Supplementary Information for

Mismatch in Covalent and Noncovalent Templating Preferences Leads to Large Coiled Coil-Templated Macrocycles

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Dynamic Combinatorial Library Setup

Peptides were dissolved in 50 mM borate buffer (pH 8.5). For acetylated peptides, concentration was determined using Trp extinction coefficient at 278 nm (5579 $(cm*M)^{-1}$).[1] For peptides containing thio-benzene moieties, a new extinction coefficient was determined by measuring the absorbance at 314 nm over multiple concentrations. These samples were prepared by weighing the desired peptide, dissolving it in 50 mM sodium borate buffer, and creating a series of samples by serial dilution. This data was plotted (**Figure S1**) as absorbance vs. concentration times path length (0.1 cm) and a trendline was used to quantify the extinction coefficient. This coefficient was subsequently used for concentration determination. To ensure disulfide equilibration, libraries with thiols were left sitting for a week.

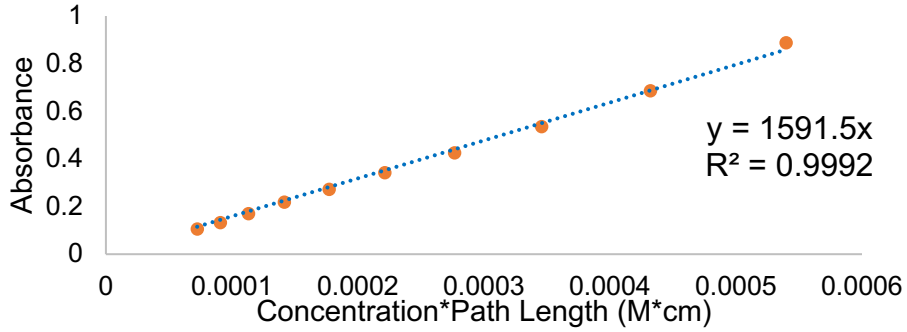

Figure S1. Serial dilution to determine the extinction coefficient of peptide containing both Trp and monomer. Measurements were taken at 314 nm. (**Extinction coefficient = 1591.5 $(M*cm)^{-1}$ @ 314 nm**). Error bars are included but cannot be seen as they are smaller than the data points.

Synthesis and Purification of Acetylated and B-Coupled Peptides

Peptides were synthesized on a 0.1 or 0.25 mmol scale using P3 Biosystems Rink Amide resin (0.56 mmol/g loading). Fmoc solid-phase peptide synthesis was done in a peptide flask or using a LibertyBlue (CEM) microwave peptide synthesizer. For peptides synthesized in a flask using 1 equiv of resin, coupling solutions were comprised of 4 equiv of Fmoc amino acid, 4 equiv of HBTU, 4 equiv HOBt, and 8 equiv DIPEA in DMF. Two of these couplings were performed sequentially for 30 minutes each. Fmoc deprotections were run for 30 minutes using 20% piperidine in DMF. Amino acid couplings performed on the microwave synthesizer were done in DMF for 2 minutes at 90 °C with 5 equiv Fmoc amino acid, 10 equiv DIC, and 5 equiv Oxyma per 1 equiv of resin. Each coupling was done once with the exception of Arg, which was coupled twice. Deprotections were done using 20% piperidine in DMF for 90 seconds at 90 °C.

Peptides were N-terminally capped while still on resin in a peptide synthesis flask either through acetylation or via the carboxylate of a tritylated monomer (Trt-B or Trt-R), which was synthesized as previously described.[2,3] For acetylation, peptides were mixed with a capping solution of 5% acetic anhydride, 6% 2,6-lutidine in DMF for 30 minutes. Coupling of monomer

B onto peptide was done using 2 equiv of Trt-B, 5 equiv DIC, 10 equiv Oxyma, and excess LiCl in NMP for 24 hrs. Cleavage and global deprotection of acetylated peptides were done using 5% TIPS, 5% water, and 90% TFA for 4 hrs. For monomer B capped peptides, cleavage was done using 5% TIPS, 5% water, 5% 3,6-dioxa-1,8-octanedithiol (DODT), and 85% TFA for 4 hrs. TFA was subsequently blown off with nitrogen.

Peptides were purified on a Waters semi-preparative HPLC outfitted with a C18 column. Two eluents were used, eluent **A** (95% water, 5% ACN, 0.1% TFA) and eluent **B** (95% ACN, 5% water, 0.1% TFA). Crude peptides were dissolved in 8:2 of **A:B** and purified using a gradient 20% - 70% **B** in 60 min.

2.10.2 ***Peptide LC-MS Analysis***

All peptides were characterized using an Agilent Rapid Resolution LC-MSD system equipped with online degasser, binary pump, autosampler, heated column compartment, and diode array detector. Samples were run with gradients of 0.2% formic acid solutions of water and ACN. Peptides were run on a C18 column. Mass spectra (ESI+) were acquired on a single quad mass spectrometer using using a drying temperature of 350 °C, a nebulizer pressure of 45 psi, a drying gas flow of 10 L/min, and a capillary voltage of 3000 V.

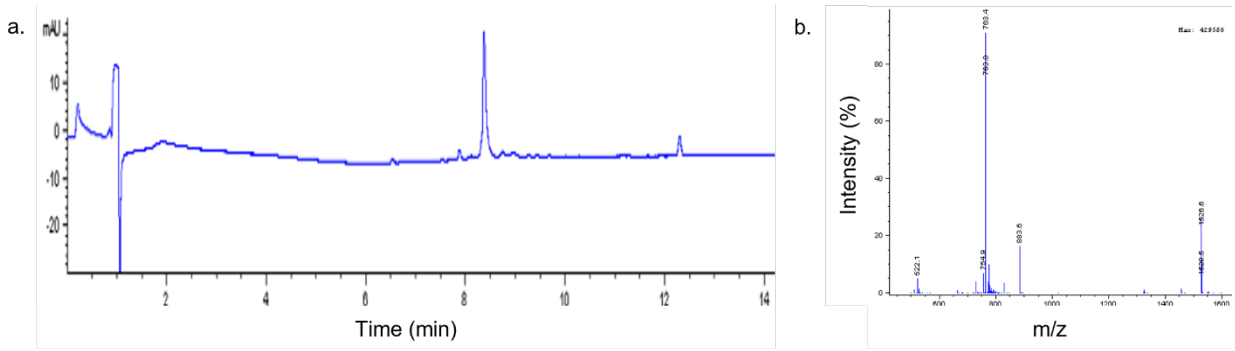

Figure S2. LC-MS data for **B-1.5Hep**. UV-Vis spectrum measured at a.) 280 nm and b.) low resolution mass spectrum of B-1.5Hep. Mass Expected: 1525.7 [M+H], 763.4 [M+2H]. Mass Observed: 1526.6 [M+H], 763.4 [M+2H].

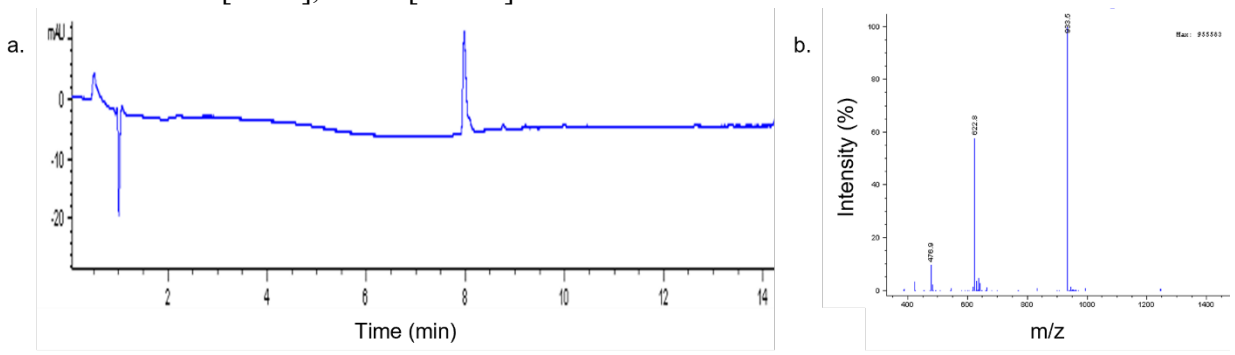

Figure S3. LC-MS data for **B-2Hep**. UV-Vis spectrum measured at a.) 280 nm and b.) low resolution mass spectrum of B-2Hep. Mass Expected: 1865.9 [M+H], 933.5 [M+2H], 622.6 [M+3H] 476.7 [M+4H+K+]. Mass Observed: 933.5 [M+2H], 622.8 [M+3H], 476.9 [M+4H+K+].

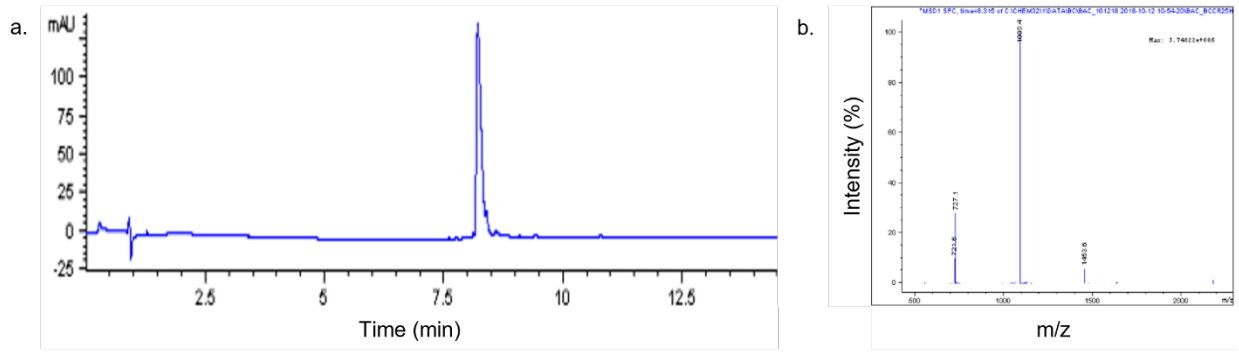

Figure S4. LC-MS data for **B-2.5Hep**. UV-Vis spectrum measured at a.) 280 nm and b.) low resolution mass spectrum of B-2.5Hep. Mass Expected: 2179.0 [M+H], 1090.0 [M+2H], 727.0 [M+3H]. Mass Observed: 1090.4 [M+2H], 727.1 [M+3H].

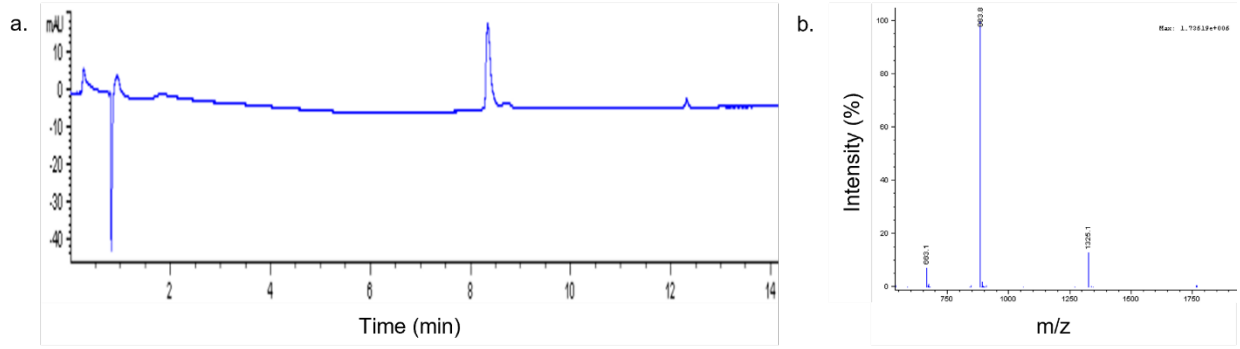

Figure S5. LC-MS data for **B-3Hep**. UV-Vis spectrum measured at a.) 280 nm and b.) low resolution mass spectrum of B-3Hep. Mass Expected: 2648.3 [M+H], 1324.7 [M+2H], 883.4 [M+3H], 662.8 [M+4H]. Mass Observed: 1325.1 [M+2H], 883.8 [M+3H], 663.1 [M+4H].

