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Supporting Information

An innovative approach for the synthesis of dual modality peptide imaging probes based on the Native Chemical Ligation

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Contents

I. Abbreviations

Fmoc (9-Fluorenylmethoxycarbonyl), AA (Amino acid), PyBOP (Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate), DMAP (4-dimethylaminopyridine), HATU (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide exafluorophosphate), DIC (N,N′-Diisopropylcarbodiimide), RP-HPLC (Reverse Phase High Performance Liquid Chromatography), UPLC (Ultra Performance Liquid Chromatography), MS (Mass Spectrometry), NMR (Nuclear Magnetic Resonance), DCM (methylene chloride), DMF (dimethylformamide), NMP (N-methyl-2-pyrrolidone), (tBu)$_4$-AAZTA-C4-COOH (6-[Bis[2-(1,1-dimethylethoxy)-2-oxoethyl]amino]-6-(5-carboxypentyl)tetrahydro-1H-1,4-diazepine-1,4(5H)-Diacetic acid α,α'-bis(1,1-dimethylethyl)ester), TIS (triisopropylsilane), DIPEA (N,N-diisopropylethylamine), TFA (trifluoroacetic acid), CH_3CN (acetonitrile), ESI-MS (Electron Spray ionization Mass Spectrometry), TCEP (Tris(2-carboxyethyl)phosphine hydrochloride), MEM (Minimun Essential Medium), MESNA (2-Mercaptoethanesulfonic acid sodium salt), NCL (Native Chemical Ligation), EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride). PET (positron emission tomography), OI (optical imaging). tBu (*tert*-butyl), Boc (*tert*-butyloxycarbonyl) Pbf (2,2,4,6,7-pentamethyIdlhydrobenzofuran-5-sulfonyl).

II. Materials and methods

Chemical Synthesis

All Fmoc protected amino acids, preloaded Wang Resin with Fmoc-Ser(*t*Bu)-OH and PyBOP were purchased by Novabiochem (Darmstad, Germany), Sigma Aldrich (Darmstad, Germany) and Iris Biotech (Marktredwitz, Germany). Fmoc-Asp-OAll was purchased by Fluka. Wang ChemMatrix® resin was purchased by Biotage (Uppsala, Sweden). Sodium acetate, acetic acid and anhydrous $GaCl_3$ were purchased by Sigma Aldrich (Darmstad, Germany). Cyanine5.5 maleimide was purchased by Lumiprobe GmbH (Hannover, Germany). All other reagents were purchased by Sigma Aldrich (Darmstad, Germany). All solvents were purchased by VWR International (Radnor, USA) and were used without further purifications. 6-[Bis[2-(1,1-dimethylethoxy)-2-oxoethyl]amino]-6-(5-carboxypentyl)tetrahydro-1H-1,4-diazepine-1,4(5H)-Diacetic acid α,α'-bis(1,1-dimethylethyl)ester ($(tBu)_4$-AAZTA-C4-COOH) was synthesized in according with Manzoni et al protocol.[1]

NMR spectra were recorded at 298 K on a Bruker AVANCE 600 spectrometer. CD_3OD and NMR tube were purchased from Sigma Aldrich.

Mass spectra with electrospray ionization (ESI) were recorded on a SQD 3100 Mass Detector (Waters) and on an LC-MS system Agilent 1200 Infinity Series (Agilent Technologies). The HPLC-MS analytical and preparative purifications were carried out on a Waters AutoPurification system (3100 Mass Detector 600 Quaternary Pump Gradient Module, 2767 Sample Manager and 2487 UV/Visible Detector) and on a HP 1200 Series (Agilent Technologies). UPLC-MS analyses were performed using a Waters Acquity UPLC *H*-Class coupled with and ESI source, a quadrupole (QDa) mass analyzer and dual-wavelength UV/Vis TUV Detector. High resolution mass analysis was performed on an Agilent 1200 Infinity Series (Agilent Technologies) equipped with ESI source and a ToF analyzer.

AE105-Cys linear peptide was synthesized automatically with a CEM Microwave Peptide Liberty Synthetizer.

UV-Vis spectra were acquired by a 6715 UV-Vis spectrophotometer (JENWAY) in the spectral range 500-800 nm using rectangular quartz cells having an optical path length of 1 cm (Hellma Analytics).

Cell culture and flow cytometry

U-87 MG human glioblastoma cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in MEM modified medium (Euroclone, Milan, Italy) supplemented with 1% glutamine, 1% sodium pyruvate, 1% Non-Essential Aminoacids, 100U/mL penicillin, 100 U/mL streptomycin and 10% (v/v) fetal bovine serum (FBS) at 310 K in humidified atmosphere containing 5% of CO_2.

In vitro binding evaluations were performed by Guava® easyCyte 8 Benchtop Flow Cytometer (Merck Millipore, Burlington, Massachusetts, USA) using RED2 filter (661/19 nm). Fluorescent profiles and binding percentages of the cell populations were obtained and analyzed using InCyte Sofware (Merck Millipore, Burlington, Massachusetts, USA).

III. Experimental synthesis procedures and analytical characterizations

Cys-AE105 (1)

The immobilized linear peptide H-Cys(trt)-Asp(*t*Bu)-Cha-Phe-D-Ser(*t*Bu)-D-Arg(Pbf)-Tyr(*t*Bu)-Leu-Trp(Boc)-Ser(*t*Bu)-Wang resin was synthetized automatically with the support of microwaves (MW) on a Wang Resin preloaded with H-Ser(*t*Bu)-OH (156 mg, 0.64 mmol/g) by standard Fmoc protocol. Cleavage of Fmoc group was achieved with 20% piperidine in DMF. Each coupling reaction was performed adding to the reaction vessel 5 equivalents of the Fmoc protected amino acid, 10 equivalents of DIPEA and 5 equivalents of the activator agent (PyBOP). The peptide was cleaved from the solid support by addition of TFA/Phenol/H_2O/TIS (88:5:2:5) overnight at room temperature. Final purification was achieved by preparative RP-HPLC by employing an Atlantis prepD® C18OBD 5μm (19X100 mm) column. Eluent: (A) 0.1% TFA in H_2O, (B) 0.1% TFA in CH_3CN. Gradient profile; isocratic at 20% of B for 8.52 min, linear gradient from 20% to 40% of B in 8.48 min, linear gradient from 40% to 100% in 2.9 min, isocratic at 100% for 1.1 minutes. Flow rate; 15 mL/min. Cys-AE105 was isolated as a homogenous peak with a retention time of ab. 18 minutes. The solvent was removed *in vacuo* and the product lyophilized from water to give **1** as a white solid (39 mg, 29%). The purity of the product was checked by analytical UPLC-MS by employing an ACQUITY UPLC® Peptide BEH C18 column (300Å, 1.7μm, 2.1X100mm). Eluent: (A) 0.05% TFA in H_2O, (B) 0.05% TFA in CH_3CN. Gradient profile; linear gradient from 5% to 50% of B in 7 min, linear gradient from 50% to 100% in 3 min, isocratic at 100% for 3 minutes. Flow rate of 0.4 mL/min and UV detection at 210 nm. Purity 98%. ESI-MS (*m/z*): calcd: For $C_{67}H_{91}N_{13}O_{21}S_2$ $(M+2H)^{2+}$ 665.37 found: 665.73.

