Gamma-AApeptides as a New Class of Peptidomimetics: Synthesis, Structures, and Functions

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Gamma-AApeptides as a New Class of Peptidomimetics: Synthesis, Structures, and Functions

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Keywords: γ-AApeptide, macrocycles, antimicrobial agents, OBOC library, beta-amyloid peptide, helical mimetic

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DEDICATION

To my parents and my wife
ACKNOWLEDGMENTS

First, I want to thank my advisor Dr. Cai for mentoring me over the past four and half years. His patience, trust, and professional dedication have always encouraged me. He has been so supportive on every decision I made. I feel great passion and motivated working with him.

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Peptidomimetics are synthetic oligomers that resemble the activities of peptides. Their advantages over peptides include high stability towards proteolysis and enormous chemical diversity. Over the past two decades, there have been extensive efforts to develop peptide mimics, such as beta-peptides, peptoids, D-peptides, etc. The research on peptidomimetics have led to many important applications in both medicinal and material science. In order to explore new functions, the discovery of peptidomimetics with novel frameworks is essential. We reported the synthesis and evaluation of a new class of peptidomimetics, termed as γ-AApeptides. Previous studies of γ-AApeptides have revealed that γ-AApeptides are highly resistant to proteolysis, and are highly amendable to chemical diversification. However, new biological activities and folding properties of γ-AApeptides still need to be explored. In order to expand the potential of γ-AApeptides in chemical biology and medicinal chemistry, I have been focusing on the development of new methods to synthesize linear and cyclic γ-AApeptides, development of one-bead-one-compound (OBOC) γ-AApeptide libraries for the discovery of inhibitors against beta-amyloid aggregation, exploring new helical foldamers for the rational design of protein-protein interaction (PPI) inhibitors, and studying cyclic γ-AApeptides for antimicrobial development.
CHAPTER 1: INTRODUCTION

1.1 Peptides and Peptidomimetics

Peptides are involved in virtually all aspects of biological processes, such as signal transduction, molecular recognition, bio-catalysis, etc. Thus, they are ideal source of drug candidates. It is indeed true, as 19 therapeutic peptides were approved by FDA between 2001 and 2012.\textsuperscript{1,2} Although the drug discovery industry is still dominated by small molecule drugs, peptides have been more successful than small molecules as therapeutics, considering much less effort has been devoted to develop therapeutic peptides than small molecule based drug discovery.\textsuperscript{3}

Compared to small molecules, peptides cover larger chemical space, thus they are more specific and less toxic. In addition, several targets that were considered as “undruggable” with small molecule drugs, such as protein-protein interactions (PPIs) and protein-nucleic acid interactions, have been tackled by therapeutic peptides.\textsuperscript{4,5} Despite these exciting advantages, there are several obstacles that prevent the development of therapeutic peptides.\textsuperscript{1} Among them, the biggest concern is the oral bioavailability and in vivo stability.\textsuperscript{6} Due to their peptidic nature, peptides are prone to degrade in the presence of proteases. In recent years, considerable research effort has been focused on improving oral bioavailability and in vivo stability of peptide drug leads. By conjugation to active transport enhancer, such as cell and tissue penetrating peptides\textsuperscript{7,8} or the immunoglobulin constant region (Fc),\textsuperscript{9-11} modified peptides exhibit enhanced pharmacological properties, such as extended half-life and improved uptake.\textsuperscript{12} In addition,
effective delivery systems can protect peptide cargoes from proteolysis and further enhance the pharmacokinetics of therapeutic peptides.\textsuperscript{13,14}

We and others have been trying to solve this problem in a fundamental way. Hence, peptidomimetics, unnatural sequence specific oligomers, were designed to not only mimic the functions of peptides, but to possess better properties than their natural analogs.\textsuperscript{15-18} Peptidomimetics, which incorporate unnatural building blocks, have much enhanced chemical diversity.\textsuperscript{15,17} In addition, they show high in-vivo stability since their modified structure cannot be recognized by proteases easily.\textsuperscript{19} Thus, they may show improved pharmacological properties.\textsuperscript{20-22}

![Chemical structures of α-peptide, peptoid, β-peptide, and γ-AApeptide.](image)

**Figure 1.1.** Chemical structures of α-peptide, peptoid, β-peptide, and γ-AApeptide.

From a chemical point of view, unnatural building blocks can be derived from α–amino acids through backbone extension,\textsuperscript{16,17,23,24} N-alkylation,\textsuperscript{7,15} N-replacement,\textsuperscript{25} Cα-substitution,\textsuperscript{26} Cα-replacement,\textsuperscript{27} and carbonyl replacement\textsuperscript{28}. Among different classes of peptidomimetics, β-peptides and peptoids are the most well-known. (Figure 1.1) They have demonstrated excellent
applications, such as mimicking antimicrobial peptides,\textsuperscript{29,30} mimicking bioactive peptides,\textsuperscript{31,32} protein-binding,\textsuperscript{33-35} biomaterials\textsuperscript{36}, etc.

1.2 $\gamma$-AApeptides

In order to further expand the chemical scope of peptidomimetics, a new type of peptide mimics has been introduced as “$\gamma$-AApeptides”.\textsuperscript{37} They contain N-acylated N-aminoethyl amino acid units derived from $\gamma$-chiral peptide nucleic acid ($\gamma$-PNA). (Figure 1.1) The synthesis of $\gamma$-AApeptide includes solution phase synthesis of $\gamma$-AApeptide monomers and solid phase synthesis based on fluorenlymethyloxycarbonyl (Fmoc) chemistry.\textsuperscript{37,38} Previous studies have revealed that they are highly stable under physiological conditions.\textsuperscript{39} Also, $\gamma$-AApeptides showed promising biological applications, such as disrupting p53/MDM2 interaction,\textsuperscript{37} mimicking RNA binding,\textsuperscript{38} antimicrobial,\textsuperscript{40-43} and transactivator of transcription (TAT) peptides,\textsuperscript{44} and agent for positron emission tomography (PET) imaging\textsuperscript{39}.

1.3 Outline of the Dissertation

This dissertation provides insight into the synthetic methodologies and secondary structures of $\gamma$-AApeptides. The high-throughput screening against A$\beta$40 peptide and the development of cyclic $\gamma$-AApeptides as potent antimicrobial agents are also discussed.

In chapter 2, novel methods to prepare both linear and cyclic $\gamma$-AApeptides are discussed.

In chapter 3, combinatorial $\gamma$-AApeptides library in a one-bead-one-compound (OBOC) fashion was first designed and prepared with methods discussed in chapter 1. The new library was demonstrated through the screening against A$\beta$40 peptide, which successfully yielded a potent inhibitor against the aggregation of A$\beta$40 peptide.
Chapter 4 focuses the design, synthesis, and biophysics studies of helical mimetics based on sulfono-γ-AAPeptides.

Chapter 5 reports the development of cyclic γ-AAPeptides as potent antimicrobial agents by mimicking antimicrobial peptides.

1.4 References


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CHAPTER 2: THE SYNTHESIS OF LINEAR AND CYCLIC $\gamma$-AAPEPTIDES

Note to Reader


2.1 Solid Phase Synthesis of $\gamma$-AApeptides Using a Novel Submonomeric Approach

2.1.1 Background

Unnatural peptidomimetics have been investigated for more than a decade, and are of increasing importance in chemical biology and drug discovery.\(^1\) Besides being resistant to protease degradation and straightforward derivatization, Many classes of peptidomimetics, such as $\beta$-peptides,\(^2\)-\(^4\) peptoids,\(^5\) $\alpha/\beta$-peptides,\(^6\),\(^7\) oligoureas,\(^8,9\) azapeptides,\(^10,11\) have shown versatile biological applications by mimicking structures and functions of bioactive peptides. One of the most important applications is to generate short peptide-like oligomeric ligands that specifically target proteins of interest, so as to facilitate the discovery of potential drug candidates\(^12\) or identification of protein-binding molecules.\(^13,14\) Such research efforts, with the development of proteomics, lead to an unprecedented need for the rapid generation of a chemically diverse combinatorial library.\(^15\) A very elegant and successful example is the development of peptoid combinatorial libraries by Kodadek’s group to identify short peptoid ligands that binds to a range
of proteins with excellent specificity and affinity. Nonetheless, there are urgent needs to develop novel combinatorial libraries with new scaffolds and functional groups in order to discover new classes of ligands with enhanced specificity, affinity, and other biological properties.

Figure 2.1.1. Representative structure of a native α-peptide and a γ-AApeptide.

We have recently developed a new class of peptidomimetics termed “γ-AApeptides”, as they comprise of N-acylated-N-aminoethyl amino acid building blocks (Figure 2.1.1), and chiral side chains are linked to the γ-carbon in the building blocks. The other half of the side chains are introduced onto the γ-AApeptide scaffold through acylation of the center N in each building block using a wide variety of commercially available carboxylic acids, which endow γ-AApeptides with limitless potential for the generation of chemically diverse library. In contrast to α-peptides, each γ-AApeptide unit is comparable to a dipeptide; and γ-AApeptides and α-peptides of the same lengths project the same number of side chains. As such, there is a strong potential to identify γ-AApeptides that can mimic the structures and functions of α-peptides.

Indeed, similar to other classes of peptidomimetics, γ-AApeptides have been shown to be highly resistant to protease degradation. More importantly, they are able to disrupt protein-
protein interactions, and mimic the Tat peptide by binding to HIV-1 RNA and facilitating membrane translocation with comparable affinity and efficiency. More recently, we have also demonstrated that γ-AApeptides are potential antibiotic agents to combat drug resistance by mimicking the mechanism of action of natural antimicrobial peptides. Thus, it is envisioned that there is great potential to identify γ-AApeptide based ligands from a combinatorial library to bind to proteins of interest with high specificity and affinity.

Figure 2.1.2. The previous method for the synthesis of γ-AApeptides.

However, the previous approach of solid phase synthesis of γ-AApeptides (Figure 2.1.2) is not suitable for the development of combinatorial libraries. In this method, a γ-AApeptide sequence is prepared by assembling γ-AApeptide building blocks on solid phase. Each building block requires a 3-step synthesis (reductive amination, acylation, and deprotection) starting from the corresponding Fmoc-amino aldehyde. For instance, in order to prepare a random library of short γ-AApeptides containing three building blocks (6 side chains,
comparable to 6-mer peptides), with the availability of 10 Fmoc-amino aldehydes (Rx = 10) and 10 carboxylic acids (Ry = 10), 100 different building blocks have to be generated, which is almost impossible to achieve.

In order to rapidly develop γ-AApeptide libraries, so as to maximize their biological potential, herein we report the development of a novel submonomeric approach for the solid phase synthesis of short γ-AApeptides by utilizing an allyl protection. This method circumvents the necessity of γ-AApeptide building block preparation, thereby it is expected to greatly facilitate the application of γ-AApeptides in biomedical sciences in the future.

2.1.2 Results and discussion

The new route for the solid phase synthesis of γ-AApeptides using the submonomeric approach is shown in Figure 2.1.3. The first two steps have been used in the microwave-assisted preparation of peptoids\textsuperscript{15} and have proven to be highly efficient. In brief, 2-1-1 is obtained through the microwave-assisted coupling of bromoacetic acid with the amino group on the Rink amide resin using DIC as the activation agent.\textsuperscript{15} With the assistance of a 1000W commercial microwave, the reaction was accomplished in 4 min (8 × 30 s). Then excess allyl amine is added as the nucleophilic agent to form a secondary amine on the solid phase to give 2-1-2, which again was assisted by microwave and finished in 4 min (8 × 30 s). We reason the introduction of the allyl protecting group is critical since it completely avoids the constant over-alkylation occurring in the reductive amination of Fmoc-amino aldehyde with the primary amino group on the solid phase.\textsuperscript{27,28} Although over alkylation can be potentially alleviated by draining out excess aldehyde remaining in the solution during the imine formation step, it does not solve the problem;\textsuperscript{27} on the contrary, incomplete imine formation is seen when the draining method is used, since the formation of the imine is not efficient.\textsuperscript{27} As such, successful preparation of
sequences employing repetitive reductive amination reactions on the solid phase is rare due to such complexity of over-alkylation and incomplete reaction.

**Figure 2.1.3.** The new route for the synthesis of γ-AApeptides by submonomeric approach. DIC = Diisopropylcarbodiimide, PMHS = polymethylhydrosiloxane, DhBtOH = 3-Hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine.

With the protection of allyl group, 2-1-3 can be obtained free of side reactions, as the reductive amination step can be repeated in order to achieve quantitative conversion. The allyl protecting group is selectively removed by using PMHS-ZnCl₂/Pd(PPh₃)₄ in THF for 4 h (twice) to provide 2-1-4. Although this method has only been used to convert allyl protected secondary
amines to primary amines, we found the deprotection of tertiary amines 2-1-3 under same condition is also extremely efficient. Followed by double coupling of carboxylic acids and deprotection of Fmoc, the first building block 2-1-5 is accomplished, which ends the first synthetic cycle on the solid phase. The desired γ-AApeptides therefore can be generated by repeating the synthetic cycles.

To demonstrate the efficiency of this approach, Fmoc-Phe-CHO was first used for the reductive amination of 2-1-2a, and CH3COOH was used to acylate 2-1-4a. Both 2-1-4a and 2-1-5a were cleaved by 95% TFA/H2O, and analyzed by HPLC. As shown in Figure 2.1.4, every step in the synthetic cycle, including reductive amination, allyl deprotection, and acylation, is highly efficient, as crude 2-1-4a and 2-1-5a shown more than 95% purity.

Figure 2.1.4. HPLC traces of crude 2-1-4a and 2-1-5a that were monitored at 215 nm.
Figure 2.1.5. a, random γ-AApeptide sequences 2-1-6a, 2-1-6b, and 2-1-6c. b, Fmoc-amino aldehydes and carboxylic acids used to prepare 2-1-6a, 2-1-6b, and 2-1-6c.
To further prove the practical application of this approach in the future development of a γ-AApeptide combinatorial library, three random γ-AApeptide sequences 2-1-6a, 2-1-6b, and 2-1-6c (Figure 2.1.5a) were synthesized from a pool of Fmoc-amino aldehydes and carboxylic acids (Figure 2.1.5b) that contain a variety of charged and hydrophobic groups. As shown in Figure 2.1.5a, 2-1-6a, and 2-1-6b are γ-AApeptides containing three building blocks, which are comparable to 6-mer peptides in length; whereas 2-1-6c is a γ-AApeptide having 5 building blocks, and thereby a 10-mer peptide mimic. We believe these γ-AApeptide sequences are sufficiently long enough to compose combinatorial libraries in the future for the identification of potential drug candidates or protein binding ligands. If the satisfactory yield of these γ-AApeptides can be achieved, this submonomeric approach will definitely be able to be used to generate γ-AApeptide libraries much more rapidly than the current building block strategy.

Table 2.1.1 Product characteristics based on the monomeric approach.

<table>
<thead>
<tr>
<th>γ-AApeptide</th>
<th>2-1-6a</th>
<th>2-1-6b</th>
<th>2-1-6c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purity</td>
<td>68%</td>
<td>62%</td>
<td>59%</td>
</tr>
<tr>
<td>Yield</td>
<td>31%</td>
<td>27%</td>
<td>22%</td>
</tr>
</tbody>
</table>

Surprisingly, the submonomeric method led to the production of these three γ-AApeptide sequences with excellent yield and purity (Table 2.1.1, Figure 2.1.6, and Figure 2.1.7). Although there are some impurities seen in crude HPLC traces, which may come from the long-time exposure of resin to air and to water moisture in the solvents during solid phase synthesis, the quality of these crude γ-AApeptides are consistent and considerably high. They can be easily purified (Figure 2.1.6) and provided with the excellent overall yields. However, as seen in Figure 2.1.8, in order to prepare same three γ-AApeptide sequence 2-1-6a, 2-1-6b, and 2-1-6c, building
blocks would have to be prepared using the previous building block strategy, which is much more tedious and time-consuming. Thus, this new submonomeric approach is a real breakthrough.

**Figure 2.1.6.** HPLC profiles of γ-AApeptide 2-1-6a and 2-1-6c. Figure 2.1.6a, top, HPLC trace of crude 2-1-6a; bottom, HPLC trace of purified 2-1-6a. Figure 2.1.6b, top, HPLC trace of crude 2-1-6c; bottom, HPLC trace of purified 2-1-6c.
2.1.3 Conclusions

In summary, we have reported a novel submonomeric method to prepare short γ-AApeptides. This strategy circumvents the needs to prepare γ-AApeptide building blocks and therefore greatly facilitates the rapid preparation of chemically diverse γ-AApeptide libraries. The application of this approach will unprecedentedly enhance the biological potential of γ-AApeptides. The preparation of the γ-AApeptide combinatorial library for specific protein targeting is currently under investigation.
2.1.4 Experimental section

**General information.** α-amino acids and Rink amide resin (0.7 mmol/g, 200-400 mesh) were provided by Chem-Impex International, Inc. All other reagents and solvents were purchased from either Sigma-Aldrich or Fisher Scientific and used without further purification. The Fmoc-amino aldehydes were synthesized following previously reported procedure.21-26,30 γ-AApeptide sequences were prepared on the Rink amide resin in peptide synthesis vessels on a Burrell Wrist-Action shaker. The steps used in the microwave were carried out in a 1000W Emerson microwave oven (model MW8119SB). The γ-AApeptides were analyzed and purified on an analytical and a preparative Waters HPLC system, respectively, and then dried on a Labcono lyophilizer. Molecular weights of the compounds and γ-AApeptides prepared on the solid phase were identified on an Agilent LC-MS or a Bruker AutoFlex MALDI-TOF mass spectrometer.

**Solid phase synthesis, purification and characterization of γ-AApeptides using the submonomeric approach.** γ-AApeptides were prepared on a Rink amide resin in peptide synthesis vessels on a Burrell Wrist-Action shaker following standard Fmoc chemistry protocol of solid phase peptide synthesis. 100 mg of Fmoc-Rink amide resin was shaken in 3 ml of 20% piperidine/DMF for 20 min, and the solution was drained. This Fmoc deprotection procedure was repeated one more time and then the resin was washed with DMF (4 × 3 mL), and CH₂Cl₂ (4 × 3 mL), respectively. 2 M Bromoacetic acid and 2 M DIC in DMF (3 ml) were shaken for 1 min and added to the beads. The vessel was placed in the microwave oven for 30 seconds and the power was set at 10%. The vessel was then taken out and gently shaken by hands and put back to oven. This step was repeated for 8 times, and the beads were washed with DMF (4 × 3 mL), and CH₂Cl₂ (4 × 3 mL), respectively. A solution of allyl amine in DMF was added and the vessel was placed into microwave oven with the power set at 10% for 30 seconds. The procedure was
repeated for 8 times again, and washed with solutions used above. An Fmoc-amino aldehyde (3 equiv) in 50% MeOH/CH₂Cl₂ was added, followed by the addition of 3 equiv of NaCNBH₃, and 100 µL of CH₃COOH in CH₂Cl₂. The reaction was shaken for 1 h and the solution was drained. This reductive amination step was repeated one more time and washed with solutions used above. To the beads in 3 mL THF were added polymethylhydrosiloxane (150 µL), Pd(PPh₃)₄ (20 mg) and ZnCl₂ (30 mg), and the reaction mixture was shaken for 4 h. The solution was drained and the deprotection procedure was repeated for one more time. The resin was washed with DMF and CH₂Cl₂ as shown above. A carboxylic acid (5 equiv.), DIC (5 equiv.), and DhbtOH (3-4-Dihydro-3-hydroxy-4-oxo-1-2-3-benzotriazine, 5 equiv.) in DMF were mixed and added to the beads, and shaken for 3 h. The coupling reaction was repeated one more time, and the beads were washed with DMF and CH₂Cl₂ as shown above.

After the desired sequences were assembled, the resin was transferred into 4 ml vials and γ-AApeptides were cleaved from solid support in 95% TFA/H₂O for 1h. Then solvent was evaporated and the residues were analyzed and purified on an analytical (1 ml/min) and a preparative Waters (20 ml/min) HPLC systems, respectively, using 5% to 100% linear gradient of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over 40 min, followed by 100% solvent B over 10 min. The HPLC traces were detected at 215 nm. The desired fraction were collected and lyophilized. The molecular weights of γ-AApeptides were obtained on Bruker AutoFlex MALDI-TOF mass spectrometer using α–cyano-4-hydroxy-cinnamic acid.
Table 2.1.2 MS analysis of molecules or γ-AAPeptides identified in the text.

<table>
<thead>
<tr>
<th>γ-AAPeptides</th>
<th>molecular weight (Actual)</th>
<th>molecular weight (found)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1-4a</td>
<td>429.2</td>
<td>430.2 (M+H⁺) (LC-MS)</td>
</tr>
<tr>
<td>2-1-5a</td>
<td>471.2</td>
<td>472.2 (M+H⁺) (LC-MS)</td>
</tr>
<tr>
<td>2-1-6a</td>
<td>862.5</td>
<td>863.4 (M+H⁺) (LC-MS)</td>
</tr>
<tr>
<td>2-1-6b</td>
<td>804.5</td>
<td>805.5 (M+H⁺) (LC-MS)</td>
</tr>
<tr>
<td>2-1-6c</td>
<td>1349.8</td>
<td>1351.2 (M+H⁺) (MALDI)</td>
</tr>
</tbody>
</table>

2.2 Quick Access to Multiple Classes of Peptidomimetics from Common γ-AAPeptide

Building Blocks

2.2.1 Background

Peptides are involved in virtually all aspects of life processes, and display remarkable biological activities. However, they also have a few intrinsic drawbacks for biological applications, including the susceptibility to proteolytic degradation. As an alternative approach, peptidomimetics have been a vibrant research area in the past two decades, as they are designed to capture the structures and functions of peptides, while possessing enhanced stability and chemical diversity. They have been used for protein surface recognition, disruption of protein-protein interactions and other important biological functions. However, as peptides and proteins display an endless diversity of structures and functions, development of new classes of peptidomimetics with novel backbones remains to be of considerable significance.

![Figure 2.2.1](image-url)  
Figure 2.2.1. The general structure of α-peptide and γ-AAPeptide.
To this endeavor, We have recently designed a new class of peptide mimics termed “γ-AApeptides” (Figure 2.2.1), as they contain N-acylated-N-α-minoethyl amino acid units (Figure 2.2.1) derived from γ-PNAs. In each unit (building block), the chiral side chain is derived from an α-amino acid, while the other side chain is introduced through acylation of the nitrogen on the backbone by any carboxylic acids or acyl chlorides. As such, each unit of γ-AApeptides is comparable to two residues in α-peptides, and γ-AApeptides essentially project an identical number of functional groups as α-peptides of the same length. Additionally, the potential of generating γ-AApeptides with chemically diverse functional groups is limitless. Furthermore, half of the side chains of γ-AApeptides remain chiral, which may impose conformational bias to promote the formation of secondary folding structures. Similar to other classes of known peptidomimetics, γ-AApeptides are highly resistant to proteolytic degradation, making them promising candidates for modulation and perturbation of biological processes. For instance, some γ-AApeptides can permeate mammalian cell membranes, bind to HIV-1 RNA with affinity and specificity akin to Tat peptide, modulate p53/MDM2 protein-protein interactions, and selectively disrupt bacterial membranes by mimicking the mechanism of natural host-defense peptides. Furthermore, γ-AApeptides can even form novel nanostructures, suggesting their potential application in biomaterial science. To further expand the versatility of γ-AApeptides in biomedical and material sciences, herein we report a method to prepare theoretically any γ-AApeptides with high efficiency. More importantly, using the same synthetic approach, a few new classes of peptidomimetics with novel backbones, including oligocarbamates, oligosulfonamides, and oligoureas, can be conveniently generated. These peptidomimetics are different from classic oligo-carbamates, -sulfonamides and -ureas, and thus they may lead to novel functions and applications in biomedical and material sciences in the future.
2.2.2 Results and discussion

The synthesis of γ-AApeptides was originally carried out on the solid phase using the building-block approach.\textsuperscript{45} Although the synthesis is well established and provides products constantly with good yields, the approach is not ideal in quickly generating derivatives with diverse groups, because each building block has to be prepared separately before the solid phase synthesis is carried out. We recently developed a submonomeric approach for the synthesis of γ-AApeptides that circumvents the necessity of preparing γ-AApeptide building blocks.\textsuperscript{52} However, the basic units Fmoc-amino aldehydes are not stable at room temperature, and as the result they have to be used immediately after being prepared. Moreover, the synthetic procedure is still tedious and takes a few steps to finish one synthetic cycle, which can significantly affect the overall yield if longer sequences are prepared. As such, it is impractical to synthesize γ-AApeptide libraries with large diversity.

To overcome the obstacle, herein we report a new method that combines both building-block and submonomeric approaches. In this approach, only a few N-alloc γ-AApeptide building blocks need to be synthesized (Figure 2.2.2) via the route 1 or the route 2,\textsuperscript{21,25,45} in order to prepare γ-AApeptides containing virtually limitless functional diversity. When R contains acid-labile protecting groups such as Boc, route 2 has to be adopted to remove the benzyl group before alloc-Cl is added. It is noted that 1% AcOH is critical in this hydrogenation step to protonate the secondary amine, which otherwise always leads to the product with the Fmoc group being removed.
**Figure 2.2.2.** Synthesis of γ-AApeptides by a combined building-block and monomeric approach. 

**a,** synthesis of N-alloc γ-AApeptide building blocks. 

**b,** synthesis of γ-AApeptide sequences. Alloc = allyloxy carbonyl.
**Figure 2.2.3.**

**a.** N-Alloc γ-AApeptide building blocks 1-5 and acylating agents used for the synthesis of the sequences. **b.** The structure of 2-2-1, and its crude and purified analytical HPLC traces. **c.** The structure of 2-2-2, and its crude and purified analytical HPLC traces.
The key step of the solid phase synthesis is the removal of the alloc protecting group. Briefly, on the solid phase, the alloc protecting group is removed by 10 mol % equiv. Pd(PPh₃)₄ and 6 equiv. Me₂NH·BH₃ in DCM. This reaction is found to be extremely efficient and only takes 10 min to give the desired product with the quantitative conversion. After the removal of the alloc group, a variety of carboxylic acids or acyl chlorides can then be used to acylate the N on the γ-AApeptide backbone. To test the efficiency of this methodology, we synthesized a tetra-block sequence 2-2-1 and a penta-block sequence 2-2-2 (Figure 2.2.3). As shown in Figure 2.2.3c, with just one N-alloc γ-AApeptide building block, 2-2-2 was synthesized to bear diverse side chains when different acylating agents were used. With the use of multiple N-alloc γ-AApeptide building blocks and different acylating agents, the sequence 2-2-1 containing a wide variety of random side chains was prepared (Figure 2.2.3b). The purity of these two crude sequences is more than 80% (Figure 2.2.3b and 2.2.3c), which demonstrates the feasibility of this approach for the efficient preparation of γ-AApeptides with diverse functional side chains. Compared to previous approaches, this new approach significantly reduces the steps and shortens the time of synthesis, and greatly improves the yield and purity of γ-AApeptides. Furthermore, N-alloc building blocks are much more stable than Fmoc-amino aldehydes, the basic units used in the synthesis of γ-AApeptides by submonomeric approach, and therefore they can be prepared in large batches and used for a long period of time. This is another versatility of this new synthetic method.

It is known that peptidomimetics such as oligocarbamates, oligosulfonamides, and oligoureas also have important biological applications, implying the promising potential of new peptidomimetics with novel functional backbones. However, the synthesis of these classes of peptidomimetics is not trivial, which might be the reason why the reports of their biological
applications are much less than those based on β-peptides and peptoids. We realized that with these same N-alloc γ-AApeptide building blocks, new backbone function entities can be introduced to prepare new classes of peptidomimetics.

**Figure 2.2.4. a.** The N-alloc γ-AApeptide building block, sulfonyl chlorides, chloroformates, and isocyanates that were used to prepare new classes of peptidomimetics. **b.** Solid phase synthesis of peptidomimetic sequence with different backbone functionalities.

To test our hypothesis, we synthesized the oligosulfonamide (2-2-3), oligocarbamate (2-2-4), oligoure (2-2-5) and the sequence 2-2-6 containing all kinds of backbone functionalities using one N-alloc γ-AApeptide building block on the solid phase (Figure 2.2.4 and 2.2.5). The synthesis was achieved by acylating the N on the backbone with a variety of commercially available sulfonyl chlorides, chloroformates, and isocyanates (Figure 2.2.4a). Both crude and purified HPLC spectra demonstrate the efficiency of the synthesis (Figure 2.2.6). It should be noted that these new peptidomimetics are different from classic oligo-carbamates, -sulfonamides, and -ureas as they are based on γ-AApeptide backbone, and therefore they may have discrete structures and functions. Our results demonstrated that with common γ-AApeptide building blocks, different classes of peptidomimetics can be simultaneously obtained.
Figure 2.2.5. The sequences of oligosulfonamide (2-2-3), oligoure (2-2-4), oligocarbamates (2-2-5), and the sequence containing all backbone functionalities (2-2-6).
2.2.6 Conclusions

In summary, we have reported a modified method for the efficient preparation of $\gamma$-AApeptides and a few new classes of peptidomimetics such as oligosulfonamides, oligoureas and oligocarbamates, using common N-alloc $\gamma$-AApeptide building blocks. Now generation of
chemically diverse libraries of $\gamma$-AApeptides with virtually limitless potential is feasible. In addition, our effort not only demonstrates the versatility of this new synthetic approach, moreover, it leads to the creation of new families of unprecedented peptidomimetics bearing diverse functional backbones and side chains. The further development of these novel peptidomimetics may find important biological applications in the future. As such, the potential scope of research on $\gamma$-AApeptides will be significantly expanded.

2.2.4 Experimental section

**General information.** All Fmoc protected $\alpha$-amino acids and Rink amide resin (0.7 mmol/g, 200-400 mesh, 1% DVB) were purchased Chemimpex. All the other solvents and reagents were purchased from vendors and used without further purification. NMR data for building blocks was obtained on a 400 MHz NMR spectrometer. High resolution masses of building blocks were determined on a Liquid Chromatography/Quadrupole Time-of Flight mass spectrometer. Masses of peptidomimetics were obtained on a Proteomics Analyzer. Solid phase synthesis was conducted in peptide synthesis vessels on a shaker. Oligomers were analyzed and purified on a HPLC system, and then lyophilized on a lyophilizer.

**Preparation of $\gamma$-AA peptide building blocks.** N-alloc $\gamma$-AApeptide building blocks 1, 2, 5 were synthesized via route 1 in Figure 2.2.2 by following previously reported methods.$^{21,54,55}$ Building blocks 3 and 4 were synthesized via route 2 in Figure 2.2.2 and the procedure for the preparation of 3 is briefly shown as follows. The benzyl ester$^{21,54,55}$ (2 g, 1.66 mmol) in 50 mL methanol containing 1% acetic acid, Pd/C (0.2 g, 10% wt) was added. Hydrogenation was conducted at atmospheric pressure and room temperature for 2 h. After filtration and evaporation, the remaining solid was suspended in 50 mL CH$_2$Cl$_2$ and N, N-diisopropylethylamine (434 µL, 2.49 mmol, 1.5 equiv.) was added. The reaction mixture was cooled to 0 °C, then a solution of allyl
chloroformate (176 µL, 1.66 mmol, 1 equiv.) in CH₂Cl₂ was slowly added over 1h. The mixture was allowed to stir at room temperature for two more hours, and then washed with saturated citric acid (30 mL x 3) and brine solution, dried over Na₂SO₄, and concentrated under vacuum. The pure building block 3 was obtained as a white foam solid after flash chromatography with 10% MeOH/CH₂Cl₂ (1.68 g, 85% yield).

Figure 2.2.7. Building blocks used for synthesizing different classes of oligomers.

Compound 1. Yield 65%. ¹H NMR (CDCl₃, 400 MHz) δ (two rotamers) 7.74 (d, J = 8 Hz, 2H), 7.56 (t, J = 8 Hz, 2H), 7.38 (t, J = 8 Hz, 2H), 7.29 (t, J = 8 Hz, 2H), 5.92-5.73 (m, 1H), 5.29-5.06 (m, 2H), 4.57-4.28 (m, 5H), 4.17-3.83 (m, 3H), 3.60-3.15 (m, 2H), 1.69-1.23 (m, 3H), 0.93-0.87 (m, 6H) ppm. ¹³C NMR (CDCl₃, 100 MHz) δ (two rotamers) 173.6, 173.2, 157.8, 156.8, 156.7, 156.6, 156.2, 143.8, 141.3, 132.3, 124.5, 119.9, 118.2, 117.4, 66.8, 66.6, 52.3, 49.8, 48.7, 47.2, 41.7, 24.8, 23.2, 22.0, 21.8, 21.7, 14.2 ppm. HR-ESI: [M+H]⁺ calc: 481.2333, found: 481.2352.
Compound 2. Yield 61%. $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ (two rotamers) 8.74 (s, 1H), 7.73 (d, $J$ = 8 Hz, 2H), 7.50-7.47 (m, 2H), 7.36 (t, $J$ = 8 Hz, 2H), 7.28-7.18 (m, 7H), 5.82-5.74 (m, 1H), 5.21-5.07 (m, 2H), 4.53-4.43 (m, 2H), 4.34-4.25 (m, 2H), 4.11-3.21 (m, 6H), 2.82 (s, 2H) ppm. $^{13}$C NMR (CDCl$_3$, 100 MHz) $\delta$ (two rotamers) 173.5, 157.5, 156.9, 156.5, 156.1, 143.8, 143.7, 141.3, 137.2, 132.2, 129.2, 129.1, 128.6, 127.7, 127.0, 126.7, 125.2, 119.9, 117.6, 66.9, 60.5, 51.7, 49.8, 49.0, 47.1, 38.8, 21.0, 14.2 ppm. HR-ESI: [M+H]$^+$ calc: 515.2177, found: 515.2196.

Compound 3. Yield 85% (from intermediate 6a). $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ (two rotamers) 8.74 (s, 1H), 7.73 (d, $J$ = 8 Hz, 2H), 7.57 (d, $J$ = 8 Hz, 2H), 7.37 (t, $J$ = 8 Hz, 2H), 7.28 (t, $J$ = 8 Hz, 2H), 5.91-5.79 (m, 1H), 5.28-5.07 (m, 2H), 4.55-4.52 (m, 2H), 4.37-3.92 (m, 5H), 3.92-3.21 (m, 3H), 3.06-3.02 (m, 2H), 1.42-1.26 (m, 15H) ppm. $^{13}$C NMR (CDCl$_3$, 100 MHz) $\delta$ (two rotamers) 172.8, 156.9, 156.7, 156.4, 156.3, 143.9, 143.8, 141.3, 132.4, 125.0, 119.9, 117.3, 79.3, 76.8, 66.7, 66.6, 52.1, 50.5, 49.8, 49.1, 47.2, 40.2, 32.1, 29.3, 28.4, 22.8 ppm. HR-ESI: [M+Na]$^+$ calc: 618.2786, found: 618.2810.

Compound 4. Yield 80% (from intermediate 6b). $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ (two rotamers) 9.08 (s, 1H), 8.11 (d, $J$ = 8 Hz, 1H), 7.70 (d, $J$ = 8 Hz, 2H), 7.62-7.21 (m, 10H), 5.84-5.61 (m, 1H), 5.21-5.07 (m, 2H), 4.55-4.50 (m, 2H), 4.35-4.15 (m, 3H), 4.02-3.71 (m, 3H), 3.48-2.60 (m, 3H), 1.61 (s, 9H) ppm. $^{13}$C NMR (CDCl$_3$, 100 MHz) $\delta$ (two rotamers) 173.5, 157.0, 156.6, 156.4, 156.2, 149.6, 143.8, 141.2, 135.4, 132.2, 132.1, 127.6, 127.0, 125.2, 125.0, 124.5, 124.0, 123.8, 122.7, 119.9, 118.9, 118.3, 117.6, 116.1, 115.3, 83.7, 83.6, 67.0, 66.8, 51.7, 51.3, 50.6, 50.5, 49.9, 49.1, 47.1, 28.1 ppm. HR-ESI: [M+H]$^+$ calc: 654.2810, found: 654.2826.

Compound 5. Yield 75%. $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ (two rotamers) 7.73 (d, $J$ = 8 Hz, 2H), 7.55 (t, $J$ = 8 Hz, 2H), 7.37 (t, $J$ = 8 Hz, 2H), 7.29 (t, $J$ = 8 Hz, 2H), 5.85-5.76 (m, 1H),
5.29-5.09 (m, 2H), 4.55-4.54 (m, 2H), 4.36-4.15 (m, 3H), 4.04-3.89 (m, 2H), 3.64-3.58 (m, 1H),
3.43-3.13 (m, 2H), 1.16-0.99 (m, 3H) ppm. $^{13}$C NMR (CDCl$_3$, 100 MHz) $\delta$ (two rotamers) 173.1,
157.0, 156.5, 143.8, 141.3, 132.3, 124.9, 119.9, 117.5, 66.9, 66.7, 53.2, 52.9, 49.8, 49.0, 47.2,

Compound 6. Yield 80%. $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ (two rotamers) 9.08 (s, 1H),
8.11 (d, $J = 8$ Hz, 1H), 7.70 (d, $J = 8$ Hz, 2H), 7.62-7.21 (m, 10H), 5.84-5.61 (m, 1H), 5.21-5.07
(m, 2H), 4.55-4.50 (m, 2H), 4.35-4.15 (m, 3H), 4.02-3.71 (m, 3H), 3.48-2.60 (m, 3H), 1.61 (s,
9H) ppm. $^{13}$C NMR (CDCl$_3$, 100 MHz) $\delta$ (two rotamers) 173.5, 157.0, 156.6, 156.4, 156.2,
149.6, 143.8, 141.2, 135.4, 132.2, 132.1, 127.6, 127.0, 125.2, 125.0, 124.5, 124.0, 123.8, 122.7,
119.9, 118.9, 118.3, 117.6, 116.1, 115.3, 83.7, 83.6, 67.0, 66.8, 51.7, 51.3, 50.6, 50.5, 49.9, 49.1,

Solid phase synthesis and characterization of $\gamma$-AApeptides and other classes of
peptidomimetics. Solid phase synthesis was conducted in peptide synthesis vessels on a Burrell
Wrist-Action shaker. 100 mg Rink amide resin (0.07 mmol) was treated with 3 mL 20%
Piperidine/DMF solution for 15 min ($\times$ 2) to remove Fmoc protecting group. The solution was
drained and beads were washed with DCM (3 $\times$ 3 mL) and DMF (3 $\times$ 3mL). A solution of N-
aloc $\gamma$-AApeptide building block (2 eqv.), HOBt (38 mg, 0.28 mmol), and DIC (44 µL, 0.28
mmol) in 3 mL DMF was shaken for 5 min, and then added to the resin. The mixture was
allowed to react at room temperature for 6 h and drained. The beads were washed with DCM (3
$\times$ 3 mL) and DMF (3 $\times$ 3 mL), followed by a capping reaction with 500 µL acetic anhydride in 3
mL Pyridine. After washing with DMF (3 $\times$ 3 mL) and DCM (3 $\times$ 3 mL), to the beads were
added Pd(PPh$_3$)$_4$ (8 mg, 0.007 mmol) and Me$_2$NH·BH$_3$ (25 mg, 0.42 mmol) in 3 mL DCM. $^{53}$
The alloc deprotection reaction was shaken for 10 min and repeated one more time. The beads
were washed with DCM and DMF, followed by the reaction with acylating agents (4 equiv.) and DIPEA (6 equiv.) in 3 mL DCM for 30 min (× 2) or with carboxylic acid (4 equiv.), HOByt (8 equiv.), and DIC (8 equiv.) for 4 h (× 2). The previous steps were repeated until the desired sequences were obtained. After that, the resin were washed with DCM and dried in vacuo.

