Supporting Information

β -and γ-Amino Acids at α-Helical Interfaces: Toward the Formation of Highly Stable Foldameric Coiled Coils

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Materials:

Fmoc-protected building blocks of canonical α-amino acid (i.e.: Fmoc-Asp(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-L-Ala-OH·H2O, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Asn(Trt)-OH, Fmoc-L-Glu(OtBu)-OH, Fmoc-L-His(Trt)-OH, Fmoc-L-Leu-OH, Fmoc-L-Met-OH, Fmoc-L-Tyr(tBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH) were purchased from Orpegen Pharma (Heidelberg, Germany). Fmoc-protected (S)-β3-Homolysine, (S)-β3-Homoleucine, were purchased from Fluka and (R)-γ4-Homoaspartic acid and (S)-γ4-Homolysine from RareChemicals (Gettorf, Germany). Fmoc-protected (S)-β3-Homoalanine was purchased from Fluka. Fmoc-3-Nitrotyrosine was purchased from Bachem. Fmoc-Glu (OtBu)- and Fmoc-Lys (Boc)-NovaSyn®-TGA resins (0.16 mmol g-1and 0.21 mmol g-1, respectively) were purchased from Novabiochem. 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetra ethyluronium (TBTU), and 1- hydroxybenzotriazole (HOBt) were purchased from Fa. Gerhardt. The following chemicals were used as purchased: acetonitrile (HPLC gradient grade, Acros), dimethylformamide (DMF, p.a., Acros), dichloromethane (DCM, Fisher), 1- Hydroxy-7 azabenzotriazole (HOAt, Iris Biotech), N,N-diisopropylethylamine (DIEA, 98%, Acros), N,N diisoporopylcarbodiimide (DIC 99%, Acros), Triisopropylsilane (TIS 99%, Acros), piperidine (99% extra pure, Acros), 1,8 diazabicyclo[5.4.0]undec-7-ene (Merck), disodium hydrogenphosphate dihydrate (DBU, p.a., Merck), and sodium dihydrogenphosphate dihydrate (ultra >99%, Fluka), acetic acid (p.a. 100%, Roth), TFA (Uvasol, Merck), GndHCl (99.5%, Acros), glutathione and glutathione-oxidized (Aldrich), Tween-20 for molecular biology (Sigma Aldrich), D(+)-Biotin (Acros), and H_2O (MilliQ-AdvantageA10 Millipore). Acetic anhydride (99%, Acros) was distilled prior to use.

Peptide synthesis and characterization: Peptides were synthesized using standard automated Fmoc solid phase synthesis (0.05 mM scale) using a SyroXP-I peptide synthesizer (MultiSyn Tech GmbH) and HOBT/TBTU activation. Manual coupling of β- and γ-amino acids was carried out by HOAT/DIC activation. The molar excess of amino acid and coupling reagents was reduced for β- and γ-residues to two fold for the first and one fold for the second coupling. Completion of these couplings was indicated by a negative Kaiser test. Prior to each deprotection step, capping of the possibly non-acylated N-termini was carried out by treatment with 10% acetic anhydride and 10% DIEA in DMF (3×10 min). N-terminal coupling of biotin was performed as double coupling with HOBt/TBTU activation. N-terminal coupling of 3-Nitrotyrosine was performed as double coupling with HOAt/DIC activation.

Peptide cleavage from resin was performed using 95% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% water. Biotinylated peptides were cleaved with TFA-TIS-EDT-H_2O (94:2.5:2.5:1). Peptides were purified by HPLC on a C18 preparative column using gradients between 0.1% TFA in water and 0.1% TFA in acetonitrile. All peptides were

>95% pure by analytical HPLC on a C8 column (Phenomenex® Luna C8, 10 μM, 250 mm × 21.2 mm). Identities of peptides were confirmed on an Agilent 6210 ESI-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA.)

