Side Chain Modifications To Improve Peptide Structure–Activity Relations

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SIDE CHAIN MODIFICATIONS TO IMPROVE PEPTIDE
STRUCTURE–ACTIVITY RELATIONSHIPS

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We certify that we have read this dissertation and that in our opinion it is adequate in scientific scope and quality as a dissertation for the degree of Doctor of Philosophy.

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I am dedicating this dissertation to my beloved parents, Vasant and Subhadra and my husband, Dr. Nandkumar. Though they are no longer part of this world, their beautiful memories continue to shape up my life.

Also, I want to dedicate this dissertation to my daughter, Radhika and my son, Kaushal for the numerous ways they have supported and encouraged me lovingly.
ABSTRACT

While natural peptides, are ideal starting points for peptide-based drug design and development, they suffer from high conformational instability, which results in susceptibility to proteolytic degradation and poor bioavailability. Peptidomimetics in recent years has helped circumvent these shortcomings by improving the pharmacological properties of polypeptides. Peptidomimetics contain essential elements (pharmacophores) that mimic a natural peptide or protein in 3D space and retain the ability to interact with the biological target producing the same biological effect. In contrast, they offer conformationally restricted structures, potentially minimizing cross-target interactions, which leads to better transport properties through biologic membranes and resistance to immune responses.

Over the years, a wide variety of side chain modified polypeptides have been developed. These modifications have been found to affect both functional as well as conformational properties of polypeptides. This important application has proven to be the motivating force behind the two research projects described in this thesis.

The site selective cleavage of peptide bonds is an essential complementary tool in protein sequencing and various bioanalytical and biotherapeutic applications of peptides and proteins. In order to cleave unreactive peptide bonds, mild and metal free, a glutamic acid selective cleavage methodology with a broad substrate scope has been developed as reported in Chapter 2 of this thesis. This methodology involves activation of side-chain carboxylate groups of glutamic acid residues followed by nucleophilic attack of the backbone amide nitrogen resulting in the formation of a cyclic pyroglutamate imide intermediate. The latter renders the scissile peptide bond susceptible to cleavage under neutral aqueous conditions. Most importantly, the strategy provides an efficient tool for peptidolysis in a wide range of peptide sequences, including Pro-Glu, disulfide bonding sites and at unnatural amino acid residues such as D-amino acids in mutated peptides. The latter provides a chemical tool for cleaving peptidomimetics
that are unsuitable substrates for proteases and may be potentially applicable for determining the mutations responsible for various age-related disorders.

Though conformational instability of peptides resulting in reduced bioavailability limits their use as promising drugs, constraining peptides by stapling (or cyclizing the side chain components in peptides) improves their pharmacological performance by imparting structural stability and increased bioavailability. In this regard, a biocompatible, mild and metal free stapling methodology with a broad substrate scope is reported in Chapter 3 of this thesis. In this method, the nucleophilic side chain of lysine is modified by reaction with an electrophilic bifunctional carbonylating agent to form urea stapled peptides with increased α-helicity and improved proteolytic stability. Additionally, the stapling strategy demonstrated the ability to synthesize bicyclic peptides with potential applications in peptide-based drug design. Most importantly, the urea moiety is anticipated to form strong H-bonds, which may be useful in catalyzing organic asymmetric transformations.

In summary, the research reported in this thesis describes design, development of peptidomimetics through side-chain modifications of glutamic acid and lysine residues and their significant potential applications in peptide based synthetic chemistry and drug design.
AKNOWLEDGMENTS

“Coming together is a beginning; keeping together is progress; working together is success.”

-Edward Everett Hale

Success is the consistent and constructive collaboration and cooperation of multiple minds that culminates into the fruitful accomplishment of an aim or purpose. In this vein, pursuing my Ph.D. was an arduous task, and I am indebted to so many of you!

I could not have imagined having a better research adviser and mentor for my PhD study than Dr. Monika Raj. Her strategic research vision offered many opportunities to challenge myself and acknowledge my potential. She encouraged me towards diligent perseverance, which I will take with me going forward. I would like to express my heartfelt gratitude to her for her boundless patience in supporting me, motivating me, imparting immense knowledge and advising in my thesis writing.

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the beginning of my PhD and the timely assistance by Dr. Yuri Kazakevich in fixing different instruments with technical problems I encountered along the way.

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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>µg</td>
<td>Microgram</td>
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<tr>
<td>µL</td>
<td>Microliter</td>
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<tr>
<td>Ala or A</td>
<td>Alanine</td>
</tr>
<tr>
<td>Arg or R</td>
<td>Arginine</td>
</tr>
<tr>
<td>Asn or N</td>
<td>Asparagine</td>
</tr>
<tr>
<td>Asp or D</td>
<td>Aspartic Acid</td>
</tr>
<tr>
<td>Boc</td>
<td>t-butyloxycarbonyl Protecting Group</td>
</tr>
<tr>
<td>C</td>
<td>Carbon</td>
</tr>
<tr>
<td>Cbz</td>
<td>Benzyloxycarbonyl</td>
</tr>
<tr>
<td>CDI</td>
<td>1,1-Carbonyldiimidazole</td>
</tr>
<tr>
<td>CNBr</td>
<td>Cyanogen Bromide</td>
</tr>
<tr>
<td>Cys or C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DCC</td>
<td>Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DIAD</td>
<td>Diisopropyl azodicarboxylate</td>
</tr>
<tr>
<td>DIC</td>
<td>Diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DIEA</td>
<td>N, N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>N, N-Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DSC</td>
<td>N, N-disuccinimidyl carbonate</td>
</tr>
<tr>
<td>DVB</td>
<td>Divinylbenzene</td>
</tr>
<tr>
<td>Equiv.</td>
<td>Equivalents</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
</tr>
<tr>
<td>FA</td>
<td>Formic Acid</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenylethoxycarbonyl</td>
</tr>
<tr>
<td>Gln or Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Glu or E</td>
<td>Glutamic Acid</td>
</tr>
</tbody>
</table>
Gly or G  Glycine
H or hr  Hour
H₂O  Water
HATU  \(O-(7\text{-azabenzotriazol-1-yl})\text{-N, N',N'-tetramethyluronium hexafluorophosphate}\)
HBTU  \(O\text{-benzotriazol-1-yl-N, N',N'-tetramethyluronium hexafluorophosphate}\)
HCTU  \(O\text{-}\(6\text{-Chlorobenzotriazol-1-yl})\text{-N, N',N'-tetramethyluronium hexafluorophosphate}\)
HF  Hydrogen fluoride or Hydrofluoric Acid
His or H  Histidine
HMBS  Heteronuclear Multiple Bond Correlation
HOAt  1-hydroxy-7-azabenzotriazole
HOBt  1-hydroxy-benzotriazole
HPLC  High Performance Liquid Chromatography
HRMS  High Resolution Mass Spectrometry
Ile or I  Isoleucine
IMS-MS  Ion Mobility Spectrometry- Mass Spectrometry
LC  Liquid Chromatography
LC-MS  Liquid Chromatography-Mass Spectrometry
Leu or L  Leucine
Lys or K  Lysine
MeOH  Methanol
Met or M  Methionine
mg  Milligram
Min  Minute
mL  Milliliter
mmol  Millimole
MS  Mass Spectrometer
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS/MS</td>
<td>Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>N₂</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NCL</td>
<td>Native Chemical Ligation</td>
</tr>
<tr>
<td>NH₂</td>
<td>Amino group</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer Wavelength</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NPCF</td>
<td>4-nitrophenyl chloroformate</td>
</tr>
<tr>
<td>0</td>
<td>Degree</td>
</tr>
<tr>
<td>OAc</td>
<td>Acetoxy</td>
</tr>
<tr>
<td>Pd (PPh₃)₄</td>
<td>Tetrakis (triphenylphosphine)palladium (0)</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>Pg</td>
<td>Protecting Group</td>
</tr>
<tr>
<td>pGlu</td>
<td>Pyro-Glutamyl</td>
</tr>
<tr>
<td>Phe or F</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Pro or P</td>
<td>Proline</td>
</tr>
<tr>
<td>PS</td>
<td>Polystyrene</td>
</tr>
<tr>
<td>PyBroP</td>
<td>Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>RCM</td>
<td>Ring-closing metathesis</td>
</tr>
<tr>
<td>RP HPLC</td>
<td>Reverse Phase High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>Ser or S</td>
<td>Serine</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid Phase Peptide Synthesis</td>
</tr>
<tr>
<td>tBu</td>
<td>Tert-butyl group</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>Thr or T</td>
<td>Threonine</td>
</tr>
<tr>
<td>TIPS</td>
<td>Triisopropylsilane</td>
</tr>
<tr>
<td>tR</td>
<td>Retention Time</td>
</tr>
<tr>
<td>Trp or W</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Trt</td>
<td>Trityl group</td>
</tr>
</tbody>
</table>
Tyr or Y
Tyrosine

UV-Vis
Ultraviolet-Visible Spectroscopy

Val or V
Valine

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NH₂

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1.1 INTRODUCTION TO AMINO ACID AND PEPTIDE STRUCTURE

Amino acids are critical to life as they are the building blocks of peptides and proteins. The twenty naturally occurring amino acids that are found within proteins contain versatile chemical structures and function in biological systems. In terms of its structure, each amino acid contains a central carbon atom, called the α-carbon, to which both a basic amino and an acidic carboxylic acid group is bound. A hydrogen atom and R group chemically bound to the central α-carbon satisfy the remaining tetravalent bonding arrangement for the tetrahedral amino acid structure (Figure 1.1). At physiological pH (about 7–7.4), amino acids exist largely as dipolar ions or zwitterions effectively making them neutral in the absence of a charged side chain group (Figure 1.1). As the carbons of standard amino acids, except glycine, are attached to four different groups, they exist as two enantiomers, D(R) and L(S). Though only L-amino acids are common, mutations, chemical and enzymatic modifications can give rise to some D-amino acids.

![General amino acid structure](image)

**Figure 1.1** General amino acid structure.

Distinguishing the naturally occurring amino acids is the composition of the side chain R group, which gives each amino acid its unique chemical, structural and biophysical properties. Based on the
nature of the R group, amino acids can be classified into the following four categories: hydrophobic (nonpolar side chains), polar (uncharged residues with hydrophilic character), acidic (side chains with a negatively charged carboxylate groups), and basic (side chains with a positively charged nitrogen) (Figure 1.2).²

![Figure 1.2. Venn diagram of amino acid properties. (https://commons.wikimedia.org/wiki/File:Amino_Acids_Venn_Diagram.png)](https://commons.wikimedia.org/wiki/File:Amino_Acids_Venn_Diagram.png)

Amino acids are joined by an amide or peptide bond to form peptides through condensation reactions. In this reaction, an amino group of one amino acid reacts with the carboxylic acid group of the other amino acid to form the peptide bond with the concomitant elimination of a water molecule (Scheme 1.1).¹ Peptides are formed with shorter (<50) amino acid sequences, whereas peptides >50 amino acids are classified as proteins. The different amino acids in a peptide and the order in which they are found from the N->C terminus is referred to as the primary peptide structure.⁵
Scheme 1.1 Peptide bond formation.

Peptides have their unique structural and biochemical characteristics, such as an isoelectric pH (pI) and ionization behavior depending on the quantity and type of each amino acid within the peptide sequence. For example, the acid-base behavior of a peptide is defined by the zwitterionic nature of the amino acid residues within the chain as well as the ionic structure of the N and C-terminal groups. All these characteristics influences peptide structure and function in a biological system.

1.2 IMPORTANCE OF PEPTIDES IN BIOLOGY

Some 7,000 naturally-occurring peptides are present in all organisms with size varying from two to twenty amino acid residues, with biological and physiological functions that impacts bodily functions and human health. Some noteworthy examples (Figure 1.3) include Carnosine, an antiglycating dipeptide found in muscle tissue promoting longevity and general well-being (1.1), Melanostatin, a tripeptide hormone that inhibits the release of melanocyte-stimulating hormone (1.2), Cholecystokinin,
a tetrapeptide hormone acting primarily in the brain as an anxiolytic (1.3), and Oxytocin, a nonapeptide which plays a role in social bonding, childbirth and milk production (1.4). Peptides like Somatostatin and Vasopressin have important roles in physiological functions. The tetradecapeptide Somatostatin not only controls the production of several hormones such as the growth hormones, thyroid stimulating hormones, insulin, glucagon and gastrointestinal hormones, but also regulates the rapid reproduction of normal and tumor cells and acts as a neurotransmitter in the nervous system (1.5). Similarly, the nonapeptide Vasopressin, an antidiuretic hormone helps water retention in the body for proper cellular function and maintains proper flow of urine from the kidneys (1.6). Thus, peptides have evolved to exhibit diverse biological roles, most prominently as signaling/regulatory molecules in a broad variety of physiological processes, including defense, immunity, stress, growth, homeostasis, and reproduction. Their diverse biological functions has enabled the development of medicinal chemistry programs to improve their therapeutic potential towards a variety of diseases and disorders.
1.3 PEPTIDE SYNTHESIS OVERVIEW

Peptide synthesis has enabled the production of many natural peptides such as hormones, neuropeptides and antibiotics, which are otherwise difficult to isolate from nature in sufficient quantities for their therapeutic applications. The first peptide synthesis of glycylglycine (Gly-Gly), as well as the term “peptide,” was first reported by Fischer and Fourneau in 1901 (Scheme 1.2).\textsuperscript{11, 12} In this method, 2,5-
diketopiperzaine was effectively hydrolyzed to Gly-Gly using acid catalyzed conditions. These harsh conditions are not applicable to other peptides containing functional side chain groups and lengthier sequences.

Scheme 1.2 The first synthesis of a dipeptide by E. Fischer

In order to overcome the synthetic challenges associated with peptide synthesis, a removable amino-protecting group was developed by M. Bergmann and L. Zervas by use of the carbobenzoxy (Cbz) group.

In addition to the transient protecting group strategies, carbodiimide-based coupling strategies developed in 1955 by J. C. Sheehan, G. P. Hess and H. G. Khorana gave the ability to form peptide bonds more rapidly and efficiently. Therefore, the combination of blocking/deblocking the N-terminus followed by activation and coupling of C-terminal amino acids enabled the production of a solution phase synthesis approach (Scheme 1.3). This resulted in synthesis of an active hormone, the octapeptide Oxytocin (Figure 1.3, peptide 1.4) leading to a new era of peptide synthesis, for which du Vigneaud was later awarded the Nobel Prize.
Scheme 1.3. Solution-phase peptide synthesis of Oxytocin
1.3.1 SOLID PHASE PEPTIDE SYNTHESIS

The most conspicuous breakthrough discovery in the field of peptide synthesis was the invention of solid phase peptide synthesis (SPPS) by R. B. Merrifield in 1963. An insoluble, polystyrene-based solid support was utilized for the synthesis of peptide sequences. After extensive research, Merrifield determined the best conditions for the solid phase synthesis, which ultimately led to the Nobel Prize in 1984 (Scheme 1.4).

![Scheme 1.4 SPPS procedure followed by Merrifield.](image)

SPPS was later modified to use the t-butyloxycarbonyl (Boc) group for Nα-protection which is labile in the presence of trifluoroacetic acid, (TFA), and hydrogen fluoride (HF) as the reagent for removal of the peptide from the resin. The Nα-protecting group, Boc is labile in the presence of TFA while side-
chain-protecting benzyl (Bn)-based groups and the peptide/resin linkage were stable in the presence of moderate acid and labile in the presence of strong acid (HF) (Scheme 1.5).

Scheme 1.5 General solid phase peptide synthesis protocol

In 1970, Carpino introduced the 9-fluorenylmethoxycarbonyl (Fmoc) group for Nα protection,¹⁹ which is removed using a mild base, piperidine. This Fmoc-SPPS strategy also utilized TFA-labile t-butyl (tBu)-based side-chain protection and hydroxymethylphenoxy-based linkers for peptide attachment to the resin, making it an “orthogonal” synthesis (Scheme 1.5). The milder conditions of Fmoc chemistry, along with improvements in basic peptide chemistry, have led to a shift in making peptides from solution-phase to solid-phase methods.
Solid-phase synthesis has many advantages over the classical solution-phase method, although there are a few drawbacks associated with peptide purification following cleavage and deprotection from the solid support and the concomitant cleavage of byproducts accumulated during synthesis. Desirably, the solid-phase synthesis method can be automated and the problem of solubilization of the peptide no longer exists since it remains attached to the solid matrix. With significant advances in the development of polymeric carriers and linkers, reversible protective groups and methods for the activation of peptide bond formation, SPPS has contributed as a powerful tool for advancement of protein and peptide research.

