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Design of Protein-based "Turn On" Molecular Probes for Intracellular Bond Cleavage

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Experimental Procedures

General Procedures

Reagents for Chemical Synthesis

All chemicals were purchased from MilliporeSigma unless stated otherwise. DBCO-PEG4-NHS was purchased from BroadPharm.

Reagents for Molecular Biology and Cell Culture

Reagents for molecular biology and cell culture were purchased from ThermoFisher Scientific unless stated otherwise. HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin Streptomycin (Pen/Strep) at 37 °C with with 5% CO_2. Live-cell imaging of cells was carried out in FluoroBrite DMEM supplemented with 10% FBS and 4 mM L-glutamine. Glass bottom dishes for flow cytometry were purchased from Cellvis.

Flash Chromatography

Flash chromatography was performed on a Teledyne ISCO CombiFlash Rf-200i chromatography system equipped with UV-Vis and evaporative light scattering detectors (ELSD).

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)

HPLC purification was performed on an Agilent 1100 Series HPLC system equipped with a UV diode array detector and an 1100 Infinity fraction collector. Semi-preparative RP-HPLC was performed with a C18 column (Agilent Eclipse XDB-C18, 9.4 x 250 mm, 5 µm). Analytical RP-HPLC was performed with a 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) unless specified otherwise. Compounds were eluted at a flow rate of either 4 mL/min (semi-preparative) or 1 mL/min (analytical) using a linear solvent gradient as specified below.

Synthesis and Biological Testing of Conjugates
Synthesis of Transferrin Probes
Synthesis of Transferrin-DBCO
1 equivalency of human holo-transferrin was reacted with 7 equivalencies of 4,7,10,13-tetraoxa-16-azaeicosanoic acid, 20-(11,12-didehydrodibenz[*b*,*f*]azocin-5(6*H*)-yl)-17,20-dioxo-, 2,5-dioxo-1-pyrrolidinyl ester (50 mg/mL in dimethyl sulfoxide) in borate-buffered saline (100 mM borate, 150 mM NaCl) pH 8.2 for 5.5 hours. The final concentration of transferrin was 250 µM. The mixture was then dialyzed overnight against ultrapure water in a Slide-A-Lyzer™ MINI Dialysis Device, 3.5K MWCO.

Conjugation of Transferrin-DBCO to Linkers
1 equivalency of transferrin-DBCO (160 µM in ultrapure water) was reacted with 2 equivalencies of linker (1 mg/mL in dimethyl sulfoxide) in PBS pH 7.4 for 24 hours. The mixture was then dialyzed for 5 hours against ultrapure water in a Slide-A-Lyzer™ MINI Dialysis Device, 3.5K MWCO.

Disulfide Cleavage Assay
Quenched linker was incubated at 50 µM in 1X PBS with 5 mM DL-dithiothreitol or 5 mM of reduced L-glutathione at room temperature. Fluorescence readings were measured at 0, 2, 4, and 24 hours using an excitation of 495 nm and an emission of 515 nm. Signal is represented as the fold increase in fluorescence upon treatment with reducing agent relative to the fold increase observed for the control linker.

Cathepsin B Cleavage Assay
Each linker or transferrin probe was incubated at 2.5 µM with 1.8 µM cathepsin B in pH 6 buffer (352mM KH_2PO_4; 48mM NaH_2PO_4; 4mM EDTA) at 40°C. Fluorescence measurements were taken at regular intervals on a TECAN M1000 Pro Plate Reader using an excitation of 490 nm and an emission of 515 nm. Signals were normalized to fluorescence of the sample incubated in a buffer control.

Flow Cytometry Analysis of Transferrin Probes
One day prior to the experiment, cells were plated into a 4-chamber 35 mm glass bottom dish with 20 mm microwell, #1.5 cover glass at 75,000 cells/well in DMEM with 10% FBS and 1% Pen/Strep. The next day, the media was removed, the cells were washed with PBS, and then incubated at 4 °C with 10 nM of the probes in DMEM with 10% FBS and 1% Pen/Strep for 1 hour. After incubation, the cells were washed twice with PBS and fresh media was added. Cells were incubated at 37 °C and 5% CO_2 for varying amounts of time. Cells were then washed with PBS, trypsinized, pelleted, and resuspended in PBS for flow cytometry analysis. Green fluorescence was measured on a BD FACS Calibur with the following instrument settings: FSC detector: E-1 Voltage, 3 Amp Gain, SSC detector: 400 Voltage, 1 Amp Gain, FL1 detector: 600 Voltage, 1 Amp Gain. Data was collected for 3 biological replicates with 2 technical replicates per biological replicate. Data processing was performed using FlowJo software.

IncuCyte Live-cell Analysis of Transferrin Probes
One day prior to the experiment, HeLa cells were plated into a 24-well tissue culture treated plate at 50,000 cells/well in DMEM with 10% FBS and 1% Pen/Strep. The media was then removed, the cells were washed with PBS, and then incubated with 150 nM of the transferrin probes in FluoroBrite DMEM with 10% FBS and 1% Pen/Strep. The IncuCyte was equipped with a 10x objective and set to image phase and green fluorescence at 30 minute time intervals. After 24 hours, the images were analyzed using the IncuCyte ZOOM software with phase filters with a minimum area of 100 μm^2 and hole fill cleanup of 100 μm^2. Green fluorescence was analyzed

with top-hat background subtraction with a radius of 30 μm and a threshold of 0.3 GCU, edge sensitivity of -59, hole fill cleanup of 10 μm^2, and minimum area filter 30 μm^2.

Synthesis of Monomers

Synthesis of Compound (1)

1 equivalency of acetyl chloride (400 mg, 5.3 mmol) was dissolved at 1.7 M in tetrahydrofuran. Separately, 2 equivalencies of 2,2'-(ethane-1,2-diylbis(oxy))bis(ethane-1-thiol) was dissolved at 150 mM in tetrahydrofuran in the presence of 6 equivalencies of triethylamine and stirred on ice for 15 minutes. The acetyl chloride solution was then added dropwise on ice over one hour. The reaction mixture was removed to room temperature for one hour. Then the reaction was quenched with 50 mL of water and extracted with ethyl acetate (40 mL, 3x). The combined organic layers were dried with sodium sulfate and concentrated under vacuum. Compound **(1)** was purified via silica gel flash chromatography. Solvent A: hexanes. Solvent B: ethyl acetate. Gradient: 0 – 30% solvent B over 40 minutes. Elution: 15% ethyl acetate. Yield: 60%, 740 mg, 3.3 mmol. The product was characterized by ^{1}H NMR.

Synthesis of Compound (2)

1 equivalency of tert-butyl allylcarbamate (106 mg, 0.67 mmol) was dissolved at 1.33 M in methanol. 3 equivalencies of (2R,3R)-1,4-dimercaptobutane-2,3-diol and 0.15 equivalencies of 2,2-dimethoxy-1,2-diphenylethan-1-one were added to this solution. The mixture was irradiated with UV light at 20 mW/cm^2 for 270 seconds. The solvent was removed under vacuum. Compound **(2)** was purified via silica gel flash chromatography. Solvent A: dichloromethane. Solvent B: methanol. Gradient: 0 – 2.5% solvent B over 25 minutes. Yield: 59%, 124 mg, 0.4 mmol. The product was characterized by ^{1}H NMR.

Synthesis of Compound (3)

1 equivalency of 3-bromopropan-1-ol (4470 mg, 32.4 mmol) was dissolved at 950 mM in water. 2 equivalencies of sodium azide were added to this solution. The resulting mixture was heated to near reflux (90°C) and stirred overnight. Then the reaction mixture was extracted with dichloromethane (35 mL, 4x). The combined organic layer was dried with sodium sulfate and the solvent was removed under vacuum. The crude product **(3)** was used without further purification. Yield: 80%, 2630 mg, 26 mmol. The product was characterized by ^{1}H NMR and ^{13}C NMR.

Synthesis of Compound (4)

1 equivalency (1430 mg, 14 mmol) of **(3)** was dissolved at 340 mM in dry chloroform. 2 equivalencies of phosphorus tribromide were added to this solution slowly. The resulting mixture was refluxed at 50°C overnight. Then the reaction mixture was removed from reflux and placed in an ice bath. The reaction was quenched by the slow addition of 125 mL of saturated sodium bicarbonate and extracted with chloroform (50 mL, 4x). The combined organic layers were dried with sodium sulfate and the solvent was removed under vacuum. The crude product **(4)** was used without further purification. Yield: 63%, 1450 mg, 8.9 mmol. The product was characterized by ^{1}H NMR and ^{13}C NMR.

Synthesis of Compound (5)

1 equivalency (1450 mg, 8.9 mmol) of **(4)** was added dropwise to a stirred solution of 10 equivalencies of prop-2-en-1-amine and 1.1 equivalencies of potassium carbonate. The mixture was reacted at room temperature overnight. The mixture was then filtered through celite and recovered by washing with a copious amount of dichloromethane. The filtered product was collected and concentrated under vacuum. The crude product **(5)** was used without further purification. Yield: 78%, 980 mg, 7 mmol. The product was characterized by ^{1}H NMR.

Synthesis of Compound (6)

1 equivalency of **(5)** (500 mg, 3.6 mmol) was dissolved at 214 mM in dichloromethane. Triethylamine (1.1 equivalencies) was added to the solution. The resulting mixture was stirred on

ice for 5 minutes. Separately, 1.1 equivalency of di-tert-butyl dicarbonate was dissolved at 550 mM in dichloromethane. The di-tert-butyl dicarbonate solution was added to the stirred solution over 5 minutes. The mixture was reacted on ice for 1 hour. The mixture was then removed to room temperature and react at room temperature overnight. The reaction was quenched with 100 mL of saturated sodium bicarbonate and extracted with dichloromethane (80 mL, 4x). The combined organic layers were dried with sodium sulfate and concentrated under vacuum. The crude product **(6)** was used without further purification or characterization. Yield: 90%, 770 mg, 3.2 mmol.

Synthesis of Compound (7)
1 equivalency of **(6)** (494 mg, 2.1 mmol) was dissolved at 1 M in methanol. 3 equivalencies of 2,2'-(ethane-1,2-diylbis(oxy))bis(ethane-1-thiol) and 0.6 equivalencies of 2,2-dimethoxy-1,2-diphenylethan-1-one were added to this solution. The mixture was irradiated with UV light at 20 mW/cm^2 for 270 seconds. The solvent was removed under vacuum. Compound **(7)** was purified via silica gel flash chromatography. Solvent A: hexanes. Solvent B: ethyl acetate. Gradient: 0 – 50% solvent B over 30 minutes. Elution: 33% ethyl acetate. Yield: 31%, 580 mg, 0.64 mmol. The product was characterized by ^1H NMR and LC-MS (calculated: 445.20, observed: 445.10 $[M+Na]^+$).

Synthesis of Compound (8)
1 equivalency of 13-bromo-2,5,8,11-tetraoxatridecane (1 g, 3.7 mmol) was added dropwise to a stirred solution of 10 equivalencies of prop-2-en-1-amine and 1.1 equivalencies of potassium carbonate. The mixture was reacted at room temperature overnight. The mixture was then filtered through celite and recovered by washing with a copious amount of dichloromethane. The filtered product was collected and concentrated under vacuum. The crude product **(8)** was used without further purification. Yield: 93%, 940 mg, 3.8 mmol. The product was characterized by ^1H NMR.

Synthesis of Compound (9)
1 equivalency of **(8)** (940 mg, 3.8 mmol) was dissolved at 214 mM in dichloromethane. Triethylamine (1.1 equivalencies) was added to the solution. The resulting mixture was stirred on ice for 5 minutes. Separately, 1.1 equivalency of di-tert-butyl dicarbonate was dissolved at 550 mM in dichloromethane. The di-tert-butyl dicarbonate solution was added to the stirred solution over 5 minutes. The mixture was reacted on ice for 1 hour. The mixture was then removed to room temperature and react at room temperature overnight. The reaction was quenched with 150 mL of saturated sodium bicarbonate and extracted with dichloromethane (50 mL, 3x). The combined organic layers were dried with sodium sulfate and concentrated under vacuum. The crude product **(9)** was used without further purification. Yield: 95%, 1250 mg, 3.6 mmol. The product was characterized by ^1H NMR.

Synthesis of Compound (10)
1 equivalency of **(9)** (660 mg, 1.9 mmol) was dissolved at 1 M in methanol. 3 equivalencies of 2,2'-(ethane-1,2-diylbis(oxy))bis(ethane-1-thiol) and 0.6 equivalencies of 2,2-dimethoxy-1,2-diphenylethan-1-one were added to this solution. The mixture was irradiated with UV light at 20 mW/cm^2 for 270 seconds. The solvent was removed under vacuum. Compound **(10)** was purified via silica gel flash chromatography. Solvent A: hexanes. Solvent B: ethyl acetate. Gradient: 0 – 100% solvent B over 30 minutes. Elution: 80% ethyl acetate. Yield: 58%, 580 mg, 1.1 mmol. The product was characterized by ^1H NMR and LC-MS (calculated: 552.27, observed: 552.20 $[M+Na]^+$).

Synthesis of Compound (11)
1 equivalency of tert-butyl 2-bromoacetate (1 g, 5.2 mmol) was added dropwise to a stirred solution of 10 equivalencies of prop-2-en-1-amine and 1.1 equivalencies of potassium carbonate. The mixture was reacted at room temperature overnight. The mixture was then filtered through celite and recovered by washing with a copious amount of dichloromethane. The filtered product was collected and concentrated under vacuum. The crude product **(11)** was used without further purification. Yield: 95%, 840 mg, 4.9 mmol. The product was characterized by ^{1}H NMR.

Synthesis of Compound (12)
1 equivalency of **(11)** (920 mg, 5.4 mmol) was dissolved at 214 mM in dichloromethane. Triethylamine (1.1 equivalencies) was added to the solution. The resulting mixture was stirred on ice for 5 minutes. Separately, 1.1 equivalency of di-tert-butyl dicarbonate was dissolved at 550 mM in dichloromethane. The di-tert-butyl dicarbonate solution was added to the stirred solution over 5 minutes. The mixture was reacted on ice for 1 hour. The mixture was then removed to room temperature and react at room temperature overnight. The reaction was quenched with 150 mL of saturated sodium bicarbonate and extracted with dichloromethane (50 mL, 3x). The combined organic layers were dried with sodium sulfate and concentrated under vacuum. The crude product **(12)** was used without further purification. Yield: 97%, 1430 mg, 5.3 mmol. The product was characterized by ^{1}H NMR.

Synthesis of Compound (13)
1 equivalency of **(12)** (800 mg, 3 mmol) was dissolved at 1 M in methanol. 3 equivalencies of 2,2'-(ethane-1,2-diylbis(oxy))bis(ethane-1-thiol) and 0.6 equivalencies of 2,2-dimethoxy-1,2-diphenylethan-1-one were added to this solution. The mixture was irradiated with UV light at 20 mW/cm^2 for 270 seconds. The solvent was removed under vacuum. Compound **(13)** was purified via silica gel flash chromatography. Solvent A: hexanes. Solvent B: ethyl acetate. Gradient: 0 – 30% solvent B over 30 minutes. Elution: 25% ethyl acetate. Yield: 48%, 640 mg, 1.4 mmol. The product was characterized by ^{1}H NMR and LC-MS (calculated: 476.22, observed: 476.20 $[M+Na]^{+}$).

Synthesis of Compound (14)
1 equivalency (375 mg, 2.7 mmol) of **(5)** was dissolved at 214 mM in dichloromethane. 1.2 equivalencies of triethylamine were added to this solution and stirred on ice for 5 minutes. Separately, 1.2 equivalencies of acryloyl chloride were dissolved at 750 mM in dichloromethane. The acryloyl chloride solution was added dropwise over 1 hour on ice. The reaction was then removed to room temperature for 1 hour. The reaction was quenched with 10 mL of water and extracted with dichloromethane (50 mL, 3x). The combined organic layers were dried with sodium sulfate and the solvent was removed under vacuum. The crude product **(14)** was purified via silica gel flash chromatography. Solvent A: hexanes. Solvent B: ethyl acetate. Gradient: 0 – 100% solvent B over 30 minutes. Elution: 33% ethyl acetate. Yield: 30%, 156 mg, 0.80 mmol. The product was characterized by ^{1}H NMR.

Synthesis of Compound (15)
1 equivalency (890 mg, 3.6 mmol) of **(8)** was dissolved at 214 mM in dichloromethane. 1.2 equivalencies of triethylamine were added to this solution and stirred on ice for 5 minutes. Separately, 1.2 equivalencies of acryloyl chloride were dissolved at 750 mM in dichloromethane. The acryloyl chloride solution was added dropwise over 1 hour on ice. The reaction was then removed to room temperature for 1 hour. The reaction was quenched with 100 mL of water and extracted with dichloromethane (50 mL, 3x). The combined organic layers were dried with sodium sulfate and the solvent was removed under vacuum. The crude product **(15)** was purified via silica gel flash chromatography. Solvent A: dichloromethane. Solvent B: methanol. Gradient: 0 – 5%

solvent B over 15 minutes. Elution: 2.5% methanol. Yield: 79%, 860 mg, 2.86 mmol. The product was characterized by ^{1}H NMR.

Synthesis of Compound (16)
1 equivalency (890 mg, 5.2 mmol) of **(11)** was dissolved at 214 mM in dichloromethane. 1.2 equivalencies of triethylamine were added to this solution and stirred on ice for 5 minutes. Separately, 1.2 equivalencies of acryloyl chloride were dissolved at 750 mM in dichloromethane. The acryloyl chloride solution was added dropwise over 1 hour on ice. The reaction was then removed to room temperature for 1 hour. The reaction was quenched with 100 mL of water and extracted with dichloromethane (50 mL, 3x). The combined organic layers were dried with sodium sulfate and the solvent was removed under vacuum. The crude product **(16)** was purified via silica gel flash chromatography. Solvent A: hexanes. Solvent B: ethyl acetate. Gradient: 0 – 50% solvent B over 30 minutes. Elution: 25% ethyl acetate. Yield: 72%, 848 mg, 3.8 mmol. The product was characterized by ^{1}H NMR.

Synthesis of Compound (17)
P-toluenesulfonyl chloride (3.5 g, 18.4 mmol) was dissolved at 175 mM in dichloromethane. Separately, 4 equivalencies of triethylene glycol (11 g, 73 mmol) were 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 (DMAP, 46 mg, 0.3 mmol), and the mixture was allowed to equilibrate for 10 minutes on ice. The solution of p-toluenesulfonyl chloride in dichloromethane 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 dichloromethane layer was collected and concentrated under vacuum in 98% yield. The crude product was used without further purification. The product **(17)** was characterized by ^{1}H NMR and LC-MS (calculated: 305.20, observed: 305.09 $[M+H]^{+}$).

Synthesis of Compound (18)
Compound **(17)** was dissolved at 739 mM in dry dimethylformamide. 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 and the residue was resuspended in diethyl ether and filtered through celite. The ether was collected and concentrated under vacuum in 96% yield. The crude product was used without further purification. The product **(18)** was characterized by ^{1}H NMR.

Synthesis of Compound (19)
Compound **(18)** (1 g, 5.8 mmol) was dissolved at 342 mM in anhydrous chloroform. To this solution was added 2 equivalencies of phosphorus tribromide (3.1 g, 12 mmol) over 5 minutes. The mixture was then 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 chloroform layer was collected and concentrated under vacuum in 30% yield. The crude product was used without further purification. The product **(19)** was characterized by ^{1}H NMR and ^{13}C NMR.

Synthesis of *Compound (20)*
1 equivalency of compound **(19)** (888 mg, 3.73 mmol) was added to 1.2 equivalencies of potassium carbonate (619 mg, 4.48 mmol) and 10 equivalencies allylamine (2.1 g, 37.3 mmol). The mixture was allowed to react overnight at room temperature. The reaction was then filtered through celite and concentrated under vacuum in 85% recovery. The crude product was used without further purification. The product **(20)** was characterized by ^{1}H NMR.

Synthesis of Compound (21)

1 equivalency of compound **(20)** (668 mg, 3.1 mmol) was dissolved at 151 mM in dichloromethane. To the solution was added 1.1 equivalencies of triethylamine (347 mg, 3.4 mmol), and the mixture was allowed to equilibrate for 10 minutes on ice. Next, 1.3 equivalencies acryloyl chloride (367 mg, 4.1 mmol) dissolved at 1.66 M in dichloromethane was added dropwise for 1 hour. The final concentration of compound **(21)** was 132 mM. The mixture was then removed from the ice and reacted at room temperature for 1 hour. The reaction was quenched with 6 mL of water and extracted with dichloromethane (80 mL, 3x). The dichloromethane layer was collected and concentrated under vacuum. The product was purified by flash chromatography (12 g silica, 0-5% methanol in dichloromethane) and recovered in 30% yield. The product **(21)** was characterized by ^{1}H NMR and LC-MS (calculated: 269.16, observed: 269.20 $[M+H]^{+}$).

Synthesis of Multifunctional Cross-linkers
Synthesis of Compounds (22 – 27)
Step 1: 1 equivalency of thiol-containing starting material **(compound 7, 10, 13)** was dissolved at 500 mM in acetonitrile. 1.1 equivalency of *N*-allylacrylamide monomer **(compound 14, 15, or 16)** and 0.1 equivalency of DBU were added. This mixture was reacted overnight at room temperature and then dried under vacuum. The crude product was then suspended at 1 M in methanol. 3 equivalencies of compound **(1)** and 0.6 equivalencies of 2,2-dimethoxy-1,2-diphenylethan-1-one were added to this solution. The mixture was irradiated with UV light at 20 mW/cm^2 for 270 seconds 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 followed by 5 minutes of isocratic 95% solvent B and characterized via LC-MS.

