DESIGN OF A SCREENING PROCESS FOR THE DEVELOPMENT OF LEAD TARGET MOLECULES VIA SPOT SYNTHESIS AND SOLID-PHASE PEPTIDE SYNTHESIS

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By

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ABSTRACT

We have initiated the development of a screening platform to design a library of small molecules on the same solid support surface. This solid support surface, and the chemistry involved, can be utilized as a means of developing lead target molecules, namely ligands and catalysts. Evidence shows the successful assembly of both simple amino acids, as well as successful employment of our synthetic compounds. We support our efforts by showing compatibility for binding studies with larger macromolecules. Thus, intrigue remains by the prospects of this project. Challenges within our efforts are highlighted and emphasis is placed on presenting solutions to current issues, in order to attain further development. Notwithstanding difficulty, the desire to establish efficient processes for the discovery of lead target molecules and to ascertain the utility of our synthesized compounds, can be captured within this body of work. Lastly, the framework for continued efforts has been set to enable future progression.

ACKNOWLEDGEMENTS

Allow me first take the time to thank Christian Schafmeister, Ph.D. for all his support within this project, serving as my advisor, and for assisting in my overall matriculation at Temple University. A great deal of learning went into my personal and professional development prior to this very work; but, surprisingly all advancements can still be captured in this, my latest efforts. A special thanks also goes to all my colleagues and friends within and outside the laboratory. Lastly, I would be remiss if not to show gratitude toward the University, the College of Science and Technology, the Chemistry Department, and to the Graduate School for providing me the opportunity for academic advancement.

PREFACE

This work is being summarized for the purpose of providing an account of all worked performed. Please allow this summary, in form of a thesis, to guide any future work related to these efforts. I afford my findings and understanding in written form, so that the laboratory and students of Christian Schafmeister, Ph.D. may continue in its development.

LIST OF FIGURES

LIST OF ABBREVIATIONS

TFA	Trifluoroacetic Acid
DCM	Dichloromethane
TsCl	Tosyl Chloride
PEG	Polyethylene Glycol
Fmoc	Fluorenylmethyloxycarbonyl
Boc	*tert*-Butoxycarbonyl
EtOH	Ethanol
Pip	Piperidine
GC/MS	Gas Chromatography/Mass Spectrometry
LC/MS	Liquid Chromatography/Mass Spectrometry
MeOH	Methanol
TIPS	Triisopropylsilane
EtOAc	Ethyl Acetate
KCN	Potassium Cyanide
IPA	Isopropyl Alcohol
NH_4OAc	Ammonium Acetate
THF	Tetrahydrofuran

CHAPTER 1.0

INTRODUCTION AND OBJECTIVE

Over recent years, there has been an increased demand to establish efficient processes for the development of improved screening platforms[1]. The development of lead targets is an essential step in the discovery of new drug therapies[2], novel catalysts, protein ligands and chemical sensors. The ability to generate high-throughput synthesis in combination with combinatorial chemistry enables such discovery(s). A means to this overall goal is the utility of Solid-Phase Peptide Synthesis (SPPS) technology on cellulose support. Strictly, we employ the fundamentals of SPOT Synthesis technology, in an attempt to assemble rationally designed compounds in a spatially-addressed manner. Involved in our efforts are concepts from peptide synthesis, medicinal, combinatorial, computational and organic chemistry, as well as biotechnology.

Numerous functions within living organisms incorporate peptides to play a vital role in the facilitation of specific functions; in addition, peptides can work in concert with other biomolecules. For instance, Calcitonin acts as an antagonist to reduce blood calcium levels (Ca^{2+}), by opposing the effects of parathyroid hormone (PTH). Also, C-peptide works to facilitate the efficient folding, assembly, and processing of insulin in the endoplasmic reticulum. An understanding of the pivotal roles that peptides play in living organisms is essential in gaining further insight into biochemical pathways. Peptide chemistry becomes an essential tool in our attempt to understand the role of natural peptides and antibodies[3], *in vivo*, as well as enabling a means for the stimulation and/or attenuation of natural biochemical pathways. Furthermore, assembling peptides allows

scientists the opportunity to explore the spatial three-dimensional presentation of functional groups. This presentation is often unique allowing specific protein-protein and protein-small molecule interactions. Chemists and biologists alike, can explore how changes in functional groups alters the effectiveness of a drug, ascertain vital information for catalyst development, and explore advancements chemical sensors.[4]

Combinatorial chemistry remains as a leading tool in many industries focused on the development of target molecules, namely pharmaceutical. At this juncture, our work expresses the same driving desires to achieve lead targets that are synthetically easy to foster and are medically useful. This chemistry is a reliable tool for ascertaining chemical and physical properties,[5] and gauging intrinsic factors involved in protein-protein and protein-ligand interactions. Combinatorial chemistry is often used for the development of catalysts as well. In a spatially addressable or "spotted" manner, we set to build libraries of molecules for such purposes.

We have also reconnoitered the capacity for binding studies on cellulose membrane. With assistance from collaborators, we employ laser electrospray mass spectrometry technology[6,7] (LEMS). By utilizing this technique, we can demonstrate that non-covalently bound macromolecules can be extricated from a solid support surface, and provide analytical evidence of an intact macrostructure. These results show promise for future binding studies. Herein, our support surface is a fully functionalized cellulose-based filter paper, via a series of required chemical reactions. To this point, we are in preparation for the design and synthesis of molecules on filter paper; subsequently, we will investigate binding studies of complimentary biomolecules (of known and unknown affinity) to demonstrate the utility of our methods.

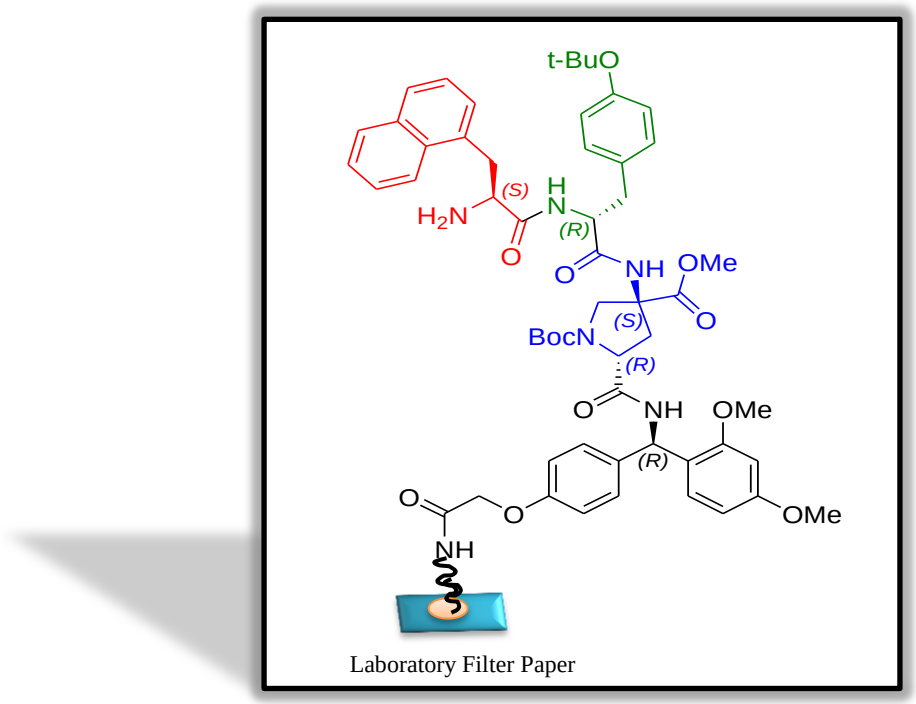

Figure 1.0 Illustrates our efforts within this project to build libraries of molecules and rationally design target compounds in a spatially addressable manner.

The current works presented here, have been inspired by the contributions of others within the realm of Spot Synthesis as well as Solid-Phase Peptide Synthesis. In our attempt to adjoin the two methodologies, we will further utilize concepts from medicinal and combinatorial chemistry as we continue exploration in developing high-throughput methods for producing lead compounds. We are excited about the prospects of this work and its applications; however, we sort through challenges that currently exist. Within this body of work, we will present the advances achieved, as well as highlight the difficulties observed, thus far. Instructively, insight will be afforded to help understand the challenges we currently face, and we offer possible solutions for future progress. Moreover, the efforts continue and the intrigue remains as new developments arise both in our lab and within related fields.

CHAPTER 2.0

BACKGROUND

2.1 Spot Synthesis

The field of Spot Synthesis is indebted to many early scientists who developed these efforts, but the field still remains an exciting, yet young area of study. The development of parallel synthesis of large numbers of "positionally addressable" peptides, in small amounts, was coined by Ronald Frank[8] in 1992. Peptide synthesis on cellulose support is becoming a common means for establishing chemical libraries of molecules. Each molecule is assembled on a particular "spot" and can be unique from other spots on the solid support. A spot is the area of the filter paper membrane (often times in the shape of a circular disk) that consists of the particular product being developed. Spot sizes can be controlled by the volume of material aliquoted on to the support.

Spot Synthesis is a technology that enables researchers to build vast combinatorial chemistry libraries on the same solid support. It is a process to assemble molecules in a spatially addressable manner. The solid support surface is typically laboratory filter paper. Part of the most notable attributes of this technology is that filter paper is very inexpensive and can usually be found in most laboratories already. These solid supports can be composed of polypropylene, polyester, and glass microfiber, but most commonly researchers utilize cellulose-based membranes (containing carbohydrates). It is important to use filter paper supports that are robust to synthesis, and determining which support is most amendable to the conditions throughout the synthetic pathway helps avoid any

hindrances in reaction rates and issues in purity. Furthermore, specialization in developing functionalized and modified versions of these supports (and others) is rapidly becoming available. Therefore, a variety of solid supports can be employed to enable a wide range of developments in chemical, physical, and biological areas of study.

