Interfacial Bioorthogonal Crosslinking

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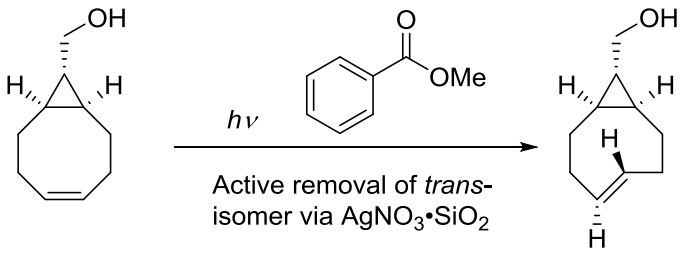
Supplementary Information

1. Synthetic Procedures

1.1. General considerations. All reactions were carried out in glassware that was flame-dried under vacuum and cooled under nitrogen. (rel-1R,8S,9R,4E)-Bicyclo[6.1.0]non-4-ene-9-ylmethanol was prepared following a known procedure[2]. *O,O'*-Bis(2-aminoethyl)octadecaethylene glycol (≥ 95% oligomer purity) and *O*-(2-Aminoethyl)-*O*-[2-(Boc-amino)ethyl] decaethylene glycol (≥ 90% oligomer purity) were purchased from Sigma Aldrich. Hyaluronic acid (sodium salt) was a generous gift from Genzyme Corporation. Dialysis membranes were purchased from Spectrum Labs (MWCO: 10 kDa). Flash Chromatography was performed using normal phase Silicycle silica gel (40-63D, 60Å). Deactivated silica gel was prepared by treating silica gel with $EtSiCl_3$[3]. An APT pulse sequence was used for ^{13}C NMR spectra, where methylene and quaternary carbons appear 'up' (u), and methine and methyl carbons appear 'down' (d). The abbreviation 'app' stands for 'apparent.' Other reagents were purchased from commercial sources without additional purification.

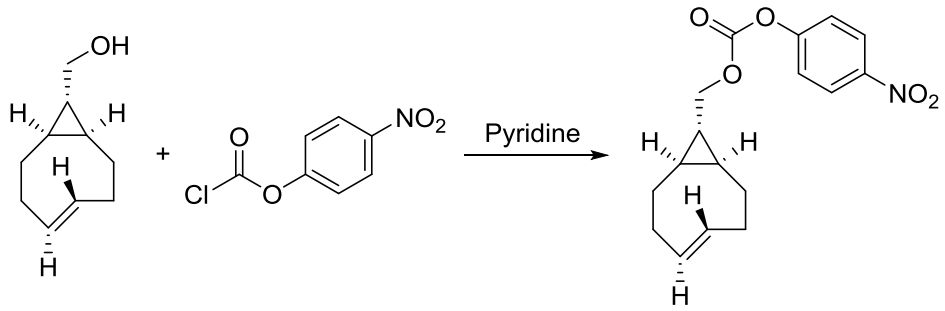
1.2. (rel-1R,8S,9R,4E)-Bicyclo[6.1.0]non-4-ene-9-ylmethanol



The continuous flow apparatus described previously[1] was used for the photoisomerization, Biotage SNAP cartridges (Biotage part no. FSK0-1107-0050) was filled with a bed of unmodified silica gel that was topped with 14.5 g of silica gel which was impregnated with AgNO3(1.45 g, 8.55 mmol). (1R,8S,9R,4Z)-Bicyclo [6.1.0]non-4-ene-9-ylmethanol(1.00 g, 6.58 mmol) and methyl benzoate (1.80g, 13.2 mmol) were placed in a quartz flask and dissolved in 500 mL of 1:1 Et_2O:hexanes. Dodecane (1.12g, 6.58 mmol) was also added to the flask to allow for GC monitoring. The solution was equilibrated through the continuous flow system at a 100 mL/min flow rate. The solution in the quartz flask was then irradiated (254nm) under continuous flow conditions (100 mL/min) for 4 hours, at which point GC analysis indicated that the reaction was complete. The SNAP cartridges were flushed with 400 mL of 1:1 Et_2O/hexanes and then dried with compressed air. To the dried silica gel was sequentially added NH_4OH (200 mL) and methylene chloride (200 mL) and the resulting mixture was filtered. The filtrate was transferred to a separatory funnel and partitioned. The aqueous layer was extracted twice with methylene chloride. The organic layers were combined, washed twice with water then dried with magnesium sulfate and filtered. The solvent was removed with a rotary evaporator, column chromatography (20% ethyl

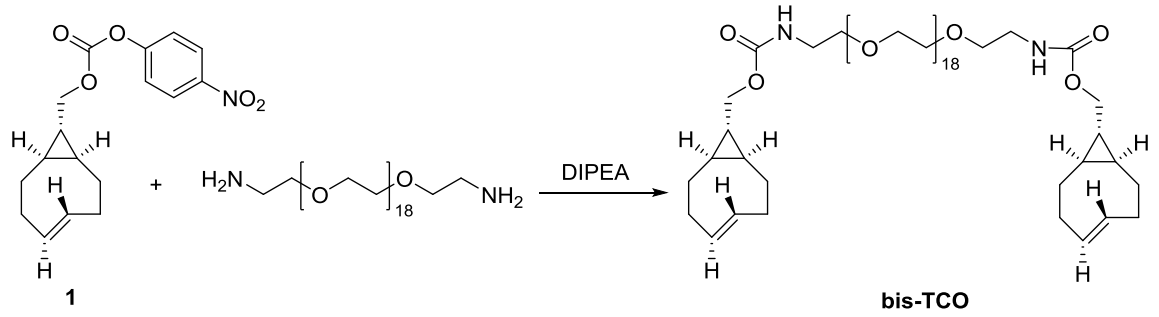
acetate/hexanes) afforded 820 mg of 7 (82%) (rel-1R,8S,9R,4E)-Bicyclo [6.1.0]non-4-ene-9-ylmethanol as a colorless oil. ^{1}H and ^{13}C NMR data agreed with the previously reported data[2].

1.3. (rel-1R,8S,9R,4E)-Bicyclo[6.1.0]non-4-ene-9-ylmethyl (4-nitrophenyl) carbonate (1)



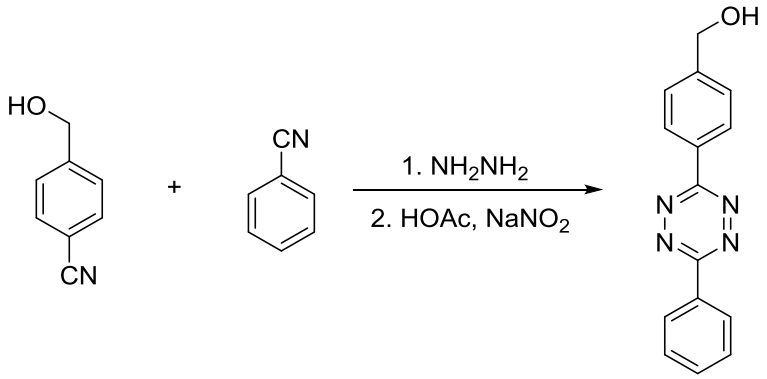
A dry round-bottled flask was charged with (rel-1R,8S,9R,4E)-Bicyclo [6.1.0]non-4-ene-9-ylmethanol (820 mg, 5.39 mmol). Anhydrous dichloromethane (100 mL) and pyridine (1.09 mL, 13.5 mmol) were added to the flask. A solution of 4-nitrophenylchloroformate (1.30 g, 6.47 mmol) in anhydrous dichloromethane (14 mL) was added to the flask via syringe and the solution was stirred for 1 h at room temperature. Saturated NH_4Cl aqueous solution was added to the mixture and the layers were separated, and the aqueous layer was extracted twice with dichloromethane. The organic layers were combined, dried with $MgSO_4$, filtered, solvent was removed using a rotary evaporator. Purification by column chromatography (5% ethyl actetate/hexanes) yielded afforded 1.18 g (3.72 mmol, 69%) of (rel-1R,8S,9R,4E)-Bicyclo[6.1.0]non-4-ene-9-ylmethyl (4-nitrophenyl) carbonate as a white solid. ^{1}H and ^{13}C NMR data agreed with the previously reported data[2]

1.4. "bis-TCO linker"



A dry round-bottled flask was sequentially charged via syringe with a solution of *O,O'*-Bis(2-aminoethyl)octadecaethylene glycol (64 mg, 0.071 mmol) in anhydrous dichloromethane (2 mL) and *N,N*-diisopropylethylamine (49.9 µL, 0.29 mmol), followed by a solution of (1*R*,8*S*,9*R*,4*E*)-bicyclo[6.1.0]non-4-en-9-ylmethyl(4-nitrophenyl) carbonate (50 mg, 0.16 mmol) in anhydrous dichloromethane (3 mL). The mixture was stirred overnight at room temperature. The solvent was removed with a rotary evaporator. Purification by column chromatography using 0-5% methanol in dichloromethane yielded 80 mg (0.064 mmol, 90%) of bis-TCO as a water-soluble clear oil. ^{1}H NMR ($CDCl_3$, 400 MHz, δ): 5.77-5.90 (m, 2H), 5.17-5.31 (m, 2H), 5.04-5.16 (m, 2H), 3.86-3.97 (m, 4H), 3.48-3.68 (m, 72H), 3.50-3.56 (m, 4H), 3.30-3.38 (m, 4H), 2.29-2.38 (m, 2H), 2.12-2.29 (m, 6H), 1.82-1.97 (m, 4H), 0.75-0.88 (m, 2H), 0.47-0.60 (m, 4H), 0.32-0.46 (m, 4H). ^{13}C NMR ($CDCl_3$, 100 MHz, δ): 157.0 (u, 2C), 138.5 (d, 2C), 131.4 (d, 2C), 70.7-70.5 (u, 34C), 70.4 (u, 2C), 70.2 (u, 2C), 69.5 (u, 2C), 40.8 (u, 2C), 38.8 (u, 2C), 33.9 (u, 2C), 32.7 (u, 2C), 27.7 (u, 2C), 24.8 (d, 2C), 22.0 (d, 2C), 21.0 (d, 2C). HRMS (ESI) [M+H]: calcd. for $C_{62}H_{113}N_2O_{23}^{+}$, 1253.7729; found 1253.7745.

1.5. (4-(6-phenyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol (2)

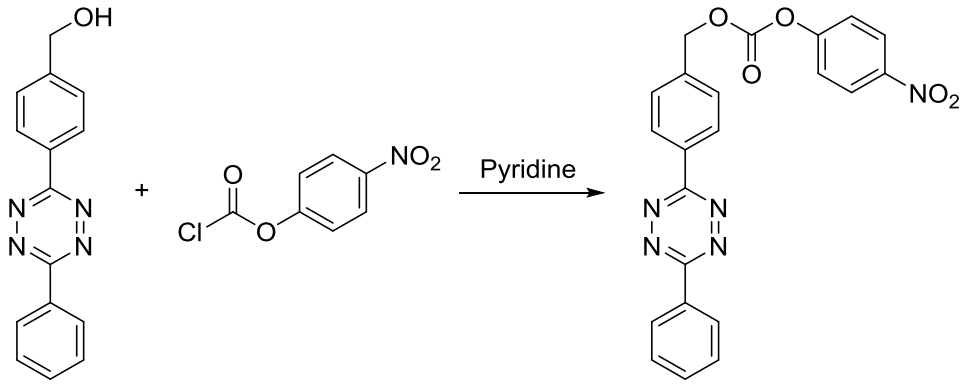


2

A dry round-bottomed flask was charged with 4-(hydroxymethyl)benzonitrile (5.80 g, 43.6 mmol), benzonitrile (18.0 g, 175 mmol) and anhydrous hydrazine (21.4 mL, 440 mmol). The flask was equipped with a reflux condenser, and the mixture was heated to 90 °C for 20 h behind a blast shield. The mixture was allowed to cool down to room temperature and was diluted with ethyl acetate (300 mL), washed twice with H_2O (150 mL), and dried over $MgSO_4$. The solution was filtered and the filtrate was concentrated under reduced pressure. The residual solid was dissolved in acetic acid (84 mL), and an aqueous solution of $NaNO_2$ (23.2 mL of a 9.40 M solution, 218 mmol) was added at 0 °C via Pasteur pipette. After stirring for 30 min at room temperature, the mixture was diluted with dichloromethane (300 mL). An aqueous solution of saturated $NaHCO_3$ was added then carefully added to neutralize the acetic acid. The mixture was partitioned and the organic phase was washed three times with saturated $NaHCO_3$ aqueous solution, and then dried over $MgSO_4$, filtered, and concentrated onto silica gel using a rotary evaporator. Column chromatography using a gradient (0-70 %) of acetone in hexanes afforded 2.46 g (9.32 mmol, 22%) of **2** as a purple solid. ^1H

NMR (DMSO-d_6, 400 MHz, δ): 8.40-8.60 (m, 4H), 7.58-7.75 (m, 5H), 5.46 (t, J=5.7 Hz 1H), 4.65 (d, J=5.6 Hz, 2H). ^{13}C NMR (DMSO-d_6, 100 MHz, δ): 163.4 (u, 1C), 163.3 (u, 1C), 147.7 (u, 1C), 132.6 (d, 1C), 132.0 (u, 1C), 130.2 (u, 1C), 129.6 (d, 2C), 127.6 (d, 2C), 127.5 (d, 2C), 127.2 (d, 2C), 62.5 (u, 1C). HRMS (ESI) [M+H]: calcd. for $C_{15}H_{13}N_4O^+$, 265.1084; found 265.1098.

