Supplementary Material

Exploiting the P-1 pocket of BRCT domains toward a structure guided inhibitor design

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Methods

Determination of K_d values from fluorescence polarization assay

The indicated amounts of His-BRCT was added to the wells of black 384-well, low-volume, v-bottom microplates in a final reaction volume of 20 µL, then the fluorescein or TMR-labeled peptides were added to the wells. The plates were shaked on the plate reader for 1 min before reading. Anisotropy values, automatically calculated by the SoftMax Pro software, were measured using the fluorescence polarization function of the Spectramax M5 (Molecular Devices) plate reader. For binding affinity analysis, a percent bound value was calculated at each protein concentration using the following equation: (anisotropy change/max anisotropy change)*100. After plotting percent probe bound versus protein concentration, an approximate Kd (dissociation constant) was determined as the receptor concentration at which 50% of the probe was bound by using SigmaPlot 11.

Determination of K_i and IC_{50} values from competitive fluorescence polarization assay

For the competition experiments, the indicated amounts of unlabeled peptides (1-4) were added to the 384-well plates. Then the mixture of His-BRCT protein and probes (Flu-short, Flu-long or TMR-long) were added to the wells. The plates were shaked on the plate reader for 1 min before reading. The IC_{50} were calculated using SigmaPlot and K_i values were determined by different equations (Coleska-Wang, Cheng-Prussof,

Kenakin, Huang, Munson-Rodbard, and Roehrl equations). For the competition experiments of peptides (1-15), the K_i values were determined by Coleska-Wang equation. All of the data represent the average of at least three independent experiments ± SEM.

Peptide synthesis and purification

The peptides were synthesized using standard Fmoc-chemistry either in house or by the Tufts peptides core. The peptides were synthesized on Rink Amide NovaGel^{TM} resin (0.25 mmol) (EMD) using N-α-Fmoc-protected amino acids (EMD) or unnatural N-α-Fmoc protected amino acids (3B Scientific Corporation or Fischer Scientific) and TBTU-HOBt coupling chemistry on a Focus XC synthesizer (Aapptec). Fmoc-acid (5eq) and TBTU/HOBt (4eq) (Chem-impex international, INC) were dissolved in 2-3 mL of NMP. DIEA (Sigma) (15 eq) was added to mixture and incubated for 5 min. This mixture was then added to Fmoc-deprotected peptide resin and allowed to couple for 1 h. Each coupling step was monitored using the Kaiser test (Sigma). To avoid derivatives with deletion, after the coupling step the N-terminal extremities were capped with a 5% acetic anhydride (Sigma), 5% DIEA, 5% HOBt, and 85% NMP. After each coupling and deprotection steps, the resin was thoroughly washed with DMF, MeOH and DCM. At the end of the synthesis, the N-terminus of the desired peptide was acetylated as described above. The peptides were then cleaved from the resin using trifluoracetic acid (TFA) (Sigma)/TIS (Sigma)/water (95:2.5:2.5) over a 3 h period. The crude peptides were precipitated in cold ether and air-dried overnight.

Purification was performed on a preparative Agilent LC system (Agilent Technologies) using an Agilent C18 reverse-phase column Zobrax 300SB-C18 (21.2X150 mm, 5 micron). Buffer A was water with 0.05% TFA and buffer B was acetonitrile with 0.05% TFA. Gradient was buffer B from 5 to 40% in 20 min then 40 to 100% in 5 min at 20 ml/min flow rate. The peptide fractions were lyophilized on a sharp freeze -110 (Aapptec). The purity of the peptides were determined by HPLC analysis with a Agilent C18 reverse phase column (4.6X50 mm, 3.5 micron) with similar buffers but a gradient from 5 to 50 B in 20 min and a gradient from 50 to 100 B in 5 min with a 1 ml/min flow rate. Electrospray mass spectrometry was carried out on an Agilent HPLC-MS system.

Peptides were dissolved in water or DMSO to make a ~10 mM stock solution and aliquoted.

Table S1. Models to determine K_i values from FP studies. [1-6]