Compound **(22):** eluted at 30.4 minutes. Calculated 894.39, Observed 894.20 $[M+Na]^+$
Compound **(23):** eluted at 27.0 minutes. Calculated 970.45, Observed 970.30 $[M+Na]^+$
Compound **(24):** eluted at 28.6 minutes. Calculated 1001.46, Observed 1001.30 $[M+Na]^+$

Step 2: Removal of the thioester protecting group was achieved by dissolving 1 equivalency of step 1 product **(compound 22, 23, or 24)** at 175 mM in methanol containing 7 M ammonia. Deprotection was performed for 1 hour at room temperature. After deprotection, methanol and ammonia were removed under vacuum. The deprotected product was then dissolved at 500 mM in acetonitrile. 1.1 equivalency of N-allylacrylamide monomer **(compound 14, 15, or 16)** and 0.1 equivalencies of DBU were added. The mixture was reacted overnight at room temperature and then dried under vacuum. The crude product was then suspended at 1 M in methanol. 3 equivalencies of 2,2'-(ethane-1,2-diylbis(oxy))bis(ethane-1-thiol) and 0.6 equivalencies of 2,2-dimethoxy-1,2-diphenylethan-1-one were added to this solution. The mixture was irradiated with UV light at 20 mW/cm^2 for 270 seconds 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 followed by 5 minutes of isocratic 95% solvent B and characterized via ^{1}H NMR and LC-MS.

Linker 1, Compound **(25):** eluted at 30.2 minutes. Calculated 1335.61, Observed 1335.40 $[M+Na]^+$
Linker 2, Compound **(26):** eluted at 29.2 minutes. Calculated 1335.61, Observed 1335.50 $[M+Na]^+$
Linker 3, Compound **(27):** eluted at 30.0 minutes. Calculated 1335.61, Observed 1335.40 $[M+Na]^+$

Synthesis of Compound (28)
1 equivalency of compound **(2)** was dissolved at 500 mM in acetonitrile. 1.1 equivalency of compound **(21)** and 0.1 equivalency of DBU were added. This mixture was reacted overnight at room temperature and then dried under vacuum. The crude product was then suspended at 1 M in methanol. 3 equivalencies of compound **(1)** and 0.6 equivalencies of 2,2-dimethoxy-1,2-diphenylethan-1-one were added to this solution. The mixture was irradiated with UV light at 20 mW/cm^2 for 270 seconds and then purified via semi-preparative RP-HPLC. The product **(28, linker 4)** was characterized via LC-MS (calculated: 826.33, observed: 826.16 $[M+Na]^+$)

Synthesis of Compound (29)
1 equivalency of compound **(2)** was dissolved at 500 mM in acetonitrile. 1.1 equivalency of compound **(21)** and 0.1 equivalency of DBU were added. This mixture was reacted overnight at room temperature and then dried under vacuum. The crude product was then suspended at 1 M in methanol. 3 equivalencies of 2-mercaptoacetic acid and 0.6 equivalencies of 2,2-dimethoxy-1,2-diphenylethan-1-one were added to this solution. The mixture was irradiated with UV light at 20 mW/cm^2 for 270 seconds and then purified via semi-preparative RP-HPLC. The product **(29, linker 5)** was characterized via LC-MS (Calculated 694.27, Observed 694.10 $[M+Na]^+$).

Synthesis of Fluorophore- and Quencher-modified Cross-linkers

Synthesis of Compound (30)

Compound **(30)** was synthesized as previously described.[1]

Synthesis of Compound (31)

1 equivalency of tert-butyl N-(2-mercaptoethyl)carbamate was mixed with 1 equivalency of 2,2'-dithiodipyridine and 2 equivalencies of triethylamine. The final concentration of tert-butyl N-(2-mercaptoethyl)carbamate was 500 mM in dimethyl sulfoxide. The mixture was reacted overnight at room temperature and purified via semi-preparative RP-HPLC. The compound was eluted at a flow rate of 4 mL/min with a linear gradient of 5% to 95% solvent B (0-15 minutes), then 95% solvent B (15-20 min) and equilibrated back to 5% solvent B (20-30 minutes). The product eluted at 12.4 minutes. The product was dissolved at 50 mM in 50% trifluoroacetic acid in dichloromethane for 1 hour. The trifluoroacetic acid and dichloromethane were then removed under vacuum to yield compound **(31)**, which was characterized via LC-MS (calculated: 187.03, observed: 187.02 $[M+H]^+$)

Synthesis of Compound (32)

1 equivalency of chloro-2,4-dinitrobenzene was dissolved at 100 mg/ml in dimethyl sulfoxide. To this solution was added 1.5 equivalencies of compound **(31)** (50 mg/ml in dimethyl sulfoxide) and 4 equivalencies of triethylamine. The final concentration of chloro-2,4-dinitrobenzene was 105 mM. The mixture was reacted overnight at room temperature and then purified via semi-preparative RP-HPLC. The product was eluted at a flow rate of 4 mL/min with a linear gradient of 5% to 95% solvent B (0-45 minutes), then 95% solvent B (45-55 min) and equilibrated back to 5% solvent B (55-60 minutes). The product eluted at 31.5 minutes in 86% yield. The product **(32)** was characterized by LC-MS (calculated: 353.04, observed: 353.00 $[M+H]^+$).

Synthesis of Compound (33)

1 equivalent of Fmoc-Val-Cit-PAB-PNP ester was dissolved at 10 mg/ml in dry dimethyl sulfoxide. To this solution, 2.2 equivalencies of N1-(2,4-dinitro-phenyl)-ethane-1,2-diamine (10 mg/ml in dry dimethyl sulfoxide) and 10 equivalencies of triethylamine were added. The final concentration of Fmoc-Val-Cit-PAB-PNP ester was 7.8 mM. The mixture was reacted for 6 hours at room temperature after which 10% v/v piperidine was added and the resulting mixture was reacted overnight at room temperature. The reaction was purified via semi-preparative RP-HPLC, and the product **(33)** was characterized by LC-MS (calculated: 632.28, observed: 632.29 $[M+H]^+$).

Synthesis of Compound (34)

Compound **(28)** was dissolved at 50 mM in 50% trifluoroacetic acid in dichloromethane for 1 hour. The trifluoroacetic acid and dichloromethane were then removed under vacuum to yield the Boc deprotected product. Removal of the thioester protecting group was achieved by dissolving 1 equivalency Boc deprotected product at 175 mM in methanol containing 7 M ammonia. Deacetylation was performed for 1 hour at room temperature. After deacetylation, the methanol and ammonia were removed under vacuum. 1 equivalency of deacetylated compound **(28)** was dissolved at 10 mg/ml in dimethyl sulfoxide. To this solution was added 5 equivalencies of compound **(32)** (39 mg/ml in dimethyl sulfoxide) and 10 equivalencies of triethylamine. The final concentration of compound **(28)** was 9.7 mM. The mixture was reacted overnight at room temperature and purified via semi-preparative RP-HPLC. The product eluted at 19.6 minutes. The product **(34)** was characterized by LC-MS (calculated: 903.29, observed: 903.10 $[M+H]^+$).

Synthesis of Compound (35)

1 equivalent of compound **(34)** was dissolved at 7.1 mg/ml in dimethyl sulfoxide. To this solution was added 2.5 equivalencies of Compound **(30)** (6 mg/ml in dimethyl sulfoxide) and 20

equivalencies of triethylamine. The final concentration of compound **(34)** was 5.2 mM. The mixture was reacted overnight at room temperature and then purified via semi-preparative RP-HPLC. The compound was eluted at a flow rate of 4 mL/min with a linear gradient of 5% to 95% solvent B (0-45 minutes), then 95% solvent B (45-55 min) and equilibrated back to 5% solvent B (55-60 minutes). The product eluted at 35.7 minutes. The product **(35)** was characterized by LC-MS (calculated: 1177.40, observed: 1177.20 $[M+H]^+$).

Synthesis of Compound (36)
Compound **(29)** was dissolved at 50 mM in 50% trifluoroacetic acid in dichloromethane for 1 hour. The trifluoroacetic acid and dichloromethane were then removed under vacuum to yield the Boc deprotected product. 1 equivalent of Boc deprotected compound **(29)** was dissolved at 10 mg/ml in dry dimethyl sulfoxide. To this solution, 1.5 equivalencies of **(30)** (6 mg/ml in dry dimethyl sulfoxide) and 15 equivalencies of triethylamine were added. The final concentration of **(36)** was 6.4 mM. The mixture was reacted overnight at room temperature and then purified via semi-preparative RP-HPLC. The product **(36)** was characterized by LC-MS (calculated: 826.33, observed: 826.30 $[M-F]^+$).

Synthesis of Compound (37)
1 equivalent of compound **(36)** was dissolved at 23 mg/ml in dry dimethyl sulfoxide. To this solution, 2.5 equivalencies of **(33)**, 4 equivalencies of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 4 equiv of N-hydroxysuccinimide, and 30 equivalencies of triethylamine were added. The final concentration of compound **(36)** was 5.1 mM. The mixture was reacted overnight at room temperature and then purified via semi-preparative RP-HPLC. The product **(37)** was characterized by LC-MS (calculated: 1459.60, observed: 1459.40 $[M+H]^+$).

Synthesis of Compound (38)
Compound **(28)** was dissolved at 50 mM in 50% trifluoroacetic acid in dichloromethane for 1 hour. The trifluoroacetic acid and dichloromethane were then removed under vacuum to yield the Boc deprotected product. Removal of the thioester protecting group was achieved by dissolving 1 equivalency Boc deprotected product at 175 mM in methanol containing 7 M ammonia. Deacetylation was performed for 1 hour at room temperature. After deacetylation, the methanol and ammonia were removed under vacuum. 1 equivalency of deacetylated compound **(28)** was dissolved at 10 mg/ml in dimethyl sulfoxide. To this solution was added 1.5 equivalencies of 1-2-((2,4-dinitrophenyl)amino)ethyl)-1H-pyrrole-2,5-dione (16.7 mg/ml in dimethyl sulfoxide). The final concentration of deacetylated compound **(28)** was 3.1 mM in 21% (v/v) phosphate-buffered saline (PBS) in dimethyl sulfoxide. The mixture was reacted overnight at room temperature and purified via semi-preparative RP-HPLC. The compound was eluted at a flow rate of 4 mL/min with a linear gradient of 5% to 95% solvent B (0-45 minutes), then 95% solvent B (45-55 min) and equilibrated back to 5% solvent B (55-60 minutes). The product eluted at 24.5 minutes. The product **(38)** was characterized by LC-MS (calculated: 968.34, observed: 968.10 $[M+H]^+$).

Synthesis of Compound (39)
1 equivalent of compound **(38)** was dissolved at 10.5 mg/ml in dimethyl sulfoxide and mixed with 2.5 equivalencies of compound **(30)** (6 mg/ml in dimethyl sulfoxide) and 20 equivalencies of triethylamine. The final concentration of compound **(38)** was 3.76 mM. The mixture was reacted overnight at room temperature and purified via semi-preparative RP-HPLC. The compound was eluted at a flow rate of 4 mL/min with a linear gradient of 5% to 95% solvent B (0-45 minutes), then 95% solvent B (45-55 min) and equilibrated back to 5% solvent B (55-60 minutes). The product eluted at 41 minutes. The product **(39)** was characterized by LC-MS (calculated: 1242.45, observed: 1242.00 $[M+H]^+$).

Results and Discussion
Synthesis of Monomers
Synthesis of Compound (1)

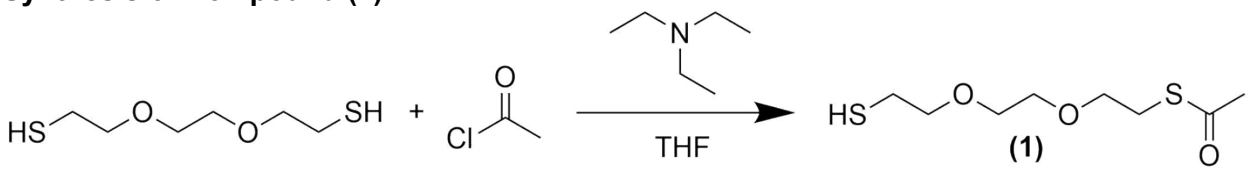


Figure S1. Synthesis scheme for compound **(1)**.

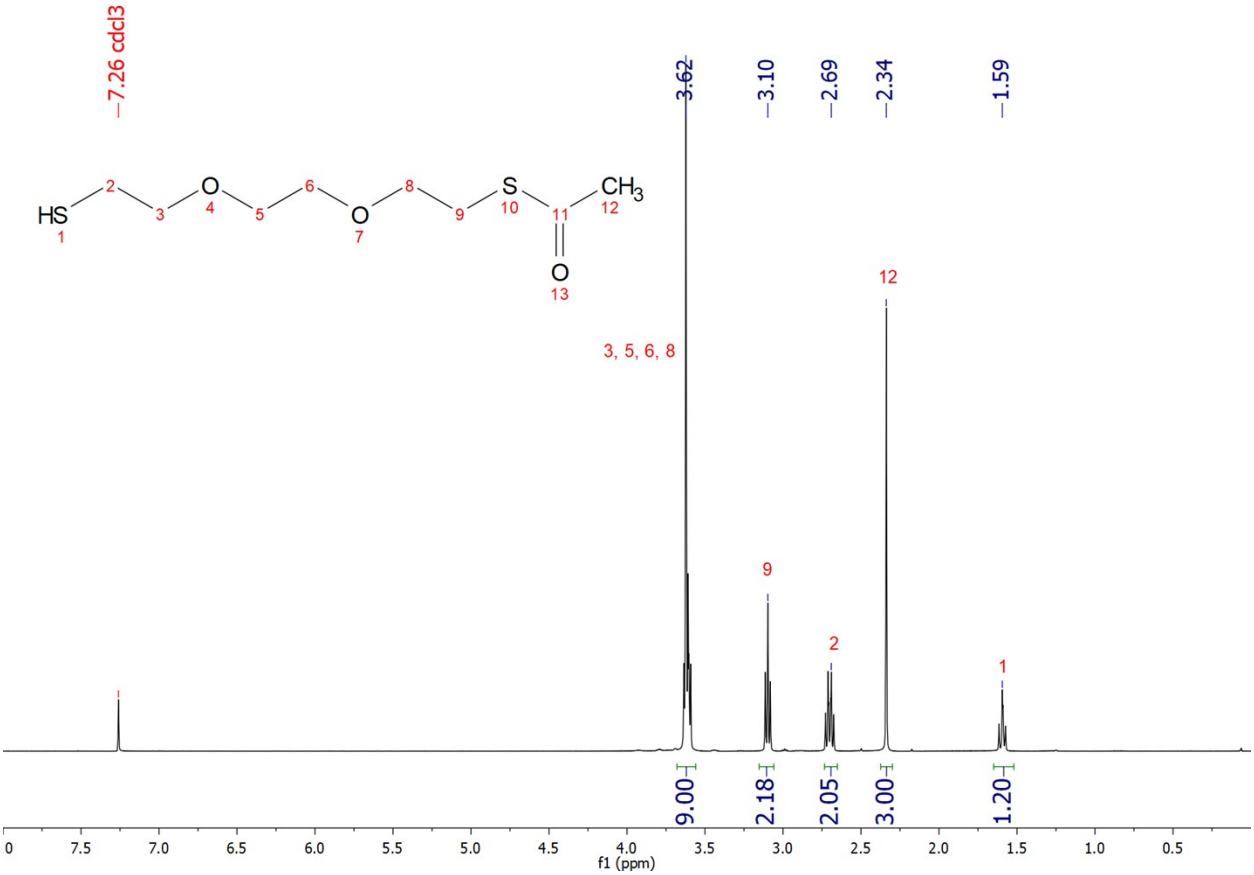


Figure S2. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **(1)**.

Synthesis of Compound (2)

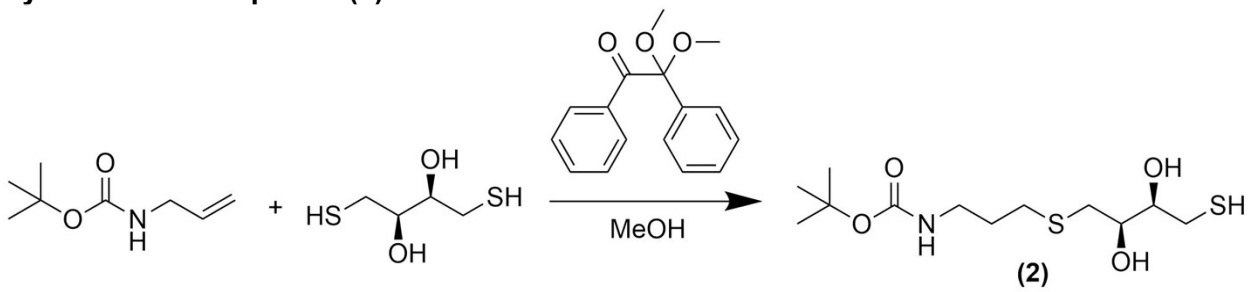


Figure S3. Synthesis scheme for compound **(2)**

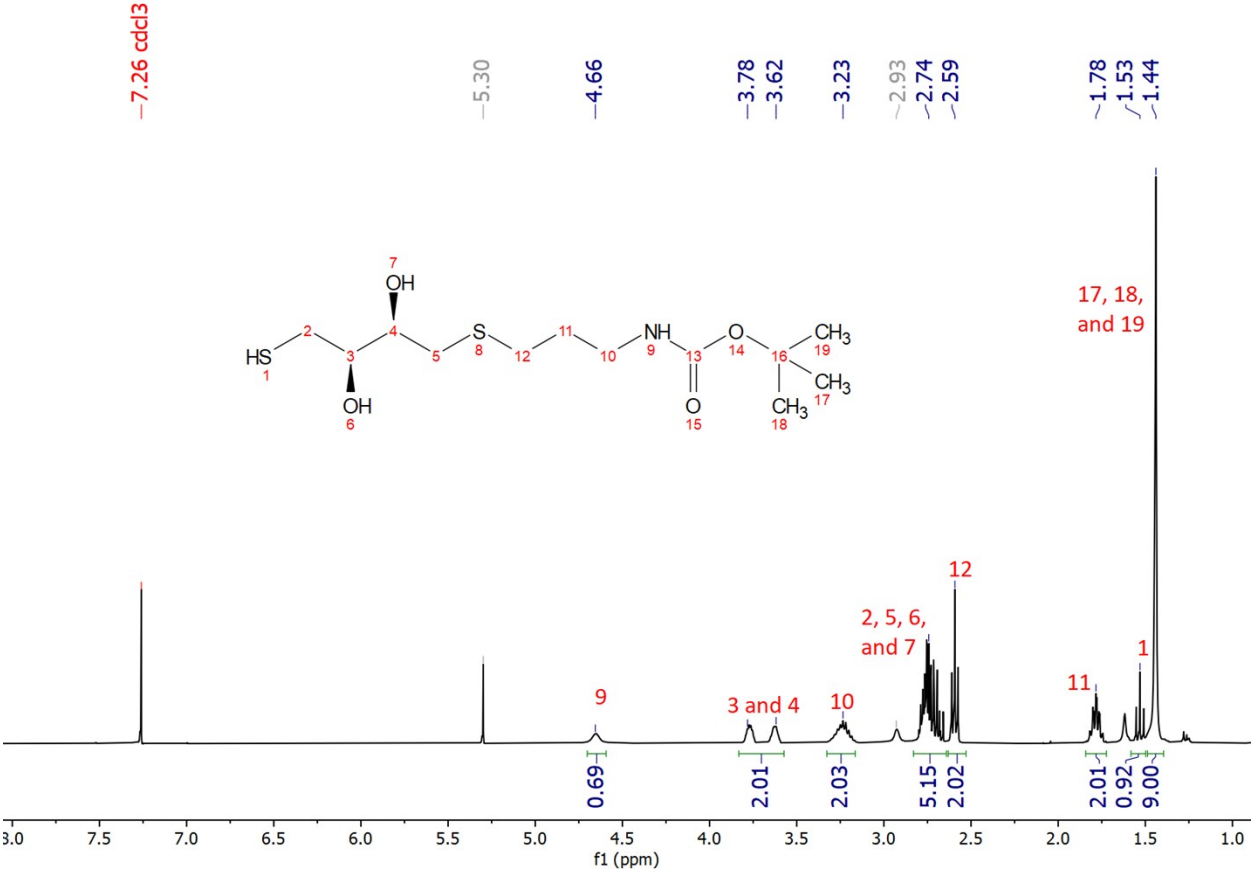


Figure S4. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **(2)**.

Synthesis of Compound (3)

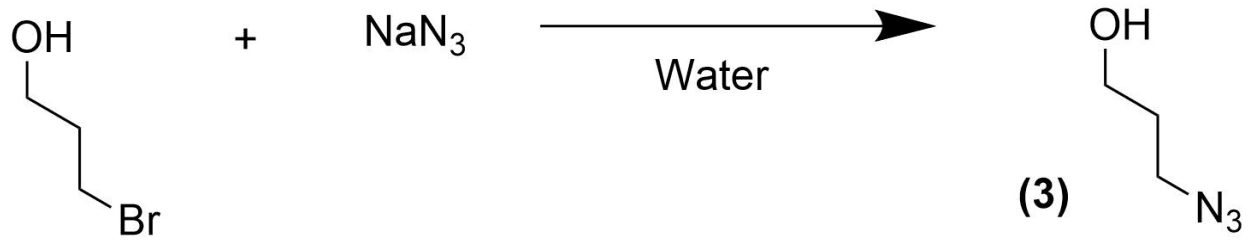


Figure S5. Synthesis scheme for compound **(3)**.

