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Supporting information

Radiolabeled Albumin through SNAr of Cysteines as a Potential Pretargeting Theranostics Agent

– A New Selective and Irreversible Conjugation Method Opening Up Pretargeting

Table of contents

Section 1: Materials and methods

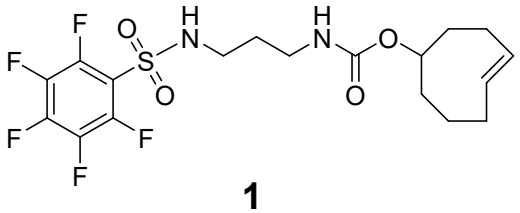
General remarks

All chemicals were acquired from commercial providers and used as received. Human serum albumin was purchased from Albumedix Ltd. (Nottingham, UK) as a 95 mg/mL stock solution. All solvents were HPLC quality. ^{1}H,^{13}C, and ^{19}F NMR spectra were recorded on a Bruker instrument at 500 MHz, 126 MHz, and 470 MHz, respectively. Chemical shift values are quoted in ppm and coupling constants (*J*) in Hz. The residual solvent peak from $CDCl_3$ (^{1}H: 7.26, ^{13}C: 77.16) or DMSO-d_6 (^{1}H: 2.50 ppm, ^{13}C: 39.52 ppm)[1] was used as reference. Dry-column vacuum chromatography (DCVC) was carried out using Merck silica gel 60A (15-40 µm Analytical HPLC measurements were recorded on an Agilent HP1100 instrument with an XBridge® column (10µm 4.6 x 100 mm), using a linear gradient of acetonitrile in water with 0.1 % TFA running from 0% to 90% acetonitrile over 10 min at a flow rate of 1.0 mL min (method A). Peaks were measured by UV absorption at 215, 230, and 254 nm. LC-MS spectra were recorded on a DionexUltiMate 3000 (Thermo Scientific) with an Acclaim™ RSLC 120 C18 column (2.2 µm, 120Å, 2.1 x 100 mm) coupled to a Bruker microTOF-QIII mass spectrometer. Detection was performed by measurement of absorbance of UV light at 214, 225, 250, and 275 nm. For LC-MS spectral analysis and protein signal deconvolution, the program Compass DataAnalysis from Bruker was used. HRMS spectra were recorded on a Bruker ESP-MALDI-FT-ICR instrument. Compound purification was performed using a semipreparative Gilson RP-HPLC with a UV detector and a stationary phase consisting of C_{18}-modified silica (XTetra RP 18 column, 10 µm, 19×150 mm). For the mobile phase, two buffers were used: buffer A (0.1 % trifluoroacetic acid in water) and buffer B (0.1 % trifluoroacetic acid in 9:1 MeCN/water).

Circular dichroism and UV-Vis spectroscopy

Solutions of HSA, HSA·**1**, and HSA·**2** in PBS buffer pH 7.4 were analyzed by circular dichroism spectroscopy using a Jasco model J-815 spectropolarimeter which was routinely calibrated with a solution of (1S)-(+)-10-camphorsulfonic acid in water. CD spectra were measured at room temperature in 1.0 mm Suprasil quartz cuvettes from Hellma. Scans were made in the region from 300 nm to 190 nm in continuous mode with a bandwidth of 2 nm and a data interval of 1 nm. The scan speed was 50 nm/min and the D.I.T. setting was 4 sec. Each spectrum represents the average of 10 scans. Spectra were analysed using the Jasco Spectra Analysis software by subtracting a corresponding reference spectrum, applying a smoothing function and converting the unit to molar residual ellipticity. Concentrations of HSA and HSA conjugates were determined by UV-Vis spectroscopy using the extinction coefficient 36500 M^{-1} cm^{-1} at 280 nm.[2] Measurements were performed using a Shimadzu UV-3600 UV-Vis-NIR spectrophotometer and 10 mm Suprasil quartz cuvettes from Hellma, or a NanoDrop One UV-Vis-NIR spectrophotometer.

Synthesis of pentafluorobenzene-TCO (1)



1

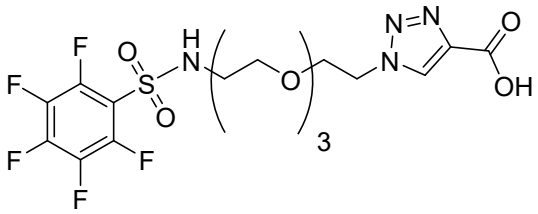
TCO-amine (CAS 1609736-43-7, 2.7 mg, MW 262.8, 0.010 mmol) was dissolved in CH_2Cl_2(150 µL)and transferred to an LC-MS vial equipped with a 200 µL glass insert. 2,6-lutidine (5.98 µL,MW 107.2, ρ 0.925, 0.051 mmol, 5 equiv.) was added and allowed to pre-react with the TCO-amine for 15 min prior to addition of pentafluorobenzenesulfonyl chloride (1.64 µL,MW 266.6, ρ 1.796, 0.0150 mmol, 1.1 equiv.). The reagents initially formed an off-white slurry. The vial was shaken at room temperature. After ½ h, the mixture had become transparent and light yellow. 1 µL portions of the reaction mixture were injected on the HPLC instrument 1, 3, 4, 5, and 22 h after reaction initiation. After 22 h, the reaction mixture was subjected to repeated extractions using 50 µL portions of Milli-Q water, saturated $NaHCO_3$ (aq), and 20% citric acid (aq) to remove the pentafluorobenzenesulfonic acid byproduct and any unreacted pentafluorobenzenesulfonyl chloride. After six extractions, no more acid could be detected in the CH_2Cl_2 phase by HPLC. The product, **1**, was used for subsequent conjugation to human serum albumin without further purification.

^{1}H NMR (500 MHz, $CDCl_3$) δ 6.46 (s, 1H), 5.53 (qdd, *J* = 16.0, 10.0, 4.1 Hz, 2H), 4.72 (s, 1H), 4.31 (t, *J*= 8.3 Hz, 1H), 3.35 – 3.23 (m, 2H), 3.23 – 3.12 (m, 2H), 2.45 – 2.25 (m, 3H), 1.93 (ddt, *J* = 21.7, 11.9, 6.1 Hz, 4H), 1.77 – 1.62 (m, 4H), 1.56 (m, 1H).^{13}C NMR (126 MHz, $CDCl_3$) δ 157.6, 144.6 (d, *J* = 253.8 Hz), 138.0 (d, *J* = 257.2 Hz), 135.0, 133.2, 117.1, 81.5, 41.2, 40.1, 38.8, 37.2, 34.4, 32.6, 31.1, 31.0 (one aromatic signal missing).^{19}F NMR (470 MHz, DMSO-d_6) δ -140.65 (d, *J* = 23.3 Hz), -150.59 (t, *J* = 22.3 Hz), -162.40 (t, *J* = 21.3 Hz).

TOF MS (ESI+) *m/z*calcd. for $C_{18}H_{21}F_5N_2NaO_4S^+$ $[M+Na]^+$ 479.1034, found 479.1028.

R_t HPLC 7.3 min (method A).

Synthesis of pentafluorobenzene-TEG-triazole-carboxylic acid (2a)



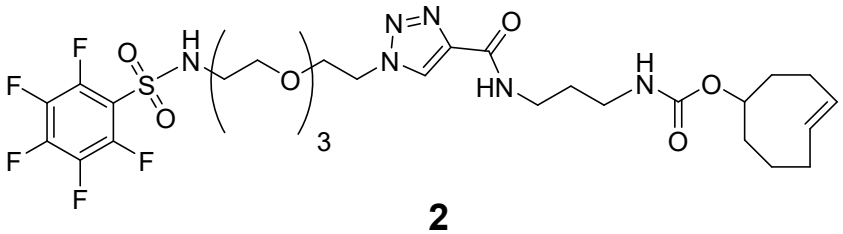
N-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-2,3,4,5,6-pentafluorobenzenesulfonamide was synthesized according to previously published procedure.[3] Propiolic acid (25.85 µL, MW 70.0, 0.42 mmol, 1 equiv.) was dissolved in THF (1 mL) purged with N_2. *N*-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-2,3,4,5,6-pentafluorobenzenesulfonamide (189.7 mg, MW 448.4, 0.42 mmol, 1 equiv.) was added. CuBr (12.20 mg, MW 143.5, 0.084 mmol, 0.2 equiv.) and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA, 22.90 mg MW 530.63, 0.042 mmol, 0.1 equiv.) were dissolved in THF/Milli-Q H_2O (1.2 mL). The solution was purged with N_2 for 10 min before being transferred to the solution of propiolic acid and azide. The reaction vial was shaken at rt for 18 ½ h. After this, 1:1 MeCN/Milli-Q H_2O with 0.1 % TFA (2 mL) and 9:1 MeCN/Milli-Q H_2O with 0.1 % TFA (1 mL) were added to the reaction mixture to quench the Cu^+ ions. The mixture was filtered, and the product was isolated using a Gilson semipreparative HPLC. The fractions containing product were mixed and freeze-dried, yielding the title compound as a **colorless gel**

(57.1 mg, MW 518.4, 0.11 mmol, **26 % isolated yield**). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.48 (s, 1H), 6.65 (s, 1H), 4.65 (d, *J* = 5.2 Hz, 2H), 3.91 (d, *J* = 5.1 Hz, 2H), 3.82 – 3.39 (m, 10H), 3.35 (d, *J* = 5.3 Hz, 2H) (carboxylic acid H signal missing).^{13}C NMR (126 MHz, $CDCl_3$) δ 144.6 (ddd, *J* = 258.1, 12.6, 4.6 Hz), 143.9 (dtt, *J* = 261.2, 13.4, 5.0 Hz), 137.8 (dtt, *J* = 257.5, 12.4, 4.8 Hz), 130.0, 116.8 (t, *J* = 15.4 Hz), 70.6, 70.6, 70.5, 70.4, 69.4, 68.9, 50.8, 43.4 (two signals missing).^{19}F NMR (470 MHz, $CDCl_3$) δ -136.36 (d, *J* = 21.6 Hz), -146.05 (t, *J* = 20.7 Hz), -158.40 (t, *J* = 21.2 Hz).Trifluoroacetic acid (δ-75.39 ppm) was added as reference.

TOF MS (ESI+) *m/z* calcd. for $C_{17}H_{20}F_5N_4O_7S^+$ $[M+H]^+$ 519.0967, found 519.0969.

R_t HPLC (method A) 5.9 min.

Synthesis of pentafluorobenzene-TEG-triazole-TCO (2)

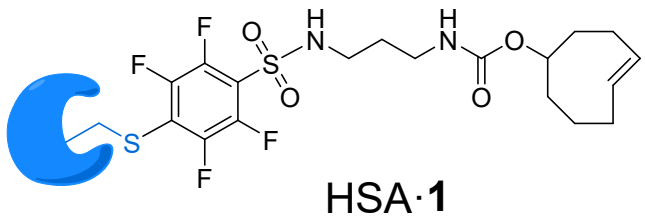


2

2.7 mg TCO-amine (MW 262.8, 0.010 mmol) was dissolved in 100 µL CH_2Cl_2 and transferred to an LC-MS vial equipped with a 200 µL glass insert. 40 µL of a 0.257 M solution of pentafluorobenzene-TEG-triazole-carboxylic acid in CH_2Cl_2 was added to the solution. PyBOP (MW 520.4, 0.010 mmol, 1 equiv.) was dissolved in 60 µL CH_2Cl_2 and added. 5.37 µL DIPEA (MW 129.2, 0.031 mmol, 3 equiv.) were added. The vial was shaken at rt. 1 µL portions of the reaction mixture were injected on the HPLC instrument 15, 30, 45, 60, 120 min, and 20 h after addition of base. A reaction time of 20 h caused severe byproduct formation. Reaction was repeated, and after 50 min of reaction, the reaction was quenched by the addition of 50 µL Milli-Q H_2O. The Milli-Q H_2O was removed, and the mixture was extracted four times using 50 µL portions of Milli-Q H_2O, three times using 50 µL portions of saturated $NaHCO_3$ (aq), and two times Milli-Q H_2O. The product, **2**, was used for subsequent conjugation to human serum albumin without further purification.

TOF MS (ESI+) *m/z* calcd. for $C_{29}H_{40}F_5N_6O_8S^+$ $[M+H]^+$ 727.2543, found 727.2542.

Conjugation of construct 1 to HSA

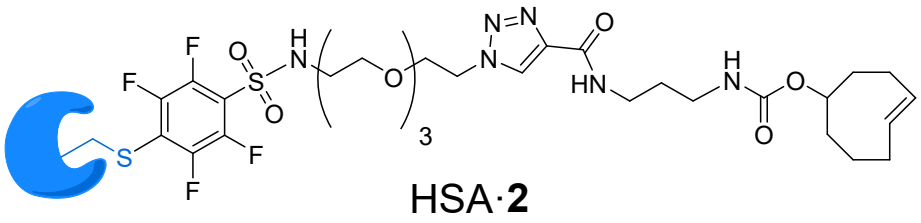


HSA·**1**

Assuming that all TCO-amine starting material had reacted in the synthesis of construct **1**, 0.010 mmol of **1** was available. This was dissolved in 10.275 mL MeCN to make a 1 mM solution. To a mixture of a solution of HSA in PBS (pH 8.5, 100 µM, 7 µL, 1 equiv.) and a solution of PBS in 30% MeCN (pH 8.5, 79 µL) that had been purged with N_2 prior to use was added **1** in

MeCN (14 µL, 20 equiv.). The vial was shaken at 400 mot/min at 31 °C for 24 h. The reaction mixture was filtered using an Amicon® Ultrafree®-MC centrifugation filter (6,000 rpm, 4 min). The HSA conjugate (HSA·**1**) was extracted from the filter using 3 × 50 µL portions of PBS buffer, pH 7.4. Expected mass of HSA·**1** $[M+H]^+$ 66438 + (456 – 20) + 1 = 66875, found 66875.

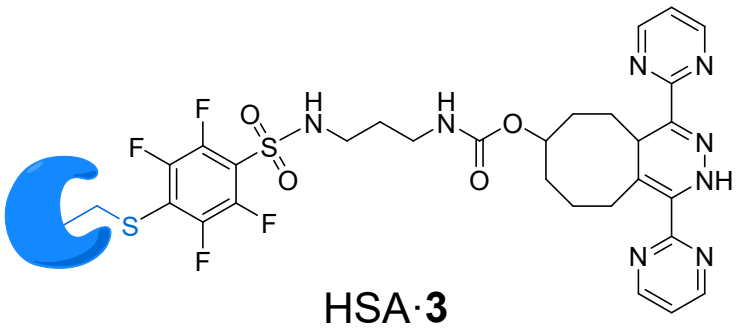
Conjugation of construct 2 to HSA



HSA·**2**

Assuming that all TCO-amine starting material had reacted in the synthesis of construct **2**, 0.010 mmol of **2** was available. This was dissolved in 10.275 mL MeCN to make a 1 mM solution. To a mixture of a solution of HSA in PBS (pH 8.5, 100 µM, 7 µL, 1 equiv.) and a solution of PBS in 30% MeCN (pH 8.5, 58 µL) that had been purged with N_2 prior to use was added **2** in MeCN (35 µL, 50 equiv.). The reaction was carried out in a LC-MS vial equipped with a 200 µL glass insert. The reaction mixture was filtered using a Zeba™ Spin desalting column with a 7 kDa cutoff by centrifugation (1500 × *g*, 2 min) to isolate the HSA conjugate (HSA·**2**). Expected mass of HSA·**2** $[M+H]^+$ 66438 + (726 – 20) + 1 = 67145, found 67148.

