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Electronic Supporting Information for

## Structural impact of thioamide incorporation into a β-hairpin

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#### **Table of Contents**

General Information	
Synthesis and Characterization of Thioamide Precursors	
Synthesis and Purification of ThioThreonine (Fmoc-Thr <sup>S</sup> -NBt) Precursor	
Peptide Synthesis, Purification, and Characterization	6
НРТ	6
YKL	7
Circular Dichroism	
HPT	
YKL	
Nuclear Magnetic Resonance Spectroscopy	
НРТ	
YKL	
HPT-Leu <sup>S</sup> 11-OH Test and Unfolded Epimerization	
Structural Models of the Thioamide-Containing Folded Control β-Hairpins	
Structural Ensembles of the Thioamide-Containing Folded Control β-Hairpins	
Previous β-Hairpin Modification Studies	56
Steric Interactions	56
π-Interactions	56
Ion-pairing	
Backbone	
References	

## **Supplemental Figures**

Figure S1. Analytical HPLC of HPT β-hairpins	9
Figure S2. Analytical HPLC of YKL β-hairpins	10
Figure S3. <sup>1</sup> H NMR overlay of thioamide-containing Test YKL β-hairpins acquired at different temperature	es 15
Figure S4. Proposed mechanism of Edman-type degradation observed for the HPT-Leu <sup>S</sup> 11-OH Unfolded an	nd Test
β-hairpins	26
Figure S5. CSD analysis ( $\delta_{H\alpha}$ ) of the HPT-Leu <sup>S</sup> <sub>11</sub> -OH epimers	27
Figure S6. Far-UV circular dichroism (CD) of thioamide-containing Test YKL β-hairpins	29
Figure S7. NOEs for Test YKL β-hairpins	30
Figure S8. NOEs for Unfolded YKL β-hairpins	31
Figure S9. $\Delta \delta_{NH}$ (Test $\delta_{NH}$ – Unfolded control $\delta_{NH}$ ) for YKL: Glu <sup>S</sup> <sub>4</sub> , Lys <sup>S</sup> <sub>9</sub> , and Leu <sup>S</sup> <sub>11</sub> -OH	32
Figure S10. Far-UV circular dichroism (CD) of thioamide-containing HPT β-hairpins	32
Figure S11. NOEs for Test HPT β-hairpins	33
Figure S12. NOEs for Unfolded HPT β-hairpins	34
Figure S13. $\Delta \delta_{NH}$ (Test $\delta_{NH}$ – Unfolded control $\delta_{NH}$ ) for HPT: Thr <sup>S</sup> <sub>2</sub> , Val <sup>S</sup> <sub>3</sub> , Ile <sup>S</sup> <sub>10</sub> and Leu <sup>S</sup> <sub>11</sub> -OH	35
Figure S14. NOEs for Folded HPT β-hairpins	
Figure S15. ROESY mixing time comparison for the HPT scaffold	37
Figure S16. Generation of HPT Folded control from YKL derivative	39
Figure S17. Constrained relax with NOEs to generate HPT Folded control structure	39
Figure S18. Modelled structure of HPT Folded control	46
Figure S19. Modelled structure of HPT-Thr <sup>S</sup> <sub>2</sub> Folded control peptide	47
Figure S20. Modelled structure of HPT-Val <sup>S</sup> <sub>3</sub> Folded control peptide	48
Figure S21. Modelled structure of HPT-Ile <sup>S</sup> <sub>10</sub> Folded control peptide	49
Figure S22. Modelled structure of HPT-Leu <sup>S</sup> <sub>11</sub> Folded control peptide	50
Figure S23. Ensemble generation of the HPT-Thr <sup>S</sup> <sub>2</sub> Folded control peptide with the backrub protocol	52
Figure S24. Ensemble generation of the HPT-Val <sup>S</sup> <sub>3</sub> Folded control peptide with the backrub protocol	53
Figure S25. Ensemble generation of the HPT-Ile <sup>S</sup> <sub>10</sub> Folded control peptide with the backrub protocol	54
Figure S26. Ensemble generation of the HPT-Leu <sup>S</sup> <sub>11</sub> Folded control peptide with the backrub protocol	55
Figure S27. Structure of amino acid derivatives	56

# Supplemental Tables

Table S1. MALDI, gradient of purification, and analytical retention time of the synthesized β-hairpins	8
Table S2. HPLC gradients for purification	1
Table S3. Concentration of NMR sample 1	4
Table S4. Amide temperature coefficient (in ppb/K) measurements for YKL and its thioamidated analogs 1	7
Table S5. <sup>1</sup> H Chemical shift assignments for HPT and YKL β-hairpins	8
Table S6. Comparison of fraction folded and $\Delta G_{Folding}$ analysis for HPT-Leu <sup>S</sup> <sub>11</sub> -OH Test and Unfolded epimers. 2	7
Table S7. <sup>1</sup> H Chemical shift assignments for HPT-Leu <sup>S</sup> <sub>11</sub> -OH Test and Unfolded epimers	8
Table S8. Fraction folded and $\Delta\Delta G_{Folding}$ analysis of HPT peptides	8
Table S9. NOE derived distance constraints for PyRosetta simulation of Folded control β-hairpins	1
Table S10. Backbone RMSD of the thioamide-containing Folded control structures in comparison to the HPT	
Folded control	-5
Table S11. Deviation, energy score and RMSD values for the backrub generated Folded control ensembles 5	1

### **Materials and Methods**

### **General Information.**

HPT peptides were synthesized, purified, and characterized at the University of Pennsylvania (Penn). Nuclear magnetic resonance (NMR) data for HPT peptides was collected at the National Taiwan University (NTU) (HPT- $\text{Thr}^{s}_{2}$  and HPT- $\text{Val}^{s}_{3}$ ) or Penn (HPT- $\text{Ile}^{s}_{10}$  and HPT-Leu<sup>s</sup><sub>11</sub>-OH). NMR data for the HPT peptide was previously collected at NTU<sup>1</sup>. All YKL peptides were synthesized, purified, and characterized at the Indian Institute of Science (IIS). NMR data for YKL peptides was collected at IIS.

*Reagents.*  $N\alpha$ -Fmoc- $N\delta$ -Boc-L-ornithine, Fmoc-D-proline,  $N\alpha$ -Fmoc- $N\omega$ -(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine, 7-Azabenzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluorophosphate (PyAOP), triisopropylsilane (TIPS) were purchased from ChemImpex (Wood Dale, IL, USA). All other Fmoc-protected amino acids and rink amide resin were purchased from Novabiochem (currently Millipore Sigma; St. Louis, MO, USA).  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) was purchased from Santa Cruz Biotechnology, Inc (Dallas, TX, USA). *N*-methylmorpholine (NMM) and 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) were purchased from Acros (currently Fisher Scientific; Waltham, MA, USA). All other reagents and solvents were purchased from Fisher Scientific or Millipore Sigma unless otherwise specified. Milli-Q filtered (18 M $\Omega$ ) water was used for all solutions. All reagents and solvents were used without further purification.

*Instrumentation*. Penn: Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were collected with a Bruker Ultraflex III (Billerica, MA, USA). High-resolution electrospray ionization mass spectra (ESI-HRMS) were collected with a Waters LCT Premier XE liquid chromatograph/ mass spectrometer (Milford, MA, USA). Low-resolution electrospray ionization mass spectra (ESI-LRMS) were obtained with a Waters single quadrupole detector (SQD) mass spectrometer with an Acquity Ultra Performance LC. NMR data were acquired with a Bruker AVANCE NEO 600 MHz spectrometer. Ultraviolet-visible (UV-vis) absorption spectra were collected on a GENESYS 150 UV-vis spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA). Circular dichroism (CD) data were acquired with a Jasco J-1500 CD spectrometer. NTU: NMR data were acquired on a Bruker AV III 800 MHz spectrometer. IIS: NMR data were acquired on a Bruker Avance 700 MHz spectrometer. CD data were acquired with a Jasco-715 spectropolarimeter

#### Synthesis and Characterization of Thioamide Precursors.



Scheme S1. General Fmoc-protected nitrobenzotriazolide thioamide precursor synthesis.

 $N\alpha$ -Fmoc-L-valine-nitrobenzotriazolide was synthesized as previously reported.<sup>2</sup>  $N\alpha$ -Fmoc-L-thioleucinenitrobenzotriazolide<sup>3</sup> and  $N\alpha$ -Fmoc-L-thioisoleucine-nitrobenzotriazolide<sup>4</sup> were synthesized as previously reported, with the following modifications.

Coupling of the Fmoc-L amino acids with 1,2-diamino-4-nitrobenzene (S1A). After addition of 4-nitrophenylenediamine the reaction was stirred under argon for 2 hours at -10 °C and then at room temperature overnight. After removing the solvent *in vacuo*, the product was dissolved in DMF and crashed out with the addition of 1:1 saturated KCl/MilliQ H<sub>2</sub>O. The precipitate was filtered and washed extensively with water. The residual water was removed overnight under high vacuum.

*Preparation of the Fmoc L-amino thioacid nitroanilides* (S1B). Phosphorous pentasulfide (P<sub>4</sub>S<sub>10</sub>) (0.75 eq) and anhydrous Na<sub>2</sub>CO<sub>3</sub> (0.75 eq) were added to dry THF under argon and left stirring for 30 minutes (or until the phosphorous pentasulfide dissolved). After which, the compound S1A was added. The reaction was purged with argon and left to stir overnight. The next day, after removing the solvent *in vacuo*, the solid was resuspended in ethyl acetate and filtered over a pad of Celite<sup>®</sup>. The filtrate was washed twice with 5% NaHCO<sub>3</sub> and once with brine. The organic layers were combined and dried with MgSO<sub>4</sub>. After filtration, the product was dry-loaded in silica or Celite<sup>®</sup> and purified over silica on a Biotage Isolera One system (Biotage, LLC, Charlotte, NC, USA) with ethyl acetate/*n*-hexanes (20-50% ethyl acetate in n-hexanes for thioisoleucine and 20-30% ethyl acetate in *n*-hexanes for thioleucine).

*Preparation of Fmoc-L-amino thioacid nitrobenzotriazolides* (S1C) After oxidation with NaNO<sub>2</sub> (1.5 eq) in 95% glacial acetic acid (v/v in Milli-Q water) the product was crashed out with cold MilliQ H<sub>2</sub>O. The filtered precipitate was lyophilized overnight and used directly for SPPS without further purification.

Synthesis and Purification of ThioThreonine (Fmoc-Thr<sup>S</sup>-NBt) Precursor.



Scheme S2. Fmoc-Thr<sup>s</sup>-Nbt (S2C) thioamide precursor synthesis.

(9H-fluoren-9-yl)methyl ((2S,3S)-1-((2-amino-5-nitrophenyl)amino)-3-(tert-butoxy)-1-oxobutan-2-yl) carbamate (S2A). Fmoc-Thr(*t*-Bu)-OH (1.987 g, 5.00 mmol) was dissolved in 40 mL of anhydrous tetrahydrofuran (THF) (dried over molecular sieves) under argon flow with stirring in an oven-dried round bottom flask. The solution was cooled to -10 °C in a 1:3 NaCl/ ice bath.*N*-methylmorpholine (NMM) (1.10 mL, 10.0 mmol, 2 eq) was added. While stirring, isobutyl chloroformate (IBCF) (0.648 mL, 5 mmol, 1 eq) was added dropwise. Residual IBCF in the syringe was deactivated by rinsing with saltwater from the ice bath. The reaction was stirred at 10 °C for 15 minutes. Afterwards, the 4-nitro-*o*-phenylenediamine (0.766 g, 5.00 mmol, 1 eq) was added. The reaction flask was purged with argon and left to stir for 2 hours at -10 °C and then at room temperature (RT) overnight under

argon. The next morning, the solution had turned orange. After removing the solvent *in vacuo*, the product was dissolved in 25 mL of DMF and precipitated upon addition to a saturated aqueous potassium chloride solution (250 mL). The precipitate was obtained by vacuum filtration with a Büchner funnel. After extensive washing with cold water, the solid was dried for 3-4 hours. Residual water was removed under high vacuum overnight. Compound **S2A** was obtained in 76.8% yield (2.045 g, 3.842 mmol).  $\delta$ H (600 MHz, DMSO) 9.20 (1 H, s), 8.26 (1 H, s), 7.88 (4 H, d, J 6.8), 7.77 (2 H, t, J 6.4), 7.41 (3 H, t, J 7.5), 7.33 (1 H, t, J 6.8), 7.25 (1 H, d, J 8.7), 6.80 (1 H, d, J 9.1), 6.54 (2 H, s), 4.39 – 4.30 (2 H, m), 4.26 (2 H, t, J 6.9), 4.07 – 4.01 (1 H, m), 3.89 (1 H, d, J 6.5), 1.17 (9 H, s), 1.13 – 1.09 (3H, m). A minor impurity due to removal of the t-butyl group resulted in the following additional peaks: 1.14 (3 H, s) and 0.93 (1 H, d, J 6.7). This impurity also resulted in the singlet at 1.17 and multiplet at 1.13-1.09 to have integrations higher than expected (10 and 6 respectively).  $\delta$ C (151 MHz, DMSO) 169.084, 156.075, 149.177, 143.891, 143.721, 140.742, 135.558, 128.911, 127.654, 127.274, 127.052, 125.371, 121.461, 121.369, 121.060, 120.106, 120.012, 113.609, 74.142, 67.394, 65.921, 59.946, 46.712, 28.319, 28.161, 27.876, 19.168, 18.904.

(9H-fluoren-9-vl)methvl ((2S,3S)-1-((2-amino-5-nitrophenyl)amino)-3-(tert-butoxy)-1-thioxobutan-2-yl) carbamate (S2B). Phosphorous pentasulfide (P<sub>4</sub>S<sub>10</sub>) (0.667 g, 1.50 mmol) and anhydrous sodium carbonate (0.159 g, 1.50 mmol) were added to an oven-dried round bottom flask. To the reaction flask, 20 mL of anhydrous THF dried over molecular sieves was added and allowed to stir under argon at room temperature until the phosphorous pentasulfide was dissolved (30-40 minutes). Compound S2A was added and stirred overnight at room temperature under argon. The next morning, the solvent was removed in vacuo to yield a yellow crystalline material. This product was resuspended in ethyl acetate and filtered over a pad of Celite<sup>©</sup> to remove insoluble phosphorous pentasulfide. The filtrate was washed twice with 5% NaHCO<sub>3</sub> (200 mL each) and once with brine (200 mL). The aqueous layer was back extracted with ethyl acetate. The organic layers were combined, dried with MgSO<sub>4</sub>, and filtered. The product was dry-loaded in silica onto a 100 g column pre-equilibrated with 20% ethyl acetate and purified on a Biotage Isolera One system (20-50% ethyl acetate in *n*-hexanes). The solvent was removed under reduced pressure. The resulting yellow solid was dried under vacuum and stored at 4 °C. Compound S2B was obtained in 55.6% yield (0.610 g, 1.10 mol).  $\delta H$  (600 MHz, DMSO) 10.89 (1 H, s), 7.98 (2 H, d, J 7.0), 7.90 (2 H, d, J 7.6), 7.75 (2 H, dd, J 19.7, 7.5), 7.46 – 7.39 (2 H, m), 7.33 (2 H, td, J 7.4, 4.0), 7.05 (1 H, d, J 8.1), 6.82 (1 H, d, J 9.7), 6.44 (2 H, s), 4.55 (1 H, dd, J 8.3, 4.3), 4.36 (2 H, d, J 6.8), 4.27 (1 H, t, J 7.1), 4.10 (1 H, p, J 5.9), 1.21 (9 H, s), 1.17 (3 H, d, J 6.2). δC (151 MHz, DMSO) 202.663, 188.230, 155.741, 150.306, 143.595, 140.732, 135.280, 127.686, 127.075, 125.331, 125.279, 122.133, 120.140, 114.017, 74.301, 68.601, 66.042, 46.685, 44.641, 36.746, 28.011, 19.181.

