Supporting Information

Selective Protein Tyrosine Phosphatase 1B Inhibitors: Targeting the Second Phosphotyrosine Binding Site with Non-Carboxylic Acid-Containing Ligands


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General Information:

Unless otherwise specified, all solvents and reagents were obtained from commercial suppliers and used without further purification. All reactions were performed under nitrogen atmosphere unless specifically noted. Flash chromatography was performed using silica gel (230–400 mesh) from E. M. Science. $^1$H NMR spectra were recorded on a Varian Mercury 300 (300 MHz), Varian UNITY 500 (500 MHz) spectrometer and are reported in ppm (δ) from tetramethylsilane (TMS: δ 0.0 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, ddd = doublet of doublet of doublet), coupling constants (Hz), integration. Mass spectral analyses were accomplished on a Finnigan SSQ7000 GC/MS mass spectrometer using different techniques, including atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), as specified for individual compounds. Exact mass measurement was performed on a Finnigan FTMS Newstar T70 mass spectrometer. The compound is determined to be "consistent" with the chemical formula if the exact mass measurement is within 5.0 ppm relative mass error (RME) of the exact monoisotopic mass.

Analytical HPLC for selected compounds were performed on two systems: System A: Samples were analyzed by a Gilson analytical HPLC system, using an YMC C-8 column (50 × 4.6 mm i.d., S-5 µM, 120 Å). Solvent system used was acetonitrile/0.1% aqueous TFA, gradient 0–70% over 15 min @ 2 mL/min. System B: Samples were analyzed by Agilent Analytical HPLC 1100 Series, SB-C8 column (Zorbax 3.5 µm, 4.6 × 75 mm). Solvent system used was acetonitrile/0.1% aqueous TFA, gradient 10–70% over 10 min.
@ 1.5 mL/min. The purity of the compounds was determined on two analytic HPLC systems to be of greater than 95% purity by UV detection.

Preparative HPLC was performed on an automated Gilson HPLC system, using a SymmetryPrep Shield RP18 prep cartridge, 25 mm × 100 mm i.d., S-7 µM, and a flow rate of 25 mL/min; λ = 214, 245 nm; mobile phase A, 0.1% TFA in H2O; mobile phase B, CH3CN; linear gradient 0–70% of B in 19 min. The purified fractions were evaporated to dryness on a ThermoSavant SpeedVac.

**Experimental Procedure:**

![Chemical Structure](image)

**Methyl 2-(4-aminobutoxy)-6-hydroxybenzoate Hydrochloride Salt (7).** To a round bottom flask was charged with tert-butyl 4-hydroxybutylcarbamate (400 mg, 2.1 mmol), 463 mg of methyl 2,6-dihydroxybenzoate (463 mg, 2.7 mmol), and triphenylphosphine (777 mg, 3.0 mmol). The flask was vacuumed and back flushed with nitrogen (3×), capped with a rubber septum, and kept under positive nitrogen atmosphere. THF was then added, followed by dropwise addition of diethyl azodicarboxylate (DEAD, 433 µL, 2.7 mmol). Most of the starting material was consumed within the first 30 min. Solvent was then removed in vacuo, and the residue was purified on a silica gel chromatography eluting with 15-30% EtOAc in hexanes to give 2-(4-tert-Butoxycarbonylamino-butoxy)-6-hydroxy-benzoic acid methyl ester (410 mg, 57%) as a colorless oil. The above-mentioned tert-butyl carbamate (410 mg, 1.2 mmol) was treated with 4N HCl in dioxane (8
mL) at ambient temperature for 4 h. The mixture was concentrated under reduced pressure, evaporated with acetonitrile twice, trituated with diethyl ether to provide the titled amine HCl salt 7 as a white solid (325 mg, 97%). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 9.99 (s, 1H), 7.91 (s, 3H), 7.16 (t, $J = 8.3$ Hz, 1H), 6.51 (dd, $J = 8.3$, 2.9 Hz, 2H), 3.96 (t, $J = 5.8$ Hz, 2H), 3.76 (s, 3H), 2.82 (t, $J = 6.4$ Hz, 2H), 1.78-1.61 (m, 4H); MS (ESI) $m/z$ 240 (M+H)$^+$. 
Synthesis of Compound (S)-8-I (R₁ = H, R₂ = NHCO₂Alloc):

Scheme 1

Reagents and conditions: (a) Diphenyliodonium carboxylate, Cu(OAc)₂, DMF, 95 °C; (b) 4N HCl/Dioxane, r.t.; (c) allyl chloroformate, sat. NaHCO₃, EtOAc, r.t., 97% over three steps; (d) CICO₂t-Bu, i-Pr₂NEt, CH₂Cl₂, 0 °C-r.t.; (e) Diphenylidazomethane, acetone, r.t., 55% over two steps; (f) TBAF, THF, r.t., 90%;
2-\{4-\[2-(S)-\text{Allyloxycarbonylamino}-2-(2\text{-trimethylsilanyl-ethoxycarbonyl})\text{-ethyl}\}\text{-phenylamino}\}\text{-benzoic acid (S-8a-I). To a stirred suspension of 3-(4-amino-phenyl)-2-\(\text{(S)-} tert\text{-butoxycarbonylamino-propionic acid 2-trimethylsilanyl-ethyl ester}^{1} \text{ (5.3 g, 13.9 mmol)}\) and diphenyliodonium-2-carboxylate monohydrate (DPIC) (4.8 g, 13.9 mmol) in \text{\(N,N\)-dimethylformamide (DMF, 50 mL)}\) was added anhydrous Cu(OAc)\(_2\) (50 mg, 0.28 mmol). The mixture was heated at 100 °C for 2 h. The reaction mixture was then concentrated \textit{in vacuo}, and the crude 2-\{4-\[2-(S)-\text{tert-butoxycarbonylamino}-2-(2\text{-trimethylsilanyl-ethoxycarbonyl})\text{-ethyl}\]\text{-phenylamino}\}\text{-benzoic acid (6.97 g, 13.9 mmol) was treated with 4N HCl in dioxane ((13.9 mL, 55.8 mmol) for 1 h. The solvent was then removed under reduced pressure. The residue was precipitated with diethyl ether (2 × 35 mL) to provide 2-\{4-\[2-amino-2-(2\text{-trimethylsilanyl-ethoxycarbonyl})\text{-ethyl}\]\text{-phenylamino}\}\text{-benzoic acid HCl salt as a light yellow solid (6.1 g, 100%). To a mixture of amine HCl salt (6.1 g, 13.9 mmol) in 35 mL of EtOAc was added NaHCO\(_3\) (4.1g, 84.7 mmol) in 25 mL of water. To the above mixture was added slowly allyl chloroformate (1.48 mL, 13.9 mmol). The resulting mixture was stirred at ambient temperature for 1 h, acidified to pH < 3 with aqueous 2N HCl and extracted with EtOAc (2 × 45 mL). The combined organic layers were washed with water (1 × 25 mL), brine (1 × 25 mL), dried
(MgSO₄), filtered and concentrated in vacuo to provide the titled compound S-8a-I (6.55 g, 97%). ¹H NMR (300 MHz, DMSO-d₆) δ 7.87 (dd, J = 7.8, 1.7 Hz, 1H), 7.72 (d, J = 8.1 Hz, 1H), 7.33 (td, J = 7.1, 1.7 Hz, 1H), 7.24-7.10 (m, 5H), 6.73 (td, J = 7.1, 1.0 Hz, 1H), 5.85 (ddd, J = 22.7, 10.5, 5.4 Hz, 1H), 5.22 (dd, J = 17.3, 1.7 Hz, 1H), 5.13 (dd, J = 10.5, 1.7 Hz, 1H), 4.42 (d, J = 5.4 Hz, 2H), 4.22-4.14 (m, 1H), 4.09 (dd, J = 9.5, 7.8 Hz, 2H), 2.96 (dd, J = 13.9, 5.4 Hz, 1H), 2.83 (dd, J = 13.6, 9.8 Hz, 1H), 0.91-0.81 (m, 2H), 0.00 (s, 9H); MS (ESI) m/z 483 (M-H⁻).