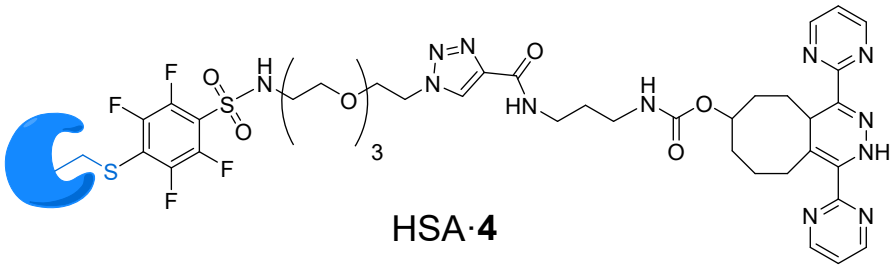
Ligation of 3,6-di(pyrimidin-2-yl)-1,2,4,5-tetrazine to HSA·1



HSA·**3**

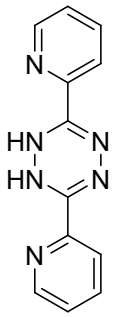
A solution in MeCN of 3,6-di(pyrimidin-2-yl)-1,2,4,5-tetrazine (MW 238.2, 0.233 mM, 1 µL, 1 equiv.) was added to a solution of HSA·**1** (5.67 µM in PBS, pH 7.4, 50 µL, 1 equiv.) in an LC-MS vial equipped with a 200 µL glass insert. The vial was shaken at 400 mot/min for 15 min at rt. Formation of the IEDDA product was confirmed by LC-MS analysis and deconvolution. Expected mass of Tz ligation product HSA·**3** $[M+H]^+$ 66438 + (666 – 20) + 1 = 67085 found 67087.

Ligation of 3,6-di(pyrimidin-2-yl)-1,2,4,5-tetrazine to HSA·2



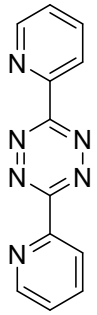
A solution in MeCN of 3,6-di(pyrimidin-2-yl)-1,2,4,5-tetrazine (MW 238.2, 0.233 mM, 1 µL, 1 equiv.) was added to a solution of HSA·**2** (5.67 µM in PBS, pH 7.4, 50 µL, 1 equiv.) in an LC-MS vial equipped with a 200 µL glass insert. The vial was shaken at 400 mot/min for 15 min at rt. Formation of the Tz ligation product was confirmed by LC-MS analysis and deconvolution. Expected mass of Tz ligation product HSA·**4** $[M+H]^+$ 66438 + (936 – 20) + 1 = 67355 found 67361.

3,6-di(pyridin-2-yl)-1,2-dihydro-1,2,4,5-tetrazine (5a)



Picolinonitrile (8.74 g, 83.94 mmol, 1 equiv.) and 5-aminopicolinonitrile (10.00 g, 83.94 mmol, 1 equiv) were dissolved in abs. EtOH (20 mL) and warmed to 60 °C. Hydrazine monohydrate (50 mL) was added to the reaction mixture and the reaction was stirred for 17 h. The reaction mixture was cooled to room temperature and diluted with H_2O (50 mL). The precipitate was filtered and the residue was washed with H_2O (3 × 50 mL) and dried under reduced pressure. The crude was purified by DCVC (0 → 60% EtOAc in heptane), which yielded a yellow solid (2.19 g, 9.22 mmol, 11%). This reaction also gave 6-(6-(pyridin-2-yl)-1,2-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-amine, as a yellow solid, in 23% yield. LCMS (ESI) *m/z* = 239.2 $[M + H]^+$; ^{1}H NMR (600 MHz, DMSO) δ 8.96 (s, 2H), 8.66 – 8.62 (m, 2H), 7.98 (dt, *J* = 8.0, 1.2 Hz, 2H), 7.93 (td, *J* = 7.7, 1.7 Hz, 2H), 7.53 (ddd, *J* = 7.3, 4.8, 1.3 Hz, 2H). ^{13}C NMR (151 MHz, DMSO) δ 148.6, 147.2, 146.3, 137.4, 125.3, 121.0. Characterization is in accordance to reference.[4]

3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine (5)



5a (2.00 g, 8.39 mmol, 1 equiv.) was suspended in dry DCM (40 mL) and the solution was cooled to 0 °C. PIDA (3.24 g, 10.07 mmol, 1.2 equiv.) was added portion wise to the reaction mixture. After complete addition, the reaction mixture was stirred at room temperature for 4 h. The reaction mixture was directly coated on Celite® and purified by DCVC (50 → 100% DCM in heptane), which yielded a pink solid (1.37 g, 5.81 mmol, 69%). LCMS (ESI) m/z = 237.2 [M + H1H NMR (600 MHz, DMSO) δ 8.95 (ddd, J = 4.7, 1.8, 0.9 Hz, 2H), 8.62 (dt, J = 7.9, 1.1 Hz, 2H), 8.17 (td, J = 7.8, 1.8 Hz, 2H), 7.74 (ddd, J = 7.6, 4.7, 1.2 Hz, 2H). 13C NMR (151 MHz, DMSO) δ 163.3, 150.6, 150.1, 137.8, 126.7, 124.3. Characterization is in accordance to reference.[5]

Section 2: Radiochemistry

Materials

Unless otherwise stated, all reagents and solvents were purchased from commercial suppliers and used without further purification. [^{111}In]$InCl_3$ (non-carrier added) was purchased from Curium. 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) -PEG_{11}-tetrazine was received from Tagworks Pharmaceuticals. All the water used was ultrapure (> 18.2 MΩ cm^{-1}). Other solvents were analytical or HPLC grade and used as received.

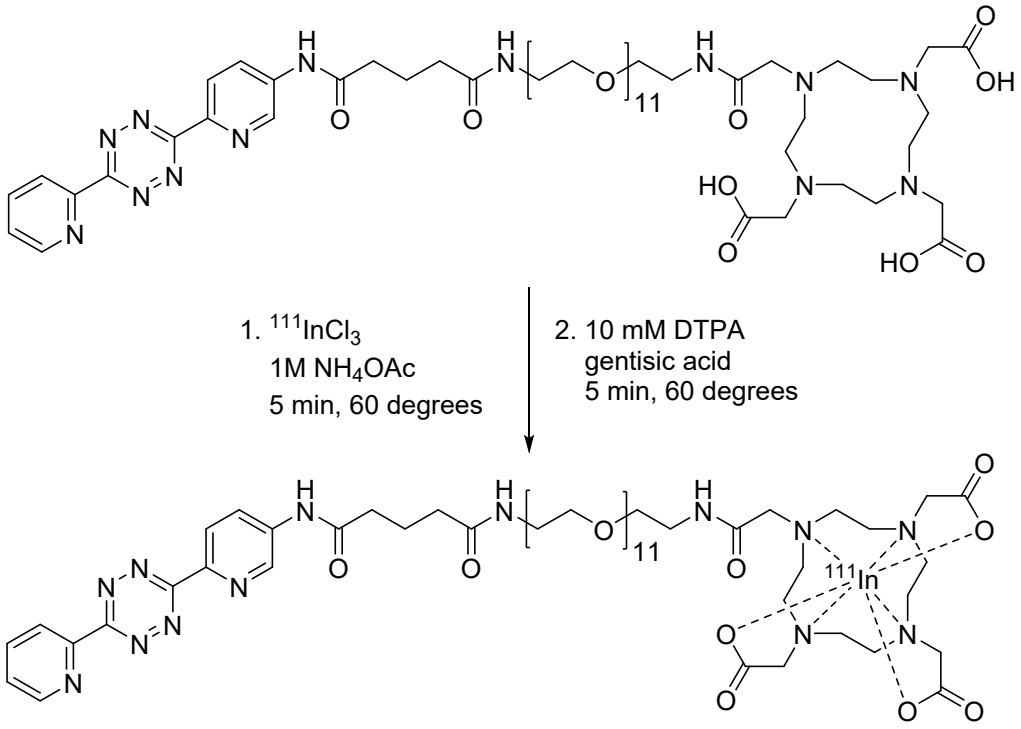
General information

Radiochemistry was performed at the Department of Clinical Physiology, Nuclear Medicine and PET, Rigshospitalet, Denmark. Analytical HPLC was performed using a DionexUltiMate 3000 HPLC system (Thermo Scientific), connected to a 170U UV-D detector, a Scansys radiodetector and a Dionex system connected to a P680A pump. The system was controlled by Chromeleon software.

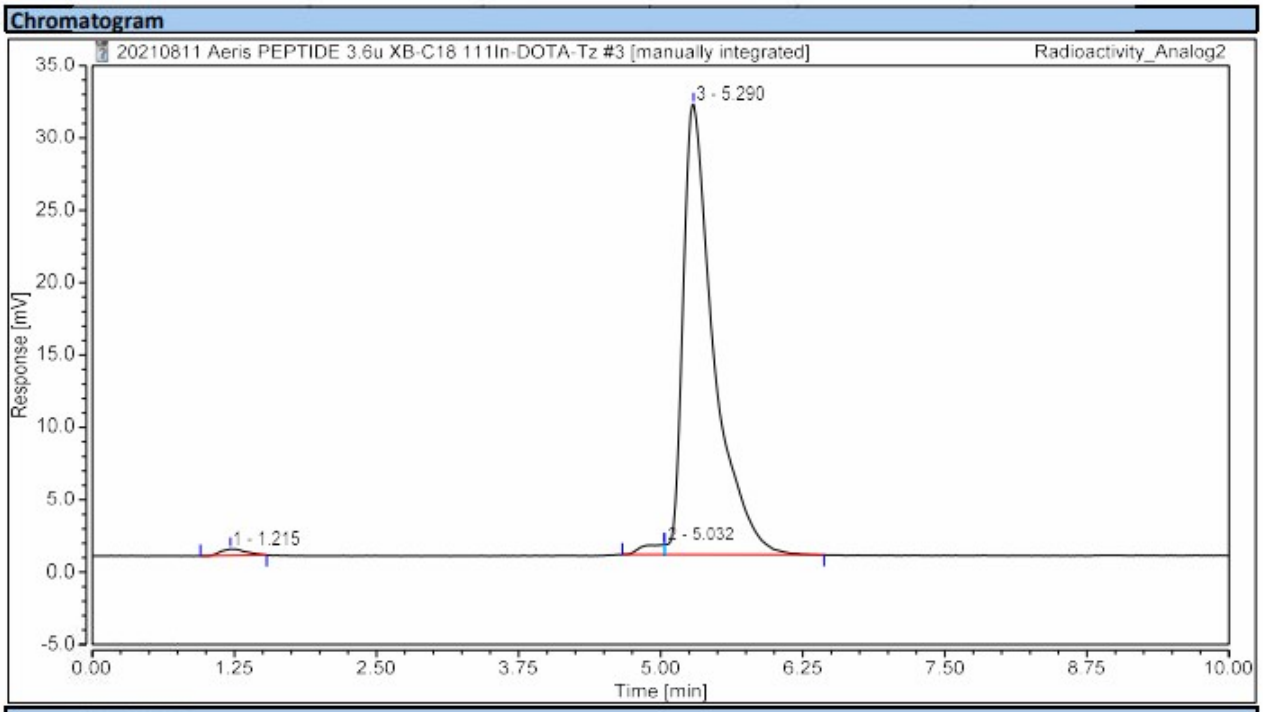
The radiochemical conversion (RCC) and the radiochemical purity (RCP) of the radiolabeled compounds were determined by analyzing an aliquot of the reaction mixture by radio-HPLC analysis followed by

integration of the radioactive peaks of the chromatogram.[6] RCC and RCP values are given as mean values.

^{111}In-labeling DOTA-tetrazine



1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-PEG_{11}-tetrazine (**6a**) was synthesized as previously published.[7] **6a** was dissolved (2 mg/mL) in metal-free water and stored at -80 °C before use. In general, an aliquot of 50-100 µL (10-30 MBq) of [^{111}In]indium chloride in 0.1 M HCl was combined with 2 µL DOTA-PEG_{11}-tetrazine and 1 M NH_4OAc buffer (pH 5.5) at a volume ratio of 1:10 was added. The mixture was shaken at 600 rpm for 5 min at 60 °C in an Eppendorf ThermoMixer C. Then, 10 mM diethylenetriamine-pentaacetic acid DTPA (volume ratio 1:10) and 2 µL 10 mg/mL gentisic acid in saline was added and the solution was shaken for an additional 5 min at 60 °C in an Eppendorf ThermoMixer C. Typically, a quantitative labeling yield and a radiochemical purity (RCP) greater than 95% were obtained with this method, as confirmed by radio-HPLC (Figure S 1).



Integration Results

No.	Retention Time min	Area mV*min	Height mV	Relative Area %	Relative Height %	Amount n.a.
1	1.215	0.111	0.440	1.12	1.36	n.a.
2	5.032	0.154	0.698	1.55	2.16	n.a.
3	5.290	9.702	31.115	97.34	96.47	n.a.
Total:		**9.968**	**32.252**	**100.00**	**100.00**	

Figure S 1. Radio-HPLC chromatogram of ^{111}In-DOTA-PEG$_{11}$-Tz

TCO titration by SDS-PAGE with ^{111}In-tetrazine

Titration experiments were conducted to quantify the amount of reactive TCOs per HSA-conjugate according to previously recorded protocols.[8] ^{111}In-labeled Tz stock was diluted accordingly to add 5 µL an excess of ^{111}In-labeled Tz (2 eq. of Tz per HSA-conjugate) to 5 µL of TCO-HSA and the mixture was shaken at 600 rpm for 1 hour at 37 °C. 3 µL NuPAGE™ LDS Sample Buffer (NP0007, Invitrogen) was added and the mixture was shaken vigorously for 10 seconds. Samples were applied to NuPAGE™ 4 to 12%, Bis-Tris, 1.0 mm, Mini Protein Gel, 12-well (NP0322BOX , Invitrogen) SDS-PAGE gels. SDS-PAGE gels were exposed to phosphor storage screens and read by a Cyclone Storage Phosphor System (PerkinElmer Inc.). Quantification of plate readings was done with Optiquant software (version 5.00, PerkinElmer Inc.).

Computational simulations

Molecular dynamics simulations of the spatial arrangement of the linkers **1** and **2** within HSA·**1** and HSA·**2** were performed in the program Molecular Operating Environment (MOE) from Chemical Computing Group using an Amber10:EHT force field. The HSA crystal structure used for the simulations was PDB 1N5U.[9] The structure of HSA was fixed except for the Cys34 residue (including the protein backbone part) and **1** or **2**, respectively. A solvent sphere with a diameter of 10 Å was placed around the Cys34 residue and **1** or **2**. Repeated cycles of heating/cooling were then conducted to elucidate the preferred conformation of **1** and **2**. The simulations showed that **1** had its olefin double bond at a distance of 17.0 Å from the Cys34 sulfhydryl (Figure S2, A-B), whereas for **2**, wrapping of the linker around itself caused the distance between the olefin double bond and the Cys34 sulfhydryl to be 15.0 Å (Figure S2, C-D).

