

**Supplemental information for: Discovery of cell active macrocyclic peptides with on-target inhibition of KRAS signaling**

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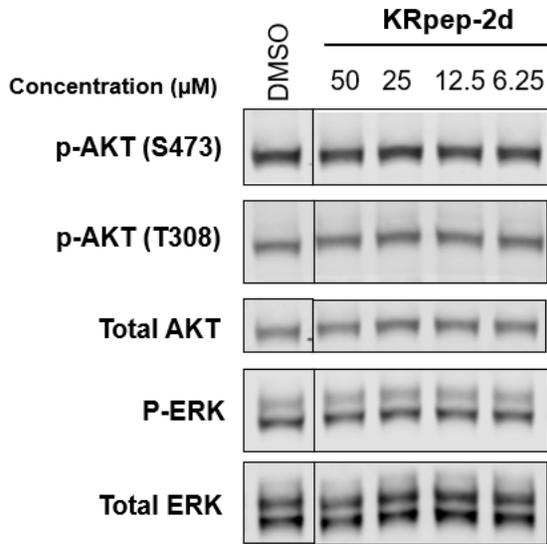
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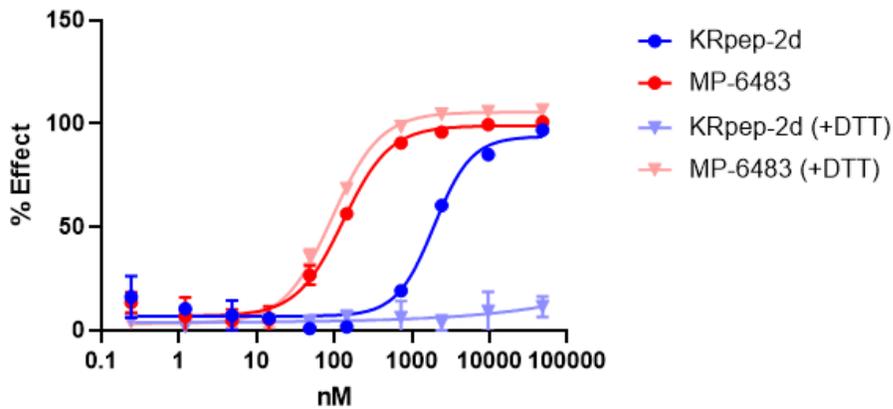
Supplementary Figures:

S1)



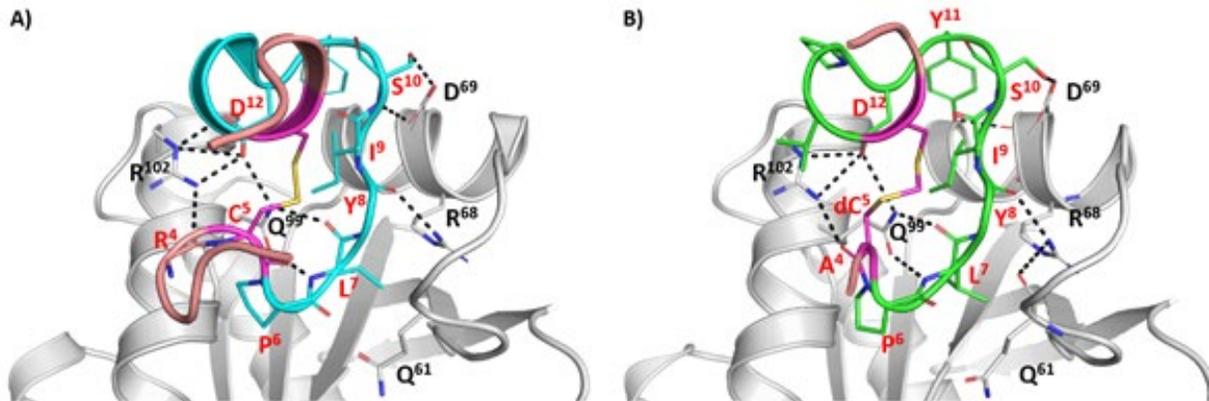
**Figure S1:** KRpep-2d does not show inhibition of phospho-ERK signaling in AsPC-1 (KRAS<sup>G12D</sup>) cells, as assessed by Western blot. Lane 1 is DMSO control.

S2)



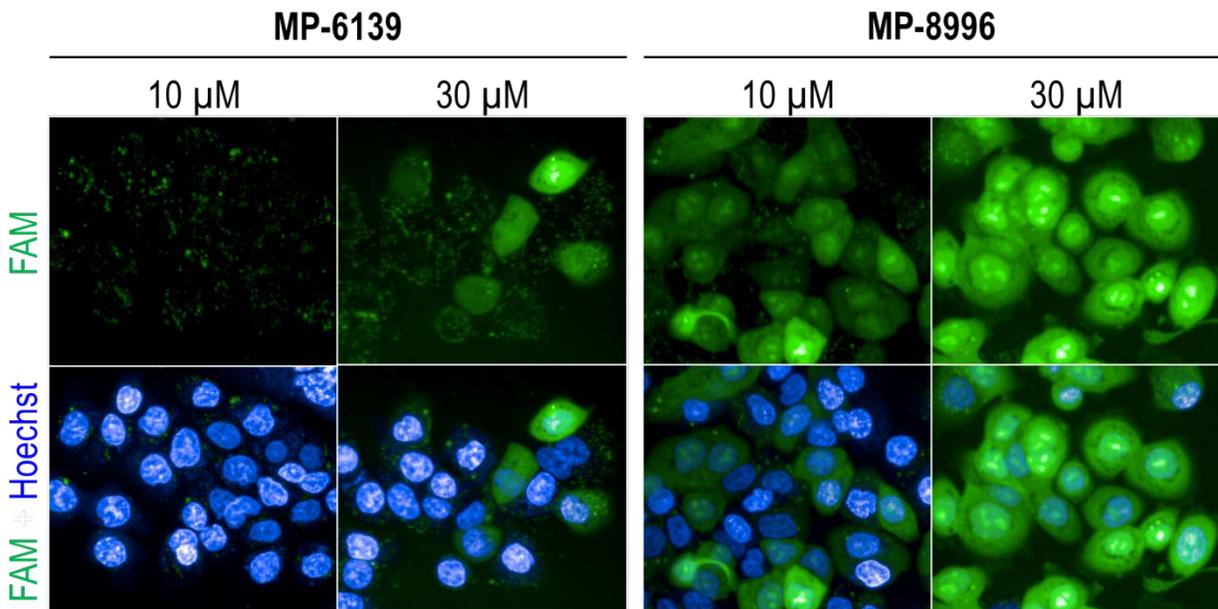
**Figure S2:** KRpep-2d (redox-sensitive macrocycle) loses binding to GDP-loaded KRAS<sup>G12D</sup> in the presence of DTT, whereas MP-6483 (redox-stable macrocycle) maintains binding, as assessed by TR-FRET assay.

S3)



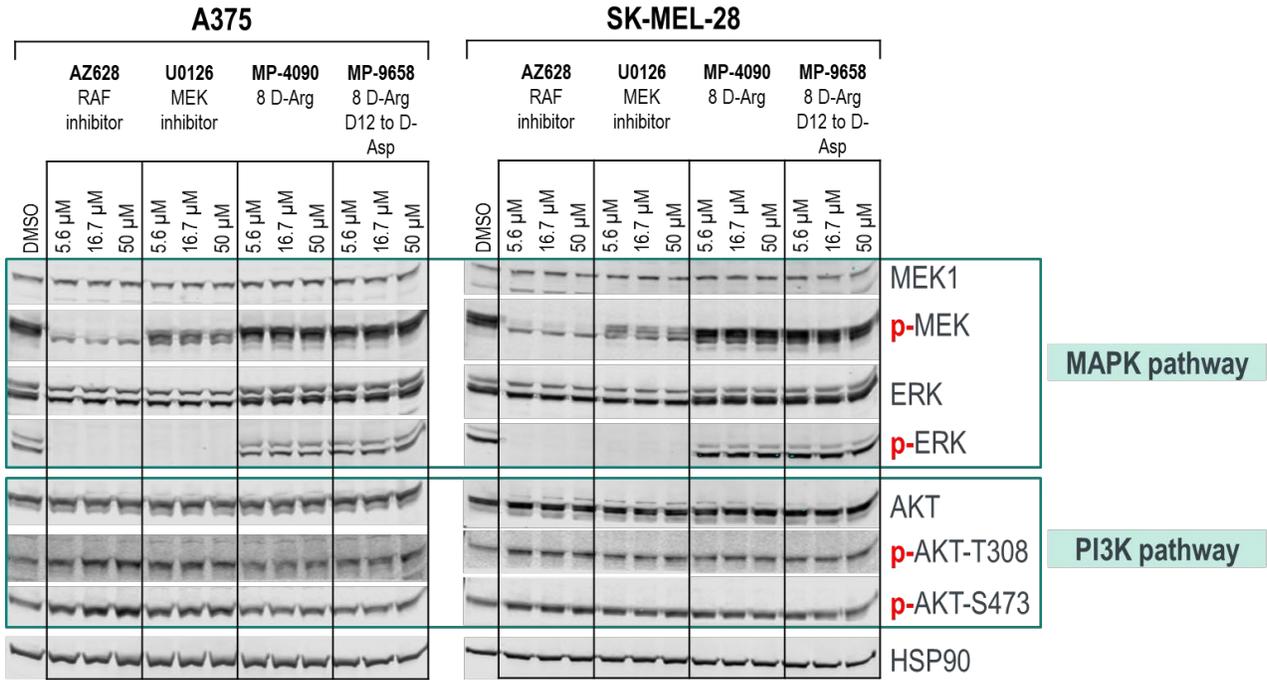
**Figure S3:** Structures of KRAS – peptide complexes, crystal structure of GDP-loaded KRAS<sup>G12D</sup> in complex with KRpep-2d (A) or molecular dynamics structure of GMPPCP-loaded KRAS<sup>G12D</sup> in complex with MP-9903 (B). KRAS is shown in grey coloured ribbon and the bound peptide is shown as cartoon (Cyan for KRpep-2d and Green for MP-9903) with the terminal Arg (Salmon), methylene bridge (magenta) and interacting residues highlighted as sticks. H-bond interactions are highlighted as dashed lines (black).

S4)

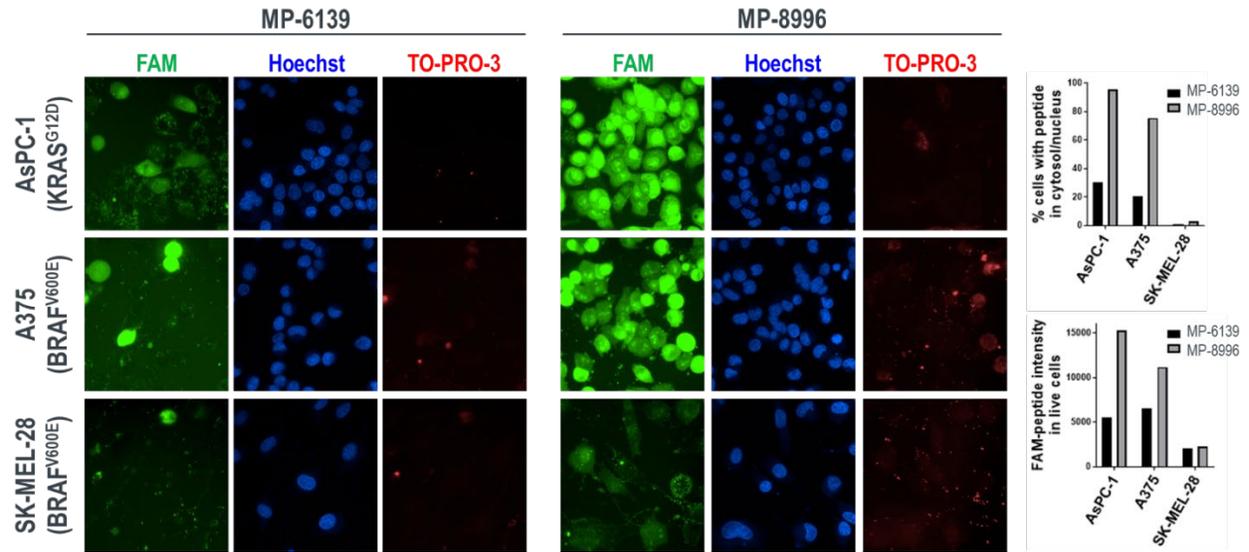


**Figure S4:** Substitution of L-arginines (MP-6139) for D-arginines (MP-8996) resulted in a marked increase in uptake in AsPC-1 cells (30 min peptide treatment), as assessed by confocal imaging using FAM-labeled peptides. See Table S4 for full sequences of the FAM-labeled analogs.

S5A)

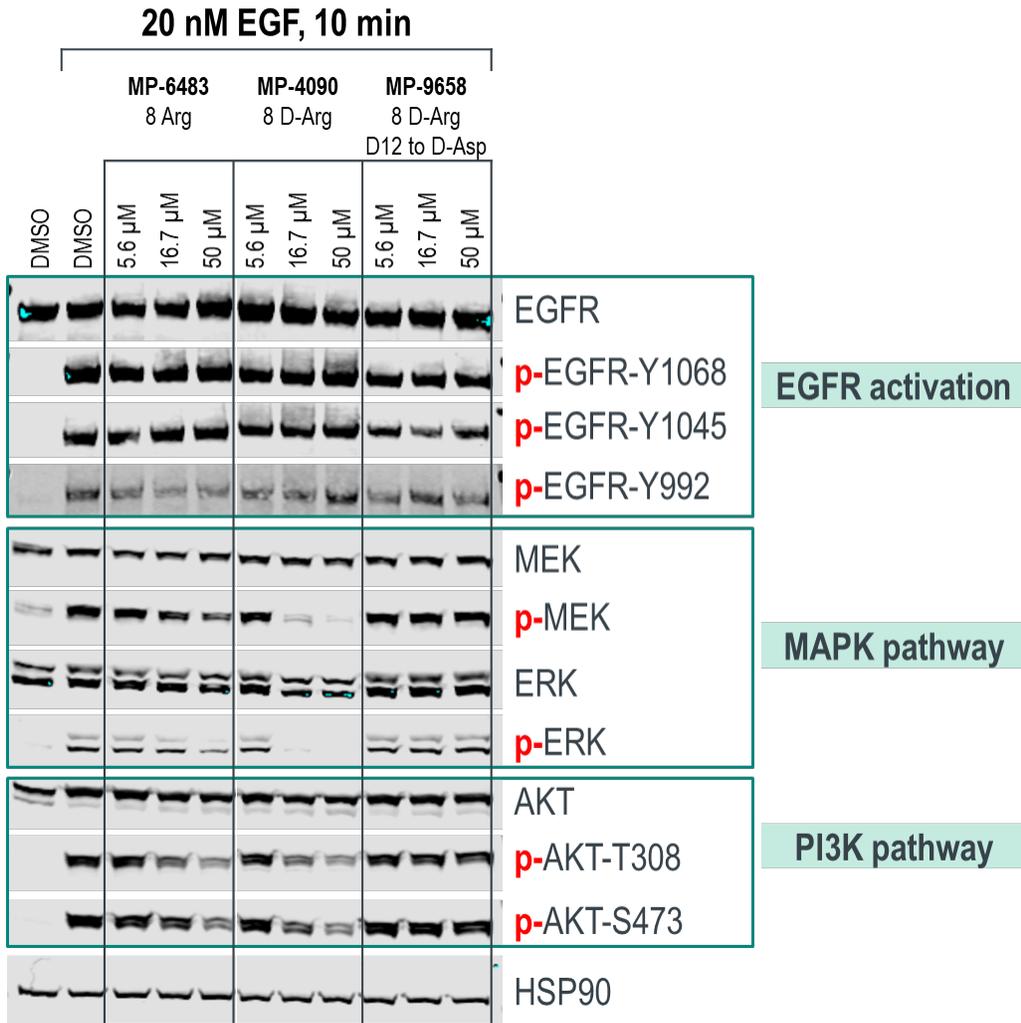


S5B)

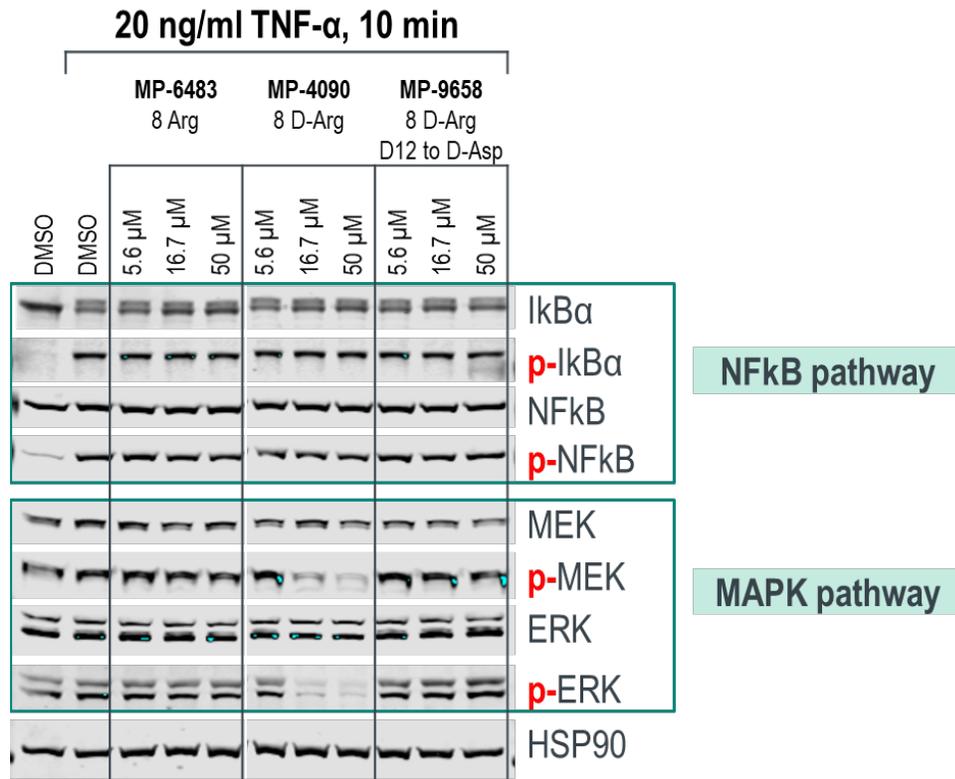


**Figure S5:** Peptides with D-arginines have increased cell permeability but do not inhibit MAPK and PI3K signaling in cancer cell lines with BRAF mutations. A) **MP-4090** does not inhibit MAPK and PI3K signaling in A375 and SK-MEL-28 cell lines. B) A FAM-labeled version of **MP-4090** (**MP-8996**) entered these cells with roughly equivalent amounts compared to AsPC-1 cells. **MP-6139** is the FAM-labeled version of the corresponding L-Arg peptide **MP-6483**.

S6A)

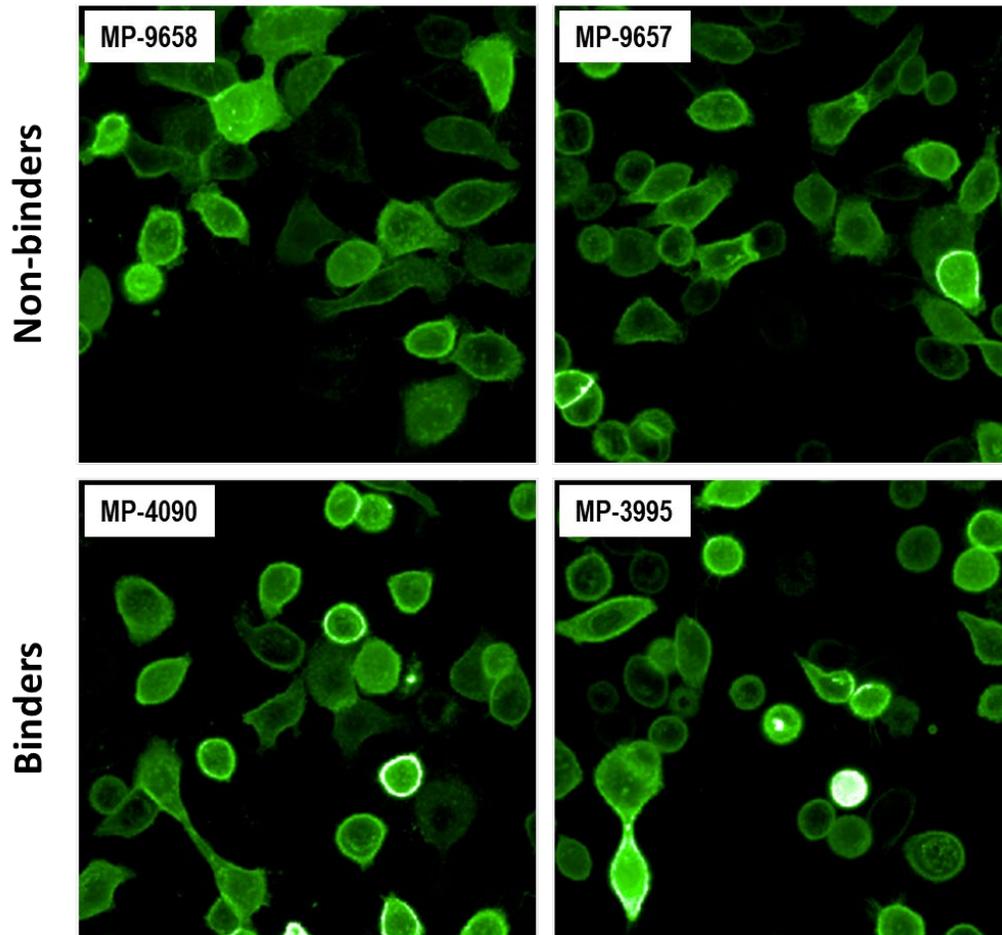


S6B)

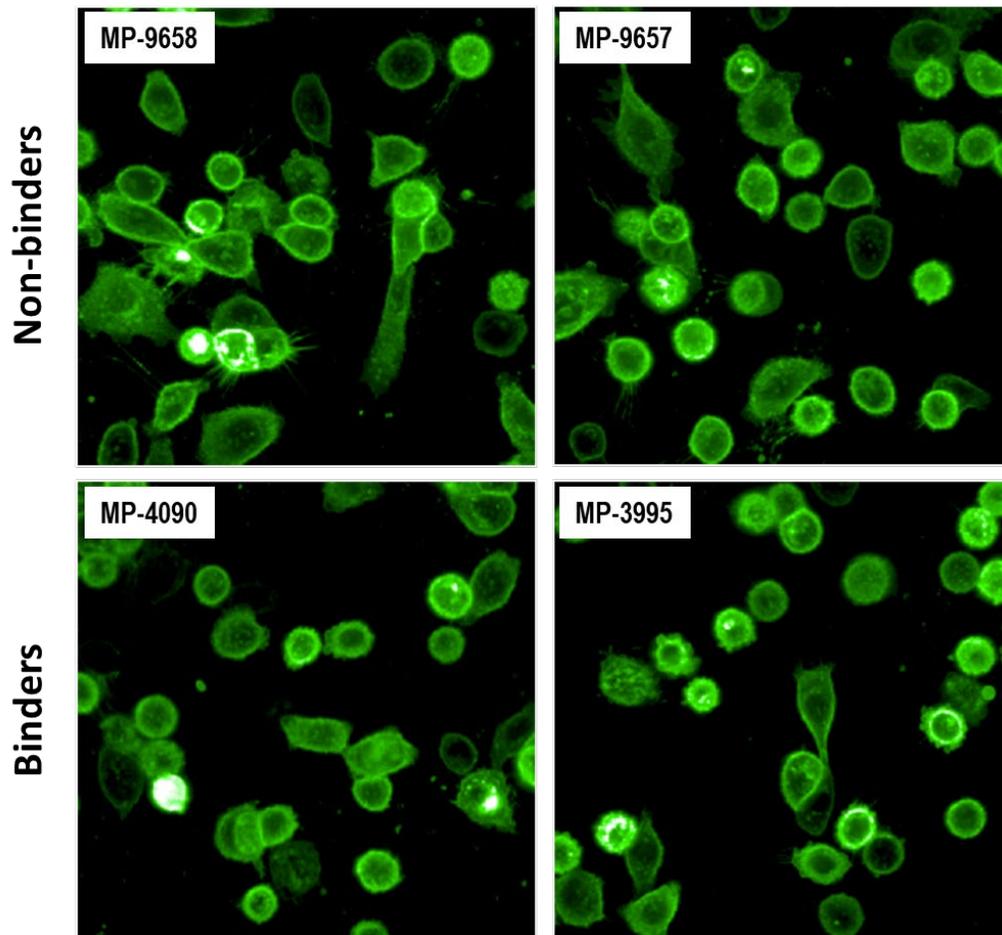


**Figure S6:** Inhibition by redox-stable KRpep-2d family members is specific to signaling events downstream of KRAS. A) **MP-6483** and **MP-4090** inhibit signaling downstream of KRAS (pERK, pMEK, and pAKT) but not upstream of KRAS (pEGFR). B) **MP-6483**, **MP-4090**, and non-binding control **MP-9658** have no effect on NFkB signaling.

S7A)

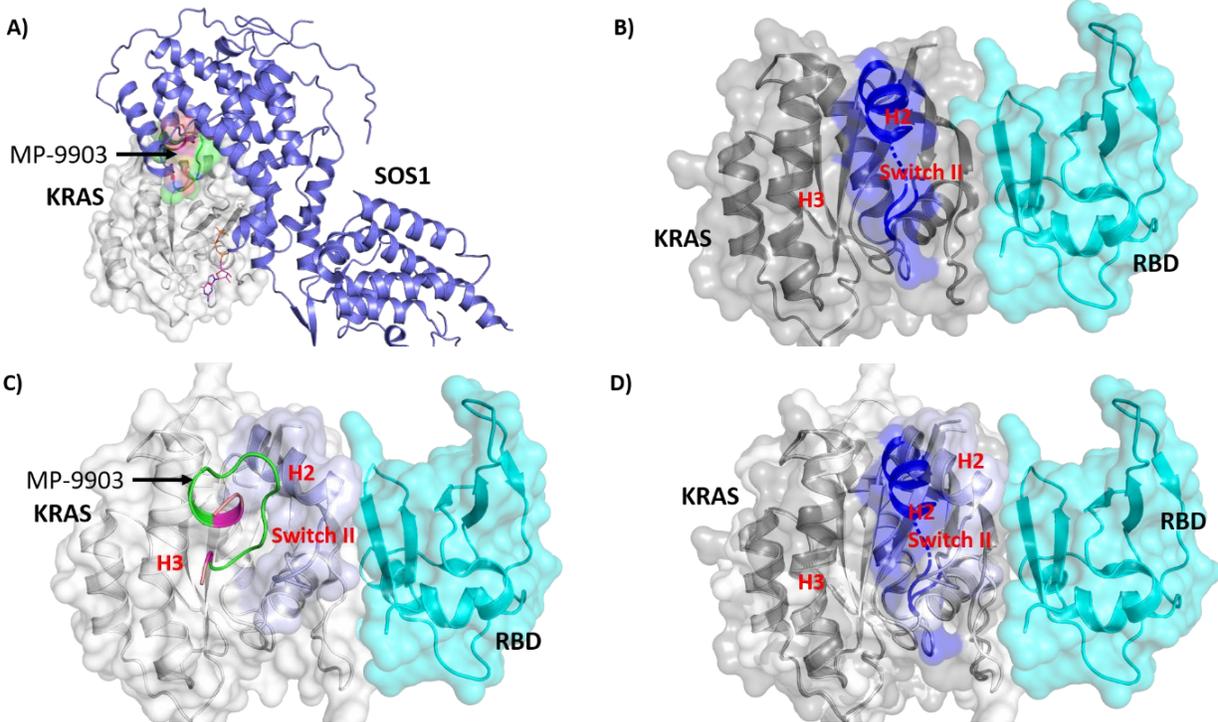


S7B)



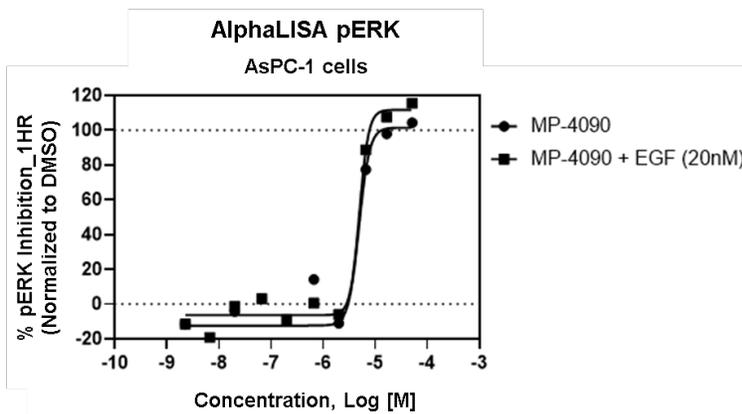
**Figure S7:** eGFP fusions whose membrane localization is not dependent on KRAS binding are not displaced from the membrane by **MP-4090**, **MP-3995**, nor by non-binding controls. A) Cells expressing eGFP fused at the C-terminal to the farnesylation signal from HRAS. B) Cells expressing a protein consisting of the *N*-terminal palmitoylation signal from Neuromodulin fused to the N-terminus of eGFP.

S8)

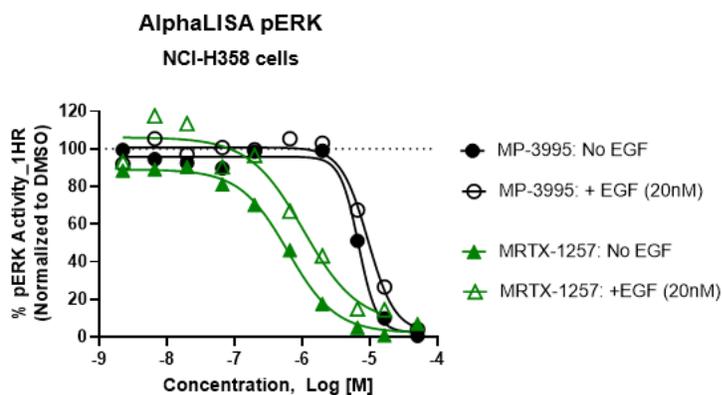


**Figure S8:** Comparison of the crystal structure of KRAS–MP-9903 with the crystal structure of KRAS–SOS/RBD complexes (PDB ID's 7KFZ and 6XHB). (A) KRAS is shown in grey cartoon/surface, **MP-9903** is shown in green cartoon/surface and SOS1 is shown in blue cartoon. (B) KRAS is shown in dark grey cartoon/surface and RBD is shown in teal cartoon/surface, highlighting the inward movement of helix H2 and switch II (C) KRAS is shown in grey cartoon/surface, **MP-9903** is shown in green cartoon, highlighting the outward movement of helix H2, and switch II, induced by the binding of **MP-9903** (D) overlay of KRAS–MP-9903 (peptide not shown) and the KRAS–RBD complex (colouring scheme same as B and C).

S9A)



S9B)



**Figure S9:** Cellular pERK activity of A) **MP-4090** on AsPC-1 (Homozygous KRAS<sup>G12D</sup>) cells and B) **MP-3995** on NCI-H358 (Heterozygous KRAS<sup>G12C</sup>) cells are not shifted by EGF treatment. In contrast, 2-fold potency shift was expected with **MRTX-1257** (KRAS<sup>G12C</sup> GDP state inhibitor) in the presence of 20 nM EGF stimulation.

#### Supplementary Tables:

**Table S1:** Effects on binding energy on in-silico alanine scanning of residues in **MP-1687** (Ac-K(N<sub>3</sub>)-RR-cyclo(c(methylene)PLYISYDPVC)-RR-NH<sub>2</sub>) in complex with KRAS<sup>G12D</sup>(GMPPCP).

Compound	Mutation	$\Delta\Delta G$ (kcal/mol)
5	Pro6Ala	5 ±0.7
6	Leu7Ala	7 ±0.9
7	Tyr8Ala	3 ±0.9
8	Ile9Ala	6 ±0.7
9	Ser10Ala	3 ±0.5
10	Try11Ala	5 ±1.2
11	Asp12Ala	9 ±1.3
12	Pro13Ala	0.9 ±0.6
13	Val14Ala	0.5 ±0.2

**Table S2:** Cell culture conditions and seeding densities for 5-day CellTiter-Glo® cell viability assay on extended 16 cell panel.

