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A protected L-bromophosphonomethylphenylalanine amino acid derivative (BrPmp) for synthesis of irreversible protein tyrosine phosphatase inhibitors

Naresh S. Tulsi, A. Michael Downey, Christopher W. Cairo *

Alberta Ingenuity Centre for Carbohydrate Science, Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

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ABSTRACT

Protein tyrosine phosphatases (PTPs) are important therapeutic targets for medicinal chemists and biochemists. General strategies for the development of inhibitors of these enzymes are needed. Several modular strategies which rely on phosphotyrosine mimics are known for PTP inhibitors. Previous strategies include phosphonomethylphenylalanine (Pmp) derivatives which act as competitive inhibitors. Pmp amino acid derivatives have been used to develop specific inhibitors by incorporation into sequences recognized by the PTP of interest. We report the synthesis of a new phosphonotyrosine analog, L-phosphonobromomethylphenylalanine (BrPmp), which acts as an inhibitor of PTPs. The BrPmp derivative was prepared as an Fmoc-protected amino acid which can be used in standard solid phase peptide synthesis (SPPS) methods. The synthesis of the protected amino acid derivative requires 11 steps from tyrosine with a 30% overall yield. Enzyme inhibition studies with the PTP CD45 demonstrate that BrPmp derivatives are irreversible inhibitors of the enzyme. A tripeptide which incorporated BrPmp had increased inhibitory potency against PTP relative to BrPmp alone, confirming that the incorporation of BrPmp into peptide sequences provides additional context to improve enzyme binding.

1. Introduction

Protein tyrosine phosphatases (PTPs) are important for the regulation of signaling pathways, acting as a biochemical counterbalance to kinases.[1,2] This mechanism plays diverse roles in biological systems, including the regulation of T cell antigen recognition and activation.[3] As a result, PTPs are an important target for both medicinal chemistry and biochemical research.[4,5] However, few general strategies have been elucidated for the determination of PTP specificity or inhibitor design. CD45 is a well studied human immune cell receptor-like PTP (RPTP), and the most prevalent membrane-associated PTP in T cells.[6] Misregulation of CD45 results in severe combined immunodeficiency (SCID), and the receptor is implicated in autoimmune diseases such as lupus and arthritis. Currently, the primary strategies to examine CD45 specificity rely on the use of phosphotyrosine-specific antibodies, or the synthesis of phosphopeptide substrates.[7] Methods that allow for detection, labeling, or inhibition of active PTP enzymes could complement or improve these strategies to expand our understanding of PTP signaling.

The design of specific PTP inhibitors remains a challenge, and new strategies that provide enhanced activity or reduce development time are of continued interest.[4] A classic strategy for designing PTP inhibitors has exploited non-hydrolyzable phosphotyrosine (pTyr) mimics, such as phosphonomethylphenylalanine (Pmp) which act as competitive inhibitors of the enzyme.[8] Modification of Pmp to a phosphonodifluoromethylphenylalanine (F_2Pmp) improves the potency of these derivatives (Fig. 1).[9] A key advantage of these compounds is their adaptability for solid phase peptide synthesis (SPPS) when prepared with the appropriate α-amino and phosphonate protection. Reported derivatives of Pmp include fluoro, difluoro, chloro, and dichloro derivatives.[9–13] These strategies have been successfully applied to develop competitive inhibitors of a variety of PTPs.[14]

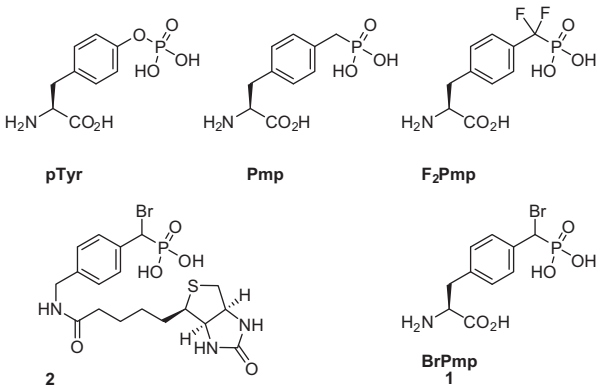

Figure 1. Synthetic phosphotyrosine mimics.

* Corresponding author. Tel.: +1 780 492 0377; fax: +1 780 492 8231.
E-mail address: ccairo@ualberta.ca (C.W. Cairo).

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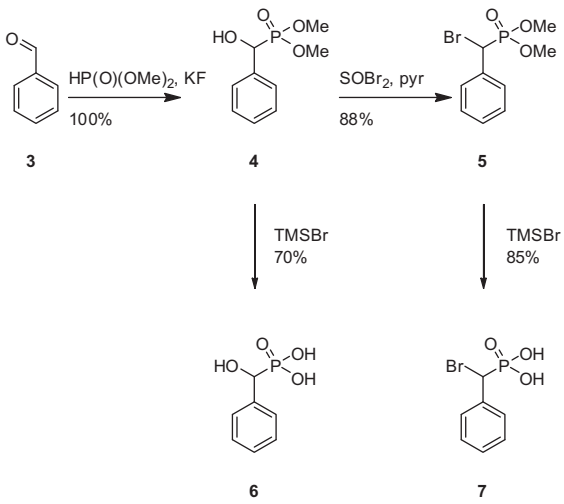
There has been sustained interest in identifying covalent inhibitors of PTPs. In addition to improved potency, covalent inhibitors (sometimes referred to as suicide substrates) can be of interest for developing enzyme labeling strategies. For example, covalent inhibitors when attached to fluorophores or affinity tags have been employed as activity-based protein probes (ABPP).[15,16] Some of the known covalent inhibitors for PTPs include quinone methides,[17–20] aryl vinyl sulfonates,[21] nitrostyrene,[22] and α-bromobenzylphosphonate (BBP) derivatives.[23] Other notable strategies have included fluorogenic substrates of PTP enzymes, which allow improved assay, detection, and imaging applications.[24]

Taylor et al. were the first to test the activity of α-bromobenzylphosphonate (BBP) analogs as inhibitors of PTPs,[25] and this was later used as a labeling strategy with biotin-tagged derivative **2**.[23] In contrast to the fluoro, difluoro, and chloro-Pmp derivatives, the BBP analogs were found to form covalent adducts with PTPs, forming the basis of proteomic strategies for PTP identification. Compound **2** was shown to covalently label a variety of PTPs, limiting its application as an inhibitor of specific enzymes. We considered that PTPs often recognize specific amino acid sequences,[26] and that additional functional groups could potentially impart specificity to the α-bromophosphonate group. Additionally, we desired an efficient route to an α-bromophoshonate that could be used for both labeling and inhibitor studies. We report here a convenient and efficient route to a phosphonobromomethylphenylalanine (BrPmp, **1**), and derivatives appropriately protected for SPPS (Fmoc-L-BrPmp(*O*Me$_2$)-OH, **16**). We found that amino acid derivatives, which incorporated BrPmp, act as irreversible PTP inhibitors. Importantly, we find that inclusion of BrPmp in a peptide sequence significantly improved its potency, suggesting that the context of the BBP functional group can improve inhibitor activity.

