Supporting Informations

F-18 Labeled RGD Probes Based on Bioorthogonal Strain-Promoted Click Reaction for PET Imaging

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1. General. Unless otherwise noted all reagents and solvents were commercially available. ADIBO-PEG_5-*N*-succinimidyl ester compound **2** was purchased from Click Chemistry Tools (Macon, GA, USA), Glu-dimeric RGD peptide (H-E[c(RGDyK)]$_2$) were purchased from Peptide International, Inc. (Louisville, KY), and cRGDyK was purchased from FutureChem, Co. (South Korea). [^{18}F]Fluoride ion was produced from a cyclotron (KIRAMS 13 MeV, South Korea) using the ^{18}O (p,n)^{18}F nuclear reaction with 13 MeV proton irradiation of an enriched [^{18}O]H_2O target. High performance liquid chromatography (HPLC) was performed with spectra system (Thermo Scientific, Waltham, MA) using semi preparative column (C18 silica gel, 10 μm, 10 × 250 mm) and analytic column (C18 silica gel, 5 μm, 4.6 × 250 mm). The eluant was simultaneously monitored by a UV detector (215 nm & 254 nm) and a NaI(Tl) radioactivity detector. Radioactivity was measured in a dose calibrator.

2. Preparation of ADIBO Substituted cRGD Peptide Precursors.

2.1. Preparation of cRGD-ADIBO. DIPEA (0.08 mg, 6.4 μmol) was added to the mixture solution of cRGDyK (2.0 mg, 3.2 μmol) and the ADIBO *N*-succinimidyl ester compound **1** (2.0 mg, 4.8 μmol, prepared according to our previous work[26]) in 0.3 mL of dried DMSO. The mixture was stirred at room temperature for 12 h and then injected onto a RP-HPLC (C18 silica gel, 10 μm, 10 × 250 mm; 0.1% TFA in H_2O/acetonitrile = 70:30 (v/v); 254 nm; 2 mL/min). The peak at the retention time of 15.4 min was collected and lyophilized overnight to obtain 1.5 mg (50%) of cRGD-ADIBO as a white powder. The product was confirmed by MALDI-TOF-MS: *m/z* 921.63 for $[MH]^+$ ($C_{47}H_{56}N_{10}O_{10}$, calculated molecular weight [MW] 921.01).

2.2. Preparation of cRGD-PEG_5-ADIBO. DIPEA (0.07 mg, 5.8 μmol) was added to the mixture solution of cRGDyK (2.0 mg, 3.2 μmol) and ADIBO-PEG_5-*N*-succinimidyl ester compound **2** (2.0 mg, 2.9 μmol) in 0.3 mL of dried DMSO. The mixture was stirred at room temperature for 12 h and then injected onto a RP-HPLC running the same condition as described above. The peak at the retention time of 16.0 min was collected and lyophilized overnight to give 1.5 mg (39%) of cRGD-PEG_5-ADIBO as a

white powder. The product was confirmed by MALDI-TOF-MS: *m/z* 1198.63 for $[M]^+$ ($C_{59}H_{79}N_{11}O_{16}$, calculated [MW] 1198.32).

2.3. Preparation of di-cRGD-ADIBO. Prepared from H-E[c(RGDyK)]$_2$ by the method used to prepare the cRGD-ADIBO, 2.0 mg (39%) of di-RGD-ADIBO was obtained as a white powder; MALDI-TOF-MS: *m/z* 1652.12 for $[MH]^+$ ($C_{79}H_{102}N_{20}O_{20}$, calculated [MW] 1651.78). Retention time (t_R) on HPLC = 14.5 min.

2.4. Preparation of di-cRGD-PEG$_5$-ADIBO. Prepared from H-E[c(RGDyK)]$_2$ by the method used to prepare the cRGD-PEG$_5$-ADIBO, 2.0 mg (33%) of di-cRGD-PEG$_5$-ADIBO was obtained as a white powder; MALDI-TOF-MS: *m/z* 1929.43 for $[M]^+$ ($C_{91}H_{125}N_{21}O_{26}$, calculated [MW] 1929.09). t_R = 15.7 min.