***Table S1**. Identification of peptides and chimera by ESI-TOF mass spectrometry**

Peptide/ Chimera	Charge	Molecular Weight Calculated [g/mol]	Molecular Weight Measured [g/mol]
Acid-pp	$[M+4H]^{4+}$	998.29	998.29
Acid-LLLL	$[M+4H]^{4+}$	990.30	990.71
Acid-LLLL-*C*Abz	$[M+4H]^{4+}$	990.30	990.41
Acid-LFYL	$[M+4H]^{4+}$	1011.29	1011.80
Acid-LFYL-*C*Abz	$[M+4H]^{4+}$	1011.29	1011.50
Acid-pp-MTER	$[M+4H]^{4+}$	1006.53	1006.94
Acid-pp-HCAN	$[M+4H]^{4+}$	983.56	983.91
B3β2γ	$[M+4H]^{4+}$	978.59	979.11
B3β2γ-GSG-*N*Bio	$[M+4H]^{4+}$	1055.67	1055.64
B3β2γ Variant1-GSG-*N*Bio	$[M+4H]^{4+}$	1027.64	1027.62
NH_2-Y(NO_2)-G-OH	$[M-1H]^{1-}$	282.04	282.07
B3β2γ-*N*Y(NO_2)	$[M+4H]^{4+}$	1000.83	1000.87

*If not stated otherwise all peptides bear an N terminal Abz label. For Acid-LLLL-*C*Abz and Acid-LFYL-*C*Abz the Abz label is attached to the primary amin of lysine 34. B3β2γ-GSG-*N*Bio and B3β2γ-variant1-GSG-*N*Bio bear a N terminal biotin and B3β2γ-*N*Y(NO_2) a N terminal 3-Nitrotyrosine, respectively.

Determination of Peptide Concentration: Concentrations were estimated by UV spectroscopy on a Cary 50 UV/Vis spectrometer (Varian) using the absorption of *o*-aminobenzoic acid (λ_{max}=320 nm at pH 7.4) acid or 3-nitrotyrosine (λ_{max}=420 nm at pH 7.4). Therefore, calibration curves were recorded using different concentrations of H_2N-Abz-Gly-COOH × HCl (Bachem) and H_2N-Tyr(NO_2)-Gly-COOH respectively in the buffer used for CD spectroscopy containing 6M guanidinium hydrochloride (Fluka). Disposable Plastibrand® PMMA cuvettes (Brand GmbH) with 1 cm path lengths were used.

Library Construction and Phage display: DNA-library coding for Acid-pp in which the library codons where randomized through the NNK-strategy[1, 2] was purchased from *biomers.net* GmbH (D-89077 Ulm), cloned into the pComb3H[2] phagemid vector (GenBank database accession number: AF268280, Barbas laboratory, TSRI) via SfiI sites, and transformed into *E.coli* K12 ER2738 (New England Biolabs E4104S) as described previously.[3] Below are sequences of purchased oligonucleotides. Randomised positions are bold and sticky ends for successful cloning into pComb3HSS plasmid underlined (N = A,C,G, or T; M = A or C; K = G or T):

Library Acid-pp sense:

5' – CGGCC CTG AGC GCG CTG GAG AAG GAG CTG GCG AGC CTG GAG AAG GAG NNK AGC GCG **NNK NNK** AAG **NNK** CTG GCG AGC CTG GAG AAG GAG CTG AGC GCG CTG GAG AAG GAG GGCCAGGC – 3'

Library Acid-pp antisense:

5' – TGGCC CTC CTT CTC CAG CGC GCT CAG CTC CTT CTC CAG GCT CGC CAG MNN CTT **MNN MNN** CGC GCT **MNN** CTC CTT CTC CAG GCT CGC CAG CTC CTT CTC CAG CGC GCT CAG GGCCGCCT – 3'

The library sequences were expressed as pIII protein fusions on M13 bacterophage surface via standard techniques. Isolation and sequencing of 20 random clones from a test infection of *E. coli* K12 ER2738 confirmed that the Acid-PP derived sequences had been inserted into the phage vector as desired, with variation at the appropriate positions. The library size was calculated to be 2.22×10^8 sequences, and thus sufficient to include every possible combination of amino acids at the four randomized positions (minimum size: $204 = 1.6 \times 10^5$). Production of the library phage was carried out as described previously.[4]

For the evaluation of the selection outcome of interaction partners, a second foldameric sequence, B3β2γ-variant1, was examined. In this control chimera the two β^3-homoleucine residues of B3β2γ were substituted with β-alanine, thereby removing two key side chains for coiled-coil interactions.[5, 6]

