Anion Recognition as a Supramolecular Switch of Cell Internalization

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Abbreviations

DEDTC: Sodium Diethyldithiocarbamate

DMEM: Dulbecco's Modified Eagle Medium

FBS: Fetal Bovine Serum

HATU: 2-(1H-7-aza-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate

HBTU: 2-[(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

PBS: Phosphate Buffered Saline

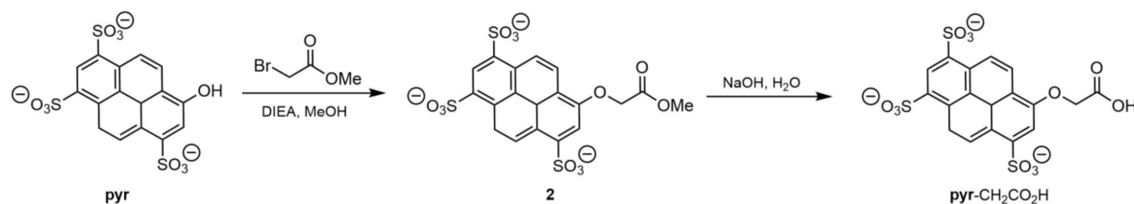
TFA: Trifluoroacetic acid

TIS: Triisopropylsilane

TMR: Tetramethylrhodamine dye

General

pyr-CH_2CO_2H was synthesized as previously described in the literature.[S1]



Cage **1** was synthesized as previously described.[S2]

General peptide synthesis procedures

All peptide synthesis reagents and amino acid derivatives were purchased from GL Biochem (Shanghai) and Novabiochem; amino acids were purchased as protected Fmoc amino acids with the standard side chain protecting scheme: Fmoc-Glu(O*t*-Bu)-OH, Fmoc-Arg(Pbf)-OH, except for the orthogonally protected Fmoc-Lys(Alloc)-OH, which was purchased from *Bachem*. Pyranine was purchased from TCI Europe. 5-Carboxytetramethylrhodamine Succinimidyl Ester (**5-TMR-NHS**) and 6-(Tetramethylrhodamine-5-Carboxamido) Hexanoic Acid Succinimidyl Ester (**6-TMR-Ahx-NHS**) was purchased from Invitrogen. All other chemicals were purchased from *Aldrich* or *Fluka*. All solvents were dry and synthesis grade, unless specifically noted.

Peptides were synthesized using an automatic peptide synthesizer from *Protein Tecnologies PS3 PeptideSynthesizer*. Peptide synthesis was performed using standard Fmoc solid-phase method on a PAL–PEG–PS resin (0.19 mmol/g) using HBTU/HOBt (4 equiv) as coupling agent, DIEA as base (6 equiv) and DMF as solvent. The deprotection of the temporal Fmoc protecting group was performed by treating the resin with 20% piperidine in DMF solution for 20 min.

Coupling of pyr-CH_2CO_2H to peptide (Scheme 1, main manuscript): The resin containing the alloc-lys peptide (75 mg, aprox. 0.015 mmol) was suspended in DMF and shaken for 1 h to

ensure a good swelling. A solution of **pyr**-CH_2CO_2H (10.5 mg, 0.018 mmol, 1.2 equiv), HATU (6.8 mg, 0.018 mmol, 1.2 equiv), pyridinium p-toluenesulfonate (4.5 mg, 0.018 mmol, 1.2 equiv) and DIEA (615 µL, 0.195 M in DMF, 0.120 mmol, 8 equiv) were added. The reaction mixture was shaken for 1 h. The resin was washed with DMF (3 ×, 5 min) and CH_2Cl_2 (2 × 5 min).

Lys-Side chain deprotection: The resulting resin-peptide containing the alloc peptide (75 mg, aprox. 0.015 mmol) was suspended in DMF and shaken for 1 h to ensure a good swelling. Then $Pd(OAc)_2$ (1 mg, 0.3 equiv), 4-methylmorpholine (16 µL, 10 equiv), PPh_3 (6 mg, 1.5 equiv), $PhSiH_3$ (18 µL, 10 equiv) and 1 mL CH_2Cl_2 were added, and the mixture shaken overnight. The resin was then filtered and washed with DMF, DEDTC and DMF again.

Coupling of acetic anhydride: The resulting lysine-deprotected peptide (50 mg, aprox. 0.01 mmol) was suspended in DMF and shaken for 1 h to ensure a good swelling. A solution of acetic anhydride (20 µL, 0.2 mmol, 20 equiv) and DIEA (400 µL, 0.195 M in DMF, 0.08 mmol, 8 equiv) was added. The reaction mixture was shaken for 0.5 h. The resin was washed with DMF (3 × 5 min) and DCM (2 × 5 min).

Coupling of TMR-NHS: The lysine-deprotected resin-linked peptide (100 mg, aprox. 0.05 mmol) was suspended in DMF and shaken for 1 h to ensure a good swelling. 5-Carboxytetramethylrhodamine Succinimidyl Ester (**5-TMR-NHS**) was coupled using 3 equiv (0.15 mmol, 64.5 mg) and 5 equiv of DIEA 0.2 M in DMF for 60 min.

Reactions were followed by analytical RP-HPLC with an Agilent 1100 series LC/MS VL G1956A model in positive scan mode, using an *Eclipse XDB-C18 analytical column (4.6 x 150 mm, 5 µm)*, 1 mL/min, gradient 5 to 75% B over 30 min.

The cleavage/deprotection step was performed by treatment of the resin-bound peptides for 1.5–2h with the following cleavage cocktail: 900 µL TFA, 50 µL CH_2Cl_2, 25 µL H_2O and 25 µL TIS (1 mL of cocktail / 40 mg resin). The crude products were purified by RP−HPLC, 4 mL/min, gradient 10 to 50% B over 40 min. (A: H_2O 0.1% TFA, B: CH_3CN 0.1% TFA) and identified as the desired peptides.

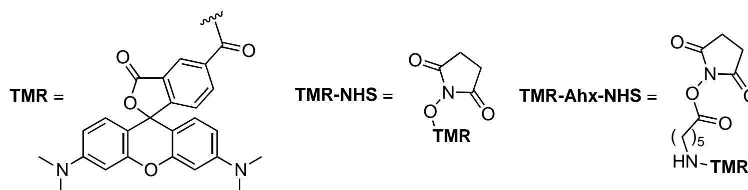


Fig. S1 Structure of **TMR**, **TMR-NHS** and **TMR-Ahx-NHS**.

pyr-R$_8$ was isolated with an approx. yield of 22%.

