Supporting Information

Controlled Supramolecular Assembly inside Living Cells by Sequential Multi-staged Chemical Reactions

Michaela Pieszka[1,2], Shen Han[1], Christiane Volkmann[1], Robert Graf[1], Ingo Lieberwirth[1], Katharina Landfester[1], David Y. W. Ng[1*] and Tanja Weil[1,2*]

[1] *Max Planck Institute for Polymer Research, Ackermannweg 10, 55128 Mainz, Germany*

[2] *Institute of Inorganic Chemistry, Ulm University, Albert-Einstein-Allee 11, 89081 Ulm, Germany*

E-mail: david.ng@mpip-mainz.mpg.de, weil@mpip-mainz.mpg.de

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1. Materials and Instruments

1.1 Materials

Reagents and solvents were purchased from commercial sources and were used without any further purification. Peptide Synthesis grade reagents were used for synthesizing the peptides. HPLC was performed using acetonitrile in HPLC grade and water for HPLC and reactions was obtained from a Millipore purification system. Thin-layer chromatography (TLC) was performed on Macherey-Nagel Alugram Sil G/UV_{254} plates and substances were visualized under UV light at 254 nm. Column chromatography was carried out using Macherey-Nagel silica gel 0.04–0.063 mm.

1.2 Instruments

1.2.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectra of dissolved compounds were recorded on a Bruker Avance II 300 MHz spectrometer. The solvent signal was used as a reference (deuterated chloroform $CDCl_3$ δ = 7.26 ppm for ^{1}H, 77.16 ppm for ^{13}C, deuterated DCM CD_2Cl_2 5.32 ppm for ^{1}H and 53.84 ppm for ^{13}C and for DMSO-d_6 2.50 ppm and 39.52 ppm respectively). The data were processed in MestReNova.

1.2.2 Microwave Peptide Synthesizer

Peptides were synthesized in a Liberty Blue Automated Microwave Peptide Synthesizer by CEM Corporation.

1.2.3 High-Performance Liquid Chromatography (HPLC)

Peptides were purified by preparative HPLC using a setup by Shimadzu. For purification either a ZORBAX Eclipse XDB-C18 HPLC column (9.4 × 250 mm, 5 µm) was used at a flowrate of 4 mL/min or an Atlantis T3 Prep OBD™ 5 µm, 19 × 150 mm column was used with a flowrate of 10 mL/min or a Phenomenex Gemini 5 µm NX-C18 110 Å 150 × 30 mm was used at a flowrate of 25 mL/min.

For analytical measurements an Atlantis T3 column (4.6 × 100 mm, 5 µm) was used at a flowrate of 1 mL/min, with the exception of analysis of cell lysates where a ZORBAX Eclipse, XDB-C18, 80Å, 5 µm, 4.6 × 250 mm column was used at a flowrate of 1mL/min. All measurements and purification steps were done using gradients of acetonitrile and MilliQ water, each acidified with 0.1% TFA. Absorbance was recorded at 190, 214, 254 and 433 nm wavelength. The software LabSolutions by Shimadzu and Powerpoint were used to process all HPLC spectra.

1.2.4 Liquid Chromatography - Mass Spectrometry (LC-MS)

Compounds were analyzed by HPLC-ESI-MS on a LC-MS 2020 by Shimadzu using a Kinetex 2.6 µm EVO C18 100 Å LC 50 × 2.1 mm column. MilliQ water, acidified with 0.1% formic acid and acetonitrile were used as solvents for all measurements. The solvent gradient started with 5% ACN and 95% water, while the ACN content was linearly increased to 95% in 12 min. Data were processed in LabSolutions and Powerpoint.

1.2.5 Matrix-Assisted Laser Desorption/Ionisation - Time of Flight Mass Spectrometry (MALDI-TOF)

All MALDI-TOF spectra were recorded on either a rapifleX MALDI-TOF/TOF from Bruker or MALDI Synapt G2-SI from Waters. Samples were mixed with a saturated solution of the matrix

α-cyano-4-hydroxycinnamic acid (CHCA) in water/ACN 1/1 + 0.1% TFA. Data processing was performed in mMass.

1.2.6 APCI-MS

APCI (atmospheric pressure chemical ionisation) mass spectra were measured on an Advion expression-L Compact Mass Spectrometer (CMS) by Advion Inc. with an atmospheric solid analysis probe (ASAP).

1.2.7 Fluorescence and Absorbance Spectroscopy

A SPARK 20M microplate reader by the company Tecan Group Ltd. was used to record fluorescence and absorbance intensity. Samples were measured in a Greiner 384 flat black well plate and data processing was done using Excel.

Binding affinity of PBA to SHA was determined by fluorescence quenching. Measurements were performed using a Monolith NT.115 instrument and data was analyzed with MO.Affinity Analysis software by NanoTemper Technologies GmbH.

1.2.8 Luminescence Intensity

Luminescence intensity was measured in a white half area 96-well plate on a Promega GloMax®-Multi Detection System using the settings for the CellTiter-Glo Luminescent Cell Viability Assay. Data were processed in Excel.

1.2.9 Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR spectra were measured on a Bruker TENSOR II spectrometer equipped with a PLATINUM ATR single reflection diamond ATR accessory. Data were processed in Excel.

1.2.10 Circular Dichroism Spectroscopy (CD)

CD spectra were recorded on a JASCO J-1500 spectrometer in a 1 mm High Precision Cell by HellmaAnalytics. Data were processed in Spectra Analysis by JASCO and Excel.

1.2.11 Solid-State NMR

CP-MAS ^{13}C (^{1}H) spectra were recorded on a Bruker Avance III 700 MHz spectrometer using a 2.5-mm $^{1}H/X$ double-resonance CP/MAS probe. Measurements were performed at MAS speeds of 25 kHz and CP/MAS measurements were done with a contact time of 3 ms and 100-kHz radio frequency nutation frequency swept-frequency two-pulse phase modulation high-power composite pulse decoupling. The CH_3 group of L-alanine with the 1.3 ppm and 20.5 ppm peak for ^{1}H and ^{13}C, respectively, were used as a secondary standard to reference the chemical shifts to tetramethylsilane.

1.2.12 Transmission Electron Microscopy (TEM)

TEM pictures of peptide solutions were taken on a JEOL 1400 transmission electron microscope at a voltage of 120 kV. Samples were prepared on Formvar/carbon-film coated copper grids (300 mesh) by Plano GmbH. In order to prepare the TEM grids, 4 µL of peptide solution were put on freshly etched (30 s at 20% oxygen content) Formvar coated copper grids. After 5 min the solution was removed using a filter paper and grids were stained with uranyl acetate 4% for 2.5 min. The grids were washed three times with MilliQ water and dried before measuring. TEM images were processed in ImageJ.

For TEM measurements of cells, sections were carefully placed onto 300-mesh carbon coated copper grid for standard bright-field imaging in a FEI Tecnai F20 200 kV transmission electron microscope. Bright-field TEM micrographs were obtained with a Gatan US1000 2k CCD camera.

1.2.13 Cell Culture

A549 cells were cultured at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, high glucose), which was supplemented with 10% FBS, 1% penicillin/streptomycin and 1x MEM non-essential amino acids.

1.2.14 Confocal Laser Scanning Microscopy

Cells were imaged on a Leica TCS SP5 and a Visitron Spinning Disc microscope with a 405 nm excitation diode and emission filter 415–515 nm, as well as at 633 nm excitation using a HeNe laser and an emission filter of 643–743 nm to monitor Coumarin 343 and the nuclear stain respectively. The same laser was used for excitation of Alexa 647 (emission 657-757 nm). Furthermore, an argon laser was used for excitation at 488 nm of Annexin V (emission 498-540 nm) and propidium iodide (emission 650-750 nm). FRET was monitored by exciting at 405 nm and measuring the emission at 550-650 nm. RFP was excited at 561 nm (emission 751-671 nm) and for imaging of lysosomes (emission 506-606 nm) and nucleoli (emission 506-606 nm) a wavelength of 488 nm was used.

1.2.15 Fluorescence Microscopy

Fluorescence was imaged using a Leica DMi8 microscope using a 100x oil objective by Leica. Coumarin 343 fluorescence was imaged with 350/50 nm excitation and a 460/50 nm emission filter cube and Proteostat fluorescence was excited at 546/10 nm and the emission was recorded at 585/40 nm.

2. Synthesis

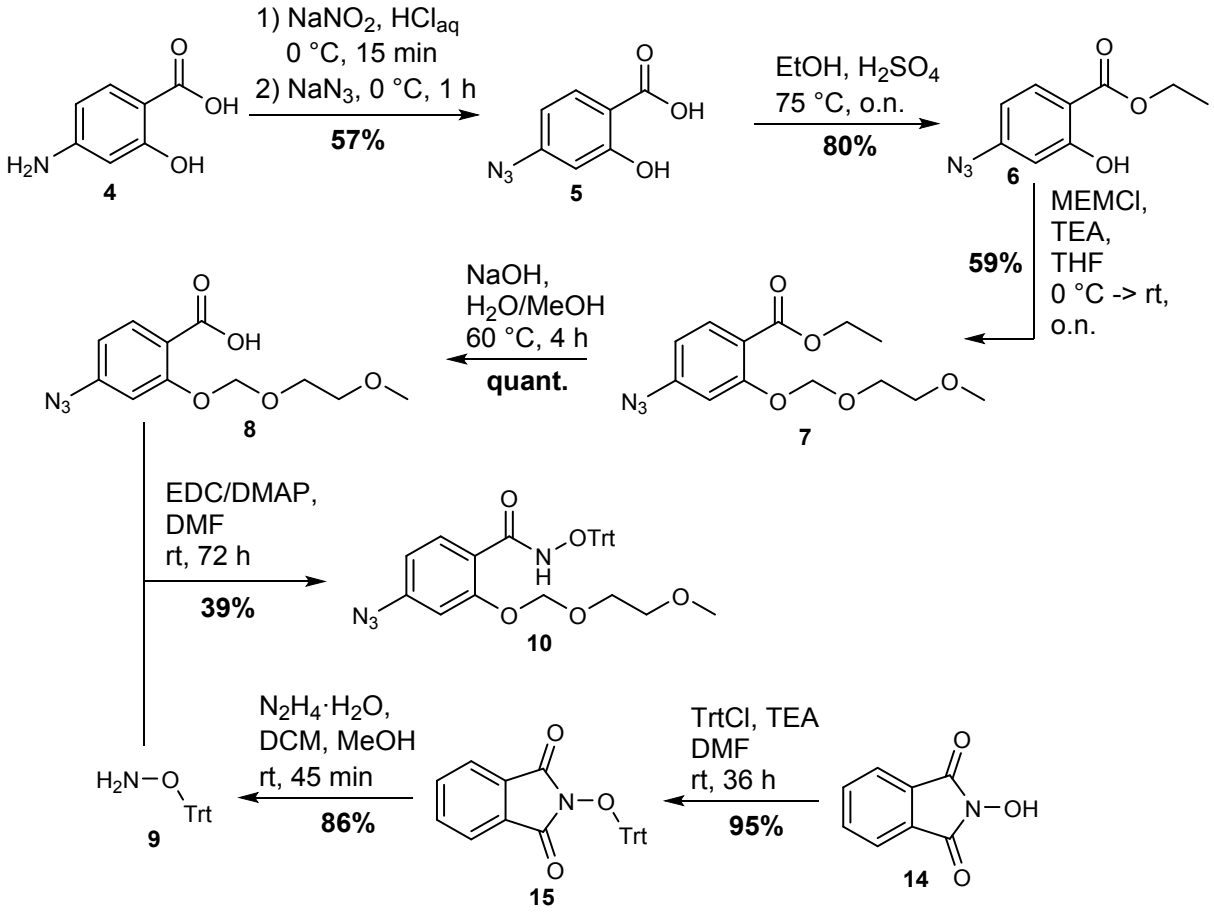
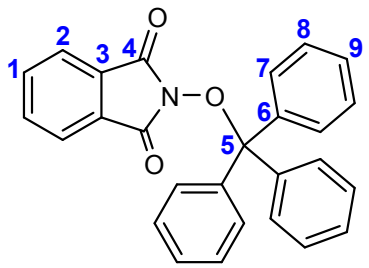


Figure S1: Synthesis of the Trt and MEM protected 4-azidosalicyl hydroxamate derivative **10** in seven steps starting from 4-amino salicylic acid **4**. After conversion of the amine into the azide **5**, the acid was protected as an ethyl ester (**6**) before the MEM protecting group was attached. Subsequently, the carboxylic acid **7** was deprotected and reacted with trityloxamine **9**, which was synthesized in two steps.

2.1 *N*-Trityloxyphthalimide



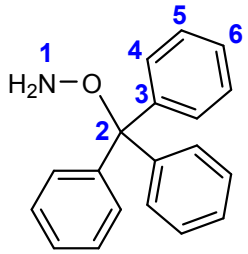
10 mL Triethylamine (72.14 mmol) were added to a solution of *N*-hydroxyphthalimide **14** (10.00 g, 61.30 mmol) in 20 mL DMF. After addition of tritylchloride (17.15 g, 61.52 mmol) the

reaction mixture was stirred for 30 min at room temperature. After the reaction proceeded for 36 h without stirring, the precipitate was suspended in 100 mL isopropanol, filtered and washed with water and saturated $NaHCO_3$ solution. After drying *in vacuo* 23.69 g (58.43 mmol, 95% yield) of the white powder **15** were received.[1]

^{1}H-NMR (300 MHz, CD_2Cl_2) δ [ppm] = 7.68-7.57 (m, 4H, 1 and 2), 7.53-7.49 (m, 6H, 8), 7.33-7.29 (m, 9H, 7 and 9).

^{13}C-NMR (75 MHz, CD_2Cl_2) δ [ppm] = 164.47 (4), 142.38 (6), 134.73 (1), 130.63 (8), 129.10 (3), 128.67 (9), 127.91 (3), 123.45 (7), 98.19 (5).

2.2 *N*-Trityloxyamine

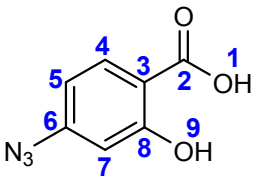


After *N*-trityloxyphthalimide (**15**, 22.07 g, 54.43 mmol) was dissolved in 135 mL DCM, a 99% hydrazine solution in water (12 mL, 0.24 mol) was diluted with 40 mL methanol and added dropwise while stirring. After 45 min stirring at room temperature, 5 M ammonium hydroxide solution was added until the precipitate dissolved. The aqueous phase was extracted three times with DCM and the organic phase was washed with brine. After drying over magnesium sulfate, the solvent was evaporated and the product **9** was recrystallized from methanol and colorless crystals (12.85 g, 46.67 mmol, 86% yield) were isolated.[1]

^{1}H-NMR (300 MHz, CD_2Cl_2) δ [ppm] = 7.46-7.43 (m, 6H, 5), 7.38-7.27 (m, 9H, 4 and 6), 4.96 (s, 2H, **1**).

^{13}C-NMR (75 MHz, CD_2Cl_2) δ [ppm] = 144.18 (3), 129.38 (5), 128.33 (4), 127.59 (6), 91.04 (2).

2.3 4-Azidosalicylic acid



4-Aminosalicylicylic acid **4** (10.00 g, 65.30 mmol) was dissolved in a mixture of 125 mL concentrated HCl and 150 mL water. After the solution was cooled with an ice bath, sodium nitrite (6.49 g, 94.09 mmol), dissolved in 38 mL water, was added slowly. The reaction mixture was stirred for 15 min. After addition of urea the solution was stirred for 2 min and then filtered over celite while cooling.

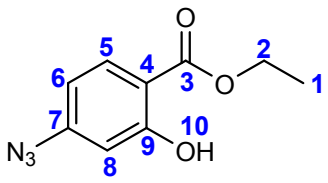
Subsequently sodium azide (6.11 g, 93.99 mmol) was dissolved in 38 mL water and added slowly to the solution. The solution was stirred for one hour and the raw product was isolated by filtration. After recrystallization from methanol, 6.66 g (37.22 mmol, 57% yield) of brown crystals of **5** were received.[2]

^{1}H-NMR (300 MHz, DMSO-d_6) δ [ppm] = 11.56 (s, 1H, 9), 7.80 (d, J = 8.3 Hz, 1H, 4), 6.69–6.64 (m, 2H, 5 and 7).

^{13}C-NMR (75 MHz, DMSO-d_6) δ [ppm] = 171.44 (2), 162.48 (8), 146.55 (6), 132.19 (4), 110.56 (5), 109.91 (3), 106.88 (7).

MS (**ESI**, 179.03 g/mol): m/z = 178 $[M - H]^-$, 379 $[2M + Na – 2H]^-$.

2.4 4-Azidosalicylic acid ethyl ester



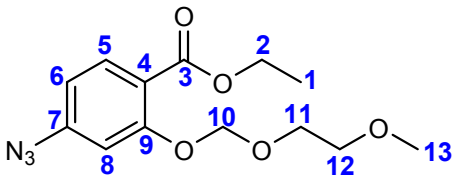
4-Azidosalicylic acid **5** (3.33 g, 18.60 mmol) was dissolved in 120 mL ethanol. After the addition of 8.5 mL concentrated sulfuric acid, the solution was stirred at 75 °C in nitrogen atmosphere overnight. After the solvent was removed *in vacuo,* the crude product was resuspended in n-hexane while stirring for 3 h. The product **6** was received after filtration and removal of the solvent, as a pale yellow powder (3.08 g, 14.88 mmol, 80% yield).[3]

^{1}H-NMR (300 MHz, $CDCl_3$) δ [ppm] = 11.03 (s, 1H, 10), 7.81 (d, J = 8.6 Hz, 1H, 5), 6.62 (d, J = 2.2 Hz, 1H, 8), 6.52 (dd, J = 8.6, 2.2 Hz, 6), 4.40 (q, 2 H, J = 7.1 Hz, 2), 1.41 (t, J = 2.1 Hz, 1).

^{13}C-NMR (75 MHz, $CDCl_3$) δ [ppm] = 169.74 (3), 163.11 (9), 147.35 (7), 131.71 (5), 110.54 (6), 109.66 (8), 107.32 (4), 61.62 (2), 14.33 (1).

MS (**APCI**, 207.0 g/mol): m/z = 208 $[M+H]^+$, 180 $[M+H-C_2H_5]^+$, 151 $[M-N_2+H]^+$.

2.5 4-Azido-2-((2-methoxyethoxy)methoxy)ethyl benzoate



4-Azidosalicylic acid ethyl ester **6** (1.21 g, 5.84 mmol) was dissolved in 18 mL dry THF in argon atmosphere. After addition of TEA (4.86 mL, 35.06 mmol) the solution was cooled by an ice bath and MEM chloride (4.00 mL, 35.06 mmol) was added slowly. The solution was stirred

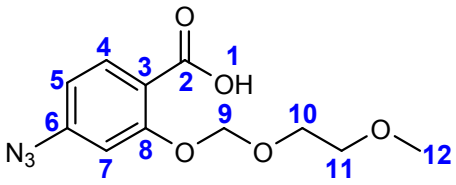
overnight, and the solvent was removed *in vacuo*. The raw product was dissolved in ethyl acetate and washed three times with water. After evaporation of the solvent the raw product was purified by column chromatography (nHex:EA 4:1). The product **7** was received as a yellow oil (1.02 g, 3.45 mmol, 59% yield).

^{1}H-NMR (300 MHz, $CDCl_3$) δ [ppm] = 7.81 (d, J = 8.4 Hz, 1H, 5), 6.88 (d, J = 2.2 Hz, 1H, 8), 6.70 (dd, J = 8.4, 2.2 Hz, 1H, 6), 5.32 (s, 2H, 10), 4.12 (d, J = 7.1 Hz, 2H, 2), 3.89–3.86 (m, 2H, 12), 3.58–3.54 (m, 2H, 11), 3.37 (s, 3H, 13), 1.36 (d, J = 7.1 Hz, 3H, 1).

^{13}C-NMR (75 MHz, $CDCl_3$) δ [ppm] = 165.30 (3), 158.44 (9), 145.23 (7), 133.21 (5), 118.12 (6), 112.18 (8), 107.65 (4), 94.32 (10), 71.58 (12), 68.24 (11), 60.94 (2), 59.14 (13), 14.33 (1).

MS (**ESI**, 295.12 g/mol): m/z = 318 $[M + Na]^+$, 334 $[M + K]^+$.

2.6 4-Azido-2-((2-methoxyethoxy)methoxy)benzoic acid



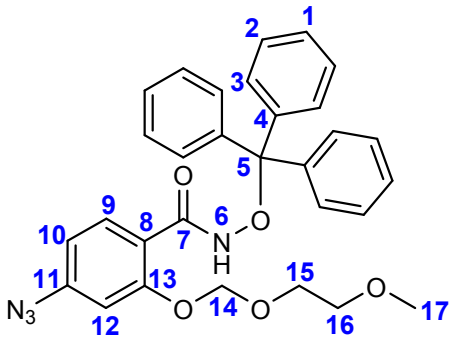
4-Azido-2-((2-methoxyethoxy)methoxy) ethyl benzoate **7** (1.02 g, 3.45 mmol) was dissolved in a mixture of methanol and 1 M NaOH (15 mL each) and heated to 60 °C for 3 h. After the solvent was removed, the raw product was extracted with ethyl acetate to remove eventually leftover educt. The aqueous phase was acidified to pH 5 using 1 M HCl and extracted with ethyl acetate. The solvent was removed and the product **8** was isolated as a yellow powder (0.92 g, 3.45 mmol, quantitative).

^{1}H-NMR (300 MHz, CD_2Cl_2) δ [ppm] = 8.11 (d, J = 8.5 Hz, 1H, 4), 6.92 (d, J = 2.1 Hz, 1H, 5), 6.86 (dd, J = 8.5, 2.1 Hz, 1H, 7), 5.49 (s, 2H, 9), 3.90-3.87 (m, 2H, 11), 3.56–3.53 (m, 2H, 10), 3.32 (s, 3H, 12).

^{13}C-NMR (75 MHz, CD_2Cl_2) δ [ppm] = 164.98 (2), 157.81 (8), 147.29 (6), 135.38 (4), 115.33 (5), 113.74 (7), 106.40 (3), 95.58 (9), 72.11 (11), 70.07 (10), 59.28 (12).

MS (**ESI**, 267.09 g/mol): m/z = 290 $[M + Na]^+$, 306 $[M + K]^+$.

2.7 4-Azido-2-((2-methoxyethoxy)methoxy)-*N*-(trityloxy)benzamide



4-Azido-2-((2-methoxyethoxy)methoxy)benzoic acid **8** (629 mg, 2.35 mmol), trityloxyamine (810 mg, 2.94 mmol), EDC·HCl (564 mg, 2.94 mmol) and DMAP (36 mg, 0.29 mmol) were dissolved in DMF. The reaction mixture was stirred for 72 h under exclusion of light at room temperature. After the solvent was removed in *vacuo* the product was purified by column chromatography (silica gel, EA:nHex 1:4). The product **10** was received as a colorless powder in a yield of 39% (476 mg, 0.90 mmol).

^{1}H-NMR (300 MHz, CD_2Cl_2) δ [ppm] = 9.62 (s, 1H, 6), 8.00 (d, J = 9.0 Hz, 1H, 9), 7.55–7.51 (m, 6H, 2), 7.39–7.30 (m, 9H, 1 and 3), 6.79–6.75 (m, 2H, 10 and 12), 4.92 (s, 2H, 14), 3.46-3.38 (m, 4H, 15 and 16), 3.27 (s, 3H, 17).

^{13}C-NMR (75 MHz, CD_2Cl_2) δ [ppm] = 162.48 (7), 156.43 (13), 145.21 (11), 133.76 (4), 129.35 (2), 128.60 (3), 128.32 (1), 117.81 (10), 113.17 (8), 106.08 (12), 94.28 (5), 93.16 (14), 71.85 (16), 69.14 (15), 59.22 (17).

MS (**ESI**, 524.21 g/mol): m/z = 243 $[Trt]^+$, 547 $[M + Na]^+$, 1071 $[2M + Na]^+$.

