Supporting Information

β-hairpin Stabilization through Interstrand Triazole Bridge

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Experimental Section

Peptide synthesis. Fmoc-protected amino acids and coupling reagents for peptide synthesis were purchased from Merck except for Fmoc-L-Dap(N3)-OH, Fmoc-L-Dab(N3)-OH, Fmoc-L-Orn(N3)-OH which were from Iris Biotech GmbH. N,N-diisopropylethylamine (DIPEA) was provided by Romil, while piperidine was purchased from Biosolve. Acetic anhydride and solvents for peptide synthesis and purification were from Sigma-Aldrich. Ascorbic acid and Copper(II) sulfate pentahydrate were purchased from Sigma-Aldrich. Analytical characterization of peptide was performed on an LC-MS system comprising an LCQ DECA XP ion trap mass spectrometer (ThermoElectron) equipped with an ESI source and a complete Surveyor HPLC system using a Jupiter C18 column (5 µm, 300 Å, 250 x 2.0 mm; Phenomenex) and HPLC Agilent 1200 equipped with a diode array using a Proteo C12 column (4 µm, 90 Å , 250 x 4.60 mm; Phenomenex).

All peptides were synthesized on solid phase by standard Fmoc chemistry using Rink amide MBHA resin (0.51 mmol/g). Resins were swollen in DMF for 30 min prior to synthesis. All the synthetic steps were performed under stirring at room temperature. For each coupling reactions (20 min, twice) were used 2.5 equivalents of the Fmoc-protected amino acid, 2.49 equivalents of Hydroxybenzotriazole (HOBt)/ O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and 5 equivalents of the base DIPEA. Fmoc deprotection was achieved with a solution of 30% v/v piperidine in DMF (5 min, twice). After each coupling, unreacted N-terminal amino groups were capped with a solution of 2 M acetic anhydride, 0.06 M HOBt, 0.55 M DIPEA in NMP (5 min). Between each reaction step, 5 wash of 1 min each in DMF were performed to remove exceeding reagents and reaction side-products. Peptides cleavage from the resin and amino acids side-chain deprotection were achieved by treatment with trifluoroacetic acid (TFA), triisopropyl silane (TIS) and water (95:2.5:2.5) at room temperature for 3 h. Cold diethyl ether was used for peptides precipitation. Crude products were collected by centrifugation, washed twice with cold diethyl ether, suspended in water/acetonitrile mixture and lyophilized. Linear peptides were purified, pooled and lyophilized.

The purified linear peptides were subjected to cyclization by CuAAC reaction. To a degassed solution of each pure linear peptide (0.5 mM) in H_2O/CH_3OH (2:1 v/v) were added Copper(II) sulfate (14 equiv.) and ascorbic acid (13 equiv.). Each reaction solution was stirred at room temperature for 60 min. All the cyclization reaction HPLC profiles showed a single main peak corresponding to the cyclic peptide. The estimated yields range from 60% to 90%, as calculated from the area integration of the HPLC traces of the crude clicked peptides. Crude peptides were directly purified by RP-HPLC and fractions were analyzed, pooled and lyophilized. All peptides showed a purity ≥ 95%, which was ascertained by analytical RP-HPLC using the following linear gradients of CH_3CN/TFA 0.1% (solvent A) in H_2O/TFA 0.1%(Solvent B) at a flow rate of 1 mL/min: Method 1: solvent A from 5% to 70% in 20 min. Method 2: solvent A from 5% to 50% in 40 min. Method 3: solvent A from 15% to 30% in 20 min. Peptide identity was verified by ESI-mass spectrometry.

For each cyclization reaction we observed the formation of a product with a significantly shorter retention time with respect to the corresponding linear peptides as expected for the intramolecular cyclization [R. Jagasia, J.M Holub, M. Bollinger, K. Kirshenbaum, M.G. Finn, *J. Org. Chem.* **2009**, *74*, 2964-2974]. To verify the presence of the covalent bridge, as representative example, cyclic NHB 2.1 peptide (100 μg in 150 μL of 50 mM TRIS HCl, 20 mM $CaCl_2$) was completely digested with trypsin (1:100 w/w enzyme:peptide; 37 °C, 24 h). The proteolysis reaction held to the formation of only one HPLC peak with a mass corresponding to the open hydrolyzed peptides (*m/z* $[M+2H]^{2+}$ calcd 693.7, found 694.7). On the contrary two peaks were observed when linear NHB 2.1 was treated with trypsin in the same conditions. Dimeric cycle formation was excluded based on the absence of the dimer-characteristic odd multicharged peaks in the ESI-MS spectra. Based on these experiments we conclude that, in our experimental condition, intramolecular cyclic peptide are formed.

