Supplementary Information

Targeted Delivery of Activatable Fluorescent Pro-Apoptotic Peptide into Live Cells

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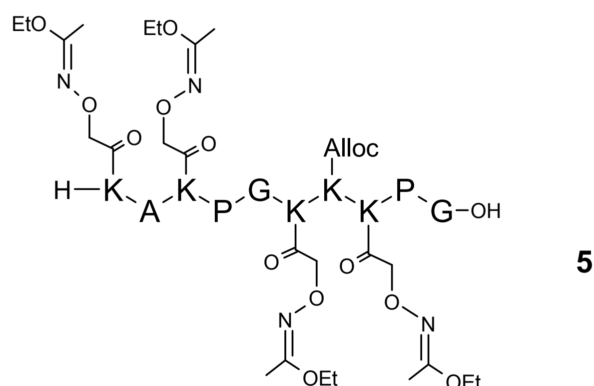
General procedure for peptide synthesis

All Fmoc amino acid derivatives and resins were purchased from Advanced ChemTech Europe (Brussels, Belgium), Bachem Biochimie SARL (Voisins-Les-Bretonneux, France) and France Biochem S.A. (Meudon, France). PyBOP was purchased from France Biochem and other reagents were obtained from either Aldrich (Saint Quentin Fallavier, France) or Acros (Noisy-Le-Grand, France). RP-HPLC was performed on Waters system equipped with a Waters 600 controller and a Waters 2487 Dual Absorbance Detector. The purity of peptide derivatives was analyzed on an analytical column (Macherey-Nagel Nucleosil 120 Å 3 µm C18 particles, 30x4.6 mm) using the following solvent system: solvent A, water containing 0.09% TFA; solvent B, acetonitrile containing 0.09% TFA and 9.91% H2O; flow rate of 1.3 mL.min-1 was employed with a linear gradient (5 to 100% B in 15 min.). UV absorbance was monitored at 214 nm and 250 nm simultaneously. Preparative column (Delta-Pak™ 100 Å 15 µm C18 particles, 200x2.5 mm) was used to purify the crude peptides (when necessary) by using an identical solvent system at a flow rate of 22 mL.min-1. ESI mass spectra were recorded on an Esquire 3000 (Bruker) spectrometer. The analysis was performed in the positive mode for peptide derivatives using 50% aqueous acetonitrile as eluent.

Peptide synthesis. Assembly of all protected peptides was carried out using Fmoc/t-Bu strategy manually in a glass reaction vessel fitted with a sintered glass frit or automatically on a synthesizer (348 Ω synthesizer, Advance ChemTech). Coupling reactions were performed using, relative to the resin loading, 1.5-2 equiv. of N-α-Fmoc-protected amino acid activated in situ with 1.5-2 equiv. of PyBOP and 3-4 equiv. of DIPEA in DMF (10 mL/g resin) for 30 min. Manual amino acid couplings were assayed by Kaiser and/or TNBS tests. N-α-Fmoc protecting groups were removed by treatment with a piperidine/DMF solution (1:4) (10 mL/g resin) for 10 min. The process was repeated 3 times, and the completeness of deprotection was verified by the UV absorption of the piperidine washings at 299 nm.

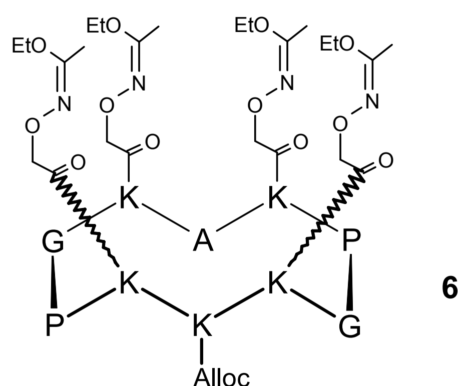
Synthesis of RGD-containing peptide 3.

Linear protected decapeptide **5**.



The linear decapeptide **5** was assembled on 2-chlorotritylchloride® resin (2.0 g, loading of 1.1 mmol/g) using the general procedure and modified amino acid Fmoc-Lys[-CO-CH_2-O-N=C(-CH_3)OEt]-OH. The anchoring of the first amino acid (Fmoc-Gly-OH) was performed following the standard procedure yielding a convenient resin loading of 0.7 mmol/g. The peptide was released from the resin using cleavage solution of TFE/AcOH/CH_2Cl_2 (2/1/7) at room temperature for 2 h. Crude product was concentrated under reduced pressure. Linear protected peptide **5** was obtained as a white solid powder (2.27 g, 1.26 mmol, 90 %) after precipitation from diethyl ether, triturating and washing with diethyl ether. Analytical HPLC t_R = 9.7 min; ESI-MS calc for $C_{75}H_{127}N_{19}O_{26}$ 1693.9, found m/z 1693.5.

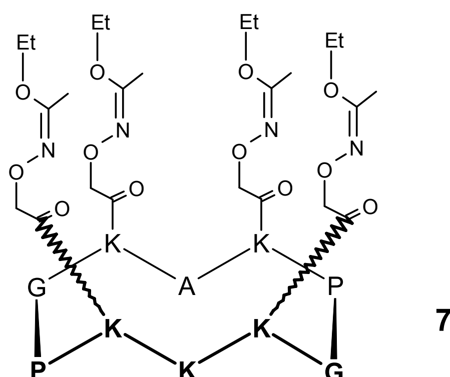
Cyclic protected decapeptide **6**.



