SYNTHESIS OF HISTONE PEPTIDES THROUGH SOLID PHASE CHEMISTRY

by

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(Under the Direction of Y. George Zheng)

ABSTRACT

Histones play an important role in gene regulation and expression. DNA is wound around these proteins and chemical modifications to the side chains of histone amino acids can result in changes in the interaction between the histones and DNA. This may cause alterations in gene expression as the DNA can become either more or less attracted to the histone which in turn alters the accessibility of the gene loci around where the modification occurs. The project undertaken in this thesis involves the synthesis of the N-terminal tail of two major histones, H3 and H4, through solid phase peptide synthesis. The N-terminal tail was chosen as it undergoes extensive modifications as it is less constrained by the secondary and tertiary structure of the protein. These peptides can later be used in biochemical assays in the presence of enzymes that catalyze histone modifications as well as in assays to screen drug inhibitors of these enzymes. This will allow for the manipulation of the post-translational modification process of histones and, potentially, the regulation of gene expression in diseases.

INDEX WORDS: peptide, solid phase peptide synthesis, histone, epigenetics

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CHAPTER ONE

Introduction and Literature Review

1.1 HISTONES

Histones are proteins in which DNA is wrapped around and condensed upon. These proteins can undergo post-translational modifications (PTM) that alter the way DNA interacts with histones and/or recruit specific proteins that recognize these chemical changes in the histones. In changing their interaction with DNA, the histones may no longer attract the negatively charged DNA which loosens the coils of DNA around the histones. This causes the DNA to become more easily accessible to cellular enzymes that are responsible for the transcription of genes. As a result, genes near the PTM marks may become overexpressed and lead to an excess of the resulting proteins. PTMs in histones may also affect other proteins that interact with histones [1]. For example, the addition of a succinyl group to a lysine residue in histones may sterically hinder an enzyme that would normally target that lysine. The succinylation would also change the charge of lysine side chains from positive to negative which would also act to deter the interaction of that specific enzyme with the histones. The opposite may occur where an enzyme that does not normally recognize that lysine residue will now interact with the histone because the negative charge on the succinyl group will recruit the enzyme. All in all, these alterations lead to a concentration of proteins that differs from typical physiological environments. This has been shown to correlate with different

diseased states, including cancers, neurodegenerative diseases, and rheumatoid arthritis [2].

The interaction between histones and DNA is based on the negative charge found on the phosphate backbone of DNA. Amino acids on the histone may attract or repel DNA depending on the side chain of the residue. Through PTMs, this interaction may change depending on the modification. For example, the acetylation of a lysine side chain will cause the DNA to be only loosely bound to the histones as the amine group will not become positively charged any more [3]. The acetyl group may also act to sterically hinder any interactions such as hydrogen bonding that may still occur with the amine on the lysine. Some of the common modifications include acetylation, acylation, methylation, phosphorylation, and ubiquitylation [4]. Modifications may also lead to the recruitment of PTM-specific protein binding that result in the activation or inactivation of specific gene expression. These proteins include YEATS domains which recognizes H3K9ac and polycomb proteins which interact with H3K27me1.

The effects of histone modifications can be studied through various techniques such as chromatin immunoprecipitation (ChIP), biochemical screening for potential inhibitors of protein modifiers, quantitative structure-activity relationship (QSAR) studies, and other biochemical assays used to validate these results [5]. With ChIP, the residues that are likely to undergo posttranslational modification can be identified along with their location in the genome. Biochemical screening allows for researchers to identify compounds that may interact and inhibit those enzymes that lead to particular PTMs. With a new compound found,

QSAR studies will follow to determine the efficacy of the compound's interaction with its target and whether optimization of the compound is needed. Synthetic histone peptides are particularly useful to set up biochemical assays to study properties and mechanisms of histone modifying enzymes and to screen inhibitors of these enzymes for therapeutic investigation.

1.2 SOLID PHASE PEPTIDE SYNTHESIS

The N-terminal tail of histones is the region of rich PTMs. Synthetic histone peptides are excellent substrates that can be used to set up biochemical assays for studying molecular mechanisms of histone modification enzymes as well as screening of inhibitors of those enzymes. This is accomplished through solid phase peptide synthesis, which involves the use of solid support resins and the necessary amino acids with the appropriate protecting groups [6]. The addition of amino acids one by one onto a resin allows for a streamlined process for peptide synthesis (Figure 1.1 and 1.2), and the protecting groups greatly reduce the possibility for any unwanted reactions as well as maintaining the option for side chain modification. The synthesis begins by coupling the first amino acid on the C-terminus of the desired peptide to the linker on a resin. The resin will generally have a protecting group on the linker so this will have to be removed before the coupling occurs. In the presence of an activating compound and a base, the carboxylic acid group on the amino acid will undergo nucleophilic substitution and obtain a better leaving group. This leaving group will allow for the linker on the resin to begin a nucleophilic attack on the activated carboxyl group, which will

cause the leaving group to be removed (Figure 1.3). Now that the amino acid has been added to the solid support, the protecting group on the N-terminus of the amino acid must be removed to couple the next amino acid (Figure 1.4). The following amino acid will be added through activation and nucleophilic substitution as stated previously. Once the proper sequence of amino acids has been added to the peptide and resin chain, the side chain protecting groups must be removed, and the peptide must be cleaved from the resin. This may occur in one step depending on the protecting groups used as well as the resin used.

In order to have a proper resin as a solid support, it must have the following characteristics: physically stable, insoluble and readily able to be filtered from solution, inert to all reagents used in the synthesis, swellable and porous, and readily available to form a covalent bond with the compound involved in the first coupling step. The use of a solid support in chemical synthesis allows for an excess in reagents to help push the reaction towards completion due to Le Chatelier's principle. Because the resin would be insoluble in the reaction solvent, the excess reagents can be simply washed away leaving only the product attached to the resin. This is especially helpful for peptide synthesis as coupling steps may be difficult due to bulky protecting groups such as the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) group or the trityl (trt) group that may be on the side chain. With the assistance of an excess of reagents, a higher yield of desired product will be found on the resin after the final cleavage.

Generally, there are two approaches to solid phase peptide synthesis: Boc-based peptide synthesis and Fmoc-based peptide synthesis [7]. For Boc-based

peptide synthesis, side chains are protecting with benzyl groups while a Boc protecting group is attached to the N-terminus of each amino acid. Boc is deprotected through 25%-50% trifluoroacetic acid (TFA) prior to each coupling step. When the synthesis is complete, a strong acid such as hydrofluoric acid (HF) or trifluoromethanesulfonic acid (TFMSA) is used to fully cleave the peptide off of the resin as well as remove any protecting groups on the amino acid side chains. The caveat for Boc-based peptide synthesis is that the target peptide must be stable under strongly acidic conditions. The alternate strategy requires milder conditions in which a Fmoc protecting group is on the N-terminus while the side chains are protected by acid-labile protecting groups such as tBu or Boc. Fmoc is removed from the amino acid through basic conditions such as 20% piperidine. This allows for the use of milder acidic conditions to remove the tBu or Boc groups as well as cleave the peptide off the resin. The Fmoc strategy was used in the synthesis of the histone peptides and, as such, will the focus of this thesis.

The use of a solid support allows for variability in peptide synthesis. By using different resins, the functional group on the C-terminus can be altered [8]. In Fmoc-based synthesis, the carboxylic acid group can be conserved using resins containing a Wang, HMPA, 2-Chlorotrityl or similar linker. The use of Rink amide, Sieber, or PAL linkers on the resin will result in the peptide's carboxylic acid group converting into an amide. The carboxylic acid can also be converted into an alcohol if the linker on the resin is the Ellman linker. For Boc-based peptide synthesis, the available linkers are limited to MBHA for peptide amides and Merrifield and PAM linkers for peptide acids resulting in another incentive for peptide chemists to opt

for the Fmoc-based route. Peptides may also undergo diversification using orthogonal protecting groups [9]. One such example includes the Dde protecting group, which is stable in piperidine and TFA, the common reagents used to deprotect Boc and Fmoc protecting groups. The use of hydrazine removes Dde and allows the deprotected residue to undergo reactions while the other amino acids are protected.

When the desired peptide has been fully synthesized, the protecting groups on the side chains need to be removed, and the peptide must be cleaved off the resin. As the peptides synthesized in this paper use the Fmoc strategy, cleavage cocktails used for Fmoc peptides will be discussed more in detail. Peptides containing traditional protecting groups such as Trt, Boc, or Pbf can be deprotected through a milder acid solution. This is also true for the cleavage of the peptide from the resin. Generally, cleavage cocktails for Fmoc peptides contain concentrated TFA as its acid and other various scavengers to prevent any unwanted side reactions. Among some of these scavengers include water for tBu cations, ethane dithiol for tBu trifluoroacetate, and triisopropyl silane for Trt cations [10]. For complete deprotection and cleavage, most peptides require anywhere from 1 to 4 hours, depending on which protecting groups are present. The Pbf group on arginine may prove to be difficult to remove if the peptide contains several of them. In this case, the peptide must be precipitated and free of the previous cleavage cocktail before adding a fresh solution of cocktail. Between 5 to 10 mL of cleavage cocktail is sufficient for every 0.5 g of resin. In the Boc strategy, the two predominating cleavage cocktails are centered around TFMSA and HF. Once the

peptide is removed from the resin, precipitation can be accomplished using cold diethyl ether and centrifugation.

The finished peptide is analyzed with high performance liquid chromatography (HPLC) as well as mass spectrometry (MS) [11]. The workflow for identification begins with the use of analytical HPLC to discern the retention time of the crude peptide given a particular method. The product is confirmed by testing each peak in the resulting chromatogram with either electrospray ionization (ESI) MS or matrix-assisted laser desorption/ionization (MALDI) MS. This is repeated for preparative HPLC to begin the purification process. After preparative HPLC, the peptide's purity can be ascertained through analytical HPLC.

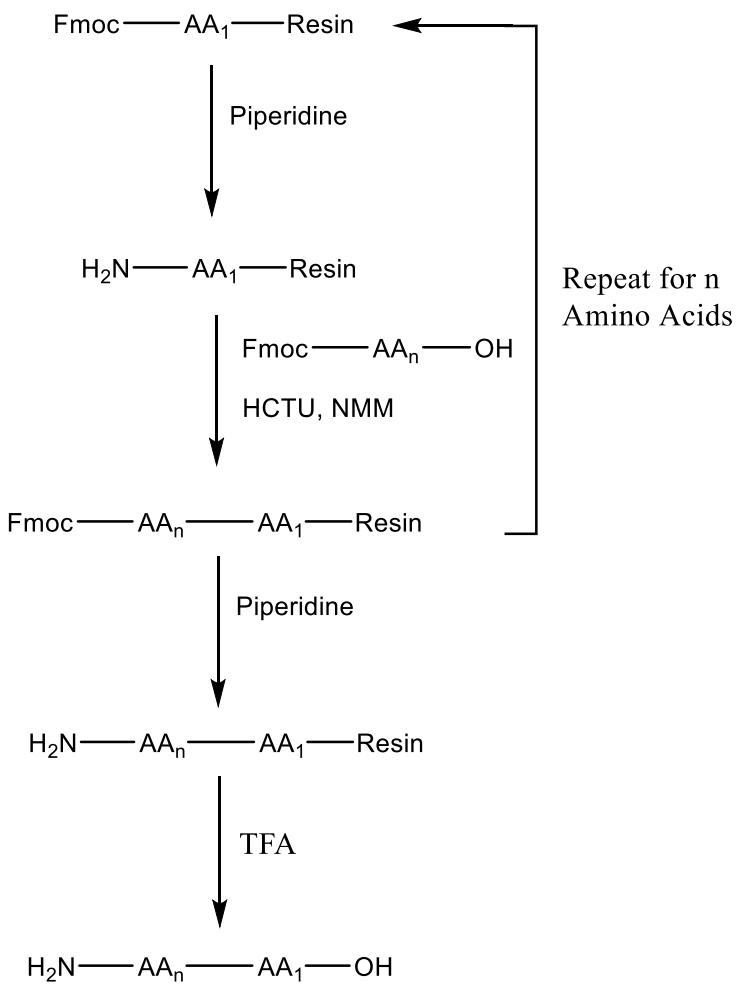

Figure 1.1 General schematic for Fmoc solid phase synthesis. Solid phase synthesis generally follows this schematic in which the Fmoc group is deprotected then coupled with an activated amino acid to continue the peptide sequence. Once the sequence has been completed, deprotection and cleavage occurs with a mild acid cocktail and the target peptide will form.

