Preparation of Phospho-L-Azatyrosine Suitably Protected for the Synthesis of Signal Transduction Related Peptides. A Correction

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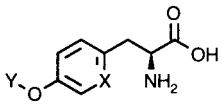
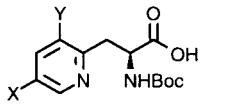
Abstract: Synthesis of N^{α}-Fmoc 4-O,O-(di-*tert*-butyl)phospho-*L*-azatyrosine (**6**) and its use in Fmoc-based solid-phase peptide synthesis are reported. The di-*tert*-butyl phospho protecting groups were removed during TFA-mediated cleavage of the finished peptide (**10**) from the resin, providing the free phosphoazatyrosine-containing peptide directly. This is in contrast to diethyl phospho protection we previously reported [Ye, B.; Otaka, A.; Burke, T. R., Jr. *Synlett.* **1996**, 459-460] in which the diethyl protection has proven impractical to remove. Due to incorrect assignment of starting material, the title compound of this latter report should be corrected to the isomeric *L*-β-(3-hydroxy-2-pyridyl)-alanine compound (**5**).

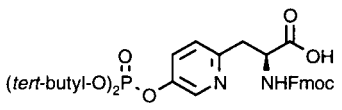
Protein-tyrosine kinase (PTK)-dependent pathways offer potentially interesting targets for therