Paintable Proteins: Biofunctional Coatings via Covalent Incorporation of Proteins into a Polymer Network

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Experimental Details

Materials. All reagents were analytical grade and obtained from Alfa-Aesar, Fisher Scientific, Oakwood Chemicals, Sigma-Aldrich, Strem Chemicals, Cambridge Isotope Laboratories, or ThermoFisher Scientific and used without further purification.

Red Fluorescent Protein (RFP) Expression and Purification. RFP was expressed from the pBAD-DsRED plasmid (addgene) in *E. coli* BL21(DE3). 1 L cultures were grown at 37°C with shaking to an OD of 0.5 and induced with arabinose at a final concentration of 1%. At induction the temperature was shifted to 18°C and cultures were grown overnight. Pellets from the overnight culture were harvested by centrifugation and stored at -80 °C until use. Cells were thawed and resuspended in 100 mL of 500 mM, Tris 8.0 buffer (NaCl 50 mM). The homogenous cell mixture was sonicated for 15 minutes at 80 amps and centrifuged at 15,000 x G for 2 hours to remove supernatant. The supernatant was then purified using a His GraviTrap TALON cobalt column (GE Healthcare, 29-0005-94). For column purification, a 500 mM NaCl, 50 mM Tris 8.0 buffer; a 500 mM NaCl, 50 mM Tris 8.0, 20 mM Imidazole buffer; and a 500 mM NaCl, 50 mM Tris 8.0, 500 mM Imidazole buffer were used for the equilibrium, wash, and elution buffers, respectively. The eluted protein was dialyzed overnight in 2 L of 250 mM NaCl, 50 mM Tris 8.0, 10% Glycerol buffer using SnakeSkin™ Dialysis Tubing, 10K MWCO, 35 mm (ThermoFisher Scientific, 88245). The purified RFP was flash frozen with liquid nitrogen and store in -80 °C. Purity was confirmed by SDS-PAGE.

RFP-mal. Flash-frozen, purified RFP was diluted to 0.1 mg/mL in phosphate-buffered saline. N-propargylmaleimide was solubilized in 100% methanol at 50 µg/mL. N-propargylmaleimide was added to dilute RFP to create a 10:1 mol ratio of maleimide to protein, and was allowed to react for 16 hours at room temperature. The reaction product was concentrated to 1 mg/mL using a 10kDa spin concentrator (Millipore Sigma, CLS431478) for application.

RFP-oPA. Flash-frozen, purified RFP was diluted to 0.1 mg / mL in phosphate-buffered saline. Compound **4** was solubilized in 100% methanol at 100 µg/mL. Crosslinker **4** was added to the diluted RFP to form either a 10:1 or 1:1 mol ratio of oPA to protein, and was allowed to react for 1 hour at room temperature. The reaction product was concentrated down using a 10kDa spin concentrator (Millipore Sigma, CLS431478) to 1 mg/mL for application.

Fluorescent microscopy. Nikon reflectance microscope was equipped with an X-Cite 120LED Boost illumination system and Hamamatsu digital CMOS camera using a TRITC filter.

Profilometry. ZETA 20 optical profiling microscope (Zeta Instruments Inc.) equipped with 20x objective lens was utilized for optical microscopy.

IR. Attenuated total reflectance-Fourier transform infrared spectroscopy was performed using a Perkin-Elmer Spectrum 100 spectrophotometer. The spectral range was selected as 4000 - 650 cm^{-1} with a resolution of 4 cm^{-1}.

TGA. Samples weighing 2-10 mg were heated at 10 °C/min from ambient to 600 °C in a stream of nitrogen in the microbalance of a TA Instruments TGA Q5000.

DSC. Samples weighing 2-10 mg were heated at 10 °C/min from -50°C to 150°C in a stream of nitrogen in the microbalance of a Mettler Toledo DSC II, and were measured on the second heating/cooling cycle.

NMR. All proton decoupled ^{13}C and ^{1}H NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer, and were taken in DMSO-d_6, D_2O, or $CDCl_3$. In ^{13}C NMR, carbon signal was set on the chloroform peak at 77.16 ppm, while in ^{1}H NMR the residual protio solvent was set at 2.50, 4.79, or 7.26 ppm for DMSO, water, and chloroform, respectively.

Mass Spectrometry. High resolution mass spectra (HRMS) were obtained on a VG Analytical VG-70S mass spectrometer with electron impact (EI) ionization and analyzed by double-focusing magnetic sectors. Low resolution mass spectra (LRMS) were obtained on a Biotage Dalton 2000 Mass Detector. Protein mass spectra were collected at the University of Illinois Roy J. Carver Biotechnology Center after trypsin digestion with liquid chromatography mass spectrometry (LC-MS).

SEM. A Thermo Scientific Scios scanning electron microscope (SEM) was used to image the particles. The samples were sputter coated with iridium to make them electrically conductive.

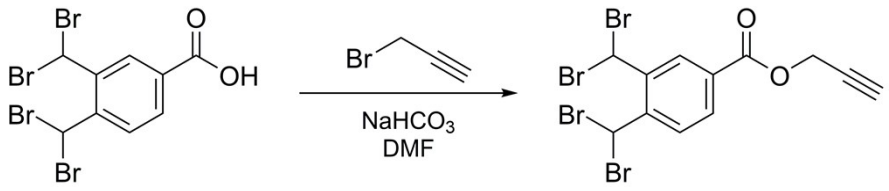
UV-vis. All UV-Vis data was obtained on a PerkinElmer Lambda 950 UV-vis spectrometer with an InGaAs detector.

Copper Painted Substrates. Stainless steel disks (Wagner) were sand blasted and primed with three coats of MIL-DTL-24441, Type III A/B (Sherwin-Williams) diluted with Polane Reducer K69 (Sherwin-Williams). Seaguard® Ablative Antifouling coating (Sherwin-Williams, Red) was applied to the primed surface in five coats and allowed to dry overnight.

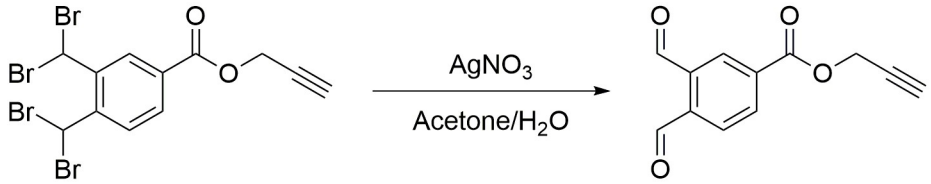
Application Process. Sodium ascorbate (5 mg, 0.03 mmol) was dissolved in RFP/buffer solution (100 μL, 1 mg/mL protein content). DMSO (100 μL) was added dropwise to the solution to limit local heating. Compounds **7** (25 mg, 0.15 mmol) and **8** (28 mg, 0.15 mmol) were added to the solution. The entire mixture was vortexed briefly and applied to substrate with a paintbrush.

Lysine-oPA conjugation. An NMR tube was charged with Boc-L-lysine methyl ester acetate salt (32.6 mg, 0.102 mmol) in DMSO-d_6 and a ^{1}H NMR spectrum was taken. To the solution **4** (21.4 mg, 0.100 mmol) was added, and the solution was vortexed for 1 min before an additional 1H NMR spectrum was taken.

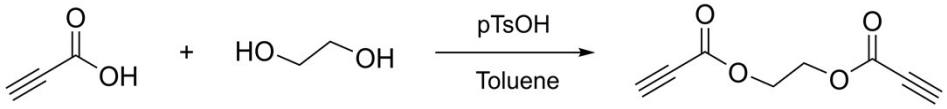
Synthetic Details



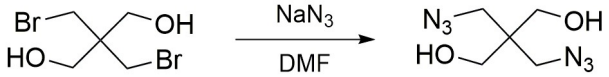
Prop-2-yn-1-yl 3,4-bis(dibromomethyl)benzoate (**3**). A 50 mL round bottom flask was charged with **2** (502 mg, 1.08 mmol), a magnetic stir bar, and was purged with nitrogen. Anhydrous DMF (5.2 mL, 0.2M) was added to the flask and the reaction mixture was cooled in an ice bath for 20 min with stirring. The septum was removed to add $NaHCO_3$ (186 mg, 32.53 mmol, 2.0 equiv) in one portion, and the septum was quickly replaced. Propargyl bromide (192 mg, 1.29 mmol, 1.2 equiv, 80% w/w in toluene) was subsequently added. The reaction mixture was stirred overnight and allowed to slowly warm to room temperature. The reaction was diluted with ethyl acetate (15 mL), and extracted with brine (30 mL). The organic layer was again washed with brine (20 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure to give **3** as crystals (525 mg, 1.04 mmols, 97% yield). An aliquot was further purified to remove trace impurities by column chromatography (SiO_2: $CHCl_3$) to yield **3** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.36 (s, 1H), 8.06 (d, 1H, *J* = 8.1 Hz), 8.01 (d, 1H, *J* = 8.3 Hz), 7.80 (s, 1H), 7.77 (s, 1H), 5.01 (d, 2H, *J* = 2.3 Hz), 3.68 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 164.0, 131.6, 131.1, 77.3, 75.7, 53.2, 35.6, 35.4. HRMS (EI) found *m/z* = 499.7273 (M^+), calculated for $C_{12}H_8Br_4O_2$: 499.7258. IR *v* (cm^{-1}): 3299, 3207, 2132, 1724.



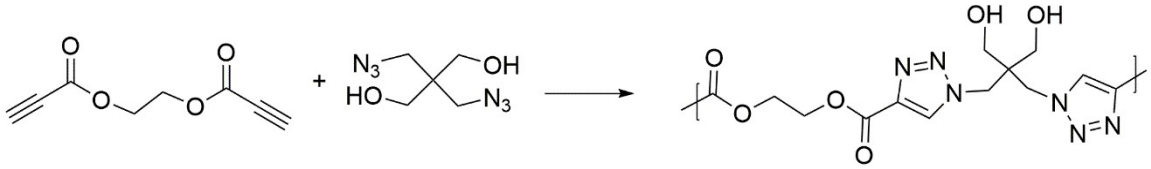
Prop-2-yn-1-yl 3,4-diformylbenzoate (**4**). A 10 mL round bottom flask was charged with **3** (53.5 mg, 0.106 mmol), acetone (4.0 mL), water (0.5 mL), a magnetic stir bar and purged with argon. Silver nitrate (76.2 mg, 0.449 mmol, 4.2 equiv) was added in one portion, and the reaction mixture was stirred overnight at room temperature. The reaction mixture was diluted with chloroform (100 mL) and passed through a pad of Celite. The organic layer was washed with potassium bromide solution (45 mg in 50 mL of water), dried over anhydrous MgSO4, filtered, and concentrated under reduced pressure. The crude product was further purified trituration with cyclohexane to yield compound **4** as a white solid (14.6 mg, 0.0675 mmol, 64% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.56 (s, 1H), 10.52 (s, 1H), 8.51 (s, 1H), 8.39 (d, 1H, *J* = 8.1 Hz), 8.10 (d, 1H, *J* = 8.0 Hz), 5.05 (d, 2H, *J* = 2.3 Hz), 3.70 (t, 1H, *J* = 2.3 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 191.7, 191.6, 164.0, 139.5, 136.6, 134.8, 134.2, 133.0, 130.9, 77.03, 75.98, 53.5. HRMS (EI) found *m/z* = 216.0428 (M^+), calculated for $C_{12}H_8O_4$: 216.0423. IR *v* (cm^{-1}): 3249, 2127, 1724, 1682.



Ethane-1,2-diyl dipropiolate (**7**). A 250 mL round bottom flask was charged with propiolic acid (10.1 mL, 11.4 g, 162 mmol), toluene (125 mL), ethylene glycol (4.59 mL, 5.11 g, 82.1 mmol, 0.5 equiv), and para-toluene sulfonic acid monohydrate (1.04 g, 5.47 mmol, 0.03 equiv), a magnetic stir bar, and a Merlic trap. The reaction mixture was stirred and heated to reflux overnight. The reaction mixture was allowed to cool, and the reaction mixture was diluted with ethyl acetate (100 mL) and hexanes (50 mL), and extracted with water (100 mL, 1x) and saturated $NaHCO_3$ (100 mL, 3x). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product was further purified by column chromatography (SiO_2: EtOAc) to yield **7** as a yellow oil (9.38 g, 56.5 mmol, 70% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.93 (s, 4H), 2.95 (s, 2H) matched previous literature reports.[1] ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.66 (s, 2H) 4.39 (s, 4H). IR *v* (cm^{-1}): 3272, 2965, 2119, 1708.

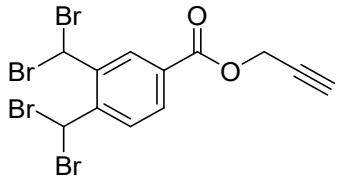


2,2-bis(azidomethyl)propane-1,3-diol (**8**). A three-neck 250 mL round bottom flask was charged with a magnetic stir bar, condenser, 2,2-bis(bromomethyl)-1-,3-propanediol (10.1 g, 38.4 mmol), and sodium azide (7.94 g, 122 mmol, 3.18 equiv). The flask was placed under argon, and anhydrous dimethylformamide (80 mL) was added. The reaction mixture was stirred and heated to 120˚C overnight. The reaction mixture was allowed to cool, and the reaction mixture was diluted with water (500 mL) and extracted with ethyl acetate (150 mL, 3x). The combined organic layers were washed with water (100 mL, 5x) and saturated NH_4Cl (150 mL, 1x), and dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product was further purified with a silica plug (SiO_2: EtOAc) to yield **8** as a yellow oil (5.38 g, 28.9 mmol, 75% yield). ^{1}H NMR (400 MHz, $CDCl_3$) 3.64 (s, 4H), 3.43 (s, 4H), 2.03 (s, 2H) was similar to previous literature reports.[2] ^{1}H NMR (400 MHz, D_2O) 3.50 (s, 4H), 3.40 (s, 4H). ^{1}H NMR (400 MHz, DMSO-d_6) 4.76 (t, 2H, *J* = 4.96 Hz), 3.29 (s, 4H), 3.27 (d, 4H, *J* = 4.96 Hz). IR *v* (cm^{-1}): 3346, 2935, 2887, 2092.



Neat polymer (**9**). A vial open to ambient conditions was charged with **7** (50 mg, 0.30 mmol) and **8** (56 mg, 0.30 mmol), vortexed briefly, and allowed to react overnight at room temperature. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.67 (br, 2H), 5.09 (br, 2H), 4.93 (br, 2H), 4.61 (br, 4H), 4.50 (br, 4H), 3.24 (br, 2H), 3.16 (br, 4H).