The cyclization reaction was carried out using crude linear peptide **5** (85 mg, 47 µmol) in DMF (0.5 mM) and the pH of the solution was adjusted to 8-9 by addition of DIPEA. PyBOP (1.1 or 1.2 equiv.) was added and solution was stirred at room temperature for 30 min. Solvent was removed under reduced pressure and residue dissolved in the

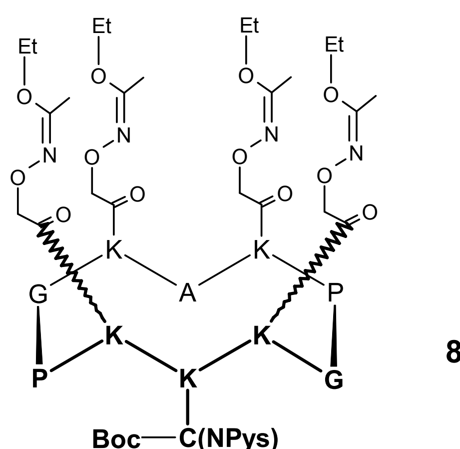
minimum of CH_2Cl_2. Precipitation and work-up in ether afforded cyclic peptide **6** as a white solid powder (79 mg, 47 µmol, quantitative). Analytical HPLC t_R = 10.8 min; ESI-MS calc for $C_{75}H_{127}N_{19}O_{26}$ 1675.9, found m/z 1675.4.

Free amino-containing cyclic decapeptide **7**.



The peptide **6** (20 mg, 12 µmol) was dissolved in 1.5 mL of anhydrous CH_2Cl_2/DMF (3/1) under argon. Phenylsilane (100 equiv.) and $Pd(PPh_3)_4$ (0.2 equiv.) were added under argon. The reaction was stirred under argon for 30 min at room temperature. The mixture was treated with 0.5 mL of methanol before evaporation of the solvents under reduced pressure. The residue was dissolved in a minimum of MeOH/CH_2Cl_2 (1/1) then finally precipitated, triturated and washed with diethyl ether affording compound **7** as a white powder (21 mg, 12 µmol, quantitative). Analytical HPLC t_R = 9.8 min; ESI-MS calc for $C_{71}H_{121}N_{19}O_{24}$ 1591.9, found m/z 1591.5.

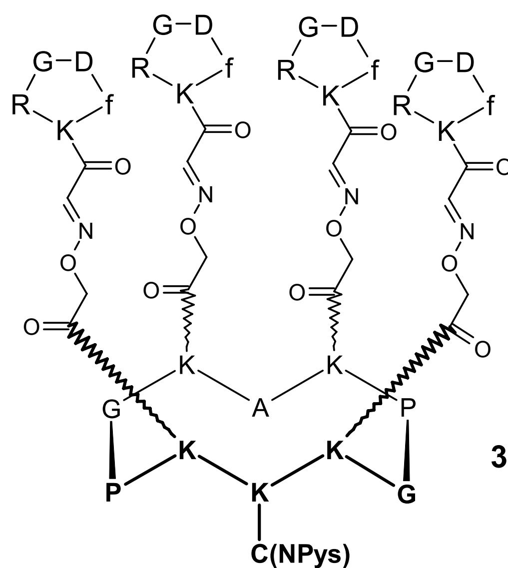
Cysteine-containing cyclic decapeptide **8**.



The crude peptide **7** (20 mg, 11.7 µmol) was dissolved in 1.5 mL of DMF and the pH was adjusted to 9 with DIPEA. PyBOP (7.9 mg, 15.2 µmol) and Boc-Cys(NPys)-OH (5.7 mg, 15.2 µmol) were added and the reaction mixture was

stirred for 30 min at room temperature. The peptide **8** was obtained as a white solid powder after precipitation, triturating and washing with diethyl ether (22.8 mg, 11.7 µmol, quantitative). Analytical HPLC t_R = 12.1 min; ESI-MS calc for $C_{84}H_{136}N_{22}O_{27}S_2$ 1948.9, found m/z 1947.8.
c[-RGDfK(COCHO)-] **9**. Cyclopentapeptide c[-RGDfK(COCHO)-] **9** was prepared as previously described.[1]

RGD-containing peptide **3**.



Peptide **8** (5 mg, 2.57 µmol) and peptide **9** (18 mg, 23.3 µmol) were dissolved in 250 µL of TFA/H_2O (7/3) solution. The mixture was stirred for 20 min and the product was purified by RP-HPLC affording conjugate **3** as a white powder (7.3 mg, 1.55 µmol, 60%). Analytical HPLC t_R = 7.9 min; ESI-MS calc for $C_{179}H_{260}N_{58}O_{53}S_2$ 4133.9, found m/z 4134.5.

1 D. Boturyn and P. Dumy, *Tetrahedron Lett.,* 2001, **42**, 2787.

RP-HPLC profile and ESI-MS analysis of 3.

