J|A|C|S ARTICLES

Published on Web 08/19/2009

Acquisition of a Potent and Selective TC-PTP Inhibitor via a Stepwise Fluorophore-Tagged Combinatorial Synthesis and Screening Strategy

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Abstract: Protein tyrosine phosphatases (PTPs) regulate a broad range of cellular processes including proliferation, differentiation, migration, apoptosis, and immune responses. Dysfunction of PTP activity is associated with cancers, metabolic syndromes, and autoimmune disorders. Consequently, small molecule PTP inhibitors should serve not only as powerful tools to delineate the physiological roles of these enzymes in vivo but also as lead compounds for therapeutic development. We describe a novel stepwise fluorophore-tagged combinatorial library synthesis and competitive fluorescence polarization screening approach that transforms a weak and general PTP inhibitor into an extremely potent and selective TC-PTP inhibitor with highly efficacious cellular activity. The result serves as a proof-of-concept in PTP inhibitor development, as it demonstrates the feasibility of acquiring potent, yet highly selective, cell permeable PTP inhibitory agents. Given the general nature of the approach, this strategy should be applicable to other PTP targets.

Introduction

Protein tyrosine phosphatases (PTPs), one of the largest enzyme families encoded by the human genome, control a broad spectrum of cellular processes including proliferation, differentiation, migration, apoptosis, and immune responses.[1] Dysfunction of PTP activity is associated with cancers, metabolic syndromes, and autoimmune disorders.[2] Given the role of PTPs in signaling and in disease formation, it is not surprising that inhibitors of these enzymes have become a sought after commodity. Unfortunately, achieving specificity for PTP inhibition is not trivial. The common architecture of a PTP active site (i.e., pTyr-binding pocket) impedes the development of selective PTP inhibitors. Fortunately, it has been recognized that pTyr alone is not sufficient for high-affinity binding and residues flanking pTyr are important for PTP substrate recognition.[3] Can potent, selective, and cell permeable PTP inhibitors be devised by tethering a nonhydrolyzable pTyr mimetic to appropriately functionalized moieties to engage both the active site and unique nearby subpockets? To address this question, we selected as our starting common molecular motif the nonhydrolyzable pTyr surrogate phosphonodifluoromethyl phenylalanine (F_2Pmp).[4]

Our PTP target is T cell PTP (TC-PTP), which is linked to the development of several inflammatory disorders including type 1 diabetes, Crohn's disease, and rheumatoid arthritis.[5] Although originally cloned from a T cell cDNA library, TC-PTP is ubiquitously expressed in all tissues. Studies with TC-PTP-deficient mice implicate a role for TC-PTP in hematopoiesis and cytokine response.[6] Accordingly, TC-PTP modulates cytokine signaling through the Jak/Stat pathways.[7] In addition, several signal molecules, including epidermal growth factor (EGF) receptor,[8] the insulin receptor,[9] Src kinase,[10] and the adaptor protein Shc[11] have also been suggested as TC-PTP

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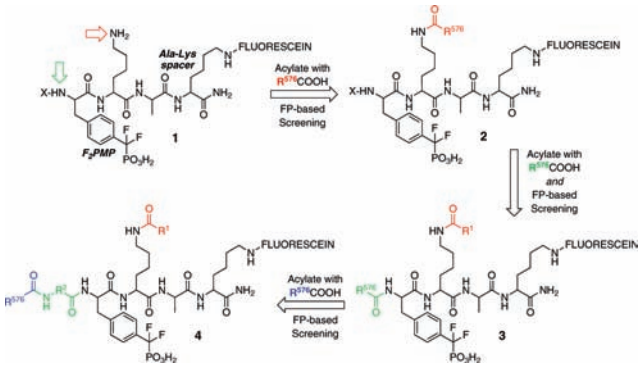
10.1021/ja903733z CCC: $40.75

Scheme 1. Stepwise Preparation and Screening of Fluorescein-Derivatized Combinatorial Libraries **2**, **3**, and **4**



substrates. Thus, TC-PTP may regulate multiple cellular processes. Despite a growing number of signaling pathways that are subject to regulation by TC-PTP, the mechanism through which TC-PTP controls cell physiology remains to be fully defined. Consequently, cell permeable TC-PTP inhibitors are unique tools for evaluating both the function of this enzyme as well as its potential as a therapeutic target.

Results and Discussion

Our combinatorial synthesis and screening design strategy for the acquisition of TC-PTP inhibitors commences with **1**, which contains four key structural elements (Scheme 1): (a) the F_2Pmp active site-directed motif common to all PTPs; (b) two amines (red/green arrows), positioned on both sides of the F_2Pmp residue that can be modified to introduce molecular diversity; (c) a fluorescein tag; and (d) an Ala-Lys spacer between the fluorophore and the F_2Pmp/diversity core. The latter is present to minimize possible interference from fluorescein with interactions between TC-PTP and the active site-directed core. The selection of Ala as part of the linker stems from its small size (i.e., lack of functionality) and synthetic simplicity. The fluorophore, an innate part of all library members, provides the means to identify high affinity active site binders via a homogeneous, high-throughput fluorescence polarization (FP) displacement assay.[12] The major advantage of the FP-based screen is that the strength of binding (increase in mA value) is independent of the concentration of the fluorophore when the fluorophore concentration is significantly lower than the protein concentration. Thus the concentration independence of the FP assay should enable one to obtain reliable structure and activity data (binding affinity) without the need for concentration uniformity for all library compounds.

In the interest of keeping the library at a reasonable size, we selected 576 carboxylic acids (see Supporting Information) that vary by molecular weight, charge, polarity, hydrophobicity, sterics, etc., which provides a high structural diversity focused within a narrow spatial window encompassing the active site. Three libraries were prepared in a stepwise fashion where the available primary amine moiety was condensed with 576 carboxylic acids (Scheme 1). Our first generation library (**2** in Scheme 1), positioned at the site immediately C-terminal to F_2Pmp, employed compound **1** as the precursor (Scheme 1). The amine on F_2Pmp is acetylated (X = Ac), leaving the only free primary amine on the neighboring Lys ready for condensation with 576 carboxylic acids. Compound **1** was prepared via

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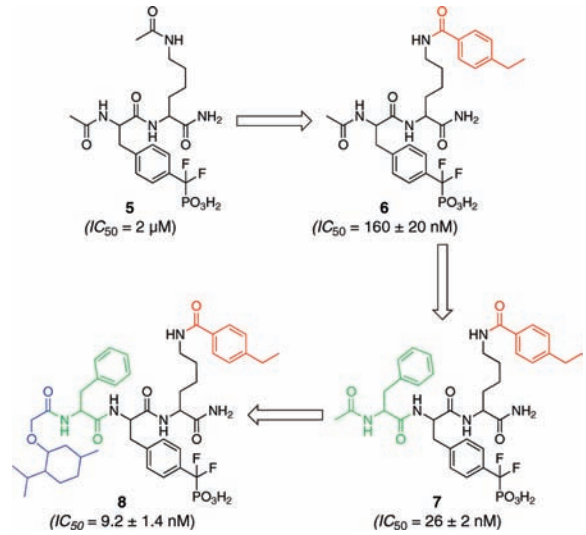
Scheme 2. Combinatorial Evolution of TC-PTP Inhibitors[a]



[a] **5** is the parent compound, leading to **6**, **7**, and **8**, which were identified via screening of libraries **2**, **3**, and **4**, respectively.

standard Fmoc solid phase peptide synthesis, cleaved, and subsequently purified by HPLC. The 576-member library was constructed from **1** on a liquid handling workstation equipped with a 96-channel pipetting head. The 576 structurally diverse carboxylic acids were introduced, in equal quantities, into individual wells of six 96-well plates, along with appropriate reagents to activate the acid functionality. A solution of **1** was then added to each well to initiate condensation. The reaction was quenched with cyclohexylamine, and the resulting library was diluted and dispensed into 384-well plates for FP-based screening.