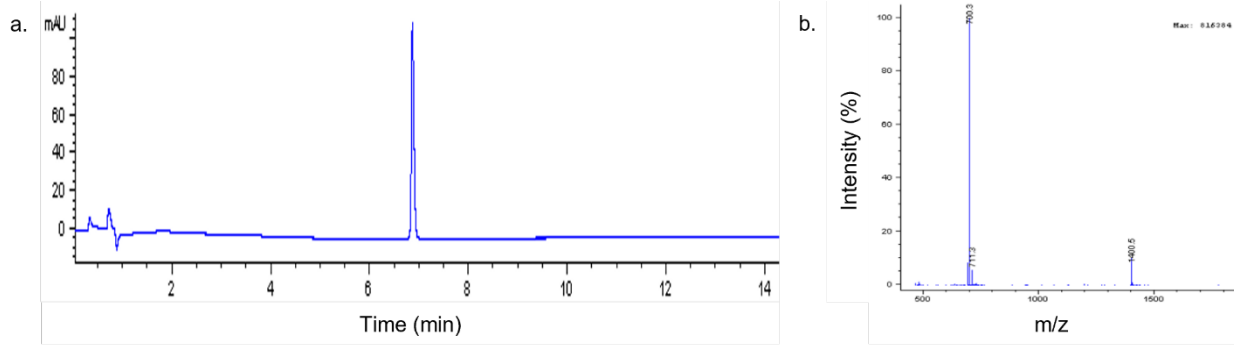

Figure S6. LC-MS data for **Ac-1.5Hep**. UV-Vis spectrum measured at a.) 280 nm and b) low resolution mass spectrum of **Ac-1.5Hep**. Mass Expected: 1399.7 [M+H], 700.4 [M+2H]. Mass Observed: 1400.5 [M+H], 700.3 [M+2H].

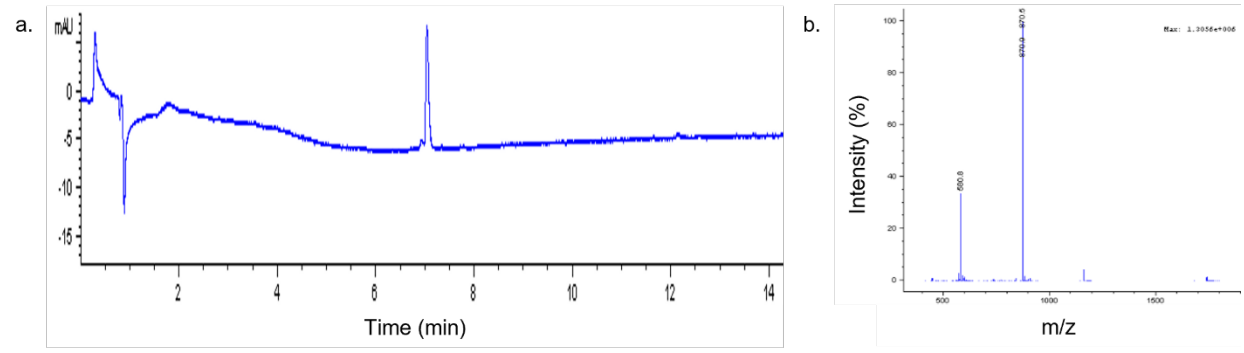

Figure S7. LC-MS data for **Ac-2Hep**. UV-Vis spectrum measured at a.) 280 nm and b.) low resolution mass spectrum of **Ac-2Hep**. Mass Expected: 1739.9 [M+H], 870.5 [M+2H], 580.6 [M+3H]. Mass Observed: 870.5 [M+2H], 580.8 [M+3H].

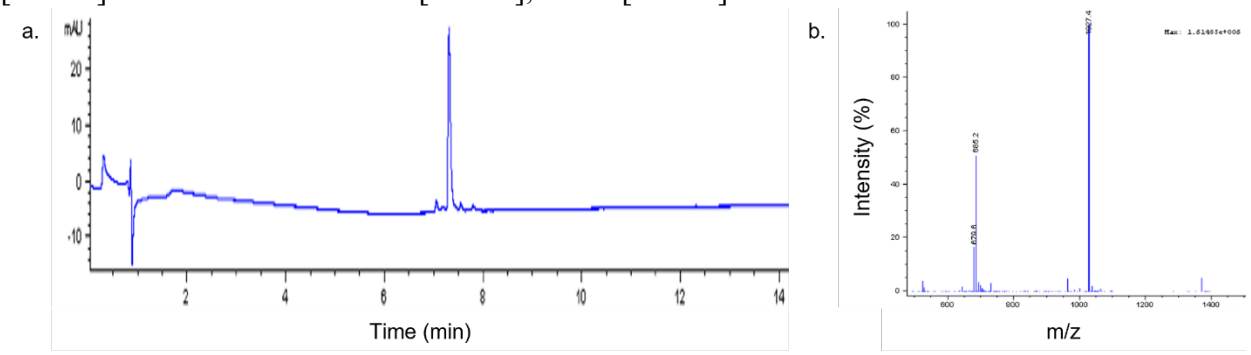

Figure S8. LC-MS data for **Ac-2.5Hep**. UV-Vis spectrum measured at a.) 280 nm and b.) low resolution mass spectrum of **Ac-2.5Hep**. Mass Expected: 2053.1 [M+H], 1027.1 [M+2H], 685.0 [M+3H]. Mass Observed: 1027.4 [M+2H], 685.2 [M+3H].

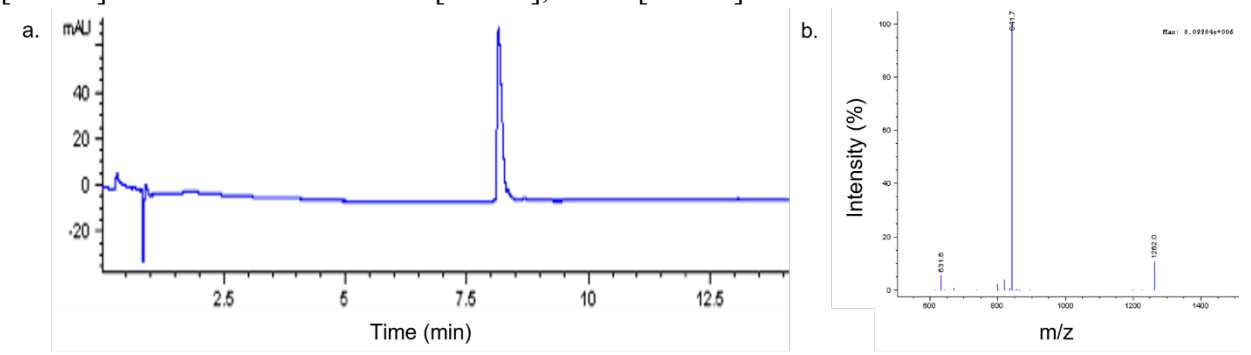

Figure S9. LC-MS data for **Ac-3Hep**. UV-Vis spectrum measured at a.) 280 nm and b.) low resolution mass spectrum of Ac-3Hep. Mass Expected: 2522.4 [M+H], 1261.7 [M+2H], 841.5 [M+3H] 631.4 [M+4H]. Mass Observed: 1262.0 [M+2H], 841.7 [M+3H], 631.6 [M+4H].

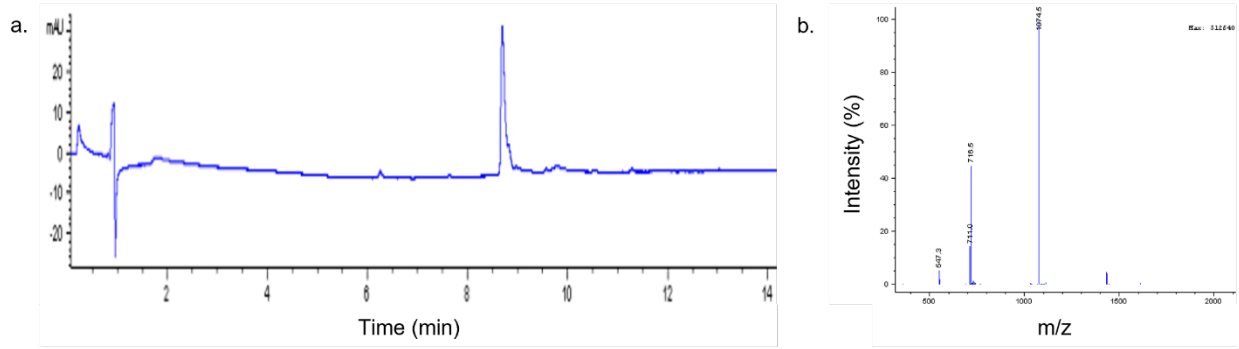

Figure S10. LC-MS data for **R-2.5Hep**. UV-Vis spectrum measured at a.) 280 nm and b.) low resolution mass spectrum of R-2.5Hep. Mass Expected: 2147.1 [M+H], 1074.1 [M+2H], 716.4 [M+3H]. Mass Observed: 1074.5 [M+2H], 716.5 [M+3H].

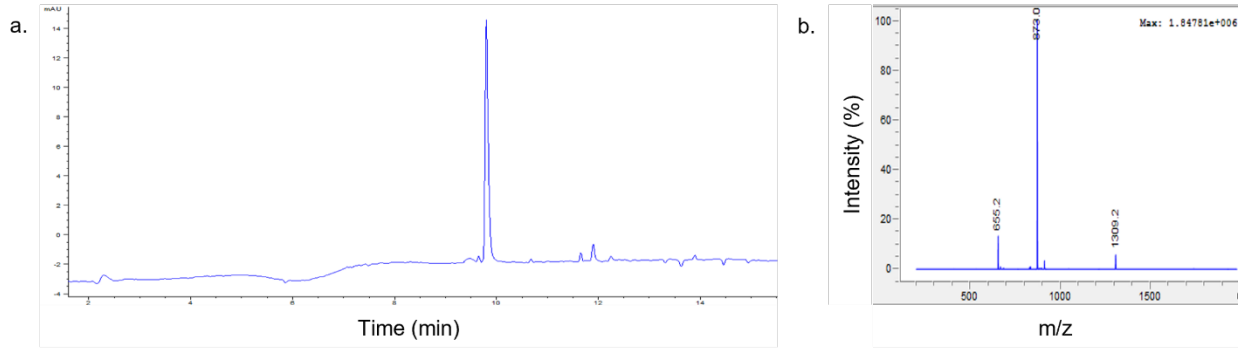

Figure S11. LC-MS data for **R-3Hep**. UV-Vis spectrum measured at a.) 280 nm and b.) low resolution mass spectrum of R-3Hep. Mass Expected: 2616.3 [M+H], 1308.7 [M+2H], 872.8 [M+3H], 654.8 [M+4H]. Mass Observed: 1309.2 [M+2H], 873.0 [M+3H], 655.2 [M+4H].

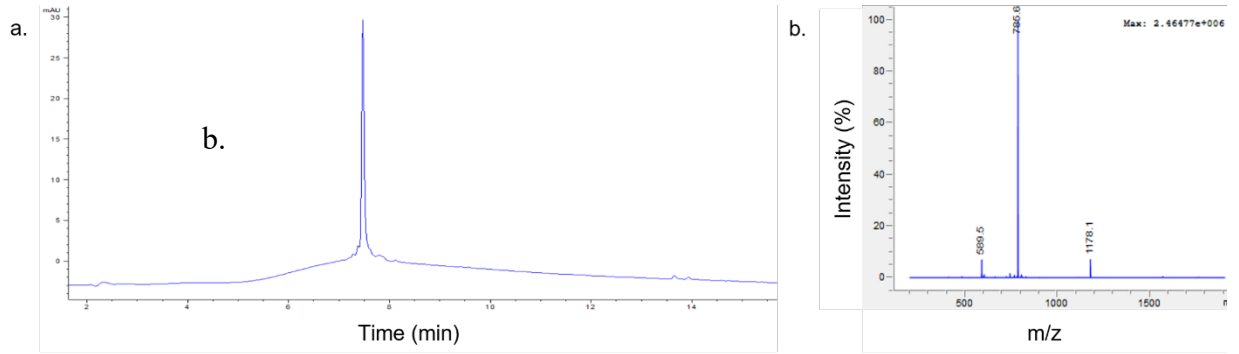

Figure S12. LC-MS data for **Ac-3Hep-Def**. UV-Vis spectrum measured at a.) 280 nm and b.) low resolution mass spectrum of Ac-3Hep-Def. Mass Expected: 2354.2 [M+H], 1178.1 [M+2H], 785.4 [M+3H], 589.5 [M+4H]. Mass Observed:1178.1 [M+2H], 785.6 [M+3H], 589.5 [M+4H].