2. Results and discussion

2.1. α-Bromobenzylphosphonate analogs

Before synthesizing the desired amino acid targets, we first undertook the synthesis of model BBP analogs based on benzaldehyde (Scheme 1). Condensation of benzaldehyde with dimethyl phosphite gave the desired hydroxy(phenyl)dimethyl phosphonate **4** in quantitative yield.[25] Initial attempts to convert **4** to the corresponding bromide were unsuccessful using triphenylphospine dibromide.[23] We then explored a variety of reported bromination conditions for related substrates. Among the conditions tested were PBr_3,[27] PPh_3/NBS,[25] and MsCl[28] followed by treatment with a bromide nucleophile (triethylammonium bromide or sodium bromide) (see Supplementary data, Table SI1). We only observed significant formation of product **5** after treatment with thionyl bromide and pyridine.[29] The reduced reactivity of the substrate may be due to the sterically congested environment of the hydroxyl. After optimization of the thionyl bromide conditions, we were able to achieve excellent yields for bromination of **4** (88%). With access to **5**, we then confirmed that deprotection of the dimethoxyphosphonate proceeded in good yield with the use of trimethylsilylbromide,[30] for both the hydroxy and bromo derivatives to give the model hydroxy(phenyl)dimethyl, **6**, or bromo(phenyl)dimethyl, **7**, respectively. Based on these results, we proceeded to develop a method for generating the desired phenylalanine targets.



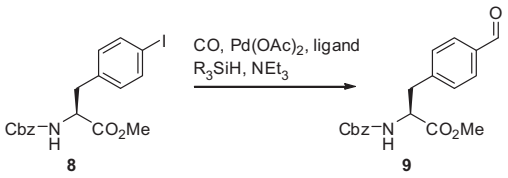
Scheme 1. Synthesis of model α-bromophosophonates.

2.2. Synthesis of Fmoc-L-BrPmp(OMe$_2$)-OH (16)

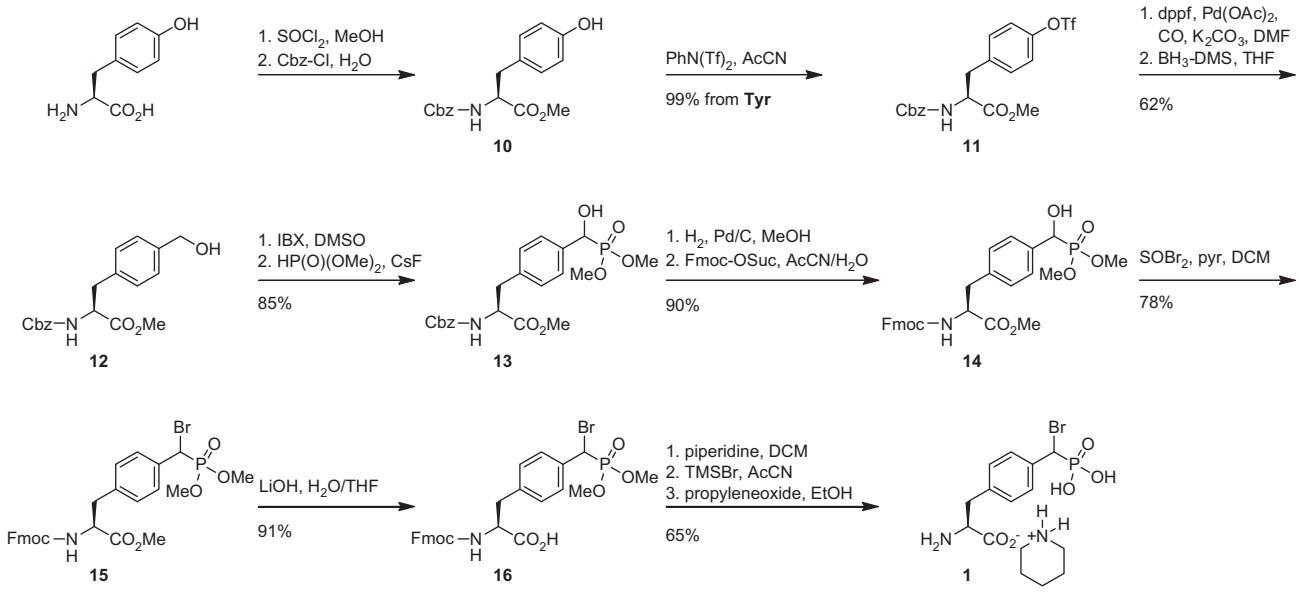
We set out to generate a protected tyrosine derivative with a benzylic aldehyde suitable for condensation to the phosphonate. We first considered a common strategy for modification of phenylalanine via iodotyrosine,[31] which could be a good substrate for oxidation to the corresponding aldehyde.[32] We selected carboxybenzyl (Cbz) as the α-amino protecting group for the first part of the synthesis, since other common protecting groups would be incompatible with intermediate steps of the synthesis. The use of a fluoride catalyst required for the initial phosphonylation would prevent the use of Fmoc, and bromination conditions with thionyl bromide would be incompatible with Boc.

In our first attempt at the route, phenylalanine was converted to iodotyrosine in good yield and subsequently protected as a methyl ester via acid-catalyzed esterification and α-amino protection as the Cbz amino acid (**8**) as previously reported.[33] We attempted conversion of **8** to the protected aldehyde **9** (Scheme 2). However, this strategy gave unreliable results in our hands (see Supplementary data, Table SI2).[32] Although we could achieve moderate yields, we found that the reaction was not scalable, with decreasing yields at larger scale. We, therefore, chose to explore alternative routes.

We chose to access the benzylic aldehyde through the conversion of the protected tyrosine **10** to the triflate followed by carbonylation (Scheme 3). Compound **10** was converted to triflate **11** using *N*-phenyl-bis-trifluoromethane sulfonamide in quantitative yield.[13] The triflate was converted to the carboxylic acid using $Pd(OAc)_2$.[34,35] The crude acid was then reduced to the benzyl alcohol (**12**) with BH_3–DMS complex.[36] Oxidation of the purified benzyl alcohol to the aldehyde using IBX was done using standard conditions.[37] We observed that prolonged storage of the intermediate aldehyde even at −20 °C resulted in partial conversion to the hydrate. To avoid this issue, the aldehyde was used immediately in the subsequent cesium fluoride-catalyzed condensation[38] reaction with dimethyl phosphite to generate compound **13**.[39] Initially, potassium fluoride was used to catalyze the condensation; however, significant amounts of starting material were still present after 24 h. We found that employing cesium fluoride gave complete conversion within 20 min, likely due to the cesium salt acting



Scheme 2. Carbonylation of iodotyrosine.