(9*H*-fluoren-9-yl)methyl ((2S,3S)-3-(tert-butoxy)-1-(6-nitro-1H-benzo[d][1,2,3]triazol-1-yl)-1-thioxobutan-2yl)carbamate (S2C, Fmoc-Thr<sup>S</sup>-NBt). Compound S2B (0.378 g, 0.690 mmol) was dissolved in 10 mL of 95% glacial acetic acid (5% Milli-Q water) and cooled to 0 °C. NaNO<sub>2</sub> (71.4 mg, 1.03 mmol, 1.5 eq) was added slowly and the solution was stirred for 30 minutes at 0 °C (the NaNO<sub>2</sub> did not dissolve immediately). (The reaction was checked on to make sure it did not freeze, if frozen the solution was warmed by hand). After 30 minutes, a precipitant formed, and the solution was transferred to 100 mL of cold MilliQ water. The precipitated product was obtained by filtration with a Büchner funnel. After extensive washing with cold MilliQ water, the solid was dried for 3-4 hours and then residual water was removed via lyophilization. Compound S2C was obtained in 85.2% yield (0.329 g, 0.588 mmol) and used directly for SPPS without further purification. (Major rotamer. Overlay of peaks resulted in integration of singlet at 0.93 to be 10. The expected integration for the major rotamer is listed below)  $\delta$ H (600 MHz, CDCl<sub>3</sub>) 9.66 (1 H, d, J 2.1), 8.44 (1 H, dd, J 8.9, 2.1), 8.31 (1 H, d, J 8.9), 7.78 (2 H, t, J 6.9), 7.68 (2 H, t, J 8.6), 7.42 (2 H, q, J 7.5), 7.38 – 7.32 (2 H, m), 7.11 (1 H, dd, J 5.6, 3.1), 4.55 (1 H, dd, J 10.8, 6.5), 4.44 – 4.34 (2 H, m), 4.28 (1 H, t, J 7.1), 0.93 (9 H, s), 0.81 (3 H, s). (Mixture of rotamers)  $\delta$ C (151 MHz, CDCl<sub>3</sub>) 206.078, 156.532, 149.758, 148.832, 143.964, 143.824, 141.415, 131.925, 127.842, 127.212, 127.192, 125.294, 125.200, 122.290, 121.593, 120.116, 120.094, 112.705, 74.154, 69.994, 67.323, 67.152, 47.347, 28.236, 21.556.

### Peptide Synthesis, Purification, and Characterization.

### НРТ

*General.* Peptides were manually synthesized via Fmoc solid phase peptide synthesis (SPPS) on rink amide resin (100-200 mesh, 0.54 mmol/g loading, NovaBioChem) in 12 mL fritted syringes. All peptides were synthesized on a 50  $\mu$ mol scale, except for the HPT-Leu<sup>S</sup><sub>11</sub>-OH peptides which were synthesized on a 100  $\mu$ mol scale.

The resin was swelled by stirring in *N*, *N*-dimethylformamide (DMF) (6 mL, 30-45 minutes). After draining and rinsing with DMF (4 or 8 mL), the resin was deprotected with 20% v/v piperidine in DMF (2 or 4 mL) for 2 x 10 minutes. In-between all deprotections, the reaction vessel was washed with Wash 1 (4 or 8 mL each: DMF x2, methylene chloride (DCM), DMF). After the last deprotection, the vessel the washed with Wash 2 (4 or 8 mL each: (DMF, DCM) x 3, DMF). The first amino acid (Fmoc-Gln(Trt)-OH) (5 eq) and PyAOP (5 eq) were dissolved in DMF (2 mL or 4 mL) and DIPEA (10 eq) was added. After vortexing, the mixture was added to the vessel. After stirring for 30 minutes at room temperature and washing with DMF (4 or 8 mL), the coupling was repeated. The Fmoc group was removed with treatment of 20% v/v piperidine in DMF (2 or 4 mL) for 2 x 10 minutes. Wash 1 and Wash 2 were performed as described. This series of couplings and deprotections continued until the thioamide was to be coupled.

*Thioamide Coupling and Deprotection.* The nitrobenzotriazolide thioamide precursor (2.5 - 3 eq) was dissolved in anhydrous DCM over molecular sieves (purchased from Fisher) (2 mL). DIPEA (3 - 3.5 eq) was added, vortexed, and the mixture was added to the reaction vessel to stir at room temperature for 1 hour. After Wash 1, the coupling was repeated. The remaining unreacted termini were acetyl capped by treatment with 5 mL (8.4 mL DMF, 1.0 mL acetic anhydride, 0.6 mL NMM) for 2 x 10 minutes, with a DMF wash in-between. Following Wash 2, the Fmocgroup was removed with 2% DBU v/v in DMF (2 or 4 mL) for 3 x 2 minutes. After the first two DBU deprotections, Wash 1 was performed, and after the last deprotection, Wash 2 was performed.

The remaining amino acids were coupled as previously described; however, deprotections were performed with 2% DBU v/v in DMF (2 or 4 mL, 3x 5 minutes) to avoid epimerization and previously published side-reactions with piperidine.<sup>5</sup> Following removal of the Fmoc-group from Arg<sub>1</sub> (Test and Unfolded peptides) or Cys<sub>1</sub> (Folded peptides), the N-terminus was acetylated (treatment with 5 mL (8.4 mL DMF, 1.0 mL acetic anhydride, 0.6 mL NMM) for 2 x 10 minutes, with a DMF wash in-between). Wash 2 was performed to clean the final product, followed by three washes with DCM (4 or 8 mL). The vessel was washed with DCM and left under vacuum to dry the resulting resin.

*Cleavage.* For the Unfolded and Test peptides: a cleavage cocktail of 95% TFA, 2.5% TIPS, 2.5% MilliQ H<sub>2</sub>O (v/v) (5 mL) was added to the vessel. For the Folded peptides: a cleavage cocktail of 90% TFA, 5% EDT, 2.5 % TIPS, 2.5% MilliQ H<sub>2</sub>O (v/v) (5 mL) was added to the vessel. The vessel rotated at room temperature for 45-60 minutes. The cleavage solution was drained from the syringe and rinsed with DCM. After removal of the cleavage solution *in vacuo*, the peptide was washed with DCM and subsequently dried *in vacuo*. Cold diethyl ether (20 mL) was added to remove residual precipitate was transferred to a 50 mL Falcon tube. A second wash with diethyl ether (10 mL) was added to remove residual precipitated peptide. A third wash was performed (10 mL) if needed. The falcon tube was cooled in dry ice for 15 minutes, and then the precipitate was collected by centrifugation at 4,000 RPM (3,313 x g) for 5 minutes. The diethyl ether was carefully poured off, and the pellet was left to dry in the hood overnight. Afterwards the pellet was stored at 4 °C.

*Purification.* The crude peptide was dissolved in a minimal volume mixture of MilliQ H<sub>2</sub>O + 0.1% TFA, methanol, and  $\leq 20\%$  acetonitrile (ACN) + 0.1% TFA and purified by RP-HPLC on either a Biotage Isolera one (Biotage, LLC, Charlotte, NC, USA) or on an Agilent 1260 Infinity II Preparative HPLC (Santa Clara, CA, USA) with a Biotage SNAP Ultra C18 column or a Luna Omega PS C18 preparative column (5 µm particle size, 250 mm length, 21.2 mm diameter) using the following gradients (**Table S2**). The desired peptide was identified with MALDI using a CHCA matrix, and was dried on a lyophilizer (Labconco; Kansas City, MO, USA). The dried peptide was

dissolved in MilliQ H<sub>2</sub>O + 0.1% TFA and subject to second-pass RP-HPLC purification on the Agilent Preparative HPLC using one of two columns: Luna Omega PS C18 preparative column (5  $\mu$ m particle size, 250 mm length, 21.2 mm diameter) or a Phenomenex Luna Omega PS C18 semi-preparative column (5  $\mu$ m particle size, 250 mm length, 10 mm diameter) using the following gradients (**Table S2**). Purity of the peptides was assessed by analytical RP-HPLC on an Agilent 1260 Infinity II series Analytical HPLC system with a Phenomenex Luna Omega PS C18 column (5  $\mu$ m particle size, 150 mm length, 4.6 mm diameter) with gradient **K**. Peptides for NMR studies were  $\geq$  95% pure (based on analytical 215 nm AUC integration).

*Cyclization.* Following second-pass purification, the dried folded peptides were dissolved in 0.1 M ammonium bicarbonate pH 8.0 until  $\leq 100 \ \mu$ M concentration (as low as 50  $\mu$ M was fine). The concentration was roughly checked by the UV-Vis absorbance measurement at 274 nm, and concentration was calculated based on the thioamide extinction coefficient ( $\epsilon_{274nm} = 10,169 \ M^{-1} \text{cm}^{-1}$ ). The product was split into multiple 50 mL Falcon tubes so that the volume per tube was  $\leq 20 \ mL$ . The solution was stirred vigorously under atmosphere and the cyclization was monitored with MALDI. The cyclization was complete within 6-9 hours and the product was dried via lyophilization. The product was subject to a final RP-HPLC purification on the Agilent Preparative HPLC with a Phenomenex Luna Omega PS C18 column using gradient **D**, **I** or **B** (**Table S2**).

### YKL

*General.* Peptides were manually synthesized via Fmoc solid phase peptide synthesis (SPPS) on Rink Amide AM resin (0.8 mmolg<sup>-1</sup>) on 200 mg scale (0.16 mmol) in 12 mL fritted syringes, except for the YKL-Leu<sup>S</sup><sub>11</sub>-OH peptides which were synthesized on 2Cl-TCP resin (1.3 mmolg<sup>-1</sup>).

The Rink Amide AM resin was swollen in DMF (20 min) and deprotected with 20% v/v piperidine in DMF (5 min x 1, 15 min x 1) followed by thorough washing with DMF (3 times). The C-terminal amino acid, Fmoc-Gln(Trt)-OH (2.5 eq) was loaded onto the resin by using standard coupling reagents (2.5 eq HOBt, 2.5 eq DIC) in DMF for 2 hours at room temperature. For the YKL-Leu<sup>S</sup><sub>11</sub>-OH peptides, the C-terminal amino acid residue was loaded onto the resin with 2.5 eq DIPEA in anhydrous DCM at room temperature. After loading the first amino acid, the remaining unreacted trityl chloride groups bound to the solid-support were capped using methanol (200  $\mu$ l/100 mg resin) for 15 min. Next, the resin was thoroughly washed with DCM (3 times), 1:1 DCM-methanol (3 times) and methanol (3 times) and finally dried under vacuum. The loading capacity was estimated from the dry weight of the resin, which ranged from 0.6-0.8 mmolg<sup>-1</sup>. This series of couplings and deprotections continued until the thioamide was to be coupled.

*Deprotections*. Fmoc-deprotections for oxo-peptides were carried out with 20% v/v piperidine in DMF (5 min + 15 min) and 10% v/v piperidine in DMF (30 sec x 2) was used for thio-peptides after the incorporation of thionated amino acid to avoid epimerization.<sup>6</sup> The resin was washed two times with 3 ml of DMF for 60 sec between each cycle.

Cleavage and Purification. The global deprotection of protected peptide was carried out with 62% TFA, 31% DCM, 3.5% TIPS, 3.5% H<sub>2</sub>O (v/v) for 30 min. After the deprotection, the deprotected peptide was precipitated in chilled ether yielding the crude product and further, it was purified by RP-HPLC with gradient **M** using a semi-preparative column (Phenomenex C18, 250 mm x 10 mm I.D., 5  $\mu$ m) at a flow rate of 4 mL min<sup>-1</sup> on a Shimadzu UFLC system (Kyoto, Japan). Purity of peptides was assessed on a C18 (Phenomenex, 100 mm x 4.6 mm) column at 1.0 ml/min flow rate using RP-HPLC with gradient **M**.

The detailed experimental procedure for the synthesis of nitrobenzotriazolide thioamide precursors and incorporation on solid support have been reported earlier.<sup>7</sup>

Dontido	[M+H	] <sup>+</sup> (m/z)	[M+Na	<b>n</b> ] <sup>+</sup> (m/z)	[M+K]	<sup>+</sup> (m/z)	Cuadiant	Analytical
Рерпие	Exp	Obs	Exp	Obs	Exp	Obs	Gradient	Time*
НРТ								
Oxo Test	1394.85	1394.76	1416.83	1416.77	1432.80	1432.76	С	22.2
Oxo Unfolded	1394.85	1395.65	1416.83	1417.64	1432.80	1433.62	ΗG	20.4
Oxo Folded	1598.80	1598.83	1620.83	1620.93	1636.81	1636.89	JВ	25.6
Thr <sup>s</sup> <sub>2</sub> Test	1410.82	1410.73	1432.81	1432.71	1448.78		AB	23.5
Thr <sup>s</sup> <sub>2</sub> Unfolded	1410.82	1410.86	1432.81	1432.84	1448.78	1448.81	AB	21.7
Thr <sup>s</sup> <sub>2</sub> Folded	1614.83	1615.06	1636.81	1637.04	1652.78		A C D	22.9
Val <sup>S</sup> <sub>3</sub> Test	1410.82	1410.94	1432.81	1432.92	1448.78		A D	25.0
Val <sup>S</sup> <sub>3</sub> Unfolded	1410.82	1411.00	1432.81	1432.99	1448.78		AB	22.9
Val <sup>S</sup> <sub>3</sub> Folded	1614.83	1615.10	1636.81	1637.08	1652.78		A C D	25.8
Ile <sup>S</sup> <sub>10</sub> Test	1410.82	1410.71	1432.81	1432.78	1448.78	1448.77	ΗI	24.6
Ile <sup>S</sup> 10 Unfolded	1410.82	1411.65	1432.81	1432.65	1448.78	1448.64	ΗG	22.2
Ile <sup>S</sup> 10 Folded	1614.83	1614.66	1636.81	1636.69	1652.78		Ι	26.0
Leu <sup>S</sup> 11-OH Test	1411.81	1411.78	1433.79	1433.76	1449.77	1449.74	ΕIΒ	25.4
Leu <sup>S</sup> 11-OH Unfolded	1411.81	1411.78	1433.79	1433.77	1449.77		FBL	22.4
Leu <sup>S</sup> 11 Folded	1614.83	1614.84	1636.81	1636.81	1652.78	1652.80	ΕB	25.9
YKL								
Oxo Test	1470.88	1470.92	1492.86	1492.96	1508.84	1508.91	М	12.7
Oxo Unfolded	1470.88	1470.95	1492.86	1492.94	1508.84	1508.90	М	11.5
Glu <sup>S</sup> <sub>4</sub> Test	1486.86	1486.89	1508.84	1508.86	1524.81	1524.98	М	16.8
Glu <sup>S</sup> 4 Unfolded	1486.86	1486.92	1508.84	1508.90	1524.81		М	13.6
Lys <sup>S</sup> 9 Test	1486.86	1486.90	1508.84	1509.02	1524.81		М	22.2
Lys <sup>S</sup> 9 Unfolded	1486.86	1486.89	1508.84	1508.97	1524.81	1524.95	М	21.1
Leu <sup>S</sup> 11-OH Test	1487.84	1487.95	1509.82	1509.91	1525.80	1525.99	М	16.9
Leu <sup>S</sup> 11-OH Unfolded	1487.84	1487.93	1509.82	1509.89	1525.80		М	15.6

**Table S1.** MALDI characterization, gradient of purification, and analytical retention time of the synthesized  $\beta$ -hairpins

\*Gradient K used for all analytical HPLC injections of HPT library and Gradient M used for YKL library.



