Substrate Design Enables Heterobifunctional, Dual "Click" Antibody Modification via Microbial Transglutaminase

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Experimental Procedures

General Procedures

Reagents for Chemical Synthesis

All chemicals were purchased from MilliporeSigma unless stated otherwise. DBCO-PEG4-Amine, (E)-cyclooct-4-en-1-yl (3-aminopropyl)carbamate (TCO-Amine), DM1, mTz-NHS ester, mTz-PEG5-NHS ester, and tert-butyl (2-(2-(2-bromoethoxy)ethoxy)ethyl) carbamate were purchased from BroadPharm. Azidopropan-1-amine was purchased from Click Chemistry Tools. OPSS-PEG24-NHS ester and mPEG24-NHS ester were purchased from Quanta Biodesign. PEG5K-SVA was purchased from Laysan Bio. DM1-S-Me was purchased from Toronto Research Chemicals.

Reagents for Protein Expression and Purification

The plasmid pDJ1-3 was kindly provided by Professor Joelle Pelletier (Université de Montréal, Montreal, Canada). pDJ1-3 encodes the proenzyme of microbial transglutaminase from *S. mobaraensis* with its N-terminal pro-sequence and a C-terminal hexa-histidine tag inserted between the *Nde*I and *Xho*I restriction sites of the vector pET20b. The plasmid pVITRO-Trastuzumab-IgG1/k for expressing trastuzumab was purchased from Addgene (Plasmid# 61883). Ni-NTA agarose resin was purchased from Qiagen. NAb protein A/G resin was purchased from ThermoFisher Scientific. Sequencing primers (T7 forward and reverse) were purchased from Integrated DNA Technologies (IDT). Sequencing was performed at the Cornell University Genomics Facility using the Applied Biosystems Automated 370xl DNA Analyzer using Big Dye Terminator chemistry and ApliTag-FS DNA Polymerase.

Reagents for Gel Electrophoresis, Molecular Biology, and Cell Culture

Sulfo-Cy5 TCO was purchased from BroadPharm. DBCO-PEG4-Carboxyrhodamine 101 was purchased from Click Chemistry Tools. Precast protein gels (4 – 20% mini-PROTEAN® TGX™) and Bio-safe Coomassie Stain were purchased from Bio-Rad Laboratories. Peptide:N-glycosidase F (PNGase F) was purchased from New England Biolabs. All cell culture reagents were purchased from ThermoFisher Scientific unless stated otherwise. HEK293F cells were cultured in FreeStyle™ 293 Expression Medium. SKOV3 and MCF7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) and DMEM supplemented with insulin (0.01 mg/mL), respectively. CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS) was purchased from Promega.

Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H and ^{13}C NMR spectra were recorded on either an INOVA 400 MHz or 500 MHz spectrometer as specified. NMR data was analyzed by MestReNova software. ^{1}H and ^{13}C NMR chemical shifts are reported in units of ppm relative to chloroform.

Liquid Chromatography Mass Spectrometry (LC-MS)

LC-MS analysis was carried out on an Agilent 1100 Series LC with a Poroshell 120 EC-C18 column (100 × 3 mm, 2.7 µm, Agilent Technologies) and an Agilent G1956B Series Single Quadripole MS in positive ion mode for mass detection. The mobile phase for LC-MS (solvent A) was water with 0.1% (v/v) acetic acid, and the stationary phase (solvent B) was acetonitrile with 0.1% (v/v) acetic acid. Compounds were eluted at a flow rate of 0.6 mL/min using a gradient of 5-100% solvent B (0-10 minutes) followed by 100% solvent B (10-12 minutes) and equilibrated back to 5% solvent B (12-15 minutes).

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) Purification

HPLC purification was performed on an Agilent 1100 Series HPLC system equipped with a UV diode array detector and an 1100 Infinity fraction collector using a semi-preparative reversed-phase C18 column (Agilent Eclipse XDB-C18, 9.4 x 250 mm, 5 µm). The mobile phase for HPLC was water with 0.1% (v/v) trifluoroacetic acid (solvent A) and acetonitrile with 0.1% (v/v) trifluoroacetic acid (solvent B) unless specified otherwise. Compounds were eluted at a flow rate of 4 mL/min using a linear solvent gradient as specified below.

Hydrophobic Interaction Chromatography (HIC)

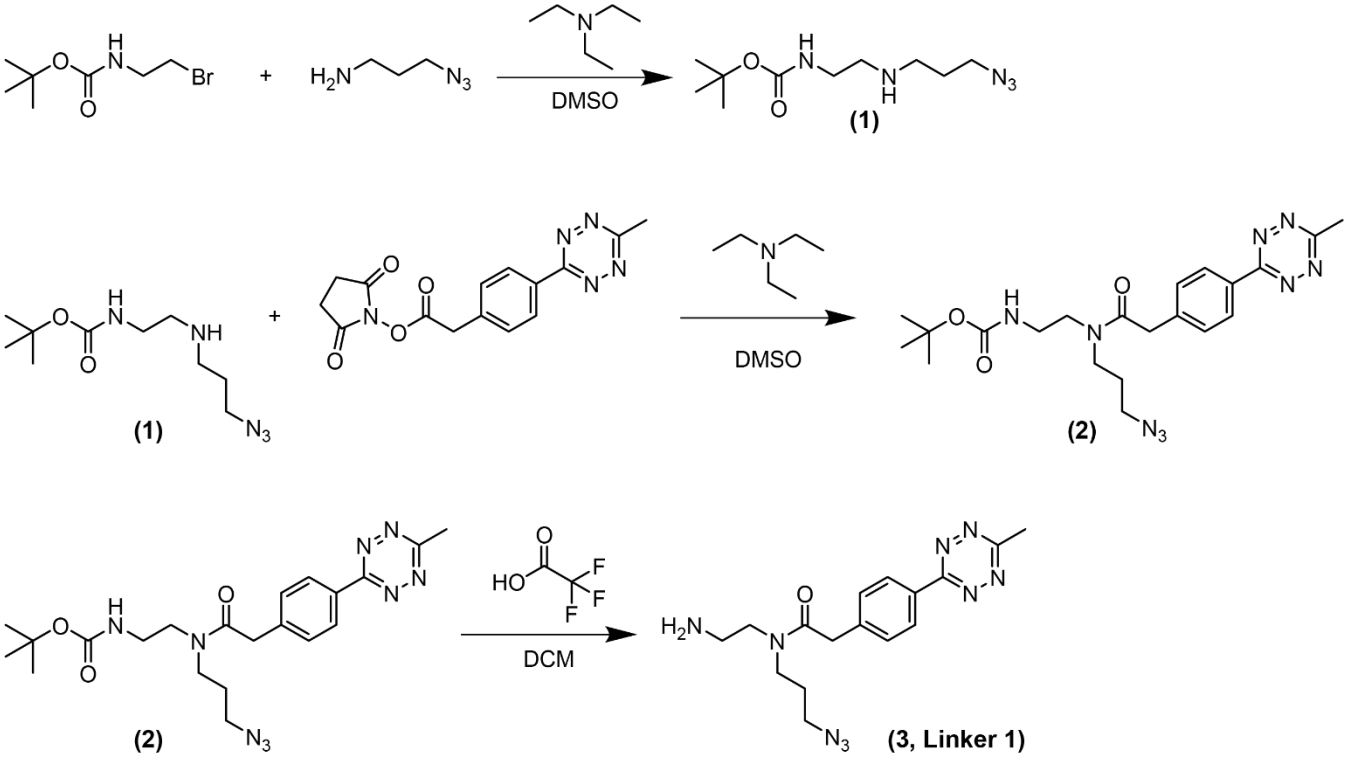
HIC was performed on an Agilent 1100 Series HPLC system equipped with a UV diode array detector and an 1100 Infinity fraction collector using a reversed-phase phenyl column (Tosoh Biosciences LLC, TSKgel Phenyl-5PW, 7.5 x 75 mm, 10 µm). The mobile phase for HIC was 25 mM phosphate, 1.5 M ammonium sulfate, pH 7.0 (solvent A) and 18.75 mM phosphate, 25% (v/v) isopropyl alcohol, pH 7.0 (solvent B). Compounds were eluted at a flow rate of 1 mL/min using a linear solvent gradient as specified below.

Gel Electrophoresis of Conjugates

All samples were denatured and reduced with 2-mercaptoethanol by boiling at 100 °C for 5 minutes. A precast 4-20% mini-PROTEAN® TGX™ gel was run for 60 minutes at 120V to separate the protein samples. Protein content was visualized using Bio-Safe Coomassie Stain according to the manufacturer's instructions and imaged using a Bio-Rad ChemiDoc™ MP Imaging System.

Synthesis of Antibody Modification Reagents

Synthesis of Linker 1 (Compound 3)



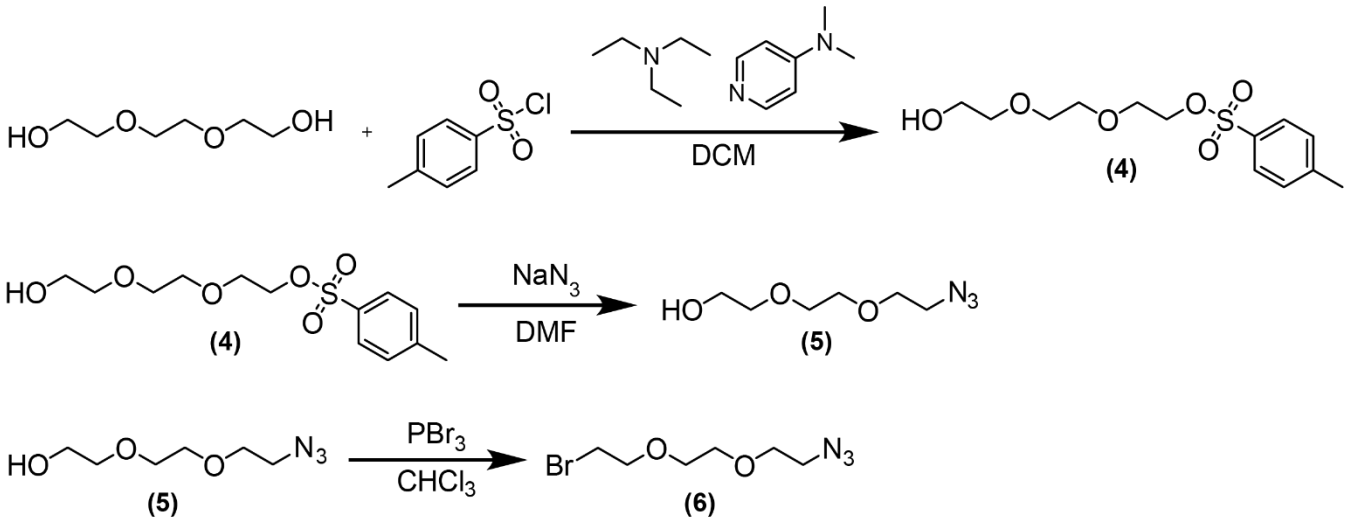
Supplementary Figure S1. Synthesis scheme for Linker 1.

Synthesis of (1): 1 equivalency (20 mg, 92 µmol) of tert-butyl (2-bromoethyl)carbamate was dissolved at 400 mM in dimethyl sulfoxide (solution 1). 2 equivalencies of azidopropan-1-amine and 2 equivalencies of triethylamine were dissolved at 400 mM in dimethyl sulfoxide (solution 2). Solution 1 was added dropwise to solution 2 at room temperature over 2 hours. The resulting mixture was reacted at room temperature overnight and then purified via semi-preparative RP-HPLC. The reaction mixture was separated using a linear solvent gradient of 5 – 45% solvent B over 20 minutes. The product **(1)** eluted at 11.5 minutes and was recovered in 94% yield (21 mg, 87 µmol). The product was characterized by LC-MS (**(1)** calculated: 244.17, observed: 244.20 $[M+H]^+$).

Synthesis of (2): 1 equivalency (10.5 mg, 43 µmol) of **(1)** was dissolved at 300 mM in dimethyl sulfoxide in the presence of 5 equivalencies of triethylamine. To this solution, 1 equivalency of mTz-NHS ester dissolved at 300 mM in dimethylsulfoxide was added. The resulting mixture was reacted at room temperature overnight and then purified via semi-preparative RP-HPLC. The reaction mixture was separated using a linear solvent gradient of 5 – 95% solvent B over 30 minutes. The product **(2)** eluted at 22 minutes and was recovered in 60% yield (11.7 mg, 26 µmol). The product was characterized by ^{1}H NMR (Figure S11) and LC-MS (**(2)** calculated: 478.24, observed: 478.00 $[M+Na]^{+}$).

Synthesis of (3): Removal of the BOC protecting group was achieved by dissolving **(2)** at 5 mM in 50% (v/v) trifluoroacetic acid in dichloromethane at room temperature for 1 hour. The solvent was then removed under vacuum, and the product was purified via semi-preparative RP-HPLC. The dried product was separated using a linear solvent gradient of 5 – 50% solvent B over 22.5 minutes. The product **(3)** eluted at 14.5 minutes and was recovered in 94% yield (8.6 mg, 24 µmol). The product was characterized by ^{1}H NMR (Figure S12) and LC-MS (**(3)** calculated: 356.19, observed: 356.10 $[M+H]^{+}$).

Synthesis of 1-azido-2-(2-(2-bromoethoxy)ethoxy)ethane (Compound 6)



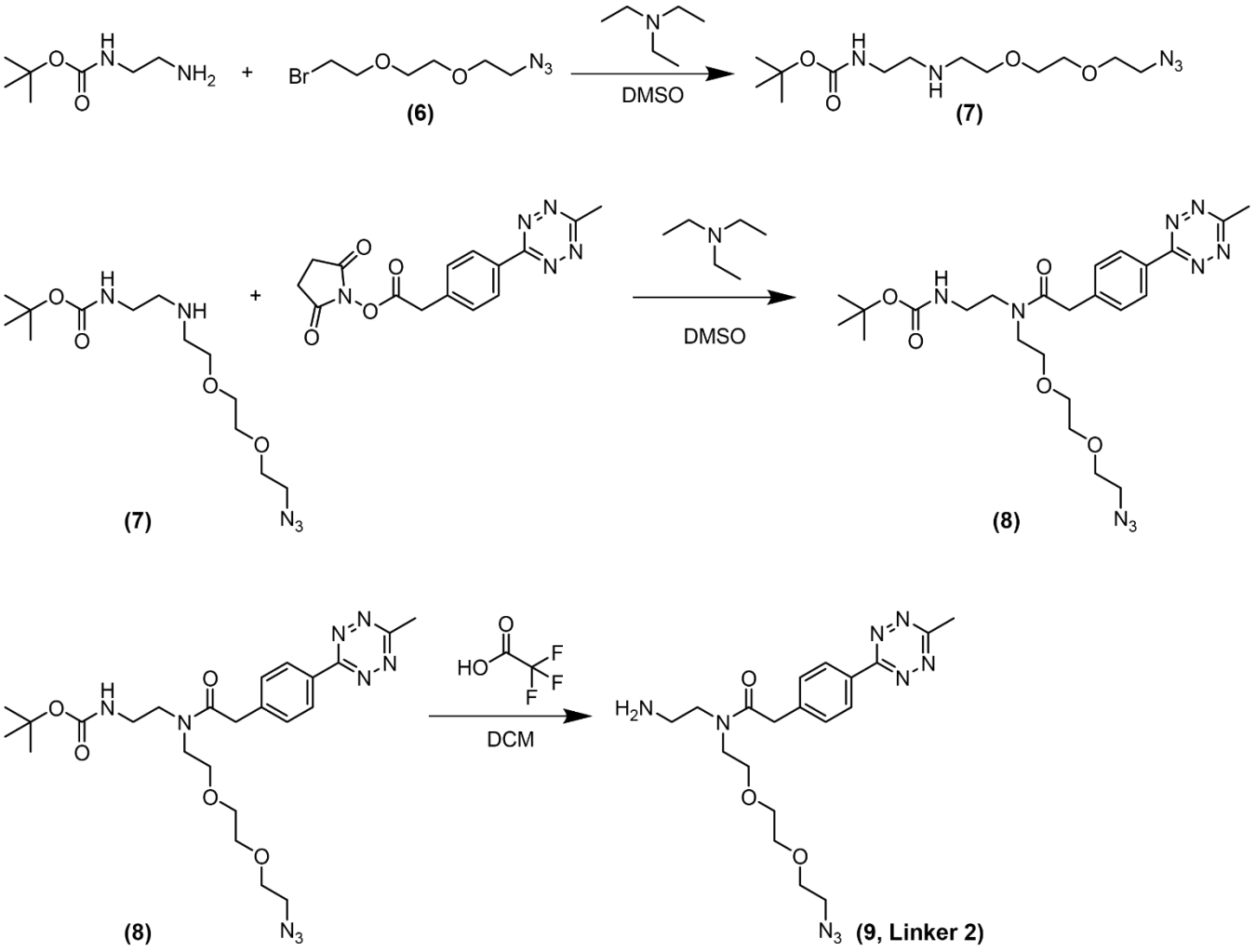
Supplementary Figure S2. Synthesis scheme for 1-azido-2-(2-(2-bromoethoxy)ethoxy)ethane.

Synthesis of (4): 1 equivalency of 4-methylbenzenesulfonyl chloride (3.5 g, 18.4 mmol) was dissolved at 175 mM in dichloromethane. Separately, 4 equivalencies of 2,2'-(ethane-1,2-diylbis(oxy))bis(ethan-1-ol) (11 g, 73 mmol) was dissolved at 110 mM in dichloromethane. To this solution was added 1.05 equivalencies of triethylamine (1.95 g, 19 mmol) and 0.02 equivalencies of 4-dimethylaminopyridine (46 mg, 0.3 mmol), and the mixture was allowed to equilibrate for 10 minutes on ice. The solution of 4-methylbenzenesulfonyl chloride in DCM was then added dropwise to the mixture over 2 hours. The mixture was subsequently removed from the ice and reacted at room temperature overnight. The reaction was quenched with 100 mL of water and extracted with dichloromethane (100 mL, 3x). The organic layer was collected, dried with sodium sulfate, and concentrated under vacuum. The crude product **(4)**, recovered in 98% yield (5.5 g, 18 mmol), was used without further purification. The product was characterized by ^{1}H and ^{13}C NMR (Figures S13 and S14).