1.3.2 PROTECTING GROUP STRATEGIES

In the last few years, more than 250 protecting groups have been found suitable for peptide synthesis. Two main protecting groups strategies have been adopted for SPPS, the t-Boc/Bzl and Fmoc/tBu strategies. In t-Boc/Bzl, the t-Boc (tert-butoxycarbonyl) group is used for the protection of the Nα amino group and a benzyl or cyclohexyl for the side chains of several amino acids. In Fmoc/tBu, the Fmoc (9-fluorenyl methoxycarbonyl) group is used for the protection of the Nα amino group and the tert-butyl group for the side chains of different amino acids in the peptide (Scheme 1.5 and Table 1.1).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Side chain group</th>
<th>Protecting group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Fmoc strategy</strong></td>
</tr>
<tr>
<td>serine</td>
<td>R-OH</td>
<td>t-butyl</td>
</tr>
<tr>
<td>threonine</td>
<td>R-OH</td>
<td>t-butyl</td>
</tr>
<tr>
<td>tyrosine</td>
<td>Ph-OH</td>
<td>t-butyl</td>
</tr>
<tr>
<td>cysteine</td>
<td>R-SH</td>
<td>trityl</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>R-COOH</td>
<td>t-butyl</td>
</tr>
</tbody>
</table>

Table 1.1 Commonly used protecting groups for the side chains in t-Boc/Bzl and Fmoc/t-Bu strategies.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>R-COOH</th>
<th>t-butyloxycarbonyl</th>
<th>2-Cl-benzylxycarbonyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>lysine</td>
<td>R-NH₂</td>
<td>t-butyloxycarbonyl</td>
<td>2-Cl-benzylxycarbonyl</td>
</tr>
<tr>
<td>arginine</td>
<td>R-NH-C(=NH)-NH₂</td>
<td>2,2,5,7,8-pentamethyl-croman-6-sulfonyl</td>
<td>tosyl</td>
</tr>
<tr>
<td>histidine</td>
<td></td>
<td>trityl</td>
<td>tosyl</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.4-dinitrophenyl</td>
</tr>
<tr>
<td>tryptophan</td>
<td></td>
<td>t-butyloxycarbonyl</td>
<td>formyl</td>
</tr>
</tbody>
</table>

### 1.3.3 SOLID SUPPORTS

Solid supports for SPPS have to meet several requirements. Notably, the resin should be of conventional and uniform size, mechanically robust, easily filterable, chemically inert, stable under the conditions of synthesis and highly accessible to the solvents, which should allow the penetration of the reagents and the lengthening of the peptide chain. They must not interact physically with the peptide being synthesized and should be capable of being functionalized by a starting amino acid residue.

Many solid supports for SPPS include composite polymer materials and functionalized surfaces. As homogeneous gel resins, such as those belonging to poly(ethylene) glycol (PEG) have shown optimal performance during solid-phase peptide synthesis. Their loading, physical and chemical properties can be varied easily, making these mechanically stable, beaded, homogeneous gel resins preferred for SPPS.\(^{27,28}\) Also, optimal properties have been obtained by radical polymerization of end group acryloylated long-chain PEG.\(^{29}\) However, as the reactivity of radicals, carbenes, carbanions, carbenium ions, or strong Lewis...
acids have to be considered for general organic synthesis, polystyrene resins have been found to be more suited (Figure 1.4).

Two types of polystyrene resins - uncrosslinked and crosslinked are available, where uncrosslinked resins dissolve in hydrophobic solvents. Moreover, they precipitate in protic solvents. So polystyrene supports used in solid phase chemistry contain 1% or 2% divinylbenzene (DVB) as a crosslinking agent and are insoluble in all common organic solvents. Typically, these resins are utilized as small, spherical beads and are functionalized with reactive linker groups such as amine or hydroxyl groups, on which peptide chains can be built. The peptide remains covalently attached to the support throughout the synthesis.

![Polystyrene based support and PEG based support](image)

**Figure 1.4** Solid support for peptide synthesis.

Polystyrene beads are available in sizes ranging from less than a micron to 750 microns in diameter. Reaction kinetics are faster on smaller beads due to the higher surface area to volume ratio. Beads in the range of 75 to 150 microns in diameter offer a good balance of reaction kinetics versus reliability. Bead size is reported in Tyler Mesh size. Two commonly used resin sizes are 100-200 mesh and 200-400 mesh.

Many polymeric supports are now available and can be derivatized with functional groups to produce highly stable linkages and peptides with different functionalities at the terminal carboxyl group including amide, acid and thioester groups. Some examples are the *p*-methoxybenzhydrylamine (MBHA), 4-hydroxymethyl-phenylacetamidomethyl (PAM) and hydroxymethyl functionalized resins used
for t-Boc/Bzl and 4-(2',4'-dimethoxyphenyl-aminomethyl)-phenoxyethyl-polystyrene (Rink), 2-chlorotrityl chloride, and diphenyldiazomethane functionalized resins used for Fmoc/t-Bu (Figure 1.5).

![Functionalized resins for t-Boc/Bzl and Fmoc/t-Bu SPPS](image)

**Figure 1.5** Functionalized resins for t-Boc/Bzl and Fmoc/t-Bu SPPS

### 1.3.4 COUPLING REAGENTS

Amide bond formation is initiated by nucleophilic attack of the N-terminus amino group of an amino acid on the electrophilic, activated carboxyl group of the neighboring amino acid (Scheme 1.5). Coupling reagents such as the carbodiimides, dicyclohexylcarbodiimide (DCC) and diisopropylcarbodiimide (DIC) have been frequently used as 'activators' to lower the activation energy barrier associated with amide bond forming reactions (Figure 1.6).
Considering carbodiimide-based coupling conditions can lead to racemization and epimerization, additives such as 1-Hydroxybenzotriazole (HOBt) and 1-Hydroxyazabenzotriazole (HOAt) have been also added to the reaction mixture to minimize epimerization via the formation of a more reactive 'active ester' species in situ (Scheme 1.6).\textsuperscript{36} Towards this effect, more reactive coupling reagents such as the phosphonium and uronium based reagents have been more commonly used in SPPS with minimal effects on peptide racemization and epimerization.
1.3.5 PEPTIDE CLEAVAGE FROM SOLID SUPPORT

Following SPPS, the crude peptide is cleaved from the solid support while the protecting groups are also removed using strong acids such as trifluoroacetic acid or a nucleophile, such as reactive thiols or amines. The crude peptide is subsequently precipitated in diethyl ether to remove organic soluble impurities and purified by reverse-phase (RP) HPLC.

1.4 PEPTIDOMIMETICS

In spite of their biological activity, native peptides are prone to proteolytic degradation, limited cell permeability, non-specific and low affinity binding to receptor targets which impedes their duration of action. These drawbacks limit their use as drugs. These limitations can be circumvented by modifying the peptides to improve their pharmacokinetic properties, which forms the basis for the field of peptidomimetics.

Peptidomimetics are synthetic or biological molecules whose essential elements (pharmacophores) mimic a natural peptide in 3D space and retain the ability to interact with the biological...
target and produce the same biological effect as the native peptide.\(^2\) In the rational design of peptidomimetics, different important factors such as binding site optimal fit, polar or hydrophobic regions and conformational stabilization\(^3\) must be considered. These structure-activity relationships (SAR) define a minimal active sequence or major pharmacophore elements responsible for the biological effect. The 3-D arrangement of the binding site key residues are used to re-assemble these critical elements on a modified scaffold.\(^4\)

Several different strategies including the cleavage of endogenous precursor peptides,\(^4^4,4^5\) L to D amino acid substitution,\(^4^6\) unnatural amino acids substitution\(^4^6\) or cyclization involving side chains have been explored in peptidomimetics (Figure 1.7).

![Figure 1.7 Peptidomimetics: different methodologies](image)

Different synthetic methodologies for making cyclic peptidomimetics include side chain-to-side chain, head-to-side chain, side chain-to-tail cyclization and backbone cyclization,\(^4^7,4^7\) peptide stapling,\(^4^8\)
and native chemical ligation. Cyclic peptidomimetics are widely used in the clinic, where chemically stable bonds, such as amide, lactone, ether, thioether, or disulfide bonds are used to confer a stable peptide backbone for improved therapeutic effects in biological systems (Figure 1.8).

![Figure 1.8 Amino acid side chain modifications.](image)

1.5 THESIS OBJECTIVES

Over the years, a wide variety of side chain modified polypeptides have been developed to affect both conformational as well as functional properties. In this thesis, the effect of cyclic conformational constraint on the peptide backbone and side chain geometry are explored as chemical tools for improving peptide SARs against biological targets.

For example, Chapter 2 of this thesis describes the activation of the side-chain carboxylate group in Glutamic acid (Glu) to form a cyclic backbone intermediate, pyroglutamate, pGlu imide moiety by nucleophilic attack of the amide nitrogen, making the C–N bond prone to hydrolysis (Scheme 1.7). Taking advantage of the susceptibility of pGlu imide moiety towards hydrolysis, we developed a new mild and metal-free methodology which cleaves the unreactive peptide bonds specifically at Glu in native as well as in mutated peptides, containing amide bonds unsuitable for enzymatic degradation.
Scheme 1.7. Cyclization and cleavage of peptide bonds by formation of reactive pGlu imide intermediate

Cyclized peptides have many advantages such as increased cell permeability,\textsuperscript{6} resistance towards proteolytic degradation, enhanced bioactivity and enhanced binding to target molecules.\textsuperscript{7} Chapter 3 of this thesis describes a new methodology for making cyclic peptides by virtue of a urea based methodology for stapling the cyclic peptide (Scheme 1.8). In this methodology, the Lysine (Lys) ε-amino group is intrinsically nucleophilic\textsuperscript{51} with pKa values of 10.4 in model compounds.\textsuperscript{52} Amino-acid nucleophiles have been reacted with electrophilic groups to create drugs.\textsuperscript{51} In this application, the nucleophilic ε-amino group of the Lys side chain is modified by a carbonyl donor to activate the peptide side chain for cyclization reaction.\textsuperscript{53,54,55}

Scheme 1.8. Synthesis of urea-bridged peptides
In this manner, a carbonylating agent behaves as an electrophilic bifunctional linker providing structural rigidity and conformational preorganization to the peptide scaffold. This method provides a new mild, metal-free stapling strategy to build cyclic peptides with improved pharmacological properties such as increased proteolytic stability and alpha helicity. Moreover, we have adapted the methodology to build bicyclic compounds, as bicyclic compounds are capable of creation of a new generation of biotherapeutics (Figure 1.9 b).5631

**Figure 1.9.** Structures of cyclic and bicyclic compound synthesized in this study (R may be same or different)

### 1.6 REFERENCES


2.1 ABSTRACT

Cleavage of peptide bonds at a specific residue is an indispensable biochemical tool to explore various biotechnological, bioanalytical and bioengineering applications of peptides and proteins. Proteases, which hydrolyze peptide bonds at specific amino acid residues, have proven to be of great importance in chemical biology applications. However, their inability to recognize and cleave modified peptides or peptidomimetics limits their potential. Many emerging applications which involve peptidomimetics necessitate the need for new chemical reagents with improved efficiency for the cleavage of peptide bonds. These chemical reagents must selectively recognize and bind to one or more amino acid residues in the peptide sequence and specifically cleave the peptide bond at the reaction site. Based on this principle, we have developed a methodology, in which bromotris (pyrrolidino)phosphonium hexafluorophosphate (PyBrOP) is used to modify the side-chain carboxylate of glutamic acid, rendering it prone to hydrolysis. In this reaction, activation with PyBrOP produced a reactive pyroglutamate imide intermediate which upon incubation in buffer led to cleavage of the scissile peptide bond at the N-terminus of the modified glutamic acid residue. The strategy presents a valuable complementary tool for peptide and protein sequencing with a broad substrate scope, including cleavage of bioactive peptides and mutated peptides with unnatural D-amino acid residues implicated in various diseases.

2.2 CHAPTER OBJECTIVES

In this chapter, the development of an artificial chemical protease for the site-selective cleavage of unreactive peptide bonds at glutamic acid is described. The site selective cleavage of a peptide bond is an essential complementary tool in peptide and protein sequencing as well as to explore various applications of peptides and proteins in chemical biology. The amide bonds, however, are extremely unreactive
towards hydrolysis with a half-life of 500-1000 years at room temperature and pH 4-8. The extreme stability of peptide bonds limits the range of appropriate peptidolysis reagents. Though different proteases, metals, self-cleaving intein sequences, and various chemical reagents like cyanogen bromide are used for proteolysis, they suffer from inherent drawbacks such as the requirement for specific temperature and pH ranges, extended cleavage reaction times, toxic metals and harsh chemical conditions that are ill-suited for the cleavage of amide bonds, particularly in modified peptides. In order to address these limitations, a mild, metal-free methodology for cleaving amide bonds in native peptides as well as peptidomimetics is described in Chapter 2 of this thesis. This methodology involves selective modification of the side-chain carboxylic acid group of a Glutamic acid (Glu) residue, followed by cleavage specifically at the modified scissile bond under neutral aqueous buffer conditions. Furthermore, this chapter highlights the rational design, method development and substrate scope for this newly applied peptide cleavage strategy. This methodology provides an effective tool for peptidolysis specifically at the Pro-Glu peptide bond found within native sequences and mutated peptides which contained unnatural D-amino acids that are typically non-substrates for digestive enzymes. Thus, this methodology has extended the scope of chemical methods used for cleaving peptide bonds while providing significant insights into the development of a synthetic self-cleaving peptidase. Respectively, these findings may lead to important contributions to the field of peptide sequencing as well as in the design of new chemical systems that can mimic enzyme activity.
2.3 GRAPHICAL ABSTRACT

Glutamic Acid Selective Chemical Cleavage of Peptide Bonds
Joseph M. Nalbone, Neelam Lahankar, Lyssa Buissereth, and Monika Raj *

Site-specific hydrolysis of peptide bonds at glutamic acid under neutral aqueous conditions is reported. The method relies on the activation of the backbone amide chain at glutamic acid by the formation of a pyroglutamyl (pGlu) imide moiety. This activation increases the susceptibility of a peptide bond toward hydrolysis. The method is highly specific and demonstrates broad substrate scope including cleavage of various bioactive peptides with unnatural amino acid residues, which are unsuitable substrates for enzymatic hydrolysis.

This article was first published on February 11, 2016.

Figure 2.1 Graphical abstract for Glutamic Acid Selective Chemical Cleavage of Peptide Bonds.
2.4 INTRODUCTION

Site-selective cleavage of peptide bonds is a valuable source of insight into human proteomics as well as to explore various biotechnological, bioanalytical and bioengineering applications of peptides and proteins.\(^1\)

Conventionally, peptidases are used quite prevalently for residue-selective hydrolysis of peptide bonds,\(^4\) such as trypsin, which is selective for cleavage at the basic of Arg and Lys residues, chymotrypsin and pepsin which are selective for cleavage at aromatic hydrophobic amino acids, Phe, Trp, and Tyr (Figure 2.2). While proteolytic enzymes cleave proteins with great accuracy, efficiency and specificity, they suffer from many disadvantages. They need high specificity at their site of action, for instance, they will attack a peptide bond, provided the appropriate category of amino acid side chain defined by lipophilicity, steric properties etc., is present for substrate recognition.\(^11\) In addition, they tend to produce short fragments that are ill-suited for sequencing and contaminate the protein digest.\(^12\) Proteases require narrow ranges of pH and temperature. Moreover, organic solvents often destabilize and inactivate peptidases.\(^13\)

![Figure 2.2 Peptidase digestion of a polypeptide](image)

\(^{11}\text{Figure 2.2 Peptidase digestion of a polypeptide}\)
To circumvent the limitations faced by biological enzymes, various chemical methods are used for controlled cleavage of peptides and proteins. One of the early methods for protein sequencing was N-terminal amino acid analysis by Frederick Sanger, where he used Fluorodinitrobenzene (FDNB), though instead, Dabsyl chloride is more commonly used now, as it forms fluorescent derivatives easy to detect with high sensitivity. Dabsyl chloride reacts with uncharged α-NH$_2$ group to form a stable sulfonamide derivative that hydrolyzes peptide bonds (Scheme 2.1) as dabsyl-amino acid, which could be identified by its chromatographic properties.

Scheme 2.1 Dabsyl chloride N-terminal amino acid analysis.

Though sensitive and powerful, the method degrades the peptide in the acid-hydrolysis step and thus all sequence information is lost. To overcome this drawback, Edman devised a method for cleaving amino-terminal residue from the peptide without disrupting the peptide bonds between the other amino acid residues (Scheme 2.2).
Edman degradation is by far the most important and widely used method due to its efficiency, sensitivity and simplicity. This method uses phenyl isothiocyanate to cleave the amino acid one by one from the amino terminal. Under mildly alkaline conditions, the thiazolinone derivative of uncharged N-terminal acid is formed, which is converted to more stable phenylthiohydantoin (PTH)-amino acid under mildly acidic conditions, cleaved and then identified using chromatographic procedures.

