Efficient Scaled-Up Synthesis of *N*-α-Fmoc-4-Phosphono(difluoromethyl)-L-phenylalanine and Its Incorporation into Peptides

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Abstract: A rapid, robust and efficient scaled-up synthetic strategy for *N*-α-Fmoc-4-phosphono(difluoromethyl)-L-phenylalanine and its direct incorporation into peptides is presented herein.

Key words: phosphorylation, inhibitors, solid-phase synthesis, peptides, bioorganic chemistry

Reversible phosphorylation of proteins is an essential principle in cell signaling.[1] The generation of phosphotyrosyl residues (pTyr) by protein tyrosine kinases (PTK) creates recognition sites for the binding of modular domains of other signaling proteins, such as Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains.[2] At the same time, these sites become high affinity substrates for their respective protein tyrosine phosphatases (PTP), which cleave the phosphate from tyrosine residues.[3] Perturbation of the concerted signaling events leads to the development of various diseases including cancer and diabetes.[1,3] Therefore, the development of tools to study and inhibit pTyr-mediated interactions is highly desirable. Hydrolytically stable pTyr mimetics have proven to be useful building blocks for the rational design of inhibitors of these interactions.[4,5] Among other pTyr analogues, phosphonodifluoromethyl-L-phenylalanine (F_2Pmp), which was developed by Burke and co-workers, has been shown to be especially valuable for the development of highly potent peptide-based inhibitors of PTPs (Figure 1).[4,5,6]

For Fmoc solid-phase peptide synthesis, *N*-Fmoc-F_2Pmp (**5**) or its respective protected diethyl-phosphonate can be used (Scheme 1).[7,8] Furthermore, an on-resin direct conversion of iodo-phenylalanine into F_2Pmp(OEt_2) has been reported.[9] However, the preferred method of F_2Pmp-incorporation into peptides uses the free phosphonate building block due to side reactions caused by the harsh conditions needed to remove the ethyl protecting groups.[8,9] Recently, *N*-Fmoc-F_2Pmp (**5**) has become commercially available, however, it is relatively expensive. Several different synthetic routes to *N*-Fmoc-L-F_2Pmp have been described so far.[4,7–13] Albeit very useful, these routes have individual drawbacks in that they either require several steps that need purification,[8,10,11] or provide relatively low overall yields.[7,10–13] Often, the highly explosive fluorinating agent diethylaminosulfur trifluoride (DAST) or diazomethane are used, which are difficult to scale-up.[9–13] In addition, these routes have only been described on small scale (mg quantities in cases where the scale was reported). Therefore, we sought to optimize and scale-up the synthesis of *N*-Fmoc-F_2Pmp.

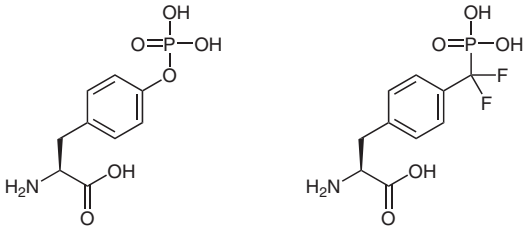

Figure 1 Comparison of phosphotyrosine (pTyr; **1**) and its non-hydrolyzable analogue phosphonodifluoromethyl-L-phenylalanine (F_2Pmp; **2**)[4–6]

Due to its high coupling efficiency, we chose to follow the approach of Qabar and co-workers,[9] adapted from Burton and co-workers,[14] as starting point. This approach is based on CuCl-promoted coupling of phosphono-difluoromethyl-CdBr (**7**) to commercially available *N*-Fmoc-L-4-iodophenylalanine (**3**; Scheme 1).

In the first step of the synthesis, the carboxylic acid moiety of **3** (4.8 g) was protected as an ester of 2-trimethylsilylethanol (TMSE). This protecting group was chosen because the ester bond can be removed under mild acidic conditions. Thus, after coupling of the phosphonogroup, the subsequent deprotection was expected to be easier to control with respect to the integrity of the Fmoc group than the previously described basic removal of a methyl ester with LiOH.[9] The esterification showed complete conversion (TLC analysis) of the starting material after four hours. Subsequent aqueous work-up and drying under high vacuum gave a brown oil, which was used in the next step without further purification.

