Electronic Supplementary Material (ESI) for Chemical Communications.
This journal is © The Royal Society of Chemistry 2023

Supplemental information for

Macrocyclic peptides as inhibitors of WDR5 -lncRNA interactions

Jen-Yao Chang, Cora Neugebauer, Stefan Schmeing, Gulshan Amrahova, Peter 't Hart

Contents

General information

All commercially available reagents and solvents were purchased and used directly without any treatment. Preparative HPLC was performed by using either BÜCHI Pure C-850 FlashPrep or Infinity Prep system with a C18 column (Macherey-Nagel, 5 µm, 125 x 21 mm, flow rate: 20 mL/min). Analytical HPLC-MS was performed by using Agilent 1200 Infinity II LC system equipped with a C18 column (Agilent Poroshell 120, 2.7 µm, 3 x 100 mm, flow rate: 0.4 mL/min, temperature: 33 ^{o}C) and Agilent InfinityLab LC/MSD G6125C. High-resolution mass spectra were recorded on LTQ-XL Orbitrap mass spectrometer coupled to an Accela HPLC System (HPLC column: Hypersyl GOLD, 50 mm x 1 mm, 1.9 µm). The gels were imaged using a Bio-Rad ChemiDoc MP imaging system. Reverse transcription (RT) was performed using an Eppendorf Mastercycler ep gradient, and quantitative polymerase chain reaction (qPCR) was performed using a Bio-Rad CFX Connect Real-Time PCR Detection System.

Preparation of peptide

In general, peptides were synthesized through the conventional Fmoc strategy. Rink Amide AM resin (0.3 – 0.6 mmol/g) was used as solid support. The reactions were conducted at room temperature unless otherwise specified.

Method A – Manual linear peptide synthesis

Fmoc was deprotected with 25% Piperidine in DMF for 10 min twice. The new amino acid was coupled by incubating resin with 4 eq amino acids, 4 eq PyBOP, and 8 eq DIPEA in DMF for 40 min. Ac capping was done after each coupling for peptides with premature termination by incubating resin with 10 eq Ac_2O, and 10 eq DIPEA in DMF for 15 min.

Method B – Automated linear peptide synthesis

Peptide synthesis was performed using an automated Syro I parallel peptide synthesizer. Fmoc was deprotected by incubating resin with 25% Piperidine in DMF for 3 min twice. The new amino acid was coupled by incubating resin with 4 eq amino acids, 4 eq PyBOP, and 8 eq DIPEA in DMF for 1 hr. Ac capping was performed by incubating resin with 10 eq Ac_2O, and 10 eq DIPEA in DMF for 15 min.

Method C – Automated linear peptide synthesis

Peptide synthesis was performed using an automated Peptide Chorus parallel peptide synthesizer. Fmoc was deprotected by incubating resin with 25% Piperidine in DMF for 3 min

twice at 50 °C. The new amino acid was coupled by incubating resin with 5 eq amino acid, 5 eq HCTU, and 10 eq DIPEA in DMF for 10 min at 75 °C. Ac capping was performed by incubating resin with 10 eq Ac_2O, and 10 eq DIPEA in DMF for 10 min.

Method D – Cyclization with Fmoc at N-terminus

Allyl/Alloc protecting groups were used for orthogonal side chain protection. The elongation of peptides was paused when the second position of cyclization was reached. The side chains were deprotected by incubating resin with 0.25 eq $Pd(PPh_3)_4$, 25 eq $PhSiH_3$ in DCM for 30 min twice[1]. Then the resin was washed with 0.5% sodium diethyldithiocarbamate in DMF for 5 min and a total of five times. The carboxylic group on the side chain was activated by incubating the resin with 2 eq PyAOP, and 2 eq HOAt in DMF for 15 min, then 4 eq 2,4,6-collidine was added directly to the reaction and continued the reaction for 48 hr. Capping of the unreacted side chain was carried out by incubating the resin in 10 eq Ac_2O, and 10 eq DIPEA in DMF for 30 min. Sequence elongation was then continued after cyclization was done.

Method E – Cyclization with *o*-Ns at N-terminus

This protocol was applied to those sequences that suffer from self-deprotection during the cyclization process. Allyl/Alloc protecting groups were used for orthogonal side chain protection. The elongation of peptides was paused when the second position of cyclization was synthesized. Fmoc was deprotected with 25% Piperidine in DMF for 10 min twice. Reprotection was performed by incubating resin in 4 eq *o*-NsCl, 5 eq 2,4,6-collidine, and 5 eq DMAP in DMF for 15 min twice.[2] The side chain was deprotected by incubating resin with 0.1 eq $Pd(PPh_3)_4$, 0.4 eq PPh_3, and 8 eq Pyrrolidine in DCM for 1 hr.[3] Then the resin was washed with 0.5% sodium diethyldithiocarbamate in DMF for 5 min and a total of five times. The carboxylic group on the side chain was activated by incubating the resin with 2 eq PyAOP, and 2 eq HOAt in DMF for 15 min, then 4 eq 2,4,6-collidine was added directly to the reaction and continued the reaction for 48 hr. Capping of the unreacted side chain was carried out by incubating the resin in 10 eq Ac_2O, and 10 eq DIPEA in DMF for 30 min. *o*-Ns group was deprotected by incubating resin with 10 eq 2-mercaptoethanol and 5 eq of DBU in DMF for 16 hr. Sequence elongation was then continued after cyclization and *o*-Ns deprotection were done.

FITC labeling at N-terminus

Sequences were further elongated with Fmoc-O2Oc-OH after the sequence was done. Fmoc was deprotected with 25% Piperidine in DMF for 10 min twice. FITC labeling was performed by incubating resin with 2 eq 6-FITC, 4 eq DIPEA in DMF for 16 hr.

Ac capping at N-terminus

After the sequence was fully elongated, Fmoc was deprotected with 25% Piperidine in DMF for 10 min twice. Ac capping was performed by incubating resin with 10 eq Ac_2O, 10 eq DIPEA and DMF for 30 min.

Global cleavage

After the sequence elongation and N-terminal modification, peptides were cleaved from the resin by incubating the resin in a solution of TFA/H_2O/TIPS (95/2.5/2.5) for 1 hr. An additional cleavage cycle was performed for peptides that were difficult to deprotect. After the cleavage was done, the resin was filtered off and the filtrate was concentrated by blowing N_2 over the surface followed by precipitation of the peptide in cold diethyl ether three times. The product was then purified by preparative LC with either acidic or basic eluent.

Preparative LC with acidic eluent

Crude peptides were resuspended in a solution of H_2O/ACN/DMSO/TFA (70/20/10/0.1), centrifuged to remove insoluble particles, then purified by reverse-phase preparative LC. Products were eluted using a linear gradient from 10% to 60% (percentage of B. A: 0.1% TFA in H_2O; B: 0.1% TFA in ACN) over 60 min. The elution was monitored using a diode array detector at 254 nm for the FITC-labeled peptides, or 210 nm for non-FITC-labeled peptides. The pure fractions were pooled together and lyophilized to give the desired products.

Preparative LC with basic eluent

The purification with alkaline eluent was used for peptides that didn't dissolve in an acidic aqueous solution. Crude peptides were resuspended in a solution of H_2O/MeOH (90/10), and the pH was adjusted by gradually mixing with 200 mM $NH_4HCO_{3(aq.)}$ until the overall pH was above 7. The crude solution was centrifuged to remove insoluble particles, then purified by reverse-phase preparative LC. Products were eluted using a linear gradient from 5% to 60% (percentage of B. A: 15 mM $NH_4HCO_{3(aq.)}$; B: MeOH) over 60 min. The elution was monitored using a diode array detector at 254 nm for the FITC-labeled peptides, or 210 nm for non-FITC-labeled peptides. The pure fractions were pooled together and lyophilized to give the desired products.

Supplemental Table 1. List of peptide's sequence, synthesis methods, purification conditions, and corresponding HRMS.

Peptide	Elongation	Cyclization	Purification	Calculated m/z	Observed m/z
1	A	-	Acidic	1769.63707 $[M+H]^+$	1769.63639
2	B	-	Acidic	1653.61013 $[M+H]^+$	1653.61042
3	B	-	Acidic	1555.54171 $[M+H]^+$	1555.54223
4	B	-	Acidic	1468.50968 $[M+H]^+$	1468.51076
5	A	-	Acidic	1939.74260 $[M+H]^+$	1939.74319
6	B	-	Acidic	1810.70000 $[M+H]^+$	1810.69995
7	B	-	Acidic	1695.67306 $[M+H]^+$	1695.67275
8	B	-	Acidic	1566.63047 $[M+H]^+$	1566.63068
9	B	-	Acidic	1437.58787 $[M+H]^+$	1437.58913
10	B	-	Acidic	763.28740 $[M+2H]^{2+}$	763.28791
11	A	D	Acidic	1506.57295 $[M+H]^+$	1506.57368
12	A	D	Acidic	1520.58860 $[M+H]^+$	1520.58958
13	A	D	Acidic	1534.60425 $[M+H]^+$	1534.60512
14	C	-	Basic	909.33916 $[M+2H]^{2+}$	909.33947
15	B	-	Acidic	1671.61554 $[M+H]^+$	1671.61547
16	A	D	Acidic	1661.66758 $[M+H]^+$	1661.66733
17	A	D	Acidic	1675.68323 $[M+H]^+$	1675.68282
18	C	E	Basic	1790.71017 $[M+H]^+$	1790.70992
18Ac	C	E	Basic	649.80916 $[M+2H]^{2+}$	649.80998
19	A	D	Acidic	902.86655 $[M+2H]^{2+}$	902.86739
20	A	D	Acidic	896.84836 $[M+2H]^{2+}$	896.85091
21	A	D	Acidic	903.85618 $[M+2H]^{2+}$	903.85858
22	A	D	Acidic	902.86655 $[M+2H]^{2+}$	902.86907
23	C	D	Basic	1790.71017 $[M+H]^+$	1790.71363
24	A	E	Basic	1790.71017 $[M+H]^+$	1790.71242
25	A	E	Basic	1790.71017 $[M+H]^+$	1790.71280
26	B	-	Acidic	619.35983 $[M+2H]^{2+}$	619.35941

Recombinant protein expression and purification

WDR5 (Δ1-21), WDR5-FLAG (Δ1-21), and $WDR5^{F266A}$-FLAG (Δ1-21) were expressed in BL21 DE RIL *Escherichia coli*. *E. coli* was grown in LB medium supplemented with 0.1% ampicillin and the expression was induced with 200 µM isopropyl-beta-D-thiogalactopyranoside (IPTG) at 20 ^{o}C for 16 hr. After centrifugation, the pellets were resuspended in Buffer A additionally supplemented with 1 mM PMSF and a spatula tip of DNase I (Roche, #04716728001), then disrupted using a sonicator CL-334 (70% amplitude, 10

s pulse with 6 min interval) while the solution was kept cold by placing it on ice. The insoluble cell components were pelleted by centrifuge at 60000 rcf under 4 °C for 45 min. The supernatant containing the desired protein was filtered with a 0.22 µm syringe filter, loaded on HisTrap HP His tag protein purification column (Cytiva, #17524801) with Buffer A and eluted by a linear gradient to Buffer B (100%, 100 min). The desired fractions were collected, pooled together, and dialysis with Prescission-3C-protease (3.5 mg/mL) at 4 °C for 16 hr. The dialyzed protein was then purified by reverse Ni-NTA with a linear gradient of Buffer A to Buffer B in 50 min, concentrated with Amicon® ultra centrifugation filter (4000 rcf, 10 min, 4 °C). The concentrated protein was cleared by centrifugation (10 min, 4 °C, 20000 rcf), and the supernatant was loaded directly on a Superdex 75 16/600 column eluting with SEC buffer (isocratic, flow = 1 mL/min). For each purification step, the protein of interest was analyzed by 15% SDS-Page polyacrylamide gels and staining by Coomassie Blue staining solution.

Fluorescence polarization

Fluorescence polarization was measured using a Tecan Spark Microplate Reader with 384-well plates (Corning, #4514). The assay buffer contained 0.1 M $K_nH_{(3-n)}PO_4$, 25 mM KCl, and 0.01% Triton-X at pH 6.5.[4]

A 15-point dose-response curve was generated for direct binding assays by titrating a twofold dilution series of protein from the highest concentration against fixed FITC-labelled peptide (5 or 10 nM). A sample of protein-free corresponding FITC-labelled peptide in the assay buffer was included as non-binding control for each set of replicates. After the plate was prepared, it was sealed and incubated at 25 °C for 1 hr before measurement. The raw value of fluorescence polarization was used, and K_D (dissociation constant) was calculated by fitting with GraphPad Prism 9 using the [Agonist] vs. Response –variable slope (four parameter) function for curve fitting.

Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry was measured using a MicroCal PEAQ-ITC, following the general instruction provided by the instrument. WDR5 was freshly dialyzed against the ITC buffer for 16 hr, and the peptide was freshly prepared by dissolving in the corresponding dialysate. The concentration of WDR5/peptide was determined by OD_{280}/ OD_{495} on Nanodrop and diluted to the target concentration with dialysate. A total of 19 injections were made at 25 °C to record the titration curve. The thermodynamic parameters were determined using the PEAQ-ITC analysis software. Measurements were performed in duplicate.

Co-Crystallization and structure determination

Co-Crystallization of WDR5 with 18Ac

Purified WDR5 was concentrated to 23.25 mg/mL and ligand X dissolved in gel filtration buffer and a 1.5-fold molar excess of peptide was added. Crystallization was set up in MRC-3 drop plates by adding 100 nL protein/ligand complex to 100 nL reservoir solution and the plates were stored at 20°C. Crystals were obtained after a few days with reservoir conditions of 0.2 M Li_3-citrate and 20 % w/v PEG3350 and were cryo-conserved in reservoir solution supplemented with 20 % glycerol.

X-ray data collection and processing

Diffraction data was collected at beamline ID30A-3 at ESRF Grenoble (acquisition date 18.11.2022). Datasets were integrated using XIA2/DIALS (ccp4).[5,6] The structure was solved using Phaser (Phenix) and an alphafold model of apo-WDR5. [7,8]

Structure solution and refinement

WDR5-**18Ac** co-crystallized in space group P 4_3 2_1 2 with dimensions 82.1737 x 82.1737 x 201.707 Å with two protein-peptide dimers in the asymmetric unit. The solved structure was refined by iterations of phenix.refine and manual model building in coot.[8,9] The structure was refined to a final Rfree of 24 % at 1.84 Å.

X-ray model analysis

The protein crystallized with two protein-peptide dimers in the asymmetric unit (Supplemental figure 2 A) and the crystal showed a dense packing of monomers (shown by the symmetry related molecules in 20 Å distance; Supplemental figure 3). We observed a big discrepancy in the quality of the $2F_O$-F_C map between the two WDR5 chains probably caused by a higher disorder and flexibility in chain B, which corresponds to the increased B-factors of this chain (Supplemental figure 2 B). Both chains share the same typical fold consisting of seven beta-propeller blades with four-stranded antiparallel sheets.[10] Overall, the two WDR5 chains still show a high similarity with an RMSD of 0.285 Å (Supplemental figure 2 C). The biggest differences of both chains were observed in the *N*- and *C*-terminal regions, where we observed a higher ordered ß-sheet conformation for chain B, while the loops of chain A point away from each other, which could be caused by additional contacts to symmetry related molecules. The presence of ligand **18Ac** was verified by a well resolved density of the central

amino acids including the position of macrocyclization, but the density of the terminal Glu and Asp was less well resolved (Supplemental figure 4 C/D). Further on, the ligands a and b bind in an identical manner to the reported native peptide of RBBP5 (Supplemental figure 4 A/B). The binding of **18Ac** to the WBM site does not lead to any significant changes of the conformation of this site compared to the reported apo-structure (PDB 2H14) (Supplemental figure 4 E). Furthermore, we observed numerous structured waters in the structure, most of them were located in the central cavity of the protein, which was also observed in previous reports (Supplemental figure 4 F).[11] Furthermore, we recognized a potential disulfide bridge of chain A and chain B with a symmetry related molecule of chain B and A, respectively (Supplemental figure 3 G).

Cancer cell line and total RNA isolation

U-2 OS (DSMZ, ACC 785) were grown in DMEM high glucose medium (Sigma-Aldrich, #D6429) supplemented with 10% Fetal Bovine Serum (FBS, Cellsera, #AU-FBSPG) and 1% Penicillin-Streptomycin (Pen-Strep, Gibco, #15140-122) in a humidified atmosphere with 5% CO_2 under 37 ^{o}C. The subcultivation ratio follows the suggestion from ATCC.

For isolating the total RNA, U-2 OS cells in a Petri dish were washed with PBS (Gibco, #10010-023), trypsinized (0.05% Trypsin, Gibco, #25300-054), followed by pelleting of the cells by centrifugation (3 min, rt, 200 rcf). The pellet was then washed with PBS, resuspended in TRIzol (Invitrogen, #15596026), and the protocol from the manufacturer was followed to obtain total RNA isolates.

RNA immunoprecipitation (RNA-IP)

Total RNA isolates, purified proteins, and inhibitors were obtained as previously described. The protocol was adapted from Fuentes-Iglesias et al.[12] 10 µL of ANTI-FLAG® M2 Magnetic Beads (Millipore, #M8823) were washed with RIP Buffer C, then loaded with target protein (0.2 mg/mL, in Buffer C) supplemented with 0.1% IGEPAL, 0.1% BSA(Serva, #11930.03) and 10 ppm yeast tRNA (Biosciences, 058Y) at 4 ^{o}C for 3 hr. After the incubation, the beads were washed with 0.1% IGEPAL in Buffer C five times. 1 µg of total RNA isolated from U-2 Os, w/wo inhibitor, and 0.1% DMSO in RNA-immunoprecipitation buffer were incubated with protein-beads complex under rt for 1 hr. After the incubation, the beads were washed with RNA-immunoprecipitation buffer 5 times and then rinsed with 50 µL of RNase free water once. The RNA/protein bound beads were resuspended in 50 µL of RNase free water. From this resuspended solution, 5 µL was transferred and mixed with 2X Laemmli buffer, loaded on a 15% SDS-PAGE polyacrylamide gel to analyze the protein loading of each sample. The other 45 µL was treated with 450 µL of TRIzol following the TRIzol protocol to extract the RNA. After the extraction process, the RNA pellet was dissolved in 10 µL of RNase free water and used for

RT-qPCR analysis. A sample of 0.5 µg RNA input underwent the same treatment starting from resuspension in 50 µL RNAse free water, including sampling for gel and TRIzol extraction, to control for sample loss between RIP product and non-treated RNA input.