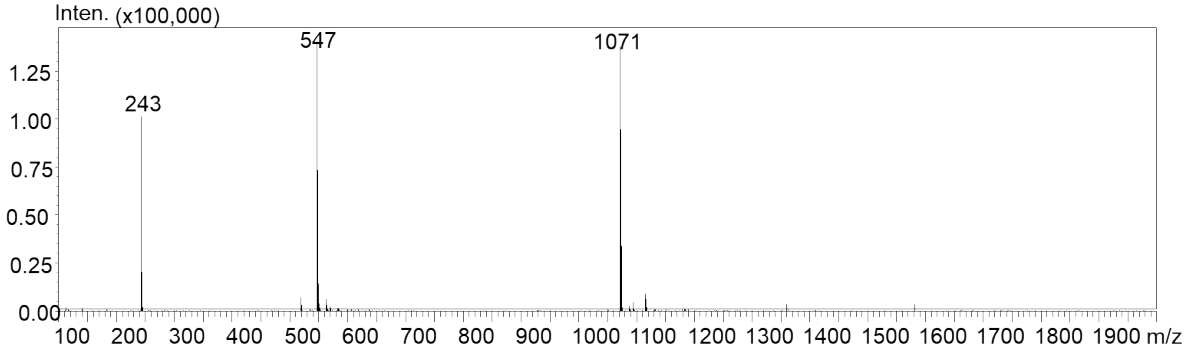


Figure S2: ESI mass (positive mode) of N_3-SHA. m/z calculated: $[M+H]^+$ = 524 g/mol, found: $[2M+Na]^+$ = 1071 g/mol, $[M+Na]^+$ = 547 g/mol, $[Trt]^+$ = 243 g/mol. Note: The acid-labile protecting group is partially removed during the measurement.

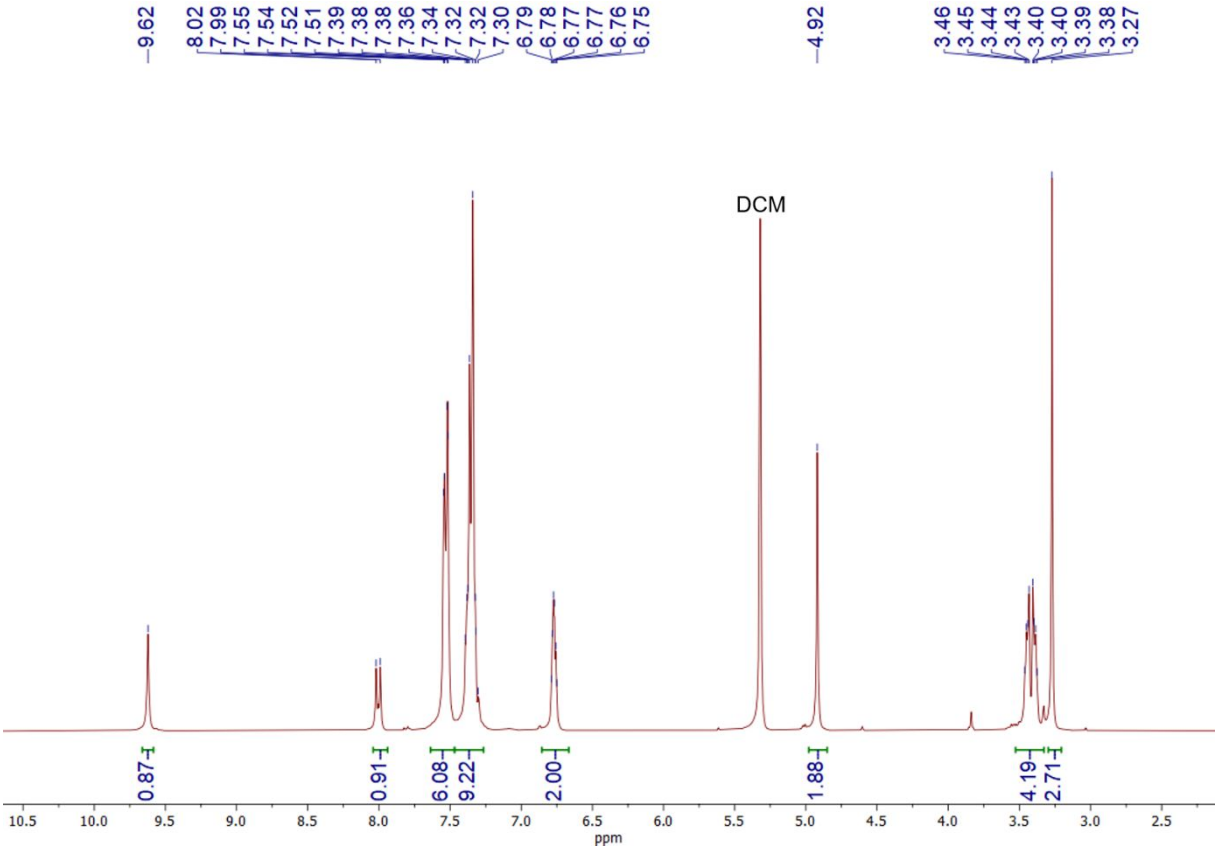


Figure S3: ^{1}H-NMR of the protected azidosalicylhydroxamate derivative measured in CD_2Cl_2.

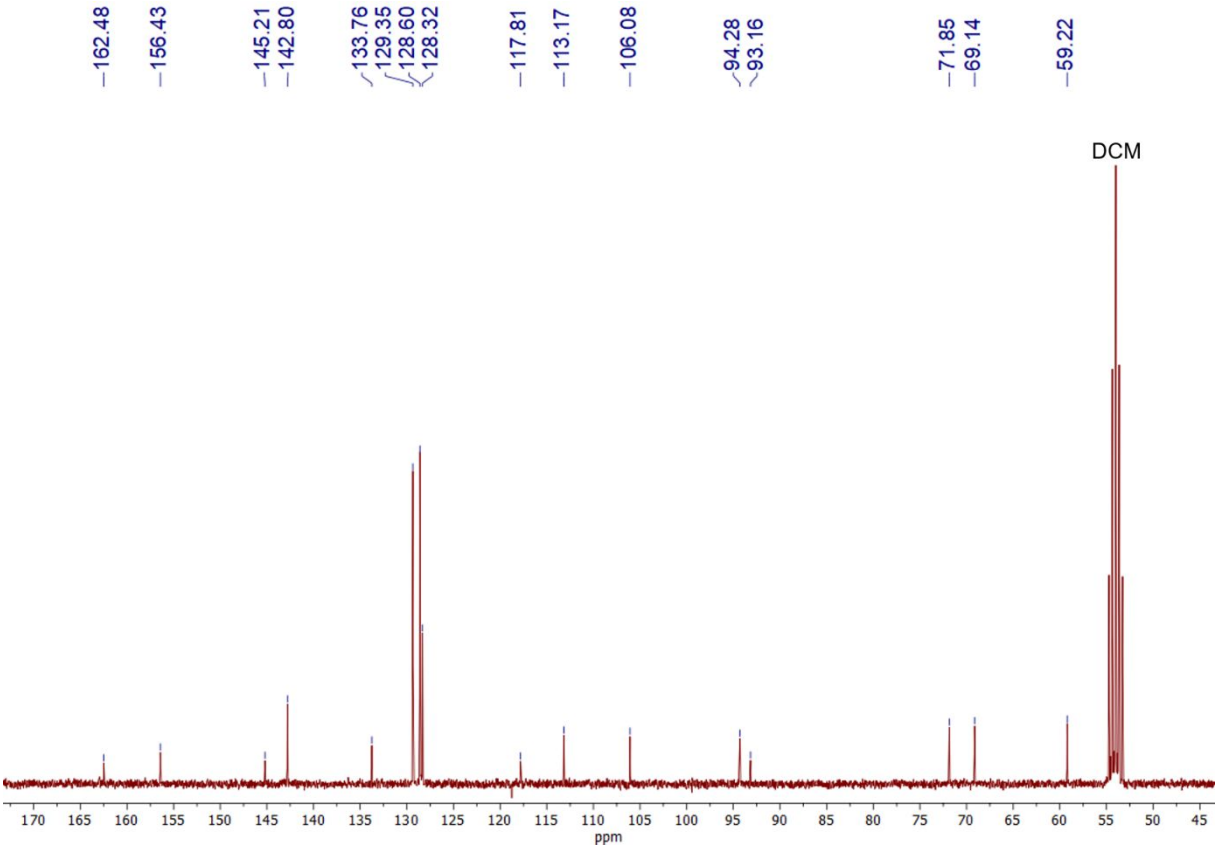


Figure S4: ^{13}C-NMR of the protected azidosalicylhydroxamate derivative **10** measured in CD_2Cl_2.

2.8 Peptide Synthesis

2.8.1 Depsi(Fmoc-I)pba-SA

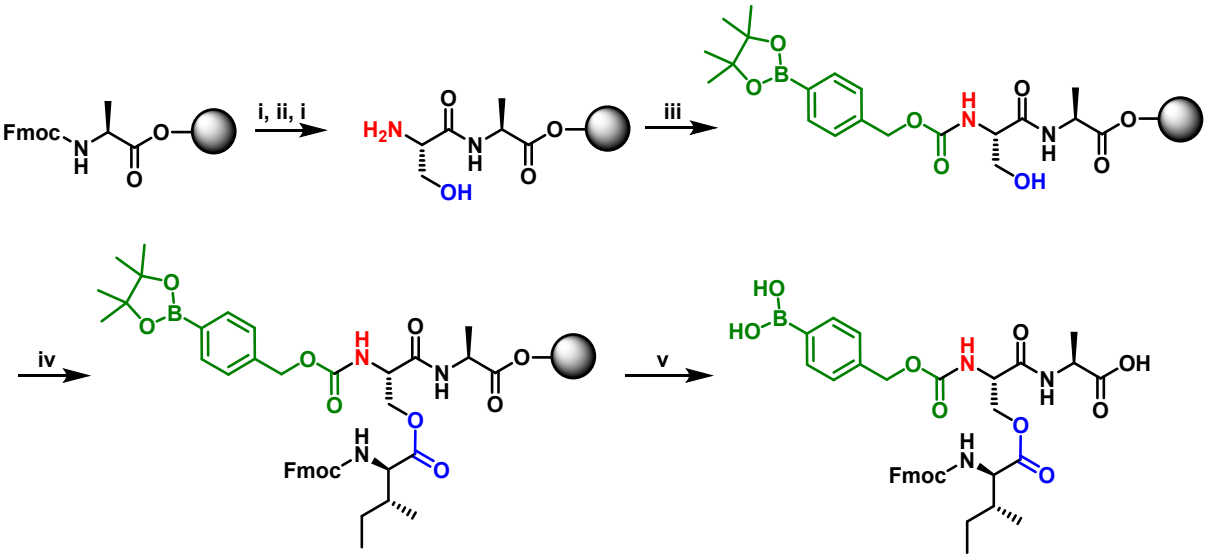
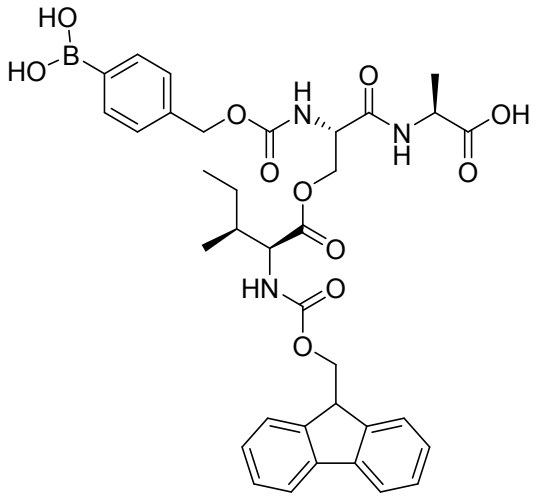


Figure S5: **i** Piperidine 20% v/v in DMF, 2 and 5 min, 75 °C, **ii** Fmoc-Ser, PyBOP, DIPEA in DMF, 20 min, 75 °C, **iii** 4-(nitrophenyl)phenylboronic acid pinacol ester, DIPEA in DMF, o.n., rt, **iv** Fmoc-Ile, DIC, 4-DMAP in DMF, 2 h and o.n., rt, **v** TFA/TIPS/H_2O 2 h, rt.

Peptides were synthesized using the Fmoc solid phase peptide synthesis strategy by Merrifield, synthesizing the peptide from *C* to *N*-terminus in a microwave assisted peptide synthesizer. Fmoc-Ala preloaded Wang resin (0.5 mmol) was swollen in DMF for 1 h before use. First, the Fmoc group was removed by two consecutive deprotection steps (2 and 5 min) with 20% piperidine in DMF (10 mL) at 75 °C (**i**). After deprotection, the resin was washed four times with DMF (7 mL). Fmoc-serine (5 equiv in 10 mL DMF) was coupled to the *N*-terminus by using the activator PyBOP (5 equiv in 4 mL DMF) and activator base DIPEA (10 equiv in 2 mL DMF) at 75 °C for 20 min (**ii**). After deprotection of the Fmoc protecting group (**i**), the *N*-terminus was modified using 4-nitrophenyl (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl) carbonate (1.25 equiv) in 5 mL DMF and DIPEA (5 equiv) overnight at room temperature (**iii**). After the resin was washed with DMF and DCM, Fmoc-Ile (10 equiv in 5 mL DMF), DIC (10 equiv) and 4-DMAP (1 equiv) were added to form the ester bond in the serine

side chain. After the reaction mixture was stirred for 2 h at room temperature, fresh Fmoc-Ile, DIC and 4-DMAP were added and stirred overnight to ensure full conversion of the coupling (**iv**). After washing with DMF and DCM, the resin was dried and half of the peptide was cleaved from the solid support by using 2 mL of a cleavage cocktail (95% TFA, 2.5% TIPS, 2.5% H_2O). This step also removed the pinacol protecting group of the PBA. After 2 hours, the cleavage cocktail was removed *in vacuo* (**v**).

The peptide was purified by HPLC with the Atlantis T3 column (flowrate 10 mL/min). The gradient started with 5% ACN, which was kept constant for 1 min, then the ACN content was increased linearly to 100% within 30 min. The retention time of the product was 25.0 min. After lyophilisation, the peptide **1a** (13.12 mg, 19.03 µmol, 7.6% yield) was received as a white powder.



Exact Mass: 689,28

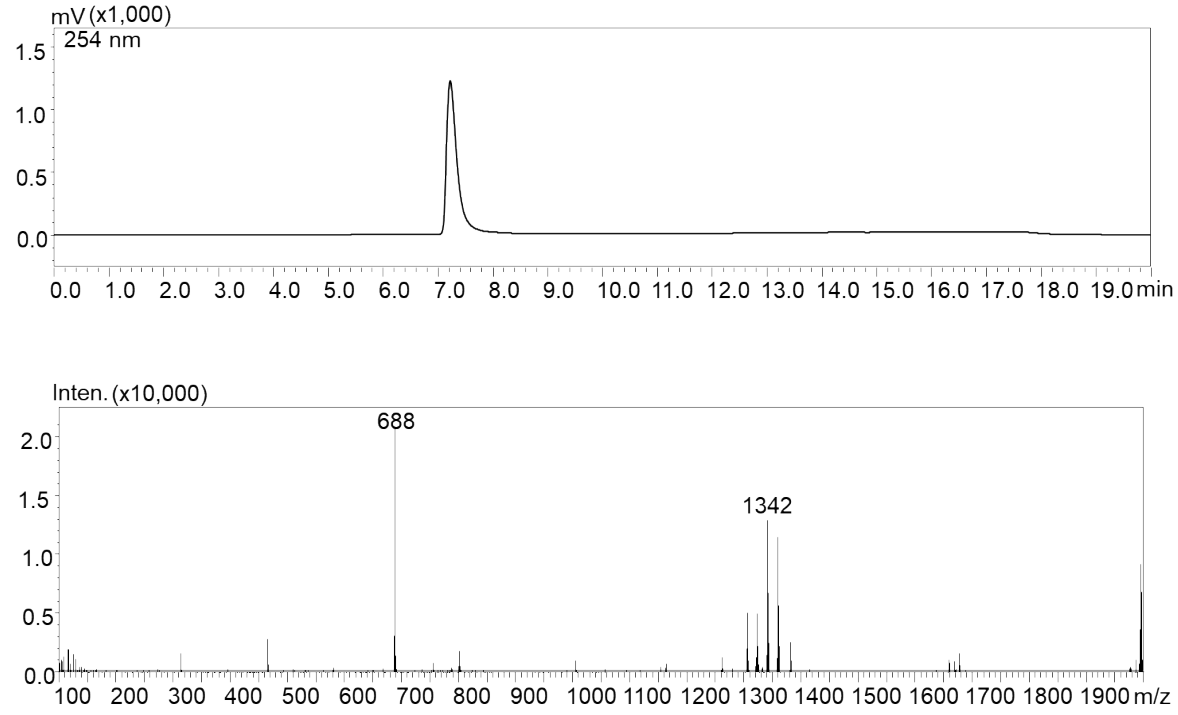
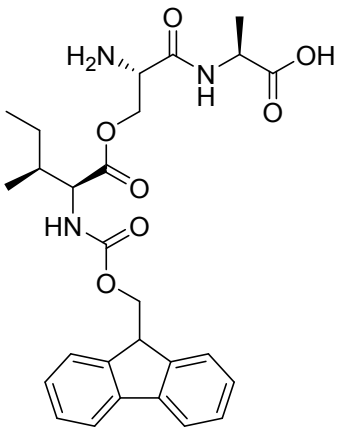


Figure S6: LC-spectrum (top) and ESI mass (negative mode, bottom) of Depsi(Fmoc-I)pba-SA. m/z calculated: $[M-H]^-$ = 688 g/mol, found: $[M-H]^-$ = 688 g/mol, $[2\ M-2\ H_2O-H]^-$ = 1342 g/mol.

2.8.2 Depsi(Fmoc-I)SA

Depsi(Fmoc-I)SA was synthesized using Boc-Ser, therefore no Fmoc deprotection was performed after the coupling of Boc-Ser. The peptide was synthesized at a scale of 0.1 mmol. After removal of the Fmoc group of alanine (3 mL of 20% v/v piperidine in DMF, 2x 5 min), Boc-Ser was coupled for 10 min at 75 °C using 5 equiv of the amino acid in 2.5 mL DMF, PyBOP (5 equiv, in 1 mL DMF) and DIPEA (10 equiv, in 0.5 mL DMF). Fmoc-Ile was coupled to the side chain of serine by heating to 75 °C for 1 h, which was repeated after addition of fresh reagents, to ensure full conversion. The same concentrations and equivalents as for Boc-Ser coupling were used. After cleavage from the solid phase as described before, the peptide was purified by HPLC using the Zorbax Eclipse column at a flowrate of 4 mL/min. The gradient started with 5% ACN in water (+0.1% TFA) and this solvent ratio was kept for 1 min, after which the ACN content was increased to 100% in 15 min. The peptide **2a** eluted from the column after 12.4 min and was received as a white powder (4.90 mg, 9.58 µmol, 9.6% yield).



Exact Mass: 511,23

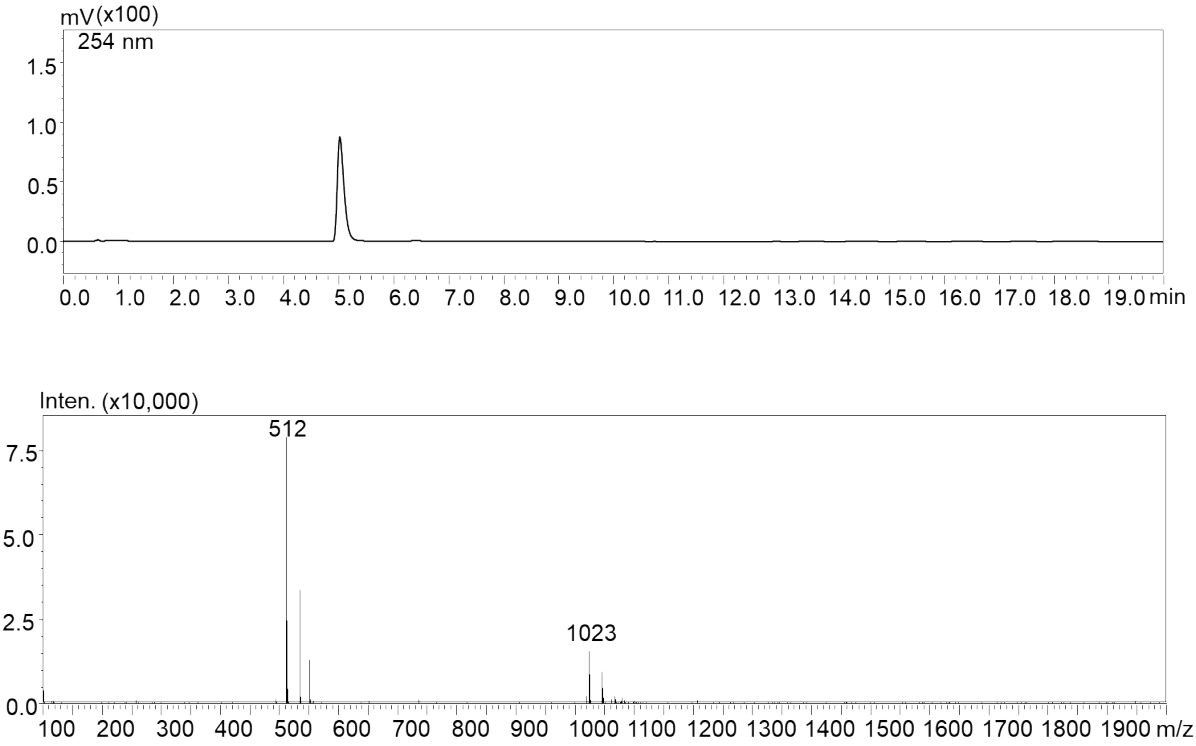
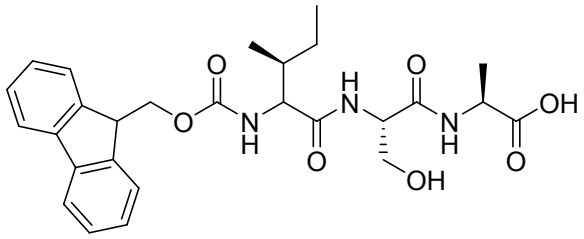


Figure S7: LC-spectrum (top) and ESI mass (positive mode, bottom) of Depsi(Fmoc-I)-SA. m/z calculated: $[M+H]^+$ = 512 g/mol, found: $[M+H]^+$ = 512 g/mol, $[2\ M+H]^+$ = 1032 g/mol.

2.8.3 Fmoc-ISA

The peptide was synthesized using a 0.1 mmol scale. After deprotection of the Fmoc group (3 mL of 20% piperidine in DMF, 2 and 5 min at 75 °C), Fmoc-Ser(tBu)/Fmoc-Ile were double coupled onto the peptide at 75 °C for 20 min. 5 equiv of the amino acids were used in 2.5 mL DMF, PyBOP (5 equiv in 1 mL DMF) and DIPEA (10 equiv in 0.5 mL) were used for the

coupling. After cleavage from the solid support, the peptide was purified by HPLC using the Atlantis T3 column (flowrate 10 mL/min) and a gradient starting from 5% ACN in water, 0.1% TFA. After keeping this solvent composition for 1 min, the amount of ACN was increased linearly within 30 min to reach 100% ACN. Fmoc-ISA **3a** eluted after 24.2 min. 13.8 mg (26.99 mmol, 27.0% yield) of the peptide were received.