In the next reaction, the cadmium-containing compound **7**, obtained by treatment of diethyl bromodifluoromethylphosphonate (**6**) with Cd, was coupled to TMSE-protected **3** in the presence of a copper halide.[9] Like Qabar and co-workers,[9] we also observed that an excess of copper (2–3 equiv) was needed for the reaction. Additionally, we found that the performance of CuBr was superior to that of CuCl and that a second addition of the cadmium re-

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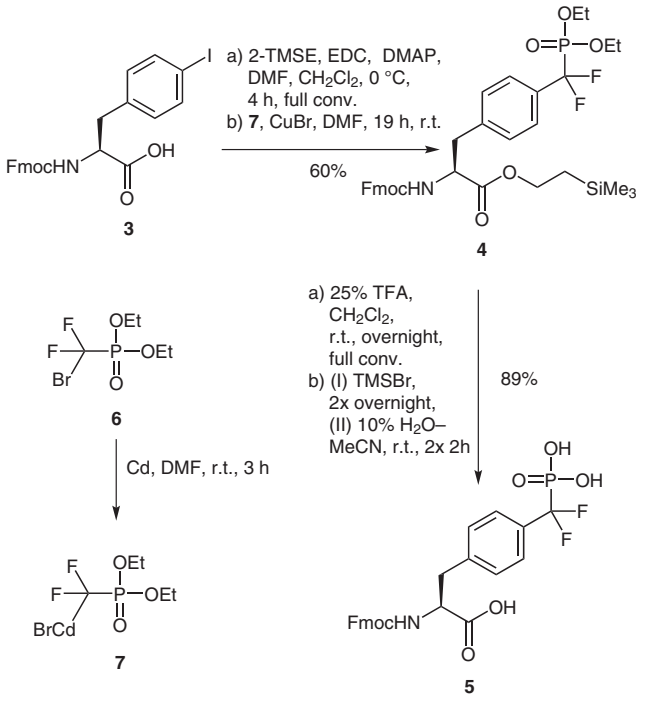
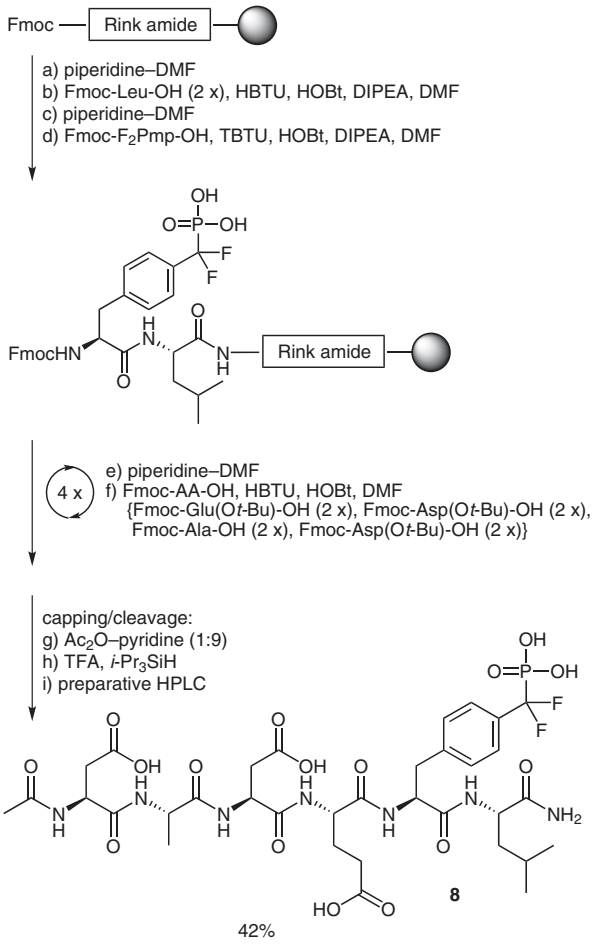


Scheme 1 Synthesis of *N*-Fmoc-F_2Pmp-OH (**5**); 2-(trimethylsilyl)ethanol (2-TMSE), *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC), 4-(*N*,*N*-dimethylamino)pyridine (DMAP), *N*,*N*′-dimethylformamide (DMF), trifluoroacetic acid (TFA), trimethylsilyl bromide (TMSBr)

agent and CuBr helped to drive the reaction faster to completion. After 19 hours, conversion into the product was more than 93% (determined by analytical HPLC). After aqueous work-up and column chromatography 3.8 g (60% over two steps) of compound **4** was isolated. Minor amounts of impurities were still detected after this purification, two species in the ^{31}P and one in the ^{1}H NMR spectra of compound **4**, which correspond to an impurity in starting material **6** and presumably to a side product derived from compound **7**. These impurities were not removed because they did not interfere with subsequent reactions of the final product *N*-Fmoc-F_2Pmp, which was to be incorporated into peptide- or peptide-mimic-based binders of PTPs or PTBs. This is evident because the impurities in the ^{31}P NMR are absent in the spectrum of the corresponding peptides (see below). If necessary, however, it is possible to remove these impurities by HPLC purification.

