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Supplemental Part

Tuning properties of a novel short cell-penetrating peptide by intramolecular cyclization with a triazole bridge

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Experimental Part

Peptide synthesis

In general, the peptides used were synthesized on Rink amide resin by automated solid-phase peptide synthesis (SPPS) on a multiple Syro II peptide synthesizer (MultiSynTech, Witten, Germany) following Fmoc/tBu-strategy utilizing a double-coupling procedure and *in situ* activation with Oxyma/DIC.

Synthesis of CAP18(106-117) (linear) and the linear precursors [Pra^{106},Lys(N_3)^{109}]-CAP18(106-117) (cyc1a), [Pra^{106},Lys(N_3)^{113}]-CAP18(106-117) (cyc2a) and [Pra^{106},Lys(N_3)^{117}]-CAP18(106-117) (cyc3a)

For linear precursor peptides, N-terminal glycine and lysine (in position 109, 113 and 117 respectively) have been replaced by the clickable unnatural amino acids Fmoc-Pra-OH and Fmoc-Lys(N_3)-OH in a manual coupling step using a 2-fold excess of amino acid, OxymaPure and DIC shaken at RT overnight. Having assembled the linear peptides, the N-terminal Fmoc protecting group was removed with piperidine in DMF (20 %, 2 x 20 min). The resin was washed with DMF, DCM, MeOH and Et_2O and dried in vacuum. For peptide cleavage from the resin and complete side-chain deprotection, a cleavage mixture consisting of TFA/TIPS/water (95:2.5:2.5 v/v/v, 1 ml) was added to the dried resin. The mixture was shaken at RT for 3 h. After filtration from the resin, the crude peptide was precipitated in 10 ml cold Et_2O and centrifuged at 5,000 g for 4 min at 4 °C. The precipitate was washed with cold Et_2O (5 x 10 ml) and finally dried in vacuum. The crude peptide was lyophilized and purified by reversed-phase HPLC. **linear:** ESI-MS (m/z) $C_{72}H_{127}N_{31}O_{13}$: calcd, 1570.97; found 786.18 $[M + 2H]^{2+}$, 524.68 $[M + 3H]^{3+}$. **Cyc1a:** ESI-MS (m/z) $C_{72}H_{127}N_{31}O_{13}$: calcd, 1635.01; found 818.16 $[M + 2H]^{2+}$, 546.00 $[M + 3H]^{3+}$. **Cyc2a:** ESI-MS (m/z) $C_{72}H_{127}N_{31}O_{13}$: calcd, 1635.01; found 818.26 $[M + 2H]^{2+}$, 546.00 $[M + 3H]^{3+}$. **Cyc3a:** ESI-MS (m/z) $C_{72}H_{127}N_{31}O_{13}$: calcd, 1635.01; found 818.15 $[M + 2H]^{2+}$, 545.98 $[M + 3H]^{3+}$.

Synthesis of CF-labeled linear peptide [Pra^{106},Lys(N_3)^{109},Lys^{117}(CF)]-CAP18(106-117) (CF-cyc1a)

Having assembled the linear peptide sequence, the N-terminal Fmoc protecting group was removed with 20 % piperidine in DMF (v/v). The resin was washed with DMF and DCM followed by a Boc-protection of the free N-terminus using di-tert-butyl-dicarbonate (Boc_2O, 10 equiv) and DIPEA (1 equiv) in 500 µl DCM left shaking for 2 h at RT. The Dde protecting group at Lys117 side-chain was then removed by successive treatment of the resin with 1 ml

of 3 % hydrazine monohydrate in DMF (v/v) (15 x 10 min). The peptide was labeled with CF at Lys117 side-chain using 5(6)-carboxyfluorescein (3 equiv), HATU (3 equiv) and DIPEA (3 equiv) in 300 µl DMF shaken for 3 h at RT. CF polymers were cleaved by treatment of the resin with 20 % piperidine in DMF (v/v) for 45 min at RT and shaking. Finally, the resin was thoroughly washed with DMF, DCM, MeOH and Et_2O and dried in vacuum. **CF-cyc1a:** ESI-MS (m/z) $C_{93}H_{137}N_{31}O_{19}$: calcd, 1993.33; found 997.34 $[M + 2H]^{2+}$, 665.38 $[M + 3H]^{3+}$.

Synthesis of CF-labeled linear peptides [CF-Pra^{106},Lys(N_3)^{113}]-CAP18(106-117) (CF-cyc2a) and [CF-Pra^{106},Lys(N_3)^{117}]-CAP18(106-117) (CF-cyc3a)

Having assembled the linear peptide sequence, the N-terminal Fmoc protecting group was removed with piperidine in DMF (20 %, 2 x 20 min). The peptide was N-terminally labeled with CF using 5(6)-carboxyfluorescein (3 equiv), HATU (3 equiv) and DIPEA (3 equiv) in 300 µl DMF shaken for 3 h at RT. CF polymers were cleaved by treatment of the resin with 20 % piperidine in DMF (v/v) for 45 min at RT and shaking. The resin was thoroughly washed with DMF, DCM, MeOH and Et_2O and dried in vacuum. **CF-linear:** ESI-MS (m/z) $C_{93}H_{137}N_{31}O_{19}$: calcd, 1929.28; found 965.32 $[M + 2H]^{2+,}$ 644.18 $[M + 3H]^{3+}$. **CF-cyc2a:** ESI-MS (m/z) $C_{93}H_{137}N_{31}O_{19}$: calcd, 1993.33; found 997.28 $[M + 2H]^{2+}$, 665.39 $[M + 3H]^{3+}$. **CF-cyc3a:** ESI-MS (m/z) $C_{93}H_{137}N_{31}O_{19}$: calcd, 1993.33; found 997.27 $[M + 2H]^{2+}$, 665.36 $[M + 3H]^{3+}$.

Synthesis of cyclic peptides

A fresh Cu(I) solution was prepared from mixing equal volumes of precooled solutions of 0.5 M $CuSO_4$ x 5 H_2O and 1 M sodium ascorbate. 3 equivalents of this *in situ* generated Cu(I) solution was immediately added to an 1 mM solution of linear peptide in 0.1 M ammonium bicarbonate. The reaction vessel was covered with argon and sealed with parafilm. The cyclization reaction was carried out for 30 min at RT and constant shaking. The reaction was quenched by the addition of TFA to adjust the pH level to approximately 2. The peptide was mostly separated from Cu-species and ascorbate by means of solid-phase extraction with an equilibrated Chromafix C18ec (M) SPE cartridge (Macherey-Nagel, Düren, Germany). The collected peptide eluate was evaporated to reduce the volume, lyophilized and purified by reversed-phase HPLC. **Cyc1:** ESI-MS (m/z) $C_{72}H_{127}N_{31}O_{13}$: calcd, 1635.01; found 818.26 $[M + 2H]^{2+}$, 546.03 $[M + 3H]^{3+}$. **Cyc2:** ESI-MS (m/z) $C_{72}H_{127}N_{31}O_{13}$: calcd, 1635.01; found 818.30 $[M + 2H]^{2+}$, 546.04 $[M + 3H]^{3+}$. **Cyc3:** ESI-MS (m/z) $C_{72}H_{127}N_{31}O_{13}$: calcd, 1635.01; found 818.23 $[M + 2H]^{2+}$, 546.01 $[M + 3H]^{3+}$. **CF-cyc1:** ESI-MS (m/z) $C_{93}H_{137}N_{31}O_{19}$: calcd, 1993.33; found 997.37 $[M + 2H]^{2+}$, 665.42 $[M + 3H]^{3+}$. **CF-cyc2:** ESI-MS (m/z)

$C_{93}H_{137}N_{31}O_{19}$: calcd, 1993.33; found 997.30 $[M + 2H]^{2+}$, 665.48 $[M + 3H]^{3+}$. **CF-cyc3:** ESI-MS (m/z) $C_{93}H_{137}N_{31}O_{19}$: calcd, 1993.33; found 997.30 $[M + 2H]^{2+}$, 665.41 $[M + 3H]^{3+}$.

Analytical methods

Peptides were analyzed by LC-MS using an agilent instrument with parallel detection at 220 nm UV-absorption and electrospray-ionization mass spectrometry (ESI-MS). The reversed-phase analytical HPLC (RP-HPLC) was performed at 1.2 ml/min flow rate on a 4.6 x 100 mm Kinetex 2.6u C18 100A column (Phenomenex, Aschaffenburg, Germany). Gradients of 10 – 45 % or 10 – 60 % acetonitrile in water over 15 min (with constant 0.1 % TFA) were used.

Purification of peptides

Peptides were purified by reversed-phase HPLC (RP-HPLC) using a Hitachi Elite LaChrom instrument (VWR, Darmstadt, Germany) at 6 ml/min flow rate and 220 nm detection. The chromatography was performed on a 15 x 250 mm Jupiter 4u Proteo 90A column (Phenomenex, Aschaffenburg, Germany) and acetonitrile/water with 0.1 % TFA gradients as needed. The collected fractions were evaporated to reduced volume, analyzed with LC-MS and lyophilized to obtain the purified peptides.

Infrared spectroscopy

Infrared (IR) spectra were recorded from solid linear and cyclic peptides without any pretreatment on an IRAffinity-1 FTIR Spectrophotometer (Shimadzu, Duisburg, Germany) by means of attenuated total reflection (ATR). Fmoc-Lys(N_3)-OH served as a reference substance for characteristic bands of the asymmetric valence vibration of azide.

Peptide fragmentation with MALDI-MS/MS

Peptide fragmentation was performed on an ultrafleXtreme matrix-assisted laser desorption/ionization mass spectrometer (MALDI-MS) instrument (Bruker Daltonics, Bremen, Germany) in TOF/TOF (time-of-flight) mode. Linear or cyclic peptide (approx. 10 pmol/µl in H_2O) was spotted together with α-cyano-4-hydroxycinnamic acid (10 mg/ml in MeCN/H_2O 1:1, v/v, with 0.1 % TFA) in equal volumes on the MALDI plate. Isolated parent ions $[M + H]^+$ were fragmented by collision with inert gas which was kept at constant kinetic energy for all measurements. The resulting fragments were detected after resolution in the second TOF and analyzed and assigned with BioTools Software (Bruker Daltonics, Bremen, Germany).

Circular dichroism spectroscopy

Circular dichroism spectra were recorded from 180 nm to 270 nm in 0.2 nm intervals at 20 °C using a JASCO J-715 spectropolarimeter in an N_2 atmosphere. The CD spectra were measured using a 1 mm quartz cuvette and the instrument parameters were set as follows: sensitivity, 100 mdeg; scan mode, continuous; scan speed, 50 nm/min; response time, 2 sec and bandwidth, 1.0 nm. Peptide solutions were dissolved in 10 mM potassium phosphate buffer (pH 7.0) containing either 0 or 50 % (v/v) trifluorethanol to a final peptide concentration of 20 μM.

In the presence of the plasmid DNA, measurements were performed at a peptide plasmid charge ratio of 17:1. In the presence of LUVs, the peptide to lipid molar ratio in the cuvette was 1/50. All CD spectra were averaged over three scans, smoothed and for each sample the background was subtracted from the signal. The ellipticity was expressed as molar ellipticity [Θ] in $deg*cm^2*dmol^{-1}$.

