Supporting Information

Structure-Based Design of Non-Natural Macrocyclic Peptides that Inhibit Protein–Protein Interactions

Dennis M. Krüger[†,‡,§,#], Adrian Glas[†,‡,#], David Bier[†,⊥], Nicole Pospiech[†], Kerstin Wallraven[†], Laura Dietrich[†,‡], Christian Ottmann[⊥,∥], Oliver Koch*[,‡], Sven Hennig*[,†,∇], Tom N. Grossmann*[,†,‡,∇]

[†] Chemical Genomics Centre of the Max Planck Society, Otto-Hahn-Str. 15, 44227 Dortmund, Germany.

[‡] TU Dortmund University, Faculty of Chemistry and Chemical Biology, Otto-Hahn-Str. 6, 44227 Dortmund, Germany.

[⊥] University of Duisburg-Essen, Department of Chemistry, Universitätstr. 7, 45141, Essen, Germany.

[∥] Eindhoven University of Technology, Department of Biomedical Engineering, Institute of Complex Molecular Systems, Den Dolech 2, 5612 AZ Eindhoven, The Netherlands.

[∇] VU University Amsterdam, Department of Chemistry & Pharmaceutical Sciences, De Boelelaan 1083, 1081 HV, Amsterdam, The Netherlands.

[§] Present address: Uppsala University, Department of Cell and Molecular Biology, BMC Box 596, 751 24 Uppsala, Sweden

[#] These authors contributed equally to this work.

* Corresponding authors: O.K. (email: oliver.koch@tu-dortmund.de), S.H. (email: s.hennig@vu.nl) or T.N.G (email: t.n.grossmann@vu.nl).

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2.1 Computational Setup

Protein structure and peptide preparation for docking

Coordinates for protein and peptide were retrieved from PDB entry 4N84. All water molecules and ions were removed. AutoDock-Tools (ADT) 1.5.6 was used to add polar hydrogen atoms and charges to the protein and peptides.[1] The peptides were further prepared by ADT assigning rotatable bonds (all substituents including the two peptide sequences were kept flexible except for amide bonds).

Library preparation

2D structures of the 18 natural (Gly and Pro are excluded) and 223 non-proteinogenic amino acids were manually created using ChemDraw 14.0 and subsequently converted to 3D structures in protonation states under neutral condition using Maestro 9.3.5.[2] The peptide library was created using in-house Python scripts by replacing single amino acids of $\beta_{SS}12$ with each amino acid of the amino acid library.

Docking engine, scoring function, and docking experiments

For conformational sampling, we used AutoDock Vina 1.1.2 as docking engine.[3] Docking parameters were chosen such that the algorithm is able to reproduce the peptide conformation as observed in the initial crystal structure (RMSD < 2.5 Å, see below). Alternatively, GOLD 5.2.2 was tested for conformational sampling, but was not able to reproduce a near native X-ray conformation.[4]

Docking of peptide library

The center of the grid box was set to x = 10, y = 13, z = 10 and the box size was set to 30 Å in each dimension. Docking parameters were chosen as follows: exhaustiveness = 12, weight_gauss1 = 0.7, weight_repulsion = 0.5, weight_hydrophobic = -0.15, weight_hydrogen = -0.6, the number of output conformations was set to maximum (num_modes = 20), all other parameters default.

During pose filtering, all poses were excluded in which the functional groups of hotspot residues L426, D427 and L428 exhibit an RMSD > 2 Å when compared to analogous residues in **2** bound to 14-3-3 (crystal structure, PDB ID 4n84). For rescoring, the remaining poses were scored with ChemScore[5] and the Astex statistical potential (ASP),[6] respectively using the simplex minimization option as implemented in Gold 5.2.2.[4]

Visual inspection

For each peptide, only the highest scoring pose was considered for the final ranking. The top five ranking peptides per scoring function and position were visually inspected (in total 60 complexes) to select one peptide per scoring function and position for experimental validation (in total twelve peptides). For selected peptides and their predicted binding modes, see Figure S4.

2.2 Peptide Synthesis and Characterization

The peptides for the alanine-scan were synthesized manually on NovaSyn®TGR Resin (loading 0.24 mmol/g; Merck KGaA) by standard Fmoc-based solid-phase peptide synthesis (SPPS).[7] The peptides containing additional variations (compared to $\beta_{SS}12$) were either synthesized manually or automated on a Syro II Synthesizer from MultiSynTech (stated in supporting table S1). Manual synthesis was performed in polypropylene (pp) reaction vessels (Bio-Rad) on a vacuum manifold (Promega). For an accurate mixing, the resin was purged with nitrogen gas. Automated synthesis was performed in pp reaction vessels from MultiSynTech. For a sufficient swelling 1 mL solvent was used per 100 mg resin in all reaction steps. After every reaction step, the resin was washed 5 times with *N*-methyl-2-pyrrolidinone (NMP), 5 times with dichloromethane (DCM) and again 5 times with NMP (1 mL solvent / 100 mg resin). Fmoc-protected, proteogenic amino acids were

purchased from Iris Biotech. The unnatural olefinic amino acids were purchased from Okeanos Tech. Coupling reagents were purchased at Carl Roth. Fmoc-protected, non-proteinogenic amino acids were purchased from Iris Biotech, PolyPeptide Group and Sigma Aldrich. Prior to every coupling, the resin was swollen NMP for 15 min and afterwards the Fmoc group was removed by treating the resin with a solution of 25 % piperidine in NMP for 15 min. Amino acids were coupled using 4 equivalents of the Fmoc-protected amino acids according to the loading of the resin. The amino acid was mixed with 4 equivalents of (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBop) and 8 equivalents of *N,N*-diisopropylethylamine (DIPEA) in NMP and added to the resin for 1 h at room temperature twice. After every double coupling remaining free *N*-terminal amino group were acetylated by treating the resin with NMP/Ac_2O/DIPEA (10/1/1, v/v/v) for 5 min twice. *N*-terminal acetylated peptides were synthesized under same conditions after Fmoc cleavage. Ring closing olefin metathesis (RCM) was performed with 4 mg mL^{-1} Grubbs catalyst 1st generation four times for 2 h followed by a washing step with a mixture of dimethyl sulfoxide (DMSO) and DCM (1:1, v/v) for 10 min. The resulting double bond was reduced using 0.6 M 2,4,6-Triisopropylbenzenesulfonyl hydrazide and 1.2 M piperidine (200 µL per 50 mg resin). The resin was treated with the solution 3 times for 100 min at 60 °C and 1000 rpm orbital shaking using a ThermoMixer from Eppendorf. For the generation of *N*-terminal fluorescent labeled peptides a spacer was introduced according to the procedure described for protected amino acids (PEG_2-linker, Fmoc-O2Oc-OH, Iris Biotech). After the removal of the Fmoc-group the resin was treated with a mixture of 4 equivalents fluorescein isothiocyanate (FITC) and 8 equivalents DIPEA in NMP 2 times for 1 h. For the cleavage of the peptides, the dry resin was treated with a solution of TFA/EDT/H_2O/TIPS (94/2.5/2.5/1, v/v/v/v) for 3 h. The peptides were precipitated with Et_2O at –20 °C. Precipitated peptides were dissolved in H_2O/ACN (1/1, v/v) and purified by reversed-phase HPLC using a Nucleodur C18 reverse-phase column (10 x 125 mm, 110 Å, particle size 5 µm, Macherey-Nagel; solvent A: water + 0.1 % TFA; solvent B: acetonitrile + 0.1 % TFA; flow rate: 6 mL min^{-1}). Obtained peptides were lyophilized and characterized by an Agilent HPLC/ESI system equipped with a Zorbax C18 reverse-phase column (4.6 x 150 mm, particle size 5 µm, Agilent; solvent A: water + 0.1% TFA; solvent B: acetonitrile + 0.1% TFA; flow rate: 1 mL min^{-1}). The Data is shown in Table S1. The Peptides were quantified by weight or by comparative HPLC at λ = 210 nm. Peptide purity was determined by RP-HPLC peak integration at λ = 210 nm.

2.3 Protein Expression and Purification

For the expression of 14-3-3ζ ΔC (aa 1-230), a preculture of *Escherichia coli Rosetta* (DE3) cells, containing pPROex HTb vector, in LB medium with 100 µg mL^{-1} ampicillin was prepared. After 17 h of shaking at 170 rpm and 37 °C, the preculture was used to inoculate 5 L TB medium containing 100 µg mL^{-1} ampicillin. The cells were grown for additional 3.5 h at 37 °C and as an OD_{600} of 0.8

was reached the protein expression was initiated through addition of 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). The culture has been shaking over night at 150 rpm at a reduced temperature of 25 °C, before the cells were centrifuged at 4500 rpm, 4 °C for 20 min. The resulting pellet was suspended in 30 mL lysis buffer (50 mM Tris, 300 mM NaCl, 5 % glycerol, 10 mM imidazole, 0.5 mM tris(2-carboxyethyl)phosphine (TCEP), 1 mM PMSF, pH 8.0), before an appropriate amount of DNase I was added and the suspension was homogenized using ULTRA TURRAX. Then, the cells were lysed by a microfluidizer. The cell fragments were removed through centrifugation at 8000 rpm, 4 °C for 30 min, before his-tagged 14-3-3ζ ΔC was purified via affinity chromatography on nickelnitrilotriacetic acid (NTA) beads (GE Healthcare). Undesired proteins were removed by washing the beads with washing buffer (50 mM Tris, 500 mM NaCl, 5 % glycerol, 25 mM imidazole, 0.5 mM TCEP, pH 8.0), before 14-3-3ζ ΔC was eluted with elution buffer (50 mM HEPES, 200 mM NaCl, 5 % glycerol, 250 mM imidazole, 0.5 mM TCEP, pH 8.0) and concentrated to 14 mg mL^{-1}. Subsequently the His_6-tag was cleaved by addition of Tobacco Etch Virus (TEV) protease (1:0.05 mg = 14-3-3:protease). 14-3-3ζ ΔC with the His_6-tag on was digested over night at 4 °C. A size exclusion chromatography (SEC) was performed on ÄKTA Pure with a HiPrep 26/60 Sephacryl S-200 HR column (GE Healthcare) to remove the His_6-tag. The pure protein was concentrated by ultrafiltration to 64 mg mL^{-1}, flash-frozen and stored at –80 °C.

