Development of Bioactive Peptidomimetics

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Development of Bioactive Peptidomimetics

by

Fengyu She

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Chemistry
College of Arts and Sciences
University of South Florida

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Keywords: γ-AApeptide, antimicrobial agents, protein interaction, OBTC library, left-handed peptidomimetic foldamers

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DEDICATION

To my wife, son, parents, parents-in-law for their understanding and support during my graduate career.
ACKNOWLEDGMENTS

It’s the fact that the support and suggestions from my committee professors, co-works and collaborators encourage and motivate me to finish my Ph.D. studying and the dissertation. My special thanks go to these people who helped me during my graduate study.

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ABSTRACT

Peptidomimetics are synthetic foldamers that are expected to be more resistant to proteolytic degradation and enormous chemodiversity when compared with peptides. To date, the functional peptidomimetics such as β-peptides, peptoids, oligoureas, etc have been developed in many science fields. In order to explore the unnatural foldameric architectures, it’s necessary to discover the novel frameworks and molecular scaffolds. γ-AApeptides were reported to be a new class of peptidomimetics that showed its potential applications in drug discovery and chemical biology. However, a wide function and property of γ-AApeptides need to be further explored. To expand the potential application of γ-AApeptides in biochemistry, I have been focusing on the development of bioactive peptidomimetics, such as exploring the antibacterial activity of helical 1:1 α-sulfono-γ-AA heterogeneous peptides, developing the helical peptidomimetic as the inhibitor of the protein Ras_Raf interaction, identifying the protein/peptide ligands by the novel one-bead-two compound macrocyclic γ-AApeptide screening library, and elucidating the de novo dragon-boat-shaped synthetic foldamers.
CHAPTER 1 INTRODUCTION

1.1 Antimicrobial Peptides and Peptidomimetics

The emerging antibiotic resistance is viewed as one of the omnipresent threat to the global public health field, due to the unrestrained abuse antibiotics. Multi-drug-resistant pathogens including E.coli, P.aeruginosa, Staphylococcus aureus (MRSA), and Staphylococcus epidermidis (MRSA) have been recognized as serious infection to life as they are no longer active to most conventional antibiotics. In order to combat those bacterial pathogens, developing the new generations of antibiotics with novel mechanisms is considered significant efforts.

Antimicrobial peptides (AMPs), also named host-defense peptides (HDPs), have attracted considerable interest. AMPs have short cationic amphiphilic peptides present in almost every living organism, and AMPs are an excellent new class of drug candidates. Although the precise mechanisms of AMPs are still under debating, it is a consensus that AMPs have their ability to generate a globally amphipathic structure, which has hydrophobic and cationic regions, to segregate and disrupt bacterial membranes.

However, The novel antibiotic agent AMPs also possess some intrinsic drawbacks, such as susceptibility to proteolytic degradation, low-to-moderate activity, and poor selectivity. To circumvent those drawbacks, over the last decades, non-natural peptidomimetics that mimic the globally amphipathic structures and mechanism of action of AMPs have been developed and investigated, such as β-peptides, peptoids, acrylamide oligomers, β-turn mimetics, and others. And these antimicrobial agents have been reported extensively. Recently, a new class of peptidomimetics termed “γ-AApeptides” were developed. In this thesis, some of our results in the development of antimicrobial γ-AApeptides that mimic the global structure, function, and mechanism of AMPs were summarized.
1.2 γ-AApeptides

γ-AApeptides are a new class of peptide mimics designed in order to facilitate drug discovery and protein surface mimicry. They are termed “γ-AApeptides”\textsuperscript{31-32} because they contain γ-substituted-N-acylated-N-aminoethyl amino acid units (Fig. 1.1)\textsuperscript{33-34} that derived from γ-chiral PNA backbones. Compared with natural peptides, the repeating unit of γ-AApeptides is comparable to a di-α-peptide residue, and γ-AApeptides are able to accommodate the same number of side functional groups on the backbone of the same length. Moreover, the different side chains, which are introduced through acylation by carboxylic acids, contribute to the limitless diverse functional groups of γ-AApeptides, and their inherent resistance to biodegradation\textsuperscript{31,32,35}.

Figure 1.1 Structures of the α-peptide and the corresponding γ-AApeptide.

1.3 Outline of the Dissertation

This dissertation is discussing the development of bioactive peptidomimetics from the following points:

In chapter 2, helical 1:1 α-sulfoxo-γ-AA heterogeneous peptides with antibacterial activity was discussed.
In chapter 3, helical peptidomimetic that mimicking the α helix structure domain from residue 78 to residue 92 of Raf protein to inhibit the protein Ras_Raf interaction. The finding is critical to further development of these agents as anti-cancer drugs.

Chapter 4 reports One-Bead-Two Compound Macrocyclic γ-AApeptide Screening Library that showed the great potential application in identifying the protein/peptide ligands with its large chemodiversity.

In Chapter 5, de novo dragon-boat-shaped synthetic foldamers has elucidated their folding conformation at the atomic level by X-ray crystal structures of a series of homogeneous L-sulfono-γ-AA foldamers.

1.4 References


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CHAPTER 2 HELICAL 1:1 α/SULFNON−γ-AA HETEROGENEOUS PEPTIDES WITH ANTIBACTERIAL ACTIVITY

Note to Reader

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2.1 Introduction

One of the greatest threats facing in 21st century is antibiotic resistance. Multidrug-resistant pathogens including K. pneumoniae, P. aeruginosa, S. epidermidis and S. aureus have caused significant mortality and health care cost. Alternative therapeutic strategies are in an urgent need to combat drug resistance. Host-defense peptides (HDPs), small natural cationic amphipathic peptides found in most life forms, are being revisited for potential antibiotic development. It is known that the outer leaflet of bacterial membranes is mainly composed of negatively charged phospholipids such as cardiolipin and phosphatidylglycerol. Moreover, other negatively charged molecules are also present on their membranes, including teichoic acids identified in Gram-positive bacteria and lipopolysaccharides in Gram-negative bacteria. As HDPs are positively charged, they could target bacterial membrane through electrostatic attraction. Another important feature of HDPs is that their global structures are amphiphatic containing cationic and hydrophobic domains, even though their secondary structures could be diverse. After docking on bacterial membranes, the hydrophobic patch of HDPs interacts with lipid bilayer, leading to bacterial membrane disruption and cell death. It is known that some HDPs may involve intracellular targets such as ribosomes and nucleic acids, however, their initial interactions with bacterial membranes are still critical for their cellular entry. It should be noted that although no therapeutic strategies are
capable of escaping the mechanism of resistance development, HDPs may be preferable to conventional antibiotics, as the membrane damage caused by HDPs is the biophysical force lacking specific membrane targets, thus it is difficult for bacteria to develop resistance. In contrast, mammalian cell membranes comprise of phosphatidylcholine, sphingomyelin, and cholesterol, most of which are zwitterionic. Negatively charged phospholipids are instead hidden in the inner leaflet of plasma membranes. As a result, the selectivity of HDPs for the bacteria over mammalian cells is considerably high.

However, there are some drawbacks associated with the development of HDP-based antibiotics, including moderate activity, low enzymatic stability, and challenge in optimization. For example, Pexiganan, known as MSI-78, is a synthetic derivative of the helical HDP magainin 2. Although it was in phase II clinical trials for diabetic foot ulcers, it failed eventually due to its moderate antimicrobial activity. Nonetheless, the amphipathic feature of magainin 2, including the crystal structure of a magainin 2 analogue obtained by Gellman’s group, inspires the design of new antimicrobial agents. It is conceived that amphipathic helical peptidomimetics may be able to mimic the mechanism of action of magainin 2, and kill bacteria via membrane disruption. Compared to magainin 2, peptidomimetics are expected to be more resistant to proteolytic degradation, and possess enhanced chemodiversity for optimization. To date, a few classes of helical peptidomimetics, including β-peptides, peptoids, and oligoureas, have been developed as antimicrobial agents that mimic magainin 2. We recently developed a class of helical foldamer termed “sulfono-γ-AApeptides”, as they are derivatives of oligomers N-sulfono-acylated-N-aminoethyl amino acids. Our previous studies suggest that sulfono-γ-AApeptides can form helical structures in solution analogous to α-peptide. Based on the structure, we designed a series of sulfono-γ-AApeptides which are expected to mimic magainin 2 and show broad-spectrum antimicrobial activity.
Figure 2.1 The general chemical structures of α-peptide, sulfono-γ-AA peptide, and 1:1 α/sulfono-γ-AA peptide.

2.2 Results and Discussion

As we recently found that 1:1 α/sulfono-γ-AA heterogeneous peptides (Figure 2.1) also adopt helical conformation in solution,\textsuperscript{23} it is compelling to study antimicrobial activity of this class of peptidomimetics. The heterogeneous scaffold could further enhance the diversity of chemical groups, and therefore potent antimicrobial agents may be identified through optimization.\textsuperscript{24} As this class of heterogeneous peptides were not investigated for their antimicrobial activity previously, our studies may shed light on the design of a new class of antibiotic agents.

In our recent report, our 2D-NMR, small angle X-ray scattering (SAXS), and CD studies suggest that the 1:1 α/sulfono-γ-AA peptides adopt well-defined helical structure in solution (Figure 2.2).\textsuperscript{23} According to the 2D-NMR analysis, this class of heterogeneous peptides project approximately four side chains per turn (Figure 2.2).\textsuperscript{23} In addition, their helical folding propensity appears to be high because the helicity is still discernable for the sequence with the length comparable to that of 9-mer peptide.\textsuperscript{23} Although their helical pitch and diameter might be different from α-peptides, we believe through manipulation of side chains, new helical sequences with cationic amphipathic structures could be properly designed. These amphipathic heterogeneous peptides are in theory should mimic structure and the mechanism of action of helical HDPs.
Figure 2.2 The schematic representation of the helical 1:1 α/sulfono-γ-AA heterogeneous peptides. The number represent the position of the residue in a sequence. a and b denote the chiral side chain and the sulfonamido side chain from a sulfono-γ-AA building block, respectively.

To test our hypothesis, we synthesized a series of 1:1 α/sulfono-γ-AA peptides with varied distribution of cationic charge and hydrophobic groups on the helical scaffold (Figure 2.3), in order to identify and develop new antimicrobial agents. These sequences were then tested for their antimicrobial activity against a few Gram-positive and Gram-negative bacterial pathogens, including multidrug-resistant pathogens.25,26 Hemolytic activity was also obtained to assess their selectivity towards bacteria cells.16

Figure 2.3 Structures of antimicrobial 1:1 α/sulfono-γ-AA heterogeneous peptides. In order to illustrate their cationic charge (colored in blue) and hydrophobic group (colored in red) distribution, the sequences are schematically shown on the helical scaffold.
Figure 2.3 continued Structures of antimicrobial 1:1 α/sulfono-γ-AA heterogeneous peptides. In order to illustrate their cationic charge (colored in blue) and hydrophobic group (colored in red) distribution, the sequences are schematically shown on the helical scaffold.
Figure 2.3 continued Structures of antimicrobial 1:1 α/sulfo-γ-AA heterogeneous peptides. In order to illustrate their cationic charge (colored in blue) and hydrophobic group (colored in red) distribution, the sequences are schematically shown on the helical scaffold.
Table 2.1 The antimicrobial and hemolytic activities of 1:1 α/sulfono-γ-AA peptides. The minimum inhibitory concentration (MIC) is the lowest concentration that completely inhibits microbial growth after 16 h.27,28 HC50 is the concentration causing 50% hemolysis. Pexiganan29,30 is included for comparison.

