Electronic Supplementary Material (ESI) for Analyst.

Interrogation of Solution Conformation of Complex Macrocyclic Peptides Utilizing a Combined SEC-HDX-MS, Circular Dichroism, and NMR Workflow

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Supplementary Information

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General Information

Acetonitrile-d_3 was purchased from Cambridge Isotope Laboratories, Inc. and used as received. All NMR spectra were recorded on a 600 MHz and referenced to the acetonitrile d_3 signal with a Bruker Avance HD III console equipped with a Prodigy TCI CryoProbe at the temperature indicated on the spectra. All NMR chemical shifts are reported in parts per million (ppm) relative Acetonitrile-d_3, 1.94 ppm for ^{1}H NMR and 29.84 ppm for ^{13}C NMR. Coupling constants are reported in Hz. All data were analyzed using MestReNova v12.1 (Mestrelab Research S.L.).

High-resolution mass spectrometry (HRMS) data were recorded on a Waters Acquity UPLC equiped with diode array detector and a Xevo G2-XS Qtof MS instrument in ESI+ (electrospray) ionization mode.

Figure S1. SEC-HDX-MS spectra for peptide **1**

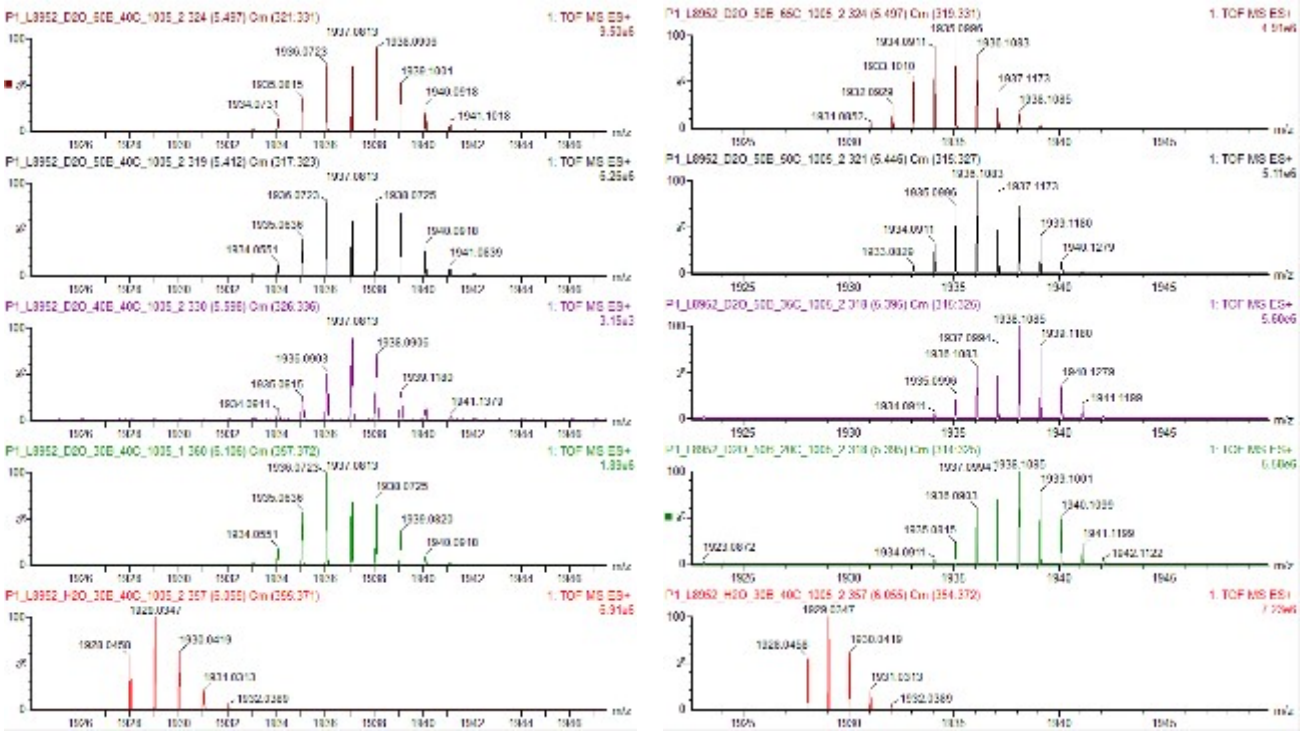


Figure S2. SEC-HDX-MS spectra for peptide **2**

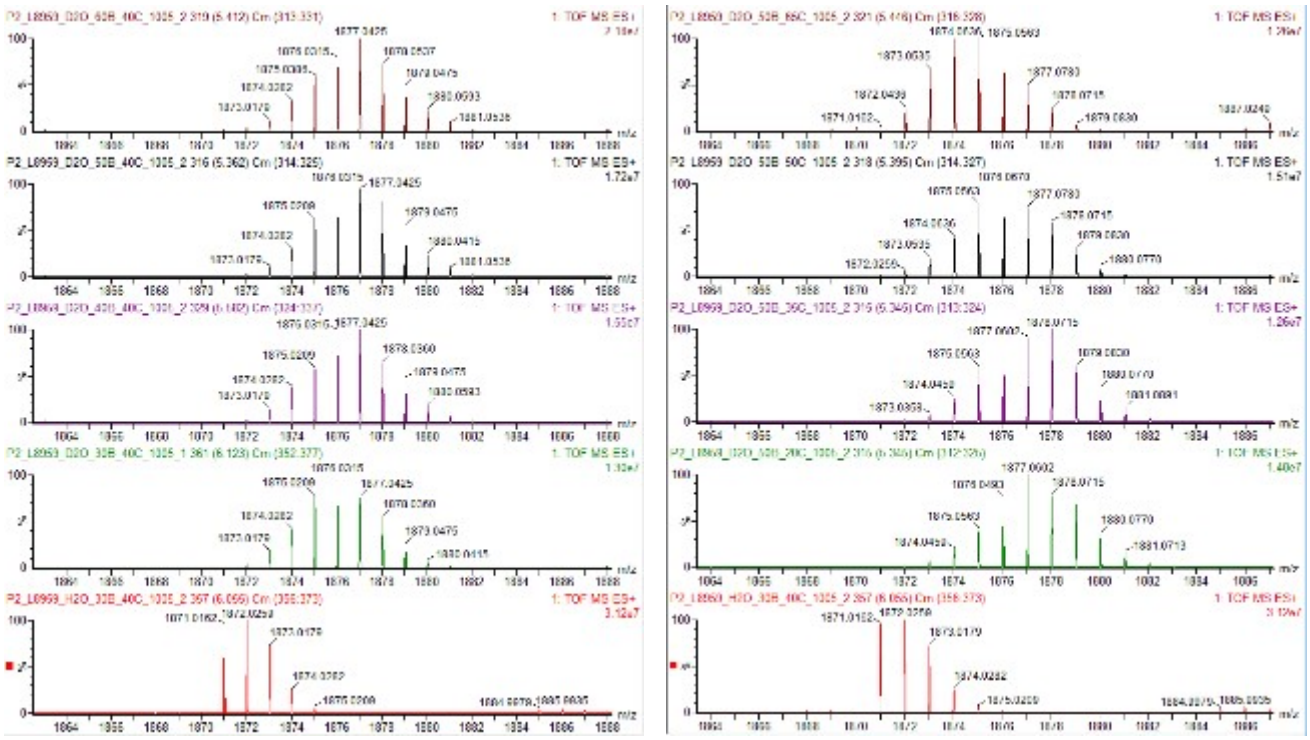


Figure S3. SEC-HDX-MS spectra for peptide **3**

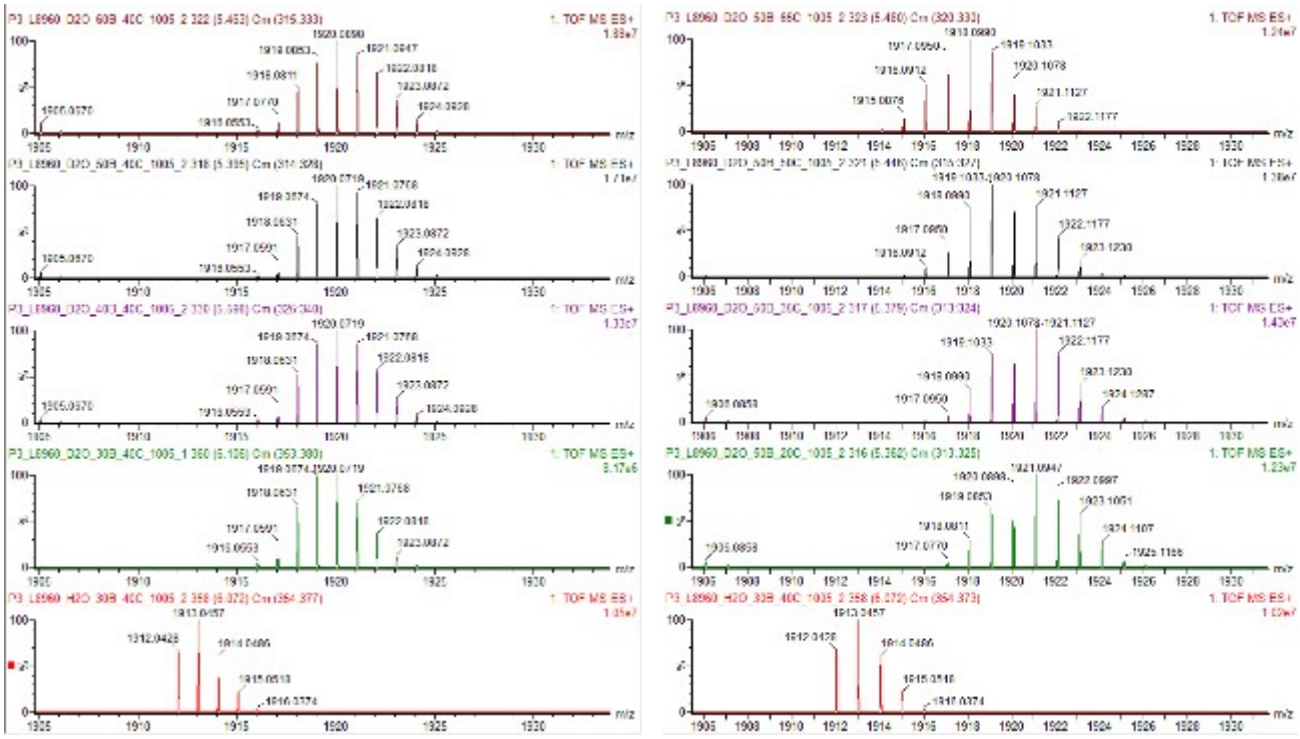


Figure S4. SEC-HDX-MS spectra for peptide **4**

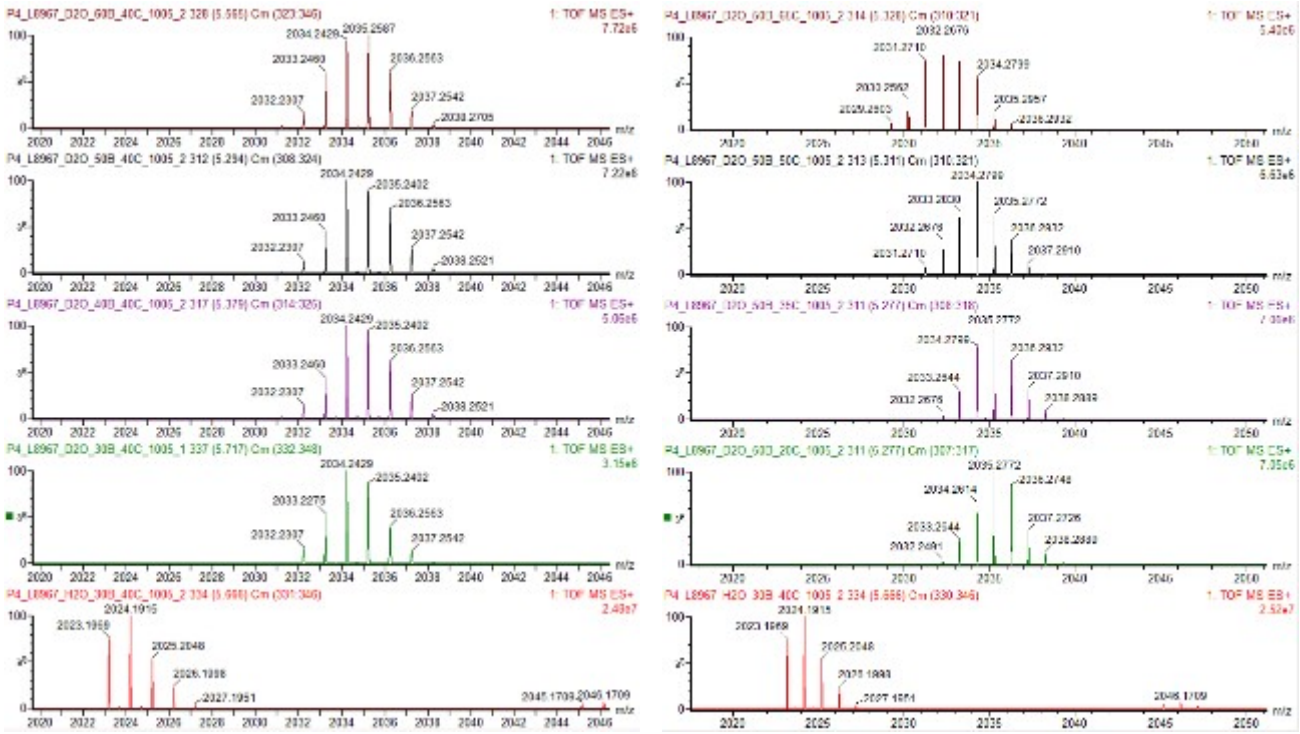


Figure S5. SEC-HDX-MS spectra for peptide **5**

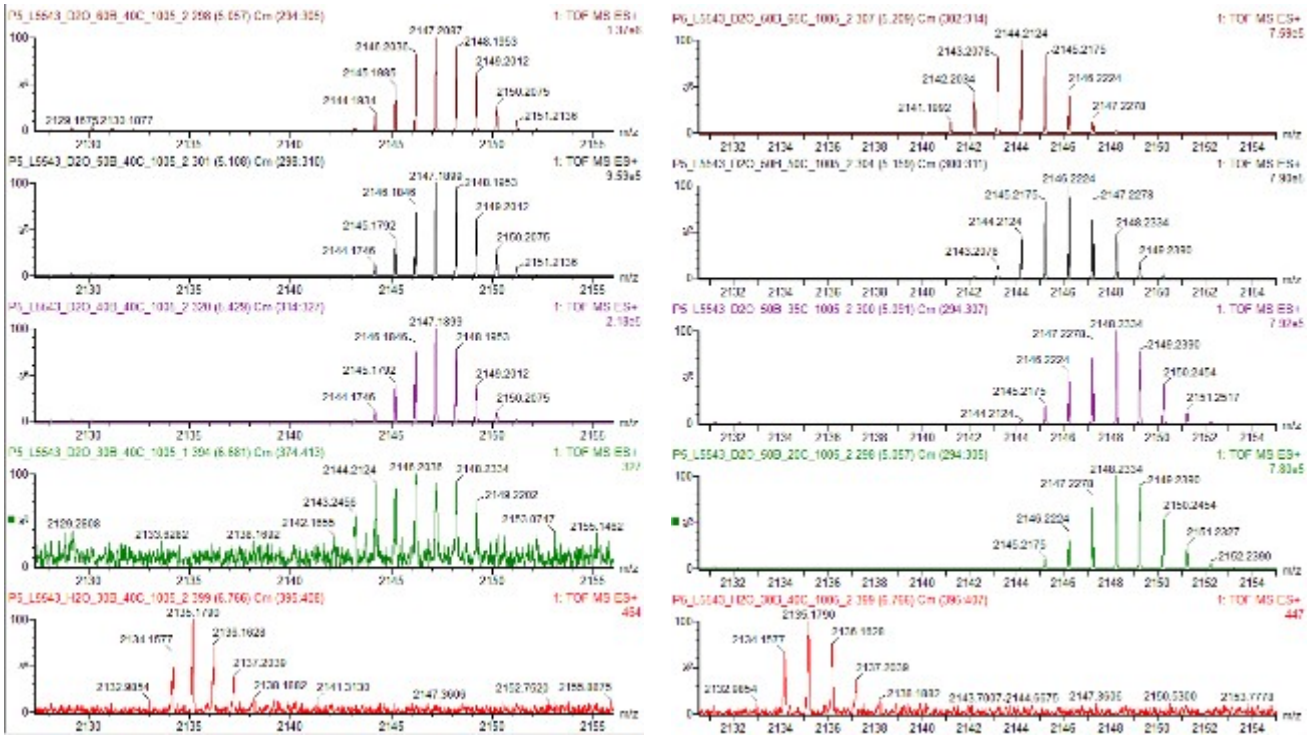


