

Supporting Information

Binding Thermodynamics and Kinetics Guided Optimization of Potent Keap1-Nrf2 Peptide Inhibitors

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S1. System Preparation for MD simulation.

The starting protein complex structures for Keap1-peptide were obtained from the modification of the correspondent crystal structures. All structures obtained from PDB were corrected using clean protein tool in Discovery Studio (DS) 3.0 package (Accelrys Inc., San Diego, CA). The Build and Edit Protein tool in DS 3.0 was used to edit the original crystal structures. For peptide **3** and **4**, the crystal structure of Keap1 Kelch domain with P62 peptide (PDB: 3ADE) was used as the template to generate the complex structures of the Keap1-peptide. The starting conformation of peptide **5** was derived from peptide **4**. Using different model structures depending on the sequence similarity was done both because it can improve the accuracy of the starting structure and make the system easily to reach the equilibrium state. The structures obtained from PDB were corrected using clean protein tool in Discovery Studio (DS) 3.0 package (Accelrys Inc., San Diego, CA). All calculations were conducted using Dawning TC2600 cluster. Except for otherwise mentioned, other parameters were set as default.

S2. Detailed Methods of MD Simulation and Trajectories Analysis.

MD simulation of Keap1 DC domain bound to peptides substrates was performed using PMEMD module of AMBER 12 with ff99SB modifications^{1, 2} of the Cornell et al. force field.³ The system was solvated with a cubic box of transferable intermolecular potential three-point (TIP3P) water molecules extending 12 Å in every direction around the solute. The counterions were added to the solvent to keep the system neutral. The detailed processes and parameters used for MD simulation have been clarified in the previous work.⁴ The geometry of the system was minimized in two steps before MD simulation. First, the water molecules were refined through 2500 steps of steepest descent followed by 2500 steps of conjugate gradient, keeping the protein fixed with a constraint of 2.0 kcal·mol⁻¹·Å². Second, the complexes were relaxed by 10000 cycles of minimization procedure (5000 cycles of steepest descent and 5000 cycles of conjugate gradient minimization). During the simulation, the particle mesh Ewald method⁵ was employed to calculate the long-range electrostatic interactions, while the SHAKE method⁶ was applied to constrain all covalent bonds involving hydrogen atoms to allow the time step of 2 fs.⁷ A 10 Å cutoff value was used for the nonbonded interactions. The whole system was heated from 0 K to 300 K running 50 ps molecular dynamics with position restraints at constant volume. Subsequent isothermal isobaric ensemble (NPT)-MD was performed for 500 ps to adjust the solvent density followed by 500 ps of constant pressure equilibration at 300 K without constraints to relax the system. The production dynamics at constant pressure achieved lengths of 10 ns of which snapshots saved at 2 ps intervals were used for further analysis. The ‘ptraj’ tool

in Amber12 was used to analyze the time-dependence of the RMSD of the backbone atoms and the hydrogen bond analysis.

S3. System Stability Examination of Molecular Dynamics Simulations.

The convergence and stability of the simulations were monitored through the examination of the root-mean-square deviation (RMSD) of backbone atoms. The detailed results can be found in Figure S1. As can be seen in the plots, the β -propeller structure of Keap1 shows good structure stability and can be used for further analysis.

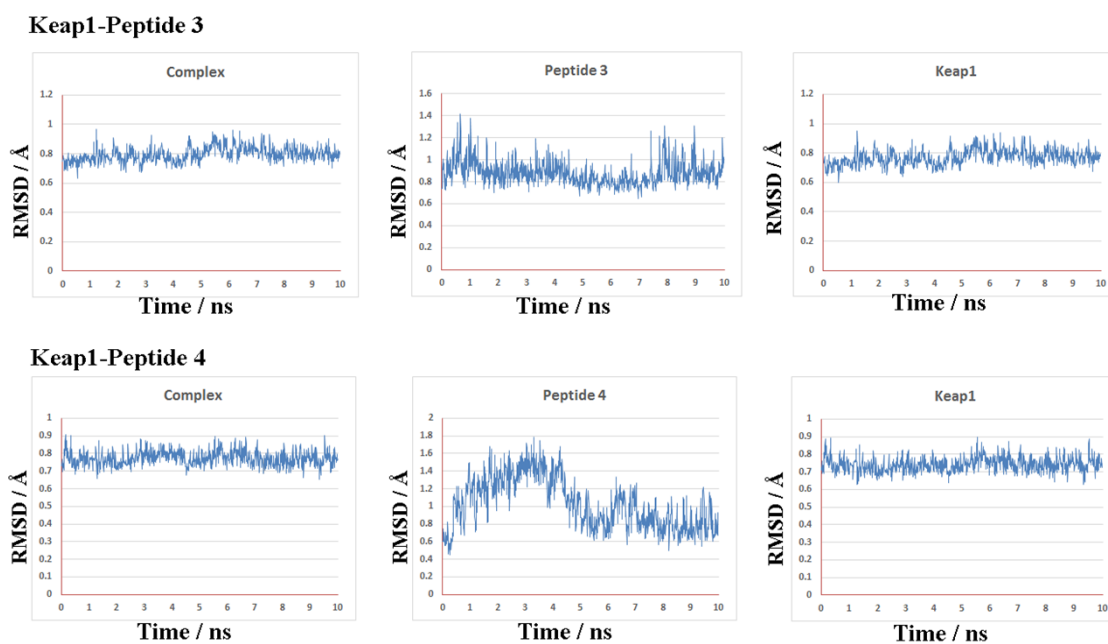
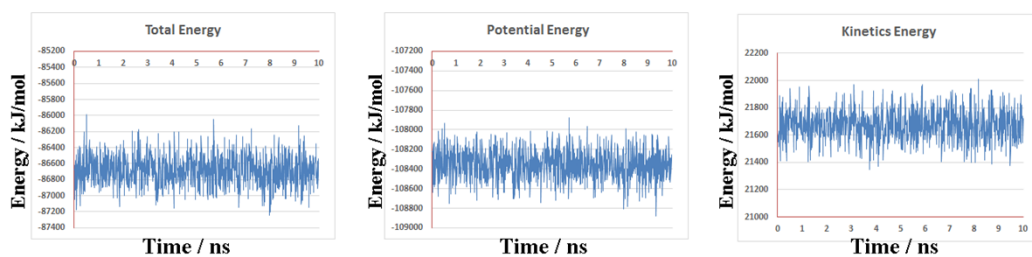


Figure S1. Stability examination for MD simulations. RMSDs of backbone atoms of Keap1-Peptide complex, peptide and Keap1 protein.