NMR spectroscopy

NMR samples. Samples of peptides **1-4** for NMR experiments were dissolved in PBS buffer (pH 6.9) to make a final peptide concentration of ca. 1 mM. The concentrations for perdeuterated SDS (d_{25}-SDS) samples resulted in a peptide:SDS molecule ratio of 1:30.

For Mn2+ experiments, a stock solution of $MnCl_2$ in H_2O was prepared and was added stepwise to the peptide/micelle sample until $MnCl_2$ concentration was 190 μM. d_{25}-SDS was purchased from Cambridge Isotopes Laboratories.

NMR experiments. Spectra were recorded at on a Bruker Avance II 600 spectrometer (^1H frequency of 600 MHz) using a 5 mm triple-resonance Z gradient probe and processed using Topspin software (Bruker). The transmitter frequency was set on the HDO/H_2O signal, and the TSP resonance was used as chemical shift reference (δTSP = 0 ppm).

One and two-dimensional spectra were acquired by standard pulse sequences using WATERGATE-based solvent suppression sequences. Two-dimensional homonuclear experiments (i.e. TOCSY and NOESY) spectra of peptides were recorded using mixing times of 80 ms and 150 ms, respectively.

The diffusion measurements were performed with a stimulated echo sequence incorporating bipolar gradients[1] with WATERGATE solvent suppression.[2] The duration of the magnetic field pulse gradients (d) and the diffusion times (D) were optimized for each sample so as to obtain a 95% dephasing of the signals with the maximum gradient strength. Gradient strengths of xx–xx ms duration were increased in 32 steps from 2 to 95% of the maximum

gradient strength in a linear ramp. 128 scans were recorded for each selected gradient strength, and 32 K complex data points were acquired. The diffusion coefficients were calculated from the Stokes–Einstein Equation. The gradient strength was calibrated by using back calculation of the coil constant from a H2O diffusion experiment with D=2.3 10^{-9} m^2 s^{-1} at 298 K.

Structure calculation. The processed data were then analyzed by using CARA software.[3] According to the general procedure described by Wüthrich,[4] the 1H resonances of the peptides were assigned by spin system identification (TOCSY) and sequential assignment (NOESY).Peak lists for the TOCSY and NOESY spectra were generated by interactive peak picking. NOESY cross-peak volumes were determined by the automated peak integration routine implemented in CARA.

Three-dimensional structures were determined by the standard protocol of the CYANA program (version 2.1),[5] using seven cycles of combined automated NOESY assignment and structure calculations followed by a final structure calculation. Since the cyclic peptides contain non-standard residues, the corresponding libraries for CYANA were built using the MOLMOL program.[6] MOLMOL was also used to visualize the three-dimensional structures. For each CYANA cycle, 1000 randomized conformers and the standard simulated annealing schedule were used. The 20 conformers with the lowest final score were retained for analysis and passed on to the next cycle. Weak restraints on ϕ/ψ torsion-angle pairs and on side-chain torsion angles between tetrahedral carbon atoms were applied temporarily during the high-temperature and cooling phases of the simulated annealing schedule in order to favor the permitted regions of the Ramachandran plot and staggered rotamer positions, respectively. The list of upper- distance bonds for the final structural calculation consists of unambiguously assigned upper-distance bonds and does not require the possible swapping of diastereotopic pairs.

Root-mean-square deviation (rmsd) values were calculated using CYANA for superpositions of the backbone N, Cα, and CO atoms; the heavy atoms over the whole peptide or the cyclic fragment. To obtain the rmsd of a structure represented by a bundle of conformers, all conformers were superimposed upon the first one and the average of the rmsd values between the individual conformers and their average coordinates was calculated. The statistics regarding the quality and precision of the 20 energy minimized conformers that represent the solution structure of peptides are summarized in Supporting Information **Table S8**.

Stability studies

For a stability assay in human blood serum (taken from a healthy volunteer), 5(6)-carboxyfluorescein-labeled peptides were dissolved to 132 µM in blood plasma and incubated at 37 °C with shaking. At the indicated time points, 30 µl aliquots were withdrawn and mixed with 60 µl of a solvent containing water, acetonitrile, ethanol and trifluoroacetic acid (7:1:1:1, v/v) and incubated at 4 °C for 6 h. Samples were centrifuged at 15,000 g for 15 min and the supernatants were analyzed by reversed-phase HPLC equipped with a C18 column. In the presence of trypsin, the proteolytic stability of the peptides was determined incubating prewarmed 150 µM peptide in 0.1 M ammonium bicarbonate together with 8.4 u/ml prewarmed trypsin (peptide/trypsin 500:1 (w/w)) at 37 °C and 1200 rpm constant shaking. An aliquot of 40 µl of the mixture was withdrawn at different time intervals (2 min to 75 h) and 20 µl 10 % TFA was added immediately to inactivate the enzyme. The mixture was analysed by analytical RP-HPLC and at 220 nm detection.

The amount of remaining peptide (%) was determined by integrating the area underneath the peptide peak and compared with that of the control reaction (no serum/trypsin).

Cell culture

The following cell lines were used: HEK-293 (human embryonic kidney cells), HeLa (human cervix carcinoma), HCT-15 (human colorectal adenocarcinoma) and MCF-7 (human breast adenocarcinoma). Cells were cultured in 10 cm petri dishes at 37 °C and 5 % CO_2 in a humidified atmosphere. For HEK-293 cells, complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15 % fetal bovine serum (FBS) was used. HeLa, HCT-15 and MCF-7 cells were grown in complete RPMI 1640 supplemented with 10 % FBS.

Cell viability assay

For a resazurin-based cell viability assay, cells were seeded in a 96-well plate, grown to 60 – 70 % confluency and incubated with various concentrations of peptides in appropriate growth medium for 24 h under standard growth conditions. For the positive control, cells were treated with 70 % ethanol for 10 min. After washing with serum-free medium, resazurin stock solution (5 % resazurin in PBS) was diluted with appropriate serum-free medium (1:10, v/v) and 100 µl of this solution was incubated with the cells for 1 h. Subsequently, the cell viability was determined relative to untreated cells by measurement of the resorufin product at 595 nm (λex = 550 nm) on a Tecan infinite M200 plate reader. The experiments were done in triplicate.

Peptide internalization studies

For peptide-uptake studies by flow cytometry, cells were seeded in a 24-well plate and grown to 60 – 70 % confluency. After incubation at either 4 °C or 37 °C for 30 min with 5(6)-carboxyfluorescein-labeled peptides in serum-free medium, the cells were treated with 300 µM trypan blue for 10 sec to quench extracellular fluorescence and washed twice with indicator-free medium, trypsinized and resuspended in appropriate medium. Analyses were performed on a BD Accuri C6 flow cytometer (Heidelberg, Germany). Cellular autofluorescence was subtracted. The experiments were performed in duplicate.

For confocal microscopical uptake studies, cells were seeded in a µ-slide 8-well (Ibidi) plate and grown to 60 – 70 % confluency. The cells were then incubated with CF-labeled peptides in serum-free medium for 30 min at either 4 °C or 37 °C. The nuclei were stained for 10 min with Hoechst33342 nuclear dye prior to the end of peptide incubation. Finally, the solution was removed and the cells were treated with a 300 µM trypan blue solution for 10 sec. After washing twice with indicator-free medium, images were taken by using a Nikon Eclipse Ti confocal laser scanning microscope equipped with a 60x oil-immersion objective. Images were recorded with Nikon EZ-C1 software and adjusted equally with ImageJ software.

Vesicle preparation

GUVs. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DOPG) were purchased from Avanti Polar Lipids (Alabaster, USA) and Atto550 labeled DOPE was from Atto Tec (Siegen, Germany). GUVs were prepared as described previously [7]. Briefly, super low melting agarose (1 %, w/v) was coated on a clean glass slide and dried on a hot plate (~50 °C) for 30 min. Afterwards, two droplets of the respective lipid solutions (10 µl each) were spread on the agarose film and dried *in vacuo* for at least 1 h to remove residual chloroform. To visualize the membranes, the lipid solution was prior doped with 0.2 mol% Atto550-DOPE. Then, a seal ring was placed onto the lipid coated areas on the slide to obtain two sealed chambers. For the preparation of GUVs encapsulating Oyster 405 (Luminaris GmbH, Münster, Germany), a buffer containing 10 mM HEPES; pH 7.4, 50 mM KCl, 50 mM NaCl, 1 mg/ml dextran (from *Leuconostoc spp.*, 6 kDa) and 5 µM Oyster 405 (300 µl each) was added to the hybrid film. The glass slide was then left in the dark for 2 h to allow hydration and swelling of the lipids. To harvest the GUV suspension, the glass slide was gently tilted in all directions to detach the liposomes from the surface. The giant

liposomes were then stored in LoBind tubes (1.5 ml, Eppendorf, Hamburg, Germany) at RT and used within three days.

LUVs. All lipids were obtained from Avanti Polar Lipids (Alabaster, USA). Depending on the desired LUV composition, the chloroform-dissolved lipid mixture was placed in a round-bottomed flask under vacuum at 37 °C for at least 1 h to remove residual solvent. The dried lipid film was hydrated with a buffer (150 mM KCl, 25 mM HEPES, pH 7.4) containing 10 % (w/v) sucrose at 45 °C to form liposomes with a final concentration of 4 mM (flow cytometry studies) or 8 mM (CF release experiments). To form LUVs, the suspension was subsequently run through 10 freeze/thawing cycles and passed 21 times through a mini-extruder equipped with a 0.4 µm polycarbonate track-etch membrane (Avanti Polar Lipids, Alabaster, USA). Liposome preparations were analyzed by dynamic light scattering indicating a range of 250 – 400 nm in diameter.

GPMVs. Either 6 x 10^5 HEK-293 cells or 5 x 10^5 MCF-7 were seeded per well in a 6-well culture plate (Sarstedt, Nümbrecht, Germany) and were grown to 70 – 80 % confluency in the appropriate medium supplemented with FBS. GMPVs were prepared as described earlier by R. E. Scott, 1976 with minor modifications.[8] The cells were washed twice with buffer containing 10 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4 (GPMV buffer). To induce the formation of cell-free vesicles, 1 ml of freshly prepared GPMV buffer supplemented with paraformaldehyde (PFA) and dithiothreitol (DTT) at final concentrations of 25 mM and 2 mM respectively was added and the cells were incubated for 2 h at 37 °C while slowly shaken (Certomat H/SII, Braun Biotech, Göttingen, Germany). After incubation, the GPMV-rich cellular supernatant was transferred into a 15 ml conical tube (Sarstedt, Nümbrecht, Germany), where the GPMVs were allowed to settle on ice for 30 min. Afterwards, 20 – 50% of the total volume was transferred from the bottom of the conical tube into a 1.5 ml Eppendorf tube. The amount of vesicles was counted using a Neubauer cell counting chamber, and in 1 ml app. 850.000 vesicles were present. For microscopical studies, GPMV membranes were stained with DiI (Invitrogen), a fluorescent dye, at a final concentration of 5 µg/ml.

Analysis of peptide/lipid interactions

Peptide-induced 5(6)-carboxyfluorescein leakage. CF-containing LUVs were prepared by hydrating a dried lipid film of desired compositions with a buffer containing 100 mM CF. The fluorescence intensity in the presence of 100 mM CF is low due to self-quenching but

increases upon dilution. Free CF outside the LUVs was separated by size exclusion chromatography using a PD10 column (GE Healthcare). Then, peptides were added to LUVs and the release of CF from vesicles was monitored by an increase in the fluorescence intensity using a fluorescence Tecan infinite M200 plate reader. At the end of each experiment, Triton X-100 (0.4% (w/v) final concentration) was applied to measure the maximum of dequenching that will be used to normalize data. The percentage of CF release was determined by

$$\%\ CF\ release = \frac{F_{(t)} - F_0}{F_f - F_0} * 100$$

where $F_{(t)}$ is the fluorescence intensity at time t, F_0 is the fluorescence intensity before peptide addition and F_f is the fluorescence intensity after the final addition of Triton X-100. Each experiment was carried out with n = 3 in duplicate.

