Cell-Permeable Bicyclic Peptidyl Inhibitors against T-Cell Protein Tyrosine Phosphatase from a Combinatorial Library

Hui Liao and Dehua Pei

Supplementary Information

Table of Contents

Experimental Methods

Materials. Fmoc-protected amino acids were purchased from Chem-Impex International (Wood Dale, IL), or Aapptec (Louisville, KY). Fmoc-F_2Pmp-OH was purchased from EMD Millipore (Billerica, MA). 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU), 1-hydroxybenzotriazole (HOBt), (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), and N-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-Osu) were purchased Advanced ChemTech. N,N-Dimethylformamide (DMF) and dichloromethane (DCM) were purchased from VWR (West Center, PA). Phenyl isothiocyanate (PITC) was purchased in 1 mL sealed ampules from Sigma-Aldrich, and a freshly opened ampule was used in each experiment. TentaGel S NH_2 resin (90 µm, 0.28 mmol/g) was purchased from Peptides International Inc. (Louisville, KY). Bio-Rad columns were purchased from Bio-Rad Laboratories (Hercules, CA). All other chemicals and solvents in library and peptide synthesis were purchased from Sigma-Aldrich (St. Louis, MO), VWR (West Chester, PA) or Fisher Scientific (Pittsburgh, PA) and used without further purification unless otherwise noted. For mammalian cell culture, Dulbecco's modified eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), 0.25% trypsin-EDTA solution, fetal bovine serum (FBS), 100x penicillin-streptomycin were purchased from Sigma-Aldrich, ThermoFisher, GE Healthcare and VWR.

Protein Expression, Purification and Labeling. TCPTP was recombinantly fused with glutathione S-transferase (GST) at its N-terminus and expressed in *Escherichia coli* BL21(DE3) Rosetta cells. The cells were grown in 3 L of LB medium supplemented with 75 mg/L ampicillin at 37 °C to reach an OD_{600} of 0.6 and induced by the addition of 0.5 mM isopropyl β-D-1-thiogalatopyranoside (IPTG). The cells were induced for 6 h at 25 °C and harvested by centrifugation. The cell pellets were resuspended in 40 mL of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 5 mM β-mercaptoethanol, 1 spatula of lysozyme) supplemented with 1x Halt Protease Inhibitor Cocktail (Thermo Scientific). After stirring at 4 °C for 20 min to get homogenous solution, the mixture was sonicated on ice with 30 short pluses of 2 sec with pauses of 8 sec to remain low temperature. The lysate was centrifuged at 15,000 rpm for 30 min in a SS-34 rotor (SORVALL) and the clear supernatant was loaded onto a glutathione-Sepharose 4B column (GE Healthcare). The unbound proteins were washed away with a washing buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 5 mM β-mercaptoethanol). GST-TCPTP was eluted with the washing buffer containing 10 mM glutathione (reduced form). Eluted protein was concentrated in an Amicon concentrator, exchanged into a storage buffer (20 mM HEPES, pH 7.4, 150 mM NaCl), and quickly frozen and stored at -80 °C. Protein labeling was carried out by incubating GST-TCPTP with 4 equivalents of NHS-PEG_4-biotin (Thermo) or NHS-Texas Red (Thermo) in HEPES buffer pH 8.0 (pH adjusted by addition of 1 M $NaHCO_3$) at 4 °C for 2 h. The protein was passed through a G25 gel filtration column to remove any unreacted labeling reagent.

Library Synthesis. The bicyclic peptide library was synthesized on 2.0 g of TentaGel S NH_2 resin (90 µm, 0.28 mmol/g). The linker sequence (β-Ala-miniPEG-β-Ala-Met) was synthesized with 4 equivalents of Fmoc-amino acids using HCTU/Cl-HOBt/N-methylmorpholine (NMM) (4,