3. Preparation of Fluorine Substituted RGD Peptide Derivatives

3.1. Preparation of cRGD-ADIBOT-F. Fluorohexaethylene glycolic azide (**3**) (0.57 mg, 2.2 μmol, prepared according to our previous work[26]) in 0.2 mL of DMSO was added to the solution of cRGD-ADIBO (1.0 mg, 1.1 μmol) in 0.1 mL of DMSO. The mixture was stirred at room temperature for 30 min and then injected onto a RP-HPLC running the same condition as described above. The peak at the retention time of 14.9 min was collected and lyophilized overnight to obtain 1.2 mg (93%) of non-radioactive cRGD-PEG$_5$-ADIBOT-F with > 95% purity as a white powder. The product was confirmed by MALDI-TOF-MS: *m/z* 1186.94 for $[M]^+$ ($C_{57}H_{76}FN_{13}O_{14}$, calculated [MW] 1186.29).

3.2. Preparation of cRGD-PEG$_5$-ADIBOT-F: Prepared from cRGD-PEG$_5$-ADIBO (1.3 mg, 1.1 μmol) by the method used to prepare the cRGD-ADIBOT-F, 1.4 mg (90%) of non-radioactive cRGD-PEG$_5$-ADIBOT-F was obtained with > 95% purity as a white powder; MALDI-TOF-MS: *m/z* 1464.61 for $[MH]^+$ ($C_{69}H_{99}FN_{14}O_{20}$, calculated [MW] 1463.60). t_R = 15.0 min.

3.3. Preparation of di-cRGD-ADIBOT-F: Prepared from di-cRGD-ADIBO (1.8 mg, 1.1 μmol) by the method used to prepare the cRGD-ADIBOT-F, 1.9 mg (91%) of non-radioactive di-cRGD-ADIBOT-F

was obtained with > 95% purity as a white powder; MALDI-TOF-MS: *m/z* 1917.61 for $[M]^+$ ($C_{89}H_{122}FN_{23}O_{24}$, calculated [MW] 1917.06). t_R = 14.2 min.

3.4. Preparation of di-cRGD-PEG$_5$-ADIBOT-F: Prepared from di-cRGD-PEG$_5$-ADIBO (2.1 mg, 1.1 μmol) by the method used to prepare the cRGD-ADIBOT-F, 2.2 mg (92%) of non-radioactive di-cRGD-PEG$_5$-ADIBOT-F was obtained with > 95% purity as a white powder; MALDI-TOF-MS: *m/z* 2195.02 for $[MH]^+$ ($C_{101}H_{145}FN_{24}O_{30}$, calculated [MW] 2194.37). t_R = 14 .8 min.

4. Representative Procedure for Preparation of di-cRGD-PEG$_5$-ADIBOT-^{18}F.

The solution of [^{18}F]**3** (930 MBq, prepared according to our previous work[26]) in 0.1 mL of EtOH/H_2O (1/1) was added to a solution of di-cRGD-PEG$_5$-ADIBO (1.5 mg, 0.87 μmol) in 0.1 mL of EtOH/H_2O (1/1). The reaction mixture was stirred at room temperature for 15 min. To remove non-reacted di-cRGD-PEG$_5$-ADIBO precursor, 50 mg of ADIBO-precursor-scavenger-resin **4** (1.05 mmol of azide portion/g, prepared according to our previous work[26]) was added to reaction mixture, and this heterogeneous solution was then stirred for approximately 20 min at room temperature. After the filtration using syringe filter (Advantec DISMIC-13, PTFE, 0.20 μm pore size) and washing with PBS solution (2 × 0.5 mL), 685 MBq of di-cRGD-PEG$_5$-ADIBO-^{18}F was obtained in 92% decay-corrected radiochemical yield (35 min total reaction time, specific radioactivity: 62.5 GBq/μmol) as a direct injectable solution for animal PET image study. Both isomers of the triazole were collected and treated as one compound. To confirm the efficacy of the treatment with the scavenger-resin **4** as an alternative purification for the removal of ADIBO precursor, and to measure the radiochemical purity and specific radioactivity, the product solution was injected onto reverse-phase HPLC (t_R = 14.8 min; Waters XTerra Prep MS C18 silica gel, 10 μm, 10 × 250 mm; 0.1% TFA in H_2O/acetonitrile = 70:30 (v/v); 254 nm; 2 mL/min). Radiochemical purity (> 98%) and specific radioactivity di-cRGD-PEG$_5$-ADIBO (62.5 GBq/μmol) was obtained on an HPLC column. di-cRGD-PEG$_5$-ADIBOT-^{18}F was characterized by HPLC chromatograph coinjected with

its non-radioactive analogue di-cRGD-PEG_5-ADIBOT-F (for HPLC study, the product solution was prepared after washing with water instead of using PBS solution).

