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Solution-phase combinatorial synthesis; Peptide derivatives of ethyl p-aminobenzoate

Andrea Scuderi

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SOLUTION-PHASE COMBINATORIAL SYNTHESIS; PEPTIDE DERIVATIVES OF ETHYL $p$-AMINOBENZOATE

Andrea Scuderi

May 2003

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry.

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Thesis Advisor

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Department Head

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SOLUTION-PHASE COMBINATORIAL SYNTHESIS;
PEPTIDE DERIVATIVES OF ETHYL p-AMINOBENZOATE

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Signature:________________________
Date: May 12, 2003
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ABSTRACT

The need to find more efficient and cost-effective methods of drug development has led pharmaceutical companies to examine combinatorial means of synthesis. The ability to carry out parallel synthesis by the hundreds of compounds is rapidly advancing synthetic, analytical, and biological research. In this thesis project, peptide derivatives of ethyl p-aminobenzoate were synthesized via solution phase combinatorial techniques. A total of 27 tri-peptide analogs were produced, using a polymer-supported carbodiimide resin as the coupling agent. The structures of all products and intermediates were confirmed by LC-MS. Two methods were used for sequential deprotection of these analogs. The traditional method using the mild base, piperidine, was used as well as a polymer-supported thiophenol method. Upon comparison of both methods, we concluded that the piperidine deprotection afforded a higher yield of the desired unprotected peptide.
1 INTRODUCTION

1.1 COMBINATORIAL CHEMISTRY

The average time it takes a company to bring a drug to market is 14 years, with a cost of $359 million\(^1\). Therefore, the speed at which a compound is produced, along with the cost of production, is critical. Thus, combinatorial chemistry has emerged as a powerful new technology, allowing chemists to synthesize large numbers of compounds for both drug design and biological evaluation.

Combinatorial synthesis involves the preparation of large numbers of structurally related compounds either as mixtures in split-pool synthesis or as individual compounds by parallel synthesis. In this manner, hundreds to millions of compounds can be synthesized in the time usually taken to prepare a small number of analogues by the orthodox methodology\(^2\).

This area of research allows for the production of diverse, yet predictable, collections of molecules using the combination and optimization of a small number of chemical reactions. In addition, the recent advances in robotics can provide fast throughput of these compounds. The field of combinatorial chemistry seems to be expanding in all areas, and these techniques are now utilized in the fields of natural products, antibiotics, catalysis, and biomaterials\(^3\).

Although combinatorial chemistry is rapidly becoming a core technology in several industries, it is still in its youth, and development continues\(^1\). Both synthetic and analytical chemists are constantly looking for ways to improve techniques in synthesis, screening, characterization, validation, and automated purification of thousands of compounds now produced in much shorter periods of time.
1.2 HISTORY OF COMBINATORIAL CHEMISTRY

Scientifically speaking, combinatorial chemistry is a relatively new branch of chemistry. In 1963, R.B. Merrifield investigated a new approach to the chemical synthesis of polypeptides through the use of solid-phase techniques\(^1\). As seen in Scheme 1.1, this technique involved the stepwise addition of protected amino acids to a specific sequence peptide chain, which was bound by a covalent bond to a solid resin particle. This new approach in peptide synthesis was the result of technical difficulties that arose during the synthesis of long chain polypeptides. This classic technique of solid-phase synthesis quickly became the key to the production of entire proteins and enzymes for the next 30 years\(^5\).

In the mid 1980’s, Mario Geysen began synthesizing peptides by the hundreds, first in parallel fashion and later as mixtures. Geysen described a procedure for the rapid concurrent synthesis on solid supports of hundreds of peptides\(^6\). Polyethylene rods were used as solid supports, where reactions were carried out at the tips of the rods. By this method a whole virus epitope was examined at a resolution of a single amino acid.

Prior to combinatorial techniques, the time required for the synthesis of hundreds of compounds was the limiting factor in production. A combinatorial approach to peptide synthesis was first introduced by Richard Houghten in 1985\(^7\). By using a combination of solid-phase and random combinatorial techniques, a large number of peptides were synthesized and for the first time, synthesis was no longer the limiting factor in drug discovery. Houghten was able to prepare and characterize 248 different 13-residue peptides in less than 4 weeks. Houghten used polypropylene mesh packets to encase the
amino acid resin. This method, also known as the “tea bag” method of synthesis, can be used for simultaneous multiple-peptide synthesis or for multiple analog peptide synthesis.

**Scheme 1.1: Merrifield’s Solid Phase Peptide Synthesis**

The next advancement in combinatorial techniques came six years later when Kit S. Lam developed a new type of synthetic peptide library for identifying ligand-binding activity. This library was created by pooling millions of beads, with each bead containing a single peptide. This complete collection of beads then represented the
universe of possible random peptides. This became known as the “one bead-one compound” technique, where one bead was capable of creating a diverse library of structurally similar molecules.

Considered a breakthrough year, it was 1998 when combinatorial chemistry was extended from peptides to small molecules. This resulted in a large increase in drug candidates in clinical trials, and the birth of two new scientific journals devoted exclusively to combichem. It also resulted in many small custom synthesis businesses developed for the rapid production of libraries of molecular analogs. Although this area of research has its roots in peptide chemistry, the ability to carry out parallel synthesis by the hundreds is spawning new excitement in synthetic, analytical, and biological research.

1.3 VARIABLE METHODS

1.3.1 Parallel Synthesis

Combinatorial parallel synthesis has become firmly established within the pharmaceutical industry as a means of rapidly producing large numbers of compounds for biological assays in a time- and resource-effective manner.

Figure 1.1: Parallel Synthesis

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
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<tbody>
<tr>
<td>A</td>
<td>AA</td>
<td>BA</td>
<td>CA</td>
<td>DA</td>
<td>EA</td>
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<tr>
<td>B</td>
<td>AB</td>
<td>BB</td>
<td>CB</td>
<td>DB</td>
<td>EB</td>
</tr>
<tr>
<td>C</td>
<td>AC</td>
<td>BC</td>
<td>CC</td>
<td>DC</td>
<td>EC</td>
</tr>
<tr>
<td>D</td>
<td>AD</td>
<td>BD</td>
<td>CD</td>
<td>DD</td>
<td>ED</td>
</tr>
<tr>
<td>E</td>
<td>AE</td>
<td>BE</td>
<td>CE</td>
<td>DE</td>
<td>EE</td>
</tr>
</tbody>
</table>
The main concept in the field of parallel synthesis can be summarized in Fig. 1. This table illustrates a hypothetical combinatorial compound library of products produced by reacting every possible combination of five starting materials. This results in a library containing 25 (5 x 5) products. This library is constructed by 25 individual reactions, with each product separate from all of the others.

Each reaction takes place simultaneously, with each reaction vessel containing the same resin. Since each reaction vessel contains only one product, isolation of the compound is simple, and most likely will be of high purity. Another key advantage to this parallel synthesis technique is that structure activity relationships can be easily determined. The diversity of such libraries is only restricted by the selection of which substituents are incorporated onto the template and the subset of products that is desired.

1.3.2 Solid-Phase Synthesis

Library generation may be accomplished using solid-phase organic synthesis or by using conventional solution-phase chemistry. Solid-phase peptide synthesis, for example, is based on sequential addition of α-amino and side-chain protected amino acid residues to an insoluble polymeric support. A protecting group is chosen for N-α-protection. After removal of this protecting group, the next protected amino acid is added using either a coupling reagent or pre-activated protected amino acid derivative. The resulting peptide is attached to the resin, and may be cleaved to yield the desired free product.
Solid-supported synthesis has several clear advantages over conventional solution-phase chemistry. A summary of the advantages and disadvantages can be seen in Table 1.1.

