Facile Incorporation of a Phosphatase Activity-Dependent Quinone Methide Generating Motif into Phosphotyrosine

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Received 23 April 2009; revised 20 July 2009

Abstract: A novel phosphotyrosine analogue that incorporates a phosphatase activity-dependent quinone methide generating motif is synthesized from tyrosine. Following orthoformylation of the phenol moiety of methyl *N*-Cbz-tyrosinate, the Fmoc-protected 3-difluoromethyl analogue of phosphotyrosine is obtained by functional group transformations.

Key words: amino acids, fluorine, hydrolyses, phosphates, quinone methides, quinomethanes

Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) are key enzymes that regulate cellular signal transduction by adding or removing a phosphate to/from a tyrosine residue of a protein, respectively.[1] It is not surprising that, like their counteracting enzymes PTKs, PTPs are involved in normal regulation as well as disease development. Investigators have used a variety of methods in characterizing the role of PTPs. Genetic approaches such as overexpression and knockout have helped to assess the role of several PTPs in cellular signaling, but may cause compensatory responses and other artifacts. DNA microarrays can provide information on gene expression at the transcription level but not necessarily at the protein expression or protein activity level, considering that posttranslational modifications such as phosphorylation can vary protein activities. While the standard (phospho)proteomic techniques do address protein expression and protein phosphorylation levels, they are often masked by abundant (phospho)proteins and not optimized for analysis of PTP activities. In terms of direct partnerships between PTPs and phosphoproteins, the substrate-trapping mutants of PTPs have achieved some success in identifying physiologically relevant substrates.[2] While PTPs can be promising biomarkers or therapeutic targets, no methods are previously available to allow direct identification of physiologically relevant PTPs for phosphoprotein substrates. In this regard, recent progress in the emerging techniques of activity-based protein profiling (ABPP) is encouraging.[3] Such techniques utilize mechanism-based inactivators, or activity-based probes, to label enzymes including phosphatases, proteases, and demethylases. Using a new variant of ABPP, our group has recently achieved activity-based selective labeling of an individual PTP by integrating a nonspecific phosphatase probe into a specific phosphopeptide substrate.[4] The integration is made possible by the synthesis of a Fmoc-protected 3-difluoromethyl analogue of phosphotyrosine **1** (Figure 1), a novel phosphotyrosine analogue that incorporates a phosphatase activity-dependent quinone methide generating motif.[5]

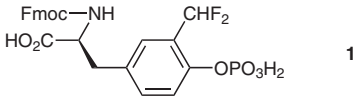
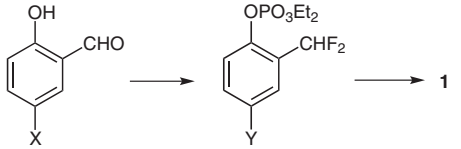


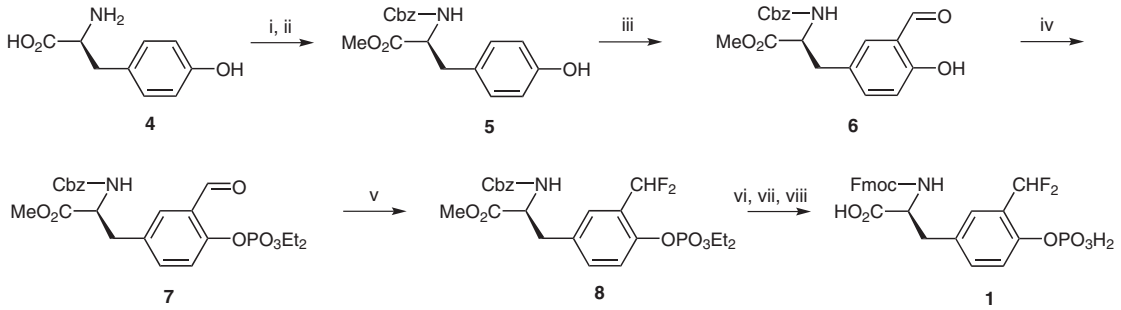
Figure 1 Fmoc-Protected 3-difluoromethyl analogue of phosphotyrosine **1**