Synthesis of (5): 1 equivalency (4.8 g, 16 mmol) of **(4)** was dissolved at 739 mM in dry dimethylformamide (DMF). To this solution was added 2 equivalencies of sodium azide (2 g, 32 mmol) and the mixture was reacted overnight at 80°C. The mixture was then concentrated under vacuum. The residue was suspended in diethyl ether and filtered through celite. The filtered product was collected and concentrated under vacuum. The crude product **(5)**, recovered in 96% yield (2.7 g, 15.4 mmol), was used without further purification. The product was characterized by ^{1}H NMR (Figure S15).

Synthesis of (6): 1 equivalency (1 g, 5.8 mmol) of **(5)** was dissolved at 342 mM in dry chloroform. To this solution was added 2 equivalencies of phosphorus tribromide (3.1 g, 12 mmol) over 5 minutes. The mixture was refluxed overnight at 50°C. The reaction was quenched on ice over 30 minutes with 75 mL of saturated sodium bicarbonate solution and extracted with chloroform (100 mL, 3x). The organic layer was collected, dried with sodium sulfate, and concentrated under vacuum. The crude product **(6)**, recovered in 30% yield (0.4 g, 1.7 mmol), was used without further purification. The product was characterized by ^{1}H and ^{13}C NMR (Figures S16 and S17).

Synthesis of Linker 2 (Compound 9)



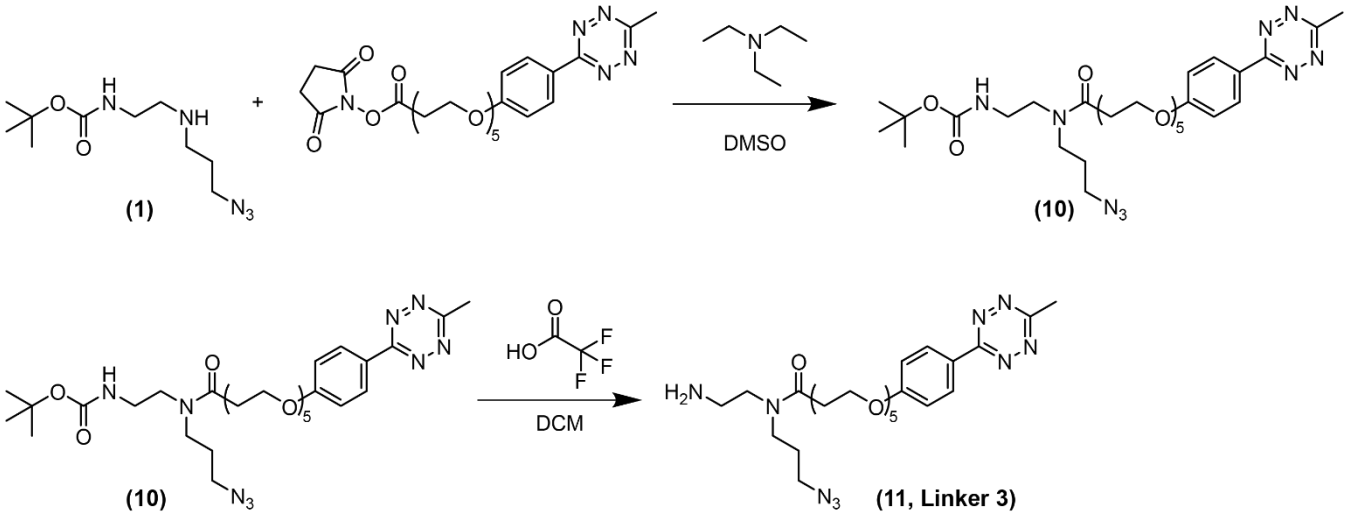
Supplementary Figure S3. Synthesis scheme for Linker 2.

Synthesis of (7): 1 equivalency (38 mg, 159 µmol) of **(6)** was dissolved at 400 mM in dimethyl sulfoxide (solution 1). 2 equivalencies of tert-butyl (2-aminoethyl)carbamate and 2 equivalencies of triethylamine were dissolved at 400 mM in dimethyl sulfoxide (solution 2). Solution 1 was added dropwise to solution 2 at room temperature over 2 hours. The resulting mixture was reacted at room temperature overnight and then purified via semi-preparative RP-HPLC. The reaction mixture was separated using a linear solvent gradient of 5 – 45% solvent B over 20 minutes. The product **(7)** eluted at 13.5 minutes and was recovered in 52% yield (26 mg, 83 µmol). The product was characterized by LC-MS (**(7)** calculated: 318.21, observed: 318.20 $[M+H]^{+}$).

Synthesis of (8): 1 equivalency (6.6 mg, 21 µmol) of **(7)** was dissolved at 300 mM in dimethyl sulfoxide in the presence of 5 equivalencies of triethylamine. To this solution, 1 equivalency of mTz-NHS ester dissolved at 300 mM in dimethylsulfoxide was added. The resulting mixture was reacted at room temperature overnight and then purified via semi-preparative RP-HPLC. The reaction mixture was separated using a linear solvent gradient of 5 – 95% solvent B over 30 minutes. The product **(8)** eluted at 21 minutes and was recovered in 28% yield (3.1 mg, 6 µmol). The product was characterized by ^{1}H NMR (Figure S18) and LC-MS (**(8)** calculated: 552.28, observed: 552.10 $[M+Na]^{+}$).

Synthesis of (9): Removal of the BOC protecting group was achieved by dissolving **(8)** at 5 mM in 50% (v/v) trifluoroacetic acid in dichloromethane at room temperature for 1 hour. The solvent was then removed under vacuum, and the product was purified via semi-preparative RP-HPLC. The dried product was separated using a linear solvent gradient of 5 – 50% solvent B over 22.5 minutes. The product **(8)** eluted at 16 minutes and was recovered in 85% yield (2.6 mg, 6 µmol). The product was characterized by ^{1}H NMR (Figure S19) and LC-MS (**(9)** calculated: 430.22, observed: 430.10 $[M+H]^{+}$).

Synthesis of Linker 3 (Compound 11)

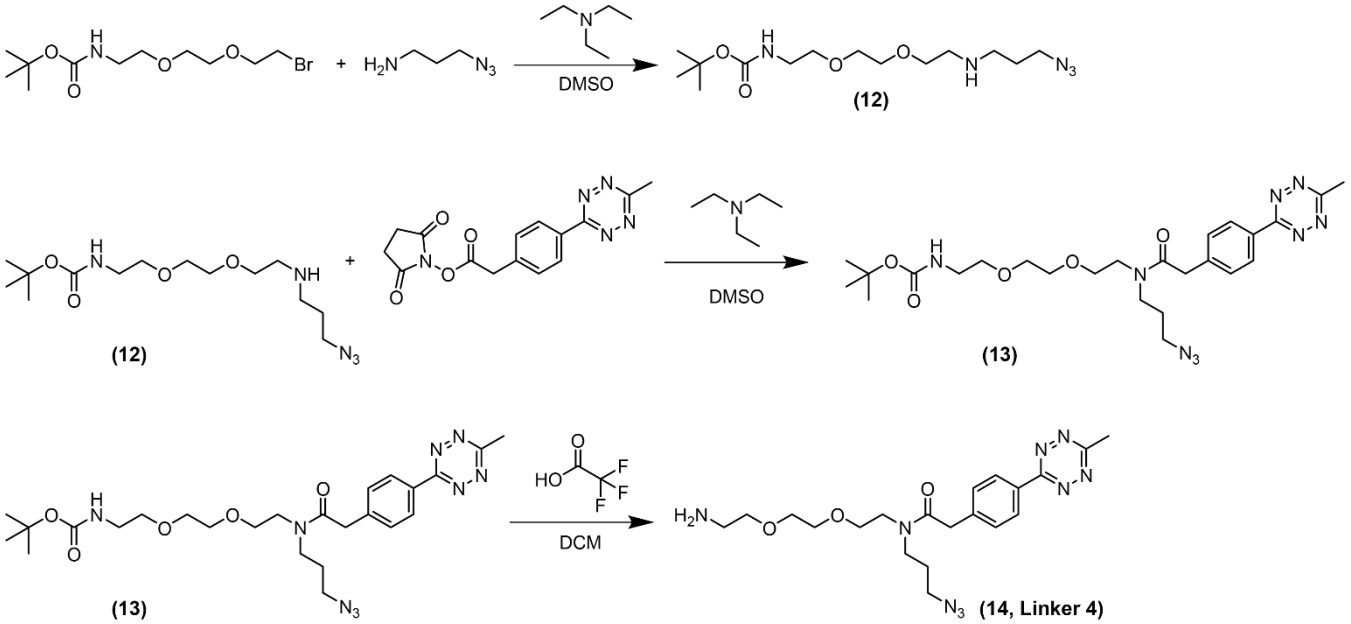


Supplementary Figure S4. Synthesis scheme for Linker 3.

Synthesis of (10): 1 equivalency (10.5 mg, 43 µmol) of **(1)** was dissolved at 300 mM in dimethyl sulfoxide in the presence of 5 equivalencies of triethylamine. To this solution, 0.36 equivalencies of mTz-PEG5-NHS ester dissolved at 300 mM in dimethylsulfoxide was added. The resulting mixture was reacted at room temperature overnight and then purified via semi-preparative RP-HPLC. The reaction mixture was separated using a linear solvent gradient of 5 – 95% solvent B over 30 minutes. The product **(10)** eluted at 22.5 minutes and was recovered in 74% yield (7.6 mg, 11.5 µmol). The product was characterized by ^{1}H NMR (Figure S20) and LC-MS (**(10)** calculated: 684.35, observed: 684.10 $[M+Na]^{+}$).

Synthesis of (11): Removal of the BOC protecting group was achieved by dissolving **(10)** at 5 mM in 50% (v/v) trifluoroacetic acid in dichloromethane at room temperature for 1 hour. The solvent was then removed under vacuum, and the product was purified via semi-preparative RP-HPLC. The dried product was separated using a linear solvent gradient of 5 – 50% solvent B over 22.5 minutes. The product **(3)** eluted at 18.5 minutes and was recovered in 79% yield (5.1 mg, 9 µmol). The product was characterized by ^{1}H NMR (Figure S21) and LC-MS (**(11)** calculated: 562.30, observed: 562.20 $[M+H]^{+}$).

Synthesis of Linker 4 (Compound 14)



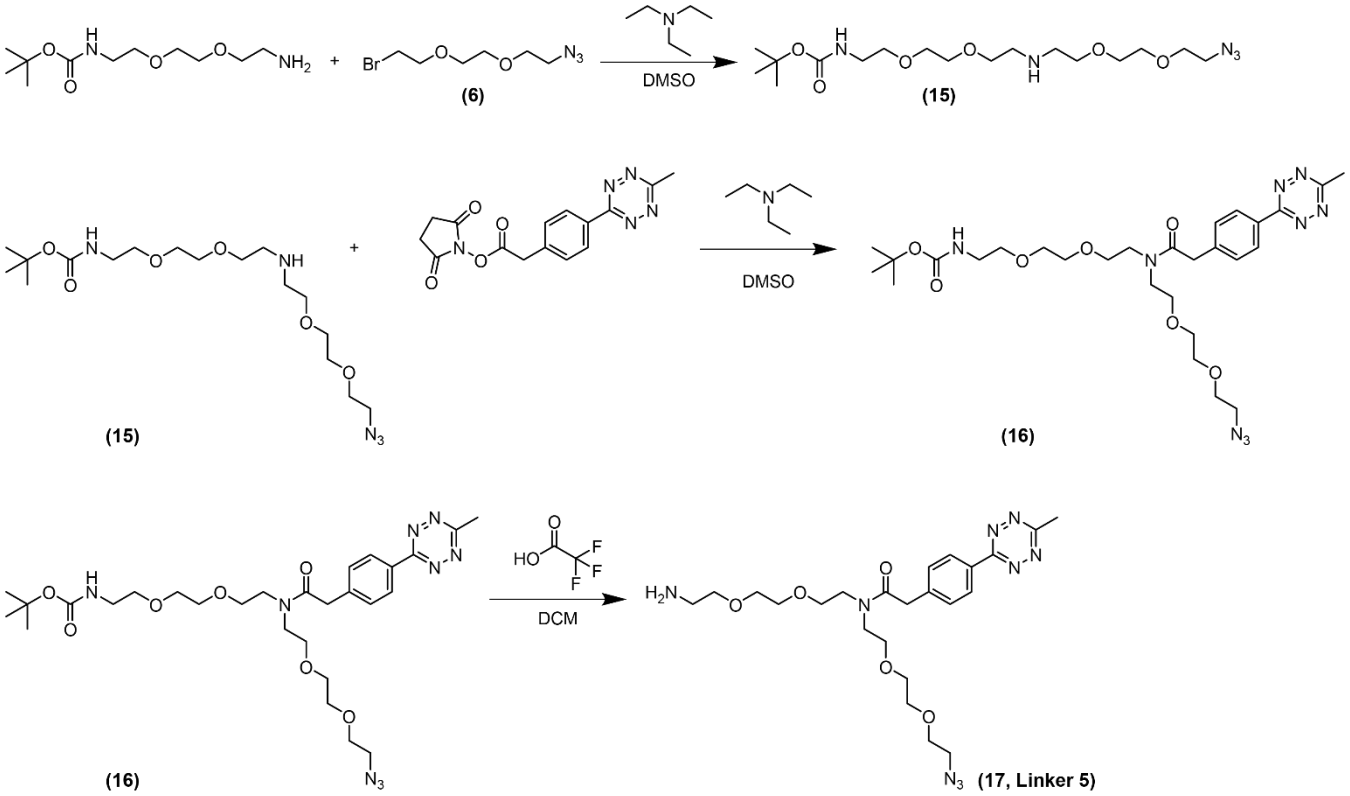
Supplementary Figure S5. Synthesis scheme for Linker 4.

Synthesis of (12): 1 equivalency (24.6 mg, 79 µmol) of tert-butyl (2-(2-(2-bromoethoxy)ethoxy)ethyl) carbamate was dissolved at 400 mM in dimethyl sulfoxide (solution 1). 2 equivalencies of azidopropan-1-amine and 2 equivalencies of triethylamine were dissolved at 400 mM in dimethyl sulfoxide (solution 2). Solution 1 was added dropwise to solution 2 at room temperature over 2 hours. The resulting mixture was reacted at room temperature overnight and then purified via semi-preparative RP-HPLC. The reaction mixture was separated using a linear solvent gradient of 5 – 45% solvent B over 20 minutes. The product **(12)** eluted at 13.5 minutes and was recovered in 75% yield (19.6 mg, 59 µmol). The product was characterized by LC-MS (**(12)** calculated: 332.22, observed: 332.20 $[M+H]^+$).

Synthesis of (13): 1 equivalency (7.4 mg, 22 µmol) of **(12)** was dissolved at 300 mM in dimethyl sulfoxide in the presence of 5 equivalencies of triethylamine. To this solution, 1 equivalency of mTz-NHS ester dissolved at 300 mM in dimethylsulfoxide was added. The resulting mixture was reacted at room temperature overnight and then purified via semi-preparative RP-HPLC. The reaction mixture was separated using a linear solvent gradient of 5 – 95% solvent B over 30 minutes. The product **(13)** eluted at 21 minutes and was recovered in 27% yield (3.3 mg, 6.1 µmol). The product was characterized by ^{1}H NMR (Figure S22) and LC-MS (**(13)** calculated: 566.29, observed: 566.10 $[M+Na]^+$).

Synthesis of (14): Removal of the BOC protecting group was achieved by dissolving **(13)** at 5 mM in 50% (v/v) trifluoroacetic acid in dichloromethane at room temperature for 1 hour. The solvent was then removed under vacuum, and the product was purified via semi-preparative RP-HPLC. The dried product was separated using a linear solvent gradient of 5 – 50% solvent B over 22.5 minutes. The product **(14)** eluted at 16.5 minutes and was recovered in 92% yield (2.5 mg, 5.6 µmol). The product was characterized by ^{1}H NMR (Figure S23) and LC-MS (**(3)** calculated: 444.24, observed: 444.10 $[M+H]^+$).

Synthesis of Linker 5 (Compound 17)



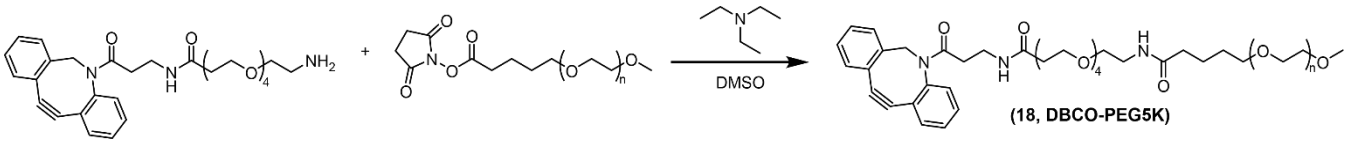
Supplementary Figure S6. Synthesis scheme for Linker 5.

Synthesis of (15): 1 equivalency (38 mg, 159 µmol) of **(6)** was dissolved at 400 mM in dimethyl sulfoxide (solution 1). 2 equivalencies of tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate and 2 equivalencies of triethylamine were dissolved at 400 mM in dimethyl sulfoxide (solution 2). Solution 1 was added dropwise to solution 2 at room temperature over 2 hours. The resulting mixture was reacted at room temperature overnight and then purified via semi-preparative RP-HPLC. The reaction mixture was separated using a linear solvent gradient of 5 – 45% solvent B over 20 minutes. The product **(15)** eluted at 15.5 minutes and was recovered in 26% yield (17 mg, 42 µmol). The product was characterized by LC-MS (**(15)** calculated: 406.25, observed: 406.10 $[M+H]^+$).

Synthesis of (16): 1 equivalency (8.6 mg, 21 µmol) of **(15)** was dissolved at 300 mM in dimethyl sulfoxide in the presence of 5 equivalencies of triethylamine. To this solution, 1 equivalency of mTz-NHS ester dissolved at 300 mM in dimethylsulfoxide was added. The resulting mixture was reacted at room temperature overnight and then purified via semi-preparative RP-HPLC. The reaction mixture was separated using a linear solvent gradient of 5 – 95% solvent B over 30 minutes. The product **(16)** eluted at 20.5 minutes and was recovered in 51% yield (6.7 mg, 11 µmol). The product was characterized by ^{1}H NMR (Figure S24) and LC-MS (**(8)** calculated: 640.33, observed: 640.10 $[M+Na]^+$).

Synthesis of (17): Removal of the BOC protecting group was achieved by dissolving **(16)** at 5 mM in 50% (v/v) trifluoroacetic acid in dichloromethane at room temperature for 1 hour. The solvent was then removed under vacuum, and the product was purified via semi-preparative RP-HPLC. The dried product was separated using a linear solvent gradient of 5 – 50% solvent B over 22.5 minutes. The product **(17)** eluted at 17.5 minutes and was recovered in 91% yield (5.1 mg, 10 µmol). The product was characterized by ^{1}H NMR (Figure S25) and LC-MS (**(9)** calculated: 518.28, observed: 518.20 $[M+H]^+$).

