Electronic Supporting Information

A novel mass spectrometry-cleavable, phosphate-based enrichable and multi-targeting protein cross-linker

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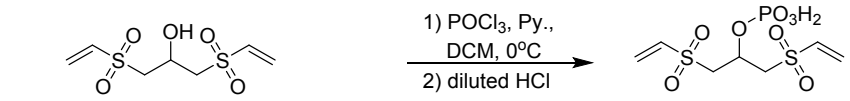
General Information

All chemical reagents were of analytical grade, obtained from commercial sources and used as supplied without further purification unless indicated.
NMR spectra were recorded on a Bruker-500 (500 MHz) instrument. The deuterated solvents employed were purchased from Energy Chemical. Chemical shifts were given in ppm with respect to referenced solvent peaks. Spectra were analyzed with MestReNova. High-resolution mass spectra (HRMS-ESI) were obtained on an ABsciex 4600 and Thermo fisher EASY1000-Fusion instruments. LC-MS^2 were recorded on Thermo fisher EASY1000-Fusion instrument. The maximum injection times for MS^1, MS^2 and MS^3 are 50ms, 100ms and 200ms respectively.

Experimental Procedures

Synthesis of 1,3-bis(vinylsulfonyl)propan-2-yl dihydrogen phosphate (pBVS)



1,3-bis(vinylsulfonyl)propan-2-ol (**BVS**) **pBVS**

A stirred solution of 1,3-bis(vinylsulfonyl)-2-propanol (**BVS**, 100 mg, 0.42 mmol) in dry DCM (5 mL) was cooled to 0 °C with ice-bath, phosphoryl trichloride (78 µL, 0.84 mmol) and pyridine (51 µL, 0.63 mmol) was then injected. After stirred at 0 °C to room temperature for 2 h, the reaction was quenched with water and adjusted to weak acidity with diluted HCl. The organic solvent was evaporated, and the resulting mixture was purified by RP-HPLC to afford white solid (8mg, 0.025 mmol), with 6% yield. **^{1}H NMR** (500 MHz, D_2O) δ 6.85 (dd, *J* = 16.5, 10.0 Hz, 2H), 6.33 (d, *J* = 16.5 Hz, 2H), 6.30 (d, *J* = 10.0 Hz, 2H), 4.93-4.87 (m, 1H)), 3.75 (d, *J* = 6.0 Hz, 4H) ppm. **^{13}C NMR** (126 MHz, MeOD) δ 135.37, 132.38, 65.29, 56.99 ppm. **ESI-HRMS** calcd for $C_{25}H_{34}N_7O_7S_3$ [$(M+H)^+$]: 320.9868, found:320.9815.

Cross-linking for BSA

Aqueous solution of **pBVS** (10 mM, 90 µL, 30 equiv) was added into 500 µL BSA in PBS (different pH), and the mixture was incubated at room temperature or 37 °C for 4 and 15 hours, respectively. Each sample was purified using a Pall™ Nanosep with 3k Omega 100/pk to remove excess reagents and the resulting mixture was characterized by UPLC−MS analysis

Protein Digestion

The above cross-linked protein was first precipitated using acetone to remove the excess crosslinking reagent, then digested using FASP method as described by Jacek R Winiewsk[1] with modifications. Briefly, the cross-linked protein was first loaded to a Nanosep filter (10 K, Pall corporation), then was washed twice with 200 uL 100 mM Tris-HCl (PH=8). The protein solution was reduced by adding 50 mM DTT in 100 mM Tris-HCl, and incubated at room temperature for two hours and then alkylated with iodoacetamide for 30 min in the dark. After reduction and alkylation, the filter was washed three times using 100 mM Tris-HCl. The protein on the filter was then digested using trypsin dissolved in 200 mM Tris-HCl at 37 °C for four hours. The protein: enzyme ratio is 50 : 1. The digestion solution was then acidified to pH = 2 to deactivate enzyme activity.

Enrichment of cross-linked peptides

The digested peptides were subsequently enriched by TiO_2 as described by Li-Rong Yu.[2] Briefly, the tryptic digested solution was diluted five times using loading buffer (1M glycolic acid, 0.5% TFA in 80% CH_3CN), then were incubated with 3 mg TiO_2 beads (GL science) at room temperature for 20 mins, the beads were washed once with loading buffer and twice with washing buffer (1% TFA in 80% CH_3CN) respectively. The peptides were eluted from TiO_2 bead by incubating with 240 ul 0.5% NH_4OH in 100 mM Tris-HCl for 6 min at room temperature. The peptides were acidified to pH = 2 immediately after the elution. The enriched peptides were then desalted using a C18 solid phase extraction column (Hypersep C18, 100 mg Thermo Scientific).

LC MS/MS analysis

The MS data was acquired on an Orbitrap Fusion MS coupled to a nano Easy LC 1000 and an Easyspray column (75 µm × 50 cm, PepMap RSLC C18 column, 2 µm, 100 Å, Thermo Scientific). The desalted peptides dissolved in 2% formic acid were loaded to a C18 trap column, then were separated on the Easy spray column, the peptides were eluted from the

Easy spray column using a gradient of 7 to 45% B (0.1% formic acid in CH_3CN) in 120 min at a flow rate of 250 nL/min. The column temperate was set to 50 °C.

The MS was operated in data dependent mode. CID-MS^2-MS^3 strategy was employed to identify cross-linked peptides. A survey scan at 120 K resolution was first conducted, then 10 most intense ions were selected (singly and doubly charged ions were excluded from the selection) for fragmentation using low collision energy dissociation (CE = 22).The MS^2 scan was performed in Orbitrap, the MS^2 resolution was set to 30 K. If a signature mass differences of 319.9789 or a signature mass differences of 222.0021 were observed, both the ion pairs were further fragmented by HCD-MS^3 with a collision energy of 30 in ion trap, the MS^3 scan speed was set to turbo. The target AGC of MS^1, MS^2 and MS^3 scan events were set to 4×10^5, 2×10^5 and 1×10^5 respectively.