Scheme 2.2 Edman degradation method for protein N-terminal sequencing.

Repeated cycles of phenyl isothiocyanate can elucidate the complete sequence of the original peptide, though it renders the method exhaustive and time consuming. In addition, as the length of the peptide increases, the efficiency of the method deceases. Even peptides less than 50 amino acids in length can become problematic in practice.
To complement these methods, some other chemicals such as cyanogen bromide,\textsuperscript{17} \( \alpha \)-iodosobenzoic acid\textsuperscript{19} are also used to fragment the original protein at specific amino acids into smaller peptides to facilitate sequencing. Cyanogen bromide hydrolyzes peptide bonds at the C-terminus of methionine residues forming a homoserine lactone, though, when methionine is followed by serine or threonine, side reactions can destroy the methionine without peptide bond cleavage, limiting the scope of the method (Scheme 2.3).

\[ \text{Scheme 2.3 Cyanogen bromide mediated cleavage of a peptide bond at methionine residue.} \]
Similarly, \(\ce{o-iodosobenzoic acid}\) cleaves peptide at tryptophan residues, but it also modifies tyrosyl residues in the peptide chain (Scheme 2.4).\(^{19}\)

**Scheme 2.4** \(\ce{o-iodosobenzoic acid}\) mediated cleavage of a peptide bond at tryptophan residue.

Different metal complexes with high catalyst turnovers, especially those containing \(\text{Zn}^{II}, \text{Co}^{II}, \text{Fe}^{IV}\) and \(\text{Cu}^{II}\) have been rationally synthesized for hydrolytic or oxidative cleavage of peptide bonds. For example, recently Kanai and Oisaki reported a serine-selective peptide-cleavage strategy that proceeds through mild aerobic oxidation promoted by a water-soluble copper\(^{II}\) - organoradical conjugate (Scheme 2.5).\(^{20}\) Still, practical applications of metals for protein analysis is still in its early stages.\(^{21-27}\)
Site-specific peptide bond cleavage methods have advanced significantly in the last 10 years. These have proven to be valuable chemical tools for biologically active peptides and peptidomimetics with their ability to hydrolyze mimetics comprised even of unnatural D-amino acids, thereby complementing enzymatic methods. However, these methods lag behind natural peptidases in activity, efficiency, cleavage site-fidelity, and substrate specificity due to different inherent shortcomings. For rapidly developing peptidomimetics, it is essential to bridge this gap with useful and practical artificial peptidases. Realizing the need for a rationally designed synthetic chemical cleavage strategy, we envisioned glutamic
acid (Glu) as a selective amino acid residue for the chemical cleavage of peptide bonds. The methodology entails activation of the side-chain carboxylate of Glu by formation of pyroglutamyl (pGlu) imide moiety (B) rendering the imide C–N bond susceptible to hydrolysis which leads to the cleavage of the peptide bond (Scheme 2.6).

2.5 RATIONAL DESIGN OF CYCLIC PYROGLUTAMYL (pGLU) IMIDE MOIETY CONTAINING PEPTIDES

In order to construct peptides with a C-N bond susceptible to hydrolysis, we decided to explore an approach of cyclizing the side chain carboxylic acid of a Glu residue in the peptide chain. We envisioned that Glu would favor formation of the kinetically preferred 5-membered pyroglutamyl (pGlu) imide ring following activation and cyclization. We proposed to activate the side chain carboxylic group of Glu into a reactive acyl chloride which would facilitate nucleophilic attack by the backbone amide nitrogen atom subsequently leading to cyclization (path A, Scheme 2.6). In principle, this cyclization reaction could lead to a 5-membered pyroglutamyl (pGlu) imide moiety B by path a or 6-membered piperidinedione B' by path b (Scheme 2.6). As the amide nitrogen of glutamic acid would have spatial proximity to its activated carboxylic group, we envisioned formation of the kinetically favored 5-membered pGlu imide moiety would be favored to render the scissile amide bond susceptible to hydrolysis, subsequently leading to desired cleavage at N-terminal of the Glu residue (Scheme 2.6).
Scheme 2.6 Rationale for the glutamic acid-selective modification and peptide cleavage in neutral aqueous solution.

2.6 RESULTS AND DISCUSSION

To achieve the generation of the pyroglutamyl imide moiety by cyclization at Glu, various activating agents were explored to activate the carboxylic acid side chain of Glu on a model hexapeptide Fmoc-Val-Ala-Glu-Arg-Phe-Ala-NH₂ (1a) (retention time \( t_R = 13.7 \text{ min} \)). The peptide was synthesized by Fmoc solid phase peptide synthesis (SPPS) with the N-terminal Fmoc protecting group present to avoid side reactions. A series of acylating agents for activating Glu were explored (Table 2.1). The % conversions from 1a to 2a were calculated by comparing peak areas of product to starting material from the HPLC data, which was further confirmed by molar mass obtained by mass spectrometry. In initial studies with N, N’ disuccinimidyl carbonate (DSC), a large excess of reagent and longer reaction times were needed for 50% conversion to the desired cyclized peptide 2a (entry 1, Table 2.1).
Table 2.1. Acylation reagents screening for formation of pGlu Imide Moiety 2a

<table>
<thead>
<tr>
<th>entry</th>
<th>reagent (equiv)</th>
<th>base (equiv)</th>
<th>additive</th>
<th>time (h)</th>
<th>conv(^d) (%)</th>
<th>conv(^b) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DSC (40)</td>
<td>DIEA (40)</td>
<td></td>
<td>48</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>PyBrop (20)</td>
<td>DIEA (20)</td>
<td></td>
<td>24</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>PyBrop (20)</td>
<td>DIEA (20)</td>
<td></td>
<td>48</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>PyBrop (40)</td>
<td>DIEA (40)</td>
<td></td>
<td>48</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>5(^c)</td>
<td>PyBrop (20)</td>
<td>DIEA (20)</td>
<td>DMAP</td>
<td>24</td>
<td>1</td>
<td>99</td>
</tr>
</tbody>
</table>

\(^a\)Reaction conditions: peptide (1 equiv) was reacted with DSC/PyBrop (20–40 equiv) and DIEA (20–40 equiv) in DMF at room temperature. \(^b\)Conversion to 2a was calculated from the absorbance at 254 nm using HPLC. \(^c\)A small crystal of DMAP was added to the reaction mixture. The entry in bold represents the optimized reaction conditions. DIEA = N, N-diisopropylethylamine, DMAP = 4-(N, N-dimethylamino)pyridine. \(^d\)Conversion to 2a\(^d\) was calculated from the absorbance at 254 nm using HPLC. Table Reprinted with permission from Nalbone, J.M.; Lahankar, N.; Buissereth, L.; Raj, M. Glutamic Acid Selective Cleavage of Peptide Bonds. *Org. Lett.* **2016**, *18* (5), 1186–1189. Copyright 2016, American Chemical Society.

When one of the more reactive coupling reagents, bromotris(pyrrolidino)phosphonium hexafluorophosphate (PyBrOP) was used for activation, with uncrystallized PyBrop, pyrrolide 2a\(^d\) as a side product was obtained with a mass 53 Da higher than the starting peptide 1a (entry 2, Table 2.1). (Figure 2.3). \(^{29, 30, 31}\)
After screening different reaction conditions, recrystallized PyBrOP (20 equiv.), DIEA \((N,N\text{-diisopropylethylamine})\) (20 equiv.), and catalytic amount of DMAP \((4-(N,N\text{-dimethylamino})\text{pyridine})\) in dimethylformamide (DMF) (0.5 mL) were selected as the reagents of choice (entry 5, Table 2.1) producing nearly quantitative conversion to the desired product 2a. Acylated hexapeptide cyclized at Glu after 17 h, with a retention time \(t_R = 13.7\) min, as analyzed by mass spectrometry (MS).

After optimization of the conditions for cyclization of Glu, NMR characterization was performed on the sequence Fmoc-Gly-Glu to confirm the formation of the five-membered cyclic ring rather than the six-membered ring. (Figure 2.4 and 2.5). Based on predicted chemical shifts values, if the six-membered ring had been formed, the methine \(^{13}\text{C}\) shift value would be 51.6 ppm (Figure 2.5). Confirmation of the five-membered cyclic pGlu ring formation was proven by the observed \(^{13}\text{C}\) shift value of 55.4 ppm, which was close to predicted \(^{13}\text{C}\) NMR chemical shift value of 55.5 ppm (Figure 2.5). The \(^1\text{H}/^{15}\text{N}\) HSQC data clearly ruled out the 6-membered ring structure due to the presence of the primary amide (six-membered ring structure would have two secondary amides) (Figure 2.4). In addition, HSQC, HMBC and \(^1\text{H}/^{1}\text{H}\) NOSEY NMR were consistent with this structure (Supporting information Figure A1).
Figure 2.4 $^{13}$C NMR and $^1$H/$^{15}$N HSQC NMR for modified Fmoc-Gly-pGlu. Reprinted with permission from Nalbone, J.M.; Lahankar, N.; Buissereth, L.; Raj, M. Glutamic Acid Selective Cleavage of Peptide Bonds. *Org. Lett.* **2016**, *18* (5), 1186–1189. Copyright 2016, American Chemical Society
Figure 2.5 Comparison of NMR chemical shifts for cyclization of Glu: Five-membered ring versus a six-membered ring.

Following the indication that the experimental NMR data matched the predicted spectra for Fmoc-Gly-pGlu, the modified peptide with pGlu moiety (2a) was subjected to incubation in phosphate buffer of pH 7.5 and the reaction progress was monitored by injecting the sample into an analytical HPLC at regular reaction time intervals (Figure 2.6). After 48 hours, the sharp peak at 13.7 min disappeared and two new peaks appeared. When analyzed by MS, the peaks at 5.6 and 22.7 min proved to be the expected hydrolyzed, cleaved peptide products. More specifically, the N-terminal fragment 3a eluted at 22.7 min, while the modified C-terminal fragment 4 eluted at 5.6 min. The successful cleavage reaction provided additional, indirect supporting evidence for the formation of the 5-membered pGlu intermediate, as the 6-membered ring cycle would not be susceptible to peptide bond cleavage.
After successful application of our Glu selective cleavage method to the model peptide Fmoc-Val-Ala-Glu-Arg-Phe-Ala-NH$_2$ (1a), we decided to probe the steric effect on the efficiency of cyclization and subsequent cleavage reaction by varying the side chain functionality of the amino acid residue preceding Glu. Also, we were interested in investigating the selectivity of our cleavage strategy towards other residues in the peptide chain with reactive functional groups. Various hexapeptides with the general sequence Fmoc-Val-X-Glu-Arg-Phe-Ala-NH$_2$ (1a–j) were synthesized to incorporate several different residues at position X (Table 2.2).
Table 2.2 Glu-Selective Amide Bond Cleavage of Fmoc-Val-X-Glu-Arg-Phe-Ala-NH$_2$ (1a–j) $^a$

![Diagram showing activation, hydrolysis, N-terminal fragment, and C-terminal fragment]

<table>
<thead>
<tr>
<th>entry</th>
<th>substrate</th>
<th>X</th>
<th>conv$^b$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1a</td>
<td>Ala</td>
<td>99</td>
</tr>
<tr>
<td>2</td>
<td>1b</td>
<td>Gly</td>
<td>99</td>
</tr>
<tr>
<td>3</td>
<td>1c</td>
<td>Arg</td>
<td>99</td>
</tr>
<tr>
<td>4</td>
<td>1d</td>
<td>Met</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>1e</td>
<td>Asn</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>1f</td>
<td>His</td>
<td>90</td>
</tr>
<tr>
<td>7</td>
<td>1g</td>
<td>Phe</td>
<td>90</td>
</tr>
<tr>
<td>8</td>
<td>1h$^c$</td>
<td>Tyr</td>
<td>90</td>
</tr>
<tr>
<td>9</td>
<td>1i$^c$</td>
<td>Ile</td>
<td>65</td>
</tr>
<tr>
<td>10</td>
<td>1j</td>
<td>Asp</td>
<td>99</td>
</tr>
</tbody>
</table>

$^a$Reaction conditions: peptide (1a–j, 1 equiv) was reacted with PyBrop, DIEA (20 equiv), and a crystal of DMAP in DMF at room temperature followed by hydrolysis with 0.1 M phosphate buffer (pH 7.5) at 25 °C for 48 h. $^b$Conversion to N-terminal fragment, Fmoc-V-X-OH (3a–j), was calculated from the absorbance at 254 nm using HPLC. $^c$Hydrolysis for 5 days. Reprinted with permission from Nalbone, J.M.; Lahankar, N.; Buissereth, L.; Raj, M. Glutamic Acid Selective Cleavage of Peptide Bonds. *Org. Lett.* **2016, 18 (5),** 1186–1189. Copyright 2016, American Chemical Society.
In all cases, after incubation in phosphate buffer, pGlu imide moiety containing C-terminal fragment 4 was obtained with the HPLC conversions ranging from 65 to 99%. Reactions proceeded smoothly in 48 h similarly to the reaction of 1a for unprotected peptides with X = Gly (1b), Arg (1c), Met (1d), Asn (1e), His (1f), and Phe (1g) (entries 2–7, Table 2.2). In contrast, substrates containing X = Tyr (1h) and Ile (1i) with bulky side groups gave the cleaved products in good yields but required longer time (5 days) for cleavage (entries 8 and 9, Table 2.2). All the above reactions proceeded cleanly without any by-products, unlike some other chemical cleavage methods,\cite{17,19} which result in the over-oxidation of Tyr, Trp, and Met containing peptides, indicating our mild method is tolerable of many side chain functional groups.

Peptide 1j, along with Glu, contains Asp, which also has free carboxylic group. This peptide under the reaction conditions could give the four membered β-lactam ring (5) or the five membered imide ring (6) by cyclization of Asp with Glu (2j). However, product (5) was not observed due to strain involved in the formation of the four membered ring. If product 6 had been formed, under the hydrolysis conditions, ring opening would occur rather than cleavage of the peptide bond. Instead, hydrolysis led to the fission of the peptide bond at the N-terminus of Glu generating two fragments as previously reported and confirmed by HPLC and MS. Based on these results, the reactions conditions were determined to be selective for Glu (Figure 2.7).
**Figure 2.7** Reactivity of Asp vs Glu toward the Backbone Activation for Peptide 1j.

The methodology was also applied to the cleavage of long peptides with multiple Glu residues and even peptides with unnatural D-amino acids, demonstrating its wide substrate scope. Longer 12-mer and 13-mer peptides (7 and 8) with two Glu residues at internal positions underwent effortless cleavage at both glutamic acids and delivered three fragments in high yield (80−85%) (entries 1 and 2, Table 2.3, and Supporting Information). Peptide 9 with unnatural amino acid residues and peptide 10, with a mixture of L- and D-amino acids, respectively, were cleaved successfully under the reaction conditions at Glu with ease and high yields (entries 3 and 4, Table 2.3). Conversion of natural L-amino acids to unnatural D-amino acids is a well-known mutation responsible for various age related disorders such as cataracts and Alzheimer’s disease, but enzymes cannot recognize and cleave these modified peptides. Thus, this method can be used as a diagnostic tool to determine different types of mutations in proteins and their role in the progression of diseases.

Furthermore, peptide 11 with an intramolecular disulfide bridge afforded the cleavage product at glutamic acid with an intact disulfide bond (entry 5, Table 2.3). Thus, this methodology can be used to determine the position of disulfide bonding in a peptide chain, which is in contrast to other chemical
Peptide 12, containing a serine residue with a reactive hydroxymethyl group at the side chain, remained unreacted under the reaction conditions, and cleavage was observed only at Glu (entry 6, Table 2.3). This again confirmed the high specificity of this methodology toward Glu.