NMR Characterization



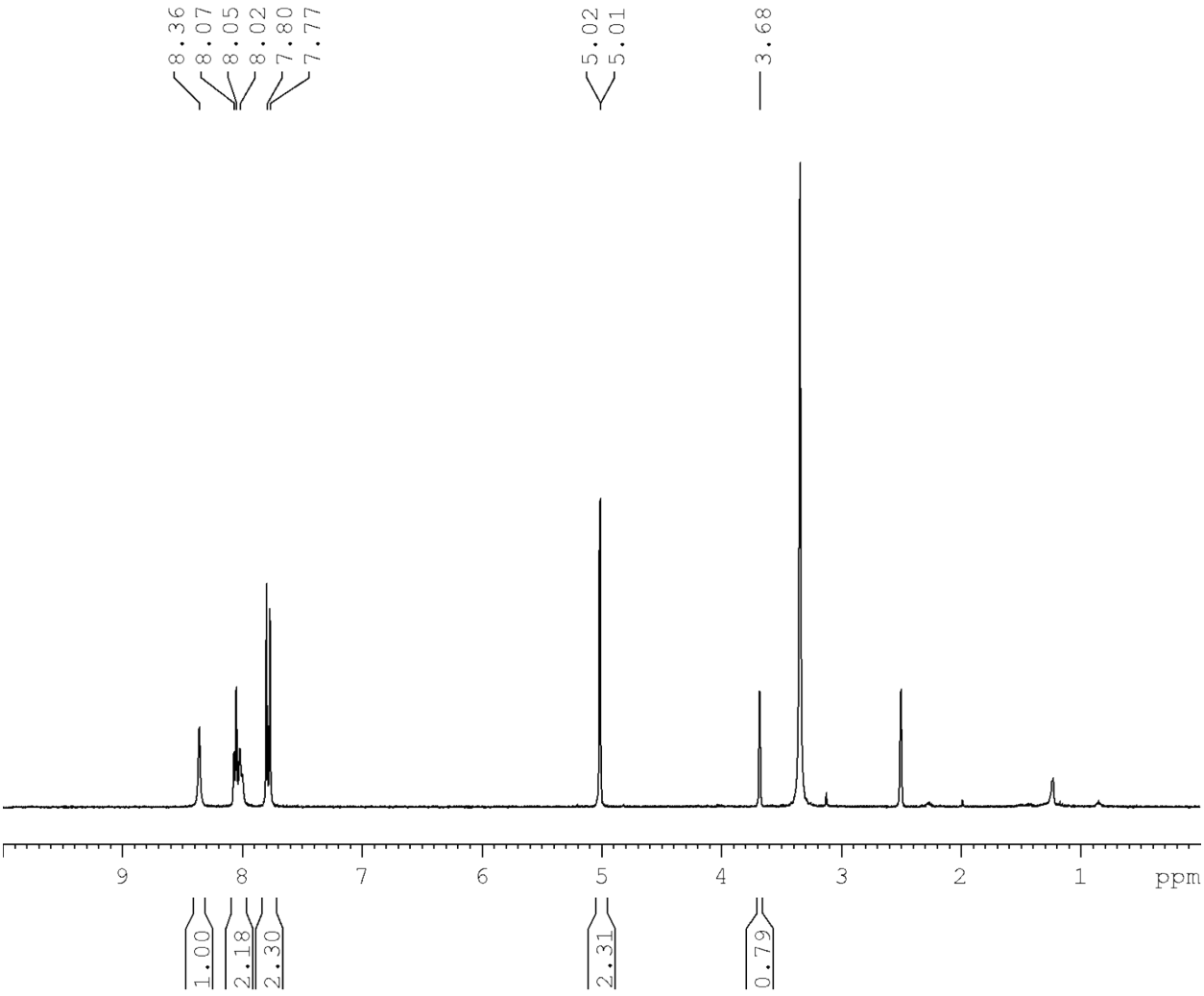


Figure S1. ^{1}H NMR spectrum of **3** (400 MHz, DMSO-d_6).

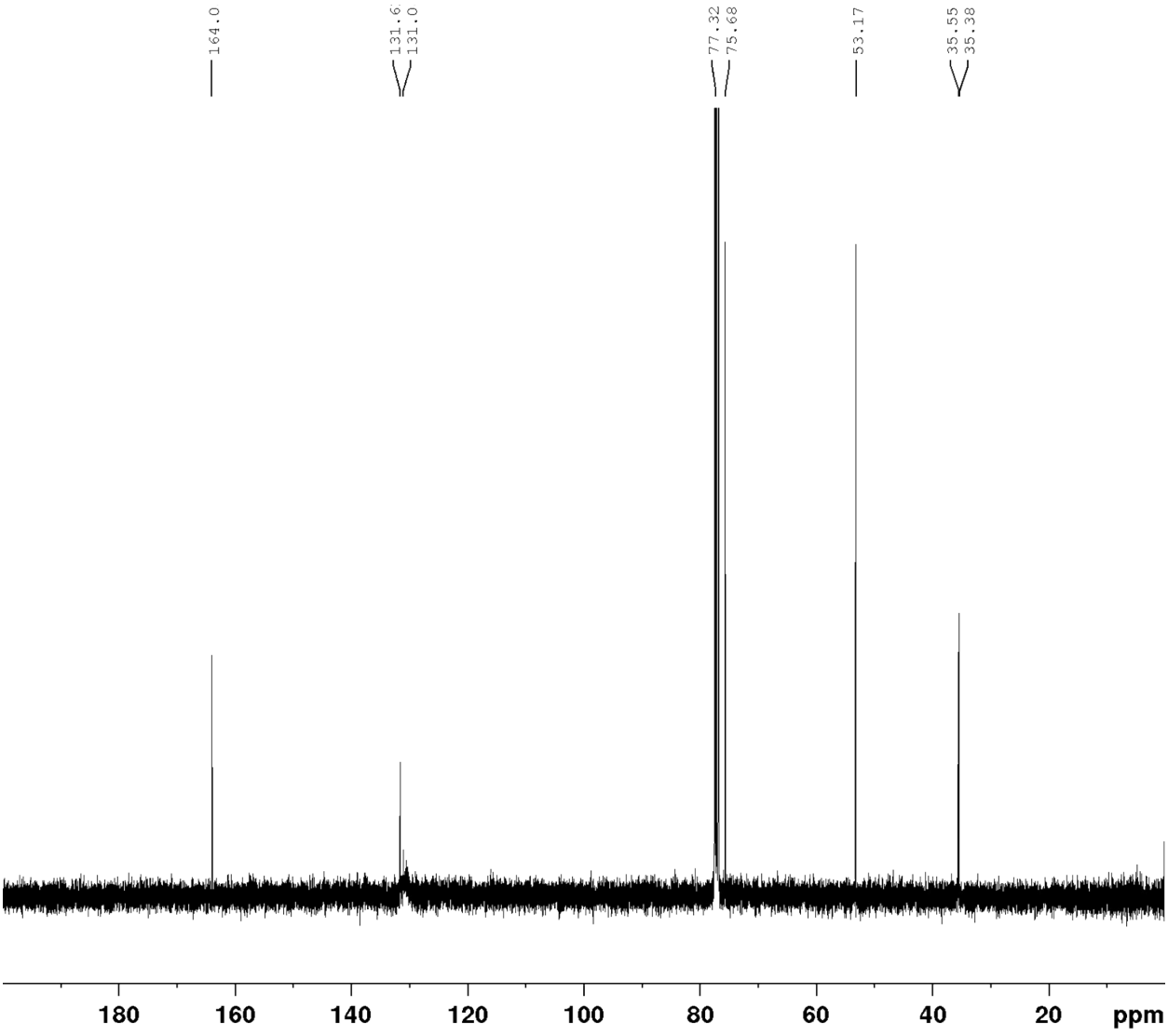


Figure S2. ^{13}C NMR spectrum of **3** (100 MHz, $CDCl_3$).

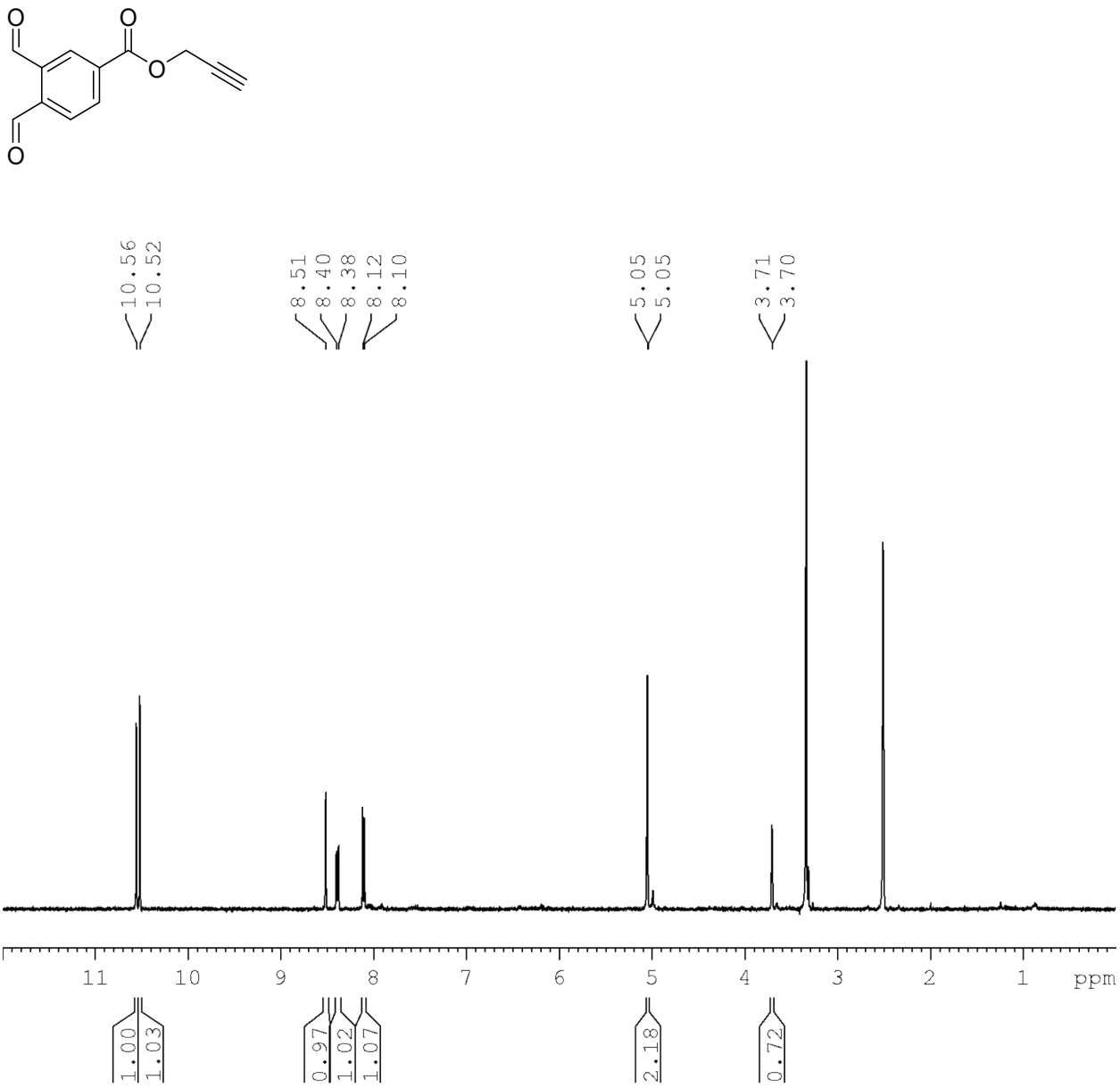


Figure S3. ^{1}H NMR spectrum of **4** (400 MHz, DMSO-d_6).

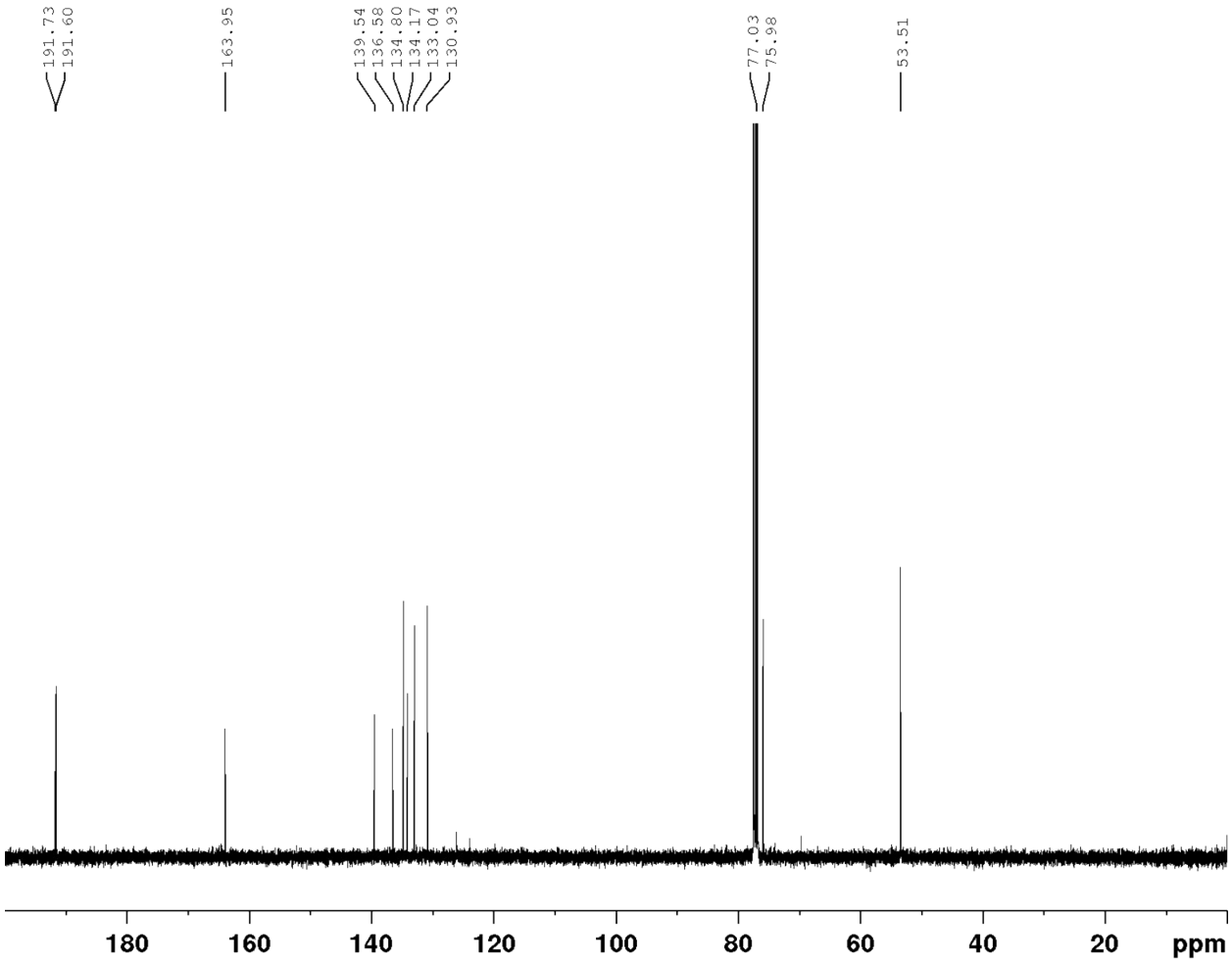


Figure S4. ^{13}C NMR spectrum of **4** (100 MHz, $CDCl_3$).

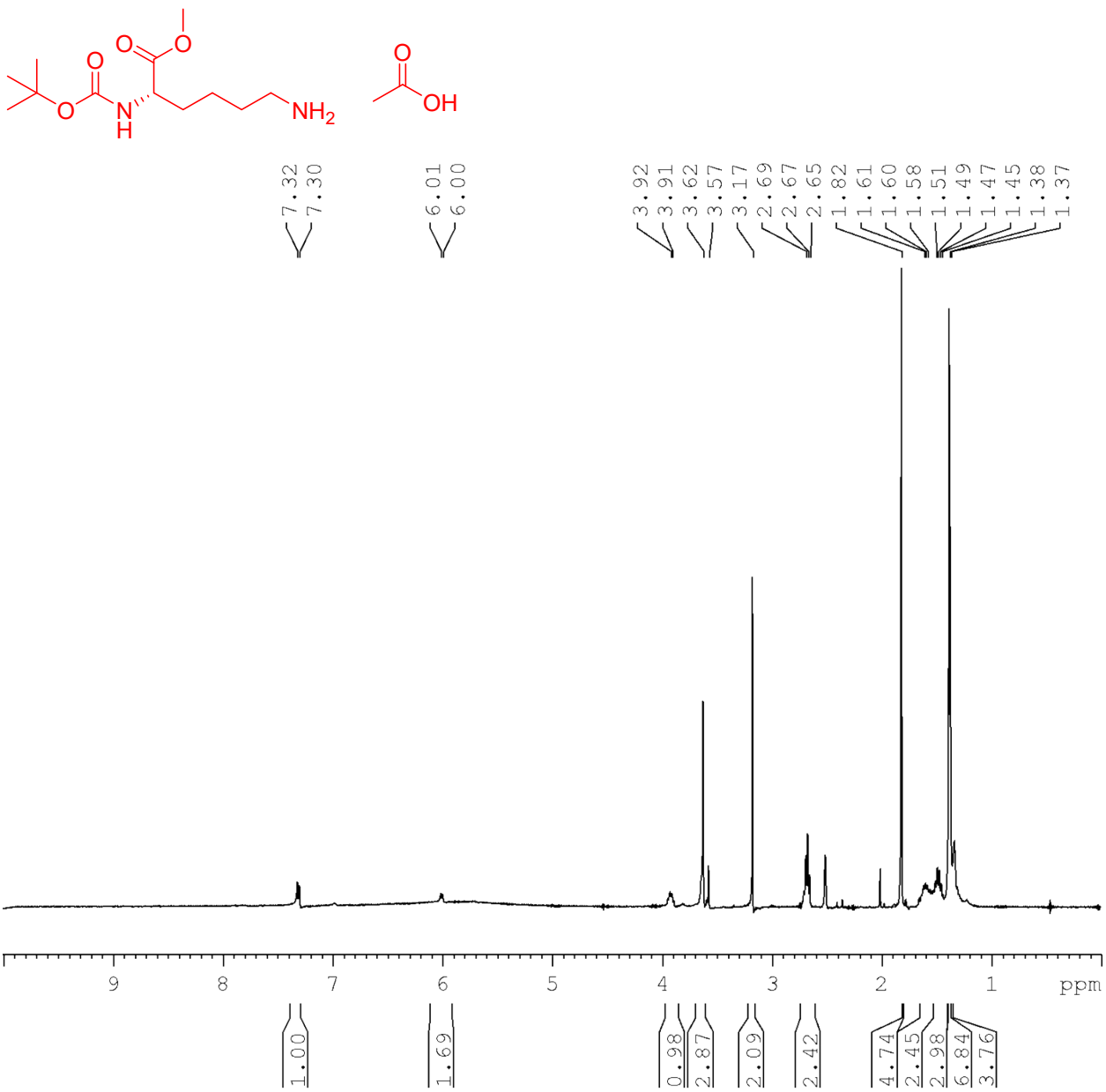


Figure S5. ^{1}H NMR spectrum of **5** (400 MHz, D_2O).

