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Supporting Information

Constraining an Irregular Peptide Secondary Structure through Ring-Closing Alkyne Metathesis

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1 Chemical Methods

Chemicals and instrumentation

Unless otherwise noted, chemicals were purchased from Sigma Aldrich, Merck, Okeanos, Roth or Alfa Aesar and were used without further purification. Protected Fmoc-amino acids and coupling reagents were purchased from Novabiochem and Iris Biotech GmbH. Building block **6** for hydrocarbon peptide stapling was purchased from Okeanos Tech. Co. LTD. All solvents were purchased from commercial suppliers and used without further purification. Analytical HPLC was performed using an Agilent 1100 Series with either a C18 HPLC column 3 µm (Macherey Nagel) or a C18 HPLC column 1.8 µm (Macherey Nagel). The system was run at a flow rate of 1.0 mL/min over 30 min using H_2O (0.1% TFA) and MeCN (0.1% TFA) as solvents. Linear gradients were run over varying periods of time. HPLC-MS analyses were performed with an Agilent 1100 Series connected to a Thermo LCQ Advantage mass spectrometer using a C18 HPLC column 3 µm (Macherey Nagel). The system was run at a flow rate of 1 mL/min over 15 min using H_2O (0.1% formic acid) and MeCN (0.1% formic acid) as eluents. Semi preparative HPLC was carried out on a Agilent 1100 Series using a SP125/10 Nuclear C18 Gravity 5 µm column (Macherey Nagel) at a flow rate of 6 mL/min. Linear gradients using H_2O (0.1% TFA) and MeCN (0.1% TFA) were run over varying periods of time. High resolution mass spectra were recorded on a QLT Orbitrap mass spectrometer coupled to an Acceka HPLC-System (HPLC column: Hypersyl GOLD, 50 mm x 1mm particle size 1.9 µm, ionization method: Electrospray Ionization). Automated peptide synthesis was performed using a CEM-Discover microwave and a CEM-Liberty peptide synthesizer. Fluorescence polarization was measured with a Tecan Safire[2]. Absorbance measurements were performed on a Tecan infinite M200 and Thermo scientific Nanodrop 2000c. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Mercury VX 500 or 400 spectrometer at room temperature. NMR spectra were calibrated to the solvent signals $CDCl_3$ (7.26 and 77.16) or DMSO (2.50 and 39.52). MicroScale Thermophoresis (MST) curves were measured on a NanoTemper Technologies Monolith NT.115.

Peptide synthesis

General

Peptides were synthesized on solid-phase using the Fmoc-strategy and Rink Amide (MBHA) resin, Rink Amide NovaSyn TGR resin or ChemMatrix Rink Amide resin as solid support. Solvents and soluble reagents were removed by suction. Washings between coupling and deprotection were carried out in DMF and DCM using 1 mL solvent per 100 mg resin. Coupling efficiency was monitored by ESI-MS and/or HPLC analyses.

Fmoc deprotection

The resin was swollen in DMF and treated with a solution of piperidine/DMF (20/80, v/v) for 2x 5 min. Afterwards the resin was washed with DMF (3x), DCM (3x) and DMF (3x).

Amino acid coupling

Fmoc-Xaa-OH (4 eq.) was dissolved in freshly prepared solution of HCTU (3.9 eq., 0.5 M) with DIEA (8 eq.). Subsequently, this mixture was added to the resin and shaken for 30 min at room temperature. For coupling of the alkyne building blocks **1** – **5**, the building blocks **12** – **14** and the subsequent amino acid: Fmoc-Xaa-OH (4 eq.) was dissolved in DMF in the presence of COMU (3.9 eq.), Oxyma (3.9 eq.) and DIEA (8 eq.), added to the resin and shaken for 1 h at room temperature. Except coupling of the alkyne building blocks **1** – **5** and the alkene building blocks **12** – **14**, all couplings were performed as double couplings. All equivalents are calculated based on theoretical loading of the resin as provided by the vendor.

N-terminal acetylation

For preparation of N-acetylated peptides and whenever a quantitative yield even after recoupling treatments was not achieved, the free N-terminal amino group was acetylated using a solution of Ac_2O/DIEA/DMF (1/1/8, v/v/v) for 2x 10 min at room temperature.

Fluorescence labelling with FITC

Prior to fluorescence labelling with FITC a PEG-linker (Fmoc-O2Oc-OH) was coupled to the free N-terminus. A mixture of Fmoc-O2Oc-OH (5 eq.), COMU (4.9 eq.), Oxyma (4.9 eq.) and DIEA (10 eq.) in DMF was transferred to the resin and shaken at room temperature for 2x 1 h. The resin was drained and washed with DMF (3x). The Fmoc group was removed as described above and the resin was treated with FITC (5 eq.) and DIEA (10 eq.) for 16 h at room temperature under exclusion of light. Afterwards, the resin was washed with DMF (3x), DCM (3x) and dried to constant weight in vacuo.

Ring-closing alkyne metathesis

The dried resin was transferred under argon into a baked out Schlenk tube and swollen and shrunken alternating in dry diethyl ether and dry toluene (3x each). Afterwards 0.5 mL of a solution of the alkyne metathesis complex **11** (2 mg·mL^{-1}) in dry toluene was added and the reaction mixture was stirred at 40°C for 1.5 h. During the reaction time argon was bubbled through the reaction mixture to evaporate the 2-butyne. After addition of 0.5 mL of fresh complex **11** solution the mixture was stirred at 40°C for 1.5 h. The resin was filtered off, washed with toluene (3x), DCM (3x) and dried to constant weight.

Ring-closing olefin metathesis

The dried resin was swollen in DCE for 15 min. A solution of Grubbs 1st generation catalyst (2 mg·mL^{-1}) in DCE was added to the resin and reacted for 2 h at room temperature. During the reaction time argon was bubbled through the reaction mixture to remove ethene. The procedure was repeated twice and the resin was washed with DCE (3x), DCM (3x), DMF (3x).

Cleavage from the resin

The dry resin was treated with a solution of TFA/EDT/TIS/H_2O (94/1/2.5/2.5, v/v/v/v) 100 µL for 10 mg resin for 2x 1 h and 1x 5 min. The solvents were evaporated and the crude peptide was precipitated by the addition of cold diethyl ether. After centrifugation (10 min, 16.100 x *g*, 4°C) the supernatant was removed. The crude product was dissolved in H_2O/MeCN (2/1, v/v) and lyophilized. The crude peptides were purified by semi-preparative HPLC.

Fmoc quantification

A defined amount of dry resin was transferred into an Eppendorf cap and treated with 0.5 mL deprotection solution for 15 min. The UV absorption of the supernatant was determined at 305 nm and the occupation density calculated using Beer-Lambert law (ε = 7800 cm^{-1}·M^{-1}).

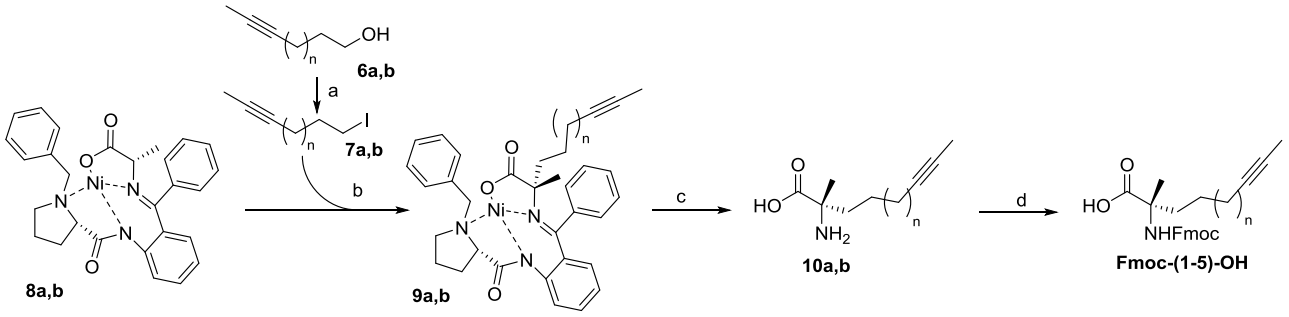
Peptide quantification

The concentration of fluorescein labelled peptides was determined by UV absorption in 20 mM phosphate buffer (pH 8.5) at 496 nm (ε = 77.000 cm^{-1}·M^{-1}). The concentration of acetylated peptides was determined gravimetrically or via UV absorption at 280 nm.