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

RNA was obtained as described above. cDNA was synthesized by using high-capacity cDNA reverse transcription kit (Applied Biosystems, 4368814) following the manufacturer's protocol. After cDNA was generated, it was analyzed by PowerUp™ SYBR™ Green Master Mix (Applied Bioscience, #A25742) via the fast-cycling mode and supplemented with 500 nM forward and reverse primer according to the target amplicon. Percentage of enrichment was calculated by normalization with 0.5 µg (50%) of RNA input. The percentage of enrichment was calculated by the method reported by Fuentes-Iglesias et al.[12] In short, the difference of Ct between the sample after enrichment and the 0.5 µg of RNA input (compensated with the percentage of dilution, 50%) was calculated.

$$Ct_{sample} - (Ct_{input} - \log_2(1/percentage\ of\ dilution)) = \Delta Ct_{corrected}$$

Then the percentage of target RNA enrichment from the RNA pool can be calculated by the following equation:

$$Percentage\ of\ enrichment = 2^{-\Delta Ct_{corrected}} \times 100\%$$

Buffer recipe list:

Buffer A:

50 mM HEPES, 300 mM NaCl, 30 mM Imidazole, 0.1 mM PMSF, 1 mM TCEP, pH = 8.0

Buffer B:

50 mM HEPES, 300 mM NaCl, 500 mM Imidazole, 1 mM TCEP, pH = 8.0

Buffer C:

25 mM HEPES, 150 mM NaCl, pH = 7.0

ITC buffer:

20 mM Tris-HCl, 200 mM NaCl, pH = 7.0

RNA-immunoprecipitation buffer[12]:

25 mM Tris-HCl, 150 mM KCl, 5 mM EDTA, 0.1% IGEPAL, 1 mg/mL BSA, 10 µg/mL yeast tRNA, 80 U/mL Recombinant RNasin Ribonuclease Inhibitor (freshly added, Promega, #N251A), pH 7.5

SEC Buffer:

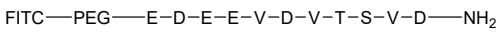
50 mM HEPES, 300 mM NaCl, 1 mM TCEP, pH = 8.0

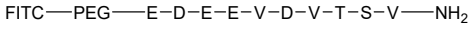
Primer List

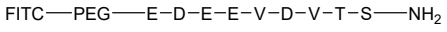
HOTTIP_Fw	TACCGGAATAGTGCTGGGGA[13]
HOTTIP_Rev	TGCGTGCTGCTCTGAGTTTA[13]
HOXC13-AS_Fw	GAAACTGCATTTCCTGGGGC[14]
HOXC13-AS_Rev	GGCTGGAGTCTTTGTCCTCC[14]

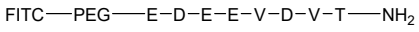
Appendix:

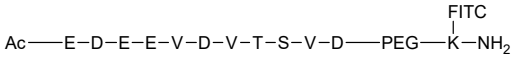
Overview of compounds

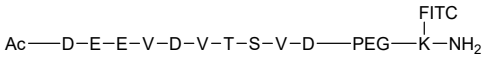
1 

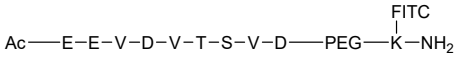
2 

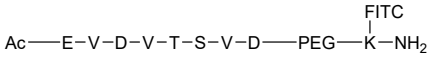
3 

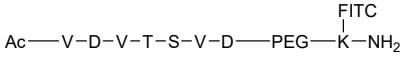
4 

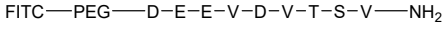
5 

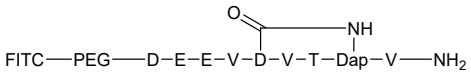
6 

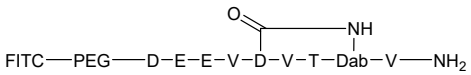
7 

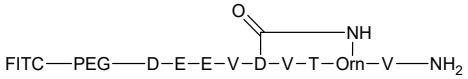
8 

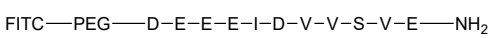
9 

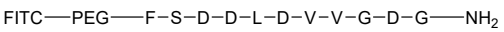
10 

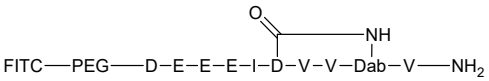
11 

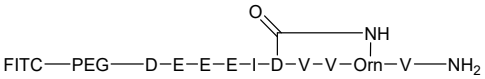
12 

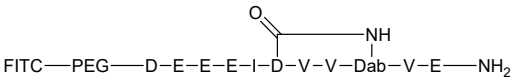
13 

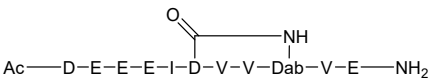
14 

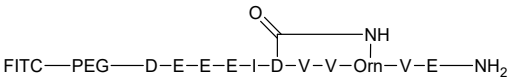
15 

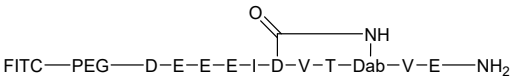
16 

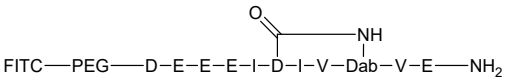
17 

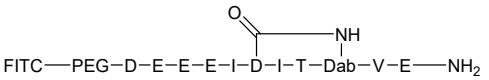
18 

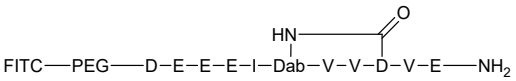
18Ac 

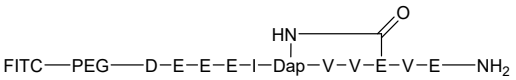
19 

20 

21 

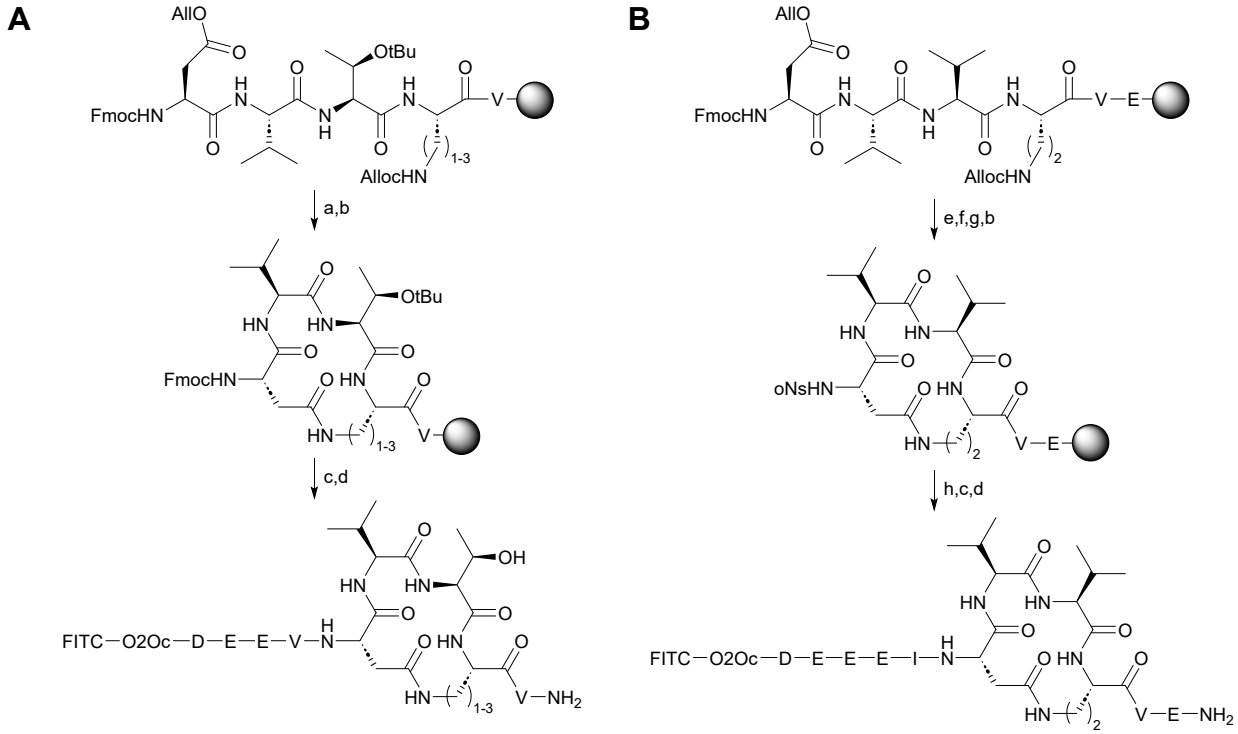
22 

24 

25 

26

Supplemental figure 1: Peptides **1** – **26**



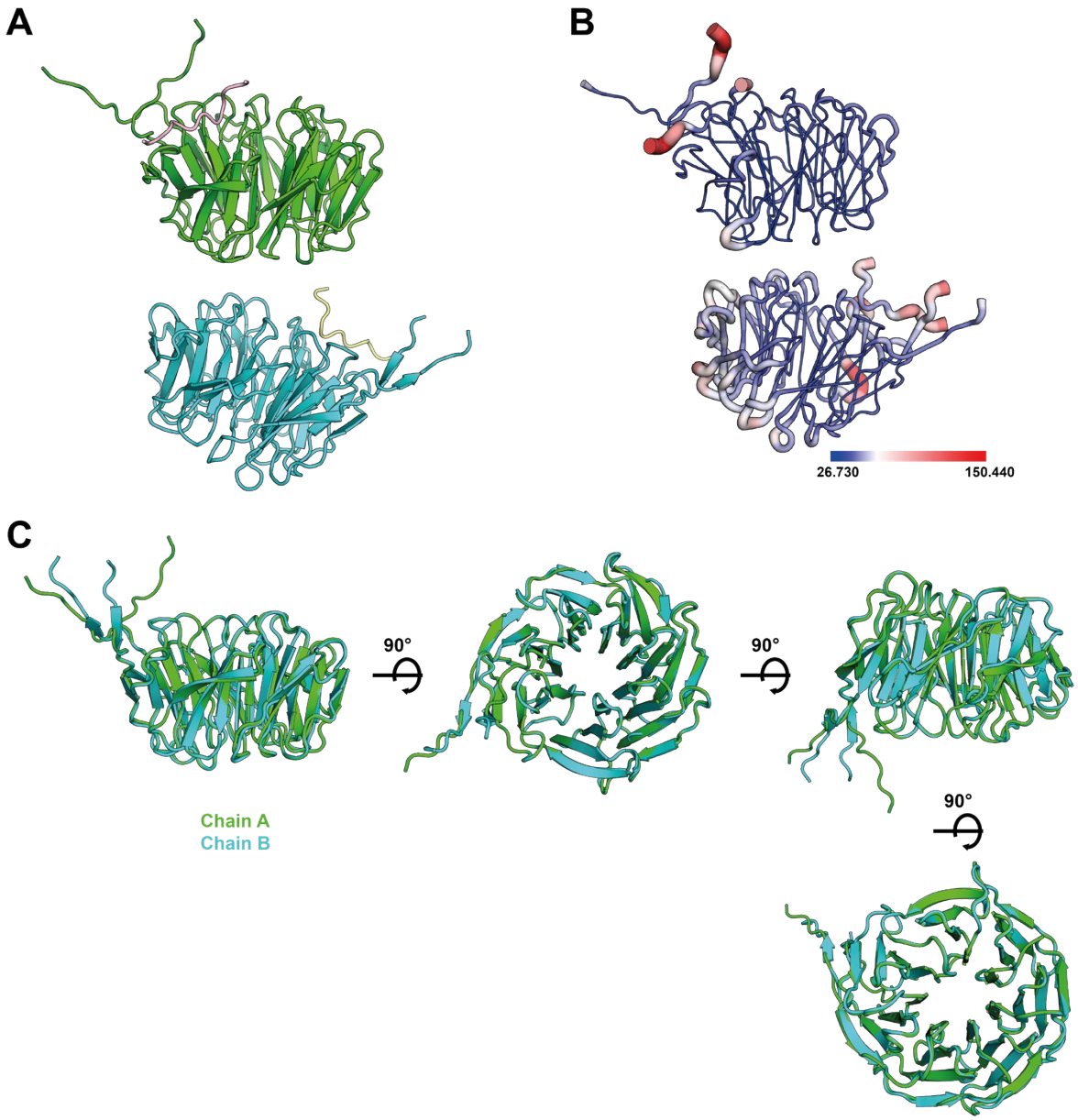
Supplemental scheme 1: Representative synthesis of peptides using (A) N-terminal Fmoc protection or (B) *o*-Ns protection (used for peptides **18**, **24**, and **25**). (a) $Pd(PPh_3)_4$, (0.25 eq), $PhSiH_3$ (25 eq), DCM, 2 x 30 min. (b) PyAOP (2 eq), HOAt (2 eq), 2,4,6-collidine (4 eq), DMF, 48 h. (c) Fmoc-SPPS. (d) TFA/TIPS/H_2O (95:2.5:2.5 v/v) 1 h. (e) 25% piperidine in DMF, 2 x 10 min. (f) *o*-NsCl (4 eq), 2,4,6-collidine (5 eq), DMAP (5 eq), DMF, 2 x 15 min. (g) $Pd(PPh_3)_4$ (0.1 eq), PPh_3 (0.4 eq), pyrrolidine (8 eq), DCM, 1 h. (h) 2-mercaptoethanol (10 eq), DBU (5 eq), DMF, 16 h.

Result of X-ray cocrystal structure

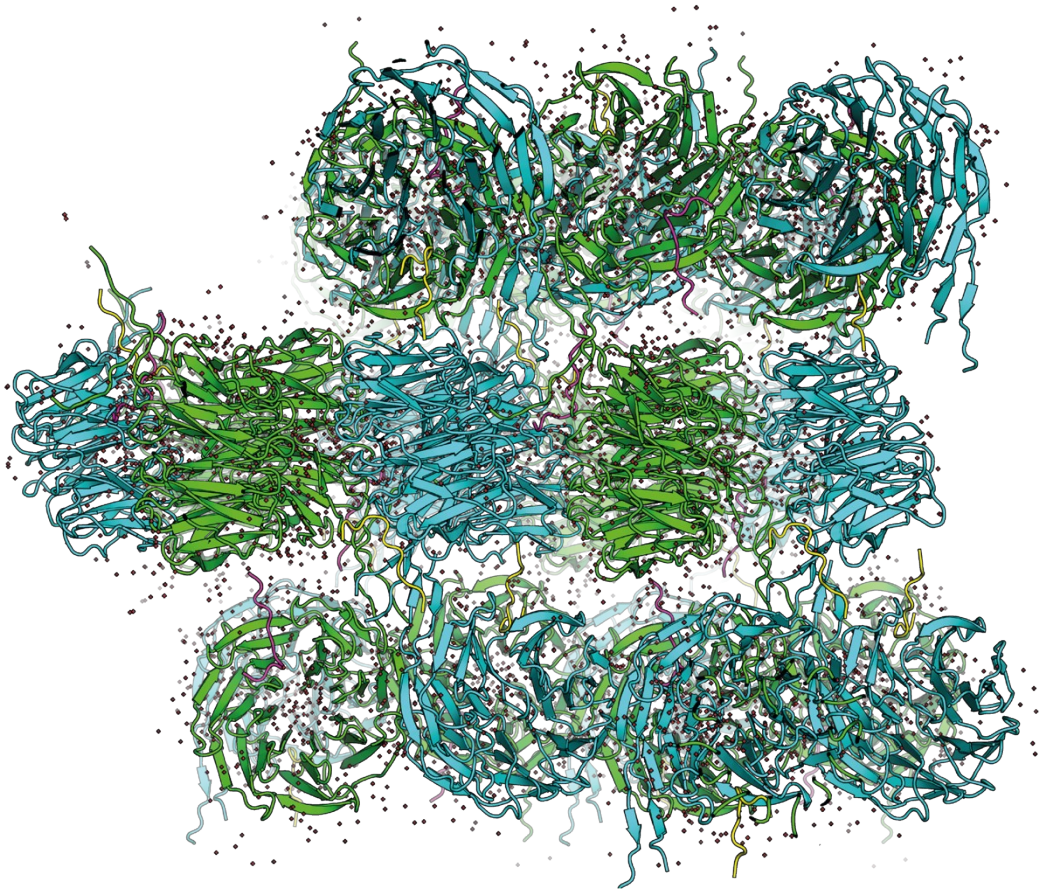
Supplemental Table 2. Data collection and refinement statistics of the WDR5-**18Ac** crystal.

	WDR5-18Ac
Data collection	
Space group	P 43 21 2
Cell dimensions	
a, *b*, *c* (Å)	82.1737 82.1737 201.707
α, β, γ (°)	90, 90 ,90
Resolution (Å)	42.98 - 1.843 (1.909 - 1.843)
R_{merge}	0.1688 (3.632)
I / s*I*	8.05 (0.26)
Completeness (%)	96.59 (66.37)
Redundancy (Multiplicity)	26.7 (27.4)
CC1/2	0.995 (0.361)
Refinement	
Resolution (Å)	1.84
No. Reflections	58517 (3960)
R_{work} / R_{free}	0.1975 / 0.2429
No. Atoms	5614
Protein	5179
Ligand/ion	0
Water	435
***B*-factors**	
Protein	56.22
Water	57.80
R.m.s. deviations	
Bond lengths (Å)	0.011
Bond angles (°)	1.25
Ramachandran favored (%)	95.19
Ramachandran allowed (%)	4.65
Ramachandran outliers (%)	0.16
Rotamer outliers (%)	2.36
Clashscore	8.32

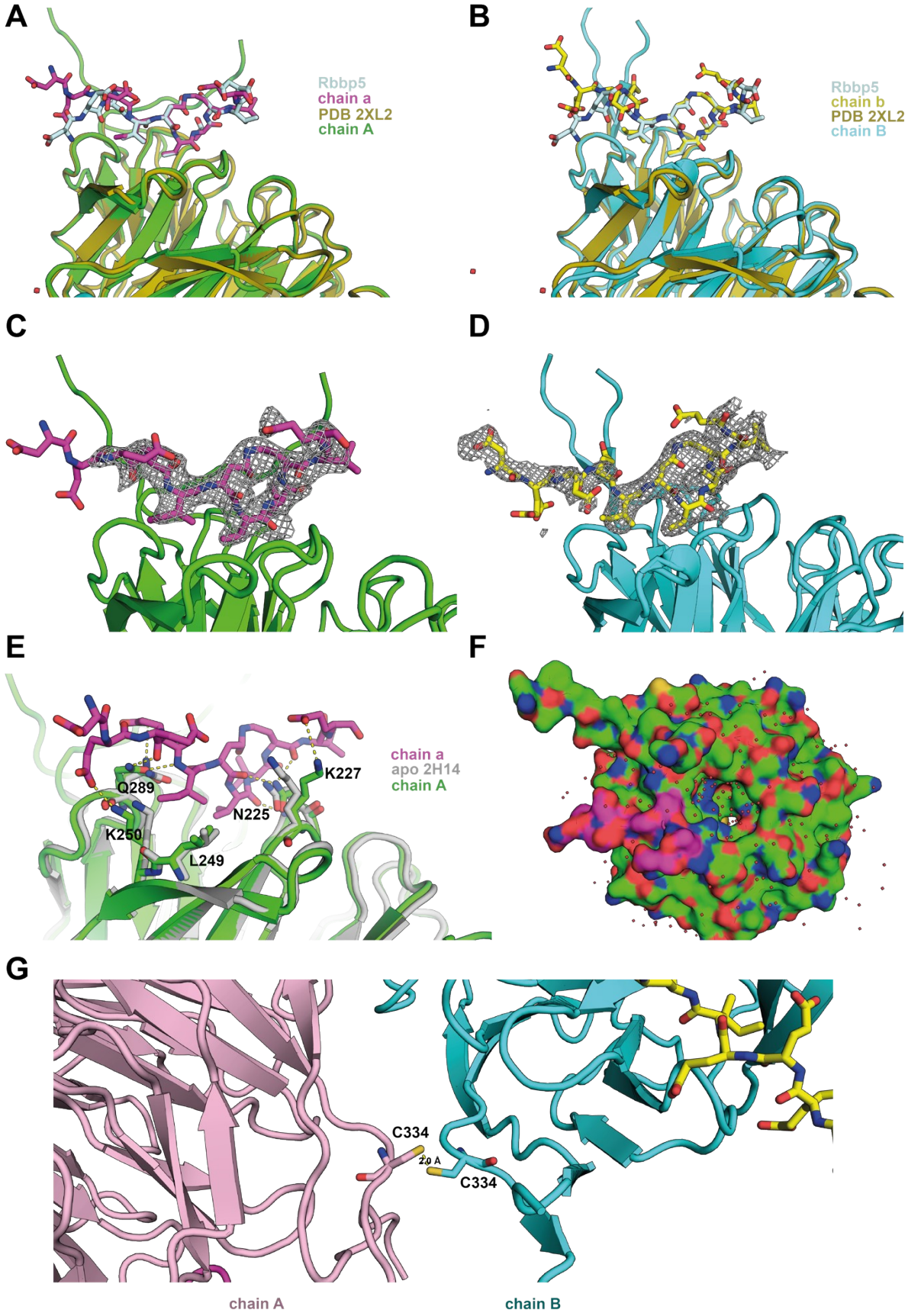
Statistics for the highest-resolution shell are shown in parentheses.



Supplemental figure 2: **(A)** Asymmetric unit (ASU) of the WDR5-**18Ac** co-crystal containing two protein-peptide dimers. **(B)** Depiction of the B-factors of both dimers A/a (top) and B/b (bottom) shows that the termini of the peptide ligands and chain B have higher B-factors than chain A which corresponds to the quality of electron density. **(C)** Overlay of the protein chains A and B indicate a high similarity of both chains except the termini.



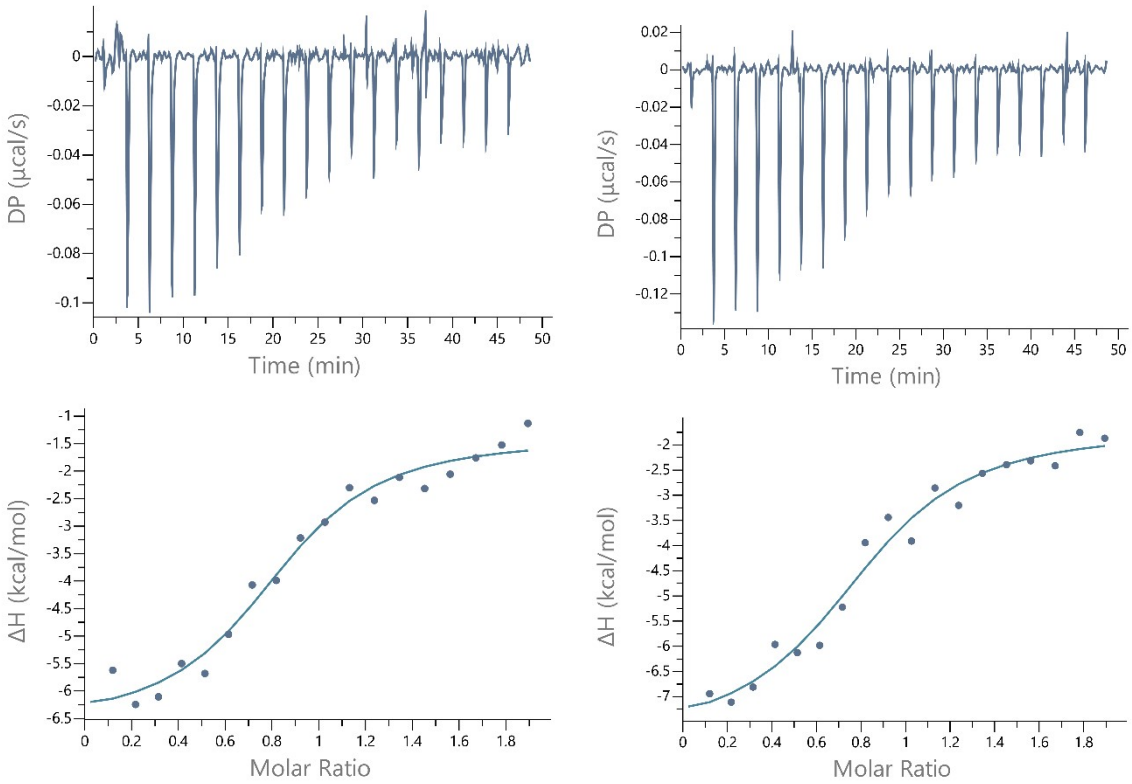
Supplemental figure 3: Symmetry mates in 20 Å distance show that the crystal packing of the structure is high.



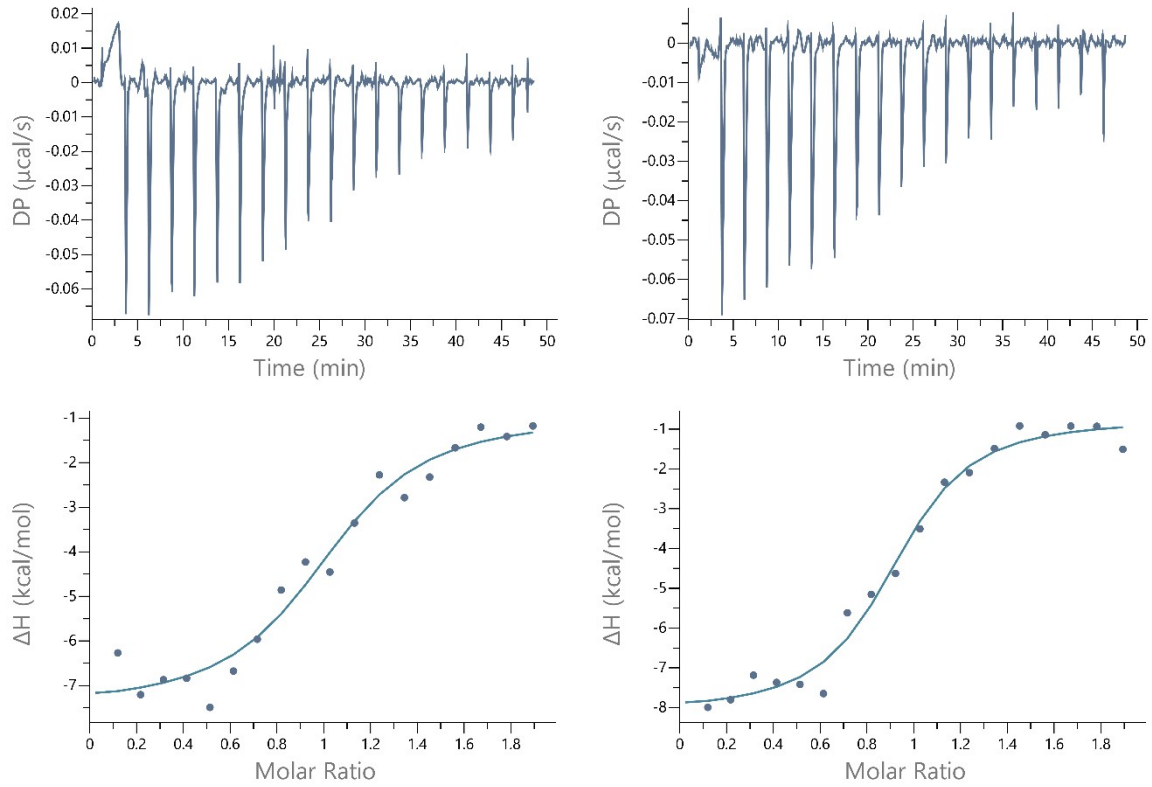
Supplemental figure 4: **(A/B)** The WBM binding site with **18Ac** bound overlays well with a peptide with similar

binding mode of Rbbp5 (PDB 2XL2) in the core regions of the peptide. **(C/D)** $2F_O$-F_C-maps of ligands a and b respectively indicate correct posing of the ligand in the WBM site. **(E)** The ligand **18Ac** binds to the WBM interface without obstructing the apo-fold compared to a published apo-structure (PDB 2H14). **(F)** Like in previous structures, the channel in the WD-fold is filled with many structured waters. **(G)** Chain A forms a disulfide bridge with a symmetry related chain B.

Isothermal Titration Calorimetry results



Supplemental figure 5: ITC thermograms of compound **14.**



Supplemental figure 6: ITC thermograms of compound **18**.

Supplemental Table 3: Thermodynamic parameters of peptides 14 and 18 as determined by ITC.

Peptide (replicate)	N	K_D (nM)	ΔG (kcal/mol)	ΔH (kcal/mol)	-TΔS (kcal/mol)
14 (1)	0.82	691	-8.40	-5.21	-3.20
14 (2)	0.81	919	-8.24	-6.12	-2.11
14 average ± sd	0.82 ± 0.01	805 ± 161	-8.32 ± 0.11	-5.67 ± 0.64	-2.66 ± 0.77
18 (1)	1	244	-9.02	-6.50	-2.52
18 (2)	0.90	153	-9.30	-7.37	-1.93
18 average ± sd	0.95 ± 0.07	199 ± 64	-9.16 ± 0.20	-6.94 ± 0.62	-2.23 ± 0.42

HPLC Analysis of compounds 1 -26

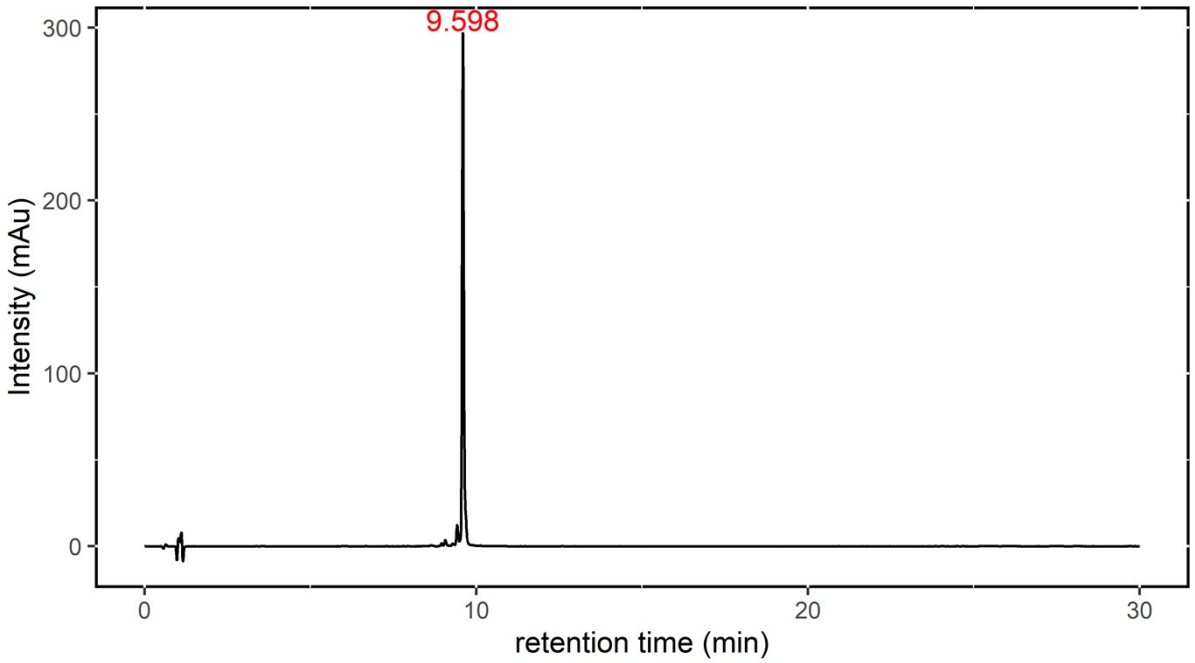
Analytical HPLC-MS was performed by using Agilent 1200 Infinity II LC system equipped with a C18 column (Agilent Poroshell 120, 2.7 µm, 3 x 100 mm, flow rate: 0.4 mL/min, temperature: 33 °C) and Agilent InfinityLab LC/MSD G6125C. Compounds were eluted with a gradient showing in the supplementary table 4. In short, a linear gradient from 5% to 95% (percentage of B. A: 0.1% TFA in H2O; B: 0.1% TFA in ACN) over 20 min. The whole UV-VIS spectrum was recorded during the run, and the selected channel (210 / 254 nm), which was indicated in supplemental table 5 for each peptide, was used to plot and calculate the purity of the sample. Isolating yield was calculated from dividing the amount of purified product by the amount of resin loading.

Supplemental Table 4. Gradient program for the analytical HPLC-MS analysis.

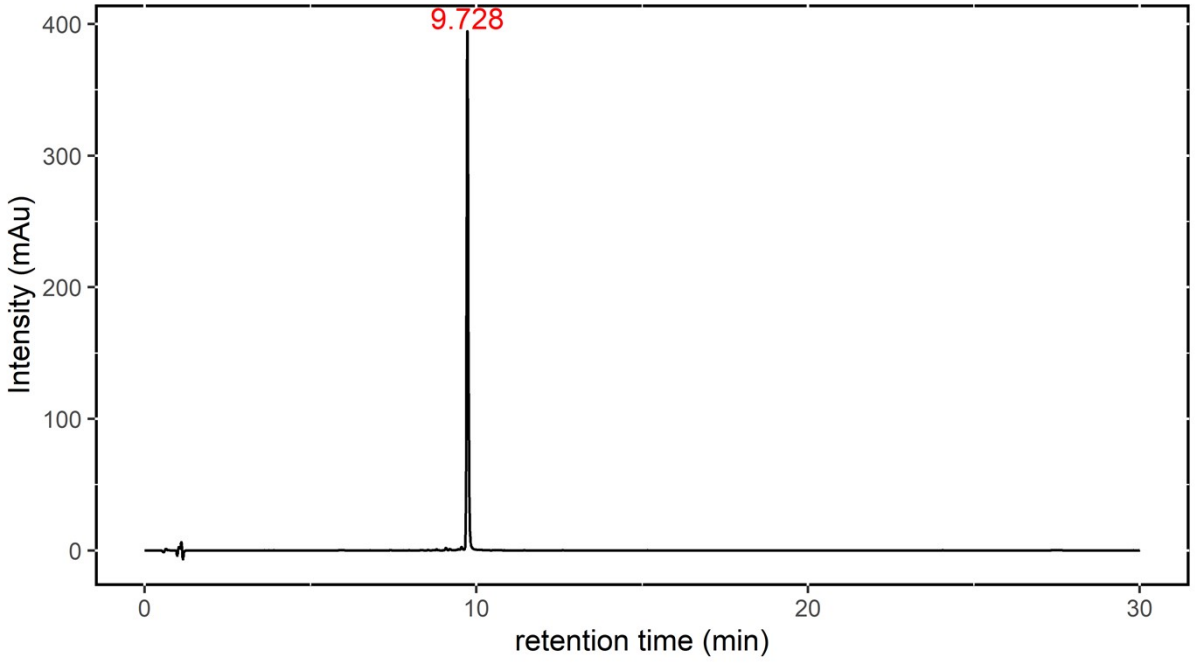
Time points (min)	0 min	1 min	21 min	25 min	27 min	30 min
B (%)	5%	5%	95%	95%	5%	5%

Supplemental Table 5. List of peptides' corresponding retention time, purity, isolating yield, and the wavelength of UV detector.