Linear peptides.

NHB 1.1: ESI-MS (*m/z*) $[M+H]^+$ calcd 1352.5, found 1355.9; RP-HPLC (method 1) t_R=13.84 min.

NHB 1.2: ESI-MS (*m/z*) $[M+H]^+$ calcd 1366.4, found 1369.7; RP-HPLC (method 1) t_R=14.22 min.

NHB 1.3: ESI-MS (*m/z*) $[M+H]^+$ calcd 1380.5, found 1383.7; RP-HPLC (method 1) t_R=19.18 min.

NHB 2.1: ESI-MS (*m/z*) $[M+H]^+$ calcd 1366.6, found 1369.7; RP-HPLC (method 1) t_R=14.01 min.

NHB 2.2: ESI-MS (*m/z*) $[M+H]^+$ calcd 1380.5, found 1383.9; RP-HPLC (method 1) t_R=14.37 min.

NHB 2.3: ESI-MS (*m/z*) $[M+H]^+$ calcd 1394.6, found 1397.8; RP-HPLC (method 1) t_R=12.58 min.

NHB 3.1: ESI-MS (*m/z*) $[M+H]^+$ calcd 1380.6, found 1384.5; RP-HPLC (method 1) t_R=17.05 min.

NHB 3.2: ESI-MS (*m/z*) $[M+H]^+$ calcd 1394.5, found 1397.8; RP-HPLC (method 1) t_R=14.79 min.

NHB 3.3: ESI-MS (*m/z*) $[M+H]^+$ calcd 1408.6, found 1411.7; RP-HPLC (method 1) t_R=13.00 min.

NHB 1.2rev: ESI-MS (*m/z*) $[M+H]^+$ calcd 1366.4, found 1369.6; RP-HPLC (method 3) t_R=12.69 min.

NHB 2.1W3: ESI-MS (*m/z*) $[M+H]^+$ calcd 1366.4, found 1369.8; RP-HPLC (method 1) t_R=14.12 min.

Trpzip2: ESI-MS (*m/z*) $[M+H]^+$ calcd 1608.7, found 1607.5; RP-HPLC (method 2) t_R=29.44 min.

W2W4W9: ESI-MS (*m/z*) $[M+H]^+$ calcd 1521.6, found 1521.1; RP-HPLC (method 1) t_R=15.94 min.

Cyclic peptides.

NHB 1.1: ESI-MS (*m/z*) $[M+H]^+$ calcd 1352.5, found 1355.7; RP-HPLC (method 1) t_R=13.42 min.

NHB 1.2: ESI-MS (*m/z*) $[M+H]^+$ calcd 1366.4, found 1369.7; RP-HPLC (method 1) t_R=13.06 min.

NHB 1.3: ESI-MS (*m/z*) $[M+H]^+$ calcd 1380.6, found 1382.8; RP-HPLC (method 1) t_R=15.93 min.

NHB 2.1: ESI-MS (*m/z*) $[M+H]^+$ calcd 1366.4, found 1369.8; RP-HPLC (method 1) t_R=13.26 min.

NHB 2.2: ESI-MS (*m/z*) $[M+H]^+$ calcd 1380.5, found 1383.8; RP-HPLC (method 1) t_R=11.11 min.

NHB 2.3: ESI-MS (*m/z*) $[M+H]^+$ calcd 1394.5, found 1397.8; RP-HPLC (method 1) t_R=12.02 min.

NHB 3.1: ESI-MS (*m/z*) $[M+H]^+$ calcd 1380.5, found 1383.9; RP-HPLC (method 1) t_R=13.39 min.

NHB 3.2: ESI-MS (*m/z*) $[M+H]^+$ calcd 1394.6, found 1397.8; RP-HPLC (method 1) t_R=11.07 min.

NHB 3.3: ESI-MS (*m/z*) $[M+H]^+$ calcd 1408.6, found 1411.7; RP-HPLC (method 1) t_R=12.50 min.