Figure S6. SEC-HDX-MS spectra for peptide **6**

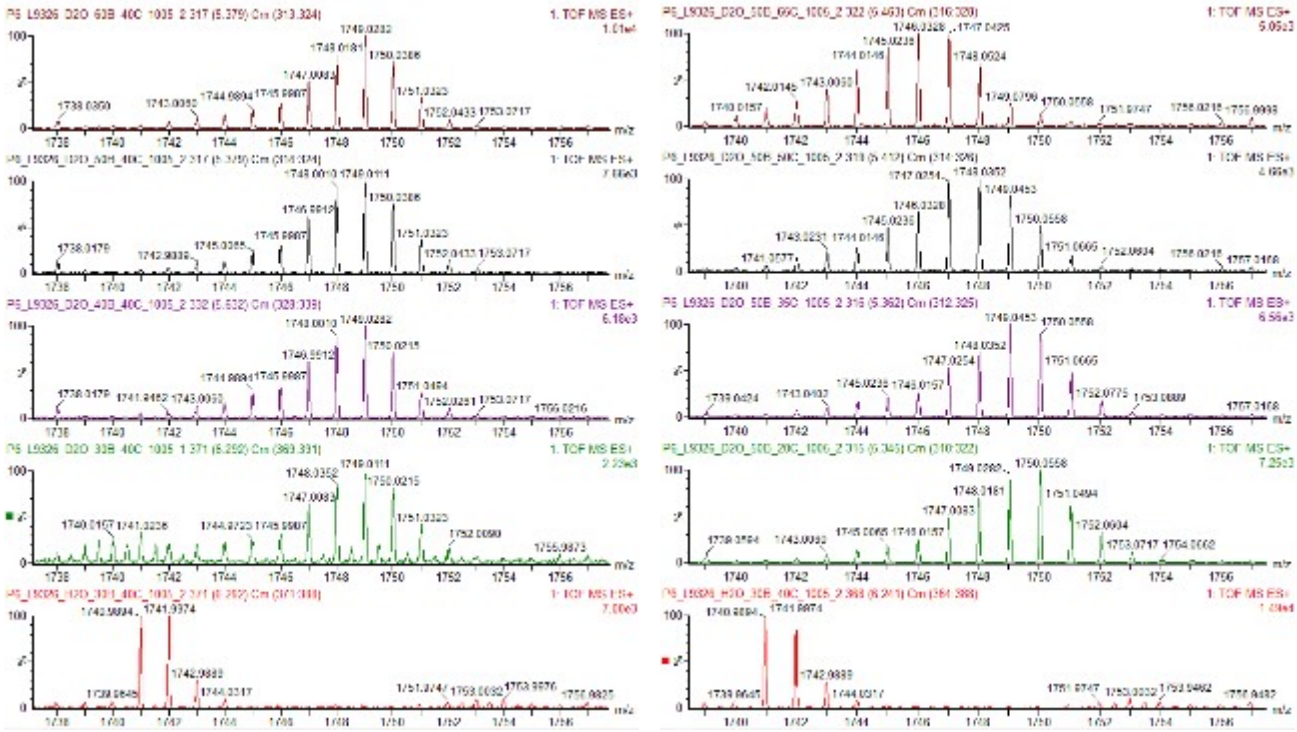


Figure S7. CD spectra of 10 % organic modifier for peptide **1-6**

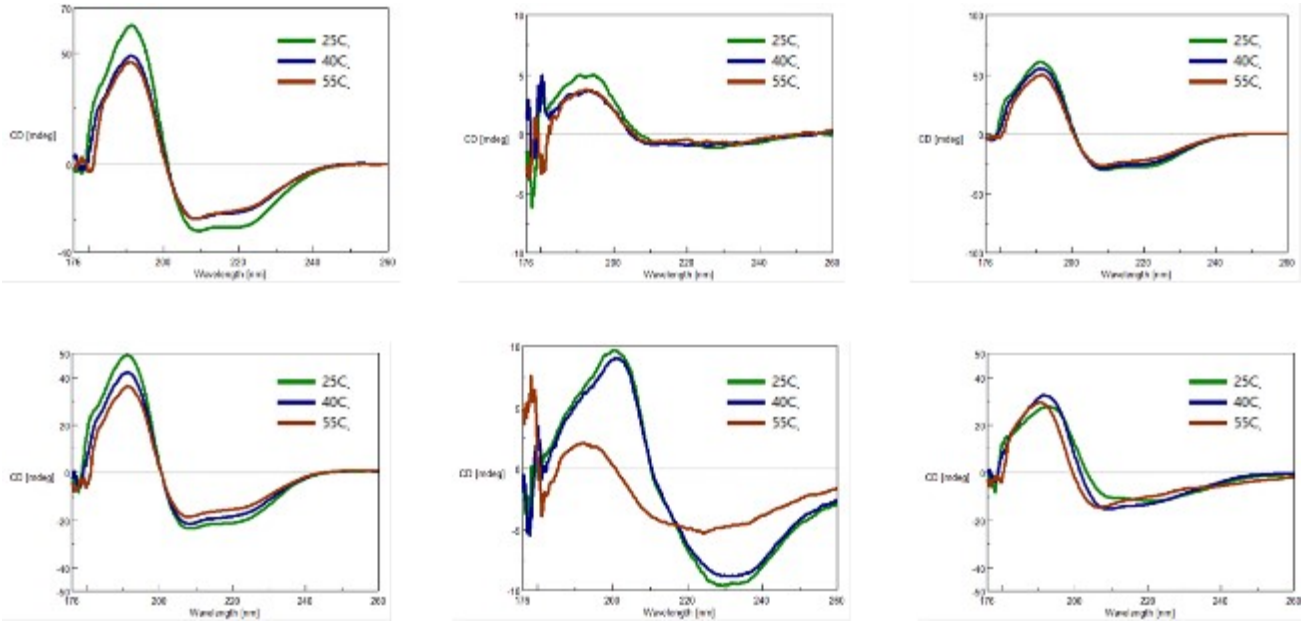


Figure S8. CD spectra of 30 % organic modifier for peptide **1-6**

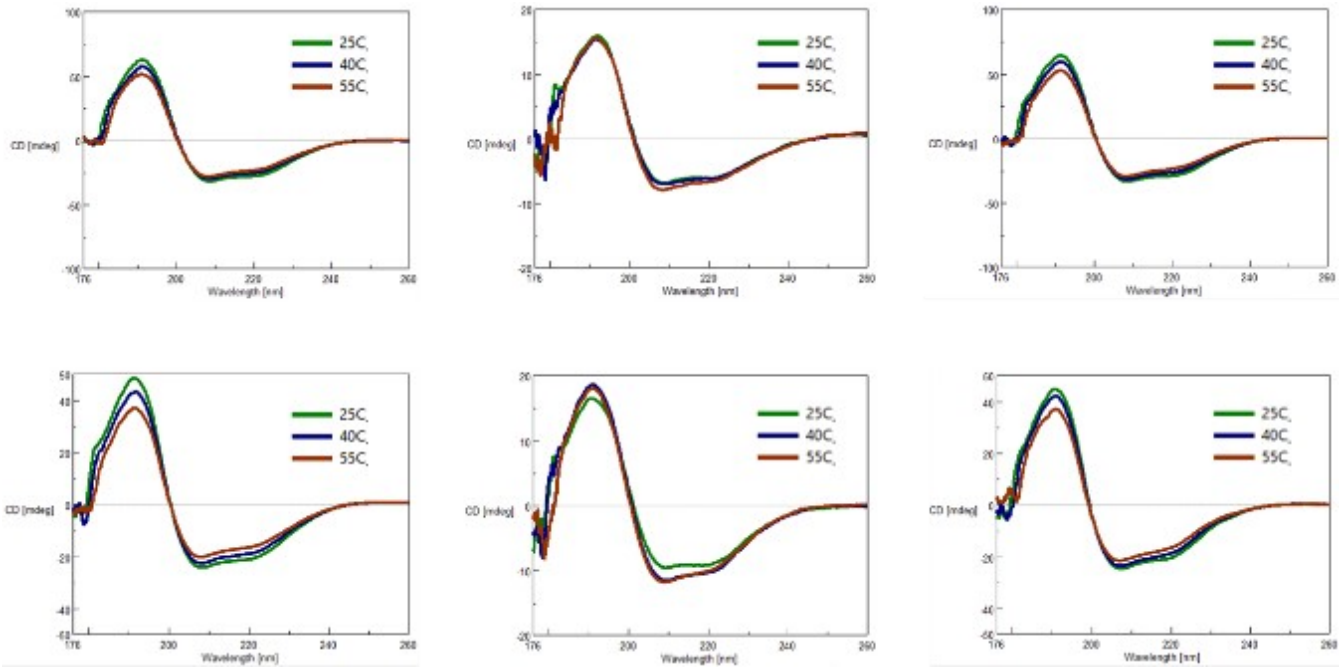


Figure S9. CD spectra of 50 % organic modifier for peptide **1-6**

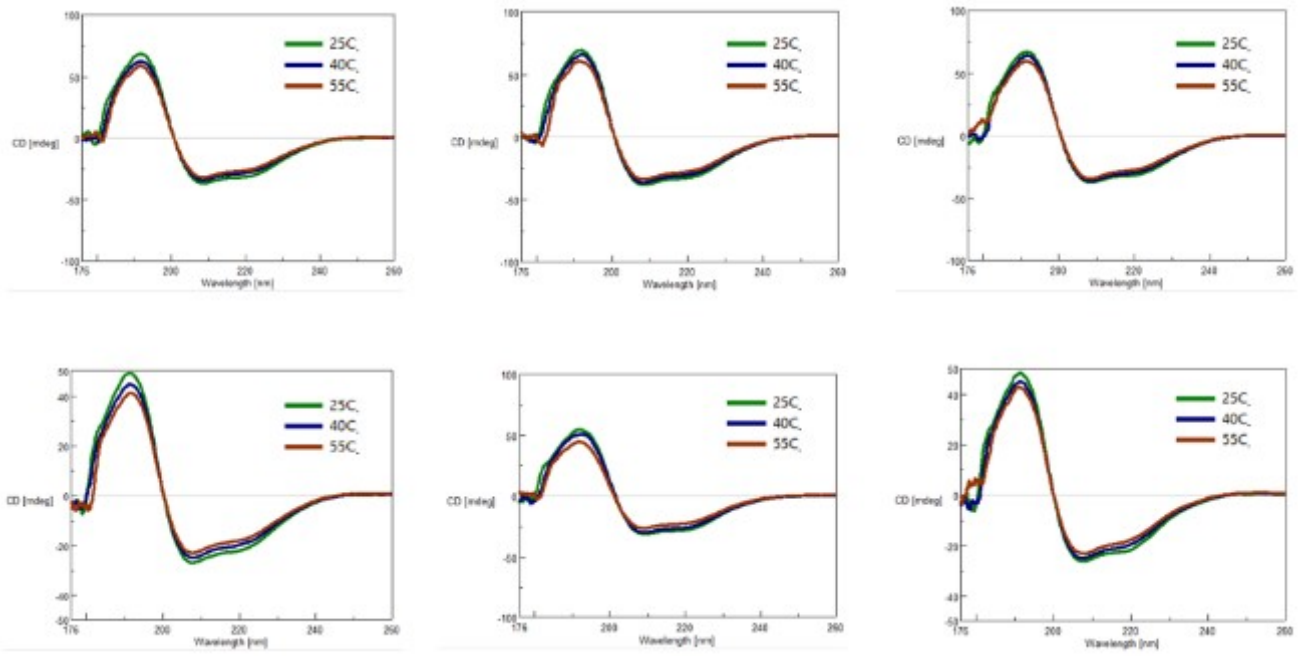


Figure S10. Structure with assignment key for **1**

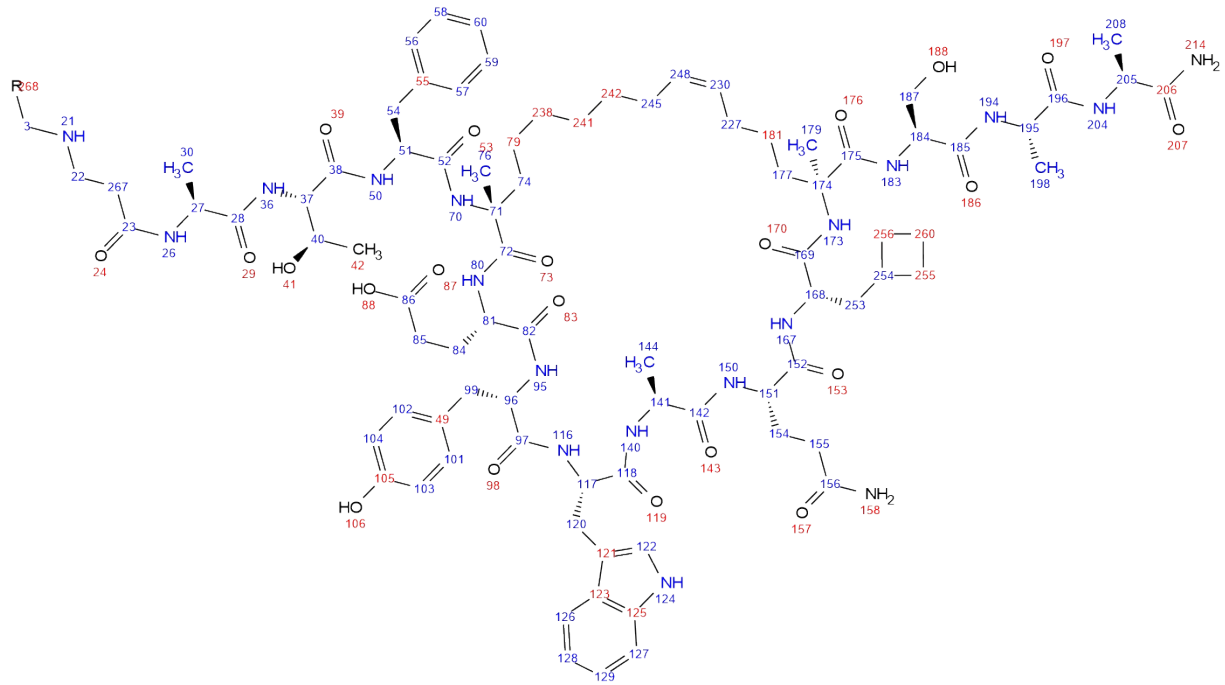


Figure S11. 1H spectra using excitation sculpting for water suppression of **1**

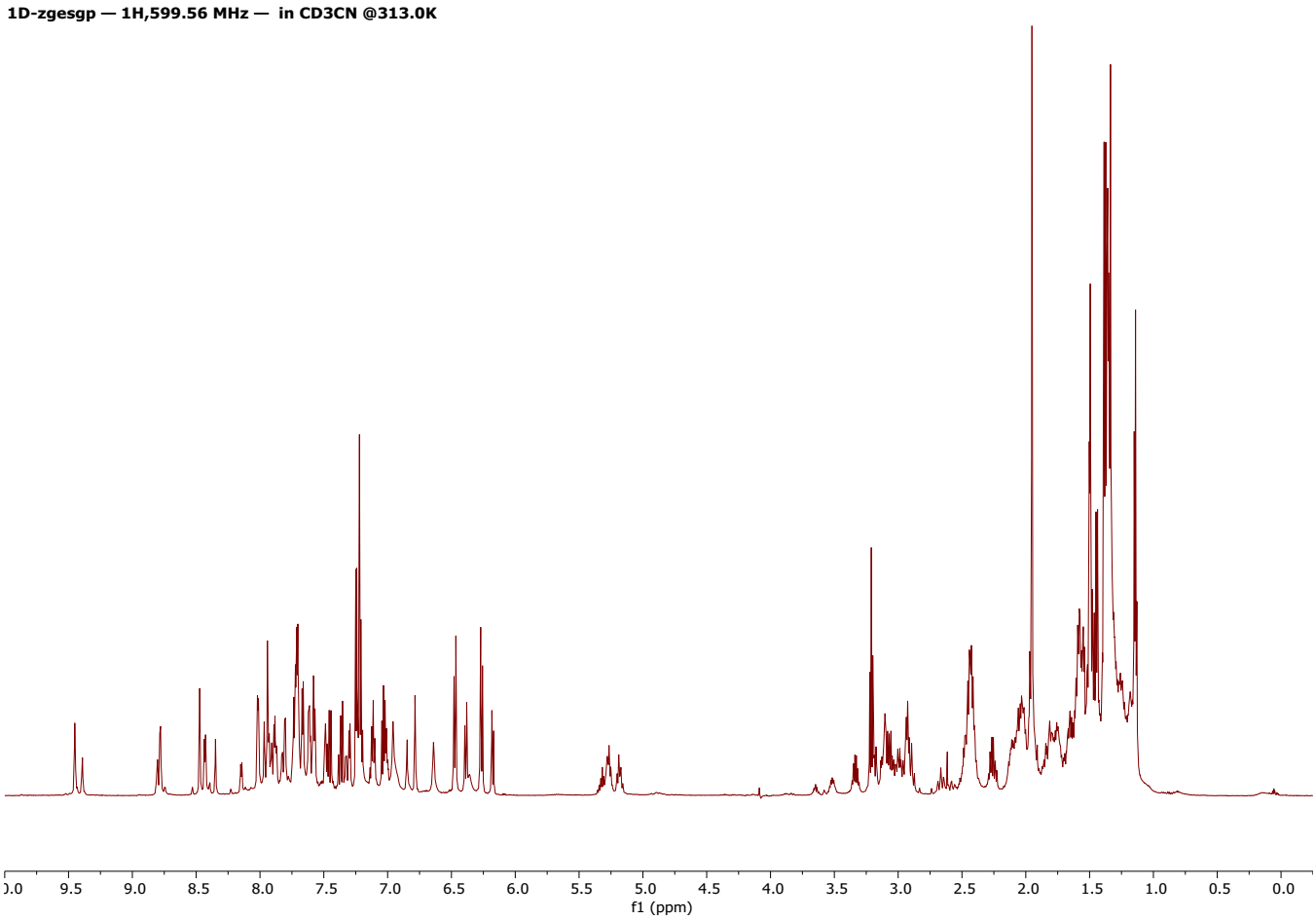


Figure S12. 1H spectra using excitation sculpting for water suppression of **1** zoomed to the amide NH region with $^{3}J_{NH/H\text{-}\alpha}$ indicated

