Supporting Information

Title

Generation and Characterization of Anti-Glucosepane Antibodies Enabling Direct Detection of Glucosepane in Retinal Tissue

Authors

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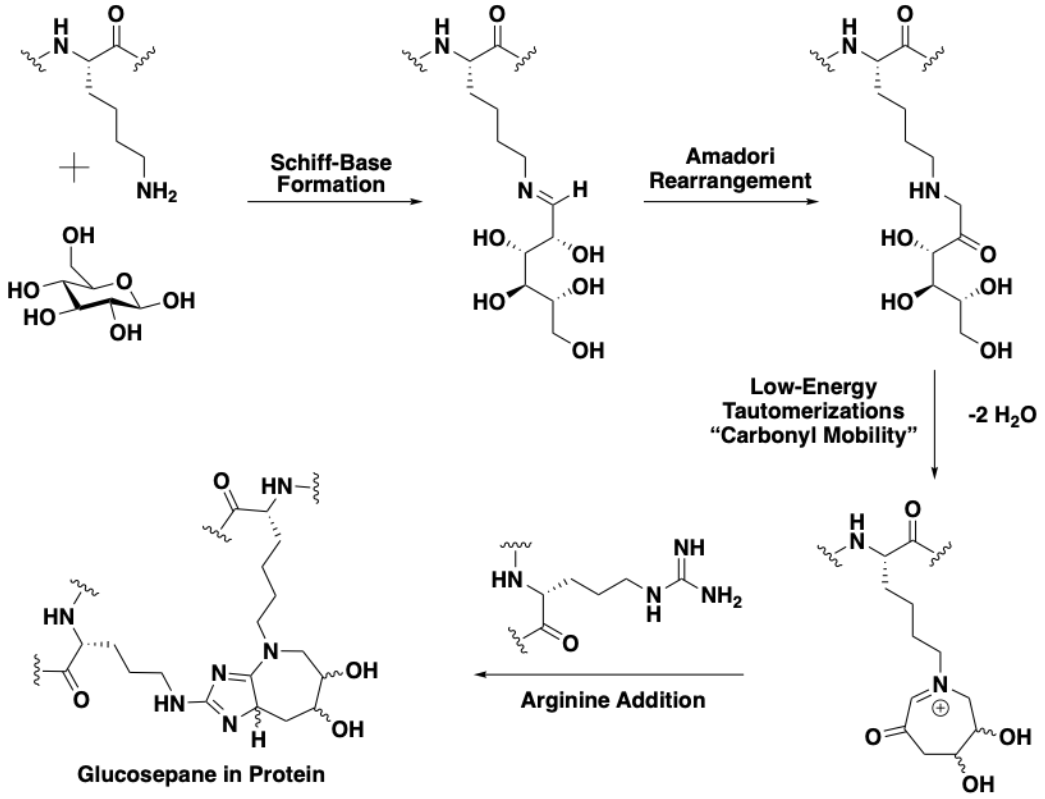
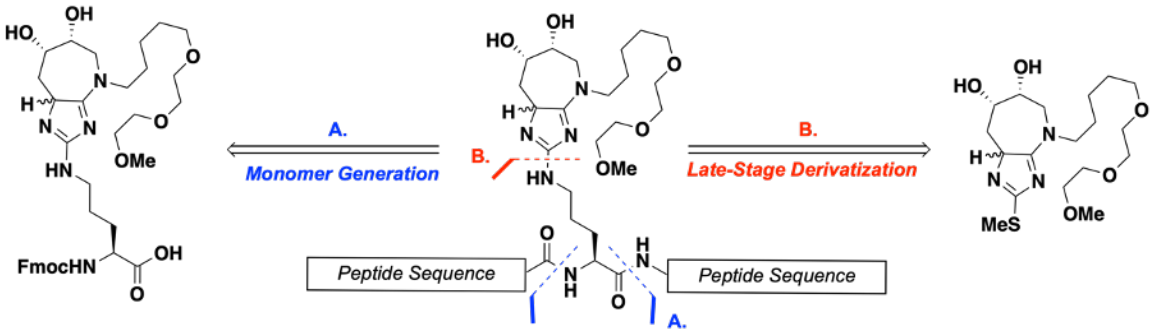
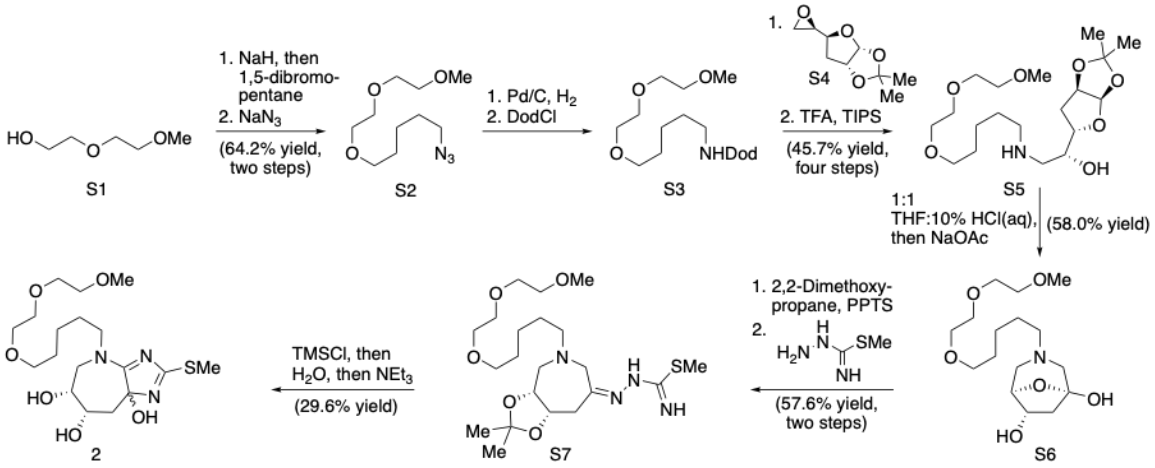
Supplementary Figures



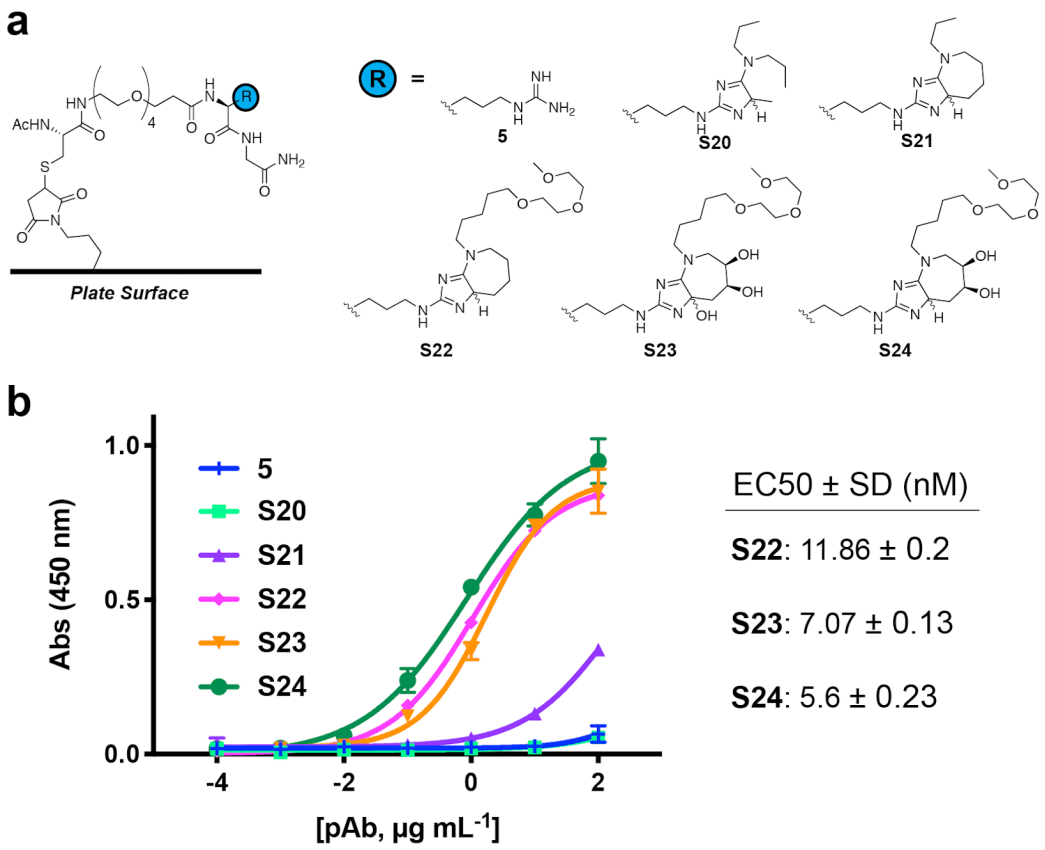
Supplementary Scheme 1. Outline of glucosepane formation pathway.



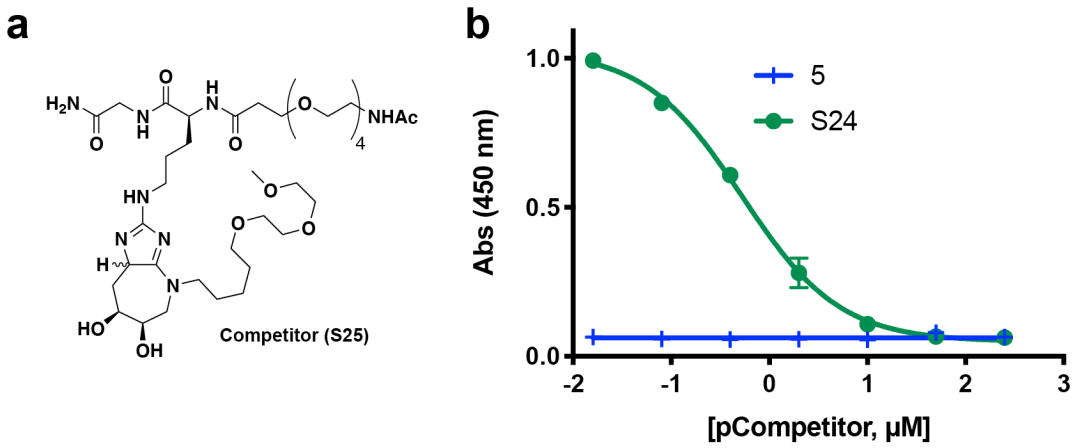
Supplementary Figure 1. Retrosynthetic planning of the proposed glucosepane immunogen. The blue retrosynthetic disconnections depict a hypothetical monomer-based approach. The red retrosynthetic disconnection represents the late-stage derivatization strategy that was used for immunogen synthesis.



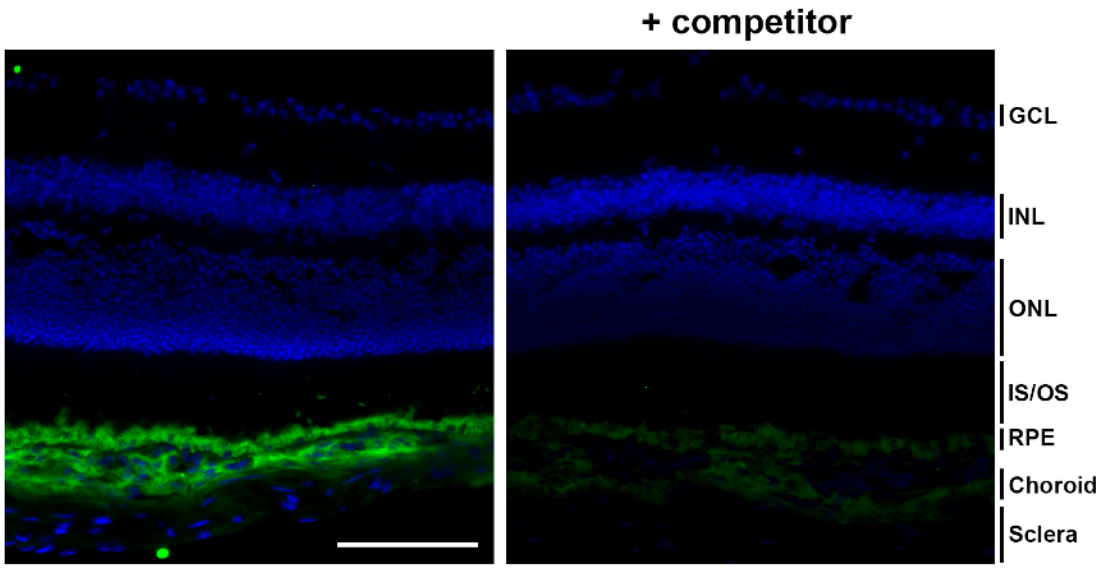
Supplementary Scheme 2. Chemical Synthesis of Glucosepane Precursor (**2**)



Supplementary Figure 2. Chemical structures of synthetic ELISA substrates and the corresponding ELISA results used to demonstrate anti-glucosepane antibody selectivity. (a) Synthetic peptides containing abiotic glucosepane analogs. (b) ELISA data towards panel of synthetic peptides. Indirect ELISAs were run on maleimide-coated plates. Error bars represent standard deviation (n=2).



Supplementary Figure 3. Competitive ELISA results for glucosepane peptide (**S24**) and arginine control peptide (**5**) incubated with antibody in the presence of varying concentrations of competitor peptide (**S25**). (a) Structure of competitor peptide (b) Competitive ELISAs were run on maleimide-coated plates. Error bars represent standard deviation (n=2).



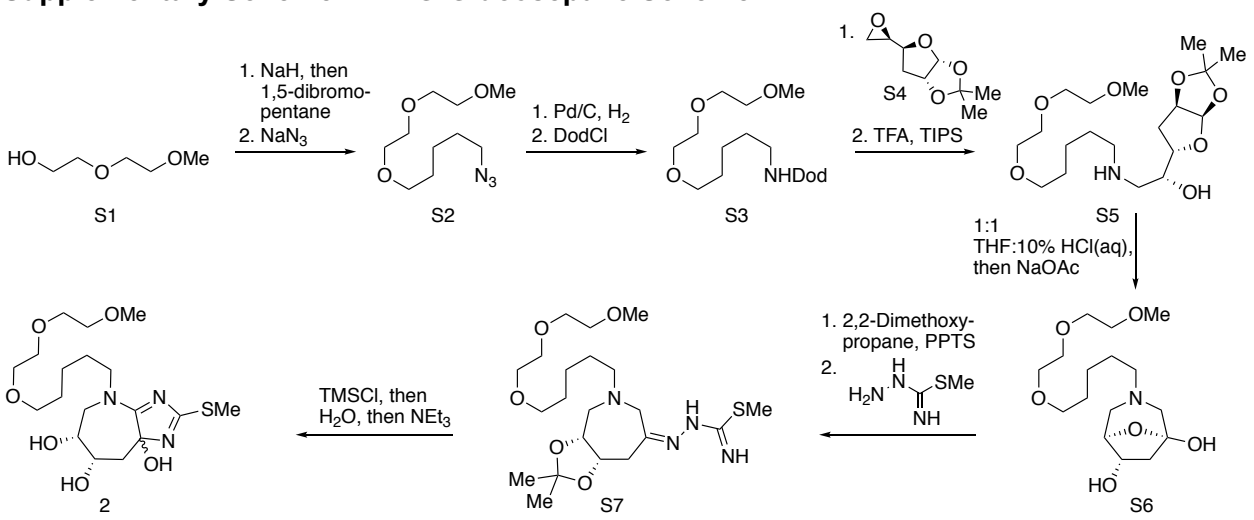
Supplementary Figure 4. Immunohistochemical competition experiment of retinal sections from 12-month-old mouse. Representative retinal sections labeled with anti-glucosepane antibody/anti-Rbt Alexa488 (Green) and DAPI (Blue). Immunohistochemical experiments were performed as previously described in the presence and absence of competitor peptide **S25**. Scale bars are 100 μm (GCL=Ganglion cell layer; INL=Inner nuclear layer; ONL=Outer nuclear layer; IS/OS=Inner/Outer segments; RPE=Retinal pigmented epithelium)

General Experimental

Starting materials were used as received unless otherwise noted. All moisture sensitive reactions were performed in an inert, dry atmosphere of nitrogen in oven dried glassware. Reagent grade solvents were used for extractions and flash chromatography. Reaction progress was checked by thin-layer chromatography (TLC, Merck silica gel 60 F-254 plates). The plates were then monitored with UV illumination followed by visualization with appropriate staining reagents such as cerium ammonium molybdate, ninhydrin, or iodine. Flash column chromatography was performed using silica gel (230-400 mesh) using Teledyne Isco CombiFlash Rf 200 unless otherwise specified. The solvent compositions reported for all chromatographic separations are on a volume/volume (v/v) basis. Infrared (IR) spectra were recorded on a Thermo Nicolet 6700 FT-IR Spectrometer. ^{1}H-NMR spectra were recorded on Agilent DD2 400 MHz, 500 MHz, and 600 MHz spectrometers and are reported in parts per million (ppm) on the δ scale relative to $CDCl_3$ (δ 7.26), Methanol-d_4 (δ 3.31), D_2O (δ 4.79) as an internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz), and integration. ^{13}C-NMR spectra were recorded on Agilent DD2 125 MHz, and 150 MHz spectrometers and are reported in parts per million (ppm) on the δ scale relative to $CDCl_3$ (δ 77.00), Methanol-d_4 (δ 49.00), ACN-d_3 (δ 1.32). LC-MS analyses were performed on a Waters UPLC/MS instrument equipped with a RP-C18 column (1.7 µm particle size, 2.1x50 mm), dual atmospheric pressure chemical ionization (API)/electrospray (ESI) mass spectrometry detector, and photodiode array detector. Optical rotations were measured at 20 °C; concentrations are in g/100 mL.

Synthetic Procedures

Supplementary Scheme 1. PEG-Glucosepane Scheme



1-Bromo-5-(2-(2-methoxyethoxy)ethoxy)pentane

2-(2-Methoxyethoxy)ethanol (S1, 7.68 mL, 65.2 mmol) was added dropwise to a stirred suspension of NaH (60% in mineral oil, 2.61 g, 65.2 mmol), in anhydrous THF (130 mL, 0.5 M) at 0 ºC. The reaction was allowed to stir at 0 ºC for 30 min. 1,5-Dibromopentane (35.5 mL, 261 mmol) was then added slowly to the suspension at 0 ºC. The reaction was then warmed to room temperature and stirred for 3 h. After this time, the reaction was carefully quenched with MeOH, vacuum filtered through a pad of celite, and concentrated. The material was then run through a pad of silica with CH_2Cl_2 and concentrated once more. The leftover dibromopentane was removed

via vacuum distillation (130 ºC, ~5 torr). The remaining material was purified by column chromatography (Silica; 120 g; Gradient: 0 to 40% EtOAc in Hexanes). The product was obtained as a clear oil (12.1 g, 68.9% yield).
^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.63-3.58 (m, 4H), 3.57-3.53 (m, 2H), 3.53-3.49 (m, 2H), 3.43 (t, 2H), 3.36 (t, 2H), 3.34 (s, 3H), 1.84 (p, 2H), 1.57 (p, 2H), 1.50-1.41 (m, 2H).
^{13}C-NMR (101 MHz, $CDCl_3$) δ 71.98, 71.04, 70.67, 70.57, 70.17, 59.05, 33.75, 32.62, 28.80, 24.88.
HR-MS: $(M+1)^+$ = 269.0749 (experimental); Exact mass = 269.0747 (theoretical)
IR *ƒ* (cm^{-1}) λ_{max}: 2864, 1455, 1352, 1249, 1199, 1105, 1028, 976, 849, 734.

1-Azido-5-(2-(2-methoxyethoxy)ethoxy)pentane (S2)
1-Bromo-5-(2-(2-methoxyethoxy)ethoxy)pentane (13.31 g, 49.45 mmol) was dissolved in DMF (25 mL, 2 M), and sodium azide (3.21 g, 49.45 mmol) was added at room temperature. The reaction was allowed to stir for 12 h at room temperature. After this time, the reaction mixture was concentrated, redissolved in EtOAc, and filtered through celite. The filtered solution was then concentrated, redissolved in a small amount of CH_2Cl_2, and purified by column chromatography (Silica; 120 g; Gradient: 0 to 50% EtOAc in Hexanes). The product was isolated as a clear oil (10.66 g, 93.2% yield).
^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.65-3.60 (m, 4H), 3.60-3.55 (m, 2H), 3.55-3.51 (m, 2H), 3.45 (t, 2H), 3.36 (s, 3H), 3.25 (t, 2H), 1.64-1.54 (m, 4H), 1.46-1.36 (m, 2H).
^{13}C-NMR (101 MHz, $CDCl_3$) δ 72.04, 71.13, 70.73, 70.64, 70.22, 59.12, 51.46, 29.24, 28.76, 23.47.
HR-MS: $(M+Na)^+$ = 254.1468 (experimental); Exact mass = 254.1475 (theoretical)
IR *ƒ* (cm^{-1}) λ_{max}: 2934, 2864, 2093, 1456, 1351, 1249, 1200, 1104, 1029, 984, 930, 851, 637, 558.

***N*-(Bis(4-methoxyphenyl)methyl)-5-(2-(2-methoxyethoxy)ethoxy)pentan-1-amine (S3)**
Azide (S2, 10.66 g, 46.1 mmol) was dissolved in MeOH (460 mL 0.1 M). This solution was purged with nitrogen for 15 min. Pd/C (1.066 g) was then added carefully under nitrogen. The reaction mixture was purged with nitrogen for an additional 10 min. Hydrogen gas was then bubbled through the suspension for 5 min, and the reaction was kept under a hydrogen atmosphere thereafter (~1 atm). The reaction was monitored by TLC until starting material had been consumed. The crude product mixture was then filtered through a pad of packed celite, being careful not to let the Pd/C become dry. The filtered solution was concentrated and used crude in the subsequent bis-(4-methoxyphenyl)-methyl (Dod) protection.

The crude material (8.41 g) was dissolved in CH_2Cl_2 (400 mL) along with NEt_3 (17.1 mL, 122.9 mmol). The mixture was cooled to 0 C. DodCl was prepared as it was previously.[1] Briefly, bis(4-methoxyphenyl)methanol (DodOH) (15 g, 61.4 mmol) was added to a flask containing Et_2O (380 mL), and the solution was cooled to 0°C. 12M aqueous HCl (30.7 mL, 368.7 mmol) was then added dropwise, which caused the solution to turn slightly pink. The heterogeneous reaction mixture was warmed to room temperature, stirred vigorously for 30 min, and then poured into a separatory funnel. After separation of layers, the organic layer was dried with a ~1:1 mixture of $MgSO_4$ and $NaHCO_3$. The solid particles were filtered to give DodCl in Et_2O solution. This DodCl solution was added dropwise into the CH_2Cl_2 solution at 0 ºC. The reaction was warmed to room temperature and stirred for an additional 3 h. The reaction was filtered through a silica plug to remove the majority of salts. The crude product was then purified by column chromatography in several batches (Silica; 120 g; 0 to 15% MeOH in CH_2Cl_2). The product was isolated as a moderately thick oil (13.33 g, 67.0% yield over two steps). Note: A large portion of the product elutes very quickly if the column is overloaded.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.28 (d, 4H), 6.82 (d, 4H), 4.71 (s, 1H), 3.76 (s, 6H), 3.66-3.61 (m, 4H), 3.59-3.56 (m, 2H), 3.56 (m, 2H), 3.44 (t, 2H), 3.37 (s, 3H), 2.54 (t, 2H), 1.57 (p, 2H), 1.52 (p, 2H), 141-1.33 (m, 2H).
^{13}C-NMR (101 MHz, $CDCl_3$) δ 158.63, 137.10, 128.32, 113.95, 72.14, 71.50, 70.83, 70.70, 70.25, 66.44, 59.13, 55.36, 48.30, 30.26, 29.72, 24.06.
HR-MS: $(M+1)^+$ = 432.2792 (experimental); Exact mass = 432.2744 (theoretical)
IR *ƒ* (cm^{-1}) λ_{max}: 2859, 1609, 1585, 1507, 1460, 1300, 1241, 1172, 1102, 1031, 817, 775, 738, 637, 576, 558.