<table>
<thead>
<tr>
<th>Sequences</th>
<th>MIC (µg/mL)</th>
<th>Hemolysis (HC50, µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MRSA (Gram+)</td>
<td>MRSE (Gram+)</td>
</tr>
<tr>
<td>1</td>
<td>12.5-25</td>
<td>6.3-12.5</td>
</tr>
<tr>
<td>2</td>
<td>6.3</td>
<td>6.3-12.5</td>
</tr>
<tr>
<td>3</td>
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<td>4</td>
<td>6.3</td>
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<td>5</td>
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<td>6</td>
<td>3</td>
<td>3</td>
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<td>7</td>
<td>12.5-25</td>
<td>5.0</td>
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<td>8</td>
<td>&gt;25</td>
<td>6-12</td>
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<td>9</td>
<td>&gt;25</td>
<td>&gt;25</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Pexiganan</td>
<td>16</td>
<td>8</td>
</tr>
</tbody>
</table>

As shown, the rational design of amphipathic sequences led to the discovery of 1:1 α/sulfono-γ-AA heterogeneous peptides with potent antimicrobial activity against a few clinically relevant multidrug-resistant bacterial strains. It is also noted that none of these sequences are toxic, as they all exhibit low hemolytic activity. This is somewhat different from our previously reported homogeneous antimicrobial sulfono-γ-AA peptides,16 which show certain hemolytic activity at high concentrations. In the meantime, it appears that the activity of these peptide-mimetics correlates with their sequences well. As shown in Table 2.1, although sequence 1 and 2 were designed to be amphipathic, and they do show antimicrobial activity against both Gram-positive and Gram-negative bacteria, the activity is quite weak. It is possible that the interaction of sequences with bacterial membrane is not strong since the sequence is not long enough.28,31

This is consistent to our previous findings.16 Compared with 1, sequences 3 and 4, with one more hydrophobic sulfono-γ-AA building block (containing two hydrophobic side chains) at the C-terminus, display enhanced antibacterial activity, particularly towards Gram-positive bacteria MRSA and MRSE. It may suggest that Gram-positive bacteria, which possess one layer of plasma membranes, are more sensitive to hydrophobic interaction of antimicrobial agents compared to Gram-negative bacteria.
containing both inner and outer membranes. Acetylation at the N-terminal end lead to augmented hemolytic activity, similar to our previously reported antimicrobial sulfono-γ-AApeptides. The same trend is observed for sequences 5 and 6. Compared with previous sequences, replacement of the hydrophobic side chain at the position 8b with a cationic side chain lead to both 5 and 6 which exhibit remarkable activity against both Gram-positive and Gram-negative bacteria. As the matter of fact, the most potent sequence 6 show MIC < 5 µg/mL against all tested strains. It should be noted that 6 is even much more potent than Pexiganan. It is interesting that sulfono-γ-AApeptide building blocks containing two cationic side chains are not favorable for antimicrobial activity of this class of sequences, as seen for sequences 7-10. For instance, in the sequence 7, the sulfono-γ-AA residue 6 contains 6a and 6b cationic side chains, whereas the sequence 7 only possesses weak antimicrobial activity, even though it has the same cationic charge/hydrophobicity ratio as the sequence 6. Although the sequence 10 largely regains activity with the cationic side chain introduced at position 8b, it is still not as active as the lead sequence 6. One plausible explanation is that the sulfono-γ-AA residue bearing two adjacent cationic charges may lead to electrostatic repulsion, which destabilizes the helical folding conformation thus preventing the formation of defined amphipathic structure.

Figure 2.4 Krakty plot of 1, 3, 4, 5 and 6 measured in pH 7.4 PBS buffer. The peak around 0.1 Å⁻¹ and the minimum in q range of 0.2-0.4 Å⁻¹ suggest 3 (red), 4 (blue), 5 (green), and 6 (magenta) form well-defined helical structures.
The helicity of lead sequences was also evaluated by SAXS (Small-Angle X-ray Scattering) studies. This is because these sequences are a new class of heterogeneous peptidomimetics, CD (circular dichroism) may provide ambiguous results, since the backbone is different from regular α-peptides. On the other hand, SAXS emerges to be an excellent technique to quickly assess the solution structures of peptides or peptidomimetics. Herein we used Kratky plot to analyze the helicity of the lead 1:1 α/sulfo-γ-AA peptide sequences 1, 3, 4, 5, and 6. The peak around 0.1 Å⁻¹ and the minimum in q range of 0.2-0.4 Å⁻¹ indicate the presence of the helix-rich globular protein, whereas a slope lacking maximum and minimum in middle q range suggests the existence of a random coil (Figure 6.8). As shown in Figure 2.4, the sequence 1 only displays limited helicity, probably due to its short length and thus incapability of helical folding. All other sequences adopt well-defined helical structures in solution, since they all show characteristic maximum around 0.1 Å⁻¹ and minimum in q range of 0.2-0.4 Å⁻¹. Consistent with our previous observation, N-terminal acetylation of the sequence could enhance helical folding propensity, as evidenced by stronger helicity of 3 and 5 compared to 4 and 6, respectively. It also suggests that the acetylation could deteriorate antimicrobial activity, as 6 is much more potent than 5.

We hypothesized that 1:1 α/sulfo-γ-AA heterogeneous peptides exert their activity by mimicking HDPs such as magainin, because they adopt amphipathic structures which lead to disruption of bacterial membranes. Therefore, fluorescence microscopy was employed to assess the ability of the peptide 6 to compromise membranes of S. aureus. The membrane permeable dye 4',6-diamidino-2-phenylindole (DAPI), and the DNA intercalator propidium iodide (PI) (Figure 2.5), were used in the study. DAPI stain membranes of both dead and live cells with blue fluorescence, but PI could only fluoresce in red color when cell membranes are damaged. As shown in Figure 2.5, bacterial cells in the control group are only visible under DAPI channel. In contrast, incubation of bacteria with 6 led to the visibility of bacteria under both PI and DAPI channel, suggesting the membranes of S. aureus were compromised.
Figure 2.5 Fluorescence micrographs of S. aureus that are treated or not treated with 10 µg/mL 1:1 α/sulfono-γ-AA peptide 6 for 2 h. a1, control, no treatment, DAPI stained; a2, control, no treatment, PI stained. b1, treatment with 6, DAPI stained; b2, treatment with 6, PI stained. Scale bar: 20 µm.

It is believed that HDPs exert bactericidal action rapidly through the disruption of bacterial membranes.30 Since 1:1 α/sulfono-γ-AA heterogeneous peptides were designed to mimic HDPs, their action were also expected to be fast. Thus, the time kill study was carried out to test the efficiency of 6 to kill MRSA. Compound 6 was studied at four, eight, and sixteen-fold of the MIC. Cell viability was determined by colony count in agar plates in a time-dependent fashion at above-mentioned concentrations (Figure 2.6). In all cases, 6 could eradicate bacteria completely in two hours, suggesting the mechanism of action is analogous to that of magainin.

Figure 2.6 Time-kill curves of 6 for MRSA. The killing activity was monitored for the first 2 h. The concentrations were 4 ×MIC, 8 ×MIC, and 16 ×MIC, respectively.
2.3 Conclusions

To summarize, we identified the first example of 1:1 \( \alpha/\text{sulfono-\( \gamma \)-AA heterogeneous peptide}
foldamers with potent antimicrobial activity towards multidrug-resistant Gram-positive and Gram-
negative bacteria. These sequences adopt defined amphipathic helical structures and are likely to kill
bacteria via disruption of bacterial membranes-based time-killing studies and fluorescence microscopy.
As their mechanism of action is similar to that of HDPs, their further development may lead to a new
class of helical foldamer combating emerging antibiotic-resistant pathogens. In addition, the effective
design of this class of antimicrobial agents suggest the potential of 1:1 \( \alpha/\text{sulfono-\( \gamma \)-AA heterogeneous}
peptides as a new class of foldamer for the interrogation of other important biological targets such as
protein-protein interactions.

2.4 Experimental Section

2.4.1 General information

All Fmoc protected \( \alpha \)-amino acids and Rink-amide resin (0.7 mmol/g, 200-400 mesh) were
purchased from Chem-Impex International, Inc. Other solvents and reagents were purchased from either
Sigma-Aldrich or Fisher Scientific and used without further purification. Solid-phase synthesis of 1:1
\( \alpha/\text{sulfono-\( \gamma \)-AA peptides were carried out in the peptide synthesis vessel on a Burrell Wrist-Action shaker.
The sequences were analyzed and purified on a Waters Breeze 2 HPLC system and lyophilized on a
Labcono lyophilizer. The molecular weight of the heterogeneous peptides was obtained on an Applied
Biosystems 4700 Proteomics Analyzer.

2.4.2 Solid phase synthesis of 1

The synthesis was conducted on 100 mg Rink amide resin (0.7 mmol/g) following our reported
protocol. The resin was swelled in DMF for 1 h before use. The Fmoc protecting group was removed by
shaking the resin in 3 mL 20% Piperidine/DMF for 15 min (x 2). The resin was then washed with DCM
(x 3) and DMF (x 3). A premixed solution of Fmoc-Lys (Boc) -OH (3 equiv.), HOBt (6 equiv.), and DIC
(6 equiv.) in 2 mL DMF was added to the resin. The mixture was allowed to shake for 4 h. After being
washed with DCM and DMF, the Fmoc protecting group was removed following the above-mentioned protocol. Next, the N-alloc γ-AApeptide building block was coupled on the resin under the same coupling condition. The introduction of sulfonamide moieties was achieved by reacting the resin with Pd(PPh₃)₄ (8 mg, 0.007 mmol) and Me₂NH·BH₃ (25 mg, 0.42 mmol) in 3 mL DCM for 10 min (× 2), followed by the reaction with the corresponding sulfonyl chlorides (4 equiv.) and DIPEA (6 equiv.) in 3mL DCM for 30 min (× 2). The reaction cycles were repeated until the desired sequence was assembled on the resin. The resin was then washed with DCM and dried in vacuo. Peptides on the resin were cleaved in a 4 mL vial using the cocktail of 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 desired fraction was collected and lyophilized and confirmed on an Applied Biosystems 4700 Proteomics Analyzer. Finally, the desired fraction was collected and lyophilized.

2.4.3 Solid phase synthesis of the sequences 2-10

The synthesis of the sequences 2-10 was carried out following the same synthetic protocol for 1.

2.5 References

(3) WHO, 2014.


CHAPTER 3 HELICAL PEPTIDOMIMETIC INHIBITOR OF PROTEIN RAS_RAF INTERACTION

3.1 Introduction

The Ras protein is a 21 kDa guanine nucleotide-binding protein, which couple extracellular, growth-promoting signals to intracellular effector pathways. It plays important roles in the control of vital cellular processes such as terminal differentiation, proliferation, and survival\textsuperscript{1,2}. The Ras protein work as molecular switches cycles between an inactive GDP-bound form (Ras-GDP) and an active GTP-bound form (Ras-GTP)\textsuperscript{3,4,5} (Fig. 3.1a). The two-nucleotide binding state of Ras proteins is balanced by the guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). In the cellular context, the collection of GEFs such as SOS, which stimulated by the activated transmembrane receptors upstream of Ras protein, act as the catalysis for the exchange of Ras-GDP to active Ras-GTP that mainly in the regions switch I and II \textsuperscript{5} (Fig. 3.1b). In the active GTP-bound state, Ras proteins could interact with a variety of effector proteins, such as Raf, PI3K, and Ral-GDS.

Figure 3.1 The protein Ras active mechanism and structure. (a) Ras cycles between an inactive GDP- and an active GTP-bound state. GEFs and GAPs enhance the intrinsically low nucleotide exchange and GTPase reaction, respectively. GTP-bound Ras proteins interact with a variety of effectors, such as Raf, PI3K and Ral-GDS, to trigger downstream signaling. In addition, the subcellular localization of Ras
proteins influences their biological activity. (b) Crystal structures of H-Ras in complex with GDP (left; purple, Protein Data Bank (PDB) code 1Q21) and ‘GTP’, a nonhydrolyzable analog of GTP (right; orange, PDB code 5P21). Switch I and II regions are highlighted in red. Residues (G12, G13 and Q61) that are most frequently mutated are shown. Mutations at positions G12 and G13 in proximity to the nucleotide-binding pocket sterically hinder the formation of the transition state required for hydrolysis101. RQ61 is directly involved in the stabilization of a crucial water molecule in the active site, rendering a mutation of this residue fatal for the hydrolysis reaction102,103. The coordinated Mg^{2+} is shown in dark gray.


It’s reported that about 20-30% of all human tumors are found in the mutations of Ras proto-oncogenes, and current therapies do not get effective response6,7. The amino acid positions G12, G13, and Q61 are found to be the main mutations in oncogenic Ras, and the mutations will impair intrinsic and GAP-mediated GTPase function. This can lead to an accumulation of GTP-bound Ras, which could promote constitutive pro-survival and pro-proliferative signaling. Therefore, mutated Ras proteins have been widely identified as one of potential anticancer targets8 and it’s necessary to develop more potent and specific K-Ras inhibitors for any practical therapeutic value13.

Currently, there are three most successful strategies in targeting Ras GTPase signaling. The first is to prevent the formation of Ras-GTP complex by locking Ras in an inactive state. The second is to develop different inhibitions of Ras-effector interactions. The third is to apply different approaches that impair correct Ras localization to prevent oncogenic signaling (Fig. 3.2). This project tends to apply the stapled peptides to inhibit the Ras-effector interactions.

![Figure 3.2 The three most successful strategies toward inhibition of mutant Ras. *](image-url)
Raf protein, a 74 kDa serine/threonine protein kinase, is one of the efforts that responsible for the signaling pathway from receptors to the nucleus. The residue and crystallographic studying of Ras protein have been reported earlier. It was proved out that Raf protein has five main β sheets and two main α helix structure from residue 57 to residue 131 (Fig. 3.3 & Fig. 3.4). By taking advantage of the chemical shift in NMR, the large chemical shift changes upon binding to Ras protein are indicated with filled circles. This purpose of this project is to inhibit the Ras_Raf interactions by mimicking the α helix structure domain from residue 78 to residue 92 of Raf protein.

**Figure 3.3** Two views of the structure of the rat Raf-1 RBD.
3.2 Results and Discussion

Development of Ras-effector interaction inhibitors has gained a lot of interesting. Based on the previous reported studying about the binding interface between Ras and Raf protein, a series of hydrocarbon-stapled α-peptides (Fig. 3.5) was designed by mimicking the helix structure domain from residue 78 to residue 92 of Raf protein (LHDCLMKALKVRGLQ). The stapled peptides with stabilized secondary structure might provide unprecedented opportunities as the renaissance in drug discovery.

