was added to remove any residual scavengers. The pellet was disrupted by vigorous shaking, and the ether solution was centrifuged again. This process was repeated for a total of three washes. After the final wash, the ether was poured off, and the peptide precipitate was allowed to dry in a hood for two hours. The peptides were then dissolved in 25 mL of 50% acetonitrile (ACN)/50% water and frozen in liquid nitrogen for overnight lyophilization. Following lyophilization, the crude material yielded 200 mg of fluffy white powdered peptide.

#### 4.4 Analysis and Purification Methods

All three LL37 related peptides were HPLC purified with the goal of achieving at least 90% purity. The instrumental system information for both the analytical and preparative HPLC is presented in Table 3. Both HPLC systems utilized a two-solvent gradient, with solvent A being composed of 99.9% water with 0.1% TFA and solvent B composed of 80% ACN, 19.9% water, and 0.1% TFA.

Analytical HPLC	Beckman System Gold HPLC
	32-Karat software ver. 5.0
	Model 125 solvent delivery module
	Model 166 UV-VIS Detector
	Model 508 Autosampler
Analytical Column	Macherey-Nagel Nucleosil 100-5 C <sub>18</sub>
	5µm particle size
	4.6 x 250 mm
	100 Å pore size
Preparative HPLC	Beckman System Gold HPLC
	32-Karat software ver. 5.0
	Model 125 solvent delivery module
	Model 166 UV-VIS Detector
	Beckman SC 100 Fraction Collector
Preparative Column	Macherey-Nagel Nucleosil 100-5 C <sub>18</sub>
	5µm particle size
	21 x 250 mm
	100 Å pore size

**Table 3: Liquid chromatography system information.**

In addition, the peptides were verified to have the correct mass by mass spectrometry. Mass spectrometry was performed using a Thermo Finnigan LCQ Duo mass spectrometer with an electrospray source and Xcaliber software.

#### 4.5 Analysis and Purification of SK29

Initially, the crude product of SK29 was analyzed by mass spectrometry to verify that it had the correct average molecular weight of 3516.15 amu. The sample was dissolved in 50% acetonitrile/50% water with 1% formic acid to promote protonation. The spectrum is shown below in Figure 14.

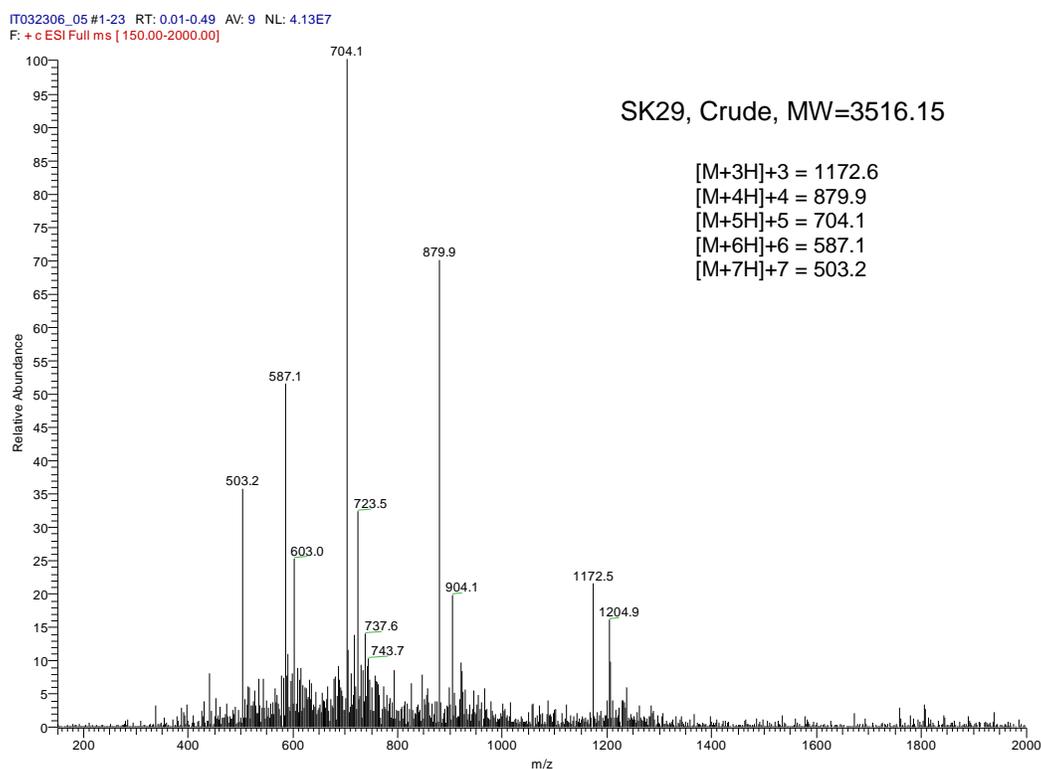


Figure 14: Mass spectrum of crude SK29.

The mass spectrum shows the mass to charge ratio ( $m/z$ ) from 150 to 2000 of all the ionized molecules present in the sample. The most abundant ion in the spectrum is seen at a mass to charge ratio of 704.1. This corresponds to SK29 ionized to +5 (MW = 3516.15 + 5H<sup>+</sup> = 3521.15). The mass to charge ratio is thus 3521.15/5 = 704.2. Four other relatively abundant ions attributed to SK29 are seen and listed in Figure 14.

Following confirmation that the crude peptide had the correct molecular weight, it was analyzed by HPLC to determine the crude purity and identify a possible gradient to use for its purification. A sample of the peptide was dissolved in 0.1% TFA in water and analyzed using a gradient of 25-85% solvent B in 25 minutes. The flow rate was set to 1.0 mL/min. and the detector monitored at the absorbance of the peptide bond at a wavelength of 215 nm.<sup>35</sup> The peptide was found to be 45% pure by the percent area of the main peak at retention time (RT)=17.433 minutes, as seen in Figure 15.

