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An Affinity-Based Fluorescence Polarization Assay for Protein Tyrosine Phosphatases

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Abstract

Protein tyrosine phosphatases (PTPs) are important signaling enzymes that control such fundamental processes as proliferation, differentiation, survival/apoptosis, as well as adhesion and motility. Potent and selective PTP inhibitors serve not only as powerful research tools, but also as potential therapeutics against a variety of illness including cancer and diabetes. PTP activity-based assays are widely used in high throughput screening (HTS) campaigns for PTP inhibitor discovery. These assays suffer from a major weakness, in that the reactivity of the active site Cys can cause serious problems as highly reactive oxidizing and alkylating agents may surface as hits. We describe the development of a fluorescence polarization (FP)-based displacement assay that makes the use of an active site Cys to Ser mutant PTP (e.g., PTP1B/C215S) that retains the wild type binding affinity. The potency of library compounds is assessed by their ability to compete with the fluorescently labeled active site ligand for binding to the Cys to Ser PTP mutant. Finally, the substitution of the active site Cys by a Ser renders the mutant PTP insensitive to oxidation and alkylation and thus will likely eliminate “false” positives due to modification of the active site Cys that destroy the phosphatase activity.

Keywords

protein tyrosine phosphatases (PTPs); high throughput screening (HTS); fluorescence polarization (FP); PTP inhibitor; assay development

1. Introduction

Protein tyrosine phosphatases (PTPs) are important signaling enzymes that control such fundamental processes as proliferation, differentiation, survival/apoptosis, as well as adhesion and motility [1]. More than 100 PTPs have been identified from the human genome, all share the common active site sequence (H/V)C(X)₅R(S/T) [2]. Evidence suggests that abnormal regulation of PTP activity causes aberrant tyrosine phosphorylation, which contributes to the development of many human diseases including cancers, autoimmune disorders and diabetes [3]. Although crucial physiological roles have been documented for a number of PTPs [1], the biological significance of the majority of the gene products in the PTP family is not completely known. One of the major challenges in the PTP field is to establish the exact functional roles

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for every PTP, both in normal cellular physiology and in pathogenic conditions. This is an important prerequisite for PTP-based drug discovery in order to minimize unwanted side effect.

Our current knowledge of PTPs has been mostly derived from experiments using gene knockout or overexpression techniques. While these studies have provided important insight into PTP biology, these approaches do have limitations. For example, overexpression may be associated with unwanted non-specificity, and gene knockouts in animals may result in compensatory effects through other mechanisms during embryonic development. Chemical approaches using small molecule inhibitors to interrogate molecular components of signal transduction pathways offers an invaluable means of analysis complementary to genetic techniques [4,5]. The main advantages of the small molecule approach include its simplicity, speed, tunability, and reversibility. In addition, small molecule inhibitors exert their effect on endogenous targets, avoiding the need for overexpression of dominant-negative or constitutively active mutants, which can cause artifacts and lead to erroneous conclusions. Indeed, the availability of agents with specificity for a number of protein kinases has greatly enhanced our ability to identify their substrates and physiological functions [6].

Obviously, small molecule PTP inhibitors can not only serve as research tools to elucidate the roles of PTPs in cellular signaling [7] but also hold promise for therapeutic development in a number of disease areas, including cancer and diabetes [8]. Until now, the most popular approach for PTP inhibitor discovery is high throughput screening (HTS) of chemical libraries. The PTP hydrolyzes pTyr with a well define catalytic mechanism, in which the active site Cys directly attacks the phosphorus atom in the substrate to form a thiophosphoryl enzyme intermediate. Hydrolysis of this covalent enzyme intermediate then completes the catalytic cycle [9]. In addition to physiological substrates such as phosphoproteins, the PTPs can also hydrolyze small molecule aryl phosphates, including: *p*-nitrophenyl phosphate (*p*NPP), 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP), and 3-O-methylfluorescein phosphate (OMFP). The ability of PTPs to hydrolyze small molecule chromogenic or fluorogenic substrates coupled with the robustness of output signals makes these assays easily amenable for high-throughput screening. Usually, the efficacy of potential PTP inhibitors is determined for their ability to inhibit the PTP-catalyzed hydrolysis of the chromogenic or fluorogenic substrates.

Although the PTP activity-based assays are widely employed in HTS campaigns for inhibitor discovery, they all suffer from one major drawback, due in large part to the reactivity of the active site Cys residue. Consequently, the PTPs are prone to inactivation by oxidizing reagents, heavy metals, and alkylating reagents commonly found in various chemical compound collections. This has presented a serious problem for the PTP activity-based inhibitor screening projects in which hits are identified based on the ability of the compounds to reduce the PTP activity. To eliminate false positives due to covalent modifications of the active site Cys, we have developed a competitive, homogeneous, high-throughput fluorescence polarization assay to identify high affinity active site binders to the PTPs. This assay is based on the observations that the catalytically inactive Cys to Ser mutant PTP (e.g., PTP1B/C215S) retains the ability to bind substrates [10,11], and with the same affinity as the wild-type enzyme [12-14]. Compounds that are capable of displacing a fluorescently labeled ligand from the active site of the Cys to Ser mutant PTP are likely competitive PTP inhibitors. In the following, we will use PTP1B as an example and describe the development of a fluorescence polarization (FP) based displacement assay that can be employed either as a primary screen for PTP inhibitor identification or as a secondary screen to validate hits generated from activity-based assays.

2. FP-based Displacement Assay

In a FP-based displacement assay, changes in the orientation of plane-polarized light brought about by a fluorophore are measured. The fluorophore undergoes significant tumbling motion during the period of time between absorption of an excitation photon and the emission of a photon through fluorescence. No significant polarization will be observed if the fluorophore is attached to a small ligand (<5000 Da) because of its rapid rotation. However, if the fluorescent ligand binds to a protein of significantly greater size, the ability of the fluorescent ligand to keep plane-polarized light is greatly enhanced, leading to increased FP signal. When the bound fluorescent ligand is displaced by a competing non-fluorescent molecule, the FP signal will drop. Therefore the decrease in FP can be used to identify competing molecules that bind to the same site occupied by the fluorescent ligand. Given its homogeneous nature and simplicity, FP is rapidly becoming the method of choice for detecting and measuring protein-protein and protein-ligand interactions [15-18].

3. Theory of FP-based Displacement Assay

The mathematical treatment for deriving binding parameters from the FP-based displacement assay is briefly described below. The polarization value (P) is defined as $(I_{//} - I_{\perp}) / (I_{//} + I_{\perp})$, where $I_{//}$ and $I_{\perp}$ are fluorescence intensities detected from the parallel and perpendicular channels, respectively, of a fluorometer. In mathematical calculation, the anisotropy value (A) is normally used instead of the polarization value (P). The relationship between A and P is shown in Equation 1.

$$A = 2P / (3 - P) \quad (1)$$

where A is a unitless value, and commonly represented as millianisotropy (mA).

In a mixture containing multiple fluorophores, the anisotropy is determined by the ratio of each fluorescent component in a linear manner (Equation 2).

$$A = \phi_1 A_1 + \phi_2 A_2 + \dots \phi_i A_i \quad (2)$$

In Equation 2, A_i is the anisotropy of the i^{th} component, and ϕ_i is the fractional fluorescence intensity of the i^{th} component.

Equation 3 describes a 1:1 binding reaction between a fluorophore (F) and a protein (E):

When $[F] \ll [E]$, the anisotropy of the system can be expressed as Equation 4:

$$A = (A_{\max} - A_{\min}) \frac{E_0}{K_d + E_0} + A_{\min} \quad (4)$$

Where, $A_{\min}$ is the anisotropy value of the free fluorophore, $A_{\max}$ is the anisotropy value of the protein/fluorophore complex, E_0 is the total protein concentration, and K_d is the dissociation constant of the protein/fluorophore complex. As shown in Equation 4, the anisotropy value of the solution increases as the protein concentration increases, but it is independent of the fluorophore concentration when the fluorophore concentration is significantly lower than the protein concentration.