4, 8 equiv, respectively) as the coupling reagents. The coupling reaction was typically allowed to proceed for 1 h and the resin was thoroughly washed with DMF and DCM. After the second β-alanine residue, the Fmoc group was removed and the beads were subjected to spatial segregation into outer and inner layers.[1] The resin was soaked in water overnight, quickly drained and suspended in 15 mL of 55:45 (vol/vol) DCM/diethyl ether containing 0.6 equivalent of Fmoc-OSu. The mixture was incubated on a rotary wheel for 30 min. Segregation was confirmed by Chloranil test of a few beads; a green colored interior and a clear ring on the surface indicate that spatial segregation is successful. The free amine group in the bead interior was protected by Boc group by incubating with Boc_2O/DIPEA/DMAP (20, 10, 0.1 equiv, respectively) for 1 h in 16 mL of 50:50 (vol/vol) DCM/DMF. The Fmoc group on the surface layer was then removed by 20% piperidine and a 1:9 (mol/mol) mixture of Fmoc-Val and Ac-Val was coupled to the surface amine. The Fmoc group was again removed and Boc-Dap(Alloc) was coupled to the exposed amine. Next, the Boc groups from both surface and interior peptides were removed by treatment with xx mL of TFA/H_2O/triisopropylsilane (TIPS) (95/2.5/2.5 vol/vol) for 1 h twice. The resin was split into 12 equal portions and a different CPP sequence (Table S1) was added to each portion of resin. To facilitate CPP sequence identification, 4% (mol/mol) of CD_3CO_2D or $CH_3CD_2CO_2D$ was added to the coupling reactions for the two N-terminal residues of the CPPs, as specified in Table S1. After that, the resin was combined and Fmoc-Dap(Mmt) was added. To synthesize the random residues, the resin was split into 24 equal portions and each portion was reacted with a different Fmoc-amino acid.[2] To differentiate isobaric amino acids during later hit identification, 4% (mol/mol) CD_3CO_2D was added to the coupling reactions of D-Ala, D-Leu, and D-Pro, whereas 4% $CH_3CD_2CO_2D$ was added to the coupling reaction of L-Nle.[3] The coupling reactions were performed for 1 h with HATU/NMM (5 and 10 equiv, respectively) as coupling reagents. The 24 portions of resin were combined, treated with 20% piperidine in DMF to remove the Fmoc group, and the split-and-pool synthesis was repeated. After the last random residue was added, the Mmt group on Dap was removed by incubation with 2%TFA/2%TIPS in DCM for 30 min. The resulting free amine group was protected with Fmoc by incubating with Fmoc-OSu/NMM (10 equiv each). The Alloc group on Dap was removed by overnight treatment with tetrakis(triphenylphosphine)palladium, triphenylphosphine, and N-methylaniline (0.5, 5, 10 equiv, respectively). The resin was washed exhaustively with 2% sodium dimethyldithio-carbamate hydrate (SDDC) in DMF to remove any residual palladium. The resulting free amine group was reacted with trimesic acid (5 equiv), HATU (5 equiv), and DIPEA (10 equiv) for 30 min (twice). The Fmoc groups on the N-terminus and the internal Dap were removed by 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF (5 min, twice). The resin was washed extensively with DMF, DCM and DMF, and the peptide was cyclized using PyBOP/HOBt/NMM (5, 5, 10 equiv, respectively) in DMF for 3 h. The library was washed with DMF and DCM, dried under vacuum, and stored at -20 °C until use. Prior to screening, the library was deprotected by incubating with a modified reagent K (7.5:5:5:2.5:1:1:78 phenol/H_2O/thioanisole/ethanedithiol/anisole/TIPS/ TFA) for 3 h and washed with TFA and DCM.

Library Screening and Hit Identification. ~400 mg of library (~1,100,000 beads) was placed in a 5-mL plastic micro-BioSpin column, extensively washed with DMF and ddH_2O, and incubated in 4 mL of a blocking buffer (30 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% NaN_3, 0.1% gelatin, 0.01% Tween-20) at 4 °C overnight. The resin was then incubated with biotinylated TCPTP (300 nM final concentration) in the blocking buffer at 4 °C for 12 h, followed by washing

with 1 mL blocking buffer once. Then it was incubated with streptavidin-alkaline phosphatase (final concentration 1 µg/mL) for 15 min in 1 mL of SA-AP binding buffer (30 mM Tris, pH 7.4, 1 M NaCl). After quick washing with 1 mL of SA-AP binding buffer twice, the resin was carefully transferred into a 6.0 cm diameter petri dish in 4 mL of staining buffer (30 mM Tris, pH 8.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.02 mM $ZnCl_2$) and 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP, 0.5 mg/mL final concentration) was added. After 30 to 40 min, turquoise color developed on some of the beads, which were removed from the library using a micropipette under a dissecting microscope. These beads were separated into two groups ("intensely" and "medium" colored). The positive beads were washed exhaustively with PBS, 6 M urea, and again PBS. The two groups of beads were blocked again overnight and then incubated with 20 nM Texas-Red-TCPTP for 2 h. The beads were examined under an Olympus SZX12 microscope equipped with a fluorescence illuminator and each group was further separated into "intensely", "medium", and "lightly" fluorescent groups. Finally, the positive beads were separated into 5 different categories and individually sequenced by partial Edman degradation-mass spectrometry (PED/MS)[3].