(*R*)-16-((3a*R*,5*S*,6a*R*)-2,2-Dimethyltetrahydrofuro[2,3-*d*][1,3]dioxol-5-yl)-2,5,8-trioxa-14-azahexadecan-16-ol (S5)
Dod-protected amine (S3, 4.02 g, 9.3 mmol) and (3a*R*,5*S*,6a*R*)-2,2-dimethyl-5-((*R*)-oxiran-2-yl)tetrahydrofuro[2,3-*d*][1,3]dioxole (S4, 2.60 g, 14.0 mmol)[1] were dissolved in isopropanol (46.5 mL, 0.2 M) and heated to 60 ºC for 18 h. The reaction was run until S3 was consumed, with additional epoxide added if necessary. The crude mixture was then concentrated via rotary evaporation. The crude epoxide-opened product was then dissolved in anhydrous dichloromethane (93 mL, 0.1 M). Triisopropylsilane (4.77 mL, 23.3 mmol) followed by trifluoroacetic acid (4.65 mL) were added at 0 ºC to facilitate the deprotection of the bis-(4-methoxyphenyl)-methyl (Dod) protecting group. The reaction was monitored closely and quenched with NEt_3 (~20 mL) upon reaction completion. The reaction was then concentrated, resuspended in a small amount of dichloromethane, and purified by column chromatography (Silica; 40 g; Gradient: 0 to 15% MeOH in CH_2Cl_2). The product eluted about halfway through the purification gradient (2.48 g, 68.2% yield over two steps).
^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.75 (d, 1H), 4.71 (t, 1H), 4.06 (m, 1H), 3.94 (m, 1H), 3.64-3.59 (m, 4H), 3.58-3.50 (m, 4H), 3.43 (t, 2H), 3.35 (s, 3H), 3.21-3.11 (br d, 1H), 3.02-2.86 (br m, 3H), 2.19 (dd, 1H), 1.78-1.65 (m, 3H), 1.57 (p, 2H), 1.48 (s, 3H), 1.41 (p, 2H), 1.28 (s, 3H).
^{13}C-NMR (101 MHz, $CDCl_3$) δ 111.62, 105.77, 80.43, 79.31, 71.99, 70.79, 70.62, 70.51, 70.12, 68.77, 59.08, 51.04, 48.38, 35.70, 28.78, 26.85, 26.22, 25.51, 23.31.
HR-MS: $(M+1)^+$ = 392.2638 (experimental); Exact mass = 392.2643 (theoretical)
IR *ƒ* (cm^{-1}) λ_{max}: 2988, 2935, 2871, 1672, 1457, 1432, 1383, 1299, 1126, 1081, 1019, 833, 799, 720, 647, 519.
$[\alpha]_D$ = -0.034º (c = 2.0, $CHCl_3$)

(1*R*,5*R*,6*S*)-3-(5-(2-(2-Methoxyethoxy)ethoxy)pentyl)-8-oxa-3-azabicyclo[3.2.1]octane-1,6-diol (S6)
Dod-deprotected product (S5, 0.77 g, 1.97 mmol) was dissolved in a 1:1 mixture of 10% HCl(aq):THF (18.2 mL, 29.5 mmol HCl). This reaction was run for 2 h at room temperature to afford clean conversion to the acetal deprotected product (352 m/z by LCMS). NaOAc (4.11 g, 50.2 mmol) and CH_3CN (40 mL) were then added to facilitate the subsequent Amadori rearrangement. The suspension was stirred at 50 ºC for 12 h. The reaction was concentrated and purified by column chromatography (Silica; 40 g; Gradient: 0 to 20% MeOH in CH_2Cl_2). The product was obtained as an oil (0.38 g, 58.0% yield).
^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.98 (br, 2H), 4.28 (d, 1H), 4.12 (s, 1H), 3.65-3.61 (m, 4H), 3.59-3.52 (m, 4H), 3.43 (t, 2H), 3.36 (s, 3H), 2.68 (dd, 2H), 2.59 (dd, 1H), 2.34-2.27 (m, 2H), 2.10 (t, 2H), 2.03 (s, H), 1.65 (d, 1H), 1.56 (p, 2H), 1.42 (p, 2H), 1.35-1.28 (m, 2H).
^{13}C-NMR (101 MHz, $CDCl_3$) δ 104.35, 82.43, 74.65, 72.06, 71.41, 70.77, 70.58, 70.18, 60.94, 59.06, 57.17, 54.09, 46.69, 29.47, 26.31, 23.81.
HR-MS: $(M+1)^+$ = 334.2228 (experimental); Exact mass = 334.2224 (theoretical)
IR *ƒ* (cm^{-1}) λ_{max}: 3391, 2936, 2864, 2811, 1451, 1351, 1287, 1185, 1107, 1061, 954, 935.
$[\alpha]_D$ = -0.175º (c = 1.0, $CHCl_3$)

(3a*R*,8a*S*)-5-(5-(2-(2-Methoxyethoxy)ethoxy)pentyl)-2,2-dimethylhexahydro-7*H*-[1,3]dioxolo[4,5-*c*]azepin-7-one

Amadori product S6 (0.38 g, 1.14 mmol) was dissolved in anhydrous toluene. PPTS (0.072 g, 0.285 mmol) and 2,2-dimethoxypropane (0.7 mL, 5.70 mmol) were added at room temperature. The reaction was heated to 95 ºC under nitrogen and monitored by LCMS (both 374 m/z and 392 m/z (hydrate) were observed). Additional 2,2-dimethoxypropane (~5 equiv) was added at room temperature when the reaction stalled. The reaction was continued at 95 ºC. The reaction was monitored closely by LCMS to minimize formation of by-product (446 m/z). When the majority of starting material had converted to product, the reaction was cooled to room temperature, quenched with NEt_3, and concentrated. The reaction mixture was then purified by column chromatography (Silica; 40 g; 0 to 10% MeOH in CH_2Cl_2). The product was obtained as a slightly unstable oil (0.346 g, 81.3% yield) and used in the subsequent reaction shortly after purification.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.25 (m, 1H), 4.11 (m, 1H), 3.63-3.58 (m, 4H), 3.57-3.48 (m, 4H), 3.42 (t, 2H), 3.34 (s, 3H), 3.16 (s, 2H), 3.02 (dd, 1H), 2.97 (dd, 1H), 2.73 (dd, 1H), 2.56 (dd, 1H), 2.51 (t, 2H), 1.56 (p, 2H), 1.44 (p, 2H), 1.38 (s, 3H), 1.38-1.31 (m, 2H), 1.29 (s, 3H).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 209.23, 108.12, 76.11, 74.04, 72.01, 71.35, 70.72, 70.61, 70.16, 68.30, 59.12, 57.78, 55.56, 43.20, 29.49, 28.37, 27.64, 25.89, 23.68.

HR-MS: $(M+1)^+$ = 374.4980 (experimental); Exact mass = 374.4975 (theoretical)

IR *ƒ* (cm^{-1}) λ_{max}: 2984, 2934, 2861, 1717, 1457, 1380, 1368, 1242, 1219, 1109, 1071, 1050, 862.

[α]$_D$ = +0.055º (c = 1.0, $CHCl_3$)

Methyl (*E*)-2-((3a*R*,8a*S*)-5-(5-(2-(2-methoxyethoxy)ethoxy)pentyl)-2,2-dimethylhexahydro-7*H*-[1,3]dioxolo[4,5-*c*]azepin-7-ylidene)hydrazine-1-carbimidothioate (S7)

(3a*R*,8a*S*)-5-(5-(2-(2-Methoxyethoxy)ethoxy)pentyl)-2,2-dimethylhexahydro-7*H*-[1,3]dioxolo[4,5-*c*]azepin-7-one (0.346 g, 0.926 mmol) was dissolved in MeOH (9.3 mL, 0.1 M). S-methylthiosemicarbazide HI salt (0.260 g, 1.11 mmol) was added at room temperature. The reaction was continued at room temperature for 3 h. The reaction was concentrated, and the crude product was purified by column chromatography (Silica; 24 g; Gradient: 0 to 15% MeOH in CH_2Cl_2). The product emerged as two peaks corresponding to E and Z isomers. The fractions were combined and concentrated, affording a mixture of isomers (0.302 g, 70.8% yield).

^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.35 (s, 2H), 4.26 (m, 1H), 4.14 (m, 1H), 3.65-3.56 (m, 5H), 3.55-3.47 (m, 4H), 3.39 (t, 2H), 3.32 (s, 3H), 3.26 (dd, 2H), 2.83 (dd, 1H), 2.76 (dd, 1H), 2.52-2.34 (m, 6H), 1.54 (p, 2H), 1.46 (p, 2H), 1.40 (s, 3H), 1.36-1.28 (m, 2H), 1.27 (s, 3H).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 160.16, 158.97, 108.01, 76.15, 73.33, 71.89, 71.33, 70.61, 70.47, 70.03, 63.03, 58.99, 56.67, 54.05, 32.26, 29.41, 27.70, 27.23, 24.96, 23.69, 12.60.

HR-MS: $(M+1)^+$ = 461.6411 (experimental); Exact mass = 461.6415 (theoretical)

IR *ƒ* (cm^{-1}) λ_{max}: 3446, 3312, 2981, 2932, 2864, 1595, 1542, 1452, 1456, 1380, 1297, 1245, 1106.

[α]$_D$ = +0.013º (c = 1.0, $CHCl_3$)

(6*R*,7*S*)-4-(5-(2-(2-Methoxyethoxy)ethoxy)pentyl)-2-(methylthio)-5,6,7,8-tetrahydroimidazo[4,5-*b*]azepine-6,7,8a(4*H*)-triol (2)

SMe-thiosemicarbazone (S7, 0.4 g, 0.868 mmol) was dissolved in anhydrous DCE (8.7 mL, 0.1 M). This solution was transferred to a flame-dried pressure vessel. TMSCl (0.55 mL, 4.34 mmol) was added, the pressure vessel was capped, and the reaction was stirred at room temperature for 15 min. The reaction was then heated to 95 ºC and monitored closely by LCMS. When SM had been consumed, the reaction was cooled to room temperature and water (0.55 mL) was added to quench the TMSCl and deprotect the acetal functionality. After 1.5 h of stirring at room temperature, the reaction was exposed to atmospheric oxygen, and NEt_3 (2 mL) was added to facilitate oxidation. The dark reaction mixture was stirred at room temperature for ~12 h until

complete oxidation had occurred. The reaction was concentrated and HPLC purified (0 to 60% CH_3CN(0.1% TFA) in H_2O(0.1% TFA). The product emerged as the main UV-active peak (254 nm). HPLC fractions were collected and lyophilized to obtain the desired product (0.114 g, 29.6% yield).

^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.44 (br, 3H), 4.37 (d, 2H), 4.22-4.14 (m, 2H), 3.93 (m, 1H), 3.67-3.42 (m, 13H), 3.37 (s, 3H), 2.64 (s, 3H), 2.53 (d, 1H), 2.14 (t, 1H), 1.71 (p, 2H), 1.61 (p, 2H), 1.39 (p, 2H).

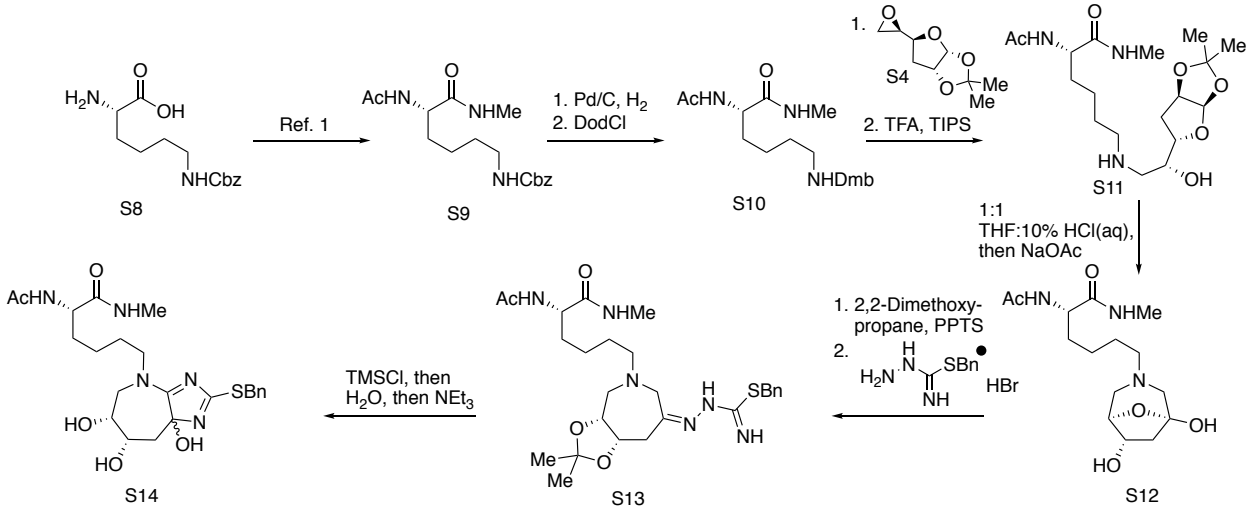
^{13}C-NMR (101 MHz, $CDCl_3$) δ 184.94, 179.25, 92.07, 71.98, 71.09, 70.68, 70.52, 70.15, 70.08, 69.74, 59.13, 55.44, 51.40, 34.19, 29.13, 26.50, 23.33, 14.18.

HR-MS: $(M+1)^+$ = 420.2167 (experimental); Exact mass = 420.2163 (theoretical)

IR *ƒ* (cm^{-1}) λ_{max}: 3124, 2870, 1673, 1509, 1253, 1265, 1199, 1132, 941, 838, 800, 720.

[α]$_D$ = -1.540° (c = 2.0, CH_3CN)

Supplementary Scheme 2. Peptide Backbone Mimetic Glucosepane Scheme



Benzyl (*S*)-(5-acetamido-6-(methylamino)-6-oxohexyl)carbamate (S9)

AcHN-Lys(Cbz)-NHMe (S9) was prepared as previously reported.[2] Briefly, Lys(Cbz)-OH (50 g, 178.4 mmol) was dissolved in 0.5 M NaOH(aq) (357 mL, 0.5 M) and NEt_3 (49.8 mL, 356.7 mmol). To this solution at 0 ºC, was added Ac_2O (21.9 mL, 231.9 mmol) in portions. The reaction was allowed to stir at room temperature for 5 h. The solution was then washed with Et_2O (~400 mL). The aqueous layer was acidified with 5 M HCl(aq) (107 mL), causing the aqueous layer to turn cloudy. The product was then extracted with EtOAc (~300 mL x3), causing solutions to become clear once more. The EtOAc layers were combined, dried (Na_2SO_4), filtered, and concentrated to a thick oil. The resulting AcHN-Lys(Cbz)-OH was of sufficient purity to move forward crude into the next reaction.

The crude product was then dissolved in THF (700 mL, 0.25 M) along with N-methylmorpholine (21.6 mL, 196.2 mmol). The solution was cooled to 0 ºC and ethyl chloroformate (18.8 mL, 196.2 mmol) was added dropwise, causing a white precipitate to form. The mixture was stirred for 30 min at 0 ºC. 40% $MeNH_2$(aq) (41.5 mL, 535 mmol) was then added dropwise, causing dissolution of the precipitate. The reaction was stirred for 30 min, with more methylamine added if the reaction did not go to completion. At the completion of the reaction, the solution was acidified with 12 M HCl. THF was concentrated away, causing the precipitation of a white solid, via rotary evaporation. The excess aqueous portion was decanted. At this point, significant impurities remained. Additional 2M HCl (aq) was added along with sufficient THF to redissolve the amorphous solid. The THF was again evaporated, and the aqueous layer was

decanted. This process was repeated until compound of suitable purity was obtained. The solid was then filtered off and dried under vacuum, yielding AcHN-Lys(Cbz)-NHMe as an off-white solid (45.2 g, 75.6% yield). Characterization data matches the reported literature values.

(*S*)-2-acetamido-6-((2,4-dimethoxybenzyl)amino)-*N*-methylhexanamide (S10)

AcHN-Lys(Cbz)-NHMe (S9, 10 g, 29.8 mmol) was dissolved in methanol (300 mL, 0.1 M). Nitrogen was bubbled through the solution for 10 min. Pd/C (2 g) was then added carefully under nitrogen, and nitrogen was bubbled through the suspension for 10 more min. The nitrogen atmosphere was then replaced with 1 atm of hydrogen by bubbling hydrogen through the suspension for 5 min and then maintaining the hydrogen atmosphere. The suspension was stirred at room temperature for 3 h and monitored by TLC. Upon consumption of starting material, nitrogen was bubbled through the suspension to remove hydrogen, and the Pd/C was filtered off with a pad of packed celite.

The solution of Cbz-deprotected material was then charged with 2,4-dimethoxybenzaldehyde (4.95 g, 29.8 mmol) and stirred at room temperature for 2 h. This solution was then cooled to 0 ºC, and $NaBH_4$ (1.13 g, 29.8 mmol) was added in portions, resulting in vigorous bubbling. The solution was concentrated and taken up in a suspension of 0.5M NaOH(aq) (~400 mL) and EtOAc (~400 mL). The aqueous layer was extracted 3x with EtOAc (~400 mL each). The organic layers were combined, dried (Na_2SO_4), filtered, and concentrated. This material was then purified by column chromatography (Silica; 80 g; Gradient: 0 to 25% MeOH (2% NEt_3) in CH_2Cl_2 (2% NEt_3)), resulting in an oil that solidified over time to a white solid (5.5 g, 52.5% yield).

^{1}H-NMR (400 MHz, CD_3OD) δ 7.12 (d, 1H), 6.53 (d, 1H), 6.47 (dd, 1H), 4.24 (dd, 1H), 3.82 (s, 3H), 3.77 (s, 3H), 3.71 (s, 3H), 2.71 (s, 3H), 2.58 (t, 2H), 1.98 (s, 3H), 1.80-1.71 (m, 1H), 1.66-1.58 (s, 1H), 1.58 -1.48 (m, 2H), 1.43-1.27 (m, 2H).

^{13}C-NMR (101 MHz, CD_3OD) δ 174.89, 173.25, 162.26, 160.13, 132.06, 119.40, 105.32, 99.29, 55.88, 55.80, 54.82, 49.02, 48.98, 32.83, 29.35, 26.31, 24.60, 22.53.

HR-MS: $(M+1)^+$ = 352.2227 (experimental); Exact mass = 352.2231 (theoretical)

IR *ƒ* (cm^{-1}) λ_{max}: 3278, 2939, 2837, 1650, 1588, 1546, 1509, 1460, 1290, 1209, 1158, 1036.

$[\alpha]_D$ = -0.065º (c = 1.0, $CHCl_3$)

(*S*)-2-acetamido-6-((2,4-dimethoxybenzyl)((*R*)-2-((3a*R*,5*S*,6a*R*)-2,2-dimethyltetrahydrofuro[2,3-*d*][1,3]dioxol-5-yl)-2-hydroxyethyl)amino)-*N*-methylhexanamide (S11)

Dmb-protected lysine (3.43 g, 9.76 mmol) was dissolved in iPrOH (20 mL, 0.5 M) along with (3a*R*,5*S*,6a*R*)-2,2-dimethyl-5-((*R*)-oxiran-2-yl)tetrahydrofuro[2,3-*d*][1,3]dioxole (S4, 2 g, 10.74 mmol)[1]. The reaction was heated to 60 ºC and monitored by TLC. When the starting epoxide had been consumed, the reaction was concentrated. The crude material was purified with a 0 to 20% gradient of MeOH in CH_2Cl_2, yielding a thick oil (4.12 g, 78.3%).

^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.12 (d, 1H), 6.51 (d, 1H), 6.45 (dd, 1H), 5.75 (d, 1H), 4.71 (s, 1H), 4.20 (dd, 1H), 4.15 (dt, 1H), 3.79 (s, 3H), 3.77 (s, 3H), 3.58-3.46 (m, 2H), 2.71 (s + rotamer, 3H), 2.56-2.35 (m, 4H), 1.97 (s + rotamer, 3H), 1.86-1.62 (m, 3H), 1.60-1.40 (m, 6H), 1.36-1.20 (m, 5H).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 175.00, 173.21, 161.84, 160.38, 132.92, 120.11, 112.11, 106.74, 105.17, 99.33, 81.80, 81.20, 69.74, 57.63, 55.80, 55.74, 55.00, 54.87, 53.92, 33.76, 32.88, 27.57, 27.20, 26.43, 26.31, 24.69, 22.50.

HR-MS: $(M+1)^+$ = 538.3122 (experimental); Exact mass = 538.3123 (theoretical)

IR *ƒ*(cm^{-1}) λ_{max}: 3278, 2944, 1653, 1615, 1541, 1511, 1458, 1373, 1294, 1210, 1161, 1134, 1026, 838, 752.

$[\alpha]_D$ = +0.133º (c = 1.0, $CHCl_3$)

(*S*)-2-acetamido-6-((1*R*,5*R*,6*S*)-1,6-dihydroxy-8-oxa-3-azabicyclo[3.2.1]octan-3-yl)-*N*-methylhexanamide (S12)

The epoxide addition product (S11, 3 g, 5.58 mmol) was dissolved in methanol (56 mL, 0.1 M) and purged with nitrogen for 10 min. Pd/C (0.3 g) was then added, and nitrogen was bubbled through the suspension for an additional 10 min. The nitrogen atmosphere was then purged with hydrogen. The suspension was stirred under a hydrogen atmosphere at room temperature for 3 h and monitored by TLC. Upon consumption of starting material, nitrogen was bubbled through the suspension to remove hydrogen, and the Pd/C was filtered off with a pad of packed celite. The crude material was concentrated and used without purification in the next reaction.

The crude material was dissolved in 90% AcOH(aq) (56 mL, 0.1 M) and heated at 100 ºC for 12 h. After consumption of starting material, the reaction was concentrated and loaded directly onto a CombiFlash column with CH_2Cl_2. The compound was purified with a gradient of 0 to 50% MeOH in CH_2Cl_2 (0.992 g, 54% yield over two steps).

^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.86 (br, 1H), 4.30-4.23 (m, 2H), 4.02 (s, 1H), 2.73 (s, 3H), 2.69 (s, 1H), 2.67 (s, 1H), 2.45 (dd, 1H), 2.33 (t, 2H), 2.08-1.96 (m, 5H), 1.84-1.72 (m, 1H), 1.68-1.54 (m, 2H), 1.54-1.28 (m, 4H).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 174.91, 173.19, 104.61, 83.17, 74.85, 62.26, 58.02, 55.46, 54.73, 46.52, 32.83, 26.93, 26.34, 24.43, 22.57.

HR-MS: $(M+1)^+$ = 330.2025 (experimental); Exact mass = 330.2023 (theoretical)

IR *ƒ* (cm^{-1}) λ_{max}: 3294, 2942, 2808, 1648, 1547, 1447, 1413, 1375, 1287, 1142, 1059, 955.

$[\alpha]_D$ = -0.278º (c = 2.0, MeOH)

(*S*)-2-acetamido-6-((3a*R*,8a*S*)-2,2-dimethyl-7-oxohexahydro-5*H*-[1,3]dioxolo[4,5-*c*]azepin-5-yl)-*N*-methylhexanamide

Amadori product (S11, 0.8 g, 2.43 mmol) was dissolved in anhydrous DMF (24 mL, 0.1 M). PPTS (0.153 g, 0.61 mmol) and 2,2-dimethoxypropane (1.49 mL, 12.1 mmol) were added at room temperature. The reaction was heated to 95 ºC under nitrogen and monitored by LCMS (both 370 m/z and 388 m/z (hydrate) were observed). Additional 2,2-dimethoxypropane (~5 equiv) was added at room temperature if needed. The reaction was continued at 95 ºC. The reaction was monitored closely by LCMS to minimize formation of by-product (444 m/z). When the majority of starting material had converted to product, the reaction was cooled to room temperature, quenched with NEt_3 (~3 mL), and concentrated. The reaction mixture was then column chromatographed (Silica; 4 g; 0 to 35% MeOH in CH_2Cl_2). The product was obtained as a slightly unstable oil (53.4% yield) and used in the subsequent reaction shortly after purification.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.31 (m, 1H), 4.26 (m, 1H), 4.18 (m, 1H), 3.20 (q, 2H), 3.04 (dd, 1H), 2.95 (ddd, 1H), 2.79 (dd, 1H), 2.72 (s, 3H), 2.62 (ddd, 1H), 2.57 (t, 2H), 1.99 (s, 3H), 1.85-1.74 (m, 1H), 1.68-1.58 (m, 7H), 1.30 (s, 3H).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 211.37, 174.95, 173.24, 109.03, 77.36, 75.33, 68.87, 58.40, 56.54, 54.80, 44.23, 32.88, 28.56, 28.27, 26.32, 25.98, 24.42, 22.53.

HR-MS: $(M+1)^+$ = 370.2336 (experimental); Exact mass = 370.2336 (theoretical)

IR *ƒ* (cm^{-1}) λ_{max}: 3292, 3099, 2984, 2937, 2859, 1716, 1648, 1548, 1411, 1370, 1242, 1219, 1134, 1050.

$[\alpha]_D$ = +0.056º (c = 1.0, MeOH)

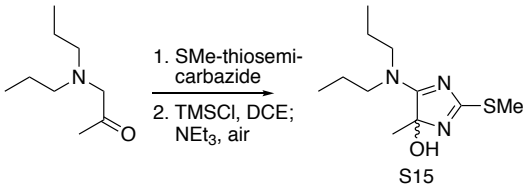
Benzyl (*E*)-2-((3a*R*,8a*S*)-5-((*S*)-5-acetamido-6-(methylamino)-6-oxohexyl)-2,2-dimethylhexahydro-7*H*-[1,3]dioxolo[4,5-*c*]azepin-7-ylidene)hydrazine-1-carbimidothioate (S13)

(*S*)-2-acetamido-6-((3a*R*,8a*S*)-2,2-dimethyl-7-oxohexahydro-5*H*-[1,3]dioxolo[4,5-*c*]azepin-5-yl)-*N*-methylhexanamide (0.5 g, 1.35 mmol) was dissolved in MeOH (13.5 mL, 0.1 M). S-benzylthiosemicarbazide HBr salt (0.355 g, 1.35 mmol) was added at room temperature. The reaction was continued at room temperature for 3 h. When the reaction had completed, 3-heptanone (0.5 mL) was added to react with excess thiosemicarbazide and enable easier

chromatographic purification. The reaction was concentrated, and the crude product was applied to an Isco column (Silica; 4 g; Gradient: 0 to 15% MeOH in CH_2Cl_2). The fractions were combined and concentrated, affording a mixture of isomers (0.431 g, 60.0% yield).
HR-MS: $(M+1)^+$ = 533.2912 (experimental); Exact mass = 533.2905 (theoretical)
IR *ƒ* (cm^{-1}) λ_{max}: 3386, 3267, 2982, 2938, 2670, 1652, 1537, 1454, 1454, 1371, 1243, 1215, 1160, 1036, 747.
[α]D = -0.064º (c = 1.0, $CHCl_3$)

(2*S*)-2-acetamido-6-((6*R*,7*S*)-2-(benzylthio)-6,7,8a-trihydroxy-6,7,8,8a-tetrahydroimidazo[4,5-*b*]azepin-4(5*H*)-yl)-*N*-methylhexanamide (S14)
Thiosemicarbazone (S13, 0.3 g, 0.72 mmol) was dissolved in anhydrous DCE (7.2 mL, 0.1 M). This solution was transferred to a flame-dried pressure vessel. TMSCl (0.46 mL, 3.6 mmol) was added, the pressure vessel was capped, and the reaction was stirred at room temperature for 15 min. The reaction was then heated to 95 ºC and monitored closely by LCMS every few h. When starting material had been consumed, the reaction was cooled to room temperature and water (0.46 mL) was added to quench the TMSCl and deprotect the acetal functionality. After 1.5 h of stirring at rt, the reaction was exposed to atmospheric oxygen and NEt_3 (1 mL) was added to facilitate oxidation. The dark solution was stirred at room temperature for ~12 h until complete oxidation had occurred. The reaction was concentrated and HPLC purified. The product emerged as the main UV-active peak (254 nm). HPLC fractions were collected and lyophilized to obtain the desired product (0.037 g, 10.5% yield).
^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.45 (d, 2H), 7.39-7.28 (m, 3H), 4.68-4.51 (m, 2H), 4.35-4.27 (m, 2H), 4.16-3.99 (m, 3H), 3.68 (dd, 1H), 3.64-3.54 (m, 1H), 3.09 (s + rotamer, 3H), 2.25 (dd, 1H), 2.10 (td, 1H), 2.01-1.94 (m, 3H), 1.92-1.73 (m, 3H), 1.72-1.60 (m, 2H), 1.54-1.28 (m, 3H).
^{13}C-NMR (101 MHz, $CDCl_3$) δ 183.86, 179.44, 173.35, 172.05, 135.62, 128.84, 128.49, 127.80, 91.25, 69.34, 55.31, 53.08, 52.03, 35.43, 33.31, 31.24, 31.15, 25.75, 24.92, 22.50, 21.11.
HR-MS: $(M+1)^+$ = 492.2272 (experimental); Exact mass = 492.2275 (theoretical)
IR *ƒ* (cm^{-1}) λ_{max}: 3391, 2958, 2483, 1647, 1497, 1455, 1350, 1272, 1201, 1138, 1043, 722.
[α]D = -0.309º (c = 1.0, MeOH)

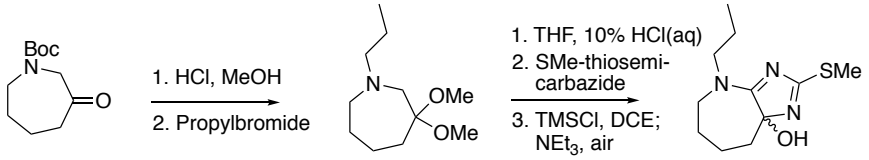
Supplementary Scheme 3. Additional ELISA Substrate Precursors



S15

5-(Dipropylamino)-4-methyl-2-(methylthio)-4*H*-imidazol-4-ol (S15)
N,N-dipropylaminoacetone (0.2 g, 1.27 mmol) was dissolved in MeOH (12.7 mL, 0.1 M). SMe-thiosemicarbazide (0.296 g, 1.27 mmol, 1 equiv) was added at room temperature, and the reaction was stirred for 4 h. The reaction was then concentrated, and the crude mixture was redissolved in CH_2Cl_2. Excess SMe-thiosemicarbazide was removed using a short pad of silica gel. The silica pad was washed briefly with CH_2Cl_2. The resulting solution was concentrated, yielding crude hydrazone. The crude material was dissolved in anhydrous DCE (4.23 mL, 0.3 M). TMSCl (0.8 mL, 6.35 mmol) was added at room temperature and stirred for 10 min. The reaction was heated, causing a slow color change to red-brown. The reaction was monitored by LCMS. After approximately 6 h, complete conversion of the starting hydrazone was observed. To the

crude solution was added MeOH (~10 mL) and NEt_3 (~4 mL) to facilitate oxidation over approximately 12 h. The dark brown suspension was then concentrated, taken up in MeOH, and purified by HPLC (0 to 50% CH_3CN(0.1% TFA) in H_2O(0.1% TFA)). The fractions were collected and lyophilized, resulting in a light brown oil (0.011 g, 3.55% yield).
^{1}H-NMR (400 MHz, CD_3OD) δ 3.84-3.62 (m, 4H), 2.70 (s, 3H), 1.89-1.72 (m, 4H), 1.80 (s, 3H), 0.99 (dt, 6H).
^{13}C-NMR (101 MHz, CD_3OD) δ 184.90, 180.41, 93.95, 53.31, 51.86, 24.03, 23.20, 21.23, 14.06, 11.35, 10.97.
HR-MS: $(M+1)^+$ = 244.1480 (experimental); Exact mass = 244.1478 (theoretical)
IR *f* (cm^{-1}) λ_{max}: 3104.8, 2970.6, 2880.1, 1674.4, 1630.4, 1515.5, 1428.7, 1384.8, 1340.8, 1306.1, 1273.2, 1199.1, 1132.5, 939.2, 799.5, 719.0.