2.4 Fluorescence Polarization Assays

To determine the affinity of the FITC labeled peptides towards 14-3-3ζ ΔC a 0.1 mM peptide solution in DMSO was diluted with FP buffer (10 mM HEPES, 150 mM NaCl, 0.1 % Tween-20, pH 7.4) to 40 nM. 14-3-3ζ Δ was also diluted with FP buffer in a 2.5-fold dilution series (80 µM – 0.5 nM) in a 384 well plate (black, low volume, non-binding surface, round bottom, Corning). To 15 µL of the protein solution, 5 µL of the 40 nM peptide stock was added leading to a final peptide concentration of 10 nM and a final protein concentration of 60 µM – 0.4 nM. After 1 h incubation time the fluorescence polarization was measured with a extinction wavelength (λ_{ex}) of 485 nm and an emission wavelength (λ_{em}) of 525 nm at room temperature. The dissociation constant (K_D) was determined from the binding curve with GraphPad from Prism.

For competition experiments, *N*-terminally acetylated peptides were diluted 1:1 in a 384 well plate (100 µM – 1 nM). 10 µL of a mixture (1:1) of 14-3-3ζ ΔC and TAMRA-labeled cRaf peptide was added leading to following final concentrations: acetylated peptides = 50 µM – 0.5 nM, 14-3-3ζ ΔC = 800 nM and TAMRA-labeled cRaf peptide = 100 nM. After 1 h, fluorescence polarization was measured with a extinction wavelength (λ_{ex}) of 530 nm and an emission wavelength (λ_{em}) of 585 nm at room temperature. The half maximal inhibitory concentration (IC_{50}) was determined from the binding curve with GraphPad from Prism.

2.5 X-Ray Crystallography and Structure Determination

The initial screening was performed with 14-3-3ζ ΔC (Residues 1−230) in 50 mM HEPES (pH 7.5), 100 mM NaCl and 2 mM $MgCl_2$. For complexation, **22** was dissolved in DMSO (11 mM) and mixed with the protein in a molar ratio of 1:2 (protein/peptide). The protein concentration in complex was adjusted to 22 mg ml^{-1}. The complex was incubated overnight at 4°C and set up for crystallization using NeXtal Screens (Qiagen).

Crystals grew within 4 weeks in the following condition: 1.36 M sodium citrate, 15 % (v/v) glycerol and sowed a diffraction to 2.34 Å. After molecular replacement, the space group was determined to be $P2_12_12_1$.

Data was collected using PXII beamline for protein crystallography at the Paul Scherrer Institute Swiss Light Source (SLS). Crystallographic analysis was performed using the XDS software package. Molecular replacement was carried out with the CCP4 package and model building was performed with COOT (Table S3). Crystal structure was deposited in the Protein Data Bank (PDB: 5jm4).

2.6 Cell Permeability Assay

Cell culture

Cell culture media and solutions were purchased from PAN Biotech (Aidenbach, Germany). Cell culture consumables were either purchased from Sarstedt (Nümbrecht, Germany) or Corning Life Sciences (Corning, NY, USA). HeLa cells were grown as a monolayer in 10 cm tissue culture dishes and cultured in DMEM supplemented with 10 % fetal calf serum, and non-essential amino acids. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. For subculturing and experiments, cells were removed from flasks by treatment with trypsin-EDTA. A subculture was performed every 3-4 days.

Fluorescence microscopy

5000 cells were plated in each well of a 96-well microplate and cultured for 24 h. Then, peptides were added at final concentration of 20 µM with 1 % DMSO to the medium and incubated for 4 h. Control reactions were performed using either 1 % DMSO only, and 20 µM Penetratin (cell-penetrating peptide as positive control). Cells were washed three times with PBS, fixed with 4 % paraformaldehyde and washed another three times with PBS. For nuclear staining, a 3 µM 4',6-diamidino-2-phenylindole (DAPI) solution in PBS was prepared and left on the cells for at least 5 min. Staining was performed at room temperature in the dark. After additional washing steps, the cells were left in PBS, and the distribution of FITC-labeled peptides was analyzed via fluorescence microscopy using a 20x air objective (Axiovert 40 CFL, Zeiss).

2.7 Quantitative Real Time PCR Analysis

U87 glioblastoma cells were cultivated in DMEM (+10 % FCS) at 37°C at 5 % CO_2. Cells were plated for 24 h and medium was changed (DMEM + 1 % FCS). After another 24 h, cells were treated with 200 nM 14-3-3ζ, the corresponding peptides in DMEM + 1 % FCS and 0.5 % DMSO. Untreated and 14-3-3ζ -treated controls were cultivated under same conditions with 0.5 % DMSO. After 24 h of incubation, total RNA was isolated (Quick-RNA MicroPrep Kit, Zymo Research) and reverse transcribed into cDNA (Quanti Tect Reverse Transcription Kit, Qiagen). Next, cDNA was used for quantitative real time PCR (SensiMix SYBR Low-ROX Kit, Bioline) in the Applied Biosystems 7500 Fast Real Time PCR machine (Thermo Fisher Scientific). For relative quantitation, $2^{-\Delta\Delta CT}$ method was used with the reference gene *GAPDH*.

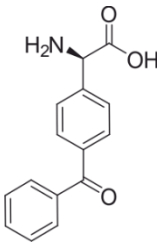
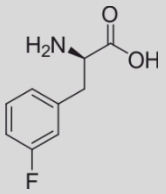
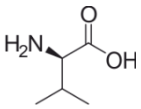
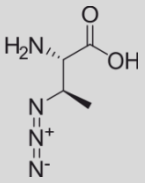
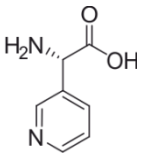
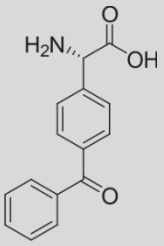
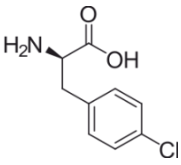
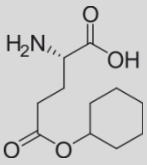
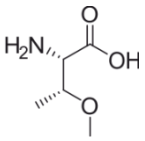
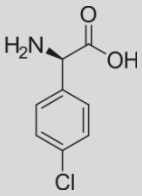
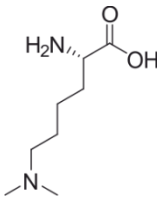
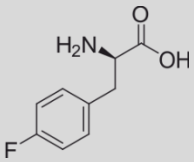
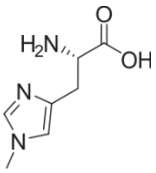
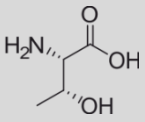
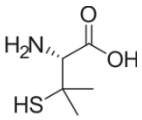
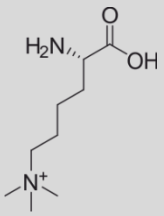
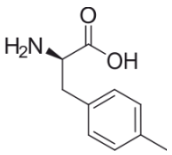
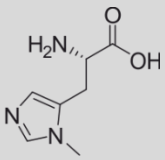
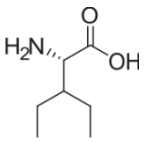
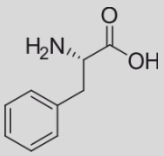
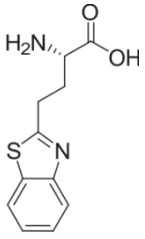
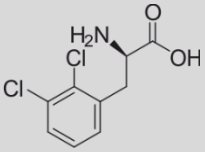
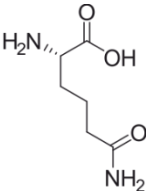
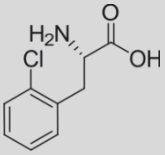
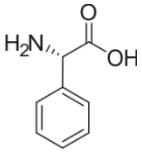
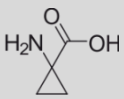
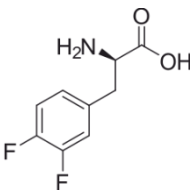
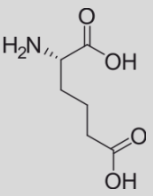
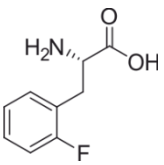
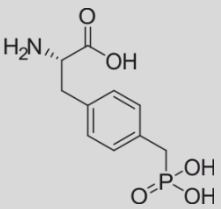
2 Supporting Tables

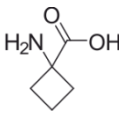
Table S1: Synthesized Peptides (with C-terminal amide). Amino acids are given in one-letter code.

