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Understanding and Designing Head-to-Tail Cyclic Peptides

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Abstract

Cyclic peptides are an exciting class of molecules with a variety of applications. However, design strategies for cyclic peptide therapeutics, for example, are generally limited by a poor understanding of their sequence–structure relationships. This knowledge gap often leads to a trial-and-error approach for designing cyclic peptides for a specific purpose, which is both costly and time-consuming. Herein, we describe the current experimental and computational efforts in understanding and designing head-to-tail cyclic peptides along with their respective challenges. In addition, we provide several future directions in the field of computational cyclic peptide design to improve its accuracy, efficiency and applicability. These advances, combined with experimental techniques, shall ultimately provide a better understanding of these interesting molecules and a reliable working platform to rationally design cyclic peptides with desired characteristics.

Introduction

Cyclic peptides (CPs) are a unique class of molecules with interesting properties. Notably, CPs have been developed for various nanotechnologies, including alternatives to carbon nanotubes,¹ transmembrane transport channels² and artificial photosynthetic systems.³ Additionally, CPs have emerged as important players in drug development.^{4–6} CPs have been shown to modulate protein–protein interactions with high affinity and specificity^{7–10} and act as potent antimicrobial peptides by disrupting cell membranes.^{11–15} On one hand, design principles of CPs for applications such as self-assembling nanotubes are reasonably well formulated.¹⁶ For example, the internal diameter of CP nanotubes can be modulated by the number of residues used and typically contain alternating D- and L-amino acids.^{1,17,18} The properties of the nanotubes can be tuned through incorporation of canonical as well as non-canonical amino acids to achieve a variety of functionalities.¹⁶ On the other hand, many of the current CP protein–protein interaction modulators and antimicrobial peptides are natural products or their derivatives.^{4,19} Their rational design remains rare, owing to our inability to predict CP structures *de novo* and our poor understanding of CP sequence–structure relationships. Consequently, the full potential of CPs is currently severely underexplored.

When designing novel CPs, numerous components must be considered. For example, various cyclization strategies are available, including head-to-tail,²⁰ side-chain to side-chain^{21,22} and side-chain to terminus bonding.^{23,24} Recently, ring expansion has been shown

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as a promising alternative to head-to-tail cyclization, mitigating the entropic barriers of cyclization using the more traditional method.^{25–30} Multiple cyclizations can also be incorporated to generate bicyclic peptides, tricyclic peptides, etc., where these additional cyclizations can provide further restraints to rigidify the peptides and afford an even greater complexity of design space.^{31–34} In this review we will focus on head-to-tail cyclized peptides. In addition to the canonical amino acids, *N*-methylated amino acids, D-amino acids, β -amino acids and other non-natural amino acids can be implemented to optimize CP properties. With many variables to consider and compounded by synthetic challenges, experimentally generating, testing and analyzing large CP libraries can be arduous and time consuming. Furthermore, CPs typically form structural ensembles in solution rather than adopting one single dominant conformation.^{35–46} This feature makes their experimental structural characterization particularly difficult and understanding how their sequences govern their conformations using a purely experimental approach almost impossible.

While using merely brute force synthesis and experimental structural characterization to understand and design CPs is challenging and likely intractable, the use of computational methods offers a promising and complementary alternative. In this review, we will provide an overview of previous experimental approaches to understand and design CPs as well as the challenges associated with them. We will also discuss recent advances in the computational design of CPs as well as provide commentary on areas of the field where further development is needed to ensure accurate and efficient structure predictions of CPs.

Experimental design of cyclic peptides

Experimental approaches for CP design often utilize non-canonical amino acids alongside a variety of cyclization strategies. Herein we focus on the use of various canonical and non-canonical amino acids with head-to-tail cyclization. With a number of systematic studies, several general rules and interesting observations have been made for CPs. For example, cyclic tripeptides are generally highly strained and contain at least one *cis* peptide bond, with the most energetically favorable conformation consisting of three *cis* peptide bonds.⁴⁷ Cyclic tetrapeptides are the smallest CPs that can adopt an all-*trans* conformation,⁴⁸ although they generally adopt either an all-*cis*⁴⁹ or alternating *cis/trans* conformation.^{50–55}