Individual Peptide Synthesis. The bicyclic peptides were synthesized on Rink Amide resin (0.3 mmol/g) or Sieber Amide resin (0.68 mmol/g) by using standard Fmoc chemistry and following the same steps as in the library synthesis. Generally, 5 equivalent of Fmoc-amino acid was used with HATU/NMM (5 and 10 equiv, respectively) as the coupling reagent, except for F_2Pmp, for where 2, 2, and 4 equiv of Fmoc-F_2Pmp/HATU/NMM were used. After the addition of the last residue, the N-terminal Fmoc group was removed and a trimesic acid (5 equiv) was coupled to the N-terminus with HATU/NMM (5 and 10 equiv, respectively). The Alloc groups on the side chains of two Dap residues were removed as previously described. The peptide was then cyclized by treatment with PyBOP/HOBt/DIPEA (5, 5, 10 equiv, respectively) for 45 min twice. The peptides were deprotected and released from the resin by treatment with 92.5:2.5:2.5:2.5 (vol/vol) TFA/H_2O/1,4-dimethoxybenzene/TIPS for 2 h. The crude peptide was triturated with cold ethyl ether three times and then purified by reversed-phase HPLC on a C18 column. The purity of the peptides was confirmed to be at least 95% by analytical HPLC monitored at 214 nm and their authenticity was confirmed by high-resolution MALDI-TOF mass spectrometry. FITC labeled peptides were obtained by incubating purified peptide and 3 equivalents of FITC in 150 µL of DMF/H_2O/0.1 M $NaHCO_3$ (1:1:1) pH 8.5 for 30 min. The labeling reaction was quenched by the addition of 20 µL of 1 M HCl. Naphthofluorescein (NF)-labeled peptide was obtained by incubating purified peptide and 2 equivalents of NF-NHS ester in $NaHCO_3$ solution (pH 8.5) for 2 h (reaction quenched by one drop of TFA). The labeled peptide was purified by reversed-phase HPLC on a C18 column.

PTP Inhibition Assay.[4] PTP assays were performed in a quartz microcuvette (total volume 120 µL). The reaction mixture contained 50 mM Tris-HCl, 50 mM Bis-Tris, pH 7.4, 100 mM sodium acetate, 1 mM TCEP, 0-2 µM peptide inhibitor, and 500 µM *para*-nitrophenyl phosphate (pNPP). The enzymatic reaction was initiated by addition of PTP (typically 50 nM final concentration) and monitored continuously at 405 nm on a UV-VIS spectrophotometer. Initial rates were calculated from the reaction progress curves (0-150 s). The half-maximal inhibition constant (IC_{50}) was obtained by plotting the rates (v) against the inhibitor concentration [I] and fitting the data against the following equation (1)

$$v = \frac{v_0}{1+\frac{[I]}{IC50}} \quad (1)$$

where v_0 is the enzymatic reaction rate in the absence of inhibitor. The inhibition constant (K_i) was determined by measuring the initial rates at fixed enzyme concentration but varying concentrations of pNPP (0-12 mM) and inhibitor (0-800 nM). The reaction rate was plotted against the pNPP concentration [S] and fitted against the equation (2) to obtain the Michaelis constant K.

$$\frac{1}{v} = \mathrm{K} \times \frac{1}{[S]} + \frac{1}{v_{max}} \quad (2)$$

The K_i value was obtained by plotting the K values against the inhibitor concentration [I] and fitted to equation (3) where K_0 is the Michaelis constant in the absence of inhibitor.

$$\frac{K}{K_0} = 1 + \frac{[I]}{K_i} \quad (3)$$

Mammalian Cell Culture. C6 (CCL-107) cell line was purchased from ATCC. HeLa cell line was a kind gift from Dr. Dmitri Kudryashov (The Ohio State University). Both cell lines were maintained in DMEM supplemented with 10% FBS and 1% Abs.

Immunoblotting. For global phosphotyrosine level, HeLa cells were seeded in a 6-well plate with standard DMEM supplemented with 10% FBS and 1% Abs at 37 ℃ in 5% CO_2 to reach 80% confluence. Then cells were treated with peptide **25** (0-6 μM) in serum free DMEM for 2 h. FBS was then added to a final concentration as 10% and cells were grown for an additional 22 h. The cells were washed twice with cold PBS, detached by treating with 0.25% trypsin-EDTA solution, and all the fractions were collected. After centrifugation (5000 rpm, 5 min), cell pellets were lysed on ice for 30 min in RIPA buffer (Thermo, #89900) containing protease and phosphatase inhibitors. Cell lysates were centrifuged at 15000 rpm for 10 min, and the extracted proteins in supernatant were collected. Protein concentrations were measured by BCA Protein Assay Kit (Thermo, #23235), and equal amounts of protein (~20 μg) were loaded into all lanes of a 10% SDS-PAGE gel. After electrophoresis, the proteins were electrophoretically transferred to a nitrocellulose membrane at 4 ℃. The membrane was first blocked with 10% nonfat dry milk in TBST [0.1% (vol/vol) Tween 20 in 1× TBS] at room temperature for 1 h, followed by incubating with an anti-pY monoclonal antibody (1:2000 dilution, ThermoFisher, #MA4-003) or anti-β-actin (1:4000 dilution, Sigma A5441) overnight at 4 ℃. The membranes were washed three times with TBST and incubated with corresponding HRP-conjugated secondary antibodies (1:2000 dilution) for 2 h at room temperature, followed by washing with TBST three times and proceeding to detection immediately. The signals were detected by using chemiluminescent HRP Antibody Detection Reagent (Denville, E-2500) by following the manufacturer's protocol.