*cyclo*RGDfK(C) (2)

Synthesis of the linear peptide. The linear peptide *H*-D-Phe-Lys(Mtt)-Arg(Pbf)-Gly-Asp-*O-All* was synthesized manually on a Wang ChemMatrix® resin (loading: 0.39 mmol/g) using Fmoc chemistry. The C-terminal Asp residue was loaded on the resin through the side chain carboxyl

group. 154.2 mg (0.39 mmol, 1 equiv) of Fmoc-Asp-OAll were dissolved in 5 mL of dry DCM, and 30.6 µL (0.195 mmol, 0.5 equiv) of DIC were added to the solution. The mixture was incubated on ice for 30 min, under stirring. Then, DCM was removed under N_2 flux, the mixture was resuspended in DMF, and added to 0.39 mmol (1 g, 1 equiv) of Wang ChemMatrix® resin swollen in DMF, with 4.7 mg (0.039 mmol, 0.1 equiv) of DMAP. The reaction mixture was stirred for 2 h at room temperature. The substitution degree of the resin was evaluated by Fmoc-test[2], resulting in 0.21 mmol/g. Peptide synthesis was completed on a 0.2 mmol scale, using a standard Fmoc protocol and the amino acids Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Mtt)-OH, Fmoc-D-Phe-OH. Coupling reactions were carried out for 30 min under stirring using 5 equiv of the Fmoc protected amino acid, 4.9 equiv of HATU as the activating agent, 10 equiv of DIPEA as base in DMF. A capping reaction was performed after each amino acid coupling by treatment of the resin with a solution of 2.0 M acetic anhydride, 0.55 M DIPEA in NMP, for 5 min under stirring. Fmoc deprotection reactions were performed by incubation of the resin with a solution of 20% piperidine in DMF, twice for 5 min. After the coupling of the Fmoc-D-Phe-OH, the resin was dried under *vacuo* and the substitution degree of the resin was evaluated by Fmoc-test, resulting in 0.072 mmol/g (1.2 g, 86.4 µmol).

Allyl group removal. The resin (1.2 g, 86.4 µmol) was swollen in dry DCM, under N_2 flux. Allyl-protecting group was removed from the α-carboxyl group of the Asp residue at the C-terminus of the linear peptide by treatment with tetrakis(triphenylphosphine)palladium(0) (25 mg, 0.25 equiv) and phenylsilane (255 µL, 24 equiv) in dry DCM, which were added to the resin under N_2 flux and gently shaken for 30 min. The procedure was repeated twice. The resin was subsequently washed with DCM and DMF and the N-terminal Fmoc group was removed by treatment with 20% piperidine in DMF (2 × 5 min).

On resin peptide head-to-tail cyclization. The cyclization reaction between the N-terminal α-amino group of the D-Phe and the C-terminal α-carboxy group of the Asp residue was carried out by adding to the resin 44.7 mg of PyBOP (86 µmol, 1 equiv) and 30.2 µL of DIPEA (172 µmol, 2

equiv) in DMF. The reaction was incubated over night at room temperature, under stirring. The resin was then washed with DMF, DCM and diethyl ether and dried under *vacuo*.

Mtt removal and coupling of a Cys residue on ε-amine group of the Lys2. After peptide head-to-tail cyclization, methyltrityl (Mtt) side chain protecting group of the Lys2 was selectively removed on solid phase by treatment with a solution of 0.5% TFA, 5% TIS in DCM, performing washes of 1 min with the solution until a uncolored eluate solution was obtained. The resin was washed with DCM and DMF and then 200 mg of Boc-Cys(trt)-OH (432 μmol, 5 equiv), 160 mg of HATU (422 μmol, 4.9 equiv) and 97 μL of 4-methylmorpholine (860 μmol, 10 equiv) were added to the resin and the mixture was incubated for 1 h at room temperature, under stirring. The resin was then washed with DMF, DCM and diethyl ether and dried under *vacuo*.

The branched cyclic peptide *cyclo*RGDfK(C) was cleaved off the resin and deprotected using a mixture of TFA/H_2O/TIS (95:2.5:2.5; v/v/v) for 2 h at room temperature. The resin was finally filtered, and the peptide *cyclo*RGDfK(C) was precipitated using cold diethyl ether. The crude product (53 mg) was purified by preparative RP-HPLC on a HP 1200 Series (Agilent Technologies) using the column Axia 50×21.2 mm, Synergi, 4 mm, Fusion RP 80 Å (Phenomenex), applying a gradient of CH_3CN (0.1% TFA) in H_2O (0.1% TFA) from 5% to 25% in 20 min, at a flow rate of 20 mL/min. Pure fractions were pooled and lyophilized. 13.5 mg of pure (>95%) *cyclo*RGDfK(C) were obtained (yield 10%). The purity of the product was checked by analytical UPLC-MS by employing an ACQUITY UPLC® Peptide BEH C18 column (300Å, 1.7μm, 2.1X100mm). Eluent: (A) 0.05% TFA in H_2O, (B) 0.05% TFA in CH_3CN. Gradient profile: linear gradient from 5% to 50% of B in 7 min, linear gradient from 50% to 100% in 3 min, isocratic at 100% for 3 minutes. Flow rate of 0.4 mL/min and UV detection at 210 nm. ESI-MS (*m/z*): calcd: For $C_{30}H_{46}N_{10}O_8S$ $(M+H)^+$ 707.33 found: 707.43, $(M+2H)^{2+}$ 354.41 found: 354.38

2-((5-(6-(bis(carboxymethyl)amino)-1,4-bis(carboxymethyl)-1,4-diazepan-6-yl)pentanoyl)thio)ethane-1-sulfonate, AAZTA-C4-CO-MES (3)

In a round bottom flask, 108.1 mg of (*t*Bu)$_4$-AAZTA-C4-COOH (1 eq, 0.161 mmol) were dissolved in 5 mL of DCM. The solution was cooled at 273 K and DMAP (0.09 eq, 1.8 mg) and EDC (1 eq, 30.8 mg) were added. After 10 minutes the ice bath was removed and MESNA (0.9 eq, 23.8 mg) were added to the solution and the reaction was stirred overnight at room temperature. The solvent was evaporated and the obtained product re-dissolved in 5 mL of TFA/TIS/H_2O (95:2.5:2.5) and the reaction was stirred overnight at room temperature. The solvent was evaporated and the final purification was achieved by preparative RP-HPLC on Waters AutoPurification system by employing an Atlantis prepD® C18OBD 5µm (19X100 mm) column. Eluent: (A) 0.1% TFA in H_2O, (B) 0.1% TFA in CH_3CN. Gradient profile: isocratic at 10% of B for 2.8 min, linear gradient from 10% to 35% of B in 5.7 min, linear gradient from 35% to 100% in 2.8 min, isocratic at 100% for 1 min. Flow rate; 15 mL/min. AAZTA-C4-CO-MES was isolated as a homogenous peak with a retention time of ab. 5 minutes. The solvent was removed *in vacuo* and the product lyophilized from water to give **2** as a white solid (35 mg, 38%). The purity of the product was checked by analytical UPLC-MS by employing an ACQUITY UPLC® Peptide BEH C18 column (300Å, 1.7µm, 2.1X100mm). Eluent: (A) 0.05% TFA in H_2O, (B) 0.05% TFA in CH_3CN. Gradient profile; linear gradient from 5% to 50% of B in 7 min, linear gradient from 50% to 100% in 3 min, isocratic at 100% for 3 minutes. Flow rate of 0.4 mL/min and UV detection at 210 nm. Purity 95%. ESI-MS (*m/z*): calcd: For $C_{20}H_{33}N_3O_{12}S_2$ $(M+H)^+$ 572.16 found: 572.31. ^{1}H-NMR (CD_3OD, 600 MHz): δ 1.63 (tr, 6H), 2.37 (t, J=7.45 Hz, 2H), 2.62 (t, J=7.45, 4H), 3.02 (tr, 4H), 3.26 (tr, 2H), 3.58 (tr, 2H), 3.74 (s, 8H).