The selection of binding partners was carried out according to previously established procedures,[4, 7, 8] of the synthesized biotinylated Base-pp peptide analogues, 5.5 µl peptide solution (5 µg μl^{-1}) was added to 30 µl *Dynabeads M-280 Streptavidin* magnetic particles (Invitrogen Dynal A.S.) in 500 µl PBS (negative control without peptide) and rotated on a *Stuart Rotator SB2* (Bibby Scientific Ltd.) for 45 min at RT. Subsequently, the supernatant was removed, 5% (w/v) non-fat dried milk in PBS was added, and the sample again rotated at RT for 45 min. After removing the milk–PBS suspension, 500 uL phage solution were added and phage–target binding was accomplished by rotating for 1.5 h at RT. Afterwards, the particles were washed four times with Tween20 in PBS (PBS buffer contained 0.1% Tween 20 (v/v) in round 1; 0.5% Tween 20 (v/v) in rounds 2–3; 0.5% Tween 20 (v/v) in rounds 4–5; in round 6 three washing steps with 1 M GndHCl (w/v) in PBS were added) and once with TBS. Bound phages were eluted from magnetic particles by adding 25 mL freshly prepared trypsin solution (10 mg mL^{-1} in TBS) and incubated for 30 min at RT. Subsequently, the reaction was quenched with 75 mL SB media. Reinfection, phage amplification and harvesting was performed as described previously.[4]

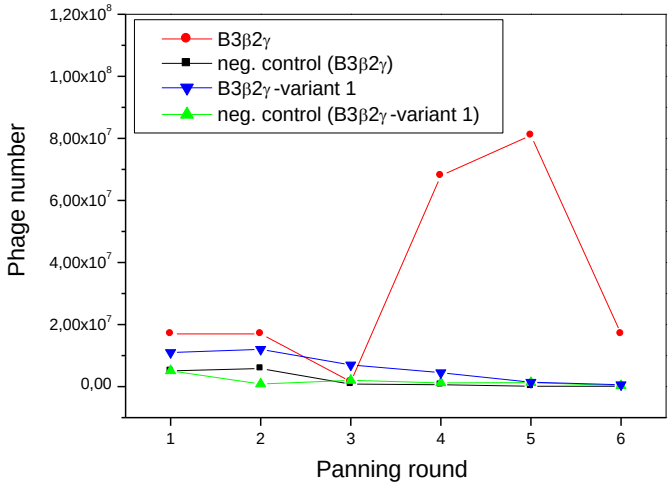

Figure S1: *Phage enrichment depicted as the phage number throughout each panning round with B3β2γ, B3β2γ-variant1, and negative controls of the respective panning round.*

The screening against the B3β2γ-variant1 control resulted in a low colony number comparable to that of unspecifically bound phages in the negative control (Figure S1). Moreover, no consensus sequence but rather a completely random distribution of amino acids occurred in all randomized positions (Table S1).

Table S1: *Sequencing results of mutation-free vectors found post panning with B3β2γ and B3β2γ-variant1 are given.*

Binding partner	**Amino acids found in randomized positions** (**Amino acid** / DNA codon)			
	Position **a'**$_{15}$	Position **d'**$_{18}$	Position **e'**$_{19}$	Position **g'**$_{21}$
B3β2γ	**Leu** / TTG	**Leu** / CTG	**Leu** / CTG	**Leu** / CTT
	Leu / TTG	**Leu** / CTG	**Leu** / CTG	**Leu** / CTT
	Leu / TTG	**Leu** / CTG	**Leu** / CTG	**Leu** / CTT
	His / CAT	**Cys /** TGT	**Ala /** GCG	**Asn /** AAT
	His / CAT	**Cys /** TGT	**Ala /** GCG	**Asn /** AAT
	Met / ATG	**Thr** / ACG	**Glu** / GAG	**Arg** / CGT
	Leu / CTG	**Phe** / TTT	**Tyr** / TAT	**Leu** / CTT
B3β2γ- variant1	**His** / CAT	**Pro** / CCT	**Leu** / CTG	**Pro** / CTT
	Ile / ATT	**Pro** / CCG	**Met** / ATG	**Trp** / TGG

SEC/SLS: Static light scattering data were collected on a Dawn Heleos 8 light scattering instrument (Wyatt Technology) coupled with an analytical gel filtration (workstation: La Chrom, VWR, Pump L-2130, UV Detector L-2400; column: WTC-015S5; 5µm, 150Å,7.8

x 300 mm, Wyatt Technology) at λ = 220 nm. All measurements were performed in PBS at room temperature with a flow rate of 0.3 ml min^{-1}. Data were analyzed using the ASTRA software version 5.3.4.20 (Wyatt Technology). Measurements were taken at pH 7.4 from a 1:1 mixture of B3β2γ and the respective Acid-pp analogue at a total peptide concentration of 60 µM and repeated two times to confirm reproducibility and give standard deviations.