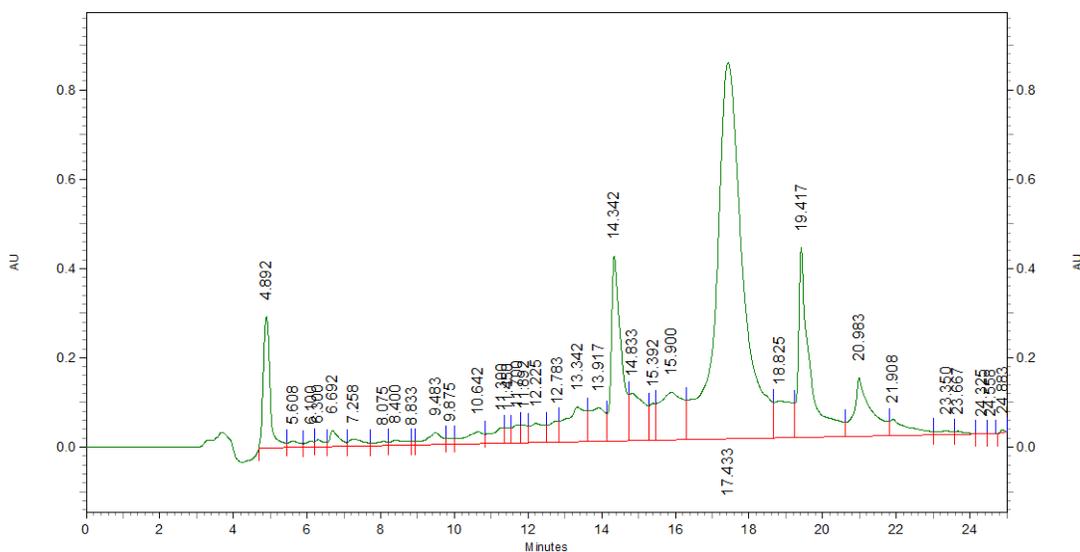
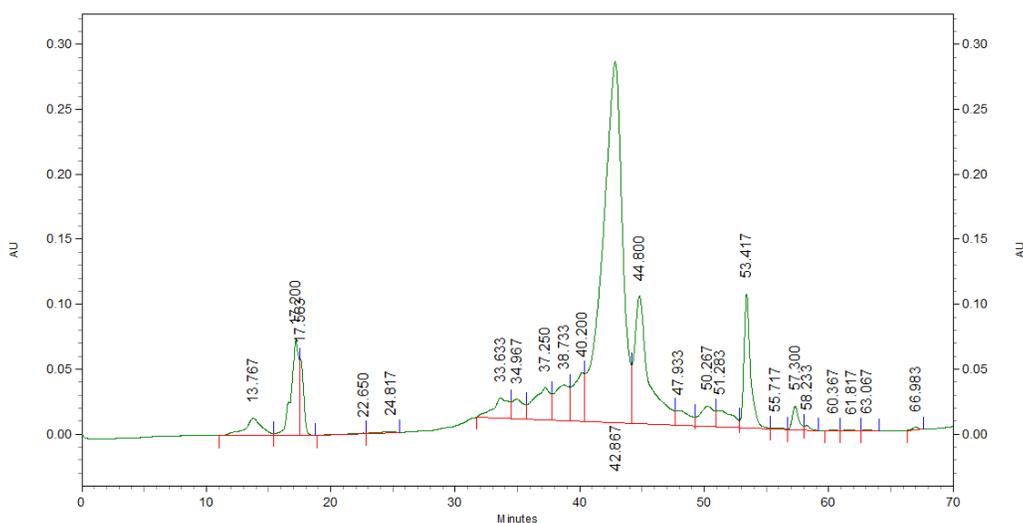


Figure 15: Crude HPLC chromatogram of SK29.

For the actual HPLC purification of SK29, the sample was dissolved in 20 mL of 20% ACN/80% water. Since the preparative HPLC had a sample loop of 5 mL, complete purification of the sample took four runs, with approximately 50 mg being purified in each run. The gradient for the analysis was 25-85% solvent B in 60 minutes with a flow rate of 5.0 mL/min. The detector monitored the absorbance at 215 nm. Fractions were collected every 30 seconds for the entire 60-minute run. A representative chromatogram of one of the four runs is shown below in Figure 16.



**Figure 16. Preparative HPLC chromatogram of SK29.**

Following each preparative run, fraction tubes were selected and run on the analytical HPLC to determine whether the desired 90% purity level was achieved. Once all the pure tubes were determined, they were combined and lyophilized overnight. The final product was analyzed by mass spectrometry and analytical HPLC to provide the

final results, which are shown below in Figures 17 and 18. The final peptide was 98.93% pure with a yield of 56 mg.