NHB 1.2rev: ESI-MS (*m/z*) $[M+H]^+$ calcd 1366.4, found 1369.7; RP-HPLC (method 3) t_R=5.50 min.

NHB 2.1W3: ESI-MS (*m/z*) $[M+H]^+$ calcd 1366.4, found 1369.7; RP-HPLC (method 1) t_R=12.04 min.

NMR spectroscopy. NMR samples were prepared dissolving the lyophilized peptides in H_2O/D_2O (9:1, v/v) at a final concentrations ranging from 0.5 to 1.0 mM. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal reference. The ^1H NMR spectra were acquired on a 400 MHz

Varian INOVA spectrometer, with the exception of the DOSY spectrum acquired on a 500 MHz Varian INOVA spectrometer. Water suppression was achieved through Double Pulsed Field Gradient Spin Echo sequences. DQF-COSY spectra was acquired with 4096 data points in the direct dimension and 500 increments with 64 scans to obtain enough resolution to measure the $^{3}J_{HNH}$ coupling constants. Spectral processing was carried out using the software Sparky and the spectra were analysed with Neasy, a tool of computer aided resonance assignment (CARA) software. (J.E. Masse, R. Keller *J Magn Reson* **2005**, *174*, 133-151).

The diffusion-ordered NMR spectroscopy (DOSY) (K.F. Morris, G.S. Johnson J Am Chem Soc 1992, 114, 3139-314) was performed using the Pulsed Gradient Spin-Echo (PSGE) NMR method (Price W.S. (1997) Concepts Magn Reson (1997), 9:299-366; Price W.S. (1998) Concepts Magn Reson 10:197-237). The following relationship exists between the translational self-diffusion parameter, D, and the NMR parameters: $I/I_0 = - \exp[D\gamma^2\delta^2G^2(\Delta-\delta/3)]$ where I is the measured peaks intensity of a particular group of resonances; I_0 is the maximum peak intensity of the same group of resonances at the smaller gradient strength; D is the translational self-diffusion constant (in m2s-1); γ is the gyromagnetic ratio of a proton (2.675×10^4 rad $G^{-1}s^{-1}$); δ is the duration (in seconds) of the gradient; G is the strength of the gradient (in G cm^{-1}); and Δ is the time (seconds) between the two gradient. Experiments were acquired by using the longitudinal eddy-current delay (PFG-LED) pulse sequence, with a post gradient eddy-current relaxation of 5 ms. Each experiments was averaged over 128 scans and the number of points was 16K. The strength of the gradient pulses was varied from 2% of the total power of the gradient coil to 95% and their shape was a sine function. The duration of the gradient was varied between 3.0 ms and 2.0 ms and the time between both gradients was changed between 100 and 150 ms.

The overall fraction fold was determined using the extent of H_α chemical shift glycine splitting (calculated from the 2D TOCSY spectrum considering the frequency difference between the centres of the two cross peaks) observed in the turn residue Gly^7 using the equation: fraction folded = $(\Delta\delta Gly_{obs}) / (\Delta\delta Gly_{100})$ where is $\Delta\delta Gly_{obs}$ is the difference in the Gly Hα chemical shifts of the clicked

peptides while $\Delta\delta Gly_{100}$ is the difference in the Gly H_α chemical shifts of the trpzip2 ($\Delta\delta Gly$ = 0.49 ppm in our experimental conditions).

The chemical shift of Hζ proton of free 1,2,3 triazolyl bridge was measured in DMSO using the reference compound 2-acetamido-5-(4-(2-acetamido-2-carbamoylethyl)-1H-[1,2,3]-triazol-1-yl) pentanamide synthesized starting from Ac-Orn(N3)-NH2 and Ac-Pra-NH_2.