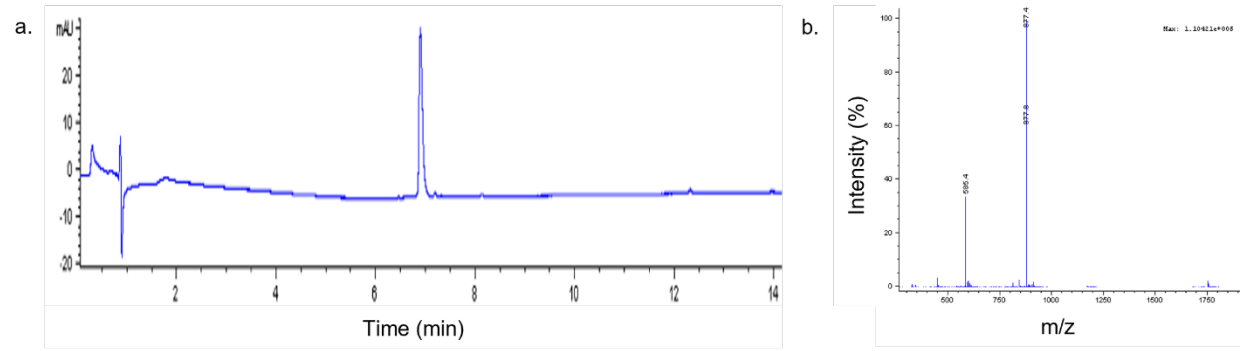

Figure S13. LC-MS data for **B-2Hep-Def**. UV-Vis spectrum measured at a.)280 nm and b.) low resolution mass spectrum of B-2Hep-Def. Mass Expected: 1753.8 [M+H], 877.4 [M+2H], 585.3 [M+3H]. Mass Observed: 877.4 [M+2H], 585.4 [M+3H].

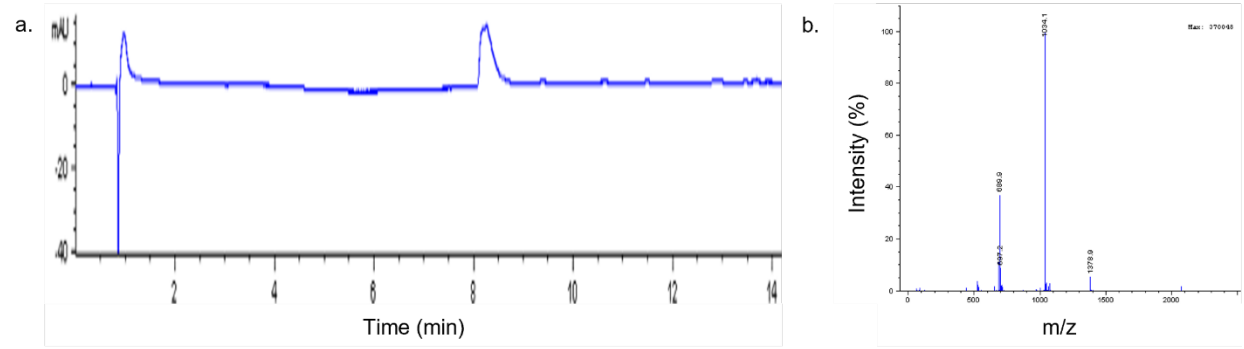

Figure S14. LC-MS data for **B-2.5Hep-Def**. UV-Vis spectrum measured at a.) 280 nm and b.) low resolution mass spectrum of B-2.5Hep-Def. Mass Expected: 2066.9 [M+H], 1034.0 [M+2H], 689.6 [M+3H]. Mass Observed: 1034.1 [M+2H], 689.9 [M+3H].

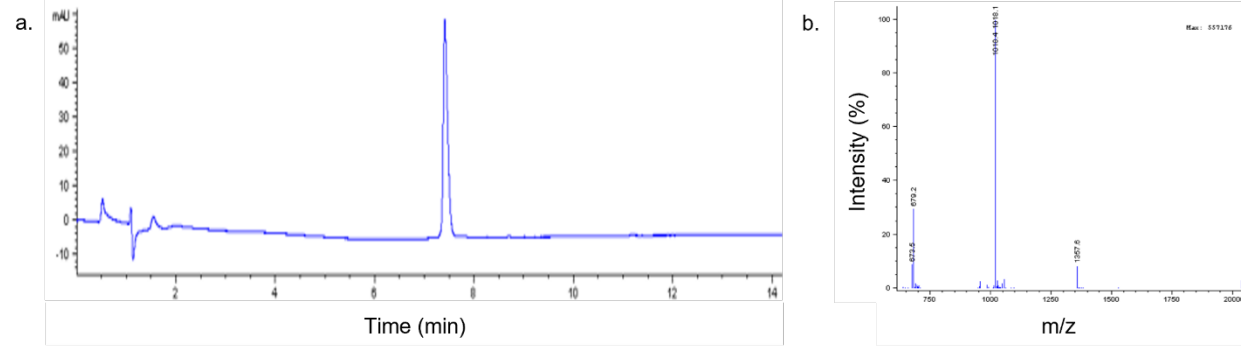

Figure S15. LC-MS data for **R-2.5Hep-Def**. UV-Vis spectrum measured at a.) 280 nm and b.) low resolution mass spectrum of R-2.5Hep-Def. Mass Expected: 2035.0 [M+H], 1018.0 [M+2H], 679.0 [M+3H]. Mass Observed: 1018.1 [M+2H], 679.2 [M+3H].

Library LC-MS Analysis

All libraries were characterized using an Agilent Rapid Resolution LC-MSD system equipped with online degasser, binary pump, autosampler, heated column compartment, and diode array detector. There were two methods used to analyze libraries (the method used for each respective library is specified in the figure captions).

Method A: Libraries were run with 0.2% formic acid solutions of water (solvent **A**) and ACN (solvent **B**). Samples were run at 0.3 mL/min using a gradient of 5 to 95% **B** in 15 minutes. Libraries were run through a C4 column held at 45 ºC. Mass spectra (ESI+) were acquired on a single quad mass spectrometer using a drying temperature of 350 °C, a nebulizer pressure of 45 psi, a drying gas flow of 10 L/min, and a capillary voltage of 3000 V.

Method B: Libraries were run using 10 mM NH_4OAc solutions of water (solvent **A**) and ACN (solvent **B**). Samples were run at 0.5 mL/min using a gradient of 0 to 100% **B** in 45 minutes. Libraries were run through a C18 column held at 40 ºC. Mass spectra (ESI-) were acquired on a single quad mass spectrometer using a drying temperature of 350 °C, a nebulizer pressure of 45 psi, a drying gas flow of 10 L/min, and a capillary voltage of 3000 V.

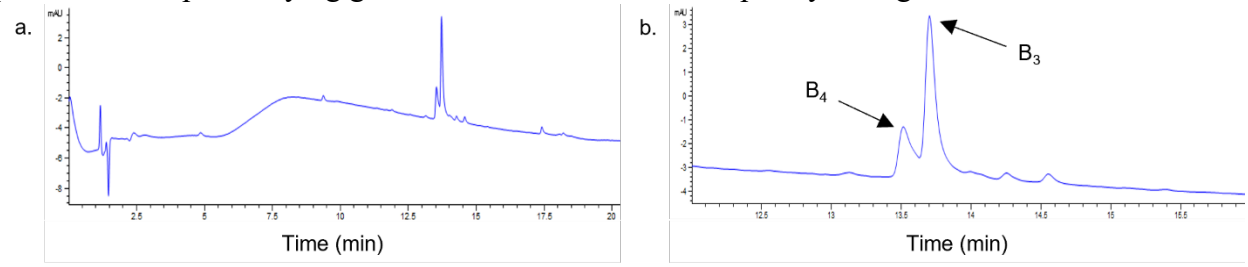

Figure S16. LC data for unmodified **B** DCL. a.) Full spectrum UV-Vis absorbance measured at 280 nm and b.) zoomed in UV-Vis spectrum at 280 nm. Spectrum measured after equilibrating for 7 days in 50 mM borate buffer (pH 8.5). Run using LC-MS **Method A**. **B_3** Mass Expected: 552.0 [M+H], Mass Observed: 552.9 [M+H]. **B_4** Mass Expected: 736.0 [M+H], Mass Observed: 736.7 [M+H].

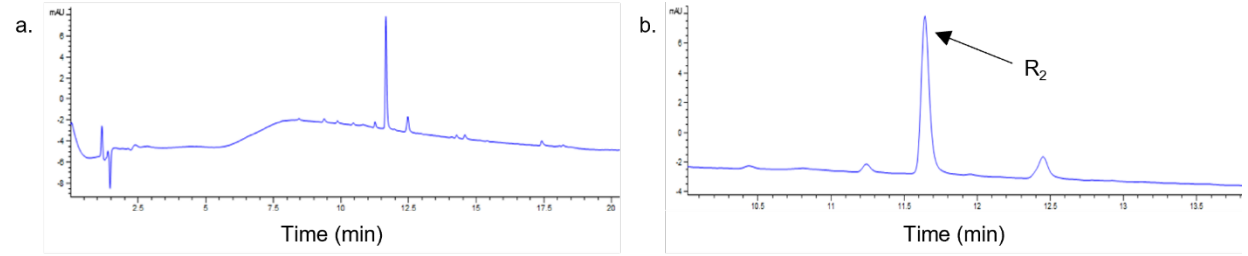

Figure S17. LC data for unmodified **R** DCL. a.) Full spectrum UV-Vis absorbance measured at 280 nm. b.) Zoomed in UV-vis spectrum at 280 nm. Spectrum measured after equilibrating for 7 days in 50 mM borate buffer (pH 8.5). Run using LC-MS **Method A**. **R_2** Mass Expected: 306.4 [M+H]. Mass Observed: 307.0 [M+H].

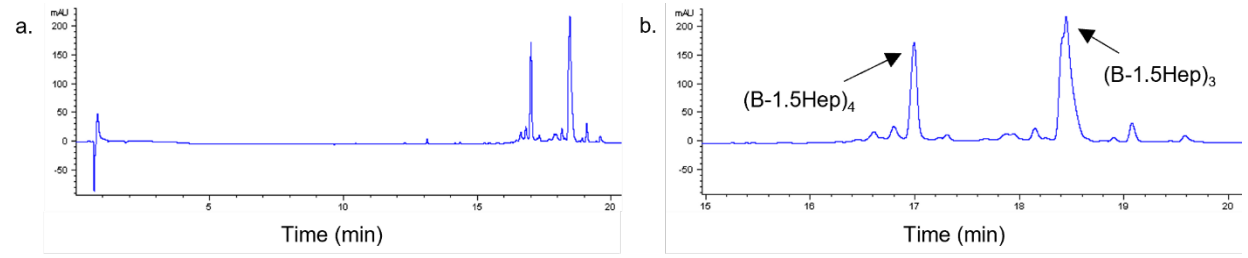

Figure S18. LC data for **B-1.5Hep** DCL. a.) Full spectrum UV-Vis absorbance measured at 280 nm and b.) Zoomed in UV-vis spectrum at 280 nm. Spectrum measured after equilibrating for 7 days in 50 mM borate buffer (pH 8.5). Run using LC-MS **Method B**. **$(B\text{-}1.5Hep)_3$** Mass Expected: 4567.1 [M-H], 1521.7 [M-3H], 1141.0 [M-4H], 912.6 [M-5H]. Mass Observed: 1522.7 [M-3H], 1141.5 [M-4H], 912.9 [M-5H]). **$(B\text{-}1.5Hep)_4$** Mass Expected: 6089.8 [M-H],

2029.3 [M-3H], 1521.5 [M-4H], 1217.2 [M-5H], 1014.1 [M-6H]. Mass Observed: 2030.2 [M-3H], 1522.7 [M-4H], 1217.8 [M-5H], 1014.5 [M-6H].