Building block synthesis

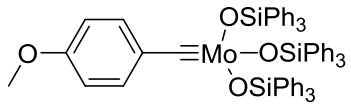
Synthetic methods

Synthesis of the alkyne building blocks **1** - **5** was performed according to adapted protocols from *Y. N. Belokon et al.*[1] and *G. H. Bird et al.*[2] Schematic representation of the synthesis is summarized below.



Synthesis of the alkyne building blocks. (a) PPh_3, I_2, Imidazol; THF, room temperature, 2 h; (b) KOH, 24 a-c; DMF, 0°C – room temperature, 2 h; (c) HCl, MeOH, reflux, 1 h; (d) Fmoc-OSu, Na_2CO_3, Dioxane/H_2O (1/1, v/v), room temperature, 7d. n = 2, 3

Synthesis of Mo-complex 11

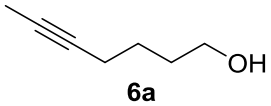


11

Chemical Formula: $C_{63}H_{52}MoO_4Si_3$
Exact Mass: 1054.22275
Molecular Weight: 1053.32000

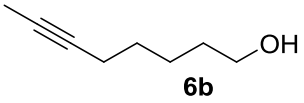
Mo-complex **11** for RCAM was prepared according to previously established procedures.[3]

Synthesis of alkyne alcohols (6a, b)



6a

Chemical Formula: $C_7H_{12}O$
Exact Mass: 112,08882
Molecular Weight: 112,17200

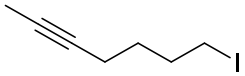


6b

Chemical Formula: $C_8H_{14}O$
Exact Mass: 126.0447
Molecular Weight: 126.9900

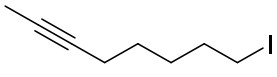
Synthesis of alkyne alcohols, hept-5-yn-1-ol (**6a**) and oct-6-yn-1-ol (**6b**), was carried out according to previously established protocols.[4]

Synthesis of iodo-alkynes (7a, b)



7a

Chemical Formula: $C_7H_{11}I$
Exact Mass: 221.99054
Molecular Weight: 222.06947



7b

Chemical Formula: $C_8H_{13}I$
Exact Mass: 236.00619
Molecular Weight: 236.09647

The alcohols **6a, b** was converted into the 7-iodohept-2-yne (**7a**) and 9-iodonon-2-yne (**7b**) following established protocols.[5]

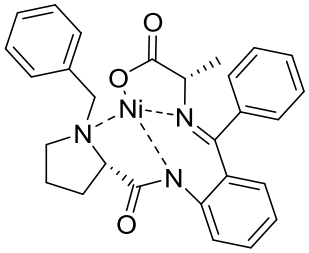
7a

^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.20 (t, J = 7.0 Hz, 2H), 2.19 – 2.13 (m, 2H), 1.98 – 1.88 (m, 2H), 1.77 (t, J = 2.6 Hz, 3H), 1.62 – 1.52 (m, 2H). **^{13}C NMR** (126 MHz, $CDCl_3$): δ = 78.5, 76.3, 32.7, 29.9, 17.9, 6.5, 3.6.

7b

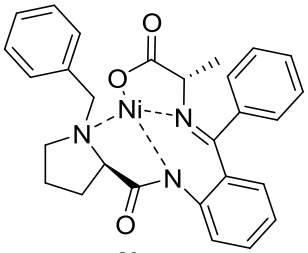
^{1}H NMR (600 MHz, $CDCl_3$): δ = 3.19 (t, J = 7.1 Hz, 2H), 2.16 – 2.11 (m, 2H), 1.87 – 1.81 (m, 2H), 1.78 (t, J = 2.6 Hz, 3H), 1.52 – 1.45 (m, 4H). **^{13}C NMR** (151 MHz, $CDCl_3$): δ = 78.94, 75.94, 33.27, 29.88, 28.08, 18.72, 6.88, 3.61.

Synthesis of (*S*, *R*)-Ala-Ni(II)-BPB (8a, b)



8a

Chemical Formula: $C_{28}H_{27}N_3NiO_3$
Exact Mass: 511.14059
Molecular Weight: 512.22568



8b

Chemical Formula: $C_{28}H_{27}N_3NiO_3$
Exact Mass: 511.14059
Molecular Weight: 512.23540

Synthesis of the Ni-complexes **8a** and **8b** was carried out according to previously established protocols starting either from *L*- or *D*-Proline.[1,2,6]

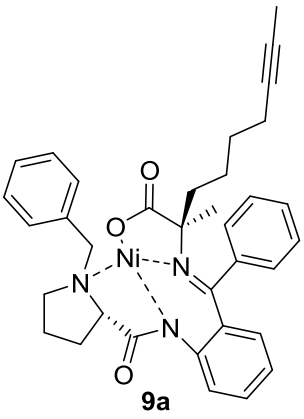
8a:

^{1}H NMR (400 MHz, DMSO) δ = 8.4 (d, J = 7.5 Hz, 2H), 8.0 (d, J = 8.7 Hz, 1H), 7.6 – 7.5 (m, 4H), 7.4 (t, J = 7.7 Hz, 2H), 7.2 – 7.1 (m, 2H), 7.1 – 7.0 (m, 1H), 6.7 – 6.6 (m, 1H), 6.5 (dd, J = 8.2, 1.5 Hz, 1H), 4.1 (d, J = 12.3 Hz, 1H), 3.7 – 3.4 (m, 4H), 3.4 – 3.3 (m, 1H), 2.5 – 2.4 (m, 2H), 2.3 – 2.1 (m, 2H), 1.4 (d, J = 7.0 Hz, 3H). **^{13}C NMR** (101 MHz, DMSO): δ = 180.9, 179.0, 142.9, 135.5, 134.2, 133.2, 132.1, 131.9, 130.2, 129.6, 129.5, 129.2, 129.0, 128.3, 128.0, 126.4, 124.0, 120.7, 70.3, 66.6, 63.2, 58.2, 31.1, 24.4, 22.0. **HRMS**: calc. $[m+H]^+$ for $C_{28}H_{27}N_3NiO_3$ = 512.14787; found = 512.14789 $[m+H]^+$. **HPLC** (flow rate 1 mL/min, 10-90% MeCN (0.1% TFA), 13 min) = 7.93 min.