Keap1-Peptide3



Keap1-Peptide4

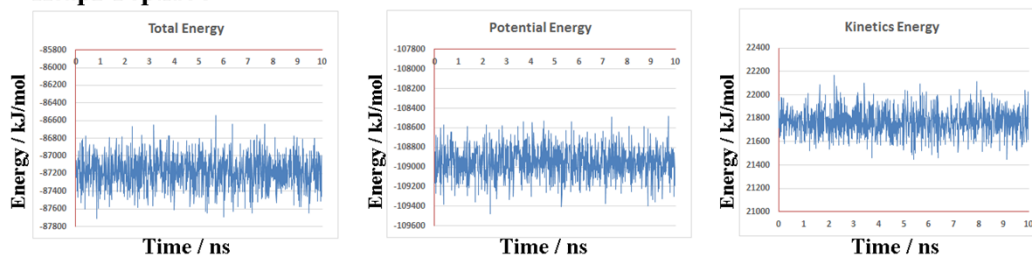


Figure S2. Total energy, potential energy and kinetics energy during the MD simulations of Keap1-Peptide. As shown in the figure, total energy, potential energy and kinetics energy of systems are constant during the MD simulation.

S4. Peptide Synthesis and Purification.

All linear peptides were synthesized manually using a standard solid phase peptide synthesis approach with Fmoc chemistry. Peptide couplings were carried out using HATU and DIPEA in DMF. The N-terminal acetyl group was attached using acetic anhydride and DIPEA in DMF. Fmoc removal was achieved with 20% piperidine in DMF for 20 min. Peptides were cleaved from the resin and side chain deprotected using TFA–TIS–H₂O (95: 2.5: 2.5). All reactions were carried out at room temperature. Cyclization was carried out in solution with 0.1 M ammonium bicarbonate, pH 8.0, for 24 h at a peptide concentration of 0.5 mM. The crude peptide was purified by semi-preparative reverse-phase high performance liquid chromatography (RP-HPLC). The purity and molecular mass of the synthesized and cyclized peptide were confirmed with liquid chromatography-mass spectroscopy (LC-MS).

S5. Expression and Purification of Keap1 Kelch Domain.

The Kelch domain (residues 322–609, UniProtKB - Q14145 (KEAP1_HUMAN)) was cloned into a pET-28a vector (Zoonbio Biotechnology) between an NcoI site and a XhoI site. The vector construct was then used to transform ArcticExpress™ (DE3) prokaryotic host, and cells were grown to an optical density of ~0.6 at 595 nm. Then isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM to induce the expression of the Keap1 Kelch domain. Cells were harvested 4 h after induction of IPTG. After that, the Kelch domain was purified on an ÄKTA™ pure 25 system (GE Healthcare, Life Sciences) using a Ni-NTA column (Novagen). The molecular weight of the purified protein was confirmed by 10% SDS-PAGE.

S6. FP Competition Assay to Determine the Inhibitory Potency of the Keap1-Nrf2 Interaction.

All fluorescence polarization (FP) assays were performed on a SpectraMax Multi-Mode Microplate Reader (Molecular Devices) using the excitation and emission filters appropriate for the fluorophore used in the binding experiment. The 9-mer Nrf2 ETGE motif derived peptide, FITC-LDEETGEFL-NH₂, was used as the fluorescent probe as previously reported.⁸ The plates used for the FP measurements were the black non-binding surface Corning 3676 384-well plates, loaded with 40 μL of assay solution per well, consisted of 10 μL of 4 nM FITC-9mer Nrf2 peptide amide and 10 μL of 12 nM Keap1 Kelch domain, 10 μL of HEPES buffer, and 10 μL of an inhibitor sample of varying concentrations. The plate was covered and rocked for 30 min at room temperature prior to FP measurements. For fluorescein, 485 nm excitation and 535 nm emission filters were used. FP was determined by measuring the parallel and perpendicular fluorescence intensity (F_{\parallel} and F_{\perp}) with respect to the linearly polarized excitation light. We elected to use polarization in our quantitative analysis. The percentage inhibition of the competitor at each concentration point was determined by using equation $\%inhibition = 1 - (P_{obs} - P_{min}) / (P_{max} - P_{min})$. The values of P_{max} , P_{min} , and P_{obs} in the equations refer to the polarization of the wells containing Keap1 and the probe, the polarization of the free probe, and the observed polarization for the wells containing the inhibitors at a range of concentrations under the assay conditions. The IC_{50} of an inhibitor was determined from the plot of $\%inhibition$ against inhibitor concentration analyzed by GraphPad Prism 6.0 software.

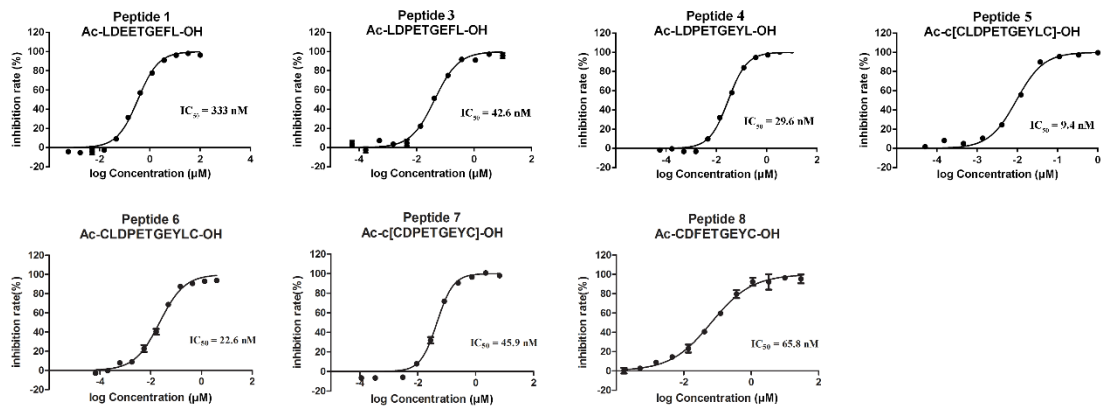


Figure S3. Dose-response inhibition curves determined by the FP-based binding assay.