Peptide	*N*-Term mod.[a]	Sequence	HPLC Grad.[b]	HPLC t_R/min	MF	Calc. m/z[c]	Found m/z	Purity[d]
1 (ESp)	F	QGLLDALDLAS	1	7.5	$C_{75}H_{105}N_{15}O_{25}S$	1649.8	1648.4	>98 %
	Ac	QGLLDALDLAS	8	11.3	$C_{50}H_{85}N_{13}O_{18}$	1157.3	1156.4	>98 %
2 (β_{SS}12)	F	QG-6$_S$-LD-6$_S$-LDLAS	2	12.2	$C_{84}H_{121}N_{15}O_{25}S$	1774.0	1773.7	96 %
	Ac	QG-6$_S$-LD-6$_S$-LDLAS	9	10.2	$C_{59}H_{101}N_{13}O_{18}$	1281.5	1280.0	>98 %
3 (β_{SS}12 S430A)	F	QG-6$_S$-LD-6$_S$-LDLAA	3	13.1	$C_{84}H_{121}N_{15}O_{24}S$	879.5	879.1	>98 %
4 (β_{SS}12 L428A)	F	QG-6$_S$-LD-6$_S$-LDAAS	3	7.4	$C_{81}H_{115}N_{15}O_{25}S$	866.5	866.1	95 %
5 (β_{SS}12 D427A)	F	QG-6$_S$-LD-6$_S$-LALAS	3	12.4	$C_{83}H_{121}N_{15}O_{23}S$	865.5	865.1	96 %
6 (β_{SS}12 L426A)	F	QG-6$_S$-LD-6$_S$-ADLAS	3	6.8	$C_{81}H_{115}N_{15}O_{25}S$	866.5	866.0	96 %
7 (β_{SS}12 D424A)	F	QG-6$_S$-LA-6$_S$-LDLAS	3	13.5	$C_{83}H_{121}N_{15}O_{23}S$	865.5	865.1	>98 %
8 (β_{SS}12 L423A)	F	QG-6$_S$-AD-6$_S$-LDLAS	3	6.8	$C_{81}H_{115}N_{15}O_{25}S$	866.5	866.0	97 %
9 (β_{SS}12 Q420A)	F	AG-6$_S$-LD-6$_S$-LDLAS	3	14.1	$C_{82}H_{118}N_{14}O_{24}S$	859.0	858.6	97 %
10 (β_{SS}12 Q420ldhw)	F	ldhw-G-6$_S$-LD-6$_S$-LDLAS	4	10.4	$C_{90}H_{125}N_{15}O_{24}S$	917.6	917.6	95 %
11 (β_{SS}12 Q420lmff)	F	lmff-G-6$_S$-LD-6$_S$-LDLAS	4	13.4	$C_{89}H_{123}FN_{14}O_{24}S$	1825.1	1824.9	95 %
12 (β_{SS}12 G421dbip)	F	Q-dbip-6$_S$-LD-6$_S$-LDLAS	5	14.0	$C_{97}H_{131}N_{15}O_{25}S$	1940.3	1939.9	>98 %
13 (β_{SS}12 G421dphe)	F	Q-dphe-6$_S$-LD-6$_S$-LDLAS	4	12.7	$C_{91}H_{127}N_{15}O_{25}S$	932.6	932.4	95 %
14 (β_{SS}12 L423lada)	F	QG-6$_S$-lada-D-6$_S$-LDLAS	6	11.2	$C_{90}H_{127}N_{15}O_{25}S$	926.6	926.5	95 %
15 (β_{SS}12 L423ltrp)	F	QG-6$_S$-WD-6$_S$-LDLAS	2	11.8	$C_{89}H_{120}N_{16}O_{25}S$	924.1	924.0	96 %
16 (β_{SS}12 D424lgln)	F	QG-6$_S$-LQ-6$_S$-LDLAS	2	11.7	$C_{85}H_{124}N_{16}O_{24}S$	1787.1	1786.8	>98 %
17 (β_{SS}12 D424rno2)	F	QG-6$_S$-L-rno2-6$_S$-LDLAS	2	14.7	$C_{86}H_{127}N_{19}O_{25}S$	1860.1	1859.7	>98 %
18 (β_{SS}12 A429dleu)	F	QG-6$_S$-LD-6$_S$-LDL-dleu-S	2	13.9	$C_{87}H_{127}N_{15}O_{25}S$	1816.1	1815.7	>98 %
19 (β_{SS}12 A429l2ce)	F	QG-6$_S$-LD-6$_S$-LDL-l2ce-S	5	6.1	$C_{87}H_{123}N_{15}O_{29}S$	1876.1	1876.7	95 %
20 (β_{SS}12 S430lmff)	F	QG-6$_S$-LD-6$_S$-LDLA-lmff	4	10.4	$C_{91}H_{126}FN_{15}O_{24}S$	933.6	933.3	>98 %
21 (β_{SS}12 S430l2ce)	F	QG-6$_S$-LD-6$_S$-LDLA-l2ce	5	8.8	$C_{87}H_{123}N_{15}O_{28}S$	1860.1	1859.8	95 %
22 (AdCe)	F	QG-6$_S$-lada-D-6$_S$-LDLA-l2ce	7	6.9	$C_{93}H_{129}N_{15}O_{28}S$	969.6	969.3	96 %
	Ac	QG-6$_S$-lada-D-6$_S$-LDLA-l2ce	7	7.1	$C_{68}H_{109}N_{13}O_{21}$	723.4	723.1	>98 %
23 (β_{SS}12 L428lnpt)	F	QG-6$_S$-LD-6$_S$-LD-lnpt-AS	6	7.3	$C_{85}H_{123}N_{15}O_{25}S$	1787.8	1787.5	96 %
24 (β_{SS}12 L428lhle)	F	QG-6$_S$-LD-6$_S$-LD-lhle-AS	5	10.6	$C_{85}H_{123}N_{15}O_{25}S$	1787.8	1788.7	96 %

[a] = F = FITC-Peg_2-, Ac = Acetylated; [b] = gradient 1: 10 % B to 90 % B in 10 min (3 min pre run 10 % B); gradient 2: 50 % B to 70 % B in 20 min; gradient 3: 50 % B to 70 % B in 10 min (3 min pre run 50 % B); gradient 4: 60 % B to 80 % B in 20 min; gradient 5: 50 % B to 95 % B in 10 min (3 min pre run 50 % B); gradient 6: 45 % B to 90 % B in 10 min (3 min pre run 45 % B); gradient 7: 60 % B to 80 % B in 20 min (3 min pre run 60 % B); gradient 8: 20 % B to 70 % B in 20 min; gradient 9: 40 % B to 70 % B in 20 min; [c] calculated molecular masses (m/z) for charged ions ($[M+1H]^{1+}$/$[M+2H]^{2+}$); [d] peptide purity was determined by RP-HPLC peak integration at λ = 210 nm.

Table S2: 241 amino acids (including four letter code) used to generate the virtual peptide library.

 1dbp	 df3f	 dval	 ldn3	 lpal
 1lbp	 df4c	 echx	 ldtm	 lpcl
 2mek	 df4f	 h1me	 ldtr	 lpen
 3mek	 df4m	 h3me	 leva	 lphe
 abbh	 dfdc	 hgln	 lf2c	 lphg
 ac3c	 dff2	 hglu	 lf2f	 lpmp



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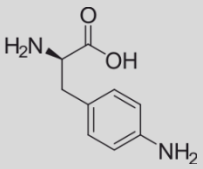

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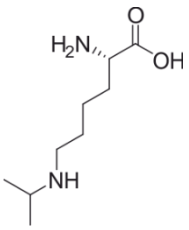

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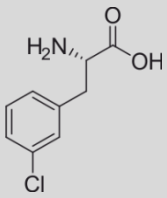

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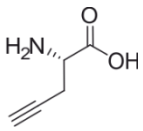

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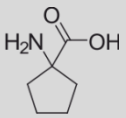

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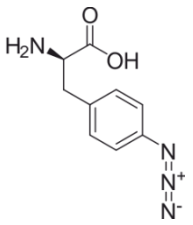

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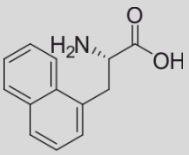

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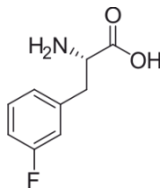

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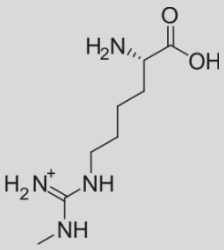

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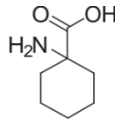

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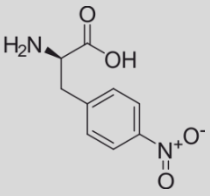

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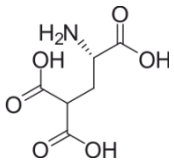

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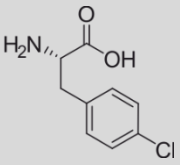

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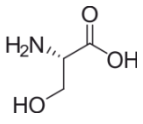

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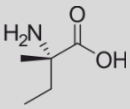

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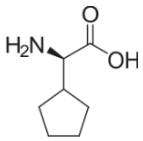

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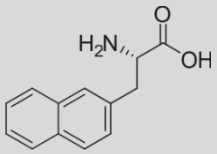

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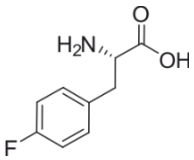

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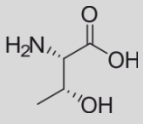

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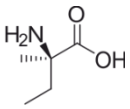

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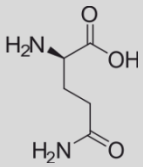

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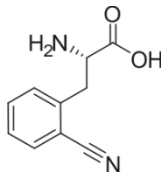

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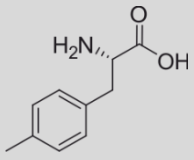

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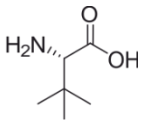

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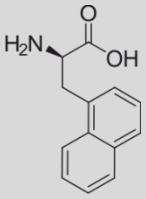

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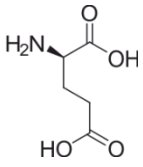

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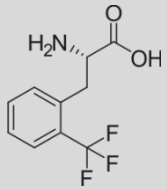

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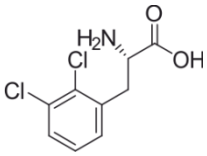

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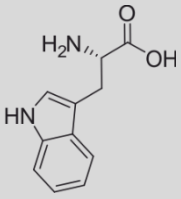

