Biophysical Matrix Cues from the Regenerating Niche Direct Muscle Stem Cell Fate in Engineered Microenvironments

Christopher M. Madl, Iris A. Flaig, Colin A. Holbrook, Yu Xin Wang, Helen M. Blau

Baxter Laboratory for Stem Cell Biology, Department of Microbiology & Immunology, Stanford University, Stanford, CA 94305, USA

Supporting Information

1. Detailed Synthetic Procedures

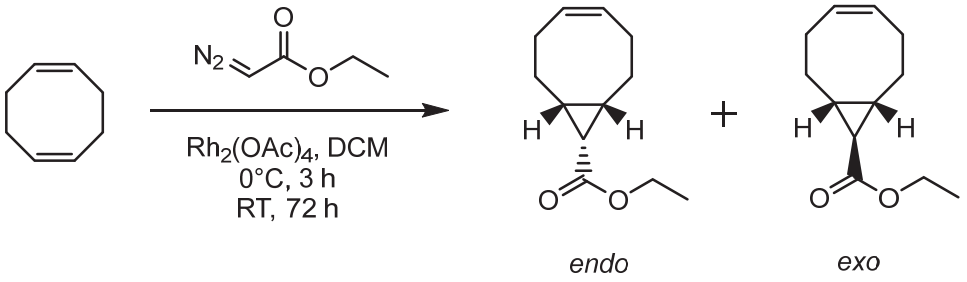
1.1. General Considerations

All reagents were purchased from Sigma-Aldrich, Fisher Scientific, or Acros Organics and used without further purification, unless otherwise noted. Multi-arm PEG starting materials were purchased from JenKem Technology USA. NMR spectroscopy was performed on Varian Mercury 400 MHz or Varian Inova 500 MHz spectrometers in the Stanford University NMR Facility. Chemical shifts were referenced to the residual solvent peak.

1.2 Synthesis of BCN-NHS

The amine reactive BCN precursor was prepared following a known synthetic route.[1–3]

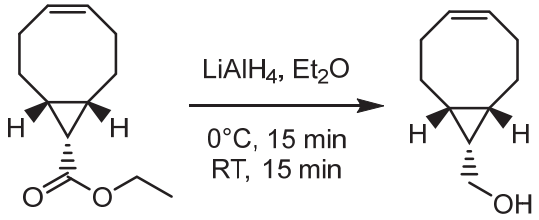
1.2.1. Ethyl (1R,8S,9s,Z)-bicyclo[6.1.0]non-4-ene-9-carboxylate



1,5-cyclooctadiene (50 mL, 44.1 g, 408 mmol, 6.2 eq.) and tetrakis(acetato)dirhodium(II) (0.75 g, 1.7 mmol, 0.026 eq.) were added to a 500 mL round bottom flask with a stir bar. The flask was purged with nitrogen while the mixture was stirred on ice. Ethyl diazoacetate (7.56 g, 66.3 mmol, 1 eq.) was diluted in dichloromethane (35 mL) and added to the stirring reaction mixture over 3 h via syringe pump, maintaining the reaction on ice under a nitrogen atmosphere. The reaction mixture was allowed to warm to room temperature and continue to react for 3 days under a nitrogen atmosphere. The reaction mixture was filtered through silica gel to remove the catalyst, and the silica gels was washed with dichloromethane followed by ethyl acetate to collect the product. Washes containing product (as visualized by TLC) were combined and concentrated *in vacuo*. The concentrate was separated by silica flash chromatography, eluting with hexanes to collect the *endo* isomer, followed by 1-3% ethyl acetate in hexanes to collect the *exo* isomer. The fractions containing mixed isomers were further separated by running a second silica column, eluting with 20:1 heptanes:ethyl acetate. Fractions containing the purified *endo*

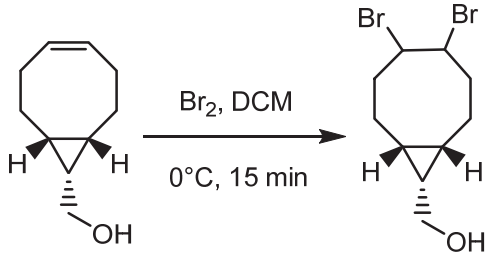
and *exo* isomers were separately combined and concentrated *in vacuo* to afford the products as colorless oils (*endo*: 3.96 g, 20.4 mmol, 30.8% yield; *exo*: 6.60 g, 34.0 mmol, 51.3% yield). *Endo*: ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 5.61 (m, 2H), 4.11 (q, J = 7.1 Hz, 2H), 2.50 (m, 2H), 2.20 (m, 2H), 2.05 (m, 2H), 1.83 (m, 2H), 1.70 (t, J = 8.9 Hz, 1H), 1.39 (m, 2H), 1.26 (t, J = 7.1 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 172.27, 129.41, 59.69, 27.04, 24.14, 22.62, 21.20, 14.38. R_f 0.53 (10:1 hexanes:EtOAc). *Exo*: ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 5.64 (m, 2H), 4.10 (q, J = 7.2 Hz, 2H), 2.30 (m, 2H), 2.20 (m, 2H), 1.56 (m, 2H), 1.47 (m, 2H), 1.25 (t, J = 7.2 Hz, 3H), 1.18 (t, J = 4.6 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 174.41, 129.89, 60.22, 28.24, 27.84, 27.71, 26.61, 14.27. R_f 0.41 (10:1 hexanes:EtOAc).

1.2.2. ((1R,8S,9s,Z)-bicyclo[6.1.0]non-4-en-9-yl)methanol



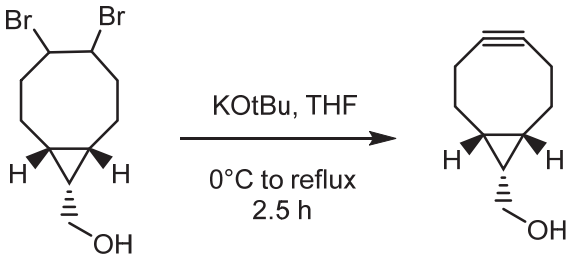
Ethyl (1R,8S,9s,Z)-bicyclo[6.1.0]non-4-ene-9-carboxylate (3.96 g, 20.4 mmol, 1 eq.) was added to a 150 mL round bottom flask with a stir bar, dissolved in anhydrous diethyl ether (50 mL) and cooled on ice, while purging the flask with nitrogen. In a separate 100 mL round bottom flask, lithium aluminum hydride (0.89 g, 23.5 mmol, 1.15 eq.) was suspended in 50 mL anhydrous diethyl ether and cooled on ice. The $LiAlH_4$ suspension was added dropwise to the stirring endo intermediate solution via cannula transfer under a nitrogen atmosphere, maintaining both flasks on ice. After addition was complete, the mixture was allowed to warm to room temperature and react for an additional 15 minutes. The mixture was again cooled on ice, and the reaction was quenched by slow, dropwise addition of 900 µL water, followed by 900 µL 15% aqueous sodium hydroxide and 2.7 mL water. The mixture was allowed to warm to room temperature and stir for 15 minutes. Magnesium sulfate was added, and the mixture was stirred for an additional 15 minutes. The mixture was filtered, and the solids were washed with diethyl ether. The flow through was combined and concentrated *in vacuo* to yield the product as a colorless oil (2.8 g, 18.4 mmol, 90.2% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 5.64 (m, 2H), 3.72 (d, J = 7.8 Hz, 2H), 2.36 (m, 2H), 2.11 (m, 2H), 2.00 (m, 2H), 1.58 (m, 2H), 1.32 (s, 1H), 1.14 (m, 1H), 1.02 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 129.72, 60.31, 27.65, 23.87, 20.76, 18.98.

1.2.3. ((1R,8S,9s)-4,5-dibromobicyclo[6.1.0]nonan-9-yl)methanol



((1R,8S,9s,Z)-bicyclo[6.1.0]non-4-en-9-yl)methanol (2.8 g, 18.4 mmol, 1 eq.) was dissolved in 140 mL anhydrous dichloromethane in a 500 mL round bottom flask with a stir bar and cooled on ice while purging with nitrogen. Bromine (1 mL, 3.12 g, 19.5 mmol, 1.06 eq.) was dissolved in anhydrous dichloromethane (15 mL) and added dropwise to the stirring reaction mixture until an orange-yellow color persisted. The reaction mixture was allowed to stir for an additional 15 minutes on ice. The reaction was then quenched by addition of 10% aqueous sodium thiosulfate with vigorous stirring. The mixture was extracted with dichloromethane (3 × 75 mL). The combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo* to yield the product as a white solid (5.34 g, 17.1 mmol, 92.9% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 4.86 (m, 2H), 3.77 (d, J = 7.6Hz, 2H), 2.69 (m, 2H), 2.28 (dddd, J = 16.3, 12.5, 5.4, 2 Hz, 1H), 2.17 (m, 1H), 1.93 (m, 2H), 1.62 (m, 2H), 1.30 (s, 1H), 1.15 (m, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 59.62, 56.16, 53.18, 34.91, 21.83, 20.07, 19.89, 18.92, 17.16.

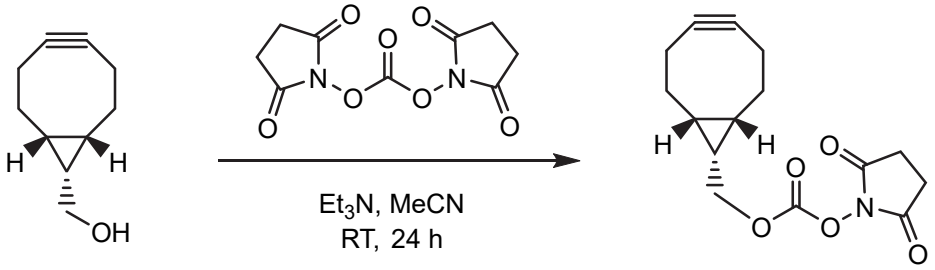
1.2.4. ((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methanol



((1R,8S,9s)-4,5-dibromobicyclo[6.1.0]nonan-9-yl)methanol (5.34 g, 17.1 mmol, 1 eq.) was dissolved in anhydrous THF (100 mL) in a 500 mL round bottom flask with a stir bar, purged with nitrogen, and cooled on ice while stirring. A solution of potassium *tert*-butoxide (1 M in 50 mL THF) was added dropwise to the stirring reaction mixture via cannula transfer while maintaining the reaction mixture on ice. After addition was complete, the flask was fitted with a reflux condenser and purged with nitrogen. The reaction mixture was brought to reflux and

allowed to react for 2.5 h. The reaction was quenched by addition of saturated aqueous ammonium chloride (120 mL) with vigorous stirring. The THF was removed by rotary evaporation, and the reaction mixture was extracted with dichloromethane (3 × 70 mL). The combined organic layers were dried over magnesium sulfate, filtered, and concentrated *in vacuo*. The resulting yellow oil was purified by silica flash chromatography, eluting with 4:1 hexanes:ethyl acetate, to yield the product as a faint yellow solid (1.43 g, 9.5 mmol, 55.6% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 3.74 (d, J = 8.1 Hz, 2H), 2.27 (m, 6H), 1.61 (m, 2H), 1.35 (m, 2H), 0.95 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 98.86, 59.99, 29.02, 21.49, 21.38, 20.00. R_f 0.38 (1:1 hexanes:EtOAc).

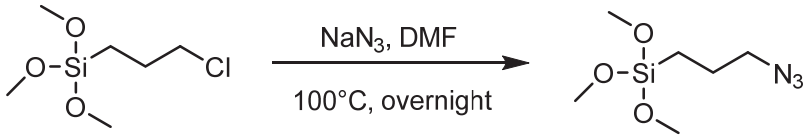
1.2.5. ((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2,5-dioxopyrrolidin-1-yl) carbonate



((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methanol (1.43 g, 9.5 mmol, 1 eq.) and disuccinimidyl carbonate (4.88 g, 19.0 mmol, 2 eq.) were dissolved in anhydrous acetonitrile (75 mL) in a 500 mL round bottom flask with stir bar and purged with nitrogen. Triethylamine (3.94 mL, 2.86 g, 28 mmol, 3 eq.) was added dropwise to the stirring reaction mixture. The reaction was allowed to proceed overnight at room temperature under a nitrogen atmosphere. The reaction mixture was concentrated by rotary evaporation. The residue was redissolved in minimal dichloromethane and purified by silica flash chromatography, eluting with 3:1 hexanes:ethyl acetate. The fractions containing the desired product were combined and concentrated *in vacuo* to afford the product as a white solid (2.09 g, 7.2 mmol, 75.8% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 4.45 (d, J = 8.3 Hz, 2H), 2.84 (s, 4H), 2.27 (m, 6H), 1.56 (m, 2H), 1.50 (m, 1H), 1.06 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 168.70, 151.59, 98.66, 70.32, 28.93, 25.44, 21.28, 20.67, 17.13. R_f 0.50 (1:1 hexanes:EtOAc).

1.3. Synthesis of (3-azidopropyl)trimethoxysilane

(3-azidopropyl)trimethoxysilane was synthesized following a published protocol.[1]

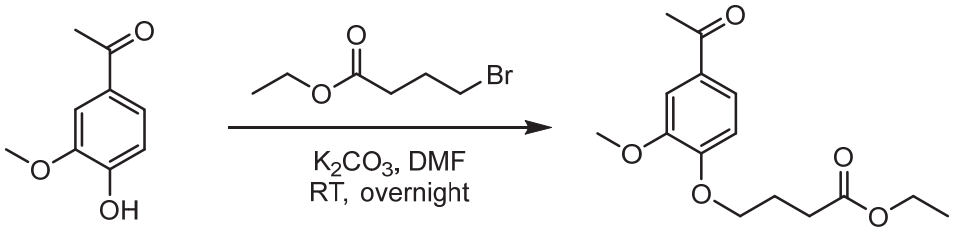


(3-chloropropyl)trimethoxysilane (12 mL, 13.1 g, 65.8 mmol, 1 eq.) and sodium azide (6.38 g, 98.1 mmol, 1.5 eq.) were added to a 150 mL round bottom flask with a stir bar. Anhydrous dimethylformamide (40 mL) was added, and the flask was purged with nitrogen. The flask was equipped with a reflux condenser, purged with nitrogen, and heated to 100°C. The reaction was allowed to proceed overnight under a nitrogen atmosphere. The reaction mixture was cooled to room temperature and then diluted with 1:1 water:diethyl ether (150 mL). The organic layer was separated, washed with water (3 × 75 mL) and brine (75 mL), dried over magnesium sulfate, filtered, and concentrated *in vacuo* to afford the product as a colorless oil (11.1 g, 54.1 mmol, 82.2% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 3.58 (s, 9H), 3.27 (t, J = 7.0 Hz, 2H), 1.71 (m, 2H), 0.70 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 53.69, 50.58, 22.42, 6.29.

1.4. Synthesis of Methyl Substituted Photocleavable Linker (N3-Me-oNB-NHS)

The ortho-nitrobenzyl photocleavable linker was prepared following a known synthetic route.[3,4]

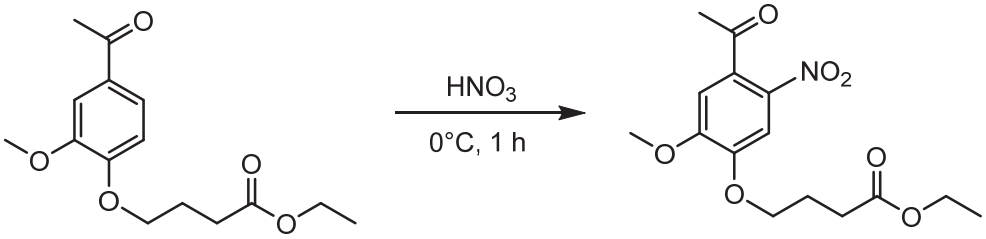
*1.4.1. Ethyl 4-(4-acetyl-2-methoxyphenoxy)butanoate (**3a**)*



Acetovanillone (30.0 g, 180.5 mmol, 1 eq.) and ethyl 4-bromobutyrate (42.3 g, 216.9 mmol, 1.2 eq.) were added to a 1 L round bottom flask and dissolved in anhydrous dimethylformamide (150 mL). After dissolution, potassium carbonate (37.4 g, 270.8 mmol, 1.5 eq.) was added, and the flask was purged with nitrogen. The reaction was allowed to proceed at room temperature under a nitrogen atmosphere for ~24 h. The reaction mixture was slowly poured into 1.5 L of stirring water at room temperature. The mixture was stirred at room temperature for 2 h and then

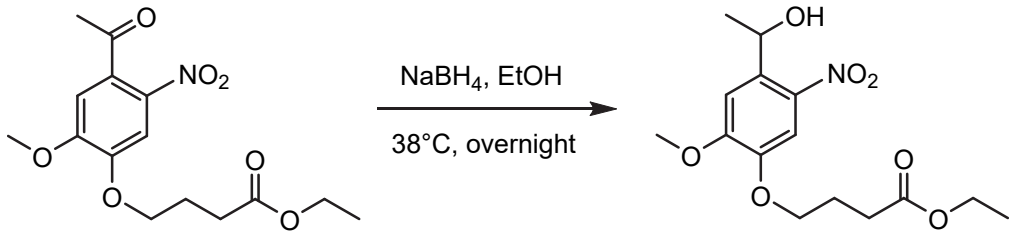
transferred to 4°C to fully precipitate the product. The precipitate was collected by vacuum filtration, and the solid was dried on the filter and *in vacuo* to afford the product as a white solid (49.1 g, 175.2 mmol, 97.1% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.53 (m, 2H), 6.89 (d, J = 8.3 Hz, 1H), 4.14 (m, 4H), 3.90 (s, 3H), 2.54 (m, 5H), 2.18 (quin, J = 6.8 Hz, 2H), 1.25 (t, J = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 196.77, 172.96, 152.55, 149.18, 130.41, 123.15, 111.15, 110.34, 67.72, 60.45, 55.93, 30.51, 26.17, 24.22, 14.16.