The FITC labeled 9-mer Nrf2 ETGE peptide, FITC-LDEETGEFL-NH₂, was chosen as the fluorescent probe. Values are shown as mean (n=3).

S7. ITC (Isothermal Titration Calorimetry) Assay.

Isothermal titration calorimetry (ITC) was performed at 25 °C with the ITC200 system (MicroCal). The Keap1 Kelch domain was lyophilized. Both the protein sample and the peptides were dissolved in 10 mM HEPES buffer (pH 7.4). 2 μ L aliquots of 0.05 mM peptide were injected 19 times at 2.5 min intervals from a stirring syringe (750 rpm) into the sample cell containing 220 μ l of 0.005 mM Keap1 Kelch domain. Data were analyzed with the computer program Origin, version 7.0, supplied by MicroCal.

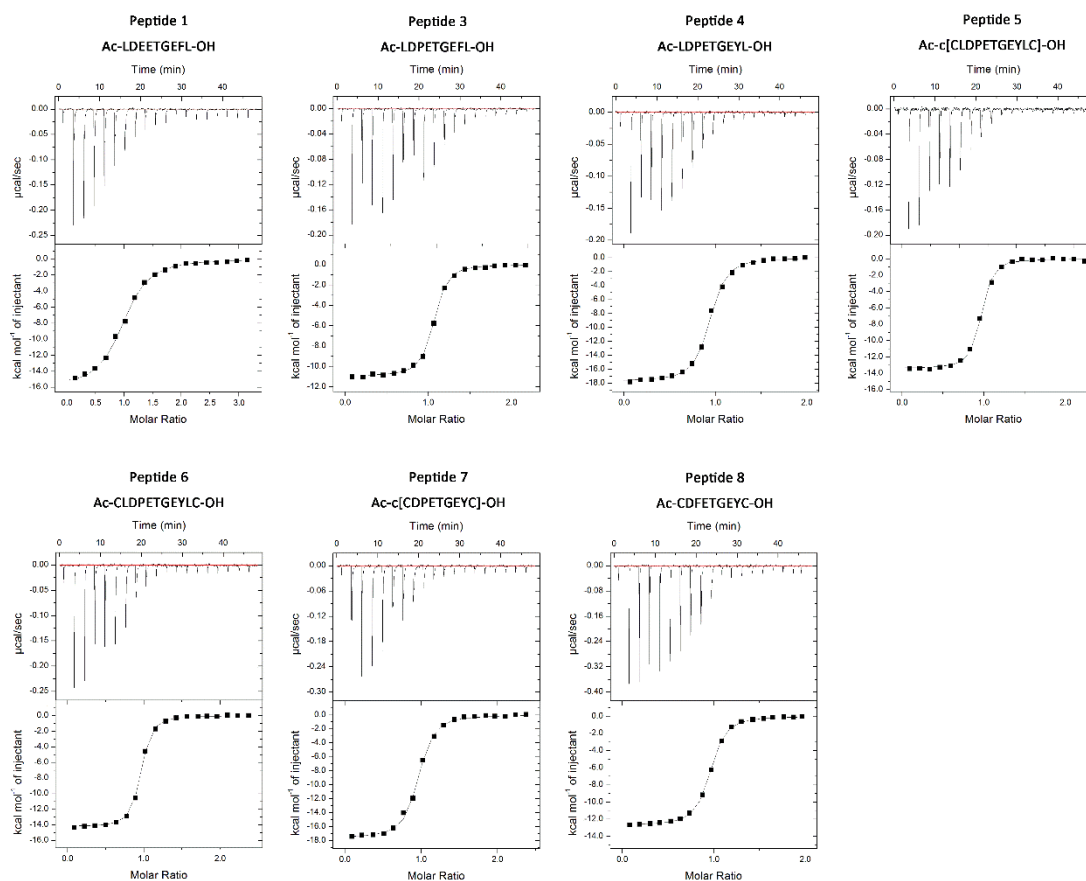


Figure S4. ITC titration profiles of Keap1 Kelch domain with seven peptides.

S8. Biolayer Interferometry.

The Keap1 Kelch domain was biotinylated in a buffer of 20 mM HEPES, pH 7.0. The interaction between the ligand and the Keap1 Kelch domain was determined by biolayer interferometry using an Octet Red 96 instrument (FortéBio Inc.). Super Streptavidin Biosensors tips (FortéBio, Inc., Menlo Park, CA) were prewetted with buffer (FortéBio) to establish a baseline before protein immobilization. Then the biotinylated protein target was immobilized onto Super Streptavidin Biosensors. All of the binding data were collected at 30 °C. The experiments comprised five steps: (1) baseline acquisition, (2) protein loading onto the sensor, (3) second baseline acquisition, (4) association of the ligand for the measurement of k_{on} , and (5) dissociation of the ligands for the measurement of k_{off} . Four concentrations of various ligands were used for detection. The association and dissociation plot and kinetic constants were obtained with FortéBio data analysis software. Equilibrium dissociation constants (K_d) were calculated by the ratio of k_{off} to k_{on} .

S9. Conformations of the Peptide 4 and 5.