**Figure S1.** Analytical HPLC (gradient K) of synthesized HPT  $\beta$ -hairpins: HPT (A), HPT-Thr<sup>S</sup><sub>2</sub> (B), HPT-Val<sup>S</sup><sub>3</sub> (C), HPT-Ile<sup>S</sup><sub>10</sub> (D), and HPT-Leu<sup>S</sup><sub>11</sub>-OH.



**Figure S2.** Analytical HPLC (gradient **M**) of synthesized YKL  $\beta$ -hairpins: YKL (**A**), YKL-Glu<sup>S</sup><sub>4</sub> (**B**), YKL-Lys<sup>S</sup><sub>9</sub> (**C**), and YKL-Leu<sup>S</sup><sub>11</sub>-OH (**D**).

A         2 CV         5         B         0.00         2 $30,9  CV$ 5-50 $3.00$ 5 $3  CV$ 50 $8.00$ 20 $1  CV$ 50 $10.00$ 20 $1  CV$ 50 $30.00$ 25 $1  CV$ 50 $30.00$ 25 $1  CV$ 50 $30.00$ 25 $0  00$ $5  0$ $30.00$ 5 $0  00$ $5  0$ $36.00$ 100 $100  00$ $5  0$ $30.00  5$ $30.00  5$ $10.00$ $20  0$ $8.00  22$ $22  0$ $10.00$ $20  0$ $8.00  22$ $22  0$ $10.00$ $30.00  5$ $30.00  27  0$ $31.00  100  0$ $41.00$ $100  0$ $31.00  100  0$ $31.00  100  0$ $43.00  5$ $5  0  000  5$ $5  000  0$ $5  000  0$ $40.00  5  0$ $5  0  000  0$ $5  000  0$ $5  000  0$ $10.00  0  20  0  0  0  0  $	No.	Time/CV	% B	No.	Time	%B
30.9  CV $5.50$ $3.00$ $5$ $3  CV$ $50$ $8.00$ $20$ $5  CV$ $100$ $0.00$ $20$ $1  CV$ $50$ $30.00$ $25$ $1  CV$ $50$ $30.00$ $25$ $ 31.00$ $100$ $ 36.00$ $100$ $ 36.00$ $5$ $  38.00$ $5$ $  30.00$ $5$ $0.00$ $5$ $0$ $0.00$ $5$ $0.00$ $5$ $0$ $0.00$ $22$ $0.00$ $20$ $0.00$ $22$ $30.00$ $30$ $30.00$ $27$ $31.00$ $100$ $30.00$ $5$ $43.00$ $5$ $38.00$ $5$ $10.00$ $20$ $100$ $15$ $5.00$ $5$ $3.00$ $5$ $10.00$ $20$	Α	2 CV	5	В	0.00	2
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5  CV         100         10.00         20           1 CV         50         30.00         25           31.00         100         31.00         100           1         1         36.00         100           1         1         38.00         5           C         0.00         5         D         0.00         5           3.00         5         1         3.00         5           C         0.00         20         8.00         22           10.00         20         10.00         22           30.00         30         30.00         27           30.00         30         30.00         27           31.00         100         31.00         100           41.00         100         31.00         100           43.00         5         38.00         5           E         0.00         5         5           10.00         20         8.00         15           10.00         20         11.00         15           15.00         20         11.00         15           5.00         100         53.00         100		3 CV	50		8.00	20
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45.00         100         53.00         100           55.00         100         55.00         5           58.00         5		40.00	40		43.00	100
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58.00         5         H         0.00         5           G         0.00         5         H         0.00         5           3.00         5         3.00         5         10.00         5           10.00         20         8.00         10           15.00         20         11.00         10           35.00         25         51.00         50           40.00         30         55.00         100		55.00	100		55.00	5
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15.00         20         11.00         10           35.00         25         51.00         50           40.00         30         55.00         100		10.00	20		8.00	10
35.00         25         51.00         50           40.00         30         55.00         100		15.00	20		11.00	10
		35.00	25		51.00	50
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>8.00         >         >         >         >         >		38.00	3		38.00	J
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<u>58.00</u> <u>100</u> <u>35.00</u> <u>20</u>		58.00	100		35.00	20
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23.00 50		23.00	50			
28.00 100		28.00	100			
33.00 100		33.00	100			
38.00 10		38.00	100			

**Table S2.** HPLC gradients for purification (solvent A = MilliQ water with 0.1% TFA, solvent B = acetonitrile with 0.1% TFA)

#### **Circular Dichroism**

### НРТ

Sample Preparation. Dried peptide was dissolved in 50:50 v/v MilliQ water/ acetonitrile and quantified by UV based on the thioamide extinction coefficient ( $\varepsilon_{274nm} = 10,169 \text{ M}^{-1}\text{cm}^{-1}$ ). Aliquots of Test, Folded, and Unfolded versions of all HPT peptides were prepared and lyophilized. The aliquot was dissolved in 50 mM sodium acetate pH 5.5 (HPT-Thr<sup>S</sup><sub>2</sub> and HPT-Val<sup>S</sup><sub>3</sub>) and 50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 5.5 (HPT-Ile<sup>S</sup><sub>10</sub> and HPT-Leu<sup>S</sup><sub>11</sub>-OH) to 130-150  $\mu$ M. The final concentration was measured based on UV. The HPT peptide was aliquoted based on mass and was dissolved in 50 mM sodium acetate pH 5.5 to 1 mM. This stock was diluted to 150  $\mu$ M.

*Data Measurement.* The wavelength absorbance scans were collected on a Jasco J-1500 CD spectrometer with a 1 mm path length Helma 110-QS CD cuvette. Measurements were completed at 25 °C, collecting between 350-190 nm with a continuous scanning rate of 50 nm/min (450 FL  $\lambda$ , 1 nm bandwidth and 1.0 nm data pitch) and a digital integration time (DIT) of 1 second. The instrument was blanked with the corresponding buffer prior to sample collection. The raw signal ( $\theta$ , mDeg) was converted to molar ellipticity ( $\theta_{Molar}$ ) (Eq. S1), where *l* is the pathlength in cm and c is the concentration (M).

$$\theta_{Molar} = \frac{\theta}{10*l*c}$$
 (Eq. S1)

The scans were smoothed with GraphPad Prism 7.01 software (San Diego, Ca) by averaging 10 neighbors on each side, and using a fourth order smoothing polynomial.

#### YKL

The Far-UV CD spectra of YKL and its thioamidated analogs were collected on a JASCO-715 spectropolarimeter with 1 mm path-length Starna 21-Q-1 CD cuvette. The data were recorded at 100  $\mu$ M concentration in 100 mM sodium acetate buffer (pH 3.8). Scans were carried out at 20 °C over the range of 190-260 nm with 0.5 nm increments and a 2 nm bandwidth. Thermal unfolding experiments were monitored at 215 nm over a temperature range of 10-90 °C. The samples were equilibrated for 5 minutes at each temperature.

### Nuclear Magnetic Resonance Spectroscopy

### НРТ

Sample Preparation. Dried peptide was dissolved in 50:50 v/v MilliQ water/ acetonitrile and quantified by UV based on the thioamide extinction coefficient ( $\epsilon_{274nm} = 10,169 \text{ M}^{-1}\text{cm}^{-1}$ ). The material was aliquoted and lyophilized. The UV measurement was compared against the mass of the dried peptide. NMR samples were prepared by dissolving the lyophilized, dried peptide ( $\geq 95\%$  purity) in 0.5-0.6 mL of 50 mM sodium deuterioacetate buffer pH 5.5 (9:1 v/v MilliQ H<sub>2</sub>O/ D<sub>2</sub>O) (HPT-Thr<sup>S</sup><sub>2</sub> and HPT-Val<sup>S</sup><sub>3</sub>) or 50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 5.5 (9:1 v/v MilliQ H<sub>2</sub>O/ D<sub>2</sub>O) (HPT-Thr<sup>S</sup><sub>2</sub> and HPT-Val<sup>S</sup><sub>3</sub>) or 50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 5.5 (9:1 v/v MilliQ H<sub>2</sub>O/ D<sub>2</sub>O) (HPT-Ile<sup>s</sup><sub>10</sub> and HPT-Leu<sup>S</sup><sub>11</sub>-OH) to 1-10 mM concentration (see exact concentration in **Table S3**). 2-Dimethyl-2-silapentane-5-sulfonate (DSS) was added to each NMR sample as the internal reference. (DSS was added from a 1 mg/mL stock in sterile Milli-Q water to ~30 µM final concentration.)

*Data Collection.* The HPT data was previously collected and published on by the Cheng lab,<sup>1</sup> however the raw data was processed and analyzed again by KEF to ensure that the analysis was consistent. NTU: NMR data were collected on a Bruker AVIII 800 MHz spectrometer. Total correlation spectroscopy (TOCSY)<sup>8</sup> and rotating-frame nuclear Overhauser effect spectroscopy (ROESY)<sup>9</sup> experiments were performed by collecting 2048 points in f 2 with 4-8 scans and 256-512 points in f 1 at 298 K. Water suppression was achieved with the WATERGATE solvent suppression sequence.<sup>10</sup> TOCSY and ROESY had a spin locking field of 10 kHz. Mixing times of 60 and 200 ms were used for the TOCSY and ROESY respectively. Penn: NMR data was collected on a Bruker AVANCE NEO 600 MHz spectrometer. TOCSY and ROESY were performed by collecting 4096 points in f 2 with 16-32 scans and 512 points in f 1 at 298 K. Water suppression was achieved with the WATERGATE solvent.<sup>10</sup> Mixing times of 80 and 250 ms were used for the TOCSY and ROESY were performed by collecting 4096 points in f 2 with 16-32 scans and 512 points in f 1 at 298 K. Water suppression was achieved with the WATERGATE solvent suppression sequence.<sup>10</sup>

#### YKL

Sample Preparation. NMR samples of YKL and its thioamidated alalogs were prepared by dissolving the lyophilized, dried peptide ( $\geq$  95% purity) in 0.5-0.6 mL of 100 mM sodium deuteroacetate buffer pH 3.8 (9:1 v/v MilliQ H<sub>2</sub>O/D<sub>2</sub>O) to obtain 1-3 mM concentration (see exact concentration in **Table S3**). Trimethylsilylpropanoic acid (TMSP) was added to each NMR sample as the internal reference.

*Data Collection*. IIS: NMR data for YKL and its thioamidated analogs were collected on a Bruker Avance 700 MHz spectrometer. Standard Bruker pulse sequences zgesgp for 1H, mlevesgpph/dipsi2rcesgpph (60 ms mixing time) for TOCSY, roesyesgpph (100 ms mixing time) for ROESY were used to acquire the NMR data. Twodimensional data were obtained using 2048 data points in the direct dimension (f 2) and 512 data points in the indirect dimension (f 1).

Peptide	Collection	Concentration (mM)
НРТ		
Thr <sup>s</sup> <sub>2</sub> Test	NTU	7.9
Thr <sup>S</sup> <sub>2</sub> Unfolded	NTU	5.9
Thr <sup>S</sup> <sub>2</sub> Folded	NTU	1.1
Val <sup>S</sup> <sub>3</sub> Test	NTU	6.8
Val <sup>S</sup> <sub>3</sub> Unfolded	NTU	3.7
Val <sup>S</sup> <sub>3</sub> Folded	NTU	2.7
Ile <sup>S</sup> <sub>10</sub> Test	Penn	3.8
Ile <sup>S</sup> 10 Unfolded	Penn	4.2
Ile <sup>S</sup> <sub>10</sub> Folded	Penn	7.7
Leu <sup>S</sup> 11-OH Test	Penn	0.8
Leu <sup>S</sup> 11-OH Unfolded	Penn	3.0
Leus <sub>11</sub> -OH Folded	Penn	1.8
YKL		
Oxo Test	IIS	2.5
Oxo Unfolded	IIS	2.8
Glu <sup>S</sup> <sub>4</sub> Test	IIS	1.9
Glu <sup>S</sup> 4 Unfolded	IIS	2.4
Lys <sup>s</sup> <sub>9</sub> Test	IIS	3.1
Lys <sup>S</sup> 9 Unfolded	IIS	2.4
Leu <sup>S</sup> 11-OH Test	IIS	2.7
Leu <sup>S</sup> 11-OH Unfolded	IIS	3.0

**Table S3.** Concentration of NMR sample

**Data Processing.** All 2D <sup>1</sup>H-<sup>1</sup>H NMR were processed and assigned with MestReNova 14.1.0 (Santiago de Compostela, Spain). Apodization of Sine Square 90° was used for both f2 and f1, and zero-fill was 2x the size of the FID. A baseline correction of a Berstein polynomial fit of order 3 was used. The spectra were referenced based on the DSS or TMSP signal. The spectra were phased manually or automatically. Peaks were manually picked with standard MestReNova settings. The TOCSY was assigned with the ROESY. NOE connectivities were determined from the ROESY with an intensity cutoff of 0.3 for the NTU and IIS collected data and 300 for the Penn collected data (**Fig. S15**). Chemical shift assignments for all HPT and YKL peptides analyzed in the main text are given in **Table S5**.



**Figure S3a.** <sup>1</sup>H NMR overlay of thioamide-containing Test YKL β-hairpins acquired at different temperatures.



**Figure S3b.** <sup>1</sup>H NMR overlay of thioamide-containing Test YKL β-hairpins acquired at different temperatures.