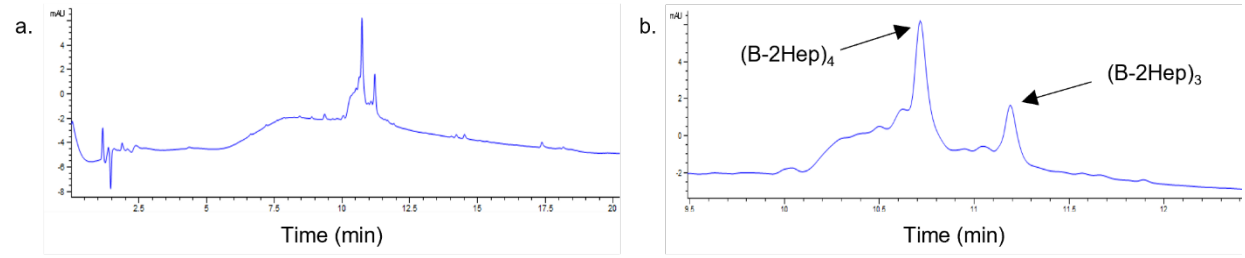

Figure S19. LC data for **B-2Hep** DCL. a.) Full spectrum UV-Vis absorbance measured at 280 nm. b.) Zoomed in UV-vis spectrum at 280 nm. Spectrum measured after equilibrating for 7 days in 50 mM borate buffer (pH 8.5). Run using LC-MS **Method A**. **(B-2Hep)$_3$** Mass Expected: 5589.7 [M+H], 1398.2 [M+4H], 1118.7 [M+5H], 932.5 [M+6H]. Mass Observed: 1398.8 [M+4H], 1119.4 [M+5H], 932.9 [M+6H]. **(B-2Hep)$_4$** Mass Expected: 7452.6 [M+H], 1491.3 [M+5H], 1242.9 [M+6H], 1065.5 [M+7H]. Mass Observed: 1492.2 [M+5H], 1243.5 [M+6H], 1066.0 [M+7H].

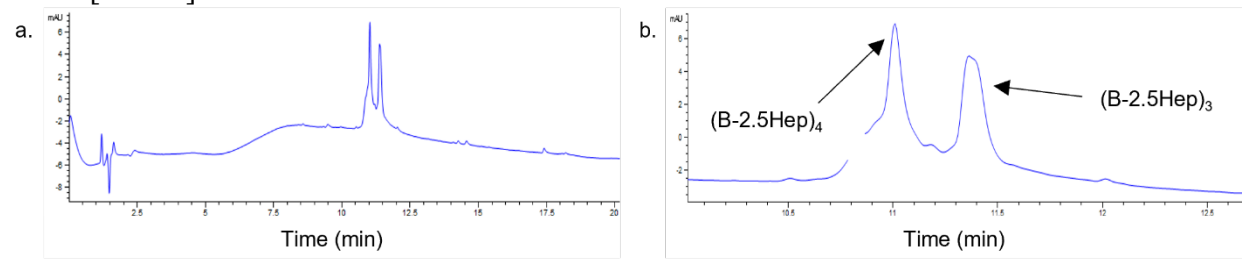

Figure S20. LC data for **B-2.5Hep** DCL. a.) Full spectrum UV-Vis absorbance measured at 280 nm. b.) Zoomed in UV-vis spectrum at 280 nm. Spectrum measured after equilibrating for 7 days in 50 mM borate buffer (pH 8.5). Run using LC-MS **Method A**. **(B-2.5Hep)$_3$** Mass Expected: 6529.0 [M+H], 1633.0 [M+4H], 1306.6 [M+5H], 1089.0 [M+6H]. Mass Observed: 1634.2 [M+4H], 1307.3 [M+5H], 1089.6 [M+6H]. **(B-2.5Hep)$_4$** Mass Expected: 8705.0 [M+H], 1741.8 [M+5H], 1451.7 [M+6H], 1244.4 [M+7H]. Mass Observed: 1742.8 [M+5H], 1452.5 [M+6H], 1245.0 [M+7H].

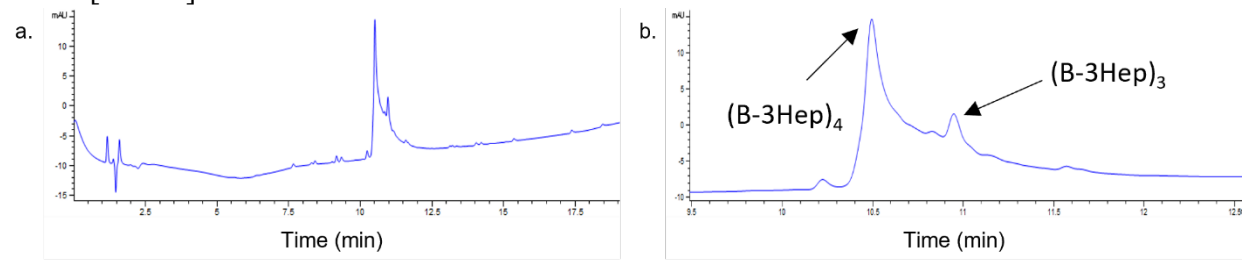

Figure S21. LC data for **B-3Hep** DCL. Full spectrum UV-Vis absorbance measured at a.) 254 nm and b.) 280 nm. Zoomed in UV-vis spectrum at c.) 254 nm and d.) 280 nm. Spectrum measured after equilibrating for 7 days in 50 mM borate buffer (pH 8.5). Run using LC-MS **Method A**. **(B-3Hep)$_3$** Mass Expected: 7936.9 [M+H], 1588.2 [M+5H], 1323.7 [M+6H], 1134.7 [M+7H]. Mass Observed: 1589.2 [M+5H], 1323.7 [M+6H], 1134.7 [M+7H]. **(B-3Hep)$_4$** Mass Expected: 10582.2 [M+H], 1764.5 [M+6H], 1512.6 [M+7H], 1323.7 [M+8H], 1176.7 [M+9H]. Mass Observed: 1765.4 [M+6H], 1513.4 [M+7H], 1324.5 [M+8H], 1177.3 [M+9H].

a. c.

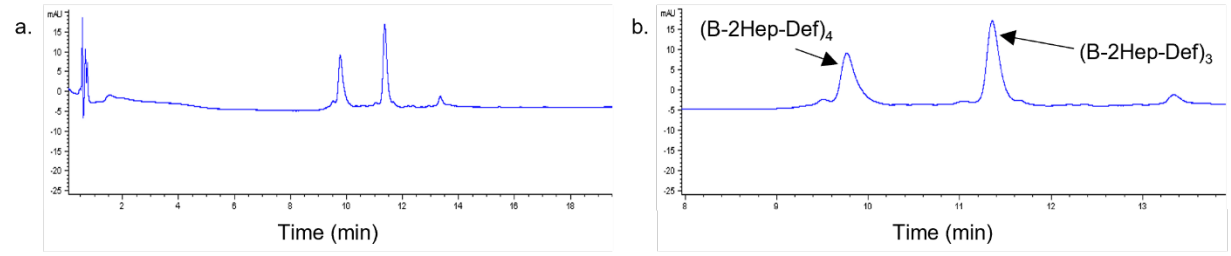

Figure S22. LC data for **B-2Hep-Def** DCL. a.) Full spectrum UV-Vis absorbance measured at 280 nm. b.) Zoomed in UV-vis spectrum at 280 nm. Spectrum measured after equilibrating for 7 days in 50 mM borate buffer (pH 8.5). Run using LC-MS **Method B**. **(B-2Hep-Def)$_3$** Mass Expected: 5254.4 [M-H], 1750.8 [M-3H], 1312.9 [M-4H], 1050.1 [M-5H], 874.9 [M-6H]. Mass Observed: 1750.3 [M-3H], 1312.6 [M-4H], 1049.8 [M-5H], 874.6 [M-6H]. **(B-2Hep-Def)$_4$** Mass Expected: 7006.2 [M-H], 1750.6 [M-4H], 1400.4 [M-5H]. Mass Observed: 1751.1 [M-4H], 1400.9 [M-5H].

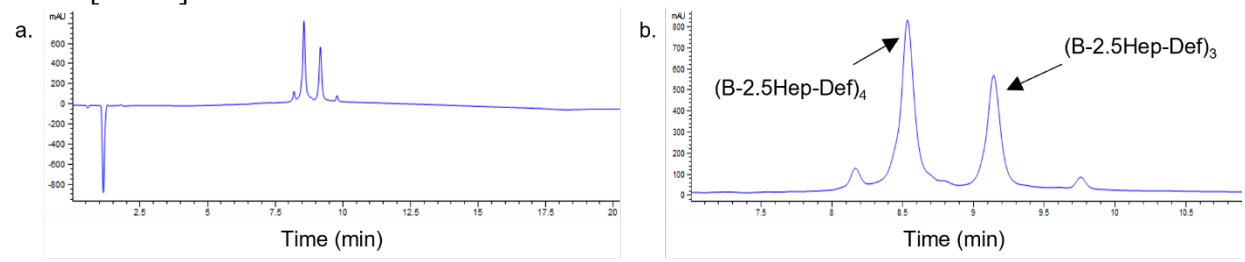

Figure S23. LC data for **B-2.5Hep-Def** DCL. a.) Full spectrum UV-Vis absorbance measured at 280 nm. b.) Zoomed in UV-vis spectrum at 280 nm. Spectrum measured after equilibrating for 7 days in 50 mM borate buffer (pH 8.5). Run using LC-MS **Method A**. **(B-2.5Hep-Def)$_3$** Mass Expected: 6192.7 [M+H], 1548.9 [M+4H], 1239.3 [M+5H], 1033.0 [M+6H]. Mass Observed: 1549.8 [M+4H], 1239.9 [M+5H], 1033.4 [M+6H]. **(B-2.5Hep-Def)$_4$** Mass Expected: 8256.6 [M+H], 1652.1 [M+5H], 1376.9 [M+6H], 1180.4 [M+7H]. Mass Observed: 1652.9 [M+5H], 1377.7 [M+6H], 1181.0 [M+7H].

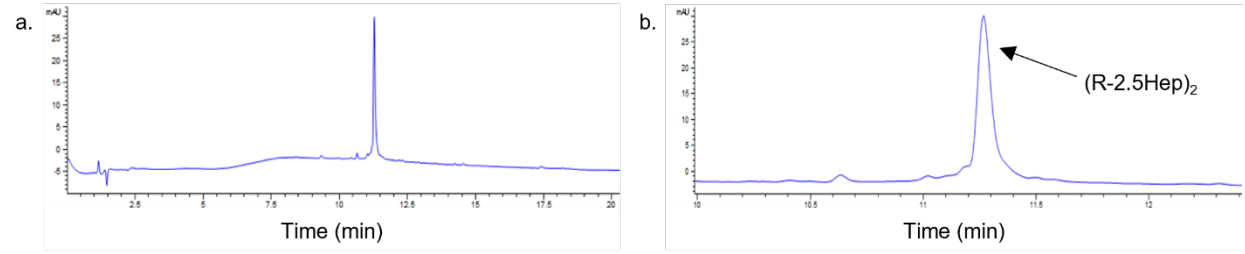

Figure S24. LC data for **R-2.5Hep** DCL. a.) UV-Vis absorbance measured at 280 nm. b.) Zoomed in UV-vis spectrum at 280 nm. Spectrum measured after equilibrating for 7 days in 50 mM borate buffer (pH 8.5). Run using LC-MS **Method A**. **(R-2.5Hep)$_2$** Mass Expected: 4293.2 [M+H], 1431.7 [M+4H], 1074.1 [M+5H], 859.4 [M+6H]. Mass Observed: 1431.7 [M+4H], 1074.0 [M+5H], 859.5 [M+6H].

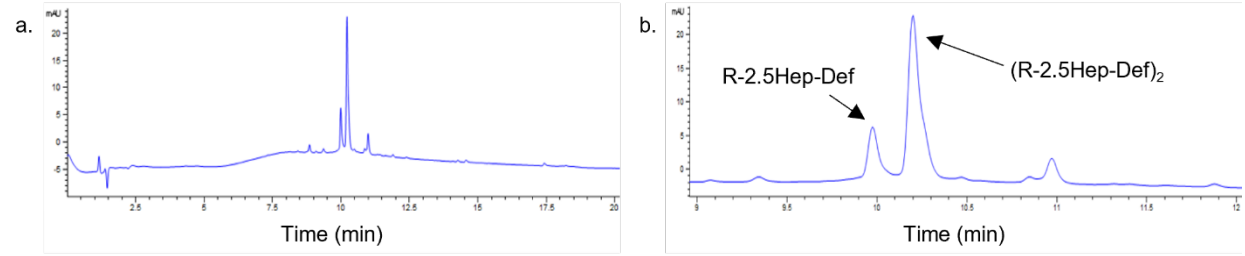

Figure S25. LC data for **R-2.5Hep-Def** DCL. a.) UV-Vis absorbance measured at 280 nm. b.) Zoomed in UV-vis spectrum at 280 nm. Spectrum measured after equilibrating for 7 days in 50 mM borate buffer (pH 8.5). Run using LC-MS **Method A**. **R-2.5Hep-Def** Mass Expected: 2035.0 [M+H], 1018.0 [M+2H], 679.0 [M+3H]. Mass Observed: 1018.4 [M+2H], 679.4 [M+3H]. **(R-2.5Hep-Def)$_2$** Mass Expected: 4069.0 [M+H], 1357.0 [M+4H], 1018.0 [M+5H], 814.6 [M+6H]. Mass Observed: 1357.0 [M+4H], 1017.9 [M+5H], 814.5 [M+6H].

