Native MS and Ligand Observed NMR Uncovers Subtle SLiM Binding Variations that Mediate HSP90–Hop PPI Modulation

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Supporting Information

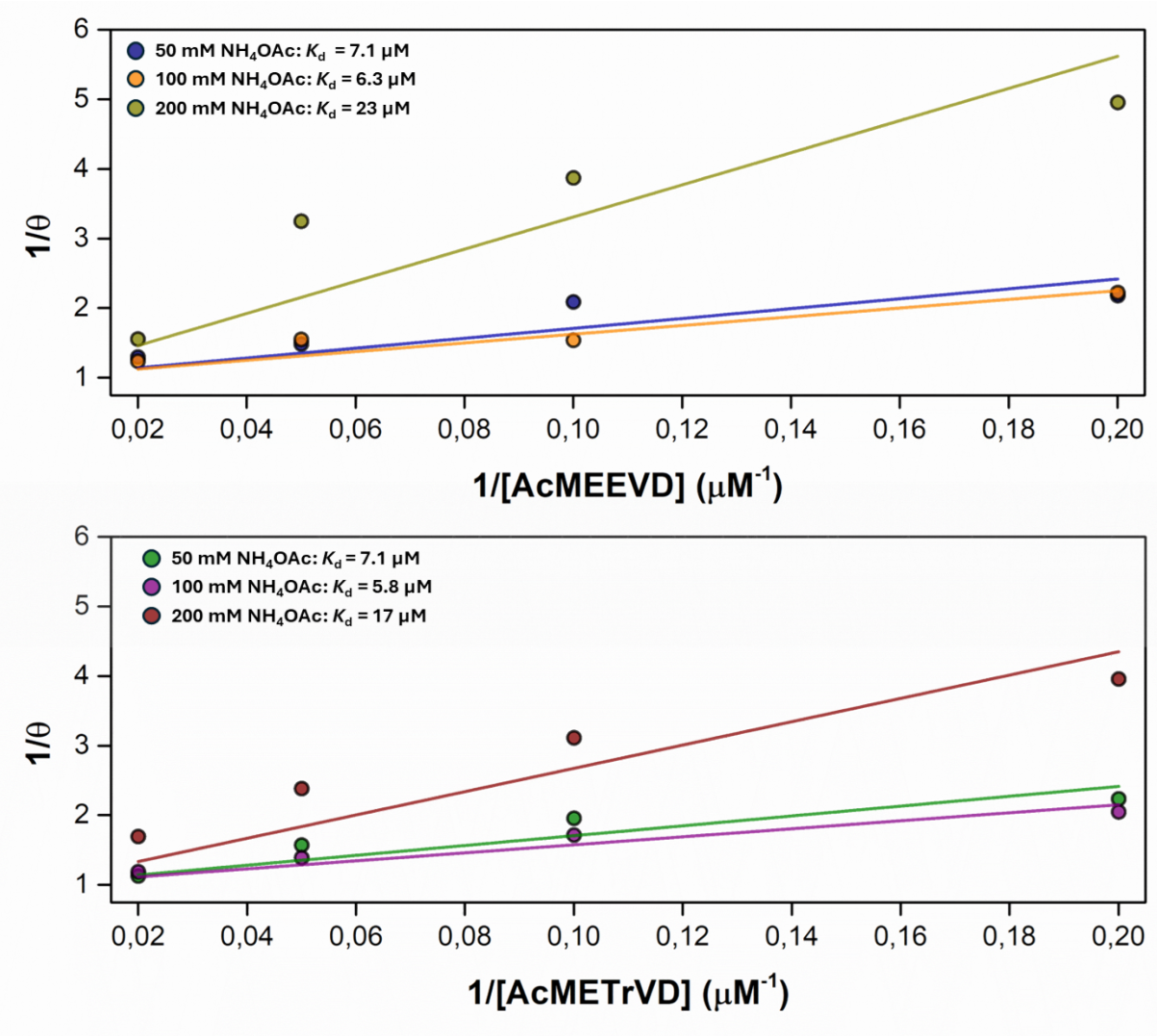

Figure S1. Comparative linear binding plots of peptides **1** and **2** to Hop_{TPR2A} derived from nMS data collected in 50, 100 and 200 mM NH_4OAc solutions. Details pertaining to *K*d calculations can be found in the experimental section.

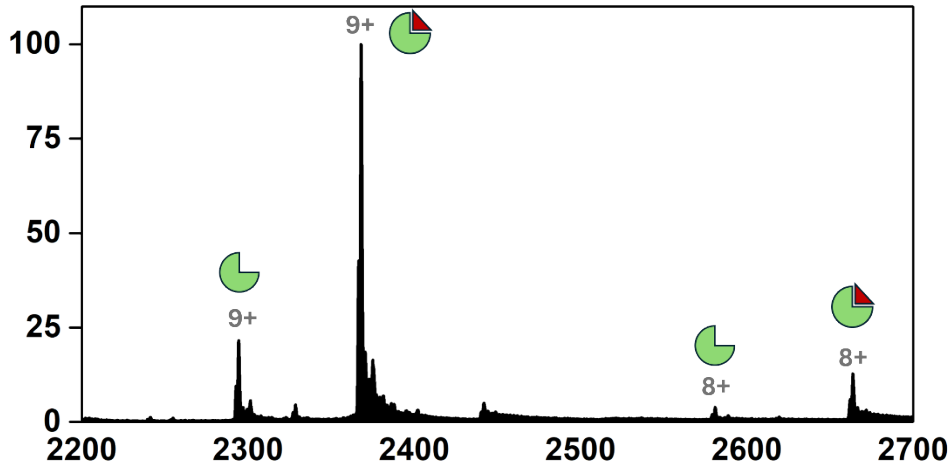

Figure S2. nMS charge state distribution of a 1 :5 mixture of Hop_{TPR2A} and peptide **1** in 50 mM NH_4OAc solution.

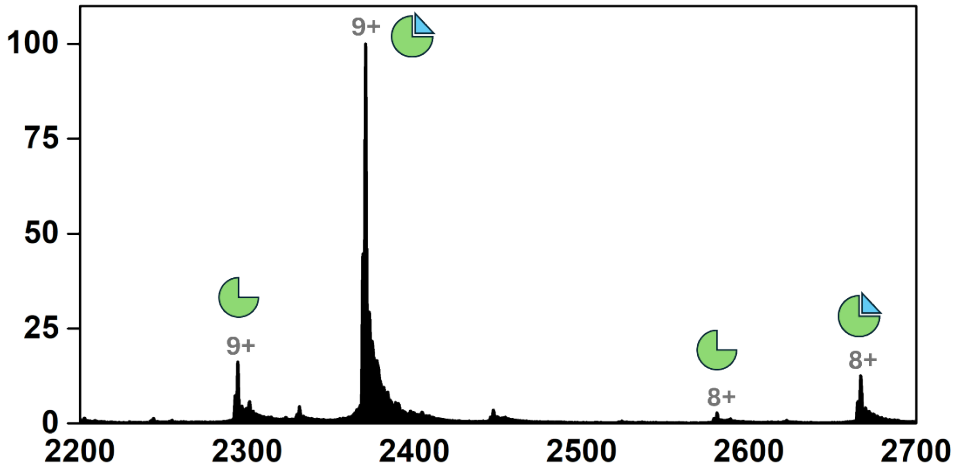

Figure S3. nMS charge state distribution of a 1 :5 mixture of Hop_{TPR2A} and peptide **2** in 50 mM NH_4OAc solution.

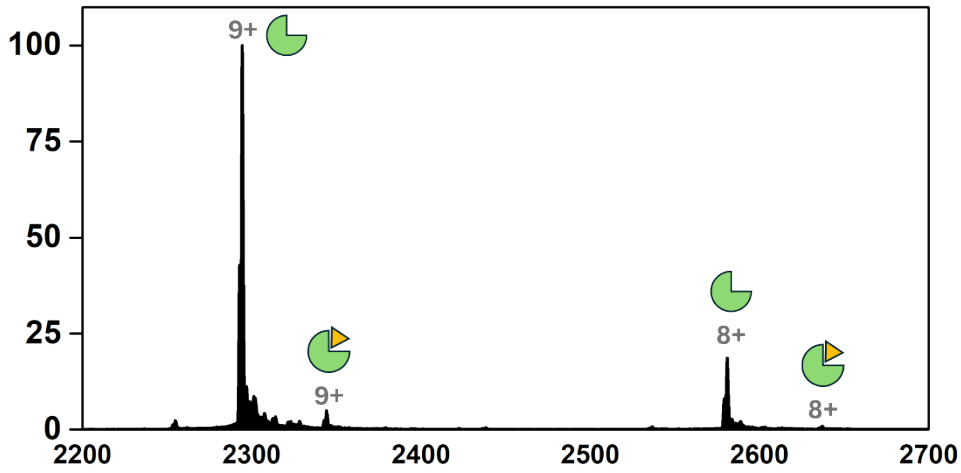

Figure S4. nMS charge state distribution of a 1 :5 mixture of Hop_{TPR2A} and peptide **8** in 50 mM NH_4OAc solution.