1.6. 4-nitrophenyl 4-(6-phenyl-1,2,4,5-tetrazin-3-yl)benzyl carbonate (3)

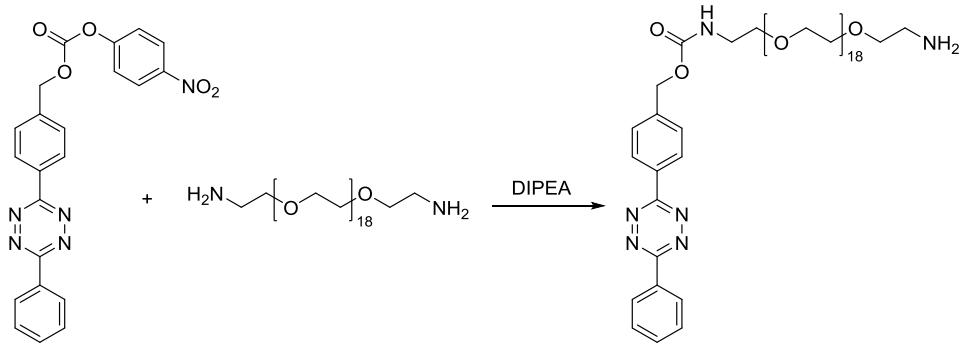


2 **3**

A dry round-bottled flask was charged with (4-(6-phenyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol **2** (157 mg, 0.595 mmol). Anhydrous dichloromethane (12 mL) and pyridine (0.12 mL, 1.5 mmol) were added to the flask. A solution of 4-nitrophenylchloroformate (180 mg, 0.892 mmol) in anhydrous dichloromethane (3 mL) was added to the flask via syringe and the solution was stirred for 2 h at room temperature. Saturated NH_4Cl aqueous solution was added to the mixture and the layers were separated, and the aqueous layer was extracted twice with dichloromethane. The organic layers were combined, dried with $MgSO_4$, filtered, and

concentrated onto silica gel using a rotary evaporator. Column chromatography using a gradient (30-70%) of dichloromethane in hexanes afforded 0.211 g (0.492 mmol, 83%) of **3** as a purple solid. ^{1}H NMR ($CDCl_3$, 400 MHz, δ): 8.63-8.76 (m, 4H), 8.26-8.34 (m, 2H), 7.60-7.75 (m, 5H), 7.38-7.46 (m, 2H), 5.43 (s, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz, δ): 164.2 (u, 1C) 163.7 (u, 1C) 155.5 (u, 1C) 152.7 (u, 1C) 145.6 (u, 1C) 138.9 (u, 1C) 133.0 (d, 1C) 132.5 (u, 1C) 131.8 (u, 1C) 129.5 (d, 2C) 129.2 (d, 2C) 128.5 (d, 2C) 128.2 (d, 2C) 125.5 (d, 2C) 121.9 (d, 2C) 70.3 (u, 1C). HRMS (ESI) [M+H]: calcd. for $C_{22}H_{16}N_5O_5^+$, 430.1146; found 430.1154.

1.7. Tetrazine-OEG-amine (4)

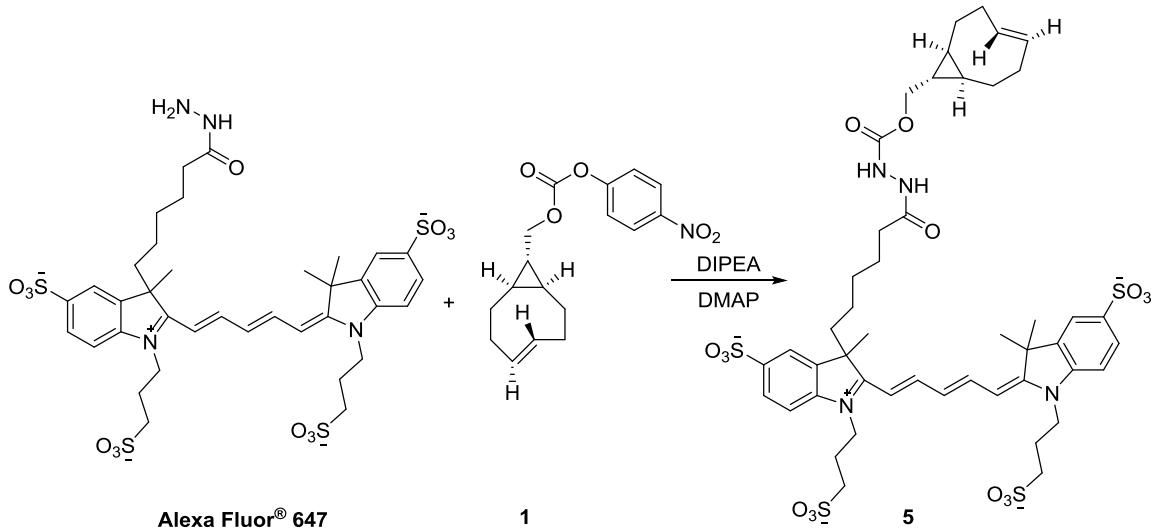


3 4

A dry round-bottled flask was charged with a solution of *O,O'*-bis(2-aminoethyl)octadecaethylene glycol (141 mg, 0.157 mmol) in anhydrous dichloromethane (4 mL) and *N,N*-diisopropylethylamine (DIPEA, 36.5 µL, 0.209 mmol). A solution of 4-nitrophenyl 4-(6-phenyl-1,2,4,5-tetrazin-3-yl)benzyl carbonate (45 mg, 0.11 mmol) in anhydrous dichloromethane (6 mL) was added to the flask via a syringe

pump over 3 h. The mixture was then allowed to stir overnight at room temperature. The solvent was removed with a rotary evaporator and the residue was washed three times with hexanes. Column chromatography on deactivated silica gel[2] using a gradient 30-100% acetone in hexanes followed by 10% methanol in dichloromethane afforded 88 mg (0.074 mmol, 71%) of **4** as a water-soluble purple solid. ^{1}H NMR ($CDCl_3$, 400 MHz, δ): 8.55-8.75 (m, 4H), 7.86-7.99 (br, 2H), 7.50-7.70 (m, 5H), 5.50-5.61 (br, 1H), 5.21 (s, 2H), 3.91 (m, 2H), 3.59-3.73 (m, 74H), 3.41 (m, 2H), 3.10-3.24 (m, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz, δ): 164.1(1C), 163.8(1C), 156.4(1C), 141.8(1C), 132.9(1C), 131.8(1C), 131.4(1C), 129.5(2C), 128.6(2C), 128.2(2C), 128.1(2C), 70.7-70.0(35C), 69.9(1C), 69.7(1C), 66.8(1C), 66.0(1C), 41.1(1C), 40.7(1C). HRMS (ESI) [M+H]: calcd. for $C_{56}H_{95}N_6O_{21}^+$, 1187.6545; found 1187.6541. UV-vis (27.5 μM in H_2O): λ_{max} 300 nm.

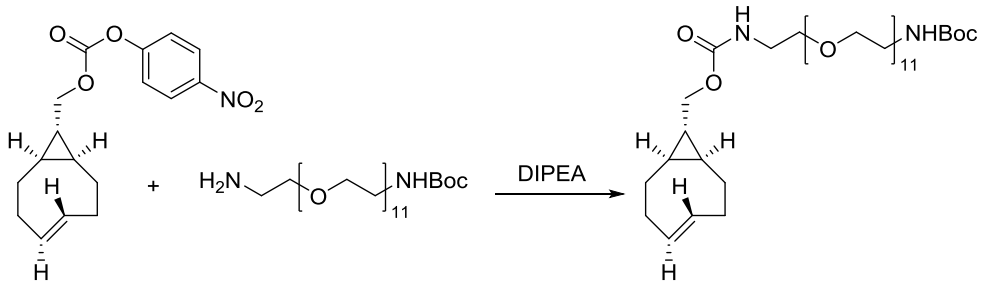
1.8. Alexa Fluor® 647-TCO (Alexa-TCO, 5)



Alexa Fluor® 647 **1** **5**

(1*R*,8*S*,9*R*,4*E*)-Bicyclo[6.1.0]non-4-en-9-ylmethyl(4-nitrophenyl) carbonate (**1**) (1.3 mg, 4.2 μmol) was added to a vial that contained Alexa Fluor® 647 hydrazide, tris(triethylammonium) salt (1.0 mg, 0.83 μmol). A DMF solution (200 μL, anhydrous) containing *N,N*-diisopropylethylamine (215 μg, 1.67 μmol) and 4-dimethylaminopyridine (DMAP, 50 μg, 0.41 μmol) was added to the vial. The mixture was stirred overnight at ambient temperature and was purified with reverse phase HPLC, generating 0.49 mg (47 μmol, 56%) of **5** as a blue solid. LC-MS (Figure S16) and HPLC (Figure S17) analyses indicated that the purity of a compound with a mass of 1047 Da. was >98%.

1.9. TCO-DEG-NHBoc (6)

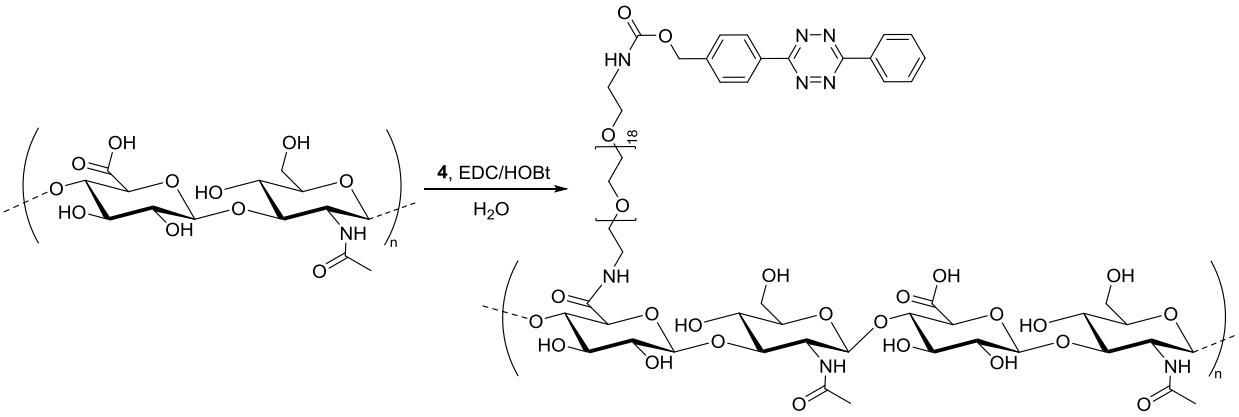


1 **6** **TCO-OEG-NHBoc**

A dry round-bottled flask was charged with an anhydrous dichloromethane (2 mL) solution of *O*-(2-aminoethyl)-*O*-[2-(Boc-amino)ethyl] decaethylene glycol (75 mg, 0.12 mmol) and *N,N*-diisopropylethylamine (30 mg, 0.233 mmol). A solution of (1*R*,8*S*,9*R*,4*E*)-bicyclo[6.1.0]non-4-en-9-ylmethyl(4-nitrophenyl) carbonate[1] (44 mg, 0.14 mmol) in anhydrous dichloromethane (2 mL) was added to the flask via a syringe. The mixture was then stirred overnight at room temperature. Solvent was removed with rotary evaporator. Purification by column chromatography using 0-5% methanol

in dichloromethane yielded 84 mg (0.10 mmol, 88%) of **6** as a water-soluble clear oil. ^{1}H NMR (MeOD, 600 MHz, δ): 5.78-5.94 (m, 1H), 5.04-5.20 (m, 1H), 3.87-3.97 (m, 2H), 3.58-3.66 (m, 42H), 3.47-3.53 (m, 4H), 3.25-3.28 (m, 2H), 3.20-3.23 (m, 2H), 2.32-2.36 (m, 1H), 2.15-2.27 (m, 3H), 1.86-1.96 (m, 2H), 1.44 (s, 9H), 0.85-0.93 (m, 1H), 0.54-0.65 (m, 2H), 0.40-0.47 (m, 2H). ^{13}C NMR (MeOD, 150 MHz, δ): 159.3 (u, 2C), 139.4 (d, 1C), 132.4 (d, 1C), 80.2 (u, 1C), 71.8-71.4 (u, 20C), 71.3-71.1 (u, 2C), 70.5 (u, 1C), 41.8 (u, 1C), 41.5 (u, 1C), 39.9 (u, 1C), 34.8 (u, 1C), 33.8 (u, 1C), 29.0 (d, 3C), 28.7 (u, 1C), 26.3 (d, 1C), 23.4 (d, 1C), 22.4 (d, 1C). HRMS (ESI) [M+H]: calcd. for $C_{40}H_{74}N_2O_{15}Na^+$, 845.4981; found 845.4982.