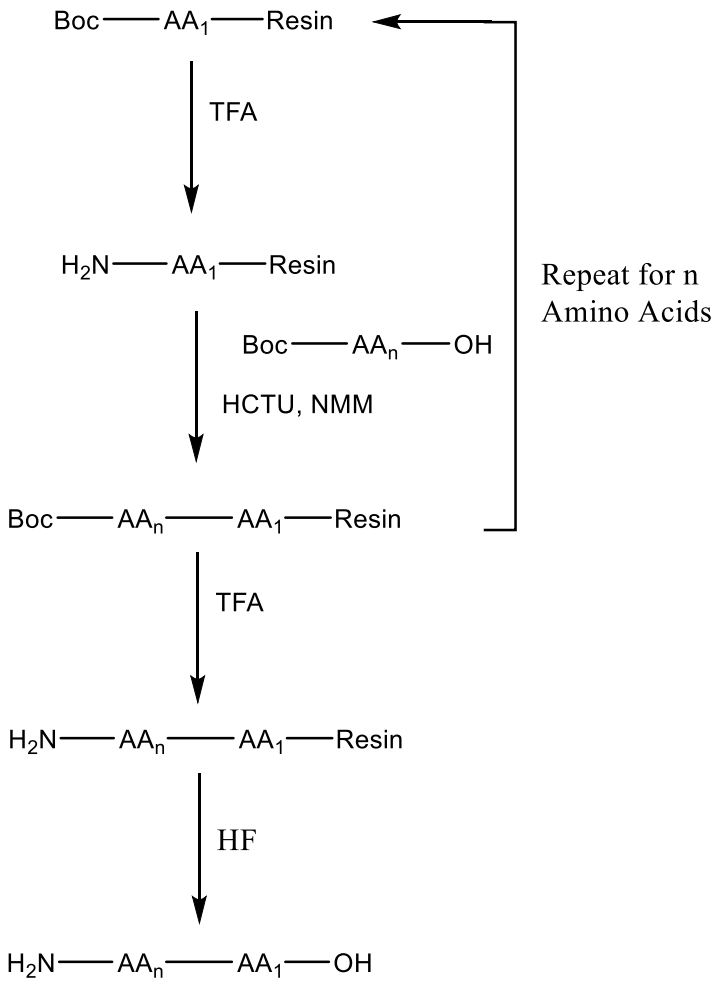

Figure 1.2 General schematic for Boc solid phase synthesis. Solid phase synthesis generally follows this schematic in which the Boc group is deprotected then coupled with an activated amino acid to continue the peptide sequence. Once the sequence has been completed, deprotection and cleavage occurs with a strong acid cocktail and the target peptide will form.

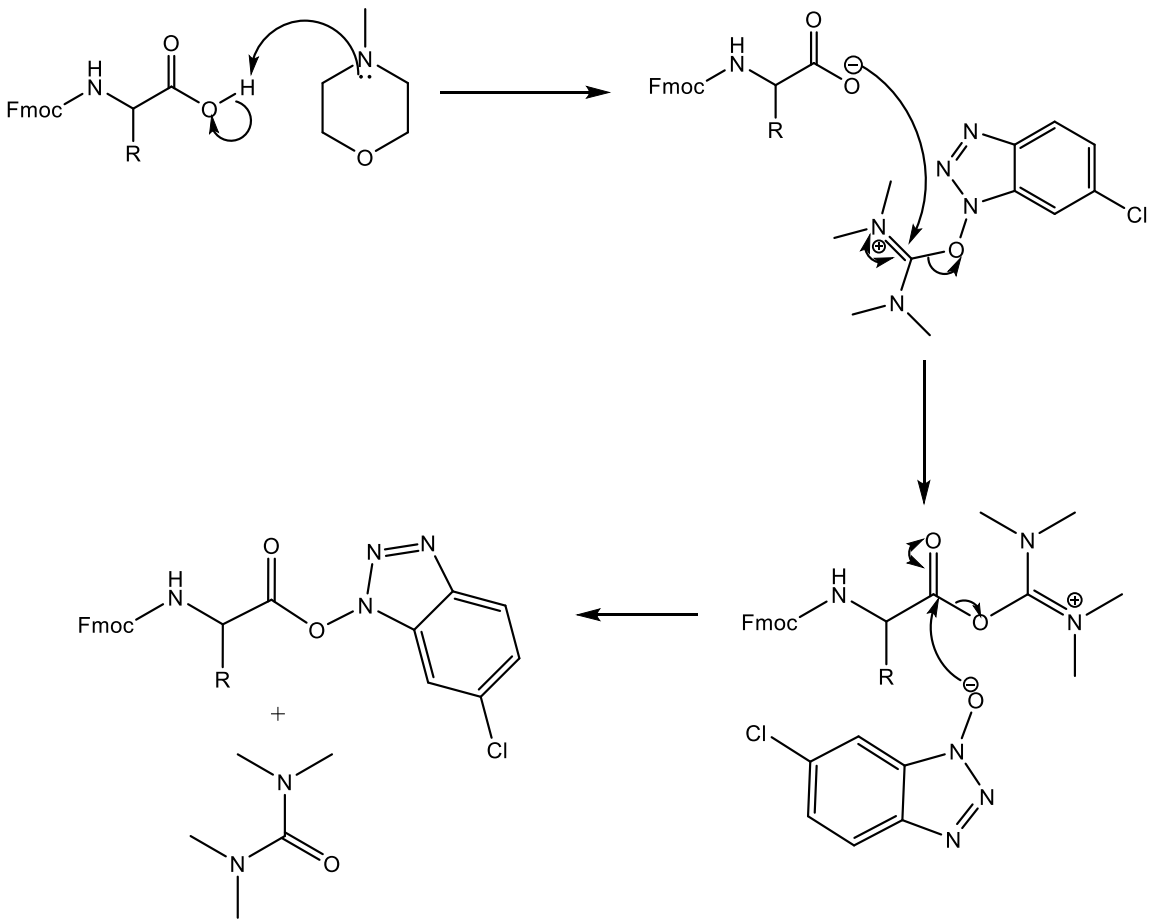

Figure 1.3 Activation mechanism for amino acids. Amino acids can be activated using 4-methylmorpholine and HCTU to allow for coupling and peptide elongation. This results in the formation of a peptide bond as the carboxylic acid is deprotonated by 4-methylmorpholine and becomes more prone to substitution with addition of the good leaving group obtained from HCTU.

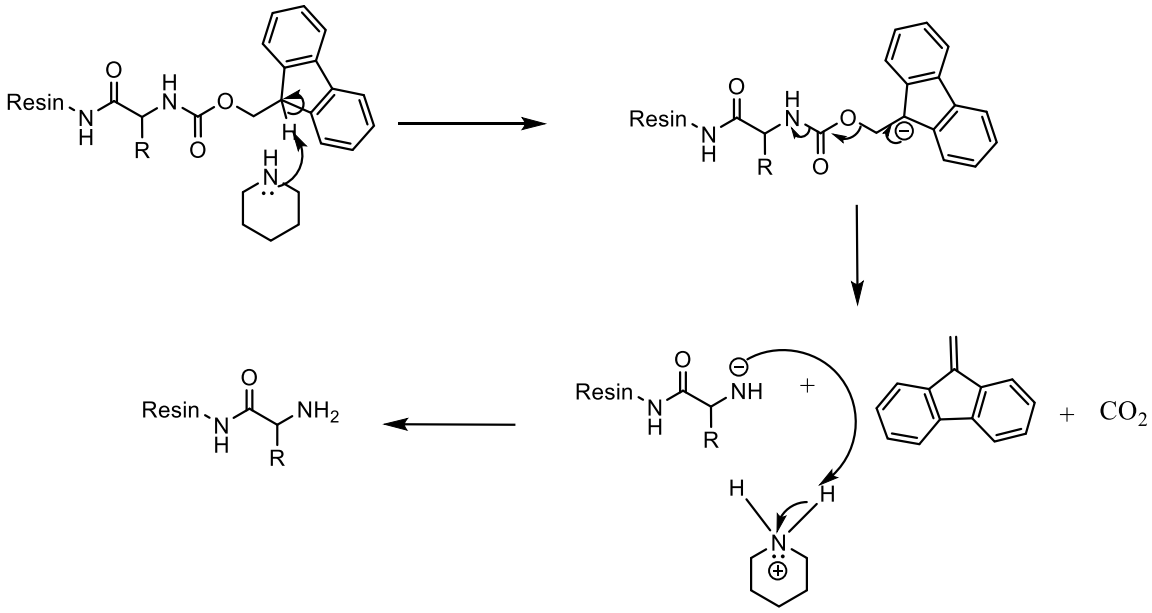

Figure 1.4 Deprotection of Fmoc using piperidine. Bases such as piperidine are used in the orthogonal deprotection of Fmoc from the N-terminus. This will allow for elongation of the peptide sequence through further coupling of amino acids without affecting other protecting groups on the peptide.

CHAPTER TWO

Synthesis of the H4 Peptide

2.1 INTRODUCTION

The amino acid sequence for Btn-H4(1-22)-OH is SRG GKG GKG LGK GGA KRH RKV L with the N-terminus having a biotin attached to the alpha amine. 0.552 g of Fmoc-Leu-Wang resin was used as the solid support with a loading scale of 0.365 mmol/g. This peptide was synthesized with the standard Fmoc strategy for solid phase peptide synthesis. Potential modifications that can be observed on this portion of the histone include acetylation of K16 and methylation and acetylation of K20 [12].

2.2 METHODS

Materials

The following N-α-Fmoc protected amino acids as well as D-Biotin were received from ChemPep Inc.: Fmoc-Ser(tBu)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Ala-OH, Fmoc-Val-OH, and Fmoc-Leu-Wang resin. Fmoc-His(Trt)-OH and HCTU were received from Chem-Impex. N-methylpyrrolidone (NMP) was received from Oakwood Chemical, ethane dithiol from Alfa Aesar, thioanisole and TFA from Acros Organics, N,N-diisopropylethylamine (DIPEA) from TCI America, dimethylsulfoxide (DMSO) and diethyl ether from Fischer Scientific, and phenol, triisopropylsilane, acetonitrile, methanol, dichloromethane, 4-methylmorpholine (NMM), dimethylformamide (DMF), and piperidine were received from Sigma Aldrich. The acetonitrile used

was HPLC grade and used in the analysis and purification of the peptides. Instrumentation includes Focus XC peptide synthesizer, Shimadzu LC-20AT HPLC and SPD-20A UV/Vis detector, Thermo Fisher Scientific Q Exactive Orbitrap mass spectrometer for ESI MS, and Labconco FreeZone 6 Plus lyophilizer. HPLC columns used in the following experiments include Aeris peptide 3.6 v XB-C18 250 x 4.6 mm for analytical HPLC, Polaris 5 C-18A 150 x 21.2 mm for preparative HPLC, and Agilent Dynamax Microsorb 100-5-C18 250 x 10.0 mm for semi-preparative HPLC.

Peptide Synthesis

The H4 peptide was synthesized on Fmoc-Leu-Wang resin through the Fmoc strategy for solid phase peptide synthesis. While on the Focus XC peptide synthesizer, the peptide was deprotected using 8 mL of 20% piperidine in DMF for 15 minutes. The activation steps for each residue consisted of a solution containing 5 mL or 10 equivalents of amino acid, 5mL or 10 equivalents of NMM, and 5mL or 10 equivalents of HCTU which was mixed for 1 minute before being added to the reaction vessel. This reaction proceeded for 1 hour and repeated for residues that require double coupling. After each of these steps, the resin was washed with 5 mL DMF three times. Once the sequence was complete, the N-terminus underwent biotinylation. The resin was swelled in DMF for 15 minutes then drained. A 2 mL solution of 0.5M HCTU was created in 1:1 DMF and DMSO. Separately, another 4 mL solution of 0.2M biotin was created in 1:1 DMF and DMSO. The two solutions were combined and 4 equivalents of DIPEA was added. The solution was quickly

mixed until it was homogenous then added to the resin to be mixed overnight. The solution was then drained and washed with organic solvents in the following order: 1:1 DMF and DMSO, DMF, dichloromethane, diethyl ether, methanol, and diethyl ether. The resin was dried under vacuum for 1 hour. To remove the peptide from the swelled resin, 4 mL of cleavage cocktail containing 200 µL of water, 200 µL of thioanisole, 100 µL of ethane dithiol, 40 µL of triisopropyl silane, 200 mg of phenol and 3.260 mL of TFA was added to the resin for 4 hours. The resin was filtered from the resulting solution, and 40 mL of diethyl ether was added to the solution. The mixture was then centrifuged at 4°C and 3214 *g* for 10 minutes. The liquid was removed, and another 40 mL of diethyl ether was added to the precipitate. The mixture was centrifuged again under the same conditions as before. The solid was collected and dissolved in water before analysis.