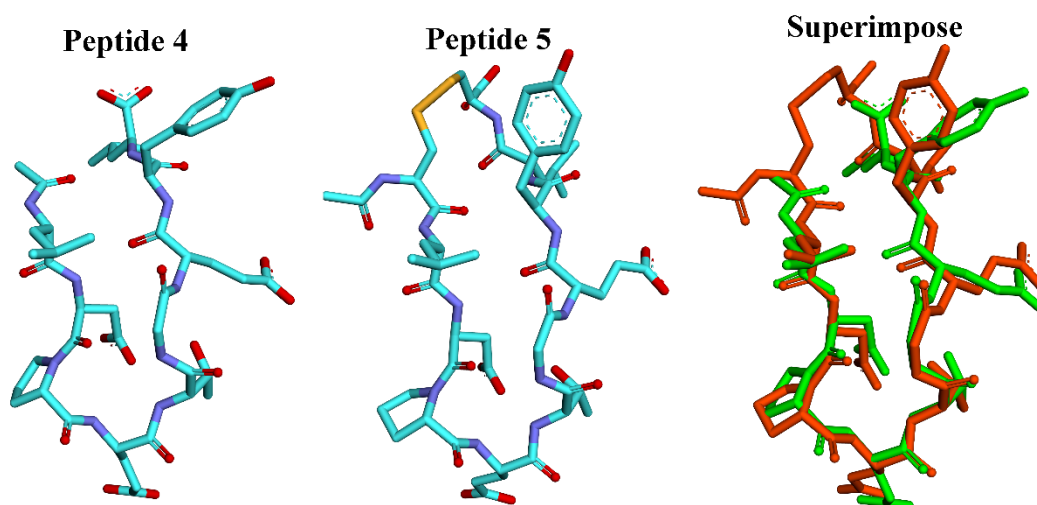
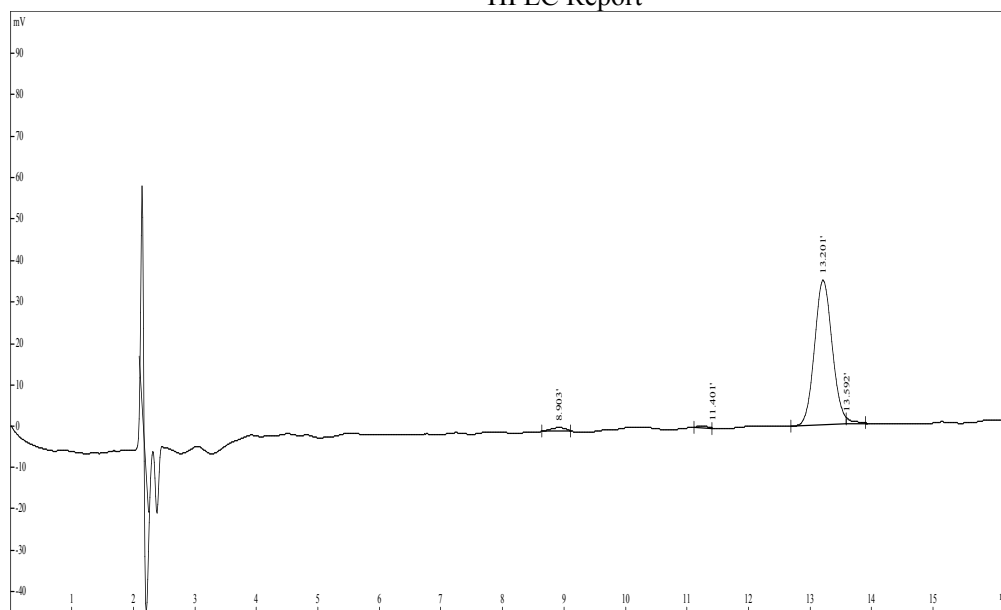


Figure S5. Conformations of Peptide 4 and 5. The two chains of the β -turn in peptide 4 is more open than peptide 5 and the conformations of core ETGE motif in peptide 4 and 5 are similar. Peptide 4 was represented as red sticks and peptide 5 was represented as green sticks in the superimposition view. The average structure along the MD simulation was used to show the peptide conformation.

S10. Characterization of the Target Peptides.

Peptide 1:

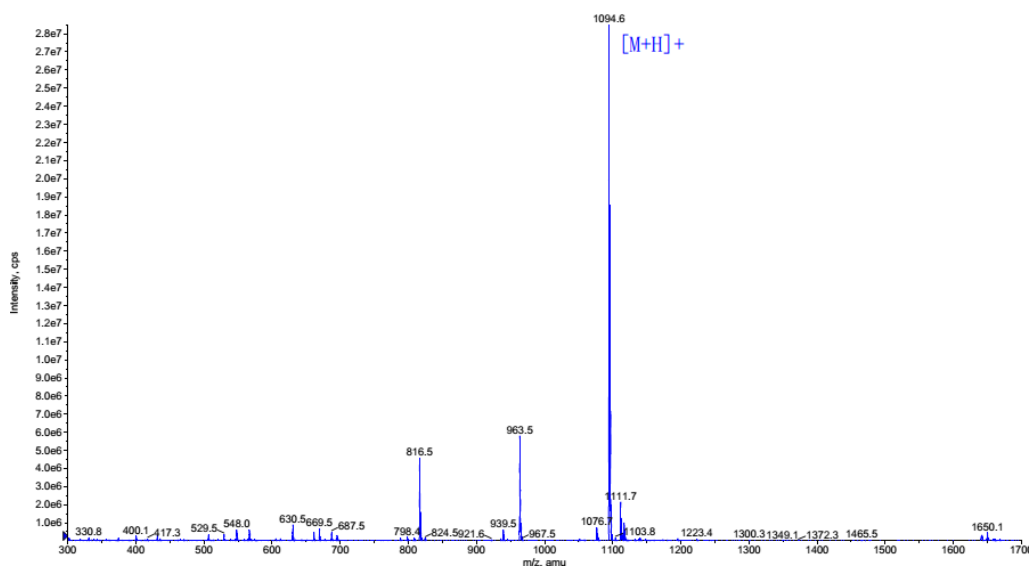
HPLC Report



Time	Quantity	Area	Height	Resolut.	T.PlateNum	Asymmetry
8.903	1.889	13668	883	12.13	7720	0.90
11.401	0.4122	2983	18	6.78	110	
13.201	96.33	697051	34934	1.47	10213	1.09
13.592	1.368	9900	1370	1.47	82554	1.09
		100	723602	37205		

