Supporting information

Enzymatic Conjugation Using Branched Linkers for Constructing Homogeneous Antibody-Drug Conjugates with High Potency

Yasuaki Anami,[1] Wei Xiong,[1] Xun Gui,[1] Mi Deng,[2] Cheng Cheng Zhang,[2] Ningyan Zhang,[1] Zhiqiang An,[1] and Kyoji Tsuchikama*[1]

[1] Texas Therapeutics Institute, The Brown Foundation Institute of Molecular Medicine, The University of Texas Health Science Center at Houston, 1881 East Road, Houston, Texas 77054, United States

[2] Departments of Physiology and Developmental Biology, The University of Texas Southwestern Medical Center, 6001 Forest Park Road, Dallas, Texas 75390, United States

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1. General Information

Unless otherwise noted, all materials were purchased from commercial suppliers (Acros Organics, Chem-Impex International, Fisher Scientific, Sigma Aldrich, and TCI America) and used as received. All anhydrous solvents were purchased and stored over activated molecular sieves under argon atmosphere.

Analytical thin-layer chromatography (TLC) was performed using silica gel plates (Merck Kieselgel $60F_{254}$, 0.25 mm for TLC) and visualization was conducted with ultraviolet light (254 nm) or by ninhydrin staining. Flash column chromatography was performed using silica gel (TCI America, spherical, particle size 60 μm).

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DPX spectrometer (^{1}H: 300 MHz) using methanol-d_4 (CD_3OD) and dimethyl sulfoxide-d_6 (DMSO) as deuterated solvent. Chemical shifts (δ) in ^{1}H NMR spectra were reported in parts per million (ppm) relative to CD_3OD (δ = 3.34 ppm) or DMSO-d_6 (δ = 2.50 ppm). Coupling constants (J) in all NMR spectra are reported in Hertz (Hz).

Analytical Reverse-phase high performance liquid chromatography (RP-HPLC) was performed using an Agilent LC-MS system consisting of a 1100 HPLC and a 1946D single quadrupole electrospray ionization (ESI) mass spectrometer equipped with a C18 reverse-phase column (50×3 mm, 2.6 micron; Accucore C18, Thermo Scientific). Standard analysis conditions for organic molecules were as follows: flow rate = 0.5 mL/min; solvent A = water containing 0.1% formic acid; solvent B = acetonitrile containing 0.1% formic acid. Compounds were analyzed using a linear gradient and monitored with UV detection at 210 and 254 nm. Preparative HPLC was performed using a Breeze HPLC system (Waters) equipped with a C18 reverse-phase column (19×150 mm, 5.0 micron; SunFire Prep C18 OBD, Waters). Standard purification conditions were as follows: flow rate = 10 mL/min; solvent A = water containing 0.05% trifluoroacetic acid (TFA); solvent B = acetonitrile containing 0.05% TFA. Compounds were analyzed using a linear gradient and monitored with UV detection at 210 and 254 nm. In all cases, fractions were analyzed off-line using the LC-MS for purity confirmation.

High-resolution mass spectra were obtained using an Agilent 6530 Accurate Mass Q-TOF LC/MS. Absorbance and fluorescence were measured on a Cytation 5 Cell Imaging Multi-Mode Reader (Biotek).

2. Supplemental Figures

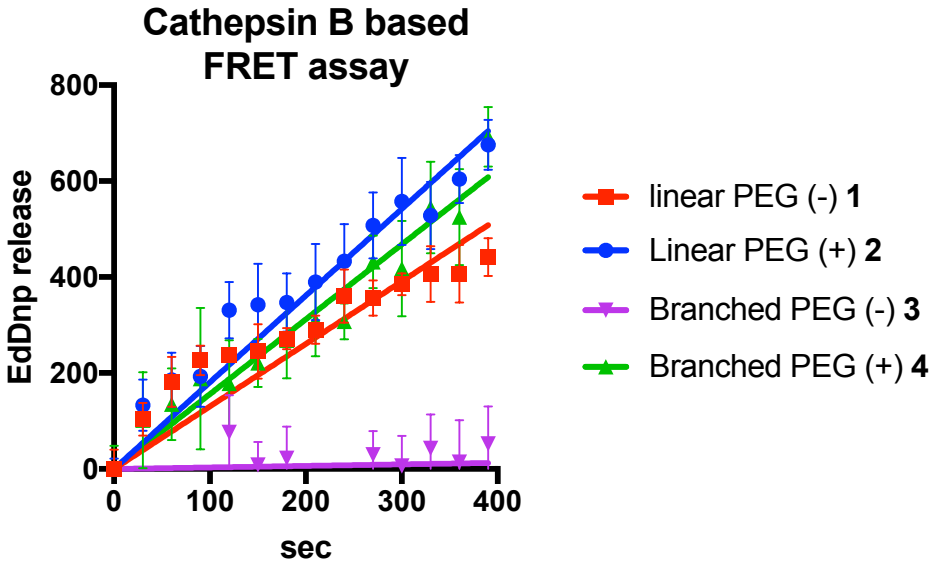


Figure S1. Quencher release rate determination in Förster resonance energy transfer (FRET) assay. Each slope reflecting drug (quencher) release rate was calculated by linear regression using Graph Pad Prism 7 software. Values of branched probes **3** and **4** were doubled for plotting because those probes possess two quencher moieties. All assays were performed in triplicate and error bars represent SEM.

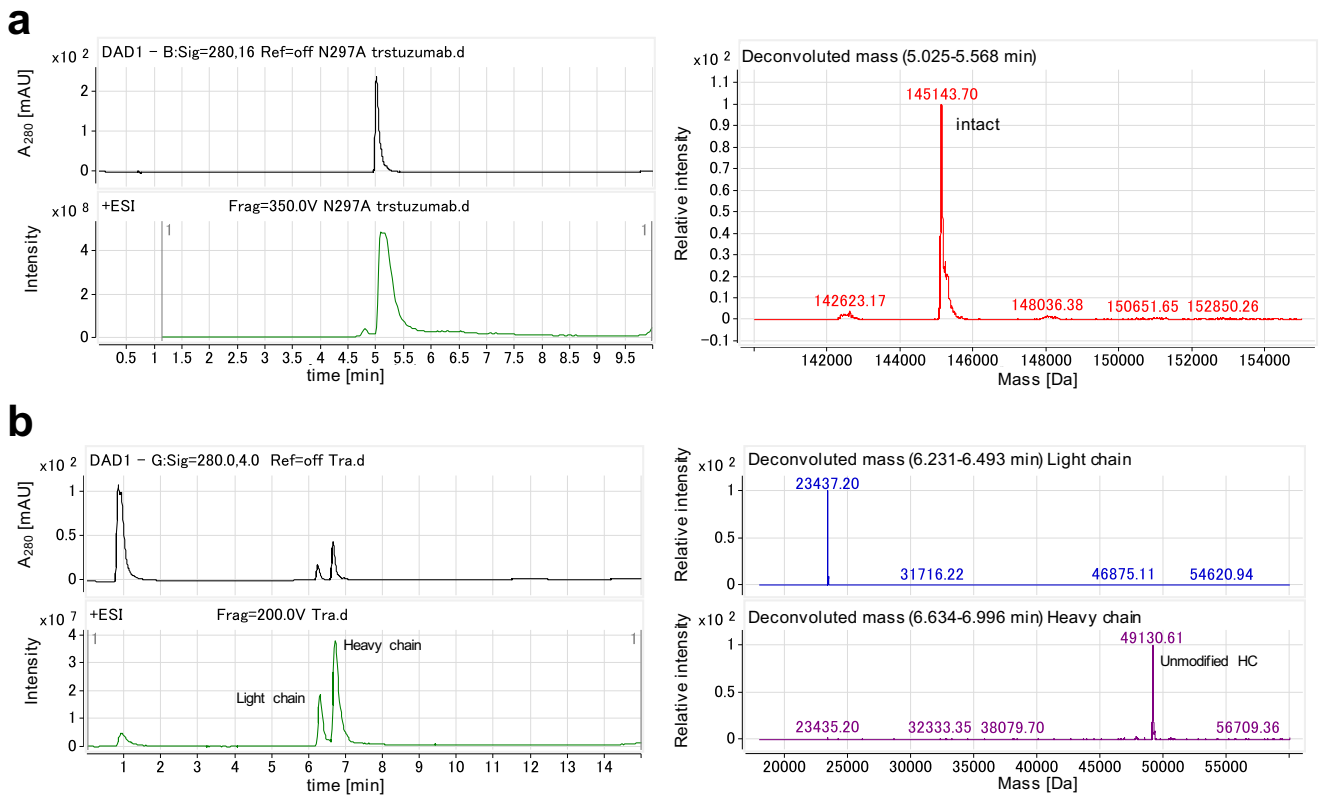


Figure S2. (a) UV trace at 280 nm, total ion chromatogram, and deconvoluted mass spectra of the intact N297A anti-HER2 antibody. (b) UV trace at 280 nm, total ion chromatogram, and deconvoluted mass spectrum of the DTT-reduced N297A anti-HER2 antibody (heavy and light chains).

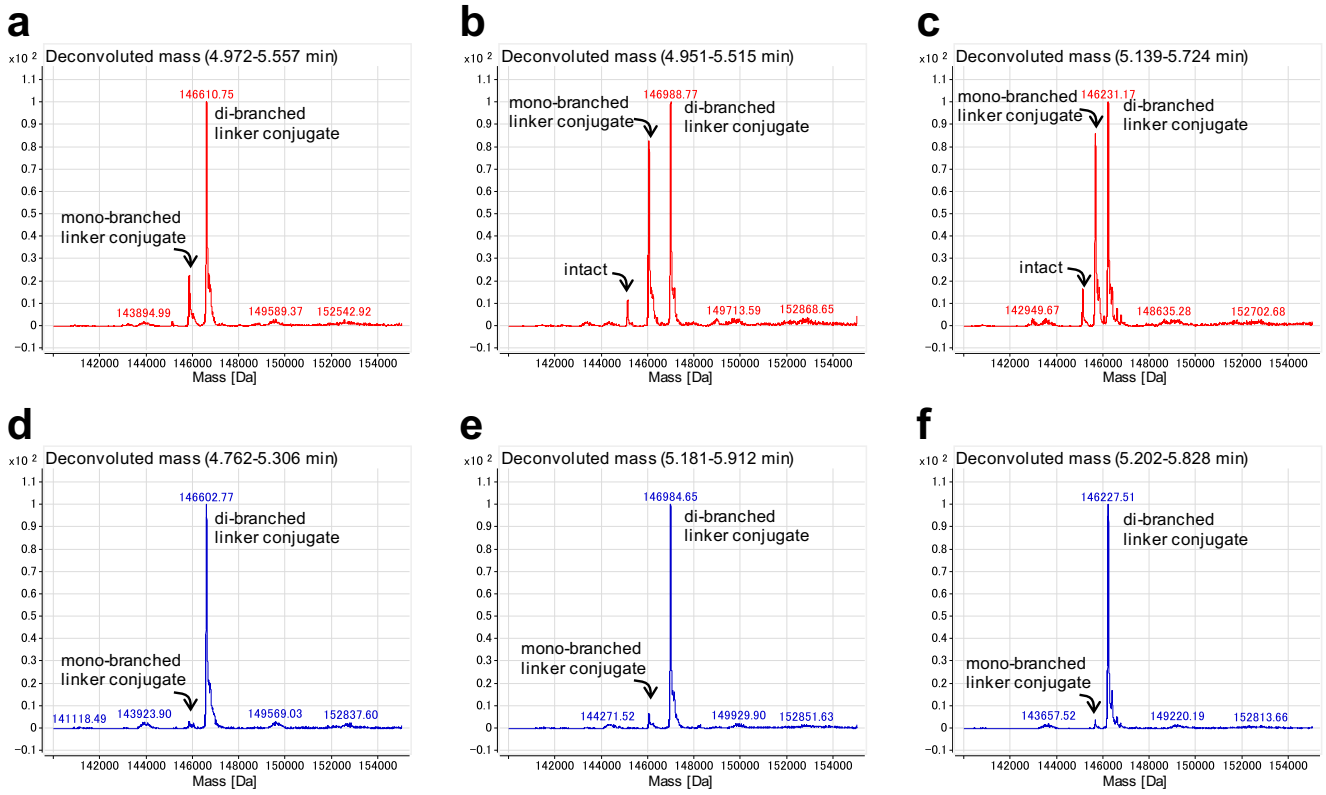


Figure S3. Deconvoluted mass spectra of the N297A anti-HER2 antibody conjugate containing branched linker **5** (a and d), **6** (b and e), or **7** (c and f) obtained under the reaction conditions reported by Schibli and coworkers[1] (a–c) or the optimal conditions reported in this article (d–f). The conversion rates shown in Table 2 were determined based on the mass intensities of these deconvoluted peaks.

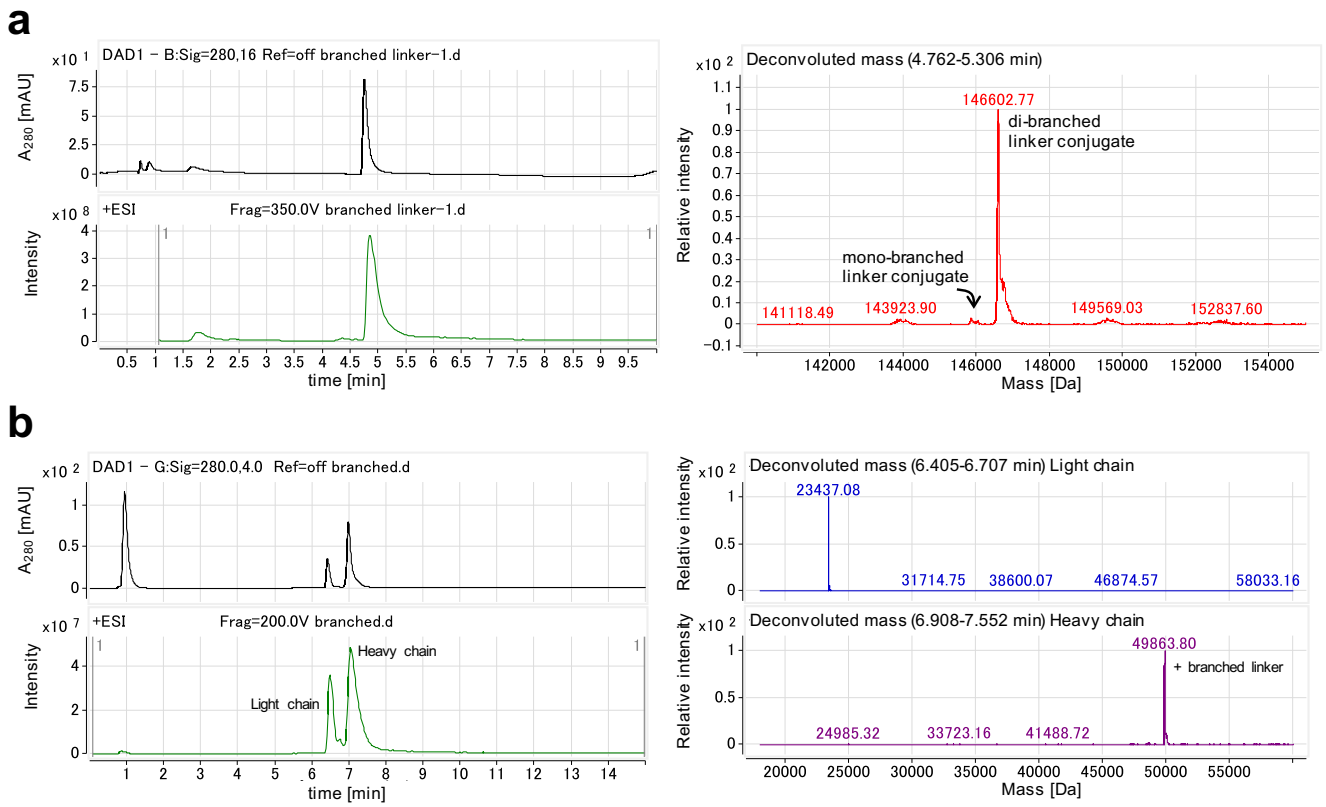


Figure S4. (a) UV trace at 280 nm, total ion chromatogram, and deconvoluted mass spectra of the N297A anti-HER2 antibody-branched linker **5** conjugate. (b) UV trace at 280 nm, total ion chromatogram, and deconvoluted mass spectrum of the DTT-reduced N297A anti-HER2 antibody-branched linker **5** conjugate (heavy and light chains).

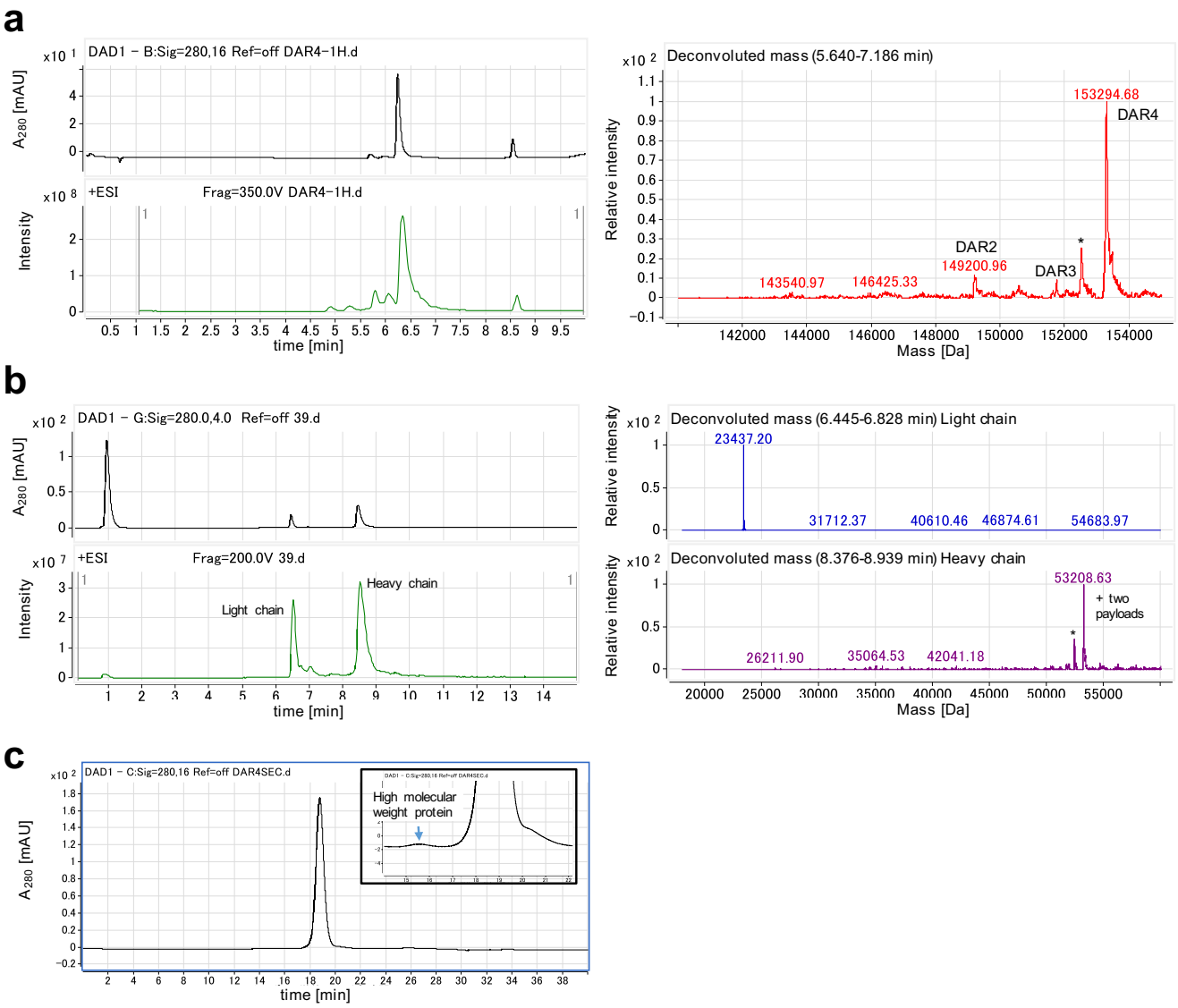
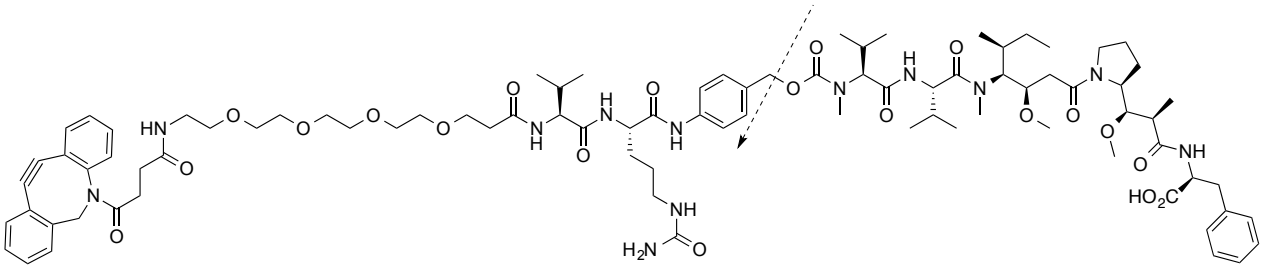
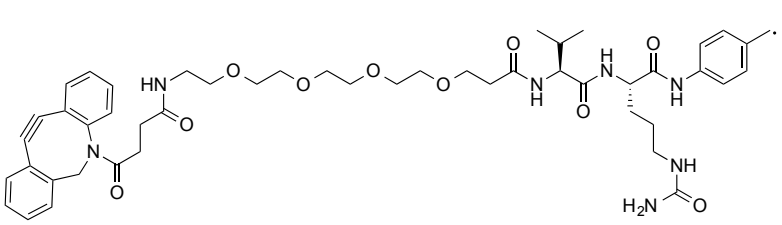


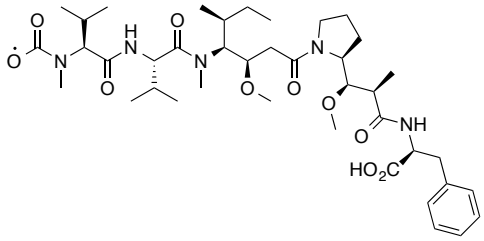
Figure S5. (a) UV trace at 280 nm, total ion chromatogram, and deconvoluted mass spectra of the N297A anti-HER2 antibody-MMAF conjugate **9**. Asterisked peak (152523.82 Da) indicates an ion fragment derived from the DAR-4 species (153294.68 Da), which corresponds to a loss of ~770.86 Da. (b) UV trace at 280 nm, total ion chromatogram, and deconvoluted mass spectra of heavy and light chain of N297A the DTT-reduced ADC **9** (heavy and light chains). Asterisked peak (52436.27 Da) indicates an ion fragment derived from the heavy chain-MMAF (53208.63 Da), which corresponds to a loss of ~772.36 Da. (c) SEC chromatogram of crude ADC **9** before purification (UV: 280 nm). The weak peak around 15.552 min is derived from high molecular weight proteins (protein aggregates). This chromatogram clearly indicates that ADC **9** was >99% monomeric.