8b:

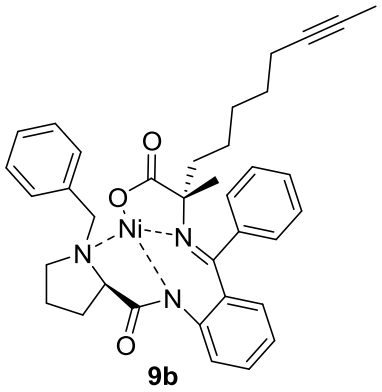
^{1}H NMR (600 MHz, DMSO) δ = 8.33 (d, J = 8.1 Hz, 2H), 7.93 (d, J = 8.7 Hz, 1H), 7.60 – 7.52 (m, 2H), 7.48 (m, 2H), 7.35 (dd, J = 10.8, 4.7 Hz, 2H), 7.13 (m, 2H), 7.05 (m, 1H), 6.64 (m, 1H), 6.49 (dd, J = 8.2, 1.6 Hz, 1H), 4.03 (d, J = 12.4 Hz, 1H), 3.58 – 3.47 (m, 4H), 3.35 – 3.30 (m, 1H), 2.47 – 2.38 (m, 2H), 2.23 – 2.10 (m, 2H), 1.42 (d, J = 7.1 Hz, 3H). **^{13}C NMR** (101 MHz, DMSO): δ = 180.2, 178.3, 169.4, 142.2, 134.7, 133.5, 132.5, 131.4, 131.6, 129.5, 128.9, 128.8, 128.5, 128.3, 127.6, 127.3, 125.7, 123.3, 120.0, 69.6, 65.9, 62.5, 57.5, 30.4, 23.7, 21.3. **HRMS**: calc. $[m+H]^+$ for $C_{28}H_{27}N_3NiO_3$ = 512.14787; found = 512.14810 $[m+H]^+$. **HPLC** (flow rate 1 mL/min, 10-90% MeCN (0.1% TFA), 13 min) = 8.12 min.

Synthesis of alkynated (*S*),(*R*)-Ala-Ni(II)-BPB (9a, b)



9a

Chemical Formula: $C_{35}H_{37}N_3NiO_3$
Exact Mass: 605.21884
Molecular Weight: 606.39240



9b

Chemical Formula: $C_{36}H_{39}N_3NiO_3$
Exact Mass: 619.23449
Molecular Weight: 620.41940

To a solution of **8a, b** in 15 mL DMF in a baked out flask under argon, freshly ground KOH (5.0 eq.) was added and the reaction mixture stirred for 20 min at 0°C. After addition of iodo-alkynes (**7a, b**) (1.2 eq.) in 2 mL DMF, the mixture was stirred for 20 min at 0°C and another 2 h at room temperature. The reaction was quenched by pouring it onto chilled acetic acid (125 mL, 5%) and extracted with DCM (3 × 80 mL). The combined organic layers were washed with water (50 mL), brine and dried over $MgSO_4$. After co-evaporation with toluene the pure product was obtained as a red solid. Yields: **9a** = 98%; **9b** = 85%.

9a:

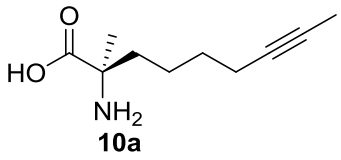
^{1}H NMR (500 MHz, DMSO) δ = 8.33 (d, J = 7.1 Hz, 2H), 7.88 (dd, J = 8.6, 1.0 Hz, 1H), 7.55 – 7.49 (m, 3H), 7.45 – 7.40 (m, 3H), 7.26 (t, J = 7.5 Hz, 1H), 7.16 – 7.12 (m, 1H), 7.11 – 7.06 (m, 1H), 6.67 – 6.61 (m, 1H), 6.61 – 6.55 (m, 1H), 4.06 (d, J = 12.3 Hz, 1H), 3.70 (d, J = 12.4 Hz, 1H), 3.52 – 3.47 (m, 1H), 3.40 – 3.34 (m, 1H), 3.13 – 2.99 (m, 1H), 2.57 – 2.51 (m, 1H), 2.50 – 2.41 (m, 1H), 2.27 – 2.20 (m, 2H), 2.17 – 2.07 (m, 2H), 2.01 – 1.91 (m, 1H), 1.63 (t, J = 2.5 Hz, 3H), 1.49 – 1.38 (m, 4H), 1.06 (s, 3H).**^{13}C NMR** (126 MHz, DMSO) δ = 180.5, 180.0, 171.7, 141.7, 136.4, 134.7, 132.8, 131.5, 131.3 130.7, 130.5, 129.3, 128.5, 128.4, 127.9, 127.8, 127.1, 126.9, 123.5, 120.0, 79.1, 76.7, 76.1, 69.7, 62.9, 56.9, 40.0, 30.2, 29.0, 28.4, 24.5, 22.7, 18.1, 3.0. **HRMS**: calc. $[m+H]^+$ for $C_{34}H_{36}N_3NiO_3$ = 606.22612; found = 606.22720 $[m+H]^+$. **HPLC** (flow rate 1 mL/min, 10-90% MeCN (0.1% TFA), 13 min) = 9.34 min.

9b:

^{1}H NMR (500 MHz, DMSO) δ = 8.33 (d, J = 7.1 Hz, 2H), 7.88 – 7.84 (m, 1H), 7.54 – 7.49 (m, 3H), 7.44 – 7.39 (m, 3H), 7.28 – 7.23 (m, 1H), 7.15 – 7.11 (m, 1H), 7.11 – 7.06 (m, 1H), 6.66 – 6.61 (m, 1H), 6.61 – 6.55 (m, 1H), 4.07 (d, J = 12.4 Hz, 1H), 3.68 (d, J = 12.4 Hz, 1H), 3.51 (m, 1H), 3.41 – 3.35 (m, 1H), 3.15 – 2.97 (m, 1H), 2.49 – 2.38 (m, 2H), 2.37 – 2.28 (m, 1H), 2.18 – 2.09 (m, 4H), 1.85 – 1.75 (m, 1H), 1.71 (t, J = 2.5 Hz, 3H),

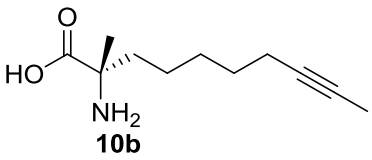
1.57 – 1.49 (m, 2H), 1.49 – 1.42 (m, 2H), 1.36 – 1.23 (m, 2H), 1.07 (s, 3H). **^{13}C NMR** (126 MHz, DMSO) δ = 180.6, 180.0, 171.7, 141.7, 136.3, 134.7, 132.7, 131.5, 131.3, 130.7, 130.4, 129.3, 128.5, 128.4, 128.0, 127.8, 127.1, 127.0, 123.6, 120.0, 79.1, 76.8, 75.8, 69.6, 62.9, 57.0, 39.9, 30.2, 29.0, 28.3, 28.2, 24.7, 22.8, 17.9, 3.1. **HRMS**: calc. $[m+H]^+$ for $C_{34}H_{36}N_3NiO_3$ = 620.24177; found = 620.24325 $[m+H]^+$. **HPLC** (flow rate 1 mL/min, 10-90% MeCN (0.1% TFA), 13 min) = 9.87 min.

Synthesis of unprotected α-methyl-α-alkynyl amino acids (10a, b)



10a

Chemical Formula: $C_{10}H_{17}NO_2$
Exact Mass: 183.12593
Molecular Weight: 183.25100

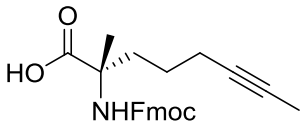


10b

Chemical Formula: $C_{11}H_{19}NO_2$
Exact Mass: 197.14158
Molecular Weight: 197.27800

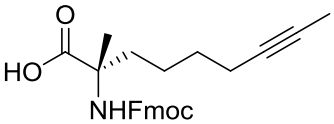
To a solution of **10a, b** in MeOH (40 mL), conc. hydrochloric acid (10 eq.) was added and the reaction mixture refluxed at 80°C for 1 h. The reaction mixture was allowed to cool to room temperature and concentrated *in vacuo*. After addition of water (20 mL) the aqueous layer was extracted with DCM (3× 20 mL). The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. Recovered BPB was purified by precipitation as hydrochloric salt from acetone solution.[7] The aqueous layer was dried by lyophilization and the crude unprotected α-methyl-α-alkynyl amino acid was used without any further purification.