Peptide cleavage was done in a 4 mL vial by treating resin with TFA/H2O/TIS (95/2.5/2.5) for 2 h. The solvent was evaporated and the crude was analyzed and purified on an analytical (1 mL/min) and a preparative (20 mL/min) Waters HPLC systems, respectively. 5% to 100% linear gradient of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over 40 min was used. The HPLC traces were detected at 215 nm. The products were confirmed by MALDI-TOF.

Table 2.2.1 MALDI analysis of γ-AAPeptides.

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<th>Purity (Based on crude HPLC trace)</th>
<th>Yield (based on loading of the resin)</th>
<th>Exact mass (Actual)</th>
<th>Exact mass (found by MAIDI-TOF)</th>
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<td>1046.5728 (+ H⁺)</td>
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<td>1129.3735</td>
<td>1130.4100 (+ H⁺)</td>
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<td>1160.6294 (+ H⁺)</td>
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<td>11%</td>
<td>1144.5045</td>
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</table>
2.3 The Synthesis of Head-to-tail Cyclic Sulfono-\(\gamma\)-AApeptides

2.3.1 Background

Cyclic peptides play an important role in the area of drug discovery.\(^{56}\) The conformational rigidity conferred by macrocyclization is often associated with increased activities compared with linear peptides, especially in the modulation of protein-protein interactions (PPIs).\(^{57-60}\) Over the years, extensive efforts have been focused on the synthesis and structural modification of cyclic peptides.\(^{61-63}\) In the meantime, a number of classes of peptidomimetics were developed to mimic the structure of peptides.\(^{32,64}\) These compounds were shown to display similar, even enhanced functions compared to peptides and possess much better stability towards proteolysis.\(^{32}\) Similar to peptides, conformational constraints, such as cyclization, have been introduced to peptidomimetics such as peptoids, further enhancing their structural rigidity and therefore potential biological activity.\(^{65-68}\)

\[\text{α-peptide} \quad \gamma\text{-A peptide} \quad \text{sulffono-} \gamma\text{-A peptide}\]

**Figure 2.3.1.** Structural presentation of a \(\gamma\)-A peptide as compared with an \(\alpha\)-peptide.

To expand the structural diversity of peptidomimetics, we have recently developed a new class of peptidomimetics termed as “\(\gamma\)-AApeptides”.\(^{54}\) \(\gamma\)-AApeptides contain N-acylated N-aminoethyl amino acid units derived from \(\gamma\)-PNAs (Figure 2.3.1). They can be efficiently synthesized by solid phase synthesis methods.\(^{54,69,70}\) Previous studies of \(\gamma\)-AApeptides have
revealed that γ-AApeptides are highly resistant to proteolysis and are highly amendable to chemical diversification.\textsuperscript{71} In addition, many γ-AApeptides were reported to bear promising biological functions.\textsuperscript{55,71-78} We thus believe that further development of γ-AApeptides will broaden the scope of their applications in the future.

Inspired by cyclic peptides and cyclic peptidomimetics, we also seek to extend the structural and functional diversity of γ-AApeptides by macrocyclization. In an initial study, an on-resin head-to-side chain cyclization method was successfully developed\textsuperscript{75} and resulted in the efficient preparation of cyclic γ-AApeptides that exhibited broad-spectrum antimicrobial activities superior to those of linear γ-AApeptides.\textsuperscript{75} However, since the cyclization was on the side chains, the resulting cyclic γ-AApeptides exhibited asymmetrical structures, and therefore structural studies and rational design of those cyclic sequences are difficult. As such, we have directed interest toward the development of the head-to-tail cyclic γ-AApeptides, especially cyclic sulfono-γ-AApeptides which may present a more rigid structure by avoiding cis-trans isomerization of tertiary amide bonds in a γ-AApeptide. Herein, we report for the first time an efficient method for the synthesis of cyclic sulfono-γ-AApeptides in a head-to-tail fashion. In order to assess the potential of cyclic sulfono-γ-AApeptides to mimic functions of peptides, structural analysis of a three-membered cyclic sulfono-γ-AApeptide 2-3-5 was subsequently conducted.

2.3.2 Results and discussion

Among successful methods for peptide macrocyclization, in-solution head-to-tail cyclization of linear peptide precursors in the presence of powerful coupling reagents has found the greatest number of applications. We initially attempted to synthesize linear sulfono-γ-AApeptide precursors by following the similar method. As such, a regular Fmoc γ-AApeptide
building block\textsuperscript{54} was first attached on the 2-chlorotrityl chloride (CTC) resin (Figure 2.3.2a). The Fmoc protecting groups were subsequently removed by 20% piperidine in DMF. However, ninhydrin test of the resulting resin showed negative, indicative of the failure of the first attempt. The LCMS analysis of Fmoc deprotection elution revealed the ketopiperazine formation during the Fmoc-deprotection process (Figure 2.3.2a). This is not surprising, as a similar phenomenon was observed on the attempted synthesis of cyclic PNAs.\textsuperscript{79} Since γ-AApeptides have the same backbone as chiral PNA, it is reasonable that synthesis of γ-AApeptides on CTC resin was unsuccessful.

![Chemical structure](image)

**Figure 2.3.2.** a Ketopiperazine formation, which prevents the sequence elongation. b Aryl hydrazine linker and 4-sulfamylbutyryl “safety-catch” linker used to eliminate ketopiperazine formation.

It is known that the formation of ketopiperazines that lead to self-cleavage off the solid support is significant under basic condition.\textsuperscript{79} Thus, we hypothesized that if the reaction is carried out under neutral condition, the potential formation of ketopiperazine would be minimized. As such, we introduced the Fmoc-N-Alloc γ-AApeptide building block (Figure 2.3.3A), with the assumption that neutral Alloc deprotection condition could bypass the autocleavage of ketopiperazines.
Figure 2.3.3. (A) Scheme for the preparation of cyclic sulfono-\(\gamma\)-AApeptide 2-3-1. (B) Structures of cyclic sulfono-\(\gamma\)-AApeptides in this study. *Acetic acid: trifluoroethanol: dichloromethane = 1:1:8 for 2h; *optimized cyclization condition: 0.5 mM linear precursors in dichloromethane with O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyl-uronium tetrafluoroborate (TBTU) (3 equiv.), hydroxybenzotriazole (HOBt) (3 equiv.), and 4-dimethylaminopyridine (DMAP) (5 equiv.) at room temperature for 6 h.
We then tested the feasibility of this method by synthesizing a four-membered cyclic sulfono-\(\gamma\)-AApeptide 2-3-1. (Figure 2.3.3A) In a general method, an Fmoc-N-Alloc \(\gamma\)-AApeptide building block (\(\gamma\)-BB-1) was first attached on the CTC resin. Fmoc protecting groups were removed by 20% piperidine/DMF solution, followed by the modification of the secondary amine by methanesulfonyl chloride. Next, the Alloc protecting groups were removed in the presence of Pd(PPh\(_3\))\(_4\) and Me\(_2\)NH.BH\(_3\) in dichloromethane,\(^{53}\) which indeed significantly prevented ketopiperazine formation. The rest of residues in the sequence were assembled with the regular \(\gamma\)-AApeptide synthesis method.\(^{70}\) Linear protected sulfono-\(\gamma\)-AApeptides were cleaved from the solid support with the regular CTC resin cleavage cocktail (acetic acid: trifluoroethanol: dichloromethane = 1:1:8). Finally, seven conditions were investigated for the efficiency of head-to-tail cyclization in solution (Table 2.3.1). We first employed PyBOP and HBTU (entry 1 and 2), which are common activating agents for peptide lactamization.\(^{86,87}\) Both of them gave modest results with 70% and 55% yields for PyBOP and HBTU, respectively. Surprisingly, an alternative method with the use of EDC\(^{88}\) (entry 3 and 4) showed even poorer yield (<5%). The most efficient cyclization was performed in dichloromethane with TBTU, HOBt, and DMAP as coupling reagents (entry 6).\(^{89}\) Under this condition, the four-membered cyclic sulfono-\(\gamma\)-AApeptide 2-3-1 was prepared with high yield (>95%) based on the analytical HPLC trace of the crude compound (Figure 2.3.4). No oligomerization was detected.
Table 2.3.1 Cyclization conditions for 2-3-1.

<table>
<thead>
<tr>
<th>entry</th>
<th>cyclization conditions&lt;sup&gt;a&lt;/sup&gt; (equiv)</th>
<th>solvent</th>
<th>yield&lt;sup&gt;b&lt;/sup&gt; (%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>PyBOP/HOBt/i-Pr&lt;sub&gt;2&lt;/sub&gt;NEt (4, 4, 8)</td>
<td>DMF</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>HBTU/HOBt/i-Pr&lt;sub&gt;2&lt;/sub&gt;NEt (4, 4, 8)</td>
<td>DMF</td>
<td>55</td>
</tr>
<tr>
<td>3</td>
<td>EDC/HOBt/i-Pr&lt;sub&gt;2&lt;/sub&gt;NEt (4, 4, 8)</td>
<td>DMF</td>
<td>&lt;5</td>
</tr>
<tr>
<td>4</td>
<td>EDC/HOBt/i-Pr&lt;sub&gt;2&lt;/sub&gt;NEt (4, 4, 8)</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;Cl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>&lt;5</td>
</tr>
<tr>
<td>5</td>
<td>TBTU/HOBt/i-Pr&lt;sub&gt;2&lt;/sub&gt;NEt (3, 3, 5)</td>
<td>DMF</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>TBTU/HOBt/DMAP (3, 3, 5)</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;Cl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>95</td>
</tr>
<tr>
<td>7</td>
<td>TBTU/HOBt/DMAP (3, 3, 5)</td>
<td>DMF</td>
<td>70</td>
</tr>
</tbody>
</table>

<sup>a</sup>The cyclization reactions were conducted with 0.5 mM linear precursor at room temperature for 6 h. <sup>b</sup>Yields were determined by analytical HPLC traces. PyBOP: benzotriazol-1-yl-oxytrypyrrolidinophosphonium hexafluoro-phosphate; HBTU: 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide.

Figure 2.3.4. HPLC trace of crude cyclic sulfono-γ-AApeptide 2-3-1.

Thus, the optimized coupling condition was selected to synthesize more cyclic sequences so as to demonstrate its generality. It is known that the ring size is another factor that affects the efficiency of synthesis. For instance, cyclization of ring sizes less than seven amino acids in peptides are sometimes problematic due to the backbone steric strain. To test the efficiency of cyclization, we investigated the effects of ring size on the cyclization of sulfono-γ-AApeptides by the preparation of a two-, a three-, and a five-membered cyclic sulfono-γ-AApeptide, 2-3-2, 2-3-3, and 2-3-4 respectively. Surprisingly, all of them showed high yields (Table 2.3.1). Even the shortest one 2-3-2, which bears same backbone size as a cyclotetrapeptide, displayed remarkable high yields. Short cyclic peptides, such as cyclotetrapeptides have attracted a lot of attention for their potent biological activities. However, the synthesis still remain a challenge.
Providing the highly efficient synthetic method, short cyclic sulfono-\(\gamma\)-AApeptides may serve as a novel scaffold to mimic the biological functions of short cyclic peptides. In addition, such method can be employed to prepare amphiphilic cyclic sequences (2-3-6 and 2-3-7) with more than 50% yield (Table 2.3.1).

The functions of peptides/peptidomimetics are tightly associated to their structures. Therefore, it is very intriguing to probe the structural conformation of head-to-tail cyclic sulfono-\(\gamma\)-AApeptides, so as to rationally design new molecules with predictable functions. To this end, we have successfully obtained a monocrystal of the three-membered cyclic sulfono-\(\gamma\)-AApeptide 2-3-5 by diffusing pentane vapor into a chloroform solution of 2-3-5. The structure was then elucidated by X-ray crystallographic study (Figure 2.3.5).

Figure 2.3.5. (A) Crystal unit cell of cyclic sulfono-\(\gamma\)-AApeptide 2-3-5. (B) Side view of the crystal structure showing spatial segregation of side chains. (C) Top view of the backbone with subunit 1, 2, and 3. (D) Superimposition of 2-3-5 backbone (red) with a turn region of a type II \(\beta\) turn (PDB: 1YCC) (yellow).
The crystal unit cell contains two 2-3-5 molecules, with each molecule displays segregated side chains on two faces. The top face is comprised of two 4-chlorophenyl sulfonyl groups and a methyl group whereas the bottom face contains two methyl groups and a 4-chlorophenyl sulfonyl group. All three tertiary sulfonamide groups adopt anti conformations. The unusual asymmetry of 2-3-5 contradicts what is predicted based on its primary structure as well as observed from other macrocycles, such as cyclohexapeptoids.\textsuperscript{65,88,92}

The backbone of 2-3-5 displays a “twisted” boat-like shape containing three trans amide bonds. (Figure 2.3.5B) Two carbonyl groups point outside the ring and one inside the ring. The dihedral angles were calculated and presented in Table 2.3.4. Six torsion angles ϕ, θ, η, ζ, ψ, and ω are defined to describe backbone dihedral angles of a γ-AApeptide (Table 2.3.4). Noticeable variations in dihedral angles ϕ, θ, η, and ζ of the three subunits present the asymmetry of this molecule. Similar to cyclic peptoids, ψ values show almost planar geometry for all three subunits.\textsuperscript{65} Three trans amide bonds are also revealed by ω values. The mean ω value is 179.2° with a standard deviation of 7.5°, which is close to what was reported for cyclic peptides\textsuperscript{93}.

The backbone also displays a hydrogen bond between C=O (subunit 1) and N-H (subunit 3), suggesting a turn-like structure. The turn contains same number of atoms as a peptide β-turn motif. Two carbon atoms (C\text{α1} and C\text{γ3}), which mimic two C\text{α} atoms in a peptide β-turn motif, show close proximity (6.103 Å). In addition, superimposition of 2-3-5 backbone at the turn region of a type II β turn (PDB: 1YCC) reveals high similarity (Figure 2.3.5D). The preference of 2-3-5 to resemble type II β turn is shown by comparison with a type I β-turn (Figure 2.3.8). The structural conformation of 2-3-5 suggests the potential of cyclic sulfono-γ-AApeptides to mimic the protein type II β-turn structure. Such β-turn mimics may find applications in various biomedical and material research.
2.3.3 Conclusions

In conclusion, we report an efficient method for the preparation of unprecedented head-to-tail cyclic sulfono-γ-AApeptides. Keto-piperazine formation was greatly reduced by introducing a unique Fmoc-N-Alloc γ-AApeptide building block for the first attachment on the CTC resin. Head-to-tail macrocyclization of the linear precursors was achieved with high efficiency by using TBTU, HOBt, and DMAP as coupling reagents. Following this method, cyclic sulfono-γ-AApeptides varying from two subunits to five subunits were readily synthesized with high yields. In order to elucidate its structural properties, we present for the first time the X-ray crystal structure of a three-membered cyclic sulfono-γ-AApeptide 2-3-5. The crystal structure shows a spatial segregation of side chains in an unusual asymmetrical pattern. More interestingly, 2-3-5 exhibits a turn-like structure with patterns similar to a peptide type II β-turn structure. By demonstrating the robust synthetic method of cyclic sulfono-γ-AApeptides, their capability to mimic peptide β-turn structure, and the ability to introduce different side functional groups, we believe that such macrocycles will find important applications soon after.

2.3.4 Experimental section

**General information.** All Fmoc protected/unprotected α-amino acids were purchased from Chem-Impex International, Inc. 2-Chlorotrityl (CTC) resin (0.98 mmol/g, 200-400 mesh) was purchased from AAPPTec LLC. All the other solvents and reagents were purchased from either Sigma-Aldrich or Fisher Scientific. NMR data for γ-AApeptide building blocks were analyzed on a Varian UnityInova400 spectrometer. Masses of AApeptide building blocks and cyclic sulfono-γ-AApeptides were obtained on an Agilent 6540 liquid chromatography/quadru-pole time-of-flight mass spectrometer. The X-ray diffraction data for 2-3-5 was collected on a Bruker D8 Venture PHOTON 100 CMOS system equipped with a Cu Kα INCOATEC Imus micro-
focus source ($\lambda = 1.54178$ Å). Solid phase synthesis was conducted in peptide synthesis vessels on a Burrell Wrist-Action shaker. Cyclic sulfono-\(\gamma\)-AApeptides were analyzed and purified on a Waters Breeze 2 HPLC system, and then lyophilized on a Labcono lyophilizer.

![Diagram of \(\gamma\)-AApeptide building blocks](image)

**Figure 2.3.6.** \(\gamma\)-AApeptide building blocks used in the study.

![Synthetic scheme of Fmoc-N-Alloc \(\gamma\)-AApeptide building block \(\gamma\)-BB-1](image)

**Figure 2.3.7.** Synthetic scheme of Fmoc-N-Alloc \(\gamma\)-AApeptide building block \(\gamma\)-BB-1.

**Synthesis and characterization of \(\gamma\)-AApeptide building blocks.** A solution of compound 1\textsuperscript{94} (4.38 g, 15 mmol) in 100 mL methanol was stirred in an ice bath. A solution of Gly-OtBu.HCl (2.52 g, 15 mmol) and triethylamine (2.09 mL, 15 mmol) in 20 mL methanol was added to the reaction flask. The reaction was allowed to stir at 0 °C for one hour, followed by the addition of acetic acid (4 mL, 3% v/v) and sodium cyanoborohydride (1.88 g, 30 mmol). The reaction was stirred at 0 °C for two more hours. After completion, methanol was removed by vacuum. The resulting slurry was dissolved in saturated sodium bicarbonate solution (150 mL) and dichloromethane (150 mL). The mixture was transferred to a separatory funnel. The bottom layer
was collected and washed with water (150 mL), then brine solution (150 mL). The organic layer was collected, dried over sodium sulfate, and concentrated in vacuo. The pure product 2 was obtained as white foam with a yield of 75% after flash column with hexane/ethyl acetate (2:1) as eluent. TLC analysis showed an Rf value of 0.1 in 1:1 hexane/ethyl acetate solution. Compound 2 (70% yield). \(^1\)H NMR (CDCl\(_3\), 400 MHz) \(\delta\) 7.26 (t, \(J = 8\) Hz, 2H), 7.22 (d, \(J = 8\) Hz, 1H), 7.16 (d, \(J = 8\) Hz, 2H), 6.11 (d, \(J = 8\) Hz, 1H), 5.84-5.75 (m, 1H), 5.20 (d, \(J = 16\) Hz, 1H), 5.13 (d, \(J = 12\) Hz, 1H), 4.50-4.38 (m, 2H), 4.20 (t, \(J = 8\) Hz, 1H), 3.75-3.65 (m, 2H), 3.38-3.08 (m, 2H), 2.98-2.78 (m, 2H), 1.39 (s, 9H) ppm. \(^1\)C NMR (CDCl\(_3\), 100 MHz) \(\delta\) 165.1, 156.6, 136.3, 132.5, 129.0, 128.7, 127.0, 117.5, 84.5, 65.9, 50.2, 49.7, 47.7, 38.8, 27.8 ppm. HR-ESI: [M+H]\(^+\) calcd: 349.2122, found: 349.2120.