Potential routes to the synthesis of the 3-difluoromethyl analogue of phosphotyrosine **1** include convergent routes and divergent routes. Potential convergent routes involve initial synthesis of an *o*-(difluoromethyl)phenyl phosphate and its subsequent integration into amino acid framework via carbon–carbon coupling, whereas potential divergent routes involve only synthetic modifications of tyrosine. On one hand, a potential convergent route may start with 2-hydroxy-5-methylbenzaldehyde (**2a**) or 2-hydroxy-5-iodobenzaldehyde (**3a**), which can undergo successive functional group transformations; phosphorylation (hydroxy) and fluorination (aldehyde), and monobromination (methyl) for **2a** only (Scheme 1). The resulting benzyl bromide **2b**/aryl iodide **3b** can then be converted into an amino acid using established carbon–carbon coupling strategies, such as alkylation with carbanionic amino acid synthons or Negishi or Suzuki coupling with amino acid synthons.[6] However, the instability of these nucleophile-sensitive phosphotriester intermediates under acidic as well as basic conditions could be troublesome in the subsequent multistep processes.[7] On the other hand, a divergent route (Scheme 2) starting with tyrosine may be more advantageous since the potentially poor yield and expensive fluorination step is placed later in the reaction sequence and the need for the amino acid



Scheme 1 Potential convergent routes to the synthesis of **1**

SYNTHESIS 2009, No. 22, pp 3765–3768
Advanced online publication: 23.09.2009
DOI: 10.1055/s-0029-1217025; Art ID: M01909SS



Scheme 2 A simple, divergent route to the synthesis of **1** from tyrosine. *Reagents and conditions:* (i) 2,2-dimethoxypropane, 36% HCl, r.t. overnight; (ii) Cbz-NHS, DMF, r.t., 3 h, 78% (from **4**); (iii) anhyd $MgCl_2$, anhyd Et_3N, paraformaldehyde, anhyd MeCN, reflux, 15 h, 43%; (iv) anhyd Et_3N, $ClPO_3Et_2$, anhyd CH_2Cl_2, ice-water bath, 1 h then r.t. overnight, 85%; (v) DAST, anhyd CH_2Cl_2, 0 °C, 1 h then r.t., 18 h, 64%; (vi) TMSBr, anhyd CH_2Cl_2, 0 °C, 1 h then r.t. 15 h; (vii) Fmoc-NHS, 1,4-dioxane–aq $NaHCO_3$, r.t., 3 h; (viii) LiOH, $CaCl_2$, THF–H_2O, r.t., 8 h, 72% (from **8**).

backbone-forming cross-coupling step is obviated. Once the difluoromethyl moiety is introduced into the structure, the diethyl phenyl phosphate is deprotected so that the instability of the phosphotriester is no longer a concern. We report herein the details of the synthesis of **1** by a simple, divergent route from tyrosine.

We started the divergent synthesis with orthoformylation of the tyrosine phenol moiety, followed by routine functional group transformations[5] (Scheme 2).

Skattebøl and co-workers recently reported orthoformylation of phenols using paraformaldehyde and magnesium chloride where a series of other functional groups including esters were tolerated.[8] We therefore speculated that a protected tyrosine like methyl *N*-Cbz-tyrosinate would be a good substrate for such orthoformylation reaction, which introduced 3-formyl group onto the tyrosine phenol moiety. First of all, tyrosine **4** was methylated at the carboxy group using a literature procedure[9] and subsequently protected at the amino group by Cbz-NHS to afford the protected tyrosine **5**, which showed NMR and MS data identical to the commercially available form from Bachem. Orthoformylation of **5** was carried out under reflux using anhydrous magnesium chloride, anhydrous triethylamine, and paraformaldehyde (dried over phosphorus pentoxide) in anhydrous acetonitrile. The presence of the aldehyde functional group was confirmed by ^1H and ^{13}C NMR and ESI-MS. While the conditions for the orthoformylation of **5** were not optimized, we found that longer reaction times (15–20 h) were needed for the protected tyrosine than for simple phenols (4 h).[8]