5. *In Vitro* Experimental

5.1. Cell Culture. U87MG human glioblastoma cell line obtained from the Korean Cell Line Bank (KCLB, Seoul). U87MG glioma cell were grown in RPMI-1640 modified medium (GIBCO, Carlsbad, CA) supplemented with 10 % (v/v) fetal bovine serum (FBS, GIBCO, Carlsbad, CA) and 1 % (v/v) penicillin streptomycin (Antibody, GIBCO, Carlsbad, CA) at 37 ^oC in humidified atmosphere containing 5 % CO_2.

5.2. Competitive Cell Binding Assay. The integrin $\alpha_v\beta_3$ *in vitro* binding affinity and specificity of cRGD derivatives (cRGDyk, cRGD-ADIBOT-F, cRGD-PEG_5-ADIBOT-F, di-cRGD-ADIBOT-F, and di-cRGD-PEG_5-ADIBOT-F) were assessed via a cellular displacement assays using ^{125}I-echiatatin (μCi/well) as the integrin-specific radioligand. For the integrin $\alpha_v\beta_3$ binding affinity assay, U87MG cells were seeded onto 96-well plates at 2×10^4 cells per well and incubated overnight at 37 ^oC. Serial dilutions of cRGD derivatives, such as cRGDyk, cRGD-ADIBOT-F, cRGD-PEG_5-ADIBOT-F, di-cRGD-ADIBOT-F, and di-cRGD-PEG_5-ADIBOT-F, were added to 96-well plates. The plates were then incubated for 30 min at 37 ^oC washed, dried and add 0.1 mL of 2*N* NaOH solution was added to each well to facilitate cell lysis. The lysates were collected and counted in a gamma counter (Perkin-Elmer, USA). Binding affinities (IC_{50}) were calculated by nonlinear regression analysis (sigmoidal dose response equation) using the GraphPad Prism 4.0 computer-fitting program (GraphPad software, San Diego, CA, USA).

6. *In Vivo* Experimental

6.1. Animal Models. Female athymic nude mice (4-wk, nu/nu) were obtained from Orient-Bio (Seoul, Korea). The animal experiment was performed in compliance with the Institutional Animal Care and Use

Committee for Animal Treatment of Chonbuk National University. After sedation, U87MG tumor was established by subcutaneous injection in the right flank with 3×10^6 tumor cells mixed in 100 μL of martrigel/PBS (1:1, v/v) (BD bioscience, Bedford, MA). After 4-5 wk after inoculation, tumor bearing mice were subjected to *in vivo* imaging and biodistribution studies when the tumor grows in 0.7-1.0 cm in length.

6.2. *In Vivo* PET/CT Imaging Study. The mice (n =3) underwent PET/CT studies when the tumor volume reached 170 - 200 mm^3 (eq. = length × W^2 × 0.5 4-5 wk after inoculation). PET and CT scans and image ananlysis were performed using a FLEX^{TM} X-PET$^{®}$/X-O^{TM} small animal imaging instrument (GE Healthcare) which combine a PET scanner and a multi-slice helical computed tomography (CT) scanner. CT scans were performed using a CT scanner with following scanning parameters: 75 kVp, 0.25 mA. The CT images were acquired with 256 projections over 2 min. About 1.8 MBq of di-cRGD-PEG_5-ADIBOT-^{18}F was intravenously injected into each mouse under isoflurane anesthesia (2.0%). PET images were acquired for 10 min at 30, 60, 90, and 120 min after injection. For each microPET scan, regions of interest (ROIs) were drawn over the tumor and muscle on decay-corrected whole-body images that were gained by 3D-OSEM iterative image reconstruction (Amira 3.1, GE Healthcare). For receptor-blocking experiments, nude mice bearing U87MG tumors were scanned at 30, 60, 90, and 120 min after coinjection with di-cRGD-PEG_5-ADIBOT-^{18}F (1.8 MBq) and non-radioactive di-cRGD-PEG_5-ADIBOT-F (10 mg/kg). The radioactivity concentration within the tumor or muscle was obtained from the mean value and then these values were compared.