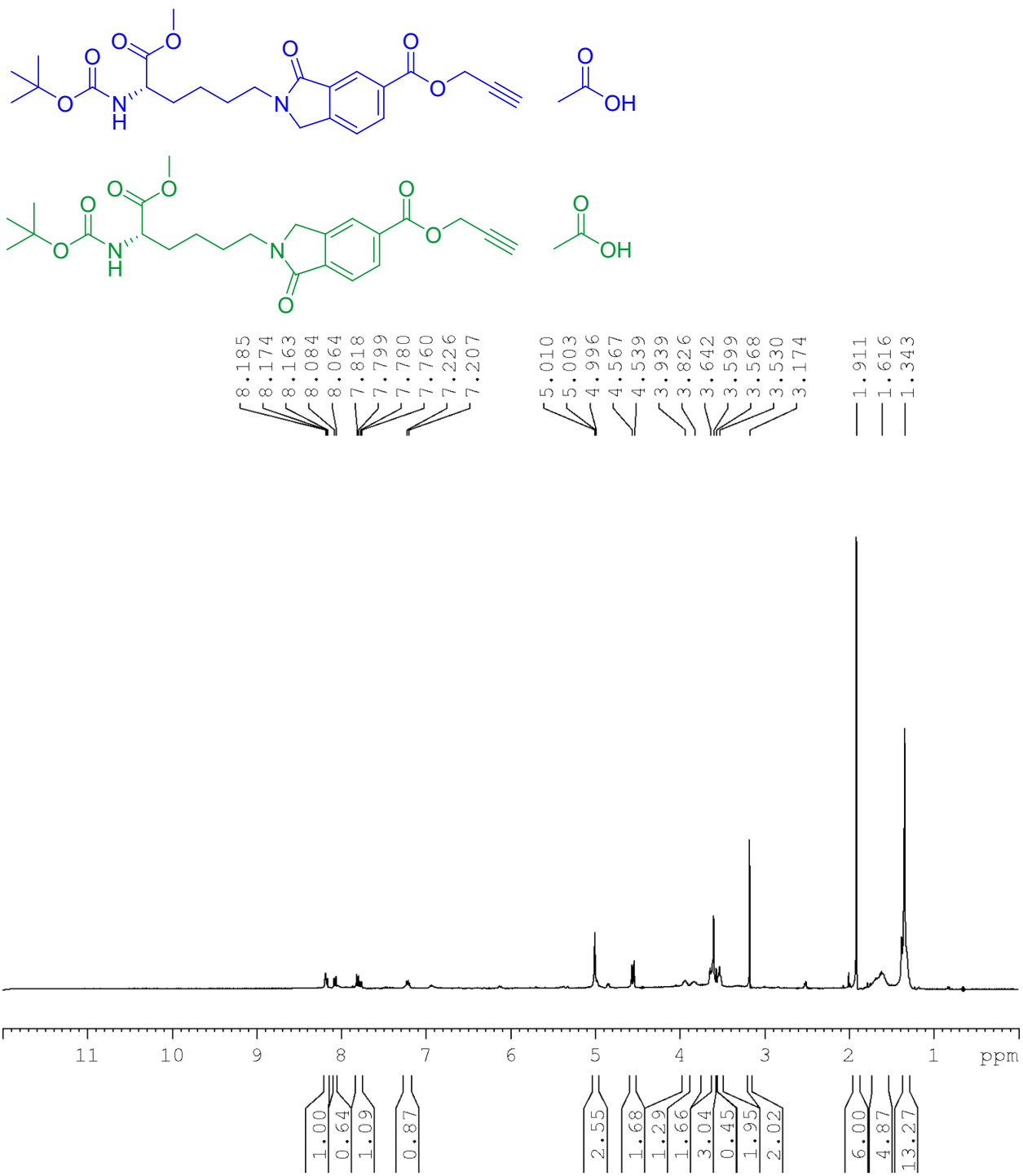


Figure S6. ^{1}H NMR spectrum of **6** (400 MHz, DMSO-d_6) showing both regioisomers.

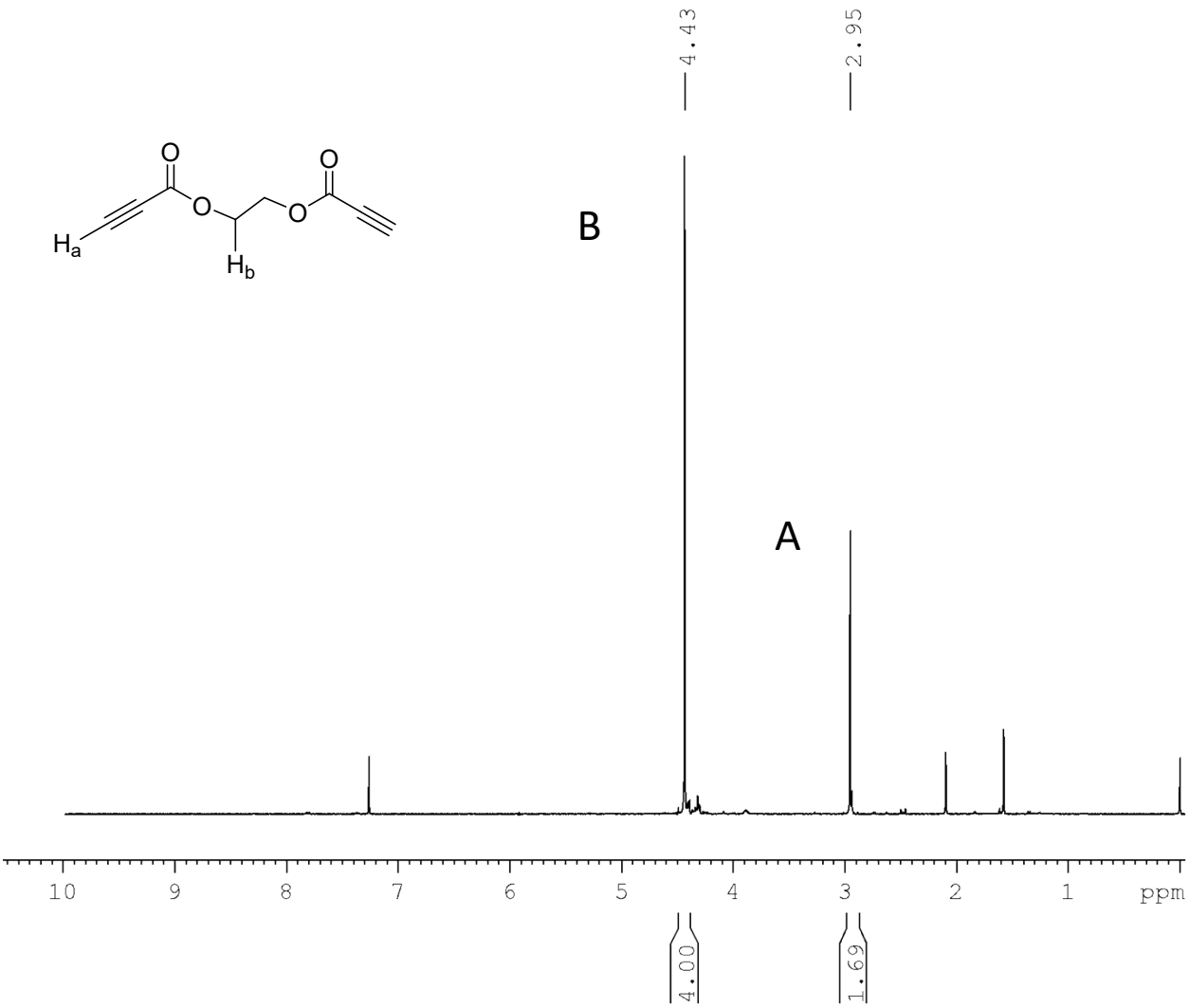


Figure S7. ^{1}H NMR spectrum of **7** (400 MHz, $CDCl_3$).

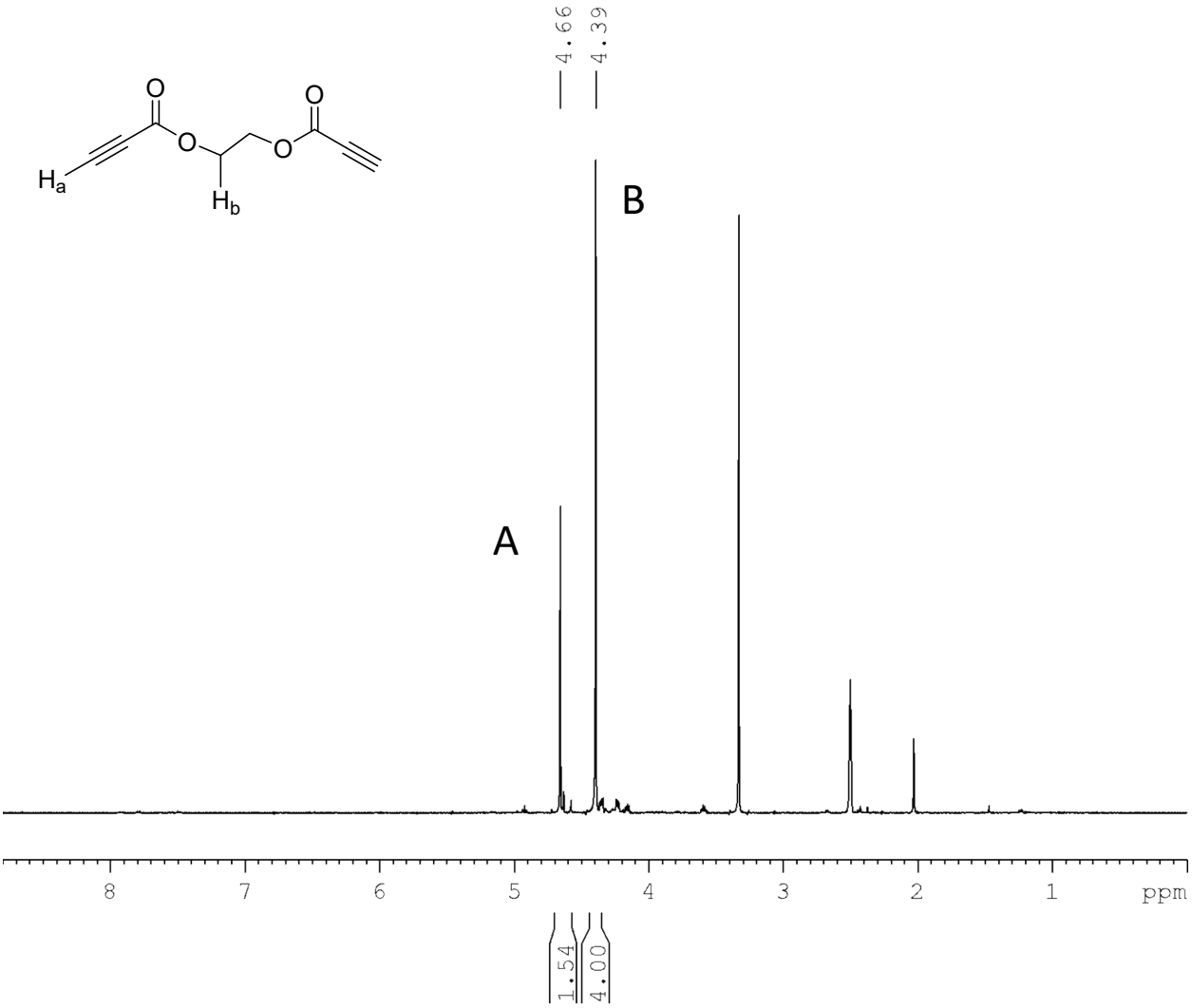


Figure S8. ^{1}H NMR spectrum of **7** (400 MHz, DMSO-d_6).

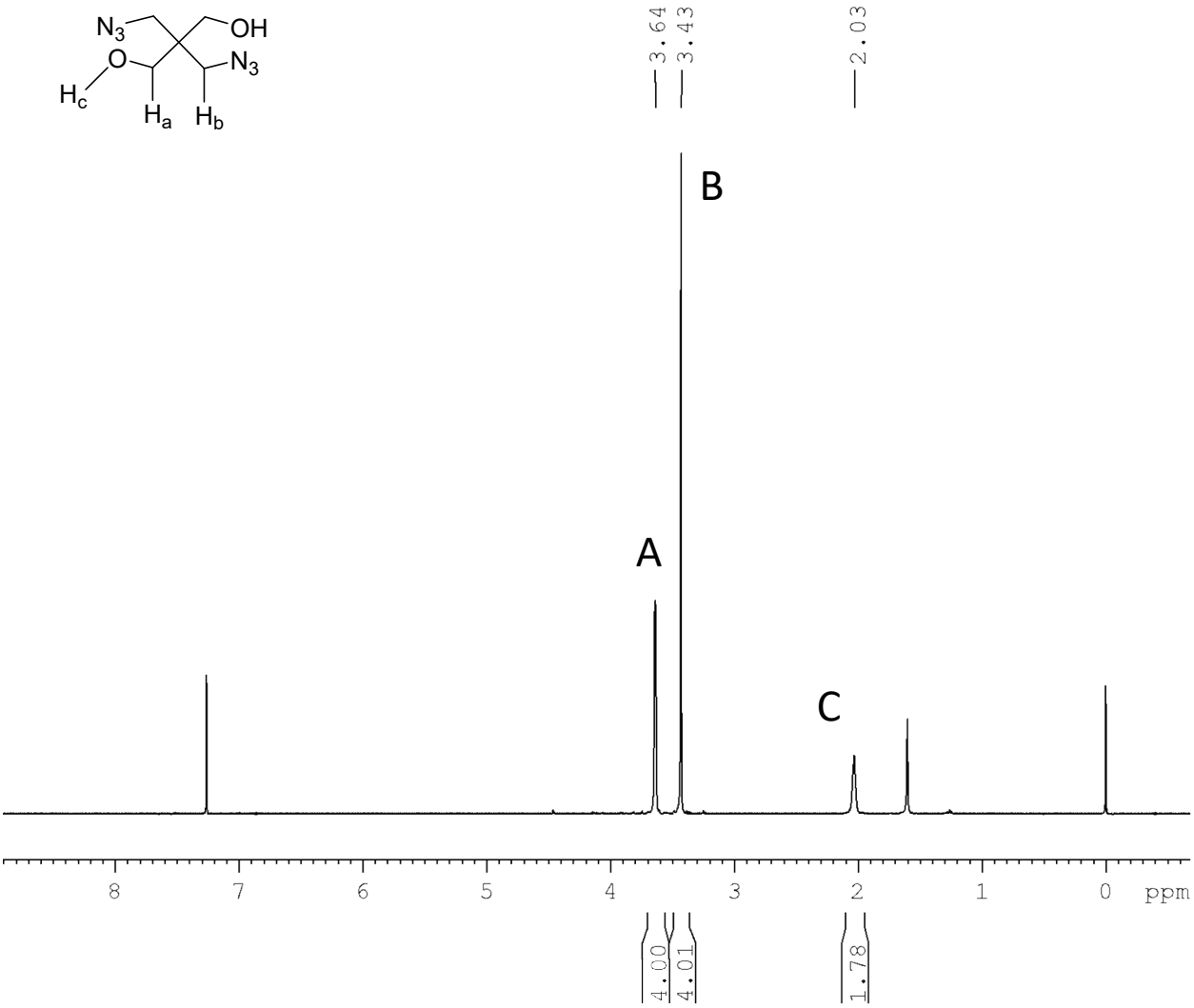


Figure S9. ^{1}H NMR spectrum of **8** (400 MHz, $CDCl_3$).

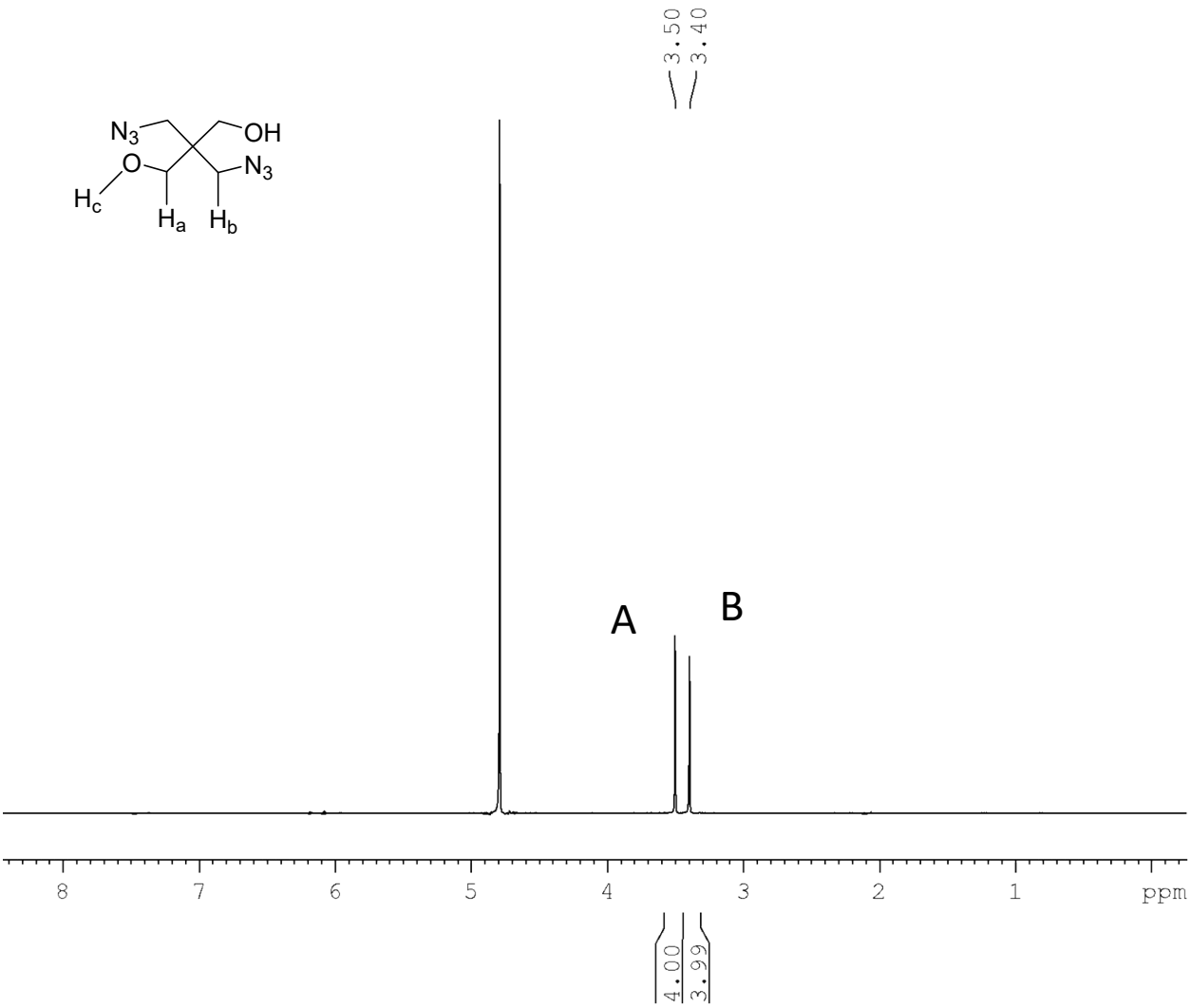


Figure S10. ^{1}H NMR spectrum of **8** (400 MHz, D_2O).

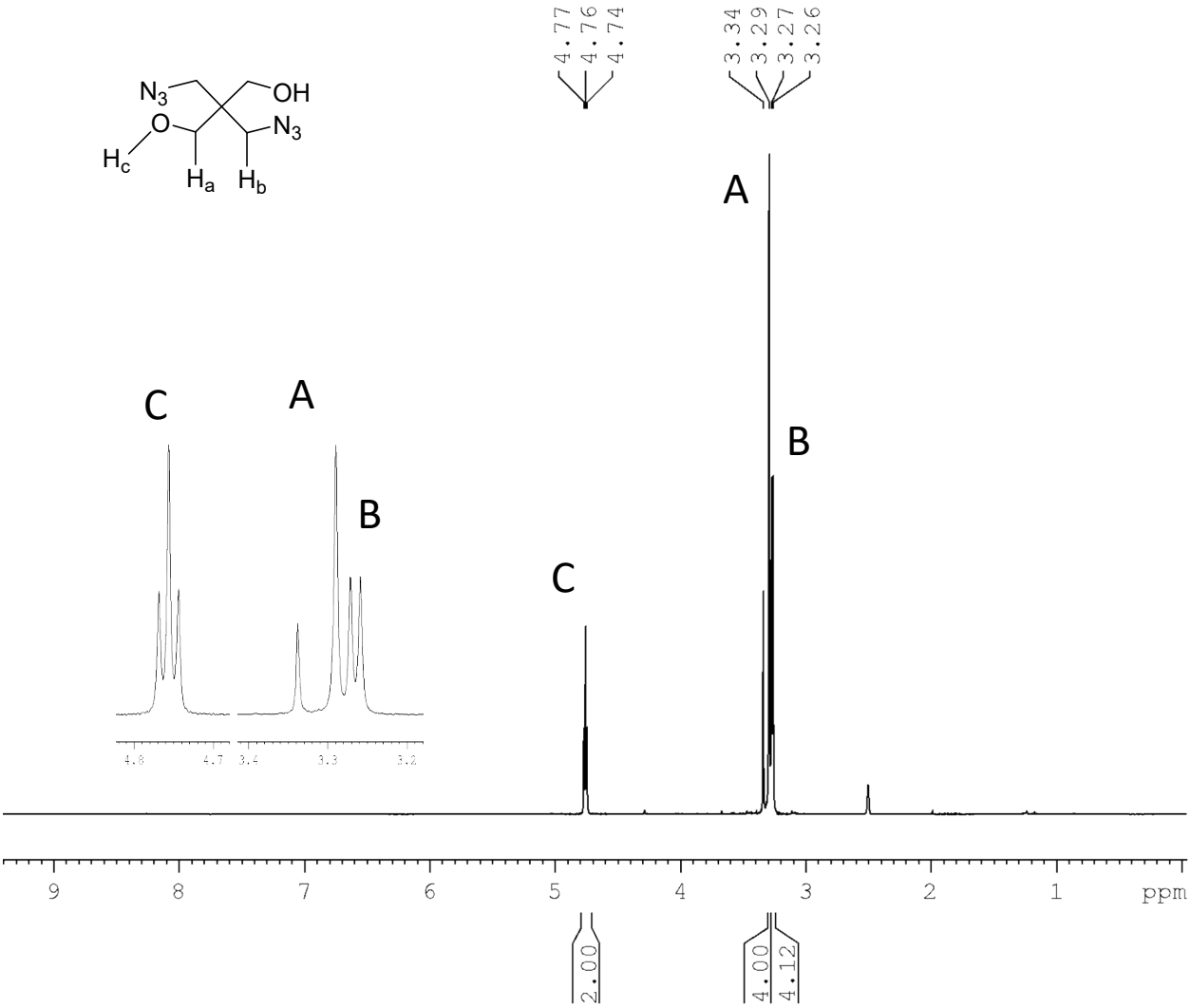


Figure S11. ^{1}H NMR spectrum of **8** (400 MHz, DMSO-d_6).

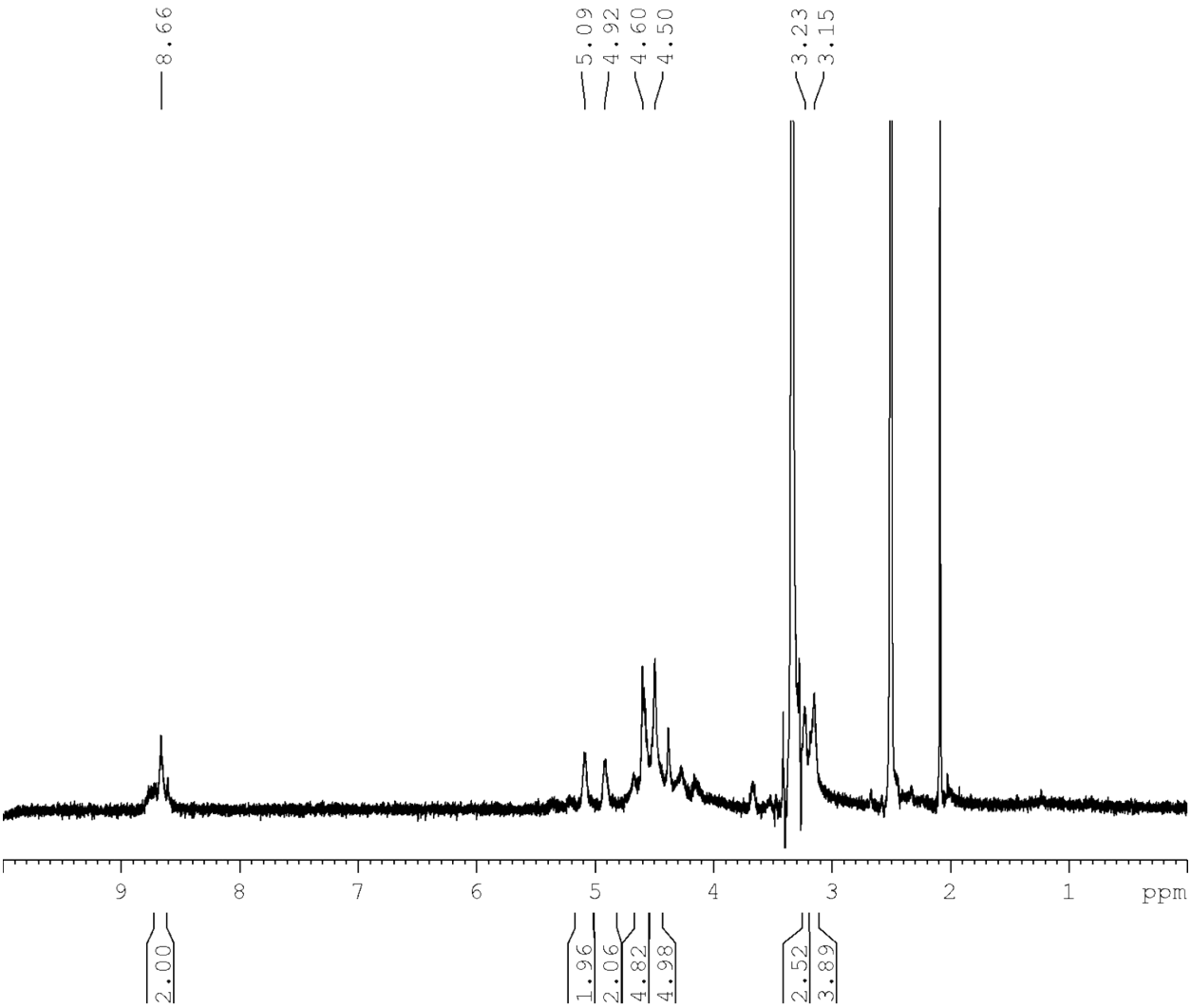


Figure S12. ^{1}H NMR spectrum of **9** made in H_2O:DMSO mixture and precipitated with water (400 MHz, DMSO-d_6).

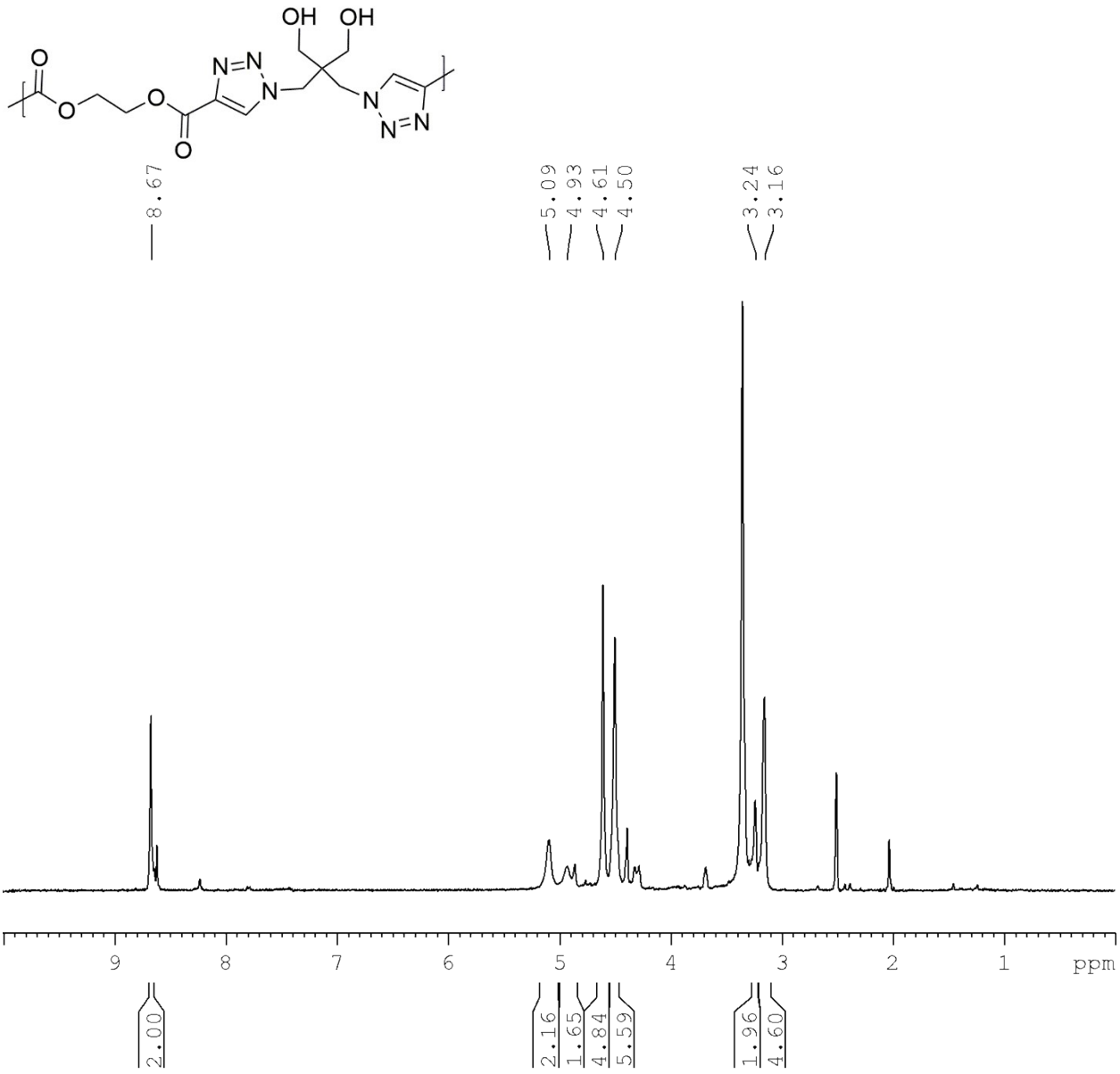


Figure S13. ^{1}H NMR spectrum of **9** (400 MHz, DMSO-d_6).