Subsequent hydrolysis of the trimethylsilylethyl ester took place quantitatively overnight in 25% trifluoroacetic acid (TFA) in dichloromethane. After coevaporation of the acid with toluene and drying, the *N*-Fmoc-F_2Pmp(OEt_2) building block was obtained and used directly in the next step without purification. The phosphonic acid diethyl esters were hydrolyzed quantitatively, without the need to add scavengers, by treatment with trimethylsilyl bromide (TMSBr) in dichloromethane. A single repetition of the treatment led to the highest purity of the final product **5**. Thus, *N*-Fmoc-F_2Pmp (**5**; 2.6 g) was isolated as a yellowish glass in more than 90% purity (determined by analytical HPLC), resulting in an overall yield of 54% based on the amount of starting material **3**.

Unpurified *N*-Fmoc-F_2Pmp (**5**) was then directly used in solid-phase peptide synthesis. The coupling took place efficiently on rink amide resin using standard coupling reagents and only three equivalents of *N*-Fmoc-F_2Pmp-OH (Scheme 2). After cleavage from the resin, HPLC analysis showed no fragments due to incomplete coupling, and model peptides **8** (Scheme 2) and **9** (Figure 2) were obtained in good yield after HPLC purification. Peptide **8** is a protein tyrosine phosphatase 1B (PTP1B) inhibitor,[6,15] and the sequence of **9** was chosen to incorporate non-polar and polar non-charged amino acids in a longer peptide.



Scheme 2 Solid-phase synthesis of model peptide **8** containing F_2Pmp; *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), *N*,*N*-diisopropylethylamine (DIPEA)

In order to probe the two peptides for their biological activity, we determined the IC_{50} values of both peptides towards PTP1B, which is a phosphatase involved in diabetes and obesity,[16] with the well-established *para*-nitrophenolphosphate (*p*NPP) competition assay.[6] First, the Michaelis–Menten constant (K_M) of PTP1B towards *p*NPP was measured (4.6 ±0.3 mM) to establish an appropriate *p*NPP concentration with which the IC_{50} values were then determined. The resulting IC_{50} value

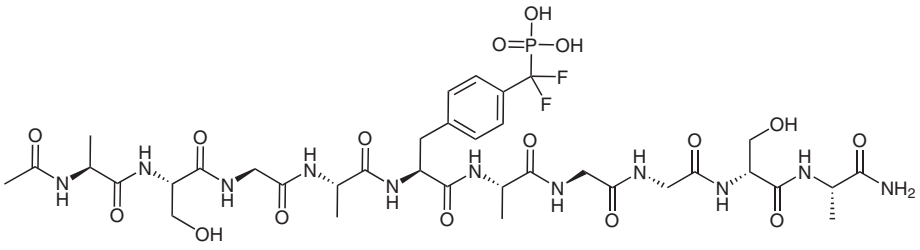


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Figure 2 F_2Pmp-containing model peptide **9**

(1.7 ±0.1 μM) of the known peptide **8**,[6,15] which contains several acidic residues, was found to be approximately ten-fold better than our model peptide **9** (15.3 ±1.9 μM), which is composed of only neutral, non-charged amino acids around F_2Pmp. These results are in agreement with the known preference of PTP1B for acidic amino acids in close vicinity to the pTyr of the substrates.[16] In addition, this confirms that, although F_2Pmp is essential for efficient binding of a peptide to PTP1B, the surrounding amino acids can effectively modulate the strength of the interaction and cause differences of up to at least one order of magnitude in inhibitory potency.

In summary, we present here a fast, robust, and efficient method to synthesize *N*-Fmoc-F_2Pmp in very good overall yield. The synthesis, which requires only one purification step, can be easily applied to obtain the final product in gram scale and in greater than 90% purity. Furthermore, the application of the unpurified building block in solid-phase peptide synthesis to generate two model peptides was demonstrated, and the biological activity of these peptides was shown. Rapid and simple access to *N*-Fmoc-F_2Pmp will enable broad use of this building block to generate inhibitors and tools for protein tyrosine phosphatase and pTyr-binding domain research.