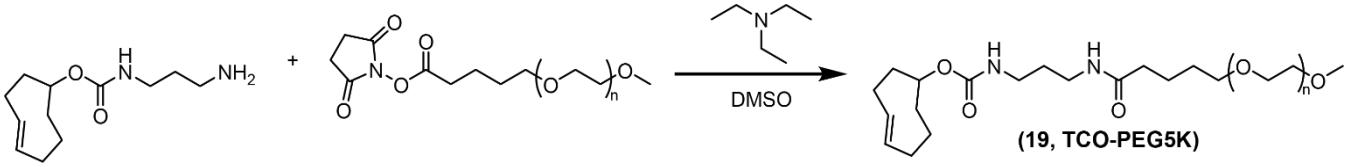
Synthesis of DBCO-PEG5K (Compound 18)



Supplementary Figure S7. Synthesis scheme for DBCO-PEG5K.

1 equivalency (1.7 mg, 3.2 µmol) of DBCO-PEG4-Amine was dissolved at 100 mM in dimethyl sulfoxide in the presence of 5 equivalencies of triethylamine. To this solution, 1.25 equivalencies of PEG5K-SVA dissolved at 50 mM in dimethylsulfoxide was added. The resulting mixture was reacted at room temperature overnight and then purified via semi-preparative RP-HPLC. The reaction mixture was separated using a mobile phase of water (solvent A) and acetonitrile (solvent B) and a linear solvent gradient of 5 – 65% solvent B over 30 minutes. The product **(18)** eluted at 25 minutes and was recovered in 62% yield (10 mg, 1.8 µmol). The product was characterized by MALDI-MS (Figure S26).

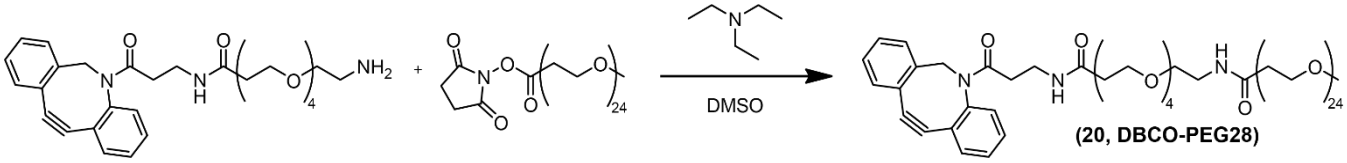
Synthesis of TCO-PEG5K (Compound 19)



Supplementary Figure S8. Synthesis scheme for TCO-PEG5K.

1 equivalency (0.7 mg, 3.2 µmol) of TCO-Amine was dissolved at 100 mM in dimethyl sulfoxide in the presence of 5 equivalencies of triethylamine. To this solution, 1.25 equivalencies of PEG5K-SVA dissolved at 50 mM in dimethylsulfoxide was added. The resulting mixture was reacted at room temperature overnight and then purified via semi-preparative RP-HPLC. The reaction mixture was separated using a mobile phase of water (solvent A) and acetonitrile (solvent B) and a linear solvent gradient of 5 – 65% solvent B over 30 minutes. The product **(19)** eluted at 24.5 minutes and was recovered in 80% yield (12.8 mg, 2.5 µmol). The product was characterized by MALDI-MS (Figure S27).

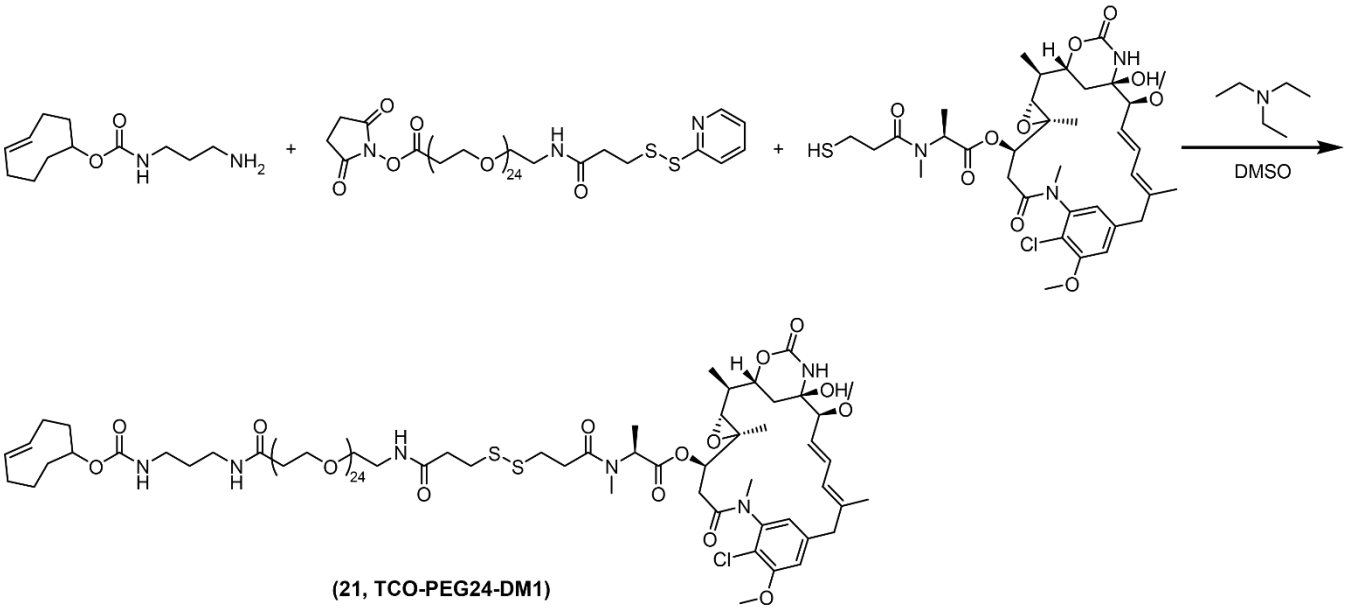
Synthesis of DBCO-PEG28 (Compound 20)



Supplementary Figure S9. Synthesis scheme for DBCO-PEG28.

1 equivalency (2 mg, 3.8 µmol) of DBCO-PEG4-Amine was dissolved at 190 mM in dimethyl sulfoxide in the presence of 5 equivalencies of triethylamine. To this solution, 1 equivalency of mPEG24-NHS ester dissolved at 100 mM in dimethylsulfoxide was added. The resulting mixture was reacted at room temperature overnight and then purified via semi-preparative RP-HPLC. The reaction mixture was separated using a linear solvent gradient of 5 – 95% solvent B over 45 minutes. The product **(20)** eluted at 24.5 minutes and was recovered in 47% yield (2.9 mg, 1.8 µmol). The product was characterized by LC-MS (**(20)** calculated: 1622.91, observed: 1622.60 $[M+H]^+$).

Synthesis of TCO-PEG24-DM1 (Compound 21)



Supplementary Figure S10. Synthesis scheme for TCO-PEG24-DM1.

1 equivalency (2.6 mg, 9.9 µmol) of TCO-Amine was dissolved at 100 mM in dimethyl sulfoxide in the presence of 5 equivalencies of triethylamine. To this solution, 0.67 equivalencies of OPSS-PEG24-NHS ester dissolved at 100 mM in dimethyl sulfoxide was added. The resulting mixture was reacted at room temperature for 1 hour. To this solution, 0.67 equivalencies of DM1 dissolved at 100 mM in dimethyl sulfoxide was added. The resulting mixture was reacted at room temperature for 4 hours and then purified via semi-preparative RP-HPLC. The reaction mixture was separated using a linear solvent gradient of 5 – 75% solvent B over 35 minutes. The product **(21)** eluted at 27 minutes and was recovered in 76% yield (10.9 mg, 5 µmol). The product was characterized by LC-MS. Details of the characteristic LC-MS fragmentation pattern are given in (Figure S28).

Protein Expression and Purification

Expression and Purification of Microbial Transglutaminase

Microbial transglutaminase (MTG) was expressed and purified as previously described with minor modifications.[1] Briefly, plasmid pDJ1-3 was transformed into *E. coli* BL21 (DE3), using standard procedures, and maintained with 100 µg/mL ampicillin. Before protein expression, correct plasmid sequence was confirmed (Table S1). A 5 mL starter culture was propagated overnight at 37°C with shaking at 240 rpm in ZYP-0.8G media. The starter culture (2.5 mL) was used to inoculate 250 mL of auto-inducing ZYP-5052 medium. The expression culture was grown for 2 hours at 37°C with shaking at 240 rpm. After 2 hours, the temperature was reduced to 22°C for 20 hours. Cells were collected by centrifugation at 3,000xg at 4°C for 30 minutes. The cell pellet was suspended in 8 mL of 0.2 M Tris-HCl, pH 6.0. The cells were disrupted by sonication at 4°C (Qsonica Model CL-18, 3 cycles of 30 second pulse at 20% intensity with 60 second pause). The N-terminal MTG pro-sequence was removed by treatment for 45 minutes at 30°C with 800 µL of trypsin at a concentration of 1 mg/mL in 0.2 M Tris-HCl, pH 6.0. Activated MTG was purified using a gravity flow column charged with 0.5 mL of Ni-NTA resin. The column was equilibrated in a buffer of 50 mM phosphate, 300 mM NaCl, and 2 mM reduced glutathione, pH 7.5. His-tagged enzyme was eluted using equilibration buffer containing increasing amounts of imidazole (0 – 200 mM). Purified enzyme was concentrated and exchanged into equilibration buffer using Amicon Ultra-0.5 mL centrifugal filters with a 10 kDa molecular weight cut off according to the manufacturer's instructions. MTG yield was quantified using absorbance at 280 nm (molar extinction coefficient of 55,408 M^{-1} cm^{-1}). The average MTG yield was 10 mg/L of *E. coli* culture. Purified MTG was snap frozen as single use aliquots containing 15% (v/v) glycerol.

Expression and Purification of Trastuzumab

HEK293F suspension cells were transfected with the plasmid pVITRO-Trastuzumab-IgG1/k using FreeStyle™ MAX transfection reagent. Transfected cells were selected with 50 µg/mL hygromycin B for two weeks to establish a stably expressing cell line. Stably expressing HERK293F cells were maintained at density of approximately 1 x 10^6 cells/mL for protein production. Trastuzumab was purified from sterile-filtered, conditioned media using a gravity flow column charged with 1 mL of protein A/G resin according to the manufacturer's instructions. Purified antibody was concentrated and exchanged into PBS buffer (100 mM phosphate, 150 mM NaCl, pH 7.4) using Amicon Ultra-0.5 mL centrifugal filters with a 30 kDa molecular weight cut off according to the manufacturer's instructions. Antibody yield was quantified using absorbance at 280 nm (molar extinction coefficient of 210,000 M^{-1} cm^{-1}). The average antibody yield was 1 – 2 mg per/L of conditioned media.

Analysis of Linker Conjugation Efficiency

Analysis of Linker Purity and Hydrophobicity via HPLC

Linker purity and relative hydrophobicity were analyzed on an Agilent 1100 Series HPLC system equipped with a UV diode array detector and an 1100 Infinity fraction collector using a reversed-phase C18 column (Agilent Eclipse Plus C18, 4.6 x 150 mm, 5 µm). The mobile phase for HPLC was water with 0.1% (v/v) trifluoroacetic acid (solvent A) and acetonitrile with 0.1% (v/v) trifluoroacetic acid (solvent B). Compounds were eluted at a flow rate of 1 mL/min with a linear gradient of 5% to 95% solvent B over 30 minutes. Linkers were analyzed at a scale of 50 µg and monitored based on their methyltetrazine-specific absorption at 360 nm.

HIC Analysis of Linker Conjugation Efficiency

1 equivalency of trastuzumab (250 µg, 1.7 nmol) at a concentration of 5 mg/mL in PBS buffer was treated with PNGase F (600 U/mg of antibody), MTG (0.75 equivalencies), and amine-bearing substrate (160 equivalencies). Conjugation reactions were carried out at 37°C for 24 hours. Excess enzyme and substrate were removed using NAb Protein A/G 0.2 mL spin columns according to the manufacturer's instructions. Conjugation efficiency was analyzed via analytical HIC using a linear solvent gradient of 0 – 60% solvent B over 60 minutes.

Gel Electrophoresis Analysis of Linker Conjugation Efficiency

1 equivalency of trastuzumab (100 µg, 0.7 nmol) at a concentration of 5 mg/mL in PBS buffer was treated with PNGase F (600 U/mg of antibody), MTG (0.75 equivalencies), and amine-bearing substrate (160 equivalencies). Conjugation reactions were carried out at 37°C for 24 hours. Excess enzyme and substrate were removed using NAb Protein A/G 0.2 mL spin columns according to the manufacturer's instructions. Purified conjugates were lyophilized and suspended at a concentration of 5 mg/mL. Conjugates were diluted to a concentration of 1 mg/mL in PBS buffer, pH 7.4 and reacted with 20 equivalencies of either DBCO-PEG5K (compound 18) or TCO-PEG5K (compound 19). Reactions were carried out at 37°C for 20 hours and analyzed by reduced and denatured SDS-PAGE. Conjugation efficiency was quantified by analyzing the relative band intensities for the IgG heavy chain and the PEG5K-modified heavy chain. Each band was quantified relative to the IgG light chain band as an internal control. Band intensities were quantified using the gel analysis tool of FIJI [2] Raw images are provided and quantified (Figure S29 and S30).

Characterization of Conjugate T5 and Antibody-drug Conjugates

Large Scale Purification of Conjugate T5

1 equivalency of trastuzumab (2.3 mg, 15.3 nmol) at a concentration of 5 mg/mL in PBS buffer was treated with PNGase F (600 U/mg of antibody), MTG (0.75 equivalencies), and Linker 5 (160 equivalencies). The reaction was carried out at 37°C for 24 hours and then purified via analytical HIC using a linear solvent gradient of 0 – 60% solvent B over 60 minutes. The product (conjugate T5) eluted at 47 minutes. The product was concentrated and exchanged into PBS buffer, pH 7.4 using Amicon Ultra-0.5 mL centrifugal filters with a 30 kDa molecular weight cut off according to the manufacturer's instructions. Conjugate was recovered in 48% yield (1.1 mg, 7.3 nmol). Conjugate purity was assessed via analytical HIC using a linear solvent gradient from 0 – 100% solvent B over 60 minutes (Figure S31). Conjugate molecular weight was characterized via MALDI-MS (Figure S32).

Gel Electrophoresis Analysis of Dual "Click" Modification

1 equivalency of conjugate T5 (50 ug, 0.33 nmol) at a concentration of 6 mg/mL in PBS buffer was reacted with 2 equivalencies of DBCO-PEG4-Carboxyrhodamine101, sulfo-Cy5-TCO, or both. Reactions were carried out protected from light at room temperature for 20 hours and analyzed by reduced and denatured SDS-PAGE. Fluorescence imaging was performed using a GE Healthcare Typhoon 9400 image system set to a photomultiplier tube voltage of 400 with the following fluorescence settings: Carboxyrhodamine101 excitation 488 nm, emission 500 – 540 nm; sulfo-Cy5 excitation 633 nm, emission 655 – 685 nm; FRET excitation 488 nm, emission 655 – 685 nm. A reaction scheme is provided (Figure S33).

One-pot Synthesis of Antibody-drug Conjugates

1 equivalency of conjugate T5 (80 ug, 0.53 nmol) at a concentration of 6 mg/mL in PBS buffer was reacted with 2 equivalencies of DBCO-PEG28 (compound 20), TCO-PEG24-DM1 (compound 21), or both. Reactions were carried out at room temperature for 20 hours. Excess reagents were removed using Amicon Ultra-0.5 mL centrifugal filters with a 30 kDa molecular weight cut off according to the manufacturer's instructions. Conjugation efficiency was assessed via analytical HIC using a linear solvent gradient from 0 – 100% solvent B over 60 minutes. A reaction scheme is provided (Figure S34). Conjugate molecular weight was characterized via MALDI-MS (Figure S35).

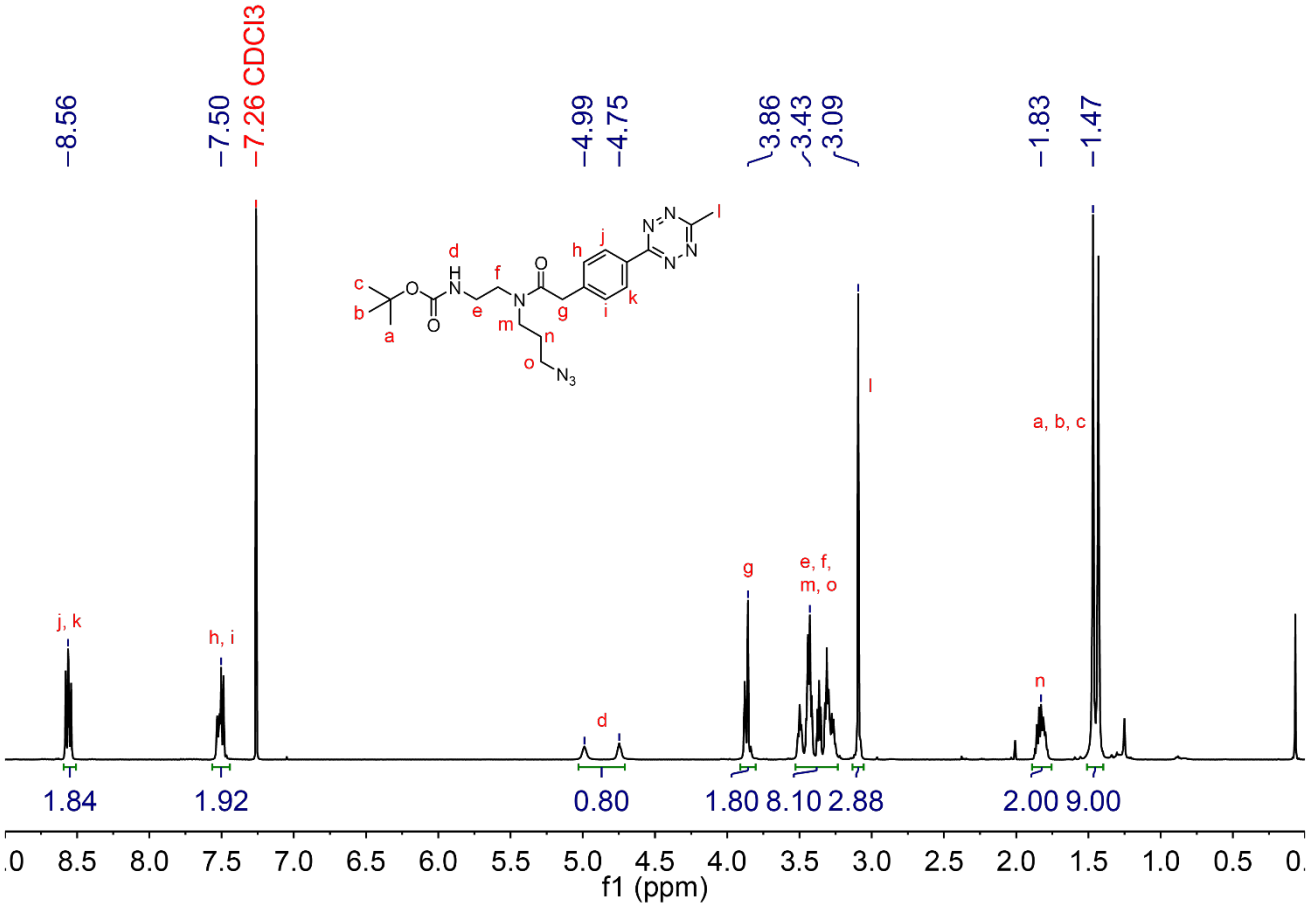
In Vitro Potency of Antibody-drug Conjugates

SKOV3 and MCF7 cells were plated at 6,000 and 1,500 cells/well, respectively, and allowed to adhere overnight. After overnight incubation, three-fold serial dilutions of the conjugates or DM1-S-Me control starting at 33 nM were added. Treated cells were then incubated for 4 days. Cell viability was measured using MTS according to the manufacturer's instructions using a Tecan Infinite M1000 Pro microplate reader. Percent viability was calculated by comparison to untreated cells and media alone. Potency data for MCF7, a Her2 negative cell line, is provided (Figure S36).