6.3. Biodistribution Study. U87MG tumor xenografted mice were subjected to the biodistribution study when the tumor reached in 170 - 200 mm^3. All mice received the intravenous (i. v.) injection through tail vein with about 1.11MBq of di-cRGD-PEG_5-ADIBOT-^{18}F. At 30, 60 and 90 min after injection, the animals were anesthetized and necropsied. The blocking experiment was performed by coinjection of radiotracer (1.11 MBq) together with nonradioactive di-cRGD-PEG_5-ADIBOT-F (10 mg/kg) and sacrificed at 90 min after injection. Tissues were weighed and counted on gamma counter. Uptake in each

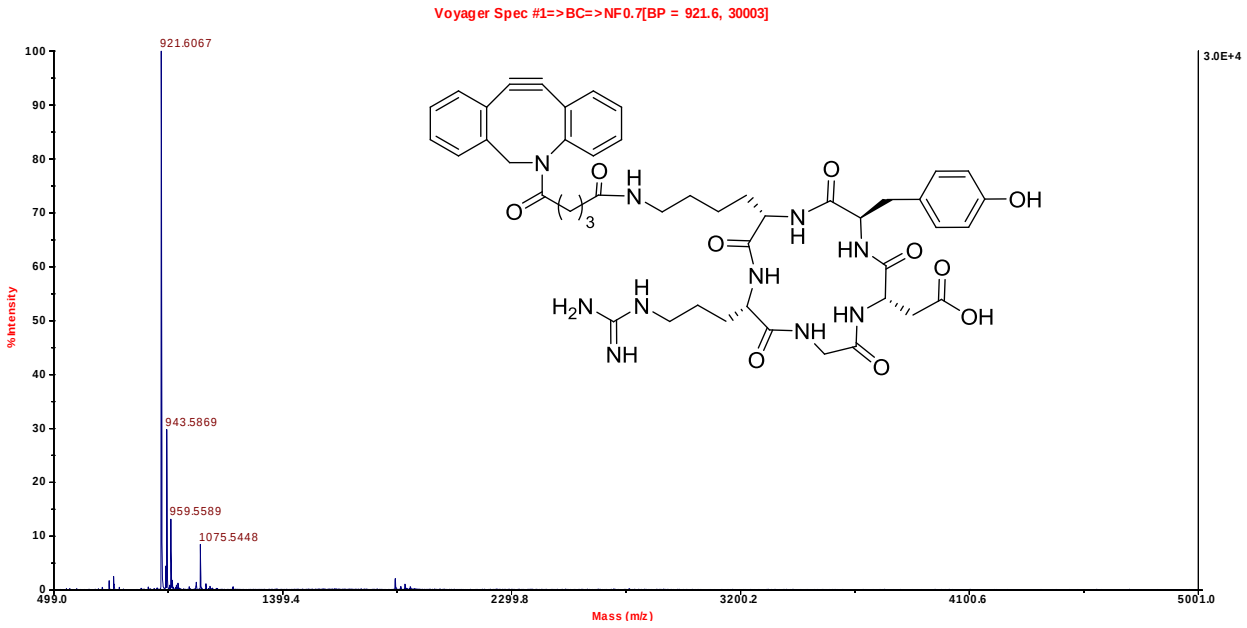
tissue was expressed as the percentage injected dose per gram of tissue (% ID/g). All experiments were repeated 4 times, and the results were presented as the mean ± standard deviation.

Table 1S. Values for all organs measured in the biodistribution

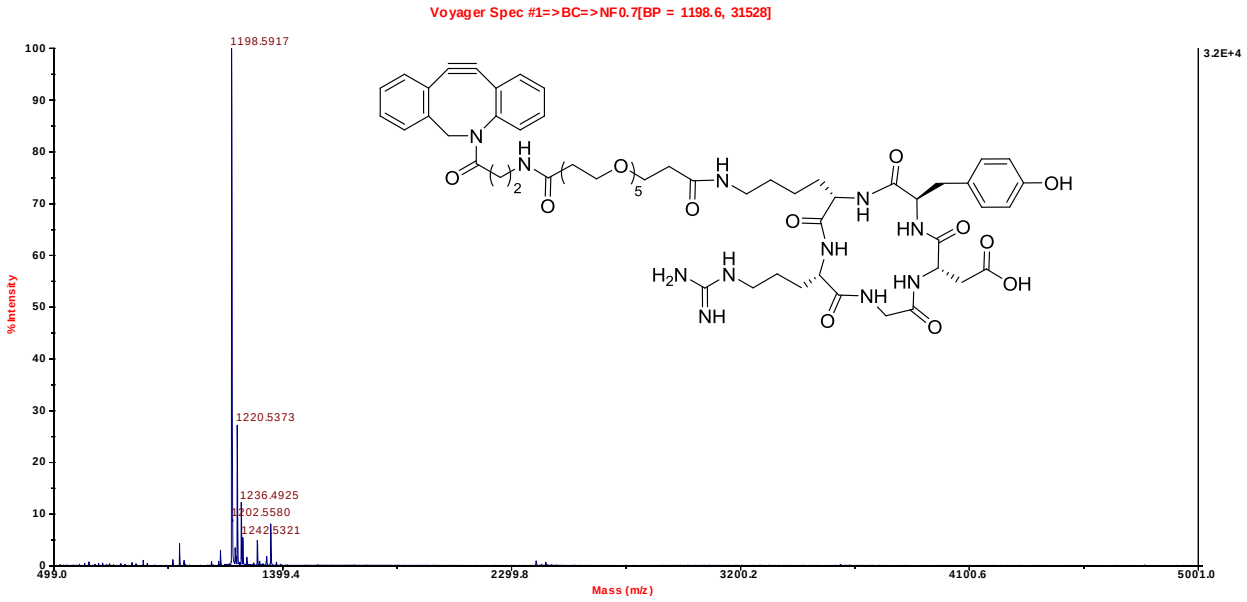
organ	30 min	60 min	90 min	90 min (Blocking)
Blood	1.19 ± 0.11	0.49 ± 0.06	0.17 ± 0.01	0.51 ± 0.10
Heart	0.65 ± 0.01	0.33 ± 0.05	0.21 ± 0.03	0.11 ± 0.02
Lung	1.16 ± 0.17	0.53 ± 0.08	0.33 ± 0.04	0.27 ± 0.06
Liver	1.33 ± 0.04	0.87 ± 0.10	0.67 ± 0.05	0.40 ± 0.10
Stoma.	1.33 ± 0.07	0.65 ± 0.08	0.63 ± 0.05	0.27 ± 0.03
Spleen	0.92 ± 0.05	0.82 ± 0.05	0.54 ± 0.03	0.16 ± 0.07
Panc.	0.28 ± 0.02	0.12 ± 0.02	0.14 ± 0.01	0.10 ± 0.01
Kidney	4.32 ± 0.29	3.58 ± 0.12	2.63 ± 0.46	2.32 ± 0.43
Lg.int	1.42 ± 0.04	0.78 ± 0.04	0.61 ± 0.03	0.19 ± 0.03
Sm.int	1.89 ± 0.19	1.47 ± 0.17	1.17 ± 0.15	0.33 ± 0.04
Bone	1.09 ± 0.14	0.43 ± 0.04	0.40 ± 0.11	0.18 ± 0.02
Muscle	0.61 ± 0.08	0.36 ± 0.13	0.14 ± 0.02	0.15 ± 0.01
Tumor	6.32 ± 0.13	2.24 ± 0.28	1.20 ± 0.10	0.43 ± 0.05
Brain	0.10 ± 0.01	0.07 ± 0.004	0.06 ± 0.007	0.04 ± 0.003

