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Developing Macrocyclic β-Hairpin Peptide Mimics to Elucidate the Structures of Aβ Oligomers in Alzheimer's Disease

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UNIVERSITY OF CALIFORNIA,
IRVINE


Developing Macrocyclic β-Hairpin Peptide Mimics to Elucidate the Structures of Aβ
Oligomers in Alzheimer's Disease


DISSERTATION


submitted in partial satisfaction of the requirements
for the degree of


DOCTOR OF PHILOSOPHY

in Chemistry


by


Tuan Dilshad Samdin


Dissertation Committee:
Professor James S. Nowick
Professor Jennifer A. Prescher
Professor Robert Spitale


2021

# DEDICATION

To

my parents

my wife

my son

my friends

At my beginning,

*"The story so far: In the beginning the Universe was created. This has made a lot of people very angry and been widely regarded as a bad move."*

Douglas Adams
*The Restaurant at the End of the Universe*

strolling through the middle,

*"Life is pleasant. Death is peaceful. It's the transition that's troublesome."*

Isaac Asimov

Somewhere before the end.

*"A day can really slip by when you're deliberately avoiding what you're supposed to do."*

Bill Watterson
*Calvin and Hobbes*

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# ACKNOWLEDGEMENTS

Thank you, James, for giving me a chance to chase my dream. A part of me always feared that I would never be able to define myself on my own terms. You have given me the opportunity to be curious and creative, the chance to disagree and fail, and the support and the environment I needed to grow and be bold. Your mentorship and kindness has helped me grow not only as a scientist, but as a person.

For my labmates and friends, thank you. There are times where it seemed as though every effort, experiment, and thought I had was doomed to end in failure. But you were always there to remind me that my beatings would only continue until my morale improved. So, despite having weeks if not months of work often crumble in failure, I knew that you would be right there with me as I tried again. And again. And again. Even when it was clear that all I had before me was a path filled with late, late nights and frustration in repairing instruments, you never allowed me to quit. Thank you, Nick, for teaching me SPPS and helping me take my first steps as an independent scientist. Thank you, Adam and Michał, for allowing me to annoy you with a hundred-and-one questions about X-ray crystallography. Thank you, Will, for keeping me grounded and giving me perspective when I felt lost and cast-off. And thank you, Sheng, Xing, Maj, Gretchen, and Chelsea for your friendship, the laughter, and the chance to share my "Tuansense".

Thank you, Mom and Dad for your love and encouragement. I could not imagine a path to where I am today without the sacrifices you've made and the support you've given me. Thank you.

And lastly, to my wife and son. I cannot ever express how grateful I am for your love and patience. Pursuing a PhD was my choice, and with that choice I have been incredibly selfish. I've missed more than a few moments and milestones, so now I hope to build us a future where the only hard choices to be made, will be deciding who gets the last cookie.

Tuan Dilshad Samdin

## Education

**University of California, Irvine** – James S. Nowick Laboratory
Ph.D. Candidate in Chemistry                                    Expected August 2021

**University of California, Berkeley** – Seung-Wuk Lee Laboratory
B.A. Molecular and Cell Biology                                              August 2016

## Research Experience

**Chemistry Department, UC Irvine -** Graduate Researcher            August 2016 – present

- Led, mentored, and trained 5 undergraduate research assistants and collaborated with a team of 9 graduate students across 5 interdisciplinary projects to design, synthesize, and assess macrocyclic peptides capable of mimicking toxic proteins in Alzheimer's and other neurodegenerative diseases using: solid-phase peptide synthesis, microwave-assisted peptide synthesis (Liberty Blue), molecular cloning, bacterial transformation, protein expression, SDS-PAGE, western blot, FPLC, SEC, HPLC, mass spectrometry, CD, X-ray crystallography, and cytotoxicity assays
- Independently planned, managed, and executed 3 projects under strict funding timelines to meet deliverables, provided regular group updates through presentations and written reports
- Communicated my scientific results and research progress in 5 scientific publications, and at 6 regional and national scientific conferences to multidisciplinary audiences
- Optimized the group's synthetic workflow by writing and obtaining a grant for $40,000 to purchase a microwave peptide synthesizer that subsequently increased crude product purity and yield, and reduced waste production and total synthesis time

**Bioengineering Department, UC Berkeley -** Undergraduate Researcher        August 2014 – May 2016

- Worked within a team of 6 interdisciplinary researchers with backgrounds in chemistry, biology, materials science, and bioengineering to design and optimize the production and chemical sensitivity of a novel bacteriophage-based biosensor for volatile organic compounds and environmental pollutants using: phage display, molecular cloning, PCR, bacterial transformation, bacterial culture, and AFM
- Presented our research to outside stakeholders, demonstrating how our biosensor could be paired with their smartwatch and smartphone products, resulting in two years of support for the laboratory
- Documented and maintained standard operating procedures for the safe and proper use of laboratory techniques, chemicals, and equipment
- Mentored and trained 3 undergraduate research assistants and 5 master's students in common laboratory techniques and use of equipment

## Publications

- <u>Samdin, T. D.</u>; Guaglianone, G.; Kreutzer, A. G.; Freites, J. A.; Wierzbicki, M.; *et.al.*; Nowick, J. S. A β-Barrel-Like Tetrameric Formed by a Macrocyclic β-Hairpin Peptide Derived from Aβ. **Manuscript in preparation**

- <u>Samdin, T. D.</u>; Kreutzer, A. G.; Nowick, J. S. Exploring Amyloid Oligomers with Peptide Model Systems. Curr. Opin. Chem. Biol. **2021**. *64*, 106–115

- Haerianardakani, S.; Kreutzer, A. G.; Salveson, P. J.; <u>Samdin, T. D.</u>; Nowick, J. S. Phenylalanine Mutation to Cyclohexylalanine Facilitates Triangular Trimer Formation by β-Hairpins Derived from Aβ. J. Am. Chem. Soc. **2020**, *142 (26)*, 20708–20716.

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- Kreutzer, A. G.; <u>Samdin, T. D.</u>; Guaglianone, G.; Spencer, R. K.; Nowick, J. S. X-Ray Crystallography Reveals Parallel and Antiparallel β-Sheet Dimers of a β-Hairpin Derived from Aβ16–36 That Assemble to Form Different Tetramers X-Ray Crystallography Reveals Parallel and Antiparallel β-Sheet Dimers of a β-Hairpin Derived from $A\beta_{16-36}$. ACS Chem. Neurosci. **2020**, *11*, 2340–2347

- Lee, J. H.; Fan, B.; <u>Samdin, T. D.</u>; Monteiro, D. A.; Desai, M. S.; Scheideler, O.; Jin, H. E.; Kim, S.; Lee, S. W. Phage-Based Structural Color Sensors and Their Pattern Recognition Sensing System *ACS Nano* **2017**, *11*, 3632–3641.

## Leadership Experience

**Graduate Safety Team, UC Irvine** – Fellow                                    January 2020 – present
- Led a team of 6 graduate students to identify and address organizational and communication deficiencies between EH&S and more than 40 research laboratories in the Chemistry department
- Implemented and executed plan to update safety training protocols, transition the department to a new chemical inventory system, and create a database of standard operating procedures for the safe handling of hazardous chemicals
- Served as a liaison between graduate students and the department's faculty and staff on matters related to laboratory safety and laboratory operations under Covid-19 restrictions

**Business & Consulting for STEM Scientists Group, UC Irvine** – Co-founder    January 2020 – present
- Organized and led a group of 30 STEM graduate students seeking opportunities to transition from the benchtop to roles in business and consulting
- Networked with and recruited industry professionals to serve as speakers, held mock case interviews, and resume workshops for students

**Chemistry TA Mentorship Program, UC Irvine**                          August 2019 – present
- Mentored and trained 12 first-year graduate students on effective classroom management and teaching strategies as part of an ongoing effort to improve undergraduate learning experiences in Chemistry

**Graduate Student Teaching Assistant, UC Irvine**                      August 2016 – present
- Taught multiple undergraduate lectures and laboratories for the Chemistry, Pharmaceutical Sciences, and Biological Sciences departments
- Prepared weekly discussion lectures, worksheets, and study guides, and held weekly office hours for students

**Berkeley Student Cooperative** - Assistant Building Manager           July 2014 – March 2016
- Managed a complex of 300 residents, maintained and oversaw a $10,000/year community fund for resident events, apartment renovations, and community outreach and service projects
- Organized and held recruitment events for students from diverse and disadvantaged backgrounds including: low-income, first-generation, disabled, undocumented, and minority students of color seeking affordable and inclusive housing

## Notations, Awards, and Fellowships

- Graduate Dean's Dissertation Fellowship, University of California, Irvine $5,000          2020
- Safety Fellow, Chemistry Department, University of California, Irvine          2020 – 2021
- Peptide Therapeutics Symposium Travel Grant Awardee – 6 out of 24 presenters          2019
- Ford Foundation Predoctoral Fellowship Honorable Mention – 350 applications out of 2000          2018
- Ford Foundation Predoctoral Fellowship Honorable Mention – 400 applications out of 2000          2017

- Amgen Scholar, Medicinal Chemistry and Pharmaceutical Sciences, UCI – 1 out of 15 candidates  2017
- Teagle Foundation Scholarship – $40,000 distributed over 4 years                              2012 – 2016

## References

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# ABSTRACT OF THE DISSERTATION

Developing Macrocyclic β-Hairpin Peptide Mimics to Elucidate the Structures of Aβ Oligomers

in Alzheimer's Disease

By

Tuan D. Samdin

Doctor of Philosophy in Chemistry

University of California, Irvine

2021

Professor James S. Nowick

Chapter 1 provides a brief review on the application and use of peptide model systems to mimic the properties and structures of oligomers formed by amyloidogenic peptides and proteins. Oligomers formed by amyloidogenic peptides and proteins are noted for the significant heterogeneity they display in their stability, structure, and stoichiometry. Obtaining high-resolution structures of the oligomers formed by amyloidogenic peptides and proteins is crucial for advancing our knowledge of the molecular basis of the diseases these assemblies are associated with.

The assembly of amyloidogenic peptides and proteins such as the β-amyloid peptide (Aβ), α-synuclein, huntingtin, tau, and islet amyloid polypeptide (IAPP) into amyloid fibrils and oligomers is directly linked to amyloid diseases, such as Alzheimer's, Parkinson's, and

Huntington's diseases, frontotemporal dementias, and type II diabetes. Although amyloid oligomers have emerged as especially important in amyloid diseases, high-resolution structures of the oligomers formed by full-length amyloidogenic peptides and proteins have remained elusive. Investigations of oligomers assembled from fragments or stabilized β-hairpin segments of amyloidogenic peptides and proteins have allowed investigators to illuminate some of the structural, biophysical, and biological properties of amyloid oligomers This chapter provides context for much of the work described in this dissertation and highlights recent advances in the study of amyloidogenic oligomers and challenges currently faced by the field.

To mimic and investigate the structures, biological, and biophysical properties of endogenous Aβ oligomers, the Nowick laboratory has developed macrocyclic β-hairpin peptides as model systems. These macrocyclic β-hairpin peptides comprise β-strand segments of residues that are derived from the full-length sequence of Aβ. To constrain the peptide to a macrocycle and stabilize a β-hairpin conformation, we use disulfide bridges and $^{\delta}$Orn turn units. To prevent the uncontrolled aggregation of these Aβ derived peptide our group typically incorporates an *N*-methyl group on one β-strand. In studying the assembly of these Aβ derived macrocyclic β-hairpin peptides, our laboratory has been able to report several crystallographic structures of dimers and trimers that further assemble to form tetramers, hexamers, octamers, and dodecamers. We believe that these unique structures reflect some of the immense variation and heterogeneity present in the structures of endogenous Aβ oligomers.

This thesis describes my efforts to build upon the archetypal macrocyclic β-hairpin model system developed by the Nowick laboratory to better mimic endogenous Aβ oligomers. In the course of my doctoral studies, I have developed and studied three new macrocyclic β-hairpin model systems. These model systems incorporate additional residues beyond the $Aβ_{16-36}$ residue

segments typically studied by our laboratory and explore the incorporation of α-methyl groups in lieu of *N*-methyl groups to limit uncontrolled aggregation. These new macrocyclic β-hairpin model systems have also provided high-resolution insight into the unique ways in which Aβ β-hairpins can assemble to form oligomers.

Chapter 2 describes the synthesis, solution-phase biophysical studies, and X-ray crystallographic structures of hexamers formed by macrocyclic β-hairpin peptides derived from the central and *C*-terminal regions of Aβ, which bear "tails" derived from the *N*-terminus of Aβ. Soluble oligomers of Aβ, are thought to be the synaptotoxic species responsible for neurodegeneration in Alzheimer's disease. Over the last 20 years, evidence has accumulated that implicates the *N*-terminus of Aβ as a region that may initiate the formation of damaging oligomeric species. Our laboratory has previously studied macrocyclic β-hairpin peptides derived from Aβ$_{16–22}$ and Aβ$_{30–36}$, capable of forming hexamers that can be observed by X-ray crystallography and SDS-PAGE. To better mimic oligomers of full length Aβ, we use an orthogonal protecting group strategy during the synthesis to append residues from Aβ$_{1–14}$ to the parent macrocyclic β-hairpin peptide **1**, which comprises Aβ$_{16–22}$ and Aβ$_{30–36}$. The *N*-terminally extended peptides **N+1**, **N+2**, **N+4**, **N+6**, **N+8**, **N+10**, **N+12**, and **N+14** assemble to form dimers, trimers, and hexamers in solution-phase studies. X-ray crystallography reveals that peptide **N+1** assembles to form a hexamer that is composed of dimers and trimers. These observations are consistent with a model in which the assembly of Aβ oligomers is driven by hydrogen bonding and hydrophobic packing of the residues from the central and *C*-terminal regions, with the *N*-terminus of Aβ accommodated by the oligomers as an unstructured tail.

Chapter 3 describes the investigation of oligomers formed by macrocyclic β-hairpin peptides derived from Aβ$_{12–40}$. Peptides **1a–i** and **2a–d**, derived from residues 12–40, demonstrate

solution-phase and crystallographic assembly dependent on β-hairpin stability and intermolecular interactions mediated by N-terminal residues 12–14. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) studies reveal that peptides **1a** and **1d** assemble to form octamers, peptides **1h** and **2a** assemble to form octamers and tetramers, and peptide **2d** assembles to form tetramers. X-ray crystallographic studies of peptide **2a** reveals the assembly of a β-barrel-like tetramer stabilized by edge-to-edge hydrogen bonding and hydrophobic packing. Additional evidence for the assembly of a β-barrel-like octamer is also present within the crystal lattice. Replica-exchange molecular dynamics (REMD) simulations show that this tetramer can accommodate residues 23–29 as a loop and additional residues from the N- and C-terminal region of Aβ. Molecular dynamics simulations reveal that this REMD modeled tetramer can disrupt a lipid membrane and facilitate water permeation in a pore-like fashion. These observations provide novel insight into the mechanisms by which endogenous Aβ oligomers may assemble and damage neuronal membranes in the Alzheimer's brain.

Chapter 4 describes studies of macrocyclic β-hairpin peptides comprising β-strands of Aβ residues 17–22 and 30–36. The incorporation of an *N*-methyl group on position 20, in peptides derived from 17–22 and 30–36, alters the hydrogen bonding edges and assembly of triangular trimers formed by these peptides. To better mimic the hydrogen boding edges of β-hairpins formed by unmodified Aβ we investigated substitution of the *N*-methyl group on position 20 with an α-methyl group on position 19. The incorporation of α-methyl amino acids into macrocyclic β-hairpin peptides derived from presents an opportunity to study the structures of oligomers that may more closely resemble endogenous Aβ oligomers.

# Chapter 1ª

# Exploring Amyloid Oligomers with Peptide Model Systems

**INTRODUCTION**

The assembly and aggregation of peptides and proteins into fibrils and oligomers is a hallmark of amyloid diseases.[1–4] Amyloid diseases are diverse in their prevalence, presentation, and symptoms, encompassing neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and Creutzfeldt-Jakob disease, as well as other diseases, such as type II diabetes and transthyretin amyloidosis.[4–8] Amyloid fibrils are common molecular assemblies associated with amyloid diseases, and are characterized by their insolubility, affinity for Congo red dye and thioflavin T (ThT), cross-β X-ray diffraction pattern, and extended networks of in-register parallel β-sheets.[9–14] The biophysical and structural properties of amyloid fibrils and their roles in disease have been studied extensively (**Figure 1.1 A–E**).[15–18] Yet, as investigations into amyloid fibrils have proceeded over the last four decades, evidence has increasingly pointed toward amyloid oligomers as the damaging species responsible for disease progression.

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Some of the initial evidence for the presence of amyloid oligomers arose from solution-phase biophysical characterization of amyloid plaques isolated from Alzheimer's disease brains.[19,20] These early studies reported the presence of soluble assemblies of the β-amyloid peptide, Aβ, in addition to insoluble fibrils. The formation of these Aβ assemblies and their relevance to disease pathology was supported by subsequent *in vitro* studies, which confirmed their assembly and neurotoxicity, and ultimately led to the formalization of the hypothesis that amyloid oligomers are causative agents in the neurodegeneration associated with Alzheimer's disease.[21–29]

Oligomers of Aβ are soluble and heterogeneous — varying significantly in their structure, stability, and stoichiometry. Antiparallel β-sheets and β-hairpins are thought to be building blocks of many amyloid oligomers. Amyloid oligomers vary vastly in size, comprising as few as two or three, or as many as dozens or more molecules. Many of these features have been observed for oligomers formed by other amyloidogenic peptides and proteins, such as α-synuclein, polyglutamine, islet amyloid polypeptide (IAPP), and tau.[4,30–33]

Only one atomic-resolution structure of an oligomer formed by the full-length sequence of Aβ$_{42}$ has been reported thus far (**Figure 1.1 F**).[34] Carulla and co-workers reported the NMR-based structure of an Aβ$_{42}$ tetramer and provided additional evidence for its assembly into an octamer. The tetramer is a six-stranded antiparallel β-sheet comprising two β-hairpins of Aβ$_{42}$ surrounding two antiparallel β-strands of Aβ$_{42}$. Although the disease relevance of this oligomer has not yet been established, the tetramer represents the first high-resolution structure of an oligomer of full-length Aβ. In light of the large number of unique amyloid fibril structures reported and deposited in the Protein Data Bank (PDB), the lack of other high-resolution structures of amyloid oligomers represents an immense gap in our understanding of amyloid diseases.[15–18]

Peptide model systems derived from the sequences of amyloidogenic peptides and proteins have emerged as useful tools to investigate amyloid oligomers and bridge this gap in our understanding. These peptides are designed to mimic the biological and biophysical properties of native amyloid oligomers. Unlike native amyloid oligomers, the oligomers formed by these peptide model systems often have the added benefits of increased homogeneity and stability, facilitating high-resolution characterization of many of the oligomers that form. This review highlights recent investigations of peptide model systems that have helped advance our knowledge of amyloid oligomers.

**A**
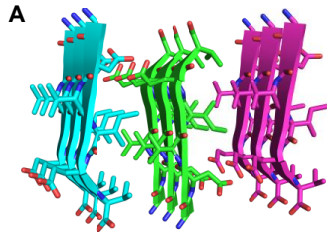
fibril steric zipper of αB crystallin$_{95–100}$
PDB 3SGS

**B**

fibril of Aβ$_{1–40}$
PDB 6SHS

**C**
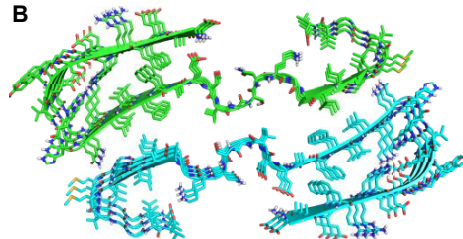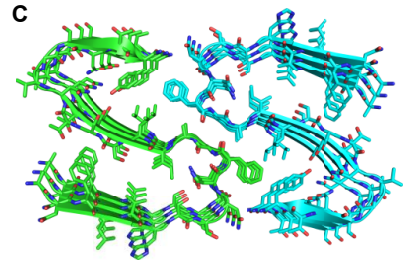
fibril of IAPP$_{13–37}$
PDB 6Y1A

**D**
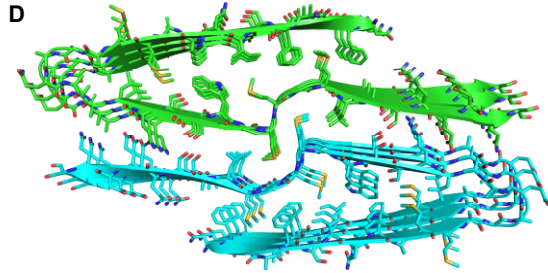
fibril of TDP-43$_{311–360}$
PDB 6N37

**E**
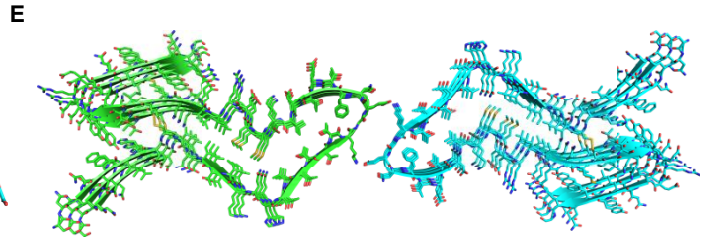
fibril of hPrP$_{170–229}$
PDB 6LNI

**F**
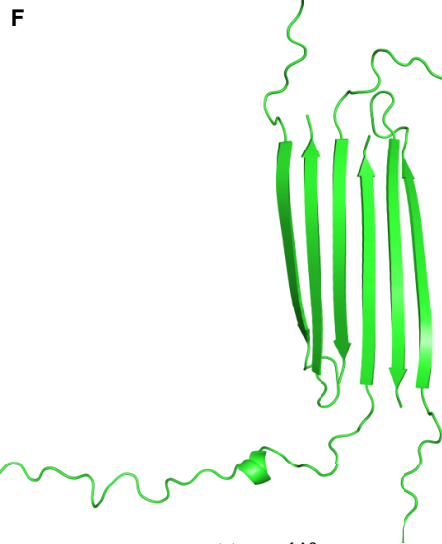
tetramer of Aβ$_{1–42}$
PDB 6RHY

octamer model of Aβ$_{1–42}$

**H**

SOD1$_{28–38}$
PDB 5IIW

**G**
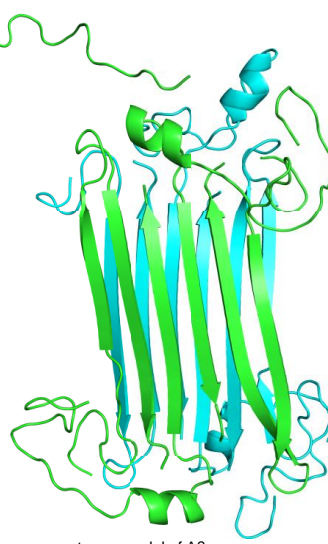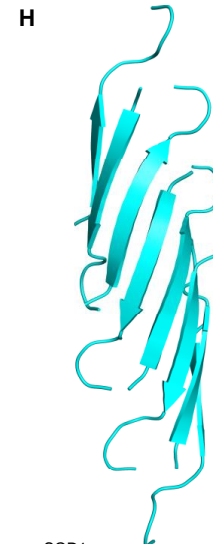
αB crystallin$_{90–100}$
PDB 3SGO

**I**

hPrP$_{177–182}$ and hPrP$_{211–216}$
PDB 4E1I

4

**Figure 1.1**. Structures of fibrils and oligomers formed by amyloidogenic peptides. **A.** Fibril-like assembly of αB crystallin$_{95-100}$; X-ray crystallographic structure. **B–E.** Fibril-like assemblies of Aβ$_{1-40}$, IAPP$_{13-37}$, TDP-43$_{311-360}$, and hPRP$_{170-229}$; Cryo-EM structures. **F.** Tetramer and octamer formed by Aβ$_{1-42}$; NMR structure and NMR-based model. **G–I.** Oligomers of αB crystallin$_{90-100}$, SOD1$_{28-38}$, and hPRP$_{177-182}$ crosslinked with hPRP$_{211-216}$; X-ray crystallographic structures.

## THE FRAGMENT-BASED APPROACH

X-ray crystallographic investigations of short fragments of amyloidogenic peptides and proteins provide one strategy for studying the molecular interactions governing fibril and oligomer assembly at high resolution. Eisenberg and co-workers reported several high-resolution structures of fibril-forming peptides that are derived from amyloidogenic peptides and proteins.[35–37] Using this fragment-based approach, Eisenberg and co-workers determined the X-ray crystallographic structures of two oligomers composed of eleven-residue peptide fragments derived from αB crystallin and superoxide dismutase 1 (SOD1) **(Figure 1.1 G, H)**.[38–40] The αB crystallin fragment assembles into a cylindrical barrel composed of six antiparallel β-strands, termed a cylindrin by the investigators **(Figure 1.1 G)**. The SOD1 fragment assembles into a corkscrew-like arrangement of antiparallel β-strands **(Figure 1.1 H)**. Surewicz and co-workers determined the structure of a hexamer composed of disulfide-linked antiparallel β-strands comprising two six-residue peptide fragments derived from human prion protein **(Figure 1.1 I)**.[41] Intermolecular hydrogen bonding between antiparallel β-strands and the close packing of hydrophobic residues are common features that stabilize each of these oligomers.

These fragment-based models are significant, because oligomers of full-length amyloidogenic peptides and proteins are thought to be composed of antiparallel β-sheets and β-hairpins.[42–45] Structures of oligomers assembled from the fragments of amyloidogenic peptides and proteins can serve as models for naturally occurring disease-relevant oligomers formed by full-length amyloidogenic peptides and proteins. Oligomers of full-length amyloidogenic peptides and proteins have not yet yielded to X-ray crystallography or CryoEM. Although CryoEM has

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emerged as a powerful tool in the structural biology of amyloid fibrils (**Figure 1.1 B–E**), thus far the oligomers of full-length amyloidogenic peptides and proteins have proven too small or too heterogenous for structural elucidation by CryoEM.[15–18,46,47]

## STABILIZED β-HAIRPINS

β-Hairpins are building blocks of some of the oligomers formed by amyloidogenic peptides and proteins.[34,48,49] Model systems consisting of stabilized β-hairpins are valuable tools for studying amyloid oligomers, because they provide control of secondary and tertiary structure while allowing quaternary structure to form through self-assembly. Härd and co-workers demonstrated that three different amyloidogenic peptides and proteins can form β-hairpins and determined the structures of these β-hairpins. In 2008, Härd, Hoyer, and co-workers elucidated the NMR structure of a β-hairpin formed by $A\beta_{40}$ by using an affibody to sequester and stabilize the β-hairpin (**Figure 1.2 A**).[42] In this β-hairpin, residues 17–23 and 30–36 of Aβ hydrogen bond to form an antiparallel β-sheet, while the intervening residues, 24–29, form a loop (**Figure 1.2 B**). The remaining *N*- and *C*-terminal residues are unstructured. Härd and co-workers also used affibodies to stabilize and determine the structures of β-hairpins formed by α-synuclein and IAPP.[43,44]

In further studies, Härd and co-workers investigated the biological, biophysical, and structural properties of oligomers formed by a covalently stabilized analogue of the Aβ β-hairpin that they previously reported.[45] In this analogue, $Ala_{21}$ and $Ala_{30}$ are mutated to cysteines to enable formation of a disulfide bridge (**Figure 1.2 C**). Oligomers formed by this disulfide-stabilized Aβ β-hairpin mimicked some of the characteristics of oligomers of unmodified Aβ — morphology by transmission electron microscopy (TEM), assembly by size-exclusion chromatography (SEC) and SDS-PAGE, and cytotoxicity toward neuronally derived SH-SY5Y cells. These oligomers were also recognized by oligomer-specific antibodies used to recognize native Aβ oligomers isolated from the brains of Alzheimer's patients and transgenic mice. These

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findings are significant, because they demonstrate that conformationally stabilized β-hairpin monomers of Aβ can assemble to form oligomers that recapitulate the properties of biologically relevant Aβ oligomers. Solid-state NMR spectroscopy revealed that a disulfide-stabilized β-hairpin comprising Aβ$_{16-42}$ forms a barrel-shaped hexamer **(Figure 1.2 D)**.[49] In this oligomer, a hydrophobic core forms at one end of the assembly by the packing of hydrophobic residues from the central and *C*-terminal regions of Aβ. Intermolecular antiparallel β-sheets form between Aβ$_{34-36}$ and Aβ$_{39-42}$ at one end of the barrel; the β-hairpin loops of each monomer comprise the other end of the barrel. This series of investigations of Aβ β-hairpins illustrates how stabilized β-hairpin peptides can be used to model and study the properties and structures of amyloid oligomers.[40,43,46]

Our laboratory has developed macrocyclic β-hairpin peptides as model systems to learn about the structure, and biological and biophysical properties of the oligomers formed by full-length amyloidogenic peptides and proteins.[50] The macrocyclic β-hairpin peptides consist of two peptide β-strands from the amyloidogenic peptide or protein that are constrained to a macrocycle by a δ-linked ornithine ($^{\delta}$Orn) turn unit and linked by a loop or a second $^{\delta}$Orn turn unit **(Figure 1.2 E–G)**.[51] An *N*-methyl group on one of the β-strands prevents uncontrolled aggregation, and thus facilitates oligomer formation. X-ray crystallographic studies of macrocyclic β-hairpin peptides derived from sequences such as Aβ, β$_2$-microglobulin, and α-synuclein have revealed the formation of dimers and trimers that further assemble to form tetramers, hexamers, octamers, nonamers, and dodecamers **(Figure 1.3)**.[51–60] Wetzel and co-workers have developed β-hairpin model systems of polyglutamine derived peptides to better understand the role of polyglutamine folding and aggregation in Huntington's disease using D-Pro-Gly turn units and *N*-methyl amino acids.[61]

In our initial investigations of Aβ oligomers, we prepared and studied macrocyclic β-hairpin peptides derived from Aβ$_{17-36}$. In 2014, we reported a macrocyclic β-hairpin peptide containing Aβ$_{17-23}$ and Aβ$_{30-36}$.[51] X-ray crystallography revealed that this peptide assembles into trimers that further assemble to form a sandwich-like hexamer and a ball-shaped dodecamer **(Figure 1.2 E & Figure 1.3 A–C)**. X-ray crystallographic studies of a homologous macrocyclic β-hairpin, incorporating the Aβ$_{24-29}$ loop, revealed that the peptide assembles to form trimers that further assemble into ball-shaped dodecamers, and five dodecamers further assemble to form an annular pore **(Figure 1.2 G & Figure 1.3 G–I)**.[52] In subsequent studies, we covalently stabilized the trimers formed by the macrocyclic β-hairpin peptide containing Aβ$_{17-23}$ and Aβ$_{30-36}$ with disulfide-bridges **(Figure 1.2 H)**.[53] These covalently stabilized trimers assemble in solution, forming hexamers and dodecamers by SEC and SDS-PAGE. The covalent trimers are toxic to SH-SY5Y cells and are recognized by the amyloid oligomer-specific antibody A11, suggesting that they may recapitulate the topology of Aβ oligomers occurring in the Alzheimer's brain.[62] X-ray crystallography revealed that the trimers form a hexamer, a dodecamer, and an annular pore comprising six dodecamers **(Figure 1.3 D–F)**. Recently, we found that incorporation of a cyclohexylalanine residue in place of a phenylalanine residue promotes folding of Aβ derived macrocyclic β-hairpins, further stabilizes trimers formed by the β-hairpins, and promotes formation of hexamers and dodecamers **(Figure 1.3 J–L)**.[56] We are now using antibodies generated against these synthetic Aβ oligomer mimics to probe biogenic Aβ oligomers from brain tissue.

We have also studied macrocyclic β-hairpin peptides derived from Aβ$_{16-36}$, in which the β-strands adopt a different alignment than the β-hairpin peptides derived from Aβ$_{17-36}$. These studies have revealed the assembly of toxic oligomers in both the crystal state and in solution, without the need for covalent stabilization through disulfide bridges.[57] A macrocyclic β-hairpin containing

A$\beta_{16-22}$ and A$\beta_{30-36}$ assembles to form dimers and trimers that further assemble into hexamers that can be observed in SDS-PAGE and by X-ray crystallography **(Figure 1.2 E & Figure 1.3 M–O)**. A related macrocyclic β-hairpin peptide containing A$\beta_{16-22}$ and A$\beta_{30-36}$ assembles in the crystal state to form trimers that further assemble into a dodecamer **(Figure 1.2 E & Figure 1.3 P–Q)**.[58]

Current efforts in our laboratory seek to incorporate more residues from full-length A$\beta_{40}$ or A$\beta_{42}$ into our macrocyclic β-hairpin model systems, to better reflect oligomers formed by full-length Aβ. We recently incorporated A$\beta_{1-14}$ as an *N*-terminally extended "tail" to the hexamer-forming macrocycle comprising A$\beta_{16-22}$ and A$\beta_{30-36}$ **(Figure 1.2 F)**. In studying a series of homologs bearing *N*-terminal tails, we found that residues from the *N*-terminus of Aβ do not disrupt oligomer assembly and likely form an unstructured tail **(Figure 1.3 R)**.[59] X-ray crystallographic studies of a macrocyclic β-hairpin peptide from A$\beta_{16-36}$ that incorporates the A$\beta_{23-29}$ loop revealed the assembly of parallel and antiparallel β-sheet dimers that further assemble to form a sandwich-like tetramer and a twisted β-sheet tetramer, with the latter packing to form an octamer **(Figure 1.2 G & Figure 1.3 S–W)**.[60]

Collectively, our studies of β-hairpin peptides derived from A$\beta_{16-36}$, A$\beta_{17-36,}$ and other amyloidogenic peptides and proteins have provided a multitude of distinct oligomer structures and revealed the unique ways in which β-hairpins can assemble to form compact oligomers stabilized by edge-to-edge hydrogen bonding and hydrophobic packing. Other laboratories have also reported various structures of Aβ fibrils, oligomers, and monomer formed by β-hairpins with different β-strand alignments.[34,42,48,63] We believe our structures reflect some of the immense variation and heterogeneity in the structures of endogenous amyloid oligomers, because many behave like oligomers of full-length amyloidogenic peptides and proteins in biological and biophysical experiments.
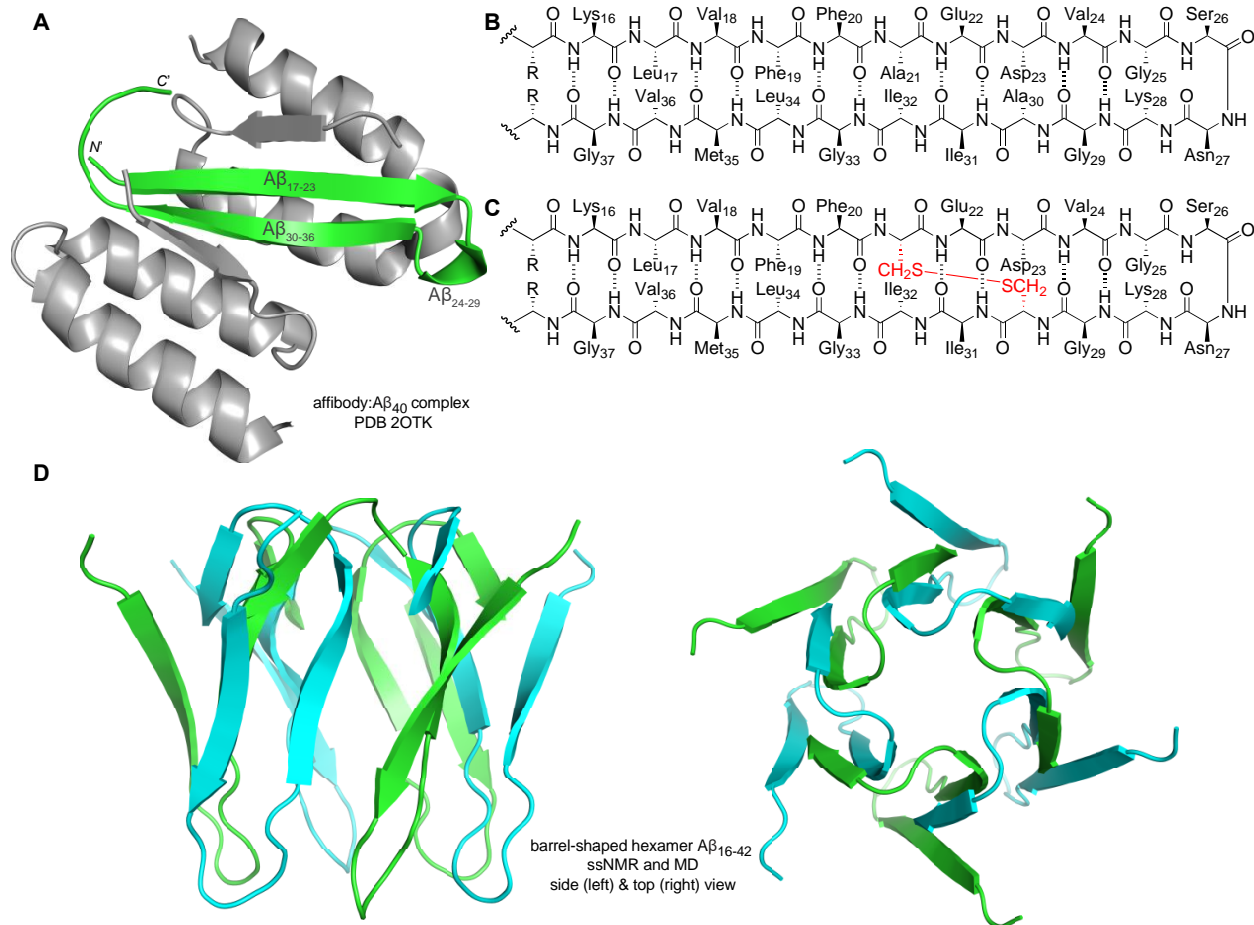
**A**

Aβ17-23
Aβ30-36
Aβ24-29
*C'*
*N'*

affibody:Aβ40 complex
PDB 2OTK

**B**

Lys16　Val18　Phe20　Glu22　Val24　Ser26
Leu17　Phe19　Ala21　Asp23　Gly25
R
Gly37　Val36　Met35　Leu34　Gly33　Ile32　Ala30　Lys28
R
Ile31　Gly29　Asn27

**C**

Lys16　Val18　Phe20　Glu22　Val24　Ser26
Leu17　Phe19　Asp23　Gly25
CH2S——SCH2
R
Gly37　Val36　Met35　Leu34　Gly33　Ile32　Ile31　Gly29　Lys28
R
Asn27

**D**

barrel-shaped hexamer Aβ16-42
ssNMR and MD
side (left) & top (right) view

δOrn　　　R1,R2 = H/Me　　　δOrn

**E**

**F**

**G**

δOrn　　　　loop

**H**
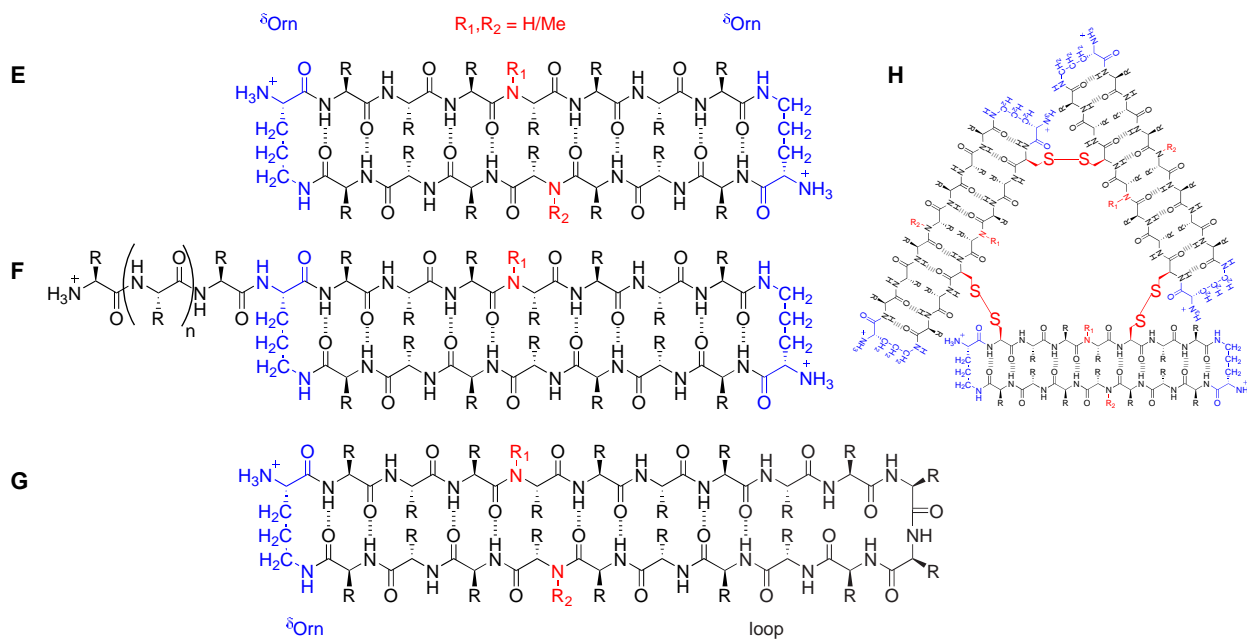
**Figure** 1.2. **A.** NMR structure of an Aβ$_{40}$ β-hairpin stabilized by an affibody. **B.** Alignment of the Aβ$_{40}$ β-hairpin. **C.** Disulfide stabilization of the Aβ$_{40}$ β-hairpin. **D.** NMR-based model of a barrel-shaped hexamer formed by a disulfide stabilized Aβ$_{16–40}$ β-hairpin. **E–H.** Macrocyclic β-hairpins and disulfide-stabilized β-hairpins derived from amyloidogenic peptides and proteins.
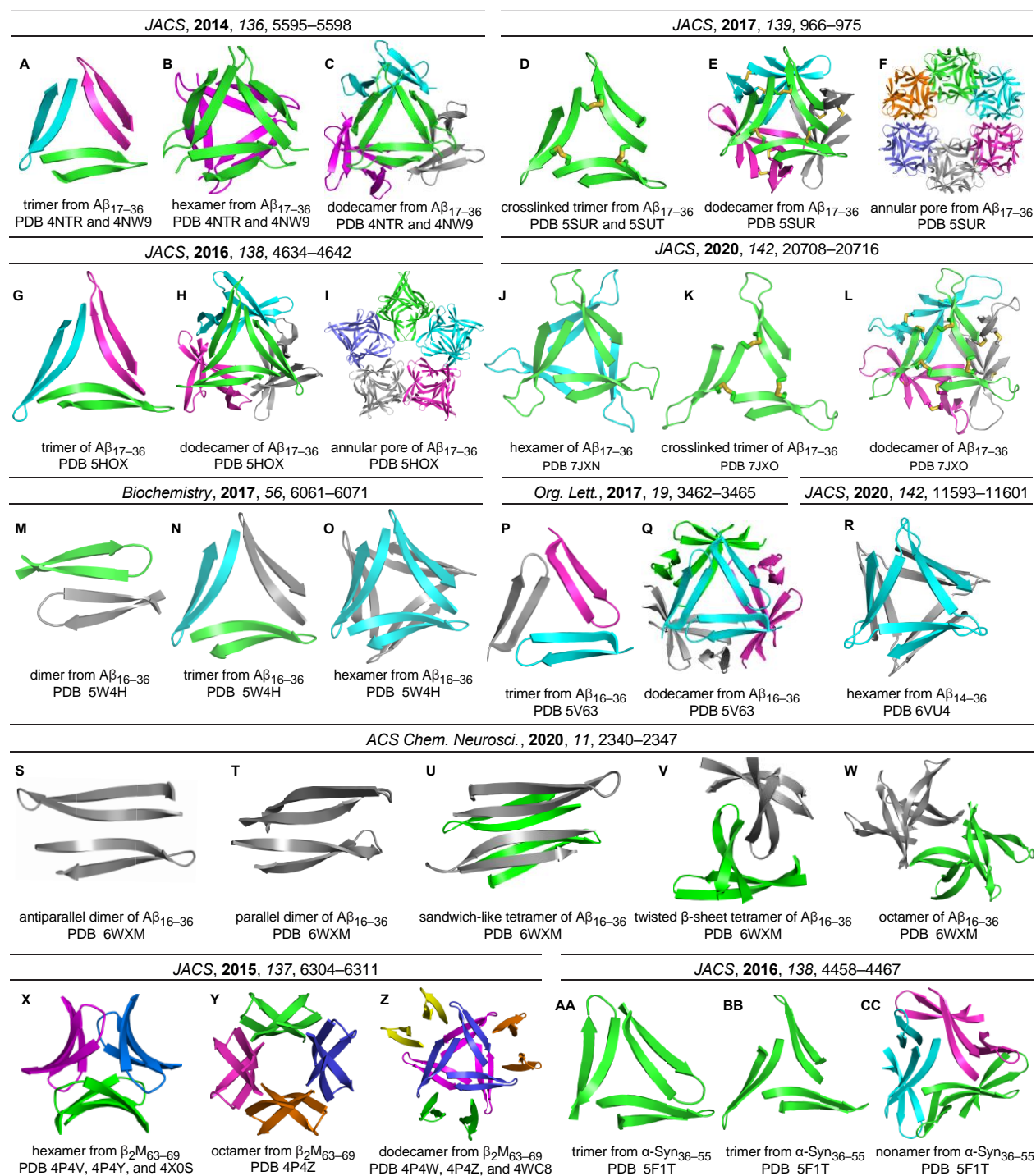
**Figure 1.3**. X-ray crystallographic structures of oligomers formed by macrocyclic β-hairpin peptides derived from Aβ, β2-microglobulin, and α-synuclein. **A–L.** Trimers, hexamers, dodecamers, and annular pores formed by macrocyclic β-hairpin peptides derived from Aβ$_{17-36}$. **M–W.** Dimers, trimers, tetramers, hexamers, octamer, and dodecamer derived from Aβ$_{16-36}$. **X–Z.** Hexamer, octamer, and dodecamer derived from β2-microglobulin. **AA–CC.** Trimers and nonamer derived from α-synuclein.

**COMPUTATIONAL TOOLS FOR STUDYING AMYLOID OLIGOMERS AND FIBRILS**

Molecular modeling can provide valuable insights into amyloid oligomer formation and structure by allowing the visualization, interpretation, and prediction of the conformations, motions, and interactions of the peptides and proteins involved.[4] These simulations allow observation of that which cannot be examined directly through experimentation and can complement experimental studies to provide deeper insights. For example, residues that had to be excluded from the peptide model systems to facilitate characterization by X-ray crystallography can be restored for study in molecular dynamics simulations. Okuno and co-workers thus used dissipative particle dynamics to restore $A\beta_{9-16}$ and $A\beta_{37-42}$ to a dodecamer-forming macrocyclic $\beta$-hairpin peptide comprising $A\beta_{17-36}$ **(Figure 1.3 H)**.[52,64] The simulations revealed that residues $A\beta_{37-42}$ can pack to form a stabilizing hydrophobic core in the central cavity of the dodecamer. Our laboratory has similarly made use of replica-exchange molecular dynamics simulations to probe whether residues absent from the design of our macrocyclic $\beta$-hairpin peptides can be accommodated by the structures of the oligomers that form.[51,55,57,60]

The protein force fields used in molecular dynamics and other forms of molecular modeling were not developed for amyloid oligomers and have limited ability to accurately model the conformation, folding, and size of amyloidogenic peptides and proteins.[65–68] Shaw and co-workers used experimental NMR and SAXS data from amyloid oligomers to improve parameters for torsion angles, and protein and water van der Waals interactions, to produce a force field, a99SB-*disp*, that more accurately simulates disordered proteins such as $A\beta_{40}$.[69] Improved algorithms for simulating the conformations of intrinsically disordered proteins and intrinsically disordered regions also promise to provide enhanced insights into amyloid oligomer formation. Recently, Petersson and co-workers reported the PyRosetta-based algorithms AbinitioVO and FastFloppyTail, which allow for the accurate prediction of protein structure across

a wide array of folds and degrees of order.[70] We anticipate that improvements in force fields and algorithms for predicting conformational ensembles will cross-fertilize other studies that use peptide model systems and full-length peptides and proteins and thus contribute to a better understanding of amyloid oligomers.

Molecular docking simulations have guided the development of ligands that bind amyloid oligomers that may ultimately lead to new imaging probes or drugs for Alzheimer's disease or other amyloid diseases. Thus, X-ray crystallographic structures of trimers and hexamers formed by macrocyclic β-hairpin peptides comprising A$\beta_{17-36}$ **(Figure 1.3 A, B, D, G)**, have been used as targets for docking studies of triphenylmethane dyes, fluorescent probes, and therapeutic ligands for Aβ oligomers.[71–74] Docking simulations of the triphenylmethane dye, crystal violet, with the structure of our covalently-stabilized trimer derived from A$\beta_{17-36}$ **(Figure 1.3 D)** produced a model for molecular recognition that guided structure-activity relationship studies.[71] Our laboratory is currently using the results of these computational and experimental studies to develop novel chemical probes for biogenic Aβ oligomers.

Computational tools are also valuable in identifying amyloidogenic regions of peptides and proteins by identifying features that drive aggregation and assembly, such as hydrophobicity, β-sheet character, a prevalence of aromatic residues, and low-charge content.[75] A number of algorithms, computational tools, and databases have been developed to assess these characteristics for a given peptide or protein sequence.[76] Tools such as TANGO, WALTZ-DB 2.0, and Cordax assess and quantify the aggregation potential of a given sequence.[77–80] Results from this type of primary sequence analysis can supplement and direct structure activity relationship studies of amyloid fibrils and oligomers.[36–38,75] These tools further our understanding of the ever-growing "amyloidome," which extends beyond disease and underlies many normal cellular, bacterial, and fungal processes.[81]

**CONCLUSION**

The amyloid state of peptides and proteins is an active and fascinating frontier of peptide and protein science for chemical and structural biologists alike. The ever-growing ties between amyloidogenic peptides and proteins and cellular function and disease inspires curiosity, and the resistance of these peptides and proteins to characterization using conventional techniques and tools drives innovation. Until the high-resolution observation of oligomers of full-length amyloidogenic peptides and proteins becomes widely feasible, peptide model systems that approximate and mimic endogenous oligomers will remain one of the best tools for dissecting their structural, biological, and biophysical properties. The growing understanding of amyloid oligomers provided by these studies will further our knowledge of amyloid diseases and bolster efforts to develop diagnostics and drugs.

## ANNOTATIONS

3**.     Ke PC, Zhou R, Serpell LC, Riek R, Knowles TPJ, Lashuel HA, Gazit E, Hamley IW, Davis TP, Fändrich M, et al.: **Half a century of amyloids: Past, present and future**. *Chem Soc Rev* 2020, **49**:5473–5509.

This review highlights investigations of pathological, functional, and artificial amyloids in disease, structural biology, microbiology, and engineering. Significant attention is given to structural and biophysical characterization of amyloid fibrils and their assembly.

4**.     Nguyen PH, Ramamoorthy A, Sahoo BR, Zheng J, Faller P, Straub JE, Dominguez L, Shea J-E, Dokholyan N V., Simone A De, et al.: **Amyloid oligomers: A joint experimental/computational perspective on Alzheimer's disease, Parkinson's disease, type II diabetes and amyotrophic lateral sclerosis**. *Chem Rev* 2021, doi:10.1021/acs.chemrev.0c01122.

This review examines *in vitro*, *in vivo*, computational, and pharmacological studies of oligomers formed by Aβ, tau, α-synuclein, IAPP, and superoxide dismutase 1 in Alzheimer's disease, Parkinson's disease, type II diabetes, and amyotrophic lateral sclerosis. The authors highlight how empirical observations of amyloid oligomers have led to and supplemented computational studies of oligomer formation and interactions.

25*.     Cline EN, Bicca MA, Viola KL, Klein WL: **The amyloid-β oligomer hypothesis: Beginning of the third decade**. *J Alzheimer's Dis* 2018, **64**:S567–S610.

This review summarizes evidence for the roles of Aβ oligomers in neurodegeneration and synaptotoxicity in Alzheimer's disease. The authors highlight *in vitro*, *in vivo*, and clinical evidence that points to Aβ oligomers as the damaging species in Alzheimer's disease.

34**.   Ciudad S, Puig E, Botzanowski T, Meigooni M, Arango AS, Do J, Mayzel M, Bayoumi

M, Chaignepain S, Maglia G, et al.: **Aβ(1-42) tetramer and octamer structures reveal edge conductivity pores as a mechanism for membrane damage**. *Nat Commun* 2020, **11**:1–14.

This paper reports the first atomic-resolution structure of a tetramer formed by full-length Aβ$_{42}$ and provides additional evidence for the formation of an octamer. The structure of the tetramer is the only structure of an oligomer of full-length Aβ that has been deposited in the Protein Data Bank (PDB).

39*.    Sangwan S, Zhao A, Adams KL, Jayson CK, Sawaya MR, Guenther EL, Eisenberg DS: **Atomic structure of a toxic , oligomeric segment of SOD1 linked to amyotrophic lateral sclerosis ( ALS )**. *Proc Natl Acad Sci USA* 2017, **114**:8770–8775.

This paper reports the structure, biophysical, and biological properties of a corkscrew-like oligomer formed by an eleven-residue peptide fragment derived from SOD1.

50**.    Kreutzer AG, Nowick JS: **Elucidating the structures of amyloid oligomers with macrocyclic β-hairpin peptides: Insights into Alzheimer's disease and other amyloid diseases**. *Acc Chem Res* 2018, **51**:706–718.

This review highlights the use of macrocyclic β-hairpin peptides by the Nowick laboratory to mimic and study the structures, and biophysical and biological properties of oligomers formed by amyloidogenic peptides and proteins. X-ray crystallographic studies of these macrocyclic β-hairpin peptides have revealed the formation of dimers and trimers that further assemble to form tetramers, hexamers, octamers, nonamers, and dodecamers.

56*.    Haerianardakani S, Kreutzer AG, Salveson PJ, Samdin TD, Guaglianone GE, Nowick JS: **Phenylalanine mutation to cyclohexylalanine facilitates triangular trimer formation by β-hairpins derived from Aβ**. *J Am Chem Soc* 2020, **142**:20708–20716.

This paper reports the X-ray crystallographic structures and solution phase behavior of trimers, hexamers, and dodecamers formed by macrocyclic β-hairpin peptides derived from Aβ$_{17-36}$. Substitution of cyclohexylalanine for phenylalanine facilitates the formation of trimers and their covalent stabilization through disulfide crosslinks.

