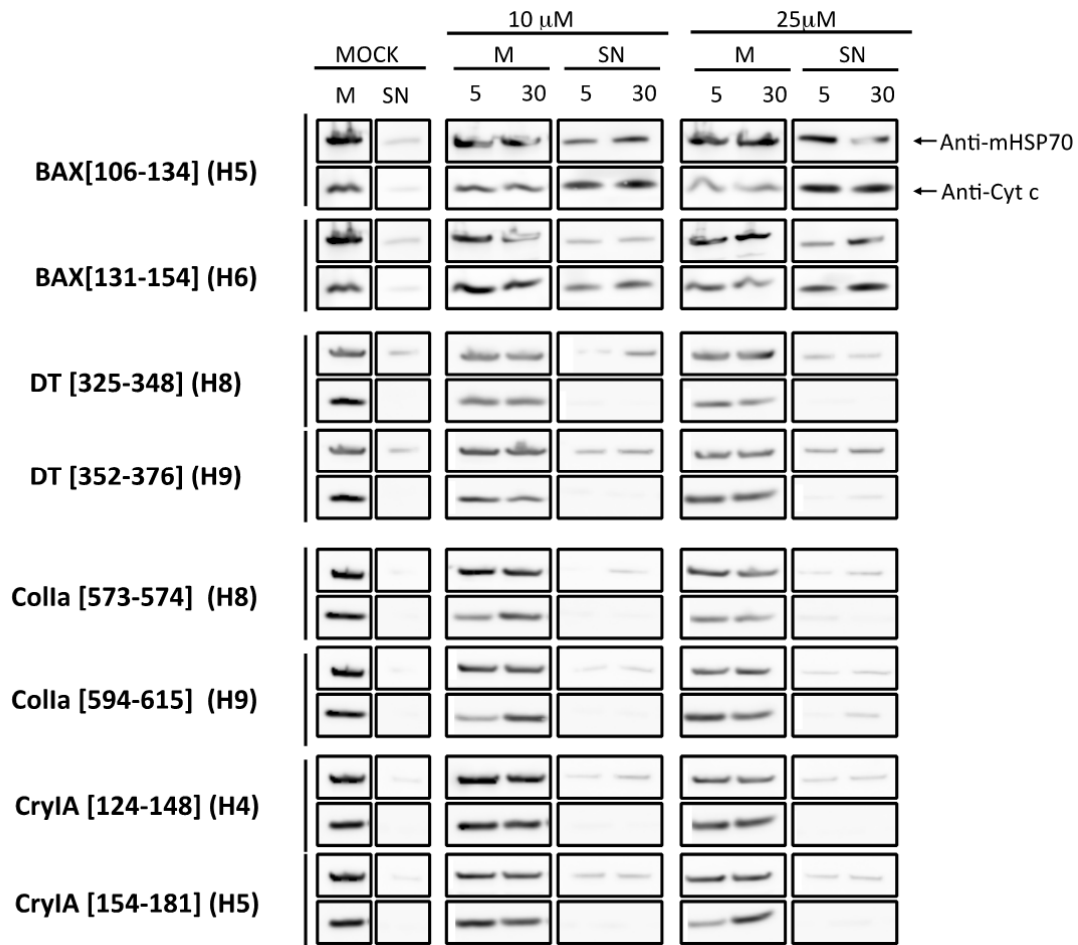


Supp. Figure 1:



Supp. Figure 1.

**Synthetic peptides corresponding to the central helical hairpin of Bax triggers cytochrome-c release from isolated mitochondria whereas analogous peptides from other globular membrane-active domains do not.**

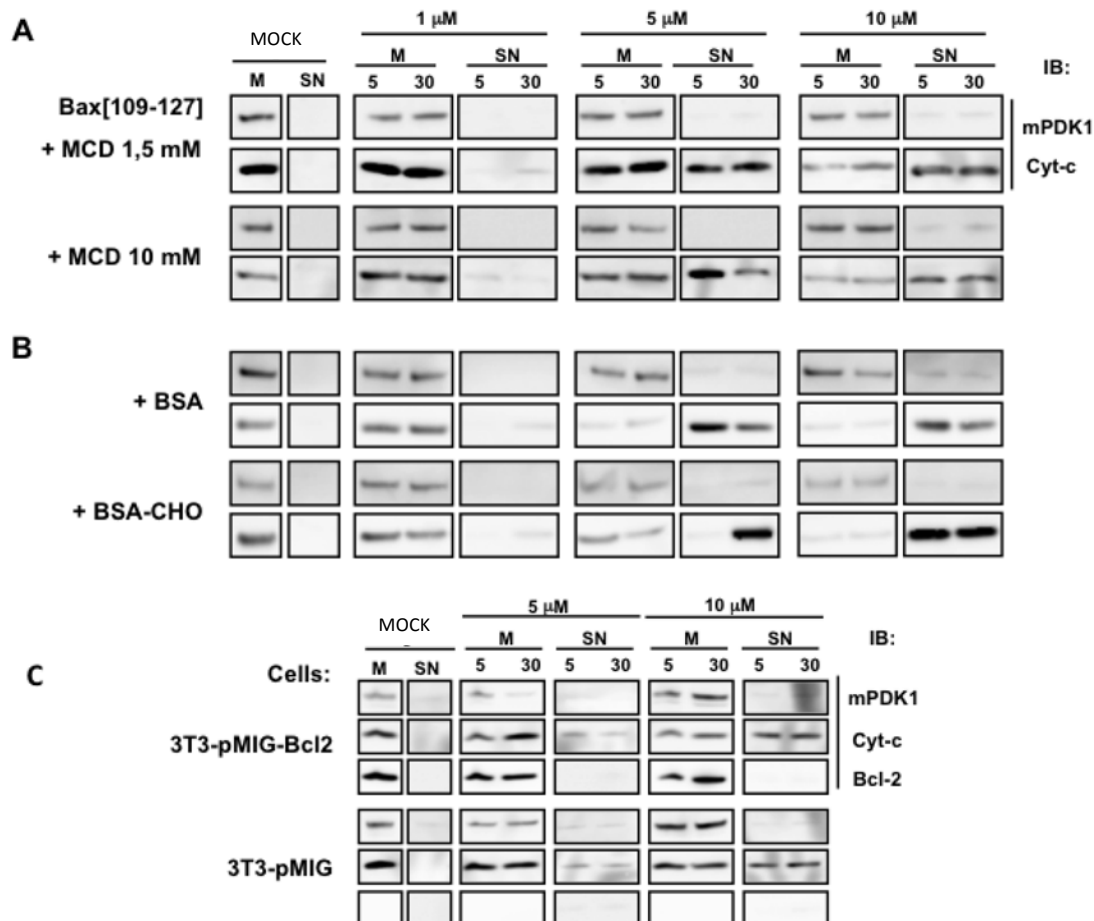
Mitochondrial cytochrome c release assays were performed with peptides corresponding to the first or second alpha-helix of the central hairpin of selected globular membrane-active domains. Peptides were synthesized that correspond to individual alpha-helices from the central hairpin of Bax (H5-H6), of the T domain of diptheria toxin (H8-H9) and of the pore-forming domains of colicin IA (H8-H9) or of *Bacillus thuringiensis* delta-endotoxin CryIA(a). The peptides were incubated at two concentrations (10 or 25  $\mu$ M) with mitochondria isolated from HeLa cells for the indicated durations (min), and the presence of mHSP70 and cytochrome c in the mitochondria (M) or in the mitochondrial supernatant (SN) was assessed by immunoblotting (IB). MOCK: control lanes with buffer-treated mitochondria. Among the assayed peptides, only Bax[106-134] (H5) and Bax[131-154] (H6) were active in inducing mitochondrial cytochrome-c release. Segments derived from analogous pore-forming toxins, which share a similar structure to that of Bax, but are active on other biological membranes, are not able to target and permeabilize the mitochondrial outer membrane. Contrary to Bax H5 and H6, these segments are also unable to target GFP to mitochondrial membranes (not shown).

**Suppl. Table 1**

Name [Residues]	Sequence (H-sequence-NH <sub>2</sub> )	Length (AA)	Series or species
Bax [106-134] (helix $\alpha$ 5)	NWGRVVALFYFASKLVLKALSTKVPELIR	29	
Bax [109-123]	RVVALFYFASKLVLK	15	
Bax [119-132]	KLVLKALSTKVPEL	14	
Bax [116-125]	FASKLVLKAL	10	
Bax [121-130]	VLKALSTKVP	10	
Bax [126-134]	STKVPELIR	9	
Bax [107-127]	WGRVVALFYFASKLVLKALST	21	L
Bax [112-127]	ALFYFASKLVLKALST	16	
Bax [107-123]	WGRVVALFYFASKLVLK	17	
Bax [109-125]	RVVALFYFASKLVLKAL	17	
Bax [109-126]	RVVALFYFASKLVLKALS	17	
Bax [109-127]	RVVALFYFASKLVLKALST	19	
Bax [115-127]	YFASKLVLKALST	13	
Bax [109-127] <sub>D</sub>	CGGRVVALFYFASKLVLKALST	22	
Bax [115-127] <sub>D</sub>	CGGYFASKLVLKALST	16	D
Scr[109-127] <sub>D</sub>	CGGLAVKVFLLTYKFSSARALV	22	
Bax [131-154] (helix $\alpha$ 6)	ELIRTIMGWTLDFLRERLLVWIQD	24	L
ColIA H8 (Colicine IA, helix $\alpha$ 8)	THAGNAATALVALVFSILTGS	22	<i>Escherichia coli</i>
ColIA H9 (Colicine IA, helix $\alpha$ 9)	SALGIIGYGLLMAVTGALIDES	22	
CryIA H4 (Delta-endotoxine CryIA(a), helix $\alpha$ 4)	PALREEMRIQFNDMNSALTTAIPLL	25	<i>Bacillus thuringiensis</i>
CryIA H5 (Delta-endotoxine CryIA(a), helix $\alpha$ 5)	QVPLLSVYVQAANLHLSVLRDVSVFGQR	28	
DT H8 (Diphtheria toxin, helix $\alpha$ 8)	TEEIVAQSIALSSLMVAQAIPLVG	24	<i>Corynebacterium diphtheriae</i>
DT H9 (Diphtheria toxin, helix $\alpha$ 9)	DIGFAAYNFVESIINLFQVVHNSYN	25	

**Supp Table 1.** Sequences of the peptides used in the study.

Supp. Figure 2:



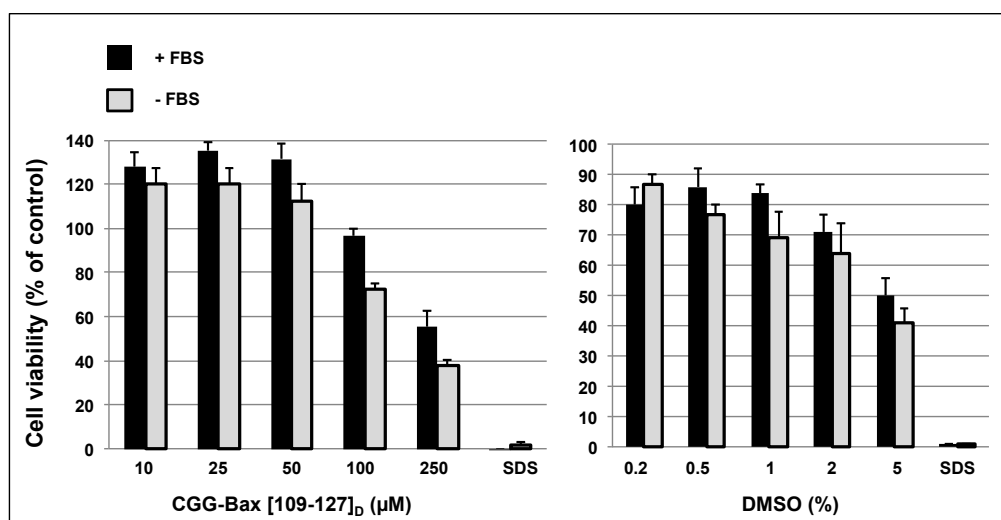
**Supp. Figure 2: Mitochondrial cholesterol content does not modulate Bax[109-127] peptide activity (Poro2)**

(A) Mitochondrial suspensions were pretreated with methyl-beta-cyclodextrin (MCD) (previously shown to induce mitochondrial cholesterol depletion {Ziolkowski, 2010 #1633}) at the indicated concentrations and were subsequently treated with the Bax[109-127] peptide. Bax[109-127] was as active in cholesterol-depleted mitochondria as in control mitochondria (see Supplementary Figure 3).

(B) The amount of cholesterol in mitochondria was increased with a BSA-cholesterol mixture, using BSA as carrier. Mitochondrial membrane poration induced by Bax[109-127] was not accelerated (and was even slightly delayed) in cholesterol-enriched mitochondria compared to mitochondria prepared with BSA alone. Mitochondrial cholesterol thus appears to be dispensable for Bax[109-127] cytochrome-c release activity.

(C) All-D Bax[109-127] triggers cytochrome-c release from mitochondria isolated from 3T3 cells regardless of expression of the anti-apoptotic protein Bcl-2. Mitochondria isolated from 3T3 cells stably expressing GFP (3T3-pMIG) or GFP-Bcl-2 (3T3-pMIG-Bcl-2) were incubated with the all-D Bax[109-127] peptide and mitochondrial cytochrome-c release and Bcl-2 expression were assayed by immunoblot.

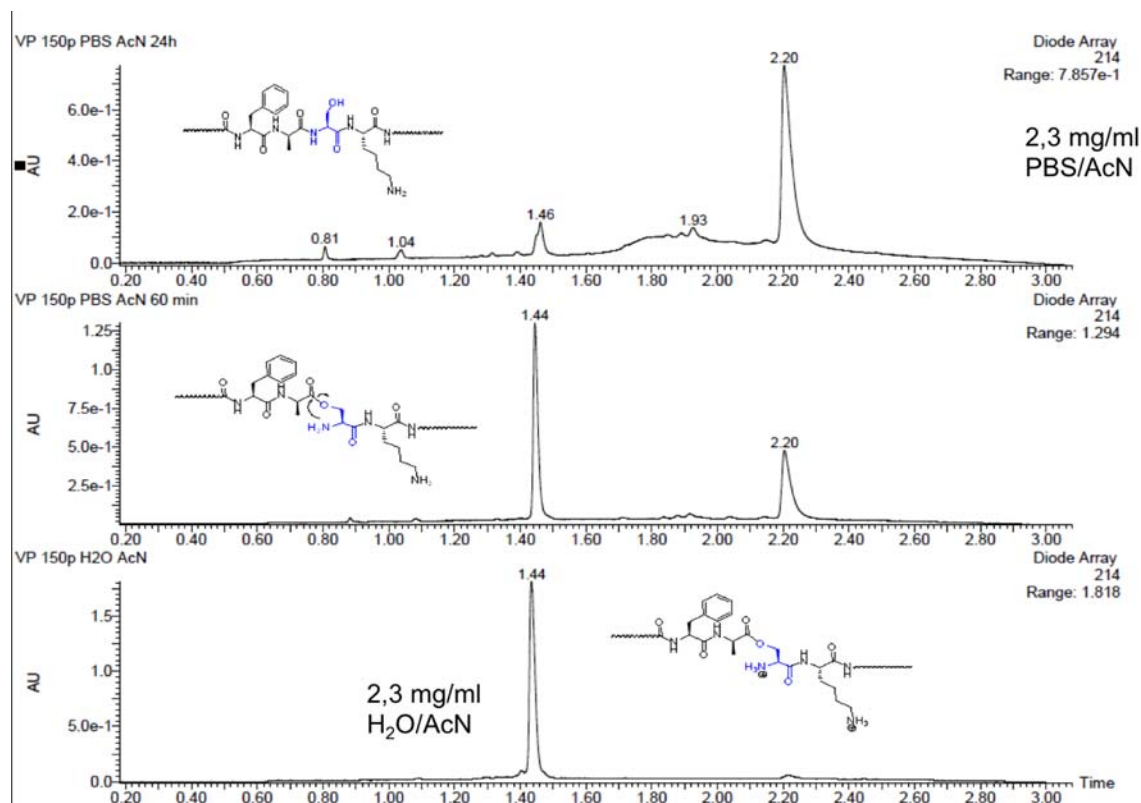
Supp. Figure 3 :



**Supp. Figure 3: Treatment of cells with unconjugated all-D Bax[109-127] does not trigger plasma membrane rupture and cell death.**

Colo829 cells were treated with various concentrations (10, 25, 50, 100 and 250 μM) of CGG-Bax[109-127]<sub>D</sub> peptide or DMSO (0.2-5%). Treatment with 0.1% SDS, which solubilizes plasma membrane of the cells, was used as a positive control for cell lysis. Cell viability was measured using the methylene blue dye exclusion technique at 24h of incubation and was expressed as percentage of control cells without treatment. Cells were cultured in presence or absence of fetal bovine serum (FBS). Data are represented as mean ± s.d (n=4). CGG-Bax[109-127]<sub>D</sub> caused non-specific cell death at high concentrations (above 100μM, left panel) that can be fully attributed to toxicity of the DMSO vehicle (right panel).

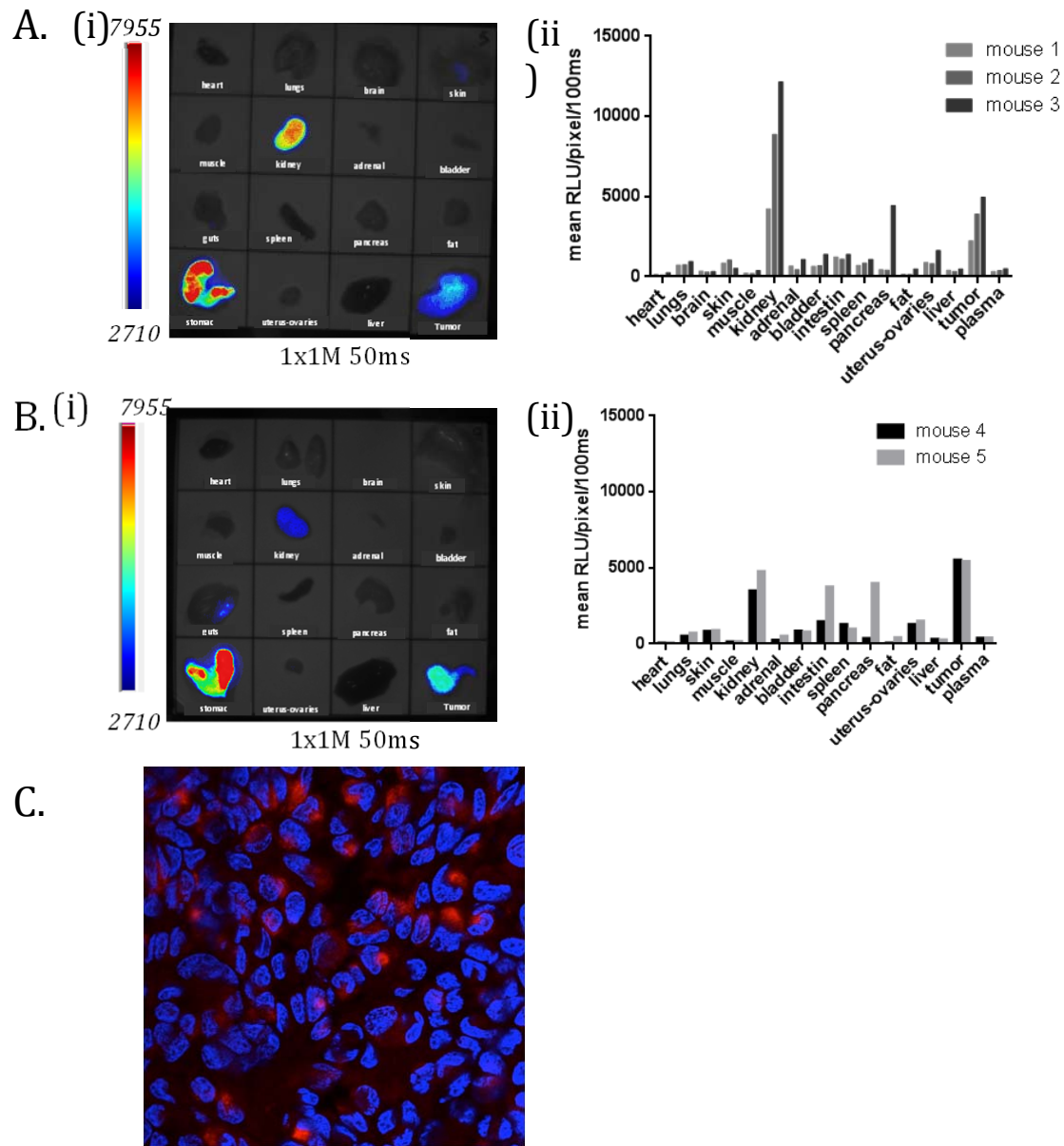
### Supp. Figure 4:



**Supp. Figure 4:** O-N shift on depsi Poro2 H-S(H-RVVALFYFA)KLVLKALST-NH<sub>2</sub> (bottom) yielding Poro2 H-RVVALFYFASKLVLKALST-NH<sub>2</sub> after 60 minutes (center) and 24 hours (top)

The synthesis proceeds on rink amide PS resin by automated microwave assisted Fmoc/tBu solid phase strategy. After coupling and deprotection of Lys<sup>119</sup>, the depsidipeptide Boc-Ser(Fmoc-Ala)-OH was coupled by DIC/HOBt activation. Noteworthy, these coupling reagents were chosen to keep the pH as low as possible to avoid hydrolysis of ester bond which was observed during our first attempts when we used tertiary amines in combination with phosphonium or uranium coupling reagents. Then, the Fmoc group at the N- $\alpha$  of Alanine was removed by DMF/piperidine 80/20 treatment and the synthesis continued at room temperature. Indeed, our first trails showed that heating such O-acylated peptide results in unwanted hydrolysis. Cleavage and removal of protecting groups proceeded in TFA/TIS/water (95/2.5/2.5 v/v/v) cocktail and the resulting crude depsi Poro2 was solubilized in water and purified by RP-HPLC using water/acetonitrile (both at 1/1000 v/v TFA) as eluents. The O-N transposition was achieved by simply adjusting the pH to 7.4 and monitored by HPLC. In 24 hours, the transposition is nearly complete. Interestingly O-acylated depsi Poro2 derivative eluted at 45% acetonitrile whenever the N-acylated Poro2 was much hydrophobic, eluting at 74% acetonitrile in HPLC gradient.