*1.4.2. Ethyl 4-(4-acetyl-2-methoxy-5-nitrophenoxy)butanoate (**4a**)*



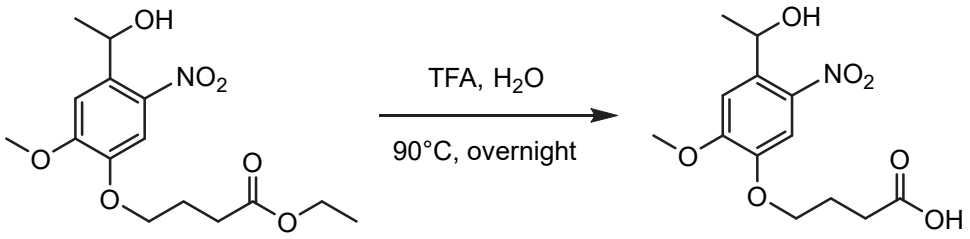
Nitric acid (140 mL) was added to a 1 L round bottom flask with a stir bar and chilled in an ice bath. Ethyl 4-(4-acetyl-2-methoxyphenoxy)butanoate (49.0 g, 174.8 mmol, 1 eq.) was added in small portions to the cold, stirring nitric acid, allowing each batch to dissolve before subsequent addition. The reaction mixture was allowed to stir on ice for an additional 1 h. The reaction mixture was then added dropwise to stirring ice-cold water (1500 mL) to precipitate the product. The mixture was stirred for an additional hour at room temperature and then stored overnight at 4°C to fully precipitate the product. The precipitate was collected by vacuum filtration and washed with ice-cold water (500 mL). The yellow powder was dried on the filter and *in vacuo*, followed by recrystallization in ethanol to afford the product as a yellow solid (19.87 g, 61.1 mmol, 35.0% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.58 (s, 1H), 6.73 (s, 1H), 4.13 (m, 4H), 3.93 (s, 3H), 2.52 (t, J = 7.2 Hz, 2H), 2.46 (s, 3H), 2.17 (quin, J = 6.7 Hz, 2H), 1.23 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 199.67, 172.69, 154.17, 148.72, 138.18, 132.69, 108.61, 107.86, 68.35, 60.45, 56.48, 30.37, 30.25, 24.05, 14.09.

*1.4.3. Ethyl 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoate (**5a**)*



Ethyl 4-(4-acetyl-2-methoxy-5-nitrophenoxy)butanoate (19.8 g, 60.7 mmol, 1 eq.) and anhydrous ethanol (300 mL) were added to a 500 mL round bottom flask with a stir bar and purged with nitrogen while stirring. The mixture was warmed to ~38°C to aid in dissolution of the starting material. After equilibrating at 38°C, sodium borohydride (2.25 g, 59.5 mmol, 0.97 eq.) was added to the stirring mixture in approximately equal portions every 5 minutes over the course of 40 minutes. The flask was again purged with nitrogen, and the reaction was allowed to proceed overnight at 38°C. The reaction mixture was then poured into 3 L of stirring room temperature water. The mixture was stirred for 1 h at room temperature and then stored overnight at 4°C to fully precipitate the product. The precipitate was collected by vacuum filtration, washed with ice-cold water (250 mL), and dried on the filter and *in vacuo* to afford the product as a yellow solid (15.47 g, 47.3 mmol, 77.9% yield). ^{1}H NMR (400 MHz, DMSO-d6) δ ppm 7.50 (s, 1H), 7.34 (s, 1H), 5.46 (d, J = 4.3 Hz, 1H), 5.24 (m, 1H), 4.04 (m, 4H), 3.88 (s, 3H), 2.46 (m, 2H), 1.96 (quin, J = 6.8 Hz, 2H), 1.34 (d, J = 6.3 Hz, 3H), 1.16 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d6) δ ppm 172.44, 153.43, 146.15, 138.85, 138.10, 109.07, 108.35, 67.81, 63.92, 59.92, 56.05, 30.05, 25.16, 24.04, 14.08.

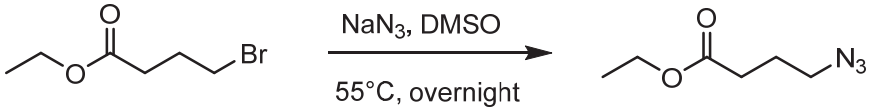
*1.4.4. 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoic acid (**6a**)*



Ethyl 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoate (15.4 g, 47.0 mmol, 1 eq.) was ground to a fine powder and added to a 1 L Erlenmeyer flask with a stir bar. Water (450 mL) and trifluoroacetic acid (45 mL) were added, and the stirring mixture was heated to 90°C. After 6 h, trifluoroacetic acid (23 mL) was added, and the mixture was stirred for an additional

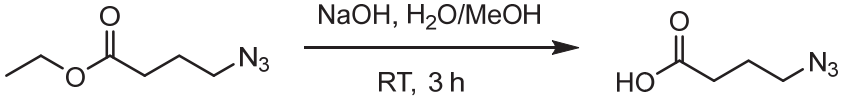
17 h at 90°C. Additional trifluoroacetic acid (23 mL) was added, and the mixture was stirred for 4 h at 90°C. The mixture was filtered hot to remove a black precipitate, and the flow through was cooled to room temperature and then on ice to precipitate the product. The precipitate was collected by vacuum filtration, re-dissolved in aqueous sodium hydroxide (1 M, ~95 mL), and re-precipitated by acidifying the solution to pH 1 with hydrochloric acid (5 N). The precipitate was collected by filtration, washed with ice-cold water (100 mL), and dried *in vacuo* to afford the product as a yellow solid (9.15 g, 30.6 mmol, 65.1% yield). ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 12.17 (br. s, 1H), 7.53 (s, 1H), 7.36 (s, 1H), 5.48 (m, 1H), 5.25 (m, 1H), 4.05 (t, J = 6.3 Hz, 2H), 3.90 (s, 3H), 2.39 (t, J = 7.3 Hz, 2H), 1.95 (quin, J = 6.9 Hz, 2H), 1.36 (d, J = 6.1 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d6) δ ppm 174.03, 153.44, 146.30, 138.89, 138.07, 109.10, 108.40, 67.89, 63.93, 58.08, 29.96, 25.18, 24.05.

1.4.5. Ethyl 4-azidobutanoate



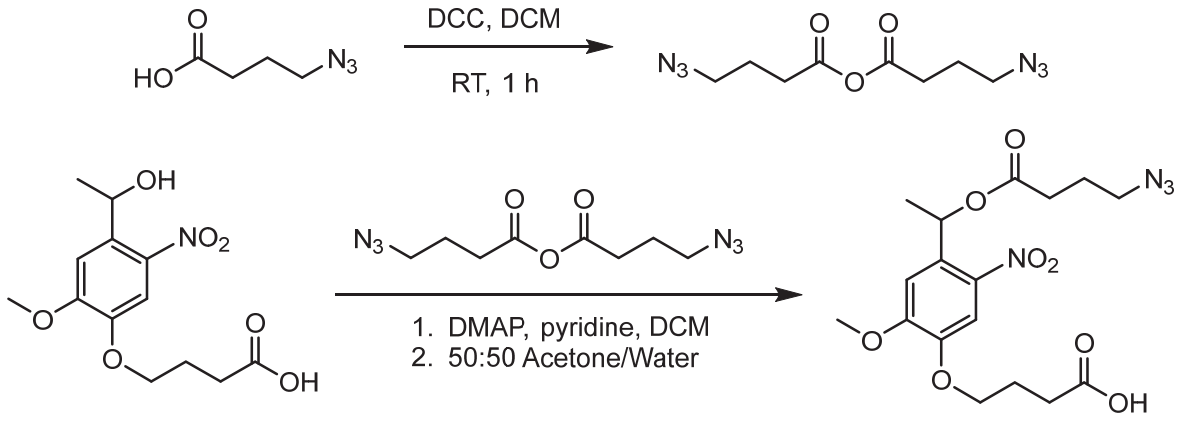
Ethyl 4-bromobutyrate (50.0 g, 256.3 mmol, 1 eq.) was added to a 500 mL round bottom flask with a stir bar and dissolved in anhydrous dimethylsulfoxide (375 mL). While stirring, sodium azide (25.0 g, 384.4 mmol, 1.5 eq.) was added. The mixture was warmed to 55°C, and the reaction was allowed to proceed overnight. The reaction mixture was diluted into 250 mL water and allowed to cool to room temperature. The mixture was extracted into diethyl ether (3 × 250 mL). The combined organic phases were washed with water (250 mL) and brine (250 mL), dried over magnesium sulfate, filtered, and concentrated *in vacuo* to afford the product as a colorless oil (38.18 g, 242.9 mmol, 94.8% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 4.14 (q, J = 7.1 Hz, 2H), 3.35 (t, J = 6.6 Hz, 2H), 2.40 (t, J = 7.2 Hz, 2H), 1.91 (quin, J = 7.0 Hz, 2H), 1.26 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 172.63, 60.52, 50.59, 31.12, 24.20, 14.15.

1.4.6. 4-azidobutanoic acid



Ethyl 4-azidobutanoate (38.1 g, 242.4 mmol, 1 eq.) was added to a 1 L round bottom flask with a stir bar and mixed with aqueous sodium hydroxide (1 M, 250 mL). Methanol (175 mL) was added to homogenize the solution. The reaction mixture was stirred at room temperature for 3 h. The methanol was removed via rotary evaporation. The remaining solution was transferred to an Erlenmeyer flask and acidified to pH 1 by dropwise addition of hydrochloric acid (5 N). The mixture was extracted into diethyl ether (3 × 250 mL). The combined organic phases were dried over magnesium sulfate, filtered, and concentrated *in vacuo* to afford the product as a colorless oil (30.66 g, 237.5 mmol, 98.0% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 6.67 (br. s, 1H), 3.38 (t, J = 6.7 Hz, 2H), 2.47 (t, J = 7.2 Hz, 2H), 1.92 (quin, J = 7.0 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 178.40, 50.42, 30.83, 23.90.

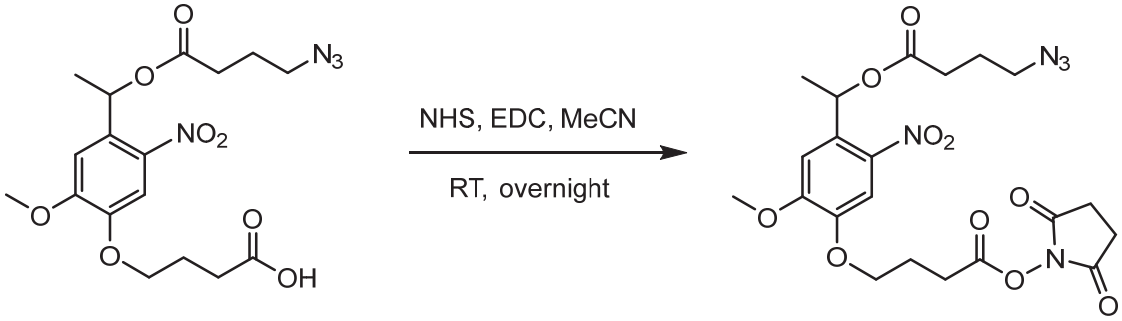
*1.4.7. 4-(4-(1-((4-azidobutanoyl)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoic acid (**7a**)*



4-azidobutanoic acid (30.5 g, 236.2 mmol, 14.9 eq.) and *N*,*N*'-dicyclohexylcarbodiimide (15.7 g, 76.1 mmol, 4.8 eq.) were added to a 500 mL round bottom flask and purged with nitrogen. Anhydrous dichloromethane (190 mL) was added, and the reaction mixture was stirred at room temperature under a nitrogen atmosphere for 1 h. The reaction mixture was filtered to remove the urea byproduct and concentrated by rotary evaporation. The concentrate was filtered to remove additional urea byproduct and was then re-dissolved in anhydrous dichloromethane (35 mL). The solution was concentrated by rotary evaporation, and no additional urea byproduct was formed. The crude anhydride was used immediately, without further purification. 4-(4-(1-

hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoic acid (4.75 g, 15.9 mmol, 1 eq.), 4-dimethylaminopyridine (95 mg, 0.78 mmol, 0.05 eq.), and the crude azido anhydride were added to a 500 mL round bottom flask with a stir bar and purged with nitrogen. The mixture was dissolved in anhydrous dichloromethane (120 mL), and anhydrous pyridine (1.3 mL, 1.28 g, 16.2 mmol, 1.02 eq.) was added. The reaction mixture was stirred at room temperature overnight under a nitrogen atmosphere. The mixture was then washed with saturated aqueous sodium bicarbonate (120 mL), hydrochloric acid (1 M, 120 mL), and brine (120 mL). The organic layer was concentrated by rotary evaporation, and the residue was re-dissolved in a 1:1 mixture of acetone and water (600 mL) and stirred overnight at room temperature. The acetone was removed by rotary evaporation, and the mixture was extracted with dichloromethane (3 × 150 mL). The combined organic phases were washed with hydrochloric acid (1 M, 150 mL) and brine (150 mL), dried over magnesium sulfate, filtered, and concentrated by rotary evaporation. The residue was purified by silica flash chromatography, using a gradient from 20-40% ethyl acetate in hexanes plus 1% acetic acid. Fractions containing the desired product were confirmed by TLC, combined, and concentrated by rotary evaporation. The residue was re-dissolved in dichloromethane (125 mL) and washed with water (3 × 125 mL) and brine (125 mL) to remove residual acetic acid. The solution was dried over magnesium sulfate, filtered, and concentrated *in vacuo*, to afford the product as a yellow solid (2.27 g, 5.5 mmol, 34.6% yield). ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 12.17 (s, 1H), 7.57 (s, 1H), 7.10 (s, 1H), 6.20 (q, J = 6.5 Hz, 1H), 4.07 (t, J = 6.5 Hz, 2H), 3.93 (s, 3H), 3.32 (t, J = 6.8 Hz, 2H), 2.43 (t, J = 7.3 Hz, 2H), 2.38 (t, J = 7.3 Hz, 2H), 1.95 (quin, J = 6.9 Hz, 2H), 1.75 (quin, J = 7.1 Hz, 2H), 1.58 (d, J = 6.6 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d6) δ ppm 173.98, 171.56, 153.53, 146.88, 139.66, 131.88, 108.65, 108.41, 67.48, 56.25, 49.86, 30.69, 29.91, 23.97, 23.71, 21.30. R_f 0.41 (1:1 hexanes:EtOAc + 1% acetic acid).