![Structure of S-8b-I](image)

2-{(4-[2-(S)-Allyloxy carbonylamino-2-(2-trimethylsilanyl-ethoxycarbonyl)-ethyl]-phenyl)-tert-butoxyoxalyl-amino}-benzoic acid benzhydryl ester (S-8b-I). To a mixture of acid S-8a-I (6.55 g, 13.5 mmol) and triethylamine (6.0 mL, 42.8 mmol) in CH₂Cl₂ (50 mL) at 0 °C was slowly added tert-butyl oxalyl chloride (4.6 g, 27.9 mmol). The reaction was allowed to warm to room temperature over 1 h and stirred at room temperature for 14 h. The resulting mixture was then acidified to pH < 3 with 1N HCl, extracted with CH₂Cl₂ (2 × 30 mL). The combined organic layers were washed with 1N HCl (30 mL), water (20 mL), and brine (20 mL), dried (MgSO₄), filtered and
concentrated in vacuo. The residue was dissolved in acetone (60 mL) with stirring, diphenyldiazomethane\(^2\) (3.16 g, 16.2 mmol) was then added. The reaction mixture was stirred for 1 h and concentrated under reduced pressure. The concentrate was purified on silica gel column eluting with 10-30\% EtOAc in hexanes to provide the titled compound \(\text{S-8b-I}\) (5.8 g, 55\% over two steps). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) [8.15 (dd, \(J = 7.8, 1.7\) Hz), 7.99 (dd, \(J = 7.8, 1.4\) Hz), 1H IN totoal], 7.60 (td, \(J = 7.8, 1.7\) Hz, 1H), 7.55-7.48 (m, 1H), 7.43-7.2.0 (m, 13H), 7.12-7.06 (m, 1H), 7.05-6.99 (m, 1H), 6.97-6.91 (m, 1H), 5.96-5.78 (m, 1H), 5.33-5.03 (m, 2H), 4.54 (d, \(J = 4.4\) Hz, 2H), 4.25-4.07 (m, 2H), 3.17-2.96 (m, 2H), 1.22&1.08 (s, 9H in total), 1.02-0.91 (m, 2H), 0.04&0.03 (s, 9H in total); MS (APCI) \(m/z\) 796 (M+NH\(_4\))\(^+\).

2-\{[4-(2-(S)-Allyoxycarbonylamino-2-carboxy-ethyl)-phenyl]-tert-butoxoyxalyl-amino\}-benzoic acid benzhydryl ester (S-8-I). To a solution of ester \(\text{S-8b-I}\) (500 mg, 0.64 mmol) in tetrahydrofuran (2.5 mL) was added tetrabutylammonium fluoride (0.71 mL, 1M in THF). The reaction mixture was stirred at ambient temperature overnight, then quenched with 1N HCl to pH < 3, and extracted with CH\(_2\)Cl\(_2\) (2 × 15 mL). The
combined organic layers were washed with 1N HCl, water, and brine, dried with MgSO₄, filtered and concentrated in vacuo. The concentrate was purified by SiO₂ flash column chromatography eluting with 200:10:1 (v/v/v) of EtOAc/hexanes/AcOH to yield the titled acid **S-8-I** as a light yellow solid (386 mg, 90%). ¹H NMR (a mixture of rotamers, 300 MHz, DMSO-δ₆) δ [8.14 (dd, J = 7.8, 1.7 Hz), 7.99 (dd, J = 7.8, 1.5 Hz), 1H in total], 7.64-7.57 (m, 1H), 7.56-7.48 (m, 1H), 7.44-7.20 (m, 13H), 7.08 (t, J = 7.8 Hz, 2H), 6.98 (d, J = 6.4 Hz, 1H), 5.95-5.78 (m, 1H), 5.27 (d, J = 15.9 Hz, 1H), 5.19 (d, J = 10.5 Hz, 1H), 5.08 (dd, J = 18.5, 7.6 Hz, 1H), 4.67-4.58 (m, 1H), 4.55 (dd, J = 5.4 Hz, 2H), 3.22-2.99 (m, 2H), 1.21&1.09 (s, 9H in total); MS (ESI) m/z 696 (M+NH₄)+.

![Methyl 2-{4-[(N-[(allyloxy)carbonyl]-4-{{2-[(benzhydryloxy)carbonyl]phenyl}[tert-butoxy(oxo)acetyl]amino}-L-phenylalanyl]amino}butoxy]-6-hydroxybenzoate (S-9-I).](image)

**Methyl 2-{4-[(N-[(allyloxy)carbonyl]-4-{{2-[(benzhydryloxy)carbonyl]phenyl}[tert-butoxy(oxo)acetyl]amino}-L-phenylalanyl]amino}butoxy]-6-hydroxybenzoate (S-9-I).** To a stirring mixture of L-phenylalanine derivative **S-8-I** (100 mg, 0.18 mmol), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU, 67 mg, 0.21 mmol), and HOBT (3 mg, 0.02 mmol) in DMF (2 mL) was added methyl 2-(4-aminobutoxy)-6-hydroxybenzoate HCl salt (7), followed by addition of triethylamine (75
µL, 0.53 mmol). The resulting mixture was then stirred at ambient temperature for 2h. The reaction mixture was then partitioned between EtOAc (10 mL) and water (10 mL), and the aqueous layer was extracted with EtOAc (10 mL) once. The combined organic layers were washed with aqueous NaHCO₃ (5 mL) and brine (5 mL), dried (Na₂SO₄), filtered and concentrated in vacuo. The resulting residue was purified on an Alltech silica gel Sep-Pak eluting with 50-60% EtOAc/hexanes to provide the titled compound (S)-9-I (89 mg, 68%). ¹H NMR (a mixture of rotamers, 500 MHz, CDCl₃) δ 11.35 & 11.33 & 11.28 (s, 1H in total), [8.12 (d, J = 7.5 Hz), 7.96 (d, J = 7.8 Hz), 1H in total], 7.61-7.46 (m, 2H), 7.43-7.18 (m, 16H), 7.08 (t, J = 8.0 Hz, 1H), 7.03-6.96 (m, 1H), 6.57 (d, J = 8.1 Hz, 1H), [6.37 & 6.32 (d, J = 8.4 Hz), 1H in total], 5.96-5.76 (m, 2H), 5.33-5.08 (m, 2H), 4.53 (d, J = 5.3 Hz, 2H), 4.31-4.19 (d, 1H), 3.89 (s, 3H), 3.32-3.14 (m, 2H), 3.12-2.97 (m, 1H), 2.93-2.83 (m, 1H), 1.77-1.52 (m, 4H), 1.24 & 1.09 (s, 9H in total); MS (ESI) m/z 900 (M+H)⁺.

Methyl 2-[4-{N-[(allyloxy)carbonyl]-4-[(carboxycarbonyl)(2-carboxyphenyl)amino]-L-phenylalanyl}amino)butoxy]-6-hydroxybenzoate (6). A mixture of ester (S)-9-I (89 mg, 0.10 mmol), 20 mg of resorcinol in CH₂Cl₂ (2.0 mL) was treated with trifluoroacetic acid (1.5 mL) at room temperature for 5 h. The reaction
mixture was then concentrated in vacuo. The crude product was purified on a Gilson preparative HPLC to provide the titled compound 6 as a white powder (35 mg, 52%). $^1$H NMR (a mixture of rotamers, 300 MHz, DMSO-$d_6$) δ 9.92 (s, 1H), 7.93-8.03 (m, 2H), 7.38-7.66 (m, 3H), 7.36 (d, $J = 8.7$ Hz, 1H), 7.29 (d, $J = 8.7$ Hz, 1H), 7.25 (d, $J = 8.7$ Hz, 1H), 6.47 (d, $J = 8.7$ Hz, 1H), 5.70-5.90 (m, 1H), 5.18 (d, $J = 18.0$ Hz, 1H), 5.08 (d, $J = 10.8$ Hz, 1H), 4.33-4.41 (m, 2H), 4.09-4.12 (m, 1H), 3.90 (t, $J = 5.85$ Hz, 1H), 3.72 (s, 3H), 2.98-3.15 (m, 2H), 2.83-2.98 (m, 1H), 2.68-2.83 (m, 1H), 1.40-1.66 (m, 4H); MS (ESI) $m/z$ 678 (M+H)$^+$; HRMS Calcd for C$_{34}$H$_{36}$N$_3$O$_{12}$ (M + H)$^+$: 678.2300, found: 678.2308; Analytical HPLC $t_R = 5.94$ min (A), $t_R = 5.08$ min (B).

Synthesis of Compound 8-II ($R_1 =$ Et, $R_2 =$ NHCOCH$_3$):
Scheme 1a

\[ \text{Benzyl (2E)-2-(acetylamino)-3-(4-amino-3-ethylphenyl)-2-propenoate (8a-II). To a} \]

stirred mixture of 2-acetamidoacrylic acid (10.3 g, 80.0 mmol) and K\textsubscript{2}CO\textsubscript{3} (10 g, 72.5
mmol) in DMF (50 mL) was added benzyl bromide (8.7 ml, 72.5 mmol) at room temperature. After stirred at room temperature for 3 h, the reaction mixture was partitioned between 1:1 (v/v) of EtOAc and water (50mL), the aqueous layer was extracted with EtOAc (2 × 45 mL). The combined organic layers were washed with brine (2 × 25 mL), dried (MgSO₄), filtered, and concentrated in vacuo to provide benzyl 2-(acetylamino)acrylate (17.5g, 100% yield). To benzyl acrylate (17.5 g, 80.0 mmol) in acetonitrile (200 mL) was added Pd(OAc)₂ (488 mg, 2.18 mmol), (α-Tol)₃P (1.32 g, 4.35 mmol), Et₃N (20 mL, 143 mmol), and 4-bromo-2-ethylaniline (14.5 g, 72.5 mmol). The reaction mixture was refluxed overnight, concentrated under reduce pressure, taken up in EtOAc (50 mL), washed with aqueous NaHCO₃, dried (MgSO₄), filtered, and concentrated in vacuo. The residue was precipitated from EtOAc/hexanes to provide 8.2 g of the titled compound 8a-I. A second crop of precipitates after concentrating provided additional 6.0 g of the titled compound 8a-I (58% yield). ¹H NMR (300 MHz, DMSO-d₆) δ 9.31 (s, 1H), 7.43-7.19 (m, 8H), 6.59 (d, J = 8.1 Hz, 1H), 5.52 (s, 2H), 5.16 (s, 2H), 2.42 (q, J = 7.5 Hz, 2H), 1.98 (s, 3H), 1.13 (t, J = 7.5 Hz, 3H); MS (ESI) m/z 339 (M+H)⁺.

Allyl 2-(acetylamino)-3-(4-amino-3-ethylphenyl)propanoate (8b-II). A mixture of benzyl ester 8a-II (5.0 g, 14.8 mmol) and 10% Pd/C (100 mg) in MeOH (50 mL) was
stirred under 4 atm. of hydrogen at ambient temperature overnight in a Parr shaker. Filtration through celite, washing with MeOH, and evaporation in vacuo provided N-acetyl-4-amino-3-ethylphenylalanine (3.7 g, 100%). A mixture of N-acetyl-4-amino-3-ethylphenylalanine (2.0 g, 8.0 mmol), Cs$_2$CO$_3$ (2.61 g, 8.0 mmol) and allyl bromide (692 µL, 8.0 mmol) in DMF (40 mL) was stirred at room temperature for 3 h. The resulting mixture was concentrated under reduce pressure and partitioned between 1:1 (v/v) of EtOAc and water (100mL). The organic phase was washed with brine (1 × 50 mL), dried (MgSO$_4$), filtered, and concentrated in vacuo. The residue was purified on silica gel column eluting with EtOAc/hexanes (5:3, v/v) to provide the titled compound 8b-II (1.44 g, 62%). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 8.23 (d, $J = 7.8$ Hz, 1H), 6.73 (dd, $J = 10.3$, 2.2 Hz, 2H), 6.50 (d, $J = 7.8$ Hz, 1H), 5.90-5.76 (m, 1H), 5.26 (dd, $J = 17.3$, 1.7 Hz, 1H), 5.18 (dd, $J = 10.5$, 1.7 Hz, 1H), 4.67 (s, 2H), 4.54-4.50 (d, $J = 5.4$ Hz, 2H), 4.38-4.30 (m, 1H), 2.82 (dd, $J = 13.7$, 5.8 Hz, 1H), 2.70 (dd, $J = 13.7$, 9.0 Hz, 1H), 2.39 (q, $J = 7.5$ Hz, 2H), 1.80 (s, 3H), 1.10 (t, $J = 7.5$ Hz, 3H); MS (ESI) $m/z$ 291 (M+H)$^+$. 

![8c-II](image_url)
2-[4-(2-Acylamino-2-allyloxy carbonyl-ethyl)-2-ethyl-phenylamino]-benzoic Acid (8c-II). A mixture of aniline 8b-II (815 mg, 2.8 mmol), DPIC (960 mg, 2.8 mmol) and copper(II) acetate (20 mg, 0.11 mmol) in DMF (5 mL) was heated to 90 °C for 6 h, then cooled to room temperature to give the crude diarylaniline. To this mixture was added N,N-diisopropylethylamine (975 µL, 5.6 mmol), benzyl oxalyl chloride (670 µL, 4.2 mmol). The resulting mixture was stirred at room temperature overnight. Acid (1N HCl, 15 mL) was added, and the mixture was extracted with EtOAc (2 × 25 mL). The combined organic layers were washed with 1N HCl (10 mL), water (10 mL), brine (10 mL), dried (MgSO₄), filtered and concentrated in vacuo. The residue was purified on silica gel column eluting with 200:10:1 (v/v/v) of EtOAc/MeOH/AcOH to give the benzoic acid derivative (700 mg, 44%). The benzoic acid was dissolved in acetone (15 mL), diphenyl diazomethane was added to the stirring mixture until all starting material was consumed as evident by TLC. The reaction mixture was concentrated under reduced pressure, purified on a silica gel column using EtOAc as eluent to provide the titled compound 8c-II (650 mg, 72%).

