Supplementary Information

Structure-Based Design and Optimization of Cyclic Peptide Ligands Targeting Delta-like Ligand 3

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Content

1. Molecular dynamics (MD)-based alanine scanning

Structural Modeling and System Preparation. The initial 3D coordinates for the peptide in complex with the DSL domain of the DLL3 receptor were generated using AlphaFold3[1] online server. The predicted interface exhibited high confidence, characterized by an interface predicted template modeling (ipTM) score of 0.9. Based on this structural model, the wild-type (WT) and alanine-scanning mutant systems were prepared.

All simulations were performed using the GROMACS 2025.3 suite with the CHARMM36 force field. Each complex was solvated in a cubic box of TIP3P water molecules, with a minimum distance of 1.2 nm between the solute and the box edges. To neutralize the system and mimic physiological conditions, sodium and chloride ions were added to a concentration of 0.15 M.

Simulation Protocol. The systems underwent energy minimization using the steepest descent algorithm until the maximum force was below 1000 kJ mol^{-1} nm^{-1}. Following minimization, the systems were equilibrated in two phases with position restraints applied to the protein heavy atoms. First, a 100 ps NVT equilibration was performed at 300 K using the V-rescale thermostat. Subsequently, a 100 ps NPT equilibration was conducted at 1 bar using the C-rescale barostat to stabilize the density.

Production molecular dynamics runs were performed for 50 ns for the WT and each variant without position restraints. The temperature and pressure were maintained at 300 K and 1 bar using the V-rescale thermostat and Parrinello-Rahman barostat, respectively. Long-range electrostatic interactions were treated using the Particle Mesh Ewald (PME) method with a cutoff of 1.2 nm. A twin-range cutoff of 1.2 nm was also applied to van der Waals interactions. Hydrogen bonds were constrained using the LINCS algorithm, allowing for an integration time step of 2 fs. Coordinates were saved every 10 ps.

Binding Free Energy Analysis (MM/GBSA). Binding free energies were calculated using the gmx_MMPBSA tool (v1.6.4) [2]. The calculations employed the GB-OBC II model (igb=5) with a salt concentration of 0.15 M. To ensure the analysis reflected the stable equilibrium state, snapshots were extracted from the production trajectory between frames 500 and 5000 (corresponding to the stable portion of the simulation). A sampling interval of 20 frames was applied to reduce data redundancy. The relative binding affinity ΔΔG for each mutant was determined by directly subtracting the binding free energy of the wild-type complex from that of the mutant, where a positive value indicates a reduction in binding affinity.

Table S1. Molecular dynamics (MD)-based alanine scanning for the PepSP1171 without C-terminal tail

Name	Peptide Sequence	ΔG (kJ/mol)	ΔΔG (kJ/mol, Absolute values)
PepSP1171 core	WCNGNSENWTCTW	-37.88	0
1	ACNGNSENWTCTW	-34.37	3.51
2	WCAGNSENWTCTW	-36.06	1.82
3	WCNANSENWTCTW	-44.27	6.39
4	WCNGASENWTCTW	-33.47	4.41

5	WCNGNAENWTCTW	-31.67	6.21
6	WCNGNSANWTCTW	-35.22	2.66
7	WCNGNSEAWTCTW	-26.08	11.8
8	WCNGNSENATCTW	-25.79	12.09
9	WCNGNSENWACTW	-41.49	3.61
10	WCNGNSENWTCAW	-46.3	8.42
11	WCNGNSENWTCTA	-32.72	5.16

2. Analytical data of the synthesized peptides

Table S2. Analytical data of the synthesized peptides **A1-A12** and **B1-B11**.

Peptides	**Formula**	**Molecular weight**	**Mass m/z calculated for $[M+2H]^{2+}$**	**HRMS**	**HPLC purity (%)**
A1	$C_{68}H_{88}N_{20}O_{22}S_2$	1600.5823	801.3	NT	95.8
A2	$C_{70}H_{91}N_{19}O_{22}S_2$	1613.6027	808.1	1614.6127	95.3
A3	$C_{70}H_{91}N_{19}O_{23}S_2$	1629.5977	816.0	NT	96.9
A4	$C_{73}H_{91}N_{19}O_{22}S_2$	1649.6027	826.1	825.8101 [a]	96.5
A5	$C_{75}H_{93}N_{19}O_{22}S_2$	1675.6184	839.3	1676.6193	95.3
A6	$C_{73}H_{94}N_{20}O_{22}S_2$	1666.6293	834.3	1667.6370	98.2
A7	$C_{73}H_{92}N_{20}O_{22}S_2$	1664.6136	833.5	1665.6204	99.3
A8	$C_{74}H_{94}N_{20}O_{22}S_2$	1678.6293	840.5	1679.6365	99.3
A9	$C_{75}H_{96}N_{20}O_{22}S_2$	1692.6449	847.3	847.3295 [a]	95.5
A10	$C_{76}H_{98}N_{20}O_{22}S_2$	1706.6606	854.6	854.3381 [c]	95.1
A11	$C_{73}H_{92}F_2N_{20}O_{22}S_2$	1702.6105	852.4	1703.6179	99.3
A12	$C_{74}H_{94}N_{20}O_{22}S_2$	1678.6293	840.5	1679.6367	98.3
B1	$C_{107}H_{134}N_{26}O_{32}S_2$	2358.9099	1180.6	2381.8964 [b]	95.6
B2	$C_{109}H_{138}N_{26}O_{32}S_2$	2386.9412	1195.3	1194.4806 [a]	95.7
B3	$C_{112}H_{144}N_{26}O_{32}S_2$	2428.9881	1215.4	810.3398 [c]	99.2
B4	$C_{111}H_{142}N_{26}O_{32}S_2$	2414.9725	1208.5	NT	95.2

B5	$C_{109}H_{136}N_{26}O_{32}S_2$	2384.9255	1193.9	2385.9436	98.7
B6	$C_{110}H_{138}N_{26}O_{32}S_2$	2398.9412	1200.7	1200.4799 [a]	95.0
B7	$C_{110}H_{138}N_{26}O_{32}S_2$	2398.9412	1200.6	1200.4811 [a]	95.0
B8	$C_{112}H_{142}N_{26}O_{32}S_2$	2440.9725	1214.8	NT	95.0
B9	$C_{111}H_{140}N_{26}O_{32}S_2$	2412.9568	1208.1	1207.4865 [a]	95.2
B10	$C_{112}H_{142}N_{26}O_{32}S_2$	2426.9725	1212.9	1214.4935 [a]	95.7
B11	$C_{114}H_{146}N_{26}O_{32}S_2$	2455.0038	1228.2	NT	98.5

[a] m/z calculated for $[M+2H]^{2+}$ with HRMS; [b] m/z calculated for $[M+Na]^{+}$; [b] m/z calculated for $[M+3H]^{3+}$; NT means not test