Figure S1 : RP-HPLC profile of **3** monitored at 214 nm

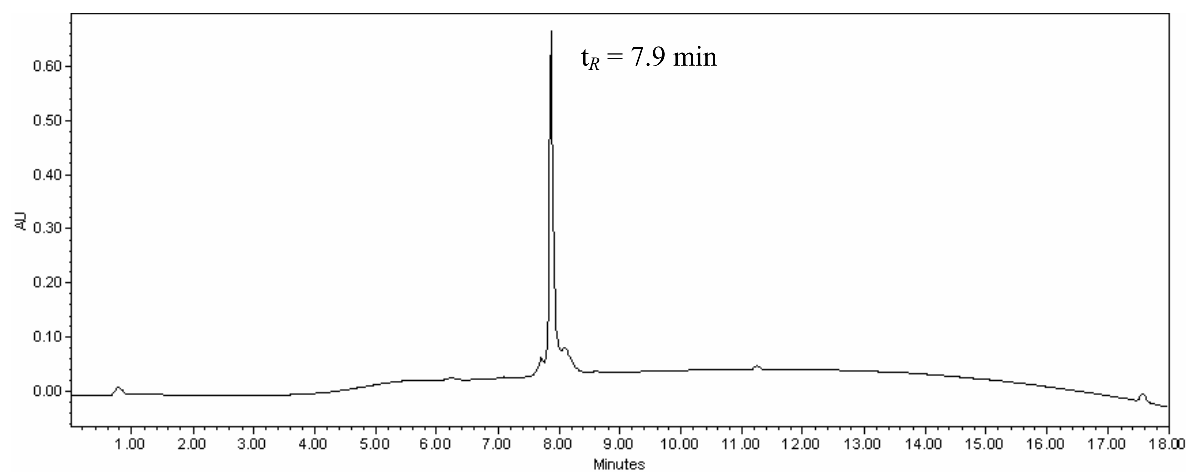
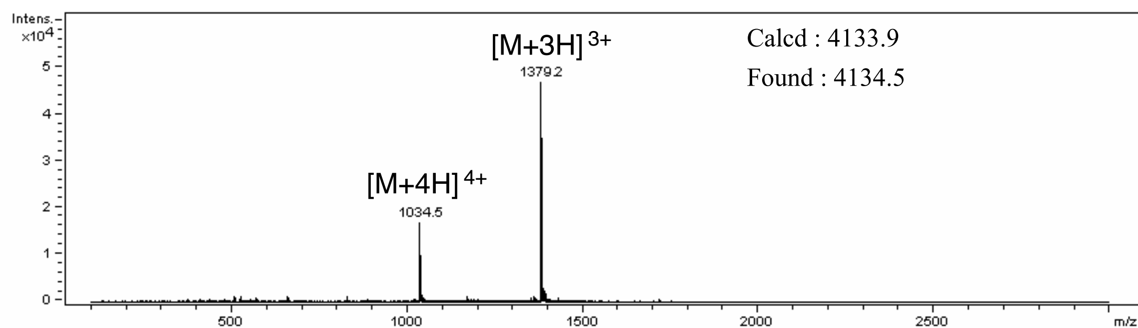
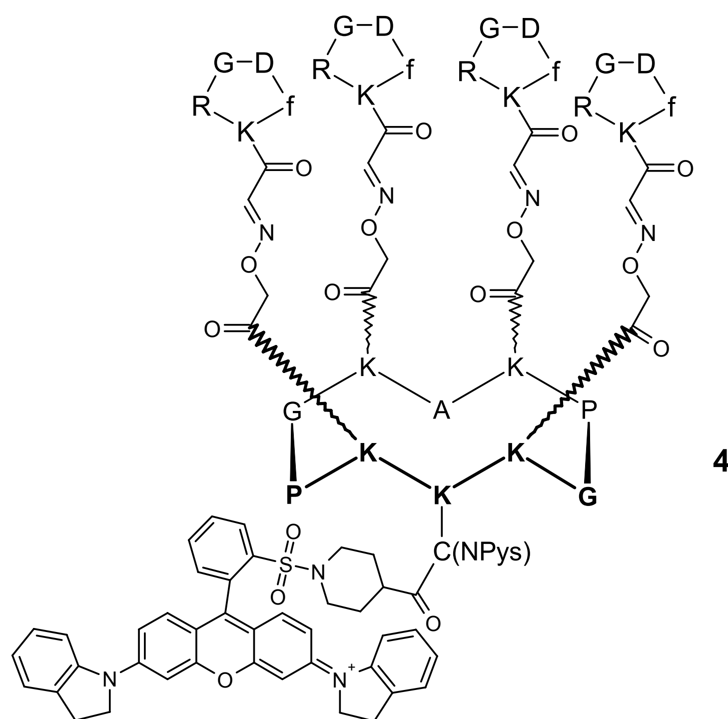


Figure S2 : ESI-MS analysis of compound **3**



Synthesis of RGD-containing peptide 4.



Peptide **3** (10.0 mg, 2.13 µmol) was dissolved in 200 µL of anhydrous DMF and the pH was adjusted to 9 with DIPEA. This solution was added to QSY®21 carboxylic acid succinimidyl ester (3.5 mg, 4.3 mmol). The mixture was stirred for 26 h at 40 °C and the product was purified by RP-HPLC. After lyophilization, the conjugate **4** was obtained as a deep blue solid powder (3 mg, 0.57 mmol, 27%). Analytical HPLC t_R = 10.2 min; ESI-MS calc for $C_{220}H_{295}N_{61}O_{57}S_3$ 4799.1, found m/z 4798.6.