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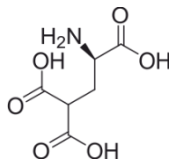
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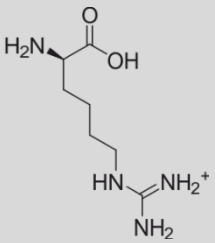
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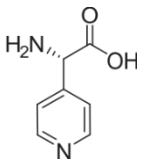
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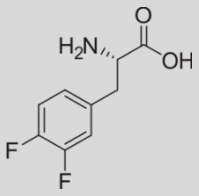
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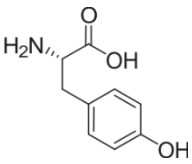
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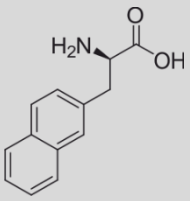
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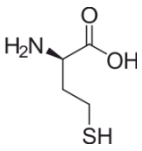
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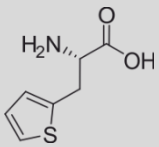
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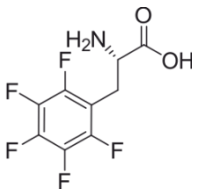
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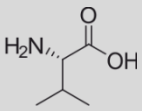
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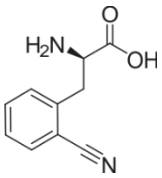
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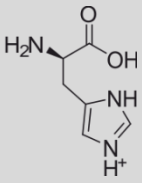
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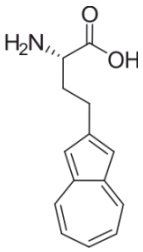
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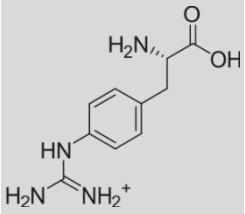
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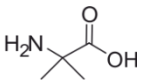
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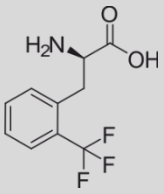
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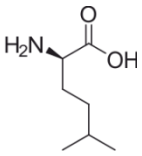
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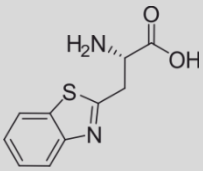
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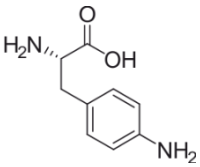
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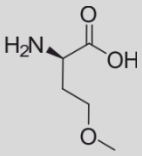
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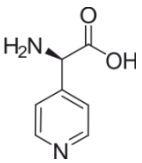
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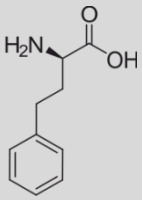
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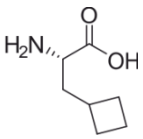
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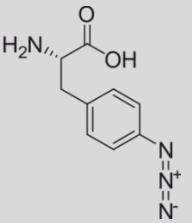
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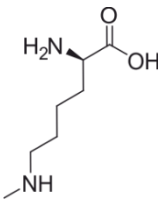
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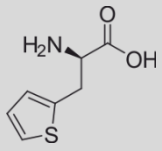
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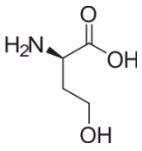
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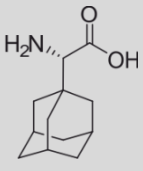
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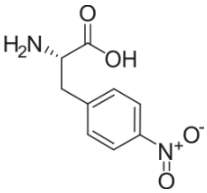
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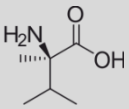
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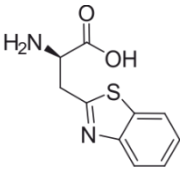
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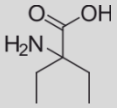
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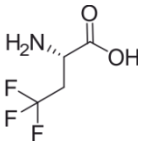
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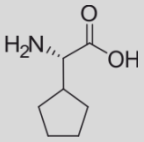
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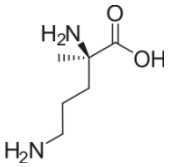
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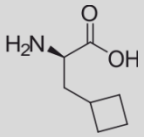
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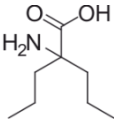
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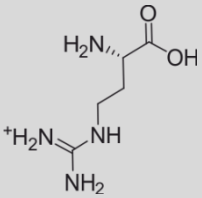
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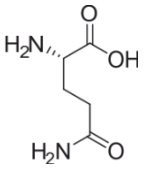
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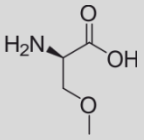
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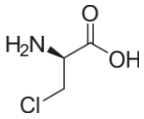
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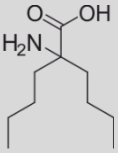
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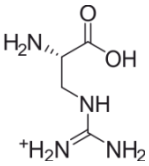
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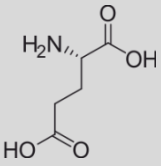
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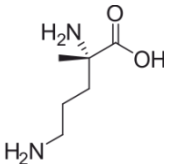
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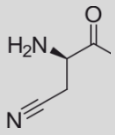
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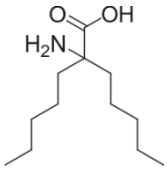
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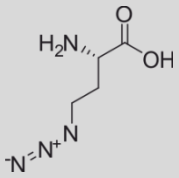
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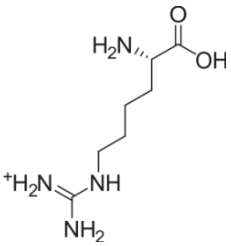
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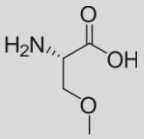
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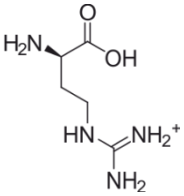
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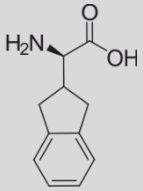
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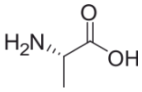
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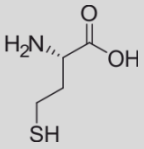
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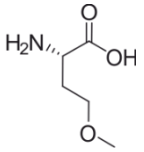
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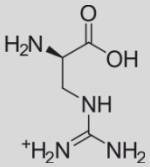
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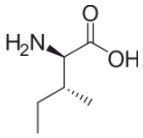
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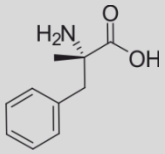
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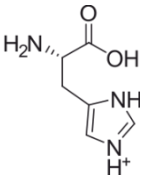
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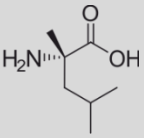
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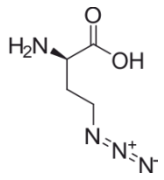

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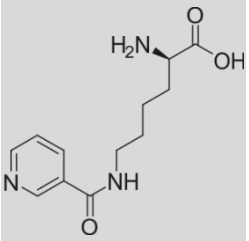

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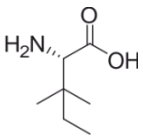

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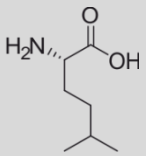

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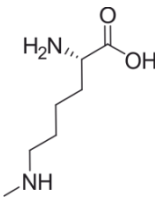

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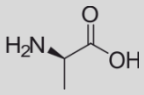

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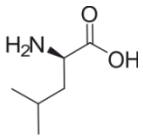

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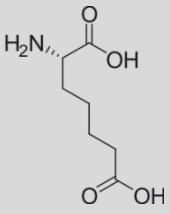

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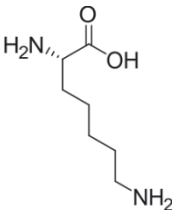

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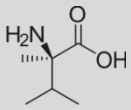

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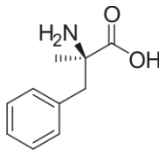

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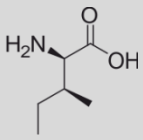

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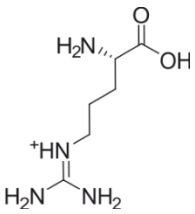

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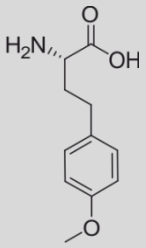

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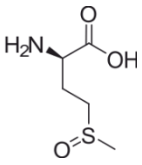

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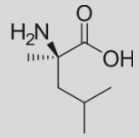

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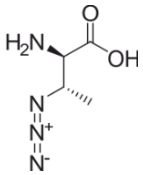

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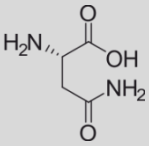

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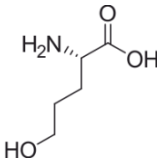

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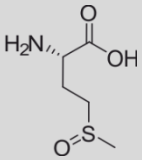

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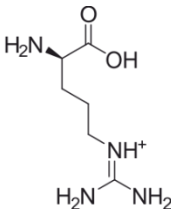

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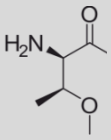

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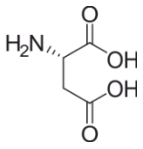

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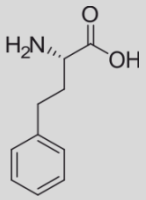

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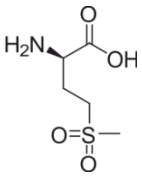

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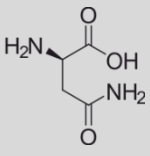

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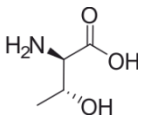

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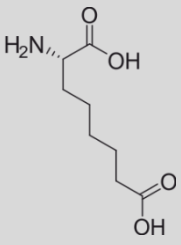

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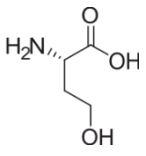

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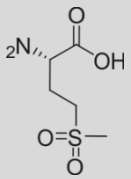

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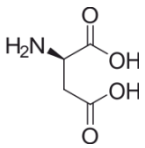

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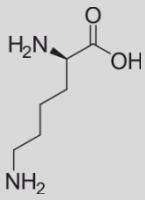

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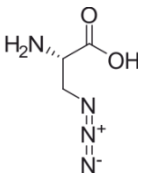

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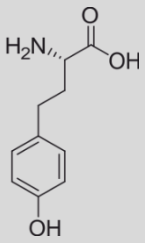

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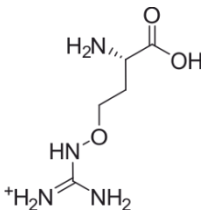

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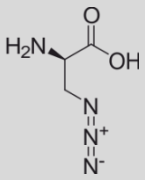

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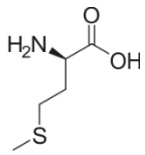

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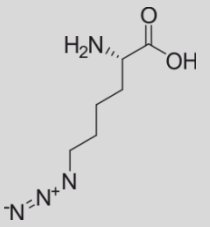

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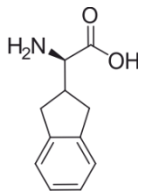

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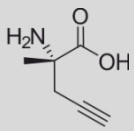

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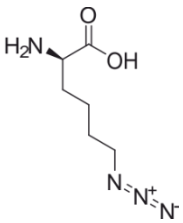

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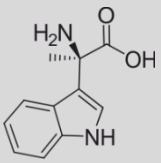

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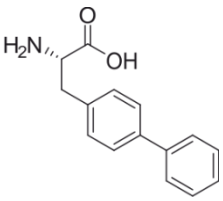

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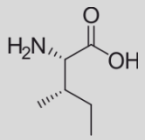

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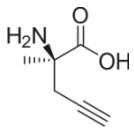

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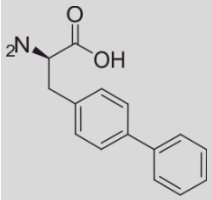

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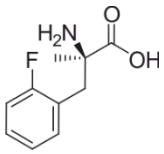