**Table 2.3. Substrate Scope of Glu-Selective Amide Bond Hydrolysis**

<table>
<thead>
<tr>
<th>entry</th>
<th>substrate</th>
<th>yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Fmoc-Ala-Val-Arg-<strong>Glu</strong>-Val-Ala-Phe-<strong>Glu</strong>-Arg-Phe-Gly-Phe-NH₂ (7)</td>
<td>80</td>
</tr>
<tr>
<td>2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Fmoc-Arg-Ala-Gly-Ala-<strong>Glu</strong>-Val-Arg-Phe-Ala-<strong>Glu</strong>-Ala-Phe-Gly-NH₂ (8)</td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td>Fmoc-d-Val-d-Ala-D-<strong>Glu</strong>-d-Arg-d-Phe-d-Ala-NH₂ (9)</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>Fmoc-d-Val-Ala-<strong>Glu</strong>-d-Arg-d-Phe-Ala-NH₂ (10)</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>Fmoc-Cys-Gly-Arg-Ala-Cys-Gly-<strong>Glu</strong>-Phe-Ala-Gly-NH₂, disulfide bond (11)</td>
<td>75</td>
</tr>
<tr>
<td>6</td>
<td>Fmoc-Arg-Ala-<strong>Glu</strong>-Ala-Gly-<strong>Ser</strong>-Gly-Phe-NH₂ (12)</td>
<td>90</td>
</tr>
</tbody>
</table>

<sup>a</sup>Reaction conditions: peptide (1 equiv) was reacted with PyBrop (20 equiv), DIEA (20 equiv), and a crystal of DMAP in DMF followed by cleavage with 0.1 M phosphate buffer (pH 7.5) at 25 °C for 48 h unless otherwise noted. <sup>b</sup>Yield of N-terminal fragment with Fmoc group.<sup>c</sup> Three fragments were detected in the HPLC trace for cleavage at both Glu residues. Table adopted from Nalbone, J. M.; Lahankar, N.; Buissereth, L.; Raj, M. Org. Lett. 2016, 18, 1186 with permission.

Subsequently, bioactive peptides: 13, a putative coproporphyrinogen III oxidase fragment, 16, and Aβ (10–19), 19, a fragment of Alzheimer’s disease associated amyloid-β peptide (entries 1–3, Table 2.4, and Figure A18-A20, Supplementary Information ) were cleaved successfully with good 79-80% conversion to the product.
Table 2.4. Glu-Selective Cleavage of Bioactive Peptides

<table>
<thead>
<tr>
<th>entry</th>
<th>substrate</th>
<th>yielda (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fmoc-Met-Gly-His-<em>Glu</em>-Glu-His-Leu-Pro-Tyr- NH₂ (13)</td>
<td>79</td>
</tr>
<tr>
<td>2</td>
<td>Fmoc-Leu-Pro-Arg-Leu-<em>Glu</em>-Glu-Ala-Trp-Gln- NH₂ (16)</td>
<td>75</td>
</tr>
<tr>
<td>3</td>
<td>Fmoc-<em>Tyr</em>-Glu-Val-His-Glu-Lys-Leu-Val-Phe-NH₂ (19)</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>Fmoc-Ala-Gly-Leu-<em>Pro</em>-Glu-Lys-Tyr-NH₂ (22)</td>
<td>82</td>
</tr>
</tbody>
</table>

*a Yield of N-terminal fragment with Fmoc group. Table adopted from Nalbone, J. M.; Lahankar, N.; Buissereth, L.; Raj, M. Org. Lett. 2016, 18, 1186 with permission.

Finally, this methodology was evaluated on a bioactive peptide, amyloid A protein fragment (Homo sapiens) 22, with a proline residue next to Glu (entry 4, Table 4). The location of proline at a neighboring position nearly blocks the cleavage by proteases completely independent of the amino acid residue, 27,28 but remarkably this method cleaved the Pro-Glu bond with 82% conversions to the peptide fragments. Interestingly, even though peptides 19 and 22 contained lysine residues with a free side-chain, cyclization was observed only at Glu to generate the kinetically favorable five-membered pGlu moiety (Figure A20, A21 Supporting Information) further confirming high specificity and broad substrate scope of the cleavage strategy.

2.7 CONCLUSIONS

In this study, a site-selective approach for the cleavage of peptides at the N-terminus of Glu under mild and metal-free reaction conditions with high specificity was developed. The method was tolerant to wide range of unprotected side chains functionalities within the tested peptides unlike some other chemical cleavage methods. In addition, as disulfide bonds are stable toward reaction conditions, this methodology can be useful for determining the position of disulfide pairing in peptides. Moreover, the
method exhibited broad substrate scope including the cleavage of peptides at proteolytically resistant Pro-Glu sites. Similarly, hydrolysis of mutated peptides with unnatural amino acid residues such as D-amino acids, unsuitable substrates for enzymes, proceeded with ease under the reaction conditions. The strategy presents a potential valuable complementary tool for peptide and protein sequencing with a broad substrate scope, including cleavage of bioactive peptides and mutated peptides with unnatural amino acid residues such as D-amino acids implicated in various diseases. The results of this study have provided a foundation for further studies aimed at developing artificial chemical proteases for the cleavage of target proteins responsible for various diseases

2.8 EXPERIMENTAL SECTION

General.

All commercial materials (Aldrich, Fluka, Nova) were used without further purification. All solvents were reagent grade or HPLC grade (Fisher). Anhydrous THF, diethyl ether, CH₂Cl₂, and DMF were obtained from a dry solvent system (passed through column of alumina) and were used without further drying. All reactions were performed under air in round bottom flask. Yields refer to chromatographically pure compounds; % yields were obtained by comparison of HPLC peak areas of products and starting material. HPLC was used to monitor the reaction progress.

Materials.

Fmoc-amino acids were obtained from Nova Biochem under (EMD Millipore Corporation) (Billerica, Massachusetts) and CreoSalus (Louisville, Kentucky). Rink amide resin was obtained from ChemPep Inc (Wellington, Florida). N,N,N',N'-Tetramethyl-O-(1Hbenzotriazol-1-yl)uronium hexafluorophosphate (HBTU) was obtained from CreoSalus (Louisville, Kentucky). N, N'-Disuccinimidyl carbonate (DSC) was obtained from Nova Biochem, under (EMD Millipore Corporation) (Billerica, Massachusetts). Bromotris(pyrrolidino)phosphonium hexafluoro-phosphate (PyBrop) was obtained from Sigma Aldrich (St. Louis, Missouri). 4-Dimethylaminopyridine (DMAP): Merck KGaA (Darmstadt, Germany). N, N-
Dimethylformamide (DMF): Macron Fine Chemicals (Center Valley, Pennsylvania). Dichloroethane (DCE), acetonitrile, N, N- Diisopropylethyamine (DIEA), N, N’diisopropylcarbodiimide (DIC), were purchased from (EMD Millipore Corporation) (Billerica, Massachusetts). Piperidine was purchased from Alfa Aesar (Ward Hill, Massachusetts).

Trifluoroacetic acid (TFA) was purchased from VWR (100 Matsonford Road Radnor, PA). Diethyl Ether: Sigma Aldrich (St. Louis, Missouri). Water was purified using a Millipore MilliQ water purification system.

**NMR:**

Proton NMR spectra were recorded on a 600 MHz spectrometer and carbon NMR spectra on a 151 MHz spectrometer at ambient temperature. All NMR chemical shifts (δ) are referenced in ppm relative to residual solvent or internal tetramethylsilane. ¹H NMR chemical shifts are referenced to residual DMSO-d₅ at 2.50 ppm, and ¹³C NMR chemical shifts are referenced to DMSO-d₆ at 39.52 ppm. Carbon NMR spectra are proton decoupled. NMR spectral data are reported as chemical shift (multiplicity, coupling constants (J), integration). Multiplicity is reported as follows: singlet (s), broad singlet (bs), doublet (d), doublet of doubles (dd), doublet of triplet (td), triplet (t) and multiplet (m). Coupling constant (J) in Hertz (Hz).

**HPLC**

**Semi-Preparative HPLC:**

Semi-preparative HPLC chromatography (HPLC) was performed on Beckman Coulter equipped with System Gold 168 detector and 125P solvent module HPLC with a 10 mm C-18 reversed-phase column. All separations involved a mobile phase of 0.1% FA (v/v) in water (solvent A) and 0.1% FA (v/v) in acetonitrile (solvent B). Semi-preparative HPLC method using a linear gradient of 0–80% acetonitrile in 0.1% aqueous FA over 30 min at room temperature with a flow rate of 3.0 mL min⁻¹. The eluent was monitored by absorbance at 220 nm and 254 nm unless otherwise noted.
Analytical HPLC:

Analytical HPLC chromatography (HPLC) was performed on an Agilent 1100 series HPLC equipped with a 4.6 x150 mm (5µm) C-18 reversed-phase column. All separations involved a mobile phase of 0.1% FA (v/v) in water (solvent A) and 0.1% FA (v/v) in acetonitrile (solvent B). Peptide compositions were evaluated by analytical reverse phase HPLC using a gradient of 0.1% FA in acetonitrile versus 0.1% FA in water. Analytical HPLC method using a linear gradient of 0–80% 0.1% FA (v/v) acetonitrile in 0.1% aqueous FA over 30 min at room temperature with a flow rate of 1.0 mL min\(^{-1}\). The eluent was monitored by absorbance at 254 nm unless otherwise noted.

LCMS:

Mass spectrometry was performed using ultra high-performance liquid chromatography-mass spectrometry using the Agilent 1100 Series LCMSD VL MS Spectrometer using positive polarity electrospray ionization (+ESI).

Fmoc Solid-Phase Peptide Synthesis.\(^{37}\)

Peptides were synthesized manually on a 0.25 mmol scale using a Rink amide resin. The Fmoc–group was deprotected using 20% piperidine–DMF for 20 min to obtain a deprotected peptide-resin. Fmoc-protected amino acids (1.25 mm) were sequentially coupled on the resin using a HBTU (1.25 mmol) and DIEA (1.25 mmol) for 2 h at room temperature. Peptides were synthesized using standard protocols.\(^{37}\) The peptide was cleaved from the resin using a cocktail of 95:2.5:2.5, trifluoroacetic acid:triisopropyl silane:water for 2 h. The resin was removed by filtration and the resulting solution was concentrated. The oily residue was triturated with diethyl ether to obtain a white suspension. The resulting solid was purified by HPLC.
General Procedure for cyclization of a peptide:

To a 5-mL round-bottom flask containing peptide (I) (20 mg, 1 equiv.) in 1 mL dimethylformamide (DMF) was added a solution of PyBrop (20 equiv.), DIEA (20 equiv.) and crystal of DMAP in DMF (0.5 mL). The mixture was stirred at room temperature for 24 h. The reaction was concentrated under vacuum and resulting peptide was dissolved in 1:1 mixture of water and acetonitrile and purified by HPLC. The purified fractions were lyophilized to afford cyclized peptide (II) as a white powder; yield: (80 %).

General procedure for the cleavage of peptides:

To a cyclized peptide (2a-j), 1 mL phosphate buffer (pH 7.5) was added. The reaction was stirred at 25 °C and monitored by analytical HPLC at regular intervals. The reaction mixture was lyophilized, purified by HPLC and isolated. HPLC: 0.1% FA (v/v) in water (solvent A): 0.1% FA (v/v) acetonitrile (solvent B); gradient 0-80 %, 0.1% FA (v/v) acetonitrile in 25 min, flow rate = 1.0 mL/min, detection wavelength 254 nm.

2.9 REFERENCES


3.1. ABSTRACT

An efficient synthesis strategy has been developed for peptide stapling onto activated lysine residues. In this approach, a two-step one pot reaction featuring activation of the side chain amino group of lysine with carbonyl diimidazole followed by urea bond formation with an adjacent side chain amino group of a second lysine residue was accomplished without the need of any metal catalyst. This methodology was used to synthesize a stapled Axin mimetic analogue, which demonstrated increased alpha helicity and improved proteolytic stability upon peptide stapling. Moreover, a bicyclic peptide was prepared with this strategy, to further extend the scope of reaction. Finally, this methodology can be potentially applied for the production of urea stapled peptides as mild and efficient synergistic catalysts for carrying out a range of organic asymmetric transformations like Michael Addition, and C-H functionalization reactions with high reactivity and selectivity.

3.2 GRAPHICAL ABSTRACT

Figure 3.1. General method for the synthesis of urea-bridged cyclic peptides

3.3. INTRODUCTION

Peptide drugs have gained a wide range of applications in treating human malignancies such as cancer (Goserelin, a synthetic gonadotropin-releasing hormone analog), multiple sclerosis (Glatiramer...
acetate, a synthetic peptide with four amino acids), and type 2 diabetes (Exenatide, a synthetic glucagon-like peptide-1 analog).\textsuperscript{1} Peptides can provide target specificity, potency and longer resident times for more effective duration of action which improves biological efficacy and minimizes side effects.\textsuperscript{2, 3} However, short synthetic peptides exhibit poor pharmacokinetic and pharmacodynamics properties which limits their translation into the clinic.\textsuperscript{4} In addition, peptides are susceptible to proteolytic degradation\textsuperscript{1} and they are poorly cell permeable which further restricts their drug-like characteristics.\textsuperscript{5}

### 3.3.1 BIOACTIVE CYCLIC PEPTIDES

As stapling linear peptides constrains them into cyclic form, the likelihood of the formation of intramolecular hydrogen bonds increases, which can decrease interactions with water, thus, increasing the potential for membrane permeability by passive diffusion.\textsuperscript{6} In addition, constrained cyclic peptides contain amide bonds which are more difficult to access by proteases, thereby improving their proteolytic resistance. Moreover, the structural stability resulting from cyclization favors biologically active conformations of cyclized peptides like Vasopressin and Desmopressin (antidiuretic hormones Eptifibatide (an antithrombolytic agent), Oxytocin (hormone related to childbirth and social bonding) (\textbf{Figure 3.2})\textsuperscript{7} with target molecules; thus decreasing the entropic cost upon binding to their target.\textsuperscript{8} For the aforementioned reasons, stapling short peptides can greatly improve their pharmacological performance.
3.3.2 SYNTHESIS OF CYCLIC PEPTIDES

Since the development of stapling techniques by Grubbs\(^9\) and Verdine using Ring-Closing Metathesis (RCM),\(^10\) various stapling methods have been explored in order to off-set some of their limitations, including the use of transition metal catalysts, un-natural amino acid residues and harsh reaction conditions that may denature peptide structure. In spite of their utility, these methods, including the use of cysteine side chains to form disulfide bridges\(^11\) and thioether formation (crosslinking with \(\alpha,\alpha'\)-dibromo-m-xylene,\(^12\) aromatic nucleophilic substitutions with perfluoroaromatic reactants),\(^13\) the use of functionalized non-natural amino acids and biaryl linkages involving the borylated phenylalanine derivatives,\(^14\)–\(^17\) or azide–alkyne cycloadditions (click chemistry)\(^18\) still required metal catalysts, usually expensive, long stepwise syntheses and the use of unnatural amino acids.

Such limitations can be overcome by new practical and general stapling methods involving modifications of naturally occurring residues. These stapling strategies are useful in modifying readily
available peptides/proteins.\textsuperscript{19-21} Chemoselective bioconjugation techniques have been developed to modify native residues such as lysine and cysteine in order to link them with reactive and functional groups that enable peptide cyclization.\textsuperscript{22} Lysine is considerably more prevalent than cysteine in naturally occurring peptides and proteins,\textsuperscript{22} and an abundance of methods to selectively modify primary amines makes them desirable reactive targets for functionalization. Building on these facts, different one-component side-chain stapling methodologies between lysine and other amino acids, like lactamization,\textsuperscript{23} have been employed to generate structurally rigid stapled peptides with improved helicity as well as biological activity (Figure 3.2, a). Additionally, by introducing a bifunctional linker between the two lysine residues, stapled peptides with different properties can be generated from linear peptides in the more versatile two-component stapling approach (Figure 3.2, b).\textsuperscript{24}

\textbf{Figure 3.3.} a-One-component stapling methodology. BOP: (benzotriazol-1-yloxy) tris(dimethylamino)phosphonium hexafluorophosphate, DIPEA: N,N-diisopropylethylamine. b- Two-component stapling methodology. 1. CuAAC- Copper-Catalyzed Azide-Alkyne Cycloaddition 2. RCM- Ring-closing metathesis
Thus, new synthetic chemical approaches for the selective and straightforward modification of lysine are of great importance for stapling methodology.

3.4. CHAPTER OBJECTIVES

In this thesis chapter, a biocompatible, two-component stapling methodology to modify the ε-amino group of lysine\(^{51}\) (pKa values of 10.4 in model compounds)\(^{52}\) with a reactive carbonyl donor leading to the formation of a urea functionality with an adjacent deprotonated lysine is reported. We hypothesized that in presence of a base and the electrophilic bifunctional linker carbonylating agent, the lysine residue will be activated in an intermolecular S\(_{N2}\) reaction to form intermediate A, which will subsequently generate the urea-stapled peptide B by an intramolecular nucleophilic attack by another lysine residue (Scheme 3.1). This method will be optimized by exploring the influence of the base and carbonylating reagent on the cyclization reaction in model peptides. With optimized conditions in hand, a series of bio-active peptides will be subjected to the proposed urea stapling strategy to investigate the influence of structure on peptide biological activity.