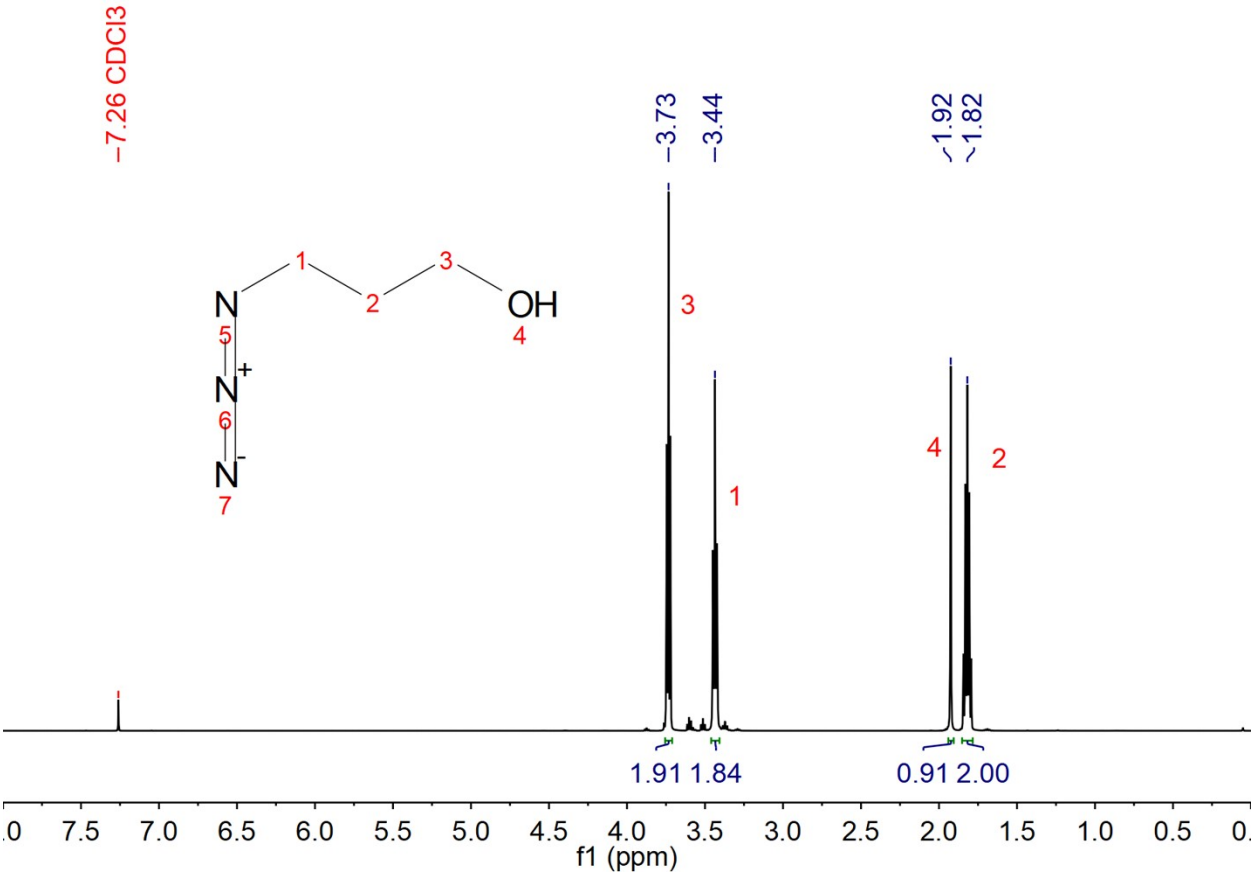


Figure S6. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(3)**.

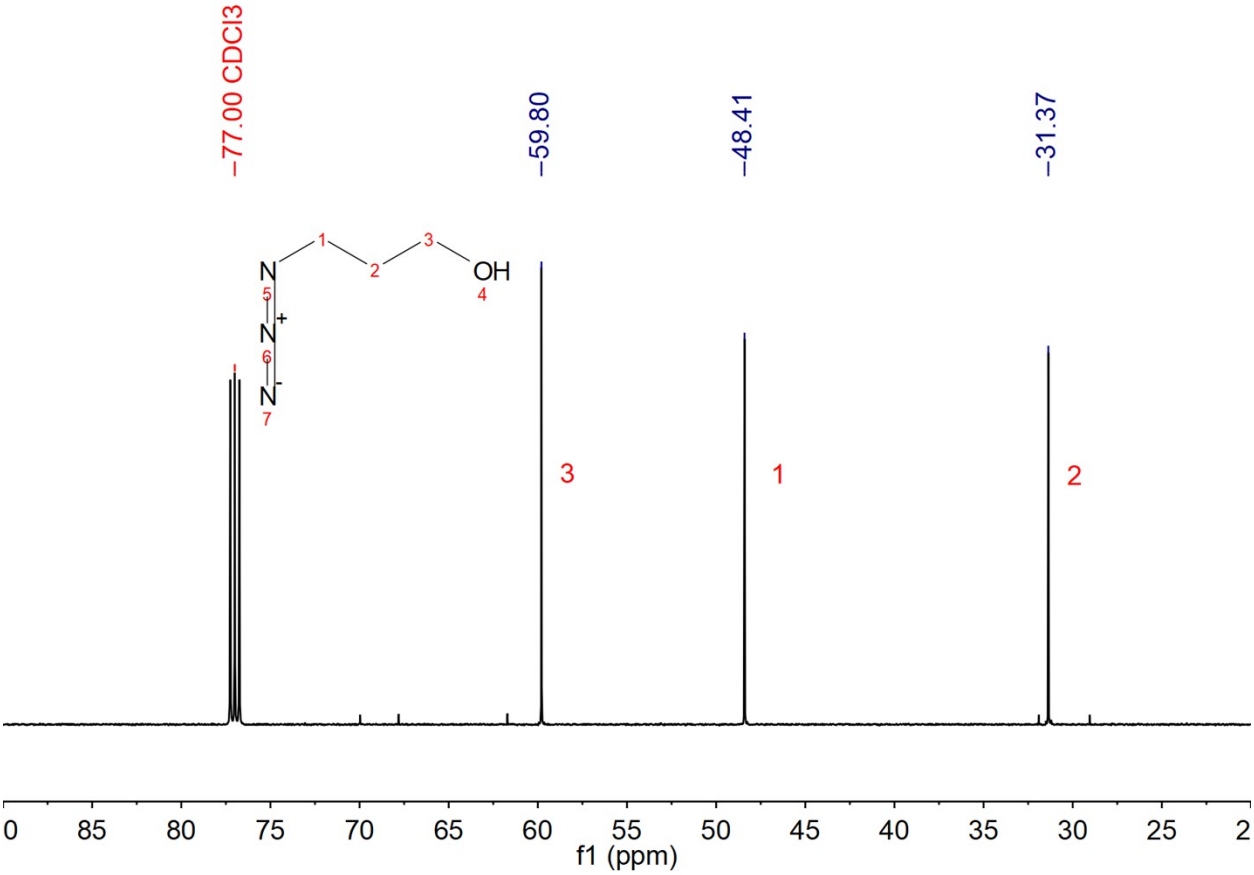


Figure S7. ^{13}C NMR (500 MHz, $CDCl_3$) of compound **(3)**.

Synthesis of Compound (4)

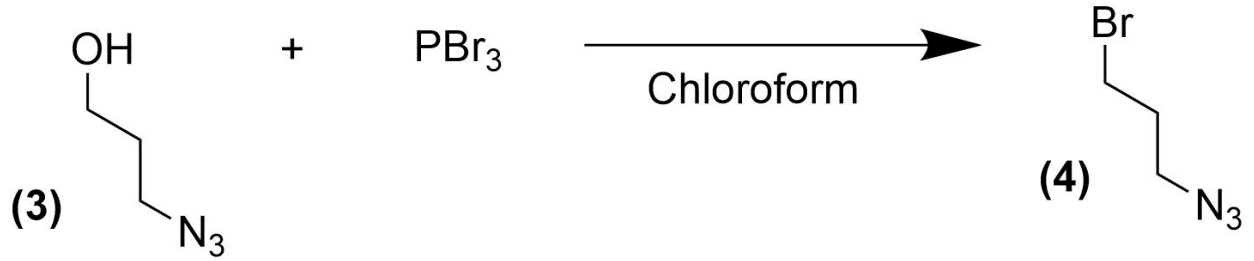


Figure S8. Synthesis scheme for compound **(4)**.

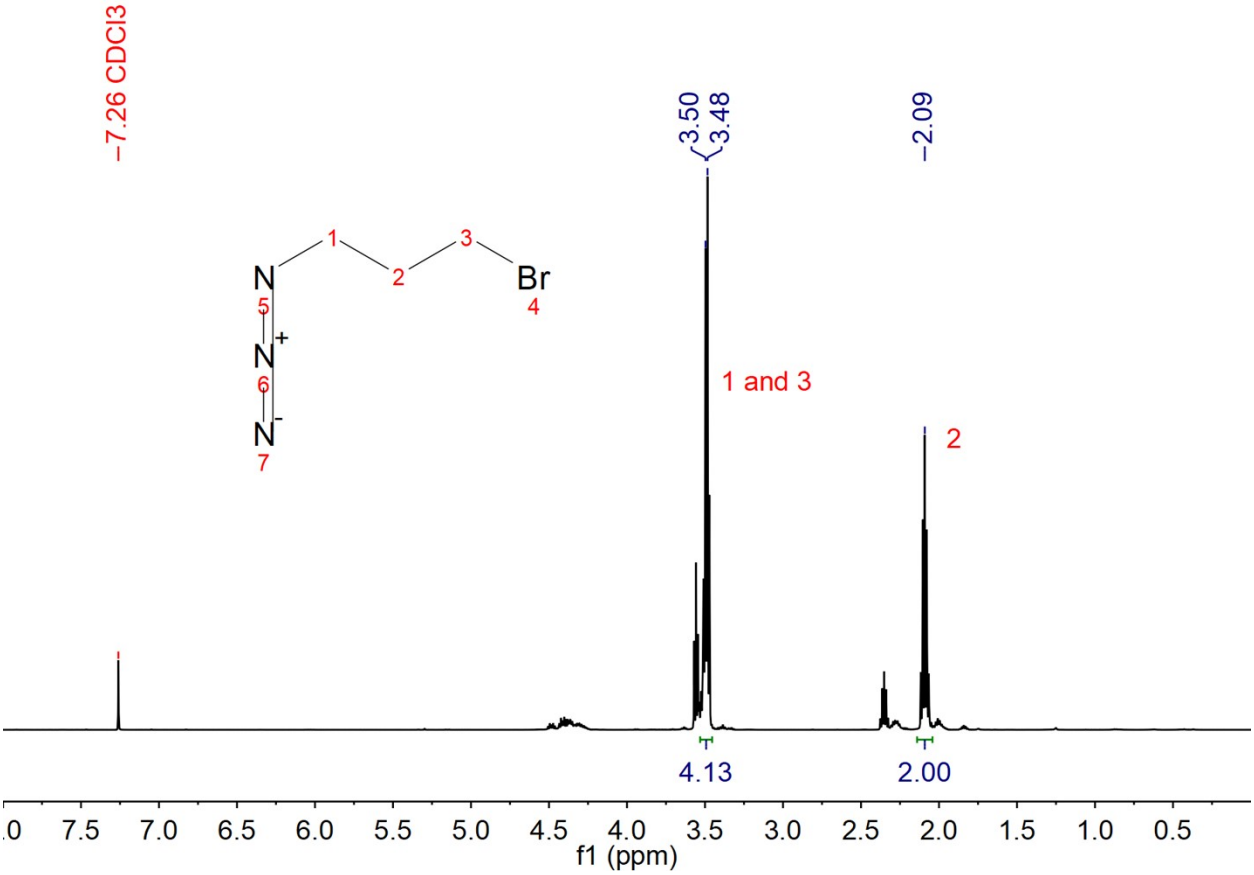


Figure S9. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(4)**.

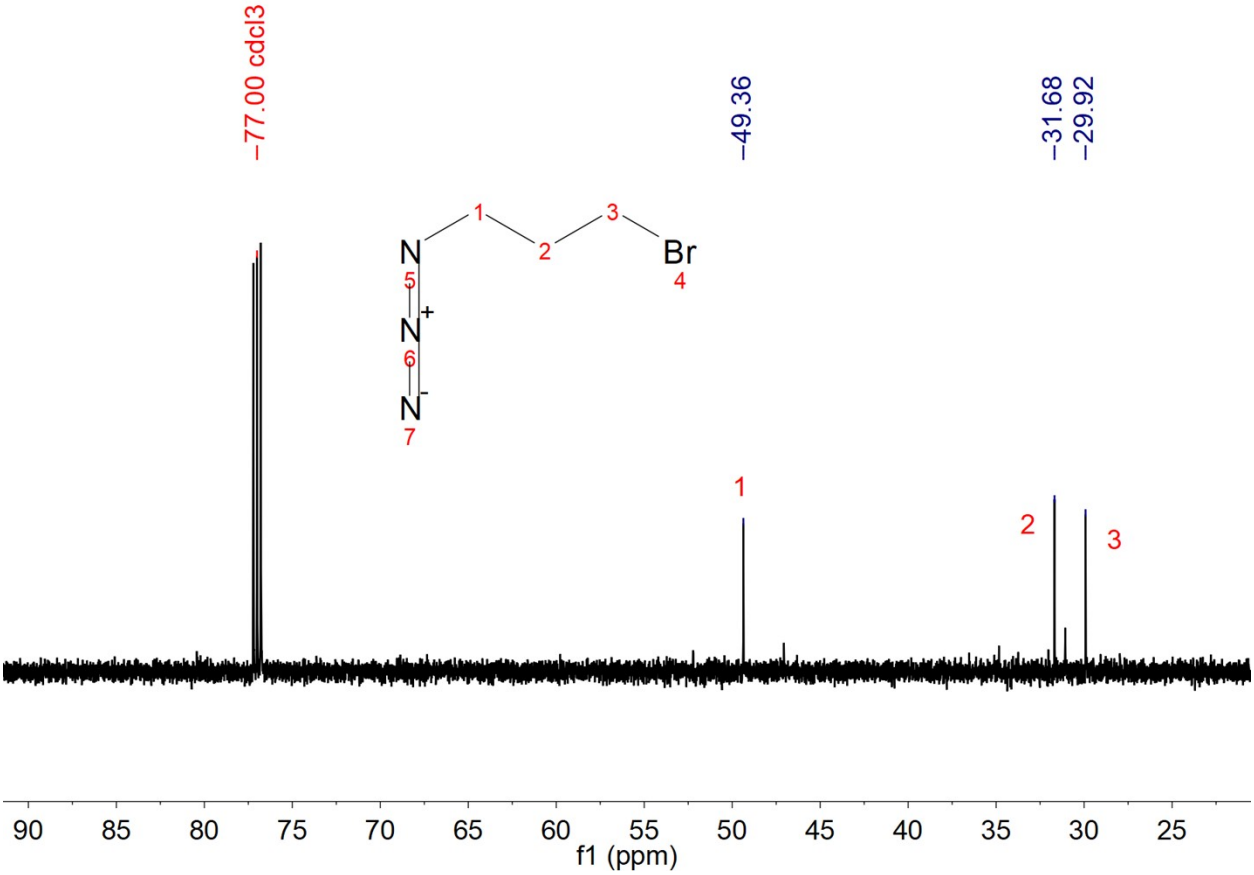


Figure S10. ^{13}C NMR (500 MHz, $CDCl_3$) of compound **(4)**.

Synthesis of Compound (5)

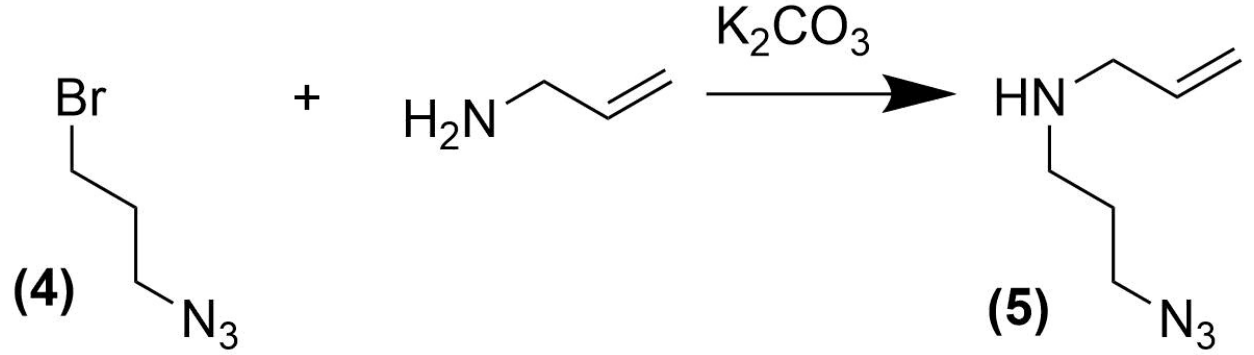


Figure S11. Synthesis scheme for compound **(5)**.

Synthesis of Compound (6)

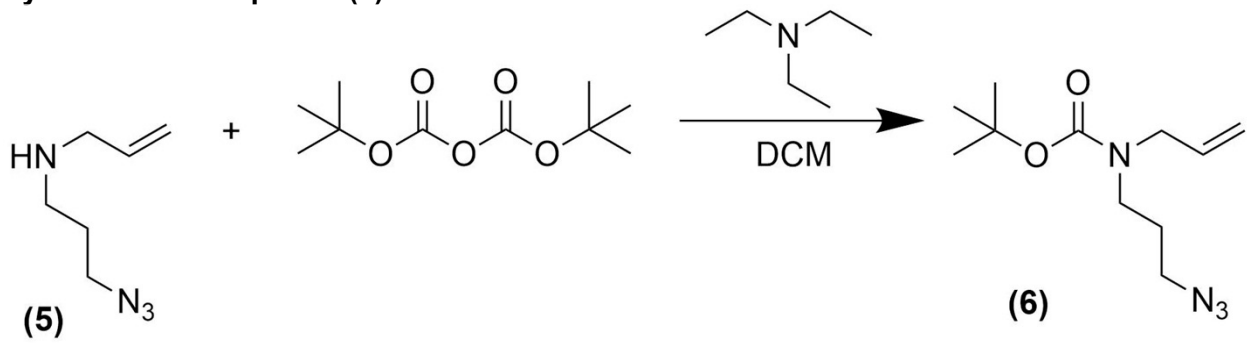


Figure S12. Synthesis scheme for compound **(6)**.

Synthesis of Compound (7)

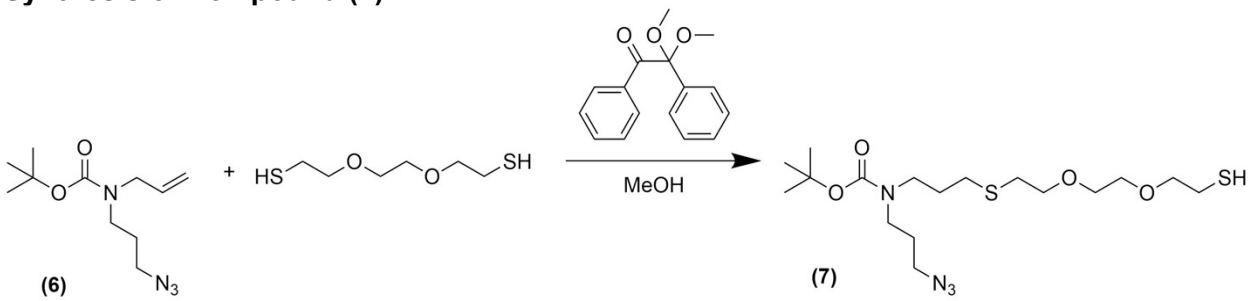


Figure S13. Synthesis scheme for compound **(7)**.

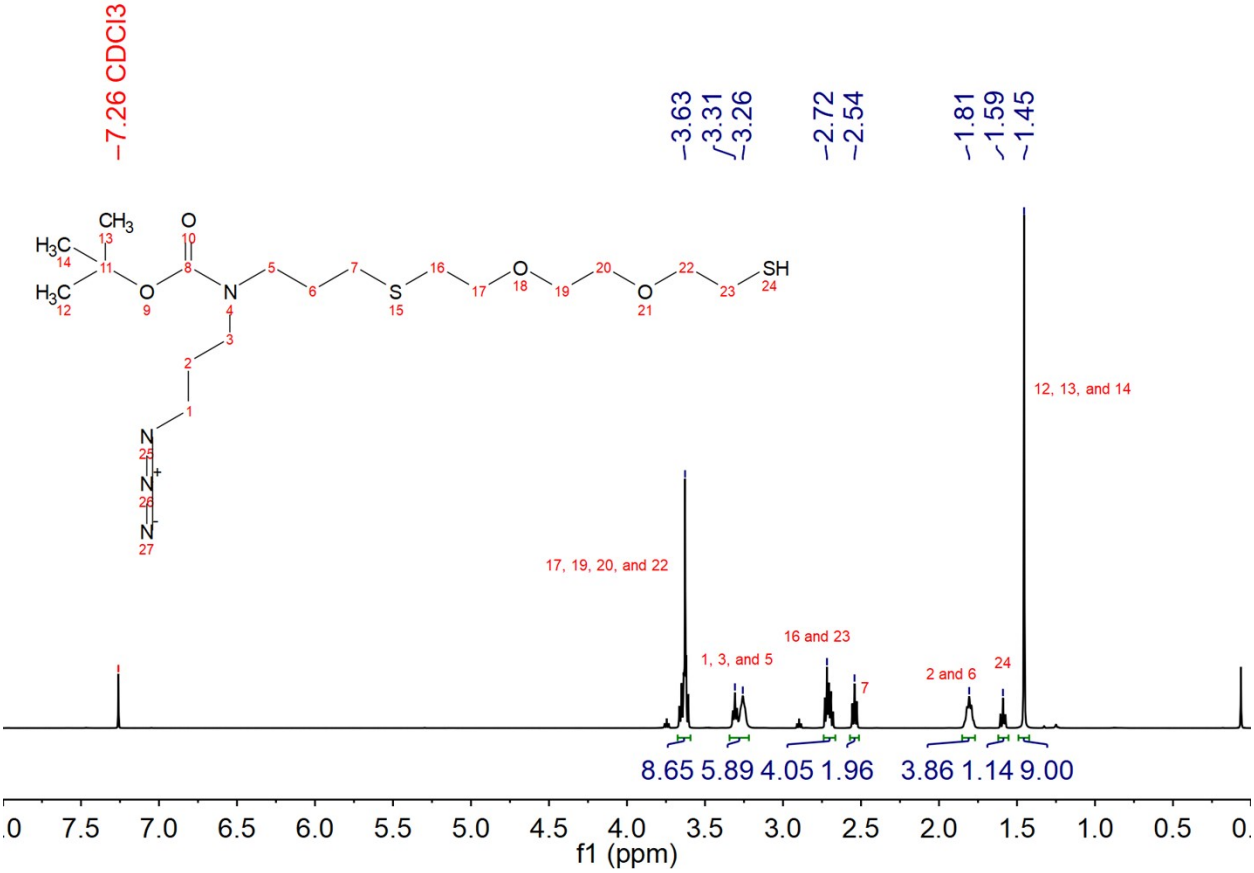


Figure S14. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(7)**.

