Supporting Information

Ternary Nylon-3 Copolymers as Host-Defense Peptide Mimics: Beyond Hydrophobic and Cationic Subunits

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Experimental section

Materials and instrumentation

Chemicals were purchased from Aldrich (Milwaukee, WI), Acros Organics, TCI America used as received, unless otherwise stated. β-Lactams (±)-**CHβ**, (±)-**COβ**, (±)-**MMβ**, (±)-**DMβ** were synthesized according to previously reported procedures.[1] Anhydrous dichloromethane was freshly distilled from calcium hydride under N_2. Tetrahydrofuran (THF, anhydrous, inhibitor free) and dimethylacetamide (DMAc, anhydrous) were purchased from Aldrich and used in the glove box without further purification. NMR spectra were obtained on a Bruker AC-300 and Bruker Avance spectrometers at 400 MHz and 500 MHz for ^{1}H and on a Bruker AC-300 at 75 MHz and Bruker Avance spectrometers at 100 MHz and 125 MHz for ^{13}C. $CDCl_3$ and D_2O for NMR were purchased from Aldrich. Polymerization reactions were carried out in an MBraun Labstar glove box under N_2. The number-average molecular weight (M_n), weight-average molecular weight (M_w) and polydispersity (PDI = M_w/M_n) for polymeric samples were obtained using a gel permeation chromatography (GPC) instrument equipped with a Shimadzu LC-10AD liquid chromatography (HPLC) pump and a Wyatt Technology miniDAWN multi-angle light scattering (MALS) detector (690 nm, 30 mW) in series with a WyattTechnology Optilab-rEX refractive index detector (690 nm). All measurements were performed using two GPC columns (Waters Styragel HR4E) linked in series, with THF as mobile phase at a flow rate of 1.0 mL/min at 40^{o}C. The data were processed using ASTRA 5.3.4.20 software (Wyatt Technology) with a dn/dc value of 0.1. For **HG** containing polymers that are not soluble well in THF, *N*,*N*-dimethylacetamide (DMAc) is used in GPC analysis. Sidechain protected HG containing polymers were analyzed on a Waters GPC instrument equipped with two Waters Styragel HR 4E columns (particle size 5 μm) linked in series and a refractive index detector (Waters 2410). DMAc containing 10 μM LiBr was used as the mobile phase at a flow rate of 1 mL/min at 80 ℃. Number-average molecular weight (M_n), weight-average molecular weight (M_w) and polydispersity index (PDI) were calculated using the Empower software and calibration curves obtained from at least nine PMMA standards with peak average molecular weight (Mp) ranging from 690 to 1944000. The observed degree of polymerization (DP) for a particular polymer was

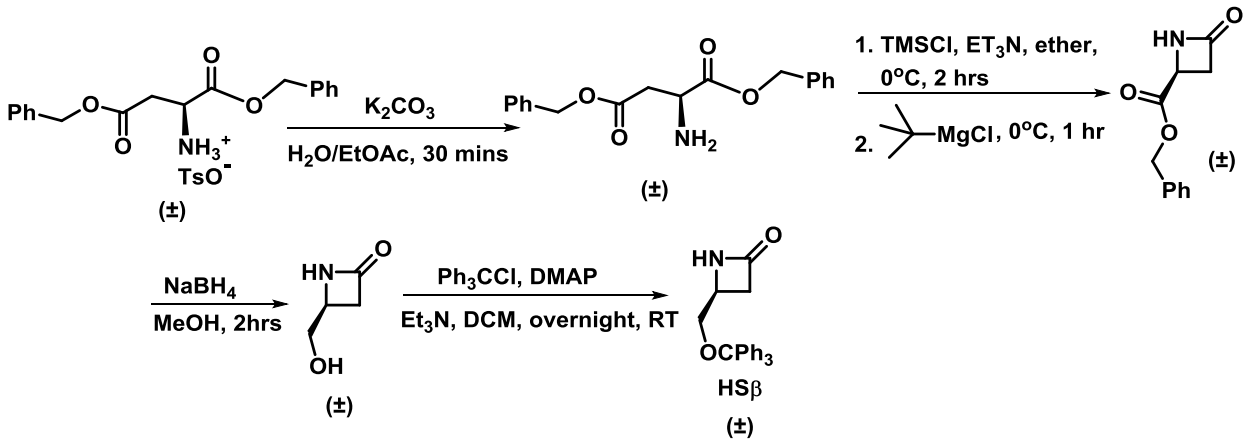
calculated based on the deduced M_n value, the initial ratio of β-lactam monomers used for the reaction, and the molecular weight of the β-lactam monomers, as described previously.[1b,c]

Polymer synthesis

All polymerizations were carried out in a N_2-purged dry box at room temperature. In a typical polymerization reaction, β-lactam monomers were weighed out in the appropriate molar ratio and placed in a reaction vial. To the vial was then added anhydrous THF or anhydrous DMAc and tbuBzCl (co-initiator) to achieve the desired monomer:co-initiator ratio and a monomer concentration of 0.1 M. The mixture was allowed to stir until all materials had dissolved. Polymerization was initiated by addition of $Li(NSiMe_3)_2$ solution (2.5 equiv. to the starting co-initiator concentration) in THF or DMAc. The resulting solution was stirred overnight at room temperature. The reaction vial was removed from glove box, and the polymerization was quenched by adding 3-4 drops of methanol. The resulting polymer was precipitated by pouring the solution into pentane. The solid was isolated by centrifugation, and the supernatant liquid was decanted off. The solid was re-dissolved in THF and the re-precipitated with pentane. After two more repetitions of precipitation/centrifugation procedure, the white pellet was dried under vacuum to constant weight.

Deprotection of the Boc was carried out by dissolving the polymer in 2 mL neat trifluoroacetic acid. For polymers containing trityl groups, 100 μL tri-isopropyl silane was added. The reaction vessel was placed on a shaker for 2 hours (room temperature). The resulting solution was poured into cold ether to cause the deprotected polymer to precipitate. The solid was isolated by centrifugation, and the supernatant liquid was decanted off. The solid was dried under a stream of N_2. The precipitate was washed with ether twice and dried under vacuum. The material was then dissolved in 5-10 mL of water and lyophilized to yield the polymer as white fluffy solid.

Synthesis of **(±)-4-(Trityloxymethyl)-azetidin-2-one (HSβ).**



(±)-Dibenzyl ester of aspartic acid: To a 500 mL round bottomed flask was added *rac*-aspartic acid dibenzyl ester p-toluenesulfonate salt (50 g, 0.1 mol) and K_2CO_3 (42.7 g, 0.31 mol) in a 2:1 mixture of H_2O:ethyl acetate (375 mL) at room temperature. The resulting solution was stirred for 2 hours, and the layers were separated. The aqueous layer was washed with ethyl acetate (3 X 30 mL). The combined organic layers were washed with brine and dried over $MgSO_4$. After the drying agent was filtered off and solvent was removed by rotary evaporation, 32.1g (>99%) of product was obtained as viscous yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.71-2.92 (m, 2H), 3.87 (dd, J = 7.1, 4.8 Hz), 5.10 (s, 2H), 5.13 (s, 2H), 7.27-7.39 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 39.1, 51.4, 66.8, 67.2, 128.4-128.7, 135.6, 135.7, 171.1, 174.1. ESI/EMM mass spectrum *m/z* calc. 314.1387 $[M+H]^+$, obs. 314.1389 $[M+H]^+$.

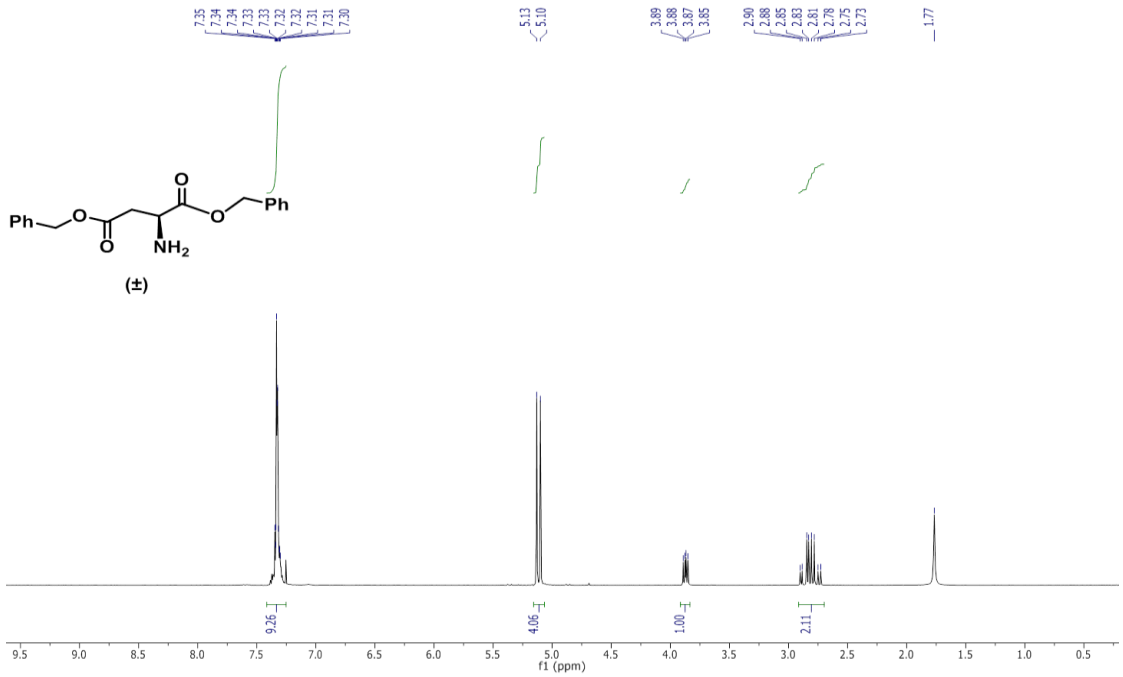


Figure S1. ^{1}H NMR spectrum of **(±)-dibenzyl ester of aspartic acid.**

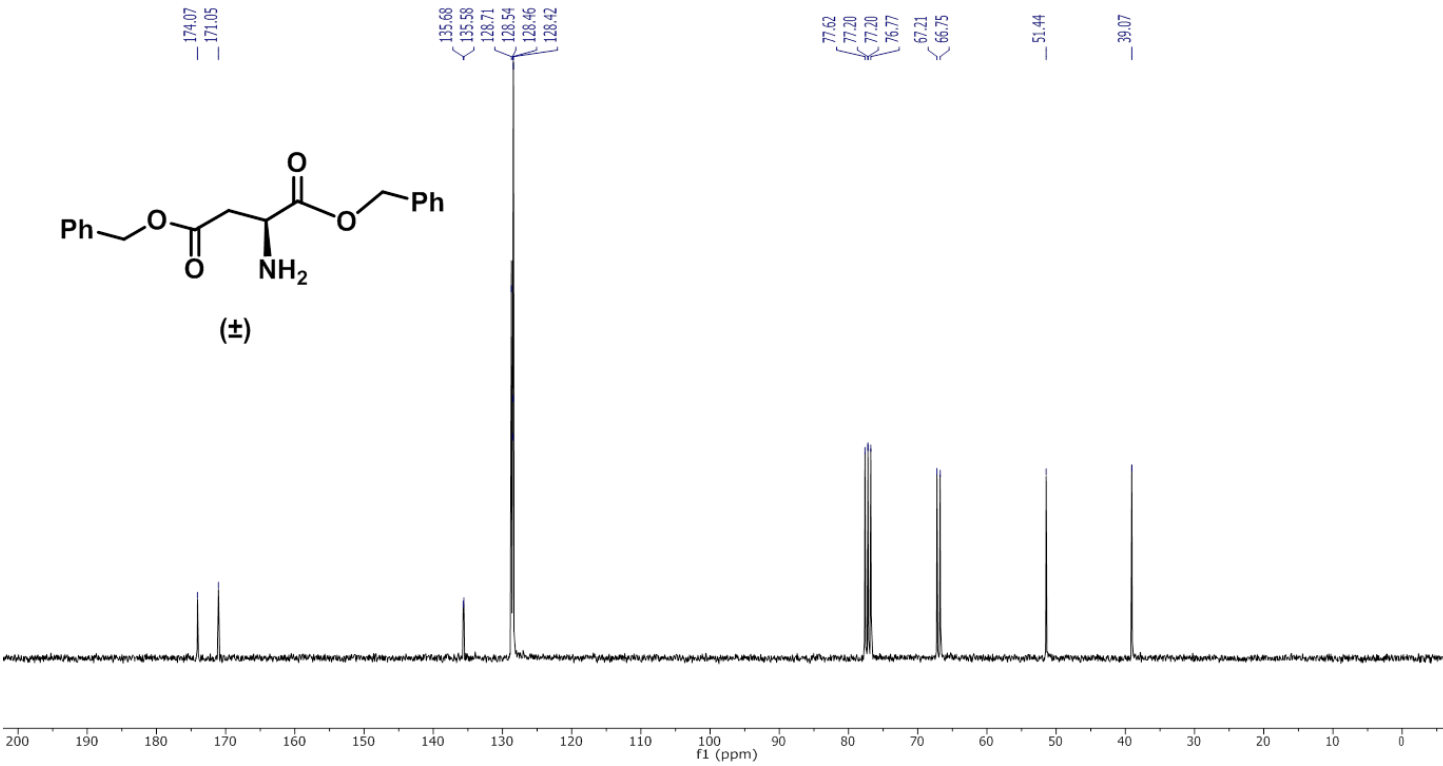


Figure S2. ^{13}C NMR spectrum of **(±)-dibenzyl ester of aspartic acid.**

(±)-Benzyl azetidin-2-one-4-carboxylate: To a solution of **(±)-dibenzyl ester of aspartic acid** (32.1 g, 0.102 mol) in 400 mL diethyl ether at 0°C was added trimethylsilyl chloride (14.3 mL, 0.113 mol) and triethyl amine (17.1 mL, 0.122 mol). The resulting suspension was stirred for 2 hours under nitrogen atmosphere at 0°C. Dropwise addition of t-butylmagnesium bromide (102 mL of a 2 M solution in diethyl ether, 0.204 mol) at 0°C yielded an immediate yellow green suspension that was stirred for one hour under nitrogen atmosphere. The suspension was quenched by careful addition of 2 N HCl saturated with NH_4Cl (300 mL), and the organic layer was separated. The aqueous part was washed with ethyl acetate thrice. The combined organic portions were then washed with brine, and dried over $MgSO_4$. After the drying agent was filtered off and solvent was removed by rotary evaporation, a pale yellow oil was obtained. SiO_2 column chromatography using 1:1 hexane:ethyl acetate as the eluent of the crude oil yielded product (14 g, 67%) as white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.08 (ddd, *J* = 14.9, 2.7, 2.0 Hz, 1H), 3.33 (ddd, *J* = 14.9, 5.9, 1.5 Hz, 1H), 4.21 (dd, J = 5.9, 2.7 Hz, 1H), 5.21 (s, 2H), 6.17 (bs, 1H), 7.30-7.43 (m, 5H); ^{13}C NMR (75 MHz, CDCl3) δ 43.8, 47.5, 67.7, 128.7-128.9, 135.1, 166.4, 171. ESI/EMM mass spectrum *m/z* calc. 223.1008 $[M+NH_4]^+$, obs. 223.1011 $[M+NH_4]^+$.

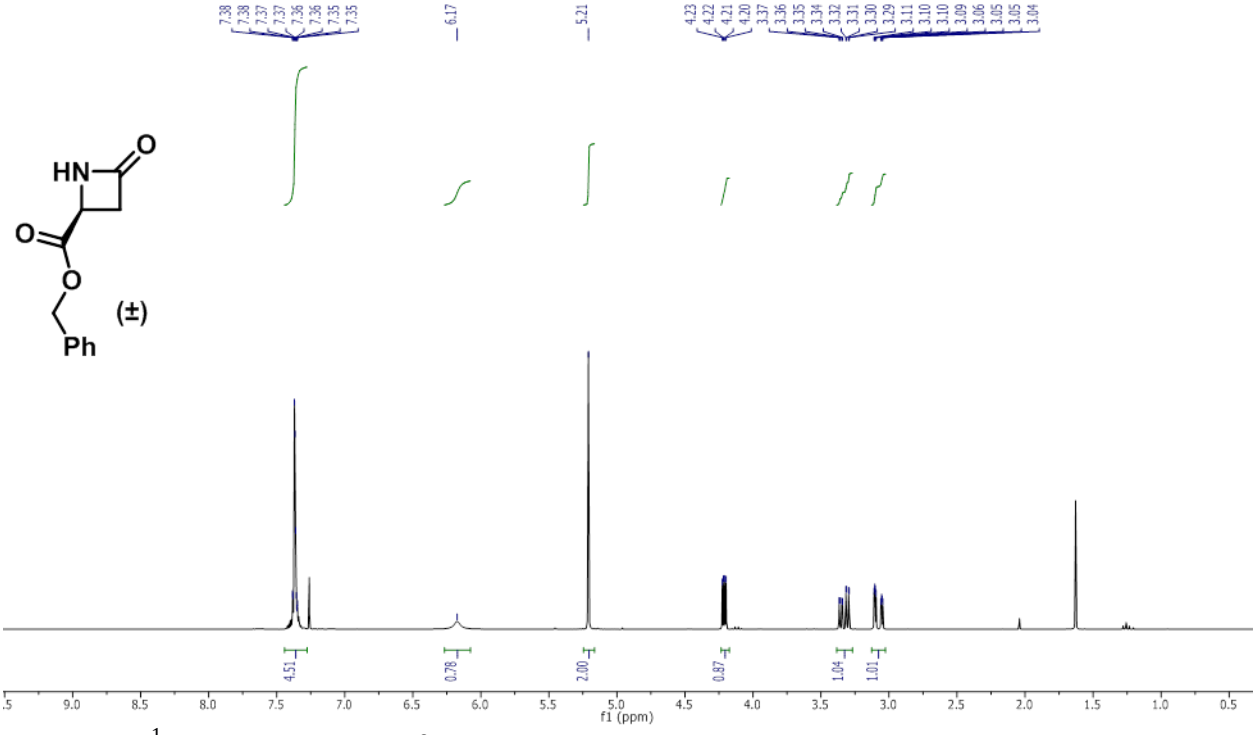


Figure S3. ^{1}H NMR spectrum of **(±)-benzyl azetidin-2-one-4-carboxylate.**

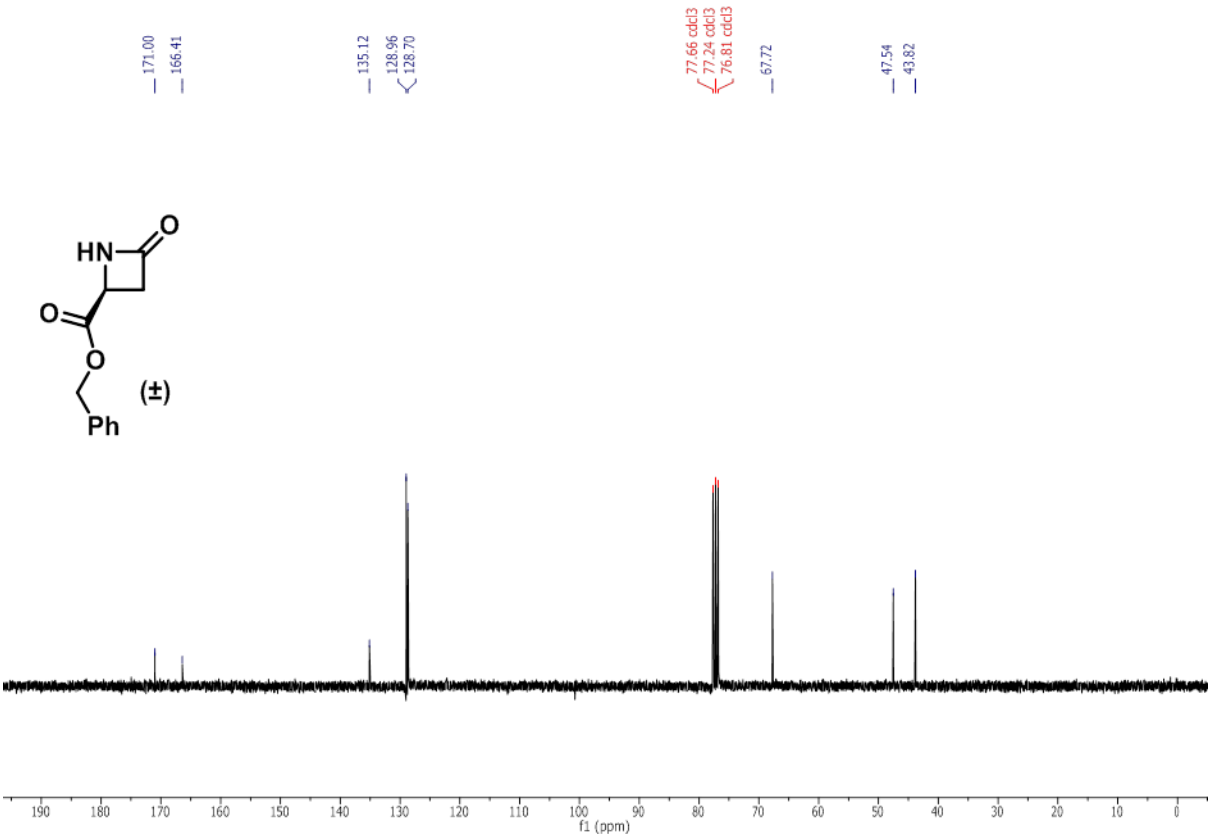


Figure S4. ^{13}C NMR spectrum of **(±)-benzyl azetidin-2-one-4-carboxylate.**

(±)-4-(Hydroxymethyl)-azetidin-2-one: To a solution of **(±)-benzyl azetidin-2-one-4-carboxylate** (14 g, 0.07 mol) in 250 mL dry methanol was added sodium borohydride (5.3 g, 0.14 mol) at 0^{o}C under nitrogen atmosphere. The mixture was stirred for 2 hours at room temperature, and then neutralized by addition of conc. HCl under cold conditions. After evaporation of the solvent, the residue was suspended in 2:1 $CHCl_3$:MeOH (450 mL) and stirred for two hours in presence of $MgSO_4$ as drying agent. The precipitate and drying agent were filtered off, and the residue was washed thoroughly with the same solvent mixture. Evaporation of the solvent from the combined filtrates by rotary evaporation provided a pale yellow oil. SiO_2 chromatography of the crude product using ethyl acetate containing 10 vol % methanol as the eluent provided product (6.3 g, 92%) as a white solid. Mp: 71.0-71.6^{o}C (literature Mp 60-66^{o}C)[2]. ^{1}H NMR (400MHz, $CDCl_3$) δ 1.86 (bs, 1H), 2.76 (ddd, *J*=14.8, 2.5, 1.3 Hz, 1H), 3.03 (ddd, *J*= 14.9, 5.1, 2.2 Hz, 1H), 3.66 (dt, *J*= 11.5, 6.0 Hz, 1H), 3.77-3.93 (m, 2H), 6.0 (bs, 1H). ^{13}C NMR

(125 MHz, $CDCl_3$) δ 39.9, 48.5, 64.6, 167.7. ESI/EMM mass spectrum *m/z* calc. 203.1027 $[2M+H]^+$, Obs. 203.1022 $[2M+H]^+$.

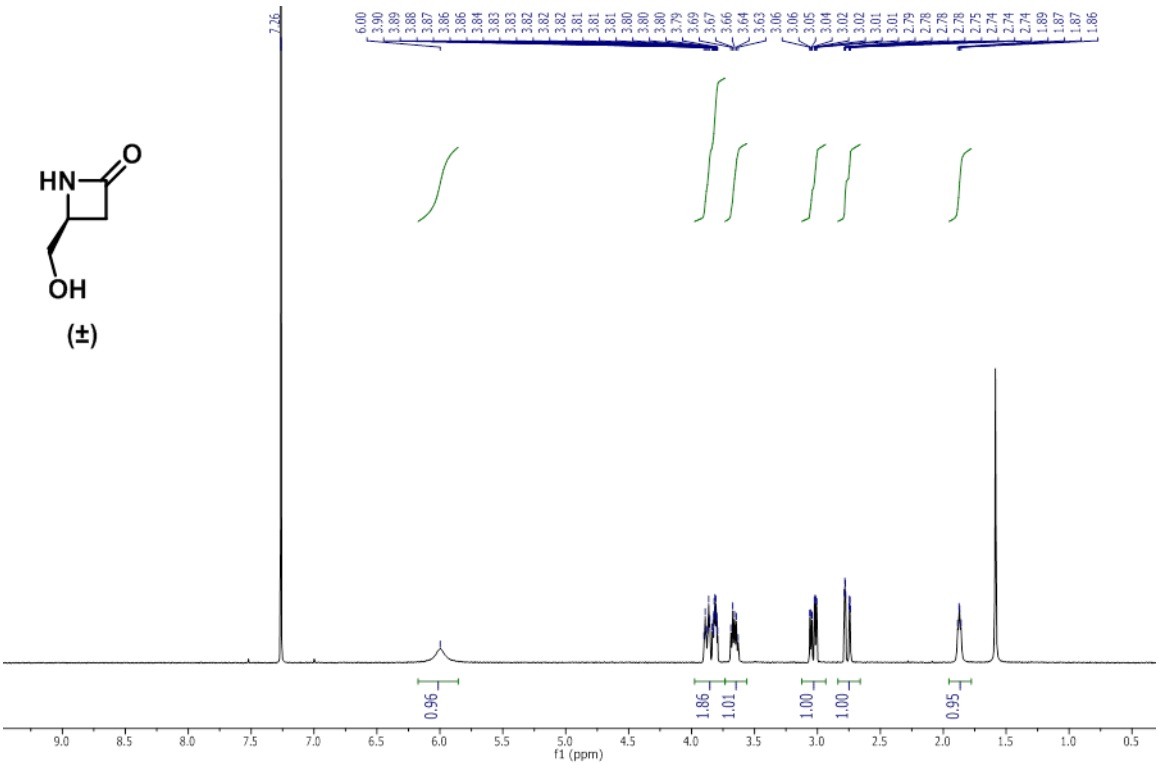


Figure S5. ^{1}H NMR spectrum of **(±)-4-(hydroxymethyl)-azetidin-2-one.**

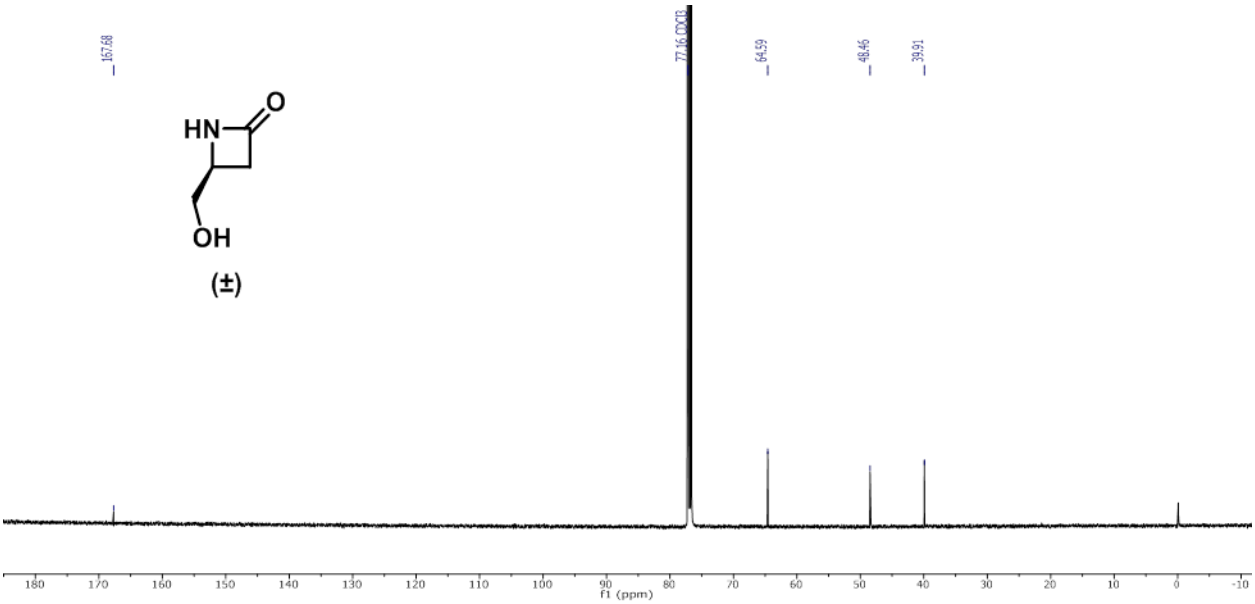


Figure S6. ^{13}C NMR spectrum of **(±)-4-(hydroxymethyl)-azetidin-2-one.**

(±)-4-(Trityloxymethyl)-azetidin-2-one (HSβ). Triphenylmethyl chloride (26.1 g, 0.093 mol) was added over 5 minutes at room temperature to a stirring mixture of **(±)-4-(Hydroxymethyl)-azetidin-2-one** (6.3 g, 0.062 mol), triethylamine (8.6 mL, 0.062 mol), DMAP (1.22 g, 0.01 mol) in dry CH_2Cl_2 (150 mL). The resulting solution was stirred at room temperature overnight under nitrogen atmosphere. The solution was washed with water thrice. The aqueous part was backwashed with CH_2Cl_2. The combined organic portions were washed with brine and dried over $MgSO_4$. After the drying agent was removed by filtration, the solvent evaporated to yield a yellow oil. The crude product was purified using SiO_2 column chromatography using 1:1 hexane:ethyl acetate to yield the product (14.5 g, 68%) as a white solid. Mp: 161.1-161.9^oC. ^1H NMR (500 MHz, $CDCl_3$) δ 2.63 (ddd, *J* = 14.9, 2.5, 1.5 Hz,1H), 2.99 (ddd, *J* = 14.8, 5.2, 1.9 Hz, 1H), 3.18 (dd, *J* = 9.8, 7.2 Hz, 1H), 3.34 (dd, J = 9.8, 4.0 Hz, 1H), 3.8 (ddt, J = 5.6, 2.6, 1.3 Hz, 1H), 5.88 (bs, 1H), 7.21-7.36 (m, 9H), 7.38-7.46 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 40.9, 47.4, 66.1, 86.9, 127.4, 128.1, 128.7, 143.7, 167.6. ESI/EMM mass spectrum *m/z* calc. 361.1911 $[M+NH_4]^+$, obs. 361.1919 $[M+NH_4]^+$.