3. BLI binding results

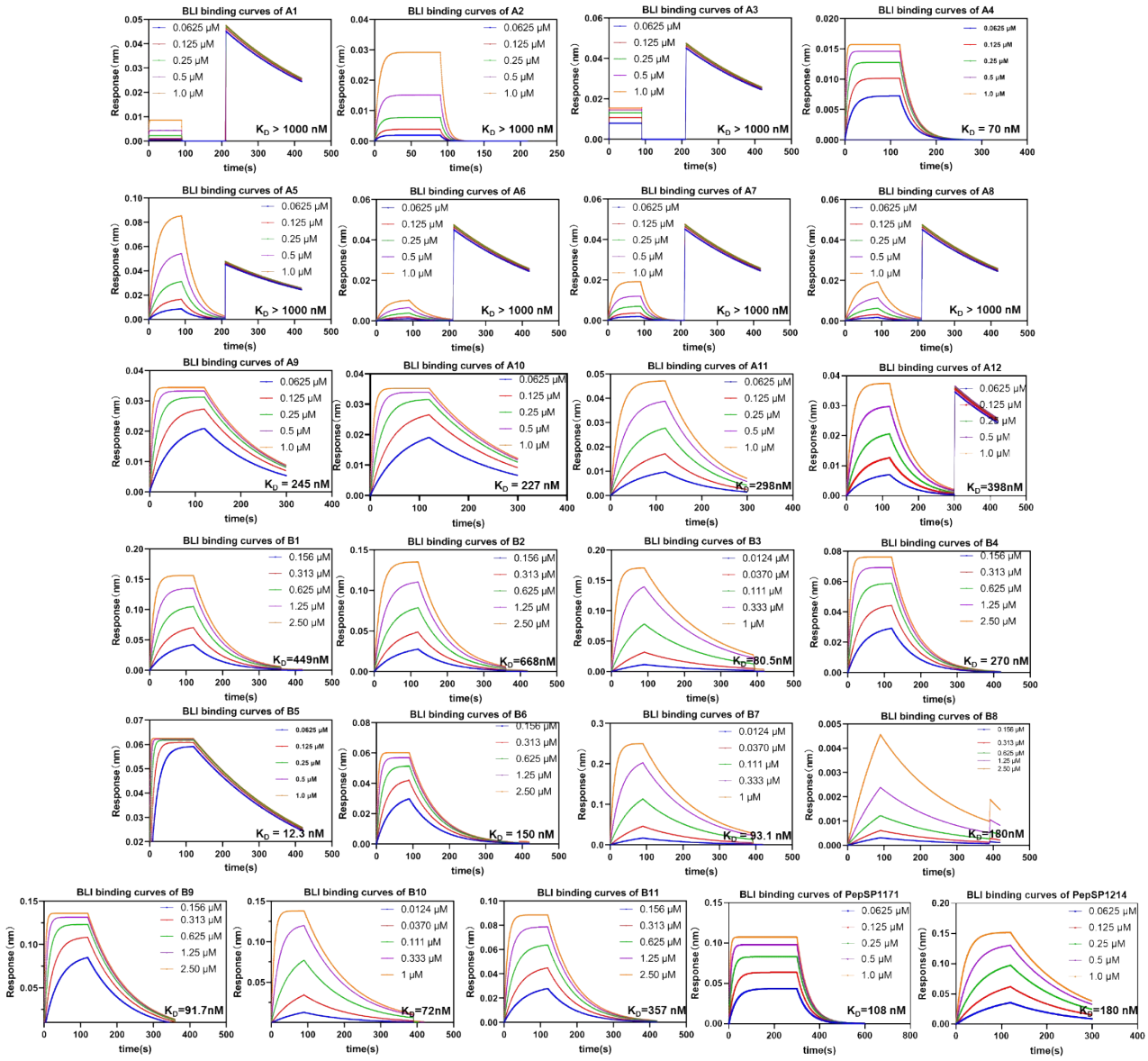
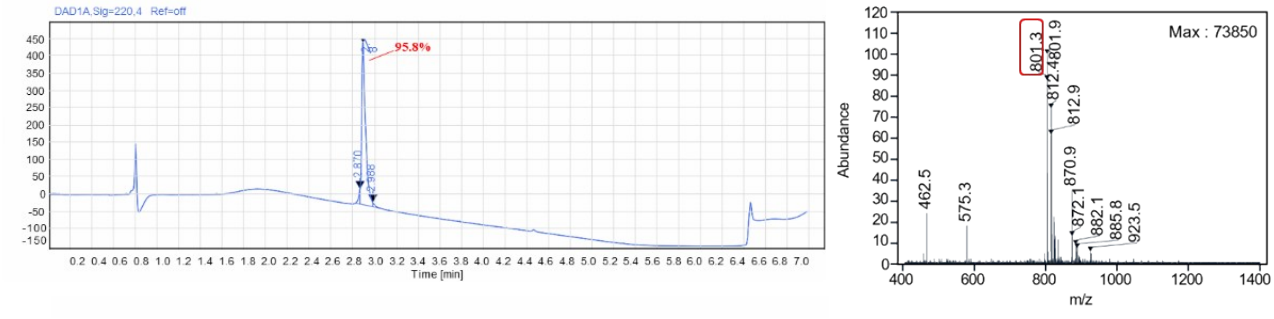
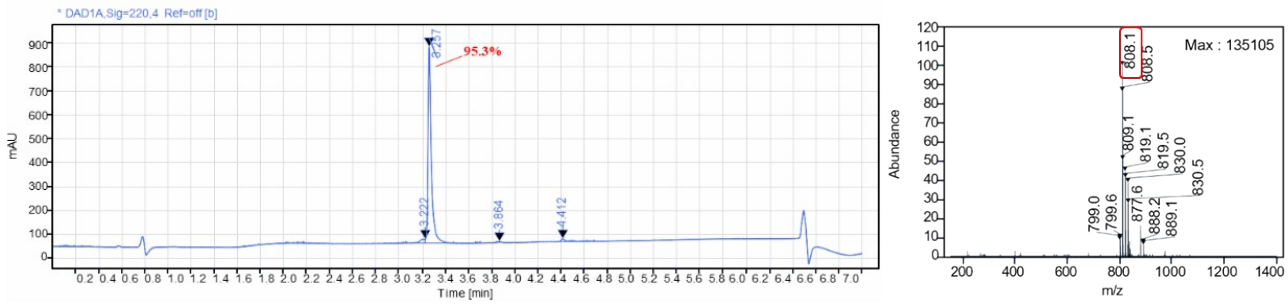


Figure S1 BLI binding kinetics curves of peptide ligands

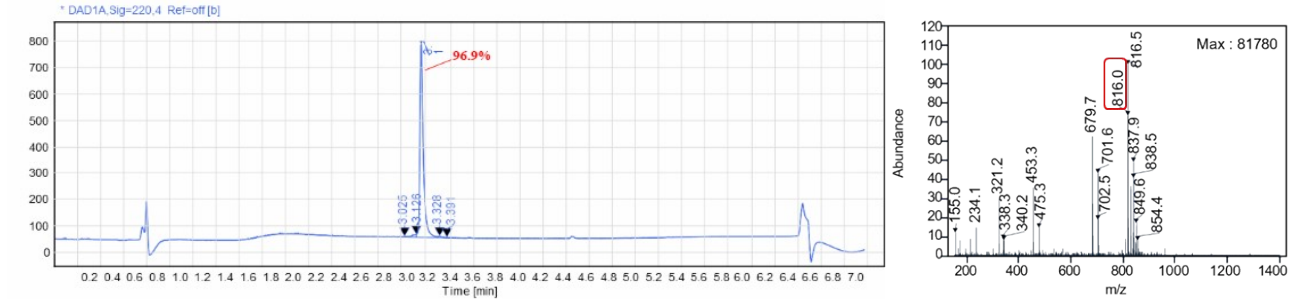
4. HPLC and LC-MS chromatograms of peptide ligands