Synthesis of Compound (8)

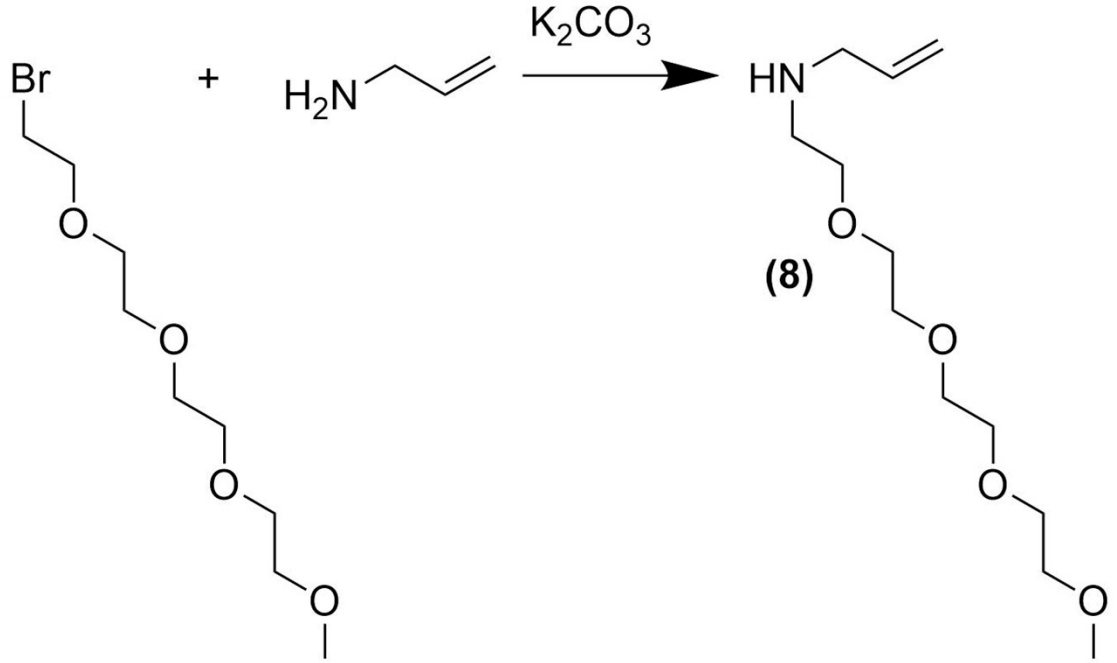


Figure S15. Synthesis scheme for compound **(8)**.

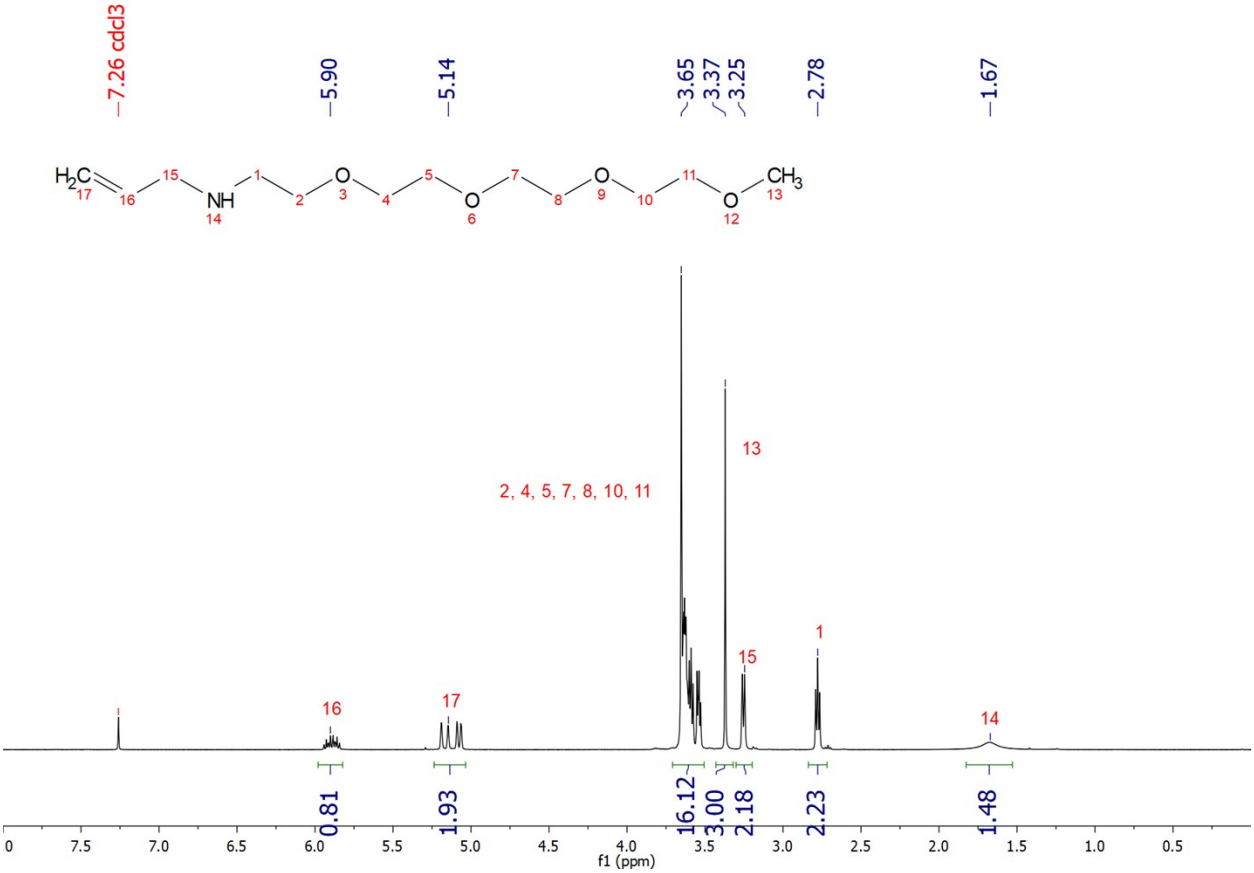


Figure S16. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **(8)**.

Synthesis of Compound (9)

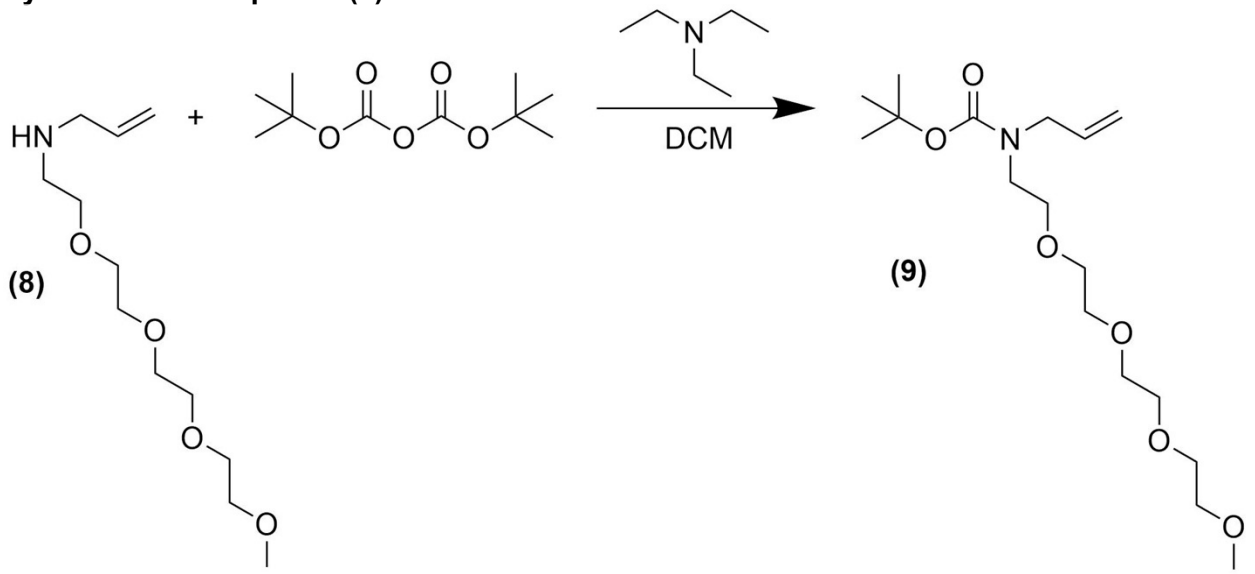


Figure S17. Synthesis scheme for compound **(9)**.

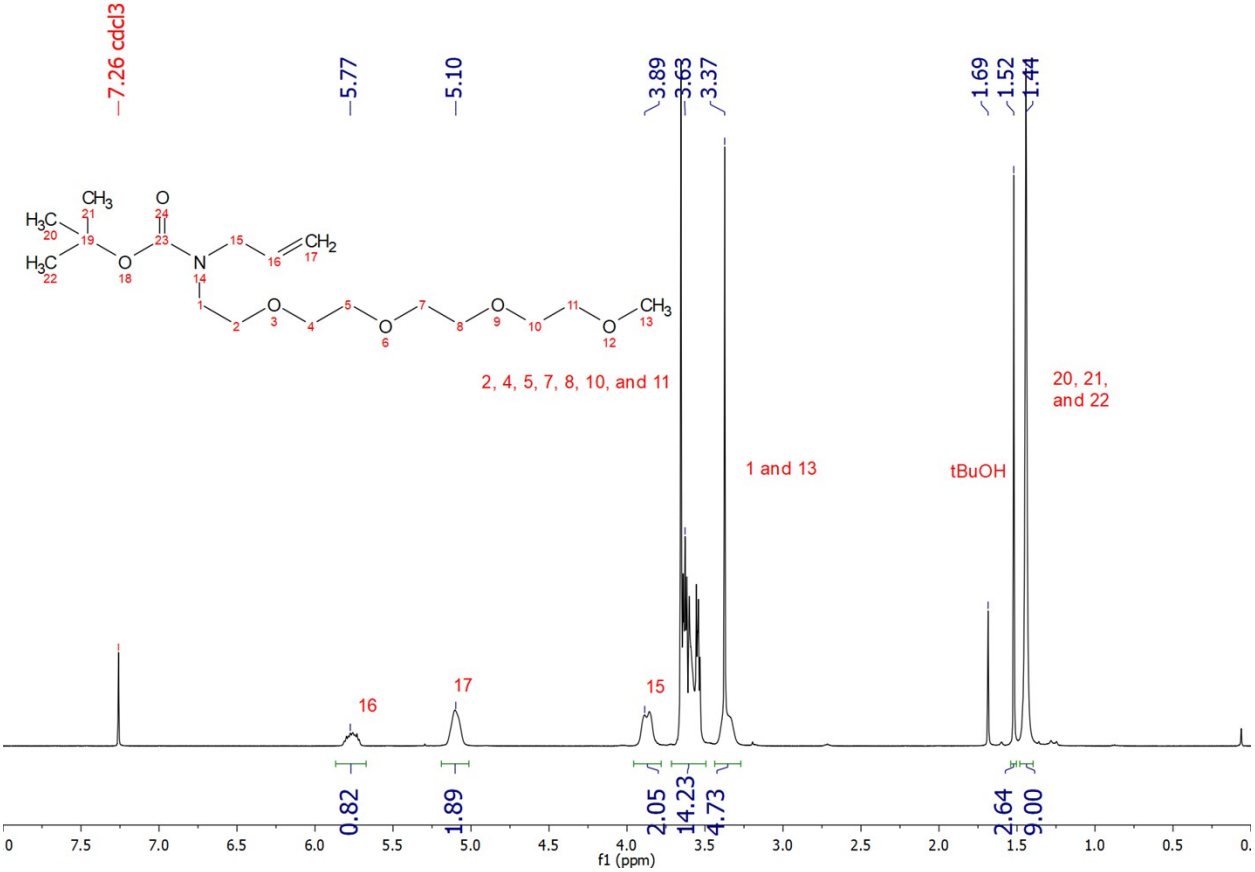


Figure S18. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **(9)**.

Synthesis of Compound (10)

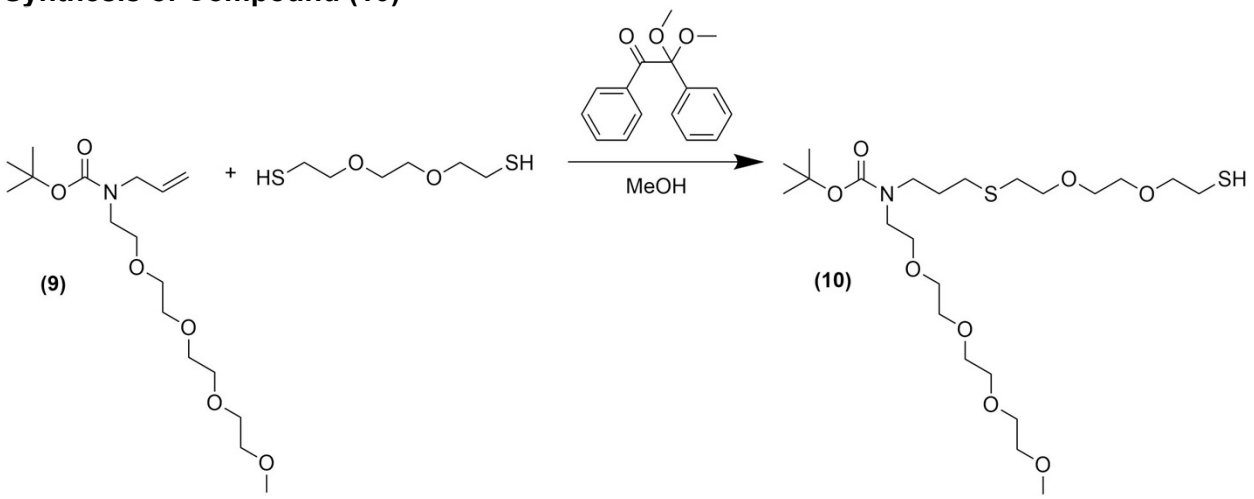


Figure S19. Synthesis scheme for compound **(10)**.

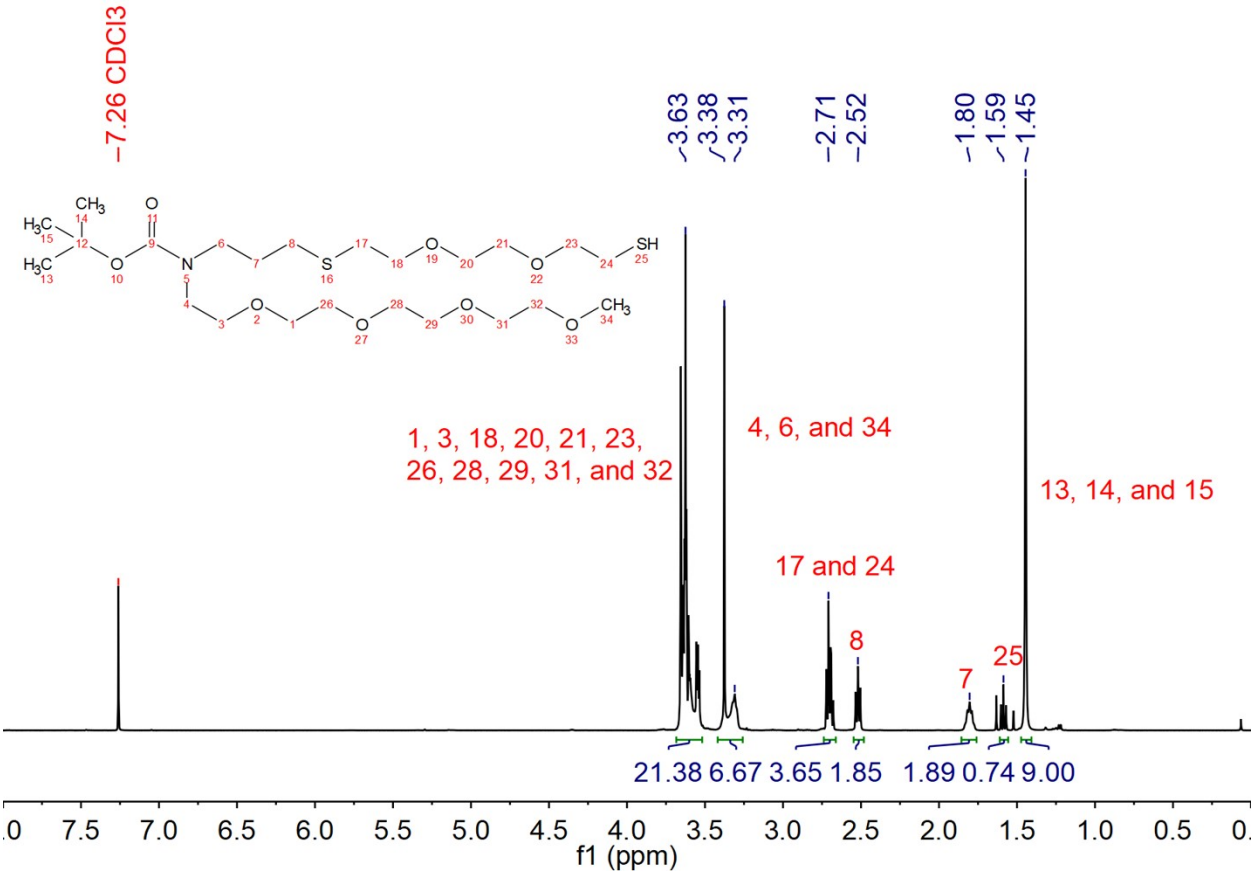


Figure S20. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(10)**.

Synthesis of Compound (11)

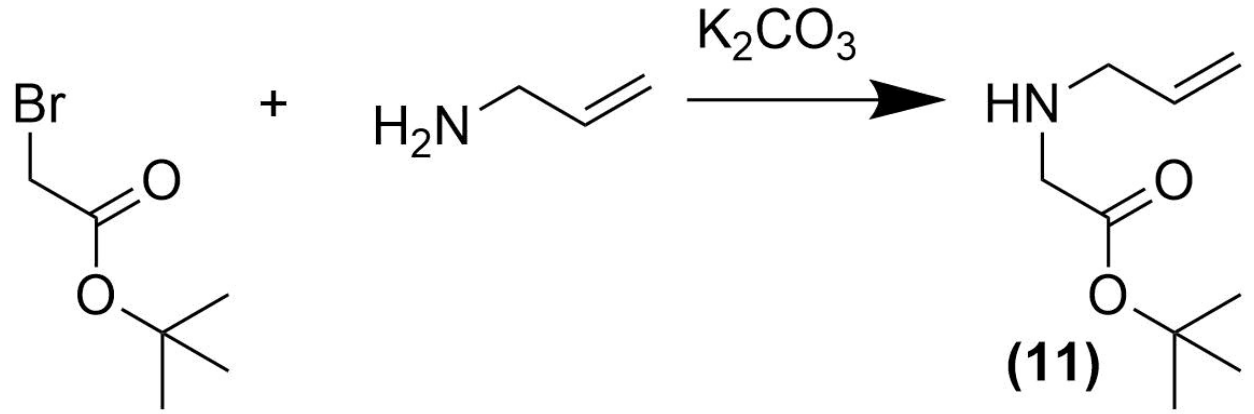


Figure S21. Synthesis scheme for compound **(11)**.

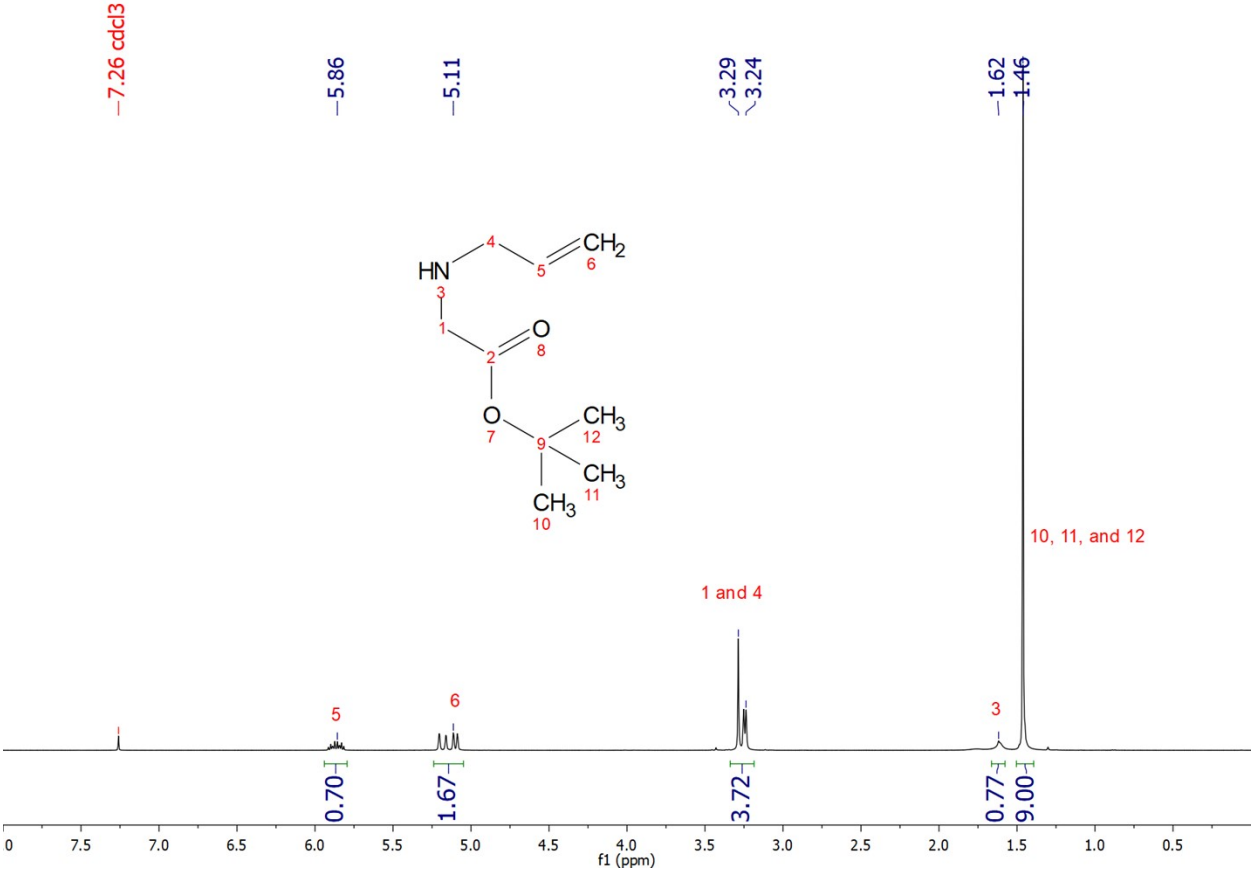


Figure S22. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **(11)**.