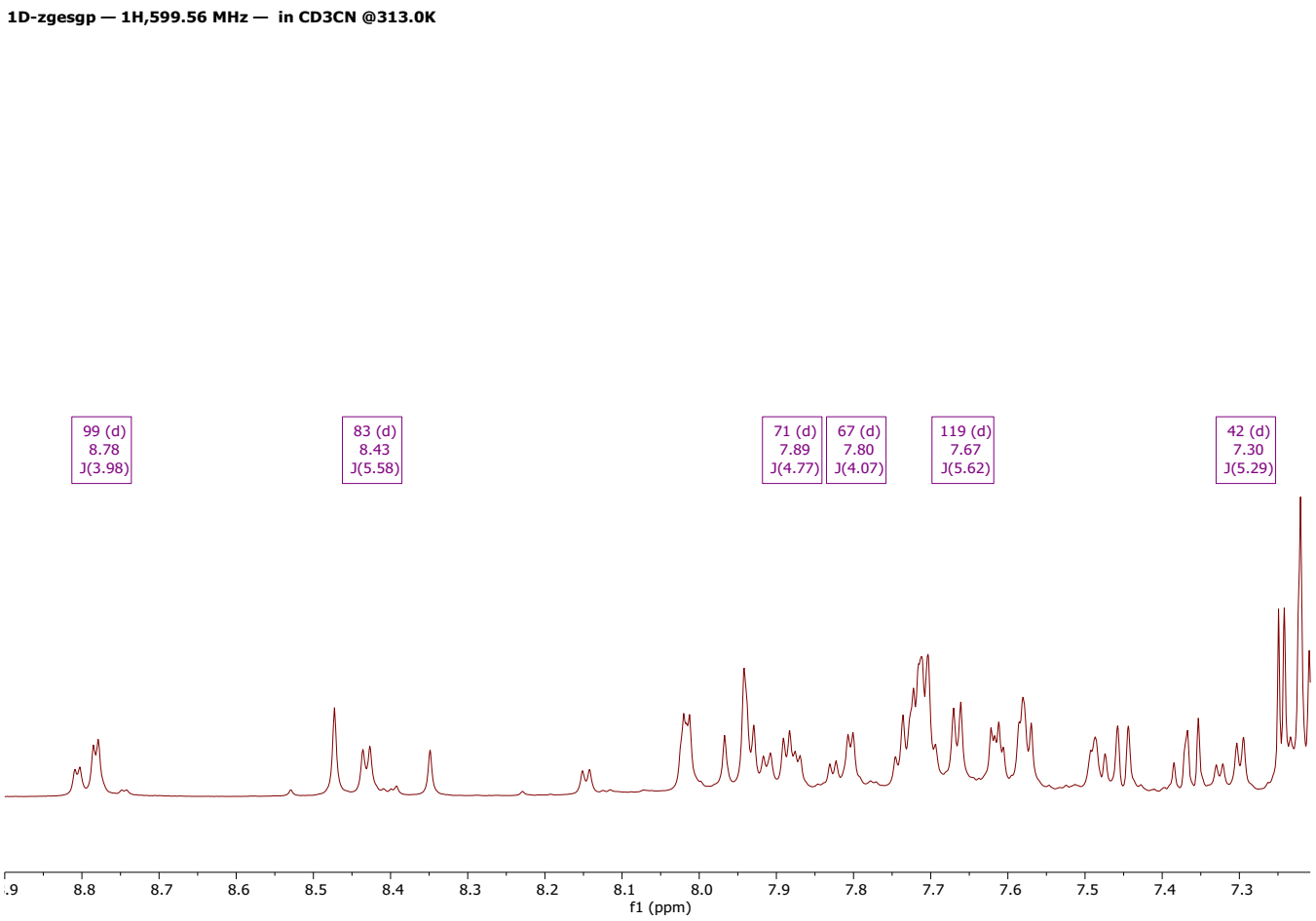


Figure S13. Broadband homo-decoupled ME-HSQC using presaturation for water suppression of **1**

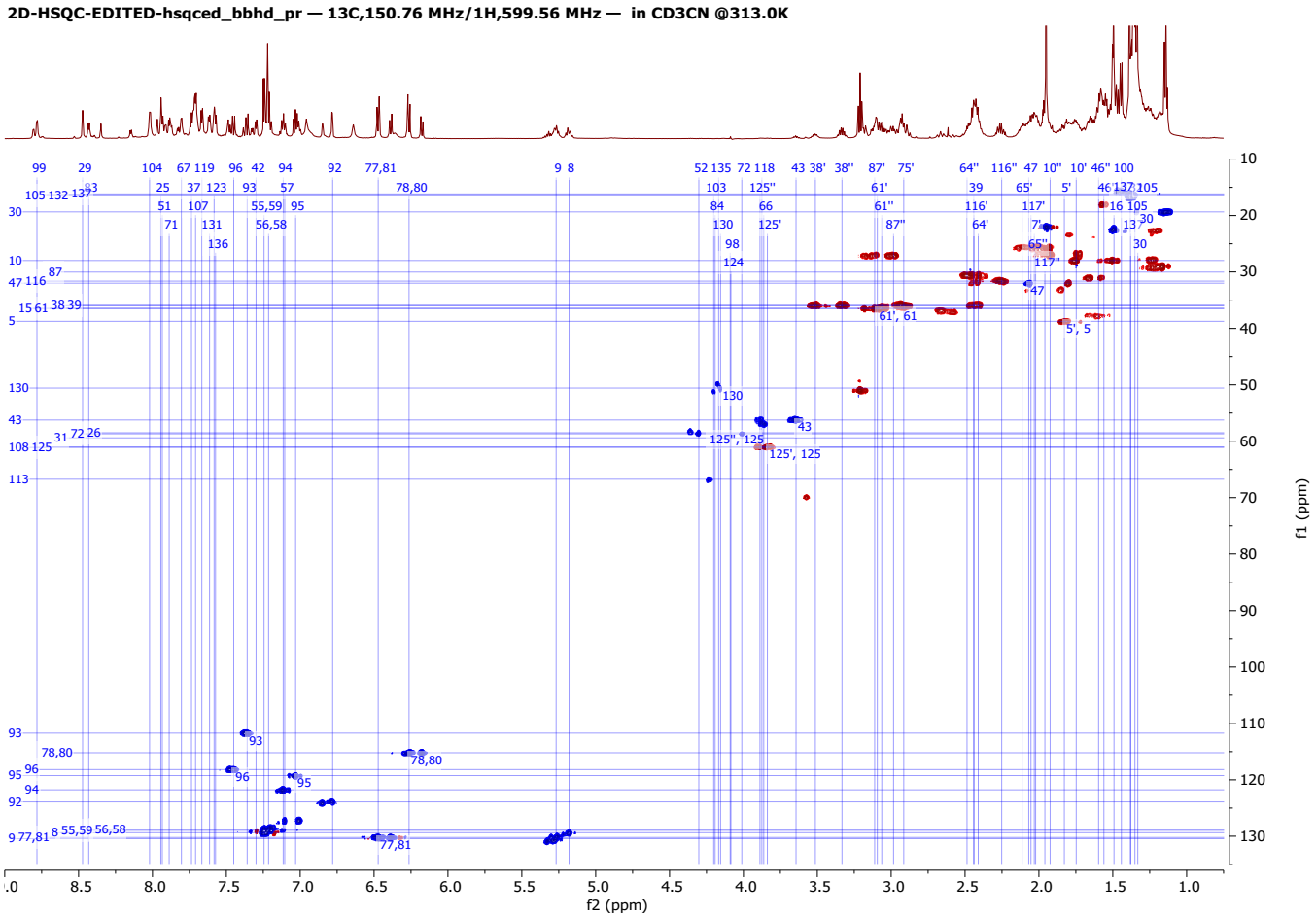


Figure S14. TOCSY using excitation sculpting for water suppression spectra of **1**

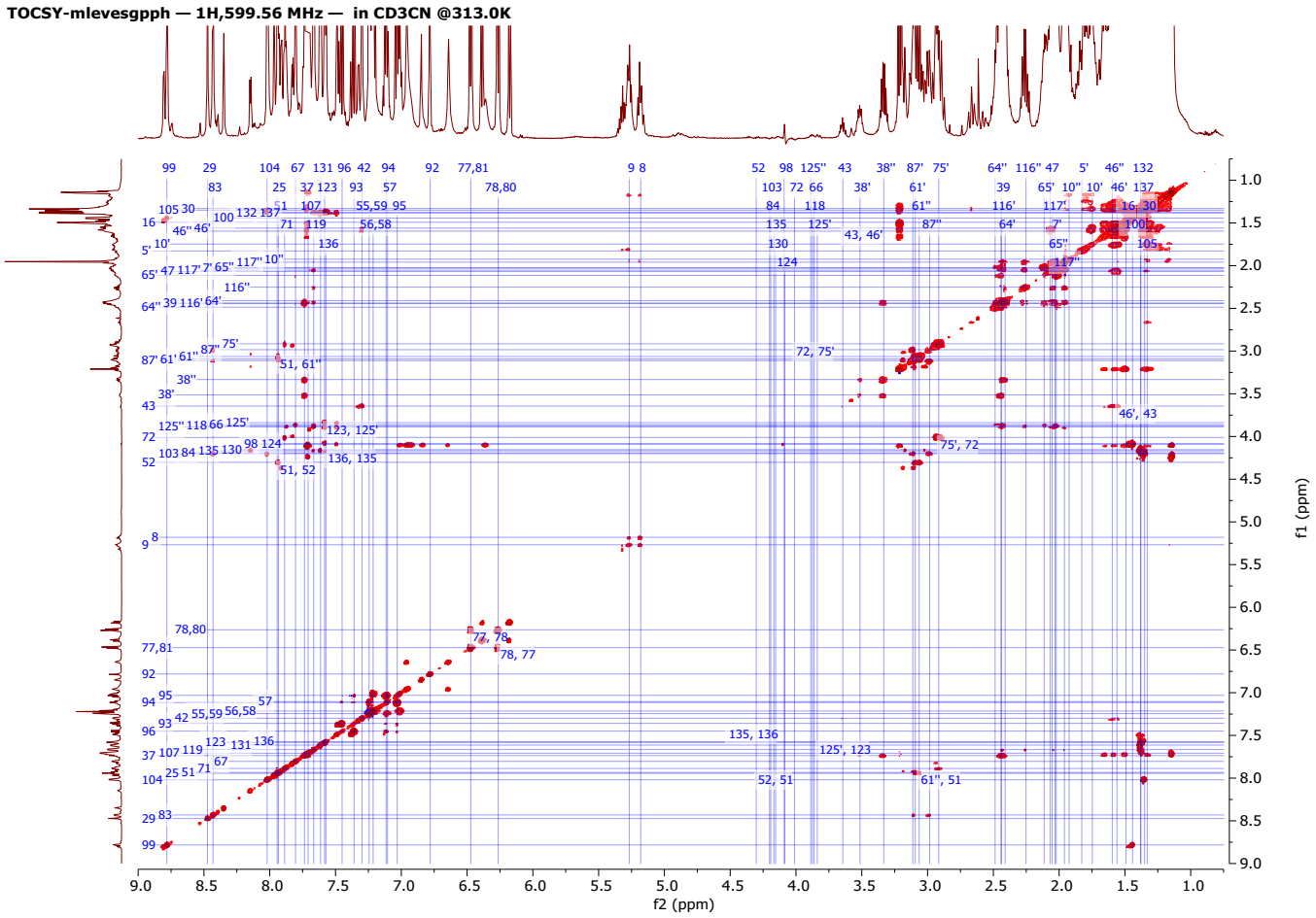


Figure S15. ROESY spectra using excitation sculpting for water suppression of **1**

