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Hydrogen Bond Surrogate Stabilization of β -Hairpins

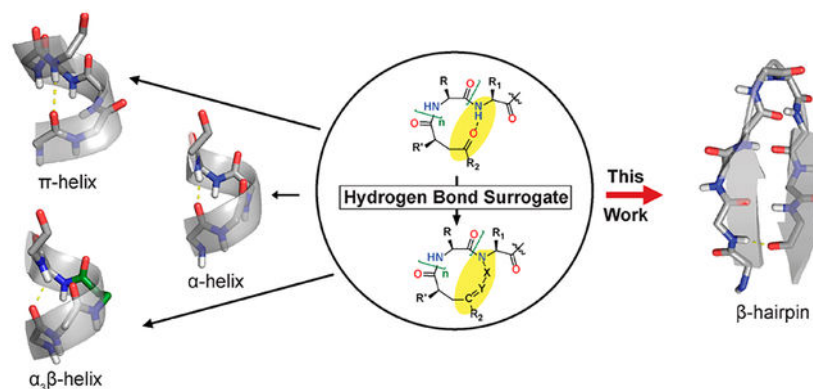
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Abstract

Peptide secondary and tertiary structure motifs frequently serve as inspiration for the development of protein–protein interaction (PPI) inhibitors. While a wide variety of strategies have been used to stabilize or imitate α helices, similar strategies for β -sheet stabilization are more limited. Synthetic scaffolds that stabilize reverse turns and cross-strand interactions have provided important insights into β -sheet stability and folding. However, these templates occupy regions of the β -sheet that might impact the β -sheet's ability to bind at a PPI interface. Here, we present the hydrogen bond surrogate (HBS) approach for stabilization of β -hairpin peptides. The HBS linkage replaces a cross-strand hydrogen bond with a covalent linkage, conferring significant conformational and proteolytic resistance. Importantly, this approach introduces the stabilizing linkage in the buried β -sheet interior, retains all side chains for further functionalization, and allows efficient solid-phase macrocyclization. We anticipate that HBS stabilization of PPI β -sheets will enhance the development of β -sheet PPI inhibitors and expand the repertoire of druggable PPIs.

Graphical Abstract



Peptides that fold into stable secondary and tertiary structures have become powerful tools to mimic molecular recognition epitopes and modulate protein–protein interactions (PPIs) to

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Author Contributions

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Supporting Information

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Methods, additional tables, and figures (PDF)

The authors declare no competing financial interest.

probe biological pathways.^{1–4} A range of stabilization strategies have been validated for common secondary (α -helix,^{5–13} β -strand^{14,15}) and tertiary structures (coiled coils,¹⁶ β -sheets^{17–22}) as well as nonregular structures.^{4,23,24}

The hydrogen bond surrogate (HBS) approach is unique among these strategies in that the resulting peptides retain all side chains to confer desirable properties. In the HBS approach, a main chain hydrogen bond is replaced with an isosteric covalent linkage.^{5,25,26} Because only a main chain hydrogen bond is replaced, all side chains can be manipulated to control binding affinity and specificity. The HBS approach was originally developed to nucleate the α -helical conformation.^{5,25–28} For HBS α -helix stabilization, compatible functional groups replace atoms involved in the N-terminal main-chain hydrogen bond, namely, the carbonyl of position i and the amide hydrogen of position $i + 4$. Reaction of these functional groups produces a 13-membered macrocycle positioned to nucleate an α -helix. HBS linkers containing olefin,²⁷ thioether,²⁹ and disulfide³⁰ groups have been validated for their ability to nucleate the α -helical conformation and inhibit a variety of α -helix-mediated PPIs.^{2,31–37}

Exploratory studies have suggested that the HBS approach can also be used to stabilize other macrocycle sizes through main chain hydrogen bond mimicry.^{26,27,38,39} For instance, peptides containing 14- and 16-membered HBS macrocycles have been synthesized. The former incorporates β -amino acids at every fourth amino acid ($\alpha_3\beta$) to promote peptides' proteolytic stability while maintaining a helical conformation.⁴⁰ The latter yields a macrocycle that is predicted to mimic a π helix—a high energy conformation rarely seen in proteins.⁴¹