To study the effect of peptide **25** on Src phosphorylation, C6 cells were seeded in a 6-well plate using complete culture medium until reaching 80% confluence. Cells were then starved in serum free DMEM for 24 h before peptide treatment. On the day of experiment, cells were washed with DPBS once, and treated with peptide **25** (0-5 μM) or **33** (0-20 μM) in serum free DMEM for 24 h, followed by stimulation with EGF (50 ng/mL final concentration) for 10 min. DMSO concentration was kept as 0.1% (vol/vol) in all wells. The cell harvest and lysis were performed as described above, except that primary antibodies against total Src (1:2000 dilution, Cell Signaling #2109), phosphoSrc Y416 (1:1000 dilution, Cell Signaling #6943), phosphoSrc Y527 (1:1000 dilution, Cell Signaling #2105) were used.

Flow Cytometry.[5] HeLa cells were seeded in a 12-well plate at a density 1.5 x 10^5 cells/well the day prior of experiment. On the day of experiment, cells in DMEM media supplemented with 1% FBS and 1% Abs were incubated with 5 μM NF-labeled peptide **25** or **33** for 2 h. The cells were washed with cold DPBS and harvested by trypsinization. The detached cells were washed twice with DPBS, resuspended in DPBS, and analyzed by flow cytometry (BD FACS Aria III), with excitation at 633 nm.

Live-Cell Confocal Microscopy. HeLa cells (12,500 approximately) were plated in one chamber of a 4-chamber glass bottom dish (35-mm diameter) the day prior of experiment. On the day of experiment, cells were washed with DPBS and treated with 5 μM FITC-labeled peptide **25** or **33** in phenol-red free, HEPES supplemented DMEM containing 1% FBS for 2 h. After removal of the medium, the cells were gently washed with DPBS twice and imaged on a Nikon A1R live-cell confocal equipped with 100X oil objective. Data were analyzed using NIS-Elements AR.

References

1. R. Liu, H. Marik, and K. S. Lam, *J. Am. Chem. Soc.*, 2002, **124**, 7678.
2. K. S. Lam, S. E. Salmon, E. M. Hersh, V. J. Hruby, W. M. Kazmierski, and R. J. Knapp, *Nature*, 1991, **354**, 82.
3. A. Thakkar, A. S. Wavreille, and D. Pei, *Anal. Chem.*, 2006, **78**, 5935.
4. W. Lian, B. Jiang, Z. Qian, and D. Pei, *J. Am. Chem. Soc.*, 2014, **136**, 9830.
5. Z. Qian, A. Martyna, R. L. Hard, J. Wang, G. Appiah-Kubi, C. Coss, M. A. Phelps, J. S. Rossman, and D. Pei, *Biochemistry*, 2016, **55**, 2601.

Table S1. Sequences of CPP motifs incorporated into the bicyclic library

CPP ID	Sequence	Capping Agent	Mass Pattern
A	FΦRRRR	none	m/z 1014, 1211, and 1358
B	RRRRΦF	none	m/z 1046, 1202, and 1358
C	FΦRRR	Propionic acid at Φ	m/z 858, 916, 1055, and 1202
D	RRRΦF	Acetic acid at R	m/z 890, 1046, 1091, and 1202
E	RΦRFRR	none	m/z 1005, 1202, and 1358
F	RRFRΦR	Propionic acid at R	m/z 1046, 1202, 1260, and 1358
G	FRRRRΦ	none	m/z 1055, 1211, and 1358
H	ΦRRRF	none	m/z 849, 1005, and 1202
I	fΦRrRr	Acetic acid at f	m/z 1014, 1211, 1256, and 1358
J	fΦRrR	Propionic acid at f	m/z 858, 1055, 1113, and 1202
K	rRrRΦf	Acetic acid at R	m/z 1046, 1091, 1202, and 1358
L	FϕrRrR	Acetic acid at ϕ	m/z 1014, 1059, 1211, and 1358

Φ = L-2-napthylalanine. Lowercase letter = D-Amino Acid, 4% of library beads were capped during synthesis at the indicated position in red with either acetic acid (+45) or propionic acid (+58) to distinguish transporter sequences with redundant masses during hit identification during PED/MS.