RP-HPLC profile and ESI-MS analysis of 4.

Figure S3 : RP-HPLC profile of **4** monitored at 214 nm

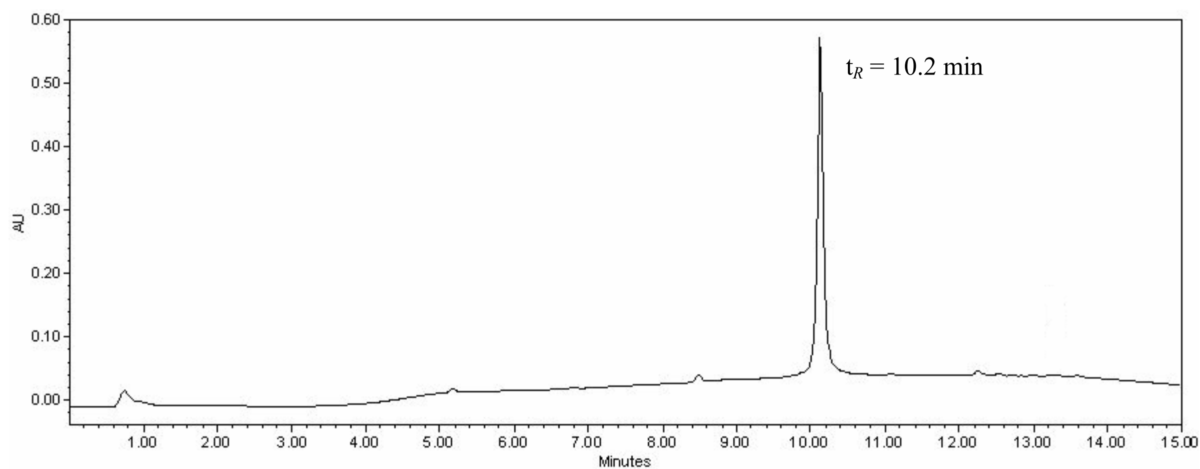
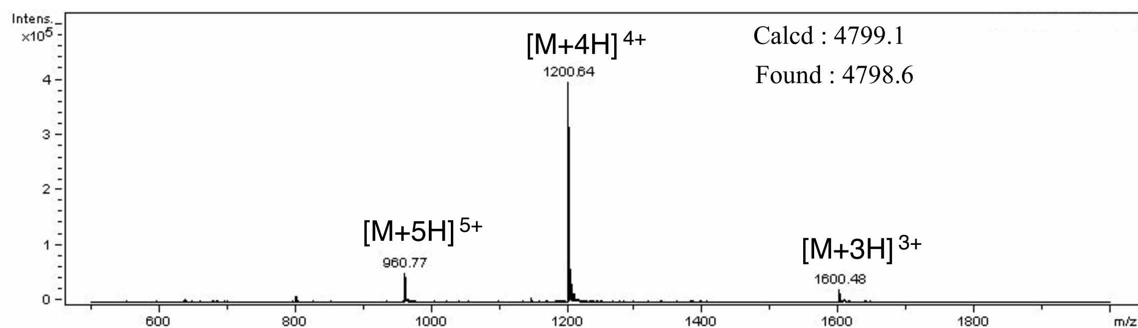
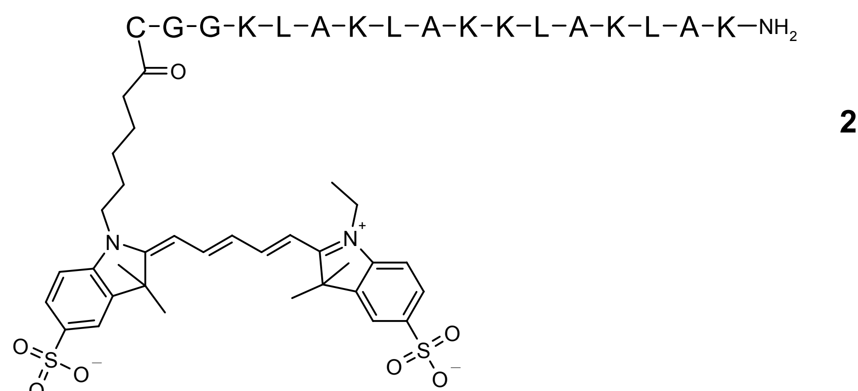


Figure S4 : ESI-MS analysis of compound **4**



Synthesis of KLA-containing peptide 2.



The linear peptide H-CGG-$(KLAKLAK)_2$-NH_2 was assembled automatically on RINK resin (140 mg, loading of 0.7 mmol/g) using the general procedure. Peptide on resin (1.0 mg, 0.7 µmol) was recovered in 50 µL of anhydrous DMF and the pH was adjusted to 9 with DIPEA. Then succinimidyl ester of Cyanine 5 (0.2 mg, 0.25 µmol) in anhydrous DMF (20 µL) was added to the resin solution. The mixture was stirred for 24 h at room temperature. Peptide **2** was released from the resin using 100 µL cleavage solution of TFA/TIS/H_2O/EDT (94/2/2). The mixture was stirred for 1 h at room temperature. Peptide **2** was obtained as a blue solid powder after precipitation, triturating and washing with diethyl ether (0.5 mg, 0.16 µmol, 63%). This crude material was used without further purification. Analytical HPLC t_R = 11.7 min; ESI-MS calc for $C_{212}H_{190}N_{26}O_{24}S_3$ 2379.4, found m/z 2377.4.