IR Characterization

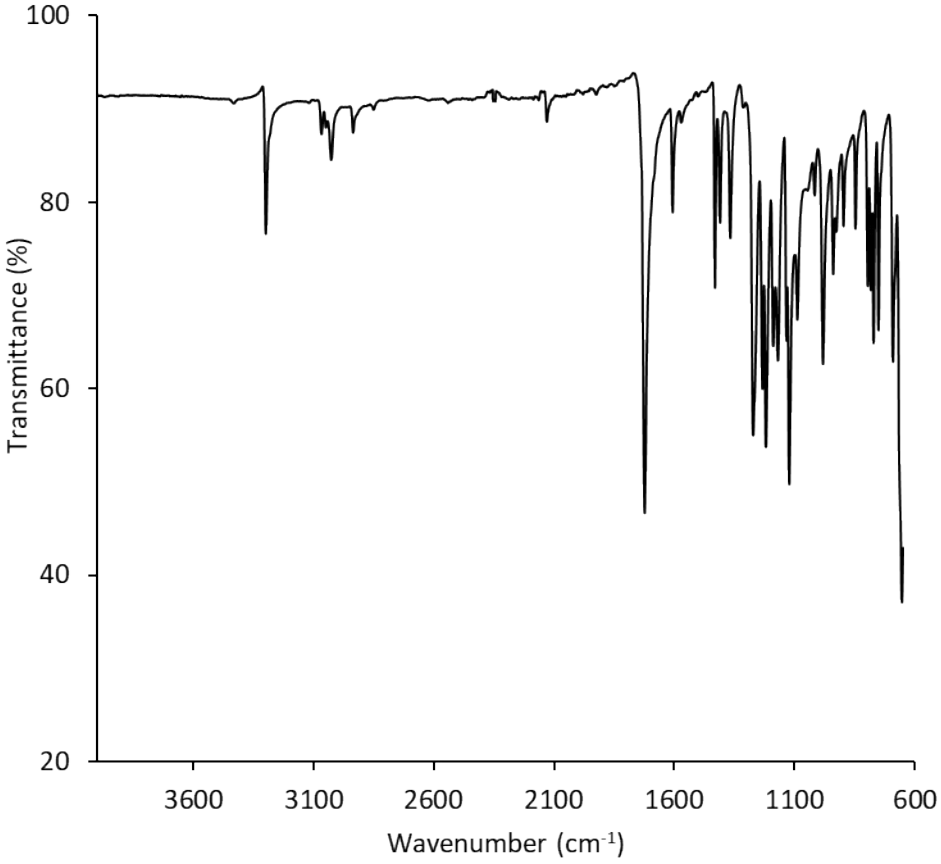


Figure S14. IR spectrum of **3**.

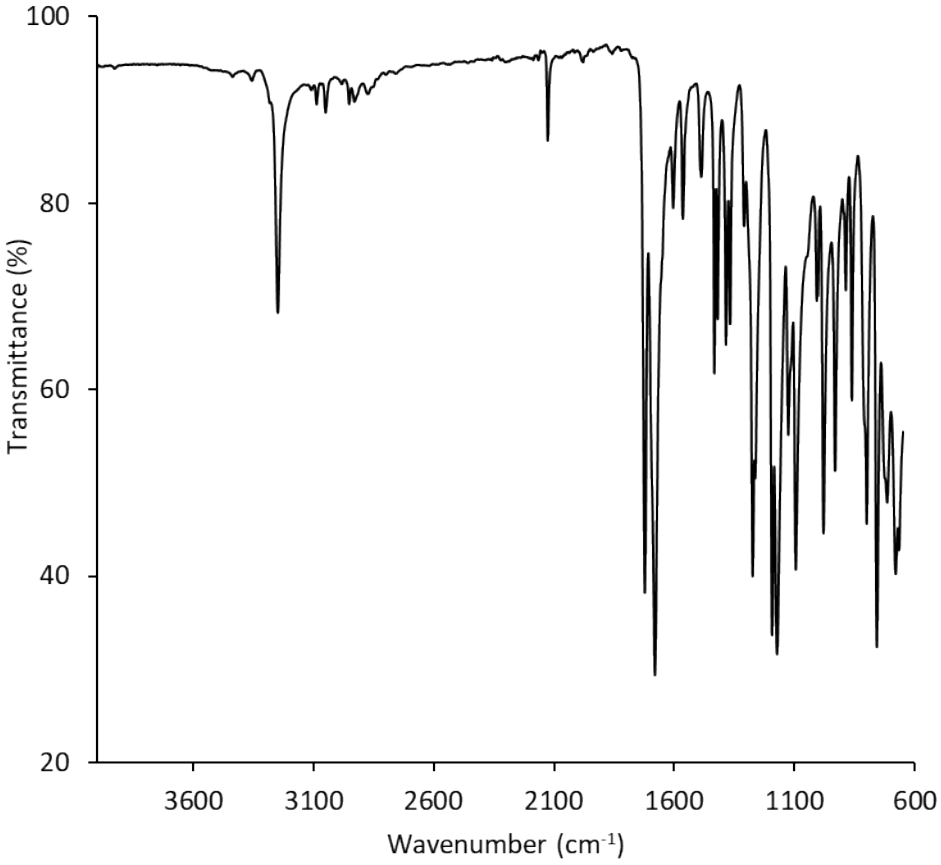


Figure S15. IR spectrum of **4**.

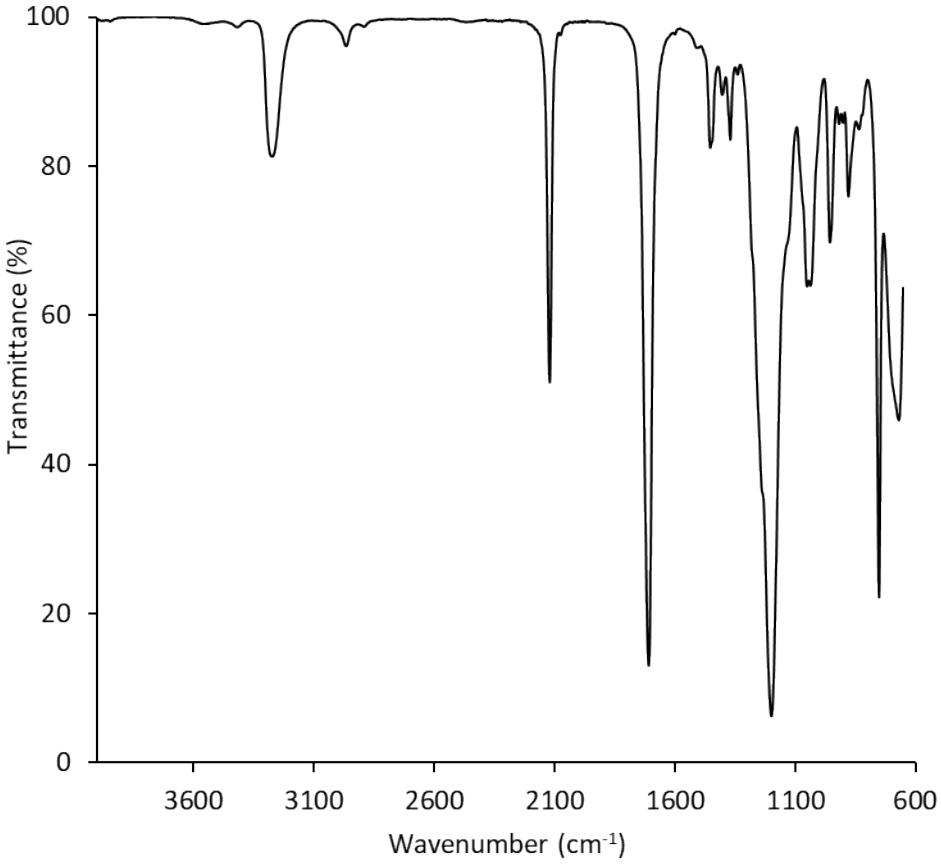


Figure S16. IR spectrum of **7.**

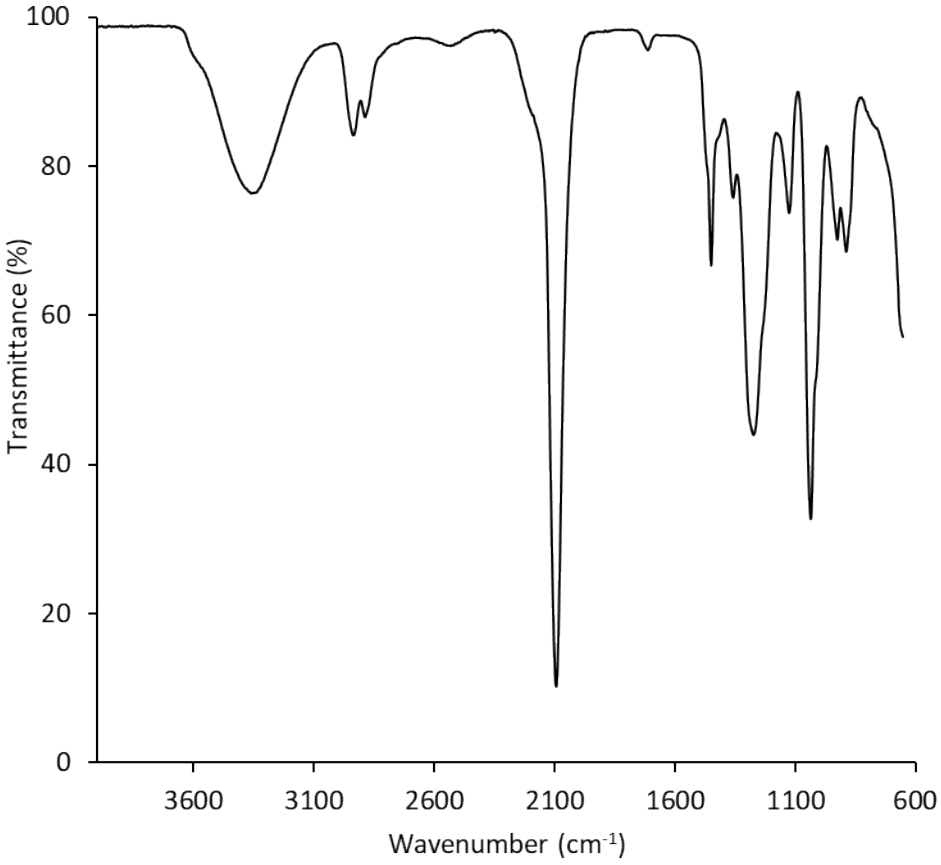


Figure S17. IR spectrum of **8**.

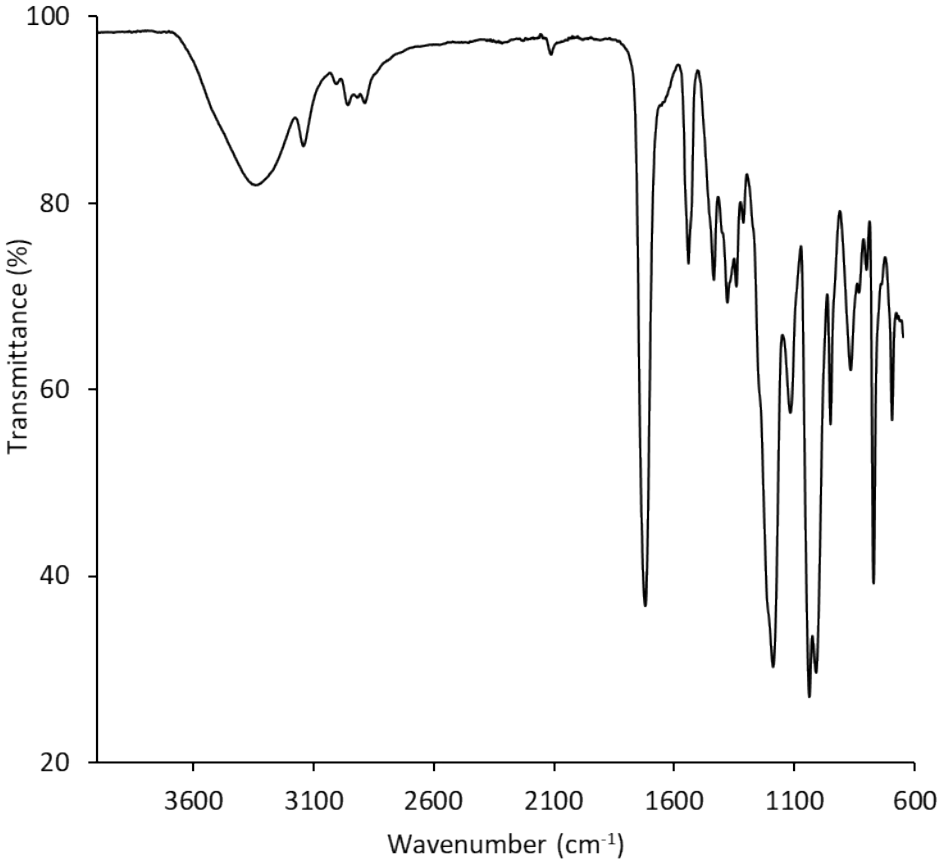


Figure S18. IR spectrum of neat **9**.

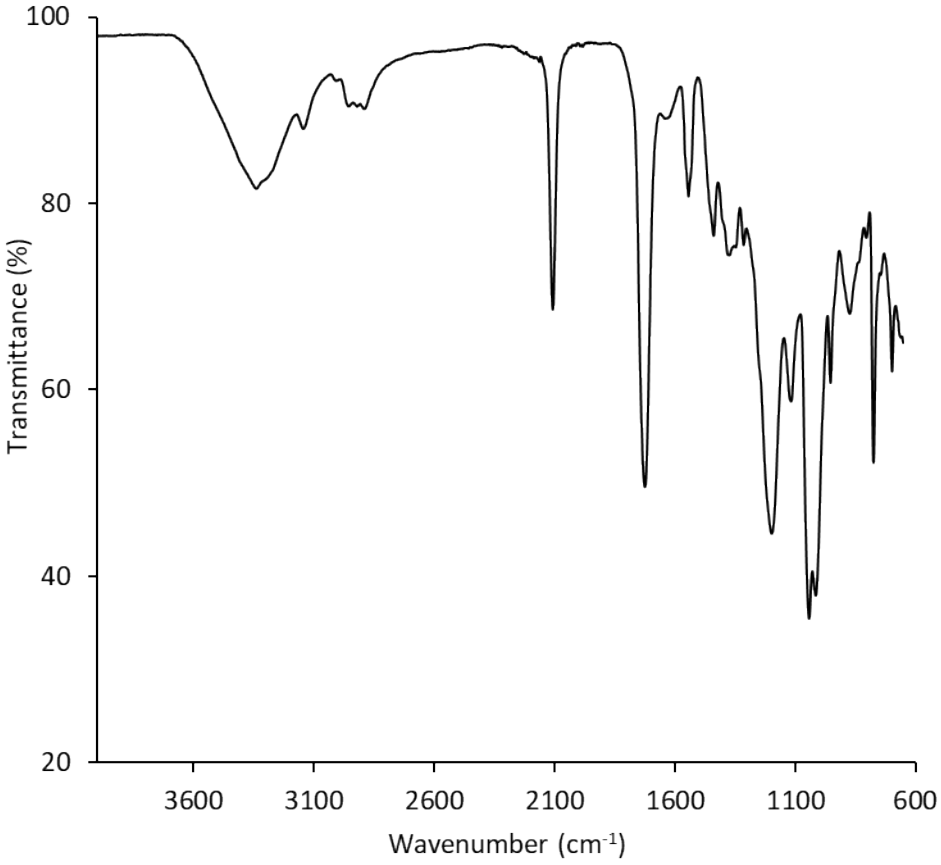


Figure S19. IR spectrum of **9** polymerized in solvents (1:1 water: DMSO).