DBCO-PEG$_4$-Val-Cit-PABC-MMAF
Exact Mass: 1670.921



DBCO-PEG$_4$-Val-Cit-PAB (fragment)
Exact Mass: 896.456



MMAF (fragment)
Exact Mass: 774.465

Figure S6. The predicted fragment. The exact mass of DBCO-PEG$_4$-Val-Cit-PABC-MMAF is 1670.921 Da and that of DBCO-PEG$_4$-Val-Cit-PAB is 896.456 Da. The difference between these two is 774.465 Da. This value is consistent to the observed values of the ion fragments in Figure S5.

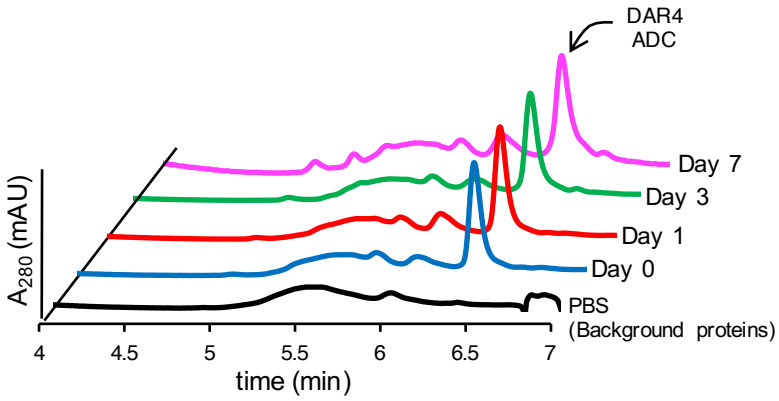


Figure S7. Stability of ADC **9** in human plasma at 37 °C (performed in duplicate). No significant reduction of the DAR was observed after 7 days (91% residual content).

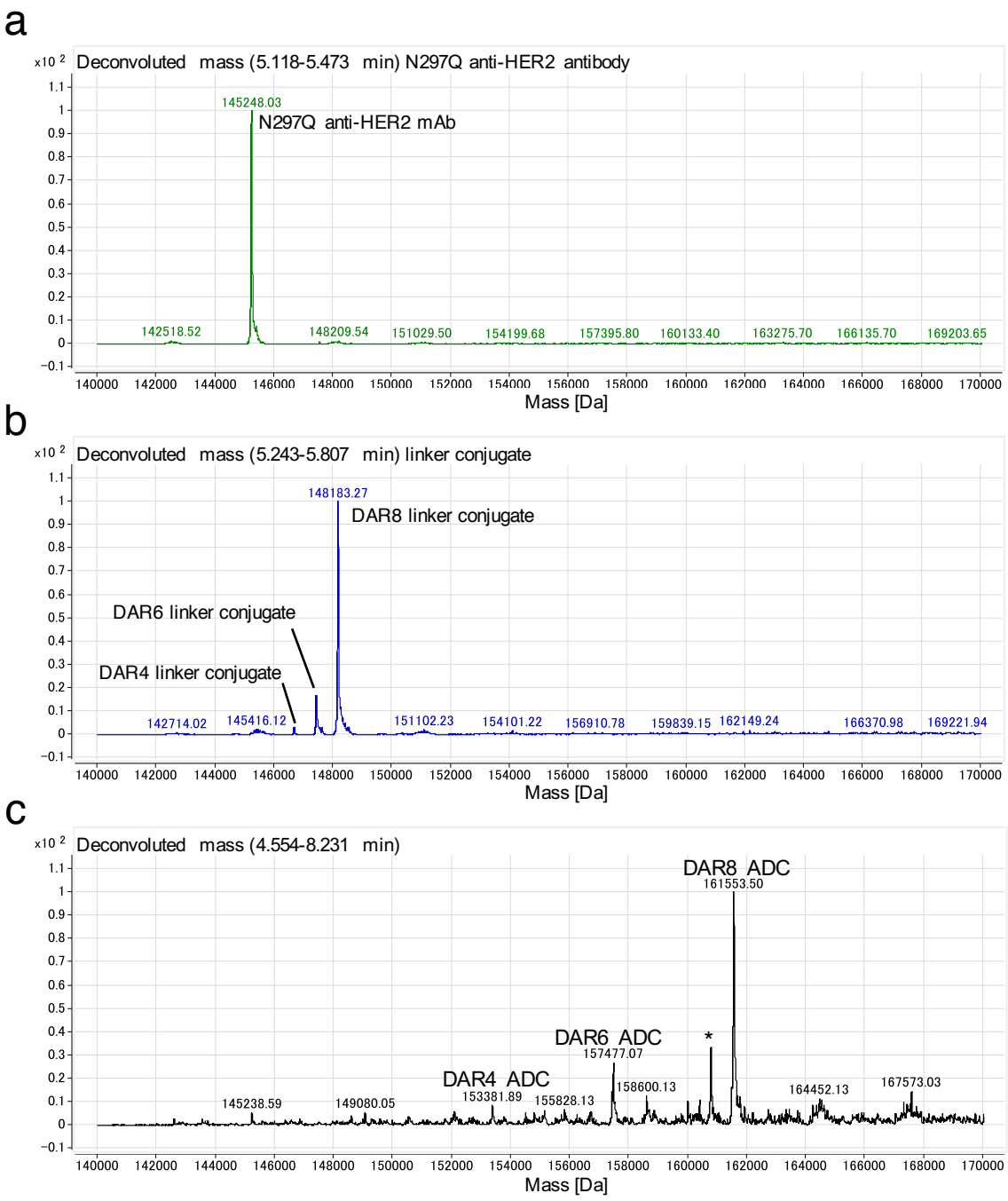


Figure S8. Deconvoluted mass spectra of (a) the N297Q anti-HER2 mAb, (b) mAb-branched linker **5** conjugate, and (c) mAb-linker **5**-MMAF conjugate. Average DAR was determined to be 7.4 based on the mass intensities of each DAR species. Asterisked peak (160779.93 Da) indicates an ion fragment derived from the DAR-8 species (161553.50 Da), which corresponds to a loss of ~773.57 Da.

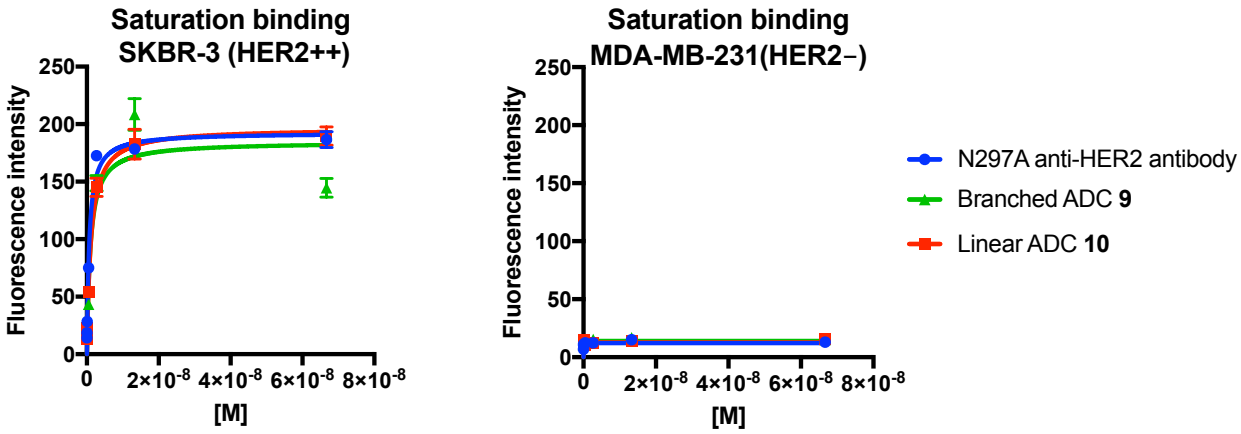
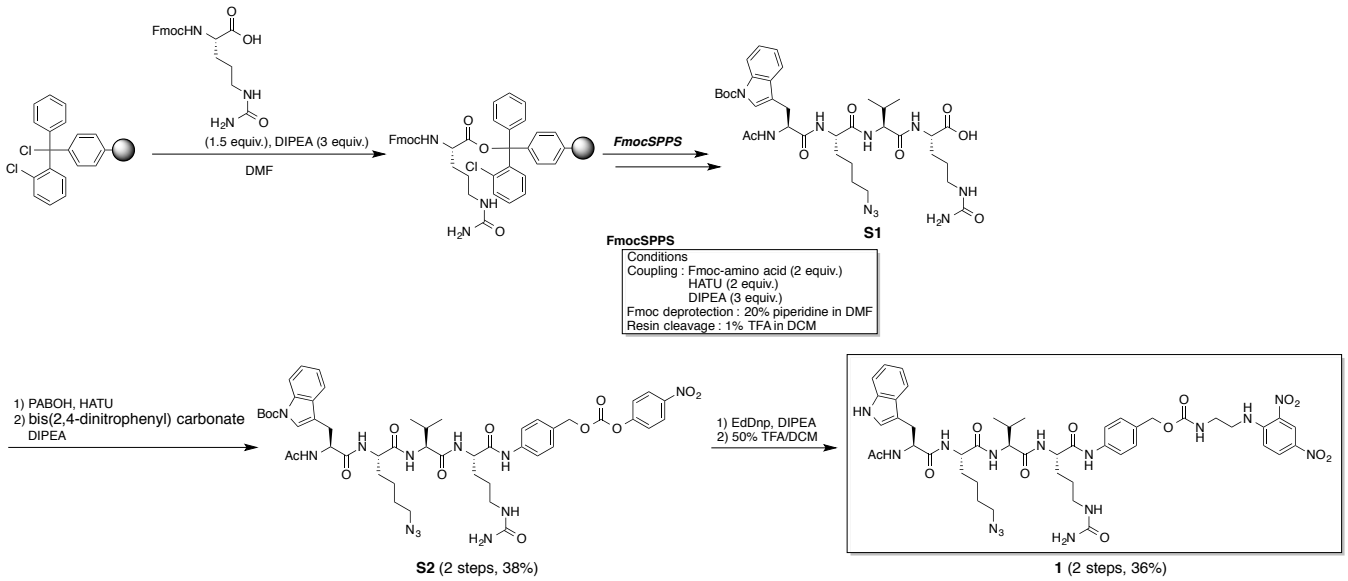


Figure S9. Saturation-binding curves obtained by cell-based ELISA All assays were performed in triplicate and error bars represent SEM. The N297A anti-HER2 antibody and ADCs **9**–**10** bound to SKBR-3 cells (HER2 positive, left) with comparable binding affinities but not to MDA-MB-231 cells (HER2 negative, right).

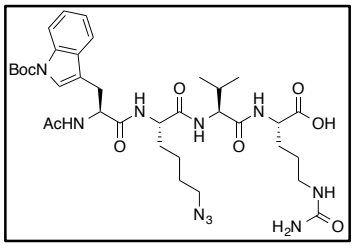
3. Experimental Details

Synthesis



Scheme S1. Synthesis of linear probe **1**.

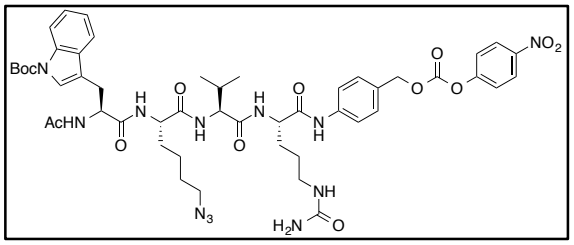
Synthesis of Ac-Trp(Boc)-Lys(N_3)-Val-Cit-OH S1



Chlorotrityl chloride resin (500 mg, 0.76 mmol) and Fmoc-citrulline-OH (453 mg, 1.14 mmol) were taken to a manual solid-phase reactor containing *N*,*N*-diisopropylethylamine (DIPEA, 397 μL, 2.28 mmol) and dimethylfolmamide (DMF, 3 mL) and agitated for 2 h. Methanol (MeOH, 600 μL, 1.52 mmol) was added to the resin and agitated for 20 min, the solution was drained and the resin was washed with DMF (5×3 mL) and dichloromethane (DCM, 5×3 mL). To remove the Fmoc-protecting group after each coupling, the resin was treated with piperidine (5 mL of 20% in DMF, 20 min) and washed with DMF (5×3 mL) and DCM (5×3 mL). Fmoc-protected amino acid (2 equiv.) was pre-activated using 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU, 2 equiv.) and DIPEA (3 equiv.) in DMF for 3 min, and the cocktail was added to the resin. The resin was agitated for 1 h at room temperature. The completion of the coupling was verified by the Kaiser test. After each coupling step, the coupling cocktail was drained and the resin was washed with DMF (5×3 mL) and DCM (5×3 mL). After elongation of the peptide, the resin was treated with acetic anhydride (2 equiv.) and DIPEA (3 equiv.) in DMF for 1 h and then washed with DMF (5×3 mL) and DCM (5×3 mL). The resulting protected peptide resin was treated with cocktail of 1% trifluoroacetic acid (TFA)/DCM at room temperature for 1 h. The

solution was concentrated in vacuo and the crude peptide was precipitated with cold diethyl ether (5–6 mL) followed by centrifugation at 500 xg for 5 min (3 times). The resulting crude peptide **S1** was dried in vacuo and then used immediately in the next step without purification.

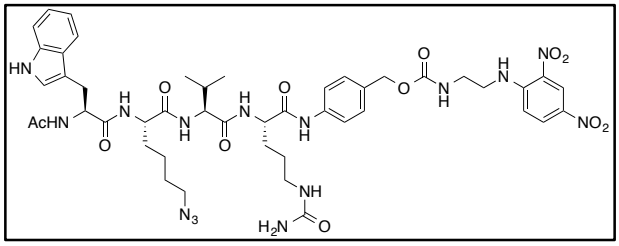
Synthesis of Ac-Trp(Boc)-Lys(N_3)-Val-Cit-PABC-PNP S2



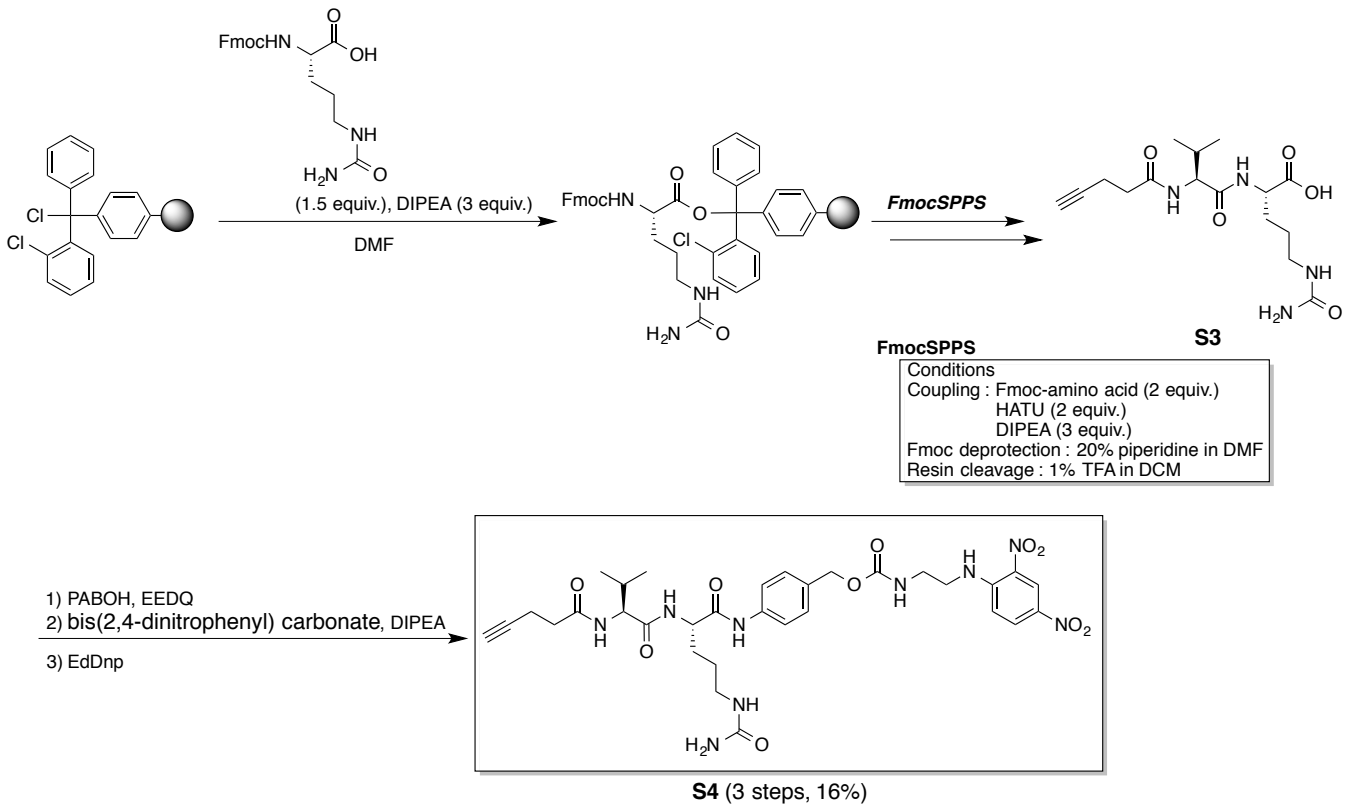
To a solution of peptide **S1** (20 mg, 0.026 mmol) in DMF (1 mL) was added HATU (13.0 mg, 0.034 mmol), DIPEA (6 μL, 0.04 mmol), and *p*-aminobenzyl alcohol (PABOH, 4.9 mg, 0.04 mmol), and the mixture was stirred at room temperature for 1.5 h in the dark. The solution was directly concentrated in vacuo and the crude peptide was precipitated with cold diethyl ether (5–6 mL) followed by centrifugation at 500 xg for 5 min (3 times). The resulting crude peptide was dried in vacuo for >90 min used immediately in the next step without purification. Note: we could not achieve a satisfying yield with EEDQ, a coupling reagent that does not promote epimerization in the citrulline–PABOH coupling. We confirmed by LC/MS analysis that about 4% epimerization occurred at the citrulline residue under the HATU coupling conditions (See the LC/MS traces of this crude product and an epimeric mixture sample attached in the HPLC and NMR section.)