### Table 1.1: Advantages and Disadvantages of Solid-Phase Synthesis

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ease of manipulation of the beads.</td>
<td>Reaction-scale restriction.</td>
</tr>
<tr>
<td>Has the ability to drive reactions to completion by the use of excess reagent.</td>
<td>Only a narrow range of reactions can be used.</td>
</tr>
<tr>
<td>The isolation of product by filtration alone.</td>
<td>Difficulty of analysis and reaction tracking.</td>
</tr>
<tr>
<td>Possibility of the split pool approach.</td>
<td>The development of new reactions on a solid-phase is frequently time consuming.</td>
</tr>
<tr>
<td>All work-up procedures can be automated.</td>
<td>Reactions at high temperatures may cause the deformation of beads.</td>
</tr>
</tbody>
</table>

### 1.3.3 Solution-Phase Synthesis

There is an unending controversy as to which of the major synthetic strategies for library synthesis is superior. Solution-phase combinatorial approaches have recently become of interest as an alternative drug discovery avenue for lead discovery and optimization. A summary of the disadvantages and advantages in solution-phase synthesis can be seen in Table 1.2.
Table 1.2: Advantages and Disadvantages of Solution-Phase Synthesis

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>The familiarity to the synthetic chemist.</td>
<td>Complicated work-up procedures have been a major hurdle to automation.</td>
</tr>
<tr>
<td>Able to produce products of high purity and easy scale-up.</td>
<td>Often slow removal of contaminants.</td>
</tr>
<tr>
<td>Any chemical reaction can be carried out even with the use of organometallic reagents.</td>
<td>Variable quality of libraries produced.</td>
</tr>
<tr>
<td>Reactions are easy to track and analyze.</td>
<td>Product isolation is associated with solvent evaporation.</td>
</tr>
<tr>
<td>High temperature reactions can be carried out with ease.</td>
<td>Excess reagents can not be recovered.</td>
</tr>
</tbody>
</table>

Both the solid support and the solution-phase synthetic techniques have advantages over each other. A solid support offers the ease of separating the product away from the reaction medium and easy manipulation of the beads using volumetric techniques. The ability to drive reactions to completion by use of excess reagent is also a key advantage to a polymer of solid-supported synthesis. However, the loading capacity of a solid support is fairly small, and therefore this limits the scale at which a reaction can occur.

Solution-phase synthetic techniques have the advantage of non-limiting scale that can be easily manipulated as well. There are several analytical techniques available for screening reaction mixtures in solution, therefore decreasing validation time. The main
disadvantage to this technique is the isolation of products away from the reaction medium may require tedious and costly isolation and purification methods, especially when a mixture of products is produced.

1.3.4 Solution-Phase Synthesis on a Solid Support

In the recent years, the attention of combinatorial chemistry has shifted to the production of “drug-like” small molecules for lead discovery and optimization11. While both solid-phase and solution-phase synthetic techniques have been employed to generate libraries, the majority of the compound libraries have been synthesized on a solid support12.

Several recently described syntheses employ both solid-phase techniques with solution-phase synthesis12,13. In one such example, polymeric reagents were used in the synthesis steps to generate reactive species for product formation. In addition, scavenging resins were used in solution reactions to remove unreacted starting materials from product mixtures affording purified product by direct filtration12. This approach combines the advantages from both techniques, and allows the chemist to have the best of both worlds.

1.3.5 Selection of the Polymer-Supported Resin

Covalently linked reagents to an insoluble organic polymer have come to acquire importance since the introduction of the solid-phase technique for the synthesis of peptides by Merrifield in 196314.

The chosen polymer must be insoluble in all of the solvents that are to be used, and have a stable physical form that will permit ready filtration. It also must contain a
functional group to which the first protected amino acid could be linked by a covalent bond. This linker must be compatible with all synthetic steps, yet labile under cleavage conditions that do not cause decomposition of compounds generated in the library.

1.3.6 Polymer-Supported Carbodiimide Reagents

The massive increase in the number of papers describing the use of polymeric supports in organic synthesis over the past decade is a vivid demonstration of its impact in the chemical community. Figure 1.2 illustrates this growing number of new and novel utilization of linkers in organic synthesis. Few other changes in synthetic chemistry methodology have displayed such a growing passion or had such a profound influence on the way synthetic chemistry is carried out.15

Figure 1.2: Number of Publications Dedicated to New or Novel Utilization of Linkers

These insoluble, polymer supports have a gel-type structure that readily allows penetration of reagents and solvents into the beads to sites where the reactions will occur.
Typical supports used for solid-phase synthesis consist of polystyrene with a 1-2% divinylbenzene cross-linking.

A few other materials are used as supports, but the polystyrene resin still dominates today. Among other materials used are Tenta Gel resins (TG) and ArgoGel (AG), specifically used when polar solvents are needed or when distancing from the resin core becomes necessary. Both resins consist of polystyrene/DVB-poly(ethylene glycol) and are graft copolymers. Crowns/Pins (CP) is another kind of support used in solid-phase synthesis, consisting of a radiation-grafted polyethylene/polypropylene support. The number of different supports used in the last two years is vast, however, polystyrene still provides one of the main workhorse resins today.

The carbodiimides are well-known condensing agents in peptide synthesis. Various polymers containing the carbodiimide functional group in their backbone have been known for some time as polymers with film- and fiber-forming capabilities. PS-Carbodiimde is a neutral, tethered carbodiimide that may be used for the synthesis of amides, esters, and activated esters. It is a 1% cross-linked poly(styrene-co-divinylbenzene) resin with a loading capacity of 0.9-1.4 mmol/g. In comparison with other carbodiimde resins, PS-Carbodiimde has been found to be highly stable at room temperature, and has given superior results relative to N-Cyclohexylcarbodiimide-N’-Me PS resin and the quaternary carbodiimide resin P-EDC. The structures of each of these resins can be seen in Figure 1.3.
Figure 1.3: Structures of Carbodiimide Resins

<table>
<thead>
<tr>
<th>Structure</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>P-EDC</td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>N-Cyclohexylcarbodiimide-N'-Me PS</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>PS-Carbodiimide</td>
</tr>
</tbody>
</table>

PS-Carbodiimide affords amides in high yield and purity without evidence of residual amine or carboxylic acid impurities. The carboxylic acid, normally used in excess relative to the amine, remains bound to the resin during work-up\(^1\).  

1.3.7 Selection of the Protecting Group

The use of selective protection of organic functional groups is a key aspect of contemporary organic synthesis. Many protective groups have been, and are being, selectively developed for the purpose of multifunctional, temporary protection\(^2\). The selective protection for the amino group has received considerable attention, specifically the use of carbamates. Carbamates are ideally suited to protect amino acids in peptide and protein synthesis due to their ability to minimize base-catalyzed racemization during synthesis\(^3\).

The most widely used carbamate in organic synthesis is the 9-fluorenylmethyl carbamate, or Fmoc. Fmoc is a base-labile protecting group with numerous advantages.
Fmoc is readily cleaved in a nonhydrolytic fashion and has excellent acid stability. Other standard peptide protecting groups, such as t-Boc (tert-butyloxycarbonyl), can also be removed in its presence. Upon the removal of the Fmoc group in solution, the amine generated is the desired free base.

**Figure 1.4: Structures of t-Boc and Fmoc Protecting Groups**

Although the most widely used protecting group in combinatorial synthesis is Fmoc, t-Boc is also a very satisfactory labile group that is stable at room temperature (see Figure 1.4). The development of Fmoc peptide synthesis arose out of concern that repetitive TFA acidolysis in Boc-group deprotection could lead to alteration of sensitive peptide bonds as well as acid catalyzed side reactions\(^2\). By contrast, Fmoc synthesis requires only the use of a mild base treatment, while cleavage and deprotection in the Boc strategy requires the use of dangerous HF and expensive laboratory apparatus.

**1.3.8 Removal of the Fmoc Group in Solution**

The Fmoc group is cleaved under mild conditions with an amine base to afford the free amine and dibenzofulvene. Figure 1.5 outlines several amine bases and their effectiveness in the removal of Fmoc\(^18\). However, incomplete Fmoc removal may occur during the course of the synthesis, leading to poor results. This may be attributed to unfavorable hydrophobic interactions of the bulky Fmoc moiety. The use of base, particularly piperidine, in the removal of Fmoc may lead to the trapping of the released
dibenzofulvene. This problem may result in alkene polymerization and problems in purification.