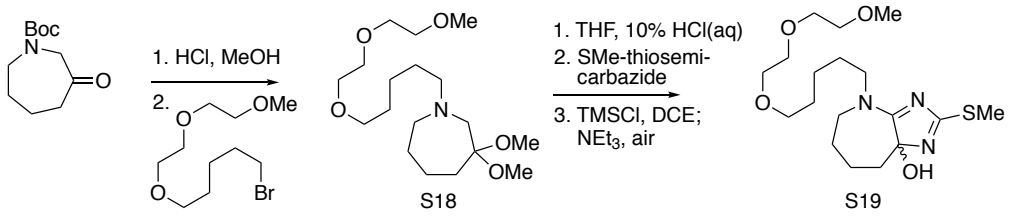


S16 S17

3,3-Dimethoxy-1-propylazepane (S16)
tert-Butyl 3-oxoazepane-1-carboxylate (1 g, 4.69 mmol) was dissolved in anhydrous MeOH (23.4 mL, 0.2 M) and 4M HCl in dioxanes (2 mL, 7.79 mmol, 1.7 equiv) was added at room temperature. The solution was heated to 55 C and monitored by LCMS. Upon complete conversion of starting material to product, triethylamine (3.3 mL, 5 equiv) was added along with 1-bromopropane (0.85 mL, 9.38 mmol, 2 equiv). The reaction was stirred at 55 C overnight. The reaction was concentrated, and the crude product was purified via column chromatography (Silica, 24g, Gradient: 0 to 70% EtOAc in Hexanes).
^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.16 (s, 6H), 2.73 (s, 2H), 2.64 (t, 2H), 2.51 (m, 2H), 1.82 (m, 2H), 1.64 (p, 2H), 1.56-1.42 (m, 1H), 0.84 (t, 3H).
^{13}C-NMR (101 MHz, $CDCl_3$) δ 103.86, 61.80, 61.64, 57.80, 48.19, 35.23, 30.31, 21.53, 19.83, 11.88.
HR-MS: $(M+1)^+$ = 202.1795 (experimental); Exact mass = 202.1802 (theoretical)
IR *f* (cm^{-1}) λ_{max}: 2932.6, 2871.4, 2828.0, 1463.4, 1096.6, 1048.7, 996.0.

2-(Methylthio)-4-propyl-5,6,7,8-tetrahydroimidazo[4,5-*b*]azepin-8a(4*H*)-ol (S17)
Dimethyl acetal (S16, 0.25 g, 0.719 mmol) was dissolved in THF (4 mL) and 2M HCl(aq) (4 mL). The reaction was heated for 1 h at 50 C. After this period, complete conversion to the aminoketone was observed by LCMS. The reaction was concentrated and dissolved in MeOH (~7 mL, 0.1 M). SMe-thiosemicarbazide (0.168 g, 0.719 mmol, 1 equiv) was added at room temperature, and the reaction was stirred for 4 h. The reaction was then concentrated, and the crude mixture was redissolved in CH_2Cl_2. Excess SMe-thiosemicarbazide was removed using a short pad of silica gel. The silica pad was washed briefly with CH_2Cl_2. The resulting solution was concentrated, yielding crude hydrazone. The crude material was dissolved in anhydrous DCE (5 mL, 0.145 M). TMSCl (0.46 mL, 3.60 mmol, ~5 equiv) was added at room temperature and stirred for 10 min. The reaction was heated, causing a slow color change to red-brown. The reaction was monitored by LCMS. After approximately 6 h, complete conversion of the starting hydrazone was observed. To the crude solution was added MeOH (~5 mL) and NEt_3 (~2 mL) to facilitate oxidation over approximately 12 h. The dark brown solution was then concentrated, taken up in MeOH, and purified by HPLC (0 to 50% CH_3CN (0.1% TFA) in H_2O (0.1% TFA). The fractions were collected and lyophilized, resulting in a light brown oil (0.0098 g, 3.27% yield).

^{1}H-NMR (400 MHz, CD_3OD) δ 4.44 (dd, 1H), 3.83-3.66 (m, 2H), 3.49 (dd, 1H), 2.70 (s, 3H), 2.28-1.66 (m, 8H), 0.99 (t, 3H),.
^{13}C-NMR (101 MHz, CD_3OD) δ 186.52, 182.28, 95.40, 56.92, 52.46, 33.65, 29.50, 25.98, 21.39, 14.13, 11.36.
HR-MS: $(M+1)^+$ = 242.1310 (experimental); Exact mass = 242.1322 (theoretical)
IR *f* (cm^{-1}) λ_{max}: 3119.6, 2964.0, 1677.8, 1646.0, 1512.0, 1363.8, 1342.8, 1272.9, 1201.3, 1135.0, 800.3, 721.9.



3,3-Dimethoxy-1-(5-(2-(2-methoxyethoxy)ethoxy)pentyl)azepane (S18)
tert-Butyl 3-oxoazepane-1-carboxylate (1 g, 4.69 mmol) was dissolved in anhydrous MeOH (23.4 mL, 0.2 M) and 4M HCl in dioxanes (2 mL, 7.79 mmol, 1.7 equiv) was added at room temperature. The solution was heated to 55 C and monitored by LCMS. Upon complete conversion of starting material to product, triethylamine (3.3 mL, 5 equiv) was added along with 1-bromo-5-(2-(2-methoxyethoxy)ethoxy)pentane (1.89 g, 7.03 mmol, 1.5 equiv). The reaction was stirred at 55 C overnight. The reaction was concentrated, and the crude product was purified via column chromatography (Silica, 24g, Gradient: 0 to 100% EtOAc in Hexanes with several column volumes eluted with 100% EtOAc). The product was isolated as an oil (0.77 g, 47.3% yield).
^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.65-3.59 (m, 4H), 3.58-3.50 (m, 4H), 3.42 (t, 2H), 3.34 (s, 3H), 3.15 (s, 6H), 2.71 (s, 2H), 2.63 (bt, 2H), 2.53 (t, 2H), 1.80 (dd, 2H), 1.66-1.40 (m, 8H), 1.28 (p, 2H).
^{13}C-NMR (101 MHz, $CDCl_3$) δ 103.77, 72.03, 71.47, 70.73, 70.61, 70.14, 61.59, 59.62, 59.10, 57.71, 48.16, 35.14, 30.17, 29.61, 26.50, 24.00, 21.49.
HR-MS: $(M+1)^+$ = 348.2734 (experimental); Exact mass = 348.2744 (theoretical)
IR *f* (cm^{-1}) λ_{max}: 2934.7, 2861.2, 2362.2, 2337.7, 1455.6, 1351.4, 1107.7, 1047.4, 995.3, 847.6, 796.6.

4-(5-(2-(2-Methoxyethoxy)ethoxy)pentyl)-2-(methylthio)-5,6,7,8-tetrahydroimidazo[4,5-*b*]azepin-8a(4*H*)-ol (S19)
Dimethyl acetal (S18, 0.77 g, 2.21 mmol) was dissolved in THF (11.1 mL, 0.2 M) and 2M HCl(aq) (11.1 mL, 0.2 M). The reaction was heated for 1 h at 50 C. After this period, complete conversion to the aminoketone was observed by LCMS. The reaction was concentrated and dissolved in MeOH (~22 mL). SMe-thiosemicarbazide (0.514 g, 2.21 mmol, ~1 equiv) was added at room temperature, and the reaction was stirred for 4 h. The reaction was then concentrated, and the crude mixture was redissolved in CH_2Cl_2. Excess SMe-thiosemicarbazide was removed using a short pad of silica gel. The silica pad was washed briefly with CH_2Cl_2. The resulting solution was concentrated, yielding crude hydrazone. The crude material was dissolved in anhydrous DCE (11 mL, 0.2 M). TMSCl (1.4 mL, 11.1 mmol, ~5 equiv) was added at room temperature and stirred for 10 min. The reaction was heated, causing a slow color change to red-brown. The reaction was monitored by LCMS. After approximately 6 h, complete conversion of the starting hydrazone was observed. To the crude solution was added MeOH (~10 mL) and NEt_3 (~4 mL) to facilitate oxidation over approximately 12 h. The dark brown suspension was then concentrated, taken up in MeOH, and purified by HPLC (0 to 50% CH_3CN(0.1% TFA) in H_2O(0.1% TFA). The fractions were collected and lyophilized, resulting in a light brown oil (0.047 g, 5.47% yield).

^{1}H-NMR (400 MHz, CD_3OD) δ 4.43 (dd, 1H), 3.79 (m, 2H), 3.64-3.46 (m, 11H), 3.36 (s, 3H), 3.15 (s, 6H), 2.70 (s, 3H), 2.25-1.60 (m, 10H), 1.44 (p, 2H).
^{13}C-NMR (101 MHz, CD_3OD) δ 186.47, 182.21, 95.39, 72.97, 71.90, 71.57, 71.36, 71.18, 59.09, 55.12, 52.37, 33.64, 30.14, 29.51, 27.66, 25.98, 24.15, 14.19.
HR-MS: $(M+1)^+$ = 388.2263 (experimental); Exact mass = 388.2265 (theoretical)
IR *ƒ* (cm^{-1}) λ_{max}: 3110.4, 2940.5, 1680.3, 1435.6, 1365.6, 1347.1, 1287.5, 1205.1, 1136.0, 842.2, 800.9, 723.5.

Peptide synthesis

Coupling Procedure

Chemicals were used as received. Pre-loaded Fmoc-Wang resins for SPPS were purchased from Novabiochem. Fmoc-Lys(Biotin)-OH was purchased from Anaspec. Fmoc-15-amino-4,7,10,13-tetraoxapentadecacanoic acid (Fmoc-NH-dPEG$_4$-CO_2H) was purchased from ChemPep. MG-H building blocks were synthesized according to previously published procedures.[1]
SPPS was performed by hand. The following procedure was used: Resin (0.1 mmol) was swelled in 3 mL DMF in a Bio-Rad Poly-Prep chromatography tube for 10 min. The resin was deprotected by adding 2 mL of 20% piperidine in DMF and rotating for 5 min at room temperature. This step was repeated, followed by washing of the resin with DMF (x2), CH_2Cl_2 (x2), and DMF (x2) again. Amino acids were coupled by adding 0.5 mmol (5 eq.) protected amino acid, 0.49 mmol (4.9 eq) HBTU, 1.0 mmol (10 eq) DIPEA, and 3 mL DMF to the resin and rotating for 2 h or overnight at room temperature. This step was repeated. Following the second coupling, the resin was washed with DMF (x2), CH_2Cl_2 (x2), and DMF (x2) again before 2 mL of capping solution (10% Ac_2O and 10% NMM in DMF) was added and the tube rotated for 10 min at room temperature. The resin was washed with DMF, CH_2Cl_2, and DMF again, then deprotected in the way described above. These steps were iterated until the peptide was completed.

General Procedure for Glucosepane/Glucosepane Analog Peptides:

For glucosepane-modified/glucosepane analog-modified peptides, the peptide sequence was synthesized as reported above. A 4-methyltrityl-protected ornithine (Orn(Mtt)) was coupled in the position that the glucosepane modification would eventually be located. The 4-methyltrityl protecting group was selectively removed with 1% TFA in CH_2Cl_2 (two subsequent 30 min incubations). The resin was washed thoroughly with CH_2Cl_2. The resin was then moved to a roundbottomed flask. S-Methyl imidazole (20 mg) was dissolved in MeCN (0.05 M) with diisopropylethylamine (5 equiv). The reaction was stirred at 50 ºC for 4 h. The resin was then transferred to a new Bio-Rad Poly-Prep chromatography tube. The resin was washed with DMF (x2), CH_2Cl_2 (x4). The resin was suspended in $CHCl_3$ and a large excess of sodium triacetoxyborohydride (~20 equiv) was added. This white suspension was stirred overnight. The tube should be vented occasionally to prevent excessive pressure buildup. The resin was then washed with methanol (x2), CH_2Cl_2 (x2), DMF (x2), and CH_2Cl_2 (x2), before being vacuum dried thoroughly for cleavage.

General Procedure for HydroxyGlucosepane Analog Peptides:

For hydroxyglucosepane-modified peptides, the peptide sequence was synthesized as reported above. A 4-methyltrityl-protected ornithine (Orn(Mtt)) was coupled in the position that the glucosepane modification would eventually be located. The 4-methyltrityl protecting group was selectively removed with 1% TFA in CH_2Cl_2 (two subsequent 30 min incubations). The resin was washed thoroughly with CH_2Cl_2. The resin was then moved to a roundbottomed flask. S-Methyl imidazole (20 mg) was dissolved in MeCN (0.05 M) with diisopropylethylamine (5 equiv). The reaction was stirred at 50 ºC for 4 h. The resin was then transferred to a new Bio-Rad Poly-Prep

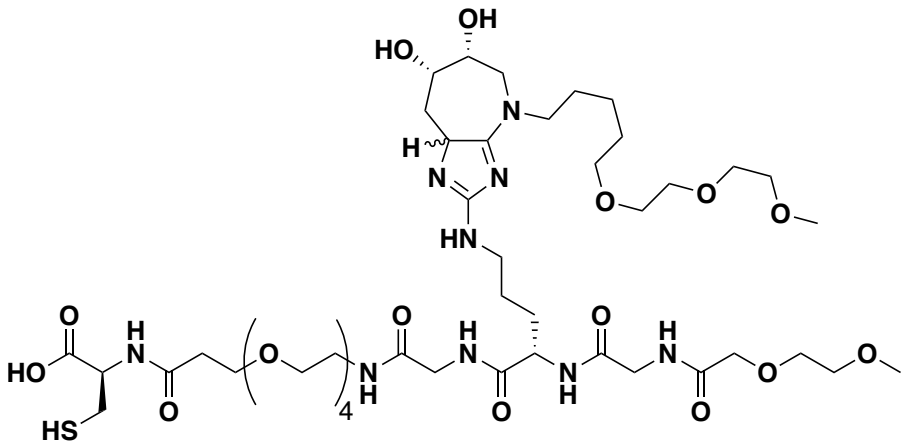
chromatography tube. The resin was washed with DMF (x2) and CH_2Cl_2 (x4) before being vacuum dried thoroughly for cleavage.

Cleavage

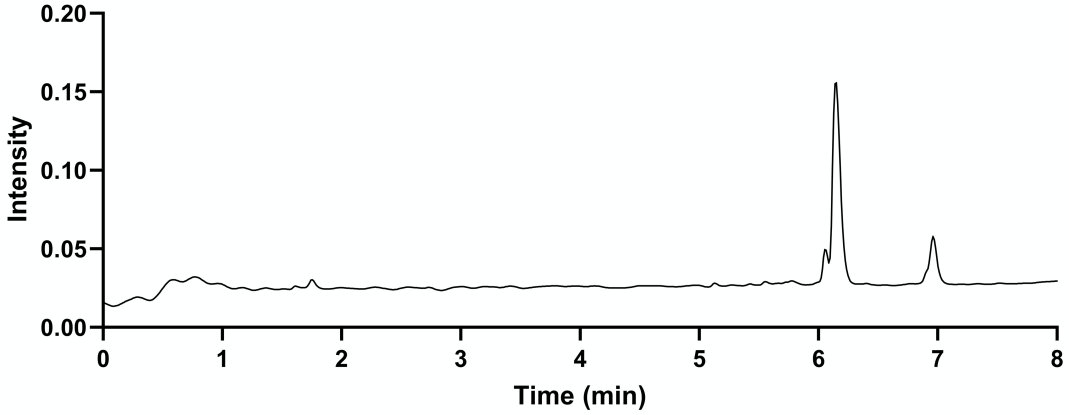
After drying, the resin was added to a round bottom flask containing cleavage cocktail (9.6 mL TFA, 0.4 mL TIPS, 0.4 mL H_2O) and stirred for the time specified below. Subsequently, the cleavage mixture was filtered through a cotton-plugged pipet and concentrated under nitrogen flow or precipitated in cold ether. The resulting residue was taken up in MeCN/H_2O solution with minimal MeCN and purified using reverse-phase HPLC in portions. The fractions were combined and freeze-dried to give the corresponding peptides.

The solvent compositions reported for all chromatographic separations are on a volume/volume (v/v) basis. Preparatory HPLC was performed with a SunFire Prep C18 OBD 10 mm 19x150 mm reverse-phase column as the stationary phase. H_2O and MeCN both buffered with either 0.1% TFA or 0.1% formic acid were used as the mobile phase. The desired HPLC fractions were combined and lyophilized to give the corresponding peptide as a TFA or formate salt. The molecular weight of the peptides was determined by High Resolution Mass Spectrometry (HR-MS), which was performed at the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

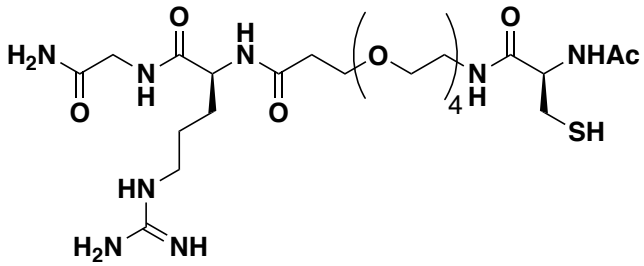
PEG_2-G-(Glucosepane(PEG))-G-dPEG_4-C (1)



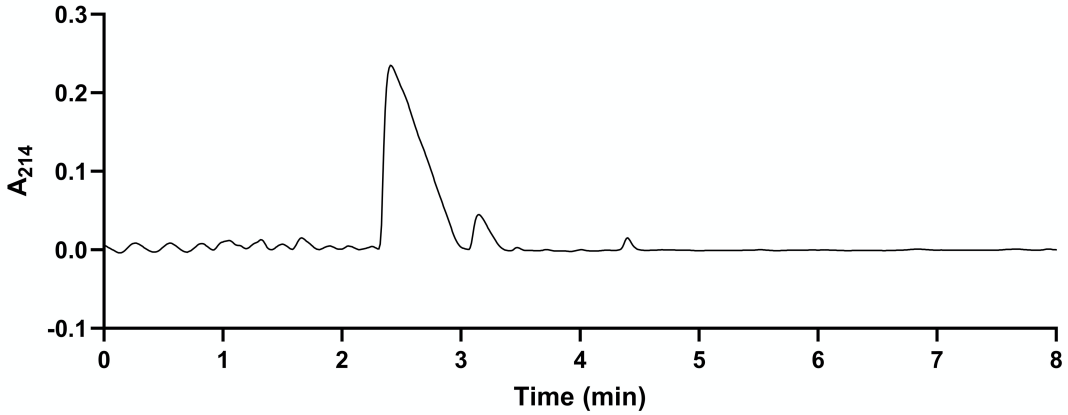
SPPS was performed by hand, starting with Fmoc-Cys(Trt)-Wang resin (100-200 mesh). After completing the peptide sequence with the general coupling procedure, the general procedure for glucosepane/glucosepane analog peptides was followed. Upon completion, the resin was treated with cleavage cocktail for 3 h. The peptide was precipitated in 10 mL of cold ether per 1 mL cleavage cocktail. HPLC conditions: 0% MeCN for 1 min, followed by 0 to 27% MeCN over 44 min, followed by 100% MeCN for 3 min, and finally 0% MeCN for 3 min. Analytical HPLC: R_t 6.1 min (5-30% B over 8 min, 0.2 mL min^{-1}, 0.1% TFA, λ = 214 nm. A small amount of disulfide with R_t 7.0 min was observed). HR-MS: (M+1)+ = 1068.5579; Exact mass = 1068.5493 (theoretical).



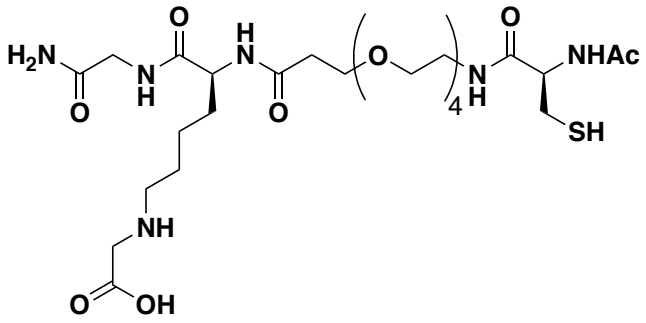
AcHN-C-dPEG$_4$-R-G (5)



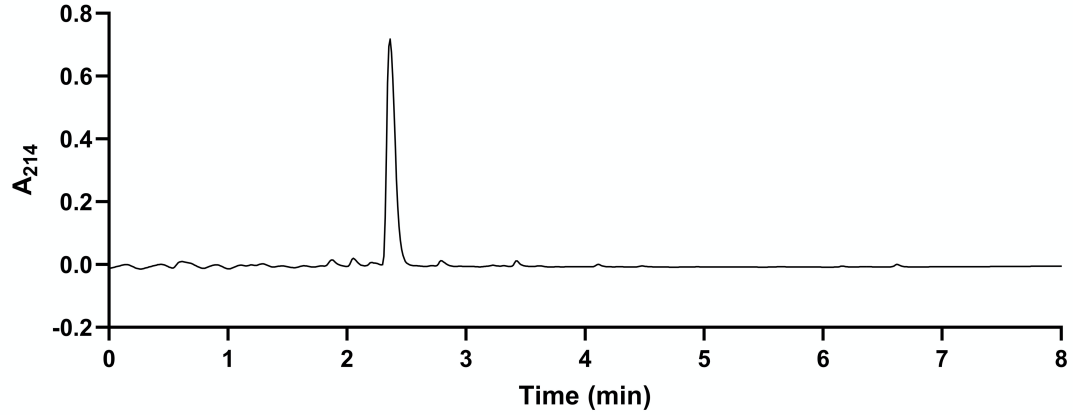
SPPS was performed using the general coupling procedure, starting with Rink Amide resin (100-200 mesh). Upon completion, the resin was treated with cleavage cocktail for 3 h. The peptide was precipitated in 10 mL of cold ether per 1 mL cleavage cocktail. HPLC conditions: 0% MeCN for 1 min , followed by 0 to 40% MeCN over 12 min, followed by 100% MeCN for 3 min, and finally 0% MeCN for 3 min. Analytical HPLC: R_t 2.4 min (5-20% B over 8 min, 0.2 mL min^{-1}, 0.1% TFA, λ = 214 nm). HR-MS: (M+1)+ = 623.3180 (experimental); Exact mass = 623.3181 (theoretical).



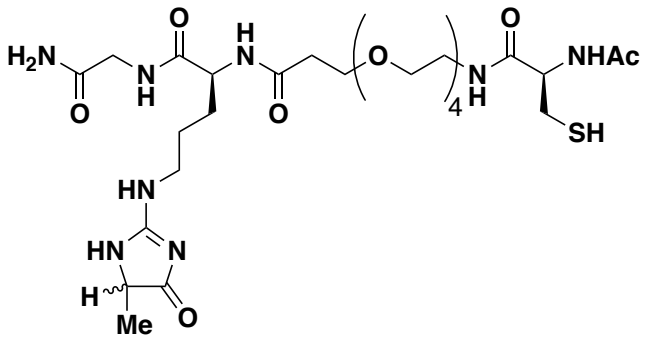
AcHN-C-dPEG$_4$-(CML)-G (6)



SPPS was performed using the general coupling procedure, starting with Rink Amide resin (100-200 mesh). The CML modification was installed as previously reported.[4] Upon completion, the resin was treated with cleavage cocktail for 3 h. The peptide was precipitated in 10 mL of cold ether per 1 mL cleavage cocktail. HPLC conditions: 0% MeCN for 1 min , followed by 0 to 15% MeCN over 39 min, followed by 100% MeCN for 3 min, and finally 0% MeCN for 3 min. Analytical HPLC: R_t 2.3 min (5-20% B over 8 min, 0.2 mL min^{-1}, 0.1% TFA, λ = 214 nm). HR-MS: (M+1)+ = 653.3171 (experimental); Exact mass = 653.3175 (theoretical).