Exact Mass: 511,23

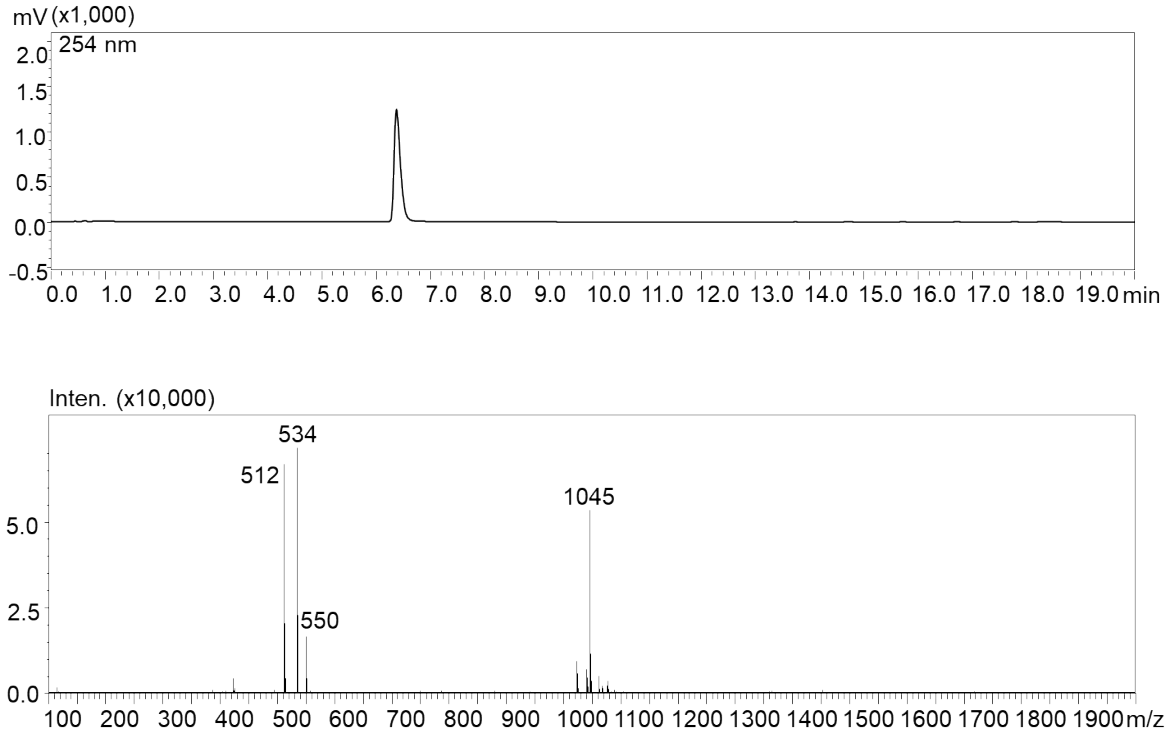
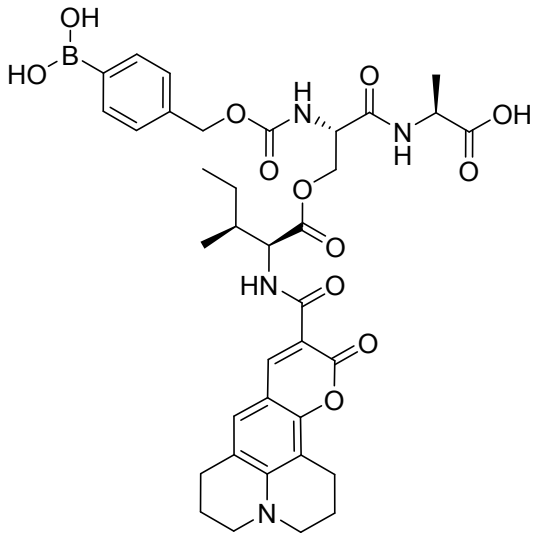


Figure S8: LC-spectrum (top) and ESI mass (positive mode, bottom) of Fmoc-ISA. m/z calculated: $[M+H]^+$ = 512 g/mol, found: $[M+H]^+$ = 512 g/mol, $[M+Na]^+$ = 534 g/mol, $[M+K]^+$ = 550 g/mol, $[2\ M+Na]^+$ = 1045 g/mol.

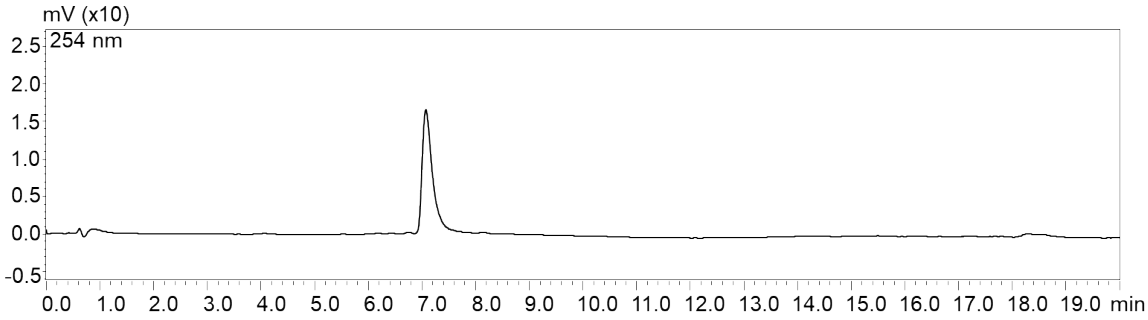
2.8.4 Depsi(C343-I)pba-SA

In order to synthesize Depsi(C343-I)pba-SA, Depsi(Fmoc-I)pba-SA (0.25 mmol) was not cleaved from the solid support, but instead the Fmoc group was removed in two deprotection

steps using 20% piperidine in DMF (10 min each, 5 mL) at room temperature. Coumarin 343 (1.2 equiv) was coupled onto the *N*-terminus overnight at room temperature, using PyBOP (2 equiv) and DIPEA (4 equiv) as coupling reagents. The resin was washed with DMF and DCM, dried and then the peptide was cleaved from the resin as described before. The peptide was purified by HPLC with the Atlantis T3 column at a flowrate of 10 mL/min using a gradient starting with 5% ACN and 95% water, both acidified with 0.1% TFA. The solvent composition was kept constant for 1 min and then the ACN content was increased linearly to 100% in 36 min. The retention time of the product was 28.8 min and the peptide **1b** was received as an orange powder (4.12 mg, 5.61 µmol, 2.2% yield).



Exact Mass: 734,30



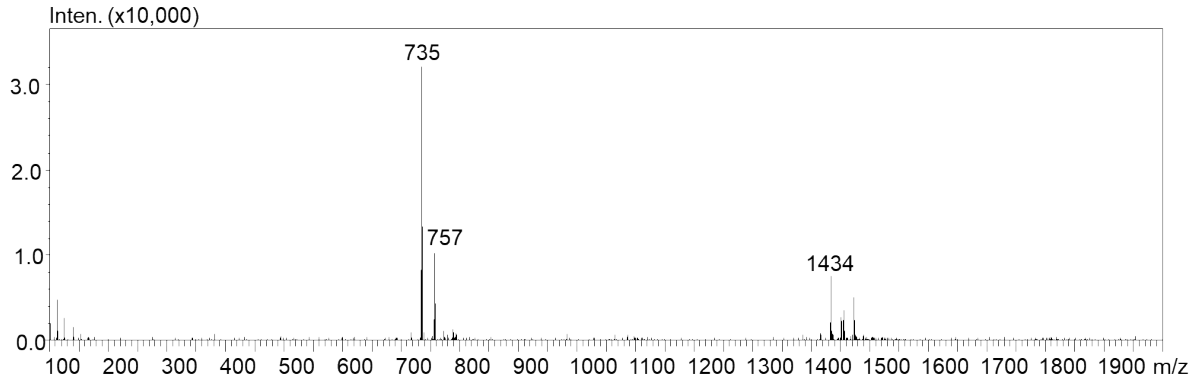
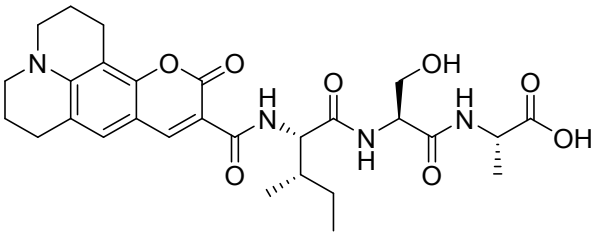


Figure S9: LC-spectrum (top) and ESI mass (positive mode, bottom) of Depsi(C343-I)pba-SA. m/z calculated: $[M+H]^+$ = 735 g/mol, found: $[M+H]^+$ = 735 g/mol, $[M+Na]^+$ = 757 g/mol, [2 M-2 H_2O+H]$^+$ = 1434 g/mol.

2.8.5 C343-ISA

Fmoc-ISA (0.05 mmol) bound to the Wang resin was treated with 20% piperidine in DMF for 2 and 5 min at 75 °C in order to remove the Fmoc protecting group. Coumarin 343 (C343) was coupled as described in section 2.8.4. The peptide was purified by HPLC using the Phenomenex column at a flowrate of 25 mL/min. The gradient started at 5% ACN in water (+0.1% TFA) and these conditions were kept for 1 min, after which the ACN concentration was increased to 100% in 36 min. The retention time of C343-ISA was 22.8 min. The yellow powdered peptide **3b** was received in a yield of 11.7% (3.25 mg, 5.84 µmol).



Exact Mass: 556,25

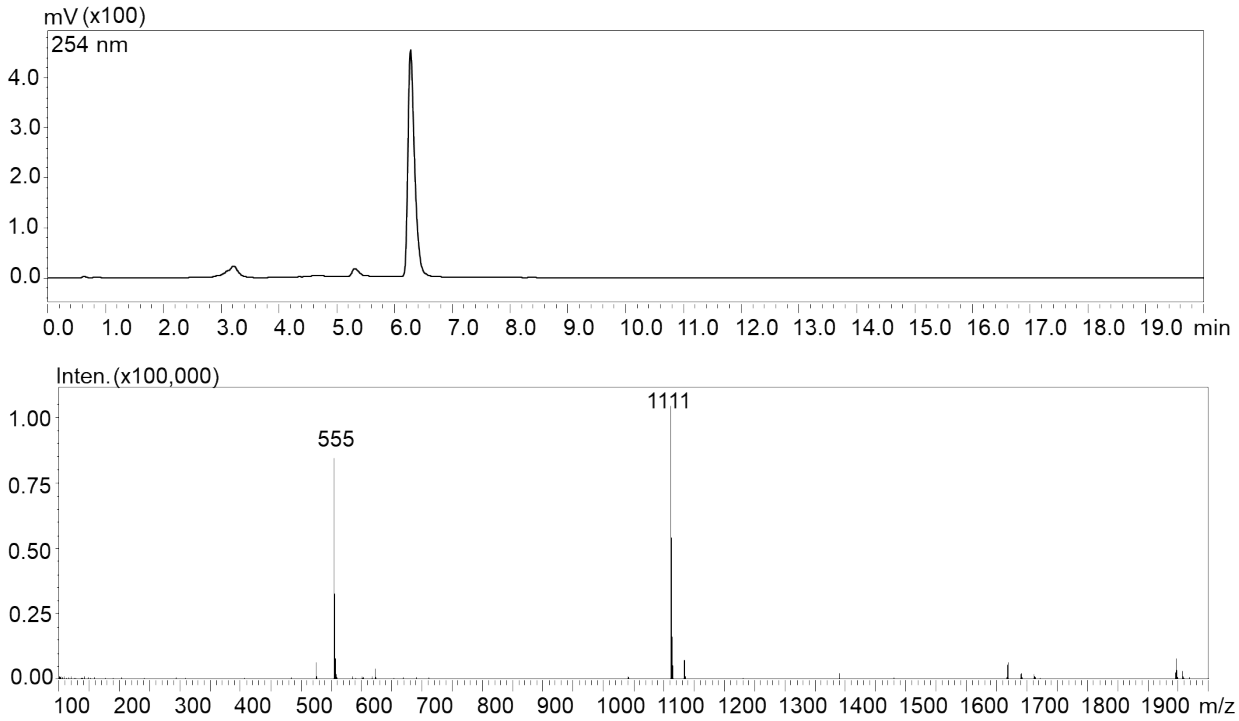


Figure S10: LC-spectrum (top) and ESI mass (negative mode, bottom) of C343-ISA. m/z calculated: $[M-H]^-$ = 555 g/mol, found: $[M-H]^-$ = 555 g/mol, $[2\ M-H]^-$ = 1111 g/mol.

2.8.6 SHA-TAT

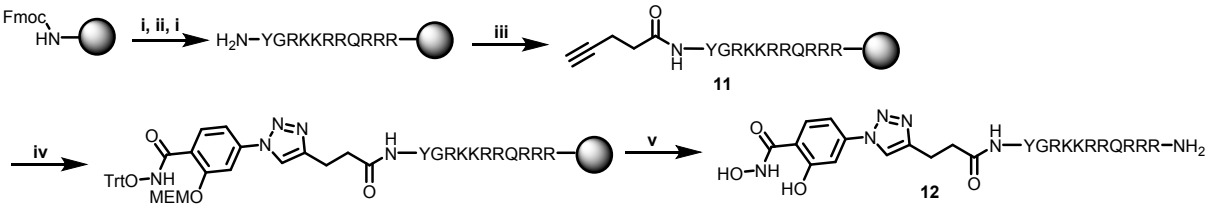
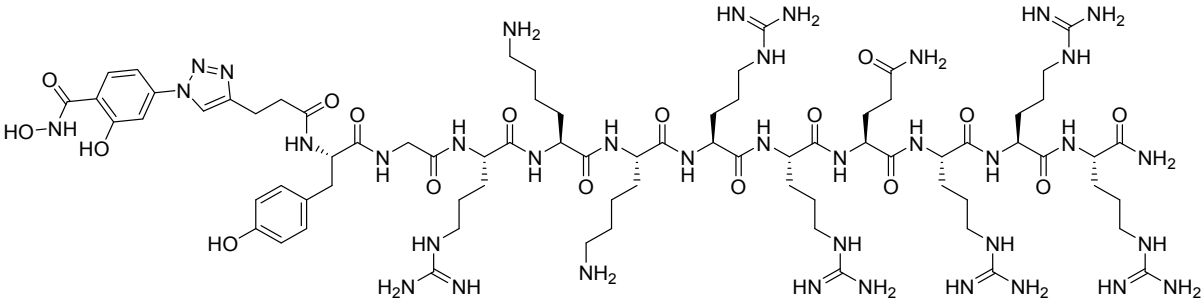


Figure S11: Synthetic scheme of the synthesis of SHA-Tat. **i** Piperidine 20% in DMF, 2 and 5 min for 75 °C, **ii** Fmoc-AA, PyBOP, DIPEA, DMF, 20 min, 75 °C, **iii** 4-pentynoic acid, PyBOP, DIPEA, DMF, overnight, rt, **iv** protected 4-azidosalicylhydroxamate, CuI, DIPEA, DMF, room temperature, overnight. **v** TFA, TIPS, H_2O, 2 h, rt.

SHA-TAT was synthesized using Rink amide resin at a scale of 0.1 mmol. Before every coupling, the Fmoc group was cleaved by two deprotection steps using 20% v/v piperidine in DMF (3 mL) for 2 and 5 min at 75 °C (**i**). Fmoc-Arg(Pbf), Fmoc-Gln(Trt), Fmoc-Lys(Boc), Fmoc-Gly and Fmoc-Tyr(tBu) (5 equiv in 2.5 mL) were coupled for 20 min using PyBOP (5 equiv in 1 mL) and DIPEA (10 equiv) in 0.5 mL DMF (**ii**). After coupling of 4-pentynoic acid using the same coupling reagents at room temperature overnight (**iii**), a CuAAC reaction was performed

overnight using protected azido-SHA (1 equiv) and CuI (0.1 equiv) as well as DIPEA (1.25 equiv) for catalysis (**iv**). The peptide was cleaved from the solid support as described before, which also removed all protecting groups (**v**). The peptide was purified by HPLC using the Atlantis T3 column at a 10 mL/min flowrate. The gradient started at a 95:5 ratio of water:ACN (+0.1% TFA) and was kept constant for 1 min, after which the ACN content was increased to 30% in 29 min. The retention time of SHA-TAT **12** was 23.0 min. The peptide was received in a yield of 7.1% (12.95 mg, 7.07 µmol).



Exact Mass: 1832,04

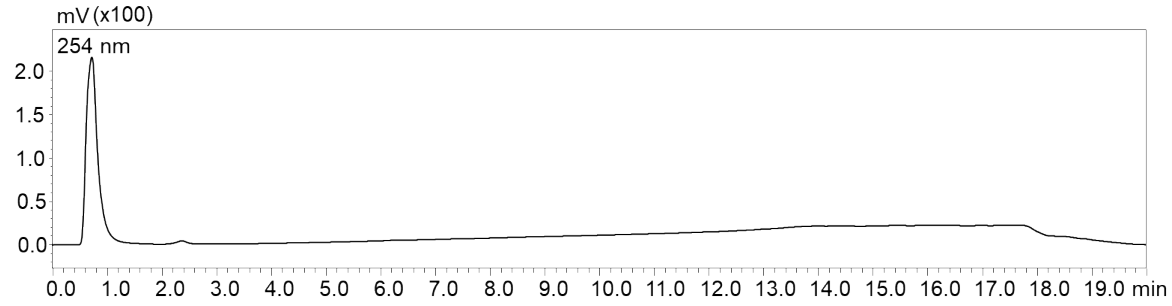


Figure S12: LC-spectrum of SHA-TAT measured at 254 nm.

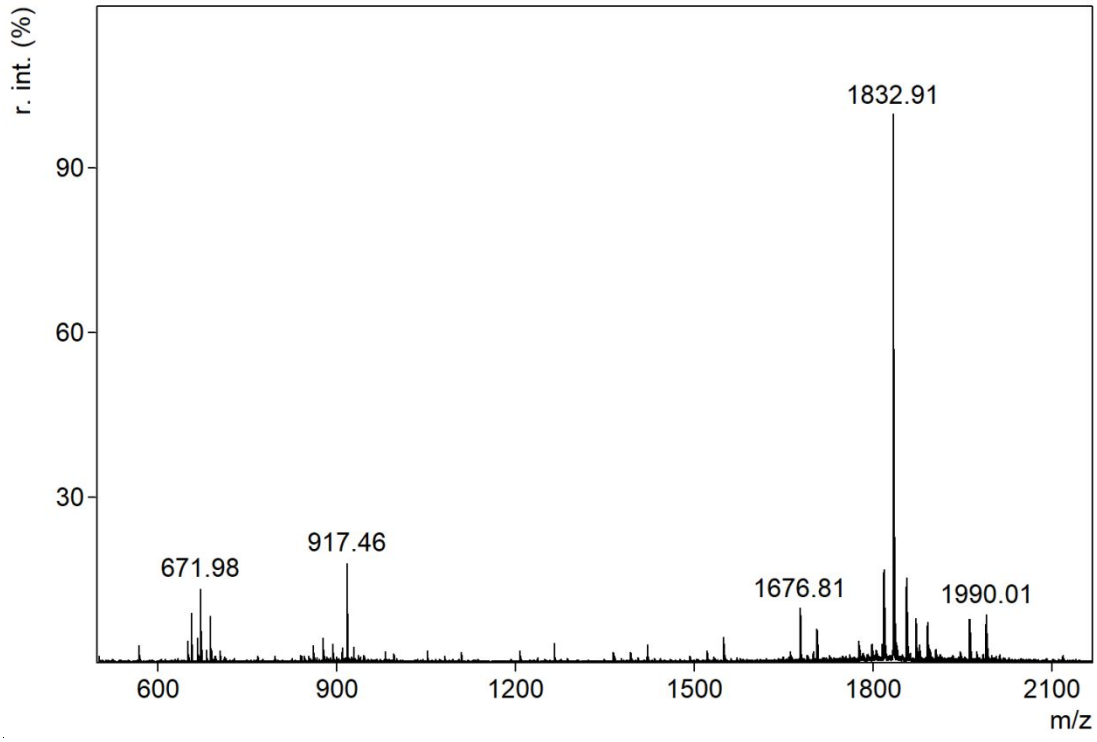
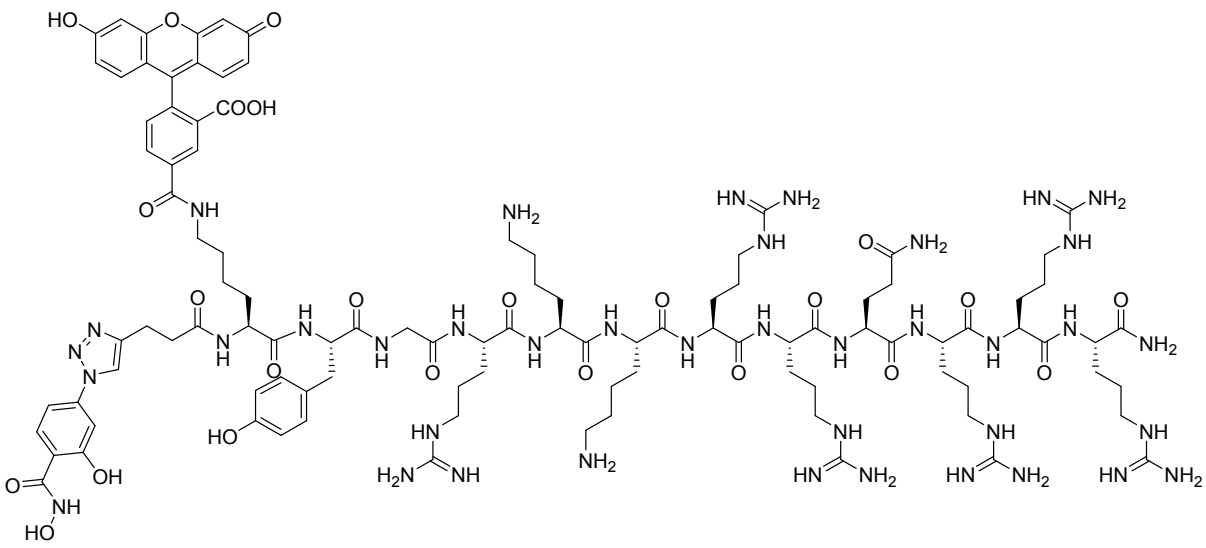


Figure S13: MALDI-TOF MS of SHA-TAT using α-cyano-4-hydroxycinnamic acid as the matrix. m/z calculated: $[M+H]^+$ = 1832 g/mol, found: $[M+TFA+3H]^{3+}$ = 671 g/mol, $[M+2H]^{2+}$ = 917 g/mol, $[M-N_3SHA+K]^+$ = 1676 g/mol, $[M+H]^+$ = 1832 g/mol, $[M+TFA+2Na-H]^+$ = 1990 g/mol. Note: SHA is partially cleaved from the peptide during the measurement.

2.8.7 SHA-Lys(5-FAM)-TAT

Fluorescent SHA-Lys(5-FAM)-TAT **14** was synthesized following the procedure described for SHA-TAT **12** in 2.8.6 at a scale of 0.025 mmol. Before coupling of 4-pentynoic acid, Fmoc-Lys(5-FAM)-OH was coupled to the TAT peptide at room temperature for 2 h using the amino acid (2.75 equiv), PyBOP (5 equiv) and DIPEA (10 equiv). After removal of the Fmoc protecting group at room temperature (2 × 20 min) using 20% piperidine in DMF and washing, 4-pentynoic acid and the salicylhydroxamate residue were attached as described before. The peptide was cleaved from the solid support with a cleavage cocktail containing TFA (95%), TIPS (2.5%) and water (2.5%) and purified by preparative HPLC using an Atlantis T3 column at a 10 mL/min flowrate. The gradient started at a 5:95 ratio of ACN: water (+0.1% TFA) and was kept constant for 1 min, after which the ACN content was increased to 30% in 29 min. The

retention time of **14** was 30.0 min. The peptide was received in a yield of 3.0% (1.74 mg, 0.75 µmol).