**Figure 1.5: Half-Lives for the Deprotection of FMOC by a Variety of Amine Bases in DMF**

<table>
<thead>
<tr>
<th>Amine</th>
<th>$t_{1/2}$</th>
</tr>
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<tbody>
<tr>
<td>20% Piperidine</td>
<td>6 s</td>
</tr>
<tr>
<td>5% Piperidine</td>
<td>20 s</td>
</tr>
<tr>
<td>50% Morpholine</td>
<td>1 min</td>
</tr>
<tr>
<td>50% Dicyclohexylamine</td>
<td>35 min</td>
</tr>
<tr>
<td>10% p-Dimethylaminopyridine</td>
<td>85 min</td>
</tr>
<tr>
<td>50% Diisopropylethylamine</td>
<td>10.1 h</td>
</tr>
</tbody>
</table>

The classical means of Fmoc deprotection employs a large excess of a secondary amine, such as piperidine, in DMF. The piperidine functions both as a base to fragment the Fmoc group and as a scavenger to trap the liberated dibenzofulvene (DBF) via a Michael-type addition thereby outcompeting reaction with the product amine (Scheme 1.2)\(^{20}\) Use of piperidine/DMF is better suited to Fmoc deprotections on solid-phase than those in solution due to the low volatility of these solvents, the solvent-dependent reversible scavenging of dibenzofulvene by piperidine, and DBF polymerization at higher concentrations\(^{21}\).
1.3.9 Use of PS-Thiophenol in Fmoc Scavenging

An alternative to Fmoc removal requires the use of polymer-supported thiophenol to scavenge alkylating agents. PS-Thiophenol (3-(3-mercaptophenyl)propanamidomethylpolystyrene) is based on an aminomethyl resin with a tethered thiophenol functionality (Fig. 1.6). PS-Thiophenol was tested and found effective in scavenging alkylating agents ranging from octyl bromide to benzyl bromide

In order to cleave disulfide linkages that may be present, the resin is pre-treated with a 0.7 M tributylphosphine solution for 1 hour, and is then washed with deoxygenated THF. Effective scavenging of alkyl halides requires the use of the potassium thiolate salt, formed with potassium trimethylsilanolate.
Ermann, et.al., recently described an efficient solution-phase synthesis of substituted benzimidazol-2-ones in which polymer-supported thiophenol was used to remove excess alkylating agents\(^2\). In previous work, the heterocycle was alkylated using a variety of alkyl halides. A deprotection step was necessary in order to give the desired product. However, using the polymer-supported thiophenol, this group was able to bypass these additional purification and isolation procedures by selective removal of excess alkylating agents. Derivatives were obtained in high yields and purities.

### 1.3.10 Analytical Evaluation of Reaction Mixtures

Compared to solution-phase organic synthesis, solid phase reactions are generally more difficult to monitor principally because the technique of following a reaction by TLC is not appropriate\(^3\). Currently, the methods used for monitoring solid-phase reactions include analyzing the molecule while it is still bound to the resin, utilizing FT-IR, \(^1\)\(^3\)C gel NMR, and magic angle NMR. It is also possible to remove a small portion of the resin and cleave the small molecule fragment. This can be accomplished using MALDI-TOF MS. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry is a technique in which a co-precipitate of a UV-light absorbing matrix and a biomolecule are irradiated by a nanosecond laser pulse. Most of the laser energy is absorbed by the matrix, which prevents unwanted fragmentation of the biomolecule.
In comparison, the primary advantage of solution-phase chemistry is its familiarity to the synthetic chemist. Reactions are generally amenable to easy tracking and analysis\textsuperscript{24}. This closely parallels general experience in the pre-combichem days with the exception that the workload is greatly magnified. Automation is called for, and LC/mass spectrometry is of particular value\textsuperscript{25}. It is also possible to monitor the reaction progress through TLC and analytical HPLC.

However, with very large libraries, analytical evaluation is usually restricted by necessity to statistical sampling and compound identification rarely goes beyond ascertaining whether the product has the correct molecular weight. If activity is found then more detailed examination takes place.

1.3.11 Informetrics

The generation of large libraries combined with robotic screening processes produce an enormous amount of data. Keeping track of these libraries is a job for high-speed computers. Many firms have developed their own programs for the data handling, and there are commercial packages that may be useful as well\textsuperscript{25}

1.4 COMBINATORIAL SYNTHESIS: PEPTIDOMIMETICS

The discovery of the physiological role of a great number of peptides stimulated researchers all over the world towards design and synthesis of peptidomimetics, or peptide-like molecules. Natural peptides can seldom be used therapeutically as drugs, because of the problems associated with low absorption, rapid metabolism, and low oral bioavailability, therefore many efforts have been aimed at modifying the natural sequence of the amino acids. This creation of entirely artificial substances for use as medicinal
agents is attributed to an interest in the elucidation of reactions found in biosynthetic and metabolic pathways\textsuperscript{26}.

Synthetic, non-natural peptides have the advantage of providing new functionalities that can circumvent natural processes in the body. For example, they become able to perform functions that are not available with the natural materials, such as binding to and penetrating cell membranes and resisting degradation by enzymes.

In solid-supported combinatorial synthesis, many of the linkers that are available require polar functional groups for binding, and the same polar groups are released after cleavage. To generate libraries with biological activity, however, such polar functionalities may possess unfavorable pharmacological properties. The synthesis of peptidomimetic libraries has become an appealing target in response to the poor oral bioavailability and enzymatic degradation of linear peptides\textsuperscript{27}.

Due to the nonpolar nature and steric bulkiness of its side chain, phenylalanine is one of the preferred residues in peptidomimetics when the biological targets are known to have hydrophobic binding sites. In order to increase the bioavailability of compounds derived from peptidomimetic approaches, amino acid residues with bulky and hydrophobic side chains are often left unchanged where other residues are modified. In this regard, phenylalanine is considered to be a key pharmacophore in many biologically important peptide-like molecules.
2 OBJECTIVE

The production of peptidomimetics, or peptide-like molecules, is a valuable tool in the pharmaceutica industry. Natural peptides can seldom be used therapeutically as drugs due to problems associated with poor absorption, rapid metabolism, and low oral bioavailability. Recent efforts have been made to synthesize bioactive peptides that are recognized by a peptide recognition site. Therefore, by mimicking important structural features of peptides, one may simultaneously maintain beneficial biological activities.

One class of synthetic non-natural peptides attaches small peptide sequences to organic molecules. This has the advantage of providing new functionalities that can circumvent natural processes in the body. Combinatorial techniques, both solution-phase and solid-phase, have allowed for the rapid production of these molecules. These techniques, combined with robotic screening, have greatly accelerated drug discovery.

The goal of this research is to synthesize a solution-phase library of PABA-based peptide analogs using a solid supported reagent. Using ethyl p-aminobenzoate as the backbone for our synthesis, it was our aim to produce a small library of tri-peptide analogs through the parallel addition of three different amino acids to this template. A secondary goal in this project was to determine the fastest route in the FMOC deprotection of these analogs, using either piperidine or a polymer-supported thiophenol. Scheme 2.1 illustrates an overview of our solution-phase peptide synthesis and deprotection sequence.
Scheme 2.1: Solution-Phase Peptide Synthesis

<table>
<thead>
<tr>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂</td>
<td>CH₃</td>
<td>CH₃OHCH₃</td>
</tr>
</tbody>
</table>

PABA \[\text{PS-Carbodiimide} \rightarrow \text{FMOC-Amino Acid} \]

\[ \text{CO}_2\text{CH}_2\text{CH}_3 \]

A

B

Piperidine/DMF

A,B

A,B
2.1 SYNTHESIS OF MONO-PEPTIDE ANALOGS

Two procedures for the production of the initial analogs were evaluated. The procedure that afforded the highest yield of protected product was used for the remaining syntheses of di- and tri-peptide analogs. A total of 27 tri-peptide analogs were produced, which included derivatives of phenylalanine, valine, and isoleucine.