Flow cytometry analysis. Various compositions of LUVs were applied to flow cytometry analysis as recently described by the Nickel group.[9] CF-labeled peptides were added to 25 µl solutions of LUVs and incubated at 25 °C for 2 h while mild shaking (450 rpm, Thermomixer compact, Eppendorf, Hamburg, Germany). After incubation, the peptide/liposome solutions were washed with 1 ml with buffer (150 mM KCl, 25 mM HEPES, pH 7.4) and centrifuged at 15,000 g for 10 min at 4 °C. The supernatants were carefully removed and the pellets resuspended in 400 µl buffer solution. Afterwards, the peptide/lipid interactions were analyzed with a fluorescence-activated cell sorter (FACS) instrument (BD Accuri C6 flow cytometer, Heidelberg, Germany).

Confocal laser scanning microscopy. Microscopical studies with GUVs were performed as recently described by us.[7b] Briefly, GUVs loaded with the membrane-impermeant fluorophore Oyster 405 were prepared as described in section 7.1. To remove untrapped Oyster 405, liposomes were centrifuged two times at 14,000 g for 10 min at RT. A 40 µl aliquot of the GUV solution was diluted in 50 µl of the respective buffer without Oyster 405 and was then transferred into a tissue culture vessel (FlexiPERM slide, 8 wells, Sarstedt, Germany). CF-labeled peptides diluted in buffer containing 10 mM HEPES; pH 7.4, 50 mM KCl, 50 mM NaCl, 1 mg/ml dextran (from *Leuconostoc spp.*, 6 kDa) were added to the outer solution of GUVs at a final concentration of 20 µM. The GUV−peptide interaction was analyzed using a confocal laser scanning system (Nikon D-Eclipse C1) consisting of an inverted microscope (Nikon Eclipse Ti) equipped with a 20× objective (NA 0.45, Plan Fluor;

Nikon). Microscope pictures were recorded in 16-bit grayscale, pseudocolored in red (channel 1), green (channel 2), and blue (channel 3) followed by processing with ImageJ.

GPMVs were analyzed by CLSM as described by the Pooga group with minor modifications.[10] Concisely, after labeling the vesicles with the fluorescence stain DiI, the CF-labeled peptides at 1 µM concentrations were immediately added to GPMV suspension and incubated at RT for 1 h. Then, 20 µl of the peptide/membrane solution was deposited onto a µ-slide 8-well (Ibidi) plate and covered with glass cover slips (Ø 9 mm, round, No 1). The GPMVs were investigated by using a Nikon Eclipse Ti confocal laser scanning microscope equipped with a 60× oil-immersion objective. Images were recorded with Nikon EZ-C1 software and adjusted equally with ImageJ software.

Electromobility shift assay

For the formation of plasmid/CPP complexes, the peptides were incubated with a peptide to plasmid charge ratio of 13:1 in 50 µl of 37 °C warm nuclease-free water for 30 min at RT. Charge ratio of 13:1 corresponds to peptide concentration of 20 µM, respectively. Immediately after complexation, 5 µl of 50 % glycerin in water was added to each of the samples which were then electrophoresed for 30 min at 100 V on a 1 % agarose gel stained with ethidium bromide in Tris-acetat-EDTA (TAE) buffer. Pure plasmid was used as control.

Peptide-mediated cell transfection

MCF-7 cells were seeded in a µ-slide 8-well (Ibidi) plate (1 x 10^5 cells/well) and grown to 60 – 70 % confluency. Formation of the pEGFP-N1 plasmid/CPP complexes in water was achieved as described above. Serum-free medium was added to the sample to give a total volume of 150 µl. The cells were incubated with the plasmid/CPP complex for 6 h at 37 °C and 5 % CO_2, or 1 h at 4 °C and then for further 5 h at 37 °C, respectively. After incubation time, the reaction solutions were supplemented with RPM1 1640 medium containing 10 % FBS. As a positive control, cells were transfected with 1.5 µl Lipofectamine 2000 according to the manufacture's protocol. As a negative control, cells were incubated with plasmid alone, respectively. The transfection was determined by fluorescence microscopy after 24 h and 48 h incubation at 37 °C.

Table S1. Analytical data and half times of the peptide conjugates synthesized. All peptides are amidated at the C-terminus. **a** indicates precursor peptides.

Peptide		Sequence	Total net charge	MW_{calc} [Da]	MW_{exp} [Da]	Purity [%]	$\tau_{1/2}$ in serum [h]
linear	CAP18(106-117)	GLRKRLRKFRNK	+8	1570.97	1570.7	≥ 99	4
cyc1a	[Pra^{106}, $Lys(N_3)^{109}$]-CAP18(106-117)	BLRXRLRKFRNK	+7	1635.01	1634.7	≥ 99	-
cyc1	cyc[Pra^{106}, $Lys(N_3)^{109}$]-CAP18(106-117)	BLRXRLRKFRNK	+7	1635.01	1634.78	≥ 99	18
cyc2a	[Pra^{106}, $Lys(N_3)^{113}$]-CAP18(106-117)	BLRKRLRXFRNK	+7	1635.01	1634.77	> 98	-
cyc2	cyc[Pra^{106}, $Lys(N_3)^{113}$]-CAP18(106-117)	BLRKRLRXFRNK	+7	1635.01	1634.89	≥ 99	12
cyc3a	[Pra^{106}, $Lys(N_3)^{117}$]-CAP18(106-117)	BLRKRLRKFRNX	+7	1635.01	1633.27	> 95	-
cyc3	cyc[Pra^{106}, $Lys(N_3)^{117}$]-CAP18(106-117)	BLRKRLRKFRNX	+7	1635.01	1634.66	≥ 99	18

B: L-propargylglycine, X: L-(Ɛ-azido)-lysine; ___: amino acids involved in cyclization

Table S2. Analytical data of the 5(6)-carboxyfluorescein labeled peptide conjugates synthesized. All peptides are amidated at the C-terminus. **a** indicates precursor peptides.

Peptide		Sequence	Total net charge	MW_{calc} [Da]	MW_{exp} [Da]	Purity [%]
CF-linear	CF-CAP18(106-117)	CF-GLRKRLRKFRNK	+7	1929.28	1929.09	> 96
CF-cyc1a	[Pra^{106}, $Lys(N_3)^{109}$,Lys^{117}(CF)]-CAP18(106-117)	BLRXRLRKFRNK(CF)	+6	1993.33	1992.93	> 95
CF-cyc1	cyc[Pra^{106}, $Lys(N_3)^{109}$, Lys^{117}(CF)]-CAP18(106-117)	BLRXRLRKFRNK(CF)	+6	1993.33	1992.74	≥ 99
CF-cyc2a	[CF-Pra^{106}, $Lys(N_3)^{113}$]-CAP18(106-117)	CF-BLRKRLRXFRNK	+6	1993.33	1992.96	> 98
CF-cyc2	cyc[CF-Pra^{106}, $Lys(N_3)^{113}$]-CAP18(106-117)	CF-BLRKRLRXFRNK	+6	1993.33	1993.78	≥ 99
CF-cyc3a	[Pra^{106}, $Lys(N_3)^{117}$]-CAP18(106-117)	CF-BLRKRLRKFRNX	+6	1993.33	1992.94	≥ 99
CF-cyc3	cyc[Pra^{106}, $Lys(N_3)^{117}$]-CAP18(106-117)	CF-BLRKRLRKFRNX	+6	1993.33	1993.05	≥ 99

B: L-propargylglycine, X: L-(Ɛ-azido)-lysine; ___: amino acids involved in cyclization

Table S3. Fragment masses of linear and cyclic peptides. Shown are experimental found masses of b- and y-ions by MALDI-MS/MS. Highlighted in grey: cyclic part of the peptide. All masses listed correspond to $[M+H]^+$ molecular ions.

cyc1a	b_1 --	b_2 209,09	b_3 365,18	b_4 519,22	b_5 675,34	b_6 788,43	b_7 944,52	b_8 1072,63	b_9 1219,74	b_{10} 1375,80	b_{11} 1490,23	b_{12} --
	y_{12} 1635,06	y_{11} 1539,98	y_{10} 1426,99	y_9 1270,84	y_8 1116,69	y_7 960,60	y_6 847,51	y_5 691,42	y_4 563,35	y_3 416,25	y_2 260,11	y_1 146,09
cyc1	b_1 --	b_2 --	b_3 --	b_4 519,29	b_5 675,33	b_6 788,42	b_7 944,49	b_8 1072,58	b_9 1219,63	b_{10} 1375,72	b_{11} 1489,75	b_{12} --
	y_{12} 1635,01	y_{11} --	y_{10} --	y_9 --	y_8 1116,59	y_7 960,50	y_6 847,44	y_5 691,38	y_4 563,32	y_3 416,20	y_2 260,07	y_1 146,07
cyc2a	b_1 96,11	b_2 209,05	b_3 365,09	b_4 493,18	b_5 649,25	b_6 762,33	b_7 918,37	b_8 1072,43	b_9 1219,45	b_{10} 1375,66	b_{11} 1490,00	b_{12} 1617,43
	y_{12} 1635,17	y_{11} 1539,85	y_{10} 1426,55	y_9 1270,65	y_8 1142,47	y_7 986,44	y_6 873,36	y_5 717,25	y_4 563,18	y_3 416,12	y_2 260,06	y_1 146,06
cyc2	b_1 --	b_2 --	b_3 --	b_4 --	b_5 --	b_6 --	b_7 --	b_8 --	b_9 --	b_{10} --	b_{11} --	b_{12} --
	y_{12} 1634,99	y_{11} --	y_{10} --	y_9 --	y_8 --	y_7 --	y_6 --	y_5 --	y_4 --	y_3 416,20	y_2 --	y_1 --
cyc3a	b_1 --	b_2 209,24	b_3 365,37	b_4 493,49	b_5 649,60	b_6 762,73	b_7 918,78	b_8 1046,75	b_9 1193,80	b_{10} 1350,06	b_{11} 1464,06	b_{12} --
	y_{12} 1635,29	y_{11} --	y_{10} 1426,89	y_9 1270,63	y_8 1142,64	y_7 986,78	y_6 873,45	y_5 717,70	y_4 --	y_3 --	y_2 --	y_1 --
cyc3	b_1 --	b_2 --	b_3 --	b_4 --	b_5 --	b_6 --	b_7 --	b_8 --	b_9 --	b_{10} --	b_{11} --	b_{12} --
	y_{12} 1635,20	y_{11} --	y_{10} --	y_9 --	y_8 --	y_7 --	y_6 --	y_5 --	y_4 --	y_3 --	y_2 --	y_1 --

Table S4. ^{1}H chemical shifts (p.p.m.) of the **linear** peptide in PBS buffer (pH 7, H_2O:D_2O 9:1) at 278K.