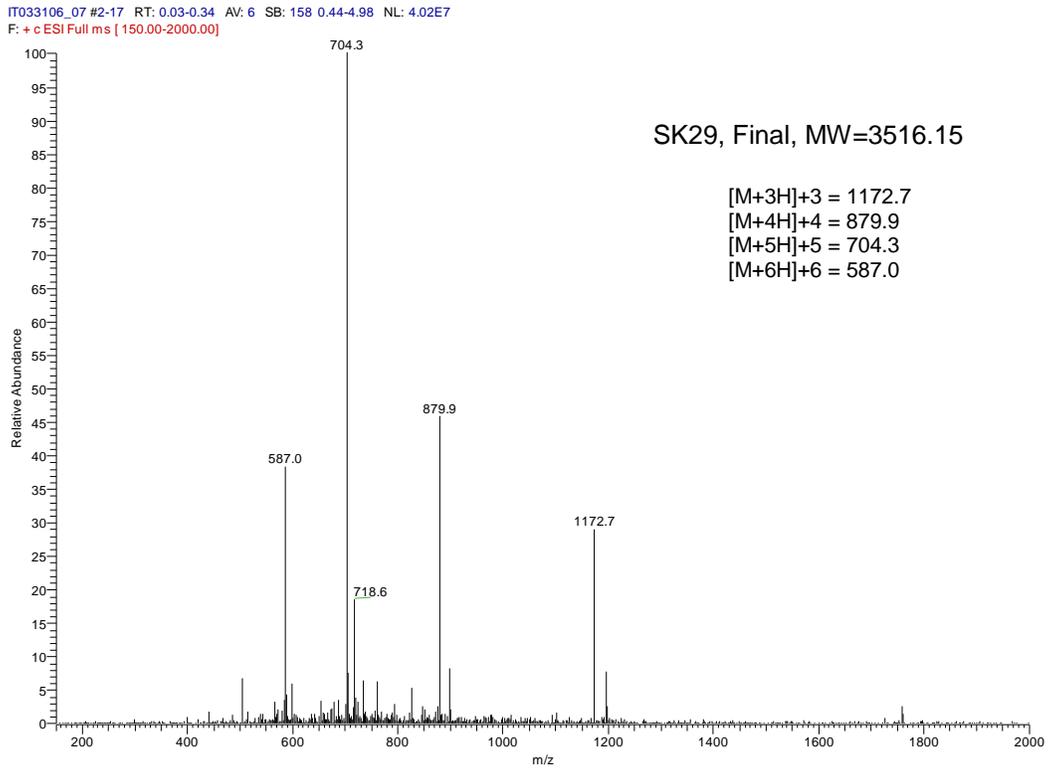
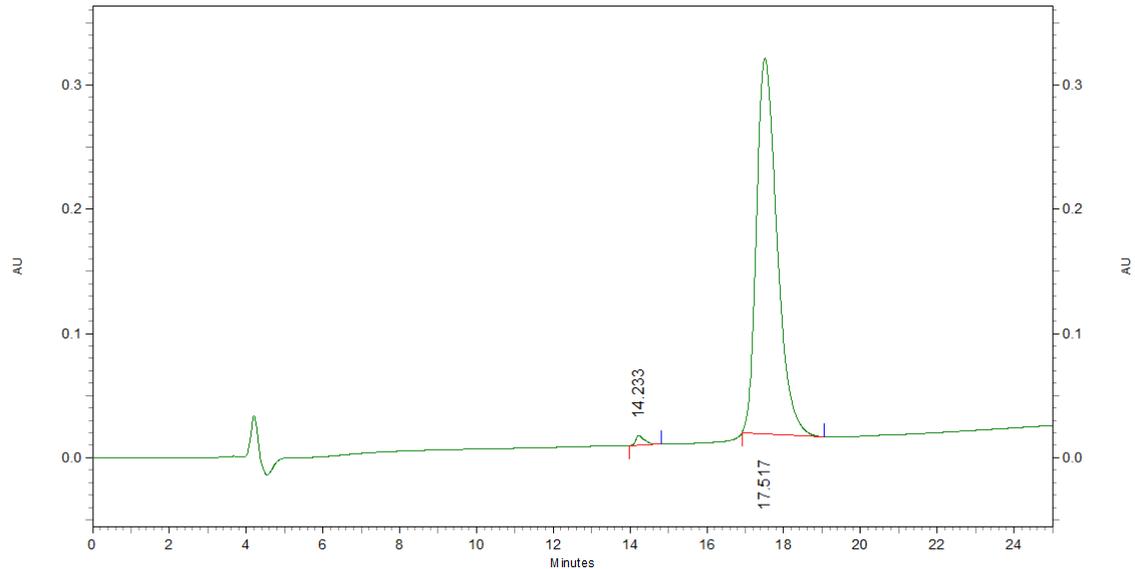


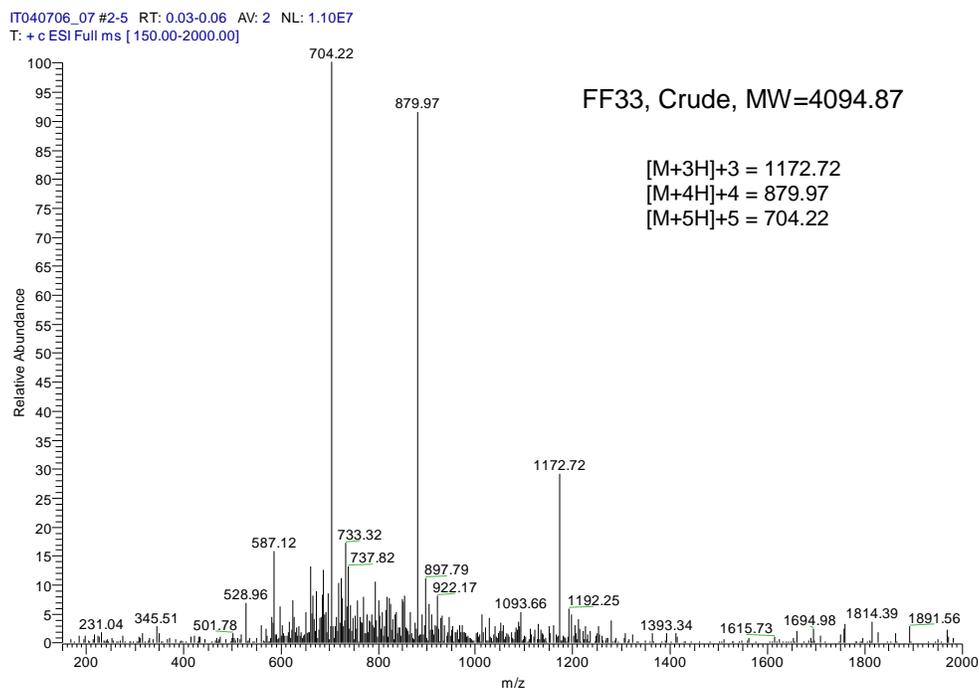
Figure 17: Mass spectrum of purified SK29.



**Figure 18: Final HPLC chromatogram of purified SK29.**

#### 4.6 Synthesis and Purification of FF33

The synthesis of FF33 was set up in the exact same manner as SK29, except with the addition of four amino acids at the N-terminus. Following lyophilization of the crude peptide, the yield was 206 mg. When the crude product was analyzed by mass spectrometry, the expected molecular weight was not found. The ionization pattern resembled the spectrum of SK29 (Figure 14) and is shown in Figure 19.



**Figure 19: Mass spectrum of crude FF33.**

Although the peptide could have been sequenced in order to confirm the incomplete coupling of the final four residues, the mass spectrum was conclusive enough. The poor coupling was most likely due to some aggregation or formation of secondary structure as the synthetic chain was lengthened past 29 residues. In fact, it has been shown that aggregation can occur beginning with the fifth residue coupled.<sup>36</sup> Since the synthesis and purification of SK29 were successful, the coupling of the final four residues of FF33 appeared to be problematic. Each residue was already double coupled for an extended time period, so extending the coupling times or adding additional couplings was ruled out. The peptide had already been cleaved from the resin, so the synthesis would have to be started over from scratch, and making modifications to the second synthesis seemed to offer the best opportunity for success.