Exact Mass: 2318,18

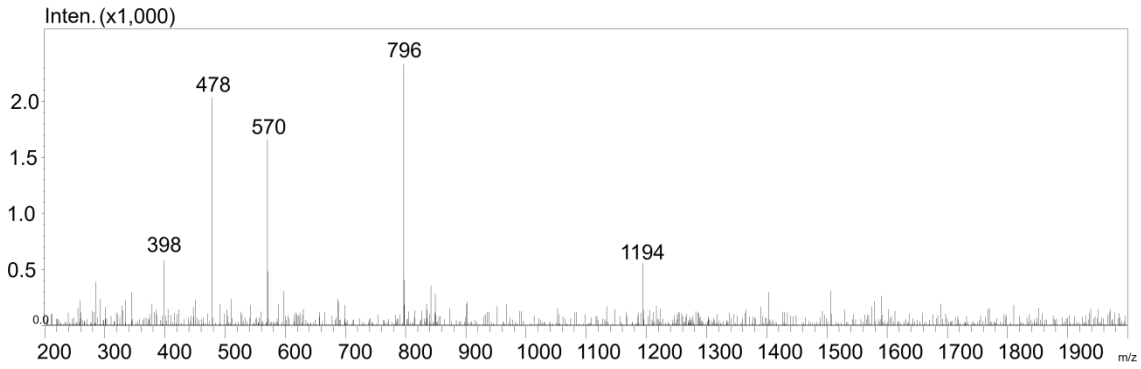


Figure S14: ESI mass spectrum (positive mode) of SHA-Lys(5-FAM)-TAT. m/z calculated: [M] = 2318 g/mol, found: $[M+3Na+3H]^{6+}$ = 398 g/mol, $[M+3Na+2H]^{5+}$ = 478 g/mol, $[M+3Na+4TFA+3Na+2H]^{5+}$ = 570 g/mol, $[M+3Na]^{3+}$ = 796 g/mol, $[M+3Na-H]^{2+}$ = 1194 g/mol.

3. Characterization

3.1 H_2O_2 induced *O, N*-Acyl Shift

Depsi(Fmoc-I)pba-SA **1a** was dissolved at a concentration of 0.1 mg·mL^{-1} in 700 µL THF and an equal amount of NH_4HCO_3 buffer 5 mM pH 7.4 was added, which either did or did not contain H_2O_2 (2 mM final concentration). At certain time points aliquots of 100 µL were analyzed using an analytical HPLC setup. The solvent gradient started at 5% ACN and 95% water, this concentration was kept for 1 min, after which the ACN content was increased to 100% linearly in 15 min. The reaction was monitored for 45 h (Figure 2 b-c).

To further confirm the identity of the product, a reaction mixture was analyzed using a LC-MS setup after incubation of 30 µg of peptide for 48 h in NH_4HCO_3 buffer 5 mM pH 7.4 mixed with methanol (1:1) with addition of 1 mM hydrogen peroxide (Figure S15).

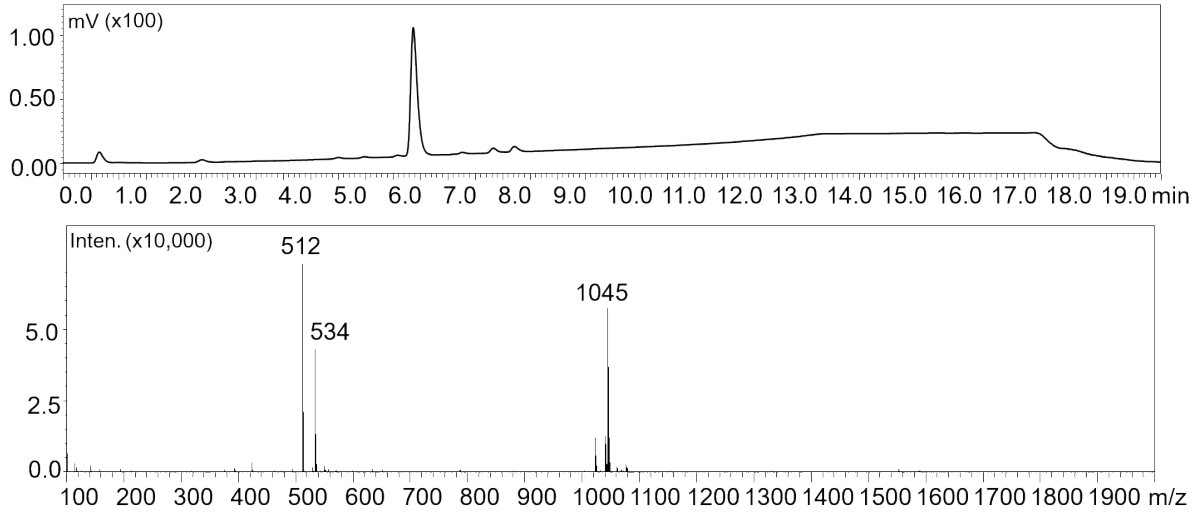


Figure S15: LC-spectrum (top) and ESI mass (positive mode, bottom) of Fmoc-ISA generated after incubation of **1a** with hydrogen peroxide. m/z calculated: $[M+H]^+$ = 512 g/mol, found: $[M+H]^+$ = 512 g/mol, $[M+Na]^+$ = 534 g/mol, $[2\ M+Na]^+$ = 1045 g/mol.

The same experiment was repeated with Depsi(C343-I)pba-SA **1b**, however the concentration and solvents were altered since a LC-MS setup was used. 30 µg of the peptide were dissolved

in 300 µL methanol and 300 µL of NH_4HCO_3 buffer 5 mM pH 7.4 were added, which either did or did not contain H_2O_2 (1 mM final concentration). At certain time points 10 µL of the solutions were analyzed by LC-MS. The solvent gradient started with 5% ACN and 95% water (+0.1% formic acid), while the ACN content was linearly increased to 95% in 12 min. The reaction was monitored for 24 h (Figure 2 d-e, Figure S16).

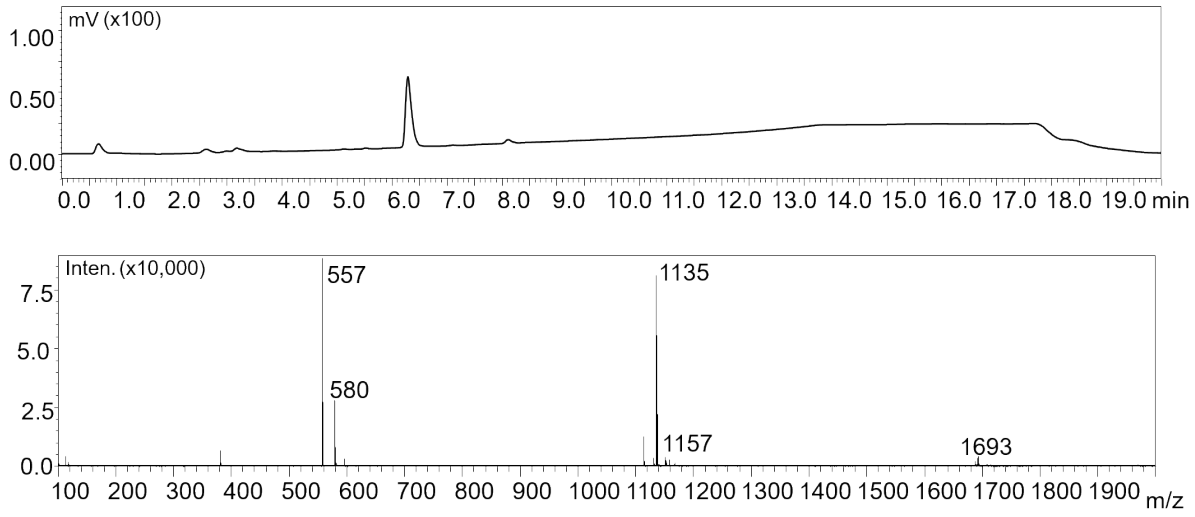


Figure S16: LC-spectrum (top) and ESI mass (positive mode, bottom) of C343-ISA **3a** generated after incubation of **1b** with hydrogen peroxide. m/z calculated: $[M+H]^+$ = 557 g/mol, found: $[M+H]^+$ = 557 g/mol, $[M+Na]^+$ = 580 g/mol, $[2\ M+Na]^+$ = 1135 g/mol, $[2\ M+2Na]^+$ = 1157 g/mol, $[3\ M+Na]^+$ = 1693 g/mol.

3.2 H_2O_2 induced Peptide Assembly

After Depsi(Fmoc-I)pba-SA **1a** or Depsi(C343-I)pba-SA **1b** were dissolved in DMSO at a concentration of 10 mM, the stock solution was diluted to 1 mM with phosphate buffer (PB) 5 mM pH 7.4, which either contained 10 mM hydrogen peroxide or did not. After 24 h incubation at room temperature, TEM measurements were performed as described above (see Figure 2 f-i). For reference C343-ISA **3b** and Fmoc-ISA **3a** were also incubated at the same conditions without addition of H_2O_2 (Figure S17 and Figure S18).

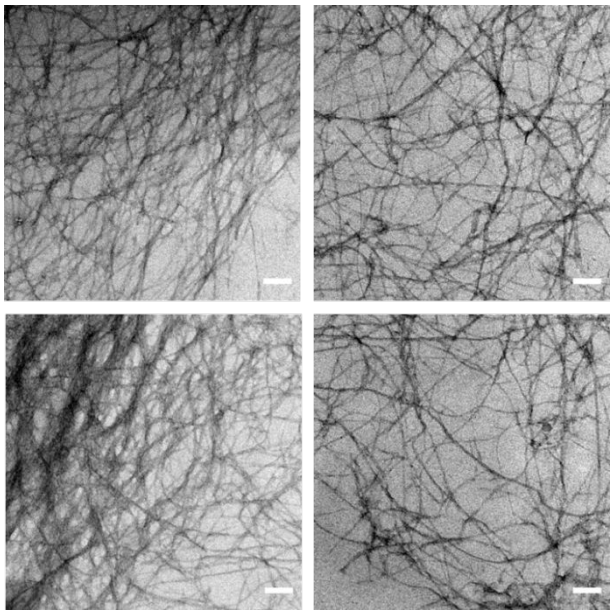


Figure S17: TEM micrographs of Fmoc-ISA **3a** incubated for 24 h in PB:DMSO 9:1. Scale bars 500 nm.

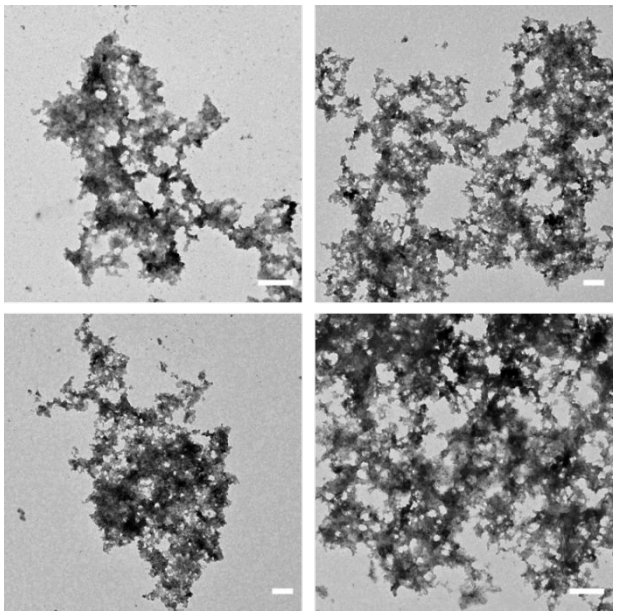


Figure S18: TEM of C343-ISA **3b** after 24 h incubation in PB:DMSO 9:1. Scale bars 500 nm.

To show that peptide fiber formation was also possible at biologically relevant hydrogen peroxide concentrations, Depsi(Fmoc-I)pba-SA **1a** was incubated with 100 µM and 10 µM H_2O_2 (Figure S19).

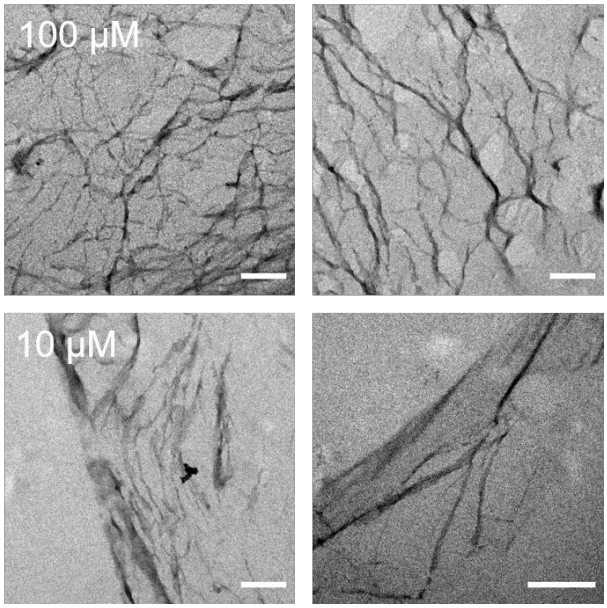


Figure S19: TEM micrographs after incubation of 1 mM Depsi(Fmoc-I)pba-SA **1a** for 24 h in PB:DMSO 9:1 with low concentrations of H_2O_2. Scale bars 500 nm.

For coincubation of the peptides, each peptide was predissolved in DMSO and they were mixed in the respective ratio. After addition of phosphate buffer pH 7.4 5 mM the solutions were incubated for 24 h at room temperature and then TEM samples were prepared as described above (Figure S20–22).

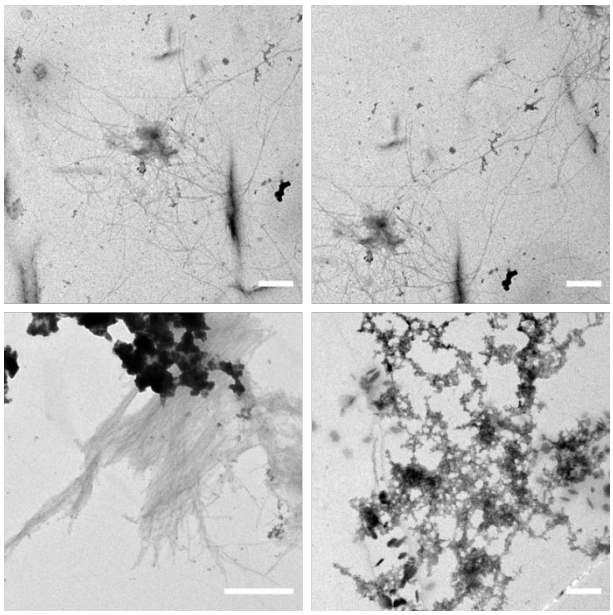


Figure S20: Incubation of Fmoc-ISA **3a** and C343-ISA **3b** at a ratio of 5:1 and a total concentration of 1 mM of peptide in PB pH 7.4 5 mM to DMSO 9:1. Scale bars 500 nm.

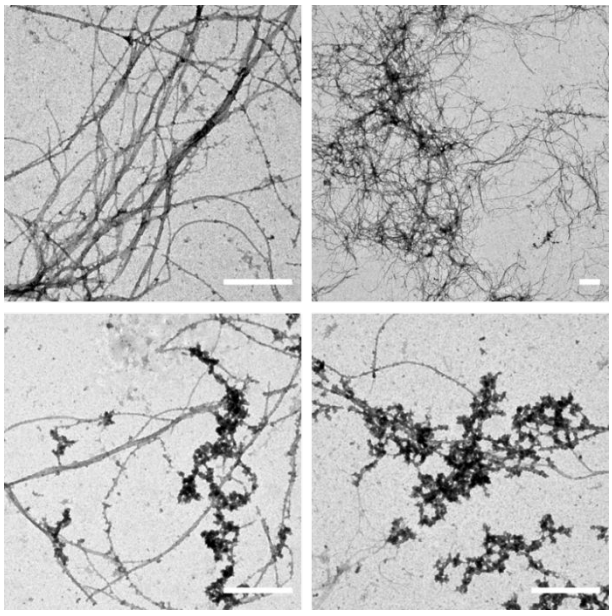


Figure S21: Incubation of Depsi(Fmoc-I)pba-SA **1a** and Depsi(C343-I)pba-SA **1b** at a ratio of 9:1 and a total concentration of 150 µM of peptide in PB pH 7.4 5 mM to DMSO 9:1 upon addition of 1 mM hydrogen peroxide. Scale bars 500 nm.

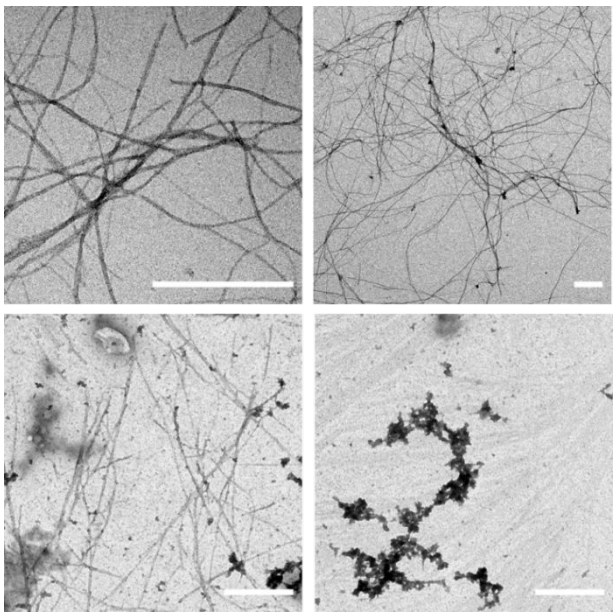


Figure S22: Incubation of Depsi(Fmoc-I)pba-SA **1a** and Depsi(C343-I)pba-SA **1b** at a ratio of 99:1 and a total concentration of 150 µM of peptide in PB pH 7.4 5 mM to DMSO 9:1 upon addition of 1 mM hydrogen peroxide. Scale bars 500 nm.

In order to see the influence of SHA-TAT **12** on structure formation, TEM images of 1 mM **13a/b** (5:1 ratio **13a**:**13b**) with or without 10 mM H_2O_2 and SHA-TAT **12** were taken after incubation overnight in phosphate buffer pH 7.4 5 mM and 10% DMSO. The results showed

that fibers were formed upon addition of hydrogen peroxide showing the conversion into **3a/b**, while small drying artifacts were observed for **12** and **13a/b**.

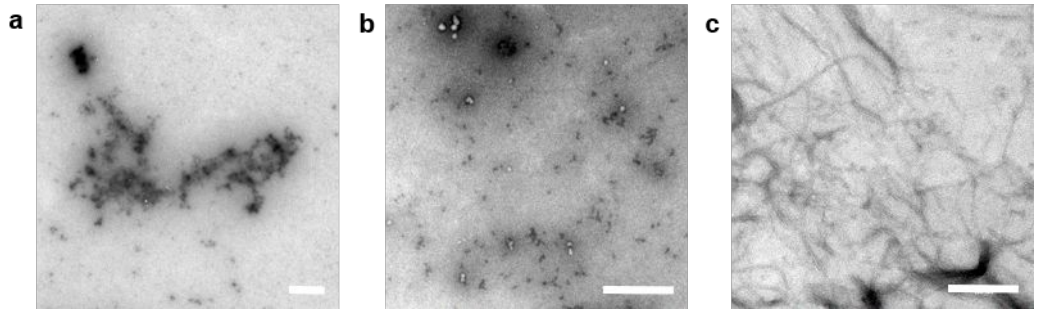


Figure S23: TEM micrograph of SHA-TAT **12** (**a**) and **13a/b** without (**b**) and with the addition of 10 mM H_2O_2 (**c**). Scale bars 500 nm.

LC-MS data confirmed the formation of the linear peptide **3a** upon incubation of **13a** for 48 h in NH_4HCO_3 buffer 5 mM pH 7.4 mixture with methanol (1:1) with addition of 1 mM hydrogen peroxide. The complex **13a** was formed 15 min before addition of H_2O_2 using **1a** (30 µg, 0.04 µmol) and **12** (80 µg, 0.04 µmol).

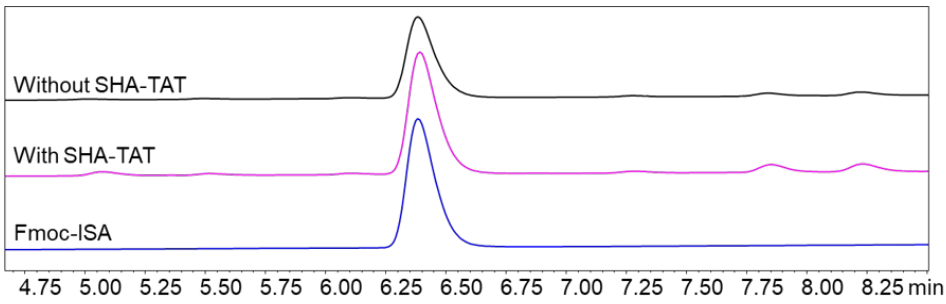


Figure S24: LC-spectra measured at 254 nm showing the conversion of **1a** (black line) and **13a** (purple line) to **3a** (control, blue line) upon treatment with hydrogen peroxide.

Furthermore, the pH dependency of the conversion of **13a/b** into **3a/b** was checked by TEM microscopy. No fibers but drying artifacts were observed upon incubation at pH 6.0 and 5.0 which indicates the pH dependency of the oxidation of boronic acids. Samples were prepared the same way as samples at pH 7.4.

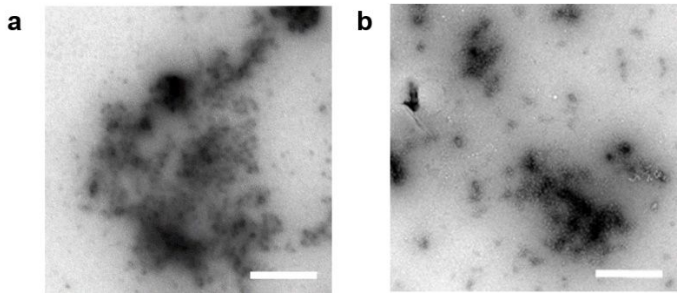


Figure S25: TEM images of structures formed after incubation of **13a/b** in phosphate buffer at pH 6.0 (a) and pH 5.0 (b).

3.3 Critical Fibrillation Concentration

A dilution series of Fmoc-ISA **3a** in PBS:DMSO (9:1) was prepared and the solutions were shaken overnight at room temperature, after which TEM grids were prepared.