Figure 3.4 A comparison of the present chemical shift differences results. *


Figure 3.5 The hydrocarbon-stapled α-peptides sequences and their brief expression.
Figure 3.5 continued. The hydrocarbon-stapled α-peptides sequences and their brief expression.
Figure 3.5 continued. The hydrocarbon-stapled α-peptides sequences and their brief expression.
Figure 3.5 continued The hydrocarbon-stapled α-peptides sequences and their brief expression.

In the GST-RBD pull-down assays that calibrated with Dr. Said M. Sebti in Moffitt Cancer Center and Research Institute, if the staple peptides could inhibit the Ras_Raf interaction, then the Raf protein could be washed away from the plate following by showing light even no trace on the plate. The current results show that FY-A-100-1 has the potent at inhibiting the ability of GST-RBD to bind mt G12D KRas from lysates of NIH-3T3 Cells that ectopically express G12D KRas (Fig 3.6.). The FY-A-90-1A, which has a CH₂ shorter side chain than FY-A-100-1, as well as FY-A-100-2, shows much less potent demonstrating the importance of lysine 85. FY-A-90-1B, FY-A-90-2A, and FY-A-90-2B had little activity compared to DMSO control.
3.3 Conclusion

Helical Peptidomimetics is one of the extremely potent candidates to inhibit the protein Ras_Raf interaction, and they have the ability to bind to Ras protein and prevent interactions with downstream target proteins such as Raf protein. The finding of helical peptidomimetics inhibitor of Ras_Raf interaction is critical to further development of these agents as anti-cancer drugs.

3.4 Experimental Section

3.4.1 General Information

All Fmoc protected α-amino acids and Rink-amide resin (0.6 mmol/g, 200-400 mesh) were purchased from Chem-Impex International, Inc. Other solvents and reagents were purchased from either Sigma-Aldrich or Fisher Scientific and used without further purification. Solid-phase synthesis of α-peptides was carried out in the peptide synthesis vessel on a Burrell Wrist-Action shaker. The sequences were analyzed and purified on a Waters Breeze 2 HPLC system and lyophilized on a Labcono lyophilizer. The molecular weight of the α-peptides was verified on an Applied Biosystems 4700 Proteomics Analyzer.

3.4.2 Solid Phase Synthesis of α-peptides

The synthesis was conducted on 200 mg Rink amide resin (0.6 mmol/g) following the standard solid phase peptide synthesis protocol. The resin was swelled in DMF for 10 min before use. The Fmoc protecting group was removed by shaking the resin in 3 mL 20% Piperidine in DMF for 15 min (× 2). The resin was then washed with DCM (× 3) and DMF (× 3). A premixed solution of Fmoc-amino acids
with protection group (3 equiv.), HOBt (6 equiv.), and DIC (6 equiv.) in 2 mL DMF was added to the resin. The mixture was allowed to shake for 1.5 h. After being washed with DCM and DMF, the Fmoc protecting group was removed following the above-mentioned protocol. The same reaction cycle was repeated until the desired sequence was assembled on the resin. For the regular cyclic sequence, the special protect groups Allyl and Alloc were removed by reacting the resin with Pd(PPh₃)₄ (8 mg, 0.007 mmol) and Me₂NH·BH₃ (25 mg, 0.42 mmol) in 3 mL DCM for 30 min (× 2), and then the cyclization was created by using PyBop/HOBT/DIPEA (4:4:8) equiv. ratio in DMF. For the olefin metathesis, the cyclization was generated according to the advantage of the Grubbs’ first-generation catalyst in DCE. After the requisite cyclization, the resin was then washed with DCM and dried in vacuo. α-Peptides on the resin were cleaved in the peptide vessel using the cocktail reagent K of 82.5% TFA, 5% Phenol, 5% H₂O, 5% Thioanisole, and 2.5% 1,2-ethanedithiol (v:v:v:v) 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 traces were detected at 215 nm. The desired fraction was collected and lyophilized and confirmed on an Applied Biosystems 4700 Proteomics Analyzer (Table 1). Finally, the desired fraction was collected and lyophilized.

Table 3.1 MALDI data of all α-peptides sequences.

<table>
<thead>
<tr>
<th>Sequences</th>
<th>MW (Theoretical)</th>
<th>MW (found)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FY-A-90-1A</td>
<td>1780.19</td>
<td>1779.66 (MALDI)</td>
</tr>
<tr>
<td>FY-A-90-1B</td>
<td>1798.21</td>
<td>1821.71 (MALDI + Na⁺)</td>
</tr>
<tr>
<td>FY-A-90-2A</td>
<td>1723.09</td>
<td>1722.67 (MALDI)</td>
</tr>
<tr>
<td>FY-A-90-2B</td>
<td>1741.11</td>
<td>1764.70 (MALDI + Na⁺)</td>
</tr>
<tr>
<td>FY-A-100-1</td>
<td>1794.22</td>
<td>1794.11 (MALDI)</td>
</tr>
<tr>
<td>FY-A-100-2</td>
<td>1737.12</td>
<td>1737.28 (MALDI)</td>
</tr>
<tr>
<td>FY-A-134-A</td>
<td>1752.20</td>
<td>1752.17 (MALDI)</td>
</tr>
<tr>
<td>FY-A-134-B</td>
<td>1552.93</td>
<td>1553.03 (MALDI)</td>
</tr>
<tr>
<td>FY-A-134-C</td>
<td>1833.34</td>
<td>1833.37 (MALDI)</td>
</tr>
<tr>
<td>FY-A-134-D</td>
<td>1805.28</td>
<td>1805.38 (MALDI)</td>
</tr>
</tbody>
</table>

3.4.3 Inhibition of Ras_Raf RBD association by α-peptides

The protein-protein interactions were tested by Glutathione-S-transferase (GST) fusion protein pull-down assay, also called "The enzyme-linked immunosorbent assay (ELISA) Test", which is a simple technique to test the interaction between a tagged protein and another protein\textsuperscript{12}. Ras_Raf protein was immobilized on a solid support (usually a polystyrene microtiter plate). After the protein was immobilized, the detection antibody was added, forming a complex with the protein. The detection antibody can itself be detected by a secondary antibody HRP that was linked to an enzyme through bio-conjugation. Between each step, the plate was typically washed with a mild detergent solution to remove any proteins or antibodies that were non-specifically bound. After the final washing step, the plate was developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of Ras_Raf binding in the sample.
Figure 3.8 The GST-RDB pull-down Assays.

3.5 Reference


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13. Wu, X.; Upadhyaya, P.; Villalona-Calero, M. A.; Briesewitz, R.; Pei, D., Inhibition of Ras- 
effector interactions by cyclic peptides. _MedChemComm_ **2013**, *4* (2), 378-382
4.1 Introduction

Nowadays, identifying molecular ligands that can recognize peptides or proteins targets with high specificity and affinity is necessary for the development of modern chemical biology and biomedical sciences, as it could help to understand the relationship between structure and function of peptides and generate to be the potential therapeutic agents. One of the powerful tools for ligand screening in medicinal chemistry is combinatorial chemistry and it has a diverse library of compounds for target screening. Peptides are viewed as the natural building blocks for the combinatorial library screening as they have module chemical diversity and favorable protein binding capabilities. Among them, macrocyclic peptides are widely applied for recognizing the interactions between ligand and receptor due to its ability in enhancing conformational restriction and binding affinity.

Non-nature sequence-specific peptidomimetics that mimic the primary structures of peptides and possess a variety of diverse functional side chains have attracted great interesting by researchers. The improvement of protease resistance, chemodiversity and bioavailability have reached by the generation of peptidomimetics when comparing with natural peptides. The examples of peptidomimetics such as β-peptides, peptoids, α-aminoxypeptides, α/β-peptides, azapeptides, and others had already make some great progress. However, it is very rare in the field of investigating the protein-ligand identification by peptidomimetic combinatorial library, especially the macrocyclic peptidomimetic combinatorial library.

γ-AApeptides, oligomers of γ-substituted-N-acylated-N-aminoethyl amino acid, have developed as a new class of peptidomimetic by our lab recently base on the backbone structure of the chiral PNA. It’s reported that γ-AApeptides are ideal candidates for molecular probes or therapeutic agents
as they resistant to proteolytic degradation, possess cellular translocation capability and improved chemodiversity.\textsuperscript{16} Certainly, these characteristics have been identified by the previously one-bead-one-compound (OBOC) linear $\gamma$-AApeptides combinatorial libraries with the ability in inhibiting $\alpha\beta$ aggregation and disrupting STAT3/DNA interaction.\textsuperscript{17,18} So the macrocyclic combinatorial library of $\gamma$-AApeptides is expected to have more rigid conformation and could be more functional in the active ligands identification.

4.2 Results and Discussion

4.2.1 Design of the cyclic library

The thioether-bridge has proven to be highly efficient in cyclization, which leads the cyclic peptides with rigidified conformational freedom and enhanced metabolic stability. The novel cyclic $\gamma$-AApeptides is taking advantage of the features to get synthesized and tested against target proteins. In the cyclic $\gamma$-AApeptides library, Dmt (4,4ˈ-dimethoxy-trityl) protected mercaptoethyl carbonyl group is introduced to the secondary amine in the first $\gamma$-AApeptide building block on the solid phase (Figure 4.1). Another 4-(bromomethyl)benzoyl group protected the N-terminal amino group after the other three $\gamma$-AApeptide building blocks attached in the out layer. Following, the $\gamma$-AApeptide could be cyclized by the SN2 reaction after the Dmt group removing.

4.2.2 Library diversity and decoding

To increase the integrality of the cyclic library, the different types of side chains were involved, such as the hydrophobic, cationic, and negatively charged side chains. In addition, based on the structural nature of $\gamma$-AApeptides, there are four chiral side chains coming from the five different N-Alloc protected $\gamma$-AApeptide building blocks (R, Figure 4.1). After deprotecting the Alloc group, seven different carboxylic acids or acyl chlorides were introduced by acylating the secondary amino group of the three side chains (Rˈ, Figure 4.1). So, by using the split and pool method, the expected theoretical diversity of the cyclic library is 320,000 (Figure 4.1), and in three copies, 960,000 beads were used for the preparation of the library.
Figure 4.1 Synthesis of the thioether bridged one-bead-two compound macrocyclic \(\gamma\)-AApeptide library.

This novel cyclic library was more feasible than the linear \(\gamma\)-AApeptide library\(^{17,18}\) in decoding part, which was analyzed by MS/MS. Here, the analyzable decoding sequences in one-bead-two-compound (OBTC) library were desired on the same beads with coding ligands and the decoding peptides that consisting \(\alpha\)-amino acids were unambiguous to be figured out by MS/MS pattern. Dde ((1-(4,4-dimethyl-2,6-dioxacyclohexylidene) ethyl) protected \(\alpha\)-amino acids were developed and used as the effective approach in coding peptides. The condition for deprotecting Dde was very mild by using NH2OH·HCl and imidazole\(^{19}\) in this thioether-bridged cyclic \(\gamma\)-AApeptide library.

4.2.3 Synthesis of the library

In this OBTC cyclic \(\gamma\)-AApeptide combinatorial library, TentaGel beads (200-250 \(\mu\)m; 1.5 nmol/bead) were soaked overnight in water and then washed by DCM/\(\text{Et}_2\)O (Figure 4.1). Following the free amine in the outer surface of the expecting separated two layers TentaGel beads coupled with 0.5 equiv. of di-tert-butyl decarboxylate (Boc\(_2\)O), and the interior layers of the beads remained in the water
phase. After washing with DMF, the interior of the beads reacted with Met amino acid, which facilitated coding peptide cleavage after cyanogen bromide (CNBr) treatment. Then the beads were split into five equal positions and respectively coupled with five different Dde protected amino acids after removing the Fmoc protecting group. Until now, the first coding tag γ-AApeptide on the outer layer can be decoded by the Met and Dde protected amino acids. Later, the Boc group in the outer layer was removed by TFA, and then the five different Alloc protecting γ-AApeptide building blocks were coupled. Next, the Dmt protected 3-mercaptopropanoic acid was attached to the secondary amino group after the Alloc group was deprotected by Pd(PPh₃)₄ and (CH₃)₂NH·BH₃. The Dde group was removed and the beads were pooled and split into five positions again. Then the second Dde protected amino acids was coupled to the decoding tag for the second γ-AApeptide building blocks on the outer layer of the beads. Those steps were repeated three more times. Since the nature of the γ-AApeptide building, which has two side chains, the two Dde protected amino acids were used to decode each building block. Finally, the N-terminal γ-AApeptides on the outer layer was capped by the 4-(bromomethyl) benzoyl chloride after removing the Fmoc protecting group, following the Dmt group with be removed from the thiol linker by 2% TFA in DCM. Under the presence of the ammonium carbonate ((NH₄)₂CO₃), the γ-AApeptides on the outer were cyclized. Then the other side chains finally deprotected in 94% TFA, 2% triisopropylsilane, 2% water and 2% thioanisole (v:v:v:v).