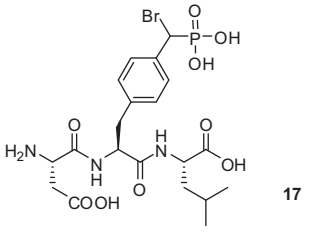
Scheme 3. Synthesis of Fmoc-L-BrPmp-OH.

as a more readily dissociated fluoride source. To make a protected amino acid derivative suitable for automated SPPS, we chose to replace the Cbz group with Fmoc using standard conditions, to provide compound **14**.

The key bromination step followed the conditions tested in our model studies. Treatment of **14** with thionyl bromide in the presence of pyridine converted the α-hydroxyphosphonate to the fully protected bromide, **15**. Subsequent cleavage of the carboxylate methyl ester with lithium hydroxide provided compound **16** in good yield. The overall yield from tyrosine to **16** was 30% in 11 steps, with only five chromatographic steps. Compound **16** was deprotected for use in enzyme assays by treatment with 20% piperidine in DMF to remove Fmoc, followed by cleavage of the phosphonomethyl groups with TMSBr.[40] Propylene oxide was added to remove residual HBr, allowing isolation of compound **1** as the piperidine salt in 65% yield. It has not escaped our attention that the final compound is isolated as a mixture of diastereomers at the α-bromo-phosphonate, and that there may be potential differences in activity between the two isomers. However, there is a large body of previous work with non-hydrolyzable phosphonate derivatives which have been successfully used for inhibition studies with diastereomeric mixtures.[41] We intend to investigate the activity of the separated diastereomers in future work.

2.3. Synthesis of peptide sequences

To test the compatibility of compound **16** with SPPS, we chose to synthesize a short peptide sequence, Asp-BrPmp-Leu (**17**) (Scheme 4). We chose a short sequence that contained an acidic residue N-terminal to the phosphotyrosine site, and a hydrophobic



17

Scheme 4. Synthetic tripeptide Asp-BrPmp-Leu (**17**).

reside at the C-terminal side—both of which are features commonly found in phosphopeptide substrates of CD45.[26] The peptide was generated using standard solid phase methods, based on the Fmoc protecting group strategy. For reactions using compound **16** we employed HBTU as a coupling agent with DIPEA as a base. Couplings were performed with a 2:1.96:4 molar ratio of **16**/HBTU/DIPEA, in two cycles. To cleave the peptide from resin, dry resin was treated with 95% TFA and 5% water for 1 h, followed by treatment with TMSI for 100 min.[40] Chromatograms of the cleavage products suggested that the coupling reactions were efficient. The isolated peptides were confirmed by HR-MS, ^{1}H and ^{31}P NMR and analytical HPLC. We initially chose to avoid commonly employed thiol scavengers in the cleavage of the methyl ester phosphonate protecting groups in anticipation of displacement of the α-bromide. However, we confirmed by NMR that compound **7** was stable to dithiothreitol (DTT) and thioanisole, suggesting that scavenger nucleophiles should not interfere with peptide cleavage and deprotection.[23,25]

2.4. Inhibition of CD45

To examine the ability of the BrPmp derivatives to act as inhibitors of a PTP, we tested compounds **1**, **7**, and **17** against CD45. Assays were conducted with the fluorogenic substrate, 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP), and are summarized in Table 1 (see Supplementary data, Figs. SI1 and SI2). The K_m for DiFMUP with CD45 was first determined, and the experiment was then repeated in the presence of compounds **7**, **1**, and **17** to provide an apparent K_m ($K_{m,\,obs}$). We found significant inhibition of CD45 for compounds **1** and **17** at micromolar concentrations; however, compound **7** did not have a significant effect on enzyme kinetics even at millimolar concentration. Previous reports of PTP inhibition using BBP analogs observed irreversible enzyme inhibition.[23] Evaluation of our kinetic data using linear transforms was not consistent with pure competitive inhibition of CD45 by **1** or **17**; however, this analysis is not conclusive on its own (see Supplementary data, Fig. SI3). To provide additional insight into the inhibition of these compounds, we obtained K_I values using a Kitz–Wilson analysis.[42,43] Compound **7** did not give a line with positive slope in this analysis, and therefore could not be analyzed by this method, consistent with its failure to alter the rate of

Table 1
Inhibition of CD45

Compd	Enzyme	Inhibitor (μM)	$K_{m,\,obs}$ ±[a] (μM)	K_I ±[b] (μM)	k_3 ± (min^{-1})
—	CD45	0	90 ± 20	na	—
7	CD45	1500	99 ± 14	na	—
1	CD45	150	141 ± 18	40 ± 8	0.041 ± 0.001
17	CD45	35	141 ± 34	16 ± 4	0.048 ± 0.003

[a] Values were determined by non-linear regression of the observed rate of reaction in the presence of inhibitor using the Michaelis–Menten equation.[43] Error is reported as the relative error from the fit.
[b] For compounds **1** and **17**, K_I was determined by Kitz–Wilson analysis. The rate of enzyme inactivation, k_3, was also determined.[42]

reaction (vide supra). Compound **7** has been previously tested as an inhibitor of the PTP Yop51, and exact kinetic constants were difficult to obtain, and we found similar difficulties for this determination with CD45.[25] This result may be due to the more hydrophobic nature of the compound. Using the Kitz–Wilson analysis, both compound **1** and **17** were found to have K_I values of 40 ± 8 μM and 16 ± 4 μM, respectively (Fig. 2). These results indicate that the tripeptide was approximately fourfold more potent than BrPmp alone, suggesting that the adjacent amino acid side chains contribute additional specificity to the inhibitor. The Kitz–Wilson analysis estimated the rate of irreversible inhibition (k_3) of CD45 at 0.05 min^{-1} for both compounds. To provide additional support for the expected mechanism of inhibition, we measured CD45 activity for an enzyme sample which was pre-incubated with the inhibitor and compared it to an enzyme sample that was only incubated with the inhibitor for a short period. We found that pre-incubation of the enzyme reduced activity, and at long incubation times completely inactivated the enzyme (see Supplementary data, Fig. SI3), confirming that the inhibitors act irreversibly at long incubation times.