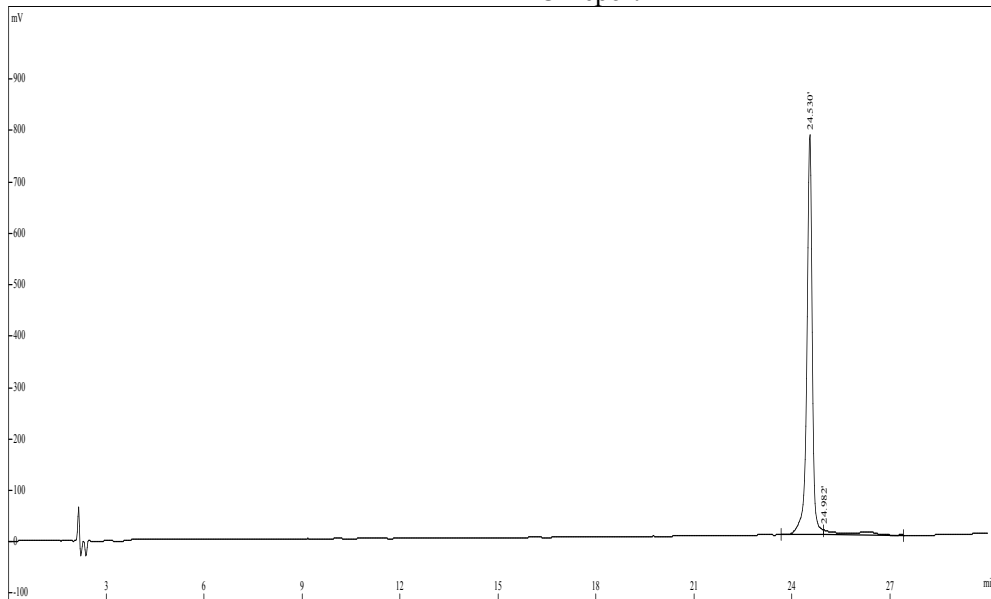
MS Report

MW: 1094.11



Peptide 3

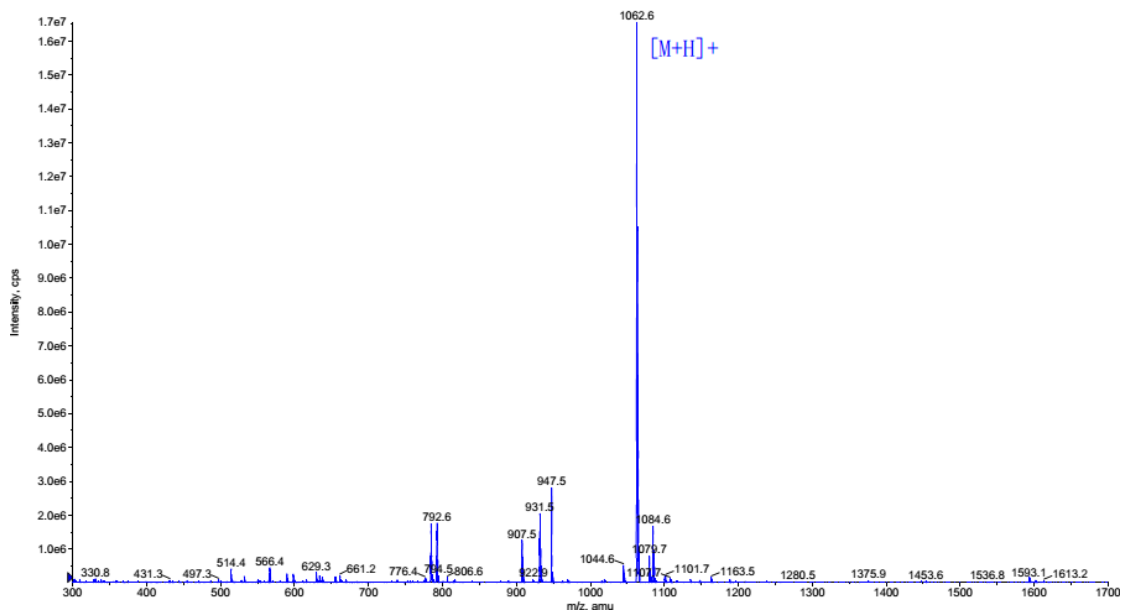
HPLC Report



Time	Quantity	Area	Height	Resolut.	T.PlateNum	Asymmetry
24.530	95.56	8875726	787501	3.01	110532	0.88
24.982	4.444	412794	8303	3.01	5892	0.88
	100	9288520	795804			

