

## Oligoalanine Helical Callipers for Cell Penetration

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### Supporting information

#### Table of contents:

Materials and methods .....	S2
Abbreviations .....	S3
General protocols.....	S3
General protocol for the SPPS.....	S3
General protocol for peptide cleavage and purification.....	S4
Synthesis of peptide <b>P</b> <sub>1</sub> .....	S4
Synthesis of peptide <b>P</b> <sub>2</sub> .....	S4
Synthesis of peptide <b>P</b> <sub>3</sub> * .....	S5
Synthesis of peptide <b>P</b> <sub>4</sub> .....	S5
Synthesis of peptide <b>P</b> <sub>5</sub> * .....	S5
Synthesis of peptide <b>P</b> <sub>6</sub> * a) and <b>P</b> <sub>7</sub> b).....	S5
Synthesis of peptide <b>P</b> <sub>8</sub> .....	S6
Synthesis of peptide <b>R</b> <sub>8</sub> .....	S6
General protocol to measure Circular Dichroism .....	S6
Cell assays.....	S7
Cell culture and live cell imaging.....	S7
Quantification of the uptake by flow cytometry.....	S7
Quantification of inhibition of the uptake by flow cytometry .....	S8
Cell viability .....	S8
Peptide stability in serum.....	S9
Supporting figures.....	S10
Supporting figures of Characterization .....	S20
Supporting references.....	S26

## Materials and methods

Commercially available Rink Amide-resin ChemMatrix, Fmoc-*L*-Ala-OH, Fmoc-*L*-Leu-OH, Fmoc-*L*-Arg(pbf)-OH, triisopropylsilane (TIS), Diisopropylethyl amine (DIEA), diisopropylcarbodiimide (DIC) were obtained from Sigma-Aldrich. Trifluoroethanol, 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP) was purchased from TCI. Egg yolk *L*- $\alpha$ -phosphatidylcholine was purchased from Avanti Polar Lipids. Ethyl(hydroxyimino)cyanoacetate (Oxyma) and 5-carboxytetramethyl rhodamine (TAMRA) were available from Carbosynth. *N*-HATU by Glenham life sciences. *N*-HBTU was obtained from Iris. Peptide synthesis grade *N,N*-dimethylformamide was purchased from Scharlau. Deuterated solvent (D<sub>2</sub>O) was from EMD Millipore Corporation. All the other solvents were HPLC grade, purchased from Sigma-Aldrich® or Fisher Scientific®, and used without further purification.

Chlorpromazine was purchased from TCI Chemicals. Ammonium chloride, Chloroquine, Heparin sodium salt and 5-(*N*-Ethyl-*N*-isopropyl)amiloride were purchased from Sigma-Aldrich. Methyl- $\beta$ -cyclodextrin was purchased from Carbosynth. Hoechst 33342 Trihydrochloride Trihydrate, and LysoTracker Deep Red were purchased in ThermoFisher. Wortmannin was obtained from Fluorochem. Dulbecco's Modified Eagle's Medium (4500 mg/L glucose, *L*-glutamine, sodium pyruvate and sodium bicarbonate) was purchased from Gibco.

A microwave assisted peptide synthesiser (Liberty Lite, CEM) was used to prepare the peptide according to standard methods developed by the manufacturers involving diisopropylcarbodiimide (DIC) 0.5 M in DMF as activator and ethyl(hydroxyimino)cyanoacetate (Oxyma) 1 M in DMF as activator base. Previous to HPLC, a quick size exclusion chromatography in sephadex G-25 eluted with Milli-Q water was carried out to remove the excess of fluorophore. High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) analyses were carried out on Agilent Technologies 1260 Infinity II associated with a 6120 Quadrupole LC-MS using an Agilent SB-C18 column or on DIONEX Ultimate 3000 U-HPLC<sup>+</sup> (Thermo Scientific) with an Acclaim RSLC 120-C18 column with *Solvent A:Solvent B* gradients between 5:95 (*Solvent A*: H<sub>2</sub>O with 0.1% TFA; *Solvent B*: CH<sub>3</sub>CN with 0.1% TFA). High-performance liquid chromatography (HPLC) semi-preparative purification was carried out on Jasco LC-4000 with an Agilent Eclipse XDB-C18 column.

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian Mercury 300 MHz. Chemical shifts are reported in ppm referenced to the following solvent signals: HOD  $\delta$  H 4.79 ppm. Spin multiplicities are reported as a singlet (s), doublet (d), triplet (t) with coupling constants (*J*) given in Hz, or multiplet (m). Accurate mass determinations (HR-MS) using ESI-MS were performed on a Bruker MicroTof mass spectrometer.

Circular Dichroism (CD) measurements were performed with a Jasco J-1100 CD Spectrometer equipped with a Jasco MCB-100 Mini Circulation Bath for temperature control.

For the acquisition of cell microscopy images an Andor Zyla 4.2 digital camera mounted on a Nikon Eclipse Ti-E epifluorescence microscope was used, and to do the images in 3D a Dragonfly confocal spinning-disk on a Nikon Eclipse Ti-E equipped with a Andor Zyla 4.2 PLUS sCMOS digital camera. The



3D projection was obtained from the different individual confocal planes with Imaris bitplane © 9.0.0 software.

A Tecan Infinite F200Pro microplate reader was used to measure directly in Costar cell culture 96-well plates UV-Vis absorbance for the MTT viability assays.

Flow cytometry was performed on a Guava easyCyte™ cytometer. Data analysis was performed with InCyte software included in GuavaSoft 3.2 (Millipore).

## Abbreviations

Aa: Amino acid; Arg: Arginine; Calcd: Calculated; CLQ: Chloroquine; CPZ: Chlorpromazine; DCM: Dichloromethane; DIC: diisopropylcarbodiimide; DIEA: *N,N*-Diisopropylethylamine; DMF: *N,N*-Dimethylformamide; DYN: Dynasore; EIPA: 5-(*N*-Ethyl-*N*-isopropyl)amiloride; EYPC: Egg yolk phosphatidylcholine; Hep: Heparin sodium salt; HFIP: 1,1,1,3,3,3-Hexafluoro-2-propanol; HRMS (ESI): High resolution mass spectrometry (electrospray ionization); Leu: Leucine; Lys: Lysine; MbCD: Methyl-beta-Cyclodextrin; Mtt: 4-Methyltrytil; *N*-HATU: *N*-[(Dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridine-1-ylmethylene]-*N*-methylmethanaminium-hexafluorophosphate *N*-oxide; *N*-HBTU: *N*-[(1*H*Benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; Pbf: 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl; SPC: spacer; SPPS: solid phase peptide synthesis; TAMRA: 5-carboxytetramethyl rhodamine; TFE: Trifluoroethanol; TNBS: 2,4,6-Trinitrobenzenesulfonic acid; TIS: Triisopropylsilane; W: Wortmannin; 6Ahx: 6-aminohexanoic acid.

## General protocols

### General protocol for the SPPS

All peptides were synthesized by automated or manual Fmoc solid-phase peptide synthesis<sup>S1</sup> using Rink Amide ChemMatrix resin (loading 0.5 mmol/g). For manual synthesis, the resin (0.5 mmol) was swelled in DMF (peptide synthesis grade, 2 mL) for 20 min in a peptide synthesis vessel prior synthesis. Coupling cycle consisted of the removal of Fmoc protecting group with a solution of piperidine in DMF (20%, 2 mL) for 10 min and then the mixture was filtered and the resin was washed with DMF (3 x 2 mL, 1 min). The amino acid coupling was carried out by treatment with a solution of  $\alpha$ -amino acids (4 equiv.), *N*-HBTU (3.95 equiv.) in DMF (2 mL), which was mixed with DIEA (0.195 M solution in DMF, 1.2 equiv.) 1 min before the addition and the resulting mixture was shaken by bubbling Ar for 15 min. Finally, the resin was washed with DMF (3 x 2 mL, 1 min). The efficiency of each amino acid coupling and deprotection was monitored employing the TNBS test.<sup>S2</sup>

For automated synthesis, a variant of the previous protocol was used instead, according to manufacturer's recommendations. 0.05 mmol of Rink Amide resin was placed into the peptide synthesiser reaction vessel, swollen in DMF, followed by cycles of Fmoc cleavage with piperidine 20% in DMF, washings (3 x 5 mL), then amino acid (5 equiv. 2M amino acid solution in DMF), DIC (10 equiv.) and Oxyma (10 equiv.) were

added into the reaction vessel and microwaved for 5 min under temperature control followed by washings (3 x 5 mL). All steps were performed under nitrogen atmosphere. After the linear peptide was finished the resin was transferred to a different reaction vessel to perform the peptide modification manually.

- A) Linker coupling:** after Fmoc cleavage with piperidine/DMF (20%, 2 mL), the linear peptide was treated with a solution of N-Fmoc-6-aminohexanoic acid (4 equiv.), *N*-HBTU (3.95 equiv.) and DIEA (0.195 M solution in DMF, 1.2 equiv.) in DMF.
- B) Fluorophore coupling:** the Fmoc-protecting group of the lineal final peptide (or the previously attached linker) was removed by using a solution of piperidine in DMF (20%, 4 mL) for 15 min and the resin was washed with DMF (3 x 3 mL). The coupling was carried out by the addition of a solution of 5-Carboxytetramethylrhodamine (1 equiv.), HATU (1 equiv.), and DIEA (0.195 M, 1 equiv.) in DMF (2 mL) and the mixture was stirred by bubbling Ar for 4 hours. Finally, the resin was washed with DMF (3 x 3 mL) and DCM (3 x 3 mL).

### General protocol for peptide cleavage and purification

Finally, peptides were deprotected and cleaved from the resin by standard TFA cleavage procedure at rt by using the TFA/DCM/H<sub>2</sub>O/TIS (90:5:2.5:2.5, 3 mL per 70 mg of resin) for 2 h. Then, the mixture was filtered, washed with TFA (1 mL) and the peptide was precipitated with ice-cold Et<sub>2</sub>O (50 mL). The precipitate was centrifuged and dissolved in H<sub>2</sub>O (5 mL).

Peptides were purified first with sephadex size exclusion chromatography (G-25, Milli-Q water) and then by semi-preparative high-performance liquid chromatography (HPLC) as previously described. Finally, the corresponding fractions were lyophilised to afford the pure peptide as pink solids.

### Synthesis of peptide P<sub>1</sub>

Following the general protocol of the SPPS followed by fluorophore coupling, **P<sub>1</sub>** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 12%. *R<sub>t</sub>* 13.0 min [RP-HPLC Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (Fig. S12). **MS** (ESI, H<sub>2</sub>O): 911.9 (35, [M+2H]<sup>2+</sup>), 608.3 (100, [M+3H]<sup>3+</sup>), 456.5 (35, [M+4H]<sup>4+</sup>). **HRMS** (ESI): Calcd for C<sub>82</sub>H<sub>126</sub>N<sub>28</sub>O<sub>20</sub> [M+2H]<sup>2+</sup>: 911.4846; found: 911.4839.

### Synthesis of peptide P<sub>2</sub>

Following the general protocol of the SPPS followed by fluorophore coupling, **P<sub>2</sub>** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 10.7%. *R<sub>t</sub>* 14.6 min [RP-HPLC Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (Fig. S13). **MS** (ESI, H<sub>2</sub>O): 953.9 (40, [M+2H]<sup>2+</sup>), 636.3 (100, [M+3H]<sup>3+</sup>), 477.6 (35, [M+4H]<sup>4+</sup>). **HRMS** (ESI): Calcd for C<sub>88</sub>H<sub>138</sub>N<sub>28</sub>O<sub>20</sub> [M+2H]<sup>2+</sup>: 953.5317; found: 953.5316.

### Synthesis of peptide **P<sub>3</sub>\***

Following the general protocol of the SPPS followed by linker and fluorophore coupling, **P<sub>3</sub>\*** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 8.7%. *R<sub>t</sub>* 14.2 min. [RP-HPLC Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (Fig. S14). **MS** (ESI, H<sub>2</sub>O): 1088.8 (5, [M+2H+TFA]<sup>2+</sup>), 1031.4 (35, [M+2H]<sup>2+</sup>), 688.0 (100, [M+3H]<sup>3+</sup>), 516.3 (40, [M+4H]<sup>4+</sup>). **HRMS** (ESI): Calcd for C<sub>97</sub>H<sub>155</sub>N<sub>29</sub>O<sub>21</sub> [M+2H]<sup>2+</sup>: 1031.0976; found: 1031.0971.