1.10. Synthesis of HA-Tz:



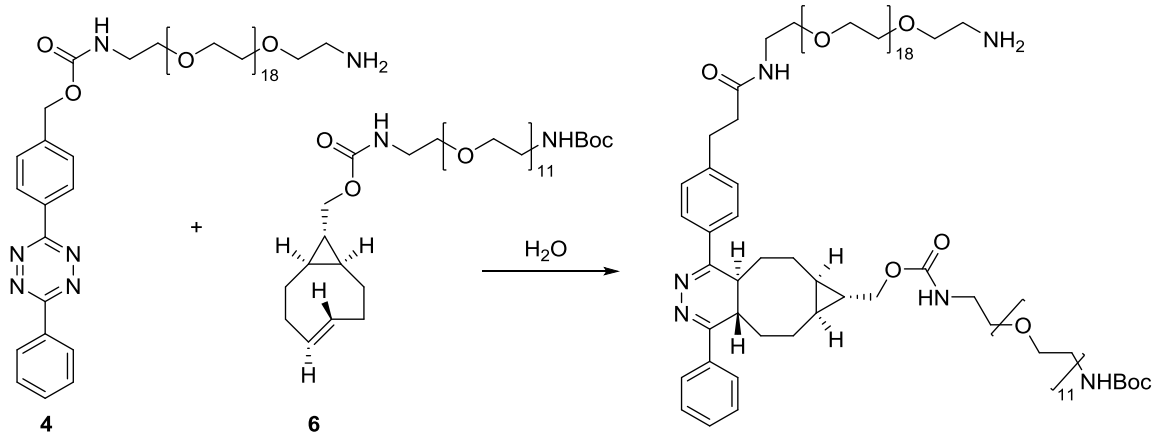
Hyaluronic acid (30.1 mg, 79.2 µmol) was dissolved in H_2O (6.7 mL) at a concentration of 4.5 mg/mL. Tetrazine-OEG-amine (**4**) (447 mg, 0.376 mmol) dissolved in H_2O (3.2 mL) was then added dropwise to the HA solution. To this mixture was slowly added a solution of 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) (57.8 mg, 301 µmol) in DMSO/H_2O (1:1, 302 µL), followed by a solution of 1-hydroxybenzotriazole (HOBt)

(40.6 mg, 301 μmol) in DMSO/H_2O (1:1, 302 μL). The resulting mixture was stirred at 37 °C for 24 h. The HA derivative was then precipitated in ice-cold ethanol (100 mL, 10 vol. excess). The precipitates were then collected by centrifugation at 4,000 rpm for 8 min. The pellet was then re-dissolved in H_2O at approximately 3 mg/mL and exhaustively dialyzed (Spectra 10 kDa MWCO) against H_2O. The purified product was lyophilized to afford 29 mg (0.063 mmol, 80%) HA-Tz as a pink fluffy solid. The product was stored at 4 °C prior to use.

2. Analytical methods

2.1. Kinetic analysis of the reaction between TCO (6) and 3,6-diphenyl-s-tetrazine (4) in H_2O

The reaction was run under pseudo-first order conditions and monitored by UV-Vis spectroscopy at 294 nm using an Applied Photophysics SX.18MV-R stopped-flow dual mixing spectrometer. The reactants were separately dissolved in H_2O and mixed in the stopped-flow device. The final concentrations of **4** and **6** were 2.50×10^{-5} M and 2.50×10^{-4} M, respectively. A spectrum was acquired every 5×10^{-4} seconds for 4×10^{-2} seconds (95% conversion). The k_{obs} was determined by fitting a linear curve to $\ln(A/A_0)$, where A = absorbance and A_0 = absorbance at t = 0, respectively. The kinetic runs were measured in triplicate, and k_{obs} was 71.6 s^{-1}(+/- 1.9). The second order rate constant (k_2) was calculated to be 71.6 s^{-1} / (2.5×10^{-4} M) = 2.86×10^5 $M^{-1}s^{-1}$ (+/- 1.3×10^4).



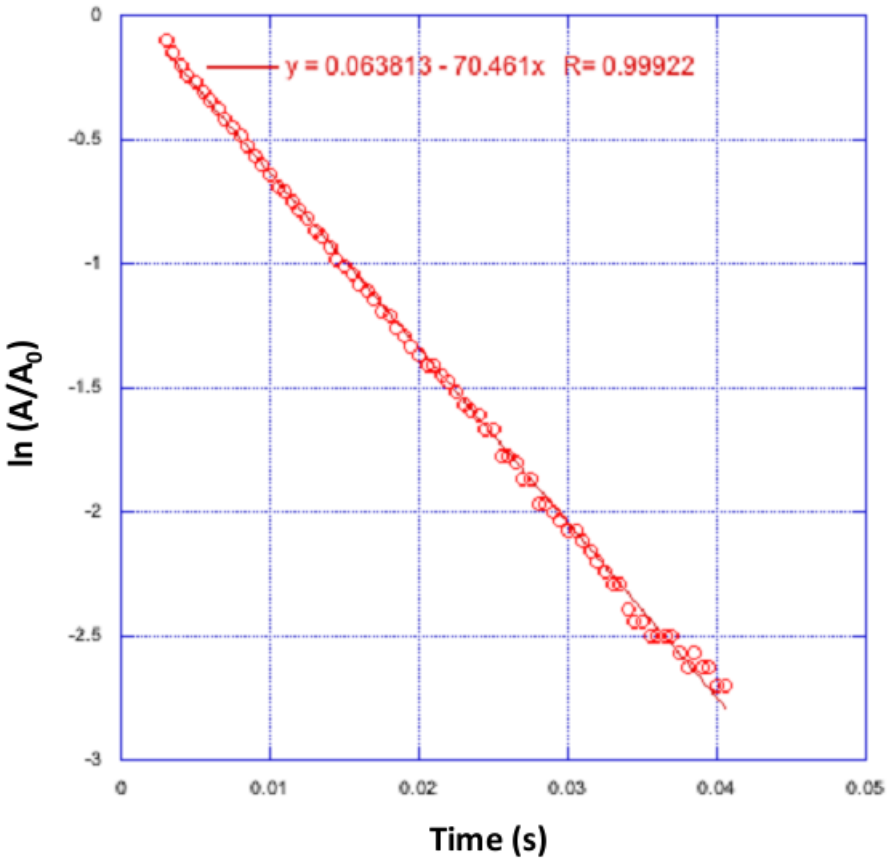


Figure S1: A representative stopped-flow kinetic run for determining the second order rate constant for the reaction between **4** and **6** in H_2O. Linear correlation was found between time (s) and $\ln(A/A_0)$.

2.2. Percent tetrazine incorporation in HA-Tz.

The percent tetrazine incorporation in HA-Tz was determined collectively by UV-vis and ^{1}H NMR analyses. UV-vis quantification was based on the tetrazine absorption at λ_{max} 300 nm employing Beer-Lambert law. Using an aqueous solution of compound **4** at a concentration of 27.5 μM as the standard (Figure S2a), the molar extinction coefficient of the tetrazine moiety (ε_{Tz}) was determined as 3.2 × 10^4 L Mol^{-1} cm^{-1}. Taking into consideration the change of the molecular weight for HA disaccharide repeats after tetrazine incorporation, the degree of tetrazine incorporation in HA-Tz was calculated as 7% (Figure S2b). By ^{1}H NMR (Figure S3), the degree of modification was analyzed by comparing the integration between the aromatic protons (7.4-8.4 ppm) to that the acetamido moiety of the N-acetyl-d-glucosamine residue of HA,

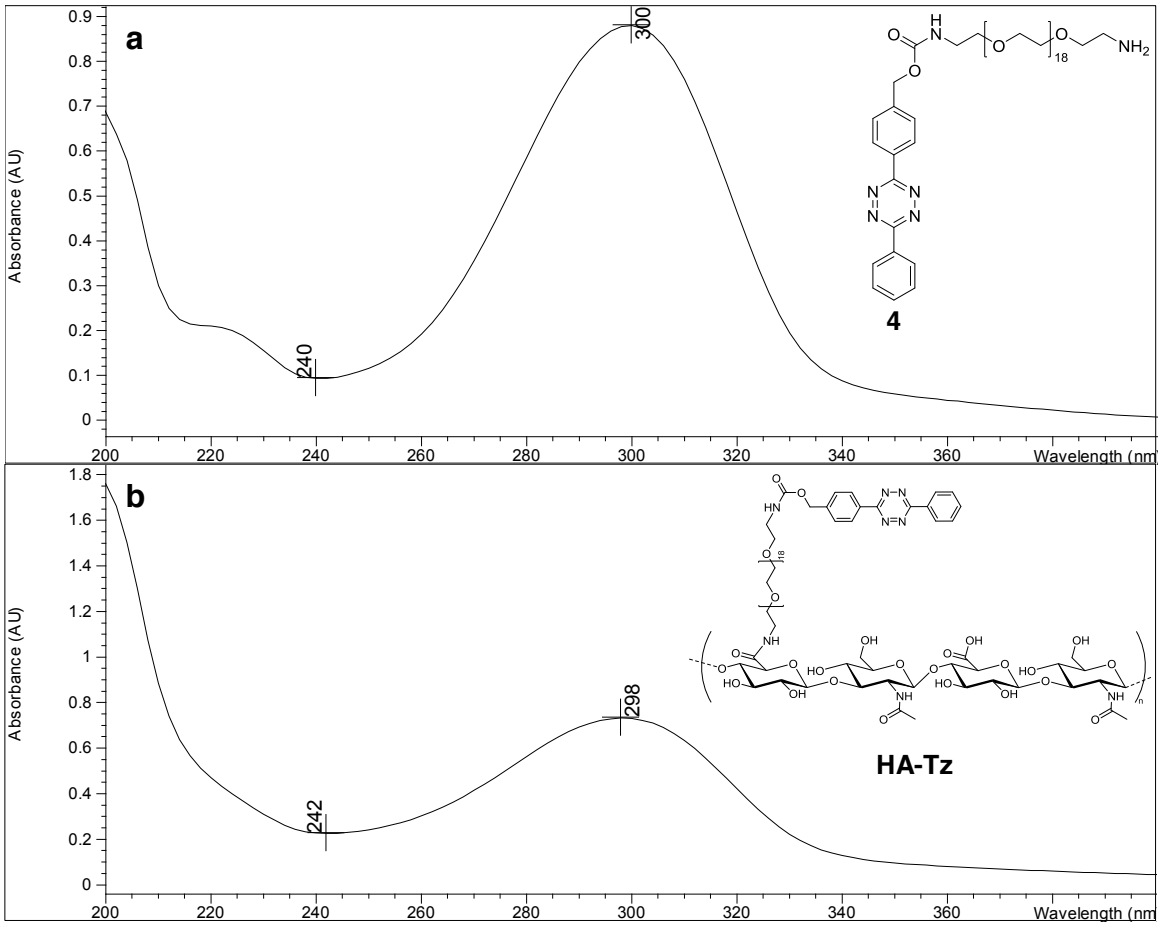


Figure S2. UV-vis spectra of aqueous solutions of (a) compound **4** at a concentration of 27.5 µM and (b) HA-Tz at a concentration of 0.153 g/L. In both measurements, a UV cuvette with a pathlength of 1 cm was used.

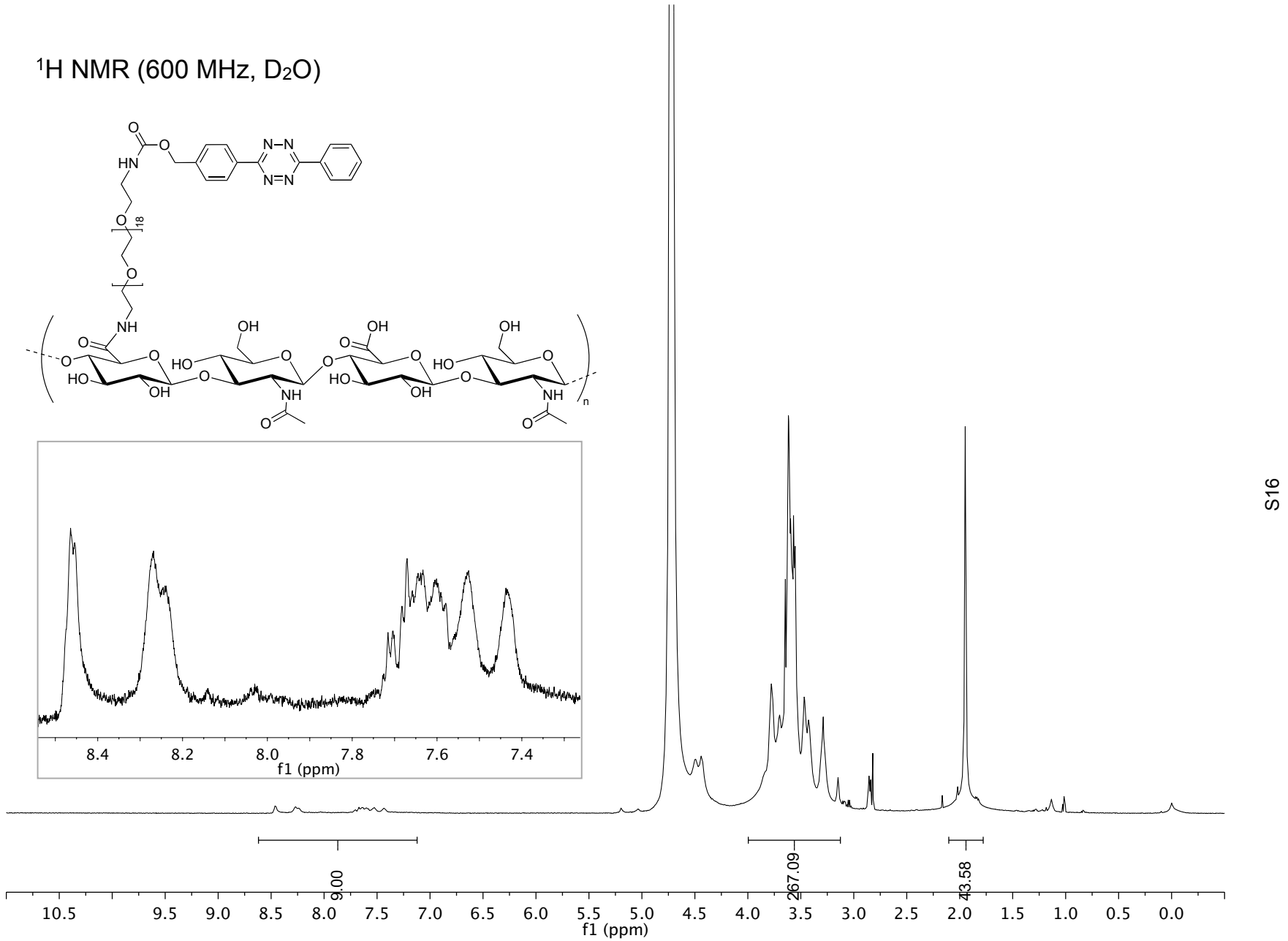


Figure S3. ^{1}H NMR spectrum of HA-Tz in D_2O.