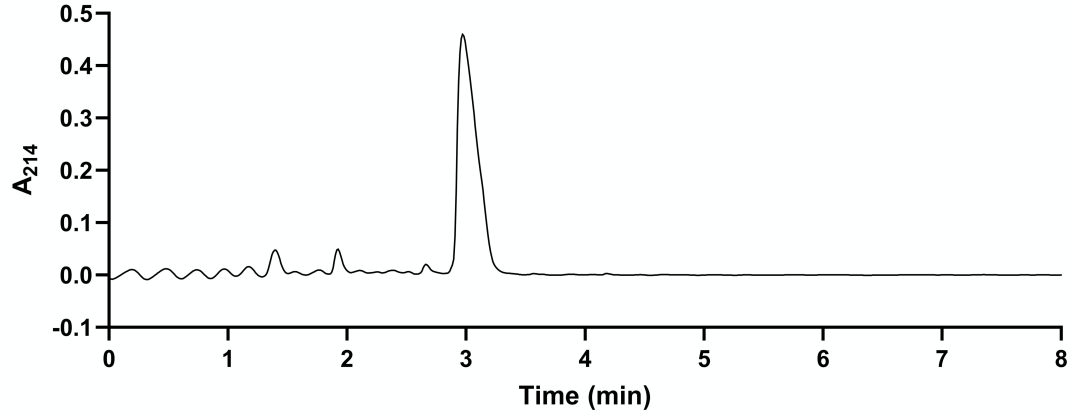


AcHN-C-dPEG$_4$-(MG-H1)-G (7)

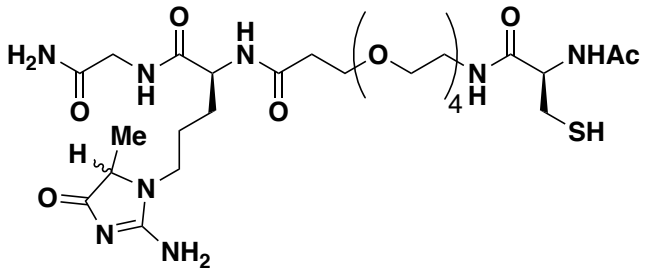


SPPS was performed using the general coupling procedure, starting with Rink Amide resin (100-200 mesh). MG-H1 monomer was synthesized and used as previously reported.[3] Upon completion, the resin was treated with cleavage cocktail for 3 h. The peptide was precipitated in 10 mL of cold ether per 1 mL cleavage cocktail. HPLC conditions: 0% MeCN for 1 min , followed

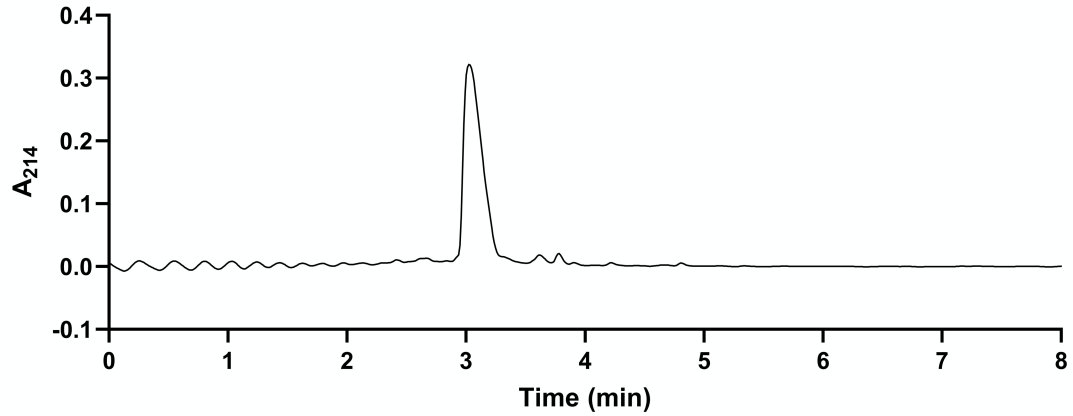
by 0 to 20% MeCN over 24 min, followed by 100% MeCN for 3 min, and finally 0% MeCN for 3 min. Analytical HPLC: R_t 3.5 min (5-25% B over 8 min, 0.2 mL min^{-1}, 0.1% TFA, λ = 214 nm). HR-MS: (M+1)+ = 677.3288 (experimental); Exact mass = 677.3287 (theoretical).



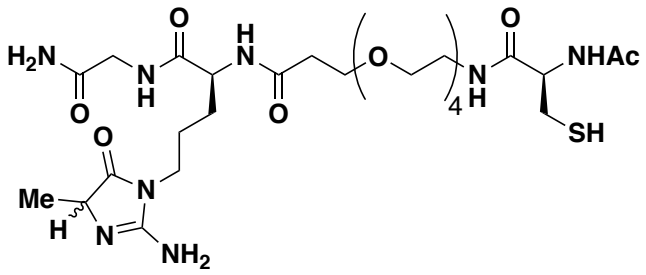
AcHN-C-dPEG$_4$-(MG-H2)-G (8)



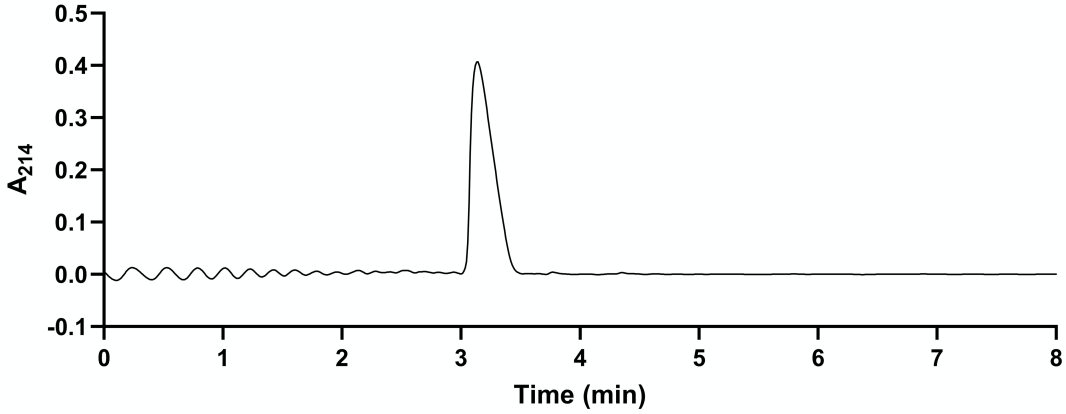
SPPS was performed using the general coupling procedure, starting with Rink Amide resin (100-200 mesh). MG-H2 monomer was synthesized and used as previously reported.[3] Upon completion, the resin was treated with cleavage cocktail for 3 h. The peptide was precipitated in 10 mL of cold ether per 1 mL cleavage cocktail. HPLC conditions: 0% MeCN for 1 min , followed by 0 to 20% MeCN over 24 min, followed by 100% MeCN for 3 min, and finally 0% MeCN for 3 min. Analytical HPLC: R_t 3.5 min (5-25% B over 8 min, 0.2 mL min^{-1}, 0.1% TFA, λ = 214 nm). HR-MS: (M+1)+ = 677.3279 (experimental); Exact mass = 677.3287 (theoretical).



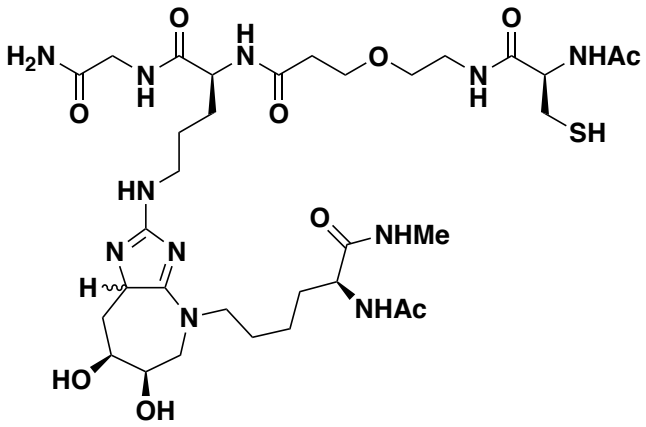
AcHN-C-dPEG$_4$-(MG-H3)-G (9)



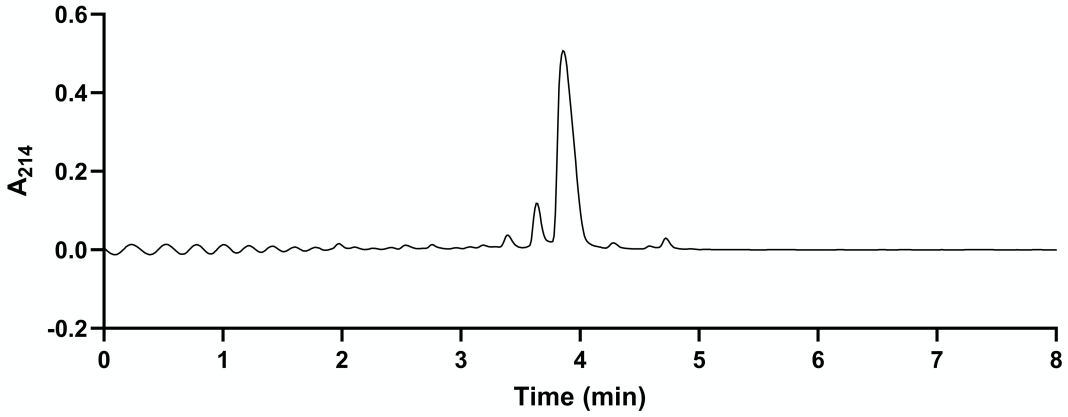
SPPS was performed using the general coupling procedure, starting with Rink Amide resin (100-200 mesh). MG-H3 monomer was synthesized and used as previously reported.[3] Upon completion, the resin was treated with cleavage cocktail for 3 h. The peptide was precipitated in 10 mL of cold ether per 1 mL cleavage cocktail. HPLC conditions: 0% MeCN for 1 min , followed by 0 to 20% MeCN over 24 min, followed by 100% MeCN for 3 min, and finally 0% MeCN for 3 min. Analytical HPLC: R_t 3.5 min (5-25% B over 8 min, 0.2 mL min^{-1}, 0.1% TFA, λ = 214 nm). HR-MS: (M+1)+ = 677.3282 (experimental); Exact mass = 677.3287 (theoretical).



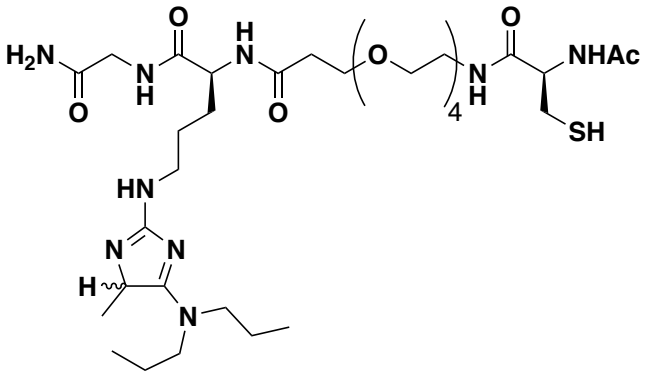
AcHN-C-dPEG$_4$-(Glucosepane(BackboneMimetic))-G (10)



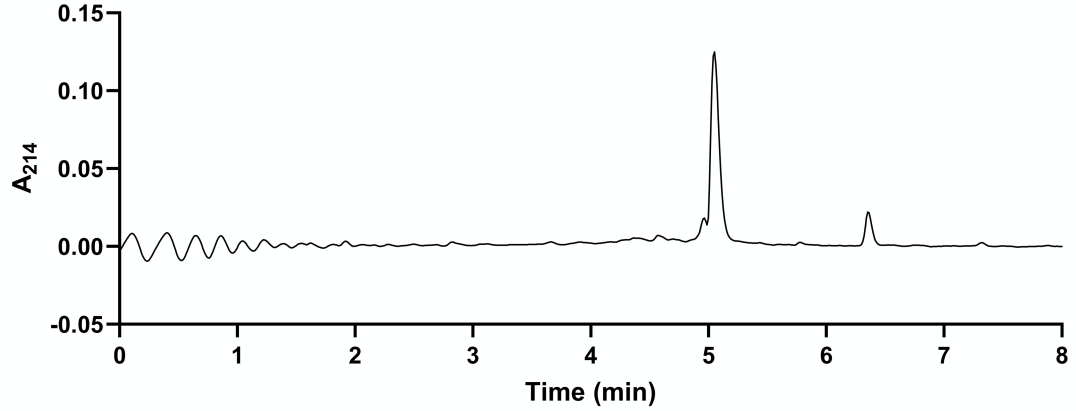
SPPS was performed by hand, starting with Rink Amide resin (100-200 mesh). After completing the peptide sequence with the general coupling procedure, the general procedure for glucosepane/glucosepane analog peptides was followed. Upon completion, the resin was treated with cleavage cocktail for 3 h. The peptide was precipitated in 10 mL of cold ether per 1 mL cleavage cocktail. HPLC conditions: 0% MeCN for 1 min , followed by 0 to 20% MeCN over 42 min, followed by 100% MeCN for 3 min, and finally 0% MeCN for 3 min. Analytical HPLC: R_t 4.4 min (5-25% B over 8 min, 0.2 mL min^{-1}, 0.1% TFA, λ = 214 nm). HR-MS: (M+1)+ = 932.4870; Exact mass = 932.4870 (theoretical).



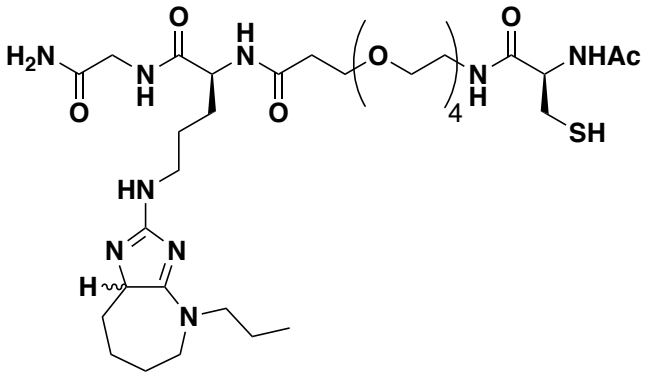
AcHN-C-dPEG$_4$-(Analog1)-G (S20)



SPPS was performed by hand, starting with Rink Amide resin (100-200 mesh). After completing the peptide sequence with the general coupling procedure, the general procedure for glucosepane/glucosepane analog peptides was followed. Upon completion, the resin was treated with cleavage cocktail for 3 h. The peptide was precipitated in 10 mL of cold ether per 1 mL cleavage cocktail. HPLC conditions: 0% MeCN for 1 min , followed by 0 to 50% MeCN over 33 min, followed by 100% MeCN for 3 min, and finally 0% MeCN for 3 min. Analytical HPLC: R_t 5.1 min (5-40% B over 8 min, 0.2 mL min^{-1}, 0.1% TFA, λ = 214 nm.) HR-MS: (M+1)+ = 760.4393 (experimental); Exact mass = 760.4386 (theoretical).

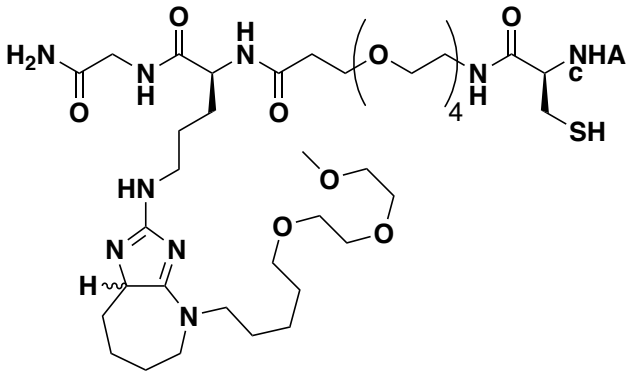


AcHN-C-dPEG$_4$-(Analog2)-G (S21)

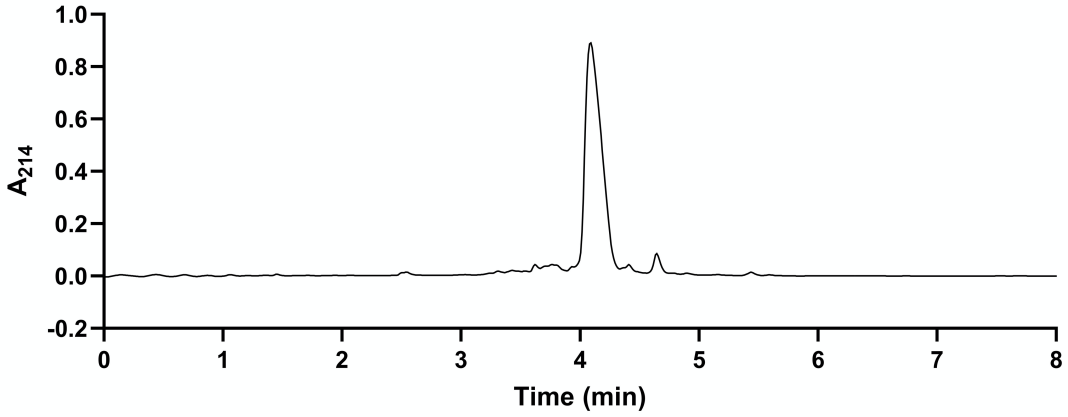


SPPS was performed by hand, starting with Rink Amide resin (100-200 mesh). After completing the peptide sequence with the general coupling procedure, the general procedure for glucosepane/glucosepane analog peptides was followed. Upon completion, the resin was treated with cleavage cocktail for 3 h. The peptide was precipitated in 10 mL of cold ether per 1 mL cleavage cocktail. HPLC conditions: 0% MeCN for 1 min , followed by 0 to 50% MeCN over 33 min, followed by 100% MeCN for 3 min, and finally 0% MeCN for 3 min. HR-MS: (M+1)+ = 758.4220 (experimental); Exact mass = 758.4229 (theoretical).

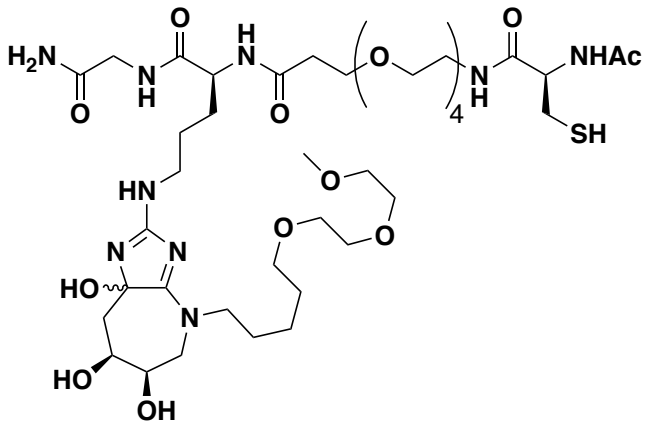
AcHN-C-dPEG$_4$-(Analog3)-G (S22)



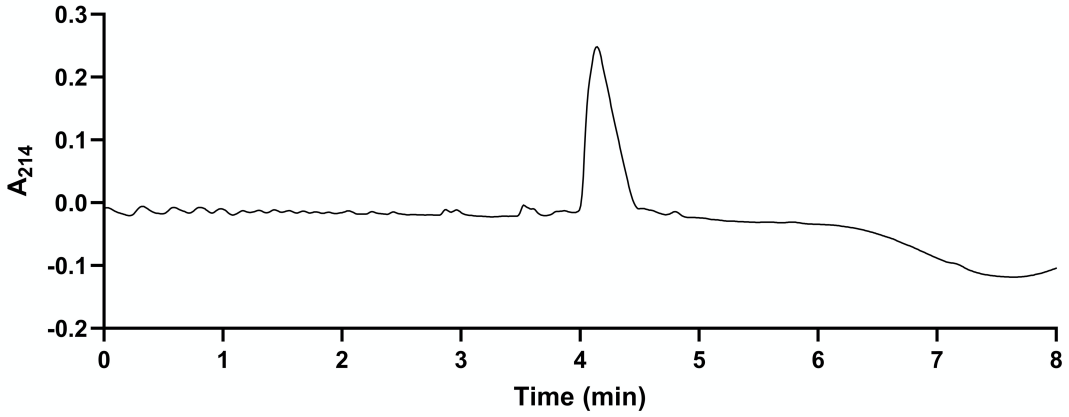
SPPS was performed by hand, starting with Rink Amide resin (100-200 mesh). After completing the peptide sequence with the general coupling procedure, the general procedure for glucosepane/glucosepane analog peptides was followed. Upon completion, the resin was treated with cleavage cocktail for 3 h. The peptide was precipitated in 10 mL of cold ether per 1 mL cleavage cocktail. HPLC conditions: 0% MeCN for 1 min , followed by 0 to 50% MeCN over 33 min, followed by 100% MeCN for 3 min, and finally 0% MeCN for 3 min. Analytical HPLC: R_t 4.1 min (5-50% B over 8 min, 0.2 mL min^{-1}, 0.1% TFA, λ = 214 nm). HR-MS: (M+1)+ = 904.5176 (experimental); Exact mass = 904.5172 (theoretical).



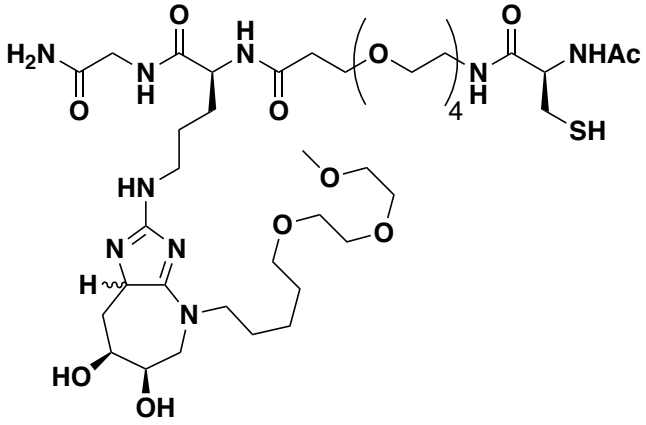
AcHN-C-dPEG$_4$-(HydroxyGlucosepane(PEG))-G (S23)



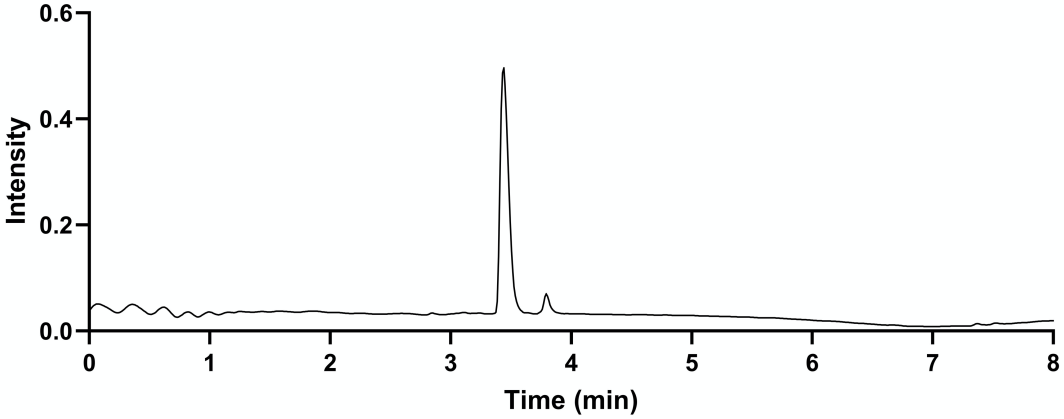
SPPS was performed by hand, starting with Rink Amide resin (100-200 mesh). After completing the peptide sequence with the general coupling procedure, the general procedure for hydroxyglucosepane/glucosepane analog peptides was followed. Upon completion, the resin was treated with cleavage cocktail for 3 h. The peptide was precipitated in 10 mL of cold ether per 1 mL cleavage cocktail. HPLC conditions: 0% MeCN for 1 min , followed by 0 to 30% MeCN over 34 min, followed by 100% MeCN for 3 min, and finally 0% MeCN for 3 min. Analytical HPLC: R_t 4.1 min (5-50% MeOH over 8 min, 0.2 mL min^{-1}, 0.1% TFA, λ = 214 nm). HR-MS: (M+1)+ = 952.5030; Exact mass = 952.5020 (theoretical).



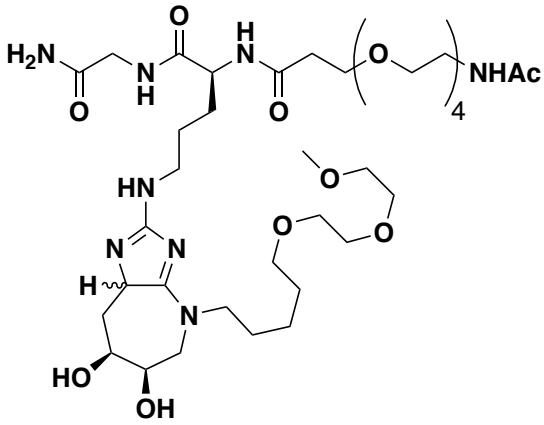
AcHN-C-dPEG$_4$-(Glucosepane(PEG))-G (S24)



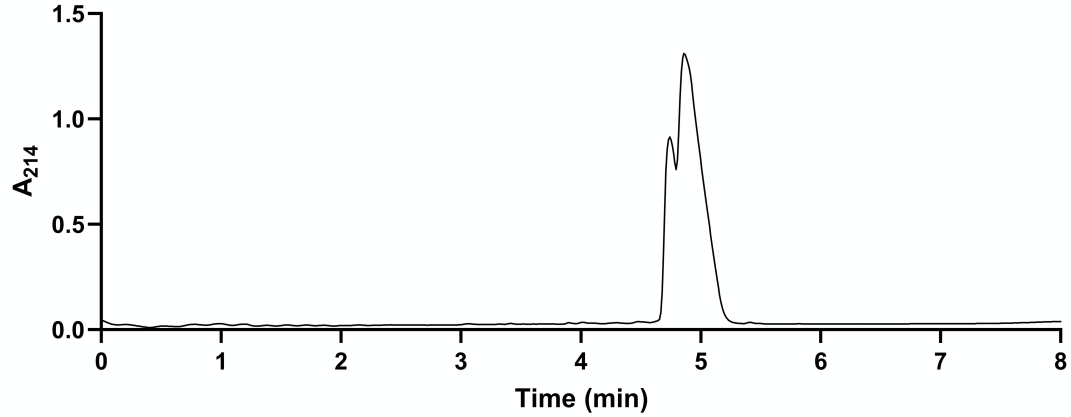
SPPS was performed by hand, starting with Rink Amide resin (100-200 mesh). After completing the peptide sequence with the general coupling procedure, the general procedure for glucosepane/glucosepane analog peptides was followed. Upon completion, the resin was treated with cleavage cocktail for 3 h. The peptide was precipitated in 10 mL of cold ether per 1 mL cleavage cocktail. HPLC conditions: 0% MeCN for 1 min , followed by 0 to 30% MeCN over 34 min, followed by 100% MeCN for 3 min, and finally 0% MeCN for 3 min. Analytical HPLC: R_t 3.4 min (5-50% B over 8 min, 0.2 mL min^{-1}, 0.1% TFA, λ = 214 nm). HR-MS: (M+1)+ = 936.5063; Exact mass = 936.5070 (theoretical).