Residue	Chemical shift (p.p.m.)[a]			
	HN	H^{α}	H^{β}	Others
G1		3.836		
L2	8.732	4.314	1.597	δ 0.890
R3	8.649	4.291	1.643, 1.804	γ 1.603, δ 3.191, ε 7.446
K4	8.583	4.240	1.769	γ 1.459, δ 1.671, ε 2.974
R5	8.583	4.298	1.788	γ 1.601, δ 3.174, ε 7.455
L6	8.518	4.335	1.647, 1.564	δ 0.939 , 0.877
R7	8.475	4.249	1.739, 1.658	γ 1.536, δ 3.156, ε 7.436
K8	8.478	4.214	1.636	γ 1.288, ε 2.932
F9	8.430	4.603	3.020, 3.113	**2H-6H** 7.259; **3-5H** 7.335; **4H** 7.301
R10	8.371	4.260	1.697, 1.777	γ 1.543, δ 3.162, ε 7.421
N11	8.600	4.623	2.758, 2.832	δ 7.074, 7.804
K12	8.517	4.234	1.861	γ 1.408, δ 1.667, ε 2.966
$CONH_2$				7.256, 7.731

[a]Chemical shifts are relative to TSP (0 p.p.m.),

Table S5. ^{1}H chemical shifts (p.p.m.) of the **cyc1** in PBS buffer (pH 7, H_2O:D_2O 9:1) at 278K.

Residue	Chemical shift (p.p.m.)[a]			
	HN	H^{α}	H^{β}	Others
Pra	-	4.216	3.300, 3.231	
L2	8.541	4.216	1.638	δ 0.883, 0.921
R3	8.337	4.074	1.901, 1.675	γ 1.573, δ 3.206, ε 7.463
Lys(N_3)4	7.906	4.270	1.780, 1.934	γ 1.161, δ 1.344, 1.485, ε 4.422, **triazole** 7.917
R5	8.331	4.254	1.768	γ 1.561, δ 3.169 , ε 7.479
L6	8.443	4.313	1.644	γ 1.559, δ 0.872, 0.933
R7	8.435	4.237	1.746	γ 1.567 , δ 3.157, ε 7.495
K8	8.488	4.210	1.671	γ 1.269, 1.309, δ 1.633**,** ε 2.931
F9	8.443	4.591	3.026, 3.113	**2H-6H** 7.257; **3-5H** 7.340; **4H** 7.303
R10	8.388	4.258	1.775	γ 1.557 , δ 3.156, ε 7.508
N11	8.625	4.616	2.755, 2.840	δ 7.078, 7.827
K12	8.539	4.227	1.856, 1.761	γ 1.399, 1.462, δ 1.665, ε 2.966
$CONH_2$				7.267, 7.750

[a]Chemical shifts are relative to TSP (0 p.p.m.),

Table S6. ^{1}H chemical shifts (p.p.m.) of the **cyc2** in PBS buffer (pH 7, H_2O:D_2O 9:1) at 278K.

Residue	Chemical shift (p.p.m.)[a]			
	HN	H^{α}	H^{β}	Others
Pra		4.089	3.269	
L2	8.700	4.372	1.572	δ 0.850 , 0.913
R3	8.766	4.371	1.644, 1.818	γ 1.553, δ 3.165, ε 7.500
K4	8.599	4.209	1.764, 1.850	γ 1.421; 1.357, δ 1.666, ε 2.929
R5	8.518	4.132	1.825, 1.869	γ 1.585, δ 3.164 , ε 7.550
L6	8.270	4.249	1.695	δ 0.921, 0.859
R7	8.296	4.257	1.749	γ 1.503 , δ 3.158, ε 7.513
Lys(N_3)8	8.340	4.183	1.856	γ 1.181, 1.237, δ 1.639, ε 4.374, **triazole** 7.915
F9	8.447	4.618	2.990, 3.111	**2H-6H** 7.244; **3-5H** 7.323; **4H** 7.299
R10	8.439	4.270	1.705, 1.791	γ 1.560 , δ 3.153, ε 7.495
N11	8.662	4.625	2.749, 2.845	δ 7.836, 7.077
K12	8.539	4.236	1.756, 1.859	γ 1.415, 1.456, δ 1.674, ε 2.966
$CONH_2$				7.759, 7.270

[a]Chemical shifts are relative to TSP (0 p.p.m.),

Table S7. ^{1}H chemical shifts (p.p.m.) of the **cyc3** in PBS buffer (pH 7, H_2O:D_2O 9:1) at 278K.

Residue	Chemical shift (p.p.m.)[a]			
	HN	H^{α}	H^{β}	Others
Pra		4.001	3.180	
L2			1.712	δ 0.918, 0.852
R3	8.874	4.313	1.840	γ 1.639, δ 3.119 , ε 7.460
K4	8.416	4.192	1.867, 1.766	γ 1.379, ε 2.940
R5	8.532	4.272	1.781, 1.859	γ 1.593, δ 3.156 , ε 7.474
L6	8.333	4.322	1.589	δ 0.856, 0.912
R7	8.346	4.169	1.800	γ 1.595, δ 3.167, ε 7.498
K8	8.319	4.150	1.170	γ 1.584 , ε 2.887
F9	8.235	4.615	3.029, 3.184	**2H-6H** 7.233 ; **3-5H** 7.318; **4H** 7.274
R10	8.255	4.264	1.746, 1.811	γ 1.568 , δ 3.159, ε 7.567
N11	8.577	4.656	2.762, 2.851	δ 7.060, 7.810
Lys(N_3)12	8.408	4.200	1.897	γ 1.400, δ 1.753, ε 4.376, **triazole** 7.882
$CONH_2$				7.258, 7.735

[a]Chemical shifts are relative to TSP (0 p.p.m.),

Table S8. ^{1}H chemical shifts (p.p.m.) of the **linear** peptide in the presence of d_{25}-SDS (pH 7, H_2O:D_2O 9:1) at 298K.

Residue	Chemical shift (p.p.m.)[a]			
	HN	H^{α}	H^{β}	Others
G1		4.110,3.957		
L2	7.993	4.153	1.770	δ 0.952
R3	8.657	4.416	1.906	γ 1.712 , δ 3.227 , ε 7.232
K4	7.742	4.149	1.796, 1.653	γ 1.337 , ε 2.964
R5	8.326	3.996	1.931, 1.758	γ 1.689, δ 3.134, ε 7.298
L6	8.147	4.229	1.819	γ , δ 0.930
R7	7.935	4.425	1.900	γ 1.716, δ 3.205, ε 7.139
K8	7.861	4.118	1.873, 1.675	γ 1.466, ε 2.994
F9	8.023	4.557	2.771, 2.667	**2H-6H** 7.325
R10	7.873	4.170	1.791, 1.949	γ 1.682 , δ 3.186, ε 7.167
N11	8.068	4.660	2.889, 2.766	δ 7.587, 6.874
K12	7.937	4.203	1.822, 1.672	γ 1.447 , ε 3.000
$CONH_2$				7.473, 7.029

[a]Chemical shifts are relative to TSP (0 p.p.m.),

Table S9. ^{1}H chemical shifts (p.p.m.) of the **cyc3** in the presence of d_{25}-SDS (pH 7, H_2O:D_2O 9:1) at 313K.

Residue	Chemical shift (p.p.m.)[a]			
	HN	H^{α}	H^{β}	Others
Pra		4.321	3.473, 3.414	
L2	7.817	4.332	1.821	γ 1.688 δ 0.980, 0.936
R3	7.832	4.326	1.907	γ 1.667, δ 3.177, ε 7.093
K4	7.998	4.176	1.818, 1.645	γ 1.346, 1.442, ε 2.994
R5	7.979	4.180	1.814	γ 1.636 , δ 3.192, ε 7.170
L6	7.799	4.365	1.812	γ 1.696 , δ 0.871
R7	7.963	4.166	1.803	γ 1.645, δ 3.176, ε 7.180
K8	7.887	4.113	1.570	γ 1.173, ε 2.923
F9	7.649	4.263	3.058, 3.255	**2H-6H** 7.198; **3-5H** 7.303; **4H** 7.281
R10	7.691	4.252	1.773, 1.844	γ 1.581, δ 3.191, ε 7.090
N11	8.262	4.683	2.874, 2.749	δ 7.504, 6.812
Lys(N_3)12	7.943	4.248	1.897	γ 1.416, ε 4.370, **triazole** 7.987
$CONH_2$				7.502, 6.938

[a]Chemical shifts are relative to TSP (0 p.p.m.),

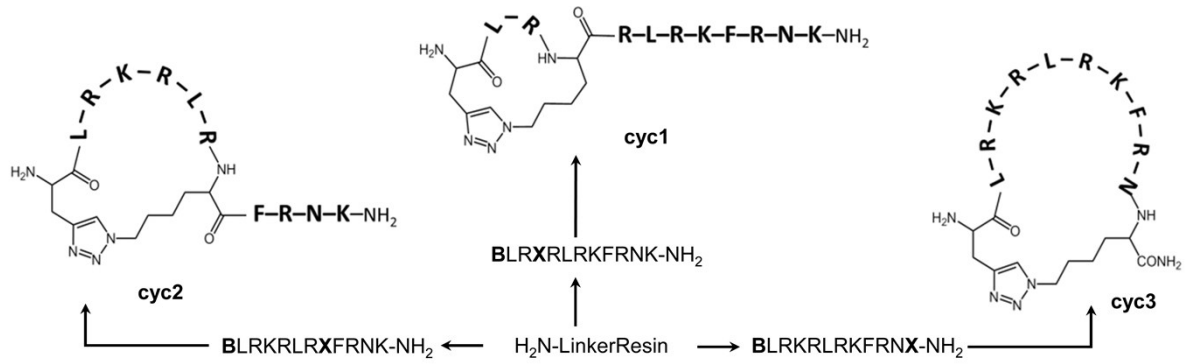
Table S10. Diffusion coefficient temperature of linear anc cyc3 peptides in the presence of SDS micelles (600MHz, 298K)

Peptide	Diffusion coefficient D ($m^2/s \times 10^{-11}$)	
	Water	**SDS**
Linear	6.1	2.14
Cyc3	3.97	2.32

Table S11. Statistics for the NMR solution structure of **linear** and **cyc1-3** peptides. Average values over the 20 energy-minimized CYANA conformers.

Parameter	Value					
	Linear /water	**Cyc1 /water**	**Cyc2 /water**	**Cyc3 /water**	**Linear /SDS**	**Cyc3 /SDS**
NOE cross-peaks	366	423	290	268	387	250
Assigned (%)	96.45	99.5	97.6	86.7	95.3	85.2
NOE upper distance limits	102	86	130	123	113	109
Short-range, |i–j|≤1	92	78	103	85	89	91
Medium-range, 1<|i–j|<5	10	8	19	20	24	16
Long-range, |i–j|≥5	0	0	8	18	0	2
Violations > 0.2 Å	0	0	0	0	0	0
CYANA target function (Å^2)	0	0	0.02	0.01	0	0.03
Rmsd to mean co-ordinates (Å)						
Backbone N, C□,C’	2.27	2.19 (0.5)[a]	0.97(0.81)[b]	0.66	1.48	1.05
All heavy atoms of residues	3.61	4.09	2.05	1.52	2.66	1.73
Ramachandran plot statistics (%)						
Residues in the most favoured	66.5	46.7	47.5	70	57.5	48.8
Residues in the additionally	33.5	53.3	52.5	30	41	50.0
Residues in the generously	0	0	0	0	1.5	1.2
Residues in the disallowed	0	0	0	0	0	0

[a]RMSD value calculated for peptide fragment 1-4. [b]RMSD value calculated for peptide fragment 1-8.