To a solution of this crude mixture in DMF (2 mL) was added bis(2,4-dinitrophenyl) carbonate (40.2 mg, 0.13 mmol) and DIPEA (23 μL, 0.13 mmol), and the mixture was stirred overnight at room temperature. The crude peptide **S2** was purified by preparative RP-HPLC to give analytically pure peptide **S2** (10.2 mg, 38% for the 2 steps). Off-white powder. HRMS (ESI) Calcd. For $C_{49}H_{62}N_{12}O_{13}Na$ $[M+Na]^+$: 1049.4452. Found: 1049.4462.

Synthesis of Ac-Trp-Lys(N_3)-Val-Cit-PABC-EdDnp 1

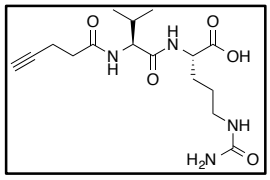


Peptide **S2** (4.6 mg, 0.0045 mmol) was treated with *N*′-(2,4-dinitrophenyl)ethane-1,2-diamine (EdDnp, 1.53 mg, 0.0068 mmol) and DIPEA (1 μL, 0.0045 mmol) in DMF (0.5 mL) at room temperature for 3.5 h. The solution was directly concentrated in vacuo to get the crude peptide. The crude peptide was then dissolved in DCM (0.5 mL) and TFA (0.5 mL) was added to the solution at 0 °C. After 1 h, the solution was directly concentrated in vacuo and purified by preparative RP-HPLC to give analytically pure peptide **1** (1.66 mg, 36% for the 2 steps). Yellow powder. HRMS (ESI) Calcd. For $C_{46}H_{59}N_{15}O_{12}Na$ $[M+Na]^+$: 1036.4360. Found: 1036.4357.



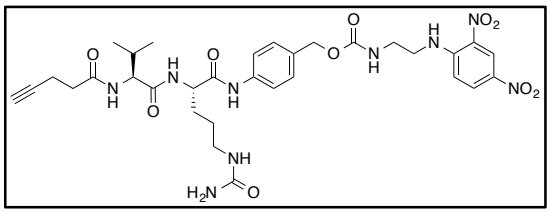
Scheme S2. Synthesis of alkyne peptide **S4**.

Synthesis of alkyne-Val-Cit-OH S3



Fmoc-Val-Cit-resin was synthesized in the same manner as preparation of **S1**. After elongation of the peptide, the resin was treated with 4-pentynoic acid (2 equiv.), HATU (2 equiv.), and DIPEA (3 equiv.) in DMF for 1 h and then washed with DMF (5×1 mL) and DCM (5x1 mL). The resulting peptide resin was treated with cocktail of 1% TFA/DCM at room temperature for 1 h. The solution was concentrated in vacuo and the crude peptide was precipitated with cold diethyl ether (5–6 mL) followed by centrifugation at 500 xg for 5 min (3 times). The resulting crude peptide **S3** was dried in vacuo and then used immediately in the next step without purification.

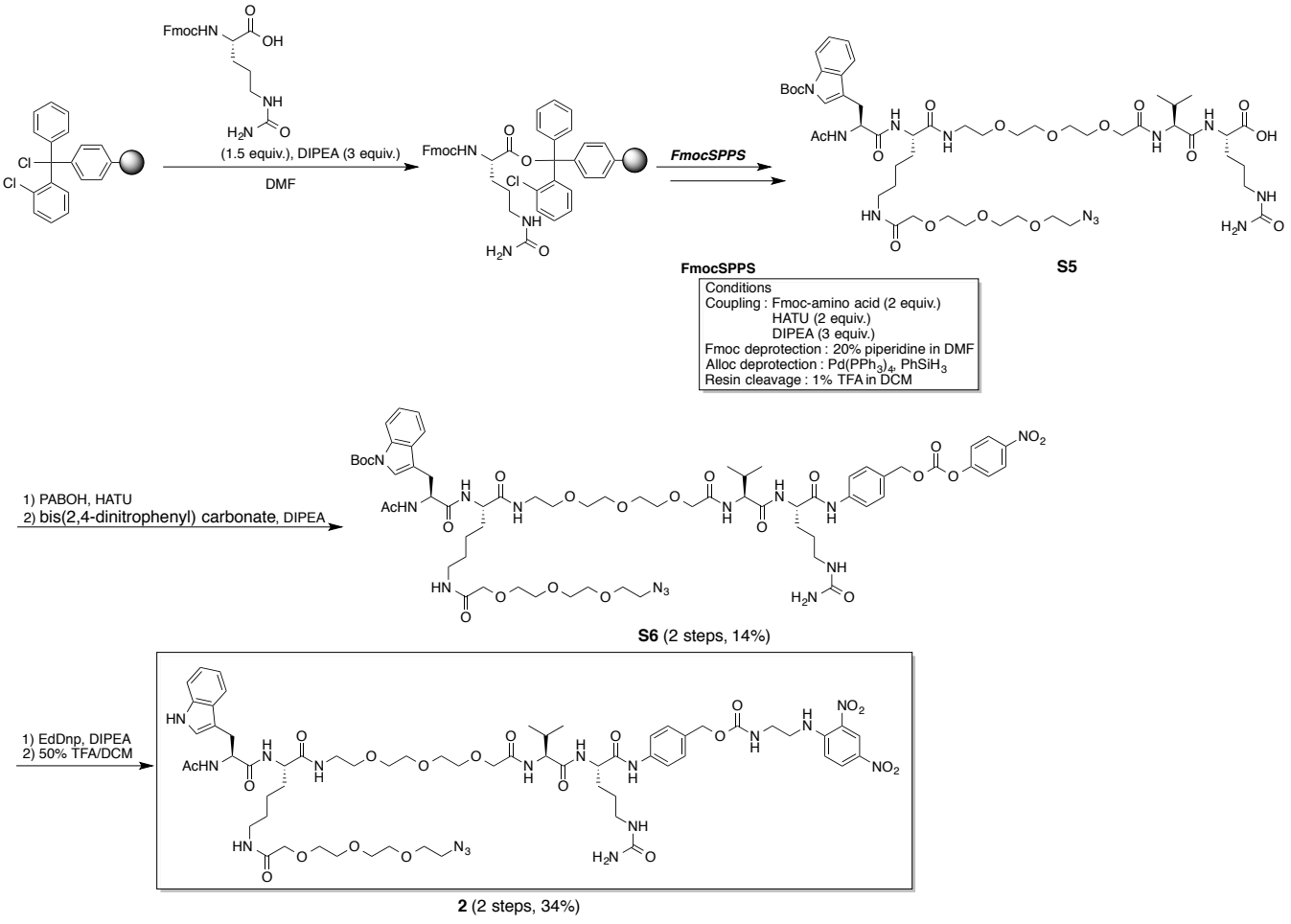
Synthesis of alkyne-Val-Cit-PABC-EdDnp S4



To a solution of peptide **S3** (21.8 mg, 0.062 mmol) in MeOH/DCM (1.5 mL, 1:2) was added PABOH (15.3 mg, 0.12 mmol) and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, 30.7 mg, 0.12 mmol), and the mixture was stirred overnight at room temperature in the dark. The solution was directly concentrated in

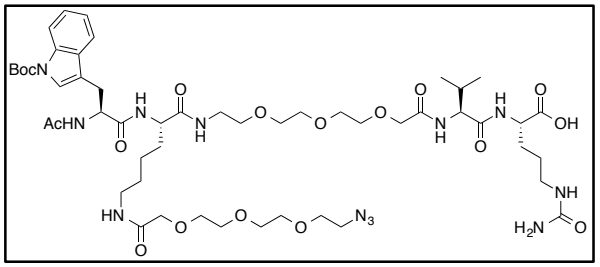
vacuo and the crude peptide was precipitated with cold diethyl ether (5–6 mL) followed by centrifugation at 500 xg for 5 min (3 times). The resulting crude peptide was dried in vacuo and then used immediately in the next step without purification.

To a solution of crude peptide in DMF (1 mL) was added bis(2,4-dinitrophenyl) carbonate (106 mg, 0.35 mmol) and DIPEA (36 μL, 0.21 mmol) and the mixture was stirred for 2 h at room temperature. Afterwards, EdDnp (31.7 mg, 0.14 mmol) was added to the mixture and the mixture was stirred for 3 h at room temperature. The additional EdDnp (55 mg, 0.25 mmol) was added and the mixture was further stirred for 1 h at room temperature. The reaction was quenched with 15% citric acid and extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The crude peptide was purified by preparative RP-HPLC to give analytically pure peptide **S4** (6.7 mg, 16% for the 3 steps). Yellow powder. HRMS (ESI) Calcd. For $C_{32}H_{41}N_9O_{10}Na$ $[M+Na]^+$: 734.2869. Found: 734.2871.



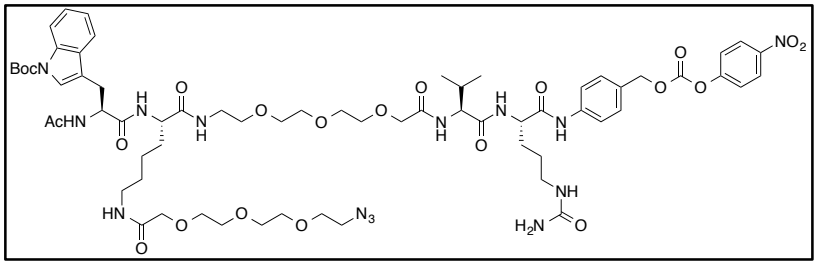
Scheme S3. Synthesis of linear probe containing PEG spacer **2**.

Synthesis of Ac-Trp(Boc)-Lys(-PEG$_3$-N$_3$)-PEG$_3$-Val-Cit-OH S5



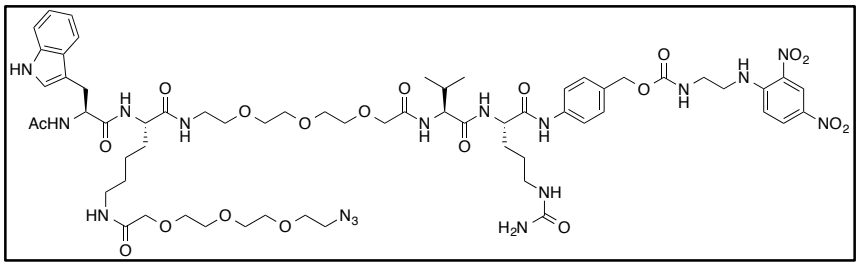
Ac-Trp(Boc)-Lys(-PEG$_3$-N$_3$)-PEG$_3$-Val-Cit-OH was synthesized in the same manner as preparation of **S1**. The resulting peptide resin was treated with cocktail of 1% TFA/DCM at room temperature for 1 h. The solution was concentrated in vacuo and the crude peptide was precipitated with cold diethyl ether (5–6 mL) followed by centrifugation at 500 xg for 5 min (3 times). The resulting crude peptide **S5** was dried in vacuo and then used immediately in the next step without purification.

Synthesis of Ac-Trp(Boc)-Lys(-PEG$_3$-N$_3$)-PEG$_3$-Val-Cit-PABC-PNP S6



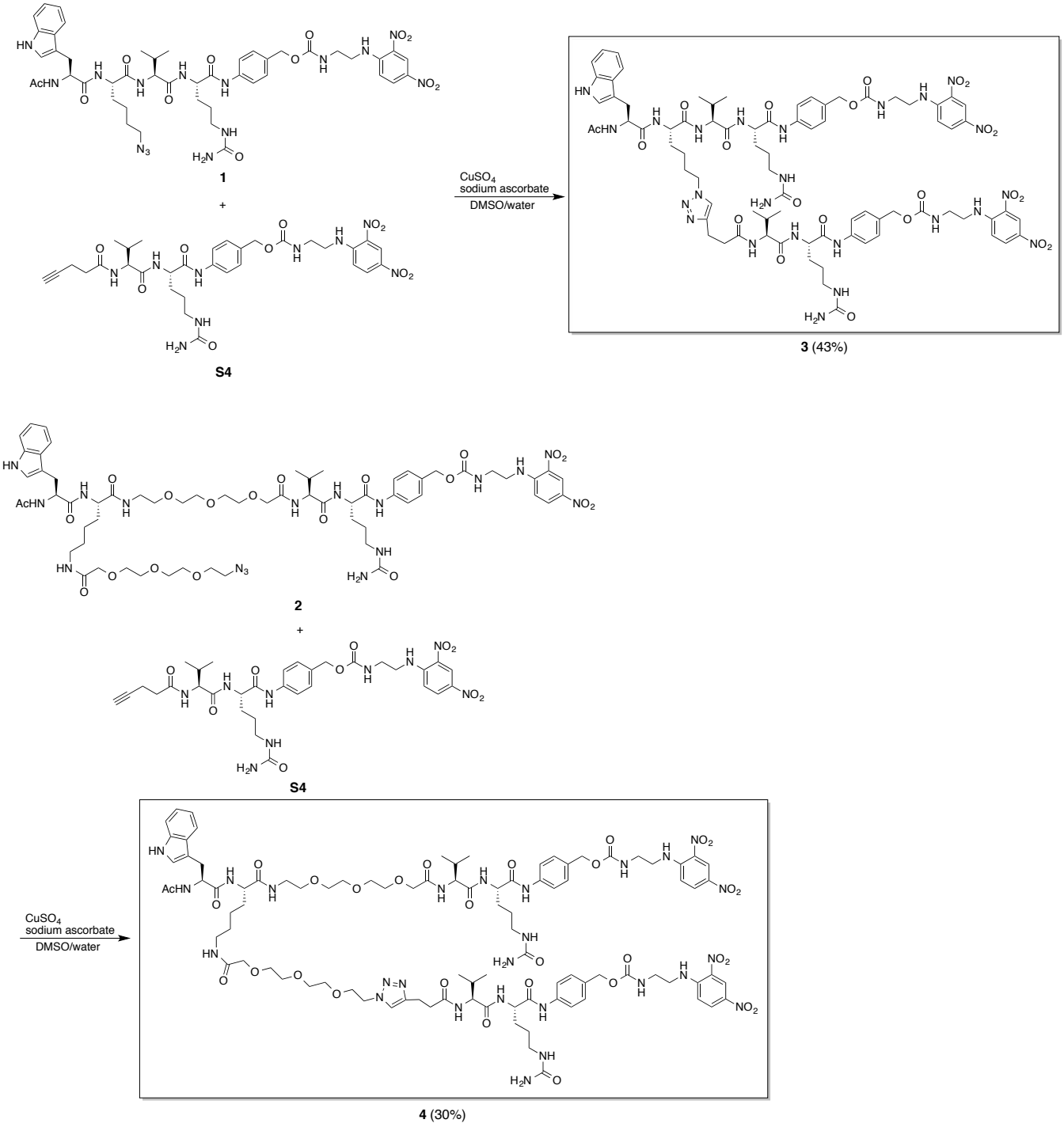
To a solution of peptide **S5** (24 mg, 0.021 mmol) in DMF (1 mL) was added HATU (10.4 mg, 0.027 mmol), DIPEA (4.8 μL, 0.027 mmol), and PABOH (3.9 mg, 0.032 mmol), and the mixture was stirred at room temperature for 2 h in the dark. The solution was directly concentrated in vacuo and the crude peptide was precipitated with cold diethyl ether (5–6 mL) followed by centrifugation at 500 xg for 5 min (3 times). The resulting crude peptide was dried in vacuo for 90 min at least and then used immediately in the next step without purification. To a solution of this crude peptide in DMF (1 mL) was added bis(2,4-dinitrophenyl) carbonate (32 mg, 0.11 mmol) and DIPEA (18 μL, 0.11 mmol), and the mixture was stirred overnight at room temperature. The crude peptide **S6** was purified by preparative RP-HPLC to give analytically pure peptide **S6** (4.0 mg, 14% for the 2 steps). Off-white powder. HRMS (ESI) Calcd. For $C_{65}H_{92}N_{14}O_{21}Na$ $[M+Na]^+$: 1427.6454. Found: 1427.6454.

Synthesis of Ac-Trp-Lys(-PEG$_3$-N$_3$)-PEG$_3$-Val-Cit-PABC-EdDnp 2



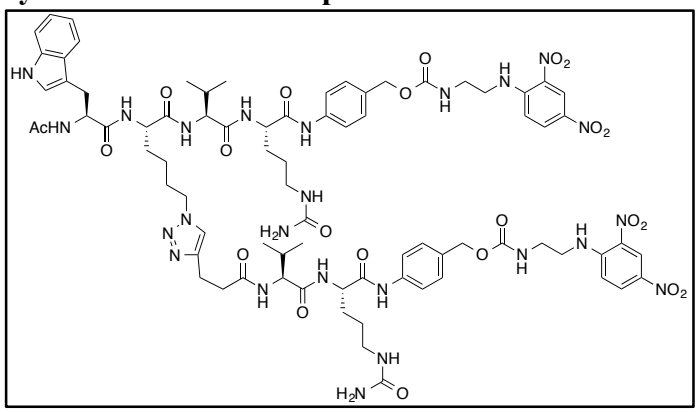
Peptide **S6** (3.2 mg, 0.0023 mmol) was treated with EdDnp (0.78 mg, 0.0035 mmol) in DMF (0.5 mL) at room temperature for 2 h and the solution was directly concentrated in vacuo. The crude peptide was then dissolved in in DCM (1 mL) and TFA (1 mL) was added to the solution at 0 °C. After 1 h, the solution was directly concentrated in vacuo and the crude peptide was

precipitated with cold diethyl ether (5–6 mL) followed by centrifugation at 500 xg for 5 min (3 times). The crude peptide was purified by preparative RP-HPLC to give analytically pure peptide **2** (1.1 mg, 34% for the 2 steps). Yellow powder. HRMS (ESI) Calcd. For $C_{62}H_{89}N_{17}O_{20}Na$ $[M+Na]^+$: 1414.6362. Found: 1414.6349.



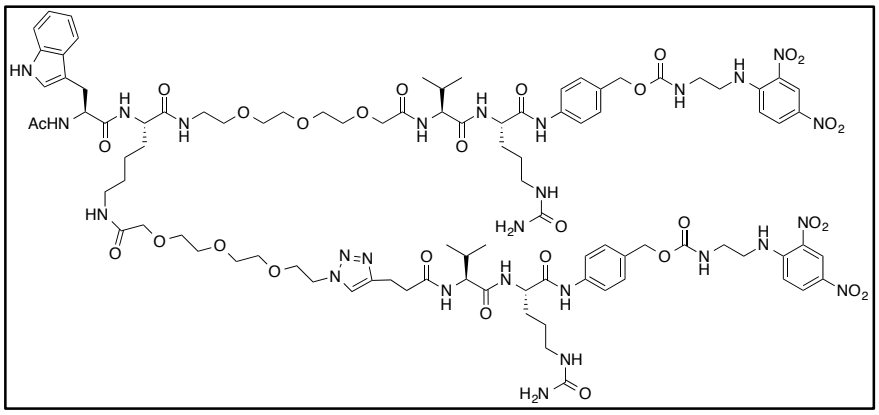
Scheme S4. Synthesis of branched probes **3** and **4**.