Establishing the most suitable functionalization, or derivatization[8], of the solid support is paramount in the overall synthetic scheme, and is undertaken first in the process. Matthew D. Bowman[9] (along with studies by Helen Blackwell) demonstrates the development of strategies to enable successful synthesis of molecules using amide bond formations on cellulose filter paper. Upon functionalization, a linker can be attached to the support prior to synthesis of the desired product. Assortments of linkers are available to allow recovery of assembled products, by employing conditions sensitive to cleavage, namely basic, acidic or photo-induced conditions.

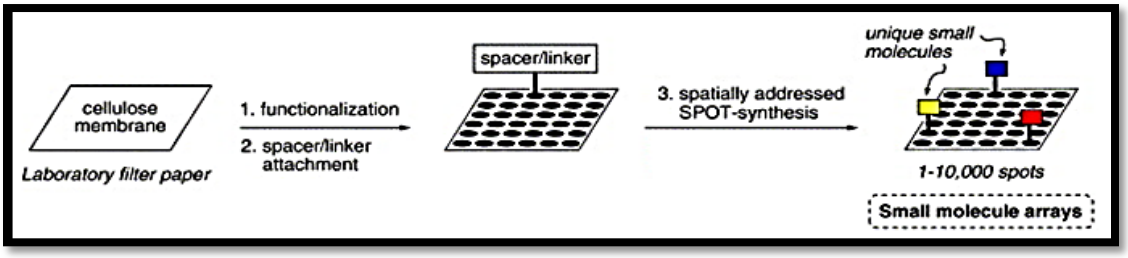

Figure 2.0 Shows the functionalization of a cellulose-based membrane, which is a key step in the process. Assembly of molecules is then undertaken in a spatially addressed manner. Adapted from reference 9.

In comparison to more traditional chemistry on solid support, i.e. polymeric resins and beads, filter paper membranes may yield less end-product, but researchers have the luxury of generating replicate spots on the same support, which affords more of the desired material. Another aspect of this technology is the advantage in time investigators save in assembling a plethora of molecules at one time on our support versus one product

at a time using other solid support systems. Additionally, filter paper membranes can be used for biological assays. Spot Synthesis began with assembling molecules using pre-activated α-amino acids and often times the same measures are still re-enacted to this day; however, more diverse approaches are being reconnoitered with different materials and the overall scope of this field is ever-increasing.

2.2 Solid-Phase Peptide Synthesis

Solid-Phase Peptide Synthesis (SPPS) is a technology introduced by Robert Bruce Merrifield[10] in 1963. Since its onset, many researchers have utilized this technology for both the manual and automated synthesis of various peptides and oligomers, as well as to develop analogues of natural peptides. We incorporate these syntheses and the assembly of both naturally occurring α-amino acids along with synthetic analogues. Assembly of short and longer chained oligomers can be generated with ease, in good yield, and with acceptable purity[10]. Furthermore, there are additional end-process measures to ensure purity, i.e. extractions, liquid chromatography, and lyophilization. We utilize such techniques in the synthesis of our functionalized oligomers.

We strive to produce various peptides and, in particular, we focus on the development of analogues of many natural oligomers in a scaffolding approach. We envision the generation of protein mimics that can serve as targets to natural proteins and other macromolecules. Moreover, we currently explore the design of three-dimensional binding pockets and forming macrostructures. Giving rise to these imaginings is our continued usage of functionalized proline monomers. With the use of chiral starting materials, we can chemically modify 4-hydroxy-proline to provide additional

stereocenters, present functionality in particular orientations, and by using polymeric resin supports further link together, or "scaffold," multiple monomeric compounds in a shape-programmable manner[11]. These molecules have been coined "building blocks."

Paramount to the rational design of combinatorial chemistry libraries is the inherent properties of each compound, particularly when assembling molecules in our scaffolding approach. Miklos Feher and Jonathan M. Schmidt[12] note an important aspect in the development of drug candidates: combinatorial chemistry libraries suffer from a lack of chirality and structure rigidity, both being widely regarded as drug-like properties. A solution we offer is that upon coupling our designed compounds, in a series fashion, we can form a rigid backbone dissimilar to natural oligomers, by forming diketopiperazine (DPK) rings. Herein, a given two monomers form two linkages via amide bonds to afford a 6-membered ring, wherein each monomer can bear protecting groups and functionalized side chains. This backbone formed can afford the appropriate rigidity to hold functional groups in particular orientations. In addition, chirality is instilled within all monomers and therefore throughout the assembly of rationally designed oligomers. Epimerization can be mitigated throughout the synthesis pathways and circular dichroism (CD) can be employed to assess secondary structures. Importantly, we can present various functional groups in defined three-dimensional presentations to control shape, simulate receptors and binding pockets, and along with Fluorescence Polarization studies ascertain binding affinities between ligands and macromolecules. Moreover, the formed diketopiperazine bonds are distinct from typical amide bonding seen in natural peptides. With this in mind, we hope to modulate endogenous biochemical processes with our peptide mimics.

Critics of traditional SPPS technology are concerned that many polymeric resins are expensive, possess loading issues, and have little to no capability with binding studies.[13] To satisfy these concerns, we feel that adjoining SPPS and Spot Synthesis technologies will grant us the capability to perform successful binding studies with macromolecules on a reliable solid support, and cellulose filter paper (along with other types of supports) is very inexpensive and common to the laboratory. Furthermore, modified versions of filter paper membranes are readily available from a number of vendors to address specific chemical and biological demands, namely Fisher Scientific, Whatman Ltd., and AIMS Scientific.

CHAPTER 3.0

THE REACTION SURFACE –

FUNCTIONALIZATION AND QUANTITATION

3.1 Introduction

In an effort to utilize common laboratory filter paper in the scope of this project, we set to amend this material in such a manner to present functional groups that are suitable to our chemistry, i.e. forming amide bonds. This chemistry, as stated above, is utilized in traditional peptide synthesis. Herein, we focus on using commercially available α-amino acids and our synthetic compounds (bis-amino acids, see Chapter 5.0).

Within the field of Spot Synthesis, researchers commonly use few different types of solid supports to engage their synthesis. This can include polypropylene and cellulose-based membranes, as well as several modified versions of these filter papers. In particular, cellulose membranes are composed of carbohydrates bearing terminal hydroxyl groups. Lastly, we show in this work the most appropriate filter paper support for our particular synthetic demands: Whatman Grade 540.

3.2 Results and Discussion

We commence functionalization by the swelling of the filter paper to ensure the membrane is ready for chemical modification.[9] This can be done with 20% TFA/DCM for 30 minutes (min). The tosylate is next formed to serve as a good leaving group, and is generated with a concentrated solution of Tosyl Chloride in Pyridine for 1-2 hours at room temperature (RT). With this in hand, we set to attach a 15-atom polyethylene glycol

(PEG) spacer. Satisfactorily, the spacer can be attached in good yield as a heat-assisted reaction at 80°C (Celsius). To achieve such, the filter paper is submerged in a pre-heated, neat solution of a diamine compound (4,7,10-Trioxa-1,13-tridecanediamine). The reaction is allowed to proceed for 30-40 minutes. We suspect that a key step in controlling the loading levels of PEG spacer maybe incumbent upon the duration of tosylate formation. With this functionalization now effected, we seek to quantify our loadings using standard quantitation methods observed in traditional solid-phase peptide synthesis on resin.

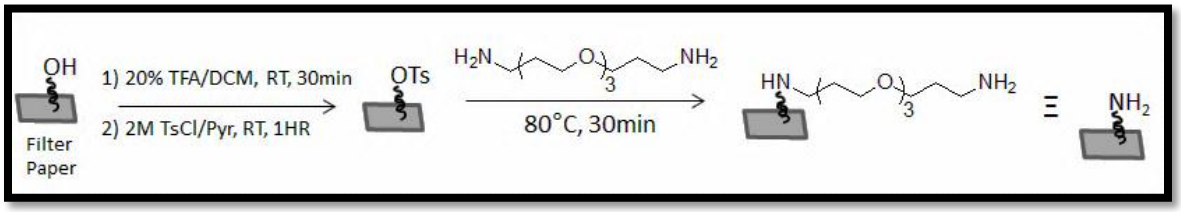

Figure 3.1 Depicts the functionalization of laboratory filter paper. The membrane is swelled then undergoes tosylation; finally, PEG Spacer is assembled. Upon this process, we prepare our solid support for peptide-array synthesis.

Using commercially available 9-fluorenylmethyl succinimidyl carbonate (FMOC-OSu), we can quantify PEG spacer loadings, and determine to which extent we want to afford amine formation. A small portion of the filter membrane can be cut out, and the area of the dimensions is ascertained. This piece of filter paper was then submerged in a prepared solution of FMOC-OSu for two hours at RT. After thorough washings and drying (see experimental section), we can use Ultraviolet-Visible (UV-ViS) Spectroscopy to measure the amount of Fmoc groups released from the solid support. A solution of 20% Piperidine in DMF will achieve release of the Fmoc group, resulting in a fulvene-Piperidine adduct for each deprotonation of Fmoc. It is well known, and can be observed

here, that this adduct absorbs quite well in the UV-Vis spectrum, possessing a λ_{MAX} at 301 nanometers (nm). Effectively, we witness the appropriate quantitation and can be confident in our results.