1H NMR (a mixture of rotamers, 500 MHz, DMSO- d₆) δ 8.41-8.31 (m, 1H), [8.12&8.06 (d, J = 7.8 Hz, 1H in total)], 7.65-6.80 (m, 20H), 5.88-5.73 (m, 1H), 5.30-5.22 (m, 1H), 5.22-5.10 (m, 1H), 4.59-4.41 (m, 5H), [3.02 (dd, J = 13.7, 7.8 Hz), 2.98 (dd, J = 14.0, 5.6 Hz), 1H in total], 2.94-2.85 (m, 1H), 2.44 (q, J = 7.5 Hz, 2H), [0.77&0.75 (t, J = 7.5 Hz), 3H in total] 1.79&1.77&1.76 (s, 3H in total); MS (ESI) m/z 756 (M+NH₄)⁺.
2-[[4-(2-Acetylamino-2-carboxy-ethyl)-2-ethyl-phenyl]-benzyloxyoxalyl-amino]-benzoic acid benzhydryl ester (8-II, R₁ = Et, R₂ = NHCOCH₃). A mixture of allyl ester 8c-II (3.4 g, 4.6 mmol), Pd(Ph₃P)₄ (166 mg, 0.144 mmol), and morpholine (0.5 mL, 5.8 mmol) in dichloromethane (25 mL) was stirred under N₂ atmosphere for 2 h. The resulting mixture was then partitioned between 1:1 (v/v) of EtOAc and water (75 mL). The organic phase was washed with 1N HCl (1 × 25 mL), brine (1 × 25 mL), dried (MgSO₄), filtered, and concentrated in vacuo to provide the titled compound 8-II (3.2 g, 100%). ¹H NMR (a mixture of rotamers, 500 MHz, DMSO-d₆) δ 12.64 (s, 1H), 8.18 (dd, J = 8.1, 4.1 Hz, 1H), [8.12&8.05 (d, J = 7.8 Hz), 1H in total], 7.67-7.02 (m, 20 H), 7.02-6.81 (m, 2H), 4.91 (dd, J = 12.2, 5.9 Hz, 1H), 4.85 (dd, J = 12.2, 4.4 Hz, 1H), 4.50-4.33 (m, 1H), [3.06 (dd, J = 14.0, 4.7 Hz), 3.00 (dd, J = 14.2, 4.5 Hz), 1H in total], 2.88-2.77 (m, 1H), 2.59-2.35 (m, 2H), 1.74&1.73 (s, 3H in total), 1.19-1.04 (m), 0.78&0.75 (t, J = 7.5 Hz), 3H in total]; MS (ESI) m/z 716 (M+NH₄)⁺.
Methyl 2-[(5-{[2-(acetylamino)-3-(4-{2-[(benzhydryloxy)carbonyl]}
[(benzyloxy)(oxo)acetyl]anilino}-3-ethylphenyl)propanoyl]amino}pentyl)oxy]-6-
hydroxybenzoate (9-II). A mixture of methyl 2-(4-aminobutoxy)-6-hydroxybenzoate 7
(42 mg, 0.12 mmol), acid 8-II (70 mg, 0.1 mmol), TBTU (32 mg, 0.1 mmol) and
diisopropylethylamine (70 µL, 0.4 mmol) in DMF (1 mL) was stirred at ambient
temperature overnight. The reaction mixture was then diluted with EtOAc and washed
with aqueous NaHCO₃ (1 × 30 mL), brine (2 × 30 mL), dried (MgSO₄), filtered and
concentrate in vacuo. The residue was purified on a silica gel column eluting with 100%
EtOAc to provide the titled compound 9-II (54 mg, 59%). ¹H NMR (a mixture of
rotamers, 500 MHz, DMSO-d₆) δ 9.90 (s, 1H), 8.15-7.89 (m, 2H), 7.61-6.80 (m, 25H),
6.50-6.40 (m, 1H), 4.91 (dd, J = 12.2, 3.7 Hz, 1H), 4.86 (dd, J = 12.2, 3.7 Hz, 1H), 4.54-
4.40 (m, 1H), 3.94-3.84 (m, 1H), 3.72 (s, 3H), 3.11-3.00 (m, 2H), [2.96 (dd, J = 13.7, 5.3
Hz), 2.89 (dd, J = 13.7, 5.2 Hz), 1H in total], 2.54-2.35 (m, 2H), 1.73&1.72 (s, 3H in
total), 1.64-1.51 (m, 2H), 1.51-1.39 (m, 2H), [1.12 (br s), 0.78&0.76 (t, J = 7.5 Hz), 3H in
total]; MS (ESI) m/z 920 (M+H)⁺.
2-[4-[2-(Acetylamino)-3-((4-[3-hydroxy-2-(methoxycarbonyl)phenoxy]butyl)amino)-3-oxopropyl](carboxycarbonyl)-2-ethylanilino]benzoic acid (10). The above-mentioned ester 9-II and 10% Pd-C (5 mg) in methanol (3 mL) was stirred under 1 atm of hydrogen at ambient temperature overnight to provide the tilted compound 10 (33 mg, 85%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.92 (s, 1H), 8.17-8.05 (m, 1H), 8.02-7.93 (m, 1H), 7.83-7.71 (m, 1H), [7.50 (t, $J = 7.9$ Hz), 7.45 (t, $J = 8.2$ Hz), 1H in total], 7.39&7.33 (t, $J = 7.6$ Hz, 1H in total), 7.30-7.09 (m, 7H), 7.04-6.98 (m, 1H), 6.81-6.75 (m, 1H), 6.47 (d, $J = 7.9$ Hz, 2H), 4.53-4.40 (m, 1H), 3.95-3.85 (m, 2H), 3.72 (s, 3H), 3.10-3.00 (m, 2H), 2.98-2.86 (m, 1H), 2.81-2.70 (m, 1H), 2.68-2.54 (m, 2H), 1.78&1.75 (s, 3H in total), 1.62-1.52 (m, 2H), 1.50-1.40 (m, 2H), [1.21 (br t), 0.93 (br t, $J = 7.9$ Hz), 3H in total]; MS (ESI) $m/\ell$ 664 (M+H)$^+$; HRMS Calcd for C$_{34}$H$_{38}$N$_3$O$_{11}$ (M + H)$^+$: 664.2507, found: 664.2514; Analytical HPLC $t_R = 5.54$ min (A), $t_R = 4.79$ min (B).
2-[4-([N-acetyl-4-[(carboxycarbonyl)(2-carboxyphenyl)amino]-3-ethylphenylalanyl] amino)butoxy]-6-hydroxybenzoic acid (11). The titled compound was prepared according to the procedure described for compound 10, substituting benzyl 2-(4-aminobutoxy)-6-hydroxybenzoate for methyl 2-(4-aminobutoxy)-6-hydroxybenzoate. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.33 (s, 1H), 8.13-7.78 (m, 3H), 7.58-6.75 (m, 7H), 6.47 (d, 2H), 4.53-4.40 (m, 1H), 3.93-3.85 (m, 2H), 3.10-2.56 (m, 6H), 1.78 & 1.75 (s, 3H in total), 1.62-1.52 (m, 2H), 1.50-1.40 (m, 2H), 1.26-0.91 (m, 3H); MS (ESI) $m/z$ 650 (M+H)$^+$; HRMS Calcd for C$_{33}$H$_{36}$N$_{3}$O$_{12}$ (M + H)$^+$: 650.2351, found: 650.2343; Analytical HPLC $t_R = 5.21$ min (A), $t_R = 4.18$ min (B).
2-[4-{2-(Acetylamino)-3-({4-[2-(methoxycarbonyl)phenoxy]butyl}amino)-3-oxopropyl}(carboxycarbonyl)-2-ethylanilino]benzoic acid (12). The titled compound was prepared according to the procedure described for compound 10, substituting methyl 2-(4-aminobutoxy)-benzoate for methyl 2-(4-aminobutoxy)-6-hydroxybenzoate. $^1$H NMR (a mixture of rotamers, 500 MHz, DMSO-$d_6$) $\delta$ 8.14-8.02 (m, 1H), 8.01-7.91 (m, 1H), [7.80 (dd, $J = 7.7$, 1.8 Hz), 7.75 (br s), 1H in total], 7.65-7.59 (m, 1H), 7.53-7.42 (m, 2H), [7.39 (td, $J = 7.4$, 1.2 Hz), 7.32 (t, $J = 7.4$ Hz), 1H in total], 7.30-7.05 (m, 4H), 7.05-6.95 (m, 2H), 4.54-4.40 (m, 1H), 4.02-3.94 (m, 2H), 3.77 (s, 3H), 3.15-3.03 (m, 2H), 3.00-2.86 (m, 1H), 2.82-2.71 (m, 1H), 2.68-2.54 (m, 2H), 1.78&1.77&1.76 (s, 3H in total), 1.71-1.59 (m, 2H), 1.59-1.47 (m, 2H), [1.21 (br t, $J = 7.4$ Hz), 0.93 (td, $J = 7.4$, 2.2 Hz), 3H in total]; MS (ESI) $m/z$ 648 (M+H)$^+$; HRMS Calcd for C$_{34}$H$_{38}$N$_3$O$_{10}$ (M + H)$^+$: 648.2558, found: 648.2564; Analytical HPLC $t_R$ = 5.67 min (A), $t_R$ = 4.98 min (B).