A solution of 2 (3.9 g, 11.2 mmol) and N, N-diisopropylethylamine (2.15 mL, 12.3 mmol) in 50 mL dichloromethane was stirred in an ice bath. A solution of Fmoc-OSU (4.15 g, 12.3 mmol) in 20 mL dichloromethane was added dropwise. The reaction was allowed to stir at 0 °C for two hours. After completion, solvent was removed by vacuum. The slurry was used directly in the next reaction.

The slurry was treated with 20 mL 1:1 dichloromethane/trifluoroacetic acid mixture for 1 h at room temperature. After completion, solvent was removed by vacuum. Trifluoroacetic acid was removed completely by co-evaporation with dichloromethane (20 mL x 5). Pure \(\gamma\)-BB-1 was obtained as white solid with a yield of 95% after flash column with hexane/ethyl acetate (1:1) as eluent. TLC analysis showed an Rf value of 0.1 in a hexane/ethyl acetate 1:1 solution. \(\gamma\)-BB-1 (65% yield, two steps from 2) \(^1\)H NMR (CDCl\(_3\), 400 MHz) \(\delta\) (two rotamers) 9.73 (s, 1H), 7.76-7.69 (m, 2H), 7.55-7.48 (m, 2H), 7.38-7.17 (m, 9H), 5.83-5.69 (m, 1H), 5.25-5.03 (m, 2H), 4.64-4.79 (m, 4H), 4.22-4.00 (m, 2H), 3.88-3.57 (m, 1H), 3.29-3.81 (m, 2H), 2.79-2.37 (m, 2H) ppm. \(^1\)C NMR
(CDCl₃, 100 MHz) δ (two rotamers) 173.4, 156.9, 156.5, 156.4, 156.0, 143.8, 143.7, 143.6, 141.4, 141.2, 137.3, 137.1, 132.6, 129.4, 129.0, 128.5, 127.7, 127.4, 127.1, 126.6, 124.9, 124.6, 119.9, 117.6, 117.5, 117.4, 68.2, 67.2, 65.5, 51.6, 51.5, 51.1, 48.9, 47.1, 38.7, 38.5 ppm. HR-ESI: [M+H]⁺ calc: 515.2177, found: 515.2165.

Figure 2.3.8. Synthetic scheme of N-Alloc γ-AApeptide building block γ-BB-2.

Compound 4 and γ-BB-2 were prepared from 3⁹⁵ following similar procedure as γ-BB-1.

Compound 4 (75% yield). H NMR (CDCl₃, 400 MHz) δ 6.01 (d, J = 8 Hz, 1H), 5.92-5.82 (m, 1H), 5.29 (d, J = 16 Hz, 1H), 5.20 (d, J = 12 Hz, 1H), 4.60-4.44 (m, 2H), 4.16-4.11 (m, 1H), 3.80 (s, 2H), 3.32-3.14 (m, 2H), 1.46 (s, 9H), 1.29 (d, J = 8 Hz, 3H) ppm. ¹³C NMR (CDCl₃, 100 MHz) δ 165.2, 156.8, 132.5, 117.8, 84.7, 66.1, 52.4, 47.9, 44.4, 27.8, 18.5 ppm. HR-ESI: [M+H]⁺ calc: 273.1809, found: 273.1820.

γ-BB-2 (70% yield, two steps from 4). ¹H NMR (CDCl₃, 400 MHz) δ (two rotamers) 9.99 (s, 1H), 7.76-7.70 (m, 2H), 7.56-7.49 (m, 2H), 7.41-7.24 (m, 4H), 5.88-5.80 (m, 1H), 5.38-5.09 (m, 2H), 4.66-4.40 (m, 4H), 4.22-4.15 (m, 1H), 4.03-3.89 (m, 2H), 3.56-2.89 (m, 2H), 1.14-0.80 (m, 3H) ppm. ¹³C NMR (CDCl₃, 100 MHz) δ (two rotamers) 173.7, 156.7, 143.7, 143.5, 141.4, 141.3, 132.5, 124.6, 120.0, 119.9, 117.7, 68.3, 67.4, 65.8, 53.2, 52.5, 49.1, 48.9, 47.1, 46.3, 45.4, 18.4, 17.9 ppm. HR-ESI: [M+H]⁺ calc: 439.1864, found: 439.1867.
γ-BB-3 was synthesized following previous reported procedure.  

**Synthesis and characterization of cyclic sulfono-γ-AApeptides. 2-3-1:** 2-Chlorotrityl chloride (CTC) resin (102 mg, 100 μmol) was swelled in 2 mL DCM for 15 min. The first attachment was conducted by adding building block γ-BB-1 (77 mg, 150 μmol) and DIPEA (26 μL, 150 μmol) to the beads in the reaction vessel, which was allowed to shake at room temperature for two hours. After that, the reaction solution was drained, followed by washing with DMF (2 mL x3) and DCM (2 mL x3). The unreacted residues were capped with 2 mL methanol for 30 min. Again, the beads were washed with DCM (2 mL x3) and DMF (2 mL x3). The Fmoc group was removed by treating beads with 20% piperidine/DMF (v/v) solution for 10 min (x2) at room temperature. The solution was drained, followed by washing with DMF (2 mL x3) and DCM (2 mL x3). N-modification was achieved by reacting beads with methanesulfonyl chloride (23 μL, 300 μmol) and DIPEA (52 μL, 300 μmol) for 30 min (x2) at room temperature. The solution was then drained. After washing with DMF (2 mL x3) and DCM (2 mL x3), the beads were treated with Pd(PPh₃)₄ (12 mg, 10 μmol) and Me₂NH.BH₃ (35 mg, 600 μmol) in 2 mL dichloromethane for 10 min (x2) to remove Alloc protecting group. After the reaction, the solution was drained. The beads were washed with DCM (2 mL x3), 0.2% TFA/DCM (2 mL 60s x3), DCM (2 mL x3), 5% DIPEA/DCM (2 mL 60s x3), and DCM (2 mL x3).

For the next coupling reaction, building block γ-BB-3 (88 mg, 200 μmol), DIC (63 μL, 400 μmol), and HOBt (61 mg, 400 μmol) were pre-mixed in 2 mL DMF for 5 min before getting transferred to the reaction vessel. The reaction was shaken at room temperature for 2 hours, and the solution was drained. The beads were washed with DMF (2 mL x3) and DCM (2 mL x3) and the coupling reaction was repeated. The beads were washed with DMF (2 mL x3) and DCM (2 mL x3). The N-Alloc was removed following the same conditions discussed above: Pd(PPh₃)₄ (12
mg, 10 μmol) and Me₂NH·BH₃ (35 mg, 600 μmol) in 2 mL dichloromethane for 10 min (x2). After the reaction, the solution was drained. The beads were washed with DCM (2 mL x3), 0.2% TFA/DCM (2 mL 60s x3), DCM (2 mL x3), 5% DIPEA/DCM (2 mL 60s x3), and DCM (2 mL x3). After that, the beads were treated with phenylmethanesulfonyl chloride (57 mg, 300 μmol) and DIPEA (52 μL, 300 μmol) for 30 min (x2) at room temperature for the second Nα-modification. After washing with DCM (2 mL x3) and DMF (2 mL x3), N-terminal Fmoc group was removed with 20% piperidine/DMF (v/v) solution for 10 min (x2) at room temperature.

The previous reaction cycle was repeated until the desired sequence was obtained. The linear sulfono-γ-AApeptide was cleaved from resin with 4 mL cleavage cocktail (acetic acid : TFE : DCM = 1:1:8). After cleavage for 2 h, the solution was collected. The remaining beads were washed with 2 mL cleavage cocktail solution for three times. All the solution was combined and concentrated by vacuum. Acetic acid was completely removed by co-evaporation with hexane.

In the cyclization reaction, crude linear sulfono-γ-AApeptide was dissolved in 200 mL DCM (~0.5 mM), followed by the addition of coupling reagents: TBTU (96 mg, 300 μmol), HOBt (46 mg, 300 μmol), and DMAP (61 mg, 500 μmol). The reaction was allowed to stir at room temperature for overnight. After completion of reaction, the solution was washed with 0.1 M HCl (100 mL x3), water (100 mL), and brine (100 mL). The DCM layer was collected, dried over anhydrous sodium sulfate, and concentrated by vacuum. Crude compound 2-3-1 was dissolved in 10 mL water/acetonitrile (1:1) solution and filtered through a Whatman filter unit (0.45 μm) before HPLC analysis. Pure 2-3-1 was obtained as white powder after preparative HPLC and lyophilization.
2-3-2 to 2-3-7: Following similar method as 2-3-1, 2-3-2 to 2-3-7 (all white powder) were synthesized and characterized by HRMS (Table 2.3.2). 2-3-6 and 2-3-7 were treated with 2 mL DCM/TFA (1:1) for 1 h, before HPLC analysis and purification.

![Image of 2-3-1](image1)

2-3-1 $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 8.23 (d, $J = 8$ Hz, 1H), 8.13-8.07 (m, 2H), 7.38-7.33 (m, 15H), 7.23-7.17 (m, 5H), 4.54-4.37 (m, 6H), 4.03-3.95 (m, 3H), 3.94-3.75 (m, 8H), 3.20-2.99 (m, 8H), 2.97 (s, 3H), 1.04 (d, $J = 8$ Hz, 9H) ppm.

![Image of 2-3-2](image2)

2-3-2 $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 7.60 (d, $J = 8$ Hz, 1H), 7.43 (d, $J = 8$ Hz, 1H), 7.41-7.36 (m, 5H), 7.29-7.22 (m, 5H), 4.64-4.49 (m, 2H), 4.09-3.84 (m, 4H), 3.67-3.51 (m, 3H), 3.26-3.13 (m, 4H), 3.00 (s, 3H), 1.00 (d, $J = 8$ Hz, 3H) ppm.
2-3-3 $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 8.30 (d, $J = 8$ Hz, 1H), 8.05 (t, $J = 8$ Hz, 2H), 7.37 (s, 10H), 7.26-7.19 (m, 5H), 4.55-4.42 (m, 4H), 3.96-3.89 (m, 3H), 3.86-3.69 (m, 6H), 3.30-3.06 (m, 6H), 3.02 (s, 3H), 1.05 (t, $J = 8$ Hz, 6H) ppm.

2-3-4 $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 8.23 (d, $J = 8$ Hz, 2H), 8.16 (q, $J = 8$ Hz, 2H), 7.35 (s, 20H), 7.23-7.12 (m, 5H), 4.52-4.35 (m, 8H), 4.10-4.00 (m, 5H), 3.96-3.80 (m, 10H), 3.25-3.03 (m, 10H), 2.98 (s, 3H), 1.00 (t, $J = 8$ Hz, 12H) ppm.
2-3-5 $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 7.89 (d, $J = 8$ Hz, 3H), 7.83-7.79 (m, 6H), 7.67-7.64 (m, 6H), 3.90 (d, $J = 16$ Hz, 3H), 3.71-3.61 (m, 9H), 3.23-3.06 (m, 6H), 0.98 (d, $J = 8$ Hz, 9H) ppm.

2-3-6 $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 7.68 (s, 6H), 7.56 (s, 2H), 7.41-7.38 (m, 10H), 4.65 (q, $J = 16$ Hz, 4H), 3.96 (d, $J = 16$ Hz, 2H), 3.73-3.68 (m, 4H), 3.18-3.12 (m, 4H), 2.75-2.71 (m, 4H), 1.51-1.46 (m, 4H), 1.33-1.24 (m, 8H) ppm.
2-3-7 $^1$H NMR (DMSO-d$_6$, 400 MHz) $\delta$ 8.53-8.07 (m, 3H), 7.68 (s, 9H), 7.38-7.29 (m, 15H), 4.61-4.41 (m, 4H), 4.20-4.08 (m, 3H), 3.94-3.72 (m, 6H), 3.19-3.11 (m, 6H), 2.75-2.71 (m, 6H), 1.49-1.27 (m, 18H) ppm.

Table 2.3.2. Cyclization of sulfono-$\gamma$-AApeptides with varying ring sizes.

<table>
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<tr>
<th>compound</th>
<th>ring size</th>
<th>yield$^a$ (%)</th>
<th>mass$^b$</th>
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<tr>
<td>2-3-1</td>
<td>4</td>
<td>95</td>
<td>1073.3595</td>
</tr>
<tr>
<td>2-3-2</td>
<td>2</td>
<td>90</td>
<td>537.1838</td>
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<tr>
<td>2-3-3</td>
<td>3</td>
<td>85</td>
<td>805.2720</td>
</tr>
<tr>
<td>2-3-4</td>
<td>5</td>
<td>85</td>
<td>1341.4466</td>
</tr>
<tr>
<td>2-3-5</td>
<td>3</td>
<td>75</td>
<td>865.1080</td>
</tr>
<tr>
<td>2-3-6</td>
<td>2</td>
<td>54</td>
<td>651.2999</td>
</tr>
<tr>
<td>2-3-7</td>
<td>3</td>
<td>50</td>
<td>976.4464</td>
</tr>
</tbody>
</table>

$^a$Yields were determined by analytical HPLC traces (Figure 2.3.8). $^b$[M+1]$^+$ determined by ESI.
Figure 2.3.9. Analytical HPLC traces for crude and purified sequences 2-3-1 to 2-3-7.
**Crystallization study.** 5 mg of 2-3-5 was dissolved in 500 μL chloroform. The solution was filtered through a 4 MM PVDF syringe filter (0.45 μm) and stored in a 2 mL vial, which was then covered with aluminum foil and placed in a sealed 20 mL vial containing 1 mL pentane. A block shaped crystal (dimension 0.3 mm x 0.3 mm x 0.2 mm) was obtained by pentane diffusion into the chloroform solution after 5 days at room temperature.

The X-ray diffraction data was measured on a Bruker D8 Venture PHOTON 100 CMOS diffractometer equipped with a Cu Kα INCOATEC Imus micro-focus source (λ = 1.54178 Å). Indexing was performed using APEX2 (Difference Vectors method). Data integration and reduction were performed using SaintPlus 6.01. Absorption correction was performed by multi-scan method implemented in SADABS. Space groups were determined using XPREP implemented in APEX2. The structure was solved using SHELXS-97 (direct methods) and refined using SHELXL-2013 (full-matrix least-squares on F2) contained in APEX2, WinGX v1.70.01 and OLEX2. All non-hydrogen atoms of the product were refined anisotropically. Hydrogen atoms of -CH, -NH, -CH₂ and -CH₃ groups were placed in geometrically calculated positions and included in the refinement process using riding model with isotropic thermal parameters: Uiso(H) = 1.2(1.5)Ueq(-CH, -NH, -CH₂(-CH₃)). Occupancies of disordered chloroform molecules have been refined as free variables or were fixed. Heavily disordered molecules of chloroform and trifluoroacetic acid (presence confirmed by F NMR studies) have been refined using restraints and isotropically in case of atoms with low occupancy. Crystal data and refinement conditions are shown in Table 2.3.3.
<table>
<thead>
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<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Identification code</strong></td>
<td>2-3-5</td>
</tr>
<tr>
<td><strong>Empirical formula</strong></td>
<td>C$<em>{70.15}$H$</em>{81.65}$Cl$<em>{15.44}$F$</em>{1.5}$N$<em>{12}$O$</em>{19}$S$_6$</td>
</tr>
<tr>
<td><strong>Moiety formula</strong></td>
<td>2(C$<em>{33}$H$</em>{39}$Cl$_3$N$_6$O$_9$S$_3$), 3.15(CHCl$_3$), 0.5 (C$_2$HO$_2$F$_3$)'</td>
</tr>
<tr>
<td><strong>Formula weight</strong></td>
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<tr>
<td><strong>Temperature/K</strong></td>
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</tr>
<tr>
<td><strong>Crystal system</strong></td>
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<tr>
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<tr>
<td><strong>a/Å</strong></td>
<td>17.1143(4)</td>
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<tr>
<td><strong>b/Å</strong></td>
<td>20.8803(6)</td>
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<tr>
<td><strong>c/Å</strong></td>
<td>26.8416(6)</td>
</tr>
<tr>
<td><strong>α/°</strong></td>
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</tr>
<tr>
<td><strong>β/°</strong></td>
<td>90</td>
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<tr>
<td><strong>γ/°</strong></td>
<td>90</td>
</tr>
<tr>
<td><strong>Volume/Å$^3$</strong></td>
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</tr>
<tr>
<td><strong>Z</strong></td>
<td>4</td>
</tr>
<tr>
<td><strong>ρ$_{calc}$/g/cm$^3$</strong></td>
<td>1.499</td>
</tr>
<tr>
<td><strong>μ/mm$^{-1}$</strong></td>
<td>5.886</td>
</tr>
<tr>
<td><strong>F(000)</strong></td>
<td>4442.0</td>
</tr>
<tr>
<td><strong>Crystal size/mm$^3$</strong></td>
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</tr>
<tr>
<td><strong>Radiation</strong></td>
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<tr>
<td><strong>2Θ range for data collection/°</strong></td>
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<tr>
<td><strong>Index ranges</strong></td>
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<tr>
<td><strong>Reflections collected</strong></td>
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</tr>
<tr>
<td><strong>Independent reflections</strong></td>
<td>17264 [R$<em>{int} = 0.0936$, R$</em>{sigma} = 0.1036$]</td>
</tr>
<tr>
<td><strong>Data/restraints/parameters</strong></td>
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<tr>
<td><strong>Goodness-of-fit on F$^2$</strong></td>
<td>1.016</td>
</tr>
<tr>
<td><strong>Final R indexes [I&gt;2σ(I)]</strong></td>
<td>R$_1 = 0.0677$, wR$_2 = 0.1595$</td>
</tr>
<tr>
<td><strong>Final R indexes [all data]</strong></td>
<td>R$_1 = 0.1073$, wR$_2 = 0.1828$</td>
</tr>
<tr>
<td><strong>Largest diff. peak/hole / e Å$^{-3}$</strong></td>
<td>0.55/-0.40</td>
</tr>
<tr>
<td><strong>Flack parameter</strong></td>
<td>0.058(9)</td>
</tr>
</tbody>
</table>

**Table 2.3.3** Crystal data and structure refinement for 2-3-5.
Figure 2.3.10. (A) Overlay of 2-3-5 (red) with a turn region of a type II β turn (yellow) and (B) a turn region of a type I β turn (blue).

Table 2.3.4 Dihedral angle measurement for 2-3-5.

<table>
<thead>
<tr>
<th>subunit</th>
<th>φ</th>
<th>θ</th>
<th>η</th>
<th>ζ</th>
<th>ψ</th>
<th>ω</th>
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</thead>
<tbody>
<tr>
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<td>-154.7°</td>
<td>48.0°</td>
<td>-134.7°</td>
<td>70.9°</td>
<td>174.9°</td>
<td>173.6°</td>
</tr>
<tr>
<td>2</td>
<td>-83.2°</td>
<td>74.9°</td>
<td>99.8°</td>
<td>-113.1°</td>
<td>6.0°</td>
<td>-171.4°</td>
</tr>
<tr>
<td>3</td>
<td>-142.3°</td>
<td>-68.1°</td>
<td>-86.8°</td>
<td>124.1°</td>
<td>3.5°</td>
<td>-179.8°</td>
</tr>
</tbody>
</table>

2.4 References

(1) Wu, Y.-D.; Gellman, S. *Accounts of Chemical Research* **2008**, *41*, 1231.


(31) Gellman, S. *Biopolymers* **2009**, *92*, 293.