When a competitor (I) which competes for the same binding site with F, is added to the solution of the protein/fluorophore complex, as shown in Equation 5,

and when $[F] \ll [P]$ and $[F] \ll [I]$, the anisotropy value of the system can be calculated by Equation 6 and 7,

$$A = (A_{\max} - A_{\min}) \frac{E_{app}}{K_d + E_{app}} + A_{\min} \quad (6)$$

$$\text{and } E_{app} = E_0 - \frac{E_0 + I_0 + K_I - \sqrt{(E_0 + I_0 + K_I)^2 - 4E_0I_0}}{2} \quad (7)$$

where $A_{\min}$ is the anisotropy value of the free fluorophore, $A_{\max}$ is the anisotropy value of the bounded fluorophore, E_{app} is the apparent protein concentration, defined by Equation 7, E_0 is the total protein concentration, I_0 is the total concentration of the competitor, K_d is the dissociation constant of protein/fluorophore complex, and the K_I is the dissociation constant of protein/competitor complex. As shown in Equation 6 and 7, the anisotropy value of the solution will decrease if the competitor concentration increases; and, at the same concentration, the tighter the binding (the smaller the K_I), the greater decrease in anisotropy will result. Therefore, when a library is screened at a fixed concentration, compounds with higher binding affinity to the protein will cause larger decreases in anisotropy, so the ΔA value (the difference of anisotropy value before and after the addition of library compounds) can be used as a parameter to evaluate the relative potency of competing ligands.

4. Materials and Reagents

The NovaSyn® TGR resin was from NovaBiochem (www.novabiochem.com). Fmoc-Dap (Boc)-OH was from Bachem (www.bachem.com). Fmoc-F₂Pmp-OH was prepared by following the published procedure [19,20]. O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt) and piperidine were from Advanced ChemTech (www.peptide.com). 6-[fluorescein-5(6)-carboxamido] hexanoic acid N-hydroxysuccinimide ester was from Invitrogen (probes.invitrogen.com). N-methylmorpholine (NMM) was from Aldrich (www.aldrich.com). N,N-dimethylformamide (DMF), isopropanol, dichloromethane (DCM) were from Fisher Scientific (www.fishersci.com). Other reagents were either from Aldrich or Fisher Scientific.

5. Development of a FP-based Displacement Assay for PTP1B

5.1 Identification of an Active Site-Directed Fluorescent-Tagged PTP1B Ligand

In order to develop a FP-based displacement assay for PTP1B, we first need to identify a fluorescently labeled small molecule that binds to the active site of PTP1B, so that the compounds that can displace it from PTP1B will mostly likely act as competitive inhibitors, which are normally desired. Ideally a ligand with an affinity at micro-molar level is preferred, so that it can bind to PTP1B at a reasonable concentration and can also be displaced by competitors during HTS. Since pTyr is the native ligand for PTP active site, a logical approach is to attach an appropriate fluorescent tag to a nonhydrolyzable pTyr surrogate. The most useful and potent pTyr surrogate thus far devised for PTPs is phosphonodifluoromethyl phenylalanine (F₂Pmp) [21]. Unfortunately, the affinity of F₂Pmp for PTP1B is in the mM range, which is not optimal for the FP-based assay. To search for an optimal PTP1B active site-directed ligand,

we screened a focused library in which the NH_2 group from the F_2Pmp residue was condensed with 720 different carboxylic acids. Compound **I** (Figure 1) was identified from this library, which exhibits a competitive inhibition pattern with a K_i of $0.6 \pm 0.1 \mu\text{M}$ against PTP1B. Attachment of fluorescein to the free amino group of compound **III** (Scheme 1), which was prepared using standard Fmoc-based solid phase peptide synthesis, produced the active site-directed fluorescent-tagged PTP1B ligand **II** (Figure 1).

The detailed procedures are described below:

- a. NovaSyn® TGR resin (100 mg, 23 μmol) was placed into a 6-ml Aldrich® fritted filter funnel with DMF (4 mL). The funnel was capped and shaken for 30 min using an Innova® 2000 shaker (New Brunswick Scientific, www.nbsc.com) with the speed setting at 200 rpm. The solvent was then removed by filtration.
- b. A mixture of Fmoc-Dap(Boc)-OH (19.6 mg, 46 μmol), HBTU (0.5 M in DMF, 92 μL , 46 μmol), HOBt (0.5 M in DMF, 92 μL , 46 μmol) and NMM (1.5 M in DMF, 92 μL , 138 μmol) in 4 mL DMF was added to the NovaSyn® TGR resin from step (a) in the Aldrich® fritted filter funnel. The funnel was capped and shaken for 2 hours. The liquid phase was then removed by filtration, and the resin was washed with DMF (4 mL, 3 times), isopropanol (4 mL, 3 times) and DCM (4 mL, 3 times).
- c. A solution of 30% piperidine in DMF (4 mL) was added to the resin from step (b) in the funnel and shaken for 30 min. After the removal of liquid phase from the reaction mixture by filtration, the resin was washed with DMF (4 mL, 3 times), isopropanol (4 mL, 3 times) and DCM (4 mL, 3 times).
- d. Step (b) and (c) was repeated for the coupling of Fmoc- F_2Pmp -OH (23.8 mg, 46 mmol).
- e. A mixture of 3,5-dihydrox-2-naphthoic acid (9.4 mg, 46 mmol), HBTU (0.5 M in DMF, 92 μL , 46 μmol), HOBt (0.5 M in DMF, 92 μL , 46 μmol) and NMM (1.5 M in DMF, 92 μL , 138 μmol) in 4 mL DMF was mixed together with the resin and then shaken for 2 hours. The resin was collected by filtration and washed with DMF (4 mL, 3 times), isopropanol (4 mL, 3 times) and DCM (4 mL, 3 times).
- f. Crude compound **III** was cleaved from the resin by treatment with a mixture of TFA/TIS/Water (95:2.5:2.5, 4 mL) for 4 hours at room temperature. The TFA solution was collected and evaporated. Compound **III** was then purified using a Breeze HPLC system (www.waters.com) equipment with an Atlantis® dC₁₈ OBD™ column (19 - 50 mm, 5 μM) (www.waters.com).
- g. Compound **III** (5mg, 8.8 mmol) in DMF (0.5 mL) was added to a solution of 6-[fluorescein-5(6)-carboxamido] hexanoic acid N-hydroxysuccinimide ester (10 mg, 17 mmol) and triethylamine (0.1 mL, 720 mmol) in DMF (0.5 mL), and the resulting mixture was stirred at room temperature for 24 hours. The solvent was evaporated under vacuum and compound **II** was purified using HPLC.

5.2 Assay Conditions and Procedures

Compound **II** was used as the fluorescent probe to bind PTP1B in the FP assay. The FP assay was carried out in black 384-well microplates and the final assay volume was 50 μL . Fluorescence intensities and the anisotropy were measured using either a TECAN Ultra 384 or a Perkin Elmer EnVision multimode plate reader at room temperature. The excitation is set at 485 nm and the emission is collected at 535 nm. The reagents for the FP assay are listed below:

- a. DMG buffer: 50 mM 3,3-dimethylglutarate, pH 7.0, 1 mM EDTA, with ionic strength adjusted to 0.15 M by adding NaCl.
- b. Stock solutions (10 mM) of each compound (including **II**) were prepared in DMSO and frozen at -20°C for storage.
- c. The proteins were stored in DMG buffer with 30% glycerol at -20°C, and were diluted using DMG buffer when necessary.

In general, the FP experiments were conducted in the following way:

- a. To check the background fluorescence intensity of the DMG buffer, an aliquot of DMG buffer (50 μ L) was added to a black 384-well microplate (Nalge Nunc International, www.nuncbrand.com), and the background fluorescence intensity was measured using the Ultra 384 (TECAN) multimode plate reader.
- b. To measure the fluorescence polarization of free compound **II**, a solution of **II** (20 nM) in DMG buffer (total 50 μ L) was added to a black 384-well microplate, and the fluorescence polarization value was measured using the Ultra 384 (TECAN) multimode plate reader.
- c. To measure the fluorescence polarization of protein/compound **II** complex, a solution of **II** (20 nM) and PTP1B/C215S mutant (0.5 μ M) in DMG buffer (50 μ L total volume) was added to a black 384-well microplate. After 5 min incubation at room temperature, the FP value was measured.
- d. To determine the displacement of the compound **II** from PTP1B/C215S, a solution of compound **II** (20 nM), PTP1B/C215S (0.5 μ M) and a competitor compound **IV** (Figure 1) (0.5 μ M) in DMG buffer (50 μ L) was added to a black 384-well microplate and incubated for 5 min at room temperature. The FP value was measured.

5.3 Evaluation of Compound **II** Binding to PTP1B/C215S

To determine the binding affinity of compound **II** to the PTP1B/C215S mutant, we measured the dissociation constant for the compound **II**/PTP1B/C215S complex. Compound **II** (20 nM) alone in the DMG buffer gave a polarization value of ~ 40 mA. As shown in Figure 2, addition of PTP1B/C215S to compound **II** (20 nM) caused a concentration dependent increase in FP. Fitting the data to Equation 4 yields a K_d value of 0.41 ± 0.06 μ M, which suggests that compound **II** is suitable for the FP based displacement assay.