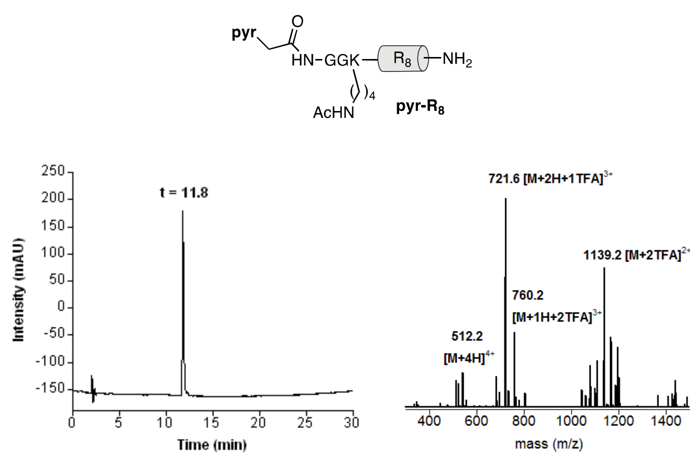


Fig. S2 Left) HPLC chromatogram of purified peptide. Gradient 5 to 75% B over 30 min. Right) Mass spectrum of the purified peptide.

EM–ESI$^+$ (m/z): Calcd. for $C_{78}H_{128}N_{37}O_{23}S_3$: 2048.28. Found: 1139 $[M+2TFA]^{2+}$; 760 $[M+1H+2TFA]^{3+}$; 721 $[M+2H+1TFA]^{3+}$; 684 $[M+3H]^{3+}$; 512 $[M+4H]^{4+}$.

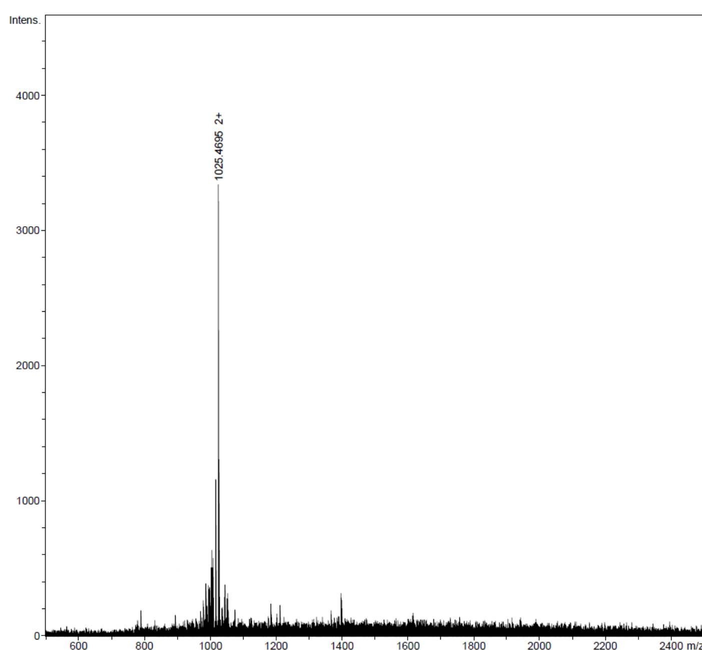


Fig. S3 High resolution ESI mass spectrum. Calcd. for $C_{78}H_{128}N_{37}O_{23}S_3$: 2048.28. Found: 1025 $[M+2H]^{2+}$.

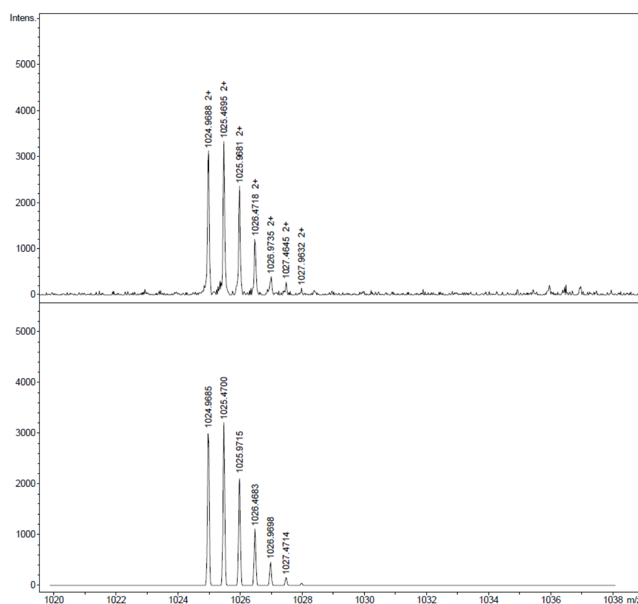


Fig. S4 Experimental (top) and calculated (bottom) isotopic distribution in the high resolution ESI mass spectrum corresponding to $[M+2H]^{2+}$.

pyr-E_1R_8 was isolated with an approx. yield of 20%.

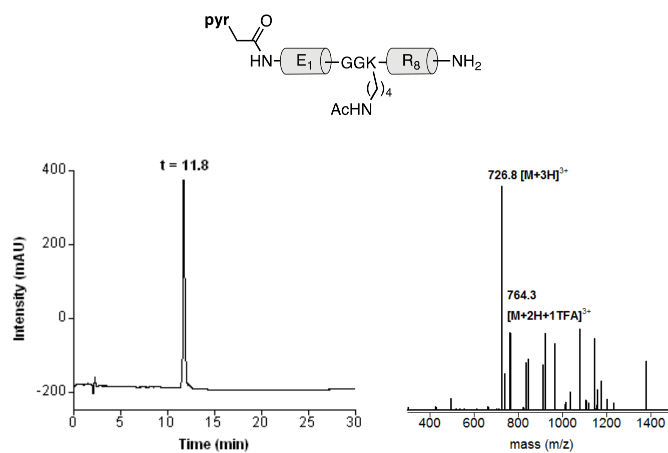


Fig. S5 Left) HPLC chromatogram of the purified peptide (gradient 5 to 75% B over 30 min). Right) Mass spectrum of the purified peptide.

EM–ESI$^+$ (m/z): Calcd. for $C_{83}H_{135}N_{38}O_{26}S_3$: 2177.40. Found: 726 $[M+3H]^{3+}$; 764 $[M+2H+1TFA]^{3+}$.

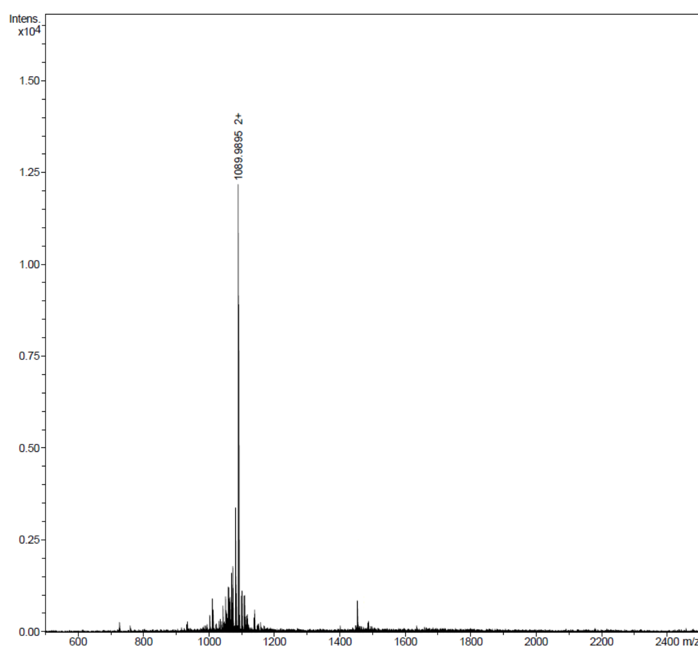


Fig. S6 High resolution ESI mass spectrum. Calcd. for $C_{83}H_{135}N_{38}O_{26}S_3$: 2177.40. Found: 1090 $[M+2H]^{2+}$.