Table S2: *SEC/SLS results of B3β2γ and Base-pp variants. Standard deviations determined from 3 independent measurements.*

	Theoretical Mass [Da]		**Determined Mass [Da]**
Acid-LFYL/ B3β2γ	15900	(tetramer)	16603 ± 790
Acid-LLLL/ B3β2γ	15732	(tetramer)	15790 ± 682
Acid-HCAN/ B3β2γ	7838	(dimer)	8222 ± 508
	3929	(monomer)	4108 ± 192
Acid-MTER/ B3β2γ	7931	(dimer)	7633 ± 326

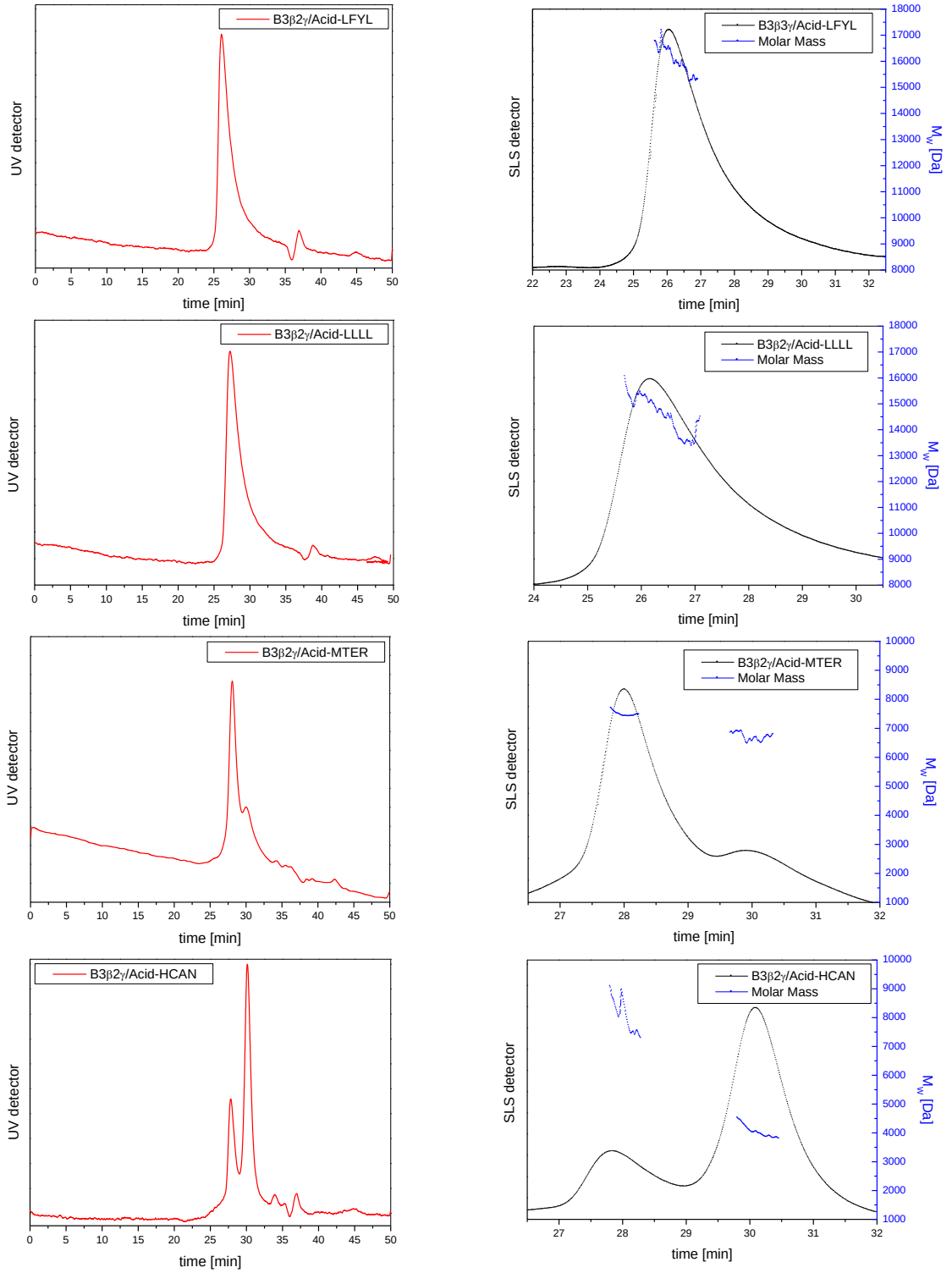

Figure S2: *SLS chromatograms depicted as the mean of 3 experiments. Respective SEC chromatograms are given to show species singularity. Peptide conc.:60 µM; eluent: 10 mM PBS; flow rate: 0.3 mL/min; pH 7.4. Measurements were taken from a 1:1 mixture of peptide analogues as indicated in the chromatograms.*