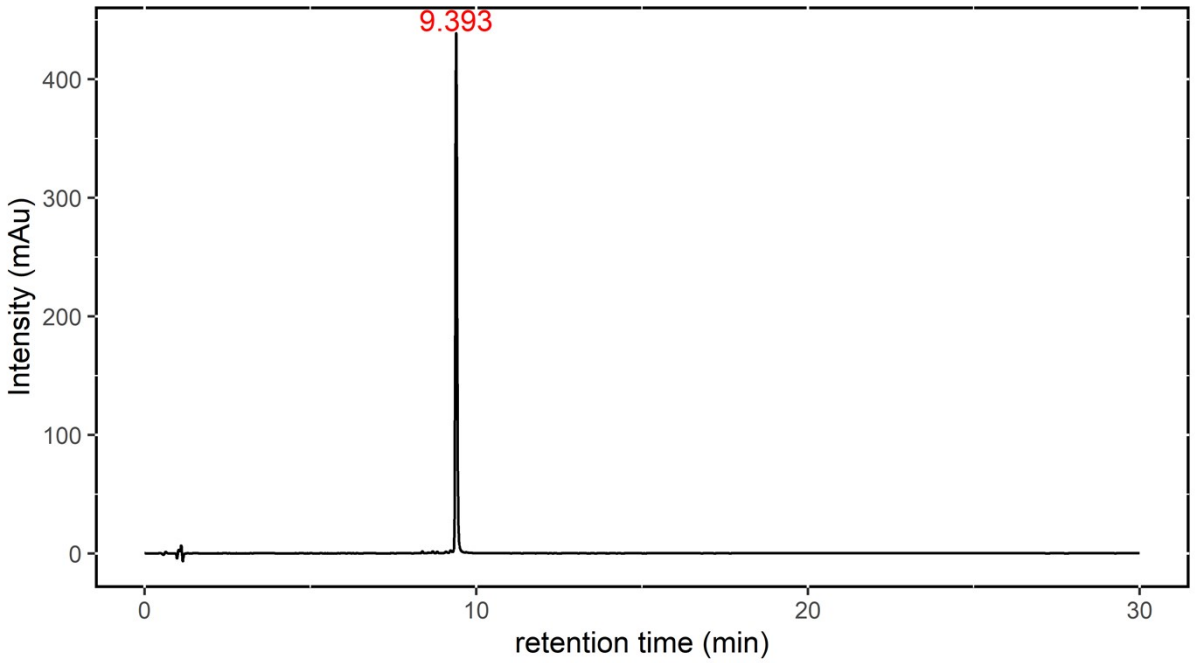
Peptide	Retention time (min)	Purity (%)	Wavelength	Isolating Yield (%)
1	9.598 min	97.2%	254 nm	5.4%
2	9.728 min	98.6%	254 nm	16.3%
3	9.393 min	98.5%	254 nm	6.2%
4	9.562 min	99.5%	254 nm	9.1%
5	9.456 min	96.7%	254 nm	28.9%
6	9.529 min	98.1%	254 nm	10.2%
7	9.592 min	97.2%	254 nm	28.7%
8	9.701 min	96.7%	254 nm	4.7%
9	9.969 min	97.4%	254 nm	3.1%
10	9.911 min	98.7%	254 nm	7.3%
11	10.17 min	97.6%	254 nm	14.7%
12	10.16 min	97.2%	254 nm	12.4%
13	10.23 min	97.8%	254 nm	10.0%
14	10.29 min	95.8%	254 nm	24.2%
15	11.09 min	99.3%	254 nm	34.2%
16	10.92 min	94.4%	254 nm	2.0%
17	10.90 min	90.3%	254 nm	1.2%
18	10.70 min	96.0%	254 nm	7.8%
18Ac	8.746 min	> 99.5%	210 nm	12.5%
19	10.64 min	97.7%	254 nm	1.9%
20	10.07 min	98.6%	254 nm	0.5%
21	11.01 min	97.8%	254 nm	0.2%
22	10.35 min	93.0%	254 nm	0.7%
23	10.05 min	78.4%	254 nm	7.3%
24	10.83 min	74.1%	254 nm	6.7%
25	10.82 min	75.9%	254 nm	3.4%
26	6.322 min	96.7%	210 nm	42.9%



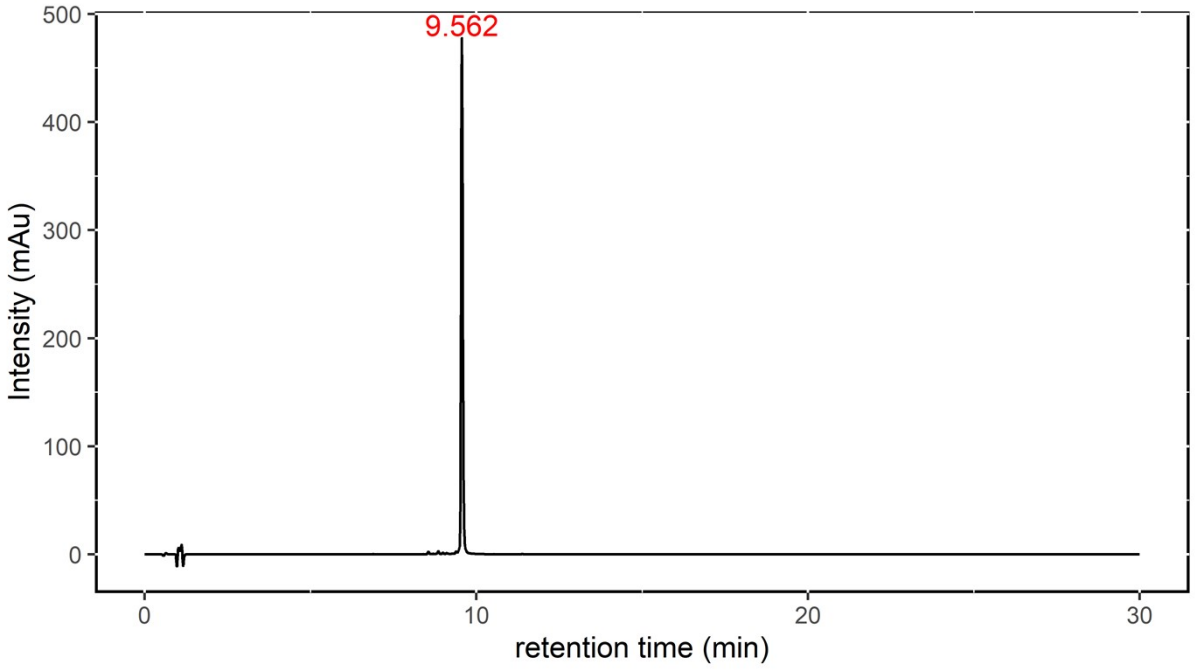
Supplemental figure 7: HPLC analysis of compound **1**



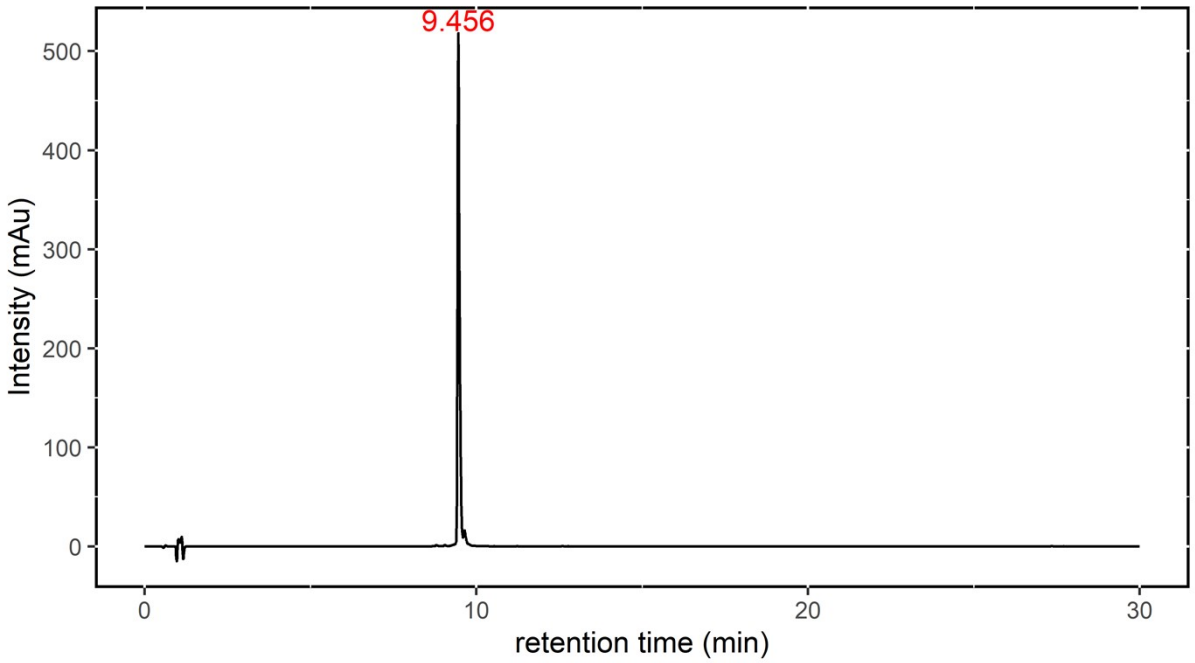
Supplemental figure 8: HPLC analysis of compound **2**



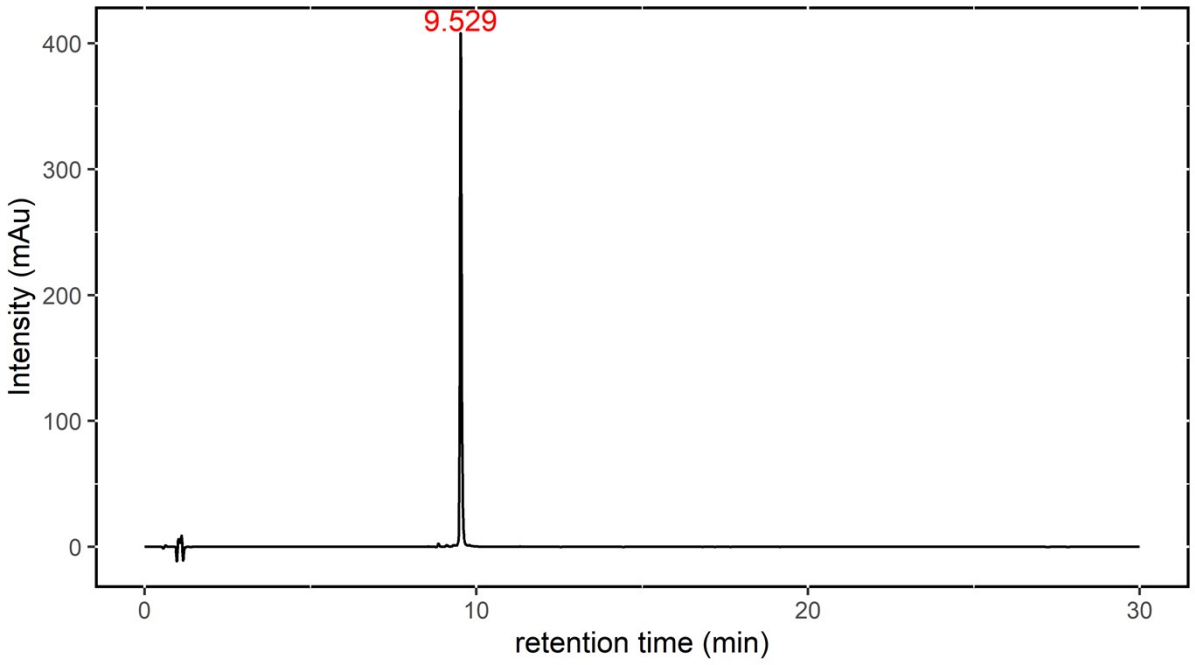
Supplemental figure 9: HPLC analysis of compound **3**



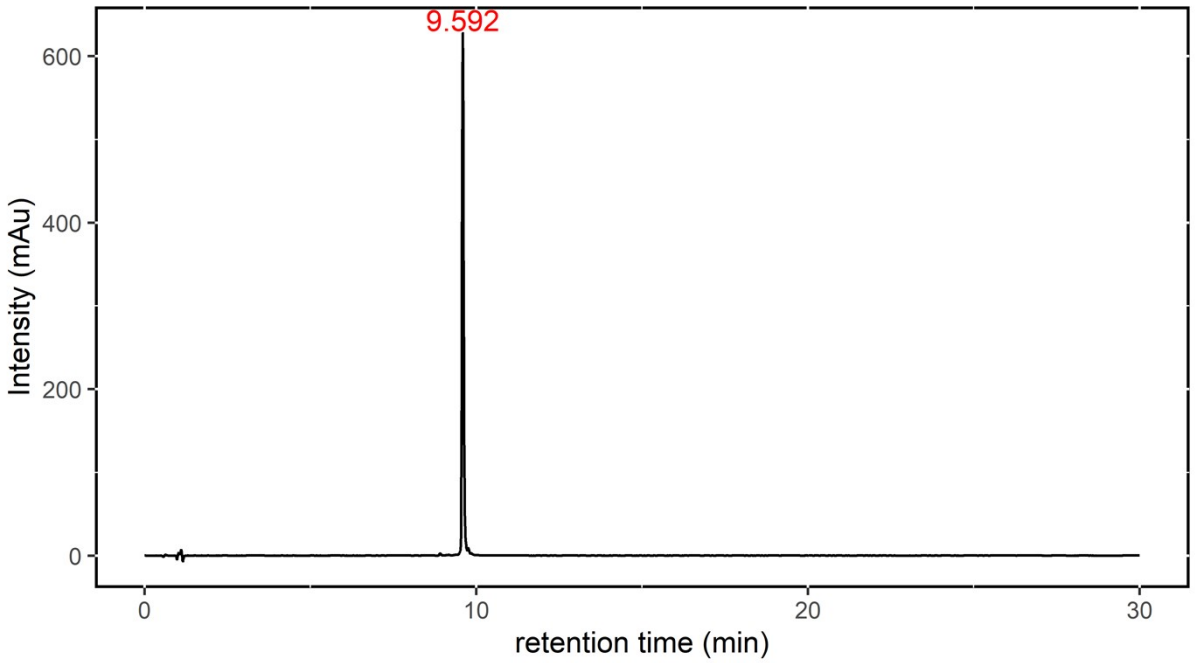
Supplemental figure 10: HPLC analysis of compound **4**



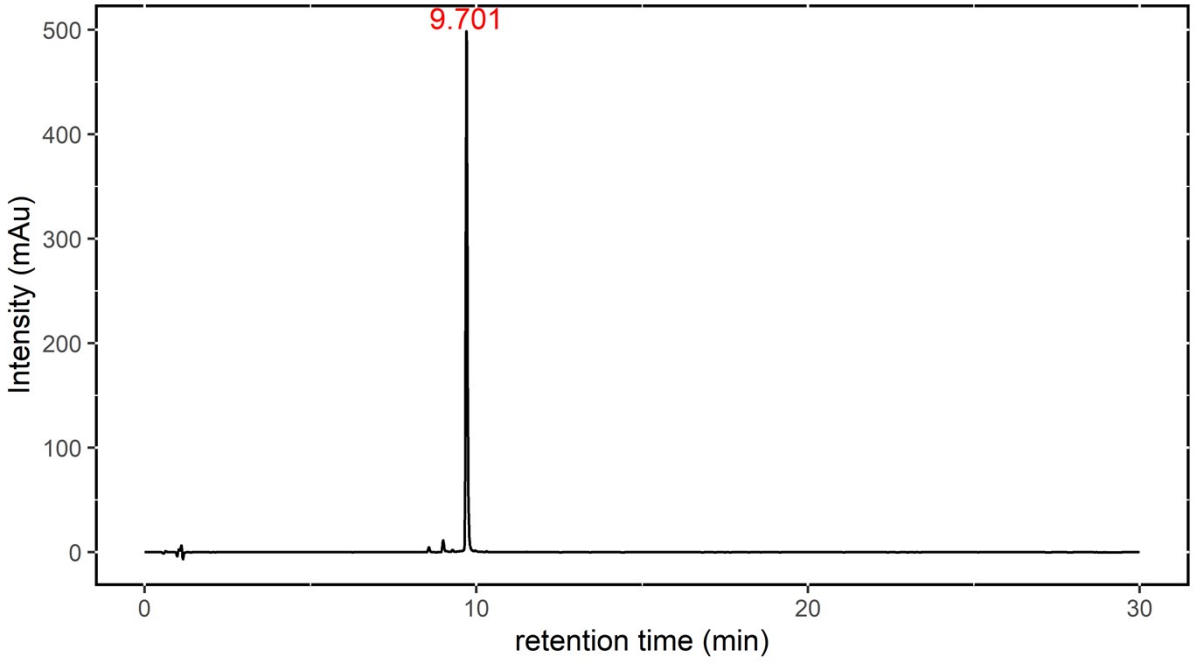
Supplemental figure 11: HPLC analysis of compound **5**



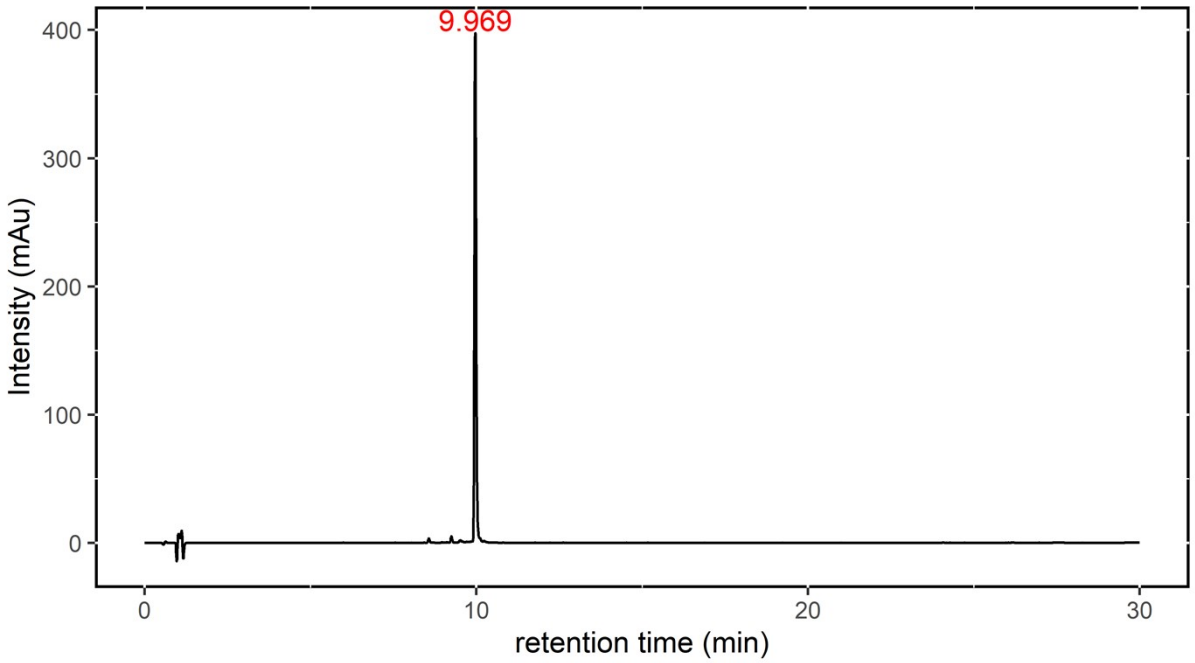
Supplemental figure 12: HPLC analysis of compound **6**



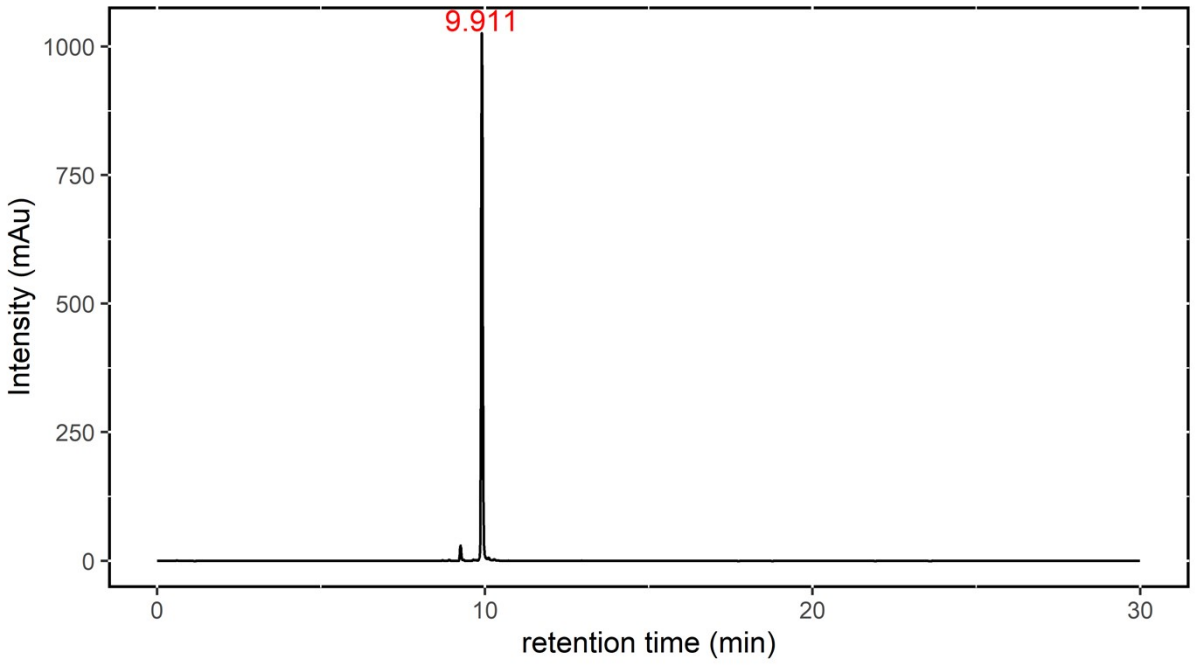
Supplemental figure 13: HPLC analysis of compound **7**