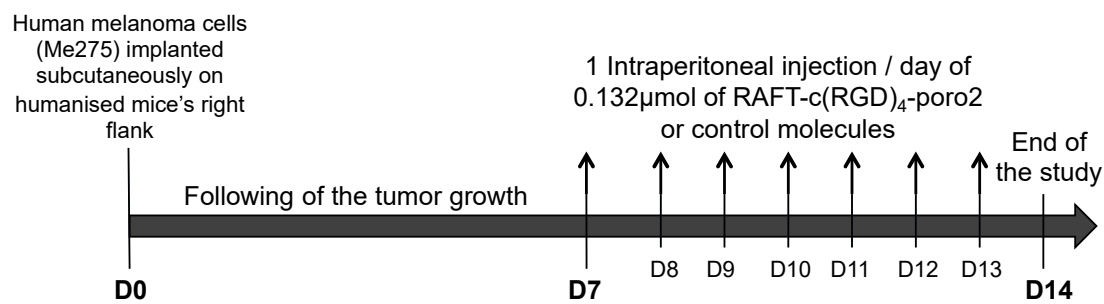
Supp. Figure 5:



**Supp. Figure 5: *in vivo* biodistribution of RAFT-c(RGD)<sub>4</sub>-A700 in subcutaneous melanoma tumor bearing mice.**

Human melanoma cells A375 (A) and Colo829 (B) were implanted on right flank of humanized mice (n=5 for A375 and n=4 for Colo829). 200 $\mu$ L of RAFT-c(RGD)<sub>4</sub>-A700 50 $\mu$ M is intravenously injected in mice. 2D-FRI is performed 24hours after the injection on Hamamatsu photonics device. Fluorescence was visualized at 660nm (i) and quantified (ii) in different organs using Wasabi® software. C) Confocal fluorescence microscopy of a Colo829 tumor section. In blue: DAPI staining of the nuclei. In Red: RAFT-c(RGD)<sub>4</sub>-A700 . Objective: 63x.

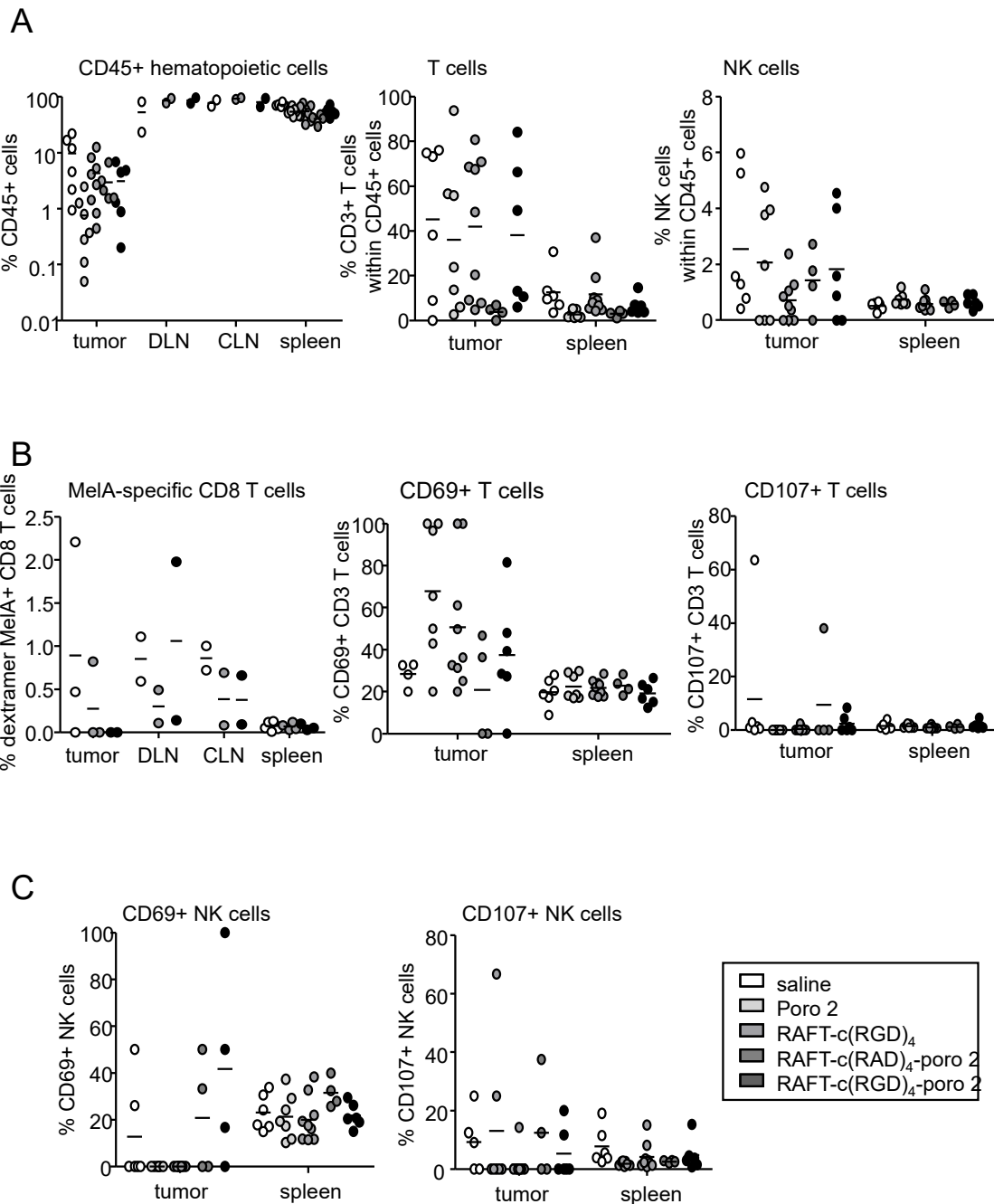
**Supp. Figure 6:**



**Supp. Figure 6: Representation of the *in vivo* experiments procedure.**

The studied molecule is RAFT-c(RGD)<sub>4</sub>-poro2 while the control molecules are saline, RAFT-c(RGD)<sub>4</sub>, RAFT-c(RAD)<sub>4</sub>-poro2 or poro2. D= day

Supp. Figure 7:



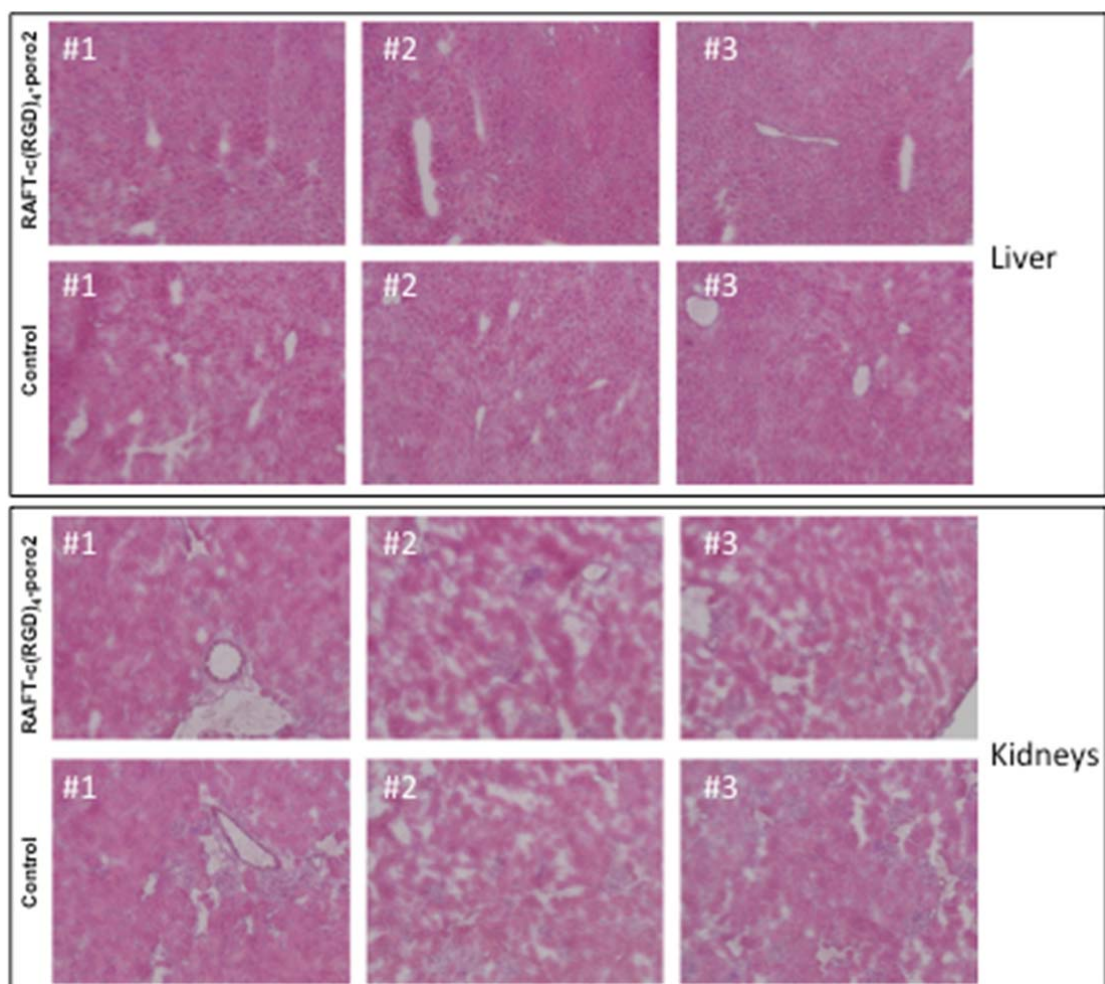
**Supp. Figure 7 : Modulation of anti-tumor immunity following RAFT-c(RGD)<sub>4</sub>-poro2 treatment**

Melanoma cells Me275 were subcutaneously implanted on Humanized mice's right flank. After 6 days, mice received one daily intraperitoneal injection of saline (n=6), RAFT-c(RGD)<sub>4</sub> (n=8), poro2 (n=7), RAFT-c(RAD)<sub>4</sub>-poro2 (n=4) or RAFT-c(RGD)<sub>4</sub>-poro2 (n=6). Seven days after the start of treatment, the infiltration and functional status of human immune cells were analyzed by flow cytometry at the tumor site, in the draining lymph nodes (DLN), in the control lymph nodes (CLN) and in the spleen. (A) Proportions of CD45+ hematopoietic cells, CD3+ T cells and CD3-CD56+ NK cells in the indicated organs. (B) Proportions of



MelA-specific CD8 T cells (within CD8 T cells), activated CD69+ T cells (within T cells) and cytotoxic CD107+ T cells (within T cells) in the indicated organs. (C) Proportions of activated CD69+ NK cells (within NK cells) and cytotoxic CD107+ NK cells (within NK cells) in the indicated organs.