Data analysis

The reagent in this method can target three different amino acids and generate three different mass tags. Previously reported software tools such as XlinkX are not applicable to the reagent reported in this study. Therefore, an in house written script was employed to do the data analysis. The raw MS^3 data were first searched against BSA sequence using Proteome discover 2.2 (Thermo Scientific), both the Sequest and MS Amanda search engine were used. The precursor mass tolerance was set to 20 ppm and fragment mass tolerance were set to 1.2 da. The following variable modifications were taken into consideration: Carbamidomethyl (C), pBVS(C/H/K), and pBVS-phos (pBVS with loss of one phosphate group). 1% FDR was used to filter away false positive hits. The psm (peptide spectra matches) results and MS/MS spectrum information were exported to .csv format. Linked scan (which scan3 is triggered by which scan2, also which scan2 is triggered by which scan1) information were generated by converting the raw MS data to a quantification file in .txt format using Raw quant software.[3] The three files obtained above were analyzed using an in house written R/R CPP script (https://github.com/wchenSHT/RCPPXLINK). The analysis is to assign each identified peptide chain sequence to its corresponding MS^1 precursor ion. If two different peptide chains were identified from the same precursor ion, and the mass error is within 10ppm, then the precursor ion is considered as a cross-linked peptide. All the results were further validated manually.

Table S1: Evaluation of the cross-linking efficiency in different reaction conditions.

Entry	Time (h)	Temperature	pH	Identified cross-linkers
1	4	r.t.	7.4	29
2	4	37 °C	7.4	36
7	15	r.t.	7.4	32
8	15	37 °C	7.4	40
9	15	37 °C	8.5	49

Table S2: The assignment of the fragment ions for crosslinked peptide pair (α-β, NECFLSHKDDSPDLPK and LCVLHEKTPVSEK) in Fig 2.

Index	m/z	Peptide sequence	Modification	Note
1	1111.424	NECFLSHKDDSPDLPK	pBVS linker	
2	1062.4364	NECFLSHKDDSPDLPK	pBVS linker-phos	
3	951.4368	NECFLSHKDDSPDLPK	None	
4	931.3991	LCVLHEKTPVSEK	pBVS linker	^{13}C monoisotopic peak

5	881.4131	LCVLHEKTPVSEK	pBVS linker-phos
6	770.4108	LCVLHEKTPVSEK	None

Table S3: The fragment ions assignment of α_{Linker} (mass error < 0.8 da) in Fig 3.

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	115.05020	58.02874	N			16
2	244.09280	122.55004	E	2107.80578	1054.40653	15
3	404.12345	202.56536	C-Carbamidomethyl	1978.76318	989.88523	14
4	551.19186	276.09957	F	1818.73253	909.86991	13
5	664.27592	332.64160	L	1671.66412	836.33570	12
6	751.30795	376.15761	S	1558.58006	779.79367	11
7	888.36686	444.68707	H	1471.54803	736.27765	10
8	1336.44073	668.72400	K-CHL-Plinker	1334.48912	667.74820	9
9	1451.46767	726.23747	D	886.41525	443.71126	8
10	1566.49461	783.75094	D	771.38831	386.19779	7
11	1653.52664	827.26696	S	656.36137	328.68432	6
12	1750.57940	875.79334	P	569.32934	285.16831	5
13	1865.60635	933.30681	D	472.27657	236.64193	4
14	1978.69041	989.84884	L	357.24963	179.12845	3
15	2075.74318	1038.37523	P	244.16557	122.58642	2
16			K	147.11280	74.06004	1

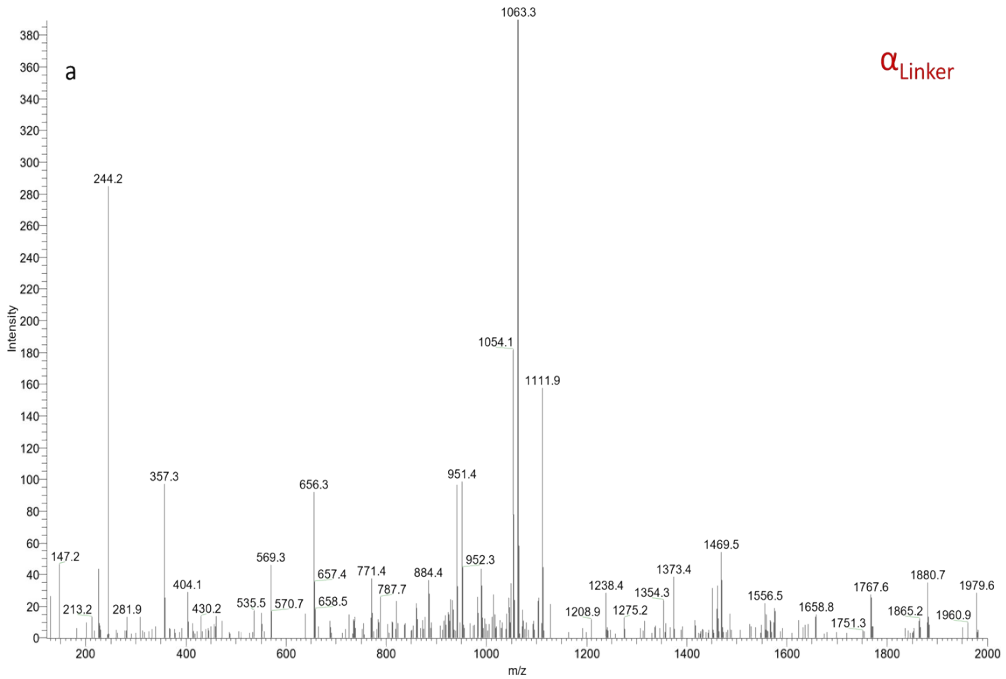


Figure S1. The ms/ms spectra of α_{Linker} in Fig 3.

