SUPPLEMENTARY INFORMATION

A β-hairpin epitope as novel structural requirement for protein arginine rhamnosylation

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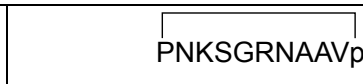
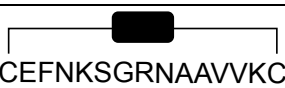
Table S1 Peptides used in this work.

Peptide name	Sequence	Exact mass calculated (m/z)	Measured (m/z)	Conversion (LC-MS)[a]
8mer	NKSGRNAA	Synthesized by GenScript		n.d.
Amide bond cyclization strategy[b]				
11mer_*Pa*	 PNKSGRNAAVp	1092.5914	1092.5906	85%
15mer_*Pa*	 PEFNKSGRNAAVVKp	1595.8658	1595.8577	14%
7mer_*Pa*	 PSGRNAAp	680.3480	680.3478	
11mer_*Rso*	 PSRSGRNAAVp	1093.5867	1093.5863	14%
11mer_*Nm*	 PIKGGRSAAKp	1079.6326	1079.6314	n.d.
11mer_-A34_*Pa*	 PNKSGRNAVp	1021.5543	1021.5535	n.d.
11mer_A34G_*Pa*	 PNKSGRNGAVp	1078.5758	1078.5749	41%
CLIPS cyclization strategy (S-alkylation[c] of Cys27-Cys37: 11mer; Cys25-Cys39: 15mer)				
11mer_CLIPS	 CNKSGRNAAVC	1265.5883	1265.5859	n.d.
15mer_CLIPS	 CEFNKSGRNAAVVKC	1768.8627	1768.8567	n.d.
Disulfide cyclization strategy (Cys27-Cys37: 11mer; Cys25-Cys39: 15mer)				
11mer_SS	 CNKSGRNAAVC	1161.5257	1161.5219	6%
15mer_SS	 CEFNKSGRNAAVVKC	1664.8001	1664.7987	17%
Tryptophan zipper peptides				
9mer_WK	NKSGRNAWV	1072.5652	1072.5649	6%
9mer_WW	NWSGRNAWV	1130.5496	1130.5498	n.d.
13mer_WF	EFNKSGRNAAVWK	1547.8083	1546.8246	n.d.
13mer_WW	EWNKSGRNAAVWK	1586.8192	1586.8173	n.d.
13mer_WKWF	EFNKSGRNAWVWK	1662.8505	1662.8492	n.d.
13mer_2WW	EWNWSGRNAWVWK	1759.8457	1759.8393	n.d.
18mer_WKWF	QKAEFNKSGRNAWVWKMK	2249.1766 1125.0920	1125.0900 (2+)	22%

		(2+)		
18mer_2WW	QKAEWNWSGRNAWVWKMK	2346.1718 1173.5901 (2+)	1173.5867 (2+)	13%
9mer_2WTW	WTWNKSGRNAAVWTW	1903.9356	1903.9356	n.d.
13mer_2WTW	WTWEFNKSGRNAAVVKWTW	2407.2100 1204.1089 (2+)	1204.1071 (2+)	n.d.
17mer_2WTW	WTWKAEFNKSGRNAAVVKMKWTW	2865.4776 1433.243 (2+)	1432.7475 (2+)	n.d.
18mer_2WTW	WTWQKAEFNKSGRNAAVVKMKWTW	2993.5361 1497.2720 (2+)	1497.2701 (2+)	n.d.

[a] Conversion in the *in vitro* rhamnosylation reaction with EarP_*Pa*

[b] Lowercase p – *D*-Pro

[c] Schematic representation of the CLIPS cyclization scaffold, based on alkylation with 1,3-bis(bromomethyl)benzene

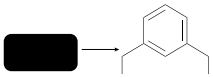


Fig. S1 Left: domain I of EF-P from Pseudomonas aeruginosa (generated with YASARA, PDB 3OYY). Arg32 loop fragment coloured in magenta. Right: linear 8mer peptide of the Arg32-loop.

Table S2 Specific amino acid interactions between EarP and domain I of EF-P (*Pseudomonas aeruginosa*), as described in literature.[1]

EF-P residue	EarP residue	Interaction type
Arg32 (side chain)	Asp16, Asp12 (side chain)	H-bonds
	Tyr112 (side chain)	H-bonds
	Tyr290, Tyr112 (side chain)	CH-pi interactions
Arg32, Ser30, Lys29 (main chain carbonyls)	Lys300, Gln292 (side chains)	H-bonds
Lys29 (side chain)	Glu294 (side chain)	Salt bridge
Ser30 (side chain)	Glu111 (side chain)	H-bond
Lys55 (side chain)	Ala87, Cys88 (main chain)	H-bonds
	Glu84(side chain)	H-bonds
	Glu89 (side chain)	Salt bridge
Val53 (main chain)	Pro126 (main chain carbonyl), Ser127 (side chain)	H-bond
Thr52 (side chain)	Pro126, Leu128(main chain)	H-bond
Asn28(side chain), Val36 (side chain)	Trp118, Phe139, Phe137 (side chain)	Hydrophobic interactions

Table S3 Calculation of RIF values for EarP peptide substrates and EF-P.

Peptide	11mer_*Pa*	11mer_*Pa*_A34G	11mer_*Rso*	EF-P
I(P)	100	27	7	11,63
I(S)	42	100	100	1,85
I(Pm)	62	10	3	9,89
I(Sm)	100	100	100	11,22
%I(Re)	70,42	21,26	6,54	86,28
%I(mix)	38,27	9,09	2,91	46,85
RIF	**1,03**	**0,98**	**0,96**	**1,00**

Fig. S2 Time-course experiment of the EarP-catalyzed conversion of 11mer_*Pa* (**A**) and EF-P_*Pa* (**B**) to the respective rhamnosylated products. Rhamnosylation of 11mer_*Pa* was performed with a ratio of enzyme : acceptor substrate of 1:2.5 (40 µM EarP, 100 µM peptide). Rhamnosylation of EFP was performed with a ratio of enzyme : acceptor substrate of 1:250 (0.4 µM EarP, 100 µM protein). **C**: combined graphs. The reactions were monitored by LC-MS (peptide) and q-TOF-LC-MS (protein), and % conversion was corrected using the RIF factor.

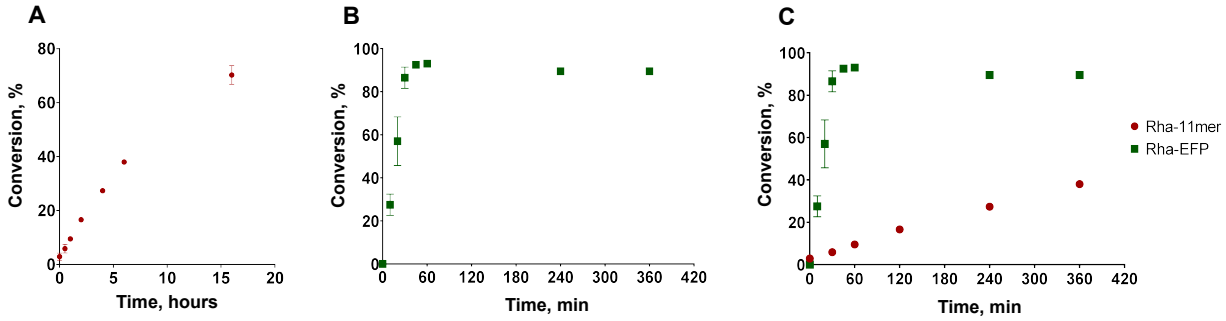


Fig. S3 Coupled HSQC of Rha-11mer_*Pa* crude reaction mixture. In the box: J_{CH} coupling of the H1-C1 (rhamnose of 11mer) determined as 167Hz, indicative of α-linked rhamnose.

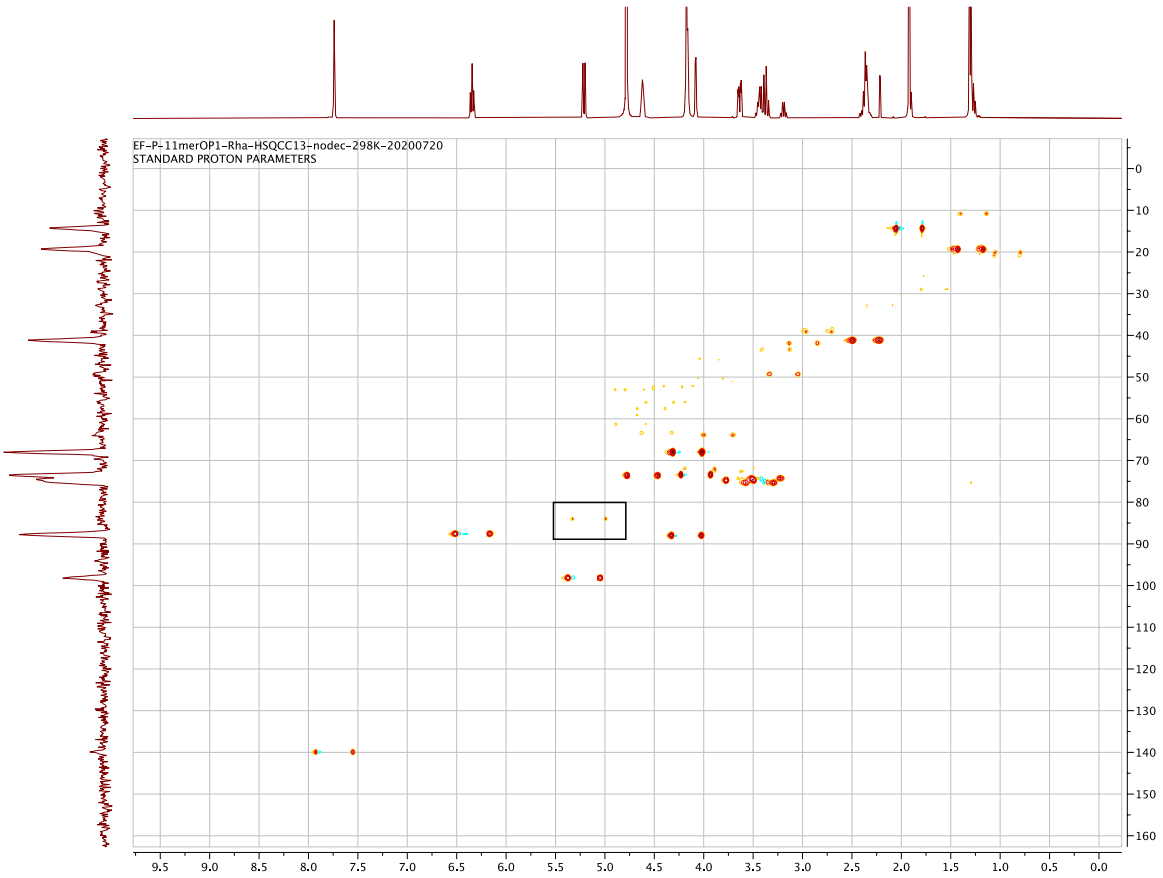


Fig. S4 Coupled HSQC of the purified Rha-11mer_*Pa* (basic conditions). In the box: J_{CH} coupling of the H1-C1 (rhamnose of 11mer) determined as 157Hz, indicative of β-linked rhamnose.

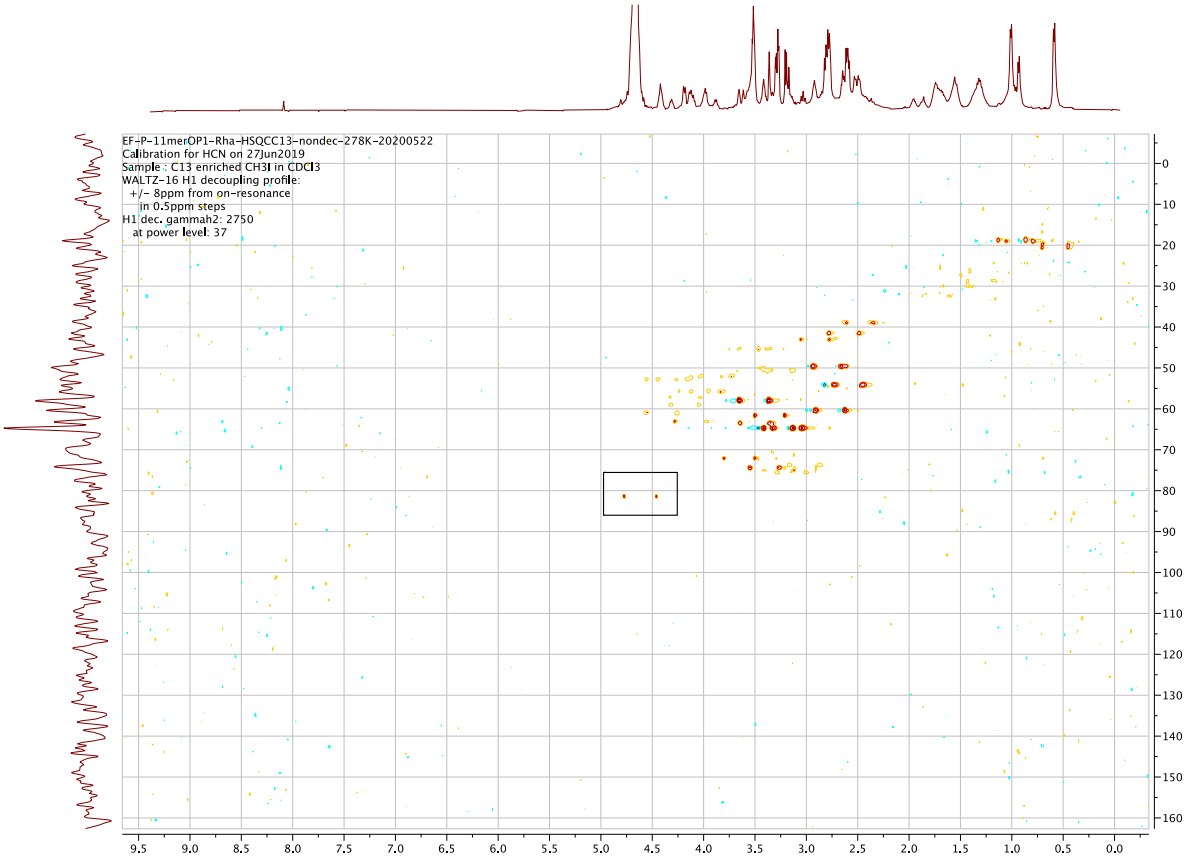


Fig. S5 Stabilization strategies used in this work to induce the β-hairpin. **A:** L-Pro-D-Pro cyclization strategy. **B:** CLIPS cyclization. **C:** Disulfide cyclization. **D:** Trp-zip stabilization.

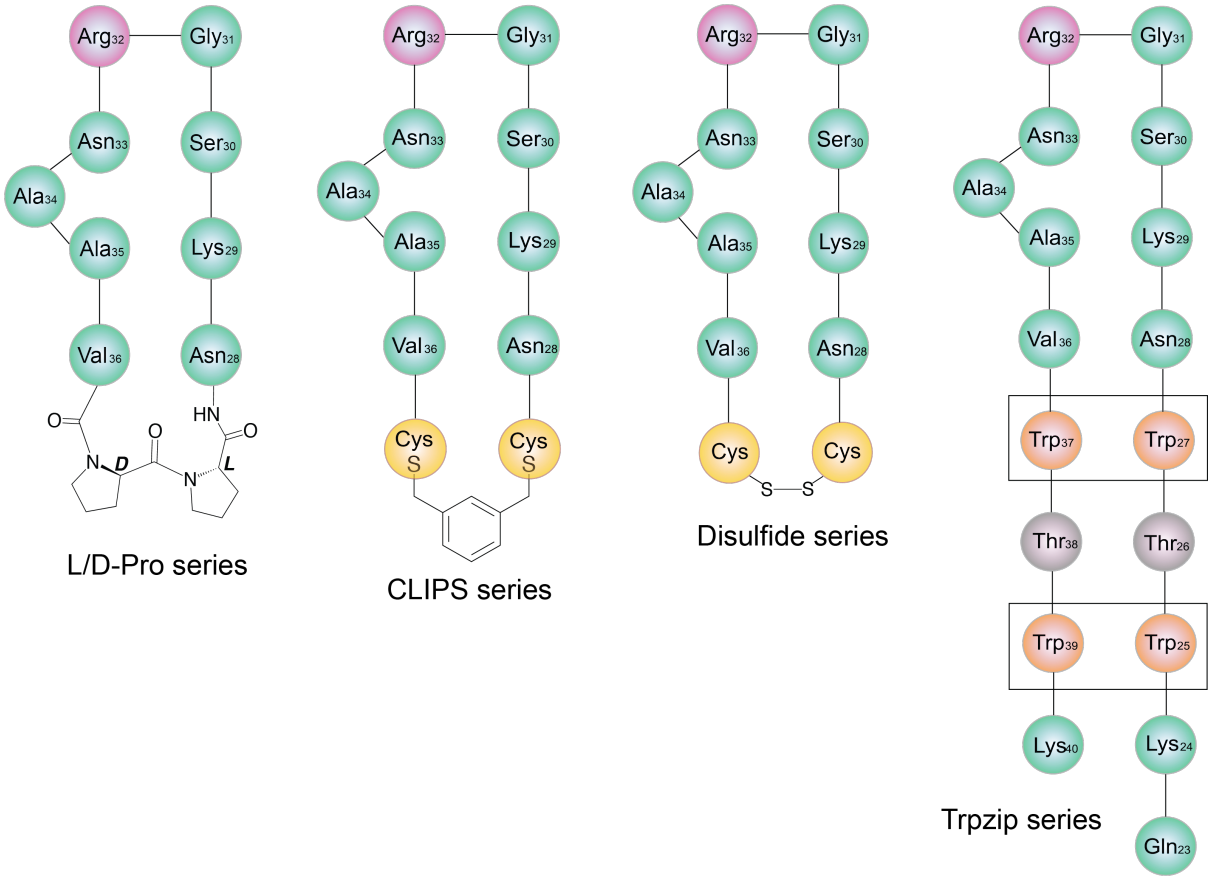


Table S4. Low temperature coefficients from NH temperature studies*:

11mer_*Pa*	11mer_*Rso*	11mer_ A34G_*Pa*	11mer_- A34_*Pa*	Linear peptide (8mer)
N28	S28	N28	N28	-
N33	N33	-	-	-

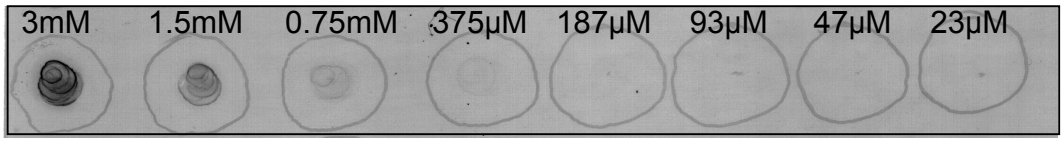
*Significantly lower than average (statistics not performed)

Table S5. Overview of the NOE signals of the peptide fragments comprising residues 28-36 of EF-P_*Pa*.

Atom pair*	11mer_*Pa*	11mer_*Rso*	11mer_A34G_*Pa*	11mer_-A34_*Pa*	Linear peptide (8mer)
P27HA-N28NH	medium	strong	medium	medium	-
N28NH-P37HA	overlap	weak	overlap	overlap	-
N28NH-V36NH	weak**	no peak	weak**	no peak	no peak
K29HA-V36NH	weak**	overlap	no peak	overlap	no peak
K29HA-A35HA	weak***	weak	weak**	no peak	no peak
S30NH-A35HA	weak	weak	no peak	overlap	no peak
S30NH-N33NH	no peak	no peak	no peak	overlap	no peak
G31HA1-N33NH	weak	weak	no peak	no peak	no peak
G31HA2-N33NH	medium	weak	weak	no peak	weak
R32NH-N33NH	medium	medium	weak	medium	medium

*Numbering and sequence according to OP1
**Unreliable, because of partial overlap, one-side of the diagonal etc.
***Only detected at one side of the diagonal in NOESY spectrum recorded with a mixing time of 500 ms.

Fig. S6 Dotblot affinity assay of a two-fold serial dilution of 3mM β-Rha-11mer binding to the anti-Arg^{Rha} antibody, and visualized using anti-rabbit Alexa488 secondary antibody.



Experimental procedures

Reagents and general methods

All chemicals were purchased from Sigma Aldrich unless otherwise stated. dTDP-β-Rha was purchased from Carbosynth. All reagents employed were of American Chemical Society (ACS) grade or higher and were used without further purification unless otherwise stated.

Preparative HPLC. Preparative HPLC runs were performed on a BESTA-Technik system with a Dr. Maisch Reprosil Gold 120 C18 column (25 × 250 mm, 10 µm) and equipped with a ECOM Flash UV detector monitoring at 214 nm. The following solvent system, at a flow rate of 12 mL/min, was used: solvent A, 0.1 % TFA in water/acetonitrile 95/5; solvent B, 0.1 % TFA in water/acetonitrile 5/95. Gradient elution was as follows: 70:30 (A:B) for 2 min, 70:30 to 0:100 (A:B) over 60 min, 0:100 (A:B) for 3 min, then reversion back to 70:30 (A:B) over 1 min, 70:30 (A:B) for 2min.

HPLC. HPLC analyses were performed on a Shimadzu Prominence-i LC-2030 system with a Dr. Maisch ReproSil Gold 120 C18 column (4.6 × 250 mm, 5 µm) at 30 °C and equipped with a UV detector monitoring 214 nm. The following solvent system, at a flow rate of 1 mL/min, was used: solvent A, 0.1 % TFA in water/acetonitrile 95/5; solvent B, 0.1 % TFA in water/acetonitrile 5/95. Gradient elution was as follows: 95:5 (A:B) for 1 min, 95:5 to 0:100 (A:B) over 25 min, 0:100 (A:B) for 2 min, then reversion back to 95:5 (A:B) over 1min, 95:5 (A/B) 1min.

HRMS. HRMS analyses were performed on a Thermo Scientific Dionex UltiMate 3000 HPLC system with a Phenomenex Kinetex C18 column (2.1 x 150 mm, 2.6 µm) at 35 °C and equipped with a diode array detector. The following solvent system, at a flow rate of 0.3 mL/min, was used: solvent A, 0.1 % formic acid in water; solvent B, 0.1% formic acid in acetonitrile. Gradient elution was as follows: 95:5 (A:B) for 1 min, 95:5 to 5:95 (A:B) over 9 min, 5:95 to 2:98 (A:B) over 1 min, 2:98 (A:B) for 1 min, then reversion back to 95:5 (A:B) over 2 min, 95:5 (A:B) for 1 min. This system was connected to a Bruker micrOTOF-Q II mass spectrometer (ESI ionization) calibrated internally with sodium formate.

Automated peptide synthesis. Peptides were synthesized by a microwave-assisted peptide synthesizer (Liberty Blue HT-12, CEM) using the following cycles of deprotection and coupling**.**

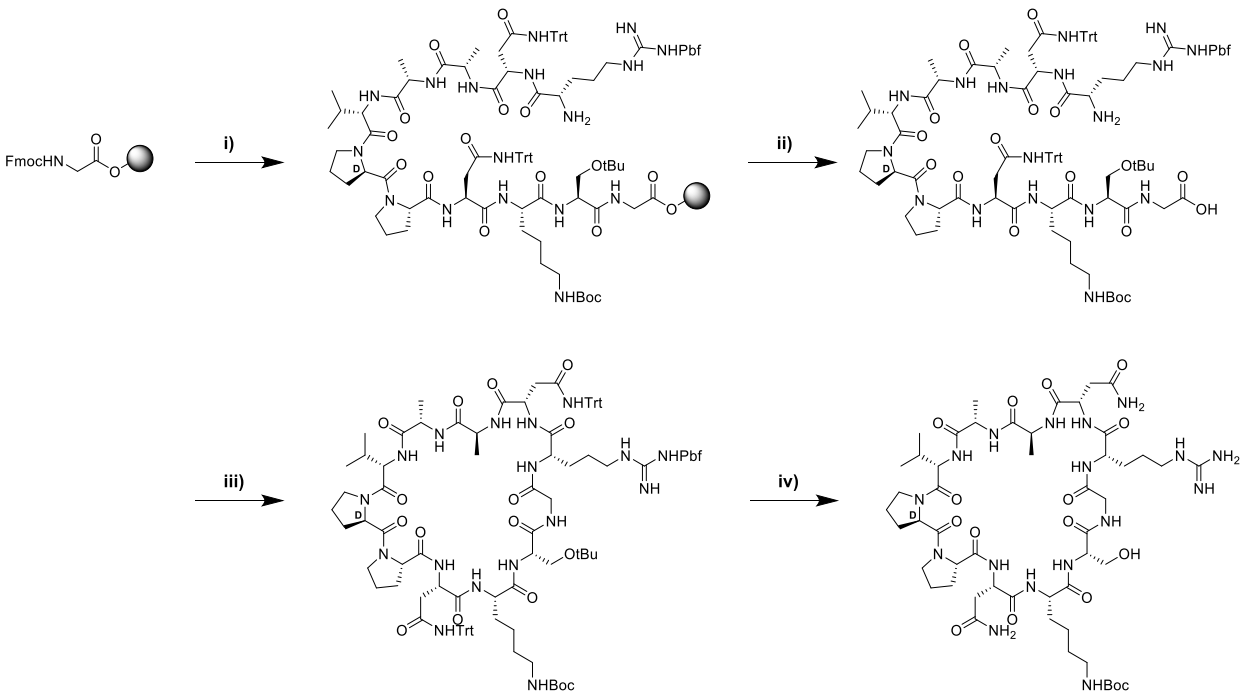
1) Fmoc deprotection: 90 ºC, 80 W, 65 s with 20% piperidine in DMF, 3 mL/deprotection

2) AA coupling: Fmoc-AA-OH (0.2 M in 2.5 mL DMF, 5 eq), DIC (1 M in 1 mL DM, 10 eq) and Oxyma (1 M in 0.5 mL DMF, 5 eq) at 76 ºC, 80 W, 15 s before the temperature was increased to 90 ºC, 80 W for 110 s.

Peptide abbreviations

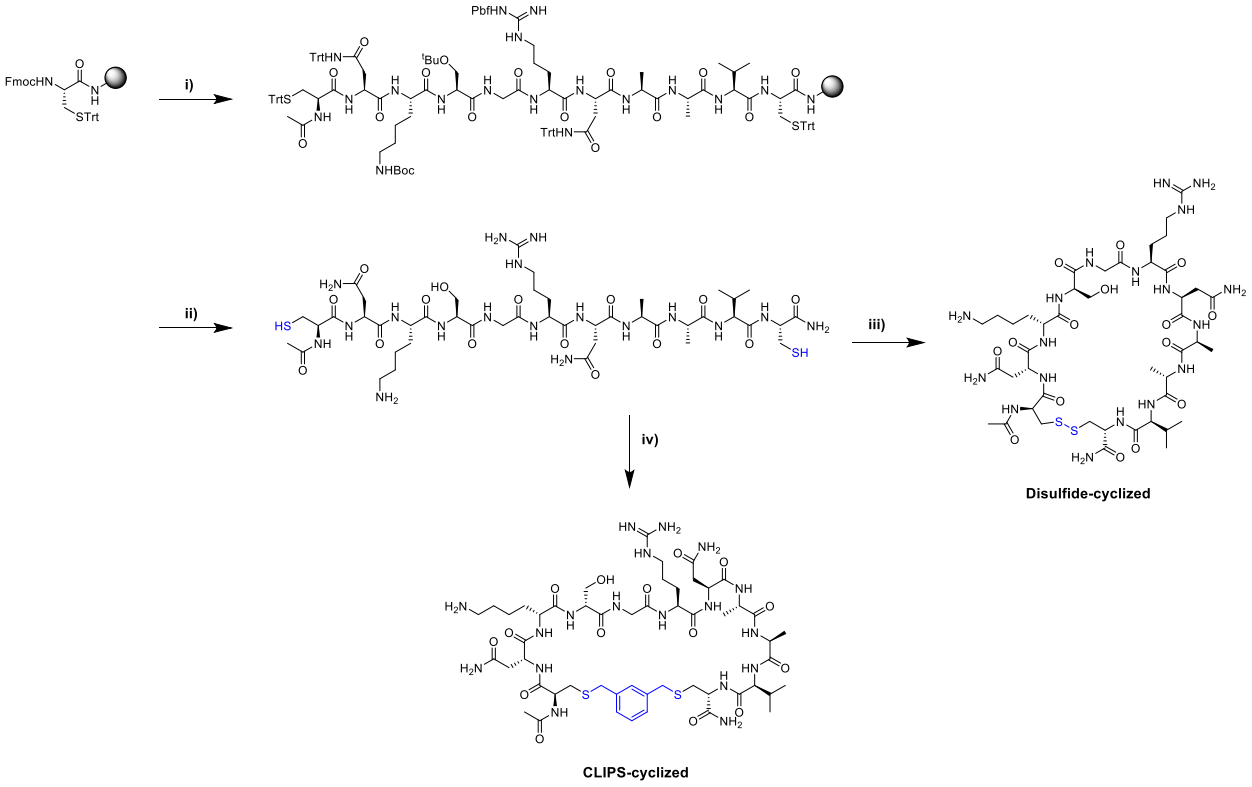
AA amino acid
Boc tert-butyloxycarbonyl
^{t}Bu tert-butyl
^{t}BuOH *tert*-butanol
Boc tert-butyloxycarbonyl
BOP (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
DIC *N,N*-Diisopropylcarbodiimide
DiPEA *N,N*-diisopropylethylamine
DMF *N,N*-dimethylformamide
Fmoc Fluorenylmethyloxycarbonyl
HFIP 1,1,1,3,3,3-hexafluoro-2-propanol
MTBE Methyl tert-butyl ether
Oxyma Ethyl cyanohydroxyiminoacetate
Pbf 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
TIS triisopropylsilane
Trt trityl

General SPPS route employed for the synthesis of all L-Pro-D-Pro cyclized peptides: i) Fmoc SPPS; ii) HFIP, CH_2Cl_2, 1h; iii) BOP, DIPEA, CH_2Cl_2, 16h; iv) TFA, TIS, H_2O, 1h.