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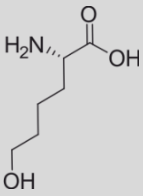

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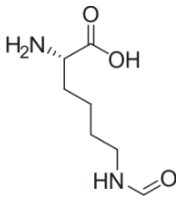

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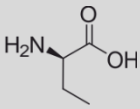

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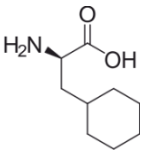

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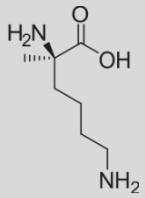

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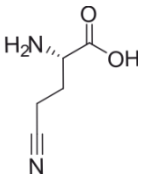

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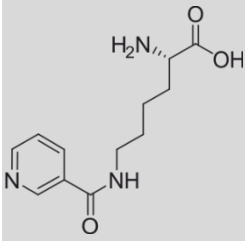

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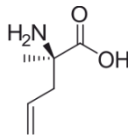

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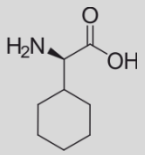

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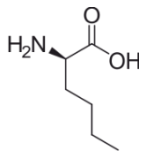

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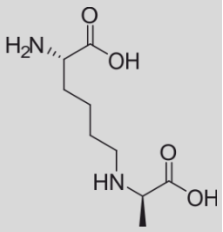

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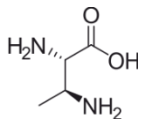

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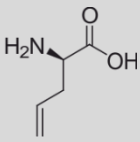

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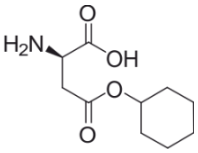
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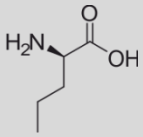
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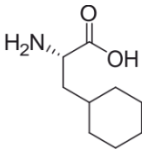
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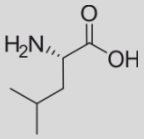
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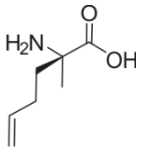
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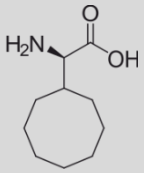
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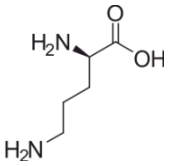
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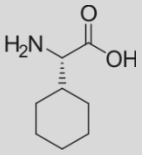
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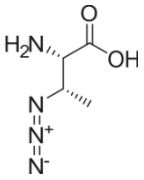
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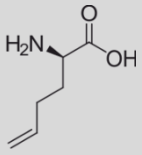
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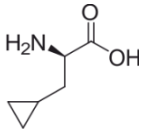
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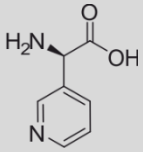
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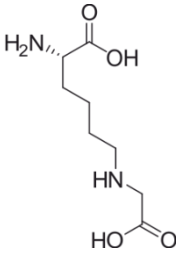
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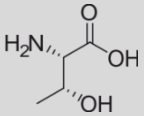
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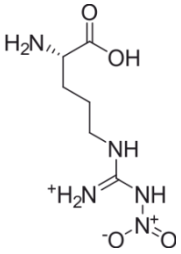
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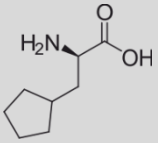
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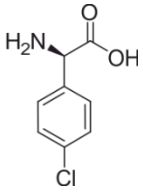
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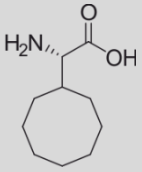
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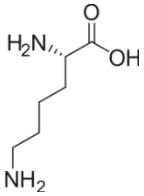
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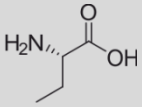
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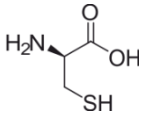
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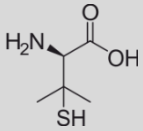
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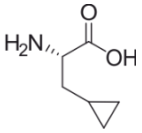
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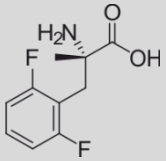
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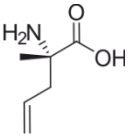
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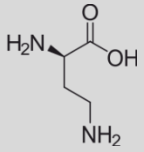
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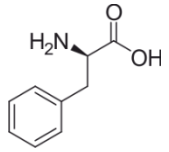
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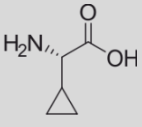
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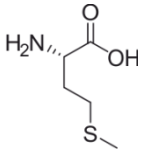
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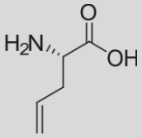
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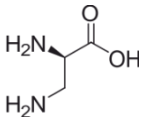
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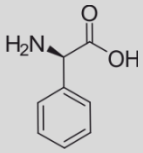
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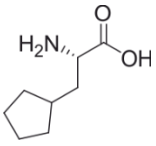
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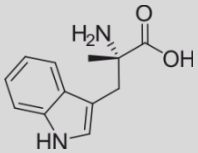
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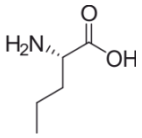
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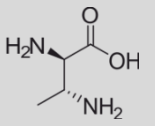
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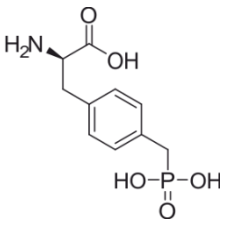
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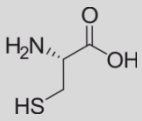
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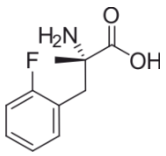
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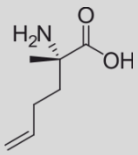
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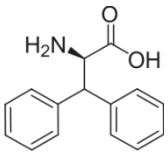
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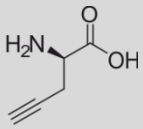
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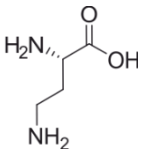
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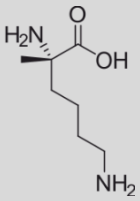
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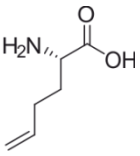
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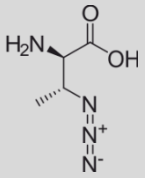
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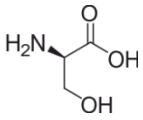
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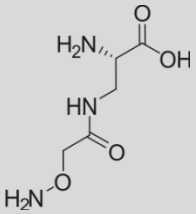
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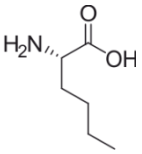
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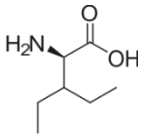
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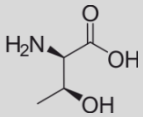
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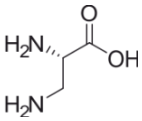
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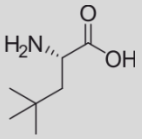
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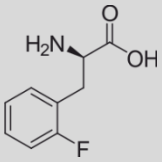
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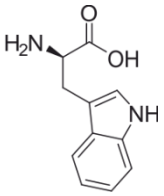
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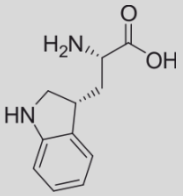
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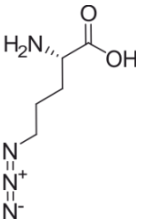
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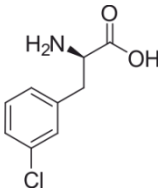
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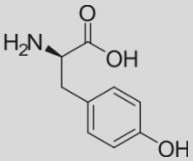
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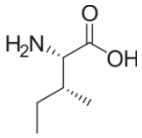
ldil

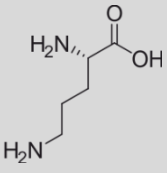
lorn

Table S3: Data collection and refinement statistics for 14-3-3/**22** complex (PDB ID: 5jm4).

	14-3-3 ζ/**22** (PDB:5mj4)
Data collection	
Space group	$P2_12_12_1$ (19)
Cell dimensions	
a, *b*, *c* (Å)	84.92, 105.74, 113.93
α, β, γ (°)	90.00, 90.00, 90.00
Resolution (Å)	47.31-2.34(3.26-2.34)
R_{meas} / $CC_{(1/2)}$	10.8(85.2) / 99.9 (59.8)
I / σI	12.52(2.78)
Completeness (%)	99.90(100.00)
Redundancy	6.52(6.51)
Refinement	
Resolution (Å)	47.31-2.34(3.26-2.34)
No. reflections	41755
R_{work} / R_{free}	0.1980/0.2436 (0.3450/0.382)
No. atoms	
Protein	3534
Ligand/ion	194
Water	103
B-factors	
Protein	57.24
Ligand/ion	62.24
Water	56.38
R.m.s. deviations	
Bond lengths (Å)	0.0190/0.020
Bond angles (°)	2.0200/1.994
Ramachandran	
Preferred Regions (%)	94.79
Allowed Regions (%)	5.21
Outliers (%)	0.00

*Values in parentheses are for highest-resolution shell.

Table S4: Filter Set-up for Fluorescence Microscopy. G: green excitation, BP: band pass, DMR: dichroic mirror, FT: color splitter, HE: high efficiency.

	DAPI	FITC
Excitation	G365	BP500/25 DMR25
Beamsplitter	FT395	FT515HE
Emission	BP445/50	BP535/50 DMR25

3 Supporting Figures

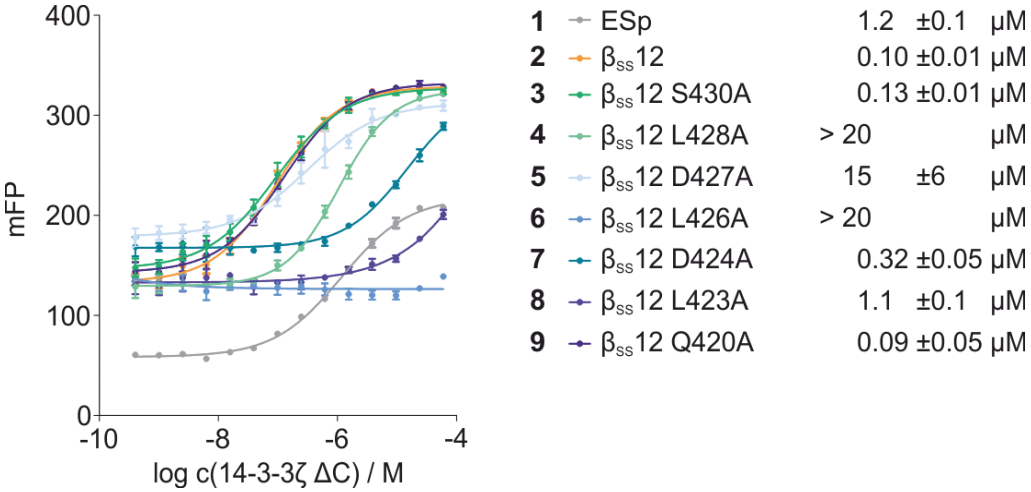

Figure S1: FP binding curves and K_D-values of peptides for alanine scanning (peptides **3** – **9**) (triplicate of runs, errors account for 1 σ).