To identify high-affinity active site-directed TC-PTP inhibitors, the fluorescein tagged library (~3 nM) was mixed with 2 μM TC-PTP, and the anisotropy values (measures of binding affinity) were recorded with a microplate reader in both the absence and presence of 500 μM Fmoc-F_2Pmp-OH, a known competitive ligand with an IC_{50} of 20 μM for TC-PTP. The fluorescein-labeled compounds that are most resistant to displacement by Fmoc-F_2Pmp-OH should possess the highest affinity for TC-PTP. Hits with highest affinity for TC-PTP were identified (Supporting Information Table S1). The structure and activity data revealed that compounds with *p*-substituted benzoic acids were preferentially selected by TC-PTP. The lead derivative from the first generation library (**2**) was resynthesized without the fluorescein tag (i.e., **6**), purified, and characterized. Compound **6** inhibits the TC-PTP-catalyzed reaction with an IC_{50} of 160 ± 20 nM, 12.5-fold better than that of the parent compound **5** (IC_{50} = 2 μM) (Scheme 2).

To build upon the lead generated from the first-generation library, the α-amino group from F_2Pmp in compound **2** (X = H) was condensed with the same set of 576 carboxylic acids to furnish a second-generation library (**3**) of 576 different analogues. Introduction of diversity at the α-amino position of F_2Pmp should maximize interactions of inhibitors with subpockets N-terminal to the pTyr-binding site in the PTPs. The library was screened as described above. A number of hits were identified based on binding affinity for TC-PTP (Supporting Information Table S2), which generally fell into two groups: aromatic amino acid derivatives and *o*-substituted benzoic acids. Once again the lead with the highest affinity was resynthesized without the linker and the fluorescein tag (**7**), and an observed

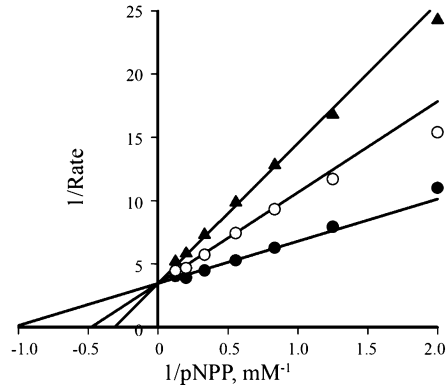


Figure 1. Effect of compound **8** on TC-PTP-catalyzed *p*NPP hydrolysis. The Lineweaver−Burk plot displayed the characteristic intersecting line pattern, consistent with competitive inhibition. Compound **8** concentrations were 0 (●), 5 (○), and 10 nM (▲), respectively.

Table 1. Selectivity of Compound **8**

PTP	K_i (nM)
TC-PTP	4.3 ± 0.2
PTP1B	34.0 ± 2.8
SHP2	>1000
He-PTP	>1000
Lyp	>1000
FAP1	>1000
PTP-Meg2	>1000
PTPα	>1000
LAR	>1000
CD45	>1000
Cdc14A	>1000
VHR	>1000
VHX	>1000
LMWPTP	>1000

77-fold enhancement in affinity relative to parent compound **5** was realized (Scheme 2).

Finally, the N-acetyl moiety at the N-terminus in compound **7** offered the opportunity for further refinement. Thus, the third generation library (**4**) was prepared by replacing the N-acetyl moiety with the same set of 576 carboxylic acids and screened. The criteria used to identify lead compounds from this library were based on both improved binding affinity and selectivity against protein tyrosine phosphatase 1B (PTP1B), the closest homologue of TC-PTP (74% sequence *identity* in their catalytic domains).[13] The (+)-menthoxyacetic acid derivative **8** emerged as the most potent TC-PTP inhibitor from the final library (Scheme 2). IC_{50} measurements of compound **8** without or with TC-PTP preincubation (30 min) yielded similar results (9.2 ± 1.4 and 8.7 ± 1.4 nM, respectively), suggesting that compound **8** is a reversible TC-PTP inhibitor and does not display slow binding kinetics. Further kinetic analysis revealed that the mode of TC-PTP inhibition by compound **8** is competitive with a K_i of 4.3 ± 0.2 nM (Figure 1). Importantly, compound **8** is more than 200-fold selective versus a panel of PTPs including the cytosolic PTPs, SHP2, Lyp, HePTP, PTP-Meg2, and FAP1, the receptor-like PTPs, CD45, LAR, and PTPα, the dual specificity phosphatase VHR, VHX, and CDC14A, and low molecular weight PTP (Table 1). In addition, a 8-fold selectivity for TC-PTP over its closest homologue PTP1B is observed. Indeed, compound **8** is the most potent and selective TC-PTP inhibitor ever described.

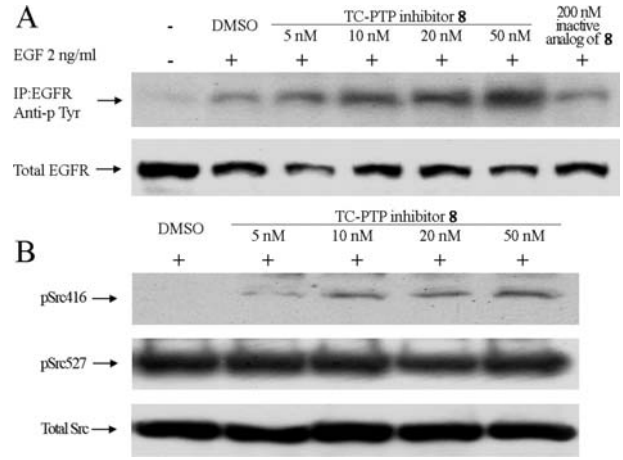


Figure 2. Cellular activity of TC-PTP inhibitor **8**. (A) Effect of compound **8** on EGF-stimulated EGFR phosphorylation. (B) Effect of compound **8** on Src phosphorylation at Tyr416 and 527.

The cellular efficacy of **8** was assessed by examining its effect on tyrosine phosphorylation of EGFR, a known substrate of TC-PTP.[8] PTP1B$^{-/-}$ mouse embryo fibroblast cells were incubated with **8** at various concentrations for 2 h and subsequently treated either with or without EGF (2 ng/mL) for 10 min. Inhibition of TC-PTP is expected to enhance endogenous levels of phosphorylated EGFR. As shown in Figure 2A, compound **8** enhanced EGFR phosphorylation in a dose-dependent fashion relative to DMSO. Remarkably, the phosphorylated form of EGFR is increased nearly 2-fold even at an inhibitor concentration of 5 nM (1.2 × K_i). The effects are even more pronounced at 10, 20, and 50 nM, with an increase in EGFR phosphorylation of 3.4-, 3.9-, and 5.0-fold, respectively. This is in contrast to the general belief that phosphonate-based PTP inhibitors are incapable of penetrating the cell membrane. As a control, a structurally related but inactive analogue of **8** (compound **9**; Materials and Methods) lacking the difluoromethylenephosphonate moiety (IC_{50} > 1 μM) has no effect on EGFR phosphorylation, even at 200 nM. This result suggests that the cellular activity displayed by **8** is unlikely due to nonspecific effects.

To further demonstrate compound **8** specificity inside the cells, we also determined its effect on Src kinase phosphorylation within the same cells. Src activity is regulated by phosphorylation at two distinct tyrosine residues. Autophosphorylation of Tyr416 in the kinase domain activates Src, while phosphorylation of Tyr527 in the C-terminal tail by the C-terminal Src kinase (Csk) blocks Src activity. Previous studies have shown that PTP1B can remove the inhibitory phosphate from pSrc527,[14] while TC-PTP is capable of dephosphorylating pSrc416.[10] Consistent with these findings, treatment of the cells with TC-PTP inhibitor **8** led to a dose-dependent increase in pSrc416 phosphorylation whereas no significant change in pSrc527, which is not a substrate for TC-PTP, was observed (Figure 2B). This result supports the conclusion that compound **8** is a selective TC-PTP inhibitor in cells.