Table S4: The fragment ions assignment of $\alpha_{Linker\text{-}phos}$ (mass error < 0.8 da) in Fig 3.

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	115.05020	58.02874	N			16
2	244.09280	122.55004	E	2009.82688	1005.41708	15
3	404.12345	202.56536	C-Carbamidomethyl	1880.78428	940.89578	14
4	551.19186	276.09957	F	1720.75363	860.88046	13
5	664.27592	332.64160	L	1573.68522	787.34625	12
6	751.30795	376.15761	S	1460.60116	730.80422	11
7	888.36686	444.68707	H	1373.56913	687.28820	10
8	1238.46183	619.73455	K-CHL-Plinker-P	1236.51022	618.75875	9
9	1353.48877	677.24802	D	886.41525	443.71126	8
10	1468.51571	734.76149	D	771.38831	386.19779	7
11	1555.54774	778.27751	S	656.36137	328.68432	6
12	1652.60050	826.80389	P	569.32934	285.16831	5
13	1767.62745	884.31736	D	472.27657	236.64193	4
14	1880.71151	940.85939	L	357.24963	179.12845	3
15	1977.76428	989.38578	P	244.16557	122.58642	2
16			K	147.11280	74.06004	1

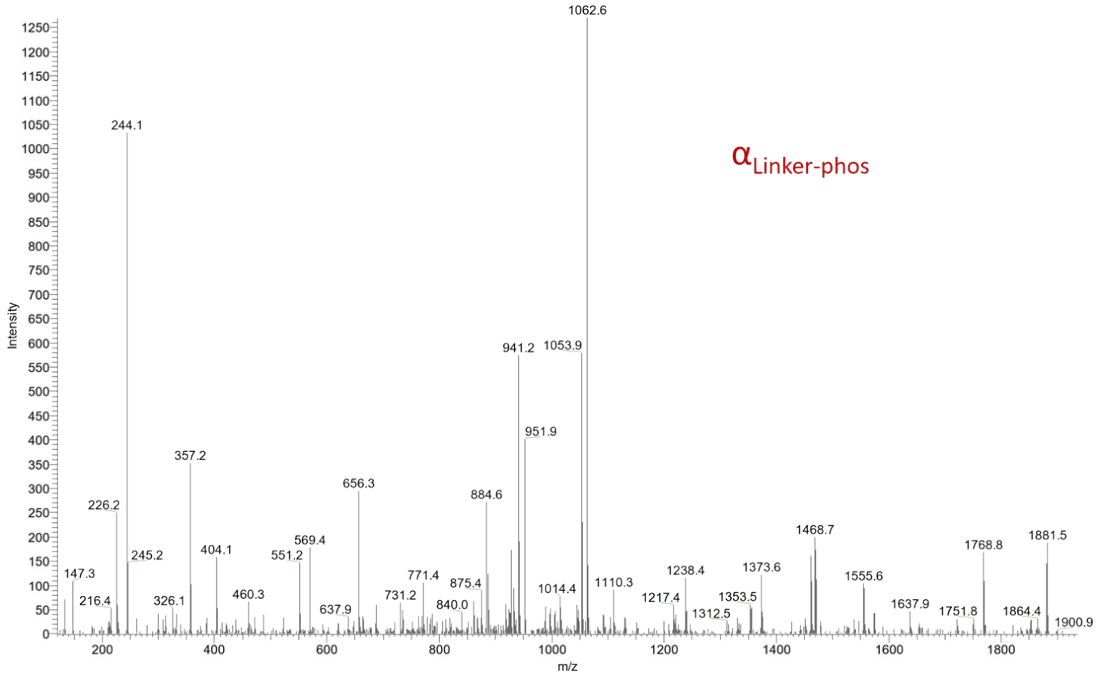


Figure S2. The ms/ms spectra of $\alpha_{Linker\text{-}phos}$ in Fig 3.

Table S5: The fragment ions assignment of α (mass error < 0.8 da) in Fig 3.

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2

1	115.05020	58.02874	N			16
2	244.09280	122.55004	E	1787.82688	894.41708	15
3	404.12345	202.56536	C Carbamidomethyl	1658.78428	829.89578	14
4	551.19186	276.09957	F	1498.75363	749.88046	13
5	664.27592	332.64160	L	1351.68522	676.34625	12
6	751.30795	376.15761	S	1238.60116	619.80422	11
7	888.36686	444.68707	H	1151.56913	576.28820	10
8	1016.46183	508.73455	K	1014.51022	507.75875	9
9	1131.48877	566.24802	D	886.41525	443.71126	8
10	1246.51571	623.76149	D	771.38831	386.19779	7
11	1333.54774	667.27751	S	656.36137	328.68432	6
12	1430.60050	715.80389	P	569.32934	285.16831	5
13	1545.62745	773.31736	D	472.27657	236.64193	4
14	1658.71151	829.85939	L	357.24963	179.12845	3
15	1755.76428	878.38578	P	244.16557	122.58642	2
16			K	147.11280	74.06004	1