2.3. Determination of molecular weight and solution viscosity.

Viscosity average molecular weight (M_v) of HA and HA-Tz was determined using a viscometer (Cannon Instrument Company, State College, Pa) in 0.2 M NaCl at room temperature. Efflux times for HA and HA-Tz solutions at concentrations of 0.2, 0.1, 0.05, 0.025, and 0 wt% were determined in triplicate. Using the Mark-Houwink equation with parameters of a=0.816 and K=0.0228 mL/g, M_v[4] was calculated to be 543 kDa and 218 kDa for the parent HA and the tetrazine derivative, respectively. The viscosity of HA and HA-Tz (both 2 wt%) was determined using a rheometer (AR-G2, TA Instrument, New Castle, DE) with a 20 mm aluminum parallel plate geometry with 100 μm gap size at ambient temperature where the shear rate was stepped from 1 to 100 s^{-1}.

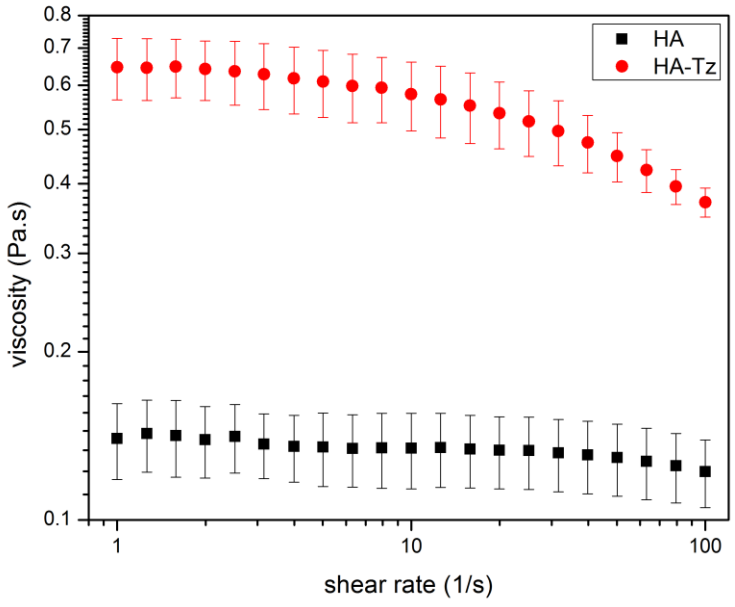


Figure S4. Viscosity profile of 2 wt% HA (black) and HA-Tz (red) in PBS

2.4. Rheological characterization.

The rheological properties of the hydrogel microspheres were evaluated using a controlled stress rheometer (AR-G2, TA Instruments, New Castle, DE) with a parallel plate geometry (8 mm diameter). After the gelation was complete, the hydrogel microsphere was loaded onto the geometry, and mineral oil was applied around the sample to prevent water evaporation during the measurement. Samples were tested at 25°C with a gap size of 175 μm. The linear viscoelastic region was determined by a strain sweep from 0.1 to 1000% at an angular frequency of 1 Hz. A time sweep experiment was conducted for 60 minutes at a frequency of 1 Hz and a strain of 1%. A frequency sweep experiment within the linear viscoelastic range was performed after the time sweep measurement at 1% strain from 0.1 to 10 Hz. All measurements were performed in triplicate.

3. Interfacial bioorthogonal crosslinking.

3.1. HA microspheres formation

HA-Tz and bis-TCO were separately dissolved in PBS at a concentration of 2 wt% and 400 μM, respectively. To prepare HA microspheres, HA-Tz was dropped via a 25G syringe into a 500 μL solution of bis-TCO solution in a 48 well plate (BD Falcon™). The interfacial crosslinking was allowed to occur at 37 °C for 2 hours without any agitation. The bis-TCO solution was then replaced with fresh PBS. The gel particles were dehydrated in graded ethanol solutions and vacuum dried. The swelling ratio,

reported as an average of three repeats, was determined as the ratio of the initial weight of the wet gel to the weight of the dry product. The microsphere wall thickness was measured by capturing an image of the microsphere during crosslinking every 3 minutes for the 2 hour gelation period. The wall thickness was then quantified by using imageJ to measure the thickness of the crosslinked wall of the microsphere as a function of time.

3.2. HA hydrogel channel formation

To prepare crosslinked hydrogel channels while simultaneously monitoring the channel formation via confocal microscopy, a glass cylinder (I.D. = 5 mm, h = 10 mm), mounted onto an imaging chamber (Lab-Tek™), was filled with ~200 μL HA-Tz (2 wt%). A syringe containing 2 mM bis-TCO and 2 μM Alexa-TCO was inserted to the bottom of the HA-Tz-filled cylinder. The syringe was pulled out of the cylinder while injecting ~ 30 μL of the solution, leaving behind a liquid channel. The channel was monitored and imaged for 60 minutes using a Zeiss 510 NLO confocal microscope (Carl Zeiss, Maple Grove, MN).

3.3. Selective interfacial tagging.

Selective interfacial tagging was achieved by timed exposure of the crosslinking HA-Tz droplet, originally dissolved in PBS at 2 wt%, to aqueous baths of bis-TCO (400 μM) alone or bis-TCO (400 μM and 1 μM Alexa-TCO) in an alternating fashion. The

total exposure time was maintained at 2 hours to ensure complete gelation. For example, alternating exposure of the HA-Tz droplet to the dye-free and dye-containing baths for 15 min each for 3 cycles, followed by a 30-min exposure to the dye-containing bath resulted in a crosslinked microsphere with 7 distinct layers. To tag the microspheres with Alexa-TCO in a gradient fashion, HA-Tz was dropped into a bis-TCO bath (1 mL, 400 μM) and Alexa-TCO was added to the bath using a syringe pump gradually over the course of 2 hours, reaching a final concentration of 0.47 μM. A control experiment was performed by dropping 2 wt% HA-Tz into bath of 400 μM bis-TCO and 0.47 μM alexa-TCO and allowed to crosslink for 2 hours. Upon completion of the tagging experiment, the bath was replaced with PBS and the gels were images using a Zeiss 510 NLO confocal microscope.

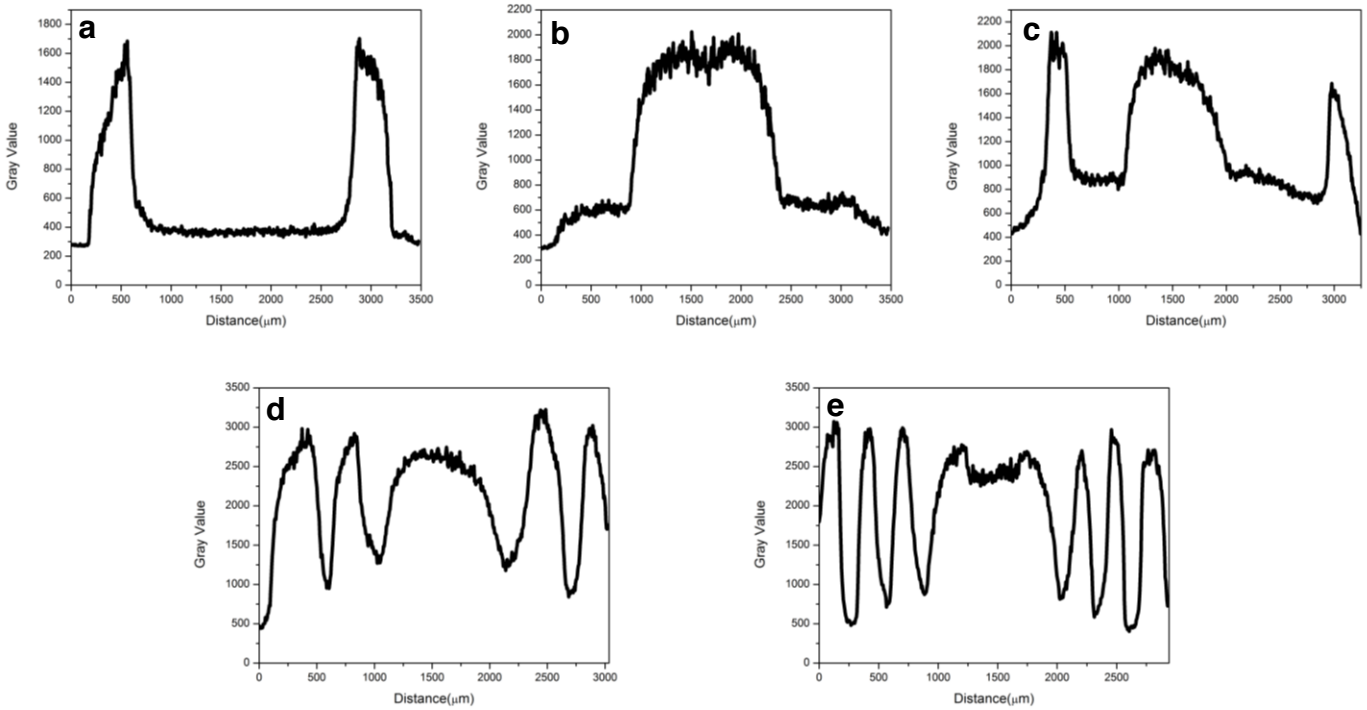


Figure S5. a-e) Color intensity plots of Figure 4b-f, respectively.

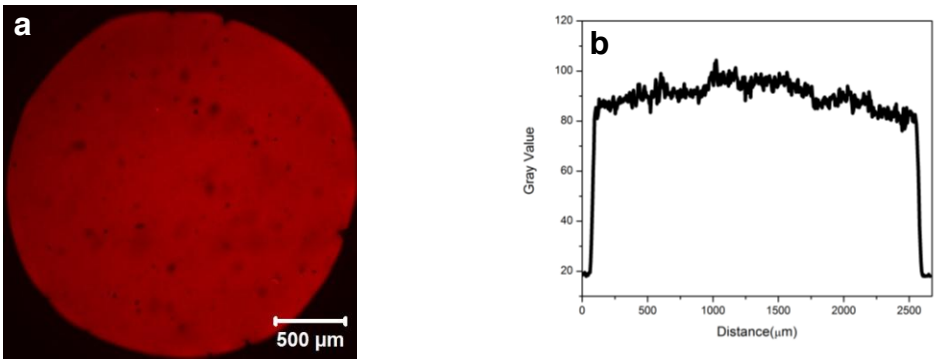


Figure S6. a) Confocal microscopy image of control experiment. b) Color intensity plot of control experiment.

4. Interfacial cell encapsulation and 3D culture.

LNCaP cells were maintained in a RPMI-1640 medium supplemented with 5% (v/v) fetal bovine serum (FBS), 100U/mL penicillin G sodium and 100 μg/mL streptomycin sulfate in 0.085% (v/v) saline (P/S), as previously described[5]. The dialyzed HA-Tz solution was sterile-filtered using a Steriflip® filter tube (EMD Millipore, Billerica, MA) before lyophilization and the dry product was dissolved in the medium at a concentration of 2 wt%. Bis-TCO (400 μM) was sterilized using a 0.22 μm Poly(vinylidene fluoride) (PVDF) sterile syringe filter (Thermo Fisher Scientific, Waltham, MA). Cell-laden HA microspheres were prepared by dropping LNCaP cells suspended in HA-Tz solution (1×10^6/mL) into a bis-TCO bath, allowing the interfacial crosslinking to occur for 2 hours at 37 °C. The bath was then replaced with fresh media and cells were incubated at 37 ºC for up to 14 days, with media refreshed every other day. Cell viability was assessed by Live/Dead staining using Syto 13 and propidium iodide (PI). Selected samples were also stained for F-actin using Alexa Fluor 488 phalloidin, with the nuclei counter stained by Draq 5, following our previous protocols[5]. Stained samples were imaged using a Zeiss 510 NLO confocal microscope. Percent viability was analyzed by taking z-stacks of 105 μm with 15 μm slices at days 1 and 5 and cell counting of the maximum intensity projections was conducted using Image J. To quantify cell proliferation, cell-laden gel constructs were treated with hyaluronidase (14.8 KU/mL, PBS) at 37 °C for 2 hours and the mixture was centrifuged at 1,500 rpm for 3 min. The collected pellets were trypsinized (0.25% Trypsin-EDTA) at 37 °C for 5 min

and the suspension was homogenous mixed. Ten microliters of the cell suspension was removed and mixed with 20 μL of 0.4% Trypan Blue (Sigma, St. Louis, MO). The live cells were counted using a hemocytometer with a light microscope (Nikon Eclipse TS100). Analyses were performed on three replicate samples and statistical significance was evaluated by analysis of variance (one-way ANOVA), followed by Tukey's post-hoc test. A P-value of <0.05 was considered to be statistically different.