Scheme 3.1: Proposed mechanism for the urea stapling reaction

3.5 RESULTS AND DISCUSSION

3.5.1 PRELIMINARY RESULTS

To investigate this one-step, two component synthetic strategy, different carbonylating agents, namely, N, N'-disuccinimidyl carbonate (DSC), 1,1'-carbonyldiimidazole (CDI), and 4-nitrophenyl chloroformate (NPCF) were screened for lysine reactivity within a model N-terminus acetyl-protected hexapeptide Ac-Phe-Arg-Lys-Gly-Lys-Ala-CONH\(_2\) in dimethylformamide (DMF) as reaction solvent and
N,N-diisopropylethylamine (DIEA) as base (Table 3.1). The desired urea-stapled peptide was observed and confirmed by LCMS, HRMS, MS/MS, and NMR spectroscopy which validated H/D exchange of the product (Figure 3.4).

H/D Exchange Data: # of exchangeable protons = 788 – 773 – 1 = 14
Figure 3.4: H/D exchange, MS, MS/MS, HRMS, NMR of stapled Ac-Phe-Arg-Lys-Gly-Lys-Ala-CONH$_2$
Good conversions (69%) were detected by HPLC conversion with CDI as the active carbonylating reagent at 25°C, (Table 3.1, entry 3), and was subsequently selected as the reagent of choice for the stapling reaction.

Table 3.1. Reagent screening for the urea stapling reaction.\(^a\)

<table>
<thead>
<tr>
<th>entry</th>
<th>reagent</th>
<th>conv(^b)(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4-nitrophenyl chloroformate</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>DSC</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>CDI</td>
<td>69</td>
</tr>
</tbody>
</table>

\(^a\) Reaction conditions: peptide (1 equiv) was reacted with CDI (3 equiv), DIEA (3 equiv) in DMF at 25°C for 24h. \(^b\) Conversion to product was calculated with HPLC by measuring absorbance at 220 nm.

The stapling reactions were conducted for 24 h since the reaction of the model peptide Ac-FKGAKF with CDI, gave optimum conversion (64%) to the product following a one-day reaction (Table 3.2, entry 3) (Figure 3.5)

Table 3.2. Optimization of conditions for the urea stapling reaction\(^a\): Time

<table>
<thead>
<tr>
<th>entry</th>
<th>time</th>
<th>conv(^b) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>13.83</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>26.73</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>63.43</td>
</tr>
</tbody>
</table>

\(^a\) peptide (1 equiv) was reacted with CDI (3 equiv), DIEA (3 equiv) in DMF at 25°C for 24h. \(^b\) Conversion to product was calculated with HPLC by measuring absorbance at 220 nm.
Next, in order to optimize the distance between two lysine residues and reaction temperature, a series of peptides with two lysine residues separated by varying amino acids were synthesized, and subsequently subjected to the optimized CDI conditions at 25°C and 65°C.

**Table 3.3** Optimization of reaction conditions for urea stapling \(^\ddagger\): Distance between lysine residues and temperature.

<table>
<thead>
<tr>
<th>entry</th>
<th>peptide</th>
<th>position of lysine residues</th>
<th>equiv of CDI</th>
<th>conv(^a) to product (%)</th>
<th>equiv of CDI</th>
<th>conv(^b) to product (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ac-Phe-Lys-Lys-Phe-NH(_2) (3)</td>
<td>i, i+1</td>
<td>3</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Ac-Phe-Arg-Lys-Gly-Ala-NH(_2) (1)</td>
<td>l, i+2</td>
<td>3</td>
<td>69</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Ac-Phe-Lys-Gly-Ala-Lys-Phe-NH(_2) (2)</td>
<td>i, i+3</td>
<td>3</td>
<td>63</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH(_2) (4)</td>
<td>i, i+4</td>
<td>3</td>
<td>86</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Ac-Arg-Lys-Ala-Leu-Gly-Ala-Lys-Phe-NH(_2) (5)</td>
<td>i, i+5</td>
<td>3</td>
<td>40</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 3.5: HPLC trace and MS for stapled Ac-Phe-Lys-Gly-Ala-Lys-Phe-NH\(_2\) at 25°C after 24 h
Ac-Phe-Lys-Val-Ala-Leu-Gly-Ala-Lys-

Phe-NH₂ (6)

7        Ac-Phe-Lys-Gly-Lys-Leu-NH₂ (7)

8        Ac-Phe-Lys-Phe-Lys-Leu-NH₂ (8)

9        Ac-Phe-Arg-Lys-Gly-Ala-NH₂ (1)

10       Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH₂ (4)

11       Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-

Val-Gln-Arg-Val-Met-NH₂ (9)

12       Ac-Thr-Ser-Phe-Lys-Glu-Tyr-Trp-His-Leu-Leu-

-Ser-NH₂ (10)

13       Ac- Ala-Lys-Leu-Gly-Phe-Lys-Leu-NH₂ (11)

14       Ac- Ile-Lys-Leu-Gly-Phe-Lys-Leu-NH₂ (12)

15       Phe- Lys-Leu-Gly-Ala-Lys-Phe-Glu (13)

a Reaction conditions: peptide (1 equiv) was reacted with CDI, DIEA in DMF at 25°C for 24 h. b Conversion to product was calculated with HPLC by measuring absorbance at 220 nm. c Reaction continued for 48 h, 23.587% intermediate.

At 25°C, high conversion to the urea stapled peptide was obtained when two lysine residues were at i and i+2, i and i+3 and i and i+4 positions with good HPLC conversions ranging from 69% to 86% (Table 3.3, entries 2-4) (Figure 3.4, 3.5, 3.6).
Figure 3.6: HPLC trace and MS for stapled Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH₂ (4) at 25°C after 24 h

Though stapled product was obtained with lysine residues at positions i and i+5, modest HPLC conversion to product was observed (40%, Table 3.3, entry 5) (Figure 3.7), while no stapled product was detected with lysine residues at i and i+6 positions (Table 3.3, entry 6).

Figure 3.7: HPLC trace and MS Ac-Arg-Lys-Ala-Leu-Gly-Ala-Lys-Phe-NH₂ (5a) at 25°C after 24 h
When the stapling reaction was carried out with 3 equiv of CDI at 65°C, it gave better conversion to product than for the reaction at 25°C (Table 3.3, entries 7 and 8), though, the amount of CDI for maximum conversion to product varied from peptide to peptide. (Table 3.3, entries 9 and 10) (Figure A23, A31, Supplementary Information).

We then proceeded to investigate the selectivity of the stapling reaction toward other residues in the peptide chain. Two model peptides, Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-CNH₂ (9) and Ac-Thr-Ser-Phe-Lys-Glu-Tyr-Trp-His-Leu-Leu-Ser-CNH₂ (10) that contained all nucleophilic residues except for cysteine, it being more nucleophilic than lysine (pKₐ around 8.5), 27 were designed (Table 3.3, entries 11 and 12 respectively) (Figure 3.8).

Both the peptides reacted cleanly without any by-products even with unprotected side chains of other amino acids in peptides. Urea stapled product was obtained for both peptides (9a and 10a) with high HPLC conversion ranging from 89% to 93%. Thus, the urea stapling methodology is chemoselective to the lysine side-chain amino groups in N-terminus Ac-protected peptides in the presence of reactive functional groups, namely, alcohols and carboxylic acids in the peptides (Table 3.3, entries 11 and 12). (Table 3.3, entries 11 and 12 respectively) (Figure 3.8).

Figure 3.8: Selectivity of peptide stapling: Stapled peptides Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH₂ (9a) and Ac-Thr-Ser-Phe-Lys-Glu-Tyr-Trp-His-Leu-Leu-Ser-NH₂ (10a)
We then aimed to explore the steric effect of side-chain functionality of the amino acids adjacent to lysine residues on the efficiency of the stapling reaction. There was only 9% HPLC conversion to the product for the peptide Ac-Phe-Lys-Phe-Lys-Leu-NH$_2$ (8) with the bulky phenylalanine in between the reactive lysine residues, whereas, 32% conversion was observed for the peptide Ac-Phe-Lys-Gly-Lys-Leu-NH$_2$ (7) at 25°C which involved substitution of Phe$^3$ for Gly$^3$ (Table 3.3, entries 8 and 7, respectively) (Figure 3.9). Thus, the bulky Phe$^3$ residue had an adverse effect on peptide stapling efficiency when compared to the sterically unencumbered Gly$^3$ residue.

![Figure 3.9: HPLC traces of stapled Ac-Phe-Lys-Gly-Lys-Leu-NH$_2$ (7a) and Ac-Phe-Lys-Phe-Lys-Leu-NH$_2$ (8a)](image)

At 65°C, HPLC conversion to product for peptide Ac-Ile-Lys-Leu-Gly-Phe-Lys-Leu-NH$_2$ (12) with bulky isoleucine residue placed outside the stapling site was 67% compared to 44% for peptide Ac-Ala-Lys-Leu-Gly-Phe-Lys-Leu-NH$_2$ (11) which substituted Ile$^1$ for Ala$^1$ (Table 3.3, entries 14 and 13 respectively), while peptide Ac-Phe-Lys-Phe-Lys-Leu-NH$_2$ (8) with bulky Phe$^3$ inside the stapling site gave 74% HPLC conversion to product, as against 63% for peptide Ac-Phe-Lys-Gly-Lys-Leu-NH$_2$ (7) (Table 3.3, entries 8 and 7 respectively), indicating that steric bulk did not affect efficiency of stapling reaction at elevated temperature, whether outside or inside the staple to be formed.
3.5.2. PROTEOLYTIC STABILITY OF THE STAPLED PEPTIDE

The proteolytic stability of the stapled peptide belonging to an Axin mimic was compared to its linear analogue (Figure 3.10). Axin analogue Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH$_2$ (9) (25 μM) widely reported in various peptide stapling papers$^{28}$ and its urea stapled analogue (9a) were subjected to *in vitro* trypsin$^{29}$ digestion in 1 M phosphate buffer (0.5 ng/μL, 25°C, pH 7.3) and monitored for degradation by LC/MS. Degradation of the linear peptide was completed in 30 min producing four detectable fragments, confirmed by MS (Figure 3.10b, 3.11a). In contrast, stapled peptide, while intact after 30 min, showed only one fragment containing the urea moiety, Ac-Glu-Asn-Pro-Glu-Lys$\hat{\text{Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH}}_2$, which did not degrade even after 90 min (9v) incubation, confirmed by MS. Thus, the urea stapled Axin peptide exhibited increased structural stability towards proteolysis. (Figure 3.9b, 3.10b).

![Figure 3.10 a) Expected cleavage sites for linear peptide and stapled peptide upon exposure to trypsin. b) Actual fragments obtained for linear peptide and stapled peptide after exposure to trypsin.](image-url)
Figure 3.11a: MS traces of linear Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH₂ (9) and its four (9i-iv) fragments after digestion with trypsin
Figure 3.11b: MS trace of stapled Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH$_2$ (9a) and fragment (9v) after digestion with trypsin.
3.5.3 SECONDARY STRUCTURE ANALYSIS OF THE STAPLED PEPTIDE

The Axin mimetic Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH₂ (9) and its urea stapled analogue (9a) were next explored to detect their secondary structures (if any) using Circular Dichroism (CD) spectroscopy (Figure 3.12). When dissolved in TFE/water, the urea stapled peptide clearly showed the α-helical signature with an observed minima at 208 nm and 222 nm and a maxima at 190 nm (Figure 31.2a). The comparison of the helical propensity indicated that the urea stapled peptide had a higher tendency (50%) to adopt α-helical character in 1:9 v/v TFE: water while the unstapled linear peptide exhibited a weaker (21%) helical structure (Figure 3.12b).

![Figure 3.12](image)

Figure 3.12: Helicity of linear and urea stapled peptide: CD spectra recorded for the peptide (5 μM) with different amounts of TFE in phosphate buffer (pH 7.3). Urea stapled peptide indicated a high tendency to adopt α-helical character.

3.5.4 SYNTHESIS OF A BICYCLIC PEPTIDE

In order to illustrate the application of the urea-stapling methodology, a bicyclic peptide was synthesized as a new class of bioactive bicyclic compounds. In this synthesis strategy, the first ring was constructed by protecting the acid side chain of glutamic acid. The free N-terminus amino group of the peptide was used for on-resin head-to-tail cyclization with the C-terminus carboxylic acid group of the model peptide NH₂-Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-COOH (13) (Scheme 3.2). Overnight reaction in the
presence of HOAT/DIC (Scheme 3.2) generated the first ring (13a), confirmed by HRMS and NMR (Figure 3.13). The second cyclization reaction was carried out by the urea stapling methodology of two internal lysine residues (Scheme 3.2, 13b) and the product was confirmed by MS and NMR (Figure 3.14). From the ion mobility mass spectrometry (IMS) data (Figure 3.15), the bicyclic peptide was shown to exist as two conformational diastereomers, 13b-1 (retention time tR 11.2min) and 13b-2 (retention time tR 12.4min). However, the conformer 13b-1 (92%) was found to dominate conformer 13b-2 (6%) (Figure 3.16) Thus, an appropriate choice of the protection scheme allowed for the synthesis of structurally diverse bicyclic peptides by our urea stapling strategy.

Scheme 3.2: Synthesis of a bicyclic compound: stapling strategy
HRMS data - Monocycle Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-CONH$_2$: [C$_{46}$H$_{69}$N$_{11}$O$_9$+2H]$^{2+}$: m/z [M+2/2]$^+$ 460.768 (calc.M+2/2)$^+$ 460.77), [M+H]$^+$ m/z 920.52 (calc[M+H]$^+$ =920.53)

Figure 3.13: HRMS and $^1$H NMR for Monocyclic peptide Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-CONH$_2$ (13a)
**Figure 3.14:** MS and $^1$H NMR of Bicycle Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH$_2$ (13b).

Figure 3.15 Ion Mobility Resolved MS/MS (Overlay) (A) 328 Å² and (B) 339 Å² Conformations for bicyclic Peptide Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH₂ (13b).
Figure 3.16: HPLC trace of two conformational diastereomers, 13b-1 and 13b-2 of the bicyclic Peptide Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH₂ (13b).

3.6 CONCLUSIONS

In conclusion, a urea-based strategy for stapling and cyclizing peptides has been developed in this thesis chapter. The methodology is chemoselective to lysine residues and utilizes an electrophilic carbonylating reagent, CDI, to generate the urea stapled peptide. This mild, one-step, two-component method works without any metal catalyst. A structural study of the urea stapled peptide displayed increased α-helicity, and also improved proteolytic stability. Moreover, this stapling strategy was successfully applied to generate bicyclic peptides as a new type of scaffold for peptide-based drug design. Finally, the unique feature of this methodology is the generation of a urea moiety that is capable of forming H-bonds and thus can play an important role in catalysis. Future studies will be used to address the application of urea stapled peptides as efficient catalysts for carrying out a range of organic asymmetric transformations such as the Michael Addition, and C-H functionalization reactions.
3.7. EXPERIMENTAL SECTION

General:

All commercial materials (Aldrich, Fluka, Nova) were used without further purification. All solvents were reagent grade or HPLC grade (Fisher). CH$_2$Cl$_2$ and DMF were obtained from a dry solvent system (passed through column of alumina) and were used without further drying. All reactions were performed under air in a round bottom flask. Yields refer to chromatographically pure compounds; % conversions were obtained by comparison of HPLC peak areas of products and starting material. HPLC was used to monitor reaction progress.

Materials:

Fmoc-amino acids were obtained from Nova Biochem under (EMD Millipore Corporation) (Billerica, Massachusetts) and CreoSalus (Louisville, Kentucky). Rink amide resin was obtained from ChemPep Inc (Wellington, Florida). N,N,N',N'-Tetramethyl-O(1H-benzotriazol-uronium hexafluorophosphate (HBTU) was obtained from CreoSalus (Louisville, Kentucky). N, N'-Disuccinimidy l carbonate (DSC) was obtained from Nova Biochem, under (EMD Millipore Corporation) (Billerica, Massachusetts). 4-Dimethylaminopyridine (DMAP): Merck KGaA (Darmstadt, Germany). N, N-Dimethylformamide (DMF): Macron Fine Chemicals (Center Valley, Pennsylvania). Acetonitrile, N, N-Diisopropylethylamine (DIEA), N, N'-diisopropylcarbodiimide (DIC), were purchased from (EMD Millipore Corporation)(Billerica, Massachusetts). Piperidine was purchased from Alfa Aesar (Ward Hill, Massachusetts). 1,1'-Carbonyldiimidazole (CDI) was purchased from Nova Biochem (Calbiochem-Novabiochem corporation, La Jolla, California) Trifluoroacetic acid (TFA) was purchased from VWR 100 Matsonford Road Radnor, PA. Water was purified using a Millipore MilliQ water purification system.