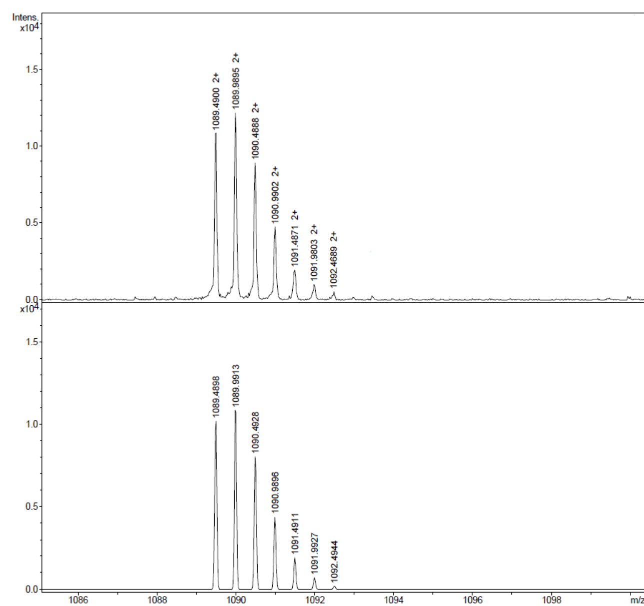


Fig. S7 Experimental (top) and calculated (bottom) isotopic distribution in the high resolution ESI mass spectrum corresponding to $[M+2H]^{2+}$.

pyr-E$_6$R$_8$ was isolated with an approx. yield of 17%.

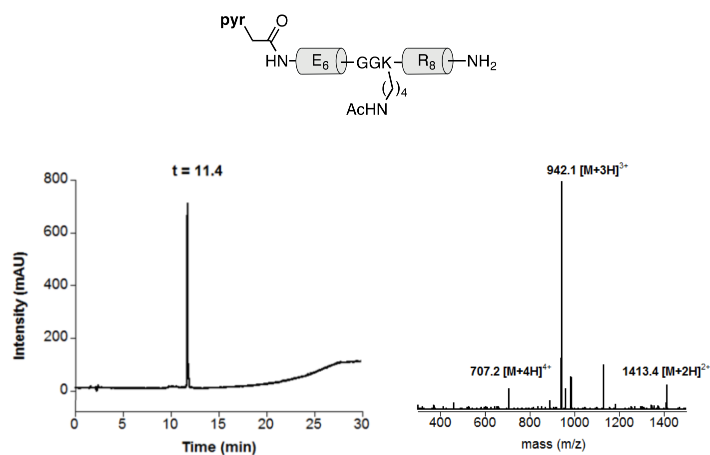


Fig. S8 Left) HPLC chromatogram of the purified peptide. Gradient 5 to 75% B over 30 min. Right) Mass spectrum of the purified peptide.

EM–ESI$^+$ (m/z): Calcd. for $C_{108}H_{170}N_{43}O_{41}S_3$: 2822.99. Found: 1413 $[M+2H]^{2+}$; 942 $[M+3H]^{3+}$; 707 $[M+4H]^{4+}$.

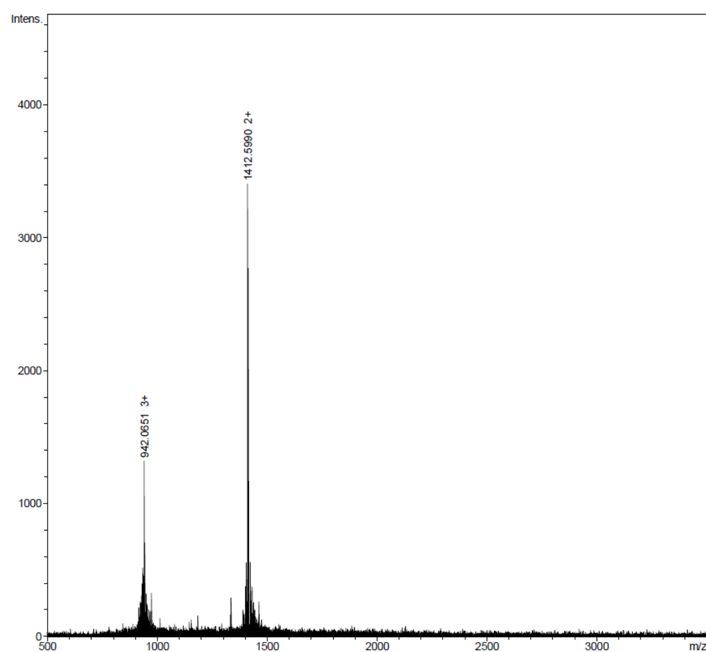


Fig. S9 High resolution ESI mass spectrum. Calcd. for $C_{108}H_{170}N_{43}O_{41}S_3$: 2822.99. Found: 1413 $[M+2H]^{2+}$; 942 $[M+3H]^{3+}$.

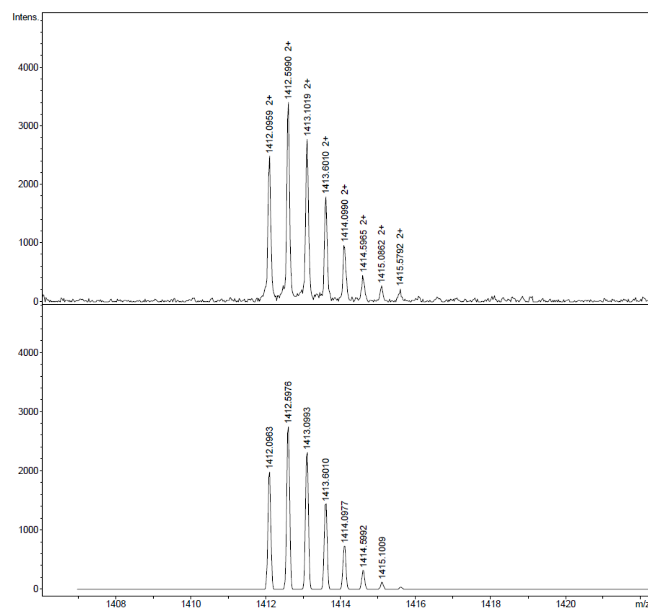


Fig. S10 Experimental (top) and calculated (bottom) isotopic distribution in the high resolution ESI mass spectrum corresponding to $[M+2H]^{2+}$.

pyr-TMR-E_6R_8 was isolated with an approx. yield of 12%.