*1.4.8. 2,5-dioxopyrrolidin-1-yl 4-(4-(1-((4-azidobutanoyl)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoate (**8a**)*

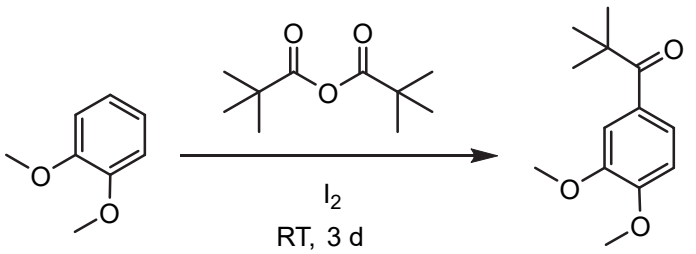


N-hydroxysuccinimide (0.95 g, 8.24 mmol, 1.5 eq.), and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (1.58 g, 8.24 mmol, 1.5 eq.) were added to a 50 mL round bottom flask with a stir bar. 4-(4-(1-((4-azidobutanoyl)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoic acid (2.25 g, 5.48 mmol, 1 eq.), dissolved in anhydrous acetonitrile (20 mL), was added, and the flask was purged with nitrogen. The reaction was allowed to proceed under a nitrogen atmosphere at room temperature for 24 h. The acetonitrile was removed by rotary evaporation, and the residue was dissolved in dichloromethane (50 mL). The solution was washed with water (2 × 50 mL), 1:1 water:brine (50 mL), and brine (50 mL), dried over magnesium sulfate, and filtered. The resulting solution was concentrated *in vacuo* to afford the product as a viscous yellow oil (2.55 g, 5.03 mmol, 91.8% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.59 (s, 1H), 7.00 (s, 1H), 6.49 (q, J = 6.4 Hz, 1H), 4.17 (t, J = 6.1 Hz, 2H), 3.97 (s, 3H), 3.33 (m, 2H), 2.89 (t, J = 7.3 Hz, 2H), 2.85 (br. s, 4H), 2.46 (m, 2H), 2.29 (quin, J = 6.7 Hz, 2H), 1.90 (quin, J = 7.0 Hz, 2H), 1.62 (d, J = 6.3 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 171.34, 169.03, 168.06, 154.01, 146.97, 139.76, 133.18, 109.25, 108.13, 68.44, 67.35, 56.26, 50.41, 31.13, 27.50, 25.54, 24.09, 21.95.

1.5. Synthesis of tert-Butyl Substituted Photocleavable Linker (N3-tBu-oNB-NHS)

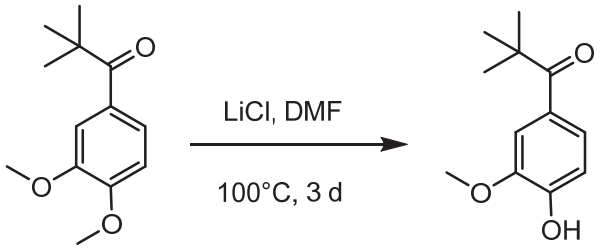
1-(4-hydroxy-3-methoxyphenyl)-2,2-dimethylpropan-1-one (**2**) was synthesized by adapting a previously published synthetic route[5] and was subsequently used to produce an azide-functionalized ortho-nitrobenzyl photocleavable linker by adapting a known synthetic route for the analogous methyl compound.[3,4]

*1.5.1. 1-(3,4-dimethoxyphenyl)-2,2-dimethylpropan-1-one (**1**)*



o-dimethoxybenzene (25.3 g, 183 mmol, 1 eq.), pivalic anhydride (41.1 g, 221 mmol, 1.2 eq.), and iodine (1.39 g, 55 mmol, 0.3 eq.) were added to a 500 mL round bottom flask with a stir bar, purged with nitrogen, and allowed to react under a nitrogen atmosphere for 3 d. The reaction mixture was diluted with chloroform (200 mL), and the reaction was quenched by stirring vigorously with 10% aqueous sodium thiosulfate. The layers were separated, and the aqueous layer was extracted with chloroform (200 mL). The organic layers were combined, washed with 200 mL brine, dried over magnesium sulfate, filtered, and concentrated by rotary evaporation. The residue was further concentrated under high vacuum (< 0.1 mbar) to removed unreacted starting material until a constant weight was reached. The product was obtained as a viscous yellow oil (35.0 g, 157 mmol, 85.8% yield) and used without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.53 (dd, J = 8.4, 2.1 Hz, 1H), 7.40 (d, J = 2.0 Hz, 1H), 6.83 (d, J = 8.5 Hz, 1H), 3.91 (s, 3H), 3.90 (s, 3H), 1.37 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 205.94, 151.70, 148.50, 129.87, 122.57, 111.96, 109.43, 55.86, 55.79, 43.83, 28.45. R_f 0.31 (6:1 hexanes:EtOAc).

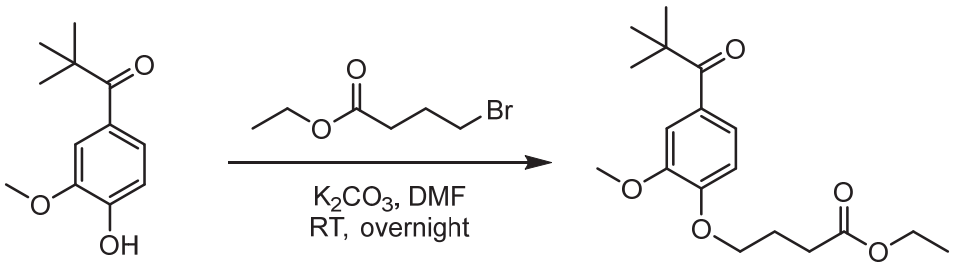
*1.5.2. 1-(4-hydroxy-3-methoxyphenyl)-2,2-dimethylpropan-1-one (**2**)*



Anhydrous-grade dimethylformamide was further dried over 4Å molecular sieves in a nitrogen-purged flask overnight. Lithium chloride was dried in a vacuum oven at 150°C overnight. 1-(3,4-dimethoxyphenyl)-2,2-dimethylpropan-1-one (35 g, 157 mmol, 1 eq.) was dissolved in the dried dimethylformamide (150 mL) in a 500 mL round bottom flask with a stir bar, and dry

lithium chloride (80 g, 1.89 mol, 12 eq.) was added. The flask was equipped with a reflux condenser, purged with nitrogen, and heated to 100°C. The reaction was allowed to proceed under a nitrogen atmosphere for 3 d. The reaction mixture was cooled and diluted with water (200 mL) and chloroform (200 mL). The organic layer was separated and washed with water (200 mL). The organic layer was subsequently extracted with 10% aqueous sodium hydroxide (100 mL). The basic aqueous phase was then washed with dichloromethane (2 × 100 mL), acidified to pH 1 with hydrochloric acid (5 N), and extracted with diethyl ether (100 mL). The ether phase was washed with water (100 mL) and brine (100 mL), dried over magnesium sulfate, filtered, and concentrated *in vacuo* to afford the product as a yellow solid (3.97 g, 19.1 mmol, 12.2% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.53 (dd, J = 8.3, 2.0 Hz, 1H), 7.43 (d, J = 2.0 Hz, 1H), 6.90 (d, J = 8.3 Hz, 1H), 6.02 (br. s, 1H), 3.93 (s, 3H), 1.39 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 205.94, 148.82, 146.29, 123.33, 113.12, 111.72, 109.61, 55.94, 43.88, 28.57. R_f 0.16 (6:1 hexanes:EtOAc).

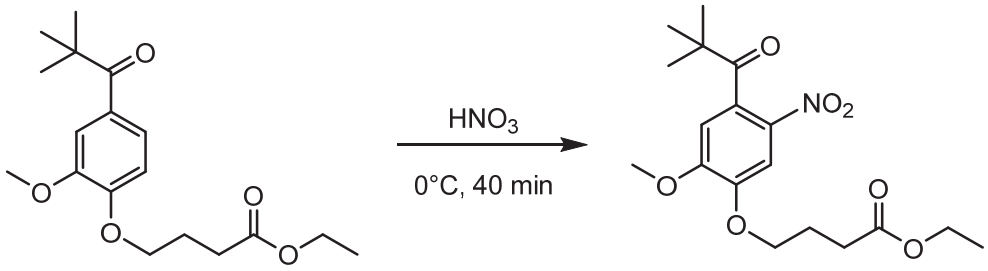
*1.5.3. Ethyl 4-(2-methoxy-4-pivaloylphenoxy)butanoate (**3b**)*



1-(4-hydroxy-3-methoxyphenyl)-2,2-dimethylpropan-1-one (3.95 g, 19.0 mmol, 1 eq.) and ethyl 4-bromobutyrate (4.40 g, 22.6 mmol, 1.2 eq.) were added to a 150 mL round bottom flask and dissolved in anhydrous dimethylformamide (20 mL). After dissolution, potassium carbonate (3.90 g, 28.5 mmol, 1.5 eq.) was added, and the flask was purged with nitrogen. The reaction was allowed to proceed at room temperature under a nitrogen atmosphere for ~24 h. The reaction mixture was slowly poured into 200 mL of stirring water at room temperature. The mixture was stirred at room temperature for 1.5 h and then transferred to 4°C to fully separate the product. The organic phase containing the product was separated from the aqueous phase and diluted with dichloromethane (50 mL). The organic phase was washed with water (2 × 50 mL) and brine (40 mL), dried over magnesium sulfate, filtered, and concentrated *in vacuo* to afford the product as an orange oil (4.68 g, 14.5 mmol, 76.3% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ

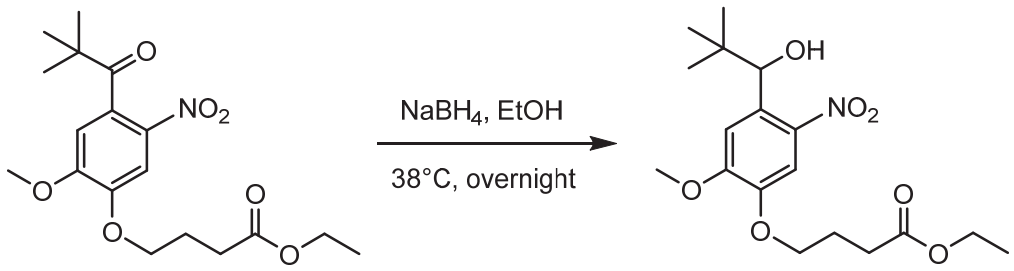
ppm 7.53 (dd, J = 8.5, 2.1 Hz, 1H), 7.42 (d, J = 2.0 Hz, 1H), 6.86 (d, J = 8.5 Hz, 1H), 4.15 (m, 4H), 3.90 (s, 3H), 2.55 (t, J = 7.2 Hz, 2H), 2.19 (quin, J = 6.8 Hz, 2H), 1.39 (s, 9H), 1.27 (t, J = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 206.04, 173.03, 151.08, 148.86, 129.98, 122.58, 112.45, 110.87, 67.66, 60.45, 55.91, 43.89, 30.54, 28.50, 24.26, 14.18.

*1.5.4. Ethyl 4-(2-methoxy-5-nitro-4-pivaloylphenoxy)butanoate (**4b**)*



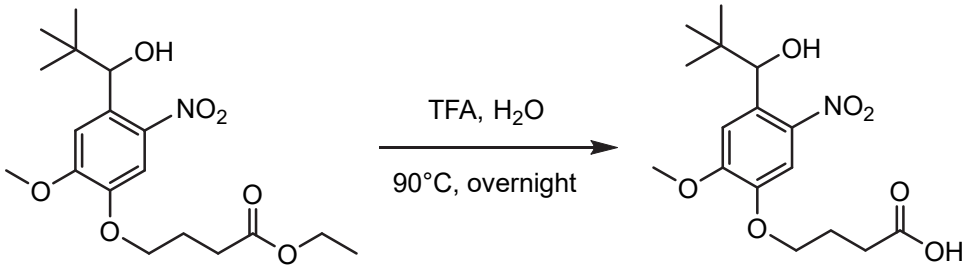
Nitric acid (15 mL) was added to a 150 mL round bottom flask with a stir bar and chilled in an ice bath. Ethyl 4-(2-methoxy-4-pivaloylphenoxy)butanoate (4.65 g, 14.4 mmol, 1 eq.) was added in small portions to the cold, stirring nitric acid, allowing each batch to dissolve before subsequent addition. The reaction mixture was allowed to stir on ice for an additional 40 minutes. The reaction mixture was then added dropwise to stirring ice-cold water (150 mL) to precipitate the product. The mixture was stirred for an additional hour at room temperature and then stored overnight at 4°C to fully precipitate the product. The precipitate was collected by vacuum filtration and washed with ice-cold water (50 mL). The yellow powder was dried on the filter and *in vacuo*, followed by recrystallization in ethanol to afford the product as a yellow solid (2.27 g, 6.18 mmol, 42.9% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.69 (s, 1H), 6.57 (s, 1H), 4.16 (m, 4H), 3.94 (s, 3H), 2.55 (t, J = 7.2 Hz, 2H), 2.20 (quin, J = 6.8 Hz, 2H), 1.27 (t, J = 7.3 Hz, 3H), 1.25 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 210.40, 172.79, 154.43, 148.11, 137.51, 132.15, 107.99, 107.75, 68.32, 60.56, 56.52, 45.32, 30.49, 27.23, 24.15, 14.19.

*1.5.5. Ethyl 4-(4-(1-hydroxy-2,2-dimethylpropyl)-2-methoxy-5-nitrophenoxy)butanoate (**5b**)*



Ethyl 4-(2-methoxy-5-nitro-4-pivaloylphenoxy)butanoate (2.25 g, 6.12 mmol, 1 eq.) and anhydrous ethanol (35 mL) were added to a 100 mL round bottom flask with a stir bar and purged with nitrogen while stirring. The mixture was warmed to ~38°C to aid in dissolution of the starting material. After equilibrating at 38°C, sodium borohydride (225 mg, 5.95 mmol, 0.97 eq.) was added to the stirring mixture in approximately equal portions every 5 minutes over the course of 30 minutes. The flask was again purged with nitrogen, and the reaction was allowed to proceed overnight at 38°C. The reaction mixture was then poured into 350 mL of stirring room temperature water. The mixture was stirred for 1 h at room temperature and then stored overnight at 4°C to fully precipitate the product. The precipitate was collected by vacuum filtration, dissolved in dichloromethane (100 mL), washed with water (100 mL) and brine (60 mL), dried over magnesium sulfate, filtered, and concentrated *in vacuo* to afford the product as a viscous yellow oil (2.22 g, 6.01 mmol, 98.2 % yield). ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 7.44 (s, 1H), 7.19 (s, 1H), 5.61 (d, J = 4.6 Hz, 1H), 5.20 (d, J = 4.9 Hz, 1H), 4.05 (m, 4H), 3.85 (s, 3H), 2.45 (t, J = 7.3 Hz, 2H), 1.98 (quin, J = 6.8 Hz, 2H), 1.18 (t, J = 7.1 Hz, 3H), 0.78 (s, 9H). ^{13}C NMR (126 MHz, DMSO-d6) δ ppm 172.44, 151.71, 146.20, 141.38, 132.14, 111.35, 108.10, 72.17, 67.70, 59.92, 55.92, 36.50, 30.06, 25.65, 24.06, 14.09.

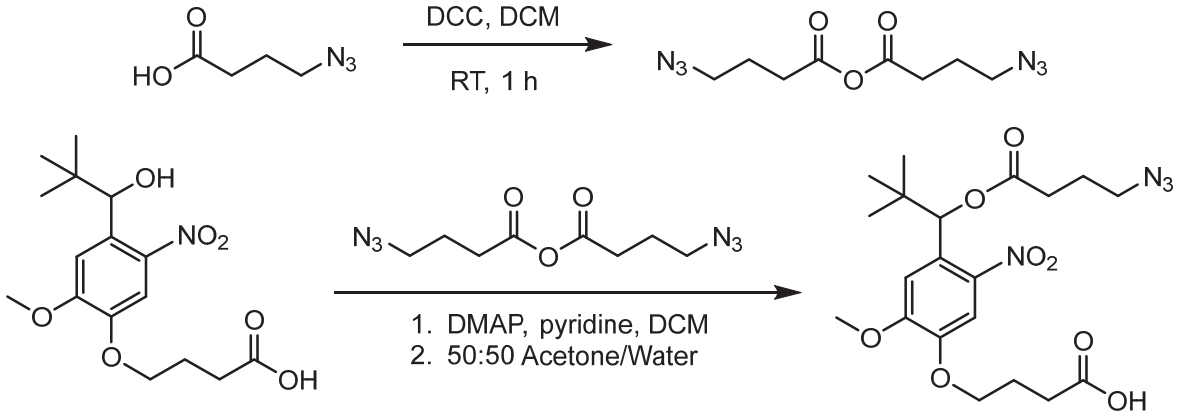
*1.5.6. 4-(4-(1-hydroxy-2,2-dimethylpropyl)-2-methoxy-5-nitrophenoxy)butanoic acid (**6b**)*



Ethyl 4-(4-(1-hydroxy-2,2-dimethylpropyl)-2-methoxy-5-nitrophenoxy)butanoate (2.20 g, 5.96 mmol, 1 eq.) was dissolved in trifluoroacetic acid (6 mL) and added to an Erlenmeyer flask

containing water (60 mL)and a stir bar. The stirring mixture was heated to 90°C. After 6 h, trifluoroacetic acid (3 mL) was added, and the mixture was stirred for an additional 18 h at 90°C. Additional trifluoroacetic acid (3 mL) was added, and the mixture was stirred for 6 h at 90°C. The mixture was cooled to room temperature and then stored overnight at 4°C to precipitate the product. The oily precipitate was separated from the aqueous layer and dissolved in dichloromethane (50 mL). The aqueous layer was extracted with dichloromethane (25 mL), and the organic phases were combined and extracted into 1 M aqueous sodium hydroxide (50 mL). The basic aqueous phase was acidified to pH 1 with hydrochloric acid (5 N), and the product was extracted into dichloromethane (50 mL, then 25 mL). The combined organic phases were washed with water (50 mL) and brine (50 mL), dried over magnesium sulfate, filtered, and concentrated *in vacuo* to afford the product as a viscous dark orange oil (1.00 g, 2.93 mmol, 49.2% yield). ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 12.19 (br. s, 1H), 7.44 (s, 1H), 7.19 (s, 1H), 5.61 (d, J = 4.6 Hz, 1H), 5.20 (d, J = 4.6 Hz, 1H), 4.04 (m, 2H), 3.85 (s, 3H), 2.39 (t, J = 7.3 Hz, 2H), 1.94 (quin, J = 6.9 Hz, 2H), 0.78 (s, 9H). ^{13}C NMR (126 MHz, DMSO-d6) δ ppm 174.74, 152.40, 146.93, 142.08, 132.78, 112.05, 108.81, 72.86, 68.46, 56.63, 37.19, 30.64, 26.35, 24.74.

