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Folding in Place: Design of β -Strap Motifs to Stabilize the Folding of Hairpins with Long Loops

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Abstract

Despite their pivotal role in defining antibody affinity and protein function, β -hairpins harboring long noncanonical loops remain synthetically challenging because of the large entropic penalty associated with their conformational folding. Little is known about the contribution and impact of stabilizing motifs on the folding of β -hairpins with loops of variable length and plasticity. Here, we report a design of minimalist β -straps (strap = strand + cap) that offset the entropic cost of long-loop folding. The judicious positioning of noncovalent interactions (hydrophobic cluster and salt-bridge) within the novel 8-mer β -strap design RW(V/H)W...WVWE stabilizes hairpins with up to 10-residue loops of varying degrees of plasticity (T_m up to 52 °C; 88 $\pm$ 1% folded at 18 °C). This “hyper” thermostable β -strap outperforms the previous gold-standard technology of β -strand- β -cap (16-mer) and provides a foundation for producing new classes of long hairpins as a viable and practical alternative to macrocyclic peptides.

Graphical Abstract

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Author Contributions

This project was conceived by S.P.R. A.D.R., S.H., and G.Z. performed the research experiments and S.P.R. with A.D.R. analyzed the data. The manuscript was written through contributions of S.P.R. and A.D.R. All authors have given approval to the final version of the manuscript.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.joc.1c01442>.

Raw CD data as well as a detailed protocol for the best-fit routine (two-state model) and the resulting Gibbs–Helmholtz equation obtained for each peptide **1–3** (XLSX)

Tables of NMR chemical shifts, structural assignments data including ^1H , TOCSY, NOESY, magnified TOCSY/NOESY overlays, and HSQC spectra, as well as CD and VT-CD spectra, and HPLC chromatograms to determine structure and purity for each peptide **1–3** and the corresponding random coils **SI-RC1–4** (PDF)

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INTRODUCTION

In the past 2 decades, the mimicry of proteins' secondary structures into peptide foldamers has emerged as a powerful modality to build novel materials¹ and bioactive macromolecules.² The identification of stand-alone foldamers such as β -hairpins and their development into macrocyclic mimics have proved to be a prolific strategy for the development of protein–protein interaction (PPI) inhibitors.³ Although pivotal advances in the mimicry of helices, turns, and β -sheets have been made,⁴ the general design principles guiding the folding of β -hairpins with long loops are still in their infancy.⁵ In fact, the folding of β -hairpin peptides is essentially limited to short turn-induced β -sheets, in which the turn-locus forces the secondary structure nucleation through chain reversal and the formation of an antiparallel strand register.^{3,6} In stark contrast, most β -hairpins typically found in the proteome are “turnless” with long and unstructured loops (β -arches) stabilized by β -pleated sheets within secondary and tertiary structures.⁷ It is proposed that the folding of these turnless long β -hairpins arises not only from loop nucleation⁸ but also from pairing-residue interactions⁹ and hydrophobic clusters formation¹⁰ along β -sheets.¹¹ From a fundamental standpoint, the current frontier in hairpin structures mimicking closely the native folds found in protein remains largely limited to short [2:2]/[2:4]-, [3:5]-, [4:4]/[4:6] hairpins.^{12,13} These synthetic hairpins are typically nucleated around a 4-residue loop having an innate turn propensity (e.g., DDATKT and NPATGK sequences, Figure 1A).^{8c,14}

Different challenges are indeed associated with length: short loops in [2:2]/[2:4]- and [3:5]-hairpins are usually centered around specific β -turns and β -bulges,¹⁵ while the foldings of [4:4]/[4:6]- and larger hairpins are exceedingly more difficult to control due to the conformational space accessible by random sequences of α -amino acids and the innate conformational strain held by their structures. Except for some rare examples of

long hairpins (10-mer loop) championed by Andersen¹⁶ by developing a β -cap- β -strand stabilizing motif of 16 amino acids length, the significant entropic penalty inherent to the nucleation of long loops and their vulnerability to fraying have basically hindered their synthetic development. For these reasons, most structural studies have only evaluated the formation of long loops built from simpler and highly flexible polyglycine chains.^{16,17} Other studies have shown that associating β -strands and β -caps of 10–12 residues is the minimal length required to stabilize hairpins with relatively short loops (2–4 residues).¹⁸ A net improvement of design is needed to create long and well-folded β -hairpins at physiological temperatures and obtain a viable and practical synthetic alternative to macrocyclic peptides of a large size (>10-mer).¹⁹

Despite the need for novel protein and antibody loop mimics, the lack of technology has hampered the synthesis of large hairpin modalities suitable to this space. Therefore, we were drawn to revisit this issue by designing some minimalist β -straps amenable to crafting loops of conceptually any length, sequence, and conformational strain (Figure 2).²⁰ To this aim, we targeted the synthesis of a [10:12]-hairpin, which mimics a highly constrained loop (RDYRFDMGFD sequence) excised from the heavy chain complementary determining region 3 (CDR-H3) of the marketed antibody drug pembrolizumab, a programmed cell-death 1 inhibitor (Figure 1B).²¹

We hypothesized that combining several types of noncovalent interactions²² at non-hydrogen-bonded (NHB) and hydrogen-bonded (HB) sites would enable long constructs of typical β -cap-strand-loop-strand- β -cap architecture to be truncated into their simplest forms β -strap-loop- β -strap and still retain a high efficacy (Figure 2). Here, we report a side-by-side comparison between three strap designs **1–3**, which demonstrates that a judicious combination of Coulombic interactions at the hairpin termini HB-site (persisting at longer distances) with van der Waals and hydrophobic contacts at NHB-sites can overcome the loop entropy to strengthen hairpin structures (Table 1).

RESULTS AND DISCUSSION

We set our aim as to construct monomeric β -hairpins soluble in water and stable at physiological temperatures. To understand the structural requirements of stabilizing motifs in the straps, we began by designing peptide constructs by including the DDATKT^{14a} and NPATGK,^{8c} d loops of known [4:6] hairpins as reversing turn loci inspired from GB1, and the B1 domain of *Streptococcal* protein G (Table 1).²³ The underlying idea was to compare peptides **1a–c** harboring an Ac-W $\cdots$ WTG-NH₂ β -strap, inspired by a capping created by Andersen (Table 1, entries 6–8)^{15,24} with constructs **2a–b** having an Ac-WVW $\cdots$ WVW-NH₂ β -strap (Table 1, entries 9 and 10) truncated mimic of the tryptophan zipper motif (Trpzip) developed by Cochran²⁵ such as the Trpzip9, and some of most stable stand-alone hairpins known to date HP7 and GB1m3 (60 T_m 92 °C, entries 3–5).^{26,27} Second, we evaluated the stability of a novel RW(V/HW) $\cdots$ (WV)WE β -strap, which combined an ionic pair at the HB-site termini in addition to the Trpzip hydrophobic core of constructs **3a–c** (entries 11 – 13) in comparison to the Coulombic capping of β -hairpin HP7 previously positioned at an NHB-site (entry 3). Here, we demonstrated the superiority of β -strap **3** and further evaluated its ability to structure longer loops (e.g., hairpins **3d–f**, entries 14–16). All peptides **1–3** were

synthesized on resins using a typical Fmoc-based solid-phase peptide synthesis (SPPS) in 15–70% yields with relatively high crude purity after cleavage (78% by RP-HPLC), which was improved by semipreparative RP-HPLC purification when needed.