### Synthesis of peptide **P<sub>4</sub>**

Following the general protocol of the SPPS followed by fluorophore coupling, **P<sub>4</sub>** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 6.5%. *R<sub>t</sub>* 14.9 min [RP-HPLC Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (Fig. S15). **MS** (ESI, H<sub>2</sub>O): 975.0 (70, [M+2H]<sup>2+</sup>), 650.3 (100, [M+3H]<sup>3+</sup>), 488.0 (15, [M+4H]<sup>4+</sup>). **HRMS** (ESI): Calcd for C<sub>91</sub>H<sub>144</sub>N<sub>28</sub>O<sub>20</sub> [M+2H]<sup>2+</sup>: 974.5551; found: 974.5550.

### Synthesis of peptide **P<sub>5</sub>\***

Following the general protocol of the SPPS followed by linker and fluorophore coupling, **P<sub>5</sub>\*** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 2.9%. *R<sub>t</sub>* 15.2 min [RP-HPLC Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (Fig. S16). **MS** (ESI, H<sub>2</sub>O): 1031.4 (38, [M+2H]<sup>2+</sup>), 688.0 (100, [M+3H]<sup>3+</sup>), 516.3 (55, [M+4H]<sup>4+</sup>). **HRMS** (ESI): Calcd for C<sub>97</sub>H<sub>155</sub>N<sub>29</sub>O<sub>21</sub> [M+2H]<sup>2+</sup>: 1031.0976; found: 1031.0886.

### Synthesis of peptide **P<sub>6</sub>\* a)** and **P<sub>7</sub> b)**

a) Following the general protocol of the SPPS followed by linker and fluorophore coupling, **P<sub>6</sub>\*** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 6.4%. *R<sub>t</sub>* 15.2 min [RP-HPLC Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (Fig. S17). **MS** (ESI, H<sub>2</sub>O): 1031.4 (40, [M+2H]<sup>2+</sup>), 688.0 (100, [M+3H]<sup>3+</sup>), 516.3 (50, [M+4H]<sup>4+</sup>). **HRMS** (ESI): Calcd for C<sub>97</sub>H<sub>155</sub>N<sub>29</sub>O<sub>21</sub> [M+2H]<sup>2+</sup>: 1031.0976; found: 1031.097.

b) Following the general protocol of the SPPS followed by fluorophore coupling, **P<sub>7</sub>** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 5.3%. *R<sub>t</sub>* 15.5 min [RP-HPLC Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (Fig. S18). **MS** (ESI, H<sub>2</sub>O): 974.8 (50, [M+2H]<sup>2+</sup>), 650.3 (100, [M+3H]<sup>3+</sup>), 488.1 (30, [M+4H]<sup>4+</sup>). **HRMS** (ESI): Calcd for C<sub>91</sub>H<sub>144</sub>N<sub>28</sub>O<sub>20</sub> [M+2H]<sup>2+</sup>: 974.5544; found: 974.5550.

## Synthesis of peptide P<sub>8</sub>

Following the general protocol of the SPPS followed by fluorophore coupling, **P<sub>8</sub>** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 1.7%. *R<sub>t</sub>* 15.2 min [RP-HPLC Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (Fig. S19). **MS** (ESI, H<sub>2</sub>O): 974.8 (40, [M+2H]<sup>2+</sup>), 650.3 (100, [M+3H]<sup>3+</sup>), 488.1 (23, [M+4H]<sup>4+</sup>). **<sup>1</sup>H NMR** (300 MHz, D<sub>2</sub>O) δ 8.53-8.45 (m, 1H), 8.18-8.10 (m, 1H), 7.57-7.47 (m, 1H), 7.21-7.10 (m, 2H), 6.96-6.86 (m, 2H), 6.68 (s, 2H), 4.38-4.15 (m, 16H), 3.30-3.10 (m, 18H), 1.94-1.59 (m, 22H), 1.55-1.35 (m, 30H), 1.05-0.84 (m, 18H). **HRMS** (ESI): Calcd for C<sub>91</sub>H<sub>144</sub>N<sub>28</sub>O<sub>20</sub> [M+2H]<sup>2+</sup>: 974.5555; found: 974.5550.

## Synthesis of peptide R<sub>8</sub>

Following the general protocol of the SPPS followed by fluorophore coupling, **R<sub>8</sub>** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 100:0→100:0 (0→5 min), 50:50→50:50 (5→35 min)] with an overall yield of 1.7%. *R<sub>t</sub>* 11.4 min (Fig. S20) [RP-HPLC Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20min)]. **MS** (ESI, H<sub>2</sub>O): 1067.7 (19, [M+2H+3TFA]<sup>2+</sup>), 1010.8 (10, [M+2H+2TFA]<sup>2+</sup>), 712.2 (45, [M+3H+3TFA]<sup>3+</sup>), 674.2 (100, [M+3H+2TFA]<sup>3+</sup>), 636.5 (75, [M+3H+TFA]<sup>3+</sup>), 598.2 (20, [M+3H]<sup>3+</sup>), 477.5 (40, [M+4H+TFA]<sup>4+</sup>), 448.9 (30, [M+4H]<sup>4+</sup>), 359.4 (27, [M+5H]<sup>5+</sup>). **HRMS** (ESI): Calcd for C<sub>79</sub>H<sub>131</sub>N<sub>36</sub>O<sub>13</sub> [M+H]<sup>+</sup>: 1792.0679; found: 1792.0691.

## General protocol to measure Circular Dichroism

Circular dichroism measurements were carried out with the following settings: acquisition range: 300-190 nm; band width: 1.0 nm; accumulation: 3 scans; data pitch: 1 nm; CD scale 200 mdeg/1.0 dOD; D.I.T. (Data Integration Time): 1 s; scanning mode: continuous; scanning speed: 200 nm/min. Measurements were done from 10 °C to 60 °C (data interval: 10 °C; temp. gradient 5 °C/min) in a quartz cell of 0.2 cm path length at a final volume of 0.5 mL (H<sub>2</sub>O or TFE) with a final peptide concentration of 100 μM. The results are expressed as the mean residue molar ellipticity [θ]<sub>MRT</sub> with units of degrees·cm<sup>2</sup>·dmol<sup>-1</sup> and calculated using the equation S1. The percentage of helicity was calculated using the equation S2. For the measurements in liposomes, samples were prepared by drying under reduced pressure L-α-phosphatidylcholine (16.7 μL, 25 mg/mL solution in CHCl<sub>3</sub>) and peptide (300 μL, 100μM) in TFE to obtain a ratio lipid/peptide of 18:1 in a 10 mL round bottomed flask. The mixture was concentrated in a rotary evaporator to dryness, forming a film. The lipid/peptide mixture were suspended in HKR buffer (300 μL) to a final peptide concentration 100 μM and sonicated for 45 min until a clear solution was obtained. Spectra were recorded in a 0.2 cm path length quartz cell.<sup>S3</sup>

$$[\Theta]_{\text{MRT}} = \frac{0.1 \times \Theta}{C \cdot l \cdot \text{number of residues}} \quad (\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}) \quad (\text{S1})$$

**Equation S1:** Formula to calculate the ellipticity. Θ is the ellipticity (mdeg), C is the peptide concentration (M) and l is the cell path length (cm).

$$[\Theta]\% = \frac{\Theta_{MRt_{222}} - 2340}{30300} \times 100 \quad (S2)$$

**Equation S2:** Formula to calculate the percent of helicity in where the molar ellipticity at 222 nm is an absolute value.

## Cell assays

### Cell culture and live cell imaging

HeLa cells were grown at 37 °C, 5% CO<sub>2</sub>, in Dulbecco's Modified Eagle's Medium (4500 mg/L glucose, L-glutamine, sodium pyruvate and sodium bicarbonate; SigmaAldrich), supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% of Penicillin-Streptomycin-Glutamine Mix.

SF9 Cells were grown at 28 °C, in mechanical stirring, in Gibco Sf-900-II medium, complemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% of Penicillin-Streptomycin-Glutamine Mix.

A-549 cells were grown at 37 °C, 5% CO<sub>2</sub>, in Dulbecco's Modified Eagle's Medium (4500 mg/L glucose, L-glutamine, sodium pyruvate and sodium bicarbonate; SigmaAldrich), supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% of Penicillin-Streptomycin-Glutamine Mix.

For live cell imaging, HeLa cells grown on glass bottom dishes were washed with HEPES-Krebs-Ringer (HKR) buffer (5 mM HEPES, 137 mM NaCl, 2.68 mM KCl, 2.05 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, pH 7.4) and incubated for 30 min with 1 µM Hoechst 33342 (ThermoFisher) in HKR to stain the nucleus. This solution was removed and cells were incubated for another 30 min with 0.5; 1; 3; 5 µM of each peptide diluted in HKR buffer, washed twice with HKR and examined on an epifluorescence microscope (Nikon Eclipse Ti-E).

For live cell imaging, SF9 cell grown on glass bottom dishes were washed with Sf-900-II medium incubated for 30 min with 1 µM Hoechst 33342 (ThermoFisher) in Sf-900-II to stain the nucleus. This solution was removed and cells were incubated for another 30 min with 1; 3; 5; 7 µM of each (Peptides) diluted in Sf-900-II medium, washed twice with Sf-900-II and examined on an epifluorescence microscope (Nikon Eclipse Ti-E).

For live cell imaging, A-549 cells grown on glass bottom dishes were washed with HEPES-Krebs-Ringer (HKR) buffer (5 mM HEPES, 137 mM NaCl, 2.68 mM KCl, 2.05 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, pH 7.4) and incubated for 30 min with 1 µM Hoechst 33342 (ThermoFisher) in HKR to stain the nucleus. This solution was removed and cells were incubated for another 30 min with 0.5; 1; 3; 5 µM of each peptide diluted in HKR buffer, washed twice with HKR and examined on an epifluorescence microscope (Nikon Eclipse Ti-E).

### Quantification of the uptake by flow cytometry

For internalization standard assays, we measured the amount of peptide by flow cytometry. Briefly, semiconfluent monolayers of HeLa cells seeded the day before were washed twice with HKR before incubation with different concentrations (1, 3, 5, 7, 10 µM) of TAMRA labelled peptides for 1 hour at 37

°C. Then, cells were trypsinized to remove membrane bound peptides<sup>S4,S5</sup> and to prepare the samples for flow cytometry using 100 µL of Trypsin-EDTA (Gibco) for 10 min at 37 °C, and trypsin neutralized with 100 µL of 2% FBS in PBS with 5 mM EDTA. Pipetting broke cell clumps and the analysis was done in a Guava easyCyte™ cytometer. TAMRA levels were determined by excitation at 532 nm and detection at 575/25 nm. For the analysis, cells with typical FSC and SSC parameters were selected, and median fluorescence intensity was calculated for each sample. Data analysis was performed with InCyte software included in GuavaSoft 3.2 (Millipore).

### **Quantification of inhibition of the uptake by flow cytometry**

To study the cellular uptake mechanisms, HeLa cells growing in a 96 well plate (100.000 cells per well seeded) were treated for 30 min with the following compounds diluted in DMEM without serum or antibiotics: Dynasore (80 µM), chlorpromazine (30 µM), methyl-β-cyclodextrin (5 mM), Wortmannin (200 nM), EIPA (50 µM), heparin (5 µg/mL), chloroquine (100 µM) or ammonium chloride (50 mM). For the incubation at low temperature, another plate was incubated on ice and ice-cold solutions were used for the washes and incubations. Cells were then incubated with the TAMRA-labelled peptides in the presence of the same amount of inhibitors in DMEM for 30 min at 37 °C. Cells were washed twice with HKR and 0.1 mg/mL of heparin in HKR and trypsinized. Trypsin was neutralized with 2% FBS in PBS with 5 mM EDTA and cell fluorescence was measured on a Guava EasyCyte™ cytometer using a green laser (532 nm) and collecting the emission at 575/25 nm. Cells with typical FSC and SSC parameters were selected and the median fluorescence intensity calculated for each sample (MFI). Each condition was done in triplicate. In the study of the uptake inhibition at different concentrations of the peptide, fluorescence values were normalized to the uptake of each untreated control (100%) after blank subtraction. In all cases, data analysis was performed with InCyte software included in GuavaSoft 3.2 (Millipore).