Cell Line	Vendor	Catalog number	Description	Complete growth medium	Seeding density (cells/well)
A375	ATCC	CRL-1619	skin, malignant melanoma	DMEM+10%FBS	250
A427	ATCC	HTB-53	lung, carcinoma	EMEM + 10%FBS	400
AsPC-1	ATCC	CRL-1682	pancreas, adenocarcinoma	RPMI1640+10%FBS	900
Capan-1	ATCC	HTB-79	pancreas, adenocarcinoma	IMDM+20%FBS	700
CFPAC-1	ATCC	CRL-1918	pancreas, ductal adenocarcinoma; cystic fibrosis	IMDM+10%FBS	600
HEK293	ATCC	CRL-1573	kidney, embryonic	EMEM+10%FBS	600
HPAF-II	ATCC	CRL-1997	pancreas, adenocarcinoma	EMEM+10%FBS	800
MiaPaCa-2	ATCC	CRL-1420	pancreas, pancreatic Carcinoma	DMEM+10%FBS	600
NCI-H2122	ATCC	CRL-5985	lung, non-small cell lung cancer	RPMI1640+10%FBS	400
NCI-H358	ATCC	CRL-5807	lung, non-small cell lung cancer, bronchioalveolar carcinoma	RPMI1640+10%FBS	500
NCI-H727	ATCC	CRL-5815	lung, bronchus cancer	RPMI1640+10%FBS	750
Panc 08.13	ATCC	CRL-2551	pancreas, adenocarcinoma	RPMI1640 + 20 Units/ml human recombinant insulin +10%FBS	900
Panc 10.05	ATCC	CRL-2547	pancreas, adenocarcinoma	RPMI1640 + 20 Units/ml human recombinant insulin +10%FBS	800
SK-CO-1	ATCC	HTB-39	colon, colorectal adenocarcinoma	EMEM+10%FBS	300
SK-HEP-1	ATCC	HTB-52	liver, adenocarcinoma	EMEM+10%FBS	600
SW480	ATCC	CCL-228	colon, adenocarcinoma	Leibovitz's L-15 Medium+10%FBS	350

**Table S3.** List of reported compounds and purity and characterization data.

Compd. #	Sequence	Method	Purity (%)	Formula	MW	Exact mass	Observed mass
1	Ac-K(N <sub>3</sub> )-RRRR-cyclo(DPLYISYDPV-Dap)-RRRR-NH <sub>2</sub>	D	92.3	C115H193N49O27	2694.13	2692.52	899.1 [M-3H]/3
2	Ac-K(N <sub>3</sub> )-RRRR-cyclo(dPLYISYDPV-Dap)-RRRR-NH <sub>2</sub>	D	93.0	C115H193N49O27	2694.13	2692.52	899.1 [M-3H]/3
3	Ac-K(N <sub>3</sub> )-RRRR-cyclo(C(methylene)PLYISYDPVC)-RRRR-NH <sub>2</sub>	E	90.2	C115H194N48O26S2	2729.26	2727.48	546.8 [M+5H]/5
MP-6483	Ac-K(N <sub>3</sub> )-RRRR-cyclo(c(methylene)PLYISYDPVC)-RRRR- NH <sub>2</sub>	E	95.3	C115H194N48O26S2	2729.26	2727.48	569.5 [M-TFA-4H]/5
4	Ac-K(N <sub>3</sub> )-RR-cyclo(c(methylene)PLYISYDPV-hC)-RR-NH <sub>2</sub>	B	81.4	C92H148N32O22S2	2118.53	2117.09	709.9 [M+3H]/3
MP-1687	Ac-K(N <sub>3</sub> )-RR-cyclo(c(methylene)PLYISYDPVC)-RR- NH <sub>2</sub>	E	96.9	C91H146N32O22S2	2104.51	2103.07	527.0 [M-4H]/4
5	Ac-RR-cyclo(c(methylene)ALYISYDPVC)-RR- NH <sub>2</sub>	F	95.7	C83H134N28O21S2	1924.3	1922.97	642.2 [M-3H]/3
6	Ac-RR-cyclo(c(methylene)PAYISYDPVC)-RR-NH <sub>2</sub>	F	90.3	C82H130N28O21S2	1908.25	1906.94	636.8 [M-3H]/3
7	Ac-RR-cyclo(c(methylene)PLAISYDPVC)-RR-NH <sub>2</sub>	F	95.0	C79H132N28O20S2	1858.24	1856.96	465.4 [M-4H]/4
8	Ac-RR-cyclo(c(methylene)PLYASYDPVC)-RR-NH <sub>2</sub>	F	94.2	C82H130N28O21S2	1908.25	1906.94	636.9 [M-3H]/3
9	Ac-RR-cyclo(c(methylene)PLYIAYDPVC)-RR-NH <sub>2</sub>	F	96.0	C85H136N28O20S2	1934.34	1932.99	645.5 [M-3H]/3
10	Ac-RR-cyclo(c(methylene)PLYISADPVC)-RR-NH <sub>2</sub>	F	95.9	C79H132N28O20S2	1858.24	1856.96	620.2 [M-3H]/3
11	Ac-RR-cyclo(c(methylene)PLYISYAPVC)-RR-NH <sub>2</sub>	F	96.5	C84H136N28O19S2	1906.32	1905.00	477.4 [M-4H]/4
12	Ac-RR-cyclo(c(methylene)PLYISYDAVC)-RR-NH <sub>2</sub>	F	94.8	C83H134N28O21S2	1924.3	1922.97	642.2 [M-3H]/3

<b>13</b>	Ac-RR-cyclo(c(methylene)PLYISYDPAC)-RR-NH <sub>2</sub>	F	96.9	C83H132N28O21S2	1922.28	1920.96	641.5 [M-3H]/3
<b>MP-4090</b>	Ac-K(N <sub>3</sub> )-rrrr-cyclo(c(methylene)PLYISYDPVC)-rrrr-NH <sub>2</sub>	F	95.1	C115H194N48O26S2	2729.26	2727.48	683.1 [M-4H]/4
<b>MP-3995</b>	Ac-K(N <sub>3</sub> )-rrrr-cyclo(c(methylene)-PLYI-aMeS-YDPVC)-rrrr-NH <sub>2</sub>	A	98.0	C116H196N48O26S2	2743.29	2741.49	915.7 [M+3H]/3
<b>MP-4956</b>	Ac-rrrr-cyclo(c(methylene)cplyisydpvc)-rrrr-NH <sub>2</sub>	F	95.3	C109H185N44O25S2	2575.09	2574.40	644.6 [M-4H]/4
<b>MP-9657</b>	Ac-K(N <sub>3</sub> )-rrrr-cyclo(c(methylene)PLYiSYDPVC)-rrrr-NH <sub>2</sub>	F	96.0	C115H194N48O26S2	2729.26	2727.48	910.5 [M-3H]/3
<b>MP-9658</b>	Ac-K(N <sub>3</sub> )-rrrr-cyclo(c(methylene)PLYISYdPVC)-rrrr-NH <sub>2</sub>	F	93.3	C115H194N48O26S2	2729.26	2727.48	910.5 [M-3H]/3
<b>14</b>	Ac-K(N <sub>3</sub> )-RR-cyclo(c(methylene)-aMeP-LYISYDPVC)-RR-NH <sub>2</sub>	A	98	C92H148N32O22S2	2118.53	2117.09	1059.9 [M+2H]/2
<b>15</b>	Ac-K(N <sub>3</sub> )-RR-cyclo(c(methylene)P-aMeL-YISYDPVC)-RR-NH <sub>2</sub>	A	96	C92H148N32O22S2	2118.53	2117.09	1059.6 [M+2H]/2
<b>16</b>	Ac-K(N <sub>3</sub> )-RR-cyclo(c(methylene)PL-aMeF-ISYDPVC)-RR-NH <sub>2</sub>	A	92	C92H148N32O21S2	2102.54	2101.09	1051.7 [M+2H]/2
<b>17</b>	Ac-K(N <sub>3</sub> )-RR-cyclo(c(methylene)PLYI-aMeS-YDPVC)-RR-NH <sub>2</sub>	A	98	C92H148N32O22S2	2118.53	2117.09	1060.0 [M+2H]/2
<b>18</b>	Ac-K(N <sub>3</sub> )-RR-cyclo(c(methylene)PLYIS-aMeY-DPVC)-RR-NH <sub>2</sub>	A	94	C92H151N32O22S2	2118.53	2117.09	1059.9 [M+2H]/2
<b>19</b>	Ac-K(N <sub>3</sub> )-RR-cyclo(c(methylene)PLYISY-aMeD-PVC)-RR-NH <sub>2</sub>	C	100	C92H148N32O22S2	2118.53	2117.09	1059.9 [M+2H]/2
<b>20</b>	Ac-K(N <sub>3</sub> )-RR-cyclo(c(methylene)PLYISYD-aMeP-VC)-RR-NH <sub>2</sub>	A	100	C92H148N32O22S2	2118.53	2117.09	1059.7 [M+2H]/2
<b>21</b>	Ac-K(N <sub>3</sub> )-RR-cyclo(c(methylene)PLYISYDP-aMeV-C)-RR-NH <sub>2</sub>	A	95.4	C92H151N32O22S2	2118.53	2117.09	1060.1 [M+2H]/2
<b>22</b>	Ac-K(N <sub>3</sub> )-RR-cyclo(c(methylene)PLYISYDPV-aMeC)-RR-NH <sub>2</sub>	A	82	C92H148N32O22S2	2118.53	2117.09	1059.8 [M+2H]/2
<b>23</b>	Ac-K(N <sub>3</sub> )-RRR-cyclo(c(methylene)PLYISYDPVC)-RRR-NH <sub>2</sub>	E	93.8	C103H170N40O24S2	2416.88	2415.28	844.5 [M-TFA-2H]/3
<b>MP-9903</b>	Ac-K(N <sub>3</sub> )-R-cyclo(c(methylene)PLYISYDPVC)-R-NH <sub>2</sub>	F	96.6	C79H122N24O20S2	1792.13	1790.87	896.7 [M-2H]/2
<b>24</b>	Ac-K(N <sub>3</sub> )-cyclo(c(methylene)PLYISYDPVC)-R-NH <sub>2</sub>	F	94.9	C73H110N20O19S2	1635.94	1634.77	818.4 [M-2H]/2

<b>25</b>	Ac-K(N <sub>3</sub> )-cyclo(c(methylene)PLYISYDPVC)-NH <sub>2</sub>	A	98.4	C67H98N16O18S2	1479.75	1478.67	740.5 [M+2H]/2
<b>MP-6139</b>	5FAM-K(N <sub>3</sub> )-RRRR-cyclo(c(methylene)PLYISYDPVC)-RRRR-NH <sub>2</sub>	F	91.2	C134H202N48O31S2	3045.54	3043.51	508.5 [M-6H]/6
<b>MP-8996</b>	5FAM-rrrr-cyclo(c(methylene)PLYISYDPVC)-rrrr-NH <sub>2</sub>	F	92.8	C128H192N44O30S2	2891.36	2889.43	723.6 [M-4H]/4

5FAM = 5-Carboxyfluorescein; Ac = acetyl; aMeC =  $\alpha$ -methyl-L-cysteine; aMeD =  $\alpha$ -methyl-L-aspartic acid; aMeF =  $\alpha$ -methyl-L-phenylalanine; aMeL =  $\alpha$ -methyl-L-leucine; aMeP =  $\alpha$ -methyl-L-proline; aMeS =  $\alpha$ -methyl-L-serine; aMeV =  $\alpha$ -methyl-L-valine; aMeY =  $\alpha$ -methyl-L-tyrosine; c = D-Cys; d = D-Asp; Dap = L-2,3-diaminopropionic acid; K(N<sub>3</sub>) = *N*- $\epsilon$ -azido-L-lysine; hC = L-homocysteine; i = D-Ile; l = D-Leu ; p = D-Pro; r = D-Arg; s = D-Ser; v = D-Val; y = D-Tyr.

## Materials and Methods:

### General procedure for the synthesis of compounds in Table S3

#### General Information:

Peptides in Table S4 were synthesized using standard solid phase synthesis using Fmoc/*tert*-Bu chemistry as exemplified in Chan, W. C.; White, P. D. "Fmoc Solid-Phase Synthesis: a Practical Approach", Oxford University Press, Oxford, **2000**; Steward, J.; Young, J. "Solid Phase Peptide Synthesis", Pierce Chemical Company, Rockford, **1984**.; N. L. Benoiton, "Chemistry of Peptide Synthesis", CRC Press, New York, **2006**; and Lloyd-Williams, P.; Albericio, F. "Chemical Approaches to the Synthesis of Peptides and Proteins", CRC Press, New York, **1997**.

The  $\alpha$ -amino group of each amino acid was protected by a 9*H*-fluoren-9-ylmethoxycarbonyl group (Fmoc) during the coupling of the carboxylic acid of the amino acid with the free amino terminus of the peptide attached to the resin. To avoid any side reactions during the coupling steps performed in DMF, the reactive side-chains of amino acids also carried acid-labile protecting groups that effectively masked the reactive groups until treatment of the resin with acid during the cleavage of the peptide from the solid support. After completion of each coupling step, the Fmoc group of the just-attached amino acid was removed with piperidine or 4-methylpiperidine and the resin was thoroughly washed to prepare for the coupling of the subsequent Fmoc-protected amino acid derivative.

The side-chain protecting groups were: *tert*-butyl (*t*Bu) for L-Asp, D-Asp,  $\alpha$ -Me-L-Ser, L-Ser, and L-Tyr; trityl (Trt) for L-Asn, L-Cys and D-Cys; *tert*-butoxy-carbonyl (Boc) for L-Dap; and, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for L-Arg and D-Arg.

Fmoc-protected amino acids were typically obtained from vendors such as Sigma-Aldrich, Novabiochem, Chem-Impex, Combi-Block.

#### Protocol A:

**Step 1. Linear peptide synthesis.** The peptide was synthesized using Fmoc/*t*-Bu chemistry on Rink Amide resin LL MBHA (NovaBioChem, 0.33 mmol/g) with a CEM Liberty automated microwave peptide synthesizer. The peptide sequence was synthesized on a 0.1 mmol scale. Typical reaction conditions were as follows: Fmoc deprotections were performed using 20% (v/v) piperidine in DMF (2 min at 90 °C). Reaction coupling conditions: double couplings with Fmoc-protected amino acid/*N,N'*-

diisopropylcarbodiimide(DIC)/Oxyma Pure (5, 10, and 5 equiv respectively; 90 °C microwave assisted heating, 2 min or 4 min for difficult couplings such as  $\alpha$ -Me amino acids, repeated twice). After the completion of linear peptide synthesis, a final acetylation step was performed using acetic anhydride (10% in DMF; 75 °C for 10 min).

**Step 2. Cleavage and Deprotection.** The linear resin-bound peptides were deprotected and cleaved from the solid support by treatment with a cleavage solution (TFA/DTT/Thioanisole/Phenol/H<sub>2</sub>O 87.5/2.5/5/2.5/2.5 or TFA/TIS/water 95/2.2/2.5, v/v, 8 mL) at 42 °C for 30 min using a Razor<sup>®</sup> peptide cleavage system from CEM Corporation. After filtration of the resin, crude linear peptides were precipitated from the TFA (Trifluoroacetic Acid) cleavage solution using cold TBME (*tert*-butyl methyl ether) (40 mL) and collected by centrifugation (4000 rpm). The crude peptide was washed with cold ethyl ether or TBME (35 mL) and air dried. The residue was semi-purified by Teledyne ISCO flash chromatography (15.5 g HP C18 Aq column) with mobile phase A: water with 0.1% TFA and mobile phase B: ACN (Acetonitrile) with 0.1% TFA, gradient 15-35% over 12 column volumes. Fractions containing the desired product were collected and lyophilized to give the linear product.

**Step 3. Peptide Cyclization.** The above linear peptide was dissolved in acetonitrile/DI water (1:1, v/v, 15 mL). (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub> (0.2 M solution in water) was added to adjust the pH to ~8. 3,6-Dioxa-1,8-octanedithiol (DODT, 2 equiv) or DL-Dithiothreitol (DTT, 2 equiv) and diiodomethane (20 equiv) were added. Additional acetonitrile (2 mL) was added to make the reaction mixture homogeneous. The resulting reaction mixture was stirred at room temperature overnight. After the reaction was complete, the reaction solution was quenched by addition of TFA (100  $\mu$ L), concentrated and lyophilized. The residue was purified by RP-HPLC on Waters XSelect Peptide CSH C18 OBD Prep column (130Å, 5  $\mu$ m, column size 150  $\times$  30 mm) using a Waters MS-Directed AutoPurification HPLC/MS system. Mobile phase: (A) 0.1 % TFA in HPLC water and (B) 0.1 % TFA in HPLC acetonitrile; flow rate: 50 mL/min; UV wavelength  $\lambda$  = 214 nm; gradient: 5% B over 17 min. The desired fractions were then lyophilized to provide the cyclized peptide as a white powder.

#### **Protocol B:**

**Step 1. Linear peptide synthesis.** The peptide was synthesized using Fmoc/*t*-Bu chemistry on Rink Amide resin LL MBHA (NovaBioChem, 0.33 mmol/g) with a CEM Liberty automated microwave peptide synthesizer. The peptide sequence was synthesized on a 0.1 mmol scale. Typical reaction conditions were as follows: Fmoc deprotections were performed using 20% (v/v) piperidine in DMF (2 min at 90 °C).

Reaction coupling conditions: double couplings with Fmoc-protected amino acid/DIC/Oxyma Pure (5, 10, and 5 equiv respectively; 90 °C microwave assisted heating, 2 min or 4 min for difficult couplings such as  $\alpha$ -Me amino acids, repeated twice). After the completion of linear peptide synthesis, a final acetylation step was performed using acetic anhydride (10% in DMF; 75 °C for 10 min).

**Step 2. Cleavage and Deprotection.** The linear resin-bound peptides were deprotected and cleaved from the solid support by treatment with a cleavage solution (TFA/DTT/Thioanisole/Phenol/H<sub>2</sub>O 87.5/2.5/5/2.5/2.5 or TFA/TIS/water 95/2.2/2.5, v/v, 8 mL) at 42 °C for 30 min using a Razor<sup>®</sup> peptide cleavage system from CEM Corporation. After filtration of the resin, crude linear peptides were precipitated from the TFA cleavage solution using cold TBME (40 mL) and collected by centrifugation (4000 rpm). The crude peptide was washed with cold ethyl ether or TBME (35 mL) and air dried. The residue was semi-purified by Teledyne ISCO flash chromatography (15.5 g HP C18 Aq column) with mobile phase A: water with 0.1% TFA and mobile phase B: ACN with 0.1% TFA, gradient 15-35% over 12 column volumes. Fractions containing the desired product were collected and lyophilized to give the linear product.

**Step 3. Peptide Cyclization.** The above linear peptide was dissolved in acetonitrile/DI water (1:1, v/v, 15 mL). (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub> (0.2 M solution in water) was added to adjust the pH to ~8. 3,6- DL-Dithiothreitol (DTT, 2 equiv) and 1,2-dibromoethane (10 equiv) were added. Additional acetonitrile (2 mL) was added to make the reaction mixture homogeneous. The resulting reaction mixture was stirred at room temperature overnight. After the reaction was complete, the reaction solution was quenched by addition of TFA (100  $\mu$ L), concentrated and lyophilized. The residue was purified by RP-HPLC on Waters XSelect Peptide CSH C18 OBD Prep column (130Å, 5  $\mu$ m, column size 150  $\times$  30 mm) using a Waters MS-Directed AutoPurification HPLC/MS system. Mobile phase: (A) 0.1 % TFA in HPLC water and (B) 0.1 % TFA in HPLC acetonitrile; flow rate: 50 mL/min; UV wavelength  $\lambda$  = 214 nm; gradient: 5% B over 17 min. The desired fractions were then lyophilized to provide the cyclized peptide as a white powder.

### Protocol C:

**Step 1. Linear peptide synthesis.** The peptide was synthesized using Fmoc/*t*-Bu chemistry on Rink Amide resin LL (NovaBioChem, 0.33 mmol/g) with a Symphony<sup>®</sup> X synthesizer (Gyros Protein Technologies). The peptide sequence was synthesized on a 0.1 mmol scale at RT. Typical reaction conditions were as follows: Fmoc deprotections were performed using 20% (v/v) piperidine in DMF (4 mL, 3 min, 3 repeats). Reaction coupling conditions: double couplings with Fmoc-protected amino acid/HATU/NMM (5, 5, and 10 equiv

respectively; 30 min or 60 min for difficult couplings such as  $\alpha$ -Me amino acids, repeated twice). After the completion of linear peptide synthesis, a final acetylation step was performed using acetic anhydride (1.0 M) and DIPEA (diisopropylethylamine) (2.0 M) in NMP (*N*-methyl-2-pyrrolidone) at room temperature for 10 min.

**Step 2. Cleavage and Deprotection.** The linear resin-bound peptides were deprotected and cleaved from the solid support by treatment with TFA/DTT/Thioanisole/Phenol/H<sub>2</sub>O (87.5/2.5/5/2.5/2.5, v/v; 8 mL) at 42 °C for 30 min using a Razor<sup>®</sup> peptide cleavage system from CEM Corporation. After filtration of the resin, crude linear peptides were precipitated from the TFA cleavage solution using cold ethyl ether (40 mL) and collected by centrifugation (4000 rpm). The crude peptide was washed with cold ethyl ether (35 mL) and air dried. The residue was semi-purified by Teledyne ISCO flash chromatography (15.5 g HP C18 Aq column) with mobile phase A: water with 0.1% TFA and mobile phase B: ACN with 0.1% TFA. Fractions containing the desired product were collected and lyophilized to give the linear product.

**Step 3. Peptide Cyclization.** The above linear peptide was dissolved in acetonitrile/DI water (1:1, v/v, 15 mL). (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub> (0.2 M solution in water) was added to adjust the pH to ~8. DODT (2 equiv) and diiodomethane (20 equiv) were added. Additional acetonitrile (2 mL) was added to make the reaction mixture homogeneous. The resulting reaction mixture was stirred at room temperature overnight. After the reaction was complete, the reaction solution was quenched by addition of TFA (100  $\mu$ L), frozen and lyophilized. The residue was purified by RP-HPLC on Waters XSelect Peptide CSH C18 OBD Prep column (130Å, 5  $\mu$ m, column size 150  $\times$  30 mm) using a Waters MS-Directed AutoPurification HPLC/MS system. Mobile phase: (A) 0.1 % TFA in HPLC water and (B) 0.1 % TFA in HPLC acetonitrile; flow rate: 50 mL/min; UV wavelength  $\lambda$  = 214 nm; gradient: 5% B over 17 min. The desired fractions were then lyophilized to provide the cyclized peptide as a white powder.

#### **Protocol D:**

**Step 1. Linear peptide synthesis.** The peptide sequence was synthesized manually using standard solid phase synthesis using Fmoc/*t*-Bu chemistry. HATU with NMM were used as coupling agents to create the amide bond between the free amino terminus of the resin-bound protected peptide and the carboxylic acid of the Fmoc-protected amino acid. Unloaded Rink amide MBHA resin (100-200 mesh, 1% cross-linked polystyrene) was used for synthesis. All the amino acids were dissolved at a 0.2 M concentration in anhydrous DMF. Reactions were typically performed at the 0.3 mmol scale.

Every synthesis cycle included: (1) Fmoc amino acid deprotection: 20% piperidine in DMF (20 mL) was added into the resin. The mixture was kept at room temperature for 30 min while a stream of nitrogen was bubbled through it. The mixture was filtered, and the peptidyl resin was washed with DMF (5 × 20 mL); (2) Coupling (potentially repeated twice for difficult couplings) with Fmoc-protected amino acid/HATU/NMM (3, 2.85 and 6 equiv, respectively; room temperature; 1 h). Cycles of Fmoc deprotection and Fmoc-protected amino acid coupling were repeated with the desired monomers until the full linear peptide was formed. Final acetylation (capping) step was performed using acetic anhydride capping solution [Capping Solution: Acetic anhydride (6 mL) and NMM (10 mL) in DMF (84 mL)].

**Step 2. Orthogonal Deprotection and Macrolactamization.** The Allyl and Alloc protecting groups on the orthogonally protected side-chains were removed by using Pd(PPh<sub>3</sub>)<sub>4</sub> (0.2 equiv) and phenylsilane (20 equiv) in DCM. The mixture was shaken for 4 h and the solution was drained. The resin was washed with 5% (w/v) sodium diethyldithiocarbamate solution and then washed successively with DMF (5 × 4 mL), DCM (5 × 4 mL) and DMF (2 × 4 mL).

The deprotected carboxylic acid and amine side-chains were coupled by using PyAOP (3 equiv), HOBt (10 equiv) and DIPEA (5 equiv) in DMF. It was shaken for 16 h at room temperature, and then the resin was washed with DMF (5 × 4 mL) and DCM (5 × 4 mL).

**Step 3. Cleavage and Deprotection.** The linear resin-bound peptide was deprotected and cleaved from the solid support by treatment with TFA/EDT/Thioanisole/Phenol/H<sub>2</sub>O (87.5/2.5/5/2.5/2.5, v/v, 30 mL) at room temperature for 2.5 h. After filtration of the resin, crude linear peptides were precipitated from the TFA cleavage solution using cold diethyl ether (270 mL) and collected by centrifugation (4000 rpm). The precipitate was washed with cold diethyl ether (2 × 270 mL). The crude was dried under vacuum overnight to give the crude deprotected linear peptide as a solid.

The residue was purified by RP-HPLC on Phenomenex Luna C18 column (100Å, 10 μm, column size 200 × 25 mm). Mobile phase: (A) 0.1 % TFA in HPLC water and (B) 0.1 % TFA in 80%ACN+20%H<sub>2</sub>O; flow rate: 50 mL/min; UV wavelength λ = 220 nm; gradient: 30% B over 60 min. The desired fractions were then lyophilized to provide the cyclized peptide as a white powder.

#### **Protocol E:**

**Step 1. Linear peptide synthesis.** The peptide sequence was synthesized manually using standard solid phase synthesis using Fmoc/*t*-Bu chemistry. HATU with NMM were used as coupling agents to create the amide bond between the free amino terminus of the resin-bound protected peptide and the carboxylic acid of the Fmoc-protected amino acid. Unloaded Rink amide MBHA resin (100-200 mesh, 1% cross-linked polystyrene) was used for synthesis. All the amino acids were dissolved at a 0.2 M concentration in anhydrous DMF. Reactions were typically performed at the 0.3 mmol scale.

Every synthesis cycle included: (1) Fmoc amino acid deprotection: 20% piperidine in DMF (20 mL) was added into the resin. The mixture was kept at room temperature for 30 min while a stream of nitrogen was bubbled through it. The mixture was filtered, and the peptidyl resin was washed with DMF (5 × 20 mL); (2) Coupling (potentially repeated twice for difficult couplings) with Fmoc-protected amino acid/HATU/NMM (3, 2.85 and 6 equiv, respectively; room temperature; 1 h). Cycles of Fmoc deprotection and Fmoc-protected amino acid coupling were repeated with the desired monomers until the full linear peptide was formed. Final acetylation (capping) step was performed using acetic anhydride capping solution [Capping Solution: Acetic anhydride (6 mL) and NMM (10 mL) in DMF (84 mL)].

**Step 2. Cleavage and Deprotection.** The linear resin-bound peptide (~1.7 g of dry resin) was deprotected and cleaved from the solid support by treatment with TFA/EDT/Thioanisole/Phenol/H<sub>2</sub>O (87.5/2.5/5/2.5/2.5, v/v, 30 mL) at room temperature for 2.5 h. After filtration of the resin, crude linear peptides were precipitated from the TFA cleavage solution using cold diethyl ether (270 mL) and collected by centrifugation (4000 rpm). The precipitate was washed with cold diethyl ether (2 × 270 mL). The crude was dried under vacuum overnight to give the crude deprotected linear peptide as a solid (~450 mg).

**Step 3. Intermediate Purification.** The crude linear peptide was purified by RP-HPLC on Phenomenex Luna C18 column (100Å, 10 μm, column size 200 × 25 mm). Mobile phase: (A) 0.1 % TFA in HPLC water and (B) 0.1 % TFA in 80%ACN+20%H<sub>2</sub>O; flow rate: 15 mL/min; UV wavelength λ = 220 nm; gradient: 30% B over 60 min. The desired fractions were then lyophilized to provide the linear peptide as a white powder.

**Step 4. Peptide Cyclization.** A portion of the above linear peptide was dissolved in phosphate buffered saline (40 mL, pH=7.2) and acetonitrile (40 mL). DTT (2 equiv) and diiodomethane (20 equiv) were added. The resulting reaction mixture was stirred at room temperature overnight. After the reaction was complete, the reaction solution was quenched by addition of TFA (100 μL) and lyophilized. The residue was purified by RP-HPLC on Phenomenex Luna C18 column (100Å, 10 μm, column size 200 × 25 mm). Mobile phase: (A) 0.1 % TFA in HPLC water and (B) 0.1 % TFA in 80%ACN+20%H<sub>2</sub>O; flow rate: 50 mL/min;

UV wavelength  $\lambda = 220$  nm; gradient: 30% B over 60 min. The desired fractions were then lyophilized to provide the cyclized peptide as a white powder.

#### **Protocol F:**

**Step 1. Linear peptide synthesis.** The peptide sequence was synthesized manually using standard solid phase synthesis using Fmoc/*t*-Bu chemistry. HATU with NMM were used as coupling agents to create the amide bond between the free amino terminus of the resin-bound protected peptide and the carboxylic acid of the Fmoc-protected amino acid. Unloaded Rink amide MBHA resin (100-200 mesh, 1% cross-linked polystyrene) was used for synthesis. All the amino acids were dissolved at a 0.2 M concentration in anhydrous DMF. Reactions were typically performed at the 0.3 mmol scale.

Every synthesis cycle included: (1) Fmoc amino acid deprotection: 20% piperidine in DMF (20 mL) was added into the resin. The mixture was kept at room temperature for 30 min while a stream of nitrogen was bubbled through it. The mixture was filtered, and the peptidyl resin was washed with DMF (5  $\times$  20 mL); (2) Coupling (potentially repeated twice for difficult couplings) with Fmoc-protected amino acid/HATU/NMM (3, 2.85 and 6 equiv, respectively; room temperature; 1 h). Cycles of Fmoc deprotection and Fmoc-protected amino acid coupling were repeated with the desired monomers until the full linear peptide was formed. Final acetylation (capping) step was performed using acetic anhydride capping solution [Capping Solution: Acetic anhydride (6 mL) and NMM (10 mL) in DMF (84 mL)].

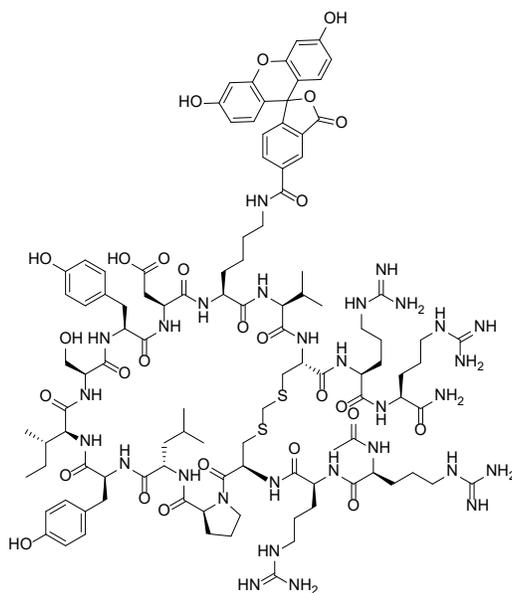
**Step 2. Cleavage and Deprotection.** The linear resin-bound peptide (~1.7 g of dry resin) was deprotected and cleaved from the solid support by treatment with TFA/EDT/Thioanisole/Phenol/H<sub>2</sub>O (87.5/2.5/5/2.5/2.5, v/v, 30 mL) at room temperature for 2.5 h. After filtration of the resin, crude linear peptides were precipitated from the TFA cleavage solution using cold diethyl ether (270 mL) and collected by centrifugation (4000 rpm). The precipitate was washed with cold diethyl ether (2  $\times$  270 mL). The crude was dried under vacuum overnight to give the crude deprotected linear peptide as a solid (~450 mg).

**Step 3. Intermediate Purification.** The crude linear peptide was purified by RP-HPLC on Phenomenex Luna C18 column (100Å, 10  $\mu$ m, column size 200  $\times$  25 mm). Mobile phase: (A) 0.1 % TFA in HPLC water and (B) 0.1 % TFA in 80%ACN+20%H<sub>2</sub>O; flow rate: 15 mL/min; UV wavelength  $\lambda = 220$  nm; gradient: 30% B over 60 min. The desired fractions were then lyophilized to provide the linear peptide as a white powder.

**Step 4. Peptide Cyclization.** A portion of the above linear peptide was dissolved in acetonitrile/DI water (1:1, v/v, 15 mL). DODT (2 equiv) was added and the mixture was stirred at room temperature for 30 min.  $(\text{NH}_4)_2\text{CO}_3$  (0.2 M solution in water) was added to adjust the pH to  $\sim 8$ . DODT (2 equiv) and diiodomethane (20 equiv) were added. The reaction mixture was stirred at room temperature overnight. After the reaction was complete, the reaction solution was quenched by addition of TFA (100  $\mu\text{L}$ ), frozen and lyophilized. The residue was purified by RP-HPLC on Phenomenex Luna C18 column (100 $\text{\AA}$ , 10  $\mu\text{m}$ , column size 200  $\times$  25 mm). Mobile phase: (A) 0.1 % TFA in HPLC water and (B) 0.1 % TFA in 80%ACN+20% $\text{H}_2\text{O}$ ; flow rate: 50 mL/min; UV wavelength  $\lambda = 220$  nm; gradient: 30% B over 60 min. The desired fractions were then lyophilized to provide the cyclized peptide as a white powder.