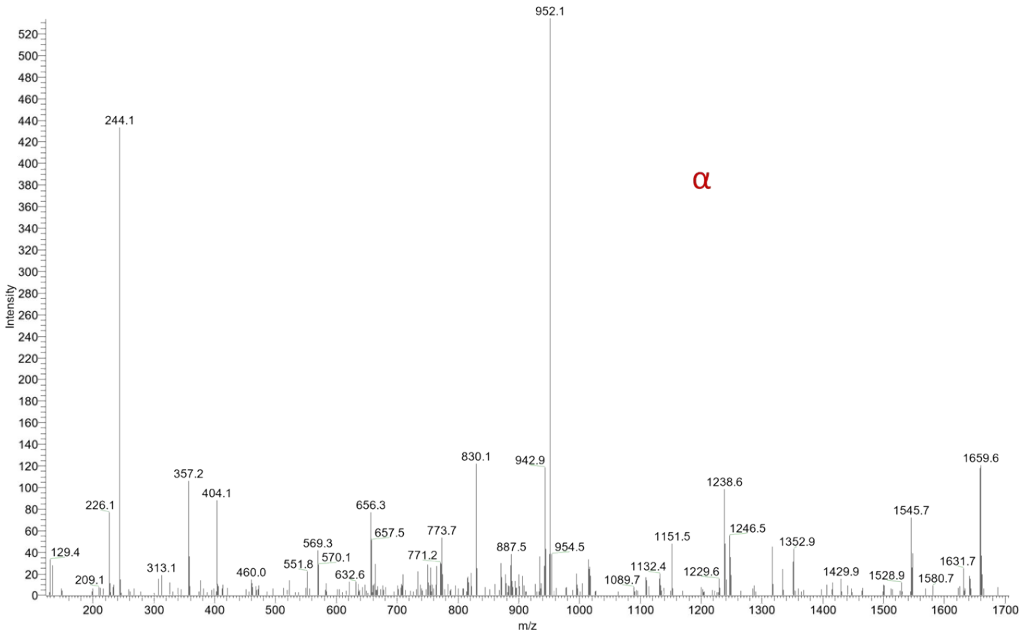


Figure S3. The ms/ms spectra of α in Fig 3.

Table S6: The fragment ions assignment of β_{Linker} (mass error < 0.8 da) in Fig 3.

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	114.09134	57.54931	L			13
2	274.12199	137.56463	C-Carbamidomethyl	1746.71478	873.86103	12
3	373.19040	187.09884	V	1586.68413	793.84570	11
4	486.27447	243.64087	L	1487.61571	744.31150	10
5	623.33338	312.17033	H	1374.53165	687.76946	9
6	752.37597	376.69162	E	1237.47274	619.24001	8
7	1200.44983	600.72856	K-CHL-Plinker	1108.43014	554.71871	7
8	1301.49751	651.25239	T	660.35628	330.68178	6
9	1398.55028	699.77878	P	559.30860	280.15794	5
10	1497.61869	749.31298	V	462.25584	231.63156	4
11	1584.65072	792.82900	S	363.18743	182.09735	3
12	1713.69331	857.35029	E	276.15540	138.58134	2
13			K	147.11280	74.06004	1

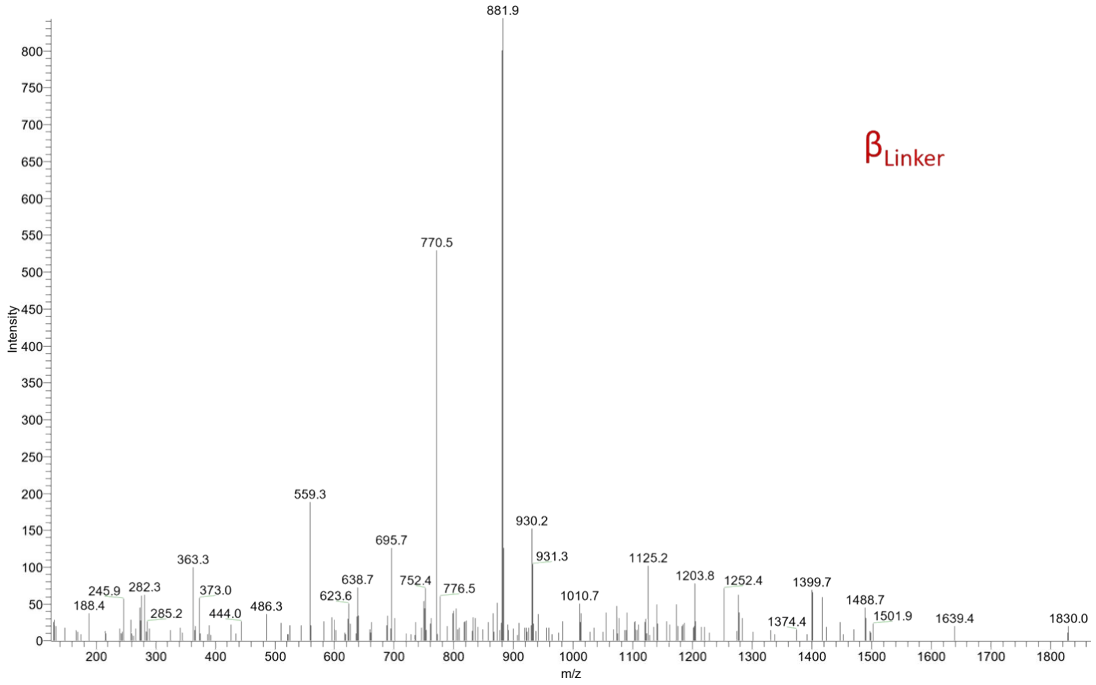


Figure S4. The ms/ms spectra of β_{Linker} in Fig 3.

Table S7: The fragment ions assignment of $\beta_{Linker\text{-}phos}$ (mass error < 0.8 da) in Fig 3.

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	114.09134	57.54931	L			13
2	274.12199	137.56463	C	1648.73588	824.87158	12

			Carbamidomethyl			
3	373.19040	187.09884	V	1488.70523	744.85625	11
4	486.27447	243.64087	L	1389.63681	695.32205	10
5	623.33338	312.17033	H	1276.55275	638.78001	9
6	752.37597	376.69162	E	1139.49384	570.25056	8
7	1102.47093	551.73911	K Plinker-P	1010.45124	505.72926	7
8	1203.51861	602.26294	T	660.35628	330.68178	6
9	1300.57138	650.78933	P	559.30860	280.15794	5
10	1399.63979	700.32353	V	462.25584	231.63156	4
11	1486.67182	743.83955	S	363.18743	182.09735	3
12	1615.71441	808.36084	E	276.15540	138.58134	2
13			K	147.11280	74.06004	1