Supp. Fig. 8



Supp. Figure 8 : Absence of toxicity in liver and kidneys

At day 14, 3 liver and kidneys samples from mice treated with RAFT-c(RGD)<sub>4</sub>-poro2 and 3 other from the saline group were analyzed by histopathology. No apparent toxicity was observed in these two organs as presented for 3 specimen.

## General procedure for synthesis

Protected amino acids were obtained from Activotec (Cambridge, UK), Bachem Biochimie SARL (Voisins-les-Bretonneux, France), France Biochem SA (Meudon, France), Merck Eurolab (Fontenay-sous-Bois, France) or Calbiochem-Novabiochem (Merck Biosciences - VWR, Limonest, France). PyBOP® was purchased from Calbiochem-Novabiochem. 2-chlorotriylchloride® resins was obtained from Advanced ChemTech Europe. Other reagents were obtained from Aldrich (Saint-Quentin Fallavier, France) and Acros (Noisy-le-Grand, France). Cyanine™ 5 Mono NHS ester was purchased from Amersham Biosciences (Orsay, France) Fonctionnalisés poly(éthylène)-glycol were purchased from Iris Biotech (Marktredwitz, Germany).

RP-HPLC analyses were performed on Waters equipment consisting of a Waters 600 controller, a Waters 2487 Dual Absorbance Detector and a Waters In-Line Degasser. The analytical column used was the Nucleosil 120 Å 3 µm C18 particles, 30 × 4 mm<sup>2</sup> operated at 1 mL.min<sup>-1</sup> with linear gradient programs in 20 min run time (classical program 5 to 100 % B in 20 min). UV monitoring was performed most of the time at 214 nm and 250 nm. Solvent A consisted of H<sub>2</sub>O containing 0.1% TFA and solvent B of CH<sub>3</sub>CN containing 9.9% H<sub>2</sub>O and 0.1% TFA. Water was of Milli-Q quality and was obtained after filtration of distilled water through a Milli-Q® cartridge system. CH<sub>3</sub>CN and TFA were of HPLC use quality.

RP-HPLC purifications were performed on Waters equipment consisting of a Waters 600 controller and a Waters 2487 Dual Absorbance Detector. Degassing of solvents was performed with 50 mL.min<sup>-1</sup> helium. The preparative column, Delta-Pak™ 300 Å 15 µm C18 particles, 200 × 25 mm<sup>2</sup> was operated at 22 mL.min<sup>-1</sup> with linear gradient programs in 30 min run time. Solvents A and B were the same than the ones used in RP-HPLC analysis.

Electron spray ionization (ESI-MS) mass spectra were obtained on an Esquire 3000 (Bruker). The multiply charged data produced by the mass spectrometer on the m/z scale were converted to the molecular weight. NMR spectra were recorded on BRUKER Avance 300 spectrometers. Chemical shifts are expressed in ppm and calculated taking the solvent peak as an internal reference.

## General Procedure for Solid-Phase Peptide Synthesis

Assembly of all linear protected peptides was performed manually or automatically by solid-phase peptide synthesis (SPPS) using the standard 9-fluorenylmethoxycarbonyl/ tertibutyl (Fmoc/tBu) protection strategy. In manual SPPS, the device consisted in a glass reaction vessel fitted with a sintered glass frit. The latter allowed elimination of excess reagents and solvents under compressed air. Before use, the vessel was treated for 12 h (typically overnight) with (CH<sub>3</sub>)<sub>2</sub>SiCl<sub>2</sub> as lubricant to prevent resin beads from sticking to the glass inner wall during the synthesis. It was then carefully washed with CH<sub>2</sub>Cl<sub>2</sub> until complete acid trace clearance. The 2-chlorotriyl resin was preloaded for 30 min in dry CH<sub>2</sub>Cl<sub>2</sub> with Nα-Fmoc-protected amino acid substituted at 1 mmol/g and capped with 6 mL of methanol/DIPEA/ CH<sub>2</sub>Cl<sub>2</sub> (2/1/17) for 5 min and then one more time for 10 min. At the beginning of the synthesis and after each ether washing, the resin was washed and swollen twice with CH<sub>2</sub>Cl<sub>2</sub> (20 mL/g resin) for 15 min and once with DMF (20 mL/g resin) for 15 min. Coupling reactions were performed using, relative to the resin loading, 1.5-2 eq. of Nα-Fmoc-protected amino acid in situ activated with 1.5-2 eq. PyBOP and 3-4 eq. DIPEA in DMF (10 mL/g resin) for 30 min. The resin was then washed twice with DMF (20 mL/g resin) for 1 min and twice with CH<sub>2</sub>Cl<sub>2</sub> (20 mL/g resin) for 1 min. The completeness of amino acid coupling reaction was checked by TNBS test. TNBS test required a solution of 1% trinitrobenzenesulfonic acid in DMF, a solution of DIPEA/DMF (1:9). Three drops of each solution were added to a small quantity of resin beads in a tube. The mixture was left at room temperature. for 1 min. Remaining of free amino functions resulted in a red coloration of the resin beads (positive TNBS test). Coupling reaction with the protected amino acid should then be repeated until the beads remain colourless (negative TNBS test).

N $\alpha$ -Fmoc protecting groups were removed by treatment with piperidine/DMF (1:4) (10 mL/g resin) for 10 min. The process was repeated three times and the resin was further washed five times with DMF (10 mL/g resin) for 1 min. The completeness of the deprotection was checked by UV measurement. Indeed, Fmoc groups releasing afforded dibenzofulvene whose adduct with piperidine absorbs light in the UV range ( $\lambda = 299$  nm,  $\epsilon = 7800$  M $^{-1}$ .cm $^{-1}$ ). Cleavage and washing solutions were thus together collected and the volume of the solution was adjusted to a known value (V) with MeOH.

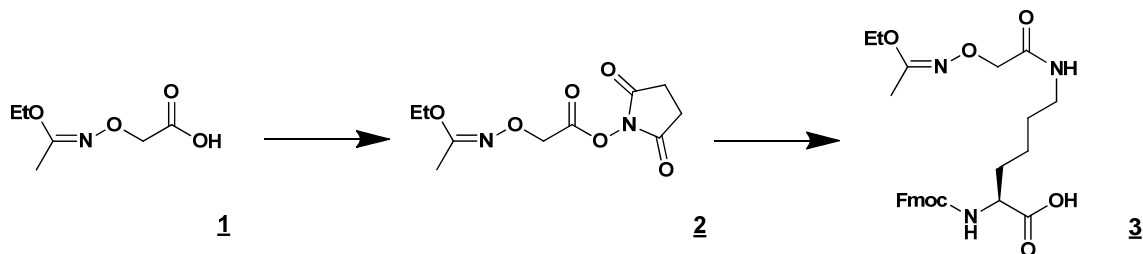
Automated peptide syntheses were performed on ABI 433 A1 Peptide Synthesizer (Applied Biosystems) or on Syro II Synthesizer (Biotage) using standard solid-phase methods.

## General Procedure for Cyclization Reactions.

All linear peptides were dissolved in DMF (0.5 mM) and the pH values were adjusted to 8-9 by addition of DIPEA. PyBOP (1.2 equiv) was added and the solution stirred at room temperature for 1 h. The solvent was removed under reduced pressure and the residue was dissolved in a minimum of CH $_2$ Cl $_2$ . Diethyl ether was added to precipitate peptides. They were then triturated and washed three times with diethyl ether to obtain crude materials that were used in the next step without further purification.

## Synthesis of modified amino-acids

### Synthesis of building block 3



### 2-(1-Ethoxyethylideneaminoxy)acetic Acid 1

To a stirred solution of iodoacetic acid (9.00 g, 48.4 mmol) in water (19 mL) at 0°C was added aqueous NaOH (3.0 mL, 40% w/w). The resulting solution was allowed to heat to room temperature, whereafter ethyl *N*-hydroxyacetimidate (6.0 g, 58.3 mmol) was added followed by aqueous NaOH (4.5 mL, 40% w/w) and water (19 mL) (pH of solution >12). After 4.5 h of stirring at 80°C and cooling to room temperature, the aqueous mixture was washed with DCM (3 x 50 mL). The water phase was cooled down to 0°C and was brought to pH 2-3 with a 1 M hydrochloride solution. The acidified water phase was then extracted with AcOEt (4 x 50 mL) (NB: After each extraction the pH was adjusted to 2-3 with a 1 M hydrochloride solution, at 0°C), and the combined organic phases from this last extraction were washed with brine (50 mL), dried over MgSO $_4$ , and concentrated under vacuum, providing compound 1 as a yellow oil (5.69 g, 35.3 mmol).