Orthoformylated tyrosine **6** was then phosphorylated using diethyl chlorophosphate to afford **7**, which was confirmed by ^1H, ^{13}C, and ^{31}P NMR and ESI-MS. Since compound **7** was sensitive to acidic conditions, its column purification was performed with triethylamine as a co-solvent.

The formyl group of **7** was subsequently converted into the difluoromethyl group using diethylaminosulfur trifluoride (DAST) to afford **8**, whose column purification also used triethylamine as a co-solvent. The presence of difluoromethyl and phosphate groups in **8** was confirmed by NMR and MS data including ^{19}F and ^{31}P NMR.

To be useful for peptide synthesis, the protective groups in the compound **8** were then removed and its amino group was Fmoc-protected. Bromotrimethylsilane treatment, which removed Cbz and ethyl protection, followed by Fmoc-NHS treatment and subsequent methyl removal by lithium hydroxide, provided the peptide-synthesis-ready, Fmoc-protected 3-difluoromethyl analogue of phosphotyrosine **1**. The identity of **1** was confirmed by ^1H, ^{13}C, ^{19}F, and ^{31}P NMR as well as by ESI-MS.

While lithium hydroxide has been used for carboxylate ester hydrolysis in the presence of N-Fmoc and/or phosph(on)ate ester protective groups,[4,10] it could remove N-Fmoc by β-elimination, even at low temperatures and for a reduced experiment time, leading to a reduced total yield. In our current procedure, we used calcium chloride as an additive in methyl group removal to avoid such a side reaction. According to Pascal and Sola,[11] the effective hydroxide ion concentration (and the undesired competing β-elimination reaction) could thus be suppressed through solubility equilibrium of calcium hydroxide. Although our modified procedure was not optimized, we indeed observed an increased yield (72%) upon using calcium chloride additive at room temperature for a prolonged experiment time (8 h), as compared to using lithium hydroxide only (60% at 0 °C for 1.5 h).[4] Although in our particular case the phosphate was not protected, lithium hydroxide treatment has been known to tolerate phosph(on)ate esters.[10] This suggests that the lithium hydroxide/calcium chloride combination could provide a useful orthogonality in the protective group chemistry of amino acids, phosph(on)ates and carboxylates. Moreover, unlike phosphotriesters, the phenyl phosphate **1** was stable enough to use in peptide synthesis and HPLC.[4]

In summary, we have described in detail the synthesis of a phosphotyrosine analogue that incorporates a phosphatase activity-dependent quinone methide generating motif. This novel amino acid has been used in peptide synthesis to generate a PTP activity-based probe that showed the first specific labeling of an individual PTP (PTP1B) in the presence of other PTPs.[4] It is expected that such amino acids, when incorporated into appropriate peptides, or proteins (in combination with expressed pro-

tein ligation),[12] will be useful in probing specific phosphoprotein-PTP interactions.

Chemicals were obtained from commercial suppliers and used without further purification. $MgCl_2$, anhydrous beads (10 mesh, 99.99%) was purchased from the Aldrich Chemical Company, Inc. Dry solvents were used for reactions wherever appropriate. NMR spectra were recorded using Bruker Avance DPX 200 or 500 spectrometers. Mass spectra (ESI) were recorded on a Thermo LCQ Advantage LC-MS spectrometer by direct injection. Column chromatography was performed using EM silica gel 60 (230–400 mesh).