	Equation	Description
Coleska-Wang	$K_i = [I]_{50}/(\frac{[L]_{50}}{K_d} + \frac{[P]_0}{K_d} + 1)$	$[I]_{50}$, the concentration of the free inhibitor at 50% inhibition $[L]_{50}$, the concentration of the free labeled ligand at 50% inhibition $[P]_0$, the concentration of the free protein at 0% inhibition K_d, the dissociation constant of the protein-ligand complex
Cheng-Prusoff	$K_i = \frac{IC_{50}}{1 + [L]/K_d}$	IC_{50}, the concentration of the free inhibitor at 50% inhibition $[L]$, free ligand concentration K_d, the dissociation constant of the protein-ligand complex
Kenakin	$K_i = \frac{L_b IC_{50} K_d}{L_0 R_0 + L_b(-R_0 L_0 + L_b K_d)}$	IC_{50}, the concentration of the free inhibitor at 50% inhibition K_d, the dissociation constant of the protein-ligand complex R_o , the total protein concentration L_o , the total ligand concentration L_b, the bound ligand concentration
Huang	$K_i = \frac{IC_{50}}{\frac{1}{1-F_0} + \frac{L_0(2-F_0)}{2K_d}} - K_d(\frac{F_0}{2-F_0})$	L_0, total concentration of the probe F_0, the fraction of ligand bound over the total ligand K_d, the dissociation constant of the protein-ligand complex IC_{50}, the concentration of the free inhibitor at 50% inhibition
Munson-Rodbard	$K_i = \frac{IC_{50}}{1 + \frac{L_T(y_0+2)}{2 \times K_d(y_0+1)} + y_0} - K_d(\frac{y_0}{y_0+2})$	IC_{50}, the concentration of the free inhibitor at 50% inhibition y_0, initial bound to free ratio for the labeled probe before perturbation of equilibrium by the added inhibitor L_T, total concentration of the labeled probe K_d, the dissociation constant of the protein-ligand complex
Roehrl-Wagner	$K_i = \frac{(IC_{50} - A)K_d X}{A}$ $A = R_T - (L_{ST}X)/(X+1) - K_C X$ $X = \frac{A_{obs} - A_{min}}{A_{max} - A_{obs}}$	IC_{50}, the concentration of inhibitor that reduces binding of the labeled probe by 50% K_d, the dissociation constant of the protein-ligand complex A_{obs}, the observed anisotropy at a particular concentration of a compound (C) A_{min}, the minimum anisotropy A_{max}, the maximum anisotropy, L_{ST}, total concentration of His-BRCT R_T, total concentration of probes

Table S2. K_i values of peptides **1-4** determined by various models.

	Peptide 1			Peptide 2			Peptide 3			Peptide 4		
	Flu-short	Flu-long	TMR-long	Flu-short	Flu-long	TMR-long	Flu-short	Flu-long	TMR-long	Flu-short	Flu-long	TMR-long
Coleska-Wang	1.07± 0.07	-13.73±0.39	1.85±0.06	-0.07±0.03	0.07±0.01	0.27±0.01	0.84±0.06	1.03±0.04	1.96±0.09	-0.57±0.04	-0.05±0.01	0.20±0.01
Cheng-Prusoff	3.18±0.12	3.63±0.10	10.82±0.36	1.12±0.06	0.83±0.05	1.93±0.05	2.76±0.11	4.12±0.14	11.39±0.49	0.21±0.07	0.40±0.05	1.52±0.05
Kenakin	-21.68±0.83	-13.73±0.39	-13.59±0.45	-7.64±0.41	-3.15±0.21	-2.43±0.06	-18.81±0.75	-15.62±0.54	-14.31±0.62	-1.44±0.49	-1.50±0.19	-1.91±0.06
Huang	0.76±0.06	0.58±0.02	-1.83±0.06	-0.22±0.03	-0.01±0.01	-0.41±0.01	0.56±0.05	0.68±0.03	-1.92±0.08	-0.66±0.03	-0.11±0.01	-0.34±0.01
Munson-Rodbard	2.46±0.12	-0.18±0.03	21.63±0.72	0.38±0.06	-0.87±0.01	3.78±0.09	2.04±0.11	-0.05±0.04	22.78±0.99	-0.55±0.07	-0.98±0.01	2.96±0.10
Roehrl-Wagner	0.44±0.10	-5.30±0.19	-6.52±0.22	8.91±2.50	-0.14±0.04	-1.04±0.03	0.95±0.14	-2.32±0.09	-6.88±0.30	0.81±0.21	0.14±0.03	-0.78±0.03

The peptides were screened with BRCT (2000 nM) and Flu-short (100 nM) or BRCT (1000 nM) with Flu-long and TMR-long (100 nM)

Flu-long = FITC-SRSTpSPTFNK
Flu-short = FITC-βA-pSPTF
TMR-long = TMR-SRSTpSPTFNK

Table S3. Computational study to explore the P-1 site on BRCT domains.

Relative Energies of BRCT bound peptides (kcal/mol)			
Protein	BRCA1	MDC1	TopBP1
	Peptide sequence		
Modification	pSPTF	pSQEY	pTPELY
none	0	0	0
14	-0.4	-0.16	-0.15
15	-0.91	-0.2	-0.53

Figure S1 Determination of K_d values from fluorescence polarization assay.

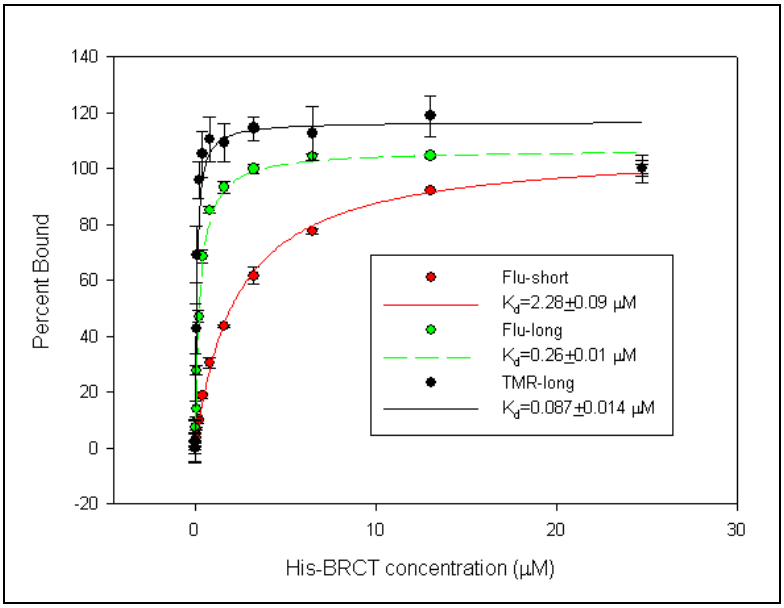


Figure S2. Determination of K_i values from competitive fluorescence polarization assay (peptide **1-4**).