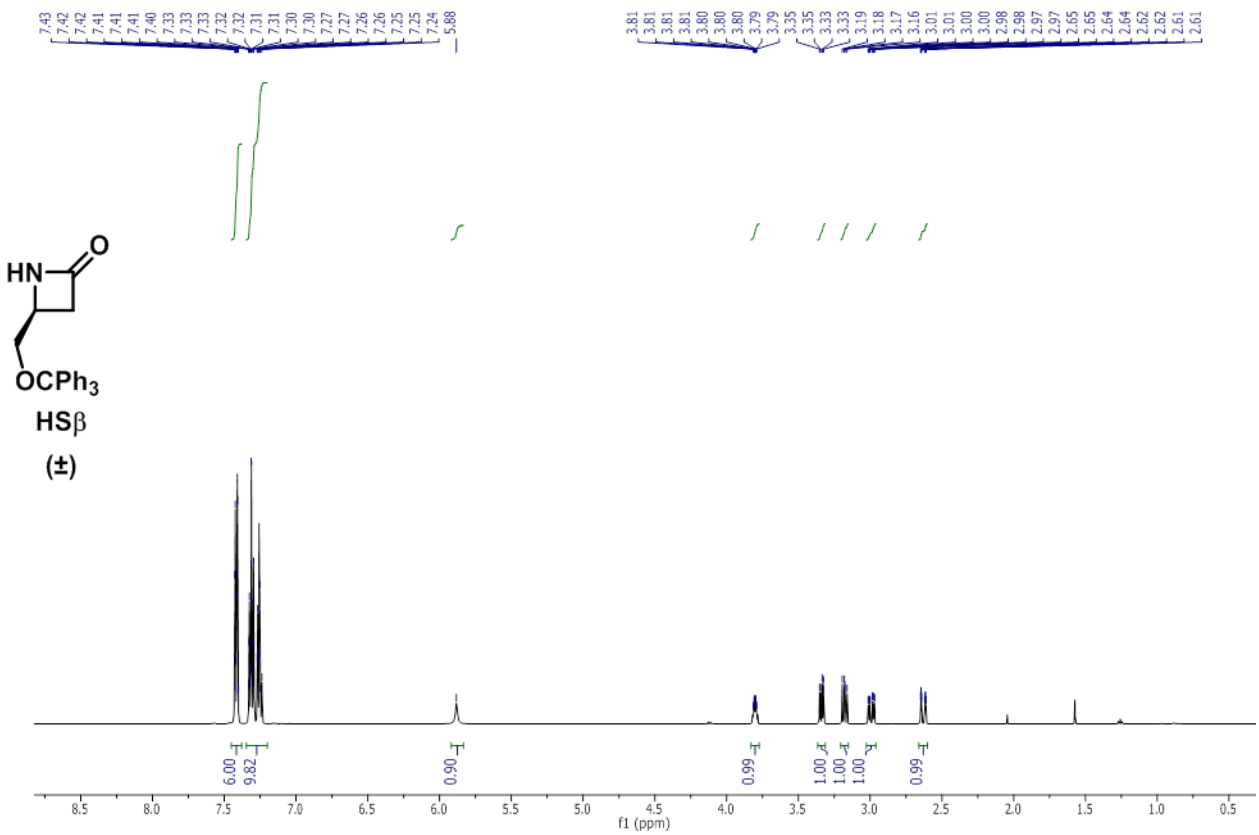
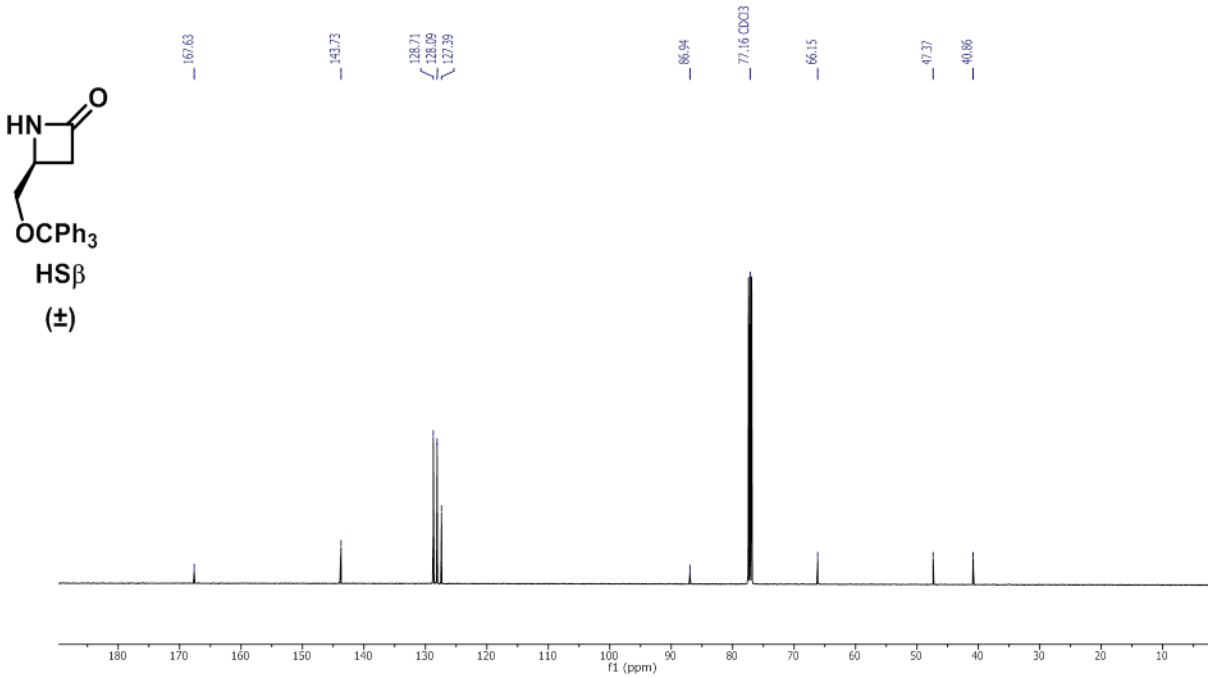
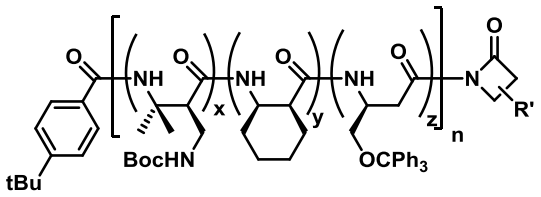


Figure S7. ^{1}H NMR spectrum of **(±)-4-(trityloxymethyl)-azetidin-2-one.**



FigureS8. ^{13}C NMR spectrum of **(±)-4-(trityloxymethyl)-azetidin-2-one.**

Table S1. Molecular weights and polydispersities of protected **DM+CH+HS** copolymers.



Ratio (x:y:z)	GPC data for protected polymer				
	Mn (Da)[a]	Mw (Da)[b]	PDI[c]	Expected DP[d]	Obs. DP[d]
50:40:10	5731	6271	1.09	20	28
50:25:25	7482	8050	1.08	20	32
40:50:10	5443	5916	1.09	20	28
40:40:20	5539	5832	1.05	20	26
45:45:10	4943	5535	1.12	20	25
50:50:0	4766	5004	1.05	20	26

[a] the number average molecular weight; [b] the weight average molecular weight; [c]polydispersity index; [d]degree of polymerization, i.e., the average number of subunits.

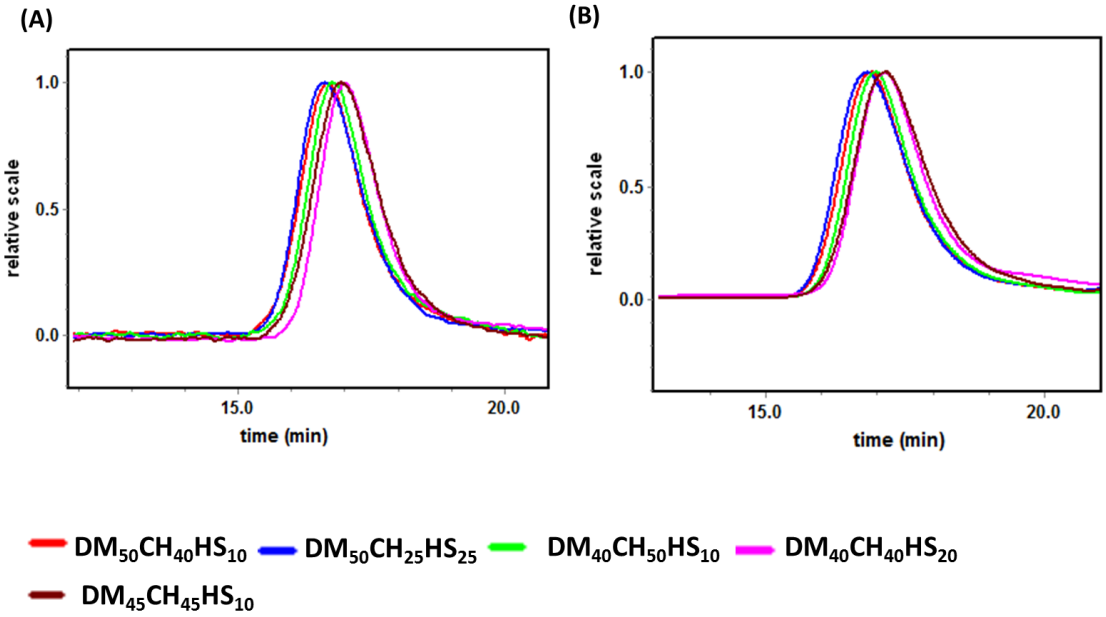
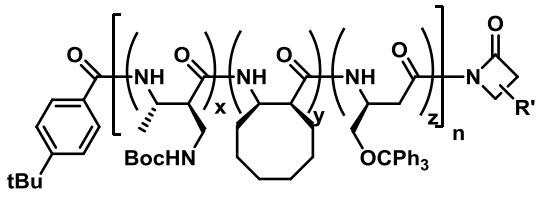


Figure S9. GPC chromatograms for Boc- and trityl-protected copolymers, with detection by light scattering (A) and refractive index (B).

Table S2. Molecular weights and polydispersities of protected **MM+CO+HS** copolymers.



Ratio (x:y:z)	GPC data for protected polymer				
	Mn (Da)[a]	Mw (Da)[b]	PDI[c]	Expected DP[d]	Obs. DP[d]
60:30:10	5441	5798	1.07	20	25
60:25:15	6296	7981	1.27	20	28
60:10:30	8873	9341	1.05	20	35
60:40:0	4402	4686	1.06	20	22

[a] the number average molecular weight; [b] the weight average molecular weight; [c]polydispersity index; [d]degree of polymerization, i.e., the average number of subunits.

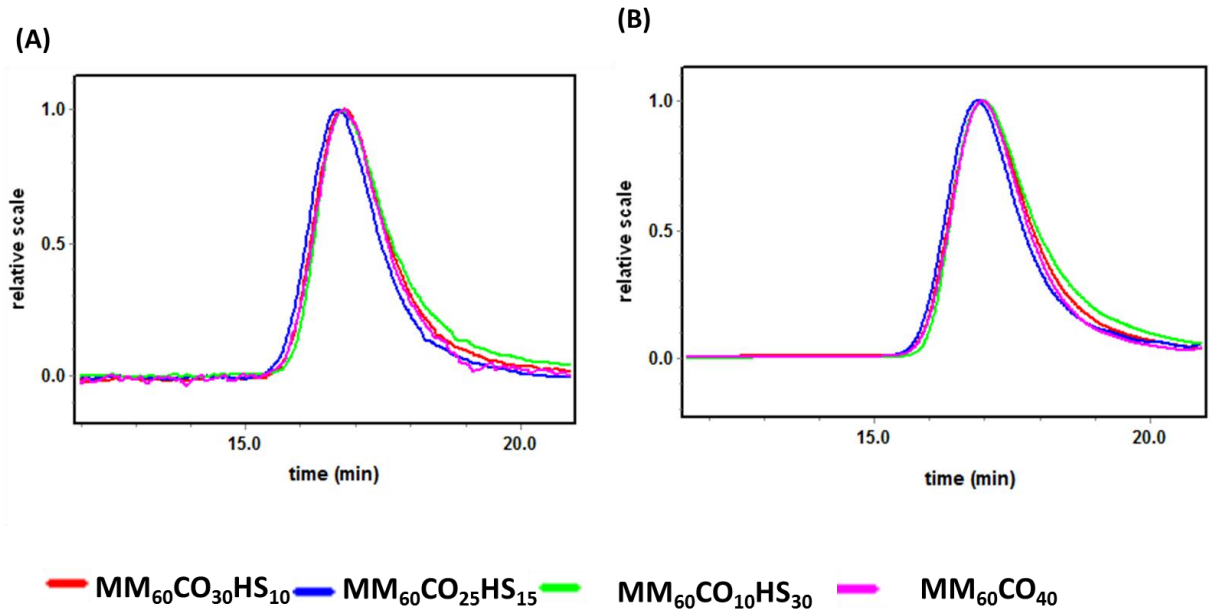
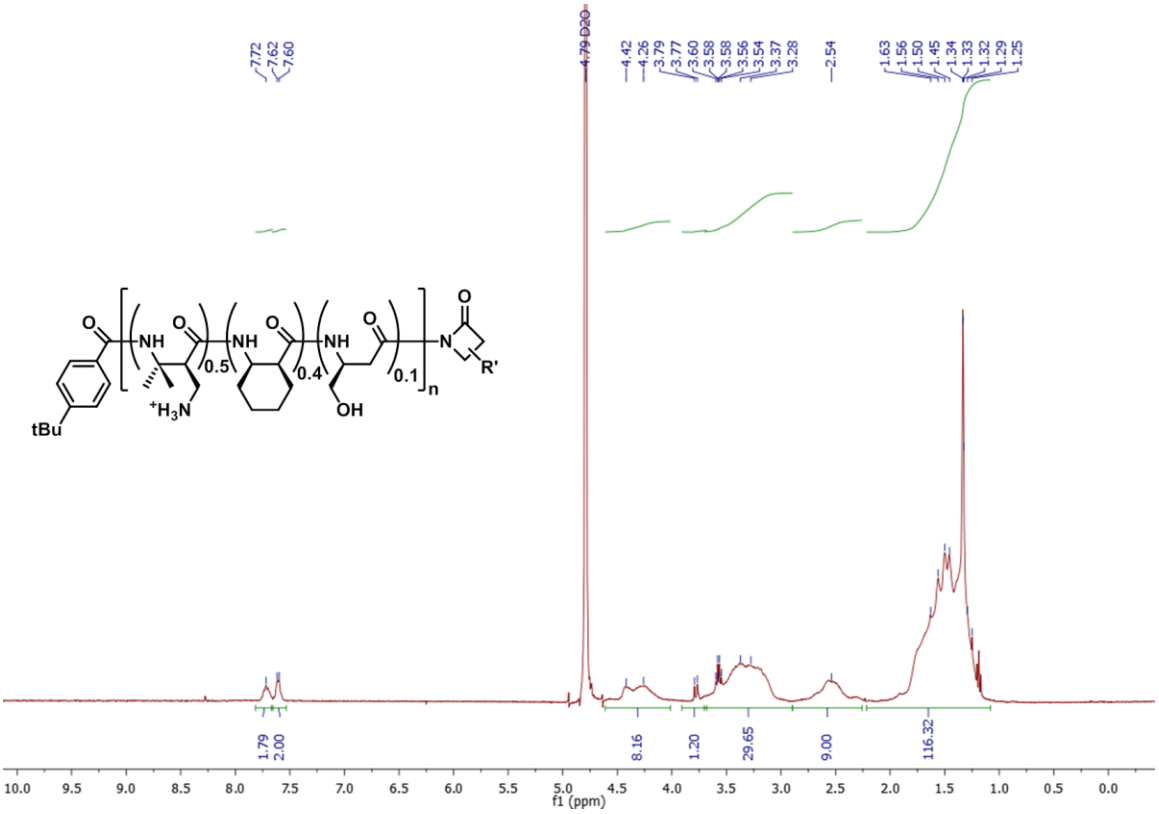
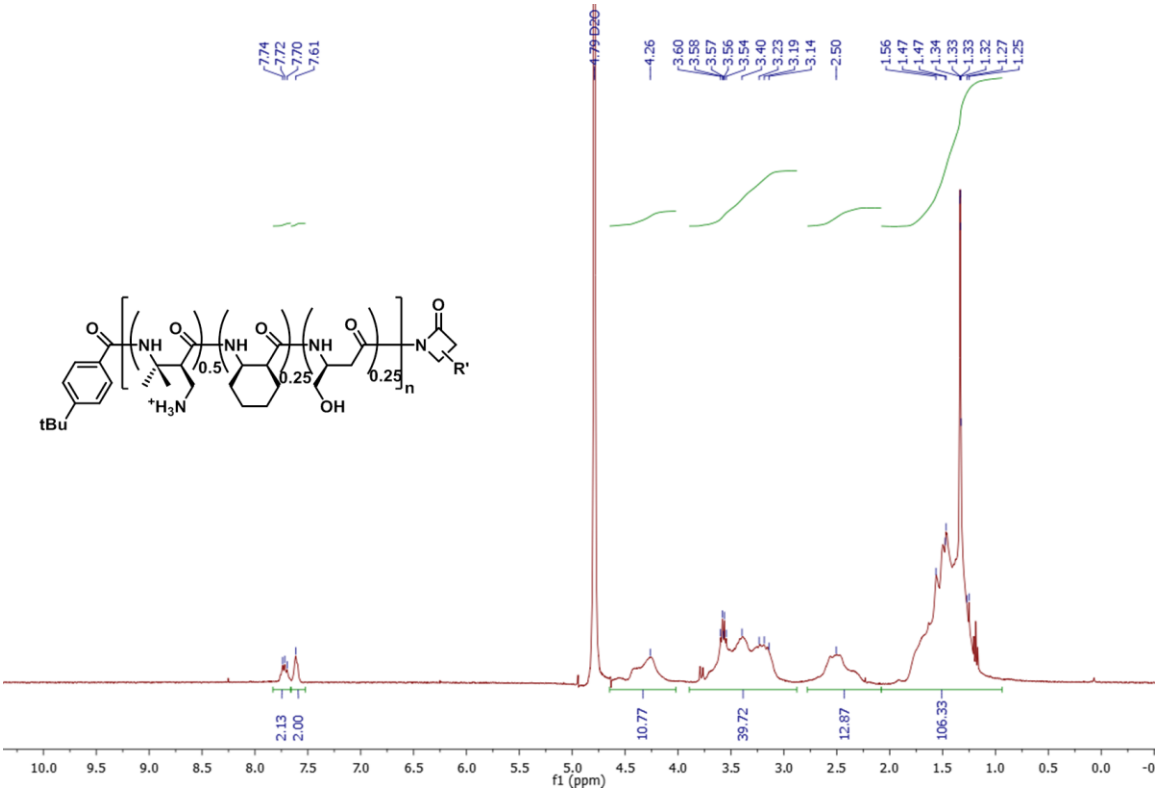