2-[(4-{2-Acetylamino-2-[4-(3-hydroxy-2-nitro-phenoxy)-butylcarbamoyl]-ethyl}-2-ethyl-phenyl)-oxalyl-amino]-benzoic Acid (13). The titled compound was prepared according to the procedure described in Example 15, substituting 2-nitroresorcinol for methyl 2,6-dihydroxy-benzoate. $^1$H NMR (a mixture of rotamers, 500 MHz, DMSO-$d_6$) $\delta$
10.81 (s, 1H), 8.13-8.03 (m, 1H), 7.99-7.93 (m, 1H), [7.93 (d, J = 8.7 Hz), 7.80 (dd, J = 7.8, 1.3 Hz), 1H in total], [7.53 (t, J = 7.2 Hz), 7.45 (t, J = 7.5 Hz), 1H in total], [7.39 (t, J = 7.6 Hz), 7.32 (t, J = 7.5 Hz), 1H in total], 7.30-7.18 (m, 4H), 7.18-6.98 (m, 2H), [6.81-6.75&6.67-6.61 (m), 1H in total]), 6.60 (d, J = 8.4 Hz, 1H), 4.52-4.39 (m, 1H), 4.08-3.94 (m, 2H), 3.14-2.99 (m, 2H), 2.99-2.87 (m, 1H), 2.81-2.71 (m, 1H), 2.69-2.51 (m, 2H), 1.78&1.76 (s, 3H in total), 1.66-1.52 (m, 2H), 1.45-1.35 (m, 2H), [1.26-1.15 (m)&0.93 (t, J = 7.2 Hz), 3H in total]; MS (ESI) m/z 648 (M+H)+; HRMS Calcd for C32H35N4O11 (M + H)+: 651.2303, found: 651.2291; Analytical HPLC t_R = 5.29 min (A), t_R = 4.45 min (B).

2-[4-[2-(Acetylamino)-3-[(4-[3-hydroxy-2-(N-methyl-amino-carbonyl)phenoxy]butyl]- amino)-3-oxopropyl](carboxycarbonyl)-2-ethylanilino]benzoic acid (14). The titled compound was prepared according to the procedure described for compound 10, substituting 2,6-dihydroxy benzoic acid N-methylamide for methyl 2,6-dihydroxybenzoate. 1H NMR (a mixture of rotamers, 500 MHz, DMSO-d6) δ 13.52 (br s, 1H), 8.96 (br s, 1H), 8.45(br s, 1H), 7.90-7.76 (m, 2H), 7.56-6.97&6.84-6.79 (m, 6H), [6.54 (dd, J = 8.4, 1.7 Hz), 6.48 (d, J = 8.3 Hz), 1H in total], 4.51-4.39 (m, 1H), 4.05-3.94 (m, 2H), 3.14-2.99 (m, 2H), 2.99-2.87 (m, 1H), 2.81-
2.71 (m, 1H), 2.69-2.51 (m, 2H), 2.86&2.85 (s, 3H in total), 1.78&1.76 (s, 3H in total),
1.72-1.59 (m, 2H), 1.59-1.46 (m, 2H), [1.12 (br s), 0.93&0.91 (t, J = 7.5 Hz), 3H in
total]; MS (ESI) m/z 663 (M+H)+; HRMS Calcd for C34H39N4O10 (M + H)+: 663.2667,
found: 663.2674; Analytical HPLC \( t_R = 5.46 \) min (A), \( t_R = 4.62 \) min (B).

![Structure](image)

Methyl 6-{4-[(\(N\)-acetyl-4-[(carboxycarbonyl)(2-carboxyphenyl)amino]-3-
ethylphenylalanyl)amino]butoxy}-3-bromo-2-hydroxybenzoate (15). Methyl 6-{4-
[(\(N\)-acetyl-4-{[2-[(benzhydryloxy)carbonyl]phenyl] [(benzylxy)(oxo)acetyl]amino]-3-
ethylphenylalanyl)amino]butoxy}-3-bromo-2-hydroxybenzoate\(^4\) (35 mg, 0.035mmol)
was treated with trifluoroacetic acid (500 \(\mu\)L)/ CH\(_2\)Cl\(_2\) (500 \(\mu\)L) at ambient temperature
for 4 h. The resulting mixture was concentrated in vacuo and evaporated with acetonitrile
(2 \(\times\) 10mL). The residue was taken up in 1N NaOH (3 eq.)/methanol (250\(\mu\)L)/THF (250
\(\mu\)L), stirred for 3 hours and concentrated under reduced pressure to provide the titled
compound 15 (15 mg, 58%). \(^1\)H NMR (a mixture of rotamers, 500 MHz, DMSO-\(d_6\))
8.08-7.92 (m, 2H), 7.45-6.94 (m, 7H), 6.64 (d, 2H), 4.43-4.38 (m, 1H), 3.90-3.86 (m,
2H), 3.78 (s, 3H), 3.10-3.05 (m, 2H), 2.90-2.85 (m, 1H), 2.75-2.62(m, 3H), 1.76(s, 3H),
1.64-1.58 (m, 2H), 1.52-1.45 (m, 2H), 1.18 (t, J = 7.5 Hz, 3H); MS (ESI) m/z 742, 743 (M+H)+; HRMS: Calcd for (M+H)+ C34H37BrN3O11: 742.1612, found 742.1606;

Analytical HPLC t_R = 5.85 min (A), t_R = 5.51 min (B).