RP-HPLC profile and ESI-MS analysis of 2.

Figure S5 : RP-HPLC profile of **2** monitored at 214 nm

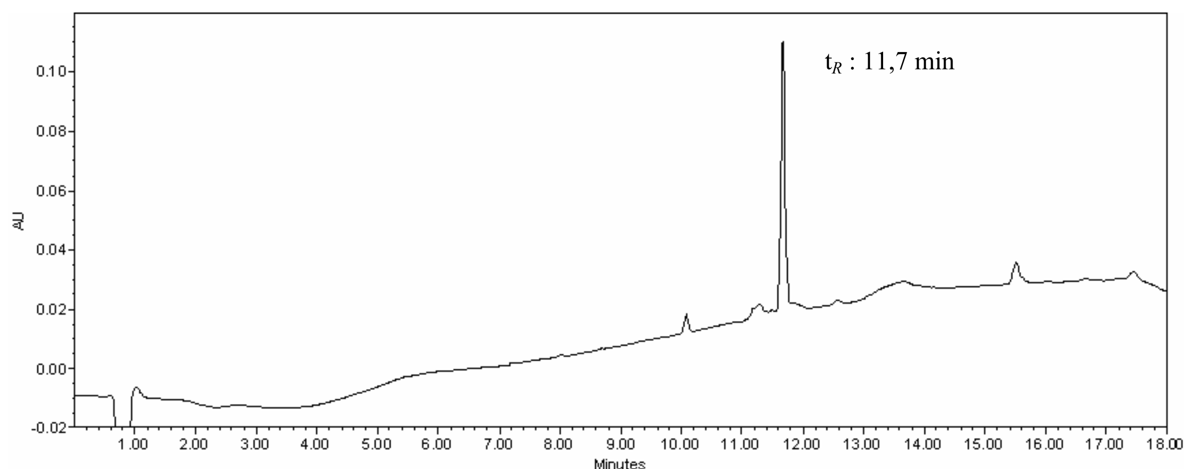
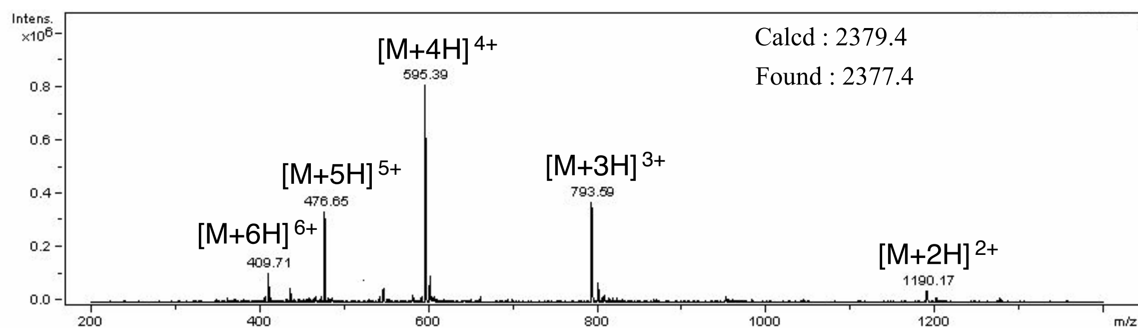
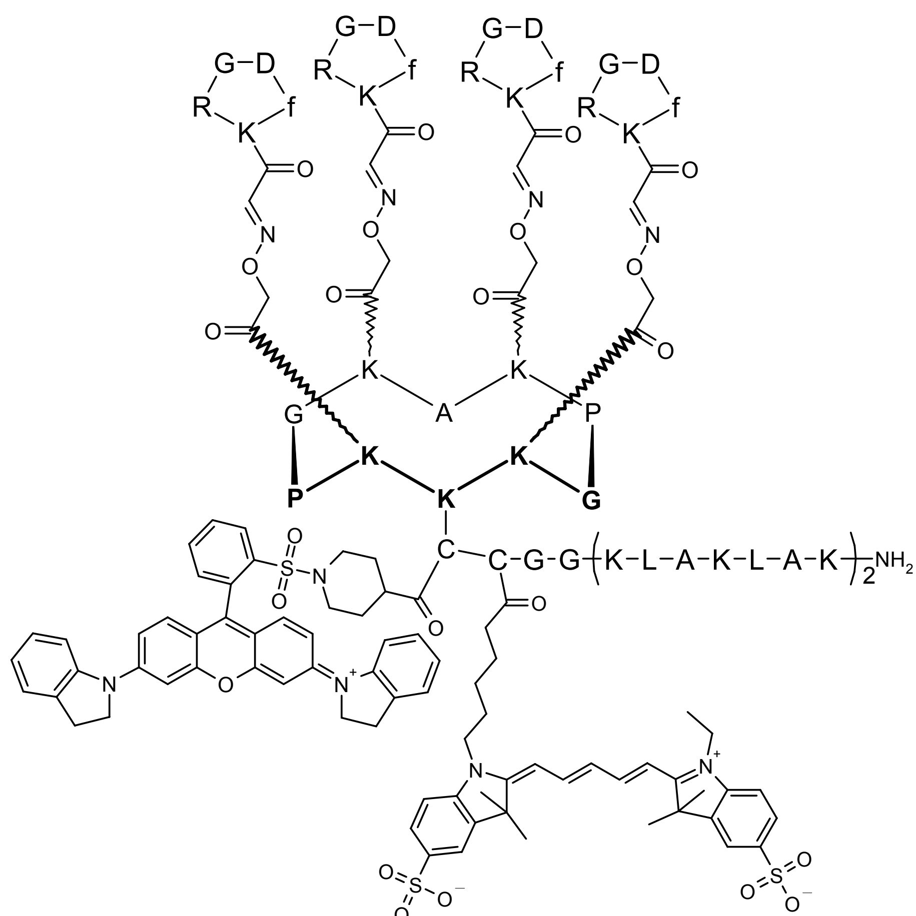