Table S2. Hit Sequences Identified from Library Screening against TCPTP

	Bead No.	X^1	X^2	X^3	X^4	CPP [a]
A	1[b]	Pro	Tyr	Ala	Pip	I
	2[b]	Pro	Ile	Asp	Pip	J
B	3	glu[c]	Nle	Asp	Gly	C
	4[b]	Asp	Asp	thr	Pip	J
	5	thr	glu	Tyr	glu	H
	6	glu	glu	Asp	Ile	D
	7[b]	Asp	glu	Asp	Ile	J
	8[b]	Gln	Asp	Abu	Ala	C
C	9	thr	Asp	Abu	Pip	J
	10	Fpa	Gly	Ser	Asp	D
	11	Ala	phe	Phg	Pip	J
	12	Ala	Ile	Gly	Pip	J
	13	Pro	Ser	Ser	Pip	J
	14	Ala	glu	Abu	Ala	C
	15	glu	glu	glu	Ala	D
	16	Ile	Fpa	His	Pip	J
	17	Ala	Ile	Ser	Ala	C
	18	Ile	glu	Asp	Ile	J
	19	Ala	phe	Asp	glu	H
	20	glu	asn	Nle	Ala	H
	21	Asp	glu	Fpa	Ile	D
	22	Ile	Tyr	Asp	Pip	J
	23	glu	Asp	Nle	Ala	D
	24	phe	Tyr	glu	Ile	D
D	25	Ile	glu	Tyr	Gln	C
	26	Ser	Phg	Asp	Ala	H
	27	Asp	Ala	Ser	glu	A
	28	Phg	Pro	glu	Gln	H
	29	Phg	Asp	glu	Ile	F
	30	Gln	phe	Asp	Ala	H
	31	thr	Asp	Gln	glu	F
	32	asn	Asp	thr	Pip	I
	33	glu	glu	Asp	Gln	C
	34	Asp	Gly	Asp	thr	C
	35	glu	Pro	asn	val	J
	36	Abu	glu	Abu	Ala	H
E	37	Phg	Pip	Asp	thr	A
	38	val	thr	glu	pro	J
	39	ala	Abu	Asp	Pip	I
	40	Asp	val	Asp	Ala	G
	41	val	glu	Trp	Ile	K
	42	Tyr	asn	Asp	Gly	F
	43	His	Ser	Phg	Asp	A
	44	Asp	Ile	asn	glu	H
	45	Ile	His	Asp	Abu	D
	46	Asp	glu	asn	Abu	I
	47	Pro	glu	Pip	Pip	J
	48	Ala	asn	glu	phe	B
	49	Phg	Ser	Asp	Ala	H
	50	val	glu	Ser	asn	H
	51	Nle	Gly	His	Pro	H
	52	glu	Pro	Asp	thr	G
	53	val	thr	Asp	Asp	J
	54	phe	Asp	thr	val	F
	55	Phg	glu	Asp	Gly	D
	56	Tyr	Ala	Asp	Ala	H
	57	Pro	Fpa	Asp	Pro	F
	58	Phg	Asp	Gln	Trp	K
	59	Pro	Ile	Asp	Phg	D
	60	Asp	glu	Asp	Ile	E

[a] CPP motif coding correspond to Table S1.
[b] Peptides selected for individual synthesis and analysis.
[c] D-amino acids are shown in all lowercase letters.