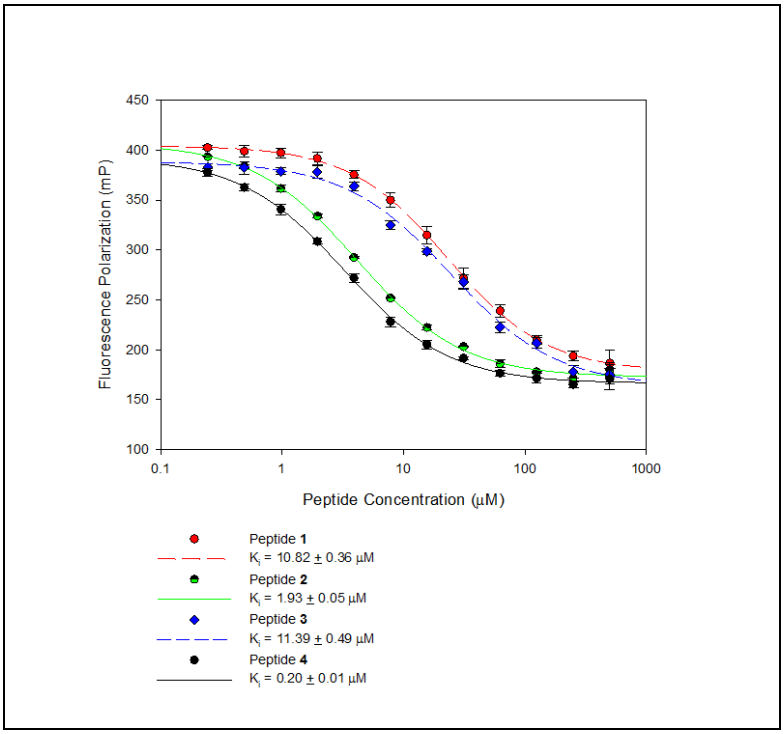


Figure S3. Determination of K_i values from competitive fluorescence polarization assay (peptide **5-10**).

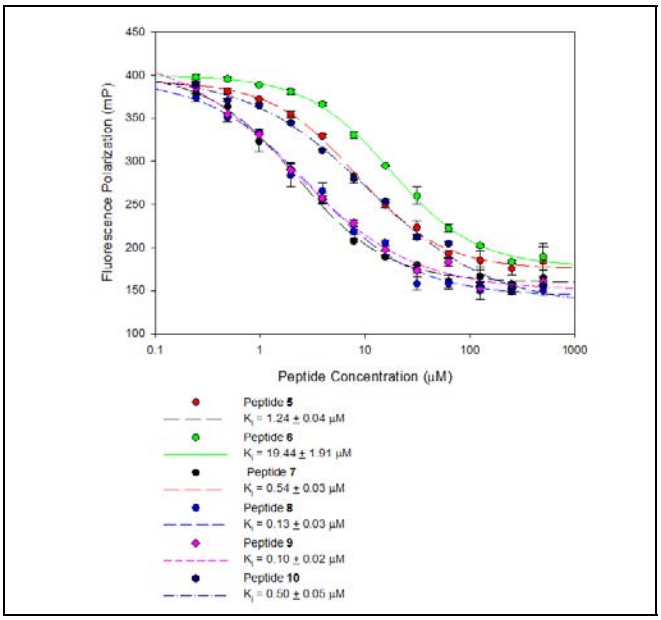


Figure S4. Determination of K_i values from competitive fluorescence polarization assay (peptide **11-15**).