The final deprotection/cleavage step proved to be challenging for constructs **2** due to the steric hindrance imparted by the resin on the nearby C-terminal Trp residue leading to side reactions and irreversible reattachment of the peptide to the resin.²⁸

Secondary Structures and Thermal Stability Analysis Determined by Circular Dichroism (CD) Spectroscopy.

Changes in molar ellipticity intensity as a function of temperature [$\theta(T)$] can be exploited at specific wavelengths of CD spectra to examine the thermodynamic folding/unfolding transition of secondary structures.²⁹ For example, the cross-strand interaction between W/W pairs positioned at NHB-sites is well known to produce a distinct and intense exciton couplet at 212 and 228 $\pm$ 1 nm (negative/positive maxima) from a $\pi-\pi^*$ transition, which can be used as a local spectroscopic probe of β -hairpin folding.^{30,31} Indeed, the edge-to-face (EtF) geometry between indole rings of facing Trp residues^{30,32} responsible for the $\pi-\pi^*$ transition is also coupled with a significant electrostatic stabilization created by multiple CH $\cdots\pi$ interactions.³³ As a result, the W/W EtF interaction was widely exploited to measure the level of backbone folding in hairpins.^{30b} The decrease in amplitude of the prototypical 228 nm exciton intensity upon heating was therefore monitored by CD spectroscopy to evaluate the thermal denaturation of β -straps **1–3** and determine the thermodynamic parameters characterizing the transition between the folded and unfolded states of hairpins (Figure 3).

To analyze these secondary structures in solution, several synthetic “random coils” **SI-RC1–4** arguably similar to the corresponding β -strap **1–3** in length, primary sequence, and compactness were synthesized and fully characterized by variable-temperature circular dichroism (VT-CD) spectra and NMR experiments.^{14d,34} The unfolded nature of these constructs **SI-RC1–4** was confirmed (see linear behavior, Figure 3A) and exploited to calibrate both CD unfolded baselines and NMR chemical shift deviations (CSDs).^{25b,26} The far-UV CD spectra of peptides **1–3** were recorded in phosphate buffer (with up to 20% MeOH added in rare cases, see the Experimental Section, Table 2) upon a gradual increase of temperature (0–95 °C). The reversibility of the thermal denaturations was ascertained by obtaining a quasi-identical recovery of spectral features for all constructs **1–3** upon cooling the temperature back to 0 °C. It is well known that the molar ellipticity intensities of the 228 nm exciton vary considerably even for fully folded hairpins ($[\theta]_{228}$ +290 000 to +560 000°) due to minor differences in perpendicularity between indole rings,^{30b,31} thus raw melting curves from $[\theta(T)]_{228}$ could not be utilized to determine fractions of folding precisely. Instead, raw data were corrected by subtracting the corresponding random coil ellipticity values obtained at 95 °C $[\theta]_{RC(95)}$ (unfolded baseline **SI-RC1–4**) and normalized (per Trp pair) for direct comparison.³⁵ The corrected and normalized raw ellipticity intensities $[\theta]_{228}$ for hairpins **1–3** were then fitted to a theoretical $[\theta(T)]_{228}$ melt curve as a function of temperature (eq S1) using the thermodynamic Gibbs–Helmholtz equation describing a two-state model (folded/unfolded states).²⁹ The best-fitted melting curves of unfolding transition

were computed for hairpins **1–3** by allowing H_m , T_m , and C_{pF}° to freely vary using a nonlinear least square fitting routine,²⁹ while using the approximation that the heat capacity is constant over the range of temperature evaluated: $C_{pF}^\circ < 0$ (Figure S2).^{34,36} Plots of %-folding (χ_F) for each hairpin **1–3** as a function of temperature were obtained (Figure 3A), along with the thermal stability curves (Figure 3B), melting temperatures (T_m),³⁷ and thermodynamic parameters characterizing the unfolding transitions (Figure 3C). As shown by the goodness of the fit determined on $[\theta(T)]_{228}$ (avg. R^2 of 0.98 and RMSD of 5,179 deg·cm²·dmol⁻¹), these melting curves indicated a temperature-induced unfolding typical from a hairpin/coil transition in agreement with a two-state model via a single transition barrier resulting from the cooperativity of fracture between the strap and loop segments. Even though the midpoint inflection of the melt curvature (at T_m) cannot be consistently observed due to the finite window of experimental temperatures (Figure 3A), both the cooperativity of the transitions and isodichroic points can be estimated from the extrapolated melts and the raw CD stacks presented in the Supporting Information. In addition to the thermal denaturation, the two-state model approximation was further validated by isothermal urea-induced denaturation titrations (0–15 M) on hairpins **2a** and **3b–d** (Figure S18).³⁴

CD melts for hairpins **1a–c**, built around an Ac-W··WTG-NH₂ β -strap motif, provided thermodynamic parameters representative of a relatively poor folding ($G_F^\circ \sim 0$). In contrast to the initial report by Andersen,^{16a} our results suggest that this strap is not extremely stabilizing for longer loops. Even so, the innate nucleation induced by the DDATKT and NPATGK loops was shown to affect the overall hairpin structures (**1a** vs **1b**, $G_F^\circ = -0.51$ kcal/mol), suggesting that a DDATKT sequence might be a more favorable loop. Adding a pair of aromatic residues F(S $\pm$ 2) to the β -strap flanking the loop in **1c** resulted in the most stable hairpin of this series despite a modest folding $\chi_F(291)$ of $63 \pm 4\%$ (Table 1, entry 8). In contrast, hairpin **2a** stabilized by the Ac-WVW··WVW-NH₂ strap and derived from a truncated Trpzip9 (Table 1, entry 5 vs entry 9) revealed some remarkable folding properties (T_m (**2a**) > 100 °C; $G_F^\circ = -2.36$ kcal/mol). This strap was further evaluated by crafting a more flexible GPGTGK loop sequence in hairpin **2b**. Surprisingly, the N4G and A6G substitutions largely abrogated the fold of hairpin **2b** (Figure 3B). The important entropic penalty associated with this flexible loop ($T \cdot S_F^\circ(\mathbf{2b/2a}) = + 3.25$ kcal/mol) could not be compensated by the noncovalent interactions within the strap. Another drawback of this short β -strap is its high hydrophobicity in comparison to the longer original Trpzip sequence,^{25d} thus creating peptides poorly soluble in both water and organic solvents (e.g., MeOH, TFE, or DMSO).

