Supporting Information

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1. Materials and methods

All chemicals and solvents were obtained from commercial sources and used without further purification. The handling of cis PCPPs was carried out in the dark and at temperatures below 5°C whenever possible, to minimize unintentional isomerization. RP-HPLC was done on an ÄKTApurifier® (GE Healthcare) using 250/4 or 250/10 Nucleosil® 100-5 C18 columns (Macherey-Nagel) with a flow of 1 mL/min or 4 mL/min. As eluents were used: 5% acetonitrile (MeCN)/0.1% trifluoacetic acid (TFA) in Water (eluent A) and 5% water/0.1% TFA in MeCN (eluent B). UV/Vis-Spectra were recorded on a Cary 50 spectrophotometer (Varian). Irradiation of the peptides was done with a mercury arc lamp (oriel), equipped with interference filters for selection of 366 nm (trans to cis) or 438 nm/495 nm (cis to trans). Nuclear magnetic resonance (NMR) spectra were recorded at room temperature on Avance III 400 instruments (Bruker). Chemical shifts are reported relative to solvent signals (DMSO: δ^{1} H= 2.50 ppm, δ^{13} C= 39.51 ppm). Confocal microscopy was done on a Zeiss LSM 780 (figure 2; figure 3b, c; figure S4) or a Zeiss LSM 510 Meta (figure 3a and figure S5) at the Bioimaging Center (BIC) of the University of Konstanz. ESI mass-spectra were recorded on an Esquire 3000 plus (Bruker). MALDI-TOF mass-spectra were measured on a Microflex MALDI-TOF (Bruker) at the proteomics facility of the University of Konstanz. Flow cytometry was executed on a BD FACSAria[™] III cell sorter (BD Biosciences).

2. Synthesis of Azobenzene building block 3



i) A suspension of 4-aminobenzoic acid (**1**, 20 mmol; 2,74 g) in 60 ml water and 14 ml $HCl_{conc.}$ was cooled to 0°C. NaNO₂ (20 mmol; 1,38 g in 5 mL water) was added dropwise under rigorous stirring and 30 min after complete addition a clear solution of the diazonium salt was obtained.

ii) 3,5-dimethylaniline (20 mmol) and 10 mL water were acidified with $HCl_{conc.}$ until pH 5-6 was reached. To this solution the diazonium salt preparation of i) was added in mL steps at 0°C whereupon an orange solid precipitated. After complete addition the reaction mixture was brought to pH 4 with 2 M NaOH_{aq.} and stirred for 2 additional hours at room temperature. After neutralization with saturated NaHCO₃ the resulting orange solid was filtered, washed with cold water and recrystallized from Toluol to give compound **2** as violet needles (3,7 g, 14 mmol, 70%)

δ¹H [ppm] (d6-DMSO, 400 MHz): 12.94 (sb, 1H), 8.07(d (J=8.6Hz), 2H), 7.78 (d (J=8.6Hz) 2H), 6.49 (s, 2H), 6.10 (s, 2H), 2.46 (s, 6H)

δ¹³C [ppm] (d6-DMSO, 101 MHz): 167.0, 156.1, 152.0, 139.4, 137.5, 130.5, 130.1, 121.3, 114.0, 21.4

b)

Fmoc-Glu(OtBu)-OH (2.5 mmol, 1.12 g), HATU (2.5 mmol, 0.95 g) und HOAt (4 mmol, 0.54 g) were dissolved in 20 mL N-methyl-pyrrolidone (NMP). After complete dissolution 50 μ L (5 mmol) Di-isopropyl-ethylene-amine (DIPEA) was added and stirred for additional 5 min.

To this solution of activated Fmoc-Glu(OtBu)-OH, compound **2** (2.5 mmol; 0.71 g) in 10 ml NMP was added and stirred at RT for 14h. The reaction mixture was poured on 200 mL ice/water, the orange precipitate was filtered off and washed with cold water. The orange solid was dissolved in ethylacetate, dried over MgSO₄ and evaporated to dryness. After column chromatography on silica gel with DCM/MeOH (10:1 -> 5:1) compound **3** was obtained as orange solid (0.51 g, 0.75 mmol, 30%).