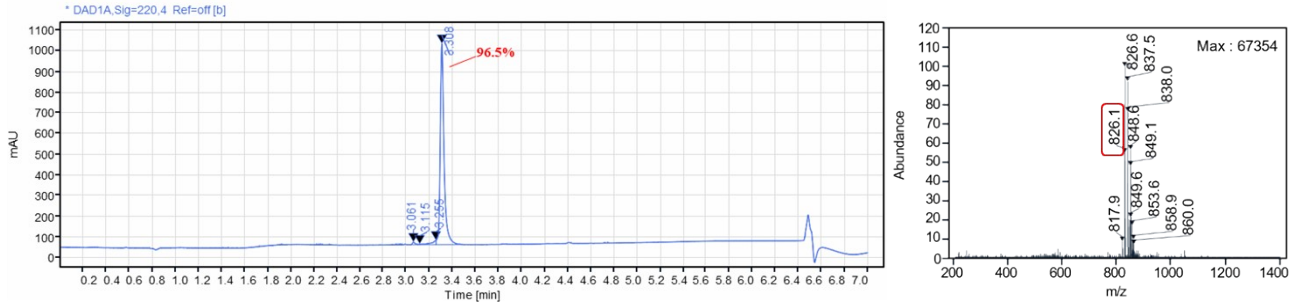
HPLC and MS chromatograms of **A1**



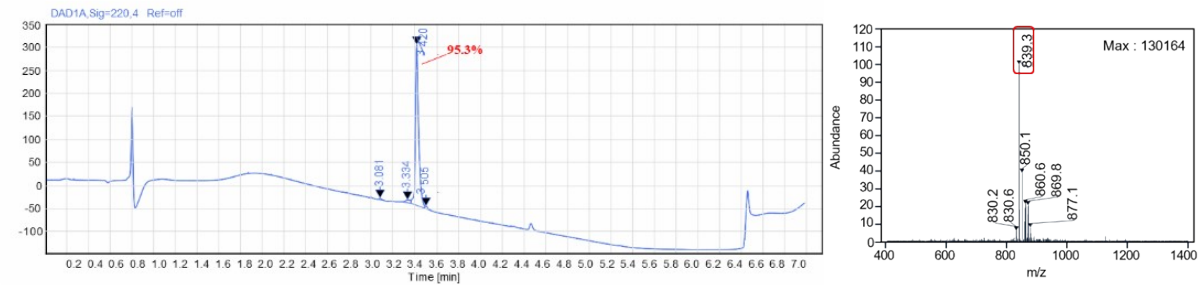
HPLC and MS chromatograms of **A2**



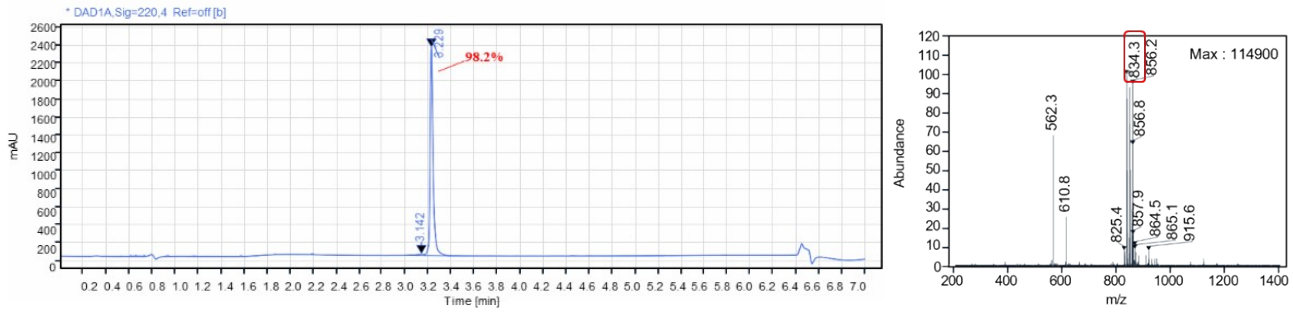
HPLC and MS chromatograms of **A3**



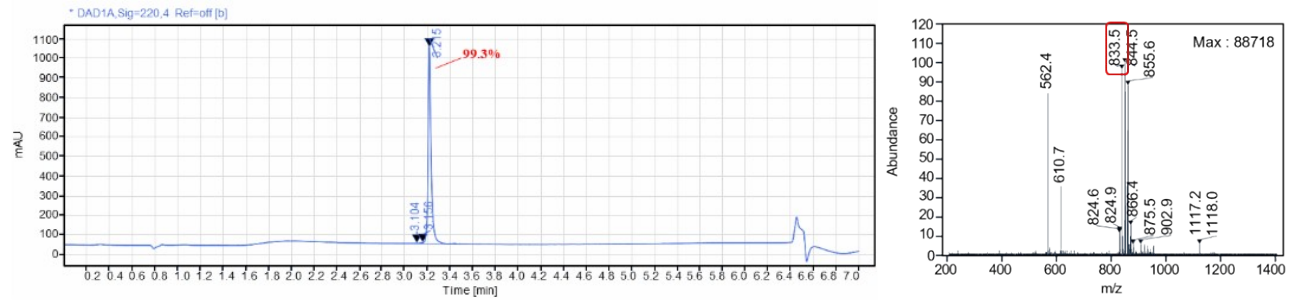
HPLC and MS chromatograms of **A4**



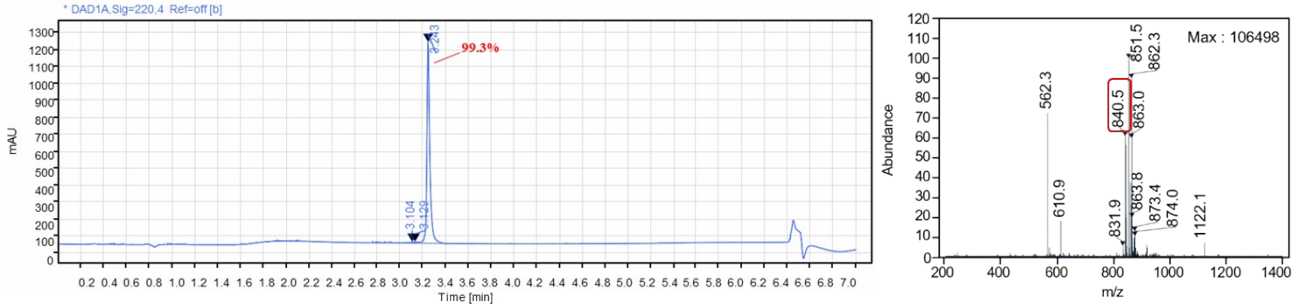
HPLC and MS chromatograms of **A5**



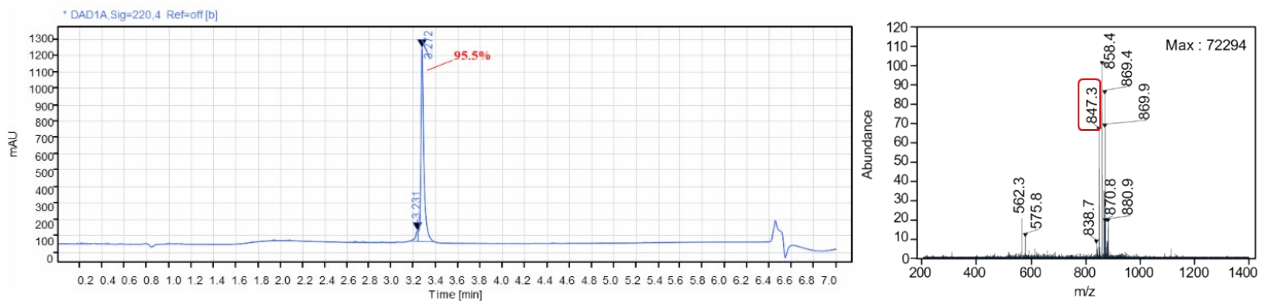
HPLC and MS chromatograms of **A6**



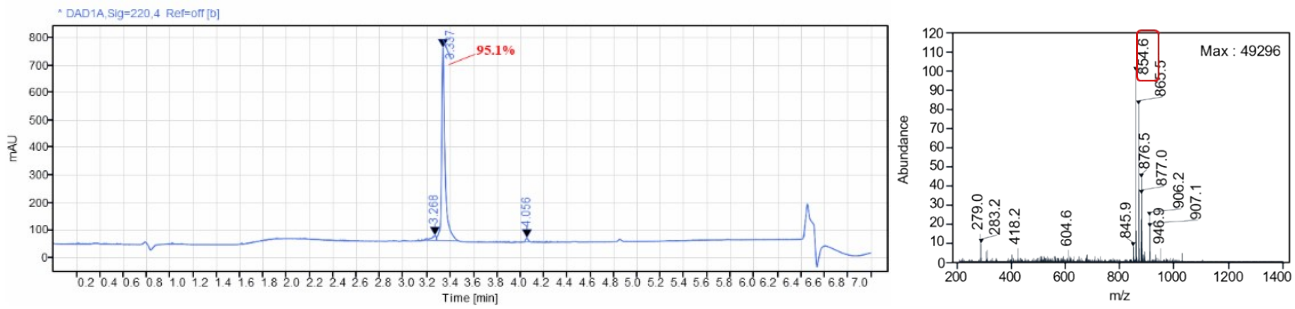
HPLC and MS chromatograms of **A7**



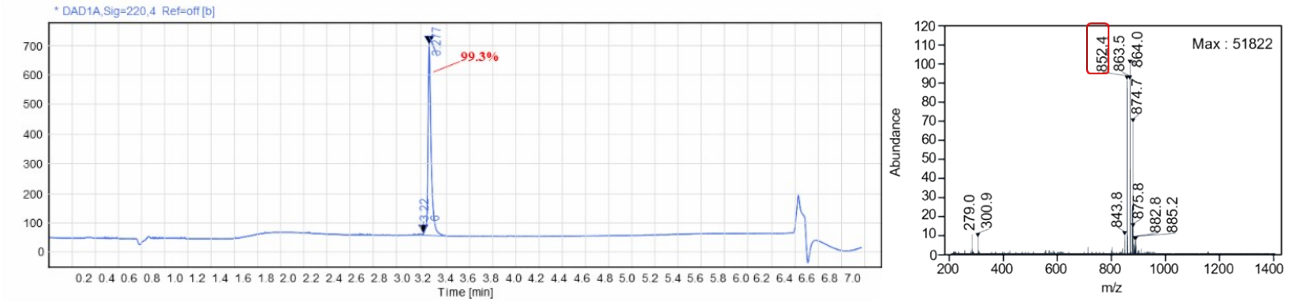
HPLC and MS chromatograms of **A8**



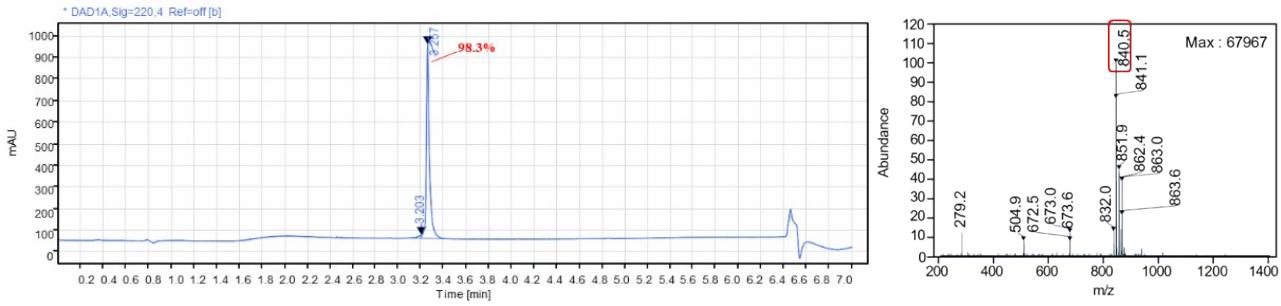
HPLC and MS chromatograms of **A9**