AAZTA-C4-CO-Cys-AE105 (4)

2 mg of **1** (1.50 µmol, 1 eq) were dissolved in 100 µL of ligation buffer (0.2 M phosphate buffer pH 7.4, 5 M Imidazole, 3 M Guanidinum chloride, 50 mM TCEP. Final pH: 7.4) and the pH is adjusted

to 7.4. A solution of **3** (3.00 µmol, 2 eq) in 100 µL of ligation buffer was added to the previous solution and the pH was adjusted to 7.4. The reaction was stirred for 1 h at room temperature. The reaction was monitored by HPLC-MS. Final purification was achieved by preparative RP-HPLC on Waters AutoPurification system by employing an XBridge™ BEH300 Prep C18 10 µm (10X100 mm) column. Eluent: (A) 0.1% TFA in H_2O, (B) 0.1% TFA in CH_3CN. Gradient profile; isocratic at 20% of B for 10 min, linear gradient from 20% to 35% of B in 10 min, linear gradient from 35% to 100% in 3.33 min, isocratic at 100% for 4 min. Flow rate; 5 mL/min. **4** was isolated as a homogenous peak with a retention time of ab. 22.5 minutes. The solvent was removed *in vacuo* and the product lyophilized from water to give **4** as white solid (1.64 mg, 62%). The purity of the product was checked by analytical UPLC-MS by employing an ACQUITY UPLC® Peptide BEH C18 column (300Å, 1.7µm, 2.1X100mm). Eluent: (A) 0.05% TFA in H_2O, (B) 0.05% TFA in CH_3CN. Gradient profile; linear gradient from 5% to 50% of B in 7 min, linear gradient from 50% to 100% in 3 min, isocratic at 100% for 3 minutes. Flow rate of 0.4 mL/min and UV detection at 210 nm. Purity 98%. ESI-MS (*m/z*): calcd: For $C_{81}H_{115}N_{17}O_{25}S$ $(M+2H)^{2+}$ 880.36 found: 880.16.

AAZTA-C4-CO-*cyclo*RGDfK(C) (5)

1.09 mg (1.91 µmol, 1 equiv) of **3** were dissolved in 283 µL of ligation buffer (0.2 M phosphate buffer pH 7.4, 5 M imidazole, 3 M guanidinium chloride, 2 mM EDTA, in ultra-gradient water. Final pH: 7.4) and the solution was immediately added to 0.98 mg (1.39 µmol, 0.75 equiv) of **2**. The mixture was incubated for 30 min at room temperature and then analyzed by LC-MS on HP 1200 Series (Agilent Technologies) using a column Aeris 100 × 2.1 mm, PEPTIDE XB-C18, 3.6 µm and applying a gradient of CH_3CN (0.05% TFA) in H_2O (0.05% TFA) from 5% to 70% in 15 min. The analysis showed the complete conversion of the cyclic peptide in the ligation product AAZTA-C4-CO-*cyclo*RGDfK(C). The reaction mixture was diluted with 0.25 mL of CH_3CN (0.1% TFA) and 4.47 mL of H_2O (0.1% TFA) (final volume of 5 mL) and purified by RP-HPLC on HP 1200 Series (Agilent Technologies) on a column Axia 50 × 21.2 mm, Synergi, 4 µm, Fusion RP 80

Å, applying a gradient of CH_3CN (0.1% TFA) in H_2O (0.1% TFA) from 5% to 60% in 10 min, at a flow rate of 20 mL/min. The product was lyophilized to give **5** (0.5 mg, 32%). The purity of the product was checked by analytical UPLC-MS by employing an ACQUITY UPLC® Peptide BEH C18 column (300 Å, 1.7 µm, 2.1 × 100 mm). Eluent: (A) 0.05% TFA in H_2O, (B) 0.05% TFA in CH_3CN. Gradient profile; linear gradient from 5% to 50% of B in 7 min, linear gradient from 50% to 100% in 3 min, isocratic at 100% for 3 minutes. Flow rate of 0.4 mL/min and UV detection at 210 nm. Purity 95%. ESI-MS (*m/z*): calcd: For $C_{48}H_{73}N_{13}O_{17}S$ $(M+H)^+$1136.25 found: 1136.64.

AAZTA-C4-CO-Cys(Cy 5.5)-AE105 (6): procedure 1

3.09 mg of **4** (1.76 µmol) were dissolved in 600 µL of 50 mM acetate at pH 6.5. 1.14 mg of Cyanine 5.5 Maleimide (0.875 eq, 1.53 µmol) were added to the previous solution and the reaction was stirred at room temperature for 3h. The reaction was monitored by UPLC-MS. Final purification was achieved by preparative RP-HPLC on Waters AutoPurification system by employing an XBridge™ BEH300 Prep C18 10 µm (10 × 100 mm) column. Eluent: (A) 0.1% TFA in H_2O, (B) 0.1% TFA in CH_3CN. Gradient profile; isocratic at 25% of B for 5.25 min, linear gradient from 25% to 50% of B in 15.77 min, isocratic at 50% of B in 5.25 min, linear gradient from 50% to 100% in 5 min, isocratic at 100% for 5 min. Flow rate; 4.5 mL/min. AAZTA-C4-CO-Cys(Cy 5.5)-AE105 (**6**) was isolated as a homogenous peak with a retention time of ab. 24 min (1.68 mg, 39%). The purity of the product was checked by analytical UPLC-MS by employing an ACQUITY UPLC® Peptide BEH C18 column (300 Å, 1.7 µm, 2.1 × 100 mm). Eluent: (A) 0.05% TFA in H_2O, (B) 0.05% TFA in CH_3CN. Gradient profile; linear gradient from 5% to 50% of B in 7 min, linear gradient from 50% to 100% in 3 min, isocratic at 100% for 3 minutes. Flow rate of 0.4 mL/min and UV detection at 210 nm and 672 nm. $Purity_{210\ nm}$: 95%. ESI-MS (*m/z*): calcd: For $C_{127}H_{164}N_{21}O_{28}S^+$ $(M+2H)^{3+}$ 822.11 found: 822.35.

Synthesis of AAZTA-C4-CO-Cys(Cy 5.5)-AE105 (6): procedure 2

2.5 mg of **1** (1.88 μmol, 1 eq.) were dissolved in 300 μL of the reaction buffer (0.2 M phosphate buffer pH 7.4, 5 M imidazole, 3 M guanidinium chloride. Final pH: 7.0) and 2.14 mg of **3** (3.75 μmol, 2 eq.) were added to the solution. The obtained solution was stirred at room temperature for 30 min. After the reaction time an UPLC-MS chromatogram shows the total conversion to the desired product.