Residue	YKL	YKL-Glu <sup>s</sup> 4	YKL-Lys <sup>8</sup> 9	YKL-Leu <sup>8</sup> 11-OH
*Arg1	-7.3	-7.3	-7.3	-6.5
Tyr <sub>2</sub>	-7	-9.3	-9.3	-7
*Val3	-5	-4	-4	-5
Glu4	-7.5	-7.3	-8	-8
*Val5	-8	-5.3	-7.8	-7.8
pro <sub>6</sub>				
Gly7	-7.3	-6.3	-7.3	-7
*Lys <sub>8</sub>	-1.3	-2	-1.5	-1.3
Lys9	-9.5	-6.3	-7.3	-9.8
*Ile <sub>10</sub>	-7.3	-7.5	-4.3	-7
Leu <sub>11</sub>	-7.3	-8.5	-9.3	-9.8
*Gln <sub>12</sub>	-6.5	-7.5	-8.8	-4.8

**Table S4.** Amide temperature coefficient (in ppb/K) measurements for YKL and its thioamidated analogs

\*Hydrogen bonded amide protons  $\Delta \delta / \Delta T$ .

## Table S5. <sup>1</sup>H Chemical shift assignments for HPT and YKL $\beta$ -hairpins

YKL	Test
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Residue	NH	Ηα	Нβ	Others
Ac		1.955		
Arg <sub>1</sub>	8.250	4.369	1.653	Hγ: 1.496, Hδ: 3.157, HNt: 7.209
Tyr <sub>2</sub>	8.548	5.121	2.727	2,6H: 6.912, 3,5H: 6.772
Val <sub>3</sub>	8.902	4.379	2.004	Ηγ: 0.861
Glu4	8.660	4.994	1.975, 1.877	Ηγ: 2.229
Val <sub>5</sub>	9.000	4.593	1.985	Ηγ: 0.930
pro <sub>6</sub>		4.359	2.376, 2.024	Ηγ: 2.112, Ηδ: 3.870
Gly7	8.693	4.007, 3.763		
Lys <sub>8</sub>	7.937	4.574	1.789	Ηγ: 1.389, Ηδ: 1.672, Ηε: 2.991, ΗΝτ: 7.612
Lys <sub>9</sub>	8.542	4.613	1.643, 1.526	Ηγ: 1.105, Ηδ: 1.330, Ηε: 2.581, ΗΝτ: 7.427
Ile <sub>10</sub>	9.114	4.486	1.907	Hγ: 1.399, 1.223, Hδ or Hγ (Me): 0.881
Leu <sub>11</sub>	8.712	4.076	1.584	Ηγ: 1.399, Ηδ: 0.685, 0.568
Gln <sub>12</sub>	8.740	4.281	2.034, 1.848	Hγ: 2.268, HNt: 7.375, 7.051
NH <sub>2</sub>	7.794, 7.199			

### YKL Unfolded

Residue	NH	Ηα	Нβ	Others
Ac		1.998		
Arg <sub>1</sub>	8.292	4.181	1.637	Hγ: 1.457, Hδ: 3.124, HNt: 7.179
Tyr <sub>2</sub>	8.382	4.619	2.968	2,6H: 7.092, 3,5H: 6.803
Val <sub>3</sub>	8.020	4.001	1.927	Ηγ: 0.870
Glu4	8.481	4.290	1.981	Нү: 2.373
Val <sub>5</sub>	8.446	4.396	2.068	Нү: 0.952
Pro <sub>6</sub>		4.392	2.318	Нγ: 2.005, 1.989, Нδ: 3.876, 3.699
Gly7	8.598	3.938		
Lys <sub>8</sub>	8.209	4.306	1.754	Hγ: 1.402, Hε: 2.974, HNt: 7.603
Lys <sub>9</sub>	8.481	4.306	1.731	Hγ: 1.410, Hε: 2.974, HNt: 7.603
Ile <sub>10</sub>	8.404	4.126	1.833	Hγ: 1.481, 1.191, Hδ or Hγ (Me): 0.878
Leu <sub>11</sub>	8.506	4.384	1.614	Ηδ: 0.859
Gln <sub>12</sub>	8.481	4.282	1.981	Hγ: 2.373, HNt: 7.630, 6.960
NH <sub>2</sub>	7.700, 7.204			

## YKL-Glu<sup>S</sup>4 Test

Residue	NH	Ηα	Ηβ	Others
Ac		1.953		
Arg <sub>1</sub>	8.261	4.337	1.650	Hγ: 1.484, Hδ: 3.145, HNt: 7.204
Tyr <sub>2</sub>	8.536	5.050	2.815, 2.757	2,6H: 6.940, 3,5H: 6.783
Val <sub>3</sub>	8.774	4.357	2.022	Ηγ: 0.849
Glu <sup>S</sup> 4	8.746	5.256	2.061, 1.992	Ηγ: 2.178
Val <sub>5</sub>	10.460	5.436	2.178	Ηγ: 0.976
pro <sub>6</sub>		4.398	2.354, 1.992	Ηγ: 2.080, 2.061, Ηδ: 4.072, 3.925
Gly7	8.521	3.937, 3.790		
Lys <sub>8</sub>	8.026	4.533	1.777	Нγ: 1.377, Нδ: 1.660, Нε: 2.979, НΝt: 7.611
Lys <sub>9</sub>	8.584	4.562	1.641, 1.533	Ηγ: 1.123, Ηδ: 1.357, Ηε: 2.637, ΗΝt: 7.452
Ile <sub>10</sub>	9.042	4.445	1.875	Hγ: 1.396, 1.201, Hδ or Hγ (Me): 0.869
Leu <sub>11</sub>	8.680	4.083	1.562	Ηγ: 1.406, Ηδ: 0.693, 0.605
Gln <sub>12</sub>	8.711	4.259	2.031, 1.846	Hγ: 2.266, HNt: 7.410, 7.040
NH <sub>2</sub>				

### YKL-Glu<sup>S</sup><sub>4</sub> Unfolded

Residue	NH	Ηα	Нβ	Others
Ac		1.982		
Arg <sub>1</sub>	8.306	4.181	1.630	Hγ: 1.464, Hδ: 3.125, HNt: 7.190
Tyr <sub>2</sub>	8.402	4.640	3.037, 2.930	2,6H: 7.102, 3,5H: 6.810
Val <sub>3</sub>	8.030	4.034	1.992	Ηγ: 0.868
Glu <sup>S</sup> 4	8.568	4.659	2.021	Ηγ: 2.363, 2.305
Val <sub>5</sub>	10.207	4.757	2.217	Ηγ: 1.054
Pro <sub>6</sub>		4.239	2.314, 1.943	Ηγ: 2.070, Ηδ: 3.678
Gly7	8.619	3.946		
Lys <sub>8</sub>	8.209	4.298	1.777	Hγ: 1.396, Hε: 2.979, HNt: 7.626
Lys <sub>9</sub>	8.457	4.288	1.757	Hγ: 1.406, Hε: 2.979, HNt: 7.626
Ile <sub>10</sub>	8.427	4.341	1.611	Hδ or Hγ (Me): 0.926
Leu <sub>11</sub>	8.404	4.356	1.650	Ηδ: 0.868
Gln <sub>12</sub>	8.428	4.288	2.109, 1.982	Hγ: 2.353, HNt: 7.653, 6.968
NH <sub>2</sub>	7.688, 7.219			

## YKL-Lys<sup>S</sup><sub>9</sub> Test

Residue	NH	Ηα	Нβ	Others
Ac		1.958		
Arg <sub>1</sub>	8.251	4.361	1.655	Hγ: 1.489, Hδ: 3.150, HNt: 7.201
Tyr <sub>2</sub>	8.532	5.143	2.759, 2.671	2,6H: 6.902, 3,5H: 6.763
Val <sub>3</sub>	8.929	4.371	1.987	Ну: 0.873
Glu4	8.736	4.996	2.007, 1.860	Ну: 2.241
Val <sub>5</sub>	9.032	4.596	1.987	Ну: 0.932
pro <sub>6</sub>		4.373	2.378, 1.985	Нγ: 2.125, 2.058, Нδ: 3.866
Gly7	8.675	4.019, 3.756		
Lys <sub>8</sub>	7.917	4.576	1.762	Нγ: 1.391, Нε: 2.974, HNt: 7.611
Lys <sup>S</sup> 9	8.684	4.879	1.792, 1.557	Ηγ: 1.079, Ηδ: 1.333, Ηε: 2.622, ΗΝτ: 7.447
Ile <sub>10</sub>	10.595	5.192	2.143	Hγ: 1.432, 1.378, Hδ or Hγ (Me): 0.912
Leu <sub>11</sub>	8.864	4.098	1.606	Ηγ: 1.411, Ηδ: 0.717, 0.561
Gln <sub>12</sub>	8.739	4.293	2.036, 1.850	Hγ: 2.271, HNt: 7.344, 7.060
NH <sub>2</sub>	7.787, 7.197			

## YKL-Lys<sup>S</sup>9 Unfolded

Residue	NH	Ηα	Нβ	Others
Ac		1.992		
Arg <sub>1</sub>	8.294	4.182	1.639	Hγ: 1.467, Hδ: 3.132, HNt: 7.191
Tyr <sub>2</sub>	8.390	4.613	2.994, 2.945	2,6H: 7.092, 3,5H: 6.805
Val <sub>3</sub>	8.010	3.995	1.928	Ηγ: 0.864
Glu4	8.480	4.274	1.966	Ну: 2.351
Val <sub>5</sub>	8.452	4.396	2.054	Ηγ: 0.958
Pro <sub>6</sub>		4.396	2.312	Ηγ: 2.007, 1.975, Ηδ: 3.882, 3.692
Gly7	8.605	3.940		
Lys <sub>8</sub>	8.178	4.300	1.764	Hγ: 1.412, Hε: 2.968, HNt: 7.601
Lys <sup>S</sup> 9	8.599	4.660	1.787	Hγ: 1.373, Hε: 2.968, HNt: 7.601
Ile <sub>10</sub>	10.125	4.660	2.014	Hγ: 1.537, 1.271, Hδ or Hγ (Me): 0.934
Leu <sub>11</sub>	8.649	4.378	1.678	Нү: 1.553
Gln <sub>12</sub>	8.452	4.284	1.975	Hγ: 2.363, HNt: 7.634, 6.963
NH <sub>2</sub>	7.712, 7.205			

### YKL-Leu<sup>S</sup>11-OH Test

Residue	NH	Ηα	Нβ	Others
Ac		1.952		
Arg <sub>1</sub>	8.34	4.385	1.727, 1.620	Hγ: 1.483, Hδ: 3.163, HNt: 7.243
Tyr <sub>2</sub>	8.532	5.176	2.778, 2.730	2,6H: 6.918, 3,5H: 6.777
Val <sub>3</sub>	8.921	4.375	1.981	Ηγ: 0.857
Glu4	8.643	5.020	1.981, 1.883	Ηγ: 2.255
Val <sub>5</sub>	9.008	4.600	1.961	Ηγ: 0.926
pro <sub>6</sub>		4.360	2.382, 2.001	Ηγ: 2.108, Ηδ: 3.867
Gly7	8.683	4.004, 3.759		
Lys <sub>8</sub>	7.927	4.580	1.786	Ηγ: 1.385, Ηδ: 1.668, Ηε: 2.987, ΗΝτ: 7.619
Lys <sub>9</sub>	8.526	4.648	1.629, 1.541	Ηγ: 1.102, Ηδ: 1.326, Ηε: 2.577, ΗΝτ: 7.426
Ile <sub>10</sub>	9.083	4.570	2.001	Hγ: 1.375, 1.170, Hδ or Hγ (Me): 0.897
Leu <sup>S</sup> 11	8.730	4.502	1.659	Ηγ: 1.512, Ηδ: 0.613
Gln <sub>12</sub>	10.199	4.648	1.942	Hγ: 2.225, HNt: 7.318, 7.016
OH				

## YKL-Leu<sup>S</sup>11-OH Unfolded

Residue	NH	Ηα	Нβ	Others
Ac		1.991		
Arg <sub>1</sub>	8.294	4.173	1.630	Hγ: 1.458, Hδ: 3.125, HNt:
Tyr <sub>2</sub>	8.369	4.627	3.006, 2.926	Нү:
Val <sub>3</sub>	8.026	4.001	1.931	Ηγ: 0.871
Glu4	8.481	4.292	1.982	Ηγ: 2.408
Val <sub>5</sub>	8.434	4.400	2.068	Ηγ: 0.949
Pro <sub>6</sub>		4.387	2.311, 1.982	Ηγ: 2.013, Ηδ: 3.875, 3.692
Gly7	8.604	3.939		
Lys <sub>8</sub>	8.192	4.322	1.755	Hγ: 1.395, Hε: 2.975, HNt: 7.605
Lys <sub>9</sub>	8.502	4.330	1.739	Hγ: 1.426, Hε: 2.975, HNt: 7.605
Ile <sub>10</sub>	8.381	4.173	1.888	Hγ: 1.172, Hδ or Hγ (Me): 0.886
Leu <sup>S</sup> 11	8.472	4.760	1.737, 1.638	Нδ: 0.922, 0.874
Gln <sub>12</sub>	9.989	4.713	2.092	Hγ: 2.350, HNt: 7.610, 6.913
OH				

### HPT Test

Residue	NH	Нα	Нβ	Others
Ac		2.019		
Arg <sub>1</sub>	8.296	4.388	1.827, 1.749	Ηγ: 1.630, Ηδ: 3.202, HNt: 7.205
Thr <sub>2</sub>	8.307	4.603	4.084	Ηγ: 1.138
Val <sub>3</sub>	8.478	4.260	2.048	Ηγ: 0.893
Glu <sub>4</sub>	8.437	4.711	1.910, 1.832	Ηγ: 2.160, 2.062
Val <sub>5</sub>	8.604	4.544	1.999	Ηγ: 0.927
pro <sub>6</sub>		4.388	2.342, 1.988	Ηγ: 2.087, 2.048, Ηδ: 3.876, 3.819
Gly7	8.511	3.986, 3.800		
Orn <sub>8</sub>	7.993	4.505	1.851, 1.798	Hγ: 1.692, Hδ: 3.016, HNt: 7.614
Lys <sub>9</sub>	8.469	4.485	1.725	Ηγ: 1.314, Ηδ: 1.451, Ηε: 2.967
Ile <sub>10</sub>	8.589	4.260	1.847	Hγ: 1.406, 1.171, Hδ or Hγ (Me): 0.868, 0.805
Leu <sub>11</sub>	8.400	4.456	1.597	Ηδ: 0.902, 0.849
Gln <sub>12</sub>	8.487	4.319	2.078, 1.941	Hγ: 2.322, HNt: 7.448, 6.878
NH <sub>2</sub>	7.648, 7.126			