The insertion of a proline or N-alkyl amino acid into the peptide sequence has been shown to reduce the formation of secondary structures and diminish aggregation.<sup>37</sup> Unfortunately, the sequence could not be modified, but two approaches that exploit this principle while still maintaining the desired sequence were found. The first method involves the use of 2-hydroxy-4-methoxybenzyl (Hmb) protecting groups.<sup>38,39</sup> Incorporating Hmb protected amino acids typically postpones the onset of aggregation for as many as six residues, and the Hmb group is effectively removed by standard cleavage procedures.<sup>40,41</sup> There are also several Hmb protected amino acids available commercially for Fmoc SPPS; however, they are very expensive.

The second method to disrupt secondary structure formation and reduce aggregation is the use of pseudoproline dipeptides in the sequence. This method, developed by Mutter, involves converting a serine, threonine, or cysteine residue into an oxazolidine dipeptide, with the serine, threonine, or cysteine on the C-terminus.<sup>42</sup> Many different configurations are commercially available with the added benefit of adding two residues in one coupling reaction. The oxazolidine is also removed by TFA, allowing for cleavage of the peptide using typical reagents.<sup>43</sup> The pseudoproline residues are less expensive but more restrictive than Hmb protected amino acids because they require either a serine, threonine, or cysteine in the sequence. Since the N-terminal amino acid of SK29 is a serine and the next residue in FF33 is a lysine, it was ultimately decided to incorporate Fmoc-Lys(Boc)-Ser(ψMe, Mepro)-OH from EMD Biosciences into FF33 and determine if that would enable the completion of the synthesis.

A second synthesis of FF33 was performed (FF33B), using the exact same conditions as the first synthesis, but substituting Fmoc-Lys(Boc)-Ser(psiMe,Mepro)-OH into the sequence prior to the last three amino acids being coupled. The pseudoproline dipeptide simply replaced the Lys-Ser in the sequence and was added with the same double coupling program as the other amino acids. Following lyophilization of the crude peptide, the yield was 230 mg. Analysis of the crude product by mass spectrometry identified the expected molecular weight of FF33, in addition to a byproduct of incomplete cleavage as shown in Figure 20.

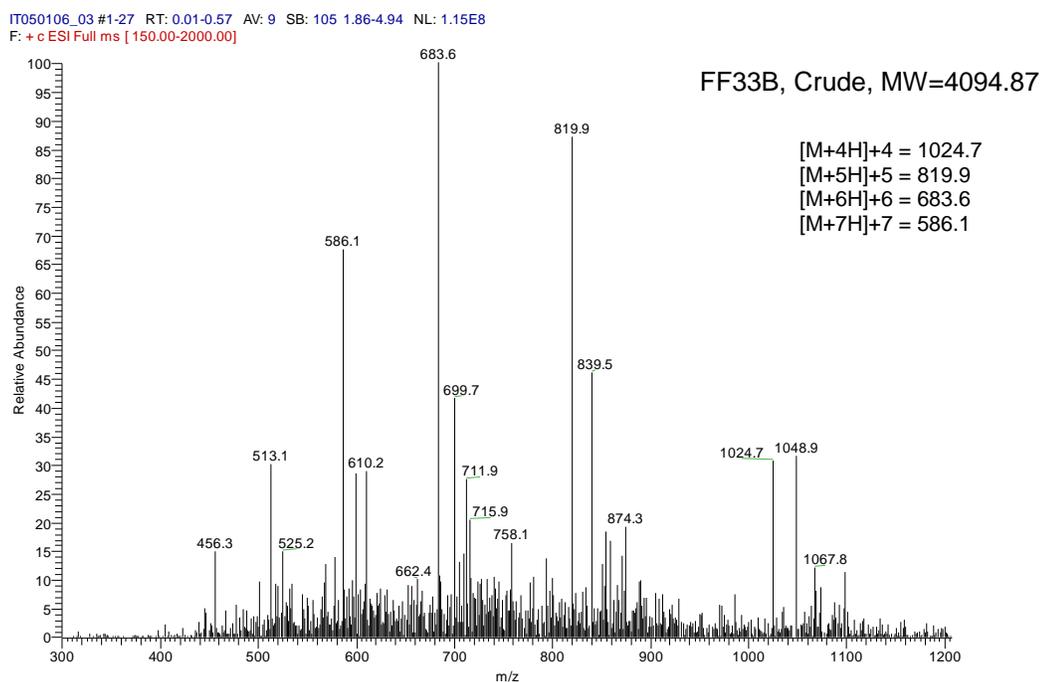
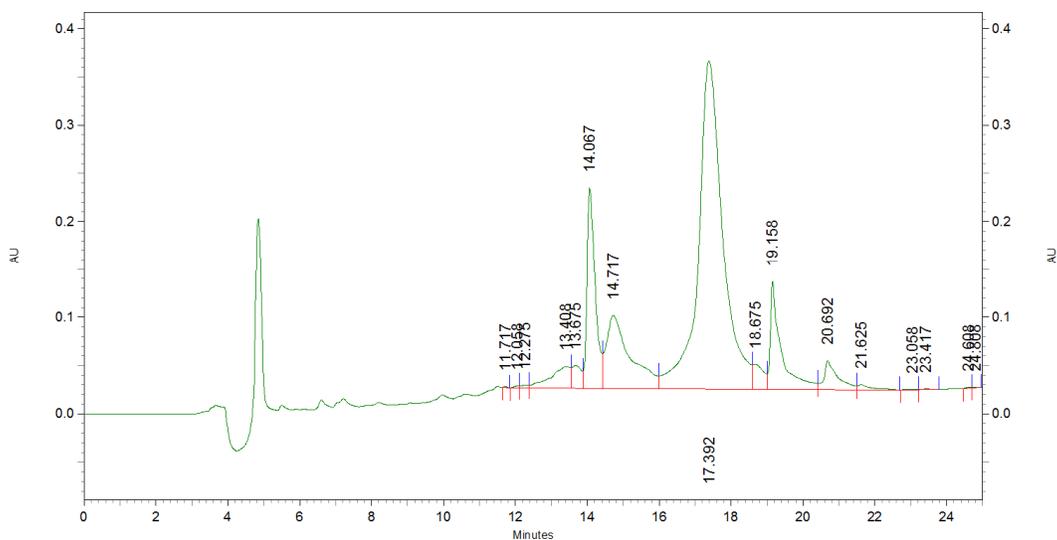


Figure 20: Mass spectrum of crude FF33B.