Subsequently, without performing any further purification, 1.39 mg of Cy 5.5 maleimide (1.87 μmol, 1 eq.) were added to the solution. After 3 h the solution was directly purified on preparative RP-HPLC (same condition of synthesis of **6**). The final product was lyophilized from water to give **6** as dark blue solid (1.0 mg, 22%). The purity of the product was checked by analytical UPLC-MS by employing an ACQUITY UPLC® Peptide BEH C18 column (300Å, 1.7 μm, 2.1 × 100mm). Eluent: (A) 0.05% TFA in H_2O, (B) 0.05% TFA in CH_3CN. Gradient profile; linear gradient from 5% to 50% of B in 7 min, linear gradient from 50% to 100% in 3 min, isocratic at 100% for 3 minutes. Flow rate of 0.4 mL/min and UV detection at 210 nm and 672 nm. $Purity_{210\ nm}$: 95%. ESI-MS (*m/z*): calcd: For $C_{127}H_{164}N_{21}O_{28}S^+$ $(M+2H)^{3+}$822.11 found: 822.15.

AAZTA-C4-CO-(Cy 5.5)-*cyclo*RGDfK(C) (7): procedure 1

0.5 mg (441 nmol, 1 equiv) of **5** were dissolved in 440 μL of 20 mM phosphate buffer, pH 7.2. 0.39 mg of Cy 5.5 Maleimide (529 nmol, 1.2 equiv) were dissolved in 25 μL of CH_3CN and added to the solution of **5**. Reaction was carried out for 1 h at room temperature, in the dark, and analyzed by LC-MS on HP 1200 Infinity Series (Agilent Technologies) on a column Aeris 100 × 2.1 mm, PEPTIDE XB-C18, 3.6 mm and applying a gradient of CH_3CN (0.05% TFA) in H_2O (0.05% TFA) from 5% to 95% in 25 min. The analysis showed the complete conversion of the **5** in the doubly labelled peptide AAZTA-C4-CO-(Cy 5.5)-*cyclo*RGDfK(C) (**7**). The reaction mixture was diluted with 0.25 mL of CH_3CN (0.1% TFA) and 4.28 mL of H_2O (0.1% TFA) (final volume of 5 mL) and purified by RP-HPLC on HP 1200 Series (Agilent Technologies) on a column Jupiter C18 250 × 10

mm, 5 µm, 300 Å (Phenomenex) applying a gradient of CH_3CH (0.1% TFA) in H_2O (0.1% TFA) from 5% to 70% in 40 min), at a flow rate of 5 mL/min. The pure **7** was quantified by UV absorption spectroscopy, using the Cy5.5 molar extinction coefficient at 684 nm of 1.98 10^5 M^{-1} cm^{-1} (0.160 mg, 86.7 nmol, 20% yield). The purity of the product was checked by analytical UPLC-MS by employing an ACQUITY UPLC® Peptide BEH C18 column (300Å, 1.7um, 2.1X100mm). Eluent: (A) 0.05% TFA in H_2O, (B) 0.05% TFA in CH_3CN. Gradient profile; linear gradient from 5% to 50% of B in 7 min, linear gradient from 50% to 100% in 3 min, isocratic at 100% for 3 minutes. Flow rate of 0.4 mL/min and UV detection at 210 nm and 672 nm. $Purity_{210nm}$: 95%.ESI-MS (*m/z*): calcd: For $C_{94}H_{122}N_{17}O_{20}S^+$ $(M+H)^{2+}$ 921.58 found: 921.37.

AAZTA-C4-CO-(Cy 5.5)-*cyclo*RGDfK(C) (7): procedure 2

1.1 mg (1.92 µmol, 1 equiv) of **3** were dissolved in 283 µL of ligation buffer (see previous procedure) and the solution was immediately added to 1.0 mg (1.41 µmol, 0.75 equiv) of **2**. The mixture was incubated for 30 min at room temperature and analyzed by LC-MS on HP 1200 Infinity Series (Agilent Technologies) showing the complete conversion of the cyclic peptide in the ligation product **5**. Without performing any purification, 2.17 mg (2.92 µmol, 1.5 equiv) of Cy 5.5 maleimide were dissolved in 50 µL of CH_3CN and added to the reaction mixture. After 30 min of reaction in the dark, at room temperature, the reaction was checked by LC-MS on HP 1200 Infinity Series (Agilent Technologies) showing the complete conversion of the **5** in the doubly labelled peptide AAZTA-C4-CO-(Cy 5.5)-*cyclo*RGDfK(C) (**7**). The mixture was diluted with 0.5 mL of CH_3CN (0.1% TFA) and 3.87 mL of H_2O (0.1% TFA) (final volume of 5 mL) and purified by RP-HPLC on HP 1200 Series (Agilent Technologies) on a column Axia 50 × 21.2 mm, Synergi, 4 mm, Fusion RP 80 Å, applying a gradient of CH_3CN (0.1% TFA) in H_2O (0.1% TFA) from 5% to 70% in 25 min, at a flow rate of 20 mL/min. After lyophilization 0.6 mg of pure **7** were recovered (yield: 23%). $Purity_{210nm}$: 95%. ESI-MS (*m/z*): calcd: For $C_{94}H_{122}N_{17}O_{20}S^+$ $(M+H)^{2+}$ 921.58 found: 921.47.

IV. Experimental labelling procedures and analytical characterizations

Ga-AAZTA-C4-CO-Cys(Cy 5.5)-AE105 (8)

0.38 mg of **6** (0.154 μmol) were dissolved in 300 μL of a solution 50:50 of 0.1 M acetate buffer at pH 3.8/CH_3CN. 10 μL of a 1000 ppm standard water solution of $GaCl_3$ were added and the solution was stirred at room temperature for 1 h. Product **8** was quantified by UV absorption spectroscopy in ethanol, using the Cy5.5 molar extinction coefficient at 684 nm of 1.98 10^5 M^{-1} cm^{-1}. The purity of the product was checked by analytical UPLC employing an ACQUITY UPLC® Peptide BEH C18 column (300Å, 1.7μm, 2.1X100mm). Eluent: (A) 0.05% TFA in H_2O, (B) 0.05% TFA in CH_3CN. Gradient profile; linear gradient from 5% to 50% of B in 7 min, linear gradient from 50% to 100% in 3 min, isocratic at 100% for 3 minutes. Flow rate of 0.4 mL/min and UV detection at 210 nm and 672 nm. $Purity_{210\ nm}$: 96%. ESI-MS (*m/z*): calcd: For $C_{127}H_{161}GaN_{21}O_{28}S^+$ $(M+2H)^{3+}$ 844.42 found: 844.25. High resolution mass spectrometry monoisotopic peak $(M+2H)^{3+}$: calcd: 843.6932 found: 843.6893.

Ga-AAZTA-C4-CO-(Cy 5.5)-*cyclo*RGDfK(C) (9)

0.54 mg of **7** (0.293 μmol) were dissolved in 450 μL of a solution 50:50 of 0.1 M acetate buffer/CH_3CN at pH 3.8. 20 μL of a 1000 ppm standard water solution of $GaCl_3$ were added and the solution was stirred at room temperature for 1h. The pure **9** was quantified by UV absorption spectroscopy in ethanol, using the Cy5.5 molar extinction coefficient at 684 nm of 1.98 10^5 M^{-1} cm^{-1}. The purity of the product was checked by analytical UPLC by employing an ACQUITY UPLC® Peptide BEH C18 column (300Å, 1.7μm, 2.1X100mm). Eluent: (A) 0.05% TFA in H_2O, (B) 0.05% TFA in CH_3CN. Gradient profile; linear gradient from 5% to 50% of B in 7 min, linear gradient from 50% to 100% in 3 min, isocratic at 100% for 3 minutes. Flow rate of 0.4 mL/min and UV detection at 210 nm and 672 nm. $Purity_{210\ nm}$:95%. ESI-MS (*m/z*): calcd: For $C_{94}H_{119}GaN_{17}O_{20}S^+$ $(M+H)^{2+}$ 954.94 found: 954.97. High resolution mass spectrometry monoisotopic peak $(M+H)^{2+}$: calcd: 953.8897 found: 953.8832.