Identification and Purification of Btn-H4(1-22)-OH

Once dissolved in water, 10 µL of the solution was analyzed through analytical HPLC and the resulting peaks were further discerned through ESI MS. This was repeated with the fractions obtained from preparative HPLC. Once the product is determined on the chromatograms, further purification was done through preparative HPLC and semi-preparative HPLC. Confirmation of purity was accomplished through analytical HPLC.

2.3 RESULTS AND DISCUSSION

The crude product after cleavage from the resin was analyzed through analytical HPLC (Figure 2.2) and found to have a retention time of 18.9 minutes as confirmed by ESI MS (Figure 2.3). After which, the peptide underwent purification through preparative reversed-phase HPLC (Figure 2.4) in which the product was observed to elute at two different times. The peptide was confirmed through ESI MS (Figures 2.5 and 2.6) to have a retention time of 25.3 minutes and 34.6 minutes. Once the crude had gone through purification, the chromatogram from the purity check of the peptide showed that both fractions of the peptide were still impure (Figures 2.7 and 2.8). To remedy this, each fraction of peptide was repurified through semi-preparative HPLC (Figures 2.9 and 2.10). The peaks that eluted around 20 minutes (Figure 2.11) in the preparative HPLC chromatogram appeared to contain the majority of the desired product whereas the peaks with retention times around 30 minutes (Figure 2.12) with the preparative HPLC column yielded very little product. The product may have been found at two different retention times due to conformational changes that would result in a different interaction with the column depending on which side chains are exposed.

The final purified peptide resulted in 13.42 mg of product with a yield of 2.74%. The low yield is largely due to the over injection of the sample after the second purification using semi-preparative HPLC. The injection loop had unknowingly been changed from 1 mL to 100 µL. Each HPLC run consisted of about 500 µL of sample which resulted in an estimated 80% loss of product from this error. The purity of the product was determined to be at 96.39% while the

purity of the secondary product was determined to be 85.23%. The majority product was then dissolved 1.104 mL of water to create a 5 mM solution of the peptide to be used in future biological experiments.

2.4 CONCLUSION

In this work, the H4 peptide Btn-H4(1-22)-OH, was successfully synthesized through solid phase chemistry and purified through reversed-phase HPLC. Despite the low yield, this synthesis resulted in a peptide with good purity and will serve to expedite the synthesis of similar histone peptides. The H4 peptide is currently being studied with protein arginine methyltransferase (PRMT) 1 in order to discern the catalytic activity of PRMT 1 as well as its properties, new PRMT 1 inhibitors, and small molecules that result in the in vitro chemical rescue of mutant PRMT 1.

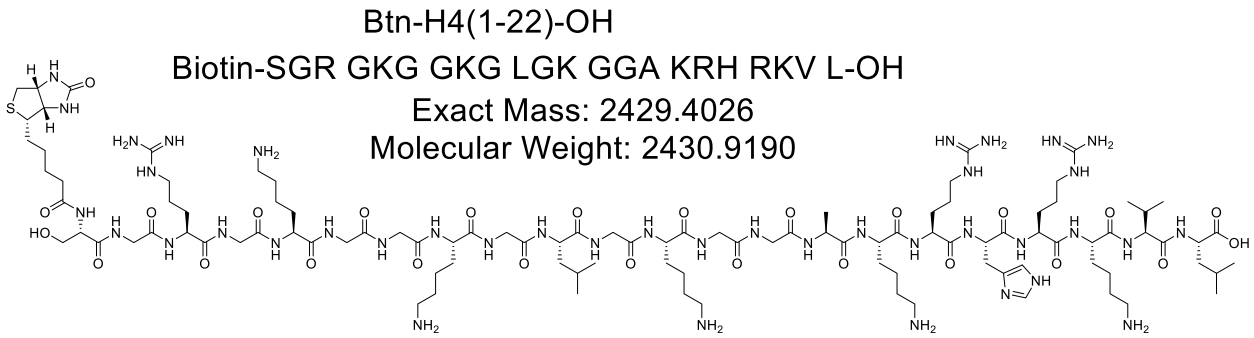

Figure 2.1 Btn-H4(1-22)-OH. The structure of the histone peptide is shown here. The peptide includes residues 1-22 from the N-terminus of the H4 histone with biotin attached to the N-terminus.

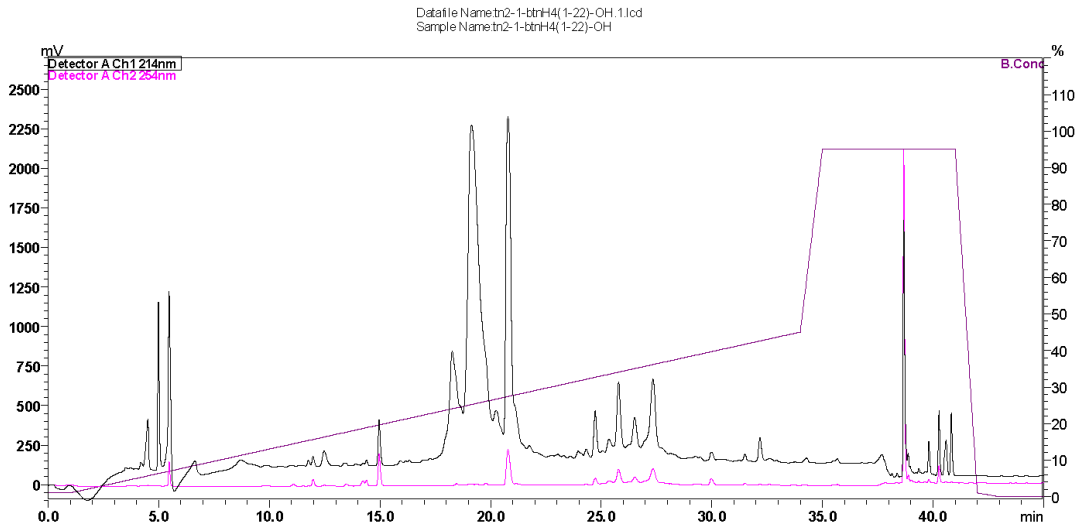

Figure 2.2 Analytical HPLC of crude Btn-H4(1-22)-OH. The analytical chromatogram shows two major peaks with retention times of 18.9 minutes and 20.6 minutes. The method used had a gradient of 1-45% of mobile phase B to mobile phase A in which mobile phase B was acetonitrile with 0.1% trifluoroacetic acid and mobile phase was water with 0.1% trifluoroacetic acid.

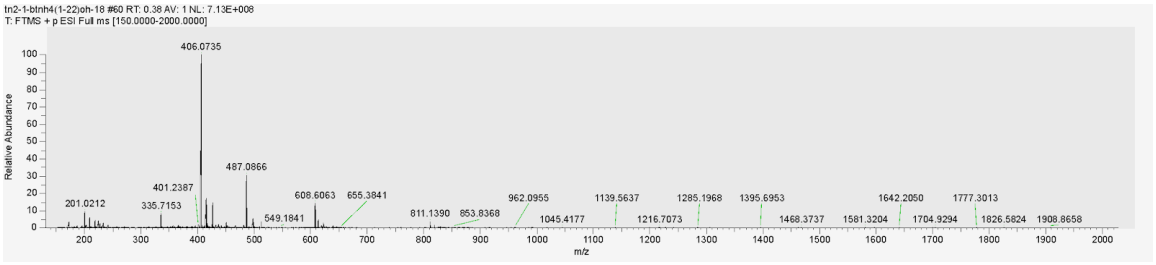

Figure 2.3 ESI MS of the peak with 18.9-minute retention time. ESI MS confirms the presence of product in this fraction. The exact mass of Btn-H4(1-22)-OH is 2429.40. The values of 406.07, 487.08, and 608.60 correlate with $[M+6H]^{6+}$, $[M+5H]^{5+}$, and $[M+4H]^{4+}$.

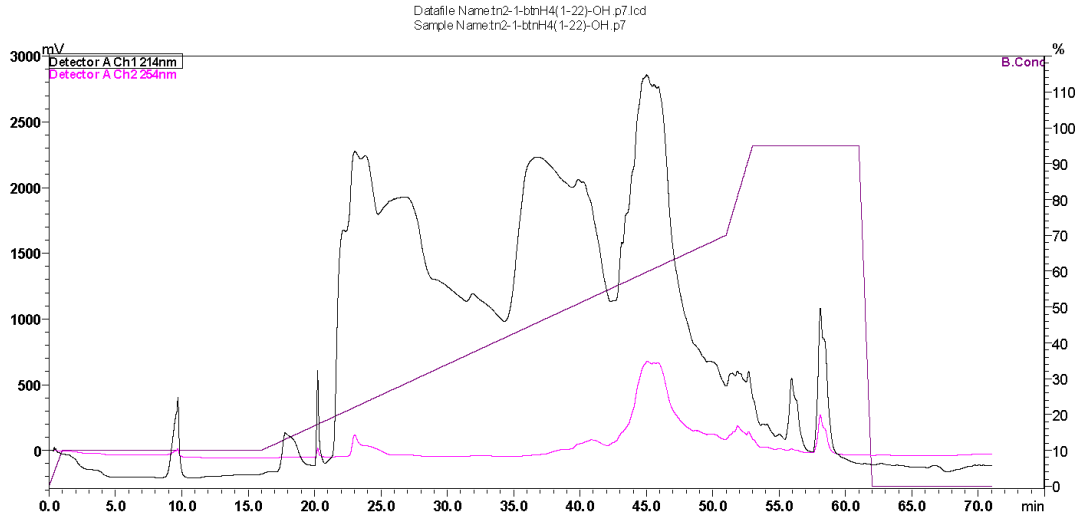

Figure 2.4 Preparative HPLC of crude Btn-H4(1-22)-OH. The product was found to be in the fraction at 25.3 minutes and 34.6 minutes and was confirmed through ESI MS. The method used had a gradient of 10-80% of mobile phase B to mobile phase A in which mobile phase B was acetonitrile with 0.1% trifluoroacetic acid and mobile phase was water with 0.1% trifluoroacetic acid. This method was later optimized for better resolution of the peaks.

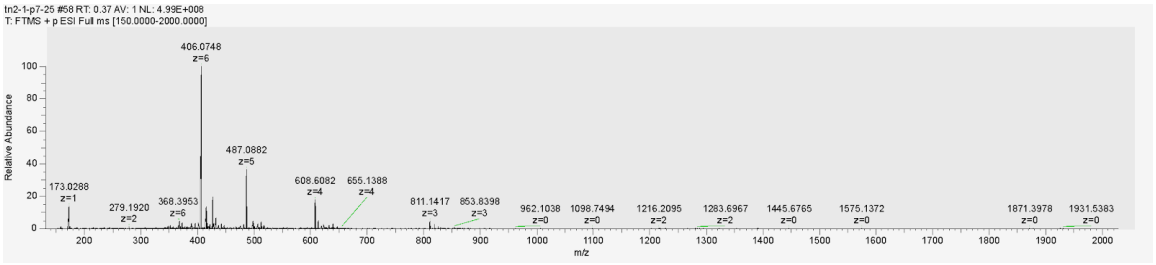

Figure 2.5 ESI MS of the peak with 25.3-minute retention time. ESI MS confirms the presence of product in this fraction. The exact mass of Btn-H4(1-22)-OH is 2429.40. The values of 406.07, 487.08, and 608.60 correlate with $[M+6H]^{6+}$, $[M+5H]^{5+}$, and $[M+4H]^{4+}$.