59*.    Samdin TD, Wierzbicki M, Kreutzer AG, Howitz WJ, Valenzuela M, Smith A, Sahrai V, Truex NL, Klun M, Nowick JS: **Effects of N-terminal residues on the assembly of constrained β-hairpin peptides derived from Aβ**. *J Am Chem Soc* 2020, **142**:11593–11601.

This paper reports the incorporation of Aβ residues 1–14 as an *N*-terminal "tail" appended to a macrocyclic β-hairpin peptide derived from Aβ$_{16-36}$ that forms a hexamer. The tailed-macrocyclic β-hairpin peptides are synthesized using an orthogonal protecting group strategy that allows incorporation of the *N*-terminal residues, and are characterized by SDS-PAGE and X-ray crystallography.

69*.    Robustelli P, Piana S, Shaw DE: **Developing a molecular dynamics force field for both folded and disordered protein states**. *Proc Natl Acad Sci U S A* 2018, **115**:E4758–E4766.

This paper describes the development of the a99SB-*disp* force field, which overcomes limitations of current protein force fields in accurately modeling amyloidogenic peptides and proteins in molecular dynamics simulations.

71*.    Salveson PJ, Haerianardakani S, Thuy-boun A, Yoo S, Kreutzer AG, Demeler B, Nowick JS: **Repurposing triphenylmethane dyes to bind to trimers derived from Aβ**. *J Am Chem Soc* 2018, **140**:11745–11754.

This paper reports the development of triphenylmethane dyes as ligands for trimers derived from Aβ. Detailed studies of the interactions of crystal violet and other triphenylmethane dyes with *C*3 symmetric trimers derived from Aβ$_{17-36}$ are described.

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# Chapter 2[b]

# Effects of *N*-Terminal Residues on the Assembly of Constrained β-Hairpin Peptides Derived from Aβ

**INTRODUCTION**

The first 16 residues of the β-amyloid peptide, Aβ, are thought to be important in the formation of fibrils and oligomers in Alzheimer's disease. These *N*-terminal residues are subject to many important mutations and post-translational modifications, and may interact with other proteins and metal cations.[1–7] These modifications and interactions have been observed to greatly affect Aβ fibril formation and oligomerization. Even without modification, these residues—$_1$DAEFRHDSGYEVHHQK$_{16}$—represent a significant number of amino acids capable of forming non-covalent interactions within and between monomers of Aβ, and should thus impact oligomerization.

Further supporting the ostensible importance of the *N*-terminus of Aβ are studies of the p3 peptide, an alternative cleavage product to full-length Aβ from the amyloid precursor protein.[8] The p3 peptide, which lacks residues 1–16 has even been described as "non-amyloidogenic", incapable of forming oligomers, devoid of any synaptotoxic effect, and

even neuroprotective.[9–13] However, a recent review from the Raskatov group has highlighted and summarized inconsistencies within the literature regarding the importance, biophysical, and biological properties of the p3 peptide.[14–16] In a subsequent investigation, the Raskatov group demonstrated that the p3 peptide is capable of assembling to form amyloidogenic fibrils and toxic oligomers.[17] These recent findings from Raskatov and others have shed some light on the importance of Aβ$_{1–16}$.

Elucidating the role of Aβ$_{1–16}$ in the formation of Aβ oligomers offers the promise of furthering our understanding of the molecular basis of Alzheimer's disease. Studying these oligomers is difficult, as these assemblies are heterogeneous—varying in structure, stability, and stoichiometry. Further compounding the challenge of studying Aβ, is its propensity to form insoluble fibrils, which are more stable than oligomers. Aβ oligomers are damaging to neurons and are thought to be present in the early stages of Alzheimer's disease before the emergence of any pathohistological hallmarks or dementia symptoms.[18–21]

The assembly of Aβ oligomers has been tied to the ability of the Aβ peptide to fold and adopt a β-hairpin conformation.[22,23] In 2008, Hard and Hoyer determined by NMR spectroscopy that Aβ$_{1–40}$ adopts a β-hairpin structure when bound to an affibody. In this structure, residues 17–23 and 30–36 hydrogen bond to form an antiparallel β-sheet, with residues 24–29 as a loop, and the *N*- and *C*-terminal residues as unstructured segments. Our own investigations of peptides derived from the central and *C*-terminal regions of Aβ have revealed that adoption of a β-hairpin conformation primes the peptide to assemble.[24–29]

In 2016, our laboratory reported peptide **1**, a β-hairpin mimic derived from Aβ$_{16–36}$ that forms hexamers that can be observed by X-ray crystallography and SDS-PAGE.[28] The peptide is cytotoxic toward SH-SY5Y cells, and cytotoxicity correlates with oligomer formation in structure-

activity studies. Peptide **1** contains the heptapeptide fragments Aβ$_{16–22}$ and Aβ$_{30–36}$ in the "top" and "bottom" β-strands, that are constrained to an antiparallel β-sheet by two turn units of δ-linked ornithine, $^{δ}$Orn (**Figure 2.1**).[30] The $^{δ}$Orn that links Glu$_{22}$ to Ala$_{30}$ replaces a loop comprising residues 23–29, while the $^{δ}$Orn that links Lys$_{16}$ to Val$_{36}$ helps constrain the peptide to a β-hairpin conformation. We incorporate an *N*-methyl group at the center of the top strand, on Phe$_{19}$, to prevent uncontrolled aggregation and fibril formation.
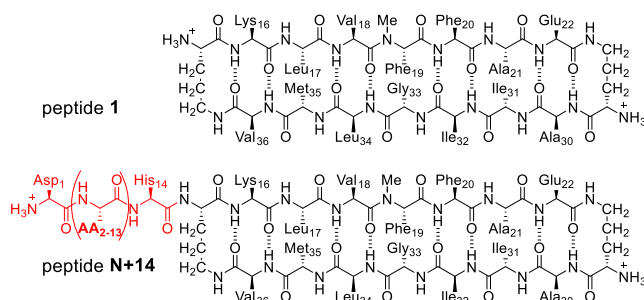


**Figure 2.1**. Extending the *N*-terminus of peptide **1** to include residues 1–14 as an *N*-terminal tail (red), giving peptide **N+14**. Residues in the tail comprise Asp$_1$, Ala$_2$, Glu$_3$, Phe$_4$, Arg$_5$, His$_6$, Asp$_7$, Ser$_8$, Gly$_9$, Tyr$_{10}$, Glu$_{11}$, Val$_{12}$, His$_{13}$, and His$_{14}$.

Although peptide **1** has provided insights into the structure, biophysical, and biological properties of hexamers formed by hydrophobic segments of Aβ*,* it lacks the important *N*-terminal region. In our initial report, REMD simulations of peptide **1** suggested that the hexamer could accommodate *N*- and *C*-terminal extensions without steric clashes.[28] In this investigation, we set out to test experimentally whether the hexamer could accommodate the *N*-terminus and to study the effects of the *N*-terminus on the structure, biophysical, and biological properties of the hexamer. Here we describe the synthesis of *N*-terminally extended homologues of peptide **1**. These *N*-terminally extended homologues bear "tails" of residues derived from Aβ$_{1–14}$ and vary in length from 1 to 14 residues. Peptide **N+14**, which contains the entire *N*-terminus, is depicted in **Figure 2.1**. Peptides **1**, **N+14**, and other *N*-terminally extended homologues form hexamers in SDS-PAGE, and trimers and dimers in size exclusion chromatography (SEC). Peptide **N+1**, which contains a tail comprising

Aβ₁₄, forms a hexamer composed of dimers and trimers in the X-ray crystallographic structure. These observations are significant because they may help bridge the gap in our knowledge of how Aβ oligomers assemble in the brain.

**RESULTS AND DISCUSSION**

*Synthesis of Peptide **N+14***. We synthesized peptide **N+14** by Fmoc-based solid phase peptide synthesis, using Fmoc-Orn(Dde)-OH to incorporate an *N*-terminal tail of residues derived from Aβ₁₋₁₄ (**Figure 2.2**). We began the synthesis by attaching Boc-Orn(Fmoc)-OH to 2-chlorotrityl resin. Residues 22 through 2 were then introduced by Fmoc-based SPPS using HCTU as the coupling reagent, with Fmoc-Orn(Dde)-OH replacing residue 15 in the natural sequence of Aβ. Residue 1 was coupled as Boc-Asp(*t*-Bu)-OH, capping the synthesis of the top strand. The Dde protecting group was then removed with 10% hydrazine in DMF, and residues 36 through 30 were coupled to residue 15 using Fmoc-based SPPS. We found microwave-assisted SPPS to be essential and used it for most of the coupling steps, other than attaching Boc-Orn(Fmoc)-OH to the resin and removing the Dde protecting group.[31] The final Fmoc protecting group was removed from residue 30, and the acyclic branched peptide was cleaved from the resin with a solution of 20% HFIP in DCM. The peptide was cyclized with PyBop in solution, and deprotected with treatment by trifluoroacetic acid (TFA). Purification by reverse-phase HPLC typically afforded approximately 12 mg peptide **N+14** as the trifluoroacetate salt.[32,33]
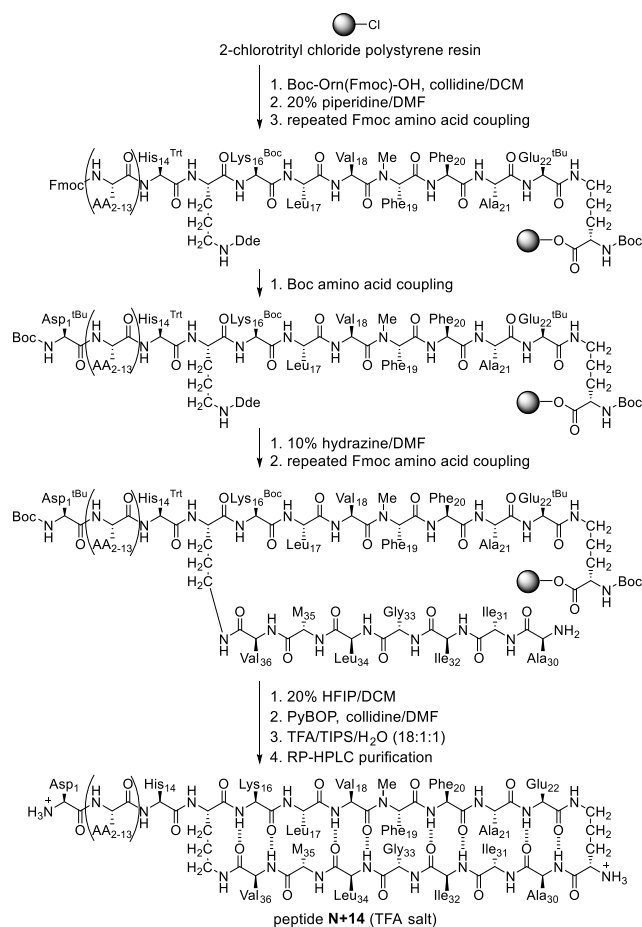
**Figure 2.2.** Synthesis of the *N*-terminally extended peptide, **N+14** derived from Aβ$_{1-36}$.

*Oligomerization and Folding of Peptides 1 and N+14.* We studied the assembly and folding

of peptide **1** and peptide **N+14** by SDS-PAGE, western blot, circular dichroism (CD) spectroscopy,

and a cytotoxicity assay with SH-SY5Y cells. Like the Aβ peptide, peptide **1** and peptide **N+14**

assemble to form oligomers in SDS-PAGE. We have previously reported peptide **1** (1.77 kDa) to

run in SDS-PAGE with a molecular weight consistent with that of a hexamer (**Figure 2.3A**).[28] The

band formed by peptide **1** is comet shaped and streaks downward from the 10 kDa ladder band,

suggesting that the hexamer may be in rapid equilibrium with lower molecular weight species. An

X-ray crystallographic structure of peptide **1** (PDB 5W4H) further corroborates the formation of a

hexamer. In contrast to peptide **1**, the *N*-terminally extended homologue peptide **N+14** (3.45 kDa)

forms two species by SDS-PAGE, which appear to be in slow equilibrium. One is an oligomer that

migrates to just below the 17 kDa ladder band, which we interpret as a hexamer. The other migrates

just above the 4.6 kDa ladder band, which we interpret as a dimer. The hexamer band formed by peptide **N+14** in silver-stained SDS-PAGE is more intense than the putative dimer band, suggesting that this oligomer predominates at the 75 µM concentration under which the gel was run. These SDS-PAGE studies show that the hexamer can accommodate residues 1–14, but that introduction of these residues appears to substantially reduce the rate of exchange between the hexamer and lower order species.
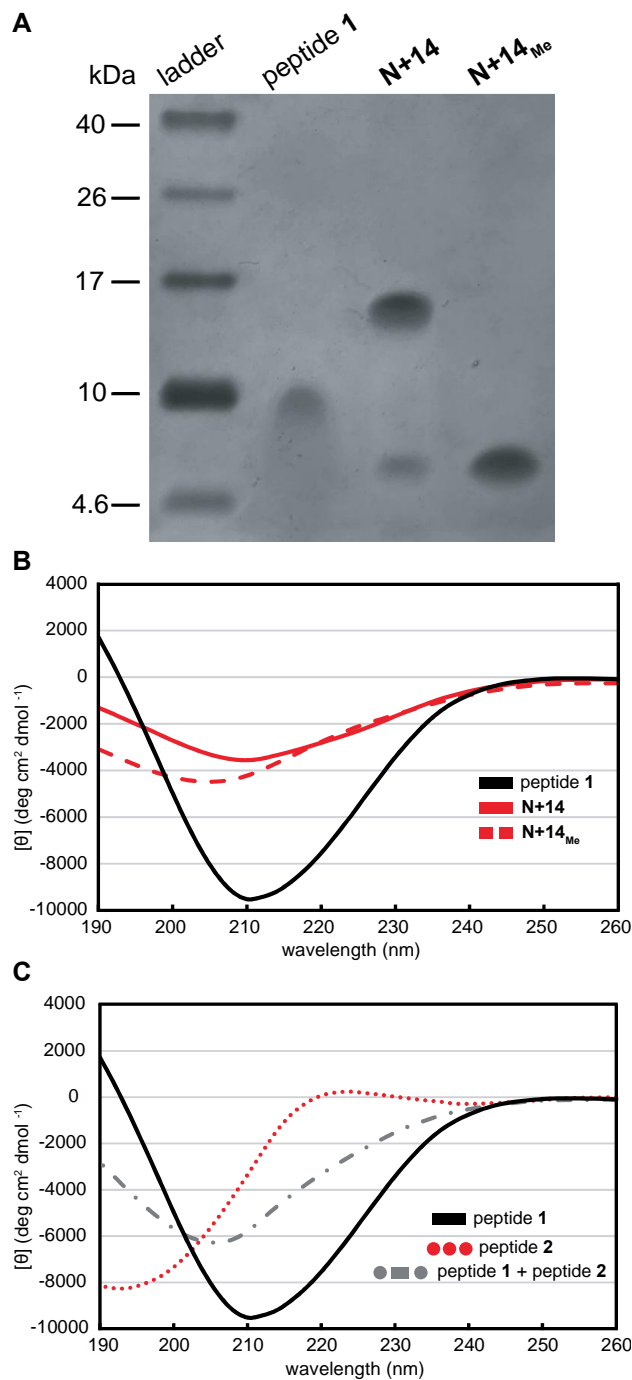
**Figure 2.3**. (A) Silver stained SDS-PAGE of peptides **1**, **N+14**, and **N+14Me**. SDS-PAGE was performed in Tris buffer at pH 6.8 with 2% (w/v) SDS on a 16% polyacrylamide gel with 75 μM solutions of peptide in each lane. (B) Circular dichroism (CD) spectra of peptides **1**, **2**, and an equimolar mixture of peptides **1** and **2**. CD spectra were acquired for each peptide at 50 μM in 10 mM phosphate buffer at pH 7.4; the ellipticity was normalized for the number of residues in each peptide.

To compare the oligomerization of peptide **N+14** to full-length Aβ, we performed

SDS-PAGE in a western blot against AβM1–42, using the 6E10 antibody to visualize the

bands generated by each peptide (**Figure S2.1**).[34–36] The 6E10 antibody recognizes the *N*-terminal epitope comprising residues $Arg_5$-$His_6$-$Asp_7$, which is shared by both peptide **N+14** and full-length Aβ.[37] $Aβ_{M1–42}$ shows a strong band for the monomer, as well as weaker bands for oligomers. In contrast, the hexamer band for peptide **N+14** predominates. This difference in oligomerization may reflect the lack of stabilizing conformational constraints in the $Aβ_{M1–42}$ peptide.[23] In peptide **N+14**, the $^δ$Orn turn units help prime the peptide for assembly by stabilizing a β-hairpin conformation.

In the hexamer formed by peptide **1**, the bottom edges of the monomer subunits hydrogen bond to each other. To test whether peptide **N+14** assembles to form a similar hexamer, we prepared and studied peptide **N+14<sub>Me</sub>**, a homologue of peptide **N+14** with an additional *N*-methyl group on $Gly_{33}$ (**Figure 2.4**). In the hexamer formed by peptide **1**, this additional *N*-methyl group has been shown to disrupt hydrogen bonding between $Gly_{33}$ of one monomer and $Ile_{31}$ of the adjacent monomer within the dimer subunit, thus interfering with assembly (**Figure S2.2**).[28,38] If peptide **N+14** assembles to form a hexamer similar to peptide **1**, *N*-methylation of $Gly_{33}$ should also disrupt assembly of the hexamer. By SDS-PAGE, peptide **N+14<sub>Me</sub>** migrates as a single band that runs parallel to the putative dimer band formed by peptide **N+14** (**Figure 2.3A**). In contrast to peptide **N+14**, peptide **N+14<sub>Me</sub>** does not form a hexamer band. The migration of peptide **N+14<sub>Me</sub>** confirms that *N*-methylation of $Gly_{33}$ in peptide **N+14** disrupts the assembly of higher order oligomers, and provides further evidence that peptide **N+14** assembles to form a hexamer similar in structure to peptide **1**.
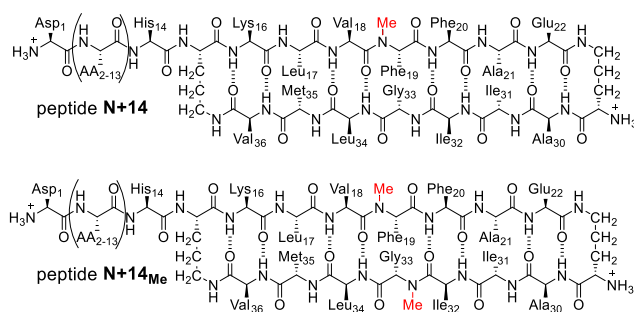
**Figure 2. 4**. Peptide **N+14<sub>Me</sub>** is a homologue of peptide **N+14** with an additional *N*-methyl group on Gly<sub>33</sub> to block hexamer formation.

Peptide **1** adopts a β-hairpin conformation in the crystallographic hexamer.[28] In aqueous solution—where size exclusion chromatography (SEC) studies have shown peptide **1** to exist as a mixture of monomer, dimer, and trimer—circular dichroism (CD) studies suggest that peptide **1** also adopts a β-hairpin conformation (**Figure 2.3B**).[28] The CD spectrum of peptide **1** displays a negative band centered at *ca.* 210 nm, with increasing ellipticity at lower wavelengths and a positive ellipticity below 192 nm. To evaluate the ability of peptide **N+14** to adopt a β-hairpin, we compared the CD spectrum of peptide **N+14** to that of peptide **1** (**Figure 2.3B**). The CD spectrum of peptide **N+14** is substantially shallower than the CD spectrum of peptide **1**, with a weaker negative band centered at a slightly lower wavelength and no positive ellipticity above 190 nm. This spectrum appears to reflect an ensemble of β-hairpin and random coil conformations. The CD spectrum of peptide **N+14<sub>Me</sub>** is similar to that of peptide **N+14**, suggesting that the additional *N*-methyl group on Gly<sub>33</sub> does not substantially alter the folding of peptide **N+14<sub>Me</sub>**.

To better understand the CD spectrum of peptide **N+14**—in particular how residues 1–14 affect the β-hairpin formed by residues 16–22 and 30–36—we synthesized peptide **2**, the *C*-terminal amide of Aβ<sub>1-14</sub> (H-DAEFRHDSGYEVHH-NH<sub>2</sub>). The CD spectrum of peptide **2** shows a strong negative band at *ca.* 190 nm and a weak positive band centered at *ca.* 220 nm, reflecting a predominantly random coil conformation (**Figure 2.3C**). To test whether the spectrum of peptide **N+14** represents a linear combination of peptide **1** and

peptide **2**, we combined both peptides in equimolar concentrations and acquired the CD spectrum of the mixture. The CD spectrum of the mixture shows a negative band centered at *ca.* 208 nm with no positive band above 190 nm. Like the CD spectrum of peptide **N+14**, the spectrum of the mixture shows a diminished β-sheet-like conformation relative to peptide **1**.

To evaluate the cytotoxicity of peptide **N+14**, we compared it to peptide **1** in an LDH release assay against neuronally derived SH-SY5Y cells. We had previously reported that peptide **1** was toxic at concentrations of 50 µM or higher.[28] Peptide **N+14** proved less cytotoxic than peptide **1**, even though it contains more residues from the sequence of full-length Aβ (**Figure S2.3**). The addition of residues 1–14 to peptide **1** does not enhance cytotoxicity, but rather diminishes the cytotoxicity. We envision that the cytotoxicity of peptide **1** is predominantly driven by the hydrophobic residues from the central region of Aβ, 16–22 and 30–36, and their interactions with cell membranes. These hydrophobic membrane interactions are likely offset by the hydrophilic tail in peptide **N+14**.

*Oligomerization and Folding of Shorter N-Terminally Extended Homologues*. To gain additional insights into the effects of *N*-terminal extension on the assembly of peptide **1**, we prepared seven additional *N*-terminally extended homologues of peptide **N+14** with shorter *N*-terminal tails: peptides **N+1**, **N+2**, **N+4**, **N+6**, **N+8**, **N+10**, and **N+12** (**Figure 2.5**). The CD spectra of these peptides all show minima between 210 and 220 nm (**Figure 2.6**). The minima are generally deeper for the peptides bearing shorter *N*-terminal extensions (**N+1** through **N+4**) (**Figure 2.6A**), and shallower for the peptides bearing longer *N*-terminal extensions (**N+6** through **N+12**) (**Figure 2.6B**). These differences reflect increased random coil character of the larger *N*-terminal extensions. The depth of the minimum of peptide **N+1** ($\Theta$ = -6190 deg cm$^2$ dmol$^{-1}$) is substantially less than that of

peptide **1** ($\Theta$ = -9516 deg cm$^2$ dmol$^{-1}$). This difference cannot be explained by a random coil conformation of the additional single residue, but rather suggests that *N*-acylation of the $\delta$-linked ornithine turn unit diminishes the β-sheet folding of the macrocycle. [39,40]
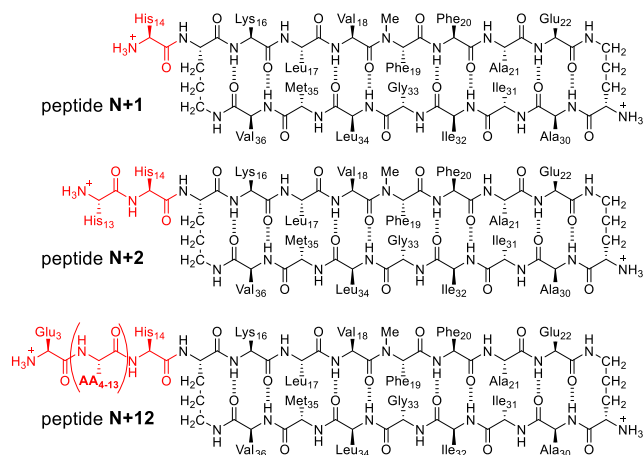


**Figure 2.5**. Peptides **N+1**, **N+2**, **N+4**, **N+6**, **N+8**, **N+10**, and **N+12** are homologues of peptide **N+14** with truncated tails.
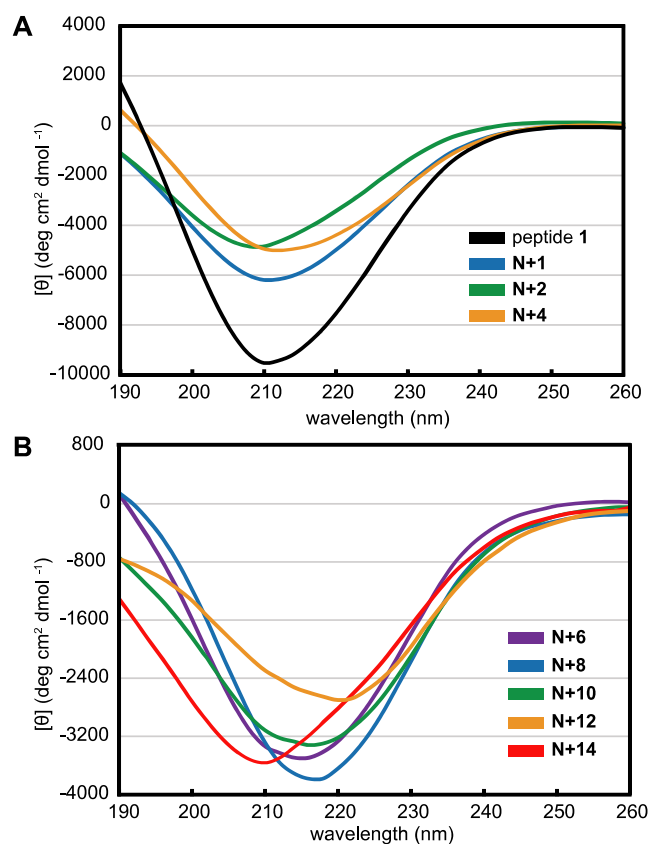
**Figure 2.6**. CD spectra of peptides **1** and **N+1** through **N+14**. CD spectra were acquired for each peptide at 50 μM in 10 mM phosphate buffer at pH 7.4; ellipticity was normalized for the number of residues in each peptide.

To determine the impact of these *N*-terminal extensions on the oligomerization of peptide **1**, we compared the assembly of peptide **1** and each *N*-terminally extended peptide using SDS-PAGE (**Figure 2.7**). We included trimers **1** and **2** as molecular-weight standards for comparison (**Figures S2.4 and S2.5**). Trimer **1** (5.3 kDa) and trimer **2** (5.3 kDa) are covalently stabilized trimers of β-hairpin peptides that we have previously reported to self-assemble and migrate respectively as 10.6 kDa hexamers and 21.2 kDa dodecamers.[26] SDS-PAGE reveals that each *N*-terminally extended peptide migrates as a hexamer. Peptide **1** migrates to approximately the same position as trimer **1**, and each of the longer *N*-terminally extended peptides migrates at a position between the hexamer formed by trimer **1** and the dodecamer formed by trimer **2**. The hexamer formed by peptide **N+1** (1.91 kDa) migrates to the top of the 10 kDa ladder band and streaks downward like peptide **1**.

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Similarly, the hexamer formed by peptide **N+2** (2.05 kDa) migrates to just above the 10 kDa ladder band and also streaks downward. The streaking observed for peptides **N+1** and **N+2** suggests that the hexamers formed by each peptide are in equilibrium with smaller oligomers. The hexamer bands formed by peptides **N+4** (2.28 kDa), **N+6** (2.49 kDa), **N+8** (2.69 kDa), **N+10** (2.99 kDa), **N+12** (3.27 kDa), and **N+14** (3.45 kDa) are in comparable or higher positions than peptide **N+2** and migrate as tight bands between the 10 and 17 kDa ladder bands. The tightness of these bands may reflect an increase in hexamer stability that arises from stabilizing non-covalent interactions from the *N*-terminal tail. Peptides **N+2** through **N+14** also show bands at or above the 4.6 kDa ladder band which correspond to the putative dimer.
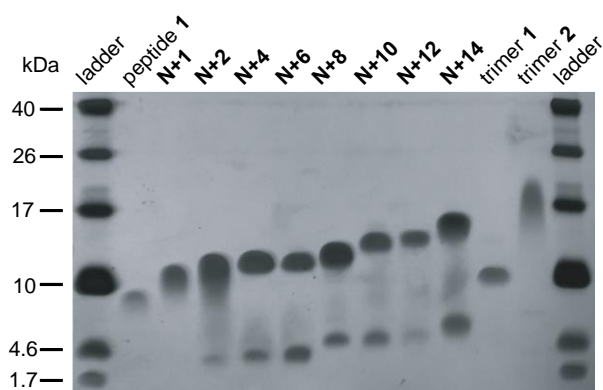


**Figure 2.7**. Silver stained SDS-PAGE and folding of peptide **1**, peptides **N+1** through **N+14**, and trimers **1** and **2**. SDS-PAGE was performed in Tris buffer at pH 6.8 with 2% (w/v) SDS on a 16% polyacrylamide gel with 75 μM solutions of peptide in each lane.

To quantitatively assess the relationship between oligomer assembly and migration, we plotted the logarithm of oligomer molecular weights against the relative migration distance ($R_f$) of the hexamer bands (**Figure 2.8**). The ladder bands exhibit an excellent correlation between log MW and $R_f$ ($R^2 = 0.999$, black), with the exception of the 1.7 kDa ladder band which was excluded from the analysis. The hexamers do not fall on this line, but rather are shifted 12–33% lower than their calculated molecular weights, thus appearing as pentamers or tetramers. The hexamer bands also exhibit a linear relationship between

41

log MW and $R_f$, albeit with a poorer fit to a straight line ($R^2 = 0.881$, red). For comparison we also plotted the log MW vs. $R_f$ for the covalently stabilized trimers **1** and **2** (green), which run on the gel as hexamers and dodecamers. These oligomers fall slightly off the line for the ladder bands.
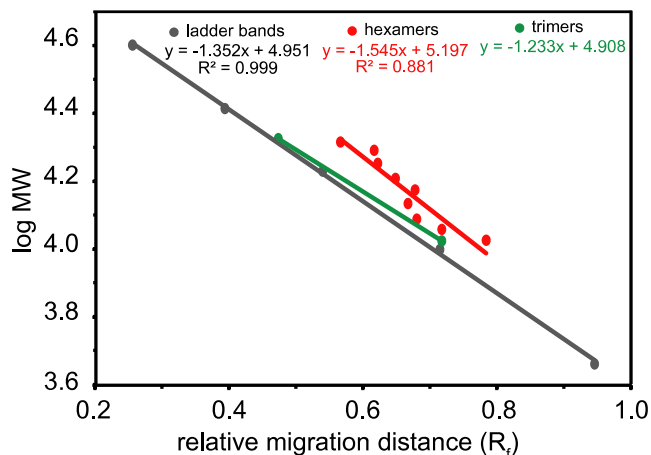


**Figure 2.8**. Oligomer molecular weights vs. relative migration distance ($R_f$) of peptide **1** and peptides **N+1** through **N+14** by SDS-PAGE (semi-log plot).

Membrane proteins often migrate by SDS-PAGE at an apparent molecular weight that does not correspond to the actual molecular weight of the protein.[41–43] Often this "gel shifting" results in a lower apparent molecular weight. This behavior has been well characterized for full-length Aβ and linear truncated variants of Aβ, where SDS binding correlates with hydrophobicity rather than the number of amino acids.[44] The low apparent molecular weights of the hexamer bands may thus reflect gel shifting, causing the hexamer bands to appear as smaller oligomers (pentamers or tetramers).

We used size exclusion chromatography (SEC) to better understand the solution-phase assembly of these *N*-terminally extended peptides. In SEC, peptide **1** does not assemble to form a hexamer (**Figure 2.9**). Peptide **1** elutes as a broad band comprising a large peak at 20.2 mL, a smaller peak at 19.1 mL, and a small peak at 17.9 mL.[28] These elution volumes fall at the low molecular weight range of the column, near the size

standards vitamin B12 (1.3 kDa, 19.7 mL) and aprotinin (6.5 kDa, 16.8 mL). Thus, the

three peaks from peptide **1** appear to correspond to a monomer, a dimer, and a trimer (1.8

kDa, 3.6 kDa, and 5.3 kDa). Peptides **N+1** through **N+14** also run as broad bands that

contain multiple features (**Figure 2.9**). The SEC traces of the *N*-terminally extended

homologues exhibit main bands between peptide **1** and the aprotinin size standard and

additional minor peaks that elute significantly after peptide **1**. The main bands are

composed of several peaks which appear to correspond to monomer, dimer, and trimer. The

*N*-terminally extended peptides also exhibit additional peaks that elute after vitamin B12,

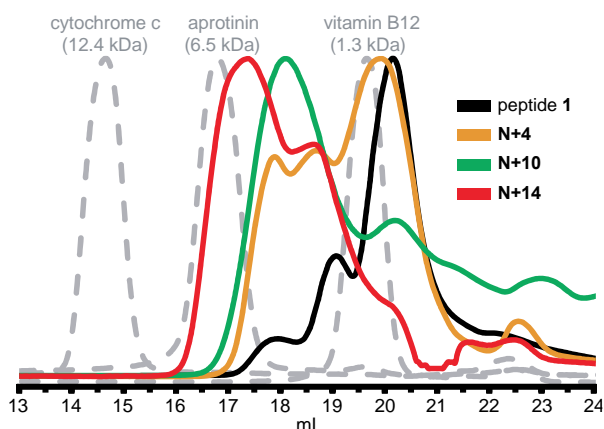which may reflect adsorption of the peptides to the column.



**Figure 2.9**. SEC traces of peptide **1** and select *N*-terminally extended peptides. SEC was performed
on a 1.0 mg/mL solution of each peptide in 50 mM Tris buffer at pH 7.4 with 150 mM NaCl on a
Superdex 75 Increase 10/300 column.

The broad band formed by peptide **N+14** shows a different distribution of monomer,

dimer, and trimer peaks than peptide **1**. Peptide **N+14** elutes with a large peak at 17.4 mL,

a smaller peak at 18.7 mL, and a small shoulder at 20.1 mL (**Figure 2.9**). We interpret these

peaks to be the trimer (10.5 kDa), dimer (6.5 kDa), and monomer (3.5 kDa) of peptide

**N+14**. The SEC trace of peptide **N+12** resembles the SEC trace of peptide **N+14** (**Figure

S 2.6**). The SEC traces of peptides **N+1, N+2**, and **N+4** resemble the SEC trace of peptide

**1** (**Figure S2.7**). The SEC traces of peptides **N+6, N+8**, and **N+10** differ from the SEC

traces of peptide **1** and peptide **N+14** (**Figure S2.8**). The SEC traces of peptides **N+6**, **N+8**, and **N+10** show two peaks, which correspond to a monomer peak and a dimer or trimer peak.

As controls we preformed SEC on peptide **2** ($A\beta_{1-14}$, H-DAEFRHDSGYEVHH-NH$_2$) and peptide **N+14$_{Me}$**. Peptides **2** and **N+14$_{Me}$** elute between the vitamin B12 and aprotinin size standards (**Figure S2.9**). Peptide **2** elutes as a narrow band at 17.6 mL that corresponds to a monomer. Peptide **N+14$_{Me}$** elutes as a broader band at 18.9 mL that may correspond to monomer and/or dimer. Elution volumes for each peptide are summarized in supplementary Table 2.

To better assess the relationship between the assembly and elution volumes amongst the *N*-terminally extended homologues, we plotted the logarithm of the oligomer molecular weight against the elution volumes for peptide **1** and peptides **N+1** through **N+14** (**Figure 2.10**). The resulting graph revealed a linear relationship between the monomer, dimer, and trimer elution volumes, with increasing oligomer size correlating with decreased elution volumes. Collectively, the SEC studies show that at hundred micromolar concentrations, peptide **1** and peptides **N+1** through **N+14** form mixtures of monomers, dimers, and trimers that equilibrate slowly on the 40-minutes time scale of the SEC experiment.[45]
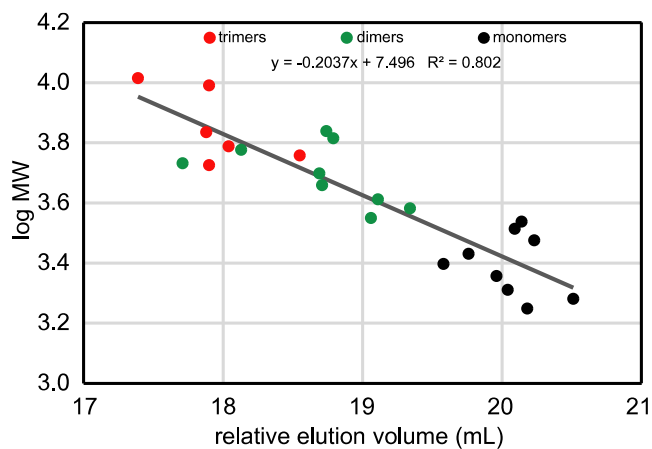


**Figure 2.10**. Oligomer molecular weights vs. elution volumes of peptide **1** and peptides **N+1** through **N+14** by SEC (semi-log plot). Elution values and oligomer molecular weight are summarized in Supplementary Table 2.

The SDS-PAGE and SEC studies reveal that *N*-terminally extended homologues of peptide **1** can assemble to form oligomers in aqueous environments in the presence or absence of sodium dodecyl sulfate. In SDS-PAGE, peptide **1** and each of the *N*-terminally extended homologues form hexamers. In SEC, peptide **1** and each of the of the *N*-terminally extended homologues do not form hexamers, but rather appear to elute as monomers, dimers, and trimers. The lipophilic environment produced by the SDS micelles in SDS-PAGE appears to drive the assembly of the hexamers.[46–48]

*X-ray crystallographic structure of peptide **N+1***.X-ray crystallography of peptide **N+1** (PDB 6VU4) corroborates the formation of the hexamers observed in SDS-PAGE (**Figure 2.11**). Peptide **N+1** afforded crystals suitable for X-ray diffraction from an aqueous solution containing 0.1 M HEPES buffer at pH 7.2, 0.2 M sodium citrate, and 25% isopropanol. X-ray diffraction data were collected at a resolution of 2.08 Å. The crystallographic phase determination was carried out through molecular replacement using the structure of peptide **1** (PDB 5W4H) as the search model. Supplementary Table 3 summarizes the crystallographic properties, crystallization conditions, data collection, and model refinement statistics for peptide **N+1**.

In the crystal structure, the asymmetric unit contains two molecules of peptide **N+1** that assemble edge-to-edge to form an out-of-register, antiparallel β-sheet dimer. Each monomer subunit folds to form a twisted β-hairpin stabilized by eight intramolecular hydrogen bonds between the top and bottom β-strands. The dimer itself is stabilized by four intermolecular hydrogen bonds between $Ile_{31}$ and $Gly_{33}$ in the bottom strands of both macrocycles (**Figures 2.11A and S2.10**). Three dimers further assemble to form a hexamer—a trimer of dimers—that is nearly identical to the hexamer formed by peptide **1** (**Figure S2.11**). The hexamer can also be interpreted as a dimer of trimers formed through face-to-face packing of two trimers (**Figure 2.11B and S2.10**). Each vertex of the trimer is stabilized by three hydrogen bonds: two between $Ala_{21}$ and $^{\delta}Orn$, and one between *N*-Me-

Phe$_{19}$ and Leu$_{17}$ (a total of nine hydrogen bonds per trimer). The hexamer is further stabilized by the packing of six sets of hydrophobic side chains from Leu$_{17}$, *N*-Me-Phe$_{19}$, Ala$_{21}$, Ile$_{31}$, and Met$_{35}$ (**Figure 2.S10**). In each of the two crystallographically independent monomers of peptide **N+1**, His$_{14}$ is clearly visible in the electron density map, further corroborating that the hexamer can accommodate the *N*-terminal Aβ residues (**Figure 2.11C**).
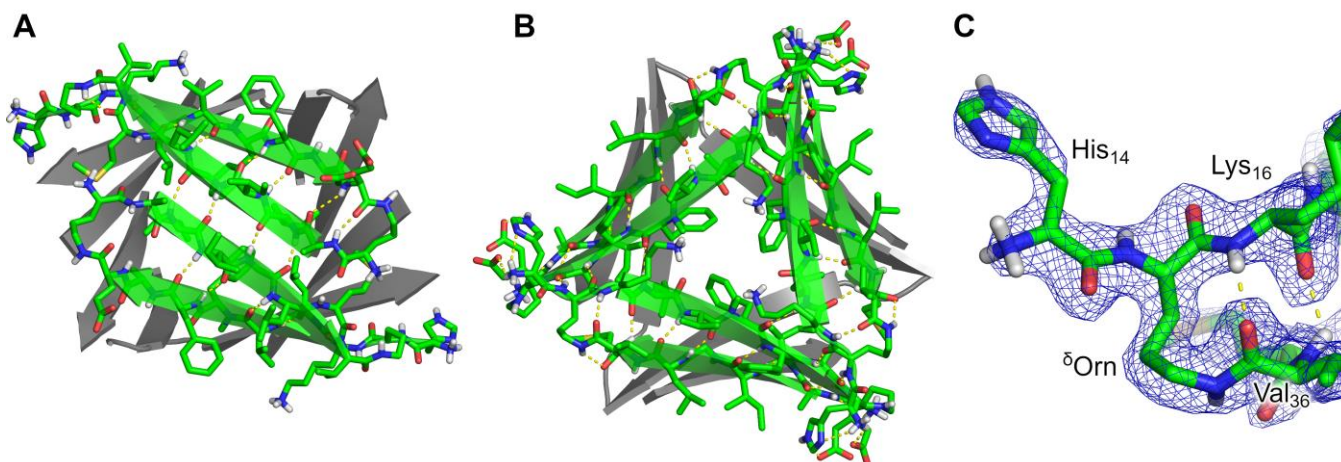
**Figure 2.11**. X-ray crystallographic structure of the hexamer formed by peptide **N+1** (PDB 6VU4). (A) The dimer subunit within the X-ray crystallographic structure of the hexamer formed by peptide **N+1**. (B) The trimer subunit. (C) 2Fo – Fc electron density map contoured at 1.3σ, showing $His_{14}$ in peptide **N+1**. $R_{work}$: 0.22, $R_{free}$: 0.26, PDB 6VU4.

## SUMMARY AND CONCLUSION

To better mimic oligomers of full length Aβ, we have appended residues from $Aβ_{1–14}$ as an *N*-terminal tail to macrocyclic β-hairpin peptide **1**, which comprises $Aβ_{16–22}$ and $Aβ_{30–36}$. The synthesis of these tailed macrocycles was achieved through an orthogonal protecting group strategy using the amino acid protecting group Dde, in addition to Fmoc and Boc. Circular dichroism revealed that peptide **1** and the *N*-terminally extended homologues adopt β-sheet-like conformations, consistent with a model in which β-hairpins are key components of Aβ oligomers. In aqueous solution, peptide **1** and the *N*-terminally extended homologues form dimers and trimers in SEC, and hexamers in SDS-PAGE. The observations of dimers, trimers, and hexamers in solution are recapitulated in the X-ray crystallographic structure of peptide **N+1**. The consistent formation of hexamers by peptide **1** and the *N*-terminally extended homologues suggests that the *N*-terminus of Aβ does not substantially modify or impede the assembly of oligomers derived from $Aβ_{1–36}$. These findings are surprising in light of the purported importance of the *N*-terminus in the aggregation of Aβ in Alzheimer's disease as they support a model in which the *N*-terminus is not critical for the assembly of Aβ oligomers.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at http://pubs.acs.org/.

> (1) Procedures for the synthesis of peptides **1**, **N+1** through **N+14**, **N+14$_{Me}$**, SDS-PAGE and silver staining, western blot analysis, circular dichroism studies, LDH-release assays, and size exclusion chromatography; (2) details of X-ray crystallographic data collection, processing, and refinement; (3) characterization data for peptide **N+1** (PDF). Crystallographic data for peptide **N+1** (cif file).
>
> Crystallographic coordinates of peptide **N+1** were deposited into the Protein Data Band (PDB) with code 6VU4.

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**Notes**

The authors declare no competing financial interest.

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Supporting information for:

## Effects of *N*-Terminal Residues on the Assembly of Constrained β-Hairpin Peptides Derived from Aβ

# Contents

## Supporting Figures and Tables



**Figure S2.1**. Western blot analysis of peptides **N+8**, **N+10**, **N+12**, **N+14**, and $A\beta_{M1-42}$ using the 6E10 antibody.
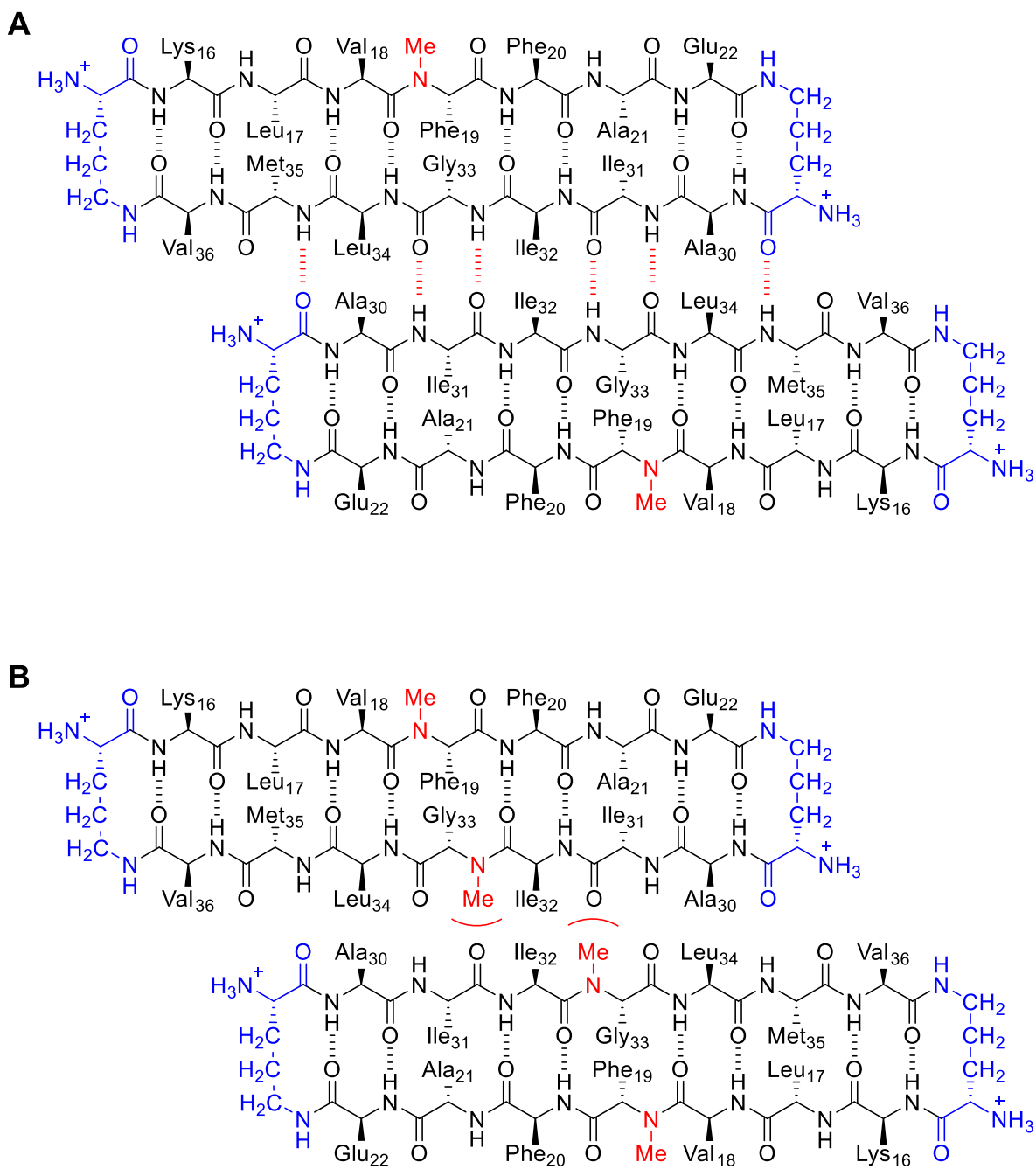
**Figure S2.2**. (A) The dimer subunit of the hexamer formed by peptide **1**. (B) *N*-Methylation of Gly₃₃ disrupts hydrogen bonding within the dimer subunit, and thus prevents assembly of the hexamer.
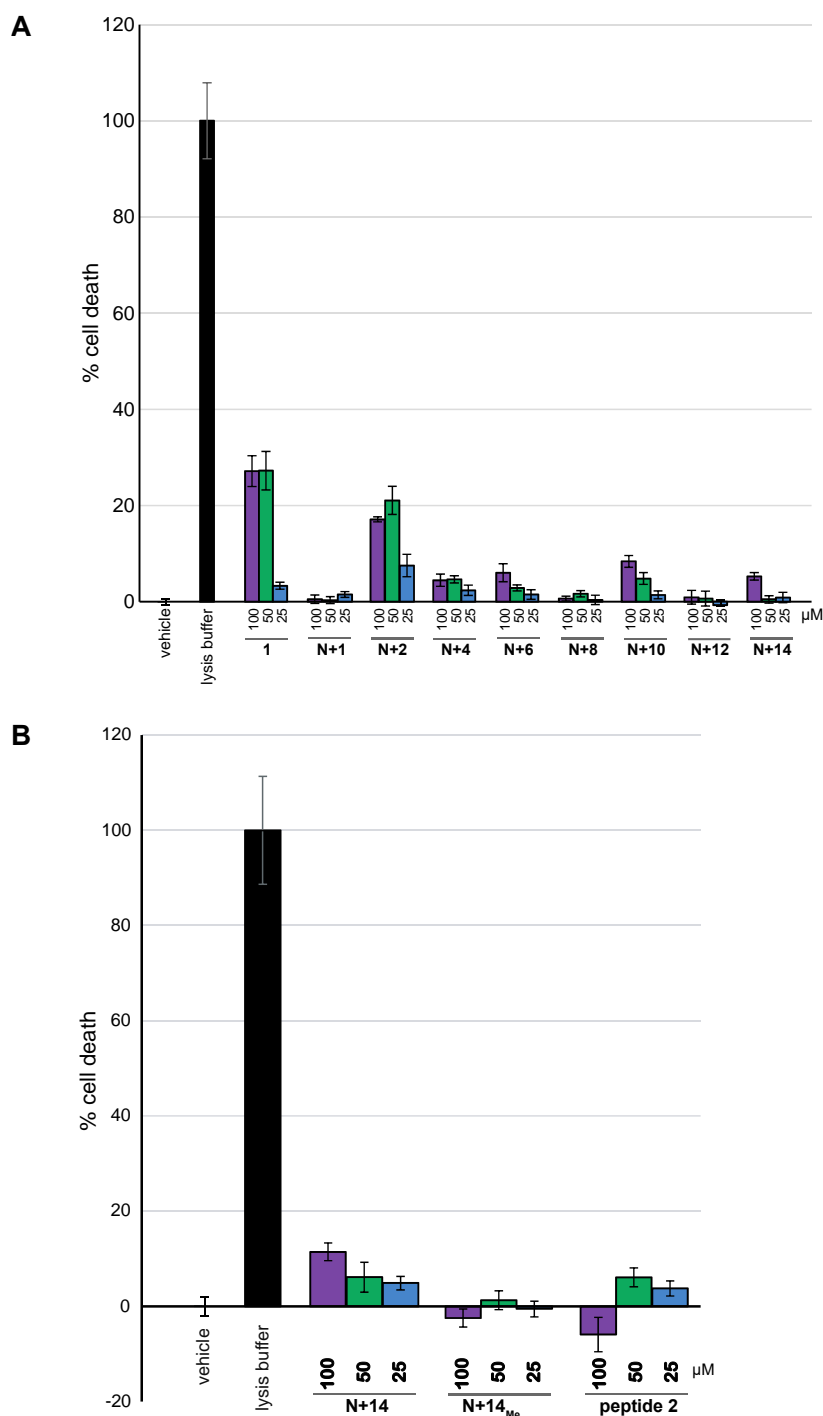
**Figure S2.3**. (A) Cytotoxicity of peptide **1** and the *N*-terminally extended homologues against SH-SY5Y cells, as assessed by an LDH release assay. Incorporation of the *N*-terminal residues of Aβ did not increase toxicity. Data represent the mean of four replicate wells ± the standard deviation. Deionized water (vehicle) was used as a negative control. (B) LDH release assay of peptides **N+14**, **N+14$_{Me}$**, and **2**.