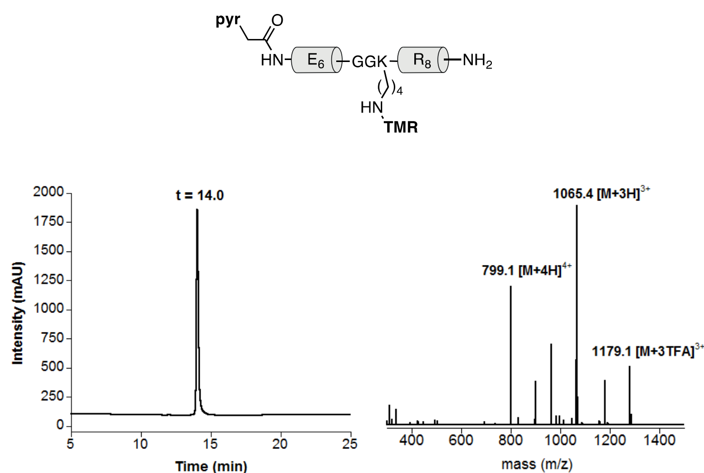


Fig. S11 Left) HPLC chromatogram of the purified peptide. Gradient 5 to 75% B over 30 min. Right) Mass spectrum of the purified peptide.

EM–ESI$^+$ (m/z): Calcd. for $C_{131}H_{189}N_{45}O_{44}S_3$: 3192.31. Found: 1179 $[M+3TFA]^{3+}$; 1065 $[M+3H]^{3+}$; 799 $[M+4H]^{4+}$.

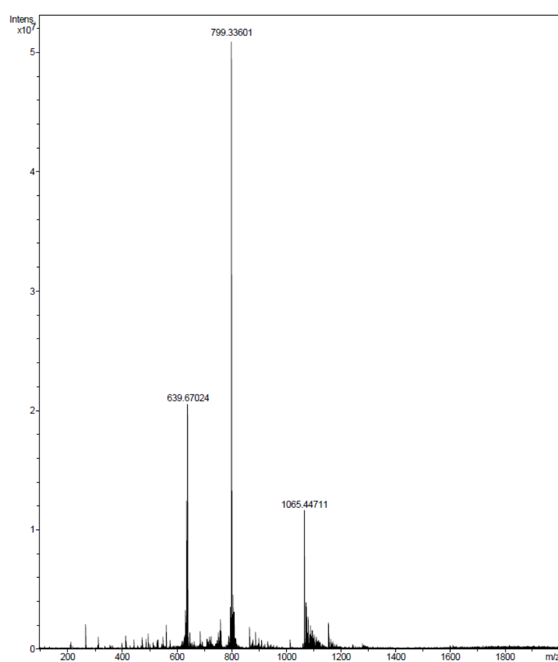


Fig. S12 High resolution ESI mass spectrum. Calcd. for $C_{131}H_{189}N_{45}O_{44}S_3$: 3192.31. Found: 1065 $[M+3H]^{3+}$; 799 $[M+4H]^{4+}$; 640 $[M+5H]^{5+}$.

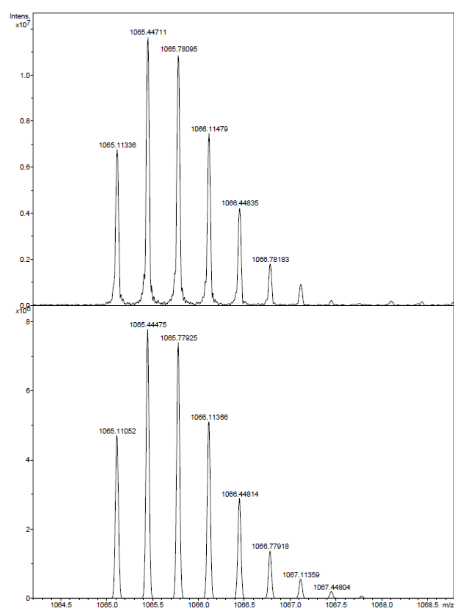


Fig. S13 Experimental (top) and calculated (bottom) isotopic distribution in the high resolution ESI mass spectrum corresponding to $[M+3H]^{3+}$.

Synthesis of TMR-1 (Figure 6, main manuscript):

TMR-Ahx-NHS (1.2 mg, 1.8 × 10^{-3} mmol) was dissolved in DMF (100 µL). Et_3N (18 × 10^{-3} mmol, 3.3 µL, 10 equiv) was added, and the mixture was stirred at rt for 3 min. Cage **1** (3.7 mg, 2 × 10^{-3} mmol, 1.1 equiv) dissolved in 30 µL of DMF was added, and the solution was stirred again at rt for 1 hour. The product was purified by RP−HPLC, 4 mL/min, gradient 10 to 50% B over 40 min. (A: H_2O 0.1% TFA, B: CH_3CN 0.1% TFA, retention time = 10.1 min), and identified as product TMR-**1** by mass spectrometry. TMR-**1** was isolated with an approx. yield of 12%.

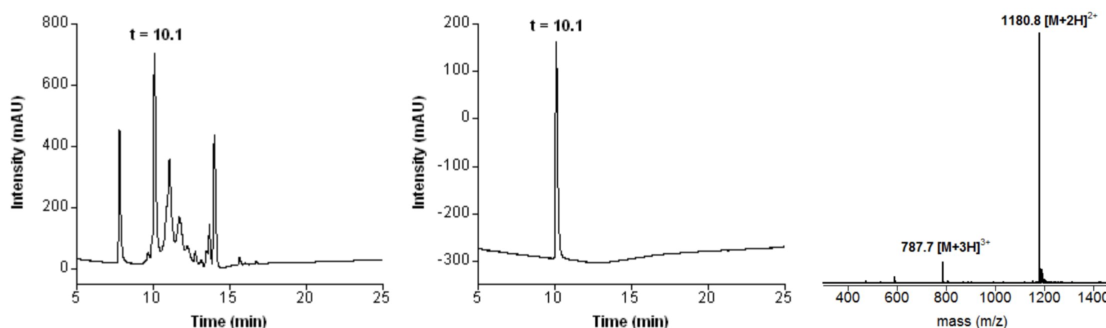


Fig. S14 HPLC chromatogram of: Left) crude residue after the synthesis; Center) purified **TMR-1**. Gradient 5 to 75% B over 30 min. Right) Mass spectrum of the purified product **TMR-1**.

EM–ESI$^+$ (m/z): Calcd. for $C_{139}H_{176}N_{31}O_5$: 2359.5. Found: 1180 $[M+2H]^{2+}$; 787 $[M+3H]^{3+}$.

^{1}H NMR (500 MHz, D_2O): δ = 8.68 (m), 8.54 (m), 8.00 (m), 7.88 (m), 7.75 (m), 7.47 (m), 7.39 (m), 6.72 (m), 6.54 (m), 4.30 (t, J = 10 Hz,12H), 3.44 (m, 2H), 3.16 (d, J = 22.4 Hz, 30H), 2.95 (m, 6H), 2.10 (m, 15H), 1.93 (m, 1H), 1.48 (m, 1H), 1.27 (m, 2H), 1.02 (m, 1H).

^{13}C NMR (500 MHz, D_2O): δ = 149.83 (C), 147.42 (CH), 136.57 (CH), 132.76 (C), 124.07 (CH), 50.59 (CH_2), 49.88 (CH_2), 43.84 (CH_2), 39.85 (CH_3), 20.50 (CH_2).