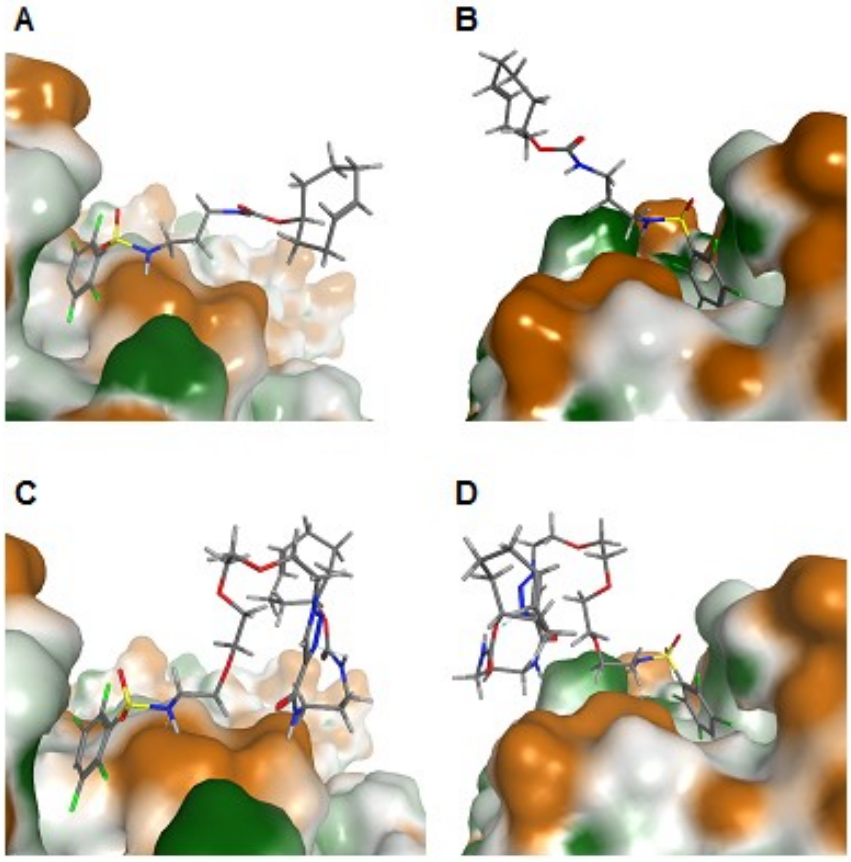


Figure S 2. Computational simulation of HSA·**1** (A-B) and HSA·**2** (C-D), conducted in Molecular Operating Environment.

Section 3: Analytical spectra

Characterization of pentafluorobenzene-TCO (1)

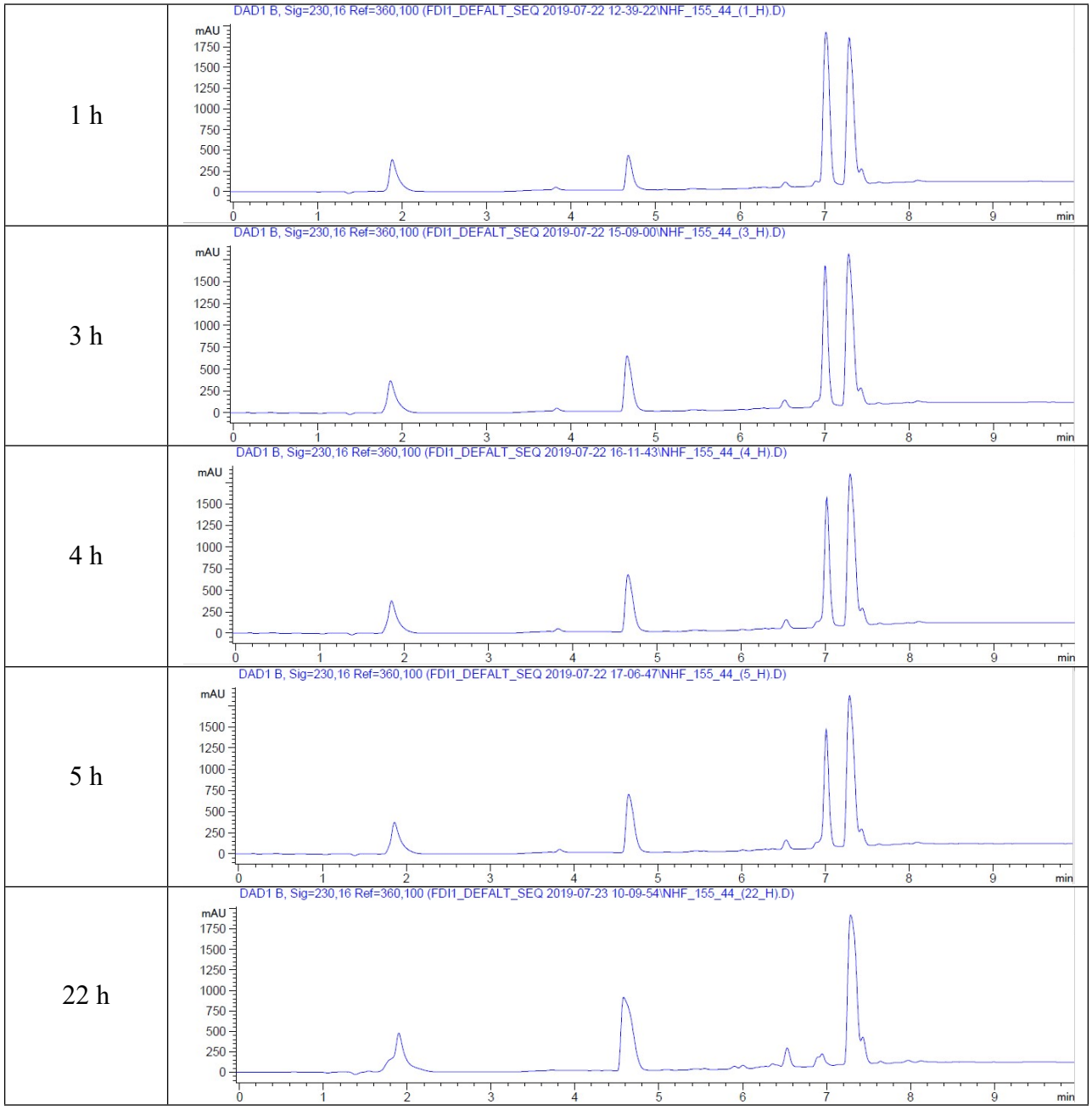


Figure S 3. Kinetic study of the synthesis of construct **1** by addition of TCO-amine to pentafluorobenzenesulfonyl chloride in CH_2Cl_2.

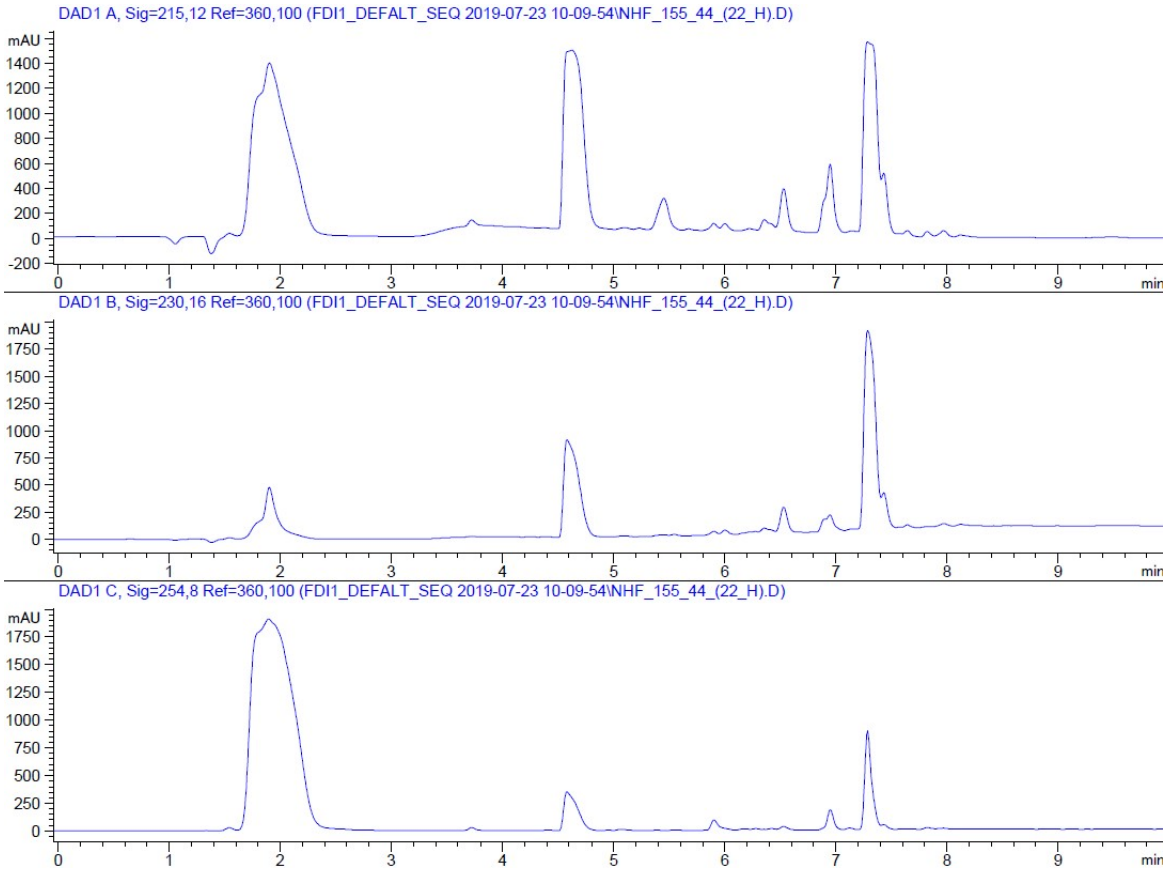


Figure S 4. HPLC spectra of reaction mixture after 22 h of reaction between pentafluorobenzenesulfonyl chloride and TCO-amine using 2,6-lutidine as base. Recorded at (from top to bottom) 215, 230, and 254 nm.

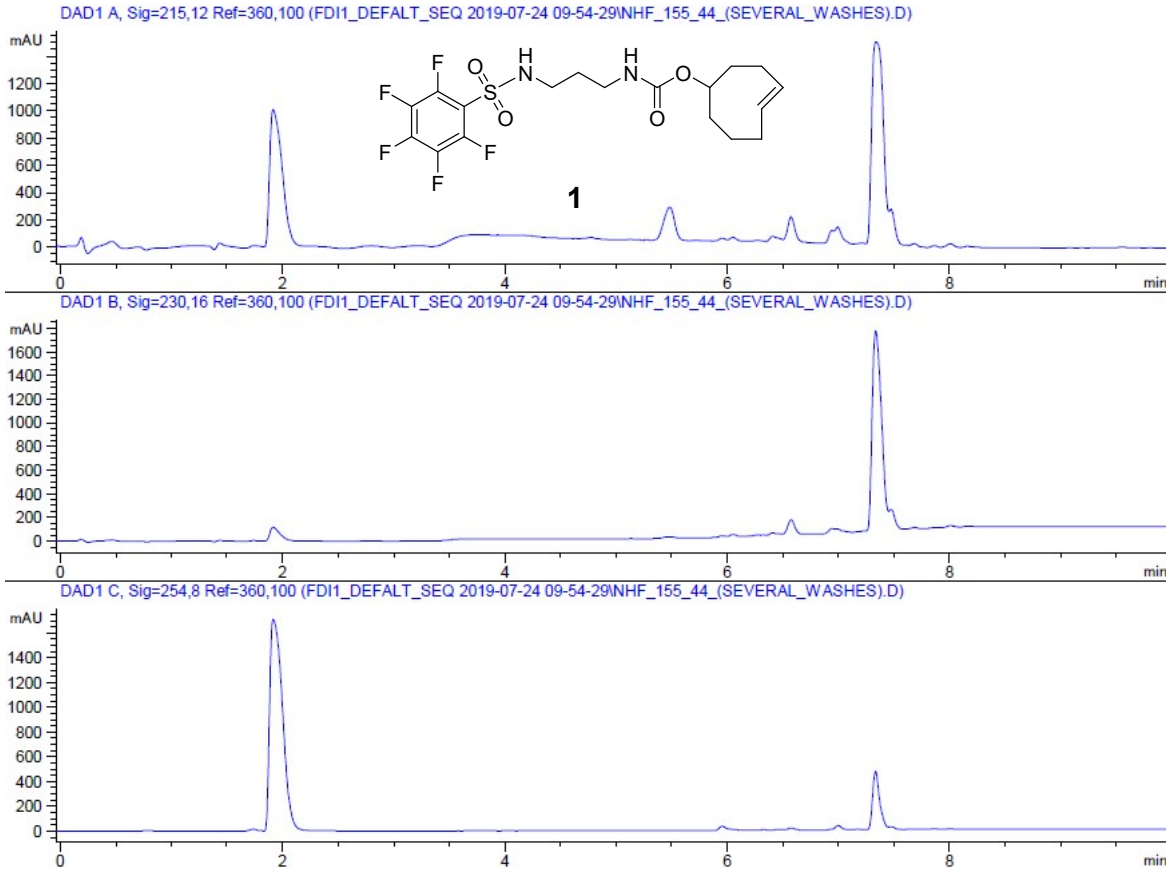


Figure S 5. HPLC spectra of construct **1** after removal of pentafluorobenzenesulfonic acid residue using six extractions with Milli-Q H_2O. Recorded at (from top to bottom) 215, 230, and 254 nm.