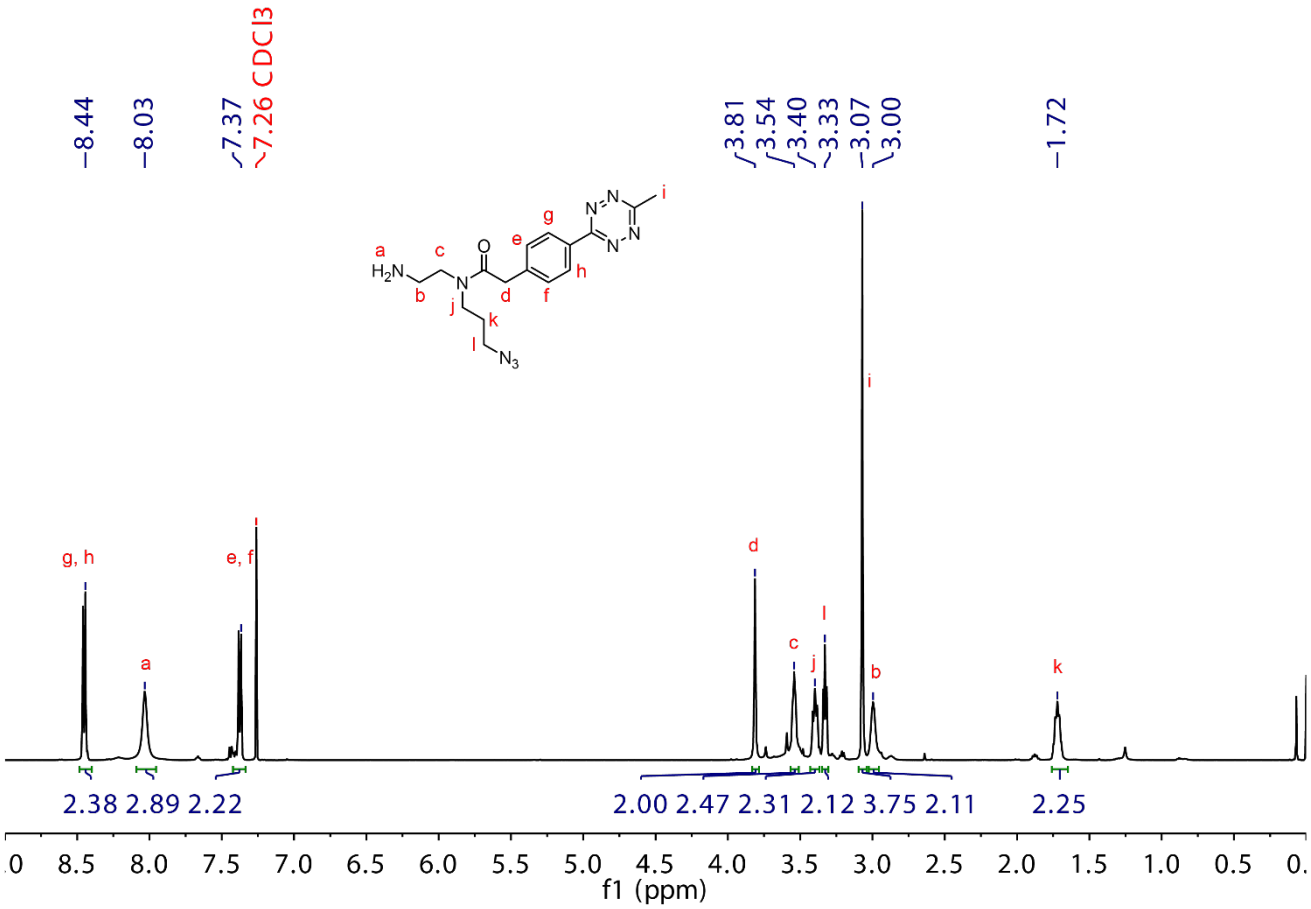
Results and Discussion

Synthesis of Antibody Modification Reagents

Synthesis of Linker 1 (compound 3)

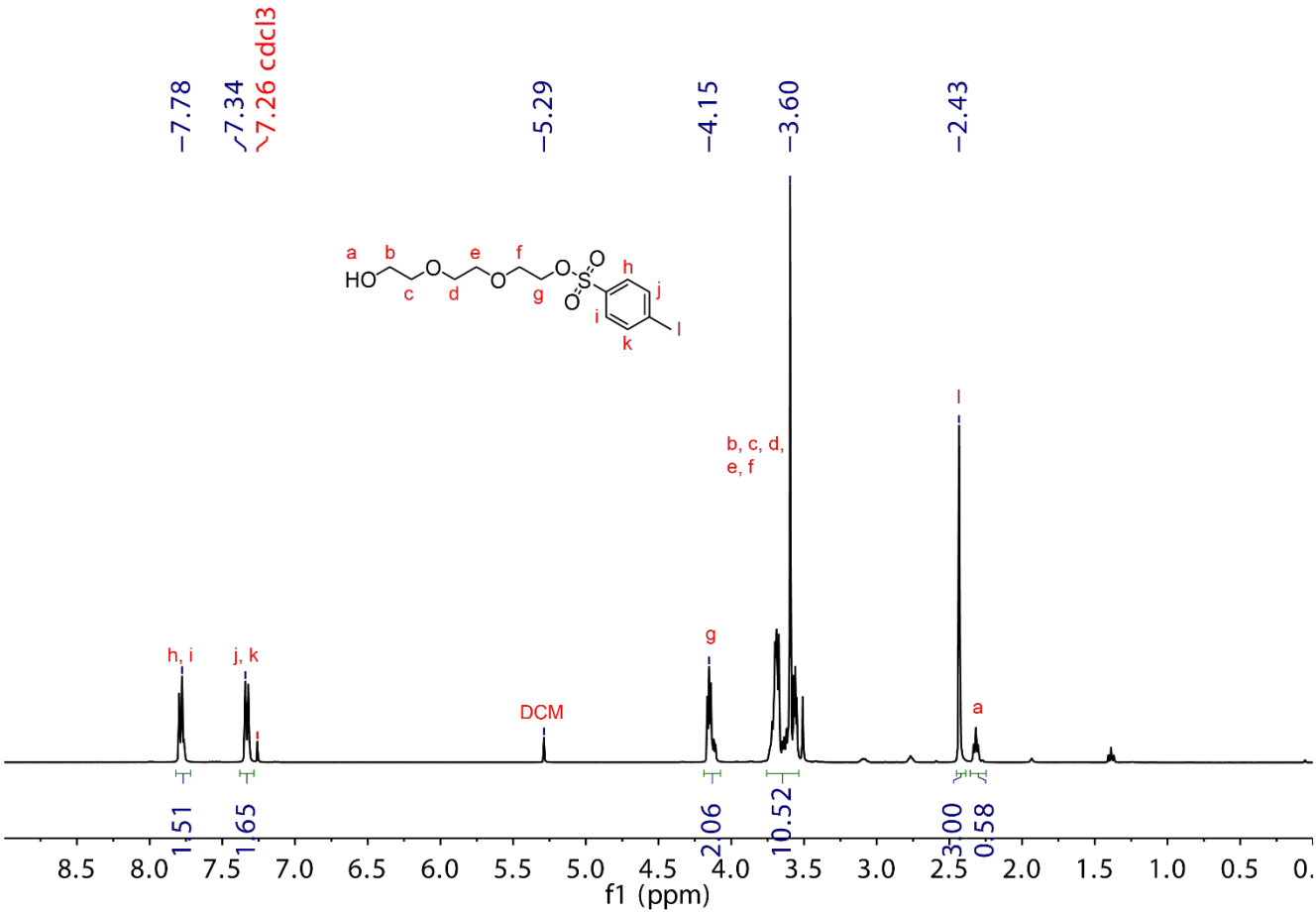


Supplementary Figure S11. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(2)**.

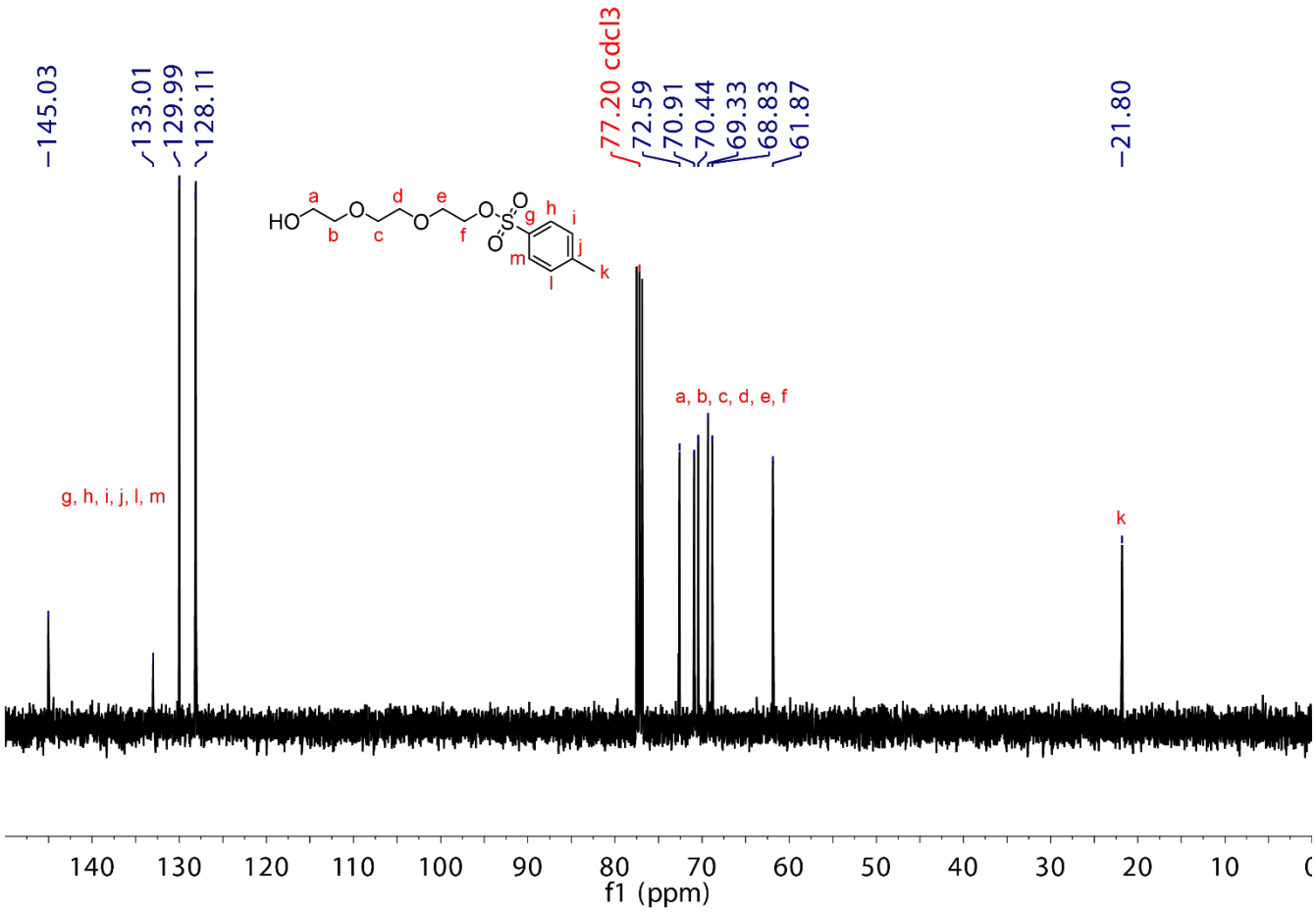


Supplementary Figure S12. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(3)**.

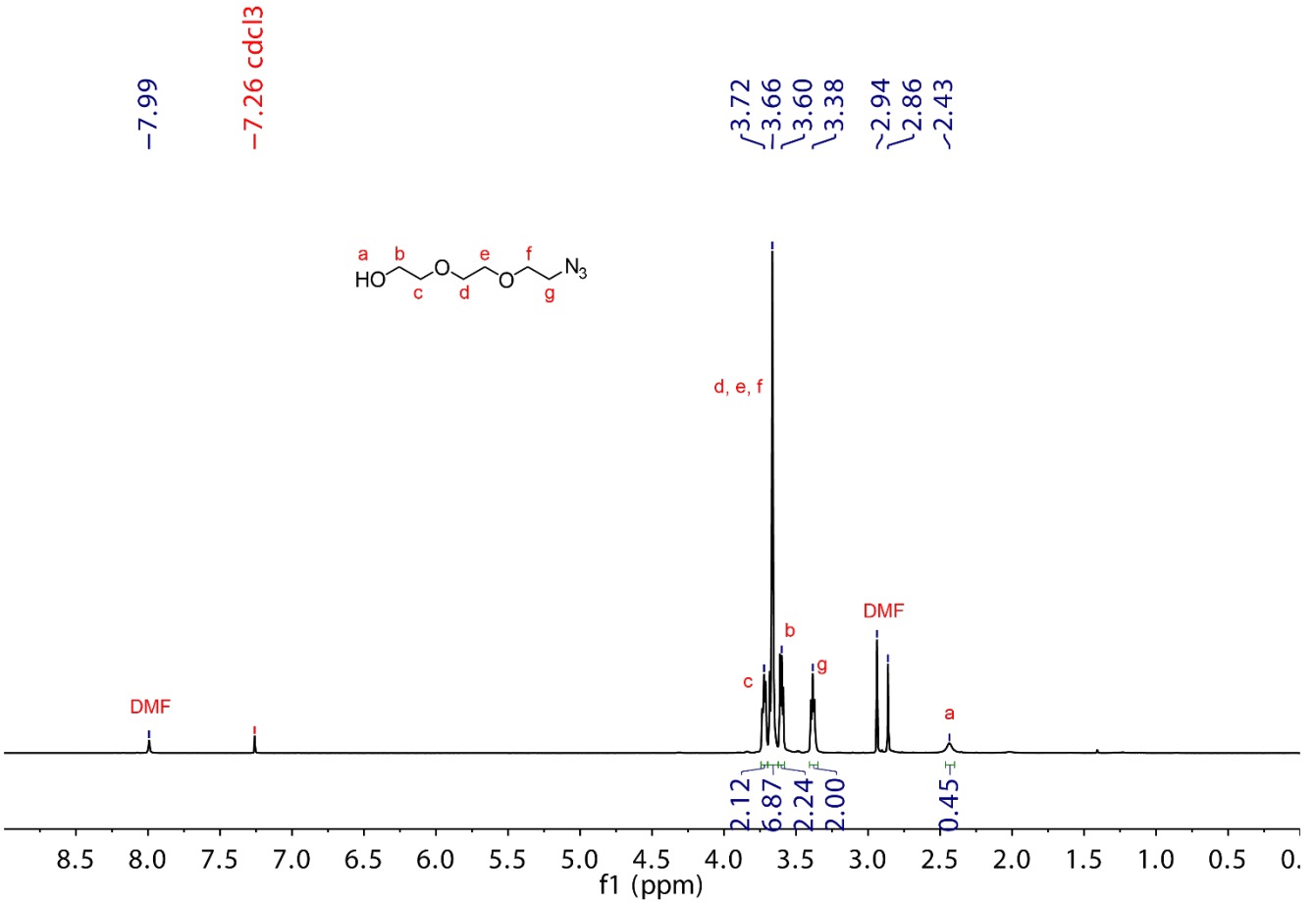
Synthesis of 1-azido-2-(2-(2-bromoethoxy)ethoxy)ethane (Compound 6)



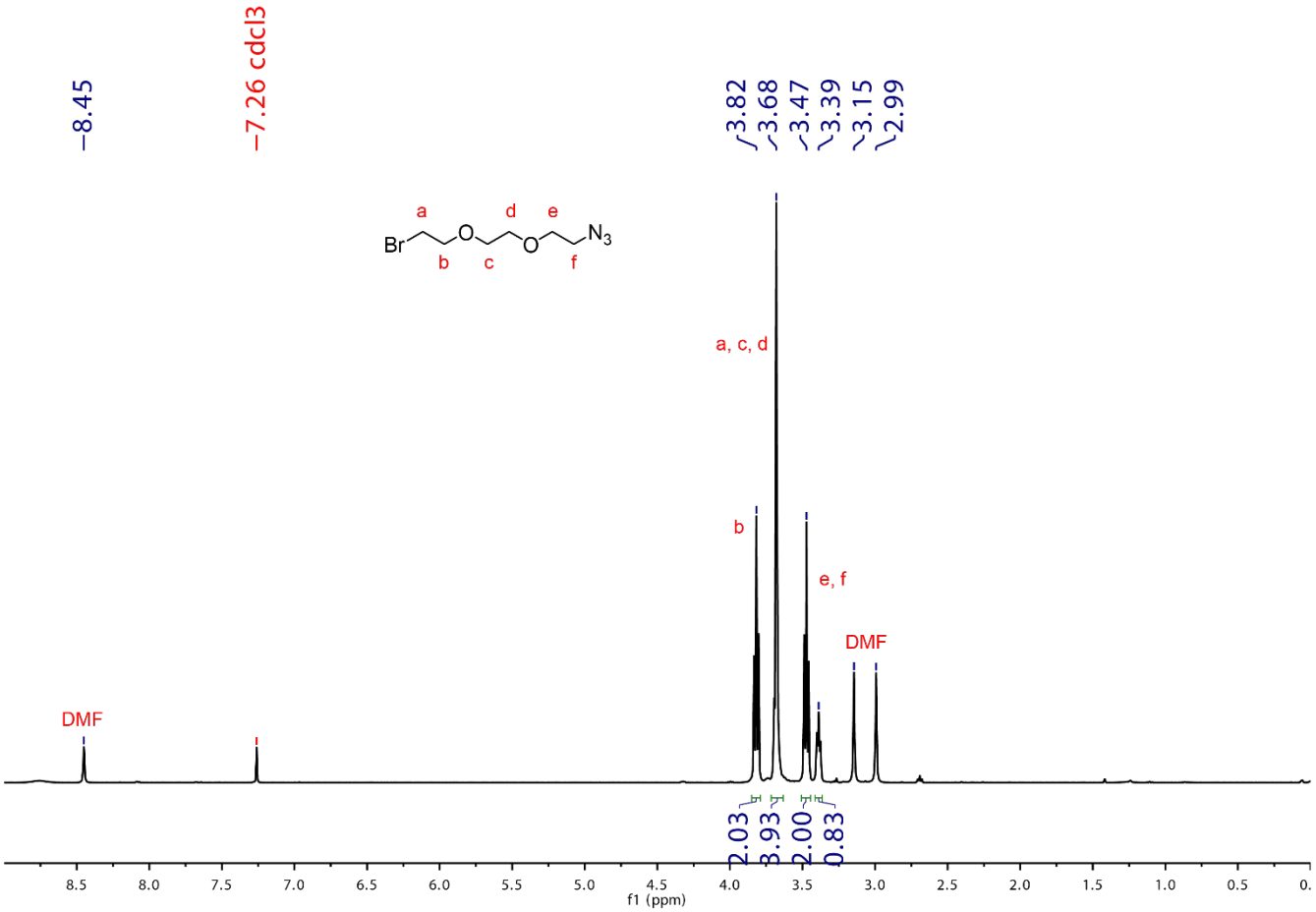
Supplementary Figure S13. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **(4)**.