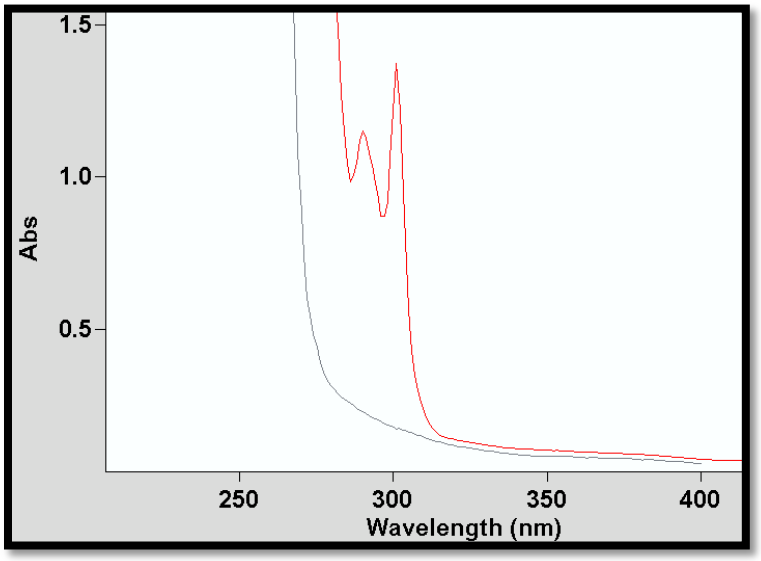

Figure 3.2 Displays the quantitation of amine loadings on filter paper support (Whatman, Grade 540). This is enacted by the release of Fmoc group from the solid support using 20% Pip/DMF for 20min. The resulting fulvenve-piperidine adduct absorbs with a λ_{MAX} at 301nm, as shown in the red line (blue line is solvent baseline from 20% Pip/DMF).

We obtain loadings within the range of 4-9 µmol/cm^2 (using Whatman Filter paper, Grade 540). Upon varying the length of time for tosylation, we can observe differences in amine yields. When tosylate formation proceeds for approximately 1 hour (HR), loadings between 4-5 µmol/cm^2 are attained. Allowing tosylation time to continue for 1.5-2 hours, we obtain loadings of 6-9 µmol/cm^2. Additional Fmoc quantitation can achieve the same results within 5.0% error. Therefore, our results show distinct differences in the quantity of fulvene released, dependent on the amount of time tosylation is allowed to occur.

3.3 Conclusion

We have achieved the successful functionalization of common laboratory filter paper, now possessing the capability to prove useful in our synthetic measures. This functionalization sets the stage for further synthesis using peptide-array chemistry. Interestingly, we anticipated loading levels to be influenced by tosylate formation, and this expectation has been witnessed throughout our experiments. Thus far, the filter paper remains intact. Originally, we could not be sure the effects that a given solvent or reagent would have on the integrity of our paper support; however, the membrane has withstood the conditions.

Importantly, the quantitation process that we utilize here, with Fmoc-protected compounds, invokes similar synthetic strategies and makes the same bonding formations that we look to employ in our attempts for peptide-array synthesis, e.g. amide bonds. The results to this point, show promise in that we can assemble compounds on our solid support, as well as quantify yields without disrupting the support itself. We remain intrigued by the straight forward nature thus far, and envision much to come.

3.4 Experimental Details

3.4.1 Functionalization

The laboratory filter paper used was a 20cm X 20cm (centimeter) Whatman paper, Grade 540 (purchased from Whatman Ltd.). Functionalization of a membrane support was conducted on an individual sheet, within a 1L (liter) Pyrex dish[14] (a second Pyrex dish was fitted to enclose the membrane).

Filter paper is submerged in 100.0mL of a 20% TFA/DCM solution, under agitation[15] for 30min. After solvent is decanted, the membrane is washed for 2min with 100.0mL of each: DCM (2X), EtOH (2X), DCM (2X), and finally dried under Argon (Ar) gas for 15 minutes. Argon gas (from Airgas, Inc.) was supplied to a vacuum chamber[16] that was purged of atmospheric air. Next, the support is submerged within 100.0mL of a 2M TsCl solution (38.13 grams TsCl dissolved in 100.0mL Pyridine), and allowed to react for 1 hour under agitation. Decant solution and wash with EtOH (2X) and DCM (2X), then dry under Ar gas. The membrane support is next submerged in a pre-heated solution: 100.0mL of 4,7,10-Trioxa-1,13-tridecanediamine (purchased from Sigma-Aldrich). This solvent is heated in an oven[17] for 30 minutes at 80°C, and then this neat solution is added to the Pyrex dish housing support. This reaction is allowed to proceed for 30 minutes in oven; wash membrane then dry under Ar gas. The final washings after diamine assembly are 100.0mL of each: DMF (2X), EtOH (2X), 1.0N NaOH, deionized (DI) H_2O, EtOH, and DCM (2X), then dried under Ar gas. This amine support is ready to be quantified. Note: longer tosylation times derive increased diamine loadings.

3.4.2 Quantitation

Individual 6cm X 6cm pieces of the functionalized amine support were cut out of the larger sheet (hole-puncher may also be used). The area of the cut out was determined, e.g. 0.36cm^2. Filter paper cut outs were isolated by enclosing each piece in its own 3.0mL glass vial (purchased from VWR International, LLC). Glass vials include a Teflon twist cap.

To the amine support, add a prepared solution of 0.60M Fmoc-OSu to the vial (0.0607 grams dissolved in 300.0µL DMF), agitate for 30sec (second), and allow 2HR reaction time. Wash with DMF (2X), Acetone (2X), then DCM (2X), and finally dry under Ar gas. Next, add 1.0mL of 20% Pip/DMF, agitate for 30 seconds, allow 20-30 minutes reaction time, and agitate well before proceeding., Aliquot 100.0µL of this solution to a 2.0mL volume of DMF in a separate glass vial; then agitate for 30 seconds. The absorbance at 301nm was measured with a Cary 50 BIO UV-Visible Spectrophotometer, Model EL02085625. A reference blank for the solvent was used by preparing 1.0mL of 20% Pip/DMF, then making a similar 20:1 dilution, which does not contain any fulvene. Subtract the background absorption from the solvent and record the OD (optical density) measured by the spectrophotometer; calculate loadings using Beer's Law (the extinction coefficient is $7800 M^{-1} \cdot cm^{-1}$). For further use, wash membrane support with DMF (2X), Acetone (2X), then DCM (2X), and finally dry under Ar gas.

CHAPTER 4.0

COMPATIBILITY FOR BINDING STUDIES

4.1 Introduction

To demonstrate the usefulness of our efforts, we employed an experiment early in this process that would allow us to gauge the scope of the overall project. We sought to test the compatibility of our support, and filter paper in general to binding studies. We want to determine if macromolecules can be released from a cellulose-based solid support, and provide analytical data to support such findings. Hence, we utilize a technique of Mass Spectrometry being developed in the field by many researchers: Laser Electrospray Mass Spectrometry[6,7] (LEMS).

LEMS is a technique that uses non-resonant femtosecond (fs) laser pulses to vaporize samples, and is coupled with subsequent electrospray post-ionization. This system enables mass analysis of macromolecules with limited sample preparation[6]. Making this technology attractive is the non-destructive nature of fs laser pulses. Unlike nanosecond (ns) laser pulses allowing materials to absorb thermal energy and cause decomposition, femtosecond laser pulses avoid this destructive behavior such that molecules remain intact. To date, utility of this technology is evident in the detection and analysis of peptides and pharmaceuticals compounds.[7,18] These advances are currently being demonstrated on solid support surfaces to include glass, wood, and steel. At this juncture, we set to complement our developing chemistry on cellulose filter paper with these exciting measures ongoing in LEMS technology.

4.2 Results and Discussion

We began our experiments using a fully functionalized amine support that we achieved (Section 3.4.1), along with an unmodified cellulose filter paper. In this, the functional groups presented in our study are primary amine and primary hydroxyl groups from our amine support and the unmodified cellulose paper, respectively. A common test protein used in such LEMS studies is the Hen Egg-White Lysozyme (HEWL). This lysozyme was dissolved TBS buffer (20mM Tris-base/100mM NaCl in Millipore H_2O, maintained at pH 7.4). A 1.0µL aliquot was spotted on to the surface twice. The protein was then subjected to different surface conditions to include maintaining a wet surface, drying the support, and re-wetting the support (post-drying). Lastly, a more common steel surface was used as well.

The LEMS system produces a profile or mass fingerprint of the macromolecule, similar to a mass spectrum trace of a small molecule in a GC/MS or LC/MS system, bearing a particular fragmentation pattern. Analysis of this fragmentation pattern allows the analyst to identify the appropriate structure, i.e. Hen Egg-White Lysozyme. Of significant importance is the fact that only the non-covalently bound macrostructure is evolved from the solid support, as the analyte contains no material from the support surface itself.