Synthesis of L-Pro-D-Pro peptides. Chlorotrityl resin (5.0 g, 1.60 mmol.g^{-1}) was loaded with Fmoc-Gly-OH. Resin loading was determined to be 0.63 mmol.g^{-1}. Linear peptide encompassing Gly31 to Arg32 (numbering based on the EF-P sequence) was assembled manually via standard Fmoc solid-phase peptide synthesis (SPPS) (resin bound AA:Fmoc-AA:BOP:DiPEA, 1:4:4:8 molar eq.) on a 0.25 mmol scale. DMF was used as solvent and Fmoc deprotections were carried out with piperidine:DMF (1:4 v:v). Amino acid side chains were protected as follows: ^{t}Bu for Ser, Trt for Asn, Boc for Lys, and Pbf for Arg. Following coupling and Fmoc deprotection of the final Arg32, the resin was washed with CH_2Cl_2 and treated with $(CF_3)_2CHOH$:CH_2Cl_2 (1:4, 10 mL) for 1 h and rinsed with additional $(CF_3)_2CHOH$:CH_2Cl_2 and CH_2Cl_2. The combined washings were then evaporated to yield the linear protected peptide with free C- and N-termini. The residue was dissolved in CH_2Cl_2 (150 mL) and treated with BOP (0.22 g, 0.5 mmol) and DiPEA (0.17 mL, 1.0 mmol) and the solution was stirred overnight after which TLC indicated complete cyclization. The reaction mixture was concentrated and directly treated with TFA:TIS:H_2O (95:2.5:2.5, 10 mL) for 90 min. The reaction mixture was added to cold MTBE:hexanes (1:1) and the resulting precipitate was centrifuged at 3500 rpm for 5 min, washed once more with MTBE:hexanes (1:1) and centrifuged at 3500 rpm for 5 min. The crude cyclic peptide was lyophilized from ^{t}BuOH:H_2O (1:1) and purified with reverse phase HPLC. Pure fractions were pooled and lyophilized to yield the desired cyclic peptide products in >95% purity as white powders, typically in 20-70 mg quantities (18-27 % yield based on resin loading).

General SPPS route employed for the synthesis of all disulfide and CLIPS peptides: i) μW Fmoc SPPS, 90ºC; ii) TFA, TIS, H_2O, 1h; iii) 100 mM NH_4HCO_3, bubble air, 16h; iv) 100mM NH_4HCO_3, MeCN, α,α′-Dibromo-m-xylene, 1h.



Synthesis of CLIPS- and disulfide-cyclized peptides. Rink Amide resin (150 mg, 0.684 mmol.g^{-1}) was loaded into the CEM Liberty Blue peptide synthesizer for a 0.1mmol scale. Linear peptide encompassing Cys1 to Cys11 were assembled using microwave irradiation (resin bound AA:Fmoc-AA:DIC:Oxyma, 1:5:5:5 molar eq.). DMF was used as solvent and Fmoc deprotections were carried out with piperidine:DMF (1:4, v:v). Amino acid side chains were protected as follows: ^{t}Bu for Ser, Trt for Asn/Cys, Boc for Lys, and Pbf for Arg. Following coupling, Fmoc deprotection and acetylation of the final Cys11, the linear peptide was removed from the peptide synthesizer, washed with DCM and directly treated with TFA:H_2O:TIS:EDT (90:5:2.5:2.5, 10 mL) for 90 min. The reaction mixture was added to cold MTBE:hexanes (1:1) and the resulting precipitate washed once more with MTBE:hexanes (1:1) and the resulting precipitate was centrifuged at 3500 rpm for 5 min, washed once more with MTBE:hexanes (1:1) and centrifuged at 3500 rpm for 5 min. The obtained pellet was split into two and dissolved in a mixture of 20 mM NH_4HCO_3 and MeCN (typically 3:1 but ratio can be varied depending on peptide solubility, total volume should be 90 ml per 0.05 mmol). *To make the disulfide peptide:* the peptide solution was stirred for 1 h at room temperature while bubbling oxygen through the mixture. *To make the CLIPS peptide:* A solution 1,3-bis(bromomethyl)benzene (0.075 mmol; 19.7 mg) in MeCN (5 mL) was added to the peptide

solution and stirred for 1 h at room temperature. Once the peptide reactions were completed MeCN was evaporated and the crude peptides were lyophilized from ^{t}BuOH:H_2O (1:1) and purified with reverse phase HPLC. Pure fractions were pooled and lyophilized to yield the desired disulfide or CLIPS products in >95% purity as white powders, typically in 10-40 mg quantities (18-30 % yield based on resin loading).

Synthesis of the trp-zip peptides. Rink Amide resin (150 mg, 0.684 mmol.g^{-1}) was loaded into the CEM Liberty Blue peptide synthesizer for a 0.1mmol scale. Linear peptide encompassing the first amino acid to the last amino acid were assembled using microwave irradiation (resin bound AA:Fmoc-AA:DIC:Oxyma, 1:5:5:5 molar eq.). DMF was used as solvent and Fmoc deprotections were carried out with piperidine:DMF (1:4, v:v). Amino acid side chains were protected as follows: ^{t}Bu for Ser/Thr/Asp/Glu, Trt for Asn/Cys/Gln, Boc for Lys/Trp, and Pbf for Arg. Following coupling, Fmoc deprotection and acetylation of the final amino acid the linear peptide was removed from the peptide synthesizer, washed with DCM and directly treated with TFA:H_2O:TIS:EDT (90:5:2.5:2.5, 10 mL) for 90 min. The reaction mixture was added to cold MTBE:hexanes (1:1) and the resulting precipitate washed once more with MTBE:hexanes (1:1) and the resulting precipitate was centrifuged at 3500 rpm for 5 min, washed once more with MTBE:hexanes (1:1) and centrifuged at 3500 rom for 5 min. The crude peptides were lyophilized from ^{t}BuOH:H_2O (1:1) and purified with reverse phase HPLC. Pure fractions were pooled and lyophilized to yield the desired peptide products in >95% purity as white powders, typically in 20-70 mg quantities (20-30 % yield based on resin loading).

Protein expression and purification. pBADSUMO plasmids harbouring *earp* or *efp* gene (from *Pseudomonas aeruginosa* PAO1, synthesized and cloned by GenScript) were used to transform chemically competent *E. coli* TOP10 cells (standard heat-shock protocol) and plated on LB-agar plates containing an appropriate antibiotic (ampicillin). A single colony was selected from the plate and used to prepare glycerol stock. To express the protein on large scale, a preculture (10 mL) in LB (100 µg/mL ampicillin) was prepared from the appropriate glycerol stock and grown at 37 ºC with shaking (200 rpm) for 16-18 h. It was then used to inoculate 500mL of Terrific Broth (TB) (100 µg/mL ampicillin) at 1:200 dilution ratio and incubated at 37 ºC with shaking until OD_{600} reached values of 0.6-0.7. Protein expression was induced by addition of 0.05% L-Ara (w/v, final concentration) and further incubation for 16-18 hours at 18 ºC with shaking (EarP) or 4 h at 37 ºC with shaking (EF-P). Cells were harvested

by centrifugation at 5000 rpm for 15 min (Sorvall centrifuge, F-12 6x500 LEX fixed angle rotor, Thermo Scientific). The supernatant was discarded and cell pellets were resuspended in ice-cold lysis buffer (20 mM Tris, 100 mM NaCl, pH 8) in the presence of the protease inhibitor cocktail (Roche, complete, EDTA-free). Cells were lysed by sonication (Branson Sonifier 450, output control 30%, 2 min) and subsequently spun down at 7000 rpm at 4 ºC for 1 hour. For His_6-tag protein purification, the cell-free extract was incubated with Ni-NTA resins (Qiagen) for 1.5 h at 4 ºC with gentle shaking. The mixture was loaded on a gravity column and the lysate was allowed to flow through, followed by a washing step (twice) with washing buffer (20 mM Tris, 100 mM NaCl, 15 mM imidazole, pH 8). The protein of interest was eluted with elution buffer (20 mM Tris, 100 mM NaCl, 400 mM imidazole, pH 8) in three steps. Column fractions were analyzed by 12% SDS-PAGE analysis and the resulting gels were stained using Instant Blue protein stain. Fractions containing protein of interest were pooled and desalted using midi PD-10 desalting columns (GE Healthcare). Proteins were routinely obtained in the yields of 20-40 mg per 1L of culture.

***In vitro* rhamnosylation of Arg-containing peptides.** Reaction mixtures routinely consisted of 100 µM peptide (from 10 mM DMSO stock to keep DMSO content low) and 1 mM TDP-Rha. Reactions were generally initiated by the addition of 20 µM EarP (usually from freshly concentrated 200-300µM stock) and incubated at 30ºC overnight. For experiments where peptide substrates were pushed to conversion, 200 µM of peptide substrate and 70µM of EarP were used. To prepare the sample for LCMS analysis, the reaction mixture was spun down in the table top centrifuge (Eppendorf) and an aliquot was taken for RP-LCMS analysis (1 µL injection, Acquity UPLC HSS T3 column (Waters, 2.1×150 mm, 1.8 µm) was used in combination with eluents A (0.1% formic acid in H_2O) and B (0.1% formic acid in acetonitrile), 20 min run (flow rate 0.3 mL/min) with a linear gradient from 5% to 50% of B in 10min with subsequent increase to 95% B for 3min).

Time course of rhamnosylation. To visualize the progress of rhamnosylation of 11mer_*Pa* and EF-P_*Pa*, the rhamnosylation reaction with respective acceptor substrates was monitored over time. For the 11mer_*Pa* reaction: mixtures containing 40 µM EarP, 100 µM peptide and 1 mM TDP-Rha were incubated at 30 ºC. For EFP_*Pa* reaction: mixtures containing 0.4 µM EarP, 100 µM peptide and 1 mM TDP-Rha were incubated at 30 ºC. At certain time points aliquots were withdrawn and quenched either by diluting the reaction fourfold and removing the EarP enzyme by centrifugation through a 10kDa MWCO spin filter (for 11mer_*Pa* reaction) or by diluting threefold and heating at 100 ºC for 10 min (EF-P_*Pa* reaction). For the 11mer_*Pa* reaction the aliquots were analyzed with RP-LCMS as described above. For the EF-P_*Pa* reaction, aliquots were further diluted tenfold with 2% ACN, 0.1% FA solution (aq.) and

analyzed with q-TOF LCMS (Waters). A BEH300 C4 column was used (Waters, 2.1×150 mm, 1.7 µm) in combination with eluents A (0.1% formic acid in H_2O) and B (0.1% formic acid in acetonitrile), 20 min run (flow rate 0.3 mL/min) with a linear gradient from 5% to 50% of B in 10 min with subsequent increase to 95% B in 1 min and isocratic flow of 95% B for 2 min). Deconvolution was performed with an open access UniDec software. Graphs were prepared using GraphPad Prism 8. Reactions were performed in duplicates.

Large-scale rhamnosylation and purification of the 11mer_*Pa* peptide. To scale up the enzymatic rhamnosylation of 11mer-L-Pro-D-Pro_*Pa* for NMR studies, 1.8 mM 11mer_*Pa* (5 mg), 3.5 mM TDP-Rha (5.17 mg) and 26.4 µM EarP (3.7 mg) were incubated overnight at 30 ºC. Reaction was pushed to full conversion over two days by addition of extra 10 µM EarP and extra 0.625 mM TDP-Rha after 16 h and 48 h. Subsequently, the reaction mixture was applied to an Amicon spin filter (MWCO 10 kDa, 15 mL, Millipore) and centrifuged at 5000 x *g* to remove the enzyme. The resulting solution was further purified from TDP and TDP-Rha by strong anion exchange on FPLC ÄKTA system (GE Healthcare). For this, 0.25 mL of the reaction mixture (approx. 1 mM peptide concentration) was applied on Q FF column (5 mL, GE Healthcare) with flow rate 1 mL/min in 5 column volumes (CV). The column was eluted with the linear gradient from 0 to 10% Buffer B in two CV with subsequent increase to 100% in four CV (Buffer B: 1M NH_4HCO_3). Elution was monitored with UV (214 nm – peptide and 280 nm – TDP, TDP-Rha). After 14 runs, the fractions containing rhamnosylated 11mer_*Pa* peptide were pooled and freeze-dried. Residual buffer salts were removed by desalting with PD10 desalting columns (GE Healthcare). The Rha-11mer_*Pa* peptide was eluted in pure H_2O and freeze-dried to yield 1.5 mg of material for NMR studies.

Isothermal titration calorimetry (ITC) experiments. All binding experiments were performed using a MicroCal PEAQ-ITC Automated microcalorimeter (Malvern). The samples were equilibrated at 20 ºC prior to the measurement. The solution of ligand in 20 mM Tris-HCl, 100 mM NaCl, pH 8, was titrated into a solution of EarP:TDP (1:3) in the same buffer. The titrations were conducted at 20 °C under constant stirring at 750 rpm. Each binding experiment consisted of an initial injection of 0.3 µL followed by 19 separate injections of 2.0 µL into the sample cell of 200 µL. The time between each injection was 150 s, the measurements were performed with the reference power set at 5 µcal s^{-1} and the feedback mode set at “high”. The calorimetric data obtained was analyzed using MicroCal PEAQ-ITC Analysis Software Version 1.20. ITC data fitting was made based on the “One set of sites” fitting model of the software. The best fit was defined by chi-square minimization. The thermodynamic parameters are reported as the average of three independent experiments.

Preparation of crude reaction mixture for HSQC experiments (glycosidic linkage determination). To prepare the crude reaction mixture of Rha-11mer_*Pa* for glycosidic linkage determination, 1.4 mg of TDP-Rha (in D_2O), 1 mg of 11mer_*Pa* (in D_2O) and 50 µM EarP (exchanged into D_2O by diafiltration) were mixed in a total volume of 600 µL (in D_2O) and incubated overnight at 30 ºC. The next day, conversion was checked by LCMS and determined to be about 78%. To push conversion further, an additional 0.6 mg of TDP-Rha (in D_2O) and 14 µM of EarP (exchanged into D_2O) were added and the reaction mixture was incubated overnight at 30 ºC. LCMS analysis determined the conversion to be about 88%. The resulting crude sample was used for the NMR analysis of the glycosidic linkage.

Antibody-based dot blot assay. Typically, 4 µL of mixture of interest was spotted on the nitrocellulose membrane and let dry for 1 h. Subsequently, the membrane was blocked in 3% BSA in TBS buffer (Tris buffer saline, 20mM Tris, 150mM NaCl, pH 7.5), followed by incubation with anti-Arg^{Rha} antibody (1:1000 in 3% BSA-TBS) for 2 h while shaking. Consequently, the membrane was washed with TBS three times (5 min each) and incubated with the secondary Alexa488 coupled Anti-rabbit antibody (Abcam, 1:7500 in 3% BSA-TBS) for 30 min. After three washes with TBS (5 min each), the membrane was dried and visualized with Typhoon fluorescent scanner (Typhoon FLA 9500, GE Healthcare) using Alexa488 settings.

RIF values determination. An experiment to determine relative ionization factor values for rhamnosylated *vs* non-rhamnosylated peptides was adopted from [2]. Briefly, reaction mixtures containing 100 µM peptide (11mer_*Pa*, 11mer_*Rso*, 11mer_*Pa*_A34G), 1 mM TDP-Rha and 40 µM EarP were prepared and incubated overnight at 30 ºC. Alongside, identical mixtures where TDP-Rha was omitted (blank) were prepared and incubated overnight at 30 ºC. To prepare LCMS samples, either the reaction sample or a 1:1 mixture of reaction and blank sample were filtered through the 10 kDa MWCO Amicon filter and analyzed by LCMS. RIF values were calculated according to the formulae from [2]:

$$\%I(Re) = \frac{I(P)}{I(P)+I(S)} \times 100\% \quad (1)$$

$$\%I(mix) = \frac{I(Pm)}{I(Pm)+I(Sm)} \times 100\% \quad (2)$$

$$RIF(P\ to\ S) = \frac{\%I(Re)\times\%I(mix)}{\%I(re)\times\%I(mix)-2\times\%I(mix)} \quad (3)$$

$$\%conversion = \frac{I(P)/RIF}{\frac{I(P)}{RIF}+I(S)} \times 100\% \quad (4)$$

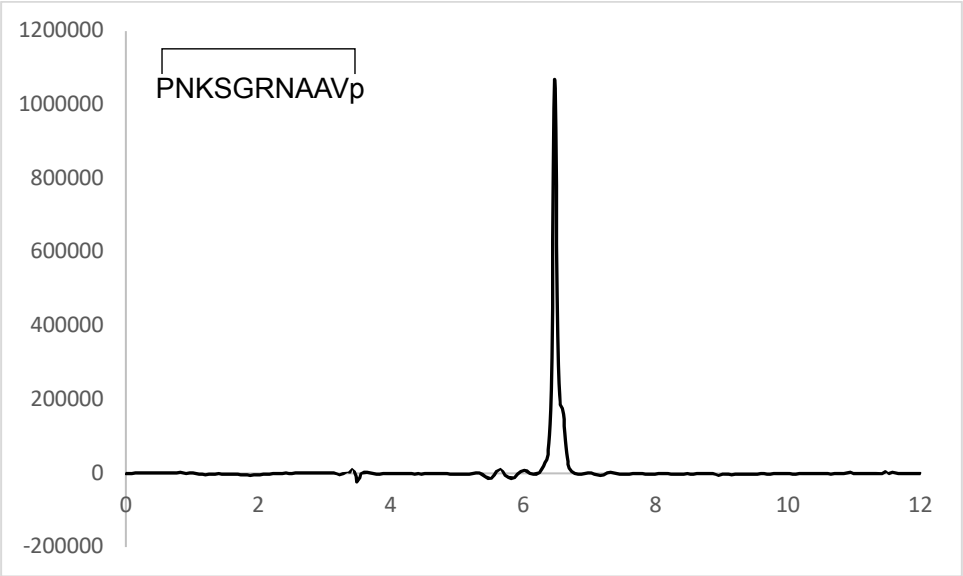
I(P) – intensity of the product ion in the reaction sample; *I(S)* – intensity of the substrate ion in the reaction sample; *I(Pm)* – intensity of the product ion in the 1:1 mixed sample; *I(Sm)* – intensity of the substrate in the 1:1 mixed sample.

NMR spectroscopy. All NMR spectra were recorded on a Varian Inova 500 MHz NMR spectrometer equipped with an HCN triple resonance probe or on a Bruker Avance NEO 600 MHz spectrometer equipped with either a broadband Prodigy CryoProbe or a SmartProbe. The peptides were dissolved in a mixture of H_2O/D_2O (9:1 v/v) with 15 mM phosphate buffer at pH = 4.7. Spectral assignments and structural analyses were accomplished by recording TOCSY spectra (mixing time 80 ms) and NOESY spectra (mixing times 300 or 500 ms) at 278K using a spectral width of 10 ppm in both dimensions and the excitation sculpting solvent suppression scheme.[3] ^{1}H-^{13}C HSQC spectra were recorded at 278K with a spectral width of 10 ppm in the ^{1}H- and 115 ppm in the ^{13}C-dimension. All data were processed with NMRPipe[4] and analyzed using Sparky.[5] Chemical shifts were referenced with respect to internal dioxane (δ_{1H}= 3.750 ppm/ δ_{13C}= 69.3 ppm).

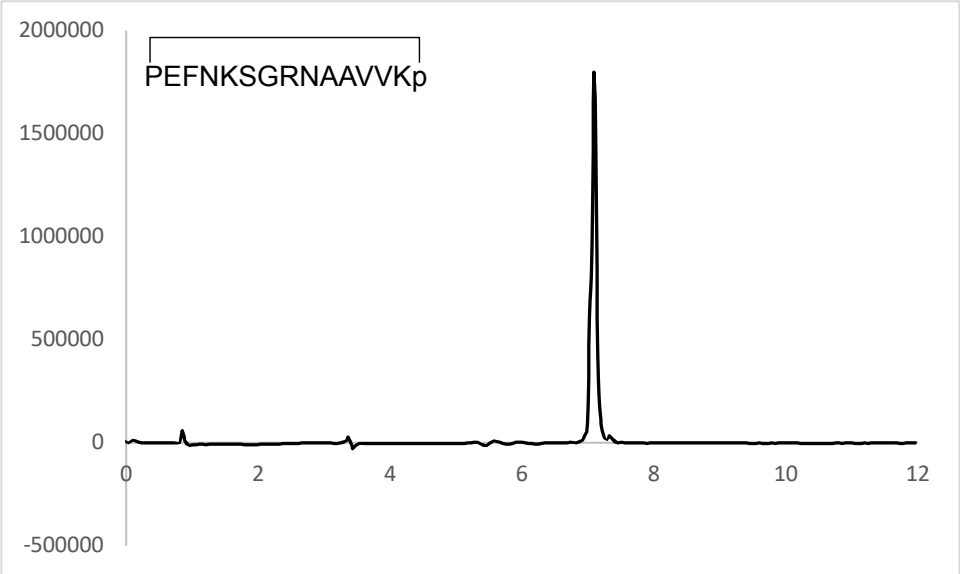
Temperature coefficients of the amide protons of selected peptides were determined by measuring 1D ^{1}H experiments in a temperature series between 273 and 303K with 5K intervals. The ^{1}J-coupling constant of the anomeric proton and carbon was determined by recording a ^{1}H-^{13}C HSQC at 298K without carbon decoupling during acquisition on a crude mixture of glycosylated substrate, EarP and dTDP-Rha. Diffusion ordered NMR spectroscopy (DOSY) measurements were performed on the same crude mixture using a stimulated spin-echo bipolar gradient pulse sequence.[6] 16 experiments of 16 scans each were recorded with gradient strengths increasing linearly between 0 and 42 G/cm with a pulse length of 1 ms (δ = 2 ms). A diffusion delay of Δ = 200 ms was used. Saturation transfer difference NMR (STD-NMR)[7] experiments were performed on samples containing mixtures of EarP, peptide and dTDP plus controls without EarP present. Saturation of the protein resonances was accomplished by a 2 s pulse train of 50 ms Gaussian pulses applied at a frequency corresponding to -0.5 ppm (on resonance) and 30 ppm (off resonance). Protein background signals were suppressed by a $T_{1\rho}$ purge pulse before data acquisition. These data were processed and analyzed using Mnova (Mestrelab Research S.L., Spain).

HPLC traces of the peptides:

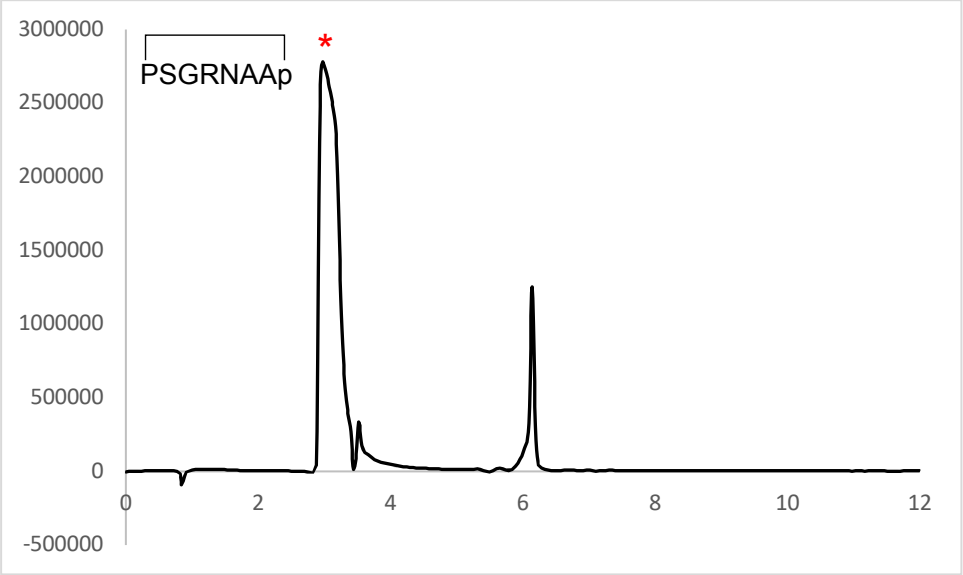
11mer_*Pa*



15mer_*Pa*

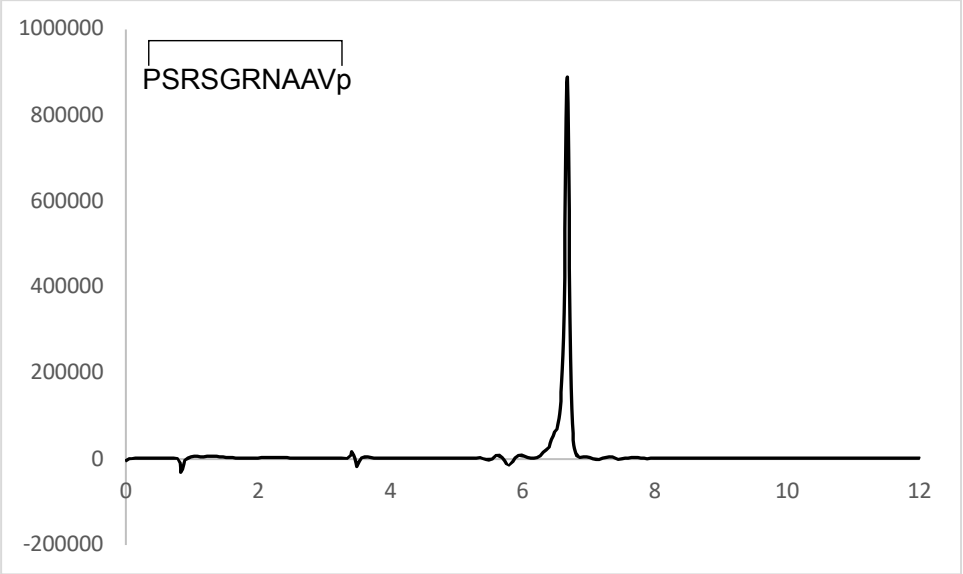


7mer_*Pa*

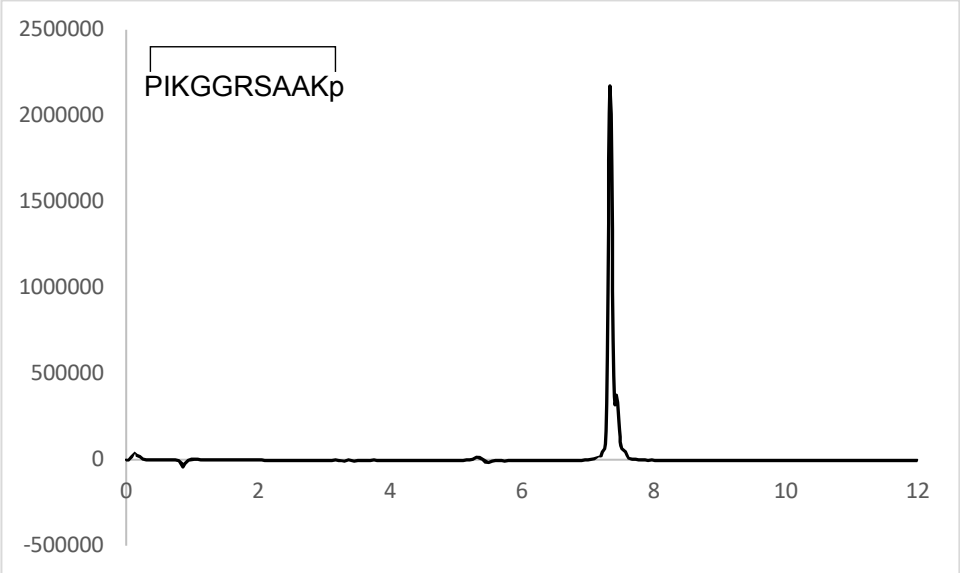


*Peak indicated with an asterisk – DMSO peak

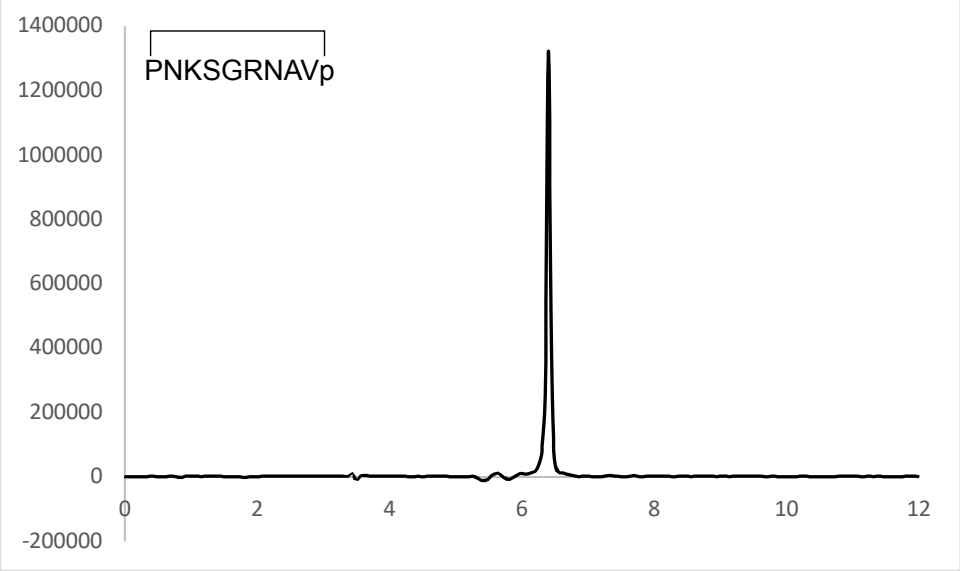
11mer_*Rso*



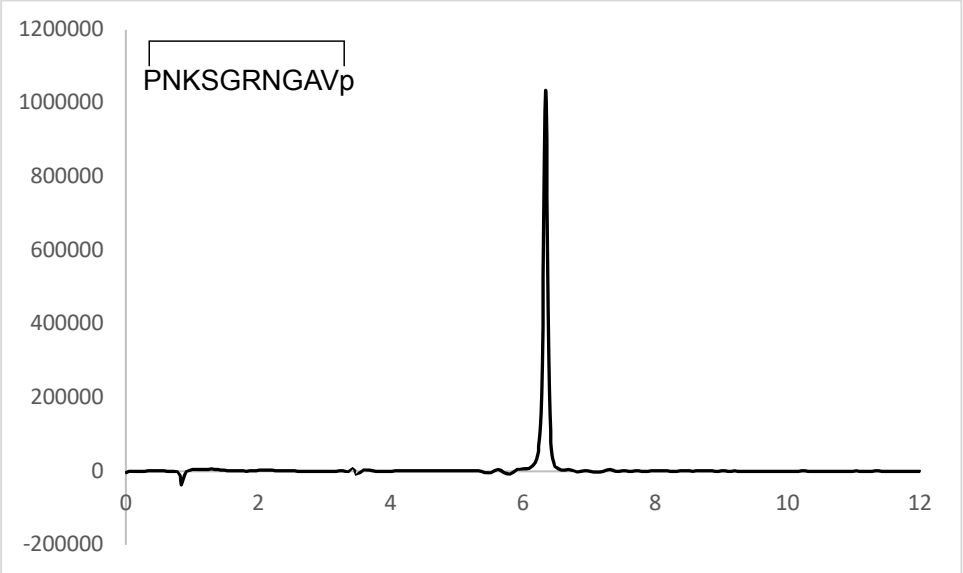
11mer_*Nm*



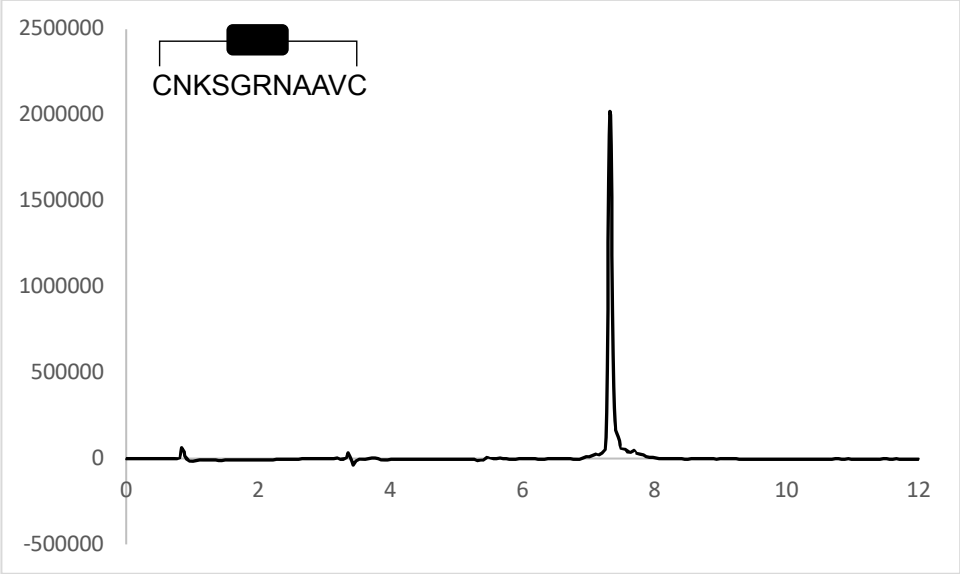
11mer_-A34_*Pa*



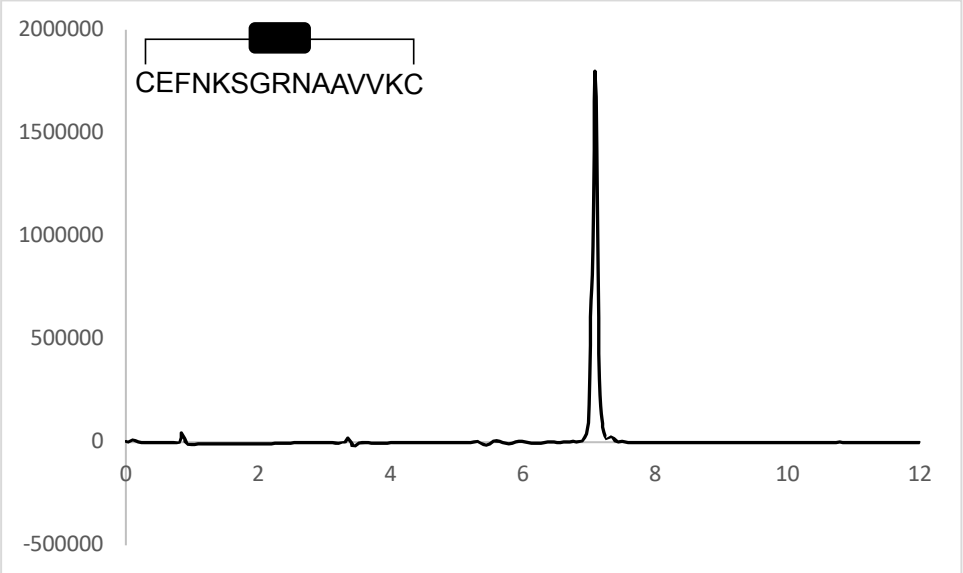
11mer_A34G_*Pa*



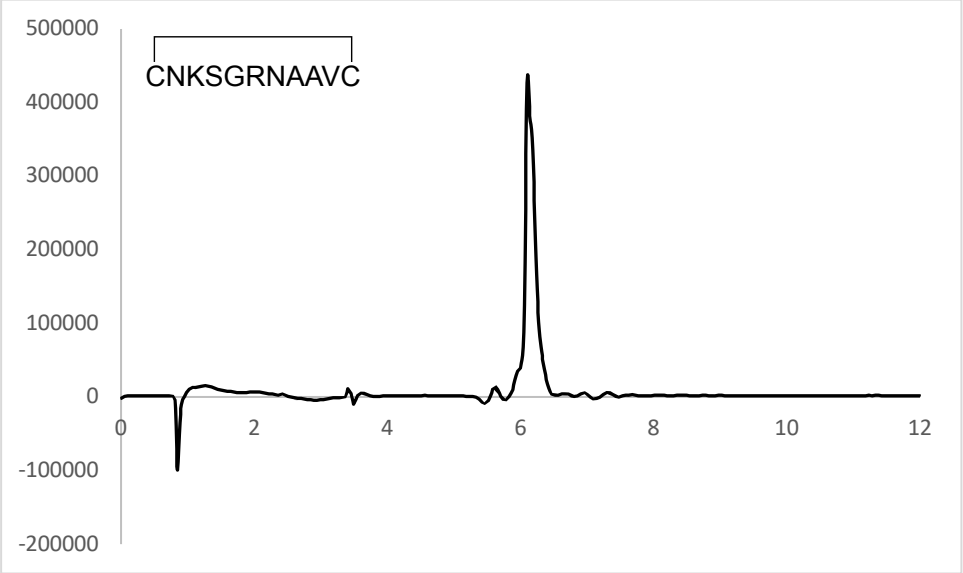
11mer_CLIPS



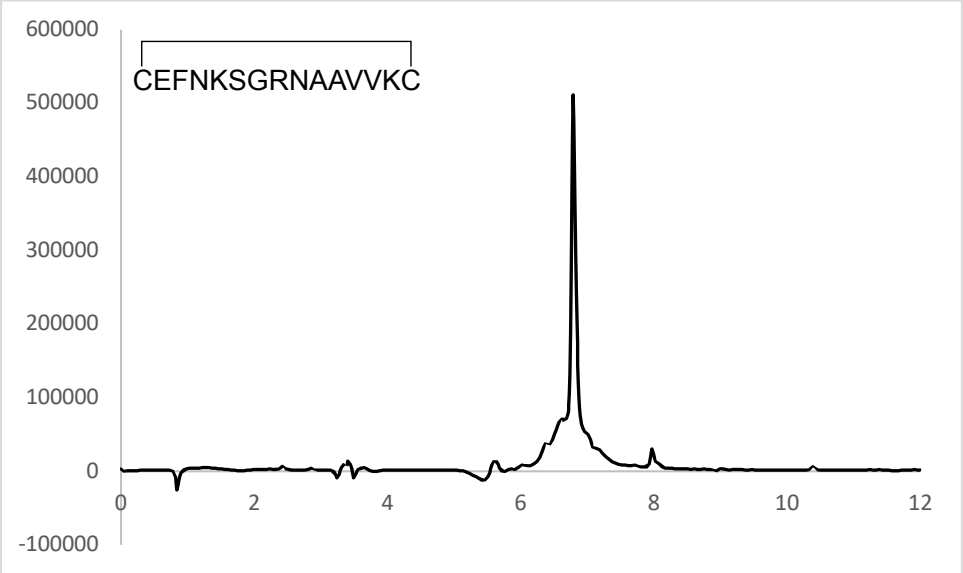
15mer_CLIPS



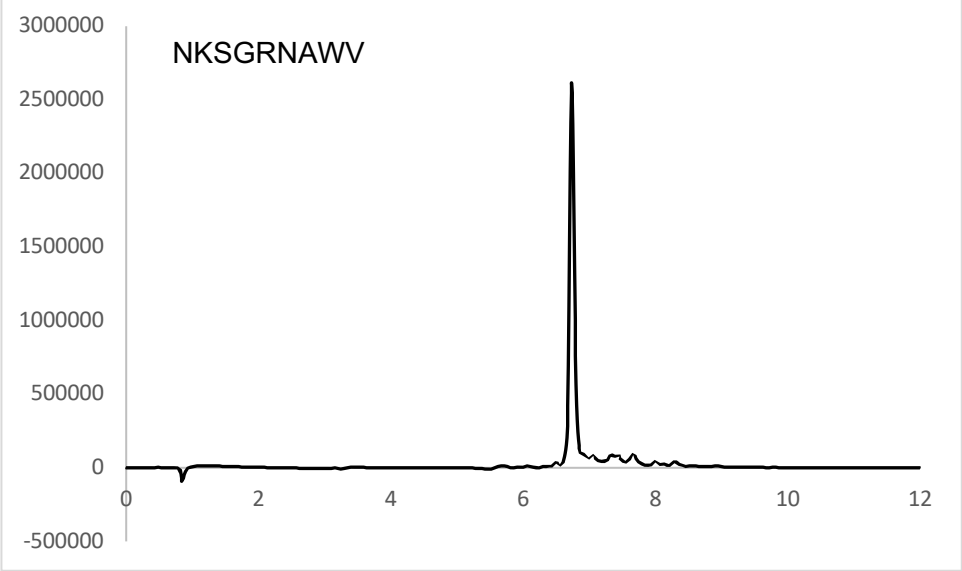
11mer_SS



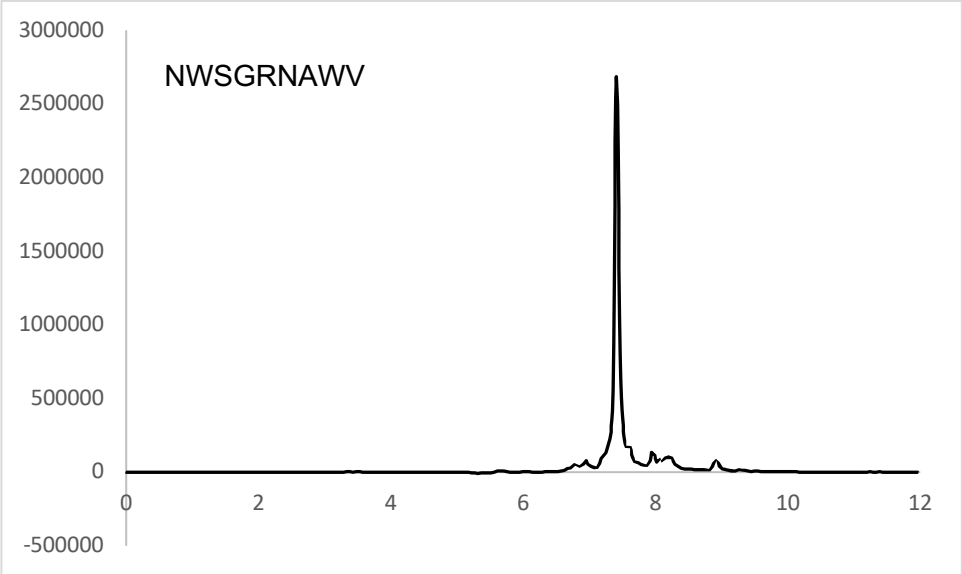
15mer_SS



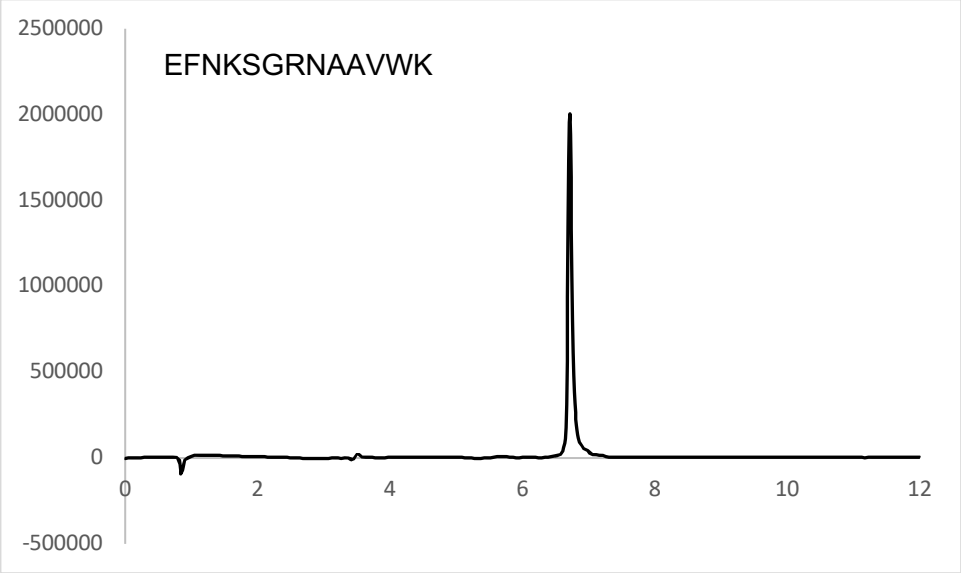
9mer_WK



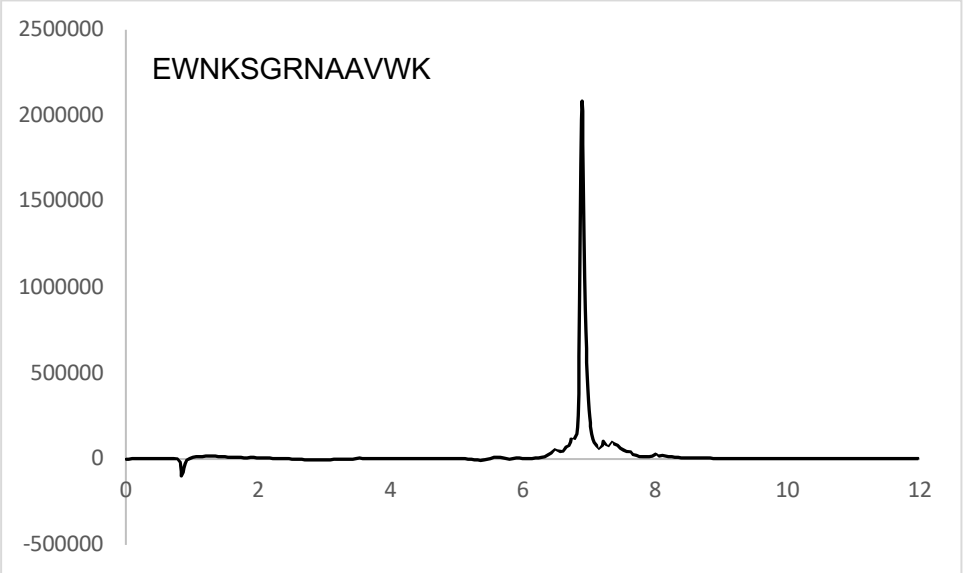
9mer_WW



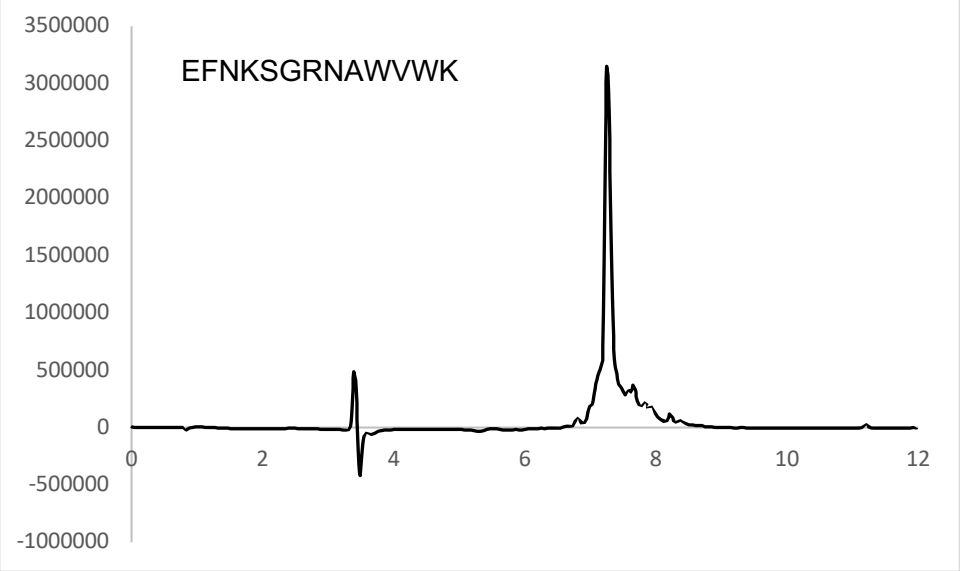
13mer_WF



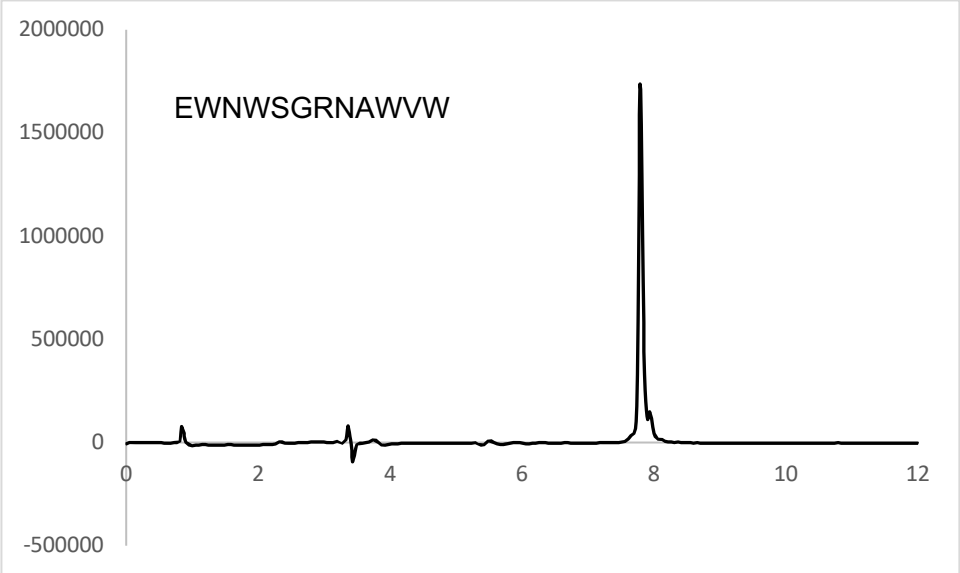
13mer_WW



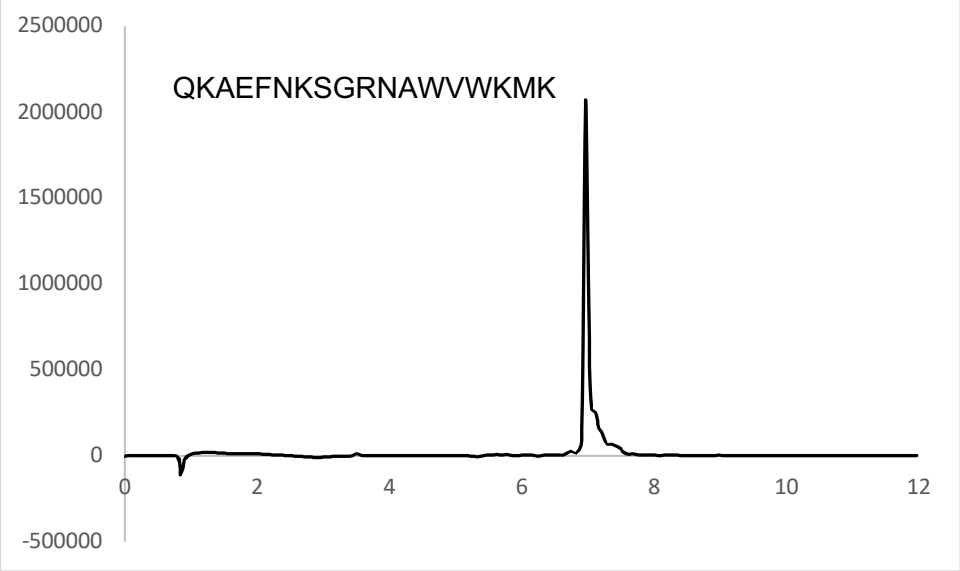
13mer_WKWF



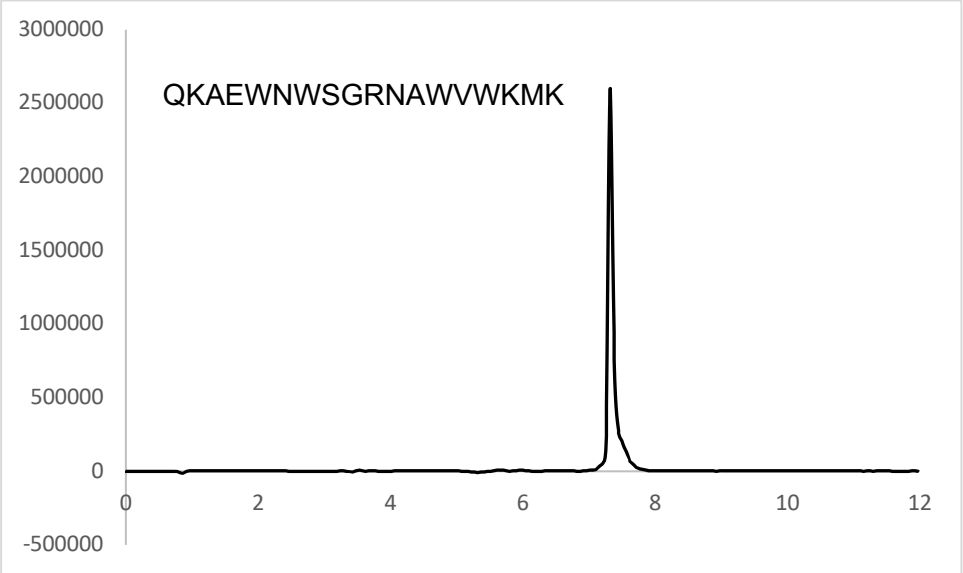
13mer_2WW



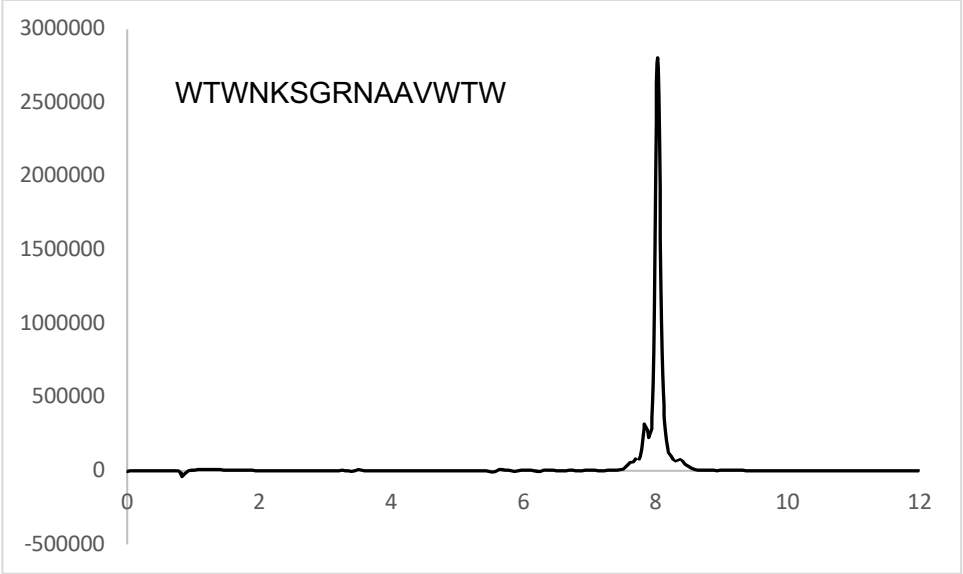
18mer_WKWF



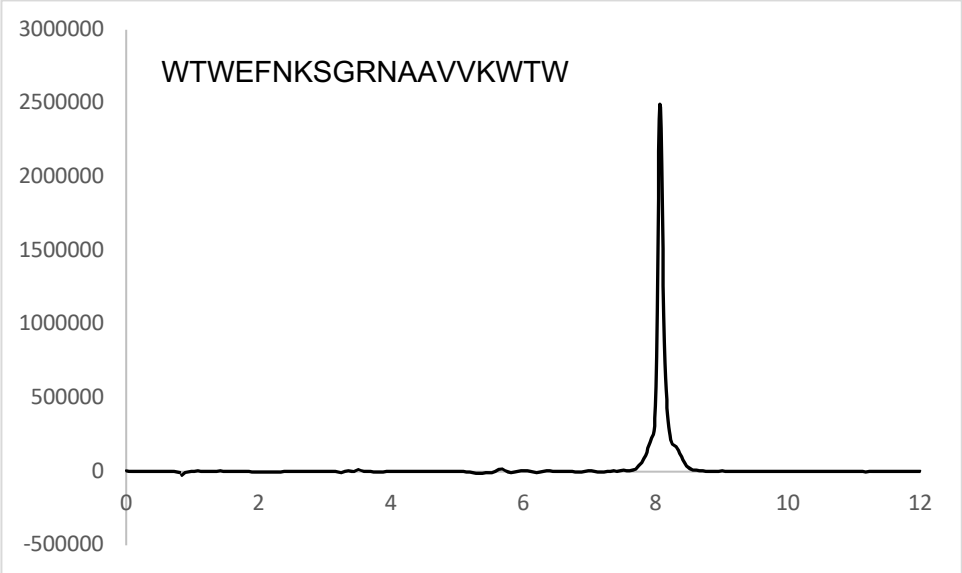
18mer_2WW



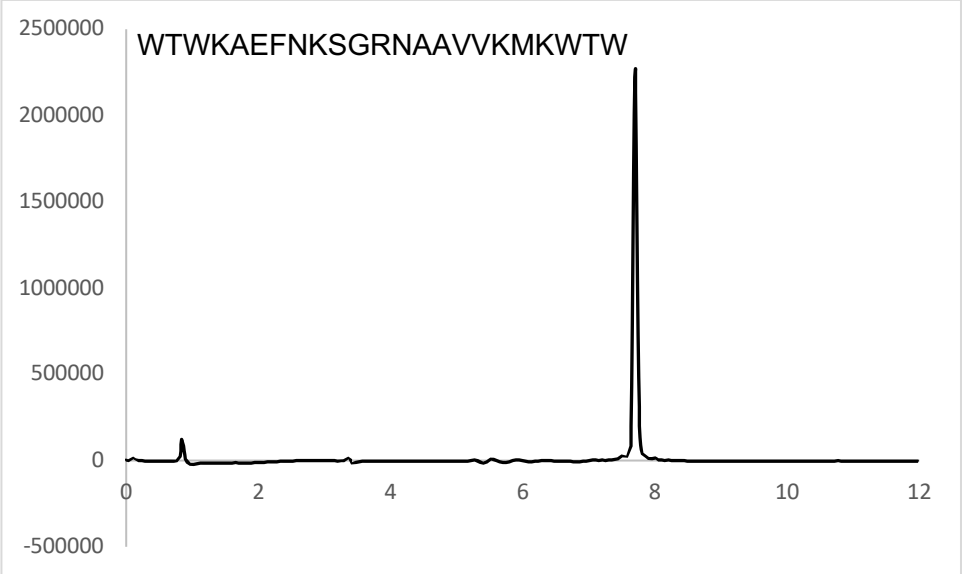
9mer_2WTW



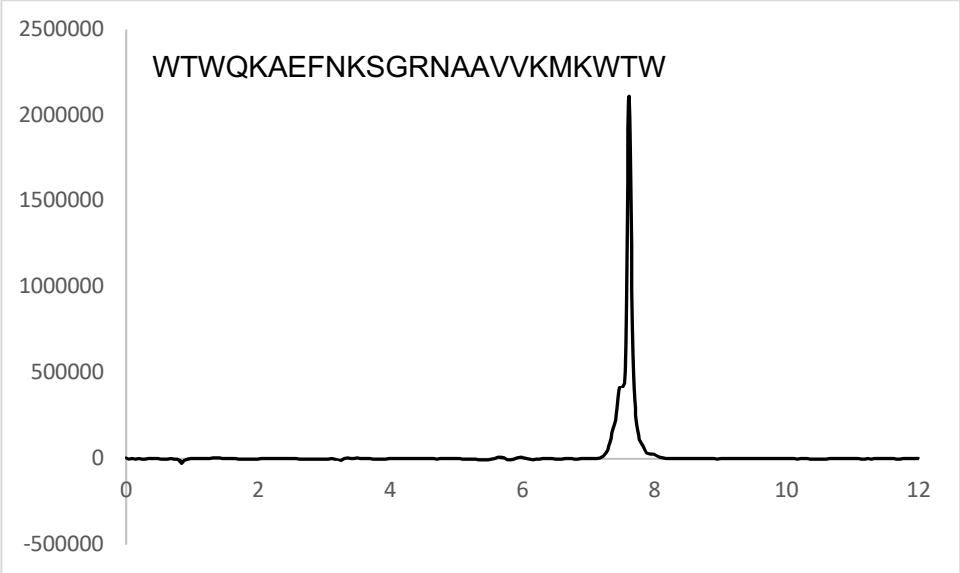
13mer_2WTW



17mer_2WTW

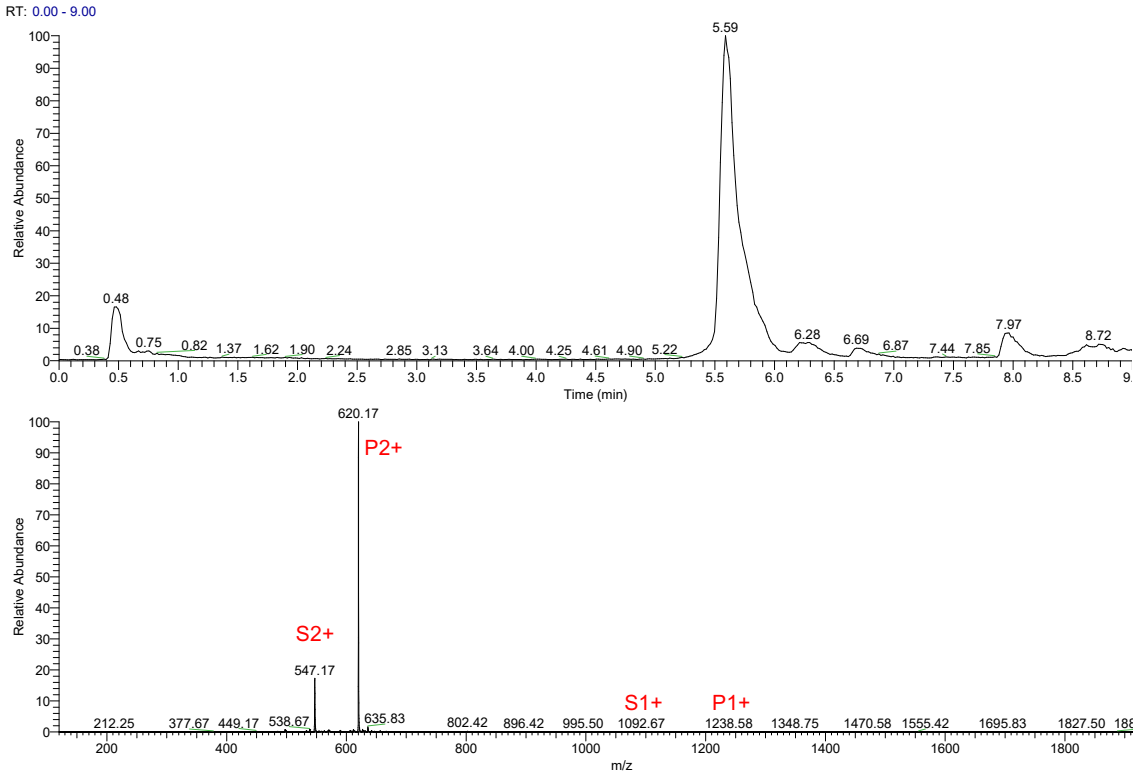


18mer_2WTW

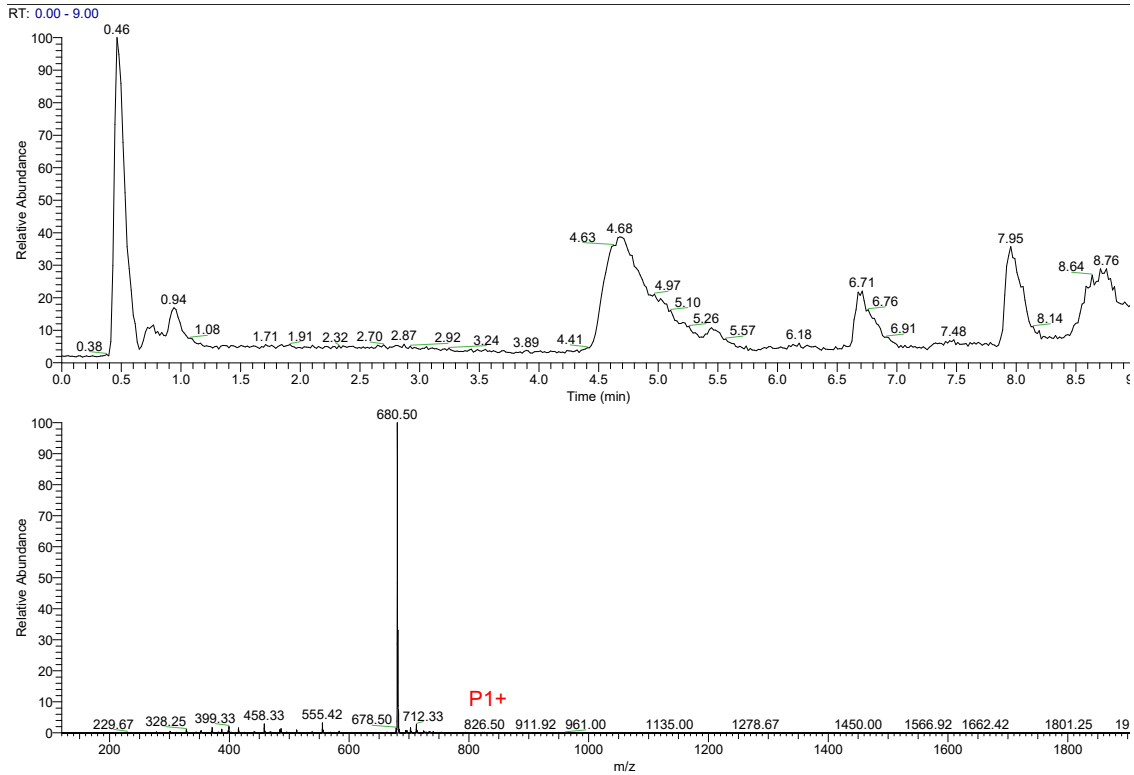

WTWQKAEFNKSGRNAAVVKMKWTW
2500000
2000000
1500000
1000000
500000
0
-500000
0
2
4
6
8
10
12

LCMS traces of *in vitro* EarP-peptide reaction mixtures:

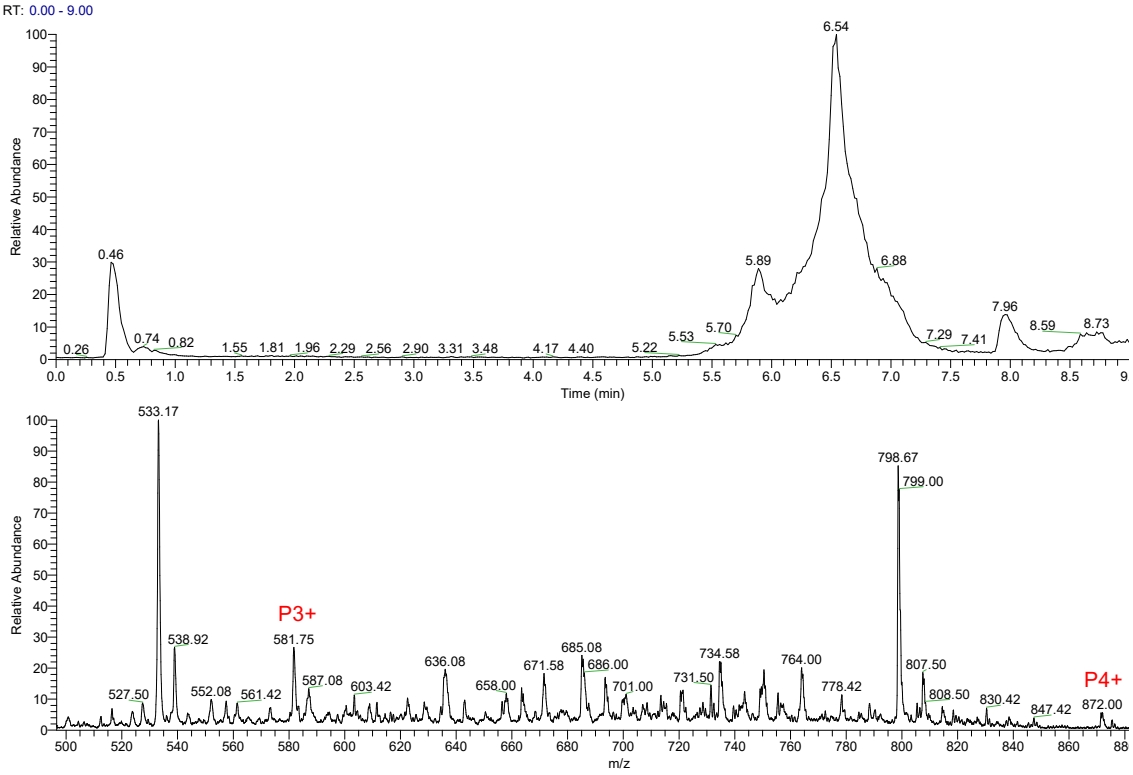
11mer_*Pa*



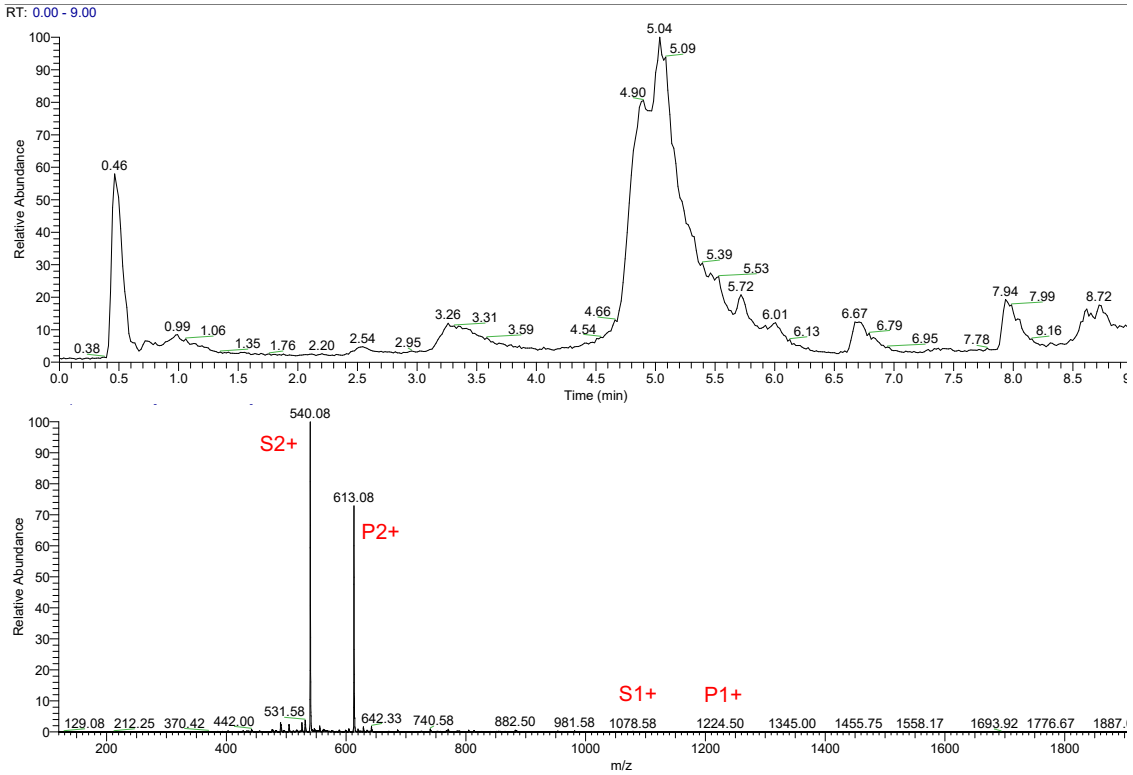
7mer_*Pa*



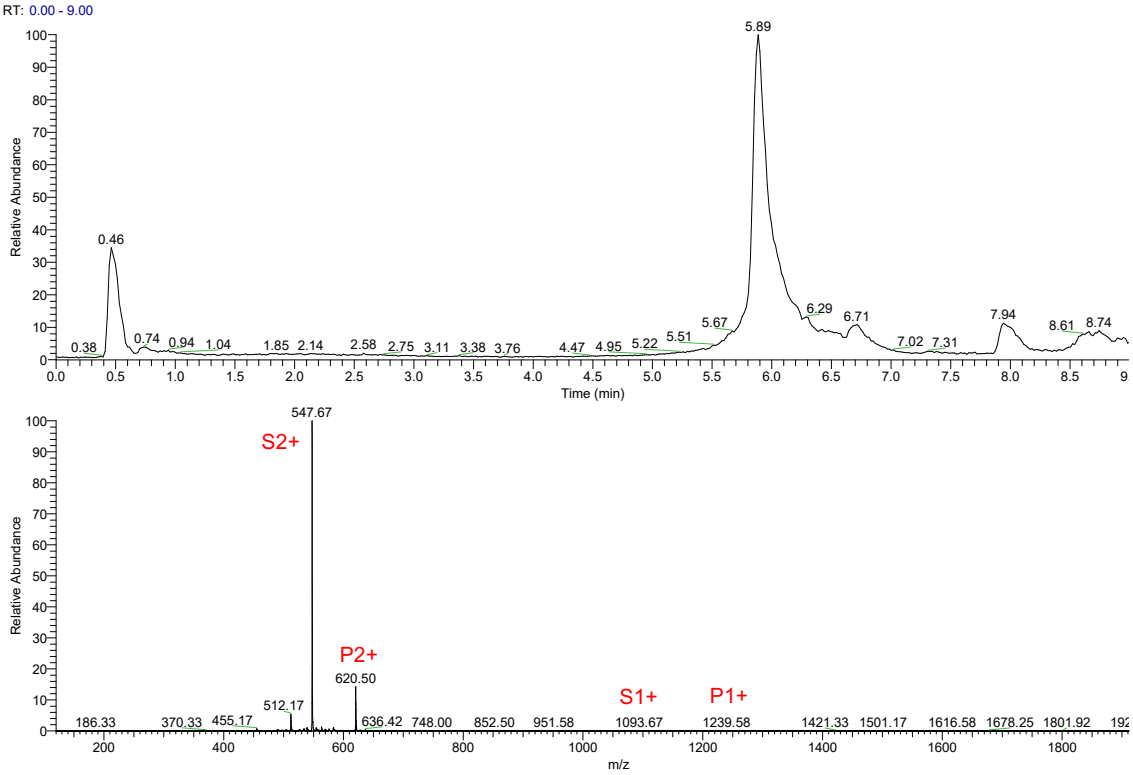
15mer_*Pa*



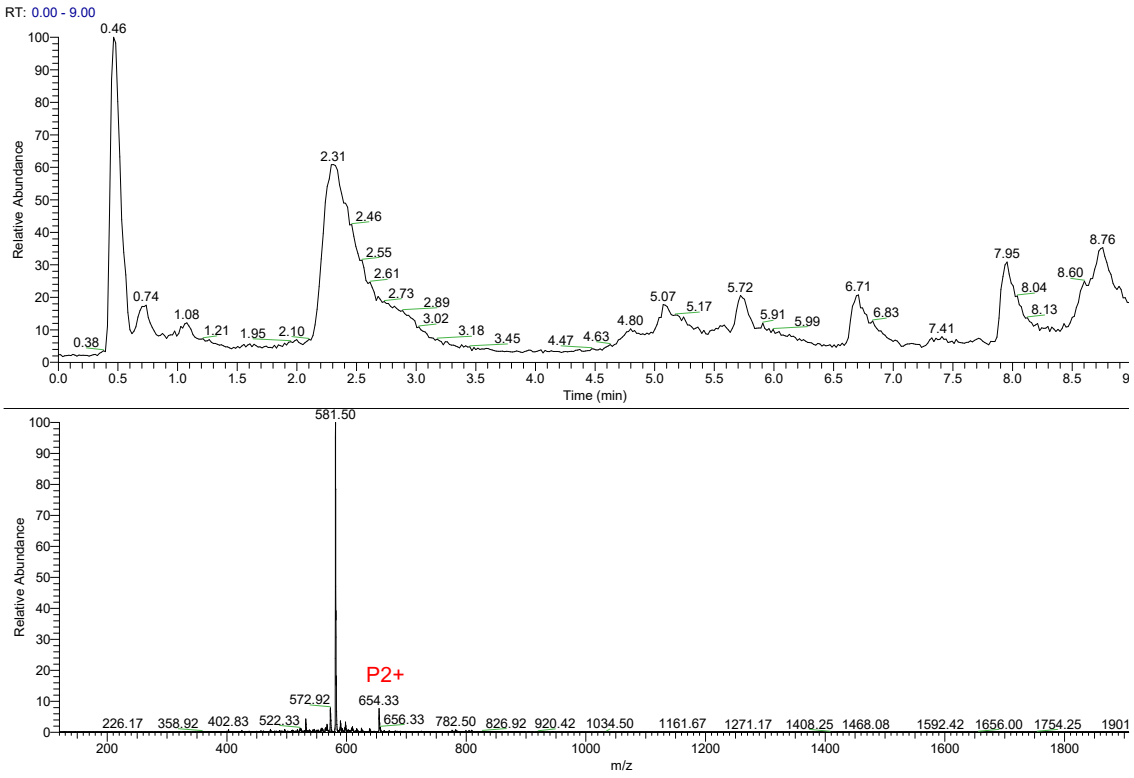
11mer_*Pa*_A34G



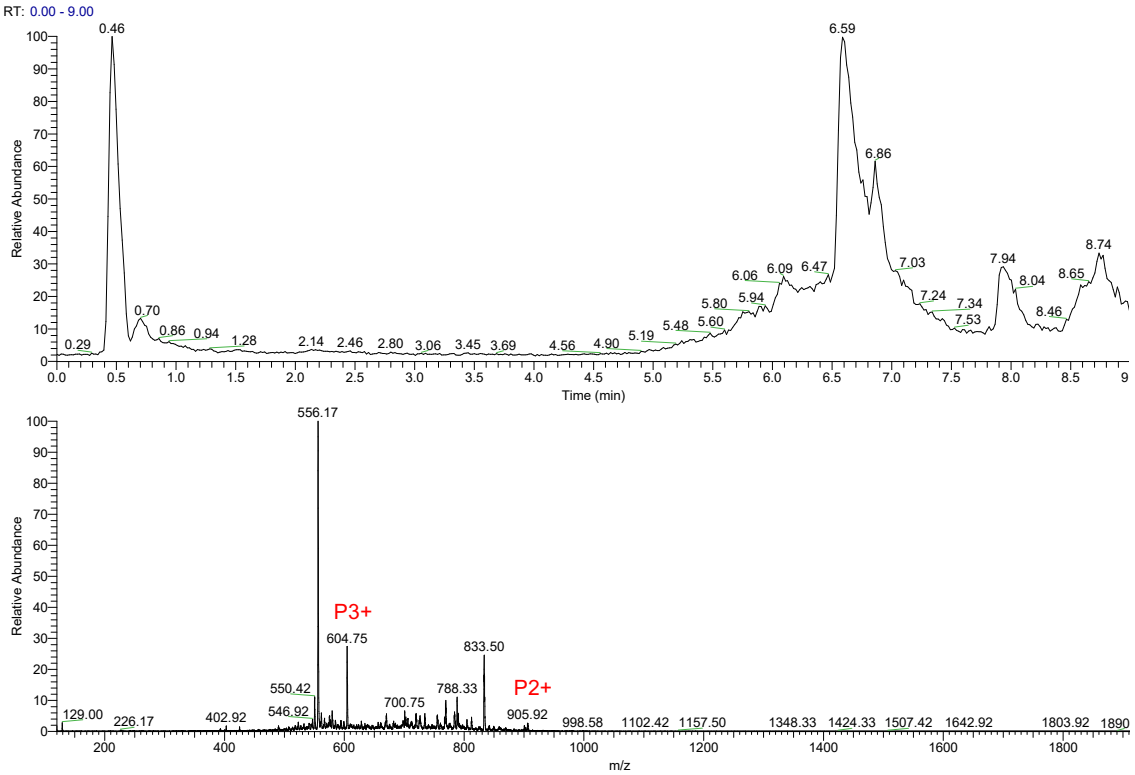
11mer_*Rso*



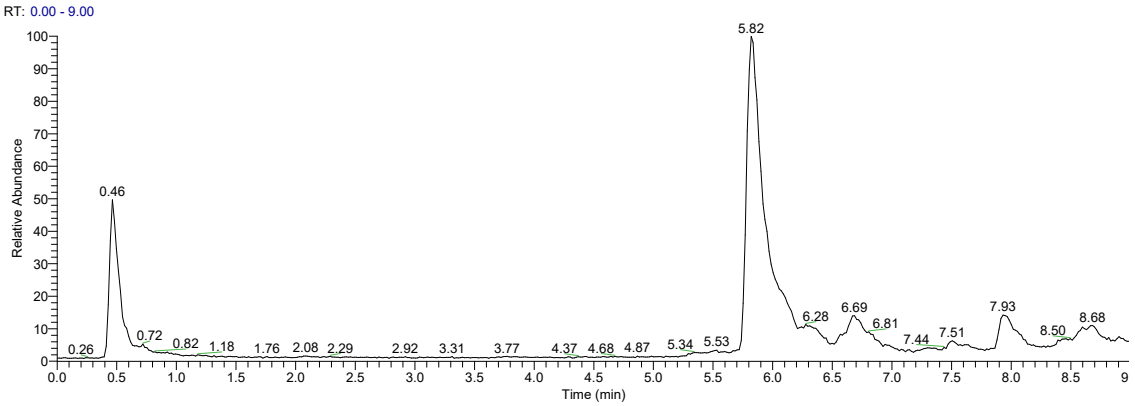
11mer_SS

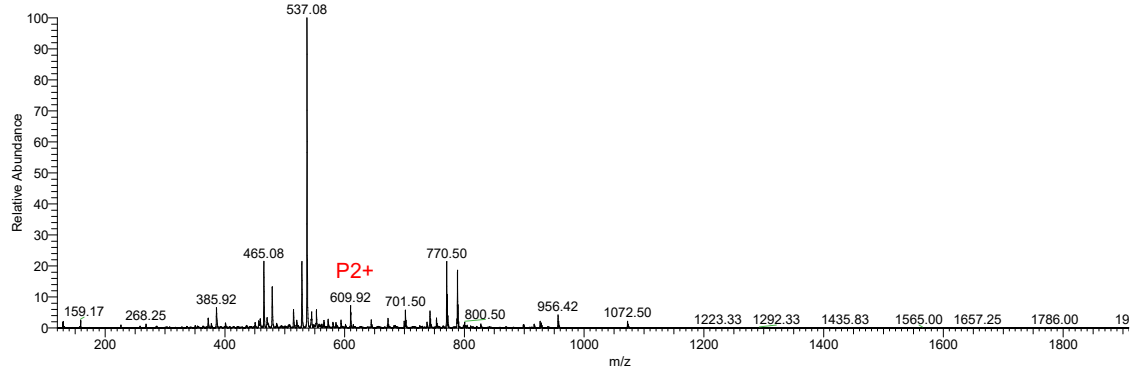


15mer_SS

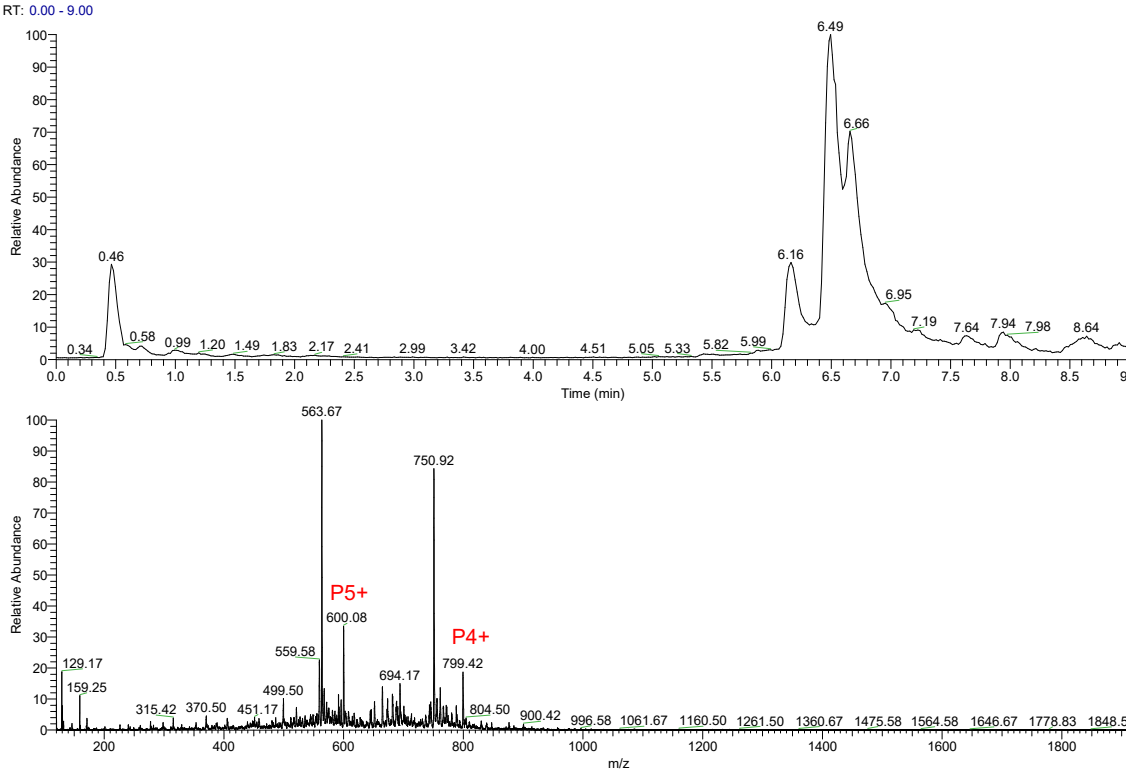


9mer_WK

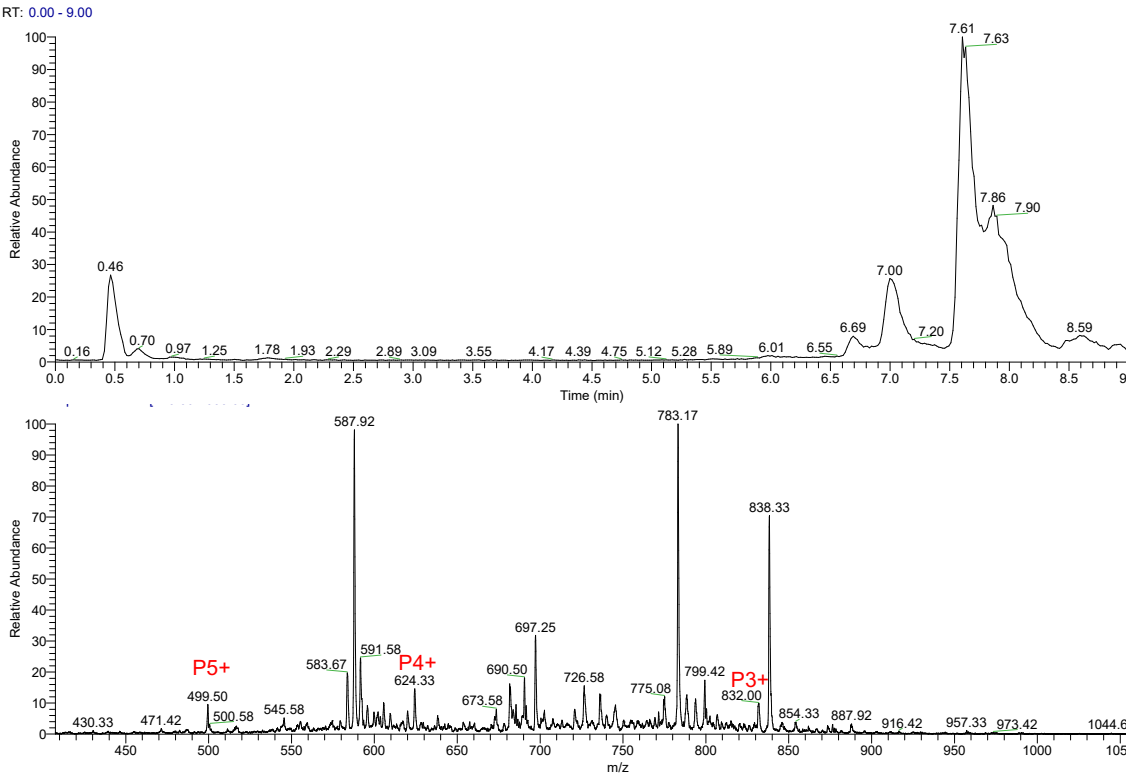




18mer_WKWF



18mer_WW



Overview of structural studies using NMR experiments

11mer_*Pa*:

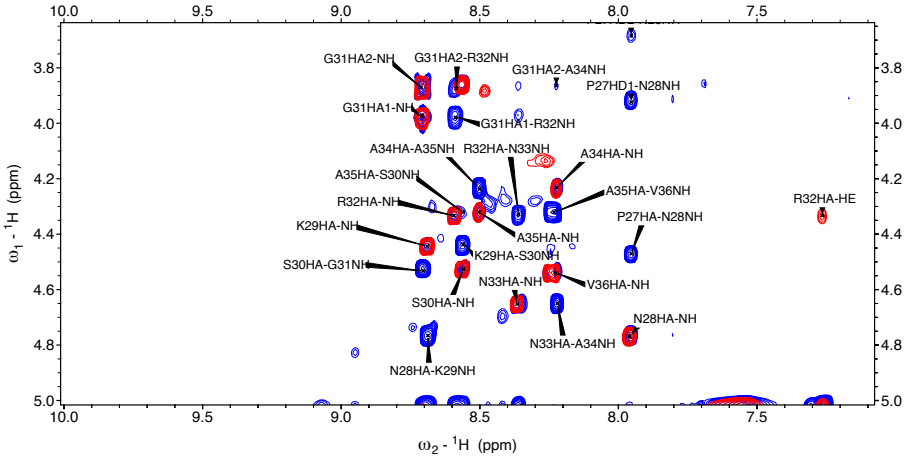
PNKSGRNAAVp[1]

$^{1}H/^{13}C$ chemical shifts of 11mer_*Pa* in H_2O/D_2O (278K)

Residue	NH	Hα	Hβ	Hγ	Hδ	Other
P27	-	4.469	2.211/2.066	2.047/1.909	3.904/3.680	
N28	7.958	4.766	2.837	-	7.799/7.166	
K29	8.683	4.442	1.884/1.637	1.411/1.361	1.667	Hε:2.998
S30	8.557	4.525	3.863	-	-	
G31	8.705	3.983/3.873	-	-	-	
R32	8.589	4.332	1.924/1.711	1.626/1.601	3.198	Hε:7.258
N33	8.361	4.232	2.875	-	7.685/7.056	
A34	8.224	4.650	1.354	-	-	
A35	8.504	4.321	1.354	-	-	
V36	8.226	4.535	2.102	0.936/0.927	-	
P37	-	4.757	2.302/1.821	2.098/2.008	3.807/3.627	

Residue	Cα	Cβ	Cγ	Cδ	Other
P27	63.45	32.24	26.30	50.32	-
N28	53.26	39.46	-	-	-
K29	56.15	33.59	24.75	29.19	Cε:42.19
S30	57.78	64.04	-	-	-
G31	45.88	-	-	-	-
R32	56.23	30.34	27.05	43.19	-
N33	53.18	39.18	-	-	-
A34	52.35	18.96	-	-	-
A35	52.66	19.40	-	-	-
V36	59.36	32.88	21.10/20.11	-	-
P37	61.35	30.58	27.48	51.16	-

NOESY: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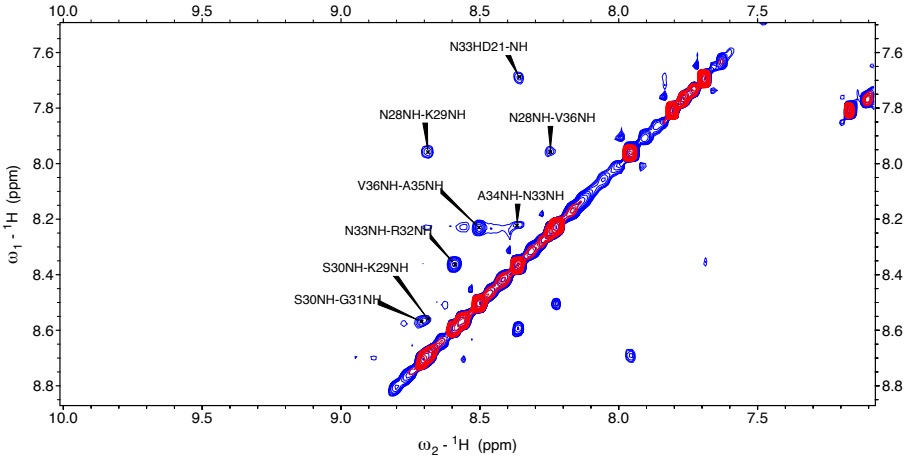
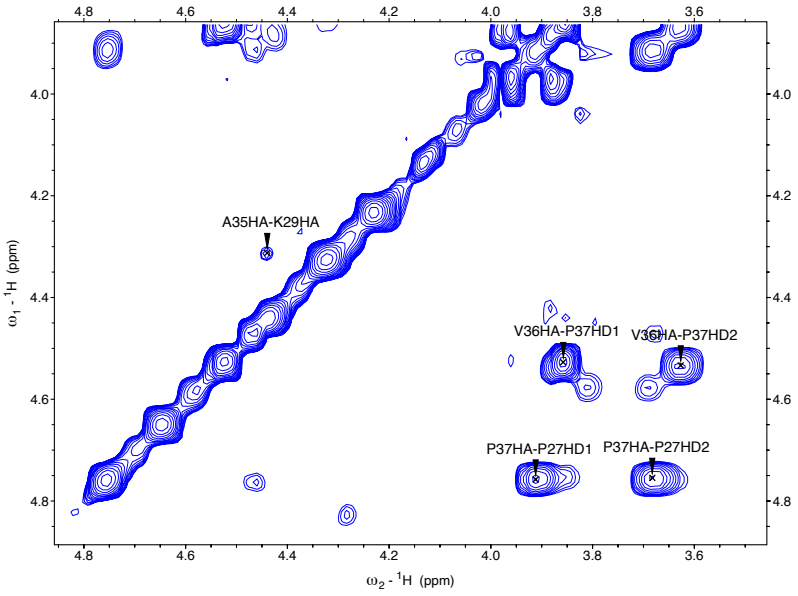
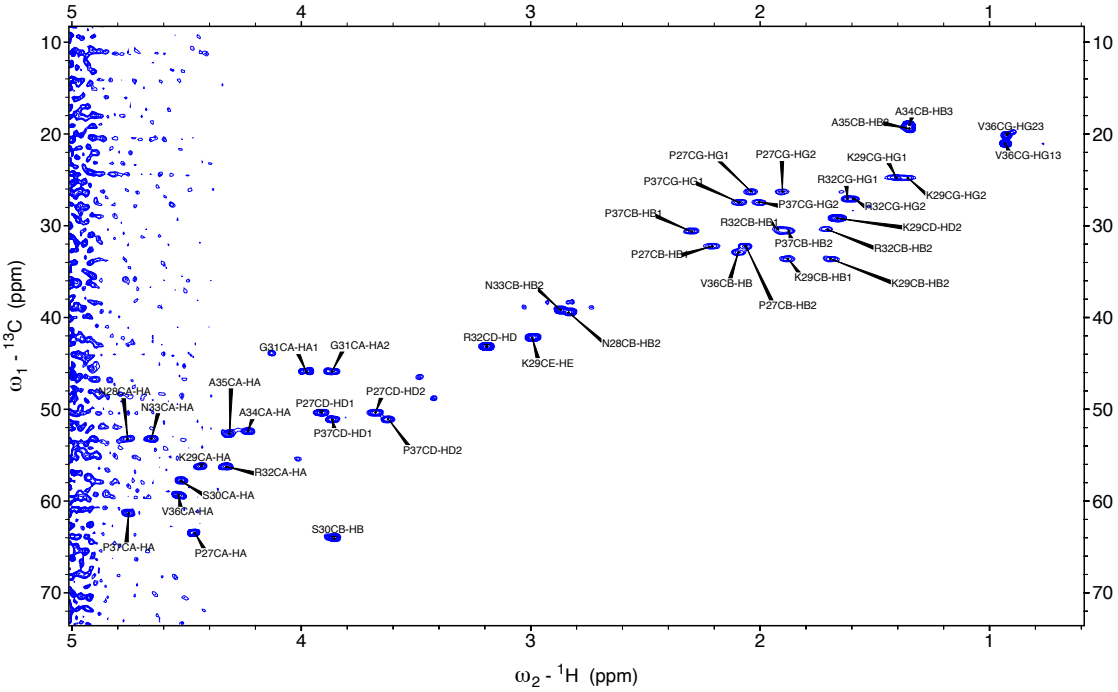
G31HA2-R32NH
G31HA2-A34NH
P27HD1-N28NH
G31HA1-NH
G31HA1-R32NH
A34HA-A35NH
R32HA-N33NH
A34HA-NH
A35HA-S30NH
A35HA-V36NH
R32HA-NH
R32HA-HE
K29HA-NH
P27HA-N28NH
A35HA-NH
K29HA-S30NH
S30HA-G31NH
N33HA-NH
V36HA-NH
S30HA-NH
N28HA-NH
N33HA-A34NH
N28HA-K29NH
ω1 - 1H (ppm)
ω2 - 1H (ppm)


N33HD21-NH
N28NH-K29NH
N28NH-V36NH
V36NH-A35NH
A34NH-N33NH
N33NH-R32NH
S30NH-K29NH
S30NH-G31NH
ω1 - 1H (ppm)
ω2 - 1H (ppm)


A35HA-K29HA
V36HA-P37HD1
V36HA-P37HD2
P37HA-P27HD1
P37HA-P27HD2
ω1 - 1H (ppm)
ω2 - 1H (ppm)

HSQC:


A34CB-HB3
A35CB-HB2
V36CG-HG23
V36CG-HG13
P27CG-HG1
P27CG-HG2
K29CG-HG1
P37CG-HG1
R32CG-HG1
K29CG-HG2
P37CG-HG2