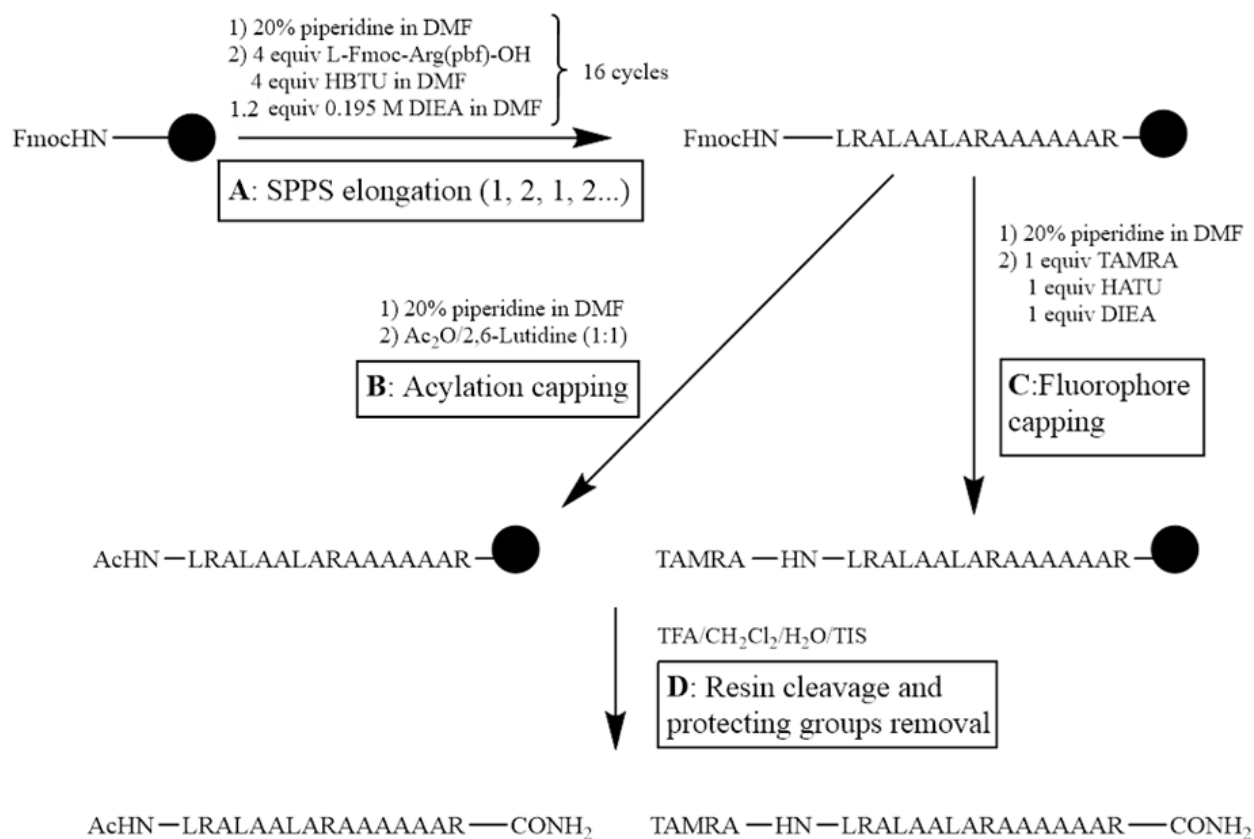
### **Cell viability**

Cell viability was established by a standard MTT assay. One day before the assay, a suspension of HeLa cells was plated in 96-well tissue culture plates (Costar 96 Flat Bottom Transparent Polystyrol) by adding 100 µl (~10.000 cells) per well. The next day, the medium was aspirated and cells were incubated in DMEM containing 10% Fetal Bovine Serum (FBS) in the presence of the different peptides (50 µl/well) at different concentrations (1, 3, 4, 5, 7, 10 µM). After 1 h of incubation at 37 °C, the viability was measured by quantifying the cellular ability to reduce the water-soluble tetrazolium dye 3-(4,5-dimethylthiazole-2,5-diphenyl) tetrazolium bromide (MTT) to its insoluble formazan salt as follows. MTT (5 mg/ml in PBS, 10 µl/well) was added to the wells and the cells were further incubated for 4 h. The supernatant was carefully removed and the water-insoluble formazan salt was dissolved in DMSO (100 µl/well). The absorbance at 570 nm was measured. Data points were collected in triplicate and expressed as normalized values for untreated as control cells (100%) after blank subtraction.

### **Peptide stability in serum**

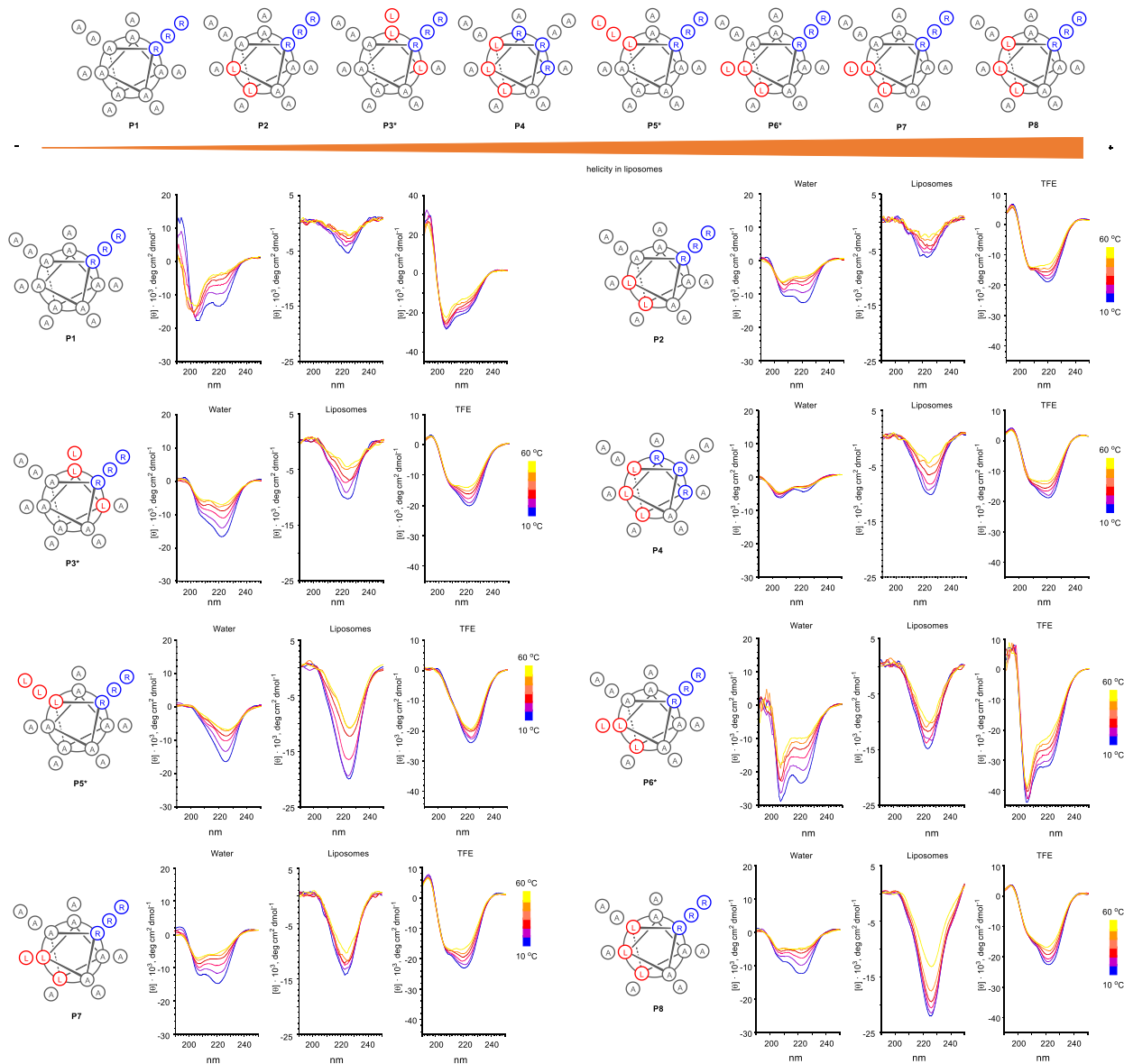
To determine the half-life of **P<sub>8</sub>** in serum, the peptide was dissolved in FBS at a final concentration of 100  $\mu$ M and incubated at 37 °C. At different time points, 40  $\mu$ L aliquots were taken. Samples were mixed with an equal volume of acetonitrile and centrifuged for 5 min at 10000 rpm to remove most serum proteins. Supernatants were freeze-dried and resuspended in 50  $\mu$ L Milli-Q water, before quantitation by HPLC, measuring TAMRA absorbance at 555 nm [RP-HPLC Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20min)].