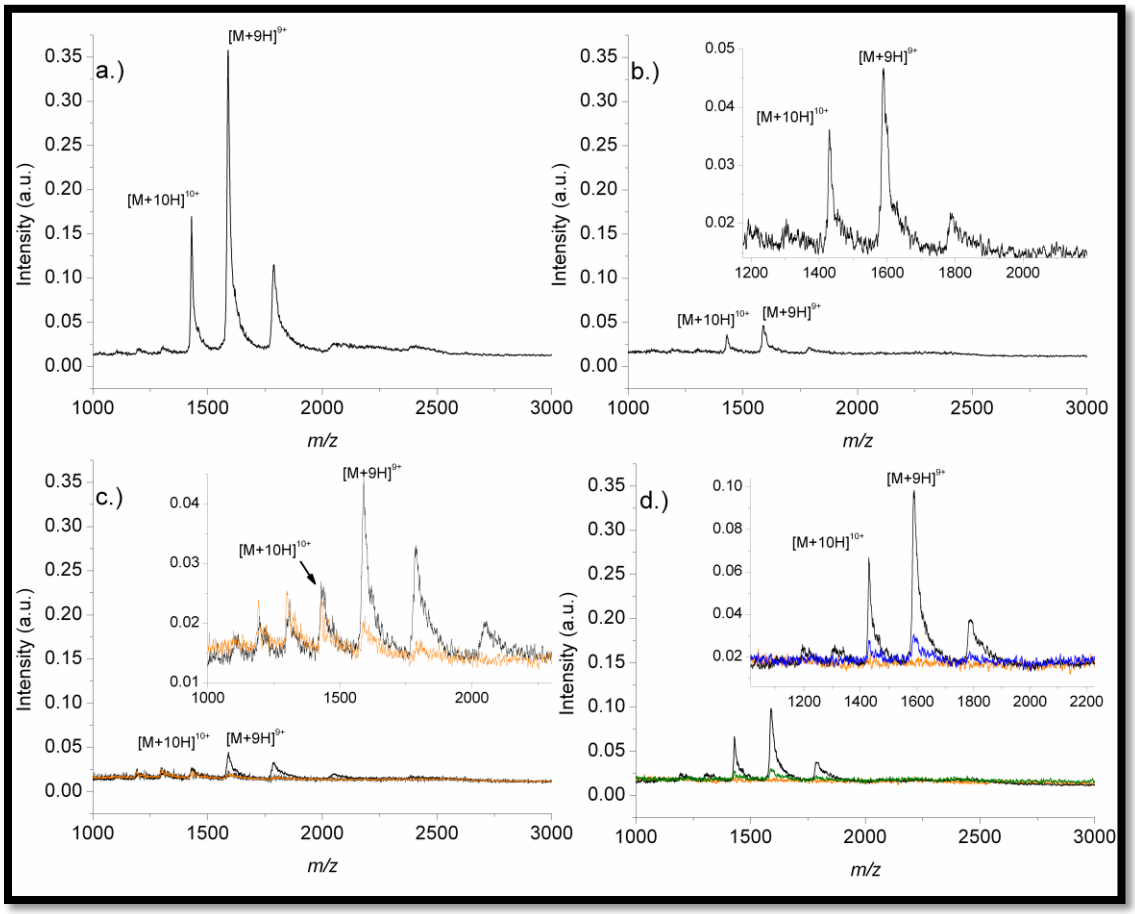

Figure 4.0 Displays lysozyme spotted on various solid surfaces, then vaporized under wet and dry conditions. a) Steel surface. b) Glass. Both steel and glass surfaces display characteristic [M+9H] and [M+10H] peaks. c) Functionalized membrane containing aqueous lysozyme (black) and dried (orange). Dried lysozyme shows reduced intensity and shifts in charged states to indicate denaturing. d) Untreated membrane contains aqueous lysozyme (black), dried (orange), and re-constituted (blue). No signal for dry conditions, but reduced signal when re-wetted.

Hen Egg-White Lysozyme (MW = 14,700g/mol) affords a mass spectrum with an expected mass fingerprint,[7] to include [M+9H] and [M+10H] peaks. We can use this data to elucidate the macromolecule. The steel surface along with both unmodified filter membrane and our functionalized cellulose support all demonstrate the necessary vaporization and subsequent mass analysis of the macrostructure. Furthermore,

maintaining wet conditions shows the most stable environment for the protein. Either when the HEWL was dried or re-constituted with buffer, major deviations were observed. As a result, this gives evidence that denaturation has occurred in the protein.

4.3 Conclusion

These findings show that the Laser Electrospray Mass Spectrometry technology is amendable to our solid-phase synthesis approach. We can effectively utilize this technique to extricate macromolecules from cellulose-based solid supports, similar to the solid support surfaces typically used. We look to employ similar measures in future binding studies to reconnoiter interactions between small molecules and proteins. Also, the macrostructure itself was the only molecule released from the solid support surface. Therefore, vaporization of molecules in this LEMS system supports our efforts in future binding studies. This technique can provide selectivity in the analysis of macromolecules non-covalently bound to cellulose-based membranes. Satisfactory conditions have been investigated to maintain intact structures in their native state; these results make for a good starting point in the process, but optimization efforts will continue. We are pleased with these findings and continue in our pursuits to design molecules on laboratory filter paper.

CHAPTER 5.0

PEPTIDE-ARRAY SYNTHESIS

5.1 Introduction

We envision the rational design of molecules on laboratory filter paper. Peptide-Array synthesis on our functionalized supports will invoke essential concepts from traditional Solid-Phase Peptide Synthesis technology on other supports, i.e. Boc and Fmoc synthesis. Herein, we make "pre-activated" compounds and assemble them on a functionalized solid support. We hypothesize that the difference in the material/make-up of our support will be a minor deviation, owing no consequence in assembling molecules in the scaffolding approach. We will undertake the assembly of simple α-amino acids along with bis-amino acid compounds bearing diverse functionality, e.g. building blocks.

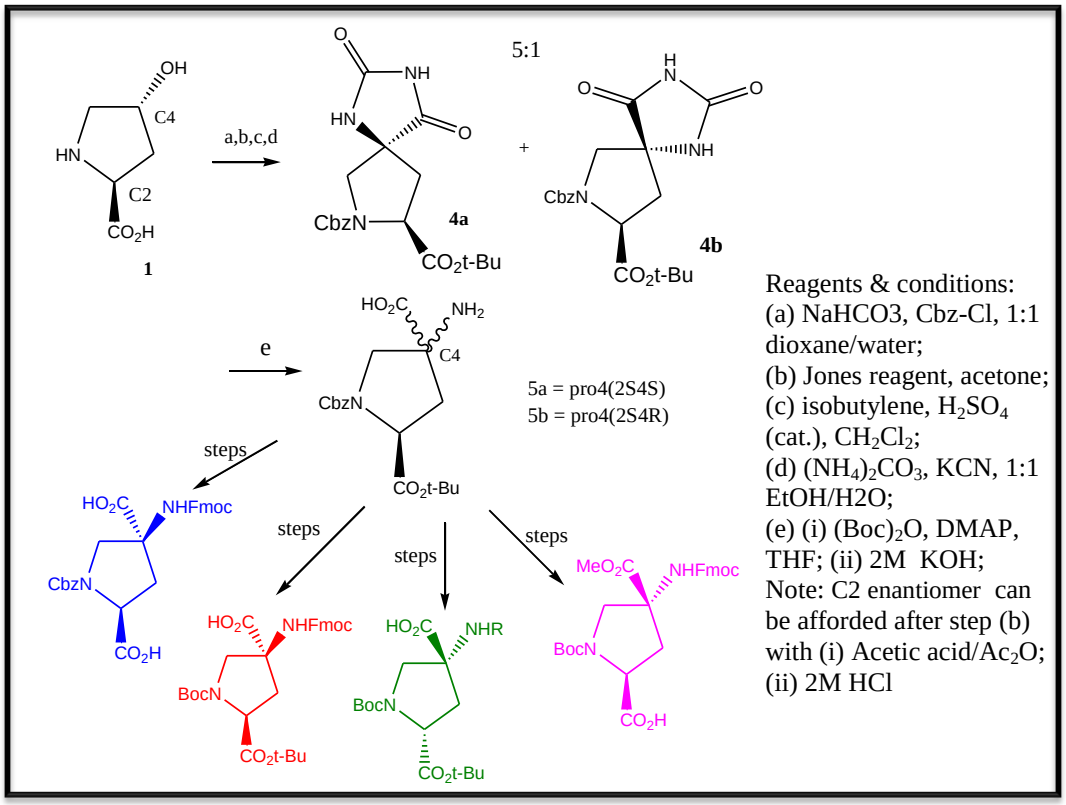

Figure 5.1 Displays the synthetic scheme in affording functionalized building blocks.

Building blocks are initially prepared in solution phase over the course of 5-6 different reactions, including purification of "hydantions" via Flash Chromatograpy[19a] (Figure 5.1, Structures 4a and 4b). Previous studies in our laboratory show that further elaboration can be conducted in solution phase or on resin support,[11] to attach protecting groups on the carboxyl and/or primary amine at C4 position. In addition, reductive alkylation with commercially available aldehydes (Sigma-Aldrich) may afford increased functionality at the primary amine, i.e. aromatic groups, hydrocarbons, haloalkanes, etc. Final products are also purified by Flash Chromatography[19b] and lyophilized[20].

Rational design is an important part of developing molecules intended to possess functionality and designed to elicit specific physiological responses in drug development.[12] Inherent properties of a given compound are particular in our scaffolding approach to assemble oligomers in a linear fashion. Forming diketopiperazine (DKP) rings can afford a rigid backbone to present various functional groups in defined three-dimensional orientations and to program overall shape of an oligomer. DKP formation is acheived by the ring nitrogen (2° amine) of a monomer attacking the ester group in the previous building block.[21,22]

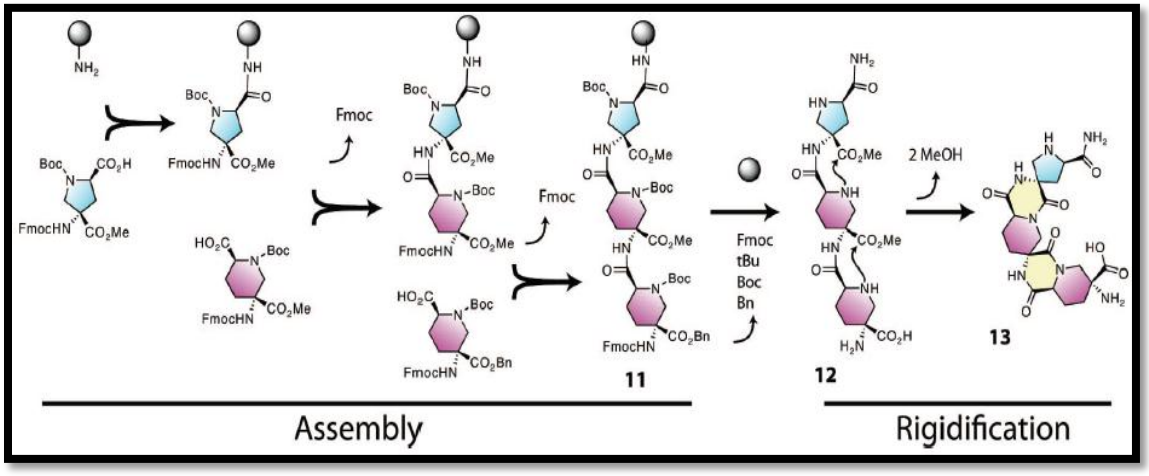

Figure 5.2 This illustrates rational design in assembling molecules in a scaffolding approach on solid support. Upon forming DKP rings, we can derive the overall shape that an oligomer adopts. Adapted from reference 11.