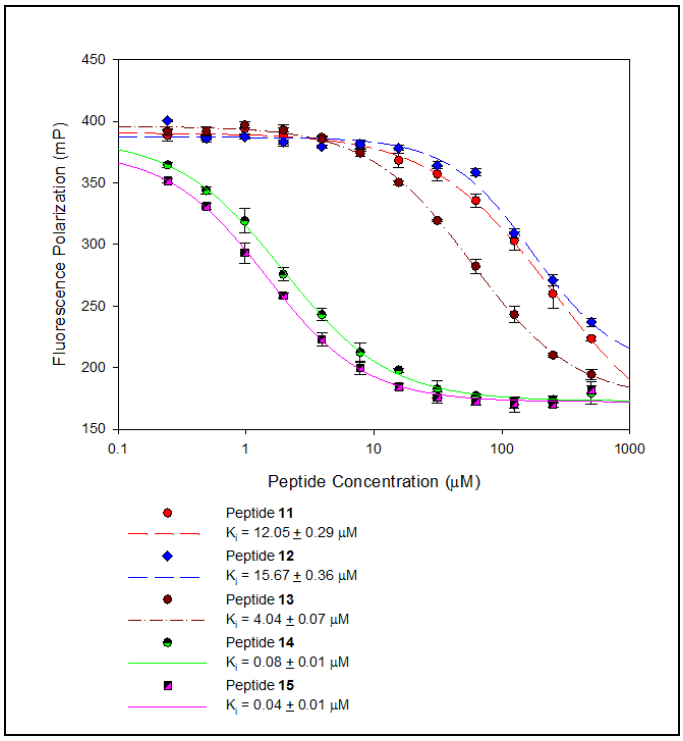


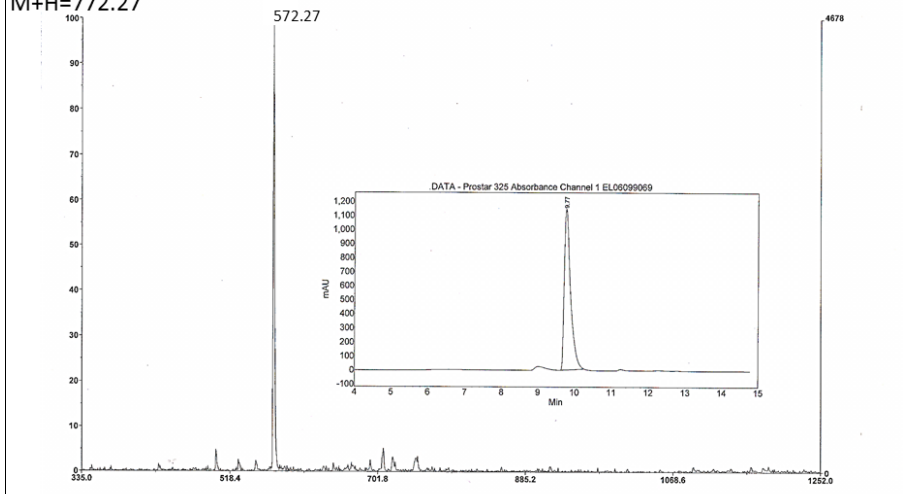
Figure S5. LC trace and mass spectrum of peptides.