### HPT Unfolded

Residue	NH	Ηα	Нβ	Others
Ac		2.030		
Arg <sub>1</sub>	8.304	4.355	1.831, 1.748	Hγ: 1.636, Hδ: 3.206, HNt: 7.215
Thr <sub>2</sub>	8.297	4.375	4.174	Нү: 1.186
Val <sub>3</sub>	8.202	4.130	2.056	Ηγ: 0.921
Glu4	8.467	4.341	1.973, 1.900	Ηγ: 2.272, 2.205
Val <sub>5</sub>	8.369	4.414	2.071	Ηγ: 0.958
Pro <sub>6</sub>		4.410	2.316, 1.934	Ηγ: 2.065, 1.999 Ηδ: 3.882, 3.706
Gly7	8.492	3.949		
Orn <sub>8</sub>	8.188	4.346	1.870, 1.773	Hγ: 1.702, Hδ: 3.010, HNt: 7.623
Lys <sub>9</sub>	8.371	4.311	1.782, 1.729	Ηγ: 1.371, Ηδ: 1.440, Ηε: 2.991, ΗΝt: 7.552
Ile <sub>10</sub>	8.276	4.135	1.836	Hγ: 1.484, 1.186, Hδ or Hγ (Me): 0.883
Leu <sub>11</sub>	8.381	4.390	1.655, 1.582	Ηδ: 0.865
Gln <sub>12</sub>	8.340	4.302	2.105, 1.978	Hγ: 2.364, HNt: 7.534, 6.870
NH <sub>2</sub>	7.599, 7.112			

### HPT Folded

Residue	NH	На	Нβ	Others
Ac		2.080		
Cys <sub>N</sub>	8.448	5.211	3.168, 2.655	
Arg <sub>1</sub>	8.778	4.672	1.833	Hγ: 1.686, 1.544, Hδ: 3.183, HNt: 7.130
Thr <sub>2</sub>	8.615	4.976	3.928	Нү: 1.079
Val <sub>3</sub>	9.093	4.467	1.999	Ηγ: 0.854, 0.803
Glu4	8.481	5.054	1.943, 1.803	Нү: 2.072
Val <sub>5</sub>	8.847	4.623	1.945	Ну: 0.903
pro <sub>6</sub>		4.337	2.390, 1.979	Ηγ: 2.141, 2.058, Ηδ: 3.867
Gly7	8.721	4.065, 3.723		
Orn <sub>8</sub>	7.890	4.653	1.818	Hγ: 1.681, Hδ: 3.007, HNt: 7.609
Lys <sub>9</sub>	8.519	4.760	1.740	Ηγ: 1.520, 1.219, Ηδ: 1.451, Ηε: 2.928
Ile <sub>10</sub>	9.143	4.467	1.852	Hγ: 1.336, 1.123, Hδ or Hγ (Me): 0.847, 0.781
Leu <sub>11</sub>	8.403	4.741	1.652	Ηγ: 1.490, Ηδ: 0.796
Gln <sub>12</sub>	9.231	4.663	2.072, 1.882	Hγ: 2.268, 2.209, HNt: 7.328, 6.833
Cysc	8.991	5.074	3.134, 3.007	
NH <sub>2</sub>	7.614, 7.247			

## HPT-Thr<sup>S</sup><sub>2</sub> Test

Residue	NH	Нα	Нβ	Others
Ac		2.032		
Arg <sub>1</sub>	8.284	4.392	1.844, 1.744	Ηγ: 1.633, Ηδ: 3.194, ΗΝτ: 7.186
Thr <sup>s</sup> <sub>2</sub>	8.463	4.832	4.081	Ну: 1.116
Val <sub>3</sub>	10.206	4.820	2.196	Ηγ: 0.959
Glu4	8.527,	4.756	1.841	Ηγ: 2.214, 2.067
Val <sub>5</sub>	8.592	4.556	1.992	Ηγ: 0.934
pro <sub>6</sub>		4.386	2.355, 1.991	Ηγ: 2.055, 2.096, Ηδ: 3.875, 3.828,
Gly7	8.533	3.998, 3.799		
Orn <sub>8</sub>	7.980	4.521	1.809	Ηγ: 1.697, Ηδ: 3.018, HNt: 7.618
Lys <sub>9</sub>	8.484	4.497,	1.727	Ηγ: 1.304, Ηδ: 1.457, Ηε: 2.965
Ile <sub>10</sub>	8.596	4.268	1.851	Hγ: 1.404, 1.175, Hδ or Hγ (Me): 0.869
Leu <sub>11</sub>	8.396	4.468	1.593	Нδ: 0.899, 0.852
Gln <sub>12</sub>	8.512	4.315	2.073, 1.938	Hγ: 2.325, HNt: 7.456, 6.873
NH <sub>2</sub>	7.660, 7.133			

## HPT-Thr<sup>S</sup><sub>2</sub> Unfolded

Residue	NH	Ηα	Нβ	Others
Ac		2.036		
Arg <sub>1</sub>	8.287	4.345	1.843, 1.743	Ηγ: 1.632, Ηδ: 3.192, HNt: 7.198
Thr <sup>s</sup> <sub>2</sub>	8.471	4.620	4.145	Ну: 1.163
Val <sub>3</sub>	9.997	4.579	2.200	Ηγ: 0.987
Glu4	8.600	4.345	1.972, 1.901	Ηγ: 2.314, 2.230
Val <sub>5</sub>	8.335	4.409	2.071	Ηγ: 0.958
Pro <sub>6</sub>		4.408	2.317, 1.937	Ηγ: 2.065, 2.001, Ηδ: 3.880, 3.705
Gly7	8.501	3.946		
Orn <sub>8</sub>	8.187	4.350	1.872, 1.773	Ηγ: 1.702, Ηδ: 3.010, HNt: 7.630
Lys <sub>9</sub>	8.376	4.309	1.732	Ηγ: 1.374, Ηδ: 1.439, Ηε: 2.987, HNt: 7.555
Ile <sub>10</sub>	8.288	4.134	1.843	Hγ: 1.48, 1.193, Hδ or Hγ (Me): 0.888
Leu <sub>11</sub>	8.389	4.386	1.655, 1.585	Ηδ: 0.935, 0.864
Gln <sub>12</sub>	8.346	4.298	2.101, 1.978	Hγ: 2.364, HNt: 7.538, 6.874
NH <sub>2</sub>	7.603, 7.115			

## HPT-Thr<sup>S</sup><sub>2</sub> Folded

Residue	NH	Ηα	Нβ	Others
Ac		2.036		
Cys <sub>N</sub>	8.455	5.260	3.172, 2.651	
Arg <sub>1</sub>	8.783	4.708	1.852	Ηγ: 1.693, 1.509, Ηδ: 3.172, HNt: 7.113
Thr <sup>s</sup> <sub>2</sub>	8.731	5.209	4.016	Ну: 1.033
Val <sub>3</sub>	10.630	5.279		Ηγ: 0.906
Glu4	8.651	5.095	1.934, 1.814	Ηγ: 2.080
Val <sub>5</sub>	8.828	4.650	1.960	Ηγ: 0.913
pro <sub>6</sub>		4.345	2.398, 1.985	Ηγ: 2.150, 2.068, Ηδ: 3.874
Gly7	8.744	4.079, 3.737		
Orn <sub>8</sub>	7.914	4.663	1.833	Ηγ: 1.693, Ηδ: 3.013
Lys <sub>9</sub>	8.535	4.771	1.744	Ηγ: 1.578, 1.217, Ηδ: 1.490, Ηε: 2.924
Ile <sub>10</sub>	9.176	4.460	1.846	Hγ: 1.354, 1.128, Hδ or Hγ (Me): 0.856
Leu <sub>11</sub>	8.472	4.733	1.674	Ηγ: 1.481, Ηδ: 0.798
Gln <sub>12</sub>	9.264	4.670	2.080, 1.871	Hγ: 2.280, 2.220, HNt: 7.353, 6.852
Cysc	9.015	5.101	3.127, 3.013	
NH <sub>2</sub>	7.595, 7.247			

## HPT-Val<sup>S</sup><sub>3</sub> Test

Residue	NH	Ηα	Нβ	Others
Ac		2.035		
Arg <sub>1</sub>	8.313	4.384	1.859, 1.754	Hγ: 1.648, Hδ: 3.207, HNt: 8.216
Thr <sub>2</sub>	8.315	4.395	4.208	Ηγ: 1.185
Val <sup>S</sup> <sub>3</sub>	8.283	4.414	2.105	Ηγ: 0.951, 0.887
Glu4	10.301	5.035	2.158, 2.023	Ηγ: 2.252
Val <sub>5</sub>	8.571	4.519	2.035	Ηγ: 0.957
pro <sub>6</sub>		4.410	2.328, 1.994	Ηγ: 2.076, 2.052, Ηδ: 3.892, 3.796
Gly7	8.413	3.962, 3.845		
Orn <sub>8</sub>	8.033	4.449	1.812	Hγ: 1.710, Hδ: 3.019, HNt: 7.616
Lys <sub>9</sub>	8.461	4.390	1.719	Ηγ: 1.355, Ηδ: 1.449, Ηε: 2.978
Ile <sub>10</sub>	7.998	4.173	1.847	Hγ: 1.449, 1.185, Hδ or Hγ (Me): 0.887
Leu <sub>11</sub>	8.367	4.390	1.648	Нγ: 1.584, Нδ: 0.928, 0.863
Gln <sub>12</sub>	8.349	4.302	2.105, 1.976	Hγ: 2.357, HNt: 7.528, 6.876
NH <sub>2</sub>	7.605, 7.112			

## HPT-Val<sup>S</sup><sub>3</sub> Unfolded

Residue	NH	Ha	Нβ	Others
Ac		2.032		
Arg <sub>1</sub>	8.308	4.370	1.857, 1.750	Hγ: 1.648, Hδ: 3.209, HNt: 7.220
Thr <sub>2</sub>	8.308	4.362	4.212	Нү: 1.185
Val <sup>s</sup> <sub>3</sub>	8.282	4.377	2.086	Ηγ: 0.950, 0.887
Glu4	10.299	4.859	2.308	Ηγ: 2.232, 2.080
Val <sub>5</sub>	8.480	4.421	2.073	Ηγ: 0.969
Pro <sub>6</sub>		4.404	2.314, 1.934	Нγ: 2.067, 1.991, Нδ: 3.888, 3.701
Gly7	8.504	3.952		
Orn <sub>8</sub>	8.187	4.351	1.870	Нγ: 1.775, 1.670, Нδ: 3.012,
Lys <sub>9</sub>	8.371	4.313	1.737, 1.675	Ηγ: 1.375, Ηδ: 1.439, Ηε: 2.987, ΗΝt: 7.558
Ile <sub>10</sub>	8.282	4.136	1.838	Hγ: 1.483, 1.185, Hδ or Hγ (Me): 0.880
Leu <sub>11</sub>	8.385	4.383	1.654	Ηγ: 1.585, Ηδ: 0.931, 0.861
Gln <sub>12</sub>	8.340	4.301	2.105, 1.978	Hγ: 2.359, HNt: 7.534, 6.870
NH <sub>2</sub>	7.600, 7.112			

# HPT-Val<sup>S</sup><sub>3</sub> Folded

Residue	NH	На	Ηβ	Others
Ac		2.053		
Cys <sub>N</sub>	8.431	4.953	3.132, 2.875	
Arg <sub>1</sub>	8.687	4.604	1.895, 1.806	Hγ: 1.634, Hδ: 3.196, HNt: 7.166
Thr <sub>2</sub>	8.522	4.680	4.084	Ηγ: 1.089
Val <sup>s</sup> <sub>3</sub>	8.279	4.528	2.104	Ηγ: 0.905, 0.873
Glu4	10.580	5.214	2.079	Ηγ: 2.161
Val <sub>5</sub>	8.515	4.639	2.015	Ηγ: 0.955
pro <sub>6</sub>		4.363	2.371, 1.990	Ηγ: 2.117, 2.047, Ηδ: 3.844
Gly7	8.675	4.014, 3.811		
Orn <sub>8</sub>	7.972	4.560	1.869, 1.736	Ηγ: 1.685, Ηδ: 3.005
Lys <sub>9</sub>	8.563	4.395	1.717, 1.634	Ηγ: 1.235, Ηδ: 1.412, Ηε: 2.945
Ile <sub>10</sub>	7.607	4.287	1.945	Hγ: 1.431, 1.133, Hδ or Hγ (Me): 0.905
Leu <sub>11</sub>	8.367	4.471	1.622	Ηγ: 1.577, Ηδ: 0.835
Gln <sub>12</sub>	8.730	4.573	2.117, 1.977	Hγ: 2.345, HNt: 7.339, 6.869
Cysc	8.748	4.877	3.189, 3.005	
NH <sub>2</sub>	7.669, 7.252			

## HPT-Ile<sup>S</sup>10 Test

Residue	NH	Ηα	Ηβ	Others
Ac		2.046		
Arg <sub>1</sub>	8.310	4.384	1.772	Hγ: 1.655, Hδ: 3.219, HNt: 7.215
Thr <sub>2</sub>	8.295	4.439	4.173	Ну: 1.209
Val <sub>3</sub>	8.156	4.251	2.124	Ηγ: 0.935
Glu4	8.386	4.721	1.905	Ηγ: 2.327, 2.234
Val <sub>5</sub>	8.536	4.549	2.030	Ηγ: 0.951
pro <sub>6</sub>		4.423	2.359	Ηγ: 2.079, Ηδ: 3.889, 3.818
Gly7	8.437	3.985, 3.845		
Orn <sub>8</sub>	7.993	4.517	1.890, 1.827	Hγ: 1.733, Hδ: 3.047, HNt: 7.619
Lys <sub>9</sub>	8.44	4.439	1.733	Ηγ: 1.373, Ηδ: 1.420, Ηε: 2.969, ΗΝτ: 7.554
Ile <sup>S</sup> 10	8.497	4.486	1.983	Hγ: 1.514, 1.264, Hδ or Hγ (Me): 0.842
Leu <sub>11</sub>	10.253	5.002	1.874	Ηγ: 1.702, Ηδ: 0.967, 0.888
Gln <sub>12</sub>	8.514	4.345	2.124, 1.991	Hγ: 2.406, HNt: 7.406, 6.884
NH <sub>2</sub>	7.605, 7.124			

### HPT-Ile<sup>S</sup>10 Unfolded

Residue	NH	На	НВ	Others
Ac		2.041	<b>F</b>	
Arg <sub>1</sub>	8.313	4.348	1.830, 1.752	Hγ: 1.650, Hδ: 3.206, HNt: 7.207
Thr <sub>2</sub>	8.282	4.380	4.176	Ηγ: 1.189
Val <sub>3</sub>	8.211	4.129	2.049	Ну: 0.923
Glu4	8.453	4.395	1.939	Ну: 2.377
Val <sub>5</sub>	8.327	4.426	2.080	Ηγ: 0.954
Pro <sub>6</sub>		4.414	2.324, 1.964	Ηγ: 2.063, Ηδ: 3.872, 3.708
Gly7	8.474	3.957		
Orn <sub>8</sub>	8.196	4.380	1.893	Hγ: 1.744, Hδ: 3.019, HNt: 7.618
Lys <sub>9</sub>	8.356	4.301	1.783, 1.713	Hγ: 1.408, Hε: 2.972, HNt: 7.539
Ile <sup>S</sup> 10	8.412	4.426	1.924	Hγ: 1.564, 1.220, Hδ or Hγ (Me): 0.845
Leu <sub>11</sub>	10.19	4.958	1.861,	Ηγ: 1.689, Ηδ: 0.954, 0.876
Gln <sub>12</sub>	8.484	4.333	2.112, 1.986	Hγ: 2.409, HNt: 7.434, 6.871
NH <sub>2</sub>	7.597, 7.120			