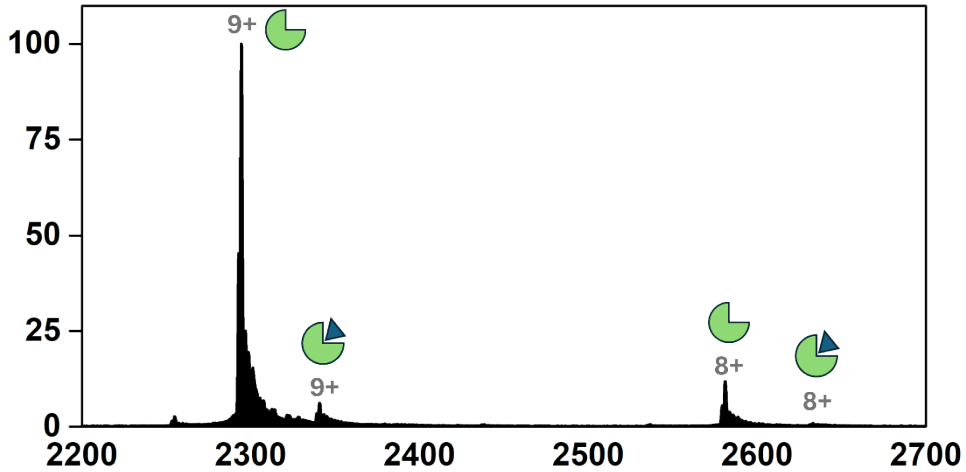

Figure S5. nMS charge state distribution of a 1 :5 mixture of Hop_{TPR2A} and peptide **9** in 50 mM NH_4OAc solution.

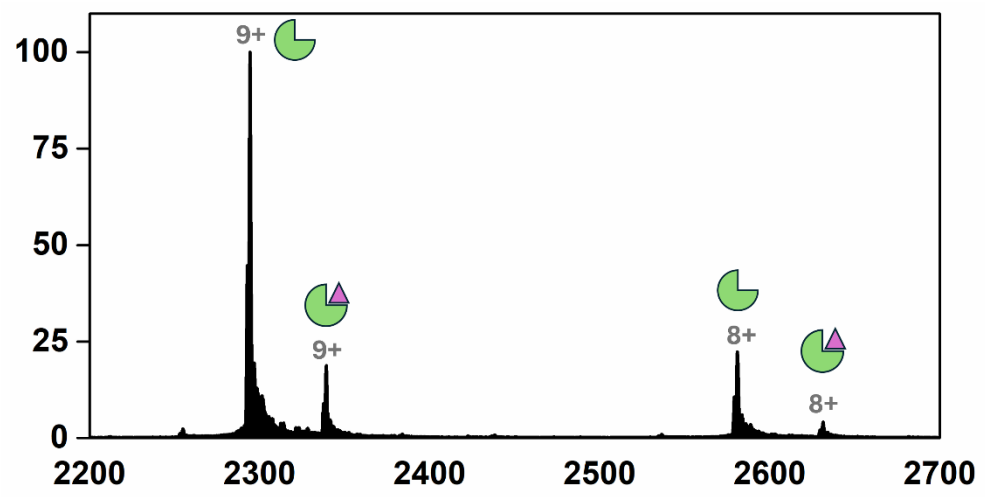

Figure S6. nMS charge state distribution of a 1 :5 mixture of Hop_{TPR2A} and peptide **10** in 50 mM NH_4OAc solution.

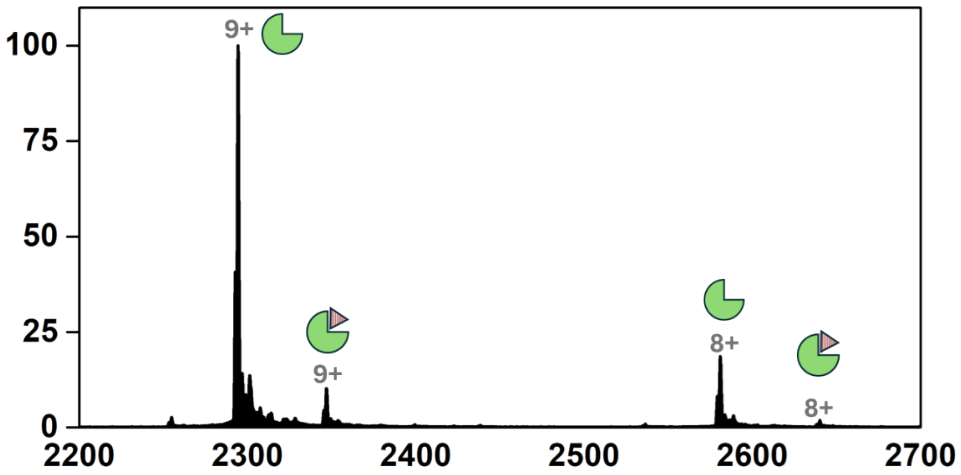

Figure S7. nMS charge state distribution of a 1 :5 mixture of Hop_{TPR2A} and peptide **11** in 50 mM NH_4OAc solution.

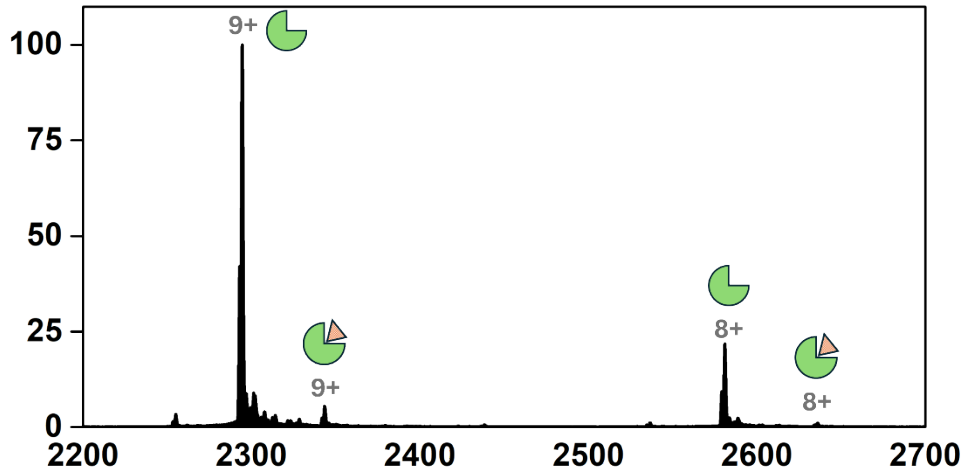

Figure S8. nMS charge state distribution of a 1 :5 mixture of Hop_{TPR2A} and peptide **12** in 50 mM NH_4OAc solution.

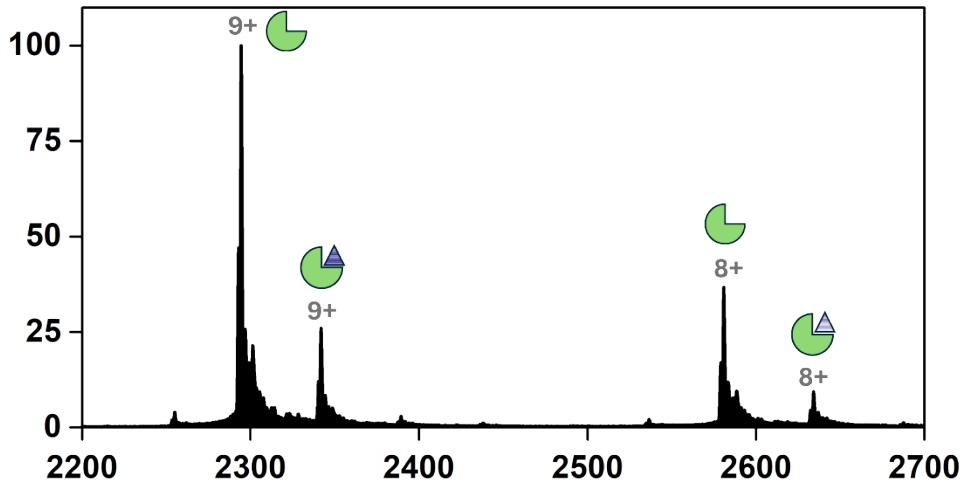

Figure S9. nMS charge state distribution of a 1 :5 mixture of Hop_{TPR2A} and peptide **13** in 50 mM NH_4OAc solution.