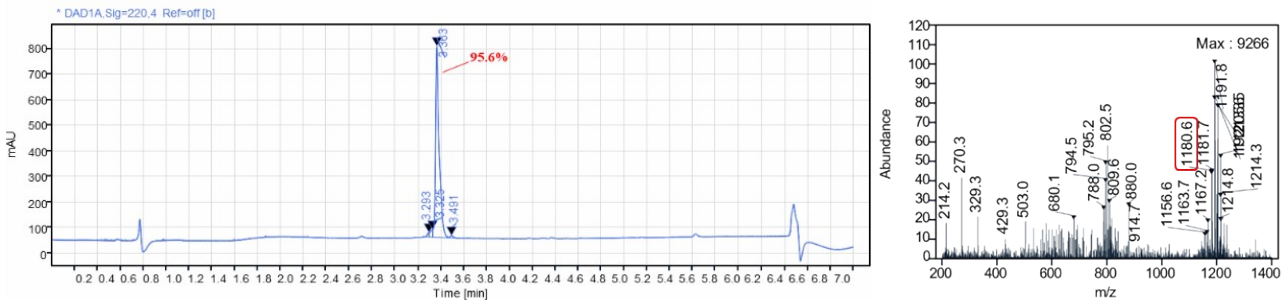
HPLC and MS chromatograms of **A10**



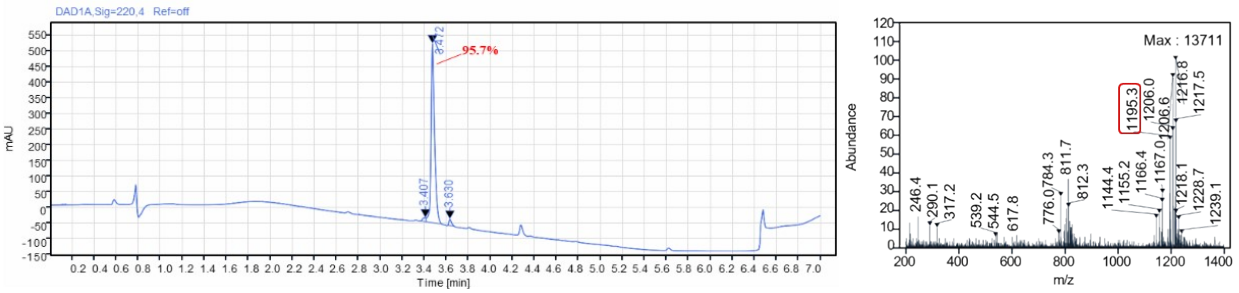
HPLC and MS chromatograms of **A11**



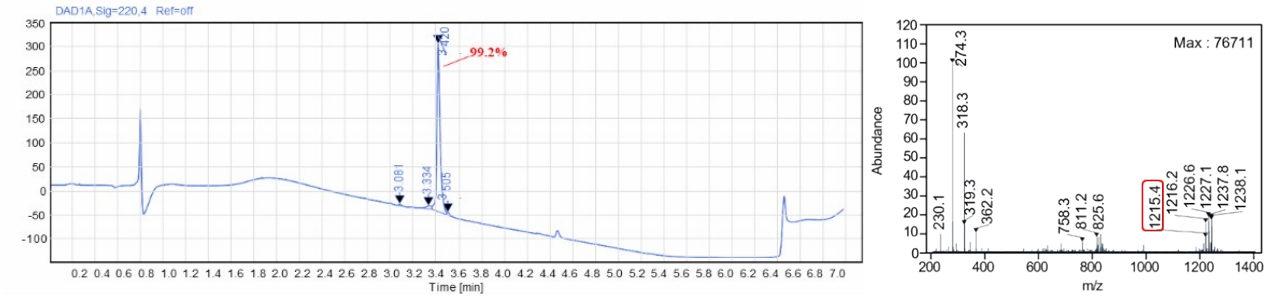
HPLC and MS chromatograms of **A12**



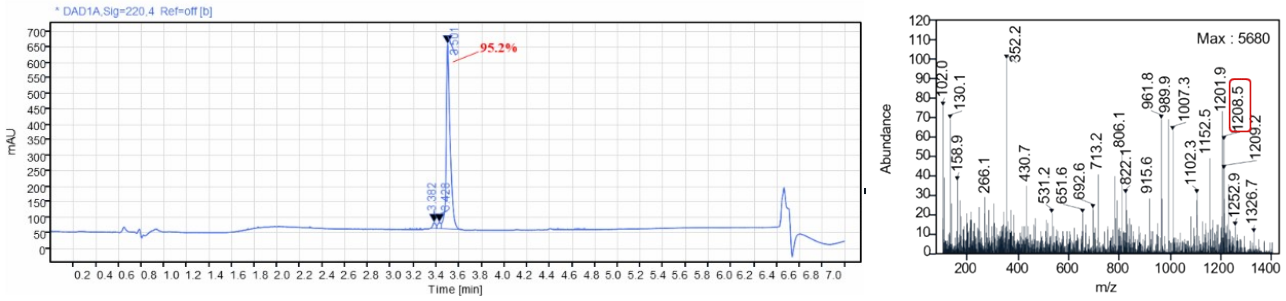
HPLC and MS chromatograms of **B1**



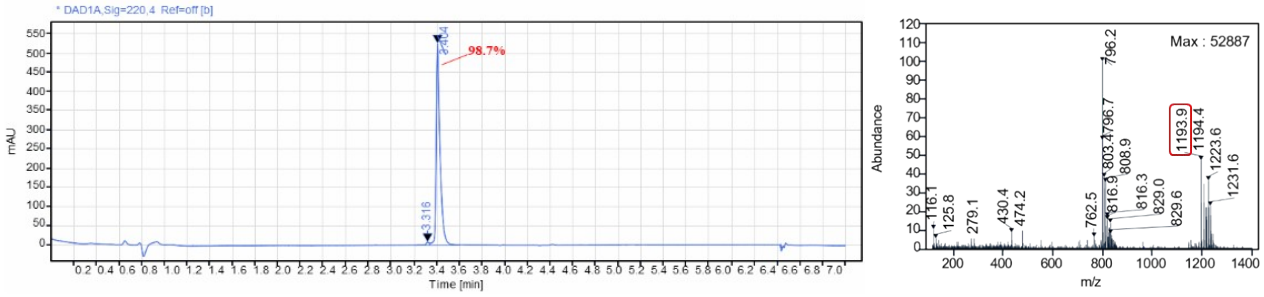
HPLC and MS chromatograms of **B2**



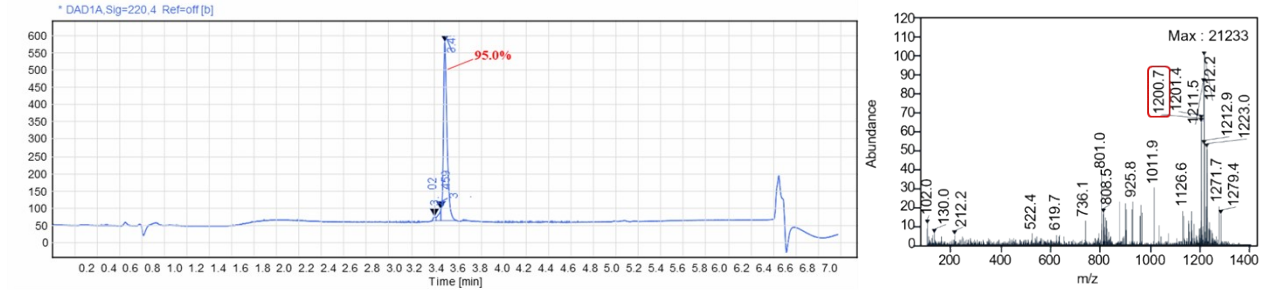
HPLC and MS chromatograms of **B3**



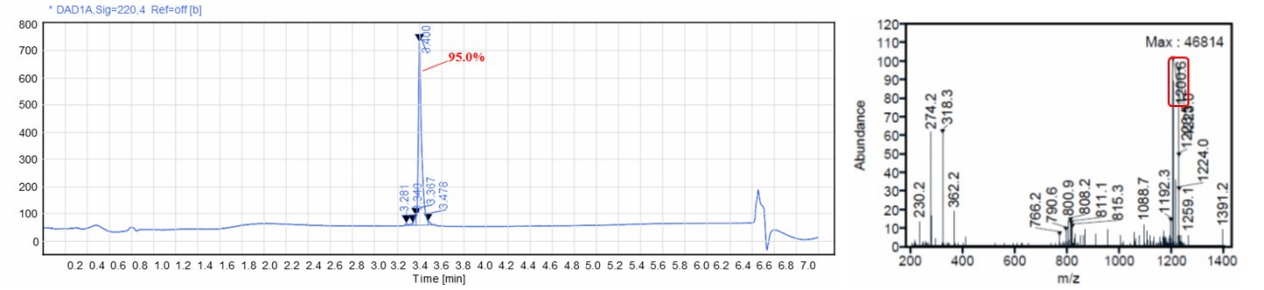
HPLC and MS chromatograms of **B4**



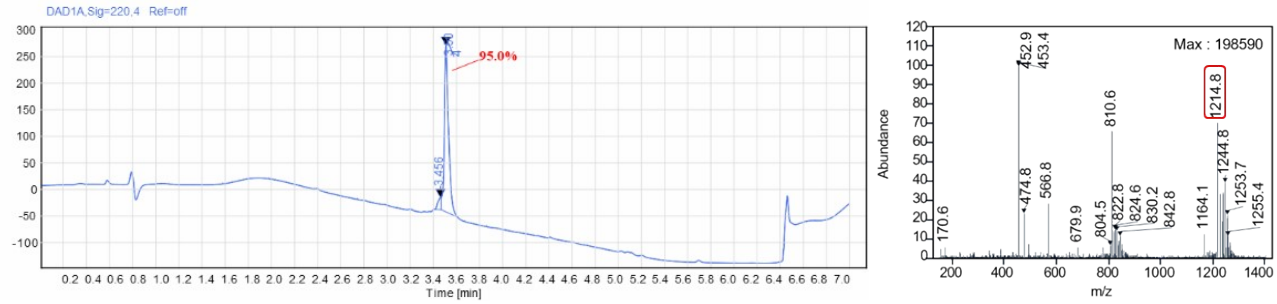
HPLC and MS chromatograms of **B5**



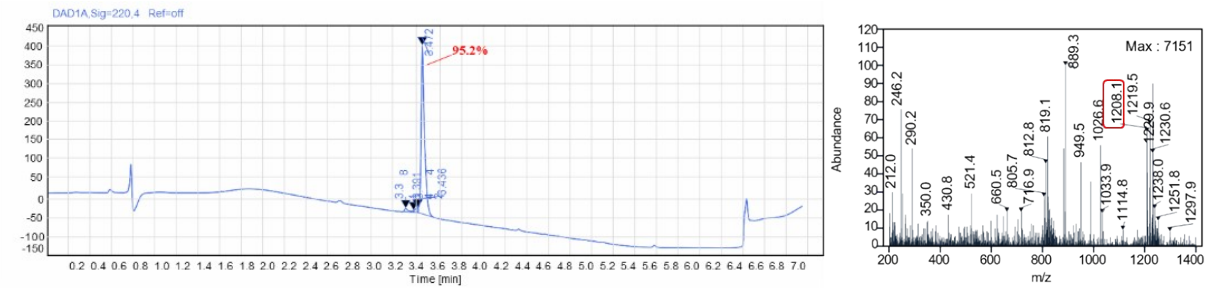
HPLC and MS chromatograms of **B6**