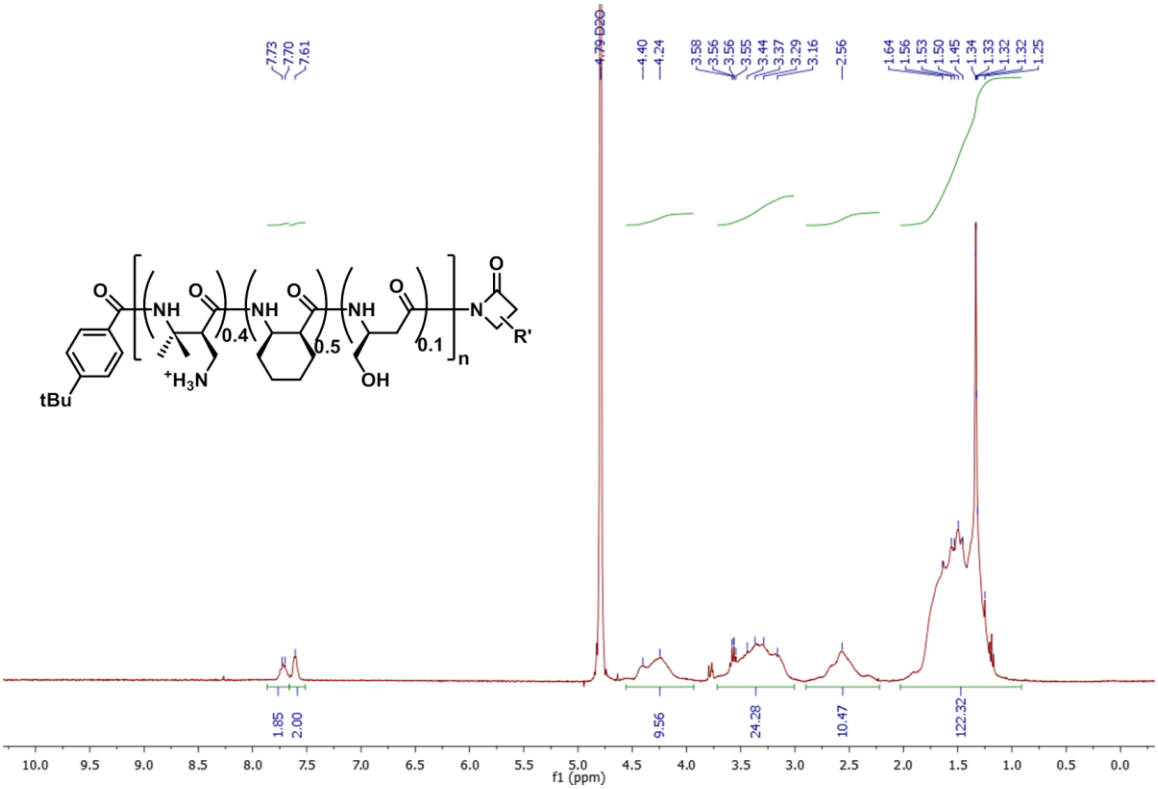
Figure S10. GPC chromatograms for Boc- and trityl-protected copolymers, with detection by light scattering (A) and refractive index (B).



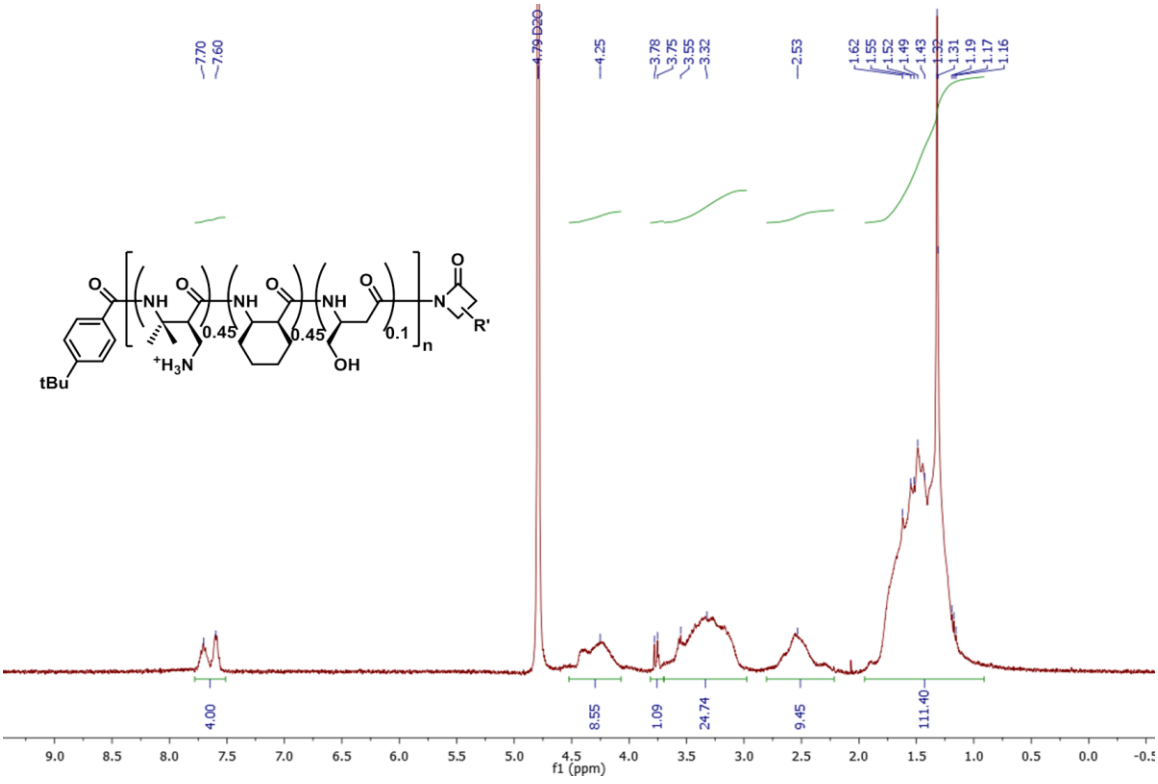
^{1}H NMR of deprotected $DM_{50}CH_{40}HS_{10}$ in D_2O.



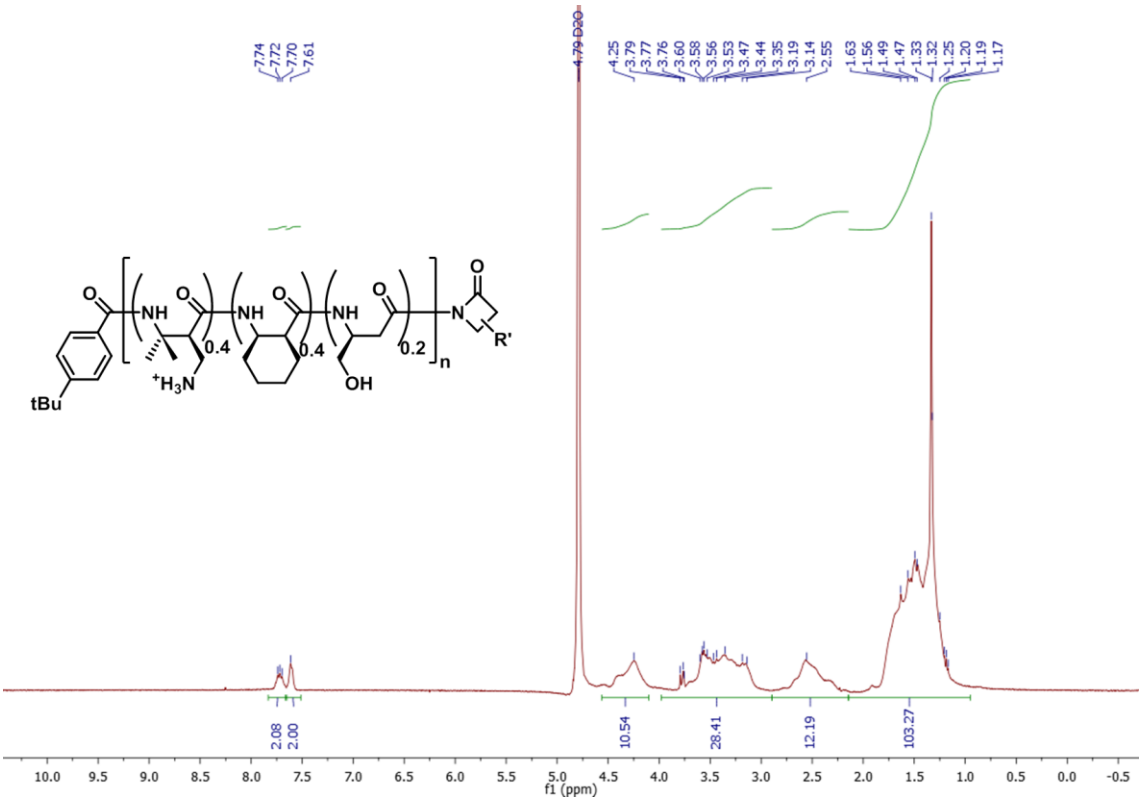
^{1}H NMR of deprotected $DM_{50}CH_{25}HS_{25}$ in D_2O.



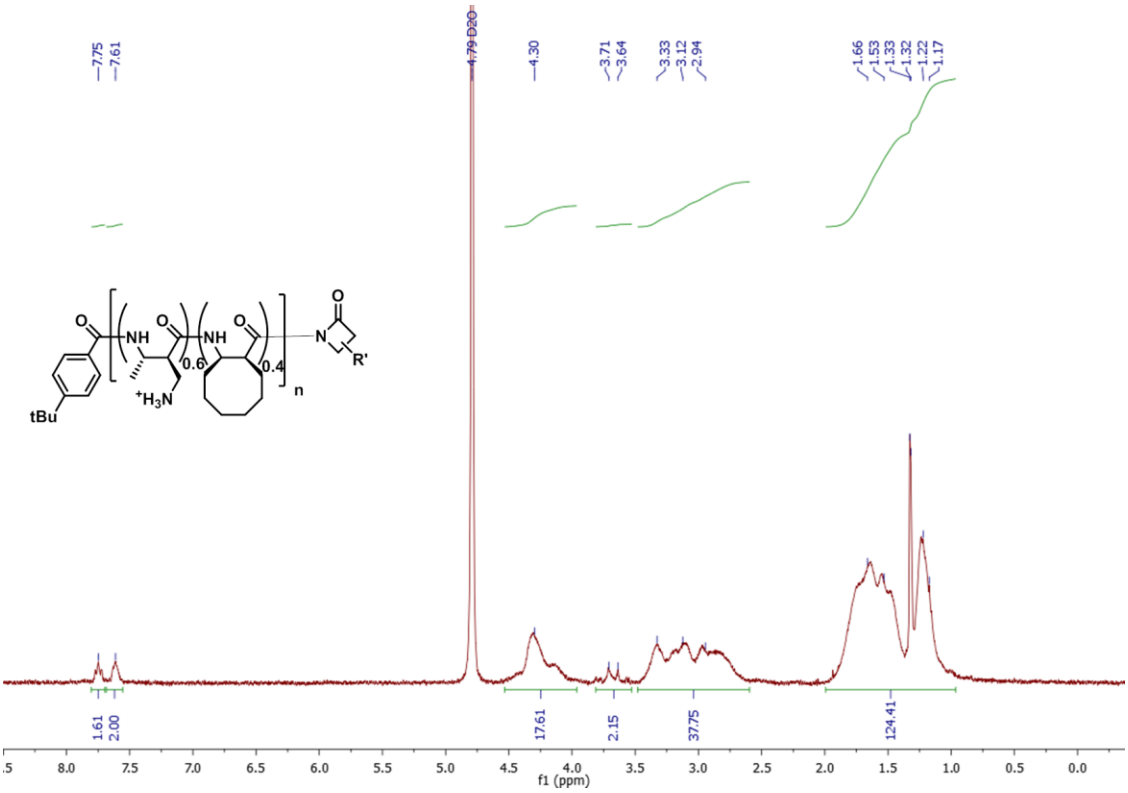
^{1}H NMR of deprotected $DM_{40}CH_{50}HS_{10}$ in D_2O.



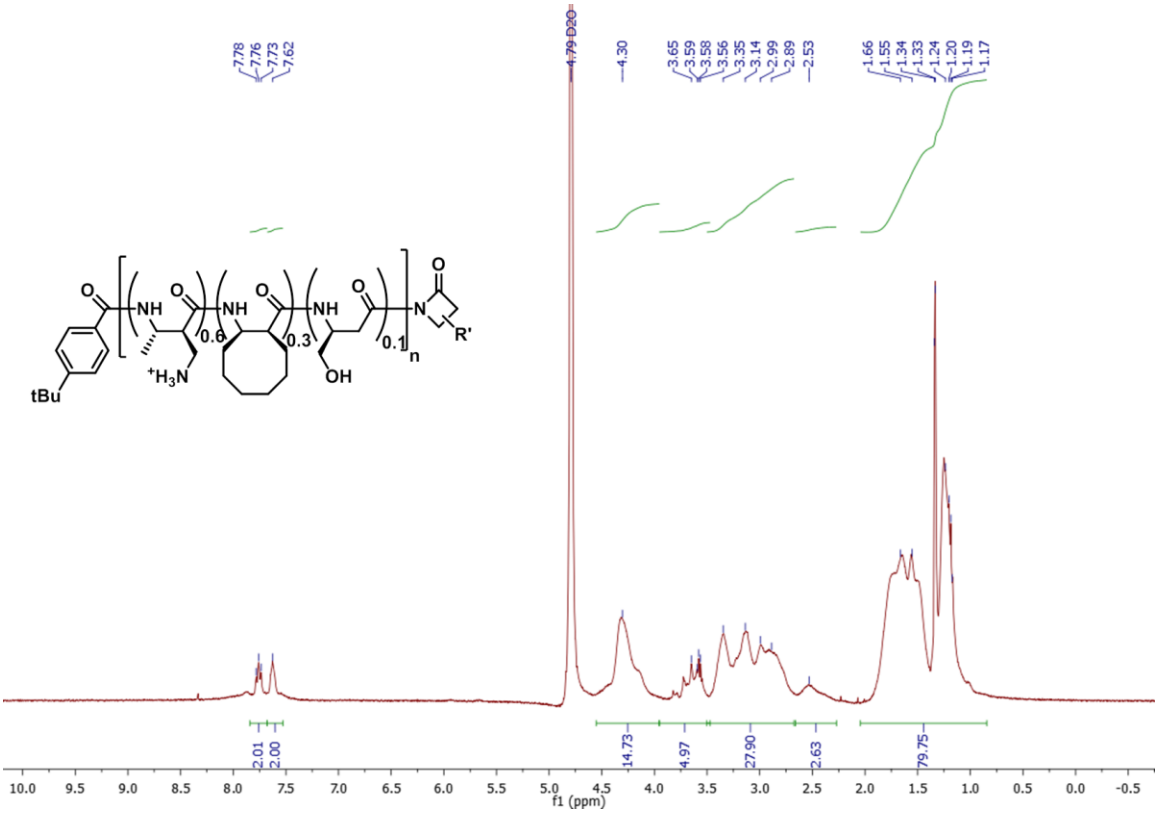
^{1}H NMR of deprotected $DM_{45}CH_{45}HS_{10}$ in D_2O.