Peptide **1**

$C_{23}H_{34}N_5O_{10}P_1$

M.Wt.=571.53

M+H=772.27

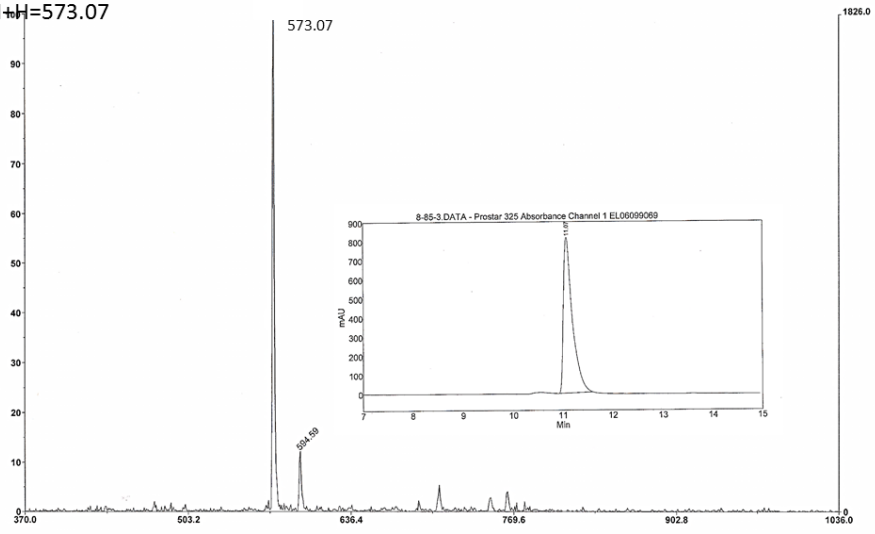


Peptide **2**

$C_{23}H_{33}N_4O_{11}P_1$

M.Wt.=572.51

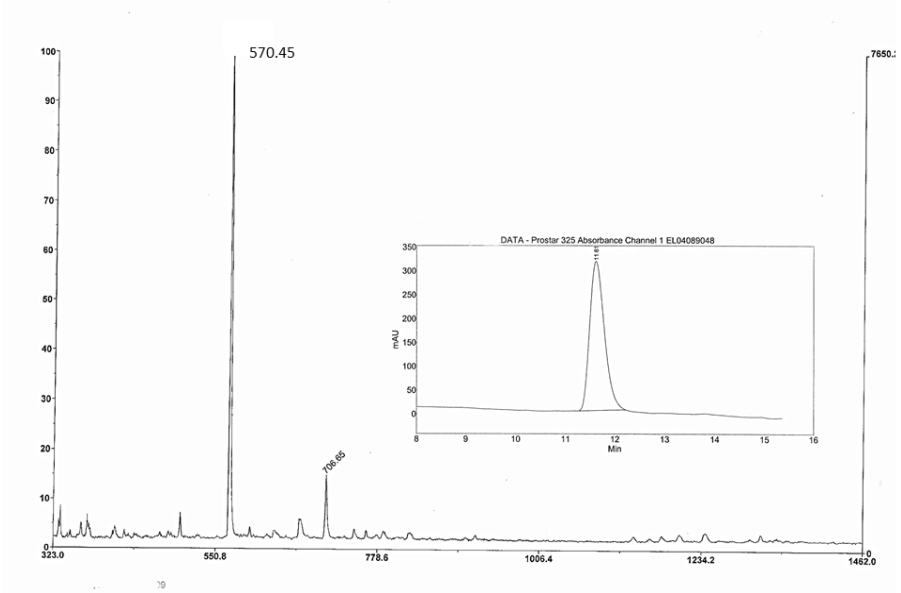
M+H=573.07



Peptide **3**

$C_{24}H_{35}N_4O_{10}P_1$

M.Wt.=570.54

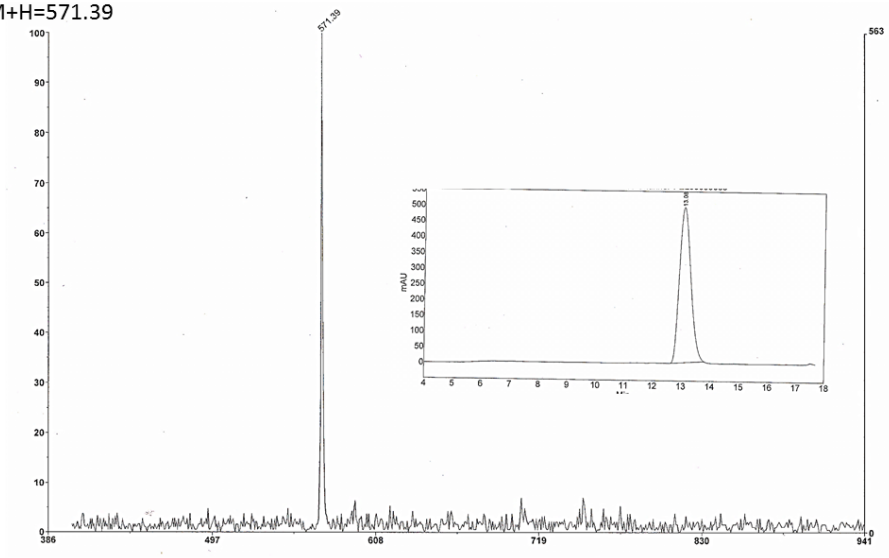


Peptide **4**

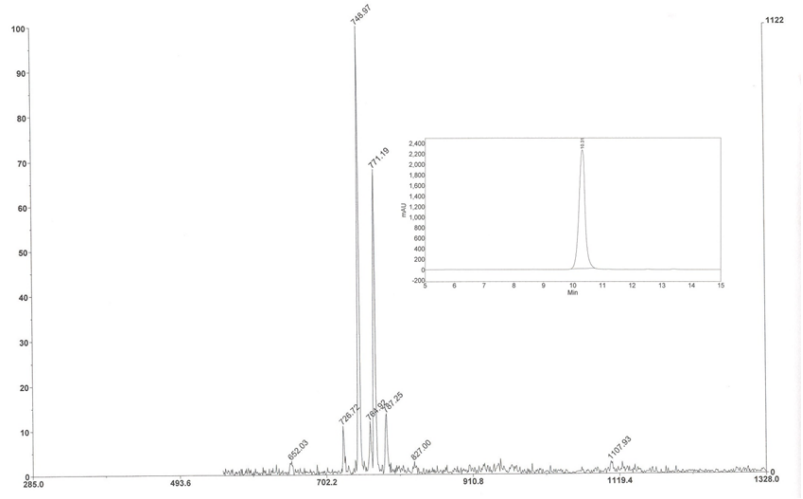
$C_{24}H_{35}N_4O_{10}P_1$

M.Wt.=570.53