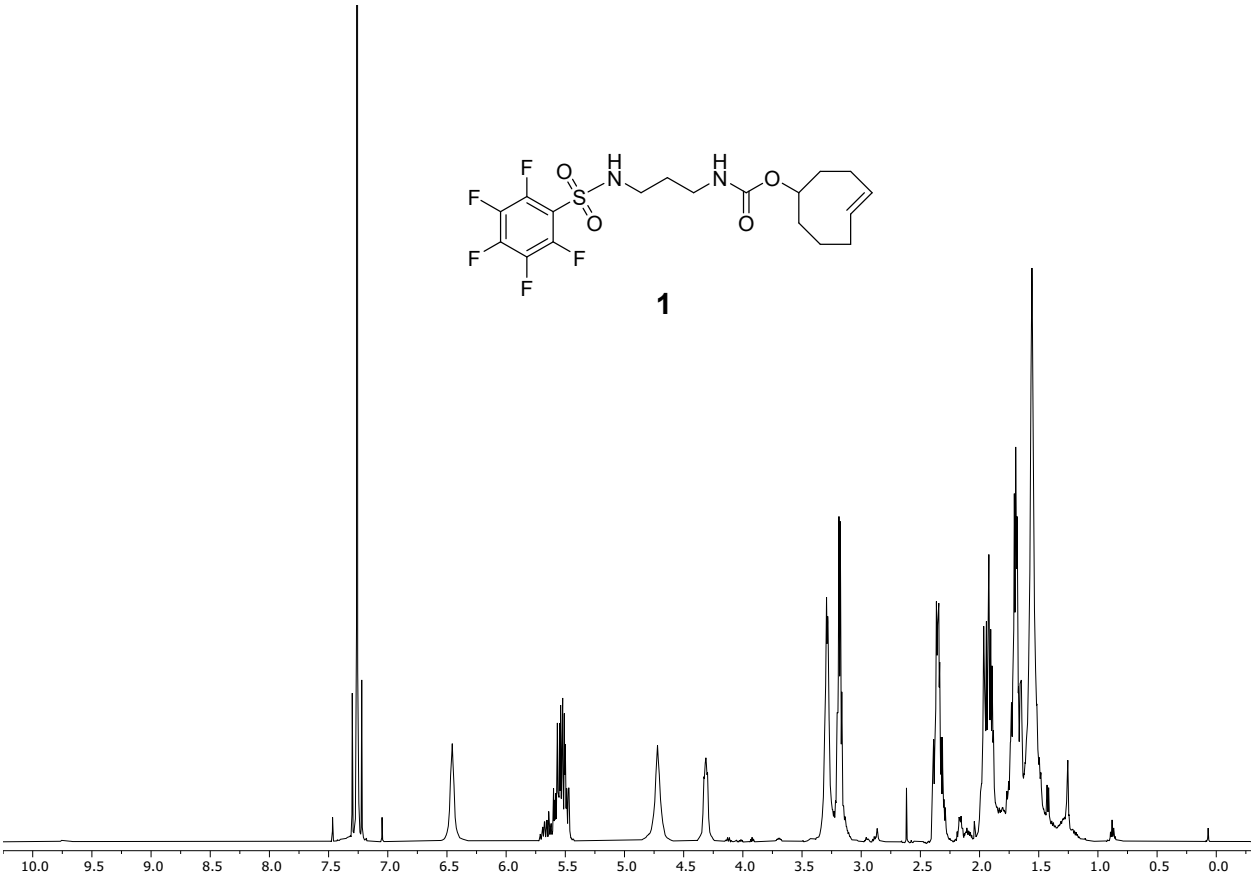


Figure S 6. ^{1}H NMR spectrum of construct **1**, recorded in $CDCl_3$ at 500 MHz.

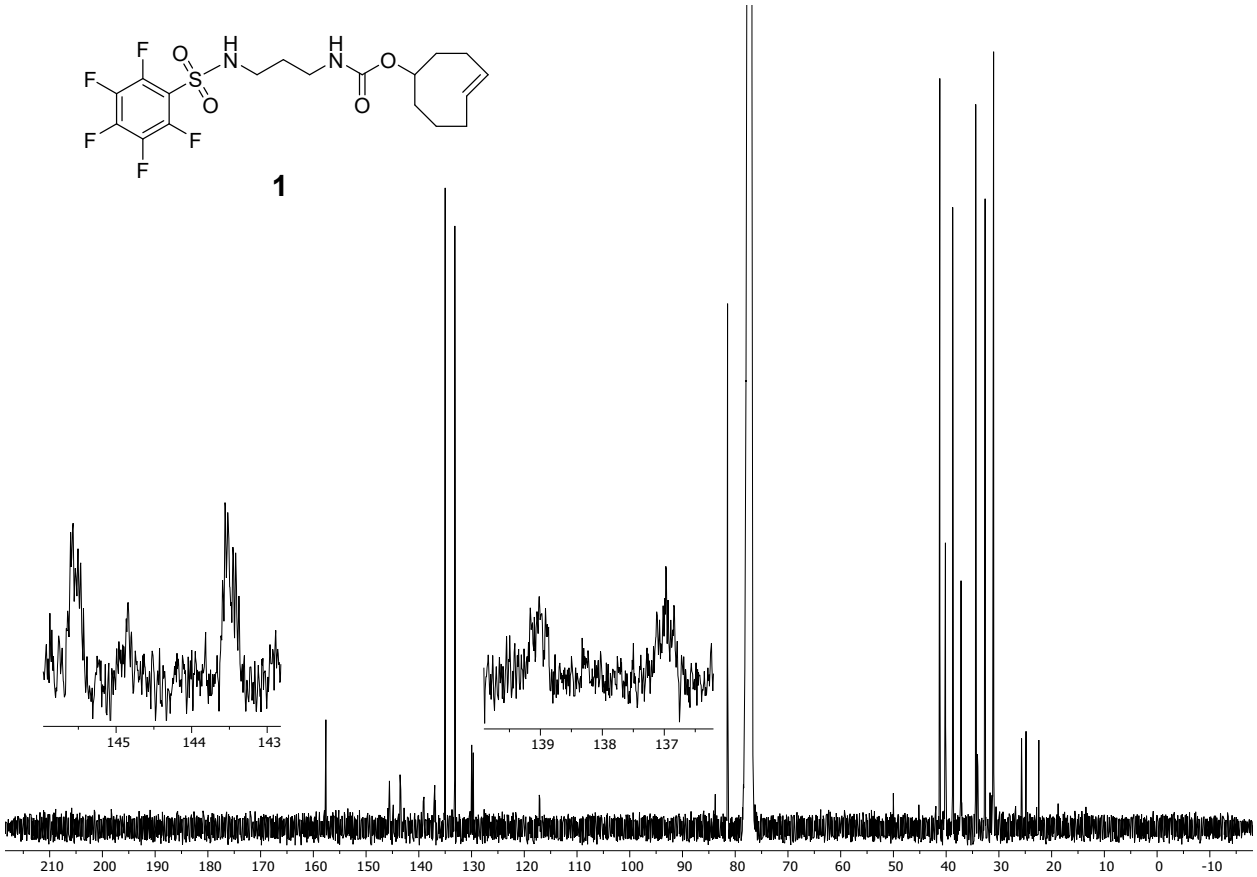


Figure S 7. ^{13}C NMR spectrum of construct **1**, recorded in $CDCl_3$ at 126 MHz.

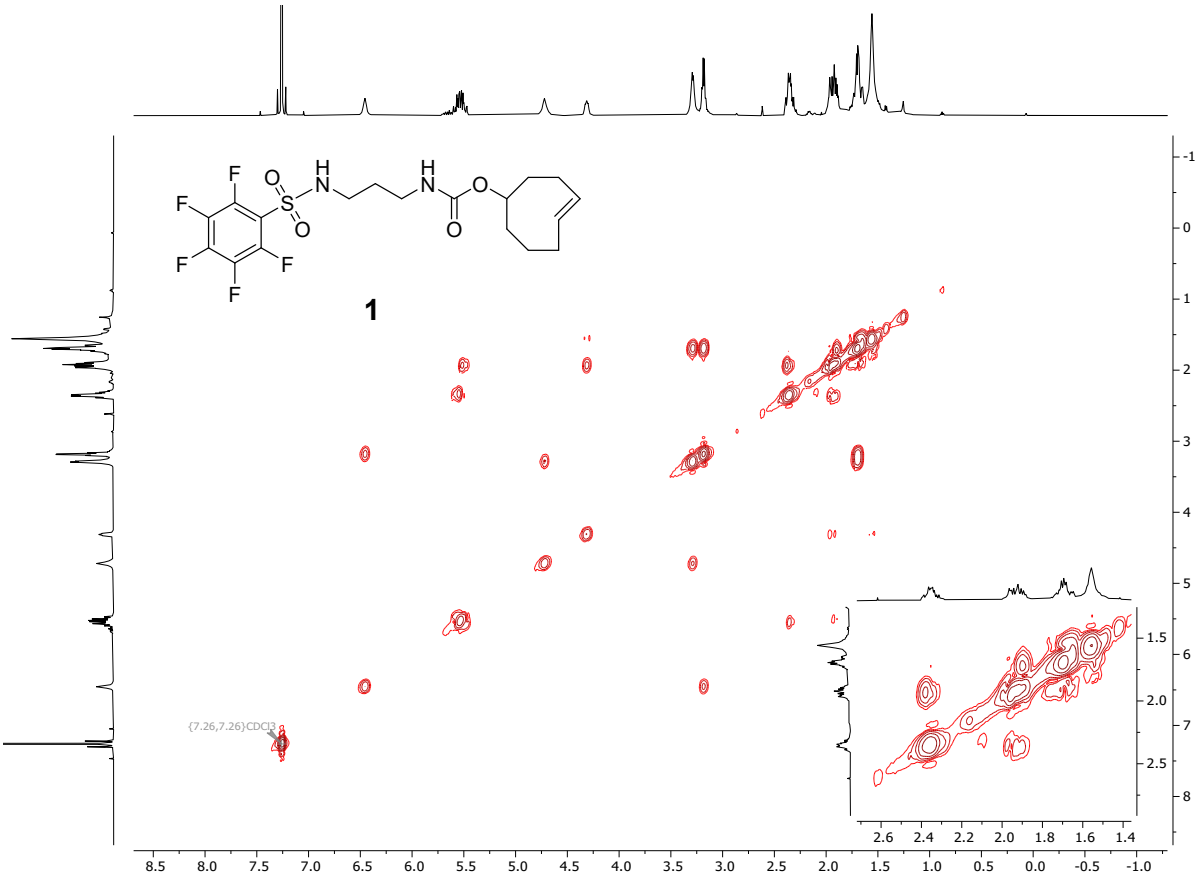


Figure S 8. ^{1}H-^{1}H COSY NMR spectrum of construct **1**, recorded in $CDCl_3$ at 500 MHz.

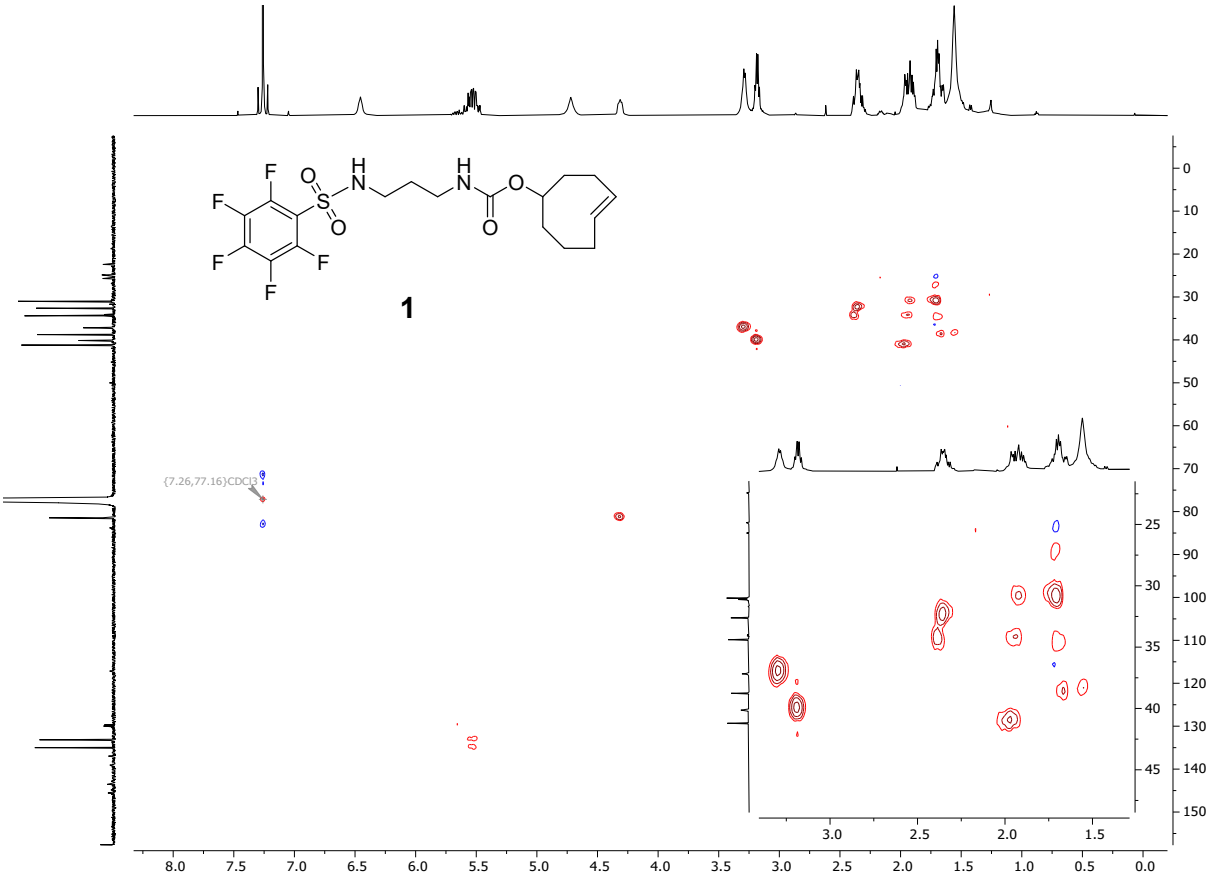


Figure S 9. ^{1}H-^{13}C HSQC NMR spectrum of construct **1**.

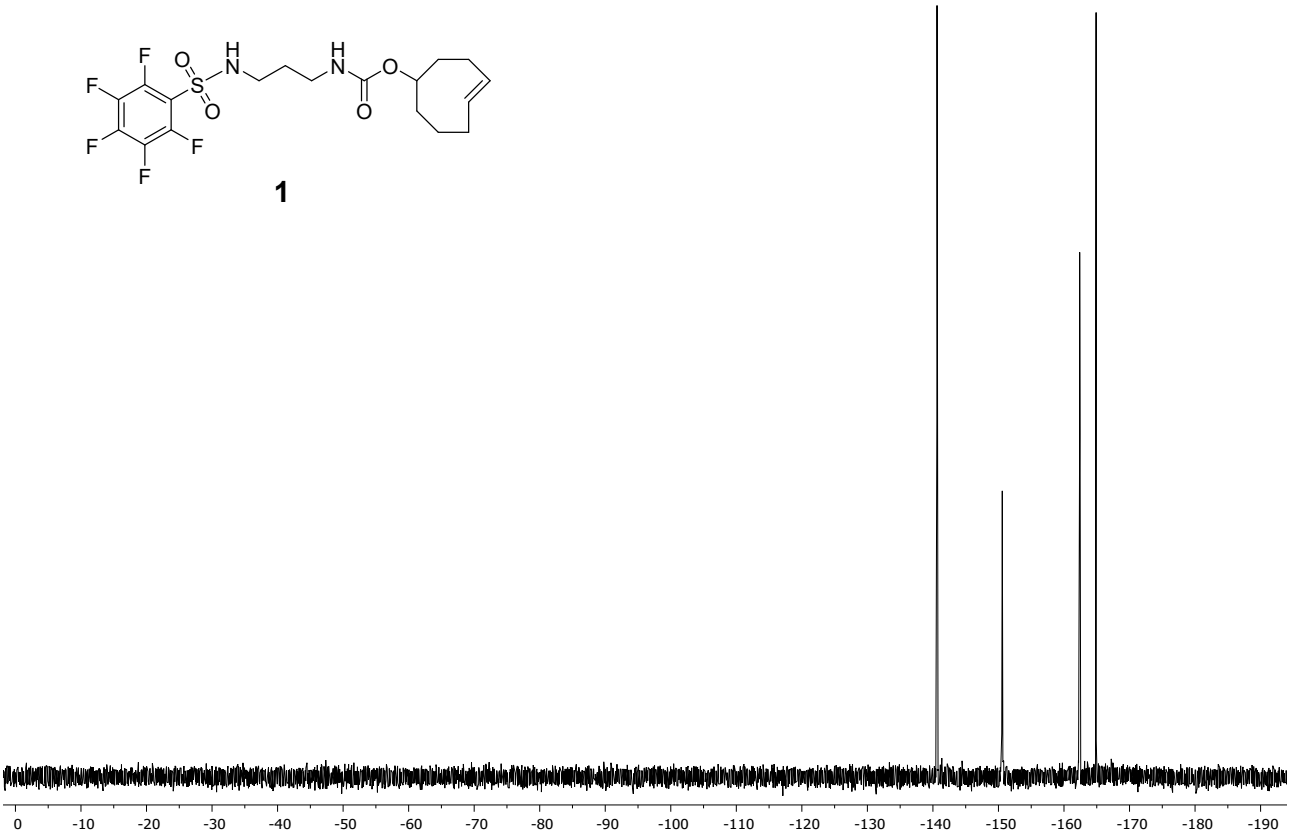


Figure S 10. ^{19}F NMR spectrum of construct **1** recorded in DMSO-d_6 at 470 MHz. Hexafluorobenzene (chemical shift -164.9 ppm) was added as reference.