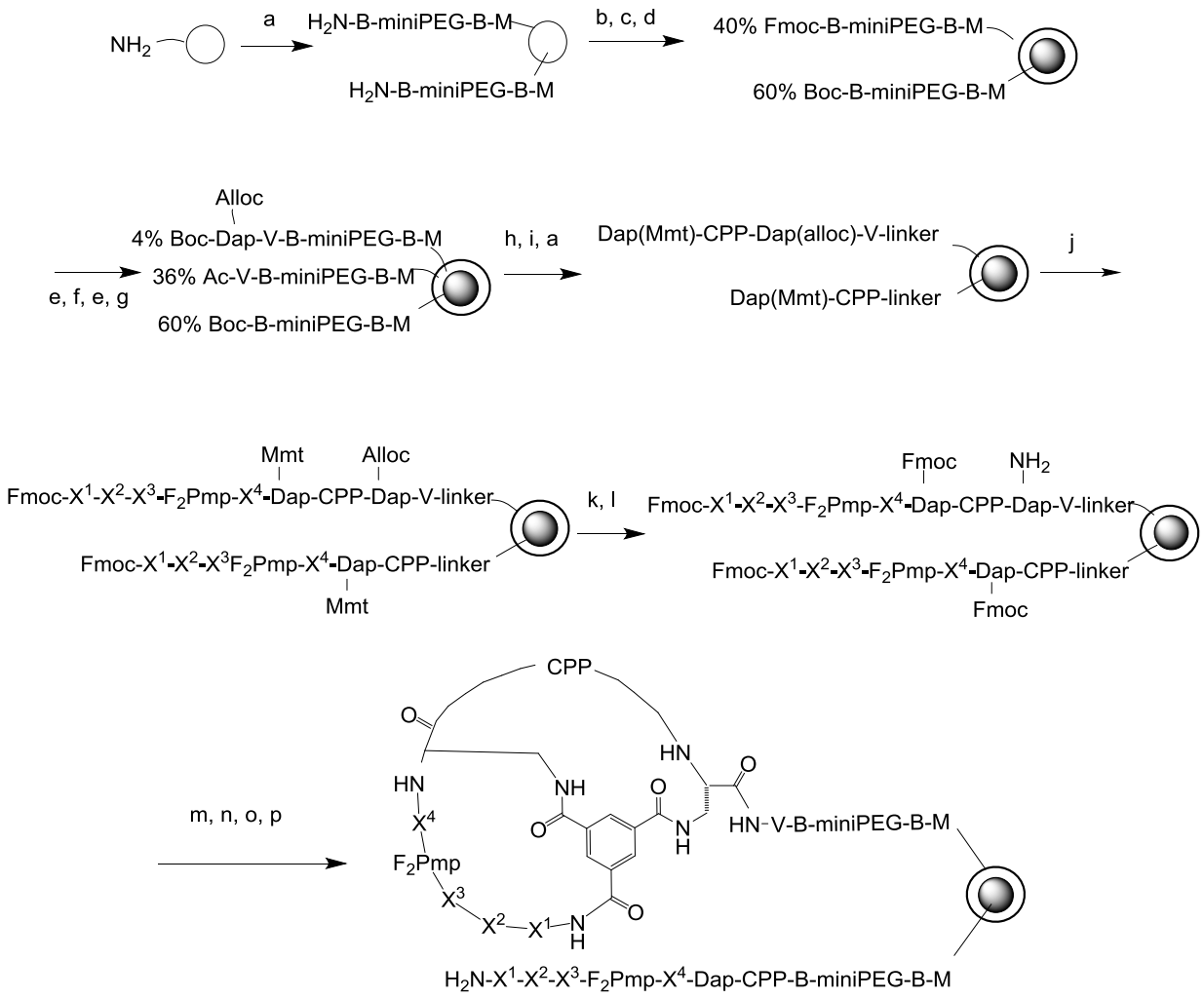


Figure S1. Library synthesis. Reagents and conditions: (a) standard Fmoc/HCTU chemistry; (b) soak in water; (c) Fmoc-OSu; (d) Boc_2O/DMAP; (e) piperidine; (f) 1:9 (mol/mol) Fmoc Val-OH/Ac-Val-OH; (g) Boc-Dap(Alloc); (h) 95% TFA, 2.5% H_2O, 2.5% TIPS; (i) split into 12 portions for CPP synthesis; (j) split-and-pool synthesis by Fmoc/HATU chemistry; (k) 2% TFA/5% TIPS; (l) $Pd(PPh_3)_4$; (m) trimesic acid/HATU/NMM; (n) 2% DBU; (o) PyBOP/HOBt/NMM; (p) Reagent K.

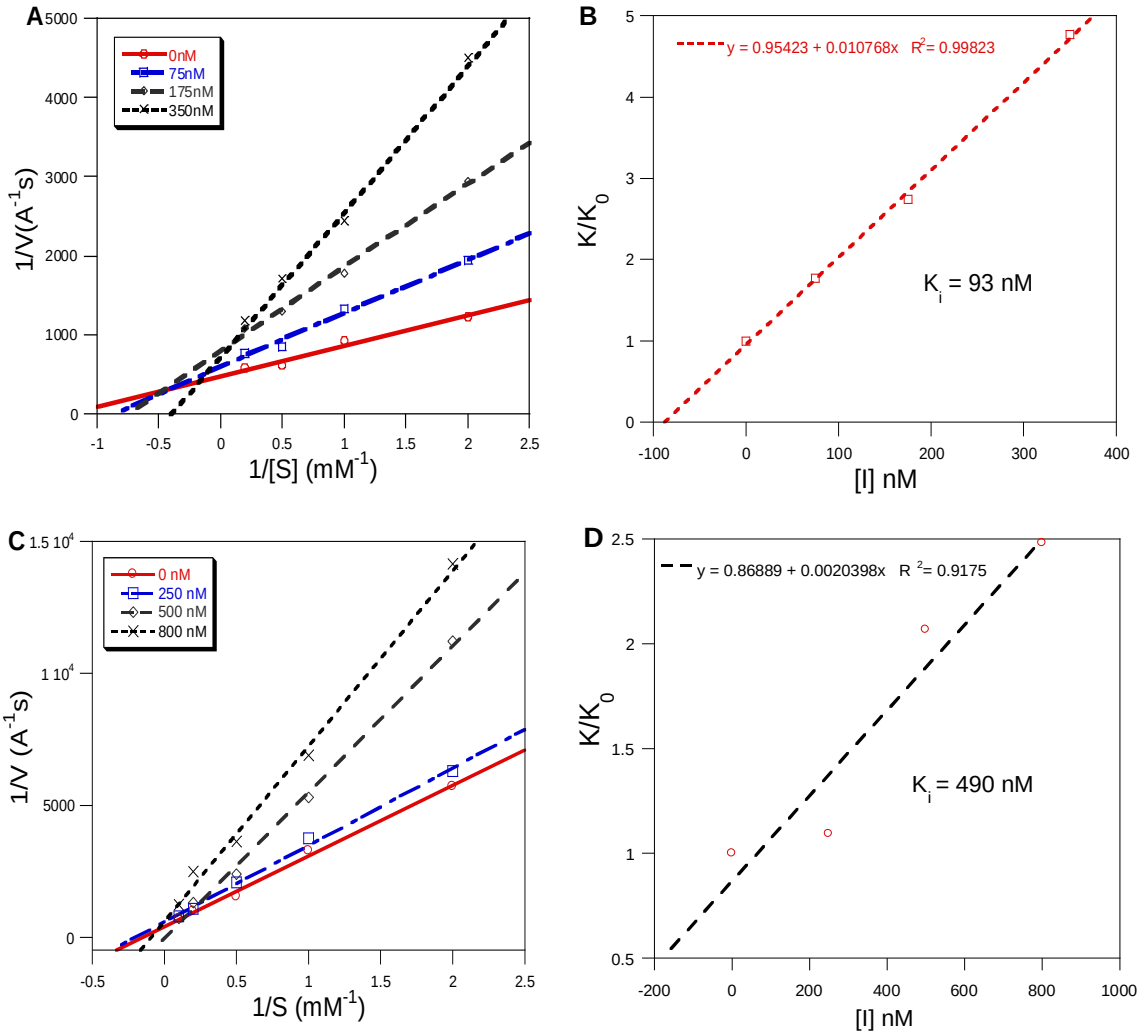
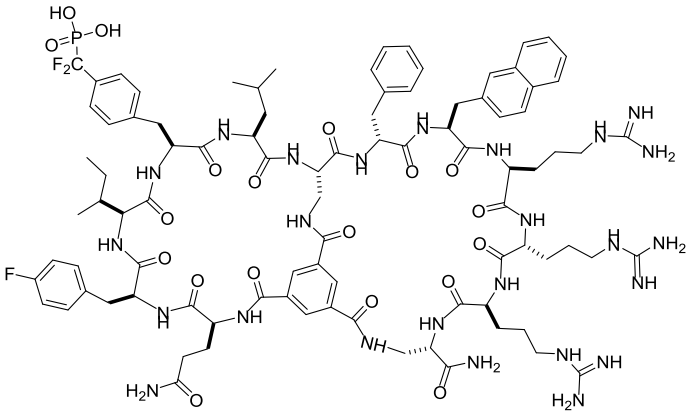