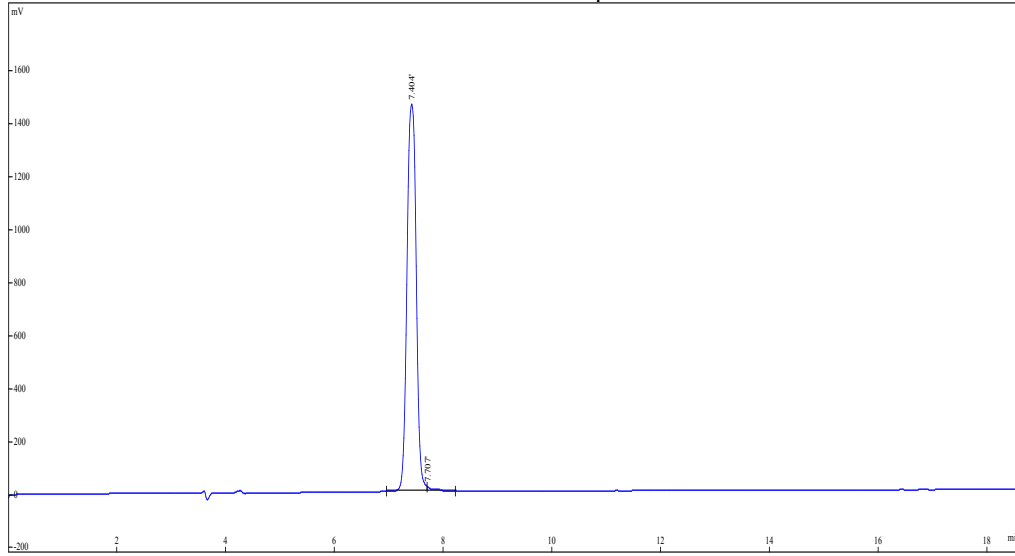
MS Report

MW: 1062.11



Peptide 4

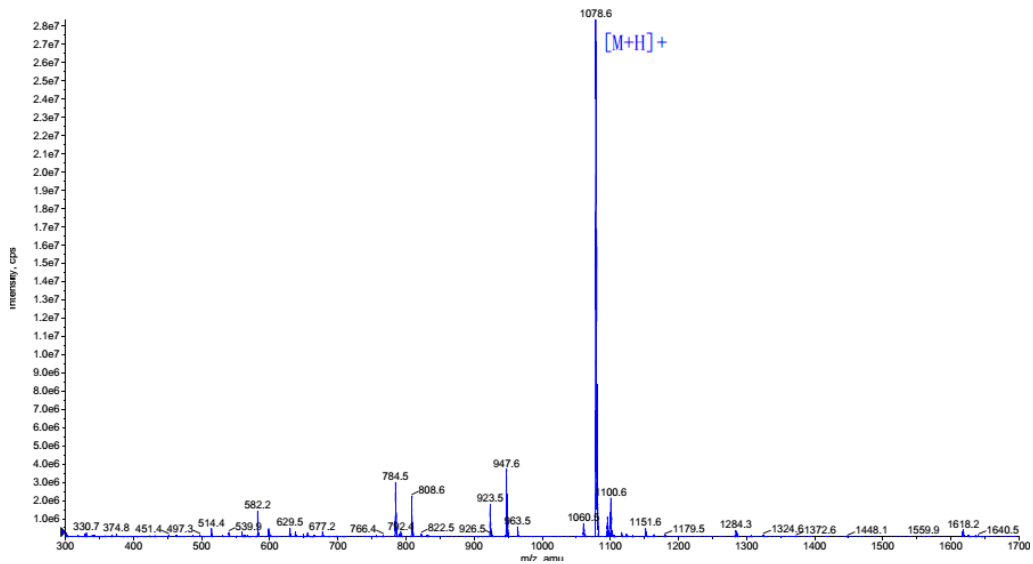
HPLC Report



Rank	Time	Name	Conc.	Area
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2	7.707		0.7308	126624
Total			100	17326826

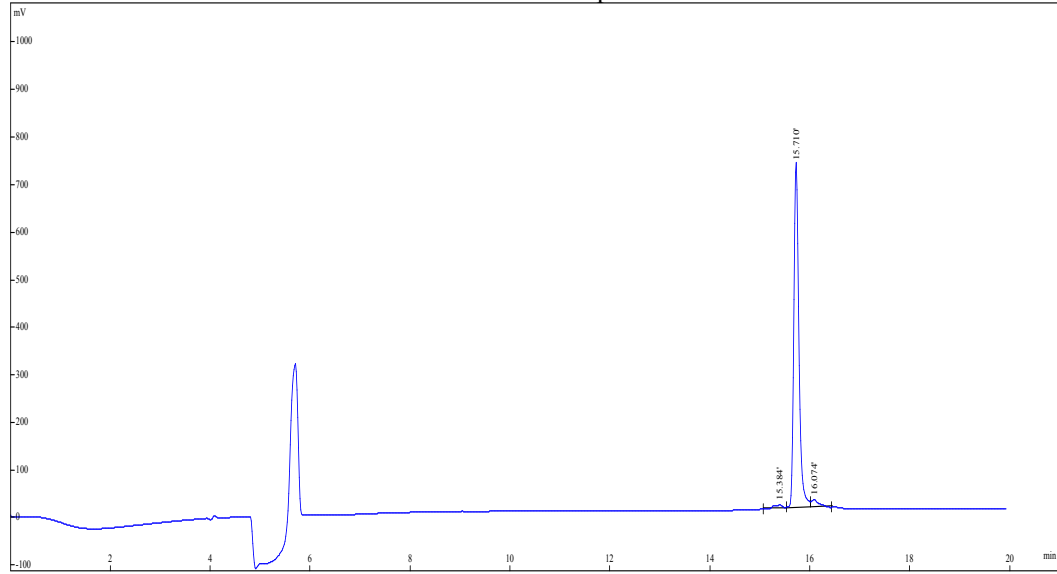
MS Report

MW: 1078.11



Peptide 5

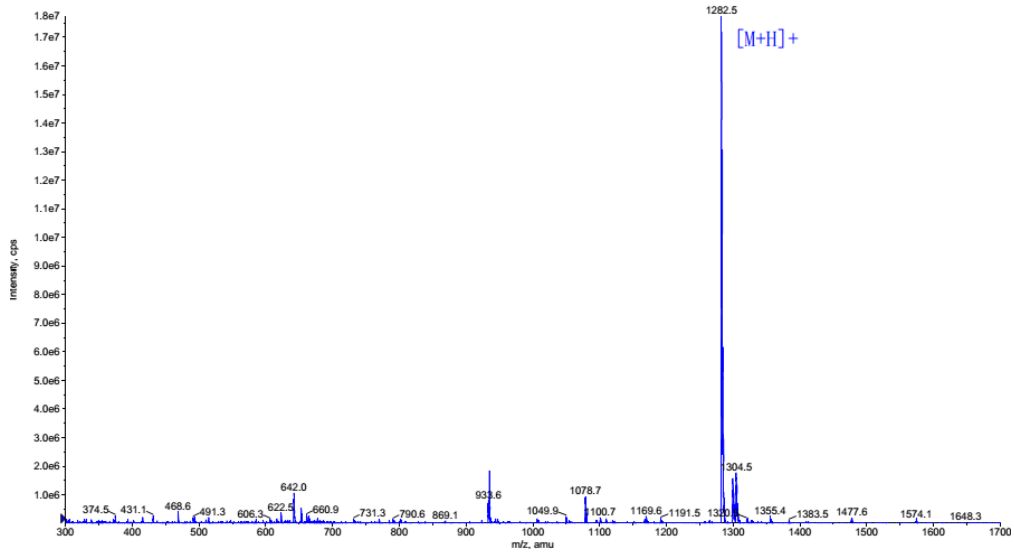
HPLC Report



Rank	Time	Name	Conc.	Area
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2	15.710		95.01	5170910
3	16.074		3.223	175418
Total			100	5442556