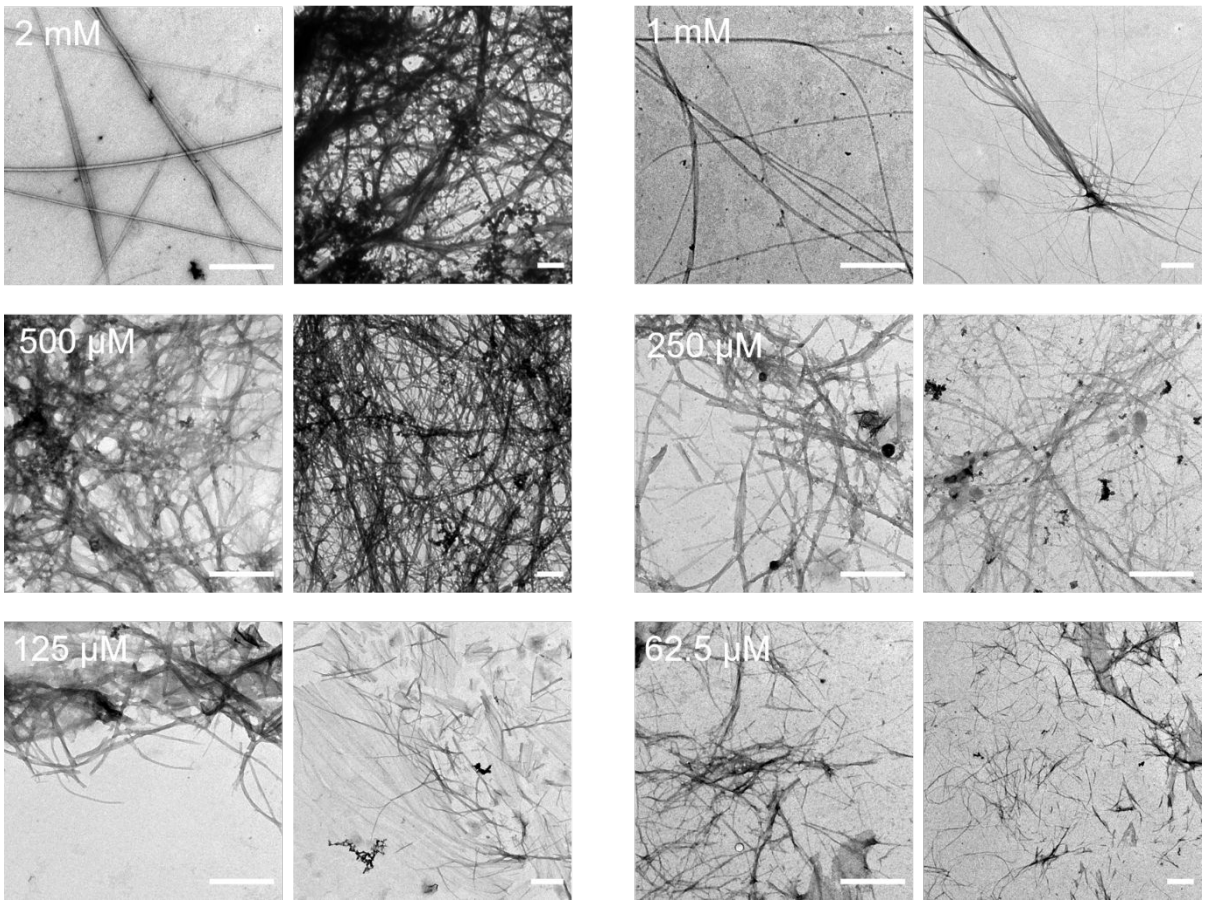


Figure S26: Determination of the critical fibrillation concentration of Fmoc-ISA **3a** by TEM. Two images of each tested concentration are displayed. Concentrations of 31.2 µM and 15.6 µM did not show fiber formation. Scale bars 500 nm.

3.4 Fluorescence Microscopy of Cofibrillized Peptides

In order to prove the staining of Fmoc-ISA peptide fibers with Proteostat, they were imaged in the fluorescence microscope (Figure S27). The sample was prepared as described before and 50 µL of 1 mM Fmoc-ISA **3a** solution (in PB:DMSO 9:1) were stained with 1 µL of Proteostat solution. The Proteostat solution was prepared according to the instructions of the supplier (*Enzo Life Sciences, Inc.*).

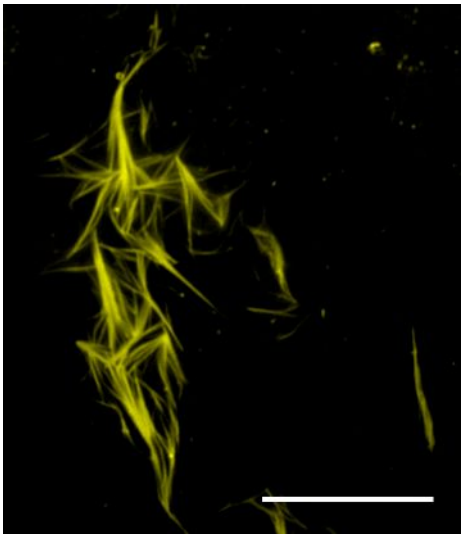


Figure S27: Proteostat stained Fmoc-ISA **3a** peptide fibers. Scale bar 20 µm.

To investigate co-assembly, Depsi(Fmoc-I)pba-SA **1a** and Depsi(C343-I)pba-SA **1b** were predissolved in DMSO and mixed at a ratio of 5:1 to achieve a peptide concentration of 10 mM. After addition of PB pH 7.4 5 mM and H_2O_2 the peptides were incubated overnight while shaking. Fibers were stained by addition of 1 µL Proteostat solution to 50 µL of fiber solution. The sample was imaged in the fluorescence microscope (Figure 2 k and Figure S28).

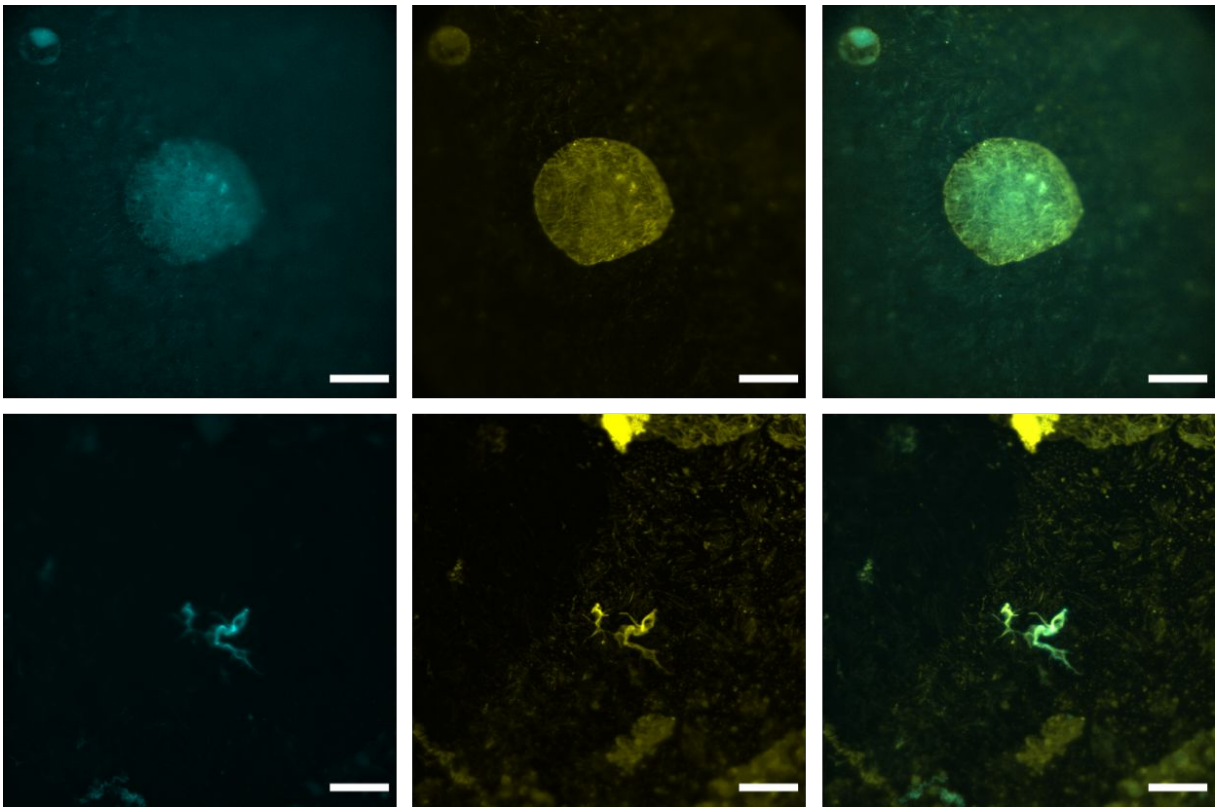


Figure S28: Cofibrillation of Fmoc-ISA **3a** and C343-ISA **3b** shown in the fluorescence microscope. Coumarin 343 fluorescence: cyan, Proteostat: yellow, colocalization: green. Scale bars 20 µm.

3.5 Fourier-Transform Infrared Spectroscopy

50 µL of either Fmoc-ISA **3a** or Depsi(Fmoc-I)pba-SA **1a** solution, incubated in DMSO:water 1:9 for 24 h, were lyophilised and FT-IR spectra were measured at wavenumbers from 400 to 4000 cm^{-1} (Figure S29). Fmoc-ISA **3a** displayed peaks typical for β-sheets (1634 cm^{-1} and either disordered or α-helical structures (1653 cm^{-1}), while the depsipeptide **1a** did not show formation of a specific structure but a very broad peak.

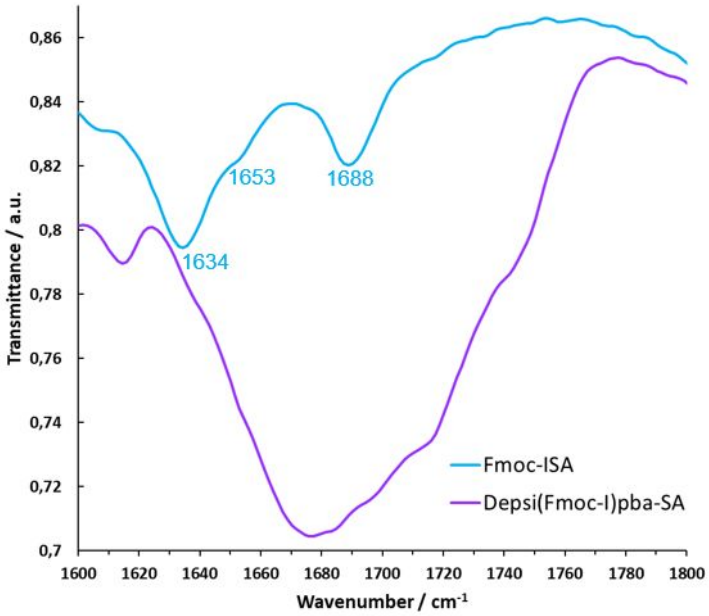


Figure S29: FT-IR spectra of Fmoc-ISA **3a** and Depsi(Fmoc-I)pba-SA **1a**.

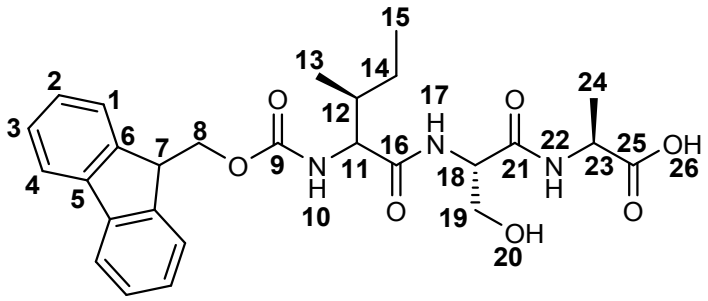
3.6 Circular Dichroism

To show co-assembly of C343-ISA **3b** with Fmoc-ISA **3a**, CD spectra were recorded in water of both peptides separately after being self-assembled overnight, as well as CD spectra of coincubated samples (5:1 ratio **3a:3b**) and peptides which were mixed before the measurement using pre-assembled **3a** and **3b** to achieve the same concentration and ratio as for the co-assembled sample (1 mM total peptide concentration). Peptides were dissolved in MilliQ water by sonication to avoid the use of DMSO which would disturb the CD measurement. After incubation overnight, the solutions were diluted to 250 µM and circular dichroism spectra were recorded at wavelengths from 320 to 180 nm with a bandwidth of 1 nm, data pitch of 0.2 nm and scanning speed at 5 nm/min. Spectra were measured three times and accumulated (Figure 2l).

3.7 Solid-State NMR

20 mg of Fmoc-ISA **3a** were dissolved in 4 mL DMSO and the solution was diluted to a concentration of 1 mM with 36 mL MilliQ water. The solution was stirred for 24 h so peptide fibers could form and the solution was subsequently lyophilised.

A ^{13}C (^{1}H) CP-MAS NMR spectrum was recorded (Figure S30), and the peaks were assigned based on the assignment of the atoms by liquid NMR spectra of the peptide in DMSO, which is displayed below. However, NMR signals may shift due to the transition from isolated molecules in solution to the solid state.



^{1}H-NMR (850 MHz, DMSO-d_6) δ [ppm] = 8.01 (d, J = 7.2 Hz, 1H, **22**), 7.95 (d, J = 7.9 Hz, 1H, **17**), 7.89 (d, J = 7.5 Hz, 2H, **4**), 7.73 (dd, J = 14 Hz, 7.5 Hz, 2H, **1**), 7.46 (d, J = 9.0 Hz, 1H, **10**), 7.41 (t, J = 7.4 Hz, 2H, **3**), 7.32 (q, J = 7.0 Hz, 2H, **2**), 4.34–4.32 (m, 1H, **18**), 4.30–4.29 (m, 1H, **8**), 4.26 - 4.17 (m, 3H, **7**, **8** and **23**), 3.95 (t, J = 8.3 Hz, 1H, **11**), 3.59–3.55 (m, 2H, **19**), 1.83–1.70 (m, 1H, **12**), 1.43–1.42 (m, 1H, **14**), 1.25 (d, J = 7.3 Hz, 3H, **24**), 1.14–1.10 (m, 1H, **14**), 0.85 (d, J = 6.8 Hz, 3H, **13**), 0.81 (t, J = 7.4 Hz, 3H, **15**).

^{13}C-NMR (75 MHz, DMSO-d_6) δ [ppm] = 174.00 (**25**), 171.23 (**16**), 169.57 (**21**), 156.07 (**9**), 143.92 (**5**), 140.72 (**6**), 127.66 (**3**), 127.10 (**2**), 125.38 (**1**), 120.11 (**4**), 65.68 (**8**), 61.70 (**19**), 59.19 (**11**), 54.85 (**18**), 47.65 (**23**), 46.70 (**7**), 36.54 (**12**), 24.36 (**14**), 17.34 (**24**), 15.35 (**13**), 10.97 (**15**).

Assignment of the peaks in the solid state was based on the following considerations: Isoleucine has many aliphatic peaks in proximity to the carbonyl position, therefore the 173 ppm peak is assigned to its C=O group. Serine contains no purely aliphatic protons, but signals above 4 ppm, which pleads for the assignment of the 171 ppm signal to the Ser carbonyl. Alanine typically displays the highest chemical shift in the carbonyl region, therefore we assign the 175 ppm to Ala. The Fmoc C=O is assigned to the two sharp and one broad peak between 157 and 160 ppm. Appearance of three peaks for Fmoc shows that there is no uniform packing of Fmoc-ISA **3a**. All carbonyl signals show multiple maxima, which further hints at the formation of different structures.

Strikingly, the serine carbonyl and the middle Fmoc carbonyl signal at 158 ppm show a correlation with the same deshielded N–H proton at 8.5 ppm (red line in Figure S25). This indicates formation of a hydrogen bond between either the N–H of Ile and C=O of serine, or alanine's N–H and the Fmoc C=O group.

The carbonyl group of alanine does not show any correlation to a deshielded proton, which together with the low intensity of the signal hints at a high mobility of the chain end (dashed red circle in Figure S25).

Comparison of the chemical shifts of Fmoc-ISA in the solid state and polypeptides to determine the secondary structure, gave the following results: The carbonyl signal of alanine (174–176 ppm) is in the region of α-helices (176 ppm) while the C_α (47 ppm) is in the β-sheet region (48 ppm). For isoleucine the carbonyl signal (172–174 ppm) covers the entire region of α-helices (175 ppm) and β-sheets (171 ppm). The chemical shift of the C_α (60–61ppm) lays between the shift of α-helices (64 ppm) and β-sheets (58 ppm) in polyisoleucine. For polyserine only the chemical shift of β-sheets (170 ppm for the carbonyl, 55 ppm for C_α) are reported, which is in accordance to the measured results (170–172 ppm for the carbonyl, 54 ppm for C_α).[4,5]

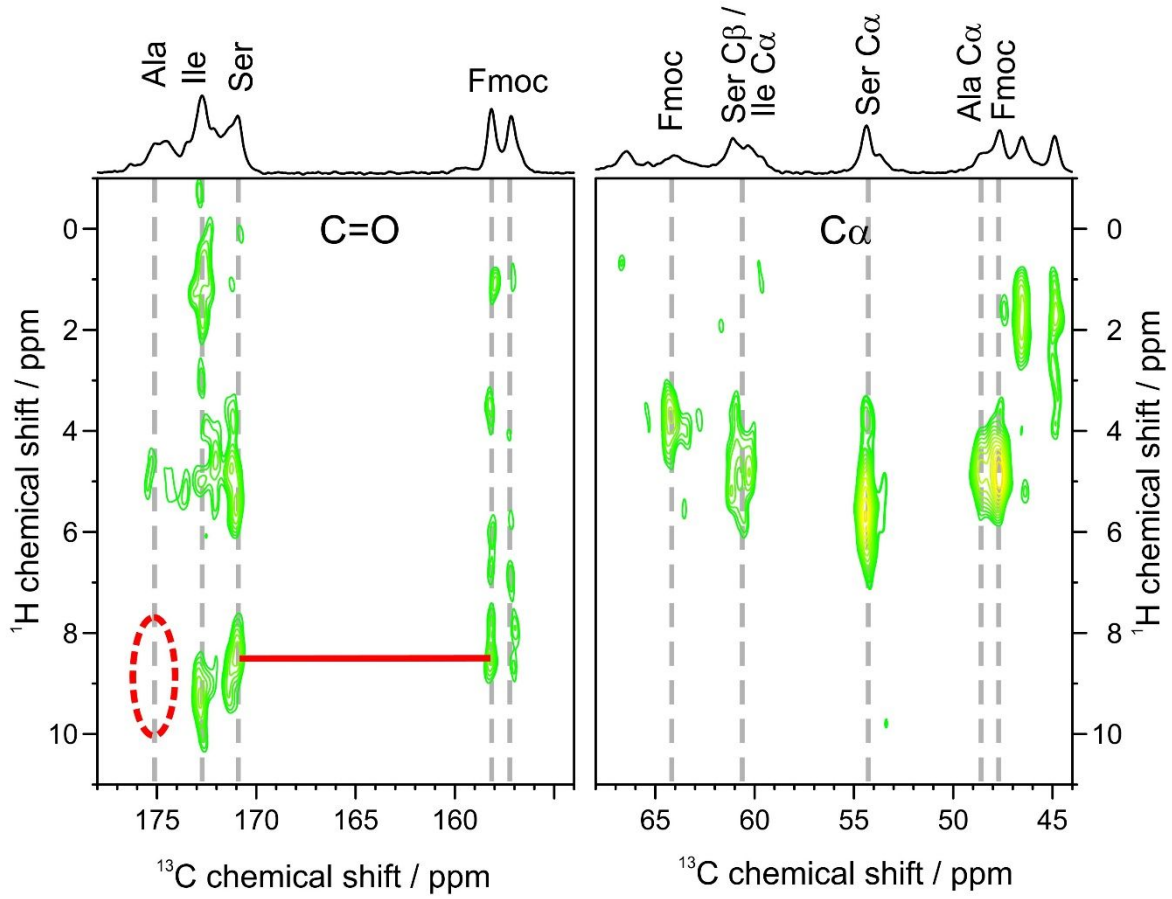


Figure S30: Carbonyl and C_α region of a ^{13}C (^{1}H) CP-MAS NMR spectrum of Fmoc-ISA **3a**.

3.8 MALDI-TOF of Depsi-TAT Complexes

SHA-TAT **12** and either Depsi(Fmoc-I)pba-SA **1a** or Depsi(C343-I)pba-SA **1b** (predissolved in DMSO) were conincubated for 30 min in NH_4HCO_3 buffer 5 mM pH 7.4 (buffer: DMSO 9:1). The concentration of both peptides was 1 mM. MALDI samples were prepared by mixing 2 µL of peptide solution at a ratio of 1:1 with saturated CHCA solution in water/acetonitrile (Figure 3 b).

3.9 Fluorescence Quenching

Depsi(C343-I)pba-SA **1b** was used as the fluorescent binding partner, dissolved in DMSO and diluted with phosphate buffer (PB, 5 mM, pH 7.4, PB:DMSO 99:1) to a concentration of 2 µM.

SHA-TAT **12** was used as the ligand and a dilution series was prepared by diluting the stock solution each time with PB 1:1 till 16 dilutions were received. Ligand concentrations ranged from 6.00 mM to 0.18 µM. For the experiment, each ligand solution (10 µL) was mixed with 10 µL of Depsi(C343-I)pba-SA **1b**, which led to a final concentration of fluorescently labelled peptide of 1.00 µM and final ligand concentrations ranging from 3.00 mM to 0.09 µM. After 1 h incubation in the dark at room temperature, approximately 8 µL of each solution was filled into Monolith NT Standard Treated Capillaries (NanoTemper Technologies GmbH). Thermophoresis/fluorescence was measured using the Monolith NT.115 instrument (NanoTemper Technologies GmbH) at 25 °C with 5 s/ 30 s/ 5 s laser off/on/off, respectively. Instrument parameters were adjusted to 60% LED power, medium MST power and fluorescence excitation and emission were performed by using the “blue channel” of the device (excitation: 465–490 nm, emission: 500–550 nm). Data of three independently pipetted measurements were analyzed (NT.Analysis software, NanoTemper Technologies GmbH) using the fluorescence signal (Figure 3 c).

In order to show the release of boronic acid compounds which are complexed to SHA-TAT **12** upon acidification, a phenylboronic acid modified BODIPY (PBA-BDP) was complexed with **12** for 15 min at a final concentration of 150 µM by being predissolved in DMSO and mixed with **12** which was dissolved in phosphate buffer pH 7.4 5 mM. Fluorescence spectra of this sample as well as a sample which was acidified to pH 5.0 with HCl_{aq} and further incubated for 10 min, and control spectra of PBA-BDP at both pH values were recorded at the emission maximum of 516 nm after excitation at 475 nm. All measurements were performed in triplicates. Complete recovery of the fluorescence and hence release of the molecules at pH 5.0 was observed (Figure S31).

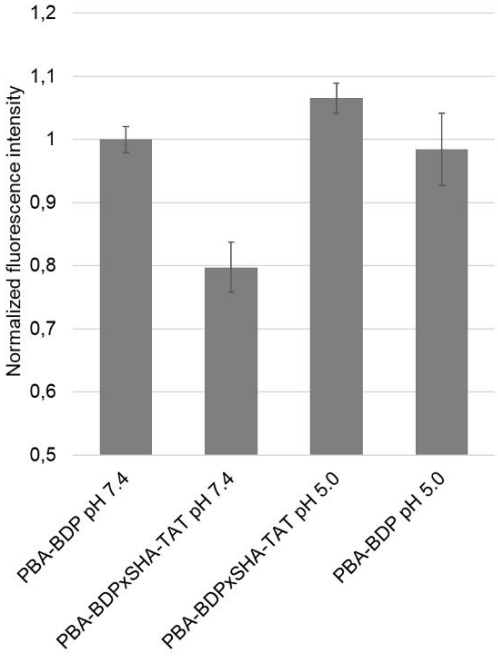


Figure S31: Fluorescence recovery of PBA-BDP upon cleavage of its complex with SHA-TAT at pH 5.0.

PBA-BDP was used as a model compound to show fluorescence recovery instead of Depsi(C343-I)pba-SA **1b** due to the quenching of fluorescence of **1b** upon acidification to pH 5.0 (Figure S32).

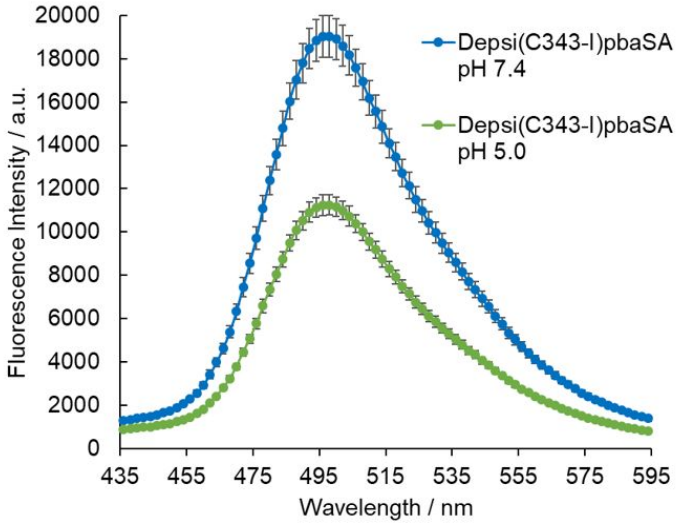


Figure S32: Fluorescence quenching of **1b** due to acidification. Spectra were recorded (excitation 405 nm) in triplicates after incubation of the peptide for 15 min at a concentration of 150 µM in a 9:1 mixture of phosphate buffer 5 mM pH 7.4 or 5.0 and DMSO.