**Supplementary Table 2.1**. Normalized absorbance values and calculated percent differences of circular dichroism samples relative to peptide **1**.

| peptide | Abs$_{214\ nm}$ | Abs$_{214\ nm}$/n | % diff. |
|---------|-----------------|-------------------|---------|
| 1 | 0.186566263 | 0.011660391 | 0 |
| 2 | 0.390456799 | 0.013015227 | 11.61 |
| 3 | 0.140225015 | 0.010016073 | -14.10 |
| N+1 | 0.195346737 | 0.011490985 | -1.45 |
| N+2 | 0.221505983 | 0.012305888 | 5.53 |
| N+4 | 0.267023077 | 0.013351154 | 14.50 |
| N+6 | 0.243538737 | 0.011069943 | -5.06 |
| N+8 | 0.311190368 | 0.012966265 | 11.19 |
| N+10 | 0.349382999 | 0.013437808 | 15.24 |
| N+12 | 0.363346815 | 0.012976672 | 11.28 |
| N+14 | 0.410958172 | 0.013698606 | 17.48 |

Out of concern that the differences observed between the circular dichroism (CD) spectra of peptide **1** and the *N*-terminally extended homologues might reflect differences in concentration across the samples, we corroborated the concentration of each sample by measuring the absorbance of each solution at 214 nm ($A_{214}$). A 10 mg/mL stock solution of each peptide was first prepared using deionized water. The stock solution was diluted with 10 mM sodium phosphate buffer at pH 7.4 to produce a 50 µM solution of each peptide, and the absorbance at 214 nm was measured in a 1 mm quartz cuvette using Cary-60 spectrophotometer. The cuvette was immediately transferred to a Jasco J-810 circular dichroism spectropolarimeter, and the CD spectrum of the peptide was acquired, as described below. The $A_{214}$ measurements were normalized for the number of residues (n) in each peptide. The $A_{214}$/n values of each peptide (peptides **N+1** through **N+14**) were all within ± 17% of the $A_{214}$/n value of peptide **1**.
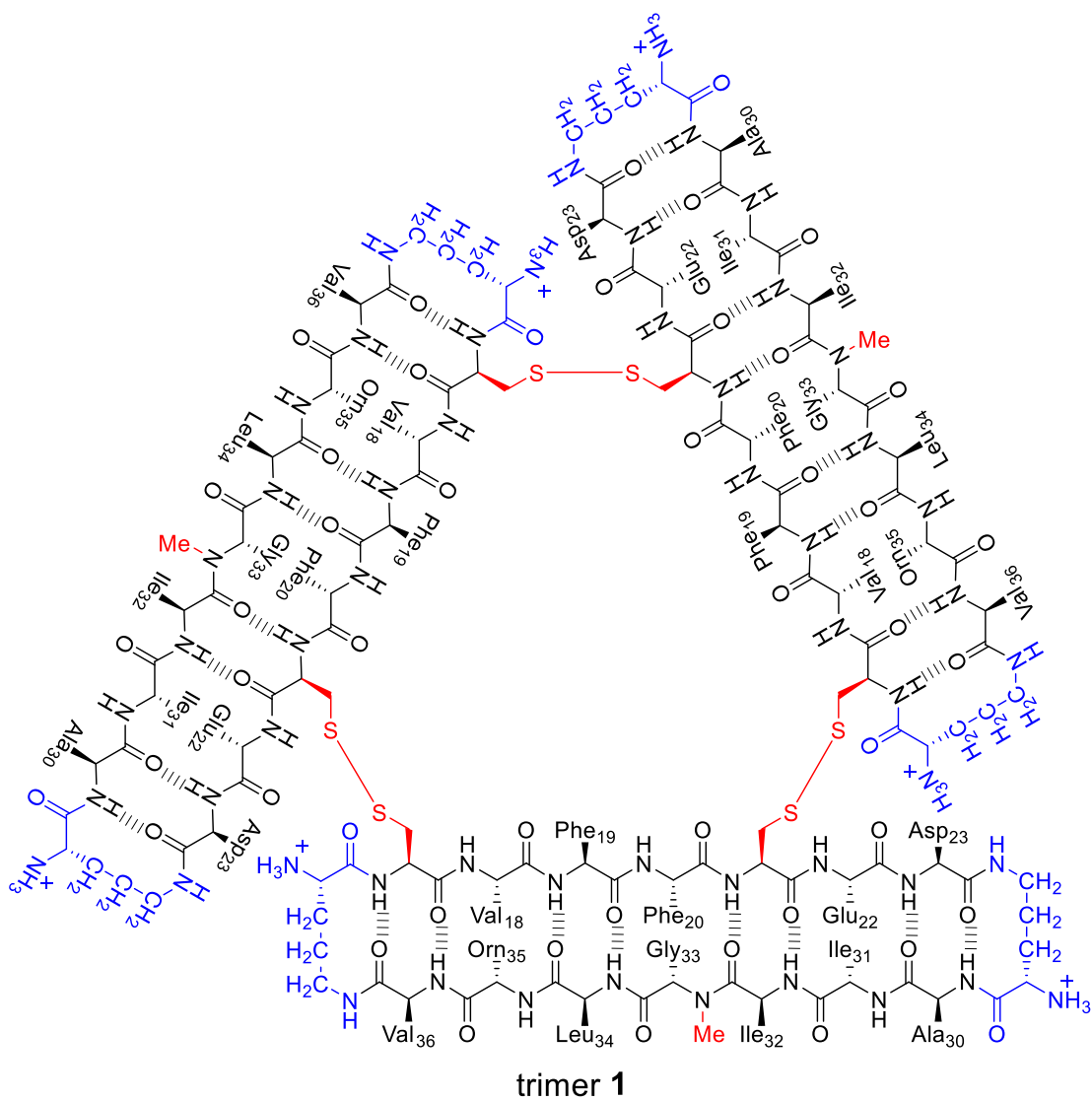
trimer **1**

**Figure S2.4**. Covalently stabilized trimer **1**. Trimer **1** assembles to form a hexamer in SDS-PAGE.
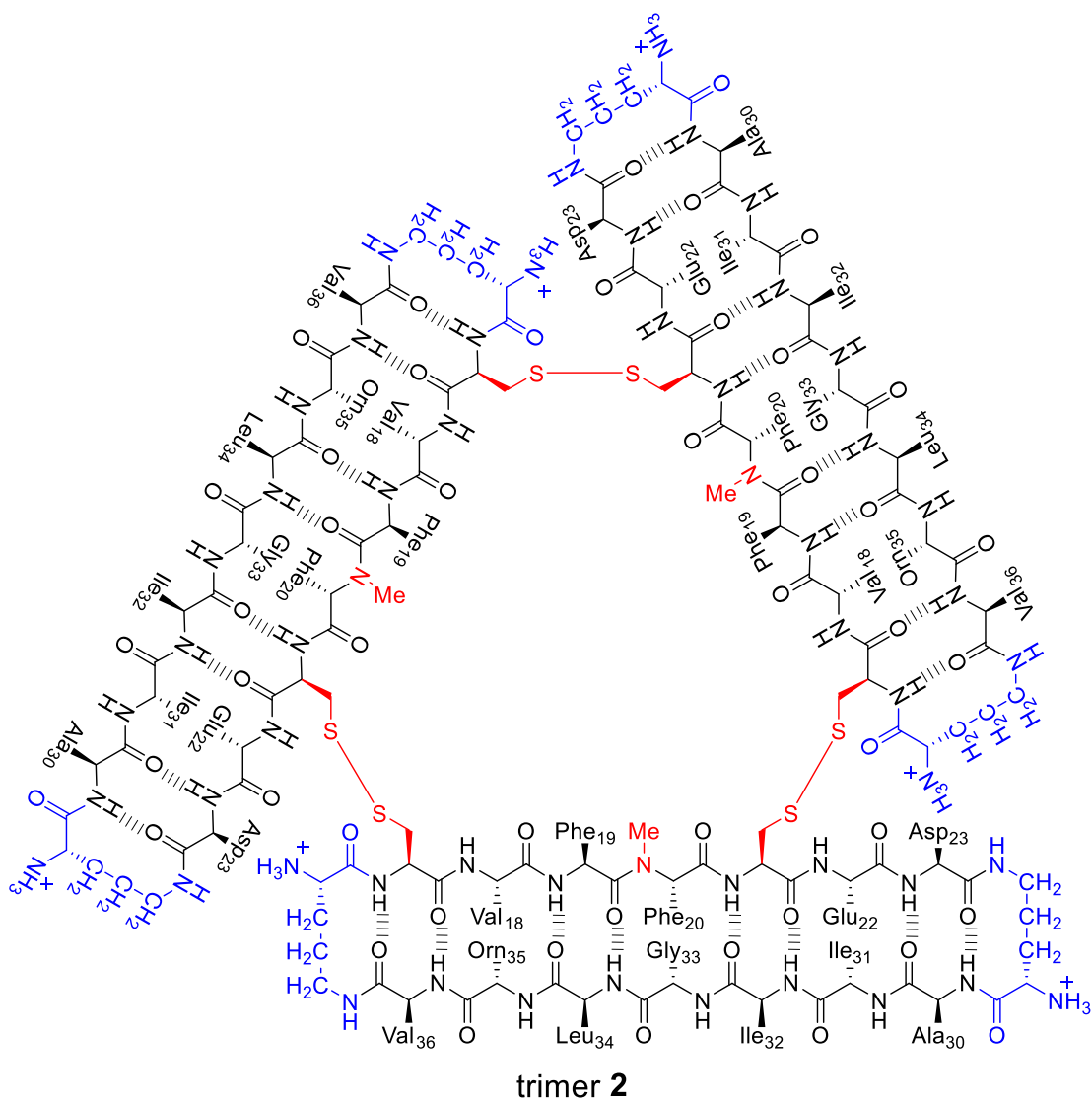
**Figure S2.5**. Covalently stabilized trimer **2**. Trimer **2** assembles to form a dodecamer in SDS-PAGE.
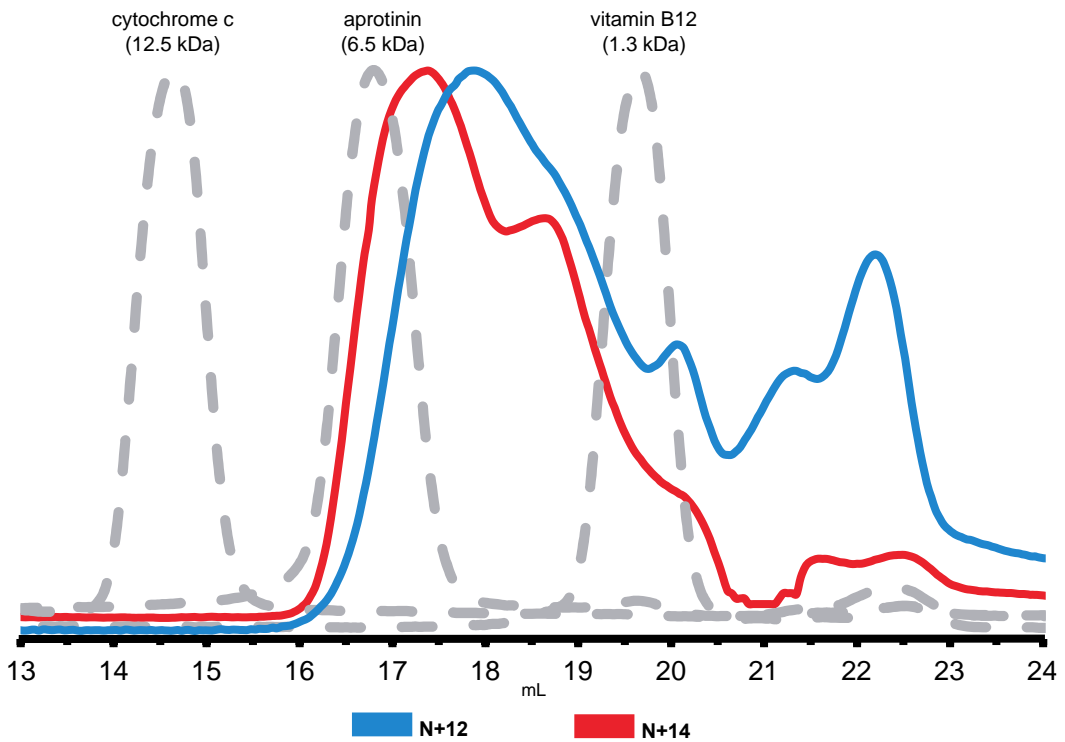
**Figure S2.6**. SEC traces of peptides **N+12** and **N+14**. SEC was performed on a 1.0 mg/mL solution of each peptide in 50 mM Tris buffer at pH 7.4 with 150 mM NaCl on a Superdex 75 Increase 10/300 column.
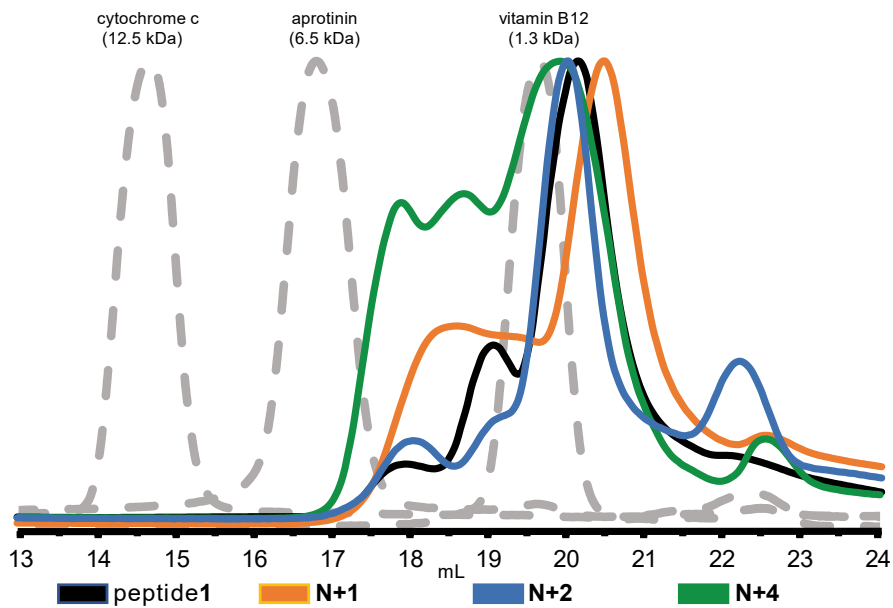


**Figure S2.7**. SEC traces of peptides **1**, **N+1**, **N+2**, and **N+4**. SEC was performed on a 1.0 mg/mL solution of each peptide in 50 mM Tris buffer at pH 7.4 with 150 mM NaCl on a Superdex 75 Increase 10/300 column.

**Figure S2.8**. SEC traces of peptides **N+6**, **N+8**, and **N+10**. SEC was performed on a 1.0 mg/mL solution of each peptide in 50 mM Tris buffer at pH 7.4 with 150 mM NaCl on a Superdex 75 Increase 10/300 column.
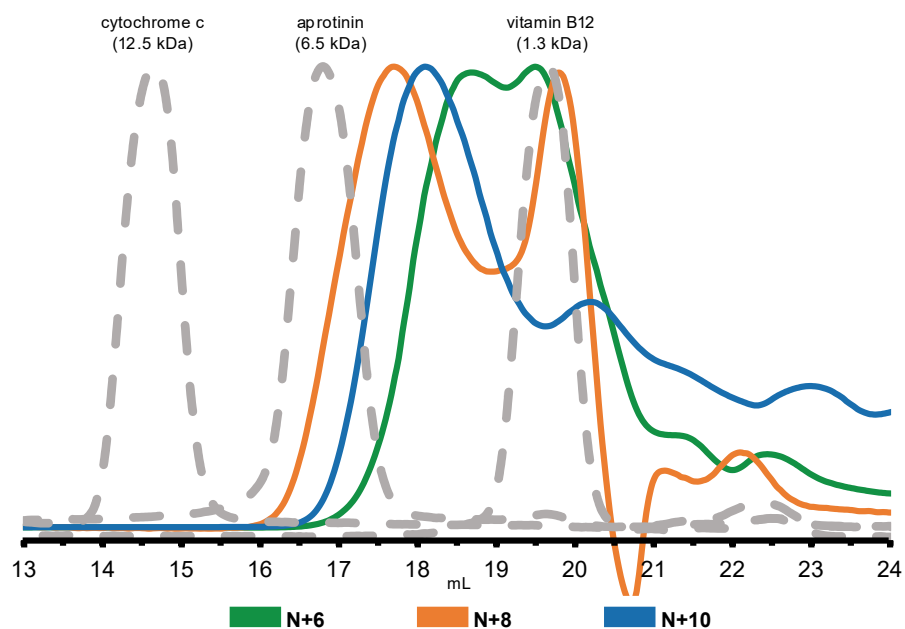


**Figure S2.9**. SEC traces of peptides **2** and **N+14$_{Me}$**. SEC was performed on a 1.0 mg/mL solution of each peptide in 50 mM Tris buffer at pH 7.4 with 150 mM NaCl on a Superdex 75 Increase 10/300 column.
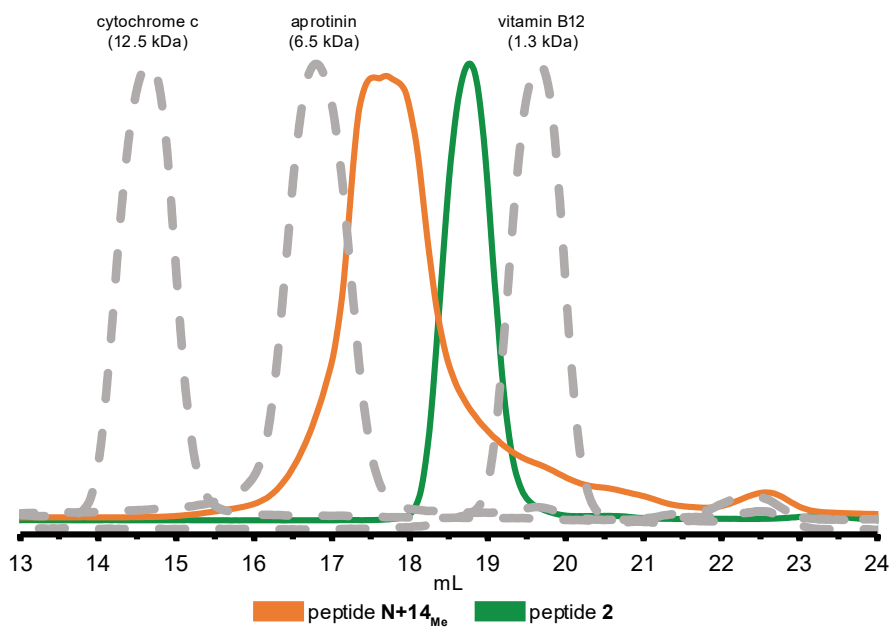
**Supplementary Table 2.2**. Elution volumes of peptides **1**, **2**, **N+1** through **N+14**, and **N+14**$_{Me}$ by size exclusion chromatography. [a]

| peptide | elution volume (mL) | putative oligomer size | molecular weight (kDa)[b] |
|---|---|---|---|
| peptide **1** | 20.18 | monomer | 1.77 |
| | 19.06 | dimer | 3.55 |
| | 17.90 | trimer | 5.32 |
| **N+1** | 20.51 | monomer | 1.91 |
| | 19.34 | dimer | 3.82 |
| | 18.55 | trimer | 5.73 |
| **N+2** | 20.04 | monomer | 2.05 |
| | 19.10 | dimer | 4.09 |
| | 18.04 | trimer | 6.14 |
| **N+4** | 20.0 | monomer | 2.28 |
| | 18.71 | dimer | 4.55 |
| | 17.88 | trimer | 6.83 |
| **N+6** | 19.58 | monomer | 2.49 |
| | 16.64 | dimer or trimer | 4.99 or 7.49 |
| **N+8** | 19.76 | monomer | 2.69 |
| | 17.76 | dimer or trimer | 5.39 or 8.09 |
| **N+10** | 20.23 | monomer | 2.99 |
| | 18.13 | dimer or trimer | 5.98 or 8.97 |
| **N+12** | 20.09 | monomer | 3.27 |
| | 18.79 | dimer | 6.54 |
| | 17.90 | trimer | 9.80 |
| **N+14** | 20.14 | monomer | 3.45 |
| | 18.74 | dimer | 6.54 |
| | 17.39 | trimer | 10.36 |
| **N+14**$_{Me}$ | 17.67 | monomer or dimer | 3.46 or 6.54 |
| peptide **2** | 18.74 | monomer | 3.45 |

a. SEC was performed on a 1.0 mg/mL solution of each peptide in 50 mM Tris buffer at pH 7.4 with 150 mM NaCl on a Superdex 75 Increase 10/300 column.
b. Molecular weights of the monomers, dimers, and trimers are calculated from the structures of the molecules.
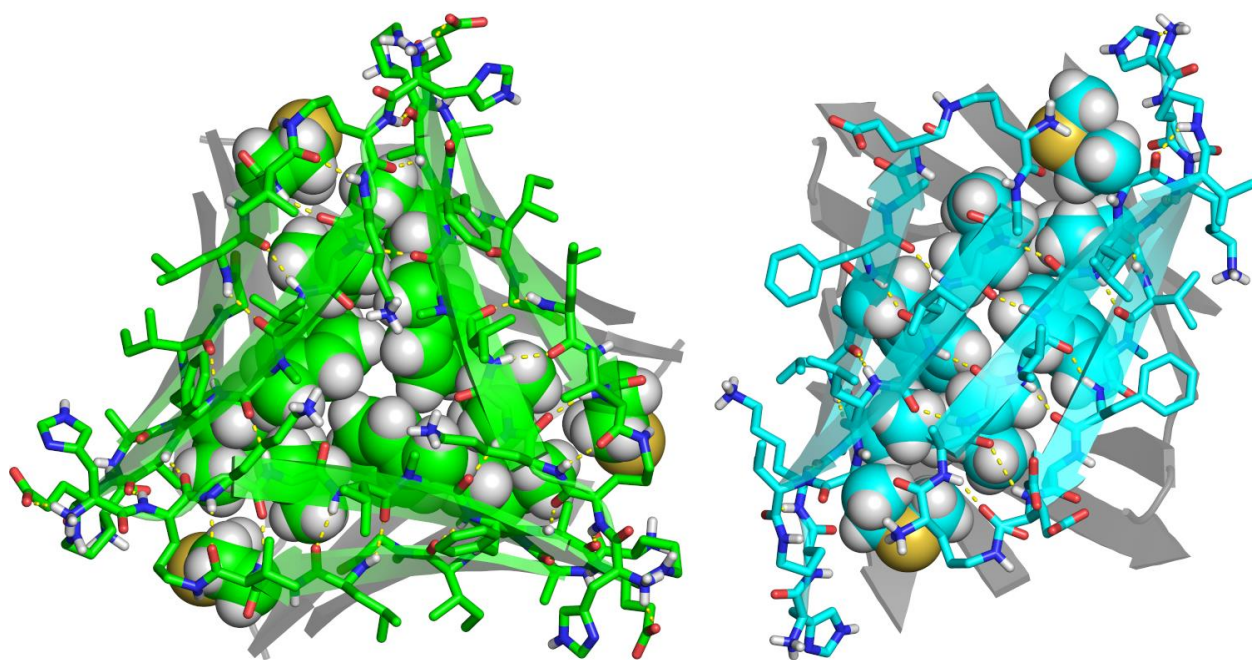
**Figure S2.10**. The trimer (left) and dimer (right) subunits within the X-ray crystallographic structure of the hexamer formed by peptide **N+1**. The trimer and dimer subunits are stabilized by backbone hydrogen bonding and the packing of hydrophobic side chain residues.
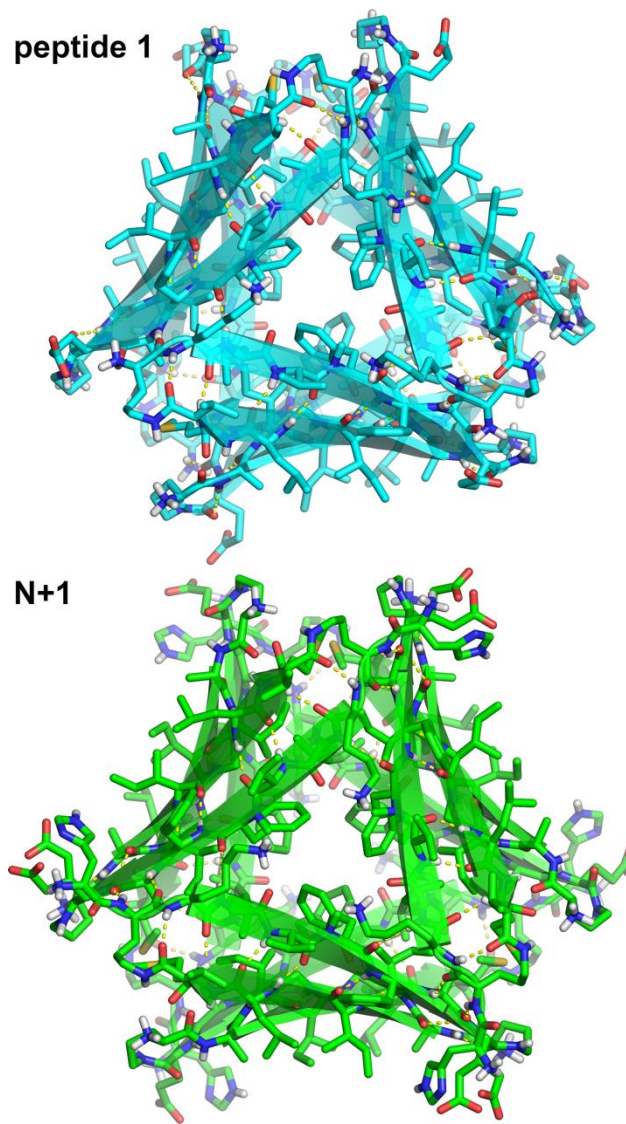
**Figure S2.11**. X-ray crystallographic structures of the hexamers formed by peptides **1** (PDB 5W4H), **N+1** (PDB 6VU4).

**Supplementary Table 2.3**. Crystallographic properties, crystallization conditions, data collection, and model refinement statistics for peptide **N+1**.

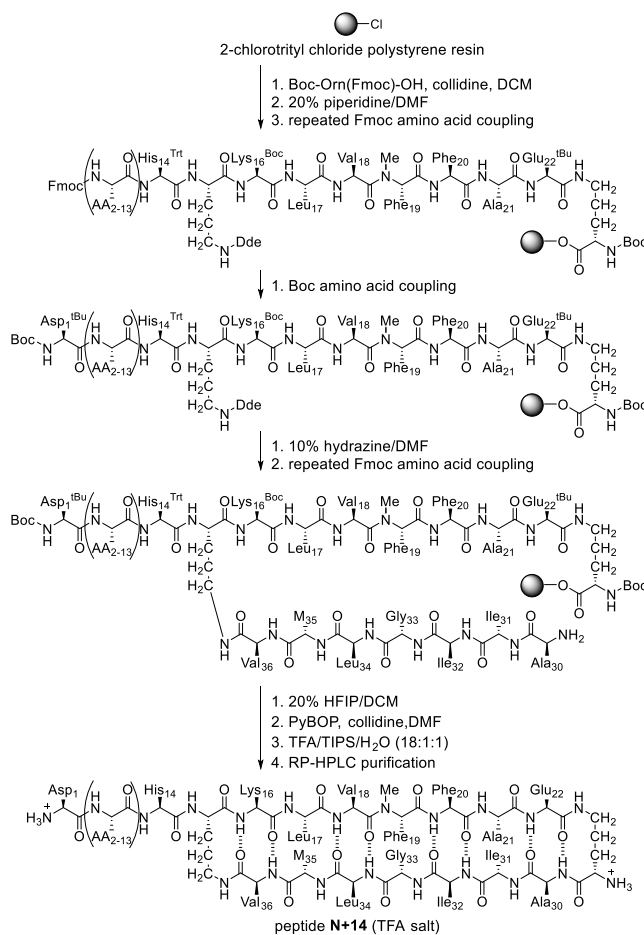| peptide | **N+1** |
| --- | --- |
| PDB ID | 6VU4 |
| space group | F*432* |
| $a$, $b$, $c$ (Å) | 96.880, 96.880, 96.880 |
| α, β, λ (°) | 90, 90, 90 |
| peptides per asymmetric unit | 2 |
| crystallization conditions | 0.1 M HEPES buffer (pH 7.2), 0.2 M sodium citrate, 25% isopropyl alcohol[a] |
| wavelength (Å) | 1.54 |
| resolution range (Å) | 24.22 - 2.08 (2.15 - 2.08) |
| total reflections | 356508 (33112) |
| unique reflections | 2632 (255) |
| multiplicity | 135.5 (129.9) |
| completeness (%) | 99.81 (100.00) |
| mean I/σ | 174.29 (14.50) |
| Wilson B-factor | 32.19 |
| $R_{merge}$ | 0.7111 (1.321) |
| $R_{measure}$ | 0.7143 (1.326) |
| $R_{pim}$ | 0.06514 (0.1154) |
| CC1/2 | 0.937 (0.906) |
| CC* | 0.984 (0.975) |
| $R_{work}$ | 0.2217 (0.2330) |
| $R_{free}$ | 0.2694 (0.4224) |
| number of non-hydrogen atoms | 318 |
| RMS(bonds) | 0.003 |
| RMS(angles) | 0.77 |
| Ramachandran favored (%) | 92.86 |
| Ramachandran allowed (%) | 7.14 |
| Ramachandran outliers (%) | 0.00 |
| Rotamer outliers (%) | 0.00 |
| clashscore | 5.43 |
| average B-factor | 39.11 |

[a]Crystals were soaked briefly in 1 M KI solution prior to data collection.

# Materials and Methods[1]

*General information*

      All chemicals were used as received unless otherwise noted. Methylene chloride ($CH_2Cl_2$) was passed through alumina under nitrogen prior to use. Anhydrous, amine-free *N,N*-dimethylfomamide (DMF) was purchased from Alfa Aesar. Deionized water (18 MΩ) was obtained from a Barnstead NANOpure Diamond water purification system. Analytical reverse-phase HPLC was performed on an Agilent 1260 Infinity II instrument equipped with a Phenomonex Aeris PEPTIDE 2.6u XB-C18 column. Preparative reverse-phase HPLC was performed on a Ranin instrument equipped with an Agilent Zorbax SB-C18 column. HPLC grade acetonitrile and deionized water, each containing 0.1% trifluoracetic acid (TFA), were used for analytical and preparative reverse-phase HPLC. All peptides were prepared and used as the trifluoroacetate salts, and were assumed to have one trifluoracetic acid molecule per amine group on each peptide.

*Synthesis of peptide N+14*



**Scheme 2.1**. Synthesis of the *N*-terminally extended peptide, **N+14** derived from A*β*₁₋₃₆.

*a. Loading the resin.* 2-Chlorotrityl chloride resin (300 mg, 1.4 mmol/g) was added to a Bio-RAD Poly-Prep chromatography column (10 mL). Dry $CH_2Cl_2$ (8 mL) was used to suspend and swell the resin for 30 min with gentle rocking. The solution was drained from the resin and a solution of Boc-Orn(Fmoc)-OH (0.78 equiv, 150 mg, 0.33 mmol) in 6% (v/v) 2,4,6-collidine in dry $CH_2Cl_2$ (8 mL) was added immediately and the suspension was gently rocked for 12 h. The solution was then drained and a mixture of $CH_2Cl_2$/MeOH/*N,N*-diisopropylethylamine (DIPEA) (17:2:1, 10 mL) was added immediately. The resin was gently rocked for 1 h, to cap the unreacted 2-chlorotrityl chloride resin sites. The resin was then washed twice with dry $CH_2Cl_2$ and dried by passing nitrogen through the vessel. This procedure typically yields 0.18 mmol of loaded resin (0.6 mmol/g loading).

*b. Manual peptide coupling of residues 22 through 18.* The resin loaded with Boc-Orn(Fmoc) was suspended in dry DMF and then transferred to a solid-phase peptide synthesis vessel. Residues 22 through 18 were manually coupled using Fmoc-protected amino acid building blocks. Each manual coupling cycle consisted of *i*. Fmoc-deprotection with of 20% (v/v) piperidine in DMF for 5 min at ambient temperature (5 mL), *ii*. washing with dry DMF (2x, 5 mL), *iii*. coupling of the amino acid (0.44 mmol, 4 equiv) with HCTU (174.0 mg, 0.44 mmol, 4 equiv) in 20% (v/v) 2,4,6-collidine in dry DMF (5 mL) for 30 min, and *iv.* washing the with dry DMF (2x, 5 mL). Residue 18, which follows N-Me-Phe19, was double coupled (4 equiv per coupling) using HATU (6 equiv) and HOAt (6 equiv) for 1 hr per coupling to ensure complete reaction. (We have found that coupling after N-methyl amino acids is difficult and requires rigorous coupling to minimize incomplete reaction.[2])

*c. Microwave-assisted coupling of residues 17 through 1 and 36 through 30.* A CEM Liberty 1 Automated Microwave Peptide Synthesizer was used to couple residues 17 to 1 and 36 to 30. Fmoc-Orn(Dde)-OH replaces residue 15 in the natural sequence. Each coupling cycle consisted of *i*. Fmoc-deprotection with 20% (v/v) piperidine with 0.1 M Oxyma Pure in DMF for 2 min. at 50 ºC, *ii*. washing with DMF (3x), *iii*. coupling of the amino acid (0.75 mmol, 5 equiv) in the presence of HCTU (0.675 mmol, 4.5 equiv) and 20% (v/v/) *N*-methylmorpholine (NMM) in DMF for 10 min. at 50 ºC, iv. washing with DMF (3x). For peptide **N+14**, residue 1 is coupled as Boc-Asp(*t*-Bu)-OH, to terminate the linear chain. For the shorter homologues (**N+12**, **N+10**, **N+8**, **N+6**, **N+4**, **N+2**, and **N+1**), the terminal residues (3, 5, 7, 9, 11, 13, and 14) were also coupled as the Boc protected amino acids (Boc-Glu(*t*-Bu)-OH, Boc-Arg(Pbf)-OH, Boc-Asp(*t*-Bu)-OH, Boc-Gly-OH, Boc-Glu(*t*-Bu)-OH, Boc-His(Trt)-OH, and Boc-His(Trt)-OH), to terminate the linear chain.

*d. Hydrazine deprotection of Dde and microwave-assisted coupling of residues 36 through 30.* The resin was transferred from the microwave synthesizer to a solid-phase peptide synthesis vessel and washed 3x using dry DMF. The Dde protecting group of residue 15 was then removed by treatment with a solution of 10% hydrazine in DMF for 20 minutes at ambient temperature. The resin was washed 8x with dry DMF and was then transferred back to the microwave synthesizer to couple residues 36 through 30. The final Fmoc group was removed with 20% (v/v) piperidine with 0.1 M Oxyma Pure in DMF (10 min 50 ºC).

*e. Cleavage of the peptide from resin.* The resin was then transferred to a 10-mL Bio-Rad Poly-Prep chromatography column, and washed 3x with dry $CH_2Cl_2$. The acyclic branched peptide was cleaved from the resin by rocking the resin for 1 h with a solution of 1,1,1,3,3,3-hexafluoroispropanol (HFIP) in $CH_2Cl_2$ (1:4, 8 mL). The suspension was filtered and the filtrate was collected in a 250-mL round-bottomed flask. The resin was washed with additional HFIP in $CH_2Cl_2$ (1:4, 8 mL). The combined filtrates were concentrated by rotary evaporation to give a white solid. The white solid was further dried by vacuum pump to afford the crude protected linear peptide, which was cyclized without further purification.

*f. Cyclization of the acyclic peptide.* The crude protected linear peptide was dissolved in dry DMF (150 mL). PyBOP (370 mg, 0.711 mmol, 6 equiv) and *N*-methylmorpholine (NMM) (0.33 mL, 1.8 mmol, 12 equiv) was added to the solution and the mixture was stirred under nitrogen for 48 h. The mixture was concentrated under reduced pressure to afford the crude protected cyclic peptide, a yellow film.

e. *Global deprotection of the cyclic branched peptide.* The protected cyclic peptide was dissolved in TFA/triisoproylsilane (TIPS)/$H_2O$ (18:1:1, 10 mL) in a 250-mL round-bottomed flask equipped with a nitrogen-inlet adaptor. The solution was stirred for 1 h under nitrogen. The reaction mixture was then concentrated by rotary evaporation under reduced pressure to afford the crude cyclic peptide. The crude cyclic peptide was immediately subjected to purification by reverse-phase HPLC (RP-HPLC).

h. *Reverse-phase HPLC purification.* The peptide was dissolved in $H_2O$ and acetonitrile (8:2, 10 mL), and the solution was filtered through a 0.2 μm syringe filter and purified by RP-HPLC. The solution of crude cyclic peptide was injected at 20% acetonitrile and eluted with a gradient of 20-60% $CH_3CN$ over 90 min, with the column heated in a water bath at 70 ºC. Each peptide eluted between 29-36% $CH_3CN$. The collected fractions were analyzed by analytical HPLC and MALDI-TOF, and the pure fractions were concentrated by rotary evaporation and lyophilized. Typical syntheses yielded between 12 and 44 mg of the peptide as the TFA salt.

### SDS-PAGE silver staining and western blot

SDS-PAGE was performed on peptides **1**, **2**, **N+1** through **N+14**, **N+14Me**, and trimers **1** and **2** using the reagents, recipes, and procedures for Tricine SDS-PAGE detailed in Schägger, H. *Nat. Protoc.* **2006**, *1*, 16–22. Each peptide was run on a 16% polyacrylamide gel with a 4% stacking polyacrylamide gel at 60 volts. A Spectra™ Multicolor Low Range Protein Ladder (ThermoFischer Scientific, catalog #: 26628) was loaded into the first lane of the gel. The remaining lanes were loaded with 5.0 μL aliquots of each peptide as 75 μM solutions in SDS-PAGE loading buffer, which were prepared as follows: A 10 mg/mL stock solution of each peptide was prepared with deionized water. Aliquots of the 10 mg/mL solutions were then diluted further

with deionized water and 6X SDS-PAGE loading buffer (G-Biosciences catalog #: 786-701) to create 75 µM working solutions of each peptide.

Staining with silver nitrate was used to visualize peptide **1** and the *N*-terminally extended homologues in the SDS-PAGE gel. Reagents for silver staining were prepared according to procedures detailed in Simpson, R. J. *Cold Spring Harbor Protocol* **2007**. [We have found it important to prepare sodium thiosulfate, silver nitrate, and developing solutions fresh each time and to use high purity sodium carbonate to prepare the developing solution.] The gel was removed from the casting glass and rocked for 20 min in fixing solution (50% (v/v) methanol and 5% (v/v) acetic acid in deionized water). The fixing solution was then discarded and replaced with 50% (v/v) aqueous methanol for another 10 min of rocking. Next, the 50% methanol was discarded and replaced with deionized water for another 10 min of rocking. Next, the water was discarded and the gel was rocked in 0.02% (w/v) sodium thiosulfate in deionized water for 1 min. The sodium thiosulfate was discarded and the gel was rinsed twice with deionized water for 1 min. The gel was then submerged in pre-chilled 0.1% (w/v) silver nitrate in deionized water and rocked at 4 ℃ for 20 min. The silver nitrate solution was discarded and the gel was rinsed twice with deionized water. The gel was incubated in developing solution (2% (w/v) sodium carbonate, 0.04% (w/v) formaldehyde) until the solution began to brown. The developing solution was then immediately discarded and fresh silver nitrate solution was added to the gel until the desired intensity of staining was reached. When the desired intensity of staining was reached, the developing solution was discarded and the gel was submerged in 5% aqueous acetic acid.

Western blot analysis was performed on peptides **1**, **N+1** through **N+14**, and $A\beta_{M1-42}$ using the 6E10 antibody (BioLegend catalog #: 803004) to visualize the bands. After the SDS-PAGE described above, the bands were electroblotted from the gel to a nitrocellulose membrane in transfer buffer (25mM Tris, 192 mM glycine, pH 8.3, 20% v/v methanol) at 400 mA over 2 h. The

nitrocellulose membrane was then rocked for 1 h at ambient temperature in a blocking solution of 10% (w/v) non-fat powdered milk in low-Tween Tris-buffered saline (TBST: 20 mM Tris, 137 mM NaCl, 0.01% Tween 20, pH 7.6). The membrane was then incubated at 4 ºC overnight in a solution of 6E10 antibody (2 µg/mL) in 5% (w/v) non-fat powdered milk in TBST.  The membrane was then washed with TBST for 5 min (3X) while rocking, and then rocked for 15 min in 5% (w/v) non-fat powdered milk in TBST at ambient temperature. The membrane was then rocked for 1 hour in a solution of horseradish peroxidase conjugated anti-mouse antibody (100 µg/mL) (Jackson ImmunoResearch catalog #: 115-035-146) at ambient temperature. The membrane was then washed with TBST for 5 min (3X) while rocking. A 10-mL portion of chemiluminescence substrate (Thermo Scientific SuperSignal West Femto Maximum Sensitivity, product #34095) was prepared according to the manufacture's protocol. The membrane was allowed to incubate in the chemiluminescence substrate for 10 minutes before imaging. The blot was imaged using a standard digital SLR camera in dark room.

### *Circular dichroism spectroscopy*

A 50 µM solution of each peptide was prepared by diluting the 10 mg/mL stock solution with 10 mM sodium phosphate buffer at pH 7.4. Each solution was transferred to a 1 mm quartz cuvette for data acquisition. Circular dichroism spectra were acquired on a Jasco J-810 circular dichroism spectropolarimeter at ambient temperature. Data were collected using 2.0 nm intervals from 260 nm to 190 nm and averaged over five accumulations with smoothing.

### *Size exclusion chromatography*

Size exclusion chromatography was performed on peptides **1**, **2**, **N+1** through **N+14**, and **N+14Me** using an AKTA Explorer 10 FPLC equipped with a GE Superdex Increase 75 10/300 GL column at ambient temperature**.** Each peptide was dissolved in deionized water to a concentration of 10 mg/ml. The peptide solutions were then diluted to 1.0 mg/mL by adding 75 uL of the 10-

mg/ml solutions to 675 uL of TBS (50 mM Tris buffer pH 7.5 and 100 mM NaCl). The peptide solutions were centrifuged at 12,000 RPM for 2 minutes to precipitate insoluble material prior to injection, soluble material was then loaded onto the column at 0.5 ml/min over 1 min. After loading, the samples were run with TBS at 1.0 ml/min. Chromatograms were recorded at 214 nm and normalized to the highest absorbance value. Standards (cytochrome C, aprotinin, and vitamin B12) were run in the same fashion.

### *LDH release assays*

The toxicity of peptides **1**, **2**, **N+1** through **N+14**, **N+14$_{Me}$** toward SH-SY5Y cells was assessed by LDH release assays. Cells were incubated in the presence or absence of equivalent concentrations of peptides **1**, **2**, **N+1** through **N+14**, **N+14$_{Me}$** for 72 h in 96-well plates. The LDH release assay was performed using the Pierce LDH Cytotoxicity Assay Kit from Thermo Scientific. Experiments were performed in replicates of four, and an additional 8 wells were used for controls. Cells were cultured in the inner 60 wells (rows B–G, columns 2–11) of the 96-well plate. DMEM:F12 media (100 µL) was added to the outer wells (rows A and H and columns 1 and 12), in order to ensure the greatest reproducibility of data generated from the inner wells.

*a. Preparation of stock solutions of peptides 1, 2, N+1 through N+14, N+14$_{Me}$.* 10-mg/mL stock solutions of peptides **1**, **2**, **N+1** through **N+14**, **N+14$_{Me}$** were prepared gravimetrically by dissolving 1.0 mg of each compound in 100 µL of  18 MΩ deionized water. The peptide stock solutions were used to create 10X working solutions of peptides **1**, **2**, **N+1** through **N+14**, **N+14$_{Me}$**.

*b. Preparation of SH-SY5Y cells for LDH release assays.* SH-SY5Y cells were plated in a 96-well plate at 30,000 cells per well. Cells were incubated in 100 µL of a 1:1 mixture of DMEM:F12 media supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL

streptomycin at 37 °C in a 5% CO2 atmosphere and allowed to adhere to the bottom of the plate for 24 hours.

*c. Treatment of SH-SY5Y cells with peptides 1, 2, N+1 through N+14, N+14$_{Me}$.* After 24 hours, the culture media was removed and replaced with 90 µL of serum-free DMEM:F12 media. 10-µL of the 10X working solutions of peptides **1**, **2**, **N+1** through **N+14**, **N+14$_{Me}$** were added to the wells on the 96-well plates. Experiments were run in replicates of four. Four wells were used as controls and received 10-µL aliquots of deionized water (vehicle). Another four wells were left untreated, to be subsequently used as controls with lysis buffer for the LDH release assay. Cells were incubated at 37 °C in a 5% CO2 atmosphere for 72 hours.

### *Crystallization of peptide N+1*

The hanging-drop vapor-diffusion method was used to determine initial crystallization conditions for peptides **N+1**. Peptide **2a** was screened in 96-well plate format using three crystallization kits (Crystal Screen, Index, and PEG/ION) from Hampton Research. A TTP LabTech Mosquito nanondisperse was used to make three 150 nL hanging drops for each well condition. The three hanging drops differed in the ratio of peptide to well solution for each condition in the 96-well plate. A 10 mg/mL solution of each peptide in deionized water was combined with a well solution in ratios of 1:1, 1:2, and 2:1 peptide:well solution at appropriate volumes to create the three 150 nL hanging drops. Crystals of peptide **N+1** grew in well conditions of 0.2 M sodium citrate, 0.1 M HEPES pH 7.5, and 20% (v/v) isopropyl alcohol.

Crystallization conditions for each peptide were optimized using a 4x6 matrix Hampton 24-well plate. For peptide **N+1** the 0.1 M HEPES buffer was varied in each row in increments of 0.1 pH units (6.9, 7.0, 7.1, and 7.2) and the percentage of isopropyl alcohol in each column in increments of 1% (v/v) (20%, 21%, 22%, 23%, 24%, 25%). Three hanging-drops were prepared

on borosilicate glass slides by combing a 10 mg/mL solution of peptide **N+1** in deionized water with the well solution in the following amounts: 1 μL:1 μL, 2 μL:1 μL, and 1 μL:2 μL. Slides were inverted and pressed firmly against the silicone grease surrounding each well. Crystals were harvested with a nylon loop attached to a copper or steel pin, soaked briefly in 1 M KI solution, and flash frozen in liquid nitrogen prior to data collection. The optimized crystallization conditions for peptide **N+1** is summarized Supplementary Table 3.

***X-ray crystallographic data collection, data processing, and structure determination of N+1***

Diffraction data for **N+1** were collected on a Rigaku Micromax-007HF X-ray diffractometer with a rotating copper anode using CrystalClear software. Datasets were indexed and integrated with XDS.[3] Scaling and merging was done with pointless and aimless in CCP4.[4] The structures were solved with molecular replacement in Phaser[5] using a dimer formed by peptide **1** (PDB 5W4H) as the search model. The refinement was done with phenix.refine module of the Phenix suite, with manipulation of the model performed using Coot.[6]

## References

1. These procedures follow closely those that our laboratory has previously published. The procedures in this section are adapted from and in some cases taken verbatim from Kreutzer, A. G.; Hamza, I. L.; Spencer, R. K.; Nowick J. S. *J. Am. Chem. Soc.* **2016**, *138*, 4634–4642, Spencer, R. K.; Kreutzer, A. G.; Salveson, P. J.; Li, H.; Nowick, J. S. *J. Am. Chem. Soc.* **2015**, *137*, 6304–6311, Spencer, R. K.; Li. H.; Nowick, J. S. *J. Am. Chem. Soc.* **2014**, *136*, 5595– 5598, and Kreutzer, A. G.; Yoo, S.; Spencer, R. K.; Nowick, J. S. *J. Am. Chem, Soc.* **2017**, *139*, 966–975.

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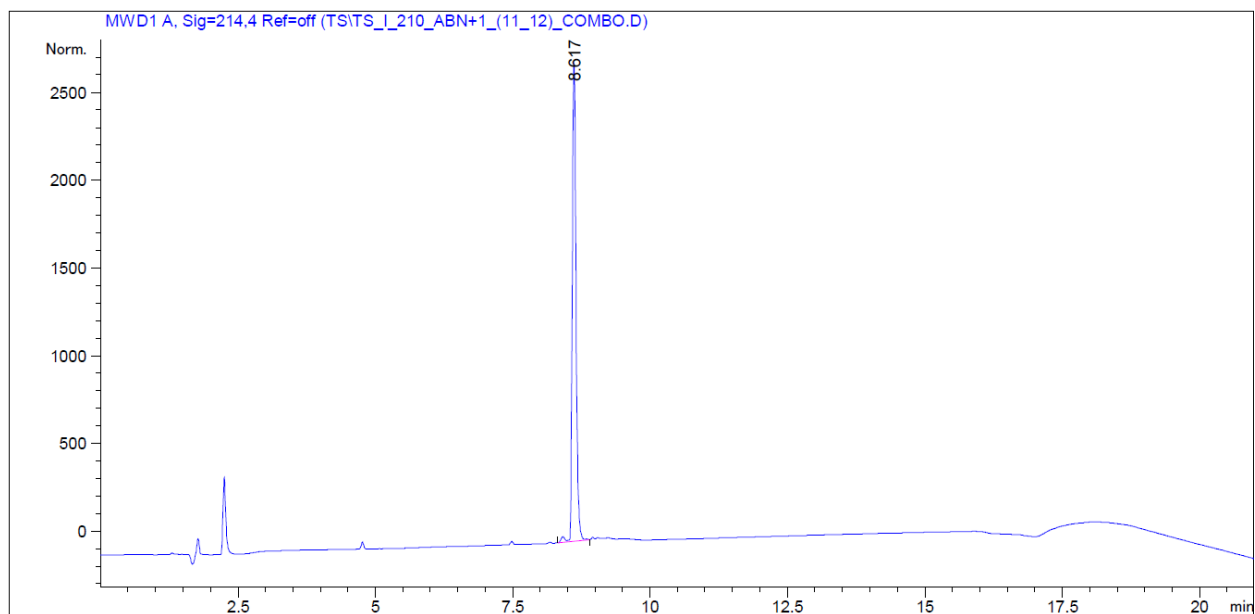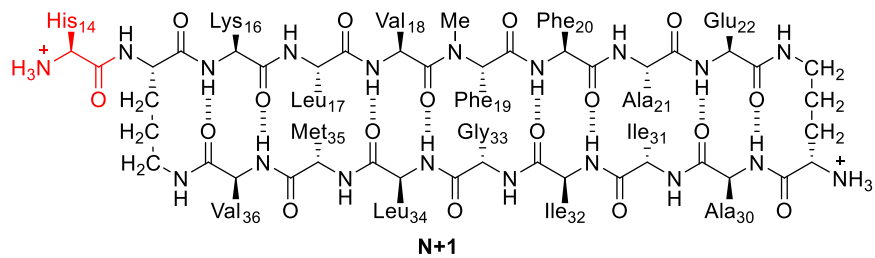
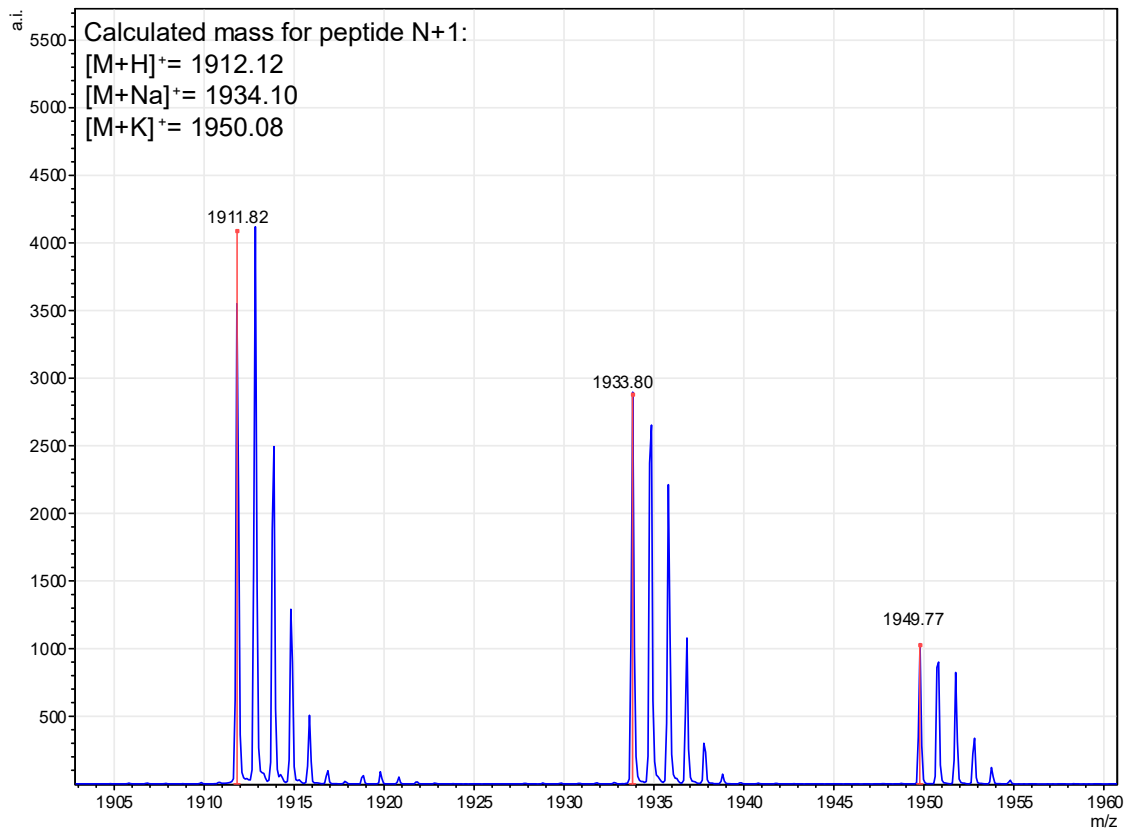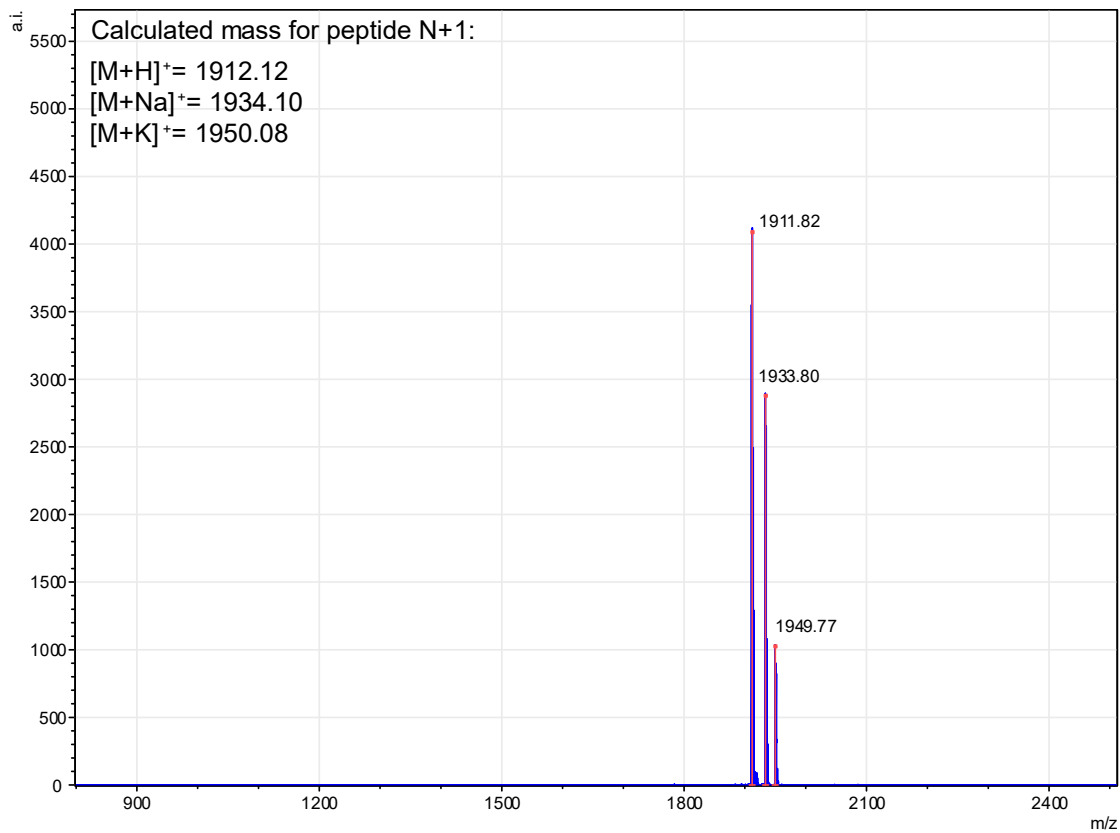# Characterization Data