Analytical HPLC:
Analytical HPLC chromatography (HPLC) was performed on an Agilent 1100 series HPLC equipped with a 4.6 x150 mm (5µm) C-18 reversed-phase column. All separations involved mobile phase of 0.1% FA (v/v) in water (solvent A) and 0.1% FA (v/v) in acetonitrile (solvent B). Peptide compositions were evaluated by analytical reverse phase HPLC using a gradient of 0.1% FA in acetonitrile versus 0.1% FA in water. Analytical HPLC method using a linear gradient of 0–80% 0.1% FA (v/v) acetonitrile in 0.1% aqueous FA, unless otherwise noted, over 30 min at room temperature with a flow rate of 1.0 mL min⁻¹. The peptide absorbance was monitored at 220 nm (peptide bond) unless otherwise noted.

**LC-MS:**

Mass spectrometry was performed on an Agilent 1100 Series HPLC with MSD VL mass spectrometer using positive polarity electrospray ionization (+ESI).

**LC-HRMS:**

Analysis was done at Merck facilities at Rahway, NJ by Ryan Cohen. High resolution MS data were acquired using an Agilent 1290 UHPLC with a 6520 Q-ToF mass spectrometer under positive polarity electrospray ionization (+ESI) with capillary and fragmentor voltages set to 3.5 kV and 175 V, respectively. The instrument was calibrated prior to data acquisition using Agilent’s reference standard solution, which provided accurate masses within 5 ppm. A reversed phase, linear gradient separation was performed at a flow rate of 1.0 mL min⁻¹ on a C18 column (Waters Acquity UPHLC peptide BEH C18, 1.7 µm particle size, 50 mm x 2.1 mm I.D.) thermostatted at 45°C and gradient from 95% solvent A (0.1% formic acid in water) to 99% solvent B (0.1% formic acid in acetonitrile) in 4 minutes, followed by 1 minute re-equilibration.

**MS/MS:**

Analysis was done at Merck facilities at Rahway, NJ by Ryan Cohen. Tandem MS analyses were performed either on an Agilent 1260 HPLC coupled with a 6530 Q-ToF or on an Agilent 1290
HPLC coupled with a 6520 Q-ToF mass spectrometer. Both mass spectrometers were operated under electrospray ionization in positive polarity (+ESI). Reverse phase C18 gradient separation conditions were used (mobile phase A = 0.1% formic acid in water and mobile phase B = 0.1% formic acid in acetonitrile). Spectra were acquired in profile mode with collision energies ranging from 25-90 V and nitrogen (N₂) as collision gas.

NMR:

NMR was done by Ryan Cohen at Merck facilities at Rayway, NJ. NMR spectra were recorded on a Bruker AVANCE III HD 600 MHz spectrometer equipped with a 5-mm HCN cryoprobe at 600 and 151 MHz for proton and carbon, respectively, spectrometer at ambient temperature. An approximately 1 mg sample was dissolved in DMSO-d6 solvent containing 0.05% v/v TMS for chemical shift referencing. 1H, 13C, gCOSY, TOCSY (80 ms mixing time), multiplicity-edited gHSQC, gHMBC, and ROESY (200 ms spin lock) experiments were performed. NMR spectra were processed using MNova (ver. 11.0.4).

All NMR chemical shifts are referenced in ppm relative to residual solvent or internal tetramethylsilane. 1H NMR chemical shifts referenced to residual DMSO-d5 at 2.50 ppm, and 13C NMR chemical shifts referenced to DMSO-d6 at 39.52ppm. Carbon NMR spectra are proton decoupled. NMR spectral data are reported as chemical shift (multiplicity, coupling constants (J), integration). Multiplicity is reported as follows: singlet (s), broad singlet (bs), doublet (d), doublet of doubles (dd), doublet of triplet (td), triplet (t) and multiplet (m). Coupling constant (J) in Hertz (Hz).

General Procedure for Fmoc Solid-Phase Peptide Synthesis:\(^{32}\):

Peptides were synthesized manually on a 0.25 mmol scale using Rink amide resin. Fmoc–group was deprotected using 20% piperidine–DMF to obtain a deprotected peptide-resin (2x 20 min). Fmoc-
protected amino acids (1.25 mmol) were sequentially coupled on the resin using a HBTU (1.25 mmol) and DIEA (1.25 mmol) for 2 h at room temperature. Peptides were synthesized using standard protocols. The peptide was cleaved from the resin using a cocktail of 95:2.5:2.5, trifluoroacetic acid (TFA): triisopropylsilane (TIPS): water (H₂O) for 2 h. The resin was removed by filtration and the resulting solution was concentrated under vacuum. The resulting solid was dissolved in ACN-water solvent system, analyzed and purified by RP HPLC.

**General Procedure for Stapling Reaction:**

To a 5-mL round-bottom flask containing peptide (1 equiv.) in 200 μL dimethylformamide (DMF) was added a solution of CDI (3-9.2 equiv.) and DIEA (3-9.2 equiv.) in DMF. The mixture was stirred at 25°C/65°C for 24 h. The reaction mixture was concentrated under vacuum and resulting peptide was dissolved in mixture of water and acetonitrile and purified by RP HPLC. The purified fractions were lyophilized to afford stapled peptide as a white powder. % conversion was obtained by comparison of HPLC peak areas of products and starting material. HPLC was used to monitor reaction progress.

**Macrocyclization of Linear Peptide, Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu, on Solid Support:**

To peptide, Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu, containing O-allyl protected glutamic acid on solid support (50 mg, 0.69 mmol/g) was added a 3 mL solution of 20 mg Pd (PPh₃)₄ and 72 μL phenylsilane in DCM. This solution was incubated on a shaker at room temperature for 40 minutes. The resin was washed with DCM (3 X 2 min). The above reaction was repeated, and then the resin was washed with DCM (3 X 2 min), MeOH (3 X 2 min), and DMF (3 X 2 min). The palladium catalyst was removed from the resin by washing with DIEA in DMF (2% v/v) (2 X 2 min) and then with DMF (2 X 2 min). Next, the peptide was treated with 20% piperdine in DMF to remove the Fmoc protection. Macrocyclization was achieved by exposing the resin to DIC, HOAt, and a catalytic amount of DMAP in DMF on a shaker for 15 h. The solution was drained, and the resin was washed with DMF. To confirm complete macrocyclization, the Kaiser test¹²
was performed, which showed yellow bead coloration indicating absence of free amino groups. The peptide was then cleaved from the resin by 2 hours incubation with a solution of TFA:TIPS:water (95/2.5/2.5, v/v/v). Resin was removed by filtration, and the resulting solution was evaporated. The peptide was purified by reversed-phase semi-preparative chromatography.
3.8 REFERENCES


CHAPTER 4: CONTRIBUTIONS TO KNOWLEDGE AND FUTURE DIRECTIONS

4.1 CONTRIBUTIONS TO KNOWLEDGE MADE IN THIS THESIS

In this thesis, side chain modified polypeptides have been developed to affect both conformational as well as functional properties. The effect of cyclic conformational constraint on the peptide backbone and the side chain geometry were explored as chemical tools for improving peptide structure–activity relationships.

4.1.1 PYROGLUTAMATE - A CYCLIC BACKBONE INTERMEDIATE FOR SELECTIVE CHEMICAL CLEAVAGE OF PEPTIDE BONDS

The second chapter of the thesis describes a site-selective cleavage at glutamic acid of unreactive peptide bonds in native peptides as well as in peptidomimetics (Figure 4.1).

Figure 4.1: Graphical abstract for Glutamic Acid Selective Chemical Cleavage of Peptide Bond.

To develop this method, we optimized the reaction conditions for activation of the peptide backbone amide bond at glutamic acid by the formation of a reactive pyroglutamyl (pGlu) imide intermediate. Next, the reactive pGlu imide moiety was subjected to hydrolysis, resulting in cleavage of the scissile peptide bond at the N-terminus of the modified glutamic acid residue. The method exhibited broad substrate scope. When we explored different substrates to study the effect of steric bulk of neighboring amino acid residues on the efficiency of chemical cleavage, there was no significant effect.
However, substrates with bulky residues such as isoleucine required longer incubation times for the cleavage reaction to proceed to completion. Moreover, the cleavage reaction proceeded without any by-products, demonstrating selectivity of the reaction. Cleavage of mutated peptides with unnatural amino acid residues such as D-amino acids, unsuitable substrates for enzymes, proceeded with ease under the reaction conditions. Moreover, disulfide bonds were stable toward the reaction conditions in contrast to other chemical reagents such as the use of cyanogen bromide. Importantly, this methodology was successful in cleaving peptide bonds next to proline, a site inaccessible to proteases. Finally, the scope of the methodology was successfully extended to different bioactive peptides such as a fragment of putative coproporphyrinogen III oxidase, amyloid-β peptide; and amyloid A protein.

The significance of this chemical cleavage strategy lies in the fact that it presents a potential valuable complementary tool for peptide and protein sequencing (Figure 4.2).

<table>
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The results of this study have provided a foundation for further studies aimed at developing artificial chemical proteases for the cleavage of target proteins responsible for various diseases (Figure 4.2B). The importance of this study was acknowledged by a publication in *Organic Letters*.

**4.1.2 PEPTIDE STAPLING VIA UREA BRIDGING-CHEMICAL INSIGHTS INTO CYCLIC PEPTIDE SYNTHESIS**

In the third chapter of this thesis, a urea-based methodology between two lysine residues for stapling and cyclizing peptides has been reported. First, we explored a suitable base and a carbonylating reagent to deprotonate and activate the lysine side chain. In our optimized conditions, 1,1'-carbonyldiimidazole (CDI) and N,N-diisopropylethylamine (DIEA) were selected as the reagents of choice. This activation step led to formation of a urea functionality with an adjacent lysine residue.
High conversion to the urea stapled peptide was obtained when two lysine residues were at i and i+2, i and i+3 and i and i+4 positions. When the steric effect of amino acids adjacent to lysine residues was investigated, we observed that bulky residues like phenylalanine in between the lysine residues had an adverse effect on the efficacy of the stapling reaction. The methodology was chemoselective to the lysine side-chain amino groups in N-terminus Ac-protected peptides in presence of reactive functional groups, namely, alcohols and carboxylic acids. Moreover, the urea stapled peptide exhibited increased structural stability towards proteolysis as well as higher tendency to adopt α-helical structure. The major advantage of this stapling strategy is that it can successfully generate bicyclic peptides. The latter was accomplished in the case of model peptide NH$_2$-Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu--COOH.

The unique feature of this methodology is the generation of a urea moiety that is capable of forming H-bonds and thus can play an important role in catalysis. Future studies will be used to address the application of urea stapled peptides as efficient organocatalysts for carrying out a range of organic asymmetric transformations like Michael Additions, and C-H functionalization reactions (Figure 4.4).
Figure 4.4: Urea stapled peptides as efficient organocatalysts: a) Michael Addition b) Design of Organic Catalyst c) Mechanistic Pathway of Peptide Catalysis

4.1.3 REFERENCES:


4.2 PUBLICATIONS, CONFERENCE PRESENTATIONS, AND AWARDS

**PUBLICATIONS**


MANUSCRIPTS IN PREPARATION

• Synthesis of Urea Stapled Helical Peptides with Lysine Carbonylation – Lahankar, N.; Pereira, Z.; Raj M.

• Proline Selective Bioconjugation – Sim, Y.; Lahankar, N.; Pereira, Z.; Buissereth, L.; Raj, M.

POSTER PRESENTATIONS


• Poster: Glutamic Acid Selective Chemical Cleavage of Peptide Bonds – Petersheim Academic Exposition at Seton Hall University, 4/2016.


AWARDS AND HONORS

• Robert De Simone Graduate Fellowship – Department of Chemistry and Biochemistry Seton Hall University 9/2017 – 6/2018.

• Outstanding Teaching Assistant, Department of Chemistry and Biochemistry, Seton Hall University 9/2017 – 6/2018.


111. Wang, Y; Chou, D. H.; Angew. Chem., Int. Ed. 2015, 54, 10931


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Figure A1: HPLC trace, $^1\text{H} / ^{13}\text{C}$ HSQC NMR, $^1\text{H} / ^{13}\text{C}$ HMBC NMR, $^1\text{H} / ^1\text{H}$ ROSEY NMR for linear and cyclized Fmoc-Gly-Glu. Product peak is labeled.
**Figure A2**: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.


**Fmoc-Val-Ala-OH** (3a). LCMS: $m/z$ 411.02 (calcd [M+H]$^+$ = 411.13). Purity: >95% (HPLC analysis at 254 nm). Retention time: 22.75 min.

**pGlu-Arg-Phe-Ala-NH$_2$** (4). LCMS: $m/z$ 503.10 (calcd [M+H]$^+$ = 503.27), 525.0 (calcd [M+Na]$^+$ = 525.27). Purity: >95% (HPLC analysis at 254 nm). Retention time: 5.67 min.
Figure A3: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.
**Fmoc-Val-Gly-pGlu-Arg-Phe-Ala-NH$_2$ (2b).** LCMS: $m/z$ 881.20 (calkd [M+H]$^+$ = 881.42), 441.0 (calkd [(M+2)/2]$^+$ = 441.21). Purity: >95% (HPLC analysis at 254 nm). Retention time: 19.10 min.

**Fmoc-Val-Gly-OH (3b).** LCMS: $m/z$ 397.20 (calkd [M+H]$^+$ = 397.17). Purity: >95% (HPLC analysis at 254 nm). Retention time: 19.69 min.

**pGlu-Arg-Phe-Ala-NH$_2$ (4).** LCMS: $m/z$ 503.12 (calkd [M+H]$^+$ = 503.27), 525.1 (calkd [M+Na]$^+$ = 525.27). Purity: >95% (HPLC analysis at 254 nm). Retention time: 5.57 min.
Figure A4: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.

**Fmoc-Val-Arg-pGlu-Arg-Phe-Ala-NH₂ (2c).** LCMS: $m/z$ 980.28 (calcd $[M+H]^+ = 980.50$), 490.42 (calcd $[(M+2)/2]^+ = 490.75$). Purity: >95% (HPLC analysis at 254 nm). Retention time: 13.9 min.

**Fmoc-Val-Arg-OH (3c).** LCMS: $m/z$ 496.12 (calcd $[M+H]^+ = 496.25$). Purity: >95% (HPLC analysis at 254 nm). Retention time: 14.2 min.

Figure A5: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.


**Figure A6**: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.
**Fmoc-Val-Asn-pGlu-Arg-Phe-Ala-NH₂ (2e).** LCMS: m/z 938.20 (calcd [M+H]+ = 938.44), 469.1 (calcd [(M+2)/2]+ = 469.72). Purity: >95% (HPLC analysis at 254 nm). Retention time: 12.95 min.

**Fmoc-Val-Asn-OH (3e).** LCMS: m/z 454.0 (calcd [M+H]+ = 454.19). Purity: >95% (HPLC analysis at 254 nm). Retention time: 18.9 min.

**pGlu-Arg-Phe-Ala-NH₂ (4).** LCMS: m/z 503.0 (calcd [M+H]+ = 503.27), 525.2 (calcd [M+Na]+ = 525.27). Purity: >95% (HPLC analysis at 254 nm). Retention time: 5.51 min.
Figure A7: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.


pGlu-Arg-Phe-Ala-NH$_2$ (4). LCMS: $m/z$ 503.0 (calcd [M+H]$^+$ = 503.27), 525.2 (calcd [M+Na]$^+$ = 525.27). Purity: >95% (HPLC analysis at 254 nm). Retention time: 5.52 min.
Figure A8: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.

Fmoc-Val-Phe-pGlu-Arg-Phe-Ala-NH₂ (2g). LCMS: $m/z$ 971.32 (calcd $[M+H]^+ = 971.47$), 486.12 (calcd $[(M+2)/2]^+ = 486.23$). Purity: >95% (HPLC analysis at 254 nm). Retention time: 15.01 min.


Figure A9: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.

**Fmoc-Val-Tyr-OH (3h)**. LCMS: $m/z$ 503.1 (calcd [M+H]$^+$ = 503.21). Purity: >95% (HPLC analysis at 254 nm). Retention time: 20.39 min.

**pGlu-Arg-Phe-Ala-NH₂ (4)**. LCMS: $m/z$ 503.09 (calcd [M+H]$^+$ = 503.27), 525.1 (calcd [M+Na]$^+$ = 525.27). Purity: >95% (HPLC analysis at 254 nm). Retention time: 5.56 min.
**Figure A10**: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.