CHAPTER 3: ONE-BEAD-ONE-COMPOUND (OBOC) γ-AAPEPTIDE LIBRARY

Note to Reader

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3.1 Introduction

One of the most important goals in modern chemical biology and biomedical sciences is to identify molecular ligands that recognize peptides or proteins of interest with high specificity and affinity. Combinatorial chemistry is a powerful approach for ligand screening as it creates a diverse library of compounds, which provides unbiased opportunity for ligand identification when the structural information of targets are not available or not helpful in the rational design. In fact, most bioactive molecules are identified through screening efforts. As peptides have favorable protein binding capabilities and well-established synthetic protocol, early efforts were dedicated to the identification of selective peptide ligands against a variety of targets. Unnatural peptidomimetic ligand libraries are of significant interest recently, as these ligands contain unnatural backbones and therefore possess enormous structural diversity and enhanced stability against proteolysis. The examples of peptidomimetic ligands include peptoids, β-peptides, and N-acylated polyamine, etc. However, except for peptoids, the applications of peptidomimetic ligand libraries are rare.
γ-AApeptides are a new class of peptidomimetics developed in our lab very recently in order to advance the application of peptidomimetics in chemical biology and biomedical sciences (Figure 3.1a). As they contain N-acylated-N-aminoethyl amino acid units (Figure 3.1a) derived from γ-PNAs, they are termed “γ-AApeptides”. Each unit (building block) of the γ-AApeptide is comparable to a dipeptide residue in a canonical peptide. As such, γ-AApeptides essentially project an identical number of functional groups as conventional peptides of the same length. Half of the side chains of γ-AApeptides are chiral, which may impose conformational bias to the molecular ligands similar to conventional peptides, and presumably lead to the identification of ligands with improved specificity and affinity. Since the other half of the side chains are introduced through acylation of the nitrogen on the backbone by carboxylic acids or acyl chlorides, there is virtually limitless potential of generating γ-AApeptide libraries with chemically diverse functional groups. Moreover, γ-AApeptides are highly resistant to proteolytic degradation, making them ideal candidates to be molecular probes or therapeutic candidates.

To further expand the biological potential of γ-AApeptides, herein we report the development of γ-AApeptide OBOC library via the split-and-pool method. N-alloc protected γ-AApeptide building blocks were used to prepare γ-AApeptides of diverse functional groups (Figure 3.1b and 3.1c).

3.2 Results and Discussion

To find out if MS/MS could be potentially used to solve the unknown γ-AApeptide structure, we analyzed the fragmentation pattern of one known γ-AApeptide to prove that MS/MS is capable of determining the sequences of γ-AApeptides. As shown in Figure 3.2, this γ-AApeptide produces fragments in clear patterns and the sequence can be deduced unambiguously.
Figure 3.1. Synthesis of the $\gamma$-AApeptides. **a**, An $\alpha$-peptide and a $\gamma$-AApeptide. **b**, the synthesis of $\gamma$-AApeptides. **c**, N-Alloc protected $\gamma$-AApeptide building blocks and acylating agents (carboxylic acids and acyl chlorides) used in the preparation of $\gamma$-AApeptide combinatorial ligand library.
**Figure 3.2.** MS/MS analysis of a known γ-AApeptide. HCD fragmentation of a double charged precursor ion was performed at collision energy of 35.

We then proceeded to the next step by establishing a one-bead-one-compound (OBOC) γ-AApeptide library. The library was prepared by the split-and-pool method which produces beads with only one compound displayed on one bead (Figure 3.3).\(^4\) A methionine residue was first attached to the TentaGel beads (160002, 150 µm, 520, 000 beads/g), which facilitates the cleavage from beads by CNBr treatment.\(^28\) Then four N-alloc-protected γ-AApeptide building blocks and five acylating agents (including carboxylic acids or acid chlorides) (Figure 3.1c) were used to generate the combinatorial library of 4-building-block γ-AApeptides (comparable to 8-mer peptides in length). In theory, this library would contain 4 \(\times\) 5 \(\times\) 4 \(\times\) 5 \(\times\) 4 \(\times\) 5 \(\times\) 4 \(\times\) 6 = 192,000 compounds.
Figure 3.3. Schematic representation of synthesizing OBOC γ-AApeptide library.
To explore the potential of this γ-AApeptide library as an excellent source of protein/peptide ligands, the library was screened against the Aβ40 peptide, which is one of the major etiological factors for Alzheimer’s disease (AD) and plays a central role in the pathogenesis of AD.29,30 Although it is of high significance to quickly identify novel anti-Aβ aggregation inhibitors, there is no effective approach to achieve it.31

We hypothesized that the γ-AApeptide library can be used to identify ligands that bind to Aβ, and their anti-aggregation efficiency can be determined by functional assay afterwards. As such, we incubated beads with the Aβ40 peptide, followed by the treatment of anti-Aβ antibody 6E10 (Figure 3.4a). Then anti-mouse IgG-dylight 549 conjugate was added. Dylight 549 produces strong orange fluorescence, in which region TentaGel beads have low background fluorescence.28 Out of ~192,000 beads, two putative hits (approximately 0.001% hit rate) were identified and collected. The low rate may suggest the high selectivity of the library. One of the structures was identified by MS/MS unambiguously (Figure 3.5). This lead, designated as HW-155-1, was re-synthesized on Rink-amide resin (Figure 3.4b). The hydrophobic core residues 16-20 of the Aβ peptide, KLVFF, which inhibits Aβ aggregation both in vitro and in vivo,32-35 was also synthesized and used for comparison.

These two sequences were investigated for their capability to inhibit the aggregation of Aβ40 by ThT assay. Consistent to previous reports, KLVFF is a weak disruptor of Aβ aggregates. 100 μM of KLVFF can only inhibit less than 50% of Aβ aggregation (Figure 3.6a). Surprisingly, 1 μM of HW-155-1 already inhibits ~50% of Aβ aggregation (Figure 3.6b), indicating it is at least 100-fold more potent than KLVFF. The similar inhibitory effect was observed even after 24 h (Figure 3.6c). As such, HW-155-1 is one of the most potent small molecules that disrupt the aggregation of Aβ40.
Figure 3.4. Screening of the γ-AApeptide library. a, Schematic representation of the on-bead screening of the γ-AApeptide library against the Aβ40 peptide. The last picture was taken under a fluorescent microscope installed with a triple filter pass. Excitation is 550 nm and emission is 605 nm. b, the identified γ-AApeptide from the on-bead screening (HW-155-1) and the control peptide KLVFF.
Figure 3.5. Structural identification of one hit by MS/MS analysis. HCD fragmentation was performed on a double charged precursor ion (587.8827) and the collision energy was set at 35.
Figure 3.6. ThT assay of compounds against Aβ40. a, the change of fluorescence in the first 2 h of incubation of KLVFF with Aβ40; b, the change of fluorescence in the first 2 h of incubation of HW-155-1 with Aβ40; c, the ratio of aggregation after 24 h. Aggregation control (100%) is set as the change of fluorescence of 2.5 μM Aβ40 in Tris (pH 7.5) buffer. The concentration of ThT is 5 μM. Excitation: 440 nm; emission: 482 nm.

The ability of HW-155-1 to prevent Aβ aggregation was further confirmed by TEM (Figure 3.7a and 3.7b). In fact, HW-155-1 can even disassemble pre-formed Aβ fibrils (Figure 3.7c and 3.7d). In our experiments, the Aβ fibrils were not seen after incubating HW-155-1 with
Aβ40 monomers (prevention of aggregation) and pre-formed Aβ40 aggregates (disassembling aggregation) for 24 h.

**Figure 3.7.** TEM images. Monomeric Aβ40 in the absence (a) and presence (b) of HW-155-1 after 24 h; and pre-aggregated Aβ40 in the absence (c) and presence (d) of HW-155-1 after 24 h. bar = 1 μM. Aβ40 is 2.5 μM and HW-155-1 is 5 μM.

We then examined the effect of HW-155-1 on the toxicity of Aβ aggregates towards N2a neuroblastoma cells. In this cellular assay, Aβ42 was chosen as Aβ42 aggregates are easier to form and are more toxic than Aβ40 aggregates towards neural cells.\(^{33,36}\) As shown in Figure 3.8, aggregated Aβ42 kills 30% of N2a cells after 24 h incubation. In contrast, addition of 0.5 equiv.
of **HW-155-1** already reduces the death of N2a cells caused by Aβ42 to 25%, while the presence of 1 equiv. **HW-155-1** virtually completely removes the toxicity of Aβ42 and eliminates the death of N2a cells. The capability of **HW-155-1** to rescue these neuroblastoma cells suggests that **HW-155-1** may prevent the formation of Aβ aggregates and thus decrease the Aβ toxicities.

![Figure 3.8](image.png)

**Figure 3.8.** Detoxification of Aβ42 aggregates by **HW-155-1**. N2a cells were cultured with 1 µM of pre-aggregated Aβ42 in the absence/presence of **HW-155-1** for 24 h.

### 3.3 Conclusions

In summary, we have developed a new class of peptidomimetic combinatorial library that shows great promise to be a rich source of protein/peptide ligands. γ-AApeptides have the unique backbone that allows introduction of diverse functional groups with limitless potential. Their similarity in size and chirality to canonical peptides also confers favorable characteristics to bind and interact with other peptides and proteins. Our initial effort with this novel library has led to the identification of a lead small γ-AApeptide that is 100-fold more effective than KLVFF to prevent and even disassemble Aβ aggregation, and removes the toxicity of Aβ aggregates.
towards N2a neuroblastoma cells. Such a compound may be used as the potential molecular probe or therapeutic agent for the treatment of Alzheimer’s disease (AD). We are currently working on the development of new γ-AApeptide on-bead libraries with novel functional diversities so as to discover new bioactive candidates for biomedical applications, as well as the optimization of the lead γ-AApeptide HW-155-1 for the intervention of AD.

3.4 Experimental Section

General information. All Fmoc protected α-amino acids and Rink amide resin (0.7 mmol/g, 200-400 mesh) were purchased from Chem-Impex International, Inc. TentaGel MB NH₂ resin (0.3 mmol/g, 140-170 µm) was purchased from RaPP Polymere GmbH. All the other solvents and reagents were purchased from either Sigma-Aldrich or Fisher Scientific. NMR data for building blocks was obtained on a Varian UnityInova400 spectrometer. High resolution masses of building blocks were determined on an Agilent 6540 Liquid Chromatography/Quadrupole Time-of Flight mass spectrometer. Masses of γ-AApeptides were obtained on an Applied Biosystems 4700 Proteomics Analyzer. MS/MS analysis was carried out with a Thermo LTQ Orbitrap XL. Solid phase synthesis was conducted in peptide synthesis vessels on a Burrell Wrist-Action shaker. γ-AApeptides were analyzed and purified on a Waters Breeze 2 HPLC system, and then lyophilized on a Labcono lyophilizer.

The amyloid beta peptide Aβ40 was used as a target for the combinatorial library screen because insoluble Aβ plaque was thought to be a pathological marker in Alzheimer’s disease (AD). The synthesized library was stored in a glass peptide synthesis vessel, and later washed and incubated in the same container. The antibodies were purchased from Fisher Scientific, and all the other chemical were provided by Sigma-Aldrich. The beads were screened and picked up under Zeiss inverted fluorescence microscope 10x43HE filter.
Solid phase synthesis of γ-AApeptides.

Figure 3.9. Solid phase γ-AApeptide synthesis.

Solid phase synthesis was conducted on Rink amide resin (0.7 mmol/g) in peptide synthesis vessels on a Burrell Wrist-Action shaker (Figure 3.9). 100 mg resin (0.07 mmol) was treated with 3 mL 20% Piperidine/DMF solution for 15 min (× 2) to remove Fmoc protecting group. The solution was drained and beads were washed with DCM (3 × 3 mL) and DMF (3 × 3mL). A solution of γ-AApeptide building block (2 equiv.),¹⁷,¹⁹,²⁰,²²,³⁸ HOBt (38 mg, 0.28 mmol), and DIC (44 µL, 0.28 mmol) in 3 mL DMF was shaken for 5 min, and then added to the resin in a peptide synthesis vessel. The mixture was allowed to react at room temperature for 6 h
and drained. The beads were washed with DCM (3 × 3 mL) and DMF (3 × 3 mL), followed by a
capping reaction with 500 µL acetic anhydride in 3 mL Pyridine. After washing with DMF (3 ×
3 mL) and DCM (3 × 3 mL), to the beads were added Pd(PPh3)4 (8 mg, 0.007 mmol) and
Me2NH·BH3 (25 mg, 0.42 mmol) in 3 mL DCM. The alloc deprotection reaction was shaken
for 10 min and repeated one more time. The beads were washed with DCM and DMF, followed
by the reaction with acid chloride (4 equiv.) and DIPEA (6 equiv.) in 3 mL DCM for 30 min (×
2) or with carboxylic acid (4 equiv.), HOBt (8 equiv.), and DIC (8 equiv.) for 4 h (× 2).

The previous steps were repeated until the desired sequences were obtained. After that,
the resin were washed with DCM and dried in vacuo. Peptide cleavage was done in a 4 mL vial
by treating resin with TFA/H2O/TIS (95/2.5/2.5) for 2 h. The solvent was evaporated and the
crude was analyzed and purified on an analytical (1 mL/min) and a preparative (20 mL/min)
Waters HPLC systems, respectively. 5% to 100% linear gradient of solvent B (0.1% TFA in
acetonitrile) in A (0.1% TFA in water) over 40 min was used. The HPLC traces were detected at
215 nm. The products were confirmed on an Applied Biosystems 4700 Proteomics Analyzer.
Then, the desired fractions were collected and lyophilized.

MS/MS analysis. The fragmentation pattern of a known γ-AApeptide was analyzed on a Thermo
Scientific LTQ Orbitrap XL mass spectrometer (Figure 3.2). Higher Energy Collision
Dissociation (HCD) was performed at collision energy of 35.

Synthesis of the OBOC γ-AApeptide library. The TentaGel NH2 resin (1.6 g, 0.48 mmol,
832,000 beads) was swelled in DMF for 1h, followed by the treatment with Fmoc-Met-OH (3
equiv.), HOBt (6 equiv.), and DIC (6 equiv.) in DMF.10 The beads were shaken at room
temperature in a peptide synthesis vessel for 4 h and repeated. The beads were washed with
DCM (× 3) and DMF (× 3). Fmoc protecting group was removed with 20% piperidine in DMF

75
for 20 min (× 2). The beads were washed and equally distributed into four peptide synthesis vessels. Each building block (2 equiv.) together with HOBt (4 equiv.) and DIC (4 equiv.) were dissolved in DMF and added to each vessel. The coupling reaction was performed at room temperature for 6 h and repeated. The beads in each vessel were then washed and mixed thoroughly by severe shaking for 1 h. The beads were equally split into five vessels. The Alloc protecting group was removed by treating beads with Pd(PPh3)4 (0.1 equiv.) and Me2NH·BH3 (6 equiv.) in DCM for 10 min (× 2). After washing, each portion was reacted with either acid chloride or carboxylic acid. The reaction with acid chloride (5 equiv.) was carried out in the presence of DIPEA (5 equiv.) and DCM for 30 min (× 2). The carboxylic acids (3 equiv.) were pre-activated with DIC (6 equiv.) and HOBt (6 equiv.) in DMF, then added to beads. The reaction was carried out by shaking the vessel for 6 hours and repeated. After that, all the beads were pooled and mixed thoroughly. The previous split-and-pool process was repeated three times. The last time, after attachment of building blocks, beads were equally distributed into six portions, five of which were treated with alloc deprotection reagents then with acid chlorides and carboxylic acids as shown previously. The sixth portion was kept unreacted. At last, all beads were combined in one peptide synthesis vessel and washed thoroughly with DMF and DCM. Beads were treated with 20% piperidine in DMF for 20 min (× 2) and then with TFA/TIS/H2O (95:2.5:2.5) for 2 h to remove all the protecting groups. The beads were washed with DCM thoroughly and dried in vacuo.
Figure 3.10. Schematic representation of synthesizing OBOC γ-AApeptide library.
**Beads screening.** The library synthesized on TentaGel beads was swelled in dimethylformamide (DMF) for 1 hour, washed with 1×TBST for five times and then equilibrated in 1×TBST overnight at room temperature. The beads were blocked in 1% BSA in TBST for 1 hour, washed and equilibrated in 1×PBST before prescreening and screening.

Prescreening: In order to avoid any possible nonspecific binding, both the Aβ and antibodies solution were made in 1% BSA/TBST blocking buffer. The library was first incubated with mouse 6e10 primary antibody (200 ng/mL) which recognizes the first 16 amino acids of Aβ1-40, followed by five times PBST wash and incubation with goat anti-mouse IgG conjugated with dylight 549 (200 ng/ml). The beads were washed with PBST completely and transferred into a 6-well plate to be observed under Zeiss inverted fluorescence microscope 10×43HE filter, and the orange bright beads were picked up for they had suspicious nonspecific binding. These bright beads were excluded for further screening.

The rest of the beads were pooled together, washed with PBST, and then treated with 1% SDS at 90 °C for ten minutes to remove any bound proteins. We used both water and TBST to wash away the SDS and then the beads were washed and swelled in DMF for 1 hour. After washing and equilibrating in TBST overnight, the beads were ready for actual Aβ screening.

Screening: The prescreened beads were equilibrated in 1% BSA/PBST for 1 hour at room temperature. Aβ solution was made by dissolving Aβ40 powder in the buffer right before the screening. After washing with PBST for three times, the beads were incubated with Aβ40 peptide at a concentration of 20 µg/mL for 4 h at room temperature. Since Aβ40 aggregates quite slowly and the screening was done in such a short time, the percentage of Aβ40 aggregation in the solution was very minimal. After thorough washing with PBST, the library beads were incubated in 5 mL of 1% BSA/PBST containing 1:5000 diluted mouse 6e10 antibodies for 2 hours at room
temperature. The beads were gently washed with PBST and incubated with 1:500 diluted goat anti-mouse IgG-dylight 549 for 1 hour at room temperature. The beads were washed with PBST and transferred into the 6-well plate to be observed under Zeiss inverted fluorescence microscope 10×43HE filter. Again the bright orange ones were picked up as candidates for further study.

**Sequence decoding.** The beads were collected and washed with 1×PBST three times. The bound fluorescent dyes, proteins, and antibodies were removed by treating beads with 1% SDS solution at 90 °C for 10 min. After being washed with water, DMSO, and acetonitrile, beads were then cleaved and analyzed using previous procedure. The structure of one hit was determined (Figure 3.5) and designated as **HW-155-1.**

**Solid phase synthesis of HW-155-1 and KLVFF peptide.** **HW-155-1** was resynthesized on rink amide resin following previous procedure. (Figure 3.9) The mass was determined to be 1091.1375 (M+H)+ on an Applied Biosystems 4700 Proteomics Analyzer. The purity was analyzed on an analytical Waters HPLC system with flow rate of 0.8 mL/min and linear gradient from 5% to 100% (CH3CN in water) in 40 min (Figure 3.11a). As a positive control, **KLVFF** sequence was synthesized manually using regular solid phase peptide synthesis method. Amino acids were assembled on rink amide resin individually using HOBT/DIC as coupling reagents. After cleavage with TFA/TIS/H2O (95:2.5:2.5) for 3 h, TFA was removed under reduced pressure. The peptide was purified and analyzed on a preparative and analytical Waters HPLC system, respectively. (Figure 3.11b)
Figure 3.11. HPLC traces of pure compounds HW-155-1 (a) and KLVFF peptide (b).

Thioflavin T (ThT) assay. Compounds in different concentrations in Tris buffer Saline (TBS, pH 7.5) containing 10 µM ThT were added into a black 96 well plate corning@3721. Aβ40 monomer was freshly thaw and used to make a stock solution in TBS with a concentration of 5 µM. Equal volume of Aβ solution was added into the 96 well plate (Final Aβ conc. = 2.5 µM). Time-dependent fluorescence change was monitored by a Synergy 2 plate reader at an excitation
wavelength of 440 nm and emission at 482 nm. After 24 h, the fluorescence change was recorded. 100% aggregation is the fluorescence change of 2.5 μM Aβ40 in TBS buffer containing 5 μM ThT.