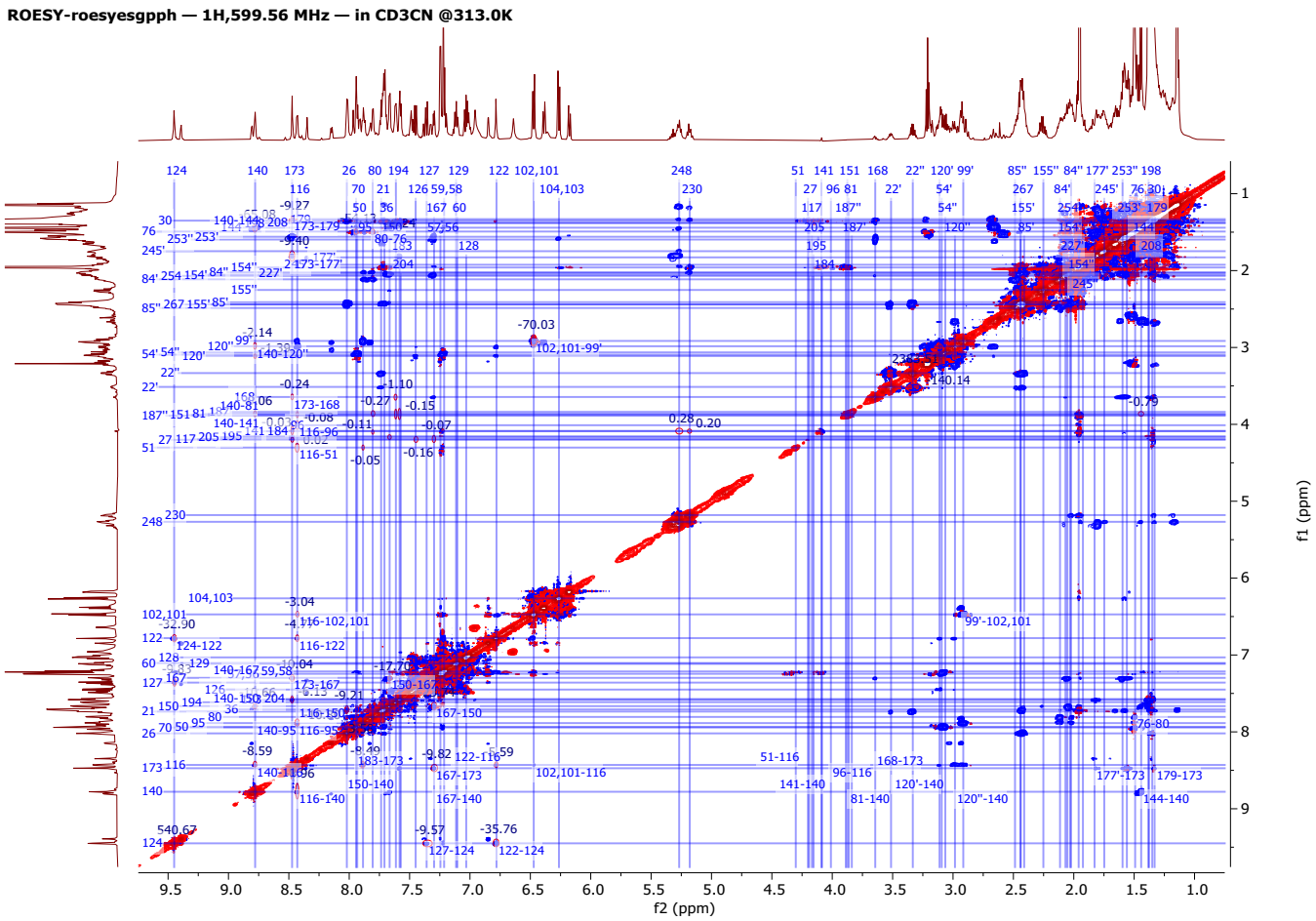


Figure S16. ROESY spectra using excitation sculpting for water suppression of **1** zoomed to the amide region demonstrating the quality of data obtained

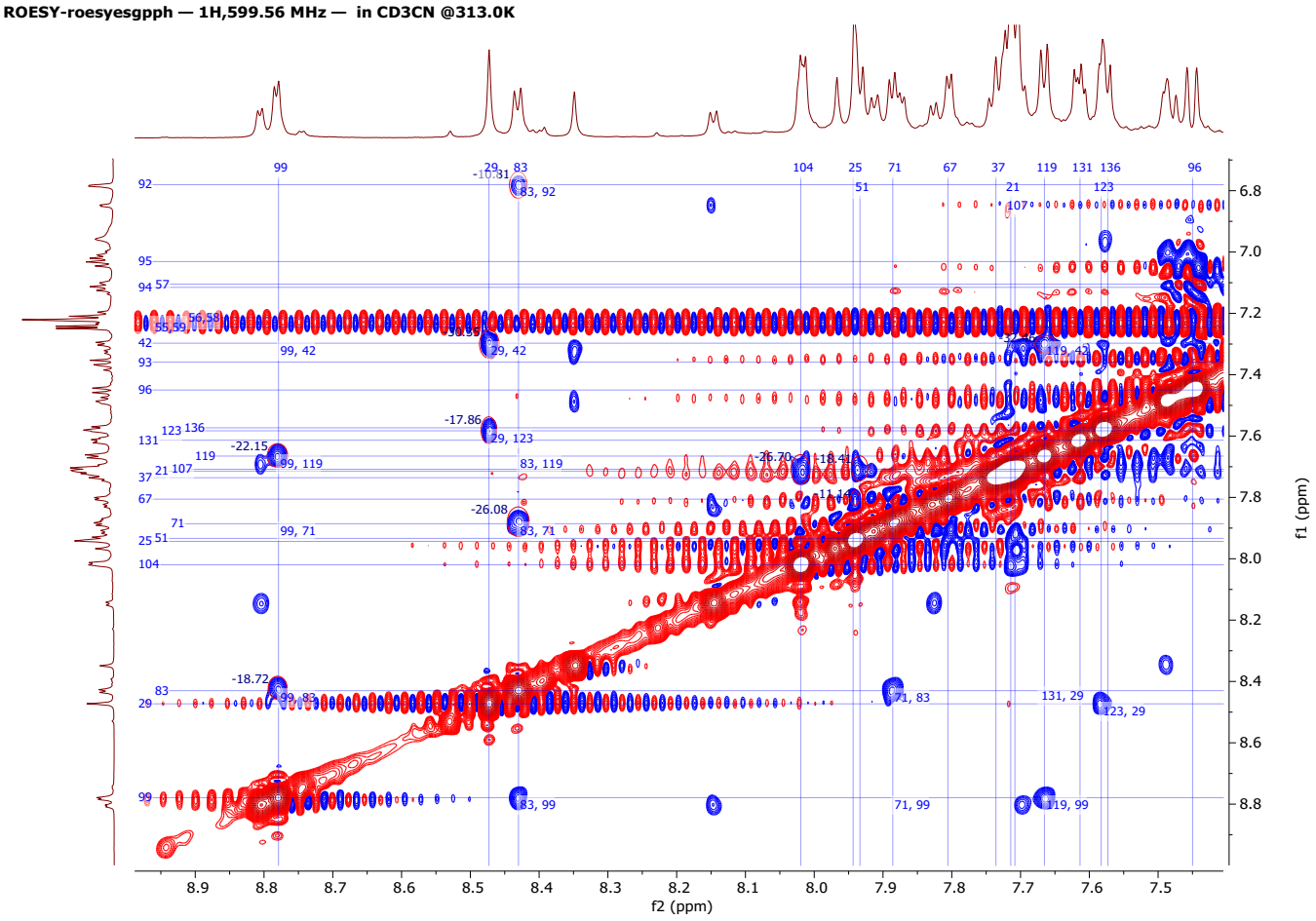
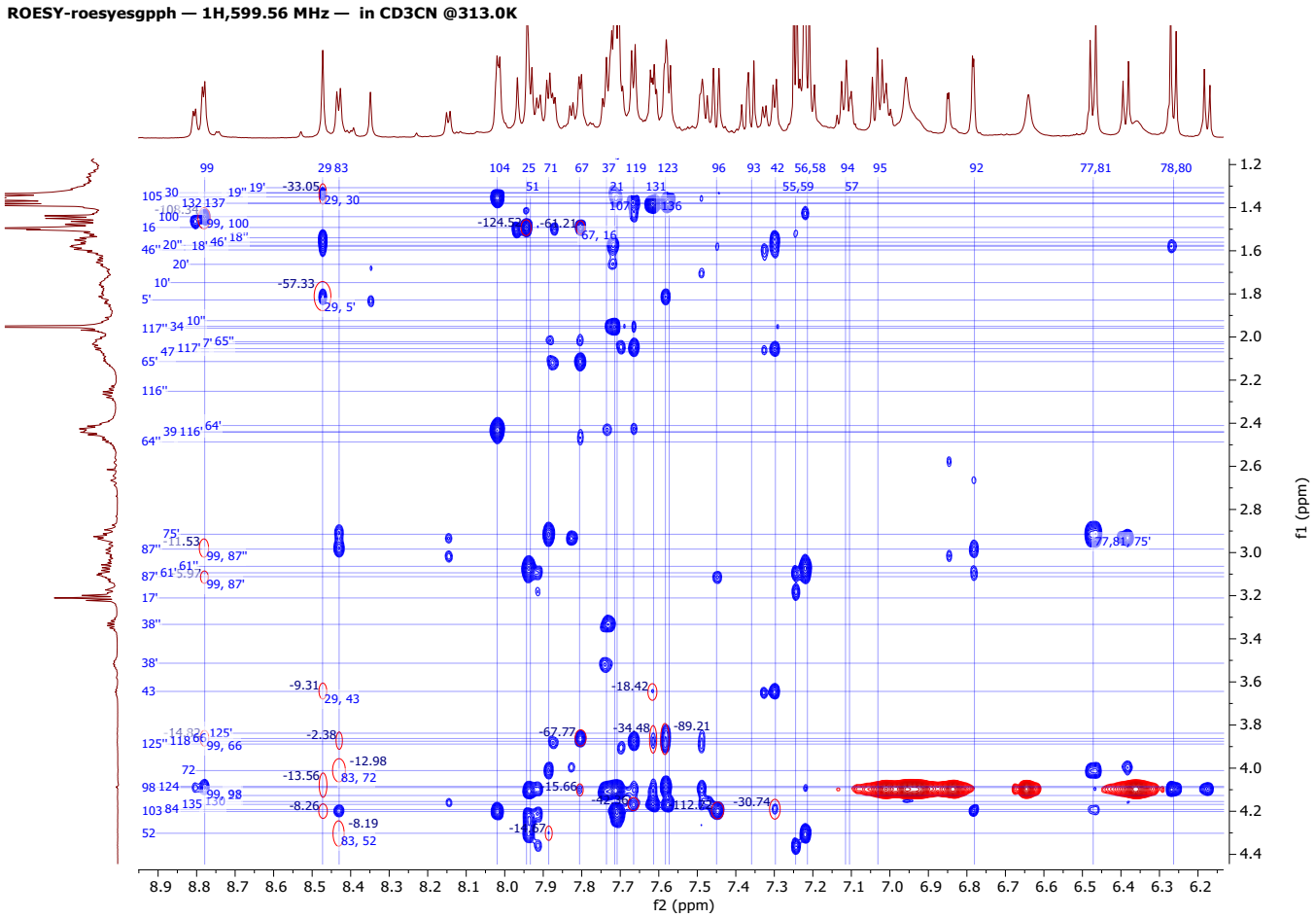


Figure S17. ROESY spectra using excitation sculpting for water suppression of **1** zoomed to the NH α-H/aliphatic intersection demonstrating the quality of spectra obtained



type	mnova numbers		NOE	NOE distance (Å)	upper limit	forcegen input
NH/NH	173	183	17.86	3.04	3.04	nmr 10 173 183 17.86 3.04
NH/NH	140	116	18.72	3.02	3.02	nmr 10 140 116 18.72 3.02
NH/NH	140	150	22.15	2.93	2.93	nmr 10 140 150 22.15 2.93
NH/NH	116	95	23.9	2.90	2.90	nmr 10 116 95 23.9 2.9
NH/NH	173	167	30.35	2.78	2.78	nmr 10 173 167 30.35 2.78
NH/NH	167	150	32.46	2.75	2.75	nmr 10 167 150 32.46 2.75
NH/Hα+3	116	51	3.25	4.04	4.04	nmr 1 116 51 3.25 4.04
NH/Hα+3	140	81	14.82	3.14	3.14	nmr 1 140 81 14.82 3.14
NH/Hα+3	194	168	18.42	3.02	3.02	nmr 1 194 168 18.42 3.02
NH/Cα+2	167	117	30.74	2.78	2.78	nmr 1 167 117 30.74 2.78
NH/NH+2	167	183	18.72	3.02	3.02	nmr 1 167 183 18.72 3.02
inter NH/Cα	173	168	9.31	3.39	3.39	nmr 1 173 168 9.31 3.39

Table ST1. ForceGen distance restraints utilized to generate a solution conformational ensemble. Distance restraints were derived as described in the text, with a 10% allowance for error added to the derived distance. The derived value is then divided between the distance and wiggle, setting the maximum distance to the derived distance with an energy penalty for exceeding this distance set to either 10 or 1 kcal/mol based on the confidence of the derived distance.

J (Hz)	NH	N	Ca	Ha	ForceGen Input
3.98	145	140	141	146	torsion 0.3 -160 -90 145 140 141 146
5.58	130	116	117	131	torsion 0.3 -160 -90 130 116 117 131
4.77	107	95	96	108	torsion 0.3 -160 -90 107 95 96 108
4.07	89	80	81	90	torsion 0.3 -160 -90 89 80 81 90
5.62	159	150	151	160	torsion 0.3 -160 -90 159 150 151 160
5.29	171	167	168	172	torsion 0.3 -160 -90 171 167 168 172
4.07	189	183	184	190	torsion 0.3 -160 -90 189 183 184 190
5.70	61	50	51	62	torsion 0.3 -160 -90 61 50 51 62

Table ST2. ForceGen restraints derived from with $^{3}J_{NH/H\text{-}\alpha}$ (column 1) with corresponding torsion atom assignment numbers included. All measured $^{3}J_{NH/H\text{-}\alpha}$ were found to be below 6 Hz corresponding to a dihedral angle which could on a peptide specific Karplus curve fall in a range between -160 to -90 degrees.[1] The torsion restraints were set conservatively for this entire range.

Cnum	NViol	MeanViol	Constraint
0	0	0.0000	nmr 10 1.52 1.52 178 189
1	0	0.0000	nmr 10 1.51 1.51 145 130
2	0	0.0000	nmr 10 1.47 1.47 145 159
3	0	0.0000	nmr 10 1.45 1.45 130 107
4	0	0.0000	nmr 10 1.39 1.39 178 171
5	0	0.0000	nmr 10 1.38 1.38 171 159
6	452	0.1561	nmr 1 2.02 2.02 130 62
7	0	0.0000	nmr 1 1.57 1.57 145 90
8	15	0.0525	nmr 1 1.51 1.51 199 172
9	858	0.3380	nmr 1 1.39 1.39 171 131
10	965	0.5415	nmr 1 1.51 1.51 171 189
11	0	0.0000	nmr 1 1.69 1.69 168 172
12	0	0.0000	torsion 0.3 -160 -90 145 140 141 146
13	0	0.0000	torsion 0.3 -160 -90 130 116 117 131
14	0	0.0000	torsion 0.3 -160 -90 107 95 96 108
15	0	0.0000	torsion 0.3 -160 -90 89 80 81 90
16	0	0.0000	torsion 0.3 -160 -90 159 150 151 160
17	0	0.0000	torsion 0.3 -160 -90 171 167 168 172
18	0	0.0000	torsion 0.3 -160 -90 189 183 184 190
19	0	0.0000	torsion 0.3 -160 -90 61 50 51 62

Table ST3. Forcegen constraint violation report for **1**

Figure S18. Backbone cartoon, colored by residue type, of the 100 lowest energy + violation energy conformers (out of 995 total conformers) generated by ForceGen based on distance and torsion restraints (Tables S1 & S2).[2, 3]

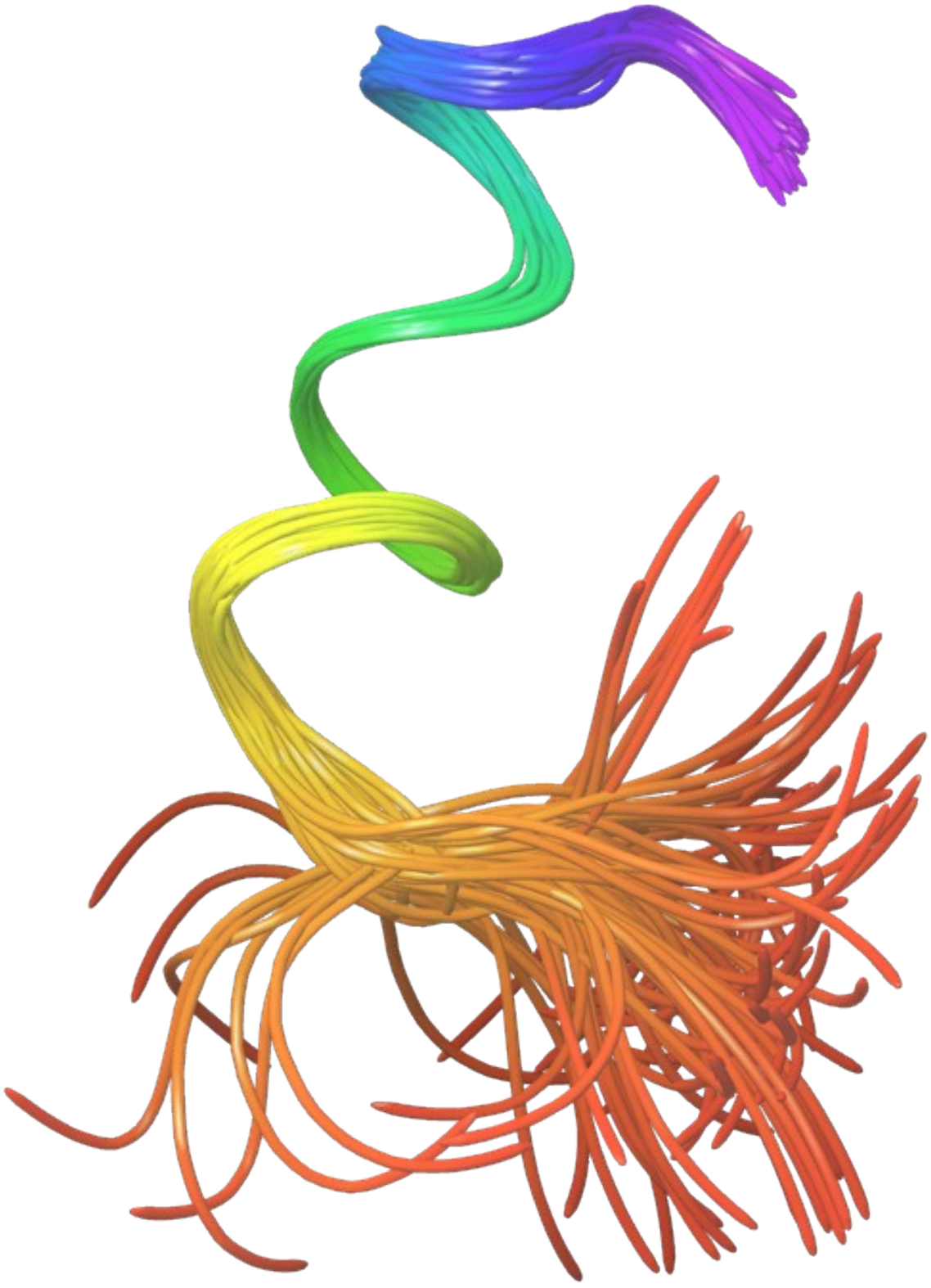


Figure S19. Best 100 conformers with lowest total energy (force field energy + restraint violation) generated by ForceGen, with line bond representation. Backbone is shown in blue and the staple linker is shown in grey.

All 100 structures are aligned using backbone heavy atoms of residues 4 through 16. Pairwise RMSD values are measured for every pair of structures within the ensemble. Mean pairwise RMSD for the backbone heavy atoms is 0.72 ± 0.38 Å and mean pairwise RMSD for all heavy atoms is 1.22 ± 0.63 Å.