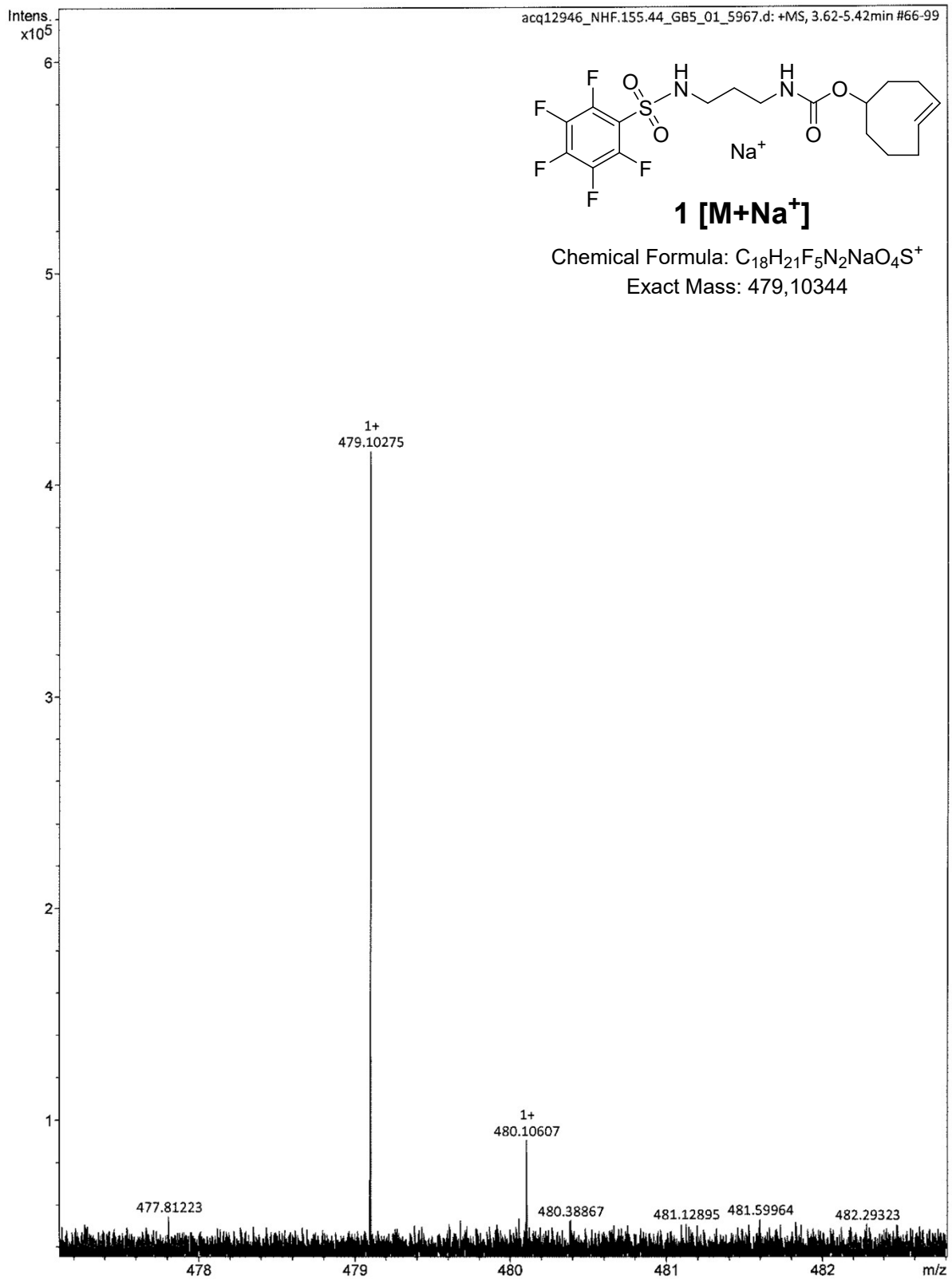


Figure S 11. HRMS spectrum of construct **1**.

Characterization of pentafluorobenzene-TEG-triazole-carboxylic acid (2a)

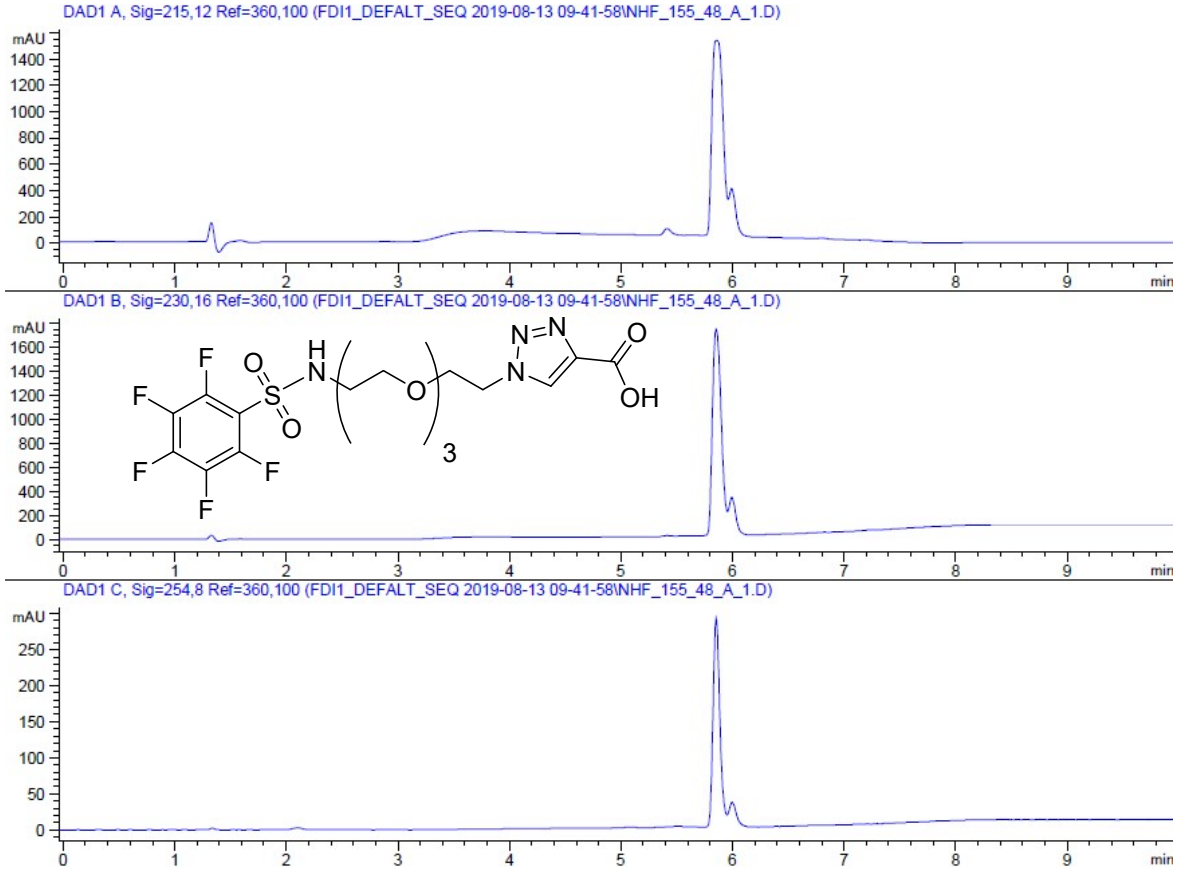


Figure S 12. HPLC spectra of pentafluorobenzene-TEG-triazole-carboxylic acid (**2a**) recorded at (from top to bottom) 215, 230, and 254 nm.

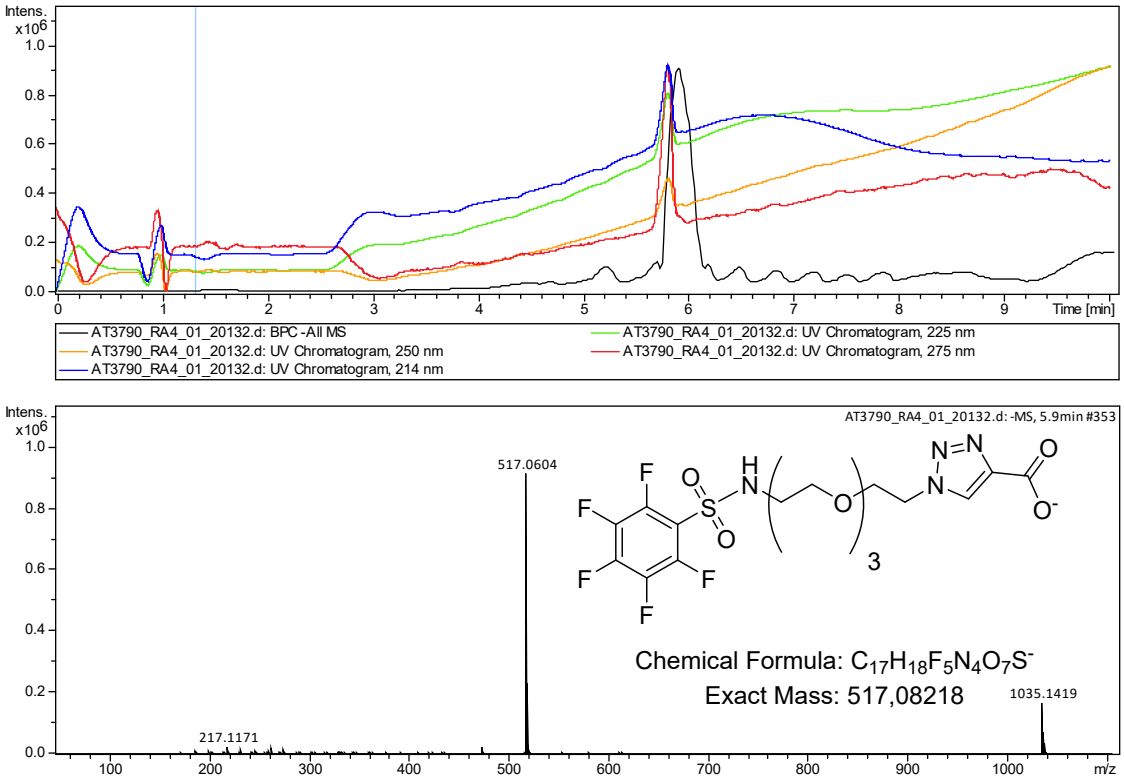


Figure S 13. LC-MS spectrum (top) and mass signal (bottom) of pentafluorobenzene-TEG-triazole-carboxylic acid (**2a**).

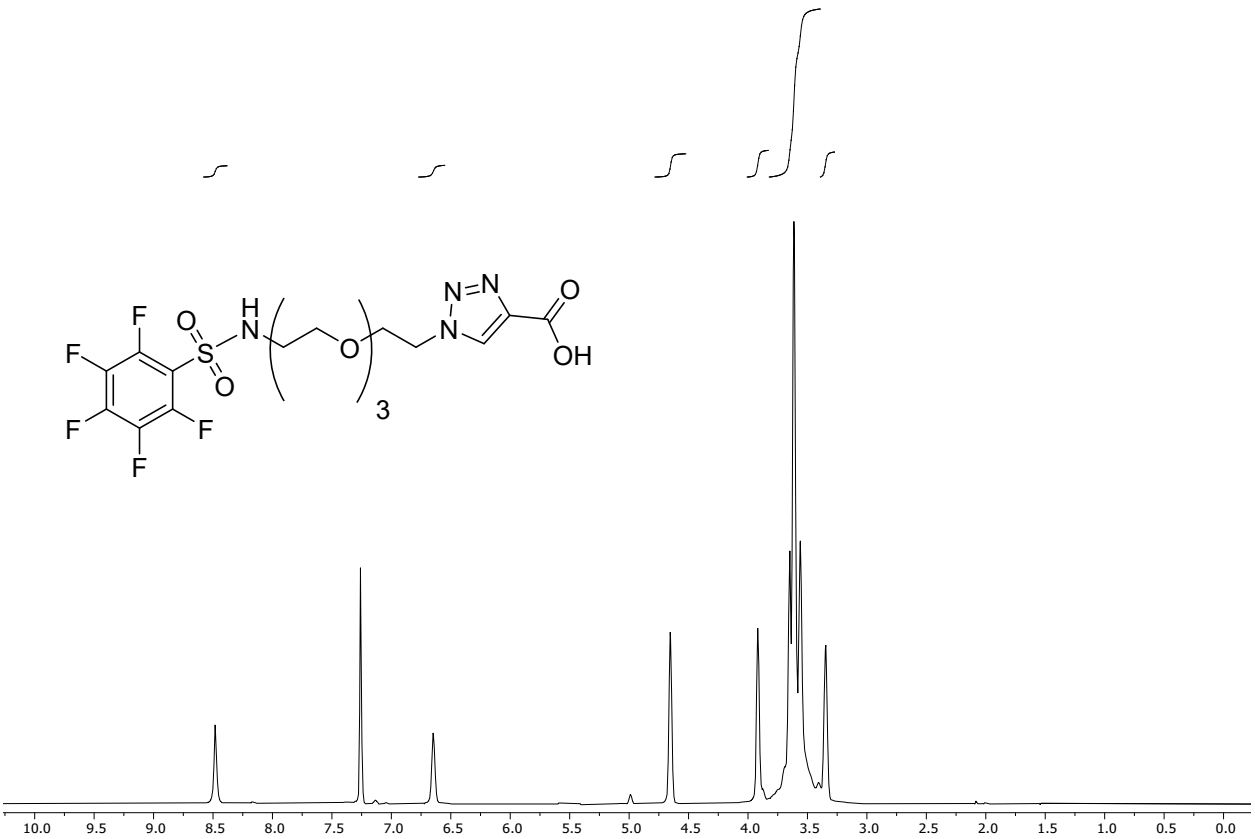


Figure S 14. ^{1}H-NMR spectrum of pentafluorobenzene-TEG-triazole-carboxylic acid (**2a**), recorded in $CDCl_3$ at 500 MHz.