4.2.4 Library Screening and binding affinity

The cyclic γ-AApeptide combinatorial library was moved forward to examine its potential for identification of valuable biological ligands. GST-Shp2 is a classical and non-receptor PTP encoded gene, and it consists of two SH2 domains, a PTP domain and a C-terminal region (Figure 4.2). Cancer-associated Shp2 mutations are prevalent in the interface between the N-SH2 domain and the PTP domains. E76 located in the N-SH2 domain is the most frequently mutated residue in human cancer, such as the lung cancer. So, it is interesting to identify ligands from the macrocyclic γ-AApeptide library that binds to Shp2E76 with high affinity.
Before the library screening, the TentaGel beads after the synthesis were swelled in DMF for 1h, then washed by Tris buffer for five times, following the beads were equilibrated in Tris buffer overnight at room temperature, and then incubated by the buffer (1% BSA in Tris buffer with the E. coli lysate) for 1h to block the wide non-specific binding.

The library screening consists of two parts: pre-screening and screening. The purpose of the pre-screening is to remove any suspicious nonspecific binding (Figure 4.3). The blocked beads were incubated with the GST tag monoclonal antibody (8-326) with Alexa Fluor 555 for 2 h at room temperature. After washing with Tris buffer three times, the beads were transferred into a 6-well plate and observed under the fluorescence microscope for picking up the red fluorescence beads.

The rest of the beads were washed by Tris buffer and treated with 8 M guanidine hydrochloride at room temperature for 1 h to remove any bound proteins. Then the guanidine hydrochloride was washed away subsequently by Tri buffer 5 times, water 5 times, DMF 5 times, and acetonitrile 5 times. The beads were later incubated in DMF 1h and equilibrated like before for real library screen.

After incubating with 10% BSA buffer and washed by Tris buffer, the beads were incubated with GST-Shp2E76K protein at the concentration of 14.28 nM for 4 h at room temperature. After being washed by Tris buffer, the library beads were incubated with 10 µL GST-antibody with Alexa Fluor 555 for 2 h at room temperature in Tris buffer. The beads were washed, then transferred into a-well plate and observed.
under the fluorescence microscope for picking up the red fluorescence beads, which were viewed as the positive hits (Figure 4.3). Fortunately, 32 possible beads were picked up from the fluorescence microscope. Those beads were denatured by treating with 8 M guanidine hydrochloride at room temperature for 1 h, and washed by Tris buffer 5 times, water 5 times, DMF 5 times, and acetonitrile 5 times. After drying by themselves from acetonitrile, CNBr was used to cleave the decoding peptides from the inner layer of the beads. Later, the peptides were analyzed by the tandem MS/MS of MALDI. However, only 4 of 32 hits can be determined unambiguously (Figure 4.4). Maybe the rest of the hits had bad quality due to the synthesis or cleavage step. Once the four different peptide structures were figured out exactly, the peptides were reverse synthesized.

**Figure 4.3** Pre-Screening and Screening of the $\gamma$-AApeptide library.
Figure 4.4 Structures of the four hits.

For the GST-Shp2E76 binding affinity testing, Dr. Jie Wu in the University of Oklahoma Health Sciences Center helped with the assay. Luckily, FS-C-118-GST-SHP2-E76K-3 showed around 30% inhibition at 100 µM (Figure 4.5).

4.3 Conclusion

In summary, the new class of macrocyclic peptidomimetic combinatorial library has been explored and developed. By taking advantage of the novel γ-AAApeptide build blocks, this one-bead-two-compound (OBTC) library has great potential application in identifying the protein/peptide ligands with
its large chemodiversity. When compared with the linear peptide library, the thioether bridged macrocyclic γ-AApeptide enhanced the conformational rigidity of the backbones. In addition, the new decoding method of Dde peptides facilitated the structure analysis. The strategy of this library could be used as a new platform for screening against various targets in the future.

Figure 4.5 GST-Shp2E76 binding affinity testing.

4.4 Experimental Section

4.4.1 General Information.

Fmoc-protected amino acids were purchased from Chem-Impex (Wood Dale, IL). TentaGel resin (0.23 mmol/g) was purchased from RAPP Polymer (Tubingen, Germany). Rink Amide-MBHA resin (0.55 mmol/g) was purchased from GL Biochem (Shanghai, China). 1-Hydroxybenzotriazole wetted with no less than 20% wt. water (HOBt), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 5,5-Dimethyl-1,3-cyclohexanedione and 4,4'-Dimethoxytrityl Chloride were purchased from Oakwood Chemical (Estill,
4-(Bromomethyl)benzoic acid was purchased from AK Scientific (Union City, CA). 3-Mercaptopropionic Acid was purchased from TCI (Tokyo, Japan). Solid phase synthesis was carried 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 purity of the compounds was determined by analytical HPLC. Masses of γ-AApeptides and the MS/MS analysis were obtained on an Applied Biosystems 4700 Proteomics Analyzer. GST Tag monoclonal antibody (8-326), Alexa Fluor, 555 was purchased from ThermoFisher Scientific (Waltham, MA). GST-Shp2E76K protein was provided by our calibrator Dr. Jie Wu in the University of Oklahoma Health Sciences Center (Oklahoma City, OK). All solvents and other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) and were used without further purification.

4.4.2 Synthesis of the Dmt protected mercaptopropionic acid.

4,4′-Dimethoxytrityl chloride (9.58 g, 28.27 mmol) was dissolved in 80 mL of CH₂Cl₂ with 3-mercaptopropionic Acid (1.642 mL, 18.84 mmol). The pyridine (15.18ml, 0.188mol) was slowly added to the solution in the ice bath. The solution was stirred at zero degrees for 30min. After that, the mixture solution was extracted with 0.5M HCl with ethyl acetate (100 mL ×3). The organic layer was dried over anhydrous Na₂SO₄, and the solvent was evaporated. The residue was purified by flash column chromatography (hexane/ethyl acetate 4:1) to afford the desired product as a light-yellow oil (90% yield).

4.4.3 Synthesis of the 4-(Bromomethyl)benzoyl Chloride

The 4-(bromomethyl) benzoic acid (5 g, 23.25 mmol) was dissolved in 100mL DCM and mixed with 3.378 mL of thionyl chloride, then the solution was refluxed overnight. The excess thionyl chloride was removed under reduced pressure to afford the desired product as a white solid and directly use without purification (85% yield)²²

4.4.4 Synthesis of 2-Acetyl-5,5-dimethylcyclohexane-1,3-dione

5,5-dimethylcyclohexane-1,3-dione (30 g, 0.214 mol), N, N-diisopropylethylamine (82mL, 0.47 mol), 4-dimethylaminopyridine (2.61g, 21.4 mmol), and 300 mL of DCM were added into 1000 mL round bottom flask. The mixture was stirred at room temperature and then acetic anhydride (60.58mL,
0.642 mol) was added. The reaction stirred overnight. The solvent was evaporated, and the mixture was purified by flash column chromatography (hexane/ethyl acetate 3:1) to afford the 2-acetyl-5,5-dimethylcyclohexane-1,3-dione as a yellowish oil (24.16 g, yield 62%).

**4.4.5 Synthesis of Dde Protected Amino Acids**

The L-amino acid (1 equiv.) was suspended in a solution of the 2-acetyl-5,5-dimethylcyclohexane-1,3-dione (1.3 equiv.) in absolute ethanol (∼50 mL). Triethylamine (1.5 equiv.) was added, and the reaction mixture was refluxed for 24 h. The resulting yellow solution was cooled and concentrated under reduced pressure. The residue was dissolved in CH$_2$Cl$_2$ (50 mL) and washed with 1 M HCl (50 mL × 2). The organic layer was dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. Addition of Et$_2$O (∼40 mL) to the residue resulted in the immediate white precipitate, which was filtered and washed with cold Et2O to afford the title compound as an off-white crystalline solid (∼50%).

**4.4.6 One-bead-two-compound (OBTC) library preparation**

TentaGel NH$_2$ resin (6.261g, 1.44 mmol, 960,000 beads) with split and pool method at room temperature were used for the one-bead-two-compound γ-AApeptide library. Based on the previously reported protocols, the formation of bi-layers on the beads could be achieved. First, the resin was soaked in water overnight. After being equally transferred into the two 100 mL peptide reaction vessels, the resin was drained and washed by 1:1 (v/v) DCM/Et$_2$O. Theoretically, each bead of the library had two layers with the free amine. A solution of (Boc)$_2$O (0.5 equiv.) in 1:1 (v/v) DCM/Et$_2$O was added to protect the free amine in the outer layer. The mixture was shaken on the Burrell Wrist-Action shaker for 3h, then washed by DCM (×3) and DMF (×3). Next, Fmoc-Met-OH (0.5 equiv.) was coupled with the free amine in the inner layer of the resin twice under the coupling reagent HOBt (2 equiv.) and DIC (2 equiv.). The Fmoc group was removed by 20% (v/v) piperidine in DMF (15 min ×2) and the beads were split into 5 portions, which were reacted with the 3 equiv. Dde-Ala-OH, Dde-Phe-OH, Dde-Leu-OH, Dde-Val-OH, and Dde-Glu(OBn)-OH in 5 peptide synthesis vessels respectively in the presence of PyBop (5 equiv.) and NEM (11 equiv.) in DMF for 4 h twice. Subsequently, the Boc protecting group on the outer layer
was removed by using 94% TFA, 2% TIS (triisopropylsilane), 2% H₂O and 2% Thioanisole, and the exposed amine was coupled with the corresponding 3 equiv. γ-AAPeptide building blocks under the coupling reagents HOBt/DIC (6:6 equiv.) in DMF for 4 h twice. Following, the Alloc protecting group on the γ-AAPeptide building blocks was removed by Pd(PPh₃)₄ (8 mg, 0.007 mmol) and Me₂NH·BH₃ (25 mg, 0.42 mmol) in 3 mL DCM (10 min × 2), and the Dmt protected mercaptopropionic acid was added to react with the secondary amine under the coupling reagents HOBt/DIC (6:6 equiv.) overnight three times. Later, all the beads were pooled and mixed thoroughly and then split into 5 portions again. The Dde group of the coding peptides in the inner layer was removed by 1.25 g (0.180 mmol) of NH₂OH·HCl, 0.918 g (0.135 mmol) of imidazole in 5 mL NMP, and 1 mL DCM before use. And 5 Dde protected amino acids were added to react as the previous step. Then the Fmoc group of the γ-AAPeptides in the outer layer was removed and the sequence was coupled with desired γ-AAPeptide building blocks. The beads were pooled and split again, and the synthetic cycle was repeated three more times. The last Dde protecting group was remained in the decoding layer, while the Fmoc group of the outer layer was removed then the beads were coupled with the 4-(bromomethyl) benzoyl chloride under DIPEA (5 equiv.) in DCM for 1.5 h three times. Later, the Dmt protecting group was removed by 2% TFA, 2% triisopropylsilane and 96% DCM for 2 min (×10) until the deprotecting solution became colorless. The cyclization of γ-AAPeptide was achieved by adding the solution of (NH₄)₂CO₃ (10 equiv.) in 1:1 (v/v) DMF/H₂O to the resin and shaking the mixture for overnight 3 times. Finally, the other protecting group on the sidechains were removed with 94% TFA, 2% triisopropylsilane, 2% H₂O and 2% Thioanisole for 1 h 3 times.

4.4.7 Library Screening

The GST-Shp2E76K protein was used as a target for the combinatorial library screening. The beads were screened and picked up under a Zeiss inverted fluorescence microscope installed with a 10x43HE filter. To avoid any possible nonspecific binding, GST-Shp2E76K protein and antibodies solution were all made in 1% BSA/TBST blocking buffer.

The TentaGel beads were swelled in DMF for 1 h. then washed by Tris buffer for five times, following the beads were equilibrated in Tris buffer overnight at room temperature, and then incubated by
the buffer (1% BSA in Tris buffer with the E. coli lysate) for 1h to block the wide non-specific binding.

4.4.8 Pre-Screening

The blocked beads were incubated with the GST tag monoclonal antibody (8-326) with Alexa Fluor 555 for 2 h at room temperature. After washing with Tris buffer three times, the beads were transferred into a 6-well plate to be observed under the Zeiss inverted fluorescence microscope installed with the 10×43HE filter. The beads emitting red fluorescence were picked up and excluded from formal screening.

The rest of the beads were washed by Tris buffer and treated with 8 M guanidine hydrochloride at room temperature for 1 h to remove any bound proteins. Then the guanidine hydrochloride was washed away subsequently by Tri buffer 5 times, water 5 times, DMF 5 times, and acetonitrile 5 times. The beads were later incubated in DMF 1h and equilibrated in Tris buffer overnight.

4.4.9 Screening:

After incubating with 1% BSA buffer and washed by Tris buffer 5 times, the beads were incubated with GST-Shp2E76K protein at the concentration of 14.28 nM for 4 h at room temperature. After being washed by Tris buffer, the library beads were incubated with 10 µL GST-antibody with Alexa Fluor 555 for 2 h at room temperature in Tris buffer. The beads were washed, then transferred into the 6-well plate and observed under the Zeiss inverted fluorescence microscope for picking up the red fluorescence beads, which were viewed as the positive hits.