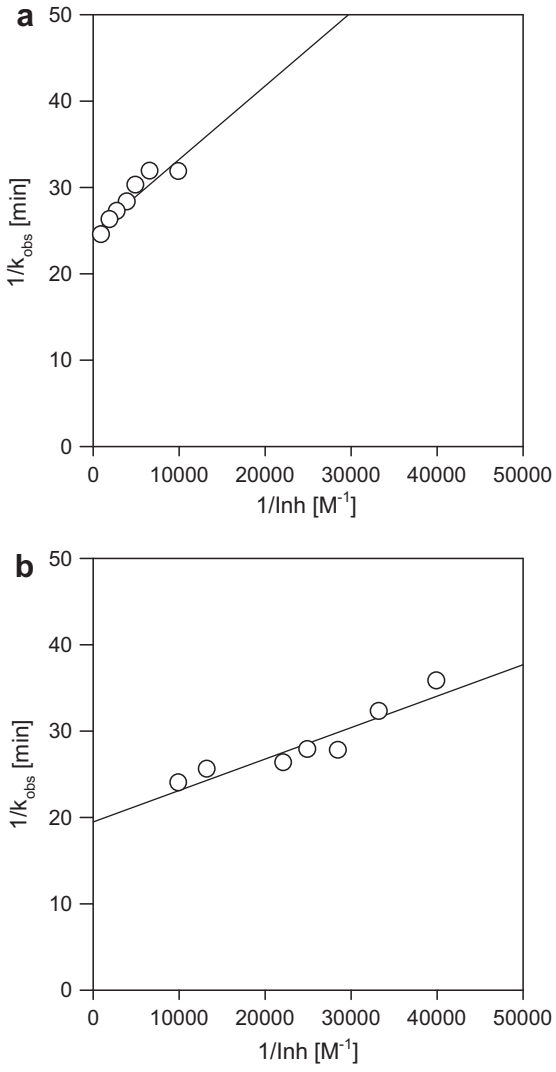


Figure 2. Kitz–Wilson analysis of compounds **1** and **17**. Compounds (a) **1** and (b) **17** were examined using a Kitz–Wilson analysis.[42] The apparent rate constant (k_{obs}) was determined for a series of concentrations of the inhibitor.[44] The data are plotted as the linear transform based on Eq. 1. Values from the non-linear regression were used for Table 1.

3. Conclusion

We present an efficient synthetic route for the preparation of BrPmp analogs that can be readily used in automated SPPS. Our method provides the Fmoc-L-BrPmp-OH derivative in 30% yield over 11 steps from tyrosine, and required only five column separations. These compounds act as irreversible inhibitors of the PTP CD45, supporting the previously observed selectivity of the α-bromobenzylphosphonate functional group.[23] Importantly, the synthetic method reported here is scalable, and the products can be easily modified using standard amino acid chemistry. The observation of improved potency for BrPmp within the context of a peptide sequence suggests that these derivatives can act as specific inhibitors of PTPs. Future work in our group will address the specificity of PTP inhibition by BrPmp derivatives, and their use as ABPP.

4. Experimental methods

4.1. Synthesis of dimethyl [hydroxy(phenyl)methyl]phosphonate (4)

Dimethyl phosphite (1.86 mL, 20.3 mmol, 1.03 equiv) was added to benzaldehyde, **3**, (2 mL, 19.8 mmol, 1 equiv) and stirred for 5 min. KF (5.75 g, 99.0 mmol, 5 equiv) was then added and the reaction mixture was stirred until it solidified (~20 min). The crude product was then dissolved in DCM, filtered, and the solvent evaporated yielding **4** as a white powder (4.38 g, 100%). The product was dried under high vacuum over P_2O_5. No further purification was required. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53–7.45 (m, 2H), 7.43–7.23 (m, 3H), 5.05 (d, J = 11.1 Hz, 1H), 3.69 (d, J = 10.4 Hz, 3H), 3.65 (d, J = 10.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 136.7, 128.6, 128.5, 127.3 (d, J_{C-P} = 5.7 Hz), 70.9 (d, J_{C-P} = 159.6 Hz), 54.3 (d, J_{C-P} = 7.0 Hz), 53.7 (d, J_{C-P} = 7.0 Hz). ^{31}P NMR (162 MHz, $CDCl_3$) δ 24.71. ESIMS calcd for $C_9H_{13}O_4P$ $[M+Na]^+$ 239.0444, found: 239.0441; mp 85–86 °C.

4.2. Synthesis of dimethyl [bromo(phenyl)methyl]phosphonate (5)

In a round bottom flask **4** (1 g, 4.63 mmol, 1 equiv) was dissolved in dry DCM (10 mL) and dry pyridine (0.47 mL, 5.78 mmol, 1.25 equiv) was added. Thionyl bromide (0.45 mL, 5.78 mmol,

1.25 equiv) was then added to the round bottom flask under inert atmosphere. The round bottom flask was sealed with a septum, cooled in an ice bath and slowly allowed to come to room temperature overnight. The solvent was then evaporated and the crude product was dissolved in ethyl acetate. The organic layer was washed with 1 M HCl, saturated $NaHCO_3$, water, brine, then dried over Na_2SO_4 and filtered. The solvent was evaporated and the crude product was purified on a silica plug 1:4 (hexanes/ethyl acetate) followed by 3:7 (hexanes/ethyl acetate) to give **12** as a viscous oil (1.14 g, 88%). The product was dried under high vacuum over P_2O_5. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (d, J = 7.8 Hz, 2H), 7.34 (d, J = 7.5 Hz, 2H), 7.26 (1H), 4.88 (d, J = 13.1 Hz, 1H), 3.85 (d, J = 10.8 Hz, 3H), 3.60 (d, J = 10.7 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 134.6 (d, J_{C-P} = 3.3 Hz), 129.7 (d, J_{C-P} = 6.7 Hz), 129.4 (d, J_{C-P} = 2.2 Hz), 129.0 (d, J_{C-P} = 1.3 Hz), 55.1 (d, J_{C-P} = 7.0 Hz), 54.7 (d, J_{C-P} = 7.0 Hz), 41.1 (d, J_{C-P} = 159.9 Hz). ^{31}P NMR (162 MHz, $CDCl_3$) δ 20.65. ESIMS calcd for $C_9H_{12}BrO_3P$ $[M+Na]^+$ 300.9600, found: 300.9596.

4.3. Synthesis of [hydroxy(phenyl)methyl]phosphonic acid (6)

Compound **4** (0.15 g, 0.69 mmol, 1 equiv) was dissolved in dry DCM (5 mL). TMSBr (0.77 mL, 5.52 mmol, 8 equiv) was added to the solution under an inert atmosphere. The reaction was stirred for 20 h at room temperature. The solvent was then evaporated, and MeOH (5 mL) was added to the reaction mixture and stirred for 1 h. The solvent was evaporated and the crude product was dissolved in water (5 mL), filtered and freeze dried, yielding **6** as a white powder (130 mg, 70%). ^{1}H NMR (400 MHz, D_2O) δ 7.52–7.31 (m, 5H), 4.99 (d, J = 12.3 Hz, 1H). ^{13}C NMR (101 MHz, D_2O) δ 137.4, 128.8 (d, J_{C-P} = 2.3 Hz), 128.5 (d, J_{C-P} = 2.9 Hz), 127.4 (d, J_{C-P} = 5.7 Hz), 71.0 (d, J_{C-P} = 158.3 Hz). ^{31}P NMR (162 MHz, D_2O) δ 20.98. ESIMS calcd for $C_7H_9O_4P$ $[M-H]^-$ 187.0166, found: 187.0162; mp 160–162 °C.