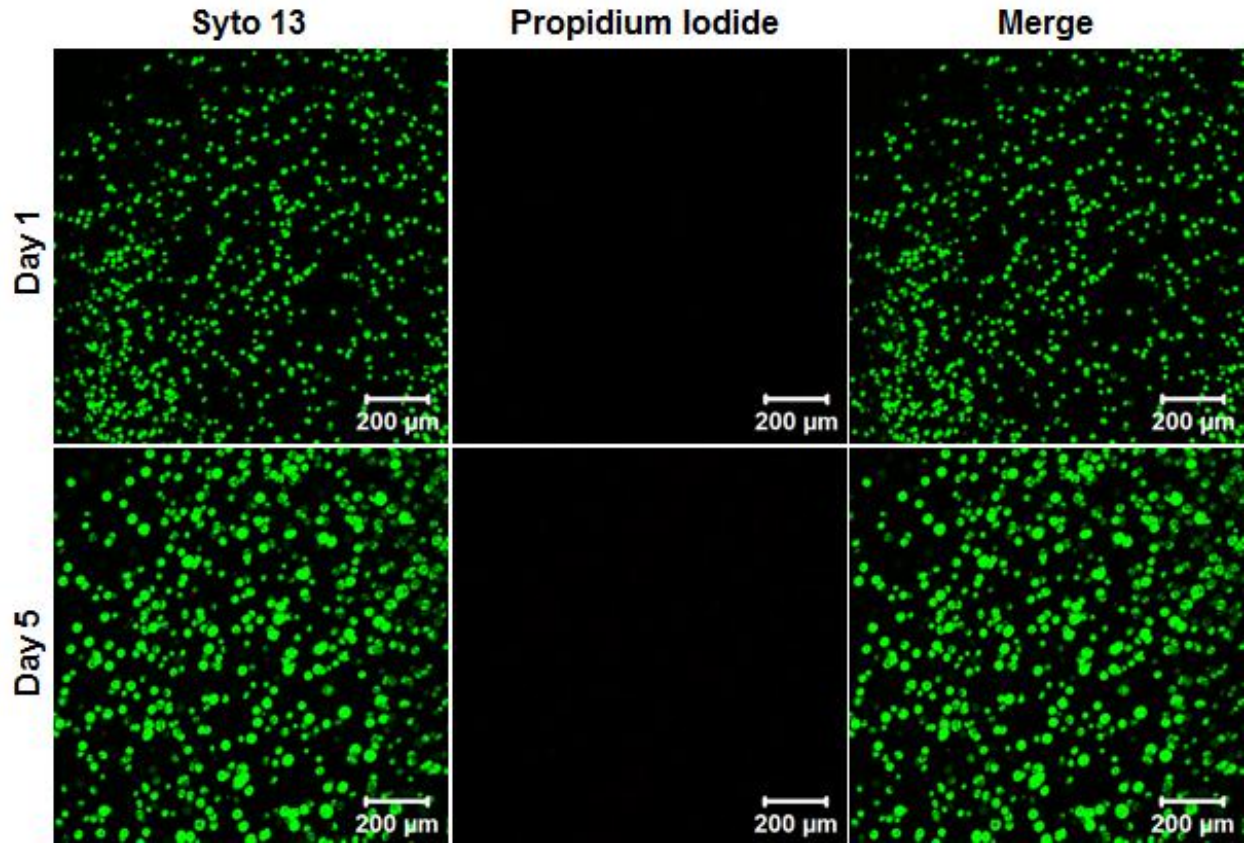


Figure S7. Live/dead staining of cells cultured in HA microspheres at day 1 and day 5. Live cells were stained green by Syto 13 and dead cells were stained red by propidium iodide.

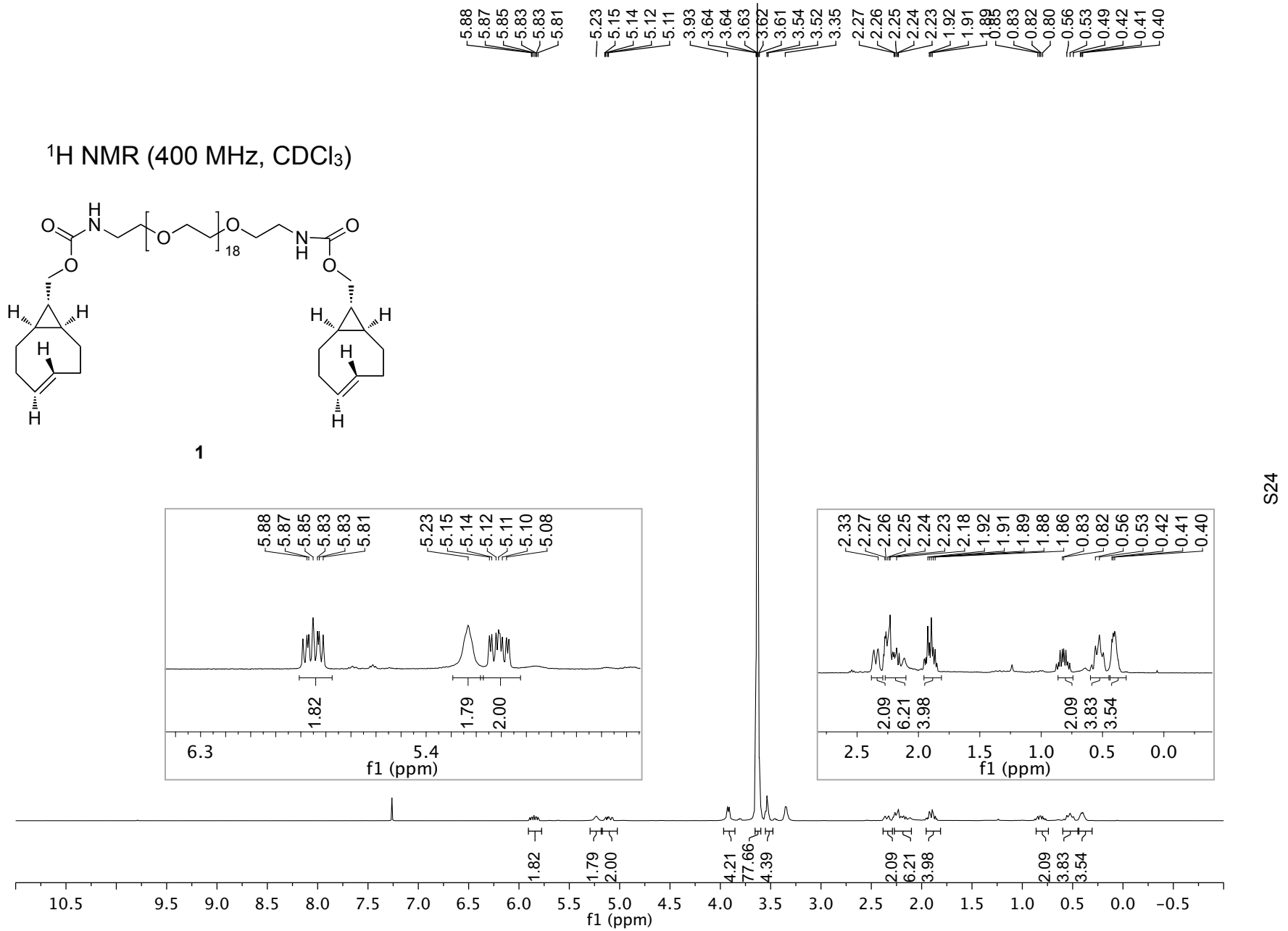


Figure S8. ^{1}H NMR spectrum of bis-TCO (**1**) in $CDCl_3$.

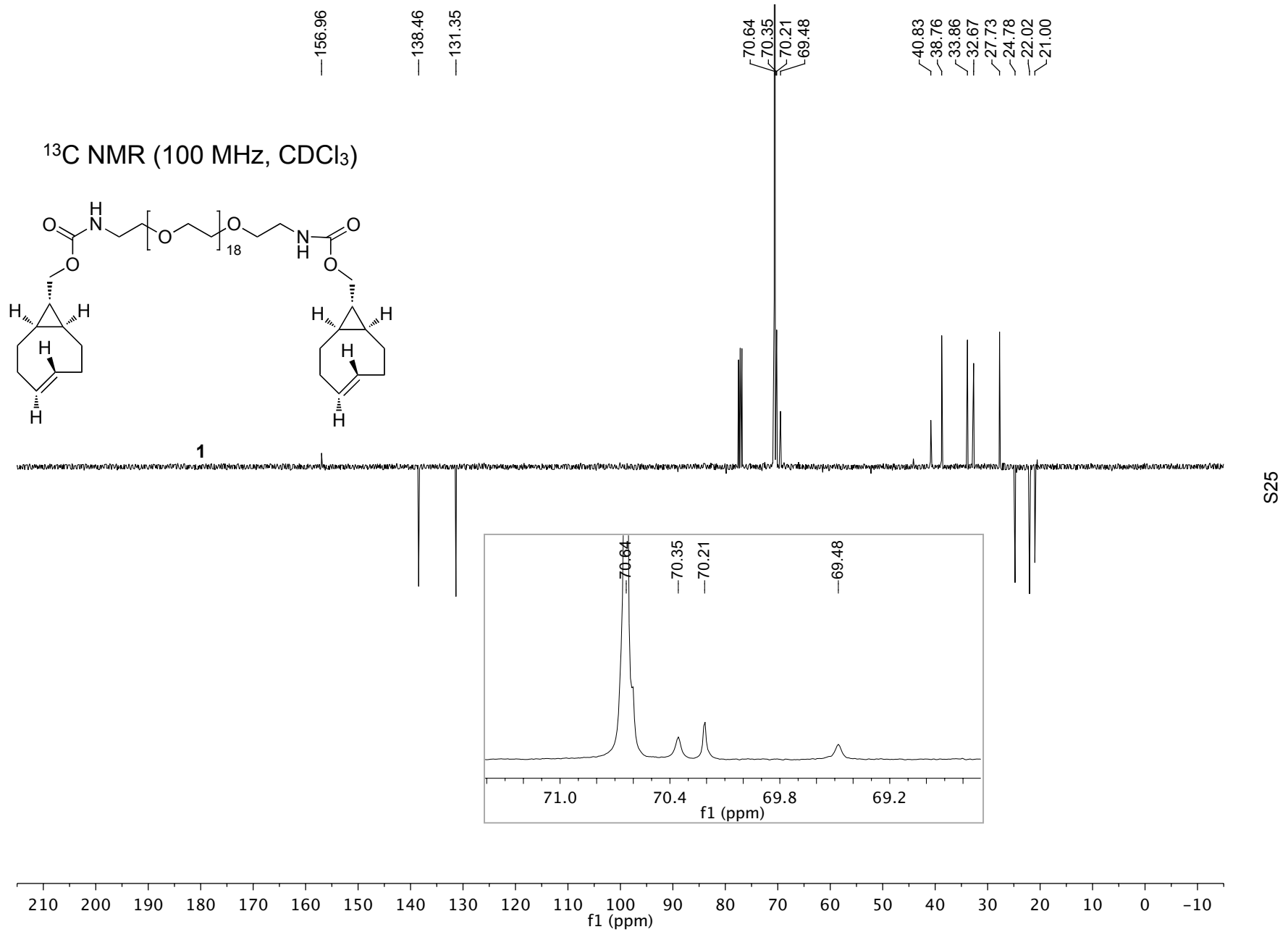


Figure S9. ^{13}C NMR spectrum of bis-TCO (**1**) in $CDCl_3$.

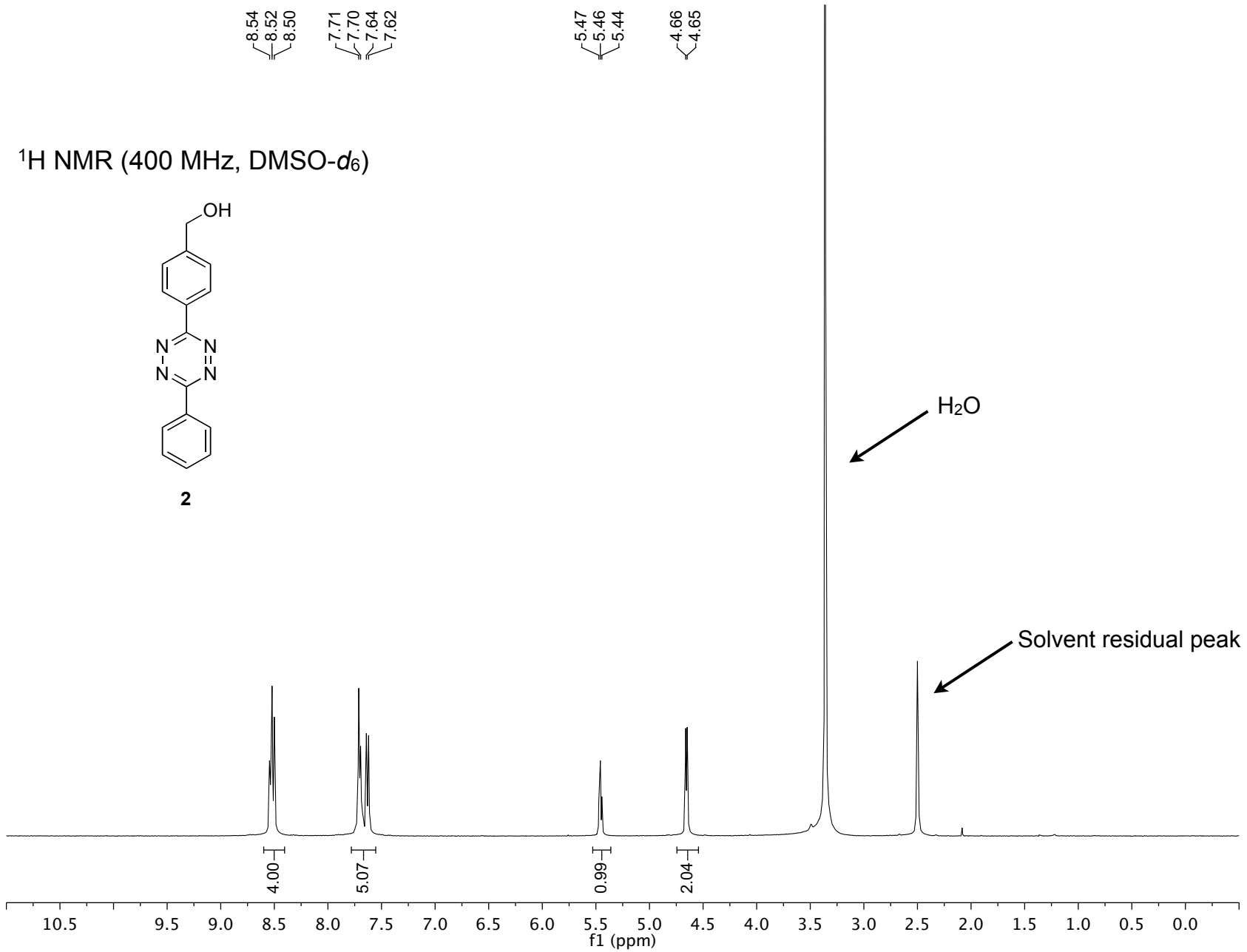


Figure S10. ^{1}H NMR spectrum of (4-(6-phenyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol (**2**) in DMSO-d_6.

