Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Supplementary Information

Application of click-click chemistry to the synthesis of new multivalent RGD conjugates

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General procedures

Materials

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% H_2O; 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.

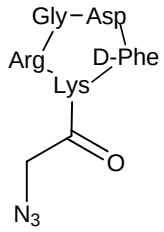
Solid-Phase Peptide Synthesis

Assembly of all protected peptides was carried out using the Fmoc/t-Bu strategy manually in a glass reaction vessel fitted with a sintered glass frit or automatically on a peptide synthesizer using 2-chlorotritylchloride. Coupling reactions were performed manually by using 2 equiv of N-Fmoc-protected amino acid (relative to the resin loading) activated in situ with 2 equiv of PyBOP and 3-5 equiv of diisopropylethylamine (DIPEA) in DMF (10 mL/g resin) for 30 min except for the first coupling on 2-chlorotritylchloride. Coupling reactions carried out on the synthesizer were performed twice. The coupling efficiency in manual synthesis was assessed by Kaiser and/or TNBS tests. N-Fmoc protecting groups were removed by treatment with a piperidine/DMF solution (1:4) for 10 min (10 mL/g resin). The process was repeated three times and the completeness of deprotection verified by UV absorption of the piperidine washings at 299 nm. Synthetic linear peptides were recovered directly upon acid cleavage. Before cleavage, the resin was washed thoroughly with methylene chloride. The linear peptides were then released from the resin by treatments with a solution of acetic acid/trifluoroethanol/methylene chloride (1:1:8, 10 mL/mg resin, 2x30 min). Hexane (5-10 volumes) was added to the collected filtrates, and the crude peptides were isolated after concentration as white solids. The residue was dissolved in the minimum of methylene chloride and diethyl ether was added to precipitate peptides. Then, they were triturated and washed three times with diethyl ether to obtain crude materials that were used in the next step without further purification.