**Fmoc-Val-Ile-pGlu-Arg-Phe-Ala-NH$_2$** (2i). LCMS: $m/z$ 937.23 (calcd $[M+H]^+ = 937.49$), 469.08 (calcd $(M+2)/2]^+ = 469.24$). Purity: >95% (HPLC analysis at 254 nm). Retention time: 14.74 min.


Figure A11: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.
**Fmoc-Val-Asp-pGlu-Arg-Phe-Ala-NH\(_2\) (2j).** LCMS: \(m/z \ 939.31\) (calcd [M+H]\(^+\) = 939.43), 470.15 (calcd [(M+2)/2]\(^+\) = 470.21). Purity: >95% (HPLC analysis at 254 nm). Retention time: 13.43 min.

**Fmoc-Val-Asp-OH (3j).** LCMS: \(m/z \ 455.2\) (calcd [M+H]\(^+\) = 455.17). Purity: >95% (HPLC analysis at 254 nm). Retention time: 19.30 min.

**pGlu-Arg-Phe-Ala-NH\(_2\) (4).** LCMS: \(m/z \ 503.09\) (calcd [M+H]\(^+\) = 503.27), 525.1 (calcd [M+Na]\(^+\) = 525.27). Purity: >95% (HPLC analysis at 254 nm). Retention time: 5.12 min.
Figure A12: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.


Figure A13: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.

Fmoc-Arg-Ala-Gly-Ala-pGlu-Val-Arg-Phe-Ala-pGlu-Ala-Phe-Gly-NH₂ (8a). LCMS: \( m/z \) 1566.32 (calcd \([M+H]^+ = 1566.41\)), 783.70 (calcd \([M+2\text{H}]^+ = 783.88\)). Purity: >95% (HPLC analysis at 254 nm). Retention time: 11.50 min.

Fmoc-Arg-Ala-Gly-Ala-OH (8b). LCMS: \( m/z \) 596.13 (calcd \([M+H]^+ = 596.28\)). Purity: >95% (HPLC analysis at 254 nm). Retention time: 11.78 min.

pGlu-Val-Arg-Phe-Ala-OH (8c). LCMS: \( m/z \) 603.1 (calcd \([M+H]^+ = 603.32\)), 625.2 (calcd \([M+Na]^+ = 625.32\)). Purity: >95% (HPLC analysis at 254 nm). Retention time: 8.15 min.

pGlu-Ala-Phe-Gly-NH₂ (8d). LCMS: \( m/z \) 404.2 (calcd \([M+H]^+ = 404.19\)), 426.0 (calcd \([M+Na]^+ = 426.19\)). Purity: >95% (HPLC analysis at 254 nm). Retention time: 8.74 min.
**Figure A14**: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.

**Fmoc-d-Val-d-Ala-d-pGlu-d-Arg-d-Phe-d-Ala-NH₂ (9a).** LCMS: \( m/z \ 895.31 \) (calcd \([M+H]^{+} = 895.44\)), 448.1 (calcd \([M+2H]^{+} = 448.22\)). Purity: >95\% (HPLC analysis at 254 nm). Retention time: 13.66 min.

**Fmoc-d-Val-d-Ala-OH (9b).** LCMS: \( m/z \ 411.06 \) (calcd \([M+H]^{+} = 411.18\)). Purity: >95\% (HPLC analysis at 254 nm). Retention time: 19.92 min.

**D-pGlu-d-Arg-d-Phe-d-Ala-NH₂ (9c).** LCMS: \( m/z \ 503.09 \) (calcd \([M+H]^{+} = 503.27\)), 525.1 (calcd \([M+Na]^{+} = 525.27\)). Purity: >95\% (HPLC analysis at 254 nm). Retention time: 5.71 min.
Figure A15: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.


pGlu-d-Arg-d-Phe-Ala-NH$_2$ (10c). LCMS: $m/z$ 503.0 (calcd $[M+H]^+ = 503.27$), 525.2 (calcd $[M+Na]^+ = 525.27$). Purity: $>95\%$ (HPLC analysis at 254 nm). Retention time: 4.96 min.
Figure A16: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.


Figure A17: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.


Figure A18: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.


Figure A19: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.


**Figure A20**: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.
**Fmoc-Tyr-pGlu-Val-His-His-Gln-Lys-Leu-Val-Phe-NH₂ (19a).** LCMS: \( m/z \) 1502.5 (calcd \([M+H]^+ = 1502.75\)), 752.14 (calcd \([M+2/2]^+ = 752.26\)). Purity: >95% (HPLC analysis at 254 nm). Retention time: 12.78 min.

**Fmoc-Tyr-OH (20).** LCMS: \( m/z \) 404.07 (calcd \([M+H]^+ = 404.14\)). Purity: >95% (HPLC analysis at 254 nm). Retention time: 22.13 min.

**pGlu-Val-His-His-Gln-Lys-Leu-Val-Phe-NH₂ (21).** LCMS: \( m/z \) 1117.57 (calcd \([M+H]^+ = 1117.78\)), 559.23 (calcd \([M+2/2]^+ = 559.39\)). Purity: >95% (HPLC analysis at 254 nm). Retention time: 6.58 min.
Figure A21: HPLC traces of cyclized starting material and the crude reaction mixture after incubation in buffer solution. Product peak is labeled.
Fmoc-Ala-Gly-Leu-Pro-pGlu-Lys-Tyr-NH₂ (22a). LCMS: m/z 980.54 (calcd [M+H]⁺ = 980.87), 490.54 (calcd [(M+2)/2]⁺ = 490.91). Purity: >95% (HPLC analysis at 254 nm). Retention time: 11.35 min.


CHAPTER 3

Ac-Phe-Arg-Lys-Gly-Lys-Ala-NH₂ Linear Peptide (1) - LCMS: m/z 747.6 (calcd \([M+H]^+ = 747.893\)), Purity: >95% (HPLC analysis at 220 nm). Retention time: 6.1 min. (HPLC method of 0–80% 0.1% FA)

Figure A22: HPLC trace and MS of Ac-Phe-Arg-Lys-Gly-Lys-Ala-NH₂ Linear Peptide

Ac-Phe-Arg-Lys-Gly-Lys-Ala-NH₂ Stapled peptide (1a) at 25°C - LCMS: m/z 773.5 (calcd \([M+H]^+ = 773.88\)), Purity: >95% (HPLC analysis at 220 nm). Retention time: Starting material 4.902 min, Product 7.848 min (HPLC method of 0–80% 0.1% FA). Reactant 31.30%, Product 68.70%.
Ac-Phe-Arg-Lys-Gly-Lys-Ala-NH₂ Stapled peptide (1a) at 65°C (9.2 equiv CDI) - LCMS: m/z 773.5 (calcd [M+H]⁺ = 773.88), Purity: >95% (HPLC analysis at 220 nm). Retention time: Starting material 5.615 min. Product 9.422 min (HPLC method of 0–80% 0.1% FA). Reactant 5.788%, Product 94.212%.

Figure A23: HPLC trace of Ac-Phe-Arg-Lys-Gly-Lys-Ala-NH₂ Stapled Peptide at 25°C and 65°C. The product peak is labeled.


13C NMR (151 MHz, DMSO-d₆) (ppm) 174, 171.6, 171.3, 171, 170.7, 169.3, 168.6, 157.9, 157, 137.9, 129, 127.9, 126.153.9, 52.2, 51.9, 51.8, 47.9, 42.3, 40.1, 38.8, 38.7, 37.3, 32.2, 31.7, 29, 28.8, 28.7, 24.8, 22.4, 22.3, 21.6, 18.2.
$^{1}\text{H}/^{1}\text{H}$ COSY NMR for Ac-Phe-Arg-Lys-Gly-Lys-Ala-NH$_2$ Stapled Peptide (1a)

$^{1}\text{H}/^{1}\text{H}$ TOCSY NMR for Ac-Phe-Arg-Lys-Gly-Lys-Ala-NH$_2$ Stapled Peptide (1a)
$^1$H/$^{13}$C HSQC NMR for Ac-Phe-Arg-Lys-Gly-Lys-Ala-NH$_2$ Stapled Peptide (1a)

$^1$H/$^{13}$C HMBC NMR for Ac-Phe-Arg-Lys-Gly-Lys-Ala-NH$_2$ Stapled Peptide (1a)
$^1$H/$^1$H ROSEY NMR for Ac-Phe-Arg-Lys-Gly-Lys-Ala-NH$_2$ Stapled Peptide (1a)

Figure A24: $^{13}$C NMR, $^1$H/$^1$H COSY NMR, $^1$H/$^1$H TOCSY NMR, $^1$H/$^{13}$C HSQC NMR, $^1$H/$^{13}$C HMBC NMR, $^1$H/$^1$H ROSEY for Ac-Phe-Arg-Lys-Gly-Lys-Ala-NH$_2$ Stapled Peptide
Ac- Phe-Lys-Gly-Ala-Lys-Phe-NH$_2$ (2) - LCMS: $m/z$ 738.9 (calcd $[M+H]^+ = 738.88$), Purity: >95% (HPLC analysis at 220 nm). Retention time: 9.9 min. (HPLC method of 0–80% 0.1% FA)

**Figure A25**: HPLC trace and MS of Ac- Phe-Lys-Gly-Ala-Lys-Phe NH$_2$ Linear Peptide
Ac-Phe-Lys-Gly-Ala-Lys-Phe-NH$_2$ Stapled peptide (2a) after 5 h: LCM: $m/z$ 764.88 (calcd [M+H]$^+$ = 764.87), Purity: >95% (HPLC analysis at 220 nm). Retention time: reactant 8.662 min., Product 11.273 min. (HPLC method of 0–80% 0.1% FA). Reactant 86.167%. Product 13.833%.

Ac-Phe-Lys-Gly-Ala-Lys-Phe-NH$_2$ peptide Stapled (2a) after 10 h: LCM: $m/z$ 764.88 (calcd [M+H]$^+$ = 764.87), Purity: >95% (HPLC analysis at 220 nm). Retention time: reactant 8.642 min., Product 11.087 min. (HPLC method of 0–80% 0.1% FA). Reactant 73.270%. Product 26.730%.

Figure A26: HPLC trace of Ac-Phe-Lys-Gly-Ala-Lys-Phe-NH$_2$ Stapled after 5h and 10 h. The product peak is labeled.
$^1$H NMR for Ac- Phe-Lys-Gly-Ala-Lys-Phe-$\text{NH}_2$ Stapled Peptide (2a).

$^1$H NMR chemical shifts referenced to residual DMSO-d$_6$ at 2.50 ppm and $^{13}$C NMR chemical shifts referenced to DMSO-d$_6$ at 39.52 ppm.

$^1$H NMR for Ac- Phe-Lys-Gly-Ala-Lys-Phe- $\text{NH}_2$ Stapled Peptide (2a)

$^1$H NMR (600 MHz, DMSO-d$_6$) δ (ppm) 8.30 (m, 1H), 8.30 (m, 1H), 7.98 (d, $J=8.4$ Hz, 1H), 7.97(d, $J=8.5$ Hz, 1H), 7.91 (d, $J=6.5$ Hz, 1H), 7.65 (d, $J=8.3$ Hz, 1H), 7.33(s, 1H), 7.17 (m, 2H), 7.17 (m, 2H), 7.13 (m, 2H), 7.11 (m, 1H), 7.11 (m, 1H), 7.02 (s, 1H), 5.76 (t, $J=5.2$ Hz, 1H), 5.29 (t, $J=4.8$ Hz, 1H), 4.45 (m, 1H), 4.34 (m, 1H), 4.19 (m, 1H), 4.18 (m, 1H), 4.12 (m, 1H), 3.93 ( dd, $J=16.4$, 6.1 Hz, 1H), 3.39 (dd, $J=16.1$, 5.8 Hz, 1H), 3.02 (m, 1H), 2.99 (m, 1H), 2.94 (dd, $J=14.2$, 5.4 Hz, 1H), 2.90 (m, 1H), 2.88 (m, 1H), 2.87 (m, 1H), 2.74 (dd, $J=14.1$, 8.0 Hz, 1H), 2.65 (m, 1H), 1.67 (s, 1H), 1.55 (m, 1H), 1.55 (m, 1H), 1.39 (m, 1H), 1.38 (m, 1H), 1.32 (m, 1H), 1.30 (m, 1H), 1.28 (m, 1H), 1.23 (m, 1H), 1.17 (m, 2H), 1.18 (m, 1H), 1.13 (d, $J=7.2$ Hz, 3H), 1.11 (m, 1H).

$^{13}$C NMR for Ac- Phe-Lys-Gly-Ala-Lys-Phe-$\text{NH}_2$ Stapled Peptide (2a).
$^{13}$C NMR (151 MHz, DMSO-$d_6$) (ppm) 172.2, 171.3, 171.2, 170.7, 169.4, 169, 157.8, 137.9, 129, 127.8, 126, 53.6, 51.9, 51.9, 48.1, 41.6, 38.2, 37.8, 37.3, 31.1, 30.5, 28.5, 27.8, 22.3, 22, 21, 17.5.

$^1$H/$^1$H COSY for Ac-Phe-Lys-Gly-Ala-Lys-Phe- NH$_2$ Stapled Peptide (2a)
$^1$H /$^1$H TOCSY NMR for Ac- Phe-Lys-Gly-Ala-Lys-Phe- NH$_2$ Stapled Peptide (2a).
$^{1}H /^{13}C$ HSQC NMR for Ac- Phe-Lys-Gly-Ala-Lys-Phe- NH$_2$ Stapled Peptide (2a).

$^{1}H /^{13}C$ HMBC NMR for Ac- Phe-Lys-Gly-Ala-Lys-Phe- NH$_2$ Stapled Peptide (2a)

$^{1}H /^{1}H$ ROSEY NMR for Ac- Phe-Lys-Gly-Ala-Lys-Phe-NH$_2$ Stapled Peptide (2a)
Figure A27: $^1$H NMR, $^{13}$C NMR, $^1$H/$^1$H COSY NMR, $^1$H/$^1$H TOCSY NMR, $^1$H/$^{13}$C HSQC NMR, $^1$H/$^{13}$C HMBC NMR, $^1$H/$^1$H ROSEY NMR for Stapled Peptide Ac-Phe-Arg-Lys-Gly-Lys-Ala-NH$_2$

Ac-Phe-Lys-Lys-Phe-NH$_2$ Linear Peptide (3): LCMS- m/z 609.9 (calcd [M+H]$^+$ = 609.752), Purity: >95% (HPLC analysis at 220 nm).
Figure A28 - MS of Ac-Phe-Lys-Lys-Phe-NH$_2$ Linear Peptide

Expected Ac-Phe-Lys-Lys-Phe-NH$_2$ Stapled Peptide (3a): (calcd $[M+H]^+ = 635.646$) was not obtained, but intermediate (3b) was obtained.

Intermediate (3b)-LCMS: $m/z$ 703.1 (calcd $[M+H]^+ = 703.823$), Purity: >95% (HPLC analysis at 220 nm). Retention time: Starting material 8.303 min intermediate - 12.582 min (HPLC method of 0–80% 0.1% FA)
Figure A29: HPLC trace and MS of Ac-Phe-Lys-Lys-Phe-NH$_2$ Intermediate. The product peak is labeled.
Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH₂, Linear Peptide (4): LCMS- $m/z$ 876.5 (calcd $[M+H]^+$ = 877.05), Purity: >95% (HPLC analysis at 220 nm). Retention time: 10.7 min. (HPLC method of 0–80% 0.1% FA)

Figure A30: HPLC trace and MS of Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH₂ Linear Peptide
Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH₂ Stapled peptide (4a) (CDI 7 equiv) at 65°C - LCMS: m/z 903.04 (calcd [M+H]⁺ = 902.6), Purity: >95% (HPLC analysis at 220 nm). Retention time: Staring material 10.141 min. Product 13.697 min. (HPLC method of 0–80% 0.1% FA). Reactant 2.042%, Product 97.958%

HRMS data: Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH₂ Stapled Peptide (4a) [C₄₁H₆₇N₁₃O₁₀+H]⁺: calc. m/z 902.5207, obs. m/z 902.5208

Observed # of exchangeable protons = 1099 – 1082 – 1 = 16. Expected # of exchangeable protons = 16
Figure A31: HPLC trace (at $65^\circ$C), MS, HRMS, H/D Exchange experiment of Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH$_2$ Stapled Peptide. Product peak is labeled.

$^1$H NMR Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH$_2$ Stapled Peptide (4a)

$^1$H NMR chemical shifts referenced to residual DMSO-d$_6$ at 2.50 ppm and $^{13}$C NMR chemical shifts referenced to DMSO-d$_6$ at 39.52 ppm.