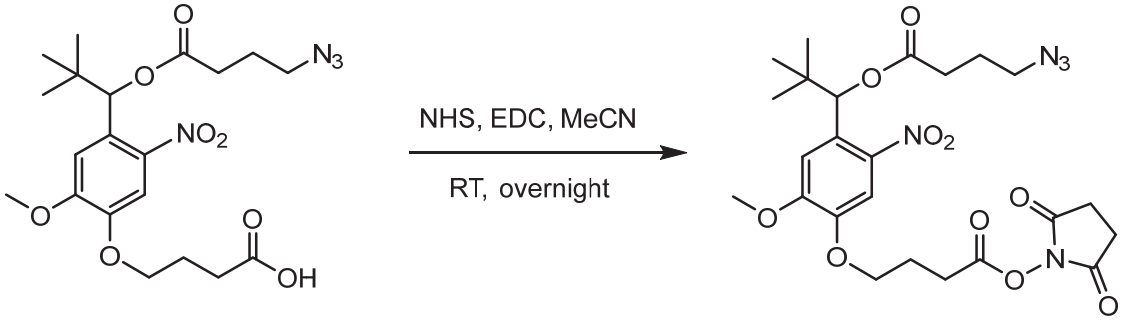
*1.5.7. 4-(4-(1-((4-azidobutanoyl)oxy)-2,2-dimethylpropyl)-2-methoxy-5-nitrophenoxy)butanoic acid (7**b**)*



4-azidobutanoic acid (5.68 g, 44.0 mmol, 15.0 eq.) was added to a 100 mL round bottom flask with a stir bar and dissolved in anhydrous dichloromethane (35 mL). *N*,*N*'-dicyclohexylcarbodiimide (2.90 g, 14.1 mmol, 4.8 eq.) was added, and the flask was purged with nitrogen. The reaction mixture was stirred at room temperature under a nitrogen atmosphere for

1 h. The reaction mixture was filtered to remove the urea byproduct and concentrated by rotary evaporation. The concentrate was filtered to remove additional urea byproduct and was then re-dissolved in anhydrous dichloromethane (8 mL). The solution was concentrated by rotary evaporation, and no additional urea byproduct was formed. The crude anhydride was used immediately, without further purification. 4-(4-(1-hydroxy-2,2-dimethylpropyl)-2-methoxy-5-nitrophenoxy)butanoic acid (1.00 g, 2.93 mmol, 1 eq.) was dissolved in anhydrous dichloromethane (25 mL) and added to a 100 mL round bottom flask with 4-dimethylaminopyridine (18 mg, 0.15 mmol, 0.05 eq.) and a stir bar. The crude azido anhydride and anhydrous pyridine (236 µL, 232 mg, 2.93 mmol, 1 eq.) were added, and the flask was purged with nitrogen. The reaction mixture was stirred at room temperature overnight under a nitrogen atmosphere. The mixture was then washed with saturated aqueous sodium bicarbonate (25 mL), hydrochloric acid (1 M, 25 mL), and brine (25 mL). The organic layer was concentrated by rotary evaporation, and the residue was re-dissolved in a 1:1 mixture of acetone and water (150 mL) and stirred overnight at room temperature. The acetone was removed by rotary evaporation, and the mixture was extracted with dichloromethane (3 × 25 mL). The combined organic phases were washed with hydrochloric acid (1 M, 25 mL) and brine (25 mL), dried over magnesium sulfate, filtered, and concentrated by rotary evaporation. The residue was purified by silica flash chromatography, using a gradient from 20-40% ethyl acetate in hexanes plus 1% acetic acid. Fractions containing the desired product were confirmed by TLC, combined, and concentrated by rotary evaporation. The residue was re-dissolved in dichloromethane (50 mL) and washed with water (3 × 50 mL) and brine (50 mL) to remove residual acetic acid. The solution was dried over magnesium sulfate, filtered, and concentrated *in vacuo*, to afford the product as a dark yellow oil (470 mg, 1.04 mmol, 35.5% yield). ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 12.20 (br. s, 1H), 7.58 (s, 1H), 6.91 (s, 1H), 6.37 (s, 1H), 4.07 (t, J = 6.5 Hz, 2H), 3.89 (s, 3H), 3.34 (t, J = 6.7 Hz, 2H), 2.50 (m, 2H), 2.38 (t, J = 7.3 Hz, 2H), 1.95 (quin, J = 7.0 Hz, 2H), 1.79 (quin, J = 7.1 Hz, 2H), 0.91 (s, 9H). ^{13}C NMR (126 MHz, DMSO-d6) δ ppm 174.01, 171.55, 152.29, 146.95, 141.32, 127.18, 110.22, 108.74, 75.03, 67.84, 56.08, 49.91, 35.82, 30.73, 29.94, 25.69, 23.77, 20.78. R_f 0.50 (1:1 hexanes:EtOAc + 1% acetic acid).

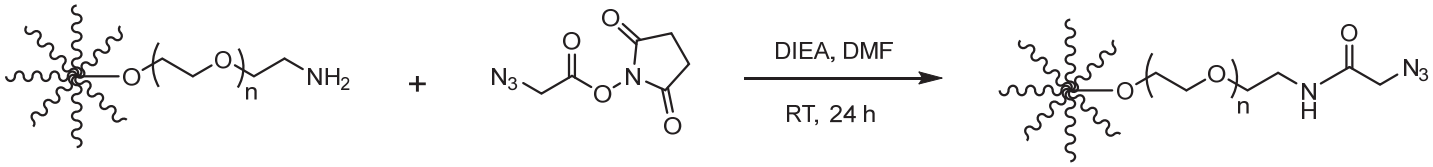
*1.5.8. 2,5-dioxopyrrolidin-1-yl 4-(4-(1-((4-azidobutanoyl)oxy)-2,2-dimethylpropyl)-2-methoxy-5-nitrophenoxy)butanoate (**8b**)*



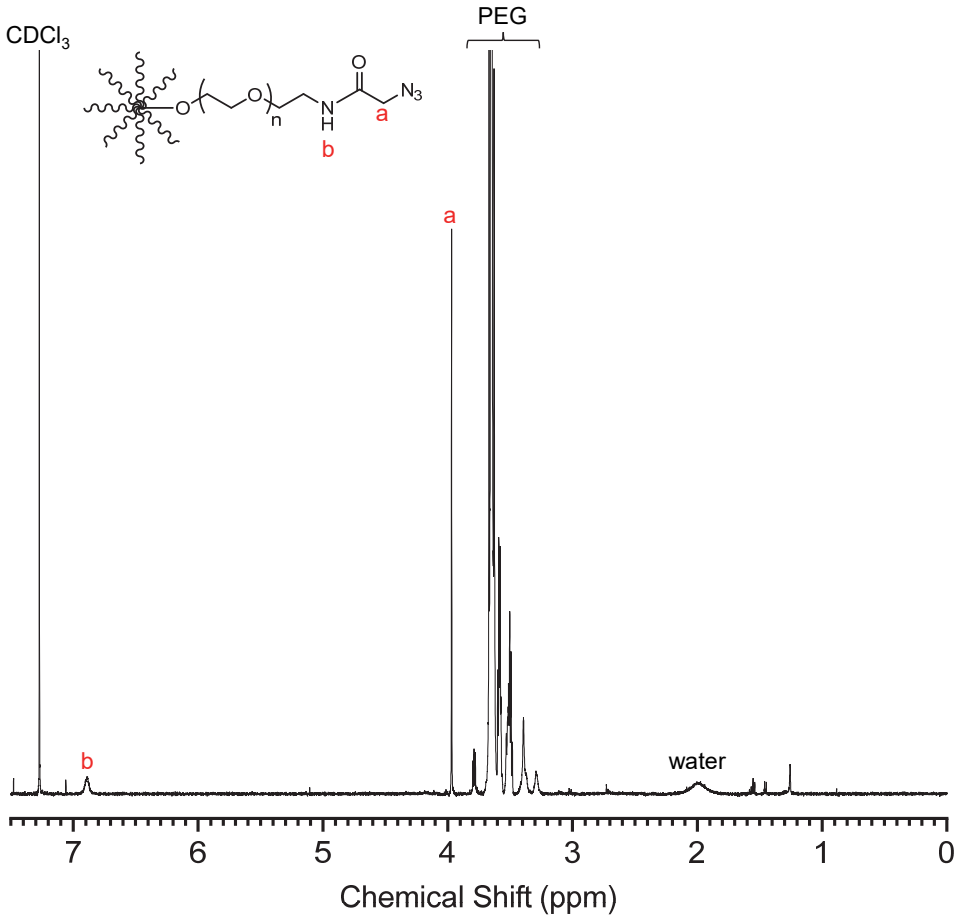
N-hydroxysuccinimide (240 mg, 2.09 mmol, 2 eq.), and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (400 mg, 2.09 mmol, 2 eq.) were added to a 25 mL round bottom flask with a stir bar. 4-(4-(1-((4-azidobutanoyl)oxy)-2,2-dimethylpropyl)-2-methoxy-5-nitrophenoxy)butanoic acid (470 mg, 1.04 mmol, 1 eq.), dissolved in anhydrous acetonitrile (5 mL), was added, and the flask was purged with nitrogen. The reaction was allowed to proceed under a nitrogen atmosphere at room temperature for 24 h. The acetonitrile was removed by rotary evaporation, and the residue was dissolved in dichloromethane (10 mL). The solution was washed with water (3 × 10 mL) and brine (10 mL), dried over magnesium sulfate, and filtered. The resulting solution was concentrated *in vacuo* to afford the product as a brown oil (414 mg, 0.75 mmol, 72.1% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.56 (s, 1H), 6.90 (s, 1H), 6.67 (s, 1H), 4.16 (t, J = 6.1 Hz, 2H), 3.93 (s, 3H), 3.33 (m, 2H), 2.89 (t, J = 7.3 Hz, 2H), 2.85 (s, 4H), 2.46 (m, 2H), 2.29 (quin, J = 7.1 Hz, 2H), 1.90 (quin, J = 6.9 Hz, 2H), 0.97 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 171.44, 169.05, 168.07, 152.64, 146.97, 141.61, 128.59, 110.26, 109.40, 76.00, 67.24, 56.15, 50.38, 36.21, 31.02, 27.50, 25.90, 25.54, 24.11, 24.09.

1.6. Synthesis of Functionalized PEGs

1.6.1. 8-arm PEG-N_3



8-arm PEG-amine (500 mg, MW ~ 10 kDa, 0.4 mmol -NH_2, 1 eq.) and azidoacetic acid NHS ester (99.1 mg, 0.5 mmol, 1.25 eq.) were added to a 25 mL round bottom flask with a stir bar and dissolved in anhydrous dimethylformamide (5 mL). *N*,*N*-diisopropylethylamine (348 µL, 2.0 mmol, 5 eq.) was added, and the reaction was allowed to proceed overnight at room temperature. The mixture was diluted to 30 mL with water and dialyzed against water (MWCO 2 kDa, 4 × 4.5 L, 4°C). The solution was flash frozen in liquid nitrogen and lyophilized to afford the product as a slightly yellow solid (490 mg, 0.046 mmol, 92% yield). ^{1}H NMR characterization is included below.

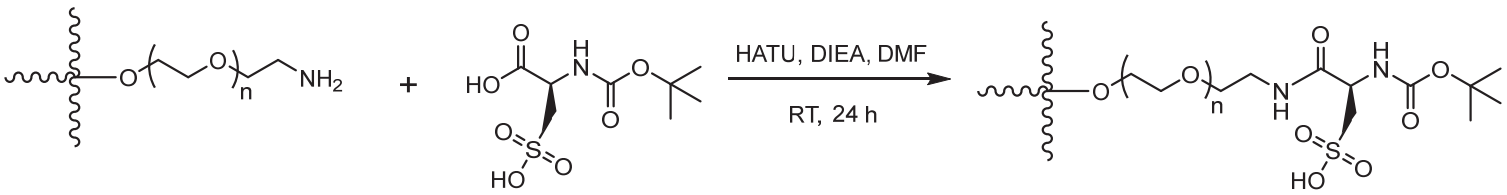


^{1}H NMR (500 MHz, $CDCl_3$) characterization for 8-arm PEG-N_3.

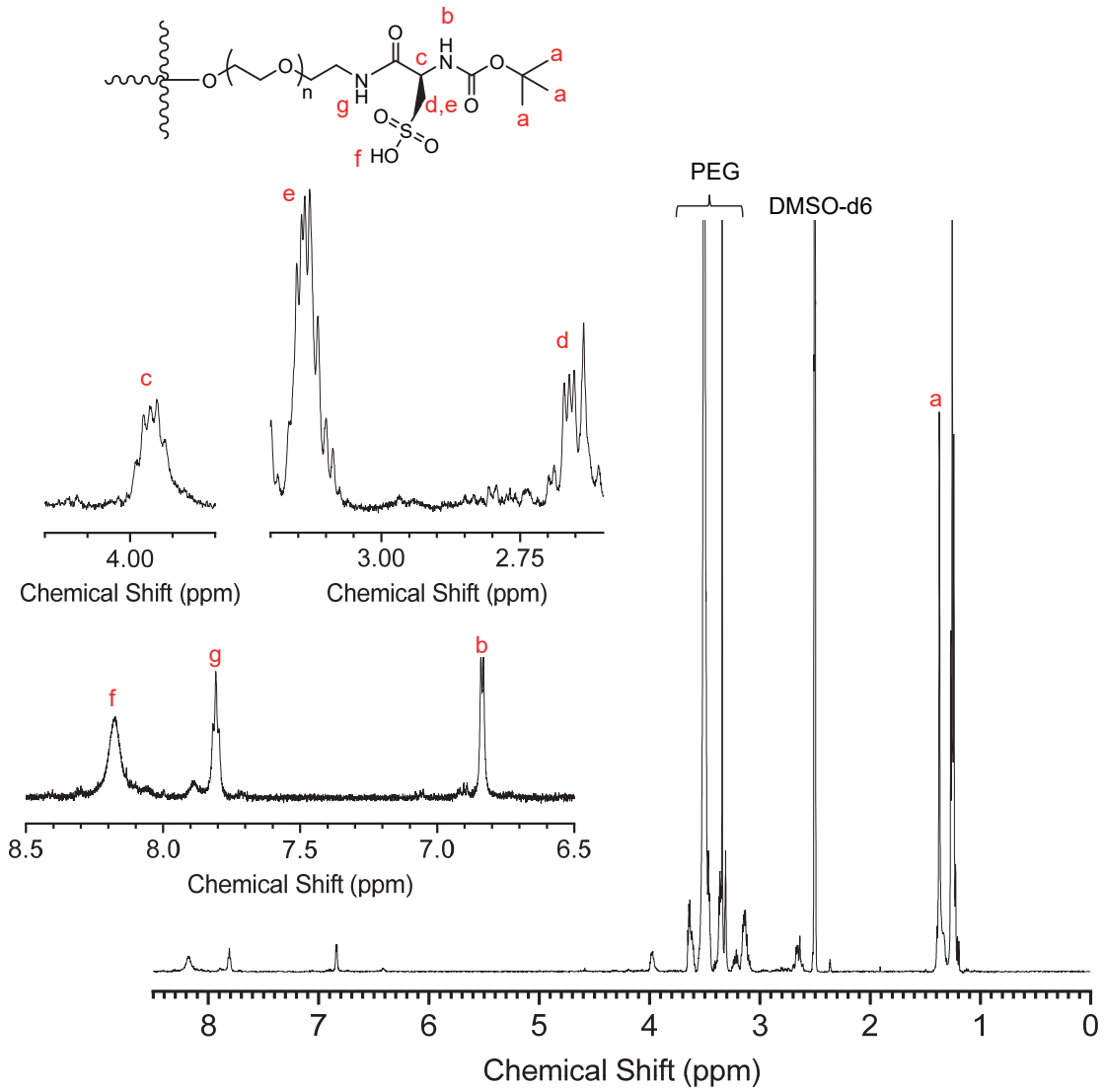
1.6.2. 4-arm PEG-Sulfo-BCN

4-arm PEGs were first sulfated to increase the aqueous solubility of BCN conjugates and to prevent aggregation.