2.2 DEPROTECTION OF PEPTIDE ANALOGS

Two different means of deprotection were evaluated: piperidine in DMF and PS-Thiophenol. The deprotection agent that afforded the highest yield of free amine was used to deprotect the final 27 tri-peptide analogs. The separation of the protecting group by-product from the reaction mixture was also a factor in choosing the ultimate agent.
3 EXPERIMENTAL

Reagents and solvents were obtained from the Sigma-Aldrich Chemical Co. All solvents were A.C.S. HPLC Grade. Polymer supported resins, including PS-Carbodiimide and PS-Thiophenol, were obtained through Argonaut Technologies Inc.

All reactions were carried out in BIO-RAD Econo-Pac Columns. These polypropylene columns were 14 cm high with dimensions of 1.5 x 12 cm. Allowing for easy filtration, each vial contains a porous 30 (μm) polyethylene bed support.

3.1 SYNTHESIS OF MONO-PEPTIDE ANALOGS

Three mono-peptide analogs were produced by linking ethyl para-aminobenzoate, or PABA, to each of the following amino acids: N-(9-Fluorenylmethoxycarbonyl)-L-phenylalanine, N-(9-Fluorenylmethoxycarbonyl)-L-valine, and N-(9-Fluorenylmethoxycarbonyl)-L-isoleucine. PS-Carbodiimide resin, or DCC, was used as the support bound reagent.

3.1.1 Procedure A

Fmoc-phenylalanine (1.2g, 3.0 mmol, 1.5 equiv) and PABA (.33g, 2.0 mmol, 1 equiv) were combined and allowed to react for 10 minutes in CH₂Cl₂:DMF (1:1, 10ml/gram of resin). PS-Carbodiimide (1.5g, 2.0 mmol, 1 equiv) was then added and the complete mixture was allowed to turn end over end for 24 hours.²

Fmoc-valine (1.0g, 3.0 mmol, 1.5 equiv) and Fmoc-isoleucine (1.1g, 3.0 mmol, 1.5 equiv) were treated in the above fashion in order to yield the remaining of the two mono-peptide analogs.
3.1.2 Procedure B

PS-Carbodiimide (2.3g, 3.0 mmol, 1.5 equiv) and Fmoc-phenylalanine (1.2g, 3.0 mmol, 1.5 equiv) were combined and allowed to react for 10 minutes in CH$_2$Cl$_2$:DMF (1:1, 10ml/gram of resin). PABA (.33g, 2.0 mmol, 1 equiv) was then added and the complete mixture was allowed to turn end over end for 24 hours.

Fmoc-valine (1.0g, 3.0 mmol, 1.5 equiv) and Fmoc-isoleucine (1.1g, 3.0 mmol, 1.5 equiv) were also treated in the above fashion in order to yield the remaining of the two mono-peptide analogs.

3.1.3 Reaction Workup

Following each procedure, the resin was removed by filtration and washed two times with CH$_2$Cl$_2$:DMF (1:1, 3ml). The filtrate was then transferred to a clean reaction vessel where the deprotection reaction occurred.

3.1.4 Deprotection of the FMOC Protecting Group

Following procedure A, piperidine in DMF (1:1, 5ml) was added to each reaction vessel. The reaction was allowed to turn end over end for two hours.

Following procedure B, piperidine in DMF (1:1, 2.5 ml) was added to each reaction vessel. The reaction was allowed to turn end over end for two hours.

3.1.5 Isolation of the Free Amine

In order to isolate the product, HPLC grade water (2 ml) was added to the solution prepared from procedure A. The precipitated product was dried in a dessicator for 1 hour and stored for analytical evaluation. Water was removed from the remaining solution as
an azeotrope using an equivalent amount of ethanol. The mixture was then stored in the freezer for analytical evaluation.

Following procedure B, the entire mixture was stored in the freezer. Upon the precipitation of product, the solution was filtered and the crystals were collected by vacuum filtration. Both the remaining solution and precipitated material were stored in the freezer for further evaluation.

### 3.2 SYNTHESIS OF DI-PEPTIDE ANALOGS

The second phase of this library was synthesized by parallel addition of each of the three mono-peptide analogs to each of the three chosen amino acids. A total of 9 di-peptide analogs were produced.

#### 3.2.1 Procedure

To a total of 9 filter reaction vessels, PS-Carbodiimide (2.3g, 3.0 mmol, 1.5 equiv) was added in CH$_2$Cl$_2$:DMF (1:1, 10ml/gram of resin). Fmoc-phenylalanine (1.2g, 3.0 mmol, 1.5 equiv), Fmoc-valine (1.0g, 3.0 mmol, 1.5 equiv), and Fmoc-isoleucine (1.1g, 3.0 mmol, 1.5 equiv) were then added three fold and allowed to turn end over end for 10 minutes.

Each of the three mono-peptide analogs was added in parallel to the protected amides. The deprotected phenylalanine analog (0.21g, 2.0 mmol, 1equiv), the valine analog (0.18g, 2.0 mmol, 1equiv) and the isoleucine analog (0.28g, 2.0 mmol, 1equiv) were added to the reaction mixture and the reaction was allowed to turn end over end for 24 hours.
3.2.2 Reaction Workup

The reaction mixture was filtered into a clean reaction vessel and the protected di-amide product was collected in the filtrate. The resin was then further washed an additional two times with CH$_2$Cl$_2$:DMF(1:1, 3 ml).

3.2.3 Deprotection of the Fmoc Protecting Group

Piperidine in DMF (1:1, 3ml) was added and allowed to react for two hours. After freezing, the solid precipitate was collected by vacuum filtration. The filtrate was stored in the freezer for further evaluation.

3.3 SYNTHESIS OF TRI-PEPTIDE ANALOGS

The third phase of this library was synthesized by parallel addition of the nine deprotected peptide analogs to each of the three chosen amino acids. A total of 27 tri-peptide analogs were produced.

3.3.1 Procedure

To a total of 27 filter reaction vessels, PS-Carbodiimide (1.4g, 2.0 mmol, 1.5 equiv) was added in CH$_2$Cl$_2$:DMF (1:1, 10ml/gram of resin). Fmoc-phenylalanine (0.77g, 2.0 mmol, 1.5 equiv), Fmoc-valine (0.68g, 2.0 mmol, 1.5 equiv), and Fmoc-isoleucine (0.71g, 2.0 mmol, 1.5 equiv) were then added nine fold and allowed to turn end over end for 10 minutes.

Each of the nine di-peptide analogs was added in parallel to the protected amino acids. The molar amounts and equivalents of each can be seen in Table 3.1.
Table 3.1: The Parallel Addition of Nine Di-Peptide Analogs

<table>
<thead>
<tr>
<th>Compound 1</th>
<th>Compound 2</th>
<th>Compound 3</th>
<th>Compound 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Compound 1" /></td>
<td><img src="image2.png" alt="Compound 2" /></td>
<td><img src="image3.png" alt="Compound 3" /></td>
<td><img src="image4.png" alt="Compound 4" /></td>
</tr>
<tr>
<td><strong>459.54 g/mol</strong></td>
<td><strong>411.5 g/mol</strong></td>
<td><strong>425.53 g/mol</strong></td>
<td><strong>377.5 g/mol</strong></td>
</tr>
<tr>
<td><strong>0.56 g</strong></td>
<td><strong>0.54 g</strong></td>
<td><strong>0.55 g</strong></td>
<td><strong>0.49 g</strong></td>
</tr>
<tr>
<td><strong>1.3 mmol</strong></td>
<td><strong>1.3 mmol</strong></td>
<td><strong>1.3 mmol</strong></td>
<td><strong>1.3 mmol</strong></td>
</tr>
<tr>
<td><strong>1 equiv</strong></td>
<td><strong>1 equiv</strong></td>
<td><strong>1 equiv</strong></td>
<td><strong>1 equiv</strong></td>
</tr>
</tbody>
</table>

*Per 1 equivalent of amino acid. Quantitative yields of di-peptide analogs are assumed as they are added in parallel to each of the three amino acids.*

25
3.3.2 Reaction Workup

The reaction mixture was filtered into a clean reaction vessel and the protected tri-amide product was collected in the filtrate. The resin was then further washed an additional two times with CH$_2$Cl$_2$:DMF(1:1, 3 ml).