Computational chemistry allows researchers the ability to utilize computer software to rationally design molecules. Christian E. Schafmeister has developed a program to predict the resulting three-dimensional structure of any assembly of monomers, i.e. oligosaccharides, building blocks and α-amino acids.[11] Using the low-energy conformations of each monomer, a design can be constructed. This program is written in C++ and Python, and is fittingly coined CANDO (Computer Aided Nanostructure Design and Optimization). We feel these tools enable our attempts at rationally designing molecules on cellulose filter paper.

5.2 Results and Discussion

Prior to the assembly of molecules, a chemically-cleavable linker is attached to the filter support. Linkers are chemically susceptible to known conditions such as with base, acid or irradiation, e.g. with photo-cleavable linkers. Undertaking these methods allow us the selective recovery of desired end-products. Often times, products are recovered with acceptable purity; however, end-process measures include liquid chromatography and lyophilization. Specifically, we use the following linkers: Wang (4-formylphenoxyacetic acid), HMBA (4-Hydroxymethyl-benzoic acid), and Rink Amide {4-[(2,4-Dimethoxyphenyl)(FMOC-amino)methyl]-Phenoxyacetic Acid}, all available from Sigma-Aldrich or EMD Biosciences. Coupling agents were also employed (see experimental section 5.4).

To quantify both the Wang and HMBA linkers, secondary measures are required. This includes reducing terminal aldehyde to resulting alcohol, and then attachment of an N-Fmoc protected α-amino acid such as glycine. It is important to note that this first amino acid will be incorporated into the end-product, therefore a wise choice is emphasized. Often times, and in particular when using the HMBA linker, we execute recovery of an assembled molecule by using this first amino acid (discussed later in Chapter 5.4). However, quantitation of the Rink Amide linker is more straight-forward, as it bears an N-Fmoc group at the distal end whereby peptide-array synthesis is enacted upon removal of the Fmoc.

Initially, we incurred much difficulty in assembling peptide arrays. The first challenge was in loading the linker. Starting with the Wang-type, we dissolved this linker in DMF along with coupling agents. Our first thought was to engage assembly with

similar practices seen in traditional SPPS: to use stoichiometric proportions based on the number of free binding sites available on a support (commercially available resins typically have known loadings in mmol/g units). Therefore, by quantifying our amine support we attempted to load a linker in 1, 2, 5, and 10 equivalents (ratio of linker and coupling agents maintained at 1:1). Unfortunately, we were unsuccessful throughout. Yields were dismal equaling 4-15nmol. We knew that such results would not be promising (observed in using both Wang and Rink Amide). We then decided to make our pre-activated solutions in greater excess. We realized the better course of action is determining optimal concentrations that the linkers could be prepared, without experiencing solubility issues. The most operative concentration range for preparing linker solutions was between 500-600mM. It is recommended that the higher molecular weight Rink Amide linker be made at the lower at end of this range (400-500mM). To our delight, dependence on optimizing concentrations delivered much higher yields equaling 100-150nmol, wherein a precedent was now set for gauging appropriate yields in the assembly of molecules. Typically, spots are made with 1.0µL volumes, but higher yields are attainable with double "spottings" and by increasing spotted volumes (thereby increasing spot size). Double spottings can also increase spot size, but this can be controlled by decreasing the volume of the subsequent spot(s) to 0.5µL. Lastly, acetylation of the support is conducted prior to Fmoc release. We used acetic anhydride (Ac_2O) to "cap" any remaining hydroxyls and/or amine groups within or outside the spotted area. Therefore, the acetylation of the support renders areas outside the spotted region inert to subsequent chemistry.

Moving toward the use of amino acids in our assembly attempts, we invoked our recent knowledge in dealing with the linkers. We focused on spotting pre-activated solutions of α-amino acids set at 400-600mM concentrations (again higher MW compounds result at the lower end of this established range). Similar measures were utilized in the assembly of building blocks on cellulose filter paper as well.

Assembling molecules and the quantitation thereof, has proven to be quite possible. Importantly, we now seek to recover our designed molecules as well. Using the HMBA and Rink Amide linkers, we set to cleave molecules from cellulose-based membranes and employed LC/MS[23] to confirm. Our attempts to successfully cleave molecules from HMBA resin were not achieved. This base-susceptible linker proved to be a challenge. However, our pursuits with acid-susceptible cleavage from Rink Amide were successful, and in this further developments were focused. Recovery of molecules from Rink Amide was made possible with the following conditions: 95%TFA/2.5%H_2O/2.5%TIPS. These molecules are attained in acceptable purity, post-lyophilization.

5.3 Conclusion

The precedent we set for suitable loadings was achieved throughout our assembly of linkers, as well as with both building blocks and α-amino acids (100-150nmol). A 1.0μL aliquot at a 400mM concentration theoretically delivers 400nmol of a pre-activated molecule to the spotted area. Therefore, it seems our yields result in delivering ≥25% of the desired compound to the membrane. At first glance, this may appear to be a disadvantage as much of the delivered material does not react/retain on support. However, it is paramount we realize that spotting compounds in our fashion, saves a

plethora of material when compared to the amount of material used in assembling molecules on resin, costing the researcher to expend between 30-60 milligrams (mg) of compound per coupling (double couplings on resin are typically conducted as well). In the spatially addressable manner we develop here, we use 10-20mg of compound, thereby vastly saving material in each assembly. Finally, Spot Synthesis is a means of generating libraries of molecules all on the same solid support.

Rendering successful peptide-array synthesis on cellulose support marks a major milestone in this body of work. All efforts experienced have led to this point and we are excited about further developments. The next stage of this work is to explore appropriate conditions to form diketopiperazine rings with monomers. With this in hand, we can continue efforts to rationally design molecules on filter paper.

5.4 Experimental Details

The primary solvent for preparing pre-activated solutions was DMF [as a substitute N-Methylpyrrolidone (NMP) may be used]. Our studies show optimal loadings of Rink Amide linker at 400-500mM concentration (Wang and HMBA both at 600mM). Pre-activated solutions contain the desired compound, coupling agent(s), and 0.9% base (volumes are typically 100.0µL or 50.0µL). A base typically chosen is N,N'-diisopropylethylamine (DIPEA). Coupling agents include N,N'-Diisopropylcarbodiimide (DIC) , N-hydroxysuccinimide (HOSu), 1-Hydroxy-7-azabenzotriazole (HOAt), 1-(Mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT), and 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU). The mechanism for pre-activation is similar for HOSu, MSNT and HOAt: the carboxyl group of the compound and DIC form an ester, then the HOSu reagent (or HOAt) attacks this carbonyl forming

an even better electrophile. Now, an incoming free amine can displace the leaving group of the new ester. HATU does not require DIC as the necessary ester formation can occur with the carboxyl group, followed by subsequent attack of amine.

A 1.0µL aliquot of a pre-activated solution is spotted on to the filter paper using either a 10.0µL Hamilton syringe or a 2.5µL Eppendorf pipette (both owing to accurate measurements). Use of the Eppendorf makes ease of the operation as syringe clogging can be avoided, and pipette tips are disposed. The reaction is allowed to proceed for 2HR. The paper will dry by this time, but it is important to always allow support to fully dry before subsequent reactions. After linker is loaded, the support is washed with DMF (2X), Acetone (2X), and DCM (2X), then finally dried under Ar gas for 15min. Prior to removal of protecting groups, the 6cm X 6cm support is acetylated by immersing the membrane in 1.0mL of 20% Ac_2O/DMF plus 0.05g DMAP (4-Dimethylaminopyridine) under agitation for 5min, and then adding 200.0µL N,N,N-triethylamine (NEt_3). Reaction continues for 25min. This process is conducted a total of four times to ensure proper acetylation. To note, Ninhydrin[24] tests can be used to ensure complete acetylation. As discussed earlier in this work, Fmoc groups can be removed with 20% Pip/DMF. Boc protecting groups are cleaved with 1.0mL of 50% TFA/DCM over 30min (2X), then the resulting amine is neutralized with the following washing: 5.0mL 5% DIPEA/DMF for 2min, then twice with 5.0mL DCM/DMF (1:1) for 2min.

Terminal aldehydes of both Wang and HMBA linkers are reduced to the alcohol, prior to assembly of monomers. Conditions used for the Wang linker are: submerge support in 1.0mL of 560mM $NaBH_4$ in THF/H_20 (5:1) at 0° Celsius for 1 hour (twice). While conditions for HMBA were: submerge support in 1.0mL of 1M LAH/THF, 16HR

at 0°C to RT. Then add 1mL DI H_20, for 30 minutes. No reduction for Rink Amide linker, but Fmoc group is removed by 20% Pip/DMF.

Assembly of all monomers is conducted in a similar fashion as linker. Most compounds may be dissolved in DMF as well, and concentrations are set between 400-600mM. Particular conditions may be required for certain compounds due to size and polarity (or significant non-polar nature). It has been observed that spotting high molecular weight compounds, e.g. above 600g/mol, experience increased difficulty due to solubility issues. However, heat, sonication and coupling agents assist in mitigating concerns; also, choosing a solvent more amendable to the demands of a compound can be suggested.