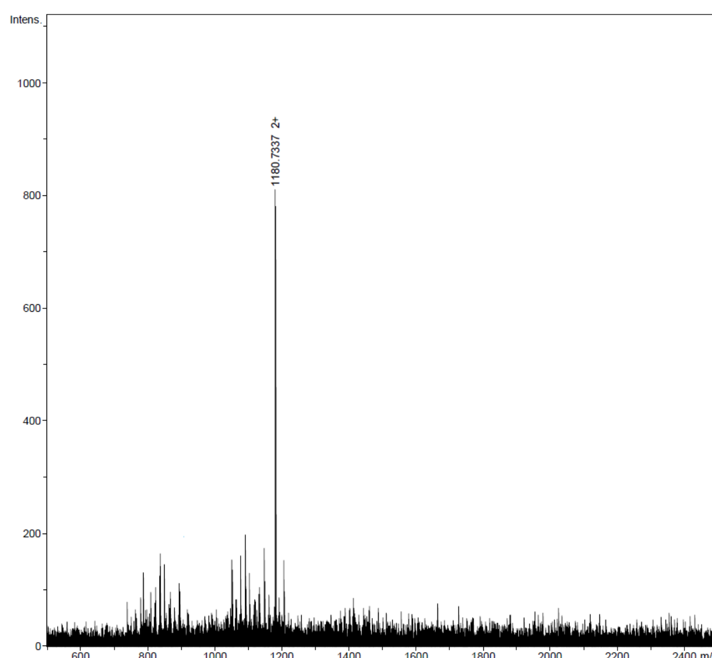


Fig. S15 High resolution ESI mass spectrum. Calcd. for $C_{139}H_{176}N_{31}O_5$: 2359.5. Found: 1180 $[M+2H]^{2+}$.

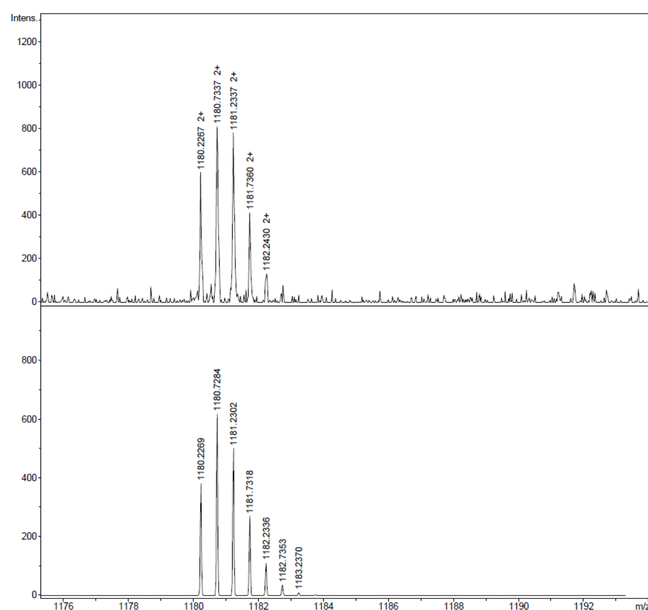


Fig. S16 Experimental (top) and calculated (bottom) isotopic distribution in the high resolution ESI mass spectrum corresponding to $[M+2H]^{2+}$.

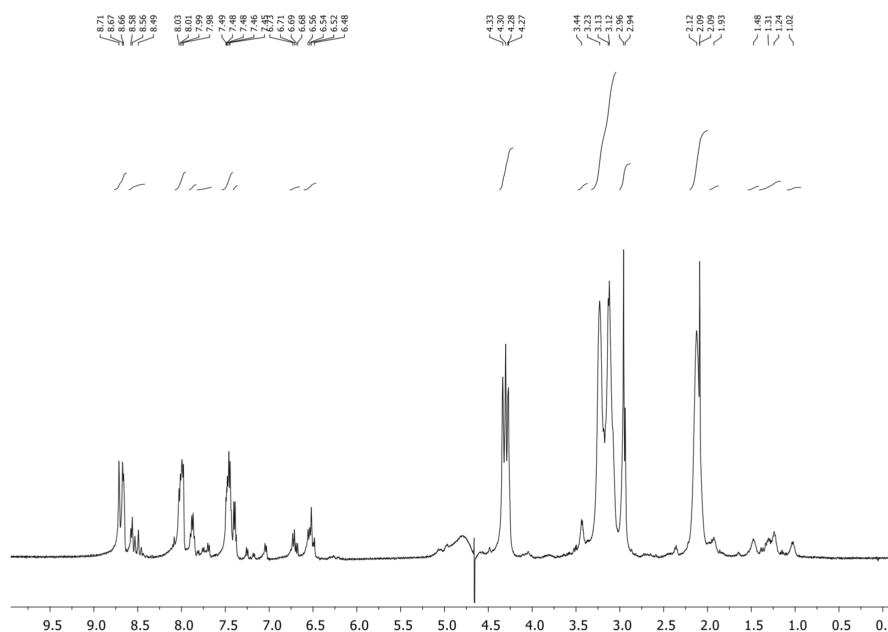


Fig. S17 ^{1}H-NMR spectrum (D_2O, 25 ºC) of **TMR-1**. The signal of the solvent has been suppressed for clarity.

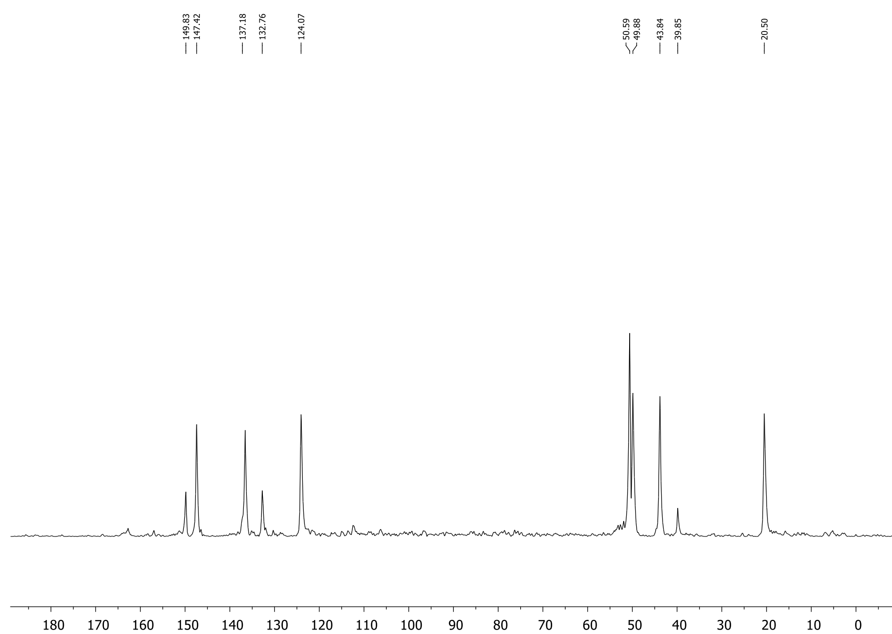


Fig. S18 ^{13}C-NMR spectrum (D_2O, 25 ºC) of **TMR-1**.