The crude peptide was then analyzed by HPLC to determine the purity and to help develop a method for purification. A sample of the peptide was dissolved in 0.1% TFA in water and analyzed using a gradient of 25-85% solvent B in 25 minutes. The flow rate

was set to 1.0 mL/min. and the detector monitored absorbance at 215 nm. The peptide was 60% pure by percent area of the main peak at RT=17.392 minutes. The chromatogram is illustrated in Figure 21.



**Figure 21: Crude HPLC chromatogram of FF33B.**

In order to ensure that the main peak was actually the desired peptide, the peak was collected and the solution was analyzed by mass spectrometry. The spectrum is shown below in Figure 22. Five mass to charge ratios attributable to FF33 are identified in the spectrum, and it is almost completely devoid of impurities.

IT050206\_04 #2-25 RT: 0.03-0.55 AV: 10 SB: 122 1.38-4.86 NL: 4.42E7  
F: + c ESI Full ms [150.00-2000.00]

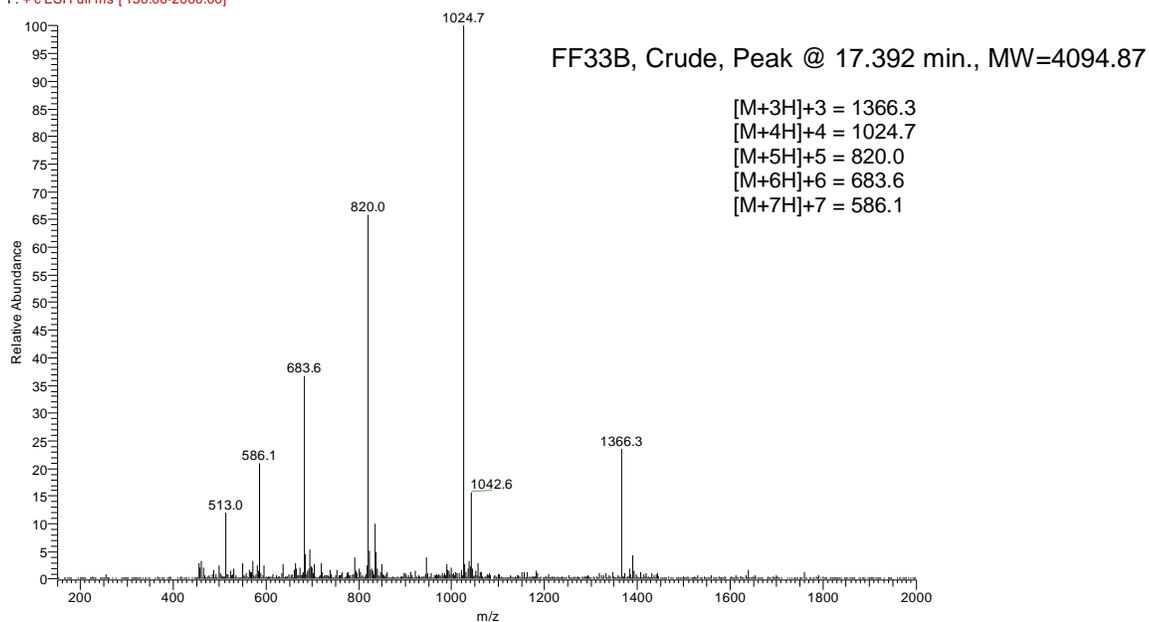


Figure 22: Mass spectrum of peak at RT=17.392 minutes in crude FF33B.

FF33B was then dissolved in 25 mL of 25% ACN/75% water and injected over the course of five runs on the same system as the SK29 purification. This accounted for approximately 45 mg per purification. A representative chromatogram of one of the five runs is shown in Figure 23.

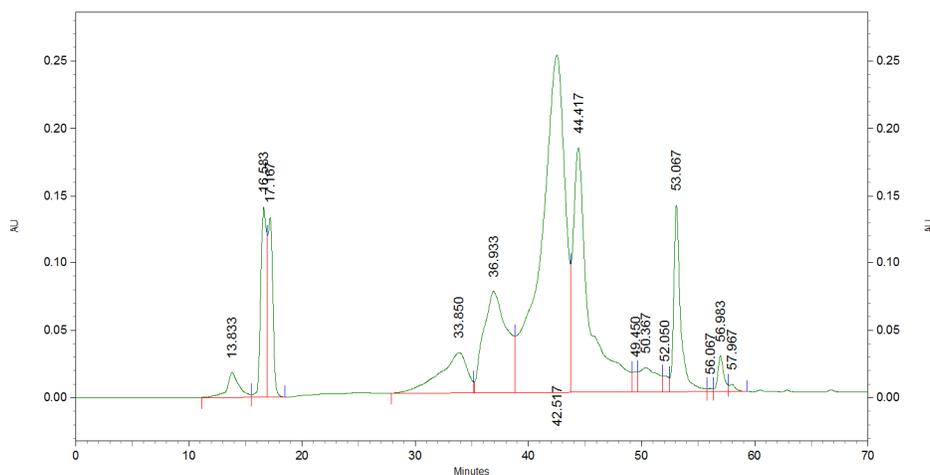


Figure 23: Preparative HPLC chromatogram of FF33B.

Following each preparative run, fraction tubes were selected and run on the analytical HPLC to determine whether the desired 90% purity level was achieved. Once all the pure tubes were determined, they were combined and lyophilized overnight. The final product was then analyzed by mass spectrometry and analytical HPLC to provide the final results, which can be seen below in Figures 24 and 25. The final peptide was 98.67% pure with a yield of 57 mg.