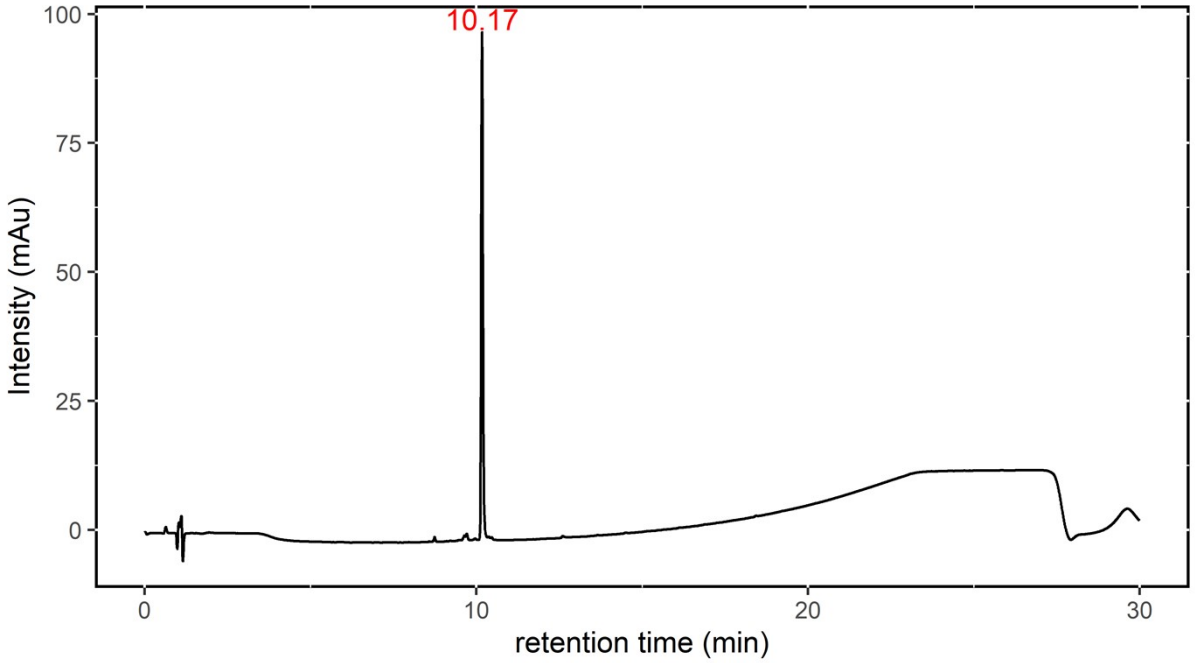
Supplemental figure 14: HPLC analysis of compound **8**



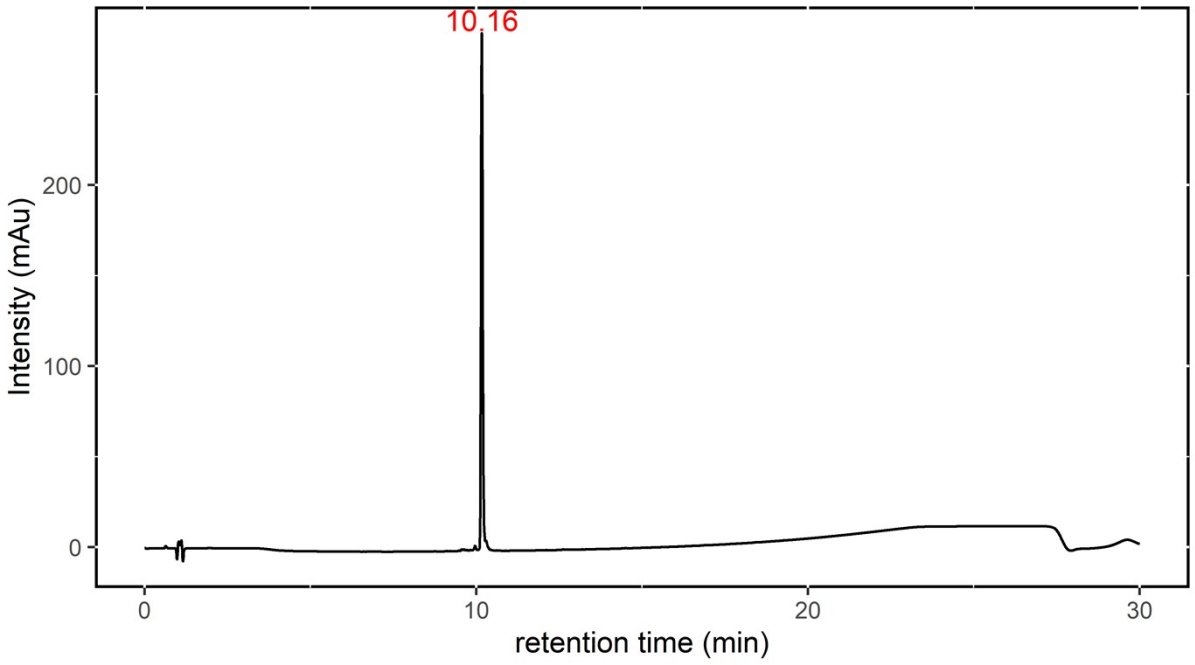
Supplemental figure 15: HPLC analysis of compound **9**



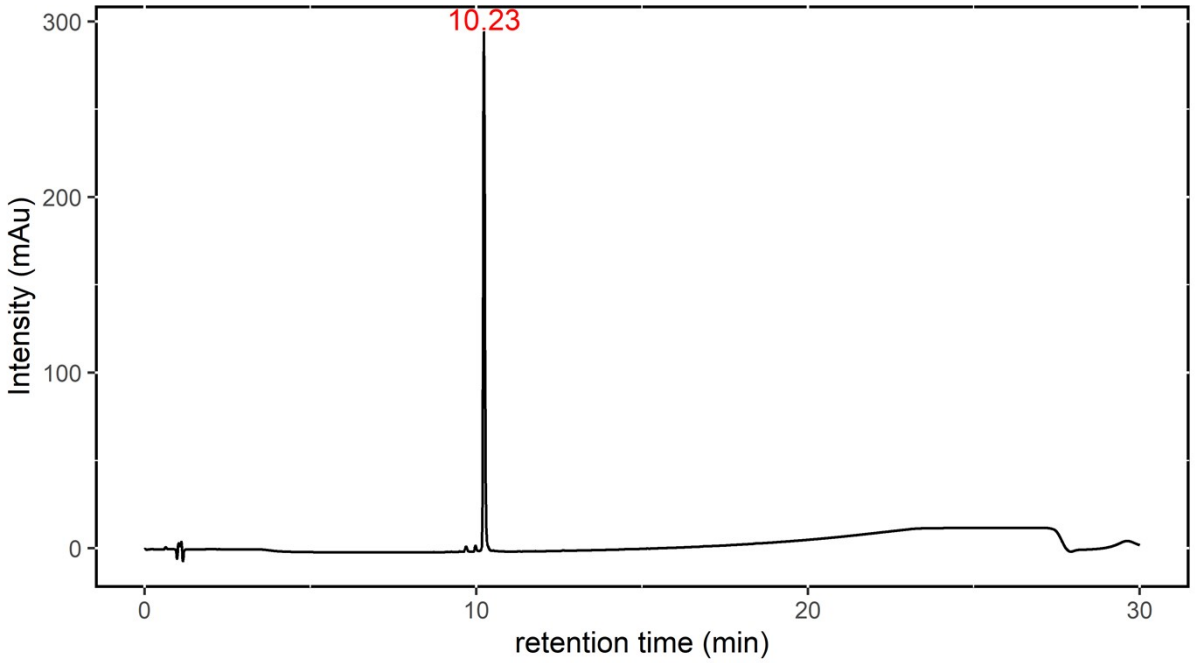
Supplemental figure 16: HPLC analysis of compound **10**



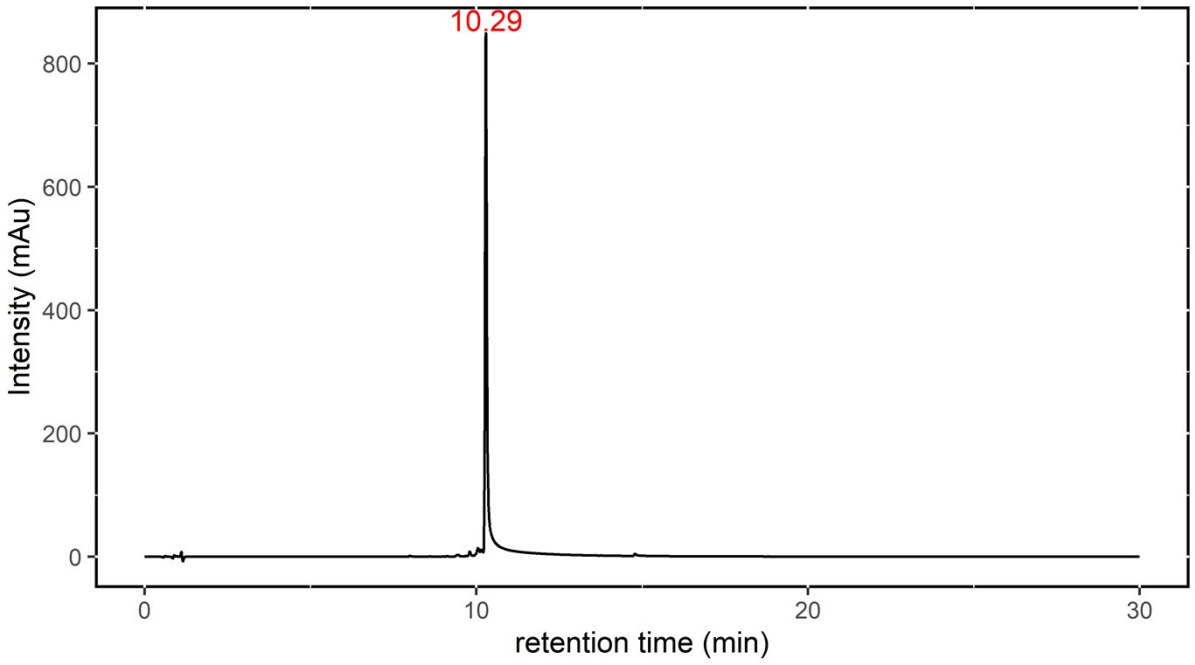
Supplemental figure 17: HPLC analysis of compound **11**



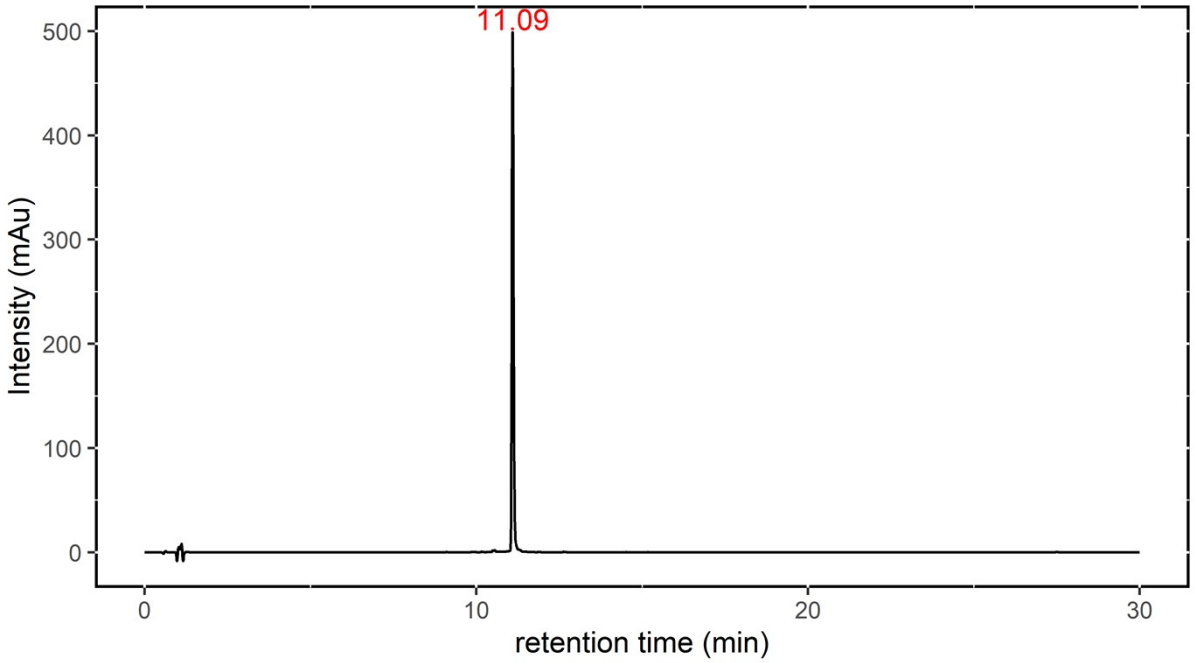
Supplemental figure 18: HPLC analysis of compound **12**



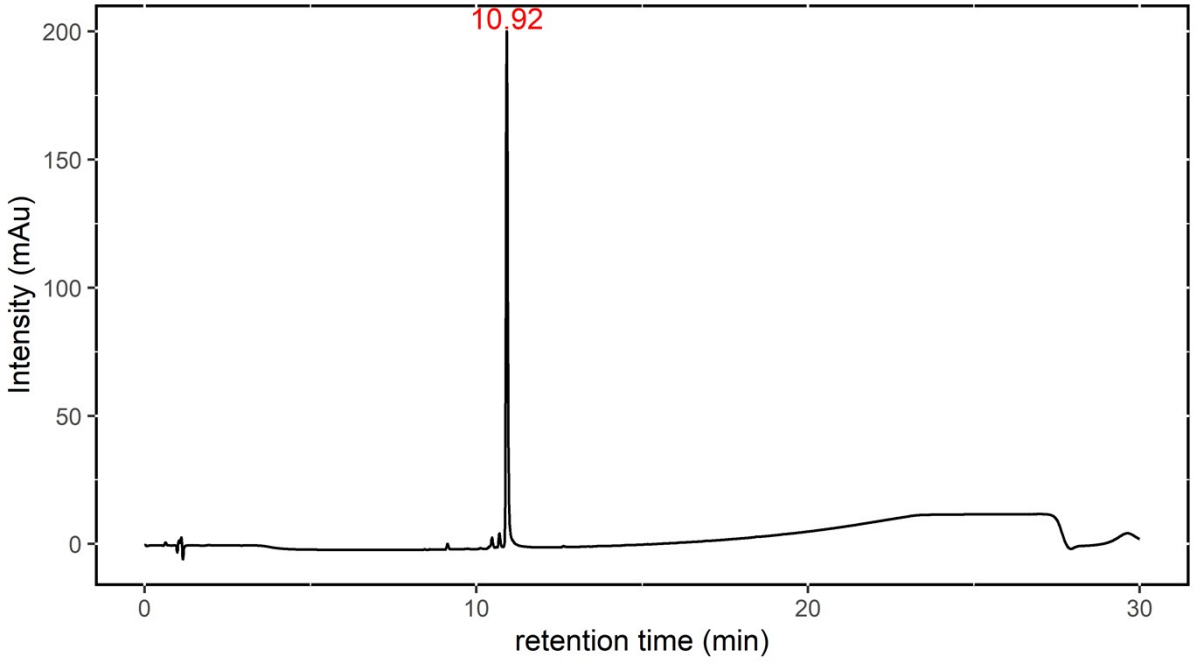
Supplemental figure 19: HPLC analysis of compound **13**



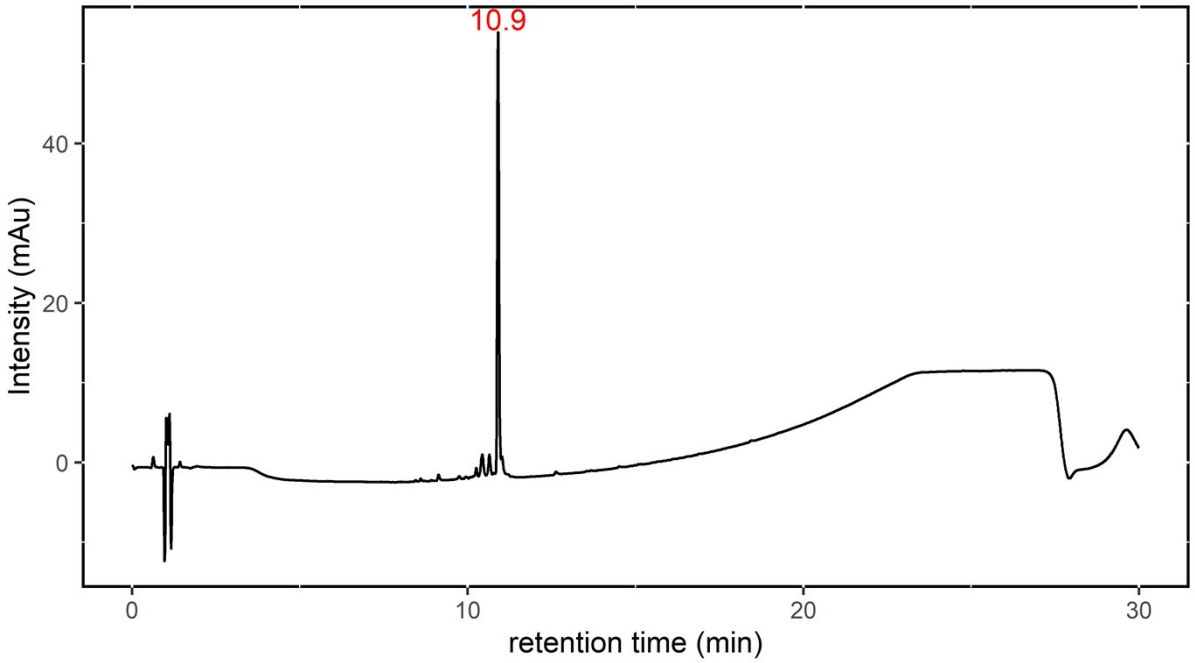
Supplemental figure 20: HPLC analysis of compound **14**