3.10 Optical Properties

To check the influence of peptide fiber formation on coumarin 343 fluorescence, the peptides were dissolved in DMSO at a ratio of 5:1 of **1a** to **1b** or **3a** to **3b** and diluted with PBS or TAT containing PBS to 150 µM (ratio PBS:DMSO 9:1). To allow fiber formation samples were incubated overnight. To check fluorescence of monomeric peptides **3a/b** the peptides were incubated only in DMSO overnight at the same concentration. The control sample contained only PBS/DMSO. Emission was recorded at 435-600 nm (step size 2 nm) with a bandwidth of 20 nm. Samples were excited at 405 nm, bandwidth 10 nm (Figure S33). Measurements were performed in triplicates.

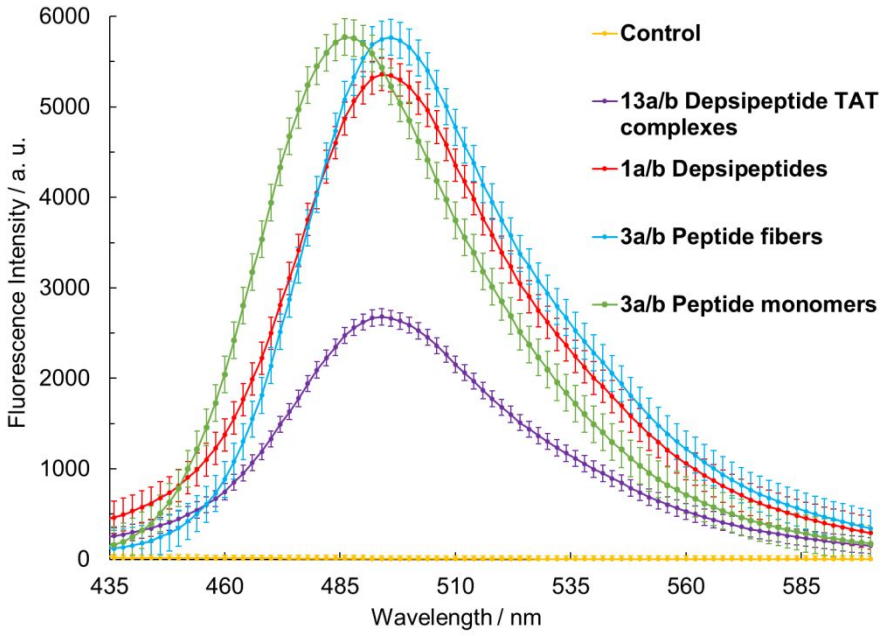


Figure S33: Fluorescence spectra of peptide mixtures after excitation at 405 nm in PBS/DMSO 9:1. In order to get a fluorescence spectrum of **3a/b** in the monomeric state, the peptides were incubated in DMSO without PBS to prevent assembly.

4. Cell Experiments

A549 cells were cultured in DMEM medium containing 10% fetal bovine serum and 1% penicillin/streptomycin as well as 1% MEM non-essential amino acids. Cells were seeded into confocal or well plates and left overnight to adhere at 37 °C, 5% CO_2.

4.1 Cell Uptake

Cells were seeded at a density of 15,000 cells/well in an 8-well confocal plate. After adhering for 24 h, cells were treated with the sample for different time points at 37 °C. Samples were preincubated to form the SHA-PBA complex **13a** and **13b** by dissolving the PBA-Depsi peptides **1a** and **1b** in DMSO and mixing with an equimolar amount of SHA-TAT **12**, which was dissolved in Dulbecco's PBS (PBS:DMSO 9:1). Sample solutions were diluted 1:4 with DMEM and incubated with the cells. Some samples were coincubated with the cells by addition of PMA (100 nM) in order to upregulate the hydrogen peroxide production in the cells. After the incubation time was over, the nucleus was stained with HCS NuclearMask Deep Red Stain for 30 min at 37 °C. The staining solution was removed, and fresh medium was added to the cells before they were imaged by confocal laser scanning microscopy (Figure 4 a and Figure S34).

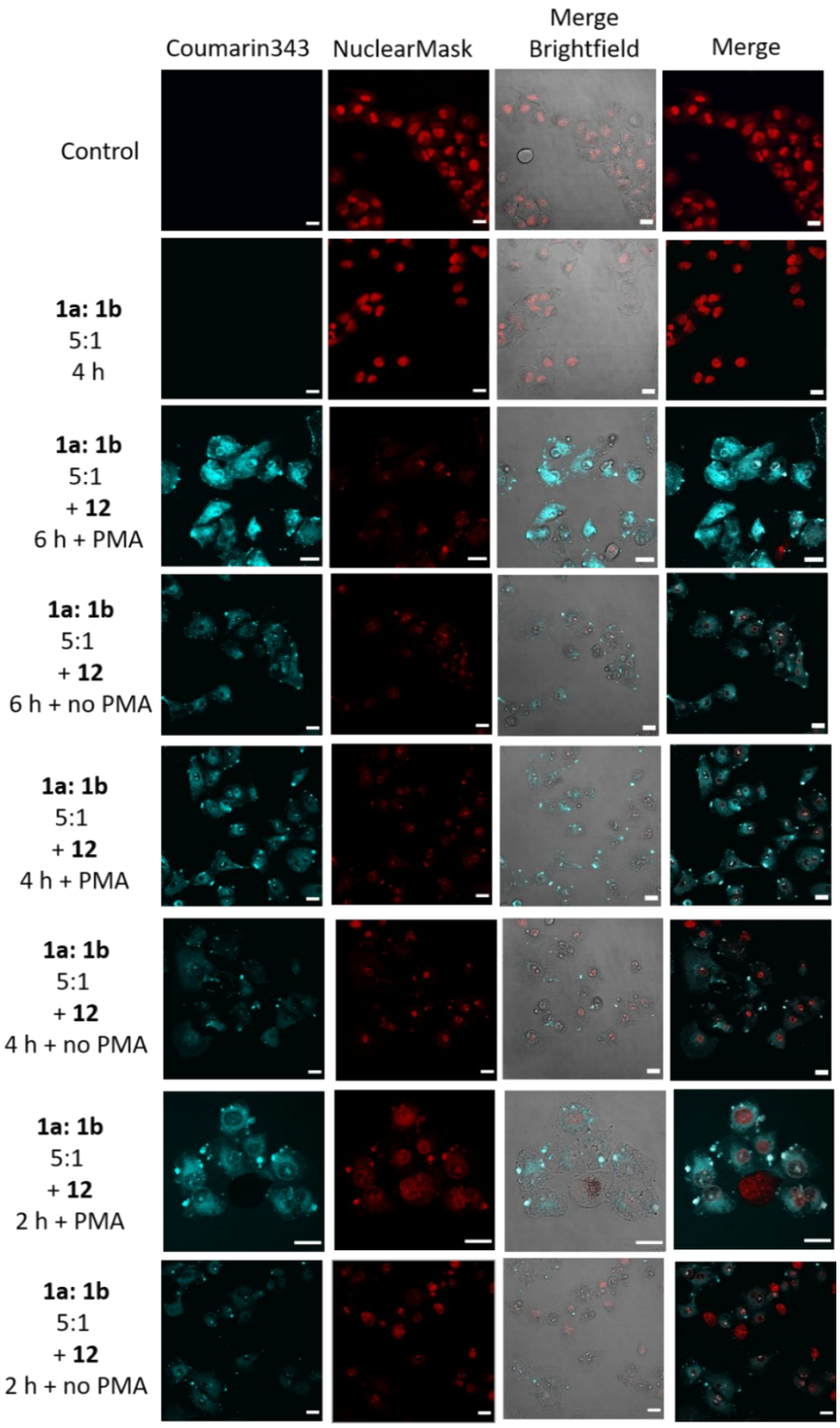


Figure S34: Confocal microscopy images of A549 cells incubated with peptides for different times with and without the addition of PMA. Peptide concentration was 150 μM in all samples. Scale bars 20 μm.

For imaging in the fluorescence microscope, cells that were incubated with the samples as described above were fixed with 4% paraformaldehyde solution for 20 min at room temperature (Figure S35).

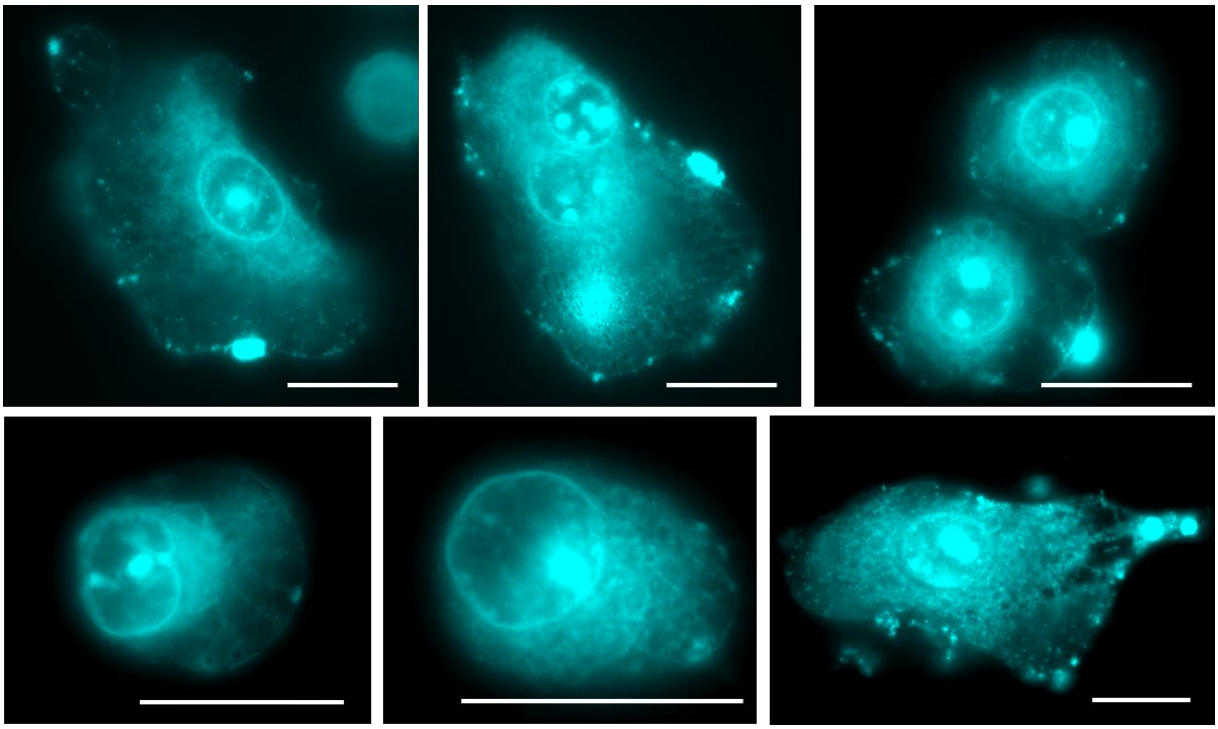


Figure S35: Fluorescence microscope images of **13a/b** treated A549 cells monitoring Coumarin 343. Scale bars 20 µm.

4.2 Staining of Cell Compartments

To elucidate the intracellular localization and uptake mechanism of the peptides confocal images were taken after staining of cell compartments. Cells were coincubated with 150 µM solutions of **13a/b** and 100 nM PMA as described before in Section 4.1. Cells were seeded at a concentration of 15,000 cells/well the day before the experiment and left to adhere, with the exception of endosomal staining, where cells were seeded two days prior to the experiment at 7,500 cells/well.

4.2.1 Staining of Endosomes and Lysosomes

After incubation with **13a/b** cells were washed and stained with 50 nM LysoTracker Green DND-26 for 5 min and washed before imaging by confocal microscopy (Figure S36). The peptides were found to be localized in lysosomes to a small extent.

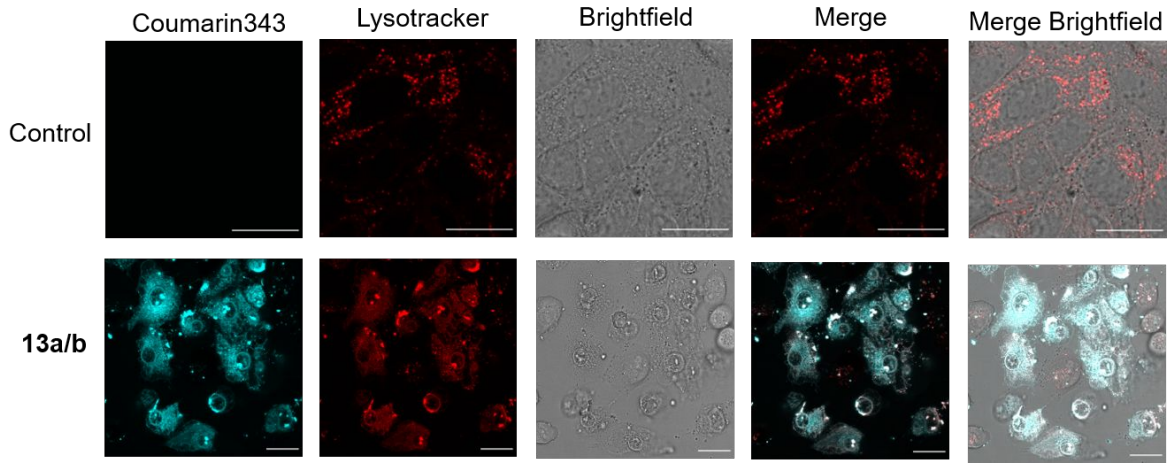


Figure S36: Confocal microscopy images of control cells and **13a/b** treated cells which were stained to visualize lysosomes. Scale bars 20 µm.

In order to proof the localization of the peptides in endosomes, adherent cells were transducted with either CellLight Early Endosomes-RFP or CellLight Late Endosomes-RFP one day after seeding and further incubated for 16 h for gene expression before the experiment was conducted. 4.5 µL of CellLight reagents were used for each well. After removal of the solution, **13a/b** was added as described before, after 4 h cells were washed with PBS once before imaging (Figure S37). Peptides were partial localized in endosomes, which indicates an endocytic pathway for cellular uptake.

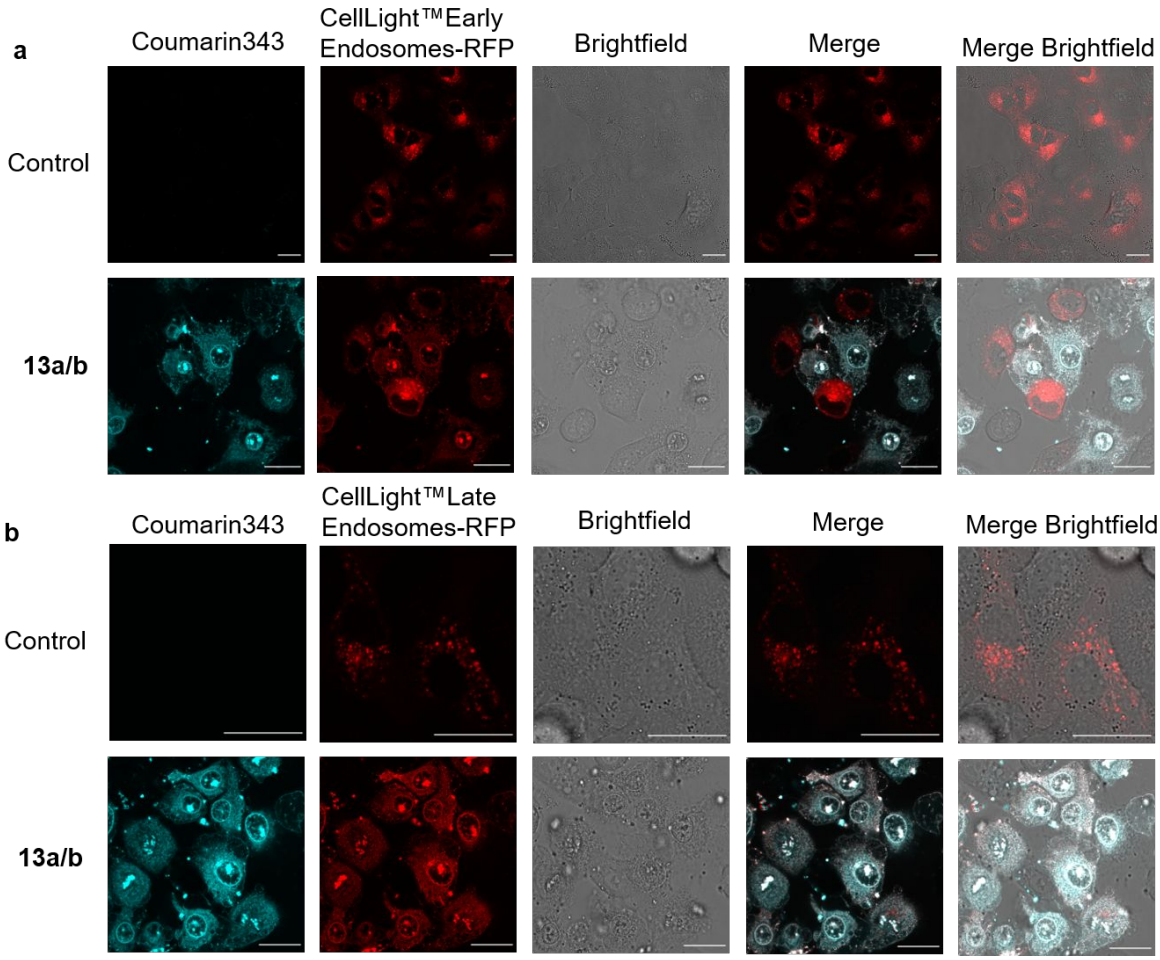


Figure S37: Confocal images of **13a/b** treated and control cell with fluorescently stained early and late endosomes. Scale bars 20 µm.

4.2.2 Staining of Nuclei and Determination of Nucleus Size

After incubation with **13a/b** cells were washed and stained with HCS NuclearMask Deep Red stain (after 250x dilution of the commercial stock solution) for 30 min and washed with PBS before imaging by confocal microscopy (Figure S38).

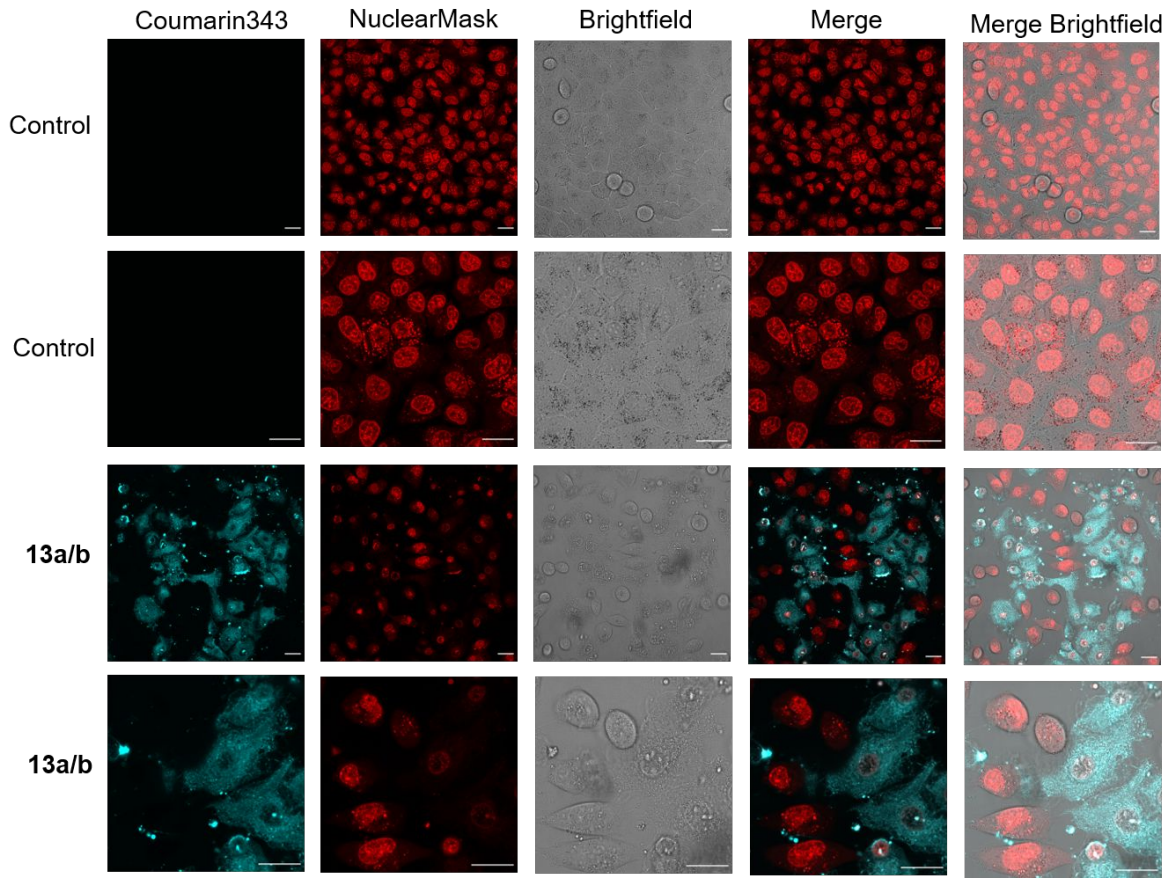


Figure S38: Confocal images of control cells and cells treated with **13a/b** for 4 h. Nuclei were visualized by staining. Scale bars 20 µm.

In order to quantify the changes in size of the nucleus upon peptide treatment, the nuclei of 20 cells of each control cells and peptide treated cells were measured at their widest point using ImageJ. The results showed that the average size of non-treated cells is 15.87±1.71 µm, while peptide treated cells had nuclei of a size of 9.28±2.23 µm (Figure S39).

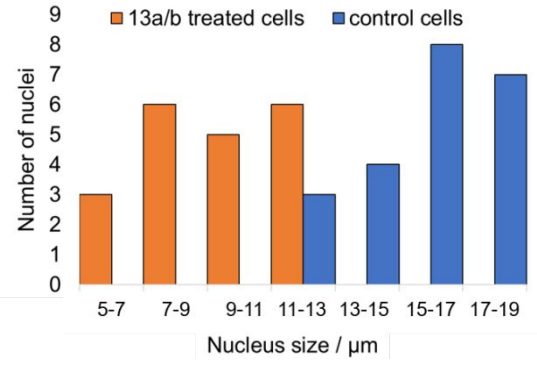


Figure S39: Distribution of nuclei size in untreated control cells and **13a/b** treated cells.

4.2.3 Staining of Nucleoli

After treatment of cells with **13a/b** and PMA as described above, the peptide solution was removed and cells were washed with PBS, after which they were fixed at -20 °C using methanol (10 min). After washing 2x with PBS, nucleoli were stained by incubation with 500 nM SYTO RNASelect Green for 20 min. Cells were imaged after washing two times with PBS. z-Stack images showed the localization of the peptides inside nucleoli (Figure S40).