Each hit was transferred to an Eppendorf microtube and denatured in 100 µL 8M guanidine•HCl for 1h at room temperature, respectively. The bead was rinsed with Tris buffer 5 × 5 min, water 5 × 5 min, DMF 5 × 5 min, and ACN 5 × 5 min. At last, the resin was placed in ACN overnight in each open microtube to allow the ACN evaporate. The decoding peptide was cleavage by 50 mg cyanogen bromide (CNBr) in the cocktail of 5:4:1 (v:v:v) of ACN: Acetic acid: H2O overnight at room temperature. The cleavage solution was then evaporated, and the cleaved peptide was dissolved in ACN: H2O (1:1) and subject to MALDI TOF-TOF analysis.
4.4.10 Synthesis of cyclic γ-AApeptides hits

After structures of hits were determined by MALDI MS/MS (Figure 4.6), the hits were re-synthesized on the Rink Amide resin by the desired building blocks under coupling reagent HOBt/DIC. The crude was purified by the Waters HPLC system and confirmed by Applied Biosystems 4700 Proteomics Analyzer.

AApeptide 1 Dde-Ala-Ala-Ala-Phe-Lys-Phe-Val-H

FS-C-114-GST-SHP2-E76K-

Figure 4.6 The determination of decoding sequences

AApeptide 2 Dde-Val-Leu-Phe-Lys-Phe-Ala-Ala-H

FS-C-116-GST-SHP2-E76K-2
AApeptide 3 Dde-Phe-Glu-Phe-Leu-Ala-Ala-Leu-H
FS-C-118-GST-SHP2-E76K-3

AApeptide 4 Dde-Ala-Glu-Phe-Lys-Phe-Glu-Phe-H
FS-C-120-GST-SHP2-E76K-4

Figure 4.6 continued The determination of decoding sequences
Figure 4.7 HPLC trace of the hits

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CHAPTER 5: *DE NOVO* DRAGON-BOAT-SHAPED SYNTHETIC FOLDAMERS

Note to Reader

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5.1 Introduction

Foldamers,1-2 which are a class of synthetic unnatural oligomers with defined and predictable structures, are capable of mimicking or complementing the three-dimensional structure and function of natural biopolymers such as proteins, peptides and nucleic acids. Endowed with enhanced functional diversity and improved resistance to proteolytic hydrolysis, foldamers hold promise in biomedical and material application.3-9 In the past two decades, synthetic oligomeric architectures such as \( \beta \)-peptides,11-12 peptoids,13-14 oligoureas,15-16 \( \beta \)-peptoids,17 aza-peptides,18-19 Aib foldamers,20 aromatic amide foldamers,21-22 oligoprolne,23-24 and others, have been characterized by crystallographic analysis, leading to various applications in molecular self-assembly and recognition.25 However, as natural macromolecules exhibit an endless set of folding structure and function, continuing exploration of unnatural foldameric architecture with new frameworks and molecular scaffolds is still in an urgent need.26 In particular, creation of new helical foldameric scaffolds with de novo helical propensity as well as elaborately designed residues and molecular entities capable of modulating specific biological processes or yielding new functional materials, is still a central goal of foldamer development.

\( \gamma \)-AApeptides (N-acetylated-N-aminoethyl amino acid oligomers, stemming from the chiral PNA backbone27-28) are receiving increasing attention as backbones of a new class of peptidomimetics, owing
to their enormous chemical diversity imparted by arbitrary side chains and their resistance to proteolytic degradation (Figure 5.1a). More recently, we have reported the crystal structures of de novo heterogeneous 2:1 α/D-sulfo-γ-AA hybrid oligomers capable of adopting right-handed 4.5 helical conformations, demonstrating that peptidomimetics containing γ-AA peptide units can be unique heterogeneous foldamers. However, the crystal structures of homogeneous sulfo-γ-AA peptides, which would be much more significant by elucidating the folding conformation of sulfo-γ-AA peptides, were not yet obtained. It could be an important addition to the foldamer development if homogeneous sulfo-γ-AA peptides, rather than heterogeneous hybrids, are identified to form defined folding structures. Although attempts were made to investigate the folding propensity of sulfo-γ-AA peptides by 2D NMR, the structure generated based on NOE-restrained molecular dynamics remains ambiguous since the helical handedness could not be derived, and the hydrogen-bonding pattern is inconclusive due to dynamic solution structures. Atomic level of structures is highly demanded to precisely elucidate the helicity and hydrogen-bonding pattern of this new helix. Herein, we report the first crystal structures of homogeneous L-sulfo-γ-AA peptide oligomers. High-resolution X-ray crystal structures of these homogeneous foldamers unambiguously delineate their sequence-structure relationships, revealing unprecedentedly well-folded, left-handed helical structures of the entire set of oligomers. These results provide a structural basis for designing de novo foldameric structure of this type as ordered biopolymers and potential therapeutic agents in the future.

5.2 Results and Discussion

5.2.1 Sequence design.

The homogeneous L-sulfo-γ-AA oligomers were initiated with five building units, with or without acetylation on the N-terminus. To exclude the potential impact of side chains on the folding propensity, initially L-methyl-sulfo-γ-AA with chlorobenzenesulfonyl group was chosen (Figure 5.1b). All together four oligomers (oligomers 1a–2b) were synthesized and feasibly obtained from solid phase Fmoc chemistry according to protocol reported previously.
To test the generality of forming helical foldamers, the side chains on the oligomeric sequences include both cationic NH₂, anionic COOH, as well as hydrophobic 4-chlorobenzenesulfonyl residues (oligomers 3a–5a). A sequence containing only methyl side chains (6a) was also synthesized.

**Figure 5.1** (a) General structures of α-peptides, L-γ-AApeptides, L-sulfono-γ-AApeptides. (b), (c), (d), (e) Homogeneous L-sulfono-γ-AA peptidic oligomers prepared for structural and spectroscopic evaluation in this study.

### 5.2.2 High-resolution crystallographic studies of oligomer 1a, 3a, 4b and 6a.

After a series of attempts, we obtained single crystals of 1a suitable for X-ray diffraction analysis at resolution of 1.1 Å from a mixture of acetonitrile and H₂O, although trials upon other homogeneous oligomers in similar solvent system failed. Surprisingly, unlike classic α-helices or recently developed 4.5 helices based on heterogeneous backbones, this homogeneous L-sulfono-γ-AA oligomer adopts an unprecedented left-handed helical structure with unanimous 4-helical fold, with radius of 2.8 Å and helical pitch of 5.1 Å (Figure 5.2a). The helix possesses four distinct helical faces, with side chains aligned at 90° intervals to form a rectangular shape when viewed down the helix axis. The side chains on the four helical faces perfectly line on top of each other, somewhat resembling dragon boat paddling. In addition to unusual folding parameters, the handedness of the crystal structure was surprising, since L-peptides are well known to generally adopt right-handed helical conformations. Moreover, oligomer 1a
shows highly consistent 14-hydrogen bonding pattern (Figure 5.2b), formed between the N-H group of the L-sulfono-γ-AA residue and the C=O group of the L-sulfono-γ-AA three residues later, namely i → i + 3 hydrogen bonding with a distance of 2.2 Å (H···O distance). This lead to the formation of a macrodipole with partial positive charge at the C-terminus and partial negative charge at the N-terminus, which is opposite to the macrodipole formed in canonical α-helix. Due to its unique hydrogen bonding pattern and arrangement of side chains, the name 4₁₄-helix is designated, indicating that four side chains (three residues) being included in the pseudo helical loop, and 14 atoms being involved in the ring formed by the intramolecular hydrogen bonds. The 4₁₄-helix is less tightly packed compared with 3₁₀-helix, α-helix, and even π-helix, while also possessing an unprecedented ordered C₂ symmetric helix. In crystal packing, the individual helical segments are arranged in a hydrogen-bonding driven head-to-tail manner to give regularly elongated helical treads (Figure 5.2c).

![Diagram](image1)

**Figure 5.2.** Single crystal structure of oligomer 1a. (a) Side and top views of crystal structure of 1a. Hydrogen bonding is shown in red. (b) The intramolecular 14-hydrogen-bonding pattern of oligomer 1a detected in the crystal structure. (c) Crystal packing or oligomer 1a viewed perpendicular and then down to the helix axis.
Direct comparison of the 414 helix with other types of natural helical peptides (Table 5.1) unveils that it resembles the left-handed counterpart of the right-handed π-helix, with similar helical pitch and diameter; however, the projection of side chains are completely distinct from that of π-helix, demonstrating a de novo scaffold. Similar to other helices, the side chains of 414-helix project away from the helical axis, but it is of particular interest that both the chiral side chains at γ-position and the sulfono side chains on the L-sulfono-γ-AA residues form a C₂ symmetric rectangle structure when viewed along the peptide axis. Both type of side chains points toward the C-terminus due to the (S)-configuration of the sulfono-γ-AA residues in the scaffold.

Table 5.1. Parameters of helical structures found in proteins and 414-helix.

<table>
<thead>
<tr>
<th>Secondary Structure</th>
<th>Handedness</th>
<th>Helical Pitch p (Å)</th>
<th>Radius of Helix r (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helix</td>
<td>Right-handed</td>
<td>5.4</td>
<td>2.3</td>
</tr>
<tr>
<td>310-helix</td>
<td>Right-handed</td>
<td>6.0</td>
<td>1.9</td>
</tr>
<tr>
<td>π-helix</td>
<td>Right-handed</td>
<td>5.0</td>
<td>2.8</td>
</tr>
<tr>
<td>4.516-14-helix</td>
<td>Right-handed</td>
<td>5.1</td>
<td>2.6</td>
</tr>
<tr>
<td>414-helix</td>
<td>Left-handed</td>
<td>5.1</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Notably, we were able to obtain more crystalline structures when incorporating both cationic NH₂ and anionic COOH groups on the chiral side chains. From solvent CHCl₃/MeOH and acetonitrile, oligomers 3a and 4b (Figure 5.3), which bear with both amino and carboxylic groups, with or without acetyl capping group at N-terminus respectively, were crystalized and resolved by high-resolution single-crystal X-ray crystallography with resolution of 1.0 Å. Oligomer 4b shows slightly different packing mode as the adjacent parallel helices are packing in different way, however, the crystal structure of oligomer 4b reveals exactly the same left-handed 414-helix, with the same helical pitch and diameter. Compared with 1a, oligomer 4b bears one more L-sulfono-γ-AA residue in length and one Lys and two Glu chiral side chains, which confer it with much better solubility in the majority of solvents, even in the presence of 10% H₂O. Further attempts furnished the crystal structure of oligomer 3a, which has one more L-sulfono-γ-AA residues in length with a Lys chiral side chain compared to 4b. Consistent with the helical conformation of 4b, oligomer 3a (Figure 5.3c) also adopts the same left-handed 414-helix.
configuration.

Taken together, the helical propensity data, with strong intramolecular hydrogen-bonding and the highly ordered, tight packing of the helical and side chains, indicates that the $4_{14}$-helix provides an unprecedented opportunity to establish a new class of secondary structures.

Furthermore, oligomer 6a, bearing just methyl side chains, also crystallized from CH$_3$CN/MeOH/CH$_2$Cl$_2$ with suitable quality for X-ray crystallography analysis. As anticipated, the crystal analysis revealed a left-handed helical structure, with the same helical pitch and diameter as those of the other oligomers (Figure 5.4). The ability of sulfono-$\gamma$-AApeptides to form ubiquitous left-handed helices regardless of side chain identity further demonstrates their unanimous folding propensity and augments their potential for applications in biological functional materials or self-assembly architectures.

Figure 5.3. Single crystal structure of oligomer 4b. (a) Side and top views of crystals 4b. (b) Crystal packing or oligomer 4b viewed perpendicular and then down to the helix axis. c) Sequence structure of oligomer 3a. (d) Sequence structure of oligomer 4b.
Figure 5.4. Comparison between crystal structures of oligomer 1a (a), 3a (b), 4b (c), and 6a (d). (e) Sequence structure of oligomer 6a.

Table 5.2. Typical torsion angles in helical structures 1a, 3a, 4b, and 6a based on single crystals.

<table>
<thead>
<tr>
<th>Angle</th>
<th>( \phi )</th>
<th>( \theta )</th>
<th>( \eta )</th>
<th>( \xi )</th>
<th>( \psi )</th>
<th>( \phi' )</th>
<th>( \psi' )</th>
<th>( \eta' )</th>
<th>( \xi' )</th>
<th>( \psi' )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>-137.9°</td>
<td>59.0°</td>
<td>-122.0°</td>
<td>97.0°</td>
<td>-136.0°</td>
<td>-137.9°</td>
<td>59.0°</td>
<td>-122.7°</td>
<td>97.0°</td>
<td>-136.0°</td>
</tr>
<tr>
<td>3a</td>
<td>-137.5°</td>
<td>70.0°</td>
<td>-121.4°</td>
<td>87.8°</td>
<td>-144.0°</td>
<td>-139.6°</td>
<td>66.5°</td>
<td>-124.2°</td>
<td>100.1°</td>
<td>-142.1°</td>
</tr>
<tr>
<td>4b</td>
<td>-136.9°</td>
<td>66.2°</td>
<td>-121.2°</td>
<td>86.1°</td>
<td>-142.8°</td>
<td>-138.0°</td>
<td>61.9°</td>
<td>-116.1°</td>
<td>88.3°</td>
<td>-146.3°</td>
</tr>
<tr>
<td>6a</td>
<td>-140.8°</td>
<td>67.2°</td>
<td>-115.5°</td>
<td>86.5°</td>
<td>-141.3°</td>
<td>-140.8°</td>
<td>67.2°</td>
<td>-115.5°</td>
<td>86.5°</td>
<td>-141.3°</td>
</tr>
</tbody>
</table>

The average backbone torsion angles \( \phi, \theta, \eta, \xi, \psi, \phi', \theta', \eta', \xi', \) and \( \psi' \) in each helical loop are quite unanimous across all structures (Table 5.2). The torsion angles of adjacent L-sulfono-\( \gamma \)-AA residues in each oligomer are also very close. Specifically, the homogeneous L-sulfono-\( \gamma \)-AA endow unique backbone torsion angles \( \phi = (-138+/-2°), \quad \theta = (66+/-5°), \quad \eta = (-120+/-5°), \quad \xi = (92+/-5°), \quad \) and \( \psi = (-141+/-5°), \) which are apparently distinct from that of heterogeneous \( \alpha/D \)-sulfono-\( \gamma \)-AA (2:1 pattern) foldamer (\( \phi, \theta, \eta, \).
ξ, ψ = 117+/−10°, -77+/−4°, 79+/−2°, 60+/−4°, -169+/−2°) with the incorporation of α-helices. The torsion angles of these residues also reasonably differ from α-helices, β-sheets and the previously reported natural or synthetic peptides. These unique torsion angles, strong hydrogen bonding and unique side chain arrangement, could pave the way to the creation of finite helical bundles in materials or the rational design of helical structure targeting membrane receptors or protein-protein interactions. While that work is beyond the scope of the current study, we have started working on such applications.