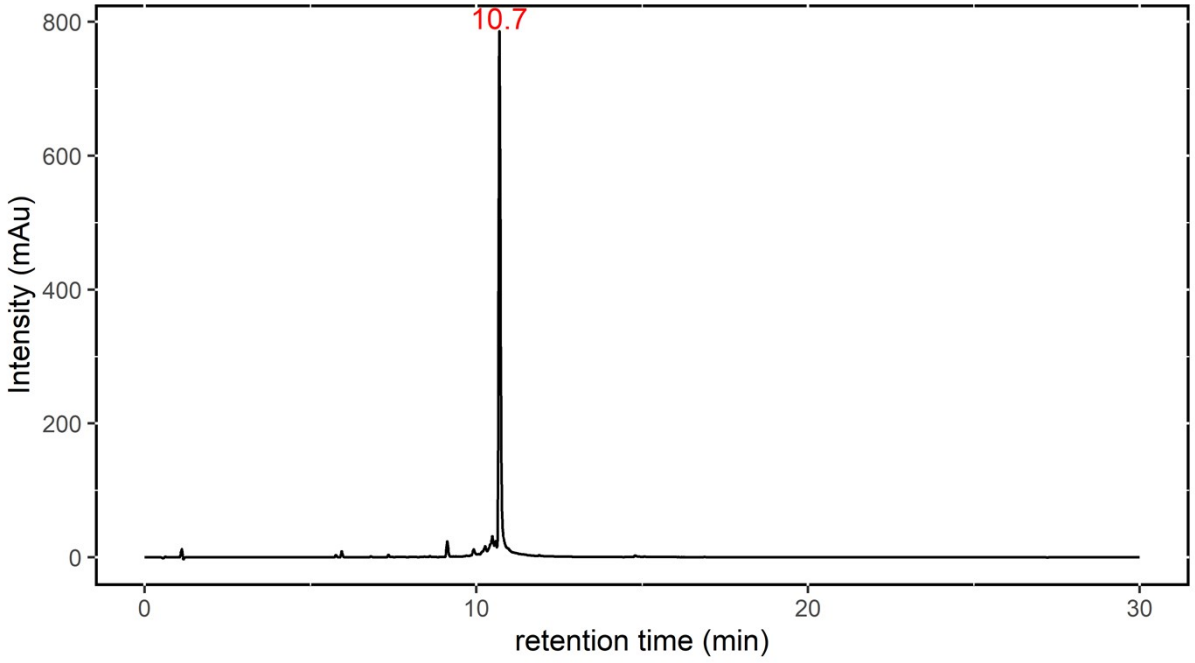
Supplemental figure 21: HPLC analysis of compound **15**



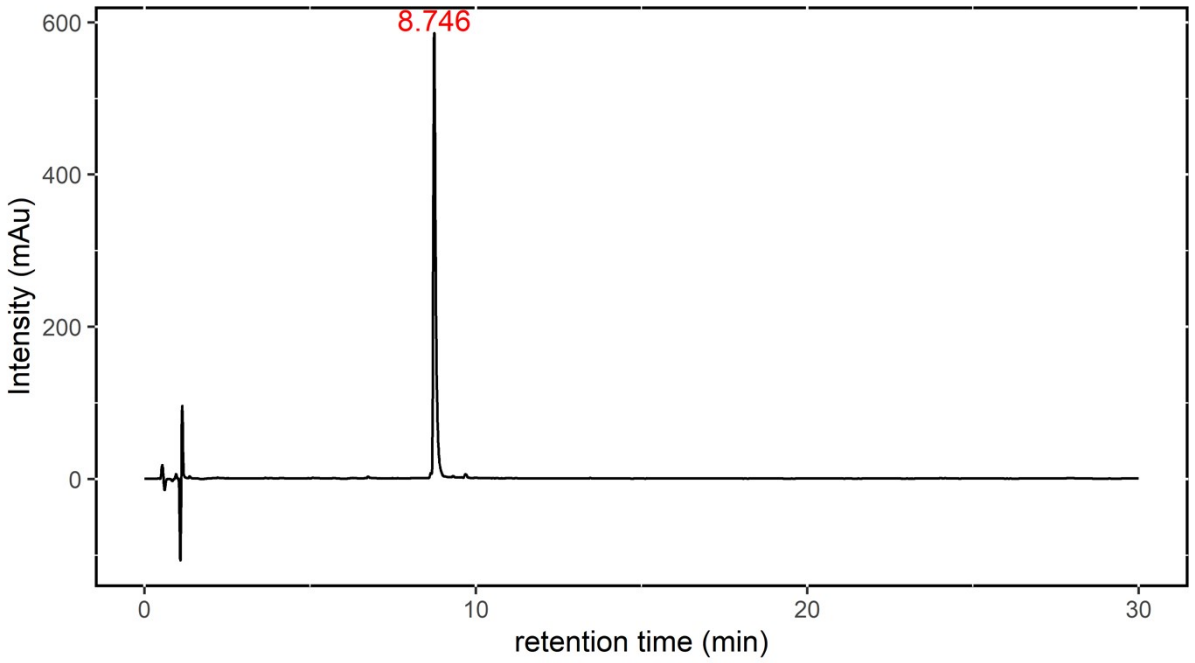
Supplemental figure 22: HPLC analysis of compound **16**



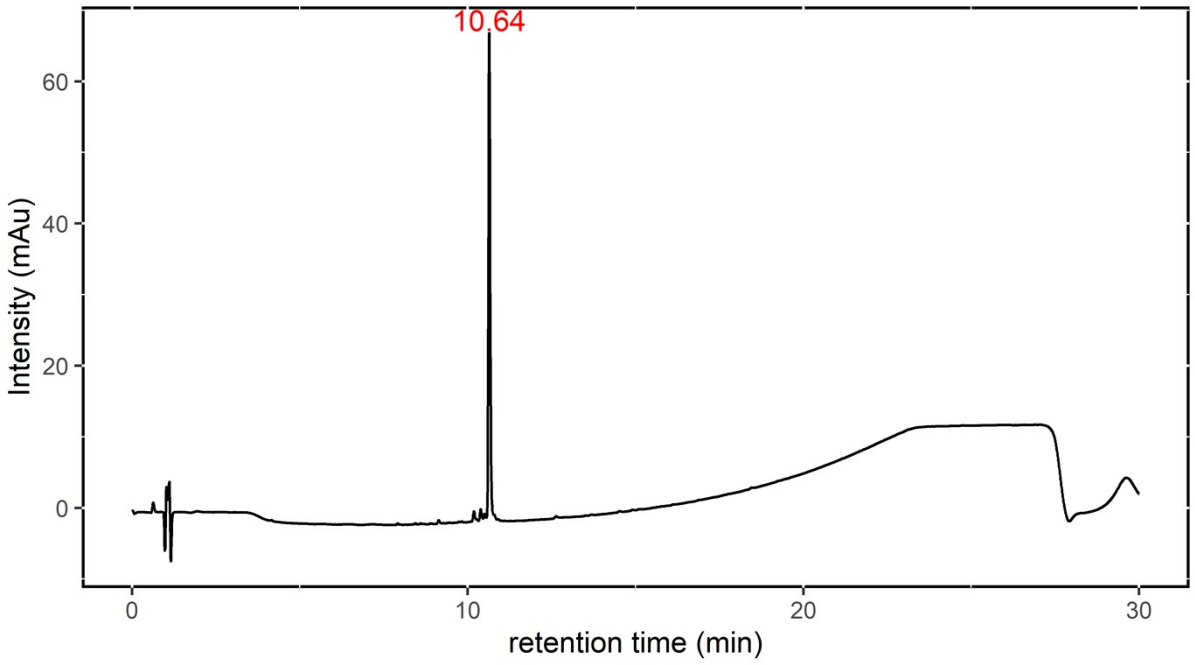
Supplemental figure 23: HPLC analysis of compound **17**



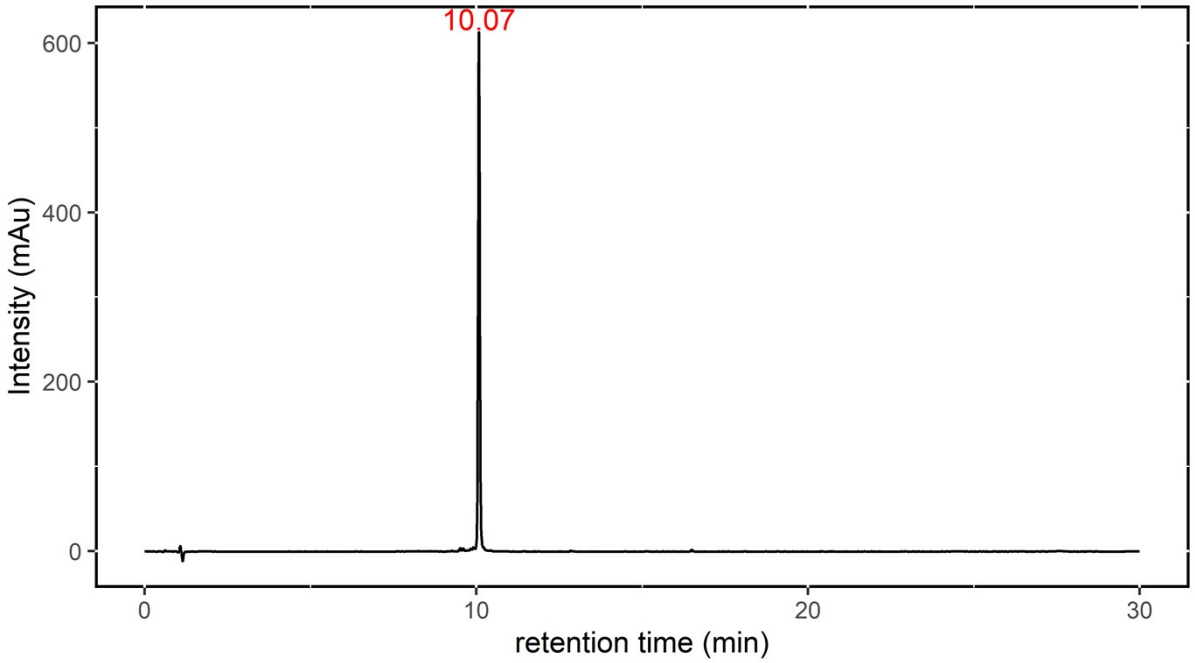
Supplemental figure 24: HPLC analysis of compound **18**



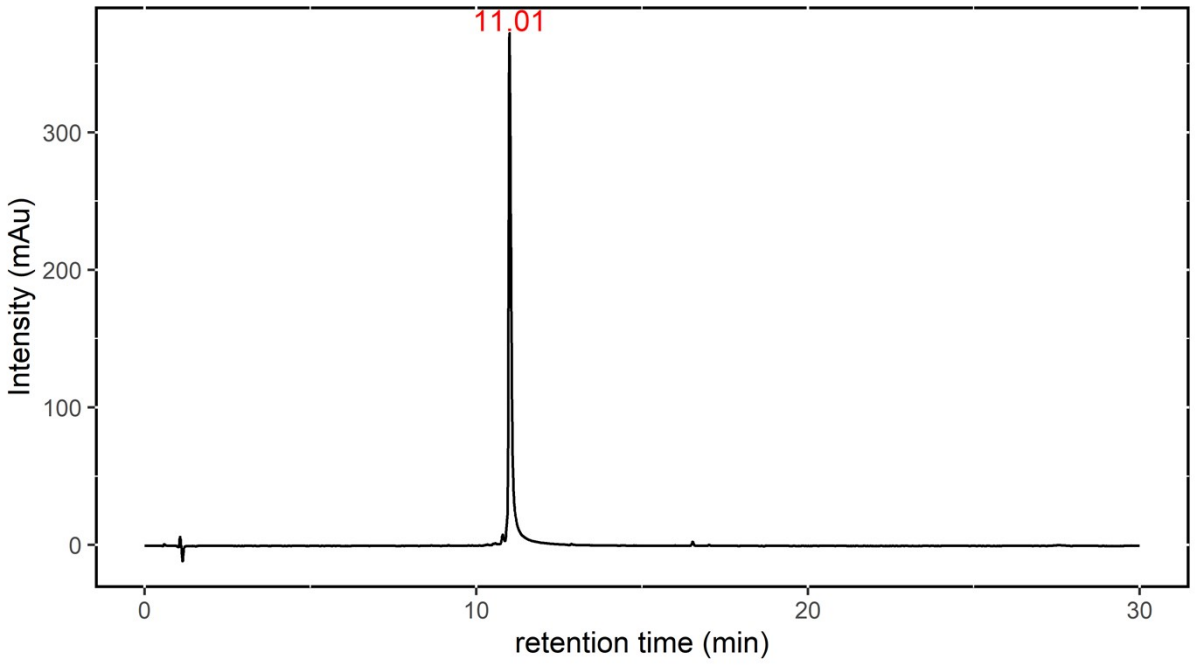
Supplemental figure 25: HPLC analysis of compound **18Ac**



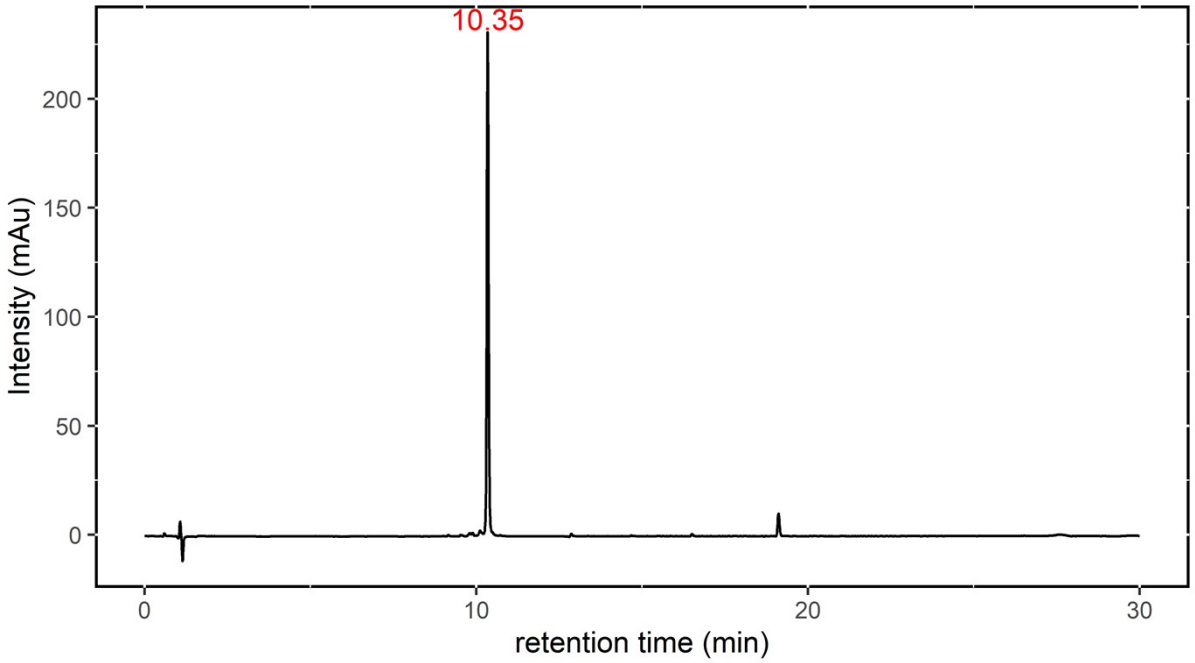
Supplemental figure 26: HPLC analysis of compound **19**



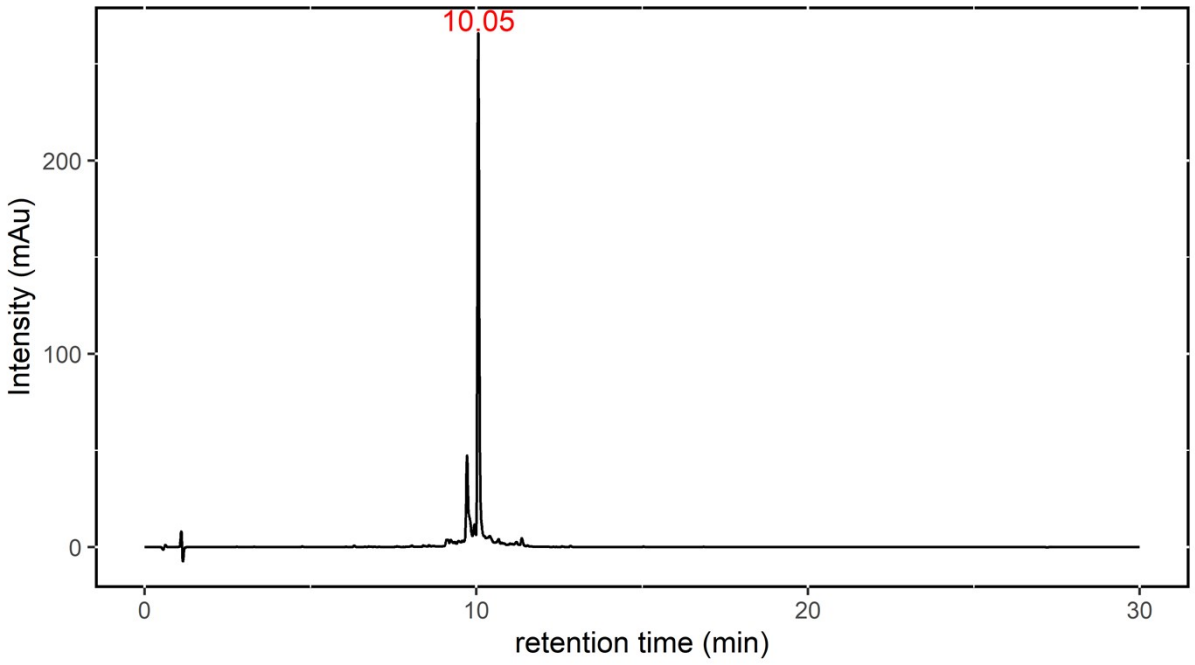
Supplemental figure 27: HPLC analysis of compound **20**



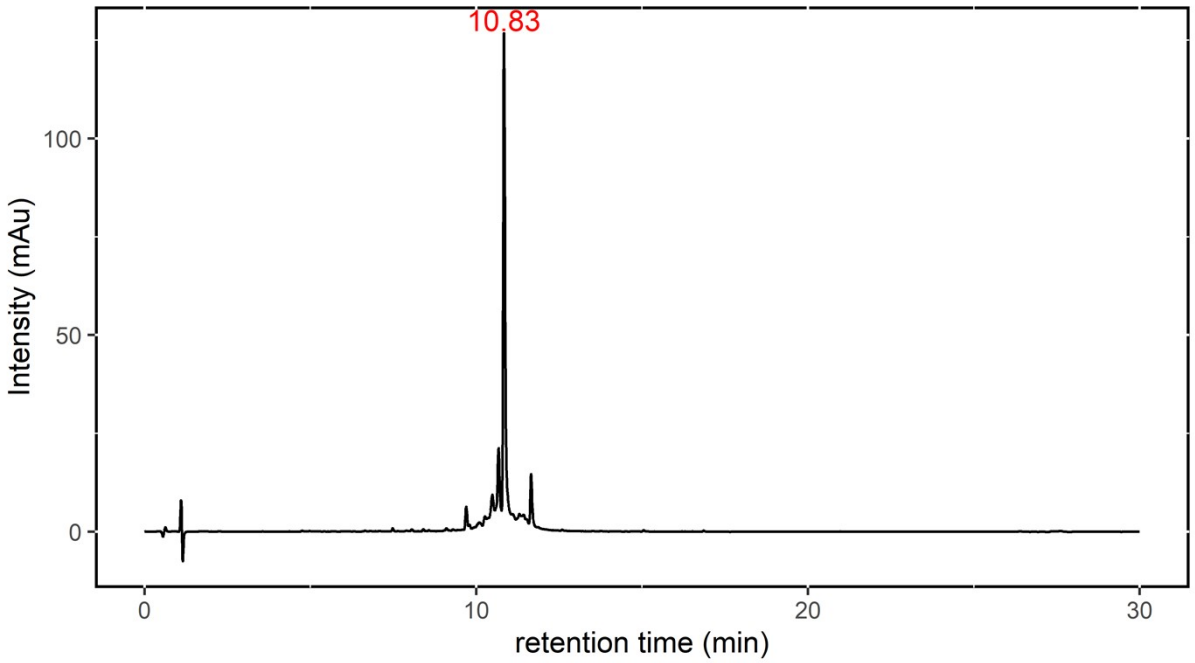
Supplemental figure 28: HPLC analysis of compound **21**