To counterbalance this hydrophobic effect, improve aqueous solubility, and increase amphipathicity, an ionic pair was inserted at the terminal HB-site resulting in the RW(V/HW)··(WV)WE strap design **3**. Hairpins **3a–c** demonstrated some of the most interesting thermodynamic properties with high melting temperatures (62 T_m 98 °C) and sizable stability (-1.63 G_F° -0.87 kcal/mol). As shown in Figure 3A, melt curves of hairpins **3a** vs **3b** highlighted some subtle differences in stability. Given the large heat capacity C_{pF}° of -158 ± 3 cal·mol⁻¹·deg⁻¹) typically associated with the burial of nonpolar moieties, the V3H substitution in **3b** appeared to enhance the overall hairpin stability, although being less resistant to the effect of temperature $T_m(\mathbf{3b}) \ll T_m(\mathbf{3a})$. A

truncated version of this strap in hairpin **3c** also demonstrated significant stability with $T_m \sim 62^\circ\text{C}$ and a $G_F^\circ = -0.87$ kcal/mol. Overall, hairpin **3c** presented the similar stability to the slightly longer HP7 construct (Table 1, entry 3), which led us to speculate that an ionic pair at the terminal HB-site might provide a new framework for building valuable β -straps. In contrast to the folding of **2a**, which is associated with an extended hydrophobic packing (highly enthalpically favorable, $H_F^\circ \sim -6$ kcal/mol), hairpin **3a** carries a smaller enthalpic contribution ($H_F^\circ \sim -4$ kcal/mol), likely to be compensated by an additional conformational flexibility from the salt-bridge.³⁷ Indeed, such Coulombic interaction persistent over long distances could conceivably tame the overall hairpin fraying and afford the appropriate flexibility to achieve an optimal geometry within the neighboring EtF W/W aromatic interaction.³⁸ Taken together, our thermodynamic results imply that the RWVW...WVWE strap sequence of **3a** was optimal in stabilizing short β -hairpins.

This strap was further evaluated on hairpins **3d–f** with long loops of variable plasticity. The CD signature from the W/W exciton maxima at 228 ± 1 nm was also observed for all constructs **3d–f** despite in lower intensity. As expected, the entropic cost associated with the folding of a polyglycyl loop in **3d** is relatively large in comparison to **3e** ($T \cdot S_F^\circ(\mathbf{3d}/\mathbf{3e}) = -3.53$ kcal/mol). Even so, hairpin **3d** is extremely well folded with a T_m of $\sim 52^\circ\text{C}$ and a χ_F of 88% at 18°C (291 K). In contrast, hairpin **3e** bearing a conformationally constrained loop of “random” sequence (pembrolizumab H3 loop model, see Figure 1B) proved less stable, as shown by the CD-melt in Figure 3A. To reduce both hydrophobicity and rigidity in the loop, an F9G substitution was evaluated at the L4 position of **3f**. Given that the heat capacities calculated for the transitions of hairpins **3e** and **3f** were comparable, the release of loop strain in **3f** likely contributed to enhance its folding. Overall, the CD melts revealed that short hairpins **2a**, **3a**, and **3b** are “hyperstable” with a long plateau of thermal stability from 0 to $\sim 50^\circ\text{C}$. Below 50°C , such foldings exist in a dynamic equilibrium of stable conformational states ($>80\%$ folded, vide infra NMR data), which affect the cooperativity of the transitions (continuum of discrete steps).^{25b} On the other hand, both hairpins bearing flexible loops **2b** and **3d** have a more ideal all-or-none transition than their constrained counterparts **2a** and **3e**. This result might be explained by the lesser change of entropy in flexible loops between folded and unfolded states.

Thermostability curves are usually applied in the analysis of protein stability to estimate both entropic and enthalpic contributions induced by mutations to the proteins’ folding free energy.⁴⁰ To rationalize the differences between β -straps **1–3** and inform their rational design, such plots of temperature dependence on unfolding free energy $G_F(T)$ were analyzed (Figure 3B).⁴⁰ The thermodynamic parameters calculated from the two-state model approximation (Figure 3C) were applied to plot these thermostability curves, which can be extrapolated outside the range of experimental temperatures. Thermostability curves have typical parabolic shapes (if $C_{pF}^\circ \neq 0$) that intercept the temperature axis ($G_F(T) = 0$) at two different temperatures characteristic of cold and thermal denaturations (T_C and T_m). At the parabolic maxima, the temperature T^* of maximal stability and free energy G_F^* can be determined for each hairpin (Figure 3B,C).⁴¹ Indeed, at T^* , $S_F^* = 0$ resulting in $G_F^* = H_F^*$. The differences in enthalpy H_F^* between hairpins of the identical loop sequence (i.e., NPATGK) were therefore readily compared to estimate the net contribution

of straps on the overall secondary structure's thermostability. As shown in Figure 3C, the RWVW...WVWE strap performed well in hairpins **3a–c** with H_F^* of -1.19 to -1.76 kcal/mol, while the Ac-WVW...WVW-NH₂ strap in **2a** was the most stabilizing motif with a H_F^* of -2.42 kcal/mol. Another significant phenomenon observed from the thermostability curves is the parabolas' curvatures.⁴⁹ Even if **2a** and **3b** are the most stable hairpins (G_F^* of -2.50 and -1.84 kcal/mol), the narrower parabolic curvature of their thermostability curves implies that these hairpins have a small range of thermal stability. On the other hand, the wider curvatures observed in the case of **3c** and **3a** suggest that these hairpins absorb more efficiently heat energy for the same increase in temperature (thermostable). This phenomenon can be explained by the fact that hairpins **3a** and **3c** have the smallest C_{pF}° of -53 and -106 cal-deg⁻¹·mol⁻¹, respectively, which ultimately is a consequence of having a smaller hydrophobic surface exposed to water. On the contrary, even if the V3H mutation created additional contact interactions in hairpin **3b** (i.e. higher fraction folded), the narrower curvature of the thermal stability parabola confirms that the enthalpic contribution of such electrostatic interactions weakened rapidly upon the effect of heat.⁴² The aforementioned entropy held by the loop also affects the thermal stability of longer hairpins and the cooperativity of the unfolding transitions. The large entropic energy contained in the polyglycyl loop of hairpin **3d** likely contributed to its sharp thermal denaturation thus resulting in a narrow range of thermal stability. Conversely, the wider curvature of the G_F^* plot for **3e–f** (shift of T^*) demonstrated how compactness and local side-chain interactions in more conformationally constrained loops can preclude the rapid thermal denaturation. This effect culminated in the stability of hairpin **3f** (slightly less rigid and hydrophobic than **3e**) with the largest G_F^* of -3.18 kcal/mol and T_m of 46.7 ± 0.1 °C.