All chemicals and anhydrous solvents were obtained from commercial sources (Sigma–Aldrich, VWR) and used without further purification. The chiral starting material *N*-Fmoc-L-4-iodophenylalanine was purchased from Amatek Chemical Co., Ltd., Zhangjiagang, China. Diethyl bromodifluoromethylphosphonate was obtained from Matrix Scientific, Columbia, USA. Fmoc-protected amino acids and Rink amide resin (200–400 mesh; 0.62 or 0.57 mmol/g) were purchased from Novabiochem, Darmstadt, Germany. Peptide synthesis was performed with an automatic peptide synthesizer Syro I from Multisyntech, Witten, Germany. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded with a 400 MHz Bruker Avance DPX spectrometer. Chemical shifts (δ) are reported in ppm and coupling constants (*J*) are given in Hz. ^{1}H and ^{13}C chemical shifts were referenced to the solvent peaks (δ = 7.26 and 77.0 ppm for $CDCl_3$). Splitting patterns are designated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet. ^{13}C and ^{31}P NMR spectra were broadband hydrogen decoupled. For ^{1}H assignment, COSY and HMQC spectra were recorded. HPLC analyses and purifications were carried out with a Shimadzu HPLC-MS LCMS-2010EV instrument fitted with a UV/Vis Photodiode array detector (SPD-M20A Prominence). For analytical and preparative HPLC, the solvent delivery module LC-20AD was used. RP-HPLC analytical runs were carried out with a Macherey Nagel C18 EC 250/4.0 NUCLEODUR 100–5 C18 ec column with a pump rate of 1.5 mL/min. For preparative separations, a Macherey Nagel C18 VP 250/10 NUCLEODUR 110–5 C18 ec column with a pump rate of 5 mL/min was used. The solvent mixtures were either H_2O–MeCN or H_2O–MeOH, if needed 0.05% TFA was added. Mass spectra were recorded with a MALDI micro MX mass spectrometer (Waters, Manchester, UK) equipped with a reflectron analyzer, used in positive ion mode with delayed extraction activated. HRMS were recorded with a LTQ Obritrap Velos (ThermoFisher Scientific, US). TLC analyses were conducted with Merck precoated silica gel plates (Merck, 60 F_{254}) using UV light (254 nm) or a solution of ceric ammonium molybdate (prepared by addition of 40 mL conc. H_2SO_4, 360 mL H_2O, 10 g ammonium molybdate and 4 g ceric ammonium sulfate). Preparative column chromatography was performed using silica gel from Merck (silica 60; grain size 0.063–0.200 mm; 70–230 mesh ASTM).

(*S*)-2-(Trimethylsilyl)ethyl 2-[(9*H*-Fluoren-9-yloxy)carbonylamino]-3-{4-[(diethoxyphosphoryl)difluoromethyl]phenyl}propanoate (4)

In a mixture of anhydrous CH_2Cl_2 (20 mL) and anhydrous DMF (30 mL), *N*-Fmoc-L-4-iodophenylalanine (**3**; 4.80 g, 9.35 mmol) was dissolved under an argon atmosphere. The solution was cooled in an ice bath, then TMSE (2.50 mL, 25.6 mmol, 2.7 equiv), DMAP (914 mg, 7.48 mmol, 0.8 equiv), and EDC (3.58 g, 23.1 mmol, 2.5 equiv) were added. The reaction was monitored by TLC. After four hours, full conversion of the starting material was observed. CH_2Cl_2 (200 mL) was added to the reaction and the mixture was extracted with aq NH_4Cl (2 × 250 mL) and once with brine (250 mL). The organic phase was dried (Na_2SO_4), the solvent was removed under reduced pressure, and the resulting brown oil [R_f = 0.35 (cyclohexane–EtOAc, 6:1)] was used in the next reaction without further purification.

For the next step, cadmium metal (6.00 g, 53.4 mmol, purum p.a. for filling reductors, Sigma–Aldrich) was washed by stirring under argon with 1 N HCl (5 mL, 15 min), H_2O (5 mL, 3 × 1 min) and acetone (5 mL, 3 × 1 min). The metal was dried overnight under high vacuum while stirring until a metallic shine was observed. While warming the reaction vessel by hand, anhydrous DMF (10 mL) and diethyl bromodifluoromethylphosphonate (12.5 mL, 31.2 mmol) were added dropwise over 15 min to the metal under argon. The slightly exothermic reaction proceeded and was allowed to stir at r.t. for 3 h.

TMSE-protected **3** was dissolved in anhydrous DMF (10 mL), then CuBr (2.70 g, 18.8 mmol, 2 equiv) and half of the supernatant solution of the Cd reagent (ca. 1.7 equiv) was added in a dropwise manner. After 3 h, further CuBr (1.40 g, 9.41 mmol, 1 equiv) and the other half of the Cd reagent solution were added. The reaction was allowed to stir at r.t. for 19 h in total. The progress of the reaction was checked by HPLC (Nucleodur C18; MeOH–H_2O, 85%; 15 min), the AUC of the peak of the starting material (R_t = 13.8 min) was 6.5% compared to the area of the product peak (R_t = 7.5 min). The reaction mixture was diluted with EtOAc (250 mL), filtered through Celite, and extracted with aq NH_4Cl (2 × 250 mL) and brine

(250 mL). After drying (Na_2SO_4), the solvents were removed in vacuo and product **4** was isolated by flash chromatography (cyclohexane–EtOAc, 6:1→2:1).