Circular Dichroism Spectroscopy: Peptide samples were analyzed in 10 mM phosphate buffer (pH 7.4). Far-ultraviolet (UV) circular dichroism (CD) spectra and thermal unfolding profiles were recorded on a J-810 spectropolarimeter (Jasco GmbH) equipped with a temperature controlled quartz cell of 0.1 cm path length. The recorded spectra were evaluated with the Jasco software package. The spectra were the averaged from three scans obtained by collecting data from 190 to 240 nm at 0.2 nm intervals, 2 nm bandwidth, and 1 sec response time. Ellipticity data in mdeg were converted to conformation parameters by the following equation: $[\theta] = [\theta]_b \times mrw / 10 \times l \times c$, where $[\theta]_b$ is the ellipticity measured in degrees, mrw is the mean residue molecular weight (molecular weight of the peptide divided by the number of amino acid residues), c is the peptide concentration in g ml^{-1}, and l is the optical path length of the cell in cm. Thermal denaturation was carried out in 0.5°C intervals with a heating rate of 3°C min^{-1}. The midpoints of the thermal melts, T_M's, were taken as the maximum of the derivative d(Fraction unfolded)/dT.

The CD spectra of the selected Acid-pp variants alone indicate α-helical folding behavior for these peptides, while isolated B3β2γ is unfolded in solution (Figure S3). When Acid-LLLL, Acid-LFYL, and Acid-MTER are individually mixed in a 1:1 molar ratio with their binding partner B3β2γ, the resulting CD spectra show considerably stronger α-helical folding behavior in comparison to the calculated average of the isolated solutions. This verifies that these Acid-pp analogs are able to undergo heteromeric α-helical coiled-coil formation with B3β2γ. However, the CD spectrum of the 1:1 solution of Acid-HCAN and B3β2γ is nearly superimposable with the calculated average of the respective isolated peptide, further verifying the primarily monomeric state of these peptides observed in SEC/SLS experiments (Figure S2). The melting points of the individual selected Acid-pp variants alone are similar to the melting points of their 1:1 mixtures with B3β2γ (Figure S4). This indicates that the Acid-pp variants have a high helix propensity. In the contrary, B3β2γ is unstructured when alone in solution. It forms α-helical conformations with the Acid-pp variants as helical templates by the formation of heteromeric coiled-coils. It is, thus, conceivable that the latter contributes to their selection during biopanning.