Synthesis of FRET compound 3

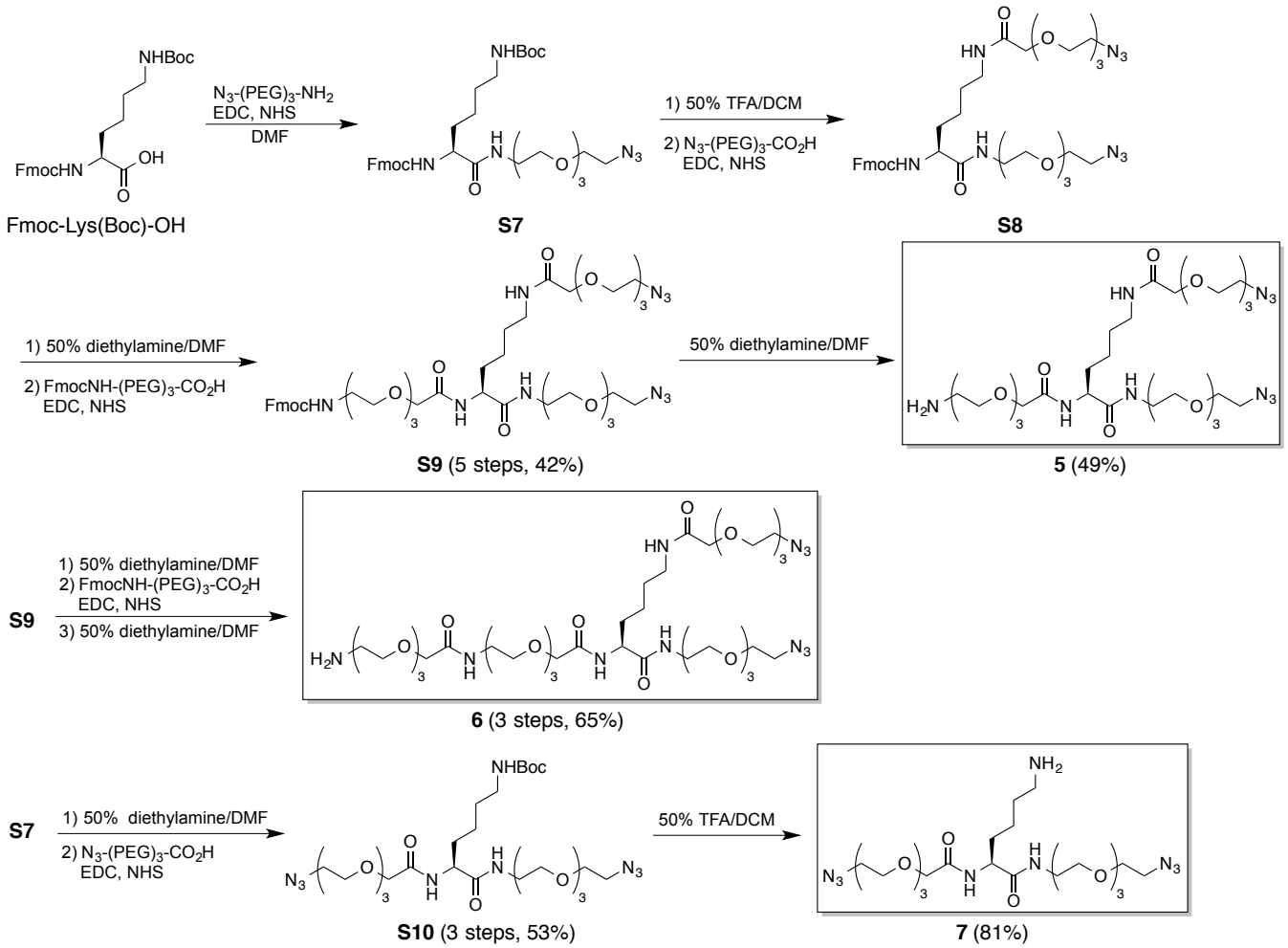


A mixture of alkyne peptide **S4** (100 μL, 10 mM in DMSO) and azide peptide **1** (130 μL, 10 mM in DMSO) in DMSO (620 μL) was treated with a mixture of $CuSO_4$ (50 μL, 100 mM in DI water) and sodium ascorbate (100 μL, 100 mM in DI water) and stirred overnight at room temperature. Additional alkyne peptide **S4** (20 μL, 10 mM in DMSO), $CuSO_4$ (50 μL, 100 mM in DI water) and sodium ascorbate (100 μL, 100 mM in DI water) were added to the reaction mixture, and further stirred at room temperature for 6.5 h. The crude peptide was purified by preparative RP-HPLC to give analytically pure peptide **3** (970 μg, 43%). Yellow powder. HRMS (ESI) Calcd. For $C_{78}H_{100}N_{24}O_{22}Na_2$ $[M+2Na]^{2+}$: 885.3614. Found: 885.3626.

Synthesis of FRET compound 4

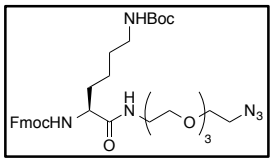


A mixture of alkyne peptide **S4** (135 μL, 10 mM in DMSO) and azide peptide **2** (79 μL, 10 mM in DMSO) in DMSO (600 μL) was treated with a mixture of $CuSO_4$ (39 μL, 100 mM in DI water) and sodium ascorbate (79 μL, 100 mM in DI water) and stirred at room temperature for 8 h. The crude peptide was purified by preparative RP-HPLC to give analytically pure peptide **4** (500 μg, 30%). Yellow powder. HRMS (ESI) Calcd. For $C_{94}H_{130}N_{26}O_{30}Na$ $[M+Na]^{+}$: 2126.9367. Found: 2126.9345.



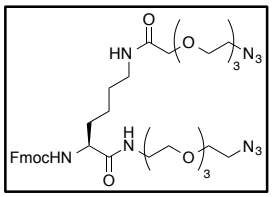
Scheme S5. Synthesis of branched linker compounds **5**–**7**.

Synthesis of Fmoc-Lys(Boc)-PEG$_3$-N$_3$ S7



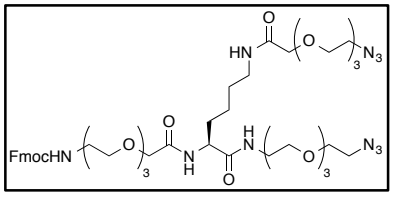
Fmoc-Lys(Boc)-OH (217.5 mg, 0.46 mmol) in DMF (3 mL) was treated with *N*-hydroxysuccinimide (NHS, 105.9 mg, 0.92 mmol) and *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC·HCl, 176.4 mg, 0.92 mmol) at room temperature. Afterwards, to this mixture was added 11-azido-3,6,9-trioxaundecan-1-amine (130.5 mg, 0.60 mmol) and stirred overnight at room temperature. The reaction was quenched with 15% citric acid and extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The crude compound **S7** was immediately used without further purification.

Synthesis of Fmoc-Lys(PEG$_3$-N$_3$)-PEG$_3$-N$_3$ S8



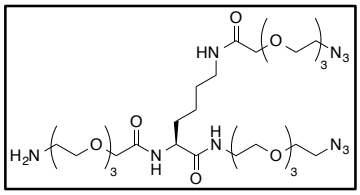
The crude compound **S7** was dissolved in DCM (2 mL) and TFA (2 mL) was added to the solution at room temperature. After 1 h, the mixture was concentrated and the Boc-deprotected compound was afforded. In other flask, a mixture of 11-azido-3,6,9-trioxaundecanoic acid (129 mg, 0.55 mmol), NHS (127.1 mg, 1.10 mmol) and EDC·HCl (211.6 mg, 1.10 mmol) in DMF (1 mL) was prepared and the mixture was added to the Boc-deprotected compound in DMF (3 mL) and DIPEA (80 μL, 0.46 mmol). After 18 h, the reaction was quenched with 15% citric acid and extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The crude compound **S8** was immediately used without further purification.

Synthesis of Fmoc-PEG$_3$-Lys(PEG$_3$-N$_3$)-PEG$_3$-N$_3$ S9



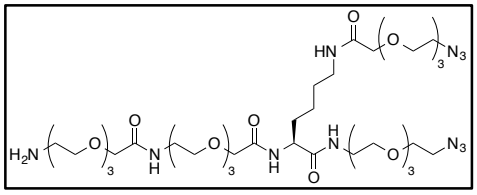
The crude compound **S8** was dissolved in DMF (1 mL) and diethylamine (1 mL) was added to the solution at room temperature. After 1 h, the mixture was concentrated and the Fmoc-deprotected compound was afforded. In other flask, a mixture of Fmoc-11-amino-3,6,9-trioxaundecanoic acid (217.3 mg, 0.51 mmol, Broadpharm), NHS (116.5 mg, 1.01 mmol) and EDC·HCl (194.0 mg, 1.01 mmol) in DMF (2 mL) was prepared and the mixture was added to the Fmoc-deprotected compound in DMF (2 mL). After 15 h, the reaction was quenched with 15% citric acid and extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The residue was chromatographed on silica gel (20 g) with DCM/MeOH (50:1 to 10:1) to afford **S9** (186.1 mg, 42% for the 5 steps). Pale yellow oil. HRMS (ESI) Calcd. For $C_{45}H_{69}N_{10}O_{14}$ $[M+H]^+$: 973.4989. Found: 973.4981. ^{1}H NMR (300 MHz, DMSO-d$_6$) δ 8.08 (t, J = 5.5 Hz, 1H), 7.89 (d, J = 7.4 Hz, 2H), 7.69 (d, J = 7.4 Hz, 2H), 7.61 (t, J = 5.9 Hz, 1H), 7.55 (d, J = 8.4 Hz, 1H), 7.41 (t, J = 7.1 Hz, 2H), 7.32 (t, J = 7.3, 1.0 Hz, 3H, aromatic-H and -NH), 4.35–4.15 (m, 4H), 3.91 (s, 2H), 3.84 (s, 2H), 3.63–3.46 (m, 28H), 3.43–3.35 (m, 8H), 3.25–3.01 (m, 6H), 1.70–1.46 (m, 2H), 1.46–1.31 (m, 2H), 1.31–1.09 (m, 2H).

Synthesis of amine-PEG$_3$-Lys(PEG$_3$-N$_3$)-PEG$_3$-N$_3$ 5



The compound **S9** (42.0 mg, 0.043 mmol) was dissolved in DMF (0.4 mL) and diethylamine (0.4 mL) was added to the solution at room temperature. After 1 h, the mixture was concentrated and the Fmoc-deprotected compound was afforded. The residue was chromatographed on silica gel (5 g) with DCM/MeOH (30:1 to 5:1) to afford **5** (15.6 mg, 49%). Pale yellow oil. HRMS (ESI) Calcd. For $C_{30}H_{59}N_{10}O_{12}$ $[M+H]^+$: 751.4308. Found: 751.4319. ^{1}H NMR (300 MHz, CD_3OD) δ 4.46 (dd, J = 8.3, 5.6 Hz, 1H), 4.09 (s, 2H), 4.01 (s, 2H), 3.87–3.61 (m, 30H), 3.58 (t, J = 5.4 Hz, 2H), 3.47–3.37 (m, 6H), 3.28 (t, J = 7.0 Hz, 2H), 3.21–3.15 (m, 2H), 1.92–1.67 (m, 2H), 1.66–1.54 (m, 2H), 1.50–1.34 (m, 2H).

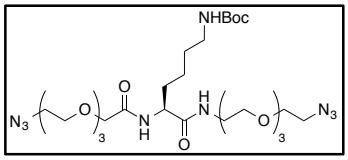
Synthesis of amine-PEG$_3$-PEG$_3$-Lys(PEG$_3$-N$_3$)-PEG$_3$-N$_3$ 6



The compound **S9** (17.1 mg, 0.018 mmol) was dissolved in DMF (0.4 mL) and diethylamine (0.4 mL) was added to the solution at room temperature. After 1 h, the mixture was concentrated and the Fmoc-deprotected compound was afforded. In other flask, a mixture of Fmoc-11-amino-3,6,9-trioxaundecanoic acid (7.6 mg, 0.018 mmol, Broadpharm), NHS (3.0 mg, 0.026 mmol) and EDC·HCl (5.1 mg, 0.026 mmol) in DMF (0.4 mL) was prepared and the mixture was added to the Fmoc-deprotected compound in DMF (0.8 mL). After 16 h, the reaction was quenched with 15% citric acid and extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The crude compound was immediately used without further purification.

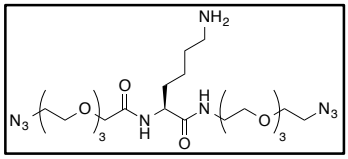
The crude compound was dissolved in DMF (0.4 mL) and diethylamine (0.4 mL) was added to the solution at room temperature. After 1 h, the mixture was concentrated and the Fmoc-deprotected compound was afforded. The residue was chromatographed on silica gel (3 g) with DCM/MeOH (30:1 to 5:1) to afford **6** (10.8 mg, 65% for the 3 steps). Pale yellow oil. HRMS (ESI) Calcd. For $C_{38}H_{73}N_{11}O_{16}Na$ $[M+Na]^+$: 962.5129. Found: 962.5129. ^{1}H NMR (300 MHz, CD_3OD) δ 4.43 (dd, J = 8.5, 5.6 Hz, 1H), 4.09 (s, 2H), 4.06 (s, 2H), 4.01 (s, 2H), 3.84–3.55 (m, 42H), 3.48 (t, J = 5.5 Hz, 2H), 3.45–3.36 (m, 6H), 3.28 (t, J = 7.0 Hz, 2H), 3.22–3.13 (m, 2H), 1.91–1.68 (m, 2H), 1.67–1.55 (m, 2H), 1.49–1.33 (m, 2H).

Synthesis of azide-PEG$_3$-Lys(Boc)-PEG$_3$-N$_3$ S10



The crude compound **S7** was dissolved in DMF (2.5 mL) and diethylamine (2.5 mL) was added at room temperature. After 1 h, the mixture was concentrated and the Fmoc-deprotected compound was afforded. In other flask, the mixture of 11-azido-3,6,9-trioxaundecanoic acid (133.0 mg, 0.57 mmol), NHS (131.7 mg, 1.14 mmol) and EDC·HCl (219.3 mg, 1.14 mmol) in DMF (1.5 mL) was prepared and the mixture was added to the Fmoc-deprotected compound in DMF (3.5 mL). After 21 h, the reaction was quenched with 15% citric acid and extracted with ethyl acetate. The residue was chromatographed on silica gel (20 g) with DCM/MeOH (50:1 to 20:1) to afford **S10** (154.7 mg, 53% for the 3 steps). Pale yellow oil. HRMS (ESI) Calcd. For $C_{27}H_{52}N_9O_{10}$ $[M+H]^+$: 662.3832. Found: 662.3836. ^{1}H NMR (300 MHz, CD_3OD) δ 4.44 (dd, J = 8.4, 5.7 Hz, 1H), 4.07 (s, 2H), 3.84–3.62 (m, 20H), 3.58 (t, J = 5.4 Hz, 2H), 3.47–3.36 (m, 6H), 3.06 (t, J = 6.9 Hz, 2H), 1.91–1.66 (m, 2H), 1.56–1.48 (m, 2H), 1.46 (s, 9H), 1.43–1.30 (m, 2H).

Synthesis of azide-PEG$_3$-Lys-PEG$_3$-N$_3$ 7



The crude compound **S10** (6.7 mg, 0.010 mmol) was dissolved in DCM (0.4 mL) and TFA (0.4 mL) was added to the solution at room temperature. After 1 h, the mixture was concentrated and the Boc-deprotected compound was afforded. The crude compound was purified by preparative RP-HPLC to give analytically pure compound **7** (4.6 mg, 81%). Colorless oil. HRMS (ESI) Calcd. For $C_{22}H_{43}N_9O_8Na$ $[M+Na]^+$: 584.3127. Found: 584.3133. ^{1}H NMR (300 MHz, CD_3OD) δ 4.47 (dd, J = 8.3, 5.7 Hz, 1H), 4.08 (s, 2H), 3.81–3.62 (m, 20H), 3.59 (t, J = 5.4 Hz, 2H), 3.47–3.37 (m, 6H), 2.95 (t, J = 7.5 Hz, 2H), 2.01–1.62 (m, 4H), 1.57–1.38 (m, 2H).

Assay protocol

Förster resonance energy transfer (FRET) assay

In a 96-well black plate, 49 μL of MES buffer (25 mM MES-Na, 1 mM DTT, pH 5.0) and 1 μL of each test compound (1 mM in DMSO, final conc.10 μM) was added. To each well was added 50 μL of human liver cathepsin B (1 ng/μL, EMD Millipore) in MES buffer. Immediately, fluorescence (Ex: 280 nm; Em: 360 nm) of each well was recorded using a plate reader at 37 °C every 30 sec. All assays were performed in triplicate.

Cell culture

SKBR-3 (ATCC) was cultured in RPMI1640 (Corning) with 10% EquaFETAL® (Atlas Biologicals), 1% glutamax (Corning), 1% sodium pyruvate (Corning), and 1% penicillin-streptomycin (Gibco). MDA-MB-453 and MDA-MB-231 (ATCC) were cultured in DMEM (Corning) with 10% EquaFETAL®, 1% glutamax, and 1% penicillin-streptomycin. All cells were cultured at 37 °C under 5% CO_2 and passaged before becoming fully confluent.

Expression and purification of N297A and N297Q antibodies

Free style HEK-293 human embryonic kidney cells (Invitrogen) were transfected with a mammalian expression vector encoding for antibody kappa light chain and full length heavy chain sequences (based on variable sequences of trastuzumab) under CMV promoter control. The antibody heavy chain constant region had a mutation of N297A or N297Q to produce aglycosylated IgG1 antibodies. The transfected HEK-293 cells were cultured in a humidified cell culture incubator with 8% CO_2, and shaking at 150 rpm for 7 days before harvesting the culture medium. The antibody secreted into the culture medium was purified using Protein A resin (GE Healthcare, Piscataway, NJ) according to the protocol previously reported from our lab.[2,3] A non-targeting N297A IgG1 (isotype control) was prepared in the same manner.

MTGase-mediated antibody-linker conjugation

A N297A antibody in PBS (32 μL, 9.3 mg/mL) was incubated with linker (7.92 μL of 100 mM stock in PBS, 400 equiv.) and microbial MTGase (purchased from Zedira, 8 μL containing 2 unit) overnight at room temperature. The reaction was monitored by LC-MS equipped with a MabPac RP column (3×50 mm, 4 μm, Thermo Scientific). The conditions were as follows: flow rate = 0.5 mL/min; solvent A = water containing 0.1% formic acid; solvent B = acetonitrile containing 0.1% formic acid. The antibody was purified by size exclusion chromatography (SEC, Superdex 200 increase 10/300 GL, GE

Healthcare) with PBS (flow rate = 0.6 mL/min). N297Q antibody-based conjugation was performed in a similar manner.

Strain-promoted azide−alkyne cycloaddition

To a solution of antibody-linker conjugate in PBS (25μL, 4.0 mg/mL) was added DBCO-PEG_4-Val-Cit-PABC-MMAF (0.96 μL of 4 mM stock solution in DMSO, 1.5 equiv. per azide, Levena Biopharma, CA, U.S.) and the mixture was incubated for 1 hour at room temperature. The reaction was monitored by LC-MS equipped with a MabPac RP column. The conditions were as follows: flow rate = 0.5 mL/min; solvent A = water containing 0.1% formic acid; solvent B = acetonitrile containing 0.1% formic acid. The antibody was purified by size exclusion chromatography (SEC, Superdex 200 increase 10/300 GL, GE Healthcare) with PBS (flow rate = 0.6 mL/min). The average DAR value was determined based on UV peak areas.

Plasma stability test[4]

Pooled normal human plasma (Innovative Research) was treated with protein A plus agarose beads (Thermo Scientific) for 1 hour at 4 °C to exclude endogenous IgG. The suspension was centrifuged (10,000 g, 4 °C, 20 min) and the supernatant was collected. ADC **9** (1 mg/mL, 4 μL) was added to the IgG-free human plasma (36 μL) to a final concentration of 100 μg/mL. Four identical samples were prepared for each time point (0, 1, 3 and 7 days). After being incubated at 37 °C for varying time, the samples were taken (40 μL each) and stored at −80 °C until use. The mixtures were incubated with protein A plus agarose beads (5 μL bed volume) for 1 hour at 4 °C, washed with PBS (3×200 μL) and the residual antibody was eluted with 50 mM, pH 2.2 glycine-HCl (3×10 μL). The eluent was analyzed by monitoring UV absorbance at 280 nm using LC-MS equipped with a MabPac RP column.

Cell-based ELISA[5]

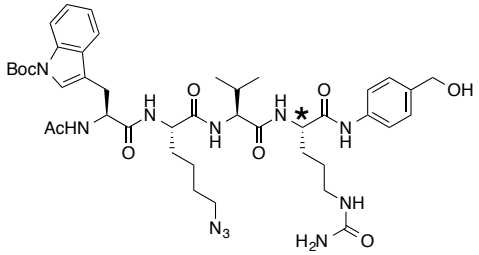
In a 96-well black with clear plate (culture treated),100 μL cells (SKBR-3 and MDA-MB-231) were seeded at 10,000 cells/well and incubated for 24 h at 37 °C with 5% CO_2. To fix the cells, 100 μL of 8% paraformaldehyde was added to each well of the plate containing culture media, and incubated for 15 min at room temperature. After incubation, the solution was aspirated and the cells were washed three times with 200 μL of sterile PBS. The solution was removed and 200 μL of freshly prepared 1x permeabilization buffer (1% TritonX-100 in PBS) was added to each well of the plate, and the plate was incubated for 30 min at room temperature. Permeabilization buffer was removed and 200 μL of 2x blocking buffer (0.2% BSA in PBS) was added to each well of the plate, and the plate was incubated for

2 h with agitation. Afterwards, serial dilutions of samples (0.1% BSA in PBS) were prepared in a separate plate and 100 µL was transferred to the plate containing the cells, and the plate was incubated overnight at 4 °C with agitation. The following day, the solution was discarded and the cells were washed three times with 200 µL of wash buffer (0.25% Tween 20 in PBS). Then 100 µL of goat anti-human IgG (H+L) secondary antibody (Alexa Fluor 647) at 5 µg/mL (0.1% BSA in PBS) was added to each well of the plate and the plate was incubated for 2 h in the dark. The plate was washed three times with wash buffer, and fluorescence (Ex: 650 nm; Em: 680 nm) of each well was recorded using a plate reader and K_D values were calculated using Graph Pad Prism 7 software. All assays were performed in triplicate.