Synthesis of Fmoc-protected α-methyl-α-alkynyl amino acids (1, 2, 4)



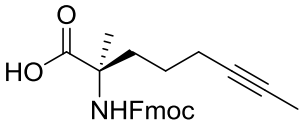
Fmoc-1-OH

Chemical Formula: $C_{24}H_{25}NO_4$
Exact Mass: 391.17836
Molecular Weight: 391.46700



Fmoc-2-OH

Chemical Formula: $C_{25}H_{27}NO_4$
Exact Mass: 405.19401
Molecular Weight: 405.49400

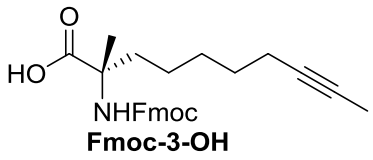


Fmoc-4-OH

Chemical Formula: $C_{24}H_{25}NO_4$
Exact Mass: 391.17836
Molecular Weight: 391.46700

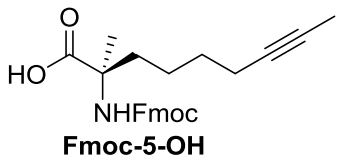
Fmoc-protected α-methyl- α-alkynyl amino acids **1**, **2** and **4** were performed according to previously established methods.[8]

Synthesis of Fmoc-protected α-methyl-α-alkynyl amino acids (3, 5)



Fmoc-3-OH

Chemical Formula: $C_{26}H_{29}NO_4$
Exact Mass: 419.20966
Molecular Weight: 419.52100



Fmoc-5-OH

Chemical Formula: $C_{25}H_{27}NO_4$
Exact Mass: 405.19401
Molecular Weight: 405.49400

To a solution of the crude unprotected α-methyl-α-alkynyl amino acid in H_2O/dioxane (40 mL, 1/1, v/v), Na_2CO_3 (4 eq.) and Fmoc-OSu (1.2 eq.) were added and stirred at room temperature for 7 d. The reaction was monitored using HPLC-MS analysis, daily and subsequently fresh Fmoc-OSu (0.5 eq.) was added. After addition of water (100 mL) the pH of the aqueous layer was set to 2-4 using aqueous hydrochloric acid and the aqueous layer was extracted with ethyl acetate (3 x 100 mL). The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified via column chromatography (R_f = 0.45, PE:EA 1:1; 0.1% AcOH) and obtained as a pale yellow solid. Yields: **3** = 79%; **5** = 90%;

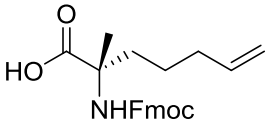
Fmoc-3-OH

^{1}H NMR (500 MHz, DMSO) δ = 12.35 (s, 1H), 7.89 (d, J = 7.5 Hz, 2H), 7.72 (d, J = 7.4 Hz, 2H), 7.41 (t, J = 7.2 Hz, 2H), 7.37 (s, 1H) 7.36 – 7.30 (m, 2H), 4.34 – 4.14 (m, 3H), 2.12 – 2.02 (m, 2H), 1.83 – 1.73 (m, 1H), 1.71 (t, J = 2.5 Hz, 3H), 1.69 – 1.61 (m, 1H), 1.43 – 1.35 (m, 2H), 1.32 (s, 3H), 1.29 (m, 2H), 1.24 – 1.13 (m, 2H). **^{13}C NMR** (126 MHz, DMSO) δ = 175.3, 154.7, 143.8, 140.7, 127.6, 127.0, 125.2, 120.0, 79.2, 75.7, 65.2, 58.3, 46.7, 36.6, 28.4, 28.4, 22.7, 22.3, 18.0, 3.1. **HRMS**: calc. $[m+H]^+$ for $C_{25}H_{28}NO_4$ = 420.21693 ; found = 420.21736 $[m+H]^+$, 442.19900 $[m+Na]^+$. **HPLC**: (flow rate 1 mL/min, 10-90% MeCN (0.1% TFA), 13 min) = 10.20 min.

Fmoc-5-OH

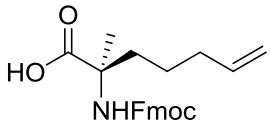
^{1}H NMR (500 MHz, DMSO) δ = 12.36 (s, 1H), 7.89 (d, J = 7.5 Hz, 2H), 7.71 (t, J = 9.2 Hz, 2H), 7.42 (t, J = 7.4 Hz, 2H), 7.39 (s, 1H), 7.33 (t, J = 7.4 Hz, 2H), 4.33 – 4.22 (m, 2H), 4.22 – 4.17 (m, 1H), 2.14 – 2.04 (m, 2H), 1.82 – 1.71 (m, 1H), 1.69 (t, J = 2.4 Hz, 3H), 1.69 – 1.60 (m, 1H), 1.43 – 1.34 (m, 2H), 1.33 (s, 3H), 1.30 – 1.17 (m, 2H). **^{13}C NMR** (126 MHz, DMSO) δ = 175.3, 154.7, 143.8, 140.7, 127.6, 127.0, 125.2, 120.0, 79.1, 75.7, 65.2, 58.2, 46.7, 36.2, 28.7, 22.6, 22.3, 18.0, 3.1. **HRMS**: calc. $[m+H]^+$ for $C_{24}H_{26}NO_4$ = 406.20128; found = 406.20170 $[m+H]^+$, 414.16581 $[m+Na]^+$. **HPLC**: (flow rate 1 mL/min, 10-90% MeCN (0.1% TFA), 13 min) = 9.89 min.

Fmoc-protected α-methyl-α-alkenyl amino acids (12, 13, 14)



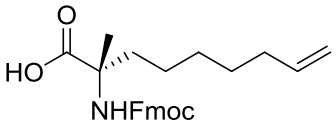
Fmoc-12-OH

Chemical Formula: $C_{23}H_{25}NO_4$
Exact Mass: 379.17836
Molecular Weight: 379.45600



Fmoc-13-OH

Chemical Formula: $C_{23}H_{25}NO_4$
Exact Mass: 379.17836
Molecular Weight: 379.45600



Fmoc-14-OH

Chemical Formula: $C_{25}H_{29}NO_4$
Exact Mass: 407.20966
Molecular Weight: 407.51000

Fmoc-protected α-methyl-α-alkenyl amino acids **12** - **14** were obtained from Okeanos Tech. Co.

2 Biochemical Methods

Fluorescence polarization assay for the determination of dissociation constants K_d

The dissociation constants (K_d) of peptide/14-3-3ζ (full-length) complexes were determined by a fluorescence polarization assay. Here, 0.1 mM stock solutions of N-terminal FITC-Peg modified peptides in DMSO were dissolved in 10 mM HEPES (pH 7.4), 150 mM NaCl and 0.1 % Tween-20 to obtain a 40 nM solution. A 200 nM 14-3-3ζ solution (15 µL per well) was diluted stepwise in a 384-multiwell plate (Corning, material no.: 4514) by a factor of 2.5 with final protein concentrations of 0.6 µM to 26 pM. After addition of peptide solution (5 µL per well, final peptide concentration 10 nM) to each well and incubation for 60 min at RT the fluorescence polarization was measured utilizing a Safire^2 plate reader (Tecan) with λ(ex) = 485 nm and λ(em) = 525 nm. The calculation of K_d values was realized by use of GraphPad Prism[9] software via non-linear regression analysis of dose-response curves.

Microscale Thermophoresis (MST)

14-3-3ζ was serially diluted in assay buffer and treated with 10 nM fluorescein-labelled peptide. After incubation for 2 h at room temperature the mixture was soaked into capillaries for microscale thermophoresis (MST) measurements. K_d values were calculated from obtained MST curves using the software Monolith Affinity Analysis (NanoTemper Technologies).