# HPT-Ile<sup>S</sup>10 Folded

Residue	NH	Ηα	Нβ	Others
Ac		2.049		
Cys <sub>N</sub>	8.429	4.880	3.113, 2.987	
Arg <sub>1</sub>	8.672	4.583	1.830	Hγ: 1.642, Hδ: 3.206, HNt: 7.171
Thr <sub>2</sub>	8.341	4.645	4.035	Ну: 1.142
Val <sub>3</sub>	7.851	4.426	2.143	Нү: 0.892
Glu4	8.355	4.927	1.877	Нү: 2.237, 2.205
Val <sub>5</sub>	8.852	4.583	2.002	Ηγ: 0.938
pro <sub>6</sub>		4.380	2.377, 1.997	Ηγ: 2.143, 2.065, Ηδ: 3.867
Gly7	8.544	4.035, 3.785		
Orn <sub>8</sub>	7.892	4.630	1.846	Hγ: 1.705, Hδ: 3.019, HNt: 7.619
Lys <sub>9</sub>	8.529	4.645	1.720, 1.611	Hγ: 1.236, Hδ: 1.423, Hε: 2.941, HNt: 7.541
Ile <sup>S</sup> 10	8.563	4.614	2.018	Hγ: 1.470, 1.236, Hδ or Hγ (Me): 0.845
Leu <sub>11</sub>	10.393	4.974	1.830, 1.752	Ηγ: 1.642, Ηδ: 0.892
Gln <sub>12</sub>	8.598	4.567	2.018	Hγ: 2.424, HNt: 7.334, 6.848
Cysc	8.583	4.864	3.222, 3.019	
NH <sub>2</sub>	7.649, 7.247			

## HPT-Leu<sup>S</sup>11-OH Test

Residue	NH	Ηα	Нβ	Others
Ac		2.036		
Arg <sub>1</sub>	8.395	4.401	1.817, 1.738	Hγ: 1.629, Hδ: 3.195, HNt: 7.232
Thr <sub>2</sub>	8.308	4.636	4.072	Нү: 1.143
Val <sub>3</sub>	8.516	4.260		Ηγ: 0.877
Glu4	8.421	4.746	1.832	Нү: 2.130, 2.036
Val <sub>5</sub>	8.636	4.558	1.973	Нү: 0.916
pro <sub>6</sub>		4.385	2.349, 2.005	Ηγ: 2.083, Ηδ: 3.879, 3.824
Gly7	8.540	3.994, 3.790		
Orn <sub>8</sub>	7.969	4.542	1.832	Ηγ: 1.691, Ηδ: 3.007
Lys <sub>9</sub>	8.495	4.542	1.723	Ηγ: 1.316, Ηδ: 1.464, Ηε: 2.96
Ile <sub>10</sub>	8.620	4.338	1.926	Hγ: 1.363, 1.128, Hδ or Hγ (Me): 0.877
Leu <sup>S</sup> 11	8.344	4.871	1.707	Ηγ: 1.629, Ηδ: 0.877
Gln <sub>12</sub>	9.980	4.793	2.020	Hγ: 2.31, 2.208, HNt: 7.414, 6.824
OH				

### HPT-Leu<sup>S</sup>11-OH Unfolded

Residue	NH	Ηα	Нβ	Others
Ac		2.049		
Arg <sub>1</sub>	8.312	4.364	1.767	Hγ: 1.658, Hδ: 3.222, HNt: 7.203
Thr <sub>2</sub>	8.276	4.380		Ηγ: 1.189
Val <sub>3</sub>	8.203	4.129	2.065	Ηγ: 0.923
Glu4	8.447	4.411	2.033, 1.955	Ηγ: 2.409
Val <sub>5</sub>	8.318	4.442	2.080	Ηγ: 0.954
Pro <sub>6</sub>		4.42	2.331, 1.971	Ηγ: 2.065, Ηδ: 3.865, 3.706
Gly7	8.469	3.957		
Orn <sub>8</sub>	8.178	4.380	1.857	Hγ: 1.743, Hδ: 3.019, HNt: 7.606
Lys <sub>9</sub>	8.400	4.333	1.814	Ηγ: 1.423, Ηδ: 1.720, Ηε: 3.003, ΗΝτ: 7.544
Ile <sub>10</sub>	8.270	4.176	1.893	Hγ: 1.470, 1.189, Hδ or Hγ (Me): 0.907
Leu <sup>S</sup> 11	8.351	4.771	1.744	Ηγ: 1.658, Ηδ: 0.923
Gln <sub>12</sub>	9.882	4.771	2.112	Hγ: 2.362, 2.276, HNt: 7.518, 6.827
OH				

### HPT-Leu<sup>S</sup>11 Folded

Residue	NH	Ηα	Нβ	Others
Ac		2.067		
Cys <sub>N</sub>	8.440	5.184	3.164, 2.647	
Arg <sub>1</sub>	8.828	4.683	1.848, 1.691	Hγ: 1.551, Hδ: 3.195, HNt: 7.136
Thr <sub>2</sub>	8.638	4.980	3.947	Нү: 1.096
Val <sub>3</sub>	9.043	4.464	1.989	Ηγ: 0.830
Glu4	8.476	5.059	1.801	Ηγ: 2.067, 1.942
Val <sub>5</sub>	8.849	4.636	1.942	Ηγ: 0.908
pro <sub>6</sub>		4.338	2.396, 1.989	Ηγ: 2.134, 2.067, Ηδ: 3.864
Gly7	8.721	4.072, 3.728		
Orn <sub>8</sub>	7.882	4.667	1.817	Ηγ: 1.691, Ηδ: 3.007
Lys <sub>9</sub>	8.529	4.793	1.738, 1.574	Нγ: 1.222, Нδ: 1.472, Нε: 2.944
Ile <sub>10</sub>	9.082	4.542	1.942	Hγ: 1.284, 1.081, Hδ or Hγ (Me): 0.846
Leu <sup>S</sup> 11	8.449	5.074	1.738, 1.613	Ηγ: 1.457, Ηδ: 0.799
Gln <sub>12</sub>	10.595	5.482	2.005	Hγ: 2.263, HNt: 7.338, 6.842
Cysc	8.993	5.106	3.132, 3.007	
NH <sub>2</sub>	7.622, 7.253			

**HPT-Leu**<sup>S</sup><sub>11</sub>-**OH Test and Unfolded Epimerization.** As noted in the main text, HPT-Leu<sup>S</sup><sub>11</sub>-OH was synthesized as a C-terminal amide on Rink amide resin, but underwent thioamide catalyzed hydrolysis during TFA cleavage, accompanied by partial epimerization (see mechanism in **Fig. S4**). Two HPLC peaks were collected for each of the HPT-Leu<sup>S</sup><sub>11</sub>-OH Test and HPT-Leu<sup>S</sup><sub>11</sub>-OH Unfolded syntheses. While it could be assumed that epimerization was partial and that the larger peak corresponded to the desired epimer (with L-chirality at Gln<sub>12</sub>), both peptides from each synthesis were characterized by NMR. CSD analysis of the HPT-Leu<sup>S</sup><sub>11</sub>-OH epimers demonstrated that there is a significant difference between the  $\beta$ -hairpin character of the Test epimers, but not the Unfolded epimers (**Fig. S5**). The more abundant Test epimer (Test A) is as folded as HPT regardless of which Unfolded peptide is used as the reference, whereas the less abundant epimer (Test B) is the least folded of any of our HPT thioamide peptides. The results of the more abundant Test epimer are consistent with the results obtained for YKL-Leu<sup>S</sup><sub>11</sub>-OH, wherein thioamide substitution was found to be non-perturbing. Based on this comparison, we assumed the most abundant Test peptide (Test A) was the L-Gln<sub>12</sub> epimer.

The more abundant Unfolded peptide (Unfolded A) was assumed to be the L-Gln<sub>12</sub> epimer and used as the reference since the ROESY data is similar for the two epimers, and the choice of Unfolded control has no effect on the CSD or fraction folded analysis. The fact that the stereochemistry of Gln<sub>12</sub> has a major effect on the spectra of the Test peptide, but a minor effect on the Unfolded control, further supports that the proGly  $\beta$ -turn is an effective Unfolded control. Fraction folded analysis for all combinations of Test and Unfolded peptides are shown in **Table S6**. For clarity, data reported elsewhere for HPT-Leu<sup>S</sup><sub>11</sub>-OH Test correspond to Test A and data for HPT-Leu<sup>S</sup><sub>11</sub>-OH Unfolded correspond to Unfolded A. Chemical shift assignments for the undesired epimers are given in **Table S7**.



**Figure S4.** Proposed mechanism of Edman-type degradation observed for the HPT-Leu<sup>S</sup><sub>11</sub>-OH Unfolded and Test  $\beta$ -hairpins. The observed masses correspond with epimerization of the C-terminal residue and a carboxylate C-terminus. As described above, the L-epimers are Unfolded A and Test A.



**Figure S5.** CSD analysis ( $\delta_{H\alpha}$ ) of the HPT-Leu<sup>S</sup><sub>11</sub>-OH epimers in comparison to YKL-Leu<sup>S</sup><sub>11</sub>-OH demonstrates that HPT-Leu<sup>S</sup><sub>11</sub>-OH Test epimer A is folded similarly to YKL-Leu<sup>S</sup><sub>11</sub>-OH Test and that HPT-Leu<sup>S</sup><sub>11</sub>-OH Test epimer B is poorly folded.

	F	raction Folde	d (%)	$\Delta G_{Folding}$ (kcal/mol)		
HPT-Leu <sup>S</sup> 11-OH	Val <sub>3</sub>	Ile <sub>10</sub>	Average	Val <sub>3</sub>	Ile <sub>10</sub>	Average
Охо	38.6%	37.7%	$38.1\pm1\%$	0.28	0.30	$0.29\pm0.0$
Test B - Unfolded A	20.3%	27.3%	$23.8\pm4\%$	0.81	0.58	$0.69\pm0.1$
Test B - Unfolded B	22.6%	26.1%	$24.4\pm2\%$	0.73	0.62	$0.67\pm0.1$
Test A - Unfolded A	39.1%	44.3%	$41.7\pm3\%$	0.26	0.14	$0.20\pm0.1$
Test A - Unfolded B	40.9%	43.3%	42.1 ± 1%	0.22	0.16	$0.19\pm0.0$

**Table S6.** Comparison of fraction folded and  $\Delta G_{Folding}$  analysis for HPT-Leu<sup>S</sup><sub>11</sub>-OH Test and Unfolded epimers

**Table S7.** <sup>1</sup>H Chemical shift assignments for HPT-Leu<sup>S</sup><sub>11</sub>-OH Test and Unfolded epimers

Residue	NH	Нα	Нβ	Others
Ac		2.036		
Arg <sub>1</sub>	8.293	4.354	1.832, 1.754	Hγ: 1.644, Hδ: 3.195, HNt: 7.232
Thr <sub>2</sub>	8.235	4.479	4.135	Нү: 1.190
Val <sub>3</sub>	8.259	4.197	2.052	Ηγ: 0.908
Glu4	8.407	4.714	1.879	Нү: 2.271, 2.177
Val <sub>5</sub>	8.527	4.526	2.005	Ηγ: 0.940
pro <sub>6</sub>		4.401	2.349, 2.005	Ηγ: 2.083, Ηδ: 3.884, 3.808
Gly7	8.441	3.978, 3.821		
Orn <sub>8</sub>	7.976	4.495	1.801	Нγ: 1.707, Нδ: 3.023
Lys <sub>9</sub>	8.502	4.417	1.754	Ηγ: 1.347, Ηδ: 1.472, Ηε: 2.976
Ile <sub>10</sub>	8.447	4.276	1.926	Hγ: 1.41, 1.175, Hδ or Hγ (Me): 0.908, 0.814
Leu <sup>S</sup> 11	8.286	4.840	1.738	Ηγ: 1.644, Ηδ : 0.908
Gln <sub>12</sub>	9.781	4.699	2.099	Hγ: 2.349, 2.255, HNt: 7.530, 6.817
OH				

HPT-Leu<sup>S</sup><sub>11</sub>-OH Test D-Gln<sub>12</sub> Epimer (Test B)

HPT-Leu<sup>S</sup><sub>11</sub>-OH Unfolded D-Gln<sub>12</sub> Epimer (Unfolded B)

Residue	NH	Ηα	Нβ	Others
Ac		2.036		
Arg <sub>1</sub>	8.308	4.354	1.825, 1.738	Hγ: 1.644, Hδ: 3.208, HNt: 7.219
Thr <sub>2</sub>	8.291	4.370	4.182	Ну: 1.19
Val <sub>3</sub>	8.204	4.119	2.052	Ηγ: 0.908
Glu4	8.469	4.338	2.255	Нү: 1.911
Val <sub>5</sub>	8.357	4.417	2.067	Нү: 0.955
Pro <sub>6</sub>		4.401	2.318, 1.95	Ηγ: 2.052, Ηδ: 3.874, 3.704
Gly7	8.490	3.947		
Orn <sub>8</sub>	8.181	4.354	1.864	Hγ: 1.707, Hδ: 3.007, HNt:
Lys <sub>9</sub>	8.418	4.323		Ηγ: 1.410, Ηδ: 1.731, Ηε: 2.991, HNt:
Ile <sub>10</sub>	8.297	4.182	1.895	Hγ: 1.457, 1.190, Hδ or Hγ (Me): 0.908
Leu <sup>S</sup> 11	8.311	4.769	1.754	Ηγ: 1.629, Ηδ: 0.908
Gln <sub>12</sub>	9.689	4.683	2.083	Hγ: 2.334, 2.240, HNt: 7.523, 6.820
OH				



**Figure S6.** (A) Far-UV circular dichroism (CD) of thioamide-containing Test YKL  $\beta$ -hairpins. All data were collected at ~100  $\mu$ M concentration in 100 mM sodium acetate buffer (pH = 3.8). (B) Temperature-dependent denaturation of thioamide-containing Test YKL  $\beta$ -hairpins. The thermal melts measured at this signature were linear and therefore T<sub>m</sub> could not be calculated. The YKL-Glu<sup>S</sup><sub>4</sub> has not been considered for the analysis due to lack of  $\beta$ -hairpin signature.









**Figure S7.** NOEs for Test YKL  $\beta$ -hairpin variants.



Figure S8. NOEs for Unfolded YKL  $\beta$ -hairpin variants.



**Figure S9.**  $\Delta \delta_{NH}$  (Test  $\delta_{NH}$  – Unfolded control  $\delta_{NH}$ ) for YKL-Glu<sup>S</sup><sub>4</sub>, YKL-Lys<sup>S</sup><sub>9</sub>, and YKL-Leu<sup>S</sup><sub>11</sub>-OH in comparison to YKL. The thioamide variants have similar values to YKL suggestive of a similar structure.