4-arm PEG-Sulfo-Boc

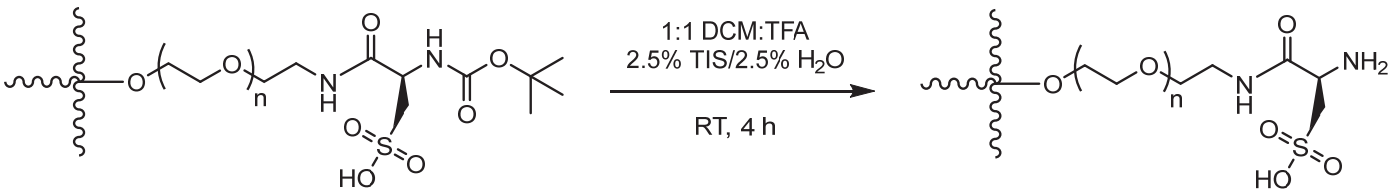


4-arm PEG-amine (400 mg, MW ~ 10 kDa, 0.16 mmol -NH_2, 1 eq.) was added to a 25 mL round bottom flask with a stir bar and dissolved in anhydrous dimethylformamide (4 mL). In a separate vial containing a stir bar, Boc-L-cysteic acid (172 mg, 0.64 mmol, 4 eq.) was dissolved in anhydrous dimethylformamide (4 mL). After complete dissolution of the Boc-L-cysteic acid, HATU (244 mg, 0.64 mmol, 4 eq.) was added and allowed to dissolve. *N*,*N*-diisopropylethylamine (280 µL, 1.6 mmol, 10 eq.) was added, and the reaction was allowed to proceed for 10 minutes at room temperature. The activated Boc-L-cysteic acid solution was added dropwise to the stirring PEG solution, and the reaction was allowed to proceed overnight at room temperature. The reaction mixture was diluted to 35 mL with water and dialyzed against water (MWCO 2 kDa, 4 × 4.5 L, 4°C). The solution was flash frozen in liquid nitrogen and lyophilized to afford the product as a slightly yellow solid (462 mg, 0.042 mmol, quantitative yield). ^{1}H NMR characterization is included below.

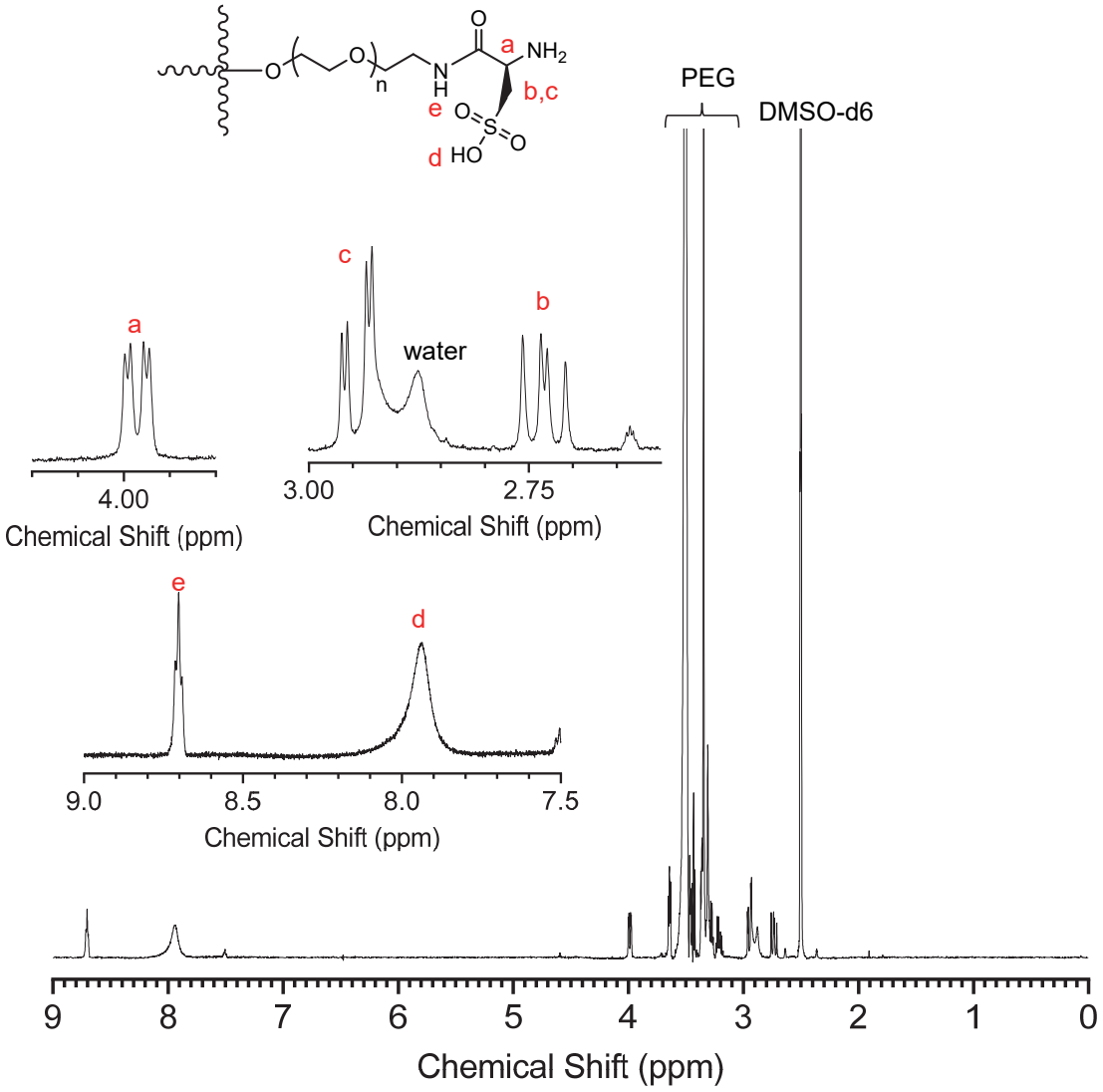


^{1}H NMR (500 MHz, DMSO-d6) characterization for 4-arm PEG-Sulfo-Boc.

4-arm PEG-Sulfo-Amine

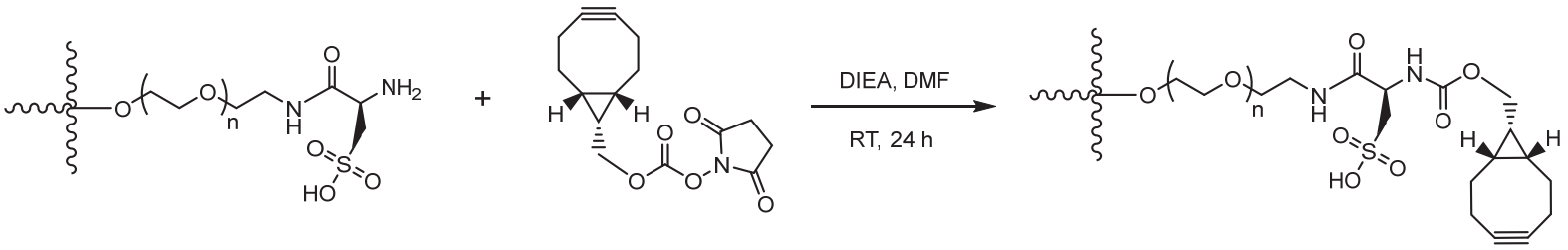


4-arm PEG-Sulfo-Boc (455 mg, MW ~ 11 kDa, 0.041 mmol) was dissolved in dichloromethane (5 mL) and added to a 25 mL round bottom flask with a stir bar. While stirring, triisopropylsilane (250 µL), water (250 µL), and trifluoroacetic acid (5 mL) were sequentially added. The reaction was allowed to proceed for 4 h at room temperature. The reaction mixture was concentrated by rotary evaporation, and the residue was added dropwise to ice cold diethyl ether (45 mL) to precipitate the PEG. The PEG was collected by centrifugation, washed with cold ether (2 × 25 mL), and dried under a stream of compressed air. The solid was re-dissolved in water (10 mL) and dialyzed against water (MWCO 2 kDa, 4 × 4.5 L, 4°C). The solution was flash frozen in liquid nitrogen and lyophilized to afford the product as a white solid (344 mg, 0.032 mmol, 78% yield). ^{1}H NMR characterization is included below.

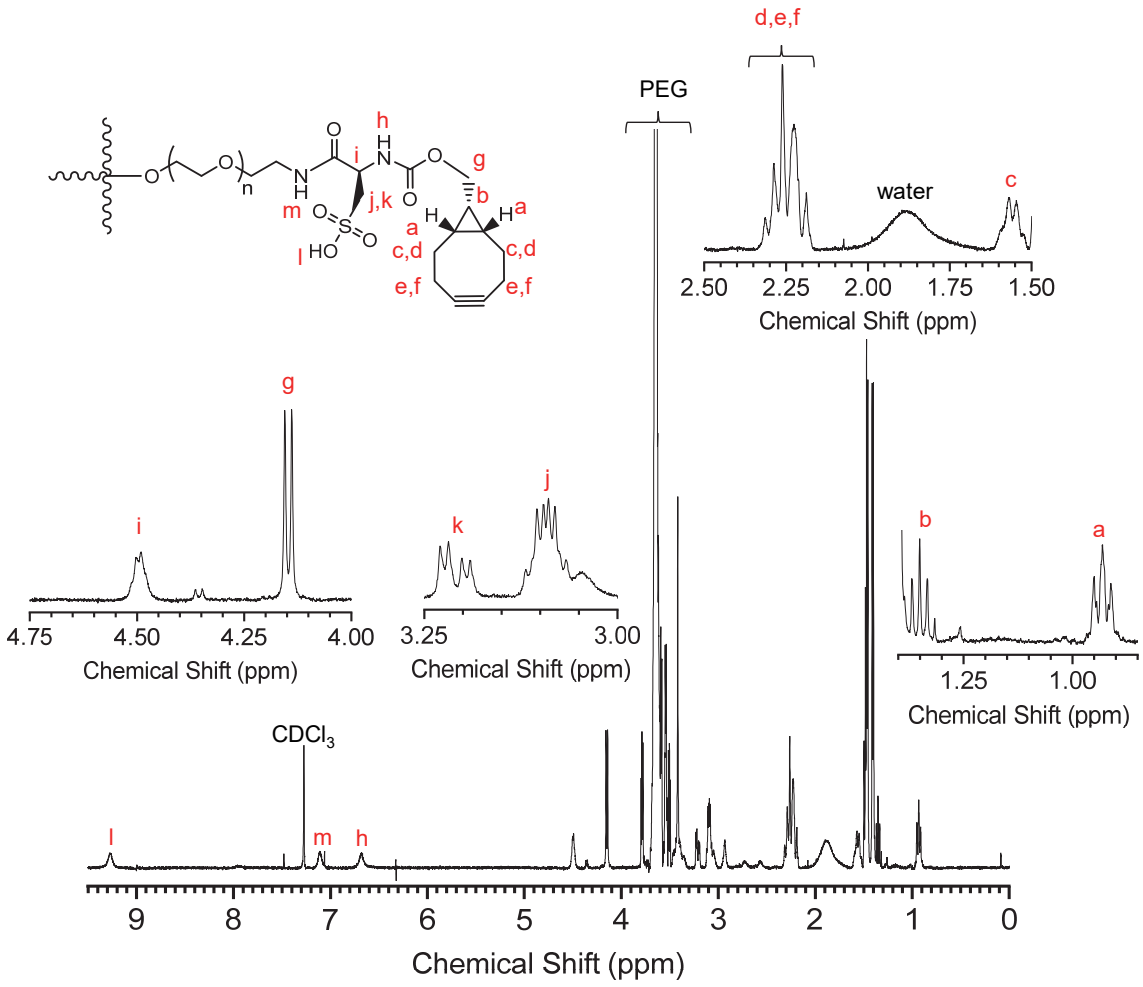


^{1}H NMR (500 MHz, DMSO-d6) characterization for 4-arm PEG-Sulfo-amine.

4-arm PEG-Sulfo-BCN

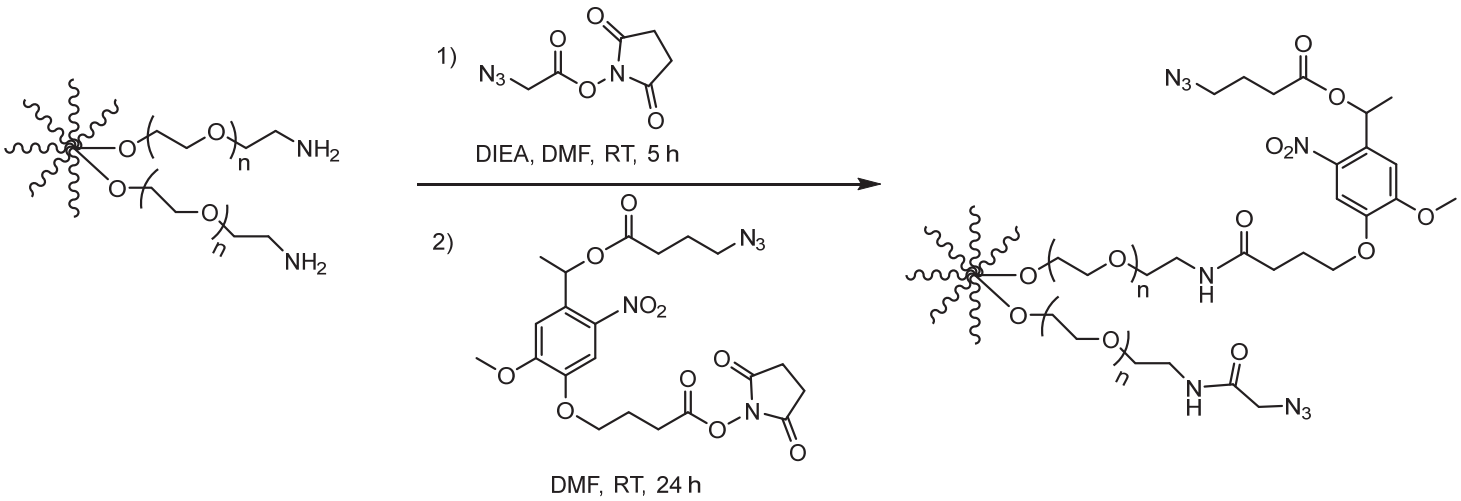


4-arm PEG-Sulfo-amine (333 mg, MW ~ 10 kDa, 0.133 mmol -NH_2, 1 eq.) was dissolved in anhydrous DMF (5 mL) and added to a 25 mL round bottom flask containing BCN-NHS (58.2 mg, 0.200 mmol, 1.5 eq.) and a stir bar. After complete dissolution of the BCN-NHS, *N*,*N*-diisopropylethylamine (139 µL, 0.800 mmol, 6 eq.) was added, and the reaction was allowed to proceed overnight at room temperature. The reaction mixture was diluted to 30 mL with water and dialyzed against water (MWCO 2 kDa, 4 × 4.5 L, 4°C). The solution was flash frozen in liquid nitrogen and lyophilized to afford the product as a white solid (305 mg, 0.027 mmol, 87% yield). ^{1}H NMR characterization is included below.