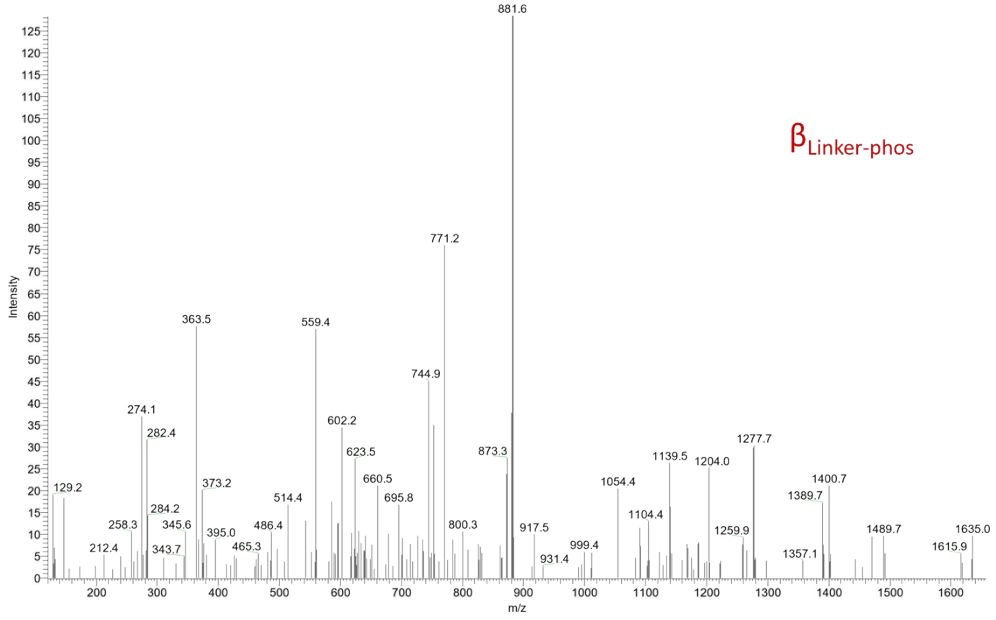


Figure S5. The ms/ms spectra of $\beta_{Linker\text{-}phos}$ in Fig 3.

Table S8: The fragment ions assignment of β (mass error < 0.8 da) in Fig 3.

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	114.09134	57.54931	L			13

2	274.12199	137.56463	C Carbamidomethyl	1426.73588	713.87158	12
3	373.19040	187.09884	V	1266.70523	633.85625	11
4	486.27447	243.64087	L	1167.63681	584.32205	10
5	623.33338	312.17033	H	1054.55275	527.78001	9
6	752.37597	376.69162	E	917.49384	459.25056	8
7	880.47093	440.73911	K	788.45124	394.72926	7
8	981.51861	491.26294	T	660.35628	330.68178	6
9	1078.57138	539.78933	P	559.30860	280.15794	5
10	1177.63979	589.32353	V	462.25584	231.63156	4
11	1264.67182	632.83955	S	363.18743	182.09735	3
12	1393.71441	697.36084	E	276.15540	138.58134	2
13			K	147.11280	74.06004	1

Figure S6. The ms/ms spectra of β in Fig 3.

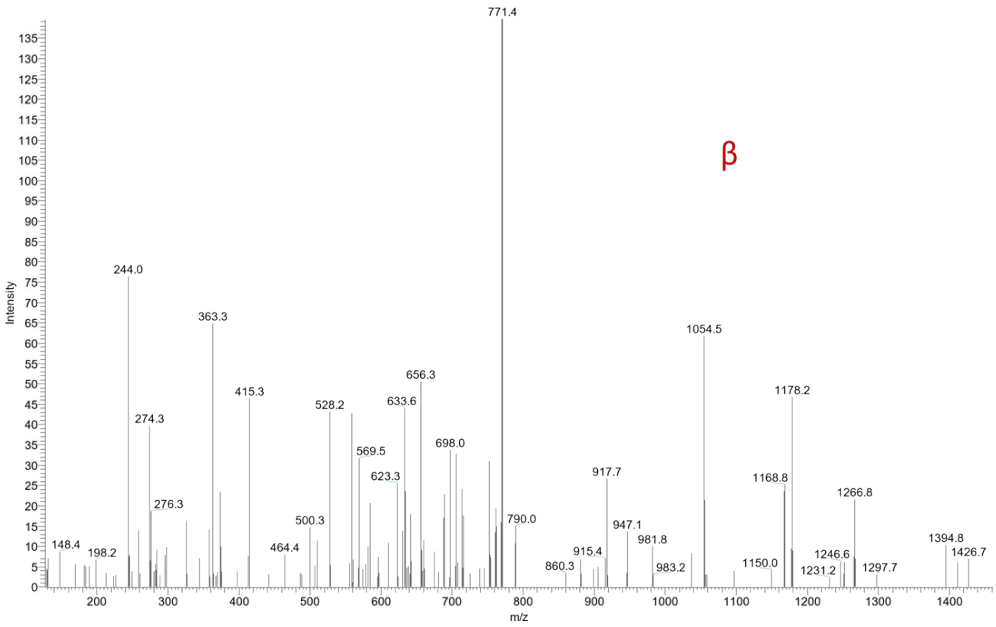


Table S9: The fragment ions assignment of the ion YLQQCPFDEHVK+pBVS (mass error < 0.8 da).

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	164.07061	82.53894	Y			12

2	277.15467	139.08097	L	1663.62015	832.31371	11
3	405.21325	203.11026	Q	1550.53608	775.77168	10
4	533.27182	267.13955	Q	1422.47751	711.74239	9
5	956.25991	478.63359	C Plinker	1294.41893	647.71310	8
6	1053.31267	527.15997	P	871.43084	436.21906	7
7	1200.38109	600.69418	F	774.37808	387.69268	6
8	1315.40803	658.20765	D	627.30967	314.15847	5
9	1444.45062	722.72895	E	512.28272	256.64500	4
10	1581.50953	791.25841	H	383.24013	192.12370	3
11	1680.57795	840.79261	V	246.18122	123.59425	2
12			K	147.11280	74.06004	1

Table s10: The fragment ions assignment of the ions LFTFHADICTLPDTEK+pBVS (mass error < 0.8 da).