Cyclization Reaction

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

Synthesis of *c*[-RGDfK($COCH_2N_3$)-] 9

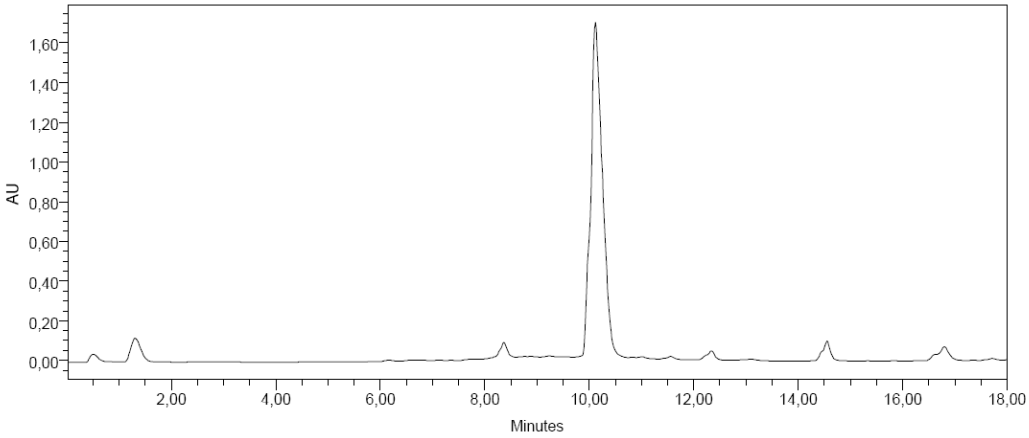


The linear peptide H-Asp(tBu)-D-Phe-Lys-(alloc)-Arg(Pmc)-G-OH was assembled on 2-chlorotritylchloride® (450 mg) using general procedure. The resin was loaded for 30 min in dry CH_2Cl_2 at 0.8 mmol/g using Fmoc-glycine, capped for 5 min with 10 mL of methanol/DIPEA/methylene chloride (2:1:17) and then one more time 10 min. Fmoc group removals, coupling reactions, and cleavage of the peptide from the support were performed as described in the general procedure. The linear pentapeptide was then obtained as a white powder (353 mg, 0.35 mmol, quantitative yield)
The cyclization reaction was carried out using the crude peptide as described above affording the cyclic peptide as a white powder (353 mg, 0.35 mmol, quantitative yield).
Alloc group was removed using the crude cyclic peptide dissolved in a solution containing 40 mL of dry CH_2Cl_2 and DMF (3:1) under argon by adding phenylsilane (100 equiv) for 3 min and then $Pd^0(PPh_3)_4$ (0.2 equiv) for 30 min at room temperature. The solvent was removed under reduced pressure. The oily residue was dissolved in the minimum of a solution containing a mixture of CH_2Cl_2 and methanol (1:1). Ether was added to precipitate the crude product. Then it was triturated and washed 3 times with ether affording the compound as a white powder (266 mg, 0.28 mmol, 82%).[1]
2-azidoacetic acid[2] (30.3 mg, 0.3 mmol) and PyBOP (156 mg, 0.3 mmol) were added to a solution containing the crude peptide (138 mg, 0.15 mmol) in 30 mL of DMF and DIPEA to adjust the pH at 8.0. The reaction was stirred for 30 min at room temperature and then concentrated under diminished pressure. The crude product was triturated and washed with ether to yield the compound as a white powder (145 mg, 0.15 mmol, 96%). ESI-MS calc for $C_{47}H_{68}N_{12}O_{11}S$ 1009.2, found 1009.4.
Full deprotection was carried out on crude material (145 mg, 0.15mmol) using 5 mL of a solution containing TFA/H_2O/TIS (95:2.5:2.5) at room temperature during 5 h. The product was isolated by RP-HPLC to yield compound **9** as a white powder (70 mg, 0.1 mmol, overall yield 55%). HPLC t_R = 5.9 min; ESI-MS calc for $C_{29}H_{42}N_{12}O_8$ 686.7, found 686.3.

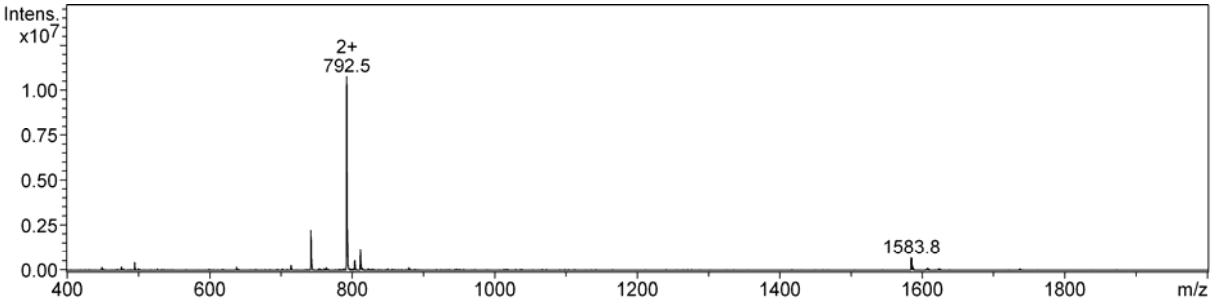
[1] D. Boturyn, P. Dumy , *Tetrahedron Lett.* **2001**, *42*, 2787.
[2] I. Akritopoulou-Zanze, V. Gracias, S. W. Djuric, *Tetrahedron Lett.* **2004**, 45, 8439.