Competition fluorescence polarization assay

The half maximal inhibitory concentration (IC_{50}) of N-terminal acetylated peptide by displacing fluorescence labelled ESp from 14-3-3ζ (full-length) were determined performing a fluorescence polarization-based displacement assay. Here, a 2.67 µM 14-3-3 (full length) solution in 10 mM HEPES (pH 7.4), 150 mM NaCl and 0.1 % Tween-20 was preincubated with 13 nM FITC-Peg modified ESp for 30 min at 4°C. DMSO stock solutions of acetylated peptide **H** and **ESp** as a reference were dissolved 10 mM HEPES (pH 7.4), 150 mM NaCl and 0.1 % Tween-20 to get 100 µM and 400 µM peptide concentrations, which were diluted stepwise in a 384-multiwell plate (Corning, material no.: 4514) by a factor of 1.5. 15 µL of preincubated 14-3-3ζ (full length) were added to each well with 5 µL acetylated peptide solution to obtain final peptide concentrations of 25 µM to 25 nM for acetylated peptide **H** and 100 µM to 101 nM for acetylated **ESp** and a final protein concentration of 2 µM for 14-3-3 with 10 nM FITC-Peg ESp. After incubation for 15 min at RT the fluorescence polarization was measured utilizing a Safire^2 plate reader (Tecan) with λ(ex) = 485 nm and λ(em) = 525 nm. The calculation of IC_{50} values was realized by use of GraphPad Prism[9] software via non-linear regression analysis of dose-response curves.

Protein Expression and Purification

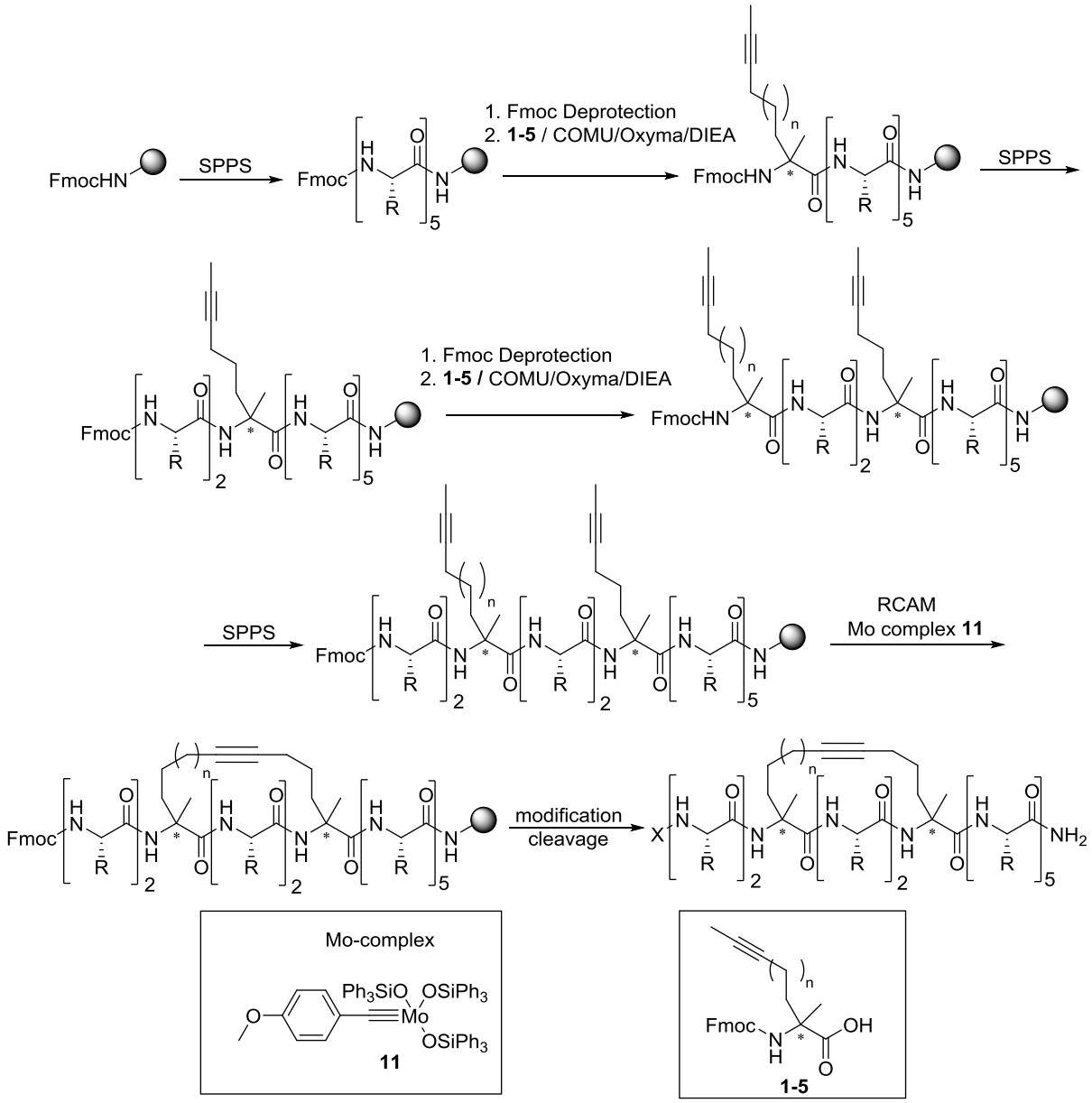
Escherichia coli Rosetta (DE3) cells (Merck, Nottingham, UK), containing the pPROex HTb vector (Invitrogen), was used to inoculate 50 mL LB medium supplemented with 100 mg·mL^{-1} ampicillin. The culture was grown for 14 h at 310 K with shaking. 5 L Terrific Broth (TB) culture containing 100 mg·mL^{-1} ampicillin was supplemented with pre-culture and shaken at 140 rpm at 310 K until an OD_{600} value of 0.6–0.8 was reached. The induction of protein expression was started by adding 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) to the culture and incubated at 295 K for 14 h. The cells were harvested by centrifugation at 4500 rpm. After resuspension of bacterial pellet in 50 mL lysis buffer containing 50 mM Tris-HCl, 300 mM NaCl, 5 % glycerol, 10 mM imidazole, 0.5 mM tris(2-carboxyethyl)phosphine (TCEP) and 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 8.0, the cells were lysed, using a microfluidizer. Cell debris were removed by centrifugation at 20 000 *g* for 30 min at 270 K and the His-tagged 14-3-3 protein was purified using a liquid chromatography system (AKTA Pure, GE), followed by affinity purification via Nickel-nitrilotriacetic acid (NTA) resin (GE Healthcare, Freiburg, Germany). The NTA resin was washed with buffer consisting of 50 mM Tris-HCl, 500 mM NaCl, 5 % glycerol, 25 mM imidazole and 0.5 mM TCEP, pH 8.0. and the protein eluted in buffer consisting of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 200 mM NaCl, 5 % glycerol, 250 mM imidazole, pH 8.0, 1.0 mM ß-mercaptoethanol. The eluted protein was dialyzed against 25 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, pH 7 cleaved by Tobacco Etch Virus (TEV) protease. For the removal of cleaved His-Tag, the protein was additionally purified by size exclusion chromatography by using AKTA Pure and HiPrep 26/60 Sephacryl S-200 HR column (GE). The 14-3-3 protein was concentrated to 103 mg·mL^{-1}, aliquoted and flash-frozen in liquid nitrogen.

Co-crystallization

The peptide **H** was mixed with 14-3-3ζ in a molar ratio of 1:1.5 (14-3-3 dimer/peptide) to a final concentration of 22 $mg \cdot mL^{-1}$ in 25 mM HEPES/NaOH (pH 7.2), 2 mM $MgCl_2$, 1.0 mM ß- mercaptoethanol and incubated on ice overnight. The initial screen was started by using NeXtal crystallization suites (Qiagen) at 4°C. Crystals were visible after 4 weeks in 1.26 M Tri-Sodium Citrate, 0.09 M HEPES pH 7.5, 10% Glycerol and grew to dimensions of 300×300×200 µm. For optimization of crystal genesis in hanging drop, EasyXtal (Qiagen) plates were used; containing the original solution (1.26 M Tri-Sodium Citrate, 0.09 M HEPES pH 7.5, 10% Glycerol) and solutions with varying Glycerol concentrations (6 to 12 %). Final crystals grew within 3 weeks to dimensions of 400×400×300 µm and showed diffraction to 2.4 Å. Data was collected using the PXII - X10SA beamline for protein crystallography at Paul Scherrer Institut (PSI) in Villigen, Switzerland. Crystallographic analysis was performed using the XDS15 software package. Molecular replacement was carried out with the CCP4 package and model building was performed with COOT. For detailed statistics see Supporting Tables 3. Crystal structure was deposited in the Protein Data Bank PDB-ID: 5J31.