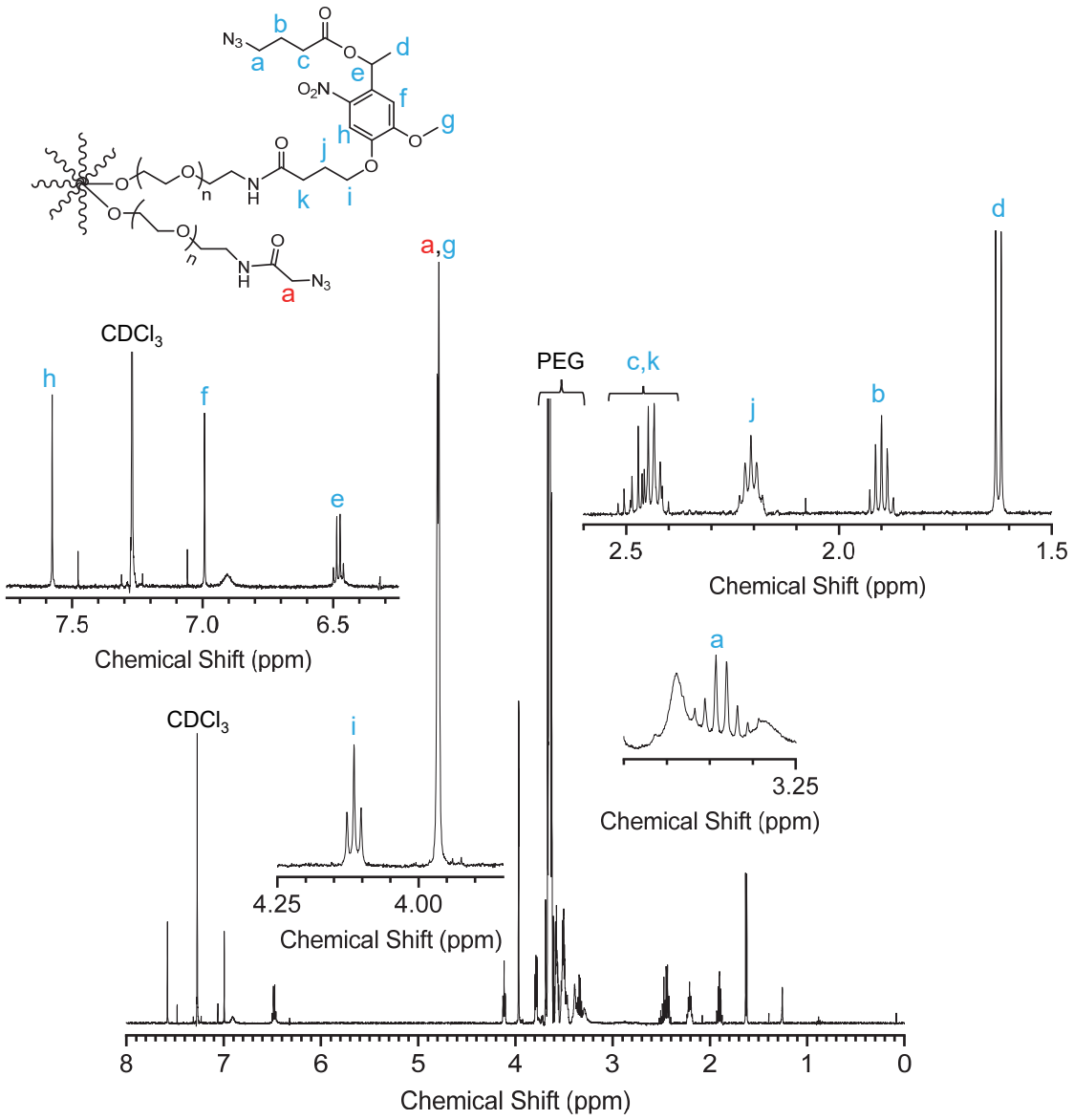


^{1}H NMR (500 MHz, $CDCl_3$) characterization for 4-arm PEG-Sulfo-BCN.

1.6.3. 8-arm PEG-[4-N₃/4-Me-oNB-N₃]

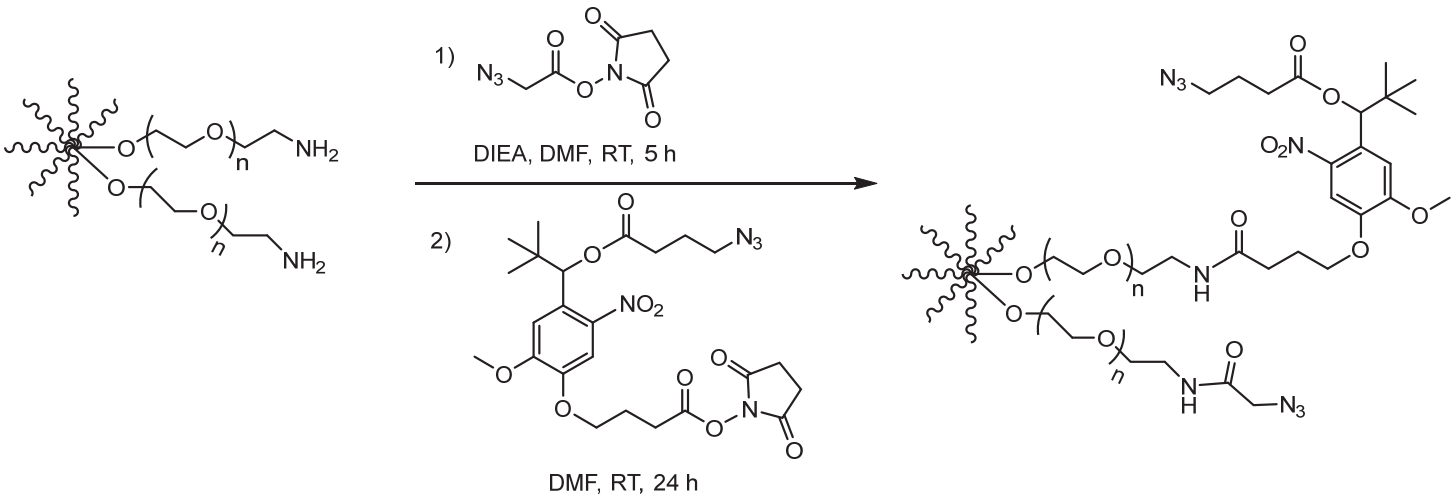


8-arm PEG-amine (200 mg, MW ~ 20 kDa, 0.080 mmol -NH_2, 1 eq.) and azidoacetic acid NHS ester (7.9 mg, 0.040 mmol, 0.50 eq.) were added to a 25 mL round bottom flask containing a stir bar and dissolved in anhydrous DMF (3 mL). After complete dissolution of the reagents, *N*,*N*-diisopropylethylamine (55.7 μL, 0.32 mmol, 4.0 eq.) was added, and the mixture was stirred for 5 h at room temperature. N_3-Me-*o*NB-NHS (30.4 mg, 0.060 mmol, 0.75 eq.) was dissolved in anhydrous DMF (150 μL) and added dropwise to the stirring reaction mixture. The reaction was allowed to proceed overnight at room temperature. The PEG was precipitated by dropwise addition to ice cold diethyl ether (45 mL) and collected by centrifugation. The supernatant was decanted, and the pellet was washed with cold ether (25 mL), dried under a stream of compressed air, and re-dissolved in water (10 mL). The solution was dialyzed against water (MWCO 2 kDa, 2 × 4.5 L, 4°C), flash frozen in liquid nitrogen, and lyophilized to afford the product as an off-white solid (193 mg, 0.0088 mmol, 88% yield). The ratio of conjugated N_3:Me-*o*NB-N_3 was estimated to be 1:0.72 based on integration of the corresponding ^{1}H NMR signals. ^{1}H NMR characterization is included below.

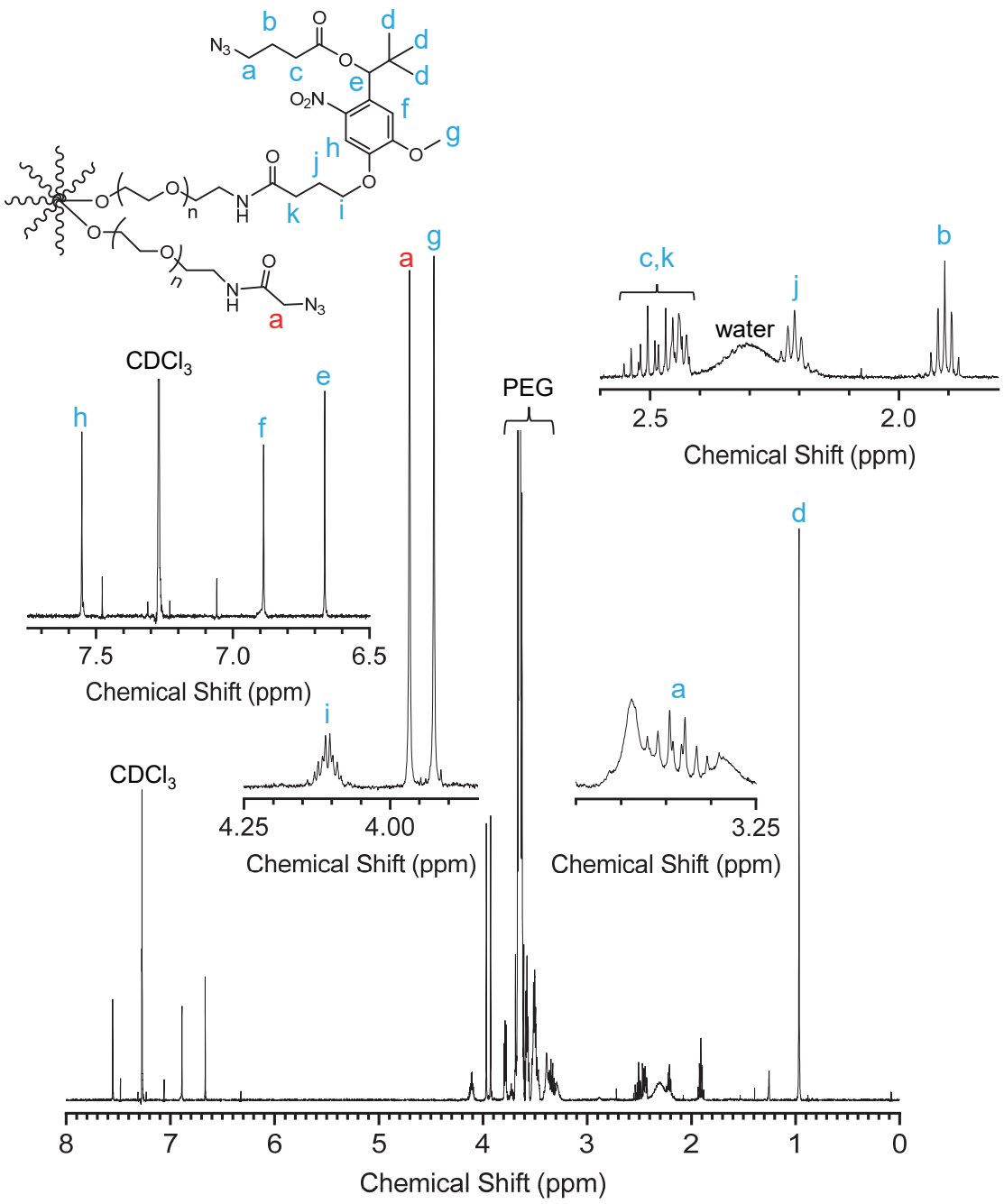


^{1}H NMR (500 MHz, $CDCl_3$) characterization for 8-arm PEG-[4-N_3/4-Me-*o*NB-N_3].

1.6.4. 8-arm PEG-[4-N₃/4-tBu-oNB-N₃]

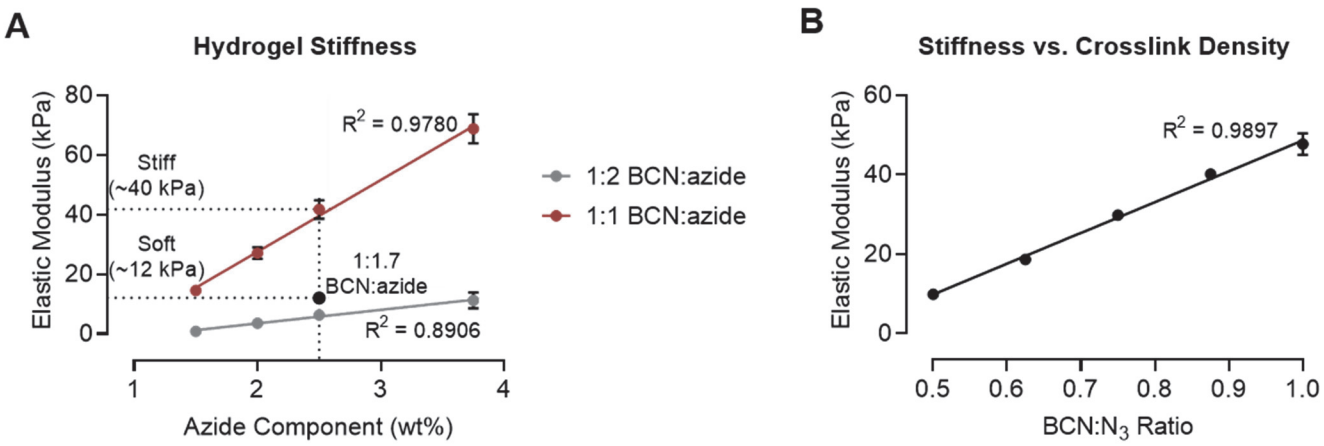


8-arm PEG-amine (200 mg, MW ~ 20 kDa, 0.080 mmol -NH_2, 1 eq.) and azidoacetic acid NHS ester (7.9 mg, 0.040 mmol, 0.50 eq.) were added to a 25 mL round bottom flask containing a stir bar and dissolved in anhydrous DMF (3 mL). After complete dissolution of the reagents, *N*,*N*-diisopropylethylamine (55.7 µL, 0.32 mmol, 4.0 eq.) was added, and the mixture was stirred for 5 h at room temperature. N_3-tBu-*o*NB-NHS (33.0 mg, 0.060 mmol, 0.75 eq.) was dissolved in anhydrous DMF (150 µL) and added dropwise to the stirring reaction mixture. The reaction was allowed to proceed overnight at room temperature. The PEG was precipitated by dropwise addition to ice cold diethyl ether (45 mL) and collected by centrifugation. The supernatant was decanted, and the pellet was washed with cold ether (25 mL), dried under a stream of compressed air, and re-dissolved in water (10 mL). The solution was dialyzed against water (MWCO 2 kDa, 2 × 4.5 L, 4°C), flash frozen in liquid nitrogen, and lyophilized to afford the product as a light brown solid (198 mg, 0.0090 mmol, 90% yield). The ratio of conjugated N_3:tBu-*o*NB-N_3 was estimated to be 1:0.73 based on integration of the corresponding ^{1}H NMR signals. ^{1}H NMR characterization is included below.

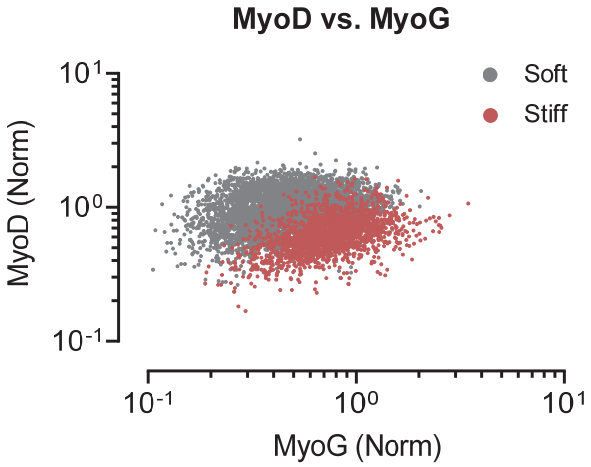


^{1}H NMR (500 MHz, $CDCl_3$) characterization for 8-arm PEG-[4-N_3/4-tBu-*o*NB-N_3].