Structural and Conformational Analysis by NMR Spectroscopy.

To confirm the secondary structures suggested by the CD study, a careful structural assignment and conformational analysis were secured by NMR spectroscopy.⁴³ Detailed NMR studies of hairpins **1–3** at 18 °C (291 K) were carried in a mixture of phosphate buffer (pH 6.5) with D₂O, with small proportions of additives (DMSO-*d*₆, or CD₃OH) in rare cases, at concentrations ca. 1–20 mM with DSS as the internal standard for chemical shifts. Signals assignments were obtained on the basis of a set of ¹H and TOCSY spectra (for intraresidue connectivities), NOESY spectra (for vicinal and interstrand backbone connectivities), and HSQC spectra (for H_α to C_α connectivities and W side-chains assignments).³⁴ First, chemical shift deviations (CSDs) were calculated for all of the H_N and H_α proton resonances in each hairpin **1–3** (Figure 4).⁴⁴ Average CSDs obtained for the synthetic unfolded constructs **SI-RC1–4** were in each case close to zero (avg. | δH | < 0.2 ppm) indicating the absence of the secondary structure as expected for coil-like structures.^{34,45} These results further validated our unfolded models used to calibrate the unfolded baselines in the CD-melt analysis. For constructs **1a,b**, the overall chemical shift dispersion of H_N and H_α signals is small, and CSDs characteristic of β -hairpins (upfield in the loop and downfield in strands) are not observed (Figure 4A). Furthermore, the W(S – 2)/W(S + 2) pair in **1a** was characterized by upfield shifts of 0.58 and 0.45 ppm for the H_{ε3} and H_{β3} signals of the “edge” W(S – 2) residue indicative of a reverse EtF arrangement characteristic of misfolding (instead of FtE interaction). In hairpin **1c**, the addition of an F/F pair at the S ± 2 positions flanking the loop enabled the β -strap to actually fold⁴⁶ in

a similar fashion to the [2:2]-hairpins reported by Andersen. An FtE W(S – 4)/W(S + 4) interaction was plainly observed in this case, with CSDs for W(S + 4) $\delta H_{\beta 3}$ and $\delta H_{\epsilon 3}$ of –0.45 and –1.62 ppm, respectively, as well as a distinctively large upfield G14H_N proton shift (δ 6.33 ppm corresponding to a CSD of –1.78 ppm from its random coil value). Overall, the backbone H_N and H _{α} CSDs from the Ac-W···WTG-NH₂ strap motif in **1c** revealed a pattern consistent with a β -sheet register. The folded conformation of hairpin **1c** was further supported by a set of cross-strand NOESY correlations (Figure S19).

Earlier studies on [2:2]-hairpins established that the extent of chemical shift splitting between glycine methylene signals in ¹H NMR spectra ($\delta H_{\alpha/\alpha'}$ (Gly)) can be exploited to quantify the %-folding of β -hairpins.^{43a,45c,47} These two H _{α} /H _{α'} diastereotopic protons experience a different chemical environment relative to the ratio of equilibrating conformers on the NMR time scale. Thus, a greater separation is indicative of a larger folded population. This local folding probe was notably applied by Horne and Del Valle to [4:4]/[4:6]-hairpins featuring the NPATGK reverse-turn locus, by measuring the $\delta H_{\alpha/\alpha'}$ (Gly) separation of G(L4).^{27,48} In a series of macrocyclic peptides, the largest $\delta H_{\alpha/\alpha'}$ G(L4) value of 0.33 ppm was attributed to 100%-folding and used as a benchmark for the “fully” folded hairpin. Strikingly, several synthetic hairpins from our study (e.g., **1c**, **2a**, **3a–c**) have shown even larger shifts 0.46–0.55 ppm (see Table 1). We therefore re-estimated the folded population for the loops of hairpins **1–3** based on a 0.55 ppm splitting for the G(L4) H _{α/α'} resonances as the >99%-folded ref 49. $\delta H_{\alpha/\alpha'}$ G(L4) values of 0.23 and 0.50 ± 0.01 ppm observed at 291 K for **1a** and **1c** correspond to a local folded population estimate of 42 ± 4 and 91 ± 2%, respectively. Although the poor solubility of constructs **2a–b** in water limited our NMR interpretations, the folding of **2b** was found minimal. Indeed, H_N and H _{α} CSDs in construct **2b** were mostly insignificant throughout, and the local loop folding was characterized by a $\delta H_{\alpha/\alpha'}$ G(L4) of 0.03 ppm corresponding to 6 ± 33% of folding at 291 K (Table 1, entry 10). On the contrary, hairpin **2a** was 93 ± 2%-folded and characterized by high CSDs for the tryptophans side-chain signals due to two FtE W(S ± 4) and EtF W(S ± 2) interstrand interactions. For the turn-flanking edge W(S – 2) residue, H _{$\epsilon 3$} , H _{$\beta 3$} , and H _{$\delta 1$} experienced upfield ring current shifts as large as 1.22, 0.95, and 0.60 ppm, due to aromatic shielding, whereas the edge C-terminal W(S + 4) residue displayed upfield shifts of 1.25, 0.99, and 0.44 ppm for the same proton signals (Figure 4, bottom panel). These NMR structuring shifts are similar to those reported for the HP7 and Trpzip4 hairpins, thus confirming the efficiency of the truncated β -strap.⁵⁰ The folded nature of hairpin **2a** was further supported by various cross-strand and long-range NOESY correlations (Figure S20). Collectively, NMR and CD data obtained at 291 K revealed that hairpin **2a** is well folded with $\chi_F > 90\%$.