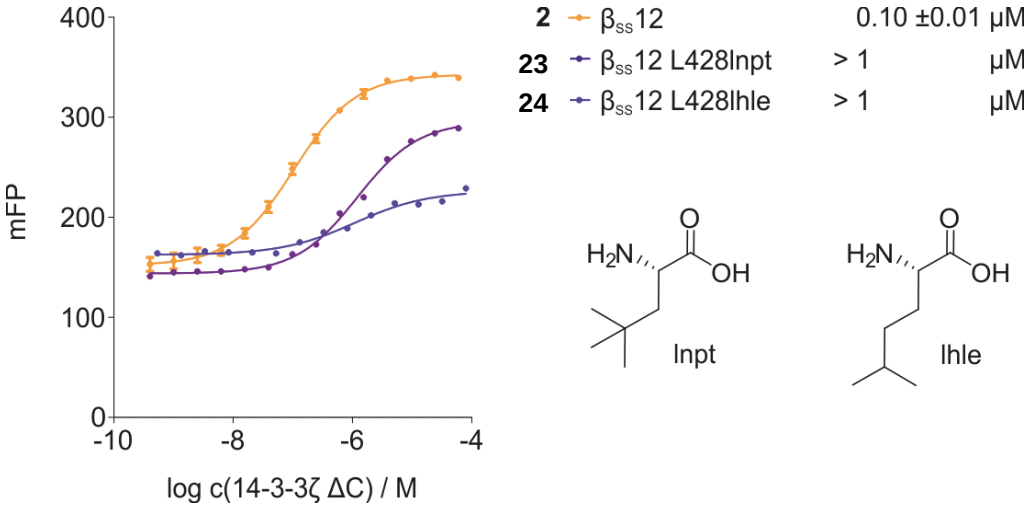

Figure S2: FP binding curves and K_D-values of peptides with hot spot variations (triplicate of runs, errors account for 1σ; binding curves for **23** β_{SS}12 L428lnpt and **24** β_{SS}12 L428lhle were determined as single measurements).

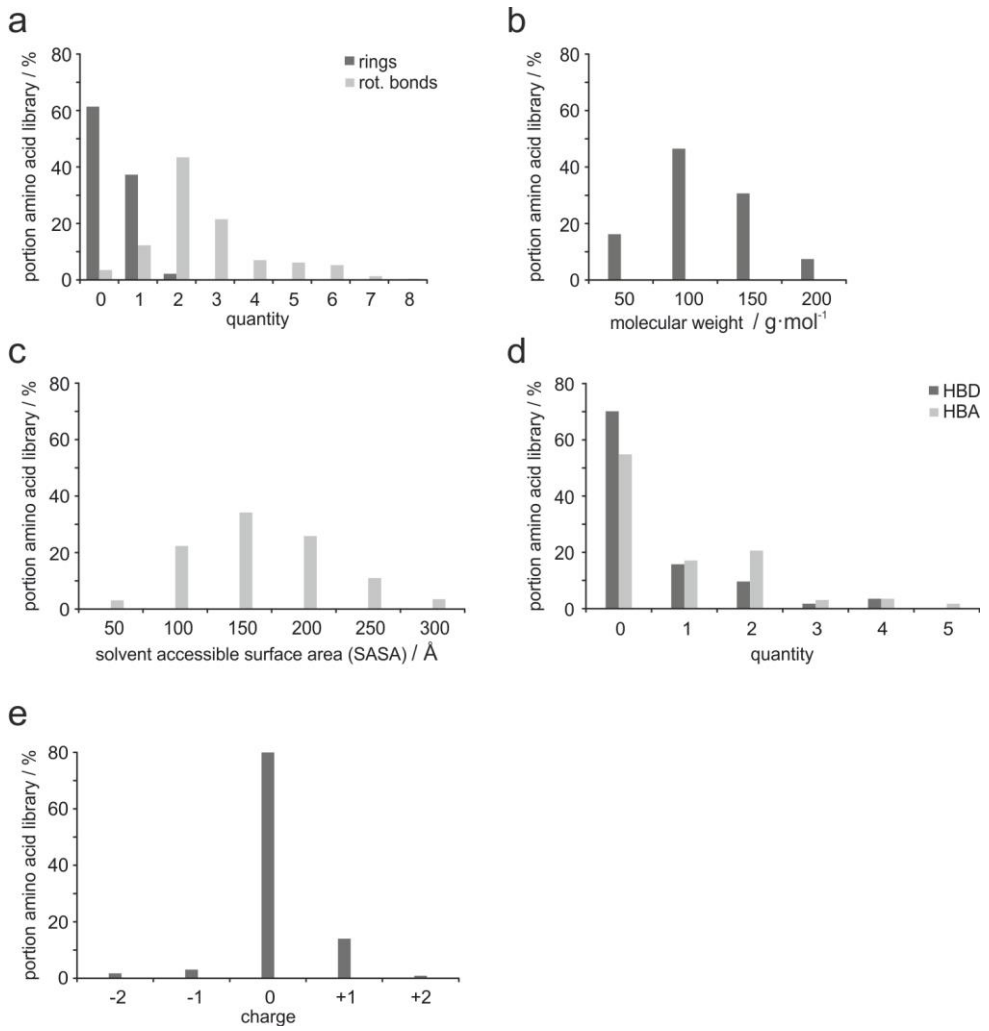

Figure S3: Distribution of physicochemical properties of residues in the amino acid library. a) The number of rotatable bonds (within the side chain) ranges from 0 to 8 with a peak at 40% of the residues containing 2 rotatable bonds. Around 40% of the residues are aromatic, they contain at least one ring system. b) Molecular weight ranges from 50 to 200 Da. c) Solvent accessible surface area (SASA) ranges from 50 to 300 $Å^2$, respectively. d) Polarity: Around 50% of the residues containing at least one H-bond acceptor (HBA) and around 30% containing at least one H-bond donor (HBD). e) Around 80% of the residues are uncharged, 15% are positively charge and 5% are negatively charged.

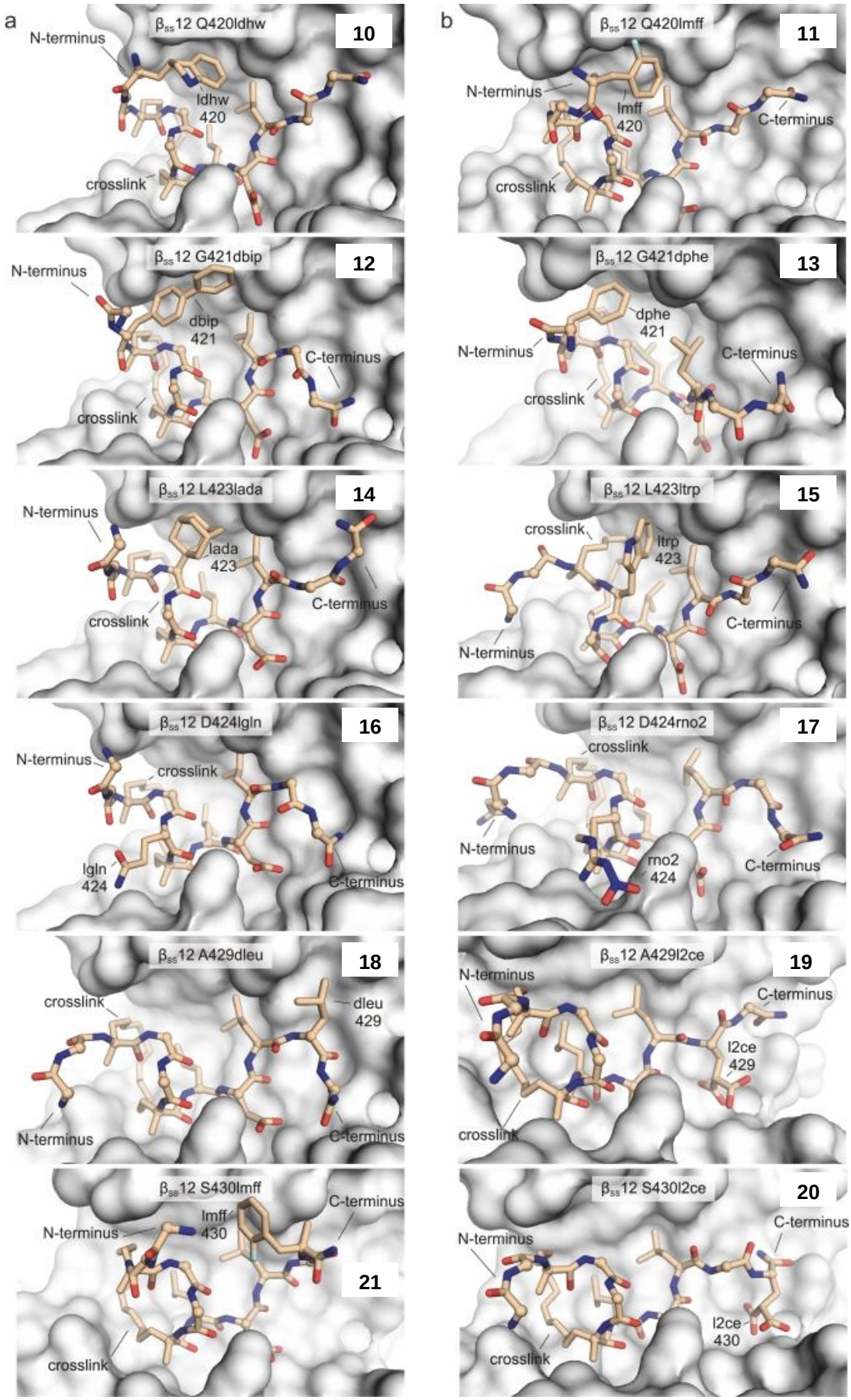

Figure S4: 12 selected peptides **10** – **21** in their highest scored binding mode (a = ChemScore; b = ASP). Atomic coordinates are available as supporting data.