Transmission electron microscopy (TEM). Aβ40 preparations were adsorbed onto 200-mesh copper grids for 1 hour (until it is dry), and then stained with 1% uranyl acetate for 20 sec. The excess fluid was removed and the grids were analyzed with FEI Morgagni 268D TEM operated at 60 kV.

MTT toxicity assay. In siliconized tubes, Aβ42 peptide of 10 μM (in F-12 medium) was pre-incubated with 0, 0.5 and 1 equiv. of HW-155-1 γ-AA peptide, respectively. These solutions were incubated on a rotating shaker (Barnstead 400100) at 8 rpm in 37 °C for 24 h. Meanwhile, N2a cells were plated in 96-well plates (10000 cells/well) with three replicates for 24 h at 37 °C. Then pre-aggregated mixtures were added into each well to make the final Aβ concentration 1 μM. The plate was incubated for another 24 h at 37 °C. Next, 10 μL MTT reagent was added to the cells. The plate was incubated for 4 h at 37 °C. After the addition of 100 μL solubilization solution (10% Triton-X 100 in acidic Isopropanol (0.1N HCl)) and incubation for overnight, OD values were read at 575 nm. The final cell viability was calculated as:

\[
\text{Cell viability } \% = \frac{(\text{OD}_{575} - \text{OD}_{\text{blank}})}{(\text{OD}_{\text{ctrl}} - \text{OD}_{\text{blank}})} \times 100\%
\]

OD_{ctrl} is the OD of the well containing cells only. OD_{blank} is the OD of the blank well.

3.5 References


(38) Wu, H.; Teng, P.; Cai, J. *Submitted*.

CHAPTER 4: SULFONO-\(\gamma\)-AAPEPTIDES AS A NEW CLASS OF UNNATURAL HELICAL FOLDAMER

Note to Reader

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4.1 Introduction

Natural biopolymers including proteins and nucleic acids adopt well-defined and compact three-dimensional folding conformations providing a structural basis for their complex biological functions.\(^1\) This paradigm suggests that unnatural foldamers with new molecular frameworks and folding propensities may also exhibit unique biomimetic properties that can be exploited in pharmaceutical development and advanced biotechnology applications.\(^2\) Specifically, unnatural foldamers offer opportunities to better understand biomolecular structure-function relationships, facilitate the design of novel nanostructures, and develop targeted diagnostic agents and potential drug candidates.\(^3\) Since unnatural monomers have an enormous diversity in size, shape and backbone structure, unnatural foldamers can theoretically be developed to display a wide variety of three-dimensional conformations and biomimetic function.\(^4\text{-}7\) In addition, many unnatural foldamers are resistant to proteolytic degradation, augmenting their potential application in biological systems. The potential importance of unnatural foldamers has led to the development of numerous foldamer systems including \(\beta\)-peptides,\(^8\text{-}10\) \(\alpha\)-aminoxy-peptides,\(^11\) peptoids,\(^12\) and oligoureas.\(^13,14\) However, unnatural foldamers have just recently begun to find biological
applications and thus the continued development of new building blocks, molecular frame works and backbones are of key interest.

We recently have developed a new class of peptidomimetics - \(\gamma\)-AApeptides, oligomers of N-acylated-N-aminoethyl amino acids (Figure 4.1).\(^{15}\) As half of the side chains are introduced through acylation, \(\gamma\)-AApeptides have virtually limitless potential in functional group diversity. Although \(\gamma\)-AApeptides are based upon a chiral PNA backbone,\(^{16}\) they are designed to capture the function of bioactive peptides rather than nucleic acids.\(^{17,18}\) For instance, certain \(\gamma\)-AApeptides display both antimicrobial activity\(^{19-21}\) and anti-inflammatory activity\(^{22}\) by mimicking host-defense peptides while others mimic the Tat peptide by binding to HIV-1 RNA with high affinity\(^{18}\) and permeating cell membranes with excellent efficiency.\(^{17}\) In addition, \(\gamma\)-AApeptides have been developed to mimic the RGD peptide\(^{23}\) and to form one-bead-one-compound libraries for the discovery of bioactive protein/peptide based ligands.\(^{24,25}\) Furthermore, \(\gamma\)-AApeptides can also form novel nanostructures akin to peptide-based biomaterials.\(^{26}\) The emerging importance of \(\gamma\)-AApeptides has heightened the interest in the folding propensity regarding biomolecular structure.

\[
\begin{align*}
\alpha\text{-peptides} & : \\
\gamma\text{-AApeptides} & : \\
\text{Sulfono-}\gamma\text{-AApeptides} & :
\end{align*}
\]

\textbf{Figure 4.1.} The general chemical structures of \(\alpha\)-peptides, \(\gamma\)-AApeptides and sulfono-\(\gamma\)-AApeptides.
4.2 Results and Discussion

One of the most attractive features of γ-AApeptides is that half of the side chains do not have to be derived from carboxylic acids. As shown in Figure 4.1, replacement of carboxylic acids with sulfonyl chlorides leads to the generation of sulfono-γ-AApeptides. As a subclass of γ-AApeptides, sulfono-γ-AApeptides possess essentially unlimited functional diversity, as a wide variety of functionalized sulfonyl chlorides are either available commercially or can be readily synthesized. Moreover, Sulfono-γ-AApeptides contain the same number of side chains as regular peptides of the same lengths, affording the potential of sulfono-γ-AApeptides to mimic bioactive peptides. The presence of protons in the second amide moieties in sulfono-γ-AApeptides indicate these polymers may exhibit folding propensities through intramolecular hydrogen bonding akin to α-peptides. In addition, the tertiary sulfonamido moieties are sufficiently bulky as to induce intrinsic curvature into the sulfono-γ-AApeptide backbone. Furthermore, half of side chains of sulfono-γ-AApeptides are chiral, which may also impose conformational bias to further promote the formation of specific secondary conformation.

To test the hypothesis that sulfono-γ-AApeptide foldamers can form discrete secondary structures, we have synthesized a series of sulfono-γ-AApeptides of differing lengths. The longest sulfono-γ-AApeptide (4-1) contains eight building blocks, comparable in length to a 16-mer peptide. The shortest sequences 4-5 and 4-6 are sulfono-γ-AApeptide monomers which are equivalent to dipeptides (Figure 4.2).
Figure 4.2. The chemical structures of the sulfono-\(\gamma\)-AApeptides 4-1 to 4-6. In each sulfono-\(\gamma\)-AApeptide, the residues from the N-terminus are numbered as 1, 2, etc. In each sulfono-\(\gamma\)-AApeptide residue, \(a\) denotes the chiral side chain derived from the cognate \(\alpha\)-amino acid, and \(b\) represents the sulfonyl side group coming from sulfonyl chlorides.

The sulfono-\(\gamma\)-AApeptides 4-1 to 4-5 were obtained through solid-phase synthesis following our previously published protocol (Figure 4.3).\(^{24,27}\) In brief, the desired N-alloc \(\gamma\)-AApeptide residues\(^{24,27}\) were attached sequentially on the solid support. After each N-alloc \(\gamma\)-AApeptide residue was added, the alloc protecting group was removed with 10 mol % equiv. of Pd(PPh$_3$)$_4$ and 6 equiv. of Me$_2$NH·BH$_3$ in DCM.\(^{30}\) Subsequently, the sulfonyl side group was introduced by reacting a sulfonyl chloride with the secondary nitrogen on the \(\gamma\)-AApeptide backbone. The synthetic cycle was repeated until the desired sequence was assembled followed by cleavage and purification by HPLC. The monomers were prepared as previously reported.\(^{24,27}\)
Figure 4.3. Synthesis of the sulfono-γ-AApeptides 4-1 to 4-5.

The crystal structure of the monomer 4-6 was successfully obtained and shown in Figure 4.4a. The crystal structure indicates that the 4-6 adopts a right-handed turn conformation. Consistent with our hypothesis, the bulky tertiary sulfonamido group appears to force the formation of the backbone curvature. In addition, there is a hydrogen bond formation between NH of N-termini and CO of C-termini. An overlay of 4-6 with a canonical α-helical scaffold reveals that this turn curvature matches that of the α-helical sense (Figure 4.4b). The demonstration that such a short sulfono-γ-AApeptide has a defined pre-organized structure due to the intrinsic folding propensity leads to the possibility that longer sulfono-γ-AApeptides can be formed with more defined and stable secondary structures.
We have then carried out NMR studies of the longest sequence, 4-1. In order to solve the structure unambiguously, different hydrophobic and hydrophilic groups are included in the 4-1 sequence. The NMR spectra were collected on an Agilent dd600 with a triple resonance cold probe. The 1D $^1$H NMR spectra were first obtained with differing concentrations (0.05-1 mM) and the chemical shifts of the backbone protons were compared. There were no obvious changes in the chemical shifts, suggesting that 4-1 does not aggregate under the experimental conditions (Figure 4.5). Next, 2D NMR was employed to investigate the solution structure of 4-1 in methanol (2 mM in CD$_3$OH, 10 ºC). Two-dimensional spectra (zTOCSY, NOESY) were collected using standard pulse sequences with the number of acquisitions typically set to 200 for the NOESY and 6 for the zTOCSY spectra. The Water suppression through Enhanced T1 Effects (WET) method was used to suppress the proton peak in the CD$_3$OH solvent. In general, a 2s
delay was applied before each scan. Experiments were collected with 2K complex data points in F2 for each of 300 t1 increments with a sweep width of 6009 Hz in each dimension. Residue-specific assignments were made based on a combination of DQFCOSY, zTOCSY, ROESY and NOESY spectra. The presence of different side chains eliminates the potential overlaps between proton signals and are helpful for the unambiguous assignment of different building blocks. The CαHs were successfully assigned based on the short-range and/or sequential NOEs with neighbor side chains or amide proton (Figure 4.6).

Medium/long range NOEs revealed clear $i-i+3$ correlations between related side chains, i.e., 1bHPA-3aHB, 2aHB-3bHPA, 3bHPA-5aHB, 4aHG-5bHPA, 5bHPE-7aHB, 6aHγ-7bHPA (Figure 4.7). The $i-i+3$ correlation pattern implies that there is a defined folding pattern in 4-1, which displays proximity between every first and third building blocks.

Figure 4.5. $^1$H NMR spectra of 4-1 in CD$_3$OH at different concentrations (0.05, 0.1 and 1 mM).
Figure 4.6. (a) Typical sequential NOEs observed for 4-1. Residue numbers are labeled. (b) Corresponding peaks in NOE spectrum.

The NMR solution structure was solved and is displayed in Figure 4.8. Schrödinger Macromodel\textsuperscript{31} was used to perform molecular dynamics calculations based upon the NOE constraints, which resulted in the 10 best structures. As shown in Figure 4.8b, the structures display very good overlap among backbone atoms (rmsd $= 0.72 \pm 0.29$ Å, Figure 4.8b and Table 4.4). The average of the 10 helical structures for 4-1 is also displayed in Figure 4.8c and 4.8d. The data demonstrate that 4-1 adopts a well-defined right-handed helical conformation in methanol, with the side chains pointing away from the helical scaffold. Further analysis of the structure of 4-1 reveals a helical radius (2.3 Å) that is the equivalent to the canonical $\alpha$-helix. In addition, the average of helical pitches is 5.7 Å, which is also very close to that of the peptide
based α-helix (5.4 Å). Furthermore, the structure indicates that each turn contains four side chains (Figure 4.8e), relative to 3.6 residues/turn found in α-helical peptides. This assignment is also consistent to the observation of i-i+3 NOE patterns. These features suggest that the sulfono-γ-AApeptide could be developed to mimic the structure and function of α-helices.

The NMR structure further suggests that 4-1 has a 10/16 helix hydrogen-bonding pattern (Figure 4.9). It is known that the α-helix is the 13-helix; however, the same hydrogen pattern cannot be formed in sulfono-γ-AApeptides due to an alternative secondary amide and tertiary sulfonamide functionalities. Nonetheless, each 10/16 helix cycle in a sulfono-γ-AApeptide is equivalent to two successive 13-helices in the α-helix. This feature may partially explain why the helical pitch and the radius of 4-1 are similar to those of the α-helix. Additionally, as expected, sulfonyl groups also contribute to the stability of the helical structure by directly participating in hydrogen bonding. SO2 groups (except the first and last one) point away from the helical scaffold and do not participate in hydrogen bonding.

Circular Dichroism (CD) spectroscopy can also provide an assessment of the folding propensity of oligomers including proteins, peptides as well as unnatural molecules such as β-peptides and peptoids. The CD spectra of 4-1 under different solvent conditions in displayed in Figure 4.10a. The spectra exhibit a maximum at ~220 nm. Interestingly, it appears that the sequence adopts a more stable helical conformation in PBS buffer relative to TFE, suggesting the potential of sulfono-γ-AApeptide for the mimicry of the α-helix and modulation of protein interactions in biological systems. The CD data further indicate that the sequence is not aggregated under the concentration range examined here, as a 10-fold dilution in TFE has little or no effect on the spectrum.
Figure 4.7. Typical medium or long range NOEs showing \( i-i+3 \) correlations in 4-1. Residue numbers are labeled. \( i \) stands for side chains.
Figure 4.8. (a) The chemical structure of the sulfono-γ-AApeptide 4-1, as well as non-sequential NOEs indicated by curved lines; (b) Overlay of the ten lowest energy three-dimensional structures of 4-1 with lowest energies calculated based on NOE constraints (2D NMR were carried out in CD$_3$OH at 10 °C) and using MD simulations; (c) The average structure based upon b; (d) A helical ribbon is drawn to guide the review; (e) Approximate positions of side chains on the helical scaffold. Residue 1, which is less ordered in solution, is omitted.

Figure 4.9. Possible hydrogen bonding pattern suggested by the average of the 10 best structures of 4-1 generated via NOE-restrained molecular dynamics. The numbers indicate the number of atoms in the hydrogen-bonded rings, respectively.
The stability of the helix was further examined by temperature-dependent CD analysis (Figure 4.10b). As expected, 4-1 forms more defined helical structures at low temperatures. However, the secondary folding structure is still discernable even up to 55 °C. To assess the general folding propensity of sulfono-γ-AApeptides, CD analysis was also carried out for the 4-2 to 4-5 sulfono-γ-AApeptide sequences. As shown in Figure 4.10c, even the shortest sequence 4-5 displayed some degree of helicity, which is consistent to the crystal structure of 4-6 foldamer. The clear trend is that longer sequences form better helical structures. The 4-4 is a trimer sequence that displays increased helicity relative to 4-5. This is similar to the helical propensity of α-peptides. Additionally, the bulky group appears to stabilize the helical conformation, as observed with another trimer sequence 4-3, which contains an aromatic group and exhibits much more discernable helicity than 4-4. Surprisingly, the pentamer sequence 4-2, which is comparable to a decamer peptide, displays almost identical helicity to the longest sequence 4-1. This indicates that the general helical propensity of sulfono-γ-AApeptides is quite high. However, as our peptidomimetics do not have canonical peptide backbone, CD data is just used as the supporting data for NMR structures, and should not be overly interpreted. For example, the dichroic bands from the arylsulfonamido chromophores may have the potentially overlapping and disrupting role. In addition, the cotton effect near 220 nm may not be the accurate indication of molecular helicity.
Figure 4.10. a, CD spectra of 4-11 in different solutions at 25 °C. (1) in methanol, 200 µM; (2) in 1:1 methanol/H2O, 200 µM; (3) in 1:1 methanol/TFE, 200 µM; (4) in 1:1 methanol/TFE, 20 µM. b, CD spectra of 4-1 at various temperatures in methanol. c, CD spectra of 4-1 to 4-5 (200 µM) in methanol at 25 °C.

4.3 Conclusions

In summary, we have identified a new class of unnatural helical foldamer- sulfono-γ- AApeptides. The crystal structure indicates that even the shortest sulfono-γ- AApeptide (monomer) possesses a pre-organized folding structure. NMR studies further suggest that sulfono-γ- AApeptides adopt well-defined right-handed helical conformations in solution similar to peptide based α-helices. Similar to α-peptides, the sulfono-γ- AApeptide 4-1 is also stabilized by intramolecular hydrogen bonding. CD studies suggest that the similar folding propensity is generally observed throughout the sulfono-γ- AApeptide library examined here, and longer sequences exhibit more pronounced helicity in their secondary structures. As a virtually endless set of functional groups can be incorporated into sulfono-γ- AApeptides, the folding propensity can be further programmed by a number of chemical approaches including inclusion of constrained residues and hydrocarbon stapling, we envision that sulfono-γ- AApeptide foldamers can be readily developed to address a variety of challenges in chemical biology.
4.4 Experimental Section

General information. All Fmoc protected α-amino acids and Rink-amide resin (0.7 mmol/g, 200-400 mesh) were purchased from Chem-Impex International, Inc. All the other solvents and reagents were purchased from either Sigma-Aldrich or Fisher Scientific and used without further purification. Solid-phase synthesis of sulfono-γ-AApeptides were conducted in a peptide synthesis vessel on a Burrell Wrist-Action shaker. The sulfono-γ-AApeptides were analyzed and purified on a Waters Breeze 2 HPLC system, and then lyophilized on a Labconco lyophilizer. The molecular weight of sulfono-γ-AApeptide was obtained on an Applied Biosystems 4700 Proteomics Analyzer. All NMR experiments were performed at 10 °C on a Varian VNMRS 600 MHz spectrometer equipped with four RF channels and a Z-axis-pulse-field gradient cold probe.

Synthesis and characterization of sulfono-γ-AApeptides.24,27 Synthesis of the sequence 4-1: Solid-phase synthesis was carried out on 100 mg Rink-amide resin (0.7 mmol/g) at room temperature. The resin was swelled in DMF for 1 h before use. The Fmoc protecting group was removed by treating the resin with 3 mL 20% piperidine/DMF solution for 15 min (x2). The resin was washed with DCM (x3) and DMF (x3). A premixed solution of N-alloc γ-AApeptide building block24 (3 equiv.), HOBt (6 equiv.), and DIC (6 equiv.) in 2 mL DMF was added to the resin. The mixture was shaken for 4 h. After being washed with DCM and DMF, the resin was treated with Pd(PPh3)4 (8 mg, 0.007 mmol) and Me2NH·BH3 (25 mg, 0.42 mmol) in 3 mL DCM for 10 min (x2), then reacted with the desired sulfonyl chloride (4 equiv.) and DIPEA (6 equiv.) in 3 mL DCM for 30 min (x2). The reaction cycles were repeated until the desired sequence was assembled on the solid phase. After that, the resin was washed with DCM and dried in vacuo. The sulfono-γ-AApeptide cleavage was achieved in a 4 mL vial by treating the resin with TFA/H2O/TIS (95/2.5/2.5) for 2 h. The solvent was evaporated and the crude was analyzed and
purified on an analytical (1 mL/min) and a preparative (16 mL/min) Waters HPLC systems, respectively. 5% to 100% linear gradient of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over 40 min was used. The HPLC trace was detected at 215 nm. The desired fraction was collected and lyophilized, and confirmed on an Applied Biosystems 4700 Proteomics Analyzer. Then, the desired fraction was lyophilized.

Other sequences: 4-2 to 4-5 were synthesized using the same synthetic protocol as 4-1. The masses of these sequences are as follows:

4-1: theoretical: 2058.7, found: 2059.6 ([M + H]⁺), (MALDI).

4-2: theoretical: 1406.5, found: 1407.9 ([M + H]⁺), (MALDI).

4-3: theoretical: 711.4, found: 712.1 ([M + H]⁺), (ESI).

4-4: theoretical: 635.2, found: 636.1 ([M + H]⁺), (ESI).

4-5: theoretical: 251.1, found: 252.0 ([M + H]⁺), (ESI).

4-6: theoretical: 508.2, found: 506.1 ([M + H]⁺), (ESI).