3.3.3 Deprotection of the Fmoc Protecting Group

PS-Thiophenol (1.3g, 2.0 mmol, 1 equiv) was treated with 20 ml of potassium trimethylsilanolate (513 mg, 4.0 mmol, 2 equiv) in THF:EtOH (1:1) and allowed to turn end over end for 30 minutes. The solution was removed by filtration and the resin was washed three times with THF:EtOH (1:1).

Three of the 27 tri-peptide reaction mixtures were added to this prepared bed of PS-Thiophenol and allowed to turn end over end for 24 hours. The reaction was filtered and the deprotected product was collected in the filtrate.

The remaining of the tri-peptides were deprotected in the same fashion as reported for each di-peptide analog.
3.4 ANALYTICAL EVALUATION OF MONO-PEPTIDE ANALOGS

3.4.1 Evaluation of Solid Precipitate

The white precipitates obtained from both procedures A and B were analyzed by melting point and NMR, using a Bruker 300MHz instrument. Proton NMR analysis was performed by dissolving 5 mg of sample in D-chloroform, with the addition of 2 drops of TMS.

3.4.2 LC-MS Evaluation of Reaction Mixture

A Hewlett Packard 1100 HPLC equipped with a diode array detector and quadrupole mass spectrometer was employed to gain quantitative and qualitative analyses of each reaction mixture.

Sample Concentration: ~1mg/ml in CH$_2$Cl$_2$/5\% TFA.

Column: Hypersil BDS-C18 5\% (3 x 50 mm).

Injection Volume: 1 \mu l.

Solvent A: 0.1 M Ammonium Acetate Buffer, pH 4.65.

Solvent B: Acetonitrile:Isopropanol (1:1).

Gradient: 90\% A/10\% B (equilabrate for 4 minutes) to 100\% B (10 minutes); hold (1 min).

Flow Rate: 2.0 ml/min

3.5 ANALYTICAL EVALUATION OF DI- AND TRI-PEPTIDE ANALOGS

All remaining precipitates were analyzed by melting point only. A Hewlett Packard (Agilent) 1100 HPLC equipped with a diode array detector and quadrupole mass spectrometer was employed to gain quantitative and qualitative analyses of each reaction mixture.

**Sample Concentration:** ~1mg/ml in CH₂Cl₂/5% TFA.

**Column:** Zorbax 18 (2.1 x 150 mm)

**Injection Volume:** 2 µl

**Solvent A:** 0.1 M Ammonium Acetate Buffer, pH 4.65.

**Solvent B:** Acetonitrile:Isopropanol (1:1).

**Gradient:** 80% B-100% B in 10 minutes; hold (4 min)

**Flow Rate:** 0.4 ml/min
4 RESULTS

4.1 ANALYSIS OF MONO-PEPTIDE ANALOGS: PROCEDURE A

Figure 4.1 shows the chromatogram of the reaction mixture following procedure A. The heating of the reaction mixture has lead to a reversal in the reaction, accounting for the large presence of PABA and amino acid starting materials.

Figure 4.1: Chromatogram of Deprotected Mono-Peptide Analog
4.2 ANALYSIS OF MONO-PEPTIDE ANALOGS: PROCEDURE B

Figures 4.2-4.4 show the LC-MS for each of the three mono-peptide analogs.

With the absence of any PABA starting material, mono-peptide analogs were synthesized with yields greater than 90%.

**Figure 4.2: LC-MS Chromatogram of Phenylalanine Analog (1A)**
Figure 4.3: LC-MS Chromatogram of Valine Analog (2A)

- TIC of -Q1: from 2a minus FMOC-.wiff

- Q1: 5.380 to 5.681 min from 2a minus FMOC-.wiff

- Q1: 4.979 to 5.246 min from 2a minus FMOC-.wiff
Figure 4.4: LC-MS Chromatogram of Isoleucine Analog (3A)

TIC of -Q1: from 3b minus FMOC-.wiff

Max. 3.8e7 cps

-Q1: 6.817 to 7.485 min from 3b minus FMOC-.wiff

Max. 3.2e6 cps
Table 4.1:  Summary of Library Results of Mono-Peptide Analogs

<table>
<thead>
<tr>
<th>Product</th>
<th>%PABA</th>
<th>%Product</th>
<th>%Other Impurities</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>0</td>
<td>96</td>
<td>4.2</td>
</tr>
<tr>
<td>2A</td>
<td>0</td>
<td>94</td>
<td>6.5</td>
</tr>
<tr>
<td>3A</td>
<td>0</td>
<td>97</td>
<td>3.4</td>
</tr>
</tbody>
</table>

*All HPLC analysis has been carried out under the assumption that all molar responses are equal.

Table 4.2:  Numbering System for Amino Acids

<table>
<thead>
<tr>
<th></th>
<th>Phenylalanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td><img src="image" alt="Phenylalanine" /></td>
</tr>
<tr>
<td>2A</td>
<td>Valine</td>
</tr>
<tr>
<td>3A</td>
<td>Isoleucine</td>
</tr>
</tbody>
</table>

![Phenylalanine](image)
4.3 ANALYSIS OF FMOC BYPRODUCT

Figure 4.5: NMR Spectra of Precipitated Fmoc

Fmoc analysis was accomplished using NMR, as seen in Figure 4.5. Melting point analysis can be found in Tables 4.3-4.4.

Table 4.3: Melting Point Results- Precipitated FMOC from Mono-Peptide Analogs

<table>
<thead>
<tr>
<th>Fmoc Precipitate</th>
<th>Melting Point (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>104-106</td>
</tr>
<tr>
<td>2a</td>
<td>94-96</td>
</tr>
<tr>
<td>3a</td>
<td>98-101</td>
</tr>
</tbody>
</table>
4.4 INITIAL ANALYSIS OF DI-PEPTIDE ANALOGS

Table 4.4: Melting Point Results- Precipitated DBF From Mono-Peptide Analogs

<table>
<thead>
<tr>
<th>Fmoc Precipitate</th>
<th>Melting Point (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a-1a</td>
<td>239-241</td>
</tr>
<tr>
<td>1a-2a</td>
<td>240-242</td>
</tr>
<tr>
<td>1a-3a</td>
<td>240-241</td>
</tr>
<tr>
<td>2a-1a</td>
<td>236-238</td>
</tr>
<tr>
<td>2a-2a</td>
<td>248-250</td>
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<tr>
<td>2a-3a</td>
<td>242-244</td>
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<tr>
<td>3a-1a</td>
<td>247-249</td>
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<tr>
<td>3a-2a</td>
<td>248-250</td>
</tr>
<tr>
<td>3a-3a</td>
<td>249-251</td>
</tr>
</tbody>
</table>

Figure 4.6: LC-MS Chromatogram of Deprotected Di-Peptide Analog
Column: Hypersil BDS-C18

Initial analytical evaluation of the di-peptide analogs was performed using a Hypersil BDS C-18 column. However, unfavorable separation conditions lead us to examine other means of evaluation. This initial chromatogram is seen in Figure 4.6.
4.5 OVERALL ANALYSIS OF DI-PEPTIDE ANALOGS

Figure 4.7 shows a general chromatogram for the intermediate di-peptide analogs. It is important to note that the presence of the PABA starting material surfaces in this data. The corresponding mass spectrum can be seen in Figure 4.8. The remaining mass spectrum for the di-peptide analogs are shown in Figures 4.9-4.16. Corresponding chromatograms can be seen in Appendix B.