Table S1. Table of ratios of **B_3:B_4** as determined by peak integrations. Ratios were only calculated for libraries that do not exhibit peak broadening.

Library	Ratio of B_3:B_4
Monomer B	2.3
B-1.5Hep	1.7
B-2Hep-Def	1.5
B-2.5Hep-Def	0.77

MALDI-TOF Analysis

All measurements were taken on a Microflex LRF MALDI-TOF System. Library samples were desalted using Waters Oasis desalting columns and eluted using MeOH. Final concentrations were estimated to be in the range of 3-8 mM. Samples were mixed in a 1:1 mixture of sample to alpha-Cyano-4-hydroxycinnamic acid (CHCA) matrix (20 mg/mL). The sample/CHCA mixture were then spotted onto a stainless steel MALDI plate and allowed to dry before analysis.

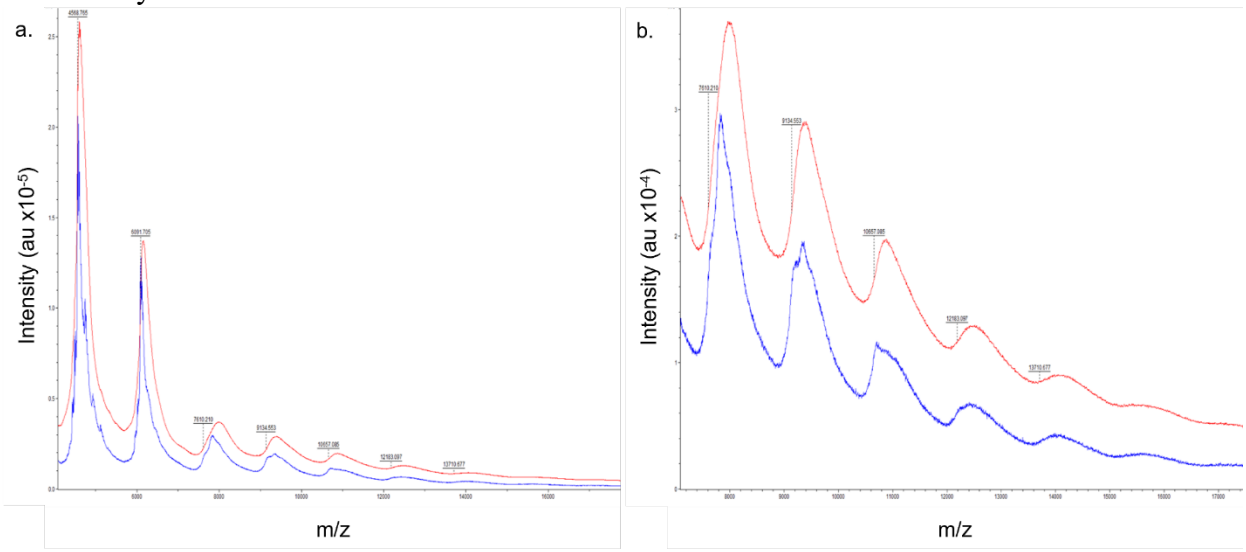

Figure S26. MALDI-TOF data for **B-1.5Hep** DCL. a.) Full spectrum of MALDI data (4-18 kDa). b.) Blown up look at the 7.5-18 kDa range of MALDI data. Red and blue spectrum represent scans of the same sample at differing laser strengths. Red spectrum was run with higher laser intensity than the blue spectrum. As laser intensity increases, peaks broaden and maxima shift to the right. Due to broadening, masses shown were manually selected to correspond to masses of expected **(B-1.5Hep)$_X$** species (**Table 3.5**). This demonstrates the patterning of mass

species that is consistent with single additions of a monomeric unit of **B-1.5Hep**. Peaks were found corresponding to **3-9 units of B-1.5Hep**. Samples were desalted, dissolved in MeOH, and prepped in CHCA matrix. Mass measurements were performed in linear mode.

Table S2. Table of expected masses for cyclic **(B-1.5Hep)$_X$** species and the corresponding masses selected on the MALDI spectrum. Peaks were identified corresponding to species with **3-9 monomeric units of B-1.5Hep**.

Oligomeric Species	Expected Mass (Da)	Observed Mass (Da)
(B-1.5Hep)$_3$	4567.8	4568.8
(B-1.5Hep)$_4$	6090.4	6091.7
(B-1.5Hep)$_5$	7613.0	7610.2
(B-1.5Hep)$_6$	9135.6	9134.6
(B-1.5Hep)$_7$	10658.2	10657.1
(B-1.5Hep)$_8$	12180.8	12183.1
(B-1.5Hep)$_9$	13703.4	13710.7
(B-1.5Hep)$_{10}$	15226.0	--------
(B-1.5Hep)$_{11}$	16748.6	--------
(B-1.5Hep)$_{12}$	18271.2	--------
(B-1.5Hep)$_{13}$	19793.8	--------

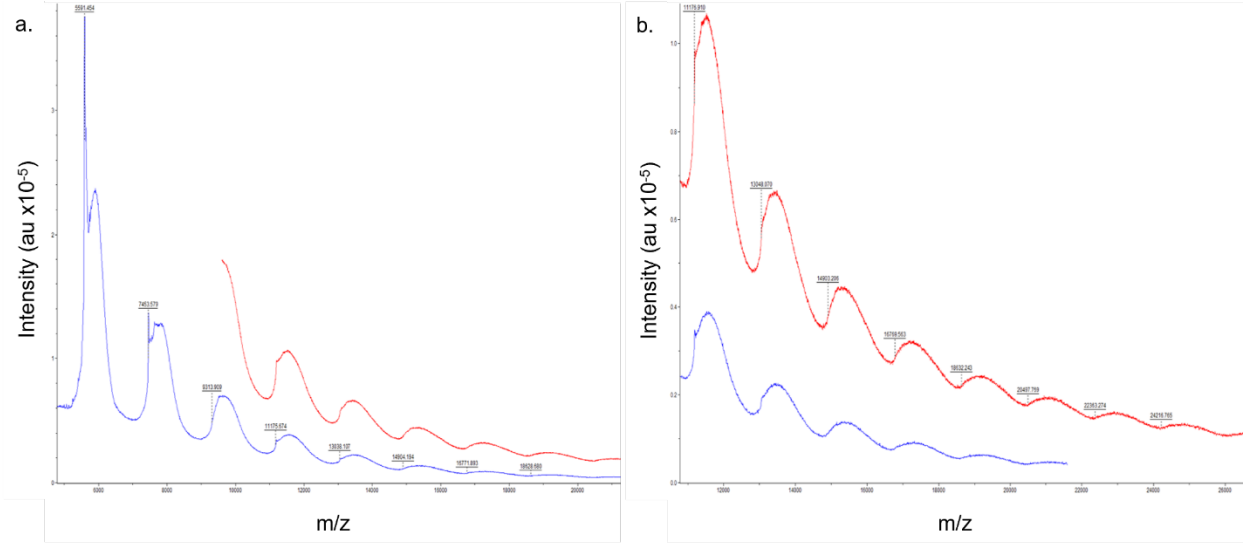

Figure S27. MALDI-TOF data for **B-2Hep** DCL. a.) Spectrum of MALDI data ranging between 5-22 kDa. b.) Blown up look at the 11-27 kDa range of the MALDI data. Red and blue spectrum represent scans of the same sample at differing laser strengths. Red spectrum was run with higher laser intensity than the blue spectrum. As laser intensity increases, peaks broaden and maxima shift to the right. Due to broadening, masses shown were manually selected to correspond to masses of expected **(B-2Hep)$_X$** species. This demonstrates the patterning of mass species that is

consistent with single additions of a monomeric unit of **B-2Hep** (**Table 3.6**). Peaks were found corresponding to **3-13 units of B-2Hep**. Samples were desalted, dissolved in MeOH, and prepped in CHCA matrix. Mass measurements were performed in linear mode.

Table S3. Table of expected masses for cyclic **(B-2Hep)$_X$** species and the corresponding masses selected on the MALDI spectrum. Peaks were identified corresponding to species with **3-13 monomeric units of B-2Hep**.

Oligomeric Species	Expected Mass (Da)	Observed Mass (Da)
(B-2Hep)$_3$	5588.7	5591.4
(B-2Hep)$_4$	7451.6	7453.6
(B-2Hep)$_5$	9314.5	9313.9
(B-2Hep)$_6$	11177.4	11176.9
(B-2Hep)$_7$	13040.3	13048.1
(B-2Hep)$_8$	14903.2	14903.2
(B-2Hep)$_9$	16766.1	16769.6
(B-2Hep)$_{10}$	18629.0	18632.2
(B-2Hep)$_{11}$	20491.9	20497.8
(B-2Hep)$_{12}$	22354.8	22363.3
(B-2Hep)$_{13}$	24217.7	24216.8

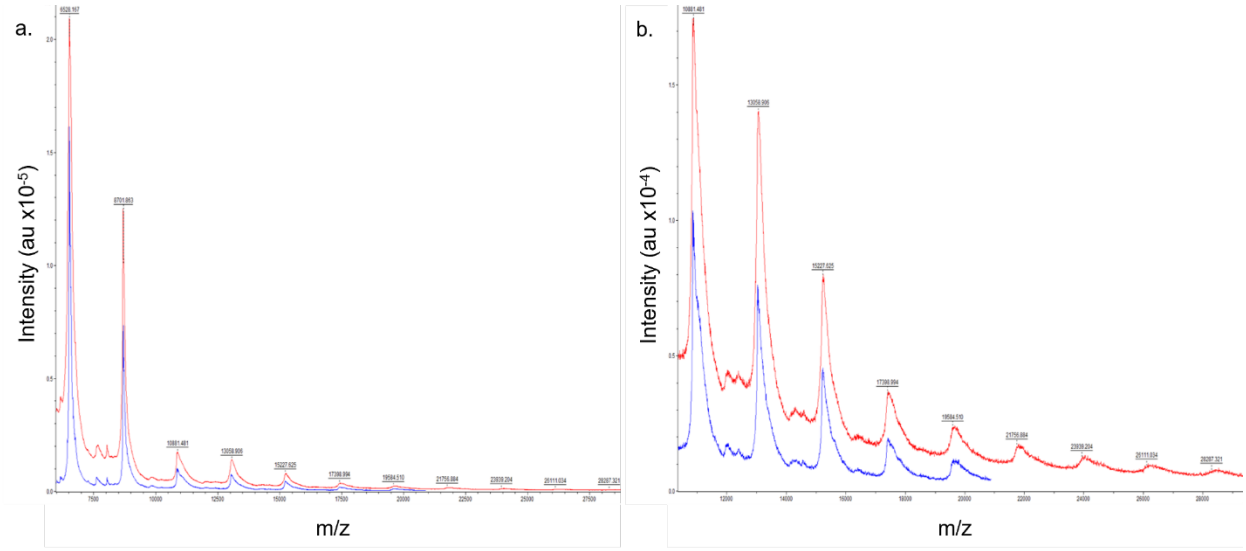

Figure S28. MALDI-TOF data for **B-2.5Hep** DCL. a.) Spectrum of MALDI data ranging between 6-30 kDa. b.) Blown up look at the 10-30 kDa range of the MALDI data. Red and blue spectrum represent scans of the same sample at differing laser strengths. Red spectrum was run with higher laser intensity than the blue spectrum. As laser intensity increases, peaks broaden and maxima shift to the right. Due to broadening, masses shown were manually selected to

correspond to masses of expected **(B-2.5Hep)$_x$** species (**Table 3.7**). This demonstrates the patterning of mass species that is consistent with single additions of a monomeric unit of **B-2.5Hep**. Peaks were found corresponding to **3-13 units of B-2.5Hep**. Samples were desalted, dissolved in MeOH, and prepped in CHCA matrix. Mass measurements were performed in linear mode.