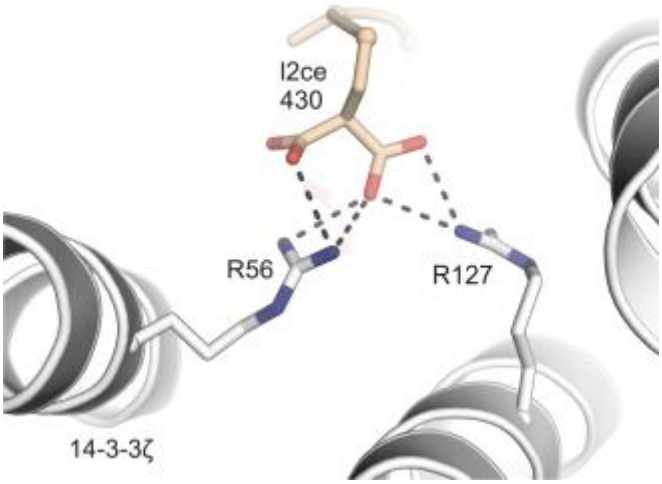

Figure S5: Highest scored binding mode for **21** (β_{SS}12 S430l2ce) with close up on predicted interaction between l2ce (orange) and 14-3-3 (grey).

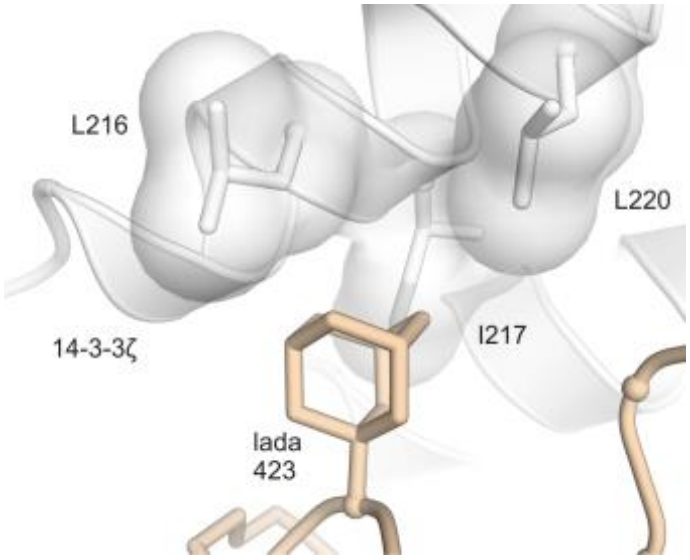

Figure S6: Highest scored binding mode for **14** (β_{SS}12 S423lada) with zoom on hydrophobic interface between lada (orange) and 14-3-3 (grey).

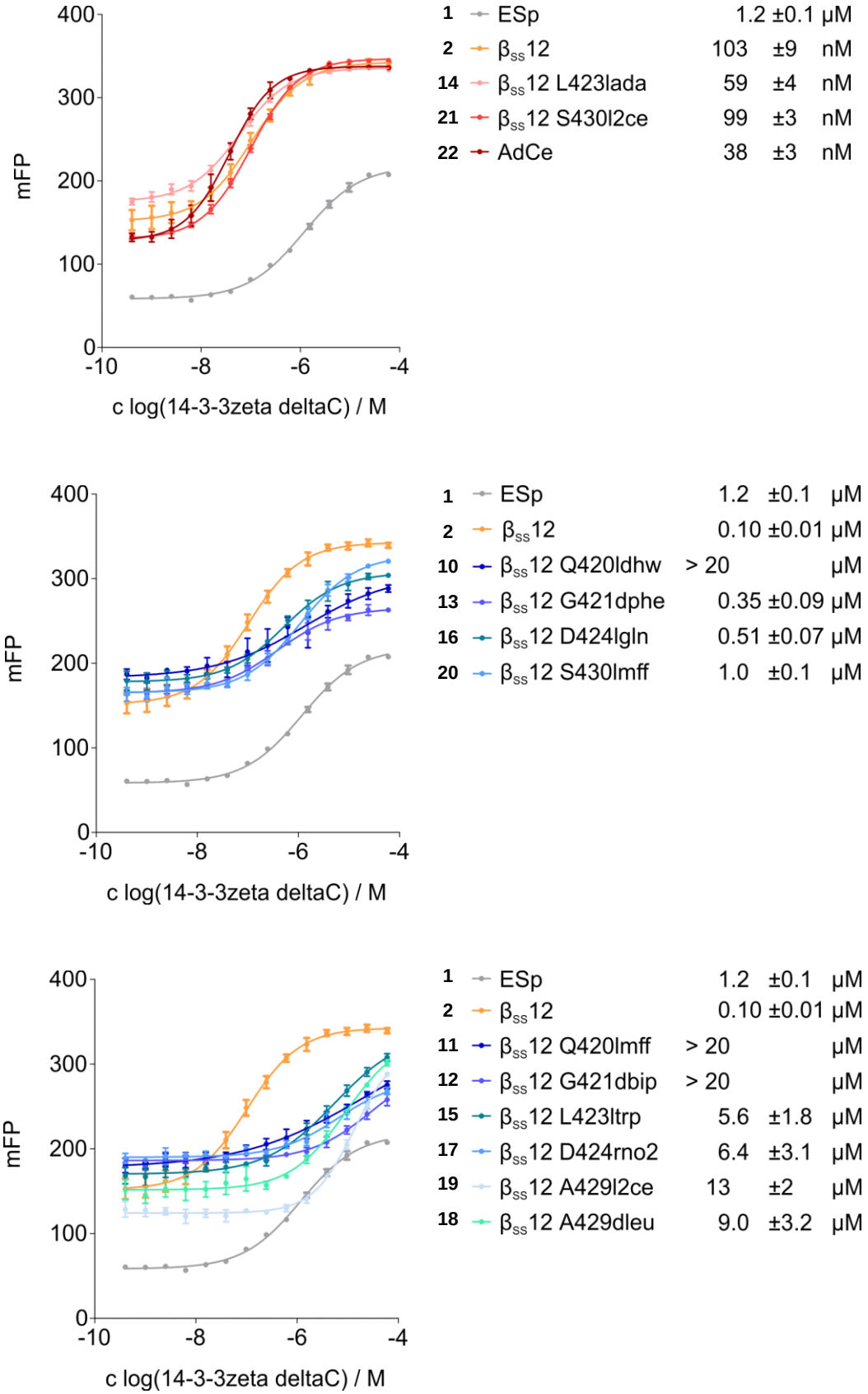

Figure S7: FP binding curves and K_D-values of peptides **10** – **21** with suggested single amino acid subsitutions and of **1**, **2** and **22** (triplicate of runs, errors account for 1 σ).

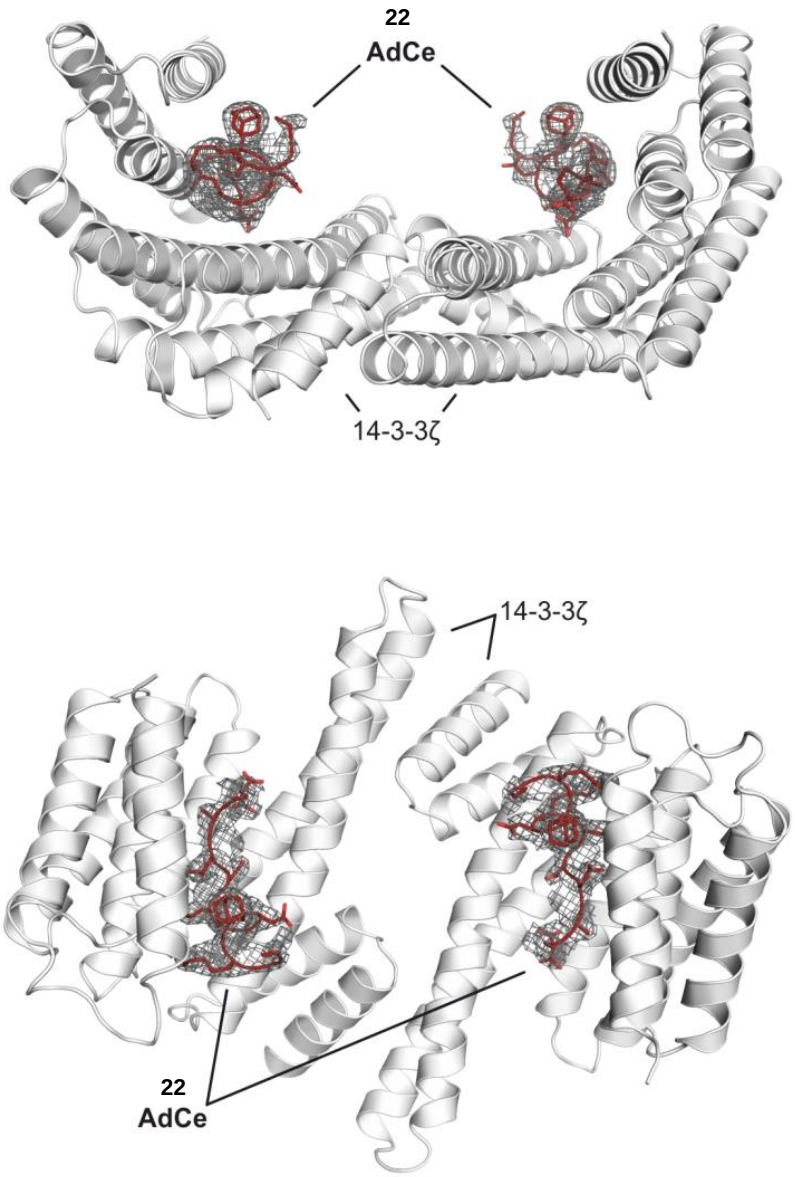

Figure S8: Overview of crystal structure of **22** (including $2F_o$-F_c electron density map) bound to 14-3-3ζ.