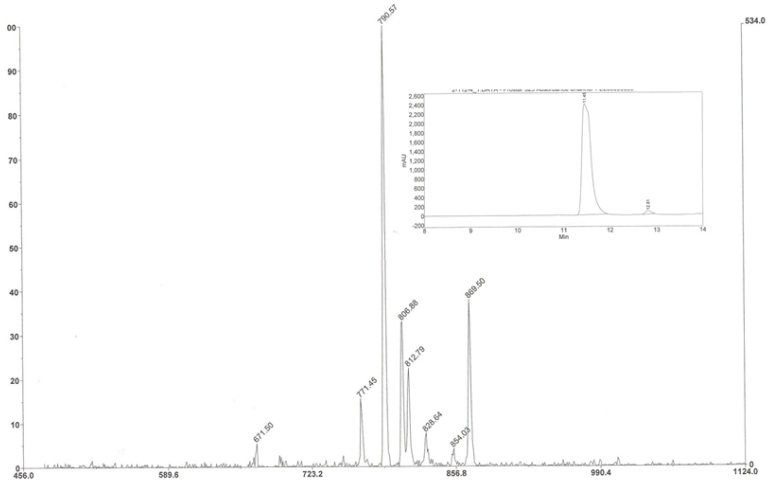
M+H=571.39



Peptide **5**
$C_{23}H_{34}N_5O_{10}P_1$
M.Wt.=770.53
M+H=771.19



Peptide **6**
$C_{23}H_{34}N_5O_{10}P_1$
M.Wt.=770.53
M+H=771.45

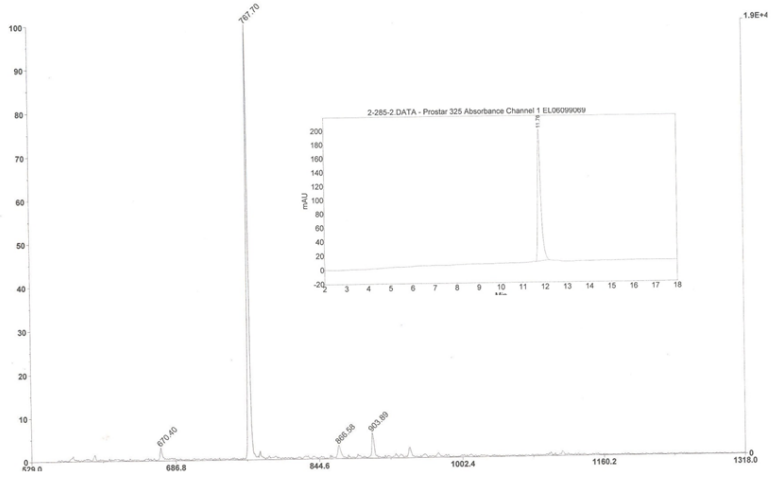


Peptide **7**

$C_{24}H_{36}N_5O_9P_1$

M.Wt.=766.55

M+H=767.70

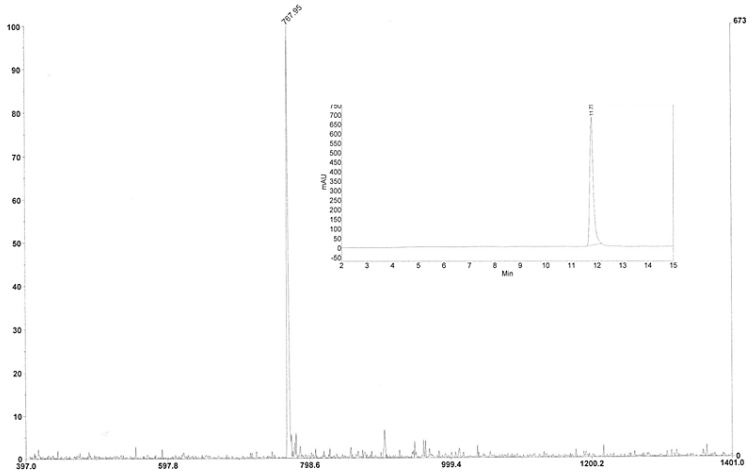


Peptide **8**

$C_{24}H_{35}N_4O_{10}P_1$

M.Wt.=767.54

M=767.95