 δ^{1} H [ppm] (d6-DMSO, 400 MHz): 13.00 (sb, 1H); 10.26(s, 1H), 8.14 (m, 2H), 7.90 (m, 4H), 7.78-7.74 (m, 2H); 7.52 (s, 2H), 7.44-7.40 (m, 2H), 7.36-7.31 (m, 2H), 6.38 (s, 1H), 4.35-4.17 (m, 4H), 2.45 (s, 6H), 2.39-2.23 (m, 2H); 2.01-1.81(m, 2H), 1.39 (s, 9H)

δ¹³C [ppm] (d6-DMSO, 101 MHz): 172.4, 171.7, 171.4, 167.3, 155.1, 145.2, 143.7, 140.8, 139.4, 133.7, 132.6, 130.5, 127.6, 127.0, 125.2, 122.0, 120.1, 119.7, 79.9, 65.7, 54.8, 46.6, 31.4, 27.7, 27.0, 20.1

3. Solid Phase Peptide Synthesis (SPPS) and peptide characterization by UV/Vis- and NMRspectroscopy

All peptides were synthesized manually on a 10-40 μ mol scale in syringes (2 mL or 5 mL, Norm-Ject® HSW) equipped with a porous polyethylene disc. Solvents and soluble compounds were removed by suction. After each reaction step the resin was washed 10x1 min with 1 mL DMF/10 μ mol peptide and 2x3 min with 1mL DCM/10 μ mol peptide. As solid support polystyrene AM RAM® (Rapp Polymere GmbH) was used and the resin was initially swollen in DMF for 1 h. Fmoc deprotection was done with 20% piperidine in DMF (5x5 min with 1mL/10 μ mol of peptide) and the resulting dibenzofulven-piperidine adduct was quantified by UV/Vis-spectroscopy (ϵ_{301} = 7800 cm⁻¹ M⁻¹)^[1]. For all coupling reactions a 5-fold excess of the activated acid was used. Fmoc-Glu(OtBu)-OH and Fmoc-Arg(Pbf)-OH and compound **4-OH** were activated with 0.95 eq HBTU and 2 eq DIPEA in 1 mL NMP/100 μ mol acid. Compound **3** was activated with 1 eq dicyclohexylcarbodiimide (DCC) and 1.5 eq 1-Hydroxybenzotriazole (HOBt) in 1 mL NMP/100 μ mol acid. After 5 minutes the active ester was added to the deprotected resin and the amide coupling was continued for 2-14 h. Unreacted amines and the N-

a)

terminus of the non-fluorescent peptides were capped 2x15min with 1 mL acetic anhydride (15% in DMF)/10 μ mol peptide. The peptides were cleaved from the resin and the side chain protection groups were removed with a mixture of 95% TFA, 2.5% triisopropylsilane (TIS) and 2.5% water. For 10 μ mol peptide 1 mL of this mixture was added to the syringe and shaken 1x2 h and 2x5 min, before the solution was poured into cold tert-butyl methyl ether (MTBE), whereupon the peptides precipitated. After centrifugation and washing with MTBE, the peptides were dried and purified by RP-HPLC. To generate the cis-peptides, they were irradiated at 366 nm for 20 min and cis and trans peptides were separated by a consecutive RP-HPLC run. The chromatograms are shown in figure S1. The peptides were further characterized by mass spectrometry and the results are summarized in table S1.



Figure S1. RP-HPLC chromatograms of the peptides after irradiation at 366 nm for 20 minutes. A_{220nm} (black line), A_{355nm} (blue line) and A_{270nm} (pink line, acetylated peptides) or A_{566nm} (orange line, fluorescent peptides) are monitored. The gradient used for purification is indicated in green. In all cases, there are two separated peaks visible: one with a strong absorption at 355nm, corresponding to the trans peptide and one that is poorly absorbing at 355 nm, corresponding to the cis form. The cis-to-trans ratio obtained after irradiation is different for every peptide ranging from about 30% cis (${}^{h}E_{6}R_{9}$) to 80% cis (${}^{A}cE_{9}R_{9}$). In case of the acetylated PCPPs the ratio of UV absorption at 355 nm and 270 nm is characteristic for each isomer (Pure cis-Form: A355/A270=0,023; pure trans Form: A355/A270=1,305). From the measured A355/A270 ratio of a cis/trans mixture the content of each isomer can be calculated (%trans = (A355/A270-0.023)*100%/1.282).