Figure S6 : ESI-MS analysis of compound **2**



Synthesis of RGD-containing peptide 1.



Peptide **2** (0.5 mg, 157 nmol) and peptide **4** (2 mg, 380 nmol) were dissolved in 400 µL of DMF/PBS (pH 4.8) under argon. The reaction mixture was stirred for 15 min at room temperature under argon. The product was then directly purified by RP-HPLC affording compound **1** as a deep blue powder (0.8 mg, 97 nmol, 62%). Analytical HPLC t_R = 9.4 min; ESI-MS calc for $C_{327}H_{481}N_{85}O_{79}S_5$ 7022.5, found m/z 7021.2.

RP-HPLC profile and ESI-MS analysis of 1.

Figure S7 : RP-HPLC profile of **1** monitored at 214 nm

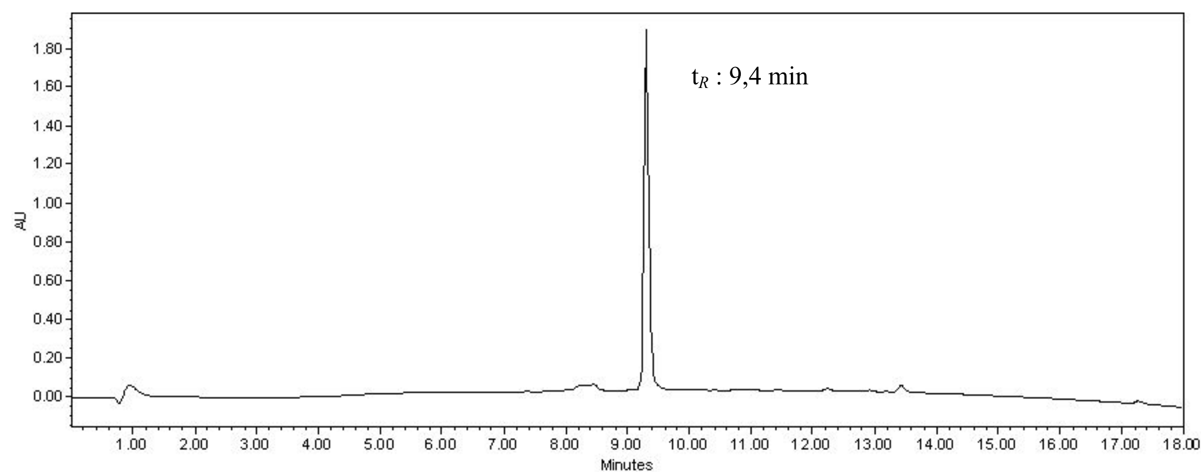
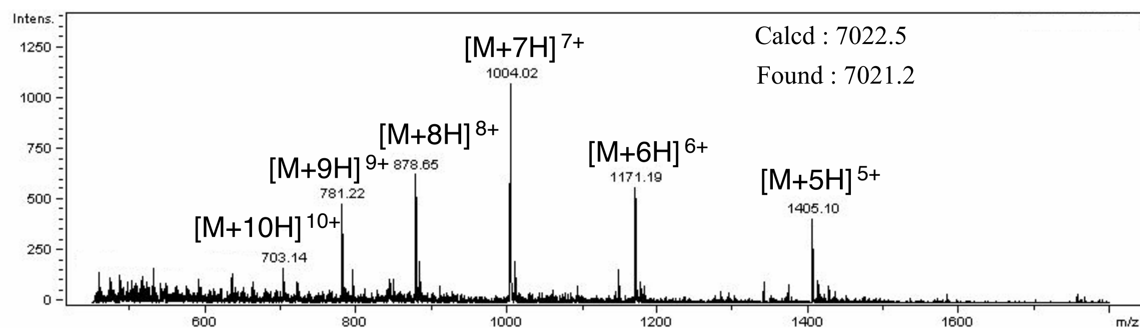