$^1$ H NMR (400 MHz, CDCl $_3$ ):  $\delta$  4.48 (s, 2H), 4.00 (q, J = 7.2 Hz, 2H), 2.01 (s, 3H), 1.27 (t, 3H, J = 7.2 Hz)

MS (ESI-MS, negative mode): C $_6$ H $_{11}$ NO $_4$ , Calcd MW = 161.2 g.mol $^{-1}$ , Found MW = 161.0 g.mol $^{-1}$

### *N*-Hydroxysuccinimidyl 2-(1-Ethoxyethylideneaminoxy)acetate 2

To a stirred solution of compound **3** (5.69 g, 35.3 mmol) and *N*-hydroxysuccinimide (5.28 g, 45.9 mmol) in ethyl acetate/ dioxane (120 mL, 1:1) at 0°C was added DCC (7.28 g, 35.3 mmol). The resulting mixture was stirred at room temperature overnight. The formed DCU was filtered off and the filtrate was concentrated under vacuum. The obtained residue was dissolved in ethyl acetate (200 mL), and the solution was washed with 5% aqueous NaHCO<sub>3</sub> (2x75 mL), water (75 mL), and brine (75 mL). The organic solution was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated under vacuum to yield **4** as a yellow solid (8.69 g, 33.7 mmol) which was used without further purification.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 4.78 (s, 2H), 4.02 (q, *J* = 7.1 Hz, 2H), 2.84 (s, 4H), 1.98 (s, 3H), 1.26 (t, *J* = 7.1 Hz, 3H).

MS (ESI-MS, positive mode): C<sub>10</sub>H<sub>14</sub>N<sub>3</sub>O<sub>7</sub>, Calcd MW = 258.1 g.mol<sup>-1</sup>, Found MW = 258.3 g.mol<sup>-1</sup>

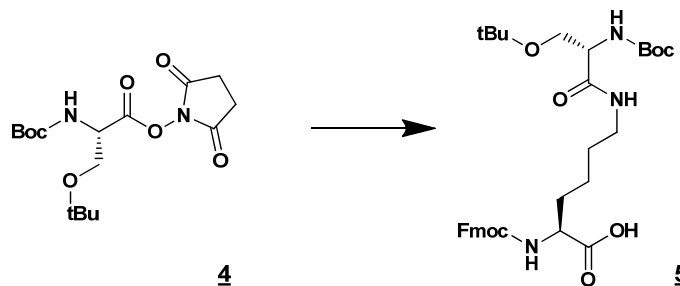
### Fmoc-Lys[*N*-EEI-Aoa]-OH **3**

To a stirred mixture of Fmoc-lysine, HCl salt (7.46 g, 20.3 mmol) and DIPEA (2-4 equiv pH=9) in DMF (100 mL) at room temperature was added dropwise over 20 min a solution of the above-prepared NHS-ester **4** (4.35 g, 16.9 mmol) in DMF (40 mL). The pH of the resulting mixture was regularly adjusted to pH 8-9 by further additions of DIPEA. After 2 h of reaction, the reaction mixture was concentrated under vacuum providing an oily residue. After addition of Ethyl acetate (100 mL), the organic phase was washed with a 10 % citric acid aqueous solution (3 x 30mL). The organic phase was then washed with brine (100 mL), dried over MgSO<sub>4</sub>, and evaporated under reduced pressure to yield the compound **5** as a light yellow powder (7.47 g, 14.6 mmol), which was used without further purification.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.60 (t, *J* = 7.1 Hz, 2H), 7.38 (t, *J* = 7.4 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 6.50 (t, *J* = 6.1 Hz, 1H), 5.68 (d, *J* = 7.8 Hz, 1H), 4.44 – 4.32 (m, 4H), 4.21 (t, *J* = 7.0 Hz, 1H), 3.96 (q, *J* = 7.1 Hz, 2H), 3.44 – 3.25 (m, 2H), 1.96 (s, 3H), 1.80 (s, 2H), 1.64 – 1.52 (m, 2H), 1.51 – 1.36 (m, 2H), 1.25 (t, *J* = 7.1 Hz, 3H)

MS (ESI-MS, positive mode): C<sub>27</sub>H<sub>33</sub>N<sub>3</sub>O<sub>7</sub>, Calcd MW = 511.2 g.mol<sup>-1</sup>, Found MW = 511.3 g.mol<sup>-1</sup>

### Synthesis of building block 5



### Boc-Ser(*t*Bu)-*N*-hydroxysuccinimide ester **4**

To a stirred solution of Boc-Ser(*t*Bu)-OH (2.8 g, 10.7 mmol) and *N*-hydroxysuccinimide (1.8 g, 16 mmol) in AcOEt/dioxan (1:1, 100 mL) cooled in an ice bath was added DCC (2.2 g, 10.7 mmol). After 5 h stirring at room temperature, the reaction mixture was filtered through a pad of Celite and washed with AcOEt and the collected filtrate was concentrated under vacuum. The obtained oily residue was taken into 150 mL ethyl acetate and washed with 5 % aqueous NaHCO<sub>3</sub> (2 x 70 mL), water (2 x 70 mL) and brine (2 x 70 mL). The organic fraction was then dried over MgSO<sub>4</sub>. Boc-Ser(*t*Bu)-NHS **1** was obtained as a white powder after evaporation of the solvent (3.5 g, 9.8 mmol).

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 5.41 (d, *J* = 9.0 Hz, 1H), 4.78 (d, *J* = 9.0 Hz, 1H), 3.92 (m, 1H), 3.66 (m, 1H), 2.82 (s, 4H), 1.46 (s, 9H), 1.20 (s, 9H)

MS (ESI-MS, positive mode): C<sub>16</sub>H<sub>26</sub>N<sub>2</sub>O<sub>7</sub>, Calcd MW = 358.2 g.mol<sup>-1</sup>, Found MW = 359.3 g.mol<sup>-1</sup>

### Fmoc-Lys[Boc-Ser(*t*Bu)]-OH **5**

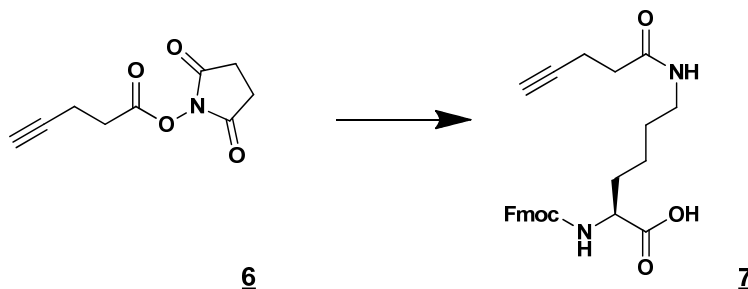
The above Boc-Ser(*t*Bu)-NHS **1** (8.00 g, 22.3 mmol) in DMF (75 mL) was added dropwise over 30 min to a stirred suspension of finely powdered HCl-salt of Fmoc-lysine (1.17 g, 29.0 mmol) and DIPEA (2-4 equiv pH=9) in DMF (130 mL). After 5 h of reaction, the solvent was evaporated under vacuum and the remaining

residue was dissolved in AcOEt (400 ml), followed by washing with a 10 % citric acid aqueous solution (70 ml), water (2 x 100 ml), and finally brine (100 mL). The organic fraction was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporation under vacuum gave **2**, as a white solid (11.85 g, 19.4mmol).

**<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):** δ 7.68 (2H, d, J = 7.5 Hz), 7.55 (2H, d, J = 7.5 Hz), 7.33-7.18 (4H, m), 6.84 (1H, broad s), 6.25 (1H, broad d), 5.62 (1H, broad s), 4.34-4.06 (5H, m), 3.63 (1H, m), 3.36 (1H, m), 3.20-3.05 (2H, m), 1.84 (1H, m), 1.65 (1H, m), 1.47-1.32 (14H, m), 1.09 (9H, s)

**MS (ESI-MS, positive mode):** C<sub>33</sub>H<sub>45</sub>N<sub>3</sub>O<sub>8</sub>, Calcd MW = 611.3 g.mol<sup>-1</sup>, Found MW = 611.2 g.mol<sup>-1</sup>

## Synthesis of building block 7



### 4-Pentynoic acid succinimidyl ester **6**

To a stirred solution of pent-4-ynoic acid (1 g, 10.2 mmol) and *N*-hydroxysuccinimide (1.53 g, 13.2 mmol) in ethyl acetate/dioxane (120 mL, 1:1) at 0 °C was added DCC (2.10 g, 10.2 mmol) in one portion. The resulting mixture was stirred at room temperature for 5h. The formed DCU was filtered off and the filtrate concentrated under vacuum. The obtained residue was dissolved in ethyl acetate (300 mL), and the solution was washed with 5% aqueous NaHCO<sub>3</sub> (2 x 75 mL), water (75 mL), and brine (75 mL). The organic layer was separated, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. Recrystallization from CH<sub>2</sub>Cl<sub>2</sub> / pentane afforded **6** as white solid which was used without further purification (1.95 g, 10 mmol).

**<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):** δ 2.03 (1H, t, J = 2.4 Hz), 2.60 (2H, td, J = 2.4, 7.0 Hz), 2.83 (4H, s), 2.87 (2H, t, J = 7.0 Hz)

**<sup>13</sup>C NMR (68 MHz, CDCl<sub>3</sub>):** δ 14.1, 25.6, 30.3, 69.6, 80.3, 167.1, 169.0.

### Fmoc-Lys[*N*-4-Pentynoic acid]-OH **7**

To a stirred mixture of Fmoc-lysine,HCl salt (4.78 g, 13 mmol) and DIPEA (3.4 mL, 20 mmol) in dimethylformamide (50 mL) at room temperature was added dropwise over 10 min a solution of **6** (1.95 g, 10 mmol) in dimethylformamide (20 mL). The pH of the resulting mixture was regularly adjusted to pH 8-9 by further additions of DIPEA. After 2 h of reaction, the reaction mixture was concentrated under vacuum providing an oily residue. After addition of ethyl acetate (50 mL), the organic phase was washed with a concentrated citric acid solution (2 x 25 mL), water (25 mL), and brine (25 mL). The organic layer was separated, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. Precipitation from CH<sub>2</sub>Cl<sub>2</sub> / pentane afforded the building block **7** as a white solid which was used without further purification (4 g, 9 mmol).