#1	b^+	b^{2+}	b^{3+}	Seq.	y^+	y^{2+}	y^{3+}	#2
1	114.09134	57.54931	38.70196	L				16
2	261.15975	131.08352	87.72477	F	2114.81561	1057.91144	705.61006	15
3	362.20743	181.60735	121.40733	T	1967.74720	984.37724	656.58725	14
4	509.27585	255.14156	170.43013	F	1866.69952	933.85340	622.90469	13
5	966.31366	483.66047	322.77607	H Plinker	1719.63111	860.31919	573.88189	12
6	1037.35077	519.17902	346.45511	A	1262.59329	631.80029	421.53595	11
7	1152.37772	576.69250	384.79742	D	1191.55618	596.28173	397.85691	10
8	1265.46178	633.23453	422.49211	I	1076.52924	538.76826	359.51460	9
9	1425.49243	713.24985	475.83566	C Carbamidomethyl	963.44517	482.22622	321.81991	8
10	1526.54011	763.77369	509.51822	T	803.41452	402.21090	268.47636	7
11	1639.62417	820.31572	547.21291	L	702.36685	351.68706	234.79380	6
12	1736.67693	868.84211	579.56383	P	589.28278	295.14503	197.09911	5

13	1851.70388	926.35558	617.90614	D	492.23002	246.61865	164.74819	4
14	1952.75156	976.87942	651.58870	T	377.20308	189.10518	126.40588	3
15	2081.79415	1041.40071	694.60290	E	276.15540	138.58134	92.72332	2
16				K	147.11280	74.06004	49.70912	1

Table S11: The fragment ions assignment of the ions FWGKYLYEIAR+pBVS (mass error < 0.8 da).

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	148.07569	74.54148	F			11
2	334.15500	167.58114	W	1520.68918	760.84823	10
3	391.17647	196.09187	G	1334.60987	667.80857	9
4	741.27143	371.13935	K Plinker-P	1277.58841	639.29784	8
5	904.33476	452.67102	Y	927.49344	464.25036	7
6	1017.41882	509.21305	L	764.43012	382.71870	6
7	1180.48215	590.74471	Y	651.34605	326.17666	5
8	1309.52474	655.26601	E	488.28272	244.64500	4
9	1422.60881	711.80804	I	359.24013	180.12370	3
10	1493.64592	747.32660	A	246.15607	123.58167	2
11			R	175.11895	88.06311	1

Table S12: Unique cross-linked linkages identified of BSA with enrichment.

No.	Cross-linking site	Cross-linked peptides(cross-linked residues in red)	Cα-Cα distance (Å)
1	BSA^{239}-BSA^{246}	LVTDLTKVHK-ECCHGDLLECADDR	10.51
2	BSA^{242}-BSA^{211}	VHKECCHGDLLECADDR-ALKAWSVAR	10.612
3	BSA^{465}-BSA^{106}	LCVLHEKTPVSEK-NECFLSHKDDSPDLPK	10.942
4	BSA^{261}-BSA^{280}	ADLAKYICDNQDTISSK-ECCDKPLLEKSH	11.232
5	BSA^{159}-BSA^{287}	FYAPELLYYANKYNGVFQECCQAEDK-SHCIAEVEK	11.749
6	BSA^{509}-BSA^{524}	HADICTLPDTEK-KQTALVELLK	12.053
7	BSA^{12}-BSA^{4}	FKDLGEEHFK-DTHKSEIAHR	12.655
8	BSA^{114}-BSA^{145}	DDSPDLPKLKPDPN-RHPYFYAPELLYYANK	12.964

9	BSA^{337}-BSA^{377}	HPEYAVSVLLR-LKHLVDEPQNLIK	13.036
10	BSA^{439}-BSA^{431}	CCTKPESER-SLGKVGTR	13.451
11	BSA^{537}-BSA^{413}	HKPKATEEQLK-KVPQVSTPTLVEVSR	11.72
12	BSA^{211}-BSA^{350}	ALKAWSVAR-LAKEYEATLEECCAK	13.625
13	BSA^{378}-BSA^{350}	HLVDEPQNLIK-LAKEYEATLEECCAK	13.694
14	BSA^{377}-BSA^{350}	LKHLVDEPQNLIK-LAKEYEATLEECCAKD	13.698
15	BSA^{67}-BSA^{242}	SLHTLFGDELCK-VHKECCHGDLLECADDR	13.988
16	BSA^{287}-BSA^{273}	SHCIAEVEK-YICDNQDTISSKLK	14.067
17	BSA^{159}-BSA^{280}	ANKYNGVFQECCQAEDK-ECCDKPLLEK	14.218
18	BSA^{116}-BSA^{136}	LKPDPNTLCDEFK-FWGKYLYEIAR	14.431
19	BSA^{520}-BSA^{116}	HADICTLPDTEKQIK-LKPDPNTLCDEFK	15.555
20	BSA^{465}-BSA^{145}	LCVLHEKTPVSEK-RHPYFYAPELLYYANK	16.041
21	BSA^{239}-BSA^{67}	LVTDLTKVHK-SLHTLFGDELCK	16.256
22	BSA^{12}-BSA^{261}	FKDLGEEHFK-ADLAKYICDNQDTISSK	16.359
23	BSA^{350}-BSA^{204}	LAKEYEATLEECCAK-CASIQKFGER	17.006
24	BSA^{93}-BSA^{106}	ETYGDMADCCEKQEPER-NECFLSHKDDSPDLPK	17.186
25	BSA^{67}-BSA^{59}	SLHTLFGDELCK-TCVADESHAGCEK	17.618
26	BSA^{350}-BSA^{474}	LAKEYEATLEECCAK-VTKCCTESLVNR	18.065
27	BSA^{221}-BSA^{280}	LSQKFPK-LKECCDKPLLEK	19.2
28	BSA^{114}-BSA^{431}	DDSPDLPKLKPD-SLGKVGTR	19.228
29	BSA^{439}-BSA^{221}	CCTKPESER-LSQKFPK	20.205
30	BSA^{537}-BSA^{471}	HKPKATEEQLK-TPVSEKVTK	19.152
31	BSA^{59}-BSA^{76}	TCVADESHAGCEK-SLHTLFGDELCKVASLR	20.467
32	BSA^{114}-BSA^{509}	DDSPDLPKLKPDPNTLCDEFK-LFTFHADICTLPDTEK	20.774
33	BSA^{221}-BSA^{242}	LSQKFPK-VHKECCHGDLLECADDR	21.527
34	BSA^{471}-BSA^{350}	TPVSEKVTK-LAKEYEATLEECCAK	21.823
35	BSA^{180}-BSA^{280}	GACLLPKIETMR-ECCDKPLLEK	21.84
36	BSA^{12}-BSA^{287}	FKDLGEEHFK-SHCIAEVEK	22.111
37	BSA^{465}-BSA^{114}	LCVLHEKTPVSEK-DDSPDLPKLKPDPNTLCDEFK	22.241

