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Authors

Guaglianone, Gretchen
Kreutzer, Adam G
Nowick, James S

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Synthesis and Study of Macrocyclic β-Hairpin Peptides for Investigating Amyloid Oligomers

Gretchen Guaglianone[a], **Adam G. Kreutzer**[a], **James S. Nowick**[a,b]

[a]Department of Chemistry, University of California, Irvine, Irvine, California 92697-2025, United States

[b]Department of Pharmaceutical Sciences, University of California, Irvine, Irvine, California 92697-2025, United States

Abstract

Chemically constrained peptides that self-assemble can be used to better understand the molecular basis of amyloid diseases. The formation of small assemblies of the amyloidogenic peptides and proteins, termed oligomers, is central to amyloid diseases. The use of chemical model systems can help provide insights into the structures and interactions of amyloid oligomers, which are otherwise difficult to study. This chapter describes the use of macrocyclic β-hairpin peptides as model systems to study amyloid oligomers. The first part of the chapter describes the chemical synthesis of the macrocyclic β-hairpin peptides and covalent assemblies thereof. The second part of the chapter describes the characterization of the oligomers formed by the macrocyclic β-hairpin peptides, focusing on SDS-PAGE, size-exclusion chromatography (SEC), and X-ray crystallography. The procedures provided focus on the β-amyloid peptide, but these strategies are applicable to a broad range of amyloid-derived peptides and proteins.

Keywords

Amyloid diseases; macrocyclic β-hairpin peptides; amyloid oligomers; SDS-PAGE; size exclusion chromatography; peptide X-ray crystallography

1. Introduction

Amyloidogenic peptides and proteins are characterized by their ability to self-assemble to form oligomers and fibrils and are involved in many neurodegenerative diseases including Parkinson's disease, Huntington's disease, and Alzheimer's disease. Aggregates of these peptides are a hallmark of amyloid diseases; however, the underlying mechanisms leading to aggregation are not well understood (Benilova et al., 2012; Lashuel et al., 2013; Li & Liu, 2020). The process of aggregation, whereby monomeric units assemble into oligomers and fibrils, is difficult to study because of the transitory and dynamic nature of assembly (Gallardo et al., 2020; Larson & Lesné, 2012; Straub & Thirumalai, 2010). Amyloid oligomers are highly heterogeneous in size and structure, hindering investigation of structure-activity relationships (Hall & Huang, 2012; Bitan et al., 2003; Ono et al., 2009). Often in the oligomeric and fibrillar state, amyloidogenic peptides and proteins adopt a β-sheet conformation, frequently known as the amyloid fold (Bitan, 2006; Celej et al., 2012;

Eichner & Radford, 2011; Gallardo et al., 2020). It has become apparent that the oligomeric assemblies contribute to the toxicity observed in amyloid diseases. Understanding the oligomerization of amyloidogenic peptides and proteins is vital for understanding the molecular basis of amyloid diseases and for discovering potential treatments of these diseases (Fantini et al., 2020; Sakono & Zako, 2010; Straub & Thirumalai, 2010).

There are limitations to studying full-length amyloidogenic peptides and proteins. Isolating soluble oligomers, the suspected toxic species, and developing therapies targeting these dynamic species has thus far been unsuccessful (Rosenblum, 2014). Model systems consisting of fragments of amyloidogenic peptides or proteins have emerged as accessible methods to increase understanding of the biophysical properties of amyloid oligomers (Benilova et al., 2012; Hawk et al., 2020; Straub & Thirumalai, 2010; Wang et al., 2018). Our laboratory has pioneered the synthesis and study of macrocyclic β-hairpin peptides derived from the β-amyloid peptide (Aβ), an amyloidogenic peptide that aggregates to form the hallmark plaques in Alzheimer's disease (Haerianardakani et al., 2020; Kreutzer, Spencer, et al., 2017; Kreutzer, Yoo, et al., 2017; Kreutzer et al., 2020; Kreutzer & Nowick, 2018; C. Liu et al., 2011; Salveson et al., 2017; Samdin et al., 2020; Spencer et al., 2015).

Here we detail methods to synthesize and study macrocyclic β-hairpin peptides (Fig.1). We describe procedures to constrain peptide fragments into a β-hairpin using the turn mimic δ-linked ornithine (Nowick & Brower, 2003; Spencer et al., 2013), with the option to incorporate additional residues onto the *N*-terminus of the macrocyclic β-hairpin peptide and modify the peptide backbone to prevent uncontrolled aggregation (Vitoux, Aubry, Cung, & Marraud, 1986). We also provide methods to incorporate cysteine mutations into the native sequence to create covalently linked, stable oligomers through disulfide bonds. Finally, we provide detailed methods to study the self-assembly of macrocyclic β-hairpin peptides, including SDS-PAGE, SEC, and X-ray crystallography. These methods are not limited to peptides derived from Aβ and can be applied to other amyloidogenic peptides and proteins to further elucidate the role of amyloid assemblies in neurodegenerative and somatic diseases.

2. Synthesis of Macrocyclic β-Hairpin Peptides

Studying the self-assembly of amyloidogenic peptides and proteins into oligomers is difficult because oligomers are heterogeneous and metastable. Our laboratory has developed techniques to synthesize and study oligomers of the β-amyloid peptide (Aβ), using macrocyclic β-hairpin peptides derived from Aβ that allow for the formation of more stable and homogeneous oligomers. In these macrocyclic peptides, δ-linked ornithine ($^\delta$Orn) turn mimics are used to constrain fragments from the central and *C*-terminal regions of Aβ into a β-hairpin. An *N*-methyl group on the backbone of the peptide prevents uncontrolled aggregation. The corresponding $^\delta$Orn and *N*-methyl amino acids are easily incorporated into the macrocyclic β-hairpin peptides using standard solid-phase peptide synthesis with Fmoc-protected amino acids. Additionally, a $^\delta$Orn orthogonally protected with Dde and Fmoc allows additional *N*-terminal amino acids to be appended to the macrocycle (Scheme 1). Solution-phase cyclization generates the macrocyclic ring (Scheme 2). Cysteine residues can be incorporated into macrocyclic peptides that exhibit well-defined self-assembly and

then oxidized to create covalently stabilized oligomers with disulfide crosslinks (Scheme 3). Detailed below is a representative procedure for the synthesis of a macrocyclic β-hairpin peptide using 2-chlorotrityl chloride resin on a 0.1 mmol scale following standard solid-phase peptide synthesis procedures using Fmoc-protected amino acids.

Peptide synthesis equipment and reagents:

- Bio-Rad Poly-Prep chromatography column (Bio-rad, cat. #7311550)

- Solid-phase peptide synthesis vessel (Chemglass, cat. #CG-1866)

- Water purification system to generate high purity (18 MΩ) deionized water (Barnstead Nanopure, cat. #50131922)

- 250 mL round-bottom flask

- Rotary evaporator

- Glass scintillation vial

- 0.2 μm syringe filter (Fisher Scientific, cat. #13100100)

- Preparative RP-HPLC instrument

- Analytical RP-HPLC instrument

- Agilent Zorbax 250 x 21.2 mm SB-C18 column (Agilent, cat. #897250-102)

- Phenomenex Aeris PEPTIDE 2.6u XB-C18 column (Phenomenex, cat. # 00B-4505-AN)

- Centrifuge

- Lyophilizer

- 2-Chlorotrityl chloride resin (Chem Impex, cat. #03498)

- DCM

- DMF (Alfa Aesar, contains a packet of amine scavenger) (Alfa Aesar, cat. #A13547)

- 2,4,6-Collidine

- DIPEA

- Fmoc-protected amino acids

- Piperidine

- HCTU

- HBTU

- HATU

- HOBt

- HOAt

- Hydrazine

- HFIP

- NMM

- PyBOP

- TFA

- TIPS

- DMSO

- Diethyl ether (anhydrous)

2.1 Loading the resin

1. Place 2-chlorotrityl chloride resin (300 mg, 1.2 mmol/g) in a Bio-Rad Poly-Prep chromatography column.

2. Add anhydrous DCM (10 mL) to the Poly-Prep column and rock for 30 minutes.

3. Drain the solution from the column using a flow of nitrogen gas and add 0.5 equivalents of Boc-Orn(Fmoc)-OH (82 mg, 0.18 mmol, 0.5 equiv) dissolved in anhydrous DCM (8 mL) with 2,4,6-collidine (0.3 mL).

4. Rock for 12 h.

5. Drain the solution after 12 h.

2.2 Capping the resin

1. Cap unreacted resin sites by adding a solution of DCM (8.5 mL), methanol (1 mL), DIPEA (0.5 mL) to the resin and rock for 1 h.

2. Drain the capping solution and wash the resin three times with anhydrous DCM (3 x 10 mL) and three times with anhydrous DMF (3 x 10mL).

3. Prepare a solid-phase peptide synthesis vessel with a line to introduce nitrogen through the frit and a line to pull vacuum to evacuate solvent into a waste collection flask.

4. Add a final portion of DMF (10 mL) to the Poly-Prep column and transfer the resin to the coupling vessel.

2.3 Coupling amino acids

Subject the loaded resin to cycles of peptide coupling. In each cycle, 20% piperidine is first used to remove the Fmoc protecting group from the amine. An amino acid is then activated with coupling agents and added to extend the amino acid chain.

1. Add 20% (v/v) piperidine in DMF (7 mL) to the coupling vessel containing the resin and bubble with nitrogen for 7 minutes to remove the Fmoc protecting group from the initial [8]Orn amino acid.

2. After 7 minutes, drain the solution from the coupling vessel.

3. Wash the resin three times with DMF (3 x 10mL), ensuring the inner walls of the vessel are well rinsed.

4. Weigh out 5.0 equivalents of the next desired Fmoc-protected amino acid (residue 23) and 5.0 equivalents of HCTU coupling agent and dissolve in 20% (v/v) 2,4,6-collidine in DMF (7 mL).

5. Add the solution to the coupling vessel and bubble with nitrogen for at least 15 minutes.

6. Drain the solution from the coupling vessel and wash the resin three times with DMF (3 x 10mL), ensuring the inner walls of the vessel are well rinsed.

7. Repeat steps 1–6 until the *N*-methyl amino acid has been coupled (residues 23–20).

2.4 Coupling the amino acid following an *N*-methyl amino acid

Note: When synthesizing a peptide containing an *N*-methyl amino acid, begin the synthesis from the $^\delta$Orn proximal to the *C*-terminal side of the desired *N*-methyl residue.

1. Following the *N*-methyl amino acid, couple the subsequent Fmoc-protected amino acid (residue 19) twice to ensure successful coupling.

2. Weigh 5.0 equivalents of the subsequent amino acid, 5.0 equivalents of HOAt, and 5.0 equivalents of HATU.

3. Dissolve in 20% (v/v) 2,4,6-collidine in DMF (7 mL).

4. Add the solution to the coupling vessel and bubble with nitrogen for 1 h.

5. Drain the solution from the coupling vessel.

6. Repeat steps 2–5. Do not wash with DMF or deprotect with 20% (v/v) piperidine in DMF.

7. Resume standard solid-phase peptide synthesis by adding 20% (v/v) piperidine in DMF (7 mL) to the coupling vessel and bubble with nitrogen for 7 minutes.

8. Continue until the top strand has been coupled (residues 23–17).

2.5 OPTIONAL: Extending the *N*-terminus with Fmoc-Orn(Dde)-OH

To extend the *N*-terminus off of the α-amino group of $^\delta$Orn, use Fmoc-Orn(Dde)-OH and follow the procedures below, then extend the bottom strand by Fmoc-based solid-phase peptide synthesis. If you do not wish to extend the *N*-terminus, use Boc-Orn(Fmoc)-OH and extend the bottom strand by standard Fmoc-based solid-phase peptide synthesis.

1. Following the synthesis of the top strand (residues 23–17), couple Fmoc-Orn(Dde)-OH following the standard solid-phase peptide synthesis procedure.

2. Continue to couple the desired *N*-terminal extended amino acids (residue 15) following the outlined solid-phase peptide synthesis procedure. Use a Boc-protected α-amino acid for the final amino acid (e.g., Boc-His(Trt)-OH).

3. Remove the Dde protecting group by adding 10% hydrazine in DMF (10 mL) for 20 minutes at ambient temperature.

4. Following deprotection, wash the resin with DMF (8 x 10 mL). It is important to remove all residual hydrazine as it can deprotect Fmoc and affect subsequent amino acid couplings.

5. Continue solid-phase peptide synthesis until the desired bottom strand is coupled (residues 36–30).

2.6 Cleaving the peptide from the resin

1. Remove the Fmoc protecting group from the final amino acid by adding 20% (v/v) piperidine in DMF (7 mL) to the coupling vessel and bubble with nitrogen for 7 minutes.