Cyclic pentapeptides commonly form structures consisting of a β -turn and a tighter turn (such as a γ/γ' - or an α_R/α_L -turn; ideal dihedral angles for these turns are shown in Figure 1A) opposite the β -turn. In these CPs, there is at least one hydrogen bond in the β -turn region, while a second hydrogen bond is only formed if the tight turn is a γ or γ' -turn.^{24,56–62} In order to understand how a D-amino acid affected the conformational preference of cyclic pentapeptides, CPs containing one D- and four L-amino acids were studied. These cyclic pentapeptides preferred a conformation with a type II' β -turn + a γ/α_R tight turn, where the D-amino acid was always at the *i*+1 position of the type II' β -turn.^{58,61–63} To further explore sequence–structure relationships in cyclic pentapeptides, Gierasch *et al.* studied variants of cyclo-(GPX₁GX₂), where X₁ was D-Phe or D-Ala and X₂ was L-/D-Ala, L-Val or L-Phe.⁴² It was found that the residues PX₁ adopted a type II β -turn and the identity of the tight turn was dependent on the amino acid composition and chirality of the rest of the sequence.^{42,64–66} For example, cyclo-(GPfGA), cyclo-(GPfGV), cyclo-(GPaGF),

and cyclo-(GPfGa) all formed a type II β -turn at $^2\text{Px}^3$, but formed a α_L , α_R , γ' , and γ -turn at residue five, respectively.⁴²

While cyclic pentapeptides commonly adopt a β -turn and a tight turn, cyclic hexapeptides generally form structures consisting of two β -turns at opposite ends with two intra-peptide hydrogen bonds. Much like the studies of cyclic pentapeptides, Gierasch *et al.* performed a systematic study of cyclo-($\text{X}_1\text{PX}_2\text{X}_3\text{PX}_4$) cyclic hexapeptides, where X's were various combinations of Gly, L/D-Ala, L/D-Phe or L/D-Val.⁶⁷ From their experiments, three trends were established: (1) PG or Px (x indicates a D-amino acid) in a cyclic hexapeptide preferred a type II β -turn, where the Pro residue was at the $i+1$ position of the β -turn, (2) PX in a cyclic hexapeptide containing GPX, where X = L-Val, formed a type I β -turn, where the Pro residue was at the $i+1$ position of the β -turn and (3) GP or xP in a cyclic hexapeptide containing GPV or xPX formed a type II' β -turn, where the Pro residue was at the $i+2$ position of the β -turn.

Besides D-amino acids, β -amino acids can be incorporated into a CP to induce desired turn types.^{68–75} For example, by incorporating two β -Ala (A_β) residues into cyclic tetrapeptides at different locations, Pavone *et al.* were able to control what types of turns those CPs would adopt.^{68,70,71} When the two A_β residues were placed next to each other, the two consecutive A_β residues tended to form a hydrogen bond in a C_{10} -like structure, a 10-membered ring stabilized by a hydrogen bond (Figure 1B), allowing for the remaining two α -amino acids in the tetrapeptide to assume a β -turn type conformation. For example, the conformation of cyclo-($\text{PFA}_\beta\text{A}_\beta$) formed a type I β -turn at residues $^1\text{PF}^2$ and a C_{10} -like structure for the two A_β residues opposite the β -turn.⁶⁸ On the other hand, if the two A_β residues were incorporated non-consecutively, then γ' -turns were observed for the remaining two α -amino acids. For example, in both cyclo-($\text{PA}_\beta\text{PA}_\beta$) and cyclo-($\text{PA}_\beta\text{VA}_\beta$), two γ' -turns were stabilized at the two α -amino acids.^{70,71} Therefore, the strategic placement of A_β residues into a cyclic tetrapeptide can significantly impact the CP's conformation.