Table S1. Mass sp	pectrometric charac	terization of the pept	tides used in this study
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Peptide	Calculated mass [Da]	Obtained mass [Da]
^{fl} E ₆ R ₉	3040.6	3040.9 ^[a]
^{Ac} E ₆ R ₉	2489.3	2489.2 ^[a]
^{fl} E ₉ R ₉	3427.4	3427.7 ^[a]
^{Ac} E ₉ R ₉	2876.4	2877.0 ^[b]







Figure S2. A) UV/Vis-spectrum of 4-OH in water at room temperature. B) Thermal cis-to-trans isomerization of [#]E₉R₉ monitored by consecutive UV/Vis-spectra. The black spectrum is obtained directly after elution from RP-HPLC and corresponds to almost pure cis-form. The half-life of thermal isomerization is about 30 h (34% MeCN; 0.1% TFA in water, 22°C). C) Photoswitching properties of [#]E₉R₉ in water at 22°C. In the dark, the trans-form of AB gives rise to an absorption band at 355 nm (red curve). Upon irradiation at 366 nm, A₃₅₅ drops and a PSS is reached after about 5 minutes (shaded purple curves). This effect is reversed when irradiating at 438 nm (dashed blue curve on top of the red curve). D) Photoswitching properties of ^{Ac}E₉R₉ in water at 22°C. In the dark, the trans-form of AB gives rise to an absorption band at 355 nm (red curve). Upon irradiation at 366 nm, A₃₅₅ drops and a PSS is reached after about 5 minutes (shaded purple curves). This effect is reversed when irradiating at 438 nm (dashed blue curve on top of the red curve). D) Photoswitching properties of ^{Ac}E₉R₉ in water at 22°C. In the dark, the trans-form of AB gives rise to an absorption band at 355 nm (red curve). Upon irradiation at 366 nm, A₃₅₅ drops and a PSS is reached after about 5 minutes (shaded purple curves). This effect is reversed when irradiating at 438 nm (dashed blue curve) or 495 nm (dotted green curve). E) Thermal cis-to-trans isomerization of ^{Ac}E₉R₉ under physiological conditions (50 mM Na₂HPO₄, 150 mM NaCl, 500 mM Arginine, pH = 7.4) monitored by consecutive UV/Vis-spectra. The half-life of thermal isomerization is about 22 h at 24°C. F) Photoswitching properties of ^{Ac}E₉R₉ under physiological conditions (50 mM Na₂HPO₄, 150 mM NaCl, 500 mM Arginine, pH = 7.4). In the dark, the trans-form of AB gives rise to an absorption band at 355 nm (red curve). Upon irradiation at 366 nm, A₃₅₅ drops and a PSS is reached after about 5 minutes (shaded purple curves). This effect is reversed



Figure S3. NMR-spectroscopic investigation of the PCPPs. Overlay of the TOCSY- (red) and NOESY-spectrum (blue) of a 0.9 mM sample of ${}^{Ac}E_6R_9$ irradiated at 366 nm (~80% of the peptides in *cis*-configuration). The presence of the highlighted NOESY-cross peaks from the H^β-and H^γ-protons of glutamic acids (2.0 to 2.5 ppm) to the Nitrogen-bound protons of the Arginine sidechain (6.8 to 7.7 ppm) is indicative for an effective pairing of the two oppositely charged amino acid sidechains as illustrated in the scheme on the right-hand side. Sample conditions: 4 mM NaN₃; 0.1 mM TSP in water (pH = 5.3); 17°C.

4. Cell culture conditions

All cells were grown in Dulbecco's Modified Essential Medium (DMEM, invitrogen) supplemented with 10 % fetal bovine serum (FBS) and antibiotics (100 units/mL penicillin; 100 μ g/mL streptomycin). Culturing was done in a 5 % carbon dioxide, water saturated incubator at 37°C.