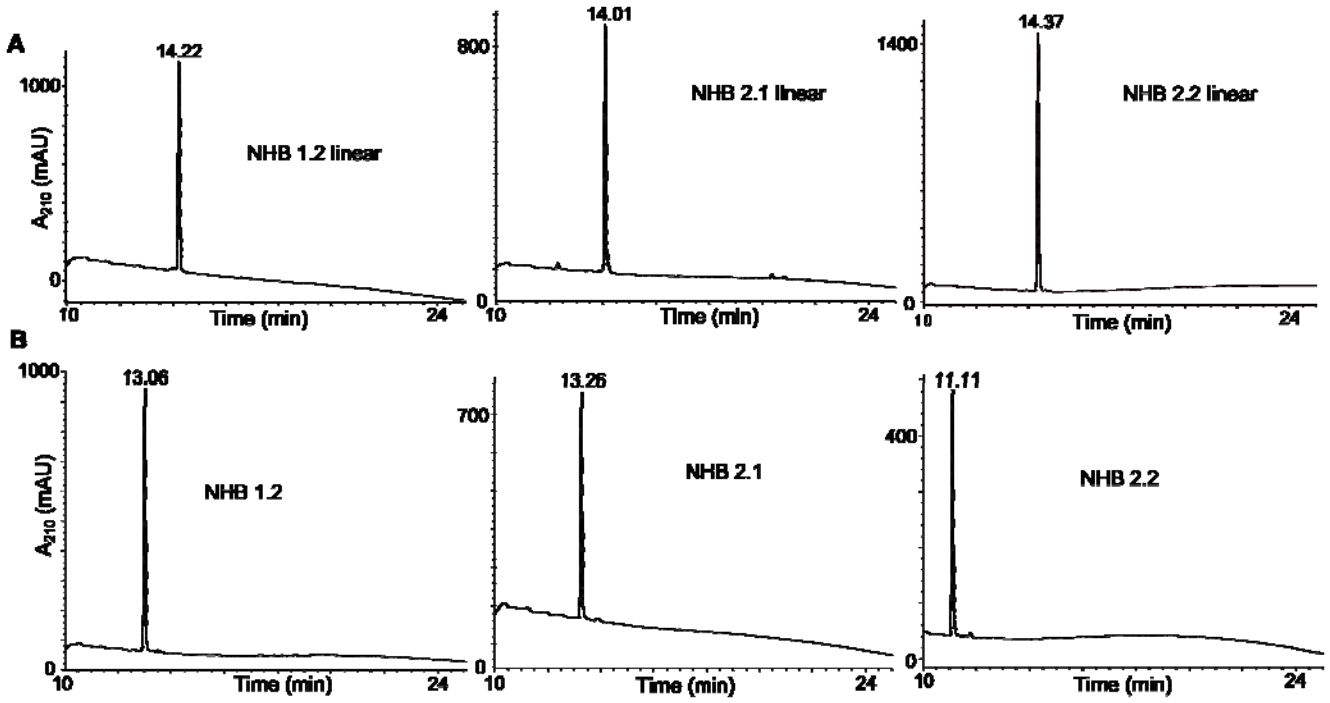

Figure S1: Peptides purity. RP-HPLC traces of selected linear (panel A) and clicked (panel B) peptides. Peptides were revealed at 210 nm (A_{210}) using methods reported in the experimental section.