We envisioned that the HBS approach could be generalized to mimic macrocycles with diverse hydrogen bonding patterns. Specifically, we hypothesized that the HBS approach could be used to stabilize β -sheet structures, which also feature prominently at PPI interfaces.⁴² Many strategies have been evaluated to generate stable macrocyclic β -sheet peptide mimics. These seminal efforts include various stabilized turn motifs (e.g., D-Pro/L-Pro,^{17,18} D-Pro/Gly,⁴³ L-ornithine⁴⁴) and covalent and noncovalent side chain bridges (e.g., cystine disulfide,⁴⁵ 1,2,3-triazole,^{22,46,47} diphenylacetylene,²¹ tryptophan zipper¹⁹) to stabilize the folded conformation (Figure 1). The salient feature of the HBS strategy is that natural sequences can be stabilized using a minimalist template by converting a key hydrogen bond to its covalent mimic. To test the hypothesis that replacement of a single hydrogen bond with a covalent bond is enough to provide a stable β -sheet conformation, we examined HBS stabilization of β -hairpins, which are the smallest unit of antiparallel β -sheets.

We chose the HP7 β -hairpin sequence as a model system. HP7 is a minimal β -hairpin designed by Andersen et al. and is one of the smallest stable β -hairpin sequences.⁴⁸ Importantly, destabilized HP7 variants have also been reported, allowing us to determine if HBS macrocyclization could rescue the conformational stability of a destabilized variant.

We began by replacing the N-to-C-terminal salt bridge, which occupies a hydrogen bonding position, with a hydrocarbon mimic enabled by a ring closing metathesis reaction (Figure S1). The olefin HBS stabilizes tertiary interactions consistent with β -hairpin formation.

Figure 2A–D shows structures and circular dichroism (CD) spectra for HP7, its destabilized variant HP7^Δ, and HP7^Δ with an HBS linking the N- and C-termini (HP7^Δ-HBS). Characteristic features of HP7 include a maximum at 228 nm resulting from exciton coupling between interacting cross-strand Trp residues arranged in an edge-to-face orientation,^{19,49} a minimum at 215 nm consistent with β -sheet formation, an inflection point near 201 nm, and a local maximum at 190 nm. Importantly, the 228 nm peak is a strong indicator of β -hairpin formation in HP7, as it requires interaction between the tryptophan residues on opposite strands. Compared to HP7, peptides that replace the terminal salt bridge with a hydrogen bond or remove the terminal Lys/Glu residues (HP7^Δ)⁴⁸ show decreased stability across the whole wavelength range (Figures 2D, S2).

The CD spectrum of HP7^Δ-HBS shows intensity recovery across the spectrum, with complete recovery of the 228 nm Trp–Trp interaction peak intensity. Significantly, the spectrum is nearly identical to the spectrum for an HP7 cystine disulfide macrocycle that serves as a traditional model for full β -hairpin folding (Figure S2C). Temperature-dependent CD spectra (Figures S2, S3) further support the hypothesis that incorporating the covalent HBS linkage into HP7^Δ improves β -hairpin stability to a greater extent than a terminal hydrogen bond or even a strong salt bridge interaction.

NMR spectroscopy studies confirmed formation of a stable β -hairpin formation in HP7^Δ-HBS. Two-dimensional TOCSY and NOESY spectra were acquired (Figures 2E, S4), showing excellent agreement with the previously reported HP7 spectra (except for protons within and adjacent to the HBS). ³J_{NHC α H} coupling constants and NOE data support the formation of the expected β -hairpin structure. Simulations using NOE and ϕ angle constraints (derived from ³J_{NHC α H} coupling constants) produced a structural ensemble that overlays very well with the original HP7 β -hairpin (Figure 2E).

One of the weaknesses of many peptides in clinical applications is their susceptibility to protease degradation, which limits their effective dose. We hypothesized that HBS macrocyclization of the HP7^Δ peptide would improve its stability relative to HP7^Δ, as observed with HBS stabilization of α -helices.³³ We measured the amount of peptide remaining at various times after incubation with high (100 μ g/mL) concentrations of the broad-specificity proteinase K. This stringent assay allowed us to calculate half-lives of each peptide. While HP7^Δ has a half-life of 36 s and HP7 has a half-life of 120 s under these conditions, HP7^Δ-HBS has a half-life of 770 s, a >20-fold improvement over HP7^Δ from which it is derived (Figure 2F).