Methyl (*S*)-2-(Benzyloxycarbonylamino)-3-(4-hydroxyphenyl)propionate (5)

To L-tyrosine (**4**, 3.30 g, 18 mmol) suspended in 2,2-dimethoxypropane (50 mL), 36% HCl (5 mL) was added. The resulting mixture was stirred overnight at r.t. and then concentrated to dryness to afford crude methyl tyrosinate, which was confirmed by ^{1}H NMR showing the formation of methyl ester. The mixture was then dissolved in a soln of DMF (20 mL) containing *i*-Pr_2NEt (8.0 mL, 46 mmol). This soln was chilled in an ice-water bath, mixed with CBz-NHS (4.78 g, 19 mmol) in DMF (10 mL) and stirred at r.t. for 3 h before concentration by rotary evaporation. The residue was dissolved in EtOAc and H_2O, acidified to pH 3 and extracted with EtOAc. The extract was washed with brine, dried (anhyd Na_2SO_4), and rotary evaporated to dryness. Flash column purification afforded a white solid; yield: 4.65 g (14 mmol, 78%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.40–7.34 (m, 5 H), 6.96 (d, J = 7.8 Hz, 2 H), 6.73 (d, J = 7.8 Hz, 2 H), 5.28 (s, 1 H), 5.14 (d, J = 12.4 Hz, 1 H), 5.10 (d, J = 12.4 Hz, 1 H), 4.65 (m, 1 H), 3.70 (s, 3 H), 3.09 (dd, J_1 = 13.8 Hz, J_2 = 5.5 Hz, 1 H), 3.02 (dd, J_1 = 13.8 Hz, J_2 = 5.7 Hz, 1 H).

^{13}C NMR (125 MHz, $CDCl_3$): δ = 172.2, 162.8, 155.7, 154.9, 136.2, 130.4, 128.6, 128.2, 128.1, 127.5, 115.5, 67.1, 55.0, 52.4, 37.5.

MS (ESI): *m*/*z* [M] calcd for $C_{18}H_{19}NO_5$: 329.1; found: 330.1 $[M + H]^+$.

Methyl (*S*)-2-(Benzyloxycarbonylamino)-3-(3-formyl-4-hydroxyphenyl)propionate (6)

To a soln of **5** (4.18 g, 13 mmol) in anhyd MeCN (100 mL) was added anhyd $MgCl_2$ (1.5 g, 16 mmol) and anhyd Et_3N (5 mL, 36 mmol) followed by paraformaldehyde (3.52 g, 120 mmol). The mixture was refluxed under argon for 15 h and allowed to cool down to r.t. After acidification to pH 5 using 1 M HCl, the mixture was extracted with Et_2O (3 × 20 mL). The combined ether extracts were washed with brine, dried (anhyd Na_2SO_4), and rotary evaporated to a sticky liquid. The desired orthoformylated tyrosine **6** was obtained as a white solid (1.97 g, 5.5 mmol, 43%) after purification by flash column chromatography (2% EtOAc–CH_2Cl_2).

^{1}H NMR (200 MHz, $CDCl_3$): δ = 10.87 (s, 1 H), 9.66 (s, 1 H), 7.27–7.21 (m, 7 H), 6.82 (d, J = 9.1 Hz, 1 H), 5.83 (d, J = 8.2 Hz, 1 H), 5.08 (d, J = 12.3 Hz, 1 H), 4.99 (d, J = 12.3 Hz, 1 H), 4.62 (m, 1 H), 3.67 (s, 3 H), 3.12 (dd, J_1 = 14.0 Hz, J_2 = 6.9 Hz, 1 H), 2.99 (dd, J_1 = 14.0 Hz, J_2 = 5.4 Hz, 1 H).

^{13}C NMR (50 MHz, $CDCl_3$): δ = 196.3, 171.7, 160.4, 155.7, 137.8, 136.3, 134.0, 128.4, 128.1, 127.9, 127.6, 120.5, 117.7, 66.8, 54.9, 52.3, 37.0.

MS (ESI): *m*/*z* [M] calcd for $C_{19}H_{19}NO_6$: 357.1; found: 358.2 $[M + H]^+$.