4.4. Synthesis of [bromo(phenyl)methyl]phosphonic acid (7)

Compound **5** (0.3 g, 1.08 mmol, 1 equiv) was dissolved in dry DCM (5 mL). TMSBr (1.2 mL, 8.64 mmol, 8 equiv) was added under an inert atmosphere. The reaction was stirred for 20 h at room temperature. The solvent was then evaporated, and MeOH (5 mL) was added to the reaction mixture and stirred for 1 h. The solvent was evaporated and the crude product was dissolved in water (5 mL), filtered and freeze dried yielding **7** as a white powder (229 mg, 85%). ^{1}H NMR (400 MHz, D_2O) δ 7.61–7.52 (m, 2H), 7.44–7.33 (m, 3H), 5.10–5.02 (m, 1H). ^{13}C NMR (101 MHz, D_2O) δ 136.5 (d, J_{C-P} = 3.3 Hz), 129.4 (d, J_{C-P} = 6.0 Hz), 129.1, 43.54. ^{31}P NMR (162 MHz, D_2O) δ 15.44. ESIMS calcd for $C_7H_7BrO_3P$ $[M-H]^-$ 248.9322, found: 248.9317 mp 158–60 °C.

4.5. Synthesis of methyl 2-{[(benzyloxy)carbonyl]amino}-3-(4-iodophenyl)propanoate (8)

Iodine (5.24 g, 20.64 mmol) and sodium iodate (2.04 g, 10.32 mmol) were added to a solution of phenylalanine (8.52 g, 51.6 mmol) in acetic acid (47 mL) and concentrated sulfuric acid (6.2 mL). The mixture was heated at 70 °C and stirred for 24 h. Sodium periodate (0.4 g) was then added and the reaction mixture was stirred for 30 min. Acetic acid was then evaporated and the crude mixture was diluted with H_2O (80 mL) and washed with Et_2O and DCM. The pH of the aqueous layer was adjusted to pH 5 with concentrated NaOH solution. The precipitate was filtered under vacuum and washed with H_2O (170 mL) and EtOH (65 mL) to afford 13.09 g of the crude product 4-iodophenylalanine. The crude 4-iodophenylalanine product (1.00 g, 3.44 mmol. 1 equiv) was then suspended in water (5 mL), $NaHCO_3$ (0.45 g, 5.35 mmol, 1.5 equiv) was then added and the reaction mixture cooled on ice. Benzyl chloroformate (0.64 mL, 4.52 mmol, 1.3 equiv) in dioxane (5 mL) was added dropwise and the reaction was stirred for 18 h at room temperature. The reaction mixture was then washed with Et_2O, and the pH of the aqueous layer was adjusted to 2 using 1 N HCl. The crude product was extracted with ethyl acetate and dried over Na_2SO_4. The solvent was evaporated to give 1.46 g of the crude Cbz protected product as a viscous yellow oil. To dry methanol (8 mL) cooled on ice, was slowly added $SOCl_2$ (0.43 mL, 5.88 mmol, 2.5 equiv). The resulting solution was warmed to room temperature and the crude Cbz protected product (1.00 g, 2.38 mmol, 1 equiv) was added and the mixture was stirred for 18 h at room temperature under argon. The solvent was then evaporated yielding 1.05 g of the methyl ester Cbz crude product as a viscous yellow oil. This product was used in the next step without further purification.

4.6. Synthesis of methyl 2-{[(benzyloxy)carbonyl]amino}-3-(4-formylphenyl)propanoate (9)

A mixture of Cbz-*N*-L-4-iodophenylalanine methyl ester **8** (439 mg, 1 mmol, 1 equiv), $Pd(OAc)_2$ (11.67 mg, 0.05 mmol, 0.05 equiv), 1,3′-bis(diphenylphosphino)propane (20 mg, 0.05 mmol, 0.05 equiv) and Et_3N (0.35 mL, 2.5 mmol, 2.5 equiv) in dry DMF (5 mL) was purged with CO for 10 min. Trioctylsilane (0.9 mL, 2 mmol, 2 equiv) was added in one portion and the mixture was stirred under a CO balloon for 8 h at 70 °C. The reaction mixture was then diluted with H_2O (20 mL), extracted with Et_2O, washed with H_2O, saturated $NaHCO_3$, H_2O, dried over Na_2SO_4 and the solvent evaporated. The compound was purified on a silica column (9:1 hexanes/ethyl acetate followed by 4:1 hexanes/ethyl acetate) to give **9** (205 mg, 60%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.98 (1H), 7.78 (d, J = 7.6 Hz, 2H), 7.53–7.13 (m, 7H), 5.35–5.25 (m, 1H), 5.13–5.05 (m 2H), 4.75–4.67 (m, 1H), 3.73 (3H), 3.28–3.10 (m, 2H). ESIMS calcd for $C_{19}H_{19}NO_5$ $[M+Na]^+$ 364.1155, found: 364.1170.

4.7. Synthesis of methyl 2-{[(benzyloxy)carbonyl]amino}-3-(4-hydroxyphenyl)propanoate (10)

Thionyl chloride (4.6 mL, 63.33 mmol, 2 equiv) was added dropwise to dry MeOH (65 mL) at 0 °C and stirred for 5 min. L-Tyrosine (5.70 g, 31.5 mmol, 1 equiv) was then added and the reaction vessel was fitted with a drying tube filled with drierite and slowly allowed to come to room temperature over 21 h. The solvent was then evaporated and crude product dried over high vacuum for 8 h. The crude product was then dissolved in a 1:1 mixture of acetone (63 mL) and a 7% solution of Na_2CO_3 in water (63 mL). Benzyl chloroformate (4.7 mL, 34.7 mmol, 1.2 equiv) was then added dropwise and the reaction was stirred for 3 h at room temperature. Ethyl acetate was then added (300 mL), and the organic layer was washed with water (100 mL), brine (100 mL) and dried over Na_2SO_4. The solvent was evaporated to give **10** as a viscous yellow oil (10.37 g). The product was used in the next step without purification.

4.8. Synthesis of methyl 2-{[(benzyloxy)carbonyl]amino}-3-(4-{[(trifluoromethyl)sulfonyl]oxy}phenyl)propanoate (11)

Compound **10** (10.37 g, 31.5 mmol, 1 equiv) and *N*-phenyl bistrifluoromethane sulfonamide (12.39 g, 34.65 mmol, 1.1 equiv) were dissolved in acetonitrile (150 mL). Et_3N (5.3 mL, 37.8 mmol, 1.2 equiv) was then added and the reaction was stirred for 3 h. The reaction mixture was then diluted with ethyl acetate (150 mL) and water (100 mL). The organic layer was washed with brine, dried over Na_2SO_4 and the solvent was evaporated. The product was purified on a silica column (3:2 hexane/ethyl acetate

followed by 2:3 hexanes/ethyl acetate) to obtain 14.38 g (99% yield) as a white solid. ^{1}H NMR (400 MHz, CDC1$_3$) δ 7.47–7.07 (m, 9H), 5.32–5.24 (m, 1H), 5.12–5.05 (m, 2H), 4.66 (dd, J = 13.7, 6.1 Hz, 1H), 3.71 (3H), 3.22–3.05 (m, 2H). ESIMS calcd for $C_{19}H_{18}F_3NO_7S$ $[M+Na]^+$ 484.0648, found: 484.0645; mp 70–73 °C.