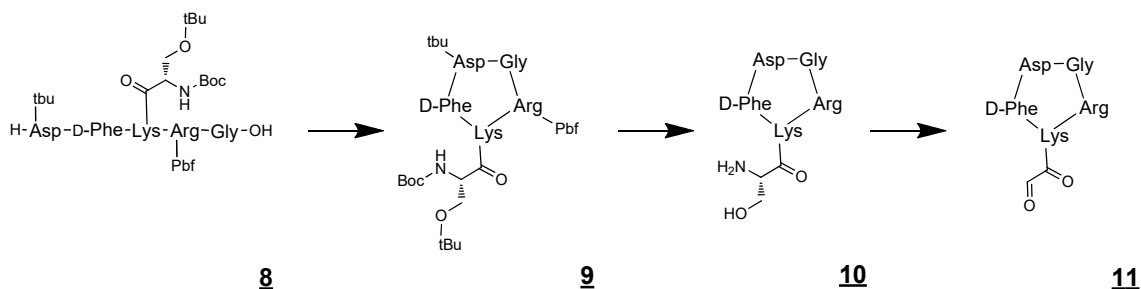
**<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):** δ 7.75 (2H, d, J = 7.4 Hz), 7.60 (2H, d, J = 7.4 Hz), 7.38 (2H, t, J = 7.4 Hz), 7.29 (2H, t, J = 7.4 Hz), 6.0 (1H, t, J = 5.6 Hz), 5.70 (1H, d, J = 7.8 Hz), 4.38-4.36 (3H, m), 4.12 (1H, t, J = 6.9 Hz), 3.19 (2H, m), 2.42 (2H, t, J = 7.0 Hz), 2.30 (2H, dt, J = 2.3, 7.0 Hz), 1.92 (1H, t, J = 2.4 Hz), 1.96 (3H, s), 1.81 (2H, m), 1.58 (2H, m), 1.45 (2H, m), 1.24 (3H, t, J = 7.1 Hz)

**<sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz):** δ 173.9, 170.0, 156.1, 143.8, 143.7, 140.6, 127.6, 127.0, 125.2, 120.0, 83.7, 71.2, 65.6, 53.7, 46.6, 38.2, 34.2, 30.4, 28.6, 23.0, 14.2

**MS (ESI-MS, positive mode):** C<sub>26</sub>H<sub>28</sub>N<sub>2</sub>O<sub>5</sub>, Calcd MW = 448.5 g.mol<sup>-1</sup>, Found MW = 449.1 g.mol<sup>-1</sup>

## Synthesis of ligands

### Synthesis of c[-RGDfK(ald)-] **11**



#### **H-D(*t*Bu)-f-K(BocS(*t*Bu))-R(Pbf)-G-OH **8****

The linear peptide **14** was assembled on 2-chlorotritylchloride<sup>®</sup> resin (1g) using the general procedure and modified amino acid **2**. The anchoring of the first amino acid (Fmoc-Gly-OH) was performed following the standard procedure yielding a convenient resin loading of 0.9 mmol/g. The peptide was released from the resin using a TFA/CH<sub>2</sub>Cl<sub>2</sub> (1/99) cleavage solution. The linear protected peptide was obtained as a white solid powder after precipitation, triturating and washing with diethyl ether (1.02 g, 0.86 mmol).

**RP-HPLC:** RT = 15.9 min (C18, 214 nm, 5-100% B in 20 min)

**MS (ESI-MS, positive mode):** C<sub>56</sub>H<sub>88</sub>N<sub>10</sub>O<sub>15</sub>S, Calcd MW = 1173.5 g.mol<sup>-1</sup>, Found MW = 1173.1 g.mol<sup>-1</sup>

#### **c[-R(Pbf)-G-D(*t*Bu)-f-K(BocS(*t*Bu))-] **9****

The cyclization reaction was carried out as described in the general procedure using crude linear peptide **14** (1.02 g, 0.86 mmol). Precipitation from Et<sub>2</sub>O afforded the cyclic peptide **15** as a white powder (1.03 g, 0.86 mmol). This crude material was used without further purification.

**RP-HPLC:** RT = 16.9 min (C18, 214 nm, 5-100% B in 20 min)

**MS (ESI-MS, positive mode):** C<sub>56</sub>H<sub>86</sub>N<sub>10</sub>O<sub>14</sub>S, Calcd MW = 1155.4 g.mol<sup>-1</sup>, Found MW = 1155.2 g.mol<sup>-1</sup>

#### **c[-R-G-D-f-K(S)-] **10****

Cyclopeptide **15** (1.03 g, 0.86 mmol). was dissolved in 30 mL of a TFA/TIS/H<sub>2</sub>O (95/2.5/2.5) solution. The reaction mixture was stirred for 3 h at room temperature. The product was concentrated under reduced pressure and the precipitation with Et<sub>2</sub>O afforded peptide **16** as a white solid powder (630 mg, 0.86 mmol). This crude material was used without further purification.

**RP-HPLC:** RT = 7,7 min (C18, 214 nm, 5-100% B in 20 min)

**MS (ESI-MS, positive mode):** C<sub>30</sub>H<sub>46</sub>N<sub>10</sub>O<sub>9</sub>, Calcd MW = 690.8 g.mol<sup>-1</sup>, Found MW = 690.5 g.mol<sup>-1</sup>

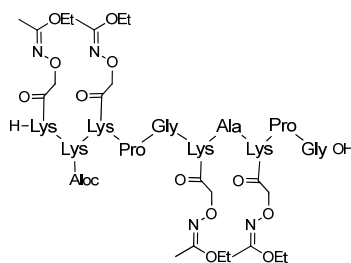
#### **c[-R-G-D-f-K(COCHO)-] **11****

Peptide **16** (100 mg, 144 μmol) and 10 equiv of NaIO<sub>4</sub> (310 mg, 1.44 mmol) were dissolved in water at 10<sup>-2</sup>M. The mixture was stirred for 30 min at room temperature and the product was directly purified by RP-HPLC affording pure aldehyde-containing cyclopeptide **17** as a white powder (55 mg, 83 μmol).

**RP-HPLC:** RT = 7.8 min (C18, 214 nm, 5-100% B in 20 min)

**MS (ESI-MS, positive mode):** C<sub>29</sub>H<sub>41</sub>N<sub>9</sub>O<sub>9</sub>, Calcd MW = 659.7 g.mol<sup>-1</sup>, Found MW = 659.4 g.mol<sup>-1</sup>

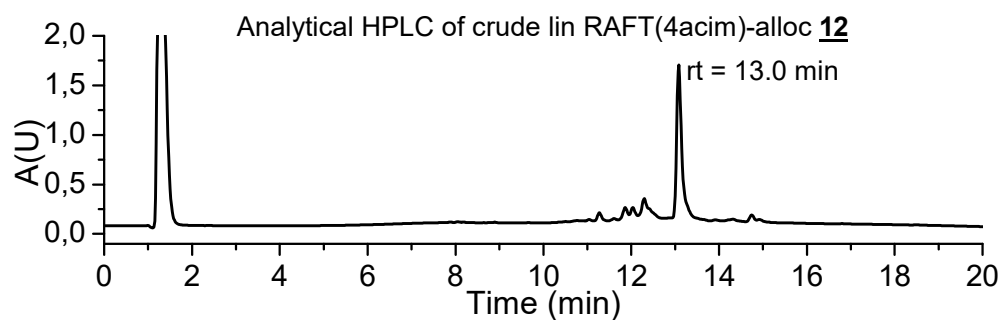
## Linear RAFT4(Acetimidate)-NHAlloc **12**



The linear decapeptide **12** was assembled on 2-chlorotritylchloride<sup>®</sup> resin (1.5 g) using the general procedure and modified amino acid **3**. 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 a AcOH/TFE/DCM (1/2/7) cleavage solution. The linear protected peptide was obtained as a light brown powder after precipitation, triturating and washing with diethyl ether (1.78 g, 1.05 mmol).

Yield: Quantitative

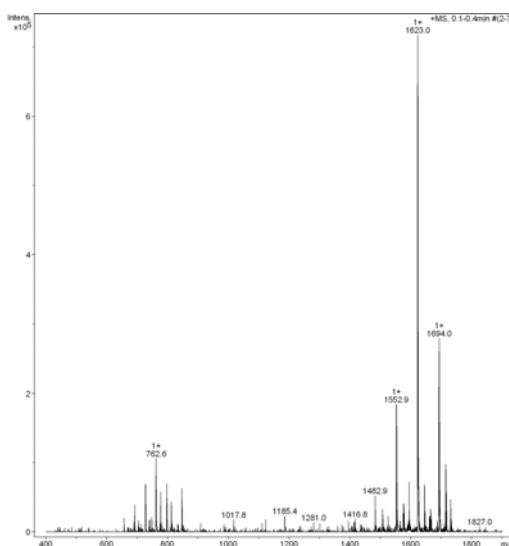
**RP-HPLC:** RT = 13.0 min (C18, 214 nm, 5-100% B in 20 min)



**MS (ESI-MS, positive mode):** C<sub>75</sub>H<sub>127</sub>N<sub>19</sub>O<sub>25</sub>

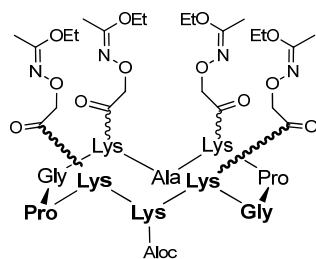
Calcd MW = 1694.9 g.mol<sup>-1</sup>

Found MW = 1695.7 g.mol<sup>-1</sup>





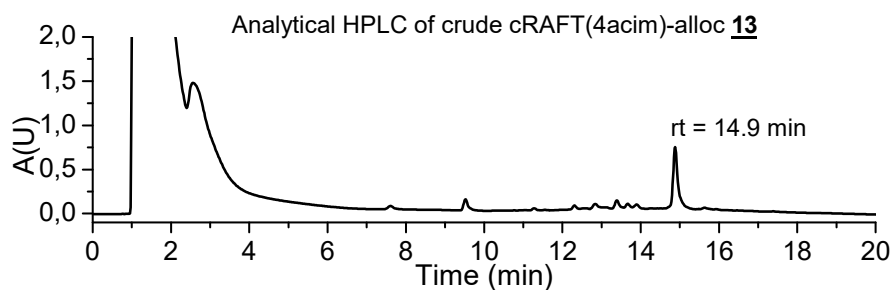
### cRAFT 4(Acetimidate)-NHAlloc **13**



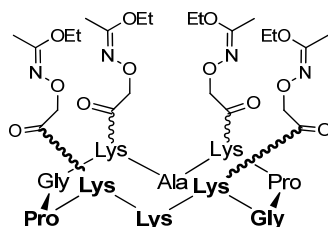
The cyclization reaction was carried out as described in the general procedure using the crude linear peptide **12** (1.78 g, 1.05 mmol). Precipitation from Et<sub>2</sub>O afforded cyclic peptide **13** as a light brown powder (1.76 g, 1.05 mmol). This crude material was used without further purification.

**Yield:** Quantitative

**RP-HPLC:** RT = 14.9 min (C18, 214 nm, 5-100% B in 20 min)



### cRAFT 4(Acetimidate)-NH<sub>2</sub> **14**

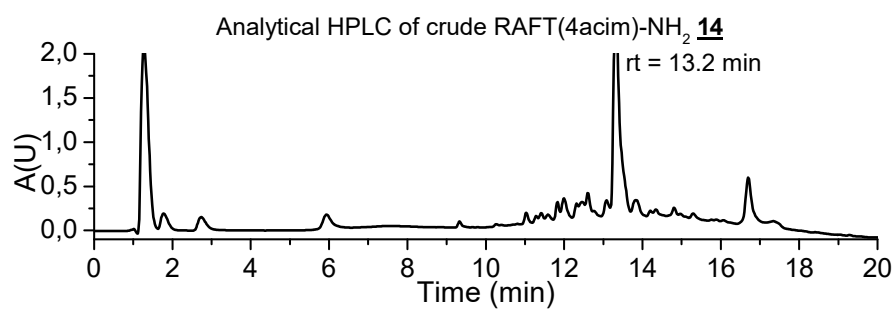


Peptide **13** (1.76 g, 1.05 mmol) was dissolved in 100 ml of anhydrous CH<sub>2</sub>Cl<sub>2</sub>/DMF (1/1) under argon. 50 equiv of phenylsilane and 0.2 equiv of Pd(PPh<sub>3</sub>)<sub>4</sub> were added under argon.