## Supporting figures

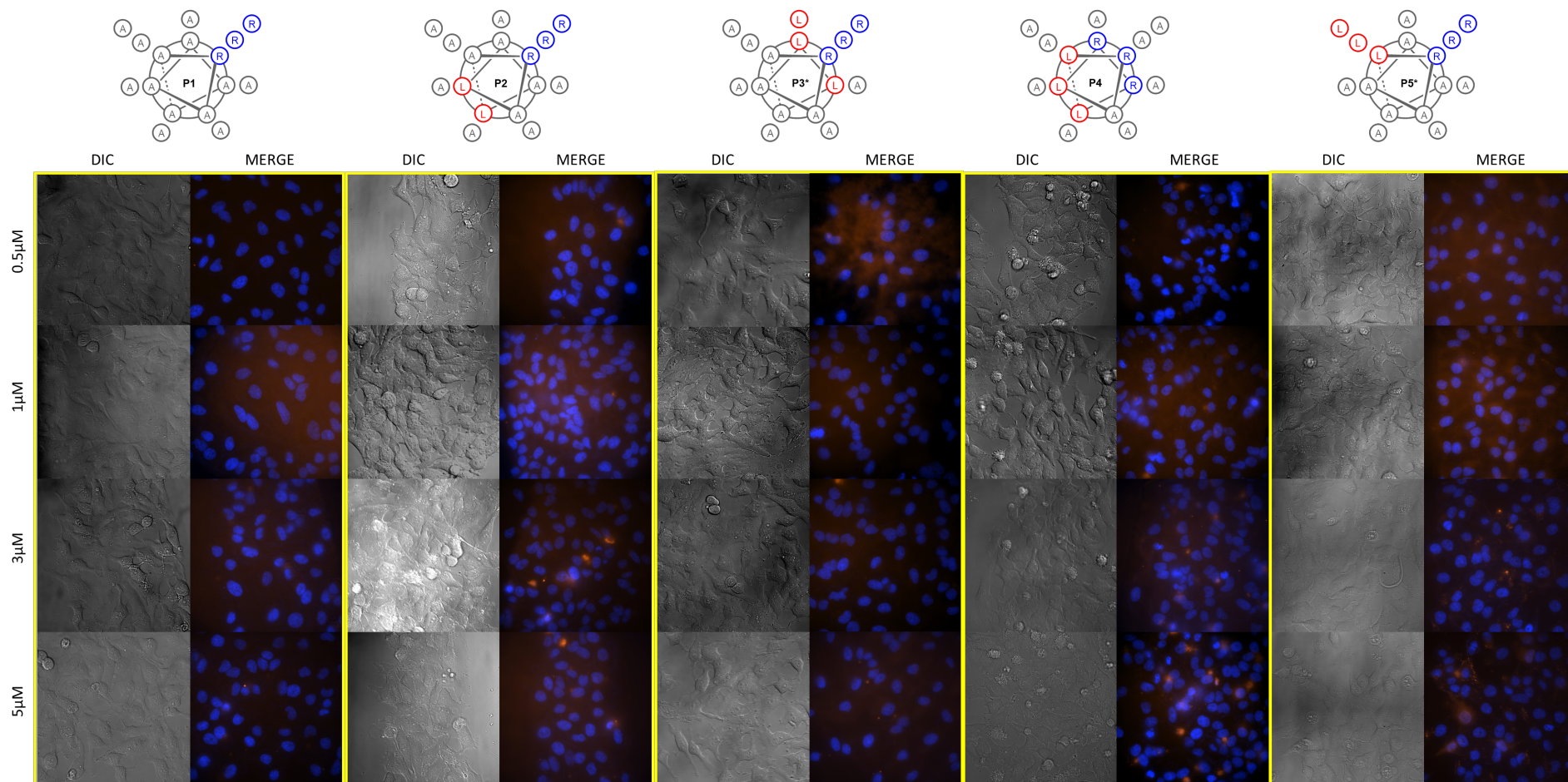


**Figure S1:** General synthetic scheme for the solid phase peptide synthesis (SPPS).



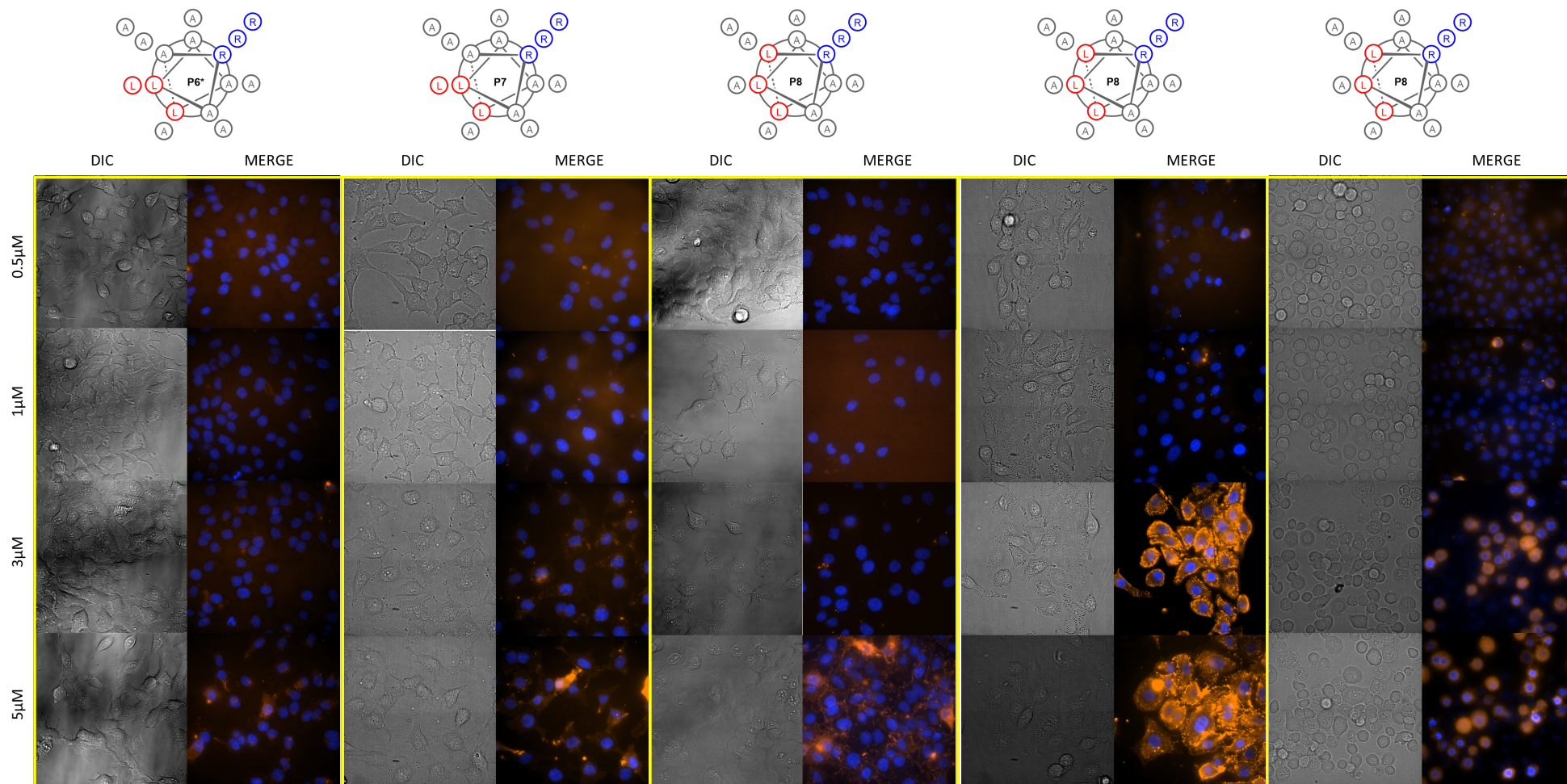


**Figure S2:** A) Heptad structures of the peptides ordered by increased helicity in liposomes. B) Circular dichroism in HKR buffer, liposomes and TFE (from left to right) of all peptides. The measurements were taken at different temperatures (10-60 °C). All samples were at 100  $\mu\text{M}$  peptide final concentration.

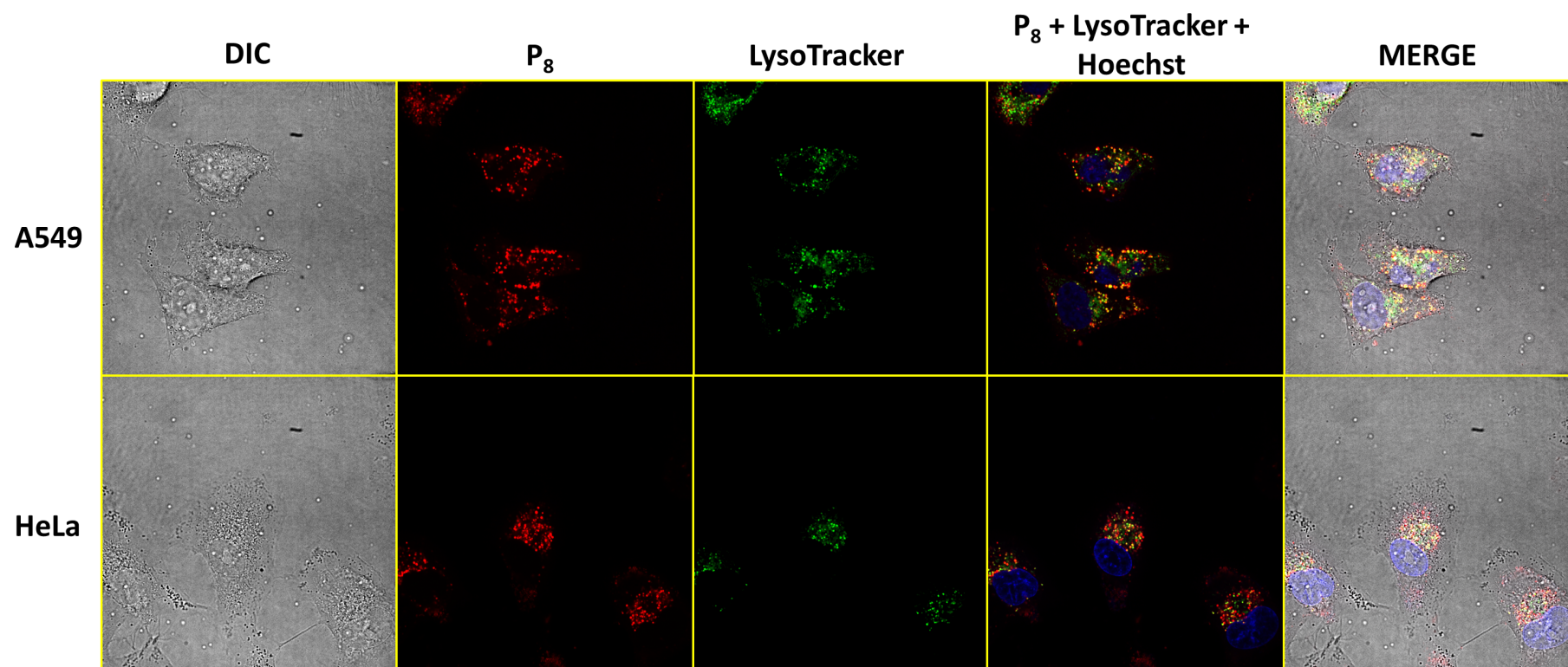


**Figure S3:** Epifluorescence image of peptides in HeLa cells. **P<sub>1</sub>**, **P<sub>2</sub>**, **P<sub>3</sub>\***, **P<sub>4</sub>** and **P<sub>5</sub>\***. DIC on the left and merge channels (peptide and nuclei) on the right. HeLa cells were treated first with Hoechst 33342 for 30 min, washed (HKR buffer, 1x) and then incubated with the peptides (0.5, 1, 3 and 5 μM) in HKR buffer for 30 min. The cells were finally washed (HKR buffer, 3x) and then observed with the epifluorescence microscope.



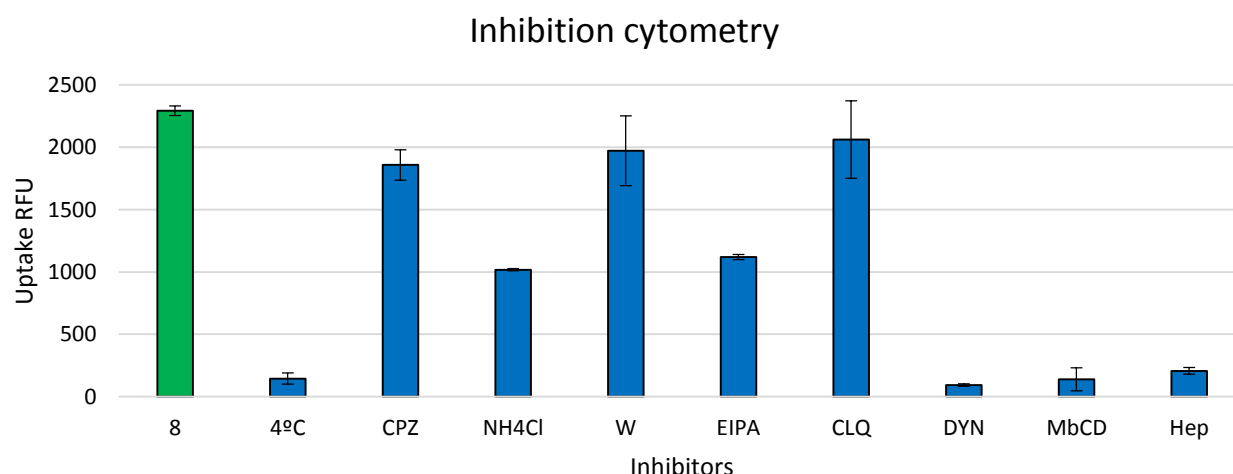


**Figure S4:** Epifluorescence image of peptides in HeLa cells. **P<sub>6</sub>** in HeLa cells; **P<sub>7</sub>** in HeLa cells; **P<sub>8</sub>** in HeLa cells; **P<sub>8</sub>** in A-549 cells; **P<sub>8</sub>** in SF9 cells. DIC on the left and merge channels (peptide and nuclei) on the right. HeLa cells were treated first with Hoechst 33342 for 30 min, washed (HKR buffer, 1x) and then incubated with the peptides (0.5, 1, 3 and 5  $\mu$ M) in HKR buffer for 30 min. The cells were finally washed (HKR buffer, 3x) and then observed with the epifluorescence microscope. In the case of SF9 the buffer used was Sf-900-II.

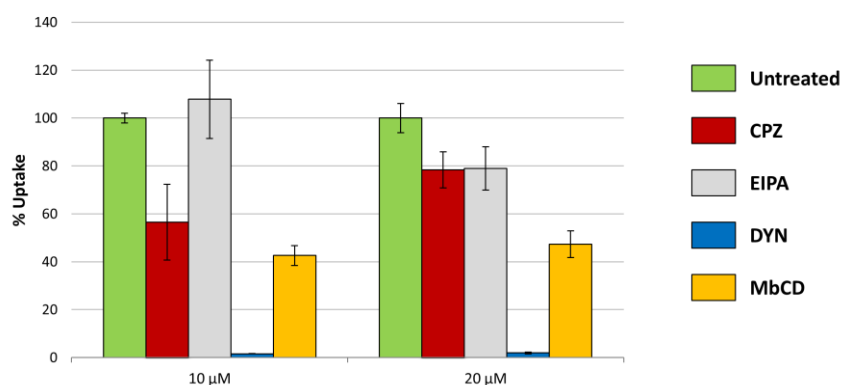


**Figure S5:** LysoTracker colocalization studies. HeLa cells and A549 cells were incubated with 5  $\mu$ M P<sub>8</sub> peptide for 30 min in HKR, washed and incubated with DMEM with 10 % serum for 3 h. Lysosomes were stained with 50 nM LysoTracker Deep Red the last 30 min of incubation before imaging in a confocal microscope. P<sub>8</sub> is shown in red, LysoTracker is shown in green. Nuclei were counterstained blue with Hoechst.