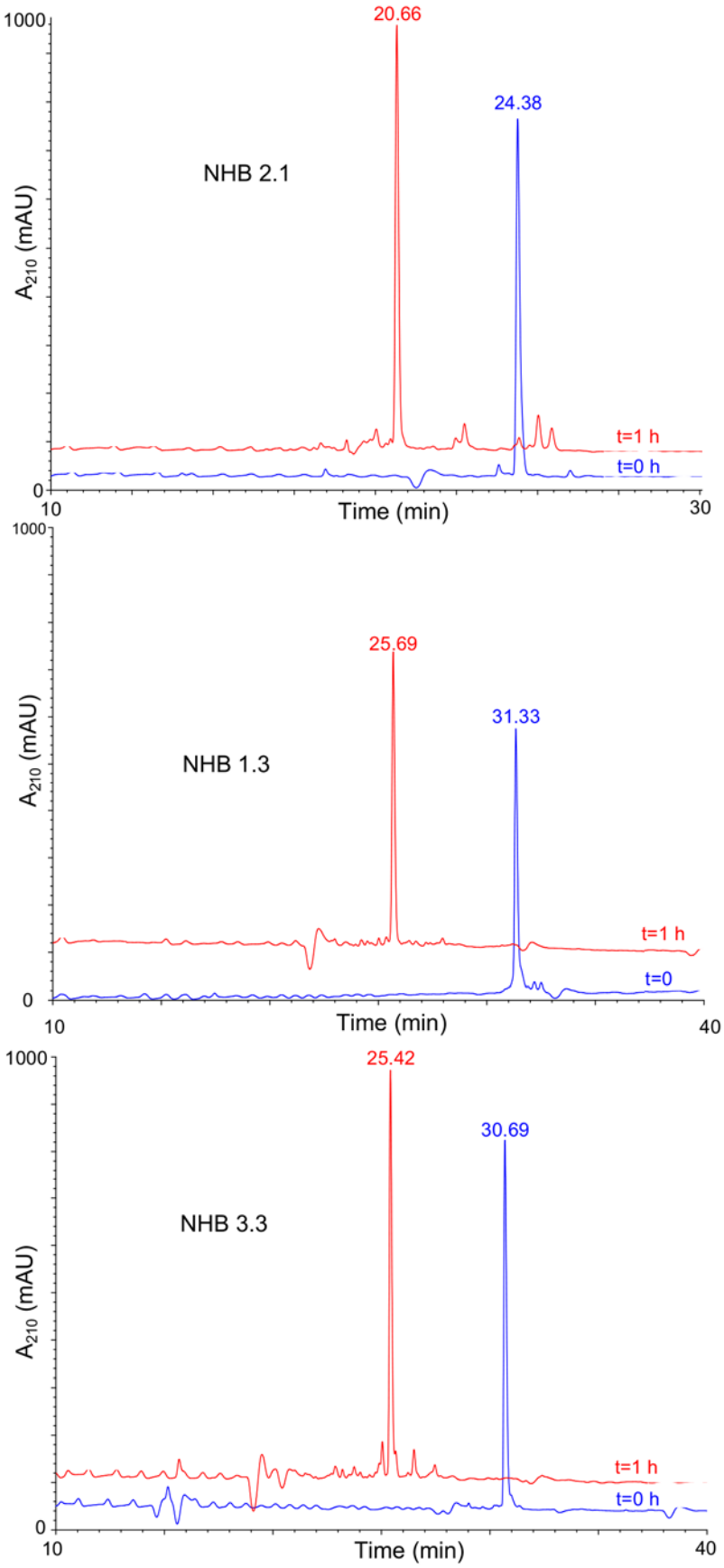

Figure S2: HPLC traces of Cu(I)-catalyzed azide alkyne reactions at two different reaction time.

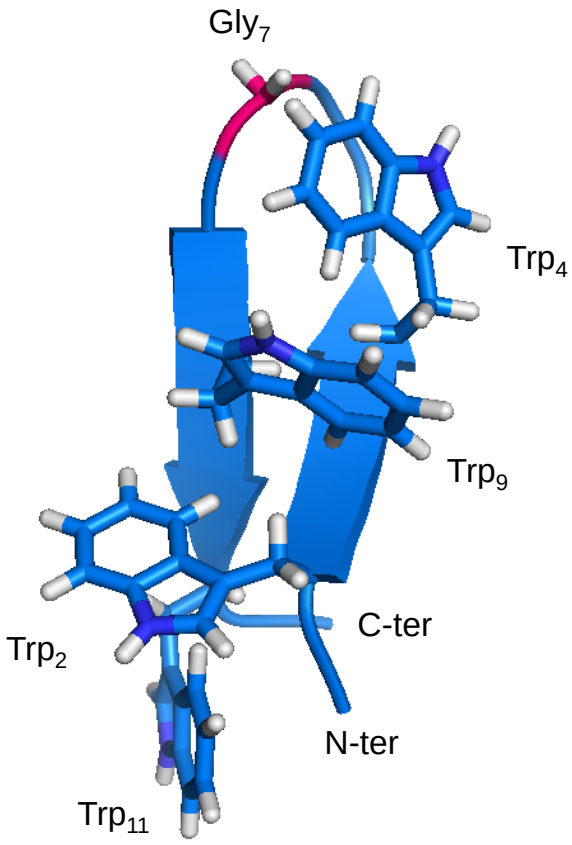

Figure S3: Trpzip2 secondary structure. Diasterotopic H_α protons of Gly 7 and tryptophans cluster are highlighted.