5.2.3 NMR studies of oligomer 4b.

To further investigate the solution conformation for these types of homogeneous foldamers, oligomer 4b was selected as a representative example for 2D NMR experiments. gDQFCOSY, zTOCSY, and NOESY spectra were recorded at a concentration of 5 mM in CD3OH at 10 °C in order to assign the backbone protons.

As shown in Figure 5.5, three types of long-range NOEs among protons on the scaffold were detected: (a) i, i+1 NOEs, correlations from methylene/γ-CH protons of L-sulfono-γ-AA, and amide protons on adjacent residue; (b) i, i+2 NOEs, correlations between amide protons of L-sulfono-γ-AA and methylene/γ-CH protons two residues down either direction of the oligomer; (c) chimeric i, i+3 NOEs, correlations between α protons of the L-sulfono-γ-AA and amide protons of the L-sulfono-γ-AA three residues earlier. These detected NOEs are consistent with the i → i + 3 hydrogen bonding pattern found in crystal structures and suggest that this homogeneous L-sulfono-γ-AA peptide foldamer is helical in methanol.

![Figure 5.5](image.png)

Figure 5.5. Summary of detected NOESY crosspeaks of 5 mM oligomer 4b between protons on nonadjacent residues in CD3OH (10 °C). Three types of NOEs are displayed in different color. Each L-sulfono-γ-AA unit is considered as two residues, since the L-sulfono-γ-AA building block is equal to two α amino acid in length.
5.2.4 Circular dichroism studies.

To further evaluate the helical propensity in solution, circular dichroism (CD) spectroscopy of sequences was conducted. As shown in Figure 5.6a, five oligomers, including a homogeneous sequence with the same side chains (1a), sequences with both cationic and anionic side chains (3a, 4b, and 5a), and an oligomer without any aromatic side chains (6a), revealed a positive cotton effect at 215–218 nm, the intensity of which is both length and side chain dependent. The hexamer 1a displayed a maximum at 218 nm, while the ellipticity of oligomers possessing NH₂/COOH sidechains (5a, heptamer 4b, and octamer 3a) also consistently exhibit the same maximum. The CD signal of 6a was considerably weaker than other oligomers due to its lack of phenyl substituents on the sulfonyl residues, nonetheless, similar pattern of cotton effect was observed. The stability of secondary structures could be slightly affected by solvents as trifluoroethanol (TFE) and acetonitrile, methanol or H₂O (Figure 6b and 6c), but overall the helical structures were very stable in various solvents. It should be noted that the peaks at 240 nm are not indicative of secondary structures, similar to what we have demonstrated for hybrid oligomers before. Only hexamer 1a displayed a dominant minimum at 240 nm, which is most likely due to the presence of homogenous aromatic residues in the side chains.

![Figure 5.6](image_url)

**Figure 5.6.** (a) CD spectra of oligomers 1a, 3a, 4b, 5a, and 6a (80 μM) measured at room temperature in TFE. (b) CD spectra of oligomer 1a (80 μM) in various solvents at room temperature. (c) CD spectra of 4b (80 μM) in various solvents at room temperature.

The helical stability of oligomer 1a and 4b at various concentrations was also investigated in
solution. As shown in Figure 7.7a and 7.7b, the CD spectra of oligomer 1a and 4a revealed consistently helical conformation from 6.25 to 100 μM. Furthermore, we also conducted the stability of the oligomers at various temperatures. Figure 7.7c and 7.7d indicate that only a slight decrease of signal intensity took place over the 5–55 °C temperature range, where 2 nm of red shifts were witnessed when temperature increased over 50 °C. These results indicated that this type of oligomer is highly stable in both diluted solution and at elevated temperatures.

Figure 5.7. (a) CD spectra of oligomer 1a in TFE at various concentrations (6.25–100 μM) at room temperature. (b) CD spectra of oligomer 4b (80 μM) in TFE at various temperatures. (c) CD spectra of 1a in TFE at various concentrations (6.25–100 μM) at room temperature. (d) CD spectra of 4b (80 μM) in TFE at various temperatures.

5.2.5 Molecular dynamics simulations.

To investigate the preference of homogeneous L-sulfonyl-γ-AA foldamers to adopt left-handed helical configurations in solution, molecular dynamics simulations were performed for 4b in the left-handed helical configuration of the X-ray structure as well as a modelled right-handed helical
conformation. These configurations were solvated in methanol, and multiple simulations were run for each system. As shown in Figure 5.8, the left-handed systems retained their helical integrity throughout the 1 μs production runs, whereas the helical structure was quickly lost in the right-handed system (Figure 5.8a, 5.8b). The average heavy atom backbone root mean squared deviations (rmsd) of the left-handed 4b systems were significantly lower than that of the right-handed systems (~1 versus ~5 Å). While the left-handed 4b remained helical, fraying of the flexible termini was intermittently observed during short intervals of the simulations (Figure 5.8c; fraying of the backbone shown in magenta). These partial unfolding events were characterized by higher rmsd, and a loss of hydrogen bonding in the termini.

The number of backbone hydrogen bonds as a function of rmsd is shown in Figure 8a–8b. For the left-handed helix 4b, all 7 hydrogen bonds were made for configurations with a rmsd below 1.0 Å. These were the dominant structures in the simulations (Figure 5.8c). A loss of one hydrogen bond occurred for rmsd values between 1.0 and 2.0 Å; these corresponded to configurations with a frayed N or C terminus. While fraying of the N terminus was more frequently observed than fraying of the C terminus, both were minor species (Figure 5.8c). A hydrogen bond at each terminus was lost when the rmsd surpassed 2.0 Å, which rarely occurred in the left-handed helical systems. The loss of hydrogen bonding and helical structure of the termini was reversible, reflected by a quick return of all rmsd peaks back to sub 1.0 Å values. In contrast, almost no hydrogen bonds were made in the right-handed system due to its inability to retain a helical structure (Figure 5.8b, 5.8d).

The preference of 4b to form a left-handed rather than right-handed helix could also be traced by energy decompositions of the minimized structures. It was found that the dihedral energy (~26 kcal/mol) contributed greatest of all bonded and nonbonded energy terms to the total potential energy difference (~84 kcal/mol). Differences in side chain dihedrals were found to be insignificant, and for the backbone, the torsion angles about the Cγ-N bond possessed the greatest total differential stability (~19 kcal/mol). The single largest contributor was the Cβ-Cγ-N-C dihedral term, which contributed 1.8 kcal/mol per dihedral or ~14 kcal/mol in total to the potential energy difference. This dihedral angle was on average -17° in the minimized left-handed helix, and 105° in the minimized right-handed structure. The Cβ-Cγ-N-
C dihedral is located at a critical position since it mediates interactions between the α carbon side chain and the backbone. For example, the carbonyl oxygen and the methyl were staggered in the minimized left-handed helix, while eclipsed in the minimized right-handed helix.

![Figure 5.8](image)

**Figure 5.8.** Molecular dynamics simulations of 4b. (a) Backbone rmsd with the energy minimized structure versus the number of backbone hydrogen bonds for the left-handed helical configuration; standard deviations shown as bars. (b) Backbone rmsd with the energy minimized structure versus the number of backbone hydrogen bonds for the right-handed helical configuration. (c) Rmsd with the energy minimized structure versus time for the left-handed helical configuration. For clarity, no hydrogen atoms are shown except for the amide hydrogen atom of the backbone (in light blue). Helical backbone is shown in black, while unfolded backbone is shown in magenta. (d) Rmsd of the right-handed helical configuration with the energy minimized structure versus time. (e) Energy profile of the dihedral angle connecting the β-carbon and carbonyl carbon atoms. The energy minimized atomic structures around the relevant torsion angle are highlighted.

### 5.3 Conclusions

We report an unprecedented left-handed helical secondary structure of homogeneous L-sulfono-γ-AA foldamers. Based on the AApeptides scaffold, these foldamers were synthesized by incrementally increasing the lengths of readily accessible L-sulfono-γ-AA units. A series of crystals adopted well-defined left-handed helical conformations with a 4,14-helix pattern in the solid state. The presence of this
secondary structure in solution was supported by CD spectroscopic data in various solvents, NMR, and molecular dynamic (MD) simulations. The preference of left-handed helix formation was rationalized by MD simulations in methanol. By showing that sulfono-γ-AApeptides form well-defined left-handed helices, our study greatly expands the repertoire of AApeptides for the design of biopolymers, materials and self-assembly architectures.

5.4 References


127, 11966–11968.


35. Baldauf, C., Günther, R., Hofmann, H-J. Mixed Helices—A General Folding Pattern in


6.1 Supporting Information for Chapter 2

6.1.1 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. Other solvents and reagents were purchased from either Sigma-Aldrich or Fisher Scientific and used without further purification. Solid-phase synthesis of 1:1 α/sulfono-γ-AA peptides were carried out in the peptide synthesis vessel on a Burrell Wrist-Action shaker. The sequences were analyzed and purified on a Waters Breeze 2 HPLC system and lyophilized on a Labconco lyophilizer. The molecular weight of the heterogeneous peptides was obtained on an Applied Biosystems 4700 Proteomics Analyzer.

6.1.2 Syntheses of 1:1 α/sulfono-γ-AA peptides 1-10.

6.1.2.1 Synthesis of the sequence 1.

**Figure 6.1.** Synthesis of the α/sulfono-γ-AA peptide 1.
6.1.2.2 Solid phase synthesis of 1.

The synthesis was conducted on 100 mg Rink amide resin (0.7 mmol/g) following our reported protocol. The resin was swelled in DMF for 1 h before use. The Fmoc protecting group was removed by shaking the resin in 3 mL 20% Piperidine/DMF for 15 min (x 2). The resin was then washed with DCM (x 3) and DMF (x 3). A premixed solution of Fmoc-Lys (Boc)-OH (3 equiv.), HOBt (6 equiv.), and DIC (6 equiv.) in 2 mL DMF was added to the resin. The mixture was allowed to shake for 4 h. After being washed with DCM and DMF, the Fmoc protecting group was removed following the abovementioned protocol. Next, the N-alloc γ-AApeptide building block was coupled on the resin under the same coupling condition. The introduction of sulfonamide moieties was achieved by reacting the resin 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), followed by the reaction with the corresponding sulfonyl chlorides (4 equiv.) and DIPEA (6 equiv.) in 3mL DCM for 30 min (x2). The reaction cycles were repeated until the desired sequence was assembled on the resin. The resin was then washed with DCM and dried in vacuo. Peptides on the resin were cleaved in a 4 mL vial using the cocktail of 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 desired fraction was collected and lyophilized and confirmed on an Applied Biosystems 4700 Proteomics Analyzer. Finally, the desired fraction was collected and lyophilized.

6.1.2.3 Solid phase synthesis of the sequences 2-10.

The synthesis of the sequences 2-10 was carried out following the same synthetic protocol for 1.
Table 6.1. MALDI data of sequences 1-10

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<th>MW (found)</th>
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Figure 6.2. HPLC analytic traces for 1-10.
Figure 6.2 continued. HPLC analytic traces for 1-10.
6.1.3. Minimum inhibitory concentrations (MICs) against bacteria.

Four bacteria strains including Methicillin-resistant *S. aureus* (MRSA, ATCC 33591), Methicillin-resistant *S. epidermidis* (MRSE, RP62A), *P. aeruginosa* (ATCC27853), and *K. pneumoniae* (ATCC 13383) were used to in the experiment for the test of antimicrobial activities of the 1:1 α/sulfono-γ-AA heterogeneous peptides. Briefly, single colonies of these bacteria were inoculated into 3 mL TSB medium and allowed to grow overnight. Then the bacteria were re-inoculated at 1:100 dilution and grew to mid-
logarithmic phase, from which 1 x 10^6 CFU/mL suspension was made. To aliquots of 50 µL of bacteria suspension 50 µL of serial dilutions of peptides starting from 25 µg/mL were added. After incubation at 37 °C for 16 h, the mixtures were read for their absorption at 600 nm wavelength by a Biotek Synergy HT microtiter plate reader. The MICs were determined as the lowest concentration at which the bacteria growth was completely inhibited. Results were repeated three times with duplicates each time.