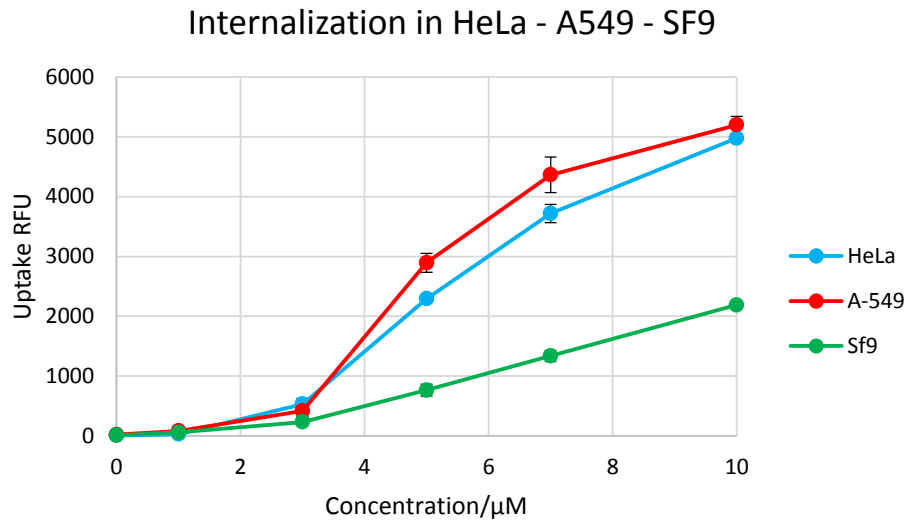
A)



B)

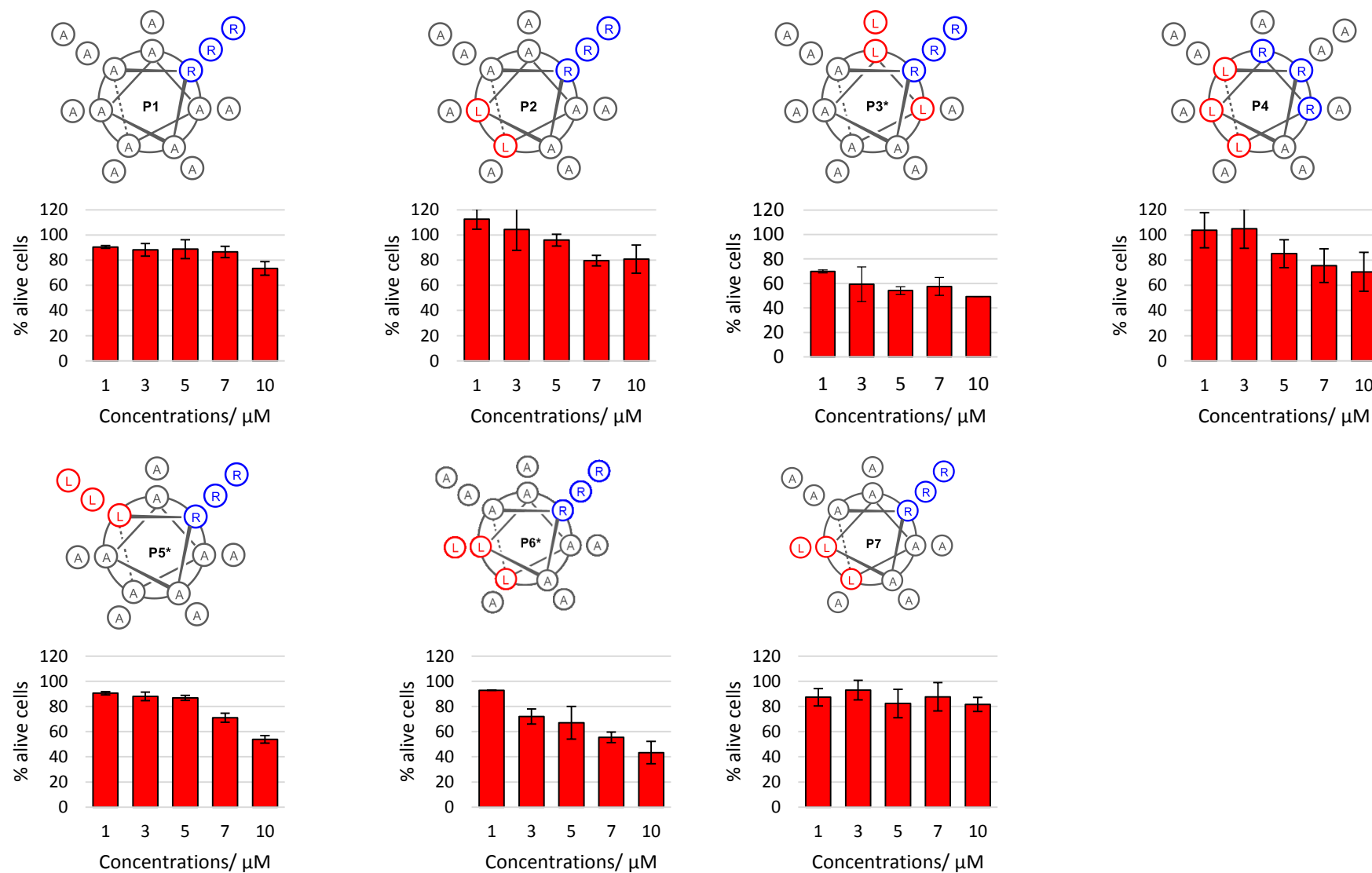


**Figure S6:** A) Histogram of the uptake in relative fluorescence units (RFU) for  $P_8$  (5  $\mu$ M) in the presence of different endocytic inhibitors. In the histogram, the green column is the peptide alone, and the blue columns are the uptake in the presence of the inhibitors: 4°C, CPZ: chlorpromazine; NH<sub>4</sub>Cl: Ammonium Chloride; W: Wortmannin; EIPA: 5-(*N*-Ethyl-*N*-isopropyl)amiloride; CLQ: chloroquine; DYN: Dynasore; MbCD: methyl- $\beta$ -cyclodextrin; Hep: heparin. Error bars indicate SD of three replicates. HeLa cells were treated in DMEM (-) for 30 min prior to trypsinization and cytometry quantification. B) Study of the endocytic mechanism at different peptide concentrations. HeLa cells were treated with the indicated inhibitors for 30 min in DMEM (-) before incubating with the peptide  $P_8$  at different concentrations for 30 min. Cells were then trypsinized and analyzed by flow cytometry. Error bars indicate SD of three replicates.

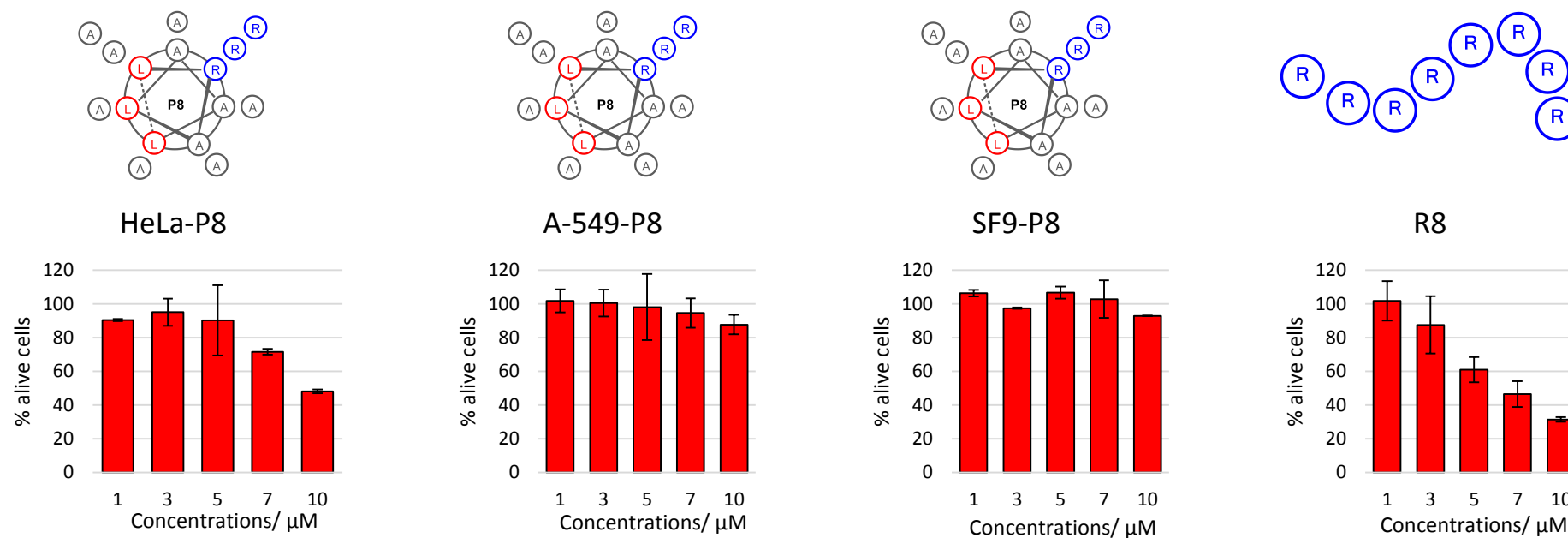


**Figure S7:** Uptake quantification by flow cytometry of the peptide  $\mathbf{P}_8$  in three different cell lines: HeLa cells in blue, A-549 cells in red and Sf9 cells in green. The points were collected at six different concentrations (0, 1, 3, 5, 7, 10  $\mu\text{M}$ ). Cells were treated in DMEM (-) or Sf-900-II in the case of Sf9 cells for 1 hour prior to trypsinization and cytometry quantification. Error bars indicate SD of three replicates.



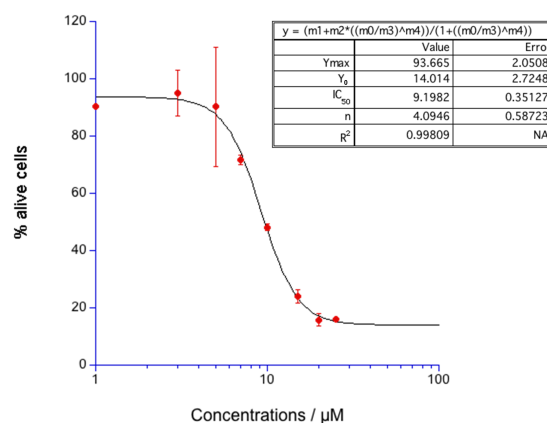


**Figure S8:** MTT assay at different concentrations of peptides:  $P_1$ ,  $P_2$ ,  $P_{3^*}$ ,  $P_4$ ,  $P_{5^*}$ ,  $P_{6^*}$ ,  $P_7$  in HeLa cells. HeLa cells were treated in DMEM (-) for 1 hour prior to MTT-assay.