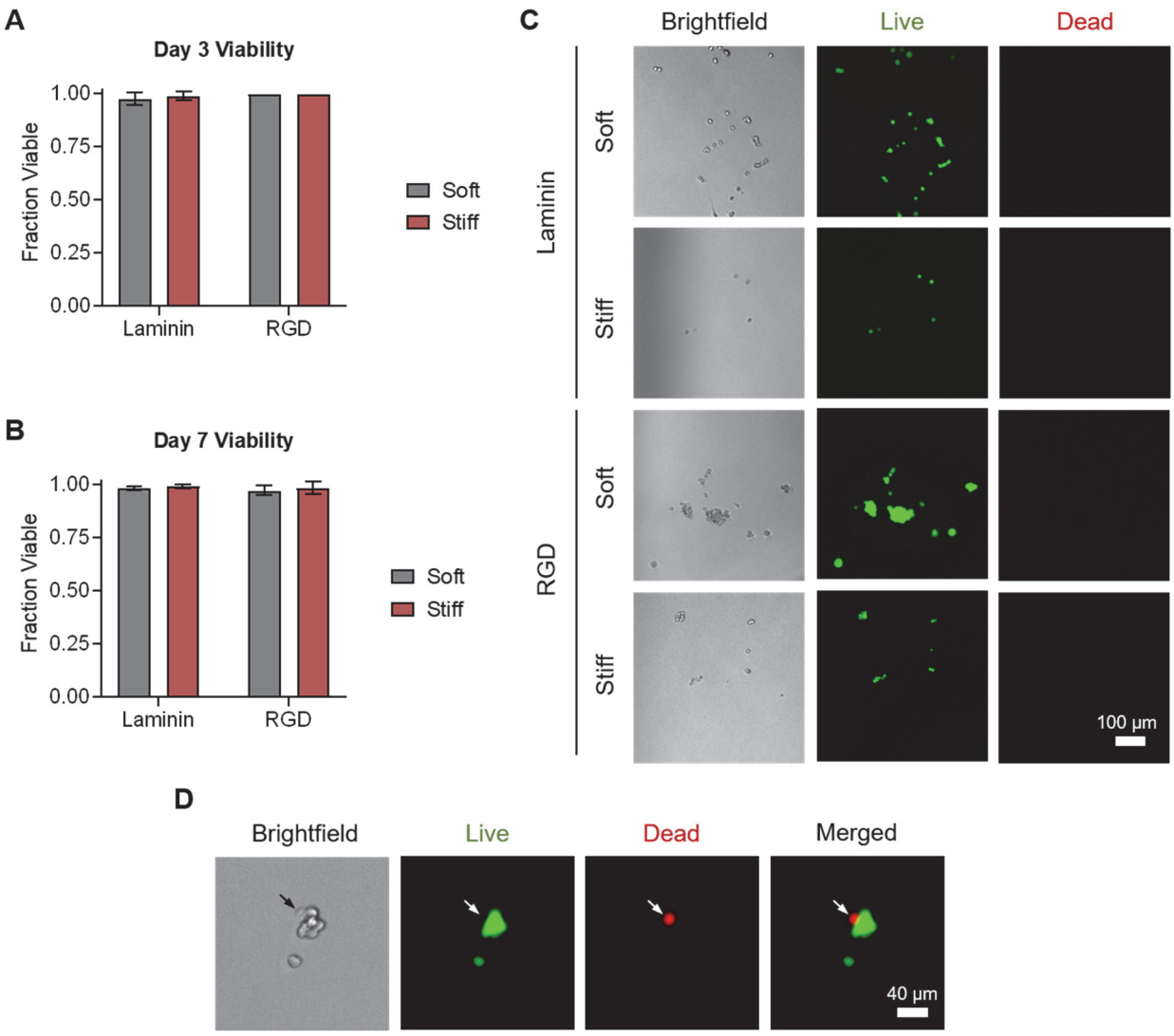
2. Supporting Figures



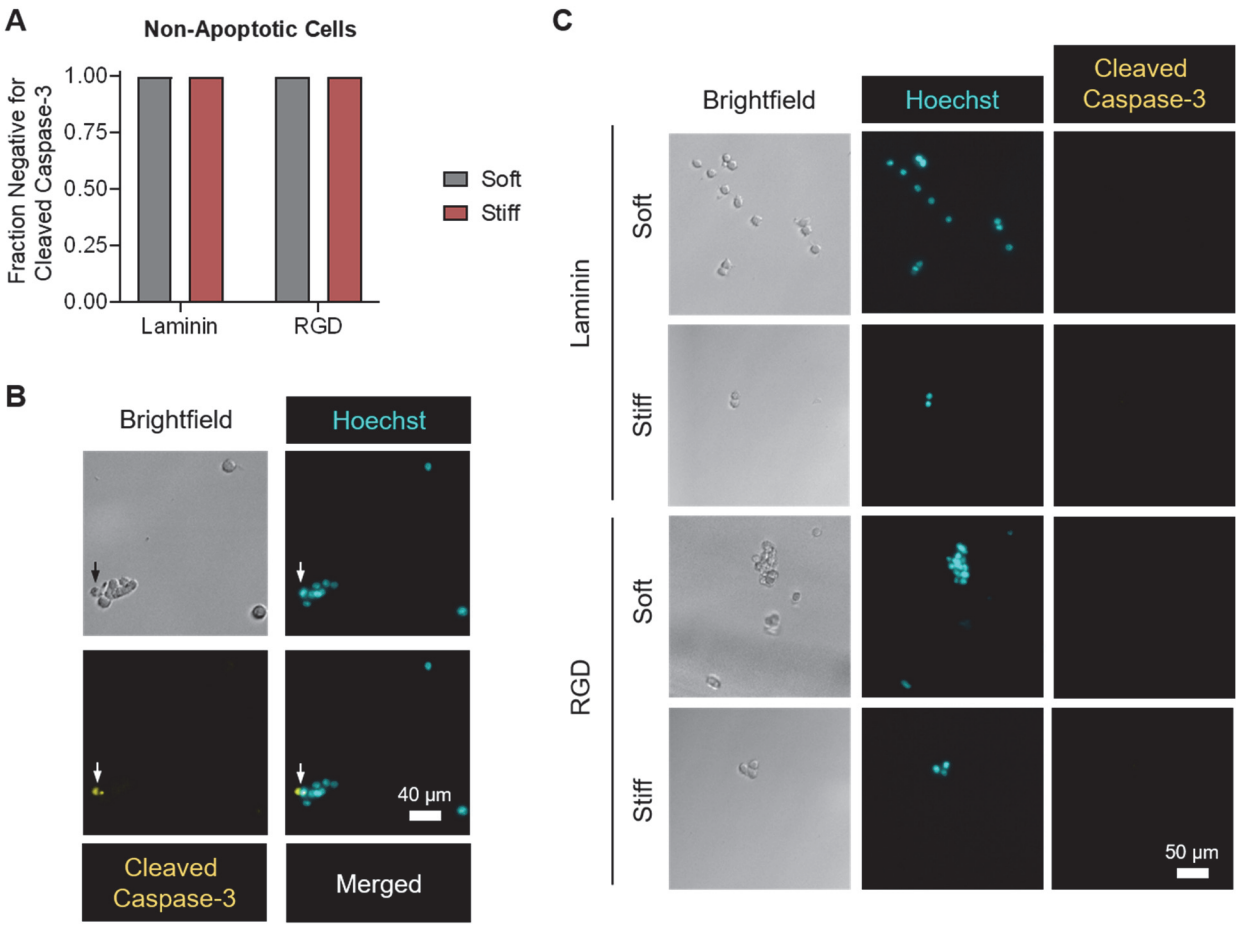
Supporting Figure S1. Characterization of hydrogel stiffness by oscillatory rheology. **(A)** The polymer content of the hydrogels was varied to identify a composition spanning a physiologically relevant range for skeletal muscle tissue. Within this composition (2.5% (w/v) 8-arm PEG-azide (MW~10 kDa)), the stoichiometric ratio of BCN:azide was varied to obtain relatively soft (*E*~12 kPa) and stiff (*E*~40 kPa) hydrogels for MuSC culture experiments. **(B)** Varying the crosslink density of the hydrogels by tuning the BCN:azide ratio enabled precise control over the stiffness of the hydrogels at a fixed composition of azide-bearing macromers (2.5% (w/v) 8-arm PEG-azide (MW~10 kDa)). Data are presented as mean ± s.d. n = 3-4.



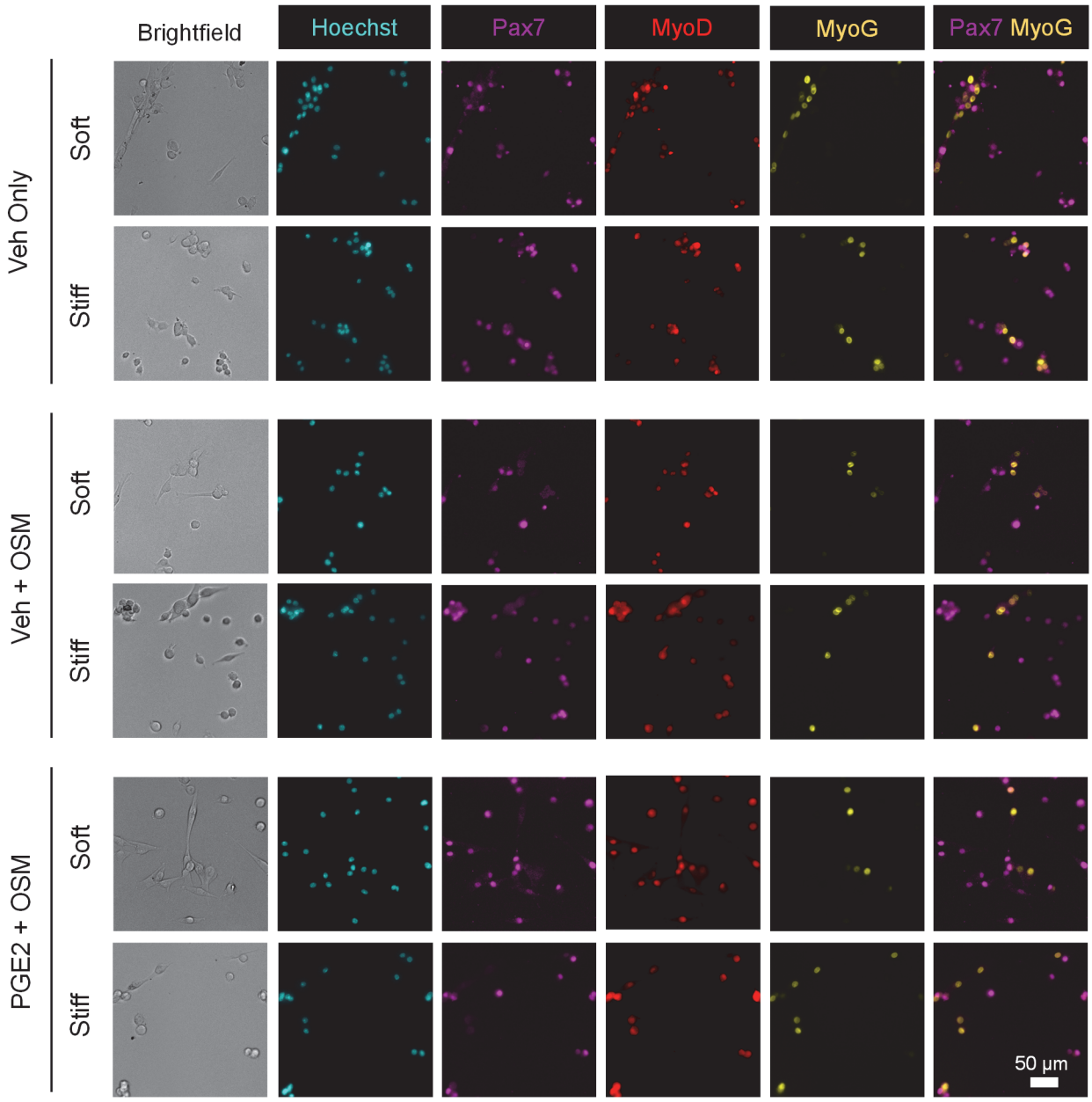
Supporting Figure S2. Single cell analysis reveals a population of activated MyoD+/MyoG- progenitors on soft, laminin-presenting hydrogels. Quantification of MyoD immunostaining and MyoG reporter intensity, normalized by Hoechst intensity. A combined 4491 cells on soft gels and 1949 cells on stiff gels from 4 independent replicates per condition were analyzed.



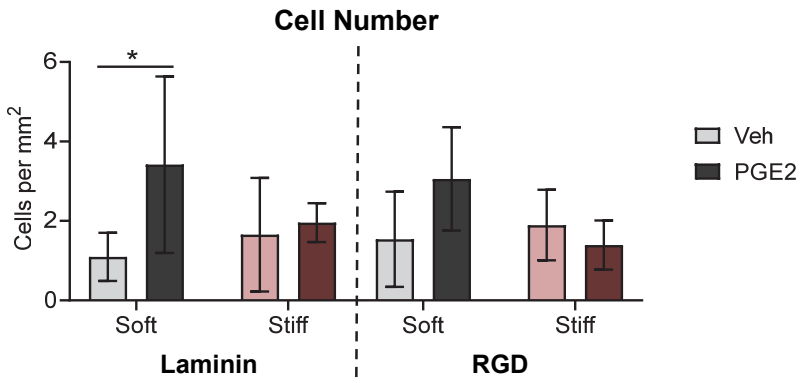
Supporting Figure S3. Viability of MuSCs on substrates with controlled stiffness and adhesive ligand presentation. MuSCs remain highly viable at **(A)** day 3 and **(B)** day 7 in culture, with no significant difference among all substrates tested. **(C)** Representative microscopy images of live/dead stained MuSCs at day 7. **(D)** Example field of view containing a MuSC stained with the dead cell marker ethidium homodimer. In A and B, data are presented as mean ± s.d., n = 3-4.



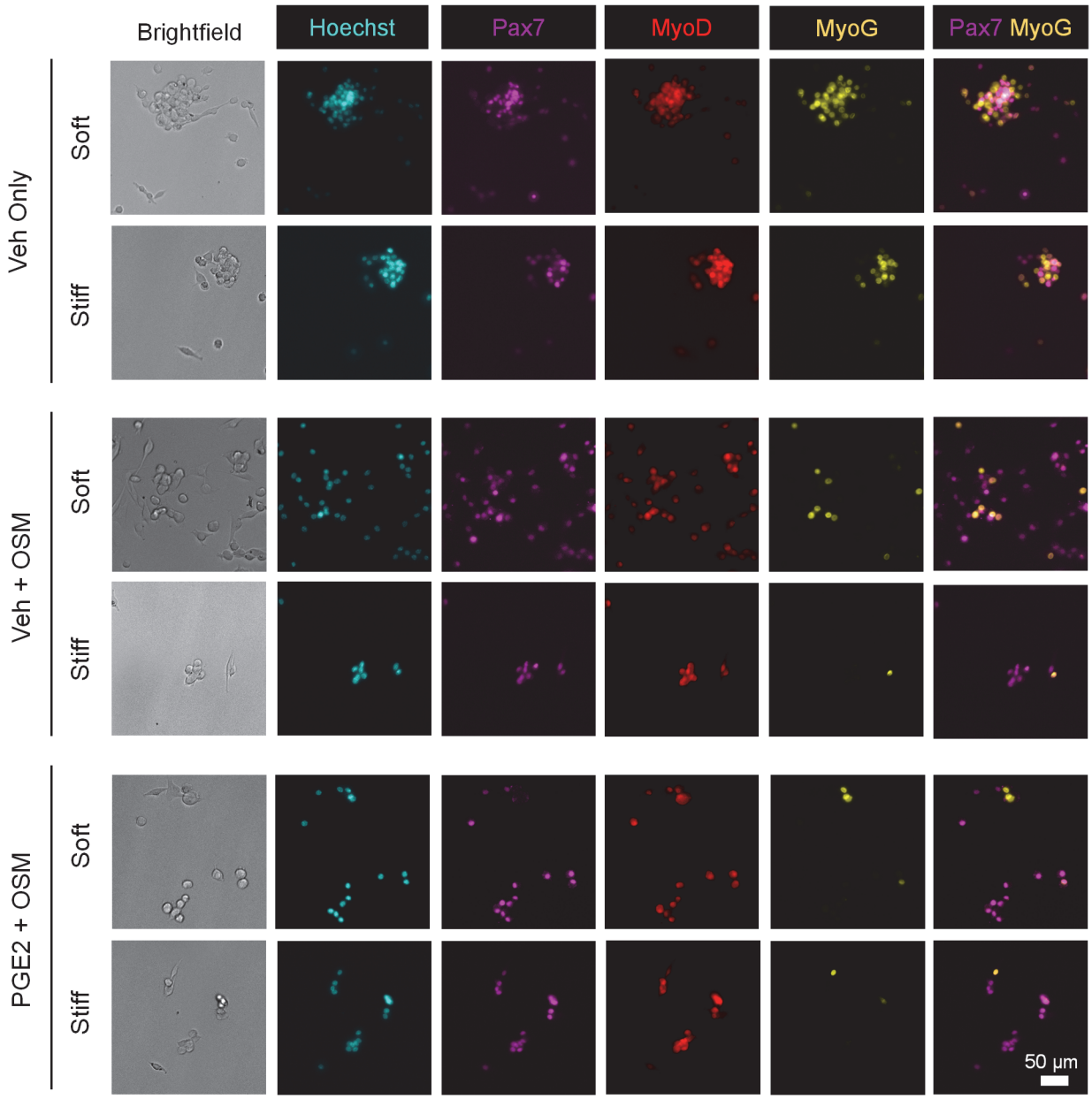
Supporting Figure S4. Identification of apoptotic cells by immunostaining for cleaved caspase-3. **(A)** The vast majority of MuSCs (>99%) are negative for caspase-3 staining after 7 days in culture on all substrates tested. **(B)** Example field of view containing a MuSC stained positive for cleaved caspase-3. **(C)** Representative immunofluorescence microscopy images of MuSCs stained for cleaved caspase-3 to mark apoptotic cells after 7 days in culture. In A, data are presented as mean ± s.d., n = 3-4.