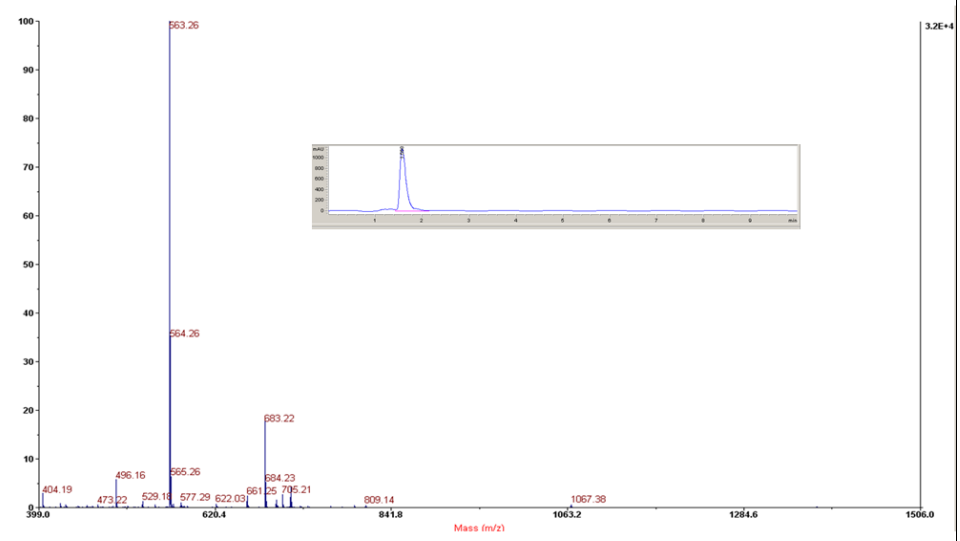


Peptide **9**

$C_{31}H_{41}N_4O_{10}P_1$

M.Wt.=660.65

M+Na=683.22

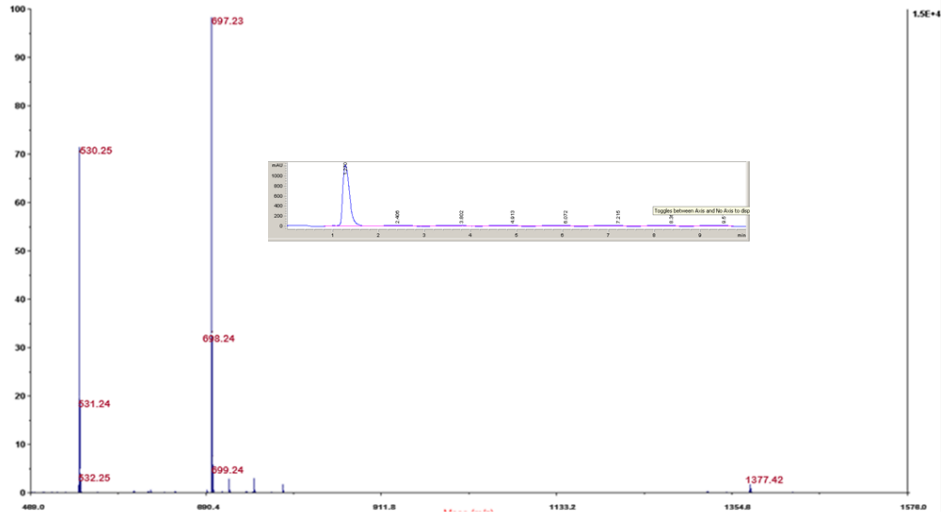


Peptide **10**

$C_{32}H_{43}N_4O_{10}P_1$

M.Wt.=674.68

M+Na=697.23

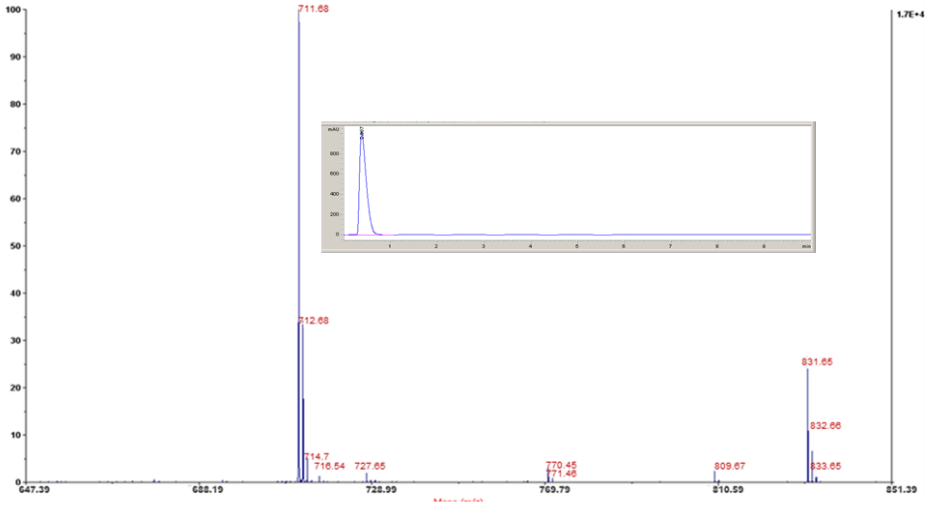


Peptide **11**

$C_{33}H_{45}N_4O_{10}P_1$

M.Wt.=688.70

M+Na=711.68

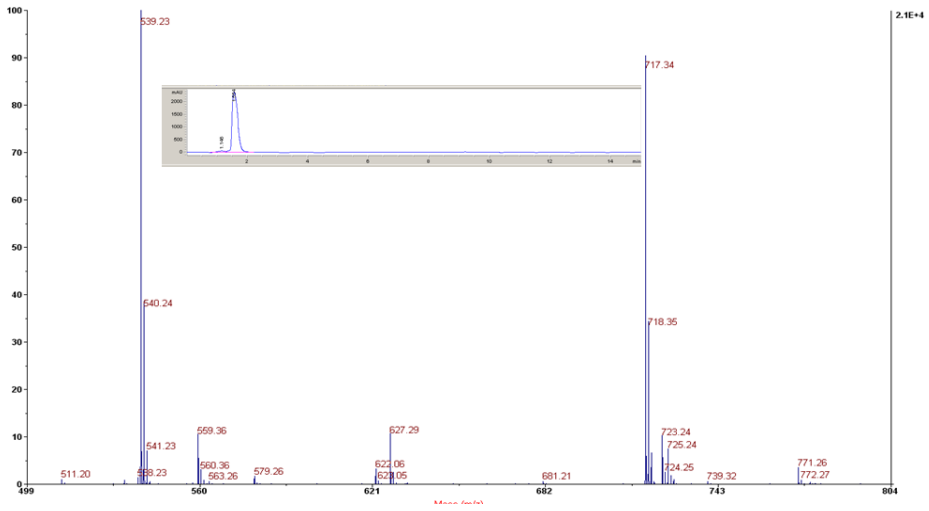


Peptide **12**

$C_{34}H_{41}N_4O_{10}P_1$