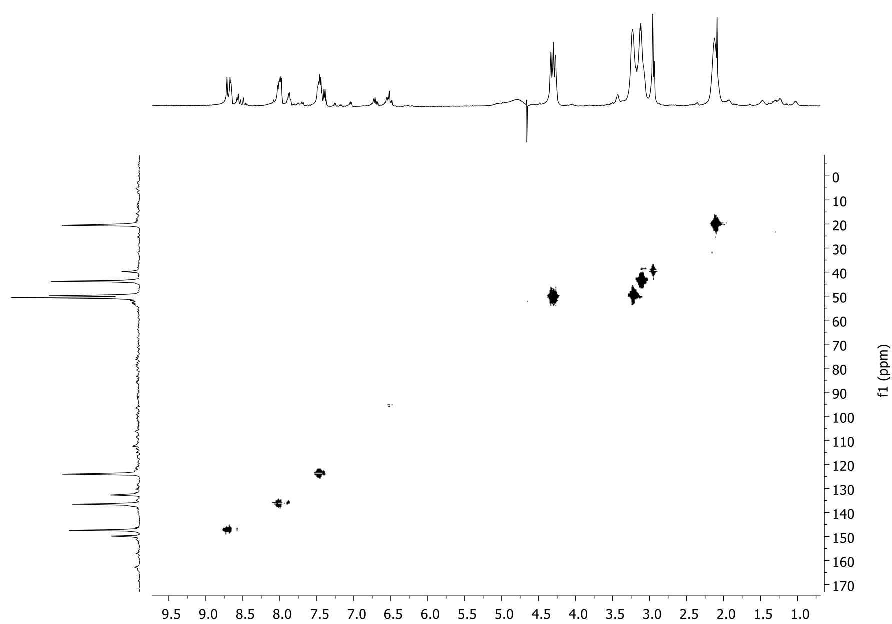


Fig. S19 HSQC spectrum (D_2O, 25 ºC) of **TMR-1**.

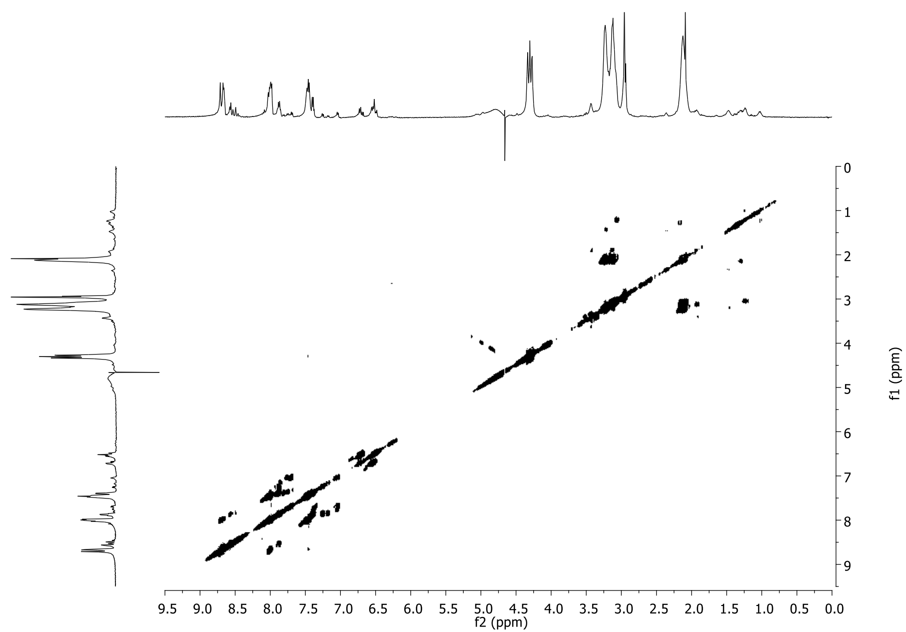


Fig. S20 ^1H-^1H COSY spectrum (D_2O, 25 ºC) of **TMR-1**.

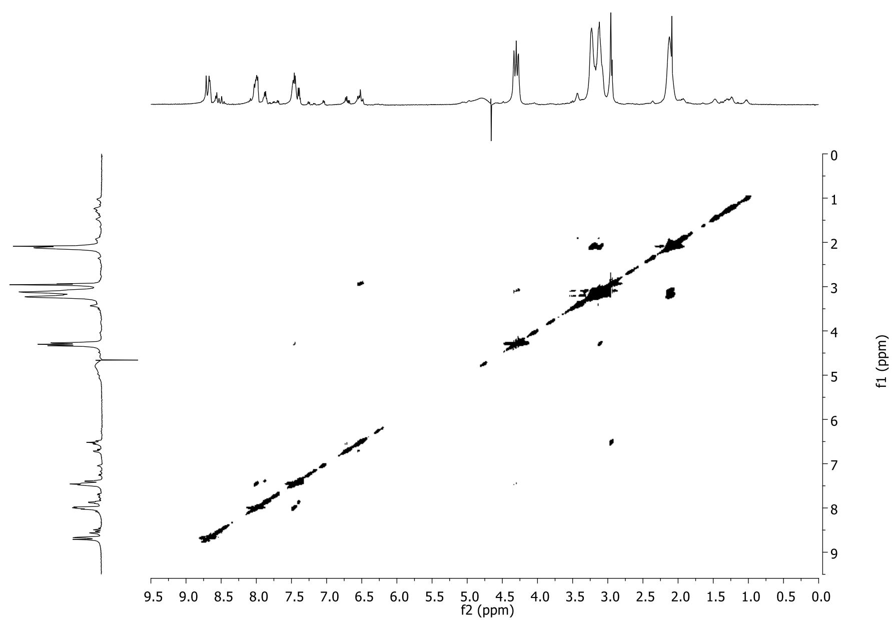


Fig. S21 NOESY spectrum (D_2O, 25 ºC) of **TMR-1**.

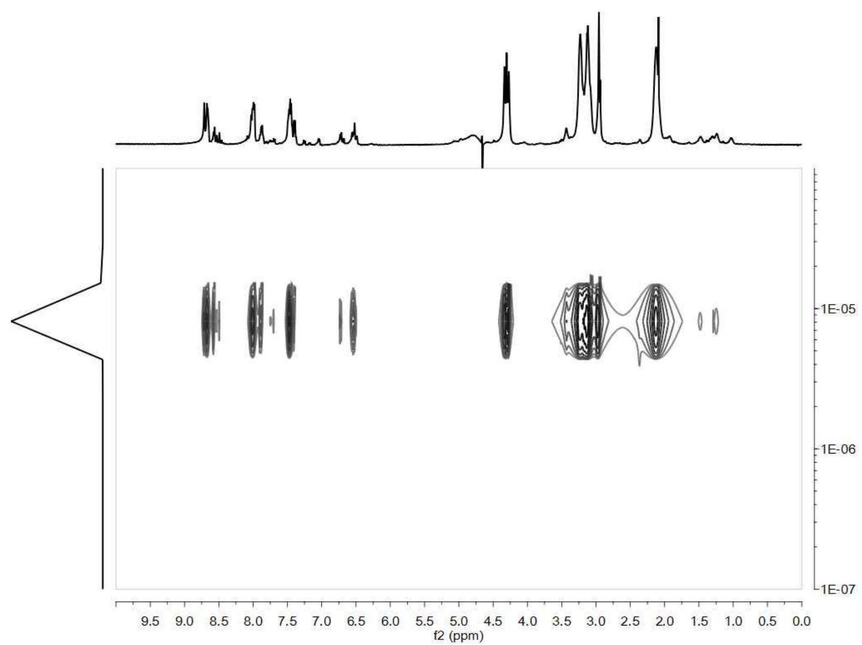


Fig. S22 DOSY spectrum (D_2O, 25 ºC) of **TMR-1**.