2. Drain the deprotecting solution and wash the resin three times with DMF (3 x 10mL).

3. Add a final portion of DMF (10 mL) to the vessel and pipet the resin back into the Poly Prep column. Additional DMF can be added to help transfer the resin.

4. Once all of the resin has been transferred, drain the remaining DMF from the Poly Prep column and wash the resin three times with dry DCM (3 x 10mL).

5. Drain the remaining DCM from the Poly Prep column.

6. Cleave the peptide from the resin by adding HFIP (1.75 mL) in DCM (5.75 mL) to the Poly Prep column. Rock for 1 h.

7. After 1 h, filter the suspension and collect the filtrate in a 250 mL round-bottom flask.

8. Add additional solution of HFIP (1.75 mL) in DCM (5.75 mL) to the remaining resin and rock for 30 minutes.

9. Filter the suspension and collect the filtrate in the same 250 mL round-bottom flask.

10. Concentrate by rotary evaporation until dry.

2.7 Cyclizing the peptide

Note: If you observe a tetramethylguanidinylated adduct by mass spectrometry after cyclization, you may need to repeat the synthesis of the macrocyclic β-hairpin peptide and use PyBOP in place of HBTU and HOBt in the cyclization step (Albericio et al., 1998).

1. Add dry DMF (150 mL) to the flask, followed by 5.0 equivalents of HOBt (114 mg, 0.75 mmol, 5 equiv) and 5.0 equivalents of HBTU (317 mg, 0.75 mmol, 5 equiv).

2. Add NMM (0.33 mL, 1.8 mmol, 12 equiv) to the solution and stir under nitrogen for 48 h.

3. After 48 h, remove the solvent by rotary evaporation.

4. The peptide can be stored under vacuum or directly subject to global deprotection and purification by RP-HPLC.

Alternatively, use PyBOP (370 mg, 0.711 mmol, 6 equiv) in place of HBTU and HOBt in Step 1.

2.8 Deprotecting amino acid side chain protecting groups

Note: Global deprotection with trifluoroacetic acid (TFA) will remove trityl (Trt),O-tert-butyl (OtBu), tert-butyloxycarbonyl (Boc), and pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) protecting groups. It does not remove other protecting groups, such as acetamidomethyl (Acm), benzyloxycarbonyl (Cbz), Alloc, or Dde.

1. To the dried, cyclized peptide, add 18 mL of TFA, 1 mL of TIPS, and 1 mL of 18 MΩ deionized water.

2. Stir at room temperature under nitrogen for 1 h.

3. After 1 h, you may remove the majority of the solution by rotary evaporation on a system dedicated for acidic solutions. It is not advised to evaporate this solution on a shared rotovap. If a rotovap dedicated to acidic solutions is not available, proceed directly to ether precipitation.

2.9 Precipitating the peptide with ether

1. Fill a flask with 100 mL of ether and chill in an ice bath for 20 minutes.

2. Divide the TFA solution containing the peptide equally into two 50 mL conical tubes.

3. Add 15 mL of cold ether to the conical tubes containing peptide. Swirl gently and incubate in the ice bath for 5 minutes.

4. After 5 minutes, centrifuge the conical tubes at 500 x g for 10 minutes. A pellet will form.

5. Decant the ether without disturbing the pellet.

6. Repeat steps 3–5 twice.

7. Transfer the peptide pellets to a 500 mL round-bottom flask by adding acetonitrile to the conical tube and pipetting into the round-bottom.

8. Remove the ether and acetonitrile by rotary evaporation. It is critical to evaporate all of the ether to avoid damaging the HPLC column in the subsequent HPLC purification. *Alternatively, the pellets can be dried under a flow of nitrogen instead of using rotary evaporation.*

9. Once all of the ether has evaporated, proceed to purification.

2.10 Purifying the peptide by HPLC

Note: Dissolve the peptide in a solution of water and acetonitrile equivalent to the ratio of water:acetonitrile you are injecting at on the HPLC instrument. Typically, this is 20% or

less. If the peptide is not soluble at this ratio, you may need to add more acetonitrile and adjust the injection water:acetonitrile ratio on the HPLC instrument to match the acetonitrile percentage in which the peptide is dissolved.

1. Dissolve the deprotected peptide in 2 mL of acetonitrile in the round-bottom flask.

2. Transfer the solution to a 15 mL conical tube.

3. Rinse the round-bottom flask with 8 mL of 18 MΩ deionized water and transfer to the 15 mL conical tube.

4. Centrifuge the 15 mL conical tube at 100 x *g* for 5 minutes to remove particulates.

5. Filter the solution through a 0.2 μm syringe filter into a new 15 mL conical.

6. The peptide is now ready to be purified using a preparative HPLC instrument with a C18 column. If available, first use analytical HPLC instrument with a C18 column to obtain a chromatogram of your crude peptide before purifying as a guide for purification.

7. Equilibrate the preparative HPLC instrument by running 20% acetonitrile with 0.1% TFA and 80% water with 0.1% TFA.

8. Load the peptide onto the column.

9. Once the peptide is loaded onto the column, increase the percent of acetonitrile by a gradient of 0.5% per minute until all peaks elute off of the column and are collected. [*Note:* Pausing the gradient and running isocratically when a peak elutes can assist in the separation of multiple peaks.]

10. Collect the fractions of interest and analyze by analytical HPLC and mass spectrometry.

11. Combine fractions containing the peptide mass with sharp analytical HPLC chromatograms and concentrate to dryness by rotary evaporation.

12. Reconstitute the peptide in 18 MΩ deionized water (10 mL) and transfer to a 15 mL conical tube.

13. Freeze the tube containing the peptide and obtain dry peptide via lyophilization.

2.11 Oxidizing macrocyclic β-hairpin peptides into crosslinked oligomers

1. To a 25 mL glass scintillation vial add 20% aqueous DMSO to lyophilized, monomeric peptide (approximately 30 mg) for a final concentration of 6 mM.

2. Rock the vial at room temperature for 48 h.

3. Monitor the formation of new species by analytical HPLC at 0, 24, and 48 h and by ion mobility mass spectrometry, if available.

4. After 48 h, purify the reaction mixture by preparative HPLC.

5.   Analyze fractions of interest by analytical HPLC and mass spectrometry for oligomeric species.

6.   Collect fractions of interest and concentrate to dryness by rotary evaporation.

7.   Reconstitute the peptide in 18 MΩ deionized water (10 mL) and obtain dry peptide via lyophilization.

3.   Methods for Studying the Self-Assembly of Macrocyclic β-Hairpin Peptides

A wide variety of methods are available to study the supramolecular assembly of amyloidogenic peptides and proteins. Below are three methods we have found to be the most reliable techniques that provide insights into the assembly and structure of macrocyclic β-hairpin peptides: SDS-PAGE, SEC, and X-ray crystallography. SEC provides information about the size and molecular weight of the peptide assembly that forms in solution. SDS-PAGE offers additional information about the peptide assembly in the lipid-like environment of SDS micelles. X-ray crystallography provides high-resolution structures of the peptide assemblies that form. A combination of these techniques can elucidate the structures and self-assembling behavior of macrocyclic β-hairpin peptides. These robust techniques are frequently supported with additional techniques such as ion mobility mass spectrometry, NMR, analytical ultracentrifugation, and dynamic light scattering, to better understand the biophysical properties of self-assembling peptides.

3.1   Tricine SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE) is a technique used to separate peptides and proteins. SDS (sodium dodecyl sulfate) is an anionic surfactant added to coat the peptides with a negatively charged layer, allowing the peptides to separate on the basis of molecular weight as they migrate in the applied electric field. Many of the Aβ-derived macrocyclic peptides and subsequent crosslinked oligomers that we have created form SDS-stable assemblies (Haerianardakani et al., 2020; Kreutzer et al., 2020; Kreutzer, Yoo, et al., 2017; Salveson et al., 2018). In fact, SDS appears to promote higher-order assembly of many of our Aβ-derived peptides and oligomers (Fig. 2). These observations are consistent with those for full-length Aβ, which also appears to form SDS-promoted assemblies (Walsh & Selkoe, 2007).

Various techniques can be used to visualize the peptide bands within the gel. The most common method we use is silver staining, which relies on the reduction of silver ions to elemental silver, in a fashion similar to the development of a black-and-white photograph (Merril, 1986; Winkler et al., 2007). We occasionally perform Coomassie staining to visualize the bands in the gel. Coomassie staining relies on adsorption of the dye to the hydrophobic side chains of the amino acids contained in the peptide to visualize the peptide assemblies (Smejkal, 2004; Winkler et al., 2007). Visualization via InstantBlue Coomassie protein stain is reversible and compatible with subsequent mass spectrometric analysis of the gel bands. While silver staining is more sensitive than Coomassie staining, it is not reversible and thus not compatible with potential subsequent mass spectrometric analysis of

the gel bands. Although other visualization methods are available, our lab has found these two to be the most effective for the visualization of our macrocyclic β-hairpin peptides.

SDS-PAGE equipment and reagents:

- Bio-Rad Mini-PROTEAN Tetra Cell (Bio-rad, cat. #1658005EDU)

- Electrophoresis power supply (Fisher Scientific, cat. #FB3000Q)

- 15 mL conical tube

- 50 mL conical tube

- Gel-loading tips (Fisher, cat. #02-707-138)

- Tris base

- Glycerol

- SDS

- TEMED

- Isopropyl alcohol

- APS

- Acrylamide

- Bisacrylamide (N,N'-methylenebisacrylamide)

- Tricine

- HCl

- Biosciences SDS-PAGE Sample Loading Buffer 6X Precision Plus Protein Standard (Bio-Rad, cat. #161-0374)

Coomassie stain equipment and reagents:

- InstantBlue Coomassie protein stain (Abcam, cat. #ab119211)

- Ethanol

- Single-edge razor blade

- Plastic container with lid (approximately 5 x 3 x 3 inches)

Silver stain equipment and reagents:

- Methanol

- Acetic acid

- Sodium thiosulfate pentahydrate

- Silver nitrate

- Sodium carbonate (99.5% pure) (Sigma Aldrich, cat. #497-19-8)

- Formalin (37% w/v formaldehyde solution)

- Single-edge razor blade

- Plastic container with lid (approximately 5 x 3 x 3 inches)

3.1.1 Tricine SDS-PAGE preparation and protocol—This protocol is adapted from
Schägger, 2006.

1. Prepare the following solutions:

    3X gel buffer: 3.0 M Tris base, 1.0 M HCl, and 0.3% SDS. Dissolve 181.7 g (1.5 mol) Tris base, 41.6 mL (0.5 mol) of 12 M HCl, and 1.5 g SDS in 18 MΩ deionized water to make 500 mL of solution. Adjust the pH to 8.45 with HCl or NaOH if necessary.

    AB-3 stock solution: Dissolve 48.0 g of acrylamide and 1.5 g of bisacrylamide (electrophoresis grade, >99% pure) in 18 MΩ deionized water to a final volume of 100 mL.

    10X cathode buffer: 1.0 M Tris base, 1.0 M Tricine, 1% SDS at pH 8.25. Dissolve 60.6 g (0.5 mol) Tris base, 89.6 g (0.5 mol) Tricine, and 5.0 g of SDS in 18 MΩ deionized water to make 500 mL of solution. Adjust the pH to 8.25 with HCl or NaOH if necessary.

    10X anode buffer: 1.0 M Tris base and 0.225 M HCl at pH 8.9. Dissolve 60.6 g (0.5 mol) Tris base and 9.4 mL (0.112 mol) of 12 M HCl in 18 MΩ deionized water to make 500 mL of solution. Adjust the pH to 8.9 with HCl or NaOH if necessary.

    10% APS solution: Dissolve 30 mg of APS in 18 MΩ deionized water to make 300 μL of solution.

2. Prepare peptide samples by dissolving dry peptide in 18 MΩ deionized water to a concentration of 10 mg/mL.

3. Dilute 10 mg/mL stock solutions with 18 MΩ deionized water to make 10 μL samples of concentrations 5.0 mg/mL, 2.5 mg/mL, 1.25 mg/mL, and 0.75 mg/mL.

4. Add 2 μL of Biosciences SDS-PAGE Sample Loading Buffer 6X to each 10 μL sample. [*Note:* Ensure SDS in sample loading buffer is fully dissolved before adding to the sample by warming in a water bath.]

5. Assemble the gel casting apparatus by sandwiching and clamping the glass panes in the vertical mount.

6. Prepare a 16% polyacrylamide running gel by adding 18 MΩ deionized water (5 mL), 3X gel buffer (5 mL), and AB-3 stock solution (5 mL) to a 50 mL conical tube and swirl gently to mix.

7. Add 100 μL of 10% APS solution and 10 μL TEMED to the polyacrylamide solution, and swirl gently to mix.

8. Quickly pipette the 16% polyacrylamide gel into the gel casting apparatus, avoiding bubbles, leaving 0.5 inches unfilled on the top for the comb and stacking gel.