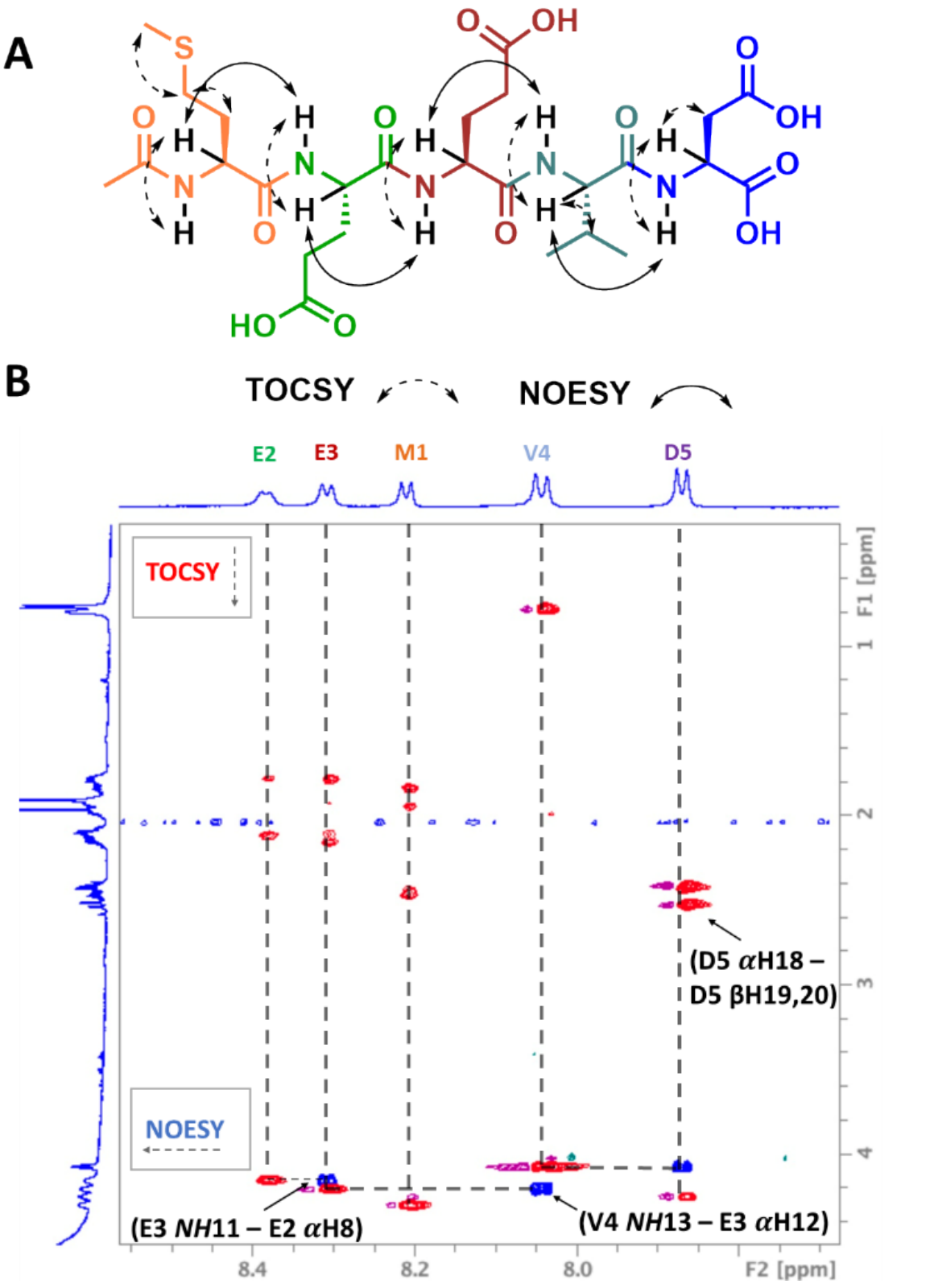

Figure S10A: Representation of some key NOESY and TOCSY correlations which allowed for the unambiguous of most proton resonances. **B:** Expanded and overlaid 2D TOCSY and NOESY spectra of peptide **1** (2mM), whilst bound to Hop_{TPR2A} (40 µM) recorded in buffer with 10% D_2O at 298 K. TOCSY correlations read vertically, indicate key intra-residue correlations. NOESY correlations, read horizontally show correlations between α and *NH* protons from adjacent amino acid residues.

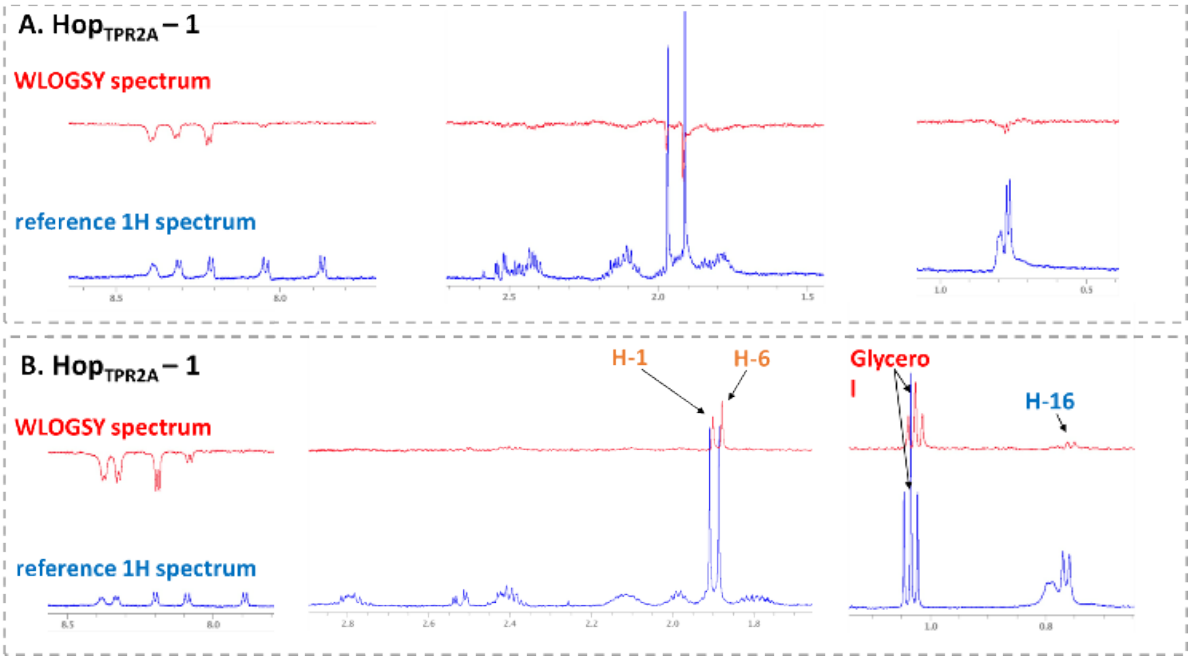

Figure S11. Stacked 1D ^{1}H expansions of the Hop$_{TPR2A}$–**1** (**A**) and Hop$_{TPR2A}$–**2** (**B**) complexes. The reference ^{1}H spectra are shown in blue and WLOGSY spectra are shown in red. The most noticeable change between the experiments is the un-inverted signals in panel B, corresponding to H-1 and H-6, respectively.