R32CG-HG2
P37CB-HB1
R32CB-HB1
K29CD-HD2
P37CB-HB2
P27CB-HB1
R32CB-HB2
V36CB-HB
K29CB-HB1
K29CB-HB2
P27CB-HB2
N33CB-HB2
R32CD-HD
N28CB-HB2
K29CE-HE
G31CA-HA1
G31CA-HA2
A35CA-HA
N28CA-HA
N33CA-HA
A34CA-HA
P27CD-HD1
P27CD-HD2
P37CD-HD1
P37CD-HD2
K29CA-HA
R32CA-HA
S30CA-HA
V36CA-HA
S30CB-HB
P37CA-HA
P27CA-HA
ω_1 - ^{13}C (ppm)
ω_2 - ^{1}H (ppm)

11mer_*Pa*_A34G

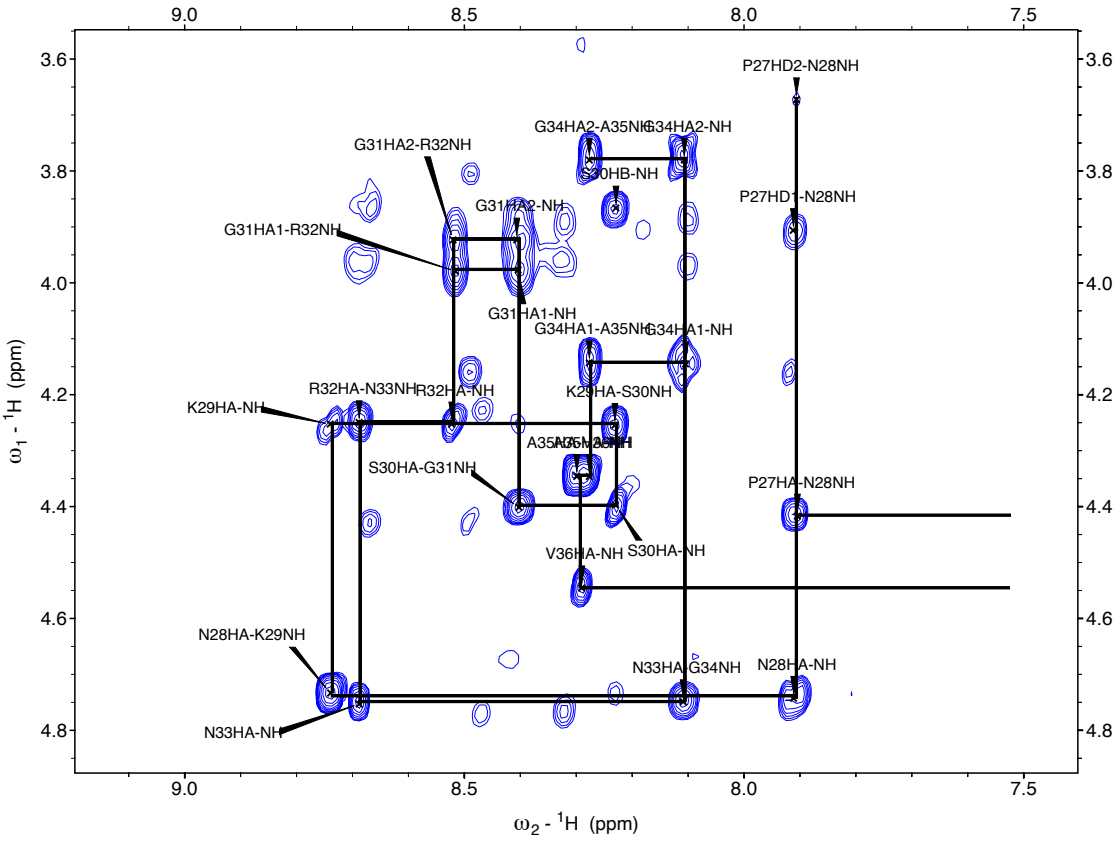
PNKSGRNGAVp

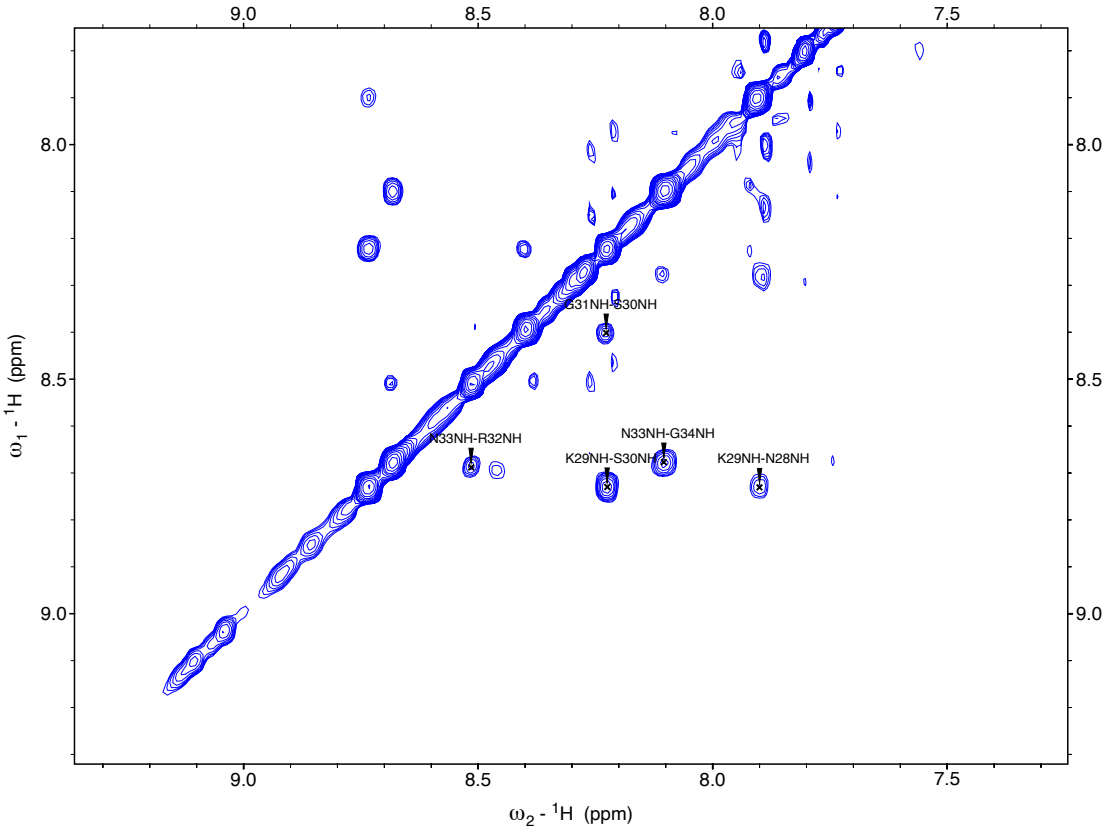
$^{1}H/^{13}C$ chemical shifts of 11mer_A34G_*Pa* in H_2O/D_2O (278K)

Residue	NH	Hα	Hβ	Hγ	Hδ	Other
P27	-	4.407	2.065/2.032	2.018/1.933	3.901/3.669	-
N28	7.906	4.726	2.989/2.779	-	7.800/7.103	-
K29	8.735	4.250	1.925/1.702	1.428	1.624	Hε:2.944
S30	8.223	4.388	3.863	-	-	-
G31	8.400	3.970/3.913	-	-	-	-
R32	8.518	4.241	1.846/1.716	1.595	3.176	Hε:7.242
N33	8.683	4.741	2.877/2.680	-	7.741/6.992	-
G34	8.103	4.135/3.770	-	-	-	-
A35	8.272	4.336	1.314	-	-	-
V36	8.289	4.537	2.058	0.906/0.886	-	-
P37	-	4.712	2.220/1.870	2.063/1.991	3.814/3.572	-

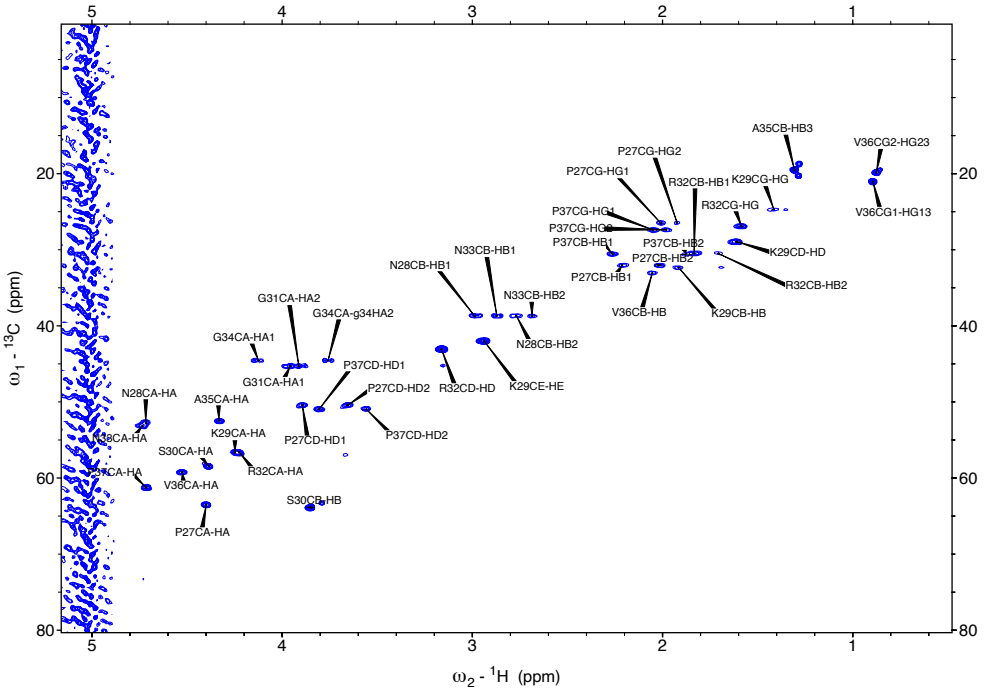
Residue	Cα	Cβ	Cγ	Cδ	Other
P27	63.59	32.03	26.59	50.46	-
N28	52.86	38.77	-	-	-
K29	56.71	32.39	24.74	28.95	Cε:42.03
S30	58.52	63.95	-	-	-
G31	45.34	-	-	-	-
R32	56.61	30.53	26.95	43.12	-
N33	53.22	38.73	-	-	-
G34	44.61	-	-	-	-
A35	52.45	19.57	-	-	-
V36	59.29	33.11	21.16/19.89	-	-
P37	61.28	30.62	27.41	51.00	-

NOESY:

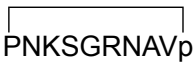

9.0
8.5
8.0
7.5
3.6
3.8
4.0
4.2
4.4
4.6
4.8
ω1 - 1H (ppm)
ω2 - 1H (ppm)
P27HD2-N28NH
G34HA2-A35NH
G34HA2-NH
G31HA2-R32NH
S30HB-NH
P27HD1-N28NH
G31HA2-NH
G31HA1-R32NH
G31HA1-NH
G34HA1-A35NH
G34HA1-NH
R32HA-N33NH
R32HA-NH
K29HA-S30NH
K29HA-NH
S30HA-G31NH
P27HA-N28NH
V36HA-NH
S30HA-NH
N28HA-K29NH
N33HA-G34NH
N28HA-NH
N33HA-NH


9.0
8.5
8.0
7.5
8.0
8.5
9.0
ω1 - 1H (ppm)
ω2 - 1H (ppm)
G31NH-S30NH
N33NH-R32NH
N33NH-G34NH
K29NH-S30NH
K29NH-N28NH

HSQC:


A35CB-HB3
V36CG2-HG23
P27CG-HG2
P27CG-HG1
R32CB-HB1
K29CG-HG
V36CG1-HG13
R32CG-HG
P37CG-HG1
P37CG-HG2
P37CB-HB1
P37CB-HB2
K29CD-HD
P27CB-HB2
N33CB-HB1
N28CB-HB1
P27CB-HB1
R32CB-HB2
N33CB-HB2
V36CB-HB
K29CB-HB
G31CA-HA2
G34CA-g34HA2
G34CA-HA1
N28CB-HB2
P37CD-HD1
G31CA-HA1
P27CD-HD2
R32CD-HD
K29CE-HE
N28CA-HA
A35CA-HA
K29CA-HA
P27CD-HD1
P37CD-HD2
S30CA-HA
R32CA-HA
V36CA-HA
S30CB-HB
P27CA-HA
ω_1 - ^{13}C (ppm)
ω_2 - ^{1}H (ppm)