9. Temporarily fill the remaining 0.5 inches with isopropyl alcohol.

10. Allow the gel to set for 30 minutes. After 30 minutes, ensure that the remaining solution in the conical tube has polymerized.

11. Decant the isopropyl alcohol off of the top of the gel.

12. Prepare the 4% polyacrylamide stacking gel by combining 18 MΩ deionized water (3.66 mL), 3X gel buffer (1 mL), and AB-3 stock solution (0.33 mL) in a 15 mL conical tube and swirl gently to mix.

13. Add 100 μL of 10% APS solution and 10 μL TEMED to the polyacrylamide solution, and swirl gently to mix.

14. Quickly pipette the 4% polyacrylamide gel into the gel casting apparatus, above the polymerized 16% polyacrylamide gel.

15. Place a clean comb between the glass panes to create wells for sample loading.

16. Allow the 4% polyacrylamide gel to set with the comb in place for 30 minutes. After 30 minutes, ensure that the remaining solution in the conical tube has polymerized.

17. Move the gel into the cassette for running electrophoresis.

18. Prepare 1X cathode and anode buffers by diluting 100 mL of 10X with 18 MΩ deionized water to make 1 L of each solution.

19. Ensure the gel is properly loaded into the cassette by adding 1X cathode buffer into the inside of the cassette and checking for leaks. If leaking is observed, adjust the glass panes until it no longer leaks.

20. Load the cassette into the electrophoresis apparatus and add 1X anode buffer to the line indicated on the apparatus.

21. Remove the comb gently after the cathode buffer is loaded into the inside of the cassette. The samples will be loaded under cathode buffer.

22. Add 1 μL of the protein standard reference ladder selected for the appropriate size range of your samples to the first well of the gel using a gel-loading tip.

23. Add 5 μL of each sample to consecutive wells using a pipette fitted with gel-loading tips.

24. Run the gel at a constant 60 volts at room temperature.

25. Monitor the band progress through the gel and end the run when bands reach the bottom of the cassette (approximately 3–4 hours).

3.2 Methods for developing SDS-PAGE

3.2.1 Coomassie protein staining—This protocol is adapted from Abcam. (https://www.abcam.com/ps/products/119/ab119211/documents/InstantBlue-Coomassie-Protein-Stain-protocol-book-v2b-ab119211%20(website).pdf).

Note: This procedure can be followed by the silver staining procedure. Wash the gel with 50 mL of 18 MΩ deionized water for 3 minutes. Repeat twice and proceed to silver staining.

1. Remove the gel from the cassette and remove the top casting glass.

2. Separate the 4% polyacrylamide gel and wells from the 16% polyacrylamide gel using a razor blade. Discard the 4% polyacrylamide gel and wells.

3. Place the 16% polyacrylamide gel in a plastic container with 25 mL of Instant Blue Coomassie protein stain.

4. Shake gently at room temperature.

5. Transfer the gel to 18 MΩ deionized water when desired staining is achieved (approximately 15 minutes).

Optional de-staining for mass spectrometry:

1. Remove the protein band of interest and transfer to an Eppendorf tube.

2. Add 30% ethanol in 18 MΩ deionized water (1 mL) and rock gently for 20 minutes.

3. Decant the solution.

4. Repeat steps 2–3 until the stain has washed away.

3.2.2 Silver staining—This protocol is adapted from Simpson, 2007.

1. Prepare the following solutions:

   fixing solution: 50% methanol and 5% acetic acid. Combine 250 mL of methanol and 12.5 mL of acetic acid to 18 MΩ deionized water to make 500 mL of solution.

   0.02% sodium thiosulfate solution: Prepare a fresh stock solution of 10.0% sodium thiosulfate by diluting 200 mg of sodium thiosulfate in 18 MΩ deionized water to make 2 mL of solution. To 98.8 mL of 18 MΩ deionized water, add 200 μL of 10.0% sodium thiosulfate stock solution.

   0.1% silver nitrate solution: Prepare fresh by adding 200 mg of silver nitrate to 18 MΩ deionized water to make 200 mL of solution. Chill to 4 °C.

   developing solution: Prepare fresh 2% (w/v) sodium carbonate and 0.04% w/v formaldehyde. Dissolve 200 mg of sodium carbonate to 18 MΩ deionized water to make 200 mL of solution. Dissolve completely. Add 214 μL of formalin. [*Note:* The use of high-purity (99.5%) sodium carbonate is crucial for successful silver staining.]

50% methanol solution: Add 250 mL of methanol to 18 MΩ deionized water to make 500 mL of solution.

5% acetic acid solution: Add 20 mL of acetic acid to 18 MΩ deionized water to make 400 mL of solution.

2. Remove the gel from the cassette and remove the top casting glass.

3. Separate the 4% polyacrylamide gel and wells from the 16% polyacrylamide gel using a razor blade. Discard the 4% polyacrylamide gel and wells.

4. Place the 16% polyacrylamide gel in a plastic container and submerge in sufficient fixing solution. Gently shake for 20 minutes.

5. After 20 minutes, decant the fixing solution while the gel remains in the plastic container.

6. Submerge in 50% (v/v) methanol and gently shake for 10 minutes.

7. Decant the 50% (v/v) methanol and submerge the gel in 18 MΩ deionized water. Shake gently for 10 minutes.

8. Decant the water and submerge the gel in 0.02% sodium thiosulfate solution. Shake gently for 1 minute.

9. Remove the sodium thiosulfate solution.

10. Rinse the gel twice with 18 MΩ deionized water for 1 minute each.

11. Add chilled 0.1% silver nitrate solution and rock gently at 4 °C for 20 minutes.

12. Rinse the gel twice with 18 MΩ deionized water for 45 s each.

13. Decant the 18 MΩ deionized water and submerge the gel in developing solution. Discard the solution if it turns yellow and add a second portion of developing solution.

14. Monitor until desired staining is achieved (approximately 2 minutes).

15. Stop the development of the gel by decanting the developing solution and adding 5% (v/v) acetic acid.

16. The gel may be stored in 1% (v/v) acetic acid.

3.3 Size-Exclusion Chromatography (SEC)

Size-exclusion chromatography offers insight into the sizes and molecular weights of the assemblies that the macrocyclic β-hairpin peptides and crosslinked oligomers form in aqueous solution. Peptide assemblies of various sizes are separated through a gel matrix with larger molecular weight peptide assemblies eluting first, followed by smaller molecular weight assemblies and peptide monomers, which better enter the pores within the gel matrix. The peptides and reference standards that elute from the column pass through a UV absorbance detector and the absorbance signal is monitored. We typically monitor absorbance at 214 nm, however, for peptides that contain tyrosine or tryptophan, absorbance at 280 nm may be used. The mass of the peptide assembly and number of peptide monomer

units can be determined by comparing the elution time of the peptide to multiple reference standards (Hall & Huang, 2012; Winzor, 2003).

It is important to identify a buffer and pH in which the peptide of interest is completely soluble and does not precipitate. Comparison of the UV absorbance of a solution of the peptide in buffer before and after centrifugation can help detect loss of peptide by precipitation or aggregation. Buffers systems that have been successful for our macrocyclic β-hairpin peptides and crosslinked oligomers include 50 mM Tris buffer at pH 7.4 with 150 mM NaCl, 10 mM glycine and 50 mM NaCl at pH 3.0, and 50 mM sodium acetate and 50 mM acetic acid at pH 4.7 (Haerianardakani et al., 2020; Kreutzer, Yoo, et al., 2017; Samdin et al., 2020). Phosphate buffers have often led to precipitation. Once an appropriate buffer system is determined, peptide samples and references standards can be run and analyzed quickly. Below is a representative procedure using sodium acetate buffer.

SEC equipment and reagents:

- FPLC instrument, such as the AKTA Explorer 10

- Cytiva or GE Superdex 75 Increase 10/300 GL (Cytiva, cat. #29148721)

- Cytochrome C

- Aprotinin

- Vitamin B12

3.3.1 SEC preparation and protocol

1. Prepare sodium acetate buffer: 50 mM sodium acetate and 50 mM acetic acid. Add 2.05 g (25 mmol) of sodium acetate and 1.43 mL (25 mmol) of acetic acid to 18 MΩ deionized water to make 500 mL of solution. Adjust the pH to 4.7.

2. Degas the sodium acetate buffer by sonicating the solution while under vacuum for 1 h.

3. Dilute lyophilized peptide sample in 18 MΩ deionized water to a concentration of 10 mg/mL.

4. Dilute sample further by adding 80 μL of the 10 mg/mL stock solution to 720 μL of sodium acetate buffer.

5. Centrifuge samples at 17000 x g for 2 minutes to remove precipitated material from solution.

6. Load the soluble material from samples onto the FPLC column at 0.5 mL/min over 1 min.

7. After loading, run the instrument at a flow rate of 1 mL/min with sodium acetate buffer. Record the absorbance at 214 nm. If saturation of the detector is observed, samples should be diluted.

8. Run reference standards at a concentration of 1 mg/mL of cytochrome C, aprotinin, and vitamin B12 by the same method.

3.4. X-ray crystallography

X-ray crystallography is a powerful technique for elucidating the three-dimensional structures of the higher-order assemblies that macrocyclic β-hairpin peptides form. These peptides are well suited for X-ray crystallography, because they adopt conformationally homogeneous β-hairpin structures that are prone to self-assemble and form a crystal lattice. We have adapted the tools of protein crystallography to crystallize and elucidate the crystal structures of macrocyclic β-hairpin peptides (Spencer & Nowick, 2015). In this section, we detail the X-ray crystallography methods *our laboratory* typically uses for crystallization of the macrocyclic peptides, collecting crystal diffraction data, processing the diffraction data, solving the crystallographic phases, building and refining the structure, and depositing the structure in the RCSB Protein Data Bank.

3.4.1 Crystallization of the macrocyclic β-hairpin peptide—To crystalize the macrocyclic β-hairpin peptide, we use the hanging-drop vapor-diffusion method. In this method, a droplet of the crystal growing solution is mixed with a droplet of the peptide and then inverted and sealed over a well containing the crystal growing solution. After a certain amount of time, the drops are examined under a microscope to look for the formation of crystals. Our macrocyclic β-hairpin peptides typically grow crystals within 24–72 hours.

3.4.1.1 Screening crystallization conditions: To identify conditions under which the macrocyclic β-hairpin peptide forms crystals, we use commercially available crystallization screens from Hampton Research. We perform the screening in 96-well plate format using a mosquito® nanoliter liquid handler. We screen 3 ratios of peptide:crystal growing solution (1:1, 1:2, 2:1). Each ratio contains 150 nL total volume. If you do not have an automated liquid handler, screening can also be performed manually.

Equipment and reagents:

- Automated liquid handling system, such as mosquito® nanoliter liquid handler

- Spool of 26,000 pipettes at 9 mm pitch (for 96+ well plates, sptlabtech, cat. # 4150-05600)

- Hampton Research Crystallization Screens (PEG/Ion cat. #'s HR2-126 and HR2-098; Index cat. # HR2-144; Crystal Screen cat. #'s HR2-110 and HR2-112)

- Axygen® 80 μm AxySeal Sealing Film (Corning, cat. # PCR-SP)

- Flat-bottom 96-well plates (Greiner BIO-ONE, cat. # 82050-760)

- V-bottom 96-well plate (Thermo Scientific, cat. # 2605)

- Viewdrop II 96-well plate seals for hanging drop (sptlabtech, cat. # 4150-05600)

- 20 μL pipette and pipette tips

- Stereo microscope

  1. Prepare the 96-well screening plates by adding 100 μL of the crystal growing solution from the Hampton Research crystallization screens to

the appropriate wells. Seal the plates with AxySeal Sealing Film. These plates can be used immediately or stored at 4 °C for weeks to months.

2. When you are ready to screen a peptide for crystallization, prepare at least 100 μL of a 10 mg/mL solution of the peptide in 18 MΩ deionized water.

3. Remove the screening plates from the refrigerator. If there is condensation on the seal, centrifuge the plates for 1 min at 1000 g.

4. Set up the program on the computer for the mosquito® LV nanoliter liquid handler.

5. Add 12 μL of the peptide solution to each well of an 8-well column of the V-bottom 96-well plate and place the plate in the appropriate position on the mosquito® nanoliter LV liquid handler.

6. Remove the seal from a crystallization screen and place the plate in the appropriate position on the mosquito® nanoliter liquid handler.

7. Remove the paper from a Viewdrop II 96-well plate seal and place the seal sticky-side-up on the seal holder, then place the holder in the appropriate position on the mosquito® nanoliter liquid handler.

8. Run the program and appropriate protocol on the computer that runs the mosquito® nanoliter LV liquid handler.

9. When the protocol has completed, remove the crystallization screen from the mosquito® nanoliter liquid handler and place it in the metal plate positioner.