Table S1. Relative ratio of *C* to *E* conformer and the normalised stability ratio

	Compound number	*C* conformer (%)	*E* conformer (%)	Stability ratio
APO TPR2A		66	34	0.69
MEEVD	**1**	88	12	0.92
MEE	**8**	66	34	0.70
EEV	**9**	75	25	0.79
EVD	**10**	68	32	0.72
METrVD	**2**	95	5	1.00
METr	**11**	85	15	0.89
ETrV	**12**	82	18	0.86
TrVD	**13**	75	25	0.79

Table S2: Chemical Shifts of Peptides **1** and **2**, alongside STD and WLOGSY intensities

MEEVD

Proton Number	Functional Group	Amino acid code	Chemical shift	STD %	WLOGSY %
1	αAc	M1	1.98	14	-14
2	αNH	M1	8.21	10	-87
3	-CH	M1	4.33	25	0
4	R-CH2	M1	2.51	18	0
5	R-CH2	M1	1.88	18	0
6	SCH3	M1	1.90	14	-52
7	αNH	E2	8.4	18	-100
8	-CH	E2	4.21	100	0
9	R-CH2	E2, E3	2.14	21	0
10	R-CH2	E2, E3	1.81	18	0
11	αNH	E3	8.31	17	-76
12	-CH	E3	4.24	23	0
13	αNH	V4	8.05	19	-14
14	-CH	V4	4.11	35	0
15	R-CH(CH3)2	V4	1.99	14	0
16	R-CH(CH3)2	V4	0.78	15	-26
17	αNH	D5	7.87	26	0
18	-CH	D5	4.30	16	0
19	R-CH2	D5	2.45	18	0
20	R-CH2	D5	2.55	18	0
			Average	23	

METrVD

Proton Number	Functional Group	Amino acid code	Chemical shift	STD %	WLOGSY %
1	αAc	M1	2.01	7	23
2	αNH	M1	8.4	4	-95
3	-CH	M1	4.33	37	0
4	R-CH2	M1	2.51	16	0
5	R-CH2	M1	1.88	9	0
6	SCH3	M1	1.96	7	36
7	αNH	E2	8.36	7	-100
8	-CH	E2	4.21	100	0
9	R-CH2	E2, Tr3	2.14	14	0
10	R-CH2	E2, Tr3	1.81	16	0
11	αNH	Tr3	8.25	6	-97
12	-CH	Tr3	4.24	100	0
13	αNH	V4	8.12	6	-28
14	-CH	V4	4.12	43	0
15	R-CH(CH3)2	V4	1.99	7	0
16	R-CH(CH3)2	V4	0.78	10	16
17	αNH	D5	7.92	10	0
18	-CH	D5	4.30	37	0
19	R-CH2	D5	2.45	11	0
20	R-CH2	D5	2.55	16	0
			Average	23	

Experimental

Peptide Synthesis

Peptide synthesis was performed manually on a 0.2 mmol scale using an Fmoc protected SPPS protocol in a Poly-Prep® column, attached to a vacuum trap for easy filtration. The synthesis was carried out on 2-CTC resin (0.95 mmol/g loading) which was swelled in anhydrous DCM for 15 min. The swelled resin was activated with a 25% (*v/v*) solution of $SOCl_2$ in anhydrous DCM (2 mL total) for 1 h. Thereafter, the resin was thoroughly rinsed with anhydrous DCM, followed by coupling of the first respective Fmoc-amino acid (2 eq.) in anhydrous DCM, in the presence of *N,N*-diisopropylethylamine (DIPEA, 10 eq.) for 1 h. Resin capping was done upon the addition of MeOH (*ca* 200 ml) for 30 min at room temperature. The remaining couplings were performed with 3 eq. of the appropriate Fmoc-amino acid, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDC.MeI) and OxymaPure in a 1:1:1 ratio for 1 h, in DMF. Fmoc protecting groups were removed with 20% piperidine in DMF in two separate reactions, the first for 1 min and the second for 7 min at room temperature, at the end of each coupling reaction. At the end of the chain elongation, the peptide was acetylated at the *N*-terminus using acetic anhydride (10 eq.) and DIPEA (20 eq.) in DMF for 35 min. Thereafter, the solvent was filtered off *via* vacuum filtration and the acetylated peptidyl resin was washed with DMF, followed by washing with DCM and then left to dry via vacuum filtration for 10 min. Final global deprotection and cleavage of the peptide was done with TFA/H_2O/TIS (triisopropylsilane) (95: 2.5: 2.5 *v/v*) for 1 h (non-tetrazole peptides) or 2.5 h (for tetrazole functionalised peptides), at room temperature. Following global deprotection, the resin/cleavage cocktail suspension was filtered through a Poly-Prep® column and the remaining resin was rinsed with 4–5 mL of TFA. The TFA was carefully removed under reduced pressure at 45 ˚C. Chilled diethyl ether was added to the remaining oil which resulted in the precipitation of the crude peptide as a white/off-white solid. The diethyl ether/peptide suspension was subsequently centrifuged for 5 min, followed by removal of the supernatant and replacement with fresh chilled diethyl ether. This process was repeated three times. The residual solvent from the final centrifugation step was removed under reduced pressure and the final crude peptide (remaining pellet) was dried *in vacuo*.

Peptide purification

Crude peptides were dissolved in 0.1% TFA/H_2O (*v/v*) solution. Semi-preparative HPLC was completed on an Agilent 1260 Infinity II HPLC using a Pursuit XRs 5-C18 S (5.0 µm, 4.6 × 150 mm) column, with a flow rate of 1.0 mL/min and UV detection at 220 nm and 254 nm, over a 20 min run. A 10 min water wash was performed after each peptide fraction was collected. Solvent A was 0.1% TFA in H_2O (*v/v*) and Solvent B was 0.1% FA in MeCN (*v/v*). An elution gradient of 95-60% H_2O-MeCN was used, according to **Table S3**. Purified peptide fractions were combined and lyophilised to afford the final pure peptides as white/off-white solids.

Table S3: Breakdown of the 95-60% gradient used for HPLC analysis and peptide purification

Time (min)	%A	%B
0.00	95.0	5.0
15.00	60.0	40.0
17.00	5.0	95.0
20.00	95.0	5.0

Peptide analysis

UPLC-MS analysis was conducted on a Synapt-G2 Q-TOF coupled to an ACQUITY UPLC (Waters) using an Acquity UPLC BEH C18 (1.7 µm, 2.1 × 100 mm) column, with flow rate of 0.3 mL/min over a 30 min run per sample. UV detection was at 220 nm. Solvent A was 0.1% (*v/v*) FA in H_2O, solvent B was 0.1% (*v/v*) FA in MeCN. An elution gradient of 95-60% H_2O-MeCN was used, according to **Table S4.** MassLynx v4.0 (Waters) was utilised for data analysis.

Table S4: Breakdown of the 95-60% gradient used for peptide analysis on the UPLC-MS

	Time (min)	%A	%B
1	0.00	95.0	5.0
2	20.00	60.0	40.0
3	21.00	5.0	95.0
4	25.00	5.0	95.0
5	26.00	95.0	5.0
6	30.00	95.0	5.0

Protein expression and Purification

Competent *Escherichia coli* BL21 codon+ cells were transformed with a plasmid encoding Hop_{TPR2A} (pQE-80L-TPR2A). Successfully transformed cells were cultured in 250 mL of 2× YT broth [1.6% (w/v) tryptone, 1% (w/v) yeast extract, and 85.6 mM sodium chloride] containing 100 µg/mL ampicillin, then incubated at 37°C with shaking at 180 rpm for 16 hours until the optical density at 600 nm (OD600) reached 0.6-0.8. At this point, protein expression was induced using 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and further incubated at 25°C with shaking at 180 rpm overnight.

After overnight incubation, the cell cultures were centrifuged at 6000×g for 15 minutes using an Avanti-JE centrifuge (Beckman Coulter, Life Sciences). The supernatant was discarded, and the pellet was resuspended in equilibration buffer (100 mM Na_2HPO_4 pH 7.5, 300 mM NaCl, and 10 mM imidazole) with phenylmethylsulfonyl fluoride (PMSF) (100 µg/mL) and 1 mg/mL lysozyme, then incubated on ice for 30 minutes and subsequently at -80°C for at least one hour. Afterwards, the cell pellet was sonicated on ice, and the lysate was centrifuged in JA-20 Beckman tubes at 10,000×g for 30 minutes at 4°C. The supernatant was collected and added to the Ni-NTA resin (Qiagen) that had been equilibrated in wash buffer (100 mM Na_2HPO_4 pH 7.5, 300 mM NaCl, and 50 mM imidazole), then incubated at 4°C overnight. Subsequently, the flow-through was collected, and the resin was washed three times with 50 mL of wash buffer. The bound protein was eluted using elution buffer (100 mM Na_2HPO_4 pH 7.5, 300 mM NaCl, and 200 mM imidazole), and stored at -80°C for subsequent experiments.