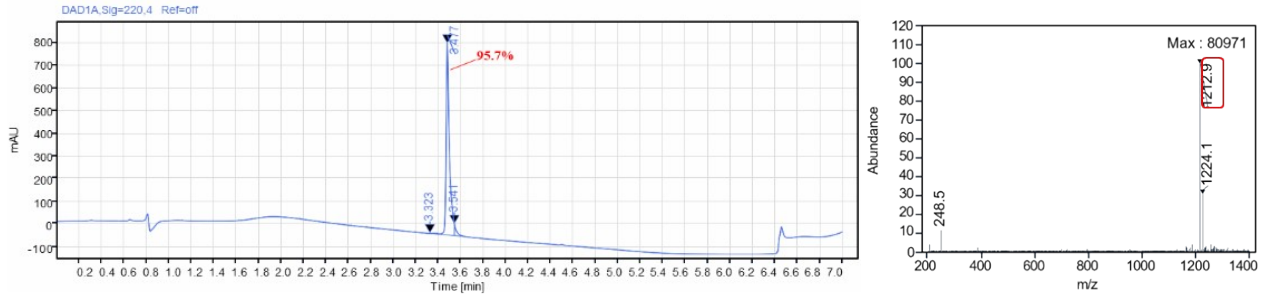
HPLC and MS chromatograms of **B7**



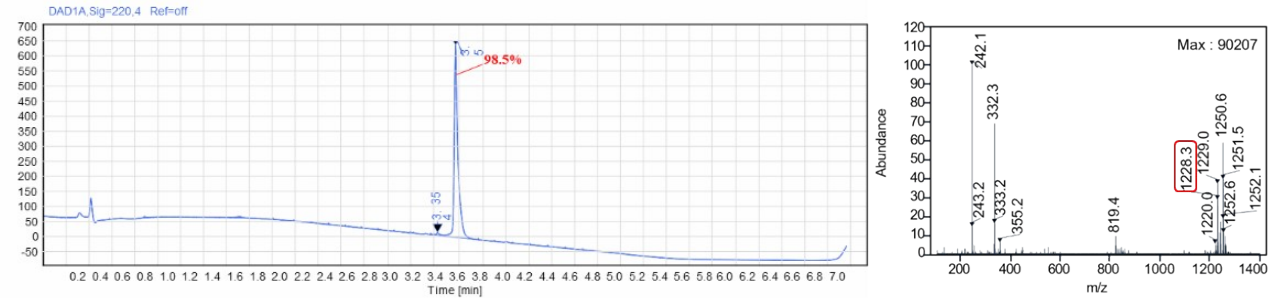
HPLC and MS chromatograms of **B8**



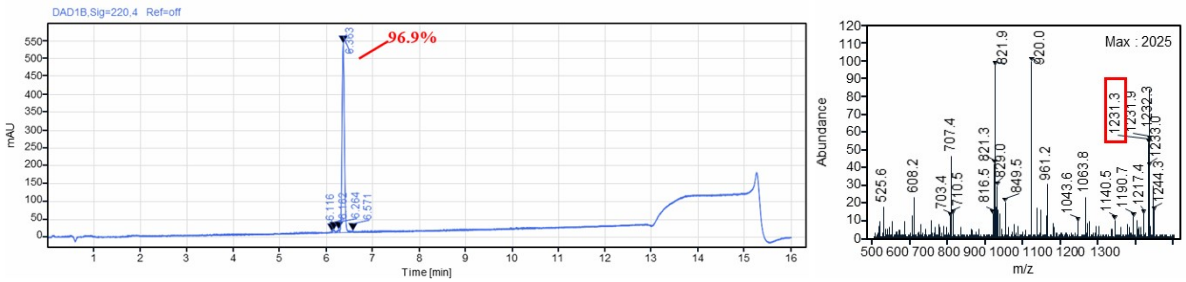
HPLC and MS chromatograms of **B9**



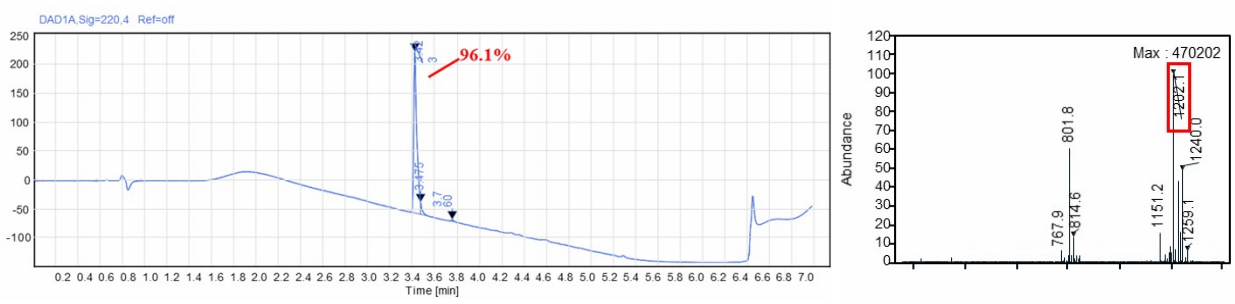
HPLC and MS chromatograms of **B10**



HPLC and MS chromatograms of **B11**



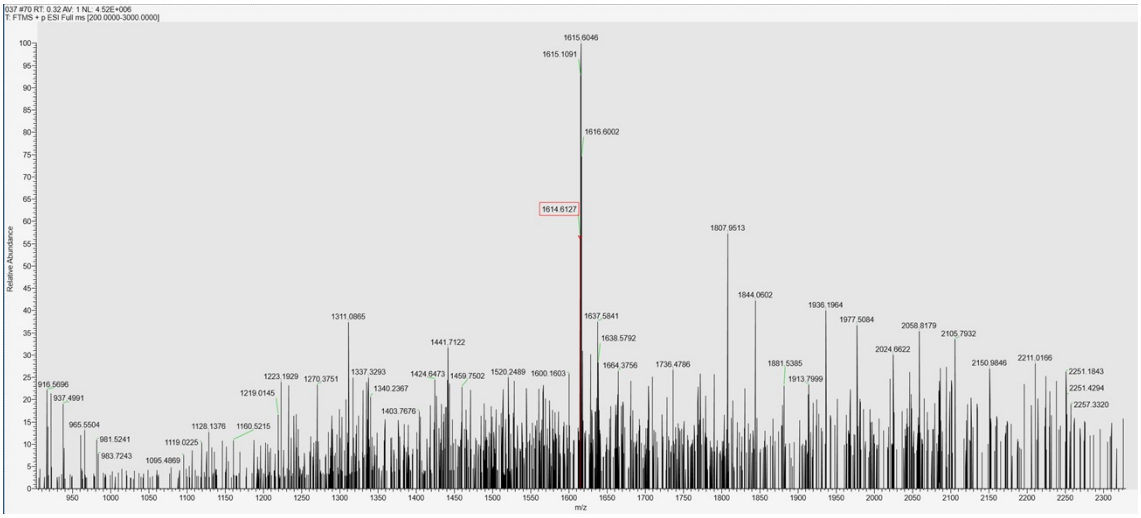
HPLC and MS chromatograms of **PepSP1171**



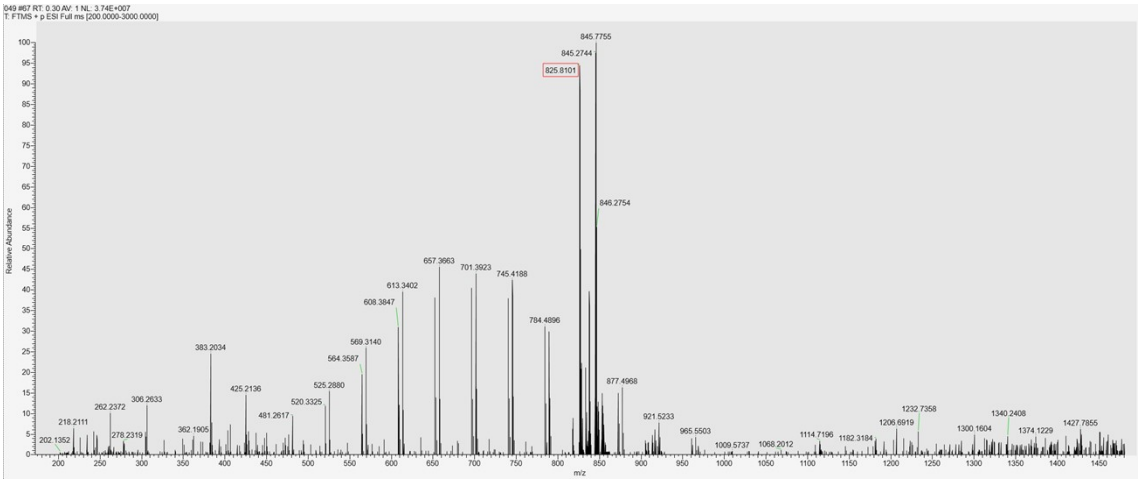
HPLC and MS chromatograms of **PepSP1214**

4. HR-MS chromatograms of the majority of the peptide ligands

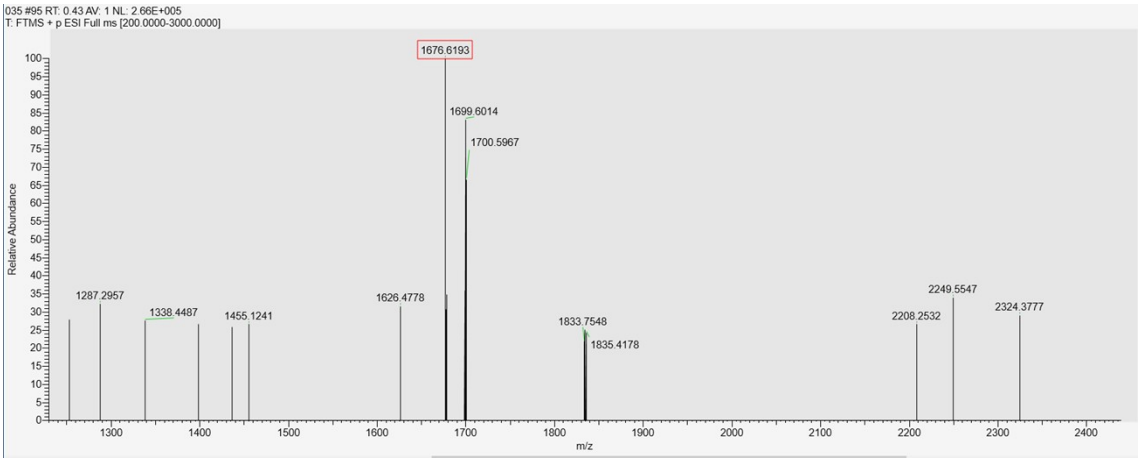
A2: HRMS (ESI$^+$, Orbitrap) m/z: $[M+H]^+$ calcd for 1613.6027, found 1614.6127