10. Remove the seal holder from the mosquito® nanoliter liquid handler and, using the plate positioner, line up the seal holder with the crystallization screen and press the seal firmly against the plate so that the seal adheres to the plate.

11. Repeat steps 6–10 for the other two crystallization screens. (You will now have three 96-well plates, with PEG/Ion, Index, and Crystal Screen conditions.)

12. After 1–24 h, examine each well of the crystallization screen using a stereo microscope to identify crystals. Reexamine the plate every 24–48 h.

13. Conditions identified that produce crystals can be optimized as described in the next section.

3.4.1.2 Optimizing crystallization conditions: Once crystallization conditions have been identified, the conditions are optimized to produce crystals suitable for X-ray diffraction. We perform crystallization optimization in 24-well plate format using 3 ratios of peptide:crystal growing solution (1:1, 1:2, 2:1 μL). For optimizing crystallization conditions, we most

commonly vary pH and the cryoprotectant concentration, however, other parameters may also be varied.

Equipment and reagents:

- VDX™ 24-well Plate with sealant (Hampton Research, cat. # HR3-170)

- 22 mm x 0.22 mm Siliconized square cover slides (Hampton Research, cat. # HR3-217)

- 30 mL luer-slip syringes

- Fisherbrand™ Syringe Filters: Nylon Membrane syringe filters (0.22 μm, 33 mm) (Fisher, cat. # 09-719-006)

- 2, 20, 200, and 1000 μL pipettes and pipette tips

- Stereo microscope

    1. Prepare at least 100 μL of a 10 mg/mL solution of the peptide in 18 MΩ deionized water.

    2. Prepare concentrated solutions of any buffer, salt, or cryoprotectant in the crystallization condition being optimized. Filter the solutions through a 0.22 μm nylon syringe filter.

    3. Create a 4X6 matrix in a 24-well plate by varying a property of one crystallization condition component (e.g., concentration of cryoprotectant) along one axis of the matrix; vary another property (e.g., pH of the buffer) along the other axis of the matrix. We typically vary pH by 0.5 units and cryoprotectant concentrations by 1–4 % per row or column of wells.

    4. Pipette the appropriate volumes of the concentrated buffer, salt, or cryoprotectant solutions to each well to achieve the desired concentration of each component. The final volume in each well of the 24-well plate is 1 mL. Make up the remaining volume with 18 MΩ deionized water.

    5. On a siliconized square cover slide, prepare 3 drops by combining the peptide solution (1 μL) with the well solution (1 μL) in a ratio of 1:1, 2:1, and 1:2 μL.

    6. Invert the slide and press firmly against the silicone grease surrounding each well of the 24-well plate.

    7. After 1–24 h, examine each well of the crystallization optimization tray using a stereo microscope to identify crystals. Reexamine the plate every 24–48 h.

    8. Crystals that grow of suitable quality are harvested and diffracted on an X-ray diffractometer or synchrotron.

3.4.2 Collecting crystal diffraction data—Once crystals have grown that are suitable for X-ray diffraction, a single crystal is harvested and flash frozen in a stream of liquid nitrogen vapor, or a bath of liquid nitrogen if the crystal will be diffracted at a later time. The crystal is then placed on a goniometer and centered relative to the incident X-ray beam on an X-ray diffractometer or synchrotron. Next, the crystal is exposed to X-rays and the quality of the diffraction is assessed. For crystals that produce high quality diffraction data, a complete diffraction data set is collected. Often, we collect multiple data sets on the same crystal at multiple angles and merge the data sets. Merging multiple data sets can improve anomalous signal as well as higher-resolution data completeness.

Typically, we first collect diffraction data sets for our crystals on an in-house X-ray diffractometer to screen for crystals that produce high quality diffraction, solve the X-ray crystallographic phases, and build a model. We then often collect diffraction data sets for our crystals on a synchrotron at the Stanford Synchrotron Radiation Lightsource (SSRL) or at the Advanced Light Source (ALS) to collect higher resolution diffraction data.

In this section, we provide instructions for harvesting crystals for diffraction on an X-ray diffractometer or synchrotron, and for collecting diffraction data on an X-ray diffractometer or synchrotron. We also provide instructions for soaking crystals in iodide or bromide salt solutions for incorporating heavy atom anomalous scatterers for single-wavelength anomalous diffraction (SAD) phasing.

3.4.2.1 Harvesting crystals for immediate diffraction on an X-ray diffractometer: A crystal is harvested by scooping the crystal into a nylon loop attached to a metal pin. Often, crystals that grow in conditions without a cryoprotectant need to be dipped in a cryoprotectant before they are flash frozen in liquid nitrogen. The most common cryoprotectants we use are glycerol, polyethylene glycol (PEG), 2-methyl-2,4-pentanediol (MPD), and 1,6-hexanediol. Many cryoprotectants will often need to be screened to determine which cryoprotectant works best for the crystals. Furthermore, the cryoprotectant may need to be mixed with the well solution for the crystal to survive cryoprotection. Once the crystal is on the loop and has been dipped in cryoprotectant, it is mounted on the goniometer and diffraction data are collected. If necessary, soaking crystals in iodide or bromide to incorporate heavy atoms into the lattice for SAD phasing is performed during the harvesting step.

Equipment and reagents:

- X-ray diffractometer (such as a Rigaku Micromax-007HF equipped with a rotating copper anode and a HyPix-6000HE photon counting detector)

- CrystalCap ALS (Hampton Research, cat. # HR4-779)

- Variably sized 18 mm Mounted CryoLoop™ - 20 micron (Hampton Research; 0.05–0.1 mm cat. # HR-945, 0.1–0.2 mm cat. # HR-947, 0.2–0.3 mm cat. # HR-970)

- Cryoprotectant

- 2 μL pipette and pipette tips

- Single-edge razor blade

- Stereo microscope

  1. Remove the lid of the 24-well optimization tray and with a stereo microscope, look at the drops and locate crystals that you intend to use for X-ray diffraction.

  2. Under the microscope, hold different sized CryoLoops over the crystals and select a loop size appropriate for scooping up a crystal.

  3. If cryoprotection of the crystal is necessary, pipette a 1–2 μL drop of cryoprotectant on to the lid of the optimization tray.

  4. When you have selected a drop to harvest crystals from, use a razor blade to gently lift the glass cover slip and remove it from the siliconized well. Set the coverslip drop-side-up on top of the lid of the optimization tray and focus the microscope on the crystals in the drop.

  5. Use the CryoLoop to scoop up a single crystal.

  6. If cryoprotection of the crystal is necessary, dip the crystal in the drop of cryoprotectant. It may be necessary to swirl the crystal around in the cryoprotectant to remove most of the aqueous mother liquor, assuming the crystal is stable in the cryoprotectant.

  7. Mount the crystal on the goniometer in a stream of liquid nitrogen and center the crystal relative to the incident X-ray beam.

  8. Move on to Section 3.4.2.3. Collecting crystal diffraction data on an X-ray diffractometer.

3.4.2.2 Harvesting crystals for diffraction on a synchrotron.: Harvesting crystals for diffraction on a synchrotron is the same as harvesting crystals for diffraction on an X-ray diffractometer, except instead of being immediately flash frozen in a stream of liquid nitrogen vapor, the crystals on the CryoLoops are submerged in a bath of liquid nitrogen and put in a liquid nitrogen Dewar for storage or put in appropriate hardware for shipment to a synchrotron, such as SSRL or ALS.

Equipment and reagents:

- Worthington Industries CX100 Cryo Express Dry Shipper (MiTeGen, #TW-CX100)

- Worthington Industries Shipping Case for CX100 or CXR100 (MiTeGen, #TW-CX10-8C00)

- CrystalCap ALS (Hampton Research, cat. # HR4-779)

- Variably sized 18 mm Mounted CryoLoop™ - 20 micron (Hampton Research; 0.05–0.1 mm cat. # HR-945, 0.1–0.2 mm cat. # HR-947, 0.2–0.3 mm cat. # HR-970)

- CrystalWand™ Magnetic, Straight (Hampton Research; cat. # HR4-729

- Universal V1-pucks, such as Universal Puck (Uni-Puck) Starter Kit 2 (MiTeGen, cat. # SKU: M-CP-UPSK002)

- Cryoprotectant (see Section 3.4.2.1)

- 2 μL pipette and pipette tips

- Single-edge razor blade

- Stereo microscope

  Note: All of the hardware needed for storing crystals and shipping crystals to the synchrotron will need to be purchased and setup for harvesting crystals. For shipping crystals to SSRL or ALS, we purchased the Universal Puck (Uni-Puck) Starter Kit 2 (MiTeGen, cat. # SKU: M-CP-UPSK002). We typically harvest crystals for shipment to SSRL or ALS the same day we ship the crystals or 1–2 days before we ship the crystals and store the crystals in the pucks in a liquid nitrogen Dewar. We typically harvest the crystals directly into the Universal V1-pucks, which are compatible with the crystal-handling robotics at both SSRL and ALS. For longer term storage, the crystals on the CryoLoops can be put into CrystalCaps, which are then stored in canes that are housed in a liquid nitrogen Dewar.

  1. Remove the lid of the 24-well optimization tray and with a stereo microscope, look at the drops and locate crystals that you intend to use for X-ray diffraction.

  2. Under the microscope, hold different sized CryoLoops over the crystals and select a loop size appropriate for scooping up a crystal.

  3. If cryoprotection of the crystal is necessary, pipette a 1–2 μL drop of cryoprotectant on to the lid of the optimization tray.

  4. When you have selected a drop to harvest crystals from, use a razor blade to gently lift the glass cover slip and remove it from the siliconized well. Set the coverslip drop-side-up on top of the lid of the optimization tray and focus the microscope on the crystals in the drop.

  5. Use the CryoLoop to scoop up a single crystal. [*Note:* If you wish to soaking crystals in iodide or bromide salt solutions, see Section 3.4.2.3.]

  6. If cryoprotection of the crystal is necessary, dip the crystal in the drop of cryoprotectant. It may be necessary to swirl the crystal around in the cryoprotectant to remove most of the aqueous mother liquor, assuming the crystal is stable in the cryoprotectant.

  7. Place the CryoLoop on the magnetic CrystalWand and submerge loop in a bath of liquid nitrogen that contains the Universal V1-pucks. Swirl

the loop around for a few seconds in the liquid nitrogen bath to try and remove any frozen aqueous mother liquor that might be on the crystal and then place the CryoLoop in the appropriate slot in the puck.

8. Repeat steps 1–7 for each crystal being sent to the synchrotron until the puck is full. Then place the lid on the puck and quickly transfer the puck to the liquid nitrogen Dewar for shipment to the synchrotron.

9. Make sure to note the location of each type of crystal if you are sending multiple samples to the synchrotron.

3.4.2.3   Soaking crystals in iodide or bromide salt solutions for incorporation of anomalous scatterers into the crystal lattice: To determine the X-ray crystallographic phases, we often soak a crystal in a iodide or bromide salt solution (e.g., potassium iodide, sodium iodide, potassium bromide, or sodium bromide) (Dauter et al., 2000). The optimal soaking conditions (i.e., concentration of salt, ratio of salt solution to well solution or cryoprotectant, soaking time, etc.) is empirically determined and will take trial and error.

Note: This step is performed after step 5 in the harvesting procedures described above.

Equipment and reagents:

- Variably sized 18 mm Mounted CryoLoop™ - 20 micron (Hampton Research; 0.05–0.1 mm cat. # HR-945, 0.1–0.2 mm cat. # HR-947, 0.2–0.3 mm cat. # HR-970)

- Iodide or bromide salt solution

- 2 μL pipette and pipette tips

- Single-edge razor blade

- Stereo microscope

    1. Prepare a solution of your salt-of-choice in water. We typically prepare 1 M aqueous potassium iodide.

    2. Pipette 1 μL of the salt solution onto the lid of the 24-well tray and then mix with 1 μL of well solution or 1 μL of cryoprotectant.

    3. Perform steps 1–5 in Sections 3.4.2.1 or 3.4.2.2.

    4. Dip the crystal in the drop of salt solution and soak the crystal in the salt solution for an appropriate amount of time. The optimal soaking time for incorporation of heavy atoms into the lattice is empirically determined and can range from 5 seconds to overnight. The soaking time will depend on the stability of the crystal in the salt solution. If the crystal appears to degrade over time, try mixing the salt solution with a different cryoprotectant or buffer.