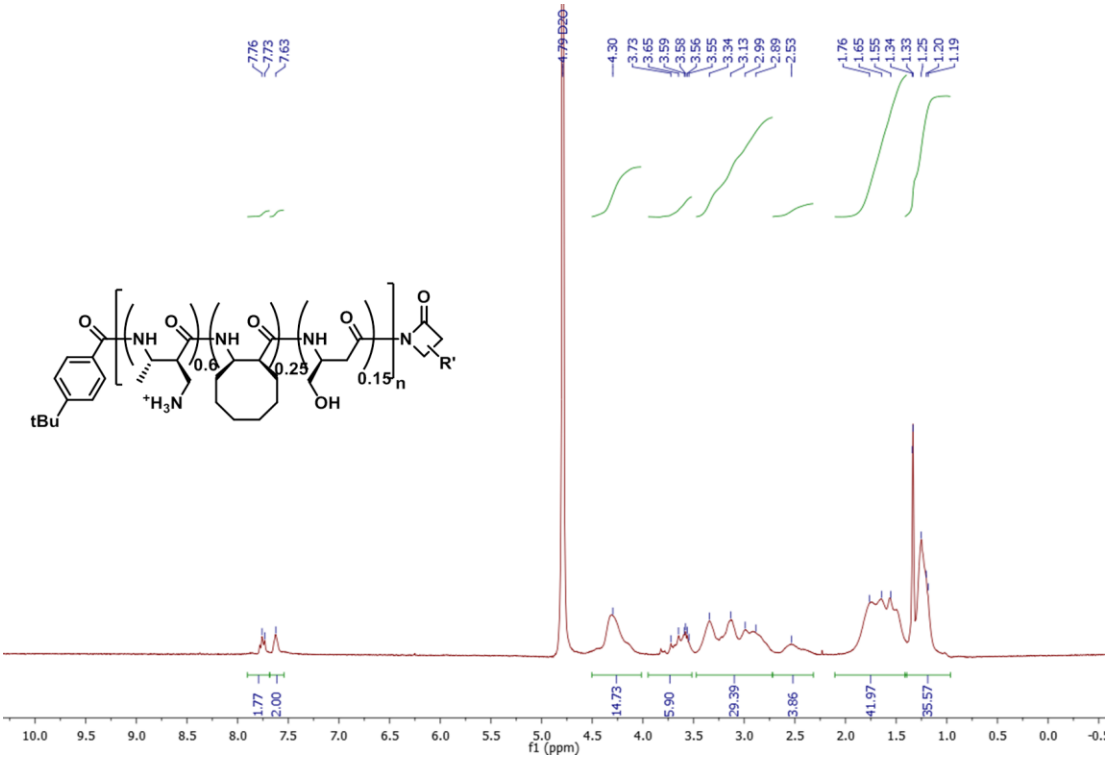
^{1}H NMR of deprotected $DM_{40}CH_{40}HS_{20}$ in D_2O.



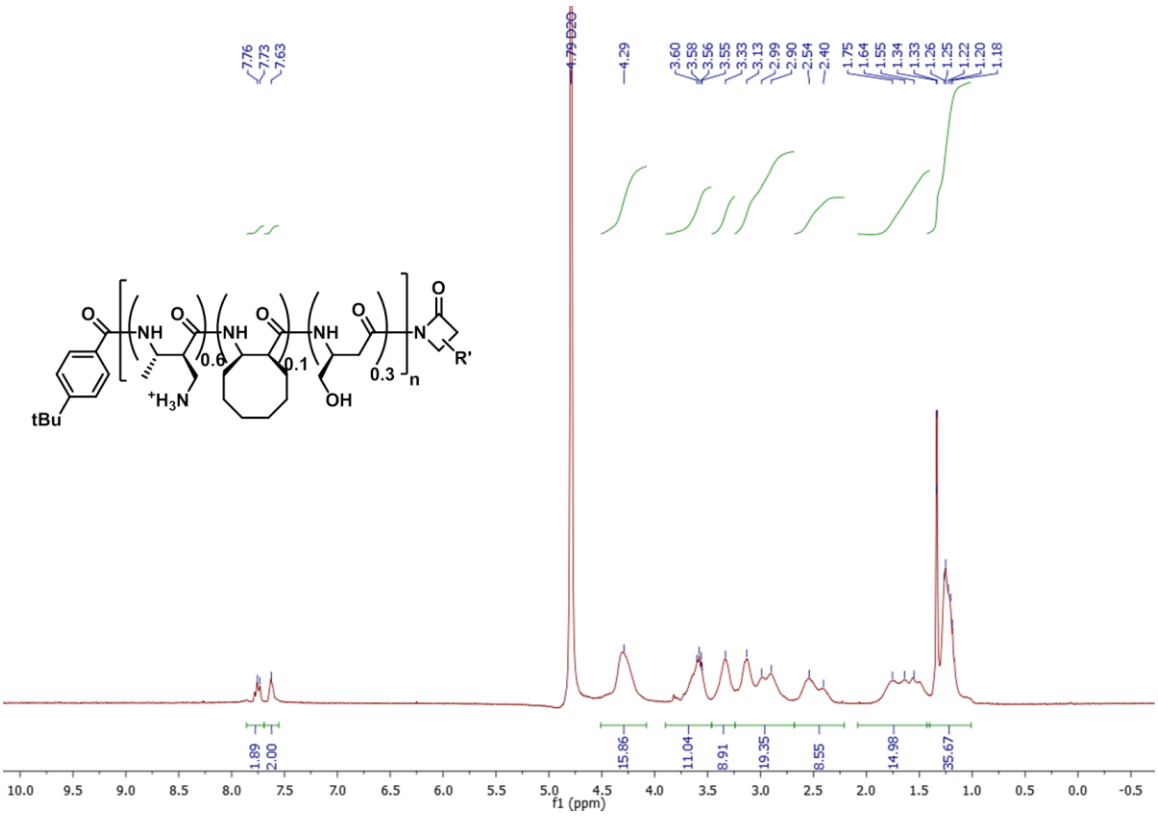
^{1}H NMR of deprotected $MM_{60}CO_{40}$ in D_2O.



^{1}H NMR of deprotected $MM_{60}CO_{30}HS_{10}$ in D_2O.



^{1}H NMR of deprotected $MM_{60}CO_{25}HS_{15}$ in D_2O.



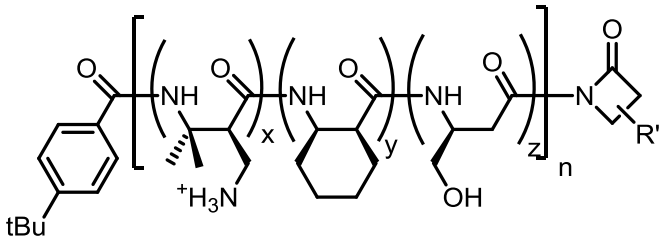
^{1}H NMR of deprotected $MM_{60}CO_{10}HS_{30}$ in D_2O.

Bioassays of polymers

Bacterial growth inhibition assays (MIC). Assays were performed as previously reported with some moderate changes in the procedure.[3] The bacteria used in these assays were *Escherichia coli* JM109,[4] *Bacillus subtilis* BR151,[5] *Staphylococcus aureus* 1206,[6] and *Enterococcus faecium* A634.[7] Antibacterial activities were determined in sterile 96-well plates (BD Falcon 353072 tissue culture plates). Bacterial cells were grown overnight at 37 ℃ on agar, after which a bacterial suspension of approximately 2 x 10^6 CFU/mL in Luria Bertani (LB) growth medium was prepared. Samples (50 µL) of this suspension were added to 50 µL of medium containing the polymer in 2-fold serial dilutions for a total volume of 100 µL in each well. The plates were then incubated at 37 ℃ for 6 h. Bacterial growth was determined by measuring the optical density (OD) at 650 nm using a Molecular Devices Emax precision

microplate reader. Positive control was OD without addition of polymer and negative control was OD of the medium without inoculum. The minimum inhibitory concentration (MIC) is defined as the lowest concentration at which complete inhibition of bacterial growth was observed (no increase in OD over the course of the experiment). MIC determinations were reproducible to within a factor of 2.

Hemolytic assay: Assays were performed as previously reported with some moderate changes in the procedure.[8] Human red blood cells (hRBC) were washed three times with Tris-buffered saline (pH 7.2, 0.01 M Tris-HCl, 0.155 M NaCl) and centrifuged at 3500 rpm, until the supernatant was clear. Two-fold serial dilutions of polymer in Tris-buffered saline were added to each well in a sterile 96-well plate (BD Falcon 353072 tissue culture plates), for a total volume of 100 μL in each well. A 2% (v/v) hRBC suspension (100 μL in Tris buffer) was added to each well. The plates were incubated at 37 ℃ for 1 h, and then the cells were pelleted by centrifugation at 3500 rpm for 5 min. The supernatant (80 μL) was transferred to a fresh plate, and hemoglobin was detected by measuring OD at 405 nm. The average OD of cells incubated with TX-100 at 1600, 800, 400, 200 μg/mL defines 100%; the OD of cells incubated in Tris buffer defines 0% hemolysis.



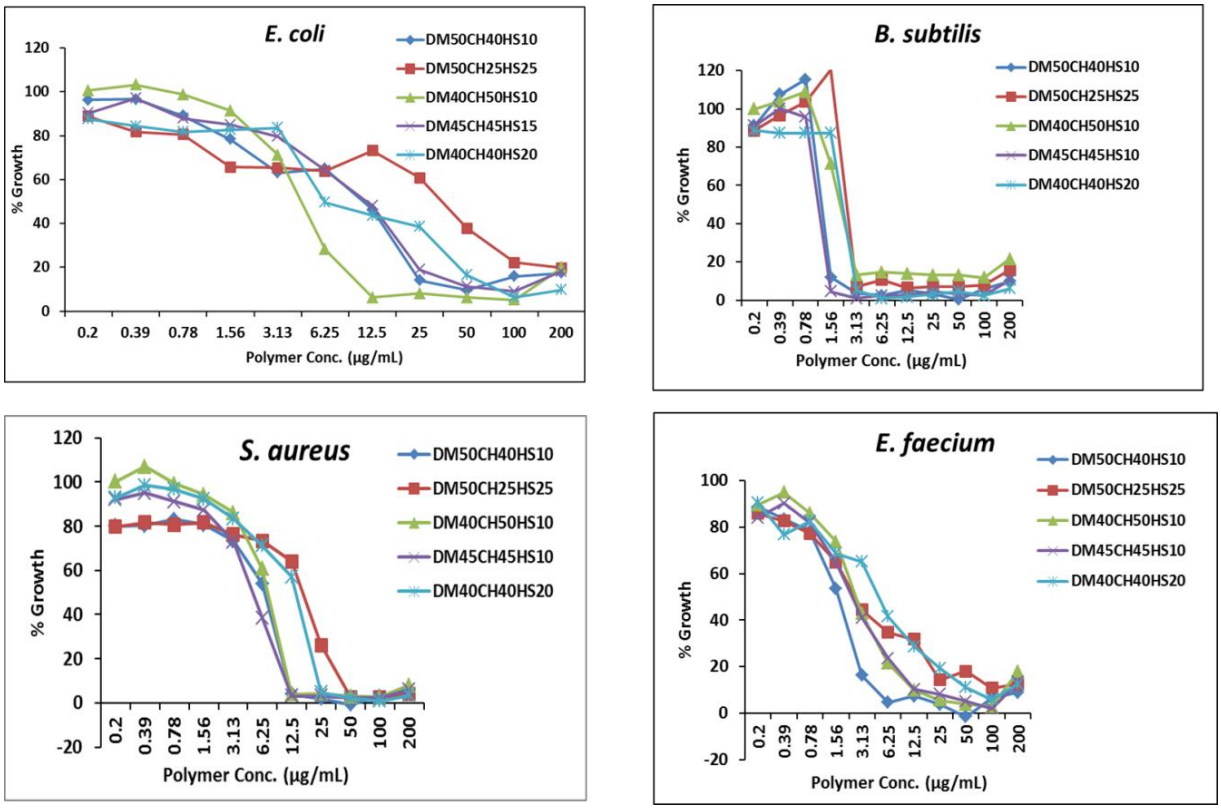
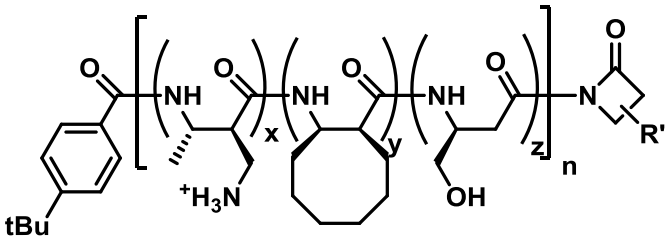


Figure S11. Representative dose-response curves of antibacterial activity of **DM+CH+HS** copolymers.



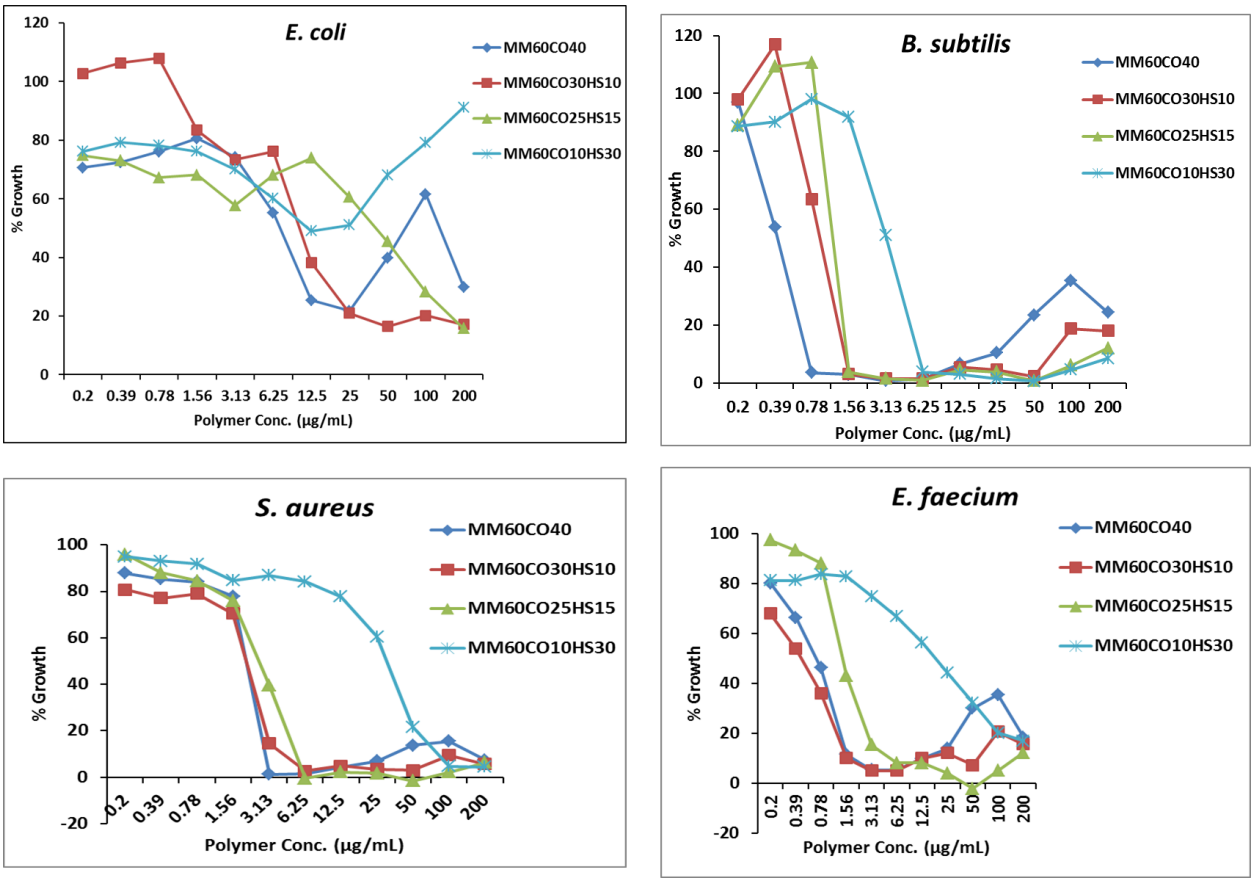
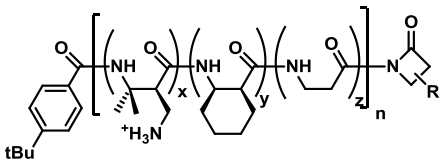


Figure S12. Representative dose-response curves of antibacterial activity of **MM+CO+HS** copolymers.

Table S3. Molecular weights and polydispersities of protected **DM+CH+HG** copolymers.



Ratio (x:y:z)	GPC data for protected polymer				
	Mn (Da)[a]	Mw (Da)[b]	PDI[c]	Expected DP[d]	Obs. DP[d]
40:50:10	4383	5324	1.21	20	26
50:40:10	4341	5406	1.25	20	24
50:25:25	5539	6927	1.25	20	33
47.5:47.5:5	3558	4280	1.20	20	20
45:45:10	3174	3928	1.24	20	18

[a] the number average molecular weight; [b] the weight average molecular weight; [c]polydispersity index; [d]degree of polymerization, i.e., the average number of subunits.