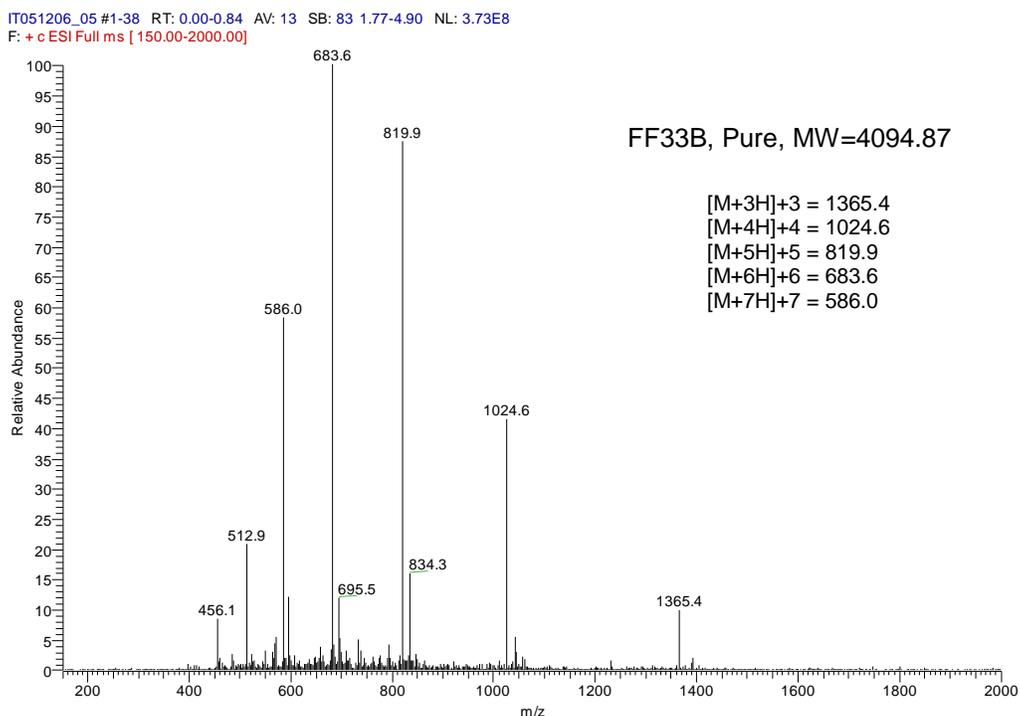
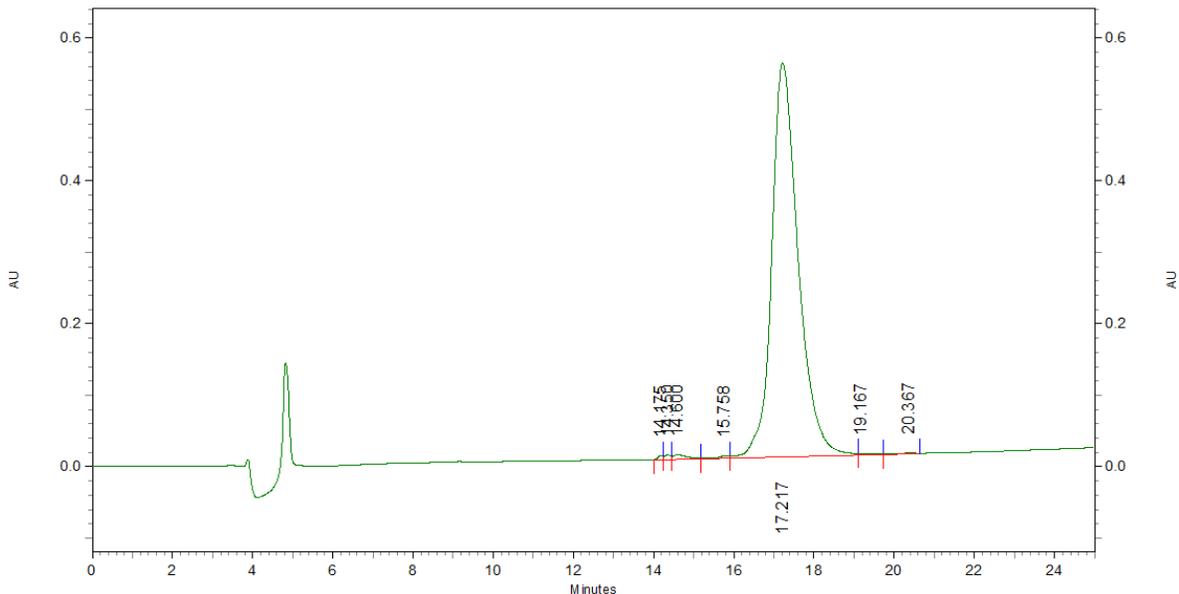


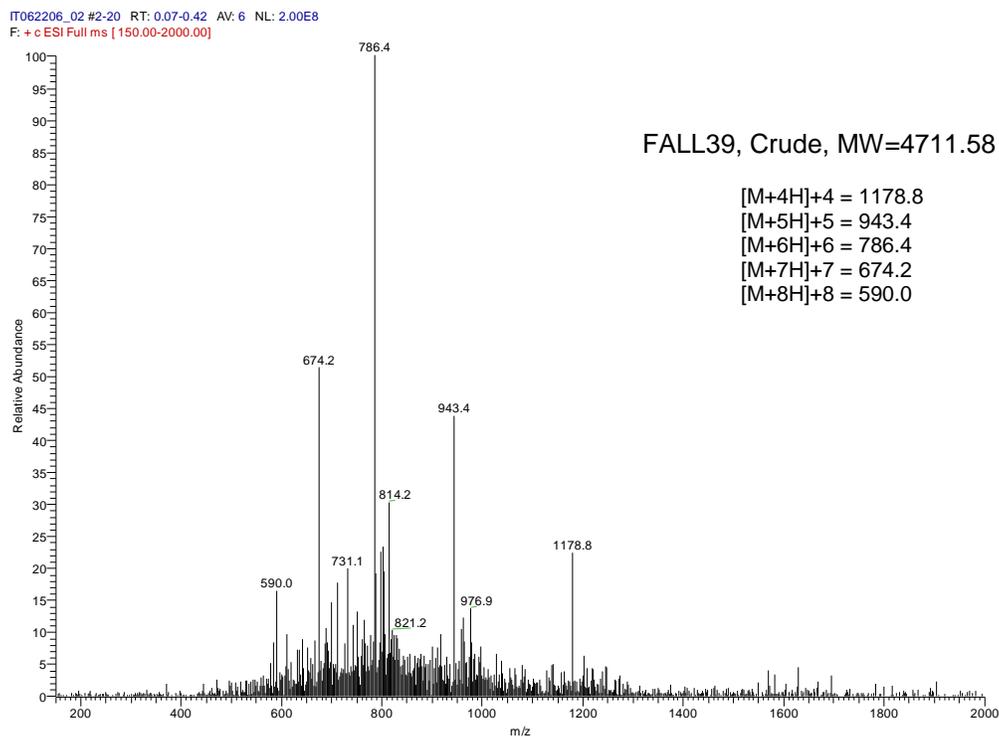
Figure 24: Mass spectrum of purified FF33B.