RP-HPLC profile and ESI-MS analysis of 5

RP-HPLC profile of **5** monitored at 214 nm

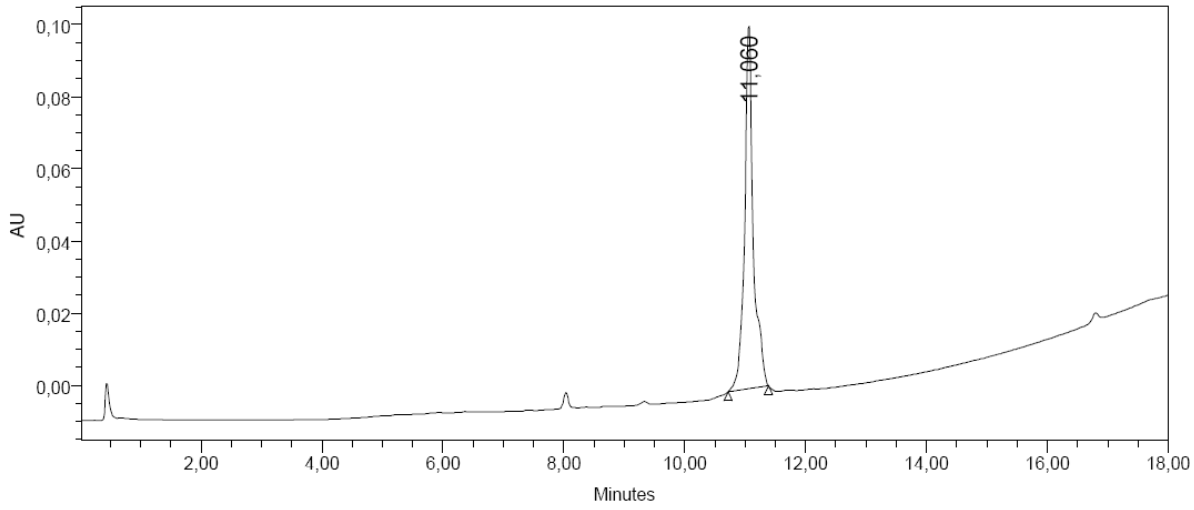


ESI-MS analysis of compound **5**

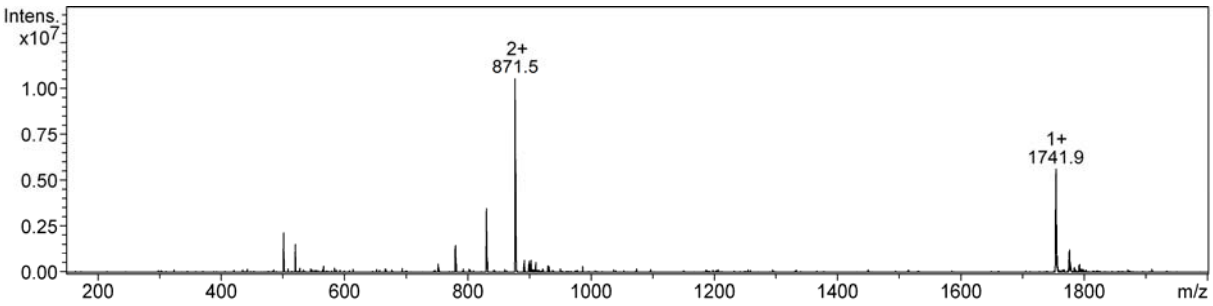


RP-HPLC profile and ESI-MS analysis of 6

RP-HPLC profile of **6** monitored at 214 nm

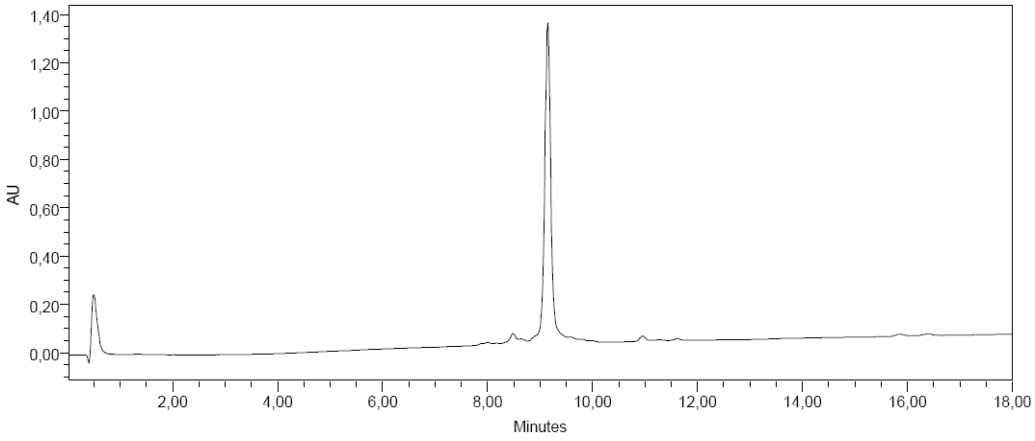


ESI-MS analysis of compound **6**

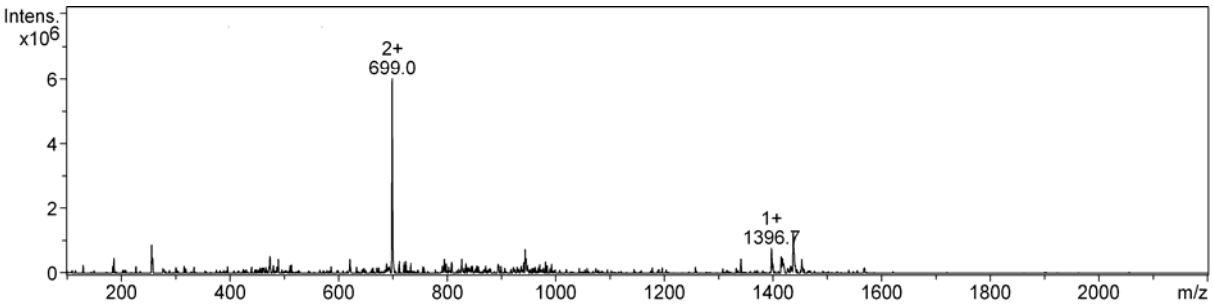


RP-HPLC profile and ESI-MS analysis of 7

RP-HPLC profile of **7** monitored at 214 nm

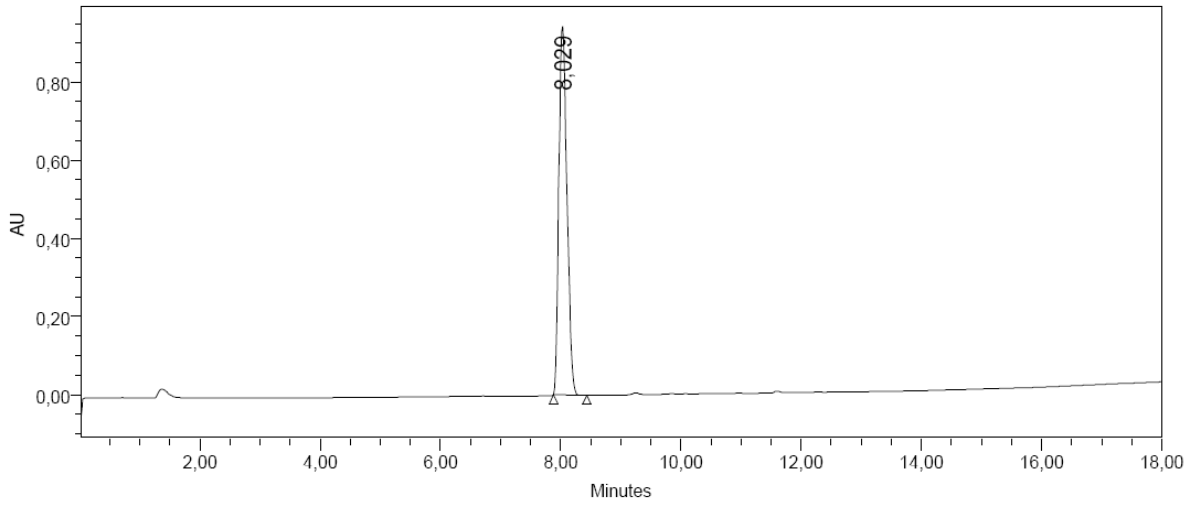


ESI-MS analysis of compound **7**

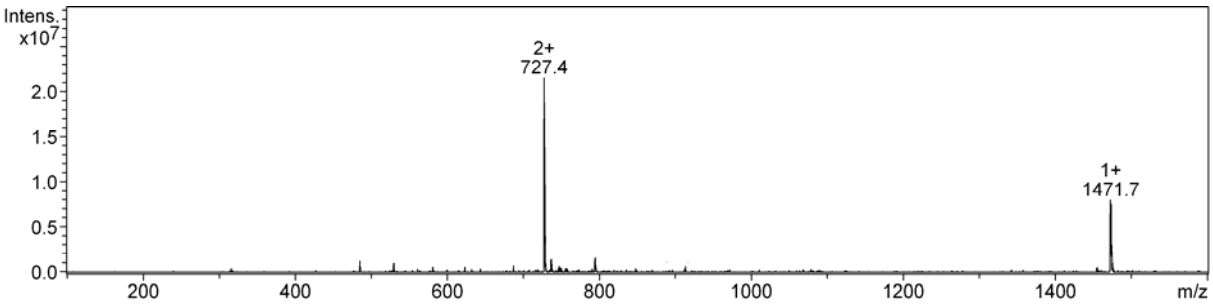


RP-HPLC profile and ESI-MS analysis of 8