Next, we investigated the effects of different HBS linker chemistry on β -hairpin folding and stability to determine if a thioether bridge, which does not require a metal catalyst for synthesis, can be installed in place of the hydrocarbon bridge (Figures 3, S5). We also explored a disulfide bridge to develop a strategy for reversibly probing β -hairpin formation. Previous studies with HBS stabilization of α -helices examined these three chemistries—olefin, thioether, and disulfide—and showed that all three HBS linkers stabilize the α -helix to promote PPI inhibition.^{29,32,50} In the olefin HBS, the C–C double bond mimics the N-terminal amide carbonyl, providing linker rigidity. The thioether and disulfide HBS linkers lack this rigidifying double bond (though disulfide bonds show specific conformational

preferences).⁵¹ CD spectra of HP7 peptides with each HBS linker reveal that all three chemistries fully restore the Trp–Trp interaction peak at 228 nm (Figure 3). Surprisingly, both thioether (HP7 -teHBS) and disulfide (HP7 -dsHBS) HBS peptides show increased spectral intensity in regions of the spectra (212–218 nm and 190–195 nm) that are characteristic of β -sheets. These data suggest that the more rigid olefin HBS may stabilize a strained β -sheet while the more flexible thioether and disulfide HBS linkers may relax any distortion.

We also examined the potential of the disulfide macrocycle HP7 -dsHBS to serve as a redox-sensitive β -hairpin. Reduction of HP7 -dsHBS to the bis-thiol peptide (HP7 -bt) using tris(2-carboxyethyl)phosphine (TCEP) showed significant loss of structure (Figure S6). The loss of structure upon reduction mirrors the difference between the structured HP7 β -hairpin and the destabilized HP7 variant (Figure 1). Temperature-dependent CD shows that HP7 -bt forms a stable β -hairpin at 5°C but rapidly loses structure, displaying ~50% loss of initial 5°C structure at physiological temperature. In contrast, HP7 -dsHBS retains >50% of its original 5°C structure even at 95°C.

Finally, we evaluated the generality of the β -hairpin HBS strategy for stabilizing protein-derived β -hairpins. We selected a β -hairpin from the Ras-binding NS1 monobody designed by Koide et al.⁵² as a prototypical PPI β -hairpin featuring a common type II' turn rather than the optimized turn in HP7. Comparison of the CD spectra for the unconstrained and HBS peptides shows dramatic stabilization of the HBS peptide in the β -hairpin conformation compared to the unconstrained peptide (Figure 4). In addition to the characteristic β -sheet minimum at 215 nm, we observed spectral signatures of cross-strand interactions between Trp and Tyr at 230 and 199 nm.^{49,53} Even at 95°C, we observed only a 25% decrease in spectral intensity at 215 nm. A cystine disulfide macrocycle of the same NS1 sequence shows similar intensity at 215 nm, suggesting that the NS1 HBS is well-folded (Figure S7).

Overall, we found that the hydrogen bond surrogate approach can be generalized to stabilize nonhelical peptide conformations, specifically the β -hairpin. Using the HP7 sequence, we confirmed β -hairpin stabilization by CD and NMR spectroscopy. Conformational stabilization also confers significant protease resistance, which is promising for designing β -hairpin HBS PPI inhibitors with potential *in vivo* applications. Comparison of the hydrocarbon, thioether, and disulfide HBS linkers suggests that the thioether and disulfide bridges provide more β -hairpin stability. This finding is consistent with earlier findings with HBS-stabilized α -helices, which showed that rigidity and the hydrogen bond isostere geometry provide subtle control over the overall peptide conformation.²⁵ Taking synthetic ease into account as well, the thioether β -hairpin HBS is preferred for generating metabolically stable β -hairpins while the disulfide β -hairpin HBS provides a redox trigger for reversible β -hairpin formation.⁵⁴

The β -hairpin HBS approach provides a generalizable scaffold for targeting β -hairpin or β -sheet-mediated PPIs. Computational analysis of the protein complexes in the Protein Data Bank (PDB) demonstrated that interfacial β -strands are key elements in thousands of PPIs.⁴² These interfacial β -strands interact with their partners in various binding modes that involve complex combinations of main- and/or side-chain interactions using one or both faces of the

β -strand. Because the β -hairpin HBS approach described here replaces only an internal hydrogen bond that cannot participate in molecular recognition, all main- and side-chain functional groups from the original β -hairpin can be incorporated and/or optimized for binding affinity and specificity.

An increasing number of designed proteins that use β -hairpins or β -sheets for molecular recognition of targets may facilitate further development of β -hairpin PPI inhibitors.^{52,55–59} As compared to native PPIs that are optimized for overall function in their biological context (not strictly binding) and constrained by their evolutionary history, designed proteins undergo focused optimization for target protein binding affinity and specificity. It is therefore likely that binding epitopes extracted from these proteins will have higher initial affinities and require less optimization than epitopes from native PPIs. Studies to generate β -hairpin HBS peptides from both native and designed β -hairpin binding proteins are underway and will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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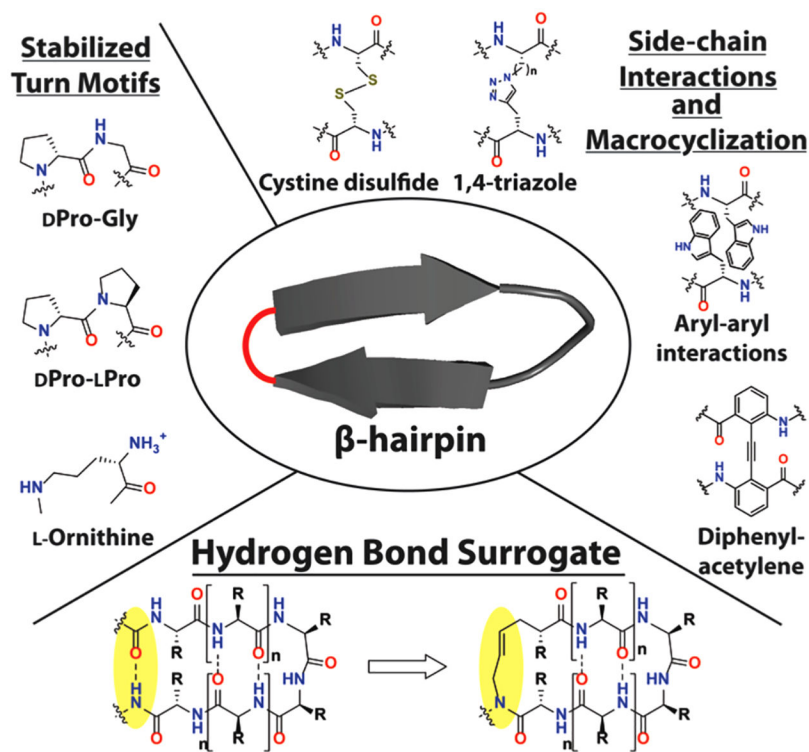


Figure 1. Strategies for stabilizing β -hairpins by macrocyclization. Previous templates to stabilize β -hairpin macrocycles include stabilized turn motifs (left) and side-chain interactions. The hydrogen bond surrogate replaces a cross-strand hydrogen bond (bottom), retaining all side chains for functionalization.