11mer_-A34_*Pa*

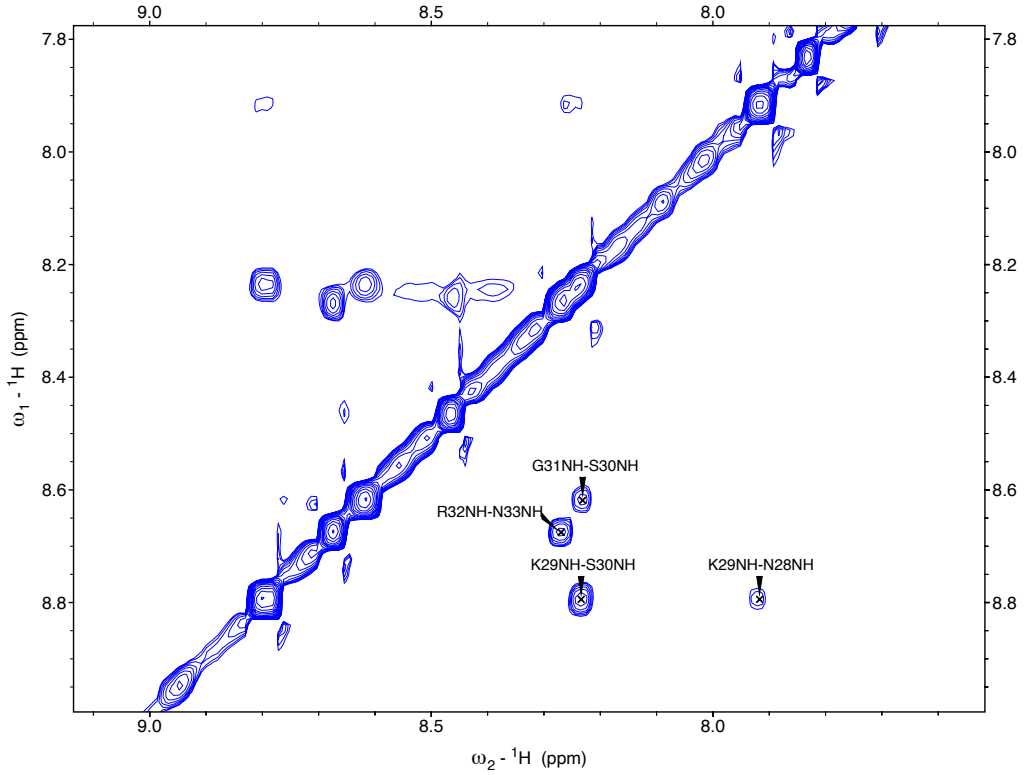


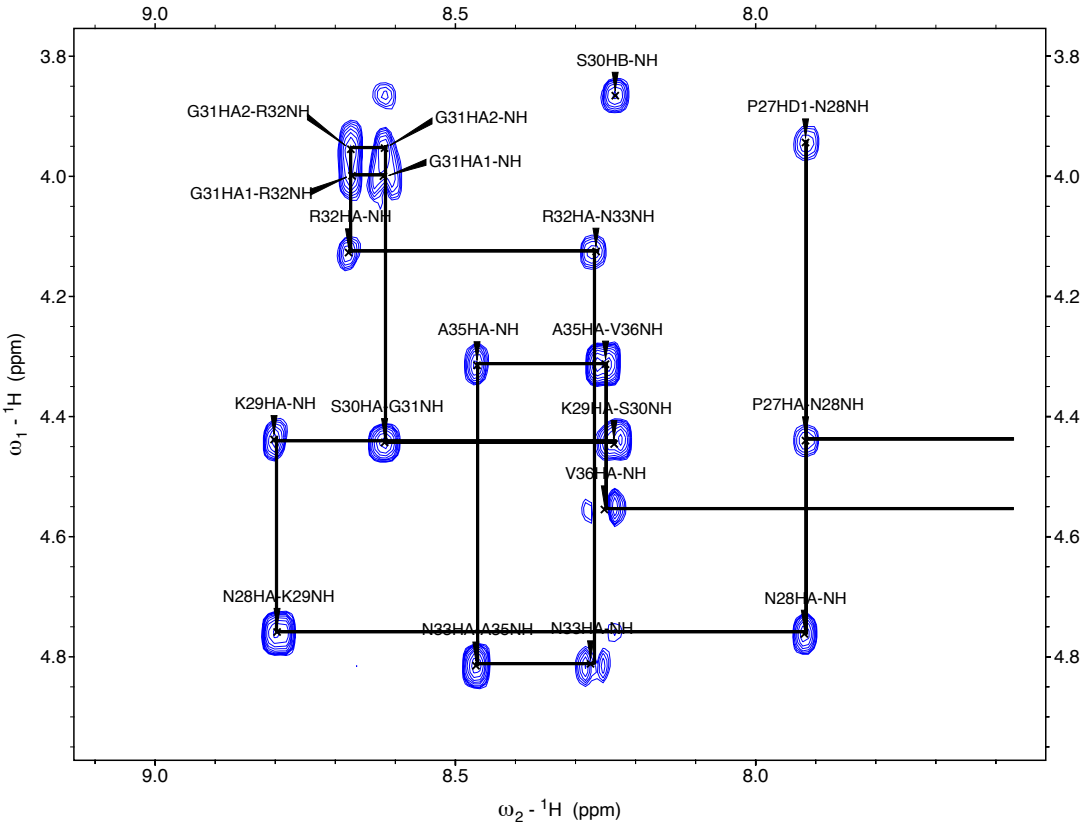
$^{1}H/^{13}C$ chemical shifts of 11mer-A34_*Pa* in H_2O/D_2O (278K)

Residue	NH	Hα	Hβ	Hγ	Hδ	Other
P27	-	4.442	2.243/2/076	2.052/1.950	3.947/3.697	-
N28	7.917	4.763	3.053/2.823	-	7.829/7.113	-
K29	8.799	4.438	1.976/1.697	1.404/1.343	1.630	Hε:2.964
S30	8.235	4.448	3.870	-	-	-
G31	8.617	3.999/3.953	-	-	-	-
R32	8.676	4.126	1.803	1.644	3.206	Hε:7.268
N33	8.269	4.814	2.766/2.675	-	7.722/7.018	-
-	-	-	-	-	-	-
A35	8.466	4.315	1.329	-	-	-
V36	8.250	4.555	2.090	0.940/0.921	-	-
P37	-	4.761	2.306/1.901	2.098/2.013	3.862/3.575	-

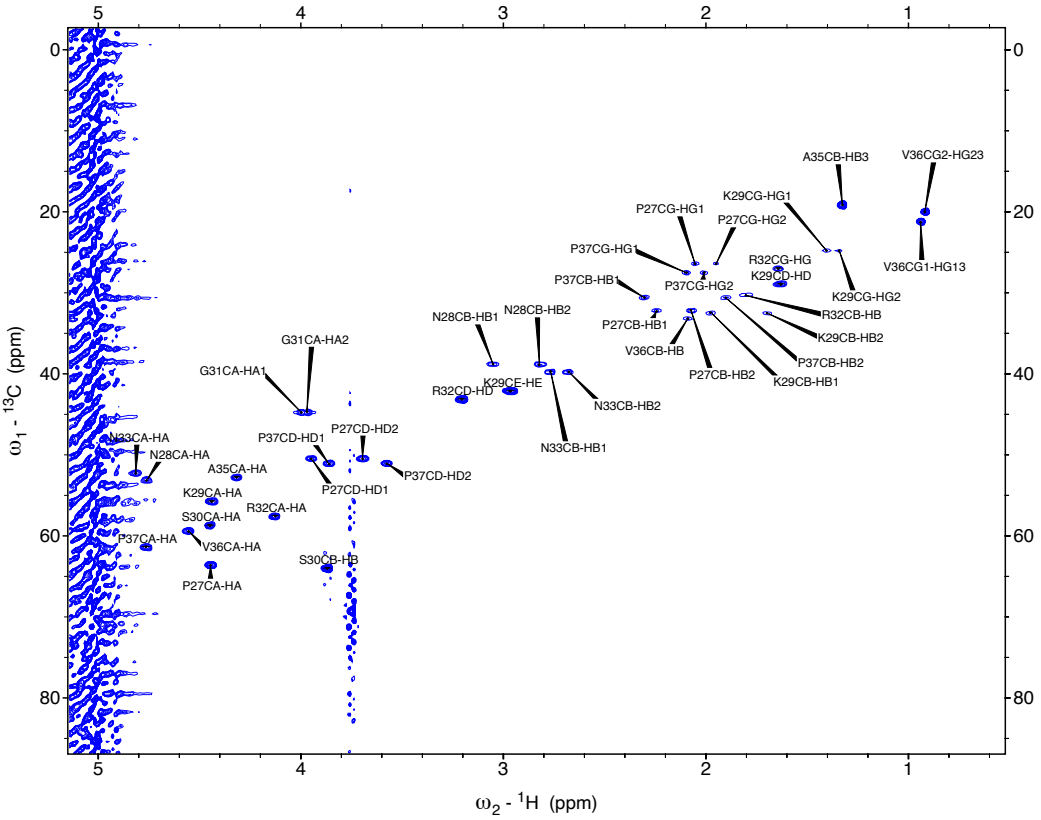
Residue	Cα	Cβ	Cγ	Cδ	Other
P27	63.62	32.16	26.35	50.48	-
N28	53.23	38.83	-	-	-
K29	55.78	32.46	24.83	28.95	Cε:42.13
S30	58.68	64.02	-	-	-
G31	44.78	-	-	-	-
R32	57.56	30.32	27.02	43.15	-
N33	52.32	39.84	-	-	-
-	-	-	-	-	-
A35	52.77	19.07	-	-	-
V36	59.44	33.23	21.31/19.99	-	-
P37	61.38	30.58	27.52	51.09	-

NOESY:


9.0
8.5
8.0
7.8
8.0
8.2
8.4
8.6
8.8
ω1 - 1H (ppm)
ω2 - 1H (ppm)
G31NH-S30NH
R32NH-N33NH
K29NH-S30NH
K29NH-N28NH


9.0
8.5
8.0
3.8
4.0
4.2
4.4
4.6
4.8
ω1 - 1H (ppm)
ω2 - 1H (ppm)
S30HB-NH
G31HA2-R32NH
G31HA2-NH
G31HA1-NH
G31HA1-R32NH
R32HA-NH
R32HA-N33NH
P27HD1-N28NH
A35HA-NH
A35HA-V36NH
K29HA-NH
S30HA-G31NH
K29HA-S30NH
P27HA-N28NH
V36HA-NH
N28HA-K29NH
N33HA-A35NH
N33HA-NH
N28HA-NH

HSQC:


A35CB-HB3
V36CG2-HG23
K29CG-HG1
P27CG-HG1
P27CG-HG2
V36CG1-HG13
R32CG-HG
P37CG-HG1
K29CD-HD
P37CG-HG2
P37CB-HB1
K29CG-HG2
R32CB-HB
N28CB-HB1
N28CB-HB2
P27CB-HB1
K29CB-HB2
V36CB-HB
P37CB-HB2
G31CA-HA2
P27CB-HB2
K29CB-HB1
G31CA-HA1
R32CD-HD
K29CE-HE
N33CB-HB2
P27CD-HD2
P37CD-HD1
N33CB-HB1
N33CA-HA
N28CA-HA
P37CD-HD2
A35CA-HA
P27CD-HD1
K29CA-HA
R32CA-HA
S30CA-HA
P37CA-HA
V36CA-HA
S30CB-HB
P27CA-HA
ω1 - 13C (ppm)
ω2 - 1H (ppm)

11mer_*Rso*

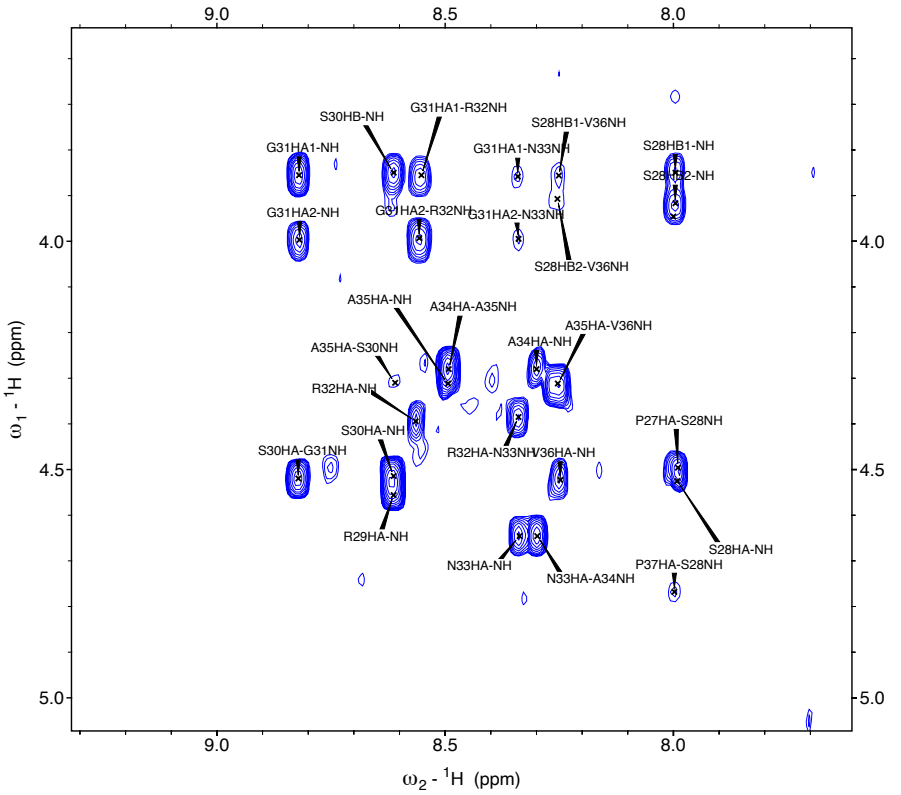
PSRSGRNAAVp

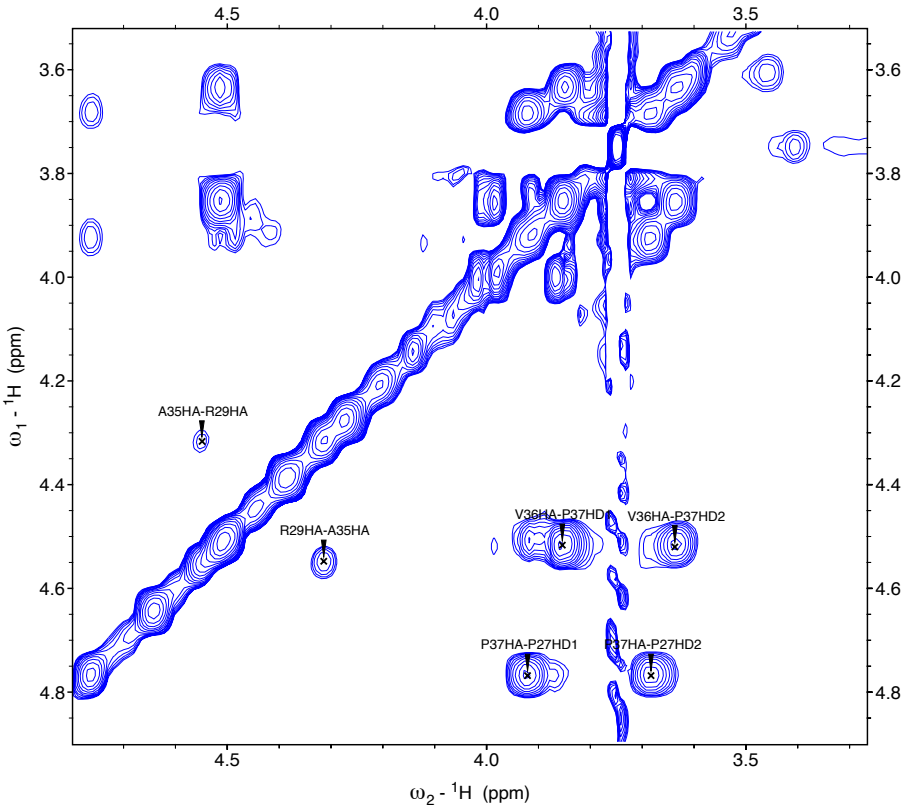
$^{1}H/^{13}C$ chemical shifts of 11mer_*Rso* in H_2O/D_2O (278K)

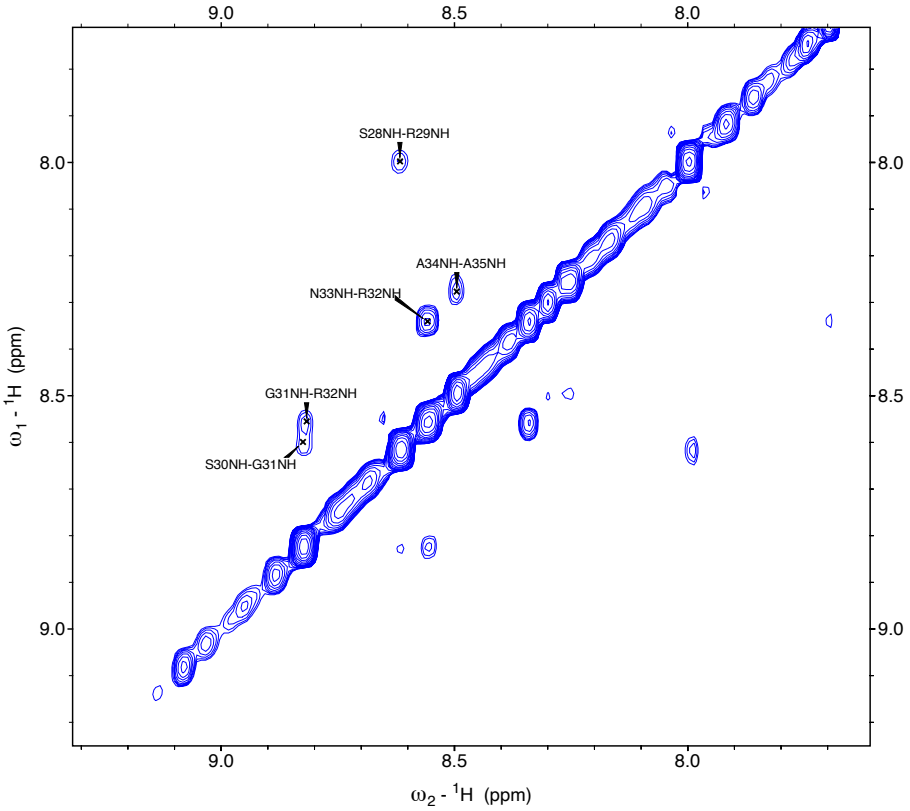
Residue	NH	Hα	Hβ	Hγ	Hδ	Other
P27	-	4.593	2.237/2.094	2.042/1.987	3.920/3.692	-
S28	7.993	4.523	3.923/3.849	-	-	-
R29	8.619	4.548	1.862/1.719	1.611	3.196	Hε:7.273
S30	8.614	4.521	3.851	-	-	-
G31	8.823	3.996/3.857	-	-	-	-
R32	8.555	4.392	1.955/1.710	1.611	3.196	Hε:7.261
N33	8.340	4.647	2.893	-	7.694/7.065	-
A34	8.301	4.280	1.349	-	-	-
A35	8.493	4.312	1.362	-	-	-
V36	8.249	4.519	2.050	0.925/0.919	-	-
P37	-	4.768	2.318/1.913	2.094/2.018	3.859/3.643	-

Residue	Cα	Cβ	Cγ	Cδ	Other
P27	63.53	32.27	26.56	50.37	-
S28	57.64	63.92	-	-	-
R29	55.79	31.60	27.09	43.28	-
S30	58.11	63.92	-	-	-
G31	45.83	-	-	-	-
R32	55.95	30.39	27.09	43.28	-
N33	53.22	39.12	-	-	-
A34	52.32	19.23	-	-	-
A35	52.74	19.23	-	-	-
V36	59.44	32.97	21.09/20.25	-	-
P37	61.42	30.60	27.38	51.10	-

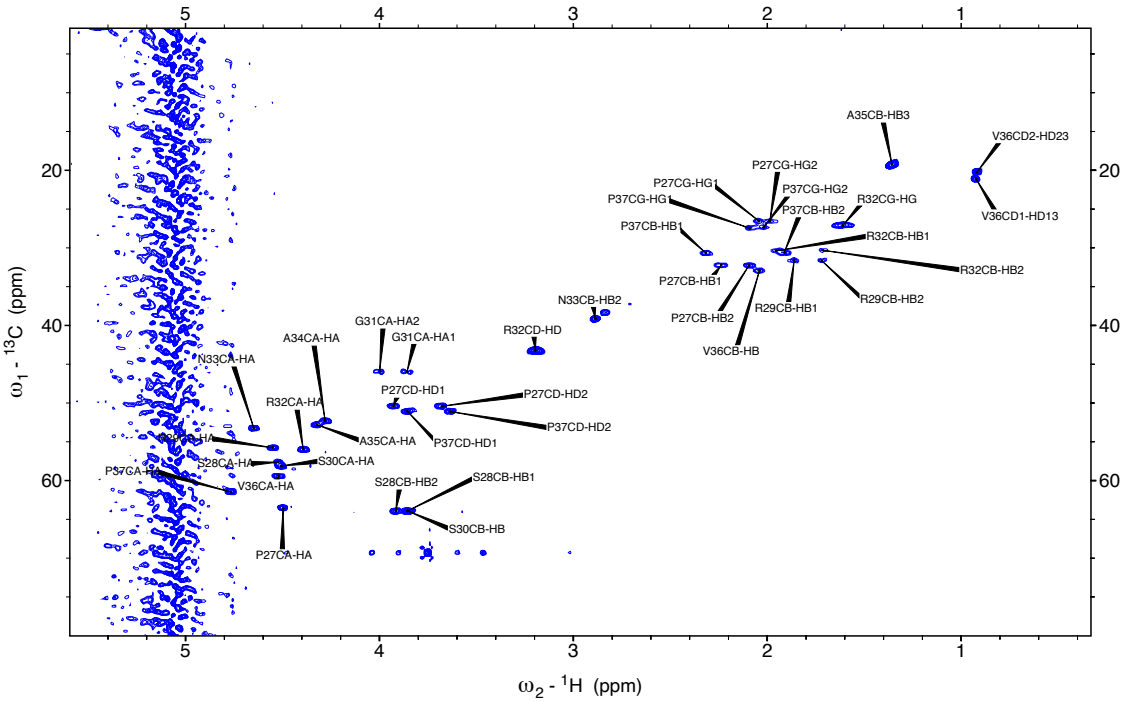
NOESY:


9.0
8.5
8.0
4.0
4.5
5.0
ω1 - 1H (ppm)
ω2 - 1H (ppm)
S30HB-NH
G31HA1-R32NH
G31HA1-NH
S28HB1-V36NH
G31HA1-N33NH
S28HB1-NH
S28HB2-NH
G31HA2-NH
G31HA2-R32NH
G31HA2-N33NH
S28HB2-V36NH
A35HA-NH
A34HA-A35NH
A35HA-V36NH
A35HA-S30NH
A34HA-NH
R32HA-NH
P27HA-S28NH
S30HA-NH
S30HA-G31NH
R32HA-N33NH
V36HA-NH
R29HA-NH
S28HA-NH
N33HA-NH
N33HA-A34NH
P37HA-S28NH


4.5
4.0
3.5
3.6
3.8
4.0
4.2
4.4
4.6
4.8
ω1 - 1H (ppm)
ω2 - 1H (ppm)
A35HA-R29HA
R29HA-A35HA
V36HA-P37HD1
V36HA-P37HD2
P37HA-P27HD1
P37HA-P27HD2


S28NH-R29NH
A34NH-A35NH
N33NH-R32NH
G31NH-R32NH
S30NH-G31NH
9.0
8.5
8.0
ω1 - 1H (ppm)
ω2 - 1H (ppm)

HSQC:


A35CB-HB3
V36CD2-HD23
V36CD1-HD13
P27CG-HG2
P27CG-HG1
P37CG-HG2
P37CG-HG1
P37CB-HB2
R32CG-HG
P37CB-HB1
R32CB-HB1
R32CB-HB2
P27CB-HB1
R29CB-HB2
N33CB-HB2
P27CB-HB2
R29CB-HB1
V36CB-HB
G31CA-HA2
A34CA-HA
G31CA-HA1
R32CD-HD
N33CA-HA
P27CD-HD1
P27CD-HD2
R32CA-HA
P37CD-HD2
A35CA-HA
P37CD-HD1
S30CA-HA
S28CA-HA
V36CA-HA
S28CB-HB2
S28CB-HB1
S30CB-HB
P27CA-HA
ω1 - 13C (ppm)
ω2 - 1H (ppm)

11mer_*Nm*

PIKGGRSAAKp

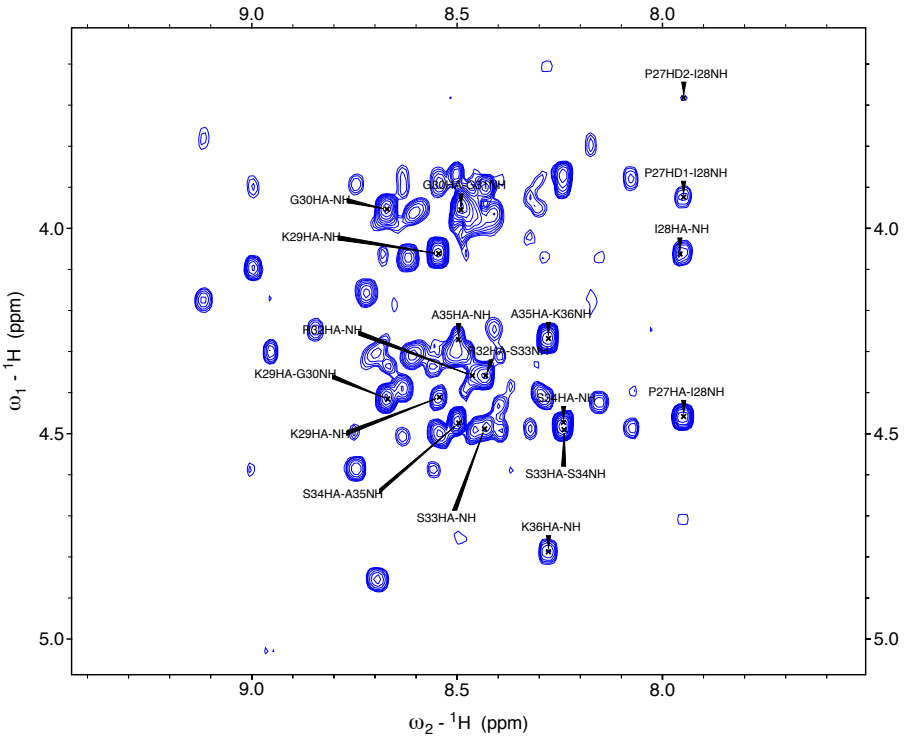
$^{1}H/^{13}C$ chemical shifts of 11mer_*Nm* in H_2O/D_2O (278K)

Residue	NH	Hα	Hβ	Hγ	Hδ	Other
P27	-	4.450	2.244/2.036	1.994	3.919/3.678	-
I28	7.948	4.062	1.973	1.534	0.8619	Hγ2:0.878
K29	8.543	4.411	1.839/1.729	1.393	1.668	Hε:2.961
G30	8.671	3.955	-	-	-	-
G31	8.491	3.935	-	-	-	-
R32	8.463	4.361	1.947/1.797	1.647	3.210	Hε:7.247
S33	8.434	4.491	3.888	-	-	-
S34	8.239	4.474	3.864	-	-	-
A35	8.495	4.273	1.398	-	-	-
K36	8.277	4.788	1.773/1.671	1.414	1.655	Hε:2.961
P37	-	4.705	2.299/1.931	2.074	3.730/3.596	-