In summary, we have transformed a weak and general PTP inhibitor into an extremely potent and selective TC-PTP inhibitor with highly efficacious cellular activity, using a stepwise fluorophore-tagged combinatorial/competitive FP-based screen-

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ing strategy. The result serves as a proof-of-concept that low molecular weight structural motifs recognized by the PTPs can be converted into potent, yet highly selective, cell permeable inhibitory agents. Given the potentially general nature of the approach, this strategy should be applicable to other PTP enzymes. Small-molecule inhibitors that are specific for individual PTPs are not only powerful chemical probes that will ultimately help to identify and decipher the physiological roles of PTPs in cell signaling but also lead compounds for the development of novel therapeutics targeting the PTPs.

Materials and Methods

Materials. Dimethylformamide (DMF), isopropanol, dichloromethane (DCM), *N*-methyl morpholine (NMM), and acetic acid (AcOH) were from Fisher Scientific. Diethyl ether, piperidine, trifluoroacetic acid (TFA), triisopropylsilane (TIS), tetrakis(triphenylphosphine)-palladium(0), 4-ethylbenzoic acid (pEBA), and (+)-menthyloxyacetic acid (MOA) were from Aldrich. The Rink amide resin, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBt), Fmoc-Phe-OH, Fmoc-Lys(Alloc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ala-OH, Fmoc-Phe(4-I)-OH, and 9-fluorenylmethyl chloroformate (Fmoc-Cl) were from Advanced ChemTech. 5-(and-6)-Carboxyfluorescein succinimidyl ester (5-(6)-FAM, SE) was from Invitrogen. Fmoc-F_2Pmp-OH was prepared following the literature procedures.[15]

Instrumentation. HPLC purification was carried out on a Waters Breeze HPLC system equipped with a Waters Atlantis dC_{18} column (19 mm × 50 mm). ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker Avance II 500-MHz NMR spectrometer. HRMS data were obtained at the Mass Spectrometry Facility at Indiana University Chemistry Department (http://msf.chem.indiana.edu) on a Waters/Macromass LCT (electrospray ionization ESI). Analytical HPLC analysis was carried out on a Waters Breeze HPLC system equipped with a Waters Symmetry C_{18} column (4.6 mm × 150 mm).

General Procedure A for Rink Amide Resin Activation. Rink amide resin was mixed with DCM (1 mL per 100 mg resin) and then shaken for 30 min. After activation, resin was washed three times with DMF (1 mL per 100 mg resin).

General Procedure B for the Removal of the Fmoc Group from the Rink Amide Resin. Rink amide resin was mixed with 30% piperidine in DMF, shaken for 30 min, and then washed with DMF (1 mL per 100 mg resin, 3 times), isopropanol (1 mL per 100 mg resin, 3 times), and DCM (1 mL per 100 mg resin, 3 times) sequentially. The removal of the Fmoc group was confirmed by the ninhydrin test.

General Procedure C for the Removal the Alloc Group from the Rink Amide Resin. The resin was washed with DCM (1 mL per 100 mg resin, 5×) and shaken under N_2 overnight with a solution of tetrakis(triphenylphosphine)-palladium(0) (10 mg), AcOH (0.5 mL), and NMM (0.2 mL) in DCM (10 mL). The resin was then washed with DMF (1 mL per 100 mg resin, 3 times), isopropanol (1 mL per 100 mg resin, 3 times), and DCM (1 mL per 100 mg resin, 3 times). The removal of the Alloc group was confirmed by the ninhydrin test.

General Procedure D for the Coupling of Carboxylic Acids to the Rink Amide Resin. Carboxylic acids (5 equiv, 0.5 M in DMF) were first mixed with HBTU (5 equiv, 0.5 M in DMF), HOBt (5 equiv, 0.5 M in DMF), and NMM (15 equiv, 1.5 M in DMF). The mixed solution was then added to the resin and shaken for 2 h. The resin was then washed with DMF (1 mL per 100 mg resin, 3 times), isopropanol (1 mL per 100 mg resin, 3 times), and DCM (1 mL per 100 mg resin, 3 times). The completion of the coupling reaction was confirmed by the ninhydrin test.

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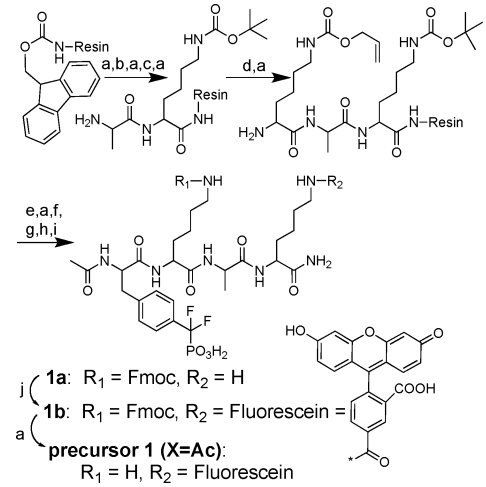
Scheme 3. Synthesis of Precursor **1** (X = Ac) for the First Generation Library[a]



[a] (a) 30% piperidine/DMF; (b) Fmoc-Lys(Boc)-OH/HBTU/HOBt/NMM; (c) Fmoc-Ala-OH/HBTU/HOBt/NMM; (d) Fmoc-Lys(Alloc)-OH/HBTU/HOBt/NMM; (e) Fmoc-F_2Pmp-OH/HBTU/HOBt/NMM; (f) AcOH/HBTU/HOBt/NMM; (g) Tetrakis(triphenylphosphine)-palladium(0), AcOH/NMM/CH_2Cl_2; (h) Fmoc-Cl/NMM; (i) TFA/H_2O/TIS (95:2.5:2.5); (j) 5-(and-6)-carboxyfluorescein, succinimidyl ester/NMM.

General Procedure E for Peptide Cleavage from the Rink Amide Resin. The resin was washed with DCM (1 mL per 100 mg resin, 5 times) and subsequently shaken with 95% TFA, 2.5% TIS, and 2.5% H_2O (1 mL per 100 mg resin). The resin was removed by filtration, and the TFA was evaporated under vacuum. The crude peptide was obtained after trituration with diethyl ether (5 mL per 100 mg resin, 2 times).

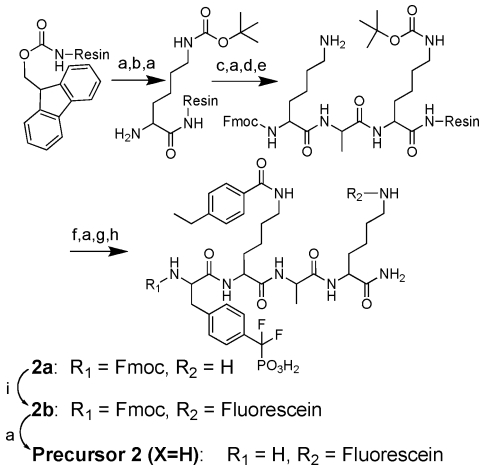
Synthesis of Compound 1a. Compound **1a** was synthesized using standard Fmoc chemistry on the Rink amide resin (Scheme 3). The resin (200 mg, 0.5 mmol/g loading) was first activated (general procedure A). The Fmoc group was removed with 30% piperidine in DMF (general procedure B). The resin was then coupled with Fmoc-Lys(Boc)-OH (general procedure D), followed by the removal of the Fmoc group (general procedure B). The resin was then sequentially coupled with Fmoc-Ala-OH, Fmoc-Lys(Alloc)-OH, Fmoc-F_2Pmp-OH, and AcOH. The Alloc group was then removed (general procedure C). The resin was shaken with Fmoc-Cl (0.2 M in DMF, 2.5 mL) and NMM (1.5 M in DMF, 0.5 mL) for 2 h. Compound **1a** was cleaved from the resin (general procedure E). Crude peptide was purified by HPLC to afford **1a** (24.3 mg, 27% yield). Mass calcd for [M] 885, found $[M + H]^+$ 886.

Synthesis of Compound 1b. Compound **1a** (24.3 mg) was treated with 5(6)-FAM SE (20 mg) and NMM (0.1 mL) in DMF (5 mL) overnight. After evaporation of the solvent, the crude product was purified by reversed-phase HPLC to afford **1b** (11.2 mg, 33% yield). Mass calcd for [M] 1243, found $[M + H]^+$ 1244.