Cell viability assay[6]

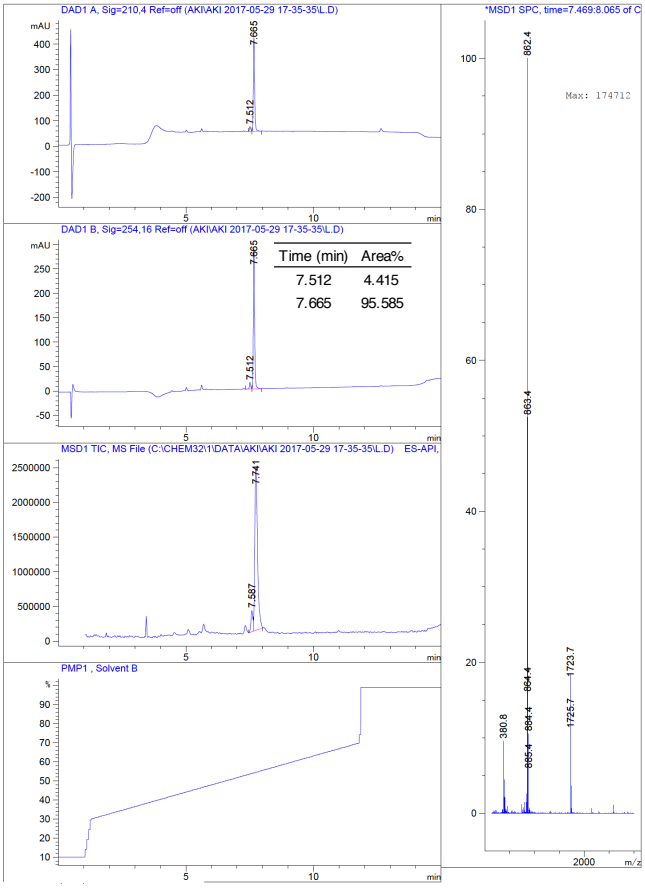
50 µL cells were seeded in a 96-well clear plate (culture treated) at 5,000 cells/well and incubated for 24 h at 37 °C with 5% CO_2. Serial dilutions of samples were prepared in a separate plate and 50 µL was transferred to each well of the plate scontaining the cells, and the cells were incubated at 37 °C with 5% CO_2. After 96 hours, 50 µL of a mixture of XTT (2,3-Bis-(2-Methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt, from Biotium) and PMS (Phenazine methosulfate, from ACROS Organics) was added to each well, and the plate was incubated for 3 h at 37 °C with 5% CO_2. The absorbance at 475 nm of each well was recorded after a gentle agitation using a plate reader, and EC_{50} values were calculated using Graph Pad Prism 7 software. All assays were performed in triplicate.

4. HPLC and NMR data

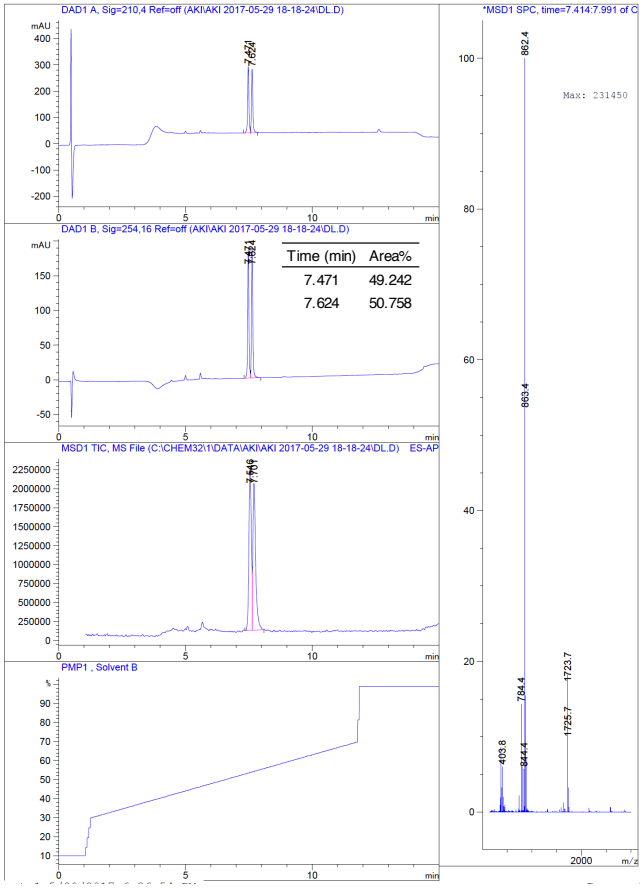


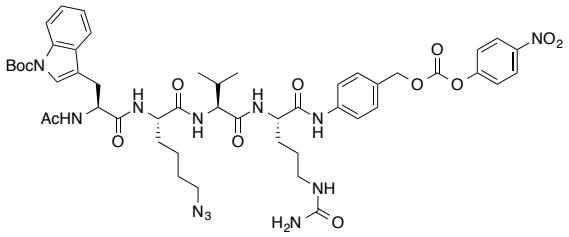
Precursor of **S2**
Chemical Formula: $C_{42}H_{59}N_{11}O_9$
Exact Mass: 861.45

Synthesized using L-citrulline. (4.4% epimer)



Synthesized using DL-citrulline. (epimeric mixture)

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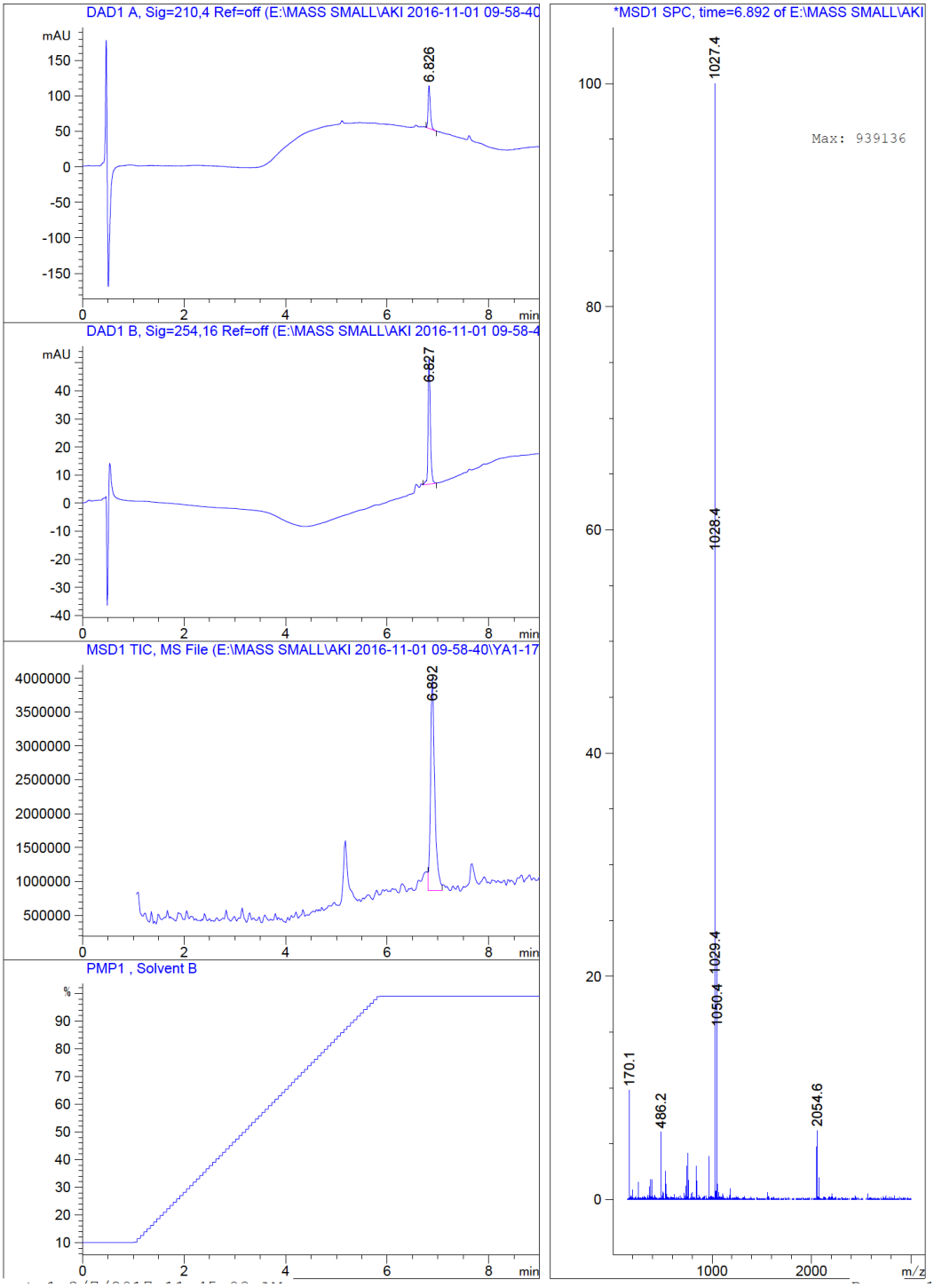
S2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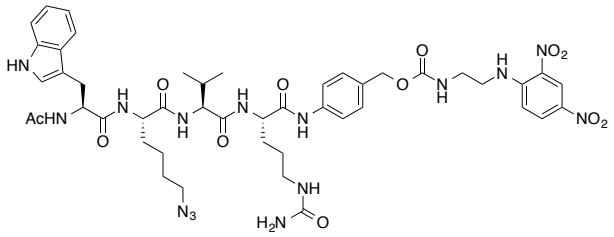

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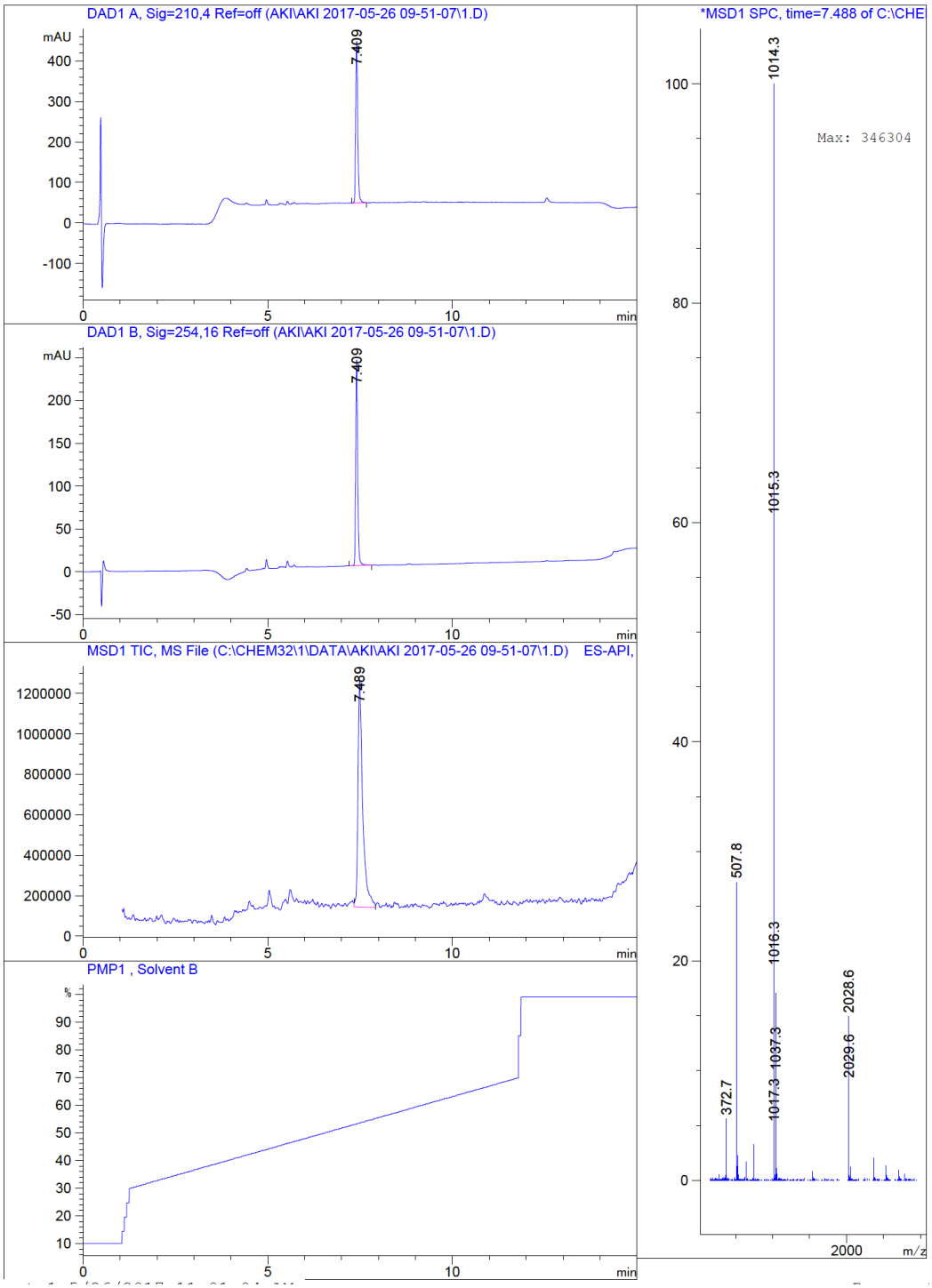
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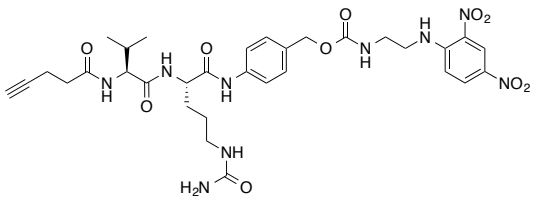
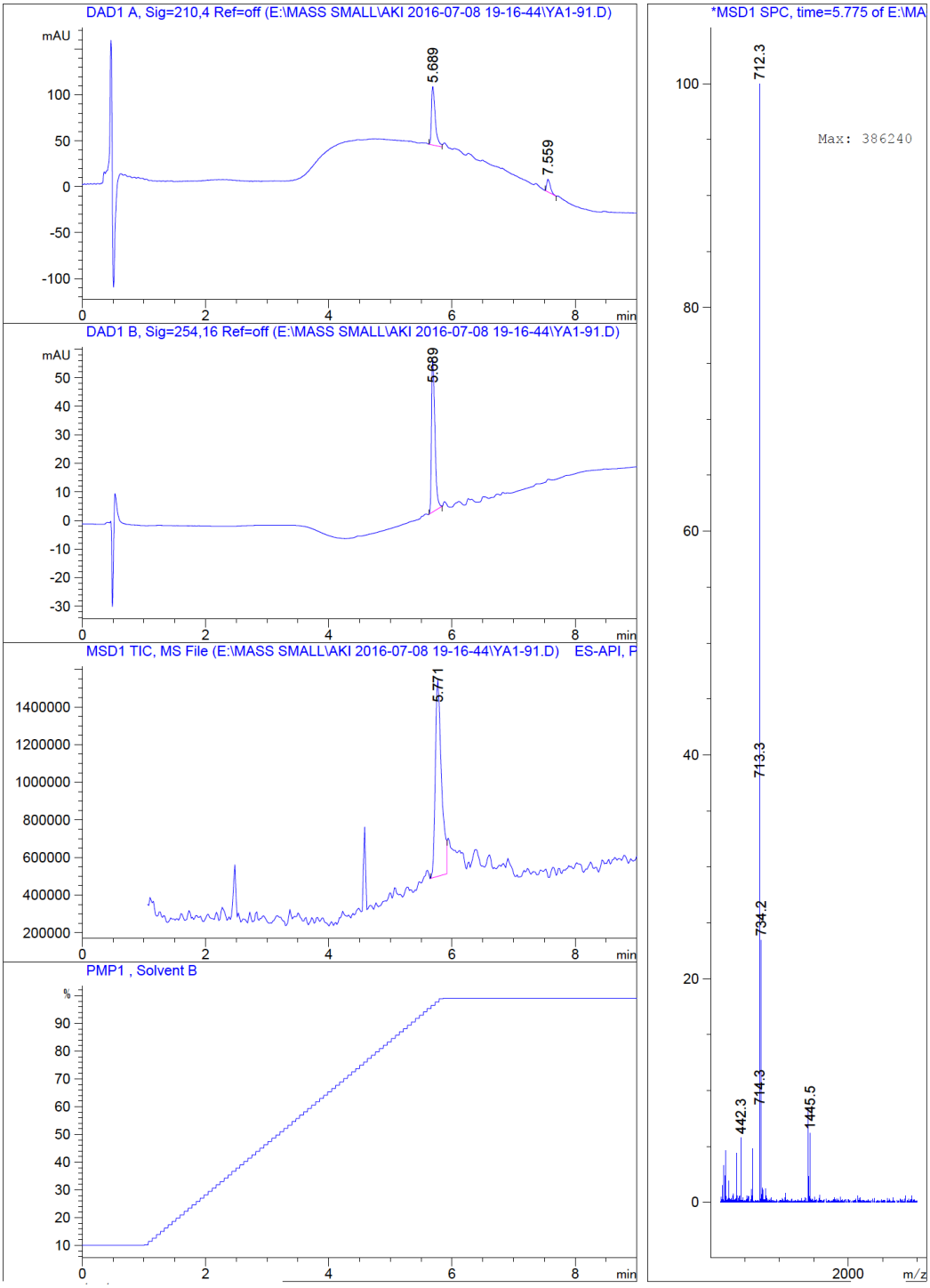