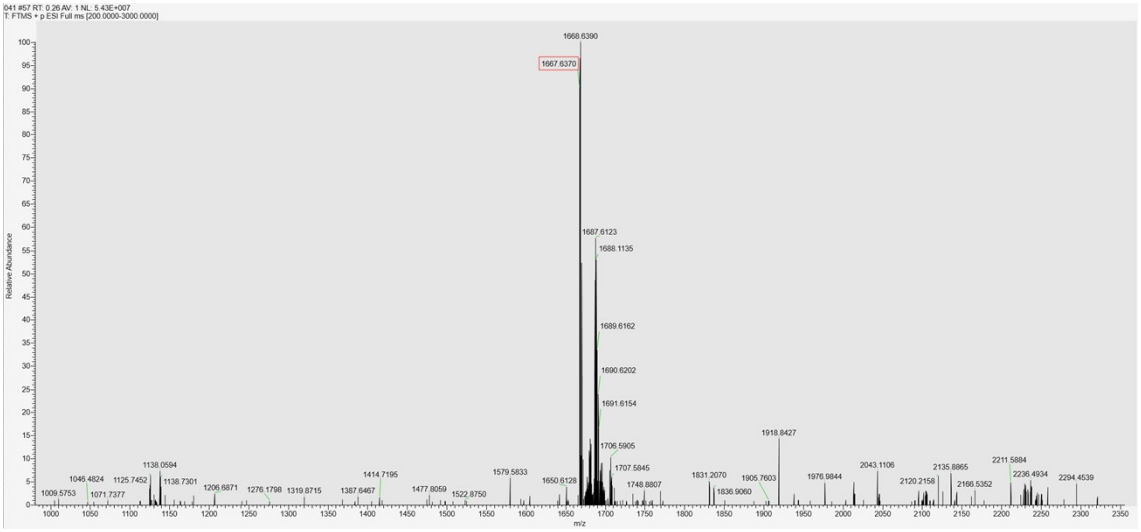
A4: HRMS (ESI$^+$, Orbitrap) m/z: $[M+2H]^{2+}$ calcd for 1649.6027, found 825.8101



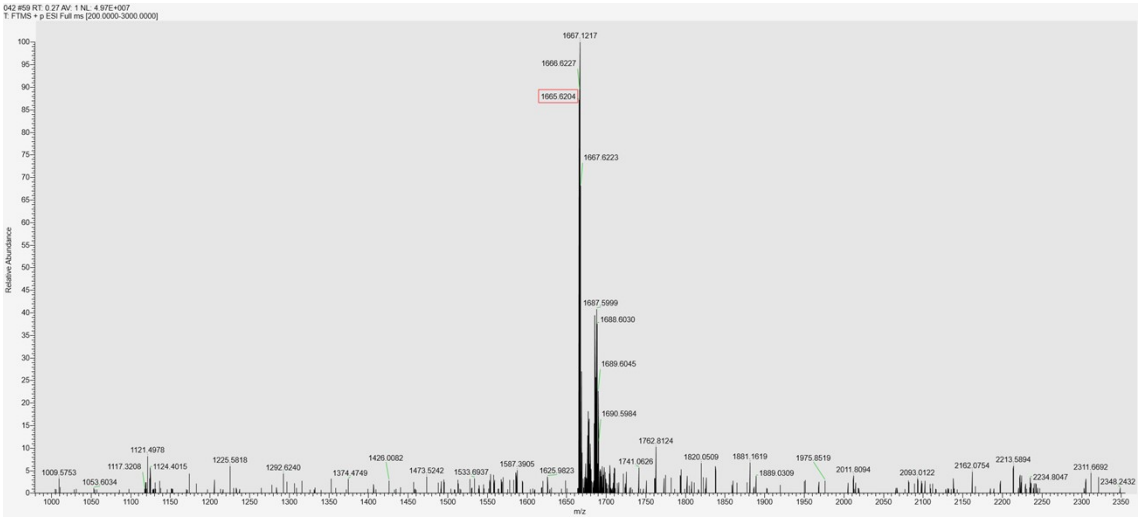
A5: HRMS (ESI$^+$, Orbitrap) m/z: $[M+H]^+$ calcd for 1675.6184, found 1676.6193



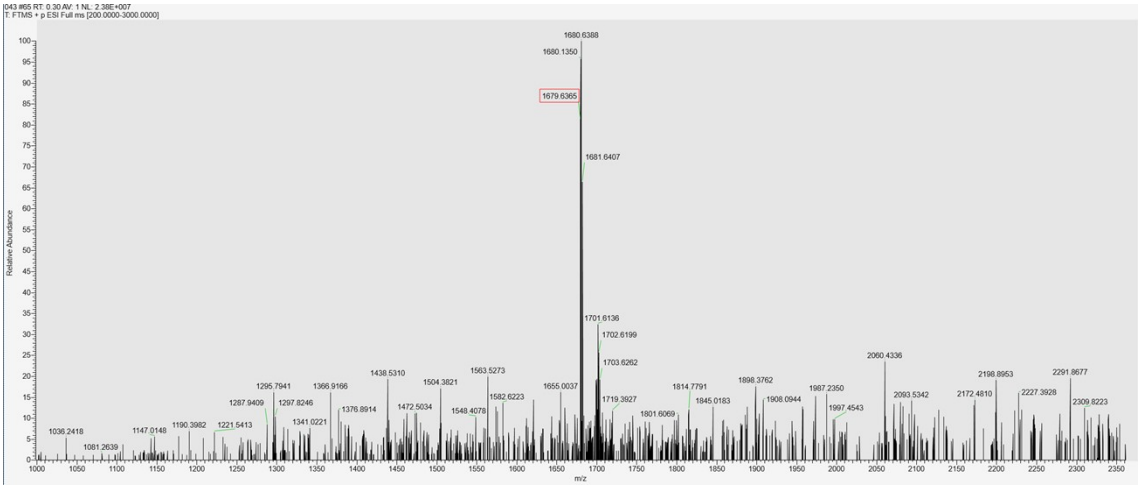
A6: HRMS (ESI$^+$, Orbitrap) m/z: $[M+H]^+$ calcd for 1666.6293, found 1667.6370



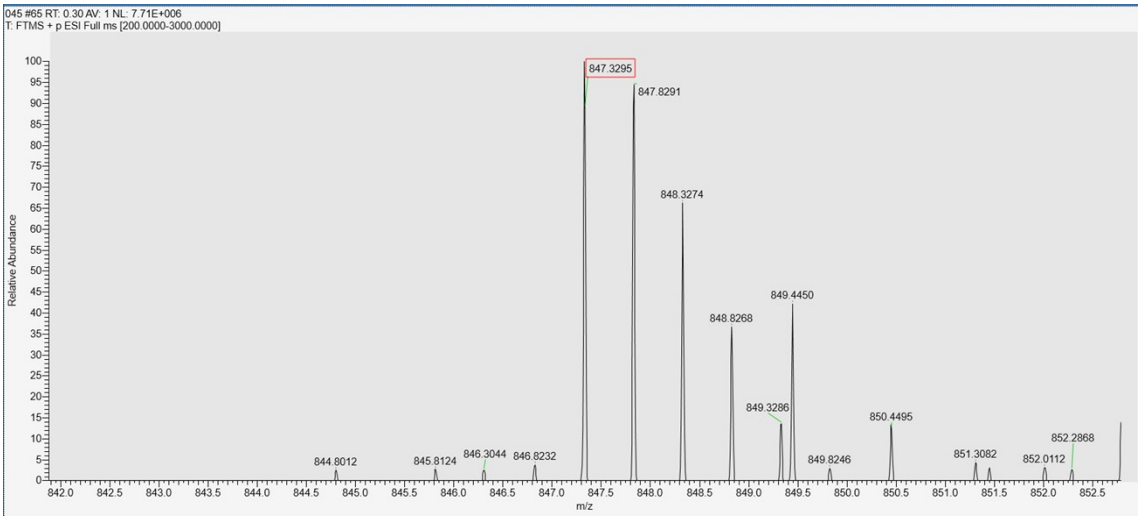
A7: HRMS (ESI$^+$, Orbitrap) m/z: $[M+H]^+$ calcd for 1664.6136, found 1665.6204



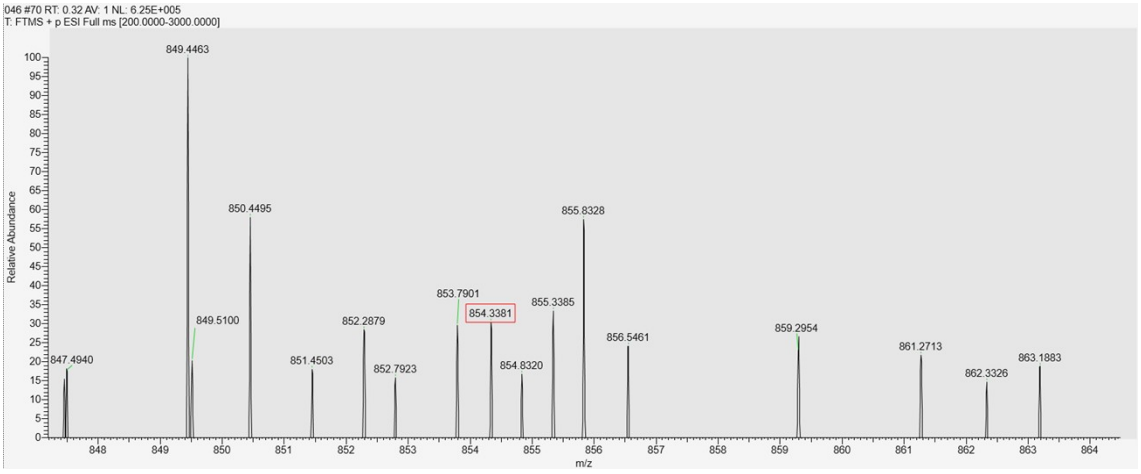
A8: HRMS (ESI$^+$, Orbitrap) m/z: $[M+H]^+$ calcd for 1678.6293, found 1679.6365