Synthesis of Precursor 1 (X = H). Compound **1b** (11.2 mg) was treated with 30% piperidine in DMF (10 mL) for 30 min. After evaporation of the solvent, the crude product was purified by reversed-phase HPLC to afford **1** (4.7 mg, 51% yield). Mass calcd for [M] 1021, found $[M + H]^+$ 1022.

Synthesis of the First Generation library. In general, the library was prepared on a Freedom EVO workstation (Tecan) with a 96-channel MCA tip block using disposable tips. The detailed procedure is as follows: the 576 different carboxylic acids (40 mM, 10 μL) in DMF were placed in six 96-well microplates. HBTU (35 mM, 10 μL), HOBt (50 mM, 10 μL), and NMM (200 mM, 10 μL) were sequentially added to each well of these plates. The library precursor **1** (2 mM in DMF, 10 μL) was then added to each well. The reactions were quenched with cyclohexylamine (87 mM in DMF, 10 μL) after 1 h. Finally, 190 μL of DMSO were added to

Scheme 4. Synthesis of Precursor **2** (X = H) for the Second Generation Library[a]



[a] (a) 30% piperidine/DMF; (b) Fmoc-Lys(Boc)-OH/HBTU/HOBt/NMM; (c) Fmoc-Ala-OH/HBTU/HOBt/NMM; (d) Fmoc-Lys(Alloc)-OH/HBTU/HOBt/NMM; (e) Tetrakis(triphenylphosphine)-palladium(0), AcOH/NMM/CH_2Cl_2; (f) 4-ethylbenzoic acid/HBTU/HOBt/NMM; (g) Fmoc-F_2Pmp-OH/HBTU/HOBt/NMM; (h) TFA/H_2O/TIS (95:2.5:2.5); (i) 5-(and-6)-carboxyfluorescein, succinimidyl ester/NMM.

each well to create the ready-for-screening format. The library was stored in a −20 °C freezer.

Synthesis of Compound 2a. Compound **2a** was synthesized using standard Fmoc chemistry on the Rink amide resin (Scheme 4). The resin (200 mg, 0.5 mmol/g loading) was first activated (general procedure A), followed by the removal of the Fmoc group (general procedure B). The resin was sequentially coupled with Fmoc-Lys(Boc)-OH, Fmoc-Ala-OH, and Fmoc-Lys(Alloc)-OH (general procedure D). The Alloc group was then removed (general procedure C), and the exposed amine was coupled with 4-ethylbenzoic acid. The Fmoc group was removed, and the exposed amine was coupled with Fmoc-F_2Pmp-OH. Compound **2a** was then cleaved from resin (general procedure E). The crude product was purified by HPLC to afford **2a** (25.5 mg, 26% yield). Mass calcd for [M] 975, found $[M + H]^+$ 976.

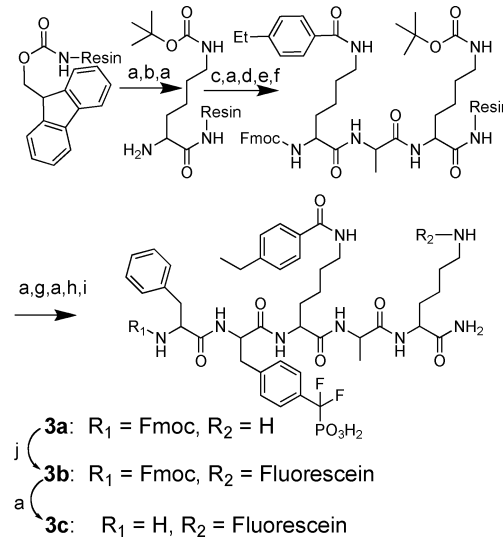
Synthesis of Compound 2b. Compound **2a** (25.5 mg) was treated with 5(6)-FAM SE (20 mg) and NMM (0.1 mL) in DMF (5 mL) overnight. After evaporation of the solvent, the crude product was purified by reversed-phase HPLC to afford **2b** (9.4 mg, 27% yield). Mass calcd for [M] 1333, found $[M + H]^+$ 1334.

Synthesis of Precursor 2 (X = H). Compound **2b** (9.4 mg) was treated with 30% piperidine in DMF (10 mL) for 30 min. After evaporation of the solvent, the crude product was purified by reversed-phase HPLC to afford **2** (3.6 mg, 46% yield). Mass calcd for [M] 1111, found $[M + H]^+$ 1112.

Synthesis of the Second Generation Library. The second generation library was prepared using the same procedure as the first generation library, except that library precursor **2** (1 mM) was used as the library precursor.

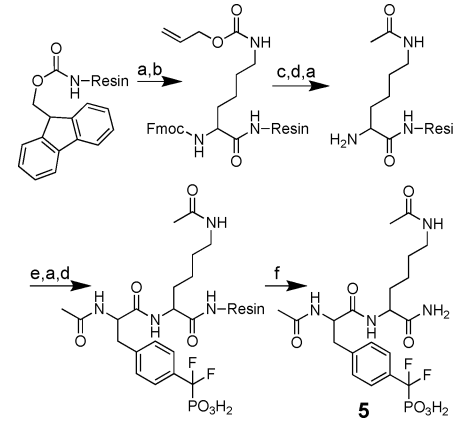
Synthesis of Compound 3a. Compound **3a** was synthesized using standard Fmoc chemistry on the Rink amide resin (Scheme 5). The resin (200 mg, 0.5 mmol/g loading) was first activated (general procedure A), followed by the removal of Fmoc group (general procedure B). The exposed amine was sequentially coupled with Fmoc-Lys(Boc)-OH, Fmoc-Ala-OH, and Fmoc-Lys(Alloc)-OH. The Alloc group was removed (general procedure C), and the exposed amine was coupled with 4-ethylbenzoic acid. After that, the Fmoc group was removed and the resin was sequentially coupled with Fmoc-F_2Pmp-OH and Fmoc-Phe-OH. Compound **3a** was then cleaved from resin (general procedure E). The crude product was purified by HPLC to afford **3a** (22.4 mg, 20% yield). Mass calcd for [M] 1122, found $[M + H]^+$ 1123.

Scheme 5. Synthesis of **3c** as the Precursor for the Third Generation Library[a]



[a] (a) 30% piperidine/DMF; (b) Fmoc-Lys(Boc)-OH/HBTU/HOBt/NMM; (c) Fmoc-Ala-OH/HBTU/HOBt/NMM; (d) Fmoc-Lys(Alloc)-OH/HBTU/HOBt/NMM; (e) Tetrakis(triphenylphosphine)-palladium(0), AcOH/ NMM/CH_2Cl_2; (f) 4-ethylbenzoic acid/HBTU/HOBt/NMM; (g) Fmoc-F_2Pmp-OH/HBTU/HOBt/NMM; (h) Fmoc-Phe-OH/HBTU/HOBt/NMM; (i) TFA/H_2O/TIS (95:2.5:2.5); (j) 5-(and-6)-carboxyfluorescein, succinimidyl ester/NMM.

Scheme 6. Synthesis of **5**[a]



[a] (a) 30% piperidine/DMF; (b) Fmoc-Lys(Alloc)-OH/HBTU/HOBt/NMM; (c) Tetrakis(triphenylphosphine)-palladium(0), AcOH/NMM/CH_2Cl_2; (d) AcOH/HBTU/HOBt/NMM; (e) Fmoc-F_2Pmp-OH/HBTU/HOBt/NMM; (f) TFA/H_2O/TIS (95:2.5:2.5).

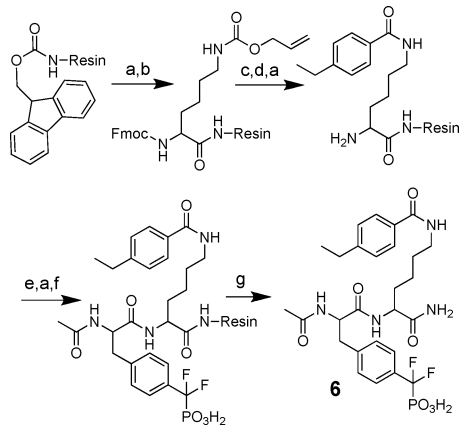
Synthesis of Compound 3b. Compound **3a** (22.4 mg) was treated with 5(6)-FAM SE (20 mg) and NMM (0.1 mL) in DMF (5 mL) overnight. After evaporation of the solvent, the crude product was purified by reversed-phase HPLC to afford **3b** (10.6 mg, 36% yield). Mass calcd for [M] 1480, found $[M + H]^+$ 1481.