Scheme S1. The synthesis of the cyclic peptide variants; (i) Solid phase peptide synthesis using Fmoc/*t*Bu strategy, (ii) Cleavage of the peptide and deprotection, (iii) Cu(I) mediated Huisgen 1,3-dipolar cycloaddition (B: L-propargylglycine, X: L-azidolysine)

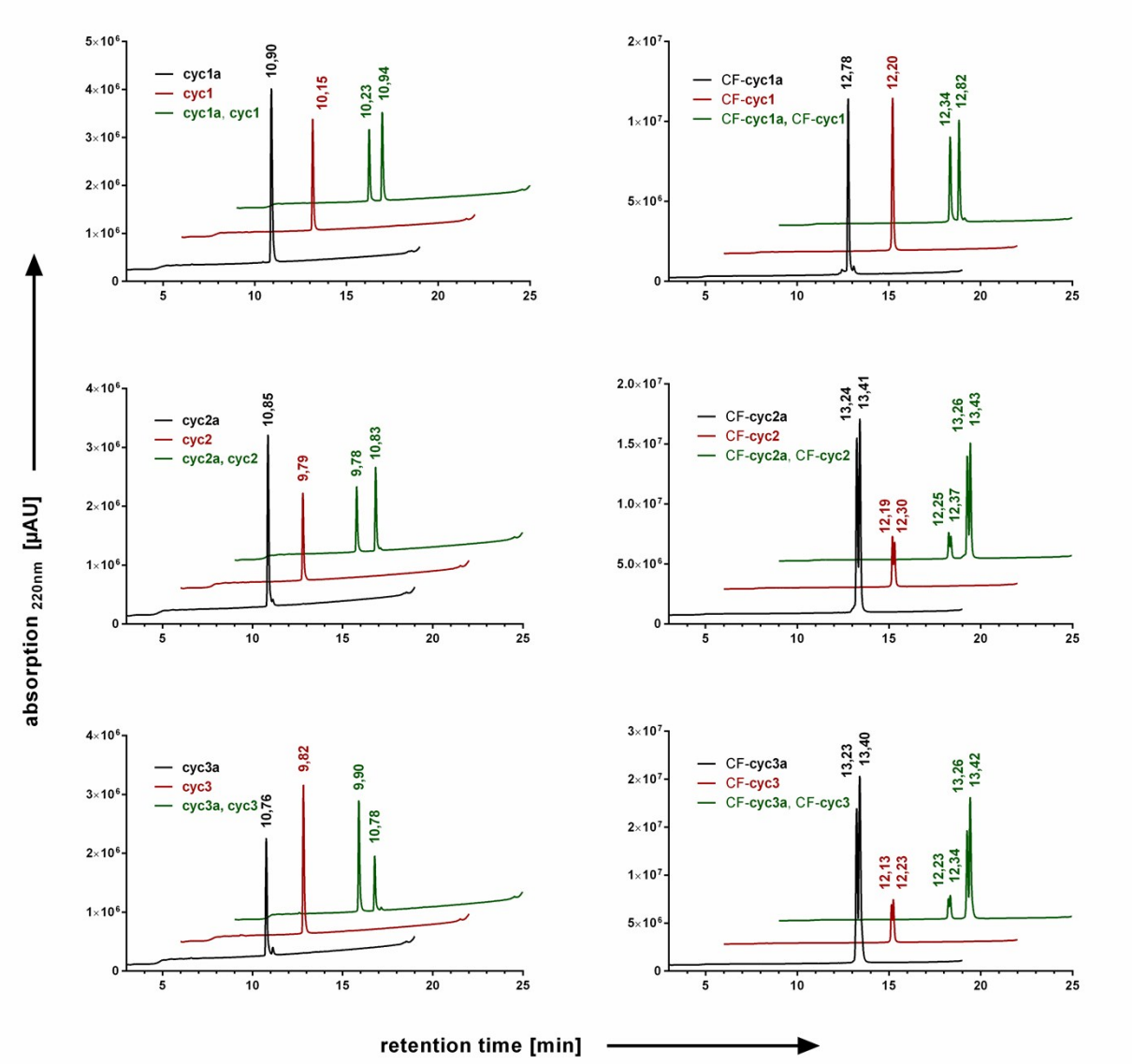


Figure S1. HPLC chromatographs of linear and cyclic peptides. Different retention times of the linear and cyclic peptides allow a differentiation and an exact determination. Black: linear precursor, red: cyclic peptide, green: coelution of both variants.

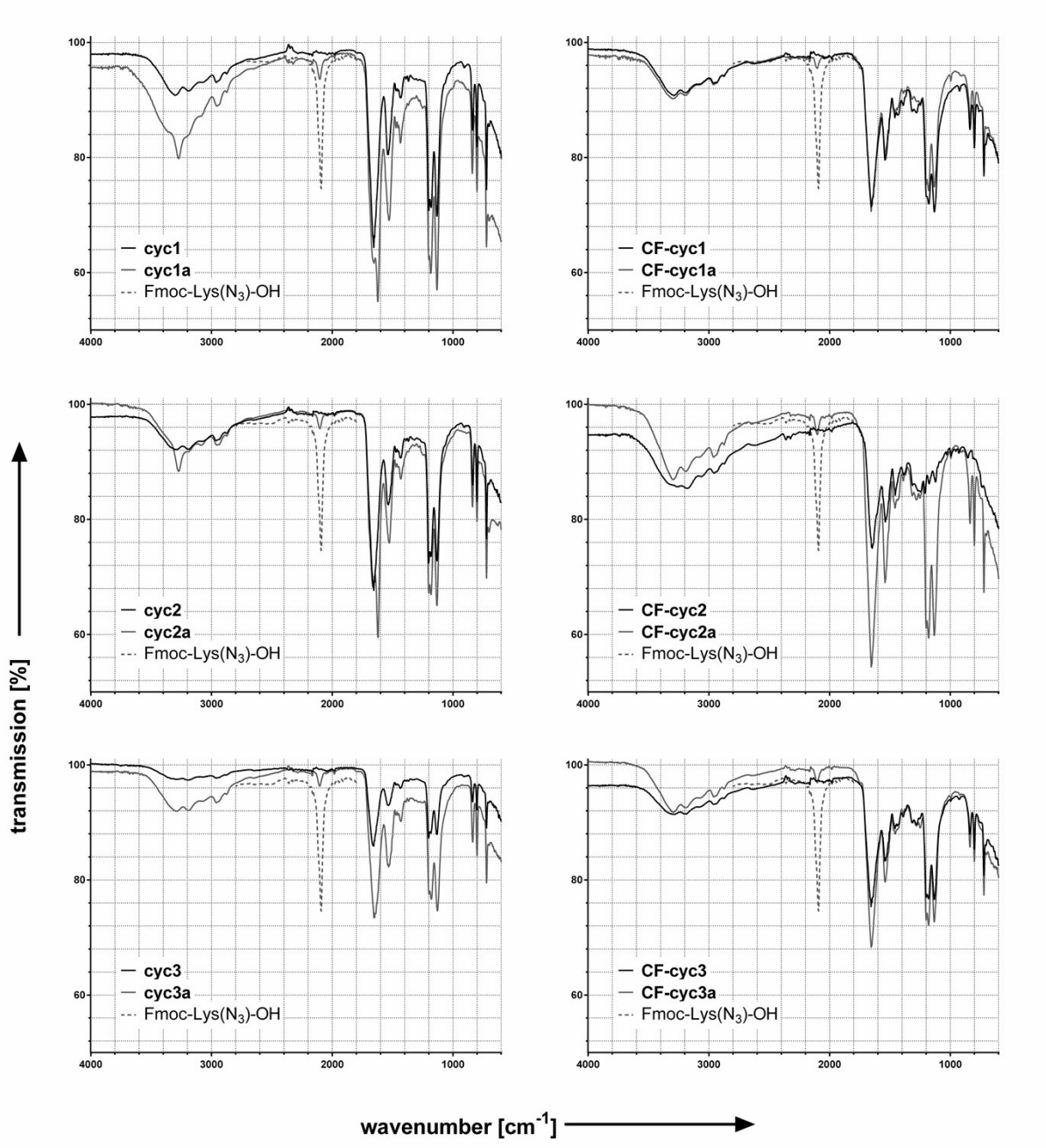
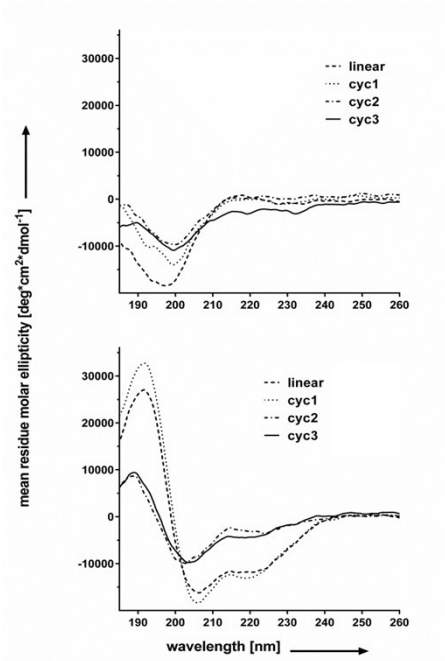


Figure S2. IR spectra of linear (grey), cyclic peptides (black) and additionally of Fmoc-Lys(N_3) in the range of the characteristic azide band (dashed).

A



B

	R-value(=$\Theta_{222nm}/\Theta_{208nm}$)			
	linear	**cyc1**	**cyc2**	**cyc3**
buffer	0.09	0.36	0.22	0.52
buffer + TFE	0.63	0.67	0.52	0.65
neutral LUVs	0.64	-	-	0.18
negatively charged LUVs	0.75	0.55	0.09	-

Figure S3. A Circular dichroism spectra of 20 µM peptides in 10 mM phosphate buffer (pH 7.0) and in 10 mM phosphate buffer with the addition of 50 % trifluoroethanol (TFE). **B** R-values (R) represent the ratio between the molar ellipticity values at 222 and 208 nm.[11]

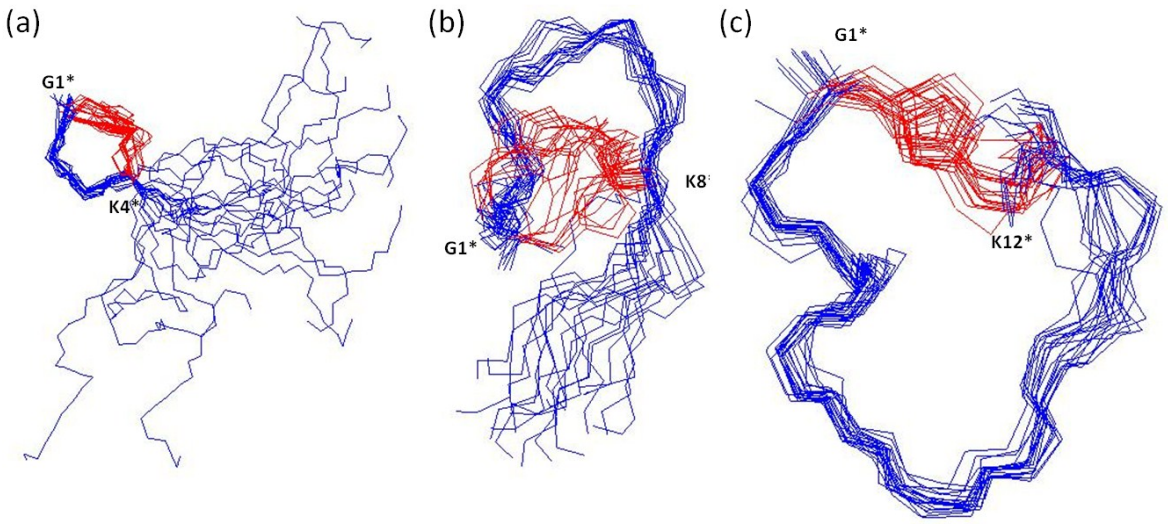


Figure S4. Overlapping of the 20energy minimized structures of (a) **cyc1** (c) **cyc2** and (d) **cyc3** peptides in water as calculated from NMR data.