V. Experimental procedure of *in vitro* receptor binding analyses

Human U-87 MG glioblastoma cells were used to determine cell binding of Ga-AAZTA-C4-CO-Cys(Cy 5.5)-AE105 (compound **8**) and Ga-AAZTA-C4-CO-(Cy 5.5)-*cyclo*RGDfk(C) (compound **9**). To minimize the non-specific uptake, incubations were performed on ice and followed immediately by flow cytometry. All cells groups (10^5) were incubated with Ga-AAZTA-C4-CO-Cys-(Cy 5.5)-AE105 or Ga-AAZTA-C4-CO-(Cy 5.5)-*cyclo*RGDfK(C) (0, 0.033, 0.133 and 0.400 μg) for 30 min at 273 K. After centrifugation of the tubes and the elimination of the supernatant, cells were washed twice with PBS 1X. 100uL of PBS 1X supplemented with 0.1% BSA were added to each tube.

1. L. Manzoni, L. Belvisi, D. Arosio, M. P. Bartolomeo, A. Bianchi, C. Brioschi, F. Buonsanti, C. Cabella, C. Casagrande, M. Civera, M. De Matteo, L. Fugazza, L. Lattuada, F. Maisano, L. Miragoli, C. Neira, M. Pilkington-Miksa and C. Scolastico, *ChemMedChem*, 2012, **7**, 1084-1093.
2. G. W. L. Mikhail F. Gordeev , Hon C. Hui , Eric M. Gordon , Dinesh V. Patel, *Tetrahedron*, 1998, **54**, 15879-15890.