*Characterization of peptide **1***



peptide **1**
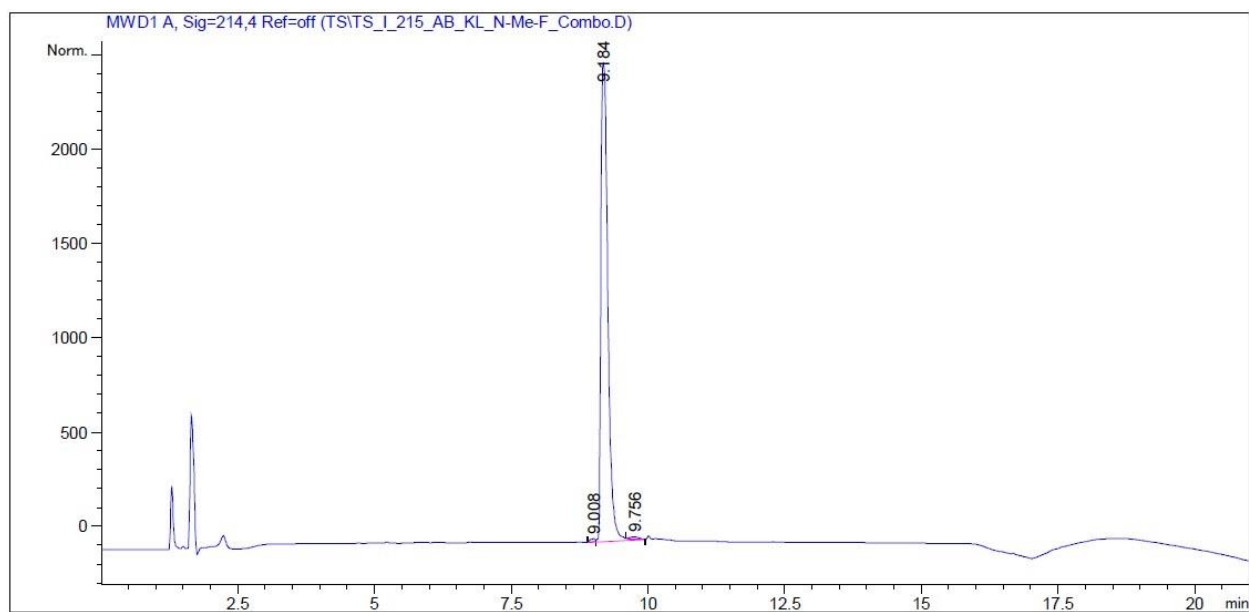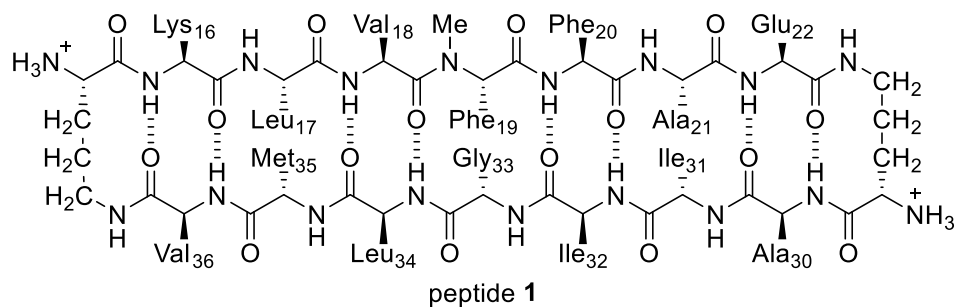


| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Height [mAU] | Area % |
|------|--------|------|--------|------------|------------|---------|
| 1 | 9.008 | BV E | 0.0814 | 78.69865 | 13.62904 | 0.3870 |
| 2 | 9.184 | VV R | 0.1387 | 2.01192e4 | 2285.21826 | 98.9293 |
| 3 | 9.756 | VB E | 0.1593 | 139.05769 | 12.54508 | 0.6838 |

Calculated mass for peptide 1:

[M+H]⁺= 1775.06
[M+Na]⁺= 1797.04
[M+K]⁺= 1813.02

Calculated mass for peptide 1:

[M+H]⁺= 1775.06
[M+Na]⁺= 1797.04
[M+K]⁺= 1813.02

*Characterization of N+1*



N+1
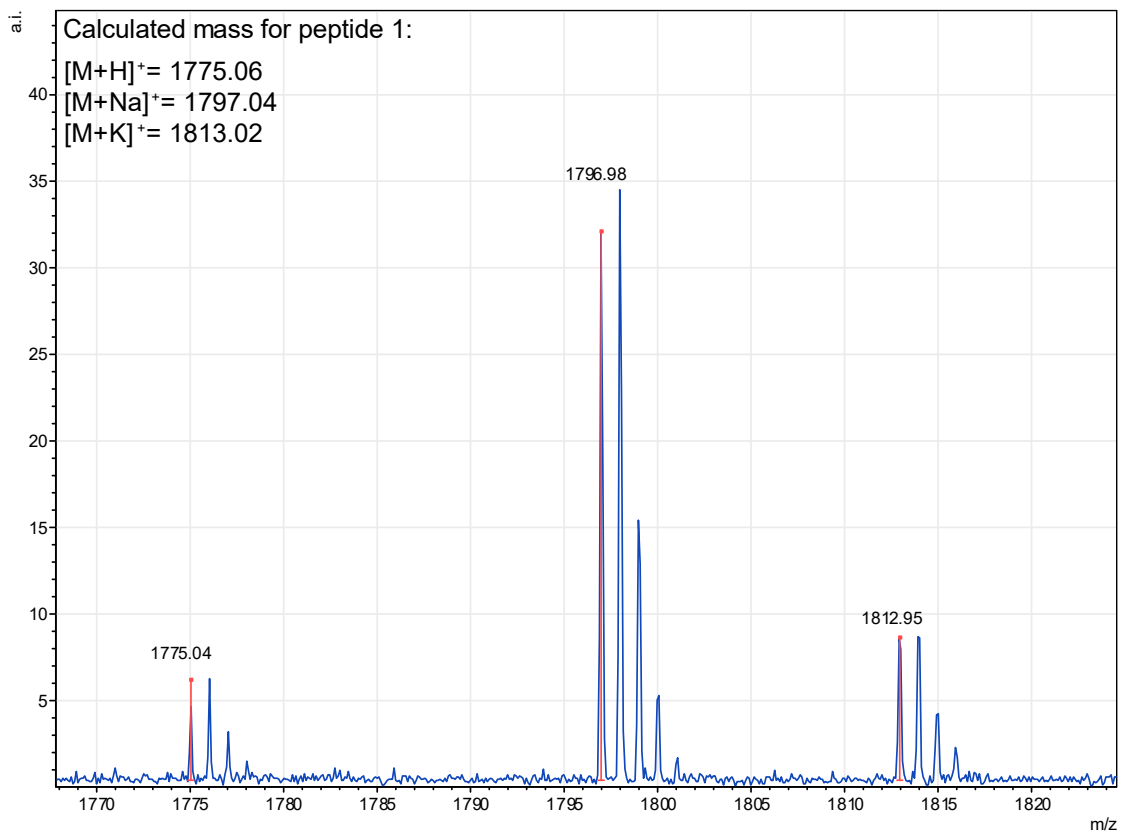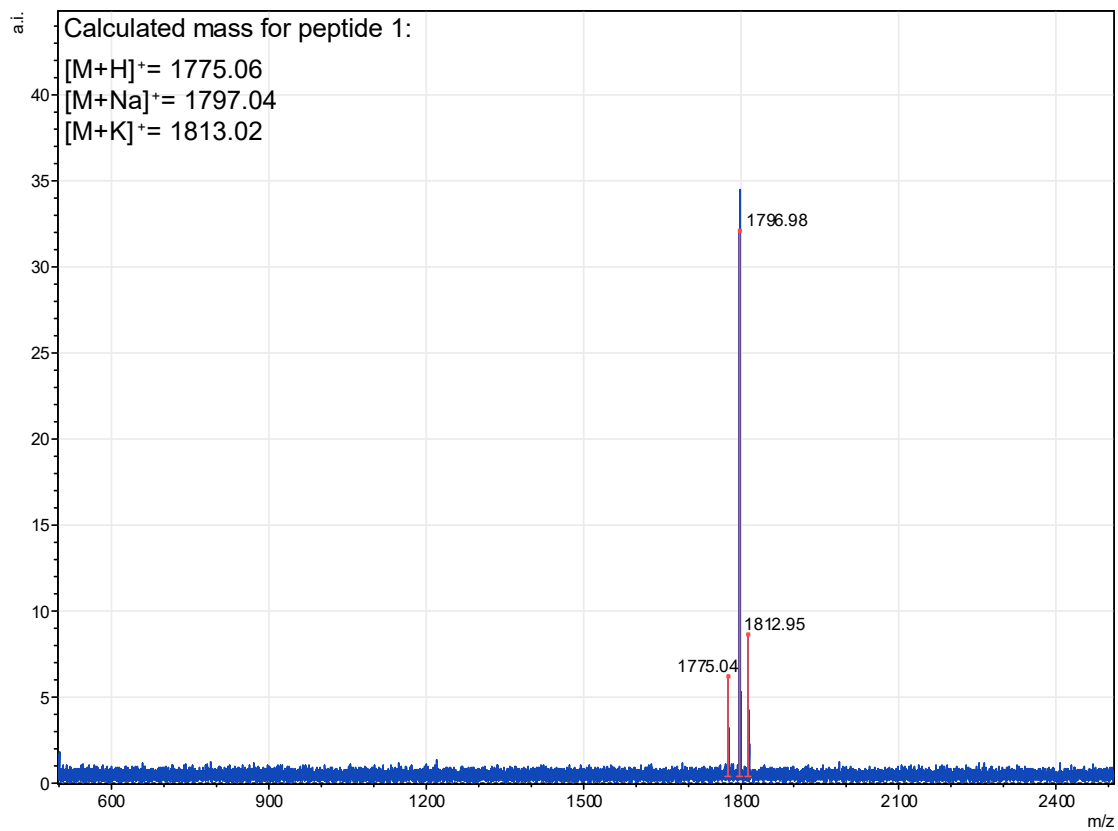


| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Height [mAU] | Area % |
|------|--------|------|--------|------------|-------------|----------|
| 1 | 8.617 | VV R | 0.0756 | 1.18688e4 | 2466.48389 | 100.0000 |

Calculated mass for peptide N+1:

$[M+H]^+$= 1912.12
$[M+Na]^+$= 1934.10
$[M+K]^+$= 1950.08

Calculated mass for peptide N+1:
$[M+H]^+$= 1912.12
$[M+Na]^+$= 1934.10
$[M+K]^+$= 1950.08

*Characterization of N+2*



**N+2**



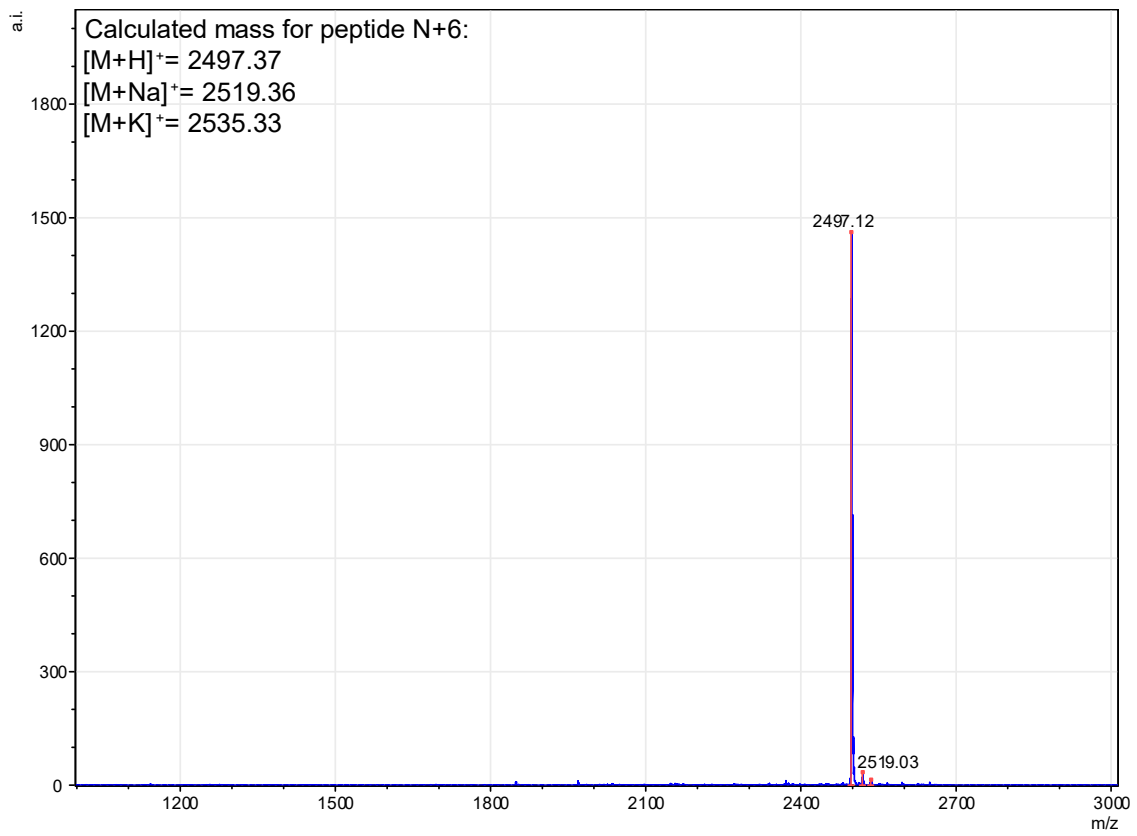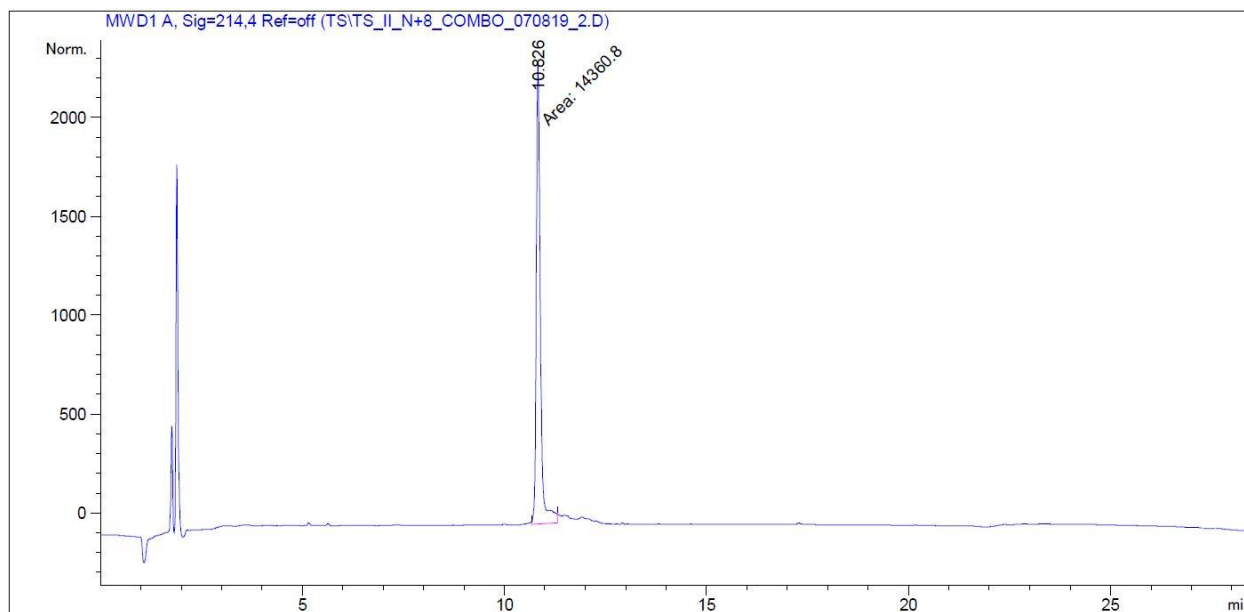| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Height [mAU] | Area % |
|------|---------|------|--------|-----------|-----------|----------|
| 1 | 8.872 | VV R | 0.2878 | 2.15888e4 | 961.62427 | 100.0000 |
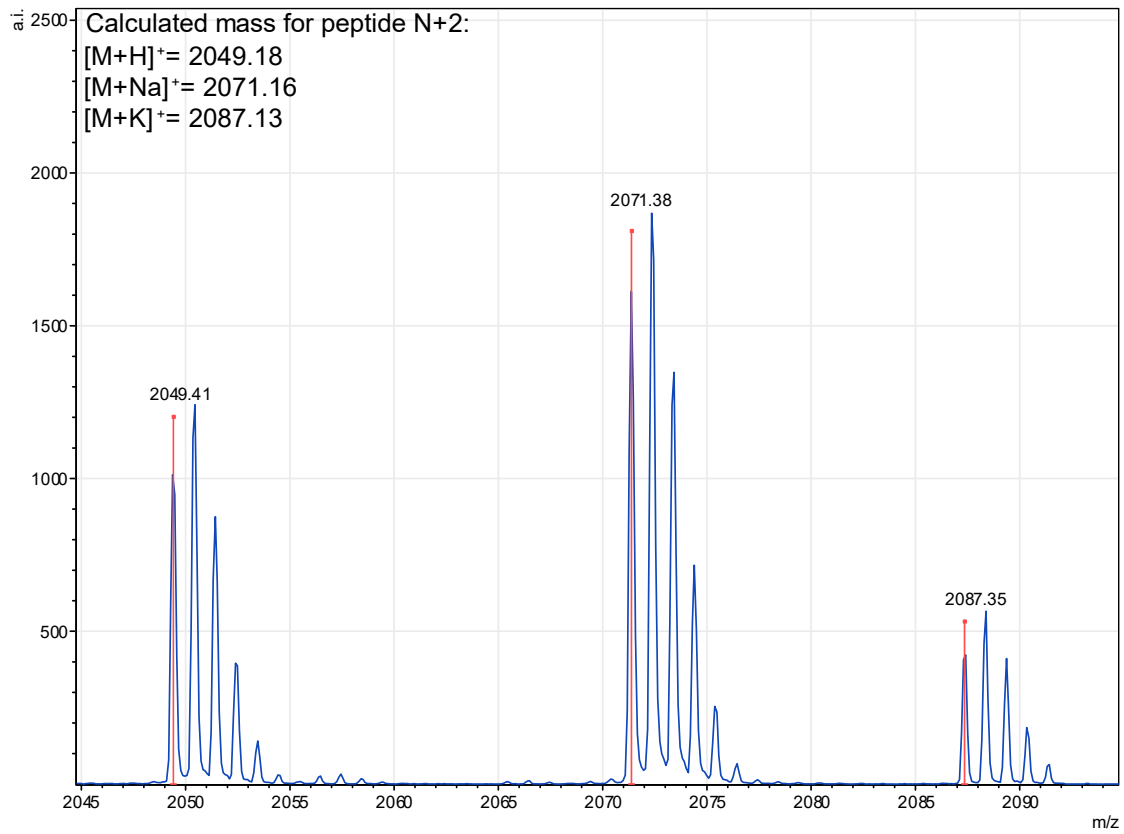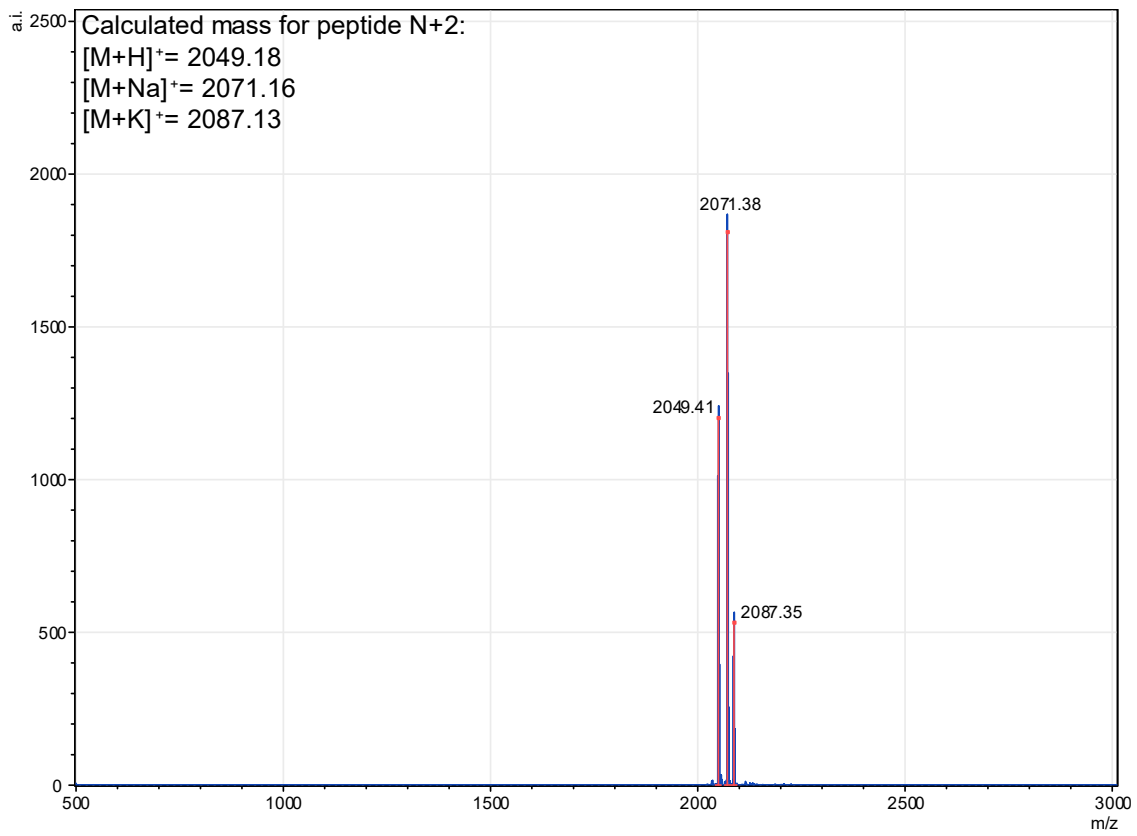
*Characterization of N+4*
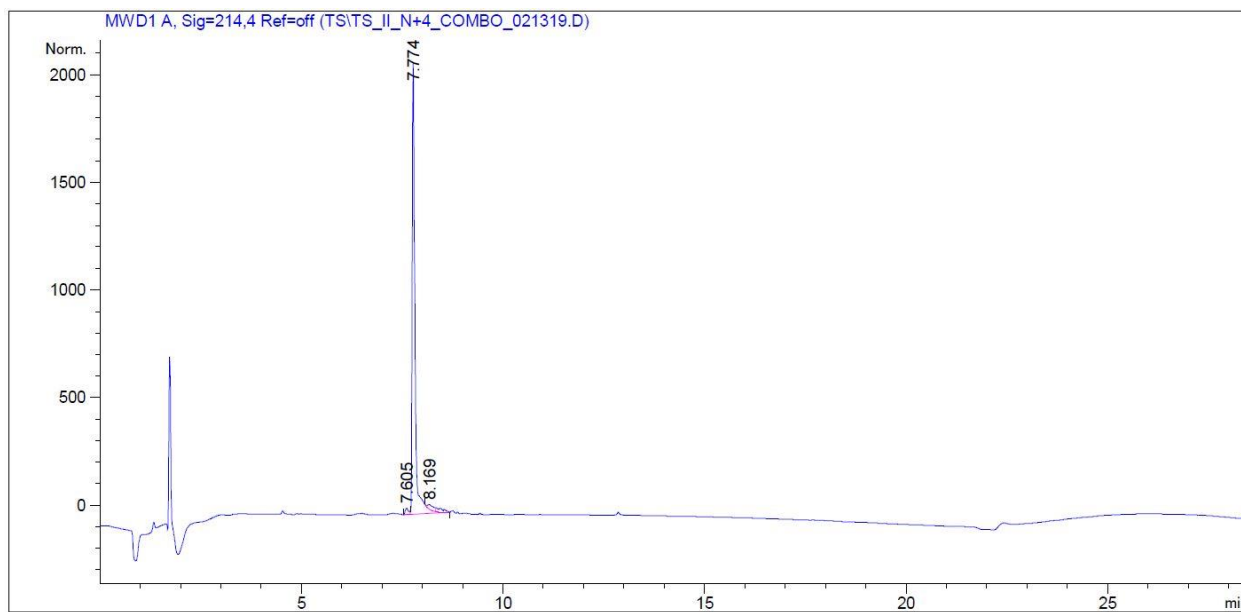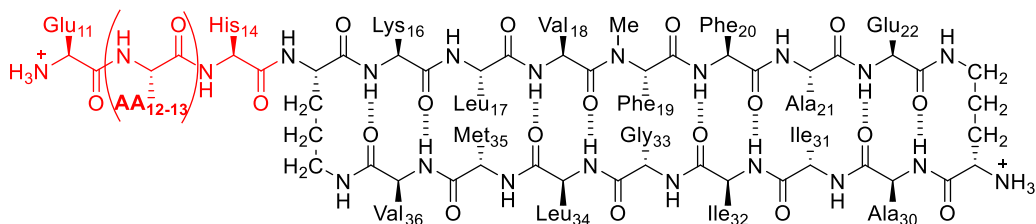


| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Height [mAU] | Area % |
|------|---------|------|--------|------------|------------|--------|
| 1 | 7.605 | BV E | 0.0805 | 151.97638 | 27.47144 | 1.5412 |
| 2 | 7.774 | VV R | 0.0701 | 9104.26660 | 1894.34375 | 96.1533 |
| 3 | 8.169 | VV E | 0.1663 | 227.32799 | 18.37850 | 2.3054 |

Calculated mass for peptide N+4:
[M+H]⁺= 2277.29
[M+Na]⁺= 2299.27
[M+K]⁺= 2315.25
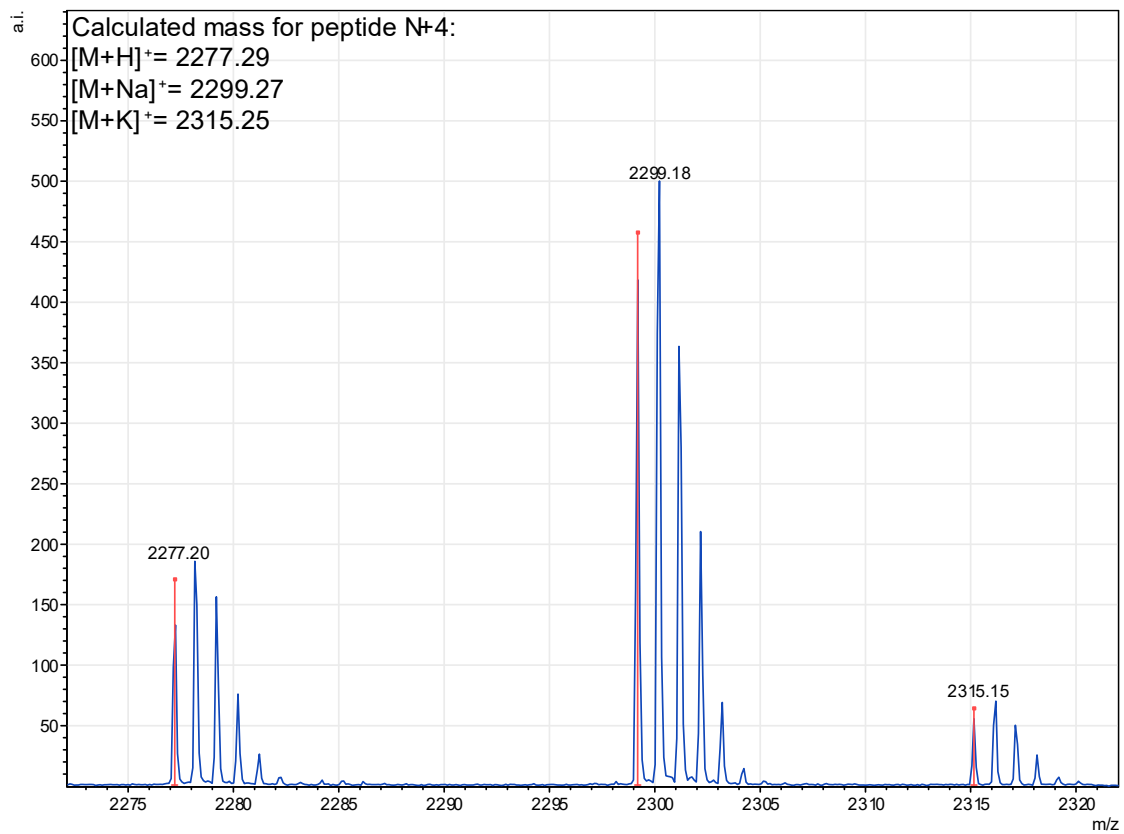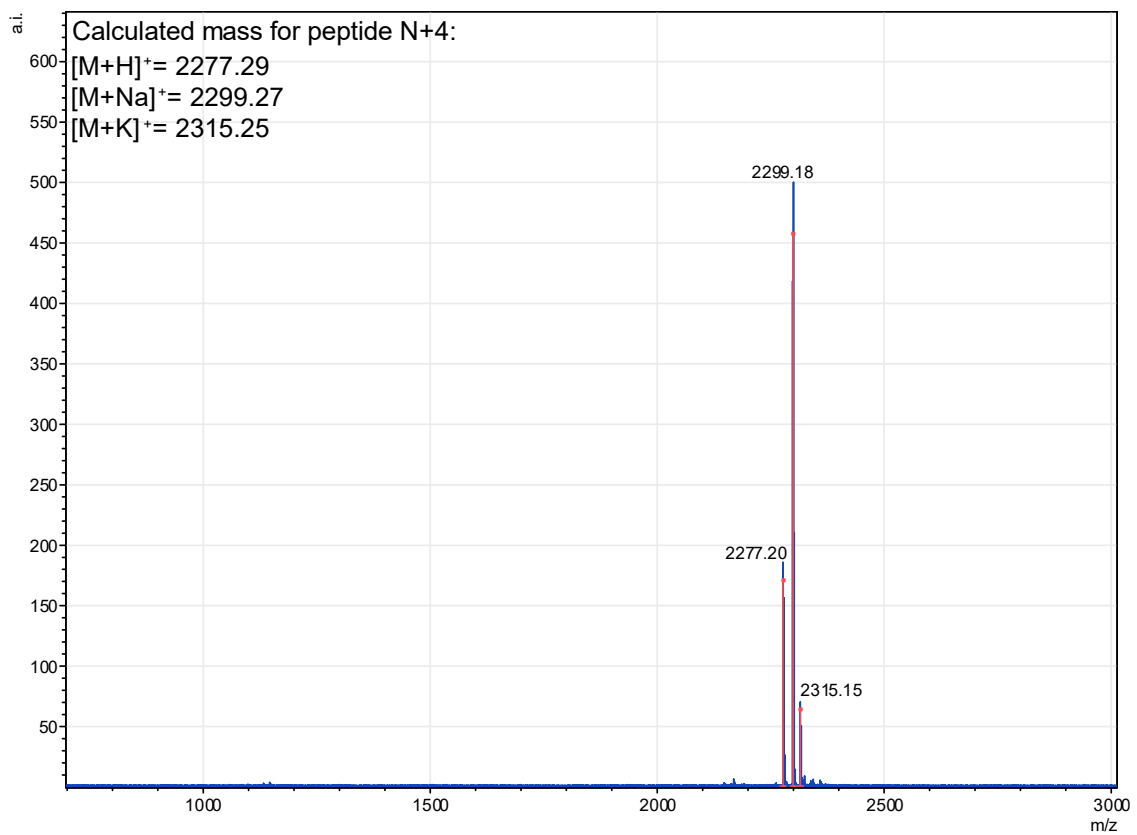
*Characterization of N+6*
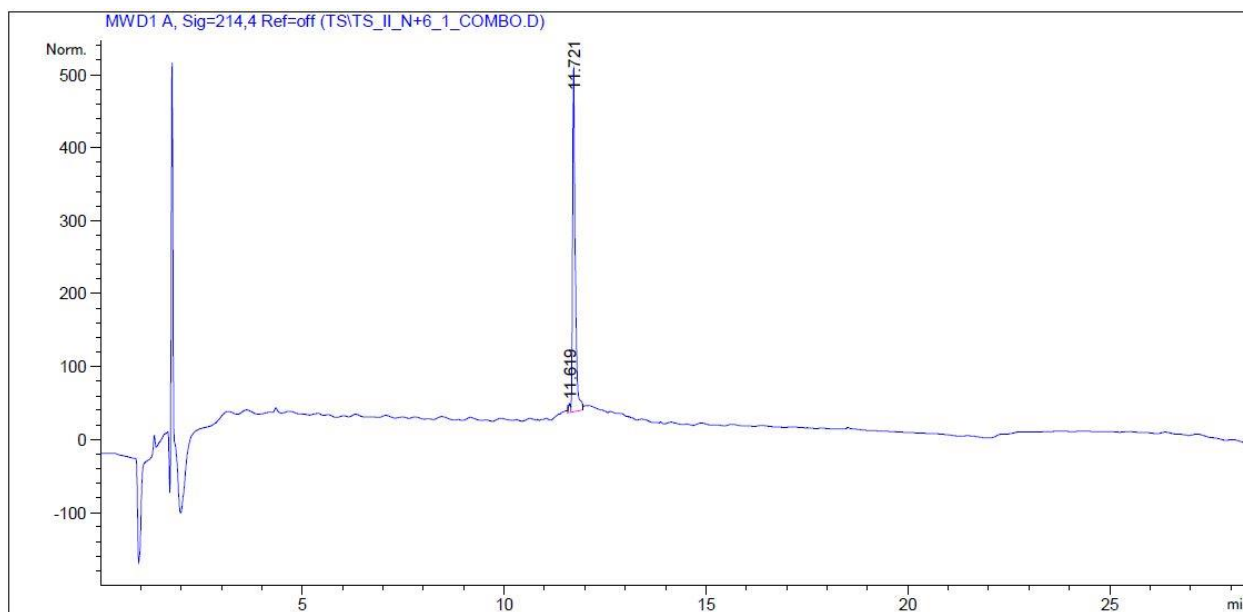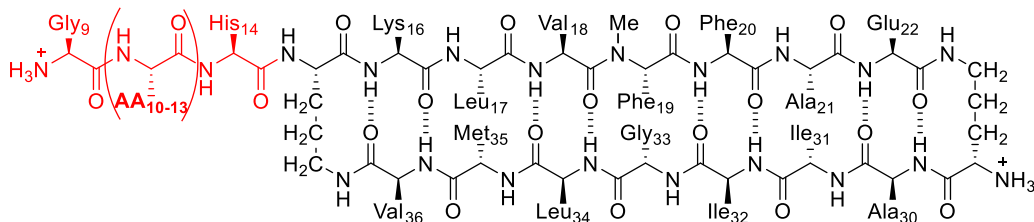


| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Height [mAU] | Area % |
|------|---------|------|--------|------------|------------|---------|
| 1 | 11.619 | BV E | 0.0462 | 21.64151 | 7.84065 | 1.6787 |
| 2 | 11.721 | VV R | 0.0664 | 1894.66553 | 421.83078 | 98.3213 |

Calculated mass for peptide N+6:
[M+H]⁺= 2497.37
[M+Na]⁺= 2519.36
[M+K]⁺= 2535.33



Calculated mass for peptide N+6:
[M+H]⁺= 2497.37
[M+Na]⁺= 2519.36
[M+K]⁺= 2535.33
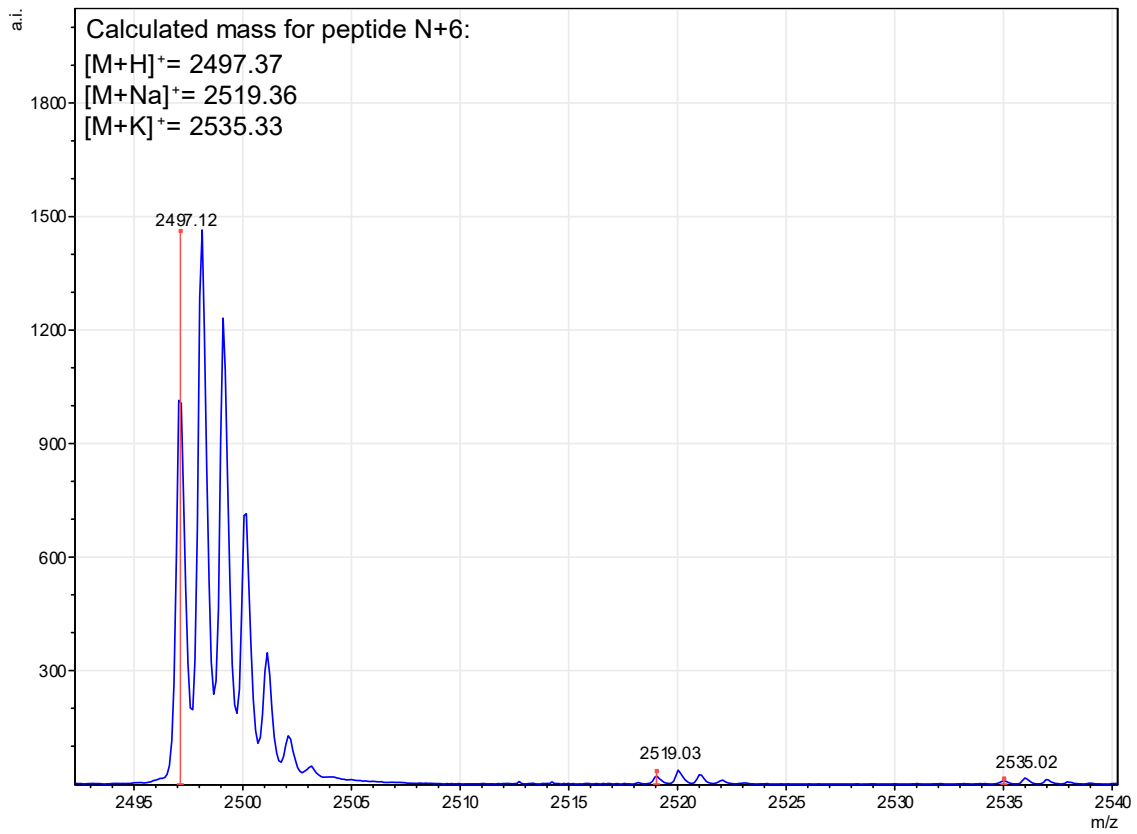
*Characterization of N+8*
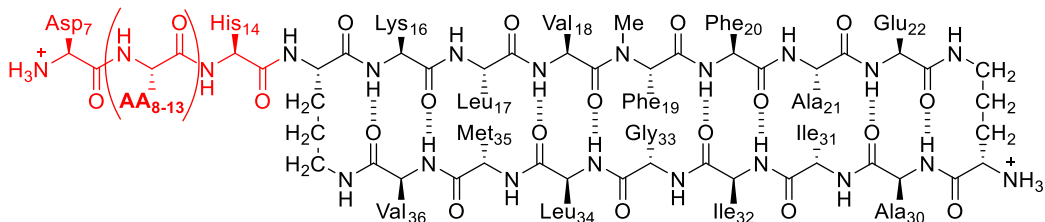


```
Peak  RetTime Type   Width     Area       Height      Area
 #    [min]          [min]    [mAU*s]     [mAU]        %
----|-------|----|-------|----------|----------|--------|
  1   10.826 MM    0.3137 1.43608e4  2105.97363 100.0000
```

Calculated mass for peptide N+8:
[M+H]⁺ = 2699.43
[M+Na]⁺ = 2721.42
[M+K]⁺ = 2737.39
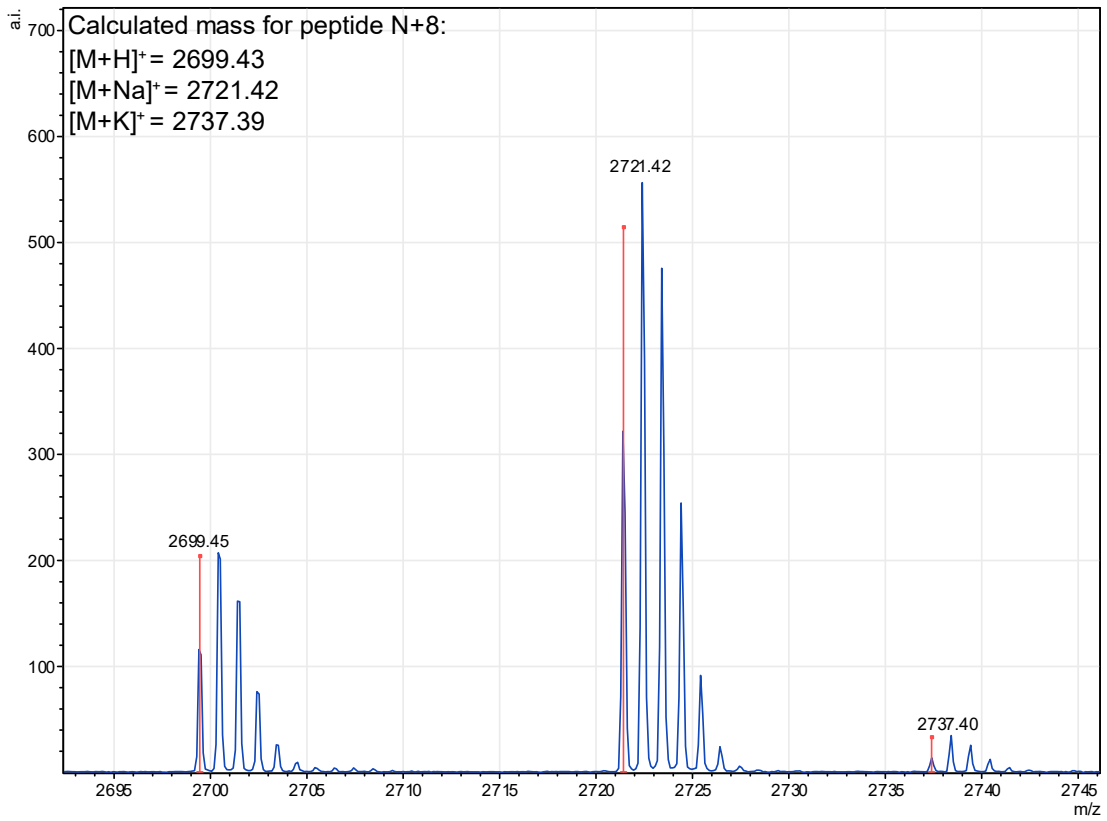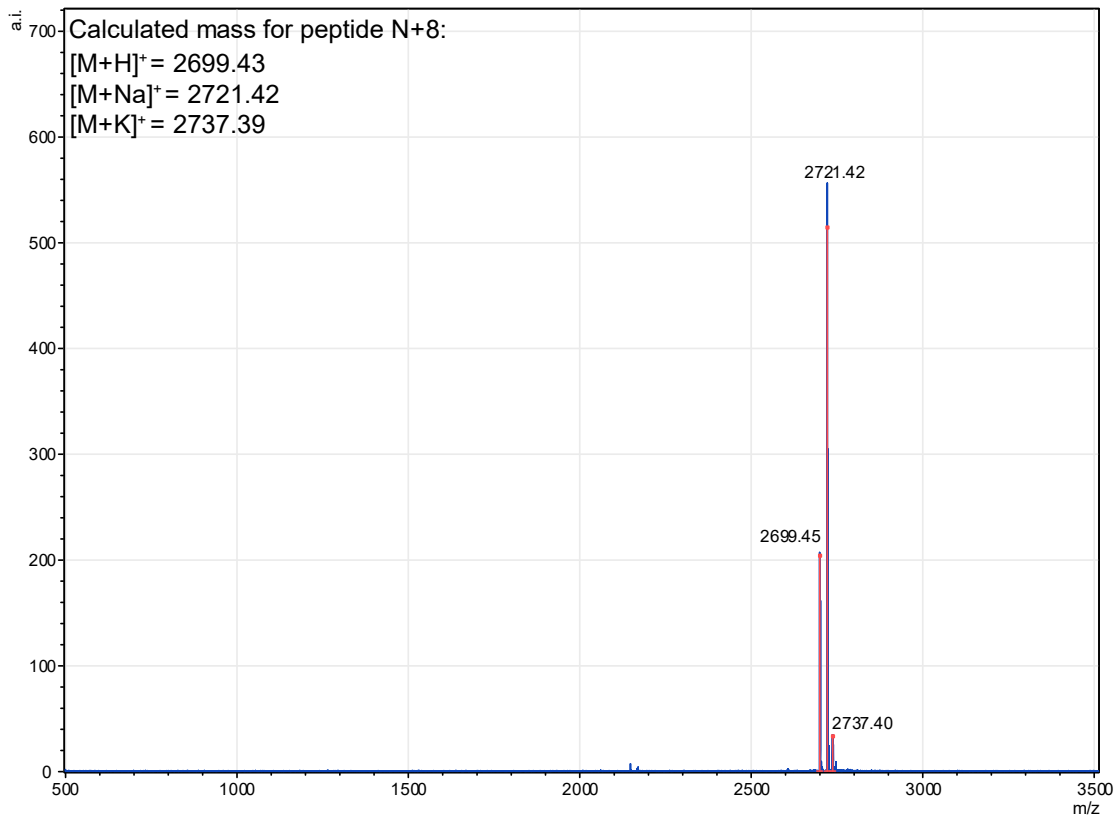
*Characterization of N+10*



```
Peak  RetTime Type  Width     Area      Height      Area
 #    [min]         [min]    [mAU*s]     [mAU]        %
----|-------|----|-------|----------|----------|--------|
  1   9.429 BV E  0.0606   346.39841   83.20280   2.8873
  2   9.580 VV R  0.1160  1.64610e4  2132.79688  97.1127
```

Calculated mass for peptide N+10:
[M+H]⁺ = 2992.59
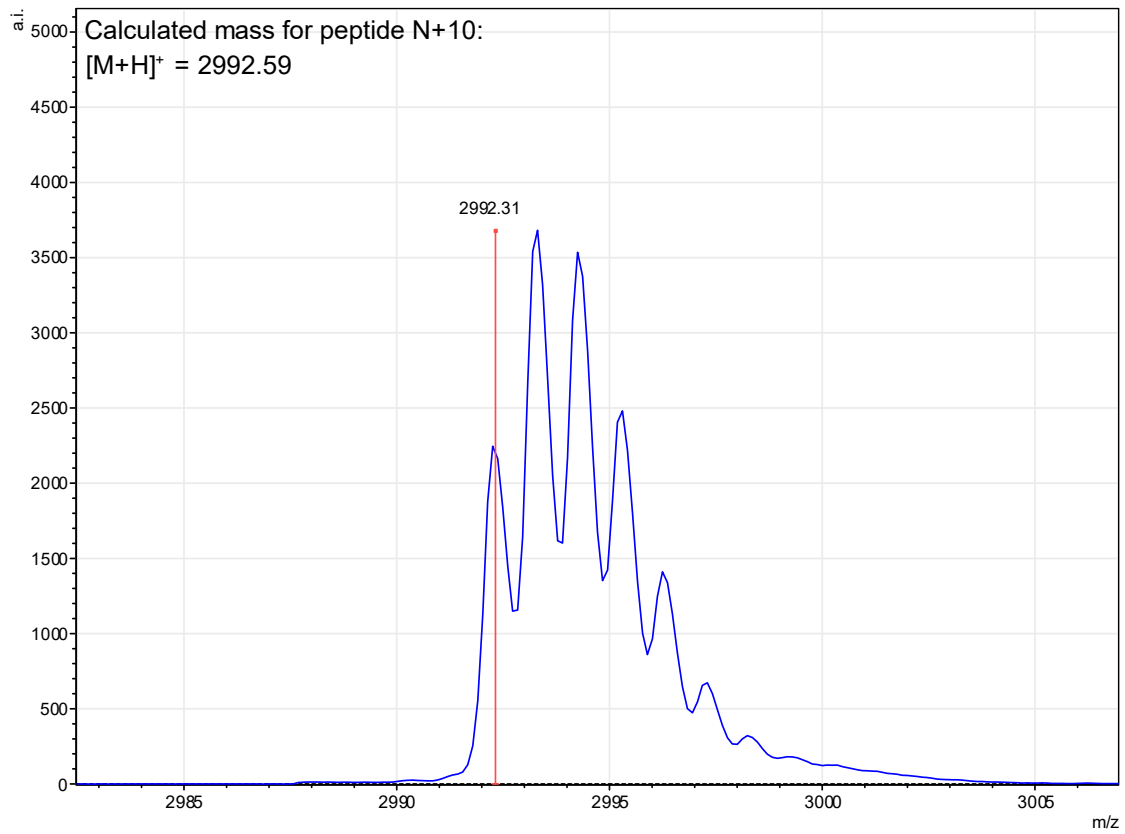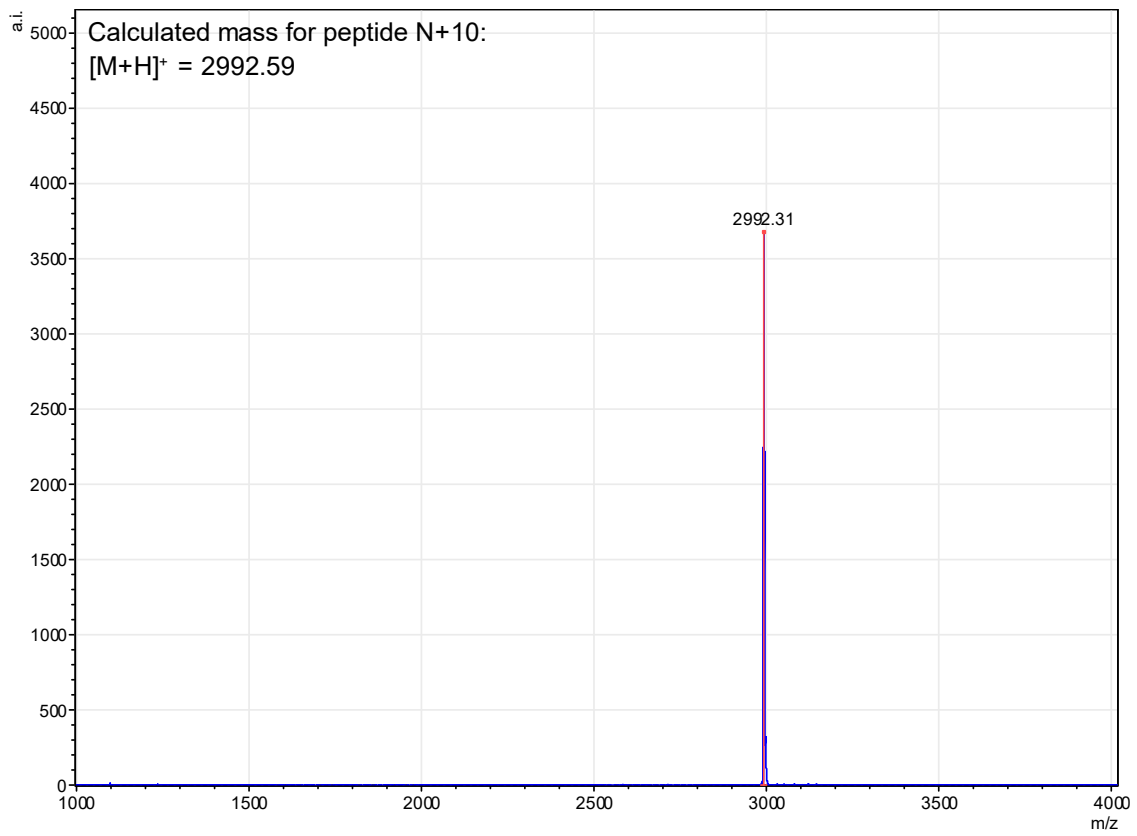
*Characterization of N+12*
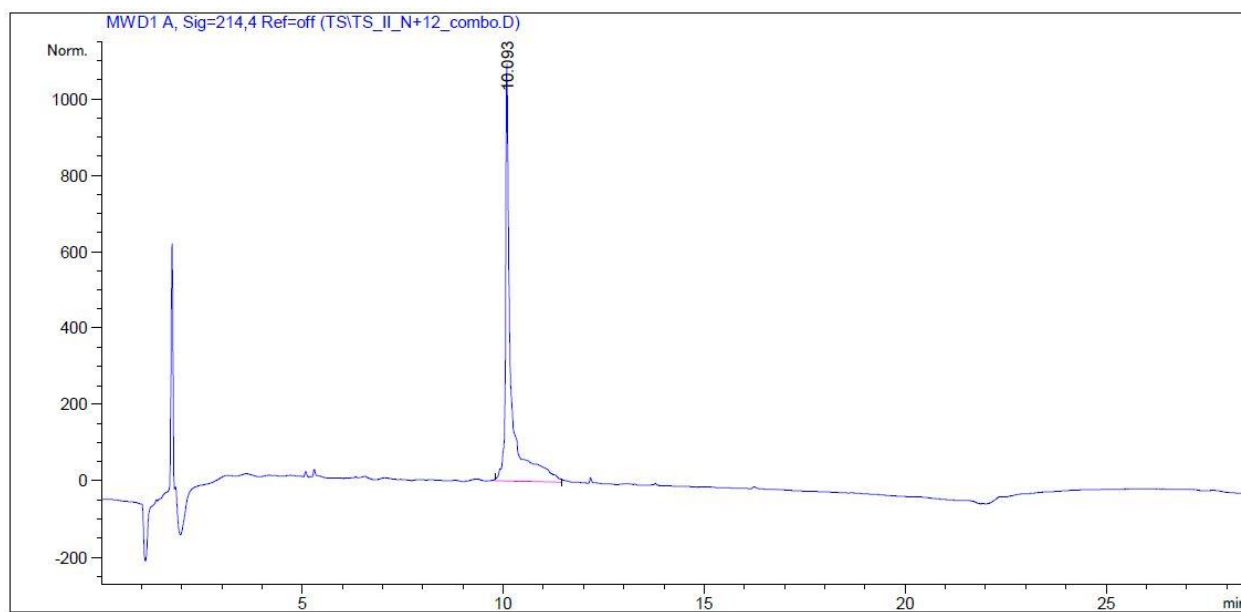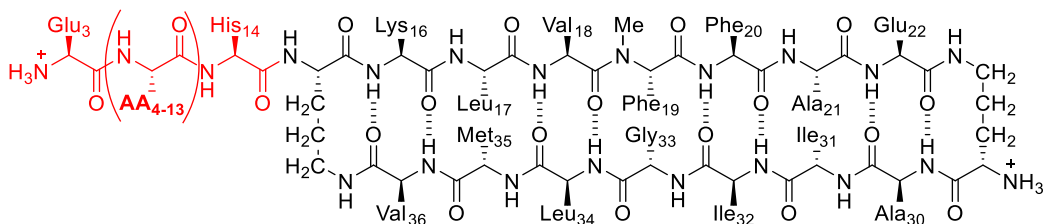


| Peak | RetTime | Type | Width | Area | Height | Area |
| # | [min] | | [min] | [mAU*s] | [mAU] | % |
|------|---------|------|-------|----------|-----------|----------|
| 1 | 10.093 | MF | 0.6067 | 6318.32568 | 986.75244 | 100.0000 |