UV-Visible Characterization

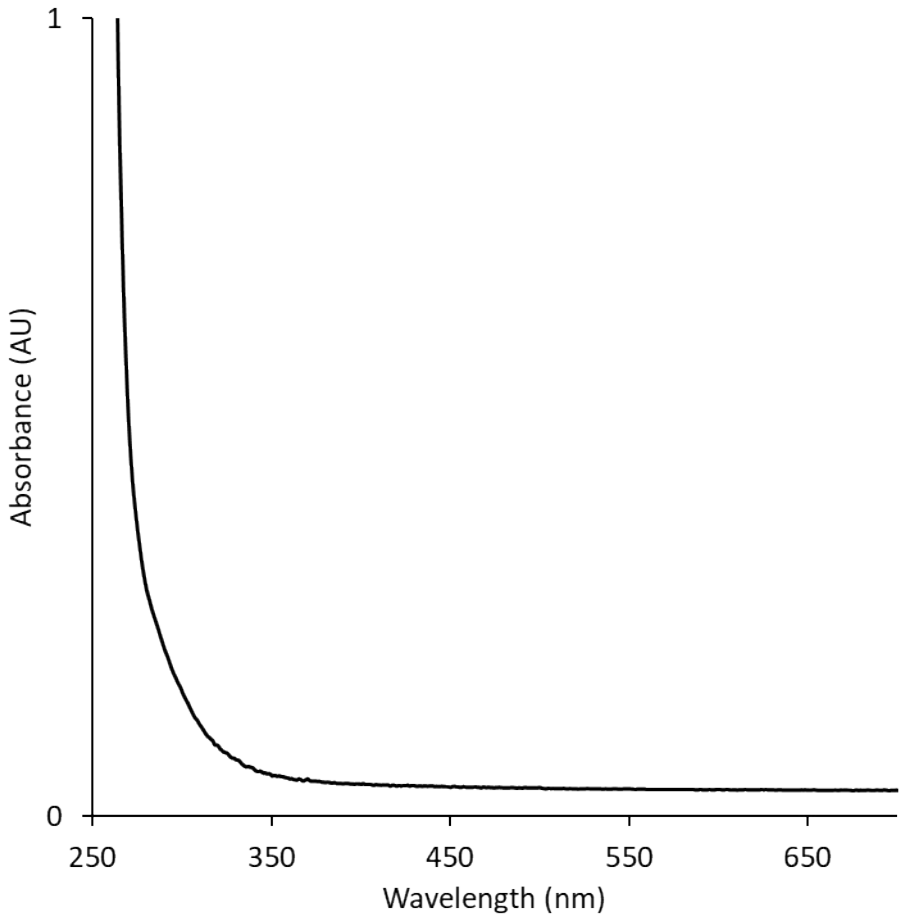


Figure S20. UV-Vis spectrum of neat **9**.

Thermal Properties

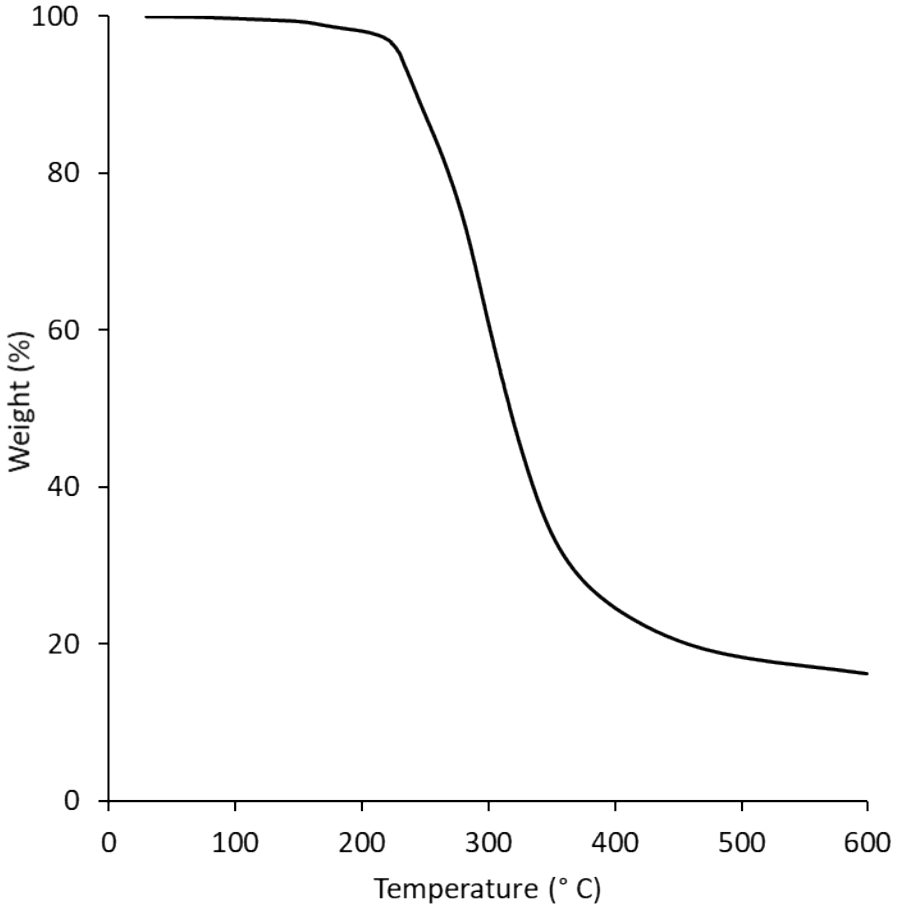


Figure S21. TGA thermogram of neat polymer **9**.

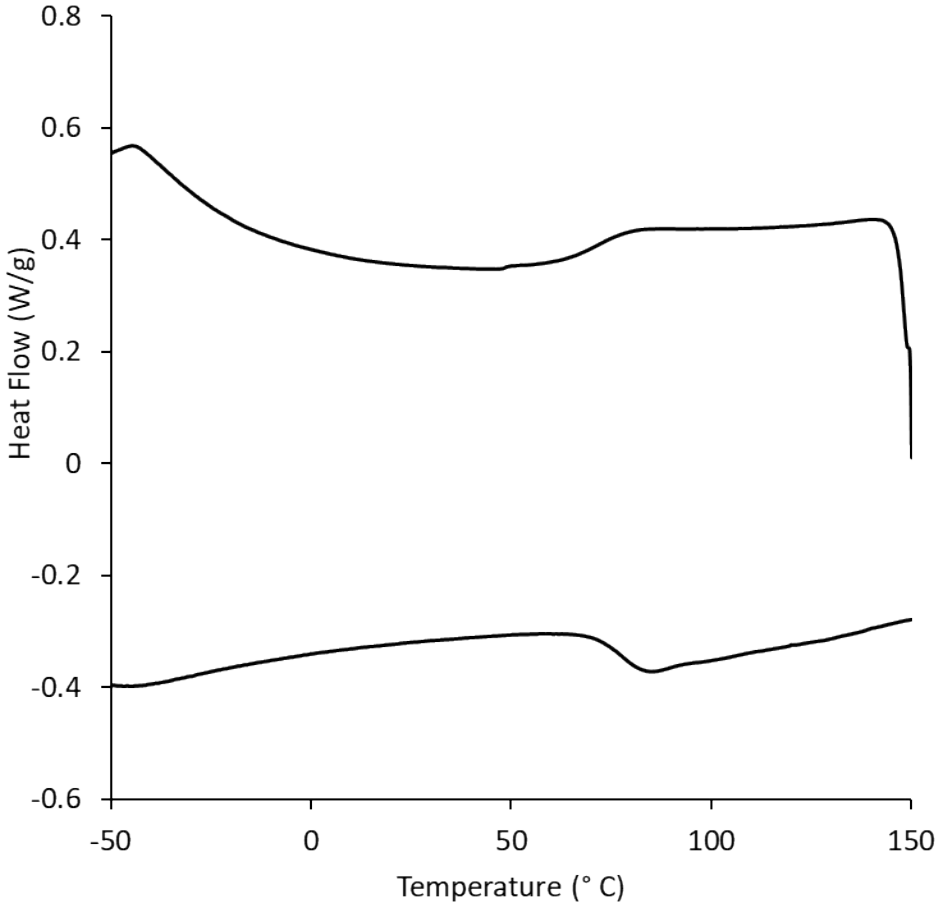


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Figure S22. DSC thermogram of neat polymer **9**.

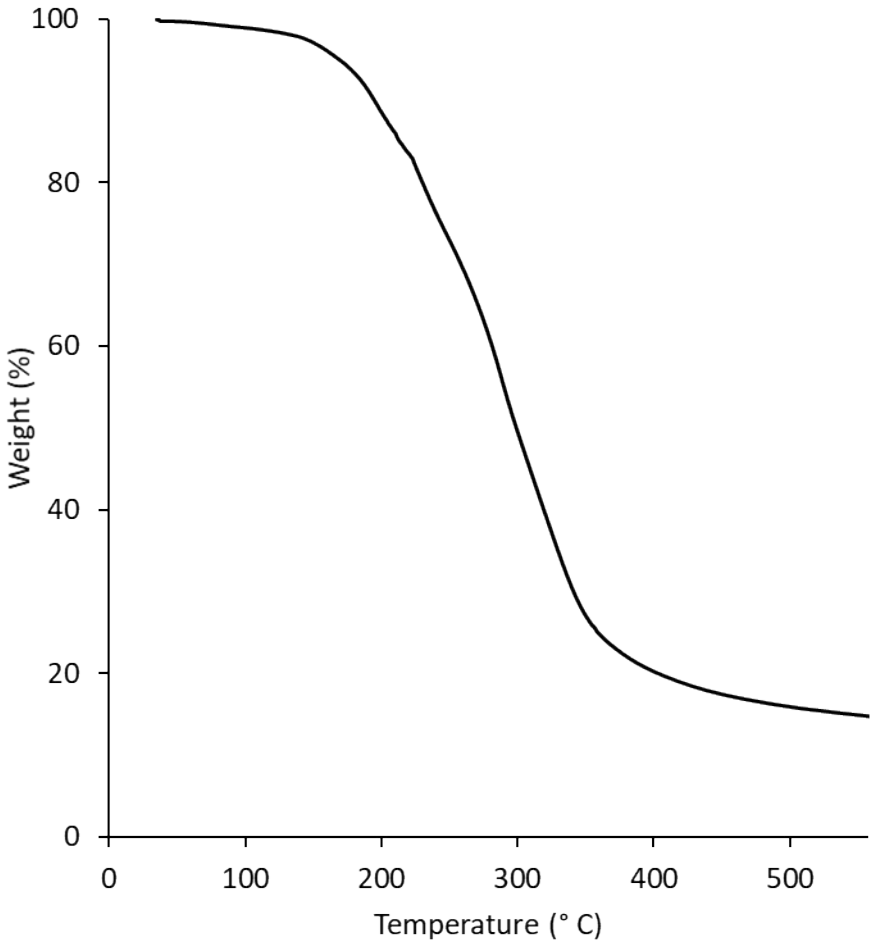

Figure S23. TGA thermogram of polymer **9** polymerized in solvents (1:1 water: DMSO).

Mass Spectrometry

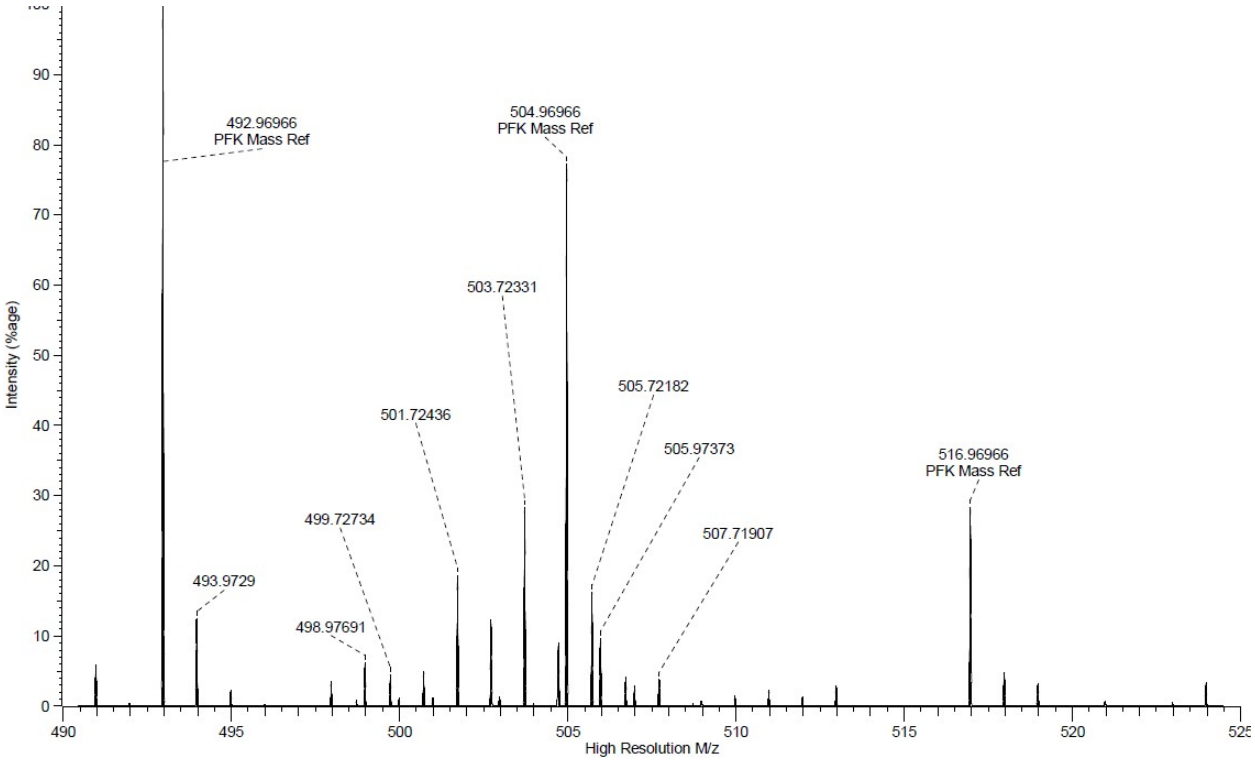

Figure S24. HRMS of **3**.

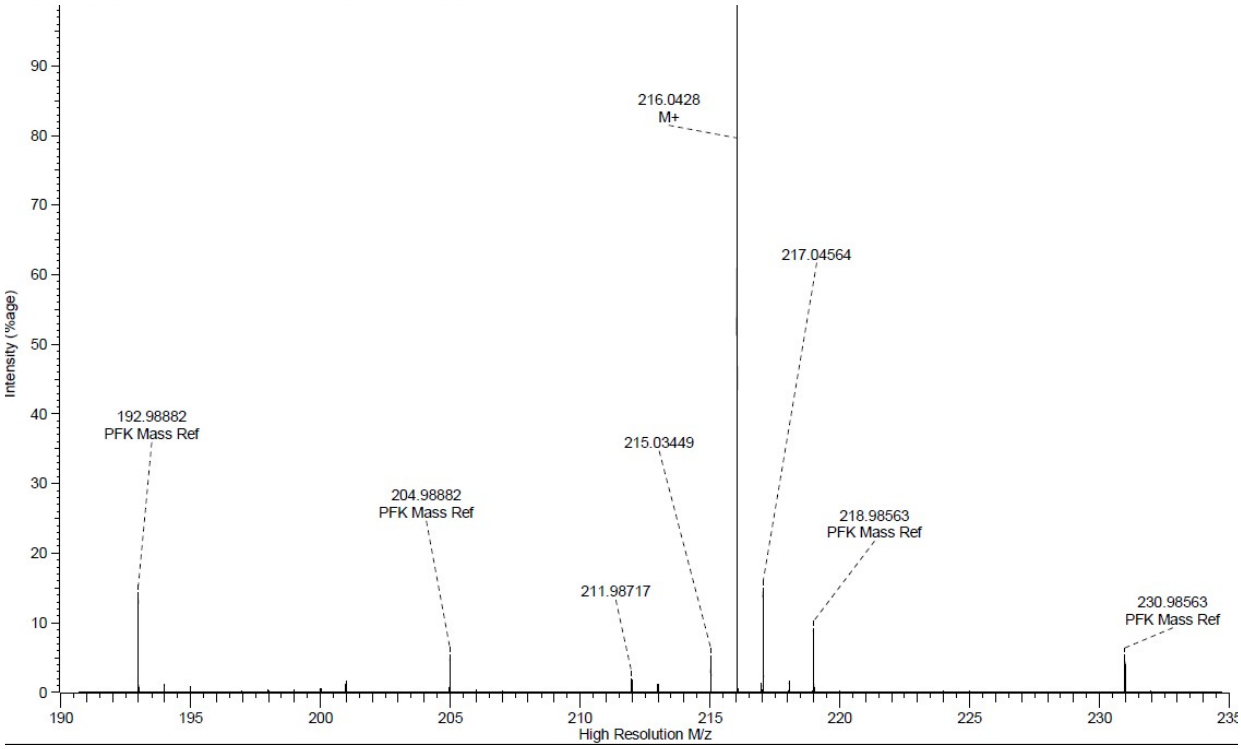

Figure S25. HRMS of **4**.