2D NMR analysis of sulfono-γ-AApeptide 4-1. The sulfono-γ-AApeptide 4-1 was dissolved in approximately 0.5 mL of CD₃OH in a 5 mm NMR tube. The ¹H shift assignment was achieved by sequential assignment procedures based on zTOCSY and NOESY measurement. TOCSY and NOESY spectra were acquired with the wet solvent suppression. All these experiments were performed by collecting 6009 points in f₂ and 300 points in f₁. A DIPSI2 spin lock sequence with a spin lock field of 6k Hz and mixing time of 80 ms were used in zTOCSY. NOESY experiment used a mixing time of 200 ms. Vnmrj was used to process the data and 2D NMR spectra were analyzed by using SPARKY program.
Figure 4.11. Analytical HPLC traces of the purified sulfono-\(\gamma\)-AA peptides 4-1 to 4-5.

Figure 4.12. \(^1\)H NMR of 4-1 in CD\(_3\)OH at 10 °C.
Figure 4.13. (Continued)
Figure 4.13. (Continued)
Figure 4.13. (Continued)
Figure 4.13. (Continued)
Figure 4.13. Overlay of 2D NMR spectra in CD$_3$OH at 10 °C (green: NOESY, red: zTOCSY).
Table 4.1 Labeling names used in 2D NMR assignment (chemical shifts of protons on the backbone). See structure below for designations of $\alpha$, $\beta$, $\gamma$ carbons. a and b denote the chiral side chain and the sulfonyl group from a sulfono-$\gamma$-AApeptide building block, respectively.

![Structure of sulfono-$\gamma$-AApeptide building block]

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Molecular dynamics (MD) calculations based on NOEs. NOE-constrained molecular dynamics calculations were carried out by using MacroModel.\textsuperscript{34} Based on signal intensities, NOEs observed for the sulfono-γ-AApeptide S1 were grouped into one of four categories, 1.7~2.5 Å, 2.5~3.5 Å, 3.5~4.5 Å, 4.5~5.5 Å. 10 best structures were identified from the MD process employing the NOE constraints.

Table 4.2 List of sequential NOEs.

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<th>H-atom</th>
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<td>7b</td>
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Table 4.3 List of non-sequential NOEs.

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<th>H-atom</th>
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<td>HPB</td>
<td>4.5-5.5</td>
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<tr>
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Table 4.3 (Continued)

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Table 4.4 Rmsd of MD calculated structures.

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**Circular dichroism analysis.** Circular Dichroism (CD) analysis was conducted on an Aviv 215 circular dichroism spectrometer using a 1 mm path length quartz. Ten scans were averaged to obtain the data of each sample. Experiments were repeated for three times and the obtained spectra were averaged. The final spectra were normalized by subtracting the average of the blank spectra. Molar ellipticity \([\theta]\) (deg.cm\(^2\).dmol\(^{-1}\)) was calculated using the following equation:

\[
[\theta] = \theta_{obs}/(n \times l \times c \times 10)
\]

In which \(\theta_{obs}\) is the measured ellipticity in millidegrees, while \(n\) is the number of side groups, \(l\) is path length in centimeter, and \(c\) is the concentration of the sulfono-γ-AA peptide in molar units.
X-ray Crystallography. The crystal of 4-6 was obtained by slow solvent evaporation of 1:1 CH₂Cl₂/isopropanol containing 5 mg/mL of 4-6. The X-ray diffraction data for 4-6 were collected on a Bruker D8 Venture PHOTON 100 CMOS system equipped with a Cu Kα INCOATEC Imus micro-focus source (λ = 1.54 Å). Indexing was performed using APEX2 (Difference Vectors method).\(^3\)\(^5\) Data integration and reduction were performed using SaintPlus 6.01.\(^3\)\(^6\) Absorption correction was performed by multi-scan method implemented in SADABS. Space groups were determined using XPREP implemented in APEX2. The structure was solved using SHELXS-97 (direct methods) and refined using SHELXL-2013 (full-matrix least-squares on F2) contained in APEX2, WinGX v1.70.01\(^3\)\(^7\) and OLEX2.\(^3\)\(^8\) All non-hydrogen atoms were refined anisotropically. Hydrogen atoms of –CH, -CH₂, -CH₃, -OH and -NH groups were placed in geometrically calculated positions and included in the refinement process using riding model with isotropic thermal parameters: Uiso(H) = 1.2Ueq(-CH, -CH₂, -NH) and Uiso(H) = 1.5Ueq(-CH₃, -OH). Disordered benzyl group has been refined using constraints (AFIX66 for phenyl group) and restraints RIGU and SADI. The ADP values for disordered C21A and C21B atoms have been set to be equal (EADP). CCDC-1018941 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data\%5Frequest/cif.  

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<td>Flack parameter</td>
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4.5 References


(2) Wu, Y. D.; Gellman, S. *Accounts of Chemical Research* **2008**, *41*, 1231.


(36) Bruker Data Reduction Software 2013.


CHAPTER 5: DESIGN AND SYNTHESIS OF UNPRECENDENTED CYCLIC γ–AAPEPTIDES FOR ANTIMICROBIAL DEVELOPMENT

Note to Reader

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5.1 Background

One of the major health concerns for eukaryotes is the infection by pathogenic bacteria, which became acute in recent years due to the bacteria’s rapid development of multi-drug resistance to conventional antibiotics.1,2 Indeed, the World Health Organization (WHO) recently identified antimicrobial resistance as one of the three greatest threats facing mankind in 21st century. While chemotherapeutic agents primarily target the specific metabolic processes in bacteria, there is also a class of “host-defense” peptides produced in the eukaryotic innate immune response, which kill invading pathogens mainly through disruption of bacterial membranes.1,2 It is generally accepted that these antimicrobial peptides (AMPs) can be induced to segregate their cationic and lipophilic side-chain functional groups upon binding to negatively charged bacterial membranes, and the resulting globally amphipathic conformation leads to the disruption of bacterial membranes.3,4 Although the details of membrane disruption remain elusive, it is widely recognized that the hydrophobic part of AMP drives the peptide’s penetration through membrane via a hydrophobic interaction.4,5 The penetration process causes the depolarization of bacterial membranes and often leads to their cell death.5 The electrostatic
interaction of AMPs with bacterial membrane is thereby considered the driving force of their selectivity for bacteria against mammalian cells, in which anionic phospholipids only exist in the inner leaflet of membrane, and the overall charge of their membranes is zwitterionic. Due to this unique antimicrobial mechanism which depends on the peptide’s global chemical properties instead of their specific sequences, AMPs are difficult for bacteria to develop resistance against. Further, AMPs exhibit broad-spectrum activities against bacteria (both Gram-positive and Gram-negative), fungi, and even viruses. Hence, they are regarded as ideal agents to supplement current antimicrobial remedy.

Despite all the promising potentials, AMPs’ intrinsic peptidic nature severely limits their practical application as therapeutics, by making them immunoreactive, and susceptible to proteolytic degradation. To circumvent the drawbacks, non-natural peptidomimetics were recently developed to mimic AMPs, in terms of both antimicrobial activities and intrinsic mechanisms, while their backbones were modified to be protease-resistant. Over the last decade, non-natural antimicrobial oligomers have been extensively investigated, such as β-peptides, peptoids, arylamides, and other synthetic polymers. Whereas initial approaches focused on peptide mimics that can adopt regular helical conformations, it was later discovered to be unnecessary for potent antimicrobial activity, as long as a global segregation of hydrophilic and hydrophobic side chains can be ultimately achievable on the molecular surface, during the interactions with bacterial membranes. Despite the growing interest and intensive studies on linear peptidomimetics, it remains a challenge to introduce a diverse set of functional groups to tune their activity and selectivity, with the reported structure-activity relationship sometimes being inconsistent. In addition, some active peptidomimetics do not exhibit good selectivity between bacteria and mammalian cells, and there is still much room to further increase their
antimicrobial activity. Exploration of antimicrobial agents with potent, broad-spectrum antibacterial activity, while maintaining an excellent selectivity against bacteria, is an urgent goal in chemical biology.

We recently reported a new class of peptidomimetics, termed “γ-AApeptides”.\textsuperscript{13} Compared to conventional natural peptides, γ-AApeptides are advantageous in both their limitless potential for diversification and their inherent resistance to biodegradation. The synthesis of γ-AApeptides is straightforward, which facilitates library development for drug screening purposes. Certain γ-AApeptides have been demonstrated to disrupt protein-protein interactions,\textsuperscript{13} and to mimic protein’s binding activity with nucleic acids.\textsuperscript{14} A small library of linear γ-AApeptides was designed, which led to the identification of a potent γ-AApeptide (γ5) that displays significant activity against both bacteria and fungi, including the multi-drug resistant clinically-relevant strains.\textsuperscript{15} In the effort of our continuous exploration of antimicrobial γ-AApeptides, we herein report for the first time the design, synthesis, and evaluation of cyclic γ-AApeptides, some of which display antimicrobial activities superior to the previously reported linear ones.

Cyclic antimicrobial peptides were commonly observed in nature, such as gramicidin S, tyrocidine, polymyxin B, and protegrin I, which generally adopt semi-rigid backbone conformations, with substituents positioned in well-defined space.\textsuperscript{16} Such a semi-rigid backbone as a result of cyclization favors the binding event in entropy, while still possessing some flexibility to optimize their conformations for binding.\textsuperscript{16,17} It was shown that the lack of disulfide bonding diminished their hairpin conformation, and reduced the membranolytic activity.\textsuperscript{18-20} Hence, the cyclic peptides may have enhanced antimicrobial activity compared to the linear ones. There has been significant effort in the development and investigation of cyclic
antimicrobial peptides.\textsuperscript{16,21-23} For instance, Robinson et al\textsuperscript{21,22} prepared several cyclic peptides or peptide-peptoid chimeras, whose structures, as revealed by NMR, were unordered in water, but readily inducible to form regular $\beta$-hairpins in the membrane-mimicking environment. It is believed that the conformational bias induced by the constrained template would stabilize the hairpin structure, leading to the cluster of functional groups to form a hydrophobic and a hydrophilic face, upon interaction with bacteria membrane.\textsuperscript{21,24} Whereas the hemolytic activity of peptide oligomers is determined by their lipophilicity, the antimicrobial effects are always mediated by peptide charges and global amphiphilicity.\textsuperscript{24} It is therefore very interesting to investigate the antimicrobial activity of the cyclic peptidomimetics, as they are expected to be more stable against proteolysis than cyclic peptides, and more antimicrobial than linear peptidomimetics.

5.2 Results and Discussion

\textbf{Figure 5.1.} Illustration of cyclic antimicrobial $\gamma$-AApeptide design. A, Basic representation of the amphiphilic $\gamma$-AApeptide building block; B, Amphipathic cyclic $\gamma$-AApeptide with globally amphipathic conformation.
Herein for the first time we designed cyclic antimicrobial \(\gamma\)-AApeptides based on the simple rationale that we previously developed to successfully generate linear antimicrobial AApeptides (Figure 5.1).\(^{15,25}\) In this rationale, potent antimicrobial activity can be achieved by joining amphiphilic building blocks together to form a globally amphipathic conformation upon the interaction with bacterial membranes. The activity and selectivity can be fine-tuned by varying the ratio of cationic/hydrophobic groups. To achieve a global distribution of cationic and hydrophobic groups along the backbone, we prepared amphiphilic building blocks with a cationic group and a hydrophobic group on either side (Figure 5.1A). By joining these building blocks together and cyclizing the resulting oligomer (Figure 5.1B), a global amphiphilicity is expected to be achieved upon binding to bacterial membranes.\(^{15}\) As such, the amphiphilic building block 2 was prepared according to previously published procedure,\(^{15}\) in which the amino acid is lysine, and the phenyl ended side chain is appended to the amine (Figure 5.2a). Given that an introduction of hydrophobic building block can tune the overall amphiphilicity of \(\gamma\)-AApeptides and improve their antimicrobial activity, we also prepared building blocks 3 (Figure 5.2b) based on the reported procedure. To facilitate the on-resin cyclization of \(\gamma\)-AApeptide, a special \(\gamma\)-AApeptide building block 1 was designed here. While the synthesis was carried out similarly to the previously reported,\(^{15}\) the mono-allyl succinate was employed to modify the amine (Figure 5.2b).
Figure 5.2. γ-AApeptide building blocks used in the preparation of cyclic γ-AApeptides. a, The structures of building blocks; b, the synthesis of building block 1 and 3.

After building block 1 was conjugated to the beads, other building blocks were sequentially assembled via the standard Fmoc chemistry. In the end, the allyl ester from the peptide was deprotected by standard Pd(PPh₃)₄/PhSiH₃ reduction, and the resulting free carboxylate group reacted with the primary amino group of the last assembled building block, which resulted in protected cyclized γ-AApeptides on the resin (Figure 5.3). The desired cyclic γ-AApeptides were obtained upon treatment with TFA and HPLC purification, which are shown in Figure 5.4.
Figure 5.3. General synthesis of cyclic γ-AApeptides via on-resin cyclization.

Cyclized γ-AApeptides (5-1, 5-2, 5-3) comprising of four, five, and six amphiphilic building blocks were prepared as an initial attempt, and tested for their antimicrobial activities against a series of Gram-positive and Gram-negative bacteria and fungi, many of which are multi-drug resistant and clinically-relevant strains (Table 5.1). The oligomers’ hemolytic activities towards human red blood cells were also measured, as an indication of their selectivity. For comparison, Pexiganan, a phase III antimicrobial peptide drug candidate,\textsuperscript{3,26-28} as well as γ5, the most potent linear γ-AApeptide,\textsuperscript{15} were both used as controls. Similar to linear γ-AApeptide, which appeared to be more potent with a longer sequence,\textsuperscript{15} the cyclic γ-AApeptide with an increasing ring size (from 5-1 to 5-3) tended to augment the antimicrobial activities (Table 5.1). The most potent cyclic γ-AApeptide 5-3 has a similar activity to the well-known Pexiganan,
though it is still inferior to $\gamma_5$. It is notable that the hemolytic activity of $5$-$3$ is much less than Pexiganan and $\gamma_5$, implying the potential to improve its anti-bacteria activities through the introduction of hydrophobic building blocks.\textsuperscript{15}

Table 5.1 The antimicrobial and hemolytic activities of oligomers. The microbial organisms used are $C. albicans$ (ATCC 10231), $B. subtilis$ (BR151), multi-drug resistant $S. epidermidis$ (RP62A), Vancomycin-Resistant $E. faecalis$ (ATCC 700802), Methicillin-Resistant $S. aureus$ (ATCC 33592), $K. pneumoniae$ (ATCC 13383), and multi-drug resistant $P. aeruginosa$ (ATCC 27853). The minimum inhibitory concentration (MIC) for bacteria is the lowest concentration that completely inhibits growth after 24 h; and MIC for fungus $C. albicans$ is the lowest concentration that completely inhibits growth after 48 h. Sequences showing the most broad-spectrum antimicrobial activity (3-6) are shaded in light grey. Pexiganan and Linear $\gamma_5$ were used as control.

<table>
<thead>
<tr>
<th>Organism</th>
<th>HW-B-3</th>
<th>HW-B-4</th>
<th>HW-B-5</th>
<th>HW-B-11</th>
<th>HW-B-12</th>
<th>HW-B-13</th>
<th>HW-B-14</th>
<th>Pexiganan$^{3,26-28}$</th>
<th>Linear $\gamma_5^{15}$</th>
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<td></td>
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<td></td>
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<tr>
<td>$B. subtilis$</td>
<td>25-50</td>
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<td>5</td>
<td>2</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>2</td>
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<td>$S. epidermidis$ (MRSE)</td>
<td>&gt;50</td>
<td>10</td>
<td>8</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>$E. faecalis$ (VREF)</td>
<td>&gt;50</td>
<td>20</td>
<td>20</td>
<td>15</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td>32</td>
<td>5</td>
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<td>&gt;50</td>
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<td>3</td>
<td>16</td>
<td>5</td>
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<td><strong>Fungus</strong></td>
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<td></td>
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<tr>
<td>$C. albicans$</td>
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<td>&gt;50</td>
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<td>2</td>
<td>4</td>
<td>124</td>
<td>8</td>
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<td>&gt;500/</td>
<td>&gt;500/</td>
<td>200/</td>
<td>150/</td>
<td>40/</td>
<td>45/</td>
<td>181/495</td>
<td>75/300</td>
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</table>
Figure 5.4. The structures of cyclic γ-AApeptides and previously prepared linear antimicrobial γ-AApeptide γ5. Underlined building blocks are hydrophobic building blocks containing two hydrophobic side chains; the rest of the building blocks in the sequences are amphiphilic with one cationic and one hydrophobic side chains.
Given that linear $\gamma_5$ bears hydrophobic building blocks, and thus exhibited much more potent antimicrobial activities than other linear $\gamma$-AApeptides completely made from amphiphilic building blocks, we attempted this similar effort to our cyclic $\gamma$-AApeptides. 5-4, 5-5, and 5-6 were thereby prepared to incorporate the same number of building blocks as 5-3, but have one or two amphiphilic building blocks replaced by the hydrophobic ones (Figure 5.4). As a result, 5-4, with the change of only one building block, showed enhanced antimicrobial activities, especially against Gram-positive bacteria, which are comparable to linear $\gamma_5$, and better than Pexiganan (Table 5.1). In spite of its weaker activity towards Gram-negative strain *K. pneumoniae*, 5-4 has a stronger inhibition of fungus *C. albicans* than both Pexiganan and linear $\gamma_5$, with a significant minimum inhibitory concentration (MIC) value of 5 µg/mL. In addition, 5-4 still possesses a low hemolytic activity, thereby making it a promising antimicrobial agent with comparable or even better selectivity than Pexiganan and linear $\gamma_5$. Following this success, 5-5 was developed to incorporate two hydrophobic building blocks, which are separated by two hydrophilic building blocks, in a way similar to the design of linear $\gamma_5$. Surprisingly, its antimicrobial activities were not improved, or even weaker with respect to 5-4. In order to assess whether the diminuendo of activity is due to the incorporation of more hydrophobic building blocks or is caused by their relative positions in the ring, 5-6 was synthesized by placing two hydrophobic building blocks adjacent to each other (Figure 5.4). As a result, 5-6 exhibited even better activities than 5-4, Pexiganan, and linear $\gamma_5$ to arrest the growth of both Gram-positive and Gram-negative bacteria pathogens, as well as in fungus (Table 5.1). Especially towards two most clinically relevant strains *S. aureus* (MRSA) and *P. aeruginosa* (PA), 5-6 achieved a MIC value of 1 µg/mL and 8 µg/mL, respectively; which is at least 5-fold more potent than the linear $\gamma_5$. Though 5-6 appears
to be more hemolytic, its overall selectivity in several important pathogens is still improved relative to linear $\gamma$5 and Pexiganan.