    5. Perform steps 6–8 in Sections 3.4.2.1 or 3.4.2.2.

3.4.2.4   Collecting crystal diffraction data on an X-ray diffractometer.: Once the crystal has been harvested and mounted on the goniometer of an X-ray diffractometer, the crystal is centered relative to the incident X-ray beam and then diffracted with X-rays and diffraction data are collected. Initially, we collect a few diffraction images at different angles to assess crystal diffraction quality. High quality crystals diffract to a high resolution and produce round, well-defined spots. We typically collect two diffraction images to index the crystal (i.e., determine the crystal cell dimensions, cell angles, and a possible space group). For crystals that produce high quality diffraction data and index to a space group other than $P1$, a complete diffraction data set is collected where the crystal is rotated around a single axis in 0.5° or 1° rotations and a diffraction image is collected at each angle until the crystal has rotated 360°.

Equipment and reagents:

- X-ray diffractometer (such as a Rigaku Micromax-007HF equipped with a rotating copper anode and a HyPix-6000HE photon counting detector)

- Goniometer tools

- Computer with software that controls the X-ray diffractometer (such as CrysAlis^Pro Software from Rigaku)

- Portable USB drive with at least 32 GB of space

    1.   Turn on the X-ray diffractometer and liquid nitrogen flow system.

    2.   If the detector on the X-ray diffractometer has a protective cover, remove the cover.

    3.   After harvesting and mounting your crystal as described in Section 3.4.2.1, use the goniometer tools to center the crystal relative to the incident X-ray beam. When centering the crystal, manually turn the goniometer 90° and use the goniometer tools to re-center the crystal. Repeat this process until the crystal is centered relative to the incident X-ray beam at all angles.

    4.   Use the X-ray diffractometer software to choose the appropriate settings for acquiring initial diffraction images for assessing crystal diffraction quality. The CrysAlis^Pro Software has preset options for screening crystals for diffraction. We rarely change these default settings except for the exposure time. The optimal exposure time is often empirically determined. For our X-ray diffractometer equipped with a rotating copper anode that produces 1.54 Å X-rays, we have found that an exposure time of 15 seconds is usually sufficient for collecting high-quality high-resolution data.

    5.   Once the appropriate collection settings have been applied, collect two diffraction images of the crystal at two different angles.

6. Visually assess the crystal diffraction quality by identifying the highest resolution at which there is spots. Visually assess the shape of the spots to further assess the diffraction quality.

7. For crystals that produce satisfactory diffraction data, the software will attempt to index the crystal.

8. If the crystal diffracts to a resolution less than ca. 3.0 Å and indexes to a space group other than P1, collect a full data set by using the "Data Collection Strategy" function of the X-ray diffractometer software.

9. The Data Collection Strategy function will most often generate parameters for collection of a single data set based on the predicted space group and unit cell dimensions. We often collect multiple 360° data sets on the same crystal to achieve highly redundant data by increasing the data redundancy in the software.

10. Note the exposure time that the Data Collection Strategy chose. For most crystals of our macrocyclic β-hairpin peptides, we have found that an exposure time of 15 seconds is sufficient for data collection.

11. The total data collection time for crystals of our macrocyclic β-hairpin peptides typically ranges from 12–24 hours.

12. Once a sufficient amount of data has been collected and data collection has concluded, using the X-ray diffractometer software, convert the image files into an appropriate format for the data processing software you plan to use. We most commonly use the data processing software XDS or iMosflm.

13. Locate the folder containing the image files on the hard drive of the X-ray diffractometer computer and transfer the folder to a portable USB drive.

14. You are now ready to process the data sets. Move on to Section 3.4.3. Processing X-ray Diffraction Data.

3.4.2.5 Collecting crystal diffraction data on a synchrotron: A synchrotron can provide X-rays of greater flux and shorter wavelength, thus enabling the rapid collection of higher-resolution data than can be collected on an X-ray diffractometer. Collection of diffraction data on a synchrotron is performed similarly to that of an X-ray diffractometer, whereby a crystal is mounted on a goniometer, centered relative to the incident X-ray beam and exposed to X-rays. Synchrotrons, such as SSRL and ALS, allow remote data collection and provide detailed instructions for shipping crystals and collecting diffraction data on their websites.

Useful links for remote data collection at SSRL:

• https://smb.slac.stanford.edu/users_guide/manual/Remote_experiments.html

- https://smb.slac.stanford.edu/users_guide/manual/Data_collection_processing.html

Useful links for remote data collection at ALS:

- https://bcsb.als.lbl.gov/wordpress/?page_id=386

- https://bcsb.als.lbl.gov/wordpress/?page_id=232

3.4.3 Processing crystal diffraction data—After collecting a crystal diffraction data set, the data set is processed by first indexing the data, and then integrating and scaling the data, and finally merging the data to make a single reflection file. We typically use the script-based program XDS to index and integrate the data, and the script-based programs Pointless and Aimless to find the most probable space group and merge and assess the quality of the diffraction data (P. Evans, 2006; P. R. Evans & Murshudov, 2013; Kabsch, 2010). There are a variety of additional free graphical user interface software packages available to process diffraction data, such as iMosflm, HKL2000, and d*TREK. Below we provide instructions for processing diffraction data collected on an X-ray diffractometer with XDS, Pointless, and Aimless. We also provide instructions for processing and merging multiple data sets together using the program blend. We process our diffraction data remotely on the SSRL server using the remote access software NX technology (NoMachine).

3.4.3.1 Processing a single diffraction data set: The instructions below are for processing a single diffraction data set. For processing and merging multiple diffraction data sets, see Section 3.4.3.2.

1. Transfer the folder containing the image files on your portable USB drive to your home folder on the /data partition of the SSRL server using an FTP client, such as FileZilla. [*Note:* The image files that comprise each data set must be in their own folder (i.e., one folder cannot contain multiple data sets).]

2. On a remote SSRL desktop, open a terminal and navigate to you home folder.

3. In your home folder, create a working directory for the crystal diffraction data you are processing. In all subsequent steps, this directory will be referred to as 'working directory'.

```
mkdir name_of_the_crystal
```

4. In the newly created working directory, create a folder called bin# for each diffraction data set that was collected. For example, if you collected 4 data sets, you would create 4 different folders called bin1, bin2, bin3, and bin4.

```
mkdir bin1
```

5. Within each bin folder, create a symbolic link called 'images' that links to the folder on the /data partition of the SSRL server that contains your diffractions images.

```
ln -s /file/path/to/images images
```

6. Using the GUI file browser, copy an appropriate XDS.INP file into each bin folder. [*Note:* XDS.INP files are specific for the type of detector you are using. Often, the X-ray diffractometer software will create an XDS.INP file when the images are converted to XDS format. The following link contains XDS.INP files for commonly used detectors (http://xds.mpimf-heidelberg.mpg.de/html_doc/xds_prepare.html). Googling "XDS.INP name of detector" can also lead you to the appropriate XDS.INP file.]

7. Open the XDS.INP file in a text editor, such as gedit.

```
gedit XDS.INP
```

8. In the 'Selection of Data Images' section of the XDS.INP file, direct the 'Name_template_of_data_frames=' to the symbolic link 'images' folder that contains your images followed by the name of your first image.

```
NAME_TEMPLATE_OF_DATA_IMAGES=./images/na
me_of_first_image_???.filetype
```

9. Close and save the XDS.INP file.

10. Run XDS

```
xds -par
```

11. If XDS runs successfully, the program creates a file in the bin folder called 'XDS_ASCII.HKL'.

12. Run Pointless on the XDS_ASCII.HKL file to create a file called 'pointless.mtz'.

```
pointless xdsin XDS_ASCII.HKL hklout
pointless.mtz
```

Note: To force pointless to process the data in a specific space group:

```
pointless xdsin XDS_ASCII.HKL hklout
pointless.mtz ≪ EOF
```

```
spacegroup P3121
```

#give space group

```
reindex h,k,l
```

#optional reindexing

```
EOF
```

13. Run Aimless on the pointless.mtz file to create a file called 'aimless.mtz' and a file called 'aimless.log'

```
aimless hklin pointless.mtz hklout
aimless.mtz | tee aimless.log

exit
```

The aimless.mtz file constitutes the scaled and merged reflection file used for the next steps, such as assessing data quality, solving the X-ray crystallographic phases, and generating an electron density map and structural model. The aimless.log file provides a record of the aimless run and contains the data processing statistics.

3.4.3.2   Processing and merging multiple diffraction data sets: Collecting multiple redundant diffraction data sets on the same crystal and then merging the data sets together can improve anomalous signal strength as well as the data completeness in the higher resolution shells. Collecting and merging multiple data sets is especially important when attempting to solve the X-ray crystallographic phases using sulfur single anomalous diffraction (S-SAD) phasing (Q. Liu et al., 2012; Sarma & Karplus, 2006). Below we provide instructions for processing and merging multiple data sets by first using the program XDS to index and integrate each data set individually, and then using the program blend to scale and merge the data sets into a single reflections file. Using blend, multiple data sets from a single crystal can be merged together, as can multiple data sets from multiple crystals.

1. Process each data set individually using XDS as described in steps 1–11 in Section 3.4.3.1 to generate an XDS_ASCII.HKL for each data set,

2. In your working directory, create a folder called 'blend'.

```
mkdir blend
```

3. Using the GUI file browser, copy each XDS_ASCII.HKL file for each data set you want to merge into the blend folder. As you copy each XDS_ASCII.HKL file into the folder, rename each file with a unique identifier (e.g., XDS_ASCII_1.HKL, XDS_ASCII_2.HKL, etc.).

4. In a terminal, navigate to the blend folder.

```
cd /file/path/to/blend
```

5. Run the analysis mode of blend.

```
blend -a .
```

6.   The analysis mode of blend creates a file called 'CLUSTERS.txt' within the blend folder. Open the CLUSTERS.txt in a text editor.

```
gedit CLUSTERS.txt
```

7.   The CLUSTERS.txt file provides values for the cluster heights for the various combinations of data sets that blend will merge. Note the value of the cluster height for the cluster that contains all of the data sets (i.e., the last number in the cluster height column).

8.   Run the synthesis mode of blend by providing a value larger than the largest cluster height value. For example, if your largest cluster height value is 4.651, type the following:

```
blend -s 4.7
```

9.   The synthesis mode of blend creates a folder called 'merged_files'. Within that folder you will find scaled and unscaled reflections files (i.e., .mtz files) for each merged data set, as well as aimless.log files and pointless.log files.

10. The scaled_###.mtz file constitutes the scaled and merged reflection file used for the next steps, such as assessing data quality, solving the X-ray crystallographic phases, and generating an electron density map and structural model.

3.4.4 Assessing data quality, solving the X-ray crystallographic phases, and generating an electron density map and structural model—We use the X-ray crystallography software suite Phenix on an SSRL remote desktop to perform all of the subsequent data analysis and operations in the following steps (3.4.4.1-3.4.4.6). The Phenix software suite contains many useful X-ray crystallography programs. Below we provide instructions for the programs our laboratory most commonly uses for our X-ray crystallographic structures. We also include a link to more detailed instructions for using each program on the Phenix website.

To solve the X-ray crystallographic phases for crystals of our macrocyclic β-hairpin peptides, we first attempt to use molecular replacement. If molecular replacement fails, we attempt to incorporate heavy atoms, such as iodide or bromide into the crystal lattice by soaking a crystal in a solution of potassium iodide or sodium bromide; or if the macrocyclic β-hairpin peptide contains disulfide cross-links or a methionine residue, we attempt sulfur single-wavelength anomalous diffraction (S-SAD) phasing. Finally, if the preceding methods fail, we incorporate a heavy atom onto the peptide by mutating an appropriate residue to a residue containing a heavy atom (e.g., phenylalanine to *para*-iodophenylalanine; leucine to (2-bromoallyl)glycine; methionine to selenomethionine).

3.4.4.1 Launching Phenix: https://www.phenix-online.org/

1.   Open a terminal on an SSRL remote desktop and type:

```
phenix
```

2. Once the software has launched, click "New Project" and provide an appropriate Project ID and set the Project Directory to the working directory for the crystal structure you are working on.

3. Recommended optional step: Provide a sequence file. To create a sequence file, navigate to your working directory in a terminal and type

```
gedit sequence.seq
```

Copy and paste or type the amino acid sequence of your peptide or protein into the gedit text window. Save and close the window. This creates the file 'sequence.seq' in your working directory, which you can provide to Phenix in the Sequence file field.

4. Click OK

5. Phenix automatically switches to the newly created project in the Projects tab.

3.4.4.2 Assessing data quality with the program phenix.xtriage: https://www.phenix-online.org/documentation/reference/xtriage_gui.html

1. Open phenix.xtriage by clicking "Xtriage" in the Data Analysis tab of Phenix.

2. In the Reflections field, load a reflections file (e.g., aimless.mtz or scaled_###.mtz) by clicking 'Browse' and then navigating to the desired reflections file. Loading a reflections file will automatically populate the Unit cell, Space group, and Data labels fields, as well as provide the high resolution and low resolution of the data.