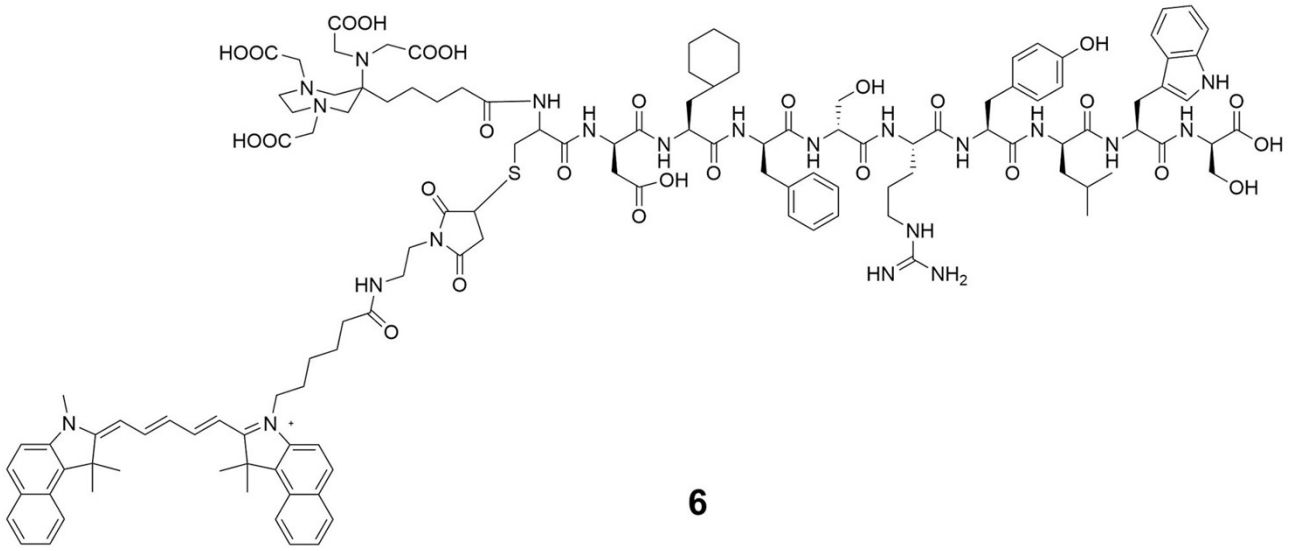

Figure S1: Chemical structure of **6**

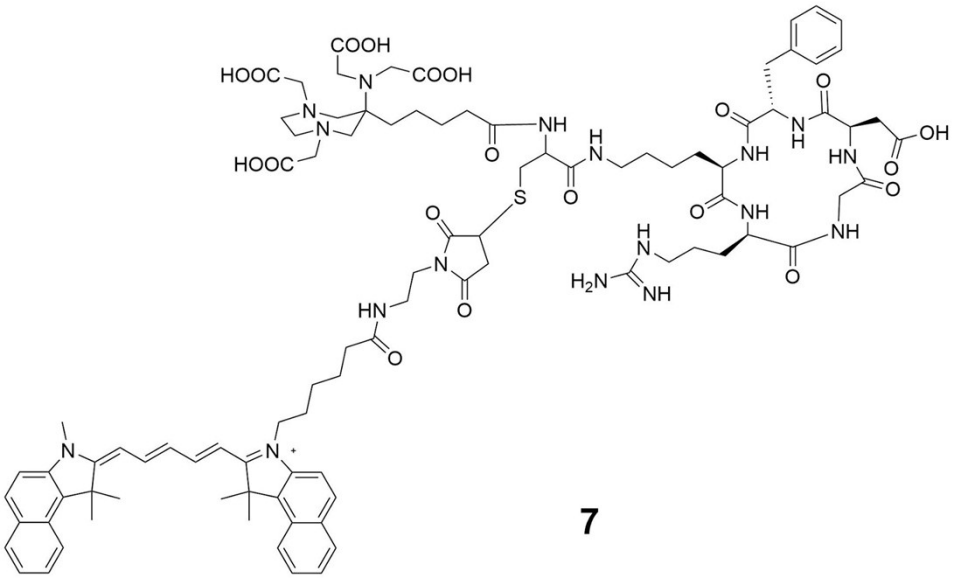

Figure S2: Chemical structure of **7**

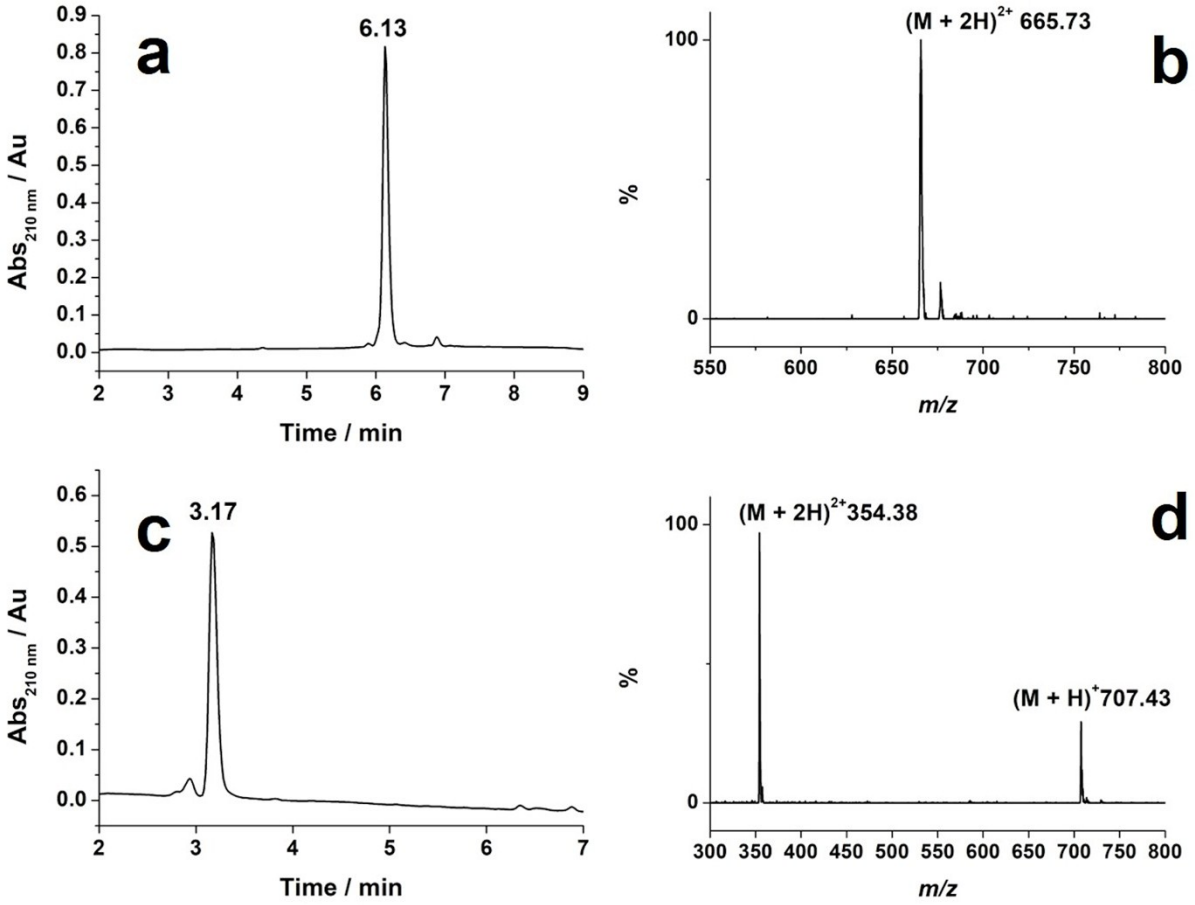

Figure S3: UPLC-MS analysis of pure compounds **1** and **2**: a) chromatographic profile of **1** revealed at 210 nm and ESI-MS spectrum (b) of the peak at 6.13 min retention time; c) chromatographic profile of **2** revealed at 210 nm and ESI-MS spectrum (d) of the peak at 3.17 min retention time

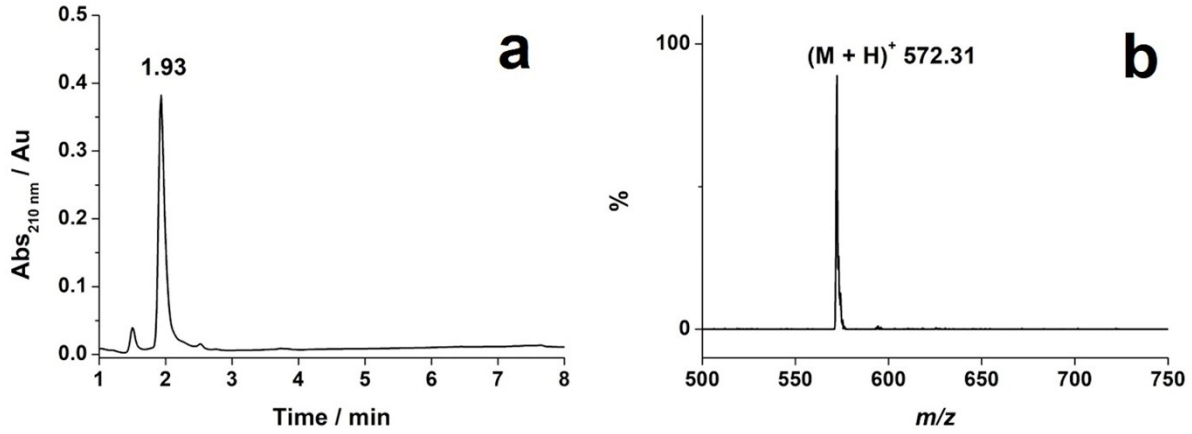

Figure S4: UPLC-MS analysis of pure compounds **3**: a) chromatographic profile of **3** revealed at 210 nm and ESI-MS spectrum (b) of the peak at 1.93 min retention time