Samples were collected at each stage of protein purification and analysed using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Gels were stained with Coomassie staining solution [0.1% (w/v) Coomassie Brilliant Blue R-250 dye, 20% (v/v) methanol, and 10% (v/v) acetic acid] for one hour and then destained overnight with destaining solution (40% methanol, 10% acetic acid in distilled water) before visualization using a ChemiDoc™ XRS+ Molecular Imager (Bio-Rad).

Protein sequence

MRGSHHHHHHGSKPPPPPKKETKPEPMEEDLPENKKQALKEKELGNDAYKKKDFDTALKHYDKAKELDPTNMTYI
TNQAAVYFEKGDYNKCRELCEKAIEVGRENREDYRQIAKAYARIGNSYFKEEKYKDAIHFYNKSLAEHRTPDVLKKC
QQAEKILKEQERLAYINPDLAKLN

Native MS

Samples of Hop_{TPR2A} were 'buffer' exchanged into 50 mM, 100 mM or 200 mM NH_4OAc solution using Micro Biospin P6 Gel columns (Biorad) prior to MS analysis. Protein concentration was determined by UV/VIS spectrophotometry (Nanodrop ND-1000 spectrophotometer, A280) and adjusted to 68.75 µM with the relevant NH_4OAc solution. 1 mg/mL stock solution of each respective peptide was prepared in LCMS grade water. Prior to MS analysis, stock solutions were diluted with the required NH_4OAc solution to a final concentration of 68.75 µM. For protein-ligand binding a 1.45 µL aliquot of Hop_{TPR2A} was incubated with an aliquot of 0.725, 1.45, 2.9 or 7.25 µL of the desired peptide made up to a final volume of 10 µL with NH_4OAc solution for a final concentration of 10 µM Hop_{TPR2A} and a 1:0.5, 1:1, 1:2 or 1:5 protein: ligand ratio.

Native MS and IM-MS data were obtained on a Synapt-G2 Q-TOF (Waters). Ionisation was achieved using nESI static spray with borosilicate emitter tip (OD 1.2 mm, ID 0.9 mm, World Precision instruments). Samples were loaded into the borosilicate tip with a platinum wire inserted into the sample to conduct a voltage. The angle and distance of the tip was adjusted manually relative to the MS inlet. For all nMS spectra, the capillary voltage was set at 1.00 kV, cone voltage was set at 50 V, extraction cone was set to 2.0 V, the source temperature was set to 70 ^{o}C and the backing pressure was 2.35 mbar. Protein-ligand complexation ratios were calculated by combining the apo and ligand bound peaks across both the 8+ and 9+ charge states and are reported as the average of three replicates samples. Error bars represent the standard deviation across all three measurements. Fractional saturation (θ) represents the average abundance of the ligand bound species. K_d values were estimated by plotting 1/ θ vs 1/[ligand] and extracting the linear gradient from the line of best fit, where the y intercept was set at 1. E.g. $1/\theta = K_d \times 1/[\text{ligand}] + 1$.

IM-MS measurements were conducted under the same nMS conditions, with the exception that a trap voltage of 5 V was applied. For IM-MS measurements, the IMS wave height and IMS velocity were set at 40 V and 650 ms respectively; IM-MS spectra were recorded for 5 minutes under each set of conditions. MS data were processed using MassLynx v4.0 (Waters), and UniDec.[1] Complexation ratios were calculated as the average of two biological replicates. Data processing to produce CIU plots was performed using CIU suite 2 (University of Michigan).[2] For the protein-ligand complexes, the drift time data for all stoichiometric complexes within the same charge state (8+) were combined. The relative abundance of the compact and extended species were determined by integrating the area under the curves and expressing the area of each peak as a percentage of the total AUC.

Ligand Observed NMR

For NMR studies, protein samples were buffer exchanged into 50 mM Na_2HPO_4 buffer using a Zeba Spin Desalting Column (Thermo Fisher Scientific) underwent buffer exchange the peptides and protein were dissolved in 540 µL of buffer (50 mM KH_2PO_4, 50 mM Na_2HPO_4, pH 7.5, 150 mM NaCl) and 60 µL of D_2O. All the spectra were acquired with a Bruker Avance III 600 MHz NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a BBO Prodigy cryoprobe and processed using Bruker processing software (Topspin 4.2.0). The 1D ^{1}H and water suppression presaturation experiments were obtained using standard Bruker pulse sequences (zg30 and zgpr). The probe temperature was maintained at 298 K for the duration of the experiments. All 1D ^{1}H spectra were obtained with 64 scans (NS) and prior 8 dummy scans (DS). A relaxation delay (D1) of 1 sec and pulse (P1) of 12 µsec were also used. Water suppression spectra were obtained with an offset (O1) of 2820.47 Hz and all reference ^{1}H spectra were acquired with a sweep width of 19.99 ppm. STD and WLOGSY NMR experiments were acquired using optimized Bruker pulse sequences with water suppression using excitation sculpting (stddifesgp and ephogsygpno.2). STD experiments were acquired at a frequency of -1.0 ppm for on-resonance acquisitions, and 30 ppm for off-resonance acquisitions. The power for the on-resonance saturation pulse (p42) was set between $1x10^{-4}$ W (40 dB) and $1x10^{-6}$ W (60 dB). The spin lock time (D29) was set to 10 ms and the saturation time (D20) was varied between 0.25 and 5 s for STD amplification build-up and optimized D20 determination. The relaxation delay (D1) used for the STD experiments was 15 s and was calculated from T_1, determined from the inversion recovery experiment using the standard Bruker pulse sequence (t1ir). The number of scans (NS) for the STD experiment were also 64, with the number of dummy scans (DS) set to 8. All STD experiments were acquired with interleaved acquisition, with a loop counter (L4) of 4. The STD amplification factor for each unique signal was calculated as the percentage of signal to noise ratio (S/N) in the on-resonance spectra over the signal to noise ratio of the STD spectra. WLOGSY Spectra were acquired with 124 scans. WLOGSY NMR experiments employed a 20 ms selective Gaussian 180° pulse at the water signal frequency (2820.47 Hz) and a NOE mixing time of 1 s. Both STD and WLOGSY spectra were phase and baseline corrected using the automatic baseline and phase correction functions in Topspin 4.2.0. To maximize the S/N ratio of the STD and WLOGSY experiments, a peptide concentration of 2.0 mM and a TPR2A concentration of 40 µM were chosen. ^{1}H NMR chemical shift assignments for the peptides, Ac-MEEVD-OH and Ac-METrVD-OH were achieved via the standard systematic application of 2D COSY, TOCSY, and NOESY experiments.

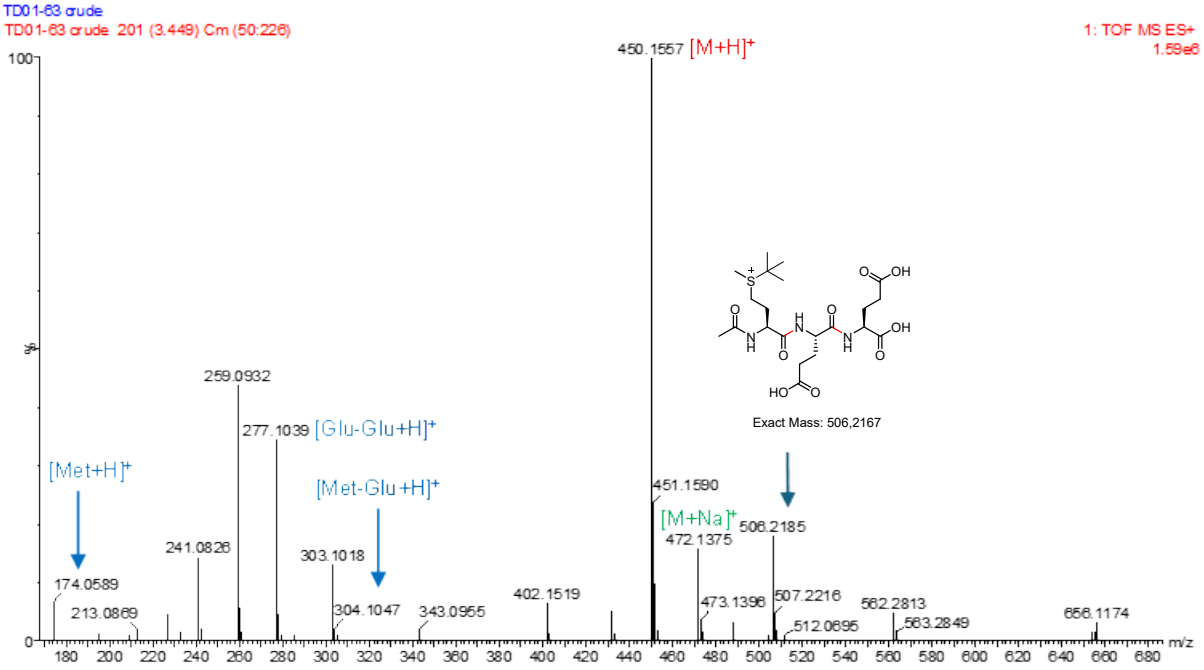

Figure S12. HR-ESI mass spectrum of peptide **Ac-MEE-OH**. Observed fragmentation products and ^{t}Bu side-product also identified.