AcHN-dPEG$_4$-(Glucosepane(PEG))-G (S25)



SPPS was performed by hand, starting with Fmoc-Cys(Trt)-Wang resin (100-200 mesh). After completing the peptide sequence with the general coupling procedure, the general procedure for glucosepane/glucosepane analog peptides was followed. Upon completion, the resin was treated with cleavage cocktail for 3 h. The peptide was precipitated in 10 mL of cold ether per 1 mL cleavage cocktail. HPLC conditions: 0% MeCN for 1 min , followed by 0 to 27% MeCN over 44 min, followed by 100% MeCN for 3 min, and finally 0% MeCN for 3 min. Analytical HPLC: R_t 4.9 min (5-30% B over 8 min, 0.2 mL min^{-1}, 0.1% TFA, λ = 214 nm). HR-MS: (M+1)+ = 833.5074; Exact mass = 832.4909 (theoretical).



Immunogen Preparation and Immunization Procedures

Preparation of Immunogen: Peptides were conjugated to Sulfo-SMCC (Thermo/Pierce # 22322) activated KLH (Thermo/Pierce # 77600) through the side chain of Cysteine via sulfhydryl chemistry in PBS, then emulsified 1:1 (volume) with FCA/IFA.

Immunization Protocol: 200-400µg of immunogen was administered subcutaneously per immunization. The initial immunizations were scheduled as follows:

Day 0 Pre-immune + Boost (400 µg immunogen with CFA)

Day 14 Boost (200 µg immunogen with IFA)

Day 28 Boost (200 µg immunogen with IFA)

Day 35 Production Bleed #1 (~20 mL antiserum per rabbit)

Day 40 Production Bleed #2 (~20 mL antiserum per rabbit)

The animals were subsequently boosted (200 µg immunogen with IFA) and bleed twice on a monthly basis thereafter for the next 12 months in order to generate the material for all required experiments.

Animals: Two female Specific Pathogen Free New Zealand White rabbits (12-16 weeks old) were immunized, with animal protocols approved by New England Peptide's Animal Care and Use committee.

Preparation of Affinity Column: The affinity column was made by coupling 4mg peptide to 2mL SulfoLink resin (Thermo/Pierce # 20402). Briefly, SulfoLink resin was washed with Tris-EDTA Buffer, then incubated with solubilized peptide for one hour. The resin was washed with Tris-EDTA Buffer and any remaining available coupling sites blocked by one hour incubation with Tris-EDTA/Cys Buffer. After two further wash steps the column (ready to bind antibody) was stored at 4°C until needed.

Modification-Specific Affinity Purification of Antibodies: Antisera was incubated with the non-modified peptide column for at least three hours with constant mixing. The pre-absorbed material was then allowed to flow-through the bottom of the column and collected. This was then incubated with the modified peptide column for at least three hours with constant mixing. After this the modified column was washed with Salt Buffer to remove non-specific material. Antibodies were eluted off the column with low pH Glycine Buffer, neutralized to pH 7.4, and dialyzed overnight in PBS.

ELISA:

Target	Bleed	Animal	Titer
Glucosepane	1/25/2016	I9778	1,837,100
	1/25/2016	I9779	1,734,500

ELISA Experiments

Synthetic Peptide ELISA

Phosphate buffered saline (pH 7.4) solutions of thiol-containing peptides (100 µL, 20 µM) were added to Sulfhydryl-BIND 96-well plates (Corning) and incubated for 1 hr RT. The wells were washed three times with PBST (0.1% tween-20) and blocked with cysteine HCl solution in PBS (300 µL, 10 µg mL^{-1}) for 1 hr. The wells were again washed three times with PBST. Anti-glucosepane antibody was added to the wells, starting with 100 µg/mL in PBST with 5-fold dilutions down each row and the last well containing 0 µg mL^{-1}. The primary antibody was incubated at RT for 1 hr and then wells were washed 3 times with PBST. (For competitive ELISAs, various concentrations of soluble competitor were co-incubated with 20 µg mL^{-1} antibody for 1 hr prior to addition to wells.) Goat anti-rabbit HRP secondary antibody (Thermo) was diluted 1:5000 in PBS (100 µL per well) and incubated at RT for 40 min. The wells were washed 5 times with PBST. TMB substrate was added to each well (100 µL per well) and allowed to develop for approximately 1 min. The reaction was quenched with 2 M H_2SO_4 (100 µL per well). The absorbance was measured at 450 nm. Data was analyzed using GraphPad Prism and curves were fit to non-linear regression (Sigmoidal, 4PL).

Protein ELISA

Carbonate buffer (pH 9.6) solutions of HSA or glycated HSA (100 µL, 200 µM) were added to Nunc MaxiSorp 96-well plates (Thermo) and incubated overnight at 4 °C. The wells were washed three times with PBST (0.1% tween-20) and blocked with 3% HSA in PBS overnight at 4 °C. The wells were again washed three times with wash buffer. Anti-glucosepane antibody was added to the wells (200 µg mL^{-1}), incubated at RT for 1 hr, and then the wells were washed 5 times with PBST. Goat anti-rabbit HRP secondary antibody (Thermo) was diluted 1:5000 in PBS (100 µL per well) and incubated at RT for 40 min. The wells were washed 5 times with PBST. TMB substrate was added to each well (100 µL per well) and allowed to develop for approximately 5 min. The reaction was quenched with 2 M H_2SO_4 (100 µL per well). The absorbance was measured at 450 nm.

Immunohistochemical Experiments

C57BL/6J wildtype female mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were fed standard chow *ad libitum* (Teklad 7012, Harlan Laboratories, Madison, WI) until the indicating ages. All animal work was performed at the Human Nutrition Research Center on Aging at Tufts University and approved by the Tufts University IACUC (H2016-92) in adherence with the ARVO statement for the use of animals in ophthalmic and vision research. Mice were killed using CO2. Enucleated eyes were immediately fixed in 4% paraformaldehyde for 2 h at 4°C and transferred to PBS. The cornea and lens were removed, and the remaining eye cup was transferred to 30% sucrose for cryopreservation and then embedded in OCT. Cryosections were obtained at a thickness of 10 µm, dried overnight, and stored at -80 °C.

For immunofluorescence, tissue sections were rehydrated in PBS containing 0.1% (v/v) Triton X-100 (PBT). For immunofluorescent detection of the RPE, slides were bleached using 10% hydrogen peroxide for 2 h at 65 °C. For the partial bleaching experiments, slides were bleached using 10% hydrogen peroxide for 1 h at 55 °C. Next, slides were blocked using normal donkey serum (Jackson Immunoresearch), incubated with primary antibodies (rabbit anti-glucosepane 2.24 µg mL^{-1}; goat-anti Lamp1 (Santa Cruz C-20) 1:200) overnight at for 4 °C, washed with PBT, and incubated with appropriate secondary antibodies conjugated to either Cy3 or Alexa Fluor 488 (Jackson Immunoresearch). For experiments with the competitor molecule, the rabbit anti-glucosepane antibody (2.24 µg mL^{-1}) was pre-incubated with 100 µM competitor **S25**) for 2 h at rt, at which point the antibody-competitor complex was added to slides and incubated overnight at 4 °C, as described above. Slides were mounted in Prolong Gold Antifade with DAPI (Molecular Probes) and photographed on a Zeiss Axiovert fluorescent microscope and digital camera.

Glucosepane Quantification

Acid hydrolysis

HSA samples were freeze dried and accurately weighed. $^{13}C_5$ Glucosepane internal standard was then added and samples freeze dried again. Acid hydrolysis was carried out by incubation overnight at 95 °C with 200µL of 7.4M HCl. Samples were then dried under a stream of nitrogen gas and resuspended in 400 µL 30% MeCN / 0.1% formic acid. After filtering through a 0.22 µm nylon filtration membrane samples were freeze dried before resuspension at 40µg/µL (dry weight) in 30% MeCN / 0.1% formic acid.

HPLC-MS method

10 µL of solution for analysis was injected onto a Cogent Diamond Hydride column (4 µm, 100 Å, 150 x 2.1 mm). A gradient of 100% (MeCN, 5% water, 0.1% formic acid, 0.005% trifluoroacetic acid) to 100% (Water, 0.1% formic acid) was used. The flow was passed into an ESI probe of a Micromass Quattro Ultima mass spectrometer and the fragmentation transitions listed below, monitored. (Mass Spectrometer parameters: source temperature 120 °C, desolvation temperature 350 °C, cone voltage 3 kV, capillary voltage 35 V, collision gas was argon, collision voltage - see table).
HPLC gradient table:

System: Waters Alliance 2795

Solvent A: (95% MeCN, 5% water) 0.1% formic acid, 0.005% Trifluoroacetic acid

Solvent B: (20% Methanol, 80% Water), 0.1% formic acid

Solvent C: Water, 0.1% formic acid

Time / min	% solvent A	% Solvent B	% Solvent C	Flow / ml/min	Curve
0	100	0	0	0.4	1
5	60	40	0	0.4	6
7	10	90	0	0.4	6
9	0	0	100	0.4	1
11	0	0	100	0.4	1
12	100	0	0	0.4	1
20	100	0	0	0.4	1

Curve 6 is a linear gradient, Curve 1 is a step change to the indicated percentage solvent.

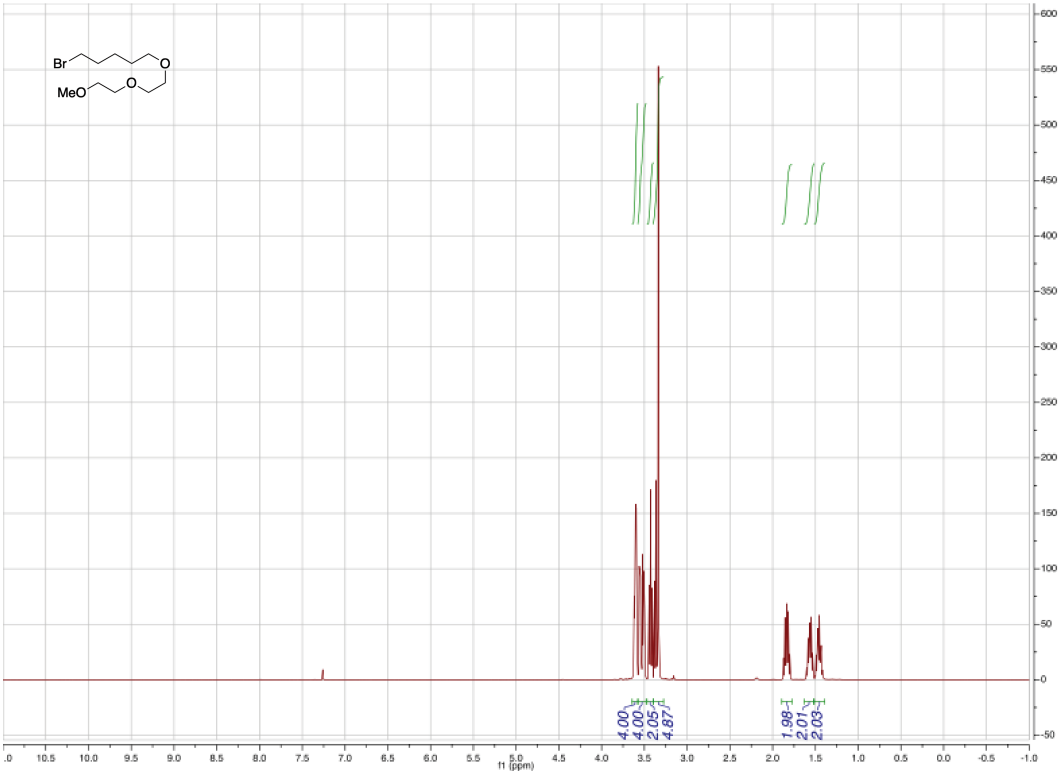
Mass transition table:

Molecule	**Q1**	**Q2**	**Collision energy**	**dwell /s**
13C Hydrolysed Glucosepane $[M+2H]^{2+}$	226.64	84.08	20	0.2
Hydrolysed Glucosepane $[M+2H]^{2+}$	224.28	84.08	20	0.2

References

1. C. Draghici, T. Wang, D.A. Spiegel, Concise total synthesis of glucosepane. *Science.* **16**, 294-298 (2015).
2. B. Schwenzer, D. Scheller, G. Losse, N-Acetyl-methylamides of amino acids as model compounds for interpretation of 13C-NMR-spectra of protected peptides. *Journal f. prakt. Chemie.* **321**(6), 1007-1016 (1979).
3. T. Wang, R. Kartika, D.A. Spiegel, Exploring post-translational arginine modification using chemically synthesized methylglyoxal hydroimidazolones. *JACS.* **134**(21), 8958-8967 (2013).
4. M. Kamalov, S. Yang, P.W.R. Harris, G.J.S. Cooper, M.A. Brimble, Expedient synthesis of peptides containing N^{ε}-carboxymethyllysine. *Synlett.* **25**, 1835-1838 (2014).