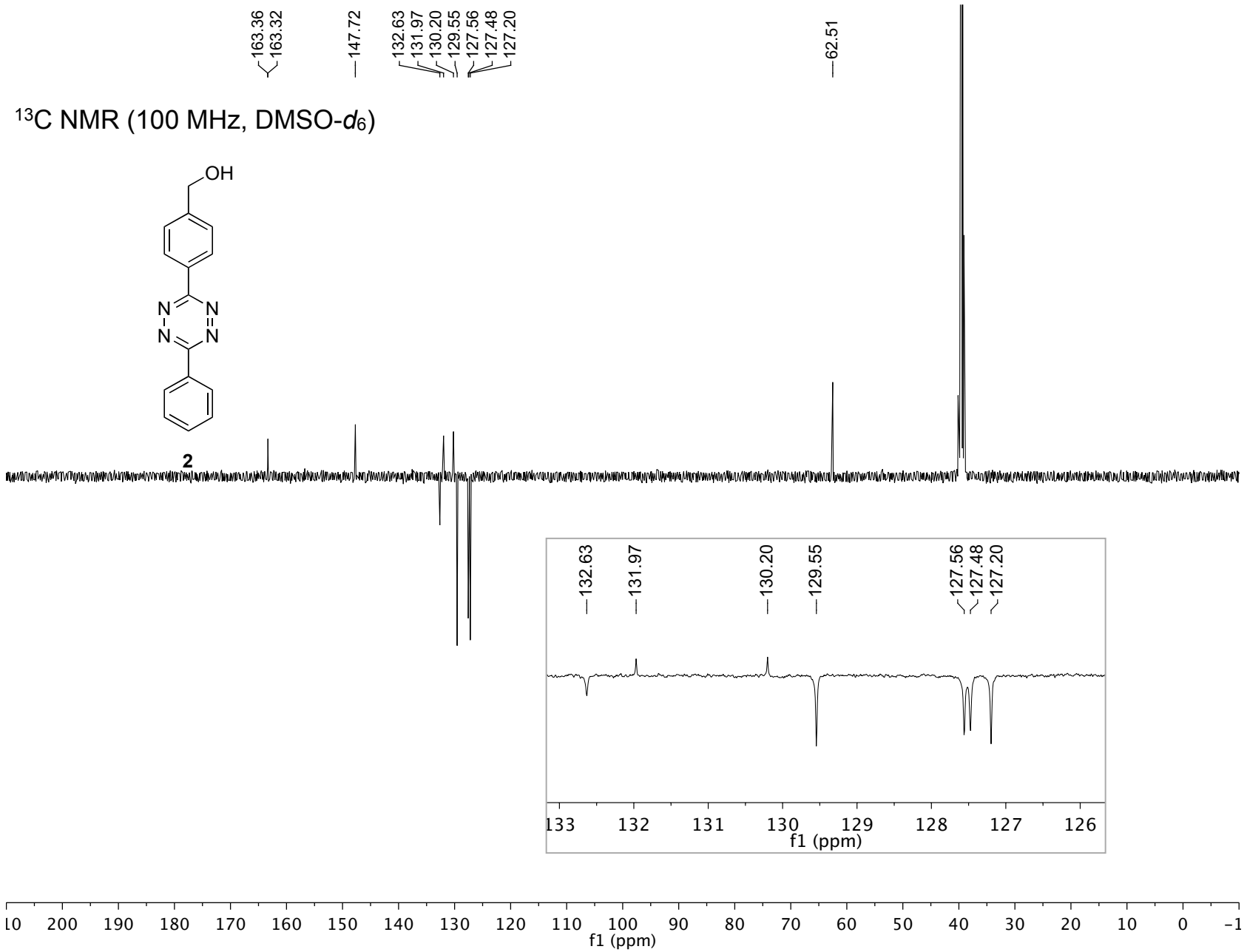


Figure S11. ^{13}C NMR spectrum of (4-(6-phenyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol (**2**) in DMSO-d_6.

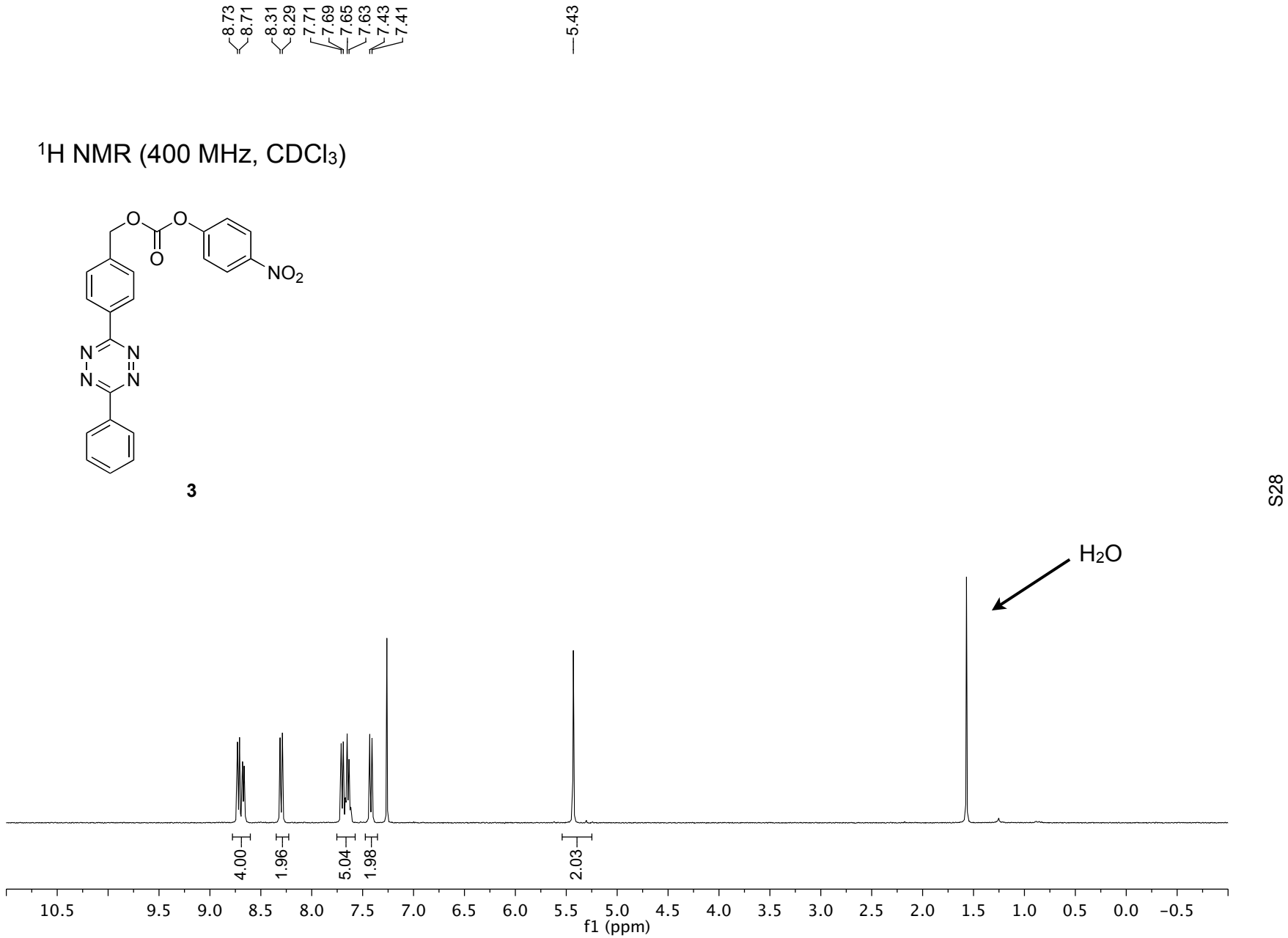


Figure S12. ^{1}H NMR of 1.3. 4-nitrophenyl 4-(6-phenyl-1,2,4,5-tetrazin-3-yl)benzyl carbonate (**3**) in $CDCl_3$.

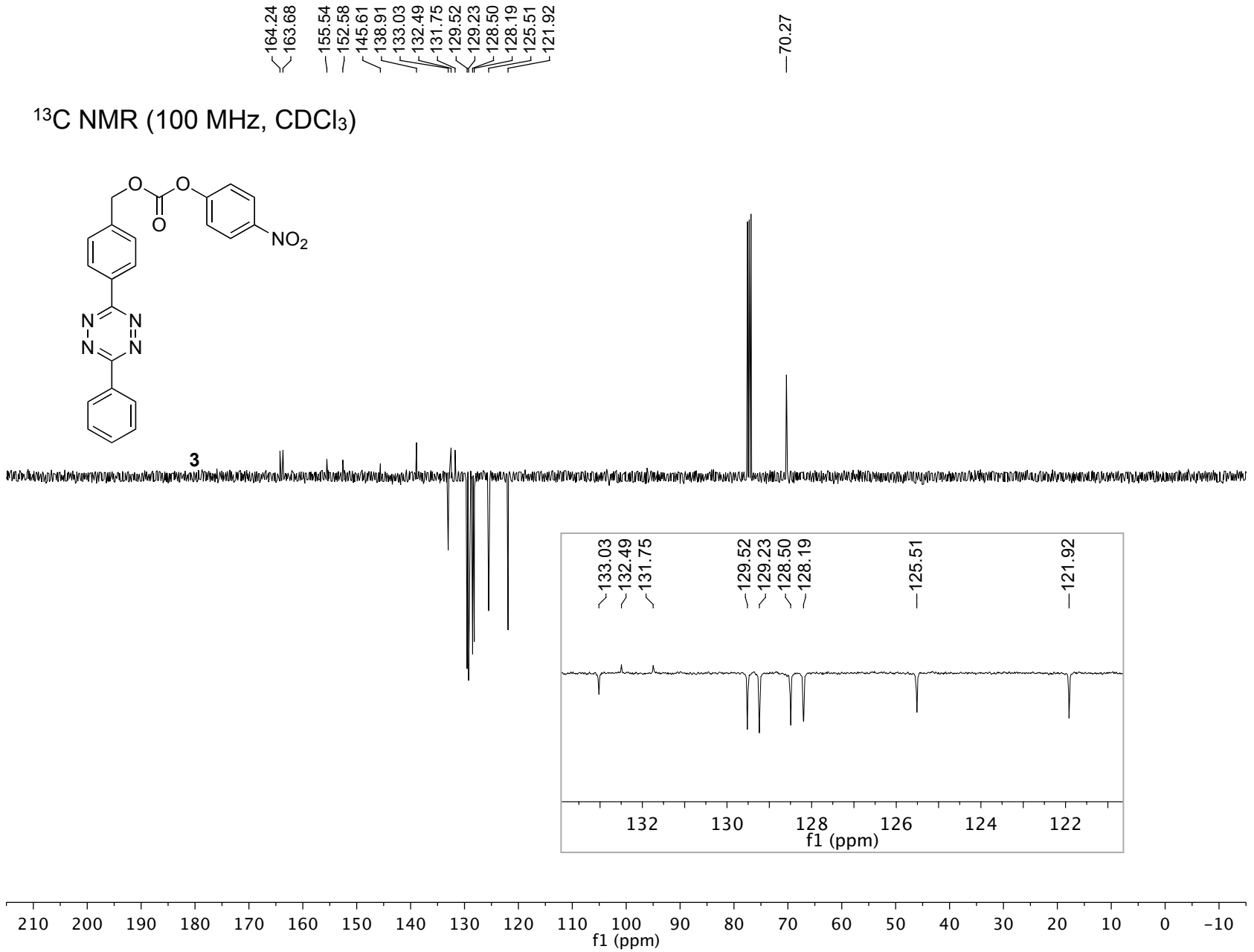


Figure S13. ^{13}C NMR of 1.3. 4-nitrophenyl 4-(6-phenyl-1,2,4,5-tetrazin-3-yl)benzyl carbonate (**3**) in $CDCl_3$.