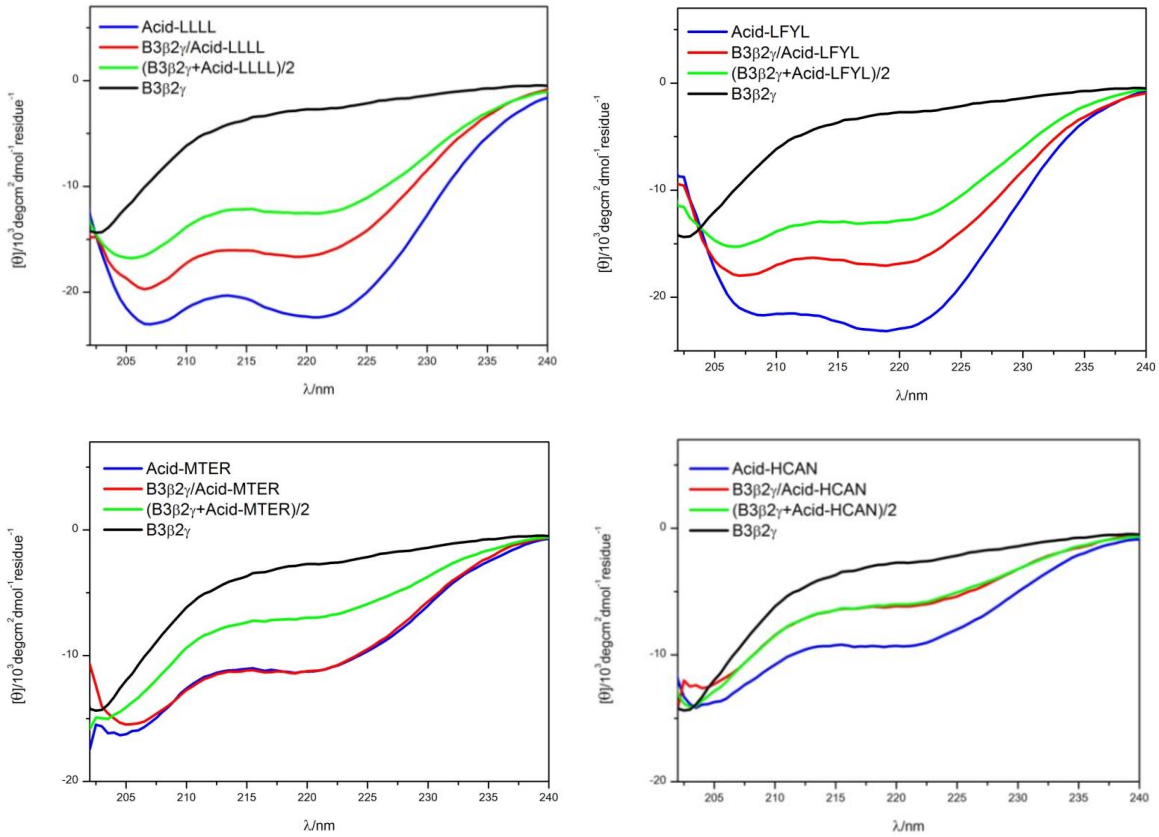

Figure S3: *CD spectra of isolated Acid-pp analogues (curves in blue) and B3β2γ (curves in black). The CD spectra of equimolar mixtures of B3β2γ/Acid-pp and its variants (curves in red) as well as the calculated average of the isolated peptides (curves in green) are given for comparison. The total peptide concentration was 20 μM. The spectra were recorded in 50 mM phosphate buffer, containing 0.25 M GndHCl at pH 7.4.*

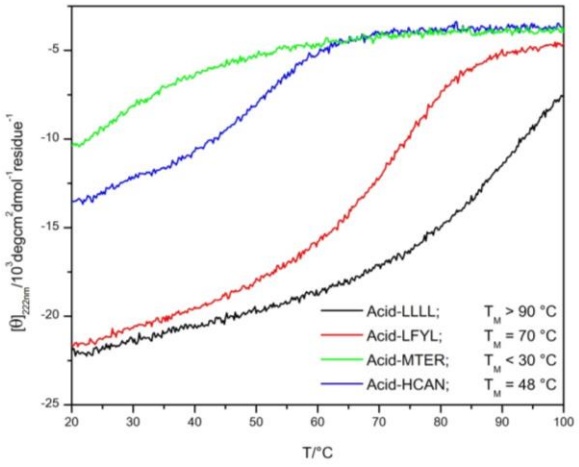

Figure S4: *Thermal denaturation spectra of selected Acid-pp variants. Melting points given in figure legend. The total peptide concentration was 20 μM. The spectra were recorded in 50 mM phosphate buffer, containing 0.25 M GndHCl at pH 7.4.*

FRET-Measurements: We carried out the FRET assay according to previously published procedures.[8, 9] Resonance energy transfer from Abz to Y(NO_2) only occurs when the donor and the acceptor are in close proximity (Förster radius = 29–31 Å).[10] In the chimeric coiled coil, this distance is about 30 Å (corresponding to its supercoil radius) which is in the range of the Förster radius, therefore, an intense quenching is expected upon hetero coiled-coil formation. We introduced the acceptor 3-nitrotyrosine (Y(NO_2); λ_{abs}=420nm) at the N-terminus of B3β2γ and the donor 4-aminobenzoic acid (Abz; λ_{ex}=320nm) was incorporated into Acid-LLLL and Acid-LFYL at either the primary amine of Lys34 or as an additional amino acid at the N-terminus. Fluorescence spectra were recorded on a luminescence spectrometer LS 50B (Perkin Elmer) using a 1 cm Quartz Suprasil cuvette (Hellma) at 20 °C. Three scans from 350 to 550 nm were performed, averaged, and the spectra were normalized to the respective maximum fluorescence.