Yield: 3.80 g (5.65 mmol, 60% yield over two steps); transparent yellowish oil; R_f = 0.62 (cyclohexane–EtOAc, 2:1).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.77 (d, ^3J = 7.3 Hz, 2 H, 2 × Ar-H-Fmoc), 7.63–7.51 (m, 4 H, 2 × Ar-H-Fmoc, 2 × Ar-H-Phe), 7.40 (t, ^3J = 7.5 Hz, 2 H, 2 × Ar-H-Fmoc), 7.32 (t, ^3J = 7.4 Hz, 2 H, 2 × Ar-H-Fmoc), 7.20 (d, ^3J = 7.7 Hz, 2 H, 2 × Ar-H-Phe), 5.26 (d, ^3J = 8.0 Hz, 1 H, NH-Phe), 4.70–4.60 (m, 1 H, α-H-Phe), 4.50–4.34 (m, 2 H, CH_2-Fmoc), 4.34–4.07 (m, 7 H, CH-Fmoc, 2 × CH_2-Ethyl, CH_2-TMSE), 3.27–3.08 (m, 2 H, CH_2-Phe), 1.30 (t, ^3J = 7.1 Hz, 6 H, 2 × CH_3-Ethyl), 0.97 (t, ^3J = 8.3 Hz, 2 H, CH_2-TMSE), 0.04 (s, 9 H, 3 × CH_3-TMSE).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.2 (O=C-TMSE ester), 155.5 (O=C-Fmoc), 143.8 (C-F_2P), 143.7, 141.3, 139.0, 131.4 (6 × Ar-C), 129.5, 127.7, 127.1, 126.5, 125.1, 125.0, 120.0 (12 × Ar-CH), 66.9 (CH_2-Fmoc), 64.7 (CH_2-TMSE), 64.1 (2 × CH_2-Ethyl), 54.7 (α-CH-Phe), 47.2 (CH-Fmoc), 38.0 (CH_2-Phe), 17.4 (CH_2-TMSE), 16.3 (2 × CH_3-Ethyl), –1.6 (3 × CH_3 TMSE).

^{31}P (126 MHz, $CDCl_3$): δ = 6.4 (t, J = 115 Hz).

HRMS (ESI): m/z [M + Na]$^+$ calcd for $C_{34}H_{42}F_2NO_7PSiNa$: 696.23284; found: 696.23318.

(*S*)-2-{[(9*H*-Fluoren-9-yl)methoxy]carbonylamino}-3-{4-[(diethoxyphosphoryl)difluoromethyl]phenyl}propanoic Acid [*N*-Fmoc-F_2Pmp(OEt_2)]

Compound **4** (3.80 g, 5.65 mmol) was dissolved in anhydrous CH_2Cl_2 (22.5 mL), then TFA (7.5 mL, 25%) was added and the mixture was stirred overnight (TLC showed complete conversion without the formation of side products). The solvents were removed in vacuo, and the remaining oil was dissolved in toluene (40 mL), again evaporated (2 ×) and dried under high vacuum. The resulting transparent brownish oil was used in the next reaction without further purification.

R_f = 0.85 (cyclohexane–EtOAc, 1:1).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (d, ^3J = 7.6 Hz, 2 H, 2 × Ar-H-Fmoc), 7.56 (d, ^3J = 7.7 Hz, 2 H, 2 × Ar-H-Fmoc), 7.51 (d, ^3J = 7.7 Hz, 2 H, 2 × Ar-H-Phe), 7.40 (t, ^3J = 7.4 Hz, 2 H, 2 × Ar-H-Fmoc), 7.30 (t, ^3J = 7.5 Hz, 2 H, 2 × Ar-H-Fmoc), 7.22 (d, ^3J = 7.8 Hz, 2 H, 2 × Ar-H-Phe), 5.49 (d, ^3J = 7.8 Hz, 1 H, NH-Phe), 4.74–4.65 (m, 1 H, α-H-Phe), 4.50–4.43 (m, 1 H, CH_2-Fmoc), 4.40–4.34 (m, 1 H, CH_2-Fmoc), 4.33–4.27 (m, 1 H, CH-Fmoc), 4.23–4.09 (m, 4 H, 2 × CH_2-Ethyl), 3.30–3.09 (m, 2 H, CH_2-Phe), 1.33–1.23 (m, 6 H, 2 × CH_3-Ethyl).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 173.2 (O=C-carboxy), 155.9 (O=C-Fmoc), 143.9 (C-F_2P), 143.7, 141.4, 139.3, 131.0 (6 × Ar-C), 129.8, 127.9, 127.2, 126.5, 125.2, 125.1, 120.1 (12 × Ar-CH), 67.1 (CH_2-Fmoc), 65.4 (2 × CH_2-Ethyl), 54.5 (α-CH-Phe), 47.3 (CH-Fmoc), 37.7 (CH_2-Phe), 16.3 (2 × CH_3-Ethyl).