3. If you want Xtriage to analyze the anomalous signal in the data, change the data labels to "some_label, merged".

4. If you want Xtriage to predict the number of copies of your peptide or protein that are in the asymmetric unit, and HAVE NOT provided Phenix with a sequence file, enter the number of residues that are in your peptide or protein.

5. Click "Run".

6. Xtriage provides a summary of the data analysis. For good quality data, each of the points in the summary should be green. In the pull-down menu, there is a variety of information given in each selection.

7. In the pull-down menu, select "Solvent content and Matthews coefficient". In this selection, Xtriage predicts the number of molecules in the asymmetric unit. This information is important for subsequent operations, such as searching for anomalous signal with HySS (Section 3.4.4.3) and molecular replacement with Phaser-MR (Section 3.4.4.5).

8. In the pull-down menu, select "Anomalous signal". In this selection, Xtriage reports the anomalous signal strength in each resolution shell. Strong anomalous signal has high measurability in the higher-resolution shells. This is valuable

information when attempting to incorporate a heavy atom into the crystal lattice to introduce anomalous signals.

9. Instructions for interpreting and understanding the other information in the pull-down menu go beyond the scope of this protocol; however, one should become familiar with the information in these selections.

3.4.4.3 Locating anomalous scatterers with the program Hybrid Substructure Search (HySS): https://www.phenix-online.org/documentation/reference/hyss.html

1. Open HySS by clicking "Hybrid Substructure Search" in the Experimental Phasing tab of Phenix.

2. In the "Anomalous data" field, load a reflections file (e.g., aimless.mtz or scaled_###.mtz) by clicking 'Browse' and then navigating to the desired reflections file. Loading a reflections file will automatically populate the Unit cell, Space group, and Data labels fields, as well as provide the high resolution and low resolution of the data.

3. In the "Wavelength of X-ray data collection" field enter the wavelength at which the X-ray data was collected (e.g., enter 1.54 for X-ray data collected on an X-ray diffractometer with a copper anode).

4. In the "Number of sites" field enter the number of anomalous scatterers expected in the asymmetric unit.

    •   If your protein or peptide contains heavy atoms, such as iodide, bromide, or sulfur, the number of sites can be determined by multiplying the number of heavy atoms in your peptide or protein by the predicted number of molecules in the asymmetric unit determined in step 7 of Section 3.4.4.2.

    •   If you have soaked your crystal in an iodide or bromide salt solution, perform multiple runs of HySS and vary the number of sites. We typically enter 1–6.

5. In the "Scattering type" field enter the symbol for the heavy atom you are searching for (i.e., "I" for iodide, "Br" for bromide, or "S" for sulfur).

6. In the "Number of processors" field enter at least 20.

7. Click "Run".

8. After HySS has completed, inspect the "Results" tab. Under "Statistics", note the number of sites that HySS found, as well as the correlation coefficient. Correlation coefficients greater than 0.3 typically generate solvable electron density maps.

9. Click "Run AutoSol" and move on to step 3.4.4.4.

3.4.4.4   Solving the X-ray crystallographic phases and generating an electron density map with the program AutoSol: https://www.phenix-online.org/documentation/reference/autosol_gui.html

1.   If you clicked "Run AutoSol" in step 9 of Section 3.4.4.3, then all of the files and fields you need to run AutoSol have been automatically populated. If not, then add the appropriate files.

2.   If a sequence file was not automatically added, add a sequence file, by clicking "Browse" and then navigating to the appropriate sequence file.

3.   In the "Options and output" tab, increase the "Number of processors" to 20 or greater.

4.   Click "Run" and then select "Run detached".

5.   AutoSol will create an electron density map and attempt to build a model to fit the electron density using the sequence provided.

6.   After AutoSol has completed, click the "Summary tab" and inspect the "Final model" category. Here you will find R-work and R-free values and the number of residues, fragments, and waters that AutoSol built.

7.   Using an FTP client, such as FileZilla, transfer the AutoSol folder created by AutoSol from your working directory on the SSRL server to your local computer.

8.   The AutoSol folder contains an electron density map file called "overall_best_final_refine_001.mtz" and structure file called "overall_best_final_refine_001.pdb". Load these files into the program Coot to build or refine the model (Section 3.4.5.1).

9.   Recommended optional step: In the Summary tab of AutoSol, click "Run AutoBuild" (see Section 3.4.4.6).

3.4.4.5   Performing molecular replacement with the program Phaser-MR: https://www.phenix-online.org/documentation/reference/phaser_mr.html

1.   Open Phaser-MR by clicking "Phaser-MR (simple one-component interface)" in the Molecular replacement tab of Phenix.

2.   Click "Add file" and load a reflections file (e.g., aimless.mtz or scaled_###.mtz).

3.   Click "Add file" and load the .pdb file to use as a search model.

4.   In the "Model RMSDs" field, enter 0.5. Note, this value can range from 0.1 to 3 and multiple molecular replacement runs can be running in parallel where this number is varied.

5.   Click "Run" and then select "Run now".

6.   When Phaser-MR has finished running, inspect the "Top LLG" and "Top TFZ" scores. LLG scores for successful molecular replacements can vary substantially. Typically, LLG scores above 100 and TFZ scores above 10 indicate that the

molecular replacement was successful. To better determine if the molecular replacement was successful, run AutoBuild (see Section 3.4.4.6) or phenix.refine (see Section 3.4.5). If the R values in AutoBuild or phenix.refine are acceptable, the molecular replacement was likely successful. Also, visually inspect the model that Phaser-MR generated by opening the .pdb file in a molecular visualization program, such as PyMOL and generating symmetry mates. For a successful molecular replacement, there should be no major clashes among the molecules in the asymmetric unit or among symmetry mates, and no wide-open spaces in the model.

7. Click "Run AutoBuild" and move on to Section 3.4.4.6.

3.4.4.6 Generating a structural model with the program AutoBuild.: https://www.phenix-online.org/documentation/reference/autobuild_gui.html

1. If you clicked "Run AutoBuild" in step 9 of Section 3.4.4.4 or step 7 of Section 3.4.4.5, then all of the files and fields you need to run AutoBuild have been automatically populated. If not, then add the appropriate files.

2. Click the "Other options" tab and increase the value in the "Number of processors" field to 30.

3. Click "Run" and select "Run detached".

4. After AutoBuild has completed, click the "Summary tab" and inspect the "Final model" category. Here you will find R-work and R-free values and the number of residues, fragments, and waters that AutoBuild built.

5. Using an FTP client, such as FileZilla, transfer the AutoBuild folder created by AutoBuild from your working directory on the SSRL server to your local computer.

6. The AutoBuild folder contains an electron density map file called "overall_best_final_refine_001.mtz" and structure file called "overall_best_final_refine_001.pdb". Load these files into the program Coot to build or refine the model (Section 3.4.5.1).

3.4.5 Building and refining the model—After generating an electron density map and a preliminary model, the map and model are loaded into the macromolecular model building program Coot. Coot is used to build and manipulate models to fit the electron density map. Once the model has been built, it is refined using the program phenix.refine in the Phenix software suite on the SSRL server. Multiple rounds of model building and manipulation and refinement are performed.

Coot can be downloaded at the following link: https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/

3.4.5.1 Building a model in Coot: To build a model in Coot, you will need to load an .mtz file and a .pdb file into Coot. If your structure contains unnatural amino acids, a CIF file will also need to be loaded for each unnatural amino acid in your molecule. The instructions

below are meant to cover the basics of building and manipulating a model in Coot. For more comprehensive instructions for using Coot, visit the Coot website.

1. Load an electron density map (.mtz file). Click "File" and select "Auto Open MTZ". Navigate to the file "overall_best_final_refine_001.mtz" in the AutoBuild or Autosol folder (see step 6 in Section 3.4.4.6 or step 8 in Section 3.4.4.4).

2. Load a model (.pdb file). Click "File" and select "Open Coordinates". Navigate to the file "overall_best_final_refine_001.pdb" in the AutoBuild or Autosol folder (see step 6 in Section 3.4.4.6 or step 8 in Section 3.4.4.4).

3. Load a CIF file for each unnatural amino acid in your molecule. Click "File" and select "Import CIF Dictionary". Navigate to the appropriate CIF file on your computer.

4. Inspect each amino acid residue of your model and ensure that each residue fills electron density.

5. Commonly used tools in Coot have icons running along the right side of the window. To fit a residue to density, click the "Real-space Refinement" tool then a select a residue or range of residues. Use the left mouse button to drag the residues to fit the density. If you are happy with the placement of the residues, click "Accept".

6. Use the Difference Maps Peaks tool to automatically find unfilled electron density. Click "Validate" and then select "Difference Maps Peaks". Change the "Find peaks above" value to 3.5 and then click "Find Peaks". A new window will pop up that contains a list of the positive and negative peaks. Examine each of the peaks found and use your best judgment for filling the electron density.

7. Save the modified model into the AutoSol or AutoBuild folder the model was originally selected from. Click "File" and select "Save Coordinates". Choose the appropriate model to save, and then click Select Filename. Navigate to the appropriate folder and then click "Save". Coot will append "-coot-#" to the file.

8. Use an FTP client to transfer the modified model file back to the appropriate folder on the SSRL server.

9. Refine the model using phenix.refine (Section 3.4.5.2).

3.4.5.2 Refining a model with phenix.refine: The following instructions detail how to run phenix.refine and are not meant as an exhaustive guide for how to improve R-free and R-work.

https://www.phenix-online.org/documentation/reference/refine_gui.html

1. Open phenix.refine by clicking "phenix.refine" in the Refinement tab of Phenix.

2. Click "Add file" and load the reflections file used for generating the initial electron density map in Phaser-MR or AutoSol (e.g., aimless.mtz or scaled_###.mtz).

3. Click "Add file" and load the model that has been modified in Coot.

4. If there are unnatural amino acids in your model, click "Add file" and load a CIF file for each unnatural amino acid.

5. Change the "Data labels" to the appropriate selection.

6. Click the "Refinement settings" tab and select the appropriate settings for your refinement.

7. Click "Run" and select "Run detached".

8. After phenix.refine has completed, click the "Results" tab and inspect the "Refinement statistics" category. Here you will find R-work and R-free values. These values provide a metric for how well your model explains the diffraction data. For a fully refined structure at a resolution of 2.0 Å, these values should be as low as possible, ideally lower 0.25. For higher-resolution structures < 2.0 Å, these values should be ideally lower than 0.25. [*Note:* Instructions for interpreting and understanding the other information provided by phenix.refine go beyond the scope of this protocol; however, one should become familiar with the information in all of the other tabs.]

9. Using an FTP client, such as FileZilla, transfer the Refine folder created by phenix.refined from your working directory on the SSRL server to your local computer.

10. The Refine folder contains an electron density map file called "something_refine_###.mtz" and a structure file called "something_refine_###.pdb". Load these files into the program Coot to perform another round of model building and manipulation in Coot (see Section 3.4.5.1).

11. Continue the steps of model building and manipulation in Coot and model refinement in phenix.refine until your refinement statistics are acceptable.

12. Once you have a fully refined model, you are ready to deposit the model in the RCSB Protein Databank (Section 3.4.6).

3.4.6 Depositing the structure in the RCSB Protein Databank.

1. In a web browser, go to rcsb.org.

2. In the "Deposit" tab, click "wwPDB OneDep".

3. Select your country of residence.

4. Fill in the required information and then click "Start deposition".

5. Login with the deposition ID (provided in the "New deposition" email sent by RCSB).

6. Click "Continue to file upload".

7. If applicable, fill in the "Previous deposition ID" and "Previous deposition password" fields and select the data items you would like transferred to the current deposition from the previous deposition.

8. Upload the appropriate files for the deposition. The deposition will require a structure file in mmCIF format and structure factor file in mtz format.

   - To generate the mmCIF file, use either the RCSB software PDB_extract (https://pdb-extract.wwpdb.org), or generate the mmCIF file in phenix.refine by selecting "Write final refined model into mmCIF file" in the Output tab of phenix.refine.

   - For the mtz file, use the .mtz file from phenix.refine associated with the refinement you are depositing.

9. After uploading the files, examine the Validation Report created by RCSB and follow the instructions on the deposition page.

4. Concluding Remarks

Macrocyclic β-hairpin peptides provide useful model systems for studying the elusive peptide and protein oligomers associated with amyloid diseases. Understanding amyloid oligomer structures and their biophysical and biological properties is critical for understanding and developing treatments of amyloid diseases. Throughout this chapter, we outline simple synthetic methods for incorporating $^{\delta}$Orn turn linkers to constrain fragments of Aβ and *N*-methyl amino acids to limit uncontrolled aggregation of the peptide. These modifications create macrocyclic β-hairpin peptides derived from amyloidogenic peptides and proteins that self-assemble to form oligomers. The structures of these oligomers can be elucidated at high-resolution using X-ray crystallography, and the oligomers can be further studied by techniques such as SDS-PAGE and SEC.