Table S4. Table of expected masses for cyclic **(B-2.5Hep)$_x$** species and the corresponding masses selected on the MALDI spectrum. Peaks were identified corresponding to species with **3-13 monomeric units of B-2.5Hep**.

Oligomeric Species	Expected Mass (Da)	Observed Mass (Da)
(B-2.5Hep)$_3$	6528.0	6528.2
(B-2.5Hep)$_4$	8704.0	8701.9
(B-2.5Hep)$_5$	10880.0	10881.5
(B-2.5Hep)$_6$	13056.0	13058.9
(B-2.5Hep)$_7$	15232.0	15227.6
(B-2.5Hep)$_8$	17408.0	17399.0
(B-2.5Hep)$_9$	19584.0	19584.5
(B-2.5Hep)$_{10}$	21760.0	21756.9
(B-2.5Hep)$_{11}$	23936.0	23939.2
(B-2.5Hep)$_{12}$	26112.0	26111.0
(B-2.5Hep)$_{13}$	28288.0	28287.3

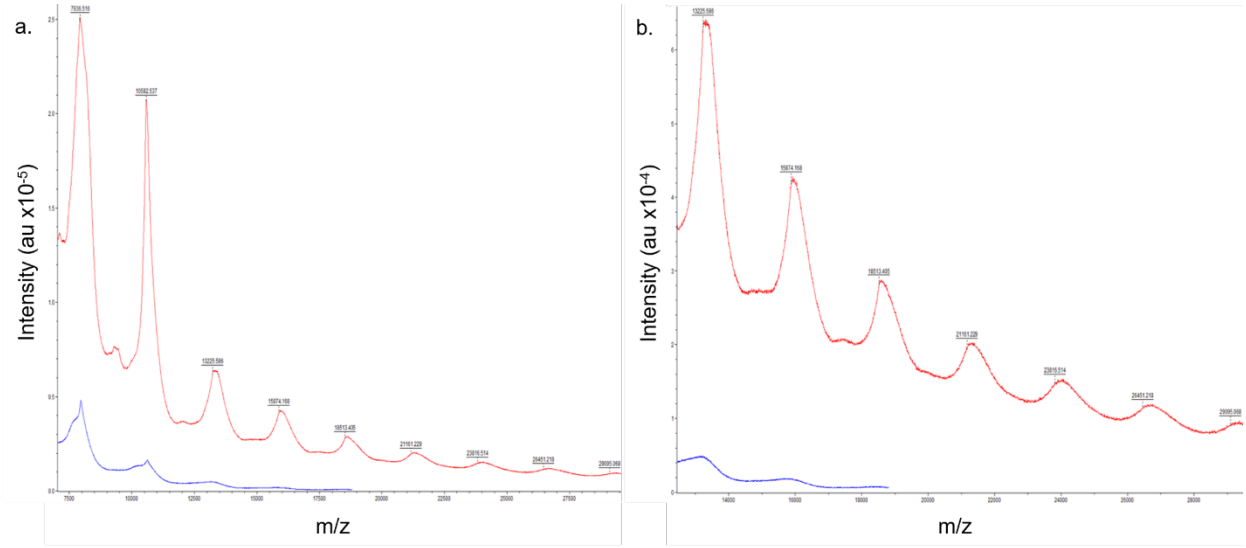

Figure S29. MALDI-TOF data for **B-3Hep** DCL. a.) Spectrum of MALDI data ranging between 7-30 kDa. b.) Blown up look at the 12-30 kDa range of the MALDI data. Red and blue spectrum represent scans of the same sample at differing laser strengths. Red spectrum was run with higher

laser intensity than the blue spectrum. As laser intensity increases, peaks broaden and maxima shift to the right. Due to broadening, masses shown were manually selected to correspond to masses of expected **(B-3Hep)x** species (**Table 3.8**). This demonstrates the patterning of mass species that is consistent with single additions of a monomeric unit of **B-3Hep**. Peaks were found corresponding to **3-11 units of B-3Hep**. Samples were desalted, dissolved in MeOH, and prepped in CHCA matrix. Mass measurements were performed in linear mode.

Table S5. Table of expected masses for cyclic (B-3Hep)$_x$ species and the corresponding masses selected on the MALDI spectrum. Peaks were identified corresponding to species with 3-11 monomeric units of B-3Hep.

Oligomeric Species	Expected Mass (Da)	Observed Mass (Da)
(B-3Hep)$_3$	7935.9	7936.5
(B-3Hep)$_4$	10581.2	10582.5
(B-3Hep)$_5$	13226.5	13225.6
(B-3Hep)$_6$	15871.8	15874.2
(B-3Hep)$_7$	18517.1	18513.4
(B-3Hep)$_8$	21162.4	21161.2
(B-3Hep)$_9$	23807.7	23816.5
(B-3Hep)$_{10}$	26453.0	26451.2
(B-3Hep)$_{11}$	29098.3	29095.1
(B-3Hep)$_{12}$	31743.6	--------
(B-3Hep)$_{13}$	34388.9	--------

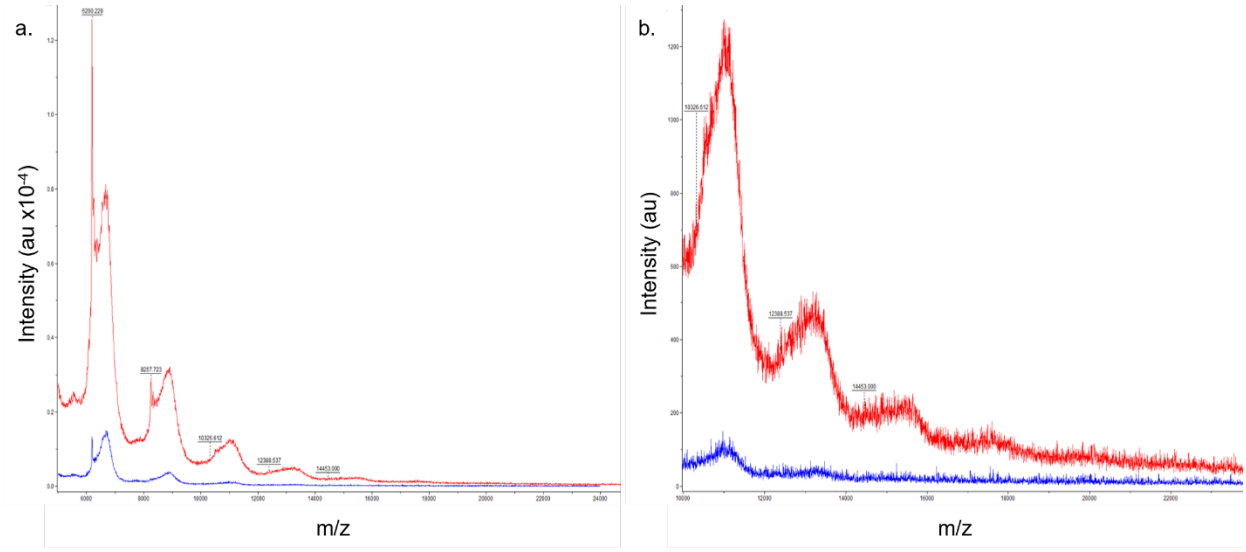

Figure S30. MALDI-TOF data for **B-2.5Hep-Def** DCL. a.) Spectrum of MALDI data ranging between 5-25 kDa. b.) Blown up look at the 10-24 kDa range of the MALDI data. Red and blue

spectrum represent scans of the same sample at differing laser strengths. Red spectrum was run with higher laser intensity than the blue spectrum. As laser intensity increases, peaks broaden and maxima shift to the right. Due to broadening, masses shown were manually selected to correspond to masses of expected **(B-2.5Hep-Def)$_X$** species (**Table S5**). This demonstrates the patterning of mass species that is consistent with single additions of a monomeric unit of **B-2.5Hep-Def**. Peaks were found corresponding to **3-7 units of B-2.5Hep-Def**. Samples were desalted, dissolved in MeOH, and prepped in CHCA matrix. Mass measurements were performed in linear mode.

Table S6. Table of expected masses for cyclic **(B-2.5Hep-Def)$_X$** species and the corresponding masses selected on the MALDI spectrum. Peaks were identified corresponding to species with **3-7 monomeric units of B-2.5Hep-Def**.

Oligomeric Species	Expected Mass (Da)	Observed Mass (Da)
(B-2.5Hep-Def)$_3$	6191.7	6200.2
(B-2.5Hep-Def)$_4$	8255.6	8257.7
(B-2.5Hep-Def)$_5$	10319.6	10326.6
(B-2.5Hep-Def)$_6$	12383.5	12388.5
(B-2.5Hep-Def)$_7$	14447.4	14453.0
(B-2.5Hep-Def)$_8$	16511.3	--------
(B-2.5Hep-Def)$_9$	18575.2	--------
(B-2.5Hep-Def)$_{10}$	20639.1	--------
(B-2.5Hep-Def)$_{11}$	22703.0	--------
(B-2.5Hep-Def)$_{12}$	24766.9	--------
(B-2.5Hep-Def)$_{13}$	26830.8	--------

SDS-PAGE gel procedures:
For all SDS-PAGE gels, a 13% acrylamide gel made with 19:1 acrylamide/bis-acrylamide solution (Bio-Rad) and ~0.33 M Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (BIS-TRIS) pH 6.8 (made using a 3X gel buffer of 1 M BIS-TRIS, pH 6.8) was used. Gels were run using a low molecular weight running buffer (50 mM 4-Morpholineethanesulfonic acid (MES), 50 mM Tris(hydroxymethyl)aminomethane (Tris), 5 mM (Ethylenedinitrilo)tetraacetic Acid (EDTA), 0.5% sodium dodecyl sulfate (SDS)), and run until the dye front reached the bottom of the gel. Samples were prepared to contain a final concentration of 31.25 mM Tris pH 6.8, 12.5% glycerol, 1% SDS, and 0.25% bromophenol blue. The gels were visualized with Coomassie Brilliant Blue and imaged using a Coomassie scan protocol on a Bio-Rad Gel Doc EZ imaging system.

Urea gels were prepared as described above with the addition of 6 M urea to the gel matrix.

Table S7. Relevant library species theoretical masses for samples analyzed by SDS-PAGE (Da).

	B-1.5Hep	B-2Hep	B-2.5Hep	B-3Hep	B-2.5Hep-Def	Ac-3Hep
Monomer	1524.6	1864.9	2178.0	2647.3	2065.9	2522.4
Dimer	3045.2	3725.8	4352.0	5290.6	4127.8	5044.8
B_3	4567.8	5588.7	6528.0	7935.9	6191.7	-
B_4	6090.4	7451.6	8704.0	10581.2	8255.6	-
B_5	7613.0	9314.5	10880.0	13226.5	10319.6	-
B_6	9135.6	11177.4	13056.0	15871.8	12383.5	-
B_7	10658.2	13040.3	15232.0	18517.1	14447.4	-
B_8	12180.8	14903.2	17408.0	21162.4	16511.3	-
B_9	13703.4	16766.1	19584.0	23807.7	18575.2	-
B_{10}	15226.0	18629.0	21760.0	26453.0	20639.1	-
B_{11}	16748.6	20491.9	23936.0	29098.3	22703.0	-
B_{12}	18271.2	22354.8	26112.0	31743.6	24766.9	-
B_{13}	19793.8	24217.7	28288.0	34388.9	26830.8	-

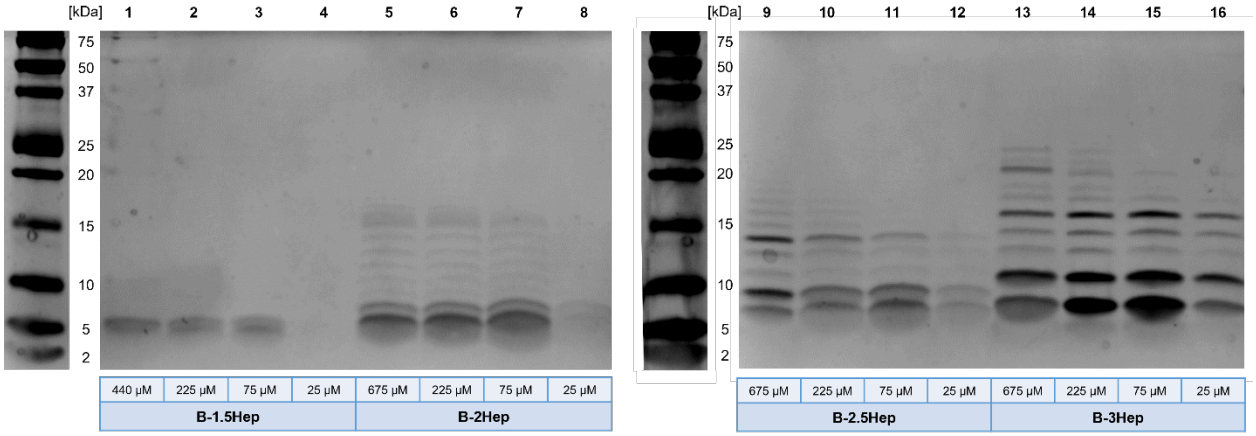

Figure S31. Bis-Tris SDS-PAGE analysis of B-*N*Hep libraries of varying peptide length (1.5-3 heptad). Each library was equilibrated at the concentrations listed in the figure, but loaded at the same concentration of 25 µM after dilution with 50 mM Tris-HCl buffer (pH 6.8) to visualize the concentration-dependence of speciation. Gels were stained with Coomassie Blue for visualization.