Calculated mass for peptide N+12:
$[M+H]^+$ = 3268.70
$[M+Na]^+$ = 3290.69

3268.69

3290.67



Calculated mass for peptide N+12:
$[M+H]^+$ = 3268.70
$[M+Na]^+$ = 3290.69

3268.69

3290.6754(1)

Calculated mass for peptide N+14:
$[M+H]^+ = 3454.77$
$[M+Na]^+ = 3476.75$

3455.27

3477.25

Calculated mass for peptide N+14:
$[M+H]^+ = 3454.77$
$[M+Na]^+ = 3476.75$

3455.27

3477.25

97
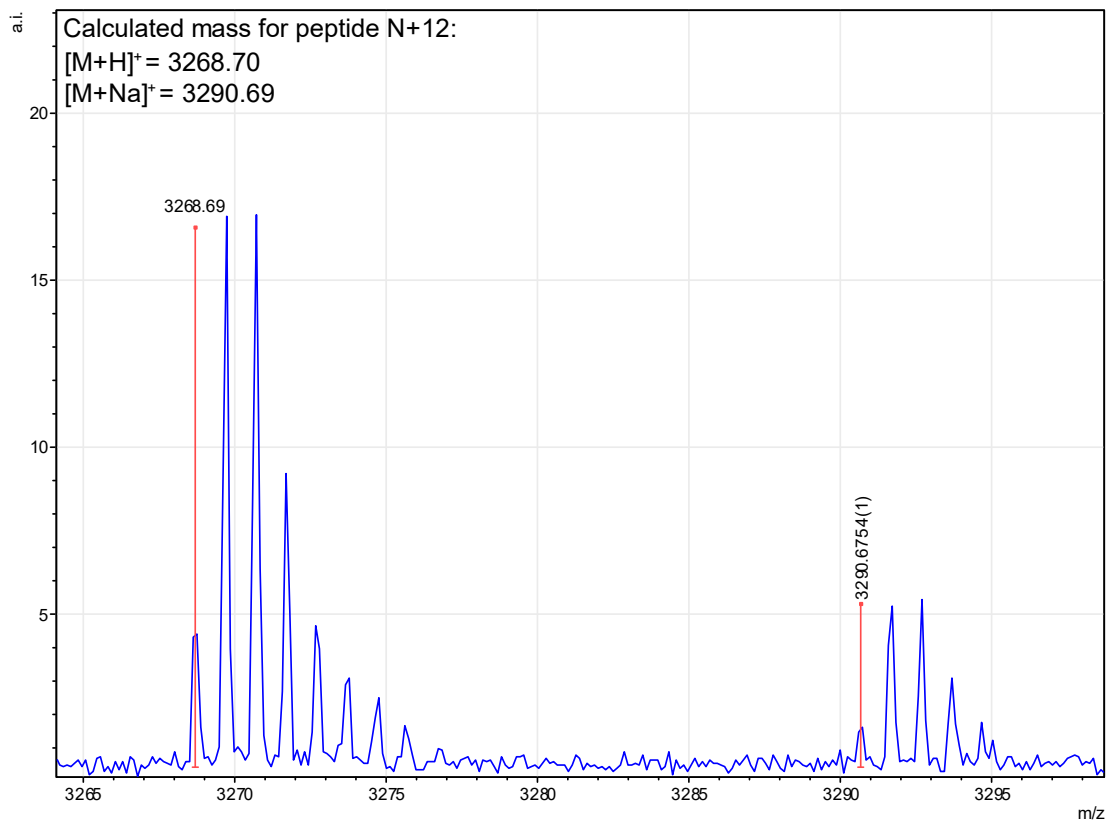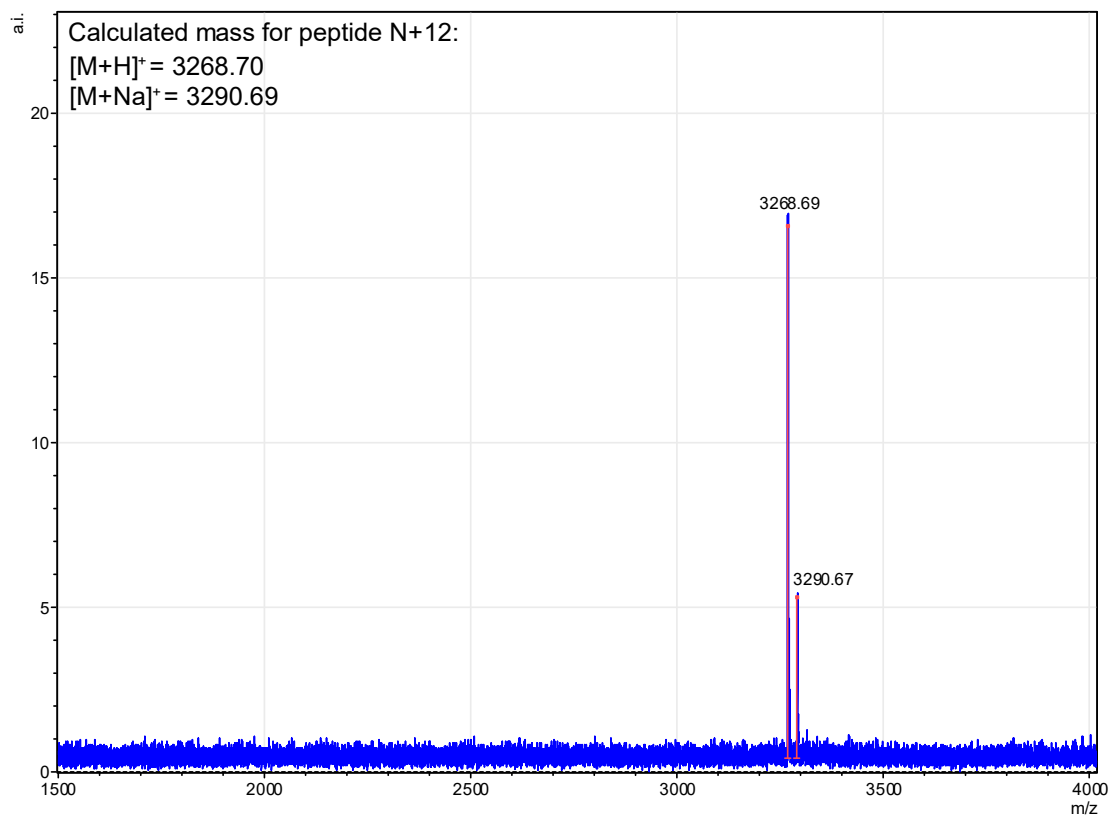
*Characterization of N+14<sub>Me</sub>*





| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Height [mAU] | Area % |
|------|---------|------|--------|-----------|-----------|----------|
| 1 | 9.954 | BV R | 0.0673 | 9244.65234 | 1702.65344 | 100.0000 |

Calculated mass for peptide N+14$_{Me}$:
[M+H]$^+$ = 3468.78
[M+Na]$^+$ = 3490.77

3468.76

3490.76



Calculated mass for peptide N+14$_{Me}$:
[M+H]$^+$ = 3468.78
[M+Na]$^+$ = 3490.77

3468.76

3490.76
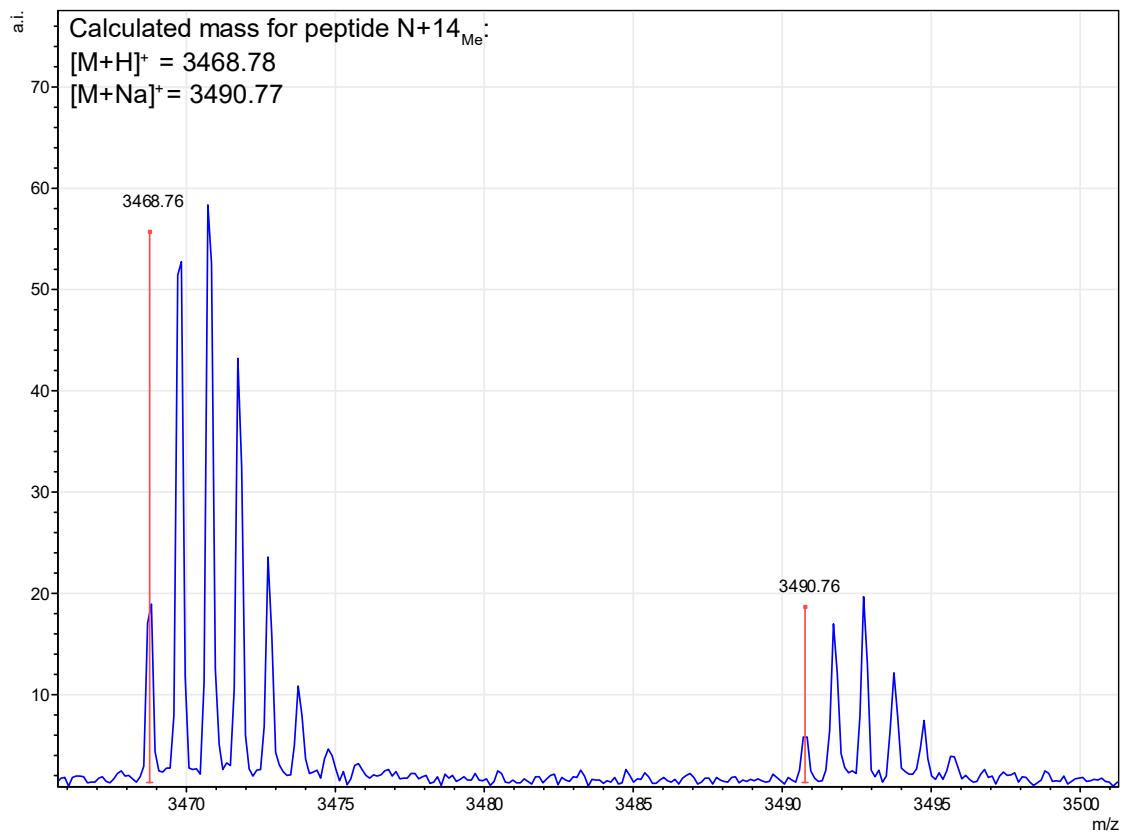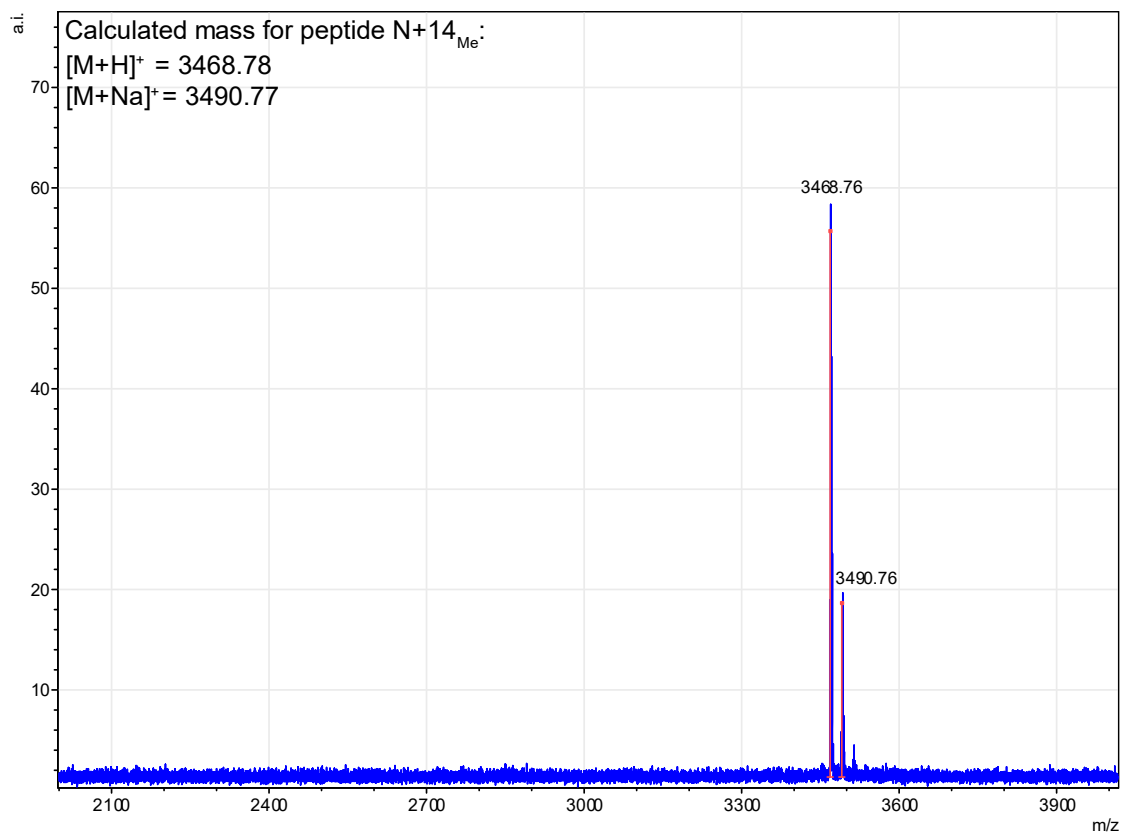
*Characterization of peptide 2*



| Peak # | RetTime [min] | Type | Width [min] | Area [mAU*s] | Height [mAU] | Area % |
|------|-------|------|--------|-----------|-----------|--------|
| 1 | 6.250 | BV E | 0.0601 | 181.10962 | 42.13483 | 0.4690 |
| 2 | 6.362 | VV R | 0.1967 | 3.82930e4 | 2528.83789 | 99.1675 |
| 3 | 6.815 | VV E | 0.0421 | 101.44323 | 39.33643 | 0.3635 |
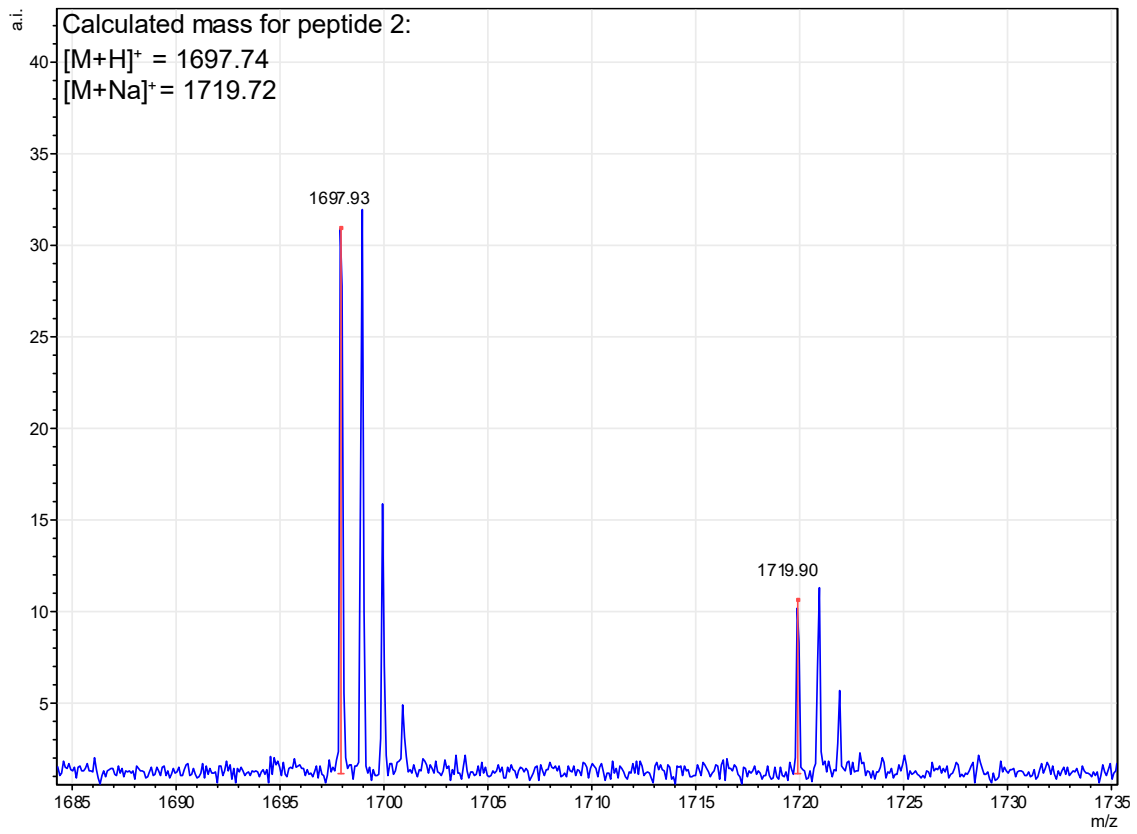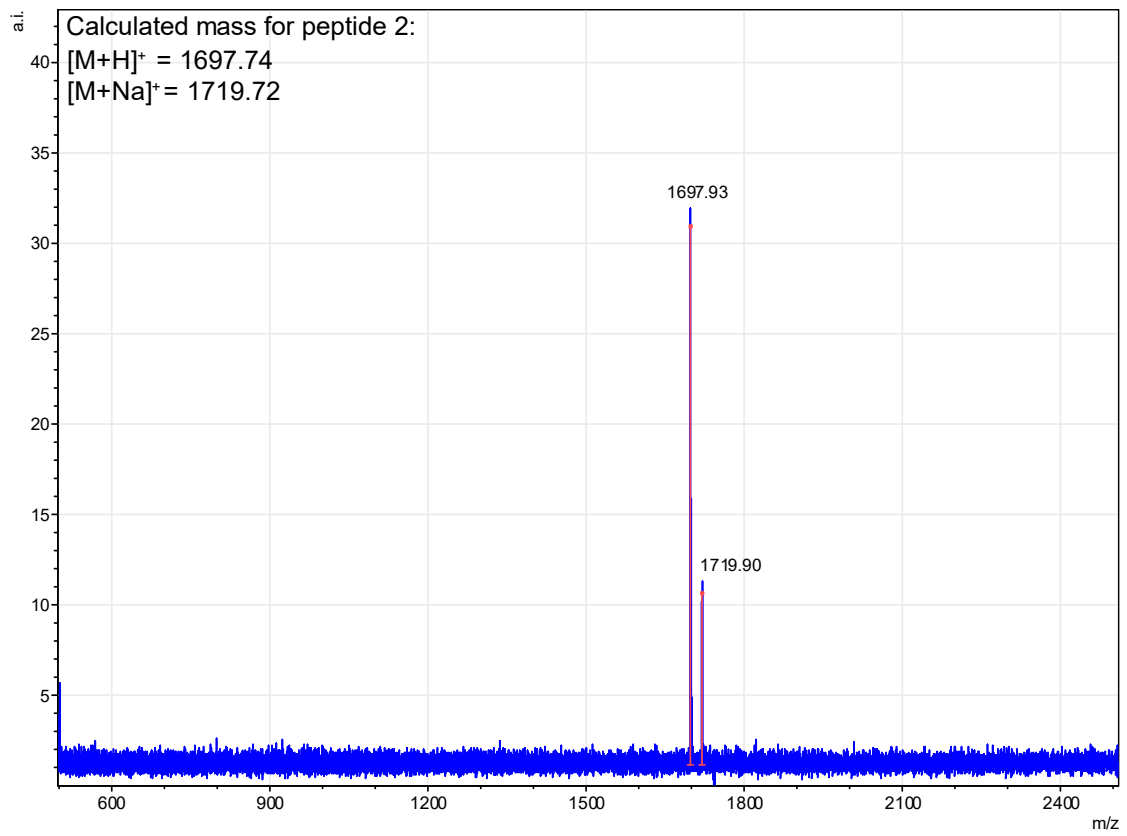
# Chapter 3

# A β-Barrel-Like Tetramer Formed by a Macrocyclic β-Hairpin Derived From Aβ

**INTRODUCTION**

The oligomers formed by the β-amyloid peptide Aβ are unstable and exhibit significant variation in their stoichiometry and structure.[1–5] This variation complicates efforts to understand how Aβ oligomers affect neurodegeneration associated with Alzheimer's disease.[6,7] Establishing structure activity relationships between Aβ oligomers and their neurotoxicity will further our understanding of Alzheimer's disease and bolster efforts to develop diagnostics and drugs.[8] Developing these connections requires expanding our library of high-resolution structures to better reflect the immense variation and heterogeneity in the structures of Aβ oligomers.

The NMR-based structure of a tetramer formed by full-length Aβ42 reported by Carulla et al. is the only atomic resolution structure of an Aβ oligomer that has been deposited in the Protein Data Bank, (PDB 6RHY).[9] The tetramer comprises a six-stranded antiparallel β-sheet, with two β-hairpins of Aβ42 that bookend two antiparallel β-strands of Aβ42 **(Sup. Figure 3.1a)**. Additional solution phase studies of this tetramer also provide evidence for the formation of an octamer. Using molecular dynamics (MD), Carulla et al. propose a model in which this tetramer, as well as the octamer, can act to disrupt a lipid membrane and facilitate water permeation. Collectively, these

studies have brought into sharp relief the importance of β-hairpins in the structures of Aβ oligomers.

Several other studies have also established the significance of the β-hairpin conformation in the formation of Aβ oligomers.[10–15] In 2008, Härd and Hoyer reported the NMR structure of a monomer of Aβ$_{40}$ adopting a β-hairpin conformation when sequestered and stabilized by an affibody.[11] Härd et al. subsequently stabilized this Aβ β-hairpin using an intermolecular disulfide-bridge and found that the stabilized β-hairpin formed oligomers that mimicked some of the properties of oligomers formed by unmodified Aβ.[12] NMR spectroscopic studies revealed that related disulfide stabilized β-hairpins derived from Aβ$_{16–42}$ assembled to form a barrel-shaped hexamer stabilized by hydrophobic packing and edge-to-edge hydrogen bonding between β-hairpins **(Sup. Figure 3.1b)**.[14]

β-Hairpins have also been reported as a key component in the structures of Aβ fibrils.[16] Tycko et al. recently reported a unique Aβ$_{40}$ fibril structure, where the typical core of parallel in-register β-sheets is coated by an outer layer of β-hairpins formed by Aβ **(Sup. Figure 3.1c)**. A model of this β-hairpin created by Tycko et al., that fits the observed electron density and other data, shows residues 16–22 and 30–36 hydrogen bonding to form an antiparallel β-sheet, with the intervening residues 23–29 forming a loop **(Figure 3.1a)**.

The β-hairpins reported by Carulla et al., Härd and Hoyer et al., and Tycko et al. all differ in the alignment of their β-strands **(Figure 3.1a)**. In the tetramer reported by Carulla et al., β-strands comprising residues 9–21 and 28–40 hydrogen bond to form an antiparallel β-sheet, with residues 22–27 forming a loop. In the barrel-shaped hexamer reported Härd et al. β-strands comprising residues 17–22 and 30–36 hydrogen bond to form an antiparallel β-sheet, with residues 23–29 forming a loop. These differences in β-strand alignment alter the overall topology of β-hairpins by shifting residue pairings across β-strands, the hydrophobicity of β-hairpin surfaces,

and the size of loop segments between β-strands. In the β-hairpin reported by Carulla et al., $Ile_{32}$ is across from $Phe_{19}$; in the β-hairpin reported by Tycko et al., $Gly_{33}$ is across from $Phe_{19}$; and in the β-hairpin reported by Härd and Hoyer et al., $Val_{34}$ is across from $Phe_{19}$ **(Figure 3.1a)**. These changes in alignment and topology may contribute to the immense variation and heterogeneity observed in the assembly and structures of Aβ fibrils and oligomers.

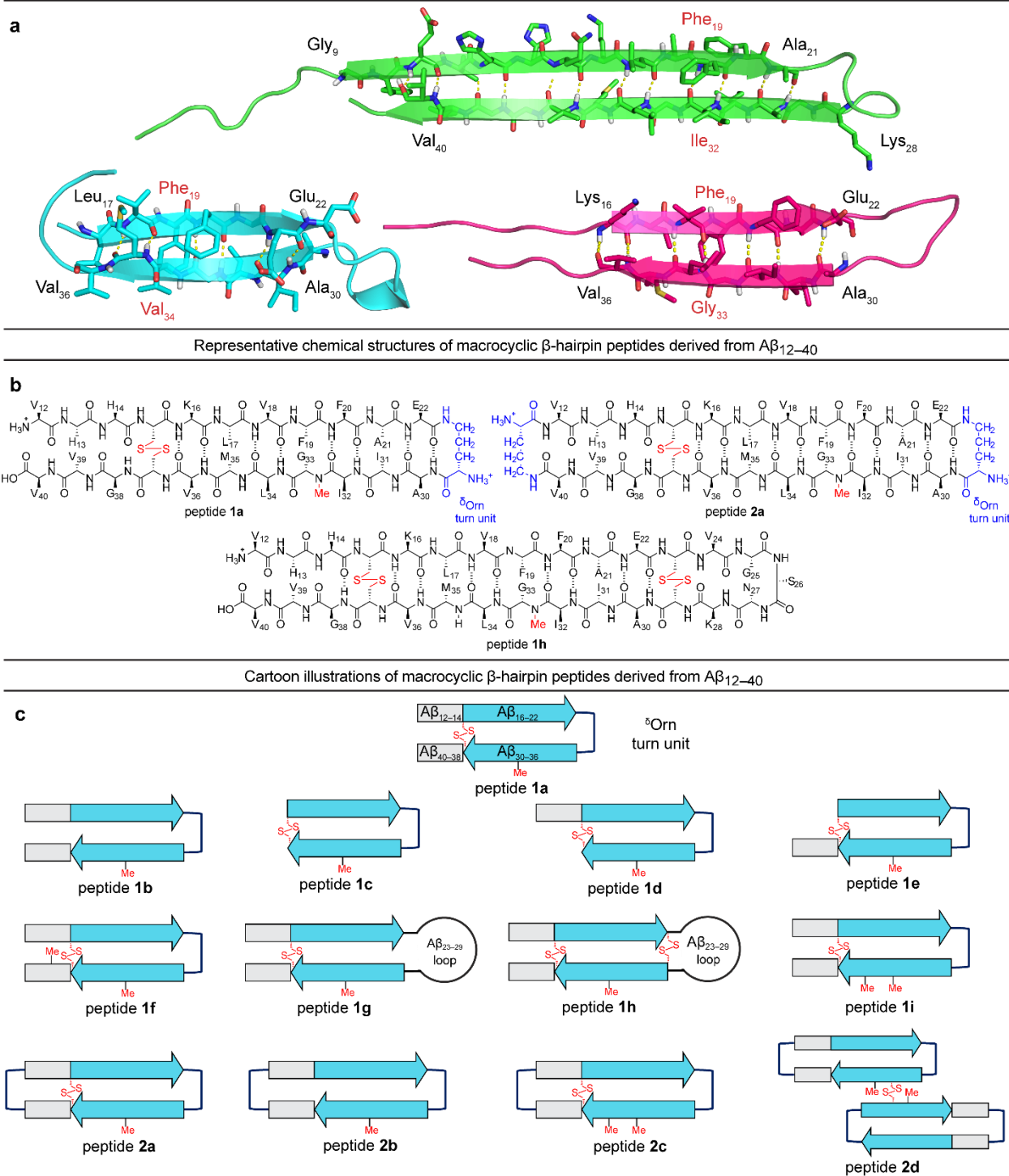**Figure 3.1**. (a) Structures of Aβ β-hairpins reported by Carulla et al. (PDB 6RHY), Härd and Hoyer et al. (PDB 2OTK), and Tycko et al. (not deposited). (b and c) Chemical structures and cartoons of β-hairpin peptides derived from Aβ$_{12-40}$.

Here we report studies of β-hairpin peptides derived from Aβ and describe their assembly to form β-barrel-like tetramers and octamers. We began our investigation by preparing and

studying peptide **1a** and observed the assembly of an octamer by SDS-PAGE. To better understand the assembly of this octamer, we synthesized peptides **1b**–**i** and **2a**–**d** which are homologues of peptide **1a** also derived from Aβ (**Figure 3.1b and 3.1c**). Solution-phase studies of peptides **1b**–**i** and **2a**–**d** allowed us to identify key intramolecular and intermolecular interactions required for β-hairpin folding and octamer assembly. X-ray crystallographic studies of peptide **2a** reveal a hitherto unprecedented β-barrel-like tetramer assembled from β-hairpins derived from Aβ$_{12–40}$. Evidence for the assembly of a concatenated β-barrel-like octamer is also present within the crystal lattice of peptide **2a**, corresponding to solution-phase observations of oligomer assembly. These studies provide unique insights into the folding of Aβ and assembly of endogenous oligomers.

**RESULTS**

*Design of peptides **1a**–**i** and **2a**–**d**.*

We designed peptides **1a**–**i** and **2a**–**d** to mimic the folding of Aβ β-hairpins and probe their assembly into oligomers. In each of these peptides, we incorporated constraints to enforce folding and stabilize an alignment in which Val$_{12}$ is across from Val$_{40}$ — an alignment which matches that of the Aβ β-hairpin recently reported by Tycko et al (**Figure 3.1a and 3.1b**). Our laboratory routinely uses Fmoc-based solid phase peptide synthesis to constrain β-strand segments derived from amyloidogenic peptides and proteins to a β-hairpin conformation using $^{\delta}$Orn turn units and disulfide bridges.[15,17–24] To prevent uncontrolled aggregation, we incorporate an *N*-methyl blocking group on one strand of the β-hairpin.

Peptide **1a** comprises two peptide β-strands of Aβ$_{12–22}$ and Aβ$_{30–40}$ linked by a $^{\delta}$Orn turn unit connecting residues 22 and 30, a cross-strand disulfide bridge replacing Gln$_{15}$ and Gly$_{37}$, and an *N*-methyl group on Gly$_{33}$ (**Figure 3.1b and 3.1c**). Peptide **1b** is a homologue of peptide **1a** that lacks the cross-strand disulfide bridge and contains the native Gln$_{15}$ and Gly$_{37}$ residues. Peptide **1c** restores this disulfide bridge but lacks the N- and C-terminal residues 12–14 and 38–40. Peptide

**1d** and **1e** are homologues of peptide **1a**, except that peptide **1d** lacks the C-terminal residues 38–40 and peptide **1e** lacks the N-terminal residues 12–14. Peptide **1f** is a homologue of peptide **1a** that bears an additional *N*-methyl group on $Gly_{38}$.

Peptide **1g**, is a homologue of peptide **1a** in which the $^{\delta}Orn$ turn unit is replaced with a loop of residues comprising $A\beta_{23-29}$. Peptide **1h** is nearly identical to peptide **1g** except that it bears an additional cross-strand disulfide bridge replacing $Asp_{23}$ and $Gly_{29}$. Peptide **1i** is a homologue of peptide **1a** that bears an additional *N*-methyl group on $Met_{35}$. Peptide **2a** is a homologue of peptide **1a** that bears an additional $^{\delta}Orn$ turn unit connecting residues 12 and 40. Peptide **2b** is a homologue of peptide **2a** that lacks the cross-strand disulfide bridge and restores $Gln_{15}$ and $Gly_{37}$. Peptide **2c** is a homologue of peptide **2a** that bears an additional *N*-methyl group on $Met_{35}$. Peptide **2d** is a dimer, comprising two monomers of peptide **2b** covalently linked by a disulfide bridge replacing $Val_{36}$ in each monomer.

*Oligomerization of peptides **1a–h** and **2a–d***

Peptide **1a** (ca. 2.5 kDa) runs as an oligomer in SDS-PAGE, migrating at a molecular weight consistent with that of an octamer (ca. 20 kDa) (Figure 2a). The octamer band streaks downward from below the 26 kDa ladder band to the 4.6 kDa ladder band, suggesting that the octamer is in rapid equilibrium with lower molecular weight species. The formation of oligomers that can be observed in SDS-PAGE is a hallmark of Aβ. Unlike the oligomer formed by peptide **1a**, the oligomers formed by full-length, unmodified Aβ are heterogeneous in size and typically display a substantial band for the monomer **(Figure 3.2a)**.[25–27] The formation of a well-defined oligomer band by peptide **1a** suggests that this constrained Aβ β-hairpin peptide forms a well-defined supramolecular assembly in the membrane-like environment provided by the amphiphilic SDS molecules.

To better understand the assembly of the putative octamer, we prepared and studied structural homologues of peptide **1a**, peptides **1b**–**h** (**Figure 3.1c and Figure 3.2a**). Peptide **1b** (2.5 kDa) lacks a cross-strand disulfide bridge and does not assemble, migrating to just below the 4.6 kDa ladder band. Peptide **1c** (1.8 kDa) lacks the N- and C-terminal residues 12–14 and 38–40 and also does not assemble, migrating as a downward streaking band from the 4.6 kDa ladder band. In contrast to peptide **1c**, peptide **1d** (2.3 kDa) lacks just the C-terminal residues 38–40 and does assemble. Like peptide **1a**, peptide **1d** migrates as a downward streaking band from below the 26 kDa ladder band to the 4.6 kDa ladder band. The band formed by peptide **1d** is consistent with the molecular weight of an octamer that is in equilibrium with lower molecular weight species. Peptide **1e** (2.1 kDa) lacks just the N-terminal residues 12–14 but does not assemble. Instead, peptide **1e** migrates as an upward streaking band from the 4.6 kDa ladder band to the 17 kDa ladder band.

The non-assembly of peptide **1b** reveals that the cross-strand disulfide bridge near the N- and C-terminal residues is necessary for assembly of the putative octamer. The assembly of peptide **1d**, in contrast to peptides **1c** and **1e**, is surprising and suggests that residues 12–14 participate in intermolecular interactions crucial for assembly of the putative octamer.

To further examine the role of interactions between the N- and C-terminal residues of peptide **1a**, 12–14 and 38–40, in the assembly of the octamer, we prepared and studied peptide **1f**. In peptide **1f**, the *N*-methyl group on Gly$_{38}$ is positioned to disrupt hydrogen bonding interactions between residues 12–14 and 38–40. In contrast to peptide **1a**, peptide **1f** (2.5 kDa) does not assemble as an octamer, but instead migrates as a band between the 10 kDa and 4.6 kDa ladder bands that streaks downward. This result suggests that hydrogen bonding interactions between residues 12–14 and 38–40 are required for assembly of the putative octamer.

To examine whether the putative octamer formed by peptide **1a** can accommodate residues 23–29 as a loop, and to better mimic endogenous Aβ β-hairpins and Aβ oligomers we prepared and studied peptide **1g (Figure 3.1c)**. In peptide **1g** a loop comprising residues 23–29 replaces the $^\delta$Orn turn unit. In contrast to peptide **1a**, peptide **1g** (3.0 kDa) does not assemble and instead migrates to just above the 4.6 kDa ladder band. This result would suggest that the putative octamer cannot accommodate residues 23–29 as a loop. We also considered an alternative premise, wherein the replacement of the $^\delta$Orn turn unit destabilizes the β-hairpin conformation of peptide **1g** thereby abrogating assembly. To test this hypothesis, we prepared and studied peptide **1h (Figure 3.1b and 3.1c)**. Peptide **1h** incorporates an additional cross-strand disulfide bridge replacing Asp$_{23}$ and Gly$_{29}$, to fortify the β-hairpin conformation of the peptide. Peptide **1h** (3.0 kDa) migrates as two oligomers, a lower molecular weight oligomer consistent with a tetramer and a higher molecular weight oligomer consistent with an octamer. The tetramer band migrates to a position between the 10 and 17 kDa ladder bands and is much greater in intensity than the octamer band, which migrates to just below the 26 kDa ladder band. The difference in intensity between the tetramer and octamer bands suggest that peptide **1h** favors assembly of the tetramer.

Our laboratory routinely prepares and studies oligomers formed by macrocyclic β-hairpin peptides derived from Aβ.[28,29] Two $^\delta$Orn turn units are used to stabilize these β-hairpin peptides to a macrocycle. Peptide **2a** is a macrocyclic homologue of peptide **1a**, that bears a second $^\delta$Orn turn unit connecting the N and C-terminal residues 12 and 40 **(Figure 3.1b and 3.1c)**. Like peptide **1h**, peptide **2a** (2.6 kDa) migrates as two oligomers, a lower molecular weight oligomer consistent with a tetramer and a higher molecular weight oligomer consistent with an octamer. The tetramer band migrates to a position just above the 10 kDa ladder band, and the octamer band migrates to a position between the 17 and 26 kDa ladder bands. Both bands are equal in their intensity, with the octamer band streaking downward toward the tetramer band, suggesting that both species are in

equilibrium with each other. Peptide **2b** is a homologue of peptide **2a** that lacks the cross-strand disulfide bridge and does not assemble, instead migrating to just below the 4.6 kDa ladder band.

The assembly of a tetramer in addition to the octamer, by peptides **1h** and **2a**, was both surprising and unanticipated. In comparison to peptide **1a**, peptides **1h** and **2a** bear additional stabilizing constraints, a second disulfide bridge and a second $^\delta$Orn turn unit respectively. Rather than stabilize the assembly of the putative octamer formed by peptide **1a**, the introduction of these additional constraints appears to have had a destabilizing effect on octamer assembly. We speculate that these tetramers may be components of the putative octamer formed by peptide **1a**. The lack of assembly by peptide **2b**, despite the second $^\delta$Orn turn unit, suggests that the cross-strand disulfide bridge replacing $Gln_{15}$ and $Gly_{37}$ is essential for octamer assembly.

*Folding of peptides **1a–h** and **2a–d***

CD spectroscopy reveals that peptide **1a** adopts a β-sheet conformation, suggesting that the peptide does fold into a conformation resembling a β-hairpin, as designed **(Figure 3.2b)**. The spectrum of peptide **1a** displays a strong negative band centered at ca. 212 nm, with increasing ellipticity at lower wavelengths that reaches a maximum at ca. 198 nm before decreasing once again. In contrast to peptide **1a**, the CD spectrum of peptide **1b** reveals that the peptide does not fold and instead adopts a predominant random coil conformation. The CD spectrum of peptide **1b** displays a strong negative band centered at ca. 198 nm and a weak positive band at ca. 220 nm **(Figure 3.2b)**. The CD spectra of peptides **1a**, **1c–f**, **1h**, and **2a** shows that each peptide folds to adopt a β-hairpin-like conformation. In contrast to peptides **1a**, **1c–f**, **1h**, and **2a**, peptides **1b**, **1g** and **2b** do not fold, and instead adopt random-coil-like conformations.

Differences in the CD spectra of peptides **1g** and **1h** reveal the impact of replacing the $^\delta$Orn turn unit with Aβ residues 23–29 **(Figure 3.2c)**. The CD spectrum of peptide **1g** displays a broad and shallow negative band (ca. $\Theta$ = -5600 deg $cm^2$ $dmol^{-1}$) centered at ca. 204 nm, suggestive of a

random-coil-like conformation. Differences between the CD spectra of peptides **1a** and **1g** likely reflect differences in the rigidity of each peptide due to the presence or absence of the $^{\delta}$Orn turn unit. In contrast to peptide **1g**, peptide **1h**, which bears an additional cross-strand disulfide bridge to restore rigidity, folds to adopt a β-hairpin-like conformation. Peptide **1h** displays a negative band (ca. $\Theta$ =-5000 deg cm$^2$ dmol$^{-1}$) that is centered at ca. 212 nm with increasing ellipticity at lower wavelengths.

The CD spectra of peptides **2a** and **2b** suggests that the addition of a second $^{\delta}$Orn turn unit connecting residues 12 and 40 does not have a substantial effect on folding relative to peptides **1a** and **1b (Figure 3.2c)**. The spectrum of peptide **2a** displays a strong negative band (ca. $\Theta$ = -7400 deg cm$^2$ dmol$^{-1}$) centered at ca. 214 nm with increasing ellipticity at lower wavelengths, reflecting a β-hairpin-like conformation. Peptide **2b** displays a strong negative band centered at ca. 200 nm and a weak positive band at ca. 220 nm, indicative of a random coil conformation. Like the CD spectra of peptide **1b**, the loss of the cross-strand disulfide bridge that replaces Gln$_{15}$ Gln$_{37}$ ablates folding.
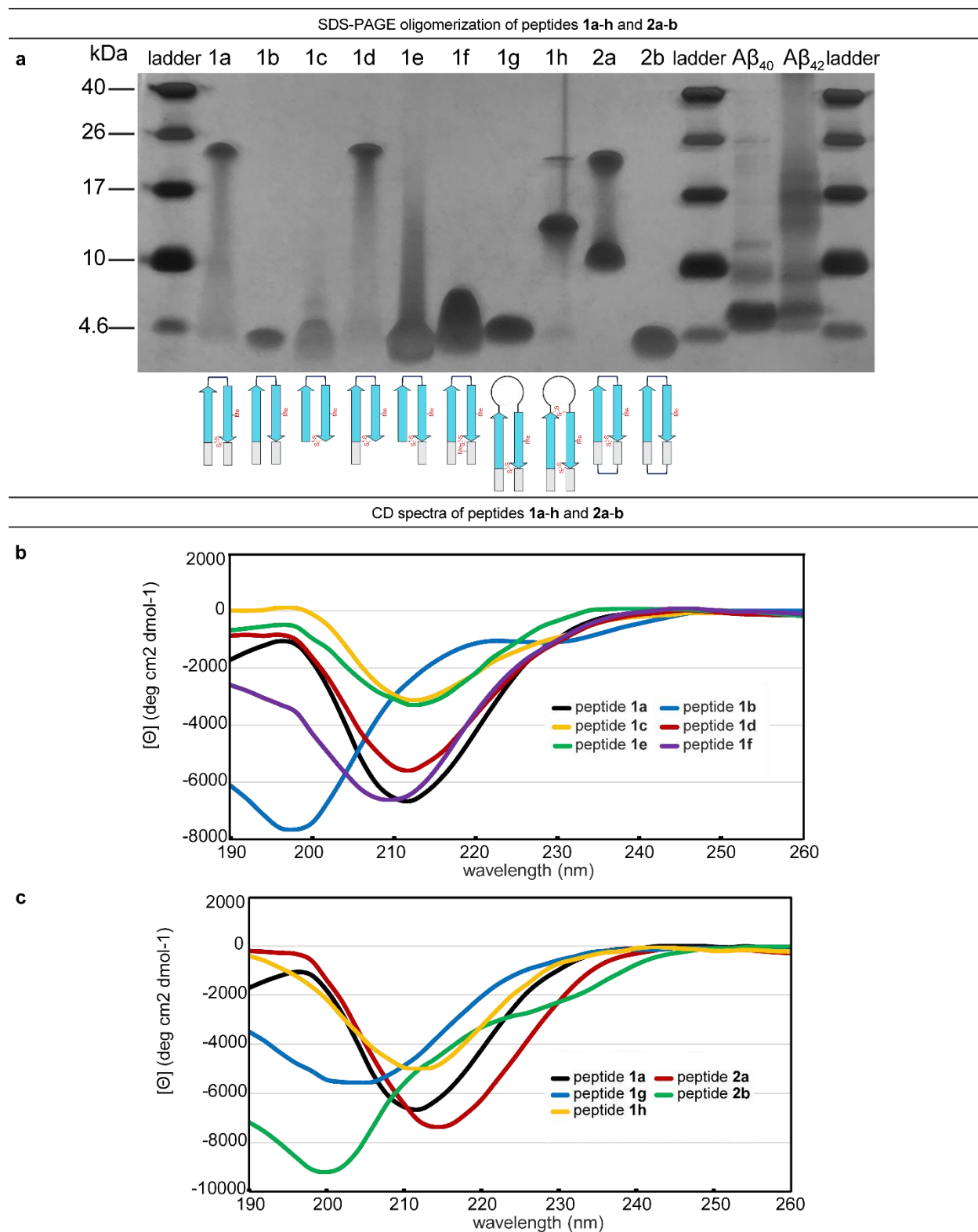
CD spectra of peptides **1a-h** and **2a-b**



**Figure 3.2**. (a) Silver stained SDS-PAGE of peptides **1a–h**, **2a–b**, and Aβ$_{40}$ and Aβ$_{42}$. SDS-PAGE was performed in Tris buffer at pH 6.8 with 2% (w/v) SDS on a 16% polyacrylamide gel with 50 $\mu$M solutions of peptide in each lane. (b and c) Circular dichroism (CD) spectra of peptides **1a–h** and **2a–b**. CD spectra were acquired for each peptide at 50 $\mu$M in 10 mM phosphate buffer at pH 7.4; ellipticity was normalized for the number of residues in each peptide.

*X-ray crystallographic and REMD studies of peptide 2a*

X-ray crystallography reveals that twisted β-hairpin monomers of peptide **2a** assemble in the crystal lattice to form antiparallel dimers, that further assemble to form β-barrel-like tetramers and octamers. The β-barrel-like tetramers and octamers are stabilized by edge-to-edge hydrogen bonding and hydrophobic packing. Peptide **2a** afforded crystals suitable for X-ray diffraction from an aqueous solution of 0.1 M sodium cacodylate trihydrate buffer at pH 6.5, 0.2 M magnesium acetate tetrahydrate, and 30% v/v 2-methyl-2,4-pentanediol. Molecular replacement, using a search model derived from the crystallographic structure of a dimer formed by a macrocyclic β-hairpin peptide derived from $A\beta_{16-36}$ (PDB 6WXM), was used to determine the X-ray crystallographic phases of peptide **2a**.[30] Supplementary table 3.1 summarizes the crystallographic properties, crystallization conditions, data collection, and model refinement statistics for peptide **2a**.

X-ray crystallography reveals that peptide **2a** folds to adopt a twisted β-hairpin conformation, corroborating the CD spectra of peptide **2a**. This β-hairpin is stabilized by eleven intramolecular hydrogen bonds formed between the β-strands comprising residues 12–22 and 30–40, and a disulfide bridge that replaces $Gln_{15}$ and $Gly_{37}$ (**Figure 3.3a**). Two monomers of peptide **2a** assemble to form a hydrogen-bonded antiparallel dimer (**Figure 3.3b**). This dimer is stabilized by two pairs of intermolecular hydrogen bonds formed between the backbone amides of $Met_{35}$ and $Cys_{37}$.

Two antiparallel dimers of peptide **2a** assemble further to form a β-barrel-like tetramer that is stabilized by edge-to-edge intermolecular hydrogen bonding and the packing of hydrophobic residues (**Figure 3.3c**). The tetramer is stabilized by four intermolecular hydrogen bonds between monomers of peptide **2a**. Two hydrogen bonds are formed between the backbone of $His_{13}$ in one monomer and the backbone of $Ala_{21}$ and $Phe_{19}$ in another monomer of peptide **2a**. The remaining

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two hydrogen bonds are formed between the backbone of $Phe_{19}$ in one monomer and the backbone of $His_{13}$ and $Cys_{14}$ in another monomer of peptide **2a**. The interior of this β-barrel-like tetramer is lined with hydrophobic residues comprising Val, Phe, Leu, Ile, Met, and Cys that pack together and provide additional stability **(Figure 3.3d)**.
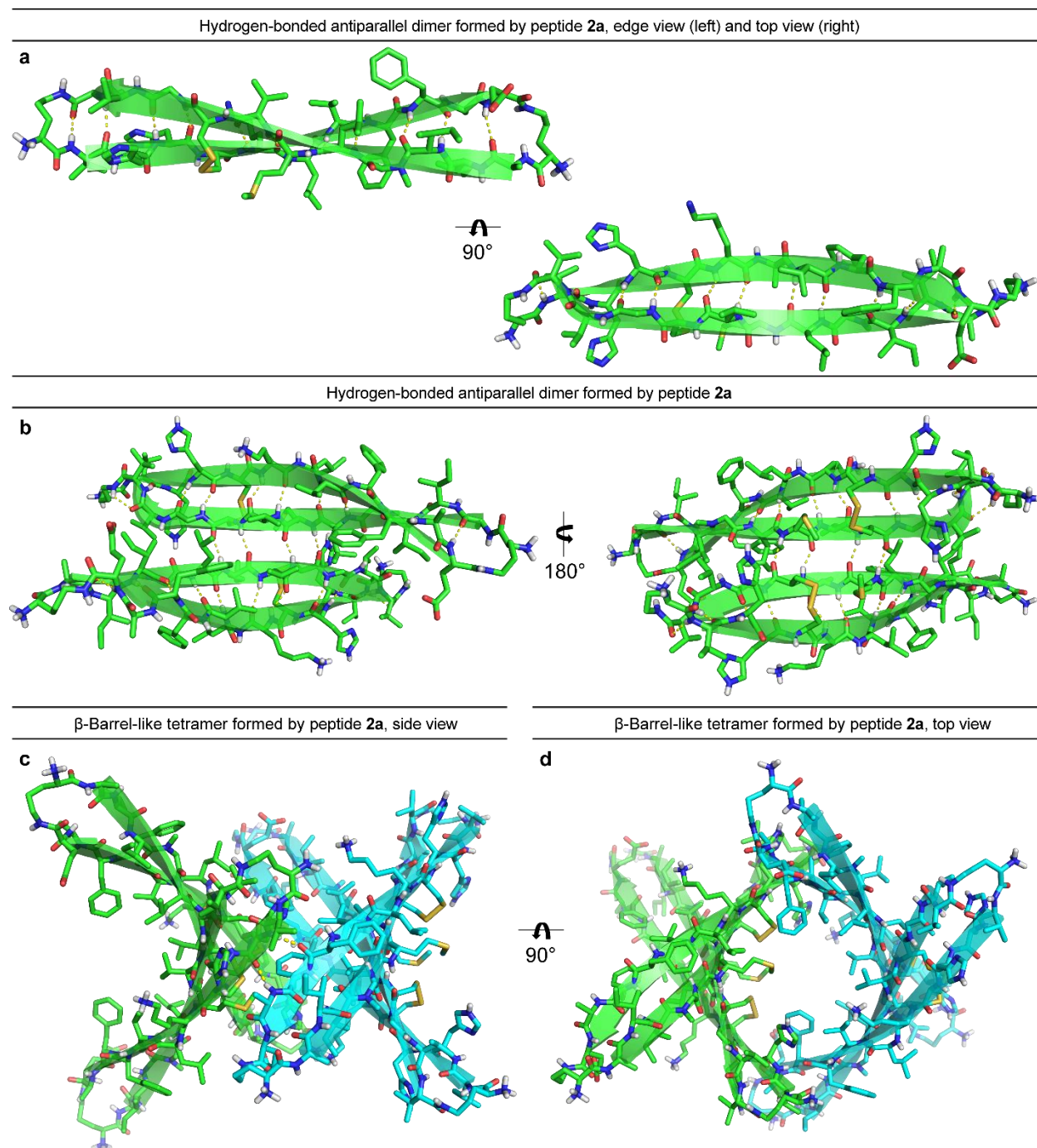
Hydrogen-bonded antiparallel dimer formed by peptide **2a**, edge view (left) and top view (right)

Hydrogen-bonded antiparallel dimer formed by peptide **2a**

β-Barrel-like tetramer formed by peptide **2a**, side view

β-Barrel-like tetramer formed by peptide **2a**, top view



**Figure 3.3**. X-ray crystallographic structure of (a) twisted β-hairpin, (b) antiparallel dimer, and (c) side and (d) top views of the β-barre-like tetramer formed by peptide **2a**.

The β-barrel-like tetramer formed by peptide **2a** is stabilized by parallel and antiparallel β-sheet interactions **(Figure 3.4a)**. Intermolecular hydrogen bonds stabilize an antiparallel dimer comprising β-hairpins of peptide **2a**, green: green and blue: blue. Intermolecular hydrogen bonds also stabilize a parallel dimer comprising β-hairpins of peptide **2a**, green: blue and blue: green.

We envision that full-length Aβ can assemble in the same fashion as peptide **2a** to form a β-barrel-like tetramer. To understand what a tetramer containing residues 23–29 and additional N- and C-terminal residues might look like, we modeled Aβ$_{9-42}$ into the crystallographic coordinates of each monomer in the tetramer formed by peptide **2a**. We built Aβ$_{23-29}$ (DVGSNKG), Aβ$_{9-11}$ (GYE), and Aβ$_{41-42}$ (IA) into the crystallographic coordinates of the four peptide **2a** monomers that comprise the tetramer. The cross-strand disulfide bridge was replaced with the native residues Gln$_{15}$ and Gly$_{38}$, and the *N*-methyl group on Gly$_{38}$ was removed. We then preformed REMD to generate realistic conformations of the loops and N- and C-terminal regions of the β-hairpins **(Figure 3.4b)**. The REMD simulations show that Aβ$_{9-42}$ could form a β-barrel-like tetramer without steric clashes.

Two β-barrel-like tetramers of peptide **2a** assemble further to form an octamer **(Figure 3.4c)**. This octamer is stabilized by the same edge-to-edge hydrogen bonding and hydrophobic packing interactions that stabilize the tetramer. The octamer comprises two concatenated β-barrel-like tetramers of peptide **2a**, containing eight monomers of peptide **2a** in total. The openings of the two tetramers lie parallel to each other on the same axis. A third β-barrel-like tetramer is formed at the center of the octamer. The opening of this third tetramer lies on an axis perpendicular to the openings of the other two tetramers and is identical to the tetramer described in figures 3.4c and 3.4d. The assembly of the β-barrel-like tetramer and octamer corroborates the assembly of peptide **2a** in SDS-PAGE.