The reaction was stirred under argon for 30 min at room temperature. The mixture was treated with 6ml of methanol before solvent removal under reduced pressure. The deprotected peptide **14** was obtained as a brown powder after precipitation, triturating and washing with diethyl ether (1.57 g, 0.99 mmol).

**Yield: 94%**

**RP-HPLC:** RT = 13.2 min (C18, 214 nm, 5-100% B in 20 min)



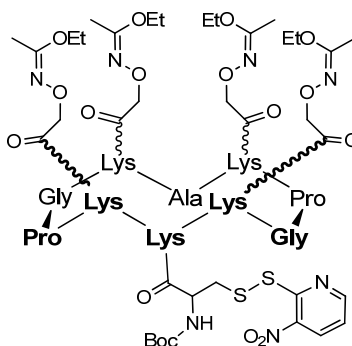
**MS (ESI-MS, positive mode):** C<sub>71</sub>H<sub>120</sub>N<sub>19</sub>O<sub>22</sub>

Calcd MW = 1591.8 g.mol<sup>-1</sup>

Found MW = 1591.9 g.mol<sup>-1</sup>



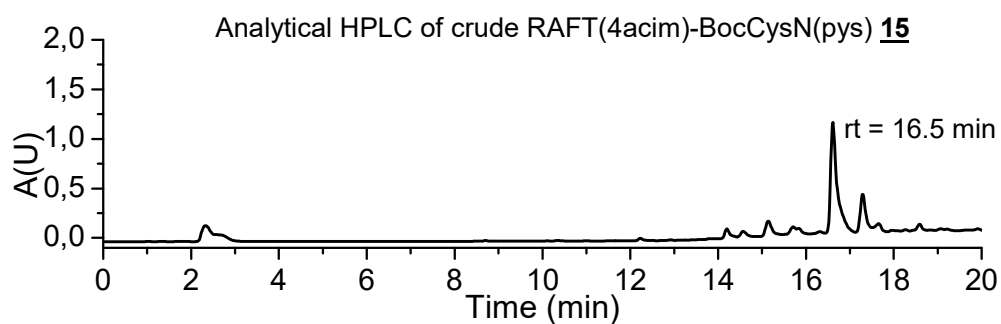
## cRAFT 4(Acetimidate)-NH-Boc-CysNpys **15**



Scaffold **14** (1.57 g, 0.99 mmol), 2 equiv of Boc-Cys(Npys)-OH (750 mg, 2 mmol) and 2 equiv of PyBop (1.04 g, 2 mmol) were dissolved in 5 mL of DMF. The pH of the solution was adjusted to 9 with DIPEA. The mixture was stirred for 2 h at room temperature. Peptide **15** was obtained as a dark brown powder after precipitation, triturating and washing with diethyl ether (1.85 g, 0.95 mmol).

**Yield: 96%**

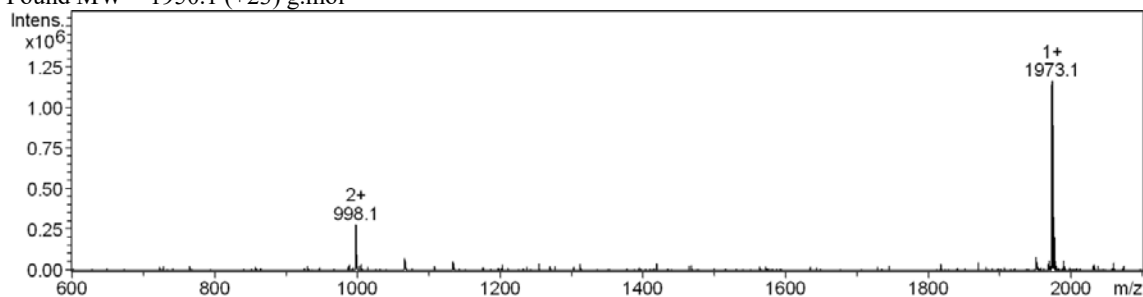
**RP-HPLC:** RT = 16.5 min (C18, 214 nm, 5-100% B in 20 min)



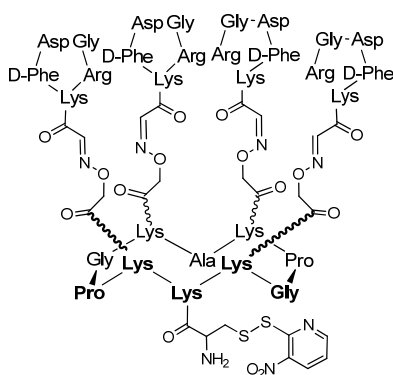
**MS (ESI-MS, positive mode):** C<sub>84</sub>H<sub>136</sub>N<sub>22</sub>O<sub>27</sub>S<sub>2</sub>

Calcd MW = 1950,2 g.mol<sup>-1</sup>

Found MW = 1950.1 (+23) g.mol<sup>-1</sup>



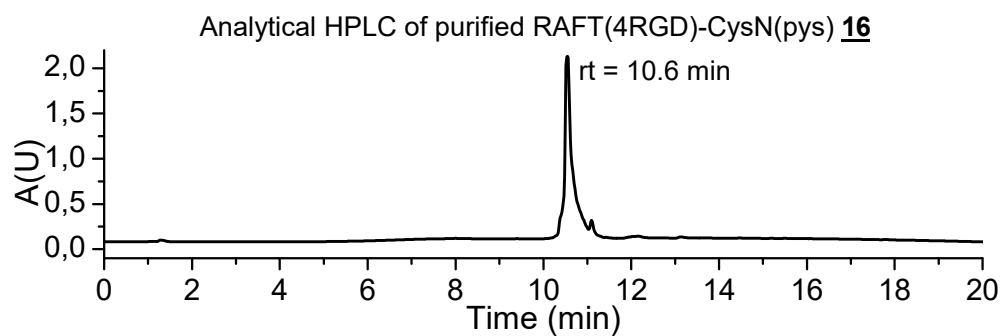
## cRAFT4(c[-RGDfK-])<sub>oxime</sub>)-NH-CysNpys **16**



Scaffold **15** (30 mg, 15.4  $\mu\text{mol}$ ) and 6 equiv of c[-RGDfK(COCHO)-] **11** (61 mg, 92.4  $\mu\text{mol}$ ) were dissolved in 2 mL of a TFA/H<sub>2</sub>O (7/3) solution. The mixture was stirred for 1 h and the product was purified by RP-HPLC affording pure conjugate **16** as a white powder (29.3 mg, 7.1  $\mu\text{mol}$ ).

**Yield: 46%**

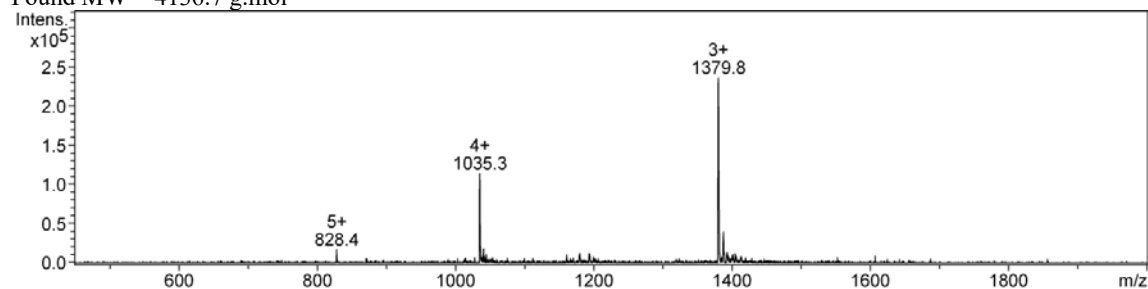
**RP-HPLC:** RT = 10.6 min (C18, 214 nm, 5-100% B in 20 min)



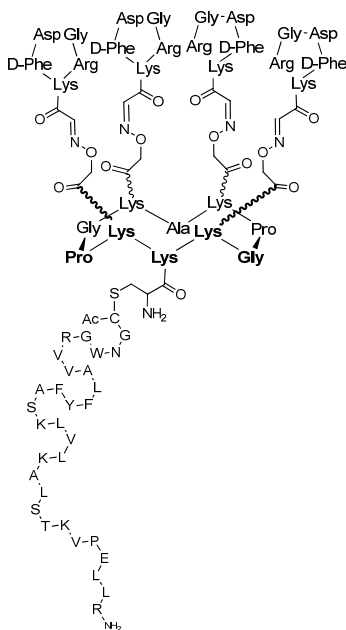
**MS (ESI-MS, positive mode):** C<sub>179</sub>H<sub>260</sub>N<sub>58</sub>O<sub>53</sub>S<sub>2</sub>

Calcd MW = 4136.5 g.mol<sup>-1</sup>

Found MW = 4136.7 g.mol<sup>-1</sup>



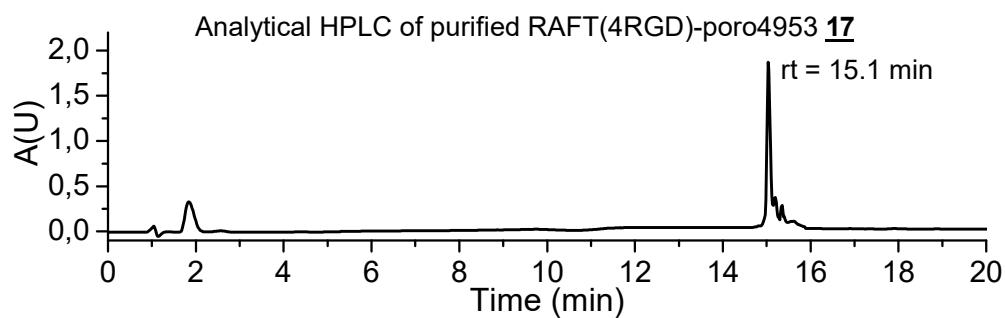
## cRAFT4(c[-RGDfK-]<sub>oxime</sub>)-NH-Cys-poro4953 **17**



Compound **16** (2 mg, 0.48  $\mu\text{mol}$ ) and JMV4953 (2 mg, 0.49  $\mu\text{mol}$ ) were dissolved in 500  $\mu\text{L}$  of DMF/PBS (pH 4.8) (3/1) under argon. The reaction mixture was stirred for 5 min at r.t under argon. The product was purified by RP-HPLC (5-100% B in 30 min) affording compound **17** as a white powder (50  $\mu\text{g}$ , 6.7 nmol).