Pavone *et al.* further performed similar types of studies for cyclic penta- and hexapeptides. For cyclic pentapeptides, they found that two consecutive A_β residues could lead to different structural motifs.^{69,74,75} For example, cyclo-($\text{PPFA}_\beta\text{A}_\beta$) was found to adopt a type VIa1 β -turn at $^1\text{PP}^2$, with a *cis* peptide bond between the two Pro residues (see Figure 1A for the ideal dihedral angles of a type VIa1 β -turn).⁶⁹ In this conformation, the type VIa1 β -turn at $^1\text{PP}^2$ adopted a C_{10} -like structure, while $^3\text{FA}_\beta\text{A}_\beta^5$ adopted a C_{13} -like structure (Figure 1B). On the other hand, cyclo-($\text{PFFA}_\beta\text{A}_\beta$) adopted an enlarged type II β -turn at $^3\text{FA}_\beta^4$ and a *cis* peptide bond at $^5\text{A}_\beta\text{P}^1$.⁷⁴ In this CP, residues $^3\text{FA}_\beta^4$ formed a C_{11} -like structure, while $^5\text{A}_\beta\text{P}^1$ formed a C_{12} -like structure (Figure 1B). For cyclic hexapeptides, it was found that A_β residues have a low propensity to participate in forming a β -turn.^{72,73} For example, cyclo-($\text{PFA}_\beta\text{FFA}_\beta$) adopted two type I β -turns at residues $^1\text{PF}^2$ and $^4\text{FF}^5$, and cyclo-($\text{PFA}_\beta\text{PFA}_\beta$) adopted two type II β -turns at each PF, and the two A_β amino acid served as the non-turning residues.^{72,73} These studies showed that β -amino acids could be used as a novel molecular tool for designing CPs with β -turns and γ -turns at specific locations.

Another example where non-canonical amino acids were incorporated into the CP design was the development of cyclo-(RGDfV) by Kessler *et al.* (lower case letters in the peptide

sequence indicate D-amino acids and underlined letters indicate *N*-methylated amino acids).⁷⁶ First, systematic studies were performed on the parent CP, cyclo-(RGDFV), where the effects of single substitutions of L- to D-amino acids on the CP structure were investigated.^{57,77} It was found that the L- to D-amino substitution at ⁴F was the most beneficial and cyclo-(RGDFV) was selective towards its binding partner (integrin $\alpha_v\beta_3$) with a 1,000 times higher activity than a linear reference peptide GRGDSPK.⁷⁶ Cyclo-(RGDFV) was then used as a template for various other modifications, including thioamide substitution,⁷⁸ incorporation of turn mimetics,⁷⁹ glycosylation,⁸⁰ synthesis of retro-inverso analogues,⁸¹ and *N*-methylation⁷⁶ in an attempt to further rigidify the peptide and improve its selectivity and activity for $\alpha_v\beta_3$. Of all these variants, *N*-methylation was found to be the most promising. In particular, cyclo-(RGDFV) demonstrated a four times higher activity for $\alpha_v\beta_3$ compared to the non-methylated cyclo-(RGDFV).⁷⁶ These systematic studies showed how incorporation of one or multiple non-canonical amino acids can be utilized to optimize the selectivity and activity of CPs.

N-methylation has also been shown as a promising way of improving the oral bioavailability of CPs, an important pharmacokinetic property of therapeutics. In general, cyclization of small peptides tends to result in increased bioavailability,^{4,82,83} which can be further improved upon by incorporating *N*-methylated residue(s), as demonstrated in the cases of somatostatin and the melanocortin family.^{84,85} To shed more light on how *N*-methylation affects CP conformation and bioavailability, libraries of *N*-methylated CPs were synthesized and characterized.^{46,85–92} For example, Beck *et al.* characterized 54 *N*-methylated derivatives of cyclo-(aAAAAA) to understand how *N*-methylation affected the CP's membrane permeability as well as its conformational preferences.⁴⁶ In their study, eight *N*-methylated CPs showed higher membrane permeability as compared to the non-methylated parent sequence, while the other 46 CPs showed little or no improvement in membrane permeability. It is noted that while it is important for a compound to have high membrane permeability, it does not inherently mean that the compound will have high oral bioavailability, which is determined by numerous other factors including compound stability, gut transit time and first pass metabolism.^{93,94} Also, *N*-methyl groups need to be implemented with caution, as *N*-methylation at some sites can have a detrimental impact on the desired structure or function of the CP.^{86–89,95}