As shown by the trend of thermodynamic stability observed in the CD melts, the RW(VW)···(WV)WE β -strap proved to be superior (e.g., **3a–c**) throughout the NMR study (Figure 4B). Indeed, ¹H NMR signals for both H_N at HB sites and H _{α} at NHB-sites within the β -strap were highly dispersed and experienced significant downfield shifts (~1.5 ppm on average).^{45a} The strong FtE and EtF W/W interactions at the S ± 4 and S ± 2 positions were further rationalized by the formation of a more compact hydrophobic cluster. Distinctive NMR shifts with CSDs of –1.49 and –1.16 ppm for H _{$\beta 3$} , –0.62 and –0.32 ppm for H _{$\delta 1$} , and –1.21 and –1.19 for H _{$\epsilon 3$} observed at the respective S – 2 and S + 4 edge sites of hairpin

3a denoted that the indole rings of these tryptophans experienced a significant ring current shift. A similar range of shifts was observed for the W(S – 2) of the shorter hairpin **3c**. As shown in Figure 5, strong NOESY correlations between the H_N protons of A(L2), T(L3), G(L4), and K(S + 1) secure the overall loop conformations in both **3a** and **3c**. Correlations between the W(S–2)H_{α2} and both G(L4) H_α and H_N were also consistent in both hairpin spectra. Both S ± 4 and S ± 2 W/W interactions were also confirmed by a combination of long-range side-chain to side-chain NOESY correlations. NOESY correlations between the W(S + 4) H_{ε3} and H_{ζ3} with the R(S – 5) H_α proton suggest that the C-/N-termini fraying for this hairpin is minimal at 291 K. More importantly, we found several intense H_N—H_N NOESY correlations between N(S – 1) and K(S + 1) residues in **3a** and **3c** and an H_N—H_α interaction N(S – 1) to W(S + 2) in **3a** supporting the formation of an interstrand hydrogen bond network in the β-strap. Finally, the NOESY correlation between the S ± 3 positions VH_N in **3a** definitely established the presence of a β-sheet register within a [4:6]-hairpin (Figure 5). Overall, folded populations of 84, 93, and >99% (±2%) were estimated from the δH_{α/α'}G(L4) values in the loops of hairpins **3c**, **3a**, and **3b**, respectively.

[10:12]-hairpins **3d–f** bearing the longest loops were also extensively characterized by combinations of NMR experiments. Excitingly, in both examples of flexible and rigid loops (**3d** and **3f**), a large positive CSD pattern across the β-strap residues unambiguously established the foundation of a β-hairpin register (Figure 4C). In the case of hairpin **3d** experiencing the most flexibility, both H_N and H_α of the strap residues resonated downfield (CSD of 0.30–2.15 ppm), while hydrogens of the loop residues witnessed small CSDs (<0.10 ppm). Except for Gly residues flanking the strap edges, δH_{α/α'}G of 0 ppm was observed in the loop due to its innate flexibility and a lack of asymmetric environment. By analogy to the shorter loops, folding of **3d** was estimated to be >99 ± 2% at 291 K given a δH_{α/α'}G(S + 1) of 0.55 ppm. CSDs of –0.94 and –1.07 ppm for H_{β3}, –0.44 and –0.31 ppm for H_{δ1}, and –1.25 and –1.15 ppm for H_{ε3} observed at the respective W(S – 2) and W(S + 4) of **3d** were comparable to the ring current shifts observed in hairpins **3a–c**, which confirmed that both W/W pairs were creating FtE arene interactions. As shown in Figure 5, NOESY correlations between R1H_α with W17H_{ε3} and W2H_{ε3} with W15H_β in hairpin **3d** are stronger than in **3a**, and the long-range interstrand correlation of W2H_α with W17H_N further supported a hairpin fold. These results suggested that strap **3** afforded enough stabilization to offset the flexibility of the polyglycyl loop. Seemingly, even if a high plasticity typically results in slower kinetics of nucleation,⁵¹ the thermodynamics of such systems (i.e., **3d** vs **3e**) are more favorable to a hairpin structuration due to a more flexible strand alignment. In regards to the more strained hairpin **3e**, ¹H NMR spectra at 291 K displayed overlapping and broader signals in the H_N region with small CSD values (avg. of 0.30 ppm) indicative of a rapid sampling of multiple conformer populations, which is not surprising considering that 298 K was the melting temperature of **3e** determined by CD.³⁴ In stark contrast, the rigid hairpin **3f** (one glycine mutation in the loop) was remarkably well folded with downfield deviations for H_N signals within the strap (CSD of 0.47–1.97 ppm) strikingly similar to **3d** suggesting a fine alignment. Large CSDs of –0.99 and –1.28 ppm for H_{β3}, –0.67 and –0.21 ppm for H_{δ1}, and –1.01 ppm for both H_{ε3} were also observed at the respective W(S – 2) and W(S + 4) positions. In addition, a number of medium to strong NOESY correlations within the strap were observed as exemplified by the V3H_N and

V16H_N long-range interaction. Taken together, these results conclusively demonstrate that **3f** is a well-structured hairpin with the stabilizing edge tryptophan side chains at residues W(S - 2) and W(S + 4) in a zipper-like motif. Along those lines, the loop %-folding in **3f** was estimated to be $\sim 55 \pm 2\%$ at 291 K based on the glycine shifts at the G(L7) position ($\delta H_{\alpha/\alpha'}$ of 0.30 ppm). Collectively, structural NMR data demonstrated that the RWVW...VVWE strap **3** was the most efficient hairpin-folding manifold for short and long loops of varying plasticity.

CONCLUSIONS

Accessing larger macrocyclic peptides is an emerging trend to build mini protein-like structures thermodynamically stable in water.⁵² However, the identification of β -hairpins that closely mimic the native fold of protein and antibody loops remain challenging due to the large entropic penalty associated with their conformational folding. In this study, a de novo design of short β -straps featuring a combination of ionic interaction with a zipper motif provided a benchmark manifold to fold hairpin architectures with long loops of variable plasticity (up to 10-mer). Several novel β -strap motifs of minimalist length were crafted by combining β -strand + β -cap to generate hairpins containing loops of variable length, plasticity, and turn propensity. Although strap Ac-WVW...WVW-NH₂ **2** was found to be highly stabilizing, the hydrophobic cluster modeled upon the Trpzip molecules (large C_{pF}° of -159 ± 17 cal-deg⁻¹·mol⁻¹) leads to a poor and unpractical solubility in water. This motif was further optimized in strap RW(VW)...(WV)WE **3** by combining the folding driving force associated with the W/W cluster hydrophobicity to the long-distance stabilizing features of a salt-bridge^{38,39} positioned at hairpins' termini. The CD-exciton signature at 228 nm from two W/W EtF interactions as well as the large NMR upfield CSDs (1.16–1.49 ppm and 1.19–1.21 ppm for H _{β} and H _{ϵ 3}) and long-range NOESY correlations reported for **3a** conclusively demonstrated that well-folded hairpins were formed (89–93% fold).

Finally, the strong enthalpic contribution from β -strap **3** (H_F^*) was able to remarkably offset the entropic penalty associated with the wealth of conformations generated by longer 10-mer loops.¹⁷ To our knowledge, this is the first observation of a short β -strap (8-mer long) able to structure loops longer than the strap itself (up to 88% fold, T_m of 25–52 °C). Strap **3** largely outperforms the technology previously developed by Andersen for long-loop closure (16-mer β -cap- β -strand).¹⁶ Collectively, these results suggest that the specific site positioning and combination of a terminal salt-bridge with van der Waals and CH— π hydrogen bond interactions from the two EtF W/W pairs within the strap are an efficient hairpin-folding manifold. Our results suggest that β -strap **3** might find useful applications to fold hairpin architectures with long loops of varying plasticity and random sequences. Ultimately, we anticipate that the present study will provide the foundation for the development of long hairpin foldings as an attractive alternative to macrocyclic peptides for the mimicry of proteins and antibodies.^{3,53}

EXPERIMENTAL SECTION

General Information.