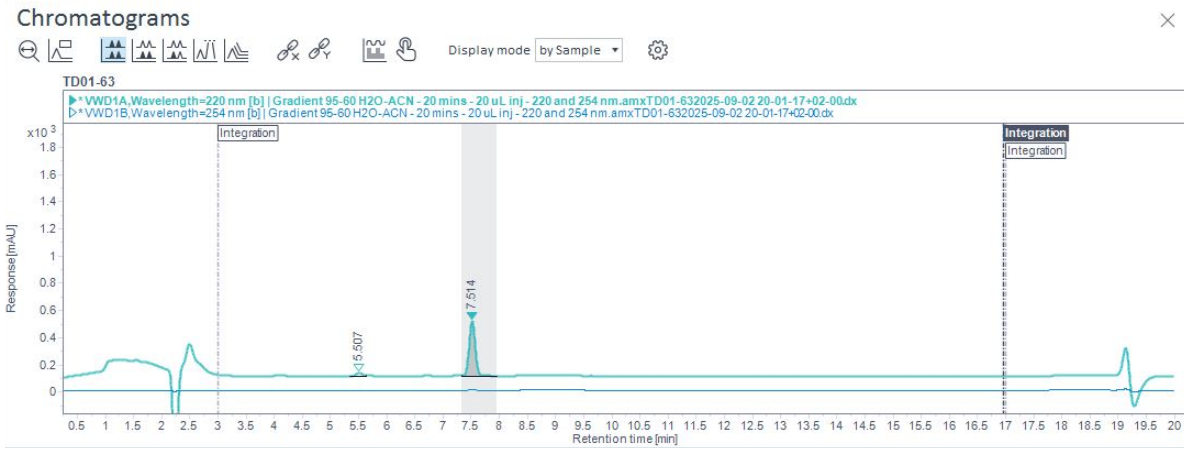

Figure S13. HPLC trace of peptide **Ac-MEE-OH**, using a solvent gradient of 95 – 60%. Compound purity determined to be 94%.

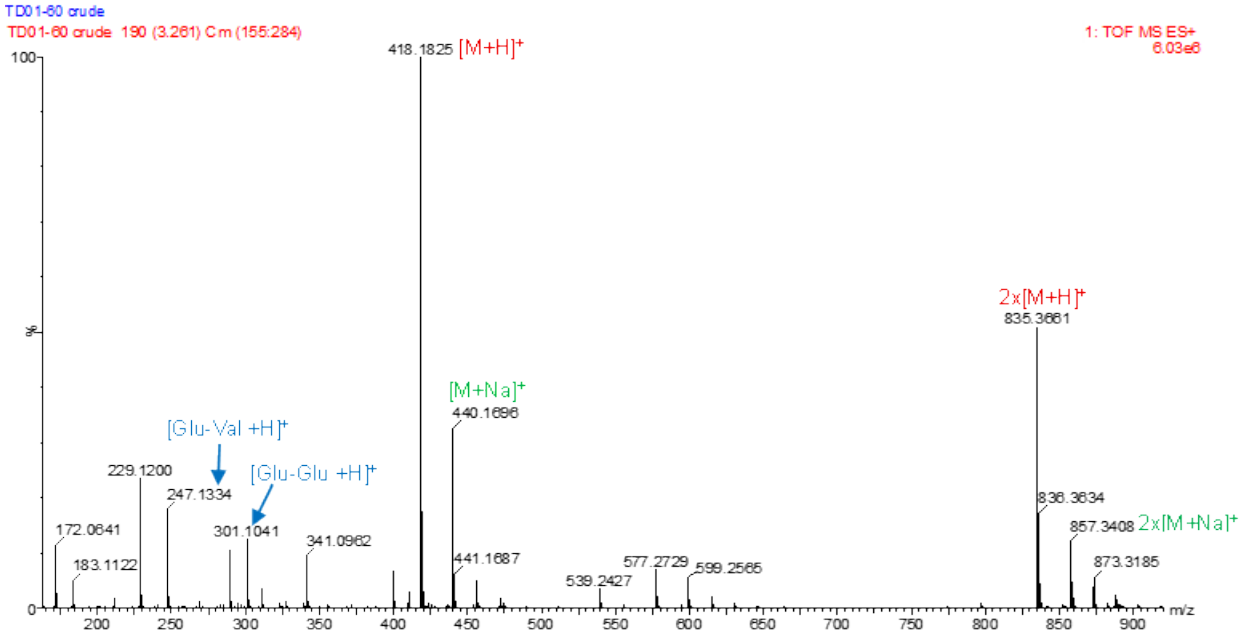

Figure S14. HR-ESI mass spectrum of peptide **Ac-EEV-OH**. Observed fragmentation products also identified.

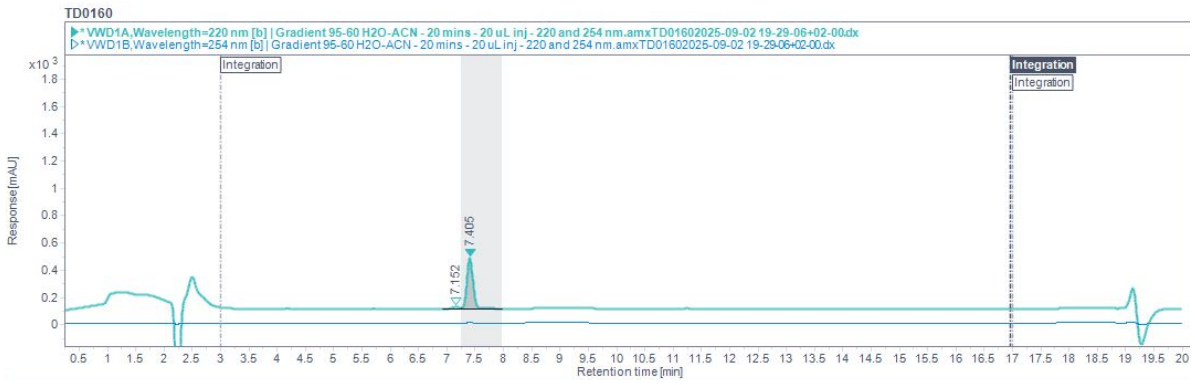

Figure S15. HPLC trace of peptide **Ac-EEV-OH**, using a solvent gradient of 95 – 60%. Compound purity determined to be 96%.

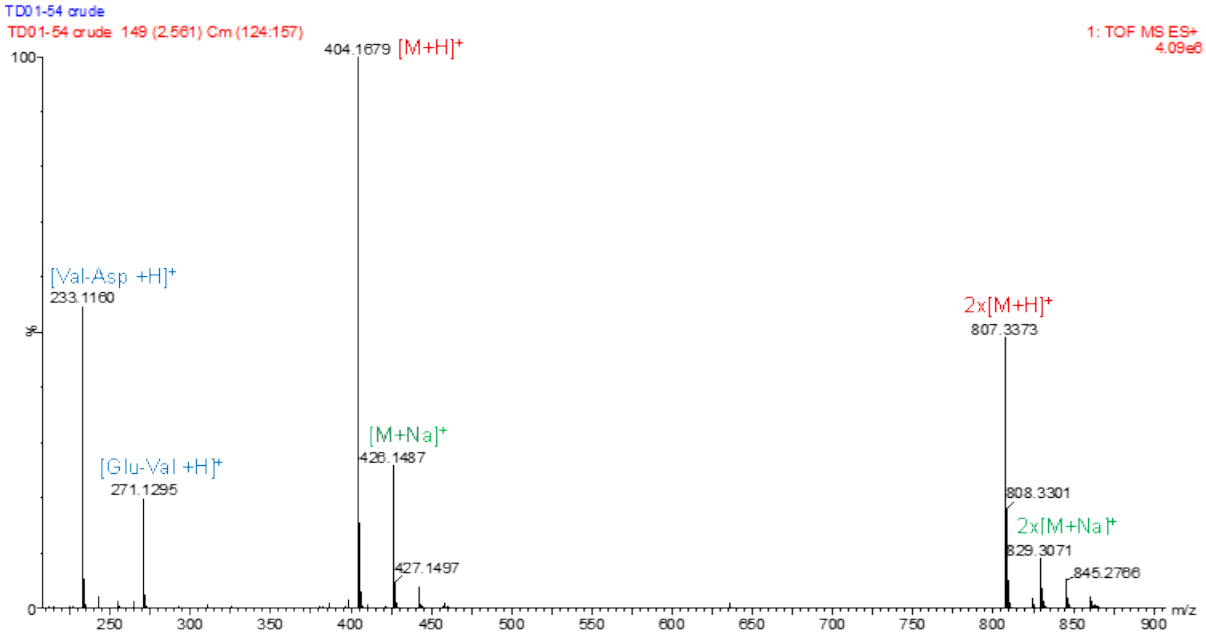

Figure S16. HR-ESI mass spectrum of peptide **Ac-EVD-OH**. Observed fragmentation products also identified.