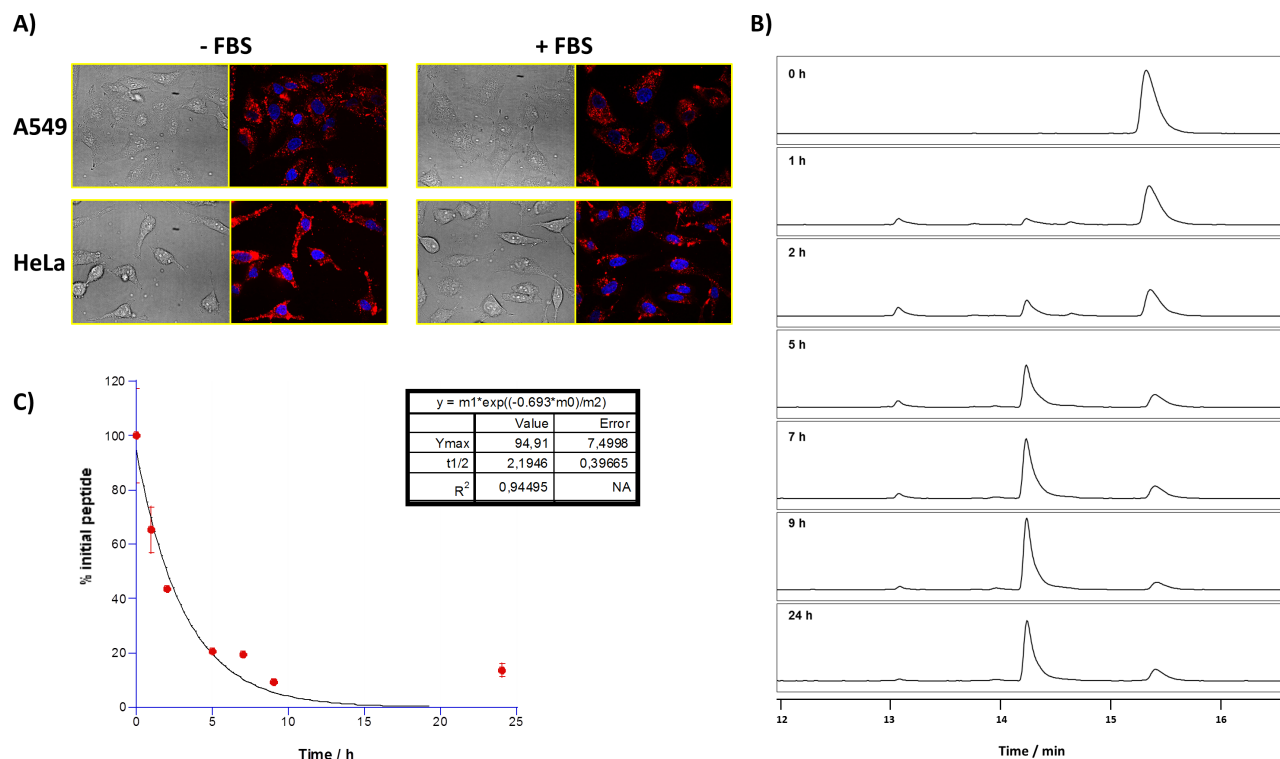


**Figure S9:** MTT assay at different concentrations of peptide  $P_8$  in three different cell lines: HeLa, A-549, SF9 and  $R_8$  in HeLa cells. Hela cells were treated in DMEM (-) for 1 hour prior to MTT-assay.



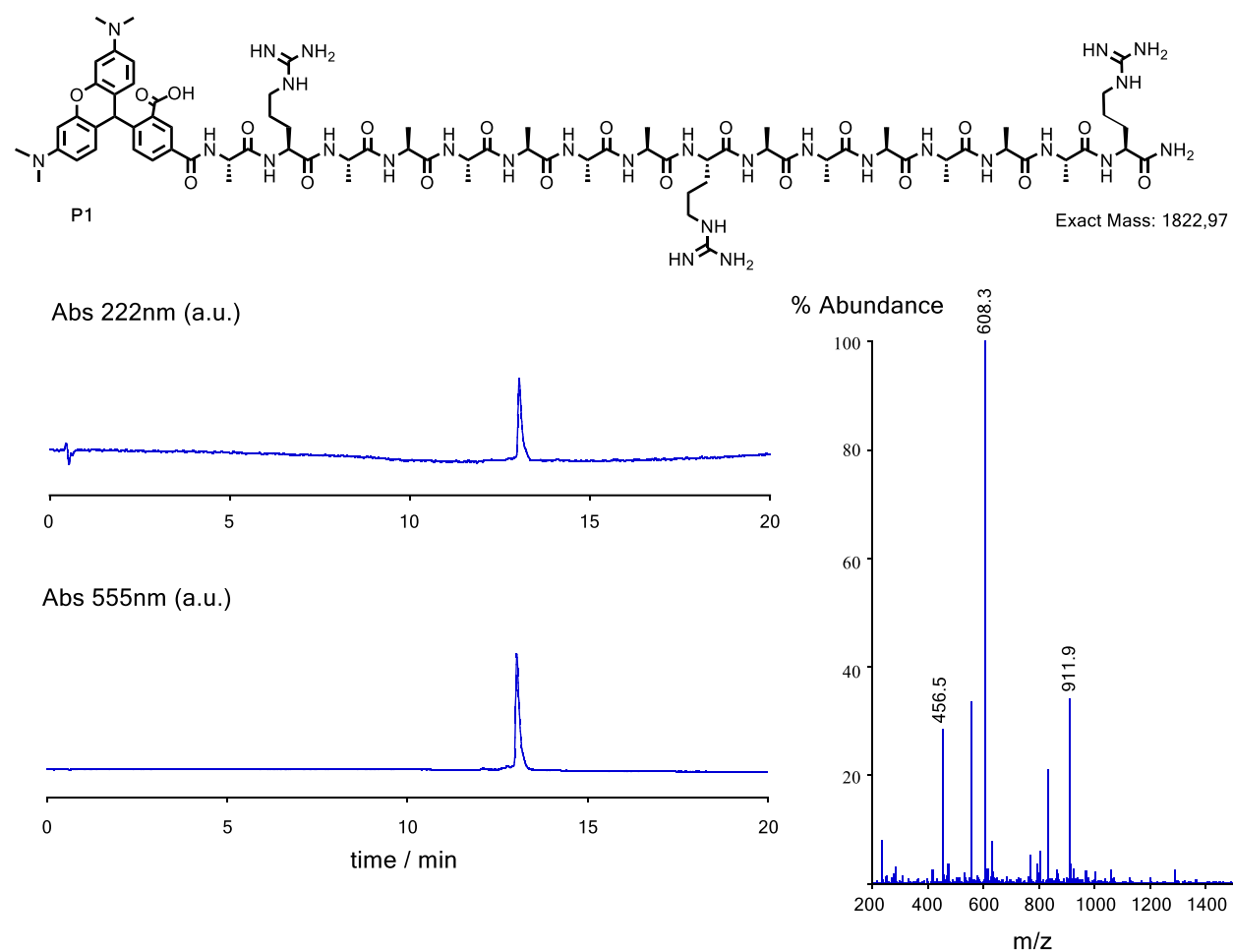


**Figure S10:** MTT assay at increasing concentrations of **P<sub>8</sub>** in HeLa cells. Cells were incubated for 1 h with the peptide in DMEM (-) before MTT assay. Curve fitting was done with KaleidaGraph software, using a 4-parameters logistic model. IC<sub>50</sub> = 9.2 μM.

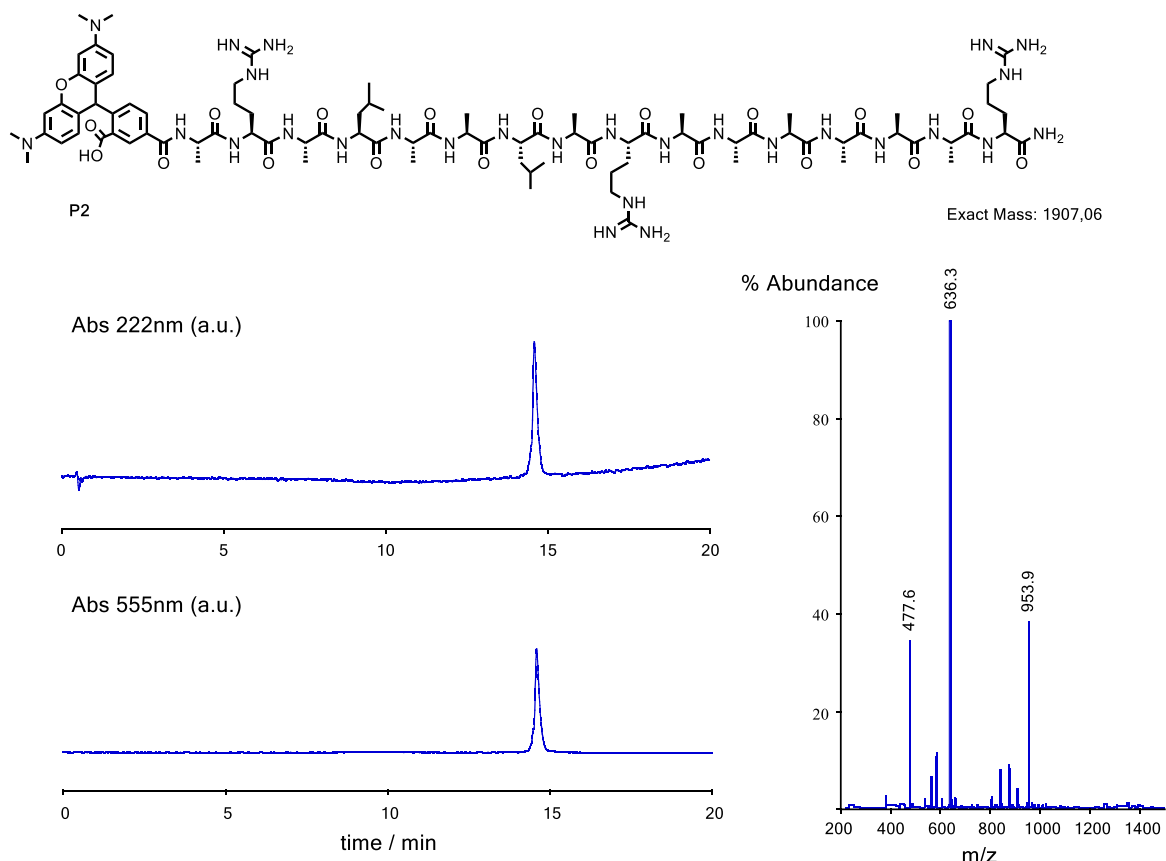


**Figure S11:** Stability in the presence of serum. A) A-549 and HeLa cells were incubated for 30 min with 5 μM dissolved in DMEM (-) or DMEM supplemented with 10 % FBS. Cells were then washed three times with DMEM (-) and imaged in a confocal microscope. B) RP-HPLC of **P<sub>8</sub>** after different incubation times with FBS (555 nm absorbance) C) Calculation of **P<sub>8</sub>** half-life in serum. Area of the peak corresponding to the intact peptide was measured for all the time points and normalized to time 0 h (100 %). Data was adjusted to an exponential decay curve using KaleidaGraph. **P<sub>8</sub>** half-life ~ 2.2 h.

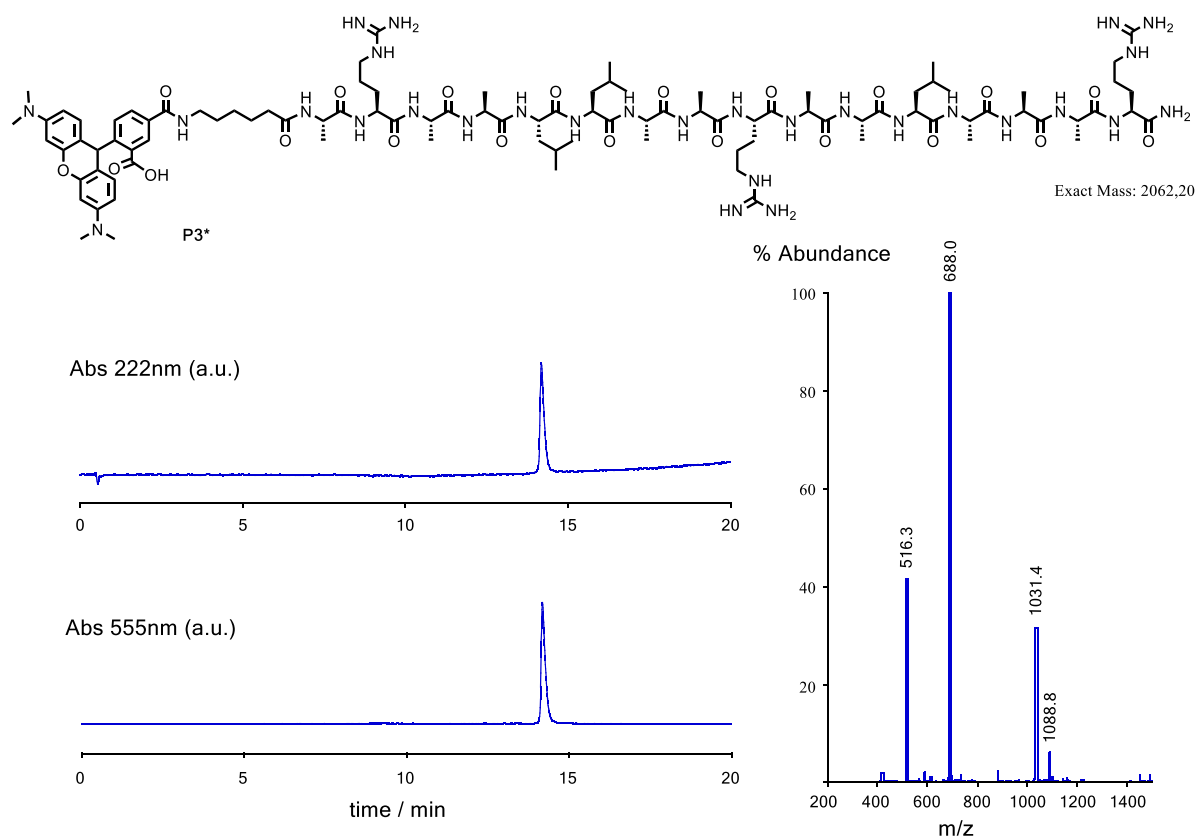
## Supporting figures of Characterization