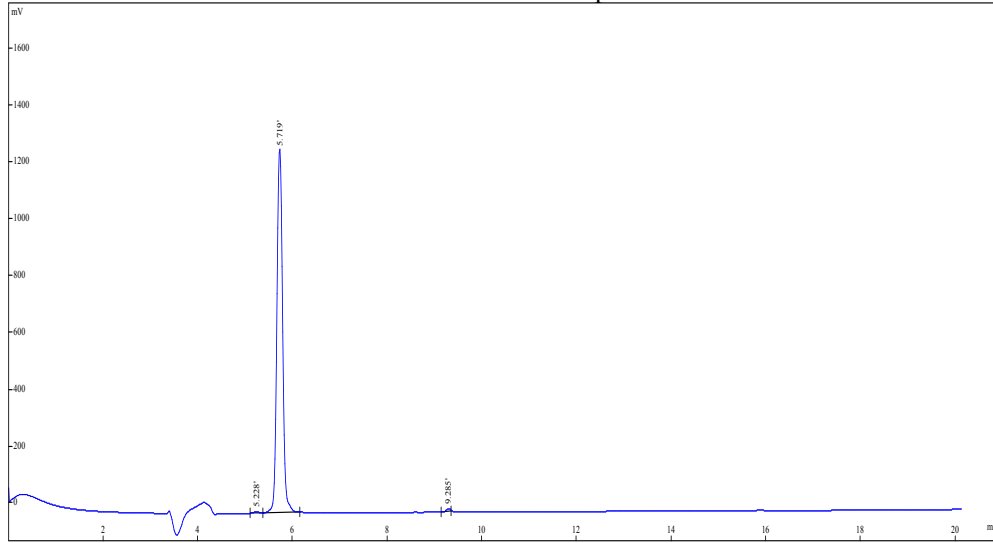
MS Report

MW: 1282.38



Peptide 6

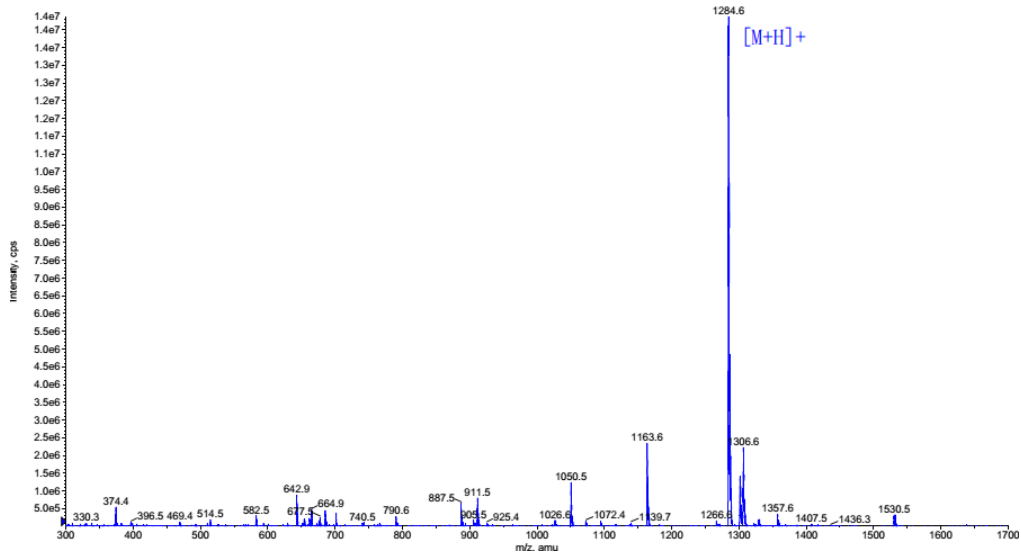
HPLC Report



Rank	Time	Name	Conc.	Area
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2	5.719		99.18	10778483
3	9.285		0.4936	53646
Total			100	10868400