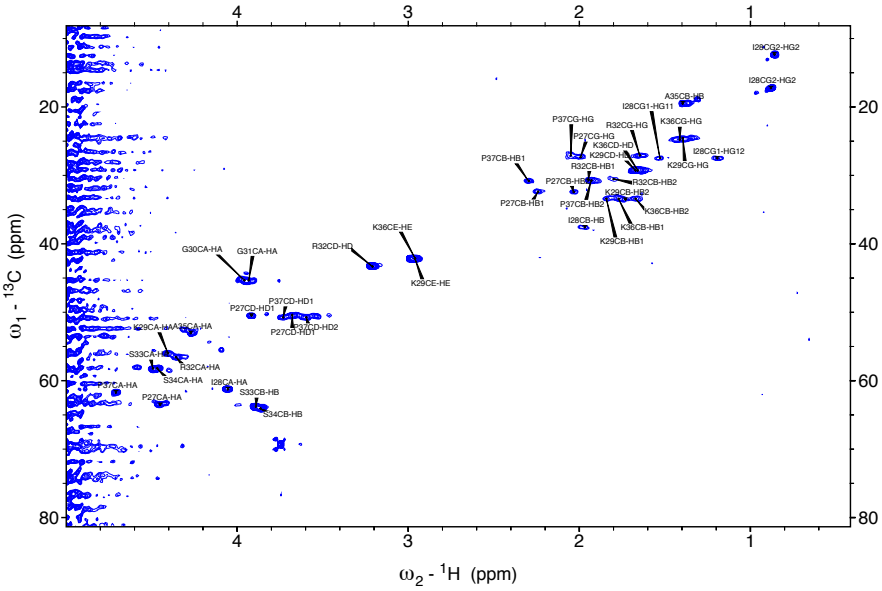
Residue	Cα	Cβ	Cγ	Cδ	Other
P27	63.51	32.52	27.33	50.57	-
I28	61.28	37.57	27.53	12.36	Cγ2:17.28
K29	56.09	33.44	24.57	29.36	Cε:42.17
G30	45.32	-	-	-	-
G31	45.32	-	-	-	-
R32	56.55	30.68	27.20	43.29	-
S33	58.32	63.77	-	-	-
S34	58.26	64.10	-	-	-
A35	53.00	19.51	-	-	-
K36	*	33.44	24.83	29.23	Cε:42.17
P37	61.74	30.87	27.13	50.71	-

*Overlap with H_2O

NOESY:


P27HD2-I28NH
P27HD1-I28NH
G30HA-NH
K29HA-NH
I28HA-NH
A35HA-NH
A35HA-K36NH
K29HA-G30NH
P27HA-I28NH
K29HA-NH
S33HA-S34NH
S34HA-A35NH
S33HA-NH
K36HA-NH
9.0
8.5
8.0
4.0
4.5
5.0
ω2 - 1H (ppm)
ω1 - 1H (ppm)

HSQC:


I28CG2-HG2
A35CB-HB
I28CG1-HG11
P37CG-HG
R32CG-HG
K36CG-HG
P27CG-HG
K36CD-HD
I28CG1-HG12
K29CD-HD
K29CG-HG
R32CB-HB1
P37CB-HB1
P27CB-HB1
R32CB-HB2
P27CB-HB2
K29CB-HB2
K36CB-HB2
I28CB-HB
K38CB-HB1
K29CB-HB1
K36CE-HE
R32CD-HD
K29CE-HE
G30CA-HA
G31CA-HA
P37CD-HD1
P27CD-HD1
P27CD-HD2
S33CA-HA
R32CA-HA
S34CA-HA
I28CA-HA
P37CA-HA
S33CB-HB
P27CA-HA
S34CB-HB
4
3
2
1
20
40
60
80
ω2 - 1H (ppm)
ω1 - 13C (ppm)

8mer linear peptide

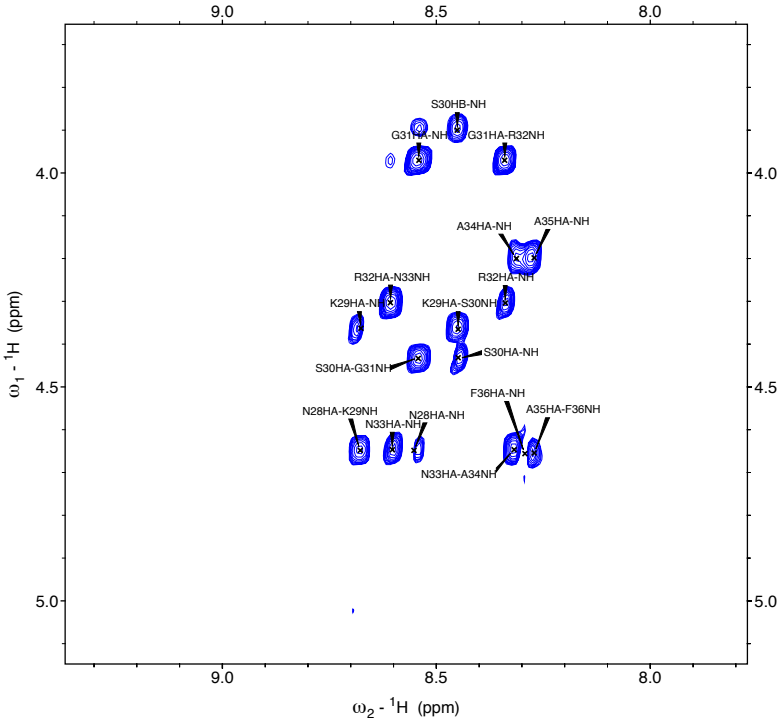
NKSGRNAA

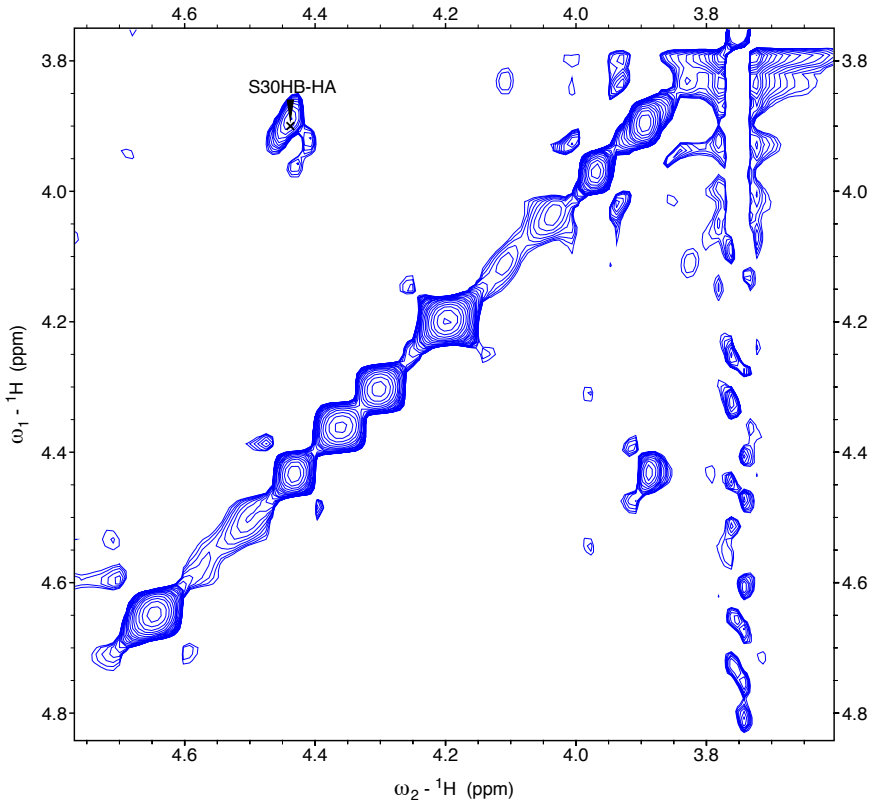
$^{1}H/^{13}C$ chemical shifts of EF-P Linear peptide in H_2O/D_2O (278K)

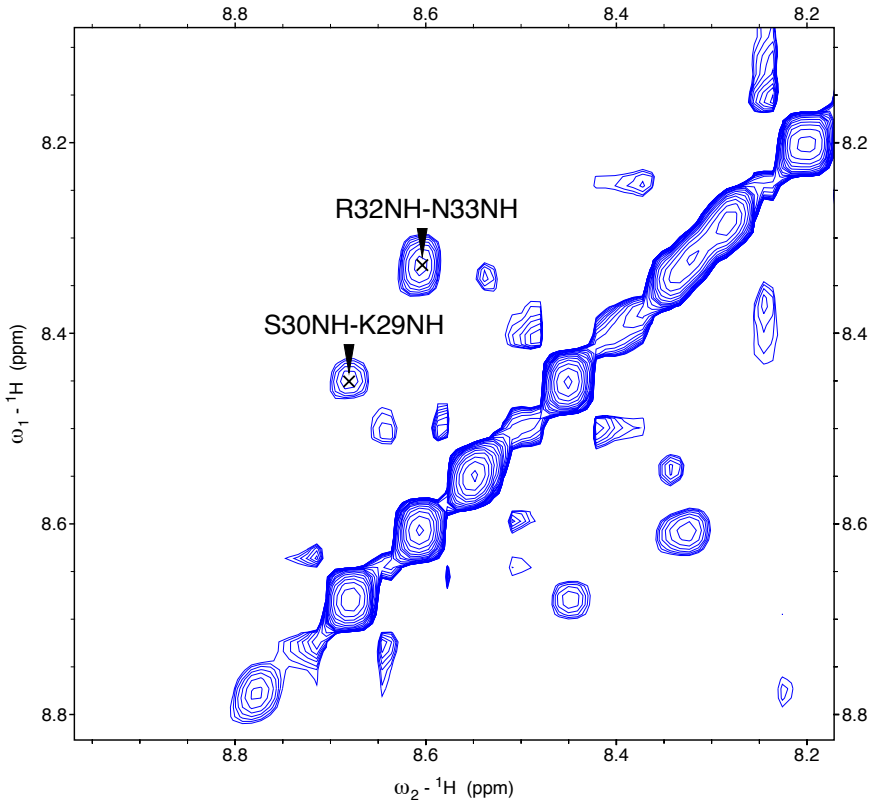
Residue	NH	Hα	Hβ	Hγ	Hδ	Other
N28	8.552	4.648	2.783	-	7.752/7.063	Ac: 2.019
K29	8.678	4.364	1.893/1.763	1.454/1.413	1.674	Hε:2.984
S30	8.447	4.431	3.893	-	-	-
G31	8.542	3.972	-	-	-	-
R32	8.333	4.301	1.831/1.745	1.595	3.166	Hε:7.232
N33	8.603	4.645	2.842/2.741	-	7.752/7.063	-
A34	8.316	4.202	1.327	-	-	-
A35	8.281	4.202	1.264	-	-	-
F36	8.295	4.659	3.329/3.153	-	7.485	Hε:8.209; NH_2:7.608/7.267

Residue	Cα	Cβ	Cγ	Cδ	Other
N28	53.49	38.91	-	-	Ac: 24.39
K29	56.56	32.77	24.81	29.09	Cε:42.1
S30	58.61	63.92	-	-	-
G31	45.44	-	-	-	-
R32	56.30	30.72	27.04	43.21	-
N33	53.47	39.93	-	-	-
A34	52.71	19.08	-	-	-
A35	52.71	18.95	-	-	-
F36	56.84	39.41	-	132.9	Cε:126.5

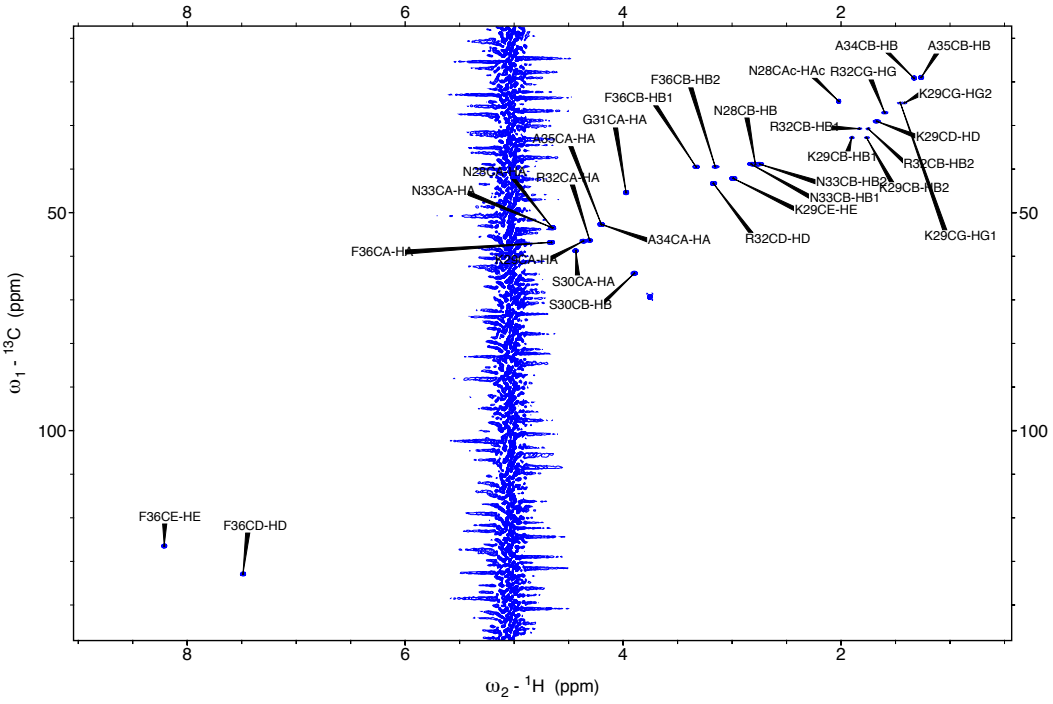
NOESY:


S30HB-NH
G31HA-NH
G31HA-R32NH
A34HA-NH
A35HA-NH
R32HA-N33NH
R32HA-NH
K29HA-NH
K29HA-S30NH
S30HA-NH
S30HA-G31NH
F36HA-NH
N28HA-K29NH
N28HA-NH
A35HA-F36NH
N33HA-NH
N33HA-A34NH
9.0
8.5
8.0
4.0
4.5
5.0
ω1 - 1H (ppm)
ω2 - 1H (ppm)


S30HB-HA
4.6
4.4
4.2
4.0
3.8
4.8
ω1 - 1H (ppm)
ω2 - 1H (ppm)

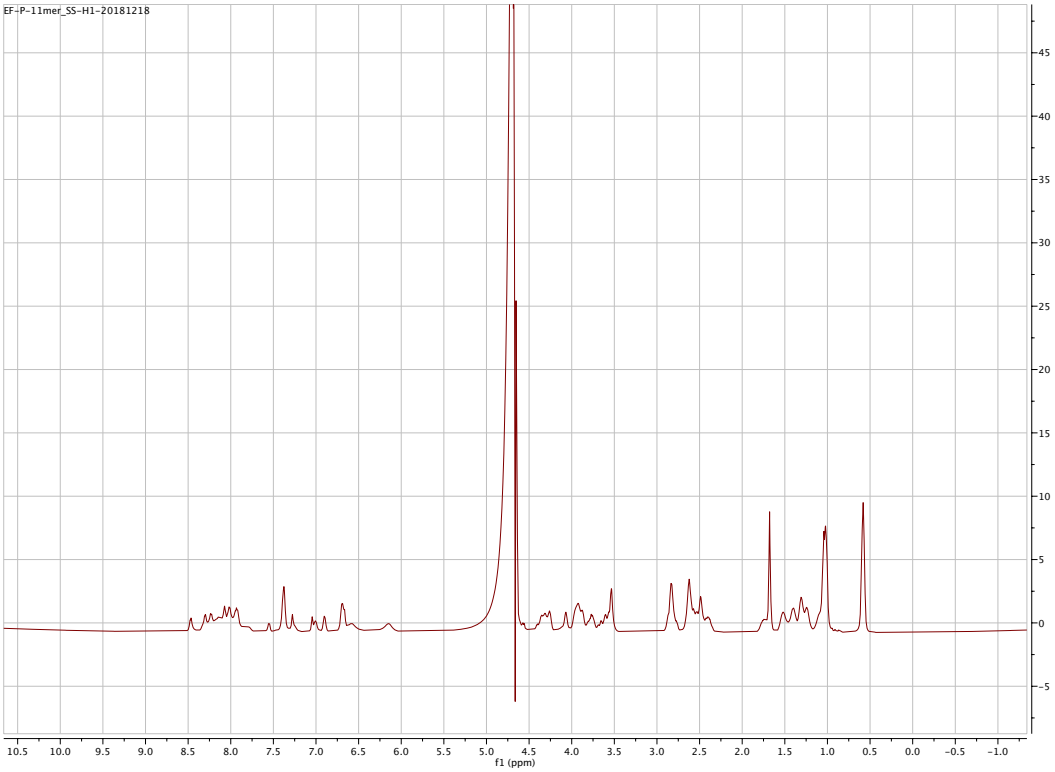

R32NH-N33NH
S30NH-K29NH
8.8
8.6
8.4
8.2
ω1 - 1H (ppm)
ω2 - 1H (ppm)

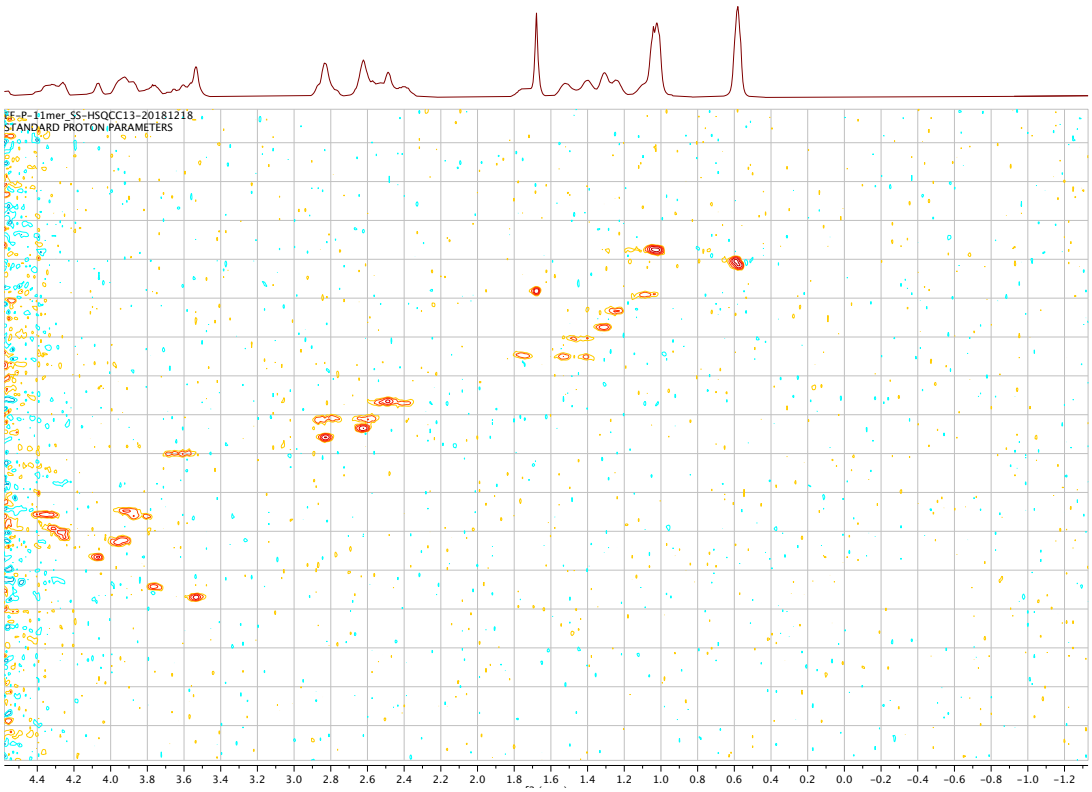
HSQC:

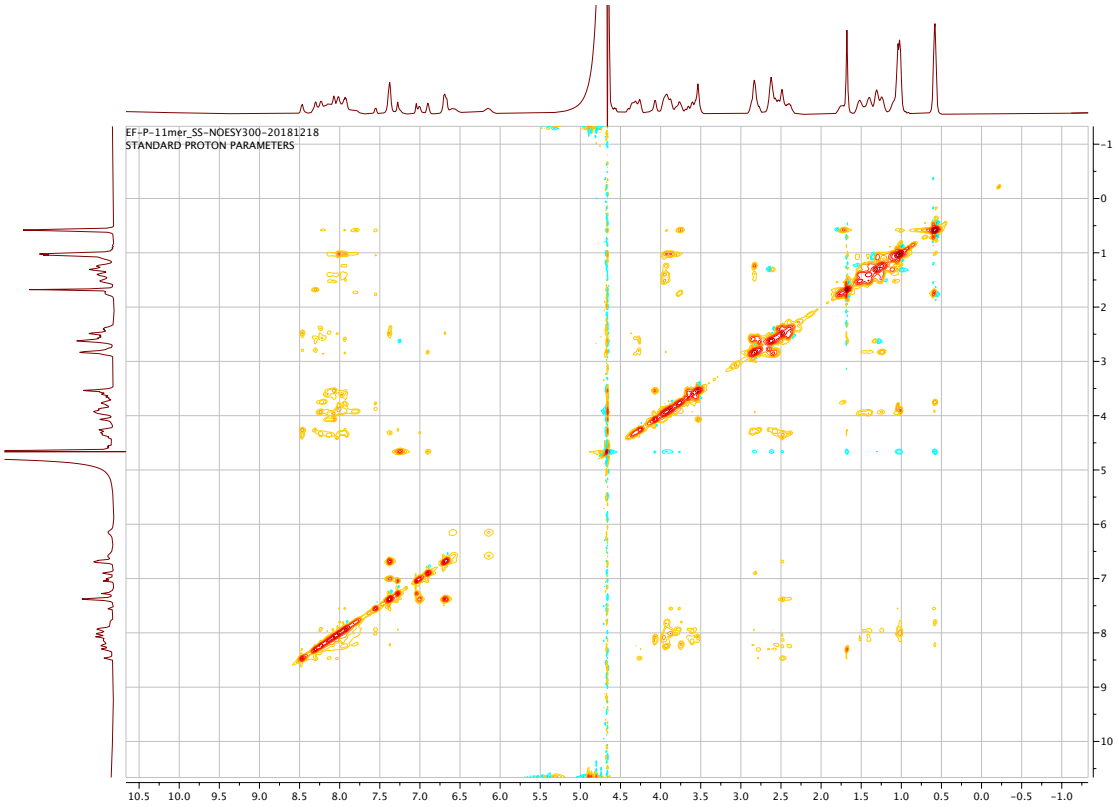

A34CB-HB
A35CB-HB
N28CAc-HAc
R32CG-HG
K29CG-HG2
F36CB-HB2
F36CB-HB1
N28CB-HB
G31CA-HA
R32CB-HB1
K29CD-HD
K29CB-HB1
R32CB-HB2
A35CA-HA
N33CB-HB2
K29CB-HB2
R32CA-HA
N33CB-HB1
N33CA-HA
K29CE-HE
A34CA-HA
R32CD-HD
K29CG-HG1
F36CA-HA
S30CA-HA
S30CB-HB
F36CE-HE
F36CD-HD
8
6
4
2
50
100
ω1 - 13C (ppm)
ω2 - 1H (ppm)

11mer_SS


CNKSGRNAAVC

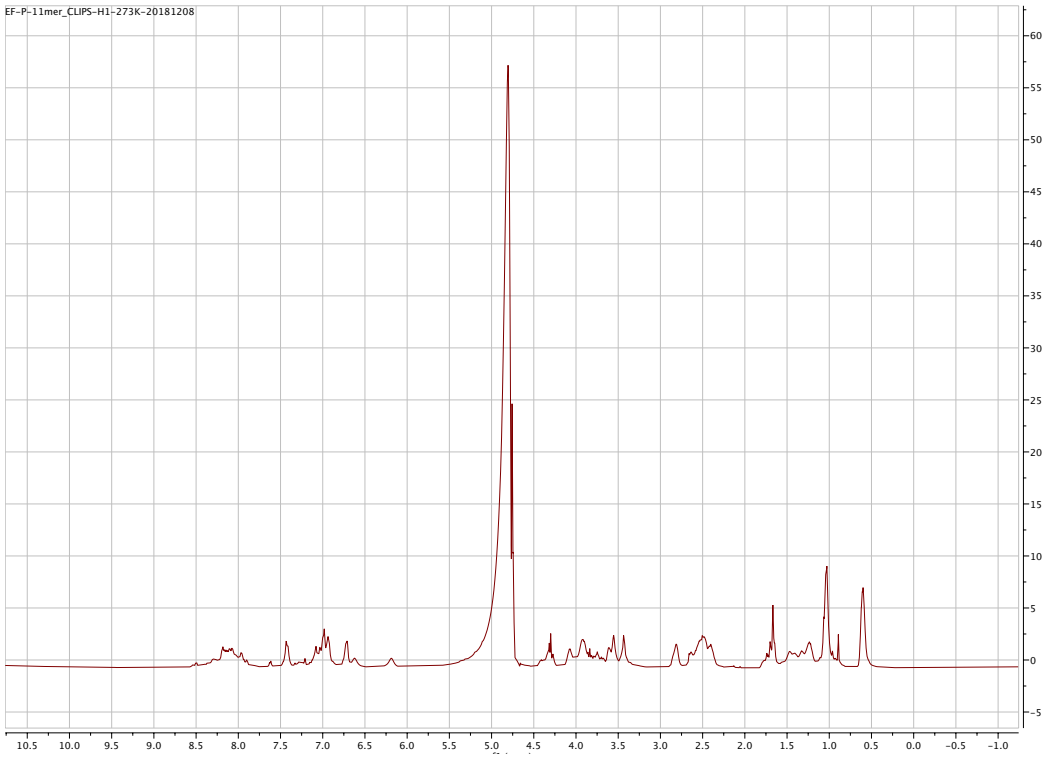

EF-P-11mer_SS-H1-20181218
f1 (ppm)