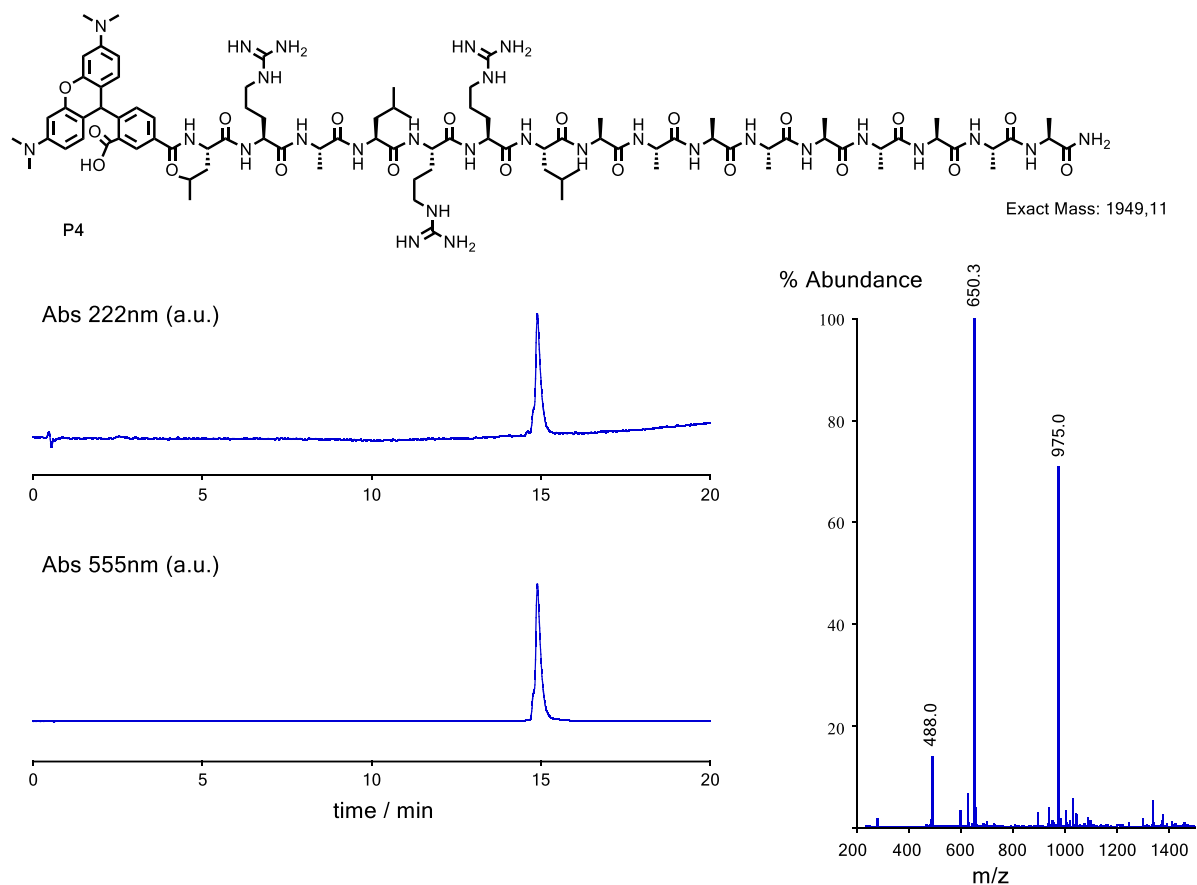
**Figure S12:** RP-HPLC [Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] ( $R_t$  13 min) absorbance at 222 nm and 555 nm and ESI-MS for Peptide **P1**.



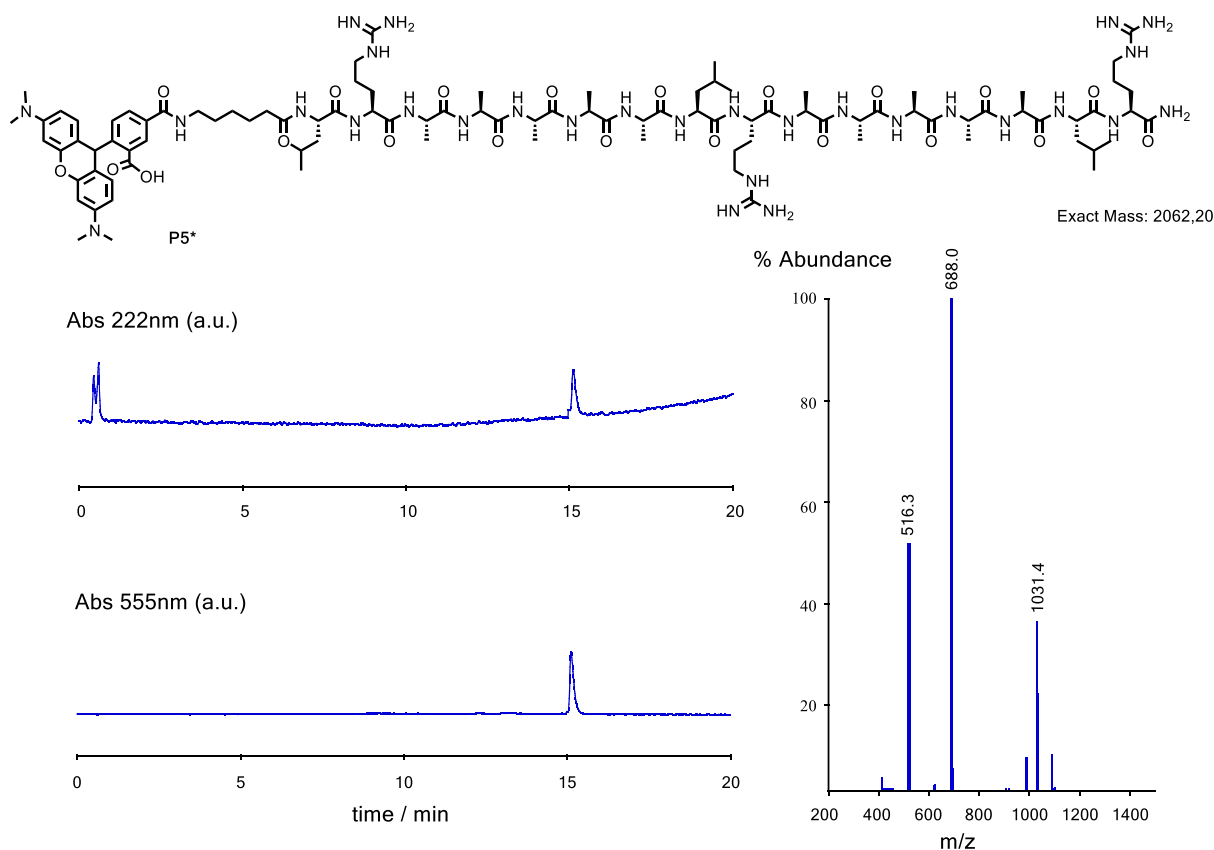
**Figure S13:** RP-HPLC [Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (*R*<sub>t</sub> 14.6 min) absorbance at 222 nm and 555 nm and ESI-MS for Peptide **P2**.



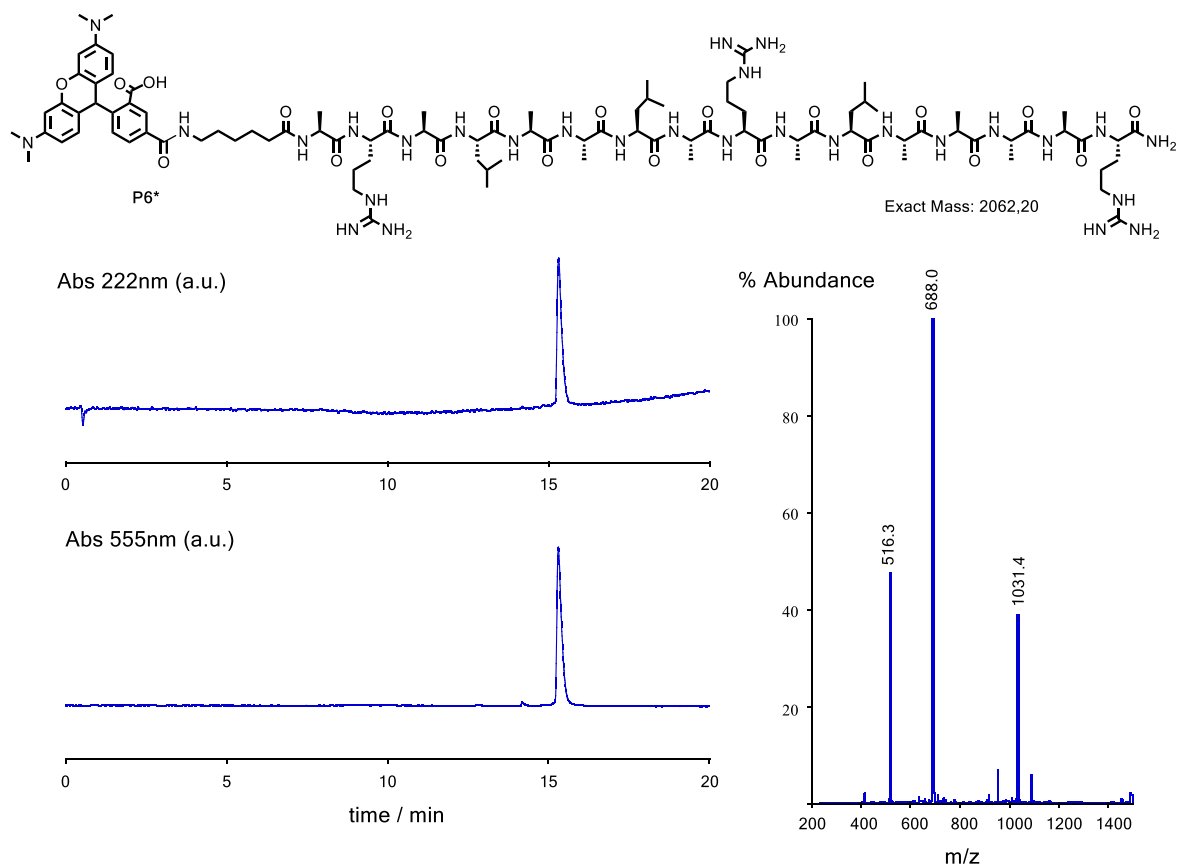
**Figure S14:** RP-HPLC [Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (*R*<sub>t</sub> 14.2 min) absorbance at 222 nm and 555 nm and ESI-MS for Peptide **P3\***.