Synthesis of Compound (12)

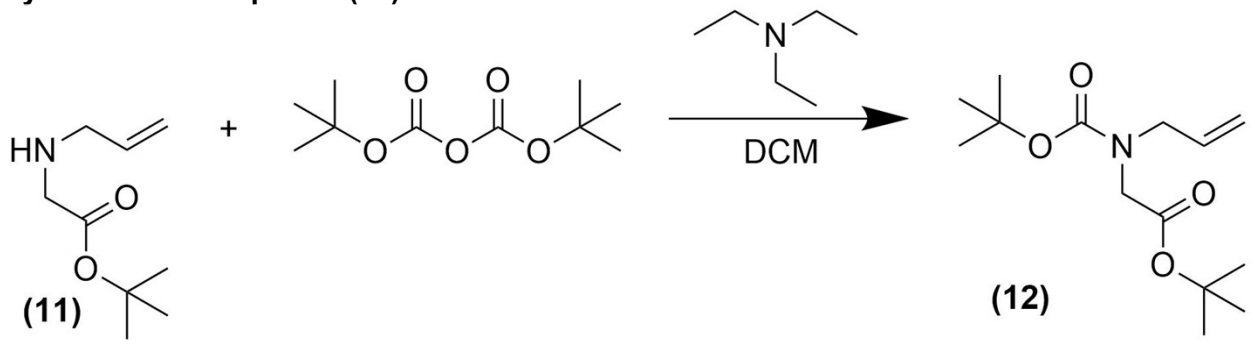


Figure S23. Synthesis scheme for compound **(12)**.

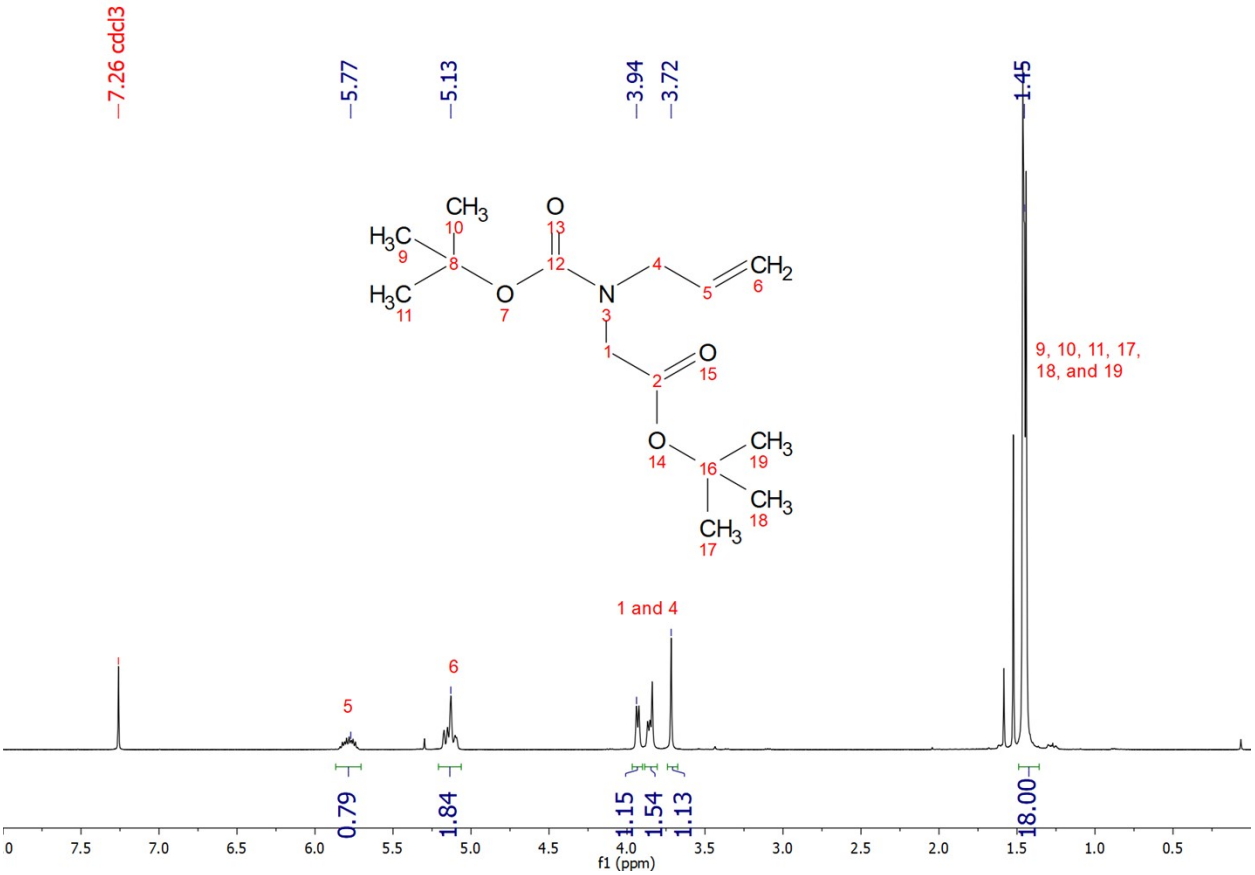


Figure S24. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **(12)**.

Synthesis of Compound (13)

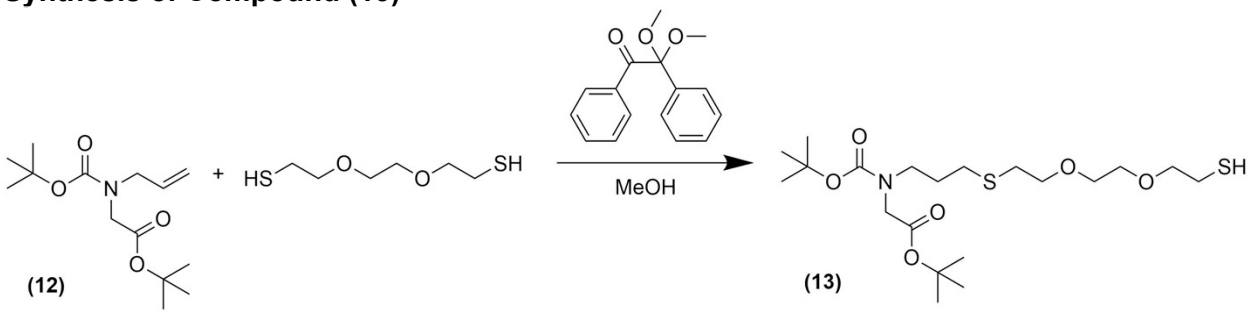


Figure S25. Synthesis scheme for compound **(13)**.

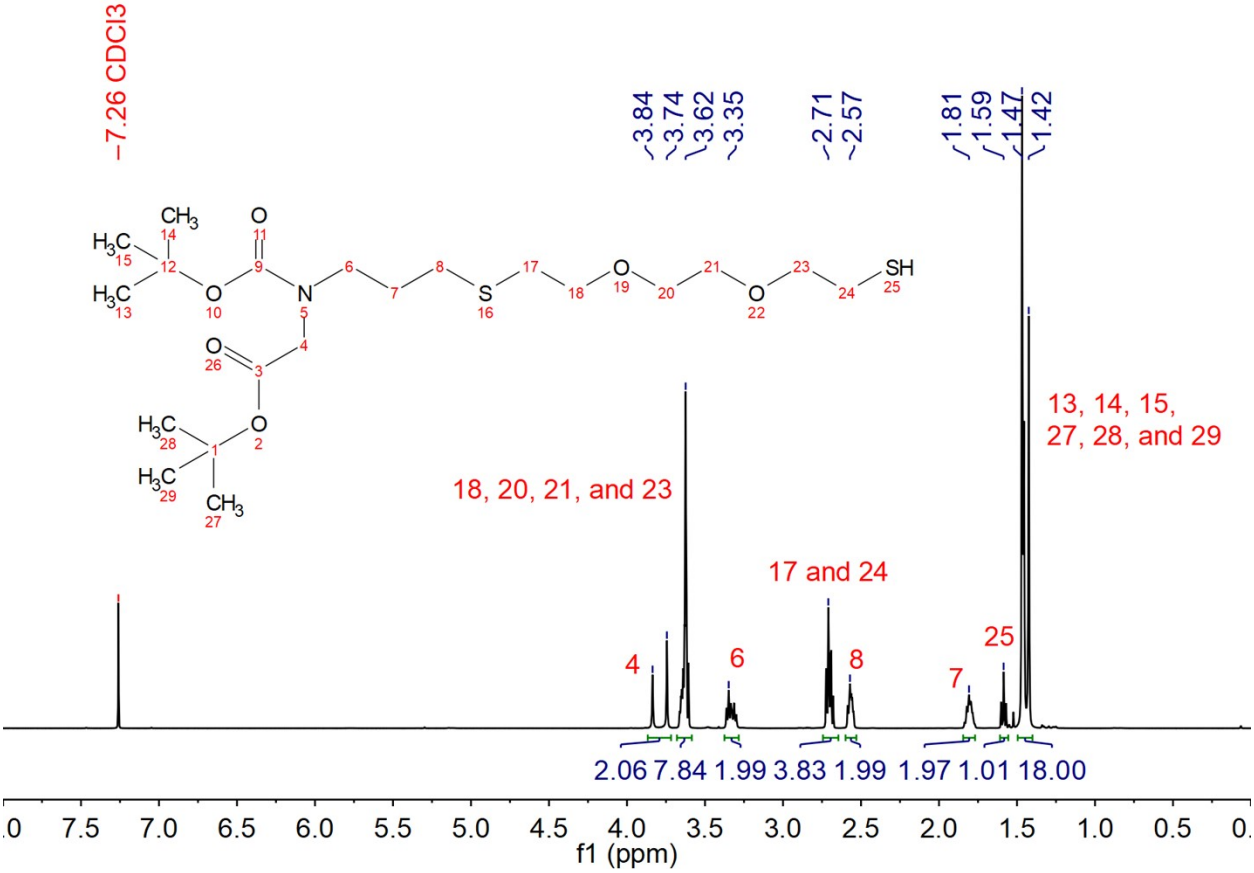


Figure S26. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(13)**.

Synthesis of Compound (14)

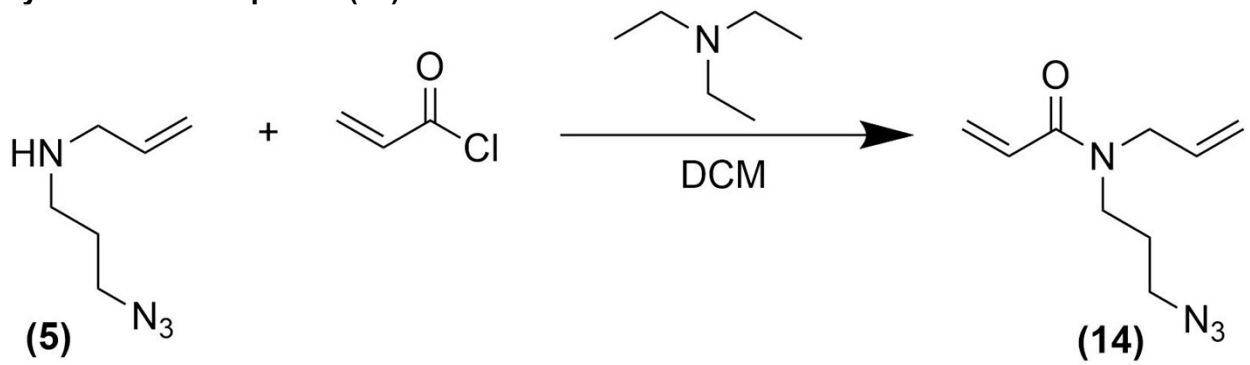


Figure S27. Synthesis scheme for compound **(14)**.

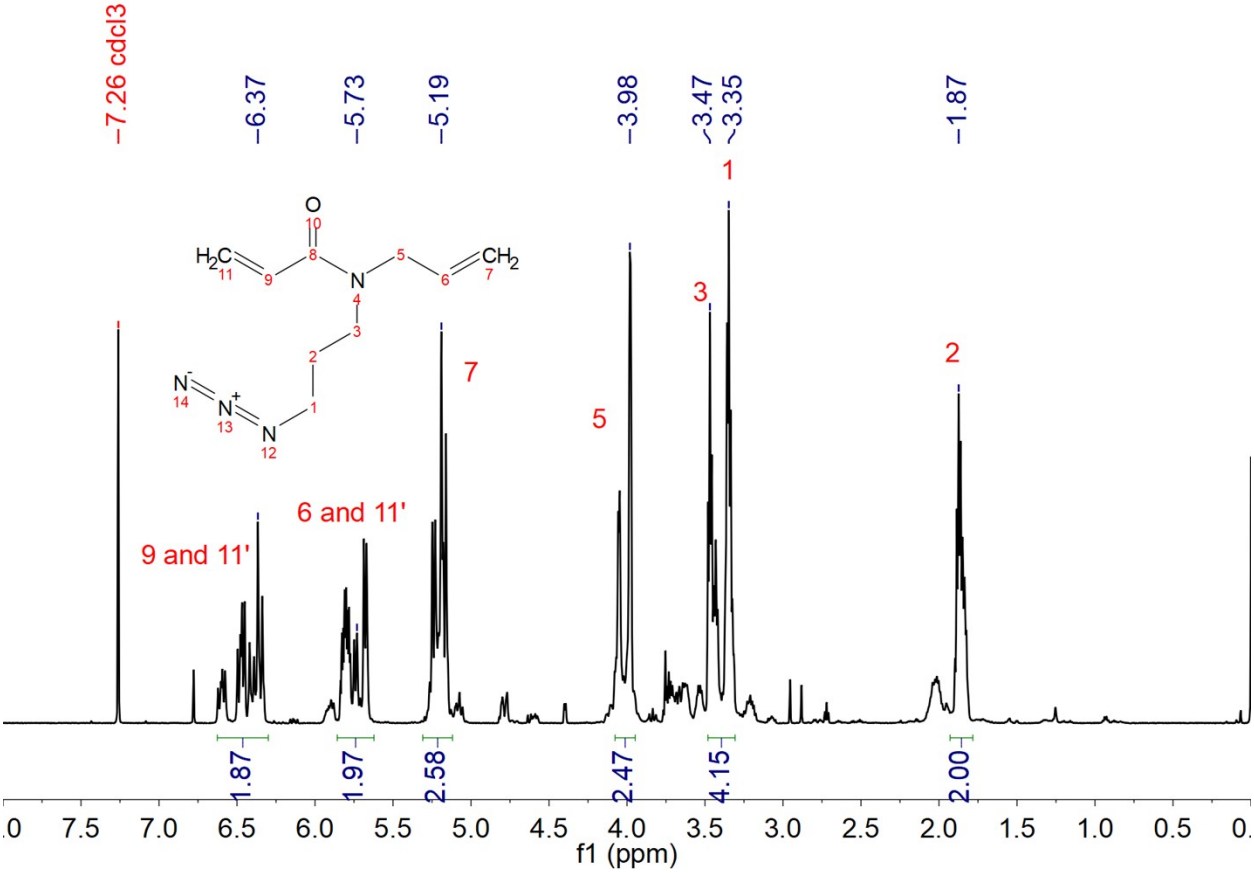


Figure S28. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(14)**.

Synthesis of Compound (15)

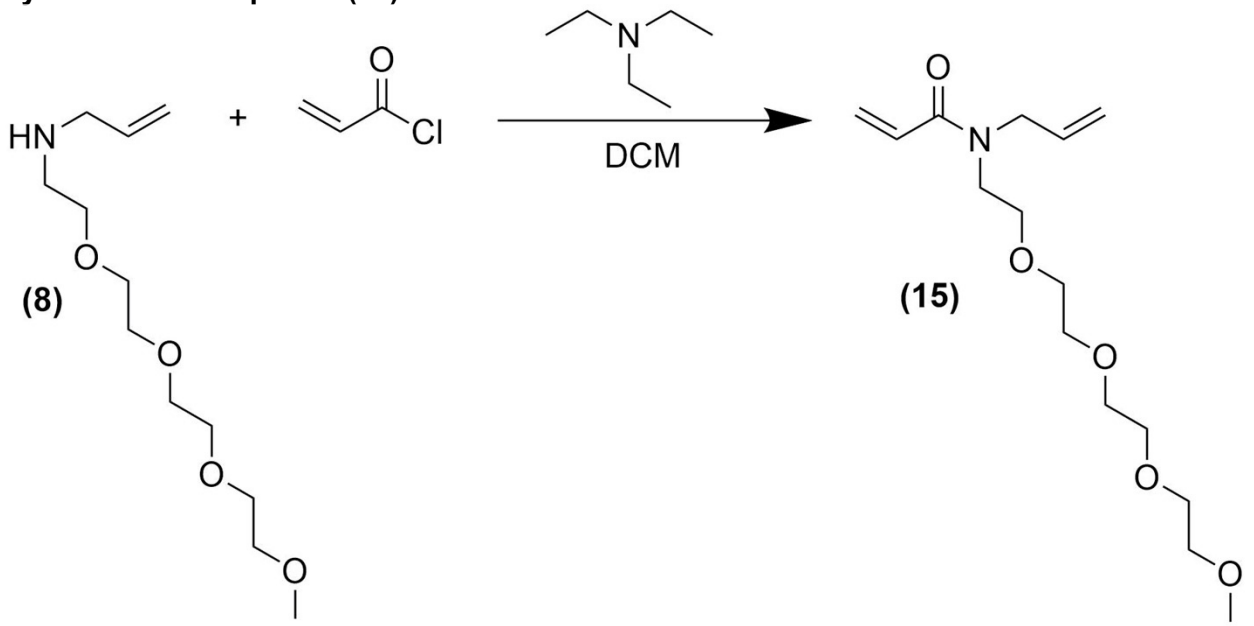


Figure S29. Synthesis scheme for compound **(15)**.

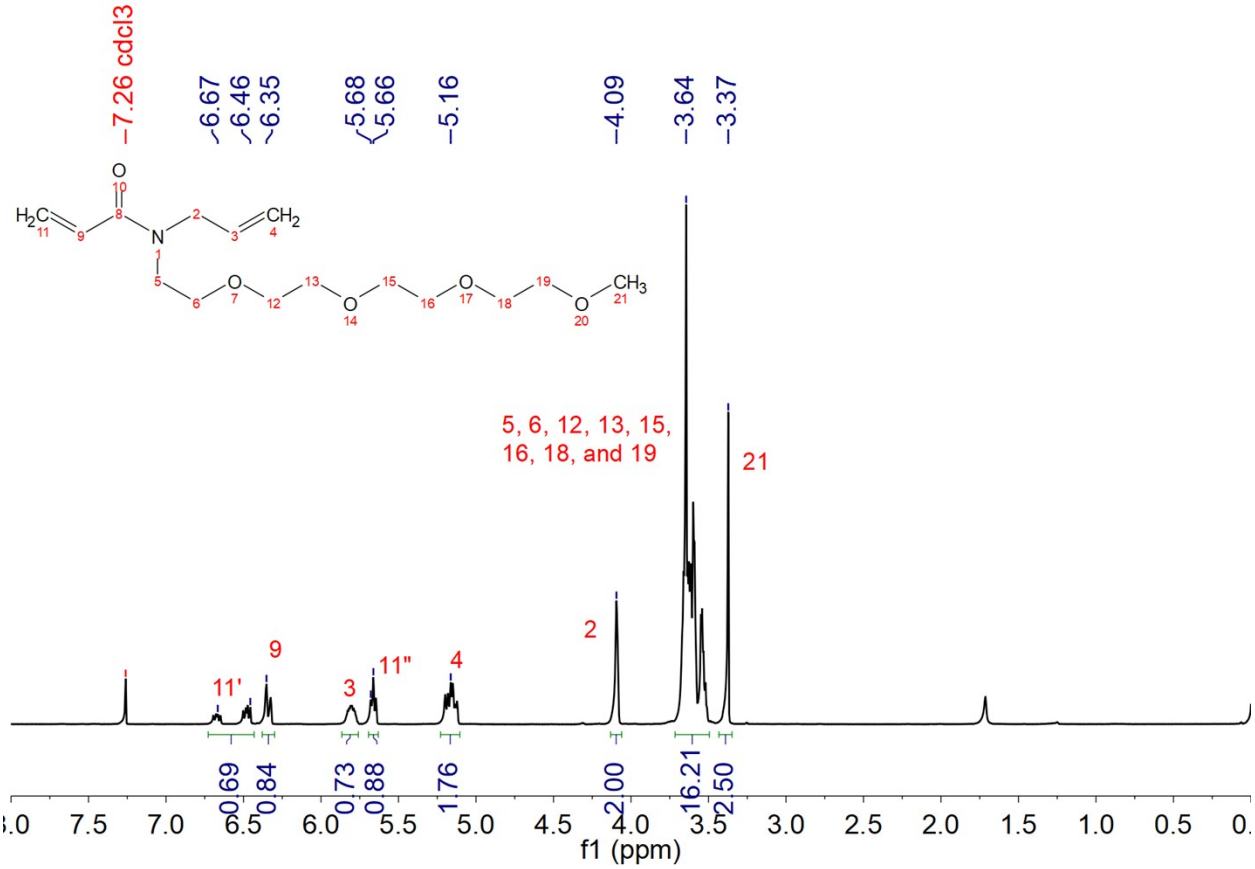


Figure S30. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(15)**.