Current cleavage conditions used for the HMBA linker were to submerge the support in 500.0µL of 20-30% DIPEA/DMF, 16HR. Successful measures to acquire proper cleavage are still in development. Recovery of molecules from Rink Amide linker (and Wang) is enacted by submerging the support in 500.0µL of a 95%TFA/2.5%H_2O/2.5%TIPS solution. The product is suspended in this solution, to be aliquoted to a clean 1.5mL vial, then dried under Argon gas. Be sure to separate product from any non-dissolvable material from the support. Liquid extractions are conducted to recover all material off the support using: 100.0µL EtOAc (2X) and 100.0µL MeOH (2X). These solutions are pooled into the same vial and dried under Argon gas. To this, 100.0µL H_2O/ACN (1:1) is added, then sample is frozen on dry ice/acetone mix, and finally lyophilized for 1HR. The resulting product is now re-constituted with 100.0µL H_2O/ACN (1:1), and a 10.0µL injection is analyzed by LC/MS.

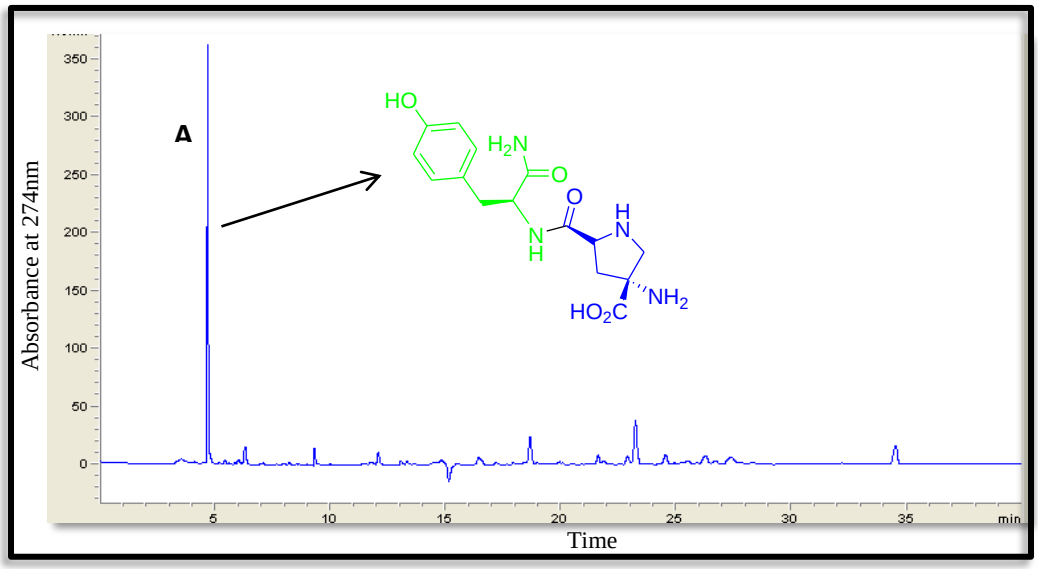

Figure 5.3 Shows the assembly of tyrosine and pro4 building block. The cleavage of this "2mer" molecule was from Rink linker on Whatman 540 membrane. Conditions were 95%TFA/2.5%H_2O/2.5%TIPS. LC/MS was used to confirm the molecule. The peak marked "A" has an m/z = 337.1 (calced for **2mer** + H^+ = 337.3).

LC/MS was used to confirm the molecule, monitoring at a wavelength of 274nm with a gradient: 5-100% ACN/H_2O with 0.1% formic acid over 40min. The molecule "A" (sample ID: **B04-W540-R-Tyr-Pro4-Isob-2**): retention time (t_r) = 4.6min.; calculated m/z for **2mer** + H^+ = 337.3; found 337.1.

CHAPTER 6.0

RING CLOSURES AND DIKETOPIPERAZINE FORMATIONS

6.1 Introduction

The next steps in our synthetic process would be to achieve ring cyclization and diketopiperazine formations. Such reactions are used in rigidifying monomers on solid support, as well as for the recovery of designed molecules from solid support. Both of these features are important aspects of our overall synthetic scheme. We set to conduct our investigations as a comparative study between traditional Solid-Phase Peptide Synthesis on polystyrene resin versus our newly designed Spot Synthesis techniques on cellulose-based membranes. In this, we would determine if there are any synthetic limitations to our attempts.

6.2 Results and Discussion

Previous studies in our laboratory express proper measures to achieve DKP ring formation.[11,12] Such measures are utilized on both the HMBA and Rink Amide resin supports. As discussed earlier, these same types of linkers are used on our functionalized membranes. Commercially available resins will also be employed in these experiments, to possess the requisite functionality as well. We shall make mention that there is no difference in the respective linker functionality, whether being utilized on resin or on filter paper. The distinction is merely the composition of the two supports, e.g. polystyrene in resin and cellulose (carbohydrates) in filter paper.

Our goals to recover molecules from the HMBA linker on filter paper were uplifted earlier in this compilation. We now re-introduce these efforts by including our

current results for resin support. Recovery of desired molecules is conducted by nucleophilic cyclization of a primary amine (of a terminal amino acid) and the susceptible ester group at the distal end of the HMBA linker. Typically, and in our studies, a Boc group is first removed from the terminal acid with 50% TFA/DCM (see Chapter 5.4).

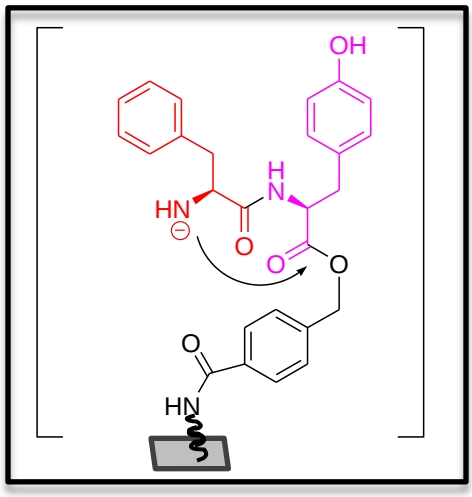

Figure 6.1 Displays the nucleophilic cyclization methods to recover molecules from HMBA linker (off cellulose support). This same mechanism is employed for resin support as well.

Cyclization of the resulting 6-membered ring thereby affords product recovery and formation of diketopiperazine. Successful conditions for cleavage of desired molecules from HMBA resin was acquired by suspending the resin in 500.0µL of 20% DIPEA/DMF for 16HR under stirring at RT. We assembled molecule "B," a 2mer of two α-amino acids on HMBA resin, and employed conditions for its cleavage from the support. The molecule consists of a single tyrosine and a phenylalanine (both enantiomerically pure, as typically used throughout all our studies).

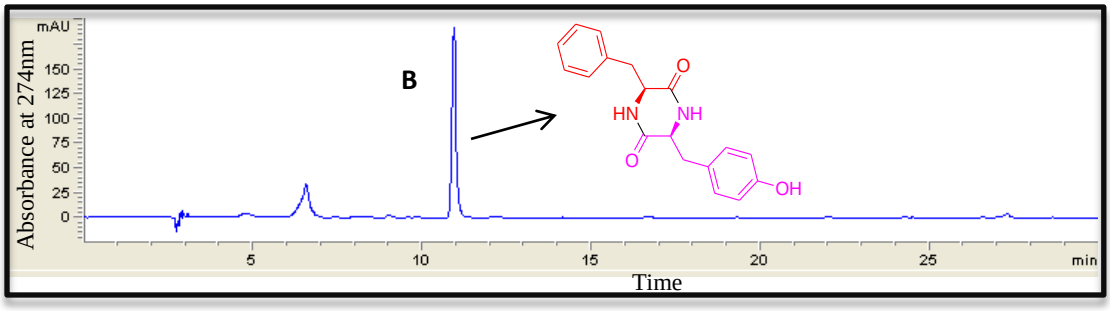

Figure 6.2 Displays the successful cleavage of a molecule from HMBA resin, using 20% Pip/DMF. LC/MS was used to confirm the molecule. The peak marked "B" has an m/z = 311.2 (calced for **2mer** + H^+ = 311.3).

LC/MS was used to confirm the molecule, monitoring at a wavelength of 274nm with a gradient: 5-95% ACN/H_2O with 0.1% formic acid over 30min. The molecule "B" (sample ID: **B52-SPPS-HMBA-Tyr-Ph_DMF**): t_r = 10.98min.; calculated m/z for **2mer** + H^+ = 311.3; found 311.2. With these conditions, we attempted to enact cyclization on our functionalized filter membrane. Despite our efforts, we were not able to reach this goal. LC/MS analysis was not conducted, as a positive Ninhydrin test reveals that product still remains on the cellulose support (no cleavage occurred).

Additional conditions were employed to recover products from HMBA-typed supports. We thought it would be prudent to ascertain a solvent system that could be concentrated after product is released. The current measures include DMF as a solvent, which is particularly difficult to remove under vacuum or inert gases. Therefore, the following conditions were utilized: 500.0µL of a 200mM NH_4OAc/ ACN (5:3) buffer solution (reacted for 16HR under stirring at RT). The same conditions were attempted when using filter paper, and comparisons are made. This water-based solution could be effectively removed by lyophilization, thereby concentrating our product for LC/MS analysis would be possible.