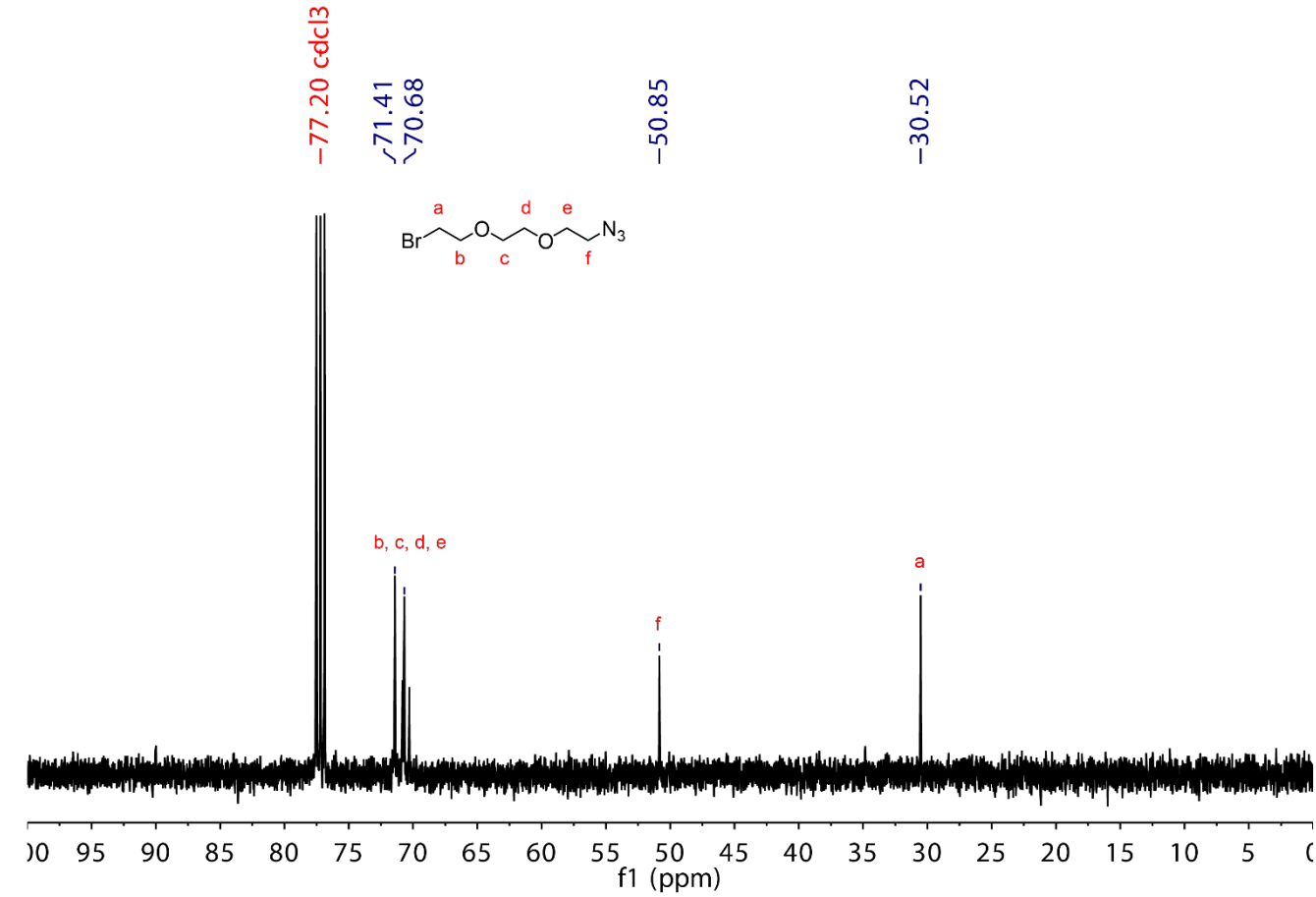
Supplementary Figure S14. ^{13}C NMR (400 MHz, $CDCl_3$) of compound **(4)**.



Supplementary Figure S15. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **(5)**.

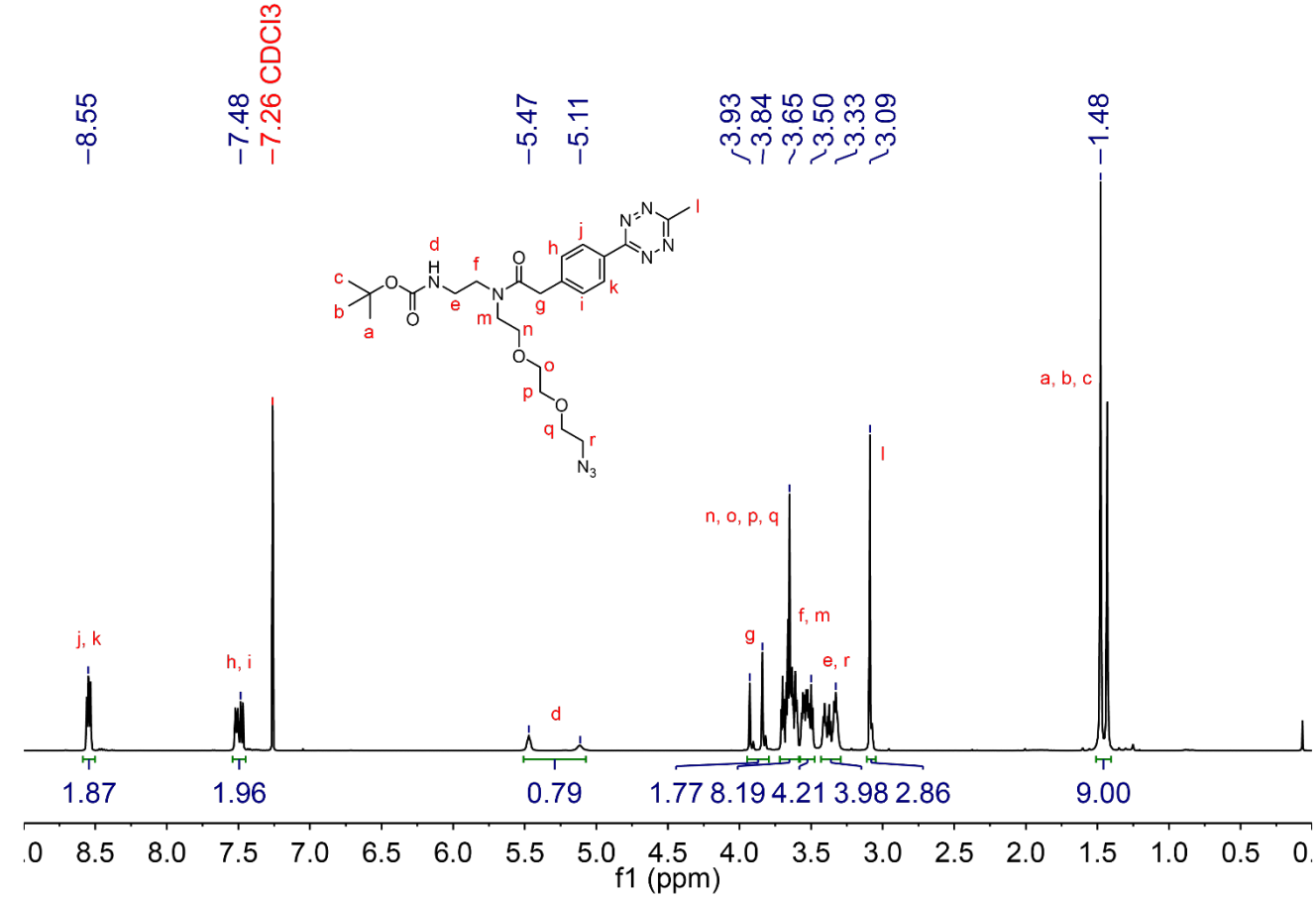


Supplementary Figure S16. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **(6)**.

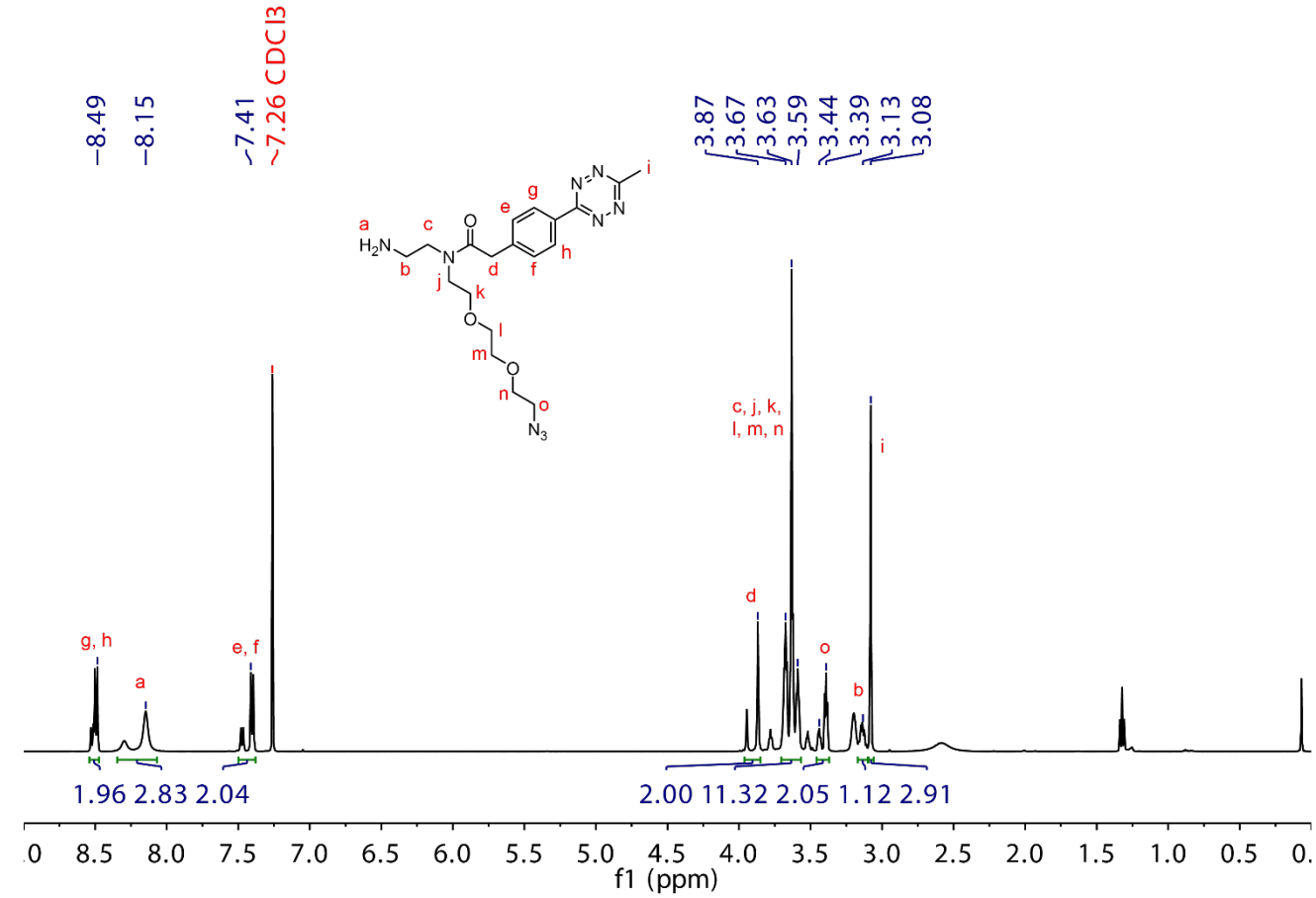


Supplementary Figure S17. ^{13}C NMR (400 MHz, $CDCl_3$) of compound **(6)**.

Synthesis of Linker 2 (Compound 9)

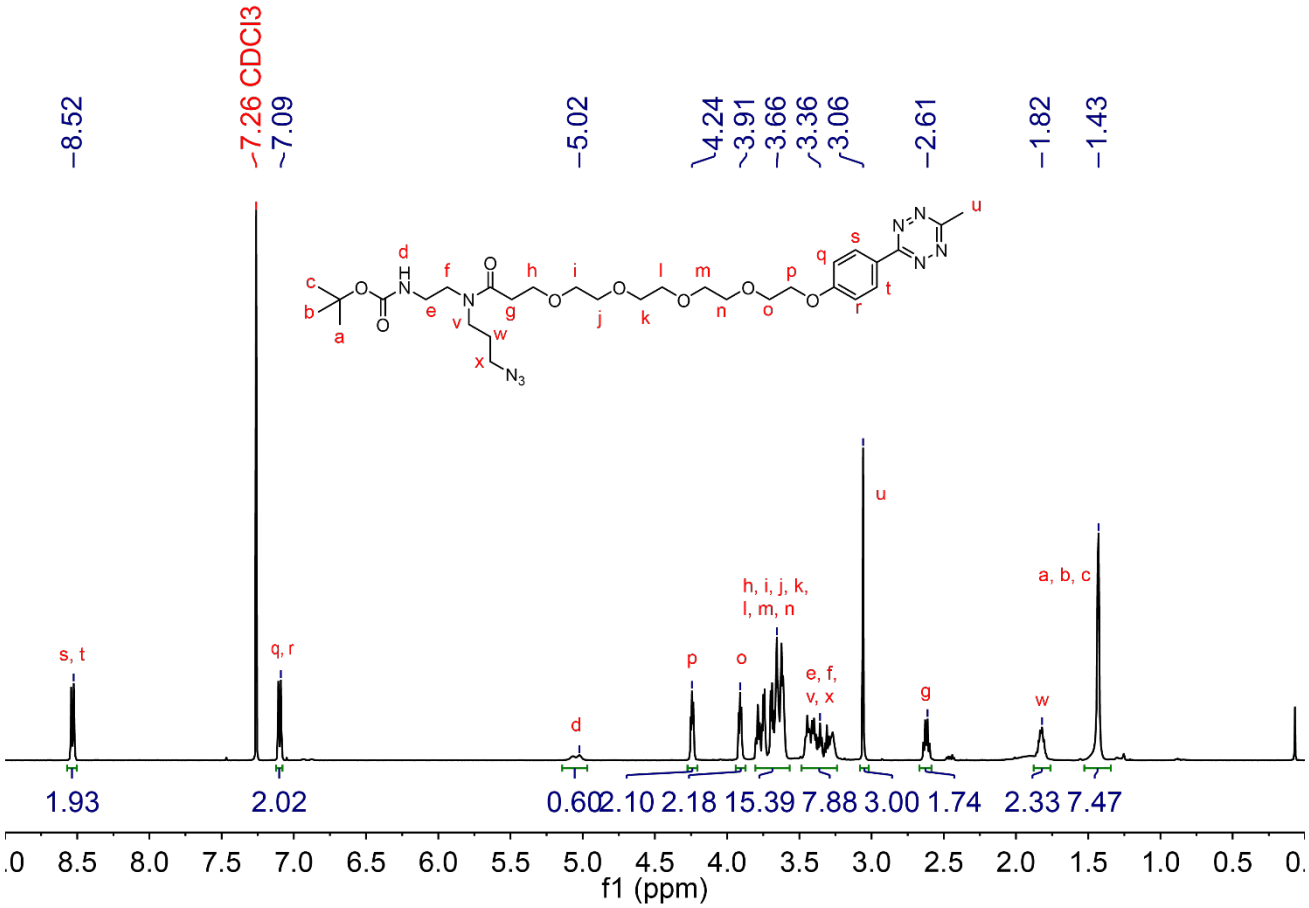


Supplementary Figure S18. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(8)**.