**Yield:** <5%

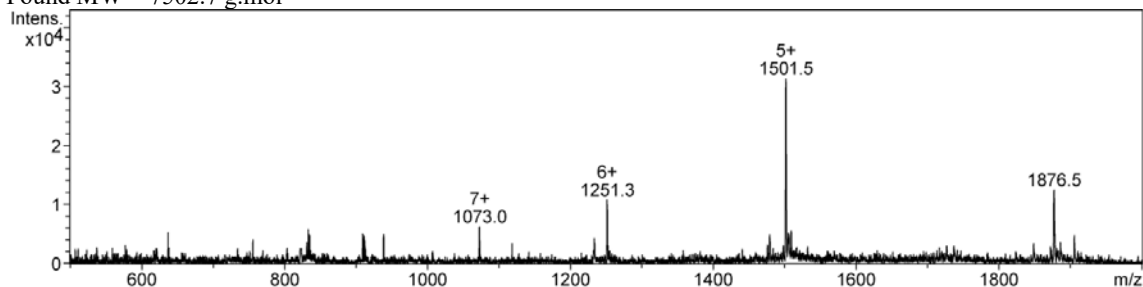
**RP-HPLC:** RT = 15.1 min (C18, 214 nm, 5-100% B in 20 min)



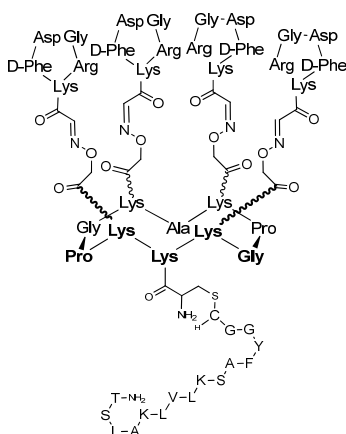
**MS (ESI-MS, positive mode):**  $\text{C}_{340}\text{H}_{523}\text{N}_{99}\text{O}_{90}\text{S}_2$

Calcd MW = 7501.7  $\text{g}\cdot\text{mol}^{-1}$

Found MW = 7502.7  $\text{g}\cdot\text{mol}^{-1}$



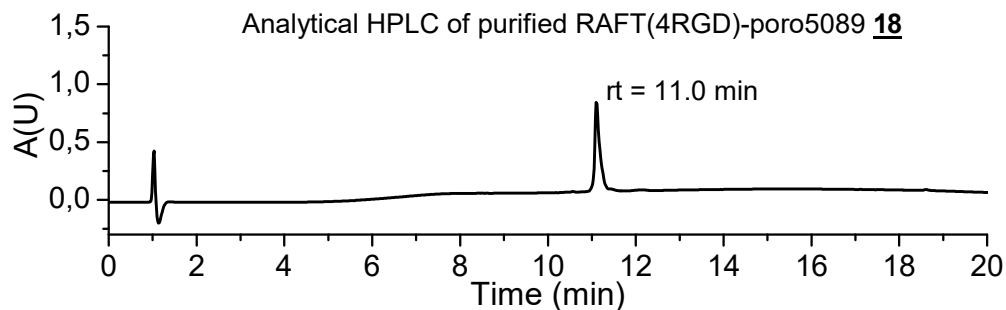
## cRAFT4(c[-RGDFK-]<sub>oxime</sub>)-NH-Cys-poro5089 **18**



Compound **16** (4 mg, 0.96  $\mu\text{mol}$ ) and JMV5089 (2.3 mg, 1.15  $\mu\text{mol}$ ) were dissolved in 500  $\mu\text{L}$  of DMF/PBS (pH 4.8) (3/1) under argon. The reaction mixture was stirred for 5 min at r.t under argon. The product was purified by RP-HPLC (5-100% B in 30 min) affording compound **18** as a white powder (2.1 mg, 0.37  $\mu\text{mol}$ ).

**Yield: 38%**

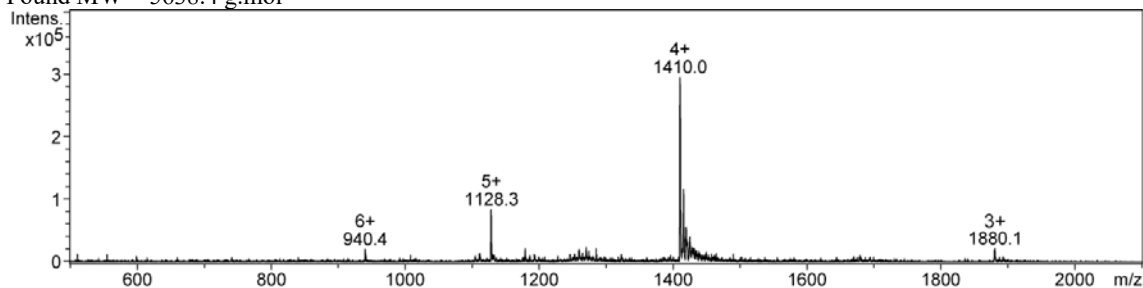
**RP-HPLC:** RT = 11.0 min (C18, 214 nm, 5-100% B in 20 min)



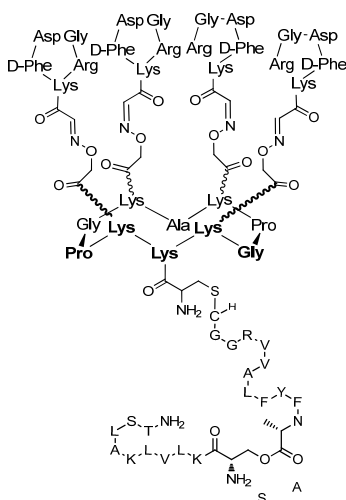
**MS (ESI-MS, positive mode):** C<sub>250</sub>H<sub>381</sub>N<sub>75</sub>O<sub>71</sub>S<sub>2</sub>

Calcd MW = 5637.4 g.mol<sup>-1</sup>

Found MW = 5638.4 g.mol<sup>-1</sup>



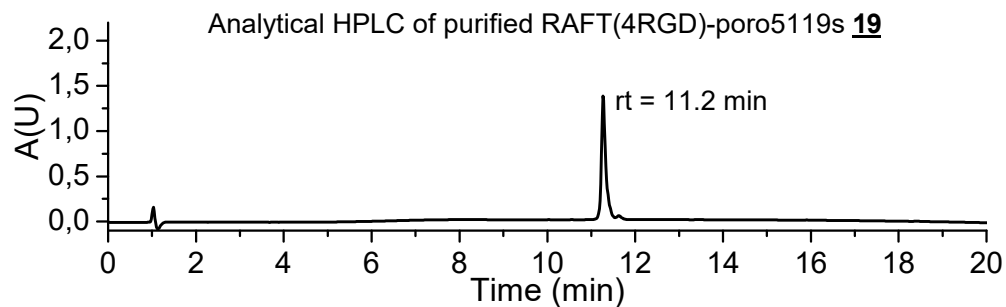
## cRAFT4(c[-RGDFK-]<sub>oxime</sub>)-NH-Cys-poro5119s 19



Compound 16 (10 mg, 2.42  $\mu\text{mol}$ ) and JMV5119 (6.8 mg, 2.43  $\mu\text{mol}$ ) were dissolved in 500  $\mu\text{L}$  of ACN/PBS (pH 4.8) (1/3) under argon. The reaction mixture was stirred for 5 min at r.t under argon. The product was purified by RP-HPLC (5-100% B in 30 min) affording compound 19 as a white powder (8.4 mg, 1.33  $\mu\text{mol}$ ).

**Yield: 55%**

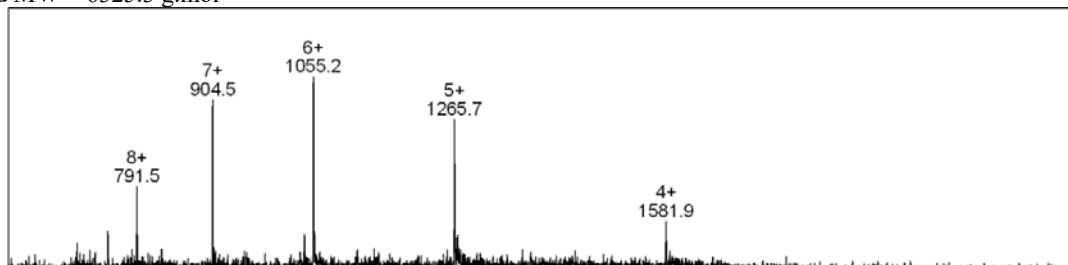
**RP-HPLC:** RT = 11.2 min (C18, 214 nm, 5-100% B in 20 min)



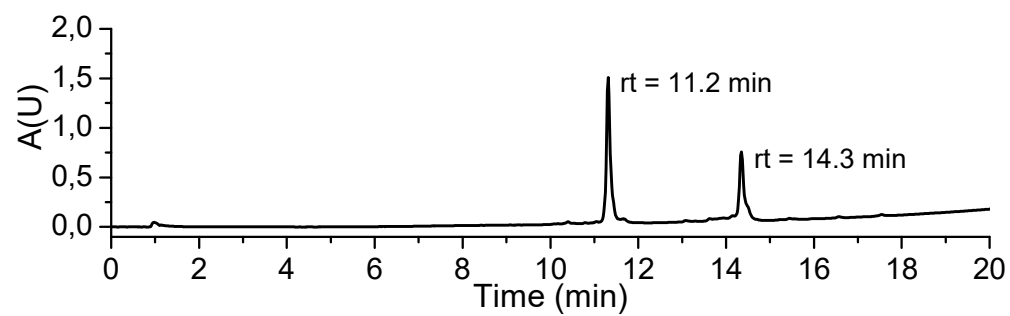
**MS (ESI-MS, positive mode):**  $\text{C}_{284}\text{H}_{436}\text{N}_{84}\text{O}_{77}\text{S}_2$

Calcd MW = 6323,3  $\text{g}\cdot\text{mol}^{-1}$

Found MW = 6325.3  $\text{g}\cdot\text{mol}^{-1}$



**cRAFT4(c[-RGDfK-]<sub>oxime</sub>)-NH-Cys-poro5119 20 : helicity recovery**



Compound 19 (1 mg, 0.16  $\mu\text{mol}$ ) was dissolved in 500  $\mu\text{L}$  of ACN/PBS (pH 7/2) (1/3). The reaction mixture was stirred for 5 min at r.t. The product was then analysed by HPLC (5-100% B in 20 min).