#### TR-FRET tracer

KRAS<sup>G12D</sup>(GDP) binding was measured by a TR-FRET competitive binding assay using a 5-FAM-labeled analog of Pro13Lys **MP-1687** with the following structure:



**Step 1. Solid Phase Peptide Synthesis.** Peptide was synthesized manually using standard solid phase synthesis using Fmoc/*t*-Bu chemistry as previously reported. HATU with NMM were used as coupling agents to create the amide bond between the free amino terminus of the resin-bound protected peptide and the carboxylic acid of the Fmoc-protected amino acid. The side chain protecting groups were: *tert*-

butyl (*t*Bu) for L-Asp, L-Ser and L-Tyr; trityl (Trt) for L-Cys and D-Cys; 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) for L-Lys; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for L-Arg. Knorr Amide MBHA Resin (0.27 mmol/g loading) was used for synthesis. All the amino acids were dissolved at a 0.2 M concentration in anhydrous DMF. The reaction was performed at the 0.2 mmol scale.

Every synthesis cycle included: (1) Fmoc amino acid deprotection: 20% Piperidine in DMF (20 mL) was added into the resin. The mixture was kept at room temperature for 30 min while a stream of nitrogen was bubbled through it. The mixture was filtered, and the peptidyl resin was washed with DMF (5 × 20 mL); (2) Coupling (potentially repeated twice for difficult couplings) with Fmoc protected amino acid/HATU/NMM (3, 2.85 and 6 equiv, respectively; room temperature; 1 h). Cycles of Fmoc deprotection and Fmoc-protected amino acid coupling were repeated with the desired monomers until the full linear peptide was formed. Final acylation (capping) step was performed using acetic anhydride capping solution [Capping Solution: Acetic anhydride (6 mL) and NMM (10 mL) in DMF (84 mL)].

**Step 2. Orthogonal Deprotection.** Deprotection of the Lys side chain Dde: 4% hydrazine hydrate in DMF (15 mL) was added to the peptidyl resin. The mixture was kept at room temperature for 30 min while a stream of nitrogen was bubbled through it. The mixture was filtered, and the peptidyl resin was washed with DMF (5 × 20 mL). Then peptidyl resin was washed with MeOH (2 × 20 mL), DCM (2 × 20 mL) and MeOH (2 × 20 mL). The resin was dried under vacuum.

**Step 3. Coupling of 5-Carboxyfluorescein (5-FAM).** After selective deprotection of the Lys side chain, the peptidyl resin was treated with a mixture of 5-FAM (0.6 mmol, 3 equiv), HATU (0.57 mmol, 2.85 equiv) and NMM (1.2 mmol, 6 equiv) in DMF (2 mL). The suspension was shaken at room temperature for 1 h while a stream of nitrogen bubbled through it. The peptidyl resin was washed with DMF (5 × 20 mL), MeOH (2 × 20 mL), DCM (2 × 20 mL) and MeOH (2 × 20 mL). The resin was dried under vacuum.

**Step 4. Cleavage and Deprotection.** The resin-bound peptide was deprotected and cleaved from the solid support by treatment with TFA/EDT/Thioanisole/Phenol/H<sub>2</sub>O (87.5/2.5/5/2.5/2.5, v/v, 34 mL) at room temperature for 2.5 h. After filtration of the resin, crude linear peptides were precipitated from the TFA cleavage solution using cold diethyl ether (150 mL) and collected by centrifugation (4000 rpm). The precipitate was washed with cold diethyl ether (2 × 170 mL). The crude was dried under vacuum overnight to give the crude deprotected cyclic peptide as a solid (475 mg).

**Step 5. Linear Peptide Purification.** Purification of the crude linear 5FAM-labeled peptide was performed by preparative reversed-phase high performance liquid chromatography (RP-HPLC) on Phenomenex Luna

C18(2) column (100Å, 10 µm, column size 200 × 21.2 mm using Waters 4000 system. Mobile phase: (A) 0.1% TFA in water and (B) 0.1%TFA in 80%ACN+20%H<sub>2</sub>O; flow rate: 15 mL/min; UV wavelength λ = 220 nm; gradient: 27–57% B over 60 min. UV absorbing fractions containing the target m/z ions were collected and the fractions containing product were confirmed by LC/MS.

**Step 6. Methylene Bridge Insertion.** The linear peptide (130 mg, 0.056 mmol) was dissolved in a 1:1 mixture of water/ACN (30 mL). DODT (0.112 mmol, 2 equiv) was added and the mixture was stirred at 30°C for 30 min. pH was adjusted to ~8 by addition of 5.8 mL of aqueous 0.2 M ammonium carbonate solution. Diiodomethane (1.12 mmol, 20 equiv) and DODT (0.112 mmol, 2 equiv) were added. The mixture was shaken at 30°C overnight.

The final crude 5-FAM-labeled peptide was purified by preparative reversed-phase high performance liquid chromatography (RP-HPLC) on Phenomenex Luna C18(2) column (100Å, 10 µm, column size 200 × 21.2 mm using Waters 4000 system. Mobile phase: (A) 0.1% TFA in water and (B) 0.1%TFA in 80% ACN+20% H<sub>2</sub>O; flow rate: 15 mL/min; UV wavelength λ = 220 nm; gradient: 22–52% B over 60 min. UV absorbing fractions containing the target m/z ions were collected and the fractions containing product were confirmed by LC/MS. The solution was collected and lyophilized to give the final tracer compound as a solid 11.0 mg with the purity of 95.3%.

Purity of fractions was confirmed by UPLC, which was measured by a reverse phase Hewlett Packard 1100 UPLC-MS system. Column: Sepax GP-C18 Column (120Å, 5 µm, column size 150 × 4.6 mm). Mobile phase: (A) 0.1 % TFA in water and (B) 0.09 % TFA in 80%ACN+20%H<sub>2</sub>O; gradient: 32–42% B in 20 min; flow rate: 1 mL/min; UV wavelength λ = 220 nm. The peptide was characterized by electrospray mass spectrometry on an Agilent 1260-6120 Quadrupole LC/MS (MW expected: 2339.7 Da; MW found: 585.8 ([M+4H]<sup>4+</sup>).

**Protein Production:** The KRAS amino acid sequence used was derived from UniProt reference P01116-2 (KRAS4B isoform) RASK\_HUMAN Isoform 2B of GTPase KRAS, Homo sapiens having a G12D mutation (VAR\_016026). It comprised the following sequence (AAs 1 to 169 plus a 4xGly motif appended to the N-terminus for sortase tagging):

GGGGMTEYKLVVVGADDGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDITAGQEEYSAMRDQYMRT  
GEGFLCVFAINNTKSFEDIHHYREQIKRVKDESDVPMVLVGNKCDLPSRTVDTKQAQDLARSYGIPFIETSAKTRQGVDD  
AFYTLVREIRKHKEK

Expression and purification of GDP and GMPPNP-loaded KRAS<sup>G12D</sup> were carried out. His-SUMO-tagged human KRAS residues 1-169 was expressed in *E. coli* Rosetta 2 (DE3). Protein expression was induced by 1 mM IPTG overnight at 18 °C. Cells were lysed in 40 mM Tris pH 7.8, 150 mM NaCl, 0.5% Triton x-100, 5 mM BME, 10 mM imidazole, protease inhibitors EDTA-free tablets (1/50mL). Clarified lysate was loaded on Ni-NTA FF prepacked columns (Qiagen) and washed with 10 column volumes (CV) of wash buffer (40 mM Tris pH 7.8, 150 mM NaCl, 5 mM BME, 10 mM imidazole). Protein was eluted by buffer containing 40 mM Tris pH 7.8, 150 mM NaCl, 5 mM BME, 300 mM imidazole. The resulting protein was tag removed by SUMO protease in dialysis buffer (40 mM Tris pH 7.8, 150 mM NaCl, 1 mM DTT, 10 mM Imidazole) overnight at 4 °C, followed by subtractive Ni-NTA purification to remove the tagged protein. KRAS protein was *N*-terminally biotinylated using SrtA and biotin-Sortag peptide [biotin-KGGGLPETGG-OHse(Ac)-amide]. The reaction was carried out at room temperature for 5 h, followed by Ni subtraction to remove His-tagged SrtA and SoftLink™ Avidin Resin purification to isolate the biotin-labeled K-Ras.

**GDP Nucleotide Loading:** KRAS<sup>G12D</sup> protein was treated with 5 mM EDTA and 5 mM GDP for a minimum of 2 h on ice. The reaction was stopped by addition of 40 mM MgCl<sub>2</sub> and incubate on ice for 1 h. The reaction was stopped by addition of 40 mM MgCl<sub>2</sub> and incubate on ice for 1 h. The resulting proteins were further purified and buffer-exchanged on a Superdex 75 26/60 size exclusion column preequilibrated in 25 mM HEPES pH 7.5, 150 mM NaCl, 2 MgCl<sub>2</sub>, 2 mM TCEP. The nucleotide loading efficiency was analyzed by HPLC. Specifically, 5 µL of the protein or nucleotide at 100 µM were injected on a RP-C18 analytical column. Nucleotides were separated through an isocratic elution in 100 mM potassium phosphate, pH 6.5, 10 mM tetrabutylammonium bromide, 0.2 mM NaN<sub>3</sub>, and 3% acetonitrile. The runs are monitored at 254 nm and compared to GDP standard.

**X-ray crystallography:** KRAS(1-168, G12D) loaded with GMPPCP was provided by Evotec at a concentration of 10mg/ml. The complex with **MP-9903** was prepared by incubating 3-fold molar excesses of the peptide on ice for 2h prior to the crystallization experiments. The complex was crystallized from a reservoir solution containing 0.1 M Bis-Tris (pH 6.5) and 25% (v/v) PEG 4000 at 18°C

via the sitting-drop vapor diffusion method. Crystals were harvested and cryoprotected with an additional 10% glycerol. X-ray diffraction data were collected at IMCA beamline ID17 and processed using the Globalphasing [1] and CCP4 [2] packages. Structures were solved by molecular replacement using 5XCO [3] and refined using BUSTER [1] and Coot [4]. Peptides were modeled using standard amino acid residue definitions or restraints generated using Flynn [5]. Coordinates and experimental data are available from the Protein Data Bank with the accession code **7ROV**.

**Surface Plasmon Resonance:** SPR experiments were performed with Biacore T100 (GE Healthcare) at 25 °C. The site-specific mono-biotinylated proteins were prepared by sortase-mediated ligation. SPR buffer consisted of 50 mM Tris pH 7.4, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 10 μM GDP, 0.05% Tween 20, and 3% DMSO. For reducing condition, 1 mM DTT was included in the SPR buffer and peptide solution. The CM5 chip was first conditioned with 100 mM HCl, followed by 0.5% SDS, 50 mM NaOH and then water, all performed twice with 6 sec injection at a flow rate of 100 μL/min. With the flow rate set to 10 μL/min, streptavidin (S4762, Sigma-Aldrich) was immobilized on the conditioned chip through amine coupling as described in the Biacore manual. Excess protein was removed by 30 sec injection of the wash solution (50 mM NaOH + 1 M NaCl) for at least 8 times. The immobilized levels are typically 4000 RU. The biotinylated KRAS<sup>G12D</sup> (GDP) protein was captured to the immobilized streptavidin to a level of ~300 RU. Flow cell consisted of only streptavidin was used as the reference surface. **KRpep-2d** (five concentrations: 0.6 nM, 1.9 nM, 5.6 nM, 16.7 nM, 50 nM) dissolved in the SPR buffer were injected sequentially in single-cycle kinetic format without regeneration (contact time 180 sec, dissociation time 300 sec, flow rate 30 mL/min). The dissociation was monitored for 300 sec. After the run, responses from the target protein surface are transformed by (i) correcting with DMSO calibration curve, (ii) subtracting the responses obtained from the reference surface, and (iii) subtracting the responses of buffer injections from those of peptide injections. The last step is known as double referencing which corrects the systematic artefacts. The resulting responses were subjected to kinetic analysis by fitting with 1:1 binding model.

#### **Time-Resolved Fluorescence/Förster Resonance Energy Transfer (TR-FRET) Assay using KRAS<sup>G12D</sup>**

**Loaded with GDP:** Macrocyclic peptide binding affinities to KRAS<sup>G12D</sup> (GDP) were characterized using a TR-FRET-based competitive binding assay. A master mixture of 30 nM KRAS<sup>G12D</sup> (GDP), 5 nM Terbium-

Streptavidin and 500 nM fluorescently labeled tracer (MP-6139) was prepared. 10  $\mu$ L of the master mixture was added to each well of the 384-well, flat bottom, non-binding, black microplate (Greiner 784900) for the respective assay. Compound dose-response titrations were prepared, and appropriate amounts of compounds were dispensed, using Echo 550 liquid handler, into the respective 384-well black microplate, containing the master mixture. The assay plate is sealed with an aluminum foil and placed on a shaker at ambient temperature for 5 min. The plate was then centrifuged at 1000g for 3 min, then incubated in the dark for 18 h at ambient temperature. Assay plates were read at ambient temperature on the Tecan M1000, with excitation at 340 nm and emission at 495 and 520 nm. Dose response curves and EC<sub>50</sub> were analyzed using a 4-parameter logistic equation in GraphPad Prism software (GraphPad, San Diego, CA).

**KRAS/RBD PPI assay:** TR-FRET based RBD assay was carried out with a final concentration of 2.5 nM Tb-Streptavidin labeled KRAS<sup>G12D</sup> (GMPPNP), 10 nM GST-RBD and 1  $\mu$ g/ml anti-GST-d2 (Cisbo) in RBD buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.01% Tween). The assay volume was 10  $\mu$ L in a 384-well plate (Greiner 784900). Inhibitors/DMSO vehicle were dispensed using Echo 550 liquid handler, into plate containing the reaction mixture. Plates were sealed and incubated at room temperature for 1h. TR-FRET signal was detected using EnVision plate reader with an excitation filter at 340 nm and emission filters for Tb at 615 and d2 at 665 nm, respectively. Dose response curves and EC<sub>50</sub> were analyzed using a 4-parameter logistic equation in GraphPad Prism software (GraphPad, San Diego, CA).

**SOS-mediated nucleotide exchange assay:** SOS-mediated nucleotide exchange assay was performed as previously reported [6] with slight modifications. Biotin-labeled KRAS<sup>G12D</sup> protein was treated with EDTA buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 10 mM EDTA, and 0.01% (w/v) Tween20) for 1 hour at room temperature. The resulting proteins were incubated with 10X excess of BODIPY-GDP in SOS assay buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.01%(w/v) Tween20) for 6 hours at room temperature. A master mixture containing 3 nM K-Ras, 30 nM BODIPY-GDP and 0.5 nM Tb-Streptavidin was prepared. Inhibitors/DMSO vehicle were dispensed to a dry plate (Corning3820) using Echo 550 liquid handler; subsequently, 3  $\mu$ L of master mixture and 3  $\mu$ L of SOS assay buffer was added to the plate and sealed. Sealed plates were incubated at room temperature for 60 min prior to the addition of 3  $\mu$ L 120 nM SOS1(618-1099 a.a.) and 9 mM GTP premix. Plates were read after 60 min incubation at room

temperature using EnVision plate reader with excitation at 340 nm and emission at 495 and 520 nm. Dose response curves and EC<sub>50</sub> were analyzed using a 4-parameter logistic equation in GraphPad Prism software (GraphPad, San Diego, CA).

**Procedure for Cellular Phospho-ERK Assay in AsPC-1 Cells (Alpha Screen):** Cellular KRAS inhibitory activity was evaluated by phosphorylation levels of ERK1/2 in AsPC-1 (homozygous KRAS<sup>G12D</sup>) cells. AsPC-1 cells (ATCC® CRL-1682™) were cultured in T175 flask in growth medium (RPMI 1640 Medium, GlutaMAX™ Supplement, HEPES (Gibco 72400-047) supplemented with 10% fetal bovine serum (Hyclone SH30071.03) and 1X Penicillin/Streptomycin (Gibco 15140-122). The cells were harvested in seeding medium (RPMI 1640 Medium, no phenol red (Gibco 11835-030) supplemented with 10% fetal bovine serum (Hyclone SH30071.03), 25 mM HEPES (Gibco 15630-080) and 1X Penicillin/Streptomycin (Gibco 15140-122) after 5 min of 0.25% Trypsin-EDTA (Gibco 25200-056) digestion and were seeded in 384-well tissue culture treated plate (Greiner 781091) at a density of 15,000 cells/20µL/well, and incubated at 37°C, 5% CO<sub>2</sub> overnight. Prior to dosing, seeding medium was removed using the BlueCatBio Bluewasher system and replaced with 20 µL of assay medium without fetal bovine serum (RPMI 1640 Medium, no phenol red (Gibco 11835-030) supplemented with 25 mM HEPES (Gibco 15630-080) and 1X Penicillin/Streptomycin (Gibco 15140-122). For assays performed in the presence of 10% serum, seeding medium was used instead of assay medium. The compound dose-response titrations were prepared, and appropriate amounts of compounds were dispensed into the 384-well cell culture assay plate using the Echo 550 liquid handler. 25 µL assay or seeding medium was added to achieve a final assay volume of 45 µL. Assay plate was incubated at 37°C, 5% CO<sub>2</sub> for the indicated length of time. After treatment, 25 µL medium was removed and transferred to an empty 384-well tissue culture treated plate (Greiner 781091) for the CytoTox-ONE™ Homogeneous Membrane Integrity (LDH) Assay. Remaining medium was removed from the plate using the BlueCatBio Bluewasher system, and cells were washed once with 25µL 1 × DPBS (Gibco 14190-144). Cells were lysed in 20 µL 1 × lysis buffer from Alpha SureFire® Ultra™ Multiplex pERK and total ERK assay kit (PerkinElmer MPSU-PTERK) containing EDTA-free Protease inhibitor cocktail (Roche 11836170001) at ambient temperature with constant shaking at 300 rpm for 10-15 min. The cell lysates were mixed for 10 cycles using the Agilent Bravo 384ST liquid handler system before 10 µL was transferred to OptiPlate-384 plate (PerkinElmer 6007680). Phosphorylated ERK and total ERK levels were detected by Alpha SureFire Ultra Multiplex pERK kit (PerkinElmer MPSU-PTERK) using 5 µL acceptor bead mix and 5 µL donor bead mix, both prepared following the manufacturer's

protocol. Plates were sealed using aluminum sealing tape (Costar 07-200-683) during incubation at ambient temperature with constant shaking at 300 rpm for 1 h (both acceptor and donor). Assay plates were read on a Envision Xcite Multilabel Reader (PerkinElmer 1040900) at ambient temperature, with emission at 535 nm (Total ERK) and emission at 615 nm (Phospho ERK). Ratio of pERK vs total ERK in each well was used as the final readout. Dose response curves and EC<sub>50</sub> were analyzed using a 4-parameter logistic equation in GraphPad Prism software (GraphPad, San Diego, CA).

**Procedure for Cellular Phospho-ERK Assay in other cell lines:** Procedure for the Alpha screen assay on other cell lines were performed according to AsPC-1 protocol, with the following change in media and cell seeding densities: NCI-H2122 (ATCC® CRL-5985™), NCI-H358 (ATCC® CRL-5807™), and Panc 08.13 (ATCC® CRL-2551™) cells were cultured in T175 flask in growth medium (RPMI 1640 Medium, GlutaMAX™ Supplement, HEPES (Gibco 72400-047) supplemented with 10% fetal bovine serum (Hyclone SH30071.03) and 1X Penicillin/Streptomycin (Gibco 15140-122). Panc 08.13 was further supplemented with 20µg/mL of insulin, human recombinant (Gibco 12585-014). SK-CO-1 (ATCC® HTB-39™) and HEK293 (ATCC® CRL-1573™) cells were cultured in (MEM Medium, GlutaMAX™ Supplement (Gibco 42360-032) supplemented with 10% fetal bovine serum (Hyclone SH30071.03) and 1X Penicillin/Streptomycin (Gibco 15140-122). A-375 (ATCC® CRL-1619™) cells were cultured in (DMEM Medium, GlutaMAX™ Supplement (Gibco 10566-016) supplemented with 10% fetal bovine serum (Hyclone SH30071.03) and 1X Penicillin/Streptomycin (Gibco 15140-122). NCI-H2122, NCI-H358, SK-CO-1 and HEK293 cells were seeded at a density of 15,000 cells/20µL/well. A375 cells were seeded at a density of 10,000 cells/20µL/well. For assays performed in the presence of EGF, cells were serum starved overnight prior to dosing, followed by 20nM of recombinant human EGF (Peprotech AF-100-15) stimulation for 10-15 minutes before cell lysates were harvested.

**CytoTox-ONE™ Homogeneous Membrane Integrity (LDH) Assay:** Transient damage to cellular membranes were measured by LDH release assay. At 1 and 18 h post dose, 25 µL medium was removed from the primary cell culture assay plate and transferred to an empty 384-well tissue culture treated plate (Greiner 781091) using the Agilent Bravo 384ST liquid handler system. CytoTox-ONE reaction mix was prepared from the CytoTox-ONE™ Homogeneous Membrane Integrity Assay Kit (Promega G7891) according to manufacturer's protocol. 25 µL of CytoTox-ONE reaction mix was added to the assay plate

containing 25  $\mu$ L medium, and plate was sealed using aluminum sealing tape (Costar 07-200-683). Assay plate was incubated at ambient temperature with constant shaking at 300 rpm for 45 min. Assay plates were read at ambient temperature on the Tecan M1000, with excitation at 560 nm and emission at 590 nm. Dose response curves and EC<sub>50</sub> were analyzed using a 4-parameter logistic equation in GraphPad Prism software (GraphPad, San Diego, CA).

**CellTiter-Glo® 2.0 Cell Viability (CTG) Assay:** Cell proliferation inhibition of compounds were determined using the CellTiter-Glo® 2.0 assay kit (Promega G9243). AsPC-1, NCI-H2122, NCI-H358, SK-CO-1 and A-375 cells were cultured in their respective growth medium containing 10% fetal bovine serum as according to the protocol described in the Alphascreen assay. AsPC-1 cells were seeded at a density of 1,000 cells/20 $\mu$ L/well into 384-well tissue culture treated plate (Greiner 781080). NCI-H2122, NCI-H358 and SK-CO-1 cells were seeded at a density of 500 cells/20 $\mu$ L/well. A-375 cells were seeded at a density of 300 cells/20 $\mu$ L/well. Assay plates were incubated at 37°C, 5% CO<sub>2</sub> overnight to allow cell attachment. Following day, compounds were dispensed into the assay plate using the Echo 550 liquid handler. 25  $\mu$ L growth medium was added to achieve a final assay volume of 45  $\mu$ L and assay plates were incubated at 37°C, 5% CO<sub>2</sub> for 120 h. At 120 h post dose, CellTiter-Glo assay was performed according to the manufacturer's protocol. 45  $\mu$ L of CellTiter-Glo® 2.0 reagent was added to the assay plate containing 45  $\mu$ L medium and incubated at room temperature for 30 minutes. Assay plates were read on a Envision Xcite Multilabel Reader (PerkinElmer 1040900) with luminescence at an integration time of 0.15 second per well. Dose response curves and EC<sub>50</sub> were analyzed using a 4-parameter logistic equation in GraphPad Prism software (GraphPad, San Diego, CA). AMG510 was prepared internally from published procedures.

**CellTiter-Glo® Cell Viability Assay (Extended cell panel):** Cell proliferation inhibition of compounds were performed on extended cell panels at fee-for-service at Shanghai ChemPartner Co., Ltd. Cell viabilities were determined using the CellTiter-Glo® assay kit (Promega G7558). Cells were cultured and seeded onto 384-well assay plate (Corning 3765) using the Thermo Scientific Multidrop, according to conditions described in Table S3. Assay plates were incubated at 37°C, 5% CO<sub>2</sub> overnight to allow cell attachment. Cells cultured using Leibovitz's L-15 medium were incubated in incubator without CO<sub>2</sub>. Following day, compounds were dispensed into the assay plate using the Tecan HPD300 system and

incubated at 37°C, 5% CO<sub>2</sub> for 120 h. At 120 h post dose, CellTiter-Glo assay was performed according to the manufacturer's protocol. Assay plates were read on a Envision Reader with luminescence at an integration time of 0.1 second per well. Staurosporine (Selleckchem S1421) was used as a positive control for the assay.

**Western Blotting:** Cells were seeded in 24-well poly-D-lysine coated plates and treated with the indicated peptides the next day. Following incubation, 100 µl of 1X Bolt™ LDS sample buffer supplemented with NuPAGE® sample reducing agent was added. Wells were scrapped using wide orifice tips and lysate was transferred into PCR-strip tubes and sonicated for 10 X 10 seconds in a chilled water bath sonicator (QSonica). 15 µl of protein extract was separated on 4-12% Bis-Tris plus gels, transferred onto nitrocellulose membranes using the Trans-Blot® Turbo™ semi-dry system (Bio-rad), and blocked for 1 hour at room temperature with tris-buffered saline (TBS) Odyssey blocking buffer (Li-Cor). Blots were probed with the appropriate primary antibodies overnight at 4°C in Odyssey blocking buffer supplemented with 0.1% Tween-20, followed by the secondary antibodies IRDye® 680RD donkey anti-mouse IgG or IRDye® 800CW donkey anti-rabbit IgG (Li-Cor) for 1 hour at room temperature. Fluorescent signals were imaged and quantified using Odyssey® CLx. Primary antibodies used were: HSP90 (BD Transduction Laboratories, 610419), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (Cell Signaling Technology, #4370), p44/42 MAPK (ERK1/2) (Cell Signaling Technology, #4695), phospho-MEK1/2 (Ser217/221) (Cell Signaling Technology, #9154), MEK1/2 (Cell Signaling Technology, #4694), phospho-AKT (Thr308) (Cell Signaling Technology, #13038), phospho-AKT (Ser473) (Cell Signaling Technology, #4060), AKT (Cell Signaling Technology, #4691 or #2920), phospho-EGF Receptor (Tyr992) (Cell Signaling Technology, #2235), phospho-EGF Receptor (Tyr1045) (Cell Signaling Technology, #2237), phospho-EGF Receptor (Tyr1068) (Cell Signaling Technology, #3777), EGF Receptor (Cell Signaling Technology, #4267), phospho-NF-κB p65 (Ser536) (Cell Signaling Technology, #3033), NF-κB p65 (D14E12) (Cell Signaling Technology, #8242), phospho-IκBα (Ser32) (Cell Signaling Technology, #2859), IκBα (L35A5) (Cell Signaling Technology, #4814), NanoLuc (Promega, N7000) and pan-RAS (Sigma, SAB1404011).

**Pull-downs:** Cells were lysed in ice-cold cell lysis buffer (Cell Signaling Technology) supplemented with 1 mM PMSF and Complete™ EDTA-free protease inhibitor cocktail (Roche) for 30 min with intermittent

vortexing. Lysates were centrifuged at 18,000 g, 4°C for 15 min and supernatants were snap frozen in liquid nitrogen. Protein concentration was determined using the BCA protein assay kit (Pierce). 200 µg protein was mixed with 10 µl anti-HA magnetic beads (Cell Signaling Technology, #11846) in the presence/absence of peptides and incubated overnight at 4°C. The next day, beads were washed three times in ice-cold cell lysis buffer. After the final wash, bound proteins were eluted with 1X Bolt™ LDS sample buffer supplemented with NuPAGE® sample reducing agent and resolved using Western blot as described above. Primary antibodies used were HA-tag (Cell Signaling Technology, #2367), b-RAF (Santa Cruz Biotechnology, sc-5284), c-RAF (Cell Signaling Technology, #12552).

**Traditional CETSA Assay:** Macrocyclic peptide cellular target engagement to KRAS<sup>G12D</sup> (GDP) were characterized using traditional western blot CETSA. AsPC-1 cells (Homozygous KRAS<sup>G12D</sup>) were seeded in 6 well plates (0.25 million cells/2 ml in RPMI + 10% FBS). The following day, cells were treated with peptides dose dependently for 1hr and then trypsinized (0.25%) and pelleted after spinning at 300 rpm for 5 min. After a PBS wash, the cell pellets were resuspended with PBS (30uL/well/dose concentration) containing protease inhibitor (Nacalai Tesque) and transferred into 96 well PCR plate (Biorad) and then heated at 71°C for 3 min followed by cooling to 25°C for 3 min using Eppendorf Mastercycler X50a PCR machine. Note that the melting temperature ( $\Delta T_m$ ) for KRAS protein is previously determined to be 71°C [7]. After this step, the cells were frozen immediately in liquid nitrogen and stored at -80°C. The cells were then thawed and 30uL of 2X stock lysis buffer (100 mM HEPES (pH 7.5), 10mM beta-glycerophosphate, 0.2 mM Sodium orthovanadate, 20 mM MgCl<sub>2</sub>, 4mM TCEP, 0.05% of w/v Rapigest (Waters) and protease inhibitor (Nacalai Tesque)) were added to each PCR well. TCEP and Rapigest were prepared freshly. After the addition of lysis buffer, the cells were frozen and thawed three times in liquid nitrogen and were spun at 21000g for 20 min, 4°C. Supernatant were collected and transferred to new tubes. Protein concentration was determined using the BCA protein assay kit (Pierce). 20 to 50 µg of protein extract was separated on 4-12% Bis-Tris plus gels, transferred onto nitrocellulose membranes using the Trans-Blot® Turbo™ semi-dry system (Bio-rad), and blocked for 1 hour at room temperature with tris-buffered saline (TBS) Odyssey blocking buffer (Li-Cor). Blots were probed with KRAS specific antibody (Thermofisher, #703345) and SOD1 as a loading control (Cell signaling, #4266) overnight at 4°C in Odyssey blocking buffer supplemented with 0.1% Tween-20, followed by the secondary antibodies IRDye® 680RD donkey anti-mouse IgG or IRDye® 800CW donkey anti-rabbit IgG (Li-Cor) for 1 hour at room temperature. Fluorescent signals were imaged and quantified using Odyssey® CLx.

**Fluorescence imaging:** Cells were seeded in 96-well poly-D-lysine coated  $\mu$ CLEAR<sup>®</sup> plates (Greiner) and allowed to attach overnight. The next day, cells were treated with the indicated FAM-labelled peptides for 30 min. Nuclei were counterstained with Hoechst and dead cells were stained with TO-PRO<sup>™</sup>-3 Iodide. For the imaging of eGFP fusion proteins, AsPC-1 cells were transfected with the corresponding mRNAs for 6 hours before treatment with the indicated peptides for 1 hour. mRNA synthesis and transfection were performed as previously described [8]. Images were acquired using the Opera Phenix<sup>™</sup> High Content Confocal Screening System under the 40X water immersion lenses and quantified using the integrated data analysis software.

**Cell Homogenate Stability and Metabolite Identification:** Stability of peptides towards intracellular proteases were evaluated using homogenized cells. Suspended THP1 cells at 1 million cells/mL were sonicated in bursts with a probe sonicator on ice until uniformly homogenized. The homogenate thus prepared was frozen and stored at -20°C until use. The peptides were incubated with the homogenate and loss of the peptide with increasing time was quantified using LC-MS/MS. Briefly, the test peptide was incubated with the homogenate at 1  $\mu$ M in final volume of 50  $\mu$ L at 37°C. At 0, 10, 30, 60 and 120 min, the reactions were quenched with 150  $\mu$ L solution of 80% (v/v) Acetonitrile in Methanol containing a known internal standard peptide. The quenched samples were centrifuged at 4000 rpm for 5 min at 10°C and supernatants injected for bioanalysis by liquid chromatography combined with tandem-mass spectrometer. Pre-boiled homogenate (prepared by incubating homogenate for 30 min in boiling water) was also used as a negative control for each test peptide. The  $k_e$  is the first-order rate constant describing the disappearance of parent drug in the incubation and obtained from regressing the initial slope ( $k_e = -(\text{slope})$ ) of the natural log of the analyte area/ internal standard area (designated as C at an appointed time t) in C versus time (min) profile. The half-life ( $t_{1/2}$ ) is calculated as:

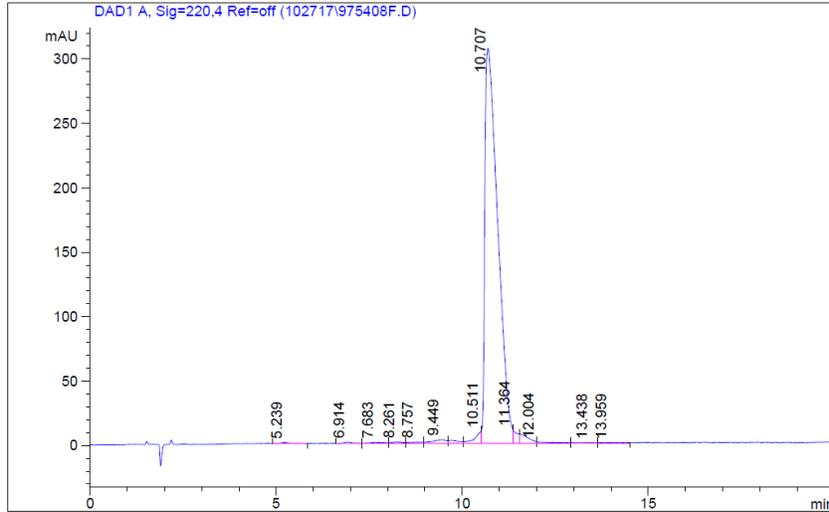
$$t_{1/2} = \frac{0.693}{k_e}$$

For metabolite identification, peptides were incubated in the homogenized cells similarly for 10 min and 30 min, and the high-resolution mass spectrometric analysis was performed, and metabolites assigned based on the mass to charge ratios.