7. MALDI-TOF-MS Spectra of ADIBO Substituted cRGD Peptide Precursors

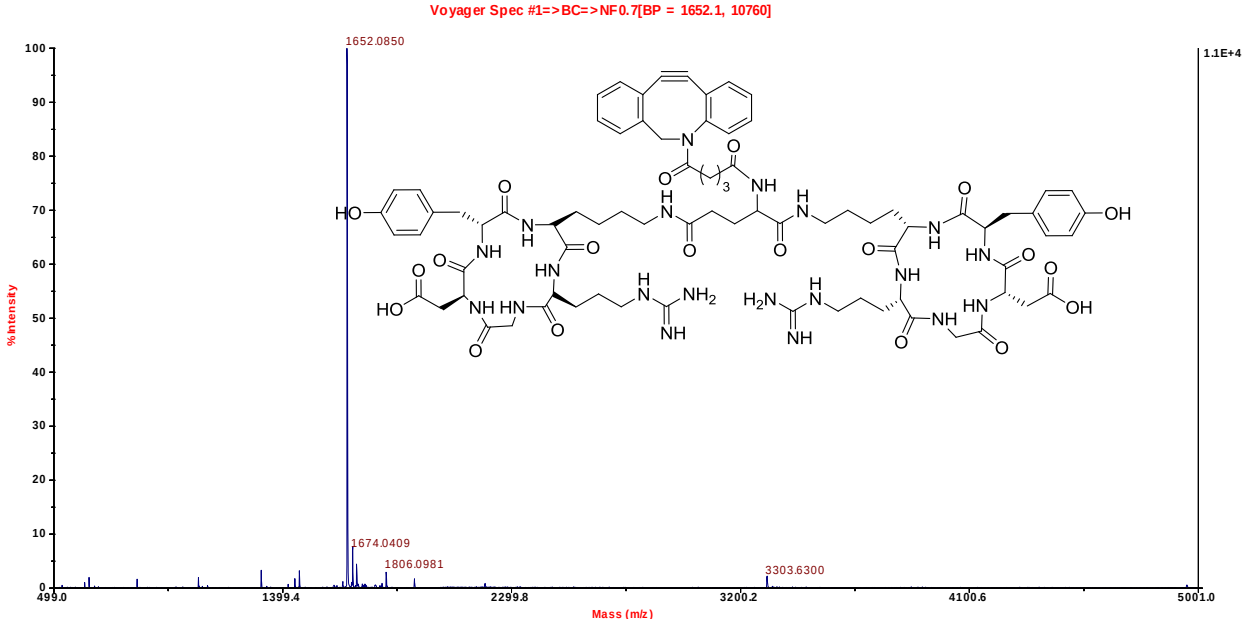
cRGD-ADIBO



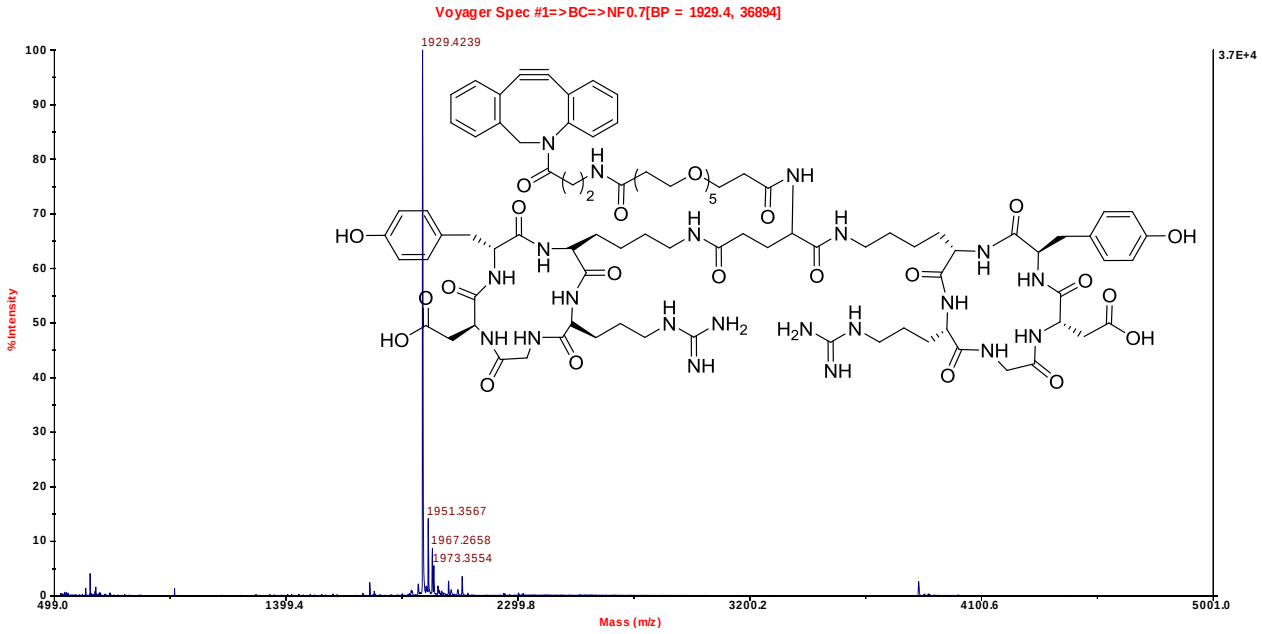
cRGD-PEG$_5$-ADIBO.



di-cRGD-ADIBO.