^{31}P (126 MHz, $CDCl_3$): δ = 6.1 (t, J = 122 Hz).

HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{29}H_{31}F_2NO_7P$: 574.18007; found: 574.18034.

(*S*)-2-{[(9*H*-Fluoren-9-yl)methoxy]carbonylamino}-3-{4-[difluoro(phosphono)methyl]phenyl}propanoic Acid (*N*-Fmoc-F_2Pmp; 5)

The material obtained in the previous step was dissolved in anhydrous CH_2Cl_2 (30 mL) and cooled in an ice bath. Under argon, trimethylsilyl bromide (12.4 mL, 94.0 mmol, 16.6 equiv) was added dropwise. The solution was stirred and allowed to warm to r.t. overnight. The solvents were removed in vacuo and the procedure was repeated once more. Then, MeCN–H_2O (10%) was added, the solution was stirred for 2 h at r.t., and the solvents were removed by evaporation (2 ×). The product **5** was obtained in >90% purity (estimated by HPLC) and used without further purification in solid-phase peptide synthesis.

Yield: 2.60 g (5.03 mmol, 89% yield over two steps); yellowish glass; t_R = 7.6 min (RP-HPLC, Nucleodur C18, MeOH–H_2O, 30 → 70% in 15 min).

^{1}H NMR (400 MHz, $CDCl_3$, drops of CD_3OD): δ = 7.76 (d, ^3J = 7.5 Hz, 2 H, 2 × Ar-H-Fmoc), 7.57 (t, ^3J = 7.1 Hz, 2 H, 2 × Ar-H-Fmoc), 7.52 (d, ^3J = 7.8 Hz, 2 H, 2 × Ar-H-Phe), 7.40 (t, ^3J = 7.4 Hz, 2 H, 2 × Ar-H-Fmoc), 7.30 (t, ^3J = 6.9 Hz, 2 H, 2 × Ar-H-Fmoc), 7.13 (d, ^3J = 7.8 Hz, 2 H, 2 × Ar-H-Phe), 4.59 (dd, ^2J = 5.8 Hz, ^3J = 8.4 Hz, 1 H, α-H-Phe), 4.41 (dd, ^2J = 6.7 Hz, ^3J = 10.5, 1 H, CHa-Fmoc), 4.32 (dd, ^2J = 6.7 Hz, ^3J = 10.6, 1 H, CHb-Fmoc), 4.18 (dd, ^2J = 6.3 Hz, ^3J = 13.8 Hz, 1 H, CH-Fmoc), 3.18–3.04 (m, 2 H, CH_2-Phe).

^{13}C NMR (100 MHz, $CDCl_3$, drops of CD_3OD): δ = 173.3 (O=C-carboxy), 155.8 (O=C-Fmoc), 143.7 (C-F_2P), 143.5, 141.2, 138.5, 131.9 (6 × Ar-C), 129.3, 127.7, 127.0, 126.3, 124.9, 124.8, 120.0 (12 × Ar-C), 66.8 (CH_2-Fmoc), 54.6 (α-CH-Phe), 47.0 (CH-Fmoc), 37.8 (CH_2-Phe).

^{31}P (126 MHz, $CDCl_3$, drops of CD_3OD): δ = 6.1 (t, J = 116 Hz).

HRMS (ESI): m/z [M + H]$^+$ calcd for $C_{25}H_{23}F_2NO_7P$: 518.11747; found: 518.11778.

Ac-Asp-Ala-Asp-Glu-F_2Pmp-Leu-NH_2 (8)

As solid support for the synthesis of hexapeptide **8**, Fmoc protected Rink amide resin (40 mg, 200–400 mesh, loading: 0.62 mmol/g) was chosen. The synthesis was performed with a Syro I automated peptide synthesizer, Fmoc deprotection was achieved by treatment with 40% piperidine in DMF (3 min) and 20% piperidine in DMF (12 min). Peptide couplings were carried out by reaction with Fmoc-protected amino acids (5 equiv), HBTU (5 equiv), HOBt (5 equiv), and DIPEA (10 equiv) in DMF for 40 min. After cleavage of the first Fmoc group, Fmoc-Leu-OH was coupled to the resin in a double coupling, and subsequently Fmoc deprotected. Next, unpurified Fmoc-F_2Pmp-OH (39 mg, 75 mol, 3 equiv) was coupled in DMF (1 mL) by manual addition of TBTU (23.8 mg, 75 μmol, 3 equiv), HOBt (10.25 mg, 75 μmol, 3 equiv), and DIPEA (43.6 μL, 150 μmol, 6 equiv) for 3 h. Fmoc deprotection was performed automatically and the next four amino acids Fmoc-Glu(O-*t*-Bu)-OH, Fmoc-Asp(O-*t*-Bu)-OH, Fmoc-Ala-OH, and Fmoc-Asp(O-*t*-Bu)-OH, were double coupled and Fmoc deprotected according to the standard protocol. The terminal amino group was acetylated by addition of acetic anhydride in pyridine (1:9, 800 μL). After thorough washing with DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min) and drying in high vacuum, the peptide was cleaved and side chain deprotected by treatment with TFA (950 μL) and triisopropylsilane (50 μL) for 2.5 h. The supernatant solution was filtered into ice-cold Et_2O (20 mL) and the colorless precipitate was centrifuged and washed with cold Et_2O (2 × 20 mL). The crude product was dissolved in MeCN–H_2O (10%) and purified by preparative RP-HPLC (Nucleodur C18; MeCN–H_2O, 10 → 75% + 0.05% TFA in 25 min). The completely deprotected peptide **8** was obtained as a colorless lyophilizate.