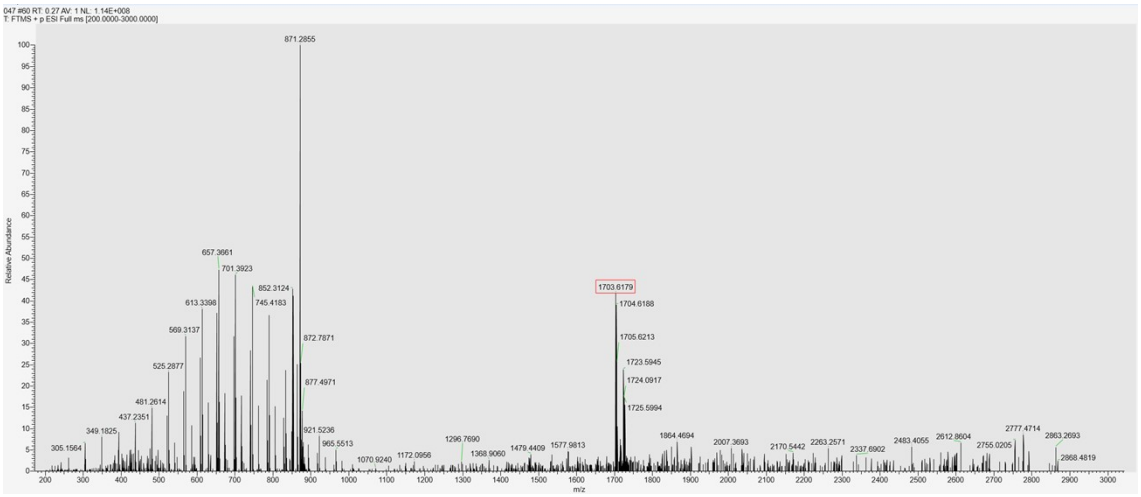
A9: HRMS (ESI$^+$, Orbitrap) m/z: $[M+2H]^{2+}$ calcd for 1692.6449, found 847.3295



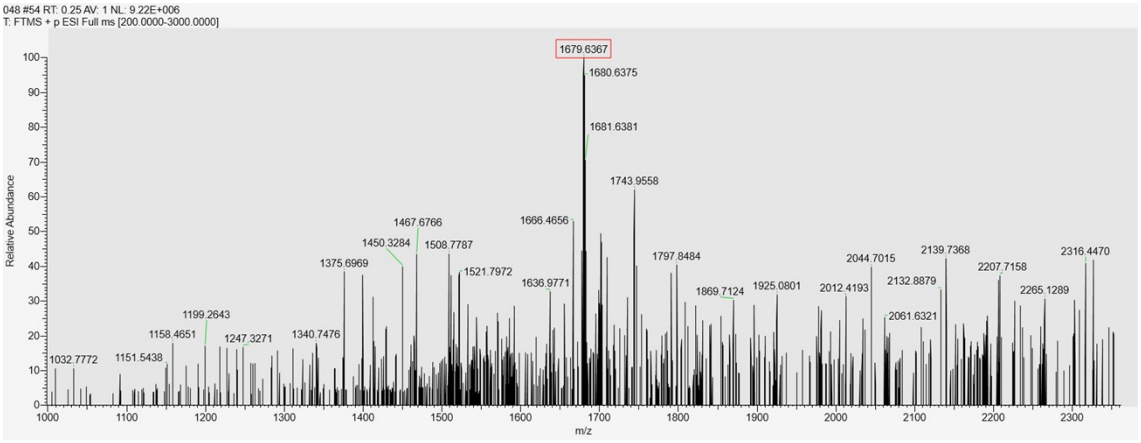
A10: HRMS (ESI$^+$, Orbitrap) m/z: $[M+2H]^{2+}$ calcd for 1706.6606, found 854.3381



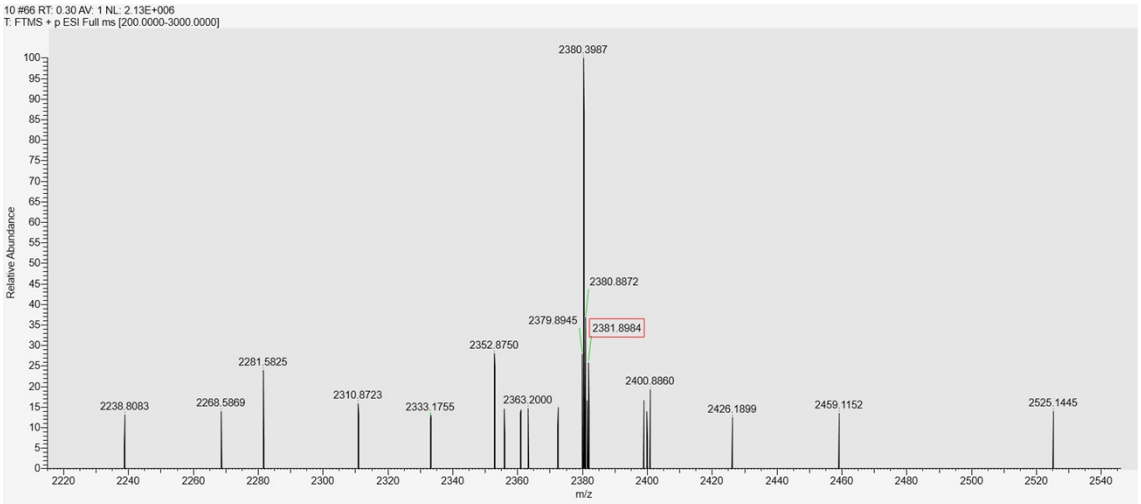
A11: HRMS (ESI$^+$, Orbitrap) m/z: $[M+H]^+$ calcd for 1702.6105, found 1703.6179



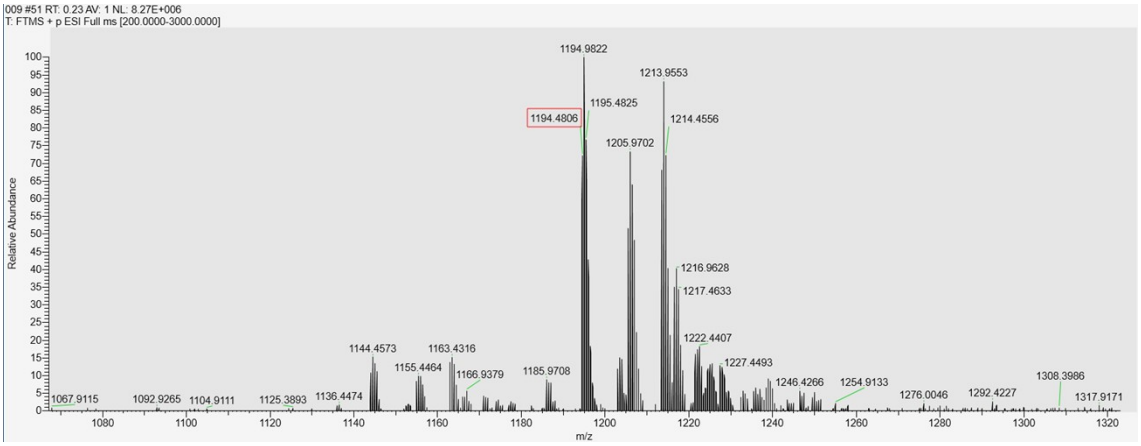
A12: HRMS (ESI$^+$, Orbitrap) m/z: $[M+H]^+$ calcd for 1678.6293, found 1679.6367



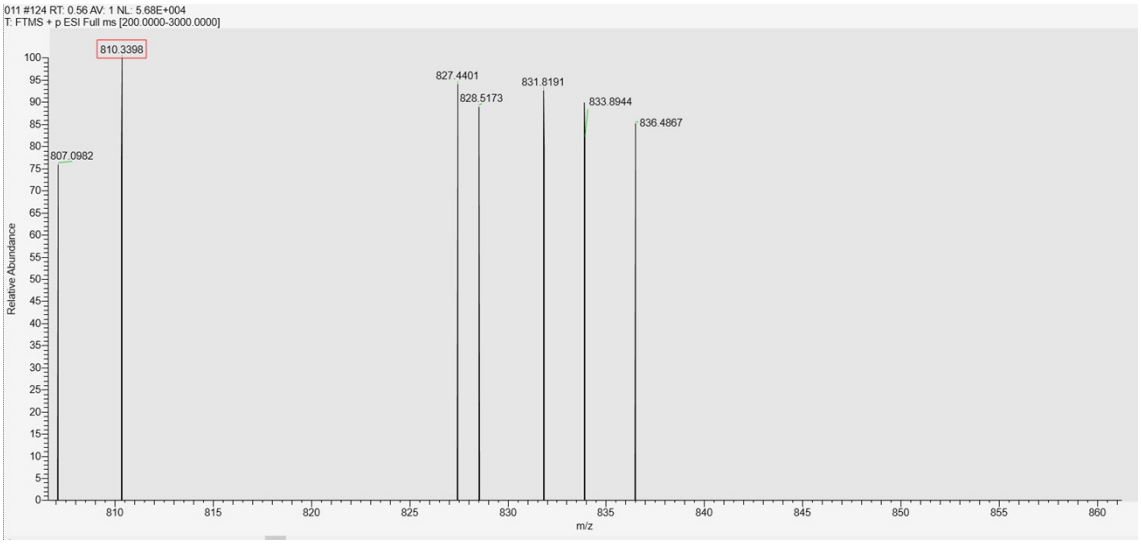
B1: HRMS (ESI$^+$, Orbitrap) m/z: $[M+Na]^+$ calcd for 2381.8997, found 2381.8984