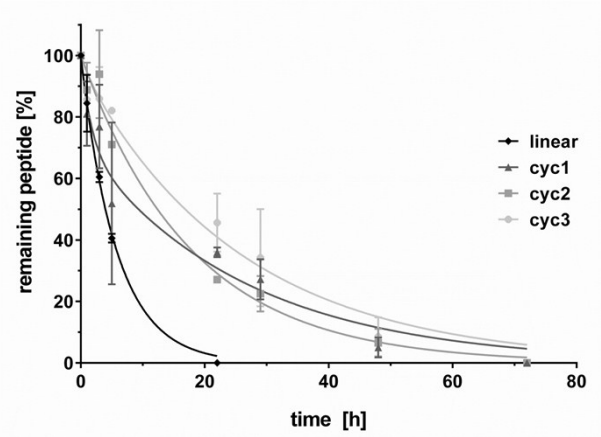


Figure S5. Stability of peptides in human serum at 37 °C measured by the percentage of remaining intact peptide. (n = 3, error bars represent standard deviations)

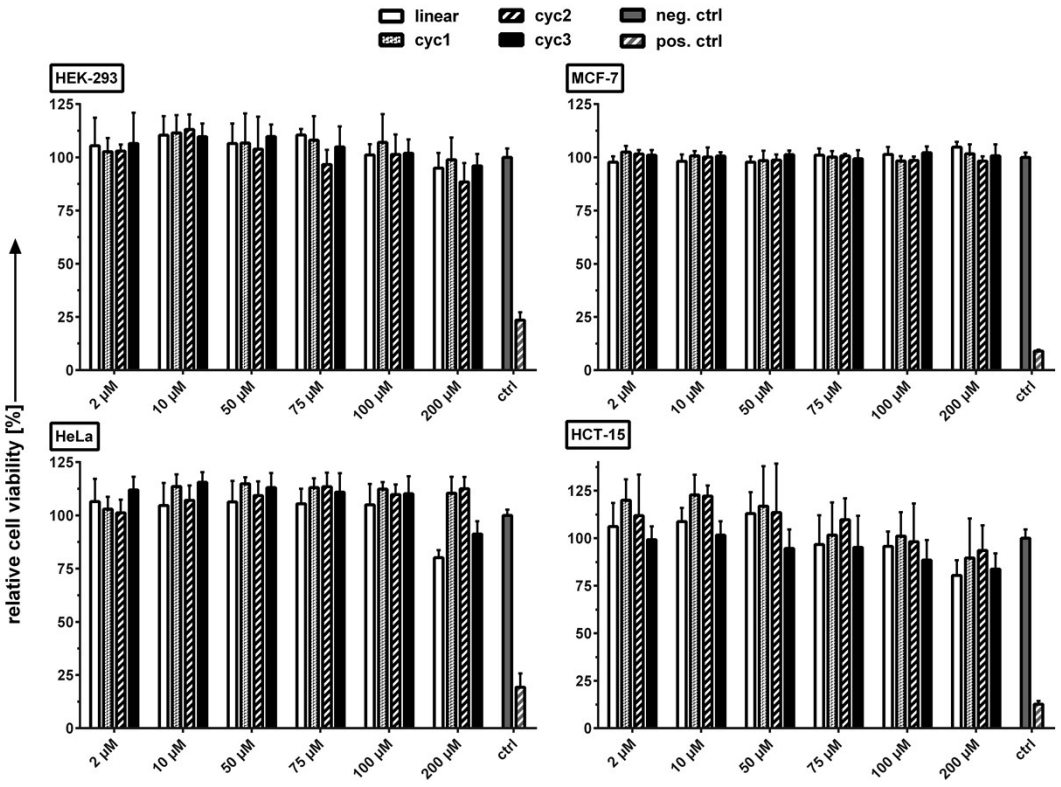


Figure S6. Cytotoxicity of CAP18(106-117) conjugates at different concentrations against various cell lines after 24 h incubation. Untreated cells served as negative control and cells treated with 70 % ethanol served as positive control. Experiments were conducted in duplicate with n = 3. Error bars represent the standard deviation.

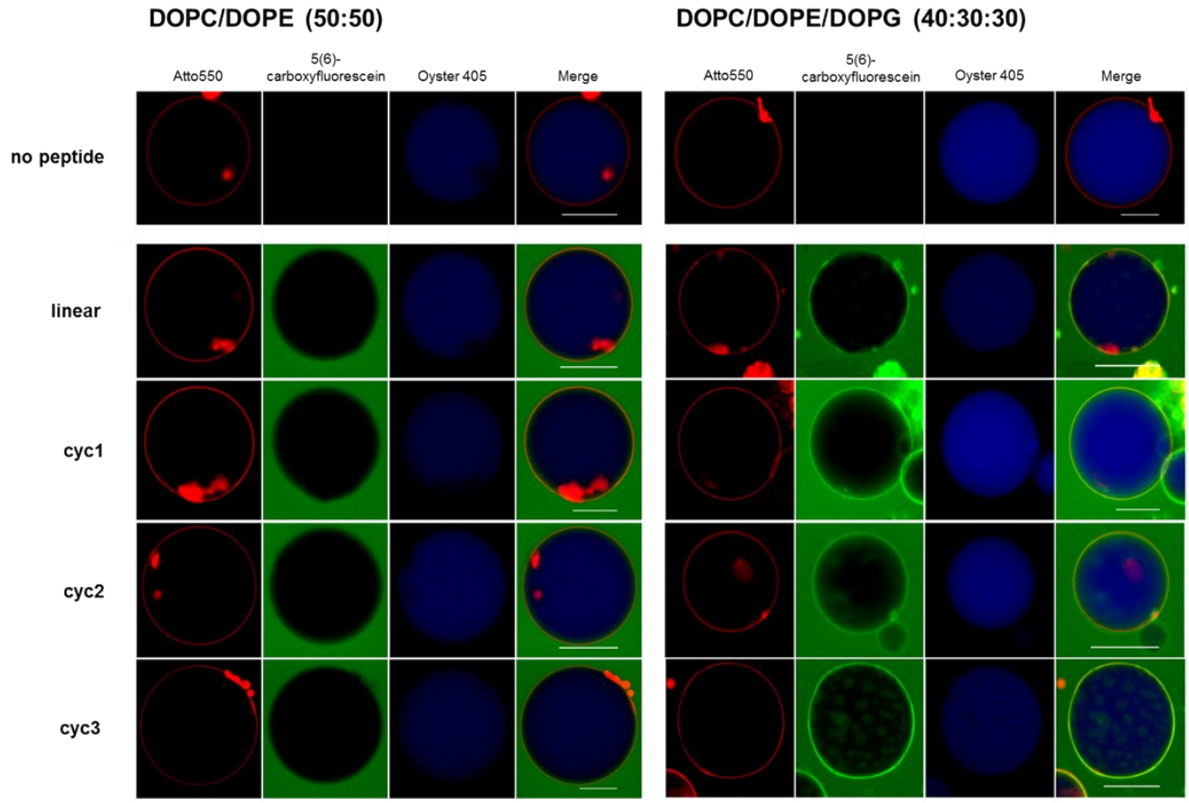


Figure S7. Interaction of CF-labeled peptides with neutral (left) and negatively charged (right) giant unilamellar vesicles (GUVs) loaded with Oyster 405 (blue). GUVs doped with 0.2 mol% Atto550-labeld DOPE to visualize their membranes (red) were incubated with CF-labeled peptides (green, 20 µM) for 90 min and analyzed by confocal laser scanning microscopy. Scale bars, 30 µm.

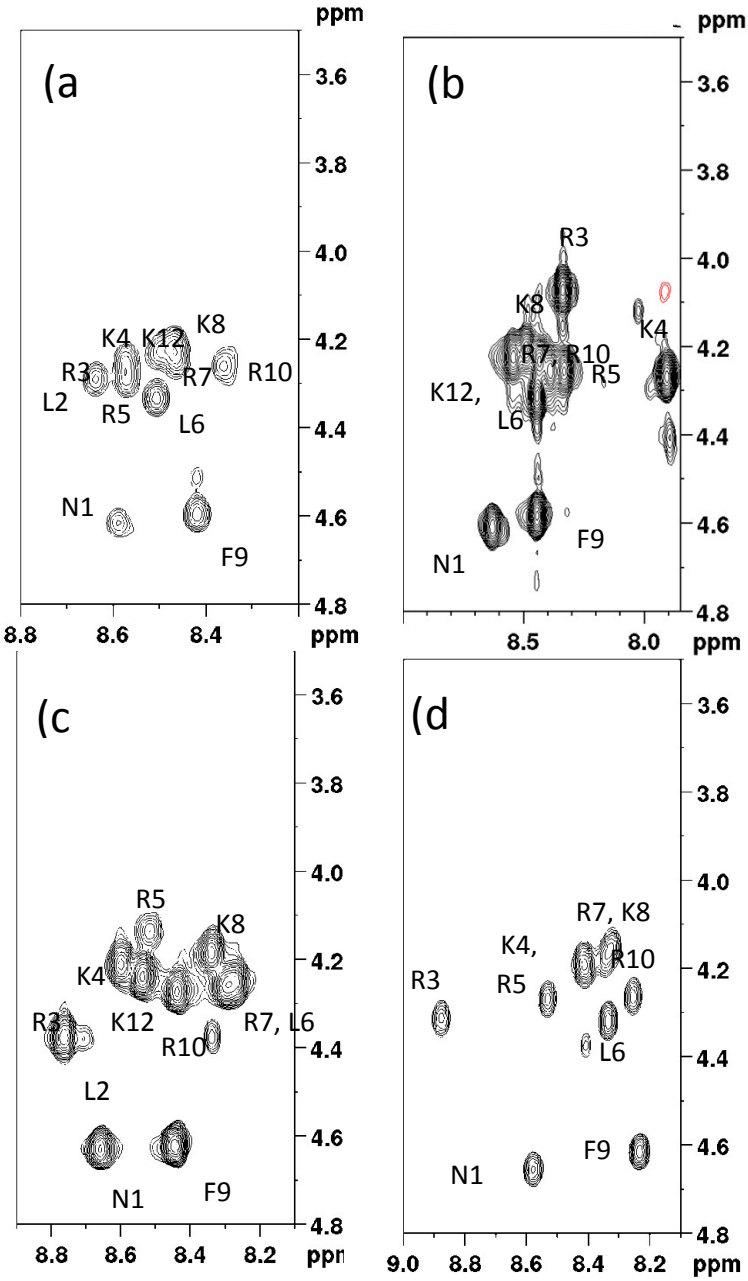


Figure S8. Expansions of the Hα-HN region of the 2D TOCSY spectra (600MHz, 80ms mixing time, 278K) of (a) **linear**, (b) **cyc1**, (c) **cyc2** and (d) **cyc3** peptides in water/PBS buffer (pH 7) K4* stands for Lys(N_3)4, K8* stands for Lys(N_3)8 and K12* for Lys(N_3)12.