All reagents, Fmoc-amino acids, and resins used in the present paper were purchased from Chemimpex and Millipore Sigma. All bulk solvents were acquired from Fischer Scientific. Peptides **1c**, **3a–c** were purchased from Peptide 2.0 Inc. Peptides, **1a–b**, **2a–b**, **3c–d**, and **SI-RC1–2** were synthesized using a standard automatized Fmoc-SPPS technique (solid-phase peptide synthesis) on a Protein Technologies PS-3 peptide synthesizer. Syntheses were accomplished either on a Rink amide resin of medium loading (0.27 mmol/g) or Fmoc-Glu-Wang resin (0.4 mequiv/g). Random coil model peptides **SI-RC3a/b** and **SI-RC4** were synthesized on a manual peptide synthesizer, using a Fmoc-FPPS (fast-parallel peptide synthesis) technique, on a Rink amide resin of medium loading (0.27 mmol/g) and Fmoc-Glu-Wang resin (0.4 mequiv/g). Our synthetic hairpin peptides have a net charge of -1 , $+1$, or $+2$ (from loop sequence) to enhance water solubility and prevent aggregation. Structural assignments were made by a set of NMR spectra including TOCSY, NOESY, HSQC, and HMBC experiments recorded on a Varian Mercury500 (500 MHz) spectrometer and processed using the Vnmrj 4.2 software.

Peptide Synthesis.

General Procedure for Peptide Synthesis Via Fmoc-SPPS.—Syntheses were carried out at room temperature in anhydrous DMF using the resins described above (200–300 mg, 1.0 equiv, as reported below for each peptide) by successive iterations of deprotection/coupling. Vials of each Fmoc-protected α -amino acid (4.0 equiv) were prepared with HBTU (4.4 equiv) and HOBt (4.4 equiv) neat. For each iteration, the deprotection/coupling sequence entails (1) wash of the resin with DMF (3×5.0 mL) for 0.5 min each, (2) Fmoc-deprotection run twice using an excess of piperidine in DMF (20% v/v, 5.0 mL) for 5 min each, (3) wash with DMF (6×5.0 mL) for 0.5 min each, (4) the cocktail from the entire vial (described above) was dissolved with *N*-methylmorpholine in DMF (3.0 mL, 4 M) and added for a 40 min coupling, (5) wash with DMF (3×5.0 mL) for 0.5 min each, finalized the sequence. After the final *N*-terminal coupling, a final Fmoc-deprotection was achieved (step 2). The resulting peptides attached to the resin were washed with CH_2Cl_2 (2×10 mL), and the resin was dried under vacuum before storage under an argon atmosphere at -78 °C until cleavage.

General Procedure for Peptide Synthesis Via Fmoc-FPPS.—Syntheses were carried out at room temperature in anhydrous DMF using the resins described above (200–300 mg, 1.0 equiv, as reported below for each peptide) by successive iterations of deprotection/coupling. For each iteration, the deprotection/coupling sequence entails (1) resin swelling in DMF (10 mL) for 10 min, (2) Fmoc-deprotection run twice using an excess of piperidine in DMF (20% v/v, 5.0 mL) for 2 min each, (3) wash with DMF (3×5.0 mL) for 0.5 min after each deprotection, (4) coupling reaction run twice for 5 min each by adding in order Fmoc-amino acid stock solution (5 equiv in 5 mL DMF), HATU stock solution (5 equiv in 5.0 mL DMF), and DIEA (10 equiv), and (5) wash with DMF (3×5.0 mL) for 5 min after each coupling reaction. After the final *N*-terminal coupling, a final Fmoc-deprotection was achieved (step 2). The resulting peptides attached to the resin were

washed with CH_2Cl_2 (2×10 mL), and the resin was dried under vacuum before storage under an argon atmosphere at -78°C until cleavage.

General Procedure for Peptide Deprotection and Cleavage from Resins.—The same protocol of simultaneous resin cleavage and sidechains' deprotection was applied to both resins. The dried resin was suspended in a cleavage cocktail (TFA/thioanisole/EDT/anisole, 90:5:3:2 v/v, 1.0 mL per 20 mg of resin) and shaken for 1.5 h at RT. The mixture was filtered to remove the resin and the mother liquor was evaporated on a rotary evaporator. The crude peptides (~250 mg) were precipitated in cold ether (40 mL), then centrifuged, and washed with cold ether (3×40 mL). The resulting crude peptides were solubilized in water and lyophilized before being stored as dry powders under an argon atmosphere at -78°C .

All lyophilized crude peptides were analyzed by analytical reverse-phase high-performance liquid chromatography (RP-HPLC) Hitachi L-7000 series equipped with an XBridge BEH C_{18} column (130 Å, 10 μm , 4.6 mm $\times$ 250 mm). HPLC grade acetonitrile and deionized water, each containing 0.1% trifluoroacetic acid, were used for analytical and semipreparative RP-HPLC. Each peptide was analyzed using a gradient from 10 to 50% of acetonitrile over 30 min with a flow rate of 1.0 mL/min at room temperature and a detection at 220 nm. Yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. If required, peptides were purified with a Hitachi L-7000 series HPLC equipped with a semipreparative XBridge BEH C_{18} column (130 Å, 10 μm , 10 mm $\times$ 250 mm) stationary phase by scaling up the analytical conditions. The pure fraction of peptides was collected and analyzed by analytical RP-HPLC and mass spectrometry using a Microflex LRF matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) instrument.

Nuclear Magnetic Resonance Data Collection.

NMR samples were prepared by dissolving the freeze-dried peptide (~1–2 mg) in a mixture of phosphate buffer (50 mM, pH 6.5) and D_2O (9:1, v/v) unless otherwise stated, using 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as the internal standard for chemical shifts (0 ppm). Samples were prepared in a range of 3–10 mM of the peptide for ^1H NMR, TOCSY (mixing time 80 ms), NOESY (mixing time 200 ms), and ^1H — ^{13}C HSQC experiments and in a range of 10–20 mM to record ^1H — ^{15}N HSQC and HMBC spectra (with the exception of **SI-RC1**, **1a**, and **1b** samples, which were prepared in a range of 10–70 mM range). A PRESAT experiment was used to suppress the H_2O solvent signal, to record initial ^1H NMR spectra. All spectra were recorded at 291 K (18°C) on a Varian Mercury500 (500 MHz) spectrometer and processed using the Vnmrj 4.2 software. Signals assignments were obtained on the basis of a set of ^1H and TOCSY spectra (for intraresidue connectivities), NOESY spectra (for vicinal and interstrand backbone connectivities), and HSQC spectra (for H_α to C_α connectivities and W side-chains assignments).