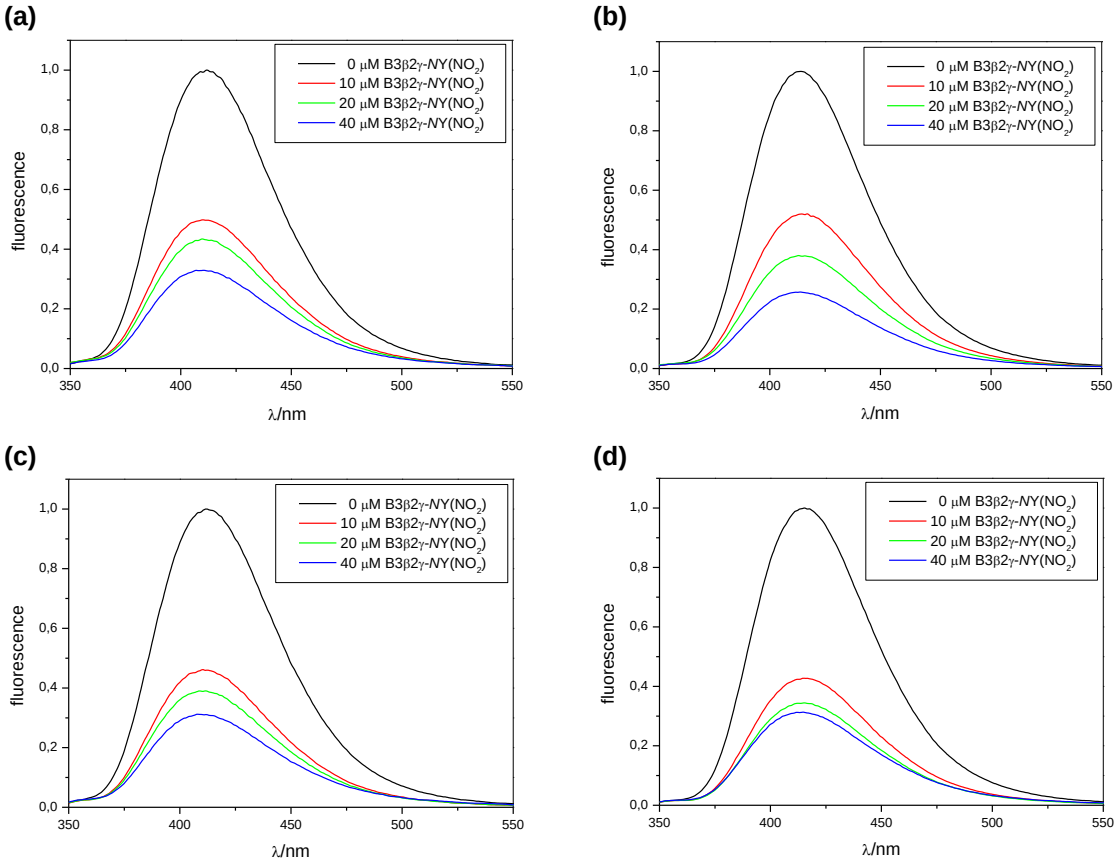

Figure S4: *Fluorescence spectra of Acid-pp variants in the presence of different concentrations of B3β2γ-NY(NO_2).* ***(a)*** *20 µM Acid-LLLL-CAbz;* ***(b)*** *20 µM Acid-LLLL-NAbz;* ***(c)*** *20 µM Acid-LFYL-CAbz;* ***(d)*** *20 µM Acid-LFYL-NAbz.*

Molecular dynamics simulations: MD-simulations were carried out with the Gromacs suite [11, 12] using the Gromos 53a6 forcefield (50 ns simulations at constant temperature and pressure, three repeats for each system).[13] A twin range cut-off for van der Waals (0.9/1.4 nm) and a smooth particle mesh Ewald algorithm for Coulomb interactions (switching distance of 0.9 nm, neighbor list update every 0.01 ps).[14] The Nosé-Hoover coupling method (T = 300 K)[15, 16] was used together with the Parrinello-Rahman coupling method (pressure of 1 bar)[17]. Bond constraints were applied with the LINCS algorithm.[18] The coiled-coil tetramers were solvated in dodecahedric boxes with approximately 13000 SPC water molecules and periodic boundary conditions were applied. The Gromos 53a6 topologies of β- and γ-amino acids are available from the authors upon request. The simulations were analyzed with VMD.