Figure 4.7: Chromatogram of Di-Peptide Analogs
Column: ZORBAX C18

Figure 4.8: Corresponding Mass Spectra: Phenylalanine-Plenylalanine (1A-1A) *Labeled with corresponding retention times.
Figure 4.9: Mass Spectra: Phenylalanine-Valine (1A-2A)

Figure 4.10: Mass Spectra: Phenylalanine-Isoleucine (1A-3A)
Figure 4.11: Mass Spectra: Valine-Phenylalanine (2A-1A)

Figure 4.12: Mass Spectra: Valine-Valine (2A-2A)
Figure 4.13: Mass Spectra: Valine-Isoleucine (2A-3A)

Figure 4.14: Mass Spectra: Isoleucine-Phenylalanine (3A-1A)
Figure 4.15: Mass Spectra: Isoleucine-Valine (3A-2A)

Figure 4.16: Mass Spectra: Isoleucine-Isoleucine (3A-3A)
Table 4.5: Summary of Library Results of Di-Peptide Analogs

<table>
<thead>
<tr>
<th>Product</th>
<th>%PABA</th>
<th>%Mono-peptide</th>
<th>%Product</th>
<th>%Other Impurities</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A-1A</td>
<td>14.46</td>
<td>7</td>
<td>75</td>
<td>3.05</td>
</tr>
<tr>
<td>1A-2A</td>
<td>0</td>
<td>0</td>
<td>96</td>
<td>4.02</td>
</tr>
<tr>
<td>1A-3A</td>
<td>0</td>
<td>8.39</td>
<td>91</td>
<td>1.06</td>
</tr>
<tr>
<td>2A-1A</td>
<td>20.28</td>
<td>0</td>
<td>78</td>
<td>2.03</td>
</tr>
<tr>
<td>2A-2A</td>
<td>0</td>
<td>5.94</td>
<td>93</td>
<td>1.05</td>
</tr>
<tr>
<td>2A-3A</td>
<td>0</td>
<td>4.92</td>
<td>94</td>
<td>1.57</td>
</tr>
<tr>
<td>3A-1A</td>
<td>14.12</td>
<td>0</td>
<td>83</td>
<td>3.24</td>
</tr>
<tr>
<td>3A-2A</td>
<td>0</td>
<td>10.9</td>
<td>87</td>
<td>10.9</td>
</tr>
<tr>
<td>3A-3A</td>
<td>0</td>
<td>11.54</td>
<td>87</td>
<td>1.65</td>
</tr>
</tbody>
</table>
Figure 4.17: LC of Tri-Peptide Analog: PS-Thiophenol Deprotection

Figure 4.17 shows a chromatogram for a tri-peptide analog deprotected using the PS-Thiophenol method. The corresponding mass spectrums are seen in Figure 4.18. Figures 4.19-4.20 show the mass spectrum for the remaining two tri-peptide analogs that underwent PS-Thiophenol deprotection. Remaining chromatograms can be found in Appendix C.

Figure 4.18: Corresponding Mass Spectra (3A-1A-1A)
Figure 4.19: PS-Thiophenol Deprotection: Mass Spectra (3A-1A-2A)
Figure 4.20: PS-Thiophenol Deprotection: Mass Spectra (3A-1A-3A)
**Figure 4.21: Chromatogram of Deprotected Tri-Peptide Analog (1A-1A-1A)**

Figure 4.21 shows a general chromatogram for a deprotected tri-peptide analog obtained by using traditional piperidine deprotection. It is important to note the large amount of PABA starting material that is present. Figure 4.22 also shows a deprotected tri-peptide analog, however, there is no PABA present in this reaction mixture. Figures 4.23-4.45 show the corresponding mass spectrum. Remaining chromatograms can be found in Appendix C.

![Chromatogram of Deprotected Tri-Peptide Analog](image)

* PABA Cleavage Present

**Figure 4.22: Chromatogram of Deprotected Tri-Peptide Analog (1A-3A-1A)**

![Chromatogram of Deprotected Tri-Peptide Analog](image)

*No PABA Cleavage Present
Figure 4.23: Corresponding Mass Spectra (1A-1A-1A)
Figure 4.24: Mass Spectra (1A-1A-2A)

Figure 4.25: Mass Spectra (1A-2A-1A)
Figure 4.26: Mass Spectra (1A-2A-2A)

Figure 4.27: Mass Spectra (1A-2A-3A)
Figure 4.28: Mass Spectra (1A-3A-1A)

Figure 4.29: Mass Spectra (1A-3A-2A)
Figure 4.30: Mass Spectra (1A-3A-3A)

Figure 4.31: Mass Spectra (2A-1A-1A)
Figure 4.32: Mass Spectra (2A-1A-2A)

Figure 4.33: Mass Spectra (2A-1A-3A)
Figure 4.34: Mass Spectra (2A-2A-1A)

Figure 4.35: Mass Spectra (2A-2A-2A)
Figure 4.36: Mass Spectra (2A-2A-3A)
Figure 4.37: Mass Spectra (2A-3A-1A)

Figure 4.38: Mass Spectra (2A-3A-2A)
Figure 4.39: Mass Spectra (2A-3A-3A)

Figure 4.40: Mass Spectra (3A-2A-1A)
Figure 4.41: Mass Spectra (3A-2A-2A)

Figure 4.42: Mass Spectra (3A-2A-3A)
Figure 4.43: Mass Spectra (3A-3A-1A)

Figure 4.44: Mass Spectra (3A-3A-2A)
Figure 4.45: Mass Spectra (3A-3A-3A)
Table 4.6: Summary of Library Results of Tri-Peptide Analogs

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<tr>
<th>Product</th>
<th>%PABA</th>
<th>%Mono-peptide</th>
<th>% Di-Peptide</th>
<th>%Product</th>
<th>%Other Impurities</th>
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</table>

* PS-Thiophenol Deprotection.
5 DISCUSSION

5.1 SYNTHESIS OF MONO-PEPTIDE ANALOGS

The synthesis of the three amino acid-bound analogs was first carried out using a procedure (A) from Argonaut Technologies\textsuperscript{17}. Procedure A required the use of only 1 equivalent of the resin per 1.5 equivalents of the protected amino acid. The ethyl \( p \)-aminobenzoate and the amino acid in \( \text{CH}_2\text{Cl}_2\text{:DMF} \) were combined in a fritted polypropylene reaction vessel and allowed to react for 10 minutes. Upon the addition of PS-Carbodiimide, the reaction mixture was turned end over end at room temperature. It is important to note that the polymer-supported DCC is added after both the \( p \)-aminobenzoate ester and the amino acid. After a reaction time of 24 hours, piperidine was added to deprotect the Fmoc group followed by the addition of water to precipitate the Fmoc byproduct from solution. The white precipitates from each of the three reaction mixtures were analyzed and found to be 9-fluorenemethanol. Structure verification can be seen in Figure 4.5. The melting point determination of each of these solids was found to be comparable with the literature melting point of 102-104 °C for 9-fluorenemethanol (Table 4.1).

Following Fmoc precipitation, it was necessary to remove the water by formation of an azeotrope from each solution by adding an equivalent amount of ethanol followed by attempted removal of the solvent \textit{in vacuo}. However, the LC of this reaction mixture shows a reversal of the reaction due to the need to heat the solution during the attempted evaporation (Figure 4.1). Although product is visible, there is a considerable amount of amino acid and ethyl \( p \)-aminobenzoate starting materials, which were not present prior to
the addition of the aminoester. Thus another method, other than the addition of water, was needed to remove the Fmoc byproduct from solution.

We also believed that the order of addition of reactants played a key role in the outcome of this reaction. Procedure A was modified to Procedure B, where equivalent amounts of carbodiimide and protected acid were allowed to react prior to the addition of the template. Our goal here was to ensure that the carbodiimide would properly activate the amino acid, prior to the addition of the template. Using this modified procedure, the reaction mixtures were then placed in the freezer where the 9-fluorenemethanol precipitated from solution over a short period of time. The LC-MS of each of the three mono-peptides synthesized using Procedure B can be seen in Figures 4.2.-4.4. The deprotected peptide analogs were synthesized with yields >90%. It was then decided to add each additional amino acid to the template using our revised Procedure B.