Synthesis of Compound 3c. Compound **3b** (10.6 mg) was treated with 30% piperidine in DMF (10 mL) for 30 min. After evaporation of the solvent, the crude product was purified by reversed-phase HPLC to afford **3c** (5.9 mg, 65% yield). Mass calcd for [M] 1258, found $[M + H]^+$ 1259.

Synthesis of the Third Generation Library. The 3nd generation library was prepared using the same procedure as the first generation library, except that **3c** (1 mM) was used as the library precursor.

Synthesis of 5. Compound **5** was synthesized according to Scheme 6, on the Rink amide resin using standard Fmoc chemistry. The resin (200 mg, 0.5 mmol/g loading) was first activated with DCM (general procedure A) and then treated with 30% piperidine to remove the Fmoc group (general procedure B). The exposed

Scheme 7. Synthesis of **6**[a]

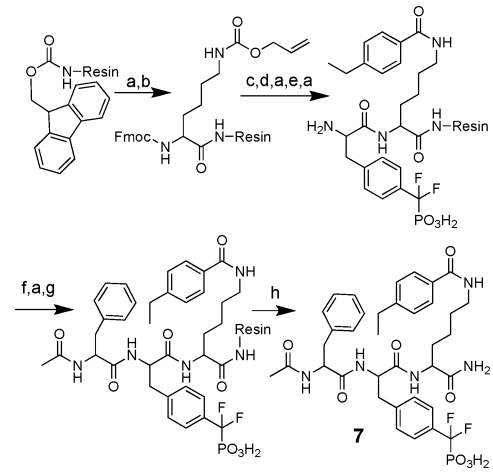


[a] (a) 30% piperidine/DMF; (b) Fmoc-Lys(Alloc)-OH/HBTU/HOBt/NMM; (c) Tetrakis(triphenylphosphine)-palladium(0), AcOH/NMM/CH_2Cl_2; (d) 4-Ethylbenzoic acid/HBTU/HOBt/NMM; (e) Fmoc-F_2Pmp-OH/HBTU/HOBt/NMM; (f) AcOH/HBTU/HOBt/NMM; (g) TFA/H_2O/TIS (95:2.5:2.5).

amine was coupled with Fmoc-Lys(Alloc)-OH (general procedure D). The Alloc group was removed using general procedure C, and the exposed amine coupled with AcOH. The Fmoc group was then removed, and the exposed amine was coupled with Fmoc-F_2Pmp-OH. The Fmoc group was removed, and the exposed amine was coupled with AcOH. Compound **5** was then cleaved from resin (general procedure E). The crude peptide was purified by HPLC to afford **5** (12.3 mg, 24% yield). The assignment of proton NMR utilized additional information from COSY. ^{1}H NMR (500 MHz, CD_3OD): δ = 7.51 (d, J = 8.0 Hz, 2 H, F_2Pmp-Ar**H**), 7.34 (d, J = 8.0 Hz, 2 H, F_2Pmp-Ar**H**), 4.59 (t, J = 7.9 Hz, 1 H, F_2Pmp-C$_\alpha$**H**), 4.28 (dd, J = 9.1 Hz, 5.3 Hz, 1 H, Lys-C$_\alpha$**H**), 3.19−3.09 (m, 2 H, Lys-C$_\varepsilon$**H**$_2$), 3.09−2.97 (m, 2 H, F_2Pmp-C$_\beta$**H**$_2$), 1.95 (s, 3 H, -NHCOC**H**$_3$), 1.93 (s, 3 H, -NHCOC**H**$_3$), 1.76−1.65 (m, 1 H, Lys-C$_\beta$**H**H′), 1.63−1.53 (m, 1 H, Lys-C$_\beta$H**H**′), 1.53−1.43 (m, 2 H, Lys-C$_\delta$**H**$_2$), 1.43−1.25 (m, 2 H, Lys-C$_\gamma$**H**$_2$). ^{13}C NMR (125 MHz, CD_3OD): δ = 176.79, 173.38, 173.23, 173.11, 140.75, 130.34, 127.59, 56.30, 53.53, 40.22, 38.69, 32.50, 29.68, 23.96, 22.46, 22.36. ^{31}P NMR (200 MHz, DMSO-d_6): δ = 3.67 (t, J = 108 Hz). HRMS (ESI): calcd for $C_{20}H_{30}F_2N_4O_7P$ [M + H]$^+$, 507.1815; found, 507.1837. RP-HPLC: t_R = 8.82 min (mobile phase: gradient from 100% H_2O with 20 mM NH_4COCH_3 to 20% CH_3CN in H_2O with 20 mM NH_4COCH_3, over 20 min), purity 97.5% (UV, λ = 254). t_R = 11.69 min (mobile phase: gradient from 100% H_2O with 0.1% TFA to 50% MeOH in H_2O with 0.1% TFA, over 20 min), purity 97.4% (UV, λ = 254 nm).

Synthesis of 6. Compound **6** was synthesized according to Scheme 7, on the Rink amide resin using standard Fmoc chemistry. The resin (200 mg, 0.5 mmol/g loading) was first activated (general procedure A) and subsequently treated with 30% piperidine to remove the Fmoc group (general procedure B). The exposed amine was coupled with Fmoc-Lys(Alloc)-OH (general procedure D). The Alloc group was removed (general procedure C), and the exposed amine was coupled with 4-ethylbenzoic acid. After removal of the Fmoc group, the resin was sequentially coupled with Fmoc-F_2Pmp-OH and AcOH. Compound **6** was then cleaved from resin (general procedure E). The crude product was purified by HPLC to afford **6** (15.8 mg, 27% yield). Structural assignment of ^{1}H NMR utilized additional information from COSY. ^{1}H NMR (500 MHz, CD_3OD): δ = 7.72 (d, J = 8.2 Hz, 2 H, pEBA-Ar**H**), 7.50 (d, J = 7.9 Hz, 2 H, F_2Pmp-Ar**H**), 7.32 (d, J = 7.9 Hz, 2 H, F_2Pmp-Ar**H**), 7.27 (d, J = 8.2 Hz, 2 H, pEBA-Ar**H**), 4.58 (t, J = 7.9 Hz, 1 H, F_2Pmp-C$_\alpha$**H**), 4.30 (dd, J = 8.9 Hz, 5.2 Hz, 1 H, Lys-C$_\alpha$**H**), 3.40−3.33 (m, 2 H, Lys-C$_\varepsilon$**H**$_2$), 3.08−2.96 (m, 2 H, F_2Pmp-C$_\beta$**H**$_2$), 2.68 (q, J = 7.6 Hz, 2 H, pEBA-C**H**$_2$CH$_3$), 1.91 (s, 3 H, −COC**H**$_3$), 1.80−1.71 (m, 1 H, Lys-C$_\beta$**H**H′), 1.68−1.55 (m, 3 H, Lys-C$_\beta$H**H**′ and Lys-C$_\delta$**H**$_2$), 1.51−1.30 (m, 2 H, Lys-C$_\gamma$**H**$_2$), 1.23 (t, J = 7.6 Hz, 3 H, pEBA-CH$_2$C**H**$_3$).^{13}C NMR (125 MHz, CD_3OD): δ = 176.80, 173.21, 173.11, 170.30, 149.50, 140.75, 133.20, 130.32, 128.96, 128.41, 127.58, 56.32, 53.59, 40.64, 38.70, 32.60, 29.94, 29.69, 24.12, 22.33, 15.85. ^{31}P NMR (200 MHz, DMSO-d_6): δ = 3.68 (t, J = 108 Hz). HRMS (ESI): calcd for $C_{27}H_{36}F_2N_4O_7P$ [M + H]$^+$, 597.2284; found, 597.2268. RP-HPLC: t_R = 15.24 min (mobile phase: gradient from 100% H_2O with 20 mM NH_4COCH_3 to 60% CH_3CN in H_2O with 20 mM NH_4COCH_3, over 30 min), purity 99.4% (UV, λ = 254 nm). t_R = 24.33 min (mobile phase: gradient from 100% H_2O with 0.1% TFA to 80% MeOH in H_2O with 0.1% TFA, over 30 min), purity 99.2% (UV, λ = 254 nm).