Though experimental design strategies have been utilized with some success, there remain several formidable challenges. Arguably the most significant challenge in CP design is our poor understanding of their sequence–structure relationships. The current majority of experimental structural information for small CPs (8 amino acids) comes from X-ray crystal structures^{39,40,48–56,64,96–141} and NMR structures in organic solvent.^{13,36,37,42,58,61,63,66,67,77,111,113,118,126–132,134,139–171} However, X-ray crystal structures lack dynamic information, providing only a single snapshot among a possible ensemble of structures available in solution. Indeed, solution NMR studies of CPs show that they often adopt multiple poorly populated conformations, existing as structural ensembles rather than one dominant structure.^{35–46} Unfortunately, even the current state-of-the-art experimental methods lack the capability to fully characterize every individual conformation in a structural ensemble.^{172–175} Additionally, the structures adopted by CPs in organic solvent may not be the same structures those CPs adopt in water, the relevant solvent for biological

studies and applications. Therefore, to experimentally establish valid sequence–structure relationships for CPs in aqueous solution, hundreds if not thousands of different sequences would first need to be synthesized to identify those rare instances where CPs adopt a single dominant conformation. Structures of these well-behaved CPs can then be obtained using multidimensional NMR spectroscopy to investigate how CP sequence controls structure. This trial-and-error approach of experimentally generating and rigorously characterizing thousands of CPs would be extremely costly and intractable. Therefore, in recent years computational simulations have emerged as being uniquely suited to aid in the development of sequence–structure relationships of CPs, as well as designing CPs to adopt desired conformations.

Computational design of cyclic peptides

Several computational methods have been used to design CPs, including virtual screening of CP libraries to identify CPs with high binding affinities for a given target,^{176–181} fragment-based algorithms to predict CP structures^{182–191} and molecular dynamics (MD) simulations to characterize the conformational ensembles of CPs.^{192–201} While virtual screening has been shown to be an effective method of surveying sequence space and identifying residues crucial for binding, it currently lacks the capability to accurately describe CP–solvent interactions, which have been shown to play a crucial role in the structures CPs adopt.^{34,129,202–205} Additionally, virtual screening methods are typically based on libraries of *linear* peptide and protein systems, and therefore may not be suitable to describe the conformations of CPs.

Fragment-based approaches and loop closure algorithms have also been developed and used to predict CP structures. For example, kinematic closure (KIC), implemented in the Rosetta package, uses a multi-stage process for loop reconstruction.¹⁸⁵ In the first stage, the backbone is optimized, with the side chains represented as centroids, and in the second stage the entire loop is optimized using an all-atom representation. This methodology was found to recapitulate the structures of many crystallographic loop conformations. However, similar to virtual screening, fragment-based approaches are also incapable of explicitly describing CP–solvent interactions. It is also noted that when fragment-based algorithms are developed to predict CP structures, they are often applicable to a limited set of CP sizes, cyclization strategies or residue types.^{182–184,186,187,189,191} Alternatively, explicit solvent MD simulations can describe CP–solvent interactions explicitly and have been used to predict the structural ensembles of various CPs.^{192–195,197–201} Herein, we will focus on how MD simulations have been used to understand and design CPs.

Several challenges are associated with using MD simulations to accurately and efficiently predict CP structures (Figure 2). (1) Both experimental and computational studies have shown that solvent plays a significant role in the structure a CP adopts.^{34,129,202–205} Therefore, to accurately account for direct CP–solvent interactions, explicit solvent simulations are necessary. However, explicit solvent simulations require substantial computational resources in order to evaluate the interactions and motions of a CP plus thousands of solvent molecules. (2) It remains challenging to efficiently explore the conformational space of a CP using conventional MD simulations. In small CPs (< 8 amino

acids) large free energy barriers between conformations caused by ring strain usually prevents efficient sampling of relevant structures by standard MD,²⁰⁶ while large CPs (> 8 amino acids) can also present significant issues, because they often have vast conformational ensembles.⁴⁷ (3) The ϕ/ψ dihedral angles of CPs, particularly of small ones, tend to fall outside the canonical regions in the Ramachandran space observed for linear peptides and proteins.²⁰⁷ Since most of the current peptide force fields are parameterized based on linear peptides and proteins, their accuracy at predicting CP structures must be verified.