Synthesis of Compound (16)

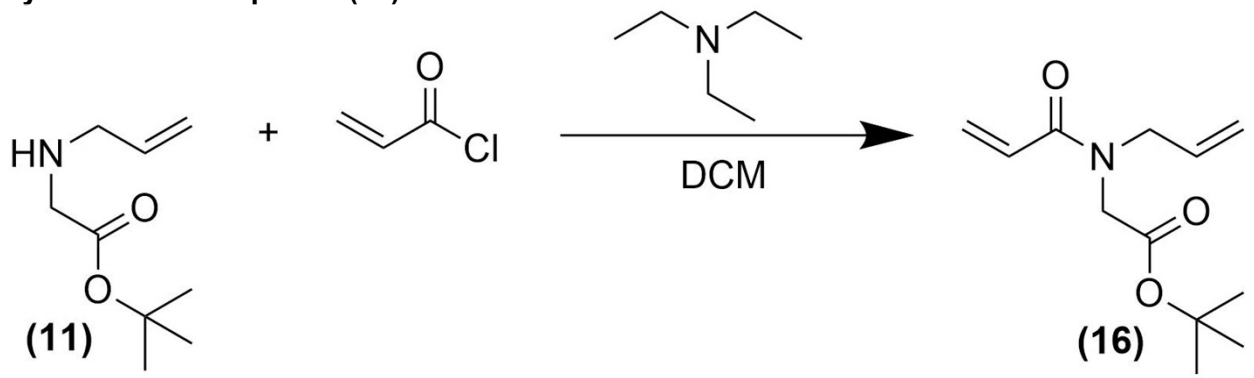


Figure S31. Synthesis scheme for compound **(16)**.

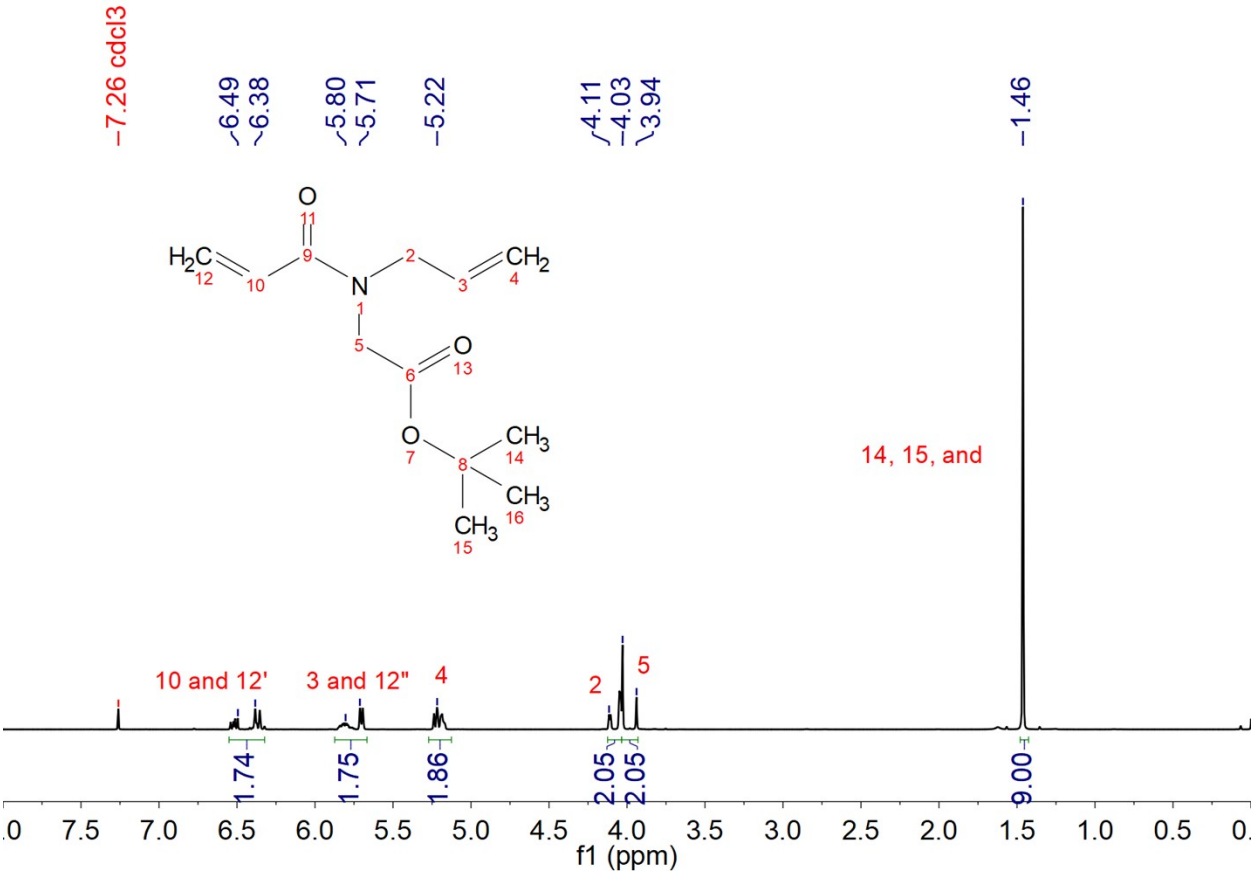


Figure S32. ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(16)**.

Synthesis of Compound (17)

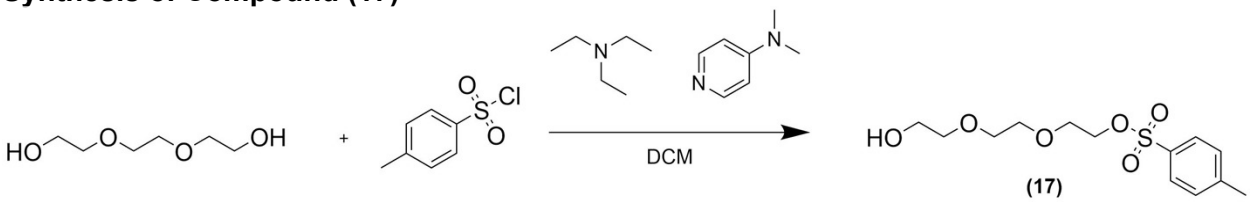


Figure S33. Synthesis scheme for compound **(17)**.

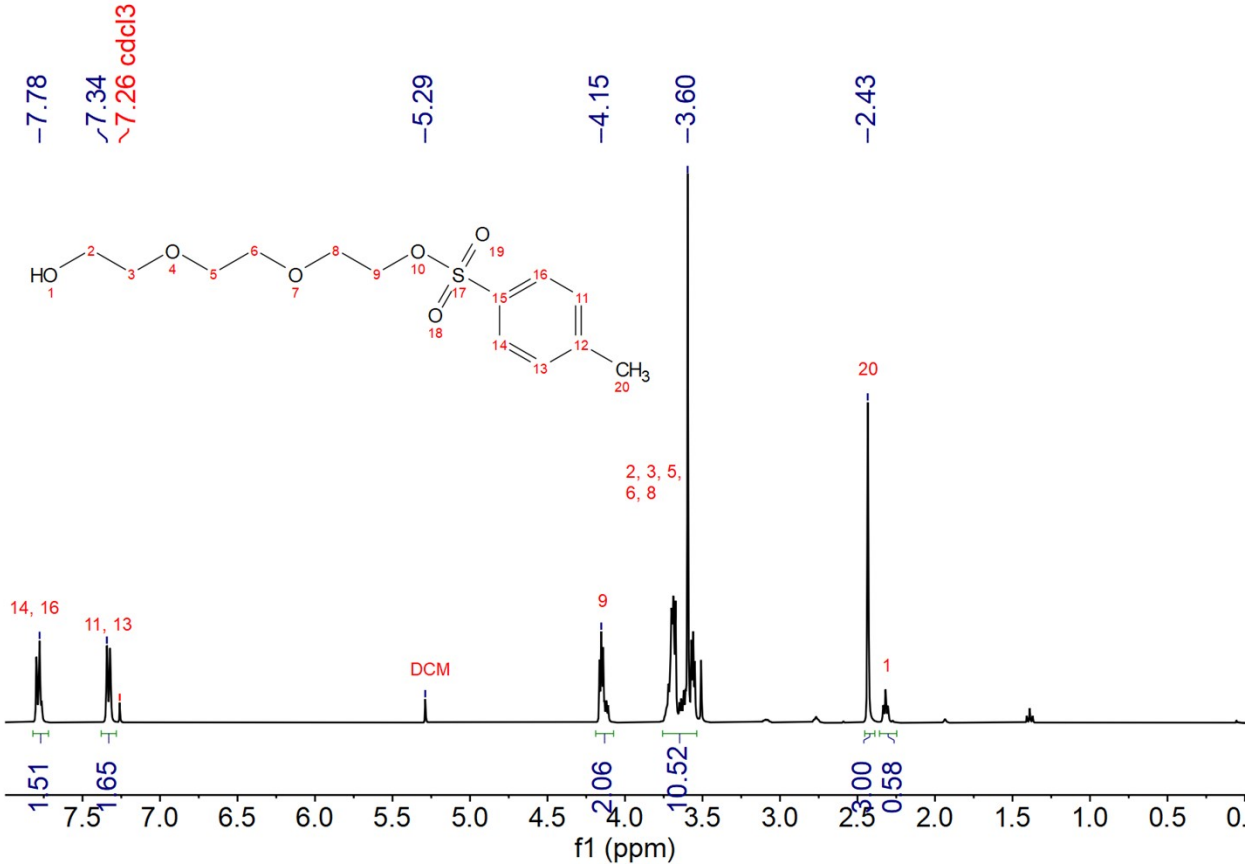


Figure S34. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **(17)**.

Synthesis of Compound (18)

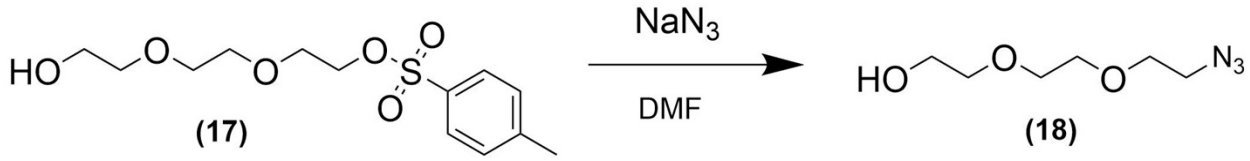


Figure S35. Synthesis scheme for compound **(18)**.

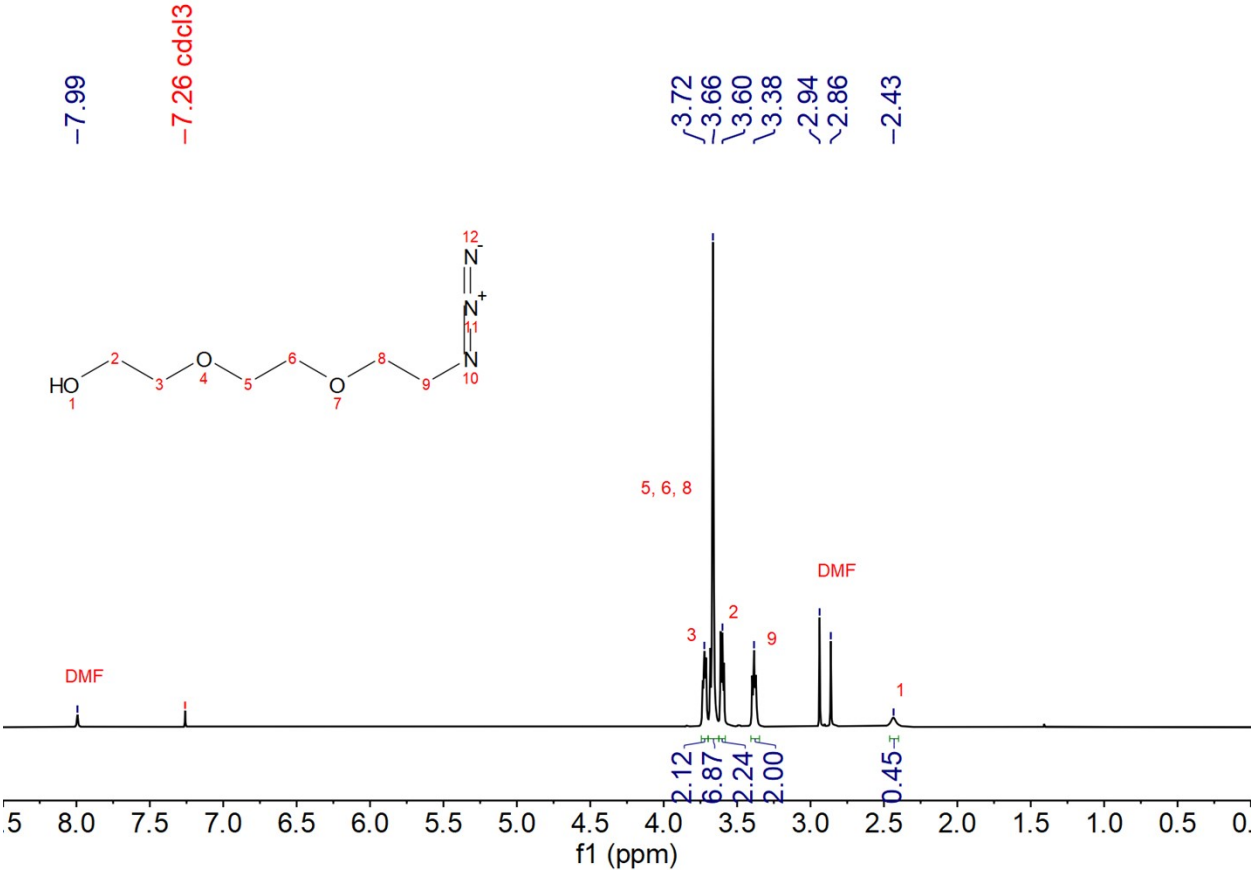
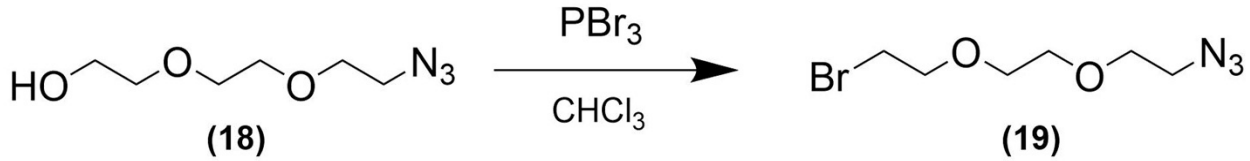


Figure S36. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **(18)**.

Synthesis of Compound (19)



(18) **(19)**

Figure S37. Synthesis scheme for compound **(19)**.

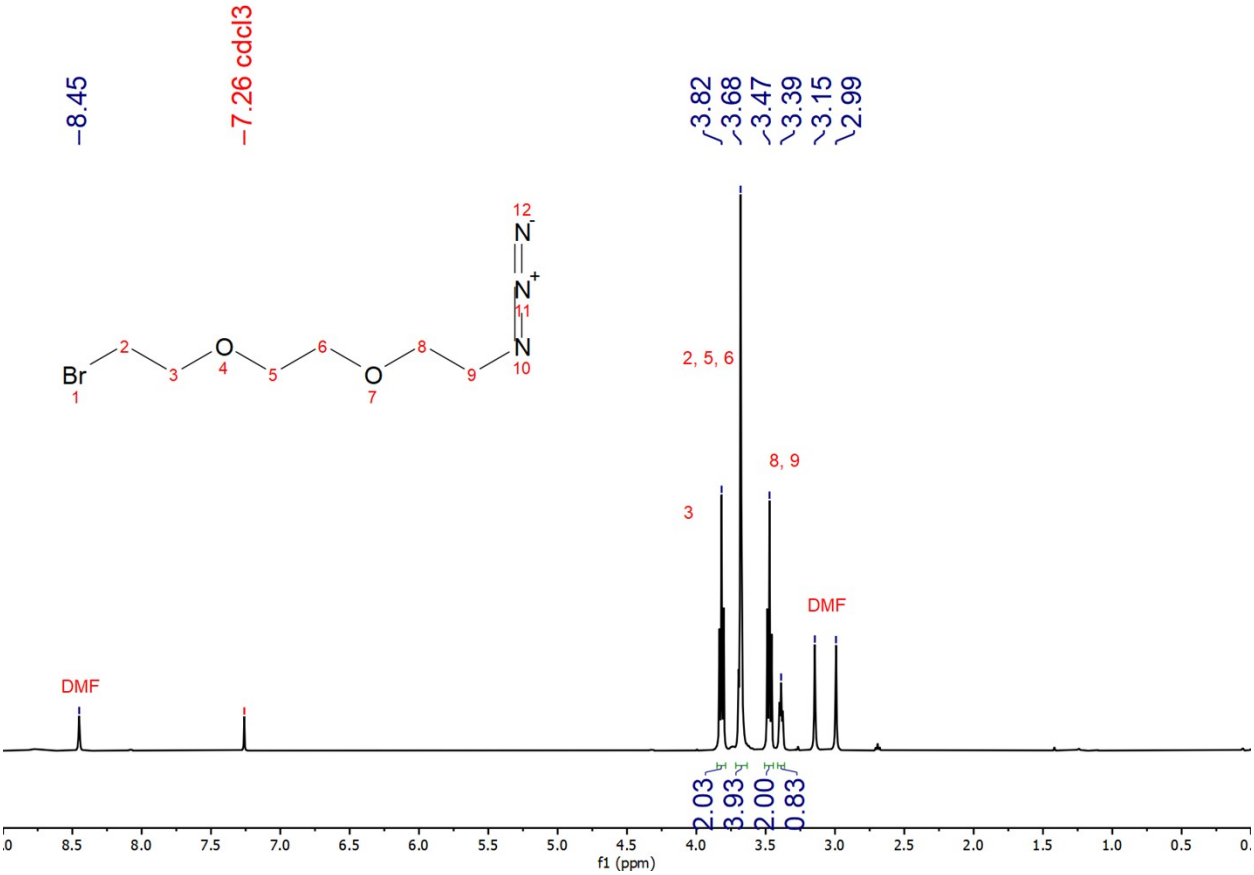


Figure S38. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **(19)**.

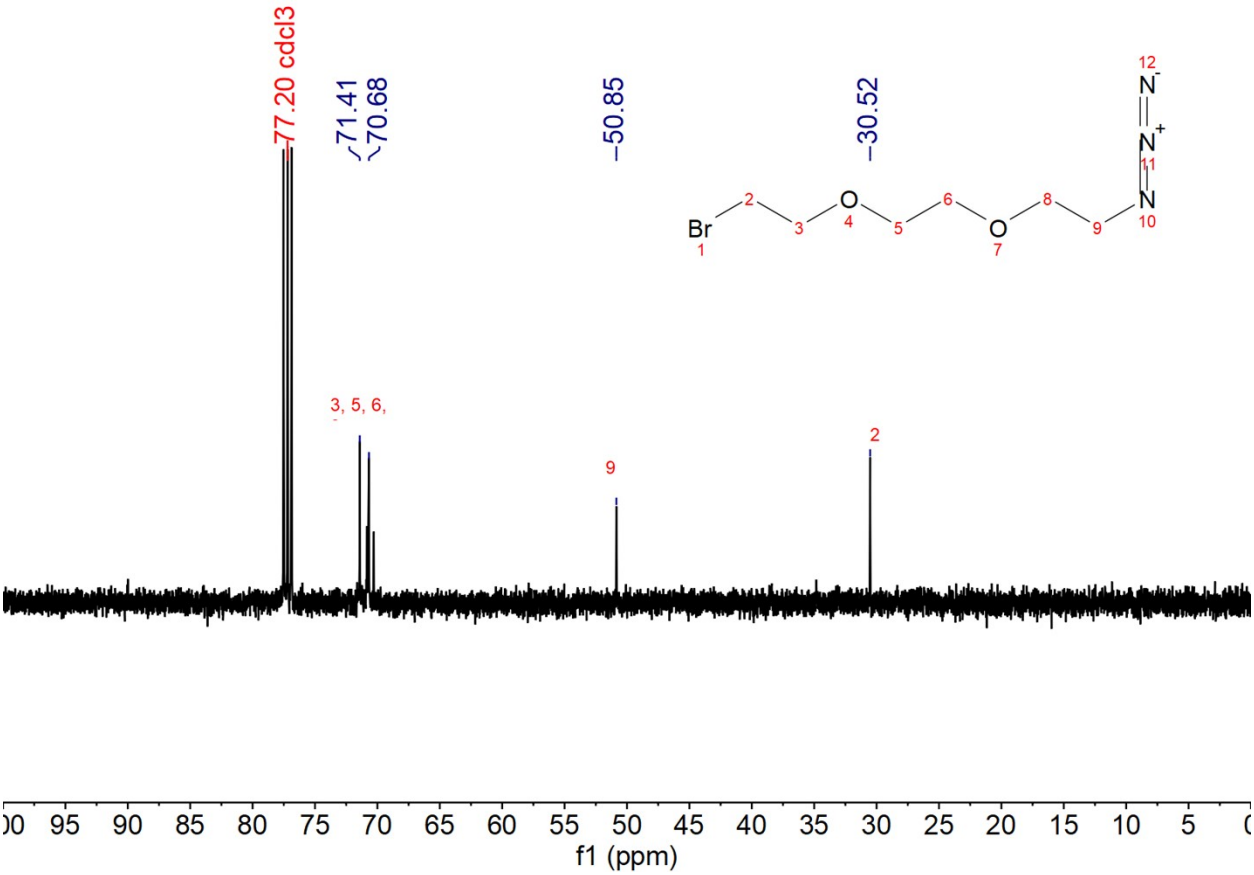


Figure S39. ^{13}C NMR (400 MHz, $CDCl_3$) of compound **(19)**.