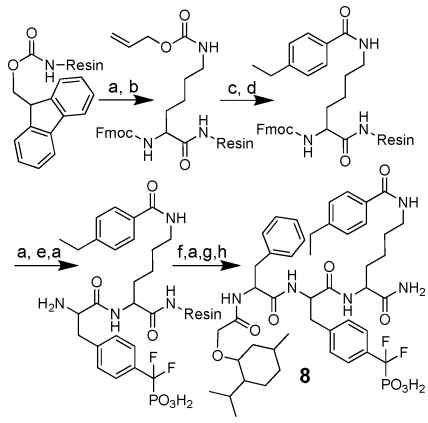
Scheme 8. Synthesis of **7**[a]



[a] (a) 30% piperidine/DMF; (b) Fmoc-Lys(Alloc)-OH/HBTU/HOBt/NMM; (c) Tetrakis(triphenylphosphine)-palladium(0), AcOH/NMM/CH_2Cl_2; (d) 4-Ethylbenzoic acid/HBTU/HOBt/NMM; (e) Fmoc-F_2Pmp-OH/HBTU/HOBt/NMM; (f) Fmoc-Phe-OH/HBTU/HOBt/NMM; (g) AcOH/HBTU/HOBt/NMM; (h) TFA/H_2O/TIS (95:2.5:2.5).

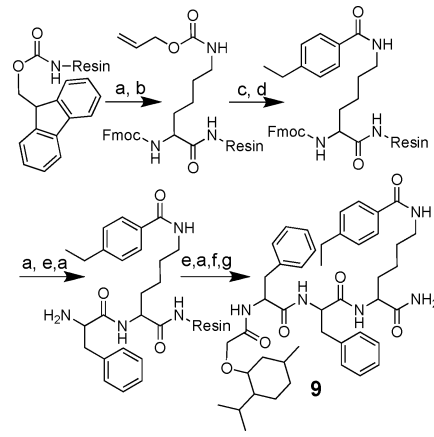
Synthesis of 7. Compound **7** was synthesized according to Scheme 8, on the Rink amide resin using standard Fmoc chemistry. The resin (200 mg, 0.5 mmol/g loading) was activated (general procedure A) and treated with 30% piperidine to remove the Fmoc group (general procedure B). The exposed amine was coupled with Fmoc-Lys(Alloc)-OH (general procedure D). The Alloc group was removed (general procedure C), and the exposed amine was coupled with 4-ethylbenzoic acid. The Fmoc group was removed, and the resin was sequentially coupled with Fmoc-F_2Pmp-OH, Fmoc-Phe-OH, and AcOH. Compound **7** was then cleaved from the resin (general procedure E). The crude product was purified by HPLC to afford **7** (18.2 mg, 24% yield). Structural assignment of ^{1}H NMR utilized additional information from COSY. ^{1}H NMR (500 MHz, CD_3OD): δ = 7.72 (d, J = 8.2 Hz, 2 H, pEBA-Ar**H**) 7.50 (d, J = 7.7 Hz, 2 H, F_2Pmp-Ar**H**), 7.30 (d, J = 7.7 Hz, 2 H, F_2Pmp-Ar**H**), 7.28−7.14 (m, 7 H, pEBA-Ar**H** and Phe-Ar**H**), 4.64 (t, J = 7.7 Hz, 1 H, F_2Pmp-C$_\alpha$**H**), 4.58 (dd, J = 9.5 Hz, 5.2 Hz, 1 H, Phe-C$_\alpha$H), 4.31 (dd, J = 8.1 Hz, 5.4 Hz, 1 H, Lys-C$_\alpha$**H**), 3.42−3.32 (m, 2 H, Lys-C$_\varepsilon$**H**$_2$), 3.14−2.97 (m, 3 H, F_2Pmp-C$_\beta$**H**$_2$ and Phe-C$_\beta$**H**H′), 2.80 (dd, J = 14.0 Hz, 9.6 Hz, 1 H, Phe-C$_\beta$H**H**′), 2.67 (q, J = 7.6 Hz, 2 H, pEBA-C**H**$_2$CH$_3$), 1.85 (s, 3 H, −COC**H**$_3$), 1.83−1.74 (m, 1 H, Lys-C$_\beta$**H**H′), 1.69−1.58 (m, 3H, Lys-C$_\beta$H**H**′ and Lys-C$_\delta$**H**$_2$), 1.50−1.33 (m, 2 H, Lys-C$_\gamma$**H**$_2$), 1.22 (t, J = 7.6 Hz, 3 H, pEBA-CH$_2$C**H**$_3$). ^{13}C NMR (125 MHz, CD_3OD): 173.54 172.68, 170.26, 149.49, 140.67, 138.38, 133.16, 130.40, 130.18, 129.41, 128.97, 128.42, 127.74, 127.56, 56.03, 55.92, 40.64, 38.69, 38.58, 32.63, 29.97, 29.68, 24.16, 22.22, 15.84. ^{31}P NMR (200 MHz, CD_3OD): 3.63 (t, J = 107 Hz). HRMS (ESI): calcd for $C_{36}H_{45}F_2N_5O_8P$ [M + H]$^+$, 744.2968; found, 744.2999. RP-HPLC: t_R = 14.23 min (mobile phase: gradient from 100% H_2O with 20 mM NH_4COCH_3 to 90% CH_3CN in H_2O with 20 mM NH_4COCH_3, over 30 min), purity 99.7% (UV, λ = 254 nm). t_R = 21.57 min (mobile phase: gradient from 25% MeOH in H_2O with 0.1% TFA

Scheme 9. Synthesis of **8**[a]



[a] (a) 30% piperidine/DMF; (b) Fmoc-Lys(Alloc)-OH/HBTU/HOBt/NMM; (c) Tetrakis(triphenylphosphine)-palladium(0), AcOH/NMM/CH_2Cl_2; (d) 4-Ethylbenzoic acid/HBTU/HOBt/NMM; (e) Fmoc-F_2Pmp-OH/HBTU/HOBt/NMM; (f) Fmoc-Phe-OH/HBTU/HOBt/NMM; (g) MOA/HBTU/HOBt/NMM (h) TFA/H_2O/TIS (95:2.5:2.5).

Scheme 10. Synthesis of **9**[a]



[a] (a) 30% piperidine/DMF; (b) Fmoc-Lys(Alloc)-OH/HBTU/HOBt/NMM; (c) Tetrakis(triphenylphosphine)-palladium(0), AcOH/NMM/CH_2Cl_2; (d) 4-Ethylbenzoic acid/HBTU/HOBt/NMM; (e) Fmoc-Phe-OH/HBTU/HOBt/NMM; (f) MOA/HBTU/HOBt/NMM; (g) TFA/H_2O/TIS (95:2.5:2.5).

to 100% MeOH with 0.1% TFA, over 30 min), purity 98.4% (UV, $\lambda = 254$ nm).