5.4 Displacement of Compound **II** from PTP1B/C215S with a Known PTP1B Inhibitor

To further confirm that compound **II** binds to the active site of PTP1B/C215S and the binding is reversible, we used compound **IV** (Figure 1), a known competitive inhibitor of PTP1B ($K_i = 0.73 \pm 0.04$ μ M) to test that if it can displace compound **II** from PTP1B. The displacement was carried out by adding different concentrations of **IV** to a solution of **II** (20 nM) and PTP1B/C215S (0.5 μ M) in DMG buffer. The anisotropy values of the resulting solution were measured and plotted versus the concentrations of **IV** (Figure 3). The displacement data was fitted to Equation 6 and 7 using SigmaPlot, yielding a K_d of 0.7 ± 0.3 μ M (averaged from 3 independent measurements). The excellent agreement between the K_i values measured by steady state kinetics and the K_d values determined from the FP-based binding assay indicate that compound **II** binds to the active site of PTP1B/C215S and a competitive inhibitor can displace it from the active site. These results also confirm that PTP1B/C215S binds ligands with the same affinity as the wild-type enzyme and validate the use of PTP1B/C215S in the FP assay. Thus the FP-based displacement assay can be used to screen for PTP1B inhibitors.

6. Optimization of the FP-based Displacement Assay for HTS

6.1 Determination of Z' Factors

To evaluate the suitability of the FP-based displacement assay for HTS, we determined the Z' factor associated with the assay, which is commonly used to assess the quality and suitability of a HTS assay [22]. The Z' factor is defined by Equation 8,

$$Z' = 1 - \frac{3(SD_p + SD_n)}{|\mu_p - \mu_n|} \quad (8)$$

Where SD_p and SD_n are the standard deviation of the signal for positive and negative controls, and μ_p and μ_n is the mean value of the positive and negative controls, respectively. Normally, an assay with a Z' factor value between 0.5 and 1 is expected to give excellent performance in high throughput screening [22]. We used the free fluorescent probe (20 nM compound **II**) as a negative control and the bound fluorescent probe (20 nM compound **II** with 0.5 μ M PTP1B/C215S) as the positive control to perform the Z' factor measurements in 384-well plates.

The experimental details are as following:

- a. For the negative control samples, a solution of compound **II** (20 nM) in DMG buffer was prepared and dispensed into a black 384-well plate (50 μ L for each well) using a MultiDrop 384 liquid dispenser (Titertek, www.titertek.com). The plate was briefly spun down using a desktop centrifuge (Centrifuge 5810 from Eppendorf, www.eppendorf.com) and then the FP signals were measured.
- b. For the positive control samples, a solution of compound **II** (20 nM) and PTP1B/C215S (0.5 μ M) in DMG buffer was prepared and aliquots (50 μ L) of the resulting solution were dispensed into a black 384-well plate. The plate was briefly spun down using the desktop centrifuge and then the FP signals were measured.
- c. The statistical analysis was performed using Excel (Microsoft Corporation, www.microsoft.com) spreadsheet.

The FP values of positive and negative controls were plotted in Figure 4. A Z' factor of 0.70 was calculated using Equation 8, demonstrating that the assay is suitable for HTS.

6.2 Time Dependence and Effect of DMSO

Other conditions such as the time dependent stability of the assay and DMSO effect were also assessed. We monitored the FP value of compound **II** (20 nM) with PTP1B/C215S (0.5 μ M) at different time point for over 3 hours, the result shows that the FP value of the solution is stable up to 1 hour. The DMSO effect on the FP displacement assay was determined by adding different concentrations of DMSO to the solution of 20 nM compound **II** and 0.5 μ M PTP1B/C215S. The result shows that DMSO has no effect on the FP displacement assay when the final DMSO concentration is < 2%.

6.3 Adaptation of the FP-based Assay for HTS

The principle of the FP-based screen is to identify compounds that displace the fluorescent labeled compound **II** from PTP1B active site by detecting the resulting decrease in FP. Based on the results described above, we devise the following FP based HTS procedure:

- a. The library compounds (20 μ M) in DMG buffer (25 μ L total volume) were dispensed to black 384-well microplates in the "one well-one compound" format. For each plate, the last two columns (column 23 and column 24) were used as controls, which means that only DMG buffer (no compounds) was added to these wells.