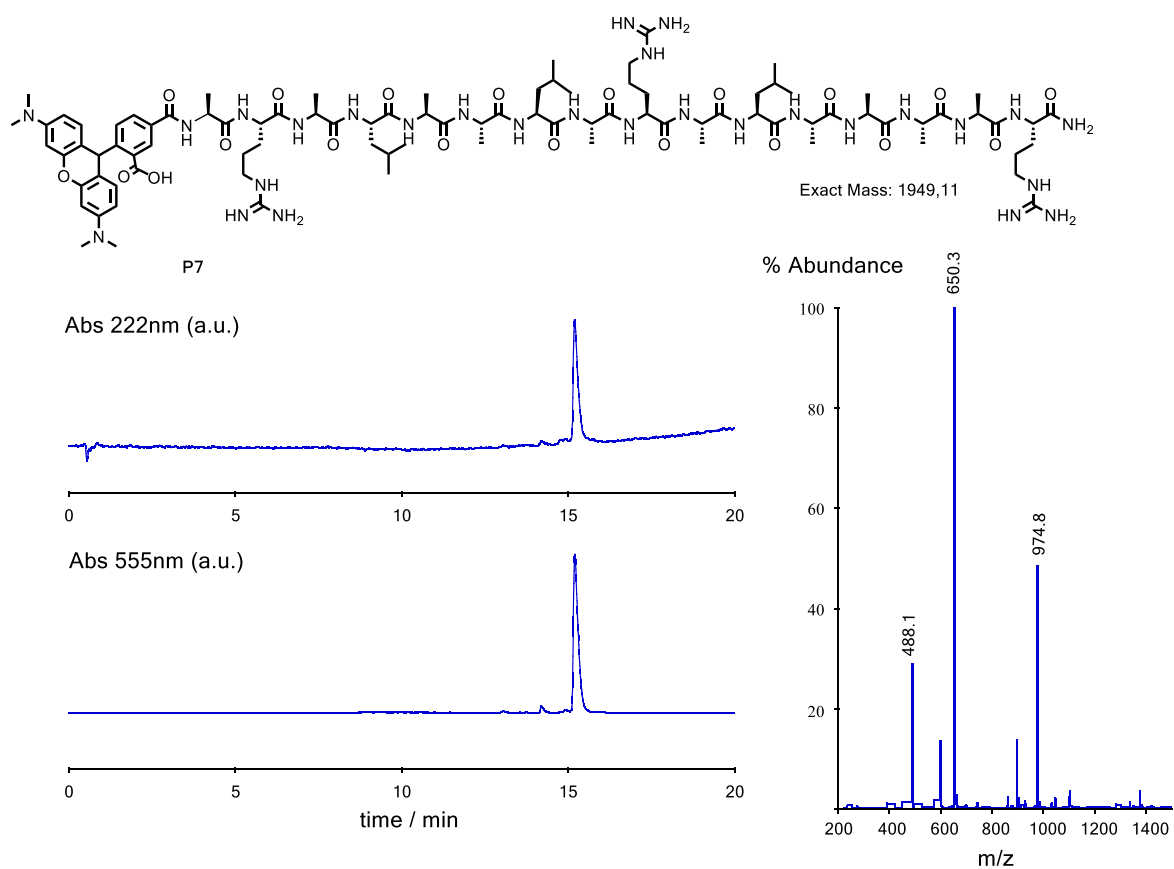
**Figure S15:** RP-HPLC [Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (*R*<sub>t</sub> 14.9 min) absorbance at 222 nm and 555 nm and ESI-MS for Peptide **P4**.



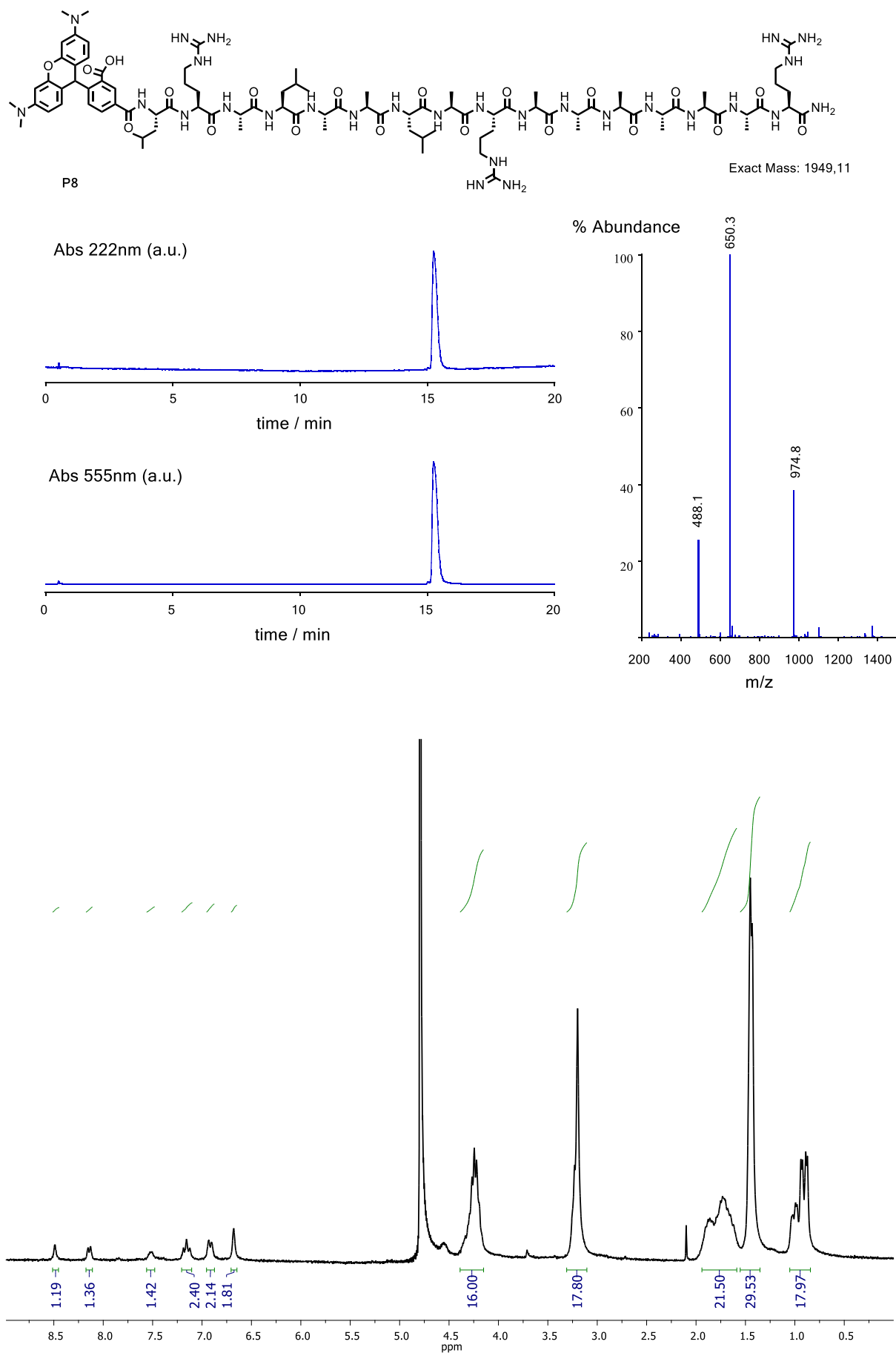
**Figure S16:** RP-HPLC [Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (*R*<sub>t</sub> 15.2 min) absorbance at 222 nm and 555 nm and ESI-MS for Peptide **P5\***.



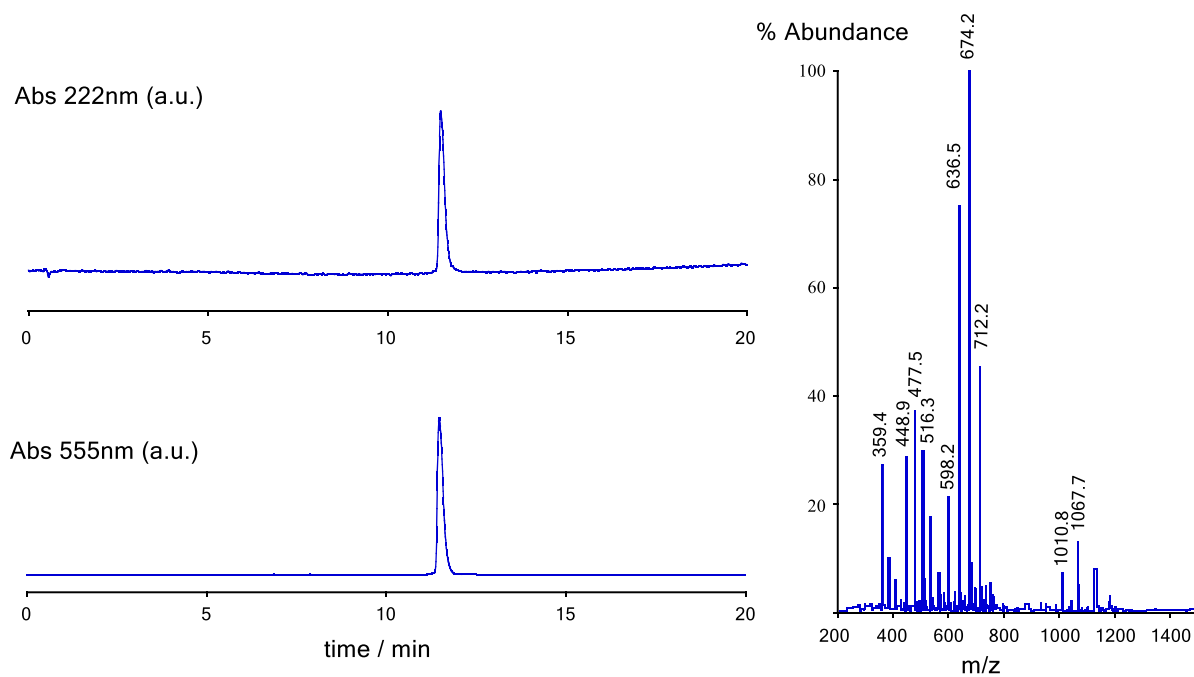
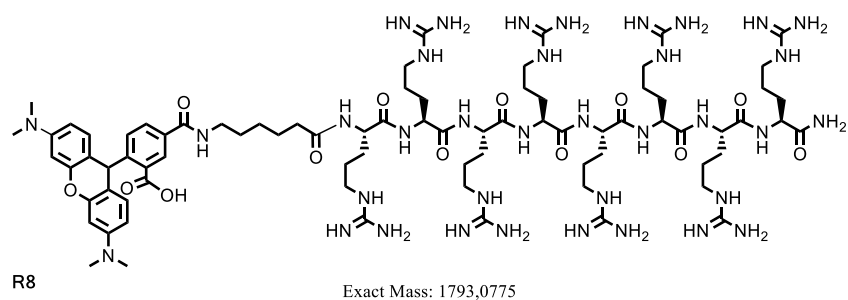
**Figure S17:** RP-HPLC [Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (*R*<sub>t</sub> 15.3 min) absorbance at 222 nm and 555 nm and ESI-MS for Peptide **P6\***.



**Figure S18:** RP-HPLC [Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (*R*<sub>t</sub> 15.5 min) absorbance at 222 nm and 555 nm and ESI-MS for Peptide **P7**.



**Figure S19:** RP-HPLC [Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (*R*<sub>t</sub> 15.2 min) absorbance at 222 nm and 555 nm and ESI-MS and NMR in D<sub>2</sub>O for Peptide P<sub>8</sub>.



**Figure S20:** RP-HPLC [Agilent SB-C18 column, H<sub>2</sub>O (0.1% TFA)/ CH<sub>3</sub>CN (0.1% TFA) 95:5→5:95 (0→20 min)] (*R*<sub>t</sub> 11.4 min) absorbance at 222 nm and 555 nm and ESI-MS for Peptide **R8**.

## Supporting references

- (S1) R. Behrendt, P. White, J. Offer, *J. Pept. Sci.* **2016**, 22, 4–27.
- (S2) C. Kay, O. E. Lorthioir, N. J. Parr, M. Congreve, S. C. McKeown, J. J. Scicinski, S. V. Ley, *Biotechnol. Bioeng.* **2000**, 71, 110–118.
- (S3) I. Louzao, R. García-Fandiño, J. Montenegro, *J. Mater. Chem. B* **2017**, 5, 4426–4434.
- (S4) T. Holm, H. Johansson, P. Lundberg, M. Pooga, M. Lindgren, U. Langel, *Nat. Protoc.* **2006**, 1, 1001–1005.
- (S5) J. P. Richard, K. Melikov, E. Vives, C. Ramos, B. Verbeure, M. J. Gait, L. V Chernomordik, B. Lebleu, *J. Biol. Chem.* **2003**, 278, 585–90.