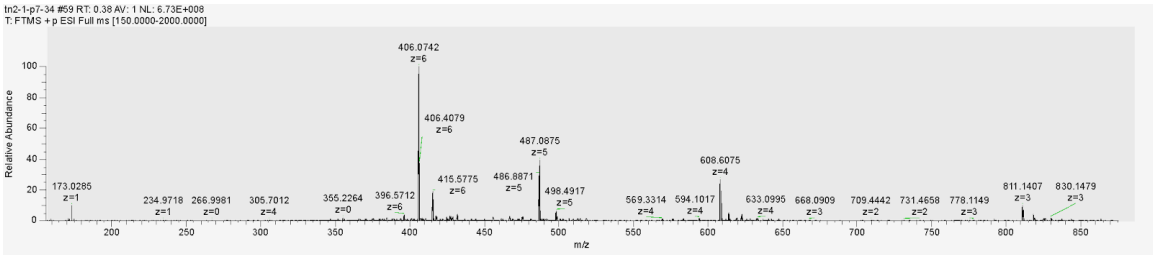

Figure 2.6 ESI MS of the peak with 34.6-minute retention time. ESI MS confirms the presence of product in this fraction. The exact mass of Btn-H4(1-22)-OH is 2429.40. The values of 406.07, 487.08, and 608.60 correlate with $[M+6H]^{6+}$, $[M+5H]^{5+}$, and $[M+4H]^{4+}$.

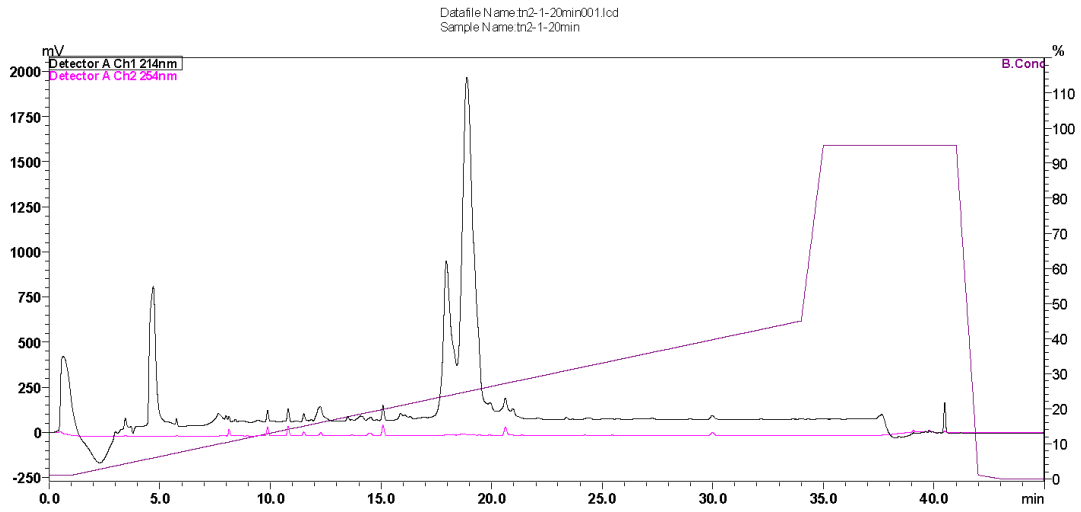

Figure 2.7 Analytical HPLC of fractions around 20-minute retention time. The analytical chromatogram shows that this fraction is not pure. The method used had a gradient of 1-45% of mobile phase B to mobile phase A in which mobile phase B was acetonitrile with 0.1% trifluoroacetic acid and mobile phase was water with 0.1% trifluoroacetic acid.

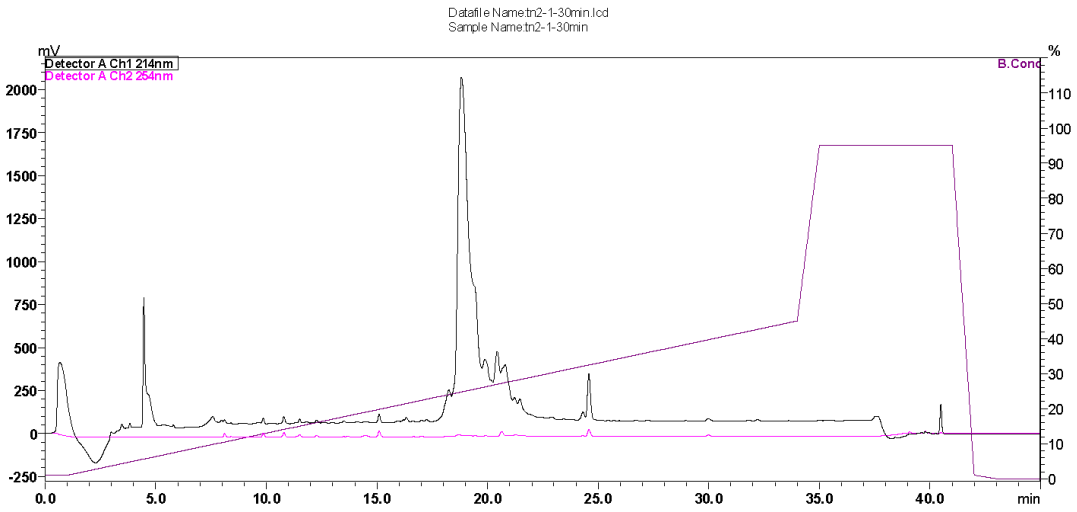

Figure 2.8 Analytical HPLC of fractions around 30-minute retention time. The analytical chromatogram shows that this fraction is not pure. The method used had a gradient of 1-45% of mobile phase B to mobile phase A in which mobile phase B was acetonitrile with 0.1% trifluoroacetic acid and mobile phase was water with 0.1% trifluoroacetic acid.

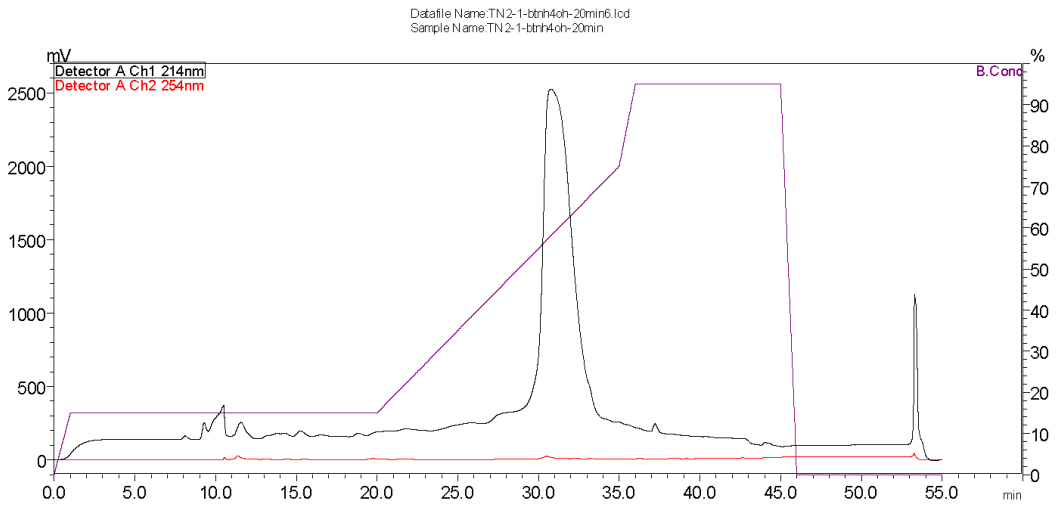

Figure 2.9 Semi-preparative HPLC of the fractions around 20-minutes.

The second round of purification showed very little impurity. The product was found to have a retention time of 30 minutes in this chromatogram. The method used had a gradient of 15-75% of mobile phase B to mobile phase A in which mobile phase B was acetonitrile with 0.1% trifluoroacetic acid and mobile phase was water with 0.1% trifluoroacetic acid.

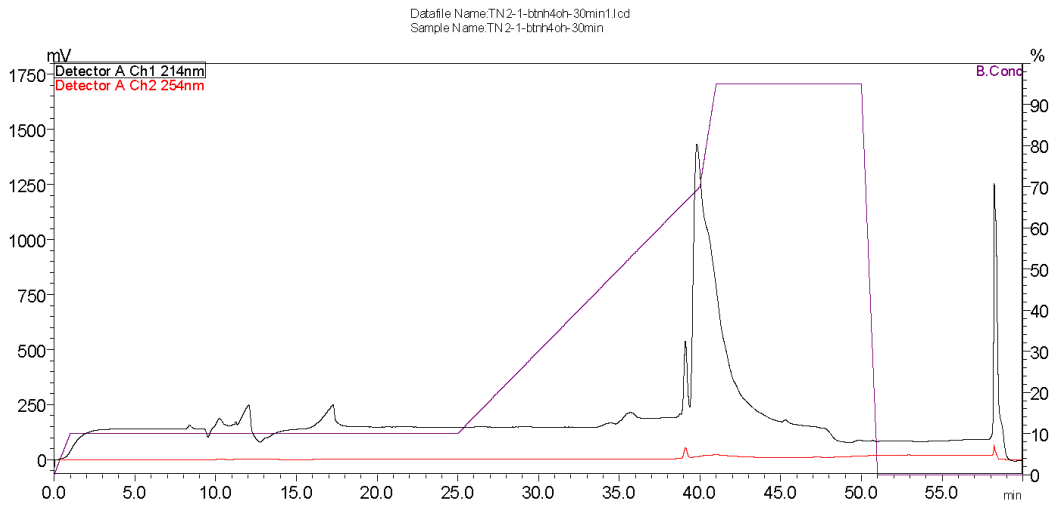

Figure 2.10 Semi-preparative HPLC of the fractions around 30-minutes. For this fraction, the product had a retention time of 39 minutes, and the chromatogram shows that this fraction had large amounts of impurities. The method used had a gradient of 15-75% of mobile phase B to mobile phase A in which mobile phase B was acetonitrile with 0.1% trifluoroacetic acid and mobile phase was water with 0.1% trifluoroacetic acid.

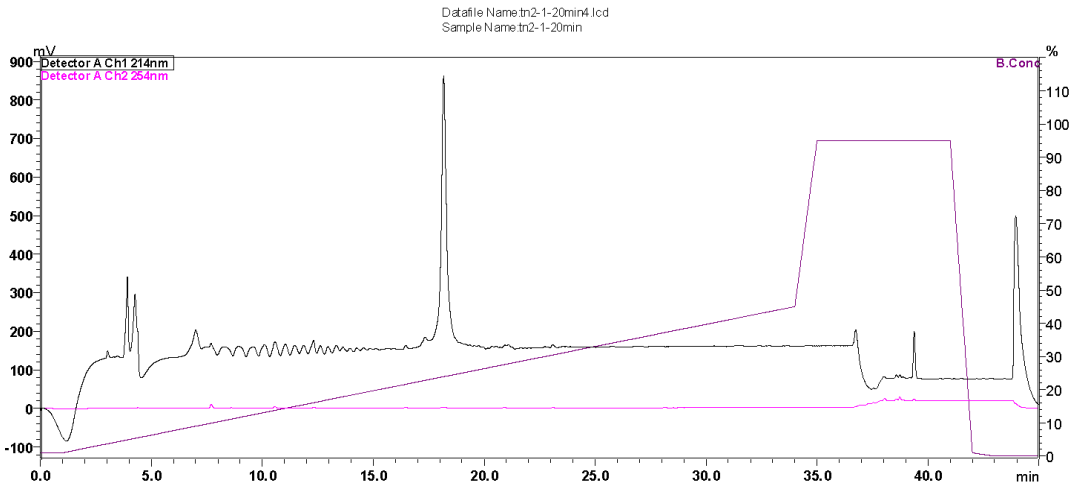

Figure 2.11 Final analytical HPLC of fractions around 20-minute retention time. The analytical chromatogram shows that this fraction is now pure. The method used had a gradient of 1-45% of mobile phase B to mobile phase A in which mobile phase B was acetonitrile with 0.1% trifluoroacetic acid and mobile phase was water with 0.1% trifluoroacetic acid.