5.1.1 Analytical Evaluation of Mono-Peptide Analogs

LC-MS of proteins and peptides is used to provide information for protein characterization, to accurately identify post-translational modifications of proteins, and to determine the molecular weight of synthetic and natural peptides\textsuperscript{30}. The characterization of the reaction mixtures for each of the mono-peptide analogs was performed using a Hypersil BDS-C18 column. This column was chosen due to the fact that it is base deactivated and endcapped to improve peak shapes of basic solutes by minimizing interaction with unreacted silanols. Literature references to use of the Hypersil BDS column include detection of basic drugs, explosives, and antibiotics.

In order to detect the molecular weights present in each reaction mixture, a quadrapole mass spectrometer was utilized and scanned from 65 to 2000 amu, alternating
positive and negative ions. The deprotected peptide analog was detected only in negative ion mode, resulting in a clean spectra and positive structure identification.

5.2 SYNTHESIS OF DI-PEPTIDE ANALOGS

The synthesis of the nine di-peptide analogs was carried out using our revised Procedure B. This second phase of the library was produced by parallel addition of each of the three mono-peptide analogs to each of the three chosen amino acids. Table 5.1 illustrates this intermediate library of nine di-peptide analogs.

5.2.1 Analytical Evaluation of Di-Peptide Analogs

Upon the addition of piperidine to deprotect the Fmoc group from solution, the reaction mixture was again frozen. Over a short period of time, a white precipitate began to form. However, upon melting point analysis, it was revealed that this was not 9-fluorenemethanol, as we had previously seen with the mono-peptide analogs. The melting points of each precipitate can be seen in Table 4.2. It was possible that our deprotected product had precipitated out of solution, and that selective recrystallization had occurred. However, LC-MS characterization revealed that this white solid was actually liberated dibenzofulvene (DBF), as a result of the addition of piperidine. Piperidine functions as both as base to fragment the Fmoc group and as a scavenger to trap the liberated DBF via a Michael-type addition thereby outcompeting reaction with the product amine. This is schematically illustrated in Scheme 1.2.

With the characterization of the DBF complete, our attention then shifted to the analysis of the reaction mixture, where our deprotected product should have then remained in solution. Initially, the same method was employed to characterize these di-
peptide analogs as was used with the mono-peptide analogs. However, separation of the remaining impurities and starting materials from the desired product could not be accomplished using a Hypersil BDS-C18 column (Figure 4.6). These remaining components in solution did not allow the deprotected product to properly ionize, thus positive structure identification for the desired product could not be achieved.

After the first attempt to characterize the di-peptide analogs failed, we then turned to an Agilent ZORBAX reversed phase C18 column. This column is based on Rx-SIL, and is aimed at providing the best peak shape and sample resolution, as well as the longest lifetimes for the low, mid, and high pH regions. Specifically, the C18 column is an efficient, silica-based column, designed for the separation of basic analytes, including peptides.

A sample chromatogram using the ZORBAX column is illustrated in Figure 4.7. Positive structure identification was achieved for all nine of the di-peptide analogs. The mass spectra for each analog can then be seen in Figures 4.8-4.16. Several mass spectra also indicate that the di-peptide reactions did not go to completion, indicated by the mass of the mono-peptide starting material. PABA is also present in several of the spectra, possibly indicating the cleavage of the PABA from the terminal peptide chain. The formation of deprotected product in solution allowed for the addition of one more amino acid to the template.
Table 5.1: Library of Intermediate Di-Peptide Analogs

<p>| | | |</p>
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5.3 SYNTHESIS OF TRI-PEPTIDE ANALOGS

The synthesis of the 27 tri-peptide analogs was also carried out using our revised procedure B. This third, and final, phase of the library was produced by parallel addition of each of the nine di-peptide analogs to each of the three chosen amino acids. Table 5.2 illustrates the complete library of tri-peptide analogs.

### Table 5.2: Library of Tri-Peptide Analogs

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<th>1A-1A-3A</th>
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5.4 DEPROTECTION OF THE FMOC GROUP

5.4.1 Use of a Polymer-Supported Thiophenol as a Scavenger

It was our goal to rapidly produce a library via parallel synthesis techniques, however, the length of time required to fully precipitate DBF from solution has been the limiting factor in the production of this library. Upon the synthesis of the final 27 analogs, PS-Thiophenol was chosen as a scavenger and added to three of the analogs. It was believed that this thiol-based Michael donor might replace piperidine as a superior DBF scavenger capable of outcompeting the product amine nucleophile.

In order for effective scavenging to occur, the use of the potassium thiolate salt is required. This salt was formed with the addition of potassium trimethylsilanolate in THF:EtOH to the bed of PS-Thiophenol. The solution was then turned end over end in a filter reaction vessel for 30 minutes. The solution was removed by filtration and the resin was washed three times with THF:EtOH. The three analogs were added to separate beds of the prepared PS-Thiophenol and allowed to turn end over end at room temperature. After a reaction time of 24 hours, the reaction was filtered and the product was collected in the filtrate.

LC-MS evaluation of the three analogs only revealed a 6-8% yield of deprotected product. Figure 4.17 shows the chromatogram for one of these analogs deprotected under scavenging conditions. In all three cases, the majority of the reaction mixture was the protected tri-peptide analog. Figures 4.18-4.20 are the corresponding mass spectra for each of the three analogs. As a precautionary measure, Argonaut Technologies suggests that PS-Thiophenol should first be prepared for use by reduction with a tributylphosphine solution in order to cleave disulfide linkages that may be present. This preparation was
not attempted in our studies due to the expense of the reducing reagent. The use of PS-Thiophenol as a method to cleave Fmoc from the desired product might have been more successful had we done the suggested preparatory reaction. The production of a very small yield of deprotected product concluded that the use of piperidine to deprotect the Fmoc group was a better method than that of PS-Thiophenol scavenging.

The remaining 24 analogs were then deprotected in the same fashion as were the mono- and di-peptide analogs. Figures 4.21 and 4.22 illustrate general chromatograms for the tri-peptide analogs. In figure 4.21, PABA is present in significant amounts, where in Figure 4.22, PABA can not be seen. The corresponding mass spectra can be seen in Figures 4.23-4.45. The positive structure identification occurred for all 24 remaining analogs. In several of the spectra, the original template, PABA, can be seen. Again, the cleavage of PABA may be occurring, contributing to lower yields of deprotected product. In several instances, remaining mono-peptide starting material can be seen, as well as di-peptide material, indicating an incomplete reaction.

5.5 COMPARISON OF LIBRARY RESULTS

The evaluation of the initial three mono-peptide analogs showed almost exclusively the expected deprotected product, with very few impurities present. The most important thing to note is that the original template, PABA, could not be seen in the mass spectra at this time. Also the addition of piperidine to deprotect the terminal amino acid resulted in isolation of 9-fluorenemethanol whose structure was confirmed by melting point and NMR.

The appearance of PABA in the LC spectra begins with the evaluation of the di-peptide analogs, and continues with the tri-peptide analogs. This appearance is both
sequential and exponential in the di- and tri-peptide analogs. It is believed that with the buildup of piperidine in solution, comes the cleavage of the original template, PABA, from the terminal peptide link. PABA, being an aromatic amine, is the weakest base and therefore the most likely piece to be cleaved from the remaining peptide upon the addition of piperidine. In addition, initial evaluation of the mono-peptide analogs concluded that the reaction does reverse upon heating. This brings us to a reliable conclusion that the PABA amide is the weakest link in the molecules.

The amount of PABA present in the tri-peptide reaction mixture results in a much lower yield for some members of that library. The huge excess of piperidine, which has been building up after each deprotection step, results in significant cleavage of the original PABA template. As more piperidine is present in the solution, more PABA is observed in the product mixture.