Methyl 4-{4-[N-acetyl-4-[(carboxycarbonyl)(2-carboxyphenyl)amino]-3-ethylphenylalanyl]amino}butoxy]-2-hydroxy-1,1'-biphenyl-3-carboxylate (16). The titled compound was prepared according to the procedure described for compound 10, substituting methyl 4-(4-aminobutoxy)-2-hydroxy[1,1'-biphenyl]-3-carboxylate for methyl 2-(4-aminobutoxy)-6-hydroxybenzoate. ¹H NMR (a mixture of rotamers, 500 MHz, DMSO-d_6) δ 10.11 (s, 1H), 8.14-8.00 (m, 2H), 7.99-7.78 (m, 3H), 7.58-6.96 (m, 11H), 6.75 (d, J = 8.4 Hz, 2H), 4.51-4.37 (m, 1H), 3.93 (s, 2H), 3.78 (s, 3H), 3.49-3.25 (m, 2H), 2.97-2.83 (m, 2H), 2.80-2.55 (m, 2H), [1.34-1.17 (m), 1.75 (t, J = 7.5 Hz), 3H in total]; MS (ESI) m/z 740(M+H)+; HRMS: Calcd for (M+H)+ C_{40}H_{42}N_{3}O_{11}: 740.2820, found 740.2825; Analytical HPLC t_R = 5.91 min (A), t_R = 5.56 min (B).
Scheme 3

Methyl 4-chloro-2,6-dihydroxy-benzoate (17a). A mixture of 5-chloro-resorcinol\(^5\) (749 mg, 5.2 mmol), KHCO\(_3\) (2.18 g, 21.8 mmol), and solid CO\(_2\) (7.0 g, 159 mmol) in glycerol (8 mL) was heated in a stainless steel bomb to 150 °C at 156-217 psi for 14 h. The reaction mixture was cooled, diluted with water, removed from the reaction vessel. The aqueous solution was carefully acidified with 1 N HCl to give a precipitate. The solids were filtered, washed with water, and dried to give the titled product as a beige solid (440 mg, 45%). A solution of the above-mentioned acid (440 mg, 2.3 mmol) in 3:1 (v/v) of diethyl ether/acetone (15 mL) was treated with a 0.3 M solution of diazomethane in diethyl ether (10 mL) and stirred for 10 min. Nitrogen was bubbled through the solution for 10 min and then AcOH (2 drops) was added to quench any remaining reagent. The reaction mixture was concentrated and purified by chromatography eluting with 5% EtOAc in hexanes to give the titled compound 17b as a white powder (330 mg, 71%). \(^1\)H
Methyl 6-{4-[(N-acetyl-4-[(carboxycarbonyl)(2-carboxyphenyl)amino]-3-ethylphenylalanyl)amino]butoxy}-4-chloro-2-hydroxybenzoate (17). The titled compound was prepared according to the procedure described in Example 10, substituting methyl 4-chloro-2,6-dihydroxy-benzoate 17a for methyl 2,6-dihydroxybenzoate. $^1$H NMR (a mixture of rotamers, 500 MHz, DMSO-$d_6$) $\delta$ 10.41 (s, 1H), 8.13-8.05 (m, 1H), 7.99-7.91 (m, 2H), 7.81 (dd, $J$ = 7.6, 1.4 Hz, 1H), 7.54 (td, $J$ = 8.1, 1.9 Hz, 1H), 7.45 (td, $J$ = 7.5, 1.6 Hz, 1H), 7.39 (t, $J$ = 7.6 Hz, 1H), 7.33 (d, $J$ = 7.5 Hz, 1H), 7.32-7.27 (m, 1H), 7.25 (d, $J$ = 7.5 Hz, 1H), 7.17-7.10 (m, 1H), 7.07 (d, $J$ = 8.1 Hz, 1H), 7.03 (d, $J$ = 4.4 Hz, 1H), 6.78 (t, $J$ = 9.1 Hz, 1H), 6.57 (dd, $J$ = 8.4, 1.6 Hz, 1H), 6.52 (br s, 1H), 4.51-4.41 (m, 1H), 3.96-3.88 (m, 2H), 3.73 (s, 3H), 3.09-3.00 (m, 2H), 2.98-2.88 (m, 1H), 2.82-2.72 (m, 1H), 2.68-2.55 (m, 2H), 1.78&1.75 (s, 3H in total), 1.62-1.52 (m, 2H), 1.49-1.39 (m, 2H), [1.27-1.18 (m), 0.93 (td, $J$ = 7.2, 1.9 Hz), 3H in total]; MS (ESI) $m/z$ 698, 700 (M+H)$^+$; HRMS: Calcd for (M+H)$^+$ C$_{34}$H$_{57}$ClN$_3$O$_{11}$: 698.2117, found 698.2108; Analytical HPLC $t_R$ = 5.87 min (A), $t_R$ = 5.01 min (B).

The titled compound 18 was prepared according to the procedure described in Example 9-II, substituting ethyl oxalyl chloride for benzyl oxalyl chloride, and trimethylsilyl diazomethane for diphenyldiazomethane. $^1$H NMR (a mixture of rotamers, 300 MHz, DMSO-$d_6$) $\delta$ 9.92 (s, 1H), 8.18-8.09 (m, 1H), 7.98 (t, $J = 5.9$ Hz, 1H), [7.92 (dd, $J = 8.0, 1.5$ Hz), 7.80 (dd, $J = 7.6, 1.5$ Hz), 1H in total], 7.66-7.53 (m, 2H), [7.49 (td, $J = 7.8, 1.7$ Hz), 7.43 (td, $J = 7.5, 1.0$ Hz, 1H in total], 7.35 (t, $J = 7.5$ Hz, 1H), 7.27 (dd, $J = 7.8, 3.1$ Hz, 2H), 7.23-7.01 (m, 2H), 7.15 (t, $J = 8.0$ Hz, 2H), 6.78 (t, $J = 6.8$ Hz, 1H), 6.47 (d, $J = 8.14$ Hz, 2H), 4.55-4.40 (m, 2H), 3.88 (q, $J = 6.4$ Hz, 2H), [3.81 & 3.76 (s), 3H in total], 3.72 (s, 3H), 3.11-2.86 (m, 3H), 2.84-2.71 (m, 1H), 2.67-2.42 (m, 2H), 1.78 & 1.76 (s, 3H in total), 1.65-1.51 (m, 2H), 1.50-1.38 (m, 2H), [1.22 (t, $J = 7.8$ Hz), 0.96 (t, $J = 7.5$ Hz), 3H in total]; MS (ESI) $m/z$ 706 (M+H)+; HRMS: Calcd for (M+H)+ C$_{37}$H$_{44}$N$_3$O$_{11}$: 706.2977, found 706.2985; Analytical HPLC $t_R = 5.46$ min (A), $t_R = 5.43$ min (B).
**Protein NMR Spectroscopy**

Human PTP1B (residues 1-288) was cloned into the pGEX-5X vector (Amersham Pharmacia) and expressed in *E. coli* BL21(DE3) cells. The final protein construct used for the NMR studies was 292 residues comprising the following amino acid sequence:

```
-4     1
GFSH MEMEKEFEQI DKSGSWAAIY QDIRHEASDF PCRVAKLPKN
50
KNRNRYRDVS
PFDHSRIKLH QEDNDYINAS LIKMEEAQRS YILTQGPLPN TCGHFWEMVW
100
EQKSRGVVML NRVMEKGSLK CAQYWPQKEE KEMIFEDTNL KLTLISEDIK
150
SYYTVRQLEL ENLTTQETRE ILHFHYTTWP DFGVPESPAS FLNFLFKVRE
200
SGSLSPEHGP VVHCSAGIG RSGTFCLADT CLLLMDKRKD PSSVDIKKVL
250
LDMRKFRMGL IQTAEQLRFS YLAVIEGAKF IMGD
288
```

Two point mutations exist in the construct (E252D and D265E, underlined), along with four additional N-terminal residues (GFSH) that remain after cleavage of the GST fusion.