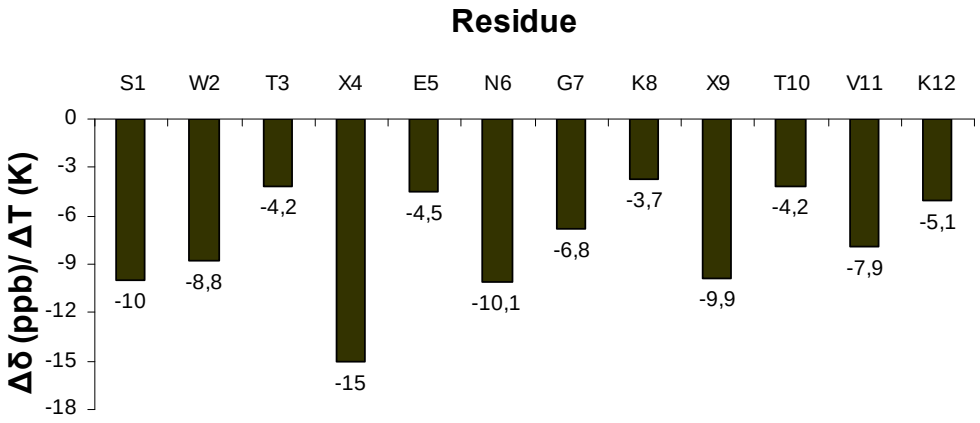

Figure S4: Plot of the temperature dependence of the amide protons for clicked NHB 2.1 peptide.

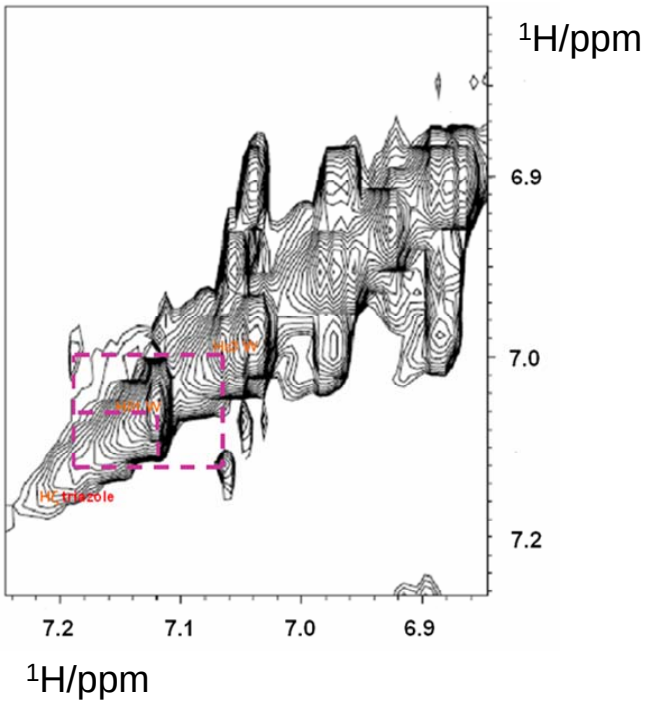

Figure S5: Aromatic region of the 2D NOESY spectrum of clicked NHB 2.1 peptide. Cross peaks between $H_{\delta 1}$ and $H_{\varepsilon 2}$ of Trp^2 side chain and Hζ of 1,2,3 triazolyl moiety are highlighted.

Table S1: $^{3}J_{HNH\alpha}$ coupling constant for trpzip2 and clicked NHB 2.1 peptides

Residue	clicked NHB 2.1	Trpzip2
1		
2	8.6	8.0
3	8.2	9.4
4	8.0	7.9
5	7.0	8.2
6	6.5	6.4
7	8.8; 5.0	9.3; 4.8
8	8.2	8.7
9		7.1
10	9.1	9.8
11	7.1	7.9
12	8.9	9.5

Table S2: nonsequential NOEs involving backbone protons of NHB 2.1 peptide.

Residue	**Proton**	**Proton**	**Residue**
Ser^{1}	H_α	H_α	Lys^{12}
Trp^{2}	H_α	H_N	Lys^{12}
Thr^{3}	H_N	H_α	Val^{11}
Thr^{3}	H_N	H_N	Thr^{10}
Hpg^{4}	H_α	H_N	Thr^{10}
Hpg^{4}	H_α	H_α	Dap^{9}
Glu^{5}	H_N	H_α	Dap9
Glu^{5}	H_N	H_N	Lys^{8}