The crystal lattice of peptide **2a** comprises concatenated chains of antiparallel dimers that form β-barrel-like tetramers and octamers **(Figure 3.4c)**. The arrangement and interaction of these dimers within the crystal lattice does not terminate in a manner that gives rise to discrete tetramer or octamer units. Instead, each edge and face of peptide **2a** is capable of participating in edge-to-edge hydrogen bonding and hydrophobic packing—enabling oligomerization. The crystallographic structure of the octamer formed by peptide **2a** provides insight toward understanding the structure of the octamer formed by peptide **1a**.
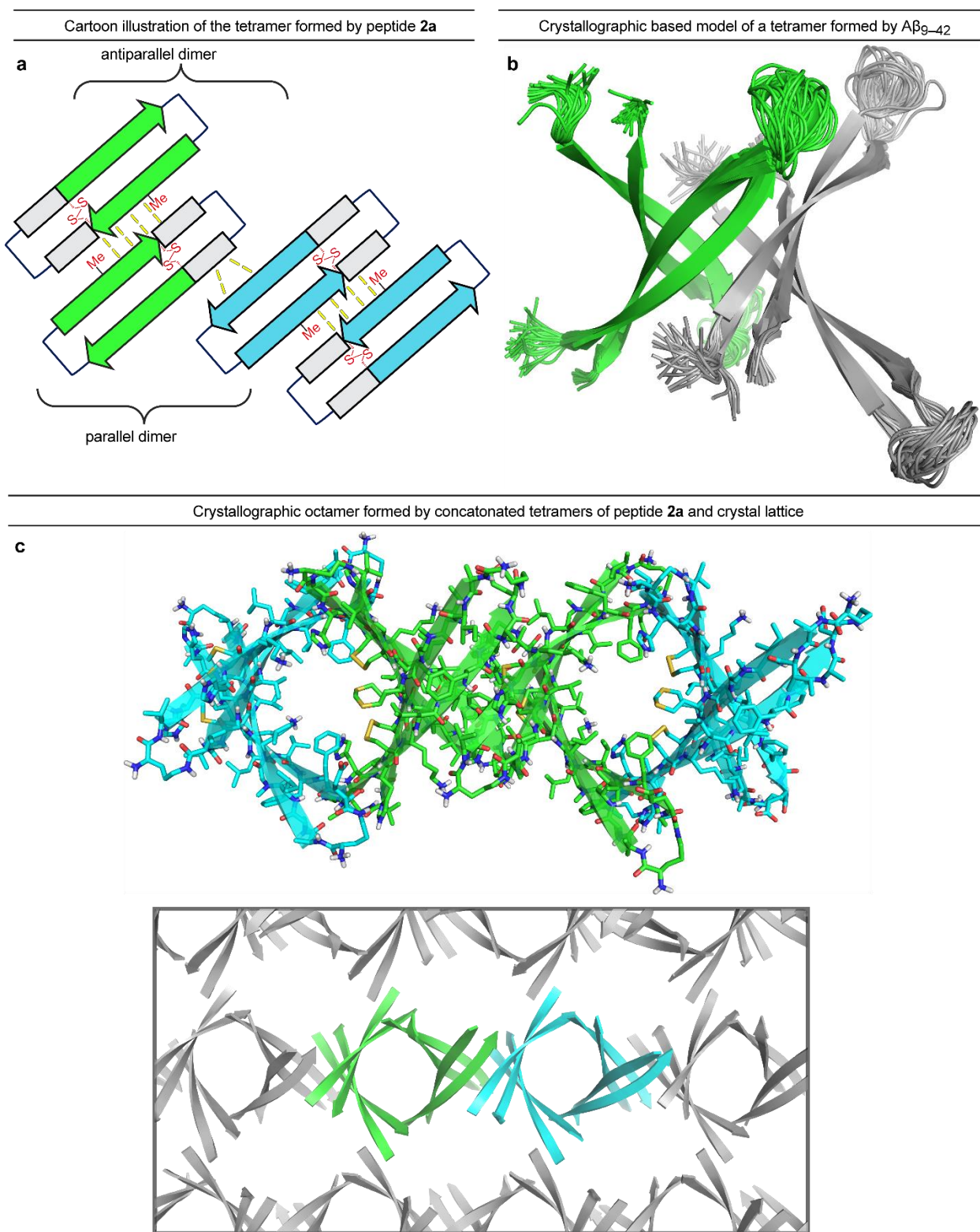
**Figure 3.4**. (a) Illustration of the parallel and antiparallel β-sheet interactions present in the β-barrel-like tetramer formed by peptide **2a**. (b) Crystallographic based model of an Aβ$_{9-42}$ β-barrel-like tetramer. Superposition of 32 structures generated by REMD. (c) β-Barrel-like octamer and crystal lattice formed by peptide **2a**.

*Reducing the disulfide bonds of peptides **1a** and **2a**, and oligomerization of peptides **1i** and **2c–d***

To better understand the importance of the cross-strand intramolecular disulfide bridge in the oligomerization of peptides **1a** and **2a**, we subjected each peptide to tris(2-carboxyethyl)phosphine (TCEP) and studied their migration by SDS-PAGE **(Figure 3.5a and 3.5b)**. After TCEP reduction, peptide **1a** does not assemble as an octamer, and instead migrates as a downward streaking band between the 4.6 and 10 kDa ladder bands **(Figure 3.5a)**. Similarly, peptide **2a** no longer migrates as an octamer following reduction by TCEP, but still migrates at a molecular weight consistent with that of a tetramer **(Figure 3.5b)**. The persistence of a tetramer for peptide **2a** may reflect an incomplete reduction of the disulfide bond. Peptide **2a** migrates as a downward streaking band from the 4.6 to the 10 kDa ladder band. Neither peptide **1a** or **2a** migrates as an octamer following treatment with TCEP, highlighting the importance of the cross-strand disulfide bridge in the oligomerization of these Aβ derived peptides.

We studied the SDS-PAGE migration of peptides **1i** and **2c–d** to corroborate the crystallographic tetramer and octamer of peptide **2a** with the SDS-PAGE assembly of peptides **1a** and **2a (Figure 3.5a and 3.5b)**. The crystallographic dimer formed by peptide **2a** is an integral component of the β-barrel-like tetramer and octamer and relies on a pair of intermolecular hydrogen bonds between Met$_{35}$ and Cys$_{37}$. If the crystallographic octamer formed by peptide **2a** is identical to the SDS-PAGE octamers of peptides **1a** and **2a**, disruption of this dimer should ablate the assembly of both peptides **1a** and **2a**. Peptide **1i** is a homologue of peptide **1a** that bears an additional *N*-methyl group on Met$_{35}$ **(Figure 3.1c)**. Peptide **1i** does not assemble as an octamer and instead migrates to the 4.6 kDa ladder band **(Figure 3.5a)**. Peptide **2c** is a homologue of peptide **2a** that bears an additional *N*-methyl group on Met$_{35}$ **(Figure 3.1c)**. Peptide **1i** does not assemble as an octamer and instead migrates to the 4.6 kDa ladder band **(Figure 3.5b)**. The lack of assembly

by peptides **1i** and **2c** corroborates the SDS-PAGE assembly of peptide **1a** and **2a** with the crystallographic β-barrel-like tetramer and octamer formed by peptide **2a**.

The SDS-PAGE migration of peptide **2d** provides further evidence that peptide **2a** forms the same oligomer by SDS-PAGE and in the crystal state. Peptide **2d** is a disulfide stabilized dimer of peptide **2b (Figure 3.1c)**. Although peptide **2b** does not assemble by SDS-PAGE to form a tetramer or an octamer, monomers of peptide **2b** may be capable of assembly if they are covalently stabilized.[19] In the antiparallel dimer formed by peptide **2a**, $Val_{36}$ in one monomer forms a non-hydrogen bonded pair with $Val_{36}$ in the other monomer. We speculated that a dimer of peptide **2b** could be stabilized by replacing this non-hydrogen bonded pair with a disulfide bond, enabling the SDS-PAGE assembly of peptide **2b**.

Peptide **2d** migrates at a molecular weight consistent with a tetramer **(Figure 3.5b)**. The tetramer band formed by peptide **2d** streaks downward from a position between the 10 and 17 kDa ladder bands to between the 4 and 10 kDa ladder bands. Reduction of peptide **2d** by TCEP produces a band that migrates to just below the 4.6 kDa ladder band, in addition to the tetramer band. The migration of this lower molecular weight band parallels the position of the band formed by peptide **2b**, highlighting that assembly of peptide **2d** is dependent on the intermolecular disulfide bond.

*Folding of peptides 1i and 2c–d*

The presence of an additional *N*-methyl group on $Met_{35}$ appears to contribute to differences in the CD spectrum of peptide **1i** relative to peptide **1a (Figure 3.5c)**. The CD spectrum of peptide **1i** is broader and shallower than peptide **1a**, displaying a negative band centered at ca. 210 nm that extends from ca. 196 nm to ca. 220 nm. In contrast to the differences between peptides **1a** and **1i**, the CD spectrum of peptide **2c** is closer in similarity to peptide **2a**. Peptide **2c** displays a strong negative band centered at ca. 214 nm with increasing ellipticity at lower wavelengths, reflecting a

β-hairpin-like conformation. The CD spectrum of peptide **2d** displays a strong negative band centered at ca. 212 nm and a strong positive band centered at ca. 196 nm, reflecting a β-hairpin-like conformation **(Figure 3.5c)**. This result suggests that covalent stabilization of dimers of peptide **2d** not only enables assembly in SDS-PAGE, but β-hairpin-like folding as well.
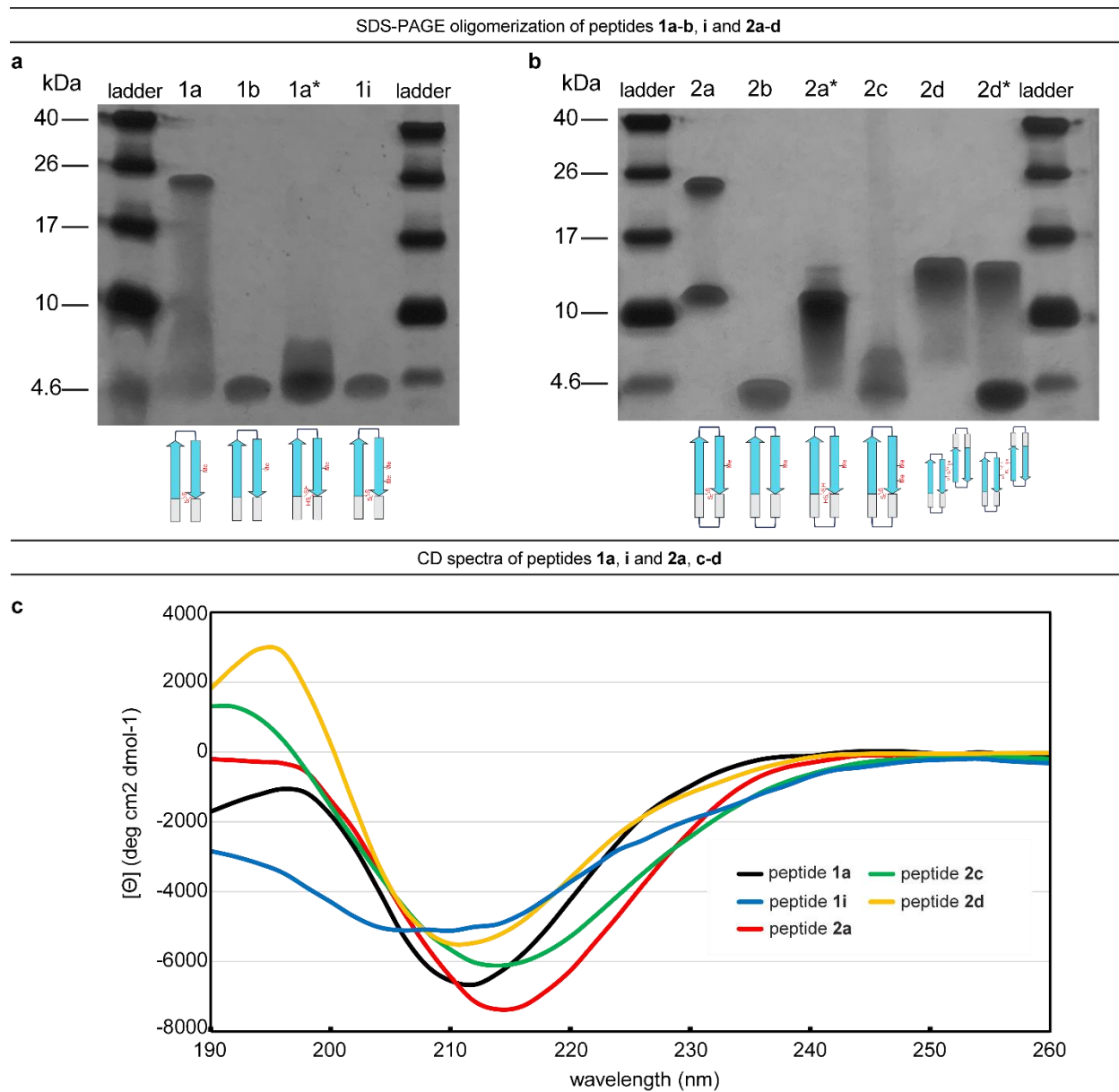


**Figure 3.5**. (a) Silver stained SDS-PAGE of peptides **1a–b, 1i,** and **2a–d**; lanes corresponding to peptides **1a\*, 2a\*,** and **2d\*** were treated with 10 mM TCEP. SDS-PAGE was performed in Tris buffer at pH 6.8 with 2% (w/v) SDS on a 16% polyacrylamide gel with 50 $\mu$M solutions of peptide in each lane. (b and c) Circular dichroism (CD) spectra of peptides **1a, 1i, 2a,** and **2c–d**. CD spectra were acquired for each peptide at 50 $\mu$M in 10 mM phosphate buffer at pH 7.4; ellipticity was normalized for the number of residues in each peptide.

*Molecular dynamics simulations of a crystallographically derived tetramer in a lipid membrane*

One mechanism by which Aβ oligomers are thought to exert toxicity is the disruption of cell membranes.[9,31–33] We used molecular dynamics to simulate interactions between a β-barrel-like tetramer formed by Aβ$_{9-42}$ and a lipid bilayer of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) molecules. We built the simulation using the CHARMM-GUI input generator and ran the simulation for 200 ns under the parameters of the CHARMM36m forcefield **(Figure 3.6)**.[34,35] Molecular graphics and simulation analyses were generated with VMD 1.9.3.[36]

Over the course of 200 ns, the β-barrel-like tetramer formed by Aβ$_{9-42}$ disrupts integrity of the POPC membrane, allowing water to cross the transmembrane space through the inner pore and along the outer face of the tetramer **(Figure 3.6a)**. Although the inner pore of the tetramer is predominantly lined with hydrophobic residues **(Figure 3.3d)**, water molecules are still able to enter and cross this space. Surprisingly, molecules of water were also able to enter the transmembrane space along the outer face of the tetramer, interacting with polar residues such as His, Gln, and Lys. As the simulation progress, each membrane leaflet experiences significant disruption, as seen by the position of the POPC headgroups (orange) **(Figure 3.6a)**. We monitored changes in RMSD for residues 12–22 and 30–40, residues from the crystallographic β-strands of peptide **2a** and observed a plateau of ca 2.6Å after 100 ns (Figure S3.2). We were also able to measure the amount of water that crossed the transmembrane space through the pore of the tetramer **(Figure 3.6b-c)**. After the simulation has reached its equilibrium point (ca. 100 ns), 10–40 molecules of water can be found within a cylindrical space defined by the pore, with an average density of 0.86 waters/ Å along the length of the pore.
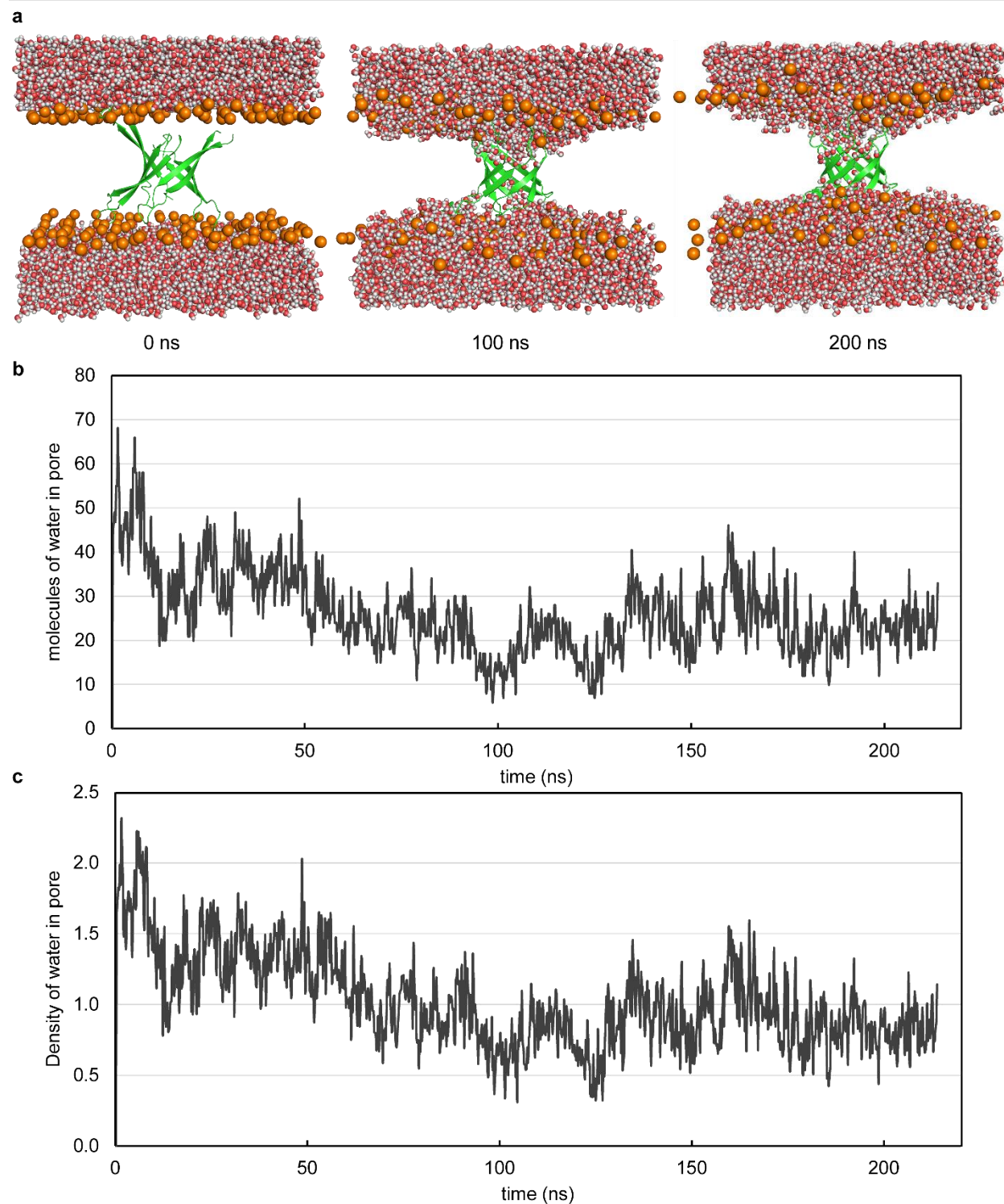
**Figure 3.6**. Molecular dynamics simulation of a β-barrel-like tetramer formed by Aβ$_{9-42}$ in a POPC lipid bilayer. (a) Simulation at 0, 100, and 200ns. (b) Total number of water molecules in the tetramer pore over the course of the simulation. (c) Density of water molecules per Å, along the length of the pore.

## DISCUSSION AND CONCLUSION

β-Hairpins are a key structural component of Aβ oligomers, with variations in β-hairpin alignment and topology likely contributing to some of the variation and heterogeneity observed in Aβ fibrils and oligomers. In this investigation, we stabilized several β-hairpin peptides derived from $A\beta_{12-40}$, using disulfide bridges and $^\delta$Orn turn units, to mimic endogenous Aβ oligomers and study the properties of the assemblies that formed. In SDS-PAGE, peptide **1a** assembles to form an oligomer that migrates at a molecular weight consistent with that of an octamer. Structural homologues of peptide **1a**, peptides **1b**–**h** and **2a**–**b**, reveal that the disulfide bridge and N-terminal residues 12–14 are required for peptide **1a** to assemble and form an octamer, and that this octamer can accommodate residues 23–29 as a loop. X-ray crystallographic studies reveal that peptide **2a** assembles to form a β-barrel-like tetramer and octamer, stabilized by edge-to-edge hydrogen bonding of antiparallel and parallel β-sheets, and hydrophobic packing. Molecular dynamics studies of a β-barrel-like tetramer assembled from a crystallographically derived model of $A\beta_{9-42}$ reveal that the tetramer can disrupt lipid membrane and facilitate water permeation. These findings contribute to our understanding of how endogenous Aβ oligomers may assemble in the Alzheimer's brain, furthering our molecular understanding of Alzheimer's disease.

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A β-Barrel-Like Tetramer Formed by a Macrocyclic β-Hairpin Derived From Aβ
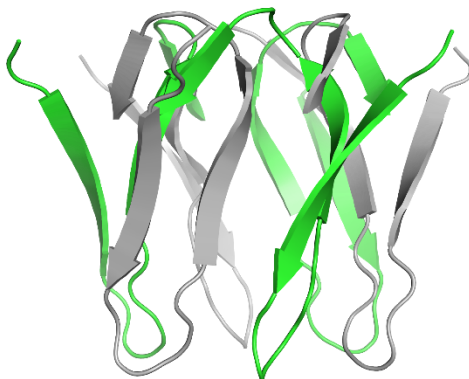
# Table of Contents

# Supporting Figures and Tables

Tetramer of $A\beta_{1-42}$, formed by the Aβ β-hairpin reported by Carulla et al. (PDB 6RHY), *Nat. Commun.*, **11**, 1–14 (2020)



a
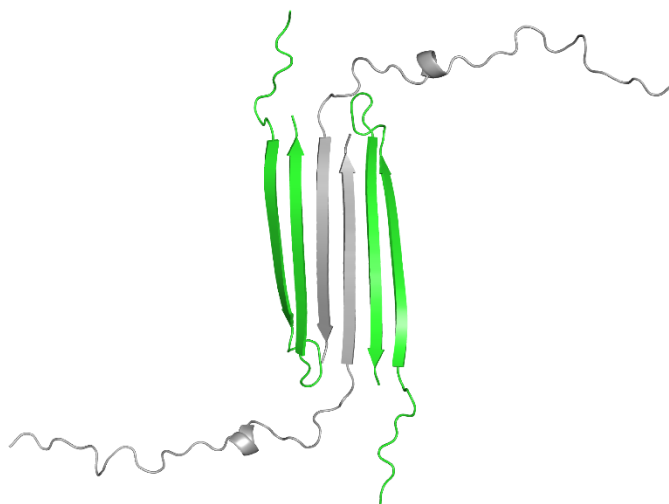
Barrel-like-hexamer of $A\beta_{16-42}$, formed by the Aβ β-hairpin reported by Härd et al., *Angew. Chemie - Int. Ed.*, **53**, 12756–12760 (2014)



b

$A\beta_{40}$ Fibrils with Aβ β-hairpins proposed by Tycko et al., *Proc. Natl. Acad. Sci.*, **118**, e2023089118 (2021)



c
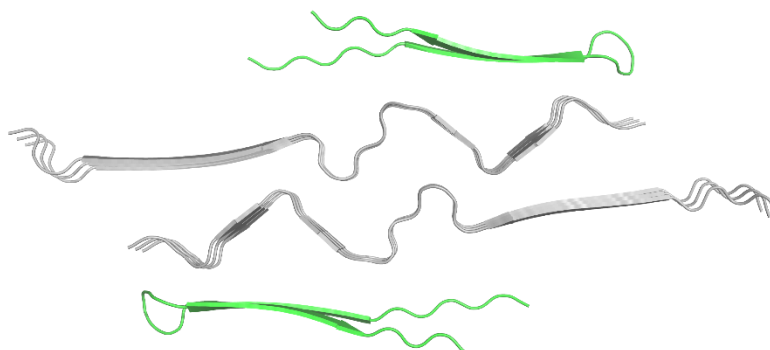
**Figure S3.1**. (a) NMR structure of the tetramer formed by the β-hairpin, reported by Carulla et al. (b) NMR structure of the barrel-like hexamer formed by an Aβ derived β-hairpin, reported by Härd et al.(c) $A\beta_{40}$ fibril structure with proposed $A\beta_{40}$ β-hairpins, reported by Tycko et al.

**Supplementary Table 3.1**. Crystallographic properties, crystallization conditions, data collection, and model refinement statistics for peptide **2a**.

| peptide | 2a |
|---|---|
| Wavelength (Å) | 1.0 |
| Resolution range | 36.52 - 2.1 (2.175 - 2.1) |
| Space group | P 41 21 2 |
| Unit cell | 44.3265, 44.3265, 64.4265 90, 90, 90 |
| Total reflections | 198405 (19442) |
| Unique reflections | 4086 (393) |
| Multiplicity | 48.6 (49.5) |
| Completeness (%) | 99.83 (100.00) |
| Mean I/sigma(I) | 21.83 (2.00) |
| Wilson B-factor | 43.80 |
| R-merge | 0.3661 (1.108) |
| R-meas | 0.37 (1.118) |
| R-pim | 0.05249 (0.1504) |
| CC1/2 | 0.998 (0.795) |
| CC* | 1 (0.941) |
| Reflections used in refinement | 4079 (393) |
| Reflections used for R-free | 406 (39) |
| R-work | 0.2639 (0.3241) |
| R-free | 0.2941 (0.3099) |
| CC(work) | 0.911 (0.810) |
| CC(free) | 0.879 (0.730) |
| Number of non-hydrogen atoms | 376 |
| RMS(bonds) | 0.016 |
| RMS(angles) | 0.89 |
| Ramachandran favored (%) | 100.00 |
| Ramachandran allowed (%) | 0.00 |
| Ramachandran outliers (%) | 0.00 |
| Rotamer outliers (%) | 0.00 |

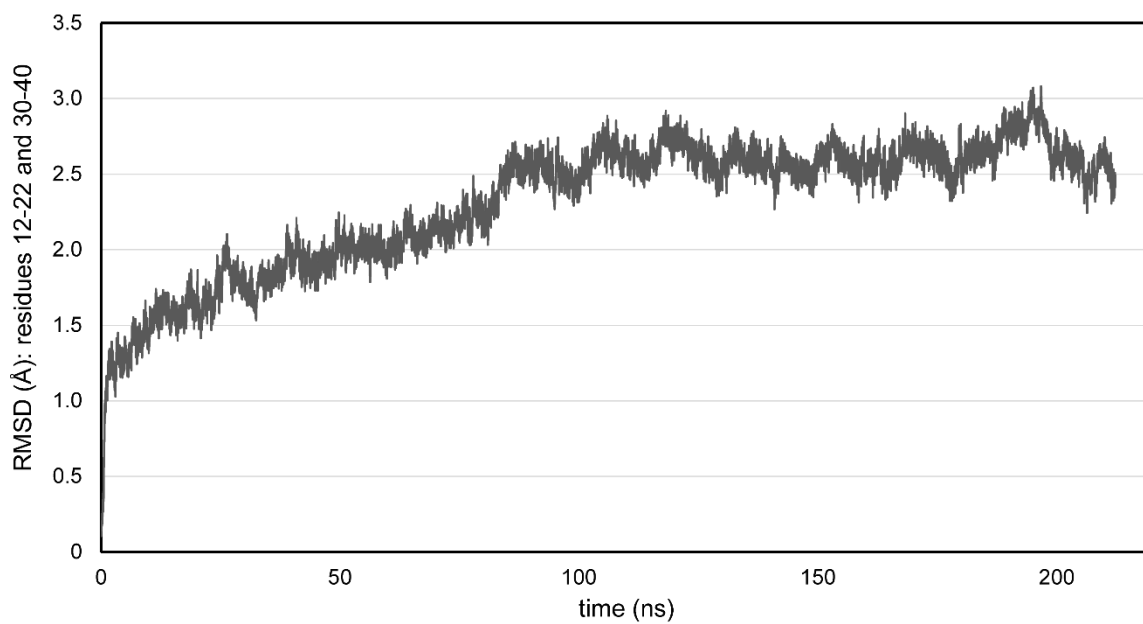| | |
|---|---|
| **Clashscore** | 5.59 |
| **Average B-factor** | 54.16 |
| **crystallization conditions** | 0.2 M Mg acetate tetrahydrate, 0.1M Na cacodylate trihydrate (pH 7.0), 30% v/v 2-methyl-2,4-pentanediol |

**Figure S2.3**. Time (ns) vs. RMSD (Å). RMSD was measured for residues 12–22 and 30–40, which were derived from the crystal structure of the β-barrel-like tetramer formed by peptide **2a**.

# Materials and Methods[1]

*General information*

 All chemicals were used as received unless otherwise noted. Methylene chloride ($CH_2Cl_2$) was passed through alumina under nitrogen prior to use. Anhydrous, amine-free *N,N*-dimethylfomamide (DMF) was purchased from Alfa Aesar. Deionized water (18 M$\Omega$) was obtained from a Barnstead NANOpure Diamond water purification system. Analytical reverse-phase HPLC was performed on an Agilent 1260 Infinity II instrument equipped with a Phenomonex Aeris PEPTIDE 2.6u XB-C18 column. Preparative reverse-phase HPLC was performed on a Ranin instrument equipped with an Agilent Zorbax SB-C18 column. HPLC grade acetonitrile and deionized water, each containing 0.1% trifluoracetic acid (TFA), were used for analytical and preparative reverse-phase HPLC. All peptides were prepared and used as the trifluoroacetate salts and were assumed to have one trifluoracetic acid molecule per amine group on each peptide.

*Synthesis of peptides* **1a–i**

*a. Loading the resin.* For peptides **1a–b** and **1e–h**, 2-chlorotrityl chloride resin (300 mg, 1.4 mmol/g) was added to a Bio-RAD Poly-Prep chromatography column (10 mL). Dry $CH_2Cl_2$ (8 mL) was used to suspend and swell the resin for 30 min with gentle rocking. The solution was drained from the resin and a solution of Fmoc-Val-OH (0.78 equiv, 50 mg, 0.33 mmol) in 6% (v/v) 2,4,6-collidine in dry $CH_2Cl_2$ (8 mL) was added immediately and the suspension was gently rocked for 12 h. The solution was then drained and a mixture of $CH_2Cl_2$/MeOH/*N,N*-diisopropylethylamine (DIPEA) (17:2:1, 10 mL) was added immediately. The resin was gently rocked for 1 h, to cap the unreacted 2- chlorotrityl chloride resin sites. The resin was then washed twice with dry $CH_2Cl_2$ and dried by passing nitrogen through the vessel. This procedure typically yields 0.18 mmol of loaded resin (0.6 mmol/g loading).

For peptides **1c–d**, rink amide AM resin (300 mg, 0.68 mmol/g) was added to a Bio-RAD Poly-Prep chromatography column (10 mL). Dry DMF (8 mL) was used to suspend and swell the resin for 30 min with gentle rocking. The solution was drained from the resin and a solution of 20% (v/v) piperidine in DMF was added immediately and the suspension was gently rocked for 1h.  The 20% (v/v) piperidine in DMF was drained from the resin, and the resin was then washed twice with dry DMF. A solution of Fmoc-Cys-Trt (0.78 equiv, 50 mg, 0.33 mmol), HATU (4.5 equiv), HOAt (4.5 equiv), in 20% (v/v) 2,4,6-collidine in dry $CH_2Cl_2$ (8 mL) was added immediately and the suspension was gently rocked for 12 h. The solution was then drained and a mixture of acetic anhydride/pyridine (3:2, 8 mL) was added immediately. The resin was gently rocked for 0.5 h, to cap the unreacted rink amide resin sites. The resin was then washed twice with dry $CH_2Cl_2$ and dried by passing nitrogen through the vessel. This procedure typically yields 0.18 mmol of loaded resin (0.6 mmol/g loading).

*b. Manual peptide coupling.* The resin loaded with Boc-Orn(Fmoc) was suspended in dry DMF and then transferred to a solid-phase peptide synthesis vessel. For peptides **1a–b** and **1e–h**, residues 40 through 32 were manually coupled using Fmoc-protected amino acid building blocks. For peptides **1c–d**, residues 37 through 32 were manually coupled using Fmoc-protected amino acid building blocks. Each manual coupling cycle consisted of *i.* Fmoc-deprotection with of 20% (v/v) piperidine in DMF for 5 min at ambient temperature (5 mL), *ii.* washing with dry DMF (2x, 5 mL), *iii.* coupling of the amino acid (0.44 mmol, 4 equiv) with HCTU (174.0 mg, 0.44 mmol, 4 equiv) in 20% (v/v) 2,4,6-collidine in dry DMF (5 mL) for 30 min, and *iv.* washing the with dry DMF (2x, 5 mL). Residue 32, which follows N-Me-Gly$_{33}$, was double coupled (4 equiv per coupling) using HATU (6 equiv) and HOAt (6 equiv) for 1 hr per coupling to ensure complete reaction. (We have found that coupling after N-methyl amino acids is difficult and requires rigorous coupling to minimize incomplete reaction.[2])

*c. Microwave-assisted coupling of residues 31 through 12.* For peptides **1a–i**, a CEM Liberty Blue Automated Microwave Peptide Synthesizer was used to couple residues 31 to 12. For peptides **1a–f** and **1i**, Fmoc-Orn(Dde)-OH replaces residues 23–29 in the natural sequence. Each coupling cycle consisted of *i.* Fmoc-deprotection with 20% (v/v) piperidine with 0.1 M Oxyma Pure in DMF for 2 min. at 50 ℃, *ii.* washing with DMF (3x), *iii.* coupling of the amino acid (0.75 mmol, 5 equiv) in the presence of HCTU (0.675 mmol, 4.5 equiv) and 20% (v/v/) *N*-methylmorpholine (NMM) in DMF for 10 min. at 50 ℃, iv. washing with DMF (3x).

*d. Cleavage and global deprotection of the peptide from resin.* The resin was then transferred to a 10-mL Bio-Rad Poly-Prep chromatography column, and washed 3x with dry CH$_2$Cl$_2$. The linear peptide was cleaved from the resin by rocking the resin for 1 h with a solution of trifluoracetic acid (TFA) (TIPS)/H$_2$O (18:1:1, 10 mL). During the 1 h deprotection, two 50-mL conical tubes

containing 40-mL of dry ether were chilled on dry ice. The solution was drained and split between the two conical tubes of ether. The tubes were then centrifuged at 800 x g for 25 mins. The ether supernatant was poured off and the pelleted peptide was dried overnight under vacuum.

*e. Oxidation of the disulfide bridge*. The crude, pelleted peptide was resuspended in a solution of 20% v/v dimethyl sulfoxide (DMSO), 50 mL in each tube. The suspension was gently rocked at room temperature for 48 h. The reaction mixture was concentrated under reduced pressure until only DMSO remained.

For ACM containing peptides a solution of 0.1 M iodine in glacial acetic acid (15 mL), 50% aq. acetic acid (250 mL), and 1 M HCl (25 mL) was used to remove the ACM protecting group and oxidize the peptide.

*g. Reverse-phase HPLC purification.* The peptide: DMSO mixture was dissolved in $H_2O$ and acetonitrile (8:2, 5 mL), and the solution was filtered through a 0.2 μm syringe filter and purified by RP-HPLC. The solution of crude peptide was injected at 20% acetonitrile and eluted with a gradient of 20-60% $CH_3CN$ over 90 min. Each peptide eluted between 29-36% $CH_3CN$. The collected fractions were analyzed by analytical HPLC and MALDI-TOF, and the pure fractions were concentrated by rotary evaporation and lyophilized. Typical syntheses yielded between 5 and 62 mg of the peptide as the TFA salt.

*Synthesis of peptides* **2a–d**

The synthesis of peptides **2a–d** generally followed the same procedures as for peptides **1a–i**, except for the following:

*a. Loading the resin.* For peptides **2a–d**, 2-chlorotrityl chloride resin (300 mg, 1.4 mmol/g) was added to a Bio-RAD Poly-Prep chromatography column (10 mL). Dry $CH_2Cl_2$ (8 mL) was used

to suspend and swell the resin for 30 min with gentle rocking. The solution was drained from the resin and a solution of Boc-Orn-Fmoc-OH (0.78 equiv, 150 mg, 0.33 mmol) in 6% (v/v) 2,4,6-collidine in dry $CH_2Cl_2$ (8 mL) was added immediately and the suspension was gently rocked for 12 h. The solution was then drained and a mixture of $CH_2Cl_2$/MeOH/*N*,*N*-diisopropylethylamine (DIPEA) (17:2:1, 10 mL) was added immediately. The resin was gently rocked for 1 h, to cap the unreacted 2- chlorotrityl chloride resin sites. The resin was then washed twice with dry $CH_2Cl_2$ and dried by passing nitrogen through the vessel. This procedure typically yields 0.18 mmol of loaded resin (0.6 mmol/g loading).

*b. Manual peptide coupling.* The resin loaded with Boc-Orn(Fmoc) was suspended in dry DMF and then transferred to a solid-phase peptide synthesis vessel. Residues 40 through 32 were manually coupled using Fmoc-protected amino acid building blocks. Each manual coupling cycle consisted of *i*. Fmoc-deprotection with of 20% (v/v) piperidine in DMF for 5 min at ambient temperature (5 mL), *ii*. washing with dry DMF (2x, 5 mL), *iii*. coupling of the amino acid (0.44 mmol, 4 equiv) with HCTU (174.0 mg, 0.44 mmol, 4 equiv) in 20% (v/v) 2,4,6-collidine in dry DMF (5 mL) for 30 min, and *iv*. washing the with dry DMF (2x, 5 mL). Residue 32, which follows N-Me-Gly$_{33}$, was double coupled (4 equiv per coupling) using HATU (6 equiv) and HOAt (6 equiv) for 1 hr per coupling to ensure complete reaction. (We have found that coupling after N-methyl amino acids is difficult and requires rigorous coupling to minimize incomplete reaction.[2])

*c. Microwave-assisted coupling of residues 31 through 12.* A CEM Liberty Blue Automated Microwave Peptide Synthesizer was used to couple residues 31 to 12. Fmoc-Orn(Dde)-OH replaces residues 23–29 in the natural sequence. Each coupling cycle consisted of *i*. Fmoc-deprotection with 20% (v/v) piperidine with 0.1 M Oxyma Pure in DMF for 2 min. at 50 ℃, *ii*. washing with DMF (3x), *iii*. coupling of the amino acid (0.75 mmol, 5 equiv) in the presence of

HCTU (0.675 mmol, 4.5 equiv) and 20% (v/v/) *N*-methylmorpholine (NMM) in DMF for 10 min. at 50 ºC, iv. washing with DMF (3x).

*e. Cleavage of the peptide from resin.* The resin was then transferred to a 10-mL Bio-Rad Poly-Prep chromatography column, and washed 3x with dry $CH_2Cl_2$. The acyclic peptide was cleaved from the resin by rocking the resin for 1 h with a solution of 1,1,1,3,3,3-hexafluoroispropanol (HFIP) in $CH_2Cl_2$ (1:4, 8 mL). The suspension was filtered and the filtrate was collected in a 250-mL round-bottomed flask. The resin was washed with additional HFIP in $CH_2Cl_2$ (1:4, 8 mL). The combined filtrates were concentrated by rotary evaporation to give a white solid. The white solid was further dried by vacuum pump to afford the crude protected linear peptide, which was cyclized without further purification.

*f. Cyclization of the acyclic peptide.* The crude protected linear peptide was dissolved in dry DMF (150 mL). PyBOP (370 mg, 0.711 mmol, 6 equiv) and *N*-methylmorpholine (NMM) (0.33 mL, 1.8 mmol, 12 equiv) was added to the solution and the mixture was stirred under nitrogen for 48 h. The mixture was concentrated under reduced pressure to afford the crude protected cyclic peptide, a yellow film.

*e. Global deprotection of the cyclic peptide.* The protected cyclic peptide was dissolved in TFA/triisoproylsilane (TIPS)/$H_2O$ (18:1:1, 10 mL) in a 250-mL round-bottomed flask equipped with a nitrogen-inlet adaptor. The solution was stirred for 1 h under nitrogen. The reaction mixture was then concentrated by rotary evaporation under reduced pressure to afford the crude cyclic peptide. During the 1 h deprotection, two 50-mL conical tubes containing 40-mL of dry ether were chilled on dry ice. The solution was drained and split between the two conical tubes of ether. The tubes were then centrifuged at 800 x g for 25 mins. The ether supernatant was poured off and the pelleted peptide was dried overnight under vacuum.

*e*. *Oxidation of the disulfide bridge*. The crude, pelleted peptide was resuspended in a solution of 20% v/v dimethyl sulfoxide (DMSO), 50 mL in each tube. The suspension was gently rocked at room temperature for 48 h. The reaction mixture was concentrated under reduced pressure until only DMSO remained.

*g*. *Reverse-phase HPLC purification.* The peptide: DMSO mixture was dissolved in $H_2O$ and acetonitrile (8:2, 5 mL), and the solution was filtered through a 0.2 μm syringe filter and purified by RP-HPLC. The solution of crude peptide was injected at 20% acetonitrile and eluted with a gradient of 20-60% $CH_3CN$ over 90 min. Each peptide eluted between 29-36% $CH_3CN$. The collected fractions were analyzed by analytical HPLC and MALDI-TOF, and the pure fractions were concentrated by rotary evaporation and lyophilized. Typical syntheses yielded between 8 and 38 mg of the peptide as the TFA salt.

SDS-PAGE was performed on peptides **1a–i** and **2a–d** using the reagents, recipes, and procedures for Tricine SDS-PAGE detailed in Schägger, H. *Nat. Protoc.* **2006**, *1*, 16–22. Each peptide was run on a 16% polyacrylamide gel with a 4% stacking polyacrylamide gel at 60 volts. A Spectra^TM Multicolor Low Range Protein Ladder (ThermoFischer Scientific, catalog #: 26628) was loaded into the first lane of the gel. The remaining lanes were loaded with 8.0 µL aliquots of each peptide as 50 µM solutions in SDS-PAGE loading buffer, which were prepared as follows: A 10 mg/mL stock solution of each peptide was prepared with deionized water. Aliquots of the 10 mg/mL solutions were then diluted further with deionized water and 6X SDS-PAGE loading buffer (G-Biosciences catalog #: 786-701) to create 100 µM working solutions of each peptide.

To reduce the disulfide bonds present in peptides **1a**, **2a**, and **2b** 50 µM solutions of each peptide were sonicated for 1h in 10mM TCEP prior to gel loading. After 1h had elapsed the solution was diluted further with 6X SDS-PAGE loading buffer (G-Biosciences catalog #: 786-701) to create 100 µM working solutions of each peptide.

Staining with silver nitrate was used to visualize peptides **1a–i** and **2a–d** in the SDS-PAGE gel. Reagents for silver staining were prepared according to procedures detailed in Simpson, R. J. *Cold Spring Harbor Protocol* **2007**. [We have found it important to prepare sodium thiosulfate, silver nitrate, and developing solutions fresh each time and to use high purity sodium carbonate to prepare the developing solution.] The gel was removed from the casting glass and rocked for 20 min in fixing solution (50% (v/v) methanol and 5% (v/v) acetic acid in deionized water). The fixing solution was then discarded and replaced with 50% (v/v) aqueous methanol for another 10 min of rocking. Next, the 50% methanol was discarded and replaced with deionized water for another 10 min of rocking. Next, the water was discarded and the gel was rocked in 0.02% (w/v) sodium thiosulfate in deionized water for 1 min. The sodium thiosulfate was discarded and the gel was rinsed twice with deionized water for 0.5 min. The gel was then submerged in pre-chilled 0.1%

(w/v) silver nitrate in deionized water and rocked at 4 ℃ for 20 min. The silver nitrate solution was discarded and the gel was rinsed twice with deionized water. The gel was incubated in developing solution (2% (w/v) sodium carbonate, 0.04% (w/v) formaldehyde) until the solution began to brown. The developing solution was then immediately discarded and fresh silver nitrate solution was added to the gel until the desired intensity of staining was reached. When the desired intensity of staining was reached, the developing solution was discarded and the gel was submerged in 5% aqueous acetic acid.

*Circular dichroism spectroscopy*

A 50 µM solution of each peptide was prepared by diluting the 10 mg/mL stock solution with 10 mM sodium phosphate buffer at pH 7.4. Each solution was transferred to a 1 mm quartz cuvette for data acquisition. Circular dichroism spectra were acquired on a Jasco J-810 circular dichroism spectropolarimeter at ambient temperature. Data were collected using 2.0 nm intervals from 260 nm to 190 nm and averaged over five accumulations with smoothing.

*Crystallization of peptide **2a***

The hanging-drop vapor-diffusion method was used to determine initial crystallization conditions for peptide **2a**. Peptide **2a** was screened in 96-well plate format using three crystallization kits (Crystal Screen, Index, and PEG/ION) from Hampton Research. A TTP LabTech Mosquito nanondisperse was used to make three 150 nL hanging drops for each well condition. The three hanging drops differed in the ratio of peptide to well solution for each condition in the 96-well plate. A 10 mg/mL solution of each peptide in deionized water was combined with a well solution in ratios of 1:1, 1:2, and 2:1 peptide:well solution at appropriate volumes to create the three 150 nL hanging drops. Crystals of peptide **2a** grew in well conditions of 0.2 M magnesium acetate tetrahydrate, 0.1 M sodium cacodylate trihydrate pH 6.5, and 30% (v/v) 2-methyl-2,4-pentanediol.

Crystallization conditions for each peptide were optimized using a 4x6 matrix Hampton 24-well plate. For peptide **2a** the 0.1 M sodium cacodylate trihydrate buffer was varied in each row in increments of 0.5 pH units (6.0, 6.5, 7.0, and 7.5) and the percentage of 2-methyl-2,4-pentanediol in each column in increments of 2% (v/v) (26%, 28%, 30%, 32%, 34%, 36%). Three hanging-drops were prepared on borosilicate glass slides by combing a 10 mg/mL solution of peptide **2a** in deionized water with the well solution in the following amounts: 1 μL:1 μL, 2 μL:1 μL, and 1 μL:2 μL. Slides were inverted and pressed firmly against the silicone grease surrounding each well. Crystals were harvested with a nylon loop attached to a copper or steel pin, soaked briefly in MPD, and flash frozen in liquid nitrogen prior to data collection. The optimized crystallization conditions for peptide **2a** is summarized Supplementary Table 3.1.

*X-ray crystallographic data collection, data processing, and structure determination of peptide **2a***

Diffraction data for **2a** were collected at the Stanford Synchrotron Radiation Light source, beamline 12.2, at 1.00 Å wavelength. Datasets were indexed and integrated with XDS. Scaling and merging was done with pointless and aimless in CCP4. The structures were solved with molecular replacement in Phaser using a dimer formed by macrocyclic β-hairpin peptide derived from Aβ$_{16-36}$ (PDB 6WXM) as the search model. The refinement was done with phenix.refine module of the Phenix suite, with manipulation of the model performed using Coot.

*Replica Exchange Molecular Dynamics Simulation of an Aβ$_{9-42}$ β-barrel-like tetramer*

A model of an Aβ$_{9-42}$ β-barrel-like tetramer was generated by replica-exchange molecular dynamics as follows: Starting coordinates for Aβ$_{9-42}$ was generated from the crystallographic coordinates of peptide **2a**. Symmetry mates of peptide **2a** were displayed in PyMOL. Each respective tetramer was selected and saved to a new PDB file. The delta-linked ornithine residues were deleted from each macrocycle. Three alanine residues were added to the N-terminus of the β-hairpin, and two alanine residues were added to the C-terminus. Seven alanine residues were

added to connect $Glu_{22}$ and $Ala_{30}$. These added residues were minimized in PyMOL using the clean function, ensuring that the crystallographic coordinates of $A\beta_{12-22}$ and $A\beta_{30-40}$ were not perturbed. After this minimization, each added alanine was mutated to its corresponding wild-type residue from $A\beta$. The mutated residues were again minimized in PyMOL using the clean function. Each N-Me-$Gly_{33}$ was replaced with the wild-type Gly. Each $Cys_{15}$ and $Cys_{37}$ was replaced with the wild-type Gln and Gly.