3 Supporting Tables and Figures

Peptide Synthesis



Supporting Figure S1. Synthesis of alkyne macrocyclized peptides. A detailed overview of all synthesized peptides (sequences, analytical data) is shown in Supporting Table S1. X = Fmoc, FITC-PEG, Ac

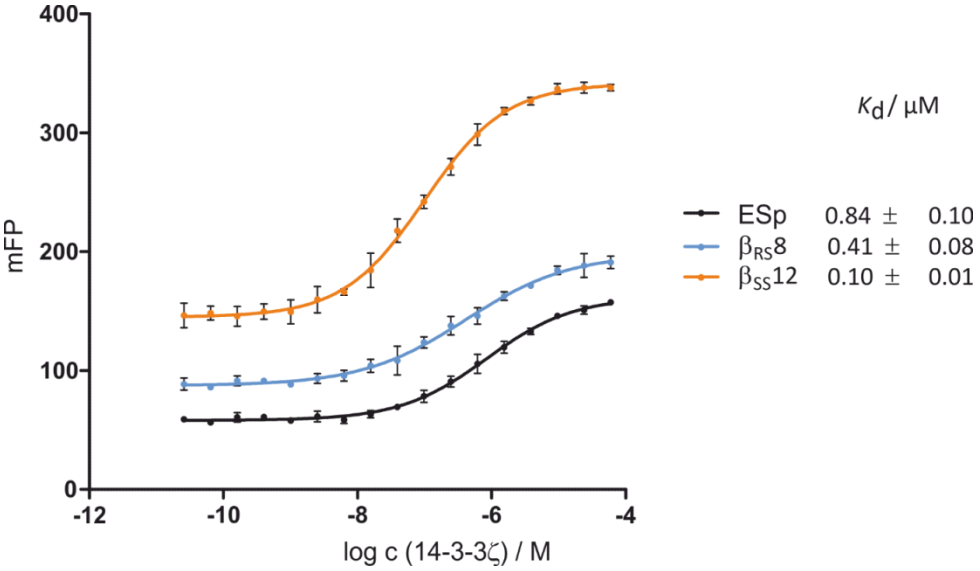
Supporting Table S1. Detailed overview of all synthesized alkyne macrocyclic peptides based on the non-modified **ESp** sequence

Entry	Peptide	*N*-Term mod. [a]	Sequence	HPLC (t_R, [min]) [b]	HRMS (Calc.)	HRMS (found)
1a	**ESp**	**F**	Q G L L D A L D L D A S	14.8	824.8642	824.8624 $[M+2H]^{2+}$
1b		**Ac**		11.3	1156.6214	1156.6215 $[M+2H]^{2+}$
2	β_{RS}**8**	**F**	Q G **13** L D **12** L D L D A S	9.4	858.8955	858.8945 $[M+2H]^{2+}$
3	β_{SS}**12**	**F**	Q G **14** L D **14** L D L D A S	12.2	886.9268	886.9263 $[M+2H]^{2+}$
4	**A**	**F**	Q G **4** L D **1** L D L D A S	9.7	870.8949	870.8954 $[M+2H]^{2+}$
5	**B**	**F**	Q G **5** L D **1** L D L D A S	9.4	863.8871	863.8883 $[M+2H]^{2+}$
6	**C**	**F**	Q G **4** L D **2** L D L D A S	9.4	863.8871	863.8878 $[M+2H]^{2+}$
7	**D**	**F**	Q G **5** L D **2** L D L D A S	8.9	856.8793	856.8804 $[M+2H]^{2+}$
8	**E**	**F**	Q G **2** L D **2** L D L D A S	9.7	870.8949	870.8950 $[M+2H]^{2+}$
9	**F**	**F**	Q G **3** L D **2** L D L D A S	9.8	1754.7982	1754.7976 $[M+H]^{+}$
10	**G**	**F**	Q G **2** L D **3** L D L D A S	10.2	877.9027	877.9033 $[M+2H]^{2+}$
11a	**H**	**F**	Q G **3** L D **3** L D L D A S	10.9	884.9106	884.9115 $[M+2H]^{2+}$
11b		**Ac**		8.3	1276.7147	1276.7184 $[M+H]^{+}$

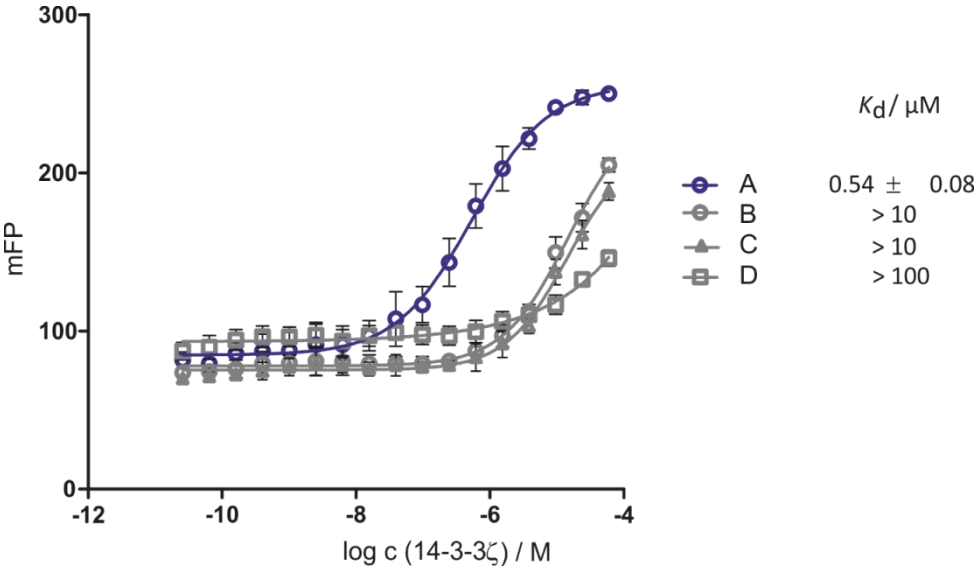
[a] **F** = Fluorescein-O2OC-, **Ac** = Acetylated

[b] Retention time of purified peptides by analytical HPLC (10-90% MeCN (0.1% TFA) for peptides **A** - **H** , 12 min)

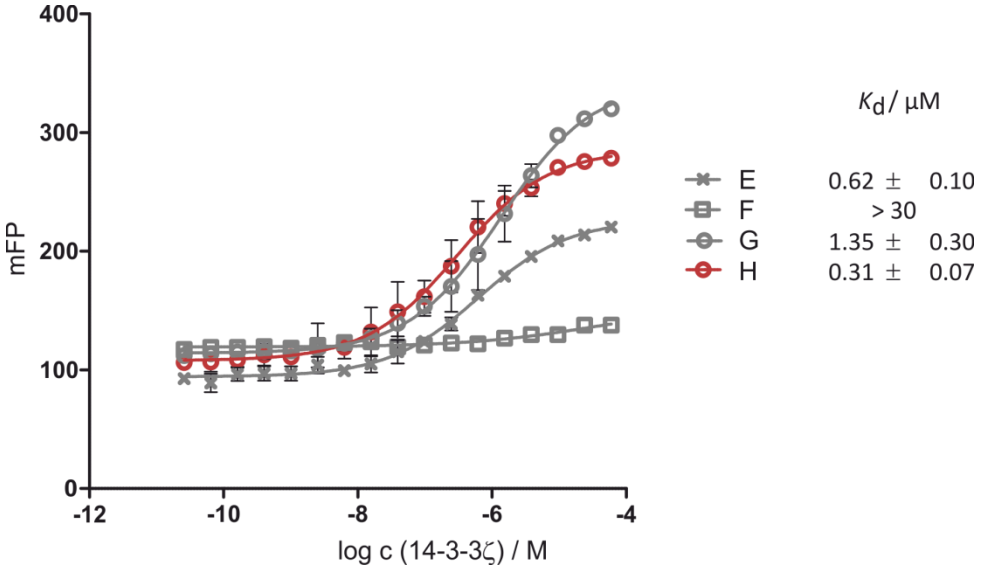
Fluorescence polarization assay for the determination of dissociation constants



Supporting Figure S2. FP graph of peptides **ESp**, **β_{RS}8** and **β_{SS}12** with 14-3-3ζ including average K_d-values (triplicate).