Synthesis of Compound (20)

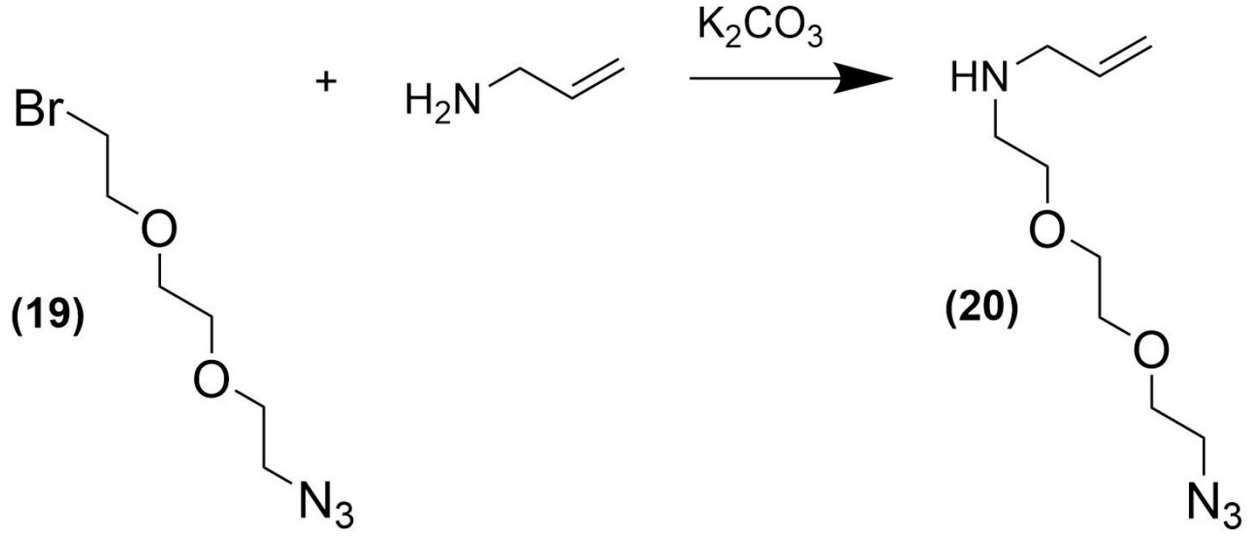


Figure S40. Synthesis scheme for compound **(20)**.

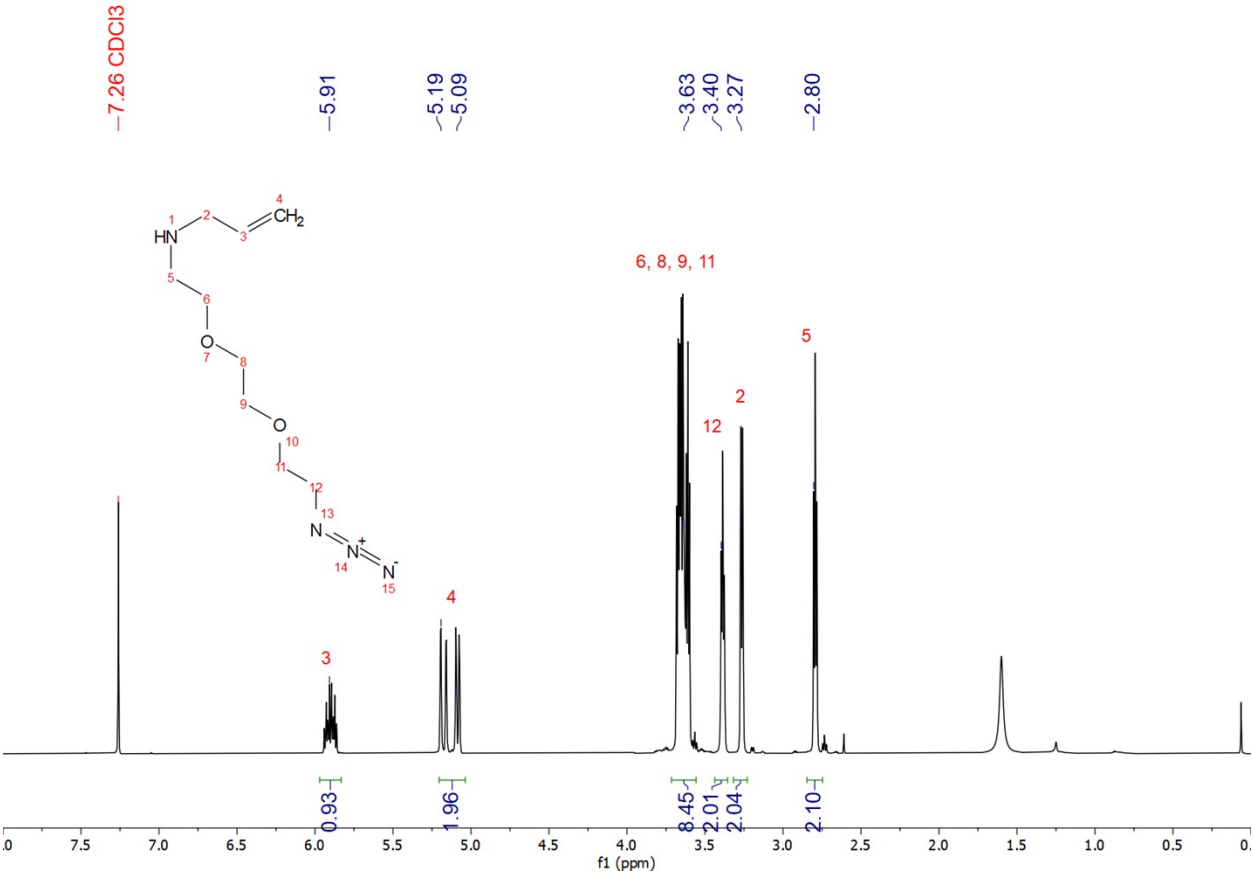


Figure S41. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **(20)**.

Synthesis of Compound (21)

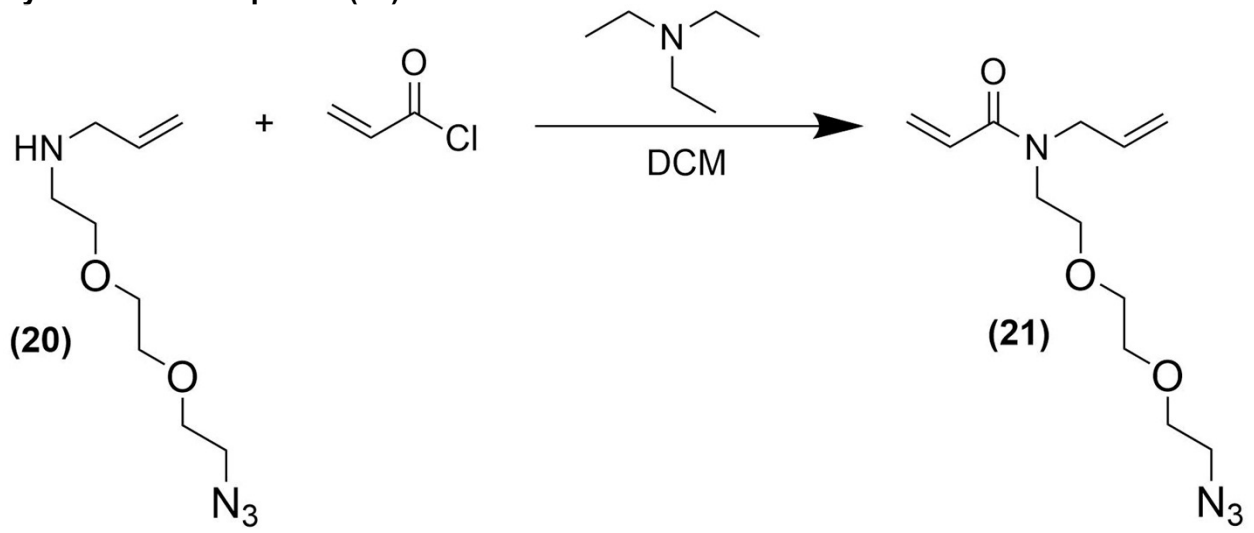


Figure S42. Synthesis scheme for compound **(21)**.

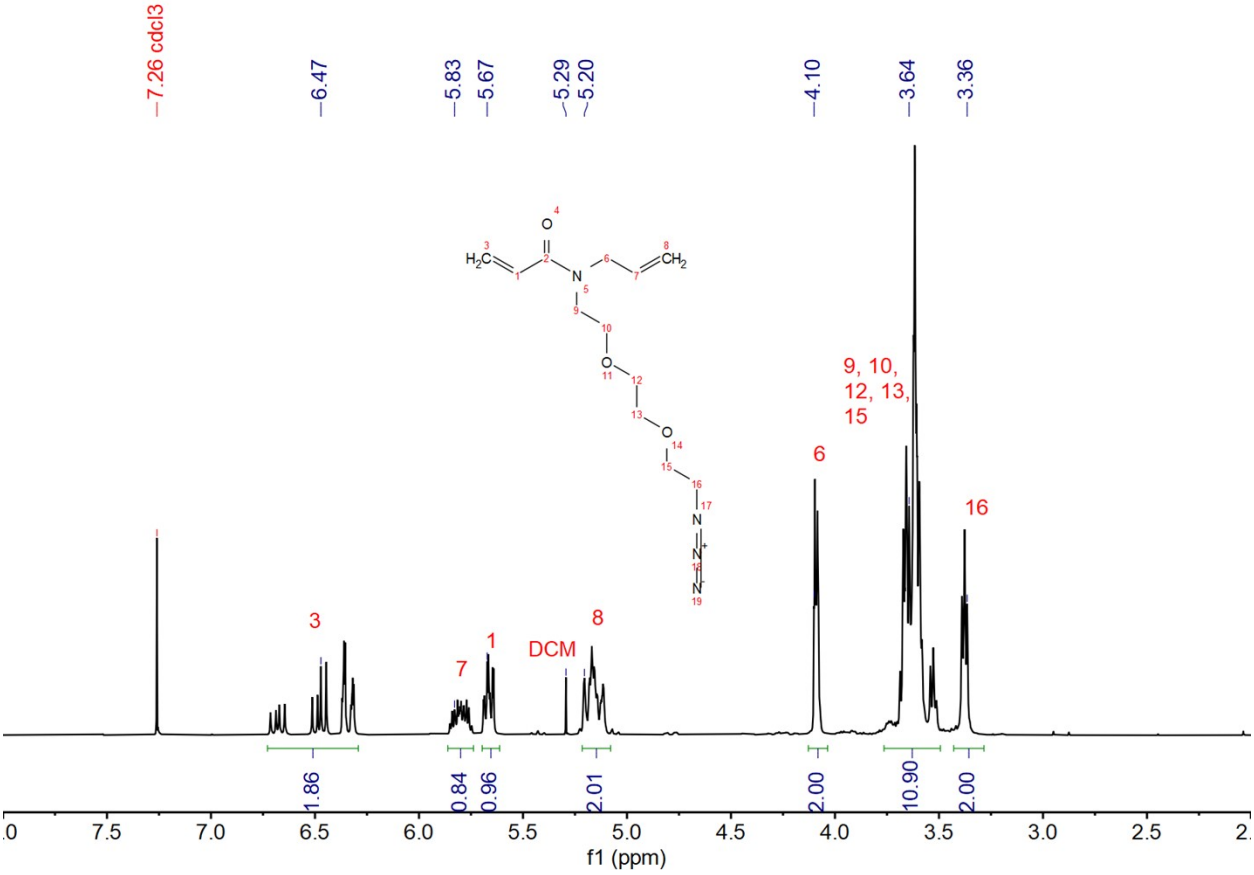


Figure S43. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **(21)**.

Synthesis of Multifunctional Cross-linkers
Synthesis of Compounds (22 – 27)

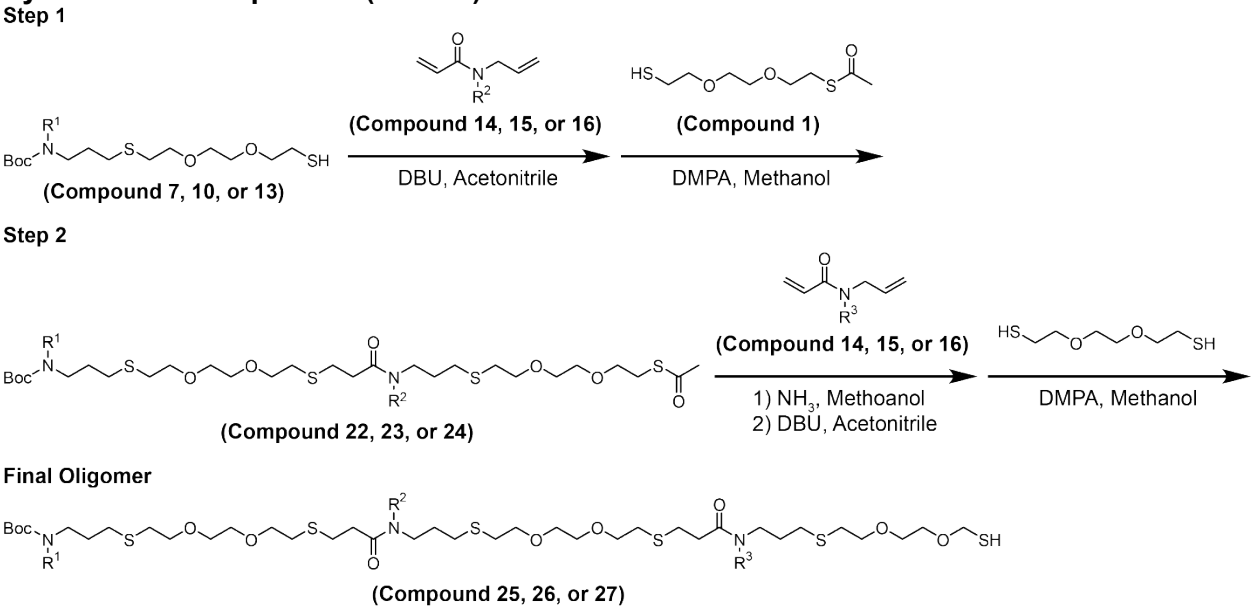


Figure S44. Synthesis scheme for compounds **(22 – 27)**.

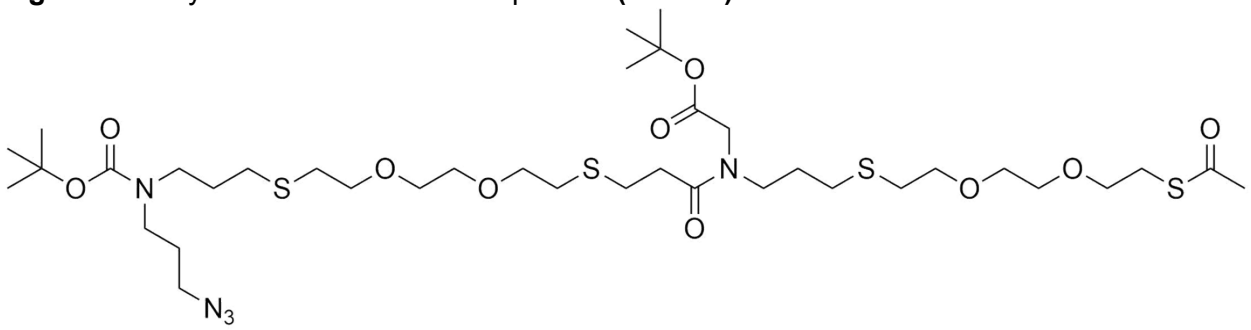


Figure S45. Structure of compound **(22)**; linker 1 intermediate.

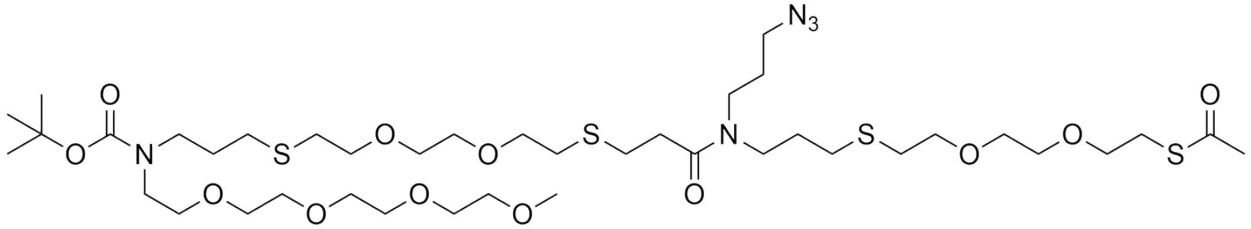


Figure S46. Structure of compound **(23)**; linker 2 intermediate.

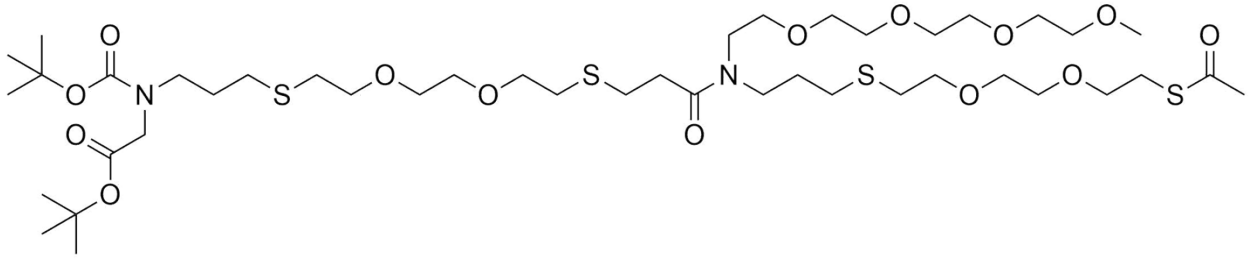


Figure S47. Structure of compound **(24)**; linker 3 intermediate.

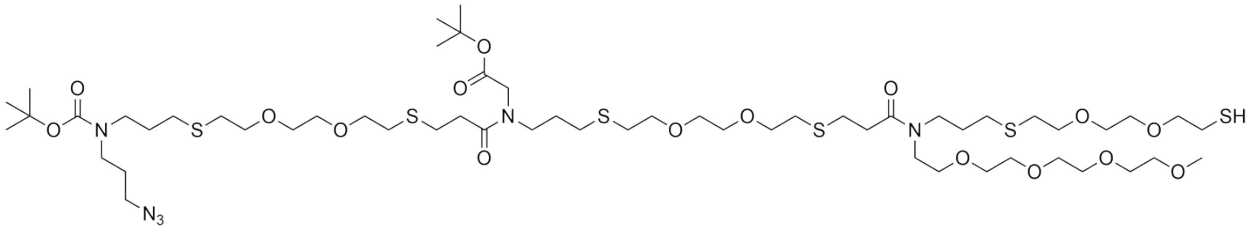


Figure S48. Structure of compound **(25)**; linker 1.

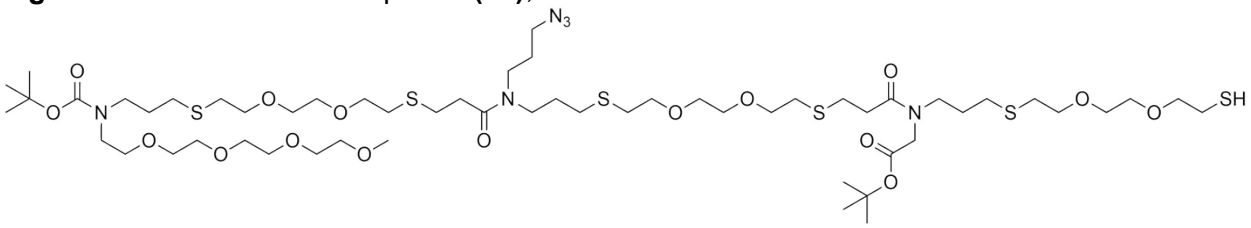


Figure S49. Structure of compound **(26)**; linker 2.

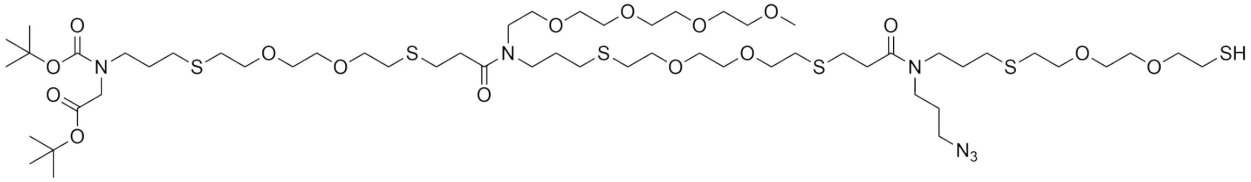


Figure S50. Structure of compound **(27)**; linker 3.