**In Vitro Rat Mast Cell Degranulation and Histamine Release Assessment:** Peritoneal lavage was isolated from Wister-Han rats as described in literature. In brief, animals were anesthetized with xylene followed by an injection of about 10 mL of Tyrode buffer (12 mM NaHCO<sub>3</sub>, 127 mM NaCl, 5 mM KCl, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 5 mM Glucose and 10 mM HEPES, pH 7.4) into the peritoneal cavity. After a gentle abdomen massage, peritoneal lavage was collected and centrifuged at 350 x g for 5 minutes. Cell pellets were resuspended in Tyrode buffer with 10% heat inactivated rat serum to reach a final concentration of 1 X 10<sup>6</sup> cells/mL. Freshly isolated rat peritoneal cells were aliquoted to a 96 well tissue-culture plate (100 µL per well). Cells (1 X 10<sup>5</sup> /mL) were incubated with titrating concentrations of various peptides for 30 minutes at 37°C. After incubation, the plate was centrifuged at 350 x g for 2 minutes at room temperature. Supernatant were collected and histamine concentration was measured by ELISA (Neogen, Lansing, MI) following vendor's instructions. Amount of histamine in the supernatant was presented as ng/mL. A detection of histamine release was defined as the histamine concentration in supernatant that was higher than the mean control values plus 3 standard deviations (typically derived from 10 vehicle-control treated samples from the same animal). The lowest concentration that a histamine release was observed for each peptide was summarized in table X (averaged from 2 rats).

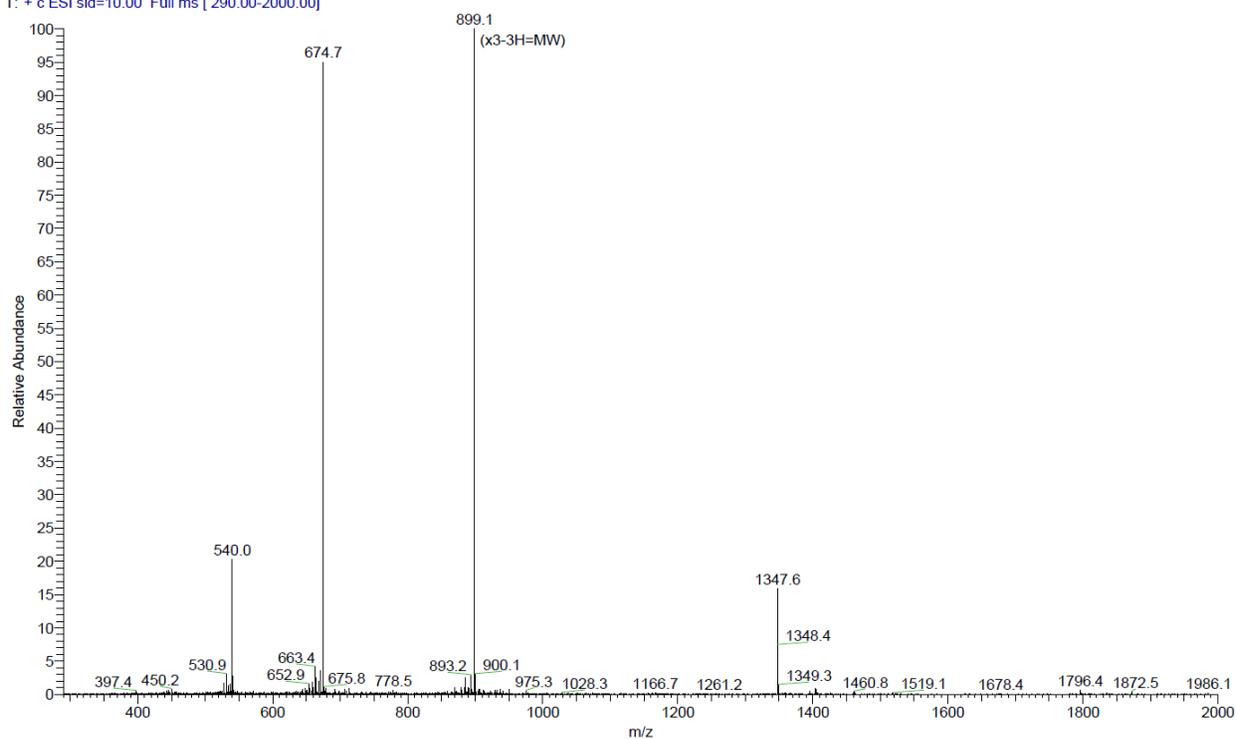
## APPENDIX: HPLC and MS spectra of peptides

### Peptide 1:

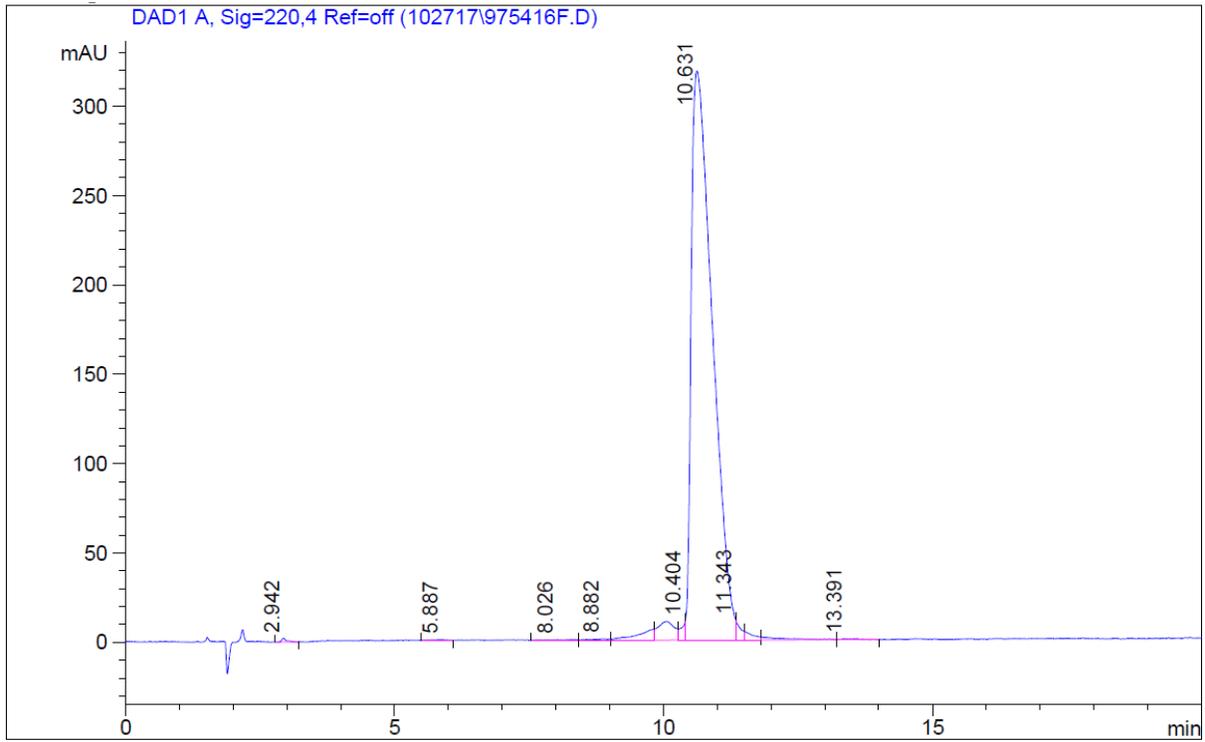


Peak #	Signal RT [min]	Type	Height	Width [min]	Area	Area %
1	5.239	VV	0.985	0.333	23.817	0.299
2	6.914	VV	1.096	0.282	21.640	0.271
3	7.683	VV	0.954	0.400	27.470	0.345
4	8.261	VV	1.137	0.306	24.169	0.303
5	8.757	VV	1.073	0.325	26.321	0.330
6	9.449	VV	2.812	0.387	76.280	0.957
7	9.718	VV	2.207	0.279	45.457	0.570
8	10.511	VV	9.356	0.188	105.630	1.325
9	10.707	VF	305.821	0.364	7358.806	92.292
10	11.364	VF	10.466	0.133	83.284	1.045
11	11.535	VF	7.063	0.276	117.096	1.469
12	12.004	VV	0.934	0.565	31.667	0.397
13	13.438	VV	0.418	0.458	15.034	0.189
14	13.959	VF	0.416	0.495	16.727	0.210

975408F #7-17 RT: 0.15-0.38 AV: 11 NL: 1.06E7  
T: + c ESI sid=10.00 Full ms [ 290.00-2000.00]



Peptide 2:

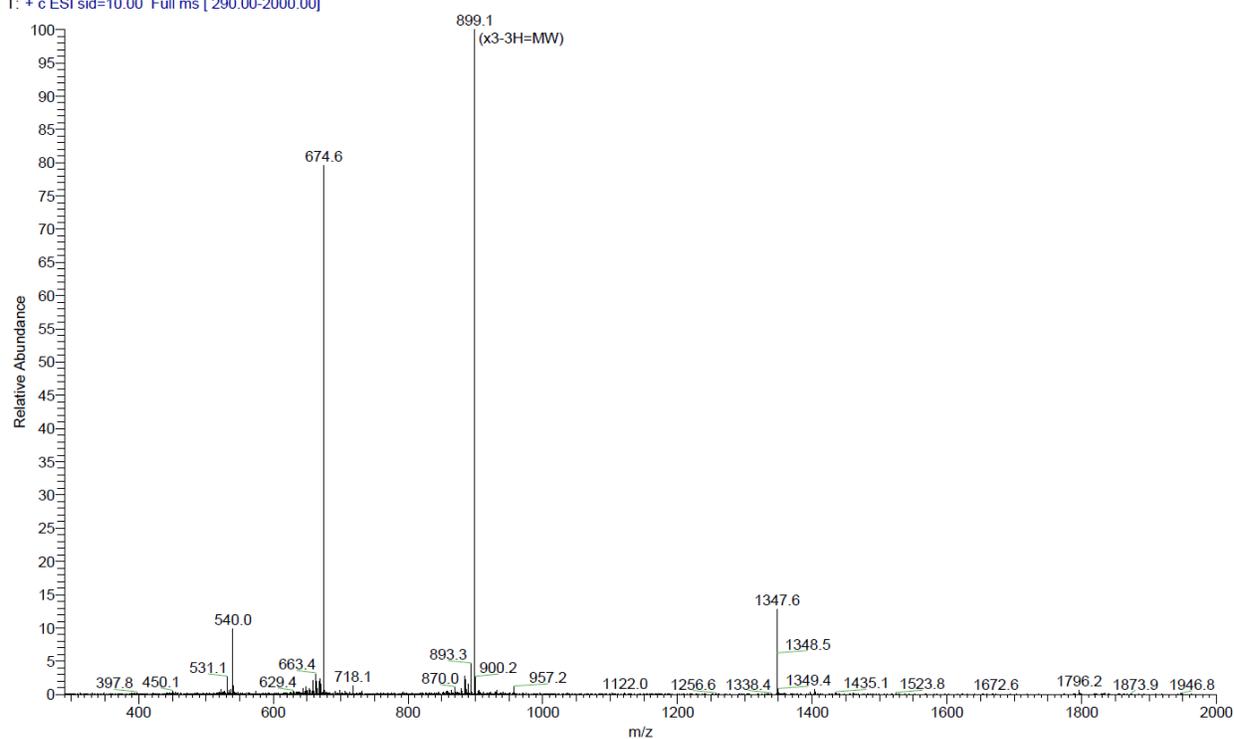


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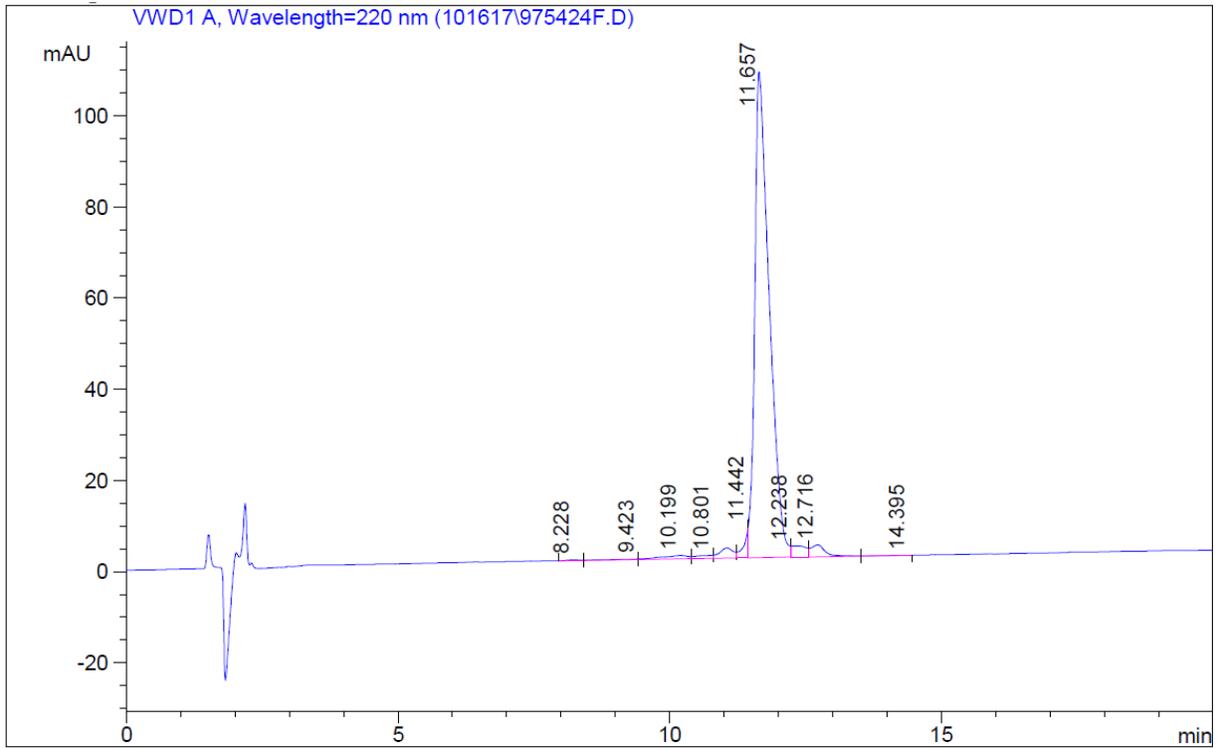
Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	2.942	BP	2.165	0.112	14.912	0.160
2	5.887	MM	0.490	0.270	7.921	0.085
3	8.026	VV	0.404	0.398	12.681	0.136
4	8.882	VV	0.705	0.338	18.123	0.195
5	9.017	VV	0.622	3.552	132.469	1.424
6	10.051	VV	10.502	0.312	222.864	2.395
7	10.404	VV	10.674	0.089	57.130	0.614
8	10.631	VF	317.070	0.421	8656.064	93.018
9	11.343	VF	11.306	0.095	64.520	0.693
10	11.492	VF	4.883	0.191	55.930	0.601
11	11.812	VV	1.456	0.582	50.864	0.547
12	13.391	VV	0.500	0.326	12.316	0.132

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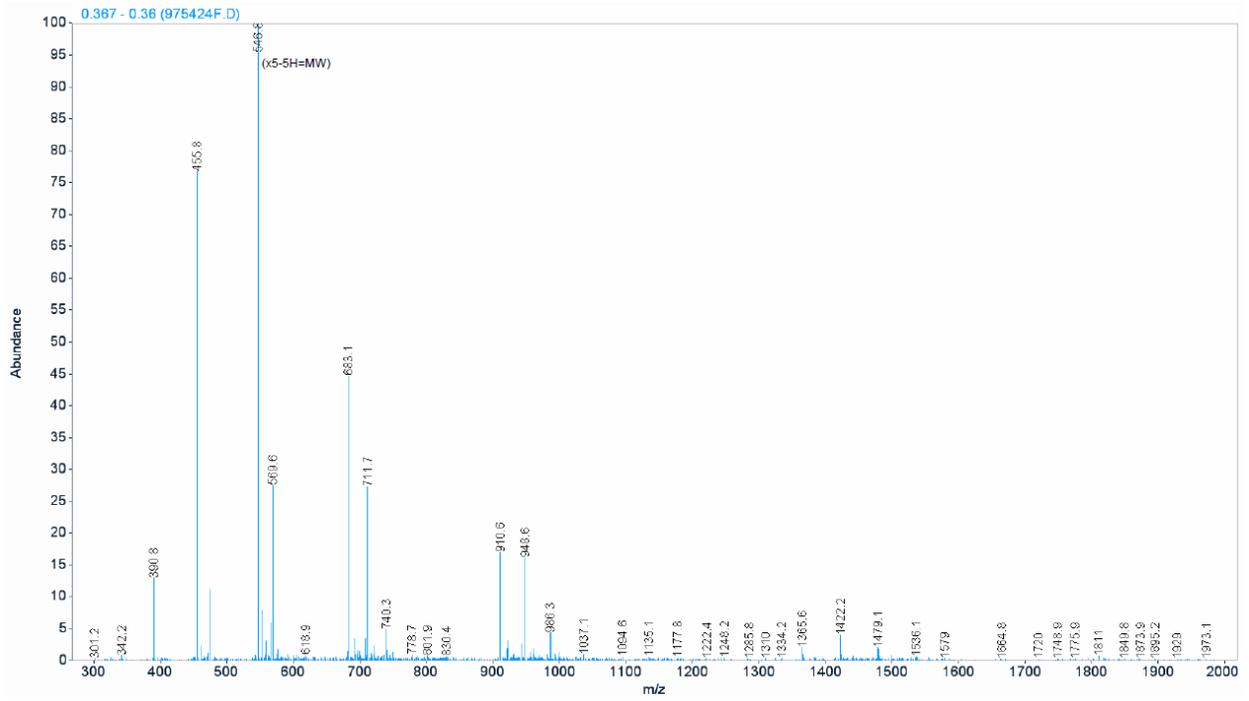
975416F #8-16 RT: 0.17-0.35 AV: 9 NL: 1.25E7  
T: + c ESI sid=10.00 Full ms [ 290.00-2000.00]



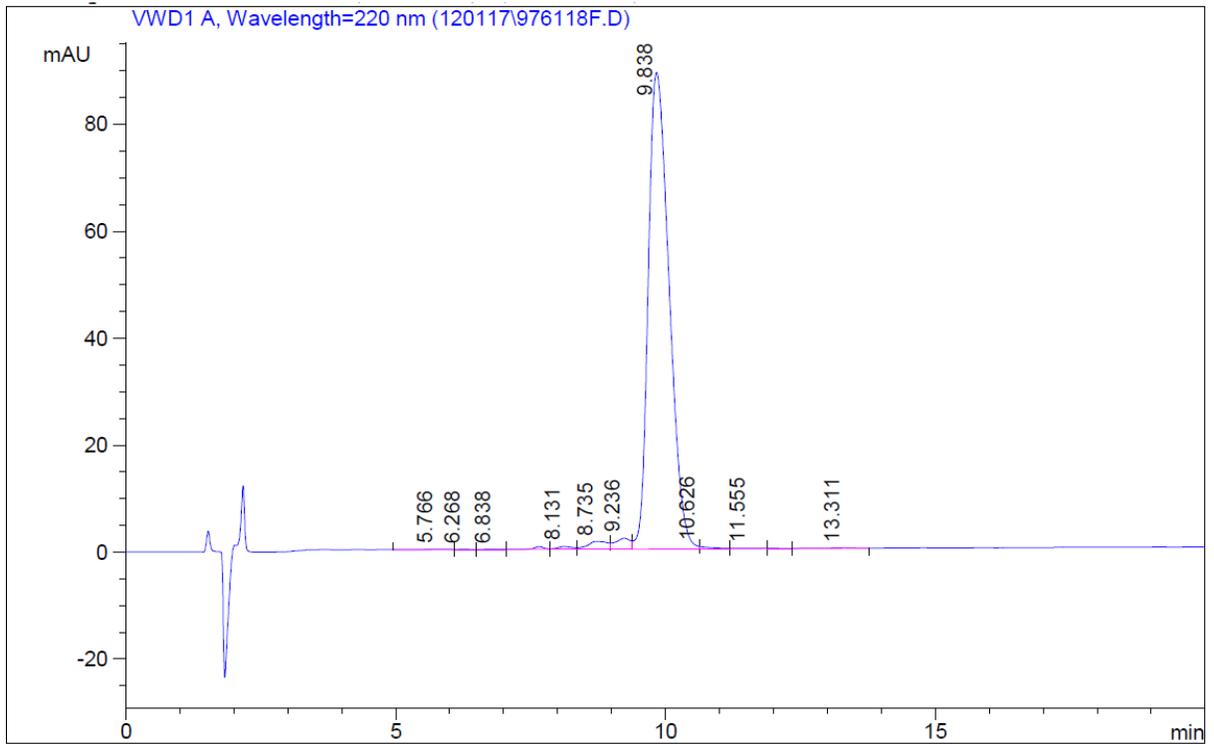
Peptide 3:



Peak #	Signal	RT [min]	Type	Height	Width [min]	Area	Area %
1	1:VWD1 A, Wavelength=220 nm	8.228	VV	0.146	0.242	2.299	0.105
2		9.423	VV	0.108	0.681	4.432	0.202
3		10.199	VV	0.753	0.463	26.127	1.193
4		10.801	VV	0.725	0.336	14.629	0.668
5		11.050	VV	2.228	0.256	38.807	1.773
6		11.442	VV	6.774	0.085	34.438	1.573
7		11.657	VF	105.664	0.276	1975.385	90.226
8		12.238	VV	2.446	0.303	44.441	2.030
9		12.716	VV	2.614	0.266	47.858	2.186
10		14.395	MF	0.016	1.001	0.964	0.044



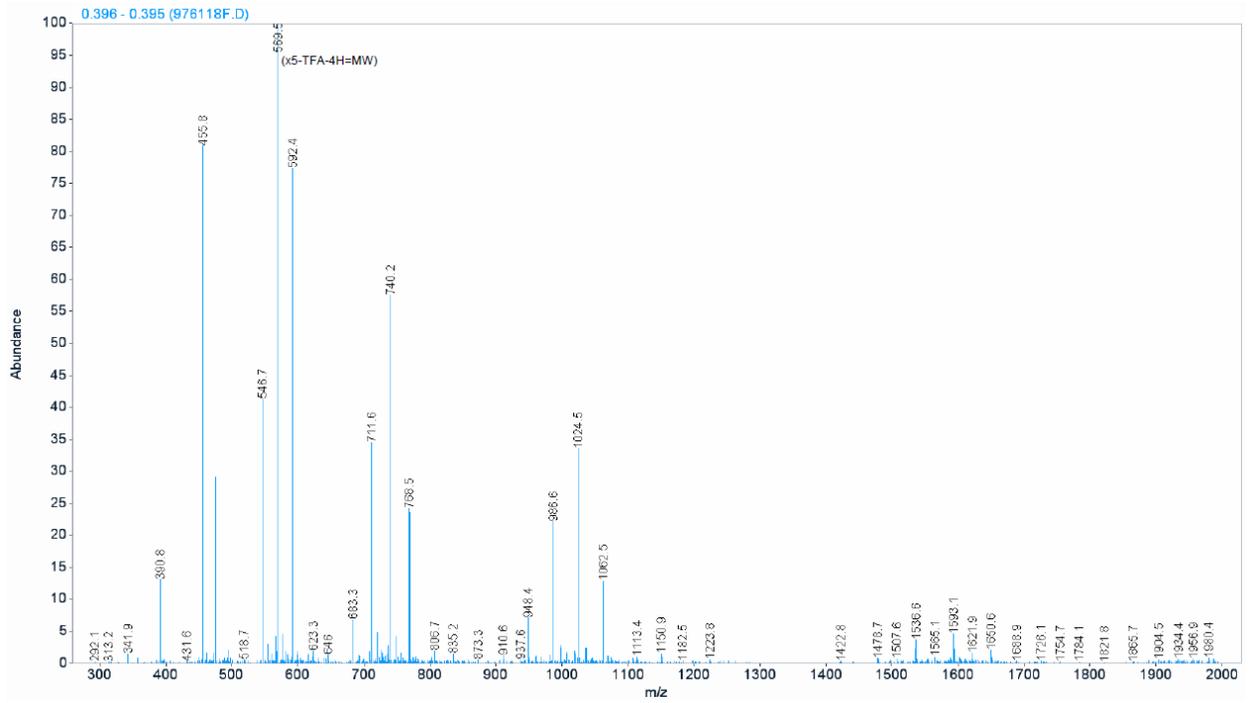
Peptide MP-6483:



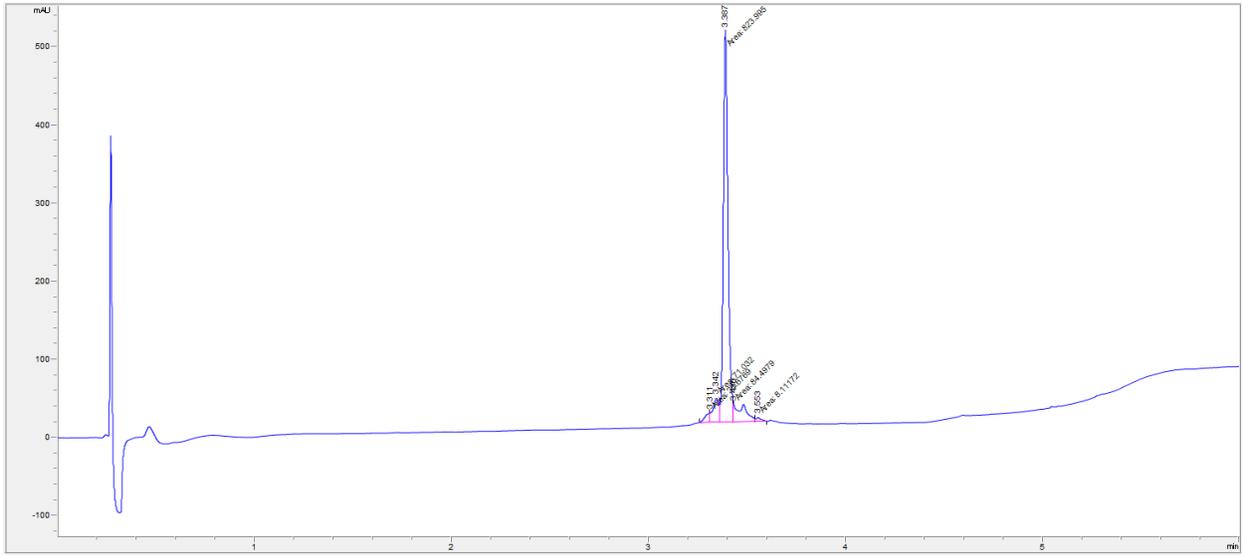
===== >

Signal 1:VWD1 A, Wavelength=220 nm						
Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	5.766	VV	0.088	0.433	2.826	0.113
2	6.268	VP	0.047	0.235	0.708	0.028
3	6.838	VV	0.040	0.354	0.843	0.034
4	7.659	VV	0.520	0.211	7.344	0.294
5	8.131	VV	0.530	0.284	9.987	0.400
6	8.735	VV	1.453	0.391	37.150	1.486
7	9.236	VV	2.050	0.274	38.869	1.555
8	9.838	VF	89.117	0.429	2383.890	95.388
9	10.626	VV	0.448	0.261	7.018	0.281
10	11.555	VV	0.098	0.500	3.521	0.141
11	12.008	VV	0.053	0.295	1.158	0.046
12	13.311	VP	0.134	0.597	5.844	0.234

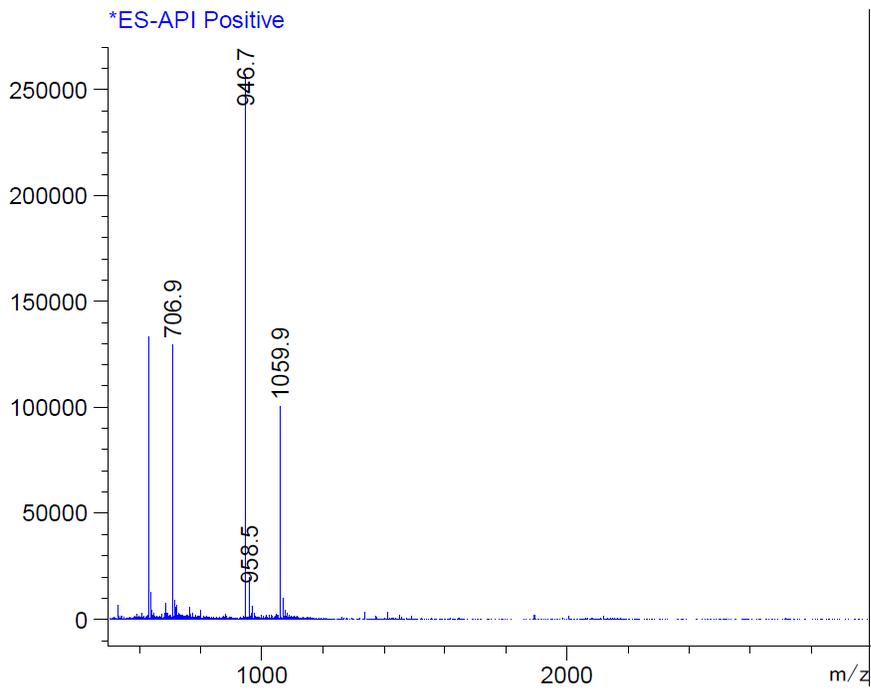
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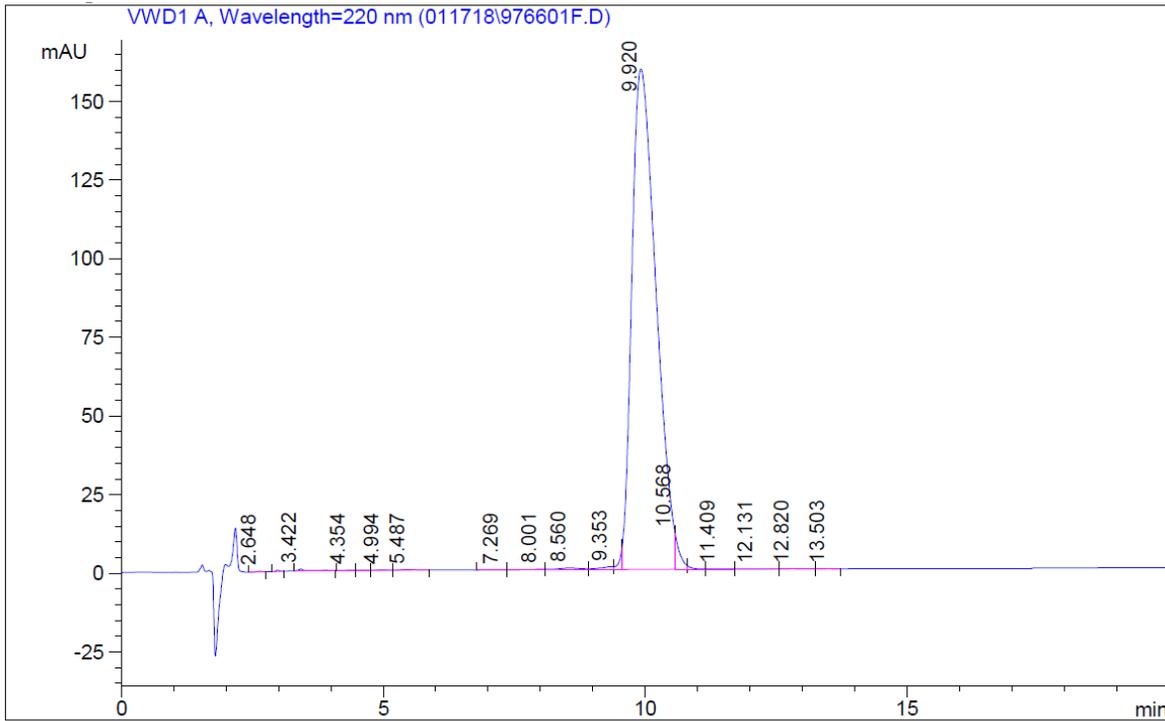
Peptide 4:



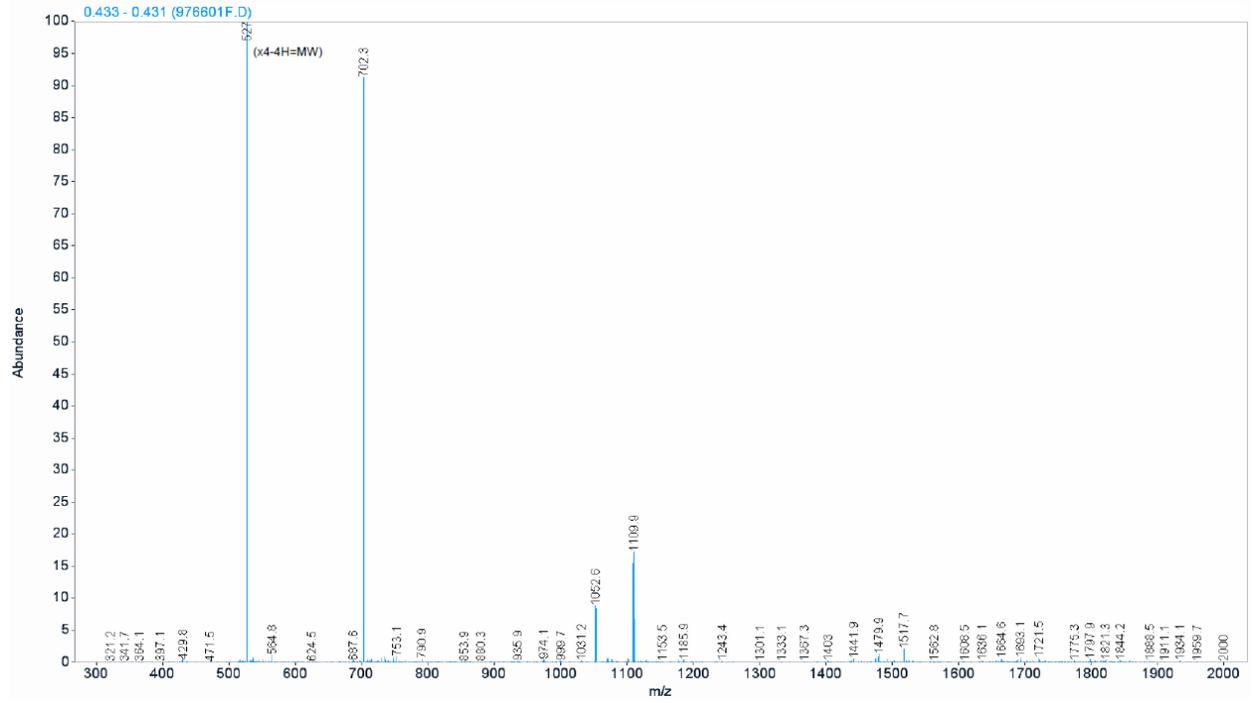
#	Time	Type	Area	Height	Width	Area%	Symmetry
1	3.311	MF	23.7	12.5	0.0315	2.341	0
2	3.342	FM	71	31.1	0.038	7.024	1.001
3	3.387	FM	824	502.5	0.0273	81.478	0.792
4	3.43	FM	84.5	22.7	0.0621	8.355	0
5	3.553	FM	8.1	5	0.0271	0.802	0.486



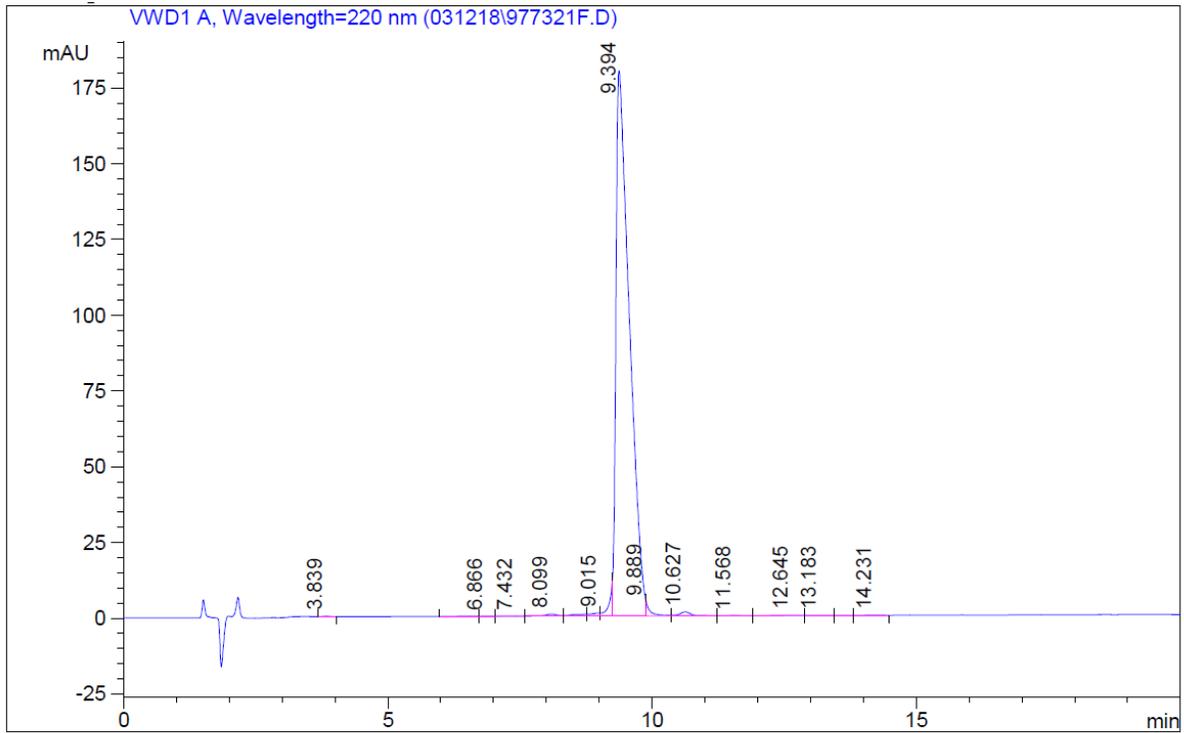
Peptide **MP-1687**:



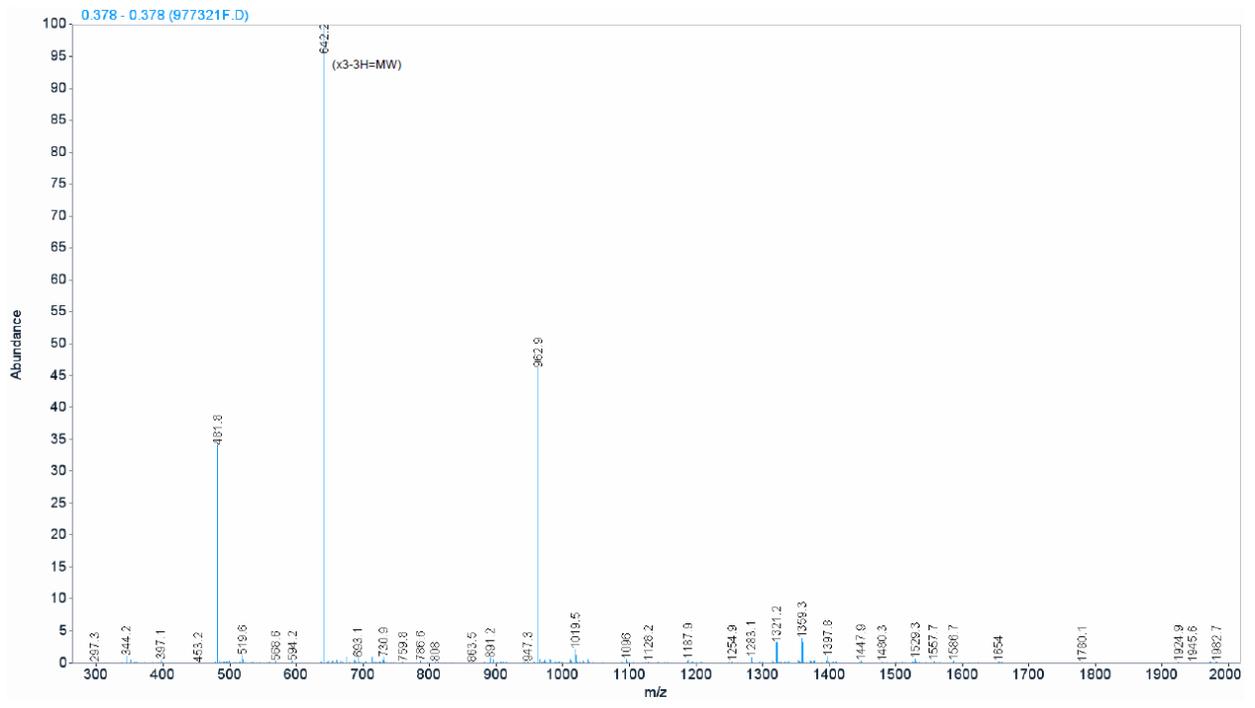
Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	2.648	MM	0.249	0.102	1.530	0.029
2	2.986	MM	0.454	0.078	2.114	0.040
3	3.422	MM	0.565	0.153	5.188	0.099
4	4.354	VV	0.133	0.134	1.132	0.022
5	4.645	VV	0.065	0.209	0.903	0.017
6	4.994	VV	0.122	0.269	2.021	0.039
7	5.487	VP	0.199	0.338	4.081	0.078
8	7.269	VV	0.022	0.318	0.559	0.011
9	8.001	VV	0.160	0.320	3.824	0.073
10	8.560	VV	0.568	0.437	17.313	0.331
11	9.353	VV	0.930	0.242	17.017	0.326
12	9.403	VV	0.887	0.442	23.536	0.450
13	9.920	VF	158.894	0.500	5065.748	96.915
14	10.568	VF	11.708	0.097	68.192	1.305
15	10.801	VV	1.181	0.092	6.520	0.125
16	11.409	VV	0.097	0.357	2.519	0.048
17	12.131	VV	0.055	0.515	2.136	0.041
18	12.820	VV	0.073	0.357	1.952	0.037
19	13.503	VV	0.032	0.309	0.712	0.014



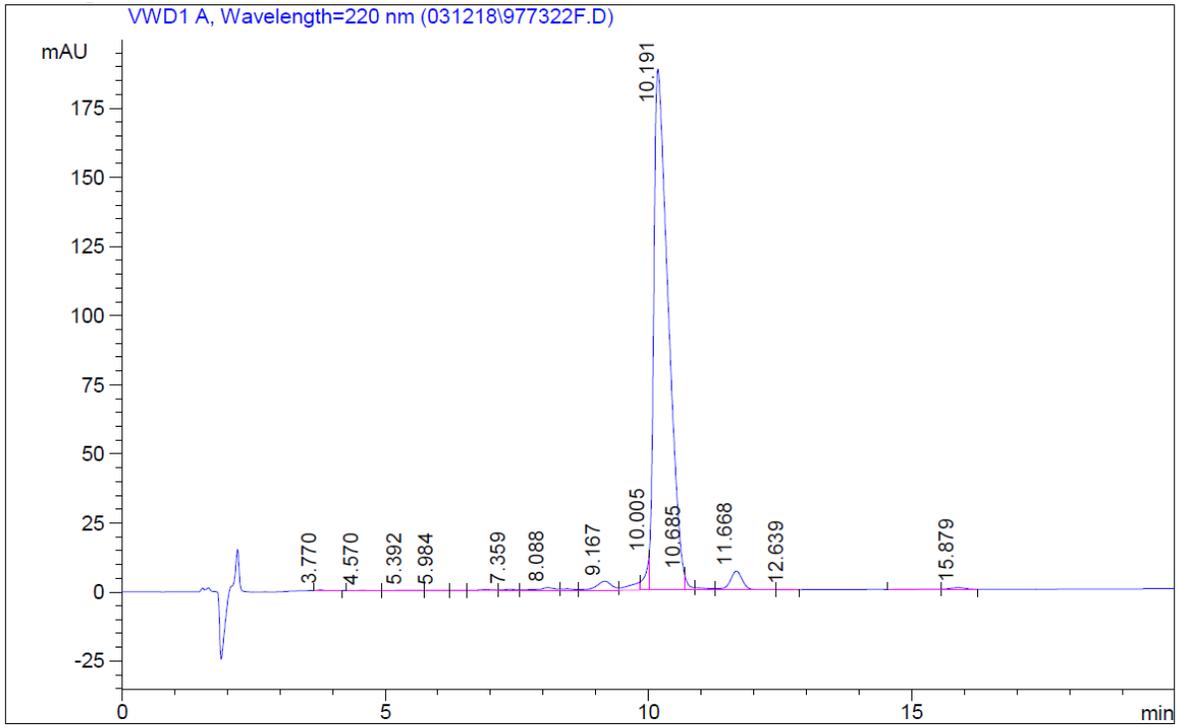
Peptide 5:



Peak #	Signal RT [min]	Type	Height	Width [min]	Area	Area %
1	3.839	BV	0.186	0.130	1.509	0.045
2	6.613	VV	0.085	0.361	2.372	0.071
3	6.866	VV	0.082	0.233	1.316	0.040
4	7.432	VV	0.108	0.312	2.466	0.074
5	8.099	VV	0.701	0.274	12.949	0.390
6	8.595	VV	0.570	0.294	12.373	0.372
7	8.763	VV	0.602	0.330	11.909	0.358
8	9.015	VV	0.899	0.816	44.012	1.324
9	9.394	VF	179.983	0.259	3181.586	95.739
10	9.889	VV	4.683	0.086	24.038	0.723
11	10.627	VV	1.287	0.234	20.038	0.603
12	11.568	VV	0.052	0.435	1.539	0.046
13	12.645	VV	0.101	0.422	2.899	0.087
14	13.183	VV	0.077	0.380	1.941	0.058
15	13.621	VP	0.034	0.249	0.561	0.017
16	14.231	VV	0.079	0.328	1.670	0.050

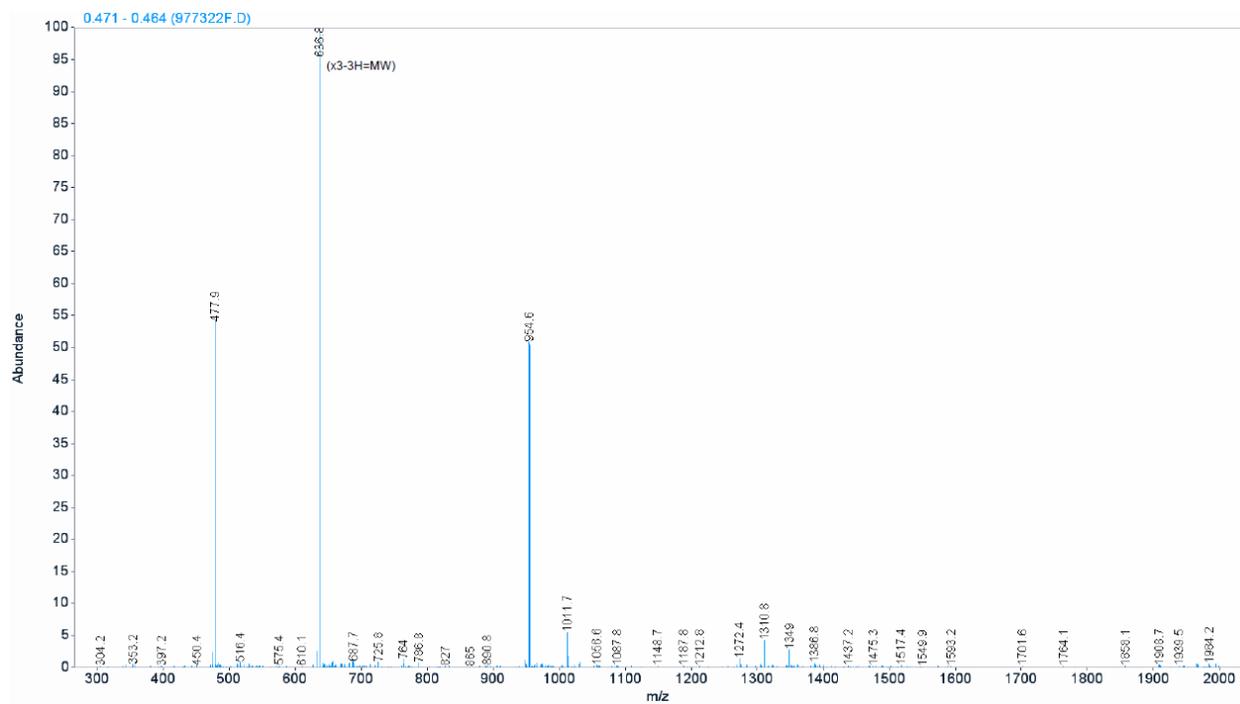


Peptide 6:

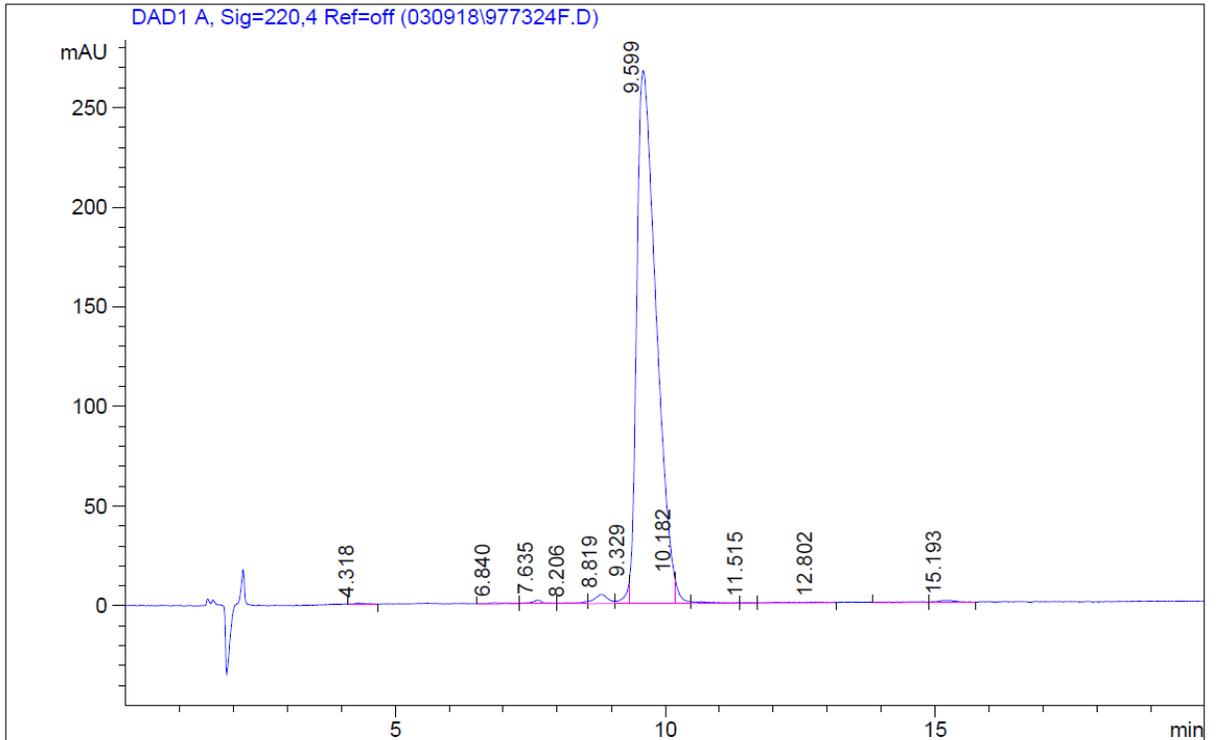


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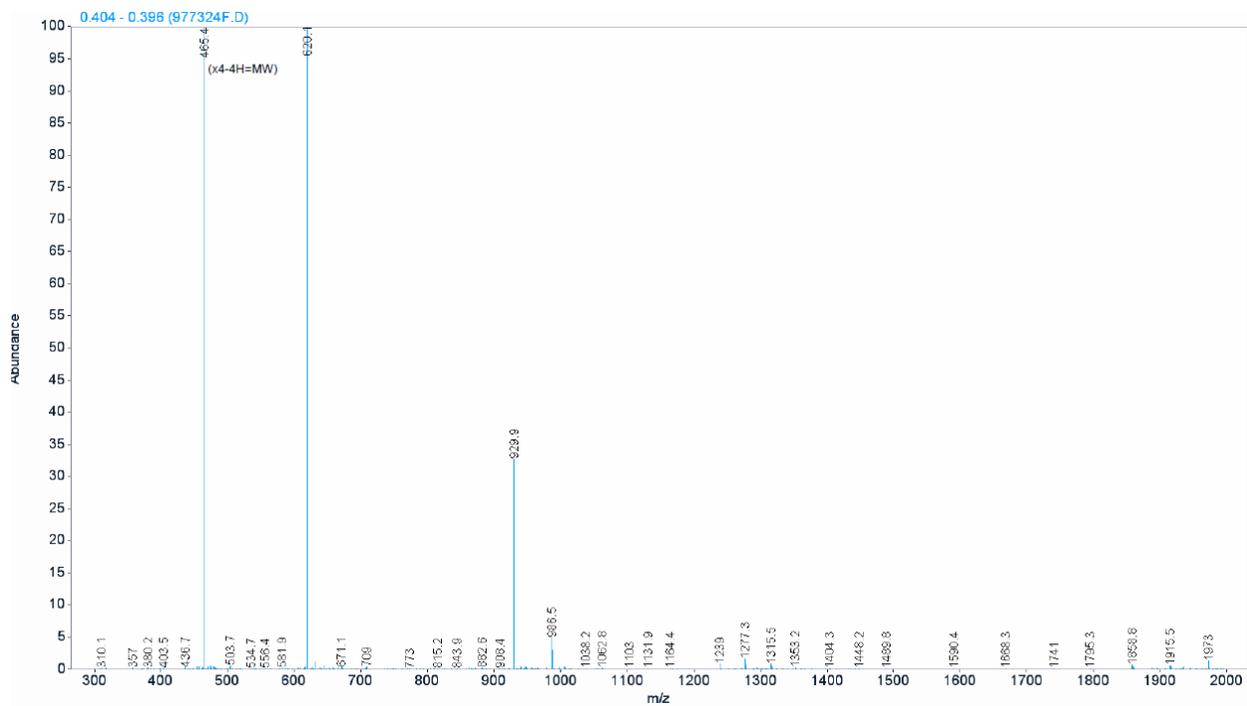
Peak #	Signal RT [min]	Type	Height	Width [min]	Area	Area %
1	3.770	MM	0.228	0.193	2.642	0.067
2	4.570	BP	0.188	0.164	2.174	0.055
3	5.392	VV	0.164	0.398	4.286	0.108
4	5.984	VV	0.110	0.311	2.433	0.061
5	6.379	VV	0.070	0.276	1.299	0.033
6	6.915	VV	0.296	0.278	5.591	0.141
7	7.359	VV	0.389	0.219	5.748	0.145
8	8.088	VV	1.000	0.287	21.137	0.534
9	8.446	VV	0.528	0.228	8.450	0.213
10	9.167	VV	3.246	0.306	65.635	1.657
11	9.830	VV	2.864	0.225	38.715	0.977
12	10.005	VV	11.830	0.081	57.277	1.446
13	10.191	VF	185.701	0.289	3578.944	90.353
14	10.685	VF	5.654	0.070	23.599	0.596
15	10.879	VV	0.818	0.292	14.313	0.361
16	11.668	VV	6.774	0.250	111.079	2.804
17	12.639	VP	0.055	0.279	1.009	0.025
18	15.557	VV	0.109	0.552	3.598	0.091
19	15.879	VV	0.675	0.297	13.149	0.332



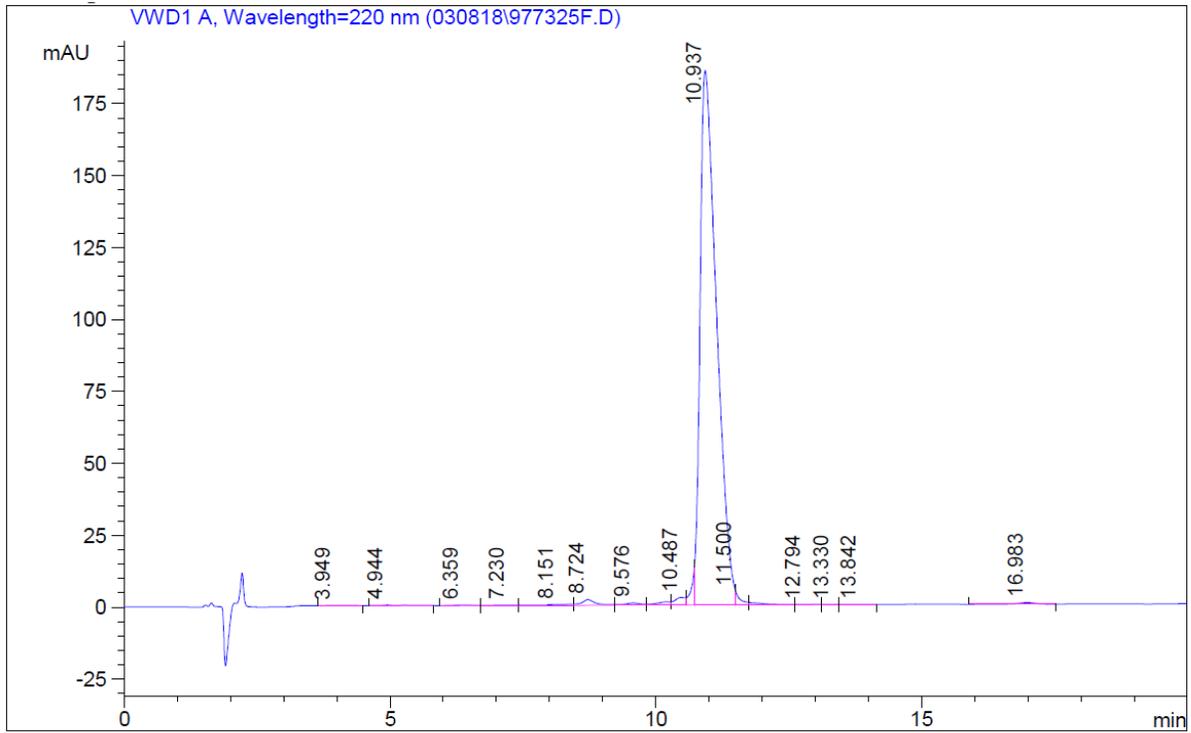
Peptide 7:



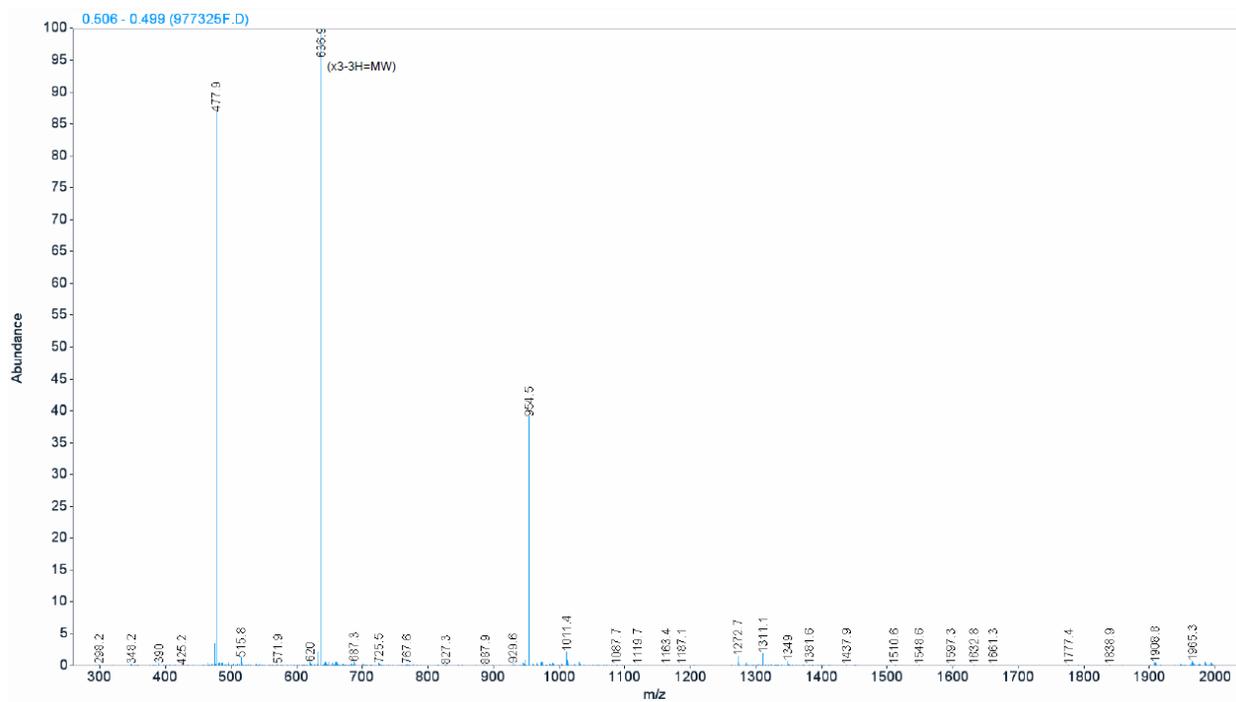
Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	4.318	VV	0.679	0.215	10.267	0.147
2	6.840	VV	0.564	0.338	14.168	0.203
3	7.635	VV	1.708	0.222	26.885	0.385
4	8.206	VV	0.351	0.521	14.492	0.208
5	8.819	VV	4.579	0.258	78.703	1.128
6	9.329	VV	10.316	0.081	50.043	0.717
7	9.599	VF	266.303	0.380	6632.005	95.034
8	10.182	VF	12.187	0.086	62.618	0.897
9	10.481	VV	0.711	0.453	19.335	0.277
10	11.515	VV	0.273	0.182	3.660	0.052
11	12.802	VV	0.454	0.640	23.171	0.332
12	14.760	VV	0.439	0.477	16.996	0.244
13	15.193	VV	1.007	0.348	26.206	0.376



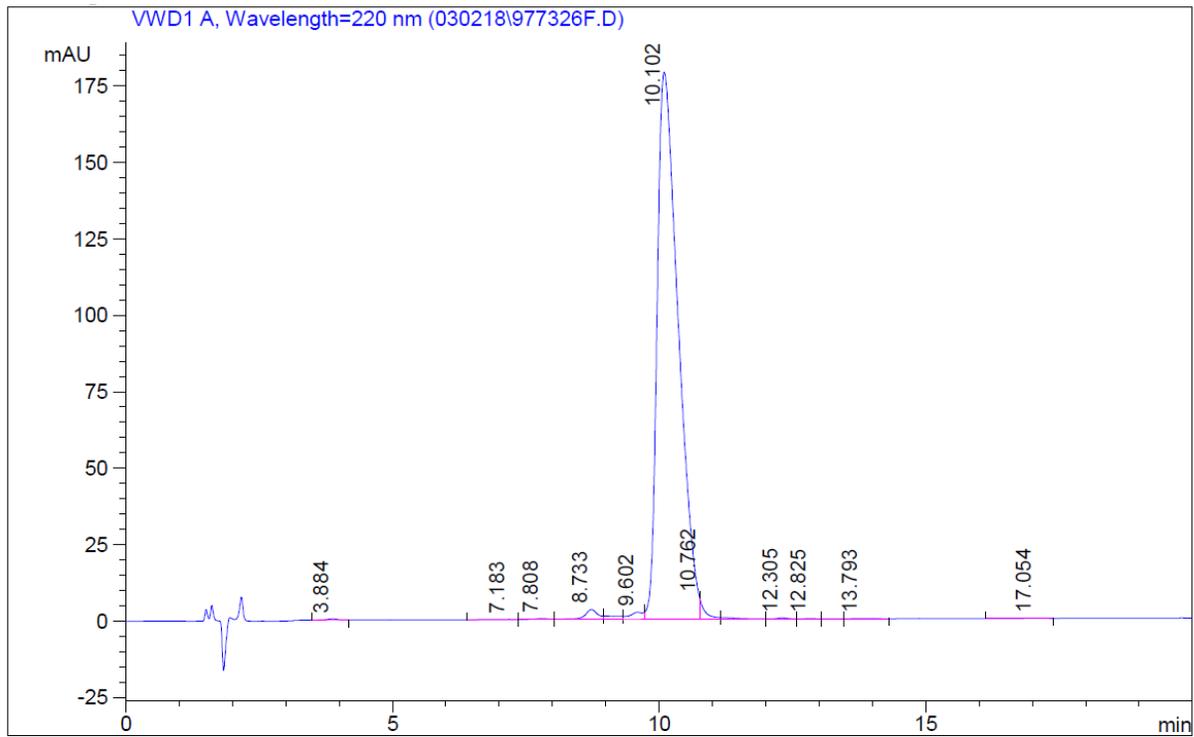
Peptide 8:



Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	3.949	MM	0.134	0.314	2.527	0.060
2	4.944	MM	0.240	0.501	7.206	0.170
3	6.359	VV	0.149	0.432	3.948	0.093
4	7.230	VV	0.126	0.360	3.513	0.083
5	8.151	VV	0.330	0.477	12.194	0.287
6	8.724	VV	1.931	0.263	34.862	0.821
7	9.576	VV	0.743	0.294	15.012	0.354
8	10.277	VV	1.042	0.307	19.211	0.453
9	10.487	VV	2.594	0.197	35.919	0.846
10	10.568	VV	2.511	0.336	50.674	1.194
11	10.937	VF	184.997	0.328	3999.248	94.212
12	11.500	VF	4.729	0.095	26.857	0.633
13	11.752	VV	0.787	0.317	14.973	0.353
14	12.794	VV	0.070	0.327	1.508	0.036
15	13.330	VV	0.030	0.211	0.443	0.010
16	13.842	VV	0.055	0.405	1.601	0.038
17	16.983	VV	0.578	0.377	15.231	0.359