Chemical Shift Deviation (CSDs).

Chemical shift deviations (or CSD) have been calculated based on a simulated unfolded reference that represents the predicted (calcd.) chemical shift values along the random coil sequence, based on the established work from Poulsen and others.⁴⁴ The calcd. random coils' chemical shifts for the backbone H_α and H_N protons were obtained from the online

software available at: https://spin.niddk.nih.gov/bax/nmrserver/Poulsen_rc_CS/. The random coil calcd. δ values were validated by comparison to the experimental values obtained for **SI-RC1-4** ($|\text{CSD}| = 0.20$ ppm). CSDs for each hairpin construct were then calculated according to $\text{CSD(H)} = \delta\text{H}_{\text{exp.}} - \delta\text{H}_{\text{calcd.}}$ and reported in tables (Supporting Information Sections 2-4). Furthermore, CSDs related to the side chains of tryptophan residues have been calculated from the list of known chemical shifts reported on the BMRB database (Biological Magnetic Resonance Bank) for Trp in random coils: http://www.bmrb.wisc.edu/ref_info/pentapeptide.tbl. This list of chemical shifts (Trp random coils) has been previously validated by Andersen et al.²⁶

Far-UV Circular Dichroism (CD) Spectroscopy.

Peptide solutions were prepared at the 20–100 μM concentration range in phosphate buffer (15 mM, pH 6.5), with the addition of MeOH (up to 50% v/v) if required to increase solubility. Each sample concentration was determined accurately by measuring the solution UV-absorbance using a JASCO V-670 spectrophotometer based on the combined molar absorptivity of Trp and Tyr residues at 280 nm ($\epsilon_{280} = 5580 \text{ M}^{-1}\cdot\text{cm}^{-1}$ per Trp, $\epsilon_{280} = 1280 \text{ M}^{-1}\cdot\text{cm}^{-1}$ per Tyr). CD spectra were recorded on a JASCO J-810 Spectropolarimeter with a temperature controller module JASCO PFD-425S. In brief, raw CD data were recorded in mdeg from 185 to 270 nm every 0.1 nm, and CD spectra of the blank solutions were subtracted for baseline correction. Spectra were smoothed with the baseline set to zero between 260 and 270 nm and the ellipticity scale was converted into molar ellipticity ($\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$) using SpectraGryph 1.2.⁵⁴ To obtain melting curves representing peptide unfolding transitions, variable-temperature CD experiments were recorded. For **SI-RC1**, **1a**, **1b**, and **2b**, VT-CD spectra were manually recorded every 5 °C from 5 to 95 °C with a 10 min stabilization between each temperature. CD spectra of the blank solution were recorded similarly and subtracted to the peptide spectra at each temperature. Molar ellipticity values at 228 nm (Trp exciton band) were plotted to obtain experimental melting curves. For **SI-RC2-4**, **1c**, **2a**, and **3a-e**, the temperature ramp was controlled automatically from 5 to 95 °C with a ramp speed of 0.75 °C/min. Intensity values at 228 nm were recorded by the instrument every 0.1 °C to obtain the desired raw melting curves. Likewise, intensities for the corresponding blank solutions were recorded and subtracted to afford the experimental melting curves' output. These thermal denaturation curves were reported in molar ellipticity ($\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$) and normalized against the number of tryptophan pairs present in the peptide construct ($[\theta(T)]_{228}/p\text{Trp}$) for comparison (see Figure S2). Finally, a nonlinear least square fitting routine program has been developed in Origin 9.0 (Originlab Corporation) to compute the best-fitted melting curves to the raw $[\theta(T)]_{228}$ data by using the thermodynamic Gibbs–Helmholtz equation of free-energy variation for a two-state model (eq S2). A detailed description for obtaining the %-folding best-fitted curves, all calculations, G° , H° , S° , C_p° , and T_m thermodynamic values is given in the Supporting Information Section 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Strand alignment and H-bonding pattern in folded [4:6]-hairpins based on DDATKT and NPATGK sequences. (B) Schematic representation and nomenclature of a [10:12]-hairpin displaying the 10-mer CDR-H3 loop excised from the pembrolizumab antibody target.

❑ **Previous approach.** Long β -strand and β -cap stabilizing motifs

Typical length (cap + strand) $\geq$ 10-12 residues
Restricted by loop sequence and length

❑ **This work.** Minimalist β -strap = β -strand + β -cap

- 1: Ac-W...WTG-NH₂ (4 residues)
- 2: Ac-WVW...WVW-NH₂ (6 residues)
- 3: RW(VW)...(WV)WE (4 or 8 residues)

β -Straps adapted to the folding entropy of long loops

Figure 2.
Design of short stabilizing β -strap motifs for the hairpin fold.

Figure 4.

Histograms of backbone H_N , H_{α} , and Trp side-chain CSD values obtained at 291 K along the hairpin primary sequences. (A) In the series of strapped β -hairpins **1a–c** and **2a–b**. (B) In the series of strapped β -hairpins **3a–c**; the arrow on the histogram points to the unusually deshielded H_N (G4) loop signal, suggesting the presence of an intramolecular H-bond as expected in a [4:6]-hairpin register. (C) In the series of strapped β -hairpins **3d–e**.

Figure 5.

Structural analysis of hairpins **3a**, **3c-d**, and **3f** by NOESY correlations establishing side-chain to side-chain interactions, the loop orientation, and the hydrogen-bonding register within β -straps^{a,b} ^aSelected key NOESY correlations recorded at 291 K, with edge arenes involved in EtF arene–arene interactions highlighted in light orange. ^bHydrogens circled (in pink) in the NOESY diagrams participate in a network of intramolecular hydrogen bonds characteristic of a hairpin register.

Table 1.