4.9. Synthesis of methyl 2-{[(benzyloxy)carbonyl]amino}-3-[4-(hydroxymethyl)phenyl]propanoate (12)

Compound **11** (7.70 g, 16.70 mmol, 1 equiv), $Pd(OAc)_2$ (378 mg, 1.68 mmol, 0.1 equiv) and 1,1′-Bis(diphenylphosphino)ferrocene (dppf) (1.86 g, 3.34 mmol, 0.2 equiv) were dissolved in dry DMF (40 mL). K_2CO_3 (11.54 g, 83.5 mmol, 5 equiv) was then added to the reaction mixture and CO gas was bubbled through for 15 min. The reaction mixture was then heated at 60 °C for 8 h under a CO balloon. The reaction mixture was then cooled and partitioned between ethyl acetate and saturated $NaHCO_3$. The aqueous layer was acidified with a 10% aqueous solution of citric acid and extracted with ethyl acetate (4 × 75 mL). The organic layer was washed with brine, dried over Na_2SO_4 and the solvent was evaporated to give the crude carboxylic acid as a tan colored solid (4.89 g). The acid was dried over P_2O_5 and was used in the next step without purification. The crude acid was dissolved in dry THF (70 mL) and cooled in an ice bath. The reaction was charged with BH_3–DMS complex (10 M, 6.96 mL, 68.47 mmol, 4 equiv) added dropwise. The reaction mixture was warmed to room temperature over 2 h. A solution of saturated $NaHCO_3$ was added dropwise until the bubbling ceased. Ethyl acetate (70 mL) was added and the organic layer was separated and dried over Na_2SO_4 and then reduced. The crude product was purified on a silica column (3:2 hexane/ethyl acetate followed by 2:3 hexanes/ethyl acetate) to give **12** (3.56 g, 62%) as a white solid. $[\alpha]_D^{25}$ +50.56 (c 0.99, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41–7.23 (m, 7H), 7.08 (d, J = 8.0 Hz, 2H), 5.23 (d, J = 7.8 Hz, 1H), 5.12–5.05 (m, 2H), 4.65 (3H), 3.72 (3H), 3.17–3.02 (m, 2H), 1.76 (1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.2, 155.9, 140.0, 136.5, 135.3, 129.7, 128.8, 128.4, 128.3, 127.5, 67.2, 65.2, 55.0, 52.6, 38.1. ESIMS calcd for $C_{19}H_{21}NO_5$ $[M+Na]^+$ 366.1312, found: 366.1311; mp 74–77 °C.

4.10. Synthesis of L-phenylalanine, 4-[(dimethyloxyphosphinyl)-hydroxymethyl]-*N*-[(phenylmethoxy)carbonyl]-, methyl ester (13)

Compound **12** (3.56 g, 10.37 mmol, 1 equiv) was dissolved in DMSO (22 mL) and 2-iodoxybenzoic acid (3.77 g, 13.48 mmol, 1.3 equiv) was added and the reaction mixture and stirred for 1 h. The reaction mixture was then diluted with water (60 mL) and diethylether (60 mL) and filtered. The organic layer was separated and washed with water (2 × 50 mL), brine, and dried over Na_2SO_4. The solvent was evaporated and the crude aldehyde was used immediately in the next step without purification. The aldehyde was dissolved in dimethyl phosphite (1.05 mL, 11.41 mmol 1.1 equiv) with mild heating. CsF (9.45 g, 62.22 mmol, 6 equiv) was added and the reaction was stirred until it solidified. The crude product was then dissolved in DCM (40 mL), filtered, and the solvent evaporated. The crude product was purified on a silica plug (first with diethylether, then 1:20 DCM/MeOH) to give **13** as a white solid (3.98 g, 85%). $[\alpha]_D^{25}$ +38.08 (c 1.55, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (dd, J = 8.1, 1.9 Hz, 2H), 7.37–7.27 (m, 5H), 7.11 (d, J = 8.0 Hz, 2H), 5.39–5.26 (m, 1H), 5.07 (2H), 5.00 (d, J = 11.0 Hz, 1H), 4.63 (d, J = 7.6 Hz, 1H), 3.69 (d, J = 2.8 Hz, 3H), 3.65 (d, J = 4.7 Hz, 3H), 3.62 (3H), 3.16–3.04 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.1, 155.9, 136.3 (d, J_{C-P} = 23.9 Hz), 135.5, 129.6 (d, J_{C-P} = 2.1 Hz), 128.8, 128.4, 128.3, 127.5 (d, J_{C-P} = 5.8 Hz), 70.6 (d, J_{c-p} = 159.4 Hz), 67.2, 55.0, 54.1 (d, J_{C-P} = 7.1 Hz), 53.9 (d, J_{C-P} = 7.1 Hz), 52.6, 38.2. ^{31}P NMR (162 MHz, $CDCl_3$) δ 24.53 (87P), 11.64 (1P). ESIMS calcd for $C_{21}H_{26}NO_8P$ $[M+Na]^+$ 474.1288, found: 474.1288; mp 68–71 °C.

4.11. Synthesis of L-phenylalanine, 4-[(dimethyloxyphosphinyl)-bromomethyl]-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-, methyl ester (14)