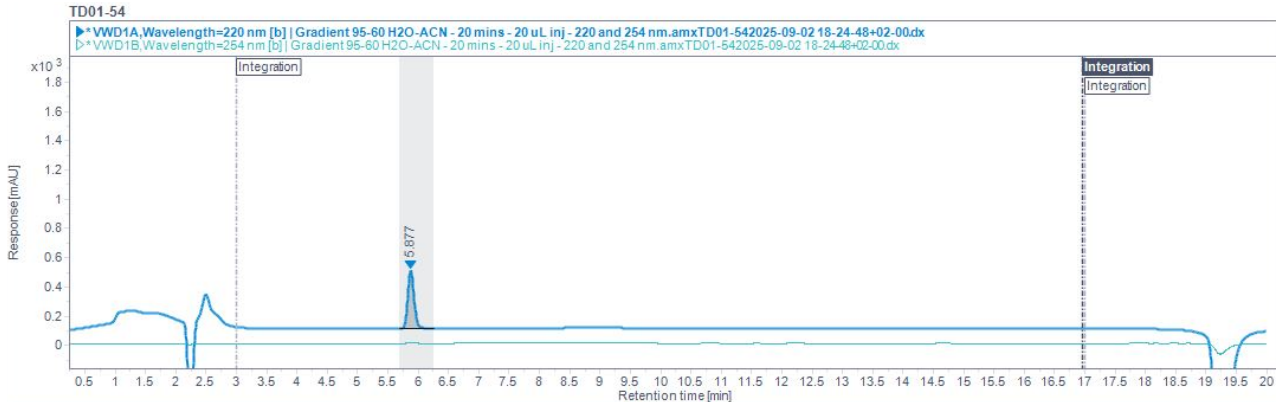

Figure S17. HPLC trace of peptide **Ac-EVD-OH**, using a solvent gradient of 95 – 60%. Compound purity determined to be 100%.

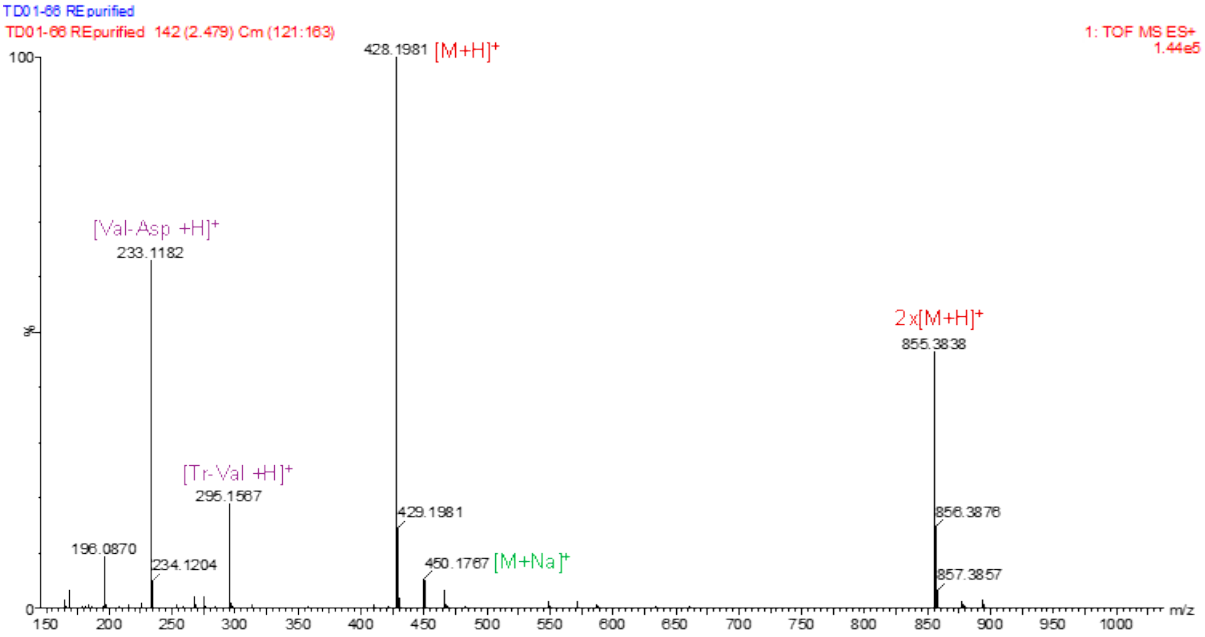

Figure S18. HR-ESI mass spectrum of peptide **Ac-TrVD-OH**. Observed fragmentation products also identified.

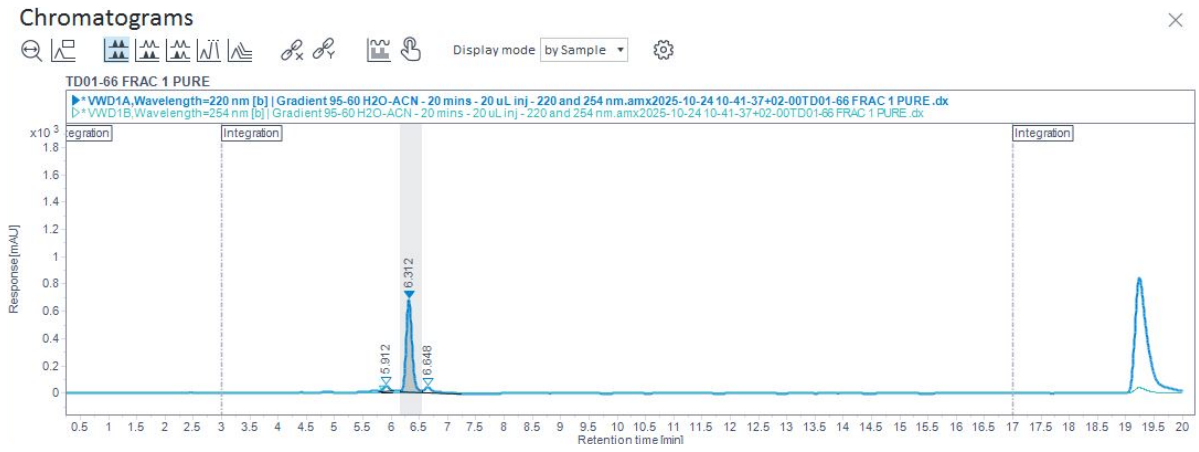

Figure S19. HPLC trace of peptide **Ac-TrVD-OH**, using a solvent gradient of 95 – 60%. Compound purity determined to be 95%.