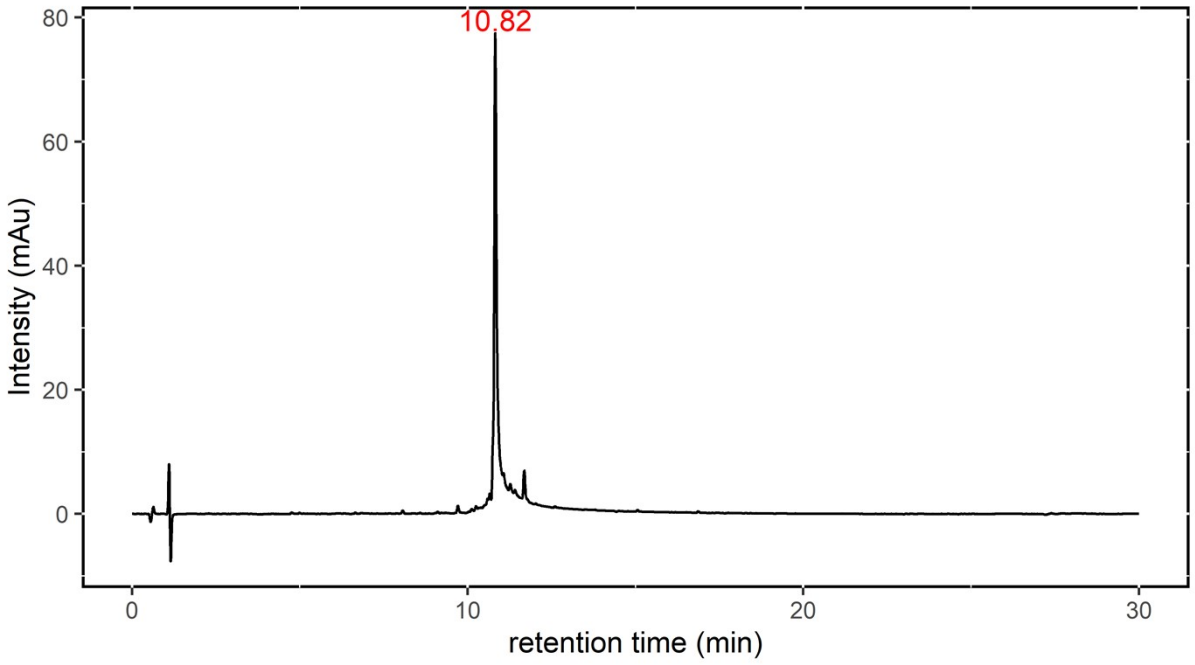
Supplemental figure 29: HPLC analysis of compound **22**



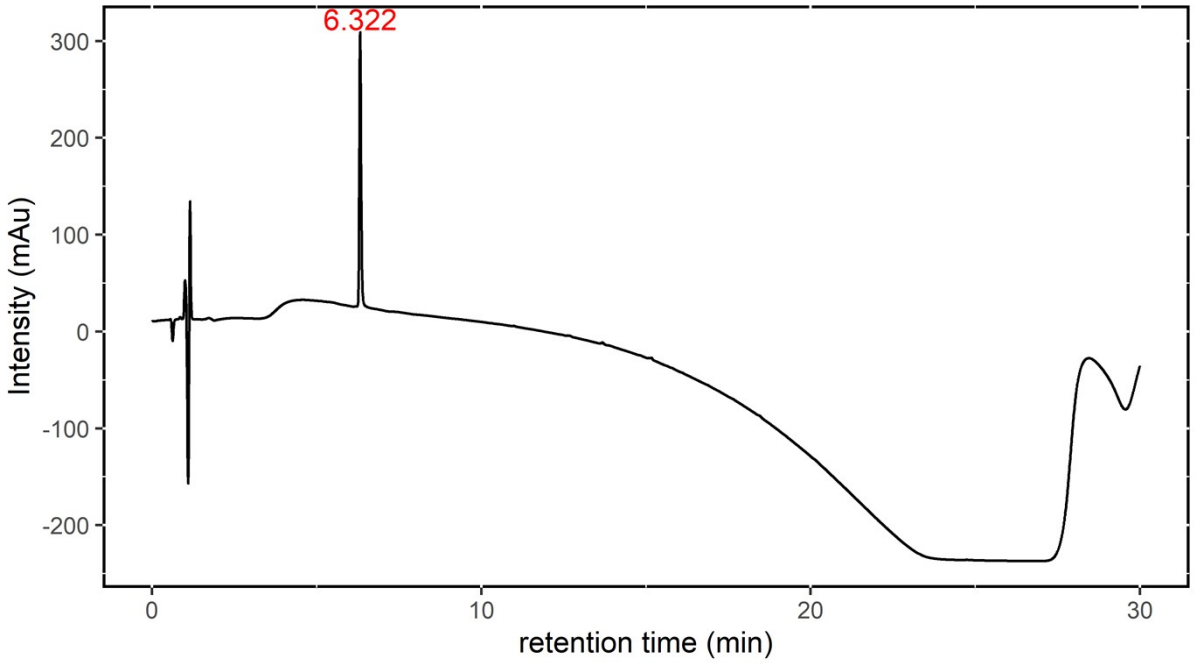
Supplemental figure 30: HPLC analysis of compound **23**



Supplemental figure 31: HPLC analysis of compound **24**



Supplemental figure 32: HPLC analysis of compound **25**

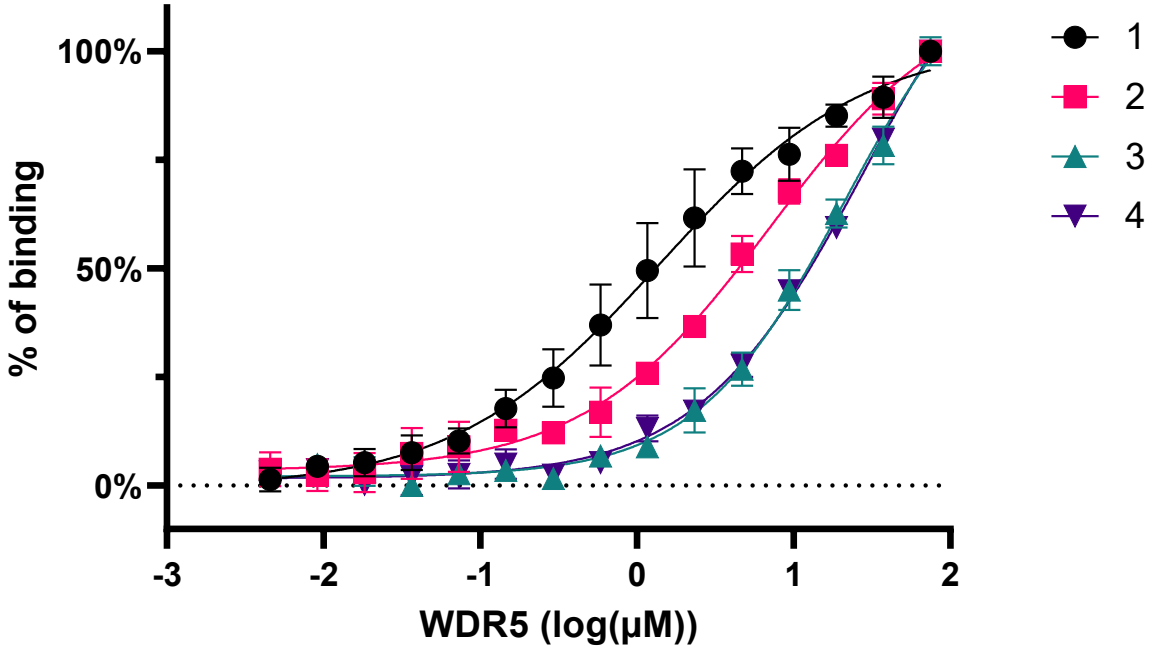


Supplemental figure 33: HPLC analysis of compound **26**

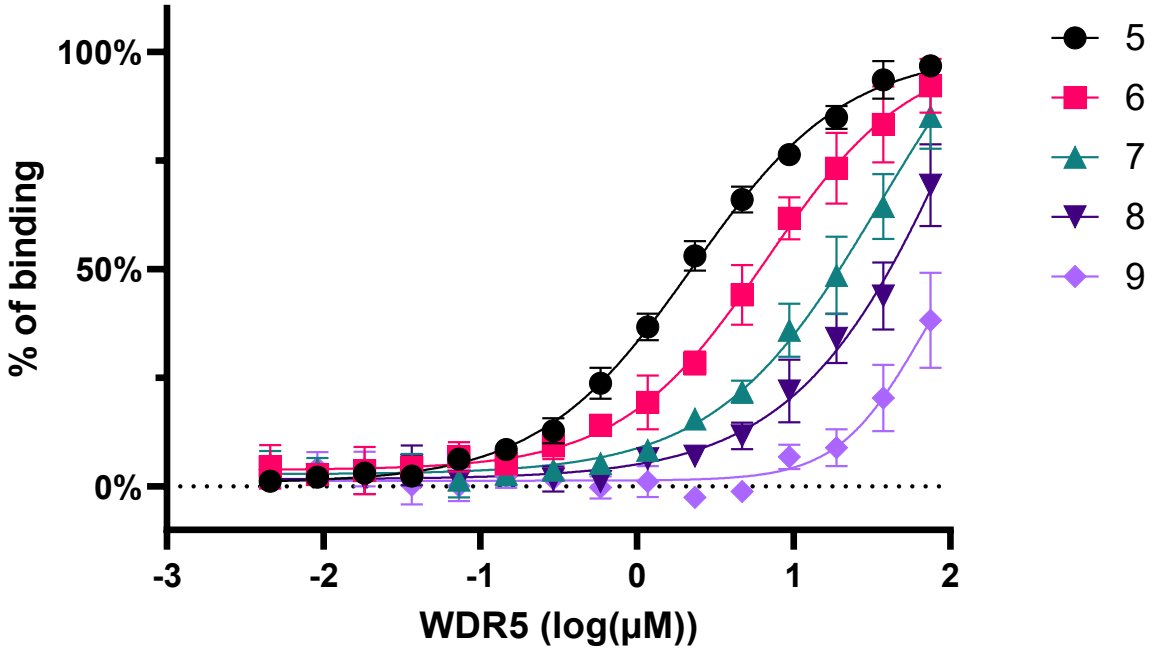
Results of Fluorescence Polarization

Fluorescence polarization measurement for direct binding assay

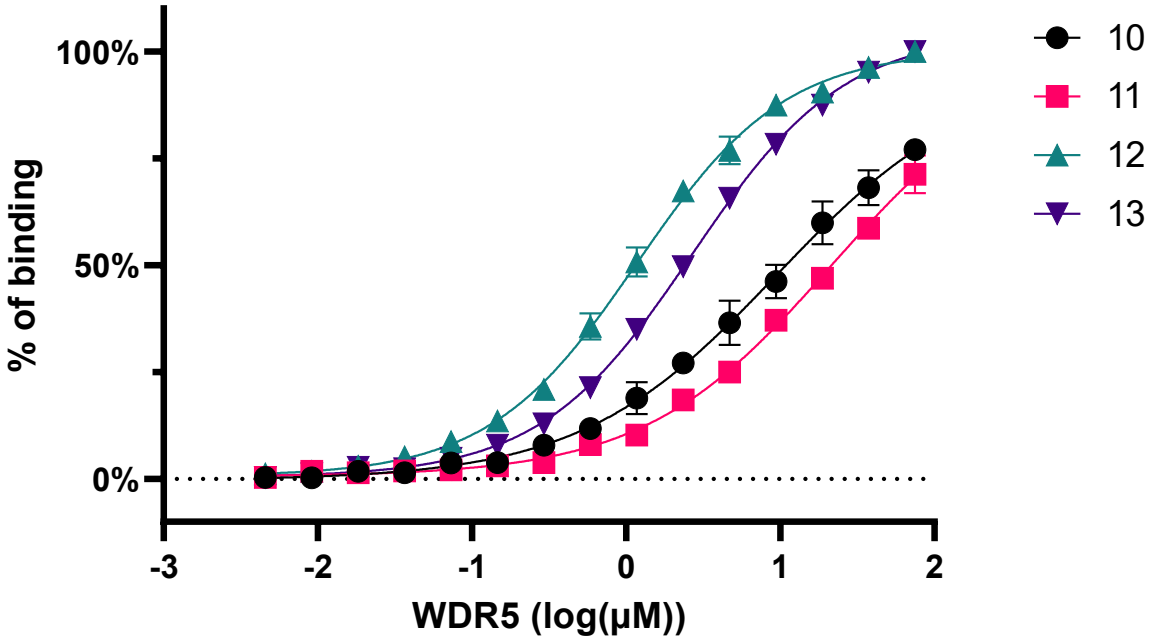
Fluorescence polarization measurements were performed by 2-fold dilution series of the protein and plotted using a logarithmic x-axis and normalized using the maximum and minimum binding values as 100 and 0% respectively.



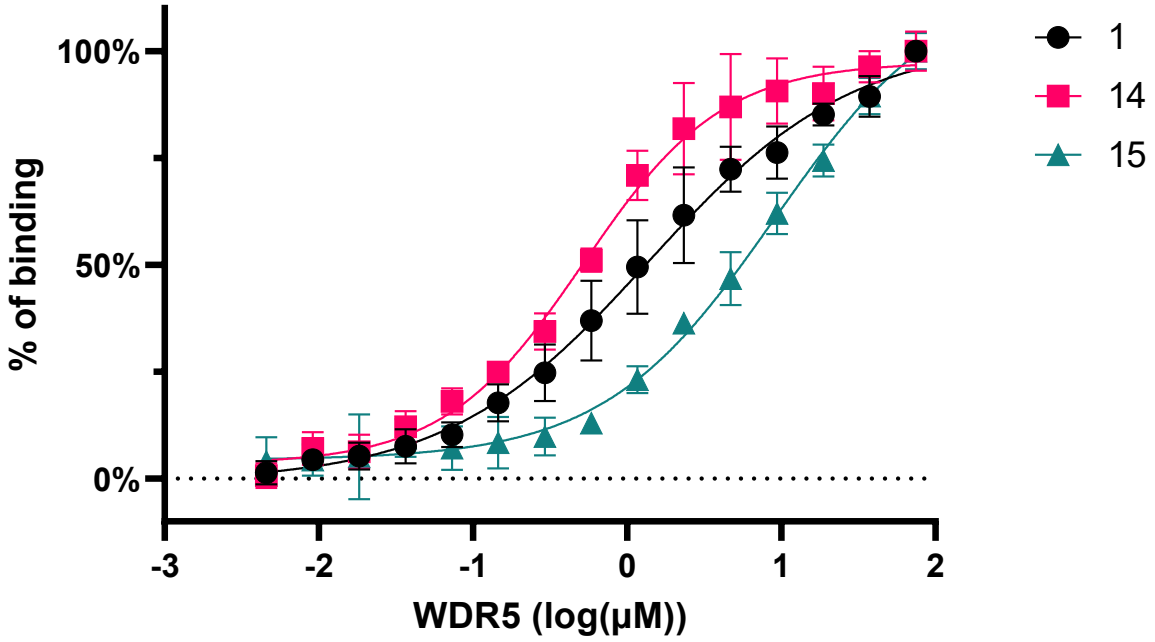
Supplemental figure 34: Direct fluorescence polarization binding curve for peptide **1** – **4**



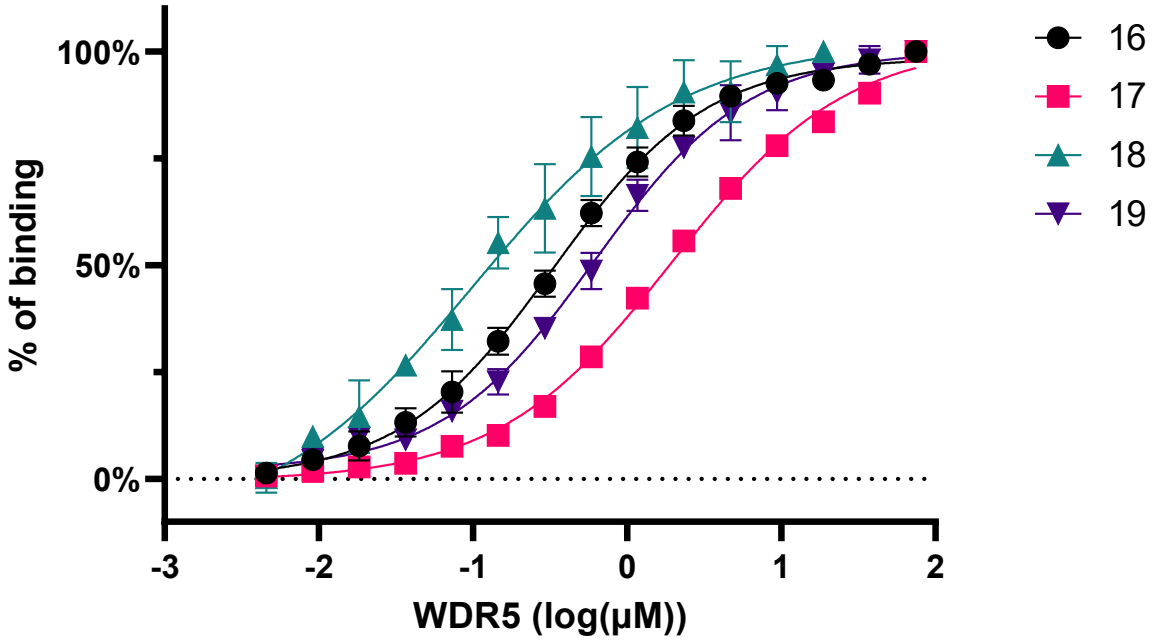
Supplemental figure 35: Direct fluorescence polarization binding curve for peptide **5** – **9**