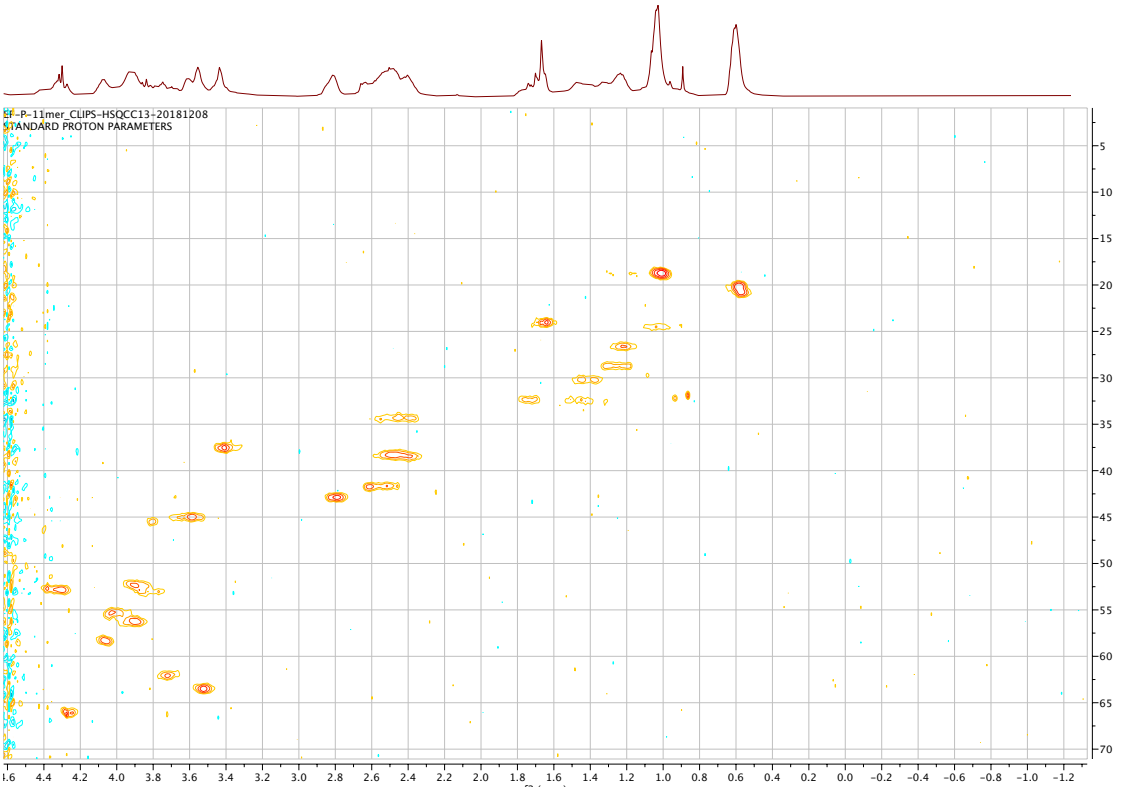

EF-P-11mer_SS-HSQCC13-20181218
STANDARD PROTON PARAMETERS

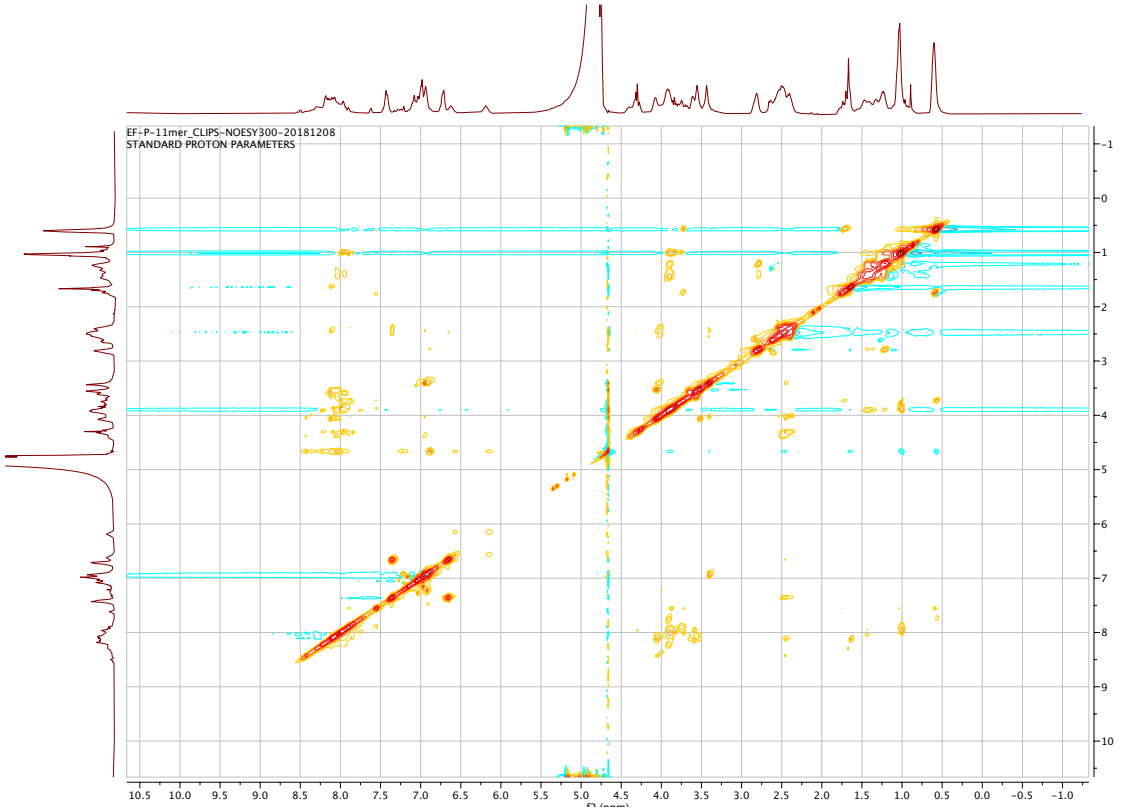

EF-P-11mer_SS-NOESY300-20181218
STANDARD PROTON PARAMETERS

11mer_CLIPS


CNKSGRNAAVC

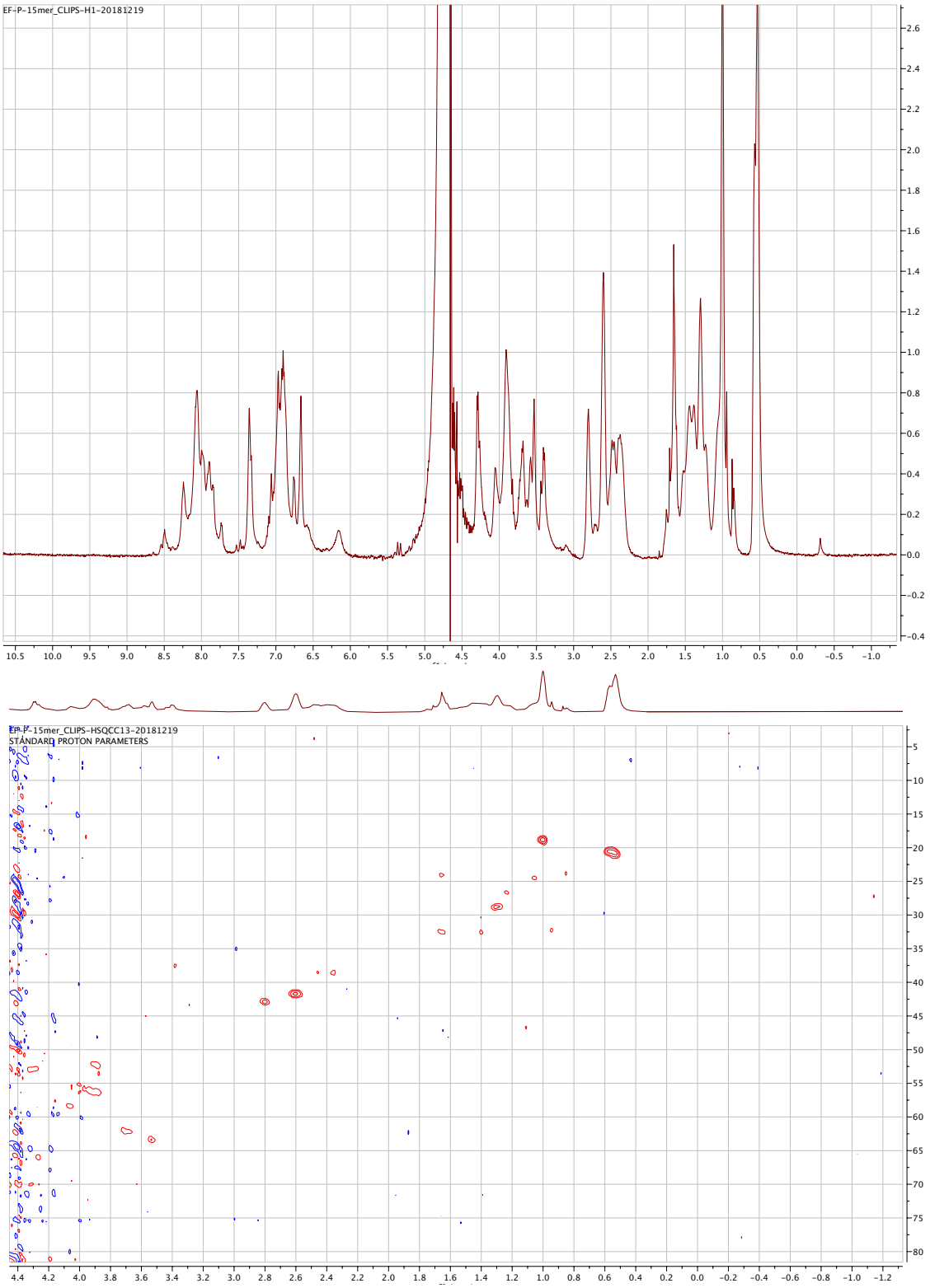

EF-P-11mer_CLIPS-H1-273K-20181208

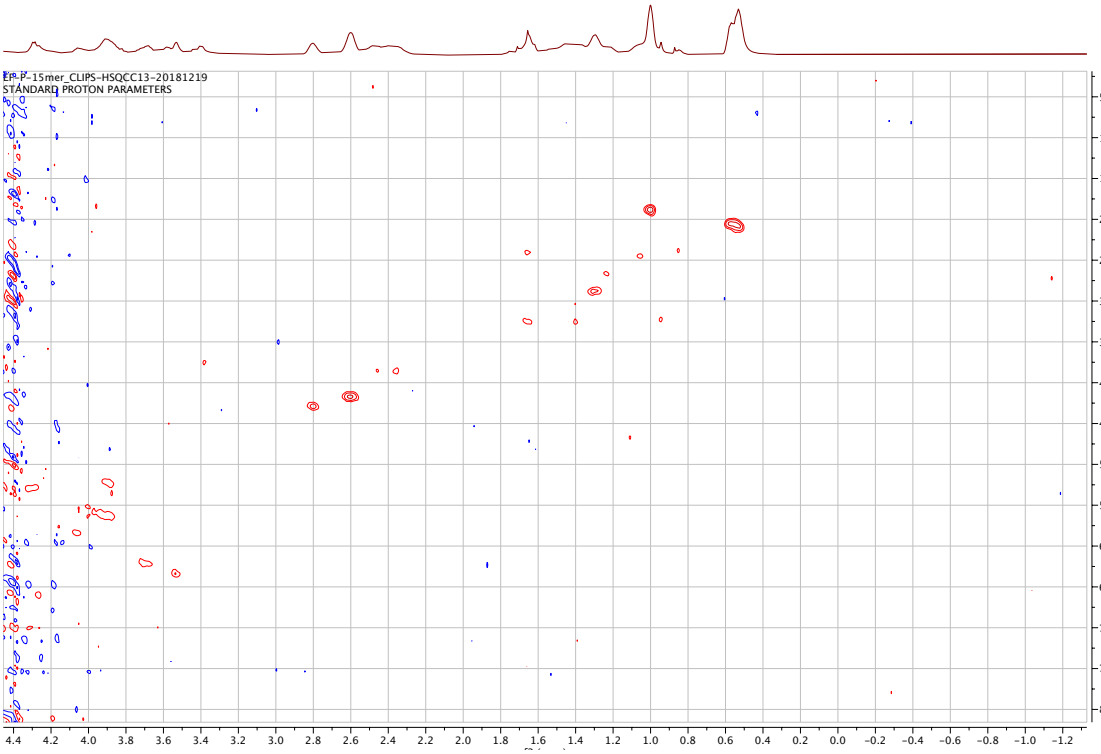

EF-P-11mer_CLIPS-HSQCC13-20181208
STANDARD PROTON PARAMETERS


EF-P-11mer_CLIPS-NOESY300-20181208
STANDARD PROTON PARAMETERS

15mer_CLIPS

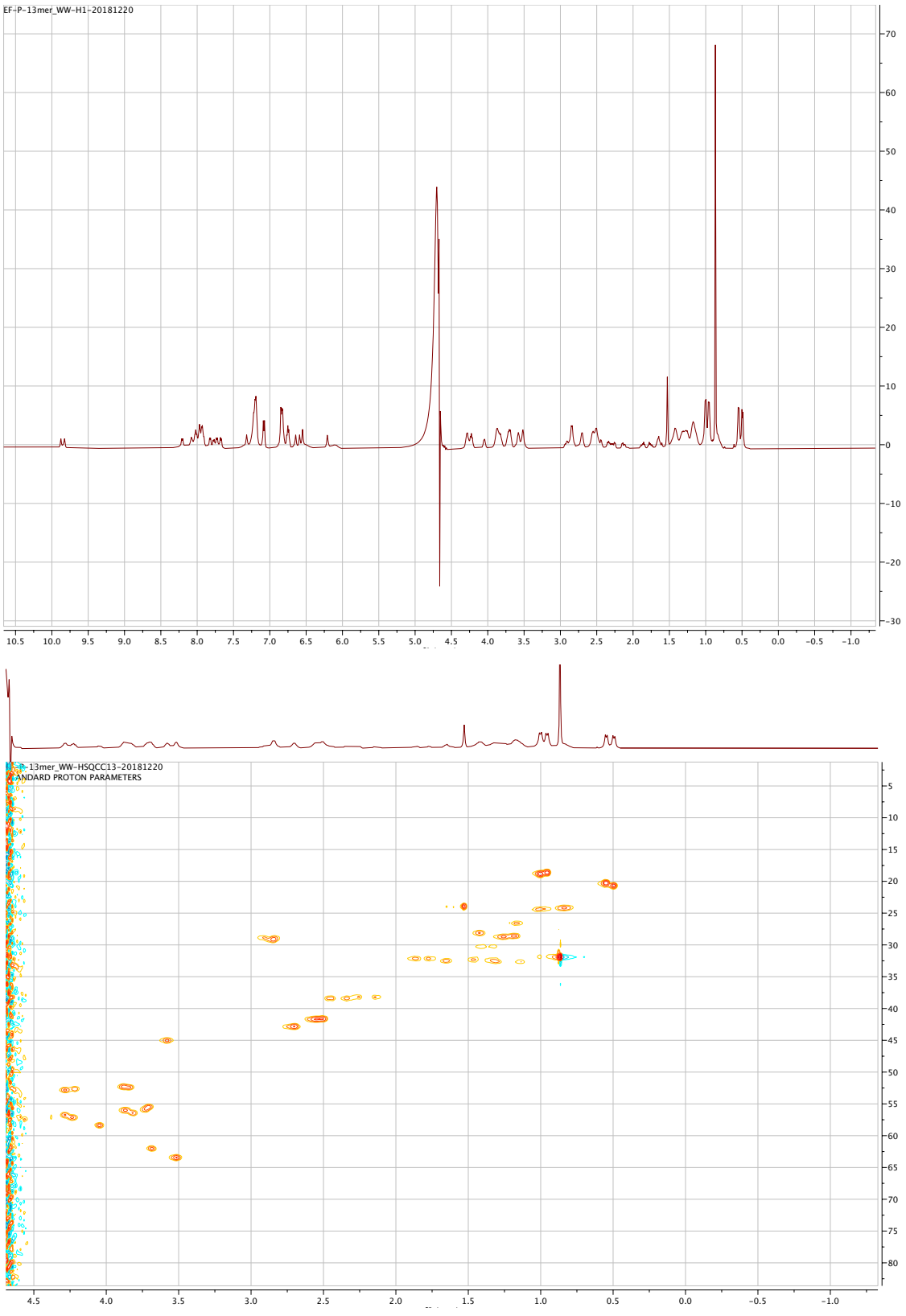

CEFNKSGRNAAVVKC


EF-P-15mer_CLIPS-H1-20181219
EF-P-15mer_CLIPS-HSQCC13-20181219
STANDARD PROTON PARAMETERS


EP-P-15mer_CLIP5-HSQCC13-20181219
STANDARD PROTON PARAMETERS

13mer_WW

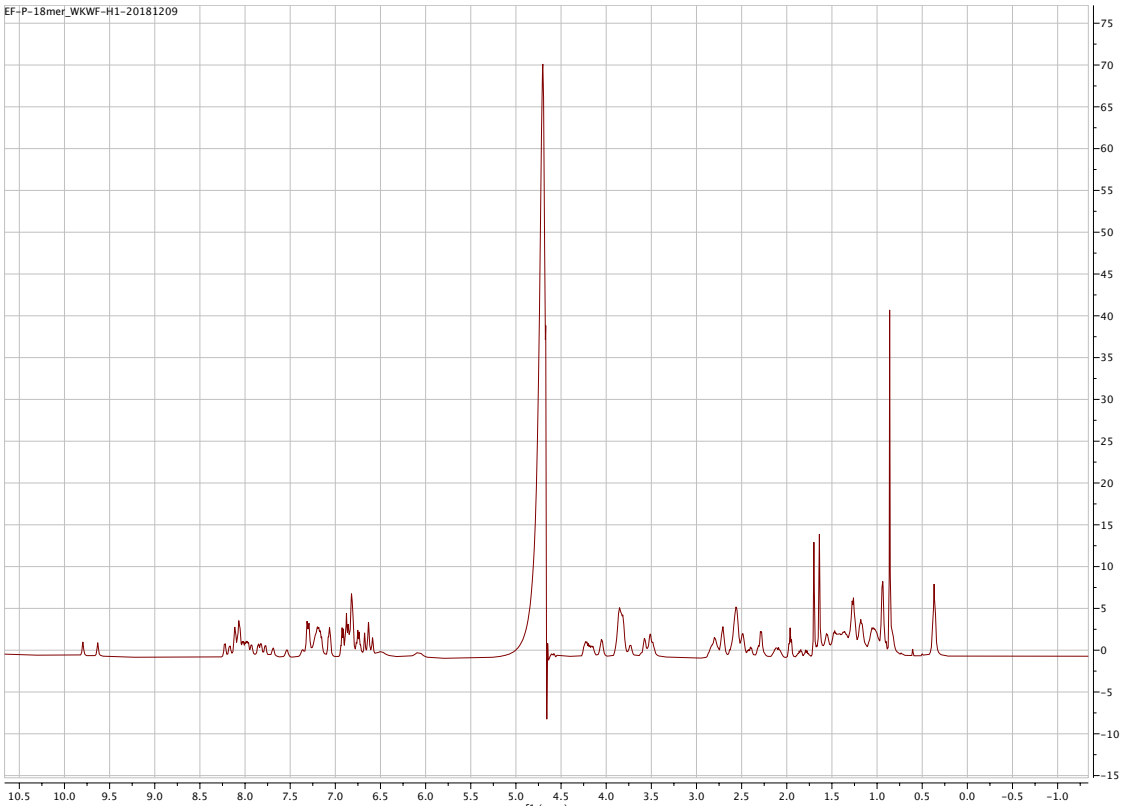
EFNKSGRNAAVWK

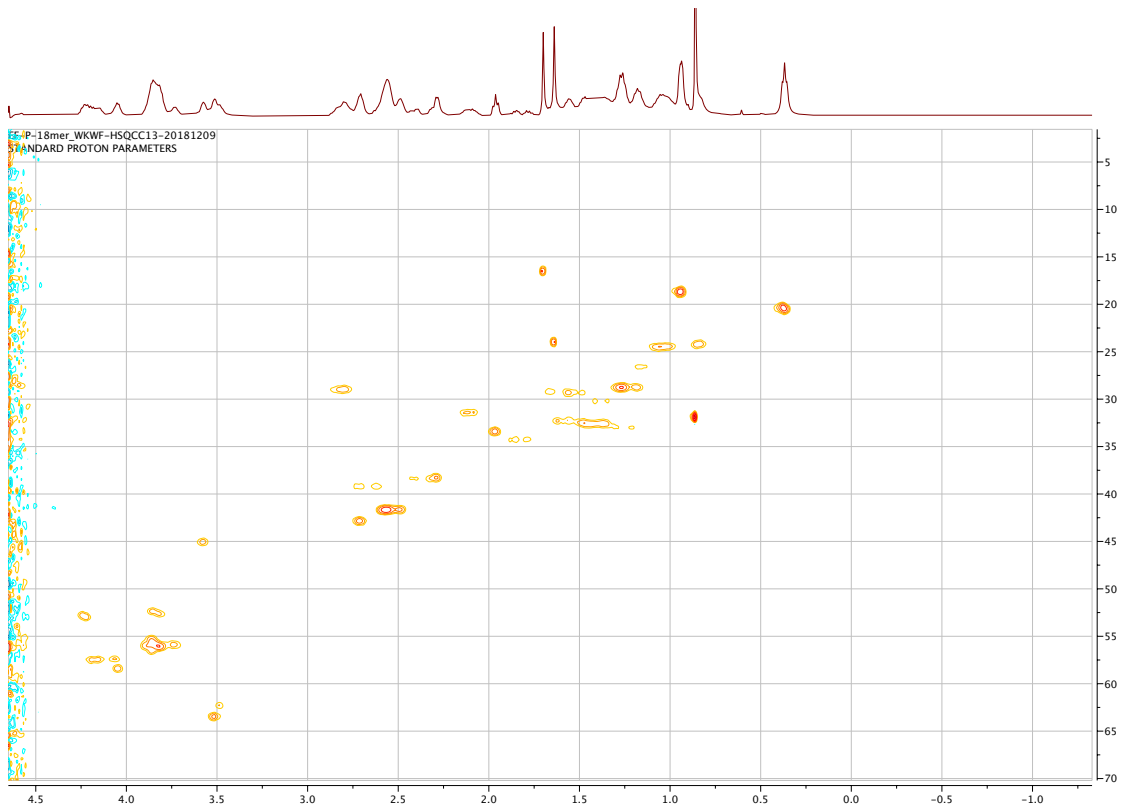


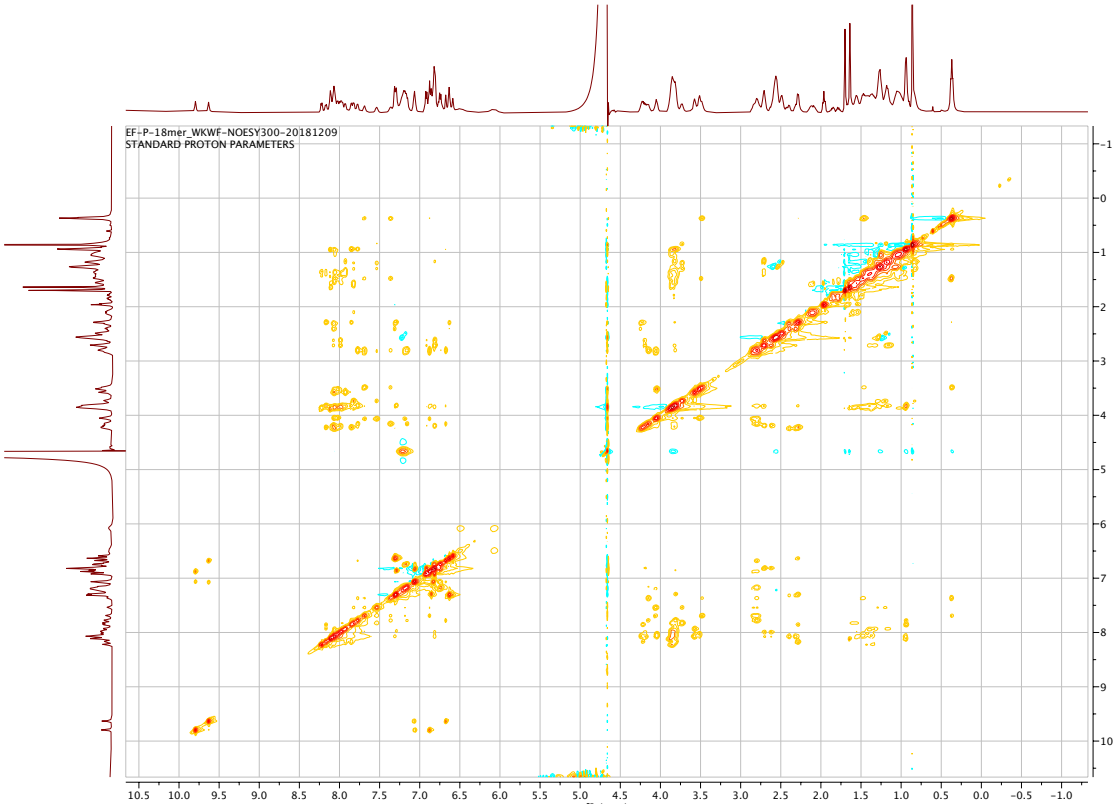

EF-P-13mer_WW-NOESY300-20181220
STANDARD PROTON PARAMETERS
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
-1 0 1 2 3 4 5 6 7 8 9 10

18mer_WKWF

QKAEFNKSGRNAWVWKMK





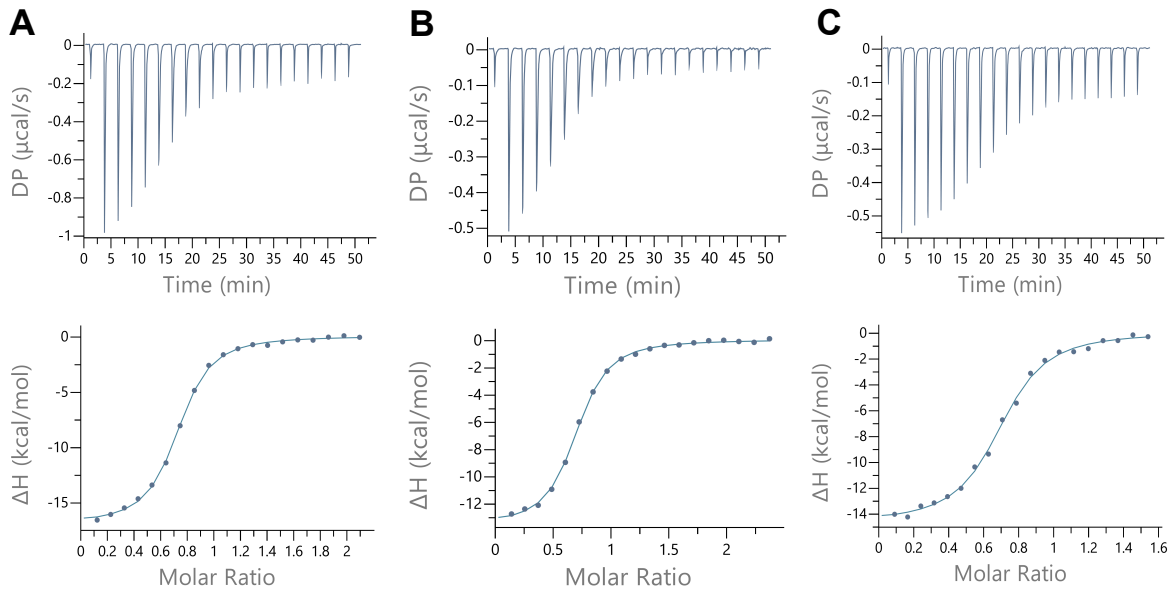

EF-P-18mer_WKWF-NOESY300-20181209
STANDARD PROTON PARAMETERS
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
-1 0 1 2 3 4 5 6 7 8 9 10

Results of ITC experiments

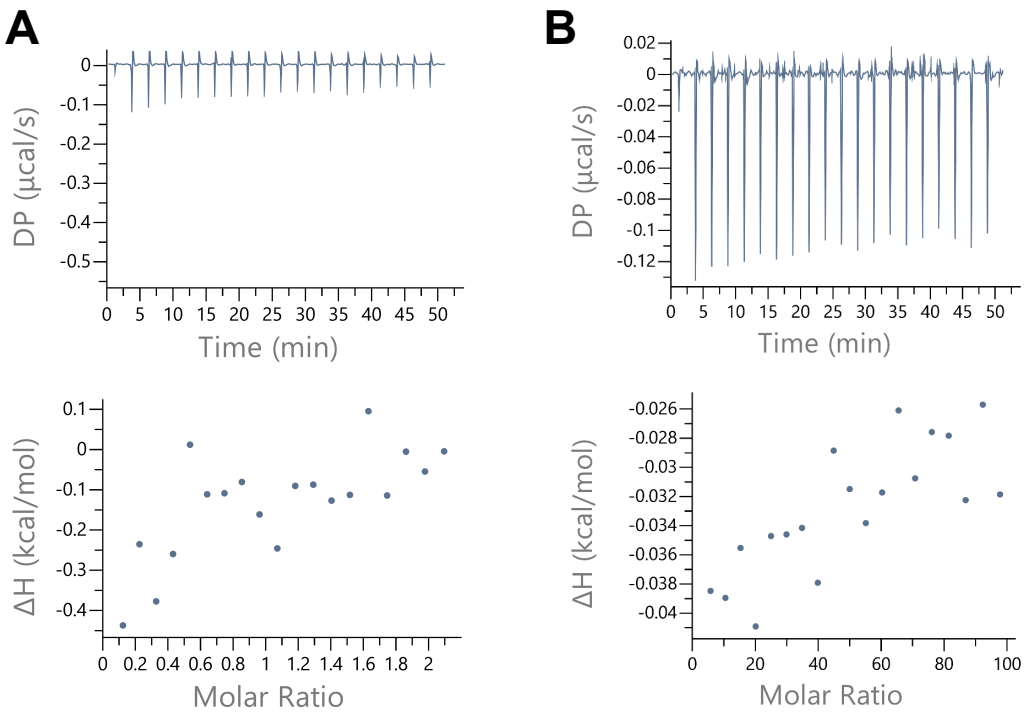
Thermodynamic parameters ± standard deviation

Ligand	Stoichiometry	Kd (μM)	ΔH (kcal/mol)	-TΔS (kcal/mol)	ΔG (kcal/mol)
EFP	0.69 ± 0.01	0.473 ± 0.094	-15.4 ± 1.2	6.9 ± 1.3	-8.5 ± 0.1
11mer_*Pa*	n/a	n/a	n/a	n/a	n/a

n/a: Parameters unable to fit in a binding model



Thermograms of EFP titrated into EarP/TDP. Experiments performed in triplicate. **A:** 283 μM EFP into 26 μM EarP, 84 μM TDP. **B:** 233 μM EFP titrated into 20 μM EarP, 60 μM TDP. **C:** 156 μM EFP titrated into 20 μM EarP, 60 μM TDP.



Thermograms of dilution titration. **A:** 283 µM EFP titrated into 84 µM TDP. **B:** 10 mM 11mer_*Pa* titrated into 60 µM TDP.

References:

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2. W. Kightlinger, L. Lin, M. Rosztoczy, W. Li, M. P. DeLisa, M. Mrksich and M. C. Jewett, *Nat. Chem. Bio*. 2018, **14**, 627-635.
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6. R. M. Cotts, M. J. R. Hoch, T. Sun and J. T. Markert, *J. Magn. Reson.* 1989, **83**, 252-266.
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