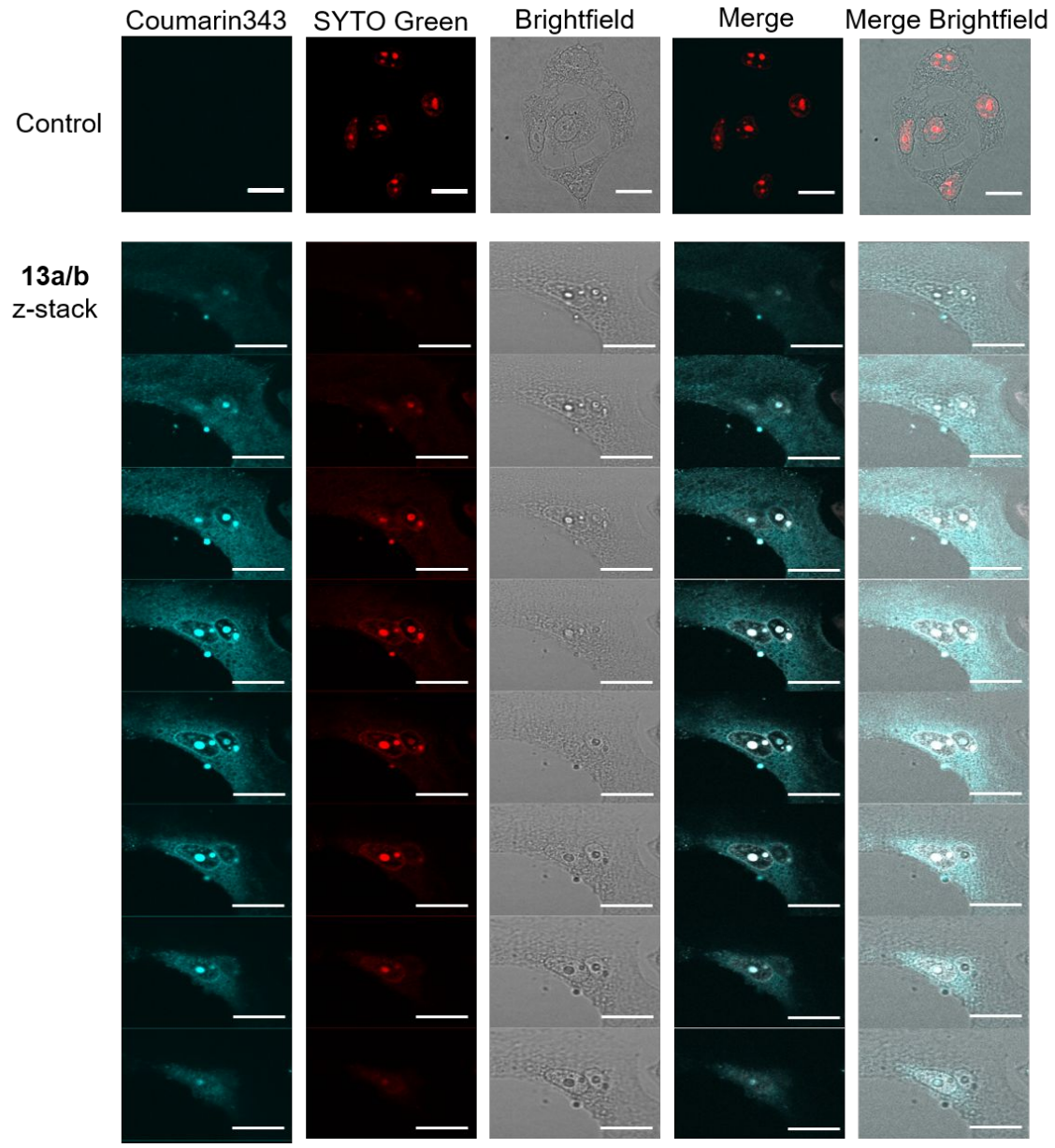


Figure S40: Confocal images of control cells and z-stack of **13a/b** treated cells showing the localization of the compounds inside nucleoli. Scale bars 20 µm.

4.2.4 Staining of the Cytoskeleton

To visualize the changes in morphology of cells upon fiber formation the cytoskeleton of control cells and **13a/b** cells were stained using Alexa Fluor 647 Phalloidin. After incubation with **13a/b** for 4 h, the cells were washed with PBS and fixed in 4% paraformaldehyde, after which the cells were incubated with the stain (330 nM in PBS) for 15 min. Cells were imaged with the confocal microscope after washing with PBS (Figure S41).

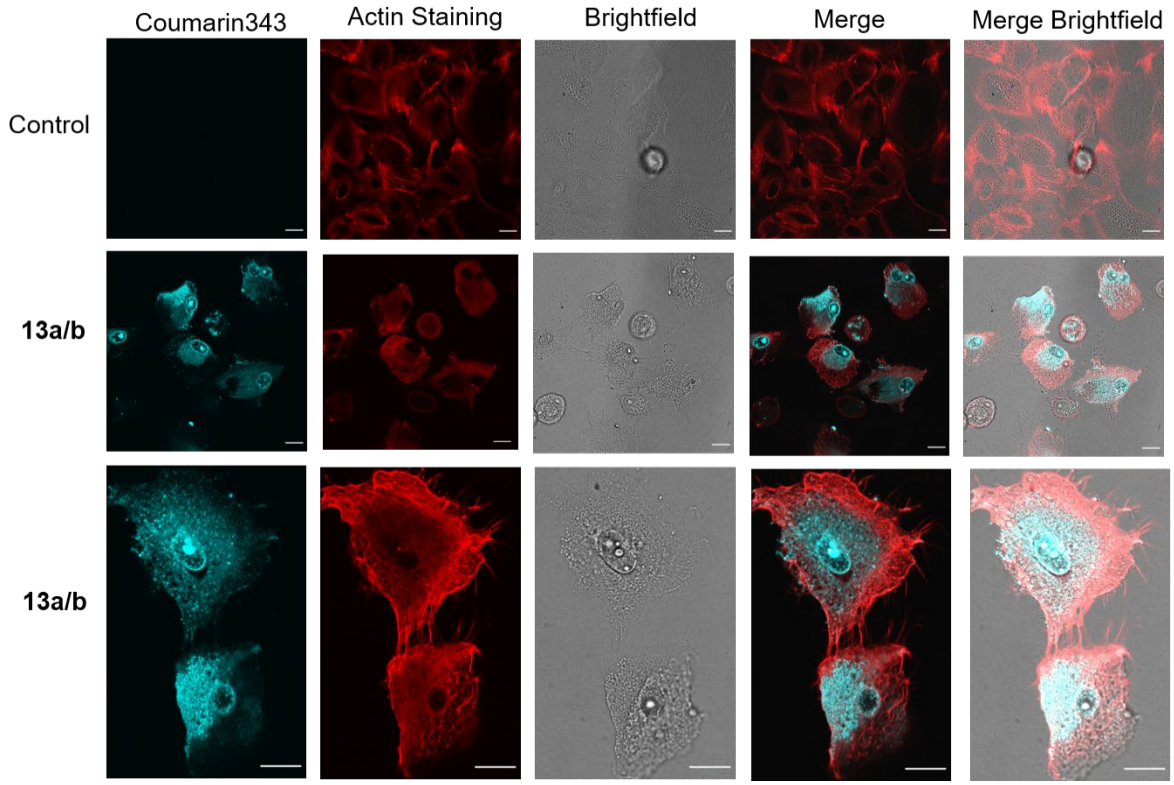


Figure S41: Confocal images of A549 cells after treatment with **13a/b** for 4 h and images of control cells. The cytoskeleton was stained using Alexa Fluor 647 Phalloidin. Scale bars 20 µm.

4.3 FRET inside Cells

For visualization of the disassembly of **13a/b** in confocal microscopy Förster resonance energy transfer between **1b** and fluorescent SHA-(Lys-5-FAM)-TAT **14** inside cells was imaged.

Cell were seeded in an 8-well confocal plate (7,500 cells/well) 2 days prior to the experiment and were left to adhere in an incubator as described before. Cells were coincubated with PMA and preformed complexes of **1a/b** with **14** to generate the boronic acid complexes with salicylhydroxamate **15a/b** following the protocol described before for generation of **13a/b**. After incubation with the peptides, cells were washed with PBS once and confocal imaging was performed (Figure S42).

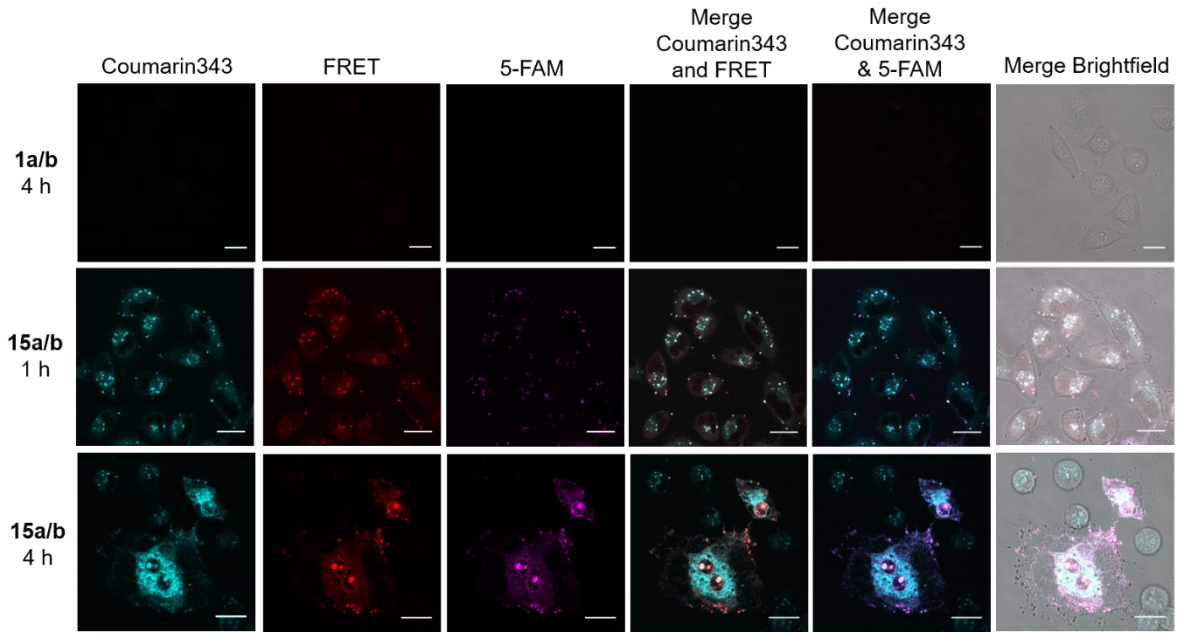


Figure S42: Confocal images of cells which were treated **1a/b** (top row) and **15a/b** (middle and bottom row). Scale bars 20 µm.

4.4 Isolation of Peptide Fibers by Cell Lysis

After cells were incubated in a confocal plate (8-well, 15,000 cells/well) and treated with a mixture of Depsi(Fmoc-I)pba-SA : Depsi(C343-I)pba-SA 5:1 (150 µM total) + 150 µM TAT as well as 100 nM PMA for 4 h, cells were washed with PBS three times and lysed using 100 µL RIPA buffer on ice for 5 min. Cells were treated using a cell scraper and the cell lysate was transferred into a PCR tube. After 5 min centrifugation, the upper part of the solution was transferred to another PCR tube to get a separation of the parts of the cell that were centrifuged down. Both fractions were analyzed in TEM (Figure 6 d and Figure S43). Grids were prepared as described above. For a control, A549 cells which were not treated with the peptides were also lysed and analyzed with TEM (Figure S44).

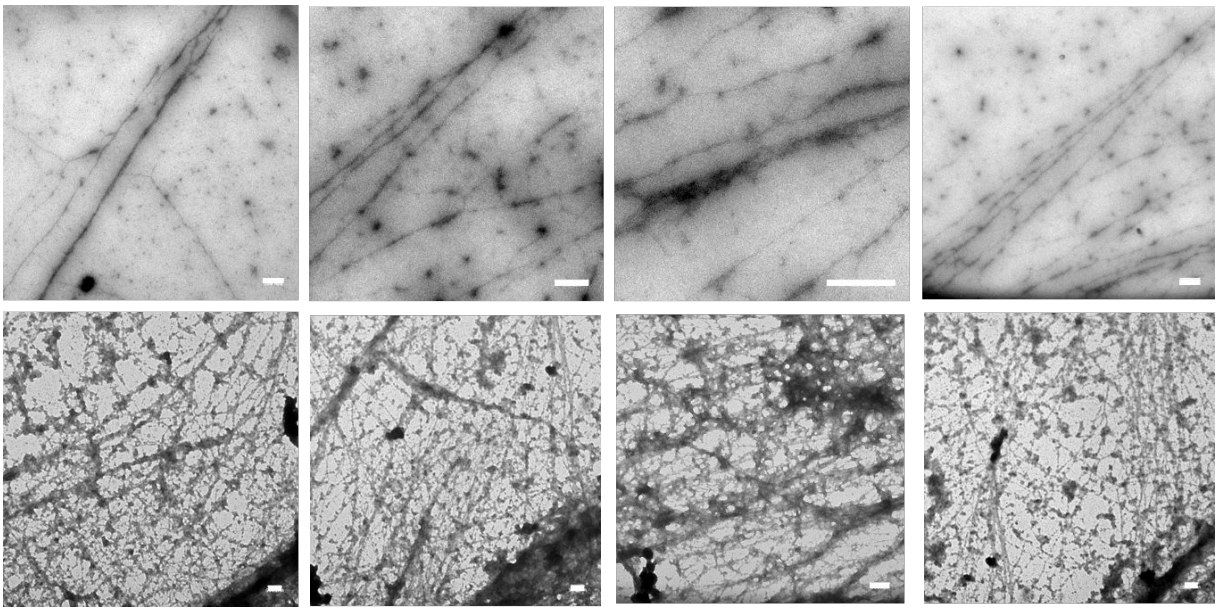


Figure S43: Peptide fibers isolated out of A549 cells. Top: lower density fraction after centrifugation. Bottom: higher density fraction. Scale bars 500 nm.

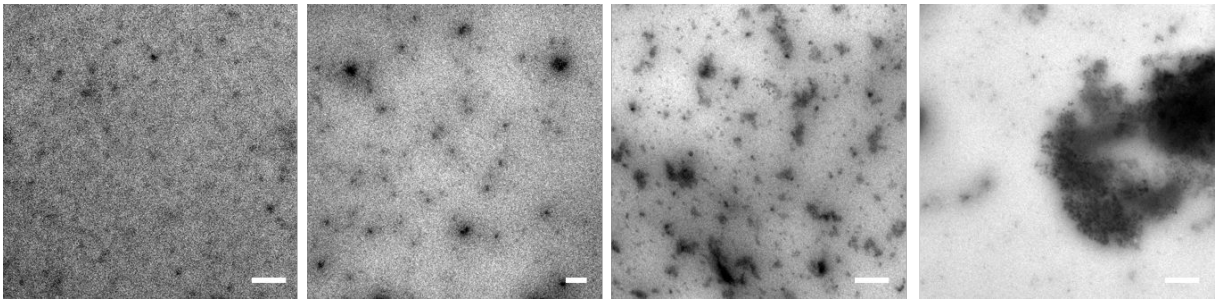


Figure S44: TEM images reflective of typical structures found in lysates of A549 cells. Scale bars 500 nm.

The fractions of peptide treated cells were also analyzed in the fluorescence microscope and the confocal microscope, with and without staining with Proteostat (1 µL of Proteostat solution, prepared as described before, in 20 µL of cell lysate).

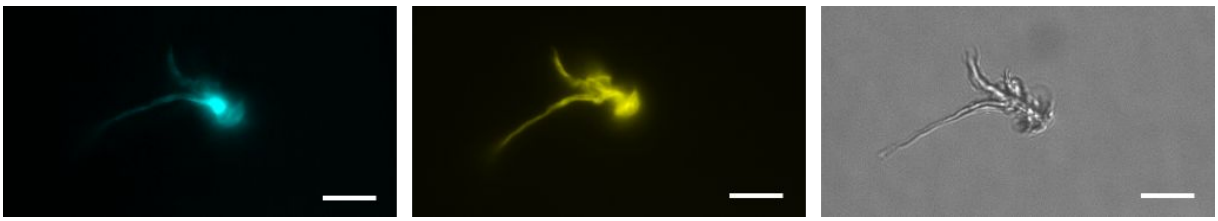


Figure S45: Fibrillar structures isolated by lysis from depsipeptide treated A549 cells. Cyan: Coumarin 343 fluorescence, yellow: Proteostat fluorescence, grey: brightfield image. Scale bars 10 µm.

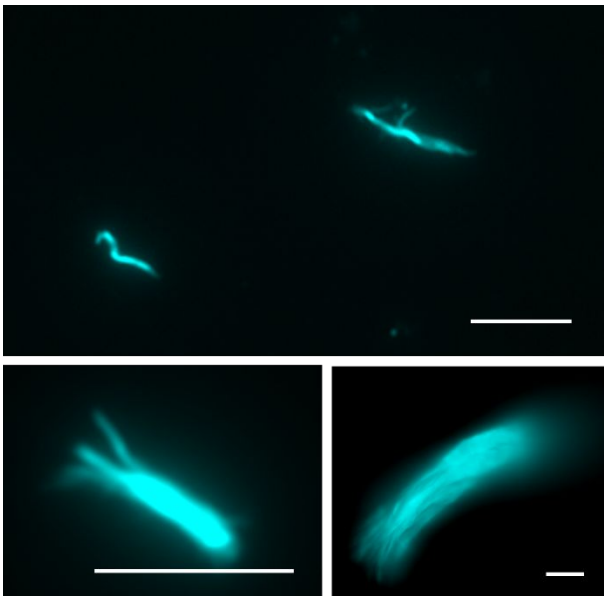


Figure S46: Fibrillar structures isolated by lysis from depsipeptide treated A549 cells. Cyan: Coumarin 343 fluorescence. Scale bar 10 µm.

The same experiment was repeated without the addition of PMA to show that the natural hydrogen peroxide production of A549 cells is sufficient to induce fiber formation (Figure S47 and Figure S48).

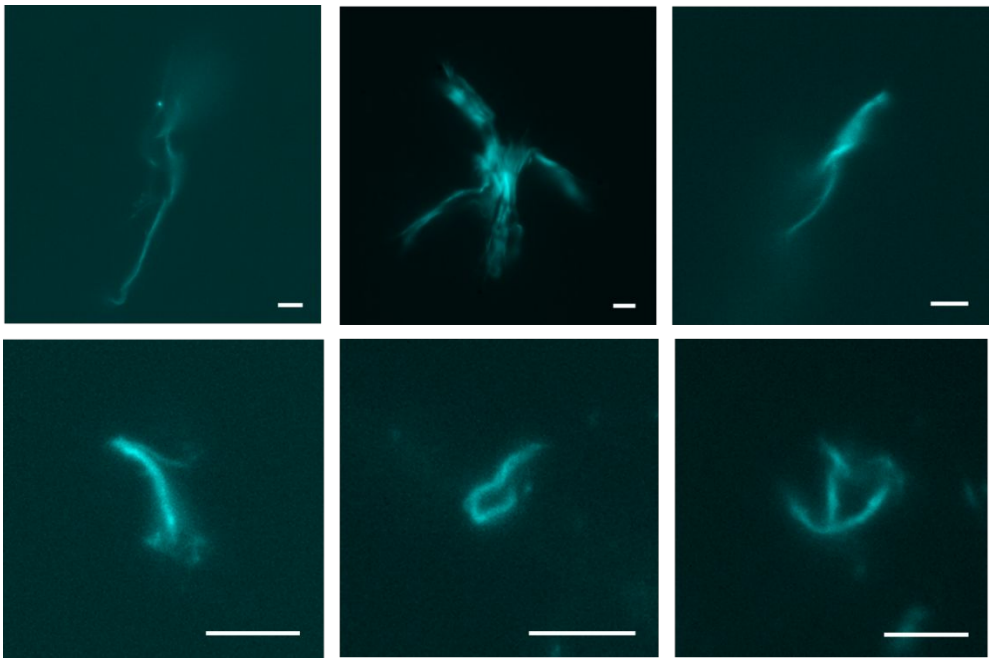


Figure S47: Fluorescence microscopy images of peptide fibers found in A549 cell lysate after incubation with **13a/b** for 4 h without addition of PMA. Images were taken in the coumarin 343 channel. Scale bars 5 µm.

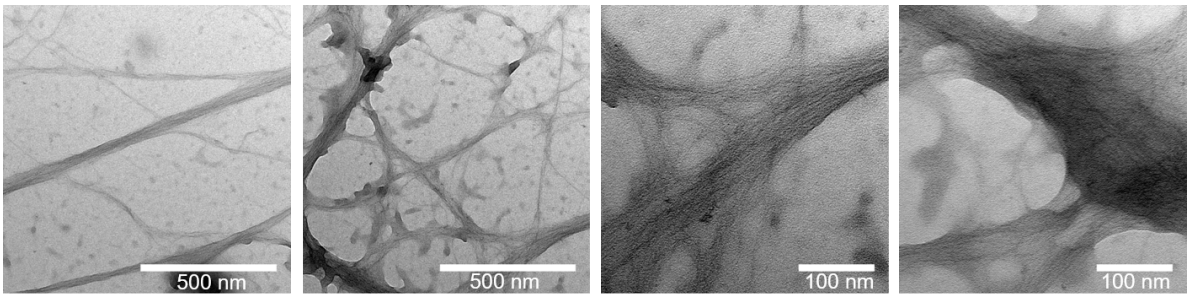


Figure S48: TEM images of peptide fibers in cell lysate of A549 cells, which were treated with **13a/b** for 4 h without addition of PMA.

4.5 Intracellular Linearization of Depsipeptides

Cells were seeded and treated with **13a/b** and PMA for 4h and lysed as described in Section 4.4. 100 µL of cell lysate were mixed with 400 µL methanol to precipitate proteins from the lysate. After centrifugation at 13,000 rpm at 4 °C for 20 min to remove the proteins and cellular debris, 180 µL of the supernatant was analyzed by analytical HPLC with a gradient starting from 5% ACN, which was kept constant for 1 min, after which the ACN content was increased linearly for 16 min to 100% ACN and kept constant for 5 min. An analytical ZORBAX Eclipse, XDB-C18, 80Å, 5 µm, 4.6 × 250 mm column was used.

The results showed that the linear peptide **3b** was formed inside cells (Figure S49).

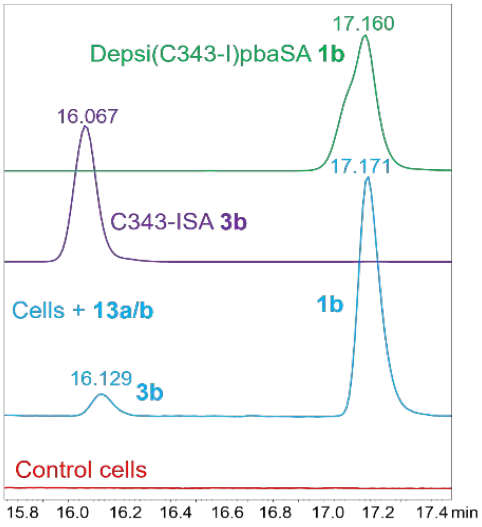


Figure S49: HPLC were spectra measured at 443 nm to monitor coumarin343 fluorescence of **1b** and **3b** in cell lysate of cells which were treated with **13a/b** (blue) or control cells which were not treated with the peptides (red)

and peptide solutions as controls to identify the peaks (green and purple). The retention times are indicated above the peaks.

4.6 TEM of Cells

Cells were seeded and grown on carbon pre-coated sapphire disks (3 mm; M. Wohlwend GmbH), which were autoclaved and freshly etched (30 s at 20% oxygen content) before use. After incubation with peptide, each sapphire disk was collected and slightly immersed into 1-hexadecene before placing them between two aluminum plates (3 mm, Plano). The aluminum plates with the sample were placed into a specimen holder for high pressure freezing in a Wohlwend HPF Compact 01 high pressure freezer with a pressure of 2100 bar for 2–3 s. The specimen holder was withdrawn from the freezer and immersed into liquid nitrogen to release the sample. The frozen sample was then labeled and stored in a container filled with liquid nitrogen. Subsequently, freeze substitution of the sample was carried out in a 0.5 mL Eppendorf tube using an AFS2 automated freeze substitution unit (Leica). Each tube contained 1 mL of freeze substitution solution, consisting of 0.2wt/vol% osmium tetroxide, 0.1wt/vol% uranyl acetate, and 5% distilled water in acetone. The tubes were firstly kept at –90 °C and slowly warmed up to 0 °C in 24 h. After keeping at room temperature for 1 h, the substitution solution was removed, and the samples were washed 3 times with acetone. Each sample was infiltrated in an ascending epoxy resin series (30%, 50%, and 75% in acetone) for 1 h before finally infiltration in 100% epoxy resin overnight. Finally, each sample was transferred into a new Eppendorf tube containing freshly prepared pure epoxy resin for polymerization at 60 °C for 24 h. After polymerization, sample blocks were kept at room temperature until their sectioning. Sample blocks for each time point were trimmed and sectioned into 100 nm sections by a 45º diamond knife (Diatome) in EM UC6 ultramicrotome (Leica). Sections were

then carefully placed onto 300-mesh carbon coated copper grid for standard bright-field imaging in Tecnai F20 200 kV transmission electron microscope (TEM) by FEI. Bright-field TEM micrographs were obtained with a Gatan US1000 2k CCD camera.