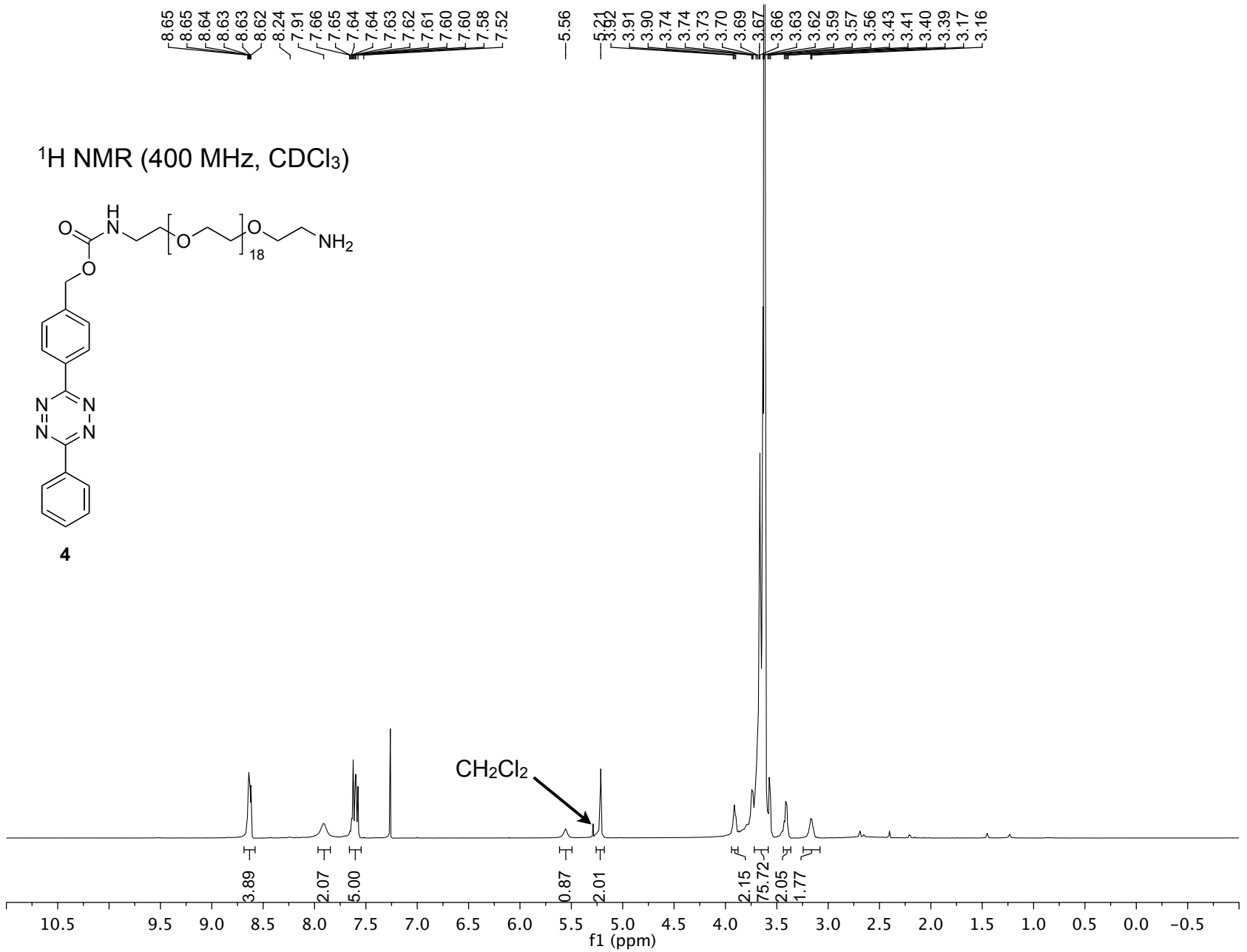


Figure S14. ^{1}H NMR spectrum of tetrazine-OEG-amine (**4**) in $CDCl_3$.

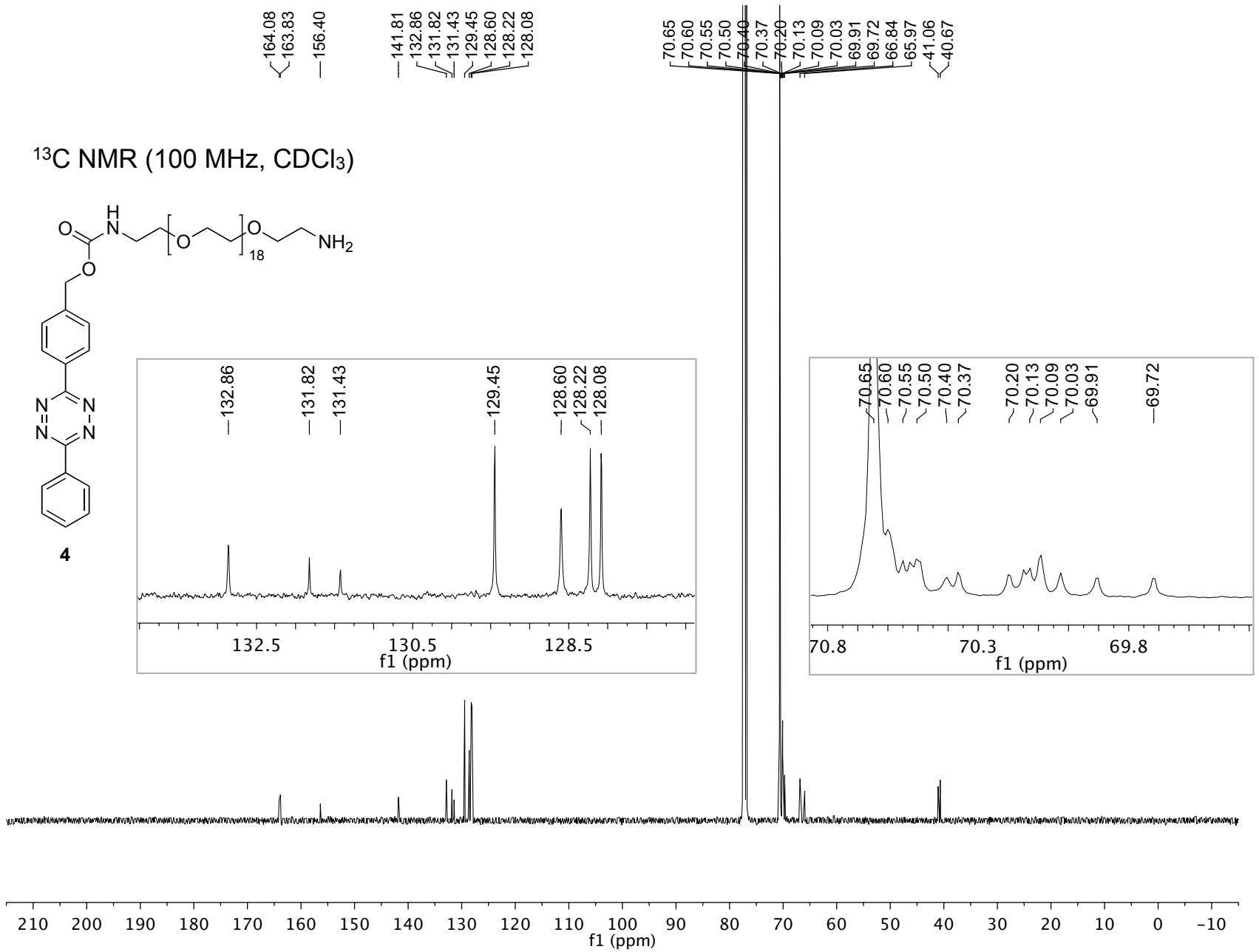


Figure S15. ^{13}C NMR spectrum of tetrazine-OEG-amine (**4**) in $CDCl_3$.

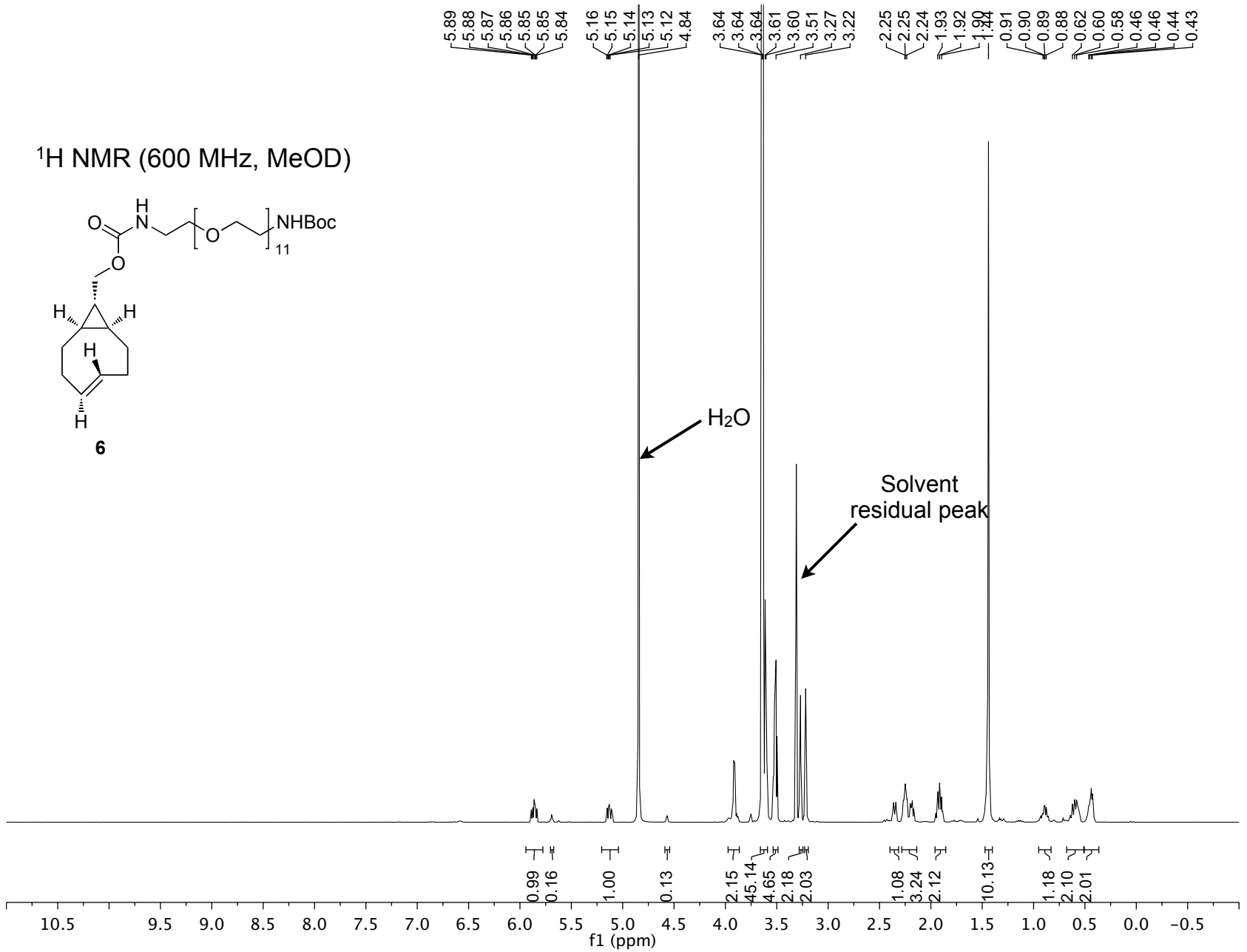


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Figure S16. ^{1}H NMR spectrum of TCO-DEG-NHBoc (**6**) in MeOD.

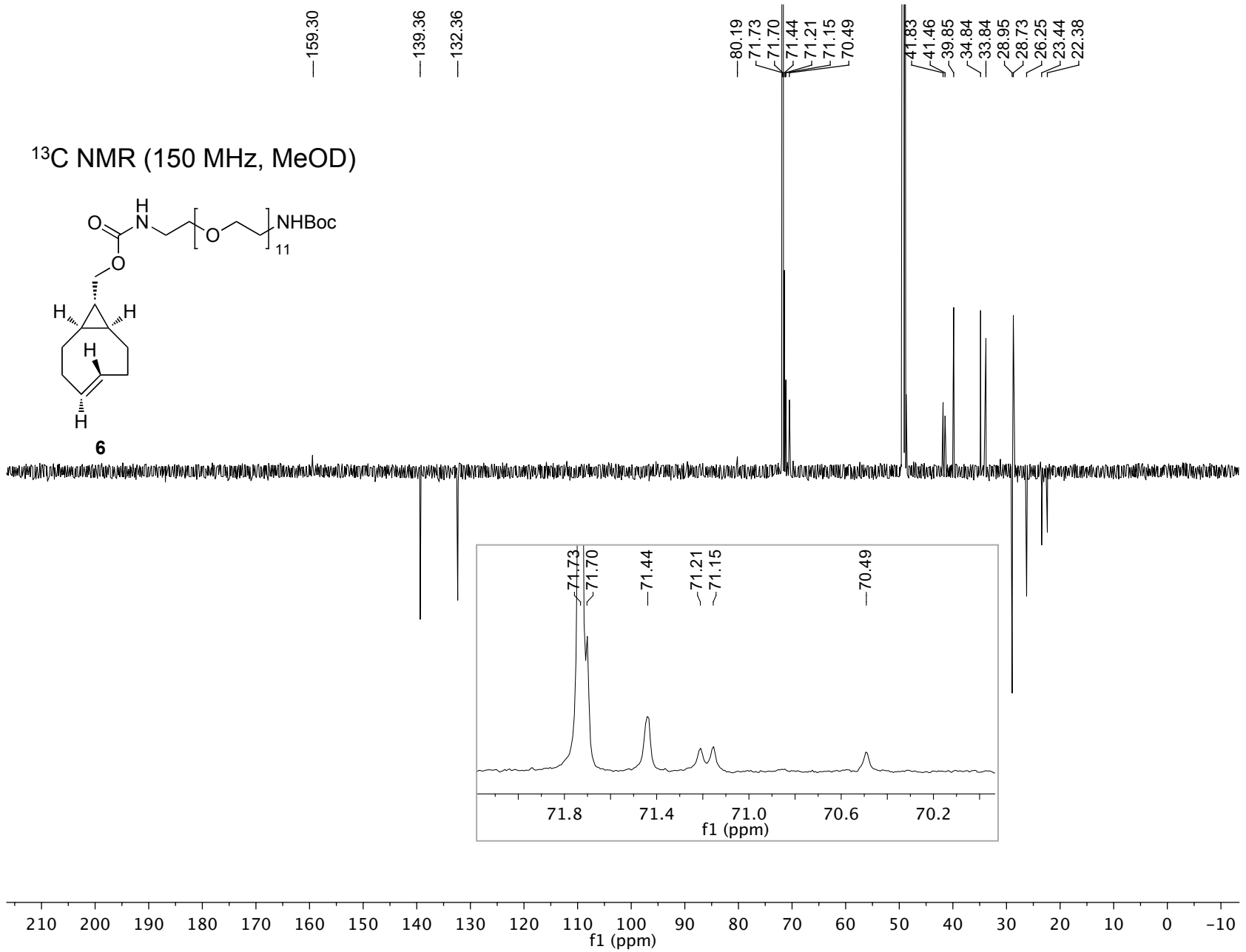

Figure S17. ^{13}C NMR spectrum of TCO-DEG-NHBoc (**6**) in MeOD.