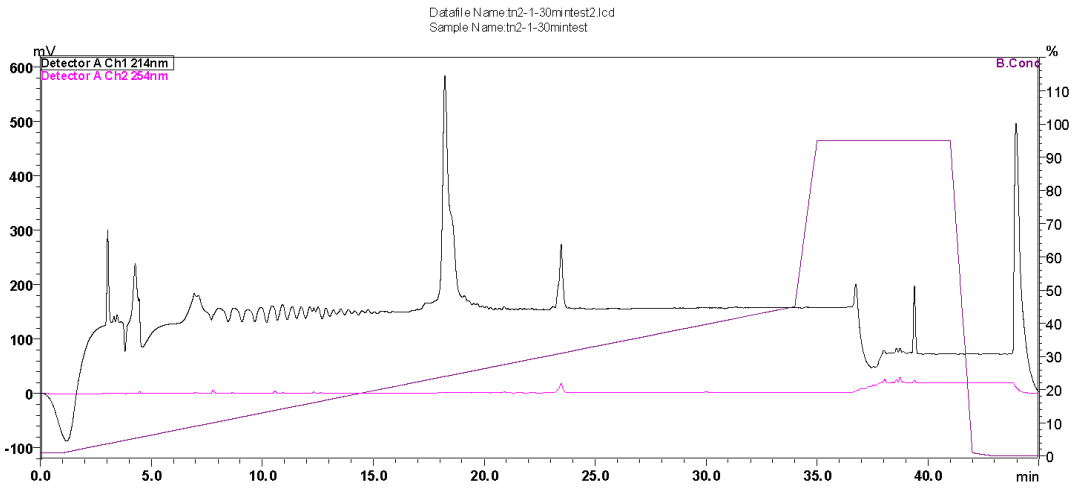

Figure 2.12 Final analytical HPLC of fractions around 30-minute retention time. The analytical chromatogram shows that this fraction still has some impurities. The method used had a gradient of 1-45% of mobile phase B to mobile phase A in which mobile phase B was acetonitrile with 0.1% trifluoroacetic acid and mobile phase was water with 0.1% trifluoroacetic acid.

CHAPTER THREE

Synthesis of the H3 Peptide

3.1 INTRODUCTION

The amino acid sequence for Btn-H3(1-25)-NH_2 is ART KQT ARK STG GKA PRK QLA TKA A with the N-terminus having a biotin attached to the alpha amine (Figure 3.1). 0.388 g of Rink Amide resin was used as the solid support with a loading scale of 0.53 mmol/g. This peptide was synthesized with the standard Fmoc strategy for solid phase peptide synthesis. As a result of using Rink Amide resin, the C-terminal was converted into an amide after the peptide was cleaved off the resin. When compared to H4, H3 has been found to have been altered with more PTMs quite consistently [12]. Some PTMs that can be found in H3 include the acetylation of K4 and methylation of K27.

3.2 METHODS

Materials

The following N-α-Fmoc protected amino acids as well as D-Biotin were received from ChemPep Inc.: Fmoc-Ser(tBu)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Ala-OH, Fmoc-Thr(tBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Pro-OH, and Rink Amide resin. NMP was received from Oakwood Chemical, HCTU from Chem-Impex, ethane dithiol from Alfa Aesar, thioanisole and TFA from Acros Organics, DIPEA from TCI America, DMSO and

diethyl ether from Fischer Scientific, and phenol, triisopropylsilane, acetonitrile, methanol, dichloromethane, NMM, DMF, and piperidine were received from Sigma Aldrich. The acetonitrile used was HPLC grade and used in the analysis and purification of the peptides. Instrumentation includes Focus XC peptide synthesizer, Shimadzu LC-20AT HPLC and SPD-20A UV/Vis detector, Bruker Autoflex mass spectrometer for MALDI MS, and Labconco FreeZone 6 Plus lyophilizer. HPLC columns used in the following experiments include Aeris peptide 3.6 v XB-C18 250 x 4.6 mm for analytical HPLC and Agilent Dynamax Microsorb 100-5-C18 250 x 10.0 mm and Agilent Eclipse XDB-C18 250 x 9.4 mm for semi-preparative HPLC.

Peptide Synthesis

The H3 peptide was synthesized on Rink Amide resin through the Fmoc strategy for solid phase peptide synthesis. While on the Focus XC peptide synthesizer, the peptide was deprotected using 8 mL of 20% piperidine in DMF for 15 minutes. The activation steps for each residue consisted of a solution containing 5 mL or 10 equivalents of amino acid, 5mL or 10 equivalents of NMM, and 5mL or 10 equivalents of HCTU which was mixed for 1 minute before being added to the reaction vessel. This reaction proceeded for 1 hour and was repeated for residues that require double coupling. After each of these steps, the resin was washed with 5 mL DMF three times. Once the sequence was complete, the N-terminus underwent biotinylation. The resin was swelled in DMF for 15 minutes then drained. A 2 mL solution of 0.5M HCTU was created in 1:1 DMF and DMSO.

Separately, another 4 mL solution of 0.2M biotin was created in 1:1 DMF and DMSO. The two solutions were combined and 4 equivalents of DIPEA was added. The solution was quickly mixed until it was homogenous then added to the resin to be mixed overnight. The solution was then drained and washed with organic solvents in the following order: 1:1 DMF and DMSO, DMF, dichloromethane, diethyl ether, methanol, and diethyl ether. The resin was dried under vacuum for 1 hour. To remove the peptide from the swelled resin, 4 mL of cleavage cocktail containing 200 µL of water, 200 µL of thioanisole, 100 µL of ethane dithiol, 40 µL of triisopropyl silane, 200 mg of phenol and 3.260 mL of TFA was added to the resin for 4 hours. The resin was filtered from the resulting solution, and 40 mL of diethyl ether was added to the solution. The mixture was then centrifuged at 4°C and 3214 *g* for 10 minutes. The liquid was removed, and another 40 mL of diethyl ether was added to the precipitate. The mixture was centrifuged again under the same conditions as before. The solid was collected and dissolved in water before analysis.

Identification and Purification of Btn-H3(1-25)-NH_2

Once dissolved in water, 10 µL of the solution was analyzed through analytical HPLC and the resulting peaks were further discerned through MALDI MS. This was repeated with the fractions obtained from semi-preparative HPLC. Once the product is determined on the chromatograms, further purification was done through semi-preparative HPLC. Confirmation of purity was accomplished through analytical HPLC.

3.3 RESULTS AND DISCUSSION

A small portion of the crude product was cleaved from the resin was analyzed through analytical HPLC (Figure 3.2) and the peak with a retention time of 17.9 minutes was observed to be $[M+106]^+$ on MALDI MS (Figure 3.3). From literature, this value may arise from the incomplete cleavage of the resin linker from the peptide [13]. Once the remaining crude peptide was cleaved from the peptide, the resulting chromatogram (Figure 3.4) did not have a peak with similar retention time to that of the test cleavage. The large peak was analyzed through MALDI MS (Figure 3.5) and may potentially be the peptide with some remaining Pbf protecting groups. The high absorbance with 254 nm may also suggest this as the peptide should not contain any residues that have high absorbance at 254 nm while Pbf with its aromatic rings will have absorbance at that wavelength.

With this hypothesis in mind, four small portions of the potentially protected peptide were allowed to react with cleavage cocktail for 1, 2, 4, and 6 more hours. These time points were chosen due to literature reporting that multiple Pbf groups may require up to 6 hours of cleavage. However, it was uncertain if this referred to 6 continuous hours or a total of 6 hours. In addition to observing the complete deprotection of the arginine residues, the multiple time points also allowed for the observation of any potential degradation or hydrolysis of the peptide. The procedure with precipitation using diethyl ether and centrifugation was repeated before analysis much like the initial cleavage. The resulting chromatogram had a peak with a retention time around 18 minutes (Figure 3.6) and was confirmed

through MALDI MS to be the desired product (Figure 3.7). In addition to removing the leftover Pbf protecting groups, the second addition of cleavage cocktail seemed to have removed the resin linker as the $[M+106]^+$ was not found in the MALDI spectrum. Because all four chromatograms showed a similar result, the crude was allowed to react with the cleavage cocktail for an additional hour. Once introduced to additional cleavage cocktail, the crude product seemed to have resulted in a different chromatogram than what was seen with the test cleavage portions (Figure 3.8). The chromatogram had two major peaks with different retention times, 15.1 minutes and 18.1 minutes. The peak with a retention time of 15.1 minutes was determined through MALDI MS to be the product (Figure 3.9) while the peak at 18.1 minutes was shown to not contain product (Figure 3.10).

Once the product was determined, it was purified through semi-preparative reversed-phase HPLC (Figure 3.11). The product was confirmed to have a retention time of 31.0 minutes (Figure 3.12), though analytical HPLC shows that the product is not pure and must undergo a second round of purification (Figure 3.13). It was determined that the semi-preparative column used had poor performance, and the Agilent Eclipse column was used. This column provided better separation and resolution of the peaks (Figure 3.14). After the second purification of the peptide, the product yield resulted in 5.5 mg or 0.94% yield with a 97.36% purity (Figure 3.15). This low yield is likely due to the poor resolution of the first semi-preparative column used as some of the product was likely in the peaks deemed as impurities.

3.4 CONCLUSION

In this work, the H3 peptide Btn-H3(1-25)-NH_2 was successfully synthesized through solid phase chemistry and purified through reversed-phase HPLC. Though the yield for this peptide was low, the newer semi-preparative column should allow for more efficient purification of future peptides. This histone peptide will be used in future experiments to test the activity of histone acetyltransferases on H3.

Btn-H3(1-25)-NH_2

Biotin-ART KQT ARK STG GKA PRK QLA TKA A-NH_2

Molecular Weight: 2851.3820

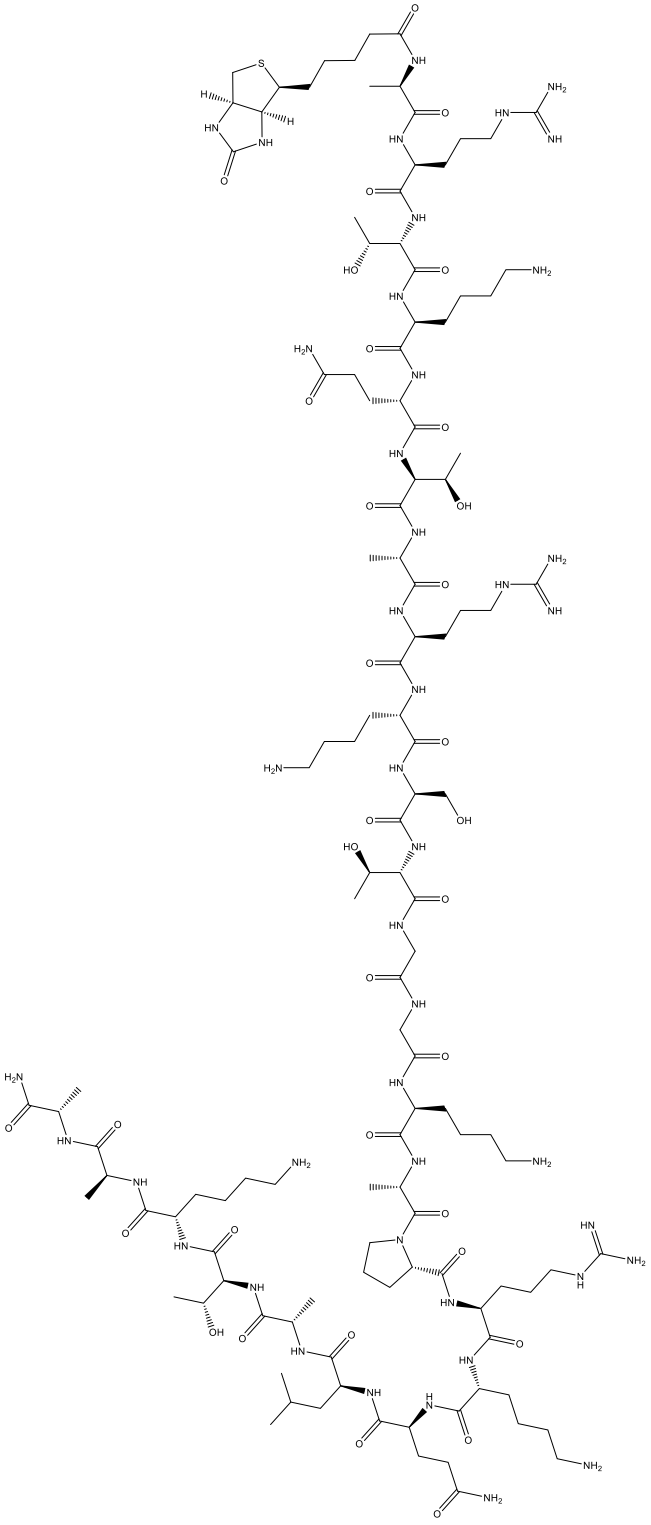

Figure 3.1 Btn-H3(1-25)-NH_2. The structure of the histone peptide is shown here. The peptide includes residues 1-25 from the N-terminus of the H3 histone with biotin attached to the N-terminus.