Compound **13** (3 g, 6.65 mmol, 1 equiv) was dissolved in dry MeOH and Pd/C (200 mg, 30 mg/mmol) was added. A three way stopcock (connected to an H_2 balloon and vacuum line) was fitted to the reaction vessel. The reaction mixture was then flushed with H_2 (3×) and stirred at room temperature for 6 h. The reaction mixture was filtered through a Celite pad and the solvent evaporated. A mixture of the residue, Fmoc-succinimide (2.35 g, 6.98 mmol, 1.05 equiv) and $NaHCO_3$ (2.34 g, 27.92 mmol, 4 equiv) in acetonitrile and water (1:1, 130 mL) was stirred at room temperature overnight. The acetonitrile was evaporated under reduced pressure and the crude product was extracted with ethyl acetate. The organic layer was dried over Na_2SO_4, evaporated under reduced pressure and purified on a silica plug (diethylether, followed by 10:1 DCM/MeOH) to give **14** as a white solid (3.59 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 8.0 Hz, 2H), 7.64–7.48 (m, 2H), 7.48–7.35 (m, 4H), 7.34–7.28 (m, 2H), 7.1 (d, J = 8.0 Hz, 2H), 5.33 (d, J = 7.8 Hz, 1H), 5.02 (d, J = 11.0 Hz, 1H), 4.70–4.60 (m, 1H), 4.45–4.29 (m, 2H), 4.21–4.18 (m, 1H), 3.70 (d, J = 7.0 Hz, 4H), 3.67–3.65 (m, 3H), 3.63–3.62 (m, 2H), 3.20–3.00 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.1, 155.8, 144.0 (d, J_{C-P} = 7.5 Hz), 141.6, 136.2, 135.7–134.3 (m), 129.7, 128.0, 127.5 (d, J_{C-P} = 5.8 Hz), 127.3, 125.3 (d, J_{C-P} = 6.1 Hz), 120.2, 70.6 (d, J_{C-P} = 159.5 Hz), 67.2, 55.0, 54.1 (d, J_{C-P} = 7.1 Hz), 54.0 (d, J_{C-P} = 7.1 Hz), 52.6, 47.4, 38.2. ^{31}P NMR (162 MHz, $CDCl_3$) δ 24.51. ESIMS calcd for $C_{28}H_{30}NO_8P$ $[M+Na]^+$ 562.1601, found: 562.1595; mp 65–70 °C.

4.12. Synthesis of L-phenylalanine, 4-[(dimethyloxyphosphinyl)-bromomethyl]-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-, methyl ester (15)

In a round bottom flask **14** (2 g, 3.70 mmol, 1 equiv) was dissolved in dry DCM (30 mL) and dry pyridine (0.38 mL, 4.63 mmol, 1.25 equiv) was added. Thionyl bromide (0.36 mL, 4.63 mmol, 1.25 equiv) was then added to the round bottom flask under an inert atmosphere. The round bottom flask was sealed with a septum, cooled in an ice bath and slowly allowed to come to room temperature overnight. The solvent was then evaporated and the crude product was dissolved in ethyl acetate. The organic layer was washed with 1 M HCl, saturated $NaHCO_3$, water, and brine, dried over Na_2SO_4 and filtered. The solvent was evaporated and the crude product was purified on a silica plug (diethylether, followed by 10:1 DCM/MeOH) to give **15** as a white solid (1.14 g, 78%). Purity (>95%) determined by analytical HPLC, C18, flow: 1 mL min^{-1}, λ: 254 nm, eluent: acetonitrile/0.1% TFA in water 10:90 (2 min) to 50:50 (22 min), retention time: 49.7 min. $[\alpha]_D^{25}$ +34.72 (c 1.02, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, J = 7.5 Hz, 2H), 7.57 (d, J = 7.4 Hz, 2H), 7.49 (d, J = 7.5 Hz, 2H), 7.41 (t, J = 7.4 Hz, 2H), 7.32 (t, J = 7.5 Hz, 2H), 7.17–6.98 (m, 2H), 5.32–5.23 (m, 1H), 4.86 (d, J = 13.1 Hz, 1H), 4.66 (dd, J = 13.4, 6.1 Hz, 1H), 4.49–4.30 (m, 2H), 4.20 (t, J = 6.9 Hz, 1H), 3.85 (d, J = 10.8 Hz, 3H), 3.72 (s, 3H), 3.59 (dd, J = 10.7, 3.8 Hz, 3H), 3.17–3.05 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.9, 155.7, 144.0 (d, J_{C-P} = 6.5 Hz), 141.6, 137.3, 133.5, 130.0 (d, J_{C-P} = 3.6 Hz), 129.9, 128.0, 127.3, 125.3 (d, J_{C-P} = 7.6 Hz), 120.3, 67.2 (d, J_{C-P} = 6.2 Hz), 54.9 (m), 52.7, 47.4, 40.8 (d, J_{C-P} = 160.0 Hz), 38.2 (d, J_{C-P} = 7.0 Hz). ^{31}P NMR (162 MHz, $CDCl_3$) δ 20.43. ESIMS calcd for $C_{28}H_{29}NO_7BrP$ $[M+Na]^+$ 624.0757, found: 624.0756; mp 68–71 °C.

4.13. Synthesis of L-phenylalanine, 4-[(dimethyloxyphosphinyl)-bromomethyl]-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]; Fmoc-L-BrPmp(*O*Me$_2$)-OH (16)

Compound **15** (3.00 g, 5.00 mmol, 1 equiv) was dissolved in THF (35 mL) and cooled in an ice bath. LiOH (240 mg, 10.00 mmol, 2 equiv) was dissolved in water (35 mL) and cooled in an ice bath. The lithium hydroxide solution was then added to the reaction mixture and stirred for 30 min. The THF was then evaporated, and the aqueous layer was washed with diethylether (30 mL). The aqueous layer was acidified to pH 2 with concentrated HCl and was extracted with ethyl acetate (4 × 75 mL). The combined organic layers were dried over Na_2SO_4, and concentrated to a white solid (2.67 g, 91% yield). Purity (>95%) determined by analytical HPLC, C18, flow: 1 mL min^{-1}, λ: 254 nm, eluent: acetonitrile/0.1% TFA in water 10:90 (2 min) to 50:50 (22 min), retention time: 35.9 min. $[\alpha]_D^{25}$ +40.24 (*c* 1.31, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.2 Hz, 2H), 7.57 (d, *J* = 6.3 Hz, 2H), 7.48–7.35 (m, 4H), 7.30 (t, *J* = 7.2 Hz, 2H), 7.16 (2H), 5.52 (d, *J* = 13.1 Hz, 1H), 4.98–4.81 (m, 1H), 4.67 (1H), 4.47 (m, 1H), 4.33 (1H), 4.19 (1H), 3.82 (d, *J* = 10.7 Hz, 3H), 3.54 (t, *J* = 10.4 Hz, 3H), 3.18 (2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.98, 144.0 (d, J_{C-P} = 10.5 Hz), 141.6, 137.8, 132.7, 130.3, 129.8 (d, J_{C-P} = 5.5 Hz), 128.0, 127.3, 125.3 (d, J_{C-P} = 7.5 Hz), 120.2, 77.6, 77.3, 76.9, 67.1, 55.6–54.9 (m), 54.8, 47.4, 41.1 (d, J_{C-P} = 7.6 Hz), 39.5 (d, J_{C-P} = 7.6 Hz), 37.8. ^{31}P NMR (162 MHz, $CDCl_3$) δ 20.59 (d, *J* = 38.6 Hz). ESIMS calcd for $C_{27}H_{27}NO_7BrP$ $[M+Na]^+$ 610.0601, found: 610.0595; mp 84–88 °C.