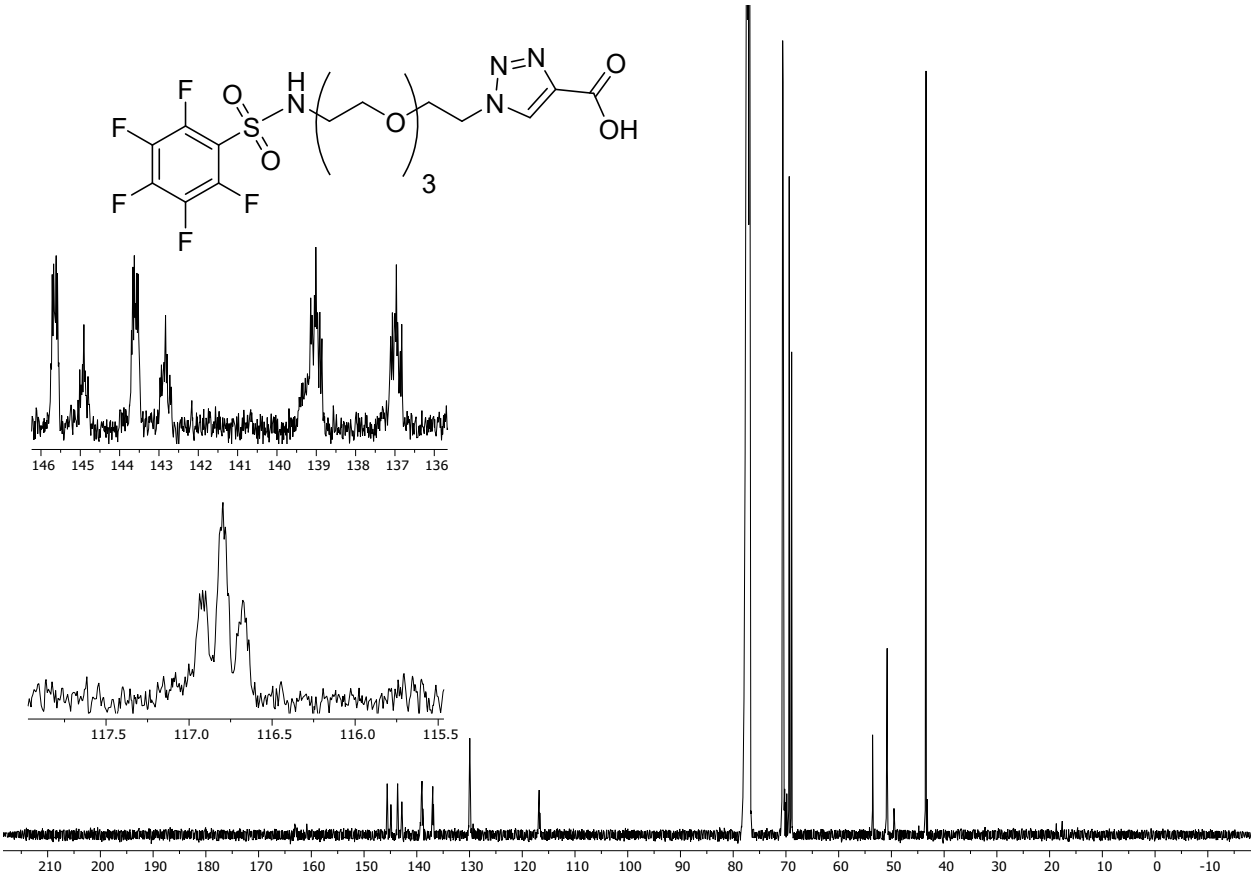


Figure S 15. ^{13}C-NMR spectrum of pentafluorobenzene-TEG-triazole-carboxylic acid (**2a**), recorded in $CDCl_3$ at 126 MHz.

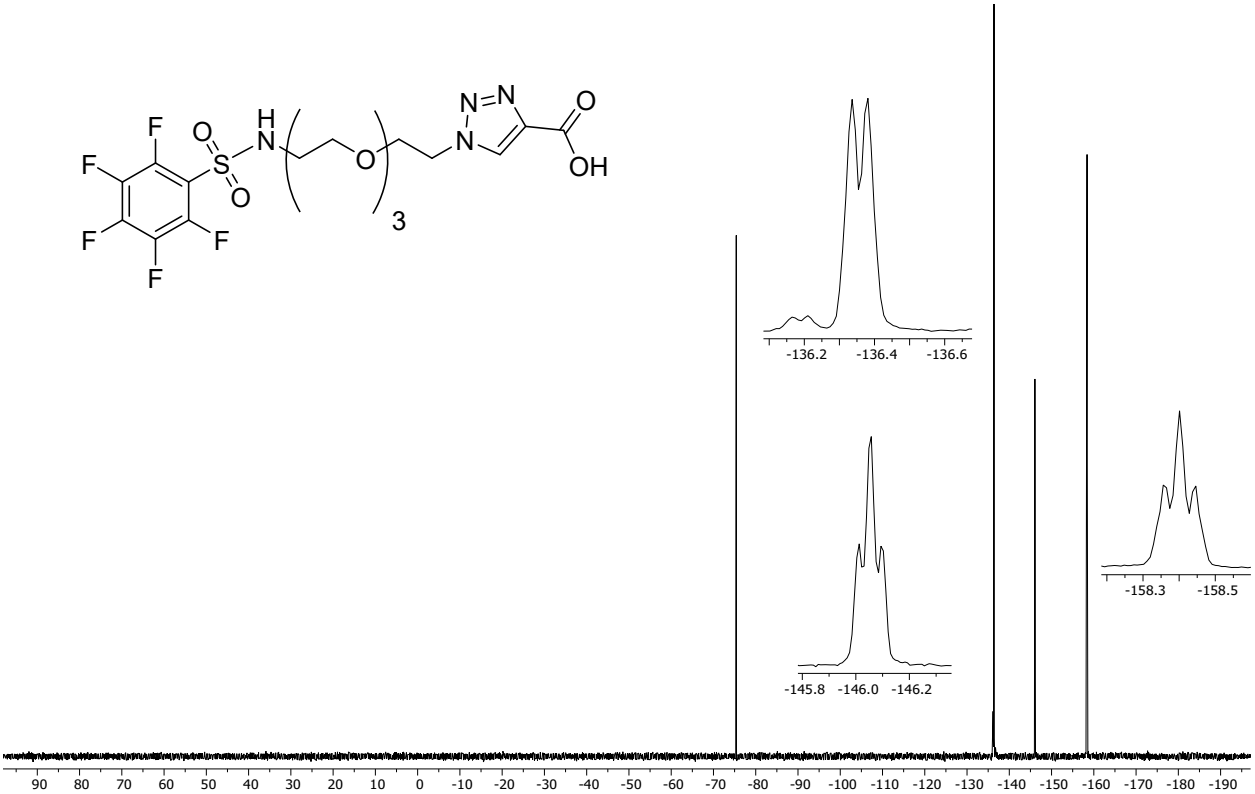


Figure S 16. ^{19}F-NMR spectrum of pentafluorobenzene-TEG-triazole-carboxylic acid (**2a**), recorded in $CDCl_3$ at 470 MHz. Trifluoroacetic acid (δ -75.39 ppm) was added as reference.

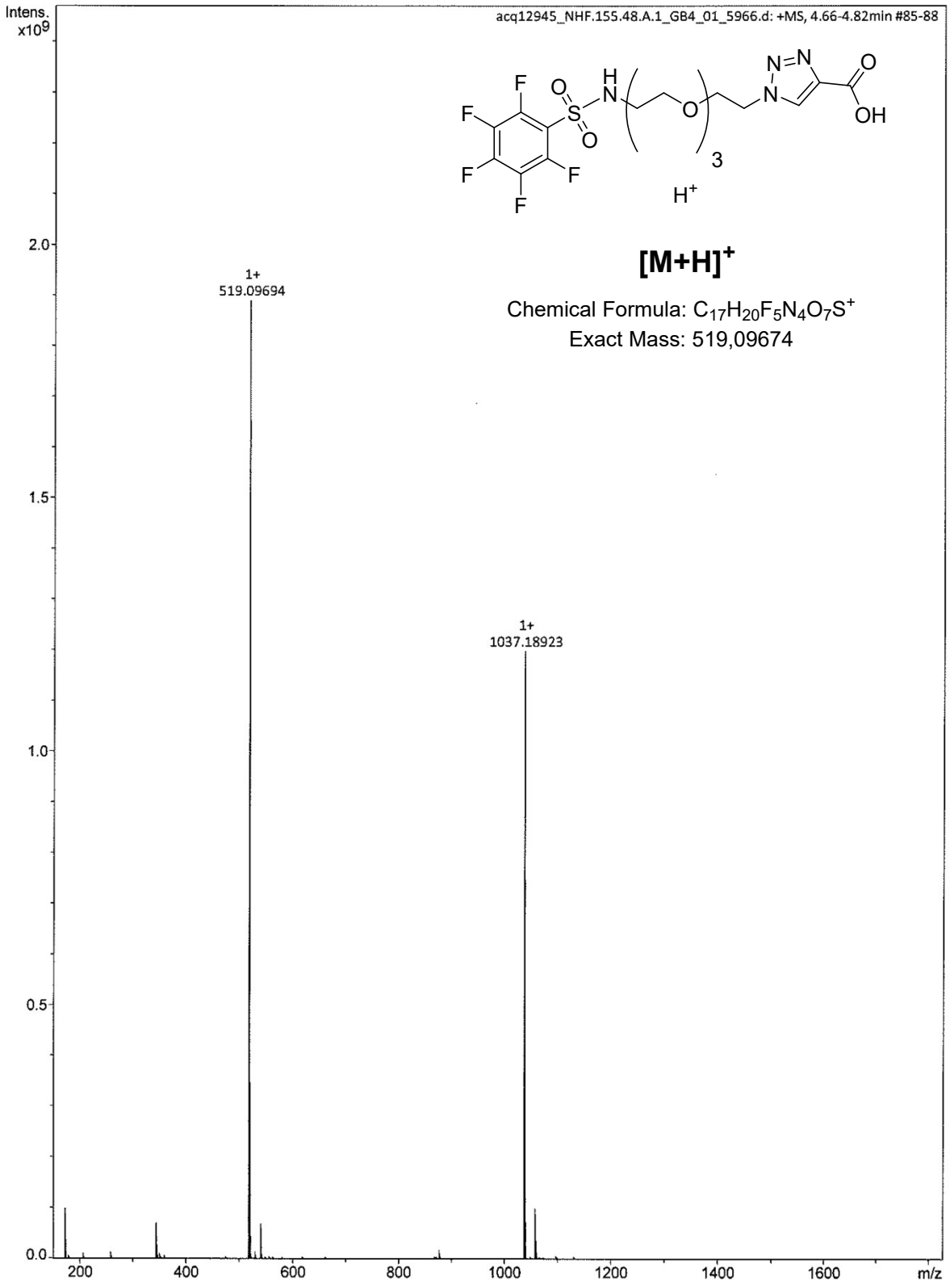


Figure S 17. HRMS spectrum of pentafluorobenzene-TEG-triazole-carboxylic acid (**2a**).

Characterization of pentafluorobenzene-TEG-triazole-TCO (2)

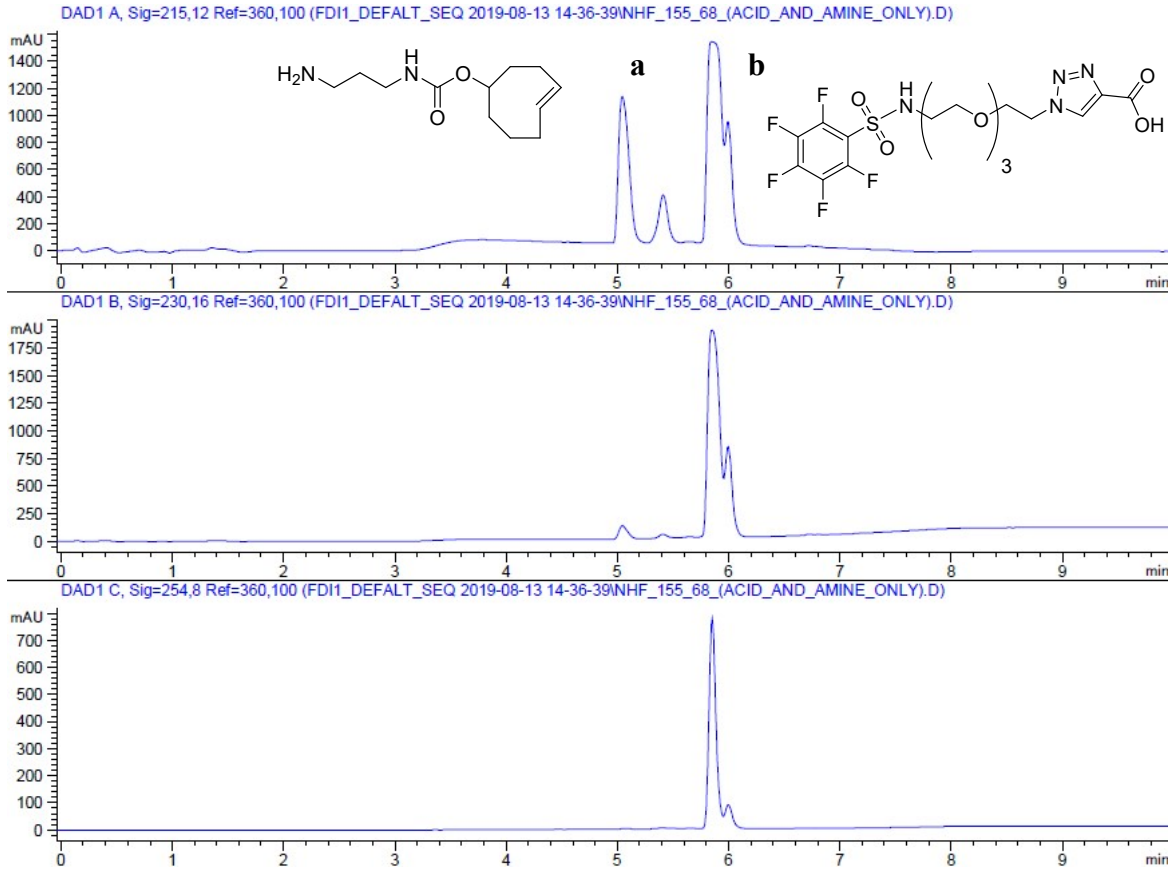


Figure S 18. HPLC spectra of TCO-amine (a) and pentafluorobenzene-TEG-triazole-carboxylic acid **2a** (b) prior to addition of PyBOP and base.