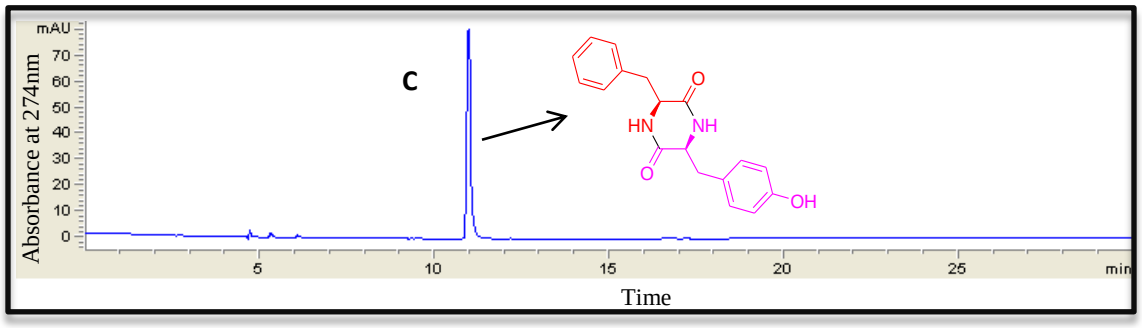

Figure 6.3 Illustrates successful recovery of a molecule from HMBA resin, using buffer conditions: 200mM NH_4OAc/ ACN (5:3). LC/MS was used to confirm the molecule. The peak marked "C" has an m/z = 311.2 (calced for **2mer** + H^+ = 311.3).

LC/MS again confirms successful recovery for a second set of conditions for HMBA resin. Product "C" is another tyrosine-phenylalanine 2mer. Monitoring was at a wavelength of 274nm with a gradient: 5-95% ACN/H_2O with 0.1% formic acid over 30min. The molecule (**B53-SPPS-HMBA-Tyr-Ph-4_NH4OAc-ACN**): t_r = 10.97min.; calculated m/z for **2mer** + H^+ = 311.3; found 311.2. However, we were still not able to retrieve products from the cellulose membrane. Having made these attempts, we are concerned with the lack of progress being made with our current challenges involving HMBA linker.

Keeping in mind the success we previously achieved with Rink Amide, we now set our efforts to conduct on-support ring cyclizations upon this linker. Formation of diketopiperazines on the Rink Amide resin was achieved with 500.0µL of 20% Pip/DMF for 2HR at RT. We realized that removal of a Fmoc group under these basic conditions would result in an anionic amine. With this in hand, cyclization would be possible.

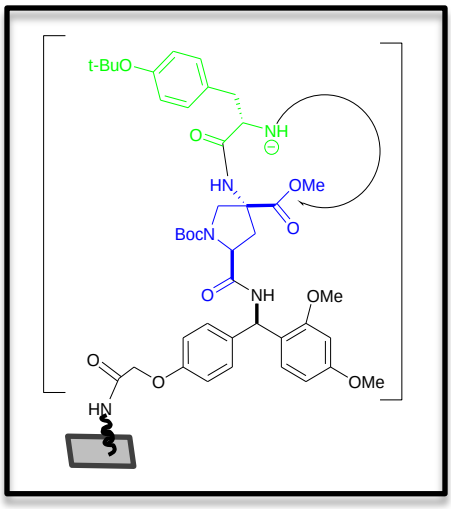

Figure 6.4 Shows the requisite ring cyclization to form diketopiperazine on cellulose support using Rink Amide.

Preference to these conditions to enact DKP formation would be favored over the aforementioned TFA-cleavage of a Boc group. Both the Rink linker and Boc groups are sensitive to acid, therefore due diligence in providing orthogonality is summoned. Molecule "D" was constructed on the Rink Amide resin, consisting of a pro4 building block and tyrosine amino acid. Basic conditions were extended over a 2HR reaction time, instead of typical 20min to ensure cyclization.

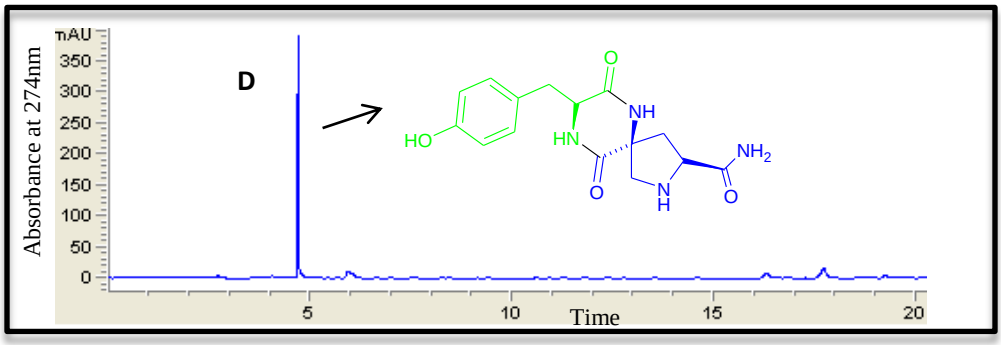

Figure 6.5 Shows successful cleavage of molecule "D" using Rink Amide resin under basic conditions. LC/MS was used to confirm this molecule. The peak marked "D" has an m/z = 318.2 (calced for **2mer** + H^+ = 319.3).

Recovery of molecule "D" was under normal conditions. Confirmation by LC/MS shows successful conditions in forming DKPs for Rink Amide resin. Monitoring was at a wavelength of 274nm with a gradient: 5-95% ACN/H_2O with 0.1% formic acid over 30min. The molecule (**B55-SPPS-R-Pro4BHFM-Tyr-4_cy_20Pip-2HR**): t_r = 4.7min.; calculated m/z for **2mer** + H^+ = 319.3; found 319.1. The same conditions were employed for DKP formation on cellulose support. In addition, basic conditions were allowed to proceed for up to 20HR. None of these conditions enabled successful formation of diketopiperazine ring. See Appendix E for LC/MS trace.

6.3 Conclusion

The recent struggles we incurred with cyclization and diketopiperazine formations have been a worthy challenge. The root cause of these concerns is still being ascertained. We suspect that the solution dynamics involved when using polymeric resin, may not be synonymous to the use of filter paper membranes. Resins are particles of polystyrene that under agitation are able to homogeneously mix in solution. Filter paper is a solid sheet that absorbs liquid, and its reactions involve the one support rather than a mixing of many particles. Therefore, reactions on membranes that require extended reaction times, an equilibrium to be set, and/or multi-component reactions may experience a burden in achieving such. If it is determined that membranes experience difficulty in paralleling the chemical flexibility of resin, then solutions to satisfy these demands are warranted. Conversely, making conditions more favorable to the dynamics of the filter paper would suffice as well, such a focusing on the function of membranes to absorb liquid. However, conducting heat-assisted reactions may aid attempts further. We currently functionalize

our support to include heat-assisted conditions. Lastly, a search for literature precedence in the field will be untaken, and time-scale studies will be conducted as well.

The advantages for assembling molecules on solid support are still prevalent. The idea to build libraries of molecules still engages us and warrants future pursuits toward achieving this goal. Thus far, we have laid the framework for the development of a platform that can be used in such efforts.

In moving forward, we have established a system to efficiently assemble molecules in a spatially addressable manner. Designed molecules can be cleanly recovered from our functionalized solid support, e.g. using the Whatman Grade 540 cellulose membrane. With this support, we have enacted ease of operation to avoid any poor mechanical handling often experienced with other filter papers. For instance, due to electrostatic factors polypropylene-based membranes can fold upon themselves or multiple sheets can bind/aggregate together under physical manipulation. In addition, the Grade 540 support has been resistant to heat as well as harsh basic and acidic conditions. Moreover, confirmation with LC/MS serves as another advantage. These factors allow us to remain confident in the utility of the cellulose-based support to conduct our syntheses.

Moreover, we have shown that researchers can assemble molecules without the risk of rapidly expelling valuable material, as seen in other technologies. This is an inspiring fact that propels interest in this work. As the demands of the many industries are ever-changing, namely pharmaceutical, lean chemical methods are pressing and development of efficient processes is ever-increasing. We are delighted to be a part of these efforts. We, also, have the capability to conduct binding assays on our support, which remains quite attractive. Therefore, the pursuit to build libraries of molecules on

the same solid support is still relevant. We look forward to future developments within our laboratory as well as in related fields.

6.4 Experimental Details

Linker and monomer assembly was conducted in the same manner expressed in the above Experimental Details section, 5.4. The assembling of compounds on resin was achieved by first swelling the support for 15-20min with DCM. If using Rink Amide resin, the Fmoc group is released after swelling with 20% Pip/DMF for 20min. After HMBA resin is swelled, it is ready for subsequent reaction. Under vacuum, washings are with 5.0mL of each: DMF (5X), IPA (5X), and DCM (5X). Pre-activated solutions are prepared in a clean 1.5mL vial to contain stoichiometric equivalents (eq) per resin loading: 3.0eq compound, 3.0eq coupling agent, and 6.0eq base. The final concentration of mixture is 150-200mM. Aliquot the entire solution to the resin, housed in a clean solid phase reaction vessel.[25] Ensure volume of the solvent (DMF) covers resin completely, and stir reaction for 1HR; then repeat process for a second coupling. Boc and Fmoc groups are removed as described previously.

Cleavage of molecules from a HMBA resin was done with either conditions: (A) 500.0µL of 20% DIPEA/DMF for 16HR under stirring at RT, or (B) 500.0µL of a 200mM NH_4OAc/ACN (5:3) solution for 16HR under stirring at RT. The same conditions were attempted when using filter paper.

Molecules were cleaved from the Rink Amide resin with 500.0µL of a solution containing 95%TFA/2.5%H_2O/2.5%TIPS. The product is suspended in this solution and aliquoted to a clean 10.0mL vial containing 5.0mL Et_2O (ether). Next, centrifuge the

product mixture, then dry under Argon gas. The resulting product was dissolved in 500.0µL of H_2O/ACN (1:1), freeze dried over a dry ice/acetone mix, and lyophilized for ≥4 hours. Product was re-constituted with 200-300.0µL of H_2O/ACN (1:1), then a 10.0µL sample was analyzed by LC/MS. Recovery of molecules from Rink linker on filter paper were the same as in Experimental Details section, 5.4.