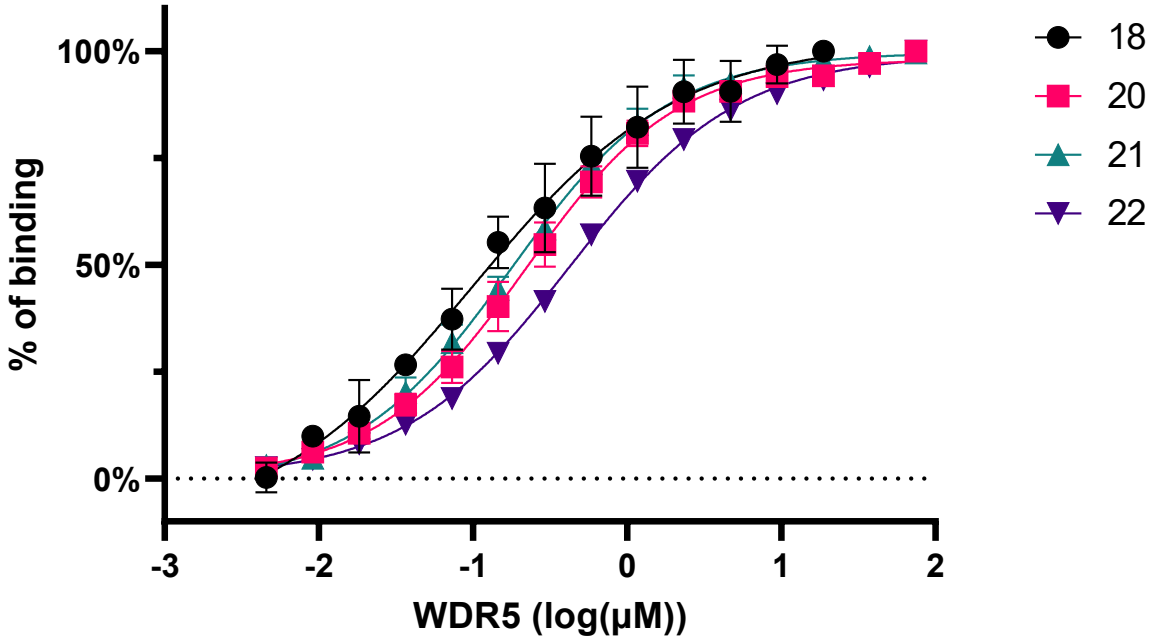
Supplemental figure 36: Direct fluorescence polarization binding curve for peptide **10** – **13**



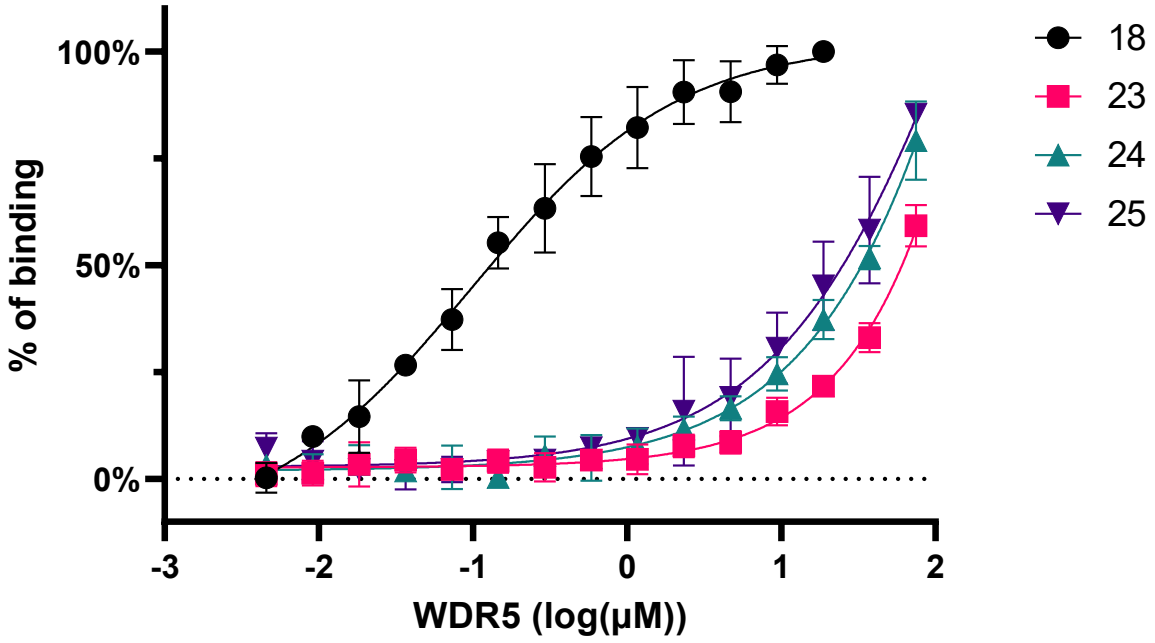
Supplemental figure 37: Direct fluorescence polarization binding curve for peptide **1**, **14** and **15**



Supplemental figure 38: Direct fluorescence polarization binding curve for peptide 16 – 19



Supplemental figure 39: Direct fluorescence polarization binding curve for peptide **18**, **20** – **22**



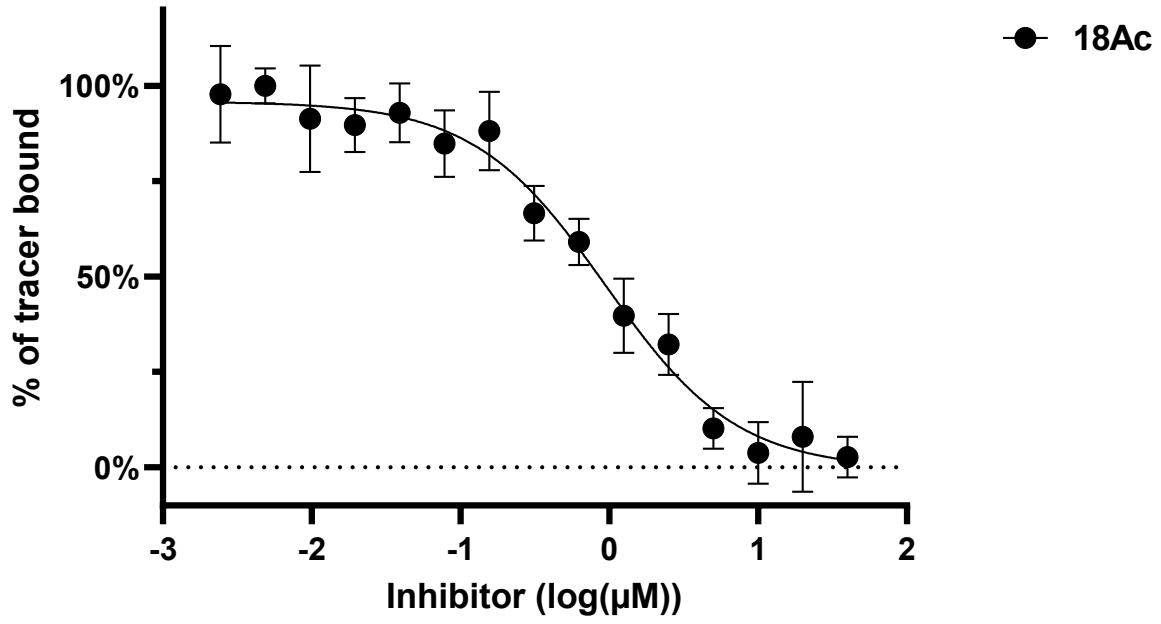
Supplemental figure 40: Direct fluorescence polarization binding curve for peptide **18**, **23** – **25**

Competitive fluorescence polarization assay

Competitive fluorescence polarization measurements were performed by 2-fold dilution series of the inhibitor against a fixed concentration of WDR5 (500 nM) and **18** (5 nM). The result was plotted using a logarithmic x-axis and normalized using the theoretical maximum and minimum binding values as 100 and 0% respectively. The raw value of fluorescence polarization was used, and IC50 was calculated by fitting with [Inhibitor] vs. Response – variable slope (four parameter) function for curve fitting. K_I was then calculated from IC_{50} by the method reported by Nikolovska-Coleska et al.[15] using the following equation:

$$K_I = [I]_{50}/(\frac{[L]_{50}}{K_D} + \frac{[P]_0}{K_D} + 1)$$

In this equation, $[I]_{50}$ is the concentration of the unbound inhibitor at 50% inhibition, $[L]_{50}$ is the concentration of the unbound tracer at 50% inhibition, and $[P]_0$ refers to the concentration of the free protein without any inhibition. The calculations were made using the excel sheet provided by the authors which can be found at: http://websites.umich.edu/~shaomengwanglab/software/calc_ki/index.html



Supplemental figure 41: Self competitive fluorescence polarization binding curve for peptide **18Ac** (Normalized)

Supplemental Table 6: IC_{50} and K_I for the **18Ac** self-competitive experiment.

Peptide	IC_{50} (nM)	K_I (nM)
18Ac	960 ± 141	97.2 ± 28.4

Results of RT-qPCR

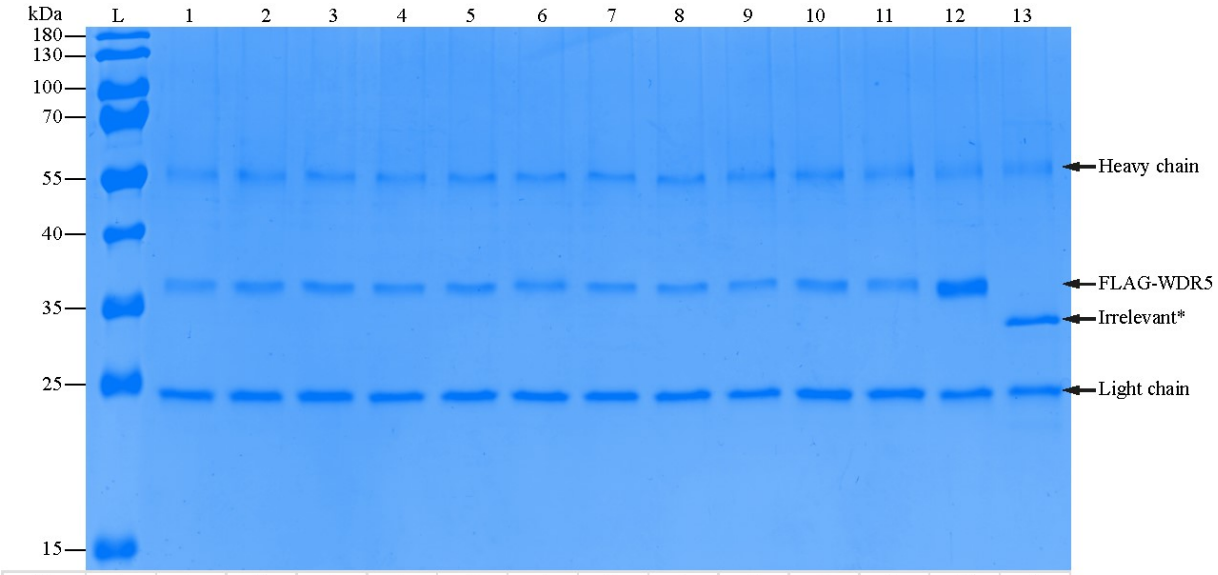
Supplemental Table 7. Details of the qPCR condition and the average Ct result.

Plate	1st					
Sample number	1	2	3	4	5	14
Sample name	DMSO	**18**	**18**	**18**	**23**	RNA input
Sample label	0.1%	1 μM	5 μM	10 μM	10 μM	0.5 μg
cDNA product input	0.5 μL	0.8 μL	0.8 μL	0.8 μL	0.8 μL	0.5 μL
Ct of HOTTIP (mean ± sd)	28.72 ± 0.04	28.75 ± 0.27	29.04 ± 0.09	29.06 ± 0.12	28.83 ± 0.09	27.42 ± 0.14
Ct of HOXC13-AS (mean ± sd)	25.01 ± 0.05	25.38 ± 0.06	25.57 ± 0.04	25.78 ± 0.12	25.21 ± 0.06	23.67 ± 0.04

Plate	2nd				
Sample number	1	8	11	12	14
Sample name	DMSO	**26**	no RNA	F266A	RNA input
Sample label	0.1%				0.5 μg
cDNA product input	0.5 μL	0.8 μL	0.8 μL	0.8 μL	0.5 μL
Ct of HOTTIP (mean ± sd)	29.93 ± 0.12	29.21 ± 0.45	n.d.*	30.74 ± 0.39	28.68 ± 0.23
Ct of HOXC13-AS (mean ± sd)	25.56 ± 0.15	24.86 ± 0.06	n.d.*	26.65 ± 0.02	24.30 ± 0.08

*n.d. not detected.

SDS-PAGE



Nr	L	1	2	3	4	5	6	7	8	9	10	11	12	13
Label	Ladder	Blank	test 1 µM	test 5 µM	test 10 µM	cyclic neg	Irrelevant*	Irrelevant*	win test	Irrelevant*	Irrelevant*	No RNA	F266A	Irrelevant*
Protein	-	FLAG-WDR5	FLAG-WDR5	FLAG-WDR5	FLAG-WDR5	FLAG-WDR5			FLAG-WDR5			FLAG-WDR5	FLAG-F266A	
RNA	-	+	+	+	+	+			+			-	+	
Peptides	-	-	**18** (1 µM)	**18** (5 µM)	**18** (10 µM)	**23** (10 µM)			**26** (10 µM)			-	-	

Supplemental figure 42: Protein loading control of RIP experiment. * Data was not discussed in this publication.

Reference:

(1) Thieriet, N.; Alsina, J.; Giralt, E.; Guibé, F.; Albericio, F. Use of Alloc-Amino Acids in Solid-Phase Peptide Synthesis. Tandem Deprotection-Coupling Reactions Using Neutral Conditions. *Tetrahedron Lett.* **1997**, *38* (41), 7275–7278. https://doi.org/10.1016/S0040-4039(97)01690-0.

(2) Chatterjee, J.; Laufer, B.; Kessler, H. Synthesis of N-Methylated Cyclic Peptides. *Nat. Protoc.* **2012**, *7* (3), 432–444. https://doi.org/10.1038/nprot.2011.450.

(3) Fukuyama, T.; Cheung, M.; Kan, T. N-Carboalkoxy-2-Nitrobenzenesulfonamides: A Practical Preparation of N-Boc-, N-Alloc-, and N-Cbz-Protected Primary Amines. *Synlett* **1999**, *1999* (8), 1301–1303. https://doi.org/10.1055/s-1999-2827.

(4) Karatas, H.; Townsend, E. C.; Bernard, D.; Dou, Y.; Wang, S. Analysis of the Binding of Mixed Lineage Leukemia 1 (MLL1) and Histone 3 Peptides to WD Repeat Domain 5 (WDR5) for the Design of Inhibitors of the MLL1−WDR5 Interaction. *J. Med. Chem.* **2010**, *53* (14), 5179–5185. https://doi.org/10.1021/jm100139b.

(5) Winter, G. *Xia2* : An Expert System for Macromolecular Crystallography Data Reduction. *J. Appl. Crystallogr.* **2010**, *43* (1), 186–190. https://doi.org/10.1107/S0021889809045701.

(6) Winn, M. D.; Ballard, C. C.; Cowtan, K. D.; Dodson, E. J.; Emsley, P.; Evans, P. R.; Keegan, R. M.; Krissinel, E. B.; Leslie, A. G. W.; McCoy, A.; McNicholas, S. J.; Murshudov, G. N.; Pannu, N. S.; Potterton, E. A.; Powell, H. R.; Read, R. J.; Vagin, A.; Wilson, K. S. Overview of the *CCP* 4 Suite and Current Developments. *Acta Crystallogr. D Biol. Crystallogr.* **2011**, *67* (4), 235–242. https://doi.org/10.1107/S0907444910045749.

(7) McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J. It Phaser Crystallographic Software. *J. Appl. Crystallogr.* **2007**, *40* (4), 658–674. https://doi.org/10.1107/S0021889807021206.

(8) Liebschner, D.; Afonine, P. V.; Baker, M. L.; Bunkóczi, G.; Chen, V. B.; Croll, T. I.; Hintze, B.; Hung, L.-W.; Jain, S.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R. D.; Poon, B. K.; Prisant, M. G.; Read, R. J.; Richardson, J. S.; Richardson, D. C.; Sammito, M. D.; Sobolev, O. V.; Stockwell, D. H.; Terwilliger, T. C.; Urzhumtsev, A. G.; Videau, L. L.; Williams, C. J.; Adams, P. D. Macromolecular Structure Determination Using X-Rays, Neutrons and Electrons: Recent Developments in Phenix. *Acta Crystallogr. Sect. Struct. Biol.* **2019**, *75* (10), 861–877. https://doi.org/10.1107/S2059798319011471.

(9) Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K. Features and Development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* **2010**, *66* (4), 486–501. https://doi.org/10.1107/S0907444910007493.

(10) Couture, J.-F.; Collazo, E.; Trievel, R. C. Molecular Recognition of Histone H3 by the WD40 Protein WDR5. *Nat. Struct. Mol. Biol.* **2006**, *13* (8), 698–703. https://doi.org/10.1038/nsmb1116.

(11) Patel, A.; Dharmarajan, V.; Cosgrove, M. S. Structure of WDR5 Bound to Mixed Lineage

Leukemia Protein-1 Peptide. *J. Biol. Chem.* **2008**, *283* (47), 32158–32161. https://doi.org/10.1074/jbc.C800164200.

(12) Fuentes-Iglesias, A.; Garcia-Outeiral, V.; Pardavila, J. A.; Wang, J.; Fidalgo, M.; Guallar, D. An Optimized Immunoprecipitation Protocol for Assessing Protein-RNA Interactions In Vitro. *STAR Protoc.* **2020**, *1* (2), 100093. https://doi.org/10.1016/j.xpro.2020.100093.

(13) Li, Y.; Liu, Y.; Chen, G.; Liu, H.; Wu, Y.; Liu, J.; Zhang, Z. HOTTIP Is Upregulated in Esophageal Cancer and Triggers the Drug Resistance.

(14) Subhash, S.; Mishra, K.; Akhade, V. S.; Kanduri, M.; Mondal, T.; Kanduri, C. H3K4me2 and WDR5 Enriched Chromatin Interacting Long Non-Coding RNAs Maintain Transcriptionally Competent Chromatin at Divergent Transcriptional Units. *Nucleic Acids Res.* **2018**, *46* (18), 9384–9400. https://doi.org/10.1093/nar/gky635.

(15) Nikolovska-Coleska, Z.; Wang, R.; Fang, X.; Pan, H.; Tomita, Y.; Li, P.; Roller, P. P.; Krajewski, K.; Saito, N. G.; Stuckey, J. A.; Wang, S. Development and Optimization of a Binding Assay for the XIAP BIR3 Domain Using Fluorescence Polarization. *Anal. Biochem.* **2004**, *332* (2), 261–273. https://doi.org/10.1016/j.ab.2004.05.055.