Our laboratory has demonstrated the utility of these synthetic modifications to make macrocyclic β-hairpin peptides derived from a variety of amyloidogenic peptides and proteins (Salveson et al., 2016; R. K. Spencer et al., 2014, 2015; Wang et al., 2017; Yoo et al., 2016). These powerful tools have led to the discovery of oligomers—elucidated through SEC, SDS-PAGE, and X-ray crystallography—ranging from dimers (2 monomers) to dodecamers (12 monomers) to large annular pore-like structures. The oligomers formed by our macrocyclic β-hairpin peptides are far more homogeneous than native oligomers formed by full-length amyloidogenic peptides and proteins, which are inherently heterogeneous. The homogeneity of the oligomers formed by macrocyclic β-hairpin peptides allows for a more direct correlation of oligomer structure with oligomer biology. In our own laboratory, we have correlated the oligomers formed by our macrocyclic β-hairpin peptides with oligomers formed by full-length Aβ using the oligomer-specific antibody A11 (Kayed et al., 2003; Kreutzer, Yoo, et al., 2017). We envision that experiments of this sort can also be done for other amyloidogenic peptides and proteins.

Current studies in our laboratory also focus on better understanding the biological significance of the oligomeric assemblies that we create and correlating these assemblies

with biogenic Aβ oligomers and other oligomers of full-length Aβ. Antibodies feature heavily among the tools that we are using in these studies, and we are looking at the cross-reactivity of antibodies generated against our macrocyclic β-hairpin peptides with biogenic Aβ oligomers. Through these studies, we aim to learn what our macrocyclic β-hairpin peptides can reveal about the structures and biology of oligomers that occur in the brain in Alzheimer's disease.

The stepwise protocols provided herein can be adapted to other amyloidogenic peptides and proteins. We hope this chapter advances the study and understanding of amyloidogenic peptides and proteins and their relationship to amyloid diseases.

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Abbreviations:

DCM	methylene chloride
DMF	*N,N*-dimethylformamide
DIPEA	*N,N*-diisopropylethylamine
HCTU	[(6-chlorobenzotriazol-1-yl)oxy-(dimethylamino)methylidene]-dimethylazanium hexafluorophosphate
HBTU	[benzotriazol-1-yloxy(dimethylamino)methylidene]-dimethylazanium hexafluorophosphate
HATU	[dimethylamino(triazolo[4,5-b]pyridin-3-yloxy)methylidene]-dimethylazanium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
HOAt	1-hydroxy-7-azabenzotriazole
HFIP	1,1,1,3,3,3-hexafluoroisopropanol
NMM	*N*-methylmorpholine
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
TFA	trifluoroacetic acid
TIPS	triisopropylsilane
DMSO	dimethyl sulfoxide
TEMED	*N,N,N',N'*-tetramethylethylenediamine

APS	ammonium persulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size-exclusion chromatography

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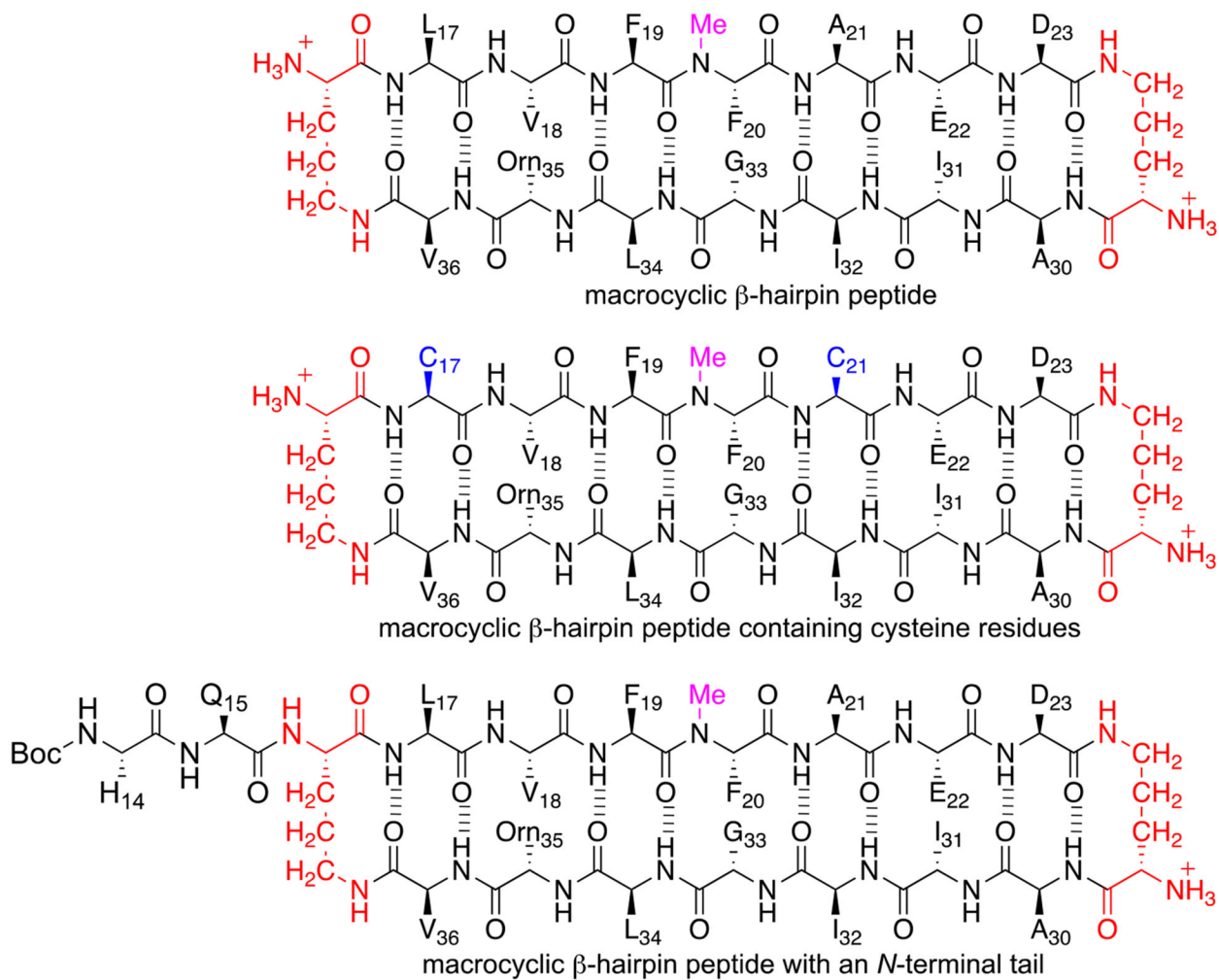
Fig. 1.
Aβ-derived macrocyclic β-hairpin peptides highlighting the use of *N*-methyl-phenylalanine at position 20 (magenta) to prevent uncontrolled aggregation, δ-ornithine turn mimics (red) to induce β-hairpin formation, and cysteine residues at positions 17 and 21 (blue) to permit disulfide crosslinking.
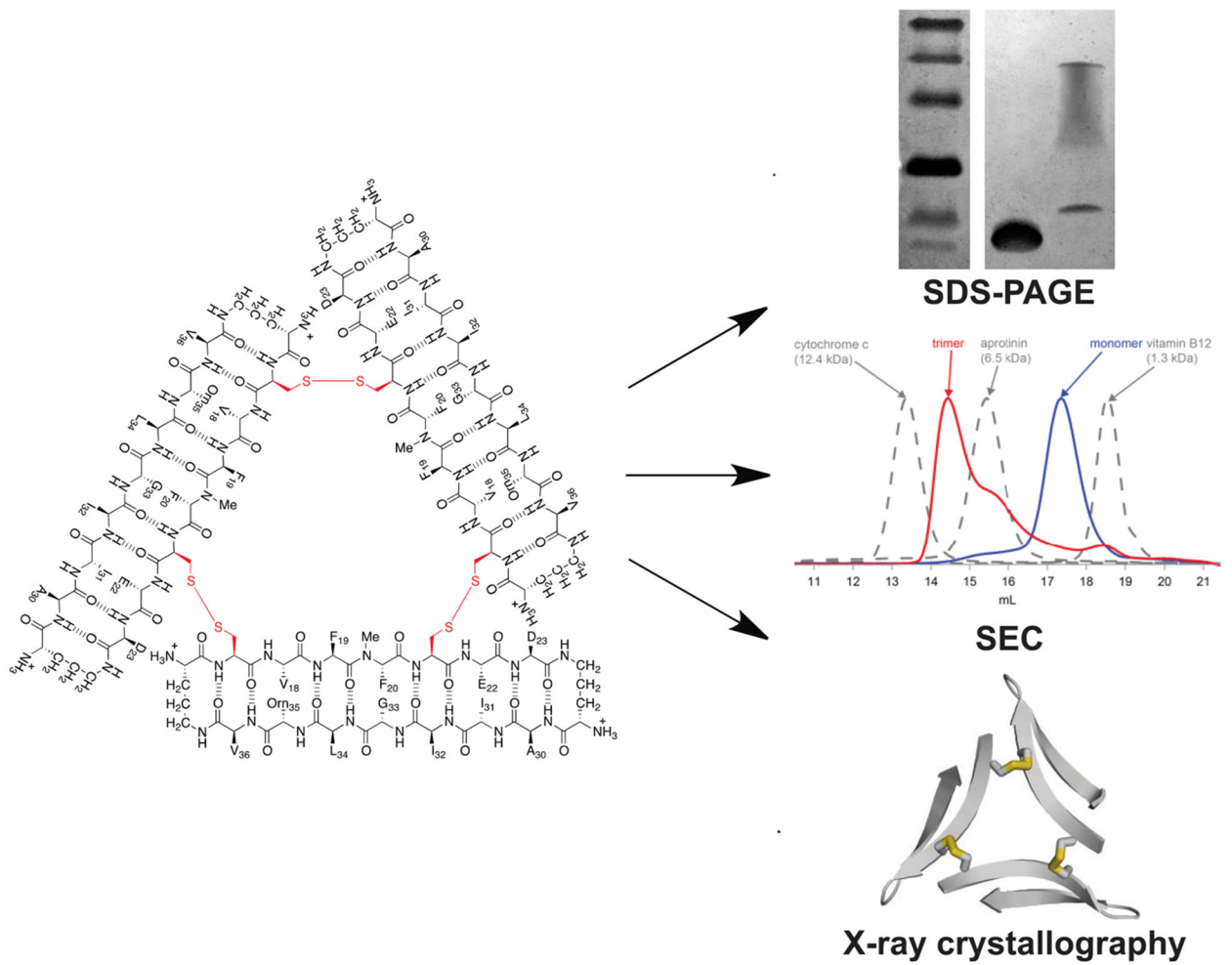
Fig. 2.
Three techniques used to study the supramolecular assembly of macrocyclic β-hairpin peptides and crosslinked oligomers: X-ray crystallography, SEC, and SDS-PAGE.
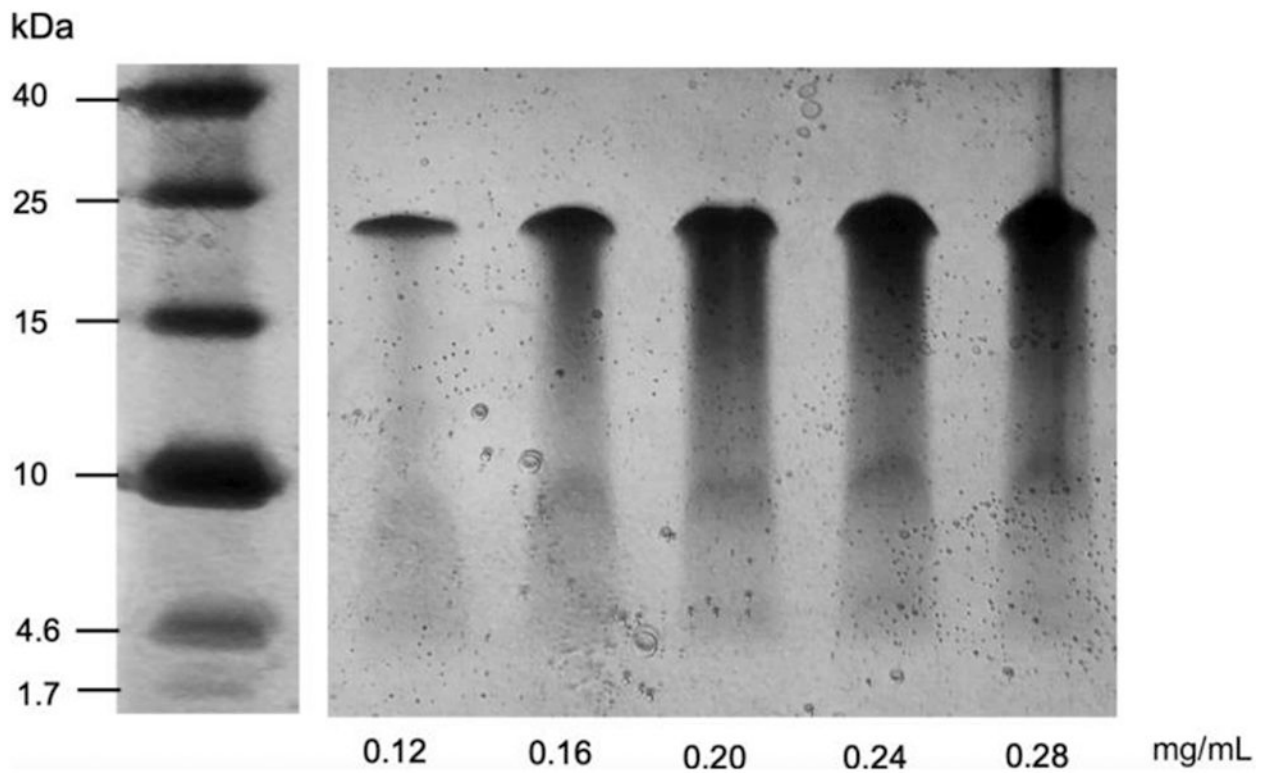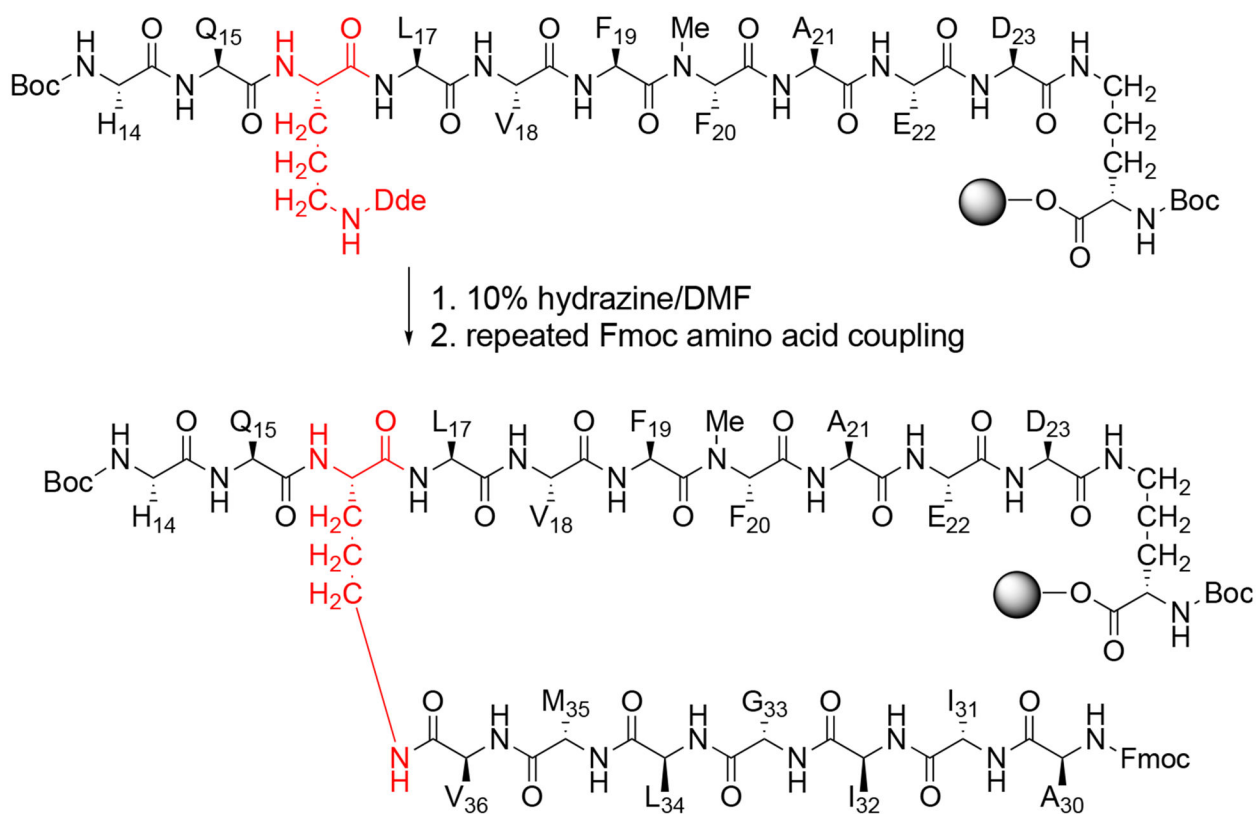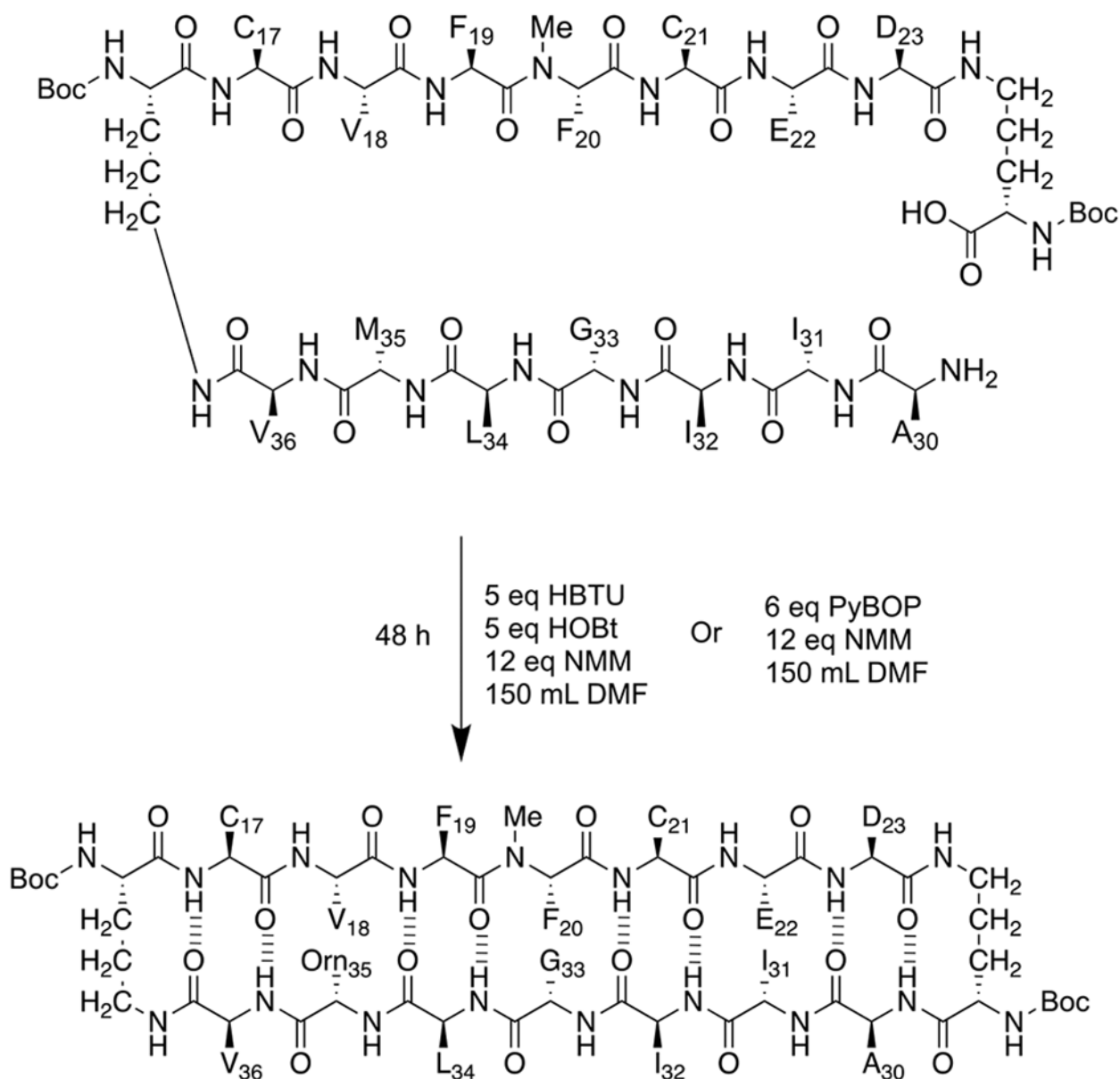
Fig. 3.
Silver-stained SDS-PAGE gel showing a macrocyclic β-hairpin peptide at varying concentrations. Streaking of the bands illustrates the equilibrium of the oligomer with monomer and lower-order oligomers.