The TEM images (Figure S51) were stitched to show the entire cell (Figure 5 a and Figure S50).

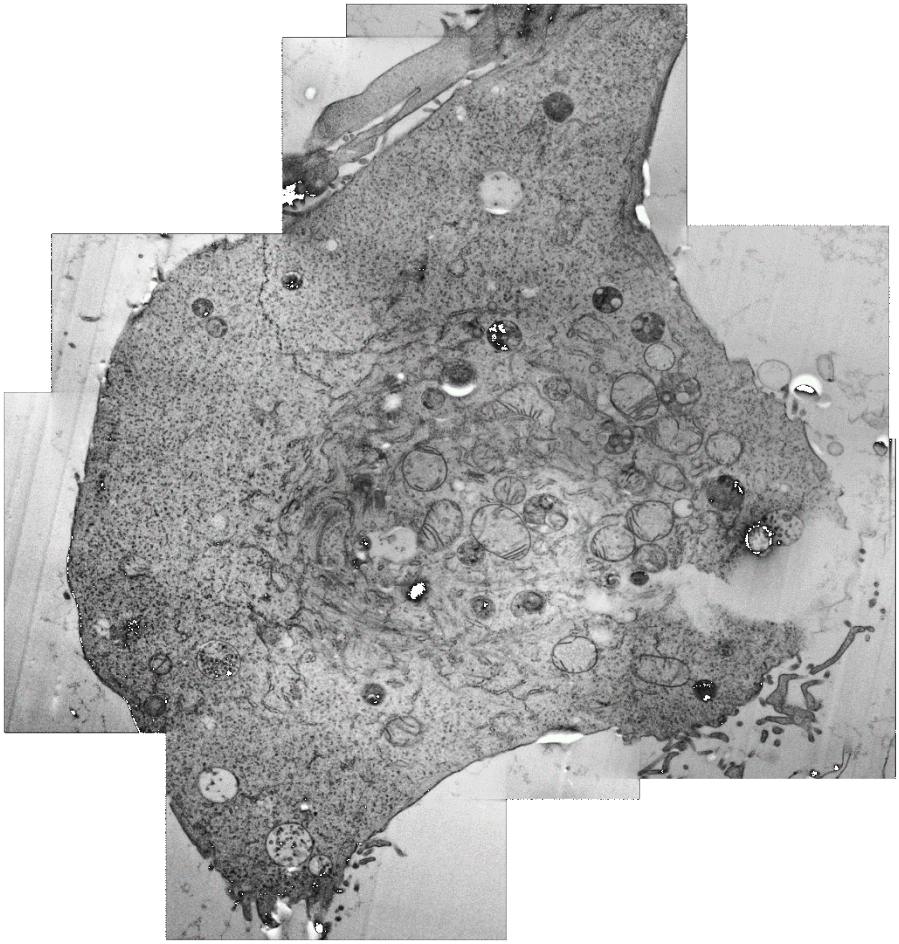


Figure S50: Stitched TEM image of an A549 cell showing intracellular peptide fiber formation.

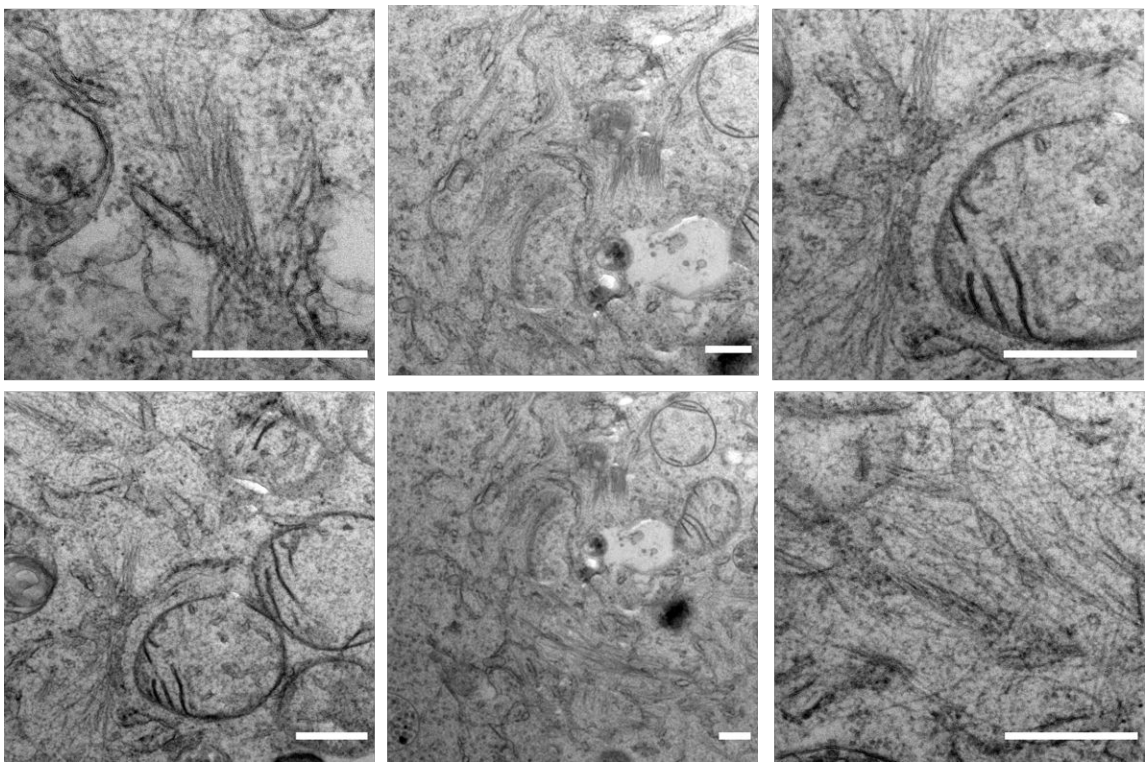


Figure S51: TEM images of peptide fibers inside an A549 cell. Scale bars 500 nm.

Furthermore, images of another cell were taken (Figure 5 b, Figure S52 and Figure S53).

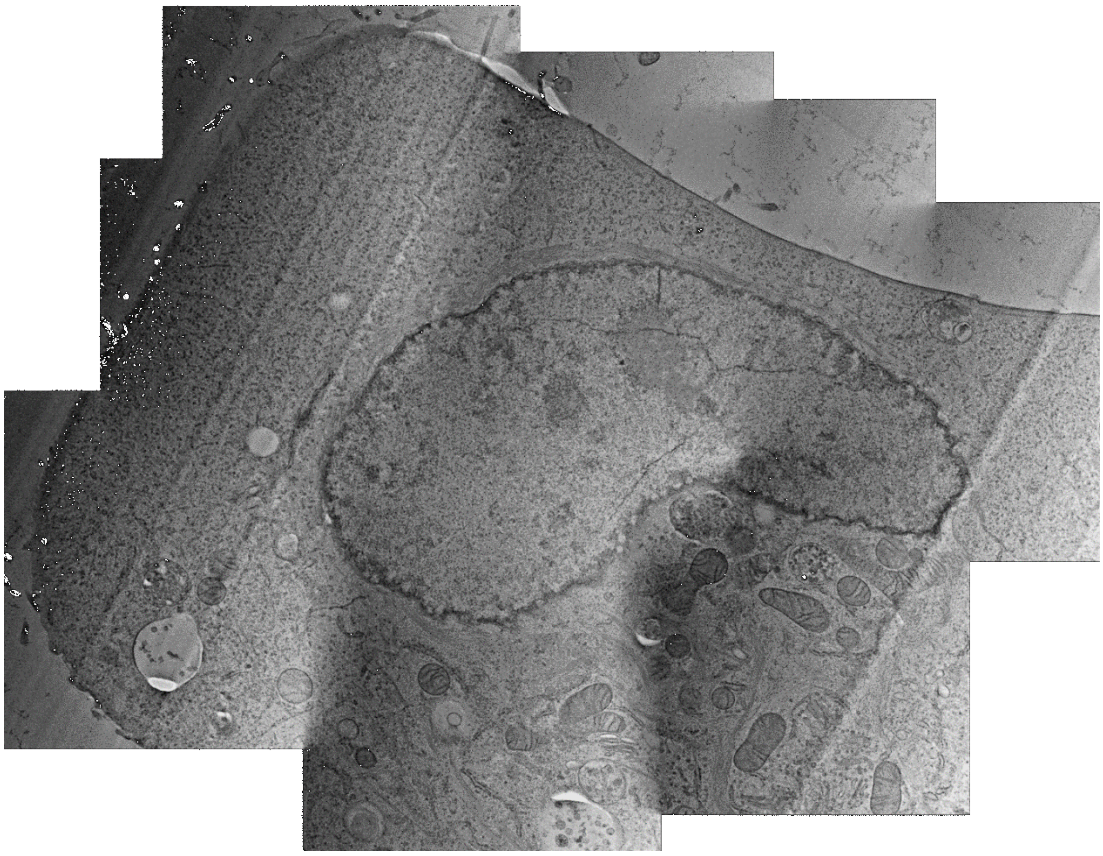


Figure S52: TEM image of the nucleus of an A549 cell, which was received after stitching TEM micrographs.

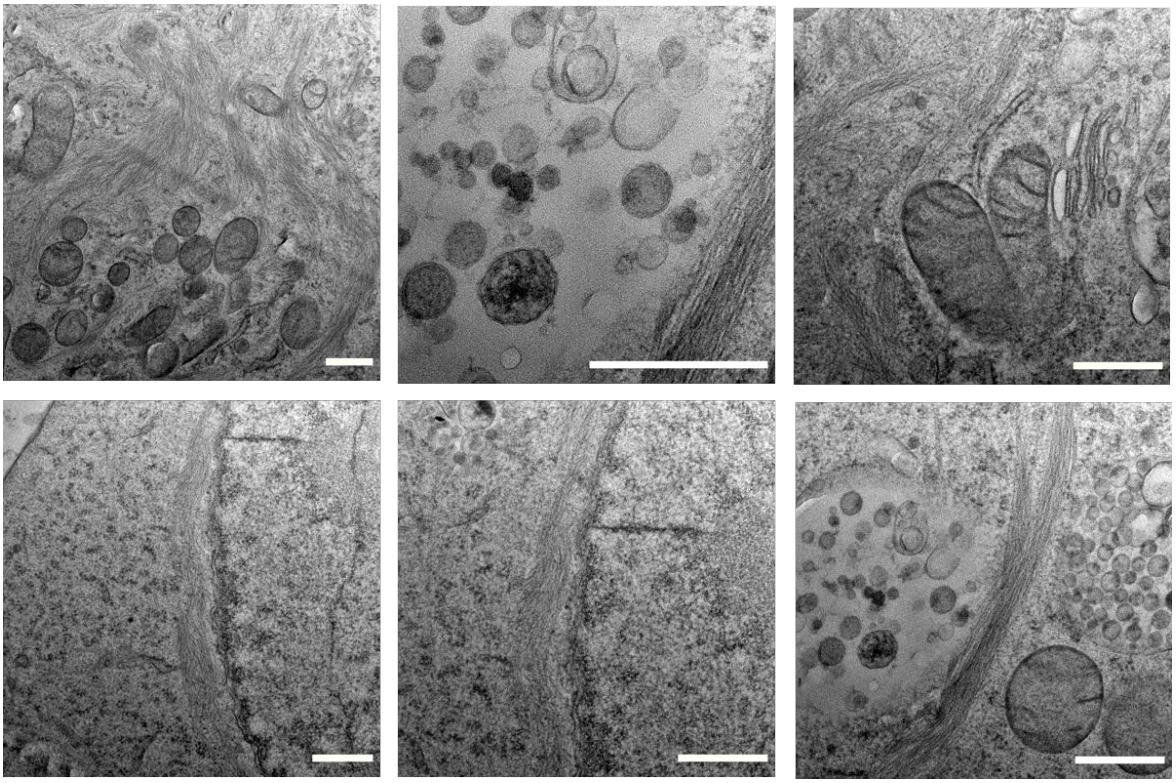


Figure S53: TEM images of peptide fibers inside an A549 cell. Scale bars 500 nm.

As a control, A549 cells which were not treated with the peptides were prepared as above and analyzed by TEM to show that the fibrillar structures derive from the peptides. No fibers were observed inside the cells (Figure S54 and Figure S55).

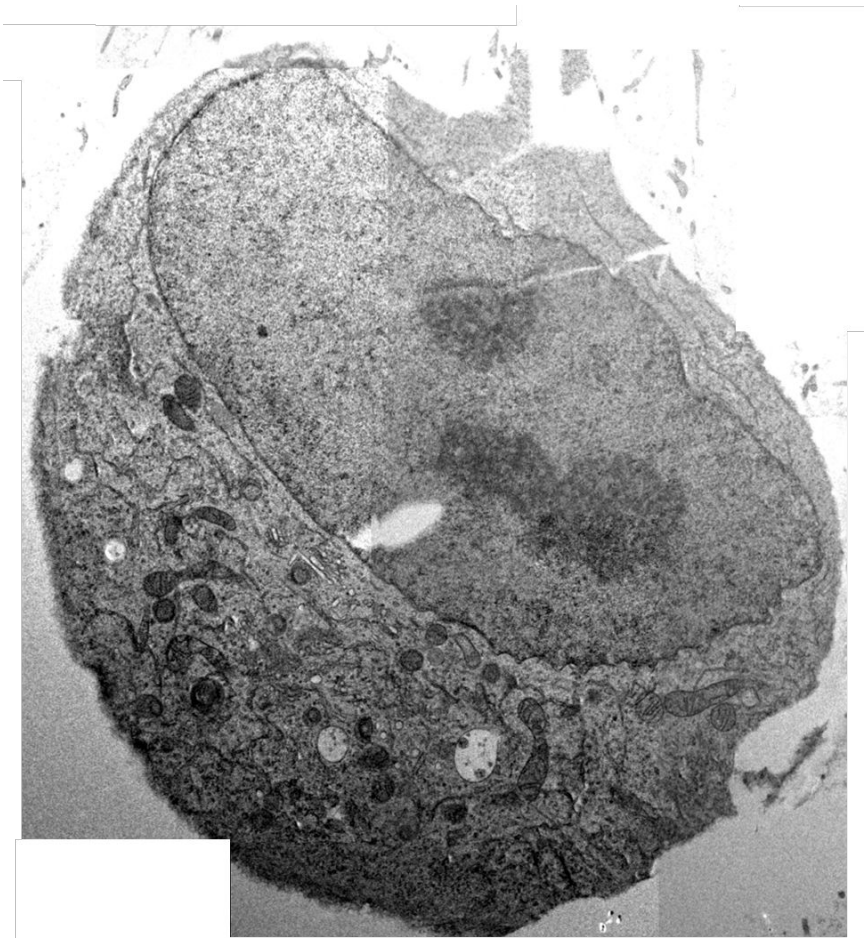


Figure S54: TEM image of a control A549 cell that was not treated with **13a/b** after stitching TEM micrographs. No fibers were observed in control cells, which were not incubated with peptides.

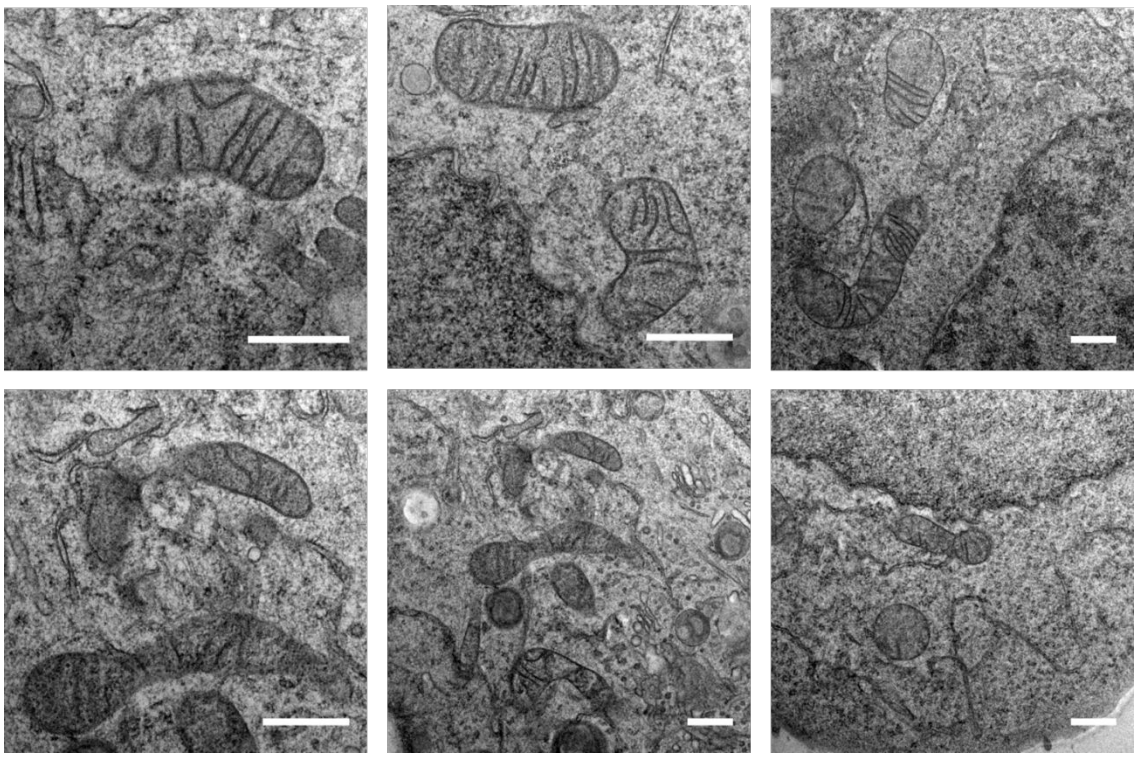


Figure S55: TEM micrographs of control A549 cells (scale bars 500 nm) which were not treated with the peptides.

4.7 Cytotoxicity

Cells were seeded at a concentration of 8000 cells/well in a white 96 half area well plate and were left to adhere overnight. Cells were treated with 50 µL sample solutions that were prepared as described before with different incubation times. After the incubation was finished, the same volume of CellTiterGlo Assay solution (prepared according to the protocol of the supplier) was added to the sample. After incubation for 10 min at room temperature, the luminescence was read out using the well plate reader Promega GloMax®-Multi Detection System, using the settings the instrument was supplied with. The assay was performed twice in triplicates (Figure 4c).

To investigate the influence of PMA coincubation on cell viability, A549 cells were incubated with 150 µM sample solutions of **13a/b** for 4 h with and without coincubation of 100 nM PMA (Figure S56). Each experiment was performed four times.

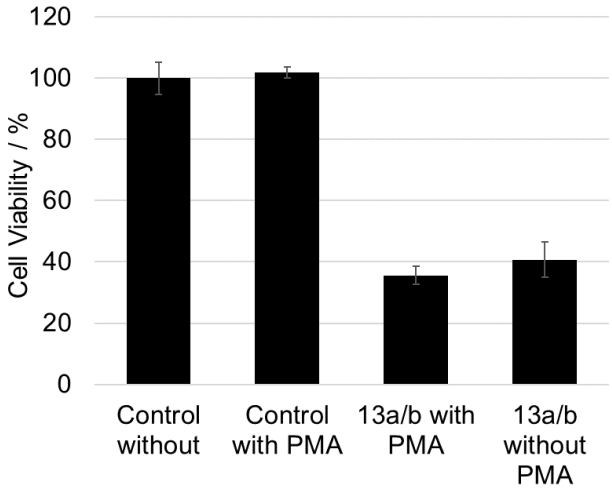


Figure S56: Cytotoxicity of **13a/b** towards A549 cells after 4 h incubation time with and without addition of PMA.

Apoptosis of **13a/b** treated cells was further visualized by Annexin V staining (Figure 5a). Here, cells were stained using Annexin V FITC and propidium iodine (PI) from the Annexin V Apoptosis Detection Kit FITC by Invitrogen. Cells were seeded at a density of 15,000 cells/well the day prior to the experiment and treated with **13a/b** and PMA following the protocol described in Section 4.1 for 2 and 4 h. After the incubation time was over, samples were removed and replaced with 200 µL of binding buffer from the assay kit and 5 µL of each Annexin V FITC and PI were added to each well. After 15 min of incubation at room temperature in the dark, the staining solution was removed and replaced with fresh binding buffer before imaging.

4.8 Intracellular hydrogen peroxide concentration

The intracellular hydrogen peroxide concentration of cells which were treated with PMA and cells which were not treated with PMA was determined using the Intracellular Hydrogen Peroxide Assay Kit by Sigma-Aldrich. Cells were seeded in a black cell culture 96 well plate

with clear bottom at a density of 8000 cells per well and were left to adhere overnight. Three wells of cells were prepared for each sample (PMA treated and non-treated control cells). To enhance the intracellular hydrogen peroxide concentration cells were treated with 100 nM PMA for 4 h. In order to measure the H_2O_2 concentration the fluorescent peroxide sensor was prepared following the protocol of the vendor and added to the cells after washing them with PBS. After incubation in the dark for 30 min cells were washed with PBS and fluorescence intensity was recorded using a well plate reader at 525 nm after excitation at 490 nm (bandwidth 10 nm each). To determine the concentration of H_2O_2 inside cells, a standard curve was generated by measuring the fluorescence intensity of a dilution series of hydrogen peroxide at the concentrations of 5, 2.5, 1.25, 0.625, 0.3125, 0.15625 and 0 µM (background blank) after incubation with the fluorescent sensor for 30 min in the dark following the vendor's protocol. All measurements for the standard curve and cells were performed in triplicates and the background blank was subtracted from all values. The intracellular H_2O_2 concentration of PMA treated cells was determined to be 2.45 ± 0.37 µM and non-treated cells had a concentration of 1.64 ± 0.16 µM (Figure S57).

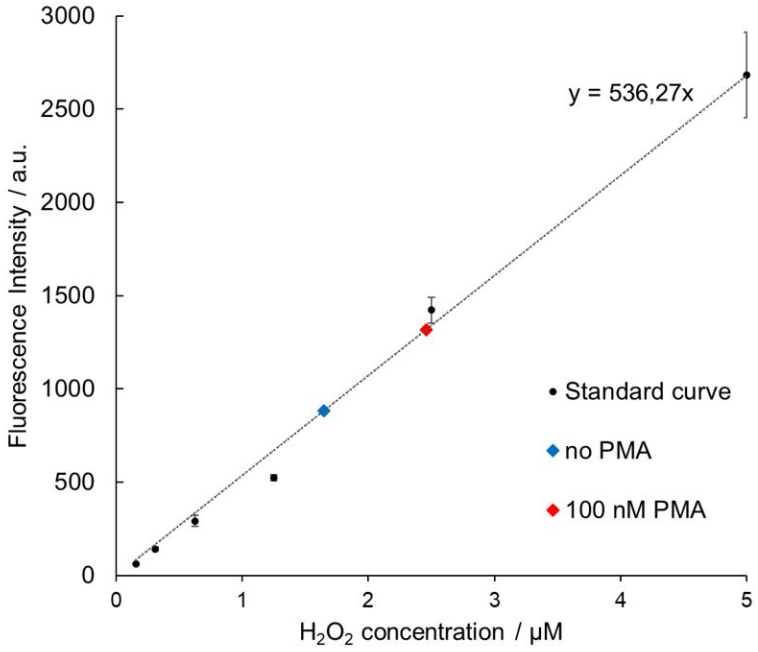


Figure S57: Standard curve for determination of the intracellular hydrogen peroxide concentration.

5. Literature

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