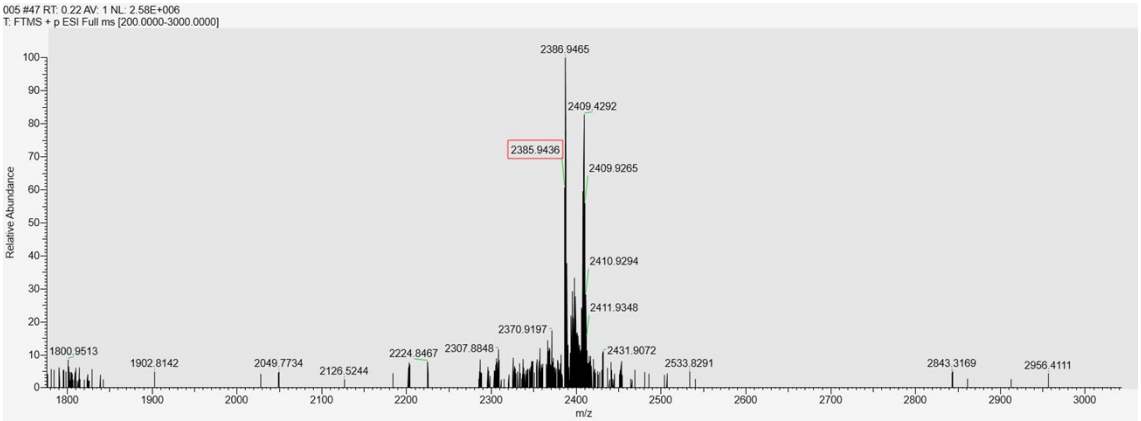
B2: HRMS (ESI$^+$, Orbitrap) m/z: $[M+2H]^{2+}$ calcd for 2386.9412, found 1194.4806



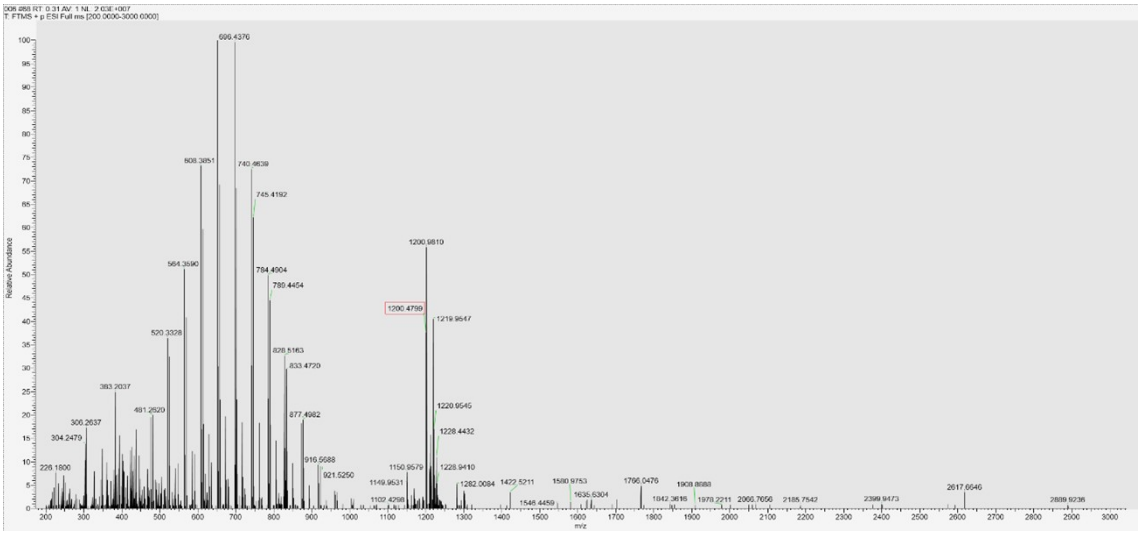
B3: HRMS (ESI$^+$, Orbitrap) m/z: $[M+3H]^{3+}$ calcd for 2428.9881, found 810.3398



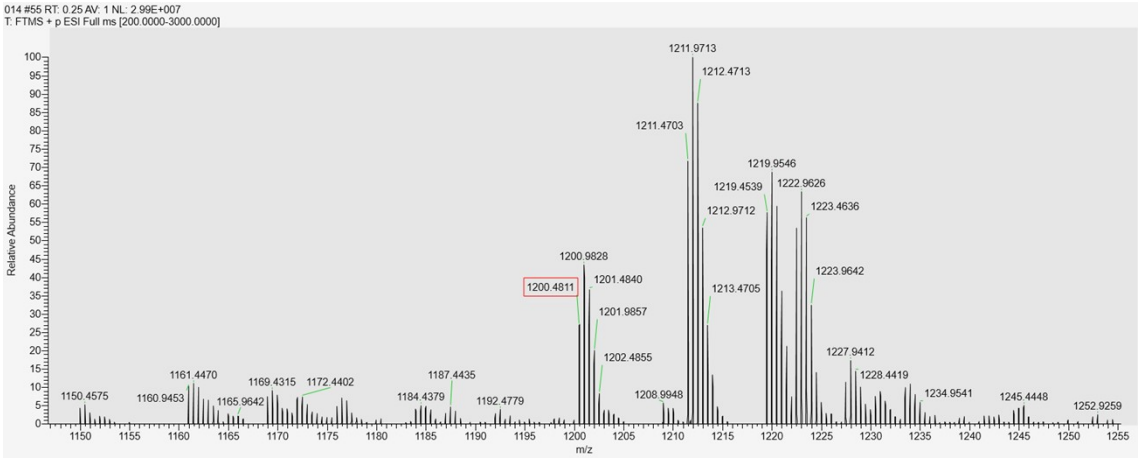
B5: HRMS (ESI$^+$, Orbitrap) m/z: $[M+H]^+$ calcd for 2384.9255, found 2385.9436



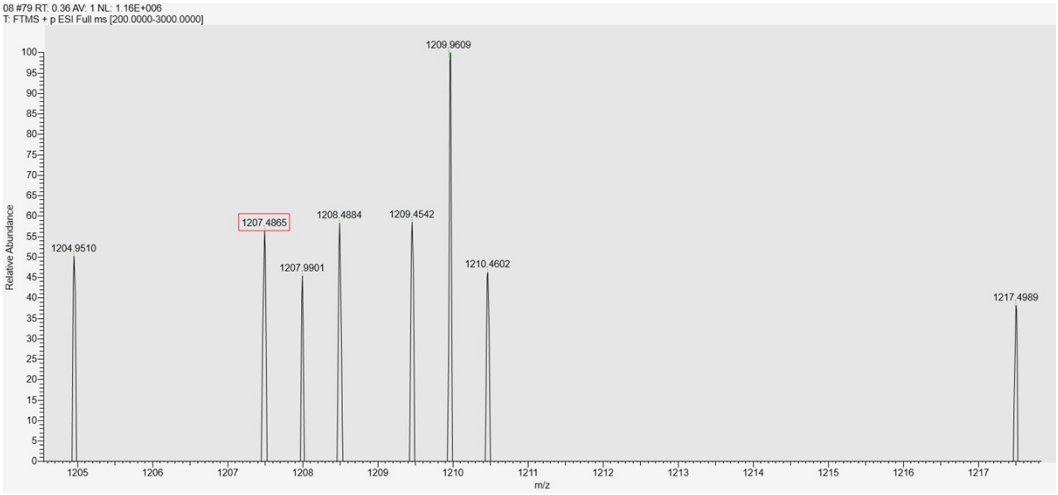
B6: HRMS (ESI$^+$, Orbitrap) m/z: $[M+2H]^{2+}$ calcd for 2398.9412, found 1200.4799



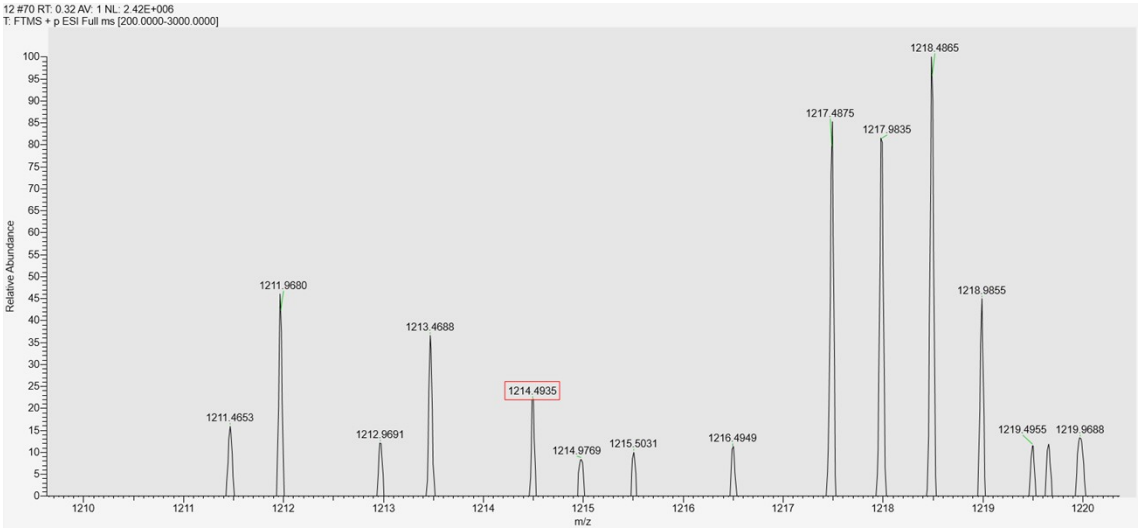
B7: HRMS (ESI$^+$, Orbitrap) m/z: $[M+2H]^{2+}$ calcd for 2398.9412, found 1200.4811



B9: HRMS (ESI$^+$, Orbitrap) m/z: $[M+2H]^{2+}$ calcd for 2412.9568, found 1207.4865



B10: HRMS (ESI$^+$, Orbitrap) m/z: $[M+2H]^{2+}$ calcd for 2426.9725, found 1214.4935



5. Reference

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(2) Valdés-Tresanco, M. S.; Valdés-Tresanco, M. E.; Valiente, P. A.; Moreno, E. gmx_MMPBSA: A New Tool to Perform End-State Free Energy Calculations with GROMACS. *J. Chem. Theory Comput.* **2021**, *17* (10), 6281–6291. https://doi.org/10.1021/acs.jctc.1c00645.