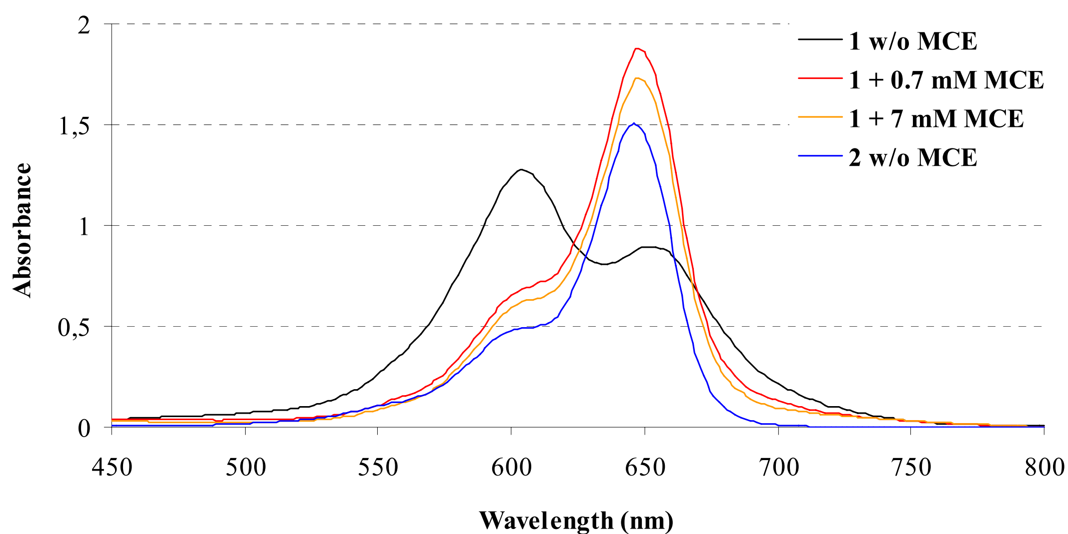
Figure S8 : ESI-MS analysis of compound **1**



UV spectra of compounds 1 and 2 with and without MCE.

UV spectra were recorded on SpectraMax Plus384 spectrophotometer (Molecular Devices) at 37 °C. Compounds 1 and 2 were dissolved in PBS (10 mM NaH_2PO_4, 50 mM NaCl, 1 mM EDTA, pH 7.0, 37° C) at concentration of 6 μM. When MCE (2-mercaptoethanol) was added, UV spectra were taken at 1 h.

Figure S9 : UV spectra of compound **1** and **2**



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Fluorescence measurements of compounds 1 and 2 with and without MCE.

Fluorescence intensities were recorded on SpectraMax Gemini XS microplate spectrofluorometer (Molecular Devices) at 37 °C. Compound **1** and **2** were dissolved in PBS (10 mM NaH_2PO_4, 50 mM NaCl, 1 mM EDTA, pH 7.0, 37° C) at concentration of 6 µM and solutions were introduced in 96-well plate with or without MCE (2-mercaptoetanol).

Figure S10 : Emission and excitation spectra of **1** with and without MCE. When MCE was added, emission and excitation spectra were taken at 1 h.

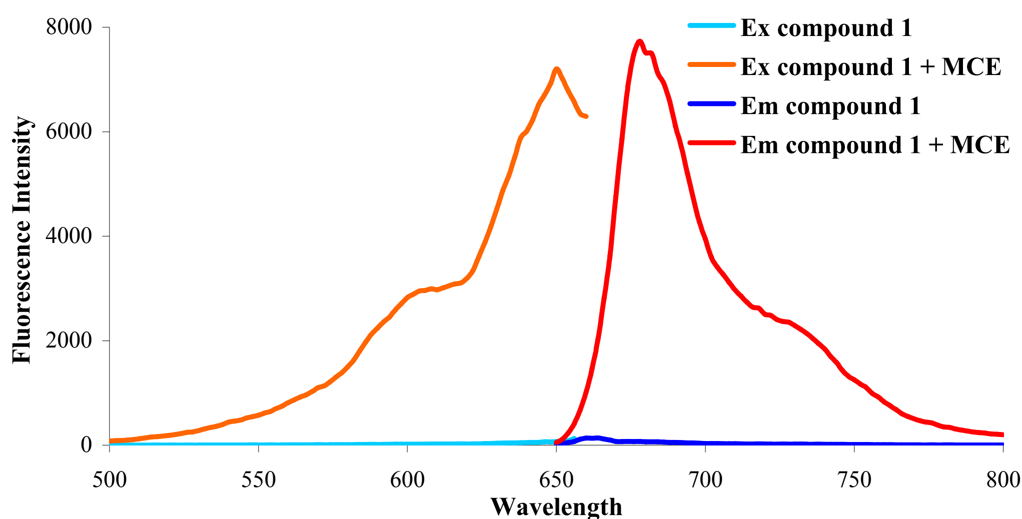
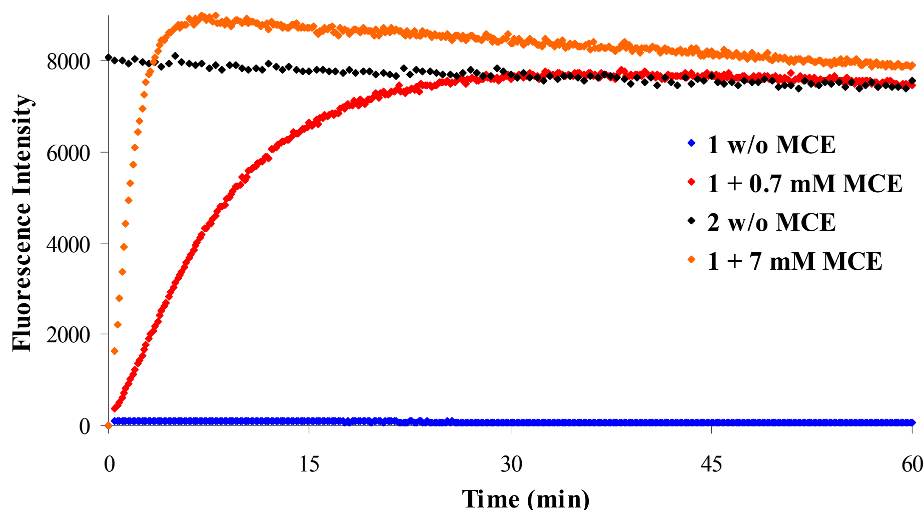