6.1.4. Hemolytic assays.

The freshly drawn, K2EDTA treated human red blood cells (hRBCs) were washed with 1X PBS buffer, and centrifuged at 1000 g for 10 min. The step was repeated several times until the supernatant was clear. After the supernatant was removed, the RBCs were diluted into 5% v/v suspension, which was subsequently incubated with equal volume of heterogeneous peptides at different concentrations at 37 °C for 1 h. The mixture was centrifuged at 3500 rpm for 10 min. Next, 30 µL of the supernatant was transferred into 100 µL PBS, and the absorbance was read at 540 nm using a Biotek Synergy TH plate reader. The hemolysis percentage was calculated by the formula % hemolysis = (Abssample-AbsPBS) / (AbsTriton-AbsPBS) x100%. Positive controls were RBCs with 2 %Triton X-100, and negative controls were RBCs with 1X PBS. Results were repeated three times with duplicates each time.

6.1.5. Fluorescence microscopy.

Fluorescence microscopy was used to assess the ability of the peptidomimetic to damage bacterial membranes. Two dyes 4’’,6-diamidino-2-phenylindole dihydrochloride (DAPI) and propidium iodide (PI), were employed in the experiment. DAPI is capable of staining bacteria cells regardless of their viabilities, whereas PI can only stain cells with damaged membranes because it is a DNA intercalator. After the bacteria grew to mid-logarithmic phase, they were incubated with peptides at 37 °C for 2 h. The mixture was centrifuged at 5000 g for 15 min, and the cell pellets were collected and washed, and were subsequently incubated with PI (5 µg/mL) then with DAPI (10 µg/mL) for 15 min on ice. Controls were bacteria without peptides treatment. After the final wash, 10 µL of the samples were placed on chamber slides and observed under Zeiss Axio Image Zloptical microscope using 100X oil-immersion objective.
6.1.6 Small-Angle X-ray Scattering (SAXS).

Small-angle x-ray scattering measurements were conducted at Beamline 12ID-B of Advanced Photon Sources (APS) at Argonne National Laboratory. The wavelength ($\lambda$) of x-ray radiation was set as 0.886 Å. Scattered x-ray intensities were measured using a Pilatus 2M detector (DECTRIS Ltd). The sample-to-detector distance was set such that the detecting range of momentum transfer $\theta = 4\pi \sin\theta/\lambda$, where $2\theta$ is the scattering angle] of SAXS experiments was 0.01-1.0 Å$^{-1}$. To reduce the radiation damage, a flow cell was used, and the exposure time was set to 1-2 seconds. The x-ray beam with size of 0.07 × 0.20 mm$^2$, was adjusted to pass through the centers of the capillaries for every measurement. In order to obtain good signal-to-noise ratios, twenty images were taken for each sample and buffer. The 2-D scattering images were converted to 1-D SAXS curves, i.e., intensity ($I(q)$) vs $q$, through azimuthally averaging after solid angle correction and then normalizing with the intensity of the transmitted x-ray beam, using the software package developed at beamline 12ID-B.

![Image](A)

**Figure 6.3.** Kratky plots for peptide models with various conformations and lysozyme. (A) Structural bundle models taken from pdb 2FEJ, amino acid residues 277–297, total 38 conformations. Only partial of the peptide (15 amino acids) was used in the calculation in (B). (B) X-ray scattering profiles, i.e., $I(q)$ vs $q$, were first calculated from structural models using program SolX, then displayed in Kratky plots, i.e., $q^2I(q)$ vs $q$. Every curve for peptides is an average of scattering profiles of all conformers. Peptide sequences used: red, 277-291; cyan, 279-293; blue, 281-295; green, 283-297; magenta, 15 amino acids all in random coil conformation generated with molecular dynamics simulation. Well-folded macromolecules with MW large mass (>10kDa) often exhibits a “Bell”-shape Krakty profile at low $q$ range, like lysozyme (MW=14kDa, orange curve). The peak position is a function of molecular size. The plateau at 0.2-0.4 Å$^{-1}$ in magenta curve arises from the random coil like conformation. The peaks in red and cyan curves around 0.3 Å$^{-1}$ arise from the helical folding.
6.1.7 Time kill study.

The kinetics of bacteria killing by the lead peptide 6 were studied. The bacteria MRSA was allowed to grow to mid-logarithmic phase in TSB medium, then the culture was diluted into 10^6 CFU/mL suspensions. The suspensions were incubated with different concentrations of heterogeneous peptides for 10 min, 30 min, 1 h and 2 h respectively. The mixtures were diluted into 10^2 to 10^4 times and then spread on TSB agar plates. After overnight incubation at 37 °C, the colonies on the plates were counted and graphed against incubation time.

![Time-kill curves of 6 for MRSA. The killing activity was monitored for the first 2 h. The concentrations were 4 ×MIC, 8 ×MIC, and 16 ×MIC.](image)

6.2 Supporting Information for Chapter 5

6.2.1 Chemistry

Chemicals and solvents were purchased from Fisher or Aldrich and used without further purification. Fmoc protected α-amino acids and Rink-amide resin (0.6 mmol/g, 200–400 mesh) were purchased from Chem-Impex International, Inc. Solid-phase synthesis of the compounds was carried out in the peptide synthesis vessel on a Burrell Wrist-Action shaker. All products were analyzed and purified on a Waters Breeze 2 HPLC system installed with both analytic module (1 mL/min) and preparative module (16 mL/min), by employing a method using 20–100% linear gradient of solvent B (0.1% TFA in
acetonitrile) in solvent A (0.1% TFA in water) over 50 min, followed by 100% solvent B over 10 min. The pure fractions were collected and lyophilized on a Labconco lyophilizer. The final products were verified by MALDI-MS on an Applied Biosystems 4700 proteomics analyzer.

**Figure 6.5.** General synthetic route to prepare oligomers 1a–6a.

**Figure 6.6.** Sequence structures of all studied oligomers.
Figure 6.6. continued Sequence structures of all studied oligomers.
Figure 6.6. continued Sequence structures of all studied oligomers.

The sulfono-γ-AApeptide building block Sulfono-γ-AApeptide1, Sulfono-γ-AApeptide2, and Sulfono-γ-AApeptide3 were synthesized as previously reported. Oligomers 1a–6a were synthesized on 100 mg Rink-amide resin, as shown in previously reported procedures.

General synthetic procedure of solid phase synthesis of oligomers. The solid phase synthesis was conducted Rink amide resin (0.06 mmol/g) for each oligomer under ambient temperature at atmosphere pressure. 200 mg of resin was soaked in DMF for 0.5 h before use, followed by treatment with 20% piperidine/DMF solution (2 mL) for 10 min (×2) to remove Fmoc protecting group, afterwards washed with DCM (three times) and DMF (three times). A premixed solution of sulfono-γ-AApeptide building block (2 equiv.), HOBt (6 equiv.), and DIC (6 equiv.) in 2 mL DMF was added to the resin and shaken for 4 h to complete the coupling reaction. After wash with DCM and DMF, the resin was treated with 20% piperidine/DMF solution for 10 min (×2). Then, the second building block was coupled on the resin under the same abovementioned reaction conditions. The reaction cycles were repeated until the desired oligomers were synthesized and then remove Fmoc protecting group under the same conditions shown above. The beads were spitted into two parts. Half of the beads were treated with acetic anhydride (0.5 mL) in pyridine (2 mL) (15 min×2) to cap N-terminus of the sequence, another half of the beads were used directly to the cleavage with uncapped N-terminus. The solid-supported oligomers were cleaved from beads by treatment with TFA/DCM (4 mL, 1:1, v/v) for 2 h. The cleavage solution was collected, and the beads was washed with TFA (1 mL×2) and DCM (3 mL×3). The solution was combined and evaporated under nitrogen flow to give the crude, which was analyzed and purified by Water HPLC system, at the 1 mL/min and 16 mL/min flow rate for analytic and preparative HPLC respectively. The gradient eluting method of 20% to 100% of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water)
over 50 min was performed. All the oligomers were obtained with moderate yield (21.13–37.61%) after prep-HPLC purification.

**Sulfono-γ-AApeptide1**  
$^1$H NMR (400 MHz, d₆-DMSO) $\delta$ 12.71 (brs, 1H), 7.81 (d, $J = 7.6$ Hz, 2H), 7.72 (d, $J = 8.4$ Hz, 2H), 7.60 (d, $J = 7.6$ Hz, 2H), 7.53 (d, $J = 8.4$ Hz, 2H), 7.33 (td, $J = 7.2$, 2.4 Hz, 2H), 7.24 (td, $J = 7.2$, 2.4 Hz, 2H), 7.10 (d, $J = 8.4$ Hz, 1H), 4.10–4.17 (m, 3H), 3.99, 3.93 (ABq, JAB = 18.4 Hz, 2H), 3.62–3.69 (m, 1H), 3.12 (qd, $J = 14.4$, 6.0 Hz, 2H), 0.96 (d, $H = 6.0$ Hz, 3H). 13C NMR (100 MHz, d₆-DMSO) $\delta$ 170.3, 155.8, 144.2 (2C), 141.1 (2C), 138.8, 138.0, 129.6 (2C), 129.3 (2C), 128.0 (2C), 127.4 (2C), 125.5 (2C), 120.5 (2C), 65.6, 52.9, 49.0, 47.1, 45.7, 18.6. HRMS (ESI), C$_{26}$H$_{26}$ClN$_2$O$_6$S [M+H]$^+$ calcld = 529.1195; found = 529.1190.

**Sulfono-γ-AApeptide2**  
$^1$H NMR (500 MHz, d₆-DMSO) $\delta$ 12.78 (brs, 1H), 7.88 (d, $J = 7.6$ Hz, 2H), 7.78 (d, $J = 8.4$ Hz, 2H), 7.67 (d, $J = 7.6$ Hz, 2H), 7.59 (d, $J = 8.8$ Hz, 2H), 7.41 (td, $J = 7.2$, 2.3 Hz, 2H), 7.32 (td, $J = 7.2$, 3.2 Hz, 2H), 7.09 (d, $J = 8.8$ Hz, 1H), 6.74 (t, $J = 5.2$ Hz, 1H), 4.18–4.30 (m, 3H), 4.07, 3.99 (ABq, JAB = 18.8 Hz, 2H), 3.57–3.59 (m, 1H), 3.27 (dd, $J = 14.4$, 5.6 Hz, 1H), 3.14 (t, $J = 8.0$ Hz, 1H), 2.87 (dd, $J = 11.2$, 5.2 Hz, 2H), 1.42–1.47 (m, 1H), 1.36 (s, 9H), 1.22–1.33 (m, 4H), 1.11–1.17 (m, 1H). 13C NMR (100 MHz, d₆-DMSO) $\delta$ 170.3, 156.2, 156.0, 144.3, 144.2, 141.2 (2C), 138.9, 138.0, 129.6 (2C), 129.3 (2C), 128.0 (2C), 127.4 (2C), 125.6 (2C), 120.5 (2C), 77.7, 65.6, 55.3, 52.0, 49.9, 48.8, 47.2, 31.7, 29.7, 28.7 (3C), 23.2. HRMS (ESI), C$_{34}$H$_{41}$ClN$_3$O$_8$S [M+H]$^+$ calcld = 686.2297; found = 686.2281.

**Sulfono-γ-AApeptide3**  
$^1$H NMR (500 MHz, d₆-DMSO) $\delta$ 7.84 (d, $J = 7.6$ Hz, 2H), 7.75 (d, $J = 8.4$ Hz, 2H), 7.64 (d, $J = 7.6$ Hz, 2H), 7.54 (d, $J = 8.8$ Hz, 2H), 7.37 (t, $J = 7.2$ Hz, 2H), 7.28 (td, $J = 7.2$, 3.2 Hz, 2H), 4.14–4.28 (m, 3H), 3.94, 3.85 (ABq, JAB = 18.0 Hz, 2H), 3.59–3.63 (m, 1H), 3.26 (dd, $J = 14.4$, 6.0 Hz, 1H), 3.15 (t, $J = 8.0$ Hz, 1H), 2.04–2.17 (m, 2H), 1.68–1.72 (m, 1H), 1.42–1.50 (m, 1H), 1.34 (s, 9H). 13C NMR (100 MHz, d₆-DMSO) $\delta$ 172.0, 170.5, 157.0, 144.0, 143.9, 140.8 (2C), 138.7, 137.5, 129.2 (2C), 129.1 (2C), 127.7 (2C), 127.4 (2C), 125.4 (2C), 120.2 (2C), 80.0, 65.4, 51.4, 48.9, 46.9, 31.5, 27.9 (3C), 27.3, 21.3. HRMS (ESI), C$_{32}$H$_{36}$ClN$_2$O$_8$S [M+H]$^+$ calcld = 643.1875; found = 643.1854.
6.2.2 Molecular weight of oligomers 1a–6a:

Oligomer 1a, MALDI, C_{68}H_{84}Cl_{6}N_{13}O_{19}S_{6} [M+H]^+ calcd = 1788.2456; found = 1788.2164.