S4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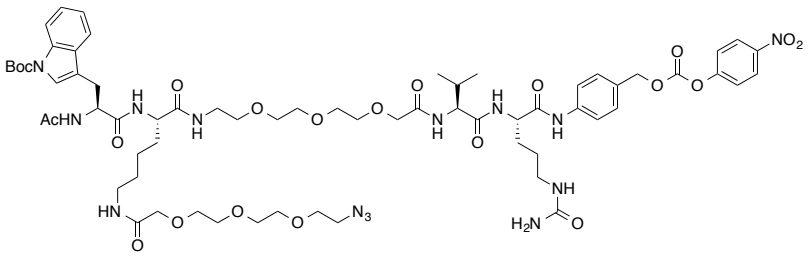
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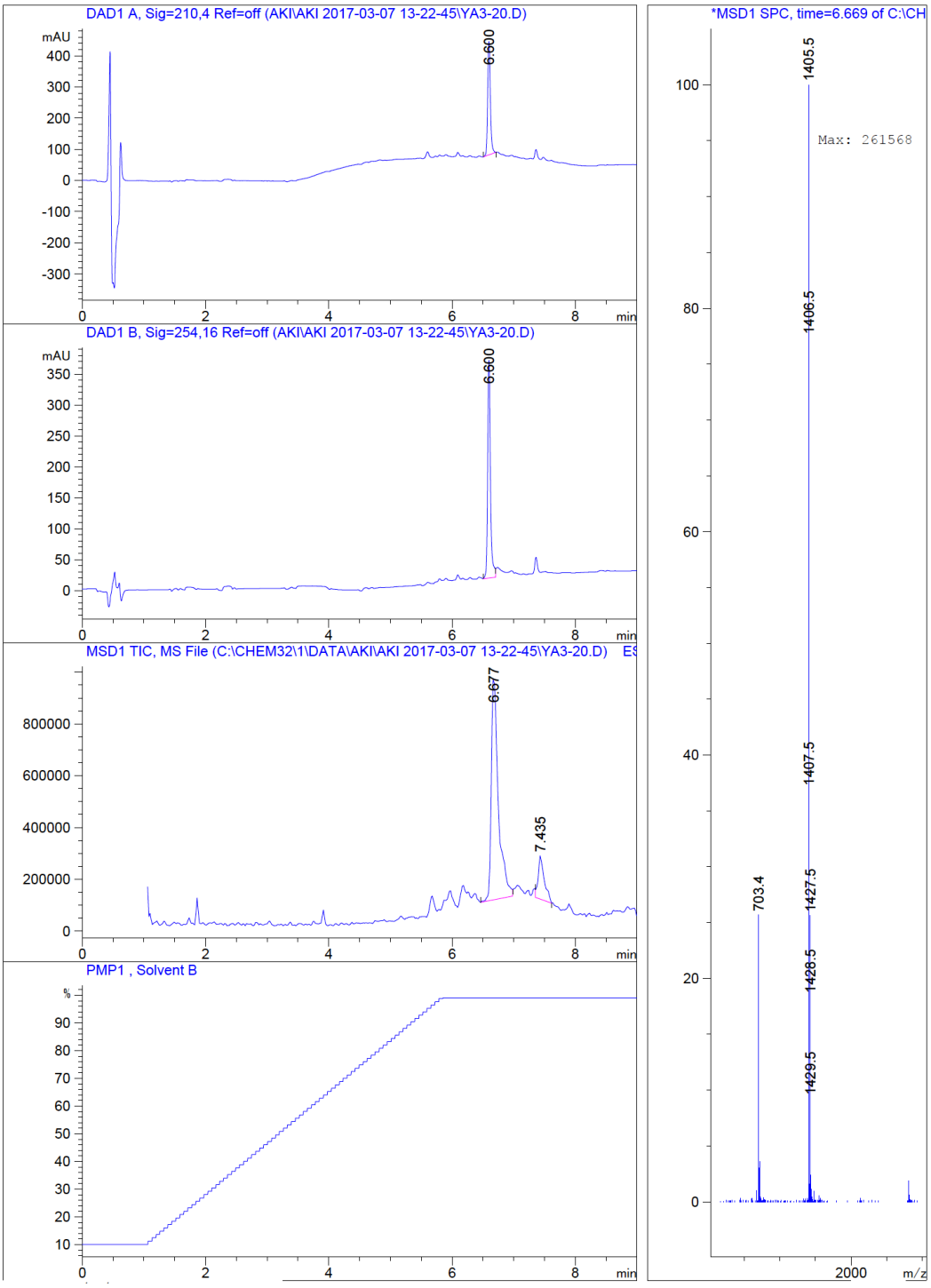
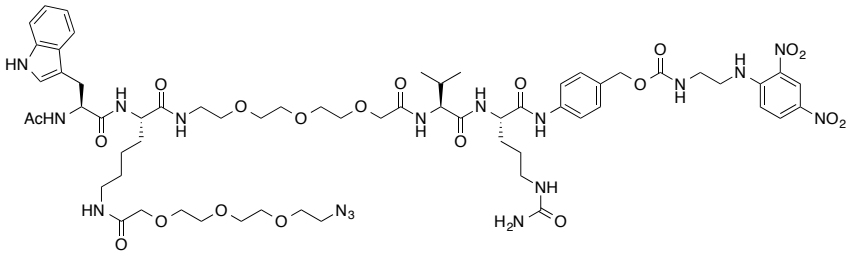
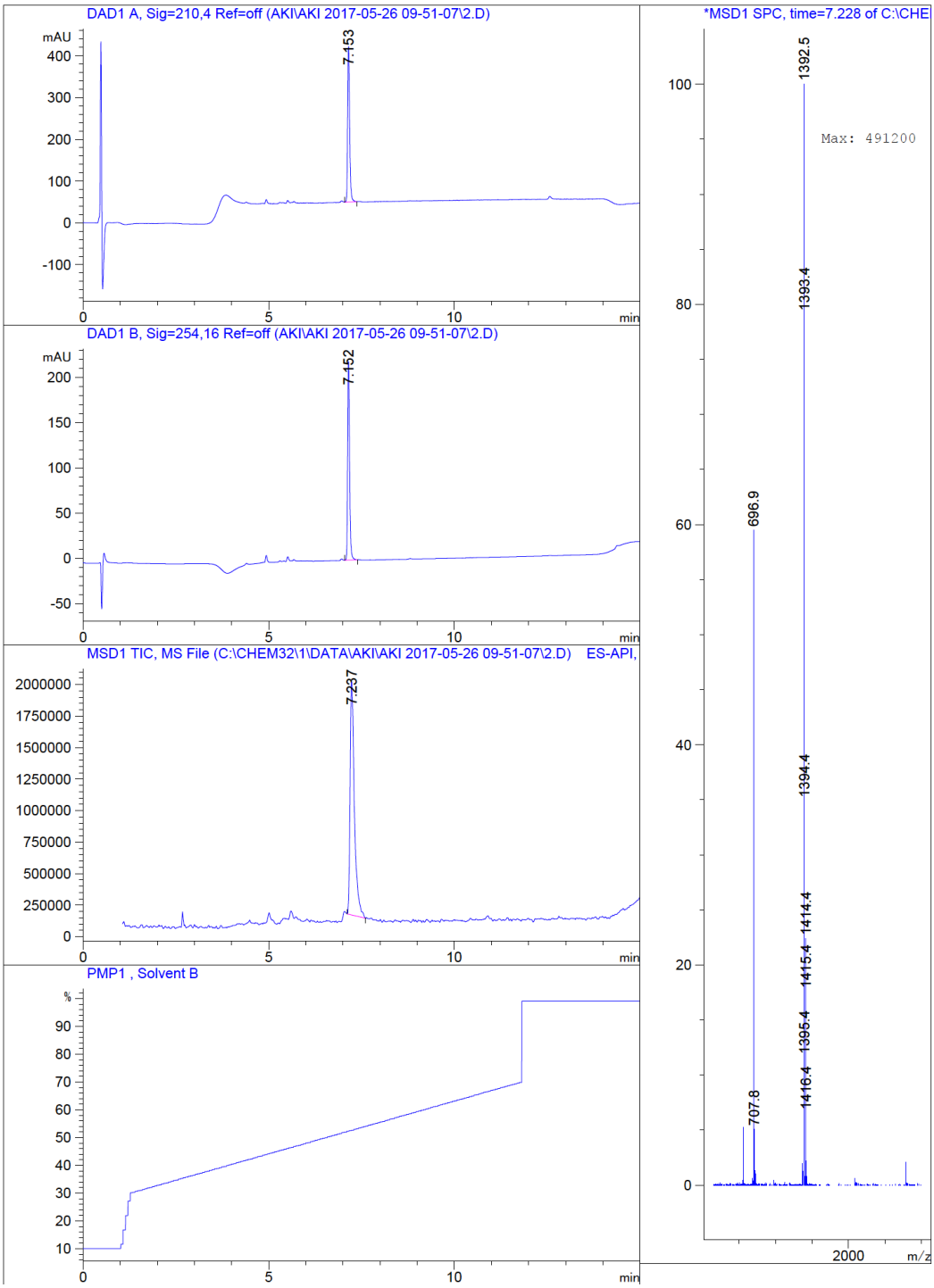
NO_2

S6


DAD1 A, Sig=210,4 Ref=off (AKI\AKI 2017-03-07 13-22-45\YA3-20.D)
mAU
6.600
min
DAD1 B, Sig=254,16 Ref=off (AKI\AKI 2017-03-07 13-22-45\YA3-20.D)
mAU
6.600
min
MSD1 TIC, MS File (C:\CHEM32\1\DATA\AKI\AKI 2017-03-07 13-22-45\YA3-20.D)
6.677
7.435
min
PMP1 , Solvent B
%
min
*MSD1 SPC, time=6.669 of C:\CH
Max: 261568
1405.5
1406.5
1407.5
1427.5
1428.5
1429.5
703.4
m/z



2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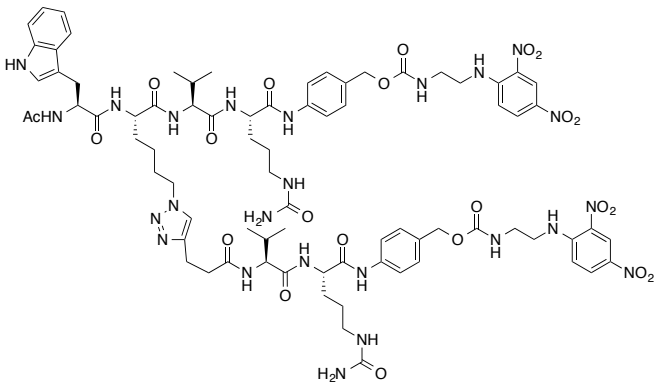
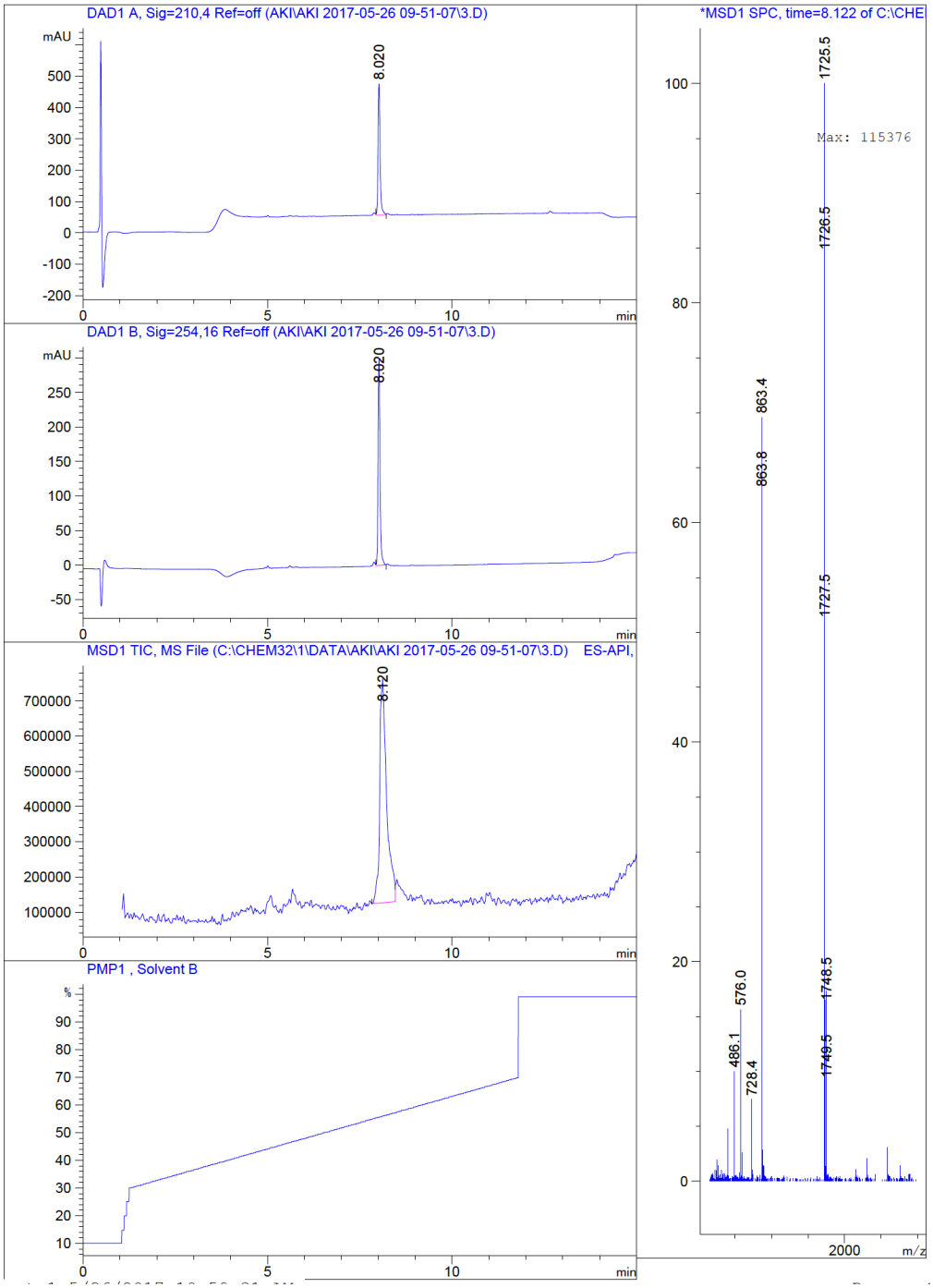
mAU
7.153
min
DAD1 B, Sig=254,16 Ref=off (AKI\AKI 2017-05-26 09-51-07\2.D)
mAU
7.152
min
MSD1 TIC, MS File (C:\CHEM32\1\DATA\AKI\AKI 2017-05-26 09-51-07\2.D) ES-API,
7.237
min
PMP1 , Solvent B
%
min
*MSD1 SPC, time=7.228 of C:\CHE
Max: 491200
1392.5
1393.4
696.9
1394.4
1414.4
1415.4
1395.4
1416.4
707.8
m/z



3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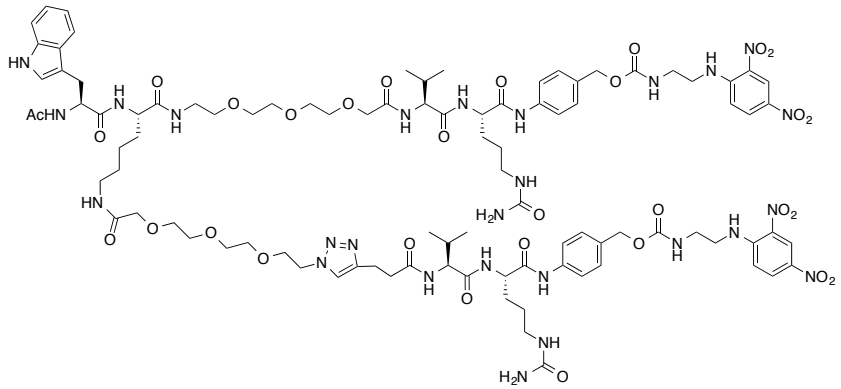
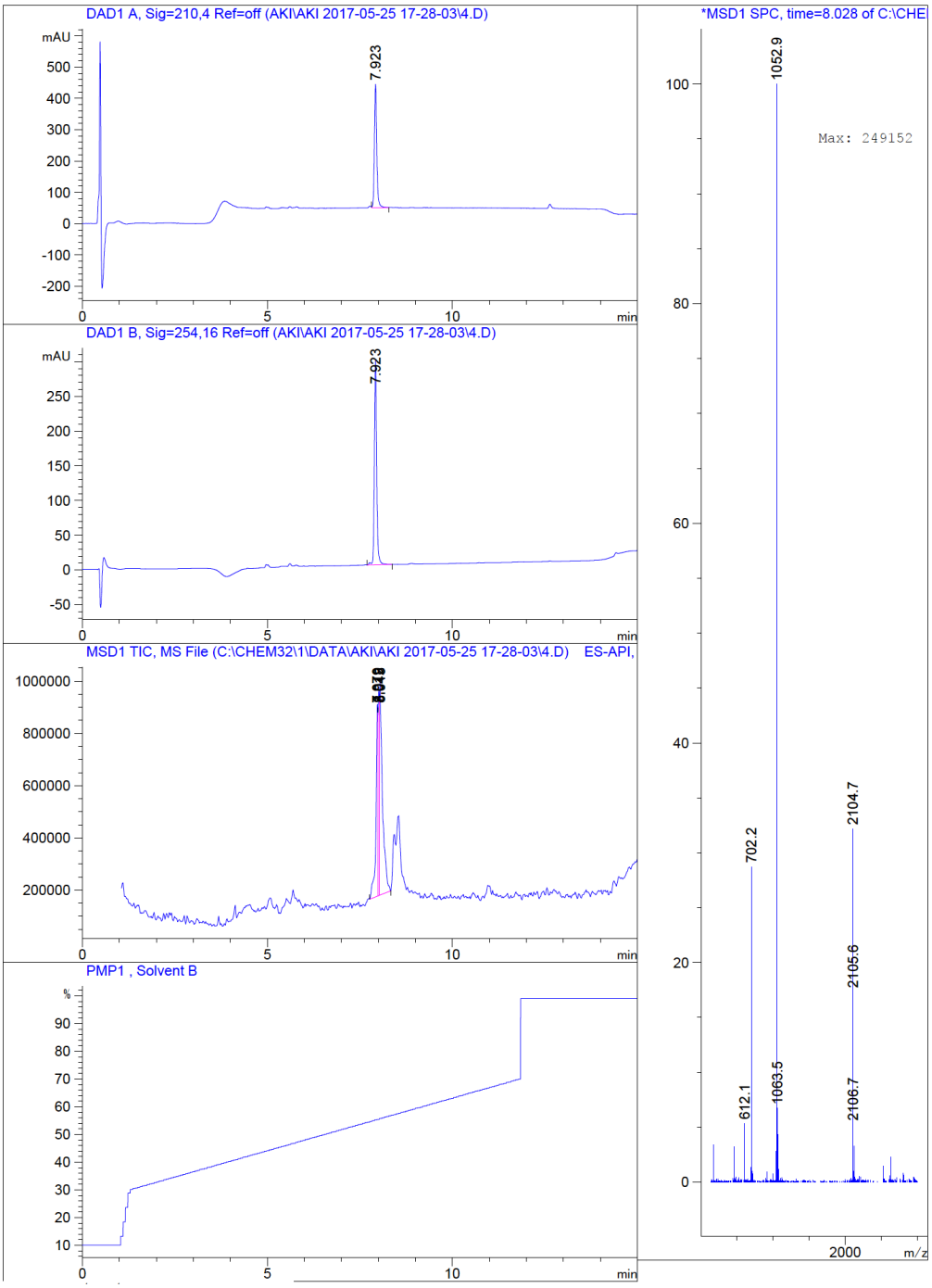
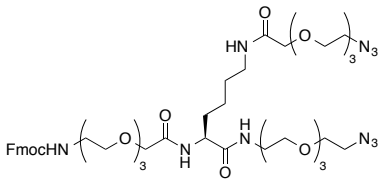
mAU
8.020
min
DAD1 B, Sig=254,16 Ref=off (AKI\AKI 2017-05-26 09-51-07\3.D)
mAU
8.020
min
MSD1 TIC, MS File (C:\CHEM32\1\DATA\AKI\AKI 2017-05-26 09-51-07\3.D) ES-API,
8.120
min
PMP1 , Solvent B
%
min
*MSD1 SPC, time=8.122 of C:\CHE
Max: 115376
1725.5
1726.5
1727.5
863.4
863.8
576.0
486.1
728.4
1748.5
1749.5
m/z