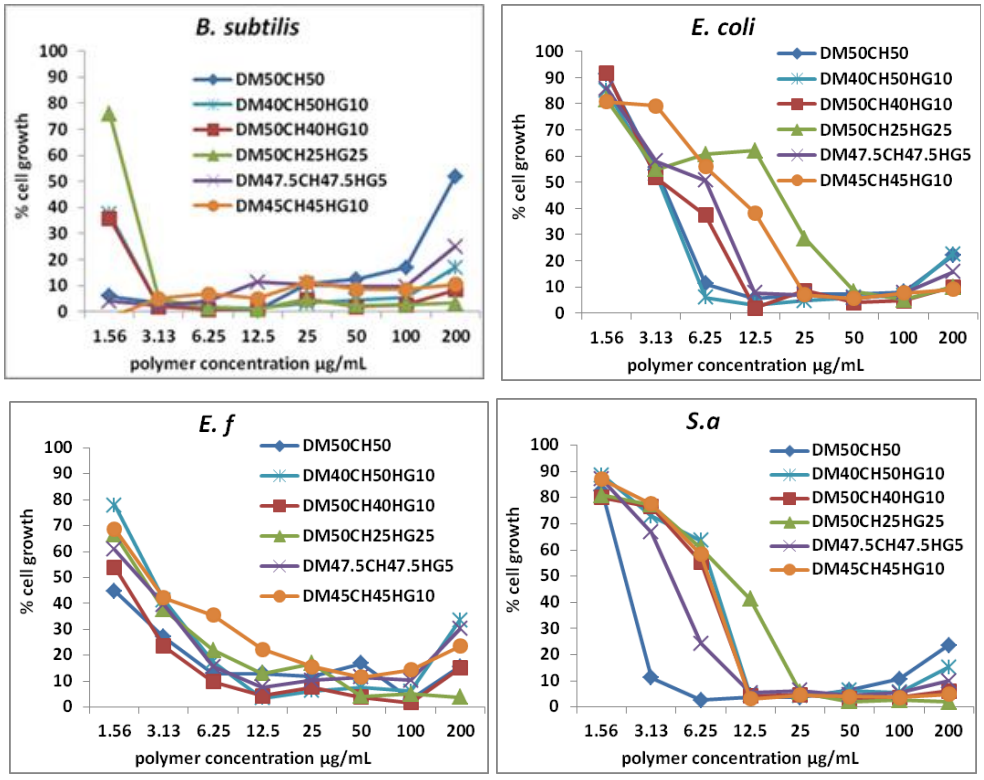
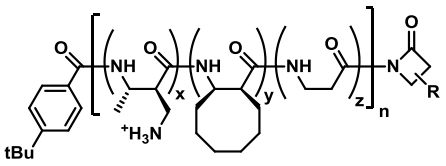


Figure S13. Representative dose-response curves of antibacterial activity of **DM+CH+HG** copolymers.

Table S4. Molecular weights and polydispersities of protected **MM+CO+HG** copolymers.



Ratio (x:y:z)	GPC data for protected polymer				
	Mn (Da)[a]	Mw (Da)[b]	PDI[c]	Expected DP[d]	Obs. DP[d]
60:30:10	5352	6888	1.29	20	29
60:25:15	5704	7485	1.31	20	31
60:10:30	5670	8077	1.43	20	33

[a] the number average molecular weight; [b] the weight average molecular weight; [c]polydispersity index; [d]degree of polymerization, i.e., the average number of subunits.

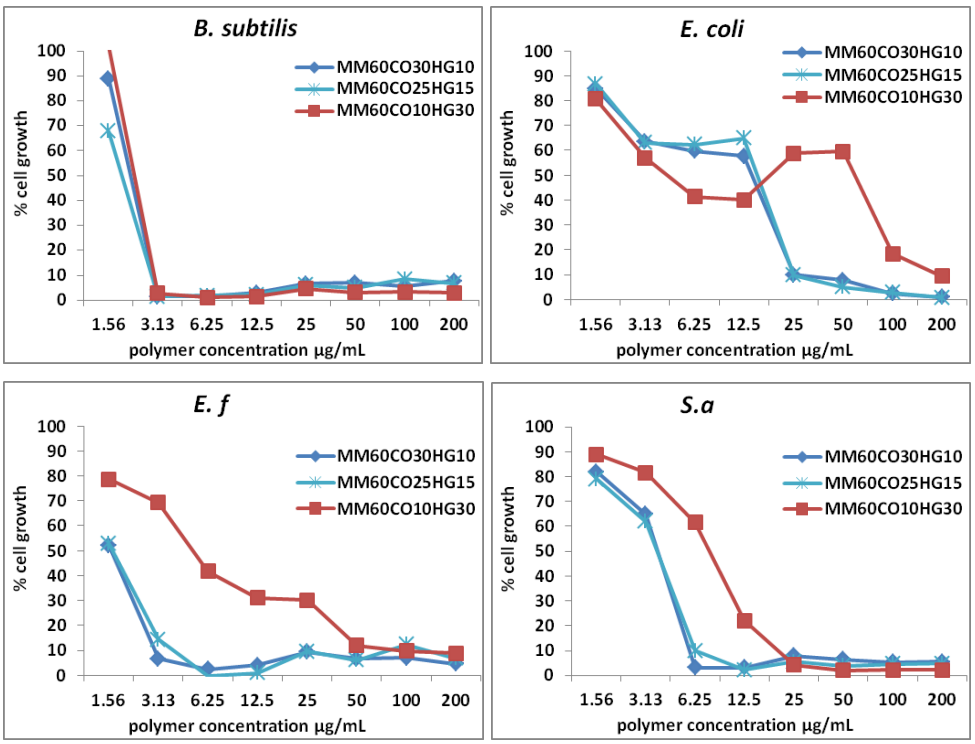
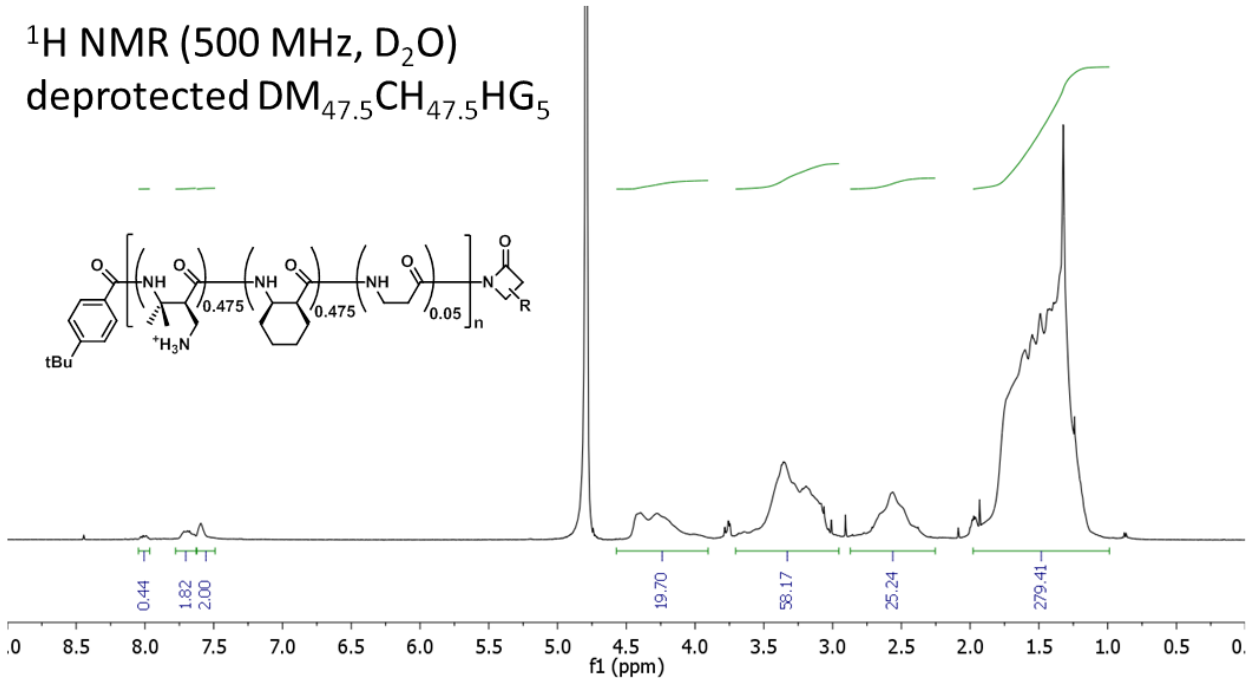
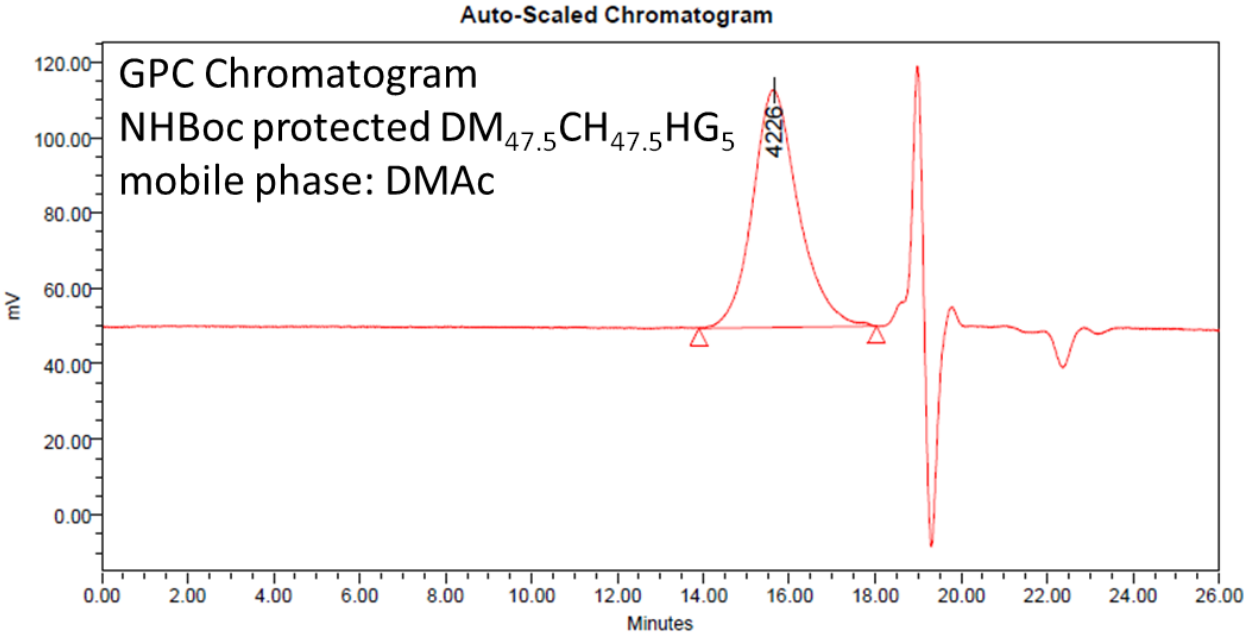


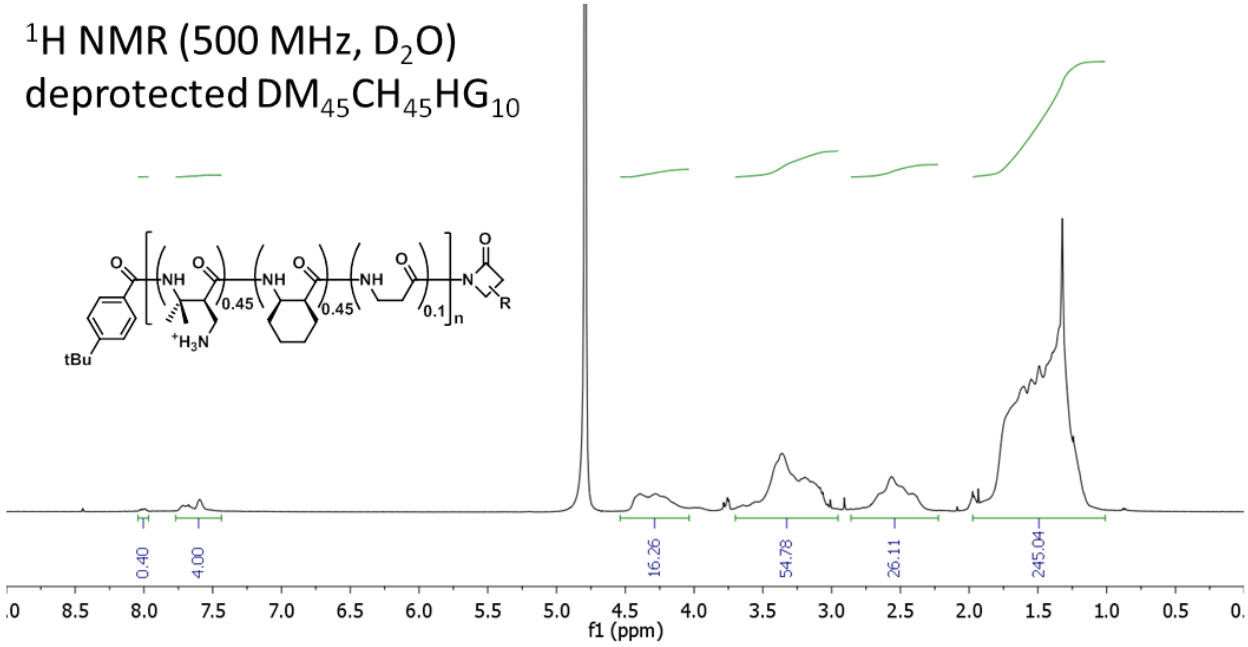
Figure S14. Representative dose-response curves of antibacterial activity of **MM+CO+HG** copolymers.

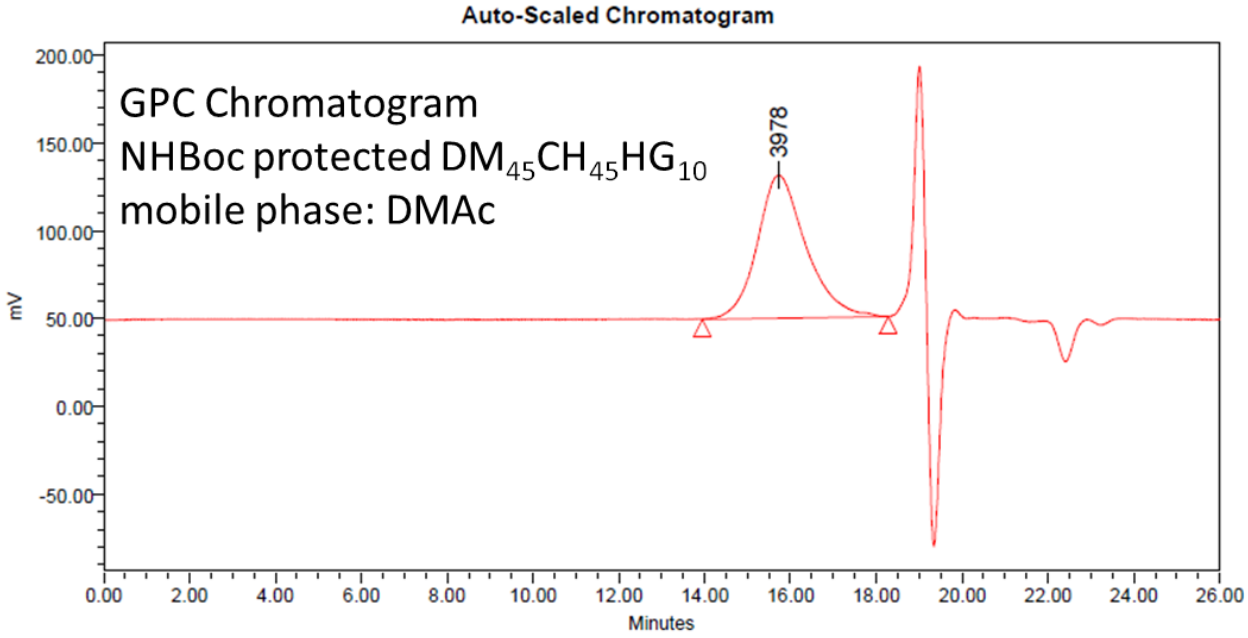




GPC Results

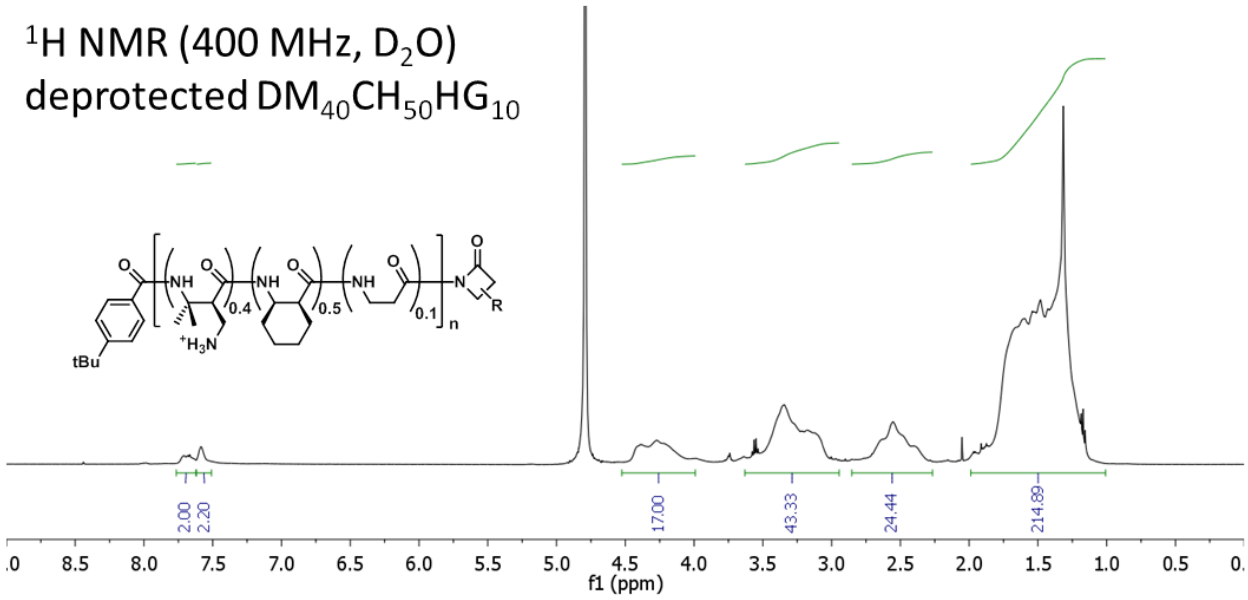
	Dist Name	Mn	Mw	Mv	MP	Mz	Mz+1	Polydispersity	K	alpha
1		3558	4280		4226	4992	5754	1.202870		