Fluorescence titration

Measurements were performed using a Varian Cary Eclipse fluorimeter. The measurements were made with the following settings: increment, 1.0 nm; integration time, 0.2 s; excitation slit width, 5.0 nm; emission slit width, 20.0 nm; excitation wavelength, 415 nm. The emission spectra were recorded from 420 to 520 nm at room temperature.

*Fluorescence titration with cage **1** in PBS buffer*

To 1 mL of a 1 μM solution of **pyr-E_6R_8** in PBS buffer pH 7,5 (rt), aliquots of a 130 μM stock solution of the cage **1** were successively added and the fluorescence spectrum was recorded after each addition.

Cell cultures and internalization studies

Cells were cultured in DMEM supplemented with 10% (v/v) FBS (Sigma), 5mM glutamine, penicillin (100 U/ml) and streptomycin (100 U/ml). Proliferating cell cultures were maintained in a 5% CO_2 humidified incubator at 37°C. For the internalization studies, cells growing on glass coverslips were incubated in PBS containing 5 μM of the corresponding peptide in the absence or presence of 1 equiv of cage **1** for 30 min.

For competitive assays, 15 μM and 50 μM Pyranine (**pyr**) were also added.

The resulting cells were then washed twice with PBS and kept in 10% FBS-DMEM supplemented with 25 mM HEPES (Life technologies) for observation *in vivo* in a fluorescence microscope equipped with adequate filters and differential interference contrast (DIC) microscopy. Digital pictures of the different samples were taken under identical conditions of gain and exposure. Images were obtained with an Olympus DP-71 digital camera mounted on an Olympus BX51 microscope (Figure 6 in the manuscript) or an Andor Zyla mounted on a Nikon TiE (Figures 2, 3 and 5 top in the manuscript). Images were further processed with Image J or NIS software. The parameters of the fluorescent channels were the following: For the Olympus BX51: filter cube U-MWU2: excitation filter (BP) 330-385 nm, emission filter (LP) 420 nm and dichromatic mirror (DM) 400 nm; filter cube U-MWB2: BP 460-490 nm, LP 520 nm and DM 500 nm; filter cube UMNG2: BP 530-550 nm, LP 590 nm and DM 570 nm. For the Nikon (Semrock): filter cube DAPI-1160B-000: BP 387/11 nm, LP 447/60 nm and DM 409 nm; filter cube FITC-3540C-000: BP 482/35 nm, LP 536/40 nm and DM 506 nm; filter cube TRITC-B-000: BP 543/22 nm, LP 593/40 nm and DM 562 nm.

As a control of reproducibility, the internalization assay was repeated in three different cell lines: monkey Vero cells (Fig. 5), A549 human lung carcinoma cells and HeLa cells.

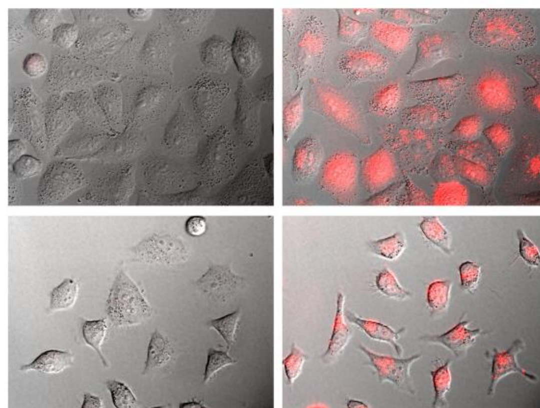


Fig. S23 Fluorescent micrographies of A549 (upper row) and Hela cells (lower row) after incubation with 5 μM **pyr-TMR-E_6R_8** in the absence (left) or presence (right) of 1 equiv of cage **1**, after incubation for 30 min at 37 ºC, followed by double washing with PBS, pH 7.5.

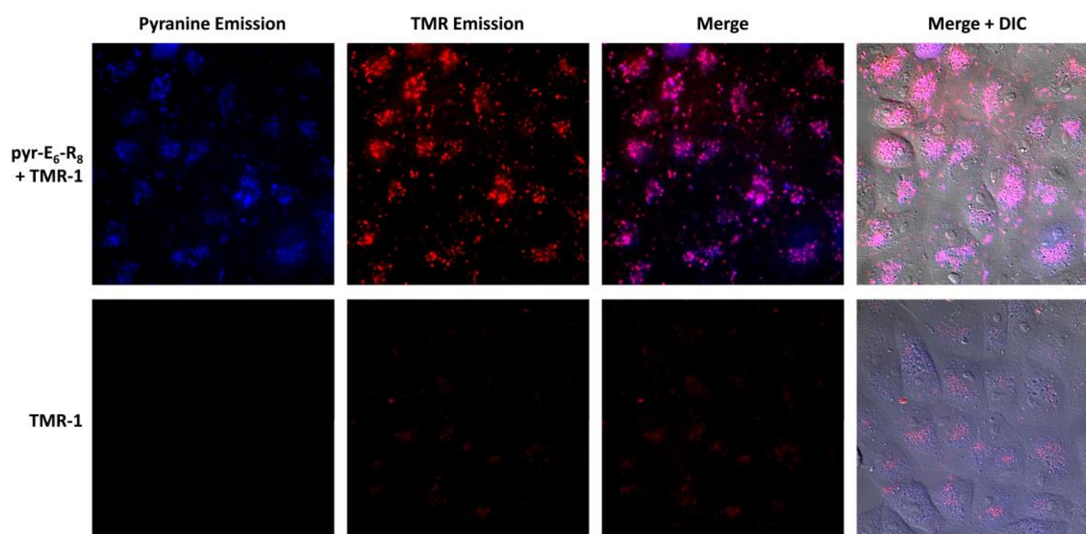


Fig. S24 Fluorescent micrographies of Vero cells after incubation with 5 μM **TMR-1** in the presence (top row) or absence (bottom row) of 1 equiv of **pyr-E_6R_8**. Cells were incubated for 30 min at 37 ºC, washed twice with PBS, pH 7.5 and observed in the fluorescence microscope. Pyranine emission (first column) is shown in blue, TMR emission in red (second column) and a color merge picture (third column) shows the colocalization of both molecules in magenta.