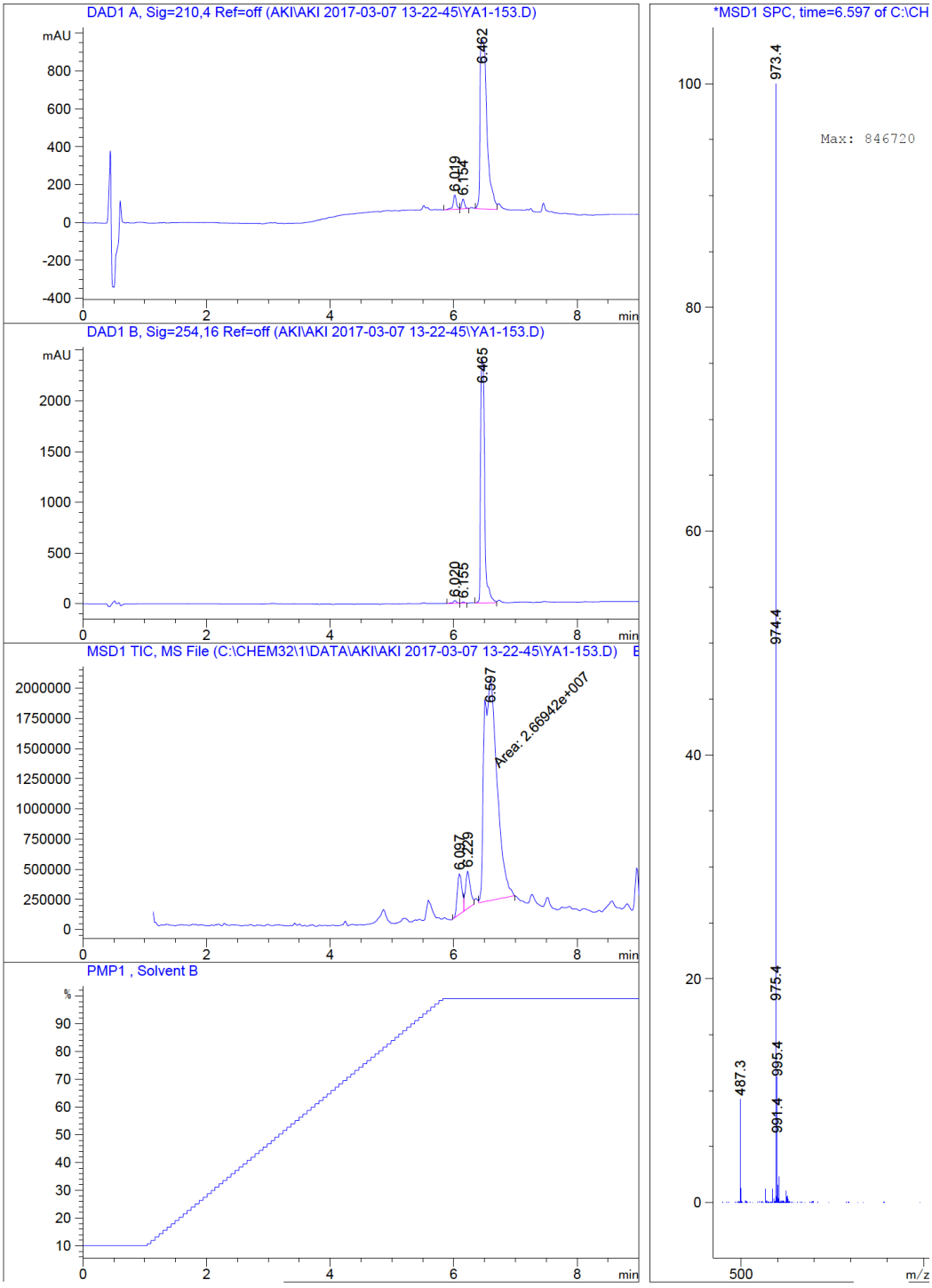


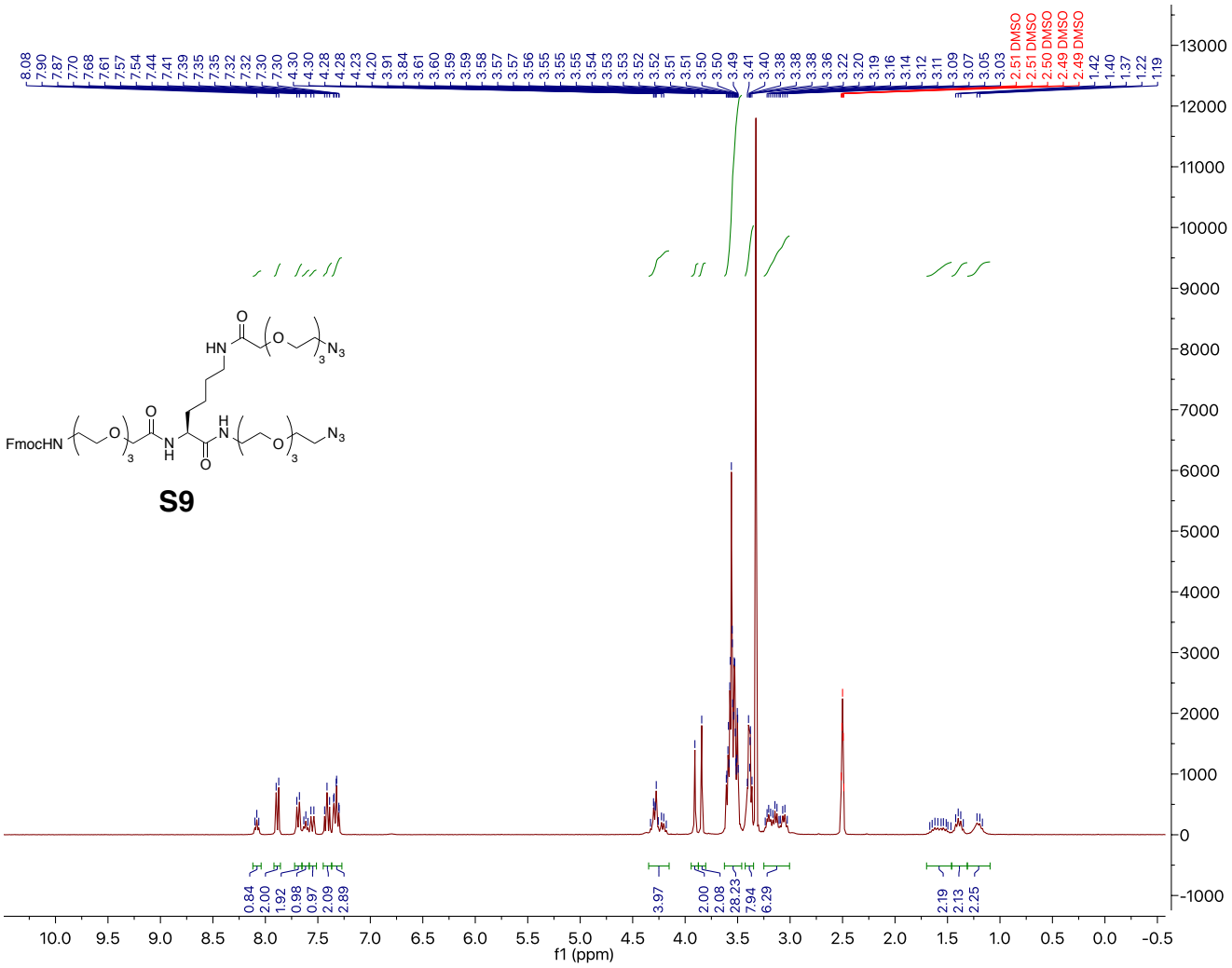
4

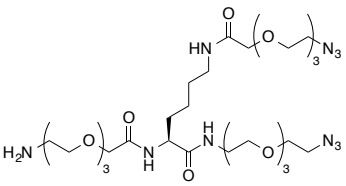

DAD1 A, Sig=210,4 Ref=off (AKI\AKI 2017-05-25 17-28-03\4.D)
mAU
7.923
min
DAD1 B, Sig=254,16 Ref=off (AKI\AKI 2017-05-25 17-28-03\4.D)
mAU
7.923
min
MSD1 TIC, MS File (C:\CHEM32\1\DATA\AKI\AKI 2017-05-25 17-28-03\4.D) ES-API,
min
PMP1 , Solvent B
%
min
*MSD1 SPC, time=8.028 of C:\CHE
1052.9
Max: 249152
702.2
2104.7
2105.6
612.1
1063.5
2106.7
m/z



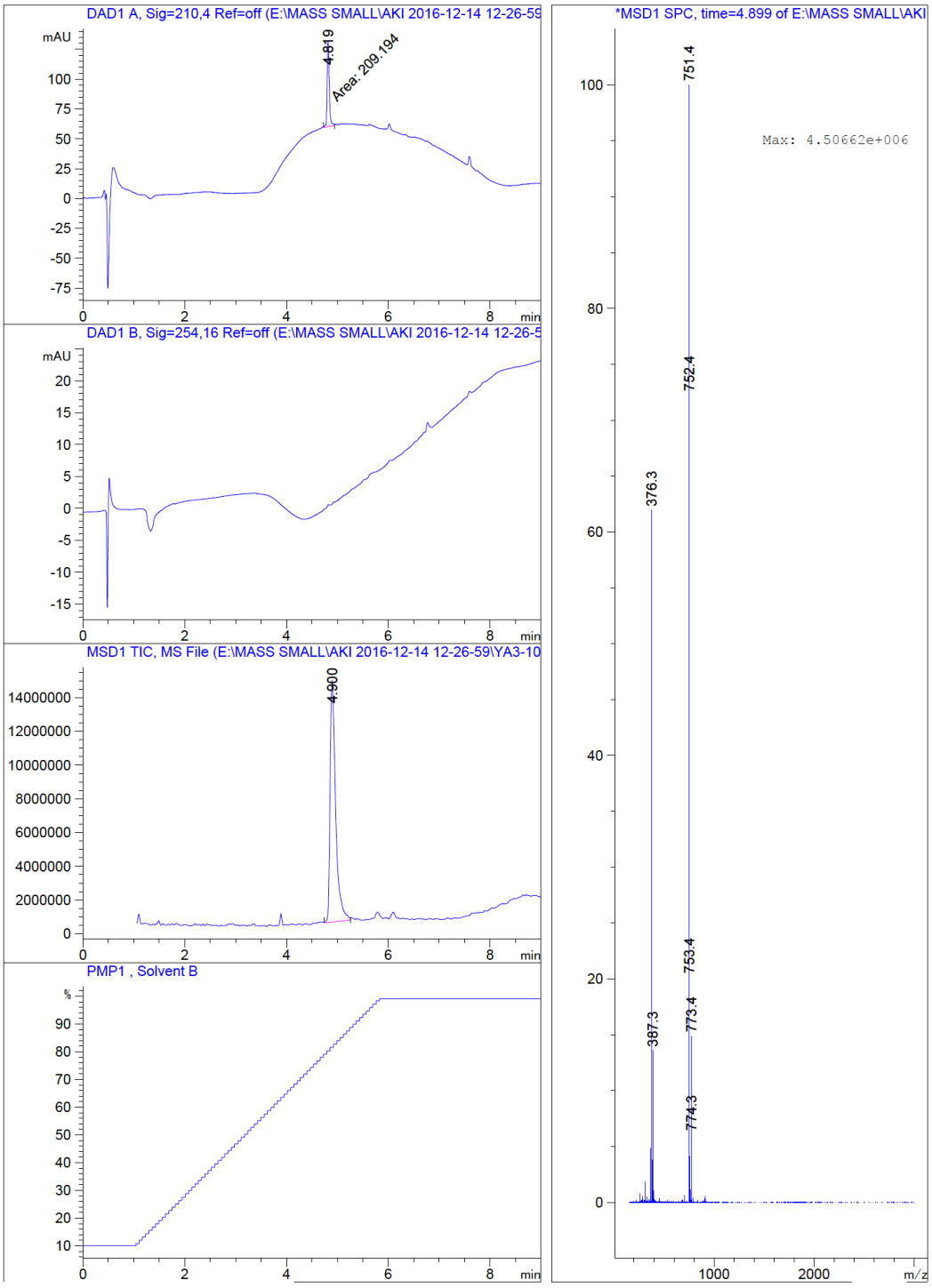
S9

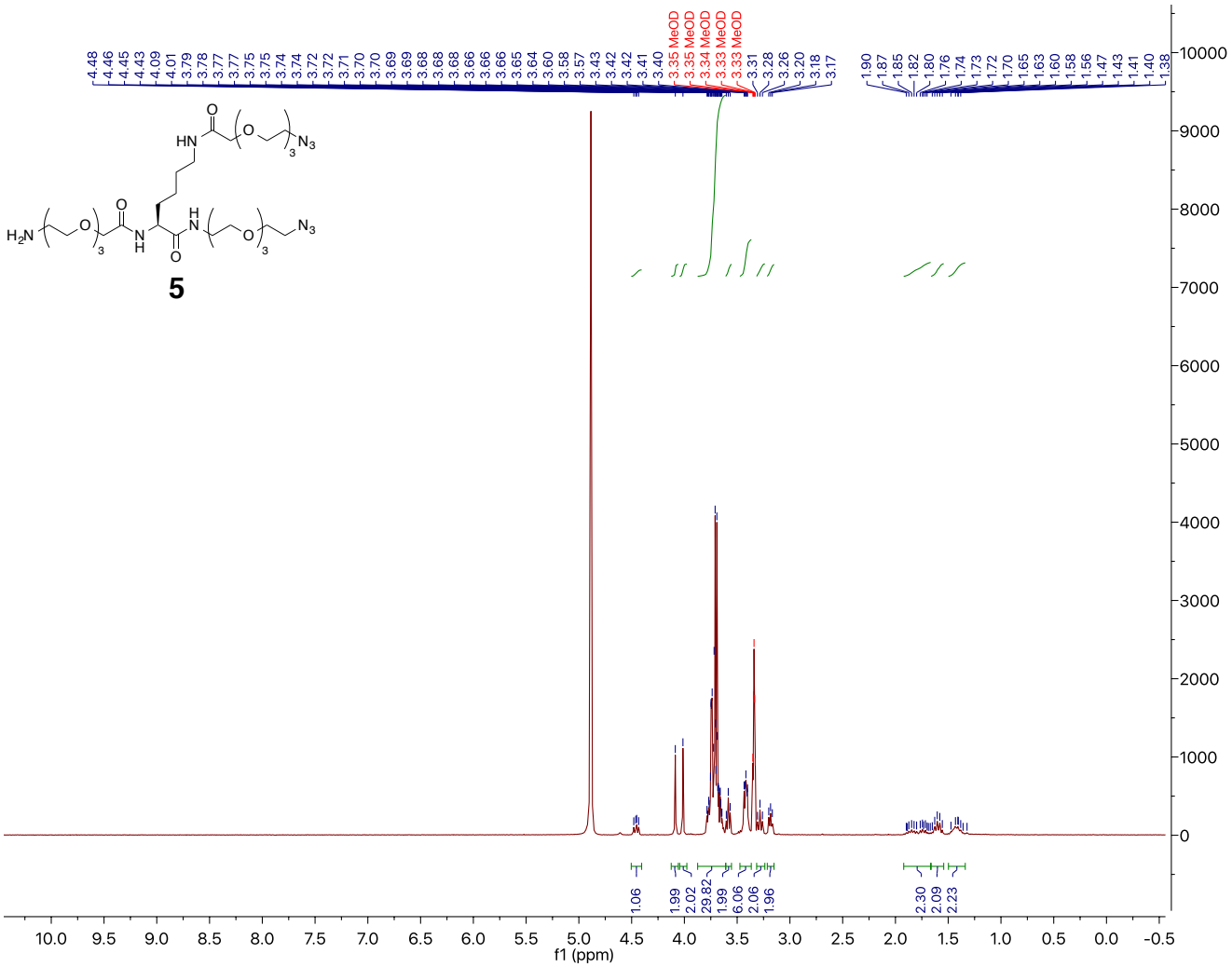

DAD1 A, Sig=210,4 Ref=off (AKI\AKI 2017-03-07 13-22-45\YA1-153.D)
mAU
6.462
6.019
6.154
min
DAD1 B, Sig=254,16 Ref=off (AKI\AKI 2017-03-07 13-22-45\YA1-153.D)
mAU
6.465
6.020
6.155
min
MSD1 TIC, MS File (C:\CHEM32\1\DATA\AKI\AKI 2017-03-07 13-22-45\YA1-153.D)
6.597
Area: 2.66942e+007
6.097
6.229
min
PMP1 , Solvent B
%
min
*MSD1 SPC, time=6.597 of C:\CH
973.4
Max: 846720
974.4
975.4
995.4
991.4
487.3
m/z

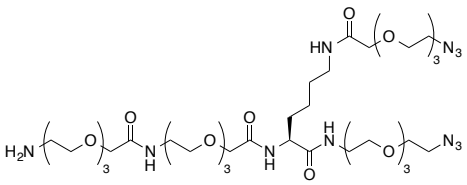

S9
FmocHN
HN
N3
f1 (ppm)



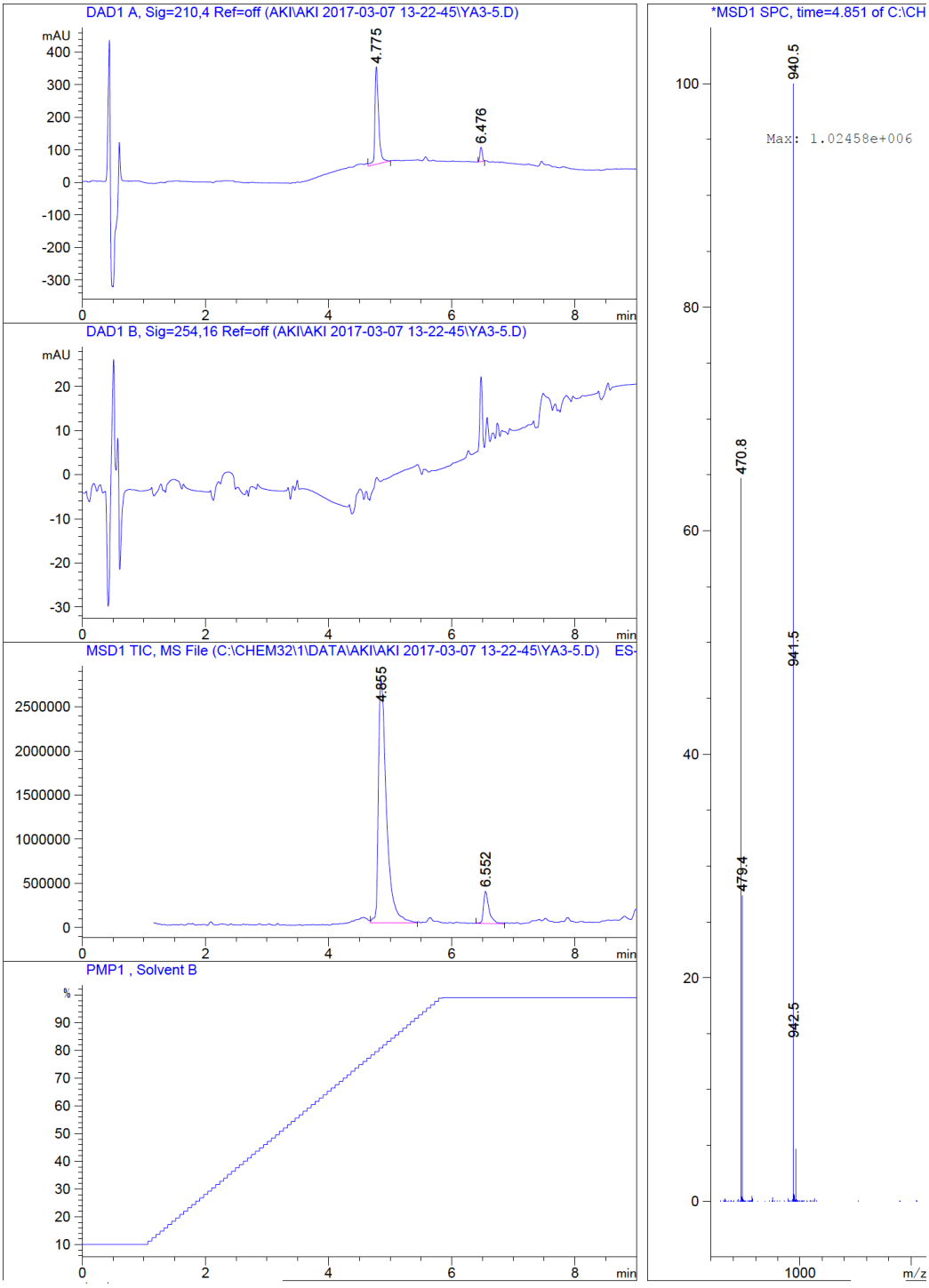
5

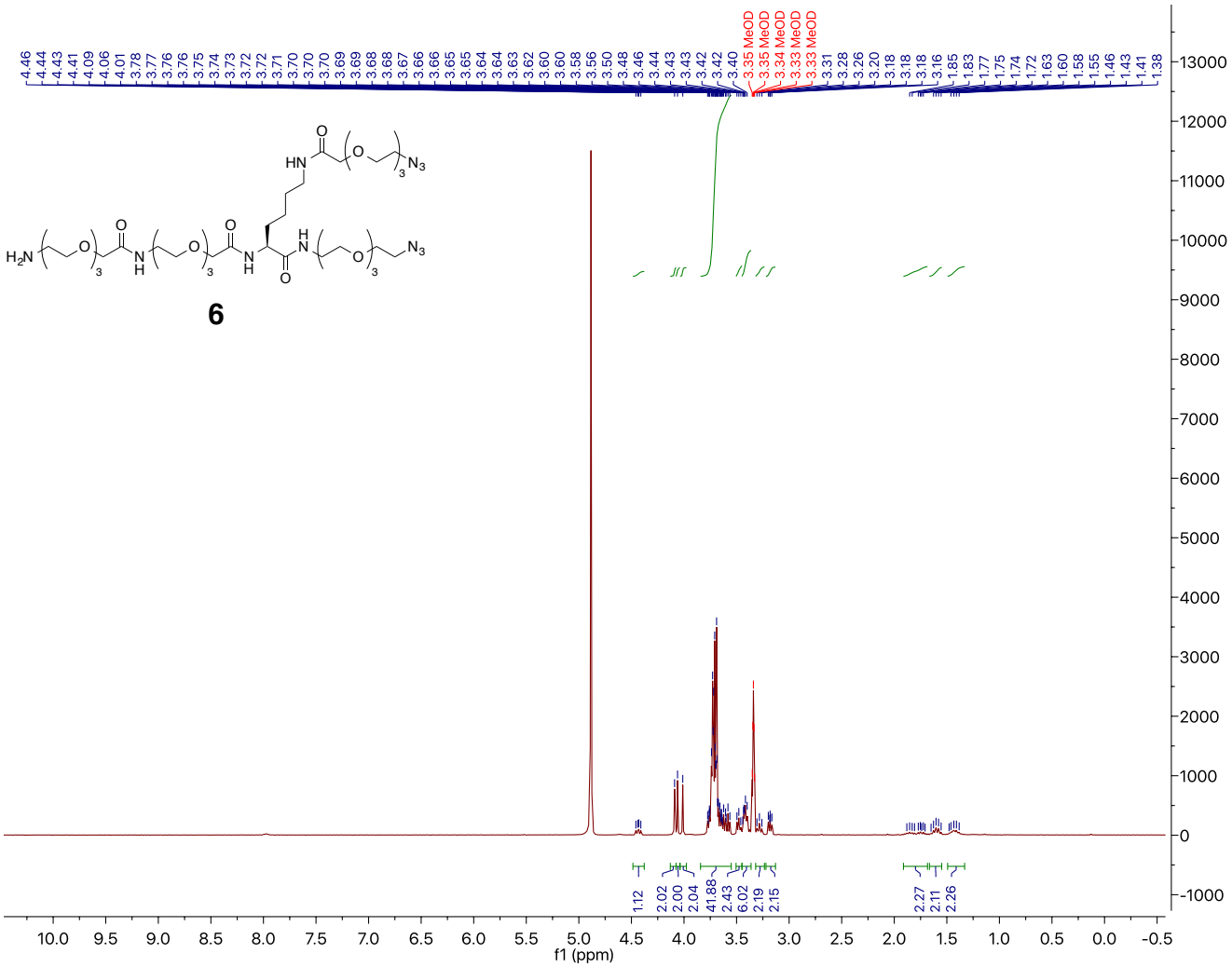

DAD1 A, Sig=210,4 Ref=off (E:\MASS SMALL\AKI 2016-12-14 12-26-59
mAU
4.819
Area: 209.194
min
DAD1 B, Sig=254,16 Ref=off (E:\MASS SMALL\AKI 2016-12-14 12-26-5
mAU
min
MSD1 TIC, MS File (E:\MASS SMALL\AKI 2016-12-14 12-26-59\YA3-10
4.900
min
PMP1 , Solvent B
%
min
*MSD1 SPC, time=4.899 of E:\MASS SMALL\AKI
Max: 4.50662e+006
751.4
752.4
376.3
753.4
773.4
387.3
774.3
m/z