5. Confocal microscopy

24h prior to use cells were seeded on a μ -Dish^{35mm, high} or μ -Dish^{35mm, high, Grid-500} (ibidi GmbH) at a density of 20,000 cells/mL and 2 mL per dish. For imaging, the medium was exchanged to CO₂-independent medium (invitrogen) supplemented with 10% FBS, antibiotics (100 units/mL penicillin; 100 µg/mL streptomycin) and the respective peptide at a concentration of 1 or 3 µM. For photoactivation the Argon⁺ laser (458nm or 488nm) was used and the output power of the Laser was determined via a FCS point measurement by the staff of the bioimaging center of the University of Konstanz. For imaging the fluorophore was excited at 543 nm (HeNe 1 laser, Zeiss LSM 510 Meta) or 561nm (Diode pumped solid state laser, Zeiss LSM 780). Fluorescence was detected from 580-660 nm using photomultiplier tubes or a GaAsP-detector array. Upon excitation of the fluorophore, some activation of the cis-peptides was also observed, since the AB-absorption is not completely diminished at these wavelengths. Therefore cells were initially selected and focused using the HeNe 2 laser (633 nm), where neither the azobenzene nor the fluorophore is absorbing. The pictures obtained were processed and optimized for brightness and contrast using the ImageJ software^[2].

 Z-pos:: 0 µm
 0.476 µm
 0.951 µm
 1.427 µm

 1,903 µm
 2,379 µm
 2,856 µm
 3.330 µm

 3,806 µm
 4,282 µm
 4,758 µm
 5.234 µm



Figure S4. Visualization of the intracellular distribution of *trans*-peptides. A) Confocal fluorescence images of HeLa cells incubated with 1 μ M *trans*-f^rE₉R₉ for 70 min (Scale bar: 10 μ m) at different Z-positions indicated at the top-left of each picture. B) Magnification of one confocal slice (Z-position: 1,902 μ m) and orthogonal cross-sections through the cells at the indicated positons (yellow lines). The intracellular distribution is inhomogeneous, indicative for a vesicular uptake of the peptides to the cytoplasm. No sticking to the plasmamebrane could be observed and especially the orthogonal x-z- and y-z-cross-sections show the most intense fluorescence inside the cells close to the nucleus. However, it cannot be excluded that a significant portion of the CPPs is enriched at the plasma membrane but remains invisible due to fluorescence quenching.^[3]



Figure S5. Peptide uptakte in different cell lines. Merged fluorescence (green) and bright field (grayscale) images (Scale bar: 50 μ m). 3 μ M cis-f^rE₉R₉ was added and after 40 minutes of incubation a picture was taken (upper panel). Then the peptide was activated by irradiation at 488 nm with high laser intensity (240 μ W) and after an additional incubation time of 40 minutes the peptide accumulated in the cytoplasm of the cells (lower panel).

6. Flow cytometry

24h prior to use cells were seeded on 6well-plates (corning) at a density of 20,000 cells/mL and 1 mL per well. The cells were incubated with the fluorescent peptides or compound **4** at a concentration of 3 μ M. After 30 minutes at 37°C the cells were washed 3 times with phosphate buffered saline, detached by trypsination for 3 minutes at 37°C and resuspended in CO₂-independent medium (invitrogen) supplemented with 10% FBS and antibiotics (100 units/mL penicillin; 100 μ g/mL streptomycin). 10,000 cells were analyzed per measurement

and 4,000 to 6,000 events were collected from healthy cells, judged by their forward and side scatter. To quantify the uptake, the fluorophore was excited at 561 nm and fluorescence was detected at 590 \pm 10 nm. The experiment was done twice with very good reproducibility. The fluorescence distribution for the respective compound is shown in figure S6.



Figure S6. Peptide uptake monitored by flow cytometry. Cells were incubated with the respective peptide at a concentration of 3 μ M for 30 minutes.

7. AlamarBlue® cytotoxicity assay

24 h prior to use cells were seeded on 96well-plates (corning) at a density of 20,000 cells/mL and 100 μ L per well. The cells were incubated with the acetylated peptides at the respective concentration for 2 days and cell viability was assessed using the alamarBlue[®] assay (invitrogen). No significant effect on cell proliferation could be seen at the highest concentrations, corresponding to the solubility limit of the respective peptide (figure S6).



Figure S7. Cytotoxicity of acetylated peptides investigated by alamarBlue®-assay. Peptides were incubated at the respective peptide concentration for 2 days. Mean value of quadruplicate experiments, error bars correspond to standard deviations.

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