Scheme 1.
Removal of the Dde protecting group in the synthesis of a macrocyclic β-hairpin peptide that incorporates additional *N*-terminal residues.

Scheme 2.
Cyclization to generate a macrocyclic β-hairpin peptide.
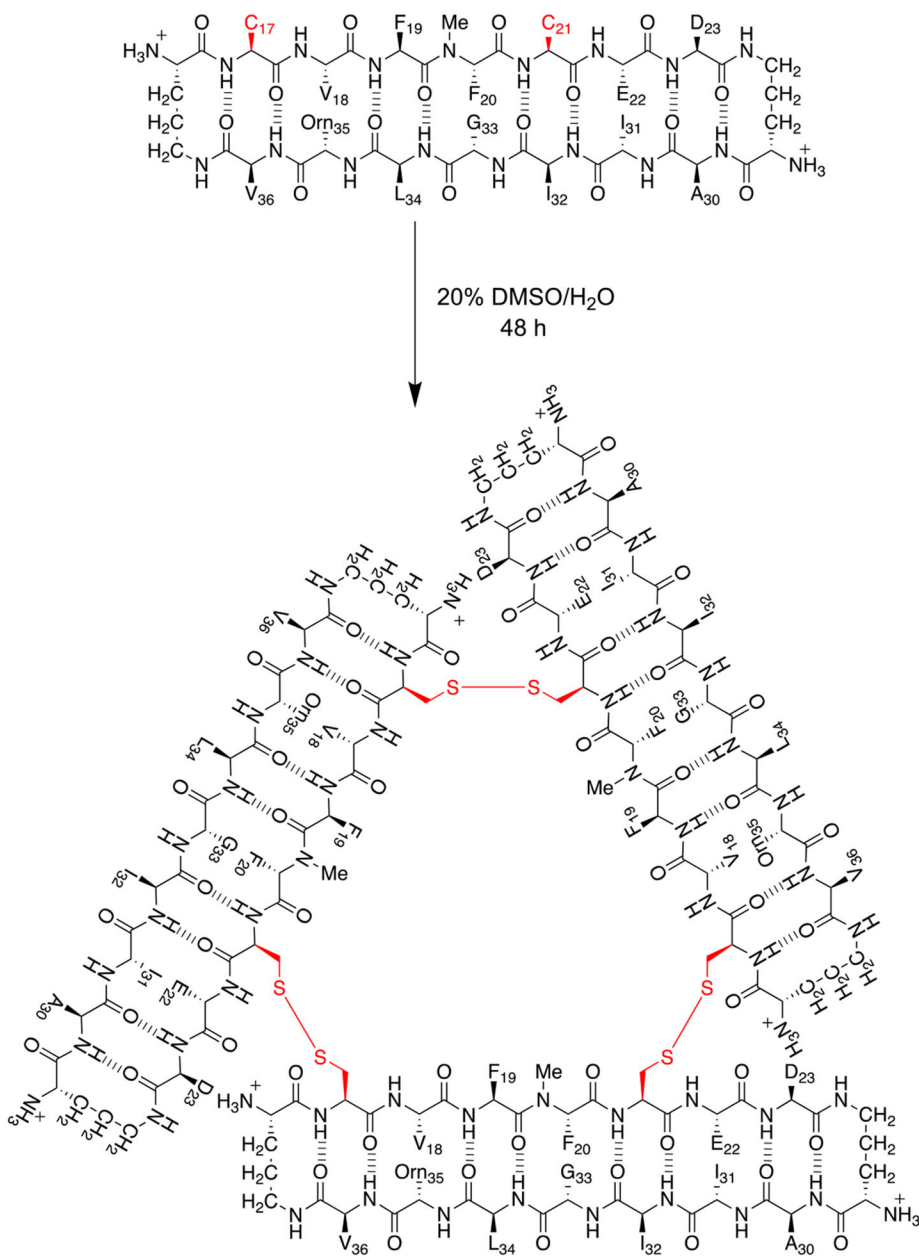
Scheme 3.
Oxidation of a macrocyclic β-hairpin peptide containing cysteine residues to generate a covalently stabilized trimer with disulfide crosslinks.