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14. Pyrex dish can be purchased from VWR International, LLC.
15. Agitation at 100RPM (revolutions per minute) was effected by the use of a desktop rotator. Two types of rotators were used: a) Lab Line Instruments, Inc., Model No. 4633, and b) VWR International, LLC., Model 1000.
16. Vacuum chamber is a Napco instrument, Model 5831.
17. Oven is a ThermoLyne instrument, Model OV19225
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19. Purification was performed on an automated flash chromatography system, ISCO (Teledyne, Inc.) with a 12gram silica packed column. Compound was dried on celite,

and chromatography was with either the following: (a) detection at a wavelength of 254nm with a gradient: 100% DCM for 5min then 0-100% MeOH for 25min. (b) monitoring at a wavelength of 220nm with a gradient: 5-95% ACN/H2O with 0.1% formic acid over 30min.

20. Lyophilization was conducted with Labconco instrument, Serial 080181632 C.
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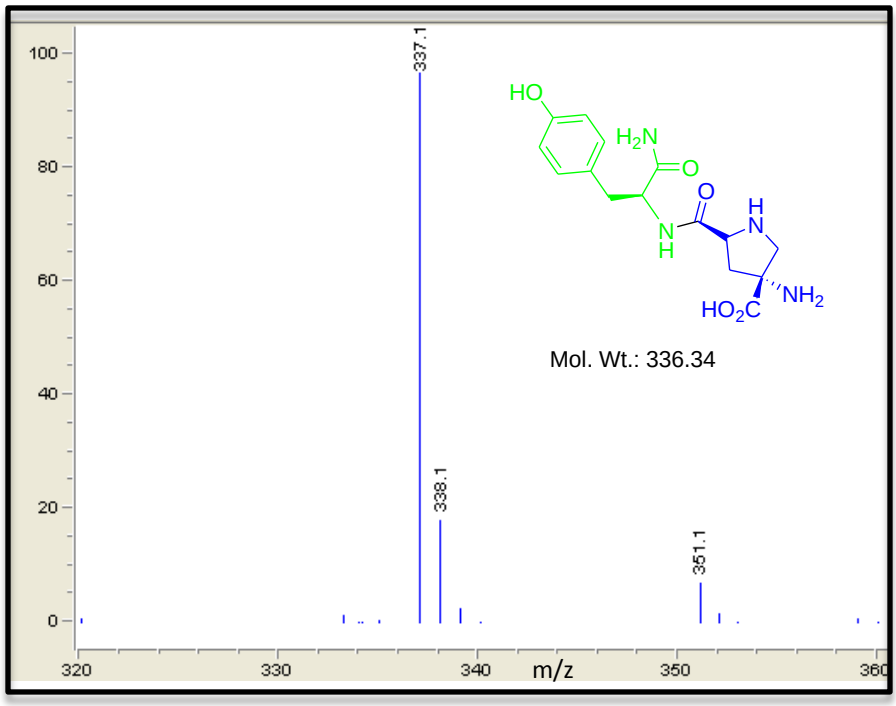
23. LC/MS instrument is a Hewlett-Packard Series 1200 with a Waters Xterra MS C18 column (3.5µm packing, 4.6 mm x 150mm) at a flow rate of 0.8mL/min.
24. Ninhydrin test is performed by mixing analyte with 100.0µL of each: KCN in H_2O/pyridine (1:1), 80% Phenol/EtOH, and 6% Ninhydrin (2,2-Dihydroxyindane-1,3-dione) in EtOH. Mixture is heated in oven at 100°C for 30 seconds to 1 minute.
25. Solid phase reaction vessels are purchased from VWR International, LLC., Product 7606-04.

APPENDICES

APPENDIX A

MASS SPECTRUM OF B04-W540-R-TYR-PRO4-ISOB-2

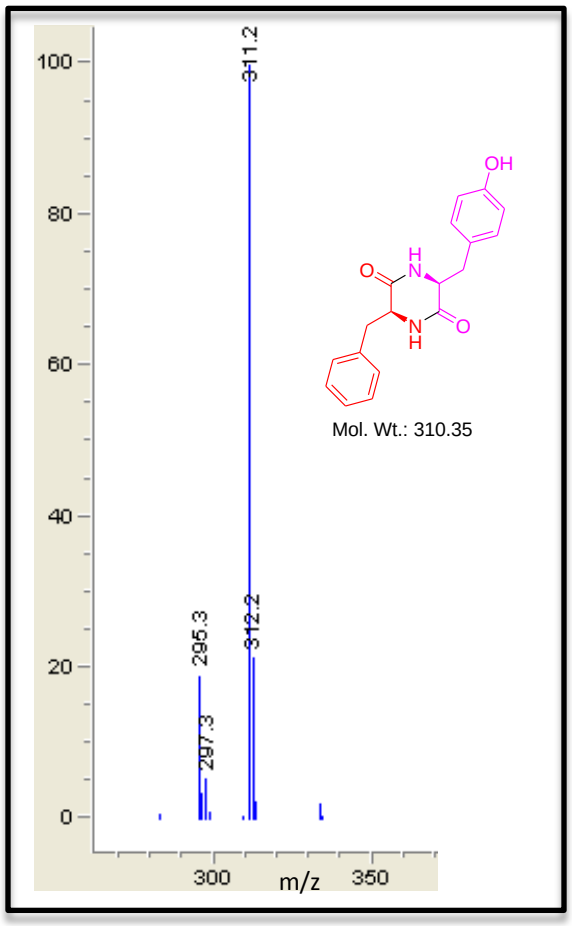
MOLECULE "A"



APPENDIX B

MASS SPECTRUM OF B52-SPPS-HMBA-Tyr-Ph_DMF

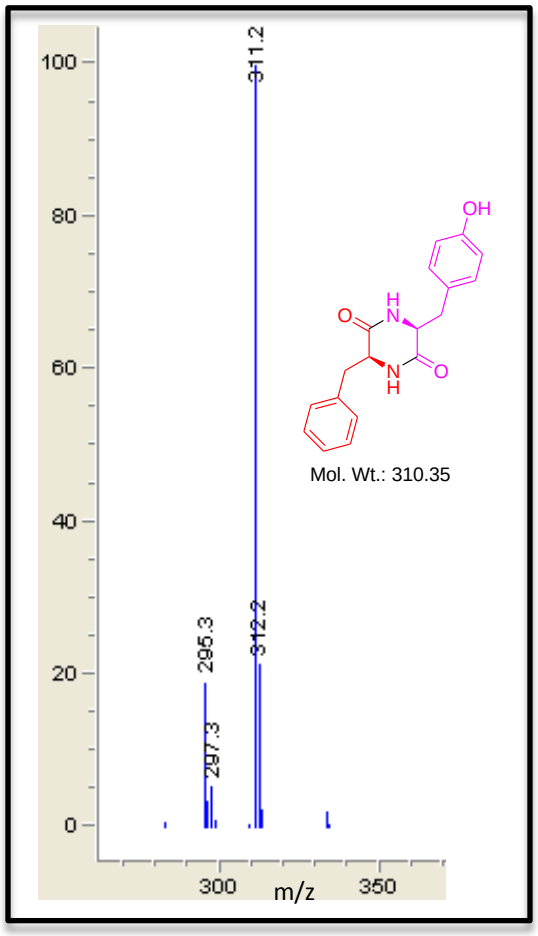
MOLECULE “B”



APPENDIX C

MASS SPECTRUM OF B53-SPPS-HMBA-Tyr-Ph-4_NH4OAc-ACN

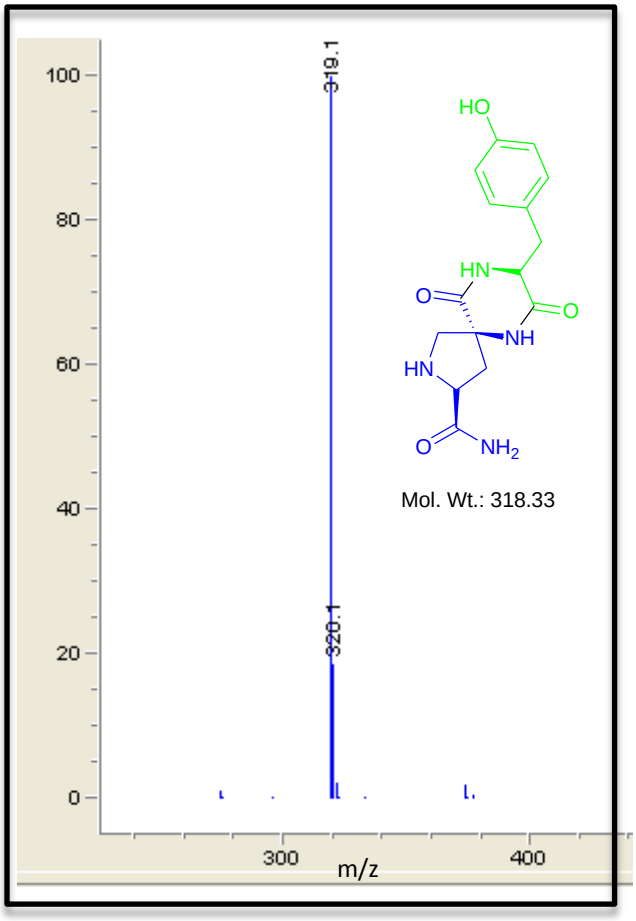
MOLECULE “C”



APPENDIX D

MASS SPECTRUM OF B55-SPPS-R-Pro4BHFM-Tyr-4_cy_20Pip-2HR

MOLECULE “D”



APPENDIX E

MASS SPECTRUM OF B53-SPPS-R-

MOLECULE “E”