RP-HPLC profile of **8** monitored at 214 nm

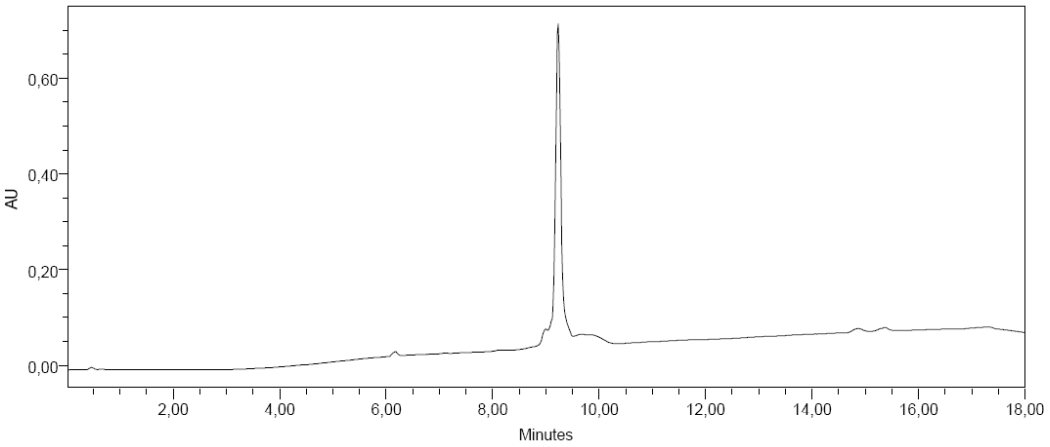


ESI-MS analysis of compound **8**

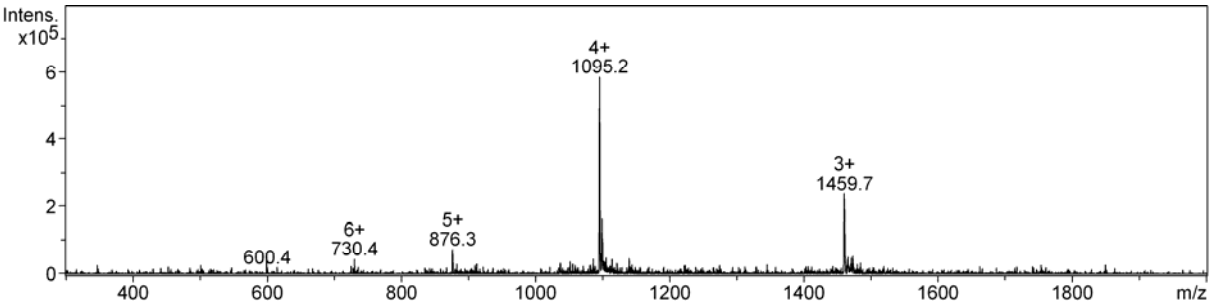


RP-HPLC profile and ESI-MS analysis of 11

RP-HPLC profile of **11** monitored at 214 nm

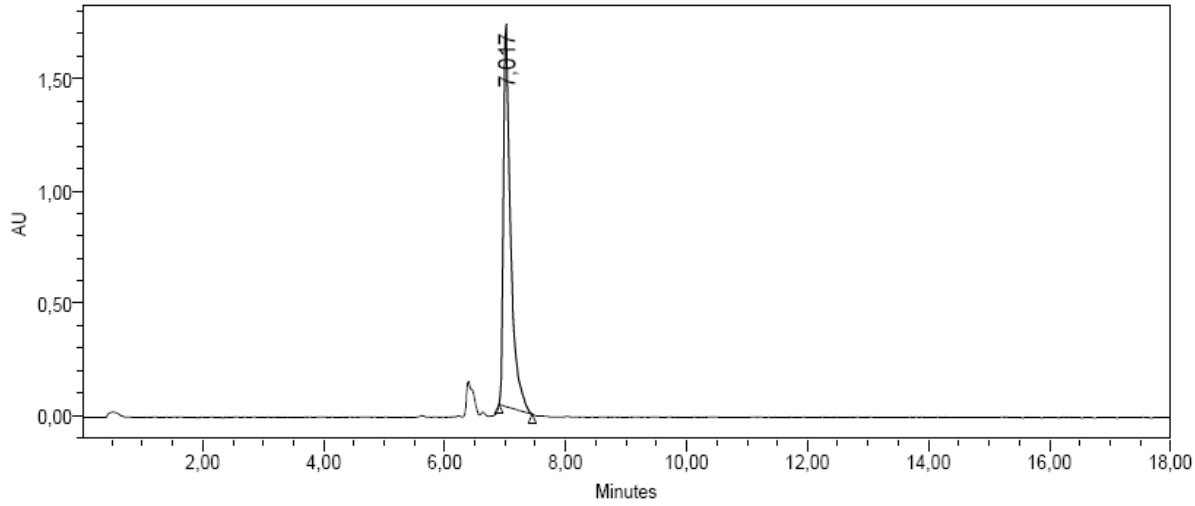


ESI-MS analysis of compound **11**

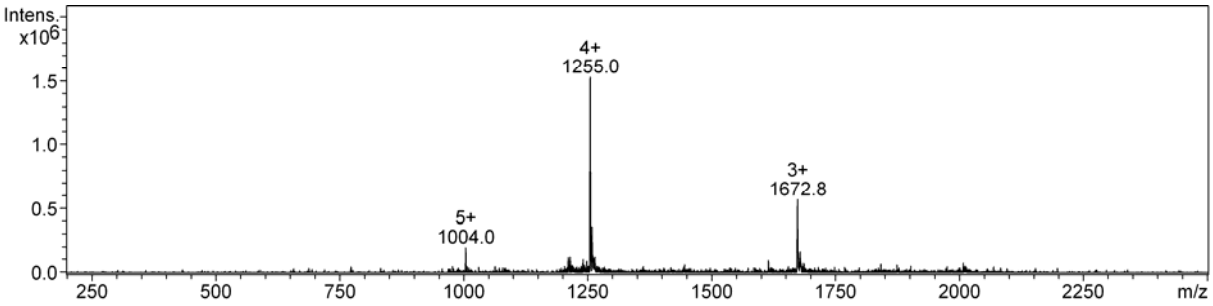


RP-HPLC profile and ESI-MS analysis of 1

RP-HPLC profile of **1** monitored at 214 nm

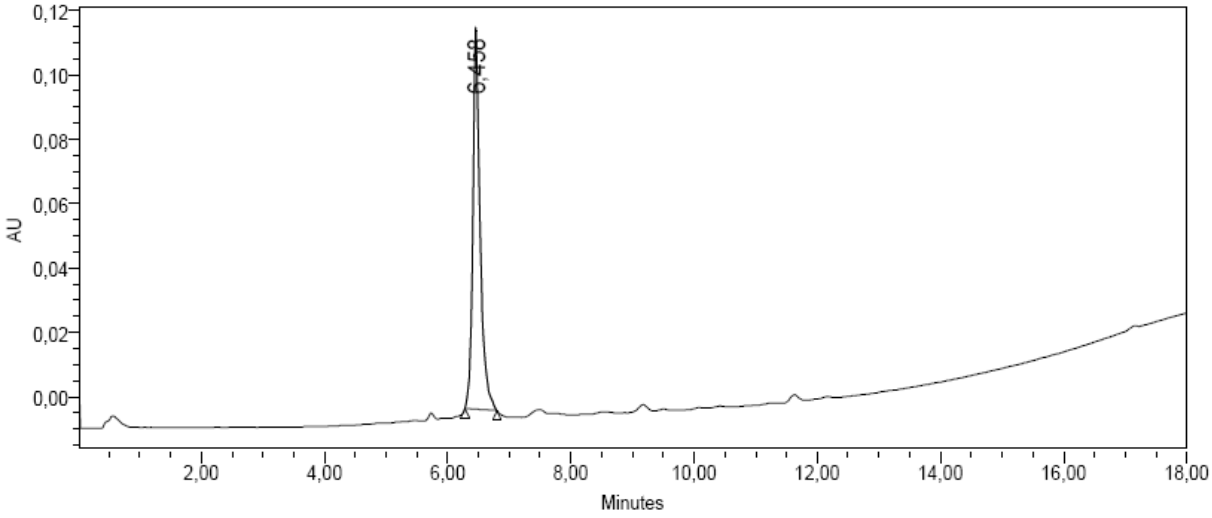


ESI-MS analysis of compound **1**

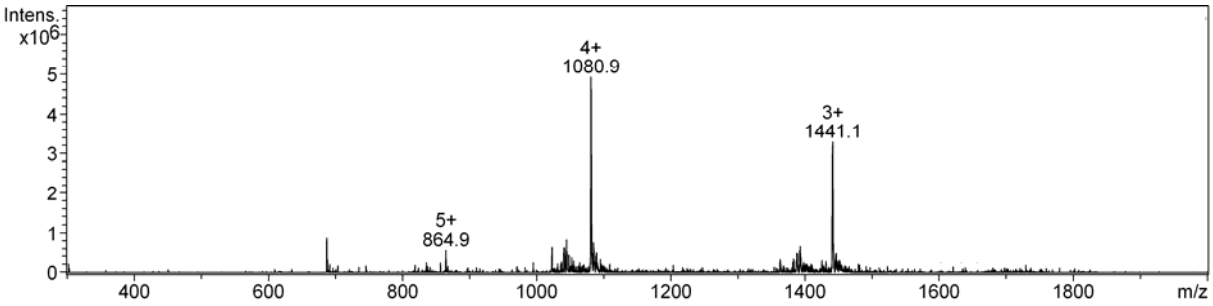


RP-HPLC profile and ESI-MS analysis of 2

RP-HPLC profile of **2** monitored at 214 nm

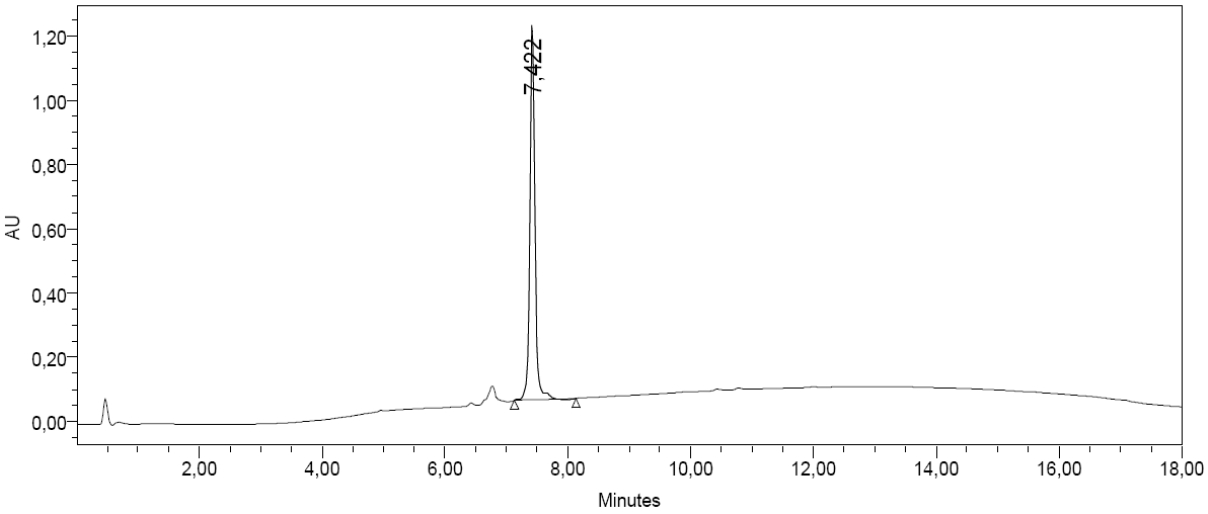