To further investigate the effect of hydrophobicity and to tune the activity, 5-7 (Figure 5.4) with three hydrophobic building blocks was developed, which, however, resulted in a slightly decreased antimicrobial activity and hemolytic activity (Table 5.1) in comparison to 5-6. Nevertheless, the activity and selectivity of 5-7 are still generally comparable, or superior to linear $\gamma$5, against several strains including MRSA and PA. Thus, it is also a promising candidate for future antibiotic development. Based on the results, it appears that the inclusion of two neighboring hydrophobic building blocks brings in the optimal antimicrobial activity. The structure-activity studies of 5-3, 5-4, 5-6, and 5-7 suggest that a higher percentage of hydrophobic groups in $\gamma$-AApeptides lead to a higher antimicrobial activity. It is well accepted that more lipophilicity would lead to an increased hemolytic activity. While the result of our structure-activity studies generally supports this rule, the slightly decreased hemolytic activity demonstrated by 5-7, which has one more hydrophobic building block than 5-6, is quite unexpected. It suggests that besides the absolute hydrophobicity, the overall conformations of molecules may also affect their hemolytic activity. Finally, the distinct activities between 5-5 and 5-6 suggest the importance of position for hydrophobic building blocks. A preliminary computer modeling of 5-6 reveals that the cyclic $\gamma$-AApeptide naturally adopts a globally amphipathic conformation, with cationic side groups clustered at the bottom left face of the ring, and the majority of hydrophobic groups at the top face of the ring (Figure 5.5). Such a constrained structure with predefined amphiphilicity may favor the binding and disruption events within bacteria membranes. On the contrary, the amphiphilic topology of 5-5 may be scrambled by the separated hydrophobic building blocks. Though linear $\gamma$-AApeptides with scrambled
amphipathicity can still be induced by membrane to adopt a global amphiphilicity, the rigid structure of cyclic γ-AApeptides compromises their conformational flexibility, thereby requiring a pre-defined sequence for interaction with bacteria membrane.

Figure 5.5. The energy-minimized structure of 5-6. The computer modeling was carried out using ChemBioOffice MM2 energy minimization.

In order to understand the antimicrobial mechanisms of cyclic γ-AApeptides, the most active ones, 5-4, 5-6, and 5-7, were used to investigate their effects in cytoplasmic membrane disruption through the depolarization of *S. aureus* membrane (Figure 5.6). The membrane potential-sensitive dye DiSC₃ was used, the distribution of which between the medium and the cell interior reflects the membrane potential. The loss of membrane potential as a result of membrane permeation/disruption will lead to a dramatic increase in fluorescent intensity. Although the oligomer concentration needed for depolarization is actually higher than the oligomers’ MIC values, which is consistent to the previous report, generally more active
antimicrobial oligomers with lower MIC values tended to reach a high percentage of
depolarization at a lower concentration. Such a trend was clearly demonstrated by 5-4, 5-6, and
5-7, which supports the membrane disruption mechanism of cyclic γ-AApeptides (Figure 5.6).

Figure 5.6. Depolarization of the cytoplasmic membrane of *S. aureus* by cyclic γ-AApeptides.

The antimicrobial mechanism of cyclic γ-AApeptide was further assessed by
fluorescence microscopy, in which *B. subtilis* was treated with the most potent 5-6, and in the
meantime stained with 4’, 6’-diamidino-2-phenylindole dihydrochloride (DAPI) and propidium
iodide dyes (Figure 5.7). DAPI stains all bacterial cells irrespective of their viability, while PI
selectively stains injured or dead cells with damaged membranes. Whereas little PI staining
(red fluorescence) was observed in the control group, *B. subtilis* incubated with 5-6 for 2 h
displayed a strong red staining by PI, indicating the significant disruption of bacterial membrane
by 5-6. The aggregation of dead bacterial cells after the oligomer treatment is consistent with
literature reports,\textsuperscript{15,31} an indication of loss of membrane potential. Collectively, these cyclic $\gamma$-AApeptides have a bacterial inhibition mechanism similar to antimicrobial peptides, but distinct from conventional antibiotics, which augments their promise for further antimicrobial development.

**Figure 5.7.** Fluorescence micrographs of *B. subtilis* treated with 5 $\mu$g/ml cyclic $\gamma$-AApeptide 5-6 for 2 h. a1, control, no treatment, DAPI stained; a2, control, no treatment, PI stained; a3, control, no treatment, the merged view. b1, 5-6 treatment, DAPI stained; b2, 5-6 treatment, PI stained; b3, 5-6 treatment, the merged view. Scale bar: 2 $\mu$m.

### 5.3 Conclusions

In summary, we have shown for the first time the design, synthesis and evaluation of a class of cyclic $\gamma$-AApeptides for antimicrobial development. Following a proper tuning of hydrophobicity, several cyclic $\gamma$-AApeptides turned out to be superior to previously reported linear $\gamma$-AApeptides and peptide drug candidate under clinic testing, in terms of antimicrobial activity and selectively. Coupled with current biocompatibility, stability, and virtually limitless side chain variation, it is conceivable that the antimicrobial activity, selectivity, and overall drugability of cyclic $\gamma$-AApeptides can be further improved in the future. With a specifically designed building block for cyclization, the corresponding synthesis is facile and straightforward, which may propel the application of cyclic $\gamma$-AApeptides to a broad field of
biomedical research. The finding of enhanced activity through cyclization of \( \gamma \)-AApeptide may also shed light on the design and optimization of other non-natural oligomers for future development of promising antimicrobial agents.

5.4 Experimental Section

**General information.** \( \alpha \)-amino acid esters and Knorr resin (0.66 mmol/g, 200-400 mesh) were provided by Chem-Impex International, Inc. All other reagents and solvents were purchased from either Sigma-Aldrich or Fisher Scientific. The \( \gamma \)-AApeptide building block was synthesized following previously reported procedure.\(^{32}\) NMR spectra of the \( \gamma \)-AApeptide building block were obtained on a Varian Inova 400 instrument. Cyclic \( \gamma \)-AApeptides were prepared on a Rink amide resin in peptide synthesis vessels on a Burrell Wrist-Action shaker. The cyclic \( \gamma \)-AApeptides were analyzed and purified on an analytical and a preparative Waters HPLC system, respectively, and then dried on a Labcono lyophilizer. Molecular weights of cyclic \( \gamma \)-AApeptides were identified on a Bruker AutoFlex MALDI-TOF mass spectrometer.

**Synthesis\(^{14,15,32}\) and characterization of the \( \gamma \)-AApeptide building blocks.** The \( \gamma \)-AApeptide building blocks (Figure 5.2a) were synthesized following previously reported procedure.\(^{14,15,32}\) The characterization of building blocks 2 has been reported.\(^{15}\) The synthesis of building block 1 and 3 is shown in Figure 5.2b.
Compound 1. Yield 60 % (two steps from 4). $^1$H NMR (DMSO-d$_6$, 400MHz) $\delta$ (two rotamers) 7.88 (d, 2H), 7.62-7.57 (m, 2H), 7.42-7.29 (m, 4H), 7.28-7.15 (m, 5H), 5.93-5.83 (m, 1H), 5.31-5.25 (m, 1H), 5.19-5.15 (m, 1H), 4.52-4.49 (m, 2H), 4.21-4.03 (m, 4H), 3.88 (d, 2H), 3.63-3.35 (m, 2H), 3.10-2.47 (m, 6H). $^{13}$C NMR (DMSO-d$_6$, 100MHz) $\delta$ 171.8, 171.7, 171.2, 171.0, 170.6, 155.6, 155.6, 143.8, 143.7, 143.7, 143.7, 140.6, 140.6, 138.7, 138.6, 132.6, 132.6, 129.0, 128.0, 127.9, 127.5, 126.9, 126.0, 125.8, 125.0, 125.0, 120.0, 117.4, 117.4, 65.3, 64.2, 64.2, 51.7, 51.4, 46.6, 46.5, 37.3, 28.9, 28.8, 27.4, 27.1. HR-ESI: [M+H]$^+$ calcd: 571.2439, found: 571.2410.

![Structure of Compound 1](image)

Compound 3. Yield 60 %. $^1$H NMR (DMSO-d$_6$, 400 MHz) $\delta$ (two rotamers) 7.83 (d, $J = 7.6$ Hz, 2H), 7.58-7.53 (m, 2H), 7.36-7.26 (m, 5H), 7.24-7.09 (m, 5H), 4.13-4.06 (m, 3H), 3.96-3.74 (m, 4H), 3.51-3.46 (m, 1H), 3.40-3.32 (m, 1H), 3.38-3.11 (m, 1H), 2.78-2.72 (m, 1H), 2.67-2.56 (m, 1H), 2.32-2.06 (m, 2H), 1.47-1.36 (m, 1H), 1.33-1.24 (m, 2H), 0.80-0.71 (m, 6H). $^{13}$C NMR (DMSO-d$_6$, 100 MHz) $\delta$ 173.7, 156.1, 144.2, 141.1, 139.5, 139.1, 129.53, 129.46, 128.4, 128.0, 127.4, 126.5, 126.3, 125.7, 125.6, 125.5, 120.5, 65.8, 65.8, 51.5, 51.0, 47.0, 38.3, 34.3, 34.1, 30.9, 27.6, 27.5, 22.8, 22.7. HR-ESI: [M+H]$^+$ calcd: 529.2697, found: 529.2700.
Compound 4. Yield 82 %. $^1$H NMR (CDCl$_3$, 400MHz) $\delta$ 7.72 (d, $J = 8$ Hz, 2H), 7.49-7.45 (m, 2H), 7.38-7.34 (m, 2H), 7.26-7.14 (m, 7H), 6.18-6.14 (m, 1H), 4.26-4.16 (m, 3H), 4.09-4.06 (m, 1H), 3.78-3.68 (m, 2H), 3.42-3.37 (m, 1H), 3.17-3.15 (m, 1H), 2.99-2.94 (m, 1H), 2.86-2.81 (m, 1H), 1.39 (s, 9H). $^{13}$C NMR (CDCl$_3$, 100MHz) $\delta$ 165.2, 165.1, 162.1, 161.7, 156.9, 156.8, 143.9, 143.6, 141.2, 141.1, 136.0, 129.0, 128.8, 127.7, 127.6, 127.1, 125.2, 119.8, 84.8, 67.3, 50.6, 49.8, 47.8, 46.8, 38.6, 36.9, 36.8, 27.8, 27.7. HR-ESI: [M+H]$^+$ cacl: 487.2591, found: 487.2565.

Solid phase synthesis, purification and characterization of cyclic $\gamma$-AApeptides. Cyclic $\gamma$-AApeptides were prepared on a Rink amide resin in peptide synthesis vessels, on a Burrell Wrist-Action shaker, following the standard Fmoc chemistry protocol of solid phase peptide synthesis. Synthesized $\gamma$-AApeptide building blocks were used (Figure 5.3a). Each coupling cycle included a Fmoc deprotection using 20% Piperidine in DMF, and 8 h coupling of 1.5 equiv of $\gamma$-AApeptide building blocks in the presence of 4 equiv of DIC (diisopropylcarbodiimide) /DhbtOH (3-4-Dihydro-3-hydroxy-4-oxo-1-2-3-benzotriazine) in DMF. The cyclization was achieved on resin via the $\gamma$-AApeptide building block 1. Briefly, 1 was first attached to the solid support, followed by standard Fmoc solid phase synthesis. After desired sequences were assembled, the allyl group was removed by treatment of Pd(PPh$_3$)$_4$ (0.2 equiv.) /PhSiH$_3$ (10 equiv.) /CH$_2$Cl$_2$ for 2h (repeated two times). The deprotection of Fmoc group was then carried out on the N-terminus. The intramolecular cyclization was accomplished using
PyBop/HOBt/DIEA/DMF. Next, the resin was transferred into 4 mL vials and cyclic \( \gamma \)-AApeptides were cleaved from solid support in 50:48:2 TFA/CH\(_2\)Cl\(_2\)/triisopropylsilane overnight. Then solvent was evaporated and the residues were analyzed and purified on an analytical (1 mL/min) and a preparative Waters (20 ml/min) HPLC systems, respectively, using 5% to 100% linear gradient of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over 40 min, followed by 100% solvent B over 10 min. The HPLC traces were detected at 215 nm. The desired fractions were eluted as single peaks at > 95% purity with yields of 6-10% (based on loading of the resin, see Figure 5.4 for sequences). They were collected and lyophilized. The molecular weights of cyclic \( \gamma \)-AApeptides (Table 5.2) were obtained on a Bruker AutoFlex MALDI-TOF mass spectrometer using \( \alpha \)-cyano-4-hydroxy-cinnamic acid.

Table 5.2 MALDI analysis of cyclic \( \gamma \)-AApeptides 5-1 to 5-7.

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<th>Cyclic ( \gamma )-AApeptides</th>
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<th>molecular weight (found)</th>
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<td>1806.6 (M+H(^+))</td>
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<td>6.8%</td>
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<td>2147.6 (M+2H(_2)O+NH(_4^+))</td>
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<td>5-5</td>
<td>6.5%</td>
<td>2078.3</td>
<td>2079.9 (M+H(^+))</td>
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<td>2078.3</td>
<td>2079.0 (M+H(^+))</td>
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<td>5-7</td>
<td>6.4%</td>
<td>2063.3</td>
<td>2117.3 (M+2H(_2)O+NH(_4^+))</td>
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Figure 5.8. HPLC traces of pure cyclic γ-AApeptides.
**Antimicrobial assays.** The microbial organisms used were *B. subtilis* (BR151), multi-drug resistant *S. epidermidis* (RP62A), *C. albicans* (ATCC 10231), Vancomcin-resistant *E. faecalis* (ATCC 700802), Methicillin-resistant *S. aureus* (ATCC 33592), *K. pneumoniae* (ATCC 13383), multi-drug resistant *P. aeruginosa* ATCC 27853. The minimum inhibitory concentration (MIC) is the lowest concentration that completely inhibits the growth of bacteria in 24 h. The highest concentration tested for antimicrobial activity was 50 µg/mL. The antimicrobial activities of the cyclic γ-AApeptides were determined in a sterile 96 -well plates by broth micro-dilution method. Bacterial cells and fungi were grown overnight at 37 °C in 5 mL medium, after which a bacterial suspension (approximately 10^6 CFU/mL) or fungal suspension *Candida albicans* (ATCC 10231) (approximately 10^3 CFU/mL) in Luria broth or trypticase soy was prepared. Aliquots of 50 µL bacterial or fungal suspension were added to 50 µL of medium containing the cyclic γ-AApeptides for a total volume of 100 µL in each well. The cyclic γ-AApeptides were prepared in PBS buffer in 2 –fold serial dilutions, with the final concentration range of 0.5 to 50 µg/mL. Plates were then incubated at 37 °C for 24 h (for bacteria) or 48h (for *Candida albicans* (ATCC 10231). The lowest concentration at which complete inhibition of bacterial growth (determined by a lack of turbidity) is observed throughout the incubation time is defined as the minimum inhibitory concentration (MIC). The experiments were carried out independently three times in duplicates.

**Lipid depolarization.** The Lipid depolarization of the bacterial cell membrane was conducted using the membrane potential sensitive dye 3, 5’-dipropylthiacarbocyanine iodide (DiSC3-5) that distributes between the cells and the medium depending on the membrane potential gradient. *S. aureus* (ATCC 33592) cells were grown in Luria broth and Trypticase soy broth medium respectively to a mid –logarithmic phase (OD600=0.5-0.6). The bacterial cells were
then collected by centrifugation at 3000 rpm for 10 min and then washed once with buffer (5mM HEPES and 5mM Glucose, pH 7.2). The cells were re-suspended to OD$_{600}$ = 0.05 with 100 mM KCl, 2 µM DiSC$_3$-5, 5 mM HEPES and 5 mM Glucose and were incubated for 30 min at 37°C for maximal dye uptake and fluorescence self-quenching. This bacterial suspension (90 µL) and 10 µL of compound stock solutions or control drug solution were added to white flat bottomed polypropylene 96-well plate (Costar) and incubated at 37°C for 30 min. The fluorescence reading was monitored using the microplate reader (Biotek) at an excitation wavelength of 622 nm and an emission wavelength of 670 nm; the fluorescence increased due to the disruption of cytoplasmic membrane. Valinomycin (final concentration 250 µg/mL) was used as a positive control, and the blank with only cells and dye was used as the background.

**Hemolysis assay.** Freshly drawn human red blood cells (hRBC’s) with additive K$_2$ EDTA (spray-dried) was washed with PBS buffer several times and centrifuged at 1000 g for 10 min until a clear supernatant was observed. The hRBC’s were resuspended in 1 × PBS to get a 5% v/v suspension. Two fold serial dilutions of γ-AAPeptides dissolved in 1× PBS from 250 µg/ml to 1.6 µg/ml were added to a sterile 96-well plate to make up to a total volume of 50 µL in each well. Then 50 µL of 5% v/v hRBC solution was added to make up a total volume of 100 µL in each well. The 0% hemolysis point and 100% hemolysis point were determined in 1 × PBS and 0.2% Triton-X-100, respectively. The plate was then incubated at 37°C for 1 h and centrifuged at 3500 rpm for 10 min. The supernatant (30 µL) was diluted with 100 µL of 1× PBS and absorption was detected by measuring the optical density at 360 nm by Biotek Synergy HT microtiter plate reader. % hemolysis was determined by the following equation:

\[
\% \text{ hemolysis} = \frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{PBS}})}{(\text{Abs}_{\text{Triton}} - \text{Abs}_{\text{PBS}})} \times 100
\]
H10 is the concentration of cyclic γ-AApeptide at which 10% hemolysis was observed. H50 is the concentration of cyclic γ-AApeptide at which 50% hemolysis was observed. The highest concentration tested in the hemolytic assay was 500 µg/mL.

**Fluorescence microscopy.** A double staining method with DAPI (4’, 6-Diamidino-2-phenylindole dihydrochloride, Sigma, >98%) and PI (Propidium iodide, Sigma) as fluorophores was used to visualize and differentiate the viable from the dead *B. subtilis* cells. DAPI as a double stranded DNA binding dye, stains all bacterial cells irrespective of their viability, whereas Ethidium derivatives such as propidium iodide (PI) is capable of passing through only damaged cell membranes and intercalating with the nucleic acids of injured and dead cells to form a bright red fluorescent complex. The cells were first stained with PI and then with DAPI. Bacterial cells were grown until they reached mid-logarithmic phase and then they (∼2 × 10⁶ cells) were incubated with the cyclic γ-AApeptide 5-6 at the concentration of 2 × MIC (10 µg/mL) for 2 h. Then the cells were pelleted by centrifugation at 3000 g for 15 min in an Eppendorf microcentrifuge. The supernatant was then decanted and the cells were washed with 1× PBS for several times and then incubated with PI (5 µg/mL) in dark for 15 min at 0 °C. The excess PI was removed by washing the cells with 1× PBS for several times. Then the cells were incubated with DAPI (10 µg/mL in water) for 15 min in dark at 0 °C. The DAPI solution was removed and cells were washed with 1× PBS for several times. Controls without the addition of 5-6 were performed following the exactly same procedure for bacteria. The bacterial cells were then examined by using the Zeiss Axio Imager Z1 optical microscope with an oil-immersion objective (100×).
5.5 References

(1) Marr, A. K.; Gooderham, W. J.; Hancock, R. E. *Current opinion in pharmacology* 2006, 6, 468.


(24) Robinson, J. A. *Accounts of chemical research* **2008**, *41*, 1278.


APPENDIX A: $^1$H AND $^{13}$C NMR SPECTRA

A1 NMR Spectra of $\gamma$-AApeptide Building Blocks
\[
\text{FmocHN} \quad \text{NBoc} \quad \text{NH} \\
\text{N} \quad \text{O} \quad \text{O} \quad \text{N}\]

\text{\(1^3\)C NMR (CDCl}_3, 100 MHz)
$^{13}$C NMR (CDCl$_3$, 100 MHz)

$^1$H NMR (DMSO-d$_6$, 400 MHz)
FmocHN

$\text{^{13}C NMR (DMSO-}$d$_{6}$, 100 MHz)$

$\text{^{1}H NMR (CDCl}_3$, 400MHz)
FmocHN

$^{13}$C NMR (CDCl$_3$, 100MHz)
A2 NMR Spectra of γ-Apeptides

2.3.1
$^3$H NMR (DMSO-d$_6$, 400 MHz)

H$_2$O  DMSO

2.3.2
$^3$H NMR (DMSO-d$_6$, 400 MHz)

H$_2$O  DMSO
2.3.7 (as TFA salt)

$^1$H NMR (DMSO-$d_6$, 400 MHz)

- DMSO
- H$_2$O
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