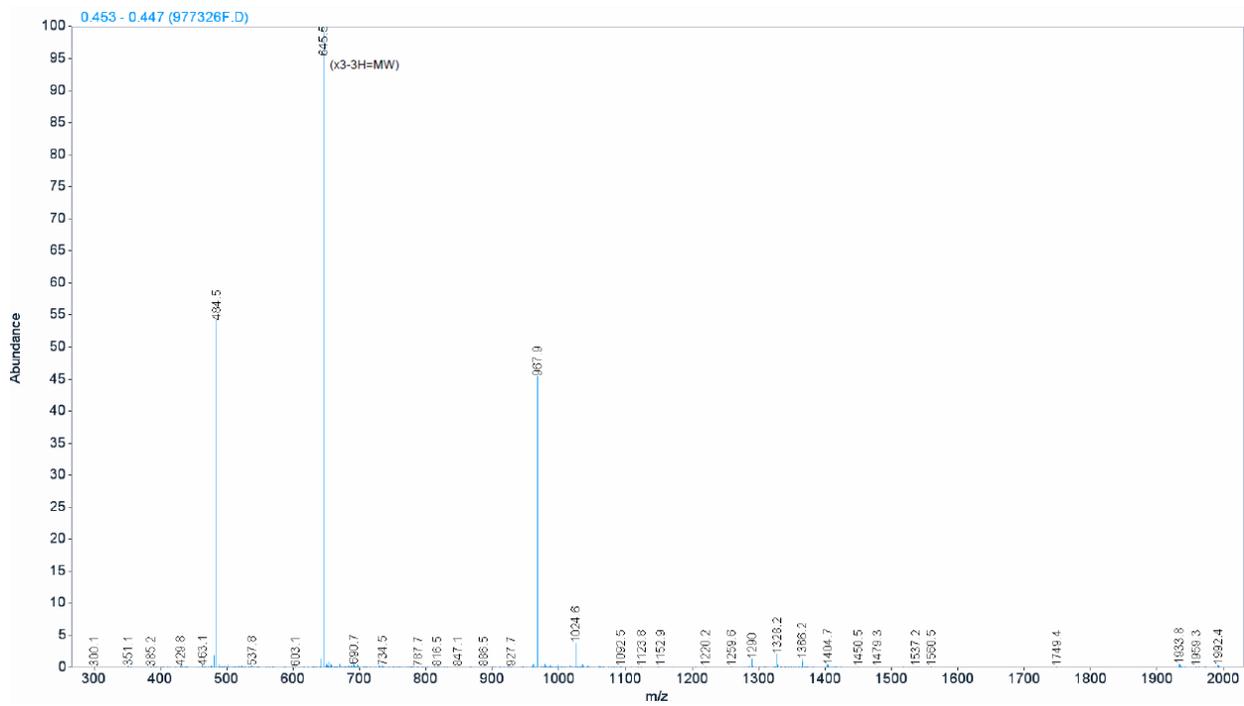


Peptide 9:

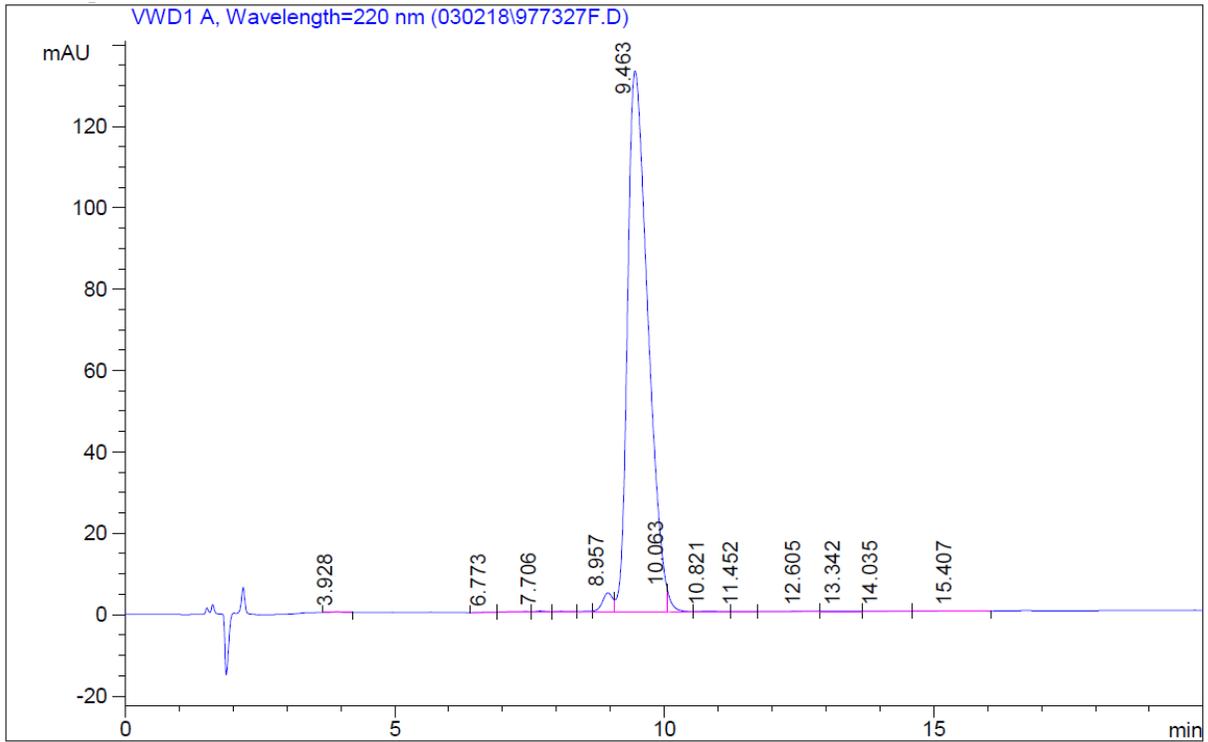


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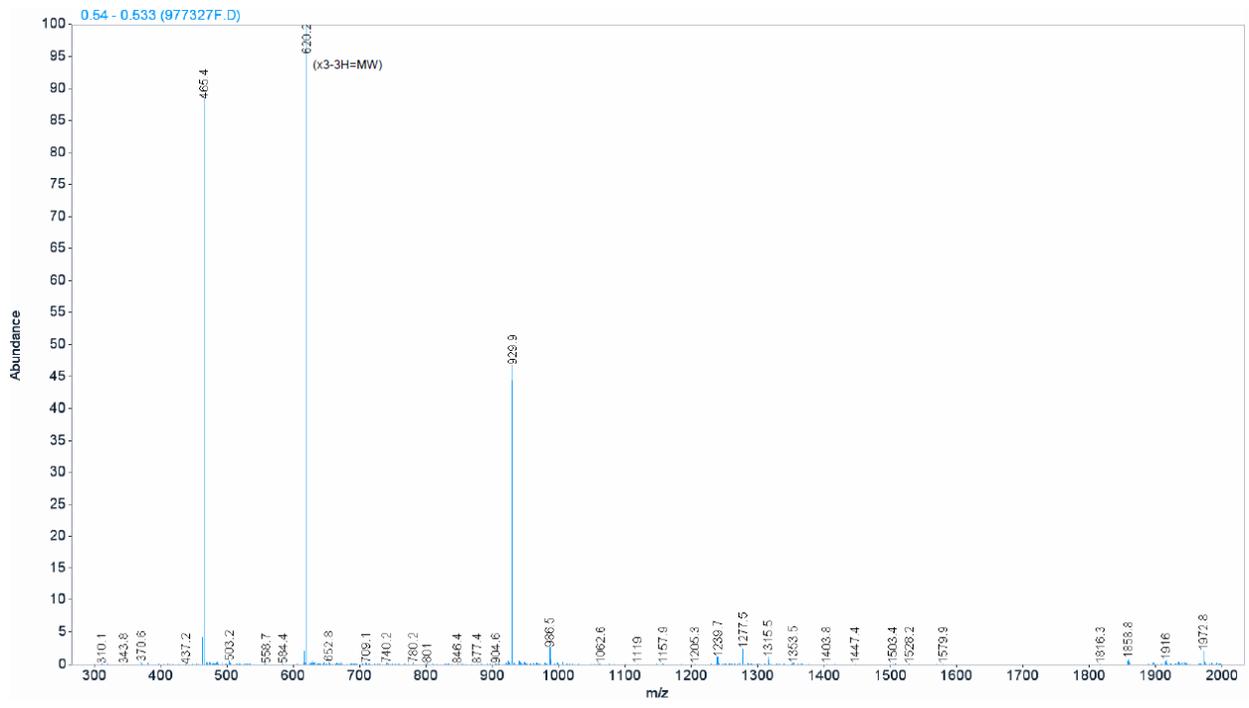
Peak #	Signal RT [min]	Type	Height	Width [min]	Area	Area %
1	3.884	BV	0.345	0.244	5.481	0.107
2	7.183	VV	0.104	0.448	3.580	0.070
3	7.808	VV	0.375	0.265	7.011	0.137
4	8.733	VF	3.266	0.251	55.675	1.091
5	8.957	VV	1.150	0.328	22.597	0.443
6	9.602	VV	2.313	0.252	41.979	0.823
7	10.102	VF	178.680	0.419	4897.575	96.004
8	10.762	VF	6.839	0.106	43.524	0.853
9	11.151	VV	0.533	0.356	11.372	0.223
10	12.305	VV	0.362	0.222	5.252	0.103
11	12.825	VV	0.090	0.317	1.954	0.038
12	13.226	VV	0.057	0.274	1.197	0.023
13	13.793	VV	0.061	0.390	1.708	0.033
14	17.054	VV	0.076	0.477	2.516	0.049



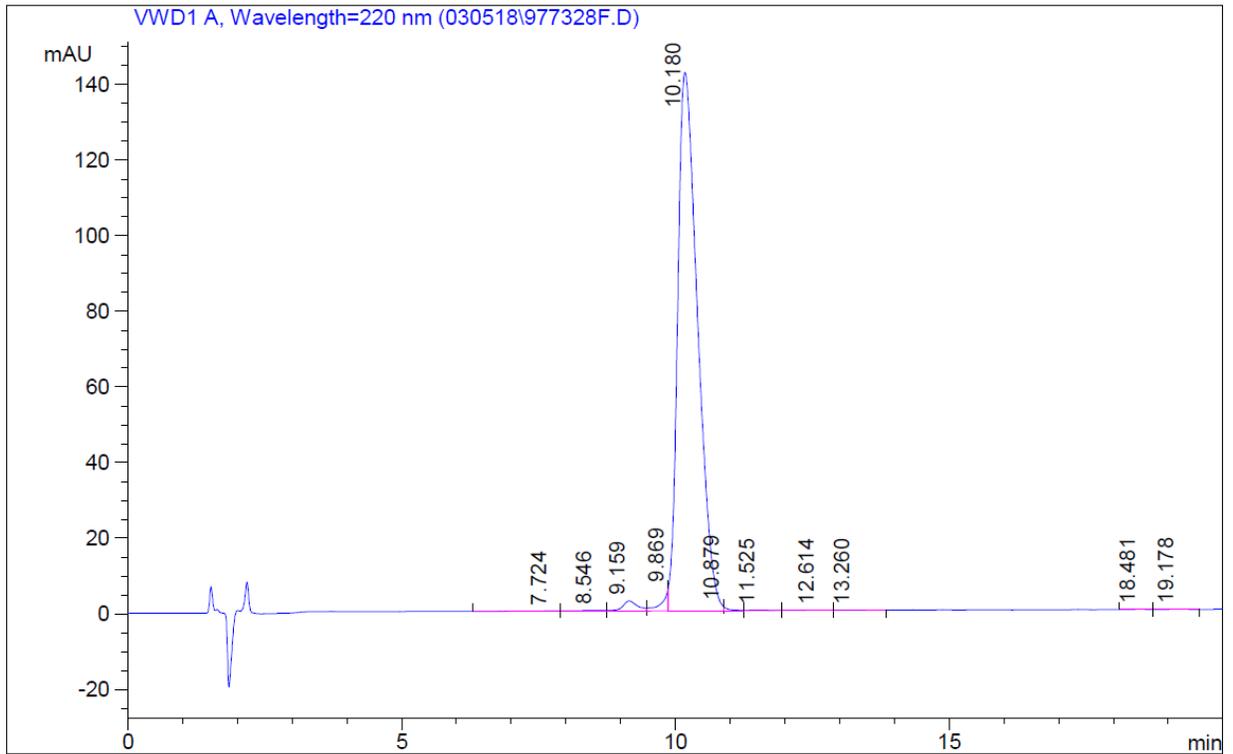
Peptide 10:



Peak #	Signal RT [min]	Type	Height	Width [min]	Area	Area %
1	3.928	MM	0.169	0.228	2.323	0.064
2	6.773	VV	0.129	0.271	2.286	0.063
3	7.430	VV	0.221	0.378	6.481	0.178
4	7.706	VV	0.353	0.235	5.692	0.157
5	8.093	VV	0.263	0.323	6.107	0.168
6	8.666	VV	0.290	0.235	4.078	0.112
7	8.957	VV	4.781	0.201	63.130	1.736
8	9.463	VF	132.943	0.405	3488.541	95.935
9	10.063	VV	5.150	0.092	28.406	0.781
10	10.821	VV	0.165	0.503	5.725	0.157
11	11.452	VV	0.118	0.366	2.934	0.081
12	12.605	VV	0.110	0.712	5.880	0.162
13	13.342	VV	0.108	0.508	4.428	0.122
14	14.035	VV	0.094	0.607	4.361	0.120
15	15.407	VV	0.106	0.719	5.982	0.165

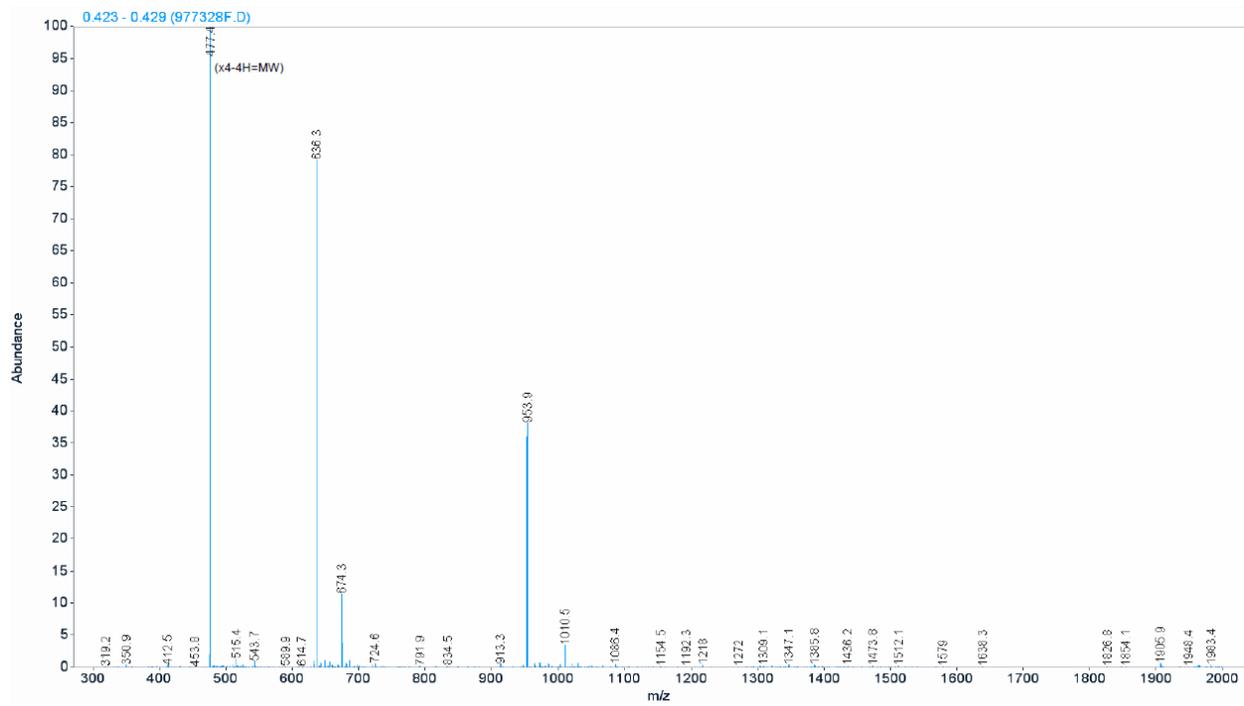


Peptide 11:

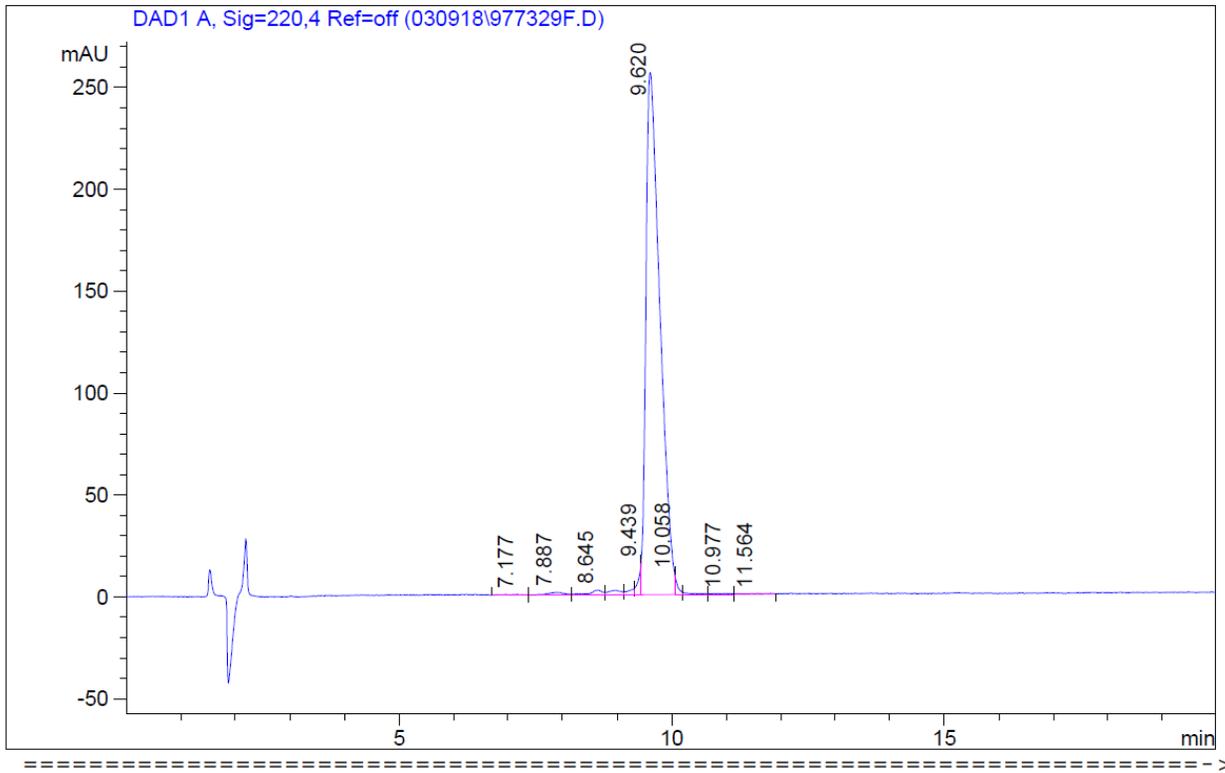


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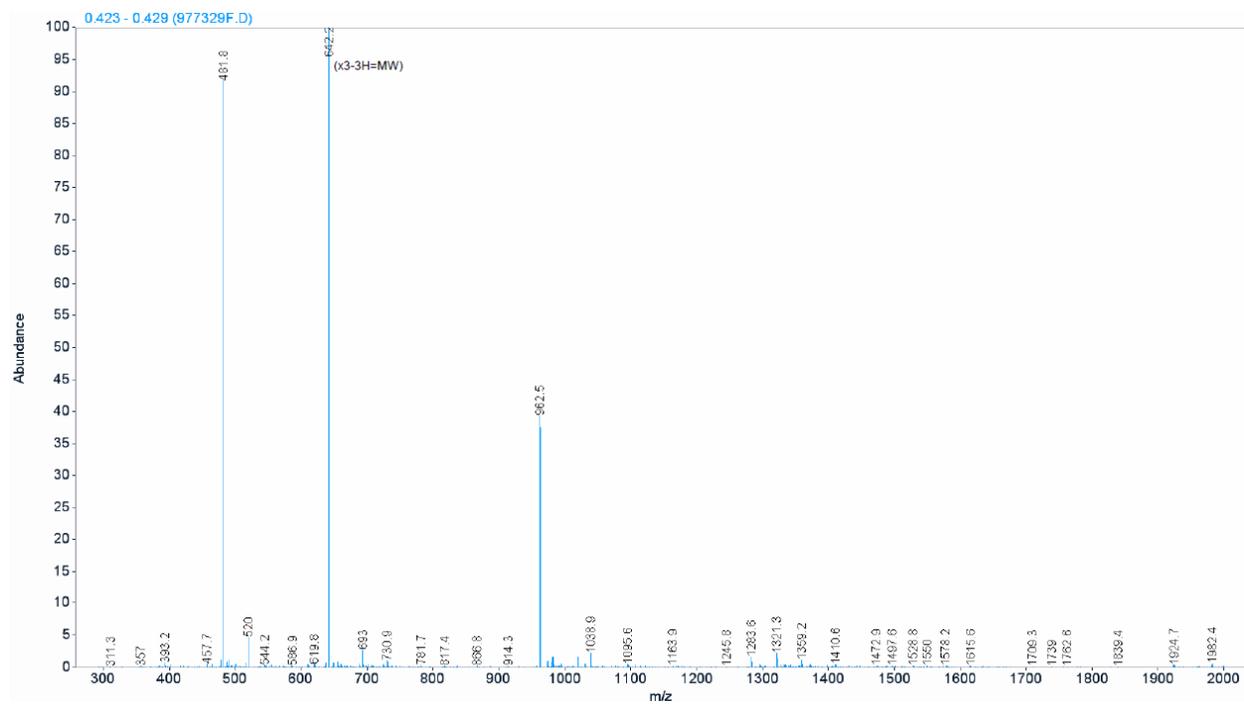
Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	7.724	VV	0.129	0.614	6.236	0.168
2	8.546	VV	0.206	0.550	7.952	0.214
3	9.159	VV	2.646	0.278	49.862	1.343
4	9.869	VV	6.118	0.119	43.804	1.180
5	10.180	VF	142.276	0.393	3582.866	96.505
6	10.879	VV	1.097	0.100	6.548	0.176
7	11.525	VV	0.136	0.448	4.216	0.114
8	12.614	VV	0.107	0.554	4.165	0.112
9	13.260	VV	0.091	0.561	3.566	0.096
10	18.481	VV	0.106	0.229	1.600	0.043
11	19.178	VV	0.058	0.413	1.807	0.049



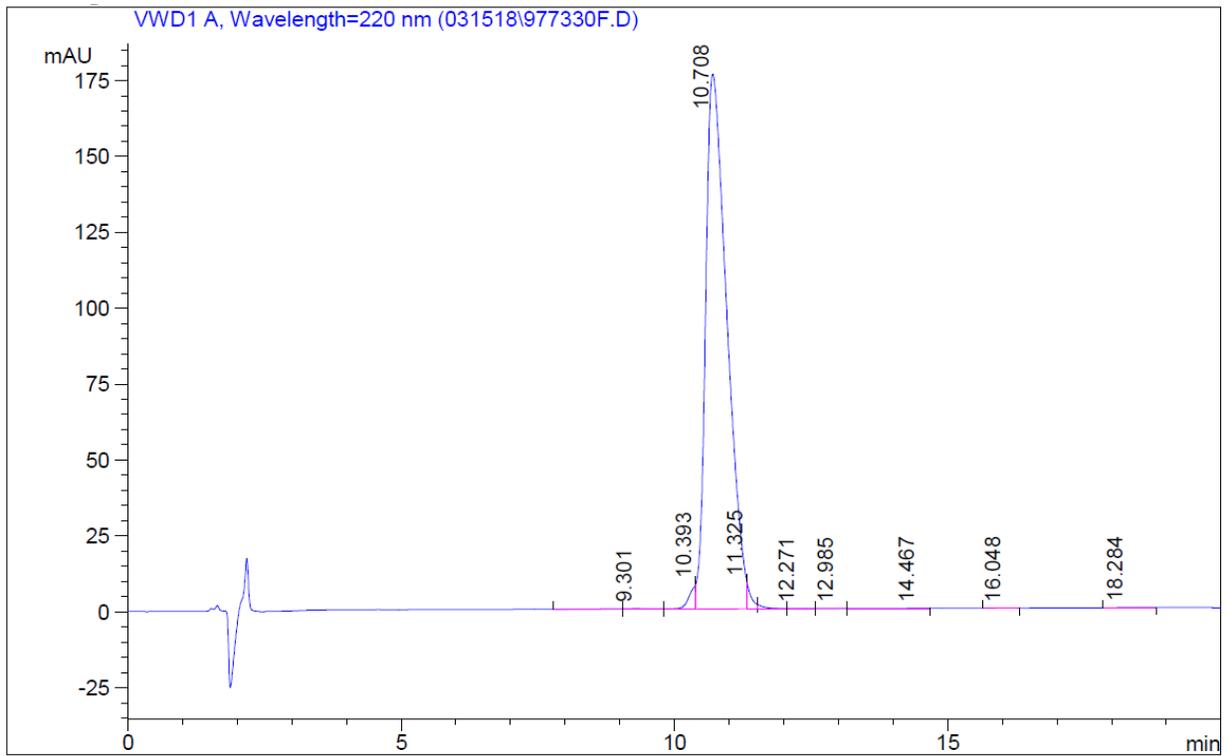
Peptide 12:



Signal 1: DAD1 A, Sig=220,4 Ref=off						
Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	7.177	VV	0.384	0.264	7.839	0.163
2	7.887	VV	1.297	0.320	27.772	0.576
3	8.645	VV	2.254	0.241	36.581	0.759
4	8.963	VV	2.008	0.258	34.458	0.715
5	9.311	VV	2.863	0.128	21.917	0.455
6	9.439	VV	15.539	0.057	52.970	1.099
7	9.620	VF	256.816	0.278	4572.042	94.863
8	10.058	VF	10.014	0.057	34.127	0.708
9	10.207	VV	0.932	0.204	11.434	0.237
10	10.977	VV	0.387	0.293	8.879	0.184
11	11.564	VV	0.428	0.355	11.609	0.241



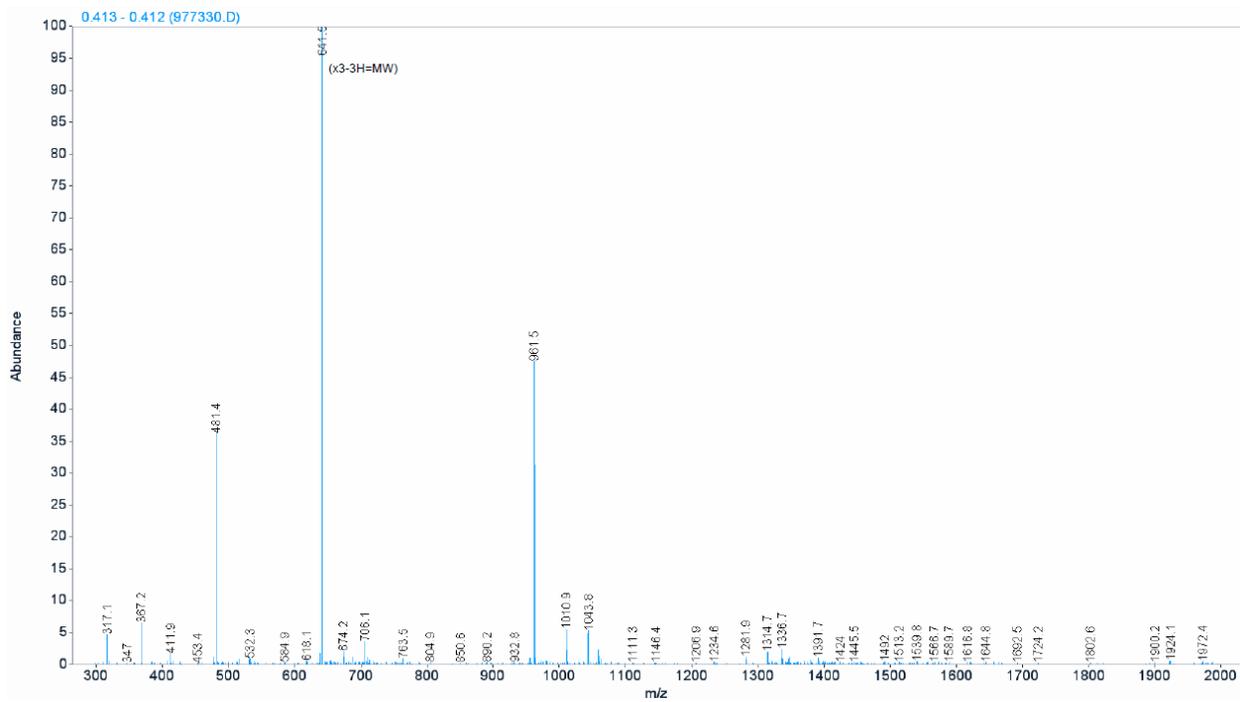
Peptide 13:



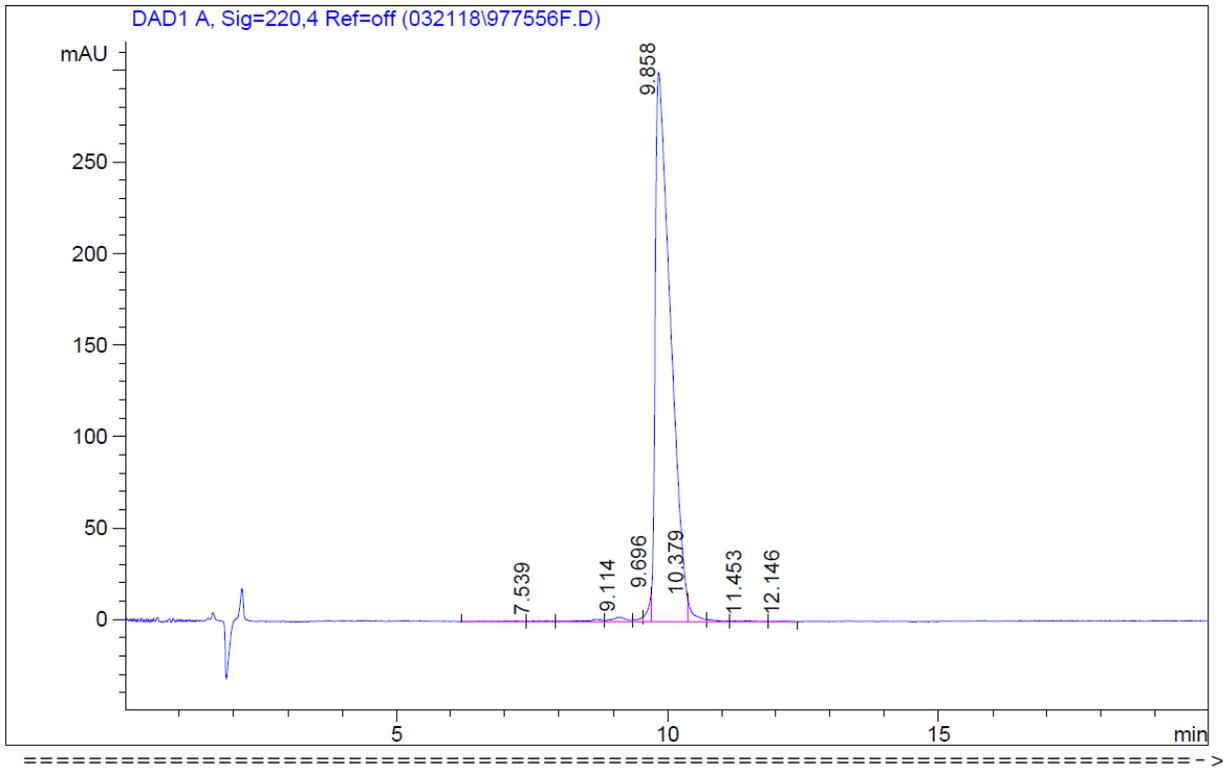
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Peak #	Signal RT [min]	Type	Height	Width [min]	Area	Area %
1	8.886	VV	0.161	0.451	5.117	0.104
2	9.301	VV	0.184	0.466	6.510	0.133
3	10.393	VV	8.370	0.133	66.660	1.360
4	10.708	VF	176.078	0.414	4752.757	96.952
5	11.325	VF	8.893	0.084	44.963	0.917
6	11.519	VV	1.408	0.146	12.343	0.252
7	12.271	VV	0.106	0.354	2.517	0.051
8	12.985	VV	0.080	0.346	1.976	0.040
9	14.467	VV	0.054	0.919	4.056	0.083
10	16.048	VV	0.086	0.405	2.090	0.043
11	18.284	VV	0.098	0.459	3.180	0.065