NMR Spectra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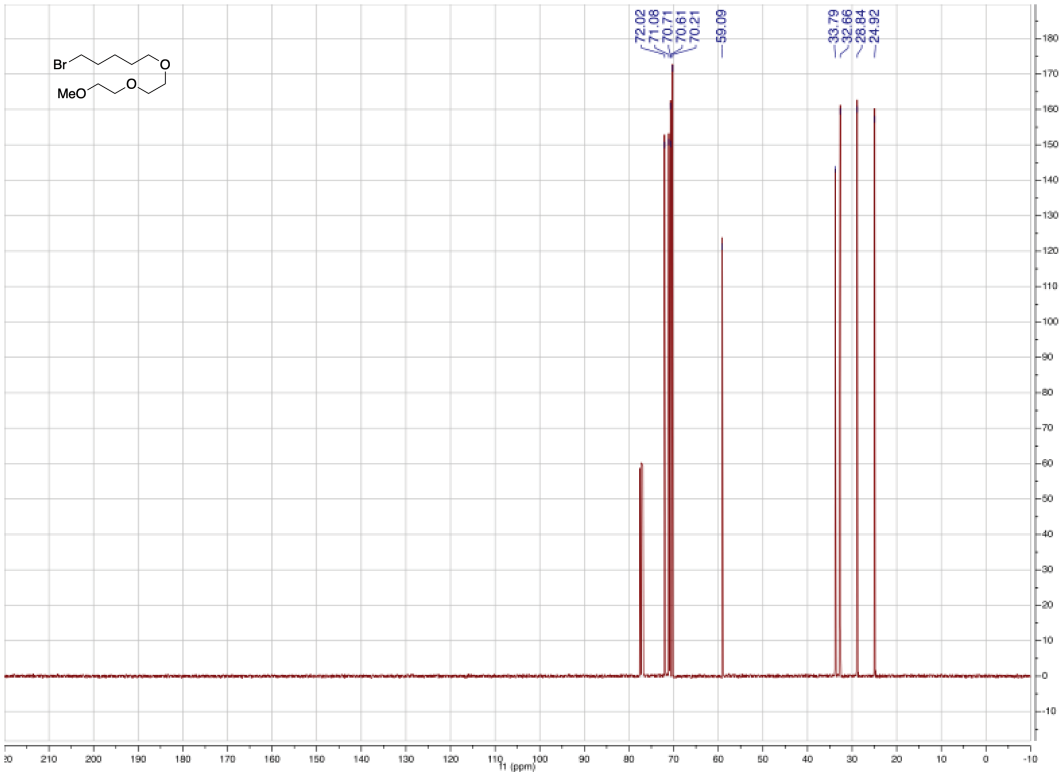
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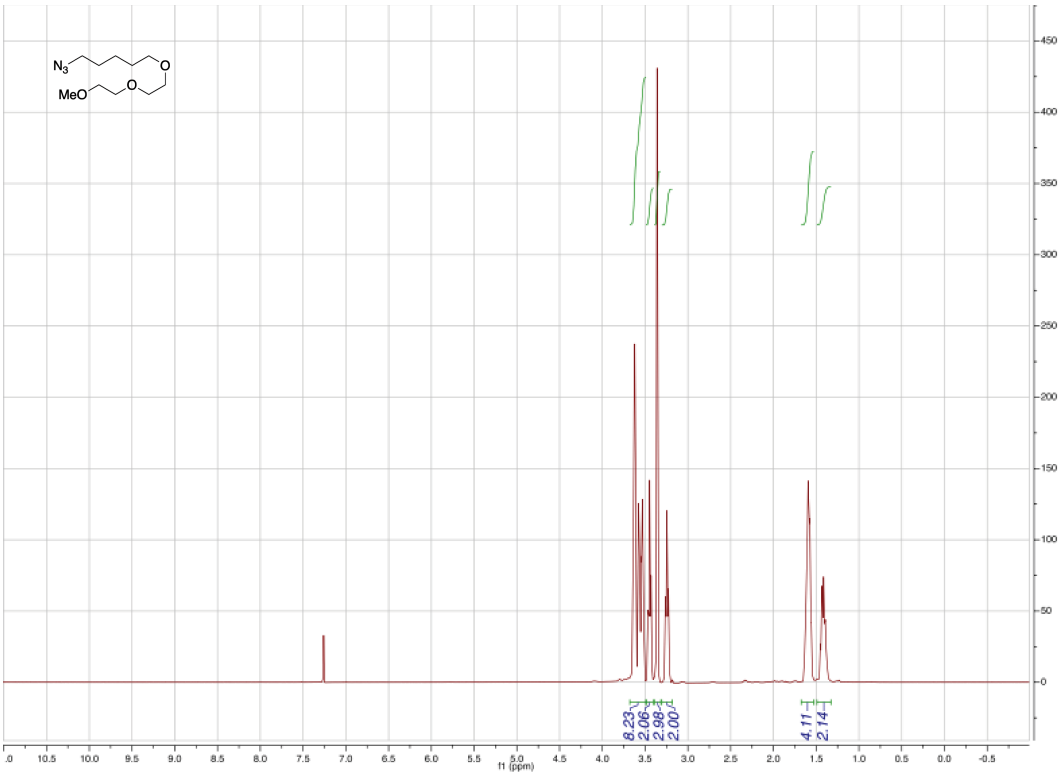
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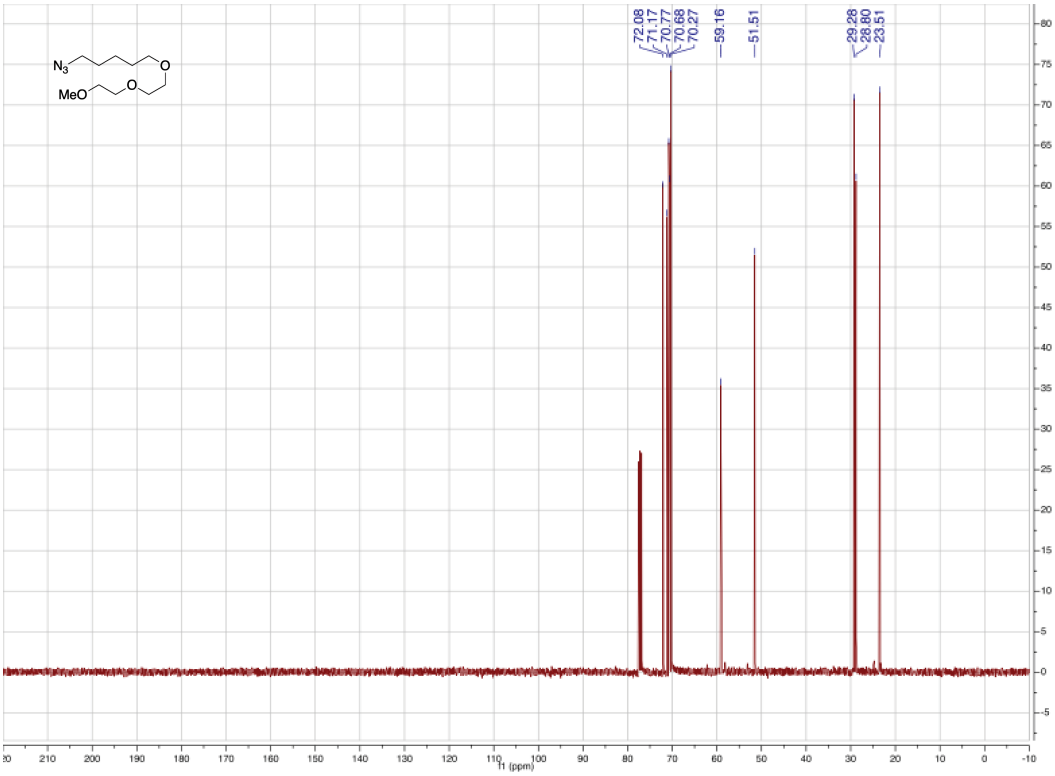
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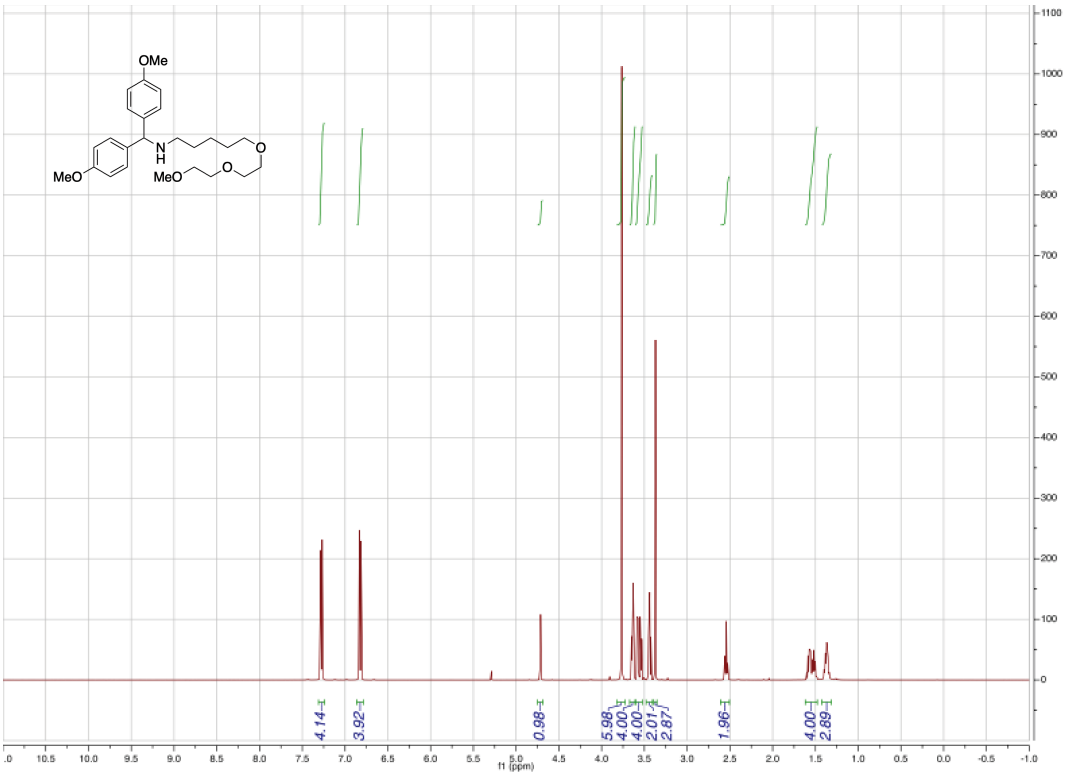
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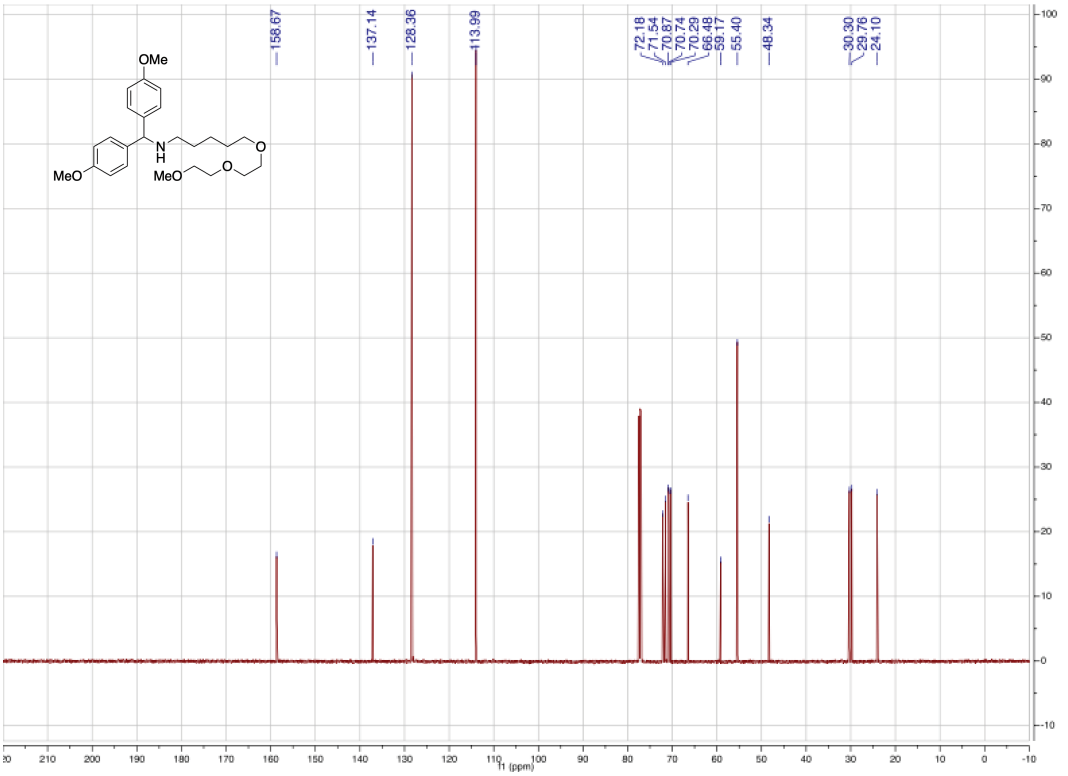
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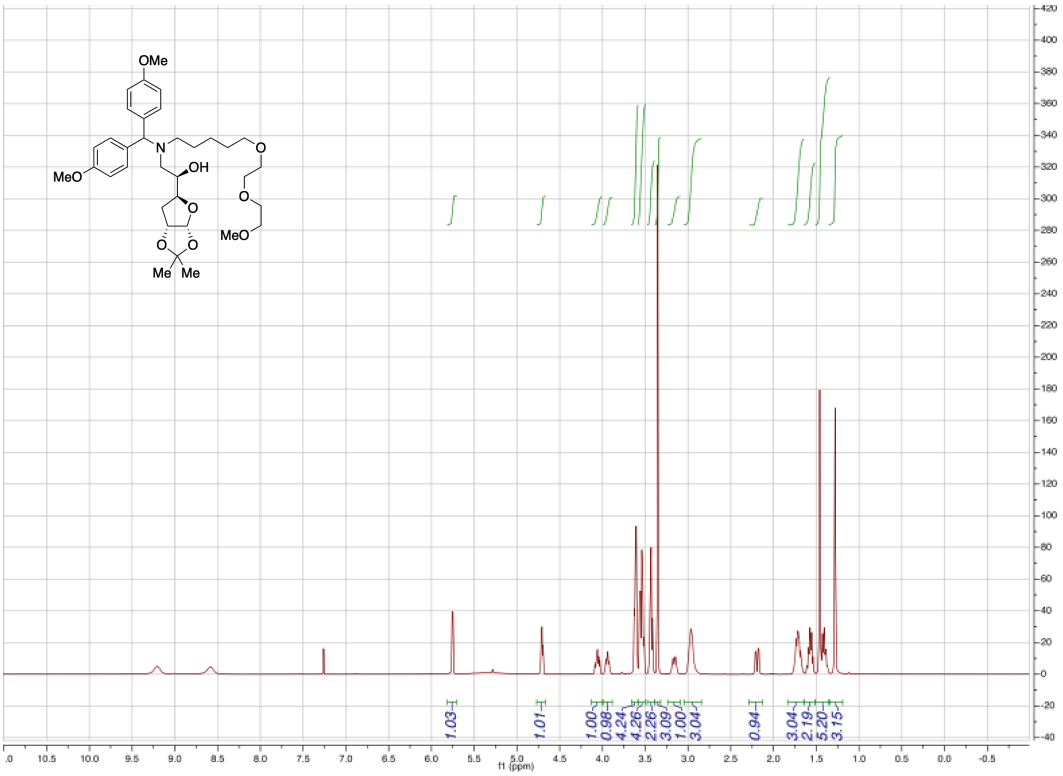
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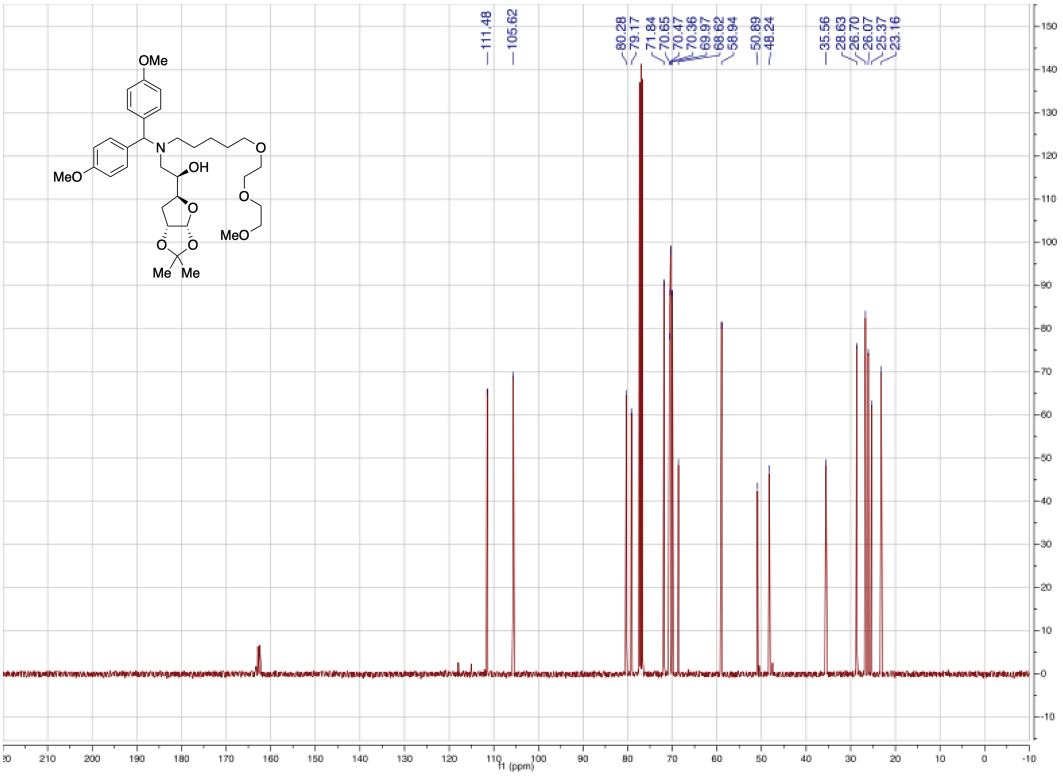
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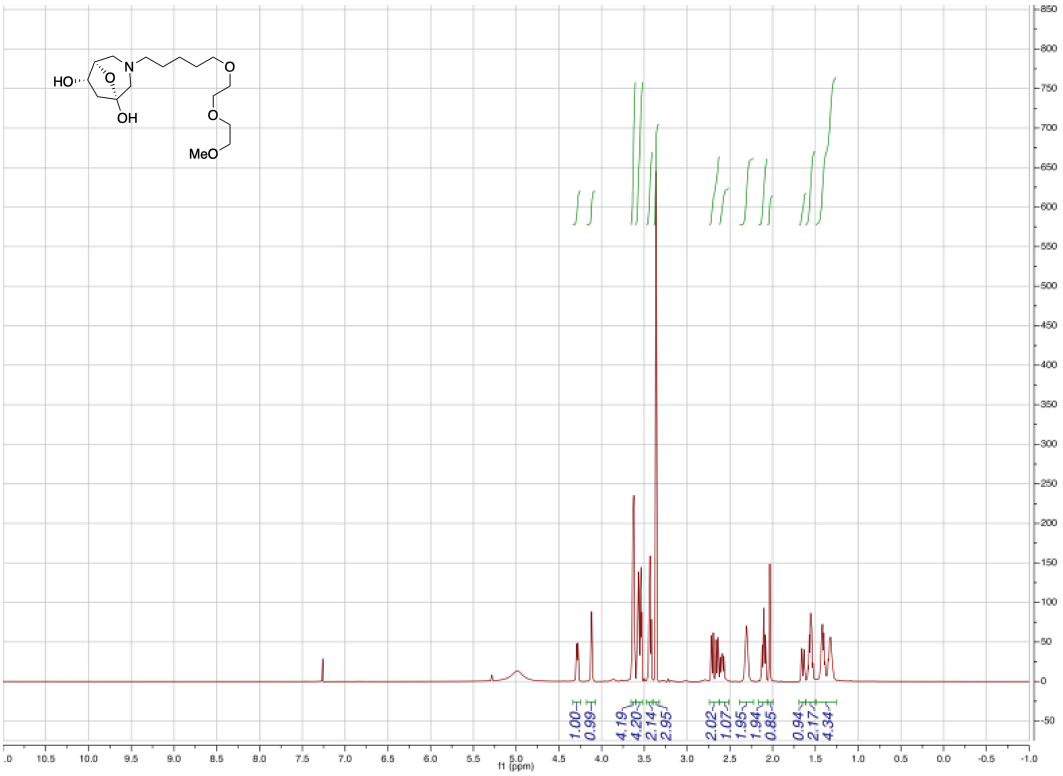
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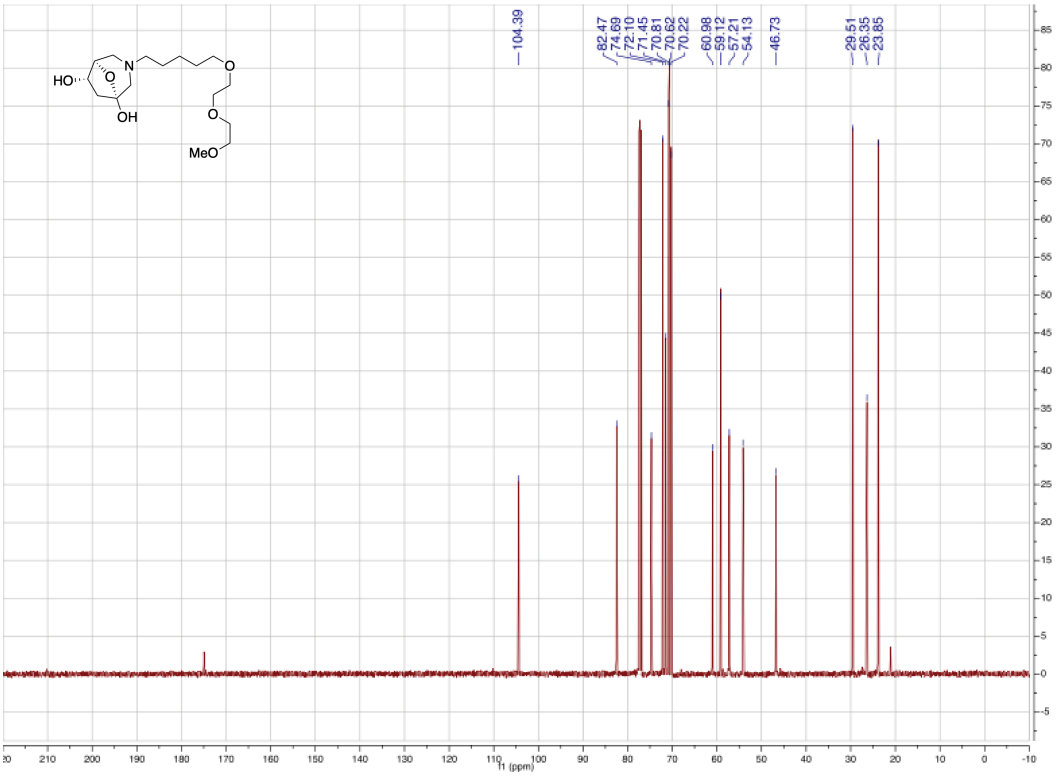
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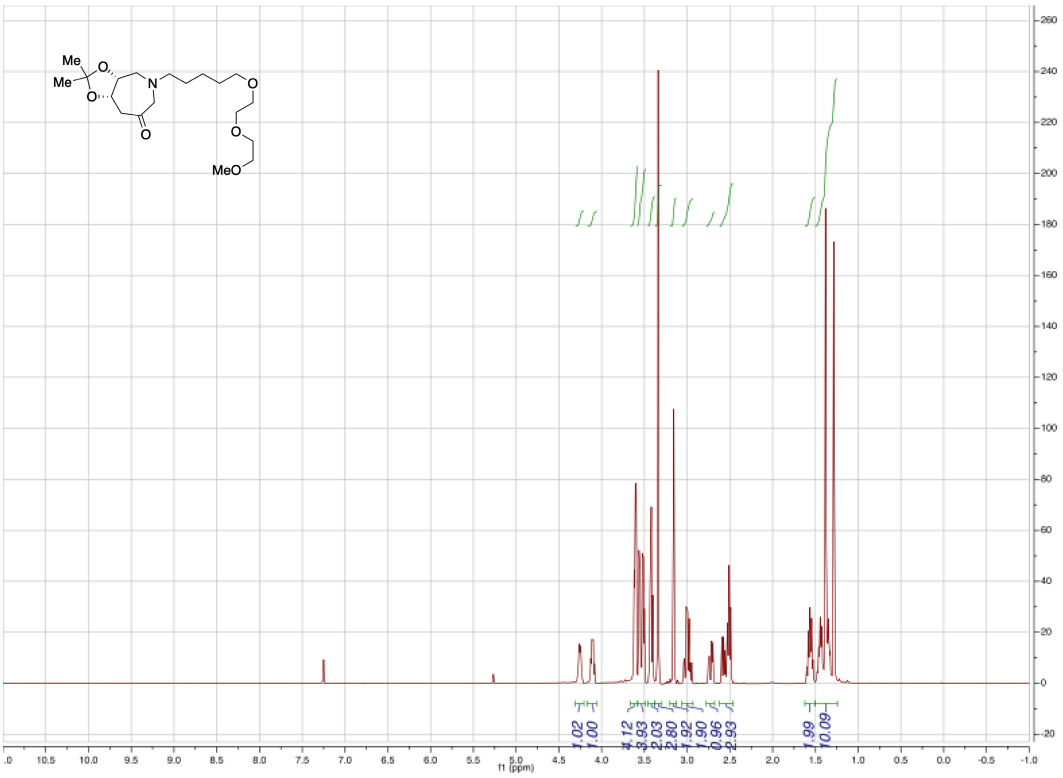
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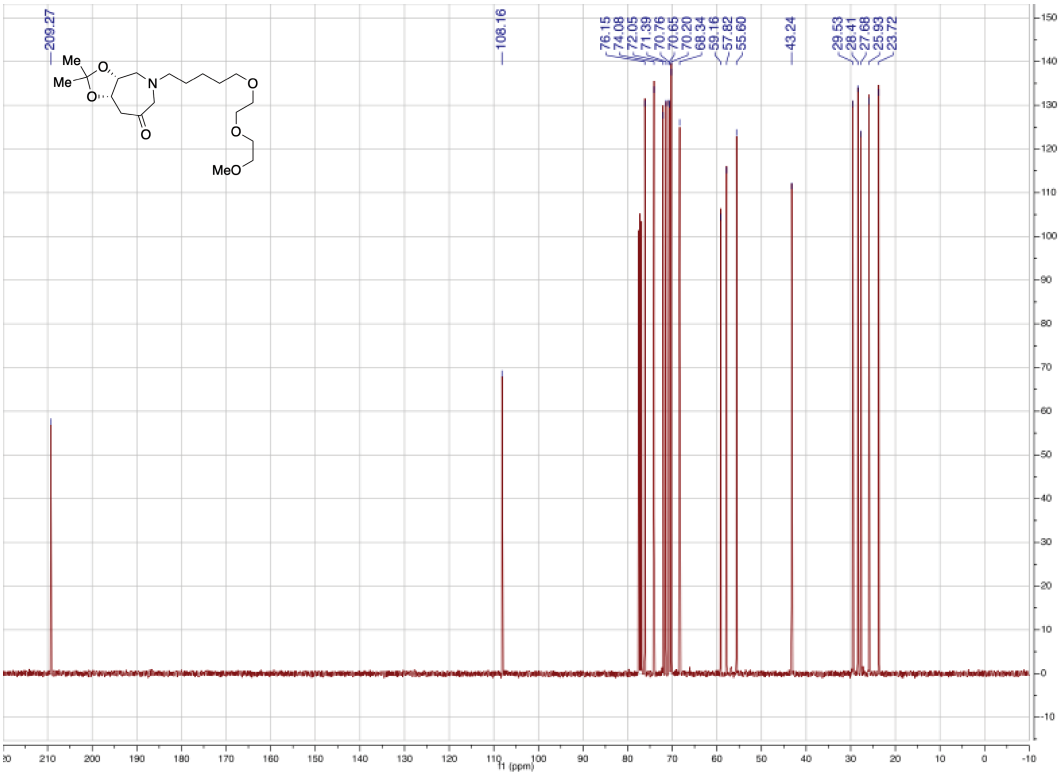
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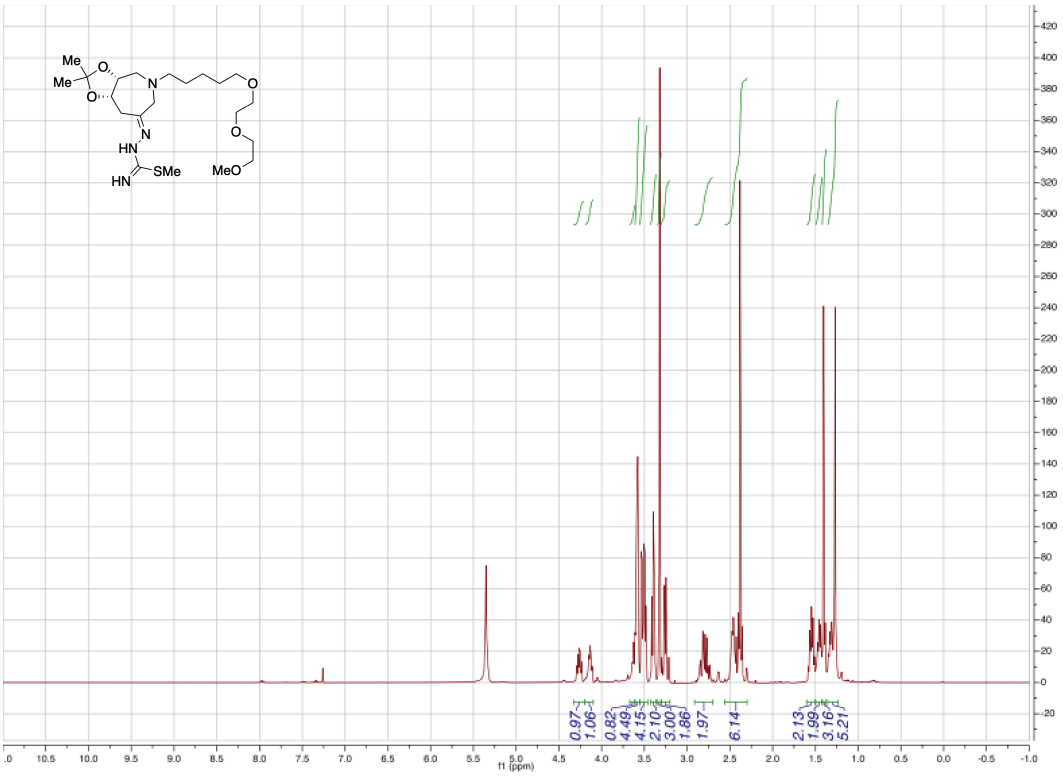
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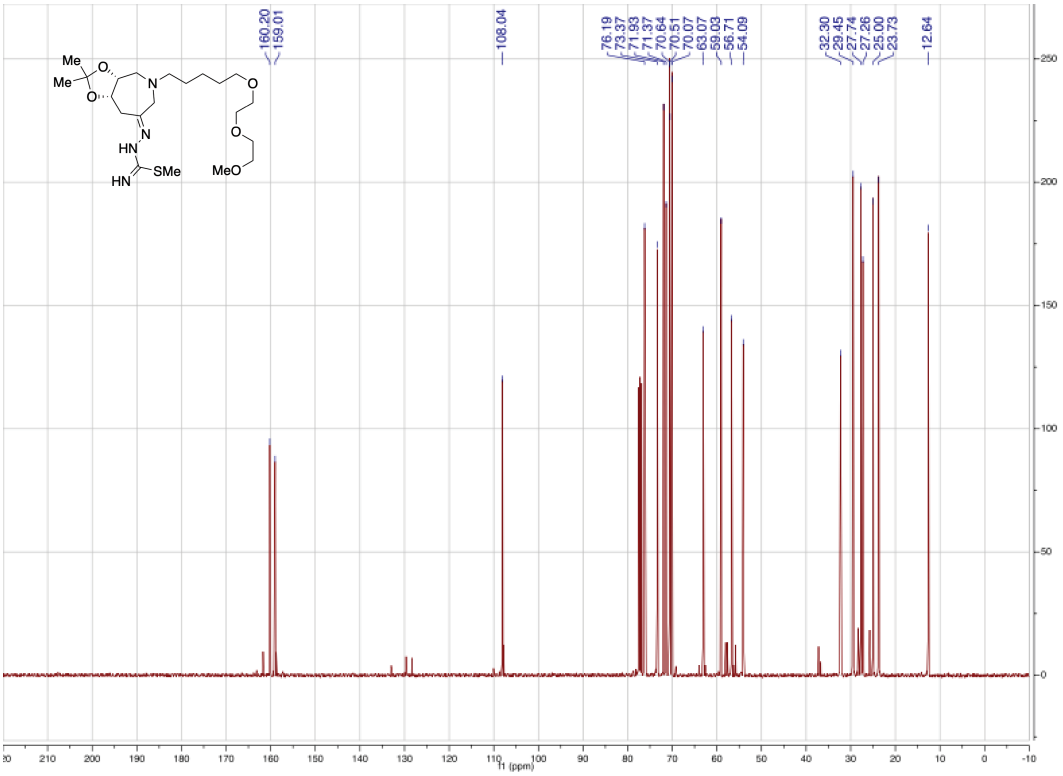
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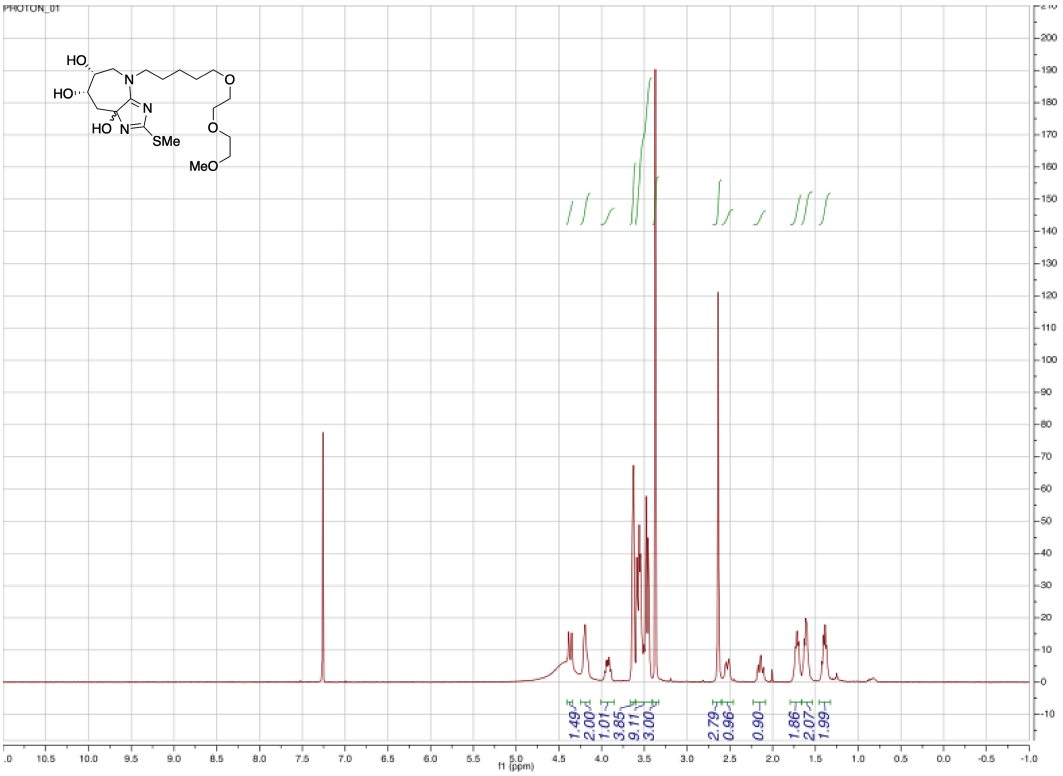