38	BSA^{378}-BSA^{474}	HLVDEPQNLIK-VTKCCTESLVNR	23.797
39	BSA^{504}-BSA^{114}	AFDEKLFTFHADICTLPDTEK-DDSPDLPKLKPDPNTLCDEFK	24.405
40	BSA^{413}-BSA^{378}	KVPQVSTPTLVEVSR-HLVDEPQNLIK	25.408
41	BSA^{337}-BSA^{366}	RHPEYAVSVLLR-DDPHACYSTVFDK	25.409
42	BSA^{280}-BSA^{337}	LKECCDKPLLEK-RHPEYAVSVLLR	29.004
43	BSA^{127}-BSA^{431}	LKPDPNTLCDEFKADEK-SLGKVGTR	35.929
44	BSA^{375}-BSA^{378}	DDPHACYSTVFDKLK-HLVDEPQNLIK	5.827
45	BSA^{520}-BSA^{524}	HADICTLPDTEKQIK-KQTALVELLK	6.491
46	BSA^{524}-BSA^{59}	KQTALVELLK-TCVADESHAGCEK	60.529
47	BSA^{261}-BSA^{285}	ADLAKYICDNQDTISSK-ECCDKPLLEKSHCIAEVEK	9.165
48	BSA^{145}-BSA^{106}	RHPYFYAPELLYYANK-NECFLSHKDDSPDLPKLKPDPNTLCDEFK	9.253
49	BSA^{280}-BSA^{287}	ECCDKPLLEK-SHCIAEVEKDAIPENLPPLTADFAEDKDVCK	9.893

Table S13: Unique cross-linked linkages identified of BSA without enrichment.

No.	Cross-linking site	Cross-linked peptides(cross-linked residues in red)	Cα-Cα distance (Å)
1	BSA^{242}-BSA^{211}	VHKECCHGDLLECADDR-ALKAWSVAR	10.612
2	BSA^{159}-BSA^{287}	RHPYFYAPELLYYANKYNGVFQECCQAEDK-SHCIAEVEK	11.749
3	BSA^{509}-BSA^{524}	LFTFHADICTLPDTEK-KQTALVELLK	12.053
4	BSA^{12}-BSA^{4}	FKDLGEEHFK-DTHKSEIAHR	12.655
5	BSA^{337}-BSA^{377}	RHPEYAVSVLLR-LKHLVDEPQNLIK	13.036
6	BSA^{159}-BSA^{280}	RHPYFYAPELLYYANKYNGVFQECCQAEDK-LKECCDKPLLEK	14.218
7	BSA^{12}-BSA^{261}	FKDLGEEHFK-ADLAKYICDNQDTISSK	16.359
8	BSA^{350}-BSA^{474}	LAKEYEATLEECCAK-VTKCCTESLVNR	18.065
9	BSA^{439}-BSA^{221}	CCTKPESER-LSQKFPK	20.205
10	BSA^{221}-BSA^{242}	LSQKFPK-VHKECCHGDLLECADDR	21.527
11	BSA^{280}-BSA^{287}	LKECCDKPLLEK-SHCIAEVEK	9.893
12	BSA^{116}-BSA^{173}	LKPDPNTLCDEFKADEK-YNGVFQECCQAEDKGACLLPK	15.436

Table S14: Unique cross-linked linkages identified of myoglobin with enrichment.

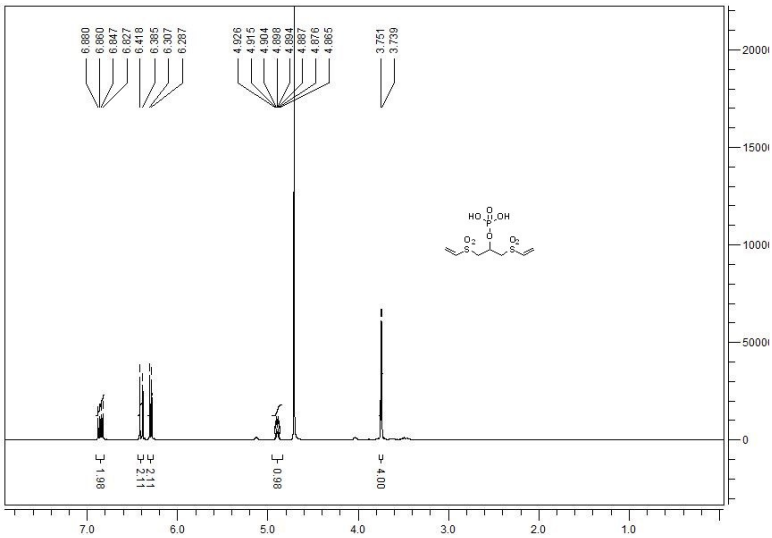
No.	Cross-linking site	Cross-linked peptides(cross-linked residues in red)	Cα-Cα distance (Å)

1	Myoglobin^{87}- Myoglobin^{145}	GHHEAELKPLAQSH-NDIAAKYK	6.996
2	Myoglobin^{98}- Myoglobin^{42}	HKIPIK-LFTGHPETLEKFDK	7.639
3	Myoglobin^{82}- Myoglobin^{145}	GHHEAELKPLAQSH-NDIAAKYK	12.88
4	Myoglobin^{36}- Myoglobin^{113}	LFTGHPETLEK-YLEFISDAIIHVLHSK	13.896
5	Myoglobin^{81}- Myoglobin^{145}	GHHEAELKPLAQSHATK-NDIAAKYK	15.202
6	Myoglobin^{36}- Myoglobin^{116}	LFTGHPETLEK-YLEFISDAIIHVLHSK	19.117
7	Myoglobin^{145}- Myoglobin^{62}	NDIAAKYK-ASEDLKK	28.253

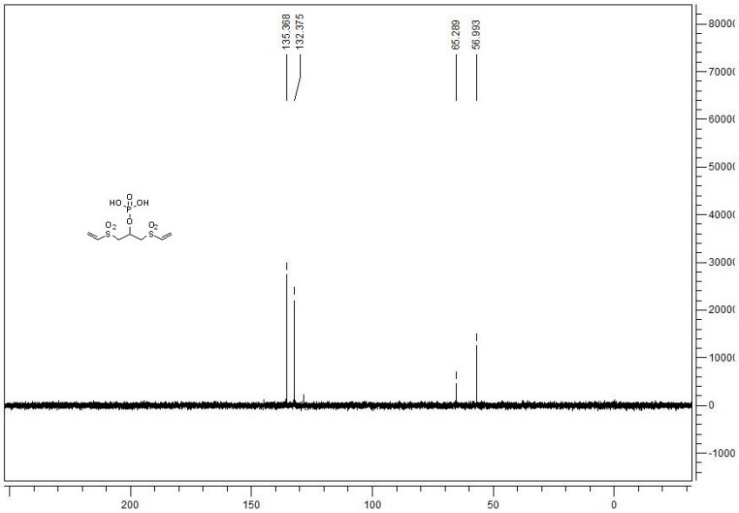
Table S15: Unique cross-linked linkages identified of Cpf1 with enrichment.

No.	Cross-linking site	Cross-linked peptides(cross-linked residues in red)	Cα-Cα distance (Å)
1	CPF^{323}-CPF^{319}	LEKLFK-NSEIFSSIKK	6.281
2	CPF^{797}-CPF^{20}	FSEDQYELHIPIAINK-FKAIPVGK	8.871
3	CPF^{707}-CPF^{20}	LYMFQIYNKDFSDK-FKAIPVGK	9.374
4	CPF^{326}-CPF^{319}	LFKNFDEYSSAGIFVK-NSEIFSSIKK	10.406
5	CPF^{370}-CPF^{374}	DKWNAEYDDIHLK-KAVVTEK	11.146
6	CPF^{707}-CPF^{720}	LYMFQIYNKDFSDK-SHGTPNLHTMYFK	11.748
7	CPF^{1216}-CPF^{1061}	IAISNKEWLEYAQTSVKH-TDADYIKK	11.933
8	CPF^{20}-CPF^{787}	FKAIPVGK-TTTLSYDVYKDKR	13.745
9	CPF^{51}-CPF^{42}	AEDYKGVKK-LLVEDEKR	14.239
10	CPF^{1277}-CPF^{1061}	IAISNKEWLEYAQTSVKH-TDADYIKK	16.24
11	CPF^{712}-CPF^{20}	DFSDKSHGTPNLHTMYFK-FKAIPVGK	17.17
12	CPF^{20}-CPF^{714}	FKAIPVGK-DFSDKSHGTPNLHTMYFK	19.392
13	CPF^{326}-CPF^{310}	LFKNFDEYSSAGIFVK-NTLNKNSEIFSSIK	20.015
14	CPF^{20}-CPF^{34}	FKAIPVGK-TQENIDNKR	22.328
15	CPF^{733}-CPF^{811}	LLFDENNHGQIR-NIFKINTEVR	24.922
16	CPF^{51}-CPF^{514}	AEDYKGVKK-NYVTQKPYSK	26.466
17	CPF^{787}-CPF^{687}	TTTLSYDVYKDKR-VSFESASKK	31.809
18	CPF^{51}-CPF^{538}	AEDYKGVKK-LYFQNPQFMGGWDKDKETDYR	33.062
19	CPF^{733}-CPF^{560}	LLFDENNHGQIR-YYLAIMDKK	34.523

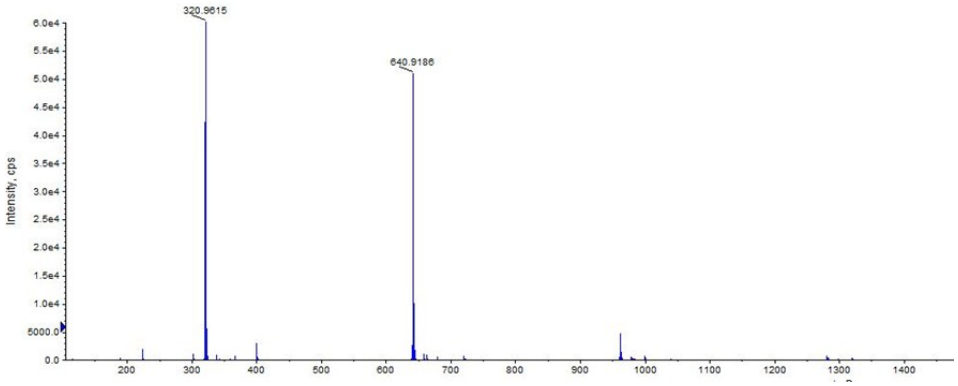
^{1}H NMR of pBVS



^{13}C NMR of pBVS



HR-MS-ESI of pBVS



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