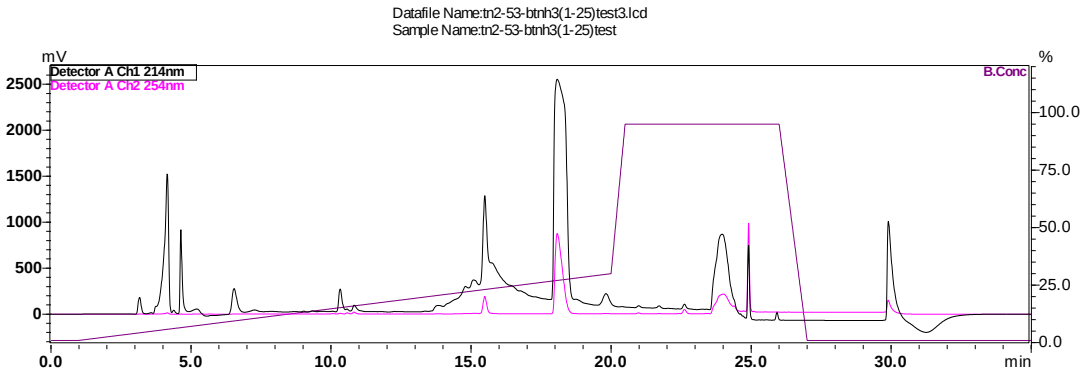

Figure 3.2 Analytical HPLC of test cleavage Btn-H3(1-25)-NH_2. The analytical chromatogram shows one major peak with a retention time of 17.9 minutes. The method used had a gradient of 1-30% of mobile phase B to mobile phase A in which mobile phase B was acetonitrile with 0.1% trifluoroacetic acid and mobile phase was water with 0.1% trifluoroacetic acid.

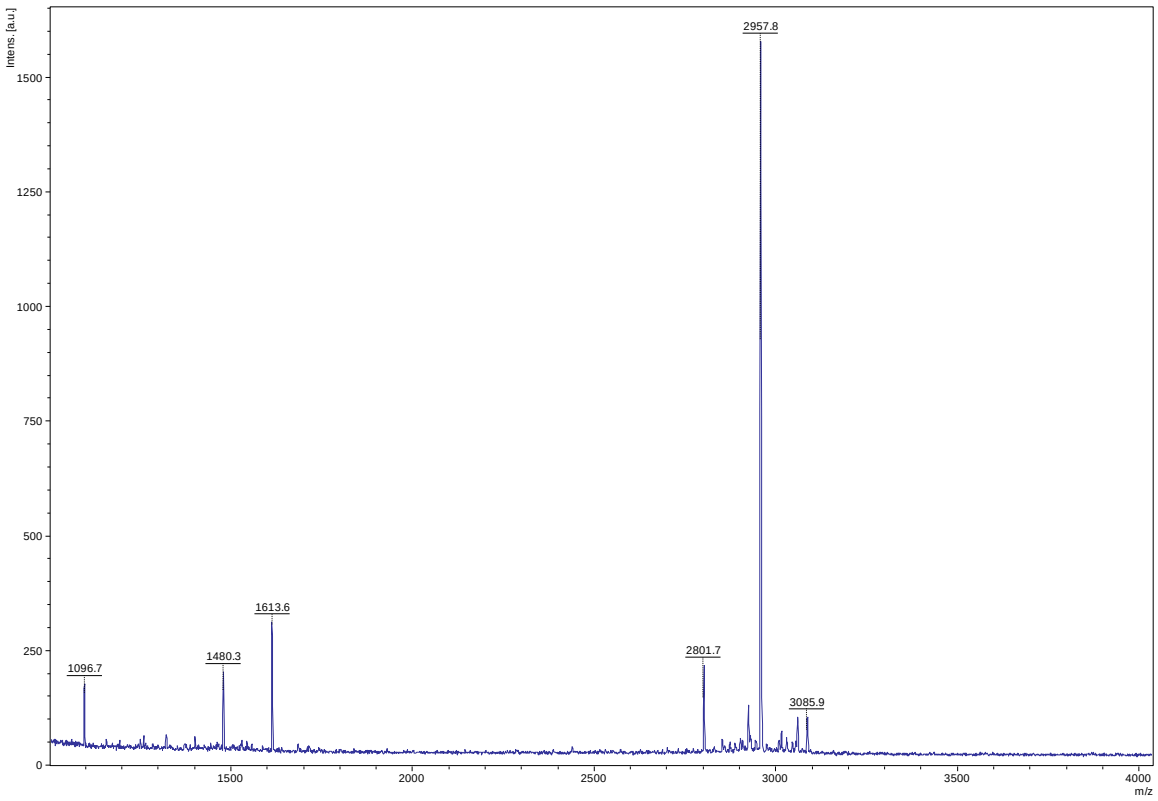

Figure 3.3 MALDI MS of the peak with 17.9-minute retention time. MALDI MS shows that this peak contains $[M+106]^+$. This may suggest that the linker from the resin has not been fully cleaved off.

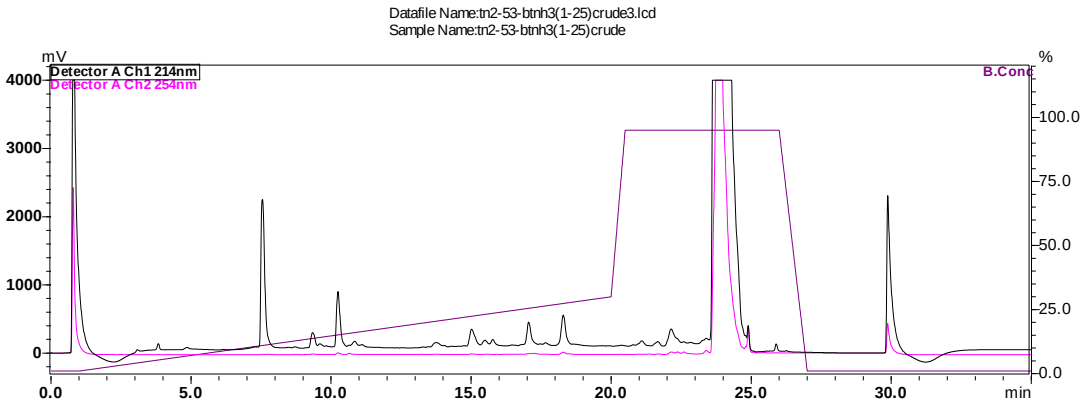

Figure 3.4 Analytical HPLC of crude Btn-H3(1-25)-NH_2. The analytical chromatogram shows one major peak that has a retention well into the wash phase of the method. The high 254 nm absorbance suggests that this may be the peptide with some protection groups on the side chains. The method used had a gradient of 1-30% of mobile phase B to mobile phase A in which mobile phase B was acetonitrile with 0.1% trifluoroacetic acid and mobile phase was water with 0.1% trifluoroacetic acid.

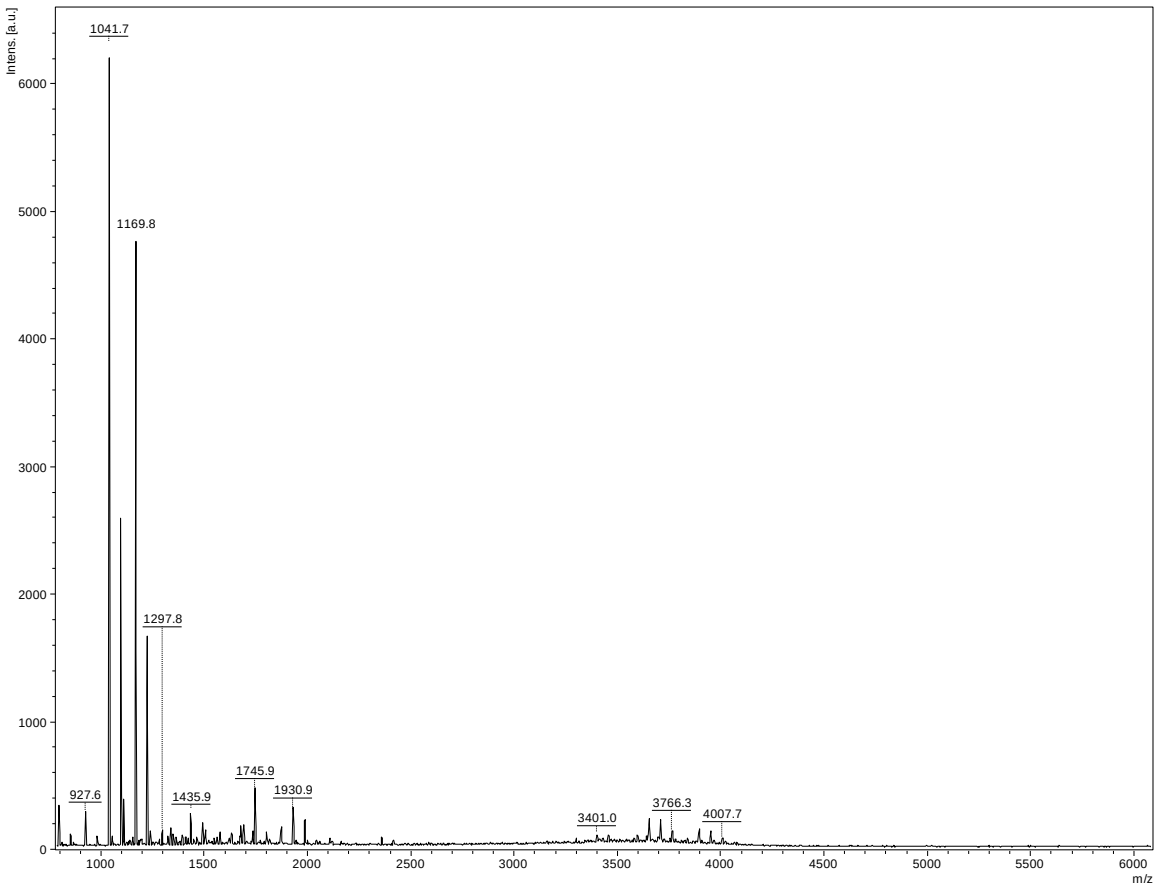

Figure 3.5 MALDI MS of the crude product. MALDI MS shows that the crude does not contain the product.

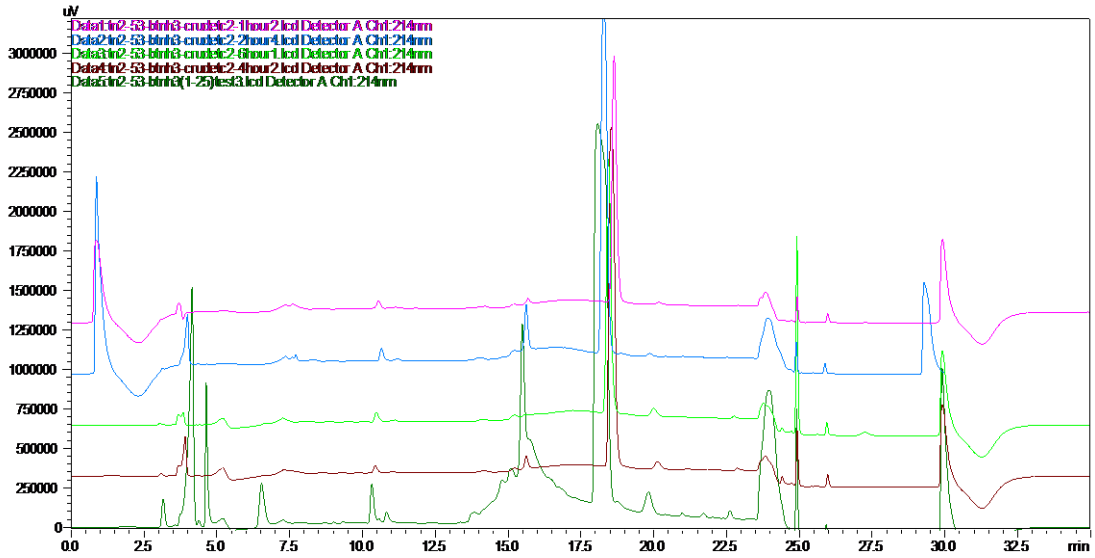

Figure 3.6 Analytical HPLC of secondary cleavage cocktail at various time points. The analytical chromatogram shows that the addition of cleavage cocktail for a second time results in a peak similar to that of the test cleavage product. There was no noticeable difference between the reaction times used. The method used had a gradient of 1-30% of mobile phase B to mobile phase A in which mobile phase B was acetonitrile with 0.1% trifluoroacetic acid and mobile phase was water with 0.1% trifluoroacetic acid.

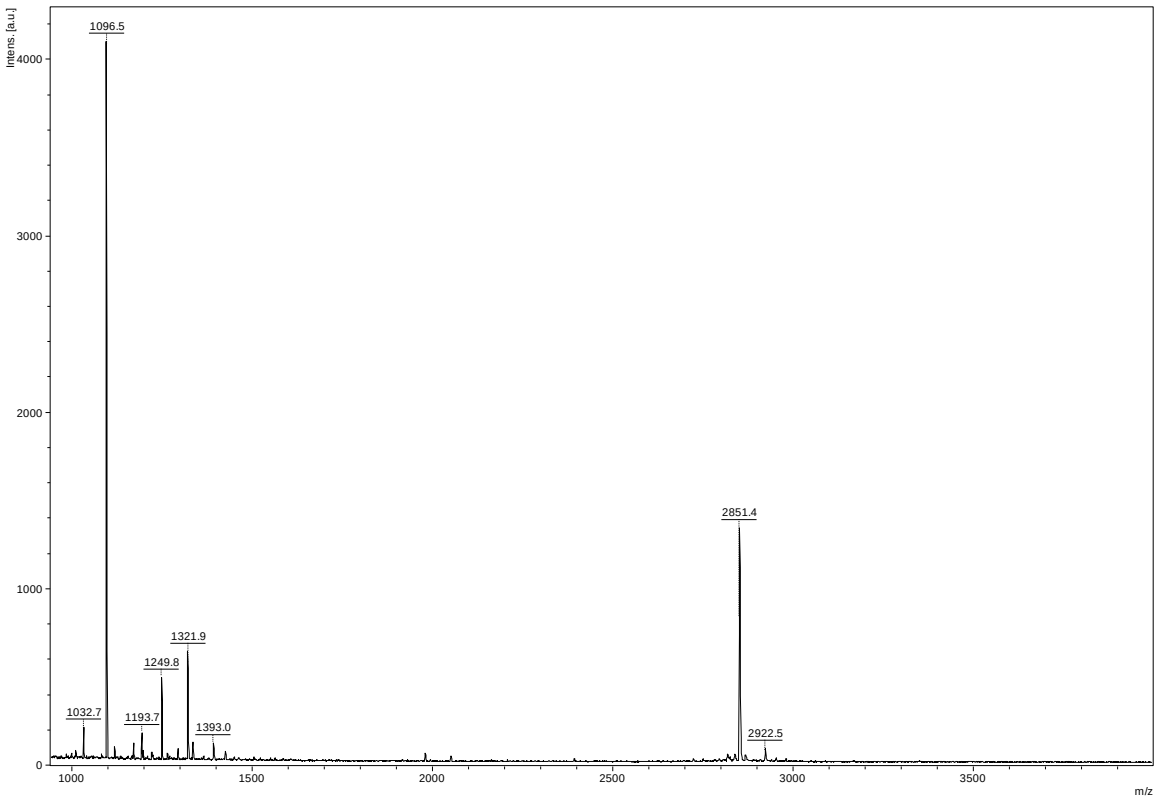

Figure 3.7 MALDI MS of the peak with 18-minute retention time after 2 hours. MALDI MS confirms that this peak contains the product. The second addition of the acidic cocktail seems to have removed what was thought to be the linker from the resin as $[M+106]^+$ is not seen in the spectrum.

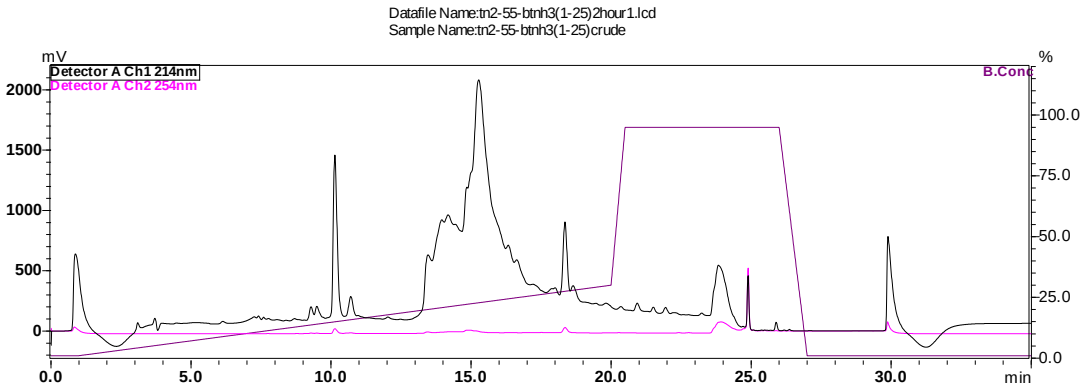

Figure 3.8 Analytical HPLC of crude Btn-H3(1-25)-NH_2 after 1 hour reaction. The analytical chromatogram shows two major peaks with retention times similar to that of the test cleavage product. However, the peak around 18.1 minutes is noticeably smaller than that of the peak at 15.1 minutes in stark contrast compared to the test cleavage product.

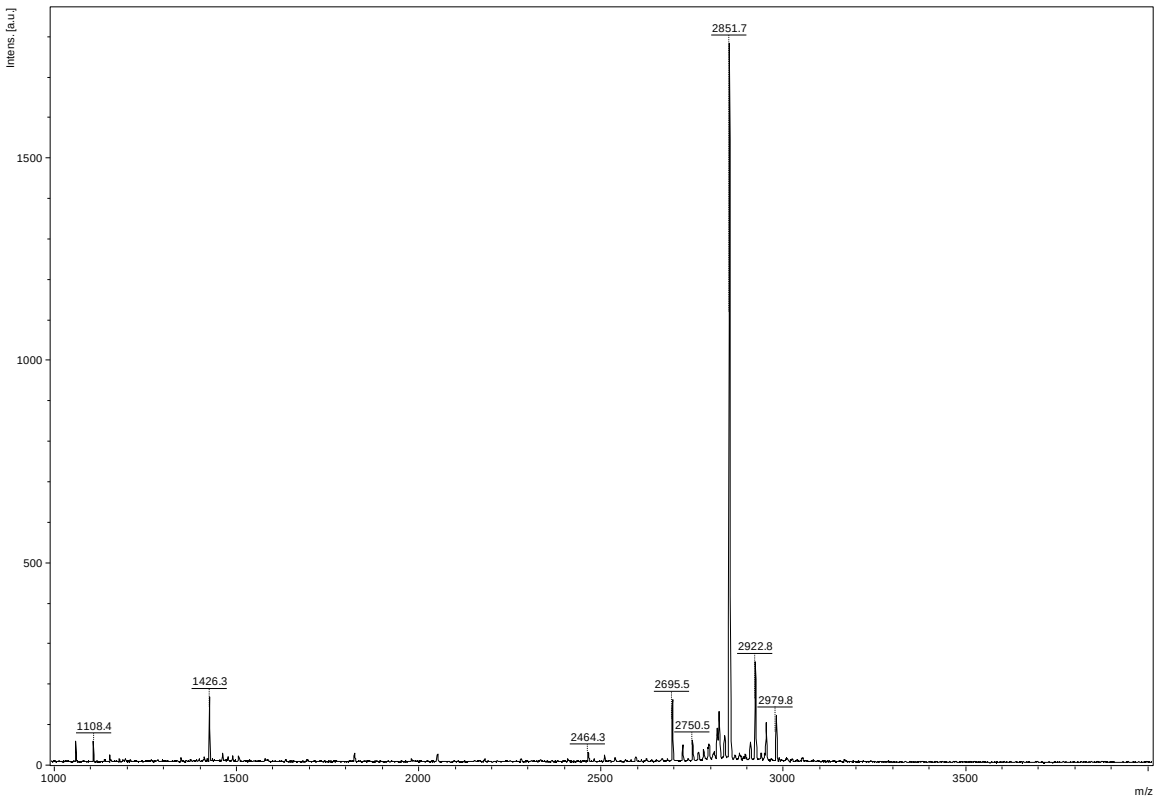

Figure 3.9 MALDI MS of the peak with 15.1-minute retention time. MALDI MS confirms that this peak contains the product.

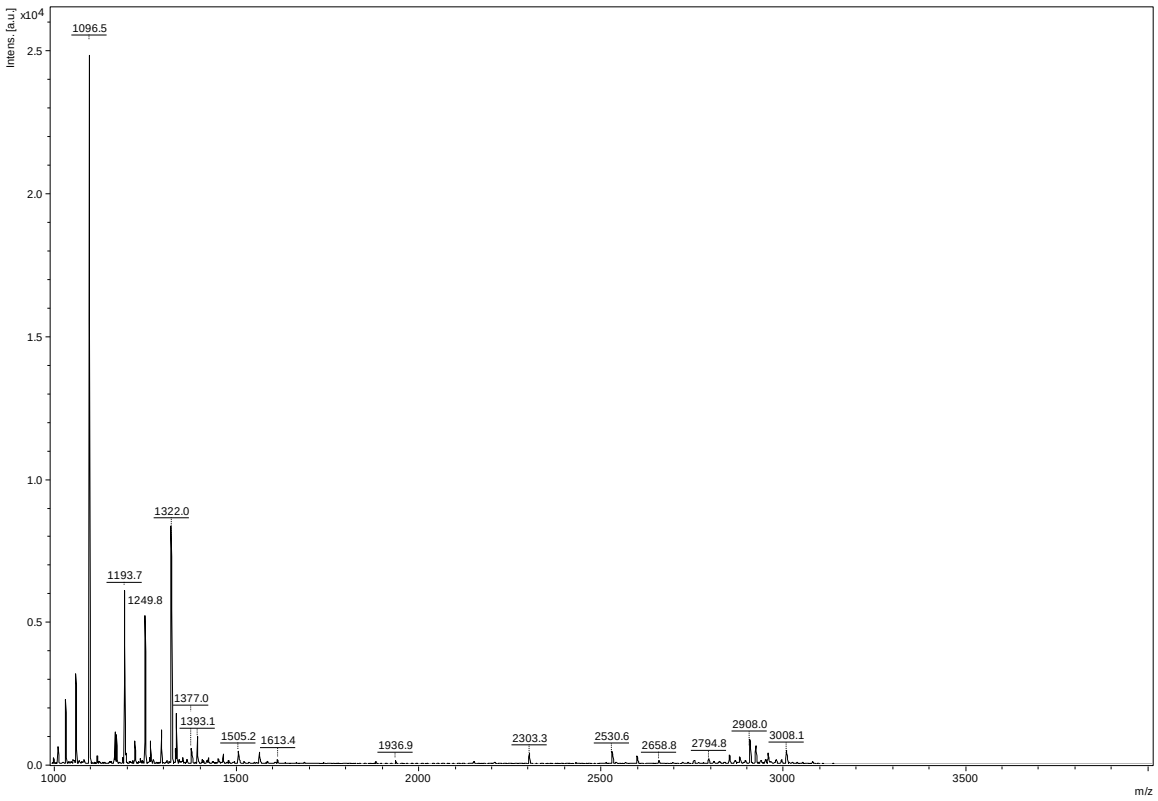

Figure 3.10 MALDI MS of the peak with 18.1 -minute retention time. MALDI MS confirms that this peak does not contain the product.

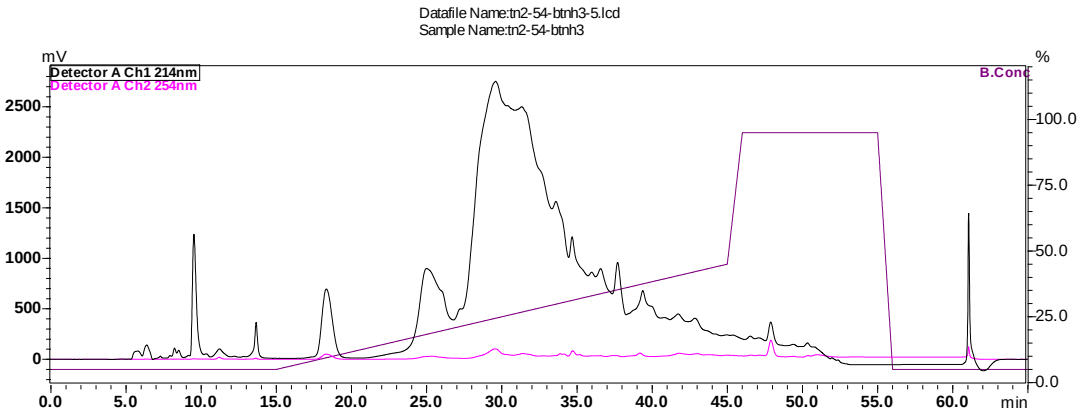

Figure 3.11 Semi-preparative HPLC of the crude product. Purification of the peptide resulted in the chromatogram shown here. The method used had an isocratic portion at 5% mobile phase B to A followed by a gradient of 5-45% of mobile phase B to mobile phase A in which mobile phase B was acetonitrile with 0.1% trifluoroacetic acid and mobile phase was water with 0.1% trifluoroacetic acid.