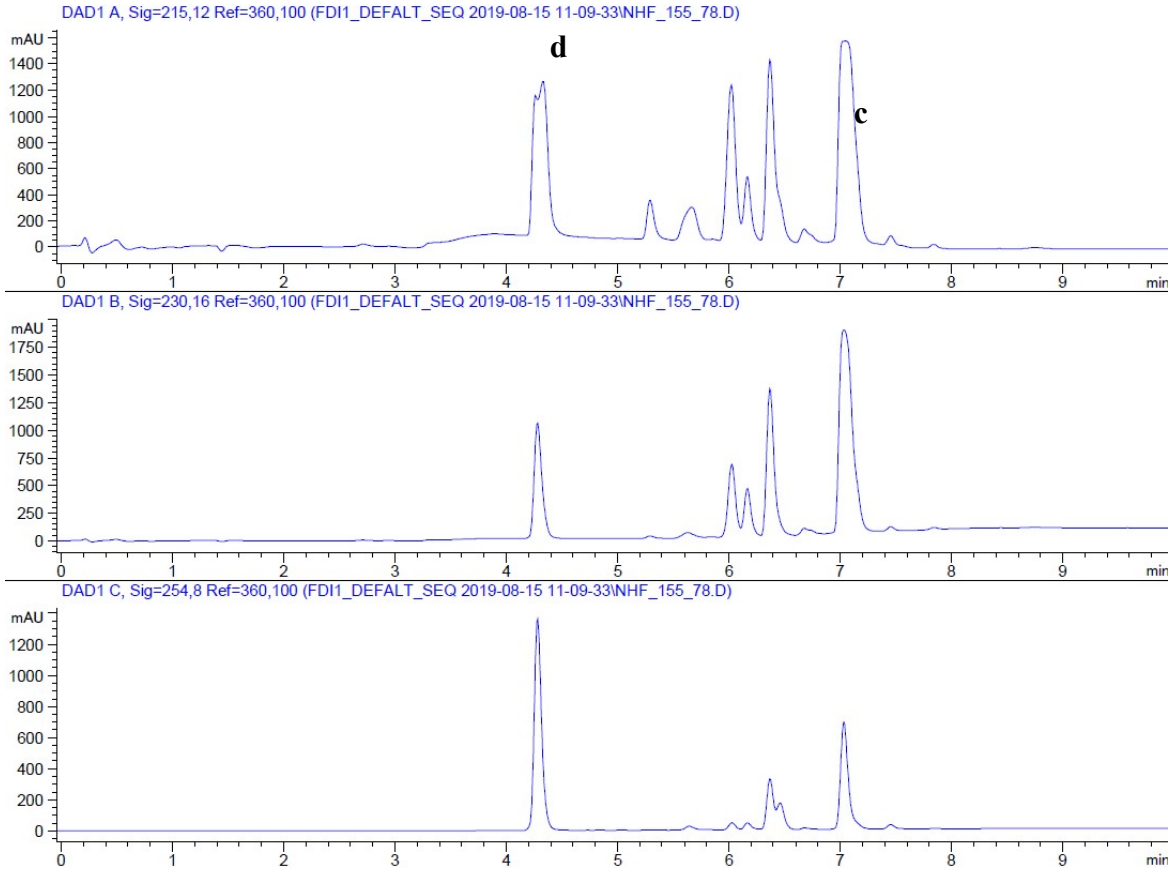


Figure S 19. HPLC spectra of reaction mixture after 50 min of reaction between TCO-amine and construct pentafluorobenzene-TEG-triazole-carboxylic acid using PyBOP as coupling reagent and DIPEA as base. Signal c is construct **2**. Signal d is HOBt. Recorded at (from top to bottom) 215, 230, and 254 nm.

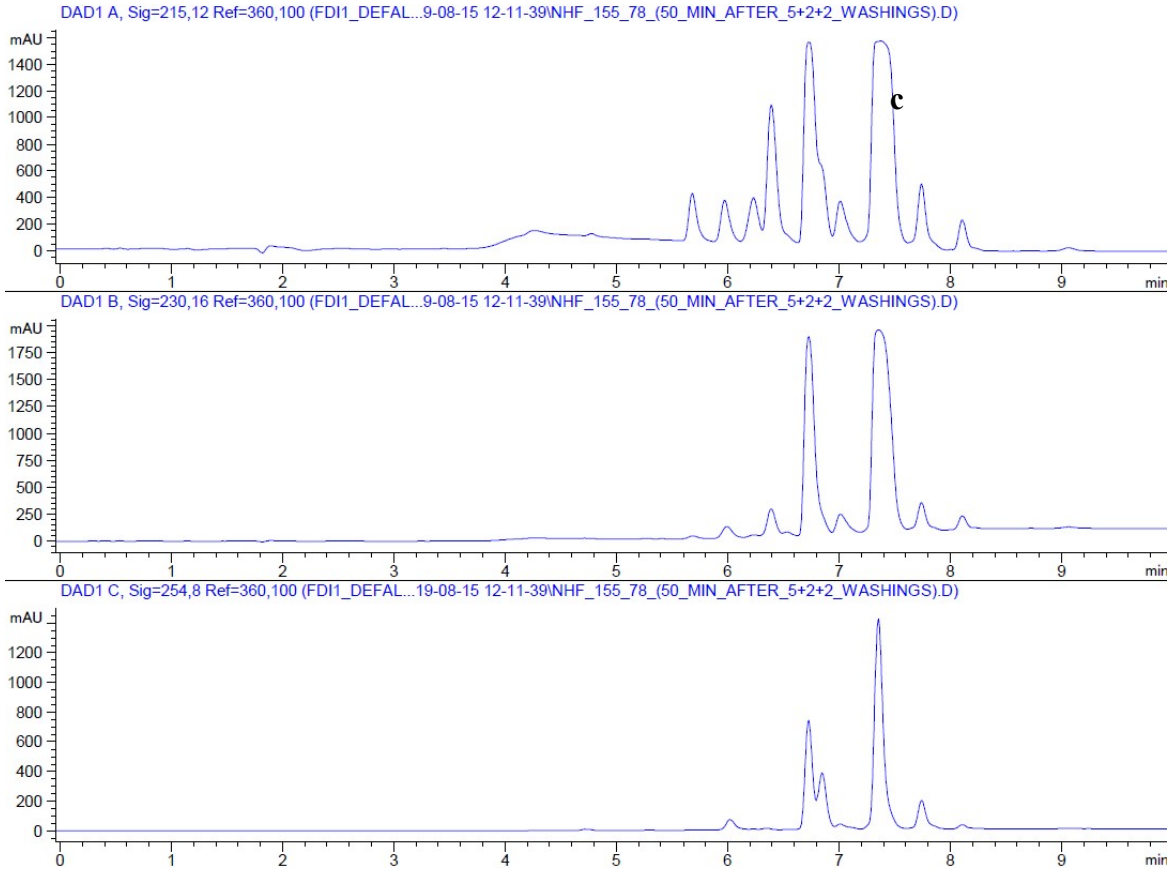


Figure S 20. HPLC spectra of reaction mixture after 50 min of reaction between TCO-amine and pentafluorobenzene-TEG-triazole-carboxylic acid (**2a**) using PyBOP as coupling reagent and DIPEA as base followed by four extractions using 50 µL portions of Milli-Q H_2O, three extractions using 50 µL portions of saturated $NaHCO_3$ (aq), and two extractions using Milli-Q H_2O. Signal c is construct **2**. Recorded at (from top to bottom) 215, 230, and 254 nm.

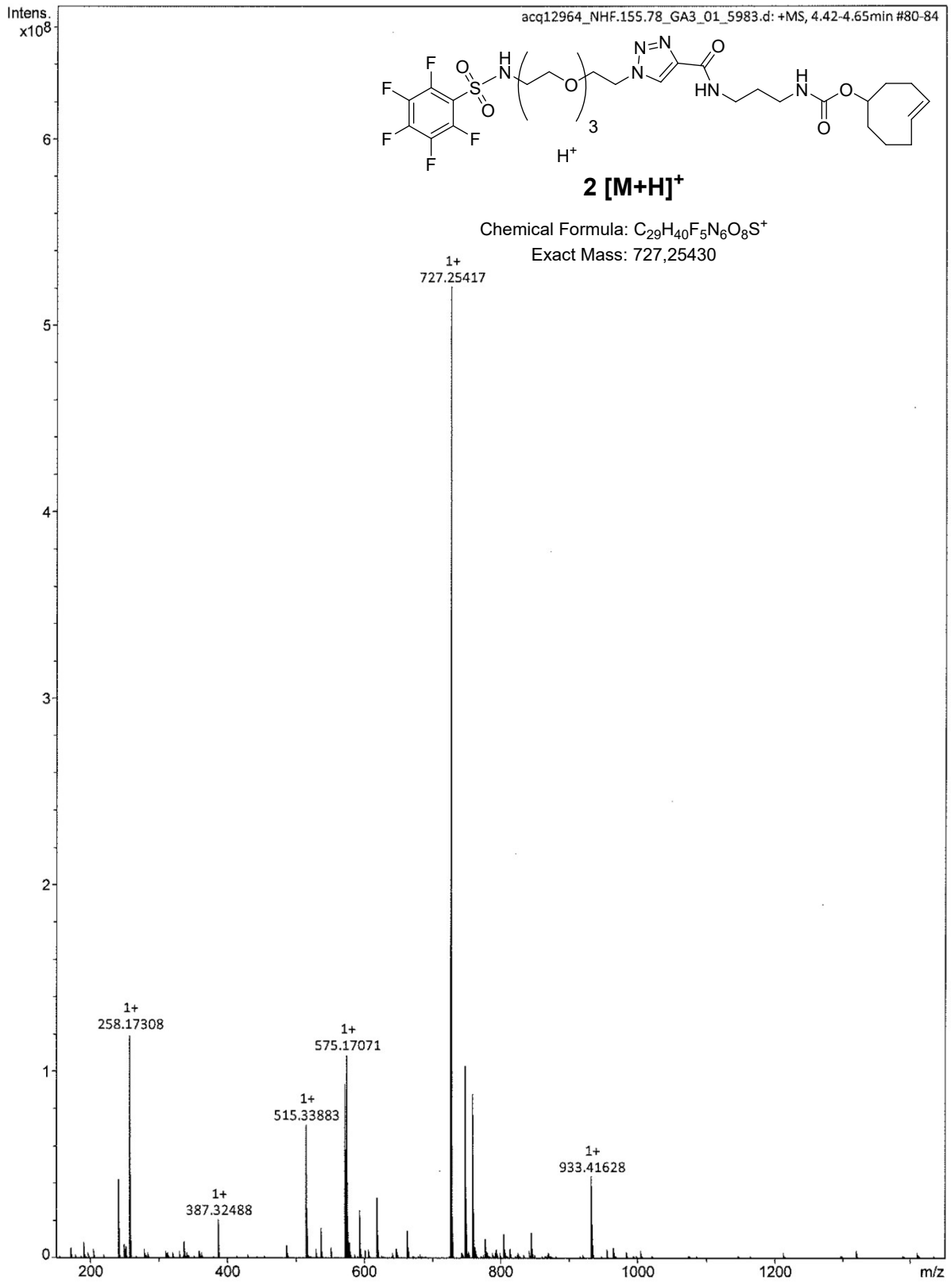


Figure S 21. HRMS spectrum of construct **2**.

LC-MS analysis of HSA

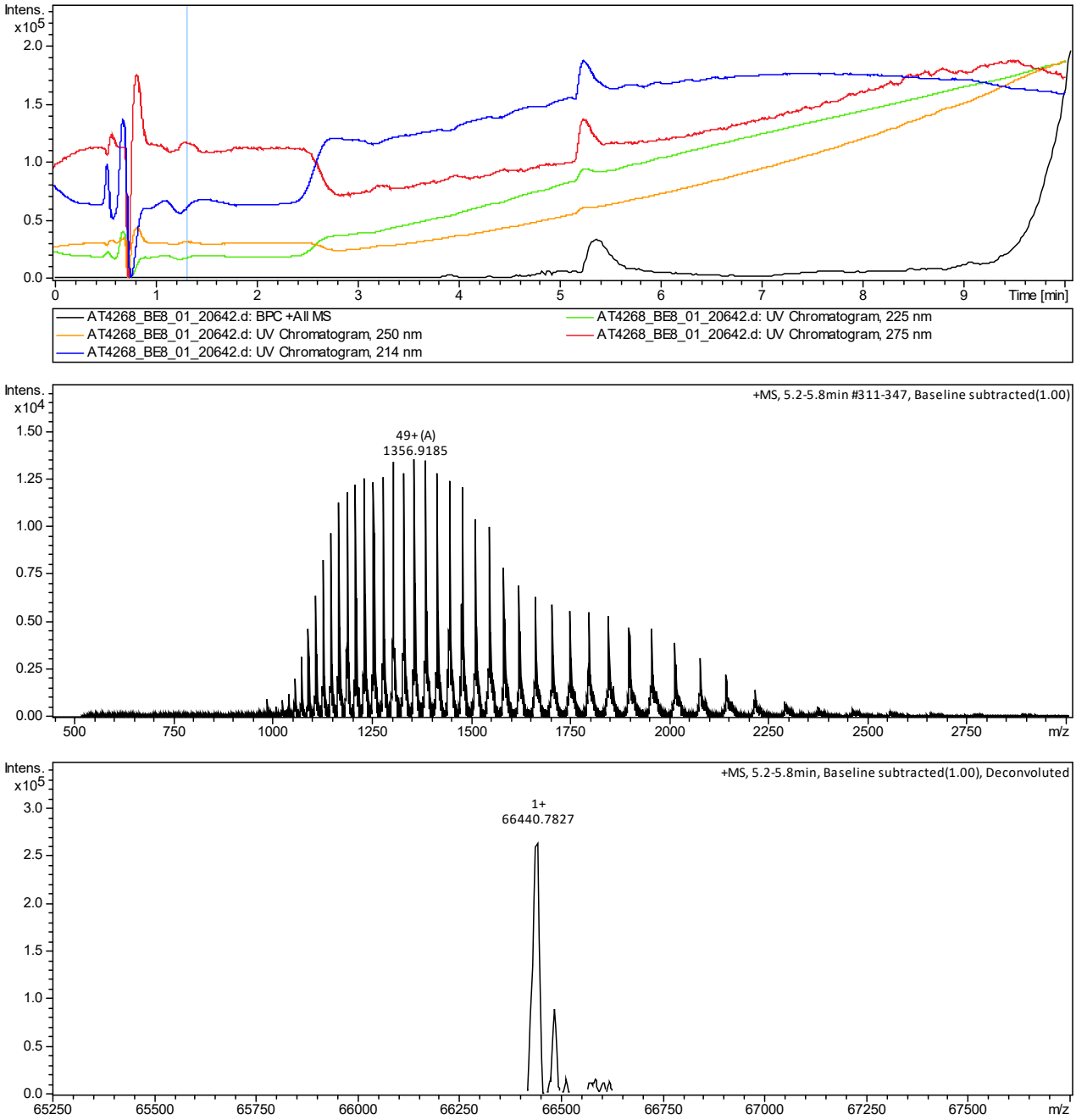


Figure S 22. LC-MS spectrum (top), MS signal (middle), and deconvoluted MS signal (bottom) of HSA reference.