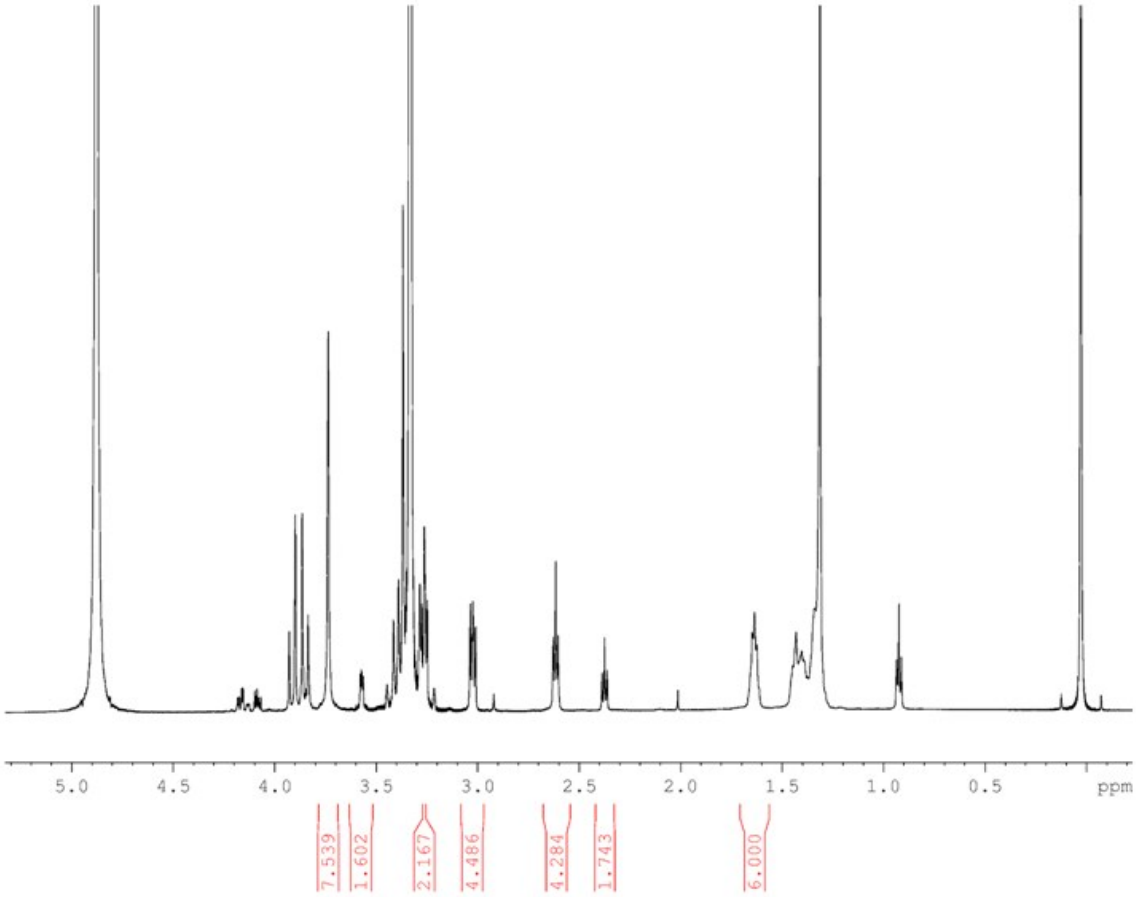

Figure S5: ^{1}H-NMR spectrum of **3** in CD_3OD at 14.6 mM

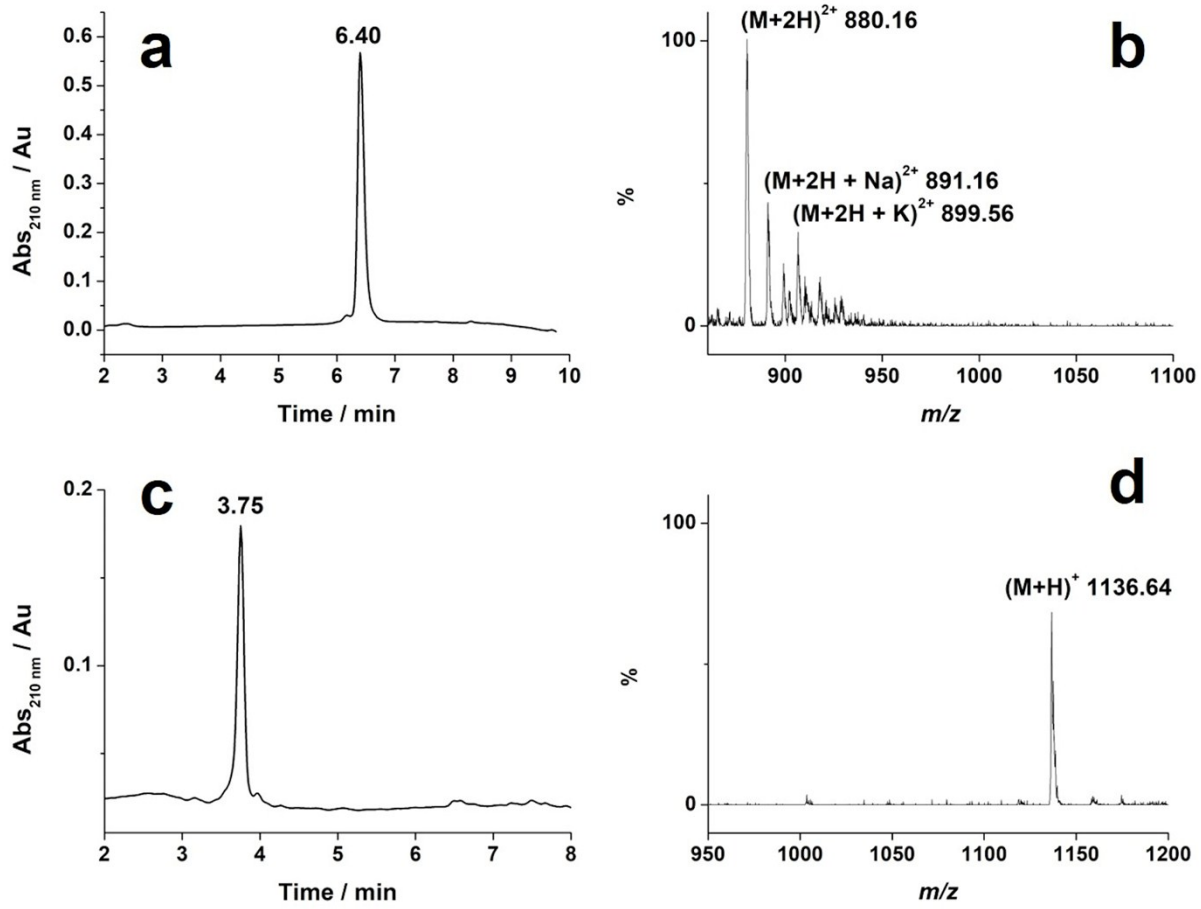

Figure S6: UPLC-MS analysis of pure compounds **4** and **5**: a) chromatographic profile of **4** revealed at 210 nm and ESI-MS spectrum (b) of the peak at 6.40 min retention time; c) chromatographic profile of **5** revealed at 210 nm and ESI-MS spectrum (d) of the peak at 3.75 min retention time

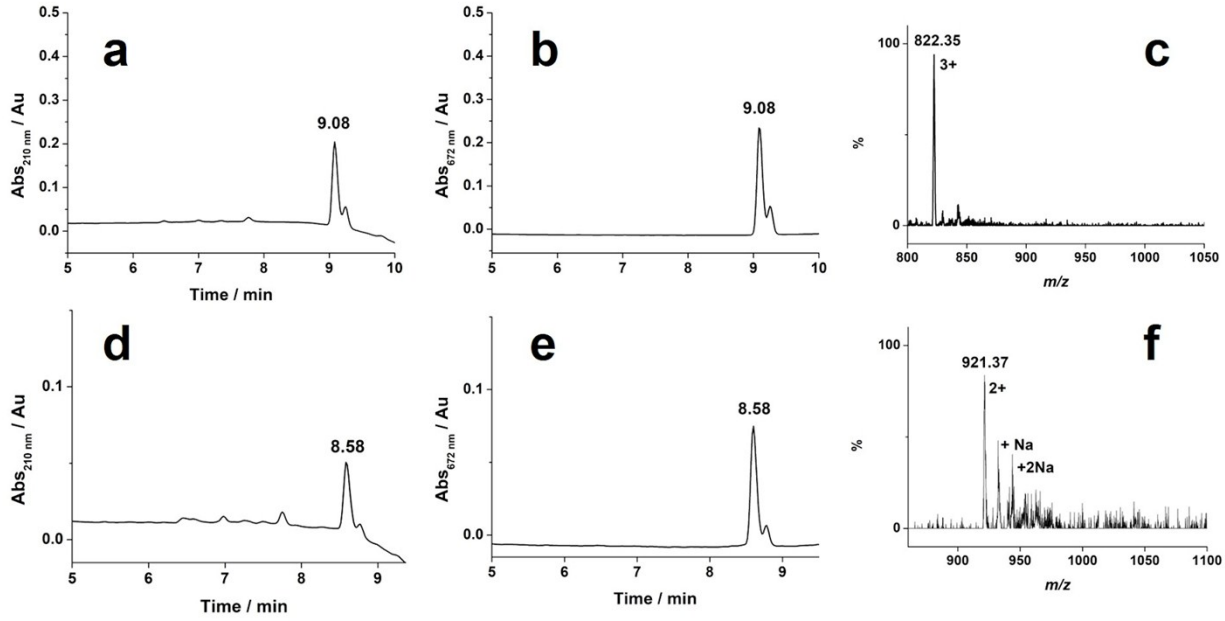

Figure S7: UPLC-MS analysis of pure compounds **6** and **7**: a) chromatographic profile of **6** revealed at 210 nm and 672 nm (b), and ESI-MS spectrum (c) of the peak at 9.08 min retention time; d) chromatographic profile of **7** revealed at 210 nm and 672 nm (e) and ESI-MS spectrum (f) of the peak at 8.58 min retention time