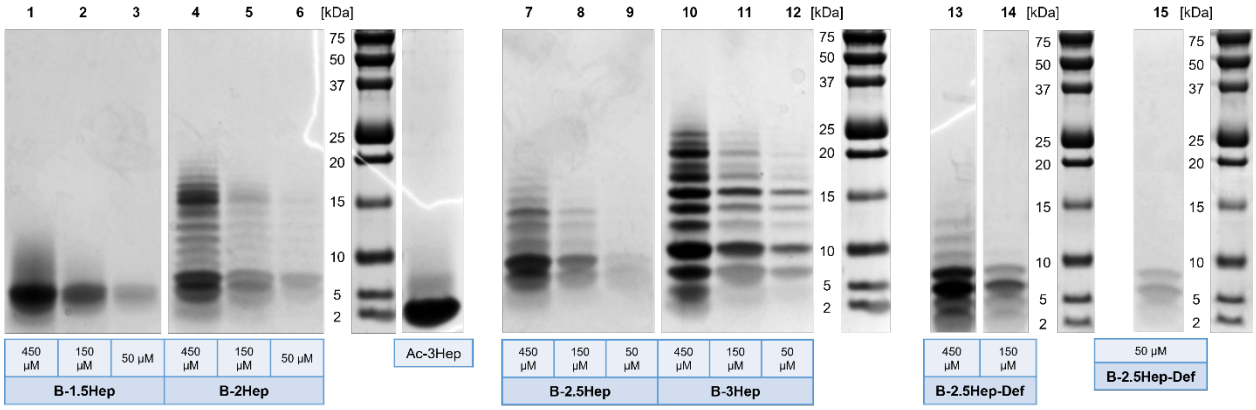

Figure S32. Bis-Tris SDS-PAGE analysis of libraries of varying peptide length (1.5-3 heptad) equilibrated at the concentrations indicated in the figure and loaded at 70% of the original

concentration, such that different intensities represent different amounts loaded on the gel. Shown in order of decreasing concentration and increasing heptad length (B-1.5Hep: 1-3, B-2Hep: 4-6, B-2.5Hep: 7-9, B-3Hep: 10-12). Ac-3Hep peptide included as a mass reference. Gels were stained with Coomassie Blue for visualization.

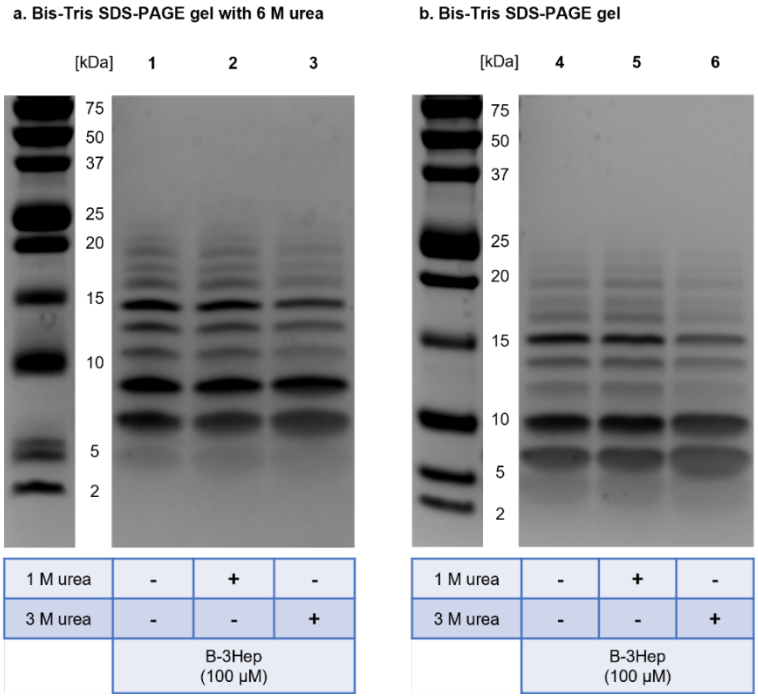

Figure S33. Denaturation of B-3Hep by urea Bis-Tris SDS-PAGE. (a) Gel containing 6 M urea; (b) Standard gel without urea (as above). The library was equilibrated for at least 7 days in 50 mM sodium borate buffer (pH 8.4). Aliquots of the same 150 µM library were used for all lanes. Before loading onto gel, aliquots of library were diluted to 100 µM containing no additional urea (lanes 1 and 4), 1 M additional urea (lanes 2 and 5), or 3 M additional urea (lanes 3 and 6). The addition of urea to a SDS-PAGE system is known to change the electrophoretic mobility of proteins, as observed here in the slight change in the spacing between bands from the standard SDS-PAGE to the 6 M urea gel.[4] Gels were stained with Coomassie Blue for visualization.

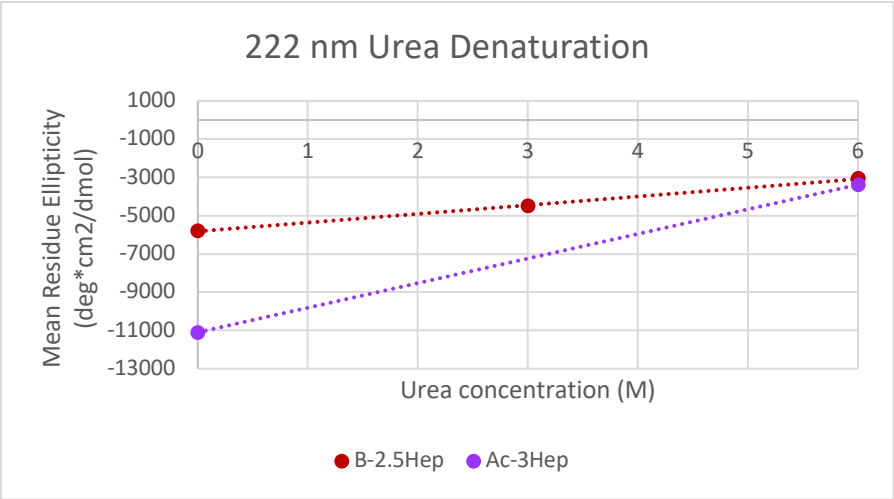

Figure S34. Comparison of the 222 nm minimum mean residue ellipticities of 150 µM B-2.5Hep and Ac-3Hep libraries equilibrated for at least 7 days in 50 mM sodium borate buffer (pH 8.4) with varied urea concentrations (0-6 M). Urea added to samples directly before CD analysis.

Table S8. Comparison of 222 nm mean residue ellipticity values between thermal and 6 M urea denaturation of 150 µM B-2.5Hep library equilibrated for at least 7 days in 50 mM sodium borate buffer (pH 8.4).

Thermal Denaturation (deg*cm^2/dmol)	6 M Urea Denaturation (deg*cm^2/dmol)
-3262.05	-3060.03

5-(iodoacetamido)fluorescein Labeling Experiments

Libraries of homodimeric monomers were prepared as previously described in 50 mM sodium borate buffer and allowed to reach equilibrium (7 days). An 80 uL aliquot of equilibrated library was used for each 100 uL reaction. For the standard samples, the library aliquot was mixed with 15 uL of a stock 5-IAF solution (in borate buffer, 5% ACN) and 5 uL of buffer acidified to pH 1 with HCl, creating a 100 uL sample with 0.25:1 5-IAF to monomer concentration at pH 7.

For the reduced samples, the library aliquot was mixed with 7.5 uL of TCEP stock solution (in borate buffer) to yield a sample with 2.1:1 TCEP to monomer concentration. This solution was put on the shake plate for 1.25 hours to allow for reduction of species in the equilibrated library, then 7.5 uL of a stock 5-IAF solution (in borate buffer, 5% ACN) and 5 uL of buffer acidified to pH 1 with HCl, creating a 100 uL sample with 0.25:1 5-IAF to monomer concentration at pH 7.

For the oxidized samples, the library aliquot was mixed with 7.5 uL of sodium perborate stock solution (in borate buffer) to yield a sample with 2.1:1 sodium perborate to monomer concentration. This solution was put on the shake plate for 1.25 hours to allow for oxidation of all monomers, then 7.5 uL of a stock 5-IAF solution (in borate buffer, 5% CAN) and 5 uL of buffer acidified to pH 1 with HCl, creating a 100 uL sample with 0.25:1 5-IAF to monomer concentration at pH 7.

All acidified samples were then left on the shake plate for 2 hours to allow for 5-IAF labeling of any free thiols.

5-IAF labeling was analyzed by SDS-PAGE and imaged using a Cy2 scan (excitation = 488 nm, emission = 525 nm) on a Typhoon Biomolecular Imager (Amersham). Subsequently, the gel was further visualized with Coomassie Brilliant Blue and imaged using a Coomassie scan protocol on a Bio-Rad Gel Doc EZ imaging system as above.

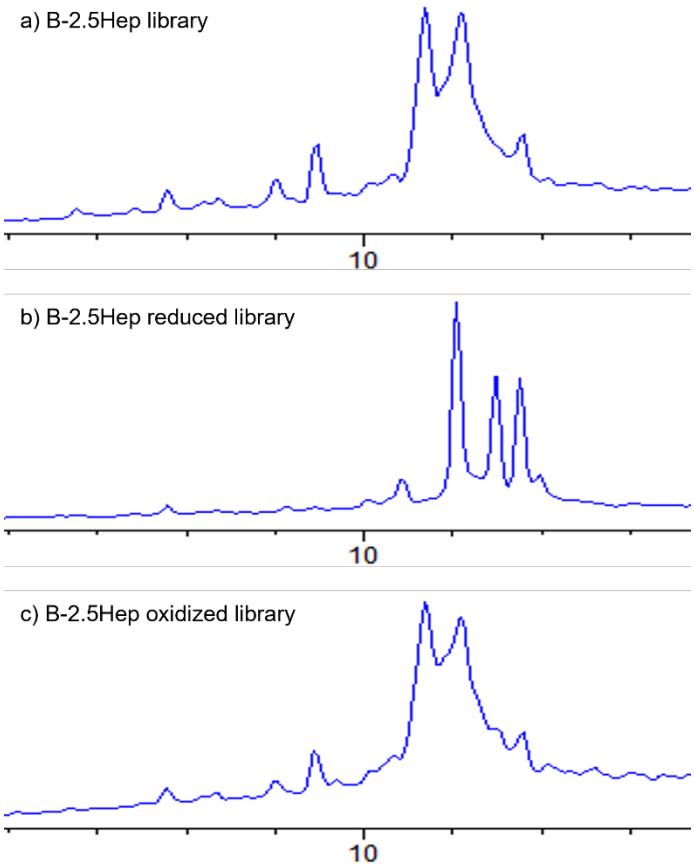

Figure S35. Partial LC-MS TIC of a B-2.5Hep library after reaction with 5-IAF under varied conditions. a) B-2.5Hep library with 0.25 equivalents of 5-IAF; b) B-2.5Hep library reduced with 2.1 equivalents of TCEP prior to addition of 0.25 equivalents of 5-IAF; c) B-2.5Hep library reduced with 2.1 equivalents of sodium perborate prior to addition of 0.25 equivalents of 5-IAF.