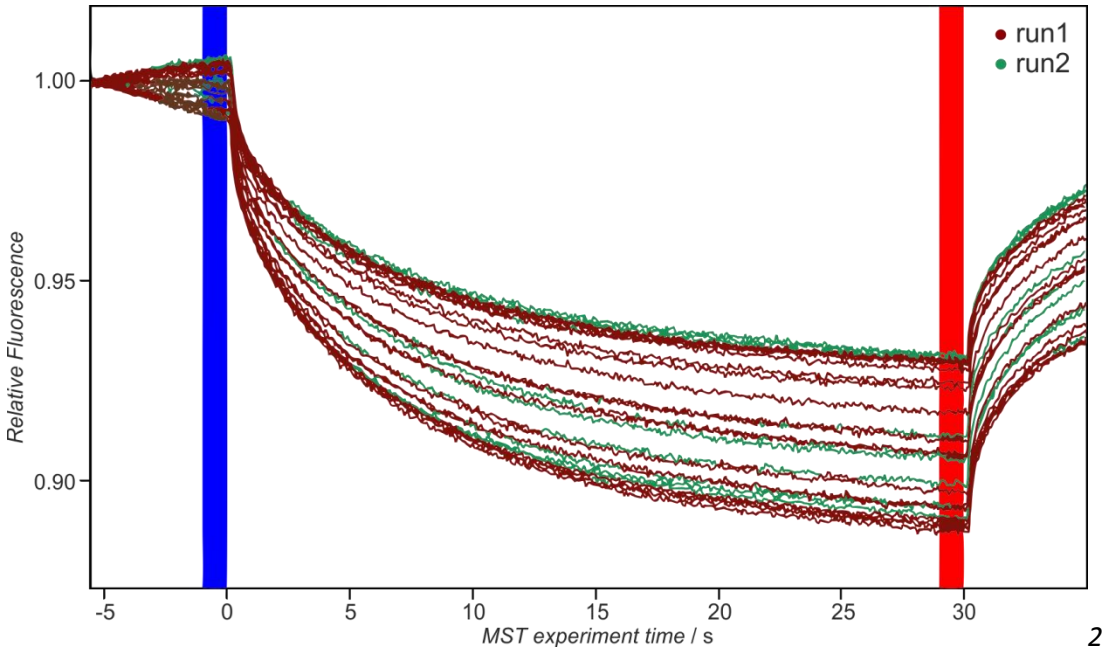


Supporting Figure S3. FP graph of peptides **A – D** with 14-3-3ζ including average K_d-values (triplicate).



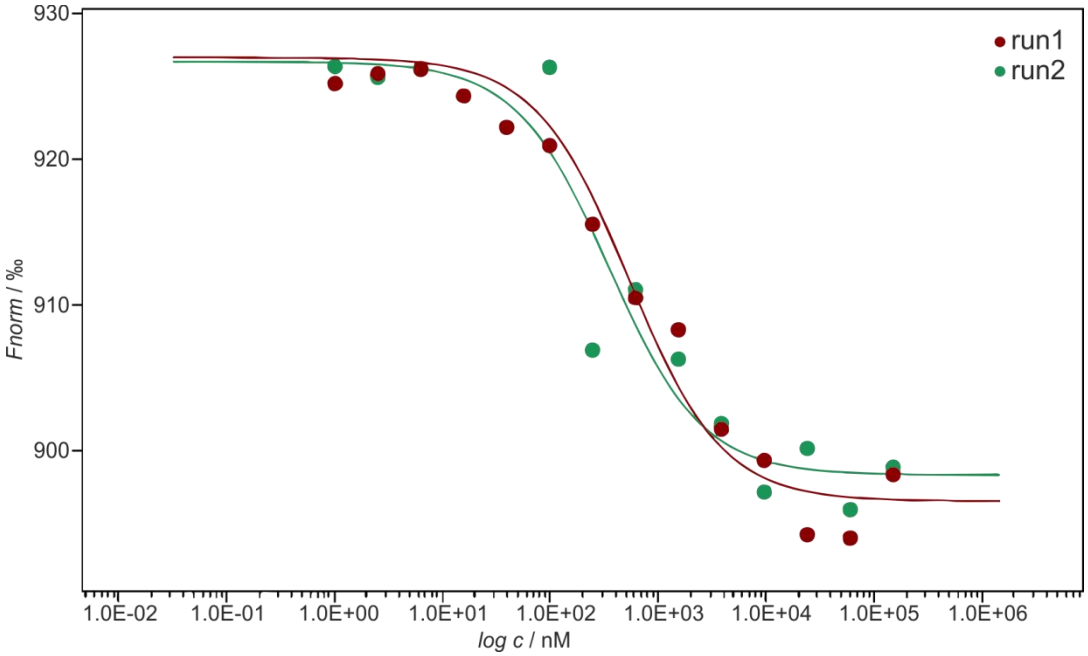
Supporting Figure S4. FP graph of peptides **E** – **H** with 14-3-3ζ including average K_d-values (triplicate).

Microscale Thermophoresis (MST)



2

Supporting Figure S5. MST curves of peptide **H**.



Supporting Figure S6. Fitted MST data of peptide **H**. (c_{max} [14-3-3ζ] = 150 µM). Calculated K_d values are summarized in Supporting Table 2.

Supporting Table S2. Results from MST experiments (Supporting Figures 4, 5). MST analysis of peptide **H**. MST data was fitted using the software Monolith Affinity Analysis (NanoTemper Technologies). Two individual runs were performed resulting in a K_d of 0.44 µM. N = 2

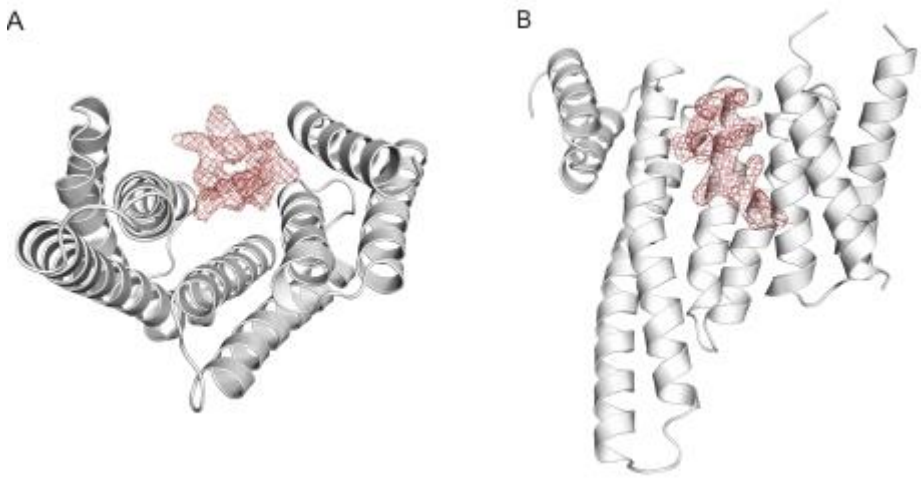
Peptide **H**	K_d
run1	0.53 ± 0.10 µM
run2	0.35 ± 0.15 µM