Truncated β -strap optimization inspired by known GB1-analog sequences and previously reported hairpin stabilities^{a-g}

Entry	Name	Peptide sequence ^a	T_m (°C) ^{b,c}	Loop %-fold χ_F at 291-298K ^{d,e}	β -Strap %-fold χ_F at 291K ^f	% Yield ^g
1 ²⁶	Loop	Ac-NPATGK-NH ₂	n.d.	6 ± 7% ^d	n.d.	-
2 ²⁶	Tr-HP7	Ac-W-NPATGK-W-NH ₂	3-7	49 ± 1% ^d	n.d.	-
3 ²⁶	HP7	KTW-NPATGK-WTE	66	89 ± 1% ^d	n.d.	-
4 ^{23,27}	GB1m3	KKWTV-NPATGK-FTVQE	60	94 ^d -97 ± 6%	n.d.	-
5 ^{25d}	Trpzip9	GEWVW-DDATKT-WVWTE-NH ₂	92	n.d.	n.d.	-
<i>Novel peptides from this study--- [4:6]-& [8:10]-Hairpins</i>						
6	1a	Ac-W-NPATGK-WTG-NH ₂	<0	42 ± 4% (70%) ^e	28 ± 1%	57
7	1b	Ac-W-DDATKT-WTG-NH ₂	20.0 ± 2.5	n.a.	54 ± 7%	32
8	1c	Ac-WTF-NPATGK-FKWTG-NH ₂	30.3 ± 0.1	91 ± 2% (151%) ^e	63 ± 4%	-
9	2a	Ac-WVW-NPATGK-WVW-NH ₂	101.1 ± 0.1	93 ± 2% (154%) ^e	99 ± 1%	15
10	2b	Ac-WVW-GPGTGK-WVW-NH ₂	23.9 ± 2.5	6 ± 33% (9%) ^e	56 ± 1%	15
11	3a	RWVW-NPATGK-WVWE	97.9 ± 0.1	93 ± 2% (154%) ^e	89 ± 14%	-
12	3b	RWHW-NPATGK-WVWE	80.7 ± 0.1	>99 ± 2% (167%) ^e	95 ± 2%	-
13	3c	RW-NPATGK-WE	61.6 ± 0.1	84 ± 2% (139%) ^e	84 ± 11%	-
14	3d	RWVW-G₄KKG₄-WVWE	51.8 ± 0.1	>99 ± 2%	88 ± 1%	70
15	3e	RWVW-RDYRFDMGFD-WVWE	25.3 ± 0.1	22 ± 8%	55 ± 1%	30
16	3f	RWVW-RDYRGDMGFD-WVWE	46.7 ± 0.1	55 ± 3%	82 ± 1%	35

^a All HB-sites from the β -strand/strap including the (S±1) positions are underlined, and residues from hydrophobic clusters and β -straps are bolded in the sequences.

^b T_m are estimates of melting transition temperatures obtained from the CD thermal denaturation curves ([θ (T)]228), based on the W/W pairs exciton couplet at 228 ± 1 nm.

^c For all novel hairpins **1-3**, T_m values were obtained from the best-fitted curves of [θ (T)]228 to the Gibbs-Helmholtz equation. The loop %-folding in the NPATGK loops were previously determined at 298K from either

^d an average of H_N CSD values at T(L3), K(S+1) and from the G(L4) $\delta H_{CD}/a'$, assuming KIR-HP7 as the fully-folded state, see reference [26]

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solely from $\delta H_{\alpha, \alpha' G(L4)}$, assuming a value of 0.33 ppm as the fully-folded state from a macrocycle, see reference [27]; Loop %-folding from this study were calculated from the same $\delta H_{\alpha, \alpha' G(L4)}$ ppm confidence on the model (reported in parentheses), and further corrected assuming hairpin **3b** as fully-folded (0.55 ppm). Standard errors for the folded populations were estimated by assuming a 0.01 ppm confidence on the chemical shift values assigned by 1H NMR.

J_{Local} %–folding calculated from the best-fitted melting curves of $[\theta(T)]_{228}$.

g Isolated yields from either crude or purified peptides (see Table 2).

Summary of Peptide Characterization

Table 2.

peptides	yield ^a (%)	HPLC %-purity ^b (net charge)	m/z [M + H] ⁺		m/z [M + Na] ⁺		solubility (mM) ^d	$[\theta]_{278}^{25} K_e$ max	NMR samples preparation ^f
			calcd	found ^c	calcd	found ^c			
SI-RC1	81 ^(a2)	88 (1)	1046.15	1046.08	1068.14	1067.94	20.0	n.a.	A1
1a	57 ^(a2)	97 (1)	1159.27	1158.91	1181.26	1181.28	2.9	120×10^3	A1
1b	32 ^(a2)	78 (–1)	1221.53	1221.59	1244.28	1244.11	35.3	77×10^3	B
1c		98 (2)	1681.84	1682.12	1703.83	1704.11	22.3	328×10^3	A1
SI-RC2	55 ^(a1)	94 (1)	1370.70	1370.52	1392.68	1392.62	78.0	n.a.	A1
2a	15 ^(a1)	96 (1)	1570.80	1570.85	1592.77	1592.92	1.2	105×10^3	C1
2b	15 ^(a1)	95 (1)	1499.76	1500.54	1521.74	1522.54	0.8	262×10^3 (e1)	C2
SI-RC3a	91 ^(a2)	98 (1)	927.49	927.91	949.47	949.85	25.0	n.a.	A1
SI-RC3b	83 ^(a2)	82 (0)	905.45	905.94	927.43	927.94	10.0		
3a		90 (1)	1815.08	1815.65	1836.89	1837.54	27.1	667×10^3	A1
3b		98 (2)	1852.90	1853.64			56.3	522×10^3	A1
3c		>99 (1)	1244.62	1244.62			39.5	301×10^3	A1
SI-RC4	45 ^(a1)	71 (2)	2273.04	2273.50	2295.03	2295.45	88.0	n.a.	A1
3d	70 ^(a1)	88 (2)	1958.97	1959.63	1980.96	1980.76	5.8	406×10^3	A2
3e	30 ^(a1)	93 (–1)	2549.16	2549.60			0.6	158×10^3 (e2)	D
3f	35 ^(a1)	99 (–1)	2459.11	2459.39	2481.09	2481.35	8.2	124×10^3	E

^a Isolated yields; (a1) of peptides obtained after semipreparative RP-HPLC purification; (a2) of crude peptides corrected based on purity determined by analytical HPLC.

^b Determined by analytical RP-HPLC from peak integration detected by UV-absorbance at 220 nm from samples of lyophilized peptides.

^c Determined by MALDI-TOF mass spectrometry.

^d Kinetic solubility measured in a phosphate buffer (PB, 50 mM) at pH 7.4.

^e Maximum molar ellipticity (deg·cm²·dmol^{–1}) recorded for peptide solutions prepared in PB (15 mM, pH 6.5) unless otherwise stated: (e1) PB (15 mM)/CH₃OH mixture (90:10 v/v) (e2) (80:20 v/v).

^f NMR data set typically recorded in A1 PB (50 mM)/D₂O (90:10 v/v) or A2 PB (15 mM)/D₂O (90:10 v/v), or B PB (50 mM)/ammonium bicarbonate (100 mM)/D₂O (84:6:10 v/v), C1 PB (50 mM)/CD₃CO₂D/D₂O (85:05:10 v/v), C2 (80:10:10 v/v), D PB (50 mM)/DMSO-*d*⁶ (70:30 v/v), E H₂O/DMSO-*d*⁶ (85:15 v/v).