The autopsf plugin in VMD was used to prepare the required files for simulation. The coordinates for $A\beta_{12-22}$ and $A\beta_{30-40}$ were fixed throughout the simulation. REMD simulations were run in NAMD with the CHARMM22 force field and generalized Born implicit solvent (GBIS) on 32 replicas. The temperatures for these replicas varied between 300 and 800K. The simulation was performed for 10 ns. Representative coordinates were selected uniformly from the last 9 ns of the simulation

*Molecular Dynamics Simulation of an $A\beta_{9-42}$ β-barrel-like tetramer in a lipid membrane*

The CHARMM-GUI input generator was used to insert a β-barrel like tetramer formed by $A\beta_{9-42}$ into a 87Åx87Å planar POPC bilayer. The tetramer was embedded in the membrane with the pore-like opening aligned to the principal Z-axis of the membrane bilayer. The protonation state of titratable amino acids were chosen for a corresponding system pH of 7.4, with the histidine sidechains left neutral. The CHARMM36m forcefield was used to parametrize the system. Molecular graphics and simulation analyses were generated with VMD 1.9.3

# References

1.  These procedures follow closely those that our laboratory has previously published. The procedures in this section are adapted from and in some cases taken verbatim from Kreutzer, A. G.; Hamza, I. L.; Spencer, R. K.; Nowick J. S. *J. Am. Chem. Soc.* **2016**, *138*, 4634–4642, Spencer, R. K.; Kreutzer, A. G.; Salveson, P. J.; Li, H.; Nowick, J. S. *J. Am. Chem. Soc.* **2015**, *137*, 6304–6311, Spencer, R. K.; Li. H.; Nowick, J. S. *J. Am. Chem. Soc.* **2014**, *136*, 5595– 5598, and Kreutzer, A. G.; Yoo, S.; Spencer, R. K.; Nowick, J. S. *J. Am. Chem, Soc.* **2017**, *139*, 966–975, Samdin, T. D.; Wierzbicki, M.; Kreutzer, A. G.; Howitz, W. J.; Valenzuela, M.; Smith, A.; Sahrai, V.; Truex, N. L.; Klun, M.;Nowick, J. S. *J. Am. Chem. Soc*. **2020**, *142*, 11593–11601.
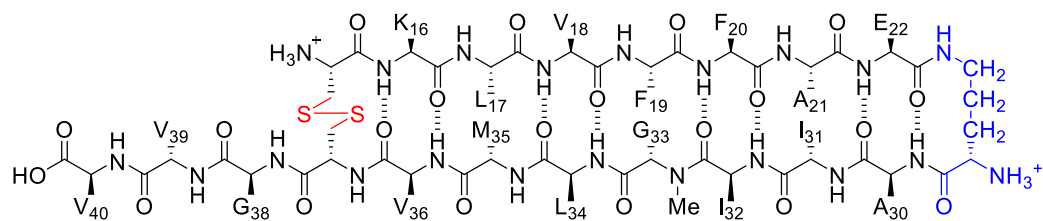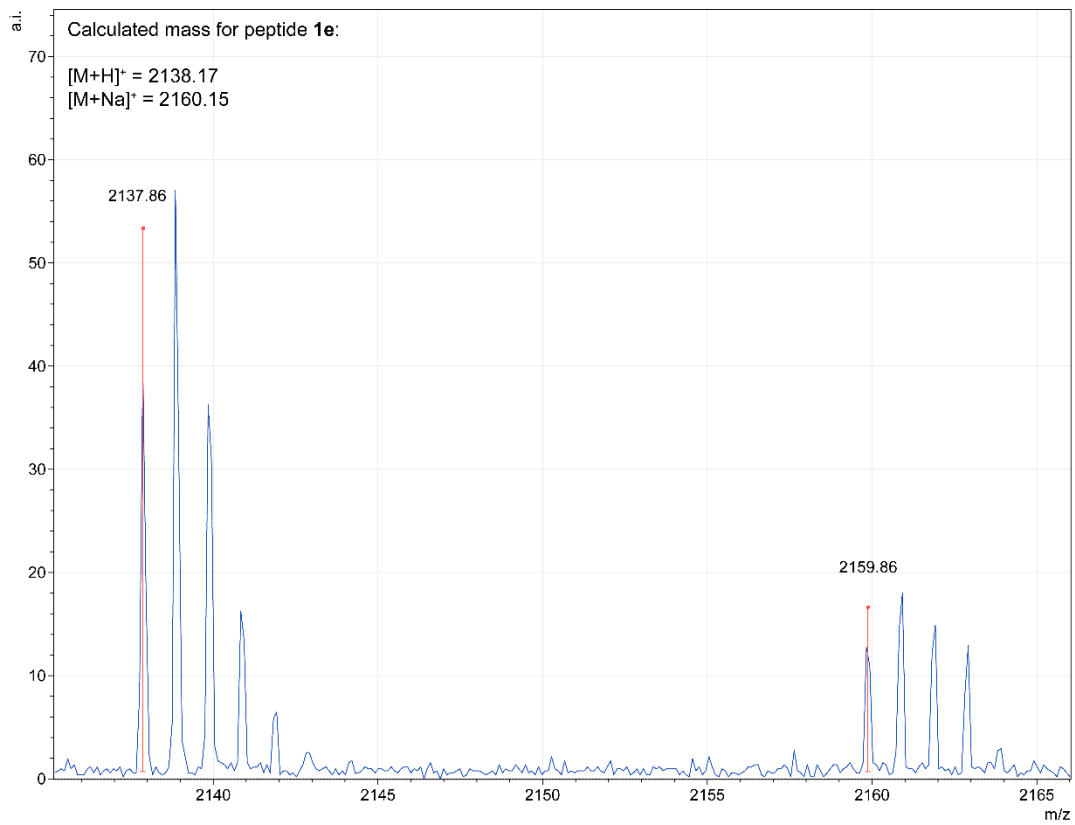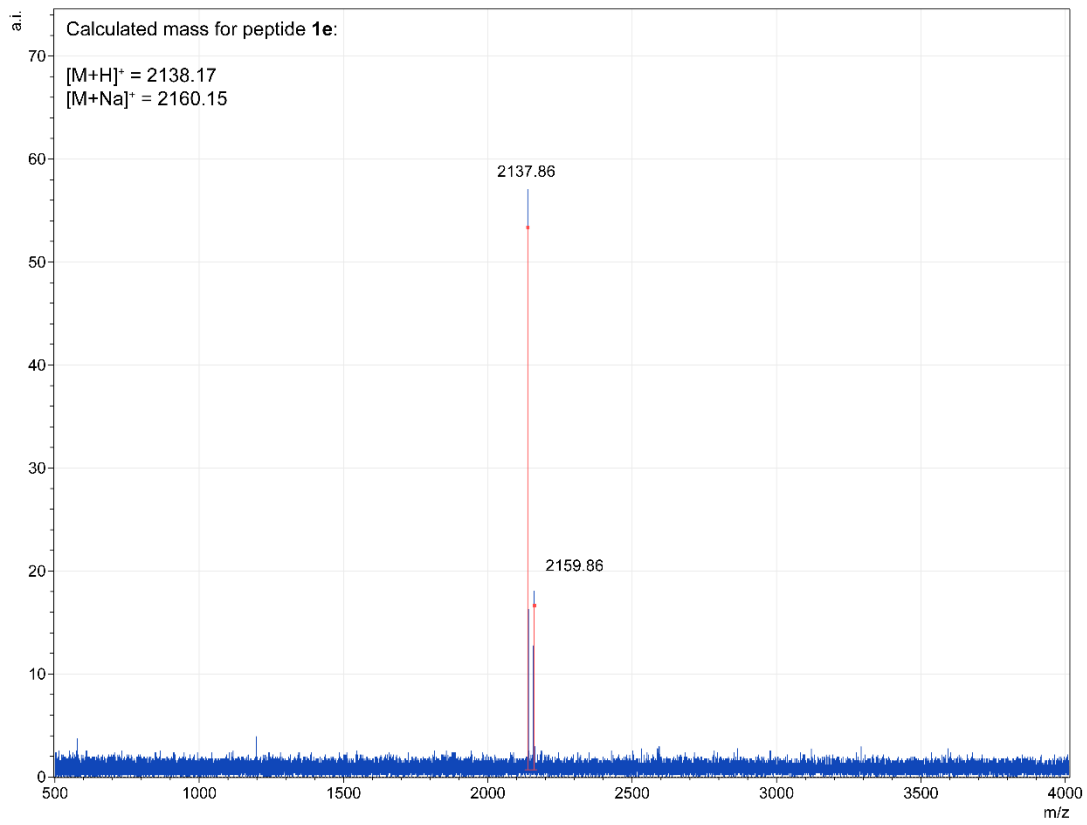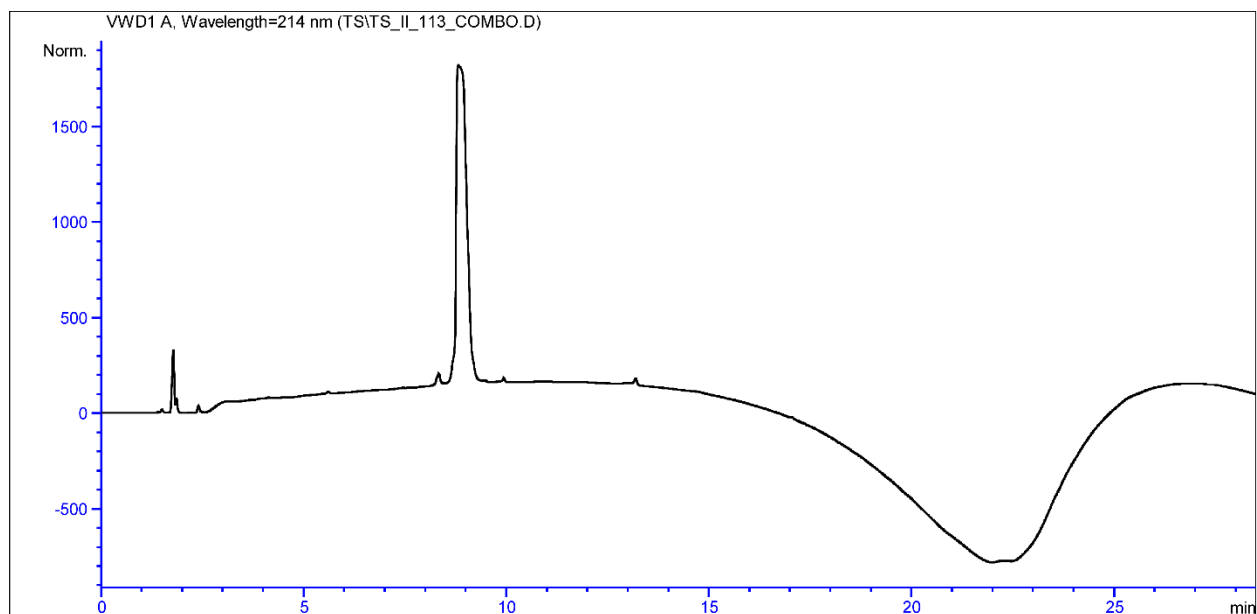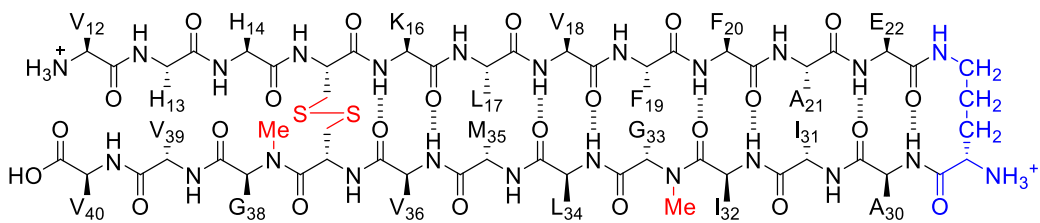
# Characterization Data

*Characterization of peptide **1a***

Calculated mass for peptide **1a**:

[M+H]⁺ = 2511.35
[M+Na]⁺ = 2533.34
[M+K]⁺ = 2549.31

2533.50
2511.53
2549.48



Calculated mass for peptide **1a**:
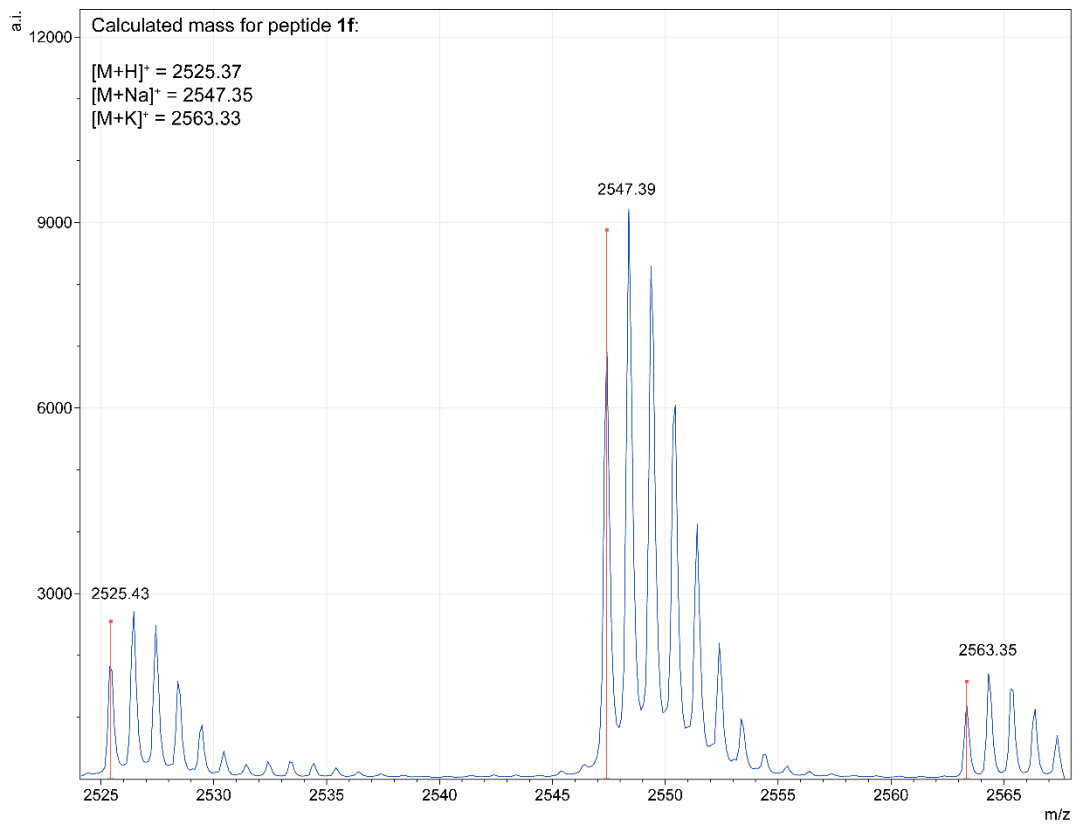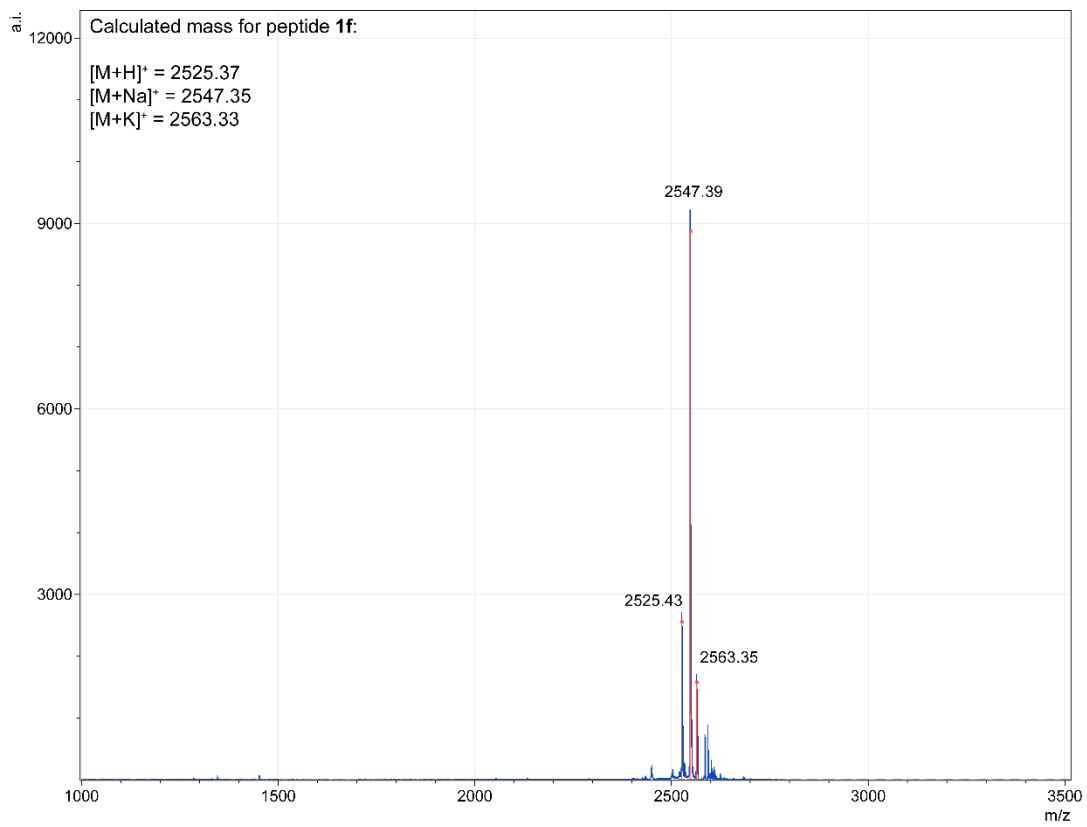
[M+H]⁺ = 2511.35
[M+Na]⁺ = 2533.34
[M+K]⁺ = 2549.31

2533.50
2511.53
2549.48

146

*Characterization of peptide **1b***

Calculated mass for peptide **1b**:

[M+H]$^+$ = 2492.42
[M+Na]$^+$ = 2514.40

*Characterization of peptide **1c***

Calculated mass for peptide **1c**:

[M+H]$^+$ = 1882.03
[M+Na]$^+$ = 1904.01
[M+K]$^+$ = 1919.98



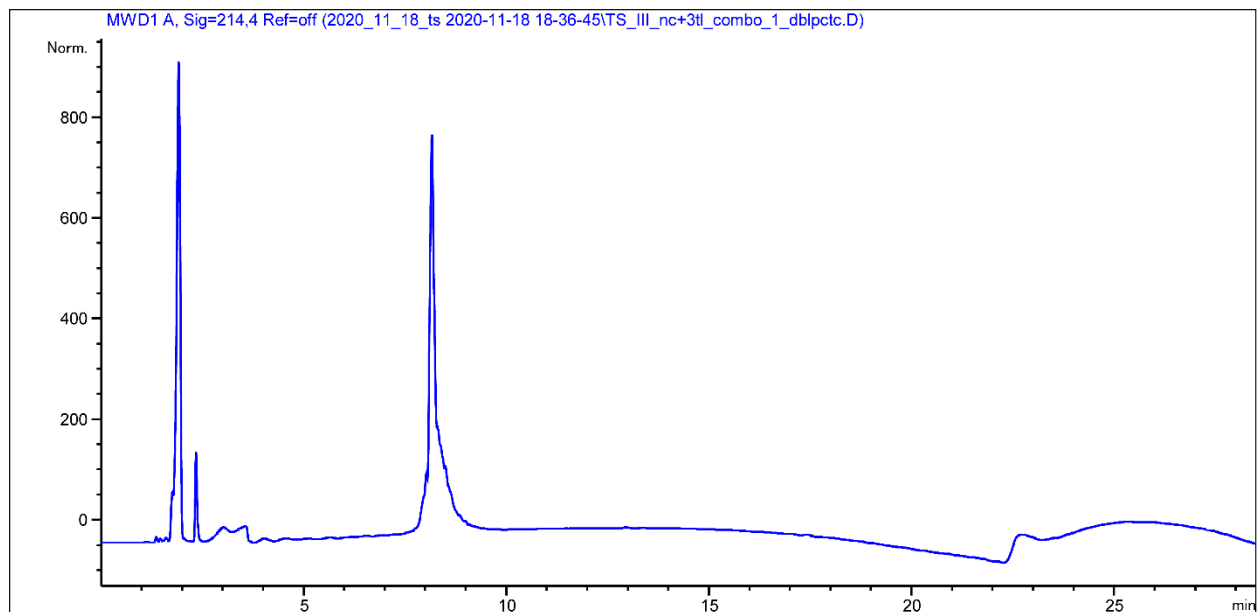Calculated mass for peptide **1c**:

[M+H]$^+$ = 1882.03
[M+Na]$^+$ = 1904.01
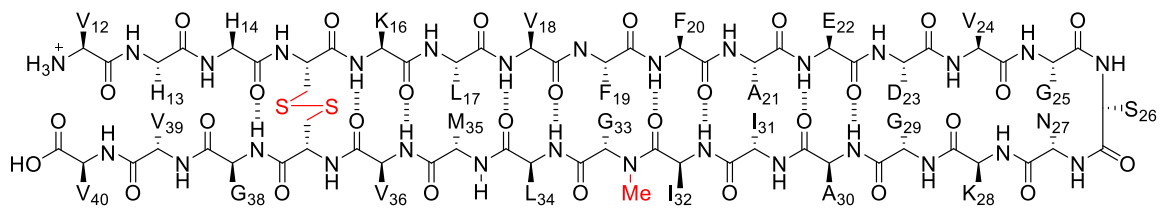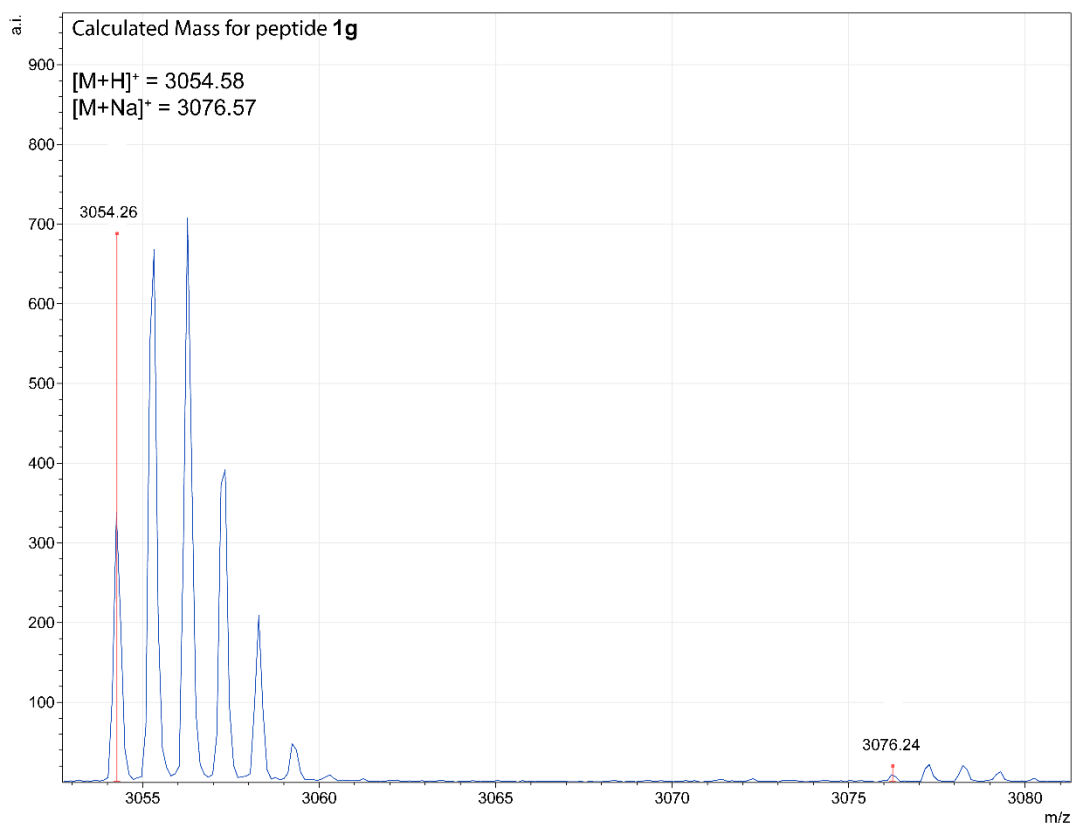[M+K]$^+$ = 1919.98

150

*Characterization of peptide **1d***

Calculated mass for peptide **1d**:

[M+H]+ = 2255.21
[M+Na]+ = 2277.19
[M+K]+ = 2293.17

2277.27

2255.31

2293.25



Calculated mass for peptide **1d**:

[M+H]+ = 2255.21
[M+Na]+ = 2277.19
[M+K]+ = 2293.17

2277.27

2255.31

2293.25

152

*Characterization of peptide 1e*

Calculated mass for peptide **1e**:

$[M+H]^+$ = 2138.17
$[M+Na]^+$ = 2160.15

2137.86

2159.86



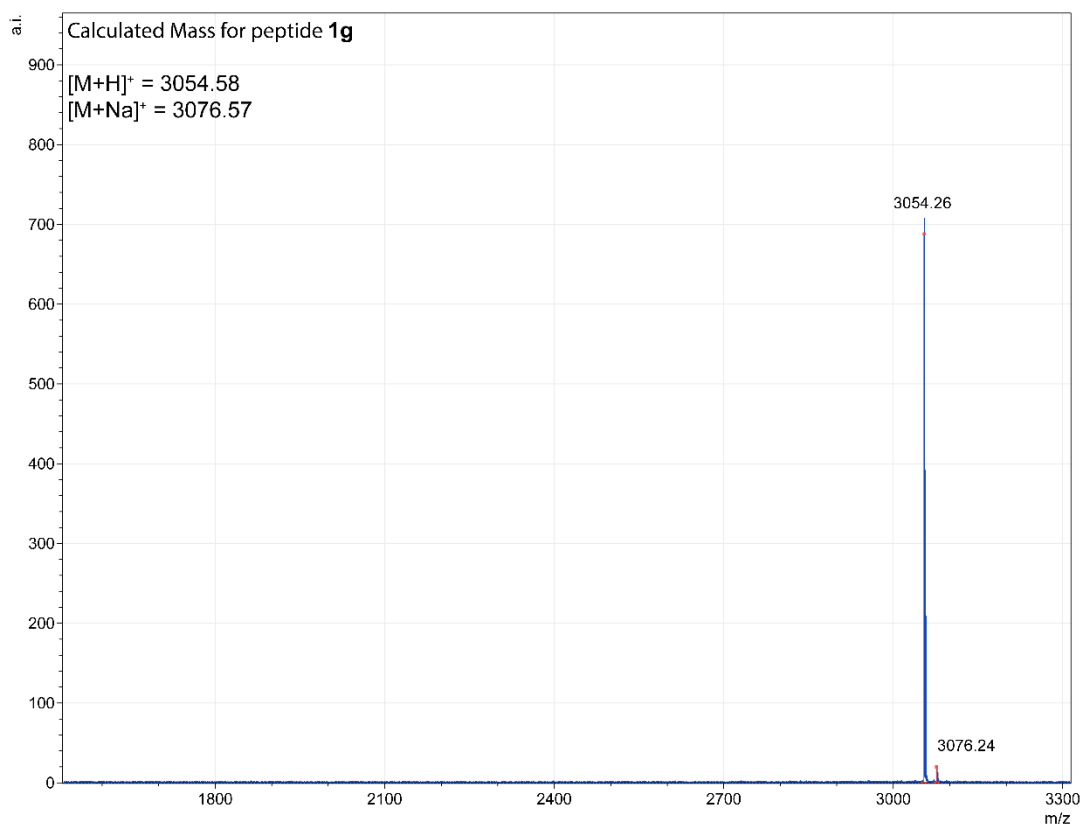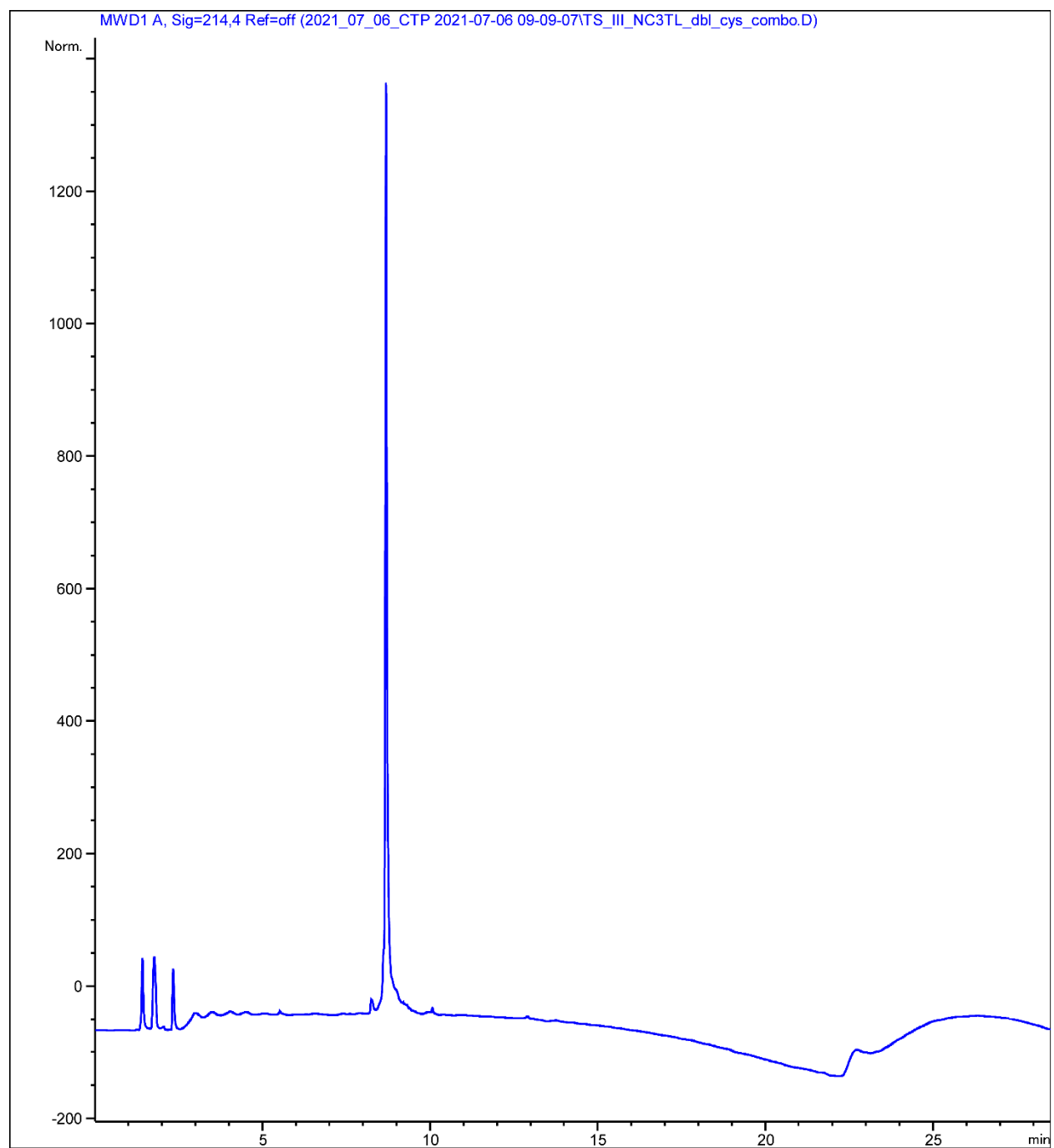Calculated mass for peptide **1e**:

$[M+H]^+$ = 2138.17
$[M+Na]^+$ = 2160.15

2137.86

2159.86

*Characterization of peptide **1f***

Calculated mass for peptide **1f**:

[M+H]$^+$ = 2525.37
[M+Na]$^+$ = 2547.35
[M+K]$^+$ = 2563.33

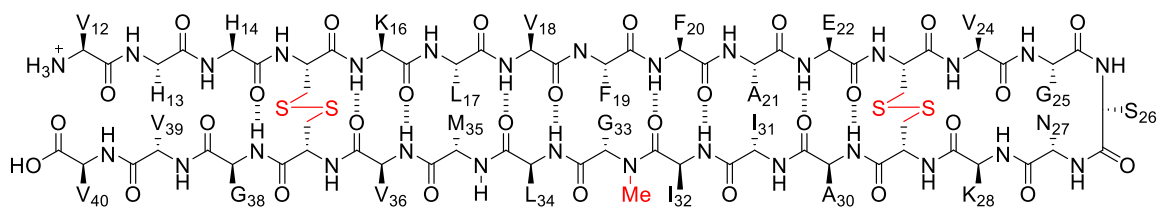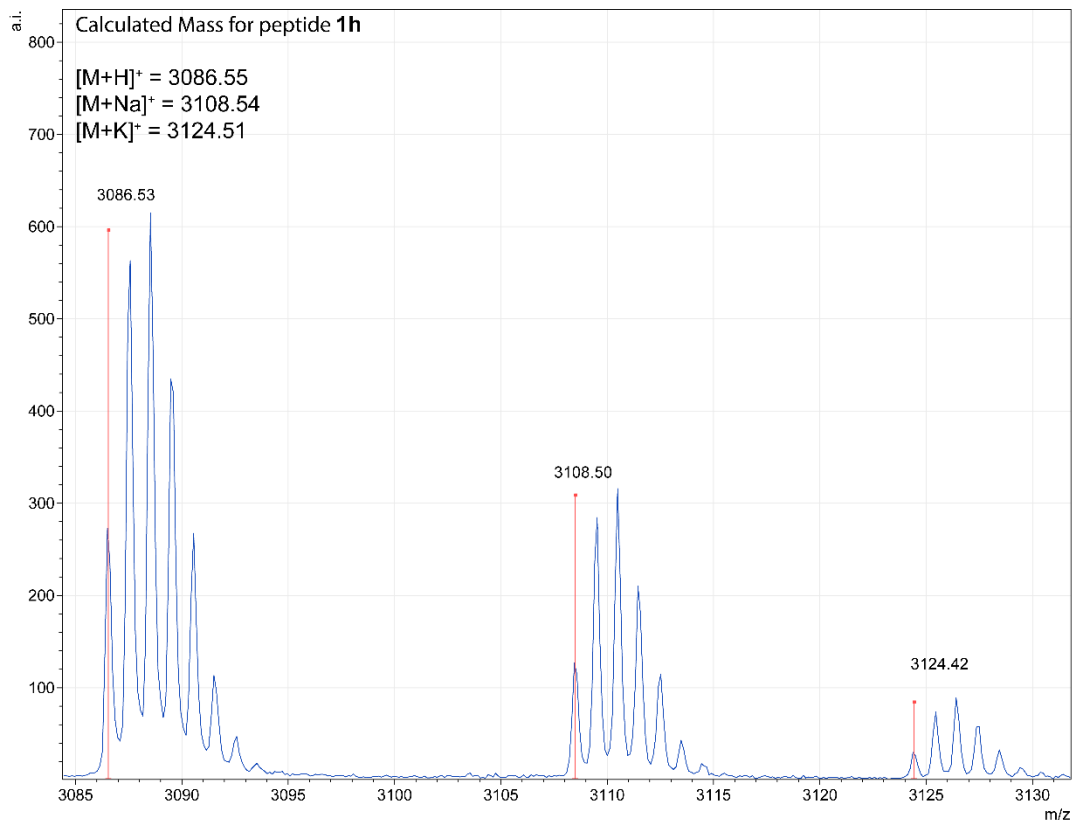Calculated mass for peptide **1f**:

[M+H]$^+$ = 2525.37
[M+Na]$^+$ = 2547.35
[M+K]$^+$ = 2563.33

156

*Characterization of peptide **1g***

Calculated Mass for peptide **1g**

$[M+H]^+ = 3054.58$
$[M+Na]^+ = 3076.57$

3054.26

3076.24



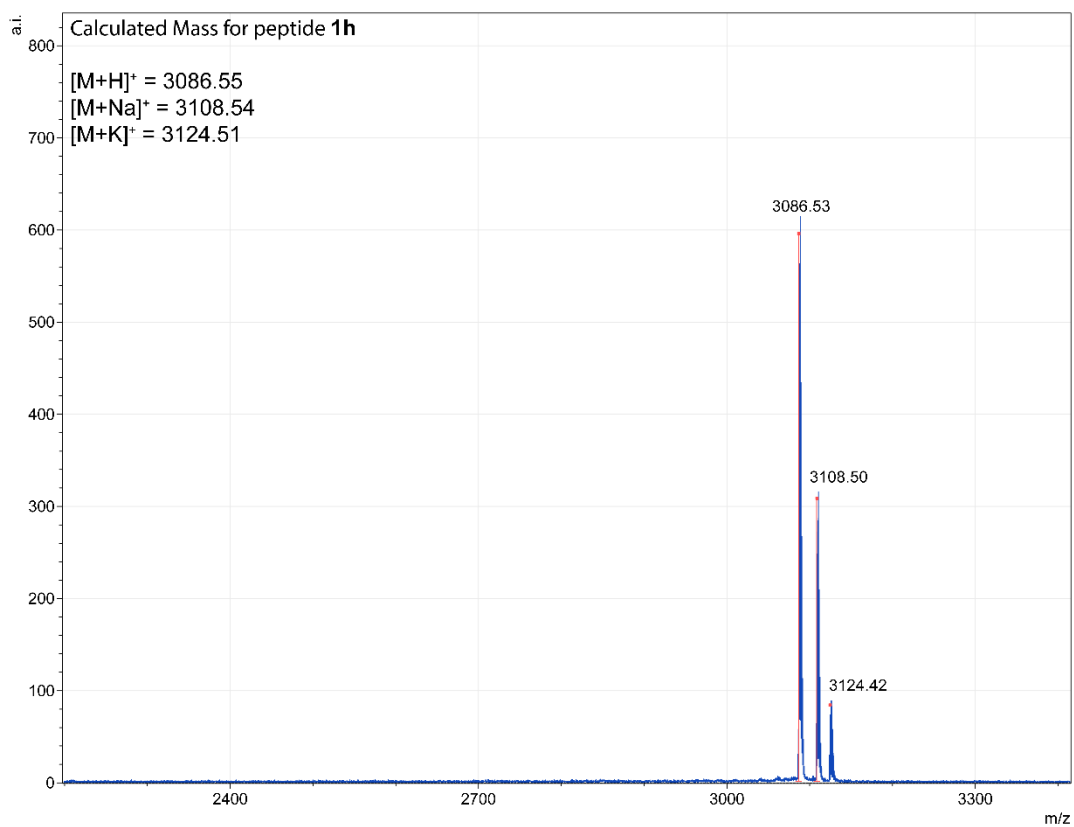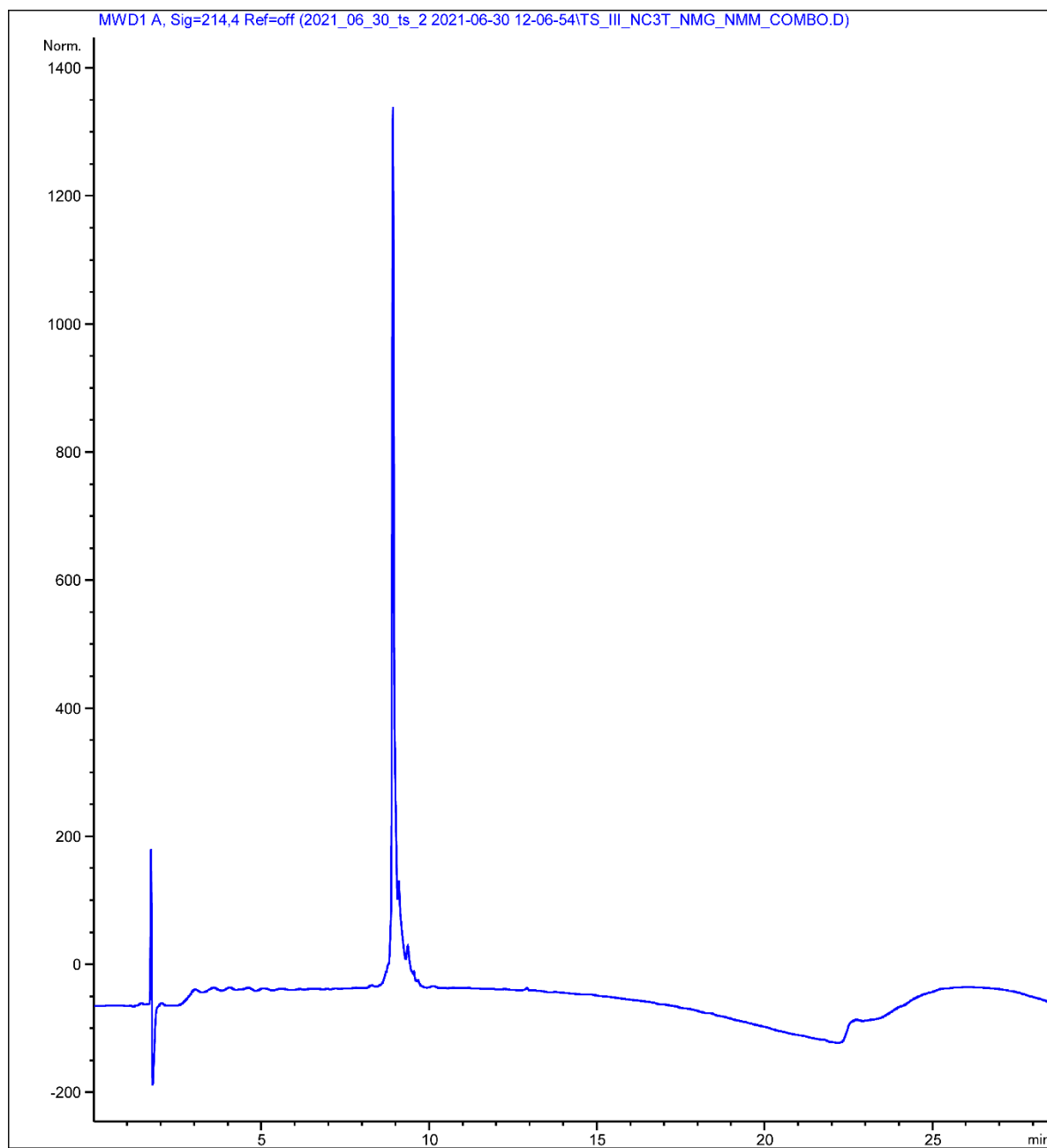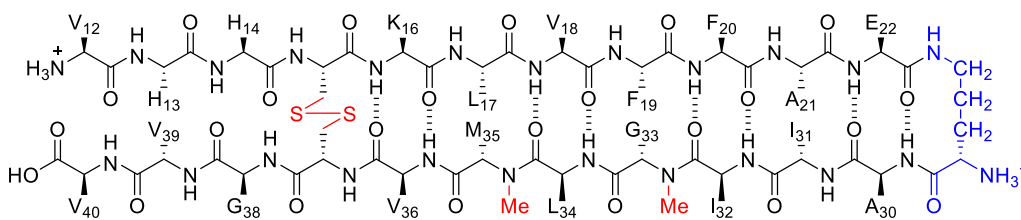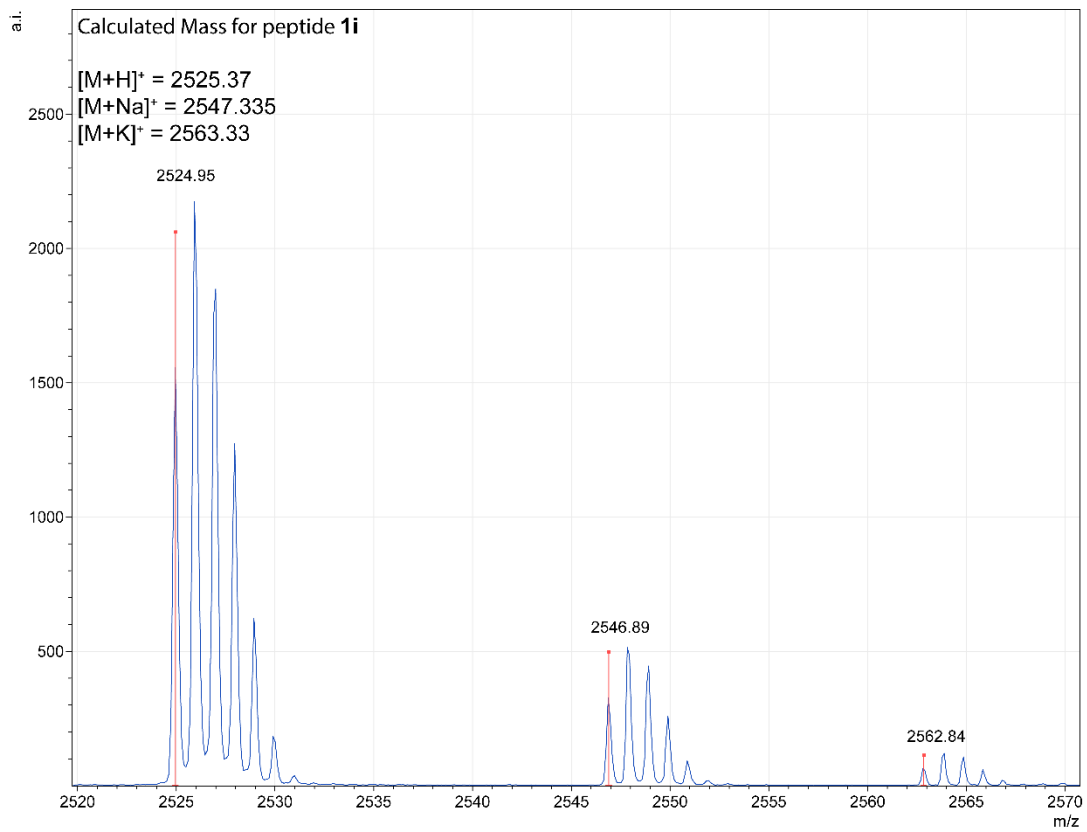Calculated Mass for peptide **1g**

$[M+H]^+ = 3054.58$
$[M+Na]^+ = 3076.57$

3054.26

3076.24

*Characterization of peptide **1h***

Calculated Mass for peptide **1h**

[M+H]$^+$ = 3086.55
[M+Na]$^+$ = 3108.54
[M+K]$^+$ = 3124.51



Calculated Mass for peptide **1h**

[M+H]$^+$ = 3086.55
[M+Na]$^+$ = 3108.54
[M+K]$^+$ = 3124.51

*Characterization of peptide 1i*

Calculated Mass for peptide **1i**

[M+H]+ = 2525.37
[M+Na]+ = 2547.335
[M+K]+ = 2563.33

2524.95

2546.89

2562.84



Calculated Mass for peptide **1i**

[M+H]+ = 2525.37
[M+Na]+ = 2547.335
[M+K]+ = 2563.33

2524.95

2546.89

2562.84

162

*Characterization of peptide **2a***

Calculated Mass for peptide **2a**

$[M+H]^+ = 2607.42$
$[M+Na]^+ = 2629.41$
$[M+K]^+ = 2645.38$

2629.46

2607.49

2645.42



Calculated Mass for peptide **2a**

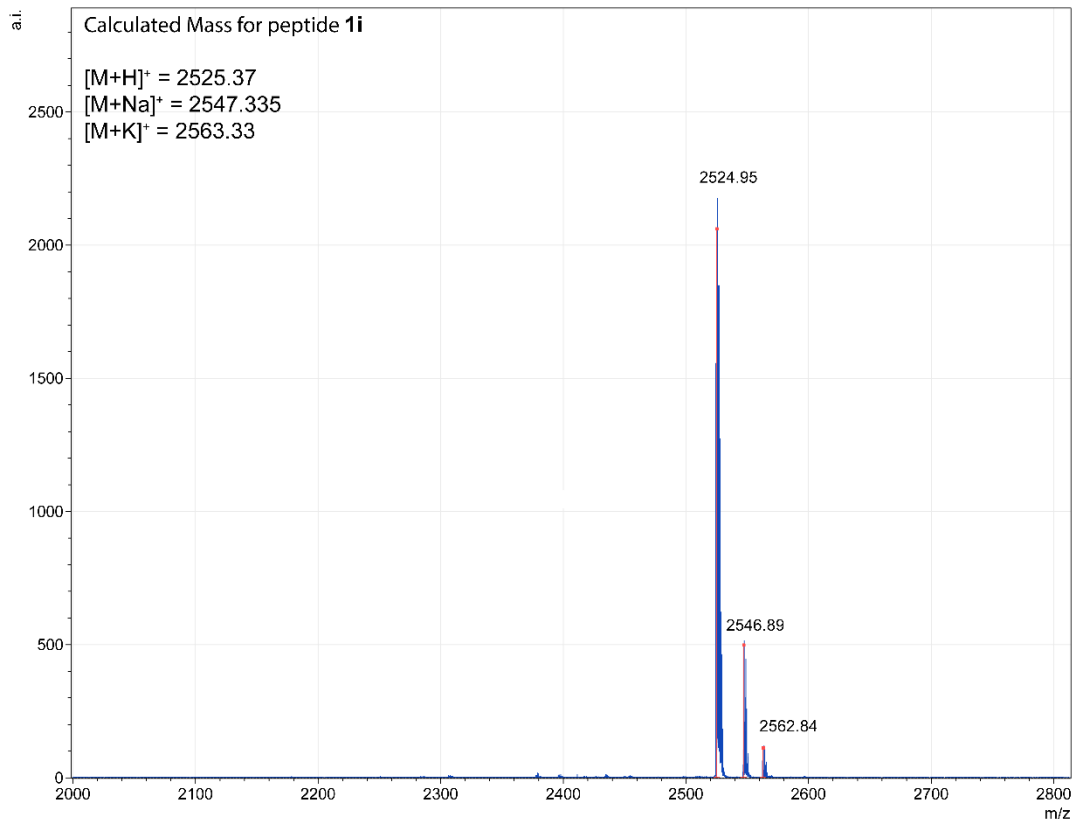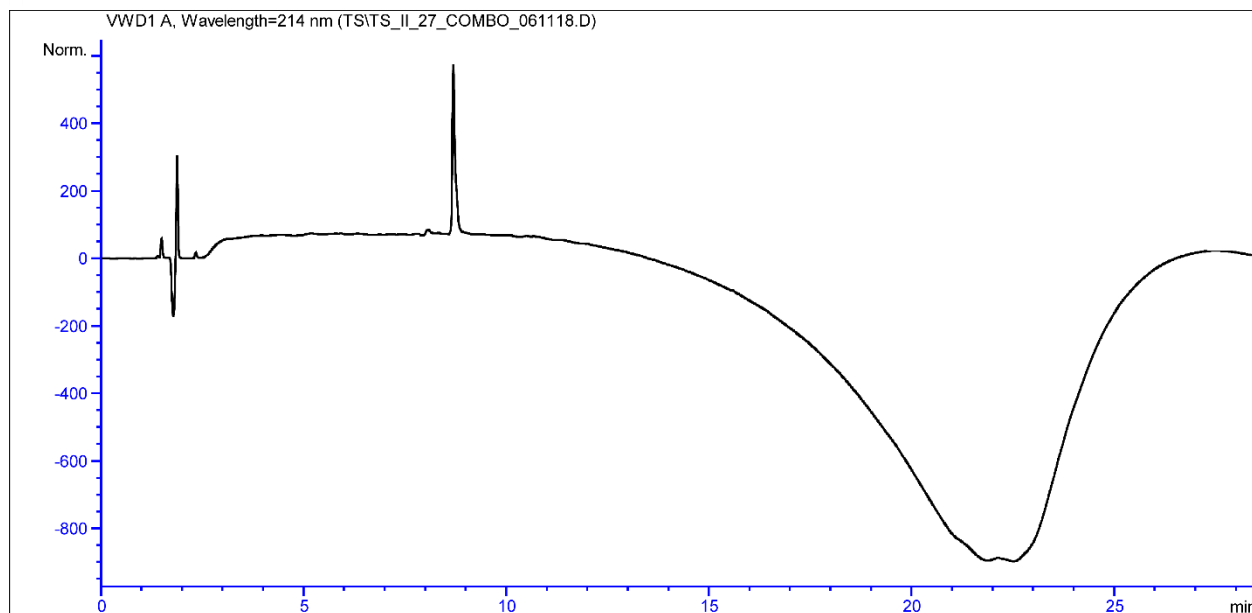$[M+H]^+ = 2607.42$
$[M+Na]^+ = 2629.41$
$[M+K]^+ = 2645.38$

2629.46

2607.49

2645.42

*Characterization of peptide **2b***

Calculated Mass for peptide **2b**

[M+H]$^+$ = 2588.49
[M+Na]$^+$ = 2610.47
[M+K]$^+$ = 2626.44



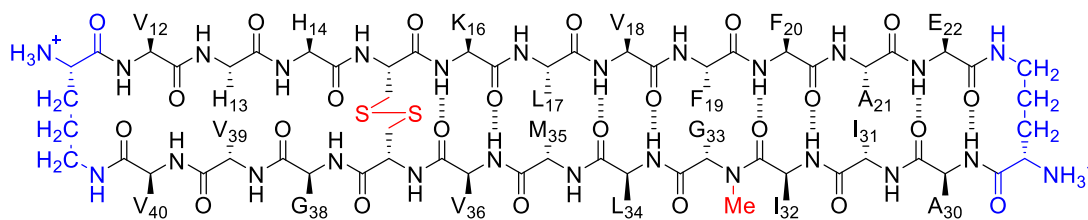Calculated Mass for peptide **2b**

[M+H]$^+$ = 2588.49
[M+Na]$^+$ = 2610.47
[M+K]$^+$ = 2626.44

*Characterization of peptide **2c***

Calculated Mass for peptide **2c**

$[M+H]^+ = 2621.44$
$[M+Na]^+ = 2643.42$

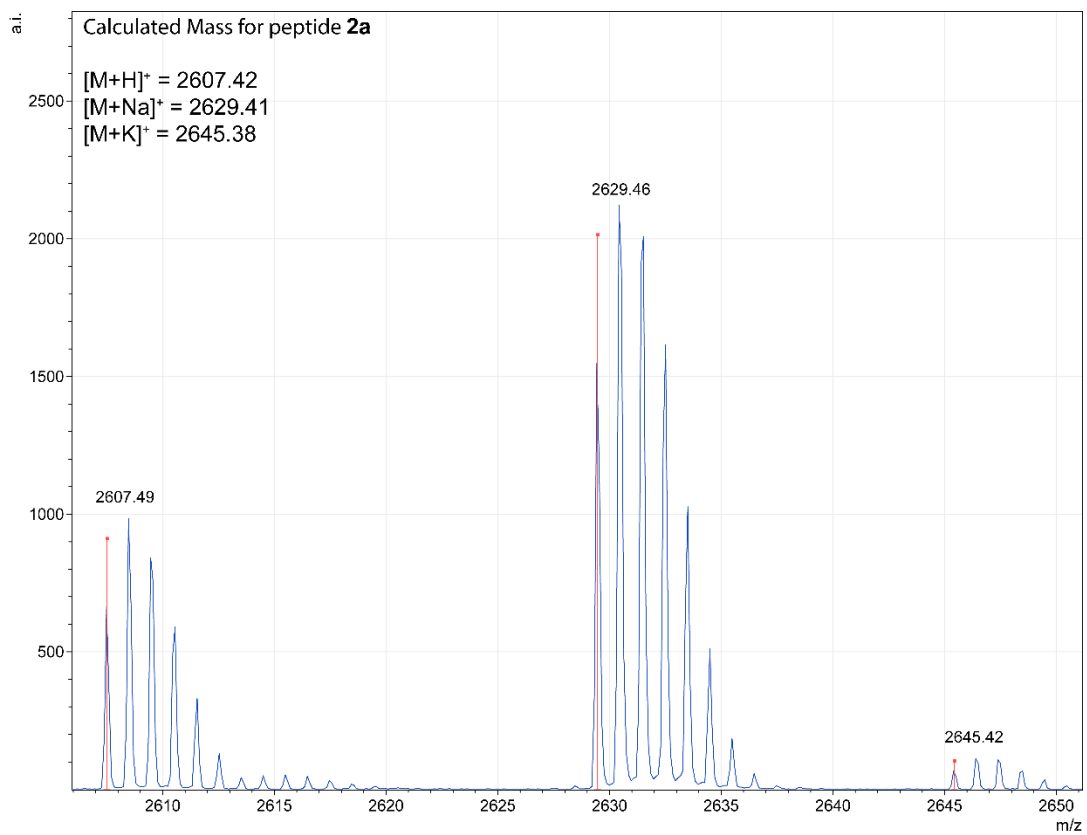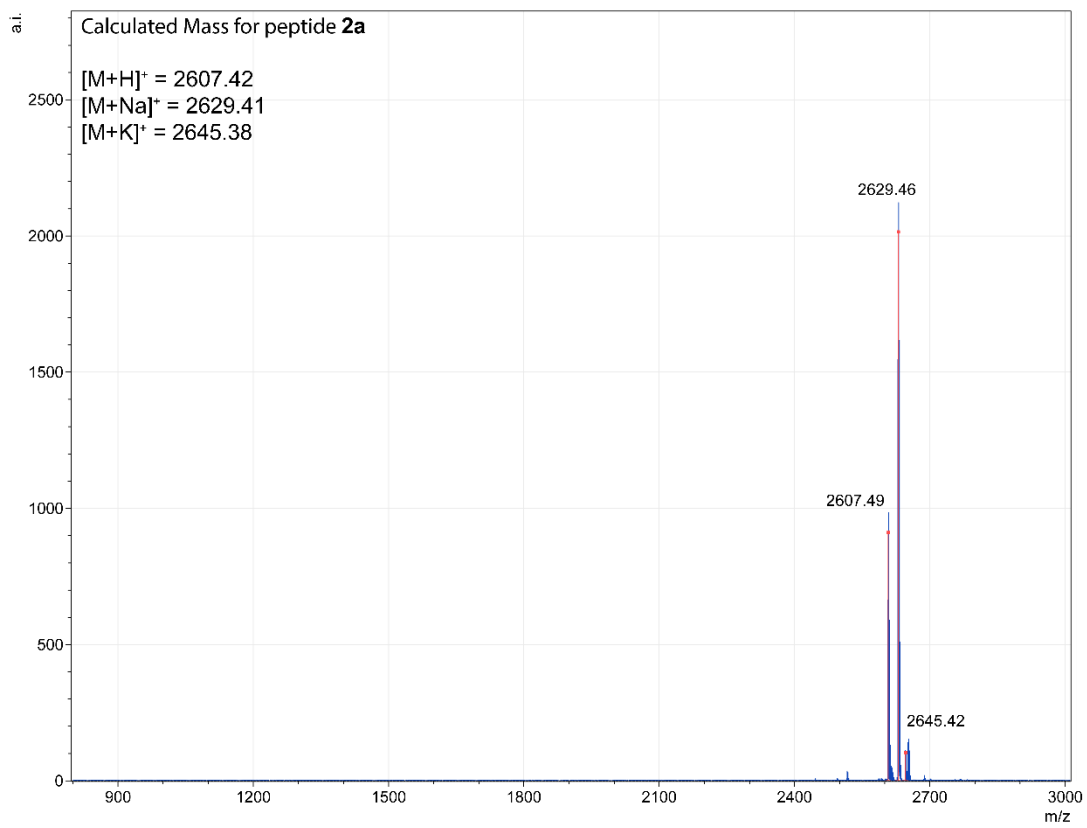Calculated Mass for peptide **2c**

[M+H]$^+$ = 2621.44
[M+Na]$^+$ = 2643.42

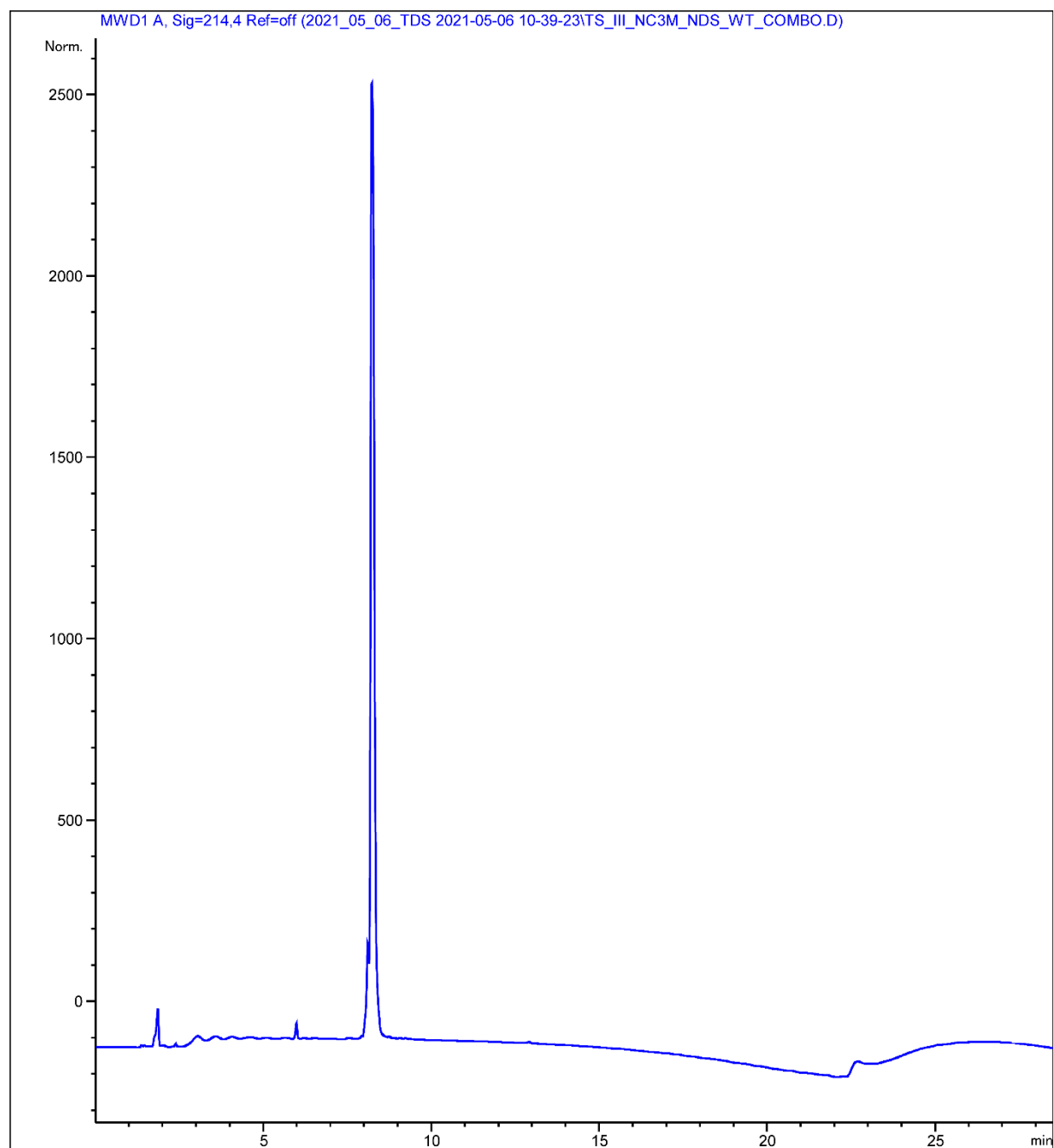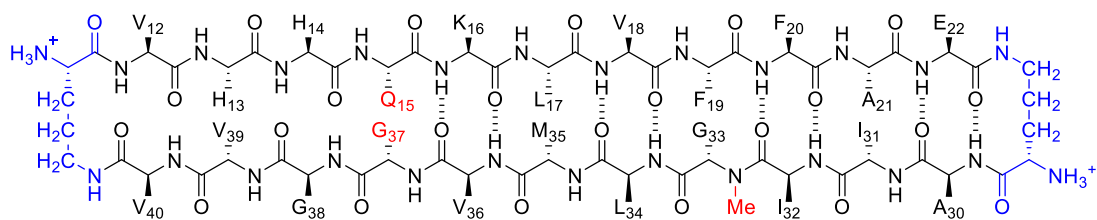*Characterization of peptide **2d***

[M+7H]⁷⁺

[M+6H]⁶⁺

[M+5H]⁵⁺

[M+8H]⁸⁺

[M+4H]⁴⁺

[M+3H]³⁺

Calculated mass for peptide 2d:

M          = 5180.73
[M+3H]³⁺ = 1727.95
[M+4H]⁴⁺ = 1296.21
[M+5H]⁵⁺ = 1037.17
[M+6H]⁶⁺ = 864.47
[M+7H]⁷⁺ = 741.12
[M+8H]⁸⁺ = 648.62

[M+3H]³⁺

Calculated mass for peptide 2d:

M          = 5180.73
[M+3H]³⁺ = 1727.95
[M+4H]⁴⁺ = 1296.21
[M+5H]⁵⁺ = 1037.17
[M+6H]⁶⁺ = 864.47
[M+7H]⁷⁺ = 741.12
[M+8H]⁸⁺ = 648.62

171

$[M+4H]^{4+}$

Calculated mass for peptide 2d:

$M$ = 5180.73
$[M+3H]^{3+}$ = 1727.95
$[M+4H]^{4+}$ = 1296.21
$[M+5H]^{5+}$ = 1037.17
$[M+6H]^{6+}$ = 864.47
$[M+7H]^{7+}$ = 741.12
$[M+8H]^{8+}$ = 648.62

$[M+5H]^{5+}$

Calculated mass for peptide 2d:

$M$ = 5180.73
$[M+3H]^{3+}$ = 1727.95
$[M+4H]^{4+}$ = 1296.21
$[M+5H]^{5+}$ = 1037.17
$[M+6H]^{6+}$ = 864.47
$[M+7H]^{7+}$ = 741.12
$[M+8H]^{8+}$ = 648.62

# Chapter 4

## Application of an α-Methyl Amino Acid in Crystallographic Studies of a β-Hairpin Derived from Aβ

### INTRODUCTION

The propensity of the β-amyloid peptide, Aβ, to aggregate into fibrils poses a challenge for investigations seeking to understand the structures and properties of Aβ oligomers.[1] Further complicating studies of Aβ oligomers, is the significant variation they display in their stoichiometry and stability—presenting as a heterogenous mixture of monomer and oligomers in biophysical sizing assays. Various protocols have emerged within the literature detailing methods and procedures for the preparation of Aβ oligomers, however, even these methods cannot overcome poor quality control in the Aβ starting material.[2–4] Alternatively, chemical crosslinking of prepared Aβ oligomers has emerged as an accessible approach to reduce some heterogeneity and enable studies of stable Aβ oligomers. [5–9] However, non-specificity in chemical cross-linking sites or variation in the secondary structure of Aβ monomers can affect the properties of stabilized oligomers.