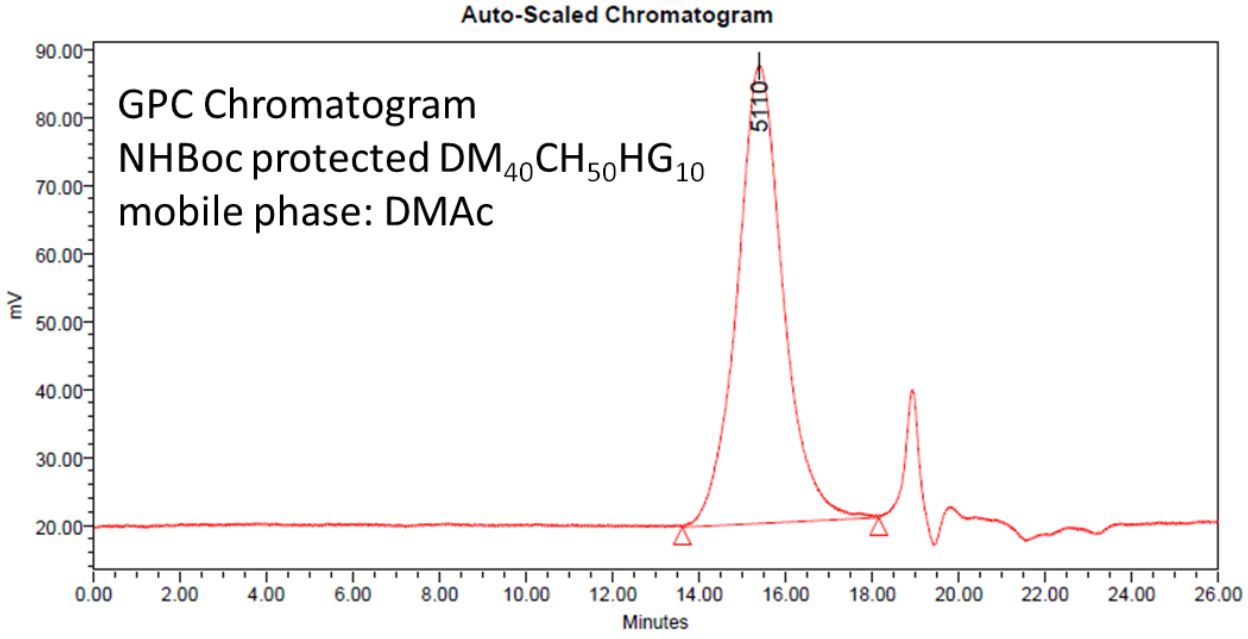




GPC Results

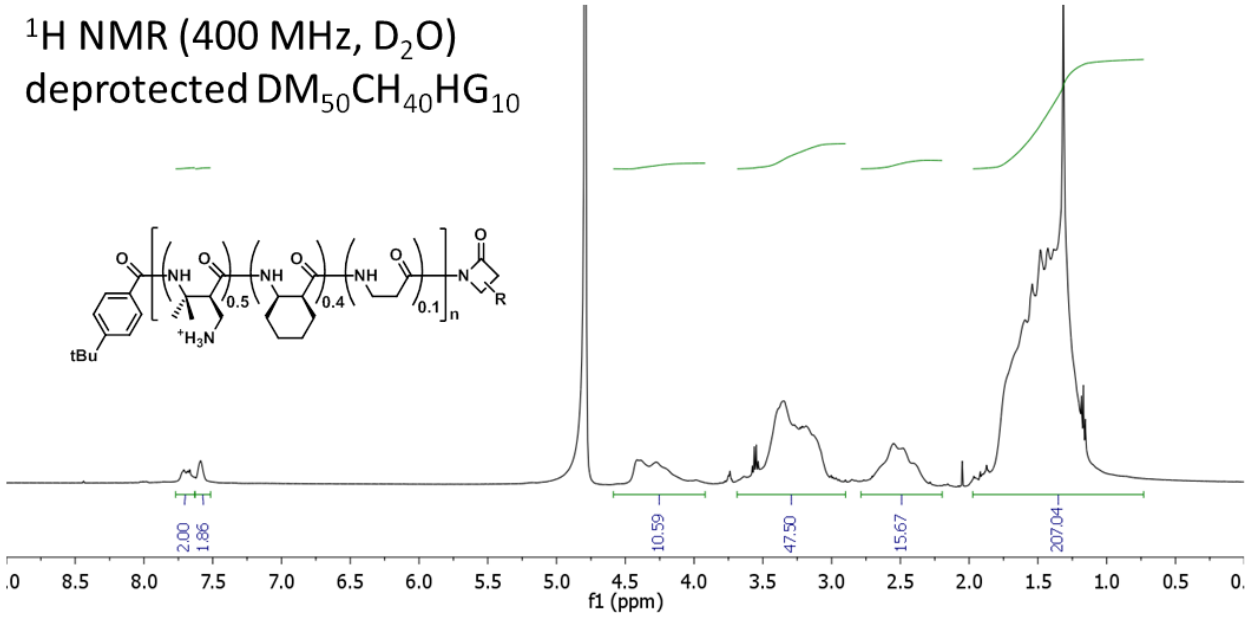
	Dist Name	Mn	Mw	Mv	MP	Mz	Mz+1	Polydispersity	K	alpha
1		3174	3928		3978	4659	5430	1.237640		

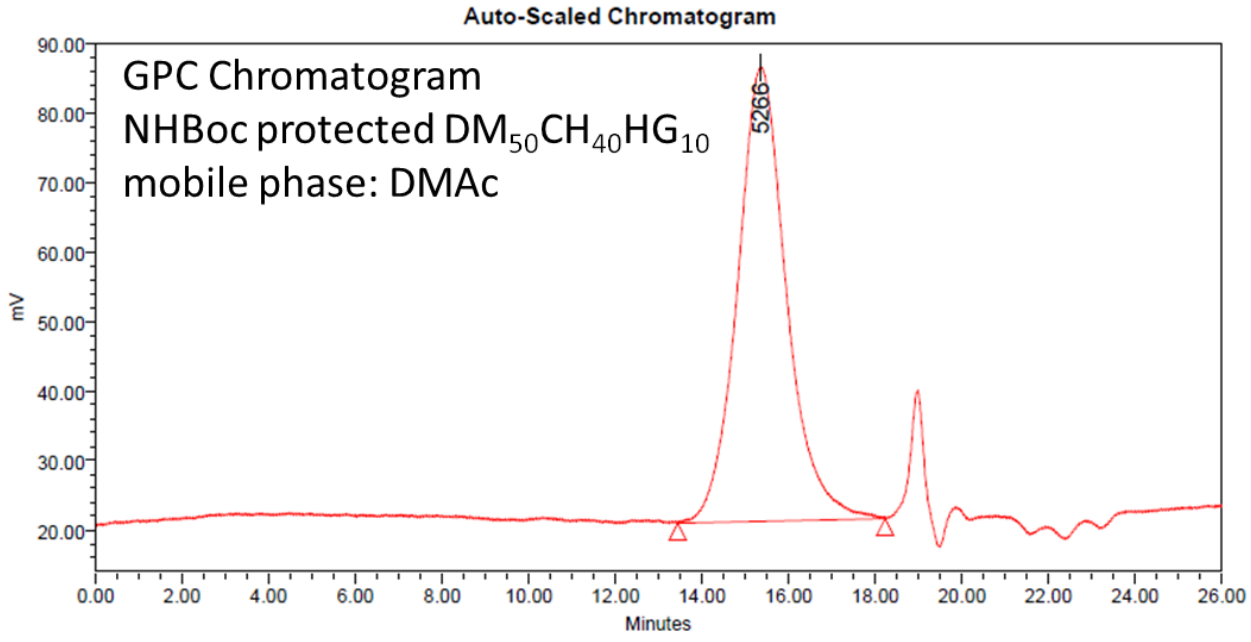




GPC Results

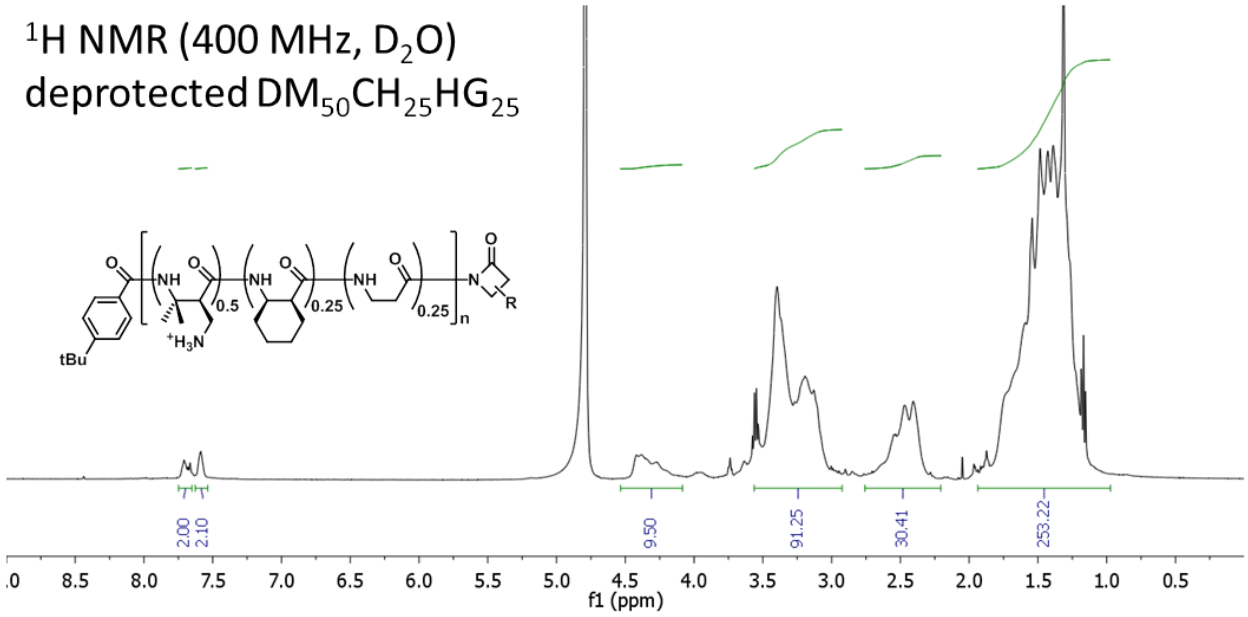
	Dist Name	Mn	Mw	Mv	MP	Mz	Mz+1	Polydispersity	K	alpha
1		4383	5324		5110	6270	7330	1.214498		

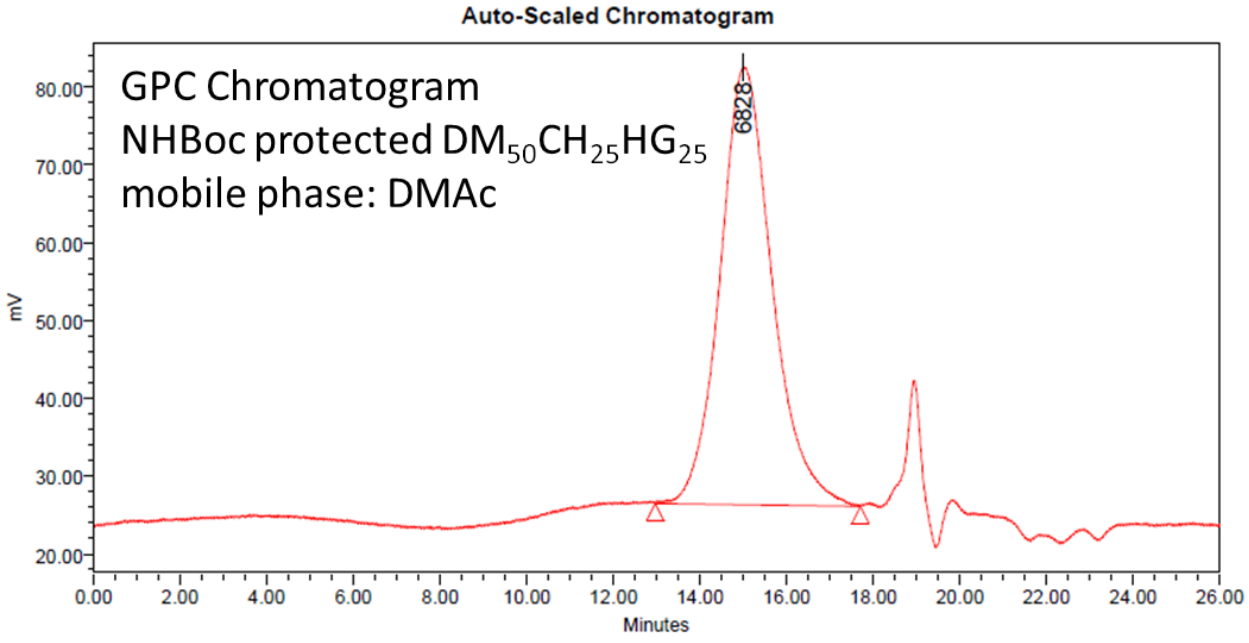




GPC Results

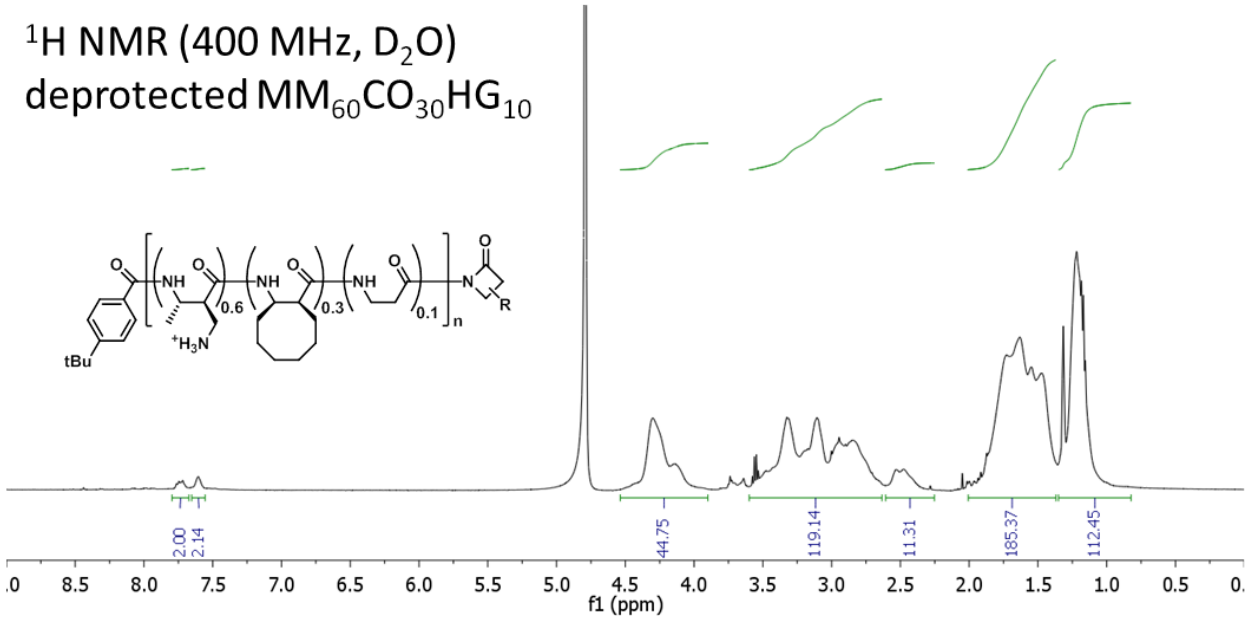
	Dist Name	Mn	Mw	Mv	MP	Mz	Mz+1	Polydispersity	K	alpha
1		4341	5406		5266	6457	7638	1.245193		