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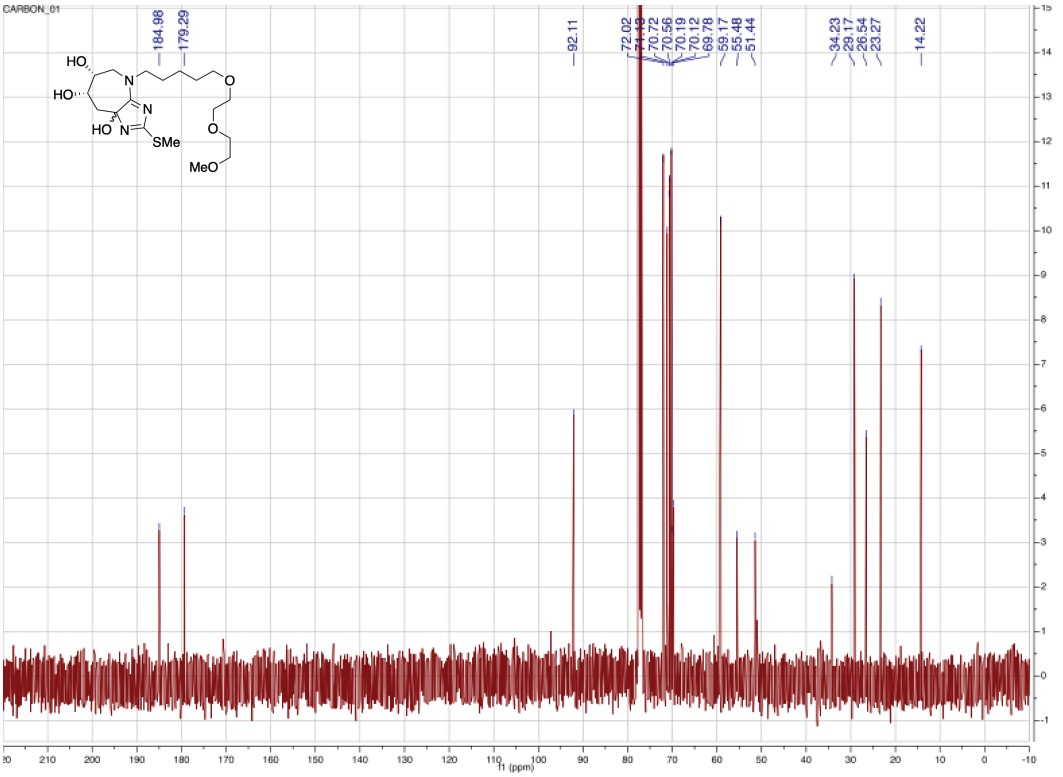
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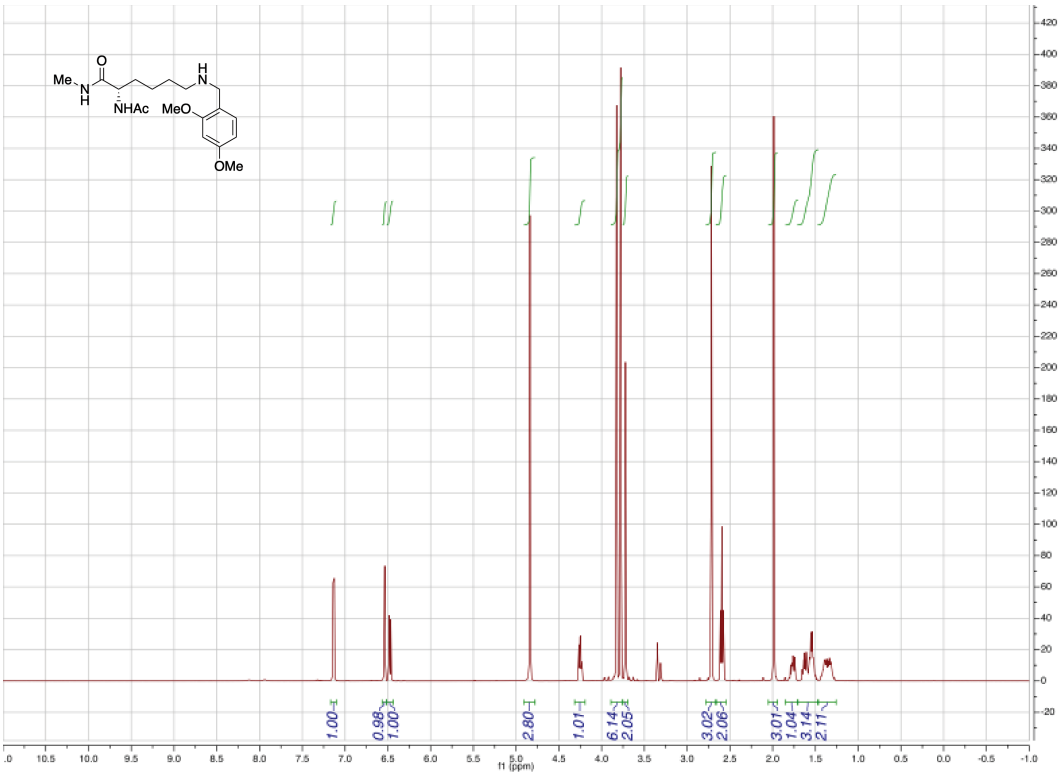
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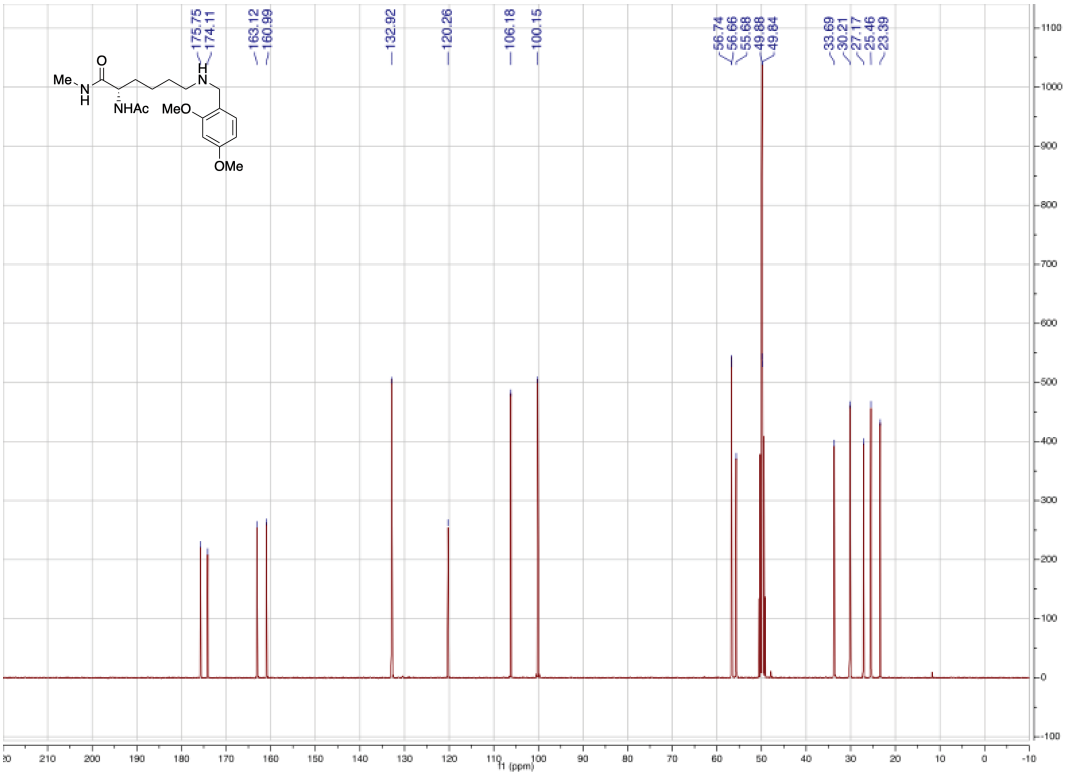
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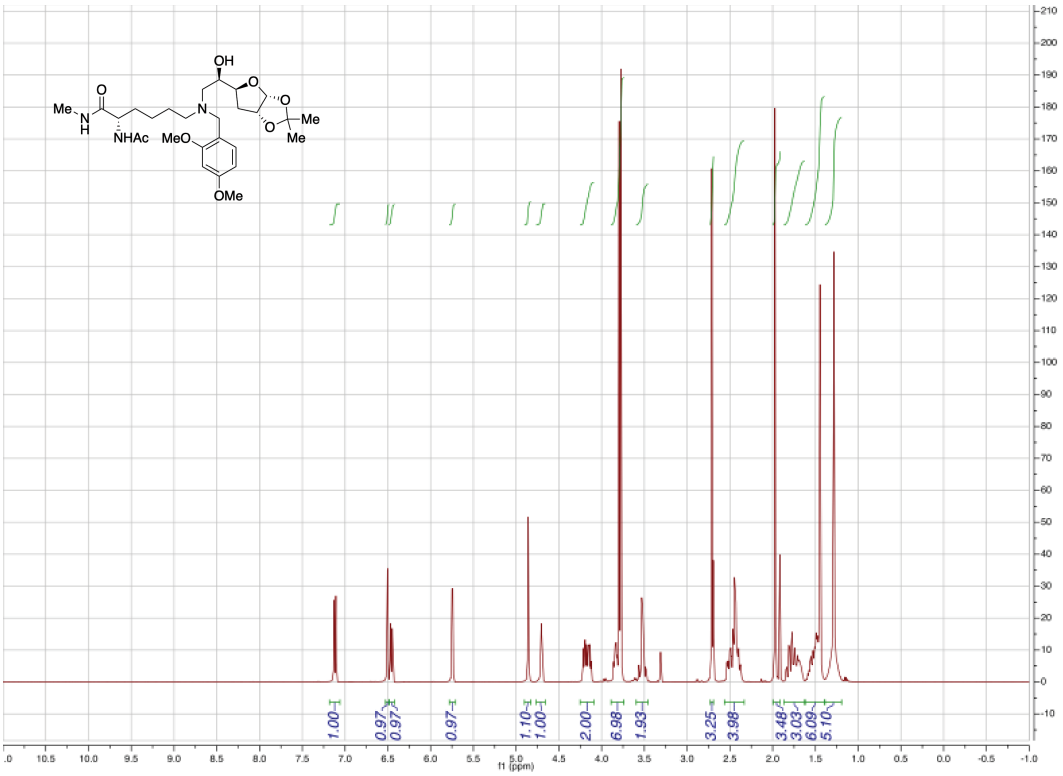
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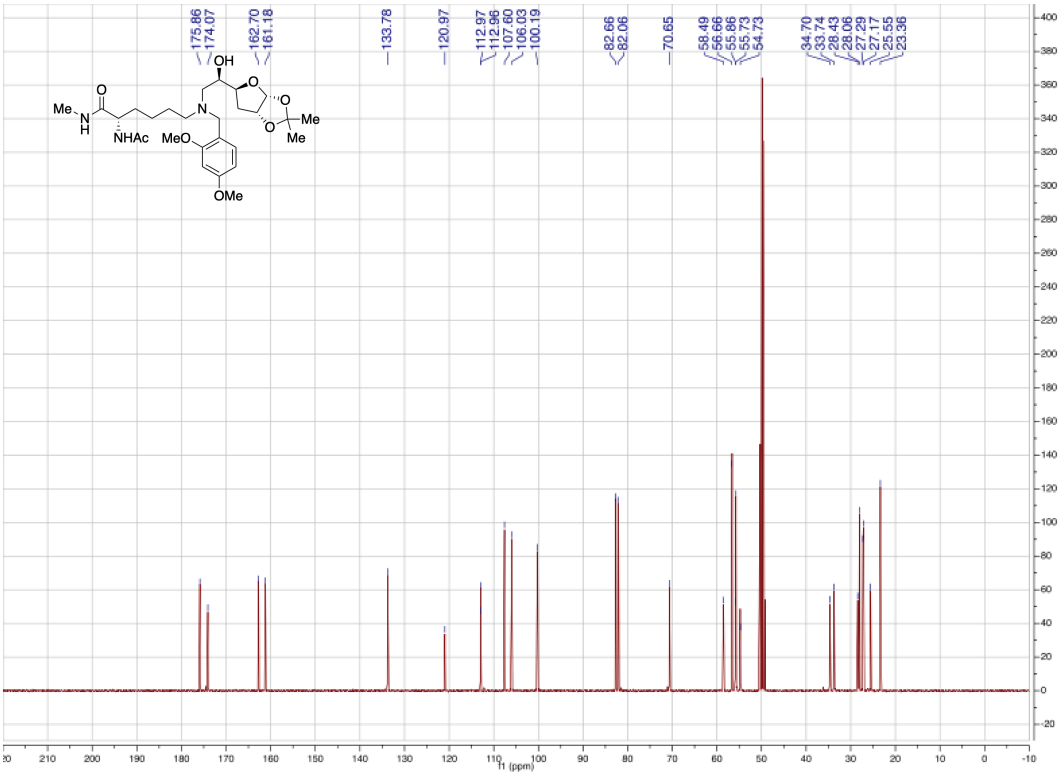
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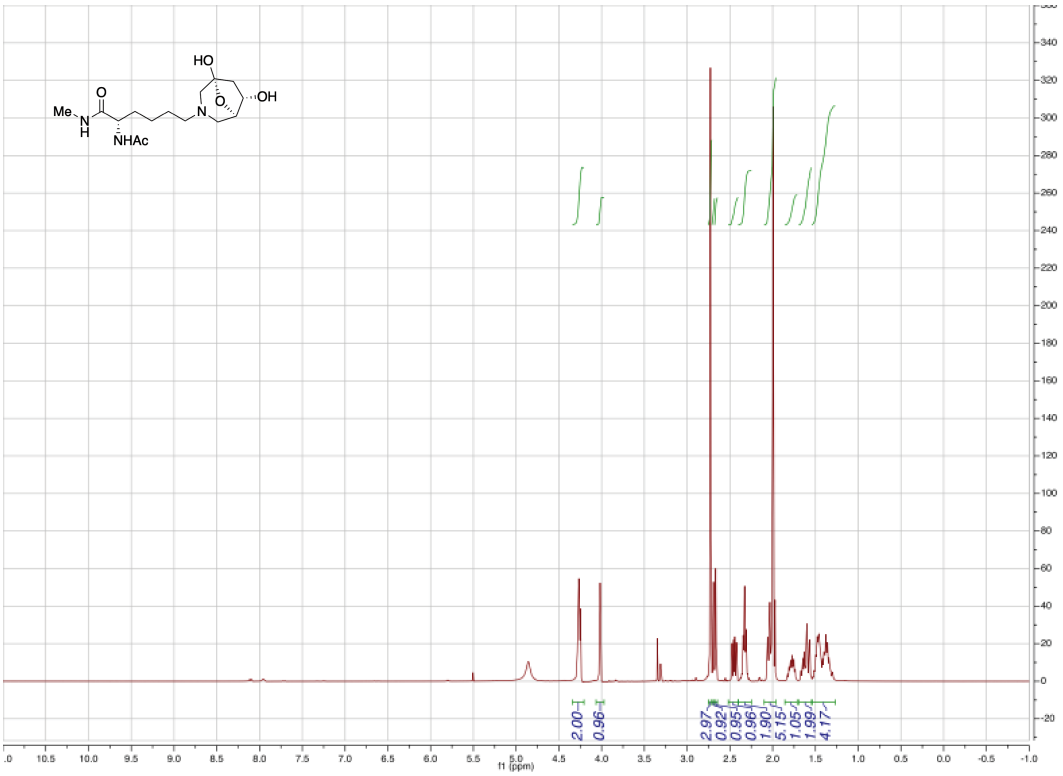
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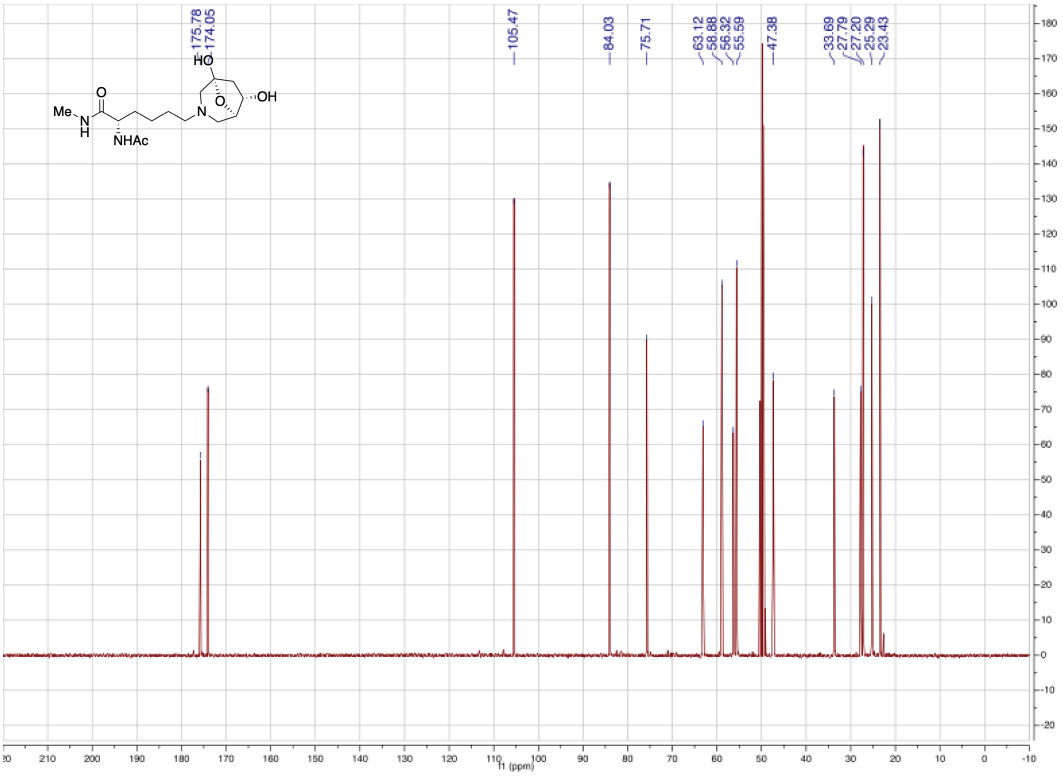
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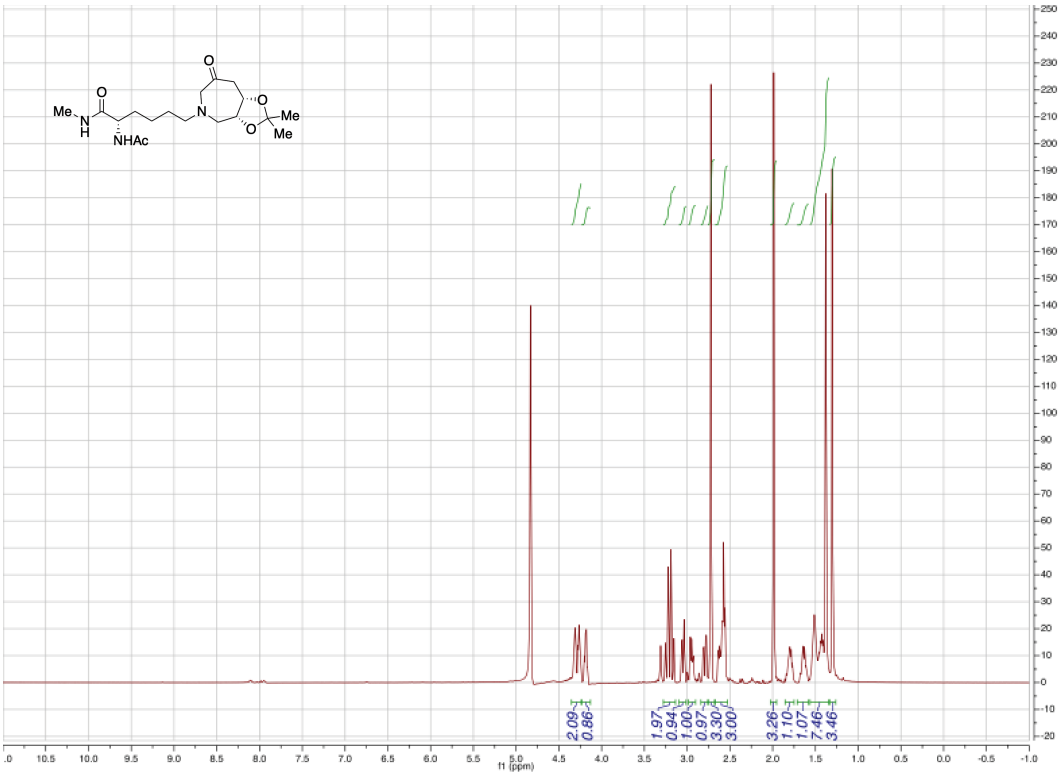
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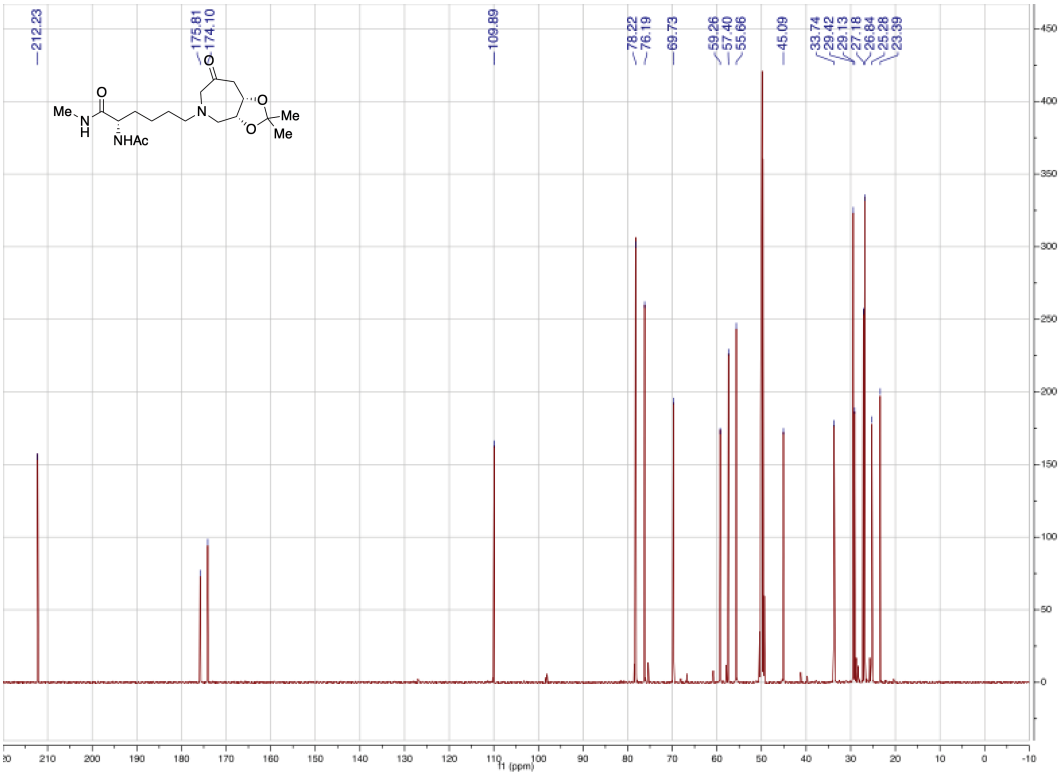
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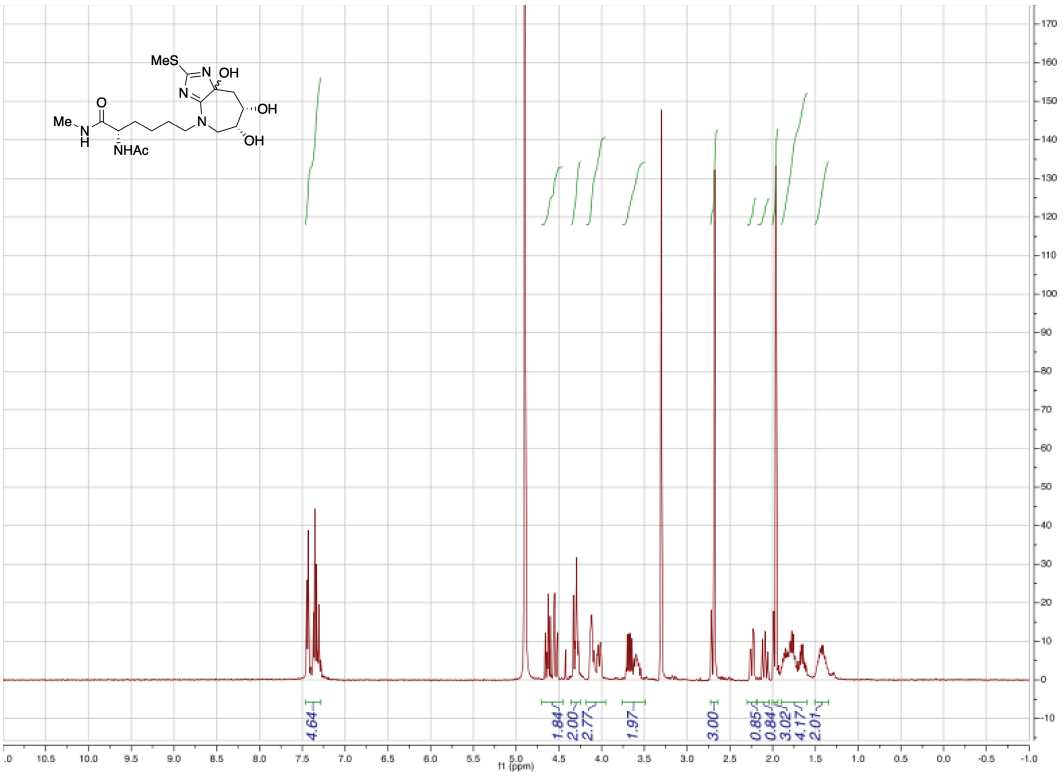
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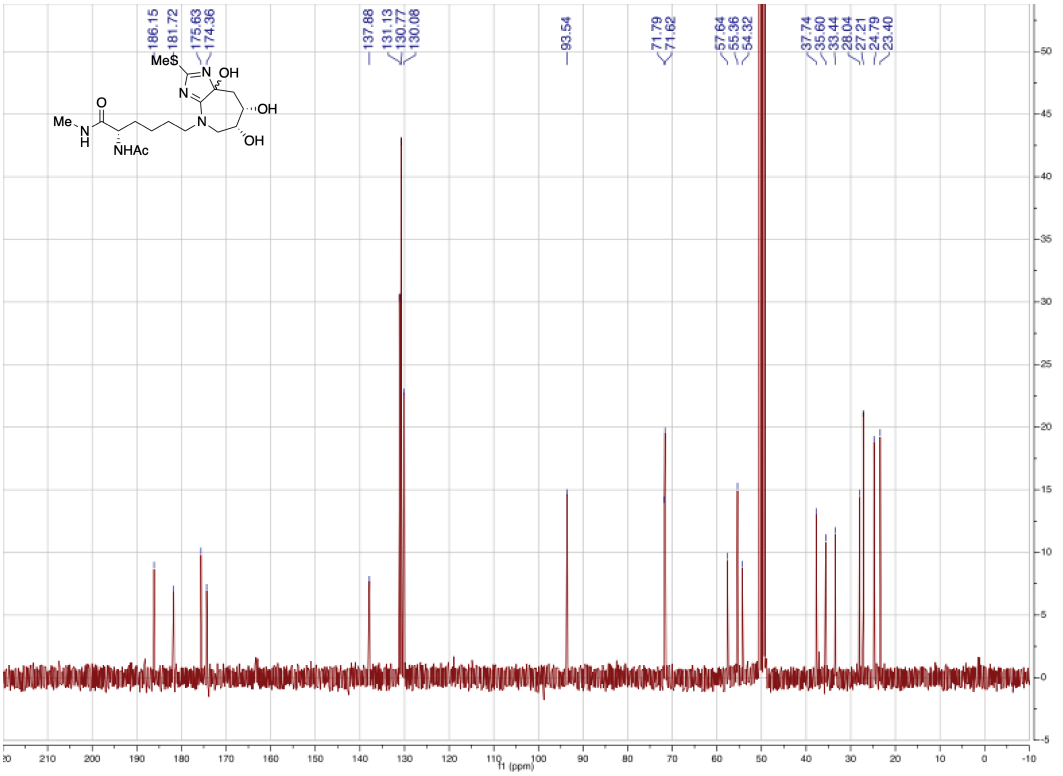
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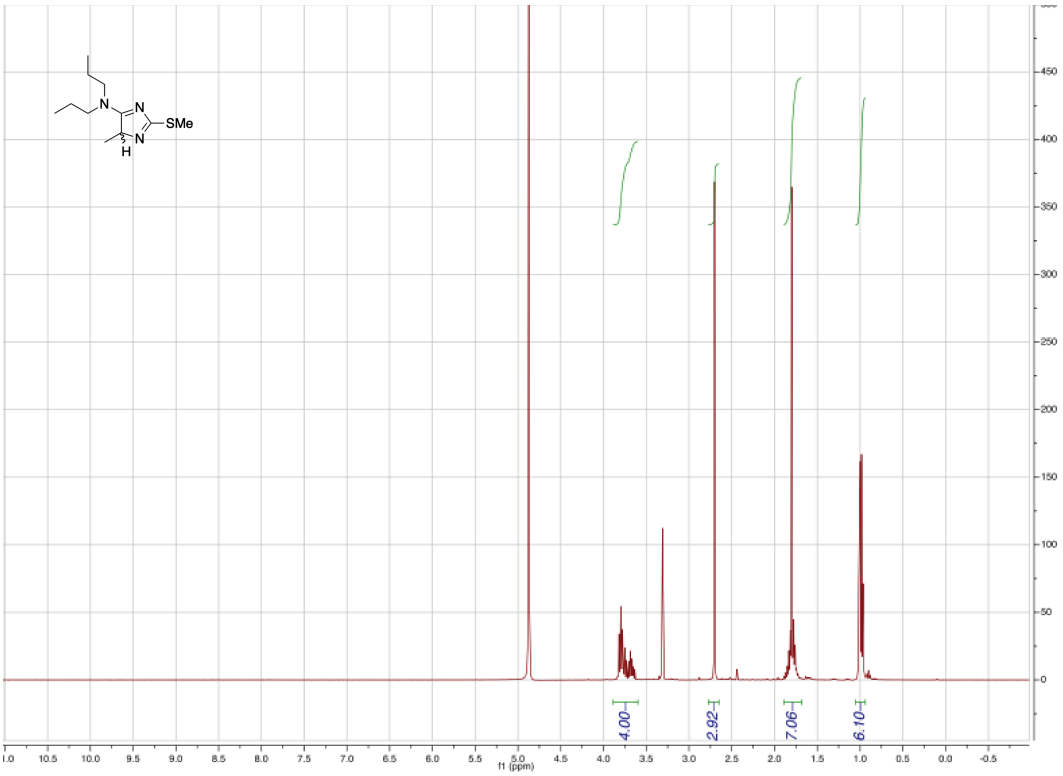
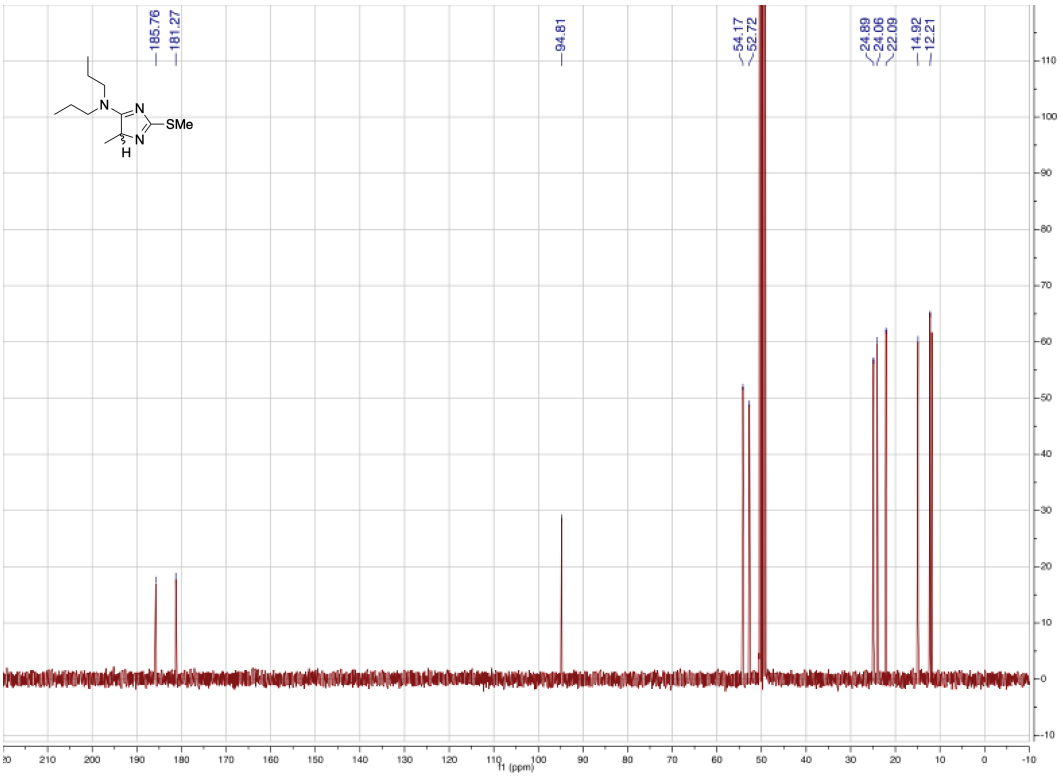
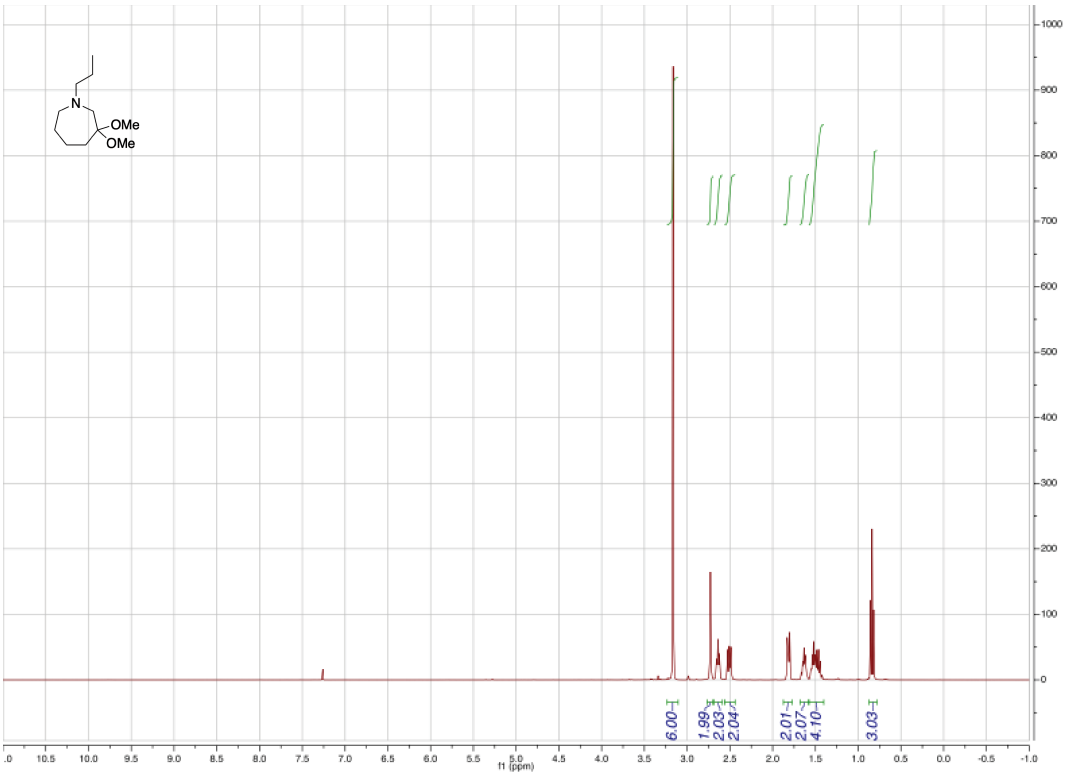
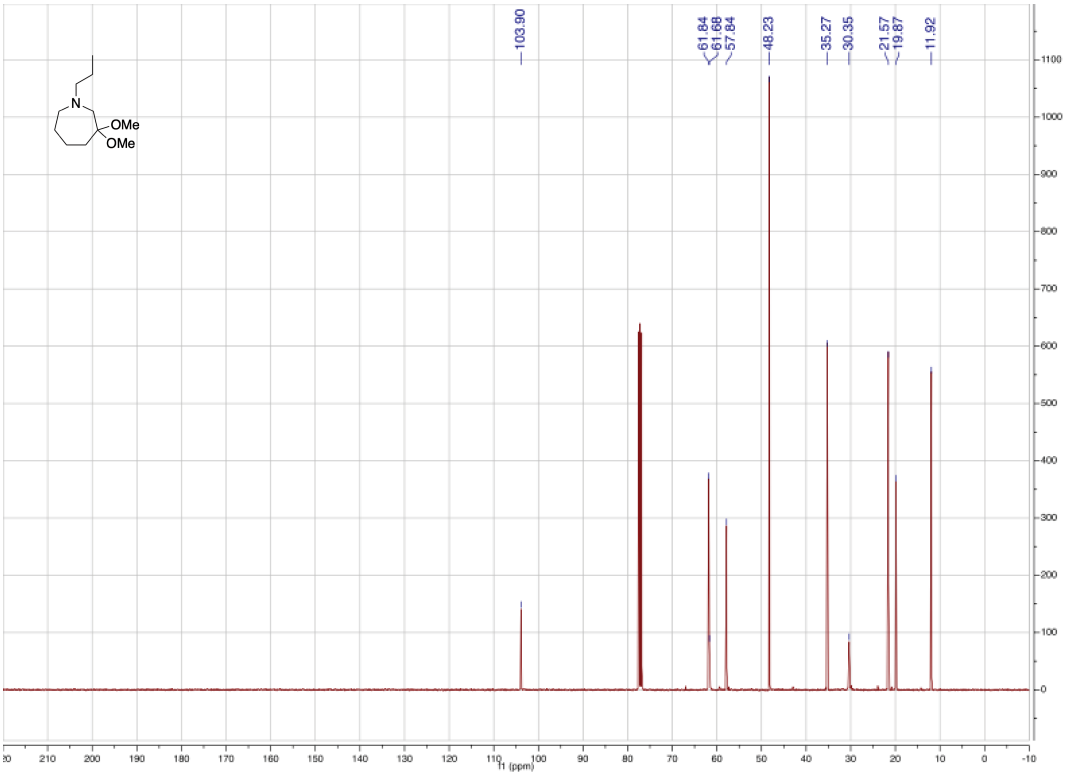
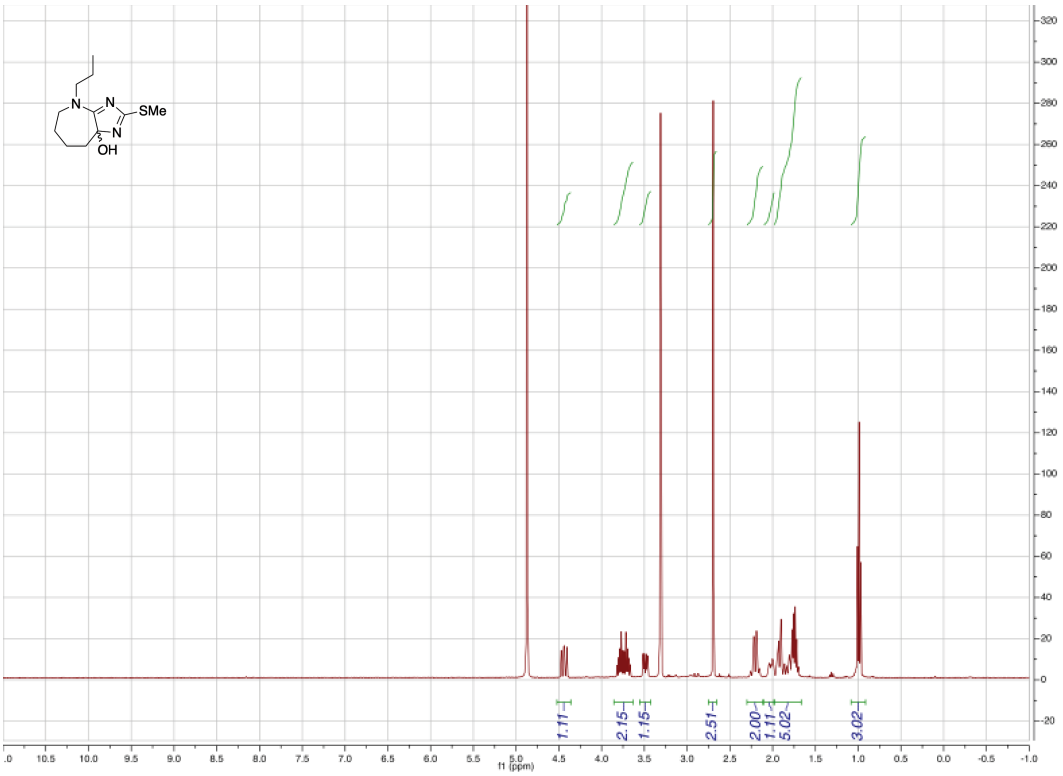
OH
f1 (ppm)