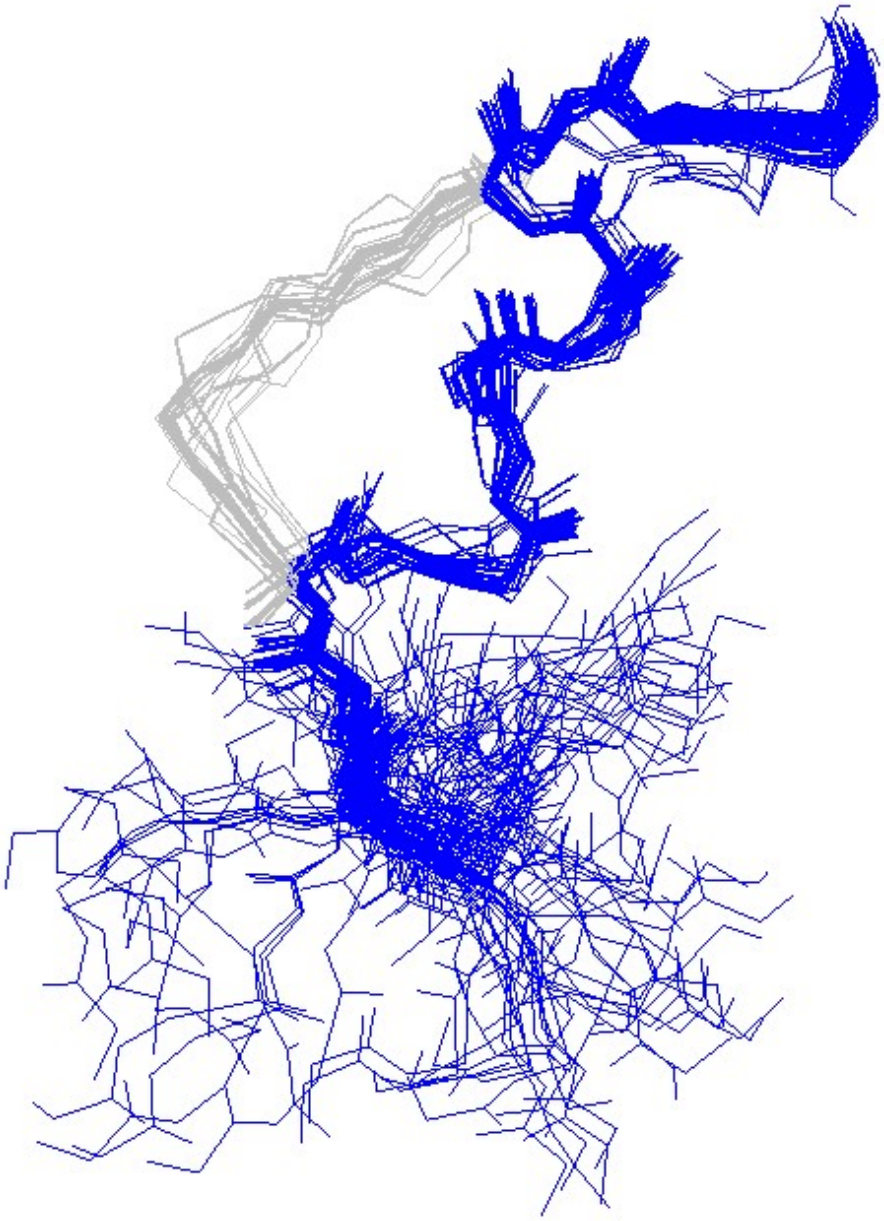
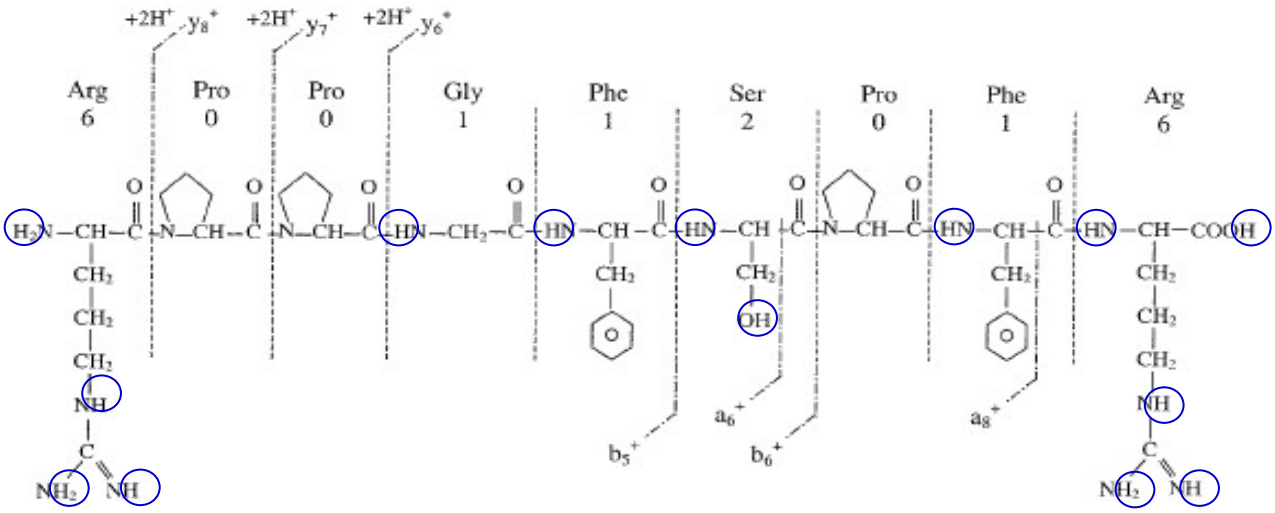


Figure S20. Bradykinin, used as a negative control for SEC-HDX-MS experiments, possesses 17 solvent accessible labile protons and has been demonstrated to possess no secondary structure.[5-7]

Compound name	Molecular weight, Dalton	Number of amino acids[1]	% α-helix in the native structure[+]	Number of solvent inaccessible labile protons[++]	**Number of solvent accessible labile protons[**]**	Calculated total number of labile protons[*]	Measured total number of labile protons[***] In 0.1%TFA/ 1.6 M GndCl at pH 7
Bradykinin	1060	9	0	0	17	17	17/17



- 17 total solvent-accessible labile protons
- No higher-order structure to restrict HDX

Figure S21. An Acquity UPLC® Protein BEH SEC-125 4.6 × 150 mm, 1.7 µm column with 125 Å pore size from Waters was used. The mobile phase A was 50 mM ammonium formate in water with an addition of TFA to adjust pH to 2.0, and organic mobile phase B was acetonitrile. Bradykinin samples were fully labelled in deuterium oxide than injected at 1µL onto the SEC column with a flow rate of 0.25 mL/min at different percentage of organic modifier (5%, 25%, 50% B) and at column temperatures (25 °C) during isocratic separation. The total run time was 10 minutes.

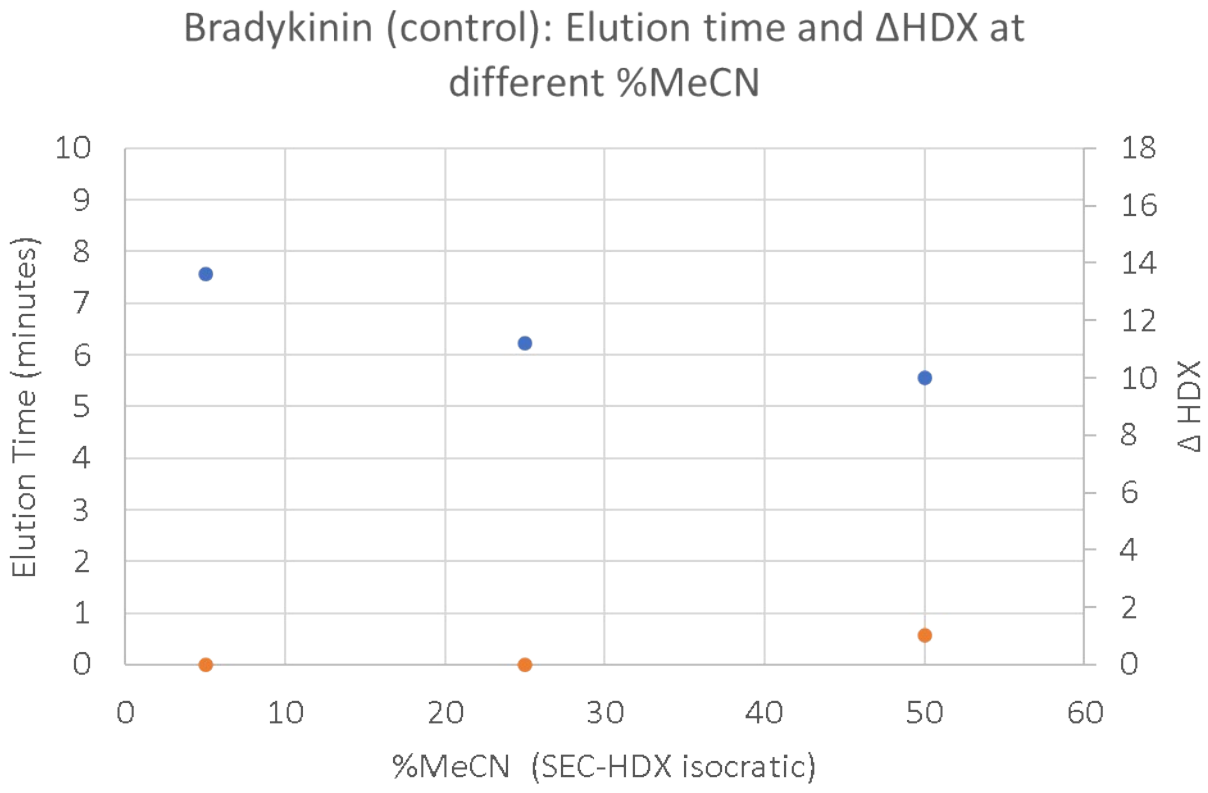


Figure S22. An Acquity UPLC® Protein BEH SEC-125 4.6 × 150 mm, 1.7 µm column with 125 Å pore size from Waters was used. The mobile phase A was 50 mM ammonium formate in water with an addition of TFA to adjust pH to 2.0, and organic mobile phase B was acetonitrile. Bradykinin samples were fully labelled in deuterium oxide than injected at 1µL onto the SEC column with a flow rate of 0.25 mL/min at 5% B and at column temperatures (25 °C) during isocratic separation. The total run time was 10 minutes.

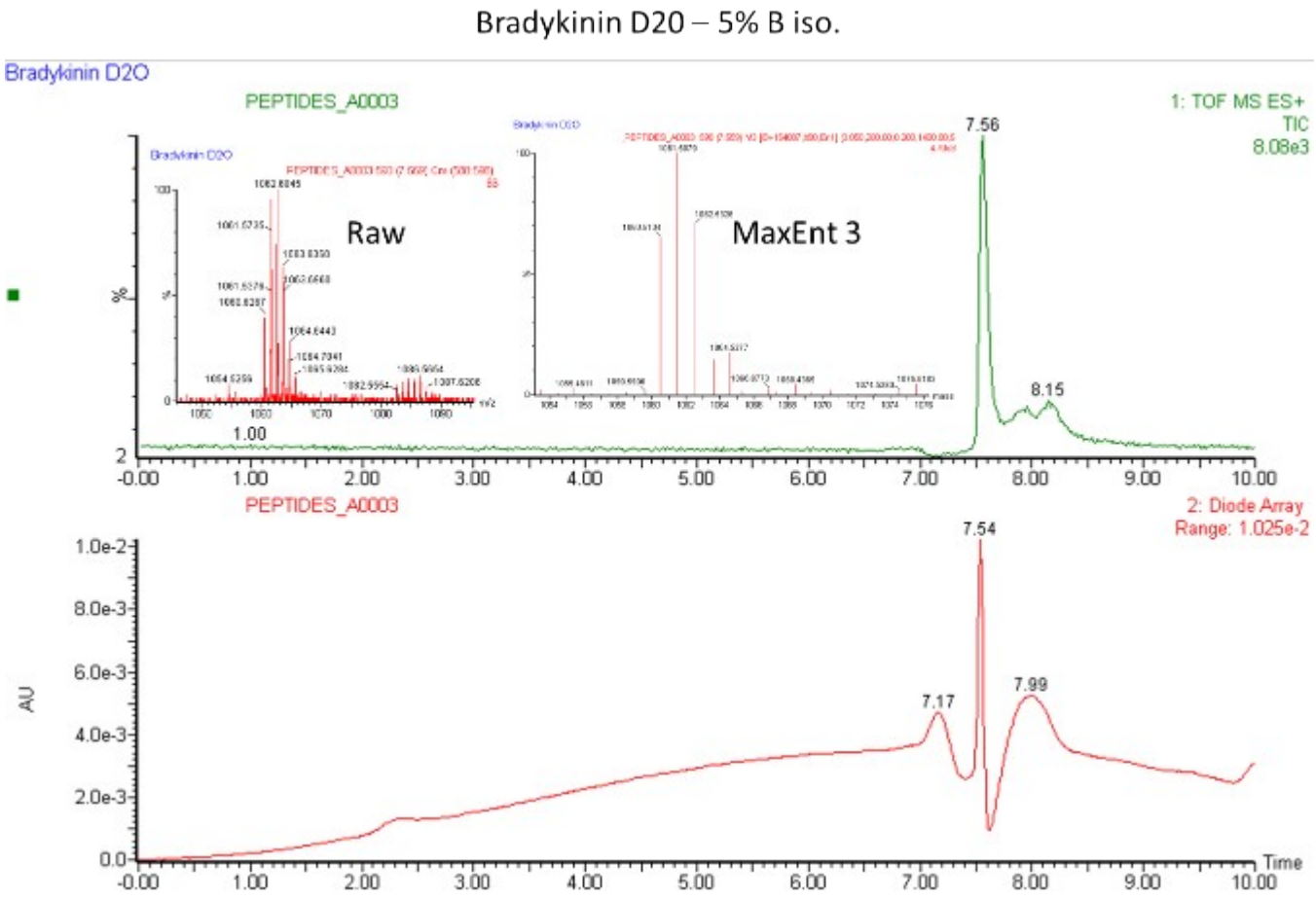


Figure S23. An Acquity UPLC® Protein BEH SEC-125 4.6 × 150 mm, 1.7 µm column with 125 Å pore size from Waters was used. The mobile phase A was 50 mM ammonium formate in water with an addition of TFA to adjust pH to 2.0, and organic mobile phase B was acetonitrile. Bradykinin samples were fully labelled in deuterium oxide than injected at 1µL onto the SEC column with a flow rate of 0.25 mL/min at 25% B and at column temperatures (25 °C) during isocratic separation. The total run time was 10 minutes.