- b. A solution of compound **II** (40 nM) and PTP1B/C215S (1.0 μ M) in DMG buffer was prepared. The solution was dispensed into the black 384-well plates (25 μ L to each well) using the MultiDrop 384 liquid dispenser. The plates were briefly spun down using Centrifuge 5810 from Eppendorf.
- c. The fluorescence polarization value was measured on a Perkin Elmer EnVision multimode plate reader. For each compound, the decrease in FP anisotropy value (the difference between the FP anisotropy of the compound well and the average FP anisotropy value of the control wells) was calculated as ΔA value and this ΔA value was used as a parameter to evaluate the displacement activity of each compound.

7. Concluding Remarks

Potent and selective PTP inhibitors should not only prove useful in dissecting the precise roles played by specific PTPs in signal transduction pathways, but also furnish a molecular foundation upon which therapeutically useful agents will be based. To identify PTP inhibitors, activity-based assays are widely used in HTS campaigns. Unfortunately, the reactivity of the active site Cys can cause serious problems in activity-based screens as highly reactive oxidizing and alkylating agents may surface as hits. We describe the development of a FP-based displacement assay that should substantially reduce the number of false positives in PTP inhibitor screening. This assay requires the use of a catalytically deficient mutant PTP that retains the wild type binding affinity. We have previously shown that the active site Cys to Ser PTP mutant exhibits similar affinity for substrates and ligands as the wild-type enzyme. Thus, in this FP-based displacement assay the binding affinity of the library members is assessed by their ability to compete with the fluorescently labeled active site ligand for binding to the Cys to Ser PTP mutant.

There are several key points to be noted concerning the FP-based assay. First, since the fluorophore-labeled ligand is designed to bind to the PTP1B active site, compounds that displace this ligand from the Cys to Ser PTP mutant most likely bind to the active site as well. Second, since the catalytically inactive Cys to Ser PTP mutant binds ligands with equal potency as the wild-type enzyme, this assay furnishes a true assessment of the PTP binding ability of the library members. Third, the substitution of the active site Cys by a Ser (e.g., PTP1B/C215S) renders the mutant PTP insensitive to oxidation and alkylation and thus will likely eliminate “false” positives due to modification of the active site Cys that destroy the phosphatase activity. Finally, in addition to utility in primary screens, we anticipate that the FP-based assay will also find use in secondary screens to validate hits identified from activity-based HTS campaigns.

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Figure 1.

Structures of compound **I**, the fluorescein-labeled compound **II**, the free amine **III** used for the preparation of compound **II**, and a known PTP1B inhibitor compound **IV**.

Figure 2.

Binding of compound **II** to PTP1B/C215S. Varying concentrations of PTP1B/C215S were added to compound **II** (20 nM) in DMG buffer. The anisotropy was measured at room temperature after a 5 min incubation. Anisotropy (mA) measurements were fitted to Equation 4 to obtain the K_d value.

Figure 3.

Displacement of compound **II** from PTP1B/C215S by a known PTP1B inhibitor **IV**. Varying concentrations of compound **IV** were added to 20 nM compound **II** and 0.5 μM PTP1B/C215S. The anisotropy was measured at room temperature after 5 min incubation. The data was fitted to Equation 6 and 7 to obtain the K_d value for compound **IV** and PTP1B/C215S.

Figure 4.

Determination of Z' factor. The Z' factor was determined with free compound **II** (20 nM) in DMG buffer as a negative control (black dots) and the PTP1B/C215S (0.5 μ M) bound compound **II** (20 nM) in DMG buffer as a positive control (open circles).

Scheme 1. Synthesis of Compound II

Reaction conditions. a) Fmoc-Dap(Boc)-OH/HBTU/HOBt/NMM/DMF, 2 h; b) (i) 30% piperidine/DMF, 30 min, (ii) Fmoc-F₂Pmp-OH/HBTU/HOBt/NMM/DMF, 2 h; c) (i) 30% piperidine/DMF, 30 min, (ii) 3,5-Dihydroxy-2-naphthoic acid/HBTU/HOBt/NMM/DMF, 2 h; d) TFA/water/TIS (95:2.5:2.5), 4 h; e) 6-[fluorescein-5(6)-carboxamido] hexanoic acid N-hydroxysuccinimide ester, Et₃N and DMF.