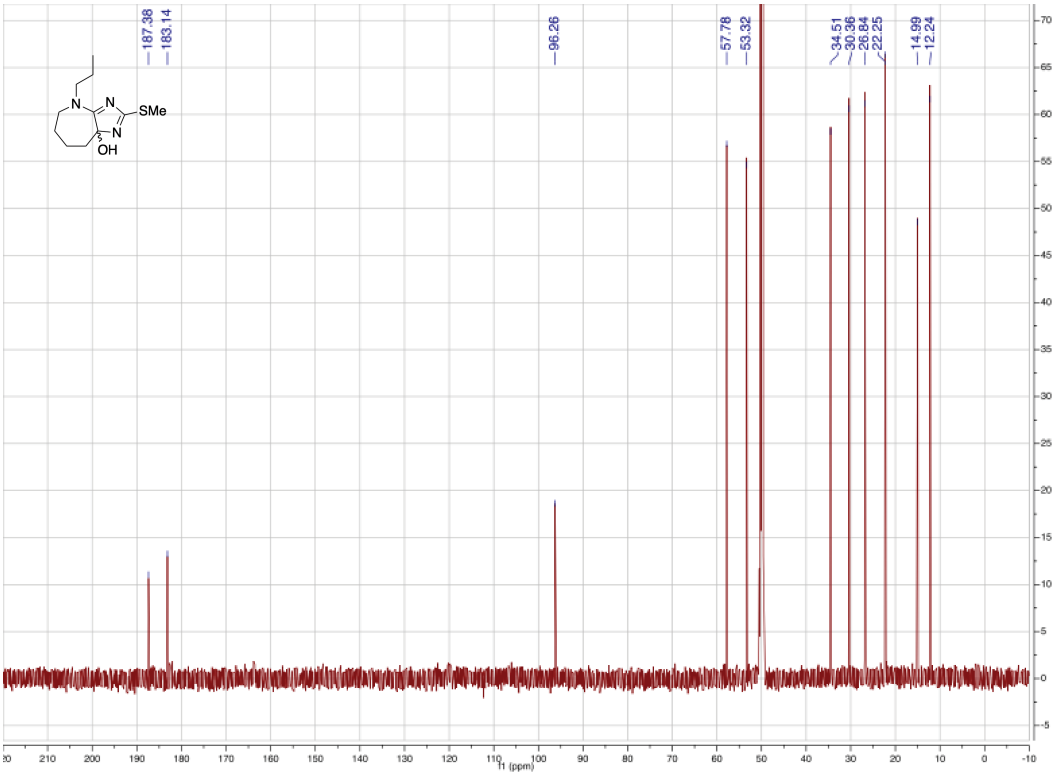

SMe
4.00
2.92
7.06
6.10
f1 (ppm)

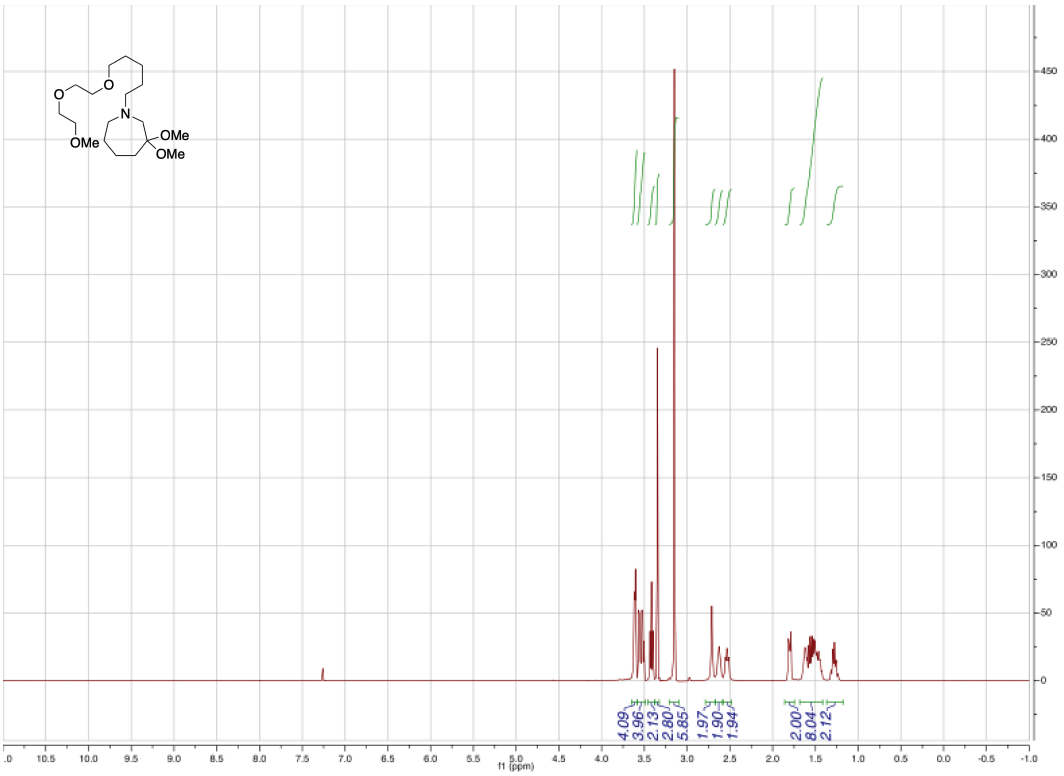

185.76
181.27
94.81
54.17
52.72
24.89
24.06
22.09
14.92
12.21
SMe
f1 (ppm)

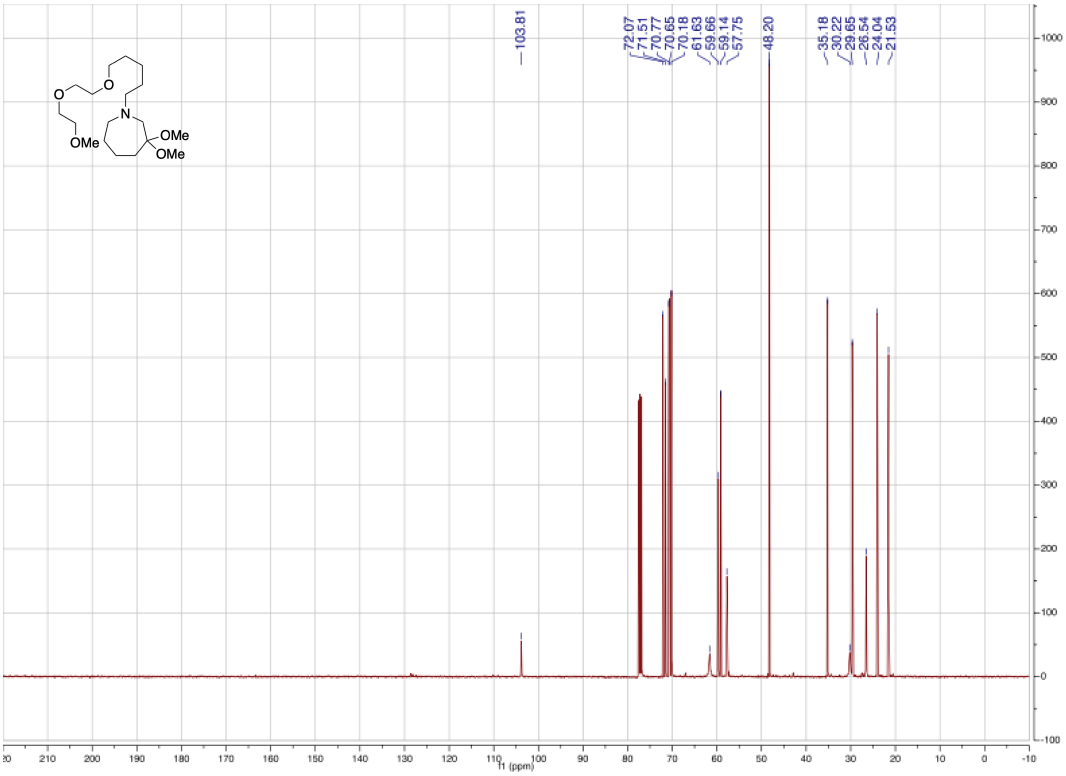

N
OMe
OMe
6.00
1.99
2.03
2.04
2.01
2.07
4.10
3.03
f1 (ppm)

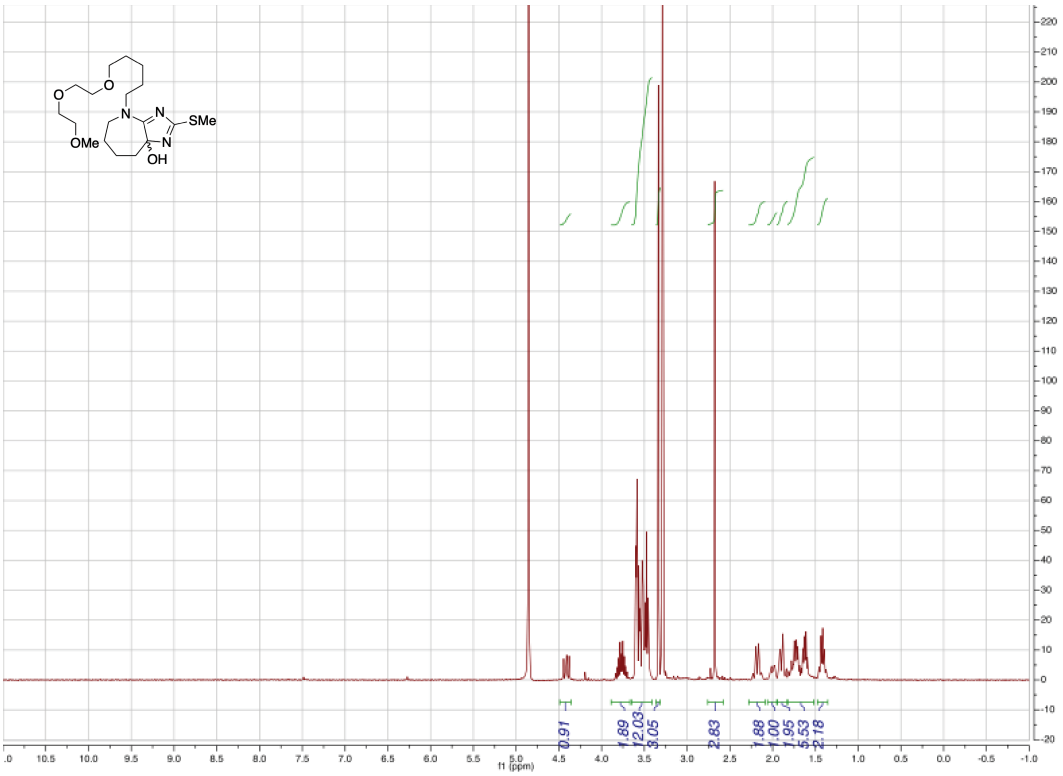

N
OMe
OMe
103.90
61.84
61.68
57.84
48.23
35.27
30.35
21.57
19.87
11.92
f1 (ppm)

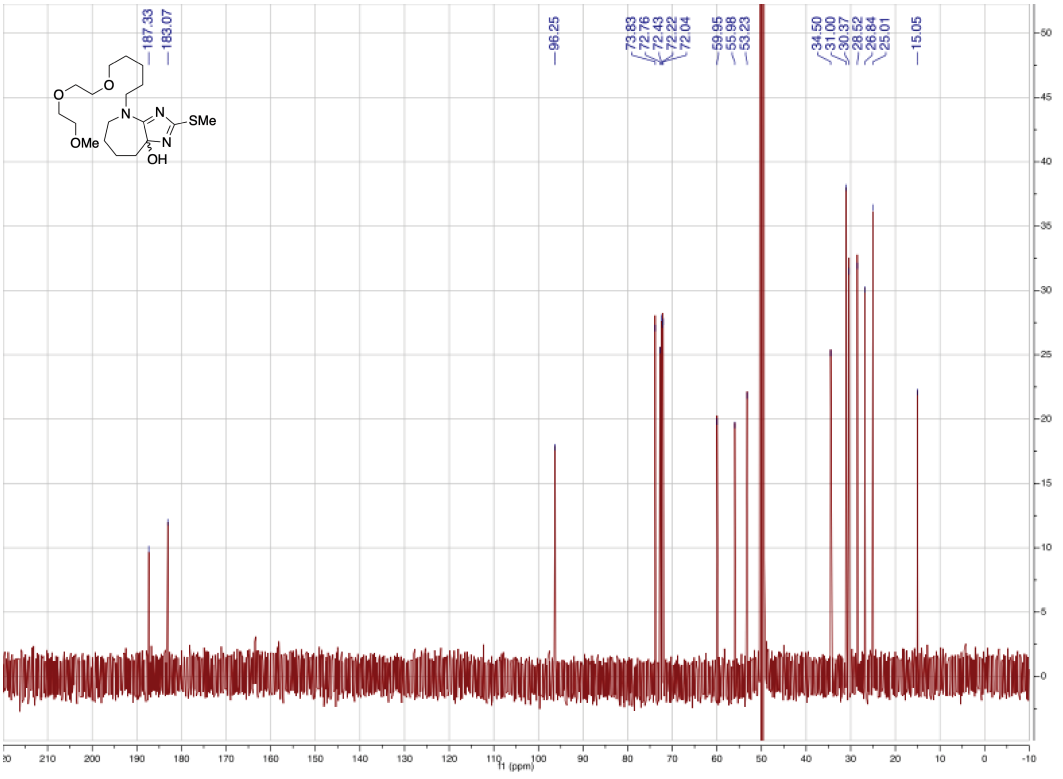

SMe
OH
1.11
2.15
1.15
2.51
2.00
1.11
5.02
3.02
f1 (ppm)


187.38
183.14
96.26
57.78
53.32
34.51
30.36
26.84
22.25
14.99
12.24
SMe
OH
f1 (ppm)


O
O
N
OMe
OMe
OMe
4.09
3.96
2.13
2.80
5.85
1.97
1.90
1.94
2.00
8.04
2.12
f1 (ppm)


O
O
N
OMe
OMe
OMe
103.81
72.07
71.51
70.77
70.65
70.18
61.63
59.66
59.14
57.75
48.20
35.18
30.22
29.65
26.54
24.04
21.53
f1 (ppm)


SMe
OMe
OH
0.91
1.89
12.03
3.05
2.83
1.88
1.00
1.95
5.53
2.18
f1 (ppm)


187.33
183.07
96.25
73.83
72.76
72.43
72.22
72.04
59.95
55.98
53.23
34.50
31.00
30.37
28.52
26.84
25.01
15.05
SMe
OMe
OH
f1 (ppm)