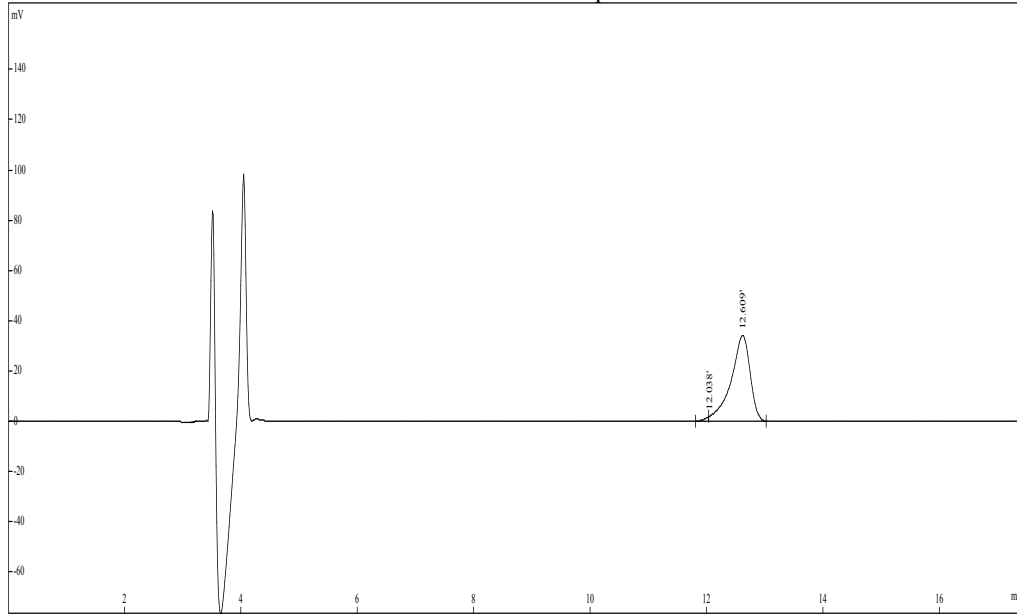
MS Report

MW: 1284.38



Peptide 7

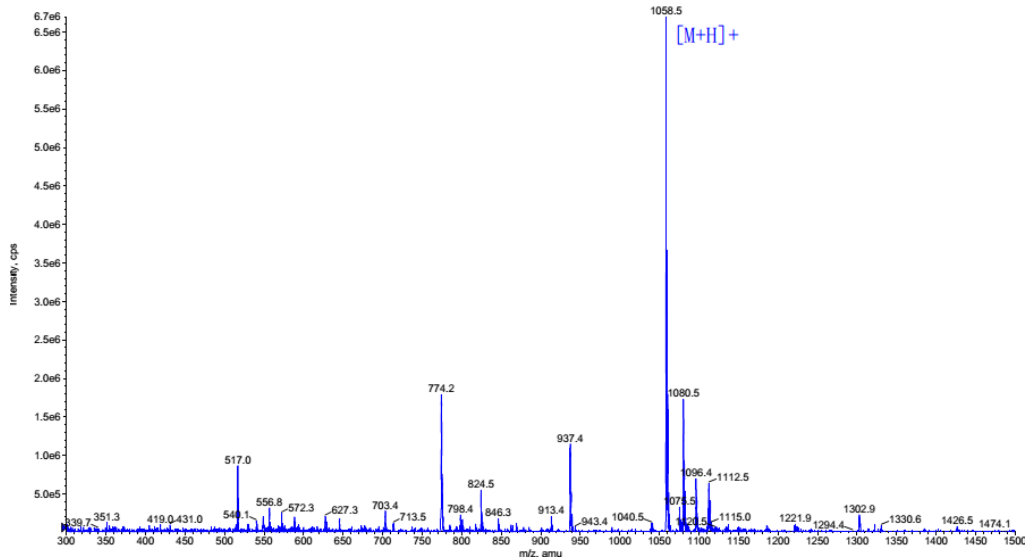
HPLC Report



Time	Quantity	Area	Height	Resolut.	T.PlateNum	Asymmetry
12.038	1.412	11203	2046	1.88	112778	
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	100	793463	36339			

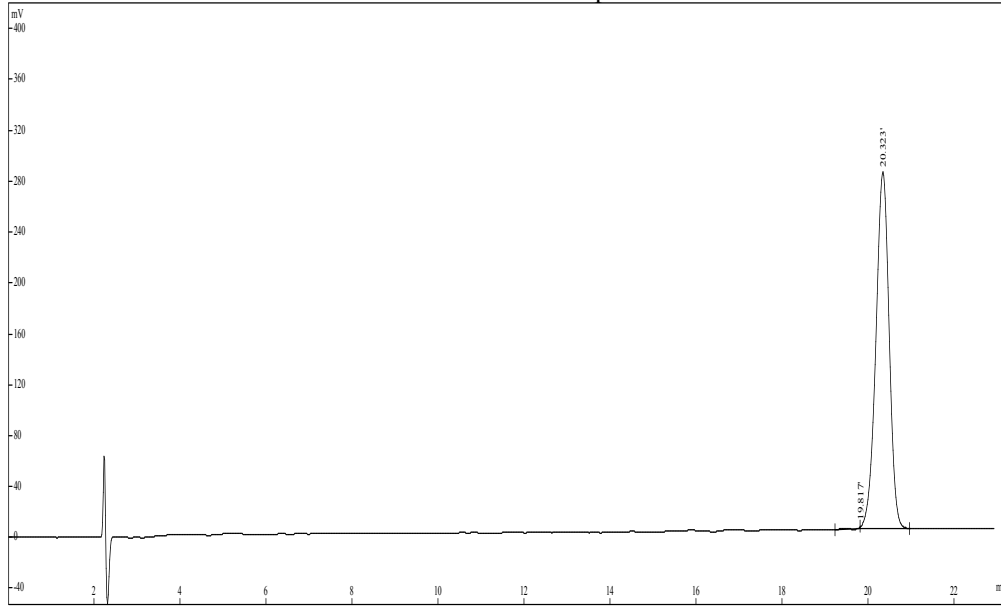
MS Report

MW: 1056.06



Peptide 8

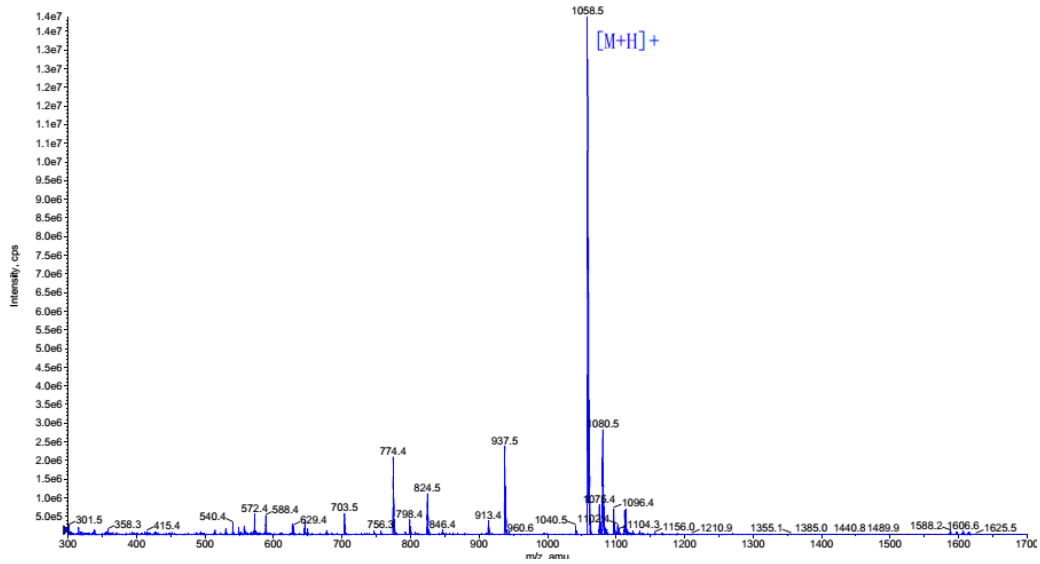
HPLC Report



Time	Quantity	Area	Height	Resolut.	T.PlateNum	Asymmetry
19.817	0.1771	10667	2315	1.77	431569	
20.323	99.82	6013002	280383	0.00	20954	0.94
	100	6023669	282698			

MS Report

MW: 1058.06



References:

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