Uniformly $^{15}$N-labeled and $^{13}$CH$_3$(IVLM)-labeled PTP-1B was prepared by growing the bacteria on a minimal medium containing 1 g/L of $^{15}$NH$_4$Cl (Cambridge Isotopes) or by adding 100/100/50 mg/L of $^{13}$C-γ-methionine/3,3'-$^{13}$C-α-ketovalerate/3-$^{13}$C-α-ketobutyrate$^6$ 1 h before induction with 1 mM IPTG. Carbenicillin (0.1 mg/mL) was used
as the selection agent. In addition to the standard salts, metals, and vitamins, the media
contained the following additives (g/L of culture): Ala (0.5), Arg (0.4), Asp (0.4), Cys
(0.05), Glu (0.65), Gln (0.4), Gly (0.55), His (0.1), Lys (0.42), Phe (0.13), Pro (0.1), Ser
(2.1), Thr (0.23), Tyr (0.17), adenine (0.5), guanosine (0.65), thymine (0.2), uracil (0.5),
cytosine (0.2), sodium acetate (1.5), and succinic acid (1.5). The cell pellets were
resuspended in a buffer containing 10 mM sodium phosphate (pH 7.4) and 150 mM NaCl
(PBS buffer). The cells were then lysed using a microfluidizer (Microfluidics
International), and the resulting lystate was clarified via centrifugation at 76,000 x g for
10 min. The clarified cell lysate was loaded onto a glutathione Sepharose 4B column
(Pharmacia), and the resin was washed with 20 column volumes of PBS buffer. The
protein was eluted with 5 column volumes of a buffer containing 50 mM Tris (pH 8.0)
and 10 mM reduced glutathione. The N-terminal GST fusion was cleaved with thrombin
(1 U of thrombin/mg of PTP1B) at 4°C for 72 h. Cleavage was monitored by SDS–
PAGE analysis. The resulting solution was then concentrated and dialyzed to remove the
 glutathione (final dilution factor of 10^{-10}), and loaded onto a glutathione Sepharose 4B
column to remove the GST. The flow-through (containing PTP1B) was collected and
dialyzed against NMR buffer (see below). Typical protein yields were 10–20 mg of
PTP1B per liter of culture.

The NMR samples were composed of uniformly ^{15}N-labeled or ^{13}CH_{3}(IVLM) PTP1B in
an H_{2}O/D_{2}O (9/1) Tris buffered solution (25 mM, pH 7.5) containing DTT (10 mM).
Ligand binding was detected by acquiring sensitivity-enhanced ^{1}H/^{15}N- or ^{1}H/^{13}C-HSQC
spectra on 400 µL of PTP1B in the presence and absence of added compound. Protein
concentrations were 300 and 25 µM for the ^{15}N- and ^{13}CH_{3}(IVLM)-labeled samples,
respectively. A Bruker sample changer was used on a Bruker AMX500 spectrometer equipped with a cryoprobe. Compounds were individually tested at concentrations of 0.1–15.0 mM, and binding was determined by monitoring changes in the HSQC spectra. Dissociation constants were obtained for selected compounds by monitoring the chemical shift changes of the protein resonances as a function of ligand concentration. Data were fit using a single binding site model. A least-squares grid search was performed by varying the values of $K_d$ and the chemical shift of the fully saturated protein.

X-ray Crystallographic Studies: Protein Production, Crystallization, and Data Collection. The final concentration of the purified PTP1B protein (1–322) was 3–4 mg/mL and containing various amounts of reducing agents, normally 2–4 mM DTT. Crystals of PTP1B belonging to space group $P\bar{3}_121$ ($a = b = 88.4$ Å, $c = 104.5$ Å) were grown routinely using the protocol described by Puius et al. with the following changes. The buffers used in protein crystallization were degassed for approximately 30 min and then purged with Argon gas prior to their use for crystallization. This simple precaution extended the lifetime of the reactive cysteine (Cys215) in the active site and provided a more stable and structurally homogeneous conformation of the active site. This protocol also allowed the direct soaking of all the PTP1B inhibitors into the crystals. Soaking times ranged from 1 to 5 h and were normally no more that 2 h. Soaking was performed using 50 µL of the reservoir in the crystallization well and mixed with a 2–4 µL of DMSO stock solution of the compound at 100 µM. The soaking protocol was validated with a control compound, 2-(oxalylamino)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-
carboxylic acid. No significant differences were observed within an extended area (~20 Å) around the active site when the structure obtained was compared with the one reported. The soaking protocol permitted the rapid structure determination of PTP1B/inhibitor complexes.

After the soaking period, crystals were transferred to a cryoprotectant solution for 5-10 seconds with rayon loops and exposed to the X-rays. The cryoprotectant solution consisted of 100 mM HEPES and 0.2 M magnesium acetate adjusted to pH 7.1 with NaOH at a 16% w/v concentration of PEG8000 with a 35% v/v concentration of glycerol. Crystallographic data were collected both in a conventional, in-house protein crystallography laboratory and occasionally at sector 17 of the Advanced Photon Source (ID and BM lines of IMCA-CAT). The data collection hardware was a Mar Research image plate (180 mm), a Mar Research 345mm image plate, or a Mar Research 165 mm CCD detector, all driven by the manufacturer’s software. For a complete data set, 80 1° frames were collected at a crystal to detector distance of 80–90 mm. Exposure times were typically 10 min/frame for in house data collection and 10 seconds for the synchrotron source. This strategy resulted in > 95% completeness in the vast majority of the data sets with reducing R factors between 5% and 9%. Data were processed with HKL2000 and scaled with SCALEPACK or within HKL2000. Phase and map calculations were performed using XPLOR or CNX. The modeling and electron-density fitting software QUANTA was used to manipulate the models.

**Biological Assay Using pNPP as Substrate.**
Protein tyrosine phosphatase 1B (PTP1B) activity was determined by measuring the rate of hydrolysis of a surrogate substrate, \( p \)-nitrophenyl phosphate (\( p \)NPP, C1907 Sigma, St. Louis, MO). The assay was carried out at room temperature in 96 well polypropylene or polyethylene plates in a total volume of 100 µL per well. Appropriate dilutions of the compounds were made in DMSO and then diluted ten fold into assay buffer (25 mM HEPES (pH = 7.5), 150 mM NaCl and 0.1 mg/mL BSA). Five different concentrations of the inhibitor (10 µL each) or 10% DMSO in assay buffer were added to individual wells containing 40 µL of 3.2, 8, 20, and 50 mM \( p \)NPP in assay buffer. The reaction was initiated by adding 50 µL of diluted PTP1B diluted in assay buffer. The phosphatase activity resulted in the formation of the colored product \( p \)-nitrophenol (\( p \)NP) which was continuously monitored at 405 nm every 30 seconds for 15 min using an appropriate plate reader. The absorbance at 405 nm was converted to nanomoles of \( p \)NP using a standard curve and the initial rate of \( p \)NP formation was calculated. For each concentration of the inhibitor or DMSO control, the initial rates were used to fit the rectangular hyperbola of Michaelis-Menten by non-linear regression analysis (GraphPad Software Prism 3.0). The ratio of the apparent \( K_m/V_{\text{max}} \) vs. inhibitor concentration was plotted and the competitive \( K_i \) was calculated by linear regression to be the negative x-intercept.

Phosphatase selectivity of PTP1B inhibitor 6 and 17 was determined using the analogous assays.