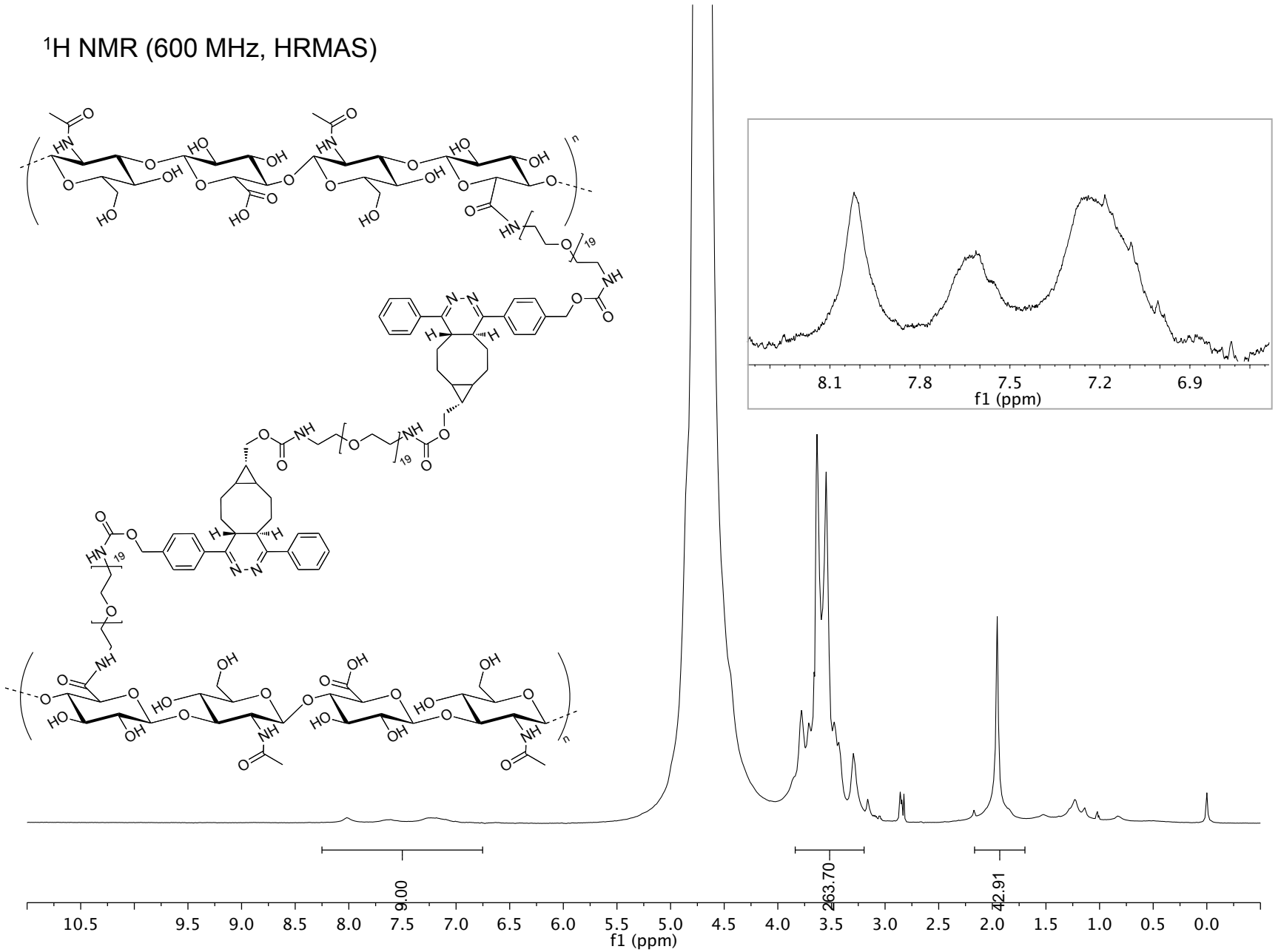

Figure S18. High resolution magic angle spinning (HR MAS) ^{1}H NMR spectrum of crosslinked HA-Tz/bis-TCO gels.

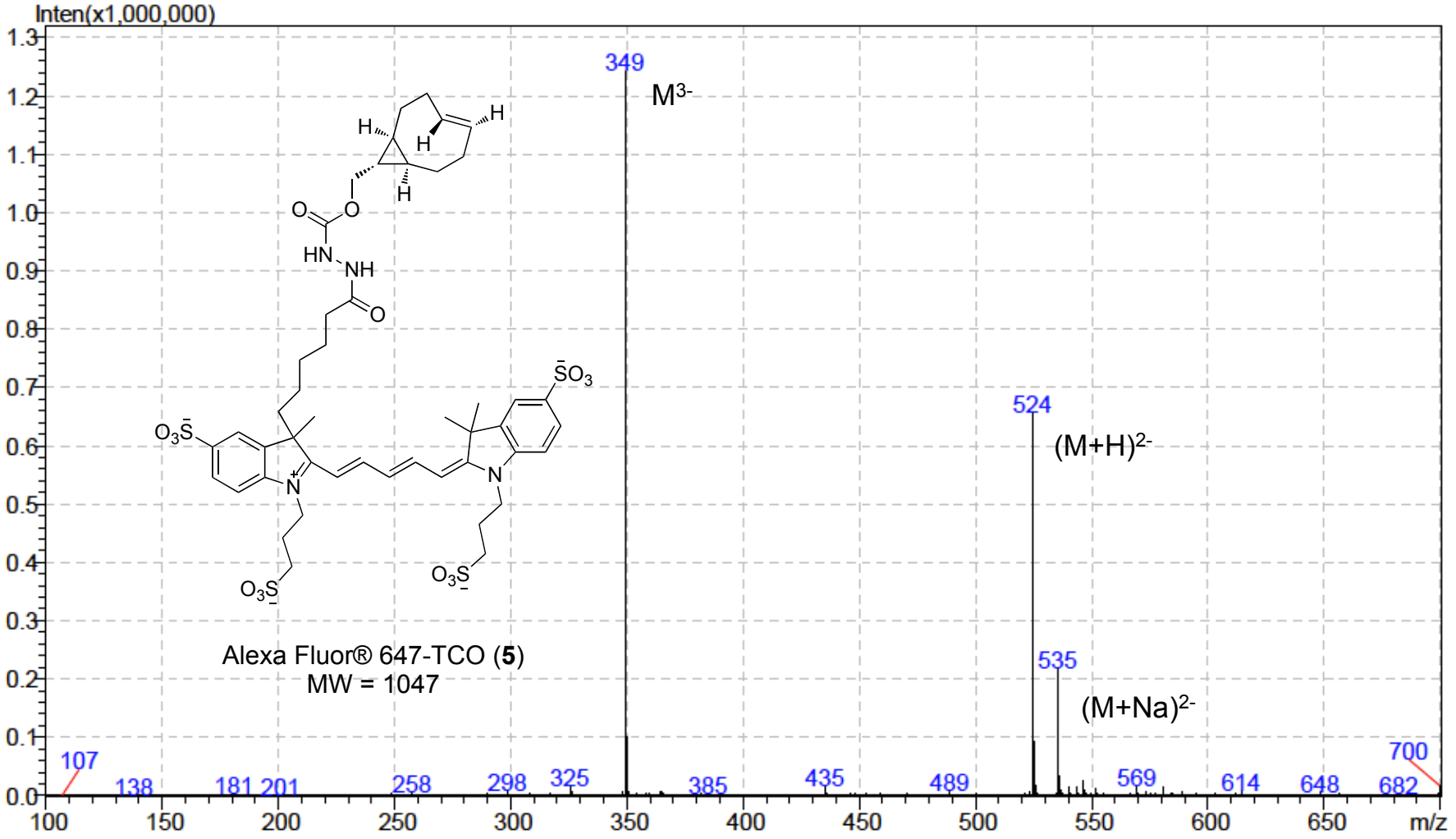

Figure S19. LC-MS analysis of Alexa-TCO (**5**) with Shimadzu LCMS-2020. (ESI negative mode, 60% ACN/H_2O)

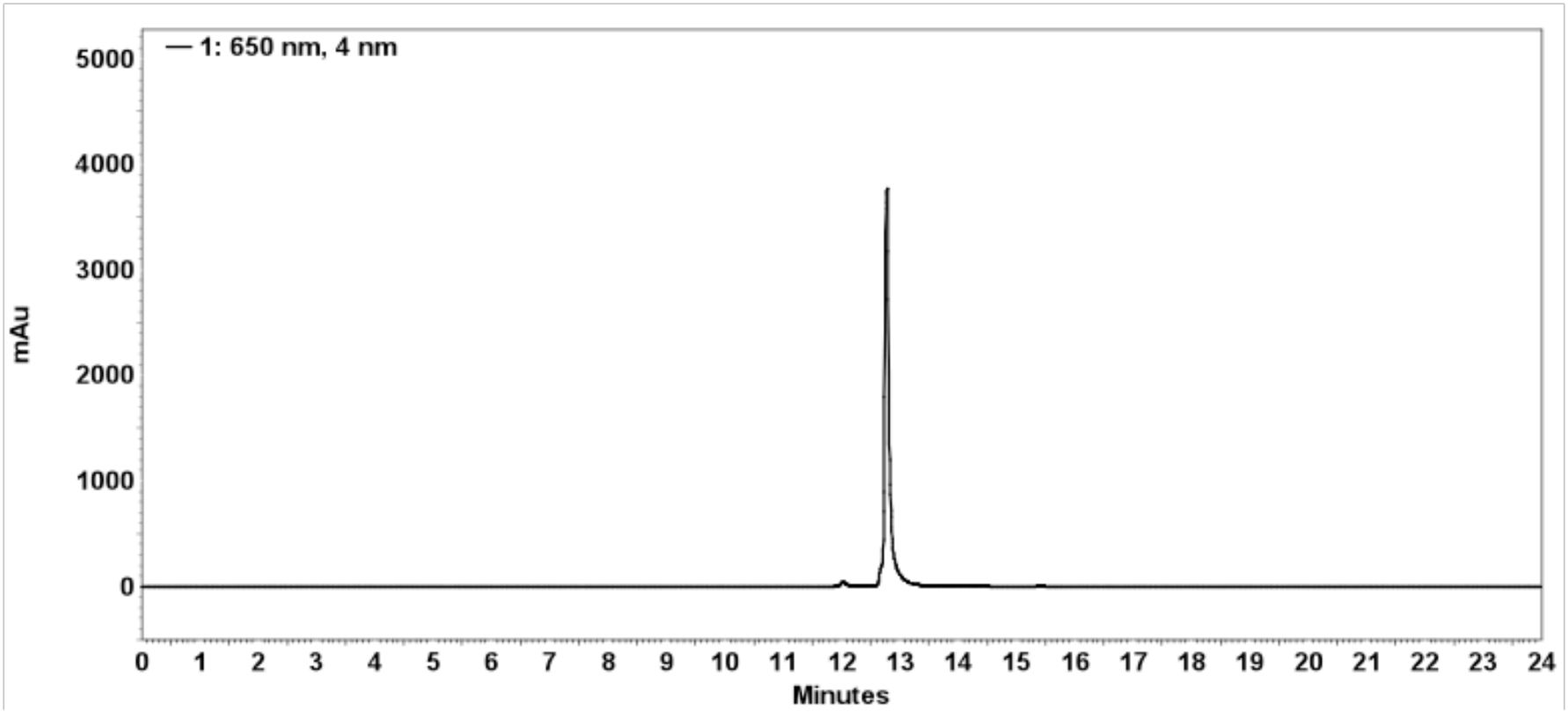

Figure S20. HPLC analysis of Alexa-TCO (**5**) with Shimadzu prominence HPLC with SPD-M20A diode array detector (5 min: 5% ACN/H_2O; 17 min: 50% ACN/H_2O; 22 min: 100% ACN; 24 min: 100% ACN).

Supplementary Movies.

Movie S1: A channel structure is produced by injecting while withdrawing an aqueous solution of bis-TCO (2 mM) into a reservoir of HA-Tz (2 wt%).

Movie S2 & S3: A water-filled channel formed instantaneously when a aqueous solution of bis-TCO (2 mM) and Alexa-TCO (2 μM) was injected into a reservoir of HA-Tz (2 wt%). At time 0, the crosslinked wall, tagged via the reaction with Alexa-TCO, reached a thickness of 155 ± 13 μm; the unreacted Alexa-TCO was retained in the channel (**Movie S2**). At t = 60 min, the wall thickness increased to 262 ± 16 μm and negligible free Alexa-TCO was found in the channel (**Movie S3**). The channels were imaged using a confocal microscope and a 789.3 μm z-stack series of 23.92 μm slices was collected and projected in the movies.

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