X-ray Crystallography

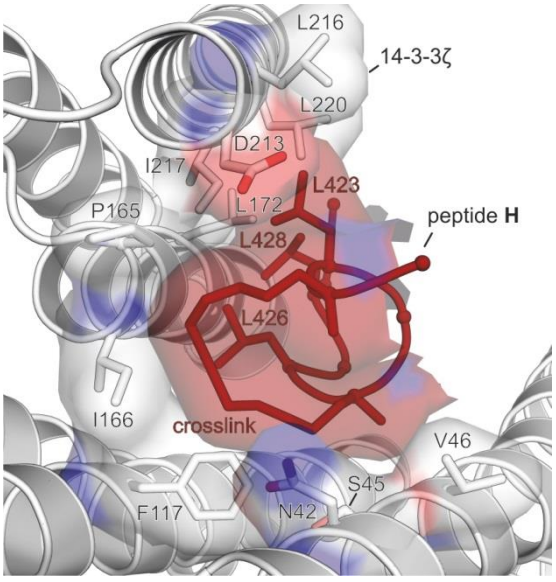
Supporting Table S3. Data collection and refinement statistics (2.5-Å complex, PDB ID 5J31**)**

	H/14-3-3ζ
Data collection	
Space group	P $2_12_12_1$ (19)
Cell dimensions	
a, *b*, *c* (Å)	83.40, 104.09, 113.80
α, β, γ (°)	90.00, 90.00, 90.00
Resolution (Å)	47.63-2.4(2.50-2.40)
R_{sym} or R_{meas}	5.2(64.4)
I / σ	19.37(3.06)
Completeness (%)	100(100)
Redundancy	13.28(13.79)
Refinement	
Resolution (Å)	47.94-2.4(2.60-2.40)
No. reflections	38425
R_{work} / R_{free}	0.163/0.219(0.183/0.230)
No. atoms	
Protein	3675
Ligand/ion	154
Water	358
B-factors	
Protein	A:65.44; B: 65.51
Ligand/ion	C:67.72; E: 68.46
Water	70.08
R.m.s. deviations	
Bond lengths (Å)	0.0206/0.0199
Bond angles (°)	2.8437/3.3327

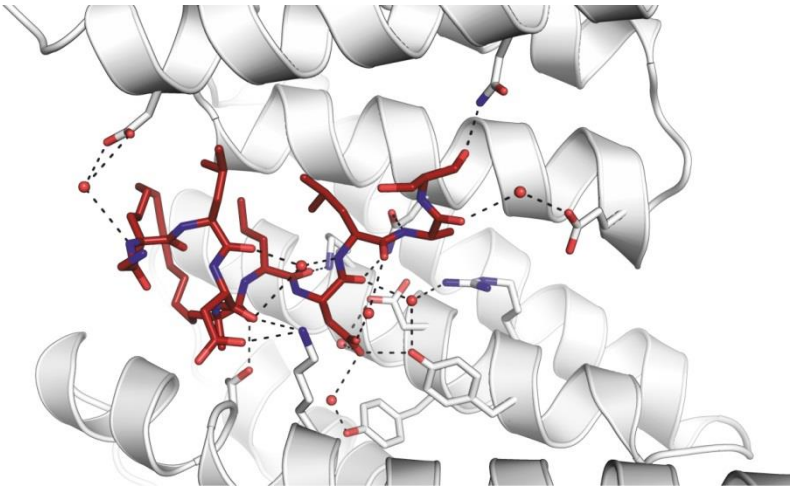
[#] Data was collected from a single crystal. Values in parentheses are for highest-resolution shell.



Supporting Figure S7. A Front view on $2F_o$-F_C electron density of peptide **H** (red) bound to 14-3-3 monomer (cartoon, white); **B** Top view on $2F_o$-F_C electron density of peptide **H** (red) bound to 14-3-3 monomer (cartoon, white).



Supporting Figure S8. Non-polar contacts between peptide **H** (ribbon presentation, red) and 14-3-3ζ (cartoon, white). Residues of 14-3-3 involved in non-polar contacts with peptide **H** are shown as sticks with semi-transparent (white = C, blue = N, red = O).



Supporting Figure S9. Polar contacts (dashed lines, black) formed between peptide **H** (ribbon presentation, red) and 14-3-3ζ (cartoon, white). Residues of 14-3-3 involved in polar contacts with peptide **H** are shown as sticks (white = C, blue = N, red = O). Water molecules are shown as red spheres.

4 Abbreviations

AcOH	Acetic acid
Ac_2O	Acetic anhydride
brine	Saturated NaCl (aqueous)
COMU	1-[(1-(Cyano-2-ethoxy-2-oxoethylidenaminooxy)-dimethylaminomorpholino)]-uronium-hexafluorphosphate
DCM	Dichloromethane
DIEA	Diisopropylethylamine
DMF	*N*,*N*-Dimethylformamide
EDT	1,2-Ethanedithiol
EA	Ethylacatate
ESI	Electrospray ionization
EtOH	Ethanol
FITC	Fluorescein isothiocyanate
Fmoc	Fluorenylmethoxycarbonyl
Fmoc-O2Oc-OH	Fmoc-8-amino-3,6-dioxaoctanoic acid
HCTU	*O*-(6-Chlorobenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
MeCN	Acetonitrile
MST	Microscale thermophoresis
NMP	*N*-Methyl-2-pyrrolidone
NMR	Nuclear magnetic resonance
Oxyma	Ethyl (hydroxyimino)cyanoacetate
PE	Petrol ether
RA	Relative Abundance
RCAM	Ring closing alkyne metathesis
RCM	Ring closing metathesis
SPPS	Solid-phase peptide synthesis
TFA	Trifluroacetic acid
TIS	Triisopropylsilane

5 References

[1] Y. N. Belokon', V. I. Tararov, V. I. Maleev, T. F. Savel'eva, M. G. Ryzhov, *Tetrahedron: Asymmetry* **1998**, *9*, 4249–4252.

[2] G. H. Bird, C. W. Crannell, L. D. Walensky, *Curr. Protoc. Chem. Biol.* **2011**, *3*, 99–117.

[3] J. Heppekausen, R. Stade, A. Kondoh, G. Seidel, R. Goddard, A. Fürstner, *Chem. Eur. J.* **2012**, *18*, 10281–10299.

[4] A. Fürstner, O. Guth, A. Rumbo, G. Seidel, *J. Am. Chem. Soc.* **1999**, *121*, 11108–11113.

[5] M. Bindl, L. Jean, J. Herrmann, R. Müller, A. Fürstner, *Chem. Eur. J.* **2009**, *15*, 12310–12319.

[6] J. Spiegel, P. M. Cromm, A. Itzen, R. S. Goody, T. N. Grossmann, H. Waldmann, *Angew. Chem. Int. Ed.* **2014**, *53*, 2498–2503.

[7] H. Ueki, T. K. Ellis, C. H. Martin, T. U. Boettiger, S. B. Bolene, V. A. Soloshonok, *J. Org. Chem.* **2003**, *68*, 7104–7107.

[8] P. M. Cromm, S. Schaubach, J. Spiegel, A. Fürstner, T. N. Grossmann, H. Waldmann, *Nat. Commun.* **2016**, 11300.

[9] H. Motulsky, A. Christopoulos, *Fitting models to biological data using linear and nonlinear regression*. *A practical guide to curve fitting*, Oxford University Press, Oxford, New York, **2004**.