**Figure 25: Final HPLC chromatogram of purified FF33B.**

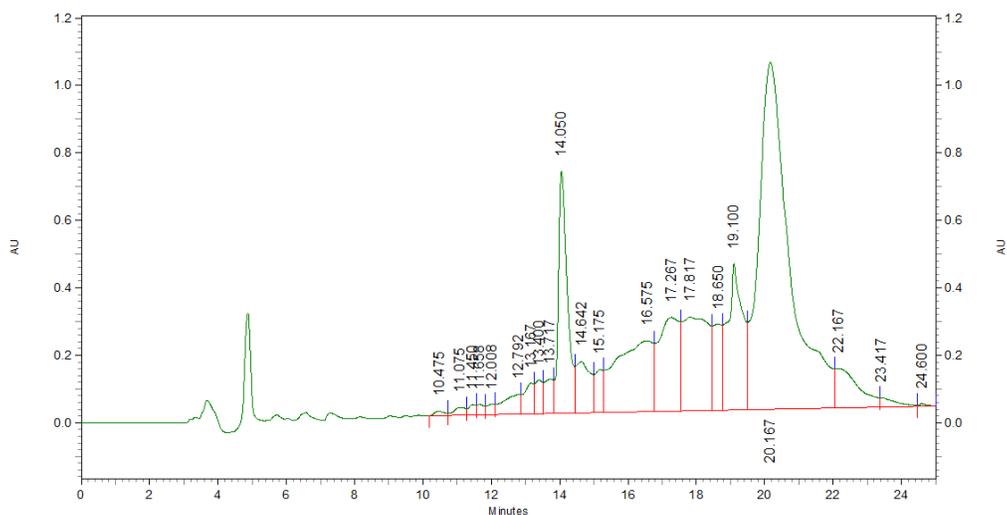
#### 4.7 Synthesis and Purification of FALL39

Following the successful synthesis of FF33B, it seemed feasible that FALL39 could be successfully synthesized in a similar manner. The synthesis was set up in the exact same manner as FF33B, except with the addition of six amino acids at the N-terminus. Once again, the Lys-Ser pseudoproline residue was incorporated into the peptide, but there was still some concern about aggregation due to the additional residues at the N-terminus. Following lyophilization of the crude peptide, the yield was only 170 mg. Despite the lower yield, the crude peptide showed the correct mass, and the spectrum is shown in Figure 26.



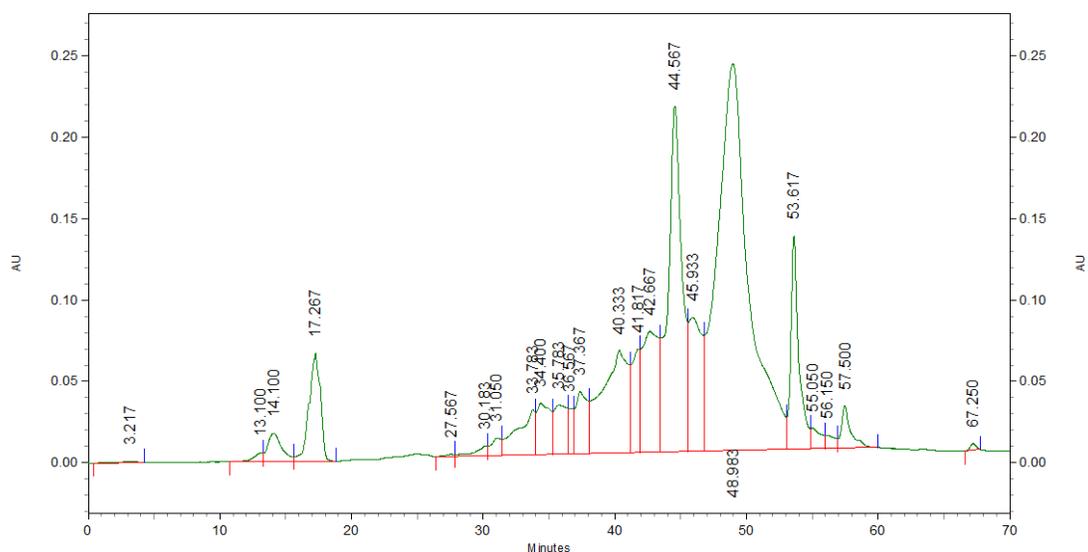
**Figure 26: Mass spectrum of crude FALL39.**

A small sample of the peptide was dissolved in 0.1% TFA and injected to determine its crude purity, which was only 40.38% by percent area of the main peak seen at RT=20.167 minutes. The chromatogram is shown below in Figure 27.



**Figure 27: Crude HPLC chromatogram of FALL39.**

FALL39 was then dissolved in 15 mL of 25% ACN/75% water and injected over the course of three runs on the same system as SK29 and FF33B. This accounted for approximately 55 mg per purification. A representative chromatogram of one of the three runs is shown below in Figure 28.