In a recent study, Quartararo *et al.* found that water was key to the structure of a bicyclic peptide.³⁴ Using MD simulations, several water molecules with long-lived hydrogen bonds to the CP backbone atoms were identified. Subsequent NOESY and ROESY data showed cross peaks between the backbone atoms and water molecules, corroborating the observed MD results. This study provides an example of how water is important to the structure that constrained peptides adopt. Additionally, Beck *et al.* identified template structures through the synthesis and characterization of a library of *N*-methylated variants of the cyclic hexapeptide cyclo-(aAAAAA) as described previously. One of these template structures, cyclo-(aAAAAA) (underlines indicate *N*-methylated residues), contained all *trans* peptide bonds and two type II β -turns at residues ⁶Aa¹ and ³AA⁴ in DMSO (Figure 3A). When simulated with explicit DMSO molecules, the simulation results accurately recapitulated the NMR structure (Figure 3B).²⁰¹ However, this is not the case when simulated with implicit solvent using a dielectric constant equivalent to DMSO ($\epsilon = 47$) (Figure 3C). The culmination of these results and similar shows that it is imperative to explicitly model solvent molecules in CP simulations.

To tackle the CP conformational sampling problem, several enhanced sampling techniques have been applied.^{192–201} Two of the most common enhanced sampling techniques that have been applied to CP systems are replica exchange molecular dynamics (REMD)^{208,209} and metadynamics (META).^{210,211} In REMD, several replicas are run in parallel, each at a different temperature. Throughout the simulation, exchanges are attempted between replicas at regular time intervals, allowing the conformational sampling of the low-temperature replicas to be enhanced by the high-temperature replicas.^{208,209} REMD simulations have been used to study the structural ensembles of several CPs.^{193,194,196,197} For example, Razavi *et al.* used a combination of computational screening and REMD simulations to design a CP that mimics a β -hairpin in LapD, a bacterial protein whose interaction with LapG is crucial to biofilm formation.¹⁹⁴ Initially, implicit solvent REMD simulations were used to rapidly screen potential CP mimics. The four most favorable designs from the screening process were subsequently studied using explicit solvent simulations, ultimately leading to designs that mimic the LapD β -hairpin.

In REMD simulations, conformational sampling is enhanced for all degrees of freedom. However, if the slowest degree of freedom of the system is known, a more efficient enhanced sampling method such as META can be applied. In META, positive Gaussian hills are deposited along a specific reaction coordinate or collective variable (CV) at regular time intervals until the free energy surface along that CV is flat.²¹⁰ To target multiple CVs at the same time, bias-exchange META (BE-META) simulations can be used, where multiple replicas are run in parallel, each biasing a different CV. At given time intervals, exchanges

between replicas are attempted and the sampling of a single replica is enhanced by the sampling of the other replicas.²¹¹ BE-META simulations are the most effective at enhancing the conformational sampling when the slowest degrees of freedom can be identified and used as CVs. In a recent study, it was found that conformational changes of small CPs generally involve coupled dihedral changes of ϕ_i and ψ_i , or ψ_i and ϕ_{i+1} , and efficient conformational sampling could be achieved by targeting these coupled two-dihedral changes using BE-META.¹⁹⁹ Using these essential transitional motions as CVs, systematic studies of cyclic hexapeptides in explicit water were performed, leading to the identification of a sequence with a single highly populated structure.²⁰⁰

As with any simulation of biomolecules, the reliability of CP structural prediction using MD simulations depends on the accuracy of the force field used. To study biomolecules, several force fields have been developed, including AMBER,^{212–219} CHARMM,^{220–222} GROMOS,^{223–225} OPLS-AA^{226–229} and residue-specific force fields.^{230,231} In general, current parameterization of protein force fields utilizes well-structured linear peptide and protein systems, whose ϕ and ψ dihedral angles typically occur in the α helix, β sheet and polyproline II regions of the Ramachandran plot. CPs, however, tend to have ϕ and ψ dihedral angles that fall outside these canonical regions, due to ring strain (Figure 2C).²⁰⁷ A recent study of a cyclic octapeptide, cyclo-(YNPFEEGG), showed that many peptide force fields tend to overstabilize these canonical regions of the ϕ/ψ space, leading to the mis-prediction of the NMR structure.¹⁹⁸