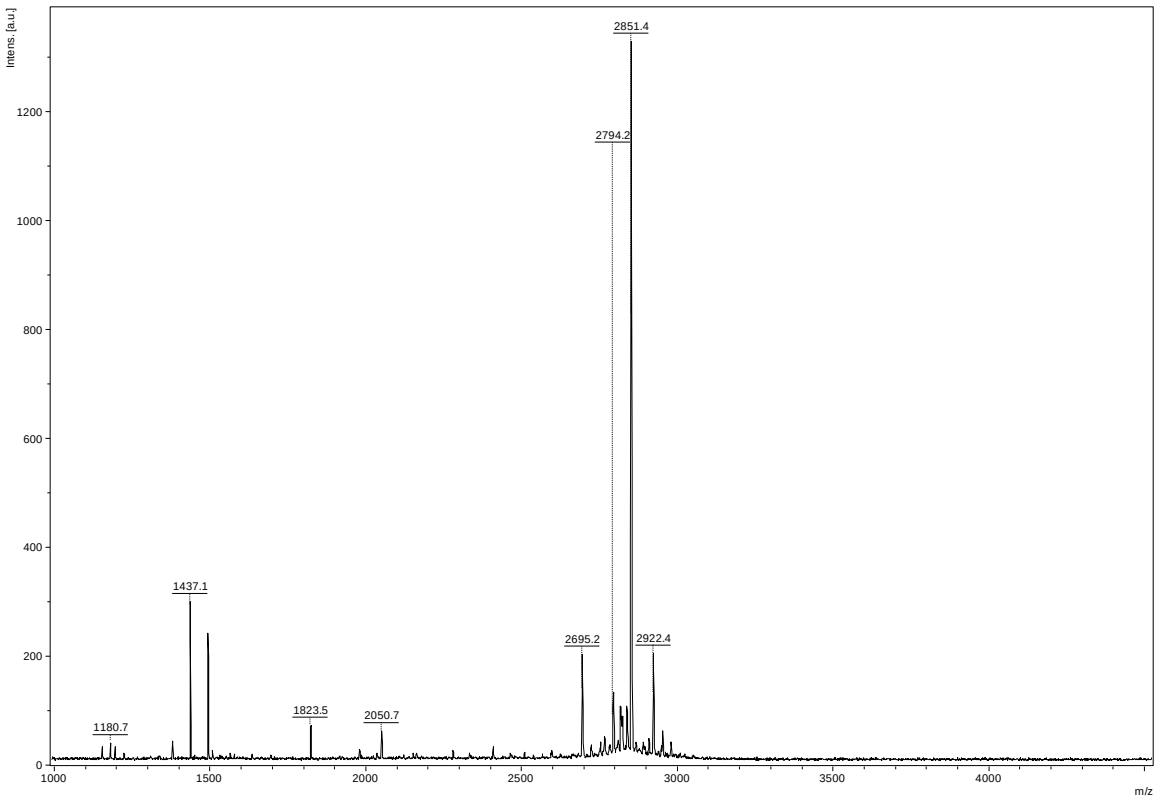

Figure 3.12 MALDI MS of the peak with 31.0-minute retention time. MALDI MS confirms the presence of the product in the second crest of the major peak.

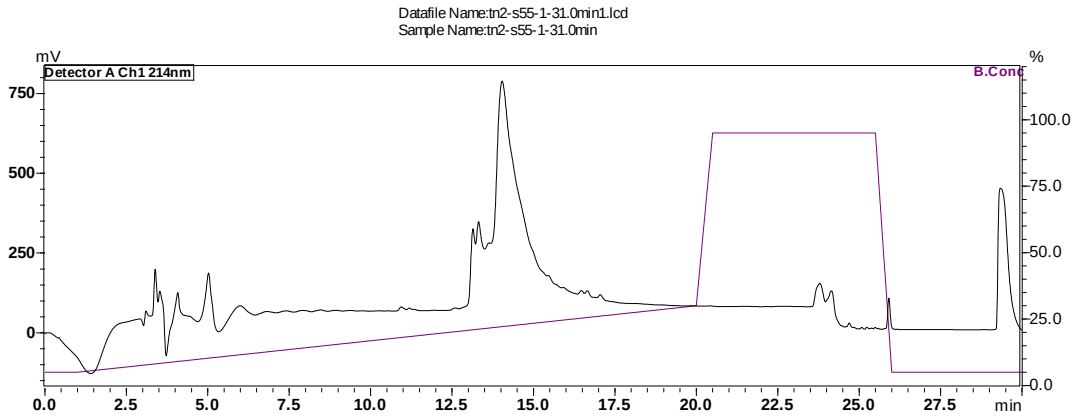

Figure 3.13 Analytical HPLC of the fractions collected around 31 minutes.

The analytical chromatogram shows that the collected fractions are not pure. The product must undergo a second round of purification.

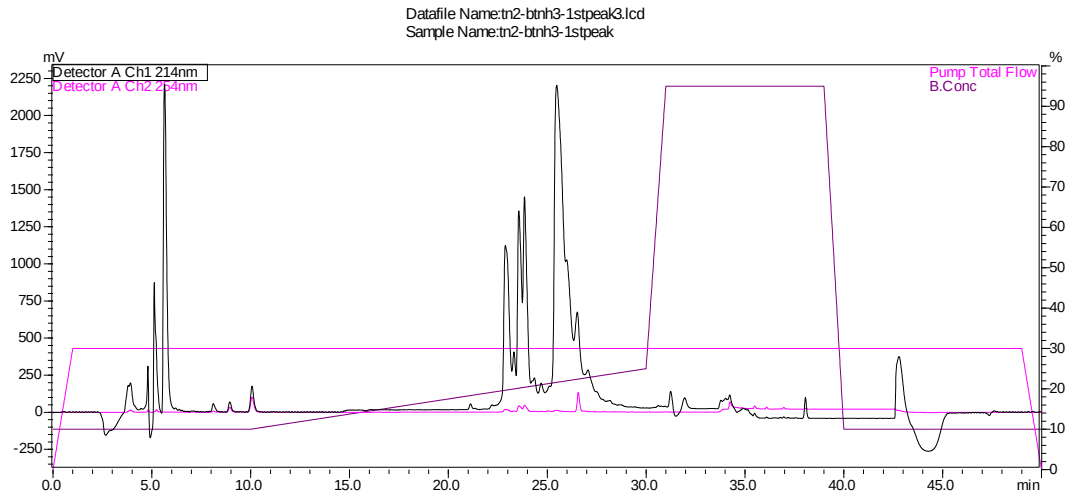

Figure 3.14 Second purification of the crude product. Purification of the peptide with the Agilent Eclipse column resulted in the chromatogram shown here. The method used had an isocratic portion at 10% mobile phase B to A followed by a gradient of 10-25% of mobile phase B to mobile phase A in which mobile phase B was acetonitrile with 0.1% trifluoroacetic acid and mobile phase was water with 0.1% trifluoroacetic acid.

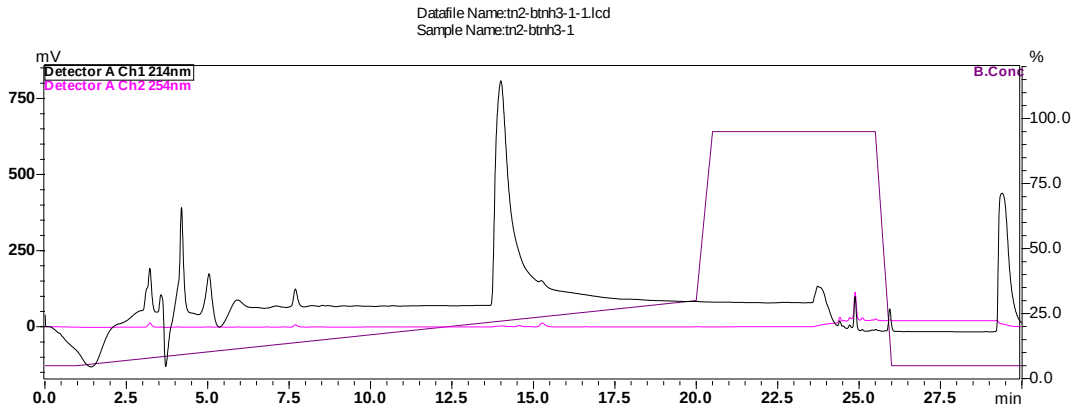

Figure 3.15 Final analytical HPLC of the product. The final product has 97.36% purity as determined by analytical HPLC. The method used a gradient of 5-30% of mobile phase B to mobile phase A in which mobile phase B was acetonitrile with 0.1% trifluoroacetic acid and mobile phase was water with 0.1% trifluoroacetic acid.

REFERENCES

1. Huang, H., Lin, S., Garcia, B. A., & Zhao, Y. (2015). Quantitative proteomic analysis of histone modifications. *Chemical Reviews*, *115*(6), 2376–2418. https://doi.org/10.1021/cr500491u

2. Chen, B.-J., Lam, T. C., Liu, L.-Q., & To, C.-H. (2017). Post-translational modifications and their applications in Eye Research. *Molecular Medicine Reports*, *15*(6), 3923–3935. https://doi.org/10.3892/mmr.2017.6529

3. Christensen, D. G., Xie, X., Basisty, N., Byrnes, J., McSweeney, S., Schilling, B., & Wolfe, A. J. (2019). Post-translational protein acetylation: An elegant mechanism for bacteria to dynamically regulate metabolic functions. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.01604

4. Bowman, G. D., & Poirier, M. G. (2014). Post-translational modifications of histones that influence nucleosome dynamics. *Chemical Reviews*, *115*(6), 2274–2295. https://doi.org/10.1021/cr500350x

5. Li, K. K., Luo, C., Wang, D., Jiang, H., & Zheng, Y. G. (2012). Chemical and biochemical approaches in the study of histone methylation and demethylation. *Medicinal Research Reviews*, *32*(4), 815–867. https://doi.org/10.1002/mrr.20228

6. Amblard, M., Fehrentz, J.-A., Martinez, J., & Subra, G. (2006). Methods and protocols of modern solid phase peptide synthesis. *Molecular Biotechnology*, *33*(3), 239–254. https://doi.org/10.1385/mb:33:3:239

7. Kates, S. A., & Albericio, F. (2000). *Solid-phase synthesis: A practical guide*. Marcel Dekker.

8. Moss, J. A. (2005). Guide for resin and linker selection in solid-phase peptide synthesis. *Current Protocols in Protein Science*, *40*(1). https://doi.org/10.1002/0471140864.ps1807s40

9. Amblard, M., Fehrentz, J.-A., Martinez, J., & Subra, G. (2007). Methods and protocols of modern solid-phase peptide synthesis. ChemInform, 38(30). https://doi.org/10.1002/chin.200730226

10. KING, D. A. V. I. D. S., FIELDS, C. Y. N. T. H. I. A. G., & FIELDS, G. R. E. G. G. B. (1990). A cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis. International Journal of Peptide and Protein Research, 36(3), 255–266. https://doi.org/10.1111/j.1399-3011.1990.tb00976.x

11. Fields, G. B. (2001). Introduction to peptide synthesis. Current Protocols in Protein Science, 26(1). https://doi.org/10.1002/0471140864.ps1801s26

12. Garcia, B. A., Hake, S. B., Diaz, R. L., Kauer, M., Morris, S. A., Recht, J., Shabanowitz, J., Mishra, N., Strahl, B. D., Allis, C. D., & Hunt, D. F. (2007). Organismal differences in post-translational modifications in histones H3 and H4. *Journal of Biological Chemistry*, *282*(10), 7641–7655. https://doi.org/10.1074/jbc.m607900200

13. Stathopoulos, P., Papas, S., & Tsikaris, V. (2006). C-terminaln-alkylated peptide amides resulting from the linker decomposition of the rink amide resin. A new cleavage mixture prevents their formation. *Journal of Peptide Science*, *12*(3), 227–232. https://doi.org/10.1002/psc.706