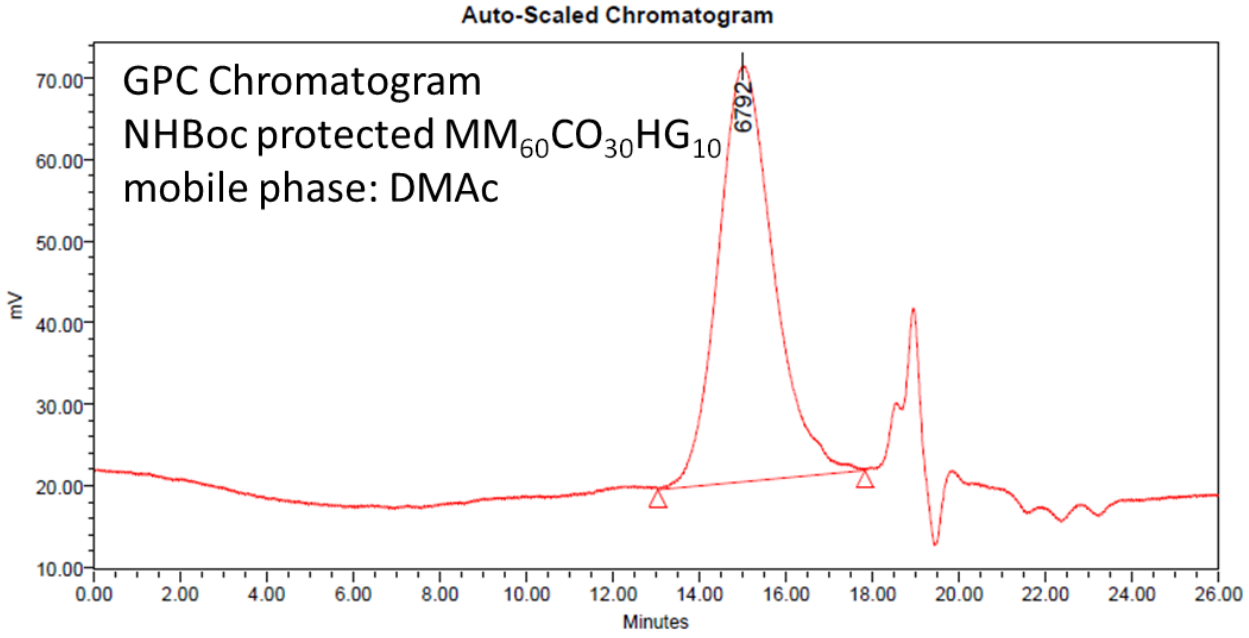




GPC Results

	Dist Name	Mn	Mw	Mv	MP	Mz	Mz+1	Polydispersity	K	alpha
1		5539	6927		6828	8427	10232	1.250613		

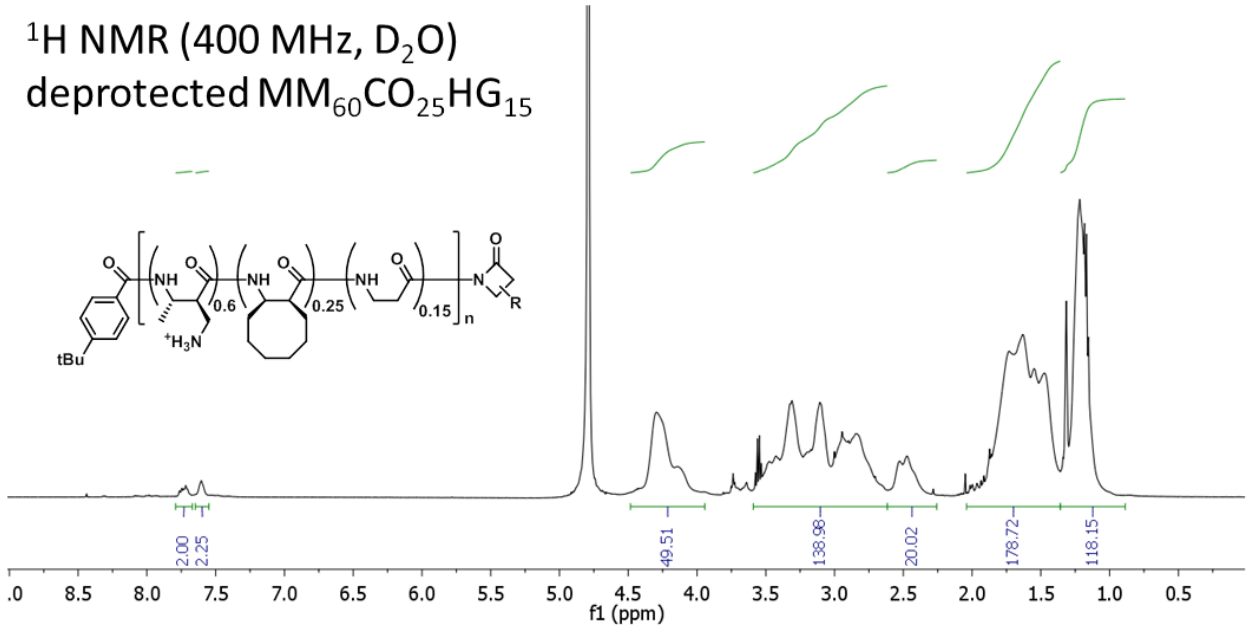


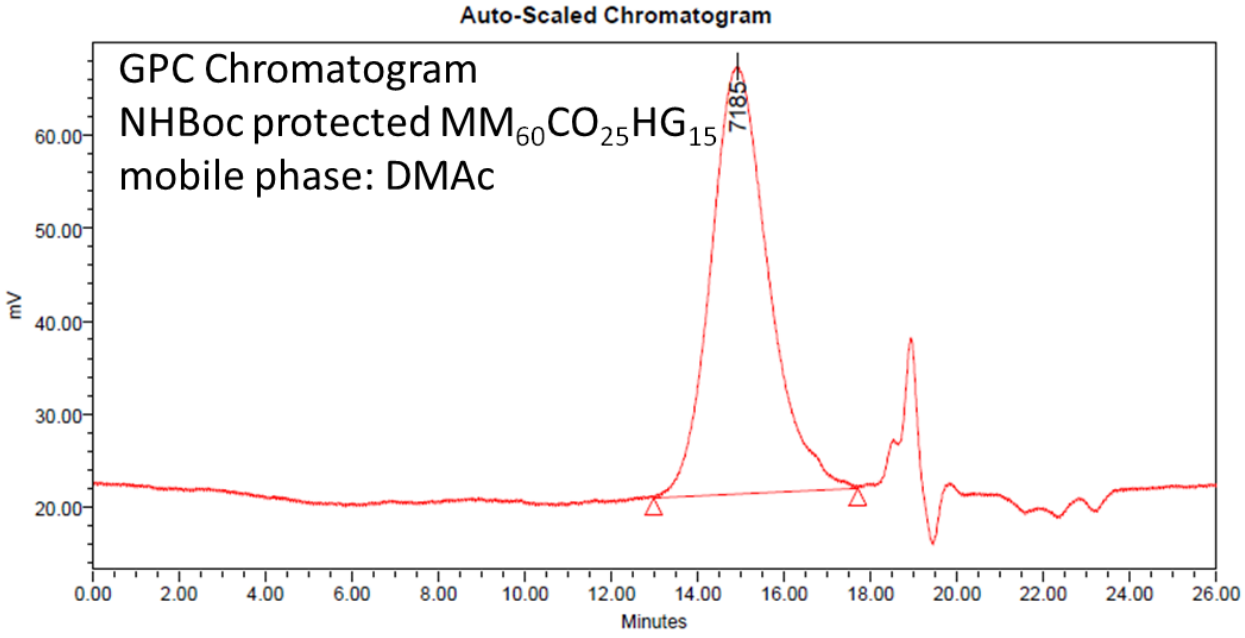


GPC Results

	Dist Name	Mn	Mw	Mv	MP	Mz	Mz+1	Polydispersity	K	alpha
1		5352	6888		6792	8472	10261	1.287060		

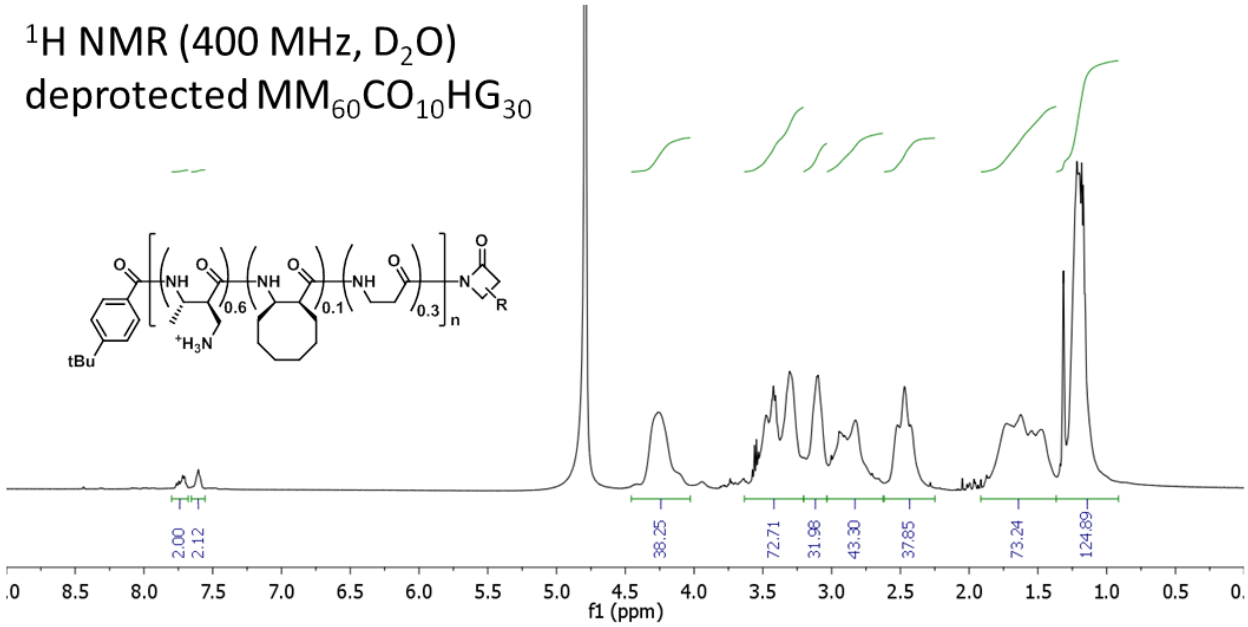
^{1}H NMR (400 MHz, D_2O)
deprotected $MM_{60}CO_{25}HG_{15}$

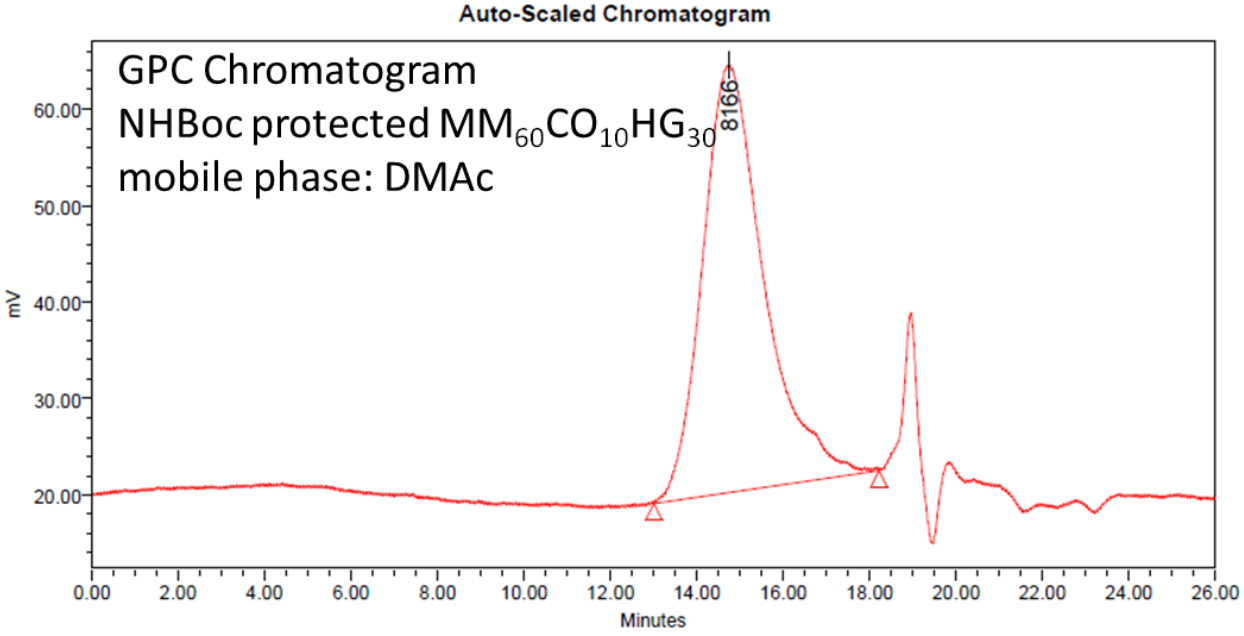




GPC Results

	Dist Name	Mn	Mw	Mv	MP	Mz	Mz+1	Polydispersity	K	alpha
1		5704	7485		7185	9358	11473	1.312383		





GPC Results

	Dist Name	Mn	Mw	Mv	MP	Mz	Mz+1	Polydispersity	K	alpha
1		5670	8077		8166	10268	12418	1.424519		

References:

1) (a) Goodgame, D. M. L.; Hill, S. P. W.; Lincoln, R.; Quiros, M.;Williams, D. J. *Polyhedron* **1993**, *12*, 2753-2762; (b) Mowery, B. P.; Lee, S. E.; Kissounko, D. A.; Epand, R. F.; Epand, R. M.; Weisblum, B.; Stahl, S. S.; Gellman, S. H. *J. Am. Chem. Soc.* **2007**, *129*, 15474−15476; (c) Zhang, J.; Kissounko, D. A.; Lee, S. E.; Gellman, S. H.; Stahl, S. S. *J. Am. Chem. Soc.* **2009**, *131*, 1589−1597.

2) (a)Salzmann, T. N.; Ratcliffe, R. W.; Christensen, B. G.; Bouffard, F. A. *J. Am. Chem. Soc.* **1980**, *102*, 6163-6165. (b) Brennan, J.; Richardson, G.; Stoodley, R. J. *J. Chem. Soc., Chem. Commun*. **1980**, 49.

3) Porter, E. A.; Weisblum, B.;Gellman, S. H. *J. Am. Chem. Soc.* **2002**, *124*, 7324-7330.

4) Yanisch-Perron, C.; Vieira, J.; Messing, *J. Gene* **1985**, *33*, 103−119.

5) Young, F. E.; Smith, C.; Reilly, B. E. *J. Bacteriol.* ***1969***, *98*, 1087−1097.

6) Weisblum, B.; Demohn, V. *J. Bacteriol.* **1969**, *98*, 447-452.

7) Nicas, T. I.; Wu, C. Y.; Hobbs, J. N., Jr.; Preston, D. A.; Allen, N. E. *Antimicrob. Agents Chemother*. **1989**, *33*, 1121-1124.

8) (a) Chambhare, R. V.; Khadse, B. G.; Bobde, A. S.; Bahekar, R. H. *Eur. J. Med. Chem.* **2003**, *38*, 89-100; (b) Chen, Y.; Mant, C. T.;Farmer, S. W.; Hancock, R. E. W.; Vasil, M. L.; Hodges, R. S. *J. Biol.Chem.* **2005**, *280*, 12316-12329.