**High Throughput Screening Caco-2 Permeability Protocol.**

Compounds 10 and 18 to be analyzed for \textit{in vitro} cell permeability were prepared as follows. DMSO stocks of 30 mM compound were diluted 1:600 into HBSS (Gibco)
containing 10 mM HEPES, pH 6.8, and 500 µg/mL Lucifer Yellow (Sigma). Samples were briefly vortexed, placed in sonicating water bath for 5 min, and then spun at ~14,000 x g in a microcentrifuge for 5 min. The supernatant was carefully removed to clean 1.5 mL eppendorf tubes. These dose solutions were labeled as T0 and used in the cell experiments as described below.

Human adenocarcinoma colon cells (Caco-2) were purchased from ATCC. Cells were plated into 24-well Transwell polycarbonate filter plates (Corning) at a density of ~400,000 cells per well and maintained in DMEM (Gibco) supplemented with 10% FBS (HyClone), 2 mM L-glutamine (Gibco), and 1xPen/Strep (Gibco) for 14-16 d, changing the medium every Monday, Wednesday and Friday. Volume of medium in the apical and basal chambers was 0.25 and 1.0 mL, respectively. Immediately prior to use in a permeability experiment, media was aspirated from the apical and basal chambers and the filters were rinsed with 1 mL of HBSS supplemented with 10 mM HEPES. The filters were preincubated in this buffer for approximately 15 min, after which time the buffer in the apical chamber was replaced with 0.2 mL of T0 stock. The buffer in the basal chamber was replaced with 0.4 mL of HBSS containing 10 mM HEPES, pH 7.4. The plates were incubated at 37 °C in 5% CO2/95% air for 1 h. After incubation, ~150 µL of apical sample and ~ 200 µL of basal samples were collected into a 96-well plate for mass spec analysis. Blank filters, which did not contain cells, were prepared the same way and run in parallel with the cell filters, except that ~40 µL apical sample and 200 µL basal samples were recovered for analysis. Radioactive caffeine (50 µM) and vinblastine (5
µM) were included in each experiment as model drug controls, and 1% DMSO as a vehicle control.

The integrity of the Caco-2 cell monolayer was evaluated by collecting a 20 µL basal sample from each well into a 96-well plate, diluting it 1:10 with HBSS and reading on a fluorescent plate reader set to detect Lucifer Yellow (Cytofluor. Applied Biosystems). The percent Lucifer Yellow transported was calculated according to Audus et al.13 Less than 0.25% Lucifer Yellow transported per h indicated an intact cell monolayer.

Quantitation of the test and blank samples was performed by LC/MS using a Sciex Pulsar Qtof system. Calibration curves for the test compounds were generated by mixing T0 stocks of up to 8 compounds and then preparing a dilution series in HBSS, pH 7.4. The T0 titration and the apical and basal samples from the filters, with and without cells, were arrayed in the 96-well plate.

Peak areas for the test compounds were imported into an Excel spreadsheet where the apparent permeability (P_{app}) was calculated by:

\[ P_{app} = \frac{(dQ/dt)}{(A*Co)} \]

Where \( (dQ/dt) \) = flux rate (nmol/sec), \( Co \) = compound concentration at \( t = 0 \), \( A \) = surface area of the filter (cm\(^2\)).

The permeability of the compounds was categorized as low (\( P_{app} < 1 \times 10^{-6} \) cm/sec), moderate (\( P_{app} = 1 - 10 \times 10^{-6} \) cm/sec), or high (\( P_{app} > 10 \times 10^{-6} \) cm/sec). These values are
predictive for human in vivo oral absorption and correspond to poorly, moderately and well-absorbed compounds, respectively.\textsuperscript{14}

**Conversion of Prodrug 18 to Parent Inhibitor 10 in FAO Cell Lysates.**

FAO (passage 19) was inoculated for three days in 2 x T-162 cm\textsuperscript{2} flasks until it reached 75\% confluent. The cells were washed twice with cold PBS, and scraped in 1mL cold 75 mM KCl containing protease inhibitors (20 µM leupeptin, 1 mM benzamidine, 2 mM AEBSF, 1 mM sodium orthovanadate, 1µM microcystin and 250 nM okadaic acid). The samples were sonicated on ice (30\% amplitude, 4 \times 1 second pulses), and then centrifuged at 14,000 g for 10 min. The supernatants were collected, and spiked with prodrug 18 in DMSO to achieve 100µM of the final concentration of the drug with 1\% of the final DMSO concentration. After incubated for 3 h, the sample was analyzed by HPLC-MS-ELSD on an Open Access Finnigan Navigator/Agilent 1100/Sedere Sedex 75 system using a Phenomenex Luna C8 column (5 µm, 2.1 \times 50mm). Solvent system used was acetonitrile/0.1% aqueous TFA, gradient 10-100\% over 4.5 min @ 1.5 ml/min. The MS was operated in the + APCI mode. Comparison of the peak corresponding to 18 and the new peak corresponding to the parent compound 10 indicated approximately 50\% conversion of the prodrug.

**PKB Phosphorylation Assay in FAO Cells.**

*FAO Cell Culture*—FAO rat hepatocytes were cultured in RPMI 1640 (with 25 mM HEPES and L-glutamine) containing 10\% fetal bovine serum (FBS), 1\times non-essential amino acids and 50 µg/mL Gentamicin (all Gibco products). Cells were inoculated at
500,000 cells/well in 24-well plates and grown for a period of 2 d prior to drug treatment. Cells were serum-starved in basal medium for 2 h and then drugs were added at 50 µM, 100 µM, and 300 µM respectively, with a 1% final concentration of DMSO. Cells were incubated with drugs for 3 h and then treated with 0.025 nM and 1.0 nM of bovine insulin (Sigma) for 15 min, respectively. A non-specific phosphatase inhibitor, BpV(pic)$^{15}$ (Alexis, San Diego, CA), at 10 mM was used as a positive control. Finally, 110 µL lysis buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, 25 mM NaCl, 1% NP-40, 10 mM NaF, 20 µM leupeptin, 1 mM benzamidine, 2 mM AEBSF, 1 mM NaVO4, 250 nM okadaic acid and 1µM microcystin) was added to each well. Lysates were stored at –80 °C prior to ELISA analysis.

**PKB ELISA**—PKB activity was measured using time-resolved fluorometry (TRF DELFIA assay, Wallac). All incubation steps were carried out on a shaker at room temperature. Protein A coated plates (Pierce) were incubated for 1-2 h with 5 µg/mL coating antibody, specific for the C-terminal end of the protein: Rabbit anti-phospho-AKT, phospho-Ser472/473/474 (BD PharMingen) diluted in TBS containing a 3% final concentration of BSA (7.5% BSA stabilizer, Wallac). Plates were washed 4 times with TBS-T and 100 µL of lysates were added and incubated for at least 1 h. Plates were washed and active PKB was detected with a Europium-tagged (Wallac) antibody (anti-AKT1/PKBα, PH Domain, Upstate Biotechnology, diluted in DELFIA assay buffer), specific for the N-terminal end of the protein, for 30 min. Plates were washed and 100 µL/well of DELPHIA enhancement solution, which causes dissociation of Europium chelate from the bound detection antibody, was added and incubated for 5 min. Fluorescence was measured using the Wallac 1420 Victor2 plate reader.
References and Notes


(4) Prepared from according to the procedure described for compound 9-II, substituting methyl 3-bromo-2,6-dihydroxybenzoate for methyl 2,6-dihydroxybenzoate.