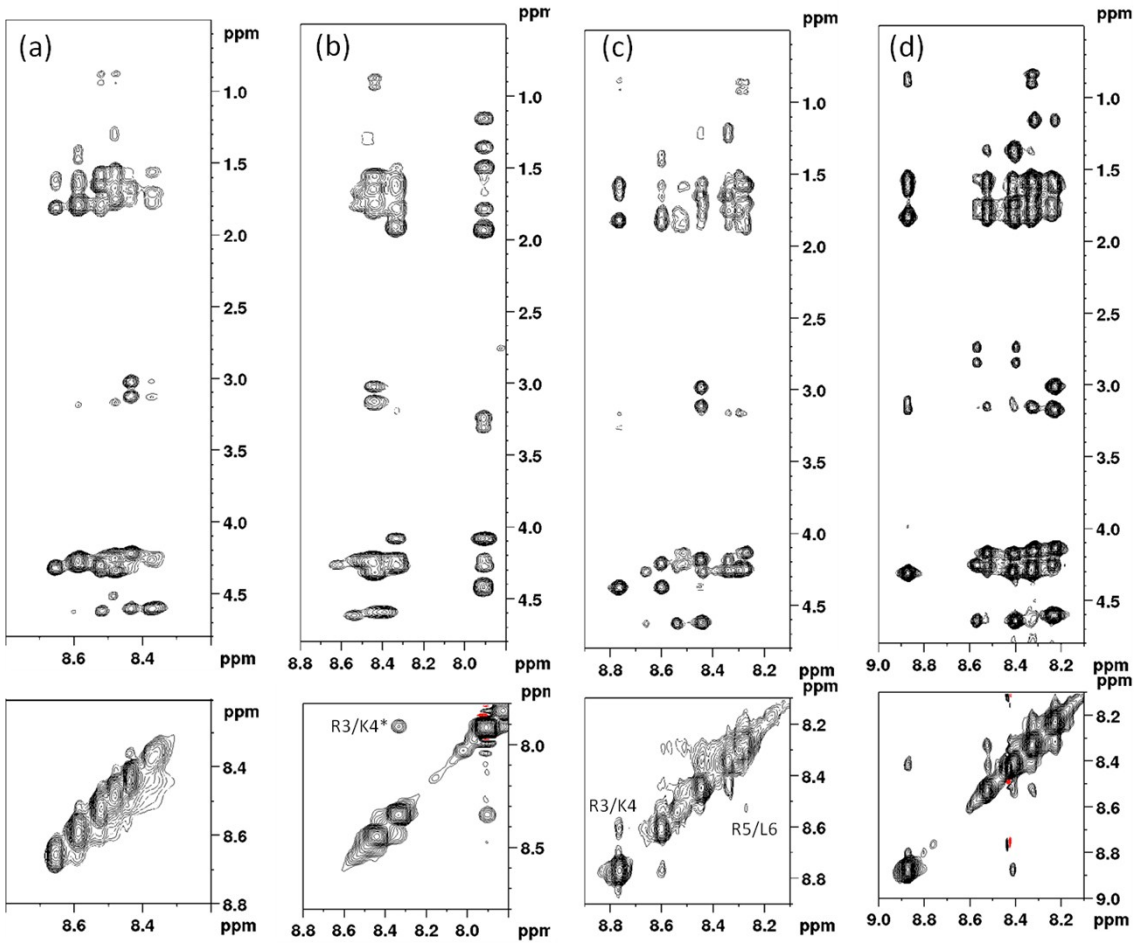


Figure S9. Expansions of selected regions of the 2D NOESY spectra (600MHz, 150ms mixing time, 278K) of (a) **linear**, (b) **cyc1**, (c) **cyc2** and (d) **cyc3** peptides in water/PBS buffer at 278K. K4* stands for Lys(N_3)4, K8* stands for Lys(N_3)8 and K12* for Lys(N_3)12.

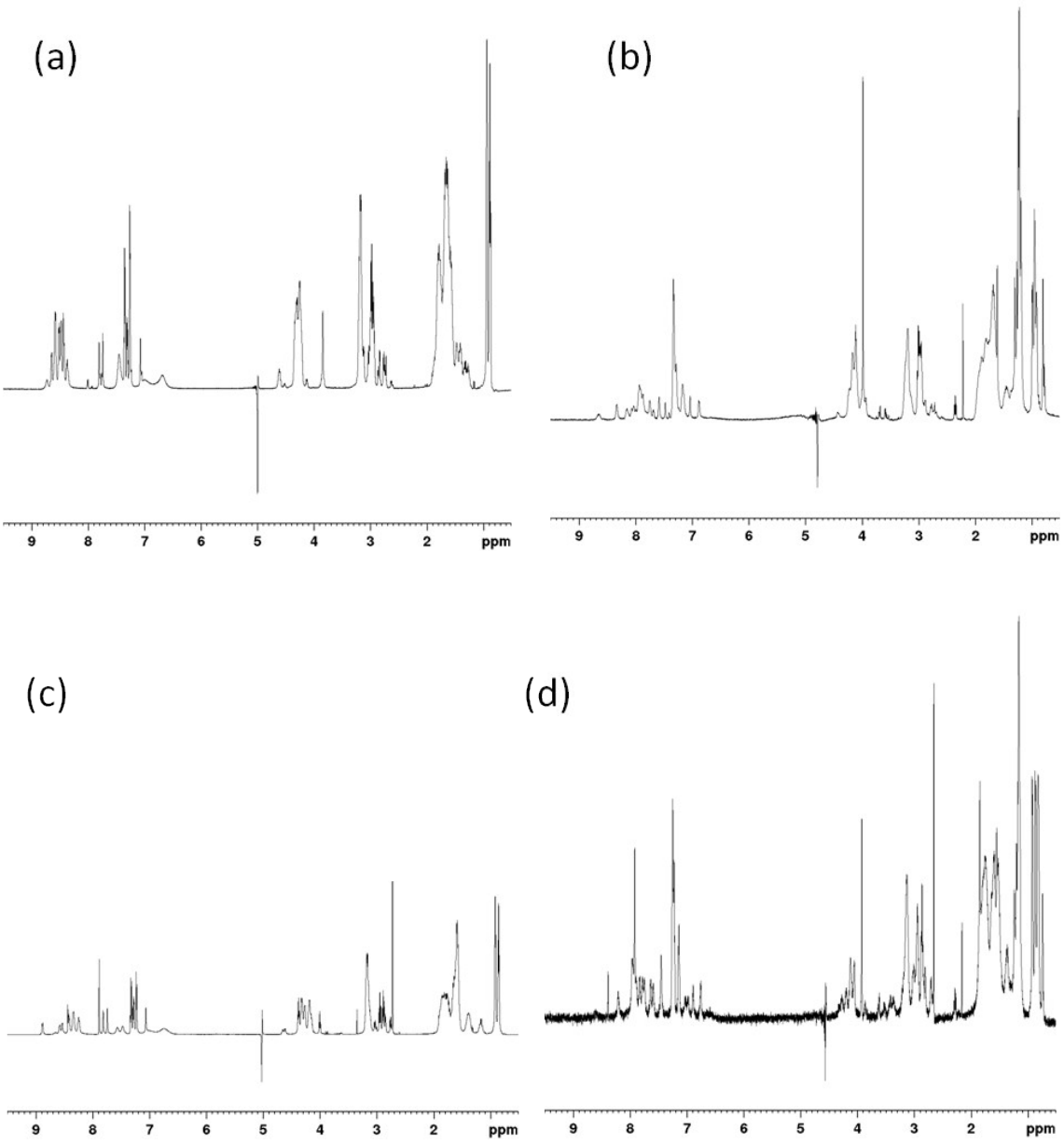


Figure S10. ^{1}H NMR spectra (600MHz,) of (upper pannel) **linear** peptide in (a) water (278K) and (b) SDS micelles (298K) and (lower pannel) **cyc3** in (c) water (278K) and (d) SDS micelles (313K).

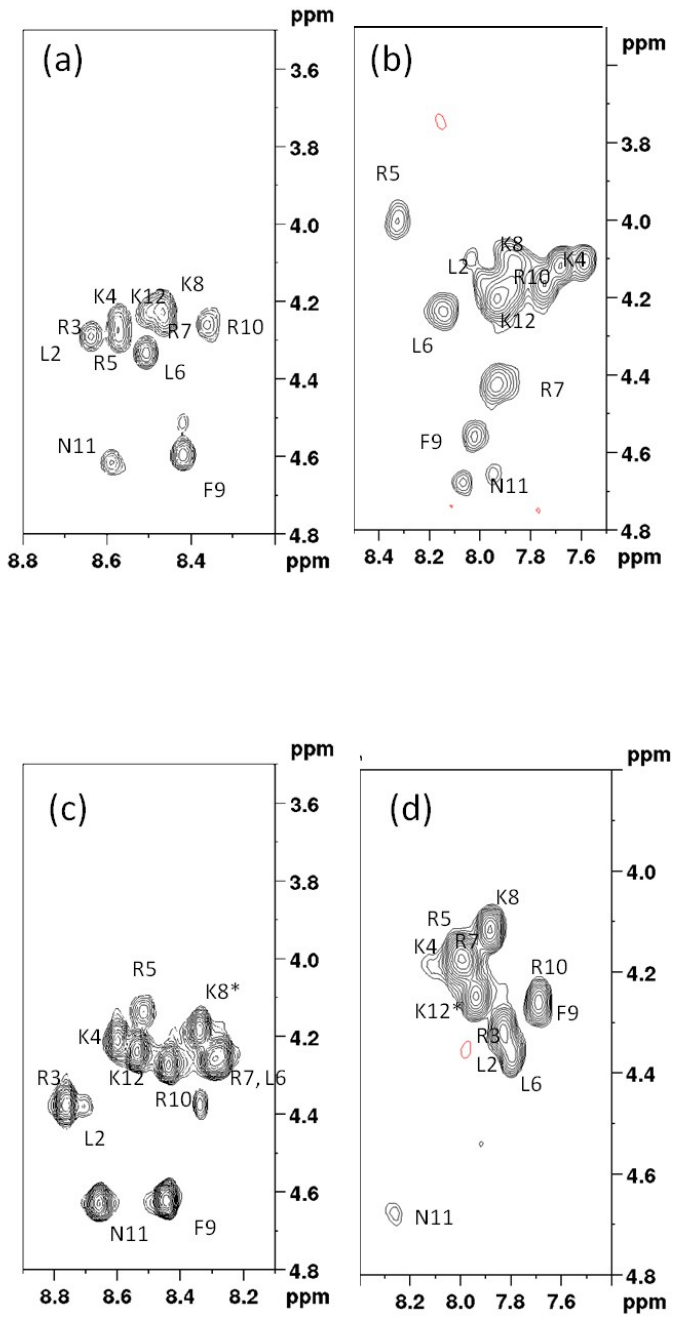


Figure S11. Expansions of the Hα-HN region of the 2D TOCSY spectra of (*upper pannel*) **linear** peptide in (a) water (600MHz, 80ms mixing time, 278K) and (b) with SDS micelles (600MHz, 80ms mixing time, 298K) and (*lower pannel*) **cyc3** in (c) water (600MHz, 80ms mixing time, 278K) and (d) with SDS micelles of (600MHz, 80ms mixing time, 313K). K12* for Lys(N_3)12.