In a 2016 study, Geng *et al.* compared how well four peptide force fields, Amber99sb-ildn,²¹⁸ OPLS-AA/L,²²⁷ RSFF1²³⁰ and RSFF2,²³¹ were able to predict the crystal structures of 20 all-*trans* CPs ranging in size from 5–12 amino acids.²³² Here RSFF1 and RSFF2 are residue specific force fields 1 and 2, both of which were parameterized based on the protein coil library and were found to perform the best at predicting the CP crystal structures. For example, RSFF2 correctly recapitulated the crystal structures of 17 out of the 20 CPs with an RMSD < 1.1 Å.²³² While RSFF2 appears promising at predicting CP structures, it is noted that all the 20 CP structures utilized in the study by Geng *et al.* were crystal structures of all-*trans* peptides composed of natural amino acids. Therefore, it remains to be tested whether RSFF2 can successfully predict solution structures of CPs, or those CPs containing *cis* peptide bonds and/or non-natural amino acids. In a recent study, it was shown that RSFF2 was not capable of reliably predicting the *cis/trans* isomers of simple *N*-methylated cyclic hexapeptides.²⁰¹ Further optimization of the force field to describe the ratio between the *cis* and *trans* conformations is thus required if *de novo* structure prediction including the *cis/trans* isomer state is desired. Additionally, modifications of the force field to include non-natural amino acids would greatly enhance its utility for CP design.

Conclusions and perspectives

Owing to our current inability to reliably and efficiently predict CP structure and our poor understanding of CP sequence–structure relationships, it is currently difficult to design CPs to adopt specific conformations or to exhibit desired properties. For example, several methods have been developed to identify crucial binding regions in protein–protein interactions. These include the identification of “hot segments” using Rosetta Peptiderive,

233–235 “hot loops” using LoopFinder,^{236–238} helical interfaces using HippDB^{239–241} and β -sheets at interfaces.²⁴² Additionally, PepComposer developed a methodology to design peptides given a target structure and binding site.²⁴³ The capability to use CPs to mimic these regions could afford development of potent inhibitors of protein–protein interactions. We expect a better understanding of and ability to design CPs will ultimately emerge from a combined computational and experimental approach. In computationally understanding and designing CPs, current challenges include the need for the use of a computationally expensive explicit water model to describe CP–water interactions, the need for an efficient enhanced sampling technique to rapidly explore CP conformational space and the need for a force field to accurately predict the solution structures of CPs with potential *cis* peptide bonds and non-natural amino acids. In experimentally understanding and designing CPs, more efficient synthetic schemes and improved methods to characterize individual conformations within the structural ensembles of CPs will be beneficial. Combined, these two approaches will complement one another and provide synergistic feedback. The resolution of these challenges shall lead to an improved understanding of CP sequence–structure relationships and ultimately the ability to efficiently design CPs *de novo*.

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Figure 1.

(A) Ideal dihedral angles for five types of β -turns and four types of tight turns. Note that for type VIa1 β -turns the peptide bond between residues $i+1$ and $i+2$ must be *cis*. (B) Schematic representations of C₁₀-, C₁₁-, C₁₂- and C₁₃-like structures. C₁₀-like structures comprised solely of β -amino acids (β_{aa} , **left**) or α -amino acids (**right**) are shown. β -amino acids are labeled in all structures.

Figure 2. Challenges in CP design using MD simulations. **(A)** Explicit solvent simulations are necessary but computationally expensive. **(B)** Trajectories of ϕ (in red) and ψ (in blue) from a conventional MD simulation of cyclo-(VGVVIVr) show that the ϕ and ψ dihedrals do not frequently change within a 500 ns simulation (~ 2.2 days of simulation time on 16 CPU cores). **(C)** NMR structure of cyclo-(aAAAAA),⁴⁶ where lowercase letters indicate D-amino acids and underlines indicate *N*-methylated residues, **(left)** and experimental ϕ/ψ angles overlaid on the typical Ramachandran plot for L-amino acids using the PDB database²⁴⁴ **(right)** show that many of ϕ/ψ angles fall outside the canonical α , β , or PPII regions.

Figure 3.

(A) NMR structure of cyclo-(aAAAAA), where lowercase letters indicate D-amino acids and underlines indicate *N*-methylated residues.⁴⁶ (B) Most populated cluster from the explicit DMSO simulation of cyclo-(aAAAAA) using RSFF2.²³¹ A representative structure and the Ramachandran plot for each residue are shown. Adapted from ref. 201 with permission from the PCCP Owner Societies. (C) Most populated cluster from the implicit DMSO simulation of cyclo-(aAAAAA) using AMBER96+GBSA/OBC ($\epsilon = 47$).^{213,245–247} A representative structure and the Ramachandran plot for each residue are shown. The ϕ and ψ angles of the NMR structure are shown as black dots in the Ramachandran plots.