**Figure 28: Preparative HPLC chromatogram of FALL39.**

Following each preparative run, fraction tubes were selected and run on the analytical HPLC to determine whether the desired 90% purity level was achieved. Once all the pure tubes were determined, they were combined and lyophilized overnight. The final product was then analyzed by mass spectrometry and analytical HPLC to provide the final results, which can be seen below in Figures 29 and 30. The final peptide was 99.26% pure, as seen by the predominantly single HPLC peak and less complex mass spectrum, with a yield of 46 mg.

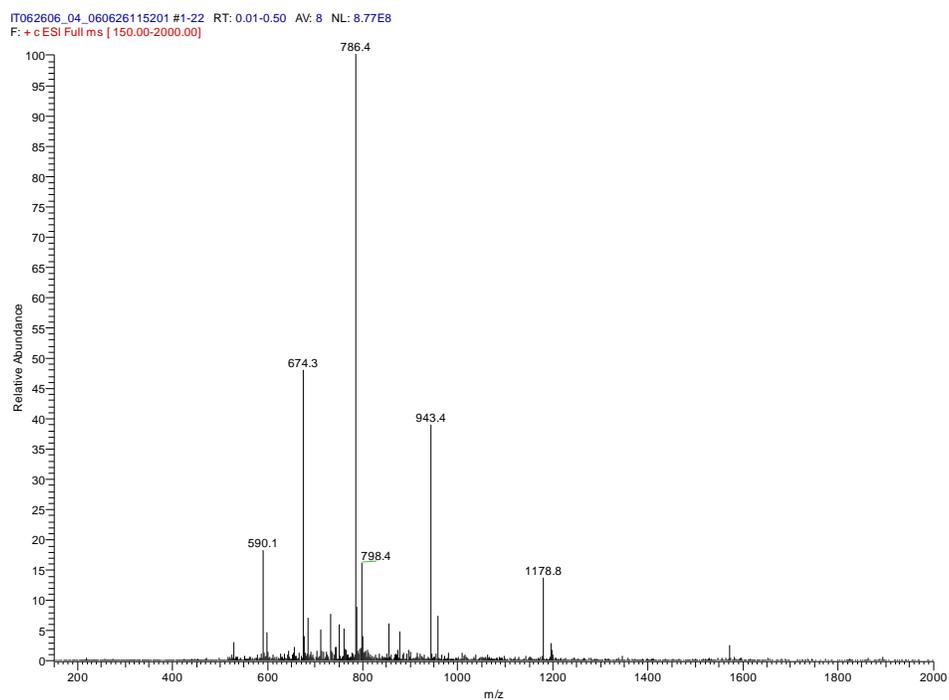
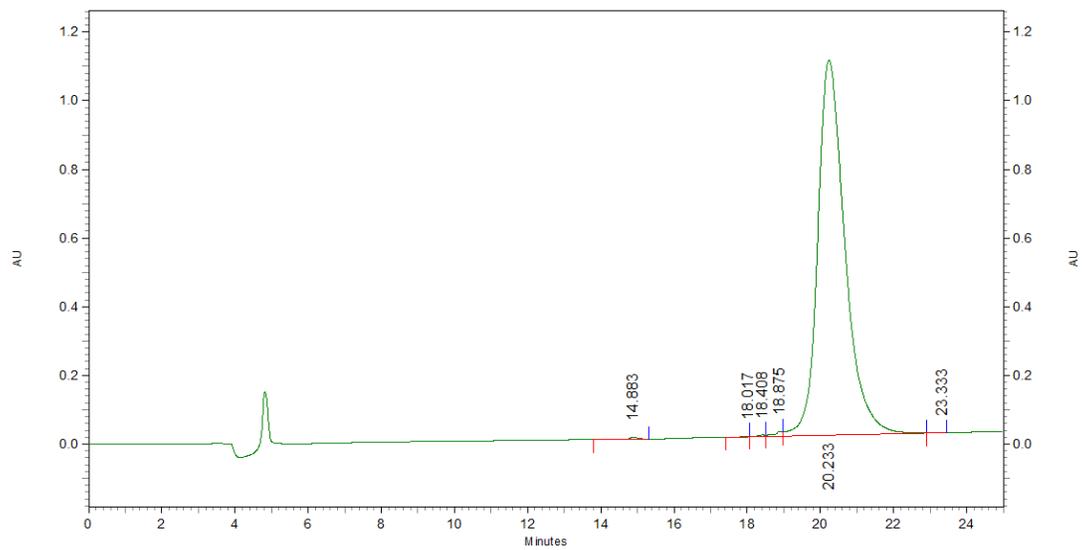


Figure 29: Mass spectrum of purified FALL39.



**Figure 30: Final HPLC chromatogram of purified FALL39.**

## 5. Future Work

With the successful synthesis of all three desired LL37 related peptides, work can begin on determining the structural and molecular principles for their antimicrobial action. It is well characterized that antimicrobial peptides kill bacteria by membrane disruption, but the exact mechanism of disruption has yet to be determined.<sup>44</sup> Once it is fully understood how these peptides are able to disrupt bacterial membranes, the development of new synthetic agents that could alleviate our current issues with antibiotic resistance may be possible.

The first objective will be the determination of secondary structure of the three LL37 related peptides using circular dichroism (CD) spectroscopy. Analyzing the structure of the peptides in water solution as well as a lipid environment will allow the determination of whether the structures vary across different conditions, such as pH, temperature, and buffer. The results of these experiments will hopefully allow optimization of the conditions for high-resolution structural studies by nuclear magnetic resonance (NMR) spectroscopy.

Since it has been shown that N-terminal truncation of LL37 to SK29 causes a loss of antibacterial activity, it is necessary to identify the reason for this loss of function and also to determine if the less truncated FF33 peptide still retains function.<sup>13</sup> In addition, the function of FALL39 needs to be compared with that of LL37 in order to determine the effects of additional N-terminal residues. This can be achieved through an examination of the high-resolution structure of LL37 and all three related peptides in solution and in

micelles using solid-state NMR methods. This will be followed by a study of the structure, dynamics, and topology of LL37 and all three related peptides in phospholipid bilayers using solid-state NMR. An investigation into the antimicrobial peptide-membrane interaction and the mechanism of membrane disruption using solid-state NMR and differential scanning calorimetry (DSC) will also be necessary.

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