LC-MS analysis of HSA·1

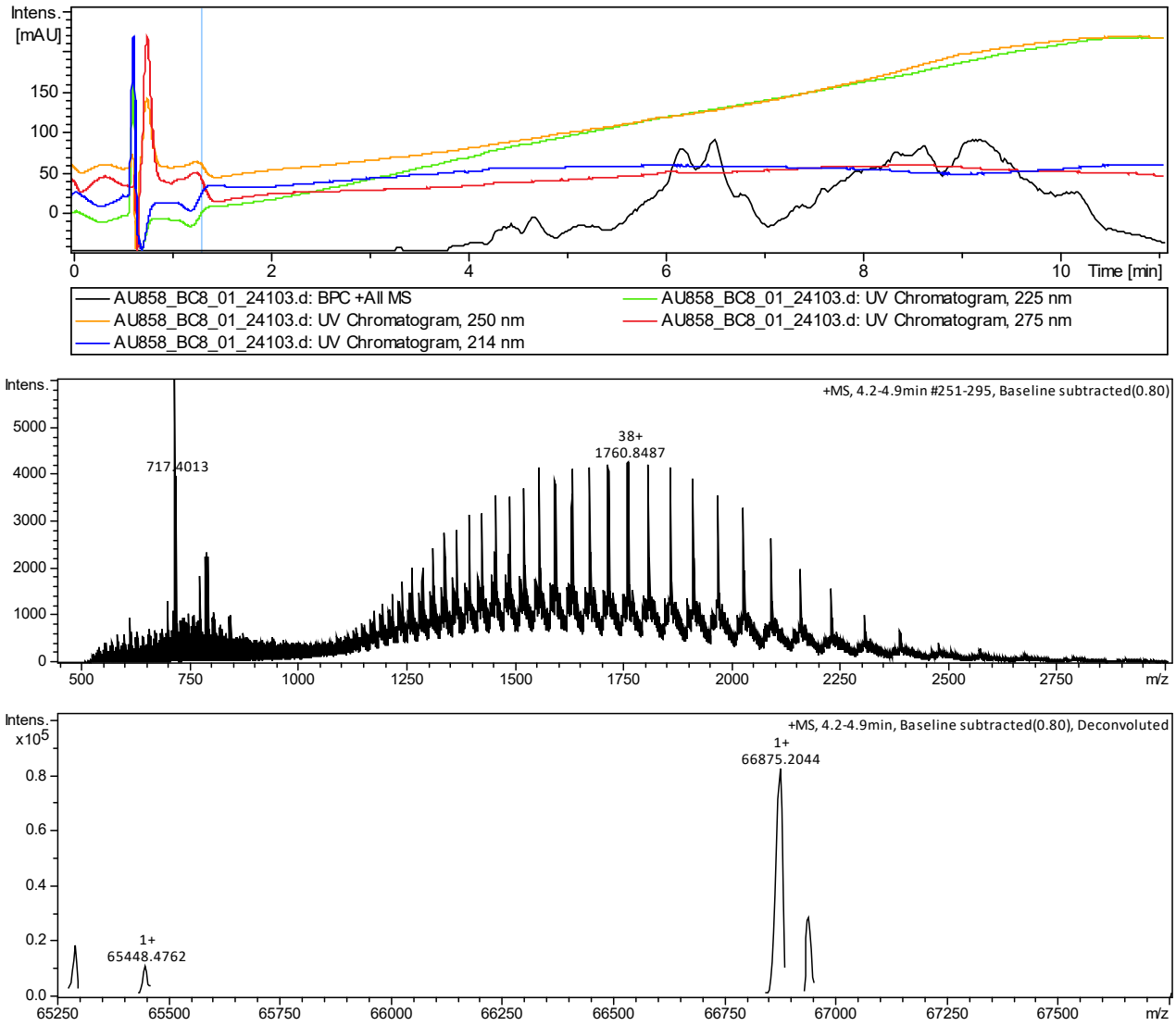


Figure S 23. LC-MS spectrum (top), MS signal (middle), and deconvoluted MS signal (bottom) of HSA·**1** formed after 24 h of reaction between HSA and 20 equiv. of construct **1**.

LC-MS analysis of HSA·2

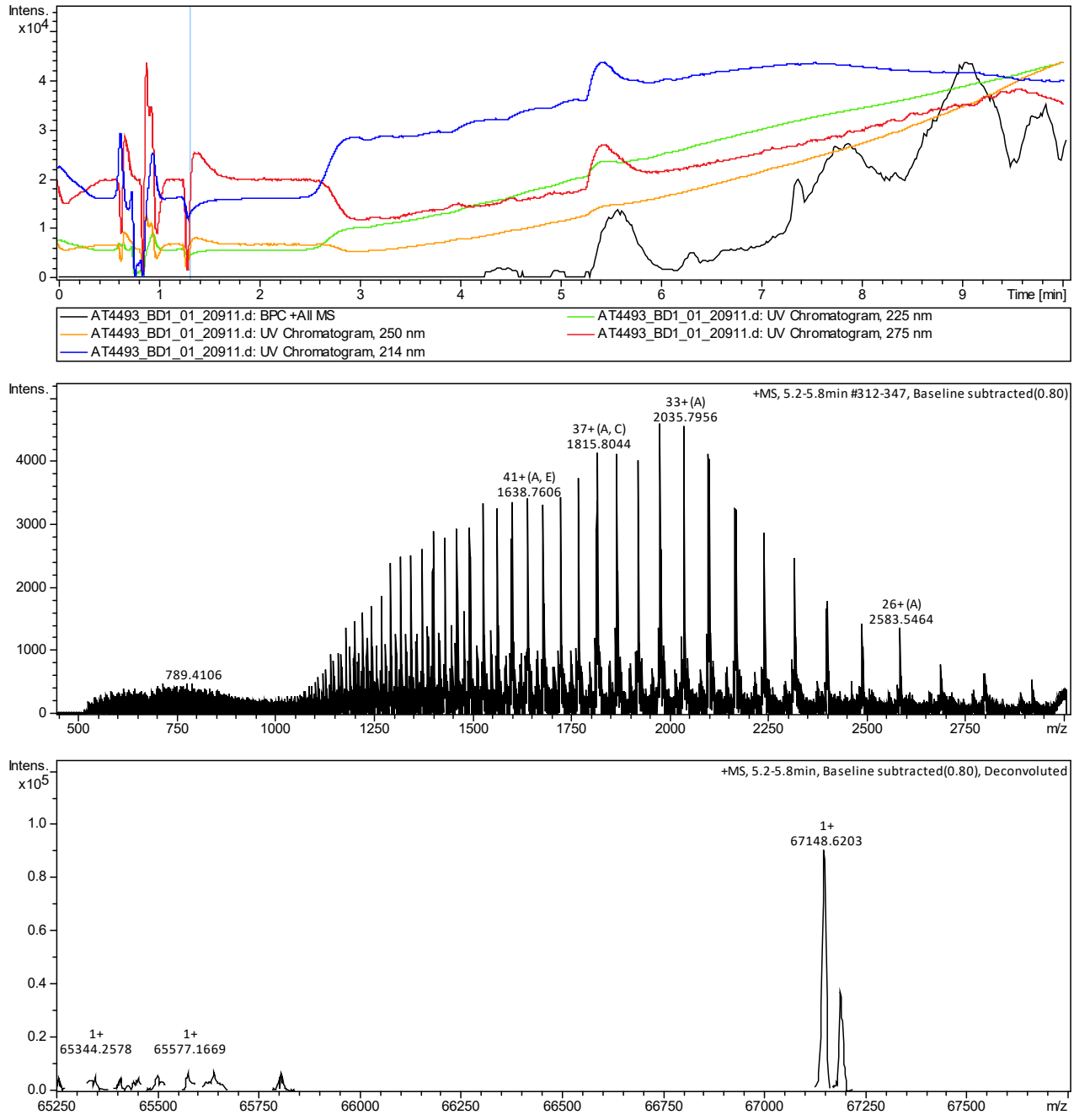


Figure S 24. LC-MS spectrum (top), MS signal (middle), and deconvoluted MS signal (bottom) of HSA·**2** formed after 24 h of reaction between HSA and 50 equiv. of construct **2**.

LC-MS analysis of HSA·3 (tetrazine ligation product of HSA·1 and 3,6-di(pyrimidin-2-yl)-1,2,4,5-tetrazine)

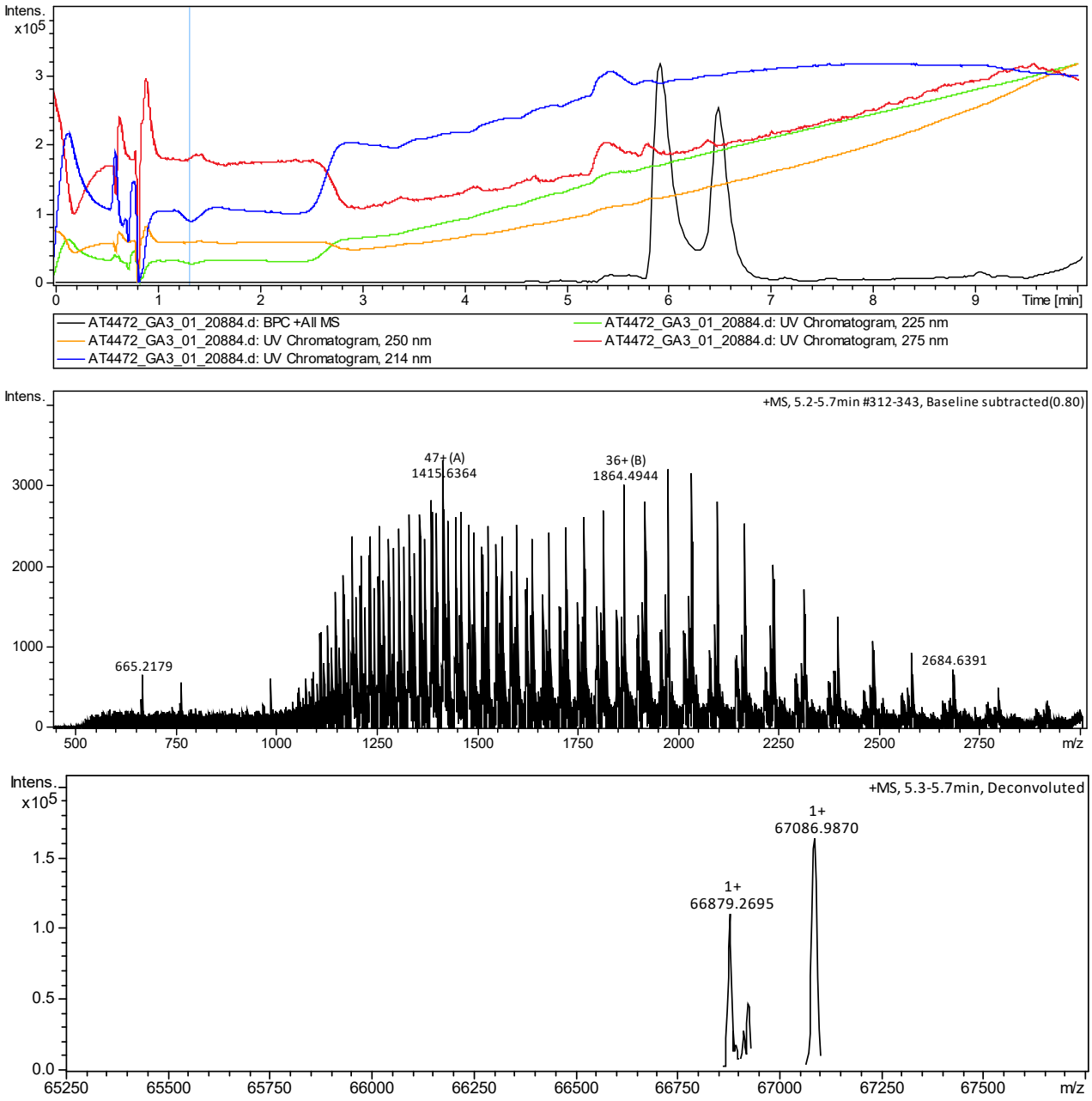


Figure S 25. LC-MS spectrum (top), MS signal (middle), and deconvoluted MS signal (bottom) after 15 min of reaction between HSA·**1** and 1 equiv. of 3,6-di(pyrimidin-2-yl)-1,2,4,5-tetrazine to form HSA·**3**.

LC-MS analysis of HSA·4 (tetrazine ligation product of HSA·2 and 3,6-di(pyrimidin-2-yl)-1,2,4,5-tetrazine)

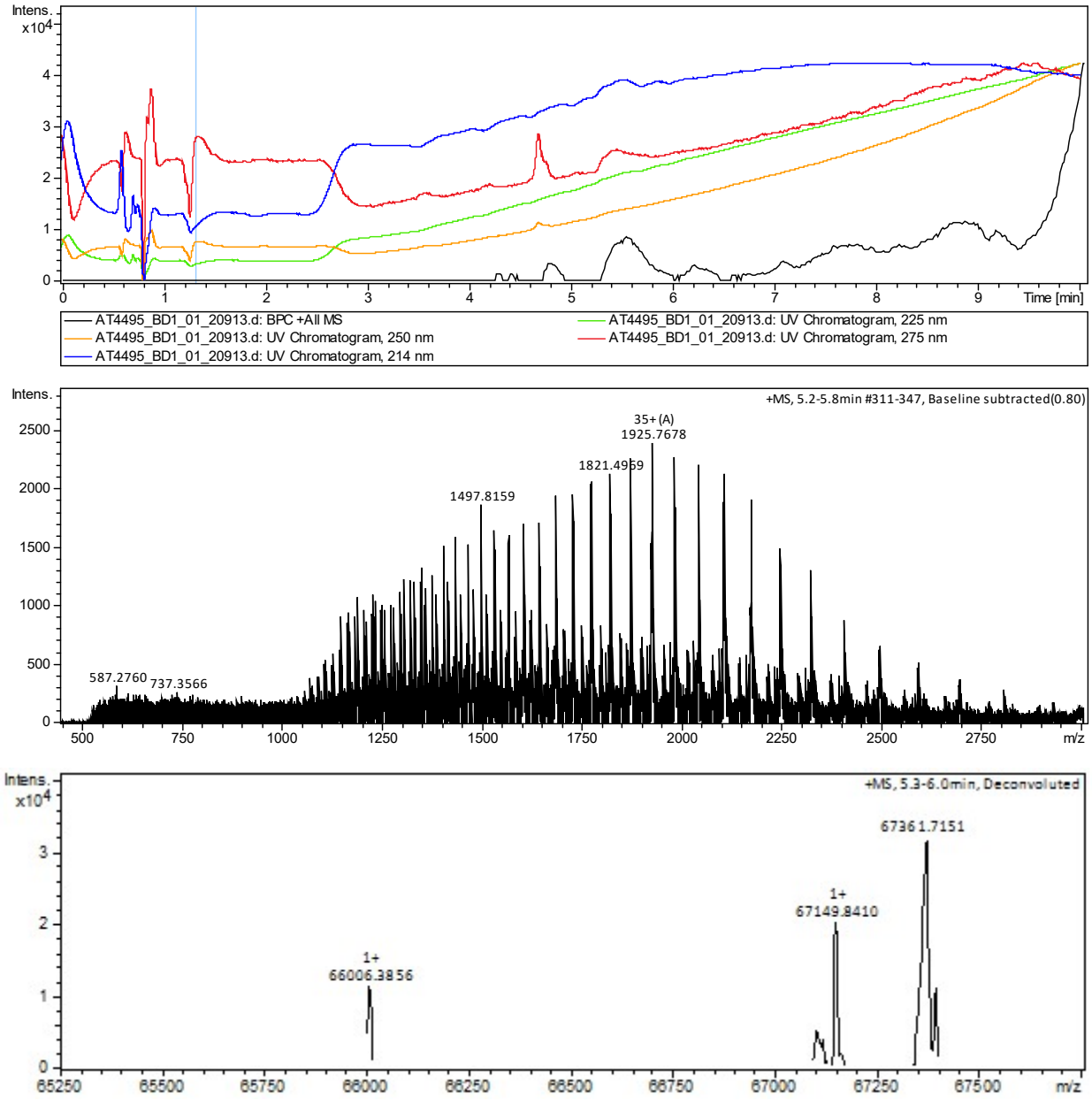


Figure S 26. LC-MS spectrum (top), MS signal (middle), and deconvoluted MS signal (bottom) after 15 min of reaction between HSA·**2** and 1 equiv. of 3,6-di(pyrimidin-2-yl)-1,2,4,5-tetrazine to form HSA·**4**.

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