4.14. Synthesis of L-BrPmp-OH (1)

Compound **16** (200 mg, 0.34 mmol, 1 equiv) was dissolved in a solution of 20% piperidine in dry DCM (10 mL) and stirred at room temperature for 30 min. The solvent was then evaporated and the residue was dried on high vacuum over P_2O_5 overnight. The residue was then dissolved in dry acetonitrile (10 mL) and TMSBr (0.47 mL, 3.4 mmol, 10 equiv) was added under an inert atmosphere and the reaction mixture was stirred overnight. The organic solvent was evaporated and the crude residue was dissolved in water and the aqueous layer was washed with diethylether (5 mL) and freeze dried. The crude bromide salt was then dissolved in anhydrous EtOH (3 mL), propylene oxide (36 μL, 0.51 mmol, 1.5 equiv) was added and the reaction mixture was stirred overnight resulting in a white precipitate the next day. Water (5 mL) was added and the EtOH was then removed under reduced pressure. The aqueous layer was filtered and freeze dried. The crude product was dissolved in water (1 mL) and passed through a C18 Sep-pak syringe column. The aqueous fractions were freeze dried yielding **1** (94 mg, 65%) as a white solid of the piperidine salt. $[\alpha]_D^{25}$ −16.56 (*c* 1.36, $CHCl_3$). ^{1}H NMR (400 MHz, D_2O) δ 7.54 (d, *J* = 7.8 Hz, 2H), 7.26 (d, *J* = 7.6 Hz, 2H), 4.95 (d, *J* = 11.6 Hz, 1H), 4.07–3.92 (m, 1H), 3.28 (d, *J* = 10.5 Hz, 1H), 3.16–3.00 (m, 5H), 1.77–1.68 (m, 4H), 1.67–1.57 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 135.6, 135.6 (d, *J* = 1.1 Hz), 129.9 (d, *J* = 5.8 Hz), 129.8 (d, *J* = 1.4 Hz), 56.0, 46.2, 44.8, 36.2, 22.5, 21.7. ^{31}P NMR (162 MHz, D_2O) δ 13.47. ESIMS calcd for $C_{10}H_{12}NO_5BrP$ $[M-H]^-$ 335.9642, found: 335.9640; decomp. 140 °C.

4.15. Synthesis of tripeptide Asp-BrPmp-Leu (17)

The tripeptide was assembled manually on Wang resin (0.6 mmol/g), preloaded with an Fmoc protected Leu residue (Fmoc-L-Leu-OH). Following a standard protocol: Fmoc-L-BrPmp(*O*Me$_2$)-OH **16** (2 equiv) was coupled to the resin using HBTU (1.96 equiv) in the presence of DIPEA (4 equiv) in NMP for 3.5 h. The reaction was monitored by Kaiser test. The coupling was repeated using the same equivalents of Fmoc-L-BrPmp(*O*Me$_2$)-OH, HBTU and DIPEA in NMP for 3.5 h. Fmoc-Asp(*t*Bu)-OH (5 equiv) was coupled using HBTU (4.9 equiv) in the presence of DIPEA (10 equiv) in NMP for 3.5 h. Fmoc deprotection was achieved with 20% piperidine in NMP. The resin was washed with NMP, AcOH, DCM, and MeOH. Immediately after washing the resin with CH_3CN, DCM and MeOH, a mixture of TFA/H_2O/TIPS (95:2.5:2.5) was added and the resin was shaken at room temperature for 3 h. The cleaved peptide was precipitated in diethylether, filtered, dissolved in a mixture of H_2O and CH_3CN and lyophilized. The lyophilized peptide was suspended in CH_3CN, and TMSI (20 equiv) was added under an inert atmosphere and the reaction mixture shaken for 100 min at room temperature. The CH_3CN/TMSI solution was evaporated under reduced pressure, and the crude product was dissolved in water and washed with diethylether (3×) and the aqueous layer was lyophilized. The peptide was purified by HPLC (C-18 semipreparative column) using a linear gradient (CH_3CN/H_2O mobile phase containing 0.1% TFA). 4 mg of pure compound was recovered from 18 mg crude product (33% of theoretical yield). Purity (>95%) determined by analytical HPLC, C18, flow: 1 mL min^{-1}, λ: 212 nm, eluent: 0.1% TFA in acetonitrile/0.1% TFA in water 10:90 (2 min) to 20:80 (22 min), retention time: 22.4 min. ^{1}H NMR (500 MHz, D_2O) δ 7.57 (d, *J* = 6.8 Hz, 2H), 7.30 (dd, *J* = 7.9, 2.2 Hz, 2H), 5.02 (d, *J* = 11.7 Hz, 1H), 4.75–4.69 (m, 1H), 4.43–4.32 (m, 1H), 4.32–4.22 (m, 1H), 3.23–3.06 (m, 2H), 2.99–2.84 (m, 2H), 1.67–1.59 (m, 2H), 0.92 (dd, *J* = 19.7, 4.7 Hz, 6H). ^{31}P NMR (162 MHz, D_2O) δ 13.78 (d, *J* = 4.8 Hz). ESIMS calcd for $C_{20}H_{28}N_3O_9BrP$ $[M-H]^-$ 564.0752, found: 564.0732.

4.16. Phosphatase inhibition assays

Enzyme assays were conducted using human CD45-cytoplasmic domain (Enzo Life Science; diluted to 4 mU/μL in 50 mM HEPES, pH 7.2, 1 mM EDTA, and 0.1% nonidet P-40). Enzyme activity was detected with a fluorogenic substrate (6,8-difluoro-4-methylumbelliferyl phosphate; DiFMUP) (Invitrogen). Assays were performed in black 96-well plates and read in a Spectra Max M2 plate reader (Molecular Devices). Stock solutions of inhibitors were prepared and stored at −20 °C. Final solutions in microplate wells contained a total volume of 100 μL consisting of 2 μL of diluted enzyme, inhibitor, and DiFMUP substrate diluted to 100 μL in the appropriate buffer. All wells were incubated for 10 min at 37 °C in the plate reader prior to the addition of DiFMUP. After addition of the substrate, the plate was read at an excitation maximum of 358 nm and an emission maximum of 450 nm. Competitive inhibition was determined by observing the initial velocity of CD45 over a range of substrate concentrations to determine K_m, or, in the presence of the inhibitor, $K_{m,\,obs}$. To determine the activity of irreversible inhibitors, the enzyme activity was monitored over 60 min in the presence of inhibitor, and the curve was fit to obtain k_{obs} as described elsewhere.[44] Values of k_{obs} were then fit to

$$k_{obs} = \frac{k_3}{1 + K_I/(I)} \quad (1)$$

by non-linear regression; where K_I was the inhibition constant, *I* was the concentration of inhibitor, and k_3 was the rate of inactivation of the enzyme.[42] Inhibitors which did not show a positive slope by Kitz–Wilson analysis were treated as competitive inhibitors for the determination of K_I.

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Supplementary data

Supplementary data (enzyme kinetic data and spectral characterization) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.09.040.

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