ESI-MS analysis of compound **2**

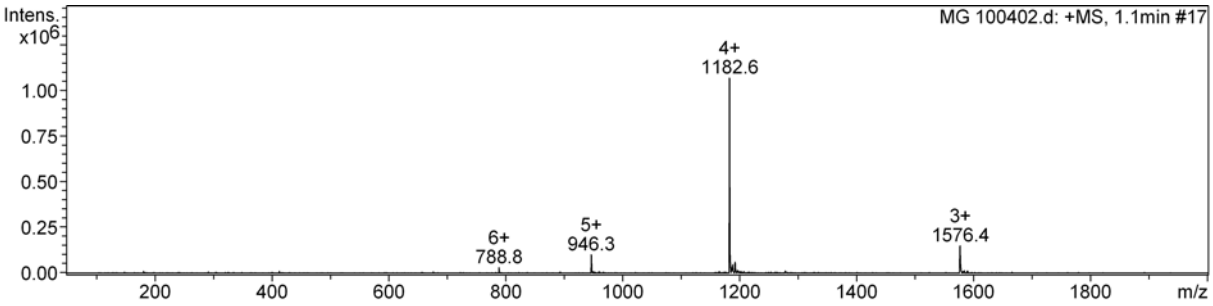


RP-HPLC profile and ESI-MS analysis of 19

RP-HPLC profile of **19** monitored at 214 nm



ESI-MS analysis of compound **19**



General biological procedures

Cell lines and culture conditions

HEK-β_3 cells, stable transfectants of human β_3 subunit from the human embryonic kidney 293 cell line, were kindly supplied by J.-F. Gourvest (Aventis, France). They were cultured in DMEM-GlutaMAX^{TM} (Gibco, Invitrogen, Cergy Pontoise, France), enriched with 4.5 g.L^{-1} glucose and supplemented with 10 % FBS, penicillin (50 U.mL^{-1}), streptomycin (50 µg.mL^{-1}) and 700 µg.mL^{-1} G418. Cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2.