Yield: 9.30 mg (42%, 10.6 nmol); RP-HPLC: t_R = 6.4 min (Nucleodur C18; 215 nm; MeCN–H_2O, 10 → 75% + 0.05% TFA in 15 min).

^{1}H NMR (400 MHz, CD_3OD): δ = 8.55 (m, NH), 8.17 (d, ^3J = 7.0 Hz, NH), 8.04 (d, ^3J = 7.0 Hz, NH), 7.91 (d, ^3J = 7.0 Hz, 2NH), 7.81 (d, ^3J = 8.3 Hz, NH), 7.52 (d, ^3J = 7.5 Hz, 2 H), 7.38 (d, ^3J = 7.4 Hz, 2 H), 4.76–4.69 (m, 1 H), 4.65–4.51 (m, 2 H), 4.37–4.29 (m, 1 H), 4.26–4.17 (m, 2 H), 3.24–3.05 (m, 2 H), 2.97–2.72 (m, 4 H), 2.37–2.24 (m, 2 H), 2.10–1.87 (m, 5 H), 1.72–1.48 (m, 3 H), 1.41 (d, ^3J = 7.4 Hz, 3 H), 0.93 (d, ^3J = 6.7 Hz, 3 H), 0.87 (d, ^3J = 5.7 Hz, 3 H).

^{13}C NMR (100 MHz, CD_3OD): δ = 179.4, 176.1, 175.1, 174.3, 173.7, 173.1, 172.7, 172.4, 172.1, 171.6, 151.2, 139.4, 133, 128.9, 126.3, 55.3, 53.8, 51.4, 51.0, 50.0, 40.2, 36.6, 35.3, 34.5, 29.8, 26.0, 24.3, 22.2, 21.1, 20.3, 15.8.

^{31}P (126 MHz, CD_3OD): δ = 5.1 (t, *J* = 112 Hz).

MS (MALDI-TOF; CHCA): *m/z* [M + Na]$^+$ calcd for $C_{34}H_{48}F_2N_7NaO_{16}P$: 902.3; found: 902.5; *m/z* [M + K]$^+$ calcd for $C_{34}H_{48}F_2KN_7O_{16}P$: 918.4; found: 918.4.

Ac-Ala-Ser-Gly-Ala-F_2Pmp-Ala-Gly-Gly-Ser-Ala-NH_2 (9)

The decapeptide **9** was synthesized on Fmoc protected Rink amide resin (40 mg, 200–400 mesh, loading: 0.57 mmol/g). The synthesis was performed with a Syro I automated peptide synthesizer, Fmoc deprotection was achieved by the standard protocol. After Fmoc deprotection of the solid support, Fmoc-Ala-OH, Fmoc-Ser(O-*t*-Bu)-OH, Fmoc-Gly-OH (2 ×) and Fmoc-Ala-OH were double-coupled and Fmoc deprotected under standard coupling conditions. Unpurified Fmoc-F_2Pmp-OH (39 mg, 75 μmol, 3.3 equiv) was coupled in DMF (1 mL) by manual addition using TBTU (23.8 mg, 75 μmol, 3.3 equiv), HOBt (10.25 mg, 75 μmol, 3.3 equiv) and DIPEA (43.6 μL, 150 μmol, 6.6 equiv) for 3 h. Fmoc deprotection and coupling of the subsequent amino acids Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Ser(O-*t*-Bu)-OH and Fmoc-Ala-OH were performed automated in the described manner. The terminal amino group was acetylated by addition of acetic anhydride in pyridine (1:9, 800 μL). After thorough washing with DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min) and drying in high vacuum, the peptide was cleaved and side-chain deprotected by treatment with TFA (950 μL) and triisopropylsilane (50 μL) for 2.5 h. The supernatant solution was filtered into ice-cold Et_2O (20 mL). The colorless precipitate was centrifuged and washed with cold Et_2O (2 × 20 mL). The crude product was dissolved in MeCN–H_2O (5%) and purified by preparative RP-HPLC (Nucleodur C18; MeCN–H_2O, 5 → 10% + 0.05% TFA in 25 min). The completely deprotected peptide **9** was obtained as a colorless lyophilizate.