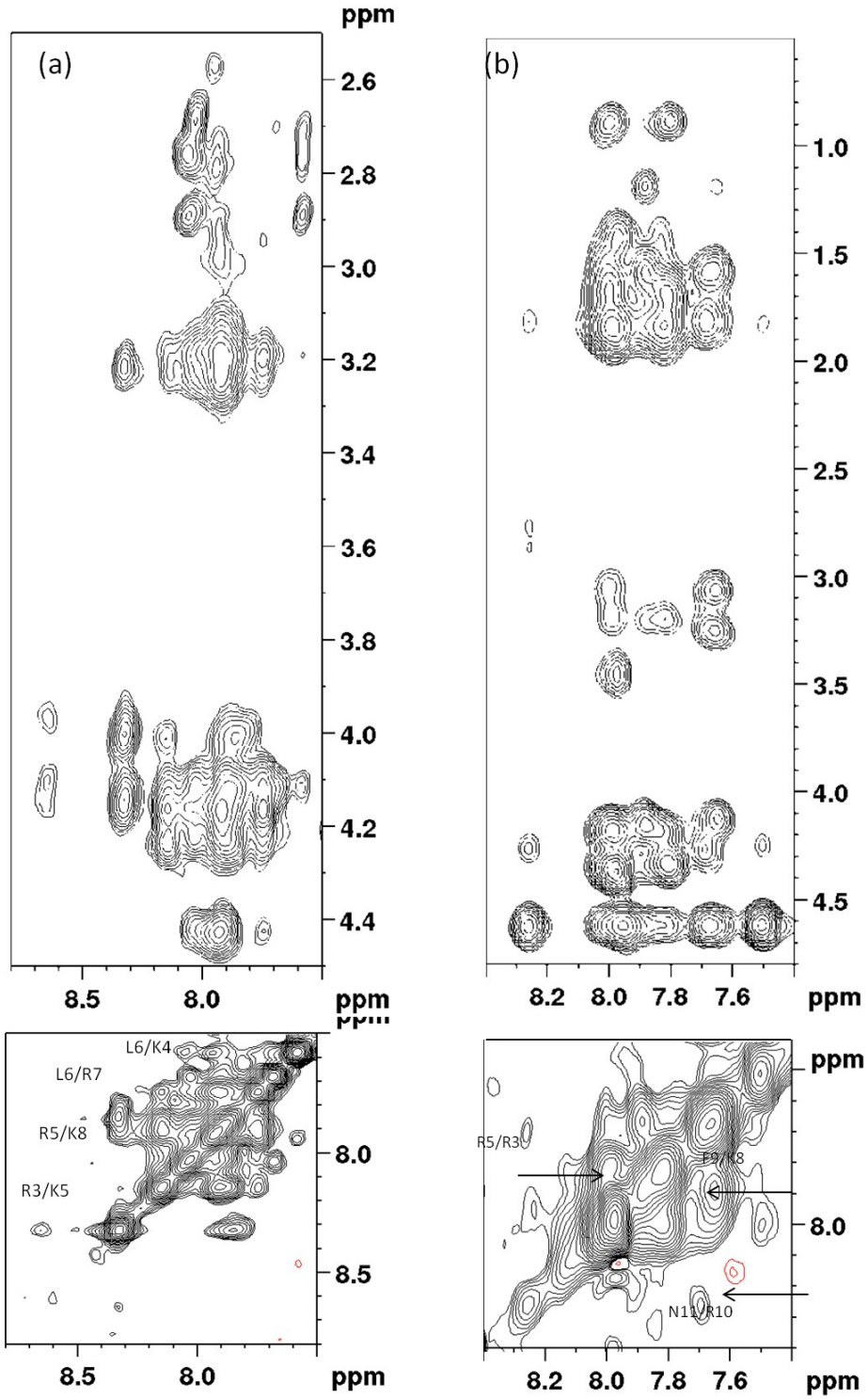


Figure S12. Sections of 2D NOESY spectra of (a) **linear** peptide and (b) **cyc3** in the presence of SDS micelles. K12* for Lys(N_3)12

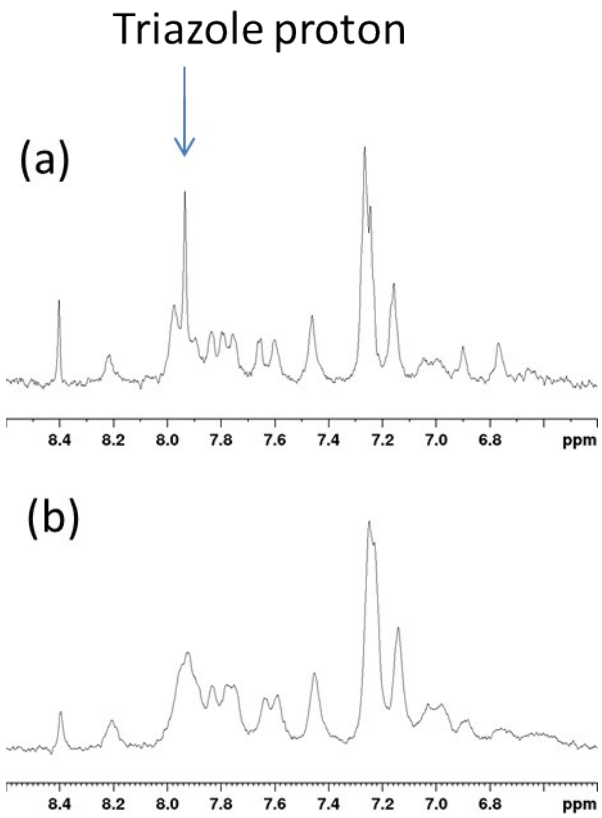


Figure S13. ^{1}H NMR spectra (600MHz,) of **cyc3** (a) with SDS micelles and (b) upon addition of $MnCl_2$.

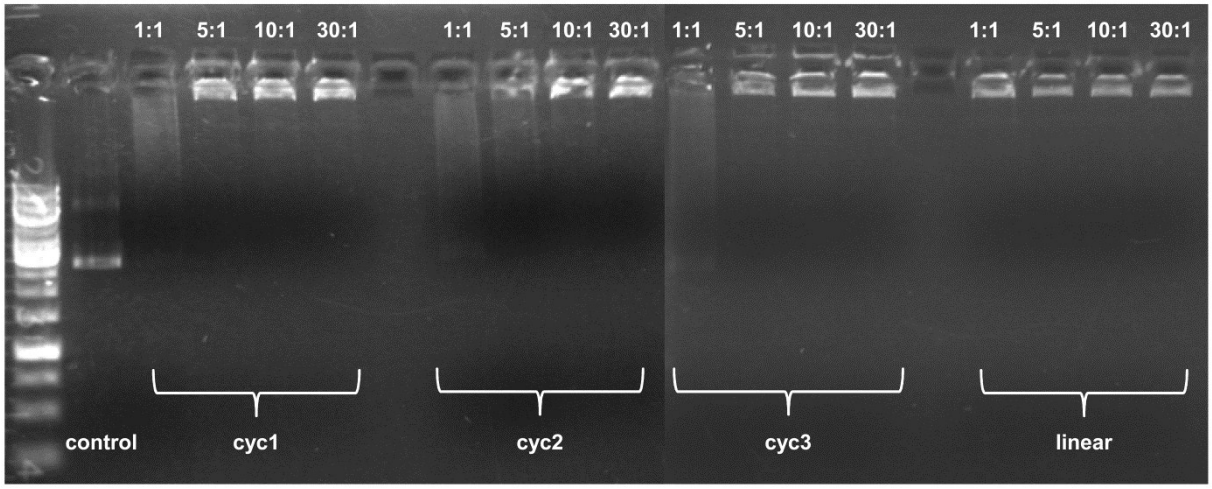


Figure S14. Electromobility shift assay. pEGFP-N1 plasmid was complexed with the peptides cyc1, cyc2, cyc3 and linear at various charge ratios specified on top. Afterwards, the samples were electrophoresed on an 1 % agarose gel. pEGFP-N1 plasmid versus control.

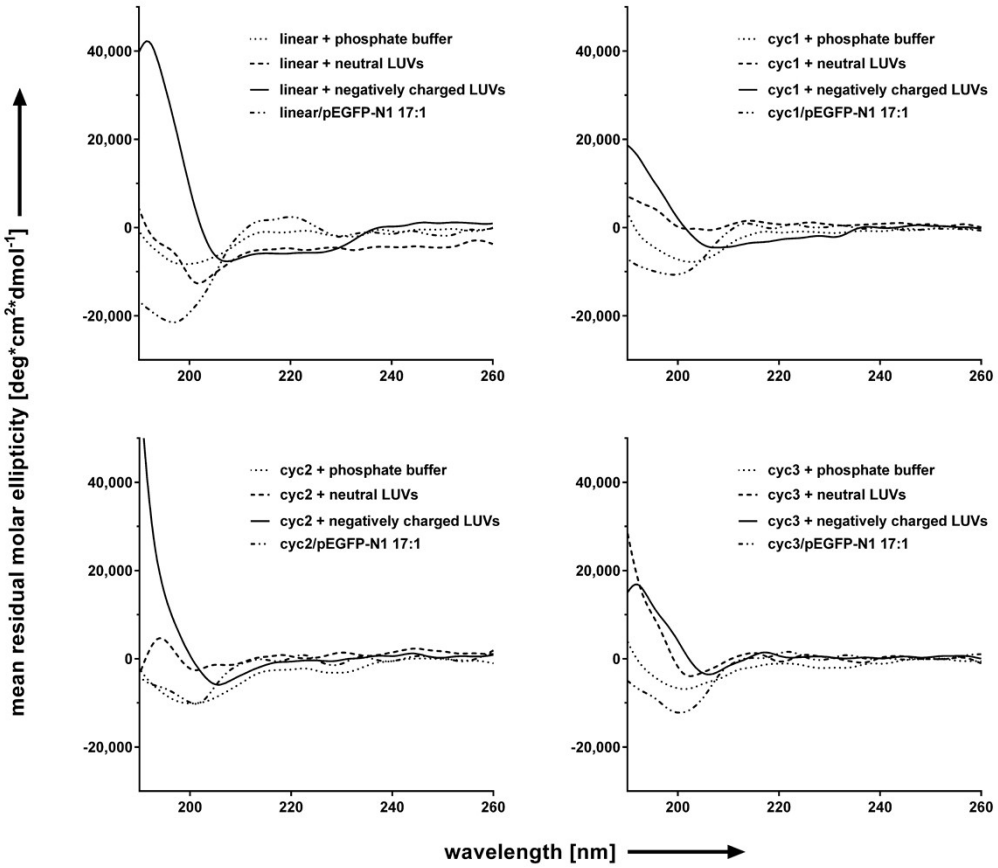


Figure S15. Circular dichroism spectra of CF-labeled peptides in 25 mM phosphate buffer (pH 7.4) and in the presence of neutral and anionic large unilamellar vesicles (LUVs) at a P/L ratio of 1/50. The peptides were dissolved at 20 μM in phosphate buffer.

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