**Figure S10.** CD scans of thioamide variants of the HPT  $\beta$ -hairpin scaffold. All data were collected at ~130-150  $\mu$ M in the buffer corresponding to the NMR buffer (HPT, HPT-Thr<sup>S</sup><sub>2</sub>, and HPT-Val<sup>S</sup><sub>3</sub> in 50 mM sodium deuterioacetate pH 5.5 and HPT-Ile<sup>S</sup><sub>10</sub> and HPT-Leu<sup>S</sup><sub>11</sub>-OH in 50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 5.5). The Folded controls exhibit the most significant anti-parallel  $\beta$ -sheet character (slight differences can be observed for the internal thioamides: HPT-Val<sup>S</sup><sub>3</sub> and HPT-Ile<sup>S</sup><sub>10</sub>). The Test  $\beta$ -hairpins have a stronger thioamide  $\pi$ -to- $\pi$ \* absorbance than any secondary structure characteristic.



0

0 0

H<sub>3</sub>N

H<sub>2</sub>N HN

**Figure S11.** NOEs for Test HPT  $\beta$ -hairpin variants.



0-

0 ∕\_0 +NH₃

 $\overset{H_2N}{\underset{HN}{\checkmark}}\overset{+}{\overset{H}}\overset{H_2}{\underset{HN}{\lor}}$ 

Figure S12. NOEs for Unfolded HPT  $\beta$ -hairpin variants.



**Figure S13.**  $\Delta\delta_{NH}$  (Test  $\delta_{NH}$  – Unfolded control  $\delta_{NH}$ ) for HPT-Thr<sup>S</sup><sub>2</sub>, HPT-Val<sup>S</sup><sub>3</sub>, HPT-Ile<sup>S</sup><sub>10</sub> and HPT-Leu<sup>S</sup><sub>11</sub>-OH in comparison to HPT. Overall, no major trends are observed. Of interest is the negative  $\Delta\delta_{NH}$  for the amide across from the HPT-Val<sup>S</sup><sub>3</sub> thioamide. This is probably due to the increased electron density of the thioamide. This negative  $\Delta\delta_{NH}$  is weakly observed for the other hydrogen bond acceptor position (HPT-Ile<sup>S</sup><sub>10</sub>).



Figure S14. NOEs for Folded HPT  $\beta$ -hairpin variants.



**Figure S15.** ROESY mixing time comparison for the HPT scaffold. HPT HPT-Thr<sup>S</sup><sub>2</sub>, and HPT-Val<sup>S</sup><sub>3</sub> were collected at NTU (200 ms mixing time). Whereas HPT-Ile<sup>S</sup><sub>10</sub> and HPT-Leu<sup>S</sup><sub>11</sub>-OH were collected at Penn (250 ms mixing time). Since the NOE connectivities are directly observed from the spectra, a comparison was needed between the two collection methods. This was done by collecting ROESY data of the HPT Test peptide with the two mixing times (**A** and **B**). No additional cross-peaks are observed with the longer mixing time (250 ms). (**C**) is the overlay of a ROESY collected at NTU versus collected at Penn. Besides a lack of Orn<sub>8</sub> signal in the Penn spectra, the cross-peaks are similar between the two collection methods. One difference between the spectra is that the intensities are on different scales. For example, the NOE between Val<sub>5</sub> H $\alpha$  and pro<sub>6</sub> H $\delta$  is ~4.5 for the NTU collection and ~5400 for the Penn collection. This 1000x fold difference is reflected in the intensity cut-off for the NOEs displayed in **Fig. S11-12**, **S14** (0.3 for NTU and 300 for Penn).

	Experimental							
	Fra	ection Fo	lded (%)	ΔΔ	GFolding (	kcal/mol)		
	Val <sub>3</sub> Ile <sub>10</sub> Average			Val <sub>3</sub>	Ile <sub>10</sub>	Average		
НРТ	38.6%	37.7%	$38.1\pm0.5\%$					
HPT-Thr <sup>s</sup> <sub>2</sub>	34.4%	41.1%	37.8 ± 3%	0.11	-0.09	$0.01 \pm 0.1$		
HPT-Val <sup>S</sup> <sub>3</sub>	24.5%	24.5%	$24.5\pm0\%$	0.39	0.37	$0.38\pm0.01$		
HPT-Ile <sup>S</sup> <sub>10</sub>	41.1%	31.9%	36.5 ± 5%	-0.06	0.15	$0.04 \pm 0.1$		
HPT-Leu <sup>S</sup> <sub>11</sub> -OH	39.1%	44.3%	41.7 ± 3%	-0.01	-0.16	$-0.09 \pm 0.1$		

Table S8. Fraction folded and  $\Delta\Delta G_{Folding}$  analysis of HPT peptides

Structural Models of the Thioamide-Containing Folded Control β-Hairpins



**Figure S16**. The structure of YKL with a Thr extension (PDB 1jy9) was used as a starting point to generate a structure for HPT. The following modifications were made using PyRosetta<sup>11</sup>: deletion of the terminal Thr residues, conversion of the penultimate Thr residues to Cys, formation of the disulfide bond, acetylation of the N-terminus, conversion of the C-terminus to a carboxamide, conversion of Tyr<sub>2</sub> to Thr, and conversion of Lys<sub>8</sub> to Orn. Upon completion of these modifications, it can be seen that the starting model of HPT (gray) exhibits similar backbone and side-chain orientations to the starting 1jy9 structure (cyan).



**Figure S17.** The HPT starting structure was subjected to ten Cartesian FastRelax cycles employing the beta\_nov16Cart score function in PyRosetta. Additionally, constrained relaxes were performed using the same conditions and the distances derived from the HPT Folded control NOEs (**Table S9**). The resulting lowest energy structure from the constrained relax differed significantly from the initial model (backbone RMSD = 2.231 Å) but was consistent with the NOEs displaying only three instances of deviation greater than 0.2 Å between the experimental and computationally derived distances (one in the  $\beta$ -turn and two between Cys<sub>N</sub> and Leu<sub>12</sub>). Of particular interest is that removal of the cross-strand cation- $\pi$  interaction of the YKL scaffold resulted in Glu<sub>4</sub> and Lys<sub>9</sub> forming a salt bridge (indicated by arrows) which was previously not observed in the HPT structure prior to the constrained relax.

Generation of Distance Constraints from NOEs. The trans-Val-pro bond has a fixed interproton distance of ~2.1 Å, which was previously used as an internal standard for converting NOE intensities to distances in a pro-Gly  $\beta$ -hairpin.<sup>12</sup> Therefore, **Eq. S2** was used to convert NOE intensities to experimental distances, where  $r_{ref}$  is 2.1 Å,  $a_{ref}$  is the NOE intensity corresponding to pro<sub>7</sub> Hδ- Val<sub>6</sub> Hα cross-peak,  $a_{ij}$  and  $r_{ij}$  correspond respectively to the NOE intensity and distance of the atom pair.<sup>13</sup>

$$r_{ij} = r_{ref} \left(\frac{a_{ref}}{a_{ij}}\right)^{1/6}$$
 (Eq. S2)

*PyRosetta Modelling of Folded Controls.* The experimental distances listed in **Table S9** were entered into PyRosetta and used for constrained relax in the following way. A flat harmonic potential was used with a standard deviation and tolerance of 0.1. The weight of the constraint term passed to the score function was 1.0. The thioamide modification was incorporated through our previously generated patches<sup>14</sup> during these simulations, which produced models of the HPT, HPT-Thr<sup>S</sup><sub>2</sub>, HPT-Val<sup>S</sup><sub>3</sub>, HPT-Ile<sup>S</sup><sub>10</sub> and HPT-Leu<sup>S</sup><sub>11</sub>-OH Folded controls. The computed interatom distances are given in **Table S9** for comparison to the NOE values. The backbone RMSD of the thioamide-containing Folded controls in comparison to the HPT Folded control are displayed in **Table S10**.

**Table S9.** NOE derived distance constraints for PyRosetta simulation of Folded control β-hairpins

The Folded control peptide numbering starts with  $Cys_1$  ( $Cys_N$ ),  $Arg_2$ ,  $Thr_3$ , etc. The atom names are represented in the PDB format. Atom names in red correspond to instances where the specific atom number could not be determined based on the chemical shifts observed, and therefore an arbitrary number was chosen. Any deviation of greater than 0.15 Å between the experimental and computational distances is highlighted in red. Note: Experimental distances were only determined to 0.1 Å precision.

Constraint number	First Res Atom Name	First Res number	Second Res Atom Name	Second Res number	Intensity	Experimental Distance	Computational Distance	Deviation
0	Н	8	1HD	7	1	3.1	3.24	0.14
1	Н	9	2HA	8	0.4	3.6	3.72	0.12
2	Н	9	1HA	8	0.5	3.5	3.35	0.15
3	Н	6	Н	9	0.4	3.6	3.48	0.12
4	Н	8	Н	9	0.8	3.2	3.25	0.05
5	Н	13	Н	2	0.3	3.8	3.70	0.10
6	Н	6	1HB	9	0.4	3.6	3.59	0.01
7	Н	2	1HD1	12	0.3	3.8	3.69	0.11
8	Н	2	1HB	13	0.3	3.8	3.91	0.11
9	Н	8	2HB	7	1.6	2.9	3.03	0.13
10	Н	8	1HG	7	0.6	3.4	3.51	0.11
11	Н	9	1HG	5	0.4	3.6	3.71	0.11
12	HA	1	HA	14	2.6	2.6	2.47	0.13
13	1HD	7	HA	6	10	2.1	2.00	0.10
14	1HD	7	1HG1	6	1.4	2.9	3.07	0.17
15	1HE	10	1HG2	3	0.4	3.6	3.70	0.10
16	2HB	1	1HD1	12	1	3.1	2.97	0.13
17	2HB	1	HG	12	1	3.1	2.94	0.16
18	2HB	1	1HB	12	0.5	3.5	3.72	0.22
19	2HB	1	HA	14	0.6	3.4	3.52	0.12
20	1HB	10	2HA	8	0.8	3.2	3.33	0.13
21	1HG2	3	1HG	10	1.8	2.8	2.90	0.10
22	Н	13	HA	3	0.3	3.8	3.68	0.12
23	Н	8	HA	7	1.4	2.9	3.02	0.12

#### **HPT** Folded Control

## HPT-Thr<sup>S</sup><sub>2</sub> Folded Control

Constraint number	First Res Atom Name	First Res number	Second Res Atom Name	Second Res number	Intensity	Experimental Distance	Computational Distance	Deviation
0	Н	9	1HA	8	0.3	3.2	3.09	0.11
1	Н	9	2HB	8	0.3	3.2	3.33	0.13
2	Н	8	2HB	7	0.5	3	3.11	0.11
3	Н	8	1HG	7	0.3	3.2	3.33	0.13
4	Н	6	Н	9	0.3	3.2	3.09	0.11
5	Н	8	Н	9	0.4	3.1	2.99	0.11
6	1HD	7	1HG1	6	0.6	2.9	3.07	0.17
7	1HD	7	HA	6	4	2.1	2.01	0.09
8	1HG2	3	HA	10	1	2.6	2.50	0.10
9	1HG2	3	1HD	10	1.1	2.6	2.70	0.10
10	1HG2	3	2HB	10	0.5	3	2.92	0.08
11	1HG2	3	Н	11	0.3	3.2	3.32	0.12
12	1HG2	3	1HE	10	0.3	3.2	3.10	0.10
13	Н	8	1HD	7	0.4	3.1	3.22	0.12
14	1HB	1	1HD1	12	0.45	3	2.90	0.10
15	1HB	1	HG	12	0.4	3.1	3.21	0.11
16	Н	8	HA	7	0.6	2.9	3.03	0.13

## HPT-Val<sup>S</sup><sub>3</sub> Folded Control

Constraint number	First Res Atom Name	First Res number	Second Res Atom Name	Second Res number	Intensity	Experimental Distance	Computational Distance	Deviation
0	Н	9	HA	7	0.6	3.8	3.61	0.19
1	Н	11	HA	5	0.4	4	4.1	0.10
2	Н	2	1HD1	12	0.4	4	4.1	0.10
3	Н	8	1HG	7	1	3.5	3.66	0.16
4	Н	8	2HB	7	1.3	3.3	3.16	0.14
5	Н	9	2HG	7	0.6	3.8	4.04	0.24
6	Н	6	Н	9	0.6	3.8	3.71	0.09
7	HA	10	HA	5	1.2	3.3	3.4	0.10
8	HB	3	2HB	10	0.4	4	3.9	0.10
9	1HD	7	HA	6	19.7	2.1	2.04	0.06
10	1HD	7	1HG1	6	2.4	3	3.17	0.17
11	2HB	1	1HD1	12	0.7	3.7	3.7	0.00
12	1HE	10	1HG2	3	0.5	3.9	4	0.10
13	2HB	1	HG	12	0.4	4	4.11	0.11
14	2HB	1	1HD1	12	0.6	3.8	3.7	0.10
15	1HG	13	1HG2	11	0.8	3.6	3.7	0.10
16	1HG2	3	HA	12	0.8	3.6	3.72	0.12
17	1HG2	3	HA	10	0.9	3.5	3.61	0.11
18	2HB	10	1HG2	3	1.3	3.3	3.19	0.11
19	Н	9	2HA	8	1.2	3.3	3.44	0.14
20	Н	9	1HA	8	1.3	3.3	3.4	0.10
21	Н	8	Н	9	0.9	3.5	3.33	0.17
22	Н	8	HA	7	2.3	3	3.15	0.15

## HPT-Ile<sup>S</sup>10 Folded Control

Constraint number	First Res Atom Name	First Res number	Second Res Atom Name	Second Res number	Intensity	Experimental Distance	Computational Distance	Deviation
0	Н	6	Н	9	506.4	4.2	4.07	0.13
1	Н	6	2HG	10	126.3	5.3	5.42	0.12
2	Н	2	1HD1	12	138	5.2	5.31	0.11
3	Н	8	1HG	7	975.2	3.8	3.92	0.12
4	Н	8	Н	9	1451.6	3.5	3.37	0.13
5	Н	8	HA	7	3459.4	3.1	3.24	0.14
6	Н	8	1HD	7	1180.6	3.7	3.56	0.14
7	1HE2	13	1HG2	11	597.2	4.1	4.2	0.10
8	HA	7	Н	9	565.6	4.1	4.21	0.11
9	HA	7	1HG1	6	1114.7	3.7	3.83	0.13
10	HB	3	2HB	10	772.7	3.9	4	0.10
11	2HA	8	Н	9	669	4	3.84	0.16
12	1HD	2	1HG1	4	478.5	4.3	4.32	0.02
13	1HB	1	1HD1	12	517.9	4.2	4.1	0.10
14	1HD	9	1HG2	11	502.3	4.2	4.32	0.12
15	1HE	10	1HD1	12	400.3	4.4	4.51	0.11
16	1HE	10	1HG2	3	702.5	4	3.89	0.11
17	1HG	5	HA	9	469.8	4.3	4.43	0.13
18	2HG	5	HA	9	414.1	4.4	4.51	0.11
19	1HG2	3	2HB	10	1443.7	3.5	3.6	0.10
20	1HG2	3	HA	12	518.8	4.2	4.22	0.02
21	1HD1	12	2HB	1	923.6	3.8	3.7	0.10
22	1HD	7	HA	6	33171.2	2.1	2.2	0.10