Yield: 8.20 mg (8.49 nmol, 37%); RP-HPLC: t_R = 10.0 min (Nucleodur C18; 215 nm; MeCN–H_2O, 5 → 10% + 0.05% TFA in 15 min).

^{1}H NMR (400 MHz, D_2O): δ = 7.45 (d, ^3J = 7.6 Hz, 2 H), 7.25 (d, ^3J = 7.6 Hz, 2 H), 4.54 (t, ^3J = 7.0 Hz, 1 H), 4.37–4.31 (m, 2 H), 4.23–4.16 (m, 3 H), 4.16–4.10 (m, 1 H), 3.90 (s, 2 H), 3.84 (s, 4 H), 3.81–3.73 (m, 4 H), 3.17–3.08 (m, 1 H), 3.02–2.94 (m, 1 H), 1.92 (s, 3 H), 1.31–1.23 (m, 9 H), 1.41 (d, ^3J = 7.1 Hz, 3 H).

^{13}C NMR (100 MHz, D_2O): δ = 180.4, 176.8, 175.6, 175.2, 174.8, 174.3, 173.3, 172.9, 171.7, 171.6, 171.3, 156.8, 138.5, 129.2, 128.5, 126.1, 61.0, 55.6, 54.4, 49.8, 49.6, 43.1, 42.5, 40.0, 21.6, 16.6, 16.5, 16.3.

^{31}P (126 MHz, D_2O): δ = 4.2 (t, *J* = 105 Hz).

MS (MALDI-TOF; CHCA): *m/z* [M + H]$^+$ calcd for $C_{36}H_{55}F_2N_{11}O_{16}P$: 966.4; found 966.4; *m/z* [M + Na]$^+$ calcd for $C_{36}H_{54}F_2N_{11}NaO_{16}P$: 988.3; found 988.4; *m/z* [M + K]$^+$ calcd for $C_{36}H_{54}F_2KN_{11}O_{16}P$: 1004.4; found 1004.3.

Expression and Purification of Recombinant PTP1B

The T7–7 vector containing the DNA sequence encoding for His-tagged PTP1B (1–321) was transformed into Escherichia coli strain BL21 DE3 using standard methods. The cells expressing the recombinant His-PTP1B were lysed by sonication in lysis buffer A [20 mM Tris-HCl, pH 8.0 containing 150 mM NaCl, 10 mM imidazole, 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. The protein was purified using a FPLC Histrap HP 1 mL column using an elution gradient of 10–500 mM imidazole gradient in buffer A. The integrity of the protein was confirmed by ESI-MS analysis.

Michaelis–Menten Constant Measurement of PTP1B for *p*NPP

In a 96 well plate, *p*NPP was added in eight different concentrations. After addition of PTP1B (40 nM) or H_2O (blank) to reach a final volume of 100 μL, absorbance at 405 nm was measured every 60 s over 1 h on a Tecan safire^2 (Tecan, Salzburg, Austria) plate reader at 37 °C. The measurements were performed in duplicate. The buffer conditions were pH 7.2, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; 25 mM), EDTA (2.5 mM), DTT (2.0 mM), NaCl (124.4 mM). After subtracting the blank, the initial reactions rates were obtained by linear regression of the data points up to 10 min, and the reaction rates were plotted over the substrate concentration. The Michaelis–Menten constant was determined by fitting the data points to the Michaelis–Menten equation using Sigma plot (Systat Software Inc., San Jose, USA).

PTP1B Inhibition Assay

In a final reaction volume of 100 μL, PTP1B (40 nM) was incubated with decreasing concentrations of the inhibitory peptides for 30 min at 37 °C. The buffer conditions were pH 7.2, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; 25 mM), EDTA (2.5 mM), DTT (2.0 mM), NaCl (124.4 mM). After the incubation time, *p*NPP (20 μL) was added to reach a final concentration of 10 mM. Using a Tecan safire^2 (Tecan, Salzburg, Austria) plate reader, the absorption at 405 nm was measured every 30 s over 15 min. All measurements were performed in triplicate. The slopes of the initial phases were obtained by linear regression of the data points up to 5 min. The slopes were plotted versus the log of the inhibitor concentrations, and the IC_{50} values were obtained by fitting the curves using the one site competition model of Sigma plot (Systat Software Inc., San Jose, USA).

Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synthesis.

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