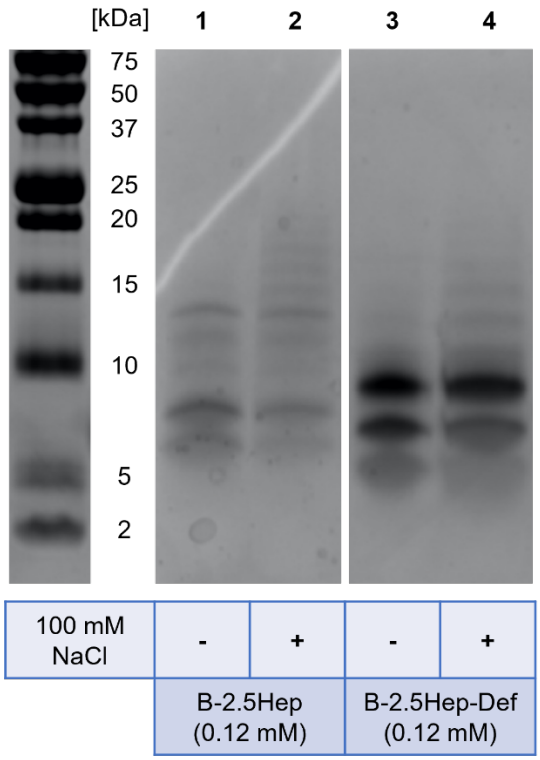

Figure S36. Bis-Tris SDS-PAGE gel analysis of 150 µM B-2.5Hep and B-2.5Hep-Def libraries equilibrated in 50 mM borate buffer (pH 8.4) with (lanes 2 and 4) or without (lanes 1 and 3) 100 mM NaCl for at least 7 days. Gels were stained with Coomassie Blue for visualization.

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy was run using a Chirascan Plus with a Peltier Temperature Control unit. Samples were run in 50 mM borate buffer (pH 8.5) to the appropriate concentration based on UV absorbance. Samples were measured at 22 °C in a 1 mm cuvette at a scan speed of 1.25 nm/sec. Measurements were taken in increments of 0.5 nm. After subtraction of background, raw ellipticity was converted to mean residue ellipticity (deg*cm^2/dmol) using the following equation:

$$MRE = \frac{\theta}{10nlc}$$

where θ is ellipticity (deg), n is # of residues, l is path length (0.1 cm), and c is concentration (M).[5]

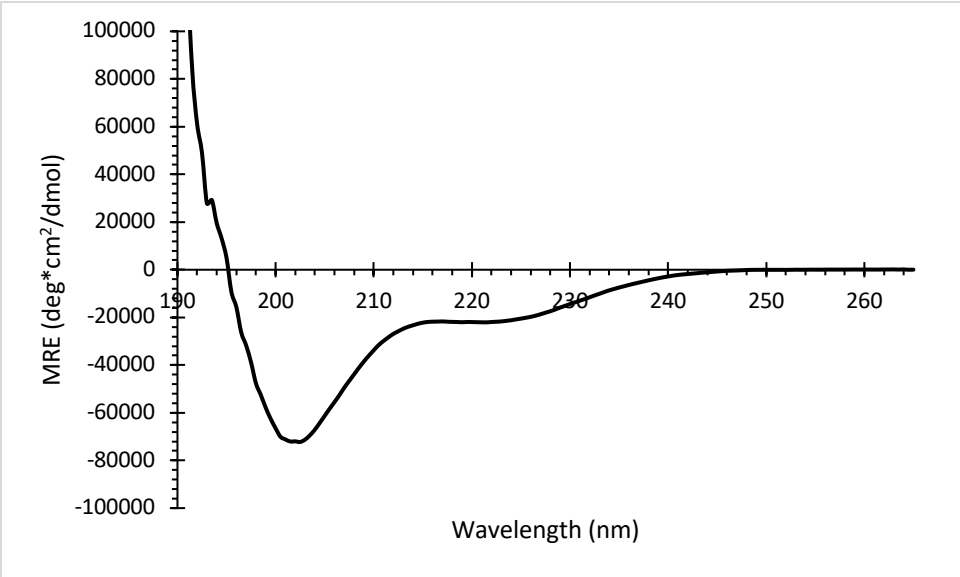

Figure S37. CD spectrum of **Ac-3Hep-Def.** Concentration of peptide is 37 μM, scans taken in 50 mM borate buffer, pH 8.5.

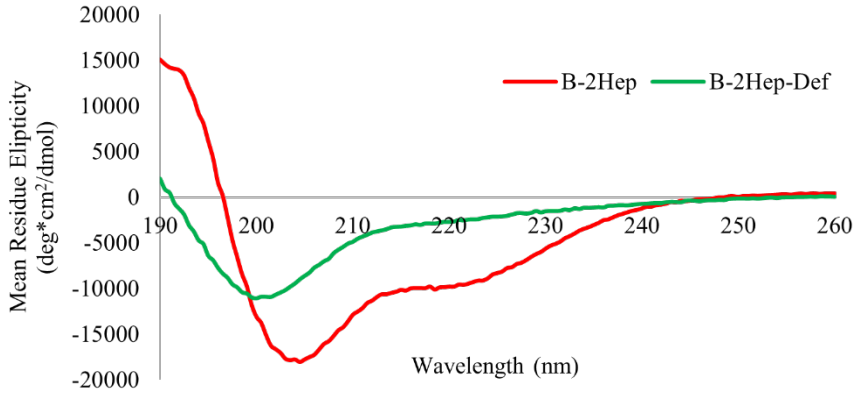

Figure S38. CD spectrum comparing **B-2Hep** to **B-2Hep-Def**. The **B-2Hep** has more helical folding, characterized by the shift of the minimum at 200 nm to 204 nm, as well as a minimum at 222 nm. The **B-2Hep-Def** is a random coil, with a prominent minimum at 200 nm. Concentration of peptides is 150 μM, scans taken in 50 mM borate buffer, pH 8.5.

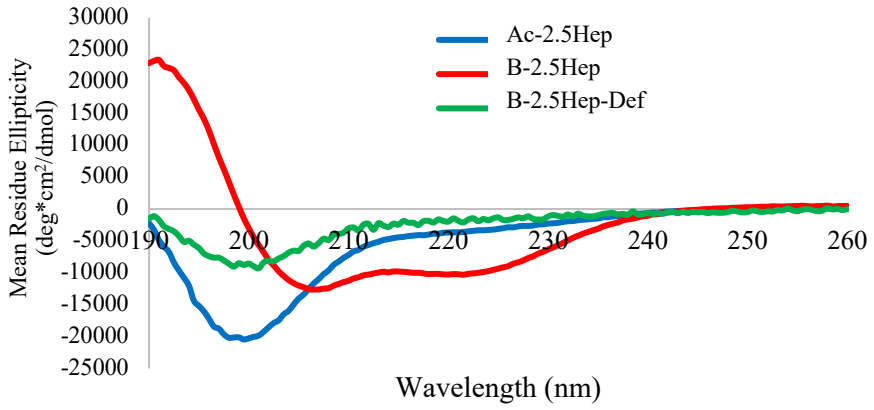

Figure S39. CD spectrum comparing **B-2.5Hep, Ac-2.5Hep, and B-2.5Hep-Def**. The **B-2.5Hep** has more helical folding, characterized by the shift of the minimum at 200 nm to 208 nm, as well as a prominent minimum at 222 nm. Both **B-2.5Hep-Def** and **Ac-2.5Hep** are random coils as they both have a prominent minimum at 200 nm. Concentration of peptides is 150 μM, scans taken in 50 mM borate buffer, pH 8.5.

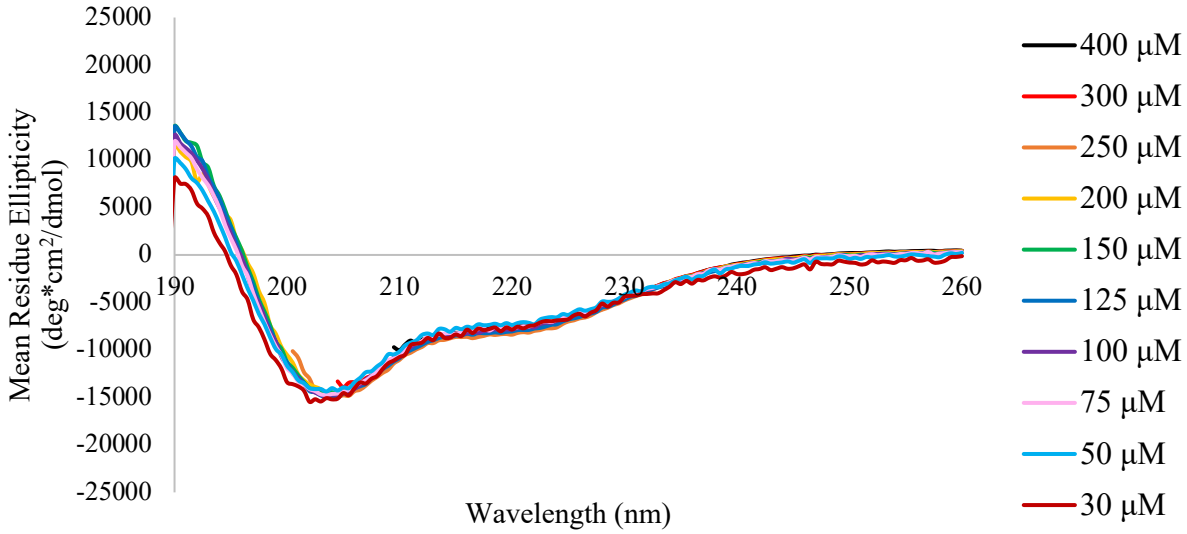

Figure S40. CD spectrum comparing **B-2Hep** at various concentrations. The overlay of spectra indicate concentration is not plying a strong role in binding. The spectra of some of the more concentrated libraries are not shown at lower wavelengths as the absorbance signal was maxed out. Scans taken in 50 mM borate buffer, pH 8.5.

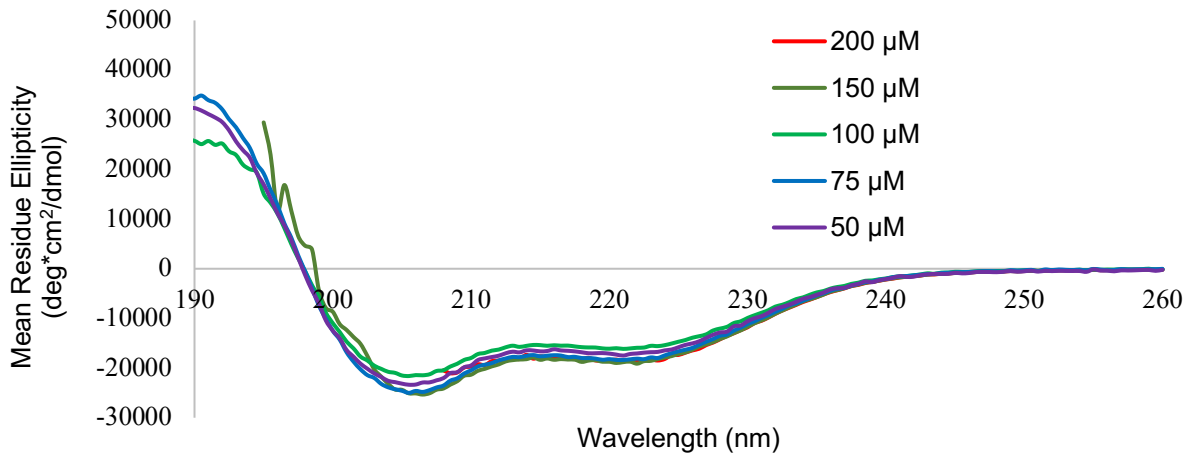

Figure S41. CD spectrum comparing **R-2.5Hep** at various concentrations. The overlay of spectra indicate concentration is not plying a strong role in binding. The spectra of some of the more concentrated libraries are not shown at lower wavelengths as the absorbance signal was maxed out. Scans taken in 50 mM borate buffer, pH 8.5.

Thermal Denaturation Experiments

Circular dichroism was run using a Chirascan Plus with a Peltier Temperature Control unit. Libraries were dissolved in 50 mM borate buffer (pH 8.5) to the appropriate concentration based on UV absorbance. Measurements were taken in a 1 mm cuvette at a scan speed of 1.25 nm/sec. The ellipticity at 222 nm was measured in 1 °C increments at 1 °C/min. Data workup involved calculating α values using the following equation:

$$\alpha = \frac{\theta - \theta_f}{\theta_i - \theta_f}$$

where θ represents the measured ellipticity value, θ_i represents the approximated max folded state, and θ_f represents the approximated ellipticity when completely unfolded. T_M was estimated as the temperature where $\alpha = 0.50$.[6]

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