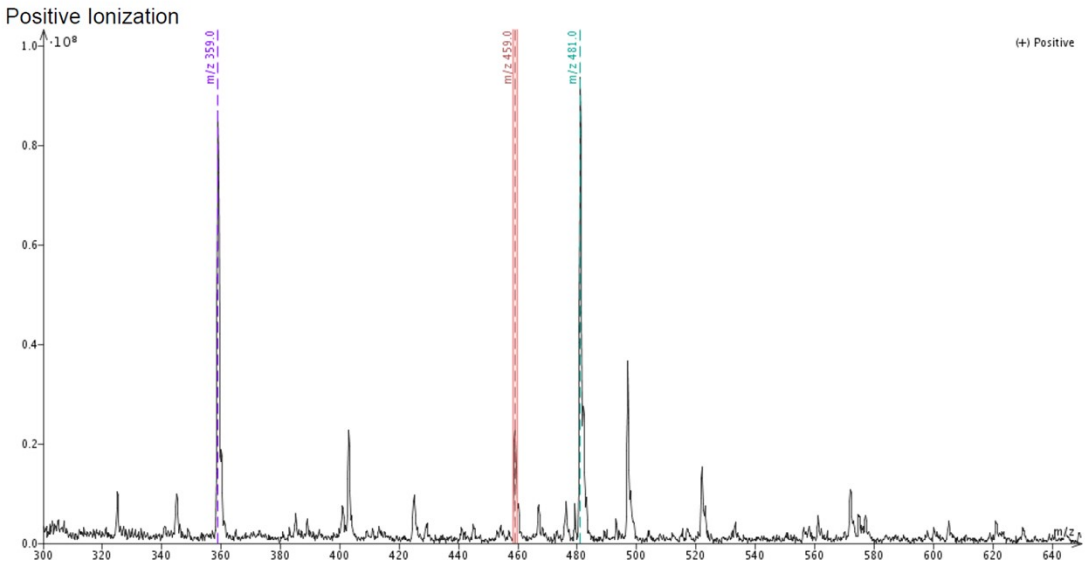

Figure S26. LRMS of **6**. LRMS (ESI) found m/z = 459.0 (M^+), calculated for $C_{24}H_{30}N_2O_7$: 458.2. LRMS (ESI) found m/z = 481.0 (M^+ + Na), calculated for $C_{24}H_{30}N_2O_7Na$: 481.2.

	Coverage	Total matches	Sequences	Sequences with modification
1:1 oPA	99%	456	40	19
1:10 oPA	61%	46	15	5
Maleimide	100%	498	77	2
No linker	99%	435	42	-

Figure S27. Summary of Mass Peptide Fingerprinting results

Lysine position	Site analysis probability	Interfering positions
15	Not covered	
45	49.95	47
47	49.95	45
50	Not detected	
70	Not detected	
74	100	
83	50	84
84	100	
92	99.99	
121	Not detected	
123	99.96	
138	89.16	
139	100	
158	99.22	
163	48.04	158
166	99.95	
168	100	
178	100	
184	Not detected	
185	99.83	
198	100	

Figure S28. Summary of site analysis used to identify lysines reactive toward oPA in 1:1 sample. Middle column shows the highest score from across all sequences. Right column shows positions causing ambiguities (i.e., appears in the same peptide fragment and not differentiated by fragmentation pattern).

Query	Residue before	Peptide Sequence	Residue After	K position in sequence	Site Probability	K position in sequence	Site Probability	K position in sequence	Site Probability
4651	R	DGVLKGETHK	A	158	72.04	163	27.96		
4652	R	DGVLKGETHK	A	158	68.08	163	31.92		
4653	R	DGVLKGETHK	A	158	86.67	163	13.33		
4654	R	DGVLKGETHK	A	158	99.22	163	0.78		
4655	R	DGVLKGETHK	A	158	99.64	163	0.36		
4656	R	DGVLKGETHK	A	158	51.96	163	48.04		
4658	R	DGVLKGETHK	A	158	84.09	163	15.91		
5910	K	KTMGWEASTER	L	139	100				
5911	K	KTMGWEASTER	L	139	99.94				
5912	K	KTMGWEASTER	L	139	100				
5913	K	KTMGWEASTER	L	139	100				
6400	K	LSFPEGFKWER	V	92	99.99	95	0.01		
6401	K	LSFPEGFKWER	V	92	99.93	95	0.07		
6404	K	LSFPEGFKWER	V	92	87.67	95	12.33		
6408	R	DGVLKGETHKALK	L	158	55.69	163	4.05	166	40.25
6409	R	DGVLKGETHKALK	L	158	89.57	163	9.87	166	0.57
6410	R	DGVLKGETHKALK	L	158	99.54	163	0	166	0.27
6411	R	DGVLKGETHKALK	L	158	95.18	163	1.39	166	3.43
6412	R	DGVLKGETHKALK	L	158	25.87	163	9.9	166	64.23
6413	R	DGVLKGETHKALK	L	158	42.17	163	56.89	166	0.94
6414	R	DGVLKGETHKALK	L	158	19.73	163	5.76	166	74.51
6491	K	LKDGGHYLVEFK	S	168	100				
6493	K	LKDGGHYLVEFK	S	168	99.99				
6494	K	LKDGGHYLVEFK	S	168	100				
6495	K	LKDGGHYLVEFK	S	168	99.1				
6496	K	LKDGGHYLVEFK	S	168	99.99				
6497	K	LKDGGHYLVEFK	S	168	99.64				
6498	K	LKDGGHYLVEFK	S	168	99.94				
6499	K	LKDGGHYLVEFK	S	168	100				
6500	K	LKDGGHYLVEFK	S	168	99.69				
6501	K	LKDGGHYLVEFK	S	168	99.84				
6502	K	LKDGGHYLVEFK	S	168	99.97				
6503	K	LKDGGHYLVEFK	S	168	99.87				
6504	K	LKDGGHYLVEFK	S	168	99.98				
6505	K	LKDGGHYLVEFK	S	168	99.83				
6507	K	LKDGGHYLVEFK	S	168	100				
6508	K	LKDGGHYLVEFK	S	168	99.95				
6509	K	LKDGGHYLVEFK	S	168	99.9				
6510	K	LKDGGHYLVEFK	S	168	99.98				
7253	K	KLSFPEGFKWER	V	84		92	94.38	95	5.62
7254	K	KLSFPEGFKWER	V	84	100	92		95	
7255	K	KLSFPEGFKWER	V	84		92	49.97	95	49.97
7256	K	KLSFPEGFKWER	V	84	0.01	92	99.9	95	0.1
7257	K	KLSFPEGFKWER	V	84		92	99.86	95	0.14
7258	K	KLSFPEGFKWER	V	84		92	99.13	95	0.87
7259	K	KLSFPEGFKWER	V	84	0.1	92	99.74	95	0.17
7260	K	KLSFPEGFKWER	V	84	8.93	92	90.95	95	0.12
7356	K	VYVKHPADIPDYK	K	74	99.45	83	0.55		
7357	K	VYVKHPADIPDYK	K	74	100	83			
7358	K	VYVKHPADIPDYK	K	74	100	83			
7359	K	VYVKHPADIPDYK	K	74	100	83			
7360	K	VYVKHPADIPDYK	K	74	99.8	83	0.02		
7361	K	VYVKHPADIPDYK	K	74	99.81	83	0.19		
7684	R	LYPRDGVLKGETHK	A	153	1.44	158	49.11	163	49.45
7685	R	LYPRDGVLKGETHK	A	153	0.13	158	69.23	163	30.64
7686	R	LYPRDGVLKGETHK	A	153	1.12	158	75.74	163	23.14
7834	K	KPVQLPGYYYVDAK	L	198	100				
7835	K	KPVQLPGYYYVDAK	L	198	100				
7836	K	KPVQLPGYYYVDAK	L	198	100				
7996	K	VYVKHPADIPDYKK	L	74		83	50	84	50
7997	K	VYVKHPADIPDYKK	L	74	98.35	83		84	
7998	K	VYVKHPADIPDYKK	L	74	99.99	83	0	84	0
7999	K	VYVKHPADIPDYKK	L	74	99.42	83		84	
8000	K	VYVKHPADIPDYKK	L	74	99.9	83		84	
8001	K	VYVKHPADIPDYKK	L	74	99.99	83	0	84	0.01
8002	K	VYVKHPADIPDYKK	L	74	100	83		84	
8221	K	ALKLKDGGHYLVEFK	S	166	99.59	168	0.41	178	
8222	K	ALKLKDGGHYLVEFK	S	166	18.33	168	81.67	178	
8223	K	ALKLKDGGHYLVEFK	S	166	0.94	168	99.06	178	
8224	K	ALKLKDGGHYLVEFK	S	166	17.36	168	82.5	178	
8225	K	ALKLKDGGHYLVEFK	S	166	13.5	168	85.99	178	
8226	K	ALKLKDGGHYLVEFK	S	166	10.27	168	89.7	178	
8227	K	ALKLKDGGHYLVEFK	S	166	6.32	168	93.28	178	
8228	K	ALKLKDGGHYLVEFK	S	166	11.89	168	87.94	178	
8229	K	ALKLKDGGHYLVEFK	S	166	17.25	168	82.73	178	
8231	K	ALKLKDGGHYLVEFK	S	166	0.48	168	99.52	178	
8232	K	ALKLKDGGHYLVEFK	S	166	6.12	168	93.87	178	0.01
8233	K	ALKLKDGGHYLVEFK	S	166	6.54	168	93.46	178	
8234	K	ALKLKDGGHYLVEFK	S	166	3.29	168	96.7	178	
8235	K	ALKLKDGGHYLVEFK	S	166	24.53	168	75.28	178	
8236	K	ALKLKDGGHYLVEFK	S	166	40.16	168	59.82	178	
8237	K	ALKLKDGGHYLVEFK	S	166	51.39	168	48.07	178	
8436	K	FIGVNFPSDGPVMQKK	T	138	67.17	139	32.83		
8437	K	FIGVNFPSDGPVMQKK	T	138	77.09	139	22.91		
8438	K	FIGVNFPSDGPVMQKK	T	138	63.83	139	36.47		
8439	K	FIGVNFPSDGPVMQKK	T	138	68.03	139	31.97		
8440	K	FIGVNFPSDGPVMQKK	T	138	89.16	139	10.84		
8441	K	FIGVNFPSDGPVMQKK	T	138	50	139	50		
8442	K	FIGVNFPSDGPVMQKK	T	138	74.3	139	25.7		
8719	K	KTMGWEASTERLYPR	D	139	99.45				
8720	K	KTMGWEASTERLYPR	D	139	99.99				
8721	K	KTMGWEASTERLYPR	D	139	99.93				
8722	K	KTMGWEASTERLYPR	D	139	99.99				
9077	K	ALKLKDGGHYLVEFK	S	166	99.95	168	99.95	178	
9078	K	ALKLKDGGHYLVEFK	S	166	98.32	168	98.32	178	1.48
9392	K	VKFIGVNFPSDGPVMQKK	T	123		138	50	139	50
9393	K	VKFIGVNFPSDGPVMQKK	T	123		138	77.41	139	22.59
9394	K	VKFIGVNFPSDGPVMQKK	T	123		138	50	139	50
9395	K	VKFIGVNFPSDGPVMQKK	T	123	99.37	138		139	
9396	K	VKFIGVNFPSDGPVMQKK	T	123	99.96	138		139	
9984	K	LKDGGHYLVEFKSIYMAK	K	168	100	178		184	
9985	K	LKDGGHYLVEFKSIYMAK	K	168		178	100	184	
9986	K	LKDGGHYLVEFKSIYMAK	K	168	99.99	178		184	
9987	K	LKDGGHYLVEFKSIYMAK	K	168		178	100	184	
9988	K	LKDGGHYLVEFKSIYMAK	K	168	99.99	178		184	
9989	K	LKDGGHYLVEFKSIYMAK	K	168	0.01	178	99.98	184	0.01
9990	K	LKDGGHYLVEFKSIYMAK	K	168	100	178		184	
9991	K	LKDGGHYLVEFKSIYMAK	K	168		178	100	184	
9992	K	LKDGGHYLVEFKSIYMAK	K	168	3.87	178	92.89	184	3.41
9993	K	LKDGGHYLVEFKSIYMAK	K	168		178	99.98	184	0.01
9994	K	LKDGGHYLVEFKSIYMAK	K	168		178	99.99	184	
13345	K	FIGVNFPSDGPVMQKKTMGWEASTER	L	138	15.44	139	75.46	149	9.09
14093	R	MEGTVNGHEFEIEGEGEGRPYEGHNTVKLK	V	36	0.64	45	49.68	47	49.68
14095	R	MEGTVNGHEFEIEGEGEGRPYEGHNTVKLK	V	36	0.09	45	49.95	47	49.95
14849	K	KPVQLPGYYYVDAKLDITSHNEDYTIVEQYER	T	185	99.83	198	0.16		
14850	K	KPVQLPGYYYVDAKLDITSHNEDYTIVEQYER	T	185	0.44	198	99.56		

Figure S29. Site analysis probabilities from mass peptide fingerprinting for all peptides.

Fluorescent Microscopy

Images were analyzed by ImageJ software with threshold range 40-250. To quantify the RFP, Analyze Particles plugin was used with size: 10-infinite and no restrictions on circularity.

	Count (Pre)	Average (Pre)	Count (Post)	Average (Post)	Retained (%)
RFP	141	93	3	2	2
	67		1		
	71		1		
RFP-mal	130	94	16	13	13
	43		11		
	109		11		
RFP-oPA	262	209	57	58	28
	145		77		
	221		39		

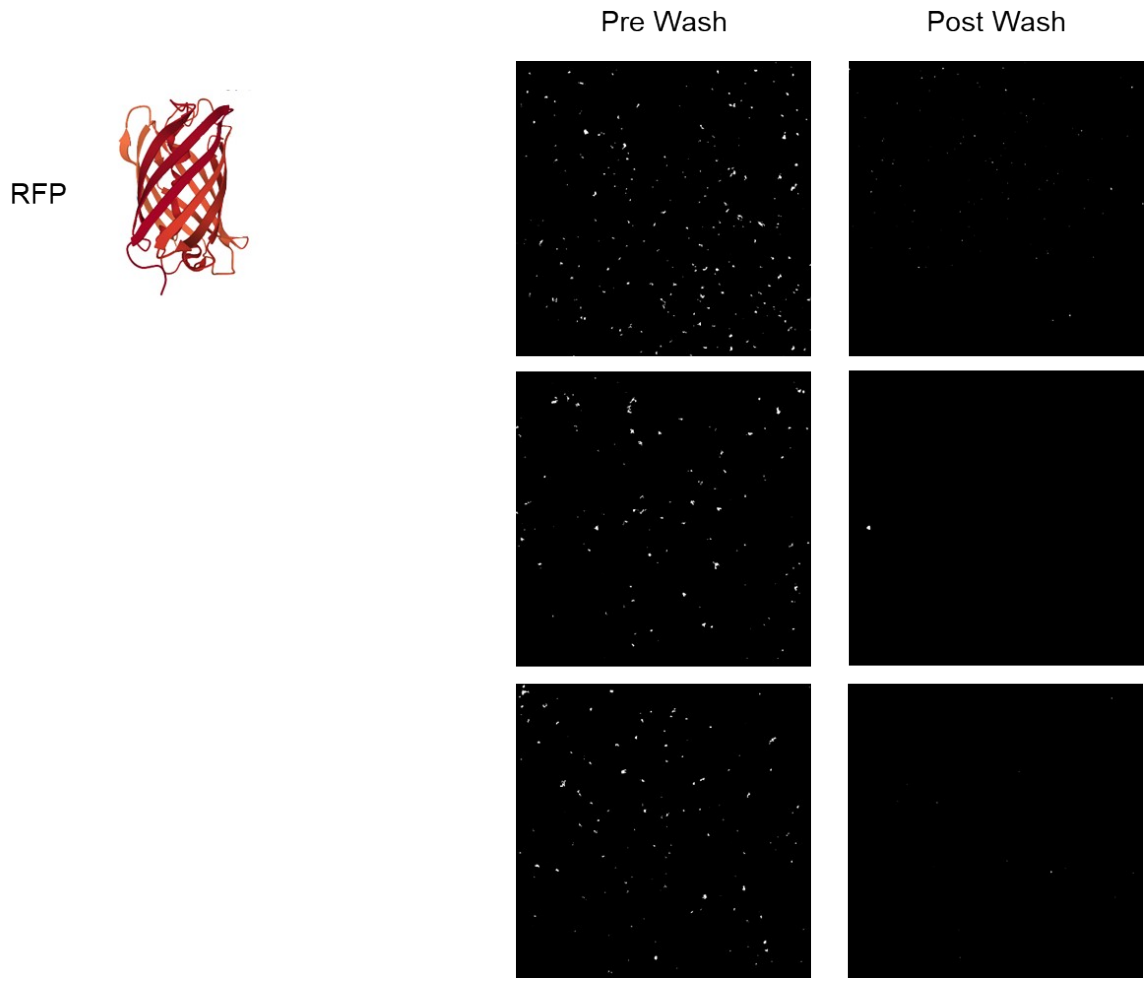

Figure S30. Post processing of representative fluorescent images taken before and after washing of copper disk without any linker on the RFP.