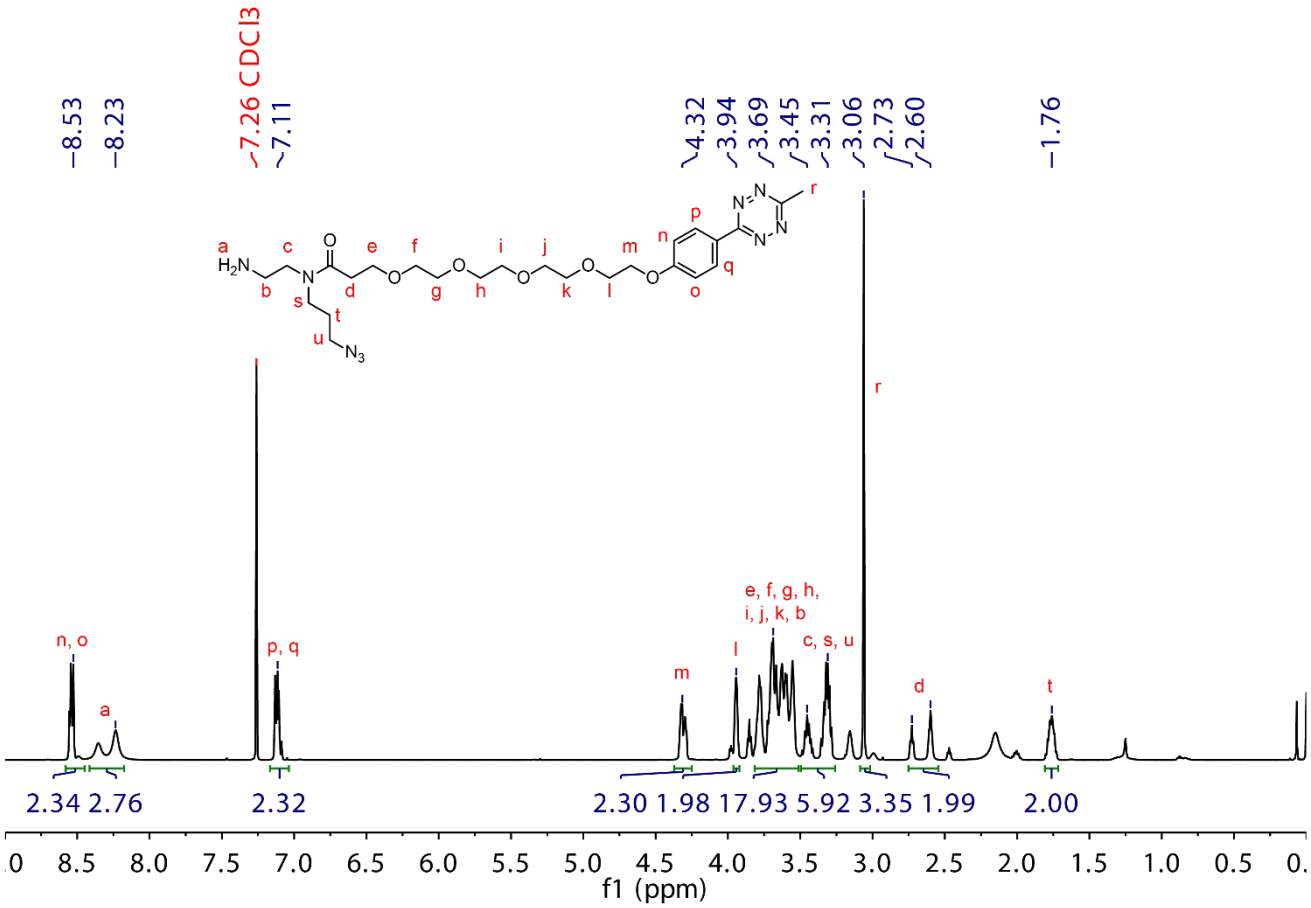


Supplementary Figure S19. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(9)**.

Synthesis of Linker 3 (Compound 11)

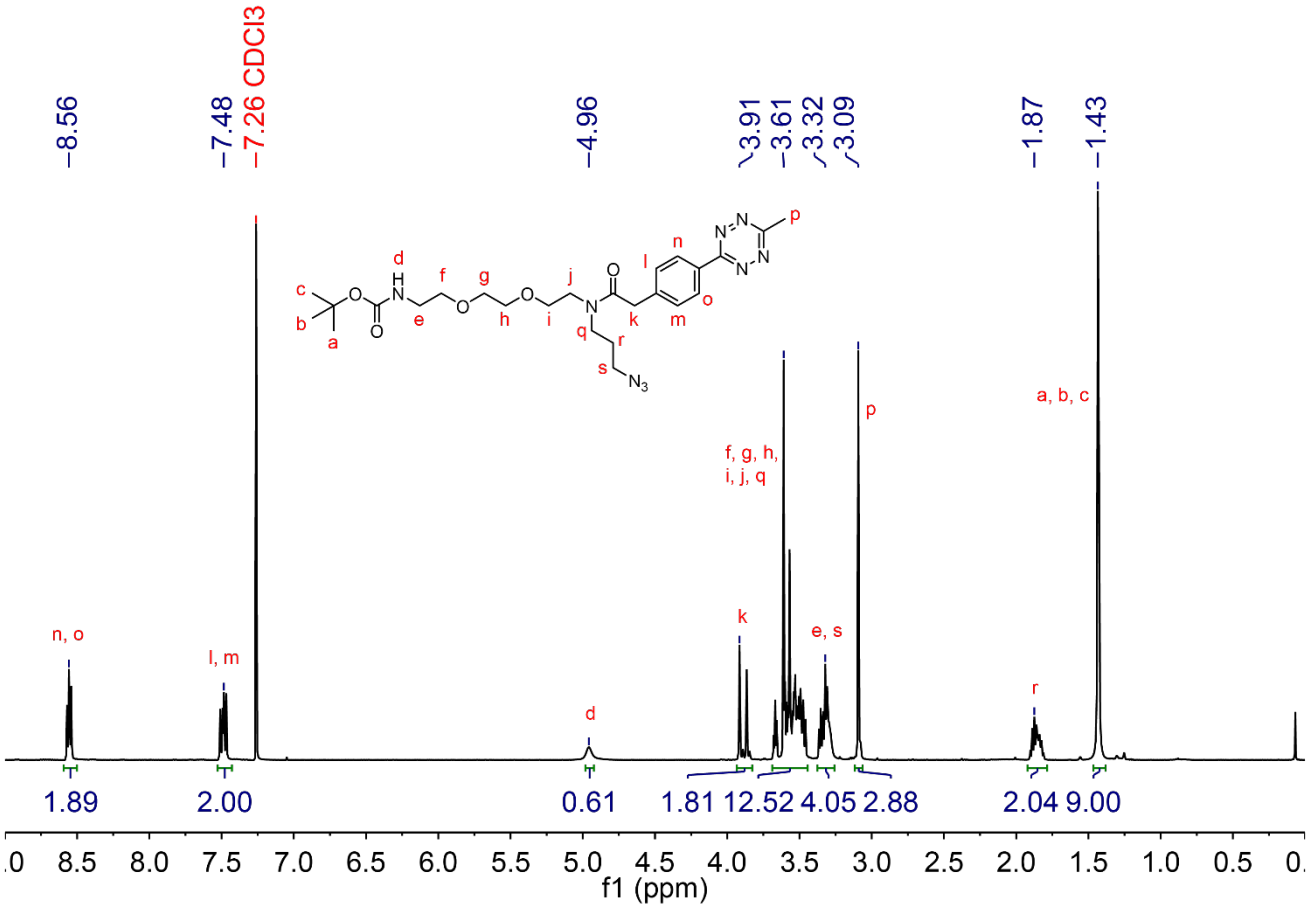


Supplementary Figure S20. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(10)**.

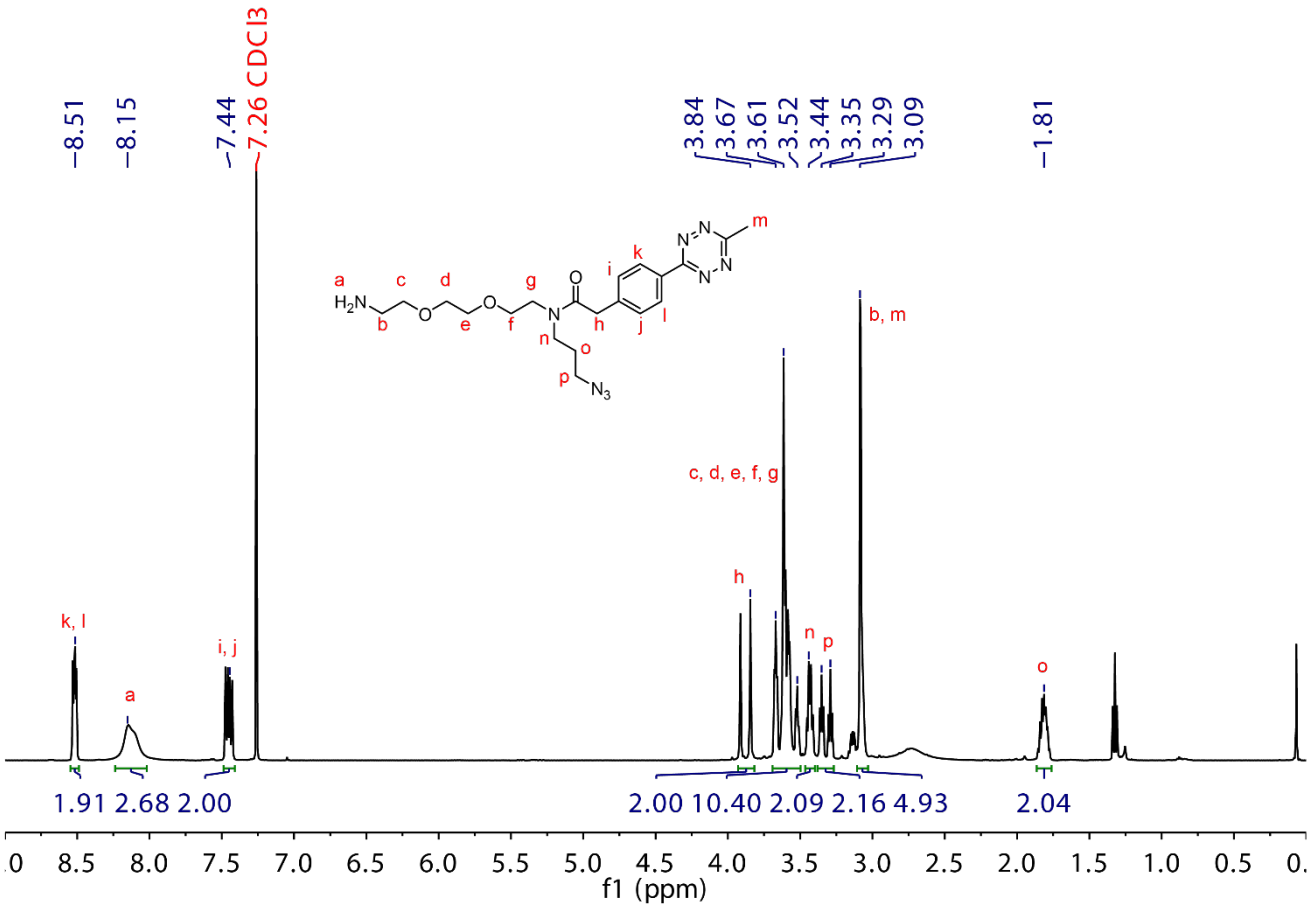


Supplementary Figure S21. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(11)**.

Synthesis of Linker 4 (Compound 14)

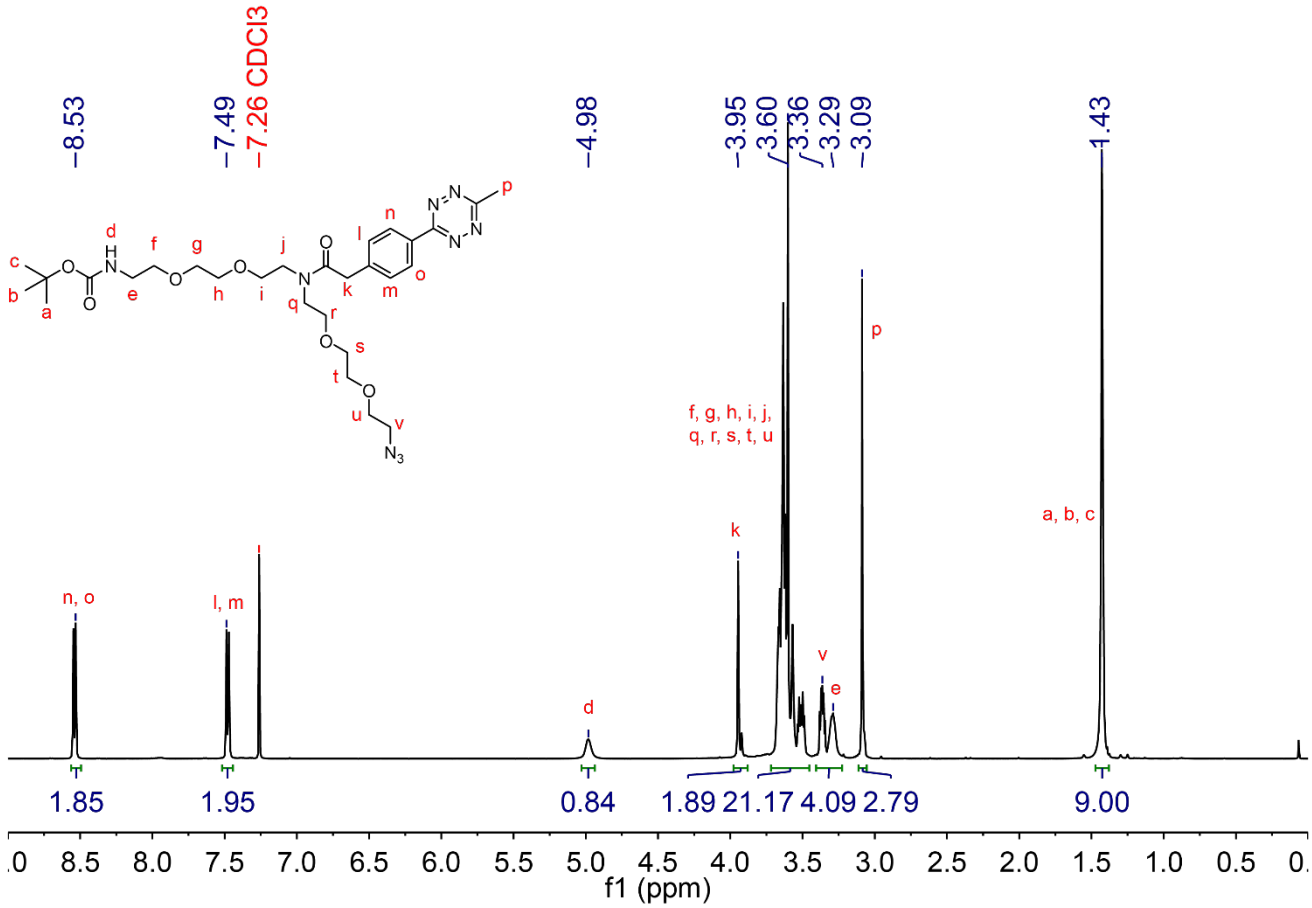


Supplementary Figure S22. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(13)**.

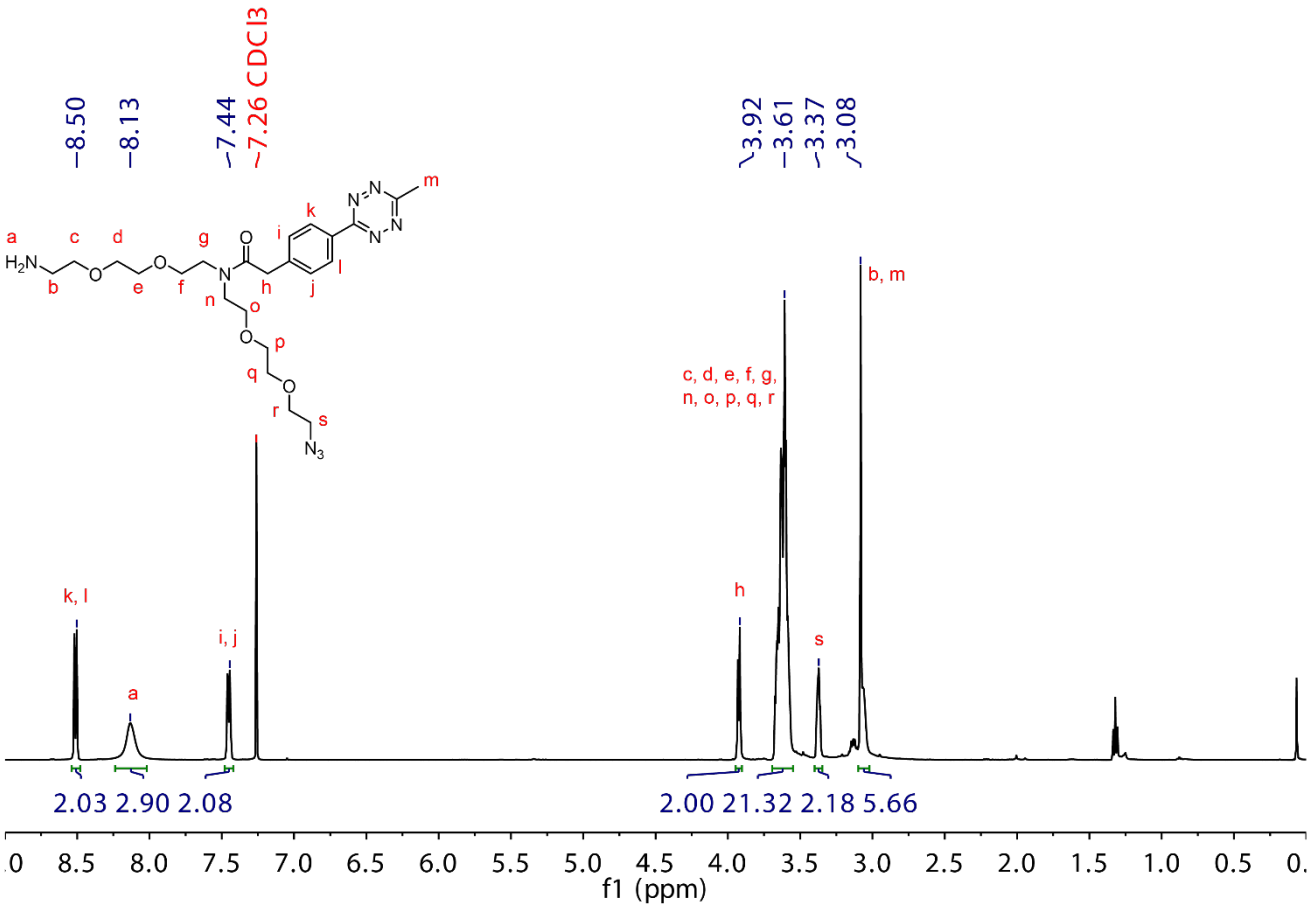


Supplementary Figure S23. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(14)**.