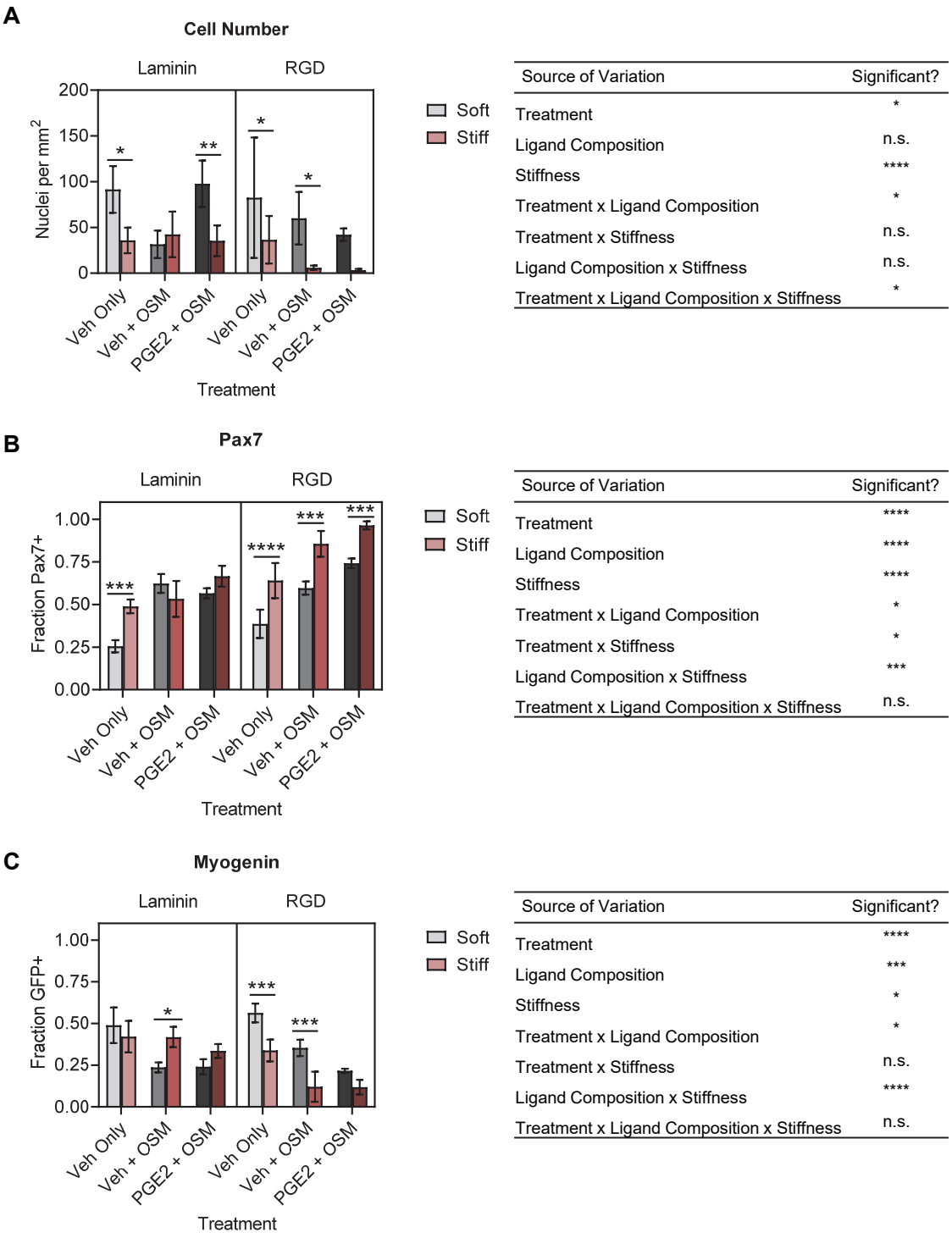
Supporting Figure S5. Immunofluorescence microscopy images characterizing the expression of Pax7 and MyoD by immunostaining and expression of the Sun1-GFP myogenin fluorescent reporter (MyoG) after 7 days of culture on soft and stiff laminin-presenting hydrogels. MuSCs were treated with vehicle only, vehicle followed by OSM on days 3 and 5, or PGE2 upon initial seeding followed by OSM on days 3 and 5.



Supporting Figure S6. PGE2 treatment enhances MuSC proliferation within the first three days of culture on soft, laminin-presenting hydrogels. Quantification of cell number after three days of culture on soft or stiff hydrogels presenting laminin or RGD peptides treated with either vehicle or PGE2 upon initial seeding. Data are presented as mean ± s.d. n = 4-8. *$p<0.05$, two-tailed Student's *t*-test.

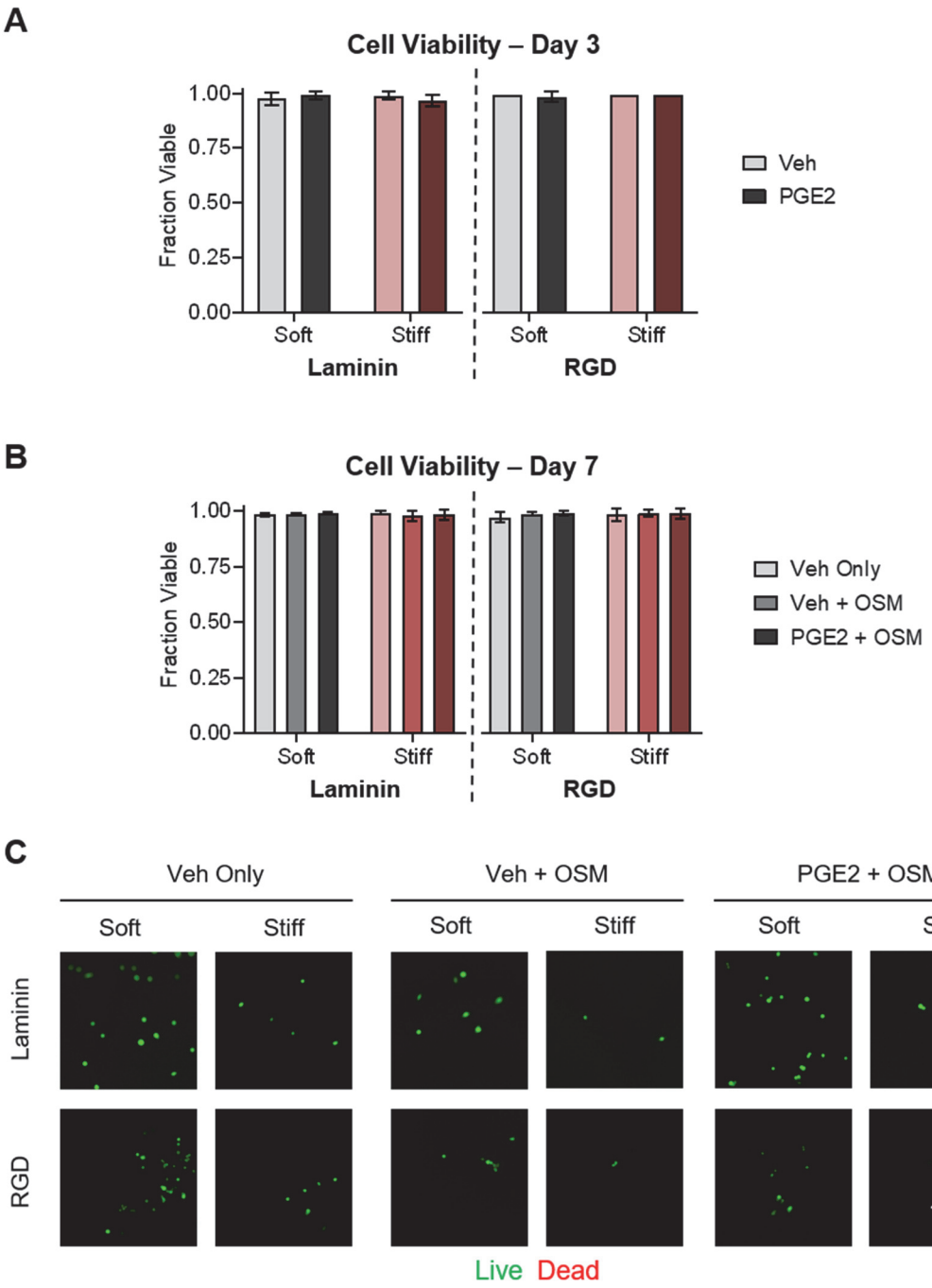


Supporting Figure S7. Immunofluorescence microscopy images characterizing the expression of Pax7 and MyoD by immunostaining and expression of the Sun1-GFP myogenin fluorescent reporter (MyoG) after 7 days of culture on soft and stiff RGD-presenting hydrogels. MuSCs were treated with vehicle only, vehicle followed by OSM on days 3 and 5, or PGE2 upon initial seeding followed by OSM on days 3 and 5.

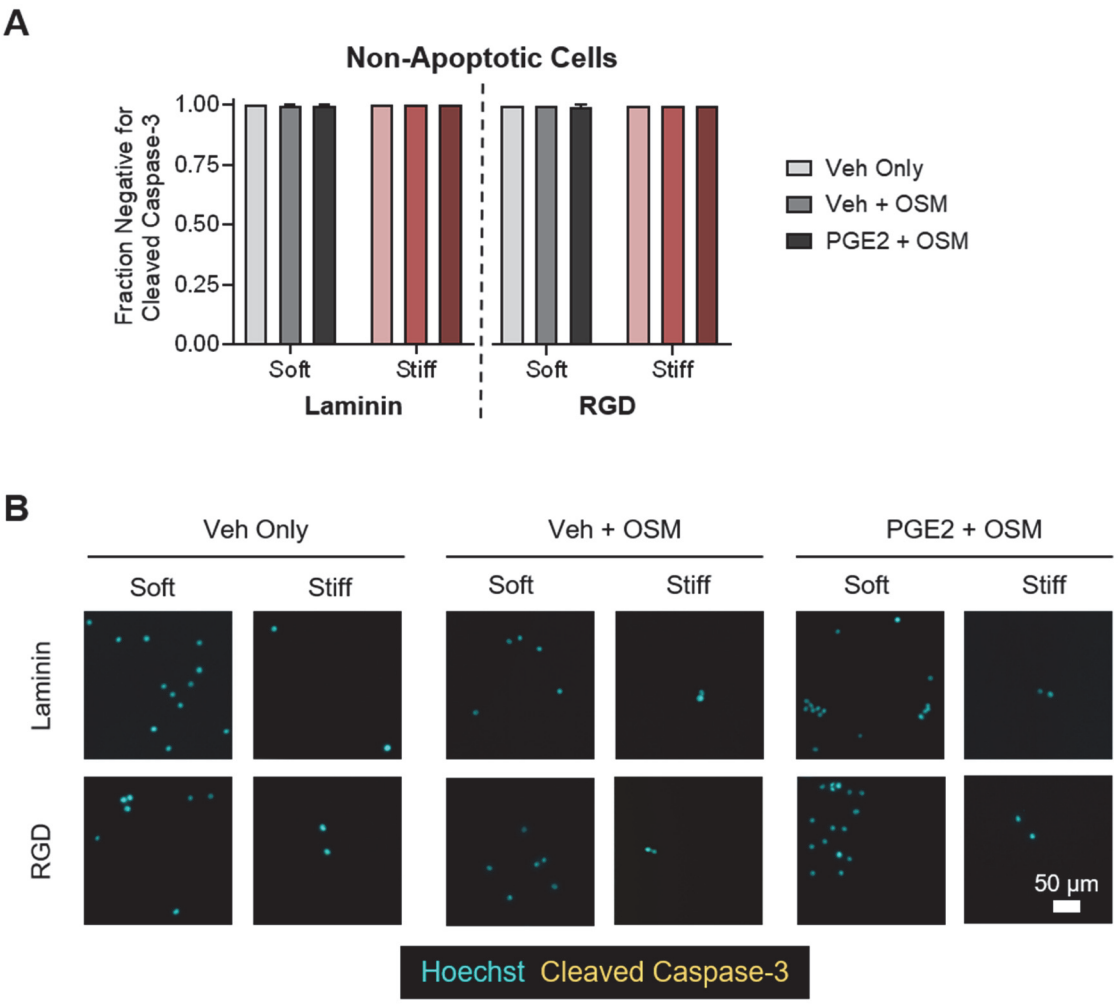


Supporting Figure S8. Three-way analysis of variance (ANOVA) reveals synergistic effects of matrix stiffness, adhesive ligand composition, and soluble factor treatment on MuSC fate. Data grouped by stiffness and ANOVA probability tables for **(A)** total cell number, **(B)** fraction of cells positive for Pax7, and **(C)** fraction of cells positive for the myogenin reporter. Data are

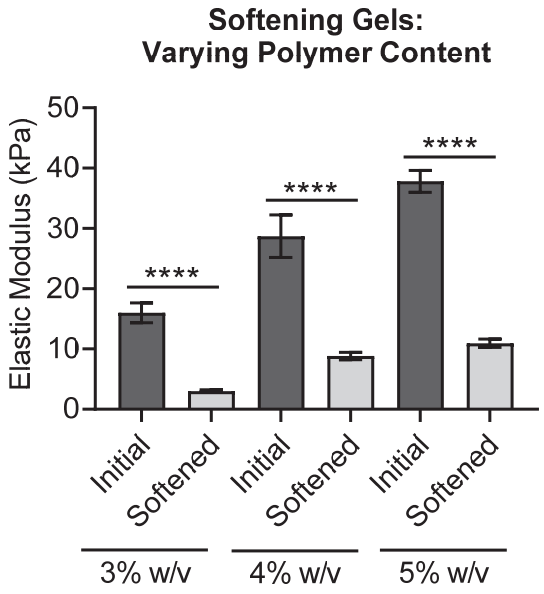
presented as mean ± s.d. n = 3-4. For pairwise comparisons, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, two-way ANOVA with Bonferroni post-hoc test. For ANOVA tables, n.s. = not significant (p>0.05), *p<0.05, ***p<0.001, ****p<0.0001, three-way ANOVA.



Supporting Figure S9. Viability of MuSCs on substrates with controlled stiffness and adhesive ligand presentation and treated with soluble niche factors. MuSCs remain highly viable at **(A)** day 3 and **(B)** day 7 in culture, with no significant difference among all conditions tested. **(C)** Representative microscopy images of live/dead stained MuSCs at day 7. In A and B, data are presented as mean ± s.d., n = 3-4.



Supporting Figure S10. Identification of apoptotic cells by immunostaining for cleaved caspase-3 in MuSC cultures treated with soluble niche factors. **(A)** The vast majority of MuSCs (>98%) are negative for caspase-3 staining after 7 days in culture on all substrates tested. **(B)** Representative immunofluorescence microscopy images of MuSCs stained for cleaved caspase-3 to mark apoptotic cells after 7 days in culture. In A, data are presented as mean ± s.d., n = 3-4.



Supporting Figure S11. Rheological characterization of *tert*-butyl substituted *o*NB ester containing hydrogels. The stiffness range spanned by the hydrogels was controlled by varying the total polymer content of the materials. Softened samples were exposed to a saturating amount of 405 nm light and allowed to equilibrate in PBS prior to rheological testing. Data are presented as mean ± s.d. n = 3. ****$p<0.0001$, two-way ANOVA with Bonferroni post-hoc test.

3. Supporting References

(1) DeForest, C. A.; Tirrell, D. A. A Photoreversible Protein-Patterning Approach for Guiding Stem Cell Fate in Three-Dimensional Gels. *Nat Mater* **2015**, *14*, 523–531.

(2) Dommerholt, J.; Schmidt, S.; Temming, R.; Hendriks, L. J. A.; Rutjes, F. P. J. T.; van Hest, J. C. M.; Lefeber, D. J.; Friedl, P.; van Delft, F. L. Readily Accessible Bicyclononynes for Bioorthogonal Labeling and Three-Dimensional Imaging of Living Cells. *Angew Chem Int Ed* **2010**, *49*, 9422–9425.

(3) Badeau, B. A.; Comerford, M. P.; Arakawa, C. K.; Shadish, J. A.; DeForest, C. A. Engineered Modular Biomaterial Logic Gates for Environmentally Triggered Therapeutic Delivery. *Nat Chem* **2018**, *10*, 251–258.

(4) DeForest, C. A.; Anseth, K. S. Photoreversible Patterning of Biomolecules within Click-Based Hydrogels. *Angew Chem Int Ed* **2012**, *51* (8), 1816–1819.

(5) Yamamoto, S.; Ikegami, H.; Yamaguchi, K.; Nakanishi, J. A Dynamic Biomaterial Based on a 2-Nitrobenzyl Derivative with a Tert-Butyl Substituent at the Benzyl Position: Rapid Response and Minimized Phototoxicity. *ChemPhotoChem* **2018**, *2* (9), 786–790.