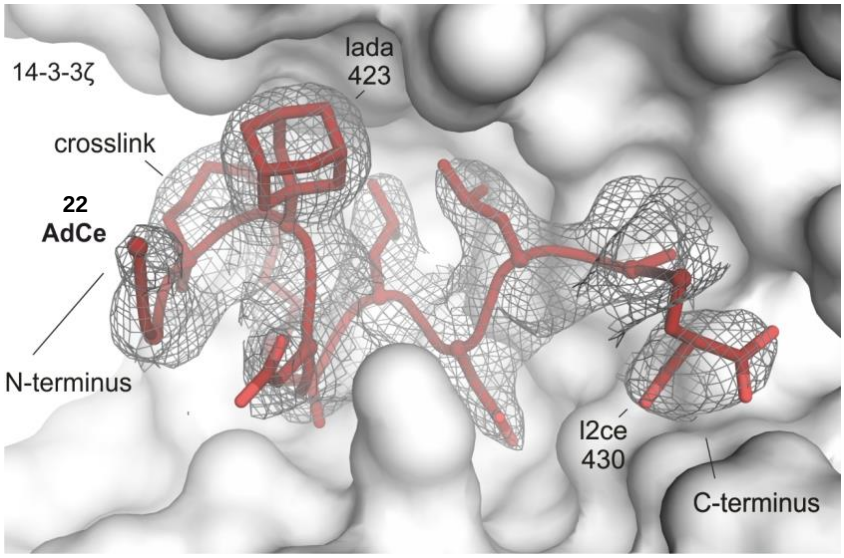

Figure S9: $2F_o$-F_c electron density map of **22** bound to 14-3-3ζ.

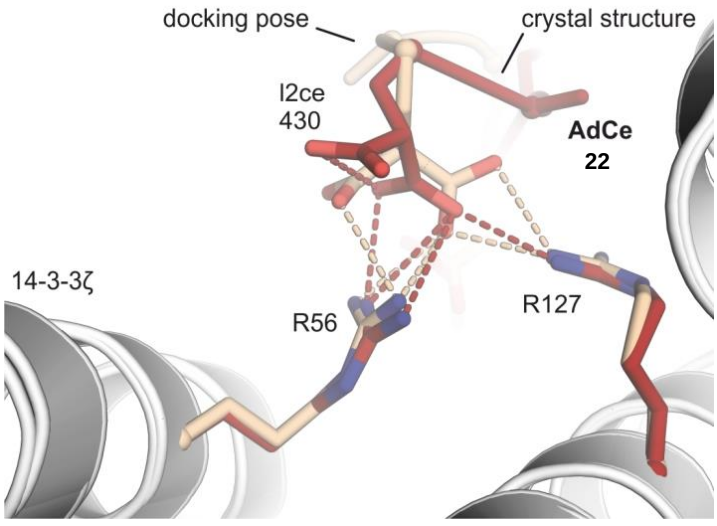

Figure S10: Overlay of crystal structure of **22** (red, PDB 5jm4) with docking pose (wheat) of l2ce in **21** interacting with two arginine residues (R56, R127) in 14-3-3ζ.

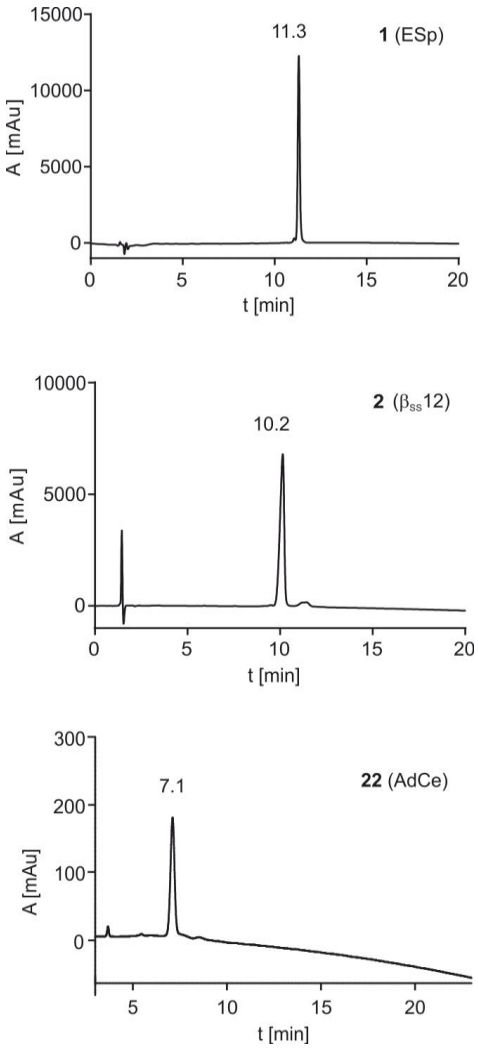

Figure S11: HPLC chromatograms of peptides used in competition and cell-based assays

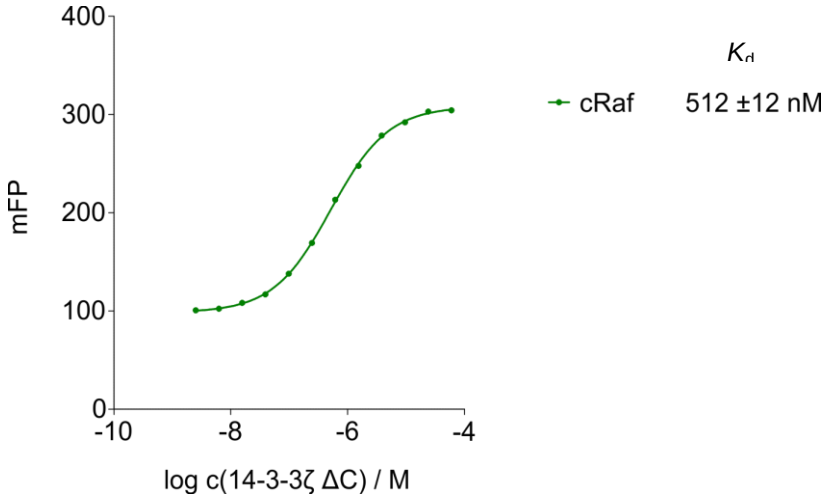

Figure S12: FP binding curves achieved for TAMRA-labeled cRaf (TAMRA-LSQRQRST{pSer}TPNVHM; purchased from *GenScript*; triplicate of runs, errors account for 1 σ).

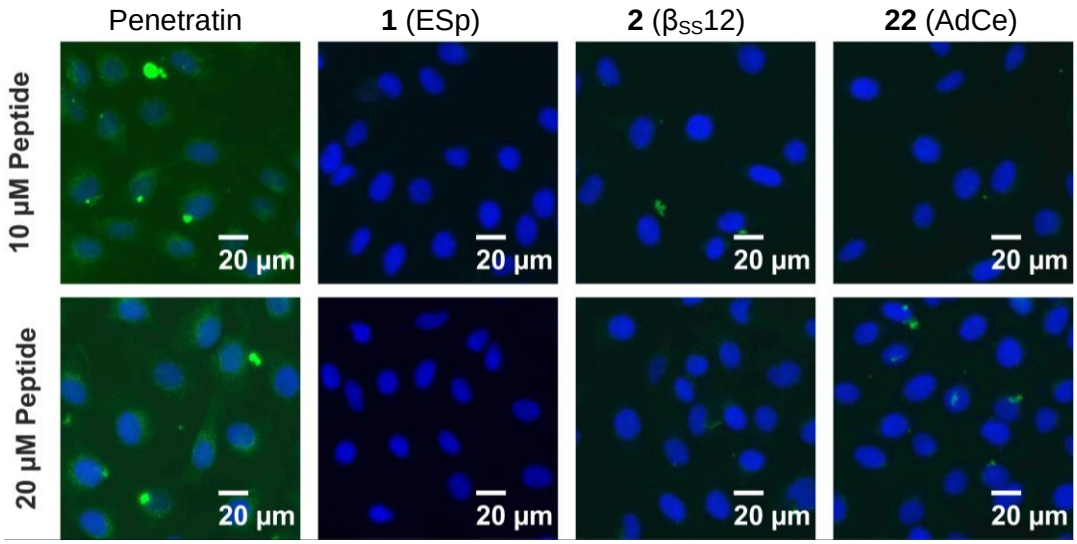

Figure S13: Wide field fluorescence microscopy depicting HeLa cells incubated with 10 and 20 µM FITC-labeled peptide (**1**, **2**, **22**) for 4 h. Pictures were taken using a 20x air lens. Scale bars represent 20 µm (blue = nuclear staining (DAPI), green = FITC-labeled peptide).

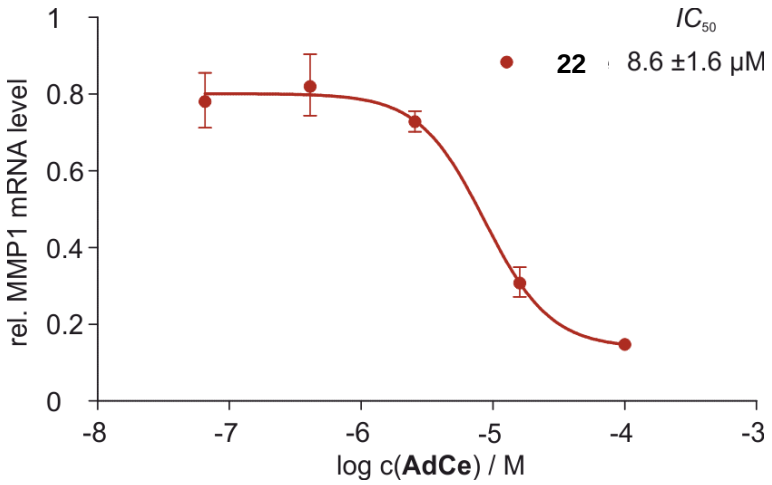

Figure S14: Quantitative real time PCR analysis. Dose-dependent **22** (AdCe**)** inhibition of 14-3-3ζ-induced MMP1 transcription (relative to DMSO treated control, triplicate, errors account for 1 σ).

4 Supporting References

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(3) Trott, O.; Olson, A. J. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. *J. Comput. Chem.* **2010,** *31* (2), 455-461.

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