Oligomer 2a, MALDI, C_{79}H_{97}Cl_{7}N_{15}O_{22}S_{7} [M+H]^+ calcd = 2076.2792; found = 2077.4841.

Oligomer 3a, MALDI, C_{100}H_{128}Cl_{8}N_{19}O_{29}S_{8} [M+H]^+ calcd = 2594.4394; found = 2597.0391.

Oligomer 3b, MALDI, C_{98}H_{126}Cl_{8}N_{19}O_{28}S_{8} [M+H]^+ calcd = 2552.4288; found = 2555.1656.

Oligomer 4a, MALDI, C_{86}H_{108}Cl_{7}N_{16}O_{26}S_{7} [M+H]^+ calcd = 2249.3480; found = 2253.7993.

Oligomer 4b, MALDI, C_{84}H_{106}Cl_{7}N_{16}O_{25}S_{7} [M+H]^+ calcd = 2207.3374; found = 2207.7856.

Oligomer 5a, MALDI, C_{111}H_{141}Cl_{9}N_{21}O_{32}S_{9} [M+H]^+ calcd = 2882.4729; found = 2885.2068.

Oligomer 6a, MALDI, C_{44}H_{90}N_{15}O_{22}S_{7} [M+H]^+ calcd = 1404.4424; found = 1404.9709.

Table 6.2. HPLC purities and retention time of pure compounds 1a–6a.

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a The gradient eluting method of 5% to 100% of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over 50 min was performed, which is different from the gradient method for other oligomers.
Figure 6.7. HPLC spectra of pure oligomers 1a–6a.
Figure 6.7. continued. HPLC spectra of pure oligomers 1a–6a.

Hydrogen bonding pattern of compounds 1a, 3a, 4b, and 6a

Figure 6.8. Hydrogen bonding pattern of 1a, 3a, 4b, and 6a.
6.2.3 NMR studies of oligomer 4b

The NMR spectra were obtained on a Varian VNMRS 600 MHz spectrometer equipped with four RF channels and a Z-axis-pulse-field gradient cold probe. Oligomer 4b was dissolved in 0.4 mL of CD$_3$OH in a 5 mm NMR tube at a concentration of 5 mM at a temperature of 10 °C. DQF-COSY, zTOCSY (80 ms mixing time) and NOESY (300 ms mixing time) spectra were recorded to assign the NMR peaks by sequential assignment procedure. COSY and NOESY spectra were acquired with the Wet solvent suppression at Varian 600 MHz at 10 °C. All experiments were performed by collecting 4096 points in f2 and 500 points in f1. A DIPSI2 spin lock sequence with a spin lock field of 6k Hz was used in zTOCSY. NOESY experiment was carried out using a mix time of 300 ms. MestReNova was used to plot 1D NMR. Vnmrj was used to process and analyze 2D NMR data.
Figure 6.9. $^1$H NMR (600 MHz) spectra of oligomer 4b in CD$_3$OH.
Figure 6.9. continued. $^1$H NMR (600 MHz) spectra of oligomer 4b in CD$_3$OH.
Figure 6.9. continued. $^1$H NMR (600 MHz) spectra of oligomer 4b in CD$_3$OH.
Figure 6.9. continued. $^1$H NMR (600 MHz) spectra of oligomer 4b in CD$_3$OH
Figure 6.10. 2D spectra for oligomer 4b. The spectra were collected at 600 MHz at a temperature of 10 °C. Blue color, zTOCSY. Red color, NOESY.
Figure 6.11. DQFCOSY spectrum for oligomer 4b.
Table 6.3. Resonance assignment of 4b in CD$_3$OH.

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6.2.4 X-ray crystallography

Lyophilized powders of oligomer 1a (5 mg) was dissolved in 5 mL of acetonitrile/H₂O (9:1, v/v) and then left for slow evaporation at room temperature to give crystals. Lyophilized powders of oligomer 3a (5 mg) were dissolved in chloroform/methanol (4 mL, 3:1, v/v), and then left for slow evaporation at room temperature to give crystals. 4b (5 mg) was dissolved in 5mL of acetonitrile and then left for slow evaporation at room temperature to give crystals. 6a (5 mg) was dissolved in acetonitrile/dichloromethane/methanol (6 mL, 1:1:1, v/v) and then left for slow evaporation at room temperature to give crystals.

The X-ray diffraction data for oligomers 1a, 3a, and 4b were measured on Bruker D8 Venture PHOTON 100 CMOS system equipped with a Cu Kα INCOATEC Imus micro-focus source (λ = 1.54178 Å). Indexing was performed using APEX (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 APEX3. Structures were solved using SHELXS-97 (direct methods) and refined using SHELXL-2017 (full-matrix least-squares on F²) through OLEX2 interface program.

In all cases the initial structure solution has led to apparent infinite “polymeric” helix with shorter
than expected unit cell parameters along the axis of helix and smaller than expected number of atoms in asymmetric unit. This can be explained through the presence of translational disorder between discrete peptide chains in the crystal. Possible explanation is that the discrete peptide helices interact through hydrogen bonds at terminal points to form one dimensional column and the shift of adjacent columns is so that there is symmetrical overlap between otherwise non-equivalent residues and terminal groups of different helices. The shift of adjacent helices so that there exists apparent extra symmetry within the helix and between helices. This makes the diffraction pattern to resemble the pattern that would be recorder if the structure was consisted of infinite helices with much shorter repetition interval along axis of helix. Although the presented models are consisted with the presence of hydrogen bonds at terminal points between different helices, the low occupancy of terminal groups would prevent the detection of alternative conformation at terminal points if they were present.

For structures 1a and 6a this leads to the model of apparent infinite helix but with every seventh -C$_3$NSO$_2$PhCl- part of sulfono-$\gamma$-AApeptide (1a) or every eight -C$_3$NSO$_2$CH$_3$ group (6a) missing – at this point peptide chains interact through NH...O hydrogen bonding. This corresponds to 0.857 (1a) and 0.875 (6a) formal occupancy of those atoms in asymmetric unit and 6:1 (1a) or 7:1 (6a) ratio of sulfono-$\gamma$-AApeptide to terminal –COCH$_3$ and –NH$_2$ groups. Crystals were small and did not diffract past ca. 1.1 Å resolution. Models were refined using restraints for geometry and ADPs to offset the effects of overfitting. Crystal data and refinement conditions are shown in Tables 6.4 and 6.5.

Similar considerations as above hold for both (3a) and (4b) structures. In those structures there are two different building units present in asymmetric unit: one corresponds to $\gamma$-AApeptide with methyl group and the second to corresponds $\gamma$-AApeptide with N-substituted side chain. Both amine and carboxylic acid side chains occupy same site in the structure and are refined as disordered. The original model is adjusted to match the formula unit through lowering the occupancies of building blocks relative to terminal –NH$_2$ and –COCH$_3$ groups. This makes occupancy of building units 0.8 for (3a) and 0.75 in case of (4b) crystals were small and did not diffract past ca. 1.0 Å resolution. Models were refined using restraints for geometry (disordered parts) and ADPs. Crystal data and refinement conditions are shown in
Tables 6.6 and 6.7.

Although presented models are consisted with the presence of hydrogen bonds at terminal points between different helices, the low occupancy of terminal groups would prevent the detection of alternative conformation at terminal points if they were present.

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Table 6.7. Crystal data and structure refinement for oligomer 4b.

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6.2.5 Circular dichroism

Circular Dichroism (CD) spectra were measured on an Aviv 215 circular dichroism spectrometer using a 1 mm path length quartz cuvette, and compound solutions in trifluoroethanol were prepared using dry weight of the lyophilized solid followed by dilution to give the desired concentrations and solvent combination. 10 scans were averaged for each sample, and 3 times of independent experiments were carried out and the spectra were averaged. The final spectra were normalized by subtracting the average blank spectra. Molar ellipticity [θ] (deg.cm².dmol⁻¹) was calculated using the equation:
\[ \theta = \theta_{\text{obs}} / (n \times l \times c \times 10) \]

Where \( \theta_{\text{obs}} \) is the measured ellipticity in millidegrees, while \( n \) is the number of side groups, \( l \) is path length in centimeter (0.1 cm), and \( c \) is the concentration of the \( \alpha \)-sulfono-\( \gamma \)-AA peptide in molar units.

**6.2.6 Molecular dynamics simulation**

Simulations of 4b were performed in the left-handed and right-handed helical configuration. Force field parameters were generated by the CHARMM General Force Field (CGenFF) program, while parameters for the methanol solvent were from the standard CHARMM force field. Due to the high penalty reported by CGenFF, the force constants of four dihedral angles were further optimized by following the CHARMM force field parameter optimization procedure (Table 6.8), using target potential energy surface data generated by Gaussian09 at the B3LYP/6-31G(d) level. Initial coordinates for the left-handed helix were obtained from the crystal structure, and solvated into a cubic box of methanol with a margin of 10 Å. After energy minimization, the solvated system was heated from 120 K to 300 K over a 1 ns time period with a 1.0 kcal mol\(^{-1}\)Å\(^{-2}\) harmonic restraint force applied to all heavy atoms. The restraints were gradually released from 1.0, to 0.5, to 0.25 and to 0.1 kcal mol\(^{-1}\) Å\(^{-2}\) in successive 0.5 ns simulations. The coordinates of the resulting structure were used for four independent equilibration runs of 10 ns with no restraints, followed by 1 μs production runs. All simulations for the left-handed helix were performed in NAMD, using Langevin dynamics for temperature (300 K) and pressure (1 atm) control.

Initial coordinates for the right-handed helix were obtained by taking the mirror image of the crystal structure, after which the positions of the substituents of the chiral centers were switched. These coordinates were subsequently energy minimized while restraining the backbone hydrogen bonds by distance restraints acting on the carbonyl oxygen and amide hydrogen atoms with a reference length of 1.9 Å and a force constant of 10 kcal mol\(^{-1}\) Å\(^{-2}\). These restraints remained active throughout minimization, heating, and release of the additional heavy atom harmonic restraints. The right-handed helix was solvated in a cubic box of methanol with 10 Å margins and energy minimized. Three replicas of the resulting coordinates were heated from 30 to 300 K over 1 ns in increments of 30 K while harmonically restraining the backbone atoms with a mass weighted force constant of 10 kcal mol\(^{-1}\) Å\(^{-2}\). Backbone
restraints were then gradually reduced to 5.0, 1.0, 0.5, and zero kcal mol-1Å⁻² in successive 0.5 ns simulations at 300 K, followed by the release of hydrogen bonding restraints at force constants of 5.0, 1.0, and 0.5 kcal mol-1Å⁻² in successive 0.5 ns simulations. These builds, minimizations, heating and restrained equilibration simulations were performed with CHARMM, and the temperature was controlled with the Nosé-Hoover thermostat. Subsequent unrestrained equilibration runs of 10 ns, and unrestrained production runs of 40 ns were performed with NAMD, using Langevin dynamics for temperature (300 K) and pressure (1 atm) control. All simulations of the left and right-handed helices used the leapfrog integrator with a time step of 2fs, SHAKE for covalent bonds involving hydrogen atoms, and the particle mesh Ewald method for long range electrostatic interactions.

Table 6.8. Optimized dihedral angle force field parameters. Atom names are shown in Figure 6.17; all other parameters were obtained from CGenFF.

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Figure 6.13. Force field names for atoms involved in optimized dihedral angles.
Figure 6.14. Molecular dynamics simulations of 4b. (a) Backbone rmsd with the energy minimized structure versus the number of backbone hydrogen bonds for the left-handed helical configuration; standard deviations shown as bars. (b) Backbone rmsd with the energy minimized structure versus the number of backbone hydrogen bonds for the right-handed helical configuration. (c) Rmsd with the energy minimized structure versus time for the left-handed helical configuration. For clarity, no hydrogen atoms are shown except for the amide hydrogen atom of the backbone (in light blue). Helical backbone is shown in black, while unfolded backbone is shown in magenta. (d) Rmsd of the right-handed helical configuration with the energy minimized structure versus time. (e) Energy profile of the dihedral angle connecting the β-carbon and carbonyl carbon atoms. The energy minimized atomic structures around the relevant torsion angle are highlighted.
Figure 6.15. $^1$H NMR (400 MHz) and $^{13}$C NMR (100 MHz) spectra of Sulfono-γ-AApeptide1.
Figure 6.15. continued $^1$H NMR (400 MHz) and $^{13}$C NMR (100 MHz) spectra of Sulfono-$\gamma$-AApeptide1.
Figure 6.16. $^1$H NMR (400 MHz) and $^{13}$C NMR (100 MHz) spectra of Sulfono-$\gamma$-AApeptide2.
Figure 6.16. continued $^1$H NMR (400 MHz) and $^{13}$C NMR (100 MHz) spectra of Sulfono-$\gamma$-Apeptide2.
Figure 6.17. \(^1\)H NMR (400 MHz) and \(^{13}\)C NMR (100 MHz) spectra of Sulfono-\(\gamma\)-AApeptide\(^3\).
Figure 6.17. continued $^1$H NMR (400 MHz) and $^{13}$C NMR (100 MHz) spectra of Sulfono-$\gamma$-AApeptide3.
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