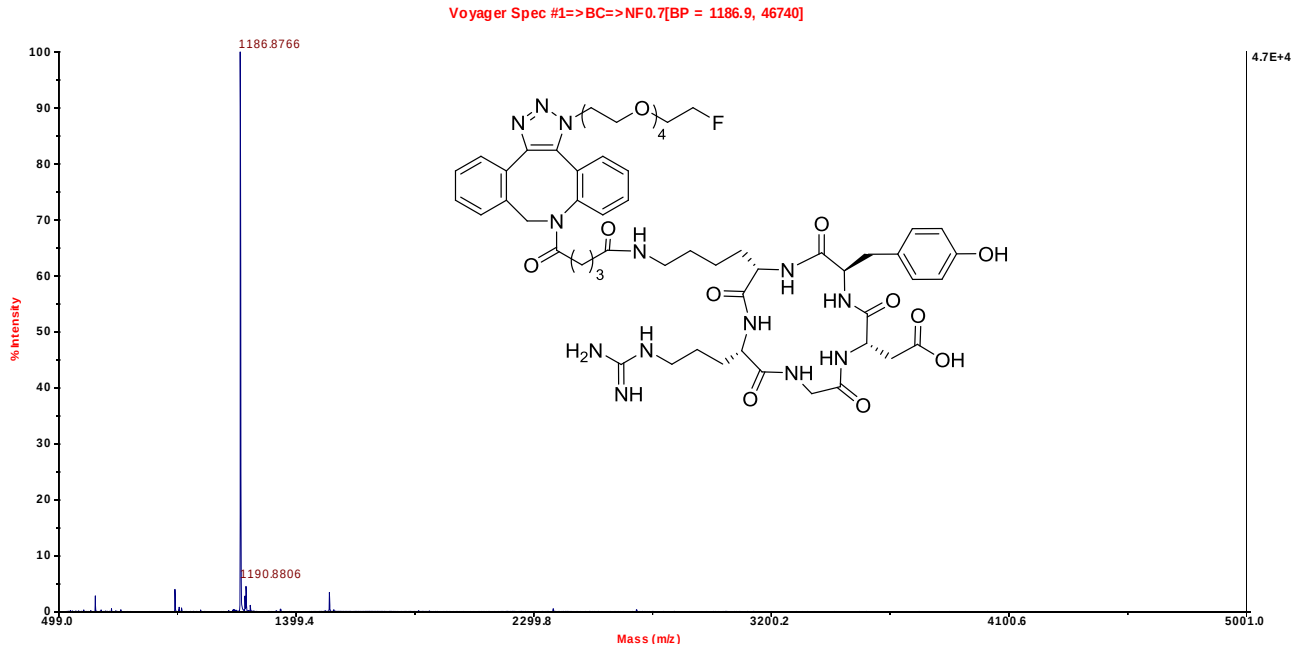


di-cRGD-PEG$_5$-ADIBO.



8. MALDI-TOF-MS Spectra of Fluorine Substituted RGD Peptide Derivatives

cRGD-ADIBOT-F.