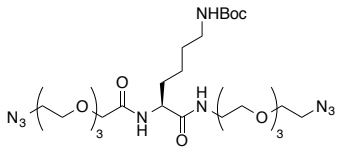

4.48 4.46 4.45 4.43 4.09 4.01 3.79 3.78 3.77 3.77 3.75 3.75 3.74 3.74 3.72 3.72 3.71 3.70 3.70 3.69 3.69 3.68 3.68 3.68 3.66 3.66 3.66 3.65 3.64 3.60 3.58 3.57 3.43 3.42 3.42 3.41 3.40 3.35 MeOD 3.35 MeOD 3.34 MeOD 3.33 MeOD 3.33 MeOD 3.31 3.28 3.26 3.20 3.18 3.17
1.90 1.87 1.85 1.82 1.80 1.76 1.74 1.73 1.72 1.70 1.65 1.63 1.60 1.58 1.56 1.47 1.43 1.41 1.40 1.38
HN
O
N3
H2N
N
H
5
10000 9000 8000 7000 6000 5000 4000 3000 2000 1000 0
1.06 1.99 2.02 29.82 1.99 6.06 2.06 1.96 2.30 2.09 2.23
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)



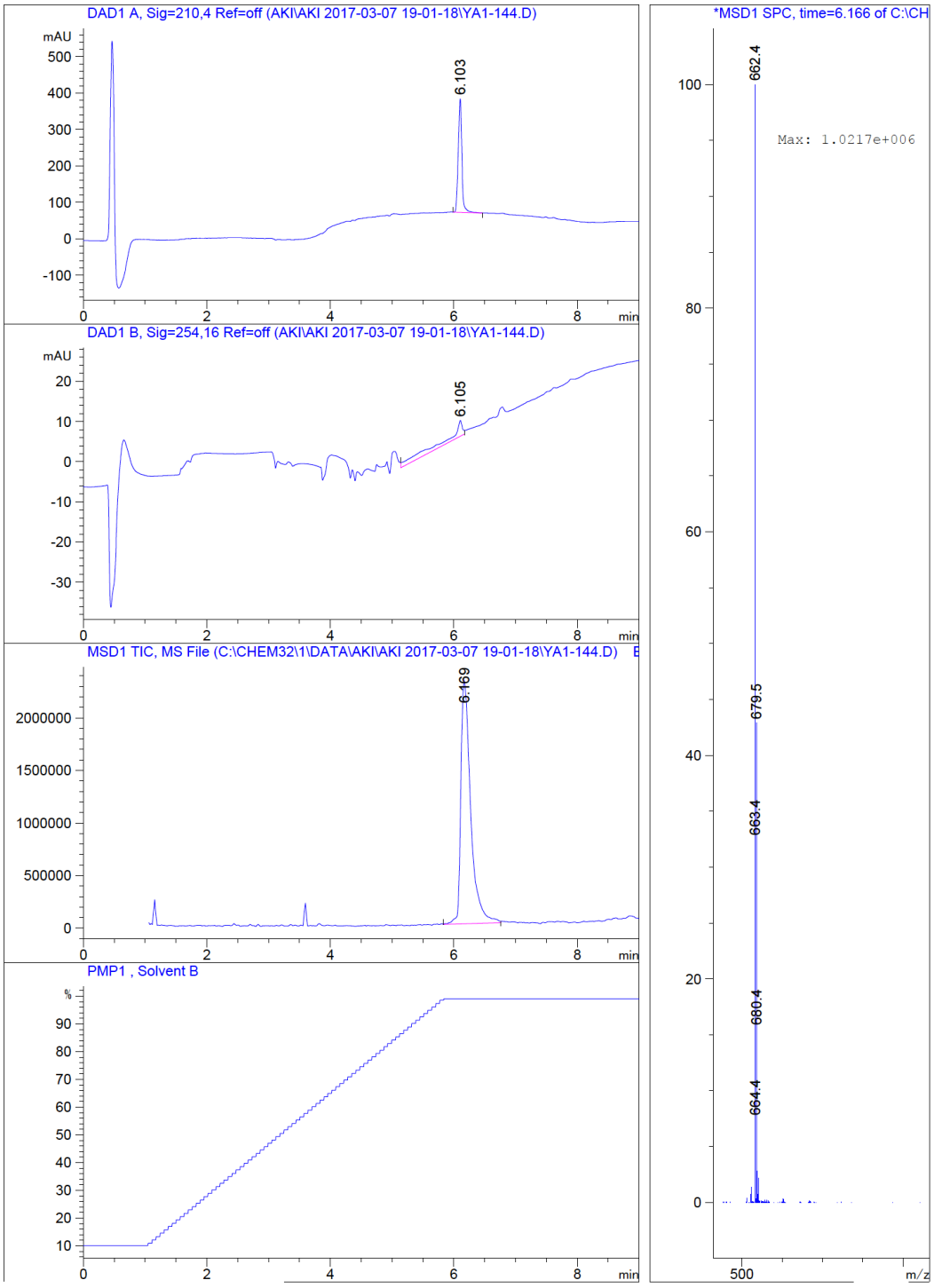
6

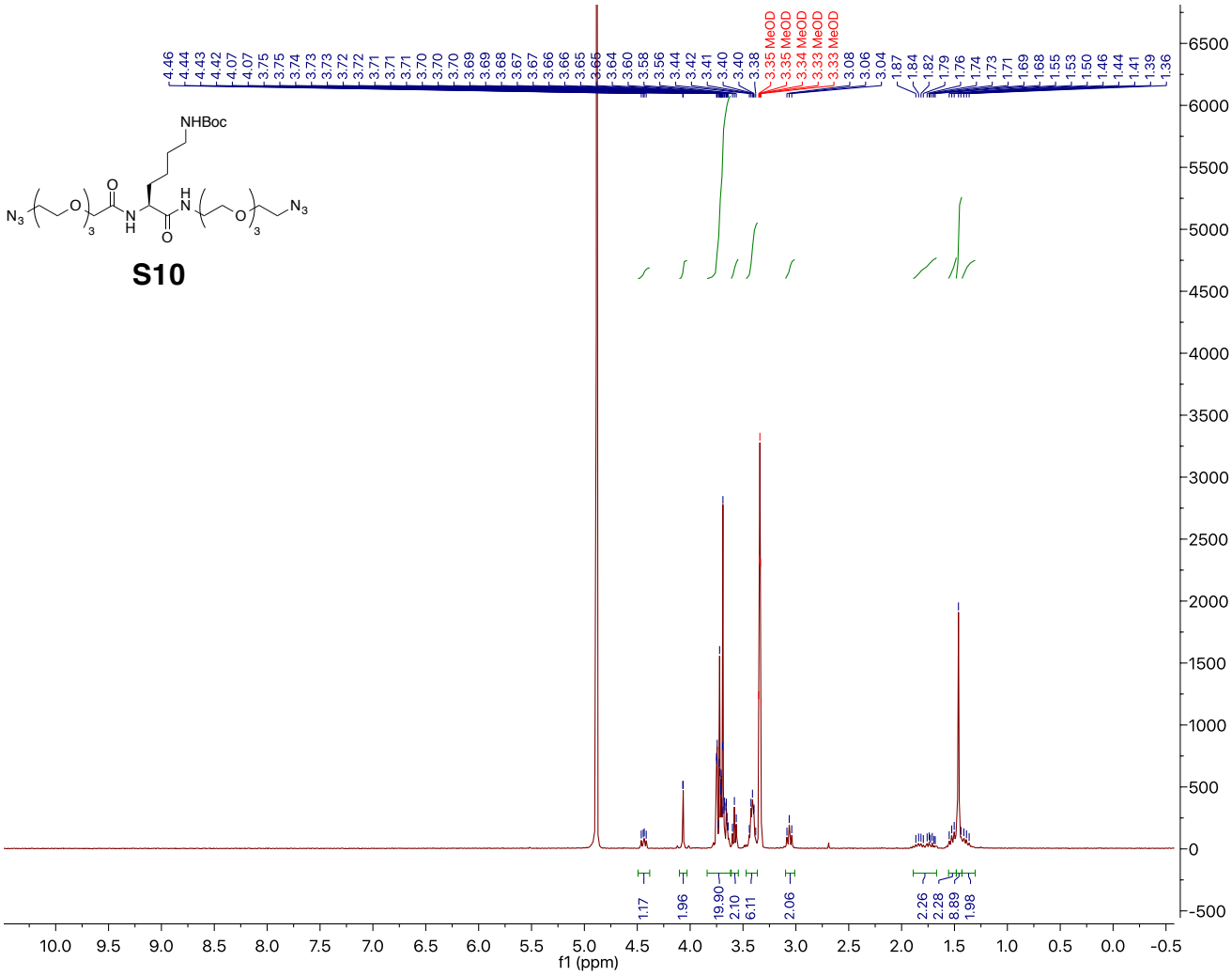

DAD1 A, Sig=210,4 Ref=off (AKI\AKI 2017-03-07 13-22-45\YA3-5.D)
mAU
4.775
6.476
min
DAD1 B, Sig=254,16 Ref=off (AKI\AKI 2017-03-07 13-22-45\YA3-5.D)
mAU
min
MSD1 TIC, MS File (C:\CHEM32\1\DATA\AKI\AKI 2017-03-07 13-22-45\YA3-5.D) ES-
4.855
6.552
min
PMP1 , Solvent B
%
min
*MSD1 SPC, time=4.851 of C:\CH
Max: 1.02458e+006
940.5
941.5
942.5
470.8
479.4
m/z

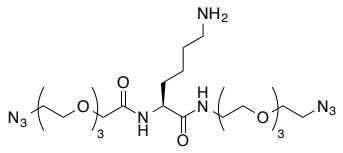

6
f1 (ppm)


NHBoc
N3
O
3
N
H
O
H
N
O
3
N3

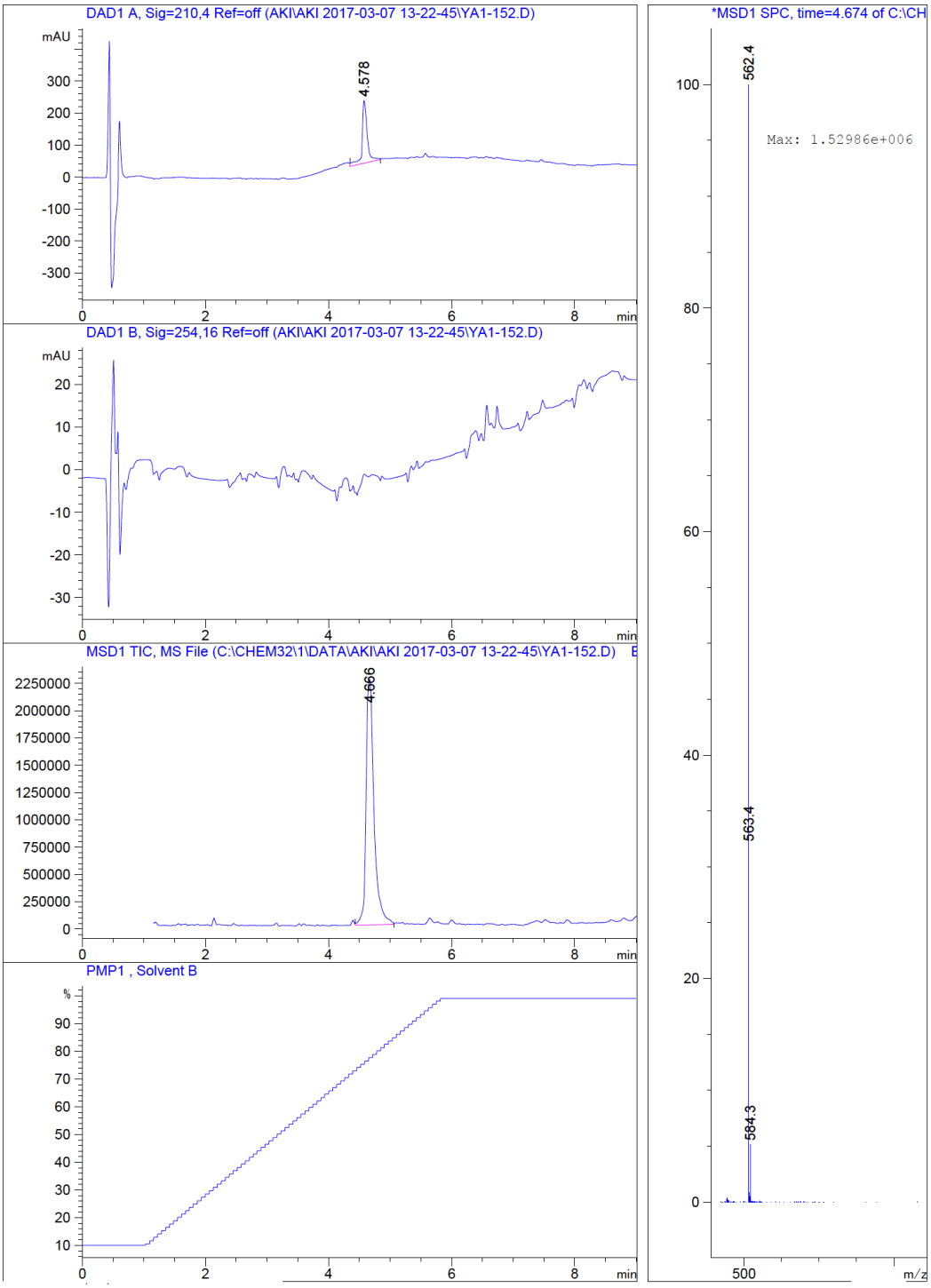
S10

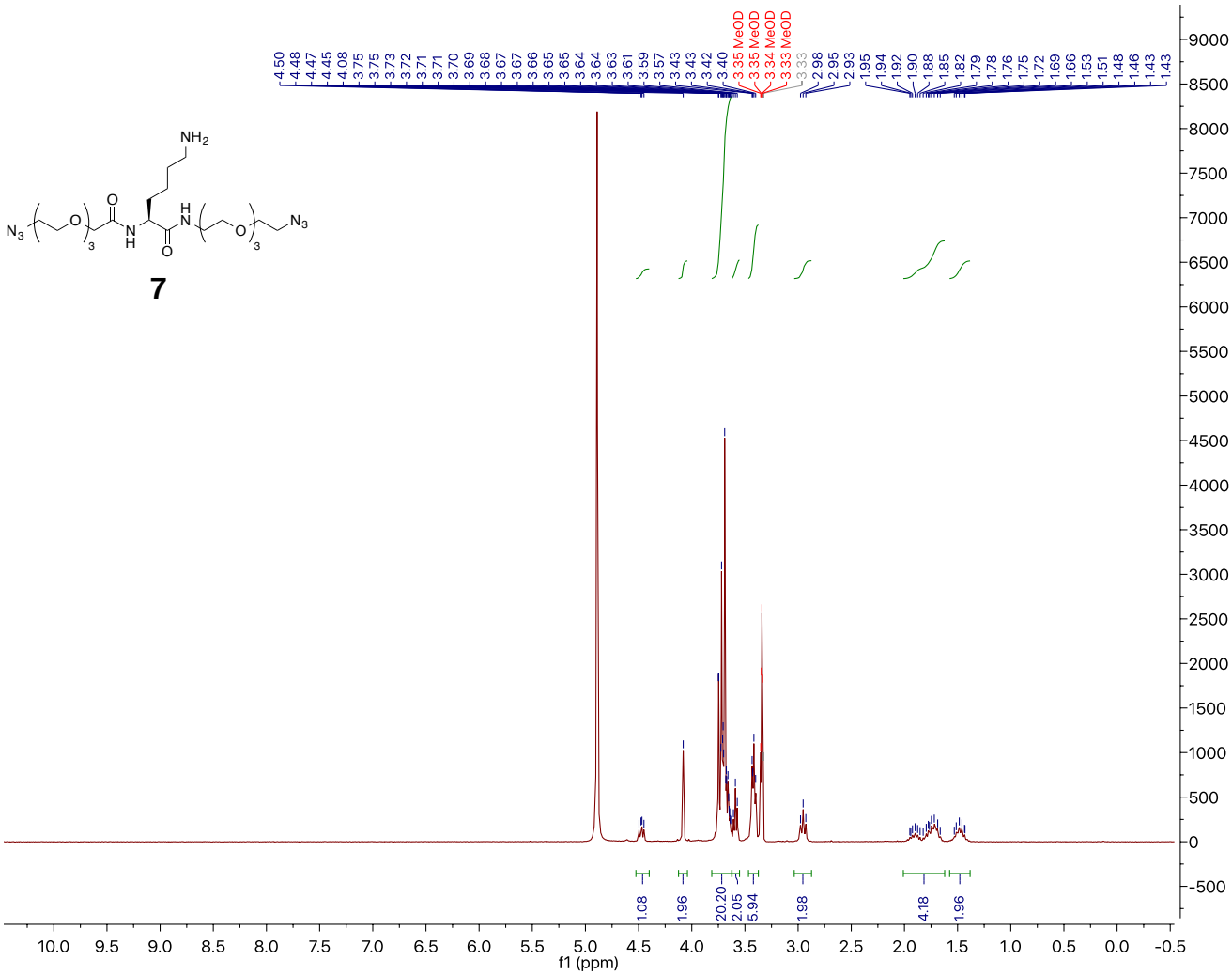

DAD1 A, Sig=210,4 Ref=off (AKI\AKI 2017-03-07 19-01-18\YA1-144.D)
mAU
6.103
min
DAD1 B, Sig=254,16 Ref=off (AKI\AKI 2017-03-07 19-01-18\YA1-144.D)
mAU
6.105
min
MSD1 TIC, MS File (C:\CHEM32\1\DATA\AKI\AKI 2017-03-07 19-01-18\YA1-144.D)
6.169
min
PMP1 , Solvent B
%
min
*MSD1 SPC, time=6.166 of C:\CH
662.4
Max: 1.0217e+006
679.5
663.4
680.4
664.4
m/z


NHBoc
N3
O
N
H
O
O
N
H
O
N3
3
3
S10
4.46 4.44 4.43 4.42 4.07 4.07 3.75 3.75 3.74 3.73 3.73 3.72 3.72 3.71 3.71 3.71 3.70 3.70 3.70 3.69 3.69 3.68 3.67 3.67 3.66 3.66 3.65 3.66 3.64 3.60 3.58 3.56 3.44 3.42 3.41 3.40 3.40 3.38 3.35 MeOD 3.35 MeOD 3.34 MeOD 3.33 MeOD 3.33 MeOD 3.08 3.06 3.04 1.87 1.84 1.82 1.79 1.76 1.74 1.73 1.71 1.69 1.68 1.55 1.53 1.50 1.46 1.44 1.41 1.39 1.36
6500 6000 5500 5000 4500 4000 3500 3000 2500 2000 1500 1000 500 0 -500
1.17 1.96 19.90 2.10 6.11 2.06 2.26 2.28 8.89 1.98
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)



7


DAD1 A, Sig=210,4 Ref=off (AKI\AKI 2017-03-07 13-22-45\YA1-152.D)
mAU
4.578
min
DAD1 B, Sig=254,16 Ref=off (AKI\AKI 2017-03-07 13-22-45\YA1-152.D)
mAU
min
MSD1 TIC, MS File (C:\CHEM32\1\DATA\AKI\AKI 2017-03-07 13-22-45\YA1-152.D)
4.666
min
PMP1 , Solvent B
%
min
*MSD1 SPC, time=4.674 of C:\CH
562.4
Max: 1.52986e+006
563.4
584.3
m/z


NH2
N3
O
N
H
O
O
3
N
H
O
3
N3
7
4.50 4.48 4.47 4.45 4.08 3.75 3.75 3.73 3.72 3.71 3.71 3.70 3.69 3.68 3.67 3.67 3.66 3.65 3.65 3.64 3.64 3.63 3.61 3.59 3.57 3.43 3.43 3.42 3.40 3.35 MeOD 3.35 MeOD 3.34 MeOD 3.33 MeOD 3.33 2.98 2.95 2.93 1.95 1.94 1.92 1.90 1.88 1.85 1.82 1.79 1.78 1.76 1.75 1.72 1.69 1.66 1.53 1.51 1.48 1.46 1.43 1.43
1.08 1.96 20.20 2.05 5.94 1.98 4.18 1.96
f1 (ppm)

5. References

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