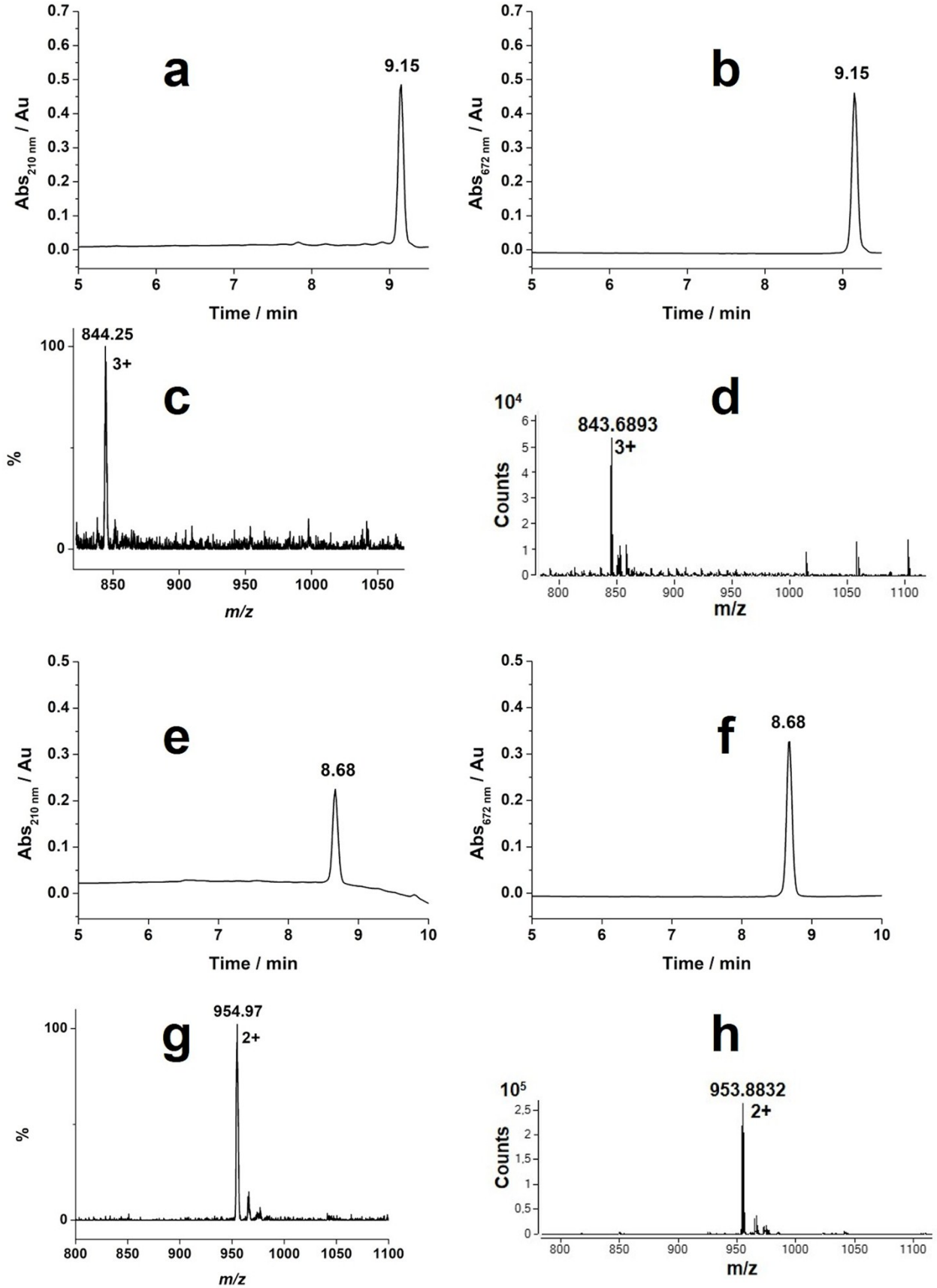
a
9.15
Abs210 nm / Au
Time / min
b
9.15
Abs672 nm / Au
Time / min
c
844.25
3+
%
m/z
d
10^4
843.6893
3+
Counts
m/z
e
8.68
Abs210 nm / Au
Time / min
f
8.68
Abs672 nm / Au
Time / min
g
954.97
2+
%
m/z
h
10^5
953.8832
2+
Counts
m/z

Figure S8: UPLC-MS and High Resolution Mass analyses of pure compounds **8** and **9**. Compound **8**: chromatographic profile revealed at 210 nm (a) and 672 nm (b), ESI-MS (c) and ESI-ToF (the monoisotopic peak is indicated) (d) spectra of the peak at 9.15 min retention time; Compound **9**: chromatographic profile revealed at 210 nm (e) and 672 nm (f), ESI-MS (g) and ESI-ToF (the monoisotopic peak is indicated) (h) spectra of the peak at 8.68 min retention time.

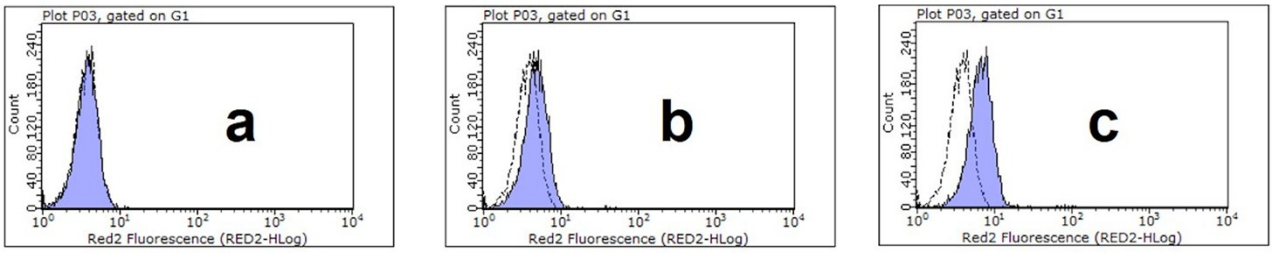

Figure S9: Flow Cytometry plot of U-87 MG cells (10^5) analysed after incubation of 30 min at 273 K with (a) 0.033, (b) 0.133 and (c) 0.400 µg of Ga-AAZTA-C4-CO-Cys(Cy5.5)-AE105 (**8**).

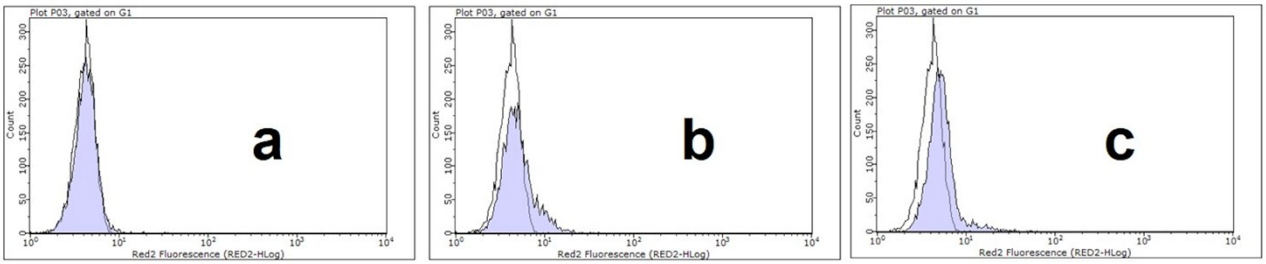

Figure S10: Flow Cytometry plot of U-87 MG cells (10^5) analysed after incubation of 30 min at 273 K with (a) 0.033, (b) 0.133 and (c) 0.400 µg of Ga-AAZTA-C4-CO-(Cy5.5)-*cyclo*RGDfK(C) (**9**).