Constraint number	First Res Atom Name	First Res number	Second Res Atom Name	Second Res number	Intensity	Experimental Distance	Computational Distance	Deviation
0	Н	6	Н	9	649	3.8	3.69	0.11
1	Н	2	1HD1	12	428	4	4.13	0.13
2	Н	8	Н	9	930.4	3.5	3.61	0.11
3	Н	8	1HG	7	549.5	3.9	4.02	0.12
4	Н	8	2HB	7	1198.5	3.4	3.52	0.12
5	Н	8	HA	7	1533.3	3.3	3.28	0.02
6	Н	8	1HD	7	794.5	3.6	3.71	0.11
7	2HE2	13	1HG2	11	306.9	4.3	4.40	0.10
8	1HD	7	HA	6	21267.6	2.1	2.00	0.10
9	1HA	8	Н	9	297	4.3	4.08	0.22
10	1HD	7	1HG1	6	2402.9	3	3.17	0.17
11	2HA	8	Н	9	282	4.3	4.14	0.16
12	1HB	1	1HE	10	2297.6	3	3.12	0.12
13	1HB	1	1HD1	12	830.3	3.6	3.49	0.11
14	HG	12	1HG	10	158.1	4.8	4.69	0.11
15	1HG2	3	2HB	10	809.6	3.6	3.74	0.14
16	2HB	1	HG	12	850	3.6	3.48	0.12
17	2HB	1	1HB	12	509	3.9	4.02	0.12

HPT-Leu<sup>S</sup>11 Folded Control

Table S10. Backbone RMSD of the thioamide-containing Folded control structures in comparison to the HPT Folded control

	Backbone RMSD (Å)
HPT-Thr <sup>S</sup> <sub>2</sub>	1.14
HPT-Val <sup>S</sup> <sub>3</sub>	2.15
HPT-Ile <sup>S</sup> <sub>10</sub>	0.63
HPT-Leu <sup>S</sup> 11	1.23



Figure S18. Modelled structure of HPT Folded control peptide based on NOE derived distance constraints



**Figure S19.** Modelled structure of HPT-Thr<sup>S</sup><sub>2</sub> Folded control peptide based on NOE derived distance constraints. In the box, the HPT-Thr<sup>S</sup><sub>2</sub> Folded control is overlaid with the HPT Folded control. An atomic detail that is different with thioamide incorporation includes flipping of the Thr side-chain. In HPT, the Thr carbonyl is within hydrogen bonding distance of the side-chain hydroxyl group. But in HPT-Thr<sup>S</sup><sub>2</sub>, the side-chain is flipped away either because of the thioamide sterics or because it is a weaker hydrogen bond acceptor. The thioamide of HPT-Thr<sup>S</sup><sub>2</sub> engages as a hydrogen bond donor with the same strength as the oxoamide in HPT (distance between the Thr<sub>2</sub> NH and the Ile<sub>10</sub> carbonyl: 2.2 Å for the thioamide and 2.3 Å for the oxoamide).



**Figure S20.** Modelled structure of HPT-Val<sup>S</sup><sub>3</sub> Folded control peptide based on NOE derived distance constraints. In the box, the HPT-Val<sup>S</sup><sub>3</sub> Folded control is aligned with the HPT Folded control around the thioamide. Despite the dramatic twist of this  $\beta$ -hairpin that presumably allows for accommodation of the internal thioamide, the thioamide has similar hydrogen bonding distances to HPT. This is unexpected but might result because Val<sub>3</sub> is conformationally restricted being closer to pro<sub>6</sub>. This agrees with theoretical studies that suggest reduced conformational flexibility of the residue proceeding the thioamide.<sup>15, 16</sup>



**Figure S21.** Modelled structure of HPT-Ile<sup>S</sup><sub>10</sub> Folded control peptide based on NOE derived distance constraints. In the box, the HPT-Ile<sup>S</sup><sub>10</sub> Folded control is overlaid with the HPT Folded control. Although the structures align well (backbone RMSD of 0.63 Å), there is an increase in the distance between the thiocarbonyl of Ile<sup>S</sup><sub>10</sub> and the NH of Val<sub>3</sub>. This corresponds with the thioamide being a weaker hydrogen bond acceptor.



**Figure S22.** Modelled structure of HPT-Leu<sup>S</sup><sub>11</sub> Folded control peptide based on NOE derived distance constraints. In the box, the HPT-Leu<sup>S</sup><sub>11</sub> is aligned with the Folded control around the thioamide. The thioamide engages as a hydrogen bond donor with apparently the same strength as the oxoamide in HPT.

#### Structural Ensembles of the Thioamide-Containing Folded Control β-Hairpins

*Ensemble Generation with Backrub Protocol.* Ensembles of the  $\beta$ -hairpin structures were simulated using a backrub protocol starting with the constrained relax structures. Backrub is an application in Rosetta which makes local rotations about an axis dictated by chosen backbone atoms.<sup>17</sup> Additionally, side-chain optimization is performed using the side chain mover according to the Dunbrack distributions. The entire  $\beta$ -hairpin structures were subjected to backrub 100 independent times using the beta\_nov16\_cart score function with constraints set to a weight of 1. A constraint weight of 1 was found to optimally model conformational flexibility without great deviation from the experimental data (**Table S11**). The ten lowest energy structures from backrub were retained and analyzed by full atom and Ca RMSD (**Table S11**). PyMOL session files are available on our GitHub at <a href="https://github.com/ejp-lab/EJPLab">https://github.com/ejp-lab/EJPLab</a> Computational Projects/tree/master/BetaHairpin/BackrubStructures.

	Average Deviation from NOE Derived Distances for All Structures	Average Score of All Structures (REU)	Average Cα RMSD for 10 Lowest Energy Structures (Å)	Average Full Atom RMSD for 10 Lowest Energy Structures (Å)
HPT-Thr <sup>S</sup> <sub>2</sub>	$0.17\pm0.13$	$498.6\pm96.9$	$0.61 \pm 0.12$	$1.77\pm0.14$
HPT-Val <sup>S</sup> <sub>3</sub>	$0.22\pm0.18$	$1241.3 \pm 106.5$	$0.54\pm0.16$	$1.77\pm0.32$
HPT-Ile <sup>S</sup> <sub>10</sub>	$0.28\pm0.21$	1191.0 ± 311.1	$1.33 \pm 0.47$	$2.37\pm0.54$
HPT-Leu <sup>S</sup> 11	$0.28\pm0.22$	917.6 ± 252.1	$0.84\pm0.13$	$1.91 \pm 0.22$

Table S11. Deviation, energy score and RMSD values for the backrub generated Folded control ensembles



**Figure S23.** Ensemble generation of the HPT-Thr<sup> $s_2$ </sup> Folded control peptide with the backrub protocol. Shown are the 10 lowest energy structures aligned. In the box, the initial orientation of the ensemble displayed, but with the side-chains shown.



**Figure S24.** Ensemble generation of the HPT-Val<sup>S</sup><sub>3</sub> Folded control peptide with the backrub protocol. Shown are the 10 lowest energy structures aligned. In the box, the initial orientation of the ensemble displayed, but with the side-chains shown.



**Figure S25.** Ensemble generation of the HPT-Ile<sup>S</sup><sub>10</sub> Folded control peptide with the backrub protocol. Shown are the 10 lowest energy structures aligned. In the box, the initial orientation of the ensemble displayed, but with the side-chains shown. The C $\alpha$  and full atom RMSD for the 10 lowest energy structures are considerably higher for this peptide. This flexibility is particularly prevalent in the  $\beta$ -turn and the  $\beta$ -strand opposite the thioamide.



**Figure S26.** Ensemble generation of the HPT-Leu<sup> $S_{11}$ </sup> Folded control peptide with the backrub protocol. Shown are the 10 lowest energy structures aligned. In the box, the initial orientation of the ensemble displayed, but with the side-chains shown.

#### Previous β-Hairpin Modification Studies

The following supplemental discussion provides a more detailed analysis of the effects of amino acid derivatives shown in Fig. 7. This discussion is generally limited to  $\beta$ -hairpins that are variations of YKL/HPT, in some cases replacing the proGly  $\beta$ -turn with AsnGly. Unless explicitly stated, all stability comparisons are made using  $\Delta\Delta G_{\text{Folding}}$  with the parent peptide as a reference.



Figure S27. Structure of amino acid derivatives.

**Steric Interactions.**  $\beta$ -branched amino acids promote  $\beta$ -sheet propensity; however, the hydrophobicity of the  $\gamma$ methyl groups of Val or Thr can cause solubility problems. Therefore, the ability of  $\beta$ -branched derivatives of charged amino acids to increase foldedness of a  $\beta$ -hairpin was explored by Gellman. TS<sup>-</sup><sub>4</sub> replacement of Glu<sub>4</sub> and TS<sup>+</sup><sub>9</sub> replacement of Lys<sub>9</sub> in the YKL scaffold stabilized the  $\beta$ -hairpin by -0.5 and -0.6 kcal/mol, respectively.<sup>18</sup> Notably, TO<sup>+</sup> was slightly destabilizing (+0.1 kcal/mol), showing that the subtle change of going from a thioether to an ether can have a significant effect on hairpin stability.

Gellman and Muir found that adding  $\beta$ -branched residues to the ends of the YKL scaffold also increased stability up to a length of seven amino acids in each strand.<sup>19</sup> The addition of two Thr to each terminus stabilized the peptide by -0.3 kcal/mol, but further residues (with the exception of an all-Thr extension) did not increase stability, suggestive of an intrinsic limit to the length of isolated  $\beta$ -sheets.

 $\pi$ -Interactions. Waters and co-workers have used the YKL system to study a variety of  $\pi$ -system interactions of aromatic amino acids as part of their studies on the structural impacts of post-translational modifications. In a 2003 study of a 14-residue derivative of the YKL scaffold, the impact of salt bridges versus aromatic/aromatic interactions on stability was analyzed.<sup>20</sup> Switching from a lateral cross-strand Phe-Phe interaction to a Glu-Lys salt bridge at various positions resulted in a loss of stability (~0.3 kcal/mol). Their subsequent studies investigated interactions with a single aromatic side-chain.

The cation- $\pi$  interaction was investigated with an AsnGly derivative of the YKL scaffold with Trp at position 2.<sup>21</sup> Methylation of Lys results in greater dispersion of the positive charge that should increase the strength of the cation- $\pi$  interaction. Each methylation of Lys<sub>9</sub> (Lys-Me, Lys-Me2, and Lys-Me3) increased the stability of the hairpin by -0.2 kcal/mol. Acetylation of Lys decreased the positive charge of the side-chain, but surprisingly did not result in a less-folded  $\beta$ -hairpin.<sup>22</sup> Acetylation changed the geometry of the interaction, where the amide NH is aimed at the face of the Trp indole ring.

Replacement of Lys<sub>9</sub> with Arg in a AsnGly derivative of the YKL scaffold with Trp<sub>2</sub> increased the stability by -0.3 kcal/mol.<sup>23</sup> This is presumably a result of the stronger interaction of the delocalized  $\pi$  system of the guanidinium

group, which can sample face-to-face and edge-to-face interactions.<sup>24</sup> Consistent with the importance of the face-to-face binding mode, methylation of Arg increased the stability by -0.6 kcal/mol.<sup>25</sup> The methylated Arg derivatives exhibit the same stability as incorporation of Lys-Me3.

Sulfur-arene interactions have also been shown to be significant stabilizing forces in biological systems.<sup>26</sup> In an AsnGly derivative of the YKL scaffold, a stabilizing diagonal interaction of -0.3 to -0.5 kcal/mol between Phe<sub>2</sub> or Trp<sub>2</sub> with Met<sub>9</sub> was observed (in comparison to Lys<sub>9</sub>).<sup>27</sup> Investigations using norleucine (Nle) at position 9 allowed Waters to identify a significant role for the methylene adjacent to the cationic Lys nitrogen and for the hydrophobic component of the Met- $\pi$  interaction.<sup>28</sup> Nle<sub>9</sub> interacted with Trp<sub>2</sub> or Phe<sub>2</sub> in a nonspecific manner, whereas the Lys<sub>9</sub> interaction is specific and tighter.

In contrast to the cation- $\pi$  and sulfur-arene interactions, Waters' investigation on the effect of phosphorylation cross-strand from an aromatic amino acid did not show evidence of a favourable anion- $\pi$  interaction.<sup>29, 30</sup> Phosphoserine, phosphothreonine and phosphotyrosine were incorporated opposite Trp<sub>2</sub> in a AsnGly derivative of the YKL scaffold. This was generally destabilizing by ~ 1 kcal/mol. C-terminal phosphoserine incorporation was less destabilizing (0.4-0.5 kcal/mol).

**Ion-pairing.** Cheng has performed a highly systematic analysis of ion-pairing interactions in  $\beta$ -hairpins using the HPT scaffold. They studied cross-strand lateral ion-pairing by changing the side-chain length and charged groups at positions 4 and 9. The greatest stability was achieved when the side-chain lengths of the negatively charged Glu/Asp derivative (position 4) and the positivity charged Lys derivative (position 9) were matched.<sup>1</sup> Variations in stability with side-chain length result from a complex interplay between alterations in charge density with distance from the backbone, increased entropic penalties for longer side-chains, and steric effects of side-chain methylene groups. Similar trends were observed with positively charged Arg derivatives at position 9, with a preference for longer side-chains due to the more conformationally restricted guanidinium group.<sup>31</sup> A follow-up study in which the positively and negatively charged amino acids were swapped revealed that the right-handed twist of the backbone played a role as well in favoring certain matched lengths.<sup>32</sup>

**Backbone**. Among backbone modifications, only aza-amino acids have been studied in the YKL/HPT scaffold, therefore we will include studies using other small hairpins for this section. Aza-amino acids were incorporated by Proulx into the YKL scaffold, aza-Val at Val<sub>3</sub> was highly disruptive to foldedness (1.26 kcal/mol) whereas aza-Gly was better accepted at this position (0.75 kcal/mol), but still less stable than YKL.<sup>33</sup> Aza-Val has a similar fraction folded to D-Val incorporation, therefore aza-amino acids were assumed to be disruptive to  $\beta$ -hairpin stability due to the adaptive chirality at N $\alpha$ .

Substitution of two adjacent  $\alpha$ -amino acids by a  $\beta$ -amino acid was performed by Horne in a  $\beta$ -hairpin sequence derived from a C-terminal segment of GB1. The peptides could fold into hairpin structures, but are destabilized by 0.5-0.6 kcal/mol per  $\alpha\alpha$ -substitution.<sup>34</sup> Incorporation of linear (E)-vinylogous  $\gamma^4$ -residues in the same scaffold were also moderately destabilizing (0.5 kcal/mol), whereas a cyclically constrained  $\gamma$ -residue was stabilizing (-0.3 to - 0.6 kcal/mol).<sup>35, 36</sup> Although N-methylated analogues were tested at non-hydrogen bonded positions, the correct isomers were still less stable than the other backbone modifications explored by Horne. This was especially prevalent for the positions near the turn where the N-methyl energetically restricts that backbone, disfavouring anti-parallel  $\beta$ -sheet structure.

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