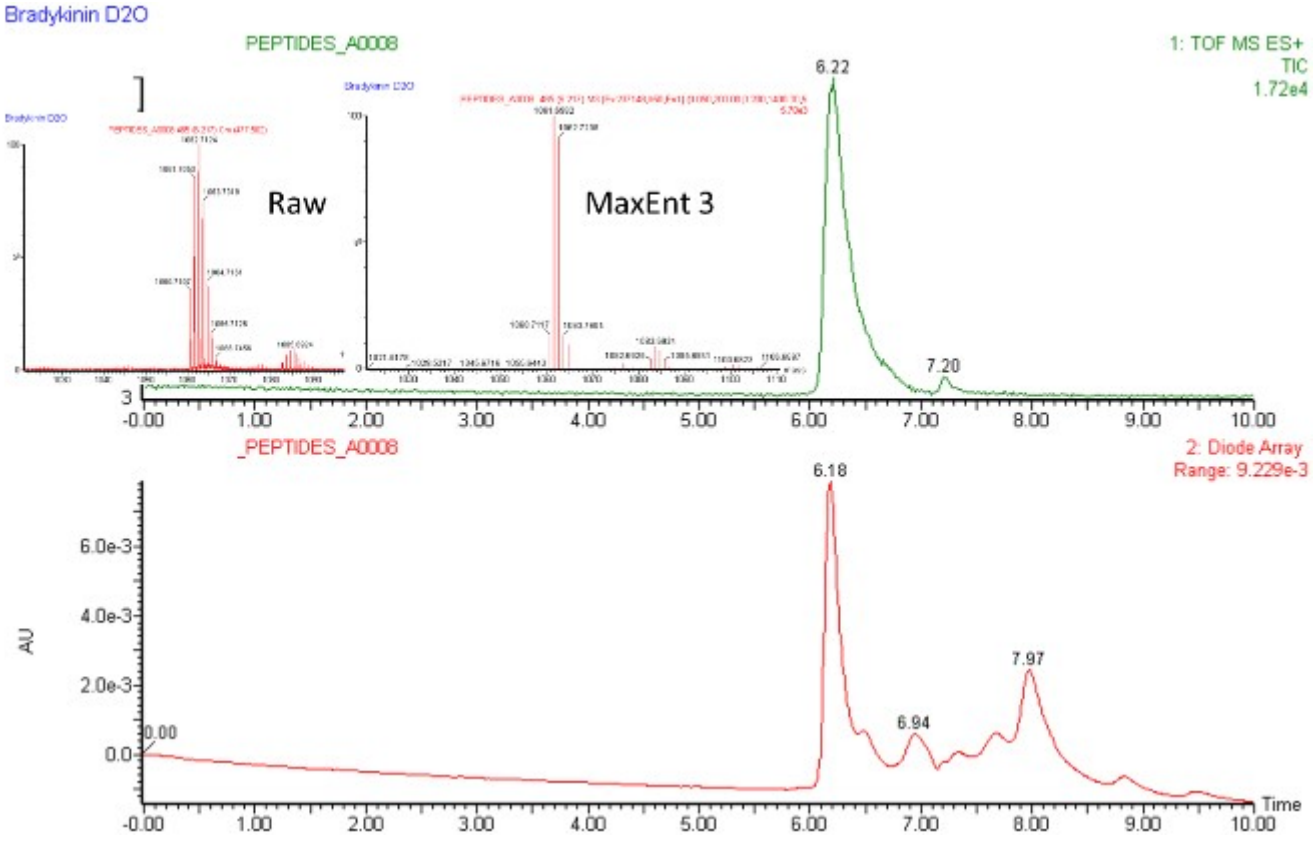


Figure S24. An Acquity UPLC® Protein BEH SEC-125 4.6 × 150 mm, 1.7 µm column with 125 Å pore size from Waters was used. The mobile phase A was 50 mM ammonium formate in water with an addition of TFA to adjust pH to 2.0, and organic mobile phase B was acetonitrile. Bradykinin samples were fully labelled in deuterium oxide than injected at 1µL onto the SEC column with a flow rate of 0.25 mL/min at 50% B and at column temperatures (25 °C) during isocratic separation. The total run time was 10 minutes.

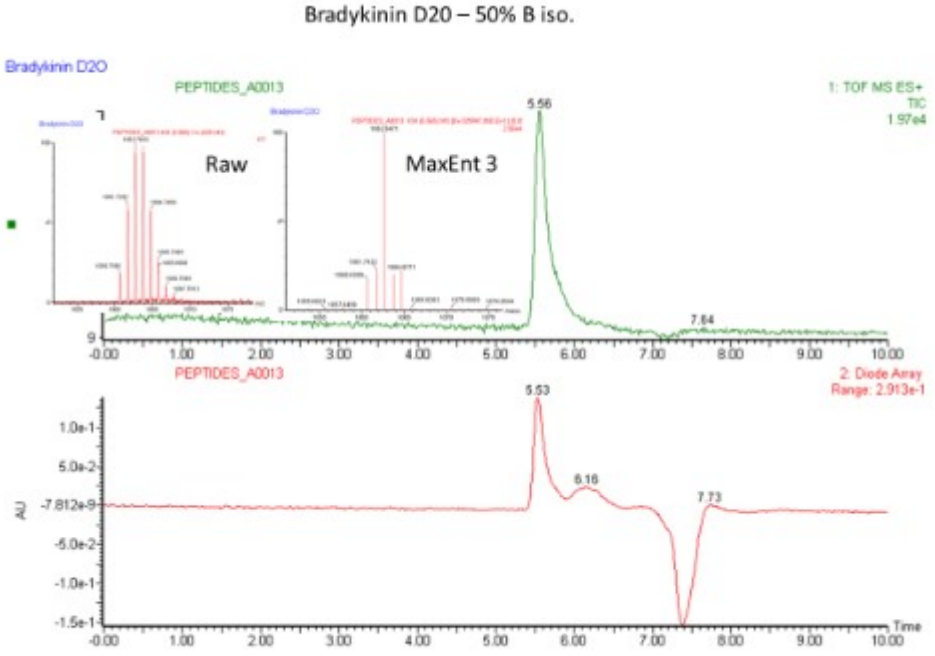
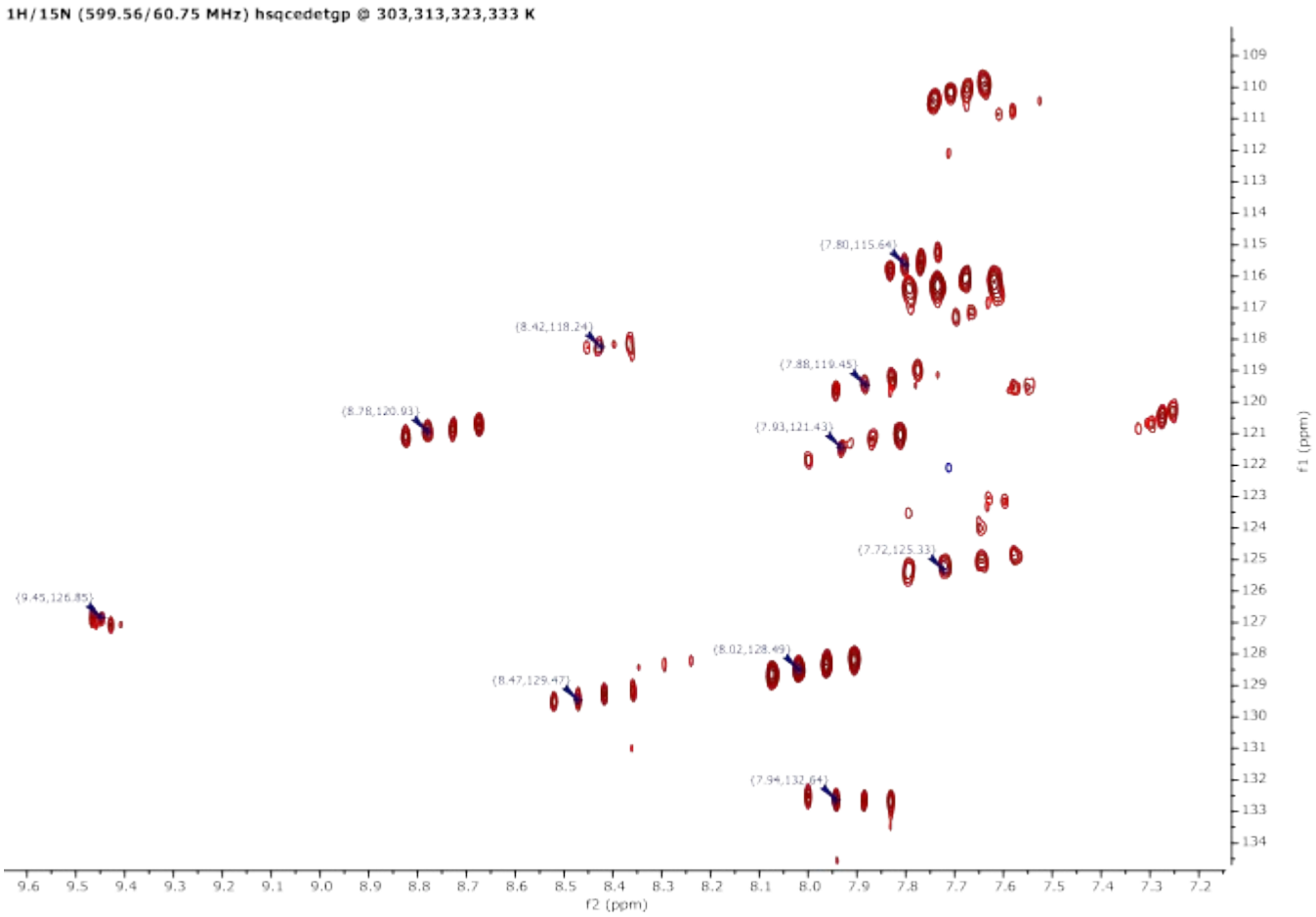


Figure S25. Overlaid $^{1}H/^{15}N$-HSQC spectra of peptide **1** at 303, 313, 323, and 333K



residue #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
residue / deg. K	R	Gly	Ala	Thr	Phe	Lnk	Glu	Tyr	Trp	Ala	Gln	Cpr	Lnk	Ser	Ala	Ala
303	7.79	7.79	8.07	7.74	8.00	8.00	7.83	7.94	8.45	8.82	7.70	7.32	8.52	7.61	7.63	7.59
313	7.72	7.73	8.02	7.71	7.93	7.94	7.80	7.88	8.42	8.78	7.66	7.30	8.47	7.58	7.61	7.57
323	7.65	7.68	7.96	7.68	7.87	7.89	7.77	7.83	8.40	8.73	7.63	7.28	8.42	7.55	7.60	7.56
333	7.58	7.62	7.91	7.64	7.81	7.83	7.73	7.78	8.36	8.67	7.62	7.25	8.36	7.55	7.60	7.56
ppb/K	-7.00	-5.67	-5.33	**-3.33**	-6.33	-5.67	**-3.33**	-5.33	**-3.00**	-5.00	**-2.67**	**-2.33**	-5.33	**-2.00**	**-1.00**	**-1.00**

Table ST4. Temperature coefficients derived from $^{1}H/^{15}N$-HSQC spectra.

References

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2. A. E. Cleves and A. N. Jain, *Journal of Computer-Aided Molecular Design*, 2017, **31**, 419-439.
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