Competitive cell adhesion assays

Competitive assay was realized as described.[3] Briefly, 96-well assay plates (Falcon, Becton Dickinson, France) were coated for 1 h at room temperature with 5 µg.mL^{-1} vitronectin in PBS and blocked for 30 min with 3 % bovine serum albumin (BSA). Varying amounts of peptides were added simultaneously with 10^5 trypsinated HEK-β_3 cells to the wells and the plate was incubated for 30min at 37°C. Wells were rinsed three times with cold PBS to remove vitronectin-unbound cells. Attached cells were then fixed with methanol, stained with methylene blue and quantified by OD reading at 630nm on Dynatech MR5000 plate reader. The activity of peptides is expressed as IC_{50} values (concentration of peptide necessary to inhibit 50 % of cell attachment to the vitronectin substrate) and determinates from triplicates in three separate experiments.

Fluorescence Reflectance Imaging (2D-FRI)

Female NMRI *nude* mice (8-10 weeks old, n=6, Janvier, Le Genest-Saint-Isle, France) were injected subcutaneously with human TS/A-pc cells ($1x10^6$ cells per mouse). After tumor growth (~10 days), anesthetized mice (isoflurane/oxygen 3.5/4% for induction and 1.5/2 % thereafter, CSP, Cournon, France) were injected intravenously with 10 nmol of Cy5-peptide. Mice were illuminated by 633-nm light-emitting diodes equipped with interference filters. Fluorescence images as well as black and white pictures were acquired during 100 ms by a back-thinned CCD camera at -80°C (ORCAII-BT-512G, Hamamatsu, Massy, France) (Josserand et al., 2007) fit with a colored glass long-pass RG 665 filter (Melles Griot, Voisins Le Bretonneaux, France). Images were adjusted between 1700-39000 RLU. At the end of the experiment, mice were euthanized to quantify the biodistribution in the different organs. Images of the organs were adjusted between 10000-65535 RLU, with an exposition time of 100 ms.

[3] E. Garanger, D. Boturyn, J.-L. Coll, M.-C. Favrot and P. Dumy, *Org. Biomol. Chem.* **2006**, *4*, 1958.