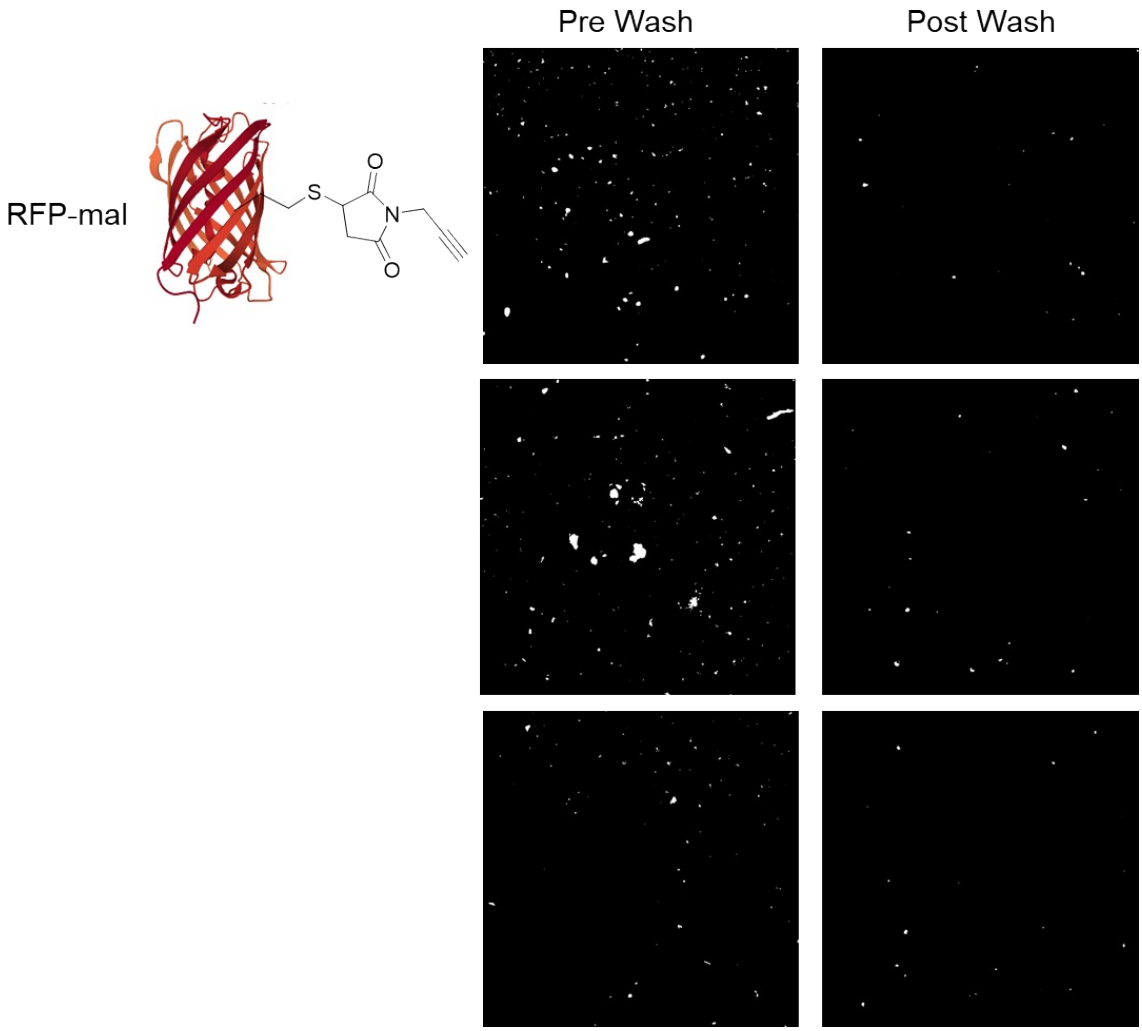

Figure S31. Post processing of representative fluorescent images taken before and after washing of copper disk with **RFP-mal**.

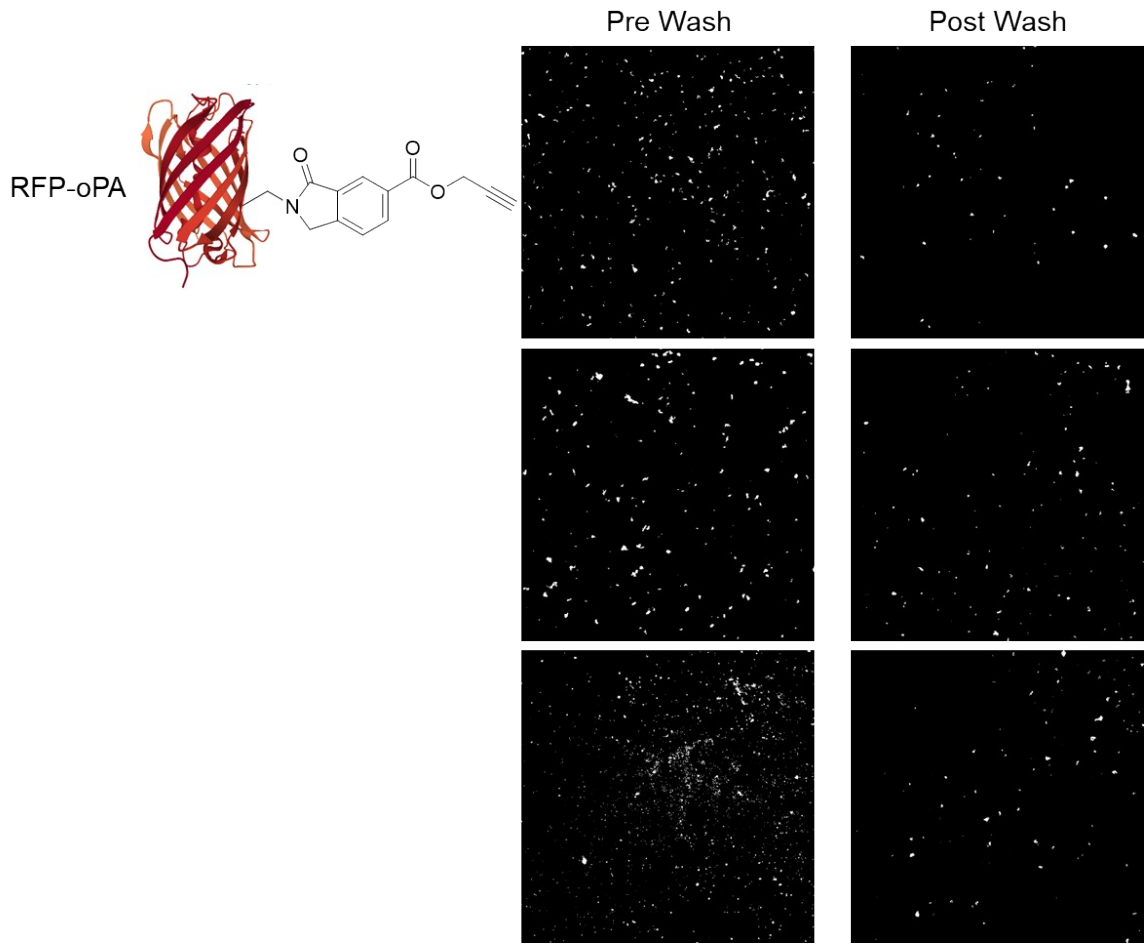

Figure S32. Post processing of representative fluorescent images taken before and after washing of copper disk with **RFP-oPA**.

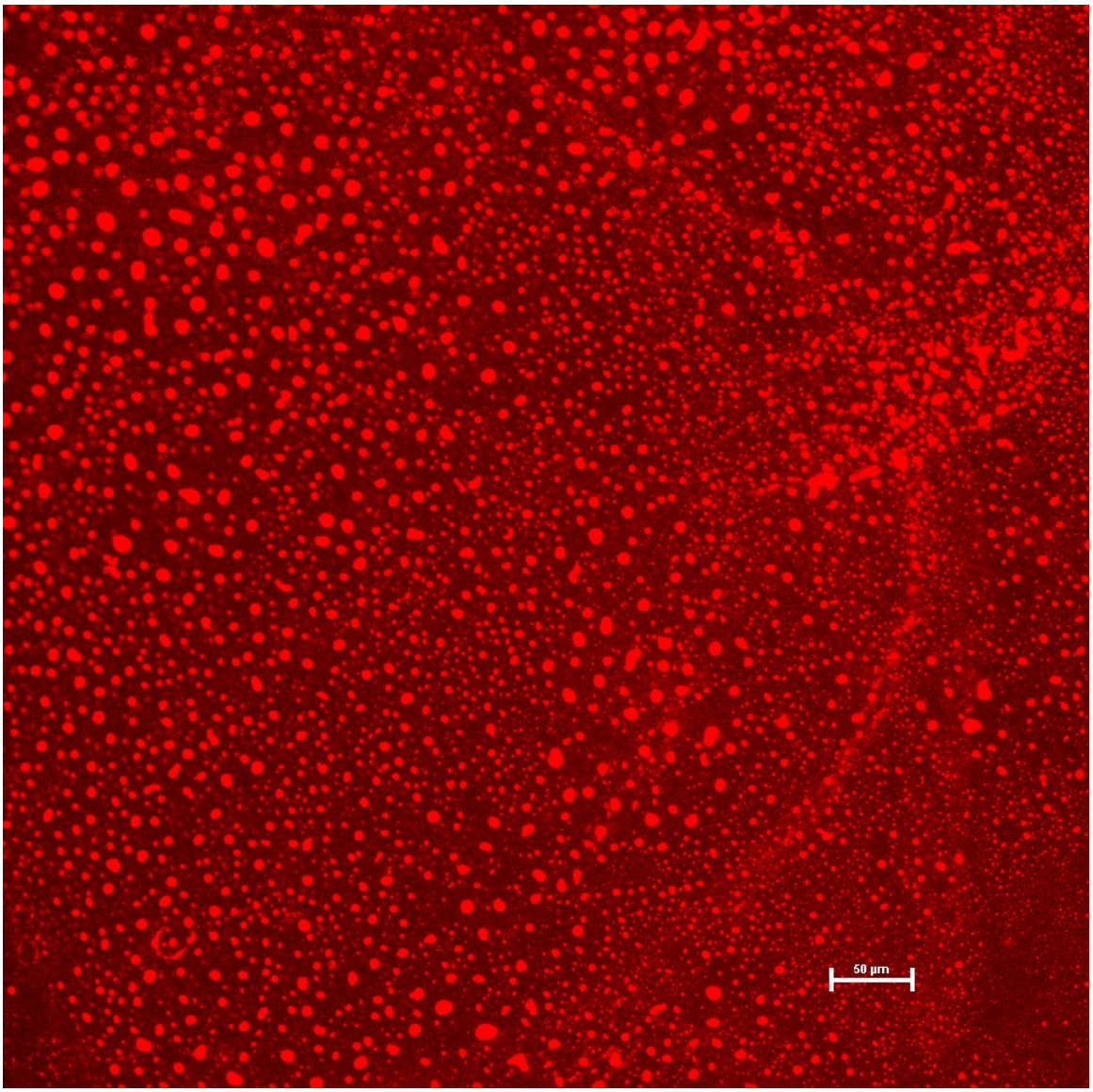

Figure S33. RFP-oPA/SA/**7**/**8** mixture applied to glass and let to sit for 30 min.

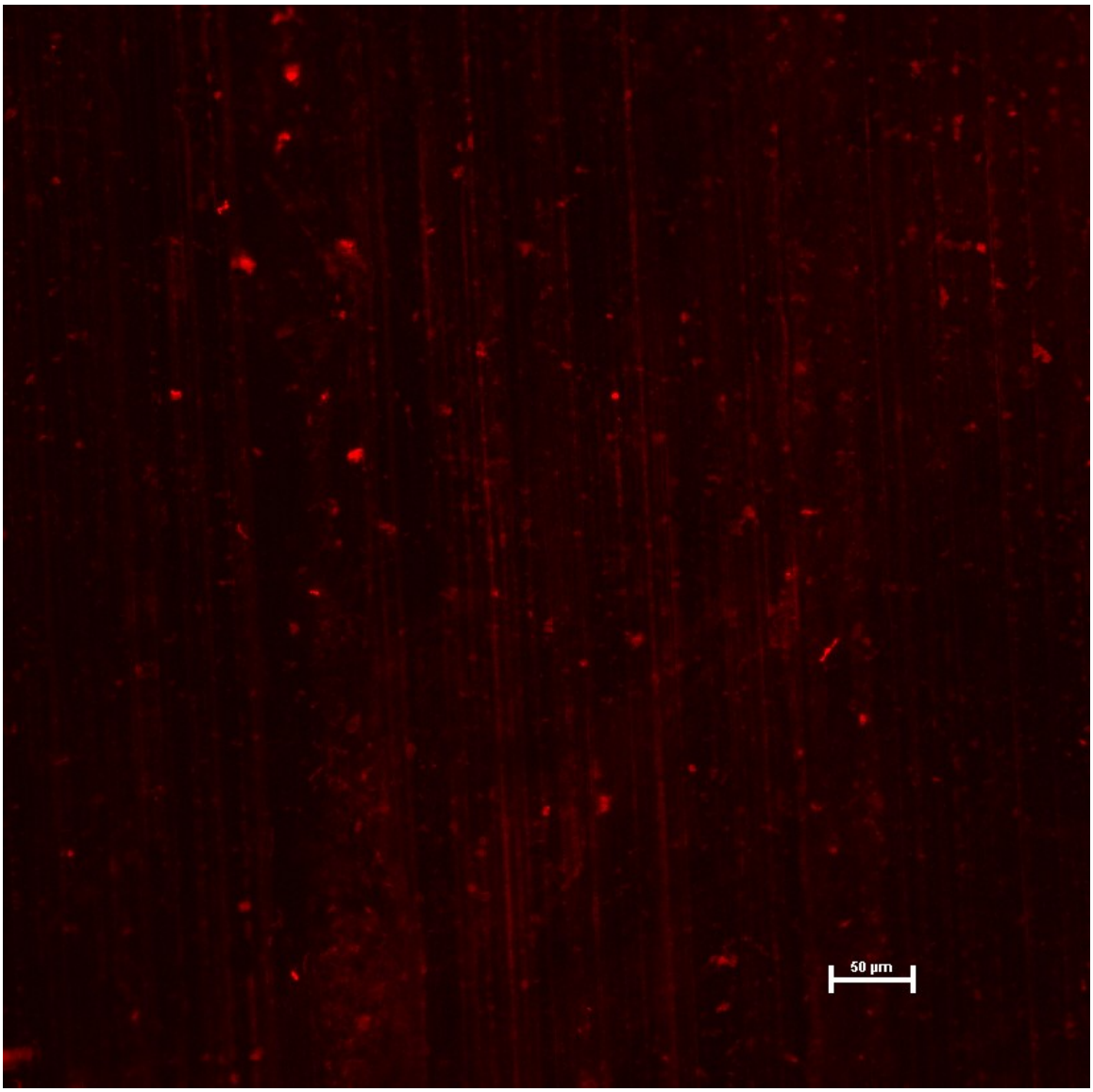

Figure S34. RFP-oPA/SA/**7**/**8** mixture applied to aluminum and let to sit for 30 min.

References

1. Henriksson, A.; Hoffmann, H., Click Coupling Reactions on Flat and Nanostructured Hydrogen-Passivated Silicon Surfaces. *physica status solidi (a)* **2018,** *216* (12).
2. Liu, X.-M.; Thakur, A.; Wang, D., Efficient Synthesis of Linear Multifunctional Poly(ethylene glycol) by Copper(I)-Catalyzed Huisgen 1,3-Dipolar Cycloaddition. *Biomacromolecules* **2007,** *8* (9), 2653-2658.