M.Wt.=696.68

M+Na-2H=717.34

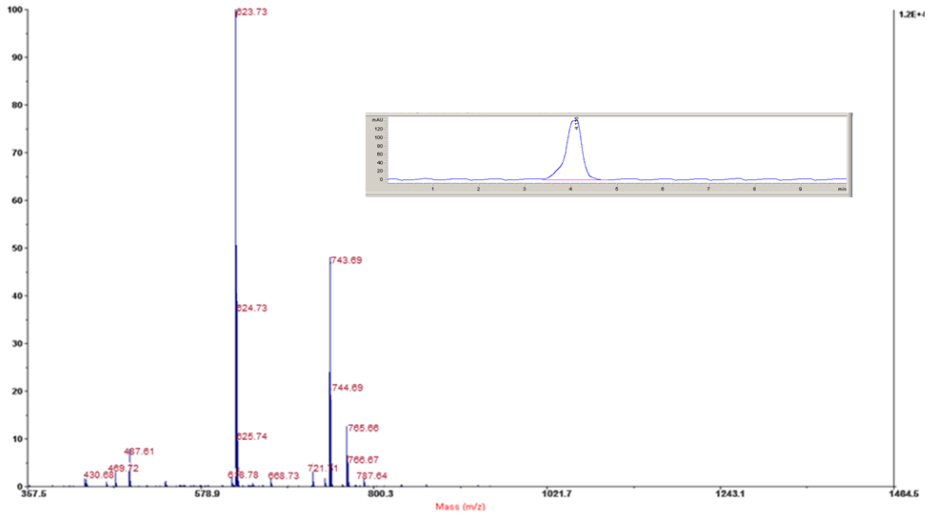


Peptide **13**

$C_{33}H_{45}N_4O_{12}P_1$

M.Wt.=720.70

M+Na=743.69

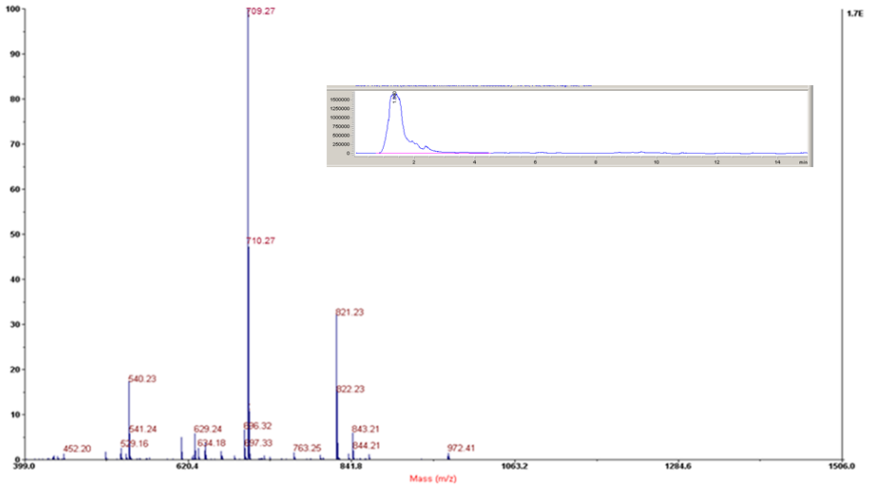


Peptide **14**

$C_{35}H_{41}N_4O_{10}P_1$

M.Wt.=708.69

M+H=709.27

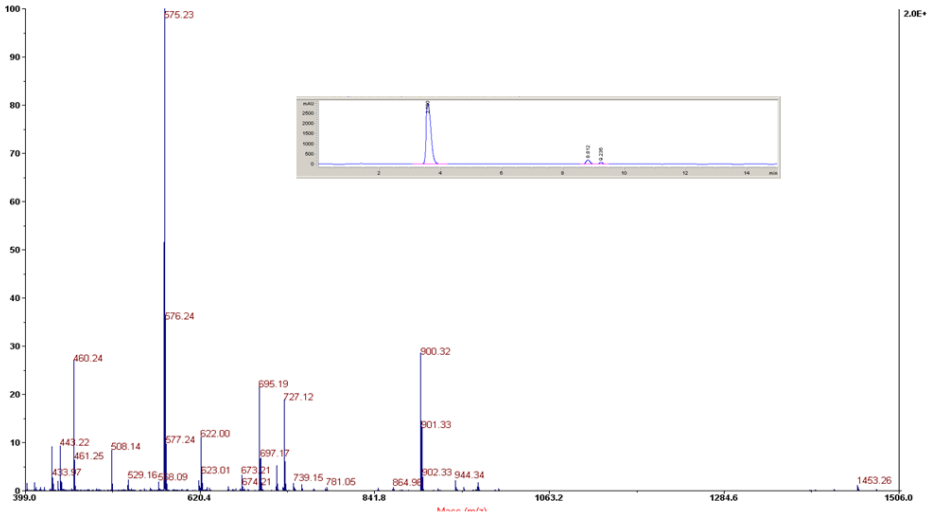


Peptide **15**

$C_{32}H_{41}N_4O_{10}P_1$

M.Wt.=672.66

M+Na=695.19



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