Synthesis of Linker 5 (Compound 17)

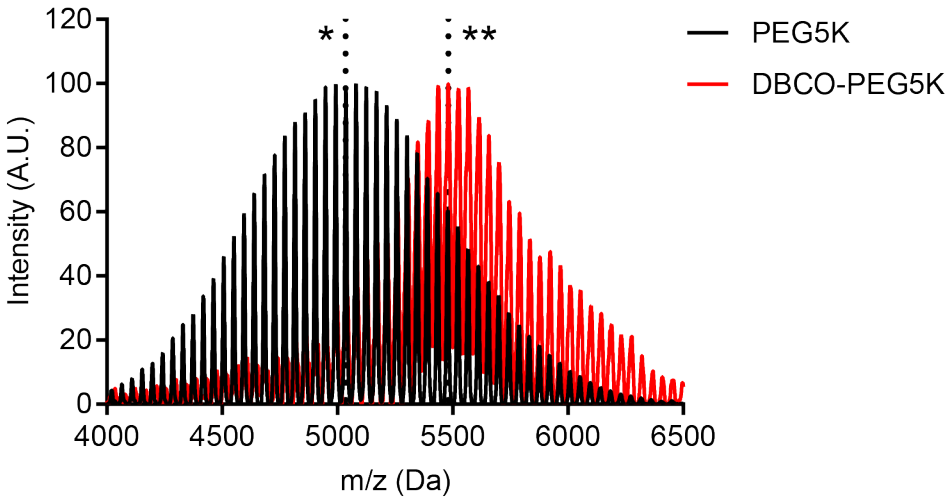


Supplementary Figure S24. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(16)**.



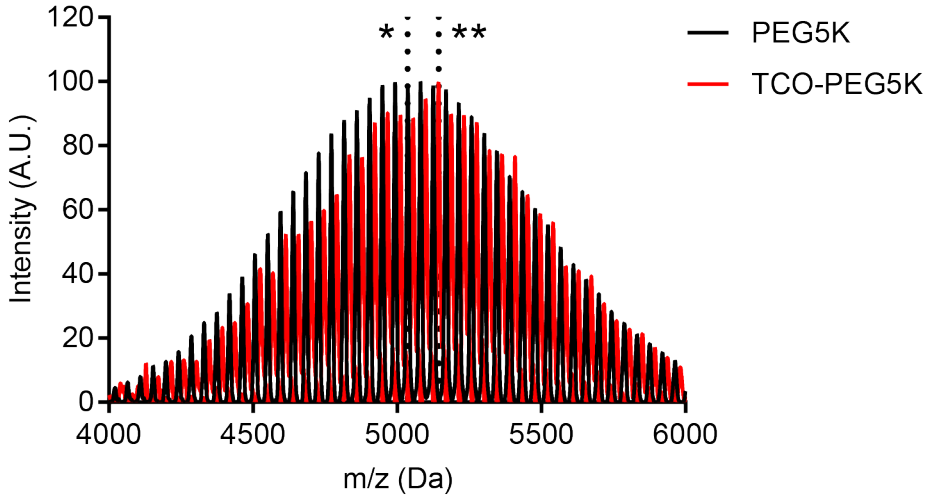
Supplementary Figure S25. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(17)**.

Synthesis of DBCO-PEG5K (Compound 18)



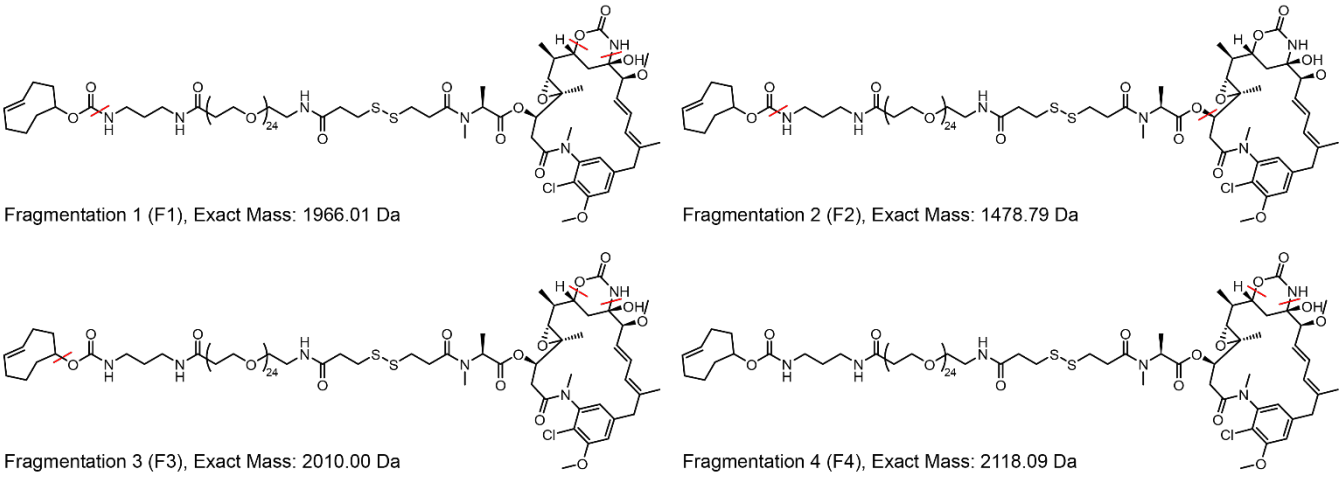
Supplementary Figure S26. MALDI-MS of compound **(18)**. Center of the distribution for the unmodified PEG5K (*) was at 5,036 Da. Center of the distribution for the DBCO-modified PEG5K (**) was at 5,481 Da. Observed difference: 445 Da, Expected Difference: 408 Da.

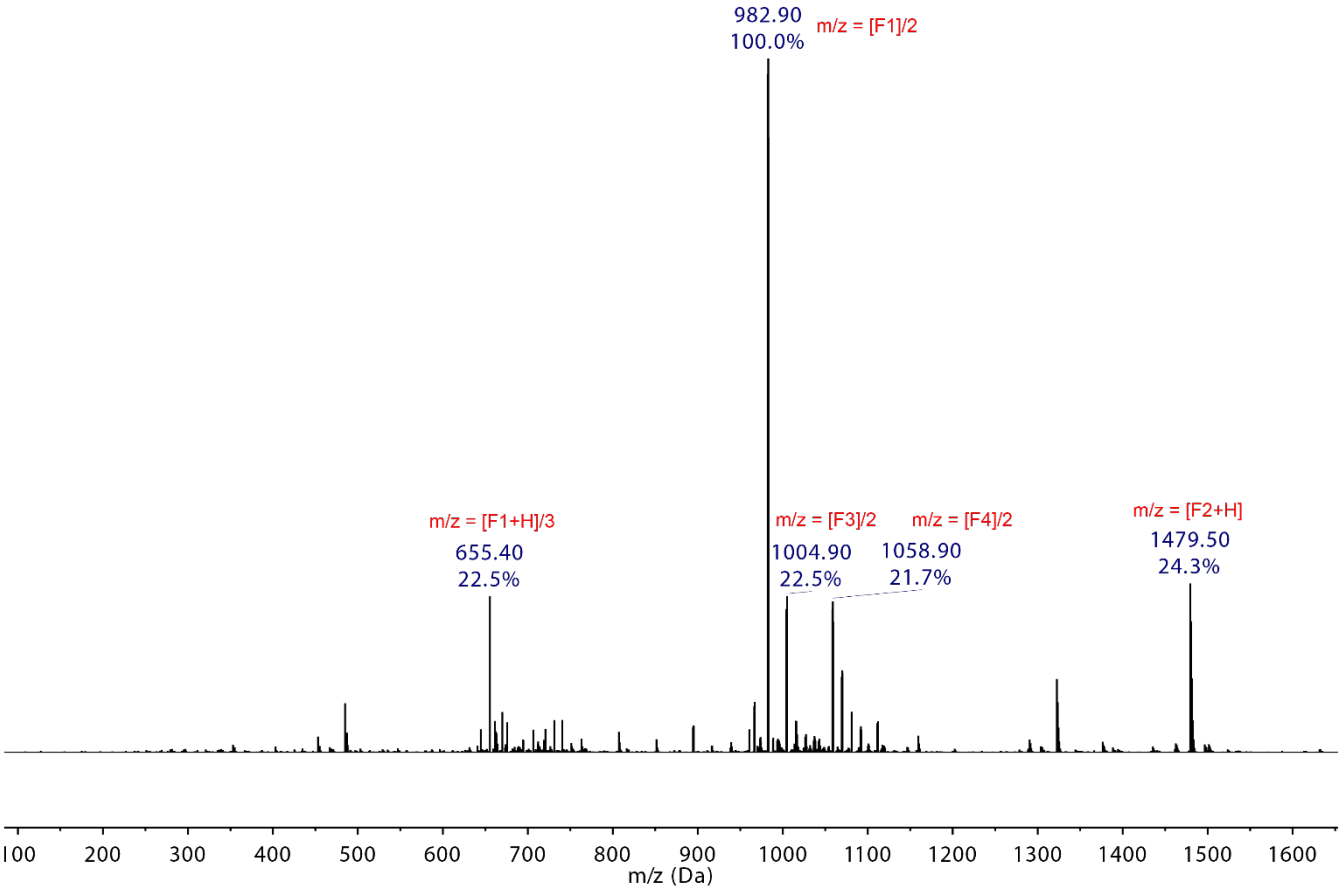
Synthesis of TCO-PEG5K (Compound 19)



Supplementary Figure S27. MALDI-MS of compound **(19)**. Center of the distribution for the unmodified PEG5K (*) was at 5,036 Da. Center of the distribution for the TCO-modified PEG5K (**) was at 5,143 Da. Observed difference: 107 Da, Expected Difference: 111 Da.

Synthesis of TCO-PEG24-DM1 (Compound 21)





Supplementary Figure S28. LC-MS of compound **(21)**.

Protein Expression and Purification

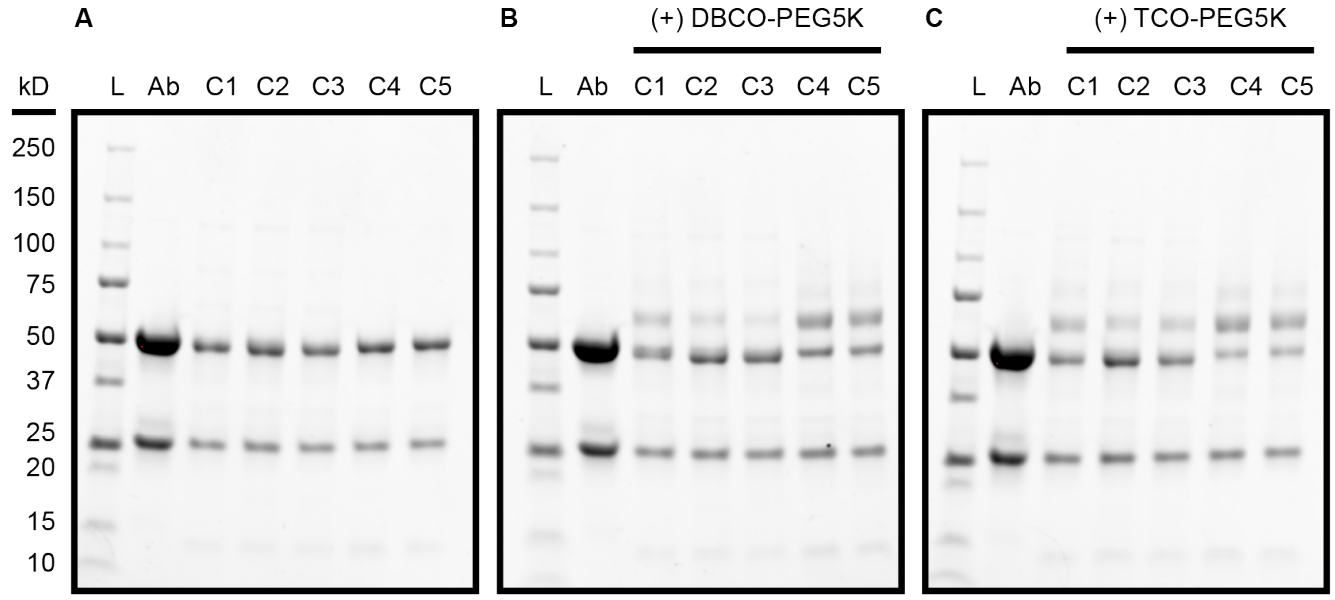
Expression and Purification of Microbial Transglutaminase

Supplementary Table 1. Sequencing information for microbial transglutaminase. Underlined text indicates the N-terminal pro-sequence.

	Sequence
Forward Primer (T7)	TAATACGACTCACTATAGGG
Reverse Primer (T7)	GCTAGTTATTGCTCAGCGG
DNA Sequence	ATGGACAATGGCGCGGGGGAAGAGACGAAGTCCTACGCCGAAACCTACCGCCTC ACGGCGGATGACGTCGCGAACATCAACGCGCTCAACGAAAGCGCTCCGGCCGC TTCGAGCGCCGGCCCGTCGTTCCGGGCCCCCGACTCCGACGACAGGGTCACCC CTCCCGCCGAGCCGCTCGACAGGATGCCCGACCCGTACCGTCCCTCGTACGGC AGGGCCGAGACGGTCGTCAACAACTACATACGCAAGTGGCAGCAGGTCTACAGC CACCGCGACGGCAGGAAGCAGCAGATGACCGAGGAGCAGCGGGAGTGGCTGTC CTACGGCTGCGTCGGTGTCACCTGGGTCAATTCGGGTCAGTACCCGACGAACAG ACTGGCCTTCGCGTCCTTCGACGAGGACAGGTTCAAGAACGAGCTGAAGAACGG CAGGCCCCGGTCCGGCGAGACGCGGGCGGAGTTCGAGGGCCGCGTCGCGAAG GAGAGCTTCGACGAGGAGAAGGGCTTCCAGCGGGCGCGTGAGGTGGCGTCCGT CATGAACAGGGCCCTGGAGAACGCCCACGACGAGAGCGCTTACCTCGACAACCT CAAGAAGGAACTGGCGAACGGCAACGACGCCCTGCGCAACGAGGACGCCCGTT CCCCGTTCTACTCGGCGCTGCGGAACACGCCGTCCTTCAAGGAGCGGAACGGA GGCAATCACGACCCGTCCAGGATGAAGGCCGTCATCTACTCGAAGCACTTCTGG AGCGGCCAGGACCGGTCGAGTTCGGCCGACAAGAGGAAGTACGGCGACCCGGA CGCCTTCCGCCCCGCCCCGGGCACCGGCCTGGTCGACATGTCGAGGGACAGGA ACATTCCGCGCAGCCCCACCAGTCCCGGTGAGGGATTCGTCAATTTCGACTACG GCTGGTTCGGCGCCCAGACGGAAGCGGACGCCGACAAGACCGTCTGGACCCAC GGAAATCACTATCACGCGCCCAATGGCAGCCTGGGTGCCATGCATGTCTACGAG AGCAAGTTCCGCAACTGGTCCGAGGGTTACTCGGACTTCGACCGCGGAGCCTAT GTGATCACCTTCATCCCCAAGAGCTGGAACACCGCCCCCGACAAGGTAAAGCAG GGCTGGCCGCTCGAGCACCACCACCACCACCACTGA
Amino Acid Sequence	MDNGAGEETKSYAETYRLTADDVANINALNESAPAASSAGPSFRAPDSDDRVTPPAE PLDRMPDPYRPSYGRAETVVNNYIRKWQQVYSHRDGRKQQMTEEQREWLSYGCV GVTWVNSGQYPTNRLAFASFDEDRFKNELKNGRPRSGETRAEFEGRVAKESFDEEK GFQRAREVASVMNRALENAHDESAYLDNLKKELANGNDALRNEDARSPFYSALRNT PSFKERNGGNHDPSRMKAVIYSKHFWSGQDRSSSADKRKYGDPDAFRPAPGTGLV DMSRDRNIPRSPTSPGEGFVNFDYGWFGAQTEADADKTVWTHGNHYHAPNGSLGA MHVYESKFRNWSEGYSDFDRGAYVITFIPKSWNTAPDKVKQGWPLEHHHHHH

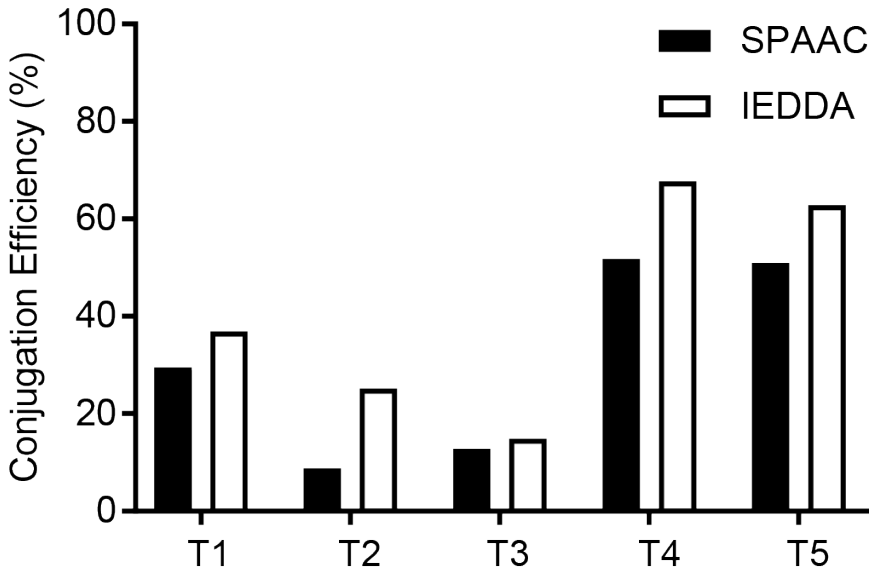
Analysis of Linker Conjugation Efficiency

Gel Electrophoresis Analysis of Linker Conjugation Efficiency



Supplementary Figure S29. SDS-PAGE analysis of conjugation efficiency. A) Linker-modified trastuzumab, B) Linker-modified trastuzumab reacted with DBCO-PEG5K, C) Linker-modified trastuzumab reacted with TCO-PEG5K.