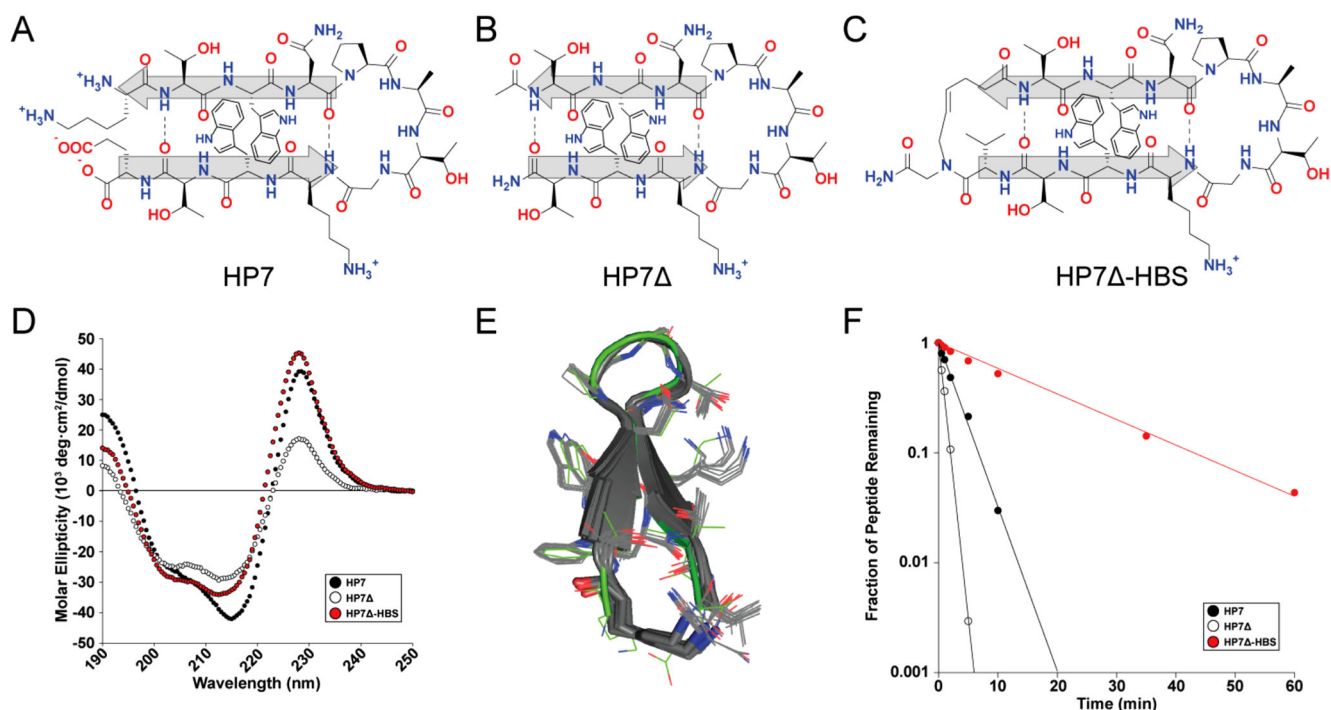


Figure 2.

HP7 β -hairpin conformational and proteolytic stability using an olefin HBS linkage. (A–C) Structures of the model β -hairpin peptide HP7, the destabilized variant HP7 Δ , and HP7 with an olefin HBS linkage (HP7 Δ -HBS), respectively. (D) Circular dichroism spectra of HP7 (black), HP7 Δ (white), and HP7 Δ -HBS (red). (E) An ensemble of the 20 lowest-energy HP7 Δ -HBS conformations derived from Monte Carlo simulations with NMR-derived torsion angle and NOE constraints (gray) overlaid with the HP7 structure (green). (F) Resistance of HP7 peptides to proteinase K digestion.

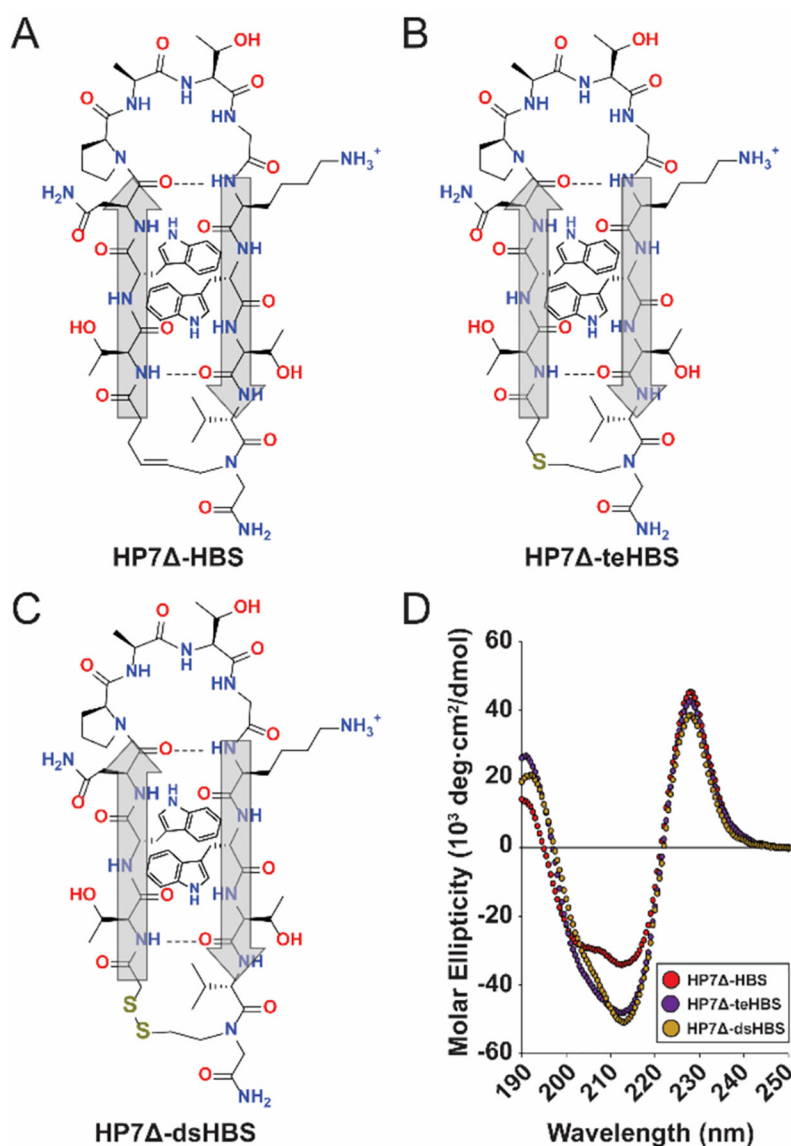


Figure 3. Comparison of β -hairpin conformational stability using different HBS linkages. (A–C) Structures of HP7 Δ -HBS peptides with olefin (HP7 Δ -HBS), thioether (HP7 Δ -teHBS), and disulfide (HP7 Δ -dsHBS) HBS linkages, respectively. (D) Circular dichroism spectra of olefin (red), thioether (purple), and disulfide (yellow) HBS peptides.

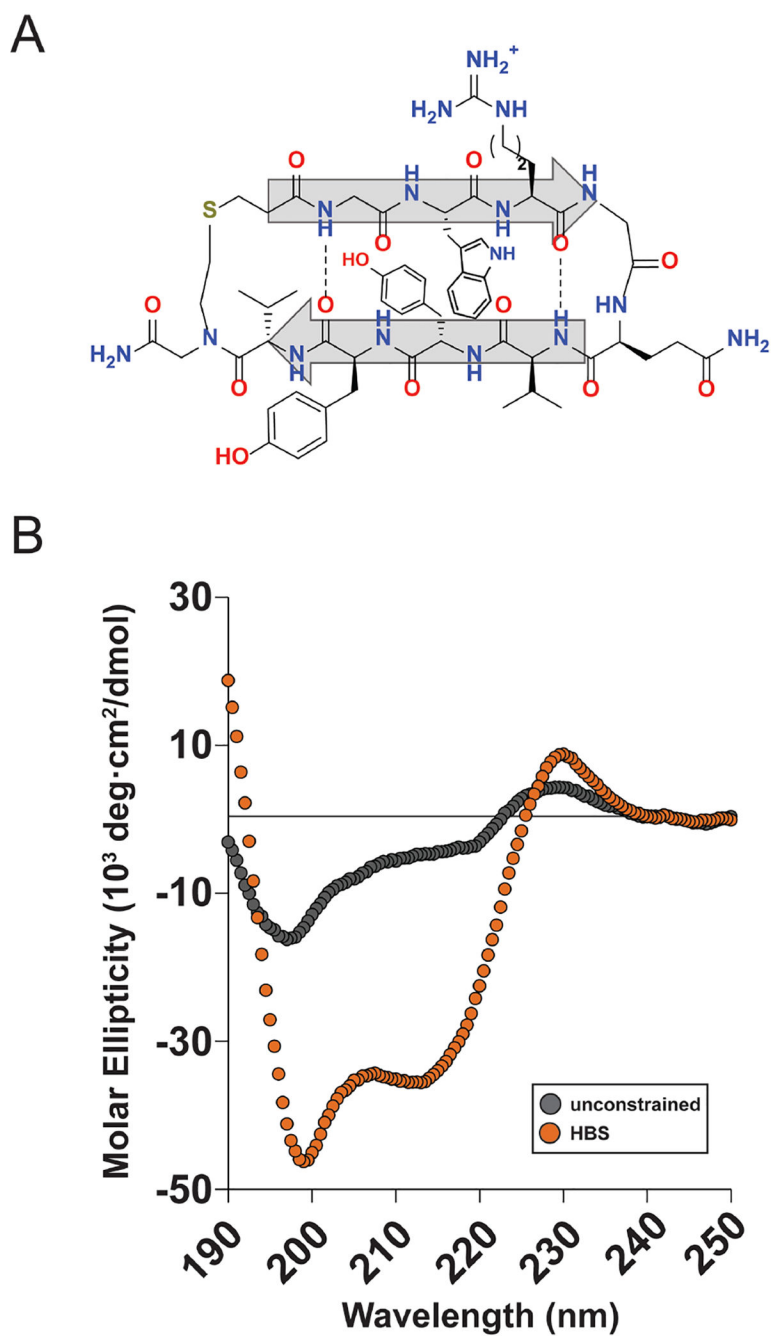


Figure 4. HBS stabilization of a protein-derived β -hairpin. (A) Structure of the NS1-derived HBS. (B) Circular dichroism spectra for NS1 unconstrained (gray, Ac-GGWKGVYYVG-CONH₂) and HBS (orange) peptides.