The covalent stabilization of Aβ β-hairpins has emerged as a strategy to increase the homogeneity and stability of Aβ oligomers.[10–13] β-hairpins are as a key structural element of Aβ oligomers.[10–12,14,15] Härd, Hoyer, and co-workers elucidated the NMR structure of an Aβ β-hairpin where residues 17–23 and 30–36 hydrogen bond to form an antiparallel β-sheet, with residues 24–

29 forming a loop, and the remaining N- and C-terminal residues left as unstructured segments.[10] In subsequent studies, Härd, and co-workers reported that a disulfide stabilized homologue of the Aβ β-hairpin that they had previously reported, mimicked properties of endogenous Aβ oligomers.[11] They further reported that this covalently stabilized β-hairpin assembled to form a β-barrel like hexamer, stabilized by edge-to-edge hydrogen bonding and hydrophobic packing.[12]

More recently, Carulla and co-workers recently reported the NMR structure of an Aβ$_{42}$ tetramer, comprising a six-stranded antiparallel β-sheet formed by two β-hairpins of Aβ$_{42}$ surrounding two antiparallel β-strands of Aβ$_{42}$.[15] In this tetramer structure, the β-hairpins are formed by residues 9–21 and 28–40 hydrogen bonding to form an antiparallel β-sheet, with residues 22–27 forming a loop, and the remaining N- and C-terminal residues left as unstructured segments. To study the stoichiometry and properties of this tetramer in other biophysical assays, Carulla and co-workers subjected them to chemical crosslinking across the acid side-chains of Asp and Glu.[16]

To reduce heterogeneity and facilitate studies of oligomers formed by Aβ derived peptides, the Nowick laboratory has developed macrocyclic β-hairpin peptides as synthetic tools to mimic endogenous oligomers **(Figure 4.1)**.[17,18] These peptides typically comprise two heptapeptide β-strand segments derived from Aβ that are covalently linked by two $^{\delta}$Orn turn units that constrain the peptide to a macrocycle. To prevent uncontrolled aggregation, we incorporate an *N*-methyl amino acid on one strand of the macrocycle. These model systems limit structural heterogeneity and provide a measure of preorganization that primes the peptide to assemble and form oligomers that can be studied using X-ray crystallography.[19–26]

In 2014, we reported the X-ray crystallographic structure of triangular trimers and ball-shaped dodecamers formed by peptide **1 (Figure 4.1)**. Peptide **1** comprises two β-strands comprising Aβ residues 17–23 and 30–36, two $^{\delta}$Orn turn units that constrain the peptide to a

macrocycle, and an *N*-methyl group on Phe$_{20}$. To improve solubility, Met$_{35}$ is replaced by an α-linked Orn. Using X-ray crystallography, we observed three monomers of peptide **1** assembling to form a triangular trimer that is stabilized by edge-to-edge hydrogen bonding between monomers **(Figure 4.2)**. Within the crystal lattice we also observed the assembly of a ball-shaped dodecamer, stabilized by the hydrophobic packing of four triangular trimers.

In the crystallographic structure of the triangular trimer formed by peptide **1**, three *N*-methyl groups on Phe$_{20}$ project inwards, towards the center of the trimer (magenta) **(Figure 4.2)**. The methyl groups prevent the formation of three additional intermolecular hydrogen bonding interactions between three pairs of backbone amides and carbonyl oxygens that would be present with the native Phe$_{20}$ residue. In the same investigation, we also studied the crystallographic assembly of a structural homologue of peptide **1** that bore an *N*-methyl group on Gly$_{33}$ instead of Phe$_{20}$. Even with the methyl group on the other strand of the macrocycle, this homologue still assembled to form triangular trimers that were nearly identical to those formed by peptide **1**. Further, this homologue was further stabilized by three additional hydrogen bonds with coordinating waters at the center of the trimer, that were absent in the structure of peptide **1**.
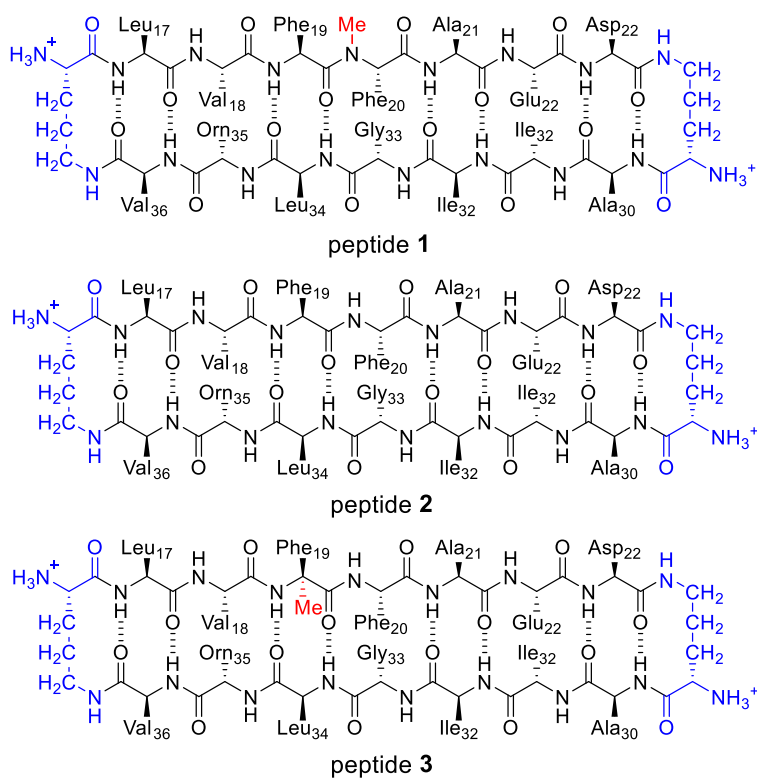
Figure **4.1**. Chemical structures of macrocyclic β-hairpin peptides derived from Aβ$_{17-23}$ and Aβ$_{30-36}$ with $^{\delta}$Orn turn units (blue) and methyl groups (red). Peptide **1** contains an *N*-methyl group on Phe$_{20}$. Peptide **2** does not contain a methyl group. Peptide **3** contains an α-methyl group on Phe$_{19}$.
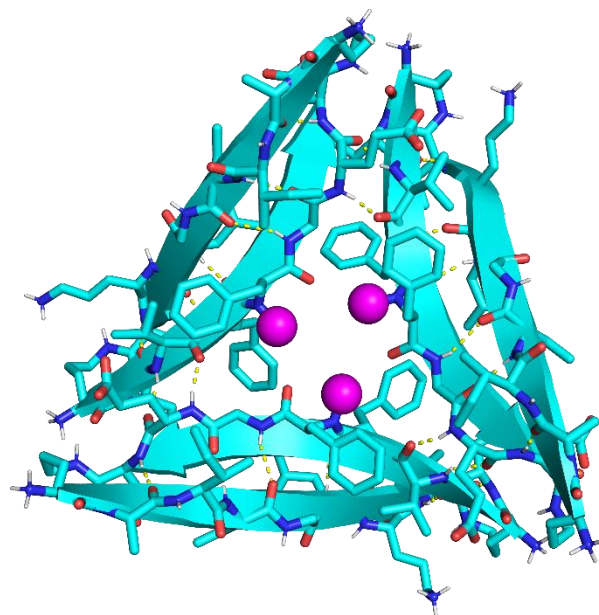


Figure **4.2**. X-ray crystallographic structure of a triangular trimer formed by peptide **1**, (PDB 4NW9).

Although the location of the *N*-methyl group does not affect the crystallographic assembly of a macrocyclic β-hairpin peptide that comprises Aβ residues 17–23 and 30–36, the presence of the *N*-methyl group does alter the hydrogen bonding edge of the macrocycle. Interactions at the hydrogen bonding edges of β-hairpins are an important facet of Aβ oligomerization. Restoring the hydrogen bonding interactions lost due to the incorporation of an *N*-methyl group on Phe$_{20}$ may alter the assembly of peptide **1**, and better mimic endogenous Aβ oligomers.

In this project, we set out to restore three intermolecular hydrogen bonds absent in the crystal structure of in peptide **1** and develop a model system that more closely mimics endogenous Aβ oligomers. **(Figure 4.1)**. We initially attempted to remove the methyl group entirely, furnishing peptide **2**. However, while peptide **2** does show evidence of oligomerization, it does not crystallize. We then turned toward incorporating an α-methyl group in the top strand of peptide **1**, in place of an *N*-methyl group, furnishing peptide **3**. We were gratified to discover that like peptide **1**, peptide **3** crystallizes, assembling to form the same triangular trimers and ball-shaped dodecamers as peptide **1**.

## RESULTS AND DISCUSSION

*Oligomerization of peptides 1–3*

Peptides **1** and **3** do not self-assemble by SDS-PAGE, migrating as single bands to just below the 4.6 kDa ladder band **(Figure 4.3)**. Peptide **2b** does self-assemble, migrating as a heterogeneous mixture of oligomers with molecular weights that correspond to trimer, tetramer, and pentamer. The difference in self-assembly between peptides **1** and **3,** and peptide **2** is likely due to the presence of the methyl blocking groups. This result shows that the α-methyl group on Phe$_{19}$ in peptide **3** can still block assembly, while allowing peptide **3** the opportunity to form the intermolecular hydrogen bonds absent in the crystal structure of peptide **1**.
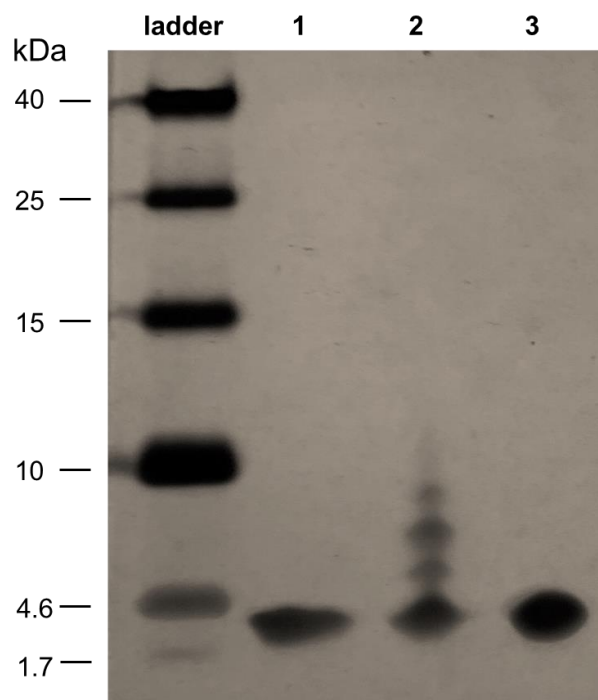


Figure **4.3**. Silver stained SDS-PAGE of peptides **1–3**. SDS-PAGE was performed in Tris buffer at pH 6.8 with 2% (w/v) SDS on a 16% polyacrylamide gel with 50 $\mu$M solutions of peptide in each lane.

*X-ray crystallographic studies of peptide 3*

      X-ray crystallography reveals that peptide **3** folds to adopt a twisted β-hairpin, stabilized by the same intramolecular hydrogen bonds that stabilize the residues 17–22 and 30–36 in peptide **1 (Figure 4.4)**. The methyl groups (violet) extend away from the β-hairpins formed by peptides **1** and **3**. An overlay of peptides **1** and **3** reveals that the monomers do not differ significantly in their structure. Molecular replacement, using a search model derived from the crystallographic structure of the trimer formed by peptide **1** (PDB 5SUR), was used to determine the X-ray crystallographic phases of peptide **3** Supplementary table 4.1 summarizes the crystallographic properties, crystallization conditions, data collection, and model refinement statistics for peptide **3**.
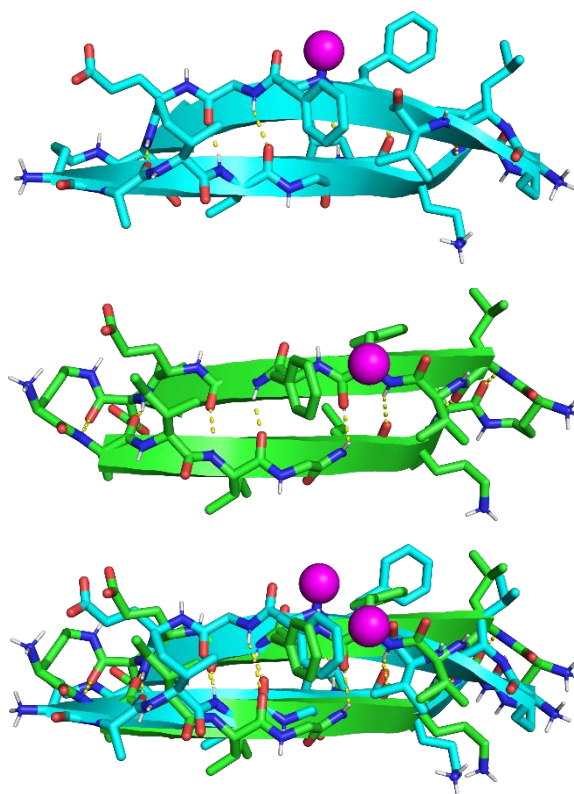


Figure **4.4**. X-ray crystallographic structure of monomer formed by peptides **1** (blue) and **3** (green) and overlay.

      Like peptide **1**, peptide **3** assembles further in the crystal lattice, forming triangular trimers and ball shaped dodecamers **(Figure 4.5** and **4.6)**. The triangular trimers formed by peptide **3**

assemble through edge-to-edge hydrogen bonding interactions and the packing of hydrophobic residues at one face of the trimer. The α-methyl groups on Phe$_{19}$ are accommodated at the center of the trimer. Unlike peptide **1b,** the backbone amide and carbonyl oxygens of Phe$_{20}$ in peptide **3** can form hydrogen bonds. Three additional hydrogen bonds are observed to form at the center of the triangular trimer formed by peptide **3**. Further, the triangular trimer formed by peptide **3** is similar but not identical to the trimer formed by peptide **1 (Figure 4.5)**. We speculate that the presence of these three additional hydrogen bonds causes a shift in the edge-to-edge registration of monomers in the trimer. Like peptide **1**, the triangular trimers formed by peptide **3** can assemble to form a ball-shaped dodecamer **(Figure 4.6)**.
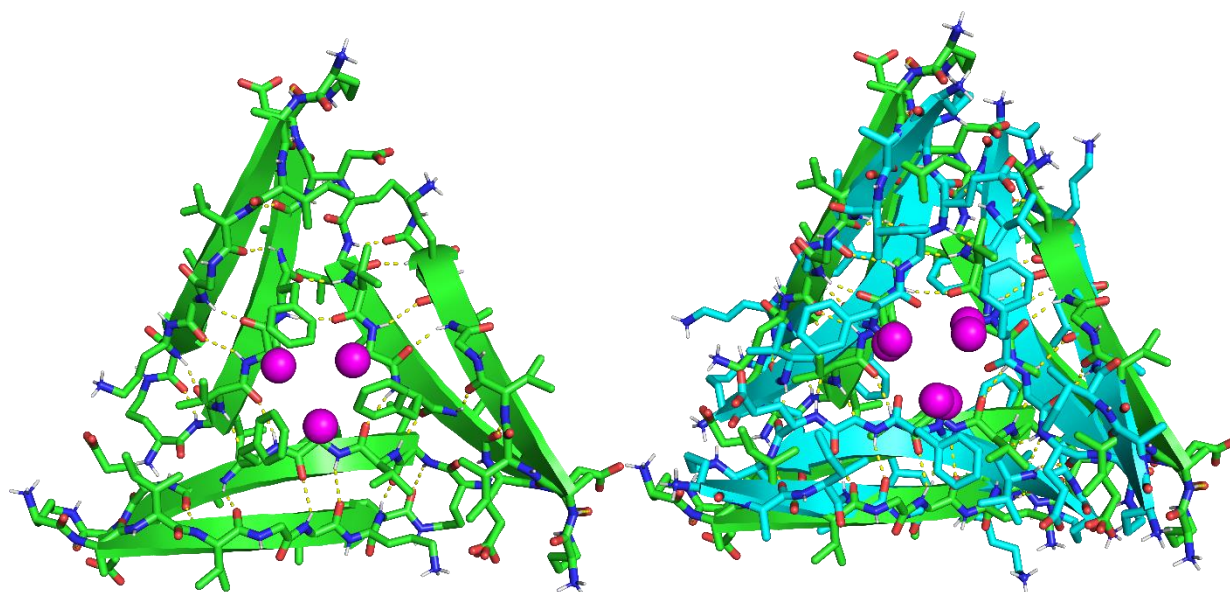


Figure **4.5**. X-ray crystallographic structure of a triangular trimer formed by peptide **3** (green) and overlay with trimer formed by peptide **1** (blue: PDB 4NW9).
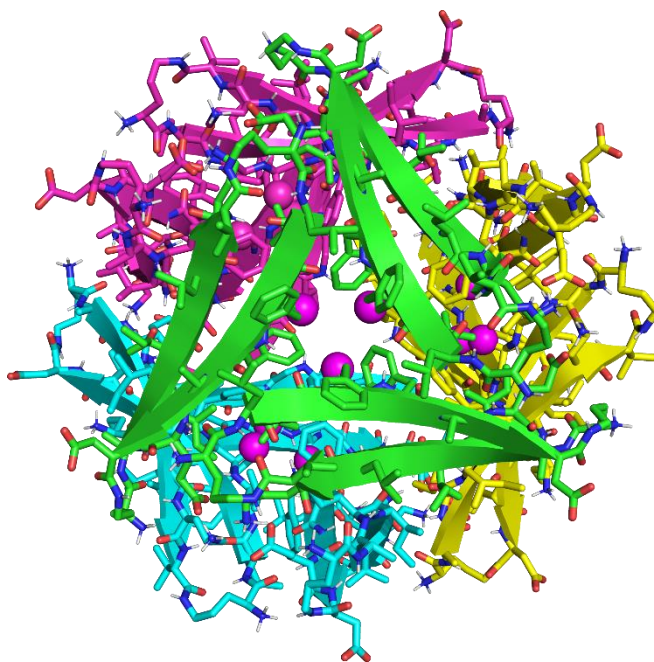
Figure **4.6**. X-ray crystallographic structure of the ball-shaped dodecamer formed by peptide **3**.

**CONCLUSION**

Differences in the assembly of peptides **1–3** reveals that the methyl group is necessary for crystallographic assembly. Replacement of the *N*-methyl blocking group with an *α*-methyl blocking group prevents uncontrolled aggregation and facilitates the formation of additional intermolecular hydrogen bonds that stabilize and slightly alter the assembly of macrocyclic β-hairpins derived from Aβ residues 17–22 and 30–36. This replacement allows our model systems to more closely mimic β-hairpins formed by unmodified Aβ, and possibly better mimic the oligomers they form in the Alzheimer's brain.

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Supporting information for:

**Application of an α-Methyl Amino Acid in Crystallographic Studies of a β-Hairpin Derived from Aβ**

## **Table of Contents**

# Supporting Figures and Tables

**Supplementary Table 4.1**. Crystallographic properties, crystallization conditions, data collection, and model refinement statistics for peptide **3**.

| peptide | **3** |
|---|---|
| Wavelength | 0.7293 |
| Resolution range | 24.09 -1.001 (1.037 -1.001) |
| Space group | F 2 3 |
| Unit cell | 83.447, 83.447, 83.447 90, 90, 90 |
| Total reflections | 1018181 (99256) |
| Unique reflections | 25968 (2555) |
| Multiplicity | 39.2 (38.8) |
| Completeness (%) | 99.72 (97.86) |
| Mean I/sigma(I) | 25.97 (0.75) |
| Wilson B-factor | 13.38 |
| R-merge | 0.06135 (6.279) |
| R-meas | 0.0622 (6.361) |
| R-pim | 0.01009 (1.014) |
| CC1/2 | 1 (0.297) |
| CC* | 1 (0.677) |
| Reflections used in refinement | 25917 (2519) |
| Reflections used for R-free | 1308 (139) |
| R-work | 0.2034 (0.3619) |
| R-free | 0.2240 (0.3662) |
| CC(work) | 0.951 (0.580) |
| CC(free) | 0.908 (0.496) |
| RMS(bonds) | 0.015 |
| RMS(angles) | 1.81 |
| Ramachandran favored (%) | 100.00 |
| Ramachandran allowed (%) | 0.00 |
| Ramachandran outliers (%) | 0.00 |
| Rotamer outliers (%) | 0.00 |
| Clashscore | 0.00 |
| Average B-factor | 16.35 |
| macromolecules | 15.47 |
| ligands | 17.22 |
| solvent | 22.45 |
| Number of TLS groups | 20 |
| crystallization conditions | 0.1 M HEPES buffer (pH 6.5), 24% (v/v) Jeffamine M-600 |

# Materials and Methods[1]

*General information*

All chemicals were used as received unless otherwise noted. Methylene chloride ($CH_2Cl_2$) was passed through alumina under nitrogen prior to use. Anhydrous, amine-free *N,N*-dimethylfomamide (DMF) was purchased from Alfa Aesar. Deionized water (18 MΩ) was obtained from a Barnstead NANOpure Diamond water purification system. Analytical reverse-phase HPLC was performed on an Agilent 1260 Infinity II instrument equipped with a Phenomonex Aeris PEPTIDE 2.6u XB-C18 column. Preparative reverse-phase HPLC was performed on a Ranin instrument equipped with an Agilent Zorbax SB-C18 column. HPLC grade acetonitrile and deionized water, each containing 0.1% trifluoracetic acid (TFA), were used for analytical and preparative reverse-phase HPLC. All peptides were prepared and used as the trifluoroacetate salts and were assumed to have one trifluoracetic acid molecule per amine group on each peptide.

*Synthesis of peptides* **1–3**

*a. Loading the resin.* 2-Chlorotrityl chloride resin (300 mg, 1.4 mmol/g) was added to a Bio-RAD Poly-Prep chromatography column (10 mL). Dry $CH_2Cl_2$ (8 mL) was used to suspend and swell the resin for 30 min with gentle rocking. The solution was drained from the resin and a solution of Boc-Orn-Fmoc-OH (0.78 equiv, 150 mg, 0.33 mmol) in 6% (v/v) 2,4,6-collidine in dry $CH_2Cl_2$ (8 mL) was added immediately and the suspension was gently rocked for 12 h. The solution was then drained and a mixture of $CH_2Cl_2$/MeOH/*N*,*N*-diisopropylethylamine (DIPEA) (17:2:1, 10 mL) was added immediately. The resin was gently rocked for 1 h, to cap the unreacted 2- chlorotrityl chloride resin sites. The resin was then washed twice with dry $CH_2Cl_2$ and dried by passing nitrogen through the vessel. This procedure typically yields 0.18 mmol of loaded resin (0.6 mmol/g loading).

*b. Manual peptide coupling.* The resin loaded with Boc-Orn(Fmoc) was suspended in dry DMF and then transferred to a solid-phase peptide synthesis vessel. Residues 22 through 17 were manually coupled using Fmoc-protected amino acid building blocks. Each manual coupling cycle consisted of *i*. Fmoc-deprotection with of 20% (v/v) piperidine in DMF for 5 min at ambient temperature (5 mL), *ii*. washing with dry DMF (2x, 5 mL), *iii*. coupling of the amino acid (0.44 mmol, 4 equiv) with HCTU (174.0 mg, 0.44 mmol, 4 equiv) in 20% (v/v) 2,4,6-collidine in dry DMF (5 mL) for 30 min, and *iv*. washing the with dry DMF (2x, 5 mL). Residue 19, which follows N-Me-Phe$_{20}$ in peptide **1**, and residue 18, which follows α-Me-Phe$_{19}$ in peptide **3**, were double coupled (4 equiv per coupling) using HATU (6 equiv) and HOAt (6 equiv) for 1 hr per coupling to ensure complete reaction. (We have found that coupling after *N*- and α -methyl amino acids is difficult and requires rigorous coupling to minimize incomplete reaction.[2])

*c. Microwave-assisted coupling of residues 36 through 30.* A CEM Liberty Blue Automated Microwave Peptide Synthesizer was used to couple residues 36 to 30. Fmoc-Orn(Dde)-OH

connects residues 17 and 36 in the natural sequence. Each coupling cycle consisted of *i*. Fmoc-deprotection with 20% (v/v) piperidine with 0.1 M Oxyma Pure in DMF for 2 min. at 50 ºC, *ii*. washing with DMF (3x), *iii*. coupling of the amino acid (0.75 mmol, 5 equiv) in the presence of HCTU (0.675 mmol, 4.5 equiv) and 20% (v/v/) *N*-methylmorpholine (NMM) in DMF for 10 min. at 50 ºC, iv. washing with DMF (3x).

*d*. *Cleavage of the peptide from resin*. The resin was then transferred to a 10-mL Bio-Rad Poly-Prep chromatography column, and washed 3x with dry $CH_2Cl_2$. The acyclic peptide was cleaved from the resin by rocking the resin for 1 h with a solution of 1,1,1,3,3,3-hexafluoroispropanol (HFIP) in $CH_2Cl_2$ (1:4, 8 mL). The suspension was filtered and the filtrate was collected in a 250-mL round-bottomed flask. The resin was washed with additional HFIP in $CH_2Cl_2$ (1:4, 8 mL). The combined filtrates were concentrated by rotary evaporation to give a white solid. The white solid was further dried by vacuum pump to afford the crude protected linear peptide, which was cyclized without further purification.

*e*. *Cyclization of the acyclic peptide*. The crude protected linear peptide was dissolved in dry DMF (150 mL). PyBOP (370 mg, 0.711 mmol, 6 equiv) and *N*-methylmorpholine (NMM) (0.33 mL, 1.8 mmol, 12 equiv) was added to the solution and the mixture was stirred under nitrogen for 48 h. The mixture was concentrated under reduced pressure to afford the crude protected cyclic peptide, a yellow film.

*f*. *Global deprotection of the cyclic peptide*. The protected cyclic peptide was dissolved in TFA/triisoproylsilane (TIPS)/$H_2O$ (18:1:1, 10 mL) in a 250-mL round-bottomed flask equipped with a nitrogen-inlet adaptor. The solution was stirred for 1 h under nitrogen. The reaction mixture was then concentrated by rotary evaporation under reduced pressure to afford the crude cyclic peptide. During the 1 h deprotection, two 50-mL conical tubes containing 40-mL of dry ether were chilled on dry ice. The solution was drained and split between the two conical tubes of ether. The

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tubes were then centrifuged at 800 x g for 25 mins. The ether supernatant was poured off and the pelleted peptide was dried overnight under vacuum.

*g*. *Reverse-phase HPLC purification.* The peptide: DMSO mixture was dissolved in $H_2O$ and acetonitrile (8:2, 5 mL), and the solution was filtered through a 0.2 μm syringe filter and purified by RP-HPLC. The solution of crude peptide was injected at 20% acetonitrile and eluted with a gradient of 20-60% $CH_3CN$ over 90 min. Each peptide eluted between 29-36% $CH_3CN$. The collected fractions were analyzed by analytical HPLC and MALDI-TOF, and the pure fractions were concentrated by rotary evaporation and lyophilized. Typical syntheses yielded between 48 and 52 mgs of the peptide as the TFA salt.

*SDS-PAGE, and silver staining*

SDS-PAGE was performed on peptides **1–3** using the reagents, recipes, and procedures for Tricine SDS-PAGE detailed in Schägger, H. *Nat. Protoc.* **2006**, *1*, 16–22. Each peptide was run on a 16% polyacrylamide gel with a 4% stacking polyacrylamide gel at 60 volts. A Spectra™ Multicolor Low Range Protein Ladder (ThermoFischer Scientific, catalog #: 26628) was loaded into the first lane of the gel. The remaining lanes were loaded with 8.0 µL aliquots of each peptide as 50 µM solutions in SDS-PAGE loading buffer, which were prepared as follows: A 10 mg/mL stock solution of each peptide was prepared with deionized water. Aliquots of the 10 mg/mL solutions were then diluted further with deionized water and 6X SDS-PAGE loading buffer (G-Biosciences catalog #: 786-701) to create 100 µM working solutions of each peptide.

Staining with silver nitrate was used to visualize peptides **1–3** in the SDS-PAGE gel. Reagents for silver staining were prepared according to procedures detailed in Simpson, R. J. *Cold Spring Harbor Protocol* **2007**. [We have found it important to prepare sodium thiosulfate, silver nitrate, and developing solutions fresh each time and to use high purity sodium carbonate to prepare the developing solution.] The gel was removed from the casting glass and rocked for 20 min in fixing solution (50% (v/v) methanol and 5% (v/v) acetic acid in deionized water). The fixing solution was then discarded and replaced with 50% (v/v) aqueous methanol for another 10 min of rocking. Next, the 50% methanol was discarded and replaced with deionized water for another 10 min of rocking. Next, the water was discarded and the gel was rocked in 0.02% (w/v) sodium thiosulfate in deionized water for 1 min. The sodium thiosulfate was discarded and the gel was rinsed twice with deionized water for 0.5 min. The gel was then submerged in pre-chilled 0.1% (w/v) silver nitrate in deionized water and rocked at 4 ºC for 20 min. The silver nitrate solution was discarded and the gel was rinsed twice with deionized water. The gel was incubated in developing solution (2% (w/v) sodium carbonate, 0.04% (w/v) formaldehyde) until the solution began to brown. The developing solution was then immediately discarded and fresh silver nitrate

solution was added to the gel until the desired intensity of staining was reached. When the desired intensity of staining was reached, the developing solution was discarded and the gel was submerged in 5% aqueous acetic acid.

*Crystallization of peptide 3*

The hanging-drop vapor-diffusion method was used to determine initial crystallization conditions for peptide **3**. Peptide **3** was screened in 96-well plate format using three crystallization kits (Crystal Screen, Index, and PEG/ION) from Hampton Research. A TTP LabTech Mosquito nanondisperse was used to make three 150 nL hanging drops for each well condition. The three hanging drops differed in the ratio of peptide to well solution for each condition in the 96-well plate. A 10 mg/mL solution of each peptide in deionized water was combined with a well solution in ratios of 1:1, 1:2, and 2:1 peptide:well solution at appropriate volumes to create the three 150 nL hanging drops. Crystals of peptide **3** grew in well conditions of 0.1 M HEPES buffer (pH 6.5), and 24% (v/v) Jeffamine M-600.

Crystallization conditions for each peptide were optimized using a 4x6 matrix Hampton 24-well plate. For peptide **3** the 0.1 M HEPES buffer was varied in each row in increments of 0.5 pH units (6.0, 6.5, 7.0, and 7.5) and the percentage of Jeffamine M-600 was varied in each column in increments of 2% (v/v) (26%, 28%, 30%, 32%, 34%, 36%). Three hanging-drops were prepared on borosilicate glass slides by combing a 10 mg/mL solution of peptide **3** in deionized water with the well solution in the following amounts: 1 μL:1 μL, 2 μL:1 μL, and 1 μL:2 μL. Slides were inverted and pressed firmly against the silicone grease surrounding each well. Crystals were harvested with a nylon loop attached to a copper or steel pin, soaked briefly in MPD, and flash frozen in liquid nitrogen prior to data collection. The optimized crystallization conditions for peptide **3** is summarized Supplementary Table 4.1.

*X-ray crystallographic data collection, data processing, and structure determination of peptide 3*
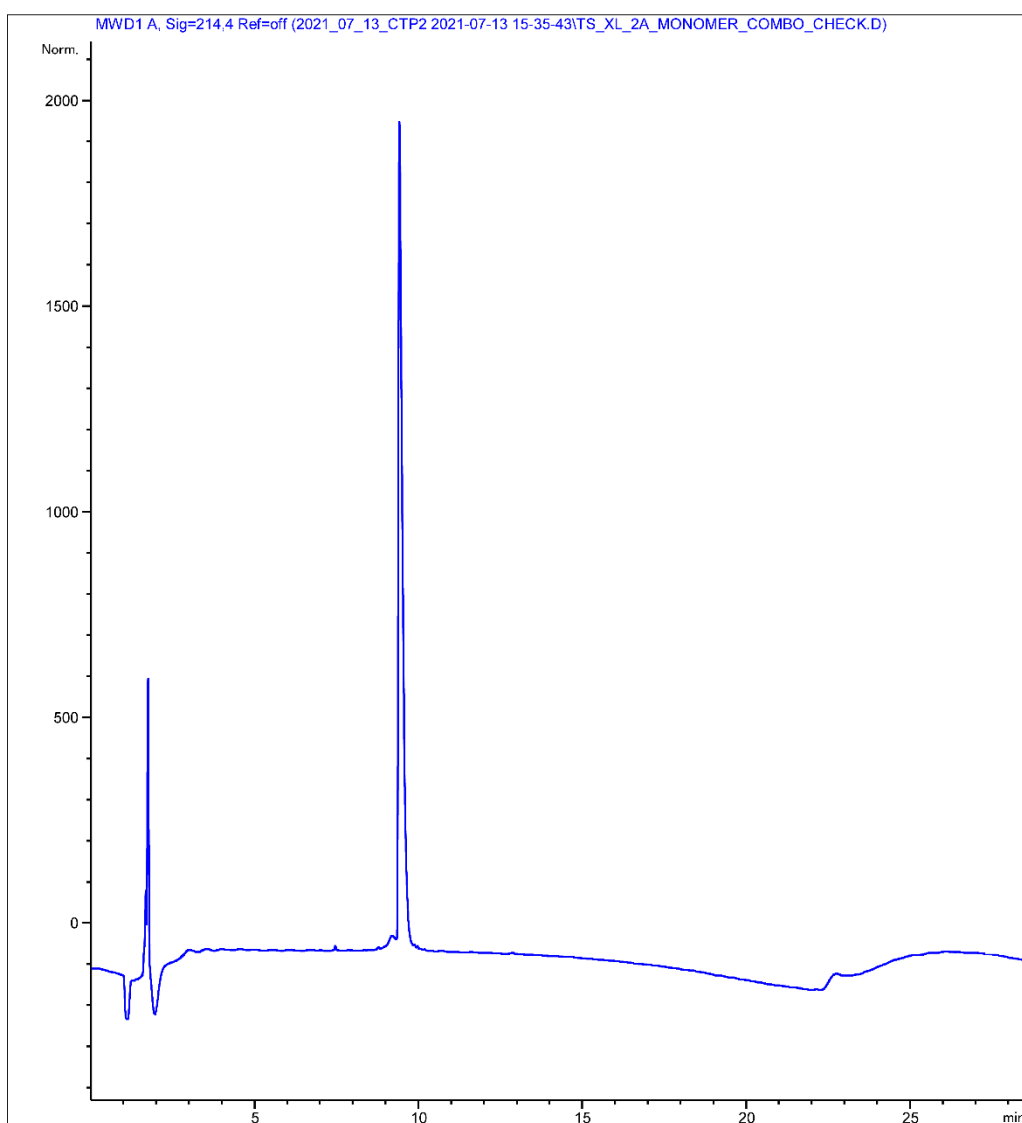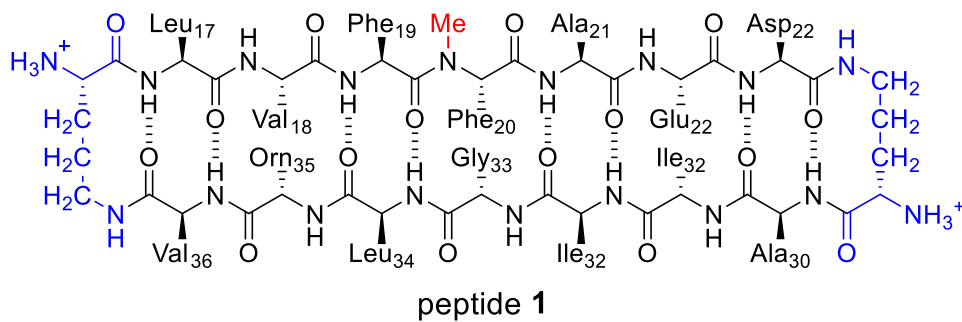
Diffraction data for **3** were collected at the Stanford Synchrotron Radiation Light source, beamline 12.2, at 1.00 Å wavelength. Datasets were indexed and integrated with XDS. Scaling and merging was done with pointless and aimless in CCP4. The structures were solved with molecular replacement in Phaser using a trimer formed by macrocyclic β-hairpin peptide derived from Aβ$_{17-36}$ (PDB 5SUR) as the search model. The refinement was done with phenix.refine module of the Phenix suite, with manipulation of the model performed using Coot.
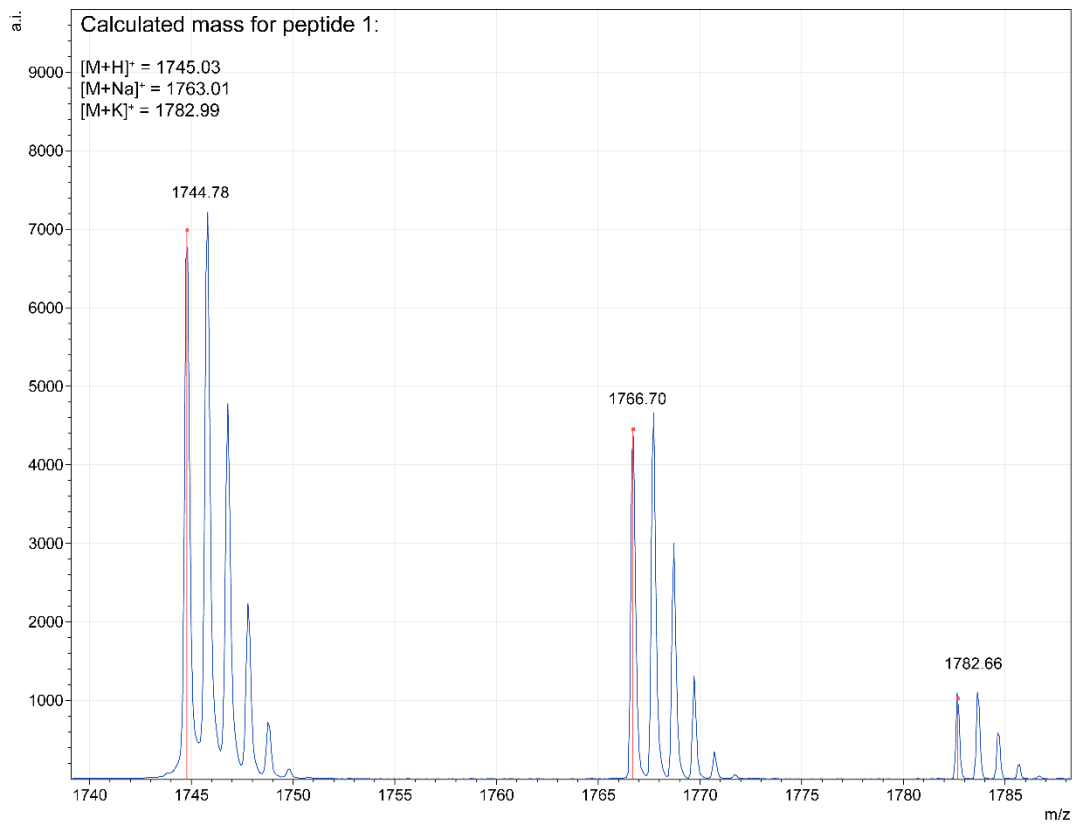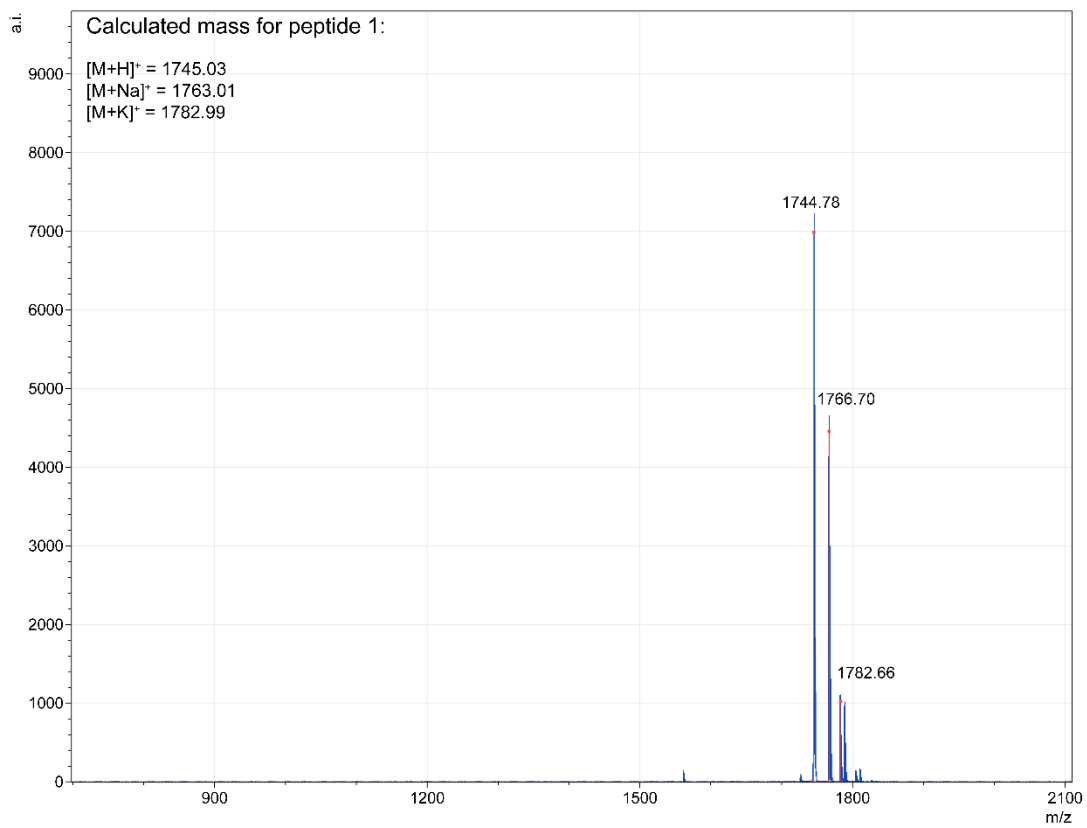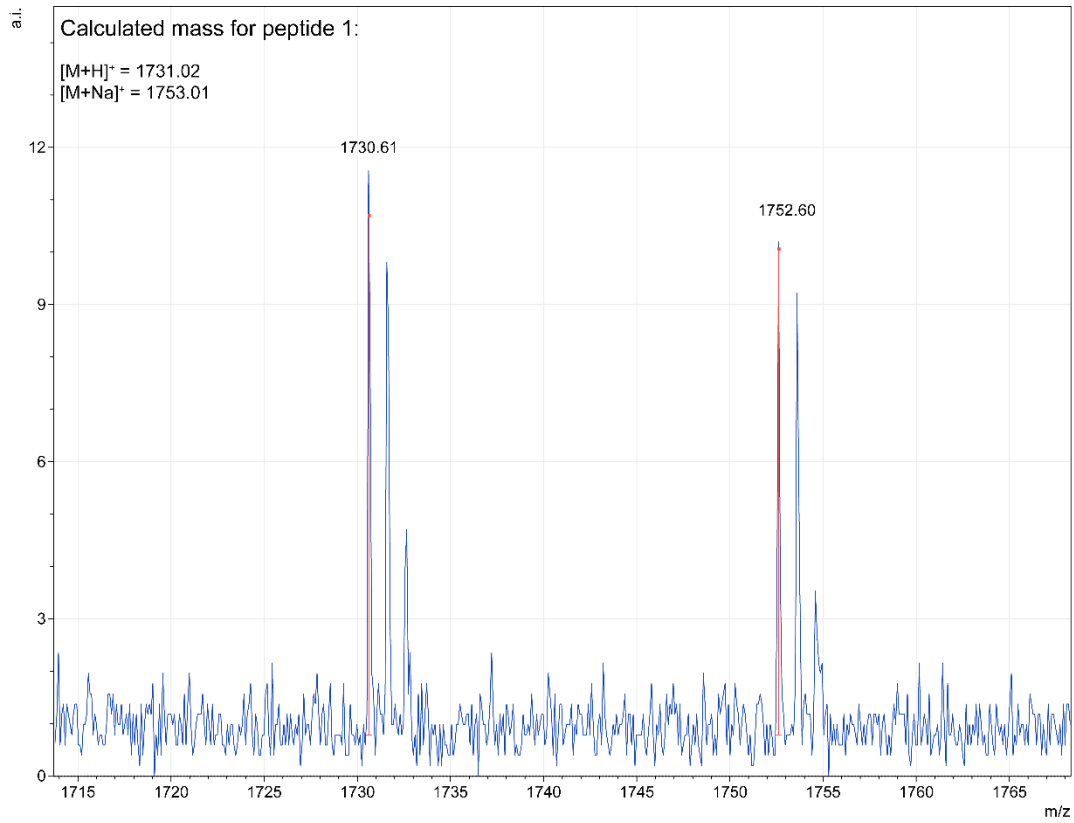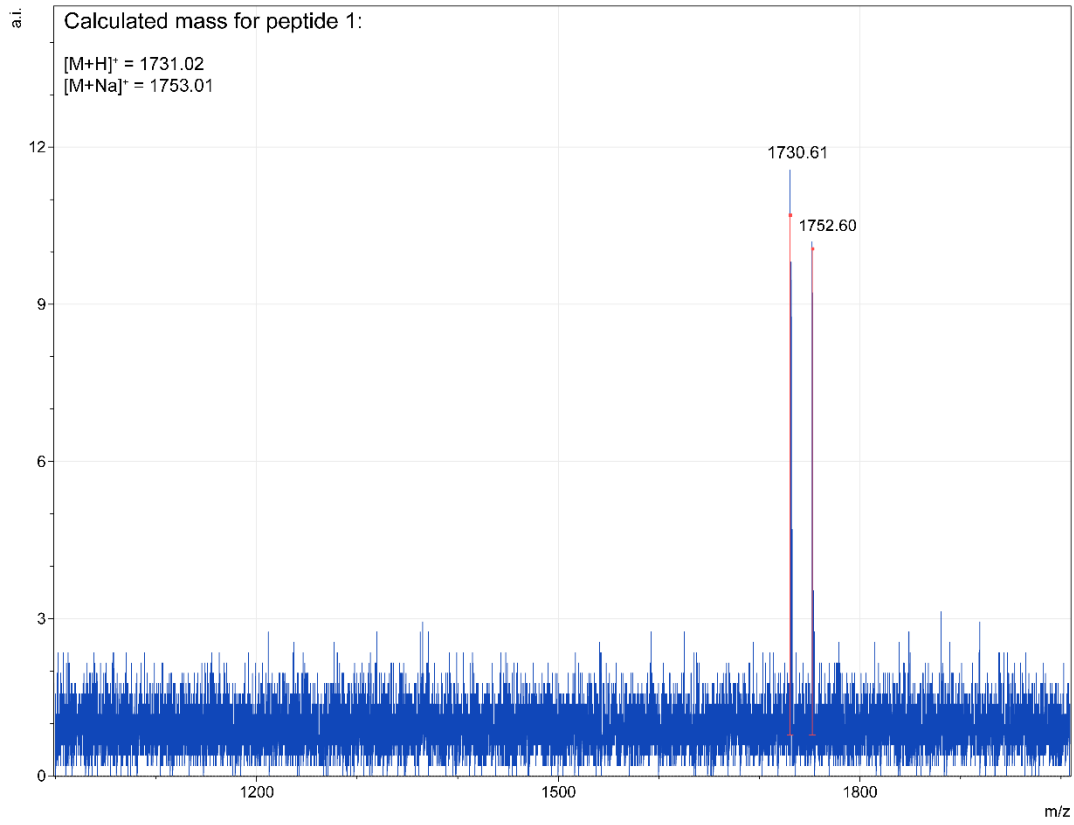
## References

1. These procedures follow closely those that our laboratory has previously published. The procedures in this section are adapted from and in some cases taken verbatim from Kreutzer, A. G.; Hamza, I. L.; Spencer, R. K.; Nowick J. S. *J. Am. Chem. Soc.* **2016**, *138*, 4634–4642, Spencer, R. K.; Kreutzer, A. G.; Salveson, P. J.; Li, H.; Nowick, J. S. *J. Am. Chem. Soc.* **2015**, *137*, 6304–6311, Spencer, R. K.; Li. H.; Nowick, J. S. *J. Am. Chem. Soc.* **2014**, *136*, 5595– 5598, and Kreutzer, A. G.; Yoo, S.; Spencer, R. K.; Nowick, J. S. *J. Am. Chem, Soc.* **2017**, *139*, 966–975, Samdin, T. D.; Wierzbicki, M.; Kreutzer, A. G.; Howitz, W. J.; Valenzuela, M.; Smith, A.; Sahrai, V.; Truex, N. L.; Klun, M.;Nowick, J. S. *J. Am. Chem. Soc*. **2020**, *142*, 11593–11601.

# Characterization Data

*Characterization of peptide **1***



peptide **1**

Calculated mass for peptide 1:

$[M+H]^+$ = 1745.03
$[M+Na]^+$ = 1763.01
$[M+K]^+$ = 1782.99

1744.78

1766.70

1782.66

Calculated mass for peptide 1:

$[M+H]^+$ = 1745.03
$[M+Na]^+$ = 1763.01
$[M+K]^+$ = 1782.99

1744.78

1766.70

1782.66

peptide **2**



MWD1 A, Sig=214,4 Ref=off (2021_06_30_ts_2 2021-06-30 12-06-54\TS_III_LV_no_methyl_combo.D)

peptide **3**