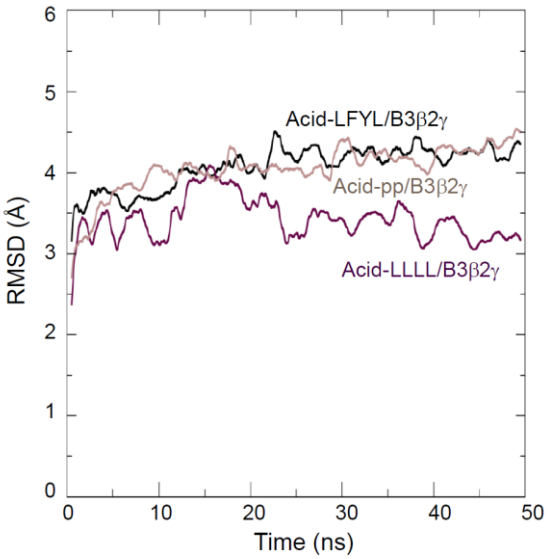

Figure S5: *RMSD to the starting conformation for the tetramers Acid-pp/B3β2γ, Acid-LFYL/B3β2γ and Acid-LLLL/B3β2γ.*

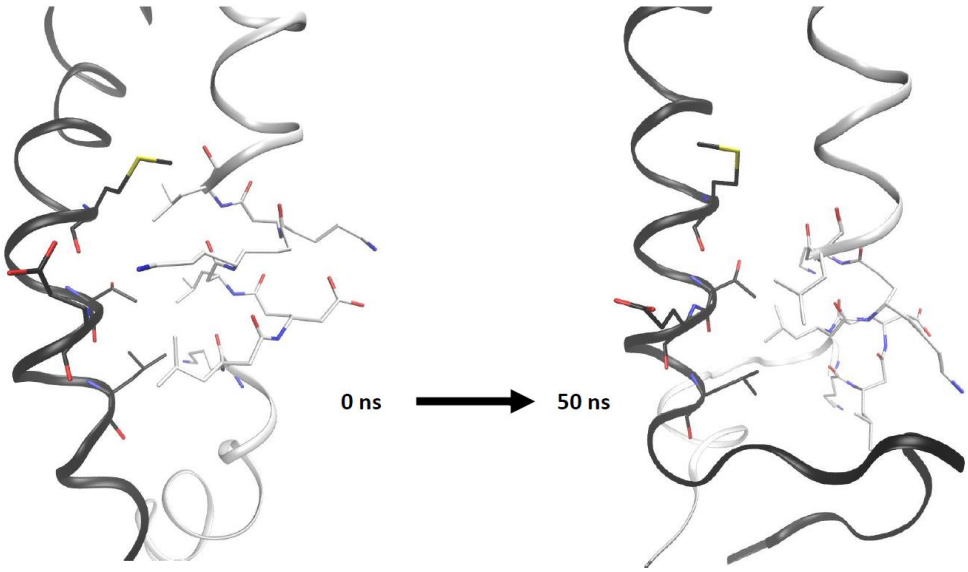

Figure S6: *Snapshots from the MD simulation of the dimer Acid-MTER/B3β2γ*

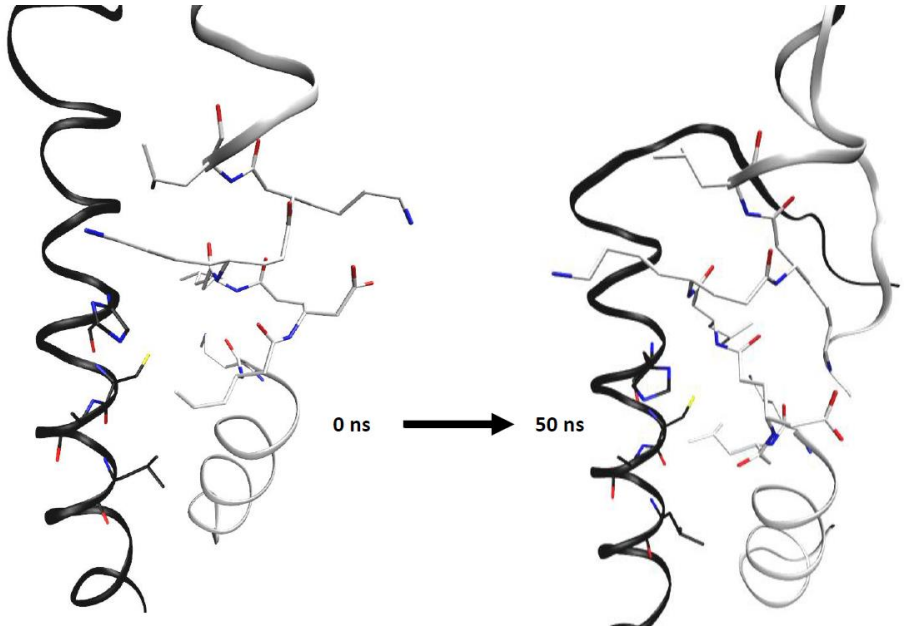

Figure S7: *Snapshots from the MD simulation of the dimer Acid-HCAN/B3β2γ*

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