Methyl (*S*)-2-(Benzyloxycarbonylamino)-3-[4-(diethoxyphosphoryloxy)-3-formylphenyl]propionate (7)

To a soln of **6** (1.03 g, 2.9 mmol) in anhyd CH_2Cl_2 (20 mL) in an ice-water bath was added Et_3N (0.60 mL, 4.3 mmol) and $ClPO_3Et_2$ (0.48 mL, 3.3 mmol). The mixture was kept in the ice-water bath for 1 h and then kept at r.t. overnight. The mixture was washed with H_2O and then brine and dried (anhyd Na_2SO_4). The mixture was concentrated to an oily liquid and then purified by flash column chromatography (20% EtOAc–CH_2Cl_2 containing 0.1% Et_3N). The desired orthoformylated phosphotyrosine **7** was obtained as colorless oil (1.21 g, 2.5 mmol, 85%).

^{1}H NMR (200 MHz, $CDCl_3$): δ = 10.37 (s, 1 H), 7.64 (s, 1 H), 7.43–7.31 (m, 7 H), 5.28 (d, J = 7.7 Hz, 1 H), 5.11 (s, 2 H), 4.66 (m, 1 H), 4.33–4.18 (m, 4 H), 3.75 (s, 3 H), 3.21 (dd, J_1 = 14.1 Hz, J_2 = 5.6 Hz, 1 H), 3.09 (dd, J_1 = 14.1 Hz, J_2 = 6.1 Hz, 1 H), 1.37 (m, 6 H).

^{13}C NMR (50 MHz, $CDCl_3$): δ = 188.3, 171.4, 155.5, 152.0 (d), 136.5, 136.1, 133.4, 129.3, 128.6, 128.3, 128.1, 127.2 (d), 121.3, 67.1, 65.2 (d), 54.7, 52.6, 37.4, 16.1 (d).

^{31}P NMR (81 MHz, $CDCl_3$): δ = –6.4.

MS (ESI): *m*/*z* [M] calcd for $C_{23}H_{28}NO_9P$: 493.1; found: 494.1 $[M + H]^+$.

Methyl (*S*)-2-(Benzyloxycarbonylamino)-3-[4-(diethoxyphosphoryloxy)-3-(difluoromethyl)phenyl]propionate (8)

To **7** (540 mg, 1.1 mmol) in anhyd CH_2Cl_2 (2 mL) at 0 °C, DAST (1.5 mL, 11 mmol) was added. The mixture was kept at 0 °C for 1 h and allowed to rise to r.t. After 18 h, the mixture was diluted with CH_2Cl_2 and added to sat. $NaHCO_3$ soln (10 mL) at 0 °C. The aqueous phase was then extracted with CH_2Cl_2 (2 × 10 mL). The combined organic phases were washed with brine, dried (anhyd Na_2SO_4), concentrated to an oily liquid, and purified by flash column chromatography (10% EtOAc–CH_2Cl_2 containing 0.1% Et_3N). The difluorinated phosphotyrosine **8** was obtained as a colorless oil (360 mg, 0.70 mmol, 64%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.37–7.25 (m, 7 H), 7.20 (d, J = 8.1 Hz, 1 H), 6.90 (t, J_F = 55.1 Hz, 1 H), 5.61 (d, J = 7.8 Hz, 1 H), 5.08 (d, J = 12.5 Hz, 1 H), 5.05 (d, J = 12.5 Hz, 1 H), 4.61 (m, 1 H), 4.24–4.16 (m, 4 H), 3.69 (s, 3 H), 3.15 (dd, J_1 = 13.8 Hz, J_2 = 5.3 Hz, 1 H), 3.06 (dd, J_1 = 13.8 Hz, J_2 = 6.4 Hz, 1 H), 1.32 (m, 6 H).