$^1$H NMR (600 MHz, DMSO-d$_6$) $^1$H NMR (ppm) 9.22 (br s, 1H), 8.28 (t, $J$ = 5.8 Hz, 1H), 8.22 (d, $J$ = 7.8 Hz, 1H), 8.12 (d, $J$ = 7.8 Hz, 1H), 8.02 (d, $J$ = 6.5 Hz, 1H), 7.99 (d, $J$ = 8.0 Hz, 1H), 7.81 (t, $J$ = 5.2 Hz, 1H), 7.61 (d, $J$ Hz = 8.1 Hz, 1H), 7.26 (s, 1H), 7.05 (s, 1H), 6.97 (d, $J$ = 8.4 Hz, 2H), 6.62 (d, $J$ = 8.4 Hz, 2H), 5.80 (t, $J$ = 5.9 Hz, 1H), 5.77 (t, $J$ = 6.1 Hz, 1H), 4.40-4.34 (m, 1H), 4.33-4.28 (m, 1H), 4.30-4.26 (m, 1H), 4.30-4.24 (m, 1H), 4.26-4.20 (m, 1H), 4.04-3.99 (m, 1H), 3.73 (dd, $J$ = 16.7, 5.5 Hz, 1H), 3.69 (dd, $J$ = 16.8, 5.7 Hz, 1H), 3.10-3.00 (m, 2H), 3.06-3.02 (m, 1H), 3.02-2.96 (m, 1H), 2.96-2.90 (m, 1H), 2.88 (dd, $J$ = 14.2, 5.1 Hz, 1H), 2.71 (dd, $J$ = 14.2, 8.5 Hz, 1H), 1.85 (s, 3H), 1.71-1.65 (m, 1H), 1.67-1.59 (m, 1H), 1.61-1.55 (m, 1H), 1.57-1.47 (m, 1H), 1.56-1.45 (m, 2H), 1.53-1.46 (m, 1H), 1.51-1.43 (m, 2H), 1.50-1.43 (m, 2H), 1.36-1.28 (m, 1H), 1.36-1.27 (m, 2H), 1.30-1.20 (m, 2H), 1.27-1.22 (m, 1H), 1.26-1.16 (m, 2H), 1.18 (d, $J$ = 6.8, 3H Hz), 0.87 (d, $J$ = 6.5 Hz, 3H), 0.81 (d, $J$ = 6.4 Hz, 3H).
\(^{13}\)C NMR Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH\(_2\) Stapled Peptide (4a).

\(^{13}\)C NMR (151 MHz, DMSO-\(d_6\)) \(\delta\) (ppm) 172.7, 172.3, 172, 171.4, 171.3, 171.2, 169.2, 168, 158.5, 157, 155.7, 130, 127.6, 114.7, 53.7, 53.2, 52.2, 51.7, 51.1, 47.8, 41.9, 40.4, 40.1, 38.9, 38, 36.6, 31.5, 30.7, 29.5, 29.4, 29.1, 24.7, 24, 22.9, 22.4, 22.2, 21.4, 18.
$^1$H/$^1$H COSY for NMR Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH$_2$ Stapled Peptide (4a)

$^1$H/$^1$H TOCSY NMR for NMR Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH$_2$ Stapled Peptide (4a)
$^{1}H/^{13}C$ HSQC NMR for Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH$_2$ Stapled Peptide (4a)

$^{1}H/^{13}C$ HMBC NMR for Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH$_2$ Stapled Peptide (4a)
\(^1H/\(^1H\) ROSEY NMR for Ac-Arg-Lys-Leu-Gly-Ala-Lys-Tyr-NH\(_2\) Stapled Peptide (4a)

**Figure A32**: \(^1H\) NMR, \(^{13}C\) NMR, \(^1H/\(^1H\) COSY NMR, \(^1H/\(^1H\) TOCSY NMR, \(^1H/\(^{13}C\) HSQC NMR, \(^1H/\(^{13}C\) HMBC NMR, \(^1H/\(^1H\) ROSEY for Stapled Peptide Ac-Phe-Arg-Lys-Gly-Lys-Ala-NH\(_2\)**
Ac-Arg-Lys-Ala-Leu-Gly-Ala-Lys-Phe-NH₂ Linear Peptide (5): LCMS- m/z 930.5 (calcd [(M+H)+] = 931.1), [(M+2)/2]+ = 465.8 (calcd [(M+2)/2]+ = 466.5) Purity: >95% (HPLC analysis at 220 nm). Retention time: 5.4 min. (HPLC method of 0–80% 0.1% FA).

Figure A33: HPLC and MS of linear peptide Ac-Arg-Lys-Ala-Leu-Gly-Ala-Lys-Phe-NH₂ Linear Peptide. Product peak is labeled.
Ac-Phe-Lys-Gly-Lys-Leu-NH₂ Linear Peptide (7):- LCMS: m/z 632.9 (calcd [M+H]+ = 633.787), Purity: >95% (HPLC analysis at 220 nm).

Figure A34: MS of Ac-Phe-Lys-Gly-Lys-Leu-NH₂ Linear Peptide. Product peak is labeled.
Ac-Phe-Lys-Gly-Lys-Leu-NH\textsubscript{2} Stapled Peptide (7a) at 65\textdegree C - LCMS: $m/z$ 659 (calcd [M+H]$^+$ = 659.781), Purity: >95% (HPLC analysis at 220 nm). Retention time: Reactant 7.984 min., Product 10.915 min (HPLC method of 0–80% 0.1% FA) Reactant 36.529%, Product 63.471%.

Figure A35: HPLC trace and MS Ac-Phe-Lys-Gly-Lys-Leu-NH\textsubscript{2} Stapled Peptide (8a) at 65\textdegree C. The product peak is labeled.
Ac-Phe-Lys-Phe-Lys-Leu-NH$_2$ Linear (8):- LCMS- m/z 723.9 (calcd [M+H]$^+$ = 723.911 ), Purity: >95% (HPLC analysis at 220 nm). Retention time: min. (HPLC method of 0–80% 0.1% FA).

Figure A36: HPLC trace and MS of Ac-Phe-Lys-Phe-Lys-Leu-NH$_2$ Linear Peptide (8). The product peak is labeled.
Ac-Phe-Lys-Phe-Lys-Leu-NH$_2$ Stapled Peptide (8a) at 65°C: LCMS- $m/z$ 749.8 (calcd [M+H]$^+$ = 749.805), Purity: >95% (HPLC analysis at 220 nm). Retention time: Reactant 10.796 min. Product 13.604 min. Reactant 26.033%, Product 73.967% (HPLC method of 0–80% 0.1% FA).

Figure A37: HPLC trace and MS of Ac-Phe-Lys-Phe-Lys-Leu-NH$_2$ Stapled Peptide (8a) at 65°C. Product peak is labeled.
Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH₂ Linear Peptide (9): LCMS:

\[(M+2)/2\] = 939.1 (calcd [(M+2)/2] = 939.58), [(M+3)/3] = 626.67 (calcd [(M+3)/3] = 626.58), Purity: > 95%. (HPLC analysis at 220 nm). Retention time: Reactant 11.305 min. (HPLC method of 0–60% 0.1% FA).
Figure A38: HPLC trace and MS of Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH₂
Linear Peptide (9)

Figure A39: HPLC trace and MS of Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH₂
Stapled Peptide (9a). Product peak is labeled.

Figure A40: HPLC trace and MS of Ac-Thr-Ser-Phe-Lys-Glu-Tyr-Trp-His-Leu-Leu-Ser-NH$_2$ (10) Linear Peptide

Stapled peptide (10a): LCMS: $m/z$ 1470.9 (calcd [M+H]$^+$ =1469.652), Purity: >95% (HPLC analysis at 220 nm). Retention time: Starting material 8.253 min. Product 18.861, 19.761 min (HPLC method of 0–60% 0.1% FA). Reactant 6.629%, Product 93.371%.
Figure A41: HPLC and MS of Ac-Thr-Ser-Phe-Lys-Glu-Tyr-Trp-His-Leu-Leu-Ser-NH₂ Stapled Peptide (10). Product peak is labeled.

Ac-Ala-Lys-Leu-Gly-Phe-Lys-Leu-NH₂ (11): Linear-LCMS: m/z 816.9 (calcd [M+H]⁺=817.025), Purity: >95% (HPLC analysis at 220 nm). Retention time: 7.826 min. (HPLC method of 0–80% 0.1% FA)
Figure A42: HPLC trace and MS of Ac-Ala-Lys-Leu-Gly-Phe-Lys-Leu-NH₂ Linear Peptide (11)

**Stapled peptide (11a)-LCMS:** m/z 844.8 (calcd [M+H]⁺ =844.01), Purity: >95% (HPLC analysis at 220 nm). Retention time: Reactant 7.891 min. Product 9.364 min, 9.692 min. (HPLC method of 0–80% 0.1% FA). Reactant 56.632%, Product 43.639%.
**Figure A43**: HPLC trace and MS of Ac-Ala-Lys-Leu-Gly-Phe-Lys-Leu-NH$_2$ (11a) Stapled Peptide. Product peak is labeled.

**Ac-Ile-Lys-Leu-Gly-Phe-Lys-Leu-NH$_2$ Linear Peptide (12)**: LCMS: $m/z$ 859.06 (calcd $[M+H]^+$ = 860.10), Purity: $>95\%$ (HPLC analysis at 220 nm). Retention time: 11.170 min. (HPLC method of 0–80% 0.1% FA)
Figure A44: HPLC trace and MS of Ac- Ile-Lys-Leu-Gly-Phe-Lys-Leu-NH₂ Linear Peptide (12). Product peak is labeled.

Stapled Peptide (12a)-LCMS: $m/z$ 885.8 (calcd $[M+H]^+ = 886.09$), Purity: >95% (HPLC analysis at 220 nm). Retention time- Reactant 12.650 min: Product 14.022 min, 14.215 min. (HPLC method of 0–80% 0.1% FA). Reactant 32.916%, Product 67.084%.
Figure A45: HPLC trace and MS of Ac-Ile-Lys-Leu-Gly-Phe-Lys-Leu-NH₂ (12) Stapled Peptide. Product peak is labeled.
**Figure A46A-1:** Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH$_2$ Linear Peptide (9): 0 min

**Figure A46A-2:** Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH$_2$ Linear Peptide (9): Fragmentation at 30 min: Fragments (9i), (9ii), (9iii), (9iv).
**Figure A46 A-3:** Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH₂ Linear Peptide(9):
Fragmentation after 90 min: Fragments (9i), (9ii), (9iii), (9iv).

**Figure A46:** Stability Study: Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH₂ Linear Peptide (9):
Fragmentation after 90 min: Fragments (9i), (9ii), (9iii), (9iv).

**Fragment Ac-Glu-Asn-Pro-Glu-Lys-OH (9i) - LCMS:** 
- Fragmentation: m/z 658.3 (calcd [M+H]+ = 657.676), Purity: >95% (HPLC analysis at 220 nm). Retention time: 5.703 min (HPLC method of 0–60% 0.1% FA).

**Fragment Ile-Leu-Asp-His-Val-Gln-Arg-Val-Met-NH₂ (9ii) - LCMS:** 
- Fragmentation: m/z 1008.8 (calcd [M+H]+ = 1008.186), (M+2/2) = 504.0 (calcd (M+2/2) = 504.593), Purity: >95% (HPLC analysis at 220 nm). Retention time: 6.648 min (HPLC method of 0–60% 0.1% FA).

**Fragment Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-OK (9iii) - LCMS:** 
- Fragmentation: (M+2/2) = 824.5 (calcd [(M+2/2) = 824.424], (M+3/3) = 549.7 (calcd [(M+3/3) = 549.949], Purity: >95% (HPLC analysis at 220 nm). Retention time: Reactant 9.831 min. (HPLC method of 0–60% 0.1% FA).

**Fragment Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-OH (9iv) - LCMS:** 
- Fragmentation: Calculated M+2/2 = 564.129 LCMS: ([M+2/2] = 564.129. Purity: >95%. (HPLC analysis at 220 nm). Retention time: 10.622 min. (HPLC method of 0–60% 0.1% FA).

**Figure A46 A:** The stability of the Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH₂ linear (9).

Fragmentation at 0 min.

Figure A46 B - 1: Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH₂ Stapled Peptide (9b):

Fragmentation at 30 min: (9a) and fragment (9v).
Figure A46 B -2: Fragmentation at 30 min: (9a) and fragment (9v).

Figure A46 B -3: Fragmentation at 90 min: fragment (9v).

Fragment stapled Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-OH (9v).


Figure A46 B: The stability of the stapled peptide.

Figure A46: The stability study of Ac-Glu-Asn-Pro-Glu-Lys-Ile-Leu-Asp-Lys-His-Val-Gln-Arg-Val-Met-NH₂. Stapled Peptide compared to its Linear Analogue.
Figure A47 A: MS of Fmoc-Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-(O-allyl)-CONH₂

LCMS: m/z 1201.4 (calcd[M+H]+=1201.43), Purity:>95%
Figure A47 B: MS of Fmoc-Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-CONH$_2$

LCMS: m/z 1162.2 (calcd [M+H]$^+$ = 1161.36), [(M+2)/2]$^+$ = 580.7 (calcd [(M+2)/2]$^+$ = 581.1) Purity: >95%

Figure A48—HPLC trace and MS of Monocyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH₂ (13a).

\[^{13}C\text{ NMR Monocyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH₂ (13a).}\]

\[^{13}C\text{ NMR (151 MHz, DMSO-}d_6\text{) (ppm) 172.9, 172.6, 172.4, 172.2, 172.1, 171.6, 171, 170.6, 169.1, 137.9, 137.4, 129.2, 129, 128, 127.9, 126.2, 126.2, 54.8, 54.1, 53.7, 52.5, 52.5, 52, 49.1, 42.1, 39.4, 38.5, 38.5, 37.3, 36.7, 32.6, 31.6, 30.3, 29, 27, 26.9, 24, 22.6, 22.2, 21.8, 21.7, 17.}\]
\(^{1}\text{H}/^{1}\text{H} \text{COSY NMR Monocyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH}_2 (13a).\)

\(^{1}\text{H}/^{1}\text{H} \text{TOCSY NMR Monocyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH}_2 (13a).\)
\( ^1\text{H} / ^{13}\text{C} \) HSQC NMR Monocyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH\(_2\) (13a).

\( ^1\text{H} / ^{13}\text{C} \) HMBC NMR Monocyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH\(_2\) (13a).
$^1$H /$^1$H ROSEY NMR Monocyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH$_2$ (13a).

Figure A49: $^{13}$C NMR, $^1$H/$^1$H COSY NMR, $^1$H/$^1$H TOCSY NMR, $^1$H/$^{13}$C HSQC NMR, $^1$H/$^{13}$C HMBC NMR, $^1$H/$^1$H ROSEY for Monocyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH$_2$ (13a).
Ion Mobility Resolved MS/MS

LCMS: m/z 946.7 (calcd [M+H]+ = 947), (M+23) + = 968.8 (calcd [(M+23)+] = 968.98), (M+39) + = 984 (calcd [(M+39)+] = 985), Purity: >95%. (HPLC analysis at 220 nm). Retention time: Reactant 8.822 min, Product 11.177 min, 12.374 min (HPLC method of 0–80% 0.1% FA). Reactant 0.946%, Product 13b-1 92.491%, 13b-2 6.563%
Figure A50: Ion Mobility Resolved MS/MS for two Conformations and HPLC trace of Bicyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-CONH₂

$^{13}$C NMR Bicyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH₂ (13b).

$^{13}$C NMR (151 MHz, DMSO-d6) δ (ppm) 173.2, 172.9, 172.4, 171.8, 171.6, 171.4, 171, 170.8, 168.7, 158.7, 138.2, 137.9, 129.3, 129.3, 128.2, 128.2, 126.4, 126.3, 54.9, 54, 52.6, 52.4, 52.2, 51.9, 49, 42.4, 39.5, 39.2, 38.4, 36.6, 36.1, 32.3, 32.1, 31.1, 28.6, 28.6, 28.2, 24.2, 22.8, 22, 21.6, 20.7, 16.2

$^{1}$H/$^{1}$H COSY NMR Bicyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH₂ (13b)
$^1$H/$^1$H TOCSY NMR Bicyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH$_2$ (13b).

$^1$H/$^{13}$C HSQC NMR Bicyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH$_2$ (13b).
$^1$H/$^{13}$C HMBC NMR Bicyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH$_2$ (13b).

$^1$H/$^1$H ROSEY NMR Bicyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH$_2$ (13b).
Figure A51: $^{13}$C NMR, $^1$H/$^1$H COSY NMR, $^1$H /$^1$H TOCSY NMR, $^1$H /$^{13}$C HSQC NMR, $^1$H /$^{13}$C HMBC NMR, $^1$H /$^1$H ROSEY for Bicyclic Phe-Lys-Leu-Gly-Ala-Lys-Phe-Glu-NH$_2$ (13a).