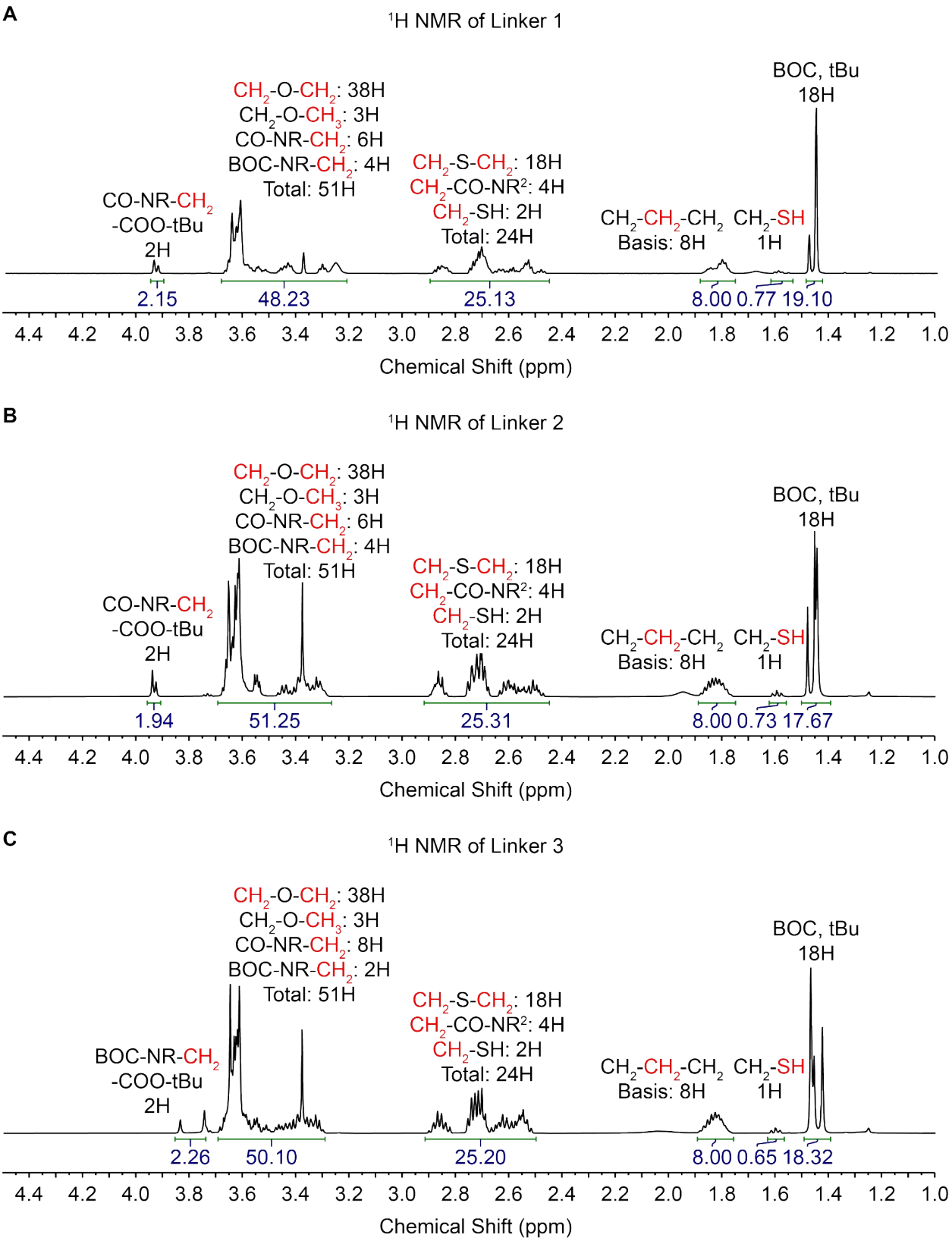


Figure S51. A) ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(25)**, B) ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(26)**, and C) ^{1}H NMR (500 MHz, $CDCl_3$) of compound **(27)**.

Synthesis of Compound (28)

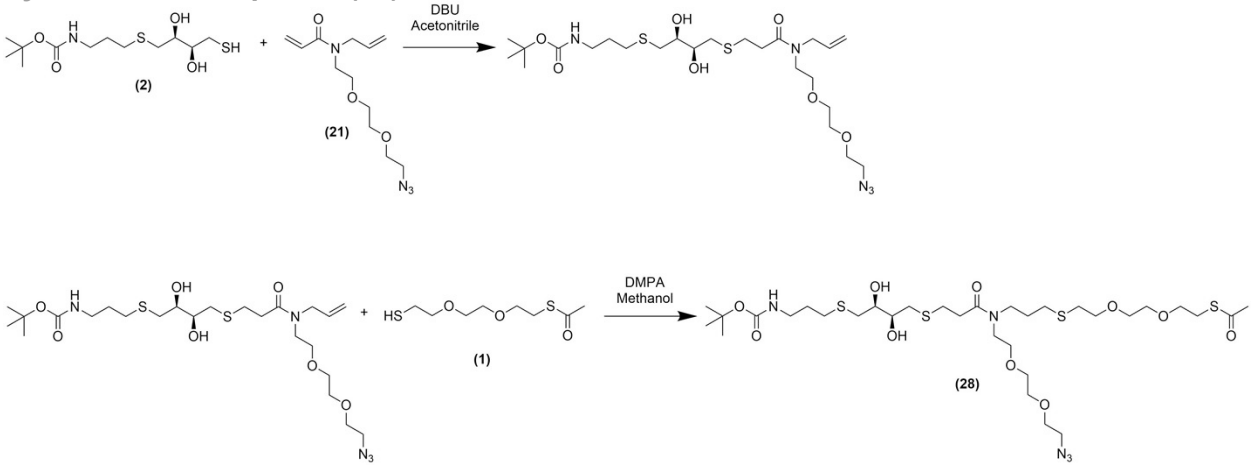


Figure S52. Synthesis scheme for compound **(28)**; linker 4.

Synthesis of Compound (29)

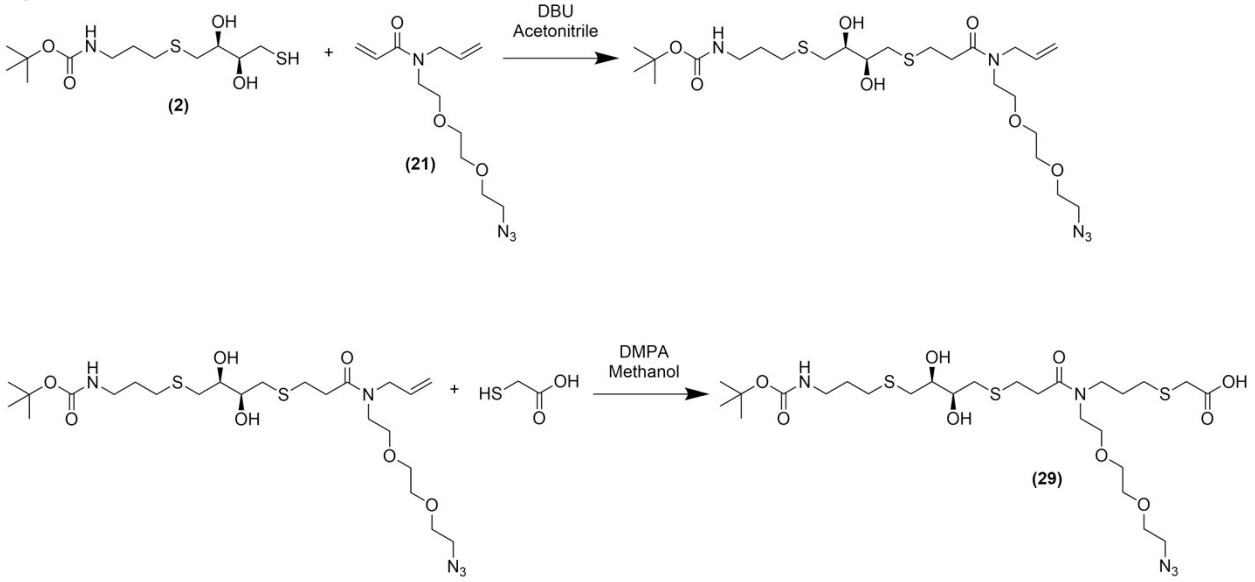


Figure S53. Synthesis scheme for compound **(29)**; linker 5.

Synthesis of Fluorophore- and Quencher-modified Cross-linkers
Synthesis of Compound (31)

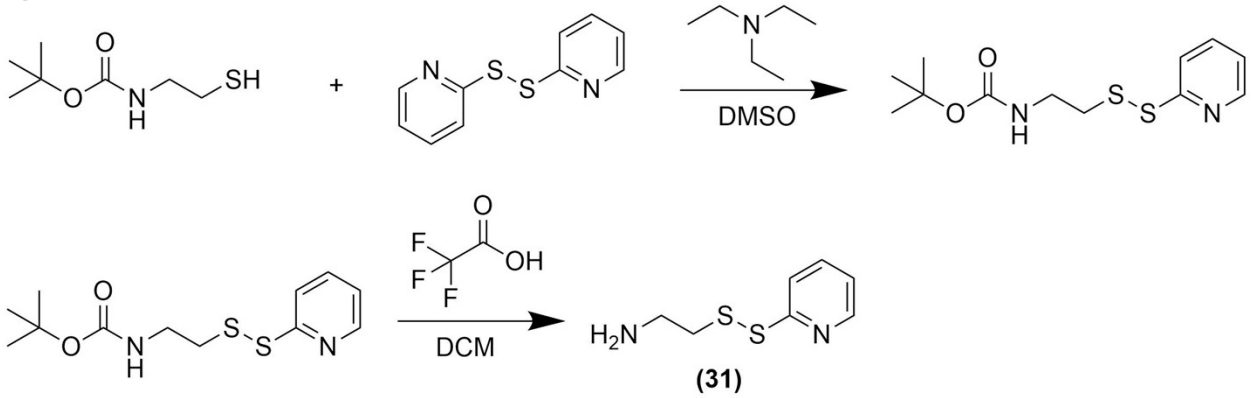


Figure S54. Synthesis scheme for compound **(31)**.

Synthesis of Compound (32)

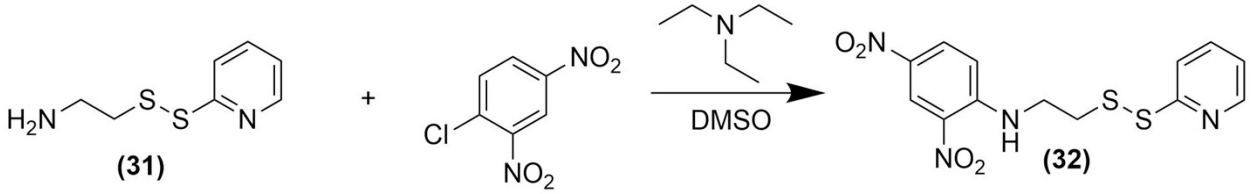


Figure S55. Synthesis scheme for compound **(32)**.

Synthesis of Compound (33)

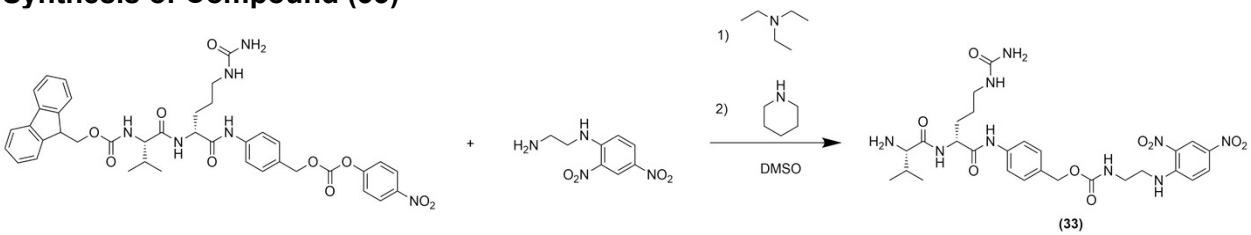


Figure S56. Synthesis scheme for compound **(33)**.

Synthesis of Compounds (34 and 35)

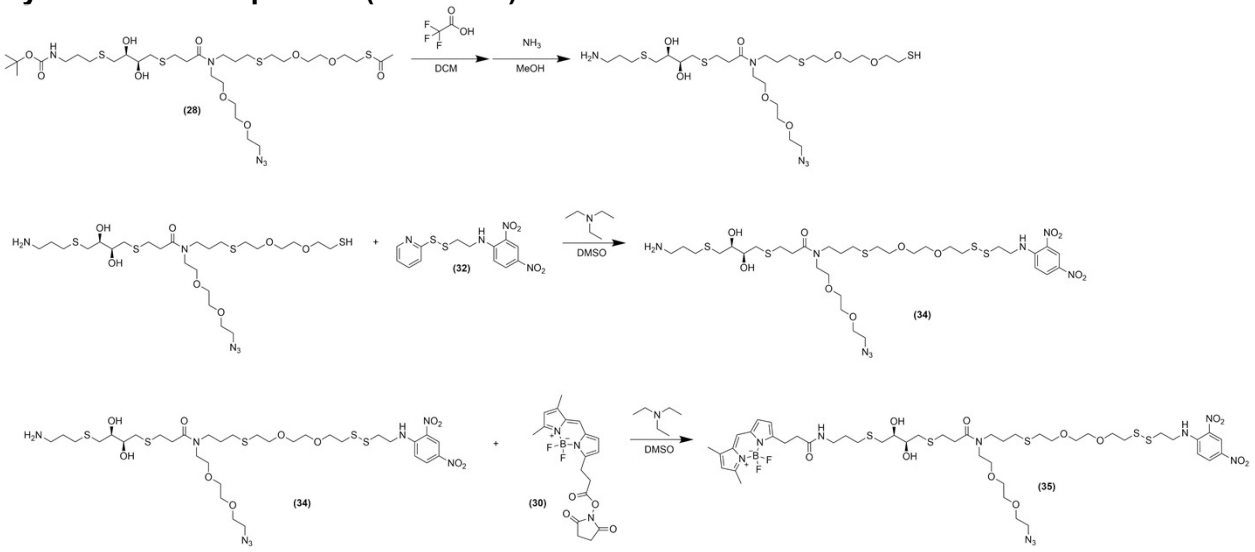


Figure S57. Synthesis scheme for compounds **(34 and 35)**.

Synthesis of Compounds (36 and 37)

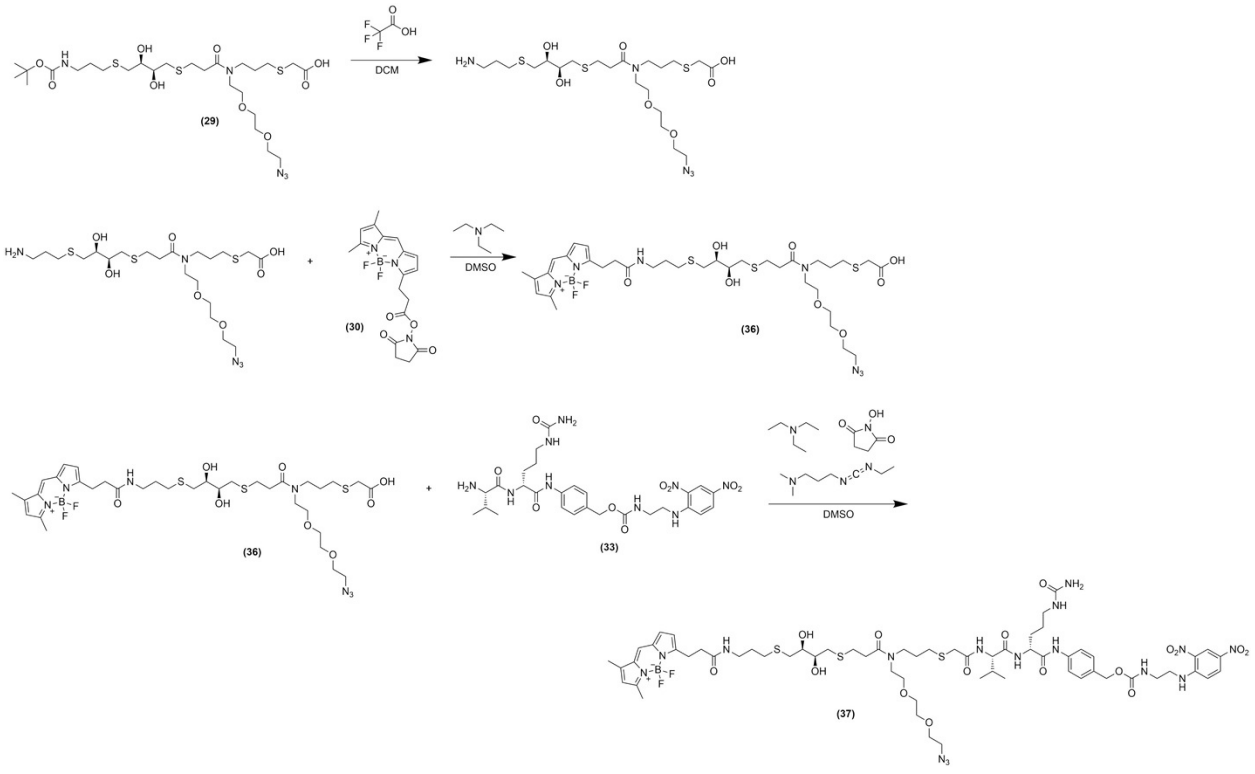


Figure S58. Synthesis scheme for compounds **(36 and 37)**.

Synthesis of Compounds (38 and 39)

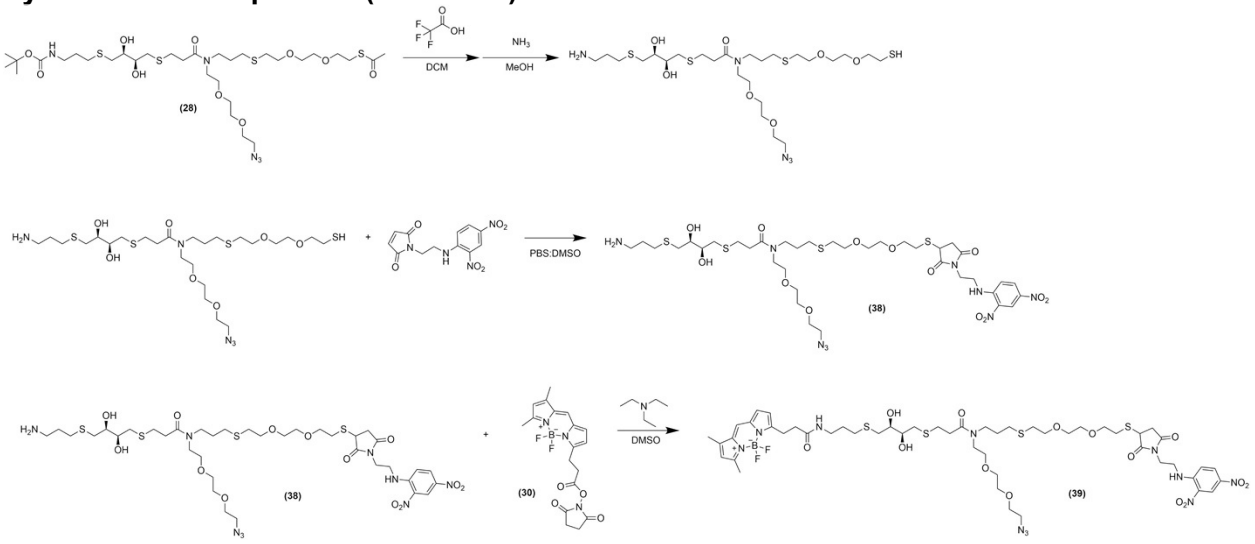


Figure S59. Synthesis scheme for compounds **(38 and 39)**.

Characterization of Molecular Probes

Characterization of Disulfide Probe

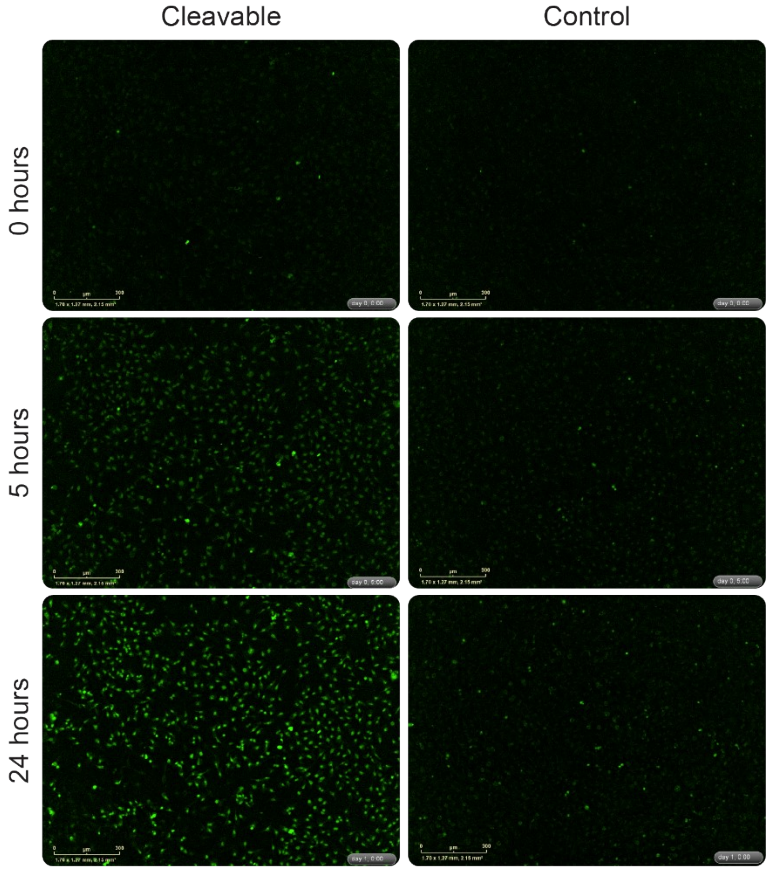


Figure S60. Visualization of disulfide bond reduction via IncuCyte ZOOM live cell imaging.

References

1 M. R. Sorkin, J. A. Walker, J. S. Brown and C. A. Alabi, *Bioconjugate Chemistry*, 2017, **28**, 907– 912.