Figure S11 : Time courses of relative fluorescence emission of **1** and **2**. MCE was added at t = 0. $t_{1/2}$ (7 mM MCE) = 80 s ; $t_{1/2}$ (0.7 mM MCE) = 6 min.



Cell and culture conditions.

HEK293(β3) are subclones of the human embryonic kidney HEK293 cell line, stably transfected by a plasmid encoding the human β3. They were cultured in DMEM (Dulbecco's Modified Eagle's Medium) enriched with 4.5 g.L-1 glucose and supplemented with 1 % glutamine, 10 % FBS (Fetal bovine serum), penicillin (50 U.mL-1), streptomycin (50 µg.mL-1) and G418 (700 µg.mL-1). IGROV-1 (Human ovarian cancer cell line) and Ts/Apc (mouse mammary carcinoma) were cultured in RPMI 1640 medium supplemented with 1 % glutamine, 10 % FBS, penicillin (50 U.mL-1) and streptomycin (50 µg.mL-1). The Ts/Apc medium was supplemented with beta-mercaptoethanol (50 nM). All cells were maintained at 37°C under an atmosphere of 5 % CO_2.

Colocalization studies.

Confocal laser scanning microscopy was performed using a LSM510 confocal laser microscope (Carl Zeiss, France) equipped with an Ar laser (457 nm, 488 nm, 514 nm), two He/Ne lasers (543 nm and 633 nm) and femtosecond Ti/Sa laser, 'Tsunami' Spectra-Physics (wavelength from 690 to 1100 nm).

Cells were cultured in four-well Lab-Tek I chambered coverglass in the appropriate medium. Medium was removed and compound **1** was added at 2 μM for at least 1 h in appropriate medium w/o FBS and red phenol. Then, MitoTracker Red (Invitrogen) 100 nM was added to cell medium for 10 minutes together with Hoechst 5 μM. Cells were carefully rinsed and medium was replaced for further hours. Cells were observed through confocal microscopy at 37°C, 5 % CO2. Confocal slices were of 0.5 or 1 μm thickness. Images were treated with ImageJ software 1.37v.

Figure S12 : Representative fluorescence confocal images from live HEK293 cells incubated with **1** (2 μM) for at least 1 h, stained with Hoechst and MitoTracker Red. Cells were imaged ($\lambda_{Ex}/\lambda_{Em}$ = 633/662 nm) at (a) 14 min, (b) 70 min, and (c) 100 min (with excitation of MitoTracker Red, $\lambda_{Ex}/\lambda_{Em}$ = 543/599 nm).

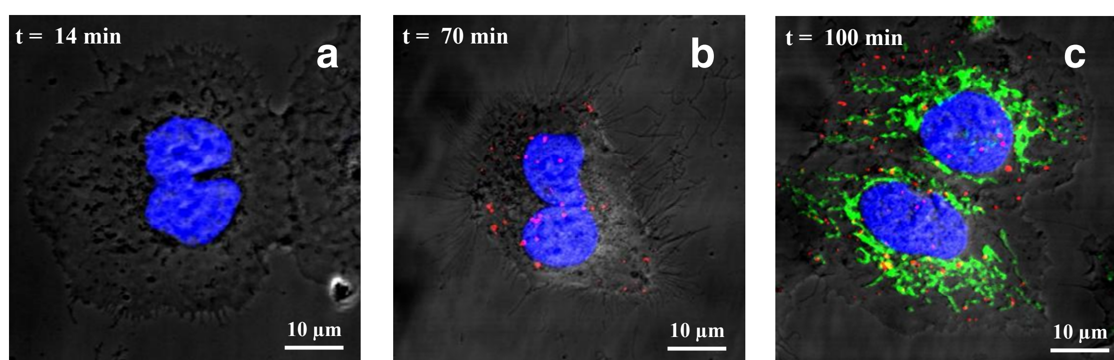


Figure S13 : Representative fluorescence confocal images from live HEK293 cells incubated with **1** (2 μM) for 60 min, stained with Hoechst and MitoTracker Red and imaged at 2 h. Cells were observed with (a) phase contrast, (b) excitation of Cy5-containing KLA peptide ($\lambda_{Ex}/\lambda_{Em}$ = 633/662 nm), (c) excitation of MitoTracker Red ($\lambda_{Ex}/\lambda_{Em}$ = 543/599 nm). (d) Images b and c were merged.