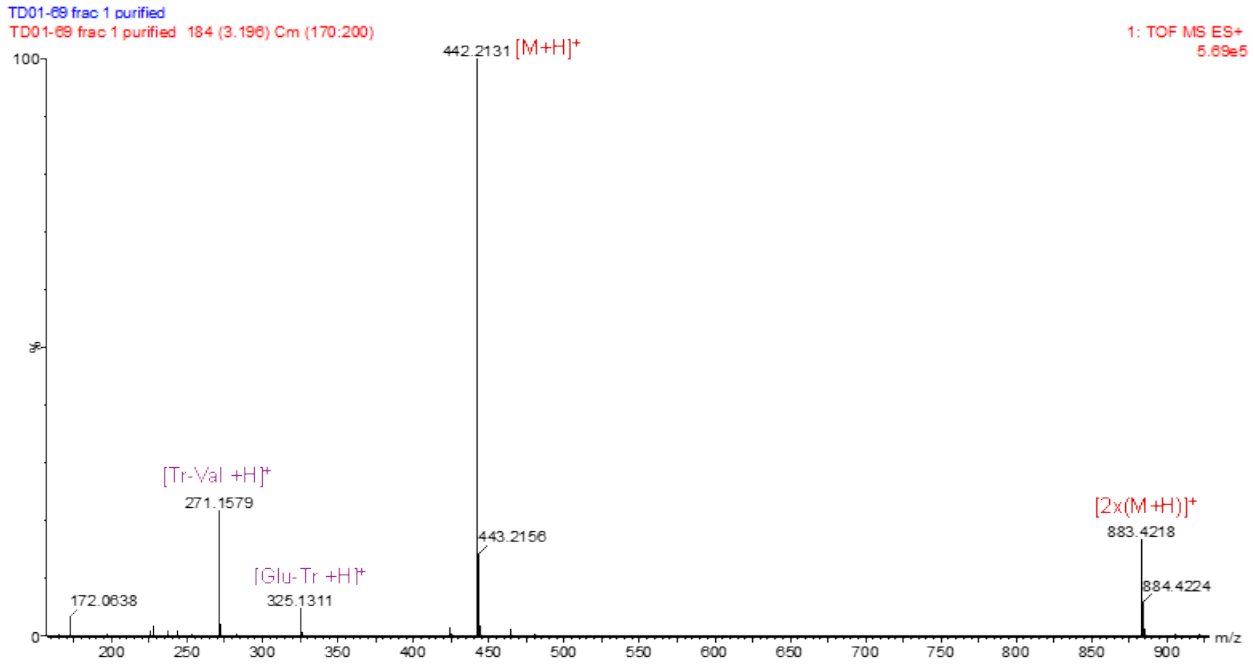

Figure S20. HR-ESI mass spectrum of peptide **Ac-ETrV-OH**. Observed fragmentation products also identified.

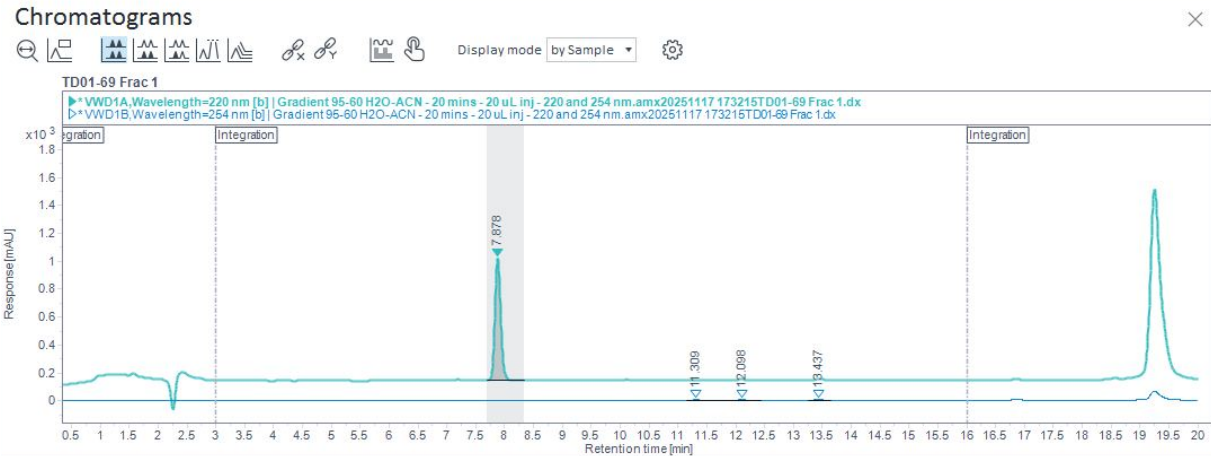

Figure S21. HPLC trace of peptide **Ac-ETrV-OH**, using a solvent gradient of 95 – 60%. Compound purity determined to be 100%.

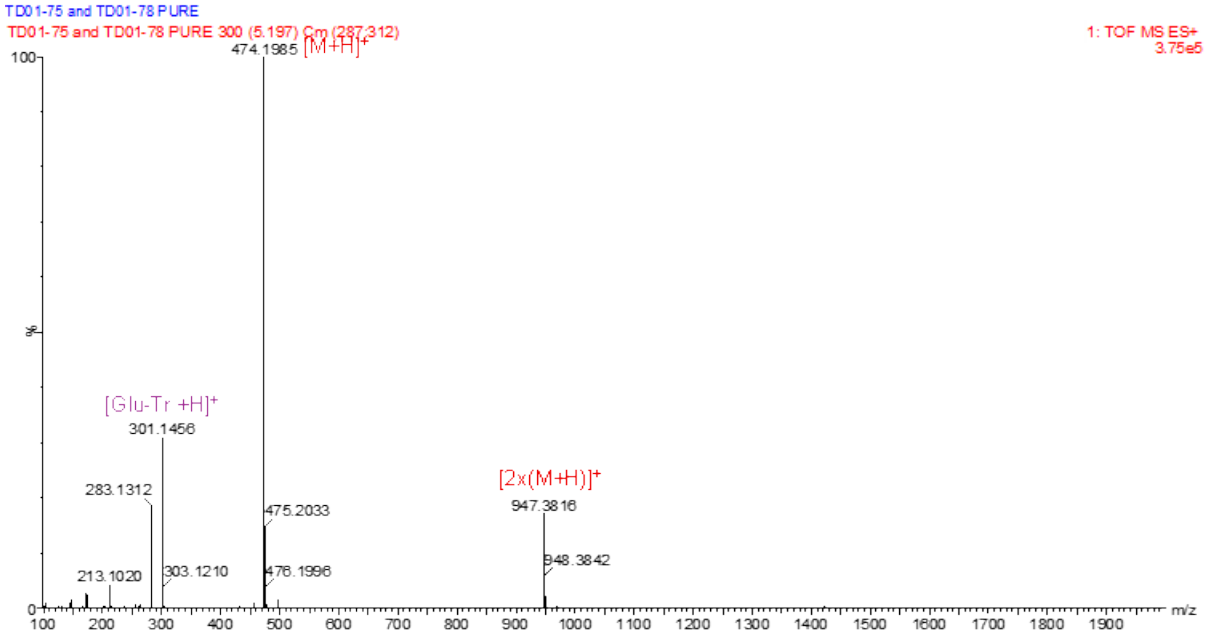

Figure S22. HR-ESI mass spectrum of peptide **Ac-TrVD-OH**. Observed fragmentation products also identified.

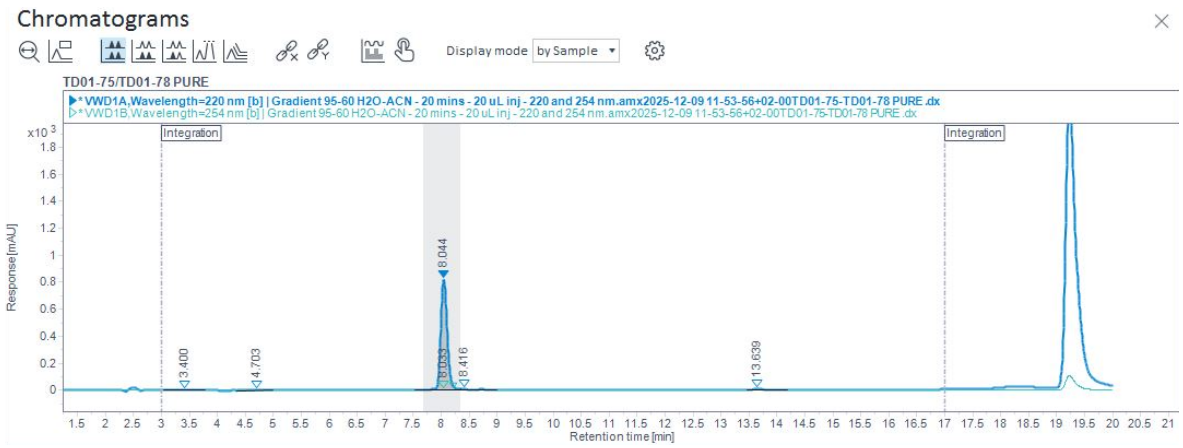

Figure S23. HPLC trace of peptide **Ac-METr-OH**, using a solvent gradient of 95 – 60%. Compound purity determined to be 95%.