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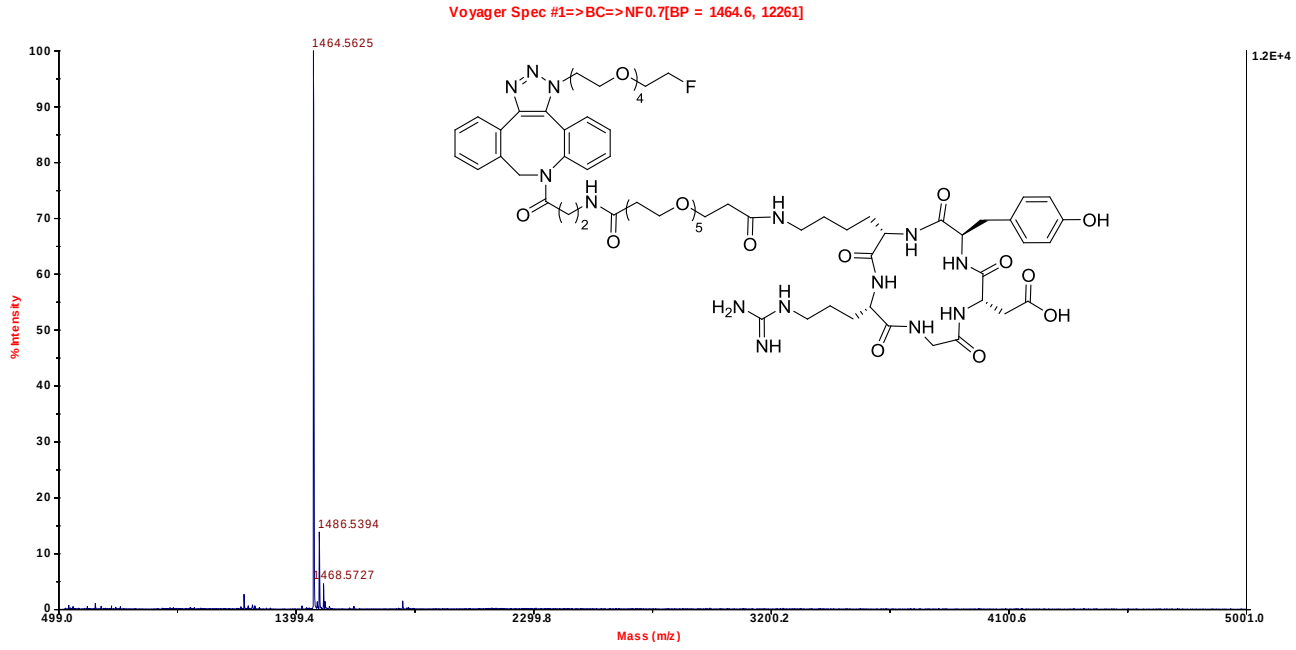

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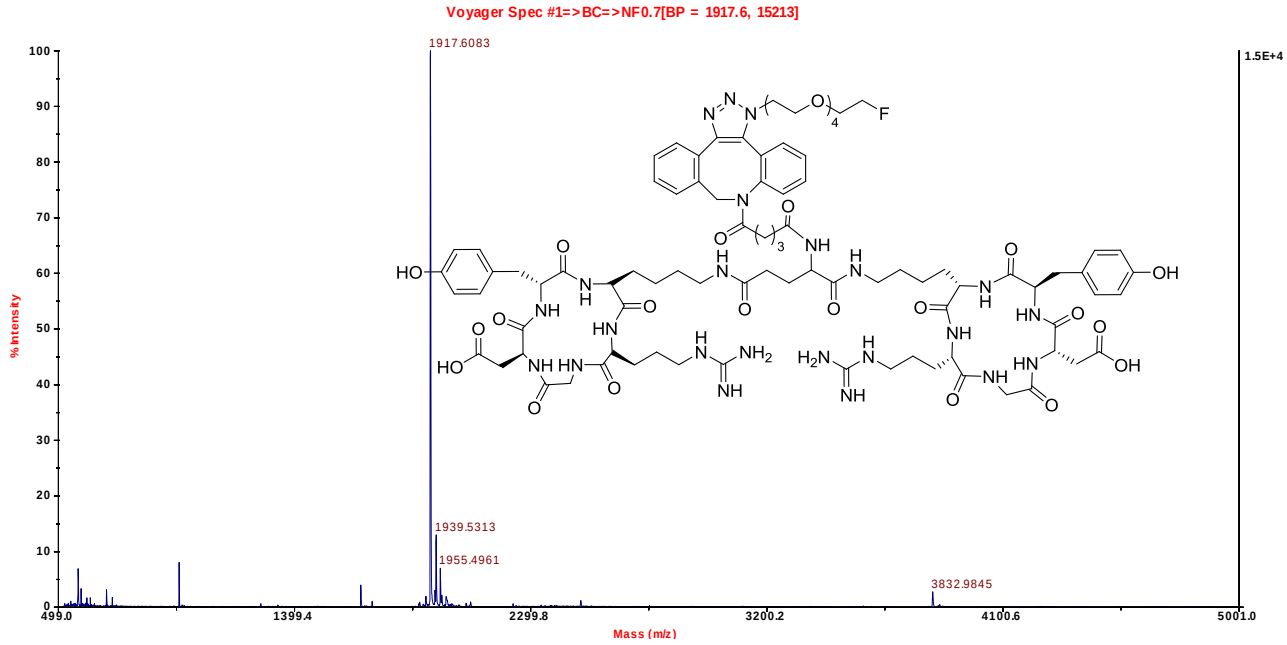

di-cRGD-PEG$_5$-ADIBOT-F.