It is also interesting to note that with the first mono-peptide reaction, 9-fluorenemethanol was produced as a by-product of piperidine deprotection. However, with both the di- and tri-peptide analogs, dibenzofulvene was released as a by-product. This may also result from the increasing and building concentration of piperidine in the solution.
CONCLUSION

It was Merrifield who stated in 1969 with regard to solid-phase organic chemistry: “A gold mine awaits discovery by organic chemists.” As a result of Merrifield’s work, combinatorial synthesis of libraries containing small organic molecules has become a rapidly evolving area of research. Both solid-phase and solution-phase strategies have been utilized to generate whole libraries of structurally related compounds.

Our current research successfully resulted in the synthesis of a solution-phase library of PABA-based analogs using a solid supported reagent. Using ethyl p-aminobenzoate as the backbone for our synthesis, we were able to produce a library of 27 tri-peptide analogs through the parallel addition of three different amino acids to this template.

Mono-peptide analogs were synthesized in solution with yields >90%. As predicted, with the addition of each amino acid to the template, the percentage of purity decreased. Di-peptide analogs were synthesized with 75-95% purity, while tri-peptide analogs gave an average yield of 45%. These yields varying from 14-54% show significant cleavage of the PABA template in some cases. It was also found that the use of a mild base, piperidine, was the fastest route to the Fmoc deprotection of these molecules, compared to the use of a polymer-supported thiophenol. However, the release of the by-product, dibenzofulvene, during Fmoc deprotection leads to some difficulty with respect to isolation and characterization of the desired product. It was also observed that with the buildup of piperidine in solution, the original template, PABA, began to cleave from the terminal peptide link. This significant cleavage resulted in lower yields of the tri-peptide analogs.
It is safe to conclude that the coupling reaction to form tri-peptide analogs worked extremely well. However, the deprotection of these analogs still needs to be optimized. The primary goal of this research was to develop a synthetic method for rapid production of a diverse set of tri-peptide analogs, with less concern for the production of compounds of high purity. This goal was achieved and these procedures can be used to synthesize analogs containing longer peptide chains, if necessary.

6.1 FUTURE WORK

In order to preserve the deprotected peptides, future work must be done in order to base scavenge the excess piperidine prior to each parallel addition. Base scavenging may lead to higher yields, eliminating significant cleavage resulting in the excess buildup of piperidine. One reagent that may be used is PS-Isocyanate, which is produced by Argonaut Technologies. This resin may be used in order to scavenge excess nucleophiles from solution, and occurs without liberation of small molecule byproducts. Once scavenged, the piperidine will be removed from the reaction mixture, allowing for the subsequent addition of amino acids to occur. In addition, further research into PS-Thiophenol scavenging may also result in less cleavage of PABA as the peptide chain is increased.

Upon the successful production of a library of tri-peptide analogs, some future applications for these compounds may exist. An efficient method for the solid-phase synthesis of individual bis-cyclic thioureas from resin bound tri-peptides has previously been examined. Substituted thioureas possess a wide variety of known biological activities, including antioxidant active compounds with potent anti-HIV activity.
Following this procedure, it may be possible to reduce PABA-bound tri-peptides in solution in order to achieve similar results (Scheme 6.1).

**Scheme 6.1: Synthesis of Bis-2-Imidazolidinethiones**
7 REFERENCES


APPENDIX A  MONO-PEPTIDE ANALOGS

A1:  LC-MS Phenylalanine Analog (1A)

TIC of -Q1: from 1a minus FMOC-.wiff
Max. 3.2e7 cps.

-Q1: 7.619 to 8.354 min from 1a minus FMOC-.wiff
Max. 2.0e6 cps.

[Graph depicting the mass spectrum with m/z values and time in minutes]

[Chemical structure diagram]
A2: LC-MS Valine Analog (2A)

**TIC of -Q1:** from 2a minus FMOC-.wiff

Max. 2.1e7 cps

**-Q1:** 5.380 to 5.681 min from 2a minus FMOC-.wiff

Max. 2.1e6 cps

**-Q1:** 4.979 to 5.246 min from 2a minus FMOC-.wiff

Max. 2.0e6 cps
A3: LC-MS Isoleucine Analog (3A)

TIC of -Q1: from 3b minus FMOC-.wiff
Max. 3.8e7 cps

-Q1: 6.817 to 7.485 min from 3b minus FMOC-.wiff
Max. 3.2e6 cps

Formula: CO₂CH₂CH₃

m/z, amu

0.0 200 400 600 800 1000 1200 1400 1600 1800 2000

33? 671.5 924.4 1020.5 1141.2 1311.1 1347.7 1546.1 1639.0 1760.2

277.0

278.1

20.0 60.0 120.0 180.0 240.0 300.0 360.0

2.0e5 4.0e5 6.0e5 8.0e5 1.0e6 1.2e6 1.4e6 1.6e6 1.8e6 2.0e6

3.2e6 3.0e6 2.8e6 2.6e6 2.4e6 2.2e6 2.0e6 1.8e6 1.6e6 1.4e6 1.2e6 1.0e6 8.0e5 6.0e5 4.0e5 2.0e5 0.0

0.0 1.0e7 2.0e7 3.0e7 4.0e7 5.0e7

1.0e7 2.0e7 3.0e7 4.0e7 5.0e7
APPENDIX B  DI-PEPTIDE ANALOGS

B1:  1A-1A

[Graphs showing retention times and mass-to-charge ratios for 1A-1A, 164, 311, and 450 with respective peaks and m/z values.]
B3: LC-MS 1A-3A

Retention Time (min)

Relative Intensity

m/z

Mono-peptide starting material, 5.048

Relative Intensity (%)

m/z
B4: LC-MS 2A-1A

Retention Time (min)

PABA, m/z 248

Relative Intensity (%)
B6: LC-MS 2A-3A

Mono-peptide starting material: 3462

2A-3A, 5592

2A-3A, 376
LC-MS 3A-2A

Mono-peptide starting material, 4.714

[Graph showing a peak at m/z 277]

3A.2A, 5.756

[Graph showing a peak at m/z 376]
B9: LC-MS 3A-3A

Mono-peptide starting material, 4.547

3A-3A, 6.217

390
APPENDIX C  TRI-PEPTIDE ANALOGS

C1:  LC-MS 1A-1A-1A
C4: LC-MS 1A-2A-2A

Retention Time (min)

Di-peptide starting material

m/z

m/z
C5: LC-MS 1A-2A-3A
C6: LC-MS 1A-3A-1A

Retention Time (min)

Retention Time (min)
C7: LC-MS 1A-3A-2A

Retention Time (min)

100
75
50
25
0

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Relative Intensity (%)

100
80
60
40
20
0

160 240 320 400 480 560 640 720 800 880 960 1040 1120 1200 1280 1360 1440 1520 1600 1680 1760 1840 1920

m/z

Retention Time (min)
C9:  LC-MS 2A-1A-1A

Retention Time (min)

Relative Intensity

m/z

PABA, 1206
165 -

2A-1A-1A, 7.344
557 -
C10: LC-MS 2A-1A-2A

Retention Time (min)

Relative Intensity

Di-peptide starting material, 5965

m/z

2A-1A-2A, 6509

m/z
C11: LC-MS 2A-1A-3A
C13: LC-MS 2A-2A-2A

- Di- peptide starting material, 5.047
  - m/z 362

- 2A-2A-2A, 5.799
  - m/z 461
C15: LC-MS 2A-3A-1A
C18: LC-MS 3A-1A-1A: PS-Thiophenol Deprotection
C19: LC-MS 3A-1A-2A: PS-Thiophenol Deprotection

**Mono-peptide starting material, 2,004**

**Protected tri-peptide analog, 7,178**

**3A-1A-1A**
C20: LC-MS 3A-1A-3A: PS-Thiophenol Deprotection
C21: LC-MS 3A-2A-1A
C22: LC-MS 3A-2A-2A
C24: LC-MS 3A-3A-1A
C24: LC-MS 3A-3A-2A
C25: LC-MS 3A-3A-3A