Synthesis of 8. Compound **8** was synthesized on the Rink amide resin using standard Fmoc chemistry (Scheme 9). The resin (200 mg, 0.5 mmol/g loading) was activated (general procedure A) and subsequently treated with 30% piperidine to remove the Fmoc group (general procedure B). The exposed amine was coupled with Fmoc-Lys(Alloc)-OH (general procedure D). The Alloc group was removed (general procedure C), and the exposed amine was coupled with 4-ethylbenzoic acid. The Fmoc group was removed, and the resin was sequentially coupled with Fmoc-F_2Pmp-OH, Fmoc-Phe-OH, and MOA. Compound **8** was then cleaved from resin (general procedure E). The crude product was purified by HPLC to afford **8** (3.8 mg, 4% yield). Structural assignment of ^{1}H NMR utilized additional information from COSY. ^{1}H NMR (500 MHz, CD_3OD): $\delta = 7.72$ (d, $J = 8.3$ Hz, 2 H, pEBA-Ar**H**) 7.52 (d, $J = 8.0$ Hz, 2 H, F_2Pmp-Ar**H**), 7.28−7.14 (m, 9 H, Phe-Ar**H**, pEBA-Ar**H**, F_2Pmp-Ar**H**), 4.73 (dd, $J = 8.0$ Hz, 5.1 Hz, 1 H, Phe-C_α**H**), 4.60 (dd, $J = 9.3$ Hz, 6.9 Hz, 1 H, F_2Pmp-C_α**H**), 4.25 (dd, $J = 9.2$ Hz, 5.2 Hz, 1 H, Lys-C_α**H**), 4.00 (d, $J = 15.2$ Hz, 1 H, MOA-O-C**H**H′-CO), 3.70 (d, $J = 15.2$ Hz, 1 H, MOA-O-CH**H**′-CO), 3.45−3.37 (m, 1 H, Lys-C_ε**H**H′), 3.25−3.18 (m, 1 H, Lys-C_εH**H**′), 3.18−3.06 (m, 3 H, F_2Pmp-C_β**H**H′, Phe-C_βH**H**′, and MOA-cyclohexane-C_1**H**), 3.02 (dd, $J = 13.0$ Hz, 9.3 Hz, 1 H, F_2Pmp-C_βH**H**′), 2.94 (dd, $J = 14.0$ Hz, 8.0 Hz, 1 H, Phe--C_βH**H**′), 2.67 (q, $J = 7.6$ Hz, 2 H, pEBA-C**H**$_2$CH$_3$), 2.10−1.99 (m, 1 H, MOA-C**H**$(CH_3)_2$), 1.99−1.92 (m, 1 H, MOA-cyclohexane-C_6**H**$_{eq}$), 1.80−1.69 (m, 1 H, Lys-C_β**H**H′), 1.67−1.50 (m, 5 H, Lys-C_βH**H**′, Lys-C_δ**H**$_2$, MOA-cyclohexane-C_3**H**$_{eq}$, and MOA-cyclohexane-C_4**H**$_{eq}$), 1.50−1.27 (m, 3 H, Lys-C_γ**H**$_2$ and MOA-cyclohexane-C_5**H**), 1.23 (t, $J = 7.6$ Hz, 3 H, pEBA-CH$_2$C**H**$_3$). 1.20−1.15 (m, 1 H, MOA-cyclohexane-C_2**H**), 1.02−0.91 (m, 1 H, MOA-cyclohexane-C_3**H**$_{ax}$), 0.91−0.85 (m, 6 H, MOA-cyclohexane-C_5-C**H**$_3$ and MOA-cyclohexane-C_2-CH(C**H**$_3$)(CH$_3$)′), 0.85−0.77 (m, 1 H, MOA-cyclohexane-C_4**H**$_{ax}$), 0.77−0.66 (m, 4 H, MOA-cyclohexane-C_2-CH(CH$_3$)(C**H**$_3$)′ and MOA-cyclohexane-C_6**H**$_{ax}$). ^{13}C NMR (125 MHz, CD_3OD): 172.82, 172.68, 172.62, 170.04, 149.45, 139.08, 137.59, 133.15, 130.52, 129.93, 129.53, 128.95, 128.50, 127.94, 127.85, 81.47, 68.43, 56.42, 54,62, 53.75, 40.90, 40.64, 39.07, 38.76, 35.55, 32.60, 32.34, 29.86, 29.70, 26.84, 24.16, 22.63, 21.46, 16.51, 15.87. ^{31}P NMR (200 MHz, DMSO-d_6): 1.41 (t, $J = 92$ Hz). HRMS (ESI): calcd for $C_{46}H_{63}F_2N_5O_9P$ [M + H]$^+$, 898.4326; found, 898.4294. RP-HPLC: $t_R = 15.43$ min (mobile phase: gradient from 35% CH_3CN in H_2O with 20 mM NH_4COCH_3 to 82.5% CH_3CN in H_2O with 20 mM NH_4COCH_3, over 30 min), purity 99.8% (UV, $\lambda = 254$ nm). $t_R = 22.76$ min (mobile phase: gradient from 70% MeOH in H_2O with 0.1% TFA to 100% MeOH with 0.1% TFA, over 30 min), purity 97.9% (UV, $\lambda = 254$ nm).

Synthesis of 9. Compound **9** was synthesized on the Rink amide resin using standard Fmoc chemistry (Scheme 10). The resin (200 mg, 0.5 mmol/g loading) was activated (general procedure A) and subsequently treated with 30% piperidine to remove the Fmoc group (general procedure B). The exposed amine was coupled with Fmoc-Lys(Alloc)-OH (general procedure D). The Alloc group was removed (general procedure C), and the exposed amine was coupled with 4-ethylbenzoic acid. The Fmoc group was removed, and the resin was sequentially coupled with Fmoc-Phe-OH, Fmoc-Phe-OH, and MOA. Compound **9** was then cleaved from resin (general procedure E). The crude product was purified by HPLC to afford **9** (13.6 mg, 18% yield). Mass calcd for [M] 767, found [M + H]$^+$ 768. ^{1}H NMR (500 MHz, DMSO-d_6): $\delta = 8.41$−8.33 (m, 2 H, Lys-N$_\xi$**H** and MOA-Phe-N**H**), 8.07 (d, $J = 8.0$ Hz, 1 H, Lys-N**H**), 7.77 (d, $J = 8.2$ Hz, 2 H, pEBA-Ar**H**) 7.30−7.10 (m, 14 H, MOA-Phe-Ar**H**, Phe-Phe-Ar**H**, pEBA-Ar**H**, Phe-Phe-N**H**, and Lys-CON**H**H′), 7.05 (s, 1 H, Lys-CONH**H**′), 4.64−4.53 (m, 2 H, MOA-Phe-C_α**H** and Phe-Phe-C_α**H**), 4.27−4.19 (m, 1 H, Lys-C_α**H**), 3.90 (d, $J = 15.0$ Hz, 1 H, MOA-O-C**H**H′-CO), 3.71−3.59 (m, 3.3 H, MOA-O-CH**H**′-CO, residue water peak), 3.31−3.16 (m, 2 H, Lys-C_ε**H**$_2$), 3.12−3.01 (m, 2 H, MOA-Phe-C_β**H**H′ and MOA-cyclohexane-C_1**H**), 2.98 (dd, $J = 13.9$ Hz, 4.3 Hz, 1 H, Phe-Phe-C_β**H**H′), 2.89−2.78 (m, 2 H, MOA-Phe-C_βH**H**′ and Phe-Phe-C_βH**H**′), 2.63 (q, $J = 7.6$ Hz, 2 H, pEBA-C**H**$_2$CH$_3$), 2.08−1.99 (m, 1 H, MOA-C**H**$(CH_3)_2$), 1.99−1.93 (m, 1 H, MOA-cyclohexane-C_6**H**$_{eq}$), 1.78−1.67 (m, 1 H, Lys-C_b**H**H′), 1.65−1.46 (m, 5 H, Lys-C_βH**H**′, Lys-C_δ**H**$_2$, MOA-cyclohexane-C_3**H**$_{eq}$, and MOA-cyclohexane-C_4**H**$_{eq}$), 1.43−1.22 (m, 3 H, Lys-C_γ**H**$_2$ and MOA-cyclohexane-C_5**H**), 1.22−1.09 (m, 4 H, pEBA-CH$_2$C**H**$_3$ and MOA-cyclohexane-C_2**H**), 0.93−0.86 (m, 1 H, MOA-cyclohexane-C_3**H**$_{ax}$), 0.86−0.80 (m, 6 H, MOA-cyclohexane-C_5-C**H**$_3$ and MOA-cyclohexane-C_2-CH(C**H**$_3$)(CH$_3$)′), 0.80−0.73 (m, 1 H, MOA-cyclohexane-C_4**H**$_{ax}$), 0.73−0.66 (m, 1 H, MOA-cyclohexane-C_6**H**$_{ax}$), 0.64 (d, $J = 6.9$ Hz, 3 H, MOA-cyclohexane-C_2-CH(CH$_3$)(C**H**$_3$)′). ^{13}C NMR (125 MHz, DMSO-d_6): 173.39, 170.75, 170.35, 168.88, 165.99, 146.92, 137.69, 137.06, 132.20, 129.30, 129.22, 128.03, 127.89, 127.52, 127.25, 126.27, 79.19, 67.29, 53.90, 52.59, 52.49, 47.44, 37.56, 37.52, 33.97, 31.91, 30.78, 28.97, 28.01, 25.14, 22.85, 22.72, 22.13, 20.86, 16.01, 15.36. HRMS (ESI): calcd for $C_{45}H_{61}N_5O_6Na$ [M + Na]$^+$: 790.4514, found: 790.4510. RP-HPLC: $t_R = 23.70$ min (mobile phase: gradient from 50% CH_3CN in H_2O with 20 mM NH_4COCH_3 to 90% CH_3CN with 20 mM NH_4COCH_3, over 40 min), purity 98.8% (UV, $\lambda = 254$ nm). $t_R = 20.44$ min (mobile phase: gradient from 70% MeOH in H_2O with 0.1% TFA to 90% MeOH in H_2O with 0.1% TFA, over 20 min, followed by 90%