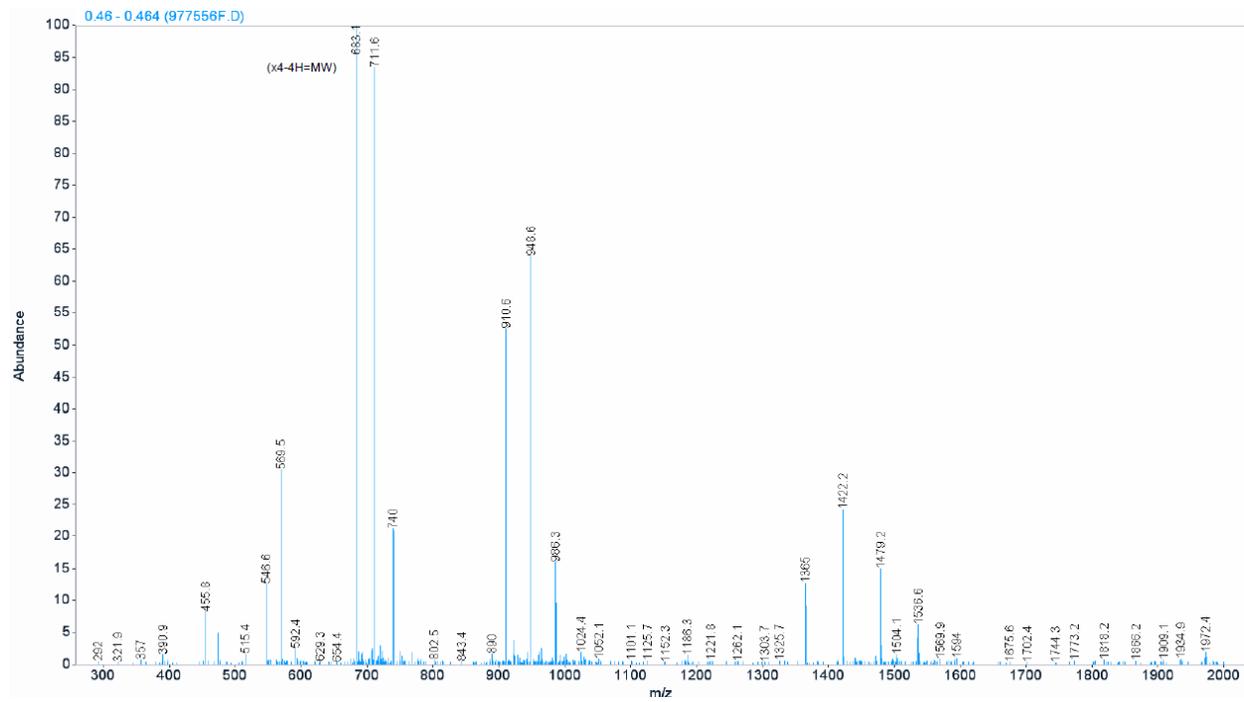
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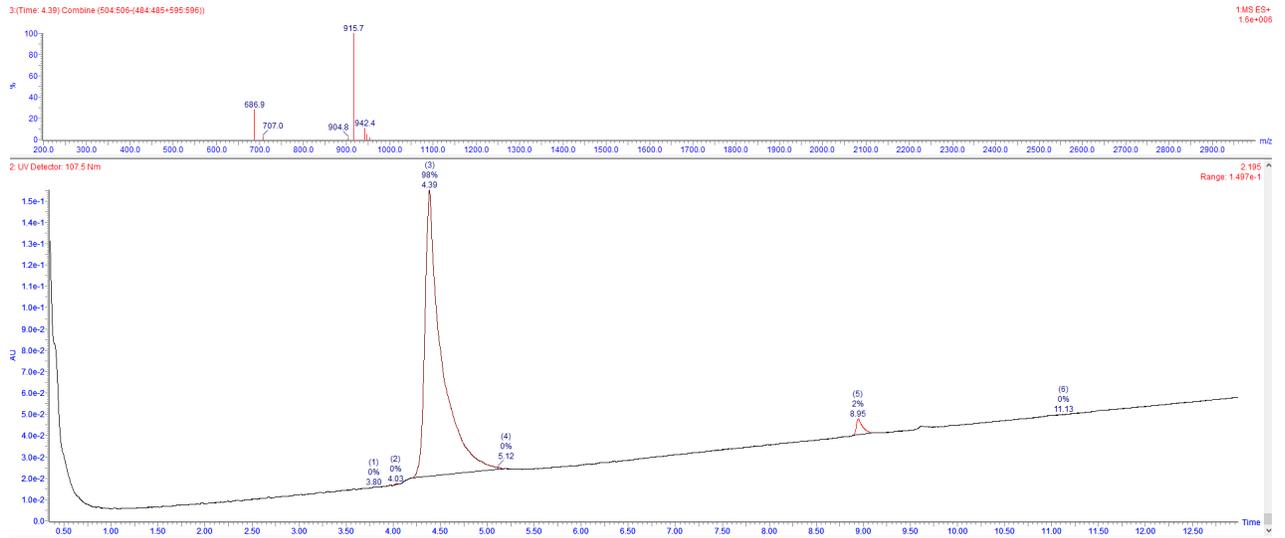
Peptide MP-4090:



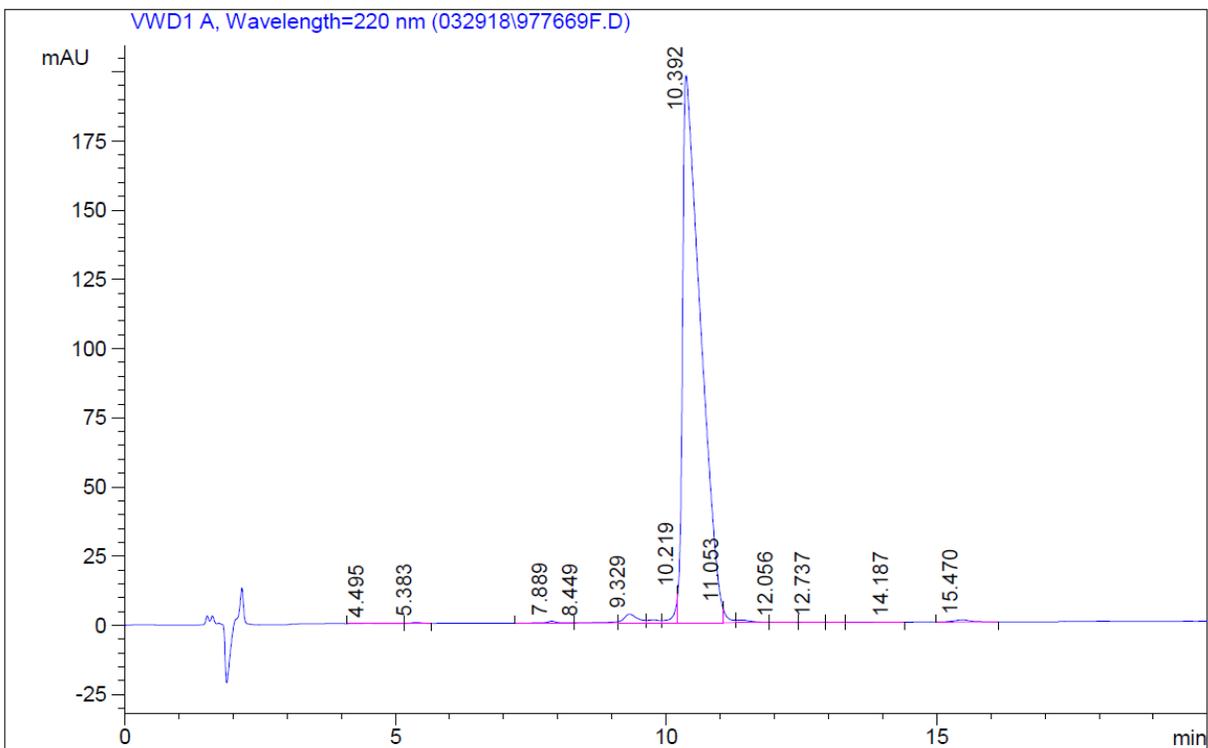
Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	7.201	VV	0.486	0.520	20.580	0.336
2	7.539	VV	0.497	0.277	11.267	0.184
3	8.696	VV	1.306	0.342	32.526	0.531
4	9.114	VV	2.392	0.259	43.948	0.718
5	9.547	VV	2.456	0.110	16.229	0.265
6	9.696	VV	14.977	0.065	58.769	0.960
7	9.858	VF	291.376	0.304	5826.002	95.150
8	10.379	VF	11.556	0.108	74.727	1.220
9	10.720	VV	1.330	0.212	16.889	0.276
10	11.453	VV	0.550	0.337	14.393	0.235
11	12.146	VV	0.408	0.243	7.621	0.124



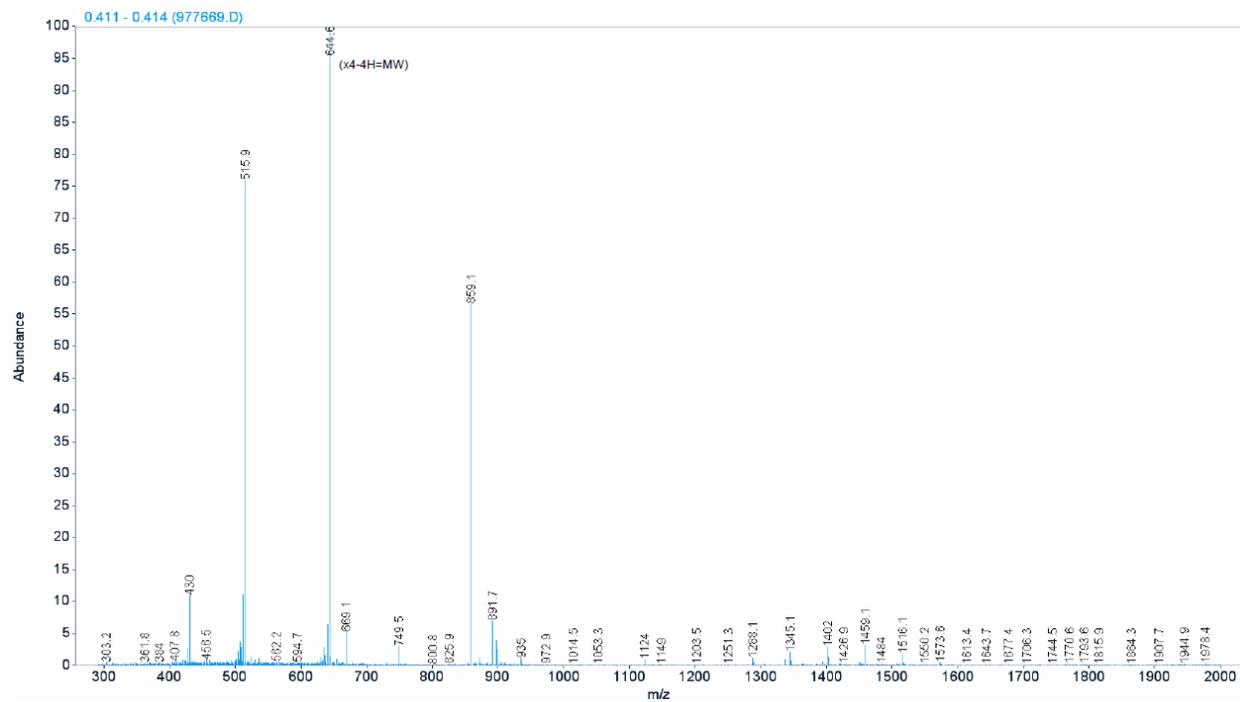
# Peptide MP-3995:



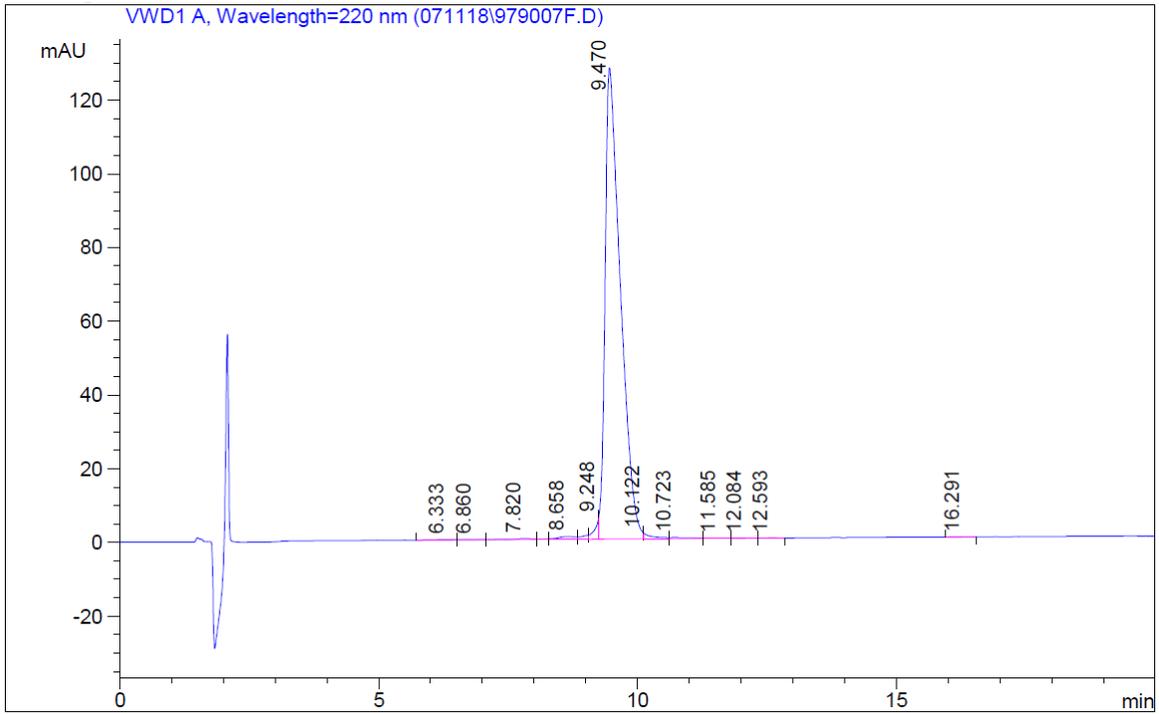
Peptide MP-4956:



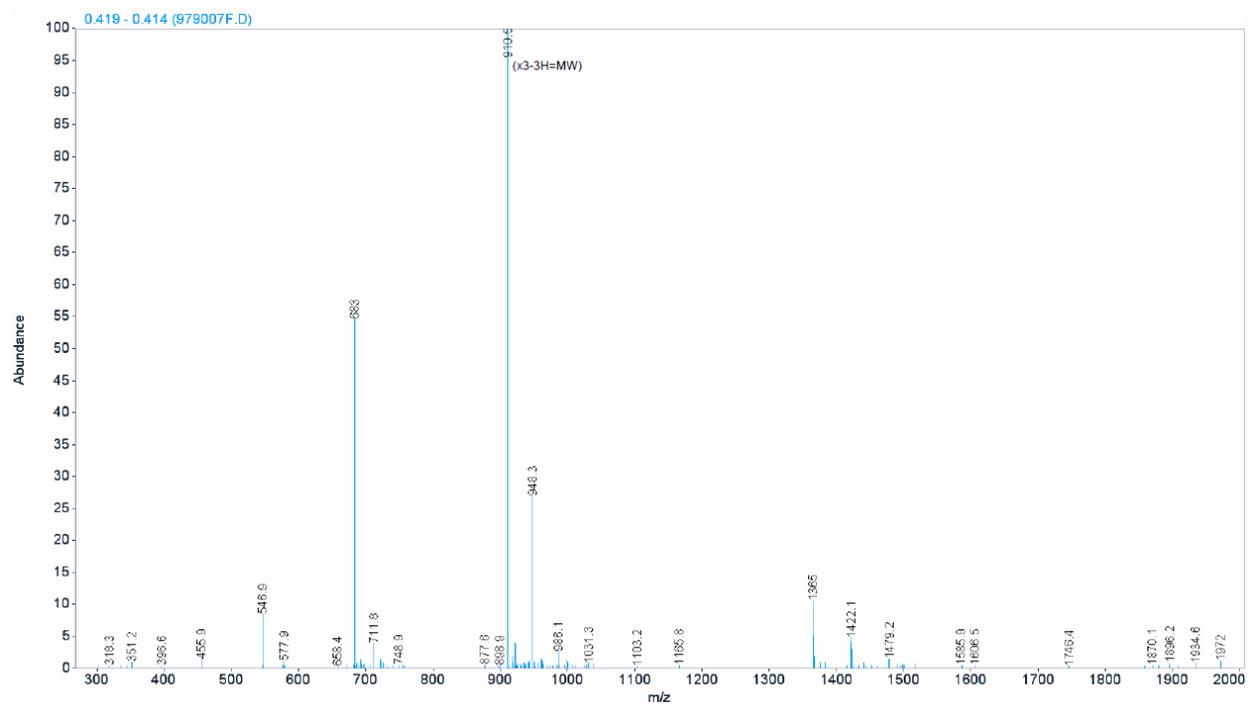
Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	4.495	VV	0.210	0.264	4.216	0.089
2	5.383	VV	0.420	0.186	5.014	0.105
3	7.889	VV	0.711	0.251	12.800	0.269
4	8.449	VV	0.151	0.807	10.101	0.212
5	9.329	VV	3.230	0.254	55.888	1.173
6	9.764	VV	1.084	0.211	15.786	0.331
7	10.219	VV	10.798	0.070	45.500	0.955
8	10.392	VF	193.803	0.332	4541.091	95.318
9	11.053	VV	5.192	0.084	26.186	0.550
10	11.402	VV	0.928	0.287	18.635	0.391
11	12.056	VV	0.158	0.361	3.946	0.083
12	12.737	VV	0.078	0.411	2.117	0.044
13	13.109	VV	0.052	0.257	0.932	0.020
14	14.187	VV	0.073	0.527	2.944	0.062
15	15.470	VV	0.813	0.342	18.976	0.398



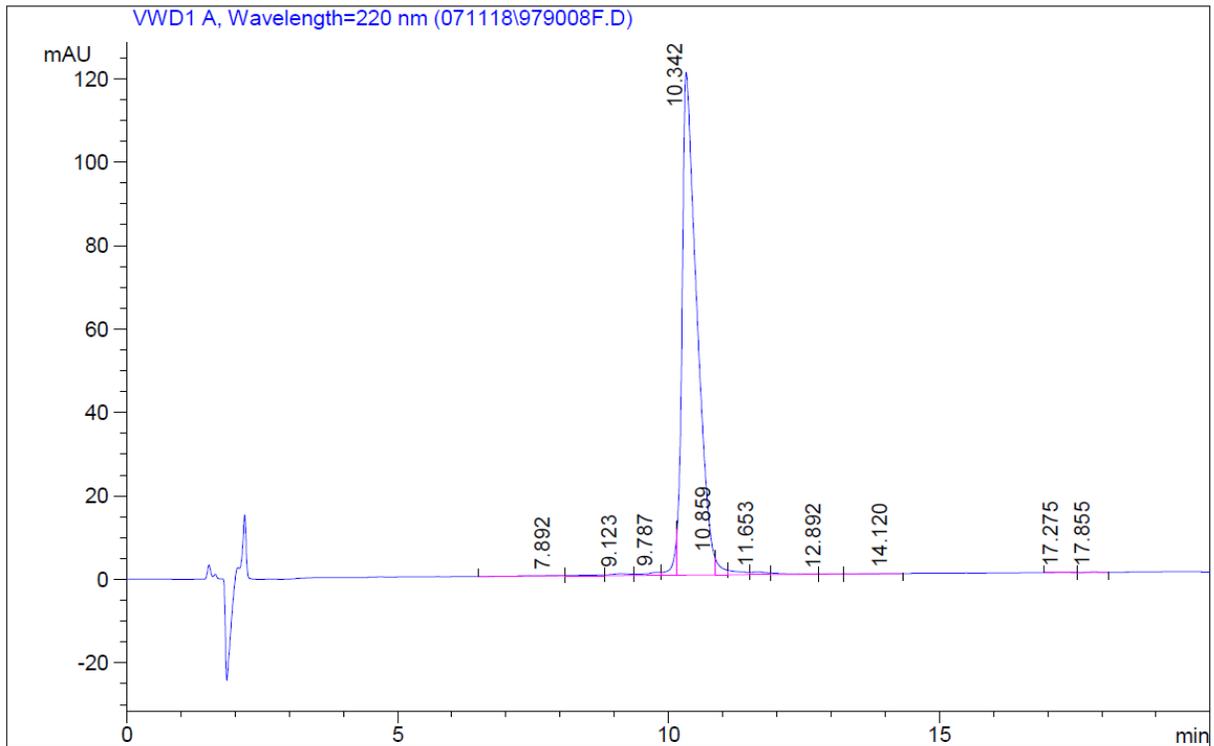
Peptide MP-9657:



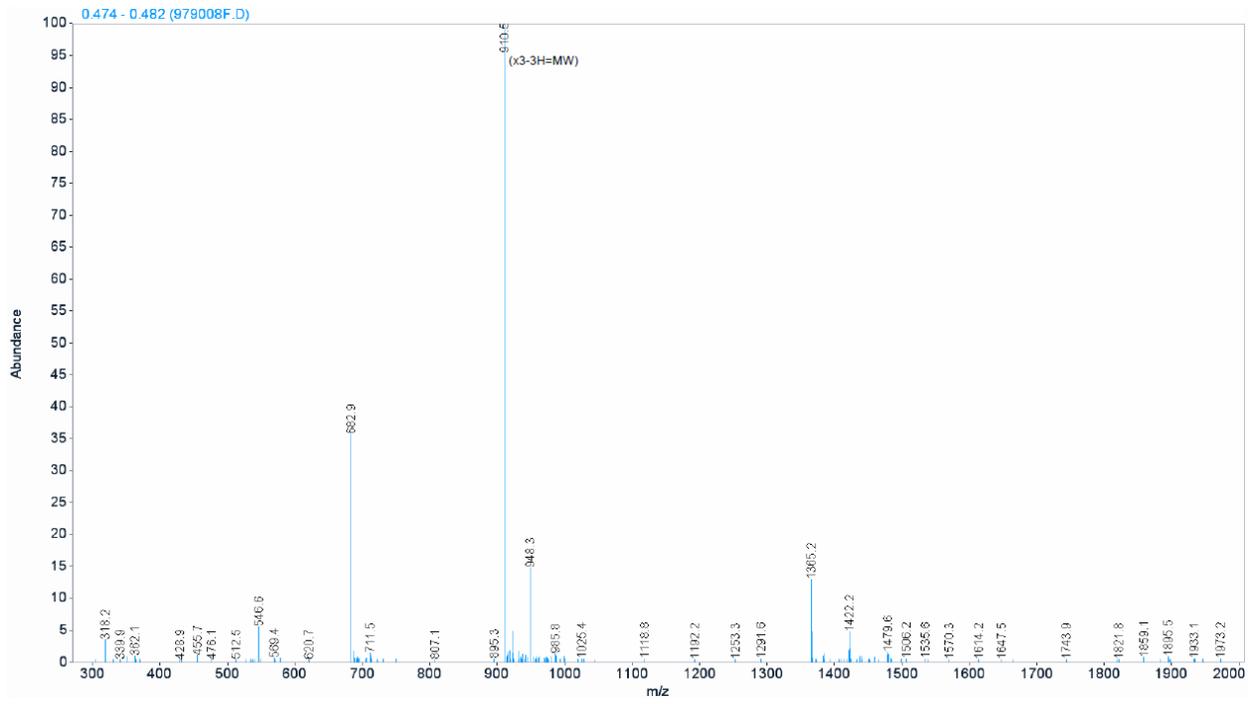
Peak #	Signal RT [min]	Type	Height	Width [min]	Area	Area %
1	6.333	VV	0.121	0.331	2.943	0.108
2	6.860	VV	0.085	0.448	2.505	0.092
3	7.820	VV	0.232	0.413	6.825	0.250
4	8.277	VV	0.160	0.199	1.904	0.070
5	8.658	VV	0.755	0.356	17.752	0.650
6	9.054	VV	1.143	0.159	10.933	0.400
7	9.248	VV	5.919	0.084	29.993	1.098
8	9.470	VF	127.895	0.303	2624.194	96.066
9	10.122	VV	1.539	0.186	17.181	0.629
10	10.723	VV	0.299	0.390	8.815	0.323
11	11.585	VV	0.133	0.413	3.916	0.143
12	12.084	VV	0.086	0.319	2.002	0.073
13	12.593	VP	0.069	0.309	1.305	0.048
14	16.291	VV	0.073	0.269	1.402	0.051



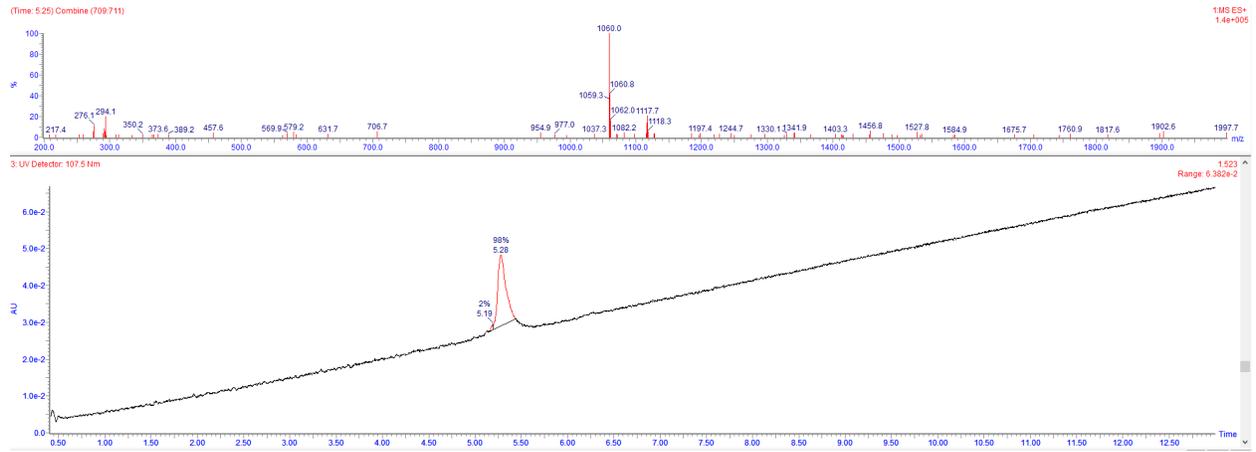
Peptide MP-9658:



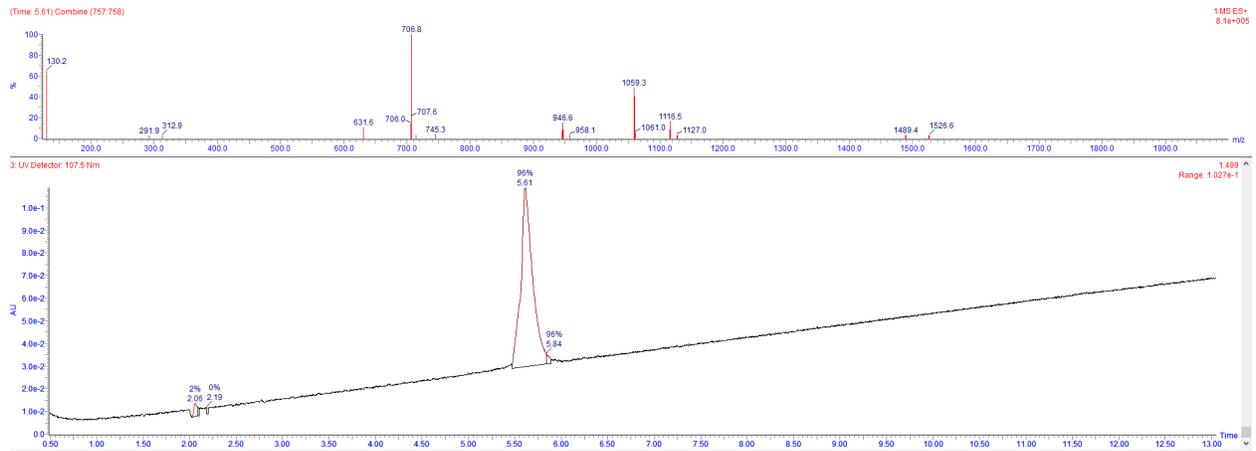
Peak #	Signal RT [min]	Type	Height	Width [min]	Area	Area %
1	7.892	VV	0.134	0.670	7.022	0.290
2	8.821	VV	0.199	0.520	6.208	0.256
3	9.123	VV	0.478	0.345	11.539	0.476
4	9.787	VV	0.763	0.270	14.964	0.618
5	9.869	VV	0.702	1.056	44.439	1.834
6	10.342	VF	120.106	0.278	2262.884	93.393
7	10.859	VF	4.170	0.114	28.621	1.181
8	11.092	VV	1.127	0.277	18.741	0.773
9	11.653	VF	0.667	0.287	13.384	0.552
10	11.888	VV	0.385	0.368	8.521	0.352
11	12.892	VV	0.069	0.282	1.498	0.062
12	14.120	VV	0.030	0.831	1.967	0.081
13	17.275	VP	0.095	0.294	1.873	0.077
14	17.855	VV	0.081	0.254	1.315	0.054



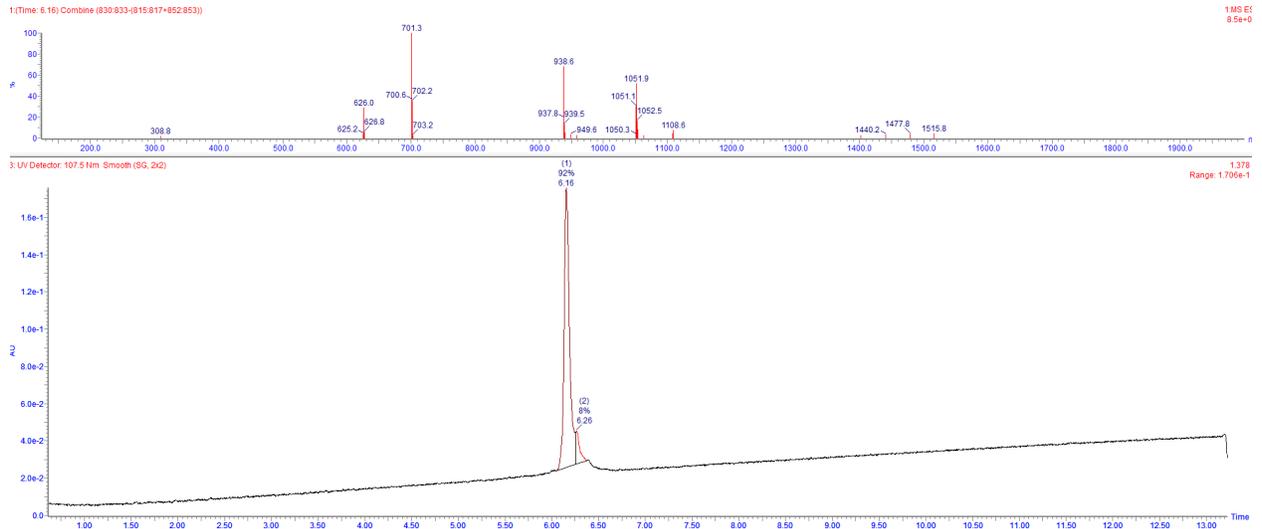
### Peptide 14:



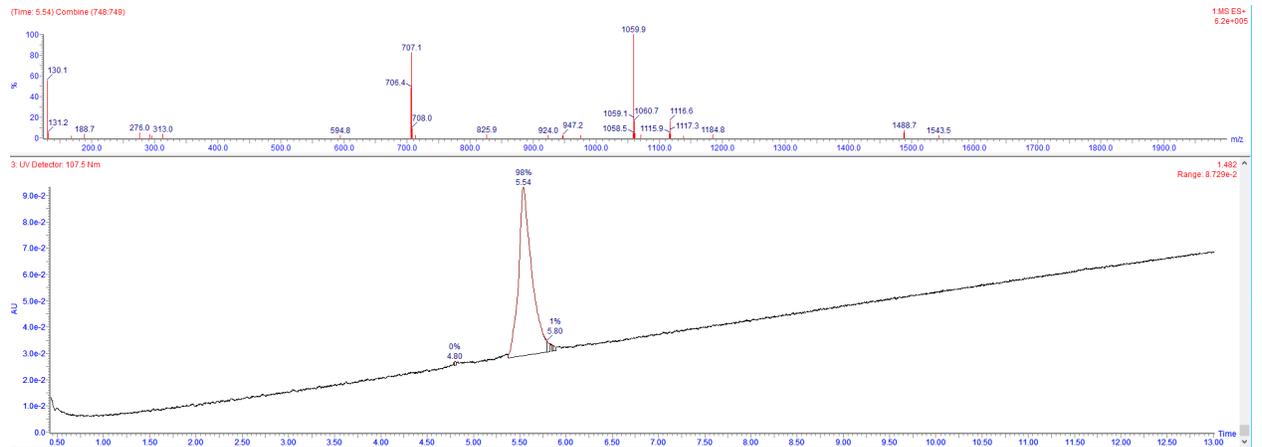
### Peptide 15:



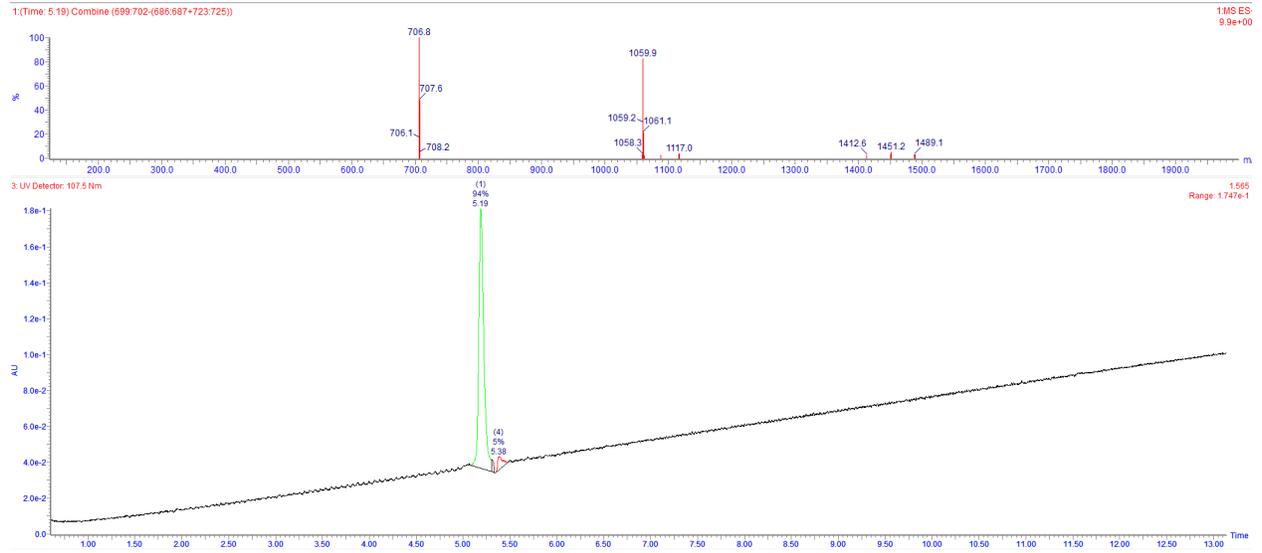
## Peptide 16:



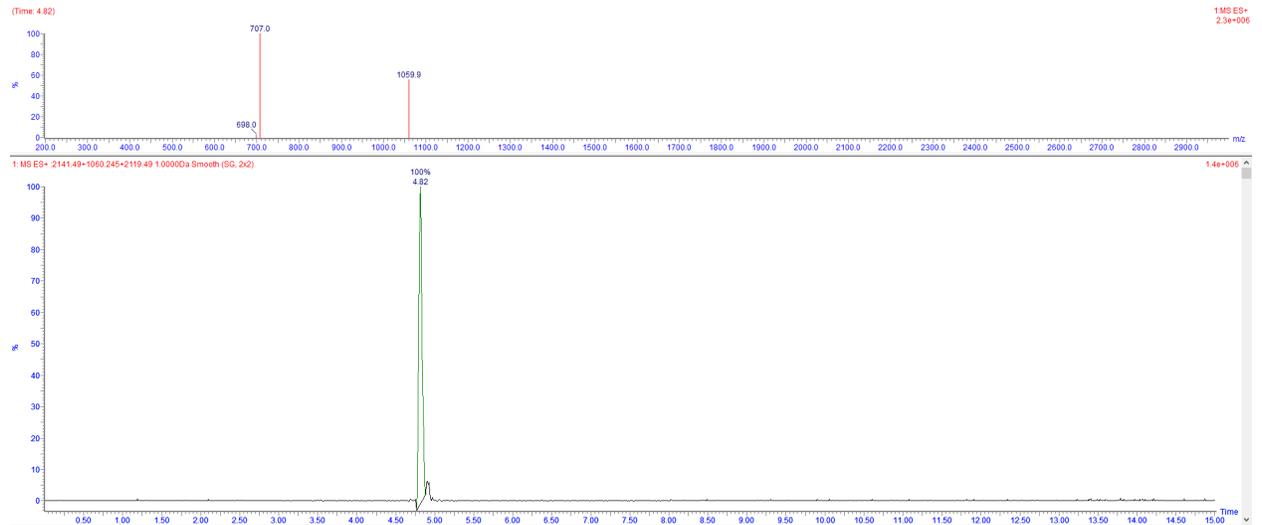
## Peptide 17:



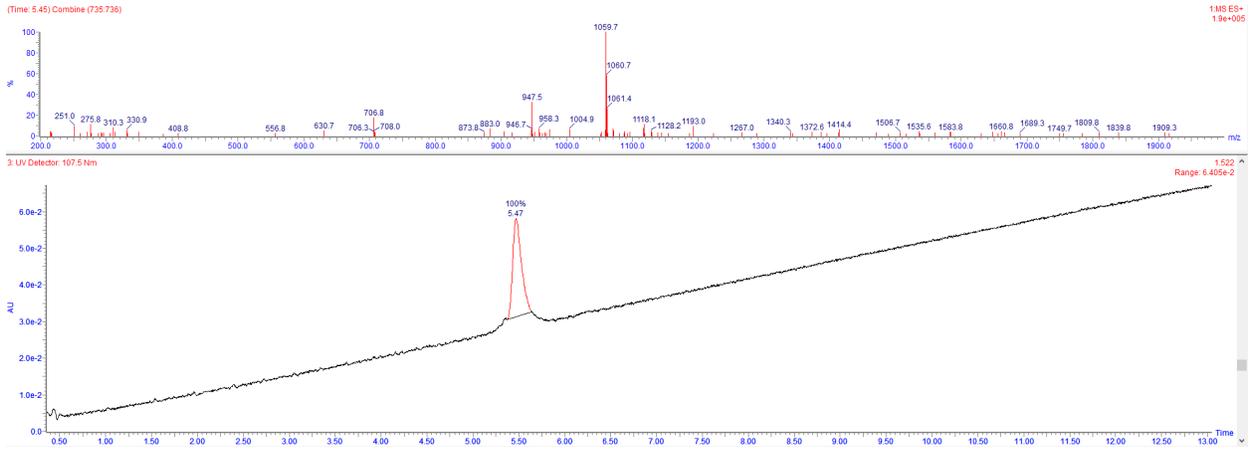
## Peptide 18:



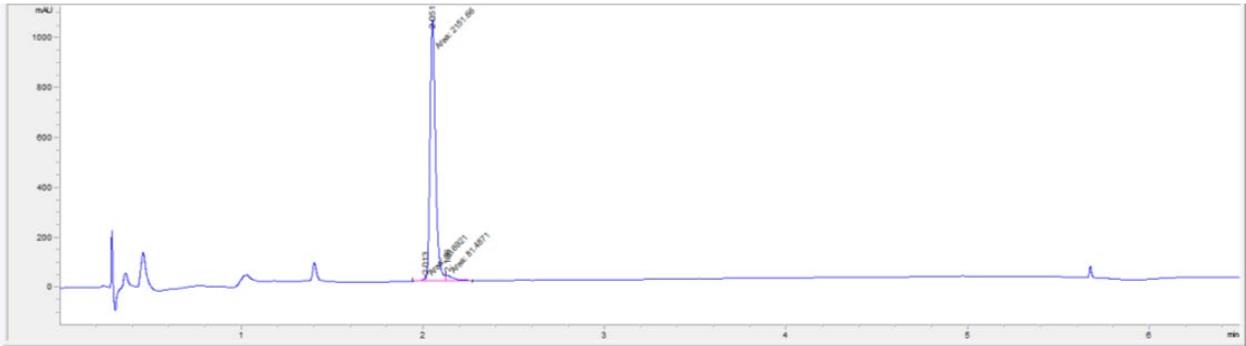
## Peptide 19:



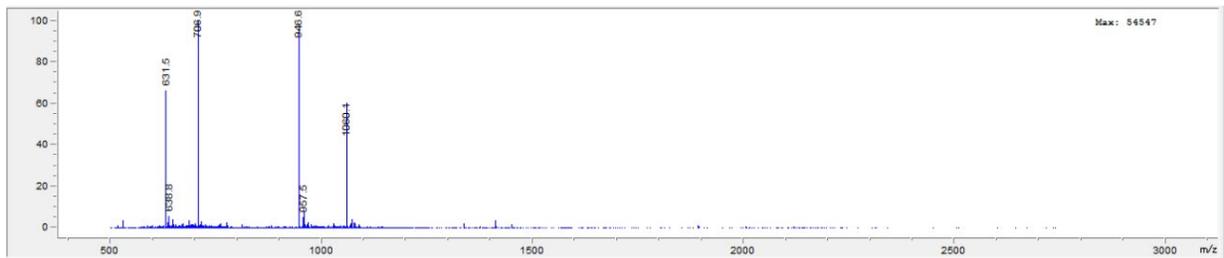
Peptide 20:



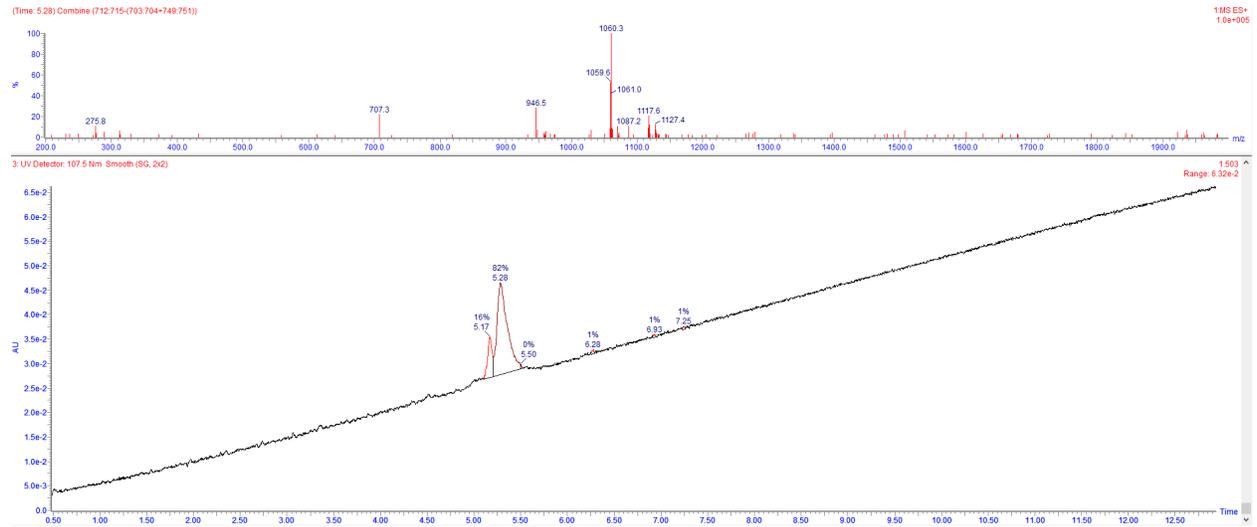
Peptide 21:



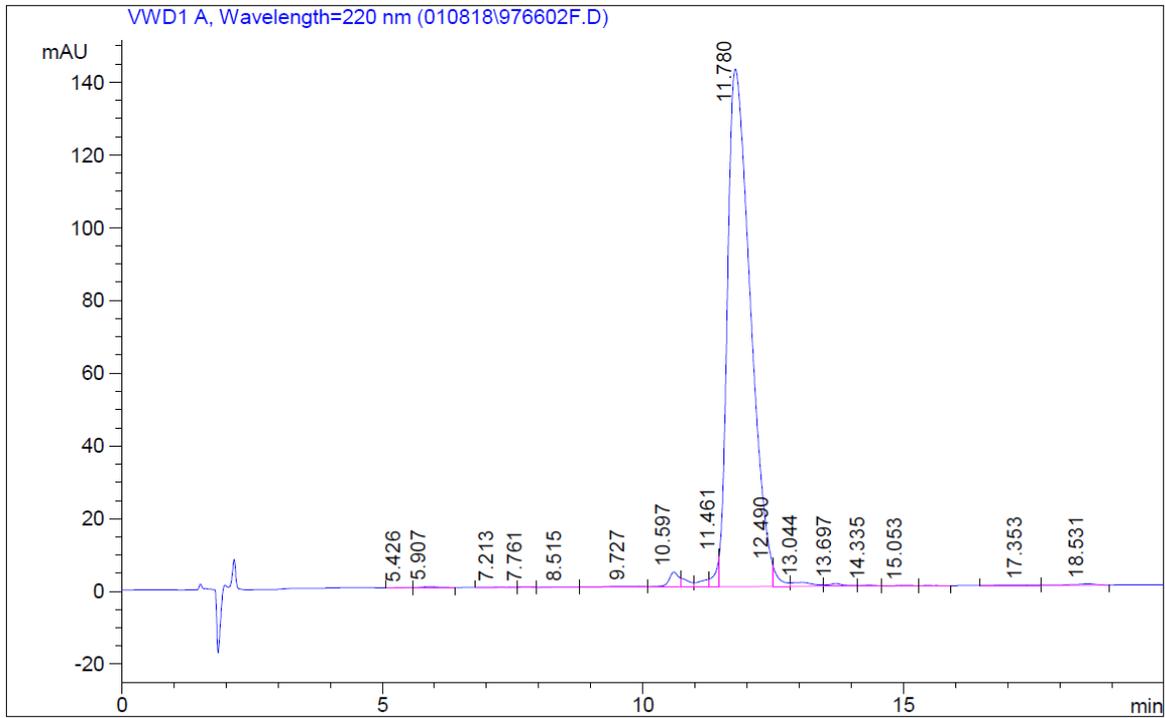
#	Time	Area	Height	Width	Area%	Symmetry
1	2.013	20.7	13	0.0265	0.918	0
2	2.051	2151.7	1047.3	0.0342	95.466	0.838
3	2.136	81.5	24.3	0.0559	3.615	0.219



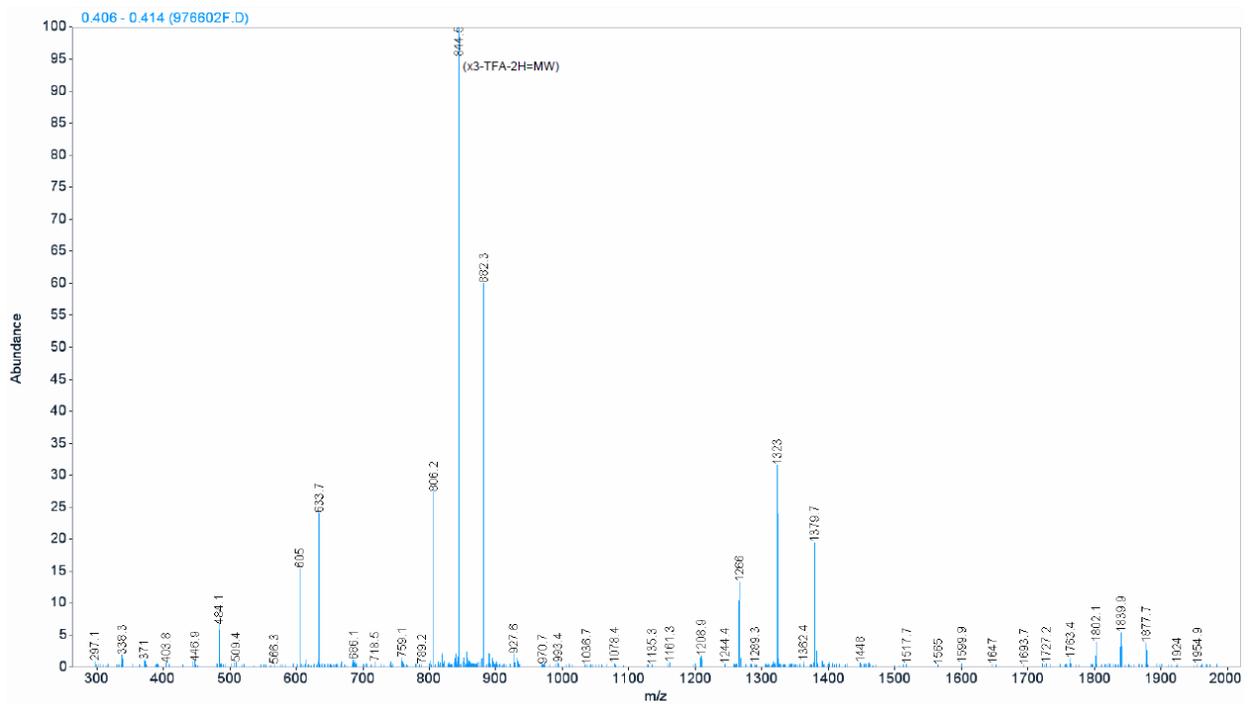
# Peptide 22:



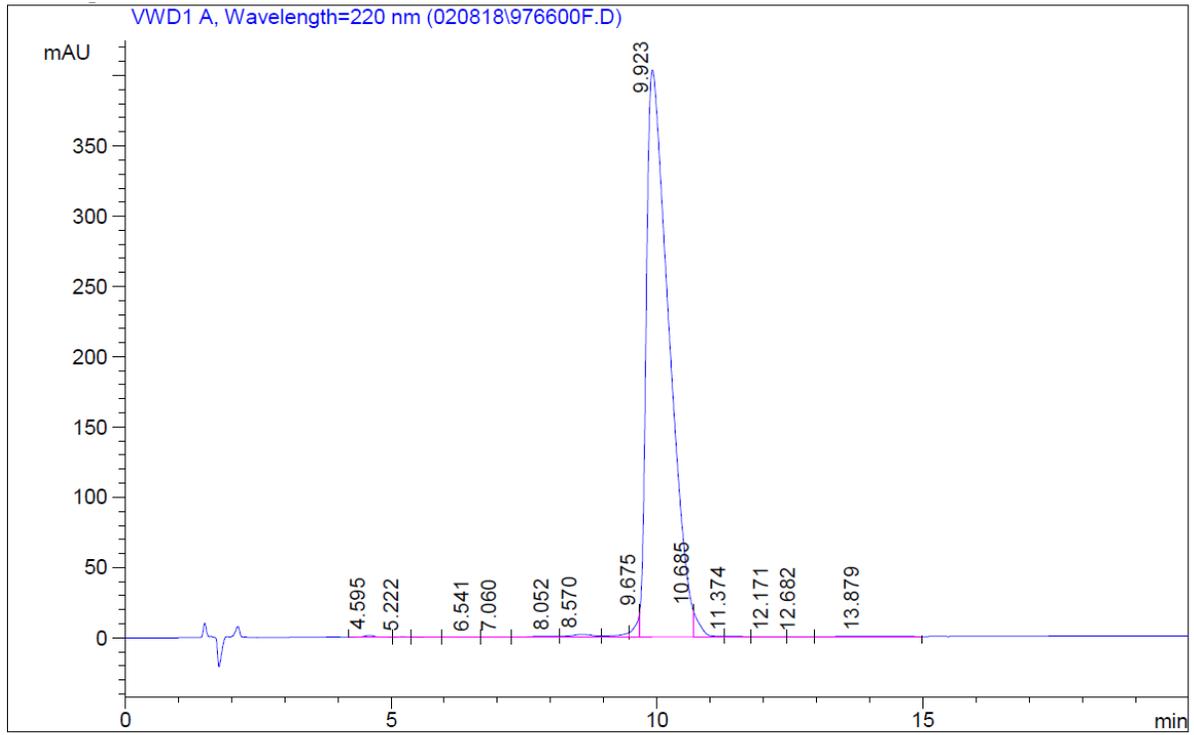
Peptide 23:



Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	5.426	VV	0.070	0.276	1.240	0.027
2	5.907	VV	0.317	0.314	6.644	0.145
3	7.213	VV	0.086	0.459	2.960	0.064
4	7.761	VV	0.080	0.227	1.236	0.027
5	8.515	VV	0.061	0.494	2.416	0.053
6	9.727	VV	0.186	0.667	9.070	0.198
7	10.597	VF	4.050	0.191	52.087	1.134
8	10.723	VV	2.397	0.181	26.024	0.567
9	11.267	VV	2.081	0.209	26.137	0.569
10	11.461	VV	8.340	0.087	43.757	0.953
11	11.780	VF	142.310	0.481	4308.594	93.821
12	12.490	VV	5.950	0.120	42.708	0.930
13	13.044	VV	1.056	0.417	29.865	0.650
14	13.697	VV	0.748	0.273	14.513	0.316
15	14.335	VV	0.227	0.270	4.228	0.092
16	15.053	VV	0.119	0.416	3.713	0.081
17	15.453	VP	0.059	0.342	1.493	0.033
18	17.353	VV	0.103	0.590	4.799	0.105
19	18.531	VV	0.337	0.452	10.881	0.237

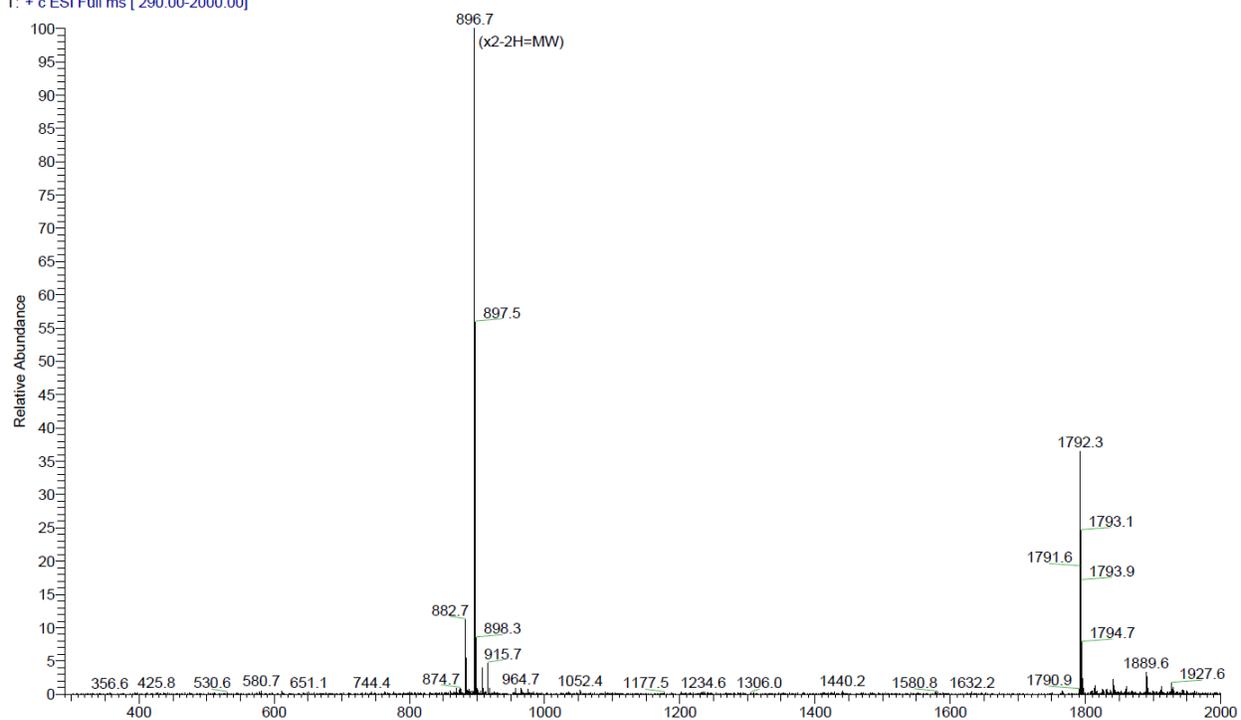


Peptide MP-9903:

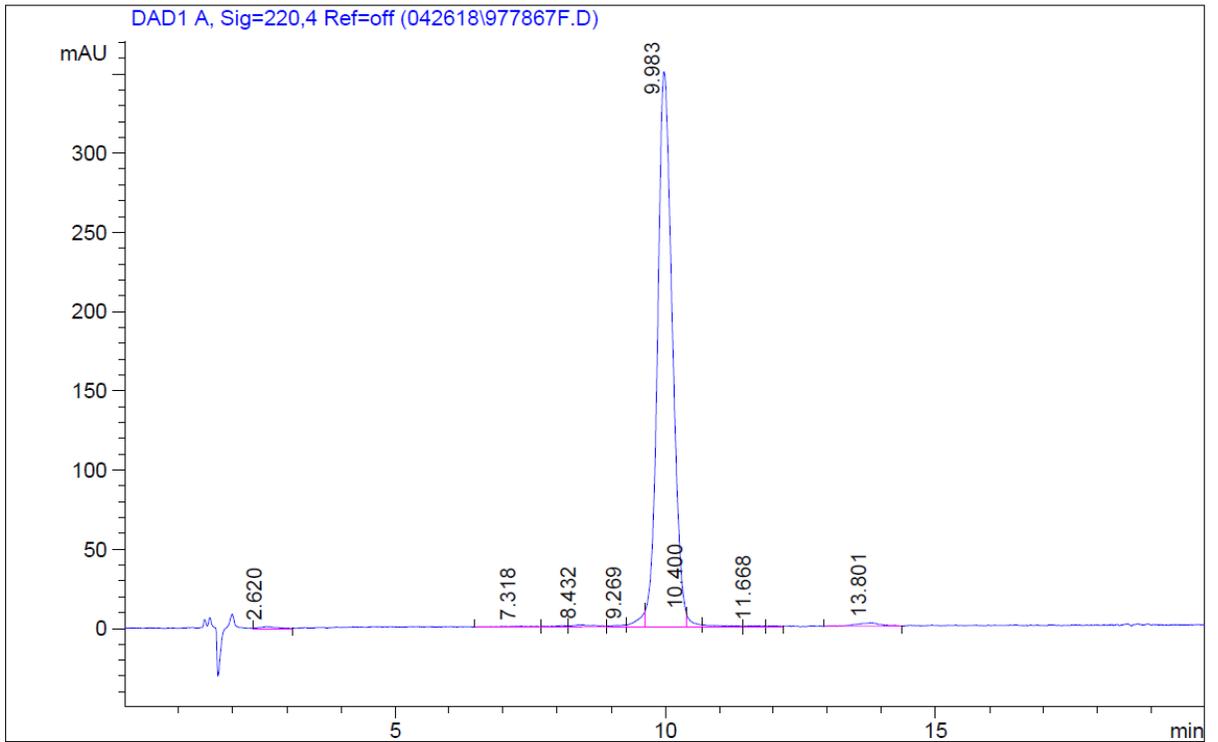


Peak #	Signal RT [min]	Type	Height	Width [min]	Area	Area %
1	4.595	VP	1.323	0.210	17.875	0.146
2	5.222	VV	0.352	0.142	3.234	0.026
3	5.481	VV	0.156	0.332	3.566	0.029
4	6.541	VV	0.111	0.409	3.273	0.027
5	7.060	VV	0.122	0.379	3.538	0.029
6	8.052	VV	0.560	0.436	18.946	0.155
7	8.570	VV	1.810	0.391	52.576	0.431
8	9.481	VV	3.078	0.213	39.388	0.323
9	9.675	VV	17.901	0.086	91.852	0.752
10	9.923	VF	401.906	0.442	11800.752	96.638
11	10.685	VV	18.306	0.131	144.115	1.180
12	11.374	VV	0.315	0.314	7.059	0.058
13	12.171	VV	0.149	0.476	5.264	0.043
14	12.682	VV	0.115	0.350	2.773	0.023
15	13.879	VV	0.295	0.788	17.128	0.140

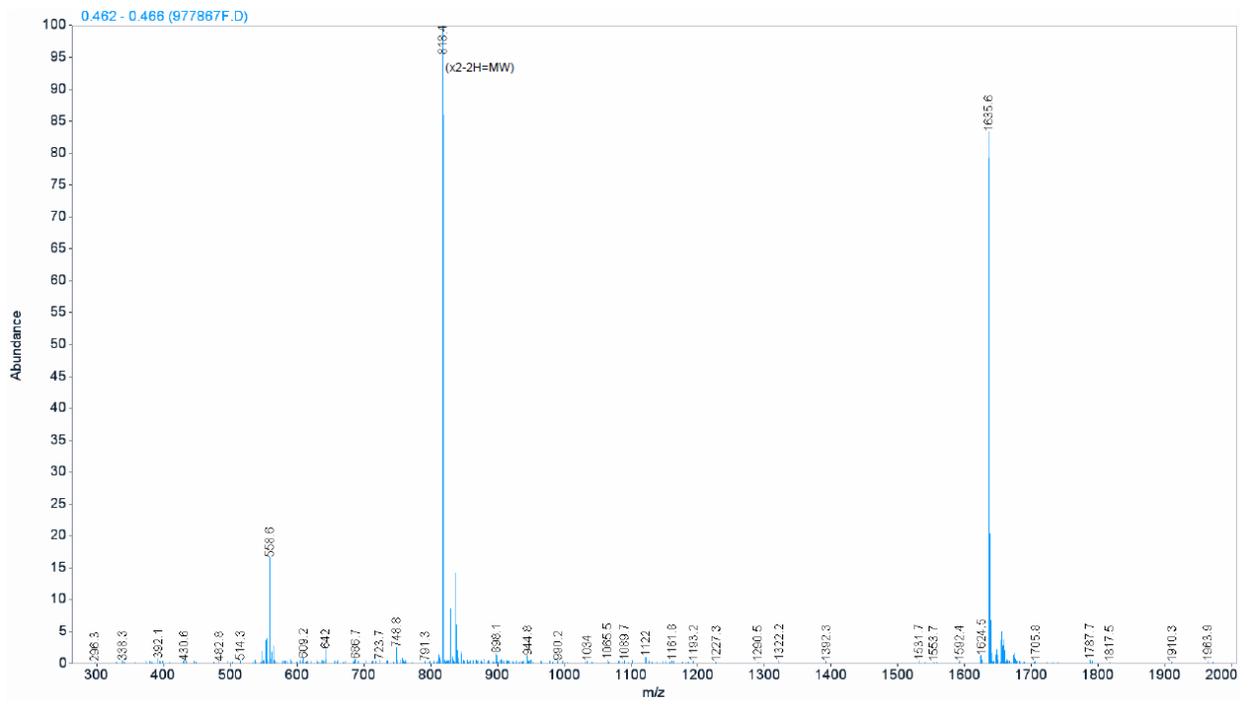
976800F #11-21 RT: 0.23-0.44 AV: 11 NL: 1.56E8  
T: + c ESI Full ms [ 290.00-2000.00]



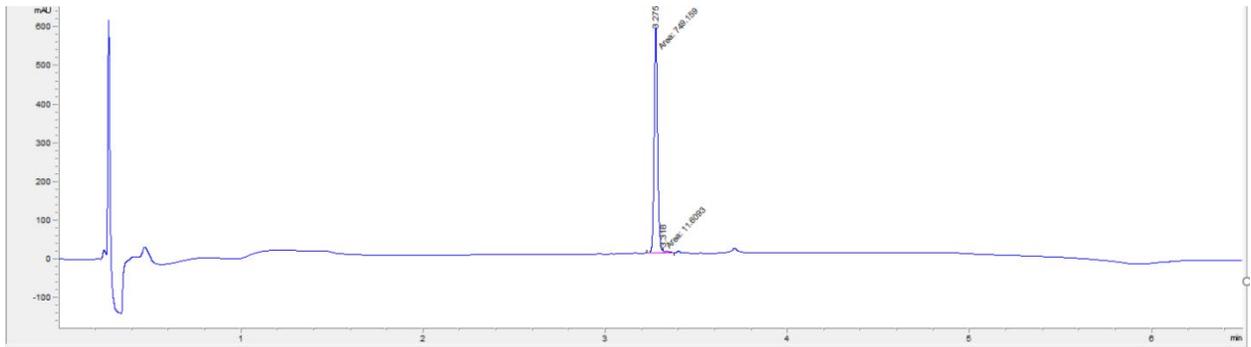
Peptide 24:



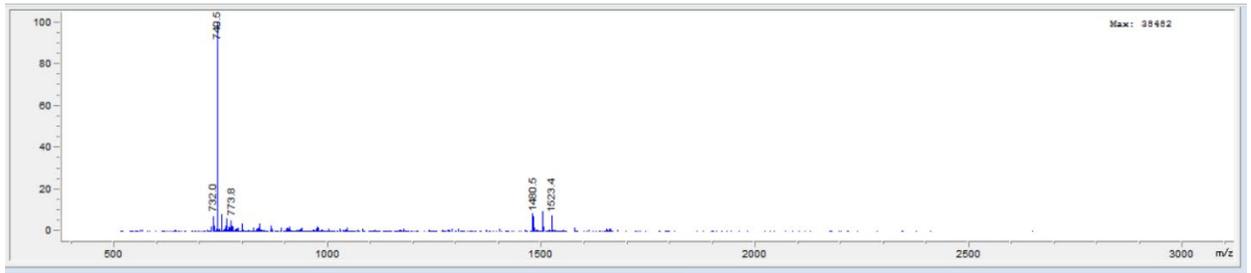
Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	2.620	MM	1.365	0.287	23.535	0.339
2	7.318	VV	0.492	0.514	20.618	0.297
3	8.095	VV	0.814	0.243	14.715	0.212
4	8.432	VV	1.176	0.401	34.607	0.498
5	9.130	VV	0.998	0.214	16.126	0.232
6	9.269	VV	1.068	1.374	88.044	1.267
7	9.983	VF	350.726	0.290	6596.114	94.912
8	10.400	VF	7.543	0.102	46.247	0.665
9	10.677	VV	1.158	0.367	25.455	0.366
10	11.668	VV	0.497	0.219	7.699	0.111
11	11.979	VV	0.323	0.192	4.593	0.066
12	13.801	VV	2.114	0.442	71.989	1.036



Peptide 25:



#	Time	Area	Height	Width	Area%	Symmetry
1	3.275	749.2	581.9	0.0215	98.474	0.973
2	3.318	11.6	5.9	0.0268	1.526	0



## Supplemental References:

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4. Emsley, P., et al., *Features and development of Coot.* Acta Crystallogr D Biol Crystallogr, 2010. **66**(Pt 4): p. 486-501.
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8. Lim, S., et al., *Exquisitely Specific anti-KRAS Biodegraders Inform on the Cellular Prevalence of Nucleotide-Loaded States.* ACS Cent Sci, 2021. **7**(2): p. 274-291.