^{13}C NMR (125 MHz, $CDCl_3$): δ = 171.8, 155.9, 147.4, 136.4, 133.9, 132.8, 128.3, 127.8, 127.7, 127.4, 125.2 (dt), 120.1, 111.2 (t, J_F = 237 Hz), 66.5, 64.9 (d), 55.0, 52.0, 36.8, 15.7 (d).

^{19}F NMR (81 MHz, $CDCl_3$): δ = –115.3 (d, J_H = 54.6 Hz).

^{31}P NMR (188 MHz, $CDCl_3$): δ = –6.5.

MS (ESI): *m*/*z* [M] calcd for $C_{23}H_{28}F_2NO_8P$: 515.1; found: 516.1 $[M + H]^+$.

(*S*)-3-[3-(Difluoromethyl)-4-(dihydroxyphosphoryloxy)phenyl]-2-[(9-fluorenylmethoxycarbonyl)amino]propionic Acid (1)

To **8** (244 mg, 0.47 mmol) in anhyd CH_2Cl_2 (5 mL) at 0 °C, TMSBr (2 mL, 15 mmol) was added. The mixture was kept at 0 °C for 1 h and allowed to rise to r.t. After 15 h, the mixture was rotary evaporated to dryness and then dissolved in MeOH (2 mL) and rotary evaporated again to dryness. After trituration with Et_2O, removal of the ethyl and Cbz protective groups was confirmed by ^{1}H NMR (D_2O). The crude product was then dissolved in a soln of $NaHCO_3$ (200 mg, 2.4 mmol) in H_2O (5 mL). This soln was chilled in an ice-water bath, mixed with Fmoc-NHS (170 mg, 0.05 mmol) in 1,4-dioxane (5 mL) and stirred at r.t. for 3 h. It was then diluted with sat. $NaHCO_3$ soln (10 mL) and washed with Et_2O. The aqueous phase was acidified to pH 3 and extracted with EtOAc. The combined extracts were washed with brine and dried (anhyd Na_2SO_4) and rotary evaporated to dryness. The resulting mixture was dissolved in THF (5 mL) and treated with LiOH (30 mg, 1.3 mmol) in aq 0.8 M $CaCl_2$ (5 mL) at r.t. for 8 h to remove the methyl protective group, and then acidified to pH 3 and extracted with EtOAc. The extract was rotary

evaporated to dryness and purified by RP-HPLC (100% MeCN–H_2O containing 0.05% TFA0) to afford the desired product **1** (184 mg, 0.34 mmol, 72%).

^{1}H NMR (200 MHz, acetone-d_6): δ = 10.45 (s, 3 H), 7.84–6.81 (m, 12 H), 4.56 (m, 1 H), 4.31–4.15 (m, 3 H), 3.32 (dd, J_1 = 13.9 Hz, J_2 = 4.7 Hz, 1 H), 3.11 (dd, J_1 = 13.9 Hz, J_2 = 9.3 Hz, 1 H).

^{13}C NMR (50 MHz, acetone-d_6): δ = 172.1, 156.0, 147.8 (m), 144.0, 141.1, 134.6, 132.9, 127.6, 127.1, 127.0, 125.5 (dt), 125.1, 120.4, 119.8, 111.5 (t, J_F = 236 Hz), 66.5, 55.1, 47.1, 36.6.

^{19}F NMR (81 MHz, acetone-d_6): δ = –115.5 (d, J_H = 54.6 Hz).

^{31}P NMR (188 MHz, acetone-d_6): δ = –5.2.

MS (ESI): *m/z* [M] calcd for $C_{25}H_{22}F_2NO_8P$: 533.1; found: 532.1 $[M – H]^–$; 1065.1 $[2 M – H]^–$; 1597.7 $[3 M – H]^–$.

Acknowledgment

We thank Dr. Narayan Hosmane for helpful discussions, Dr. Heike Hofstetter for help with NMR experiments and Northern Illinois University (Department of Chemistry and Biochemistry and Center for Biochemical and Biophysical Studies) for financial support.

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