MeOH in H_2O with 0.1 TFA for another 20 min), purity 99.5% (UV, $\lambda = 254$ nm).

Screening of the First Generation Library. The library was screened using the Tecan Genesis workstation with a 96-channel tip block with fixed tips. Before screening, the library compounds were diluted from the DMSO stock solution into DMG buffer (50 mM 3,3-dimethylglutarate buffer, pH 7.0, containing 1 mM EDTA with an ionic strength of 0.15 M adjusted by addition of NaCl), resulting in a set of six daughter plates with an ~75 nM concentration of each compound in each well. In the first screen, TC-PTP (2 μM in DMG buffer, 50 μL) was dispensed into each well of a 384-well plate, and then 2 μL of the fluorescein-tagged library compounds were transferred from four 96-well intermediate plates to the 384-well plate (final compound concentration ~3 nM). The fluorescence polarization values (A_1) were determined on Envision 2021 Multilabel Microplate Reader (Perkin-Elmer). In the second screen, 50 μL of a mixture of 2 μM TC-PTP and 500 μM Fmoc-F_2Pmp-OH (as a competitive ligand) in DMG buffer were dispensed into each well of another 384-well plate, followed by the addition of 2 μL of the fluorescein tagged library compounds (75 nM in DMG buffer). The fluorescence polarization values (A_2) were again measured. A displacement percentage was calculated for each library compound as $(A_1 - A_2)/(A_1 - A_0) \times 100\%$, where A_1 and A_2 are the fluorescence anisotropy values of each sample in screen 1 and screen 2, respectively, and A_0 is the fluorescence anisotropy of free library compounds in DMG buffer. To simplify the calculation, A_0 was set to 30. The binding affinity ranking of each compound was determined on the displacement percentage: the smaller the displacement percentage, the higher the binding affinity. The best hits were selected based on affinity and are listed in Table S1 (Supporting Information).

Screening of the Second Generation Library. The library was screened using the same protocol as the first generation library, except that 0.4 μM TC-PTP and 1.5 mM competitive ligand Fmoc-F_2Pmp-OH were used. The best hits were selected based on affinity and are listed in Table S2 (Supporting Information).

Screening of the Third Generation Library. The library was screened using the same protocol as the first generation library, except that 0.5 μM TC-PTP and 5 μM compound **7** (as a competitor) were used. The library was also screened against PTP1B under the same conditions. The best hits were selected based on both affinity and selectivity against PTP1B (Table S3, Supporting Information).

Determination of Inhibition Constant (K_i) and IC_{50} Value. PTP activity was assayed using *p*-nitrophenyl phosphate (*p*NPP) as a substrate in DMG buffer (50 mM DMG, pH 7.0, 1 mM EDTA, 150 mM NaCl, 2 mM DTT, 0.1 mg/mL BSA) at 25 °C. The assays were performed in 96-well plates. Normally, to determine the IC_{50} values, the reaction was initiated by the addition of enzyme (final concentration at 10 nM) to a reaction mixture (0.2 mL) containing 2 mM (K_m for the substrate) *p*NPP with various concentrations of inhibitors. The reaction rate was measured using a SpectraMax Plus 384 Microplate Spectrophotometer (Molecular Devices). For Compound **8**, the final TC-PTP concentration was 0.4 nM. The reactions were incubated at room temperature for 30 min and quenched with 5 N NaOH. The absorbance at 405 nm was read on the SpectraMax Plus 384 Microplate Spectrophotometer. For the reversibility test, the enzyme was incubated with the inhibitor at room temperature for 30 min before *p*NPP was added to initiate the reaction. The reaction was then incubated at room temperature for 30 min and quenched and read. IC_{50} values determined were the same with or without enzyme preincubation with the inhibitor (8.7 ± 1.4 and 9.2 ± 1.4 nM, respectively). To determine the mode of inhibition, the reactions were initiated by the addition of enzyme (final concentration for TC-PTP was 0.4 nM and for PTP1B was 2 nM) to the reaction mixtures (0.2 mL) containing various concentrations of *p*NPP with different concentrations of the inhibitor. Data were fitted using SigmaPlot Enzyme Kinetics Module (Systat Software, Inc.).

Cellular Activity of TC-PTP Inhibitor 8. PTP1B$^{-/-}$ mouse embryo fibroblast cells (a generous gift from Dr. Michel L. Tremblay) were treated with TC-PTP inhibitor at the indicated concentration for 2 h prior to EGF treatment. After EGF treatment (2 ng/mL) for 10 min, cells were lysed in 10 mM HEPES, pH 7.4, 150 mM NaCl, 10% Glycerol, 10 mM sodium phosphate, 10 mM sodium fluoride, 1 mM sodium pervanadate, 1 mM benzamidine, 1% Triton X-100, 10 μg/mL leupeptin, and 5 μg/mL aprotinin. Cell lysates were cleared by centrifugation at 15 000 rpm for 10 min. The lysate protein concentration was estimated using the BCA protein assay kit (Pierce). To detect EGFR phosphorylation levels, 10 μg of anti-EGFR antibody (Cell Signaling, #2232) were added to 1 mg of cell lysate and incubated at 4 °C for 2 h. 20 μL of protein A/G-agarose beads were then added and incubated for another 2 h. After extensive washing, the protein complex was boiled with sample buffer, separated by SDS-PAGE, transferred electrophoretically to a PVDF membrane, and immunoblotted with anti-pTyr antibodies (Millipore, #05-1050) followed by incubation with horseradish peroxidase conjugated secondary antibodies. The blots were developed by the enhanced chemiluminescence technique (ECL kit, Amersham Biosciences).

To study the effect of compound **8** on Src phosphorylation, PTP1B$^{-/-}$ mouse embryo fibroblast cells were grown to 80% confluence before being treated with TC-PTP inhibitor **8** for 2 h. Cells were lysed, and the lysates were cleared by centrifugation at 15 000 rpm for 10 min. 30 μg of total proteins were boiled with sample buffer, separated by SDS-PAGE, transferred electrophoretically to a PVDF membrane, and immunoblotted with anti-pSrc416, anti-pSrc527 antibodies (Invitrogen 44-660G and 44-662G) and anti-Src antibody (Cell Signaling Technology #2108) followed by incubation with horseradish peroxidase conjugated secondary antibodies. The blots were developed by the enhanced chemiluminescence technique (ECL kit, Amersham Biosciences).

Acknowledgment. We are thankful to the NIH (RO1 CA126937 and RO1 CA079954) for financial support.

Supporting Information Available: Primary screening results, NMR spectra, HPLC chromatographs, and the structures of 576 carboxylic acids. This material is available free of charge via the Internet at http://pubs.acs.org.

JA903733Z