For the quantification of the rate of internalization, cells were incubated in 5 μM solutions of the corresponding peptides in the presence or absence of 1 equiv of cage **1** for 20, 45, 90 and 300 minutes. Since longer incubations couldn't be done in PBS due to cellular detachment, all incubations were carried out in serum-free DMEM supplemented with 25 mM HEPES for these experiments. We have observed that the internalization is slower in DMEM than in PBS (used in the other experiments) which is positive for quantification purposes. 10 micrographies of each condition were taken and quantified for pyranine fluorescence intensity and cell number using the NIS software package (Nikon). In this manner, more than 500 cells were quantified for each condition.

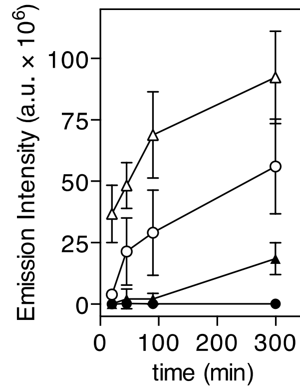


Fig. S25 Assay of relative rate of internalization in Vero cells after incubation with 5 μM of the indicated peptides in the absence or presence of 1 equiv of cage **1**. **pyr-E$_6$R$_8$** (●), **pyr-E$_6$R$_8$** in the presence of cage-**1** (▲),**pyr-ER$_8$** (○), **pyr-ER$_8$** in the presence of cage-**1** (△).

To confirm that the internalization triggering mechanism involves the interaction between the cage **1** and peptide **pyr-E$_6$R$_8$**, we carried out a competition assay. In these experiments, Vero cells growing on glass-bottom coverslips were incubated for 30 min in PBS solutions containing 5 μM of peptide **pyr-E$_6$R$_8$**, 1 equivalent of cage **1** and growing amounts of free pyranine (**pyr**) and observed in the fluorescence microscope. The procedure to prepare the incubation mixtures was as follows: 5 μM solutions of peptide **pyr-E$_6$R$_8$** were first mixed with different amounts of free pyranine. Then, 1 equivalent of **TMR-1** was added to these mixtures immediately before incubation with cells. We concluded from these experiments that the addition of 3 equivalents of free pyranine was enough to prevent the internalization of peptide **pyr-E$_6$R$_8$** or cage **1** almost completely.

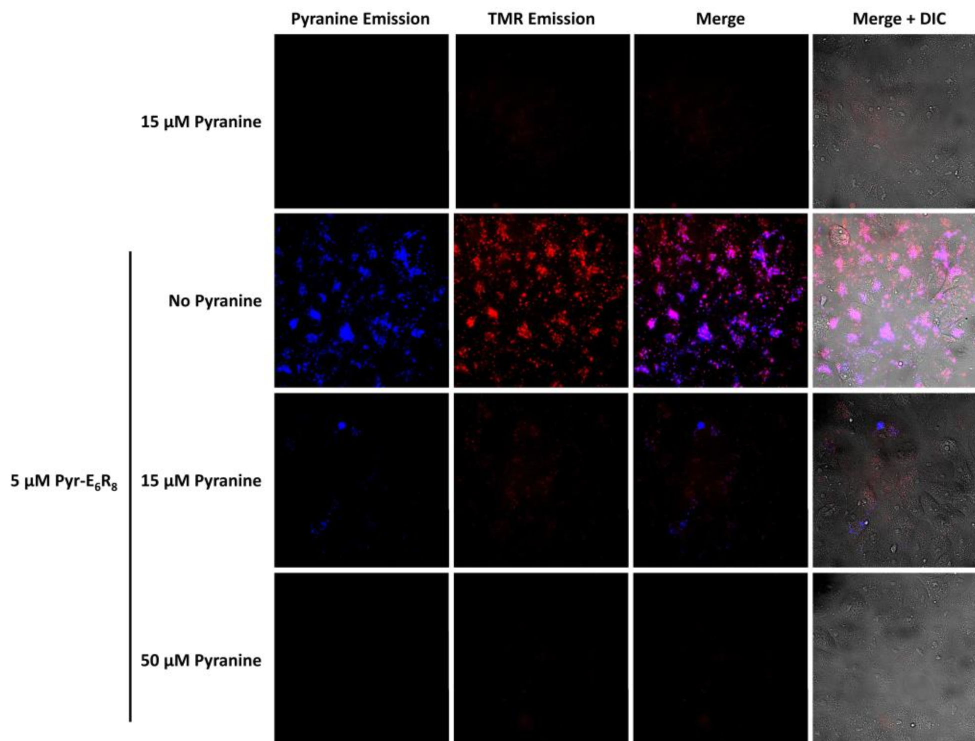


Fig. S26 Competition assay in Vero cells. Top row: 5 μM of cage **1** in presence of excess of pyranine. The other three rows: **pyr-E$_6$R$_8$** and cage TMR-**1** in presence of different amounts of pyranine. Only in the absence of pyranine we observed internalization (second row).

Cell Viability assays

To evaluate the toxicity of the treatment of cells with the peptides and cage **1**, Vero cells were submitted to an MTT assay 24 h after the incubation described in the Cell culture and internalization studies section. The MTT assay was performed as follows: Thiazolyl Blue Tetrazolium Bromide (Sigma) was added to the cell culture medium to a final concentration of 0.5 mg/mL. Cells were then incubated for 4 h to allow the formation of formazan precipitates by metabolically active cells. A detergent solution of 10% SDS (sodium dodecyl sulfate) and 0.01 M HCl in water was then added and the plate was incubated overnight at room temperature to allow the solubilisation of the precipitates. The quantity of formazan in each well (directly proportional to the number of viable cells) was measured by recording changes in absorbance at 570 nm in a microtiter plate reading spectrophotometer (Tecan Infinite 200 PRO).

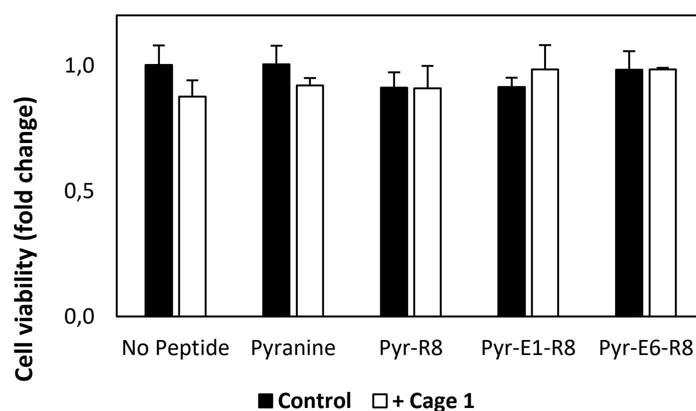


Fig. S27 Viability assay of Vero cells treated with the indicated pyranine-conjugated peptides in the presence or absence of cage **1**.

[S1] E. K. Nyren-Erickson, M. K. Haldar, Y. Gu, S. Y. Qian, D. L. Friesner, and S. Mallik *Anal.Chem.* **2011**, *83*, 5989 – 5995.

[S2] J. Mosquera, S. Zarra, and J. R. Nitschke *Angew. Chem. Int. Ed.* **2014**, *53*, 1556 –1559.