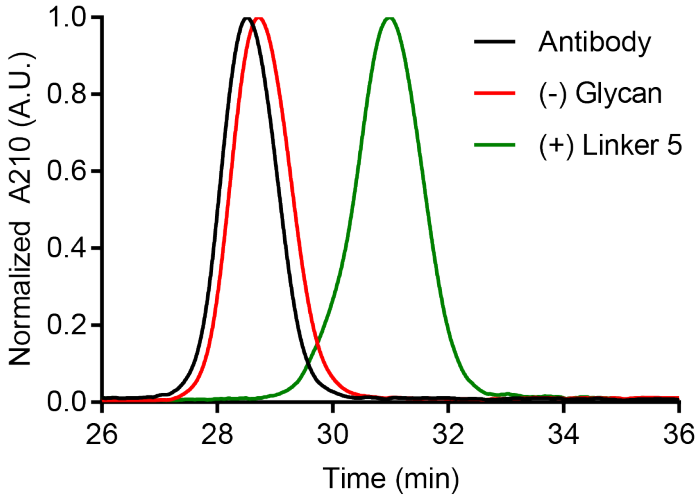
Quantification of Electrophoresis Analysis of Linker Conjugation Efficiency



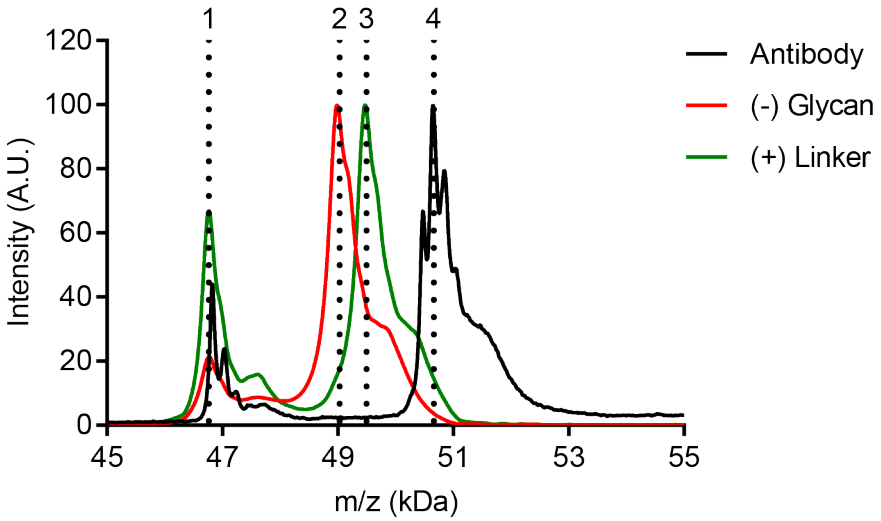
Supplementary Figure S30. Quantification of relative band intensity from SDS-PAGE analysis of linker conjugation efficiency.

Characterization of Conjugate T5 and Antibody-drug Conjugates

Large Scale Purification of Conjugate T5

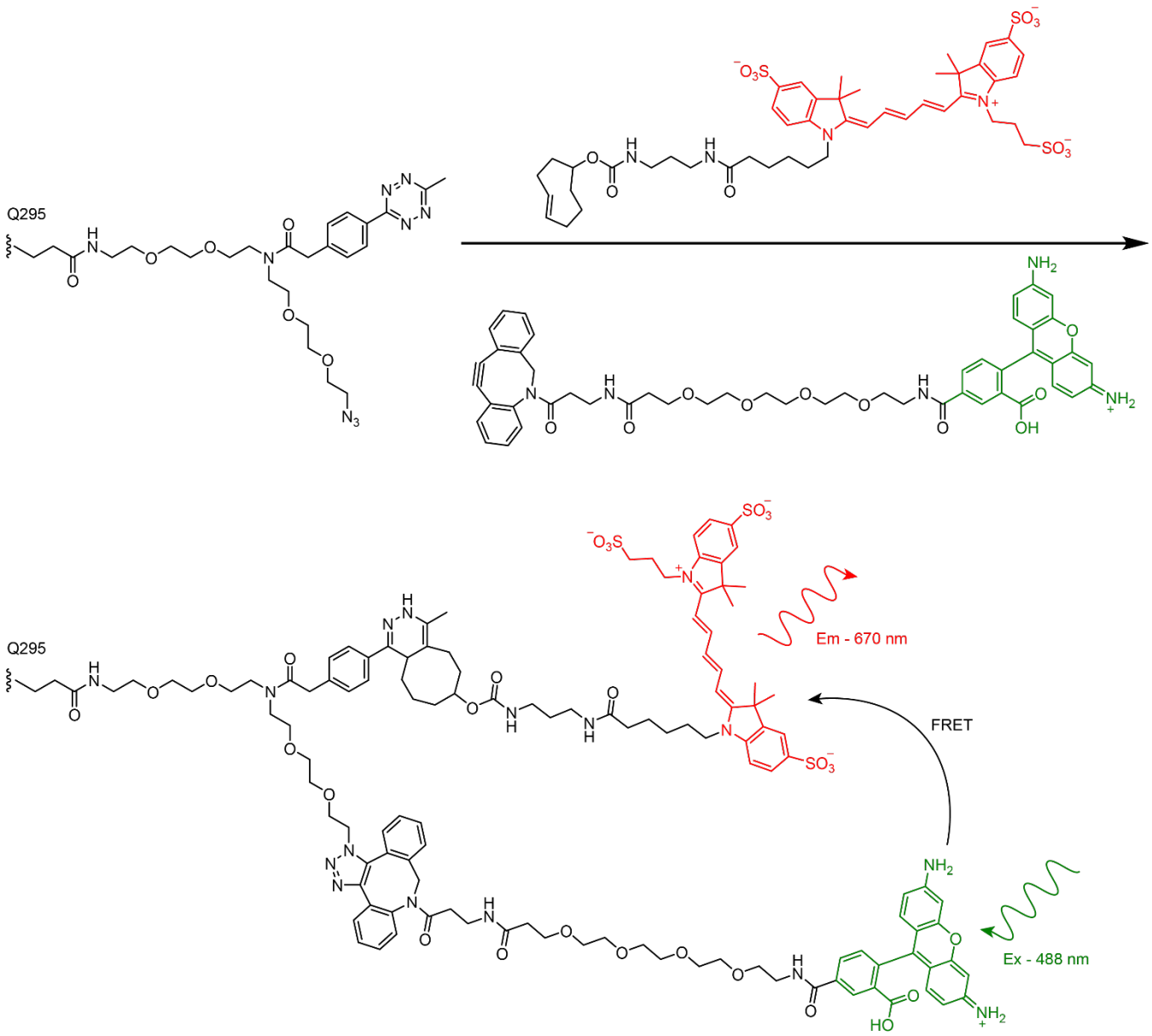


Supplementary Figure S31. HIC analysis of conjugate T5 purity.



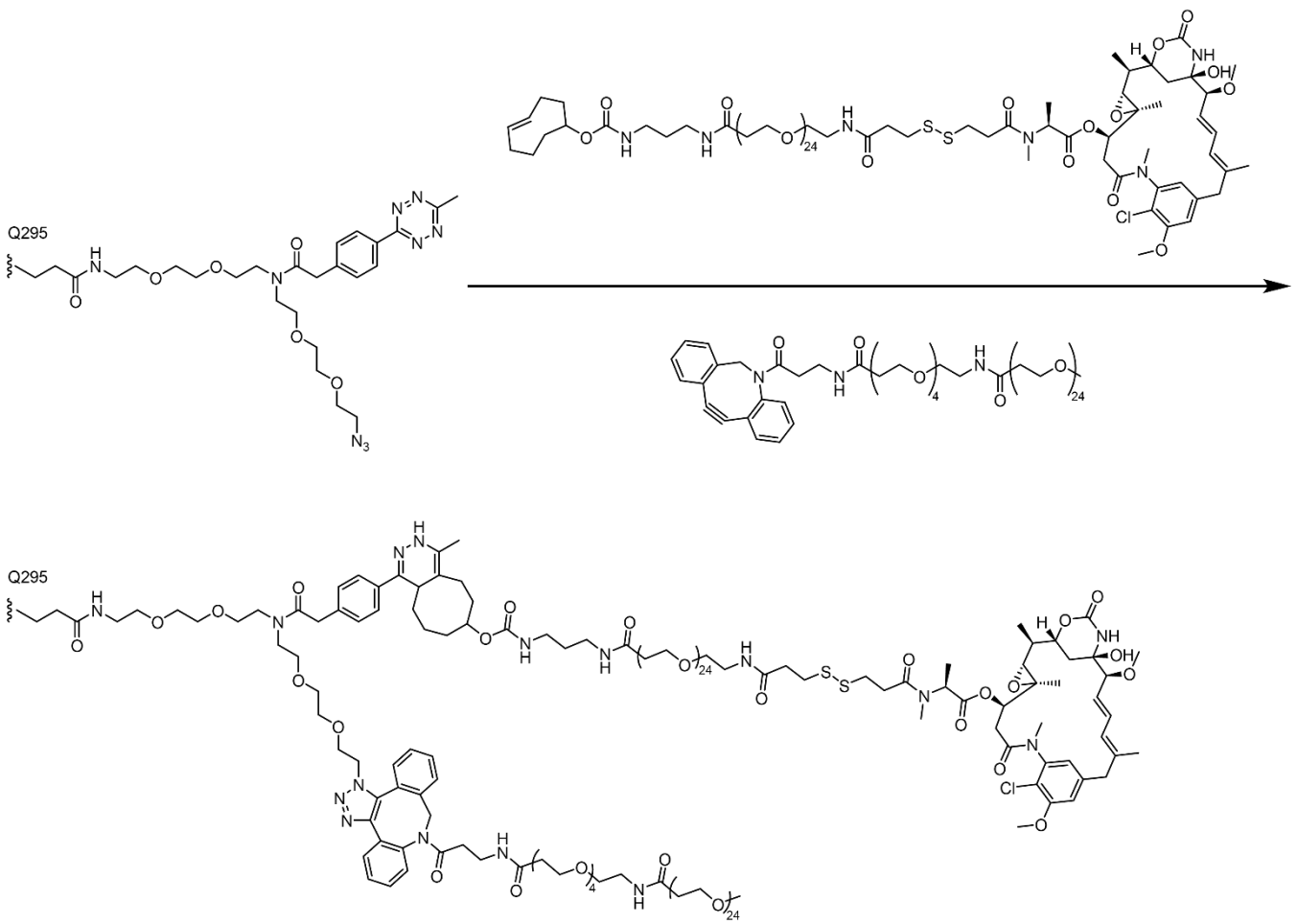
Supplementary Figure S32. MALDI-MS analysis of conjugate T5 molecular weight. Conjugate was reduced before analysis to confirm attachment of linker 5 to the heavy chain of the antibody. A double mass (1) of the light chain was observed at 46,792 Da. The heavy chain of the untreated antibody (4) was observed at 50,651 Da. Upon treatment with PNGase F (2), the aglycosylated heavy chain was observed at 49,021 Da, consistent with loss of a 1,630 Da consistent with removal of the glycan at position 297. Upon treatment with MTG in the presence of linker 5 (3), the linker-modified heavy chain was observed at 49,502 Da, consistent with one addition of linker 5 to the heavy chain. Observed difference: 481 Da, Expected difference: 500 Da.

Gel Electrophoresis Analysis of Dual "Click" Modification

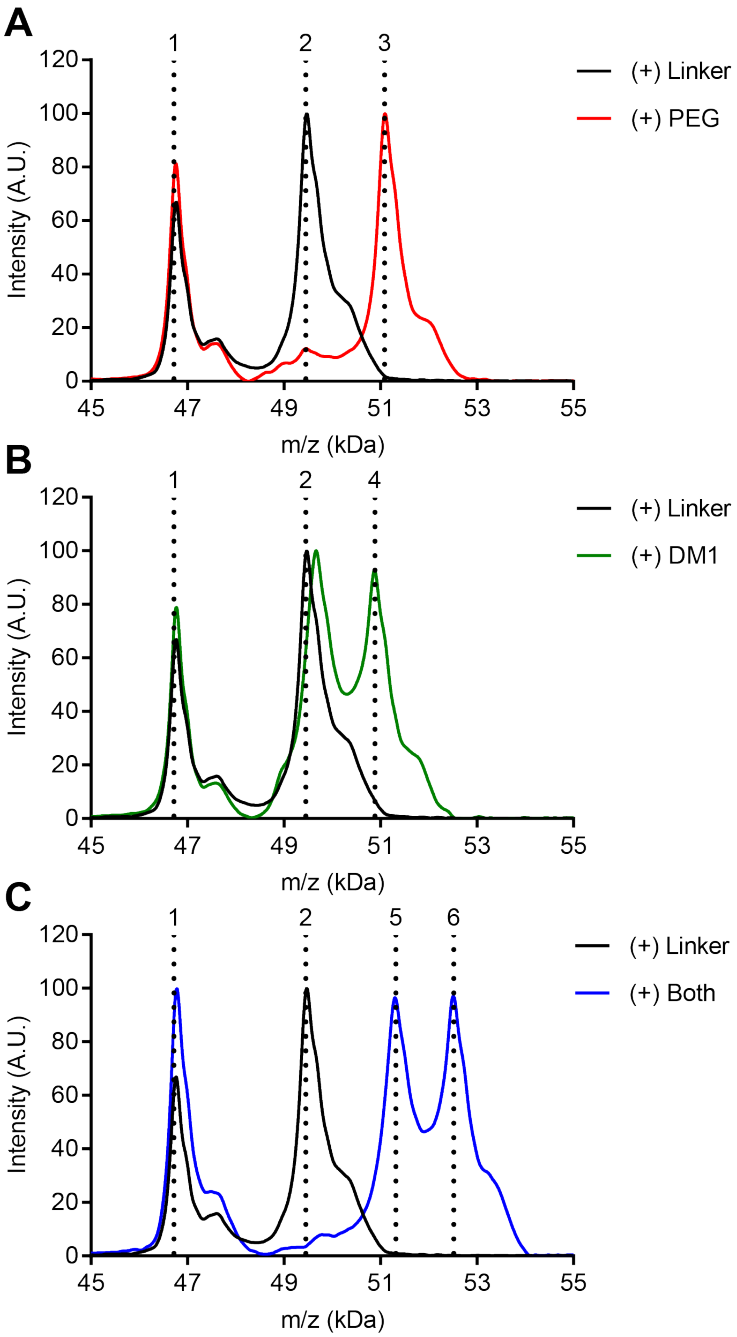


Supplementary Figure S33. Reaction scheme for dual "click" modification with FRET pair of fluorophores.

One-pot Synthesis of Antibody-drug Conjugates

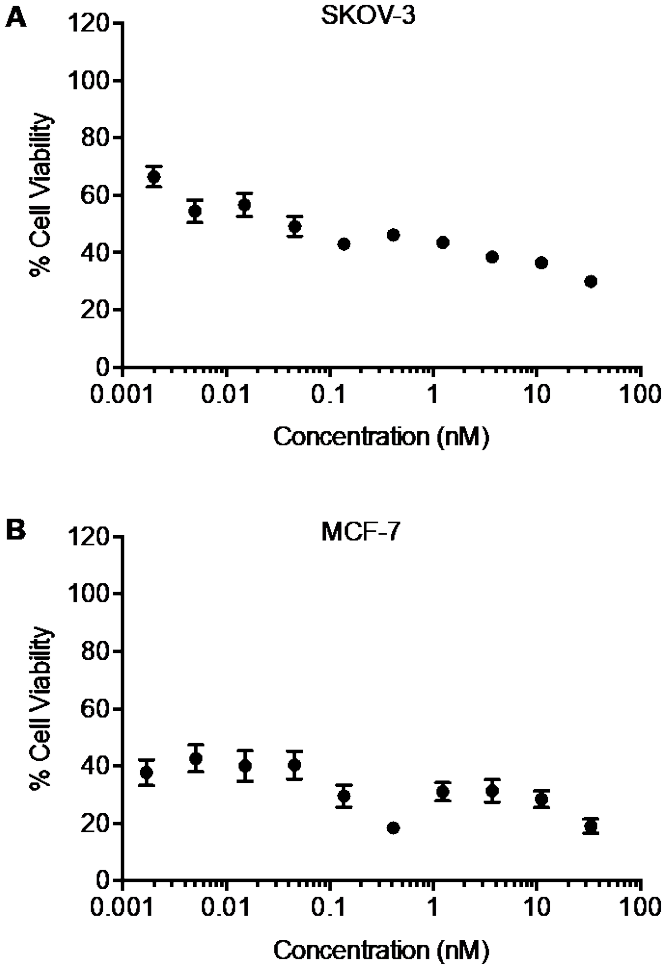


Supplementary Figure S34. Reaction scheme for dual “click” synthesis of antibody-drug conjugates.



Supplementary Figure S35. MALDI-MS analysis of antibody-drug conjugate molecular weight. Conjugates were reduced before analysis to confirm attachment to the heavy chain of the antibody. A double mass (1) of the light chain was observed at 46,786 Da in all samples. The heavy chain of the linker 5-modified antibody (2) was observed at 49,502 Da. A) Upon addition of DBCO-PEG28, complete conversion was observed with the modified heavy chain (3) appearing at 51,124 Da; Observed difference: 1,622 Da, Expected difference: 1,622 Da. B) Upon addition of TCO-PEG24-DM1, partial conversion was observed with the modified heavy chain (4) appearing at 50,900 Da; Observed difference: 1,398 Da, Expected difference: 1,414 Da. C) Upon addition of both DBCO-PEG28 and TCO-PEG24-DM1, partial conversion was observed. The first product (5) appeared at 51,331 Da corresponding to addition of DBCO-PEG28 (observed difference: 1829 Da, expected difference: 1622 Da). The second product (6) appeared at 52,536 Da corresponding to addition of both DBCO-PEG28 and TCO-PEG24-DM1 (observed difference 3,034 Da, expected difference: 3,036).

In Vitro Potency of Antibody-drug Conjugates



Supplementary Figure S36. *In vitro* potency of DM1-S-Me (drug alone) on A) SKOV-3 and B) MCF7 cells.

References

(1) Gundersen, M. T., Keillor, J. W., and Pelletier, J. N. (2013) Microbial transglutaminase displays broad acyl-acceptor substrate specificity. *Appl Microbiol Biotechnol 98*, 219–230.

(2) Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods 9*, 676–682.