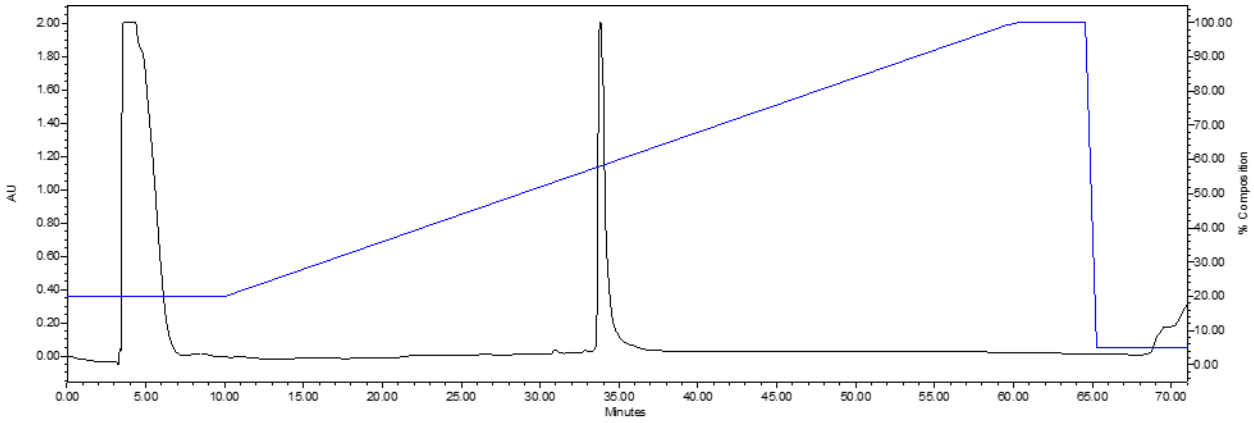
Figure S2. Determination of inhibition constant (K_i) of Peptide **25** against TCPTP (A and B) and PTP1B (C and D). (A, C) Lineweaver-Burk plots in the presence of different concentrations of peptide **25**. (B, D) Secondary plots of the ratio of Michaelis constants (K/K_0) as a function of peptide **25** concentration [I].

Figure S3. Representative HPLC tracings and high-resolution MALDI-TOF mass spectra of peptides used in this study.

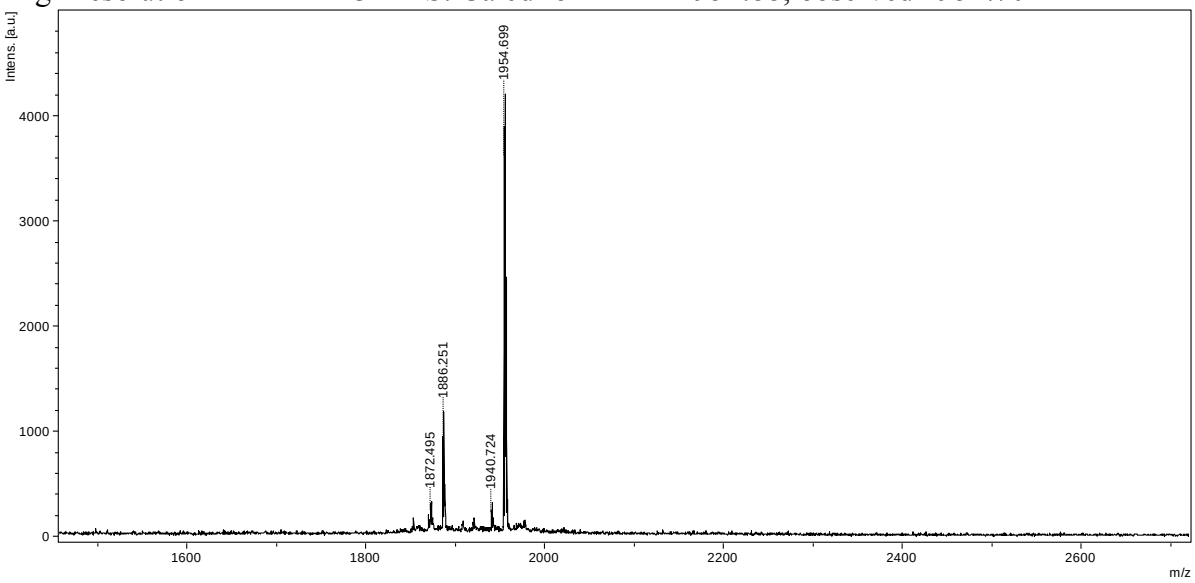
Peptide **25:**



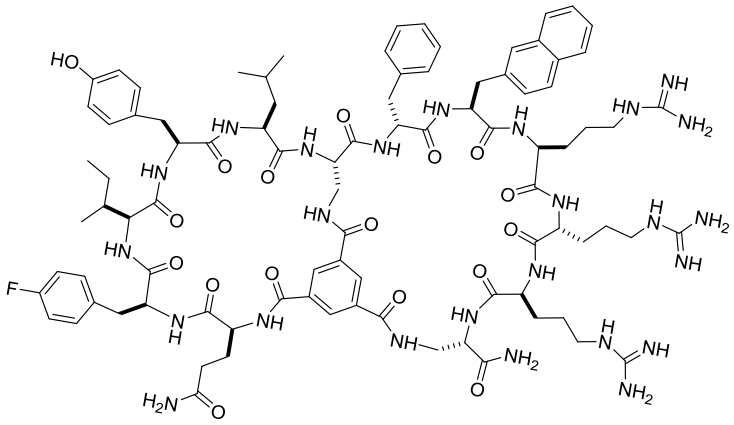
Analytical HPLC trace for peptide **25:**



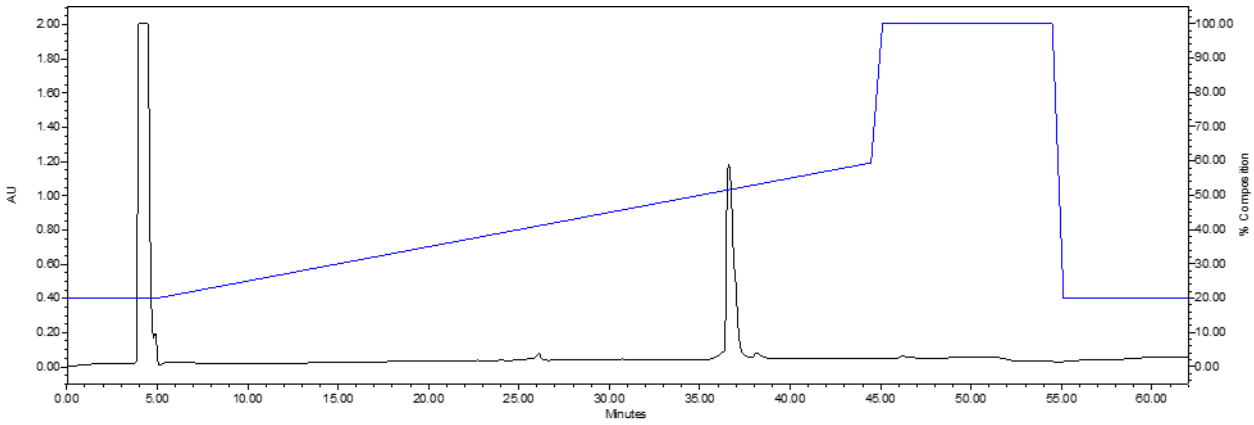
High-resolution MALDI-TOF MS: Calcd for $M+H^+$ 1954.88; observed 1954.70



Peptide **33**:



Analytical HPLC trace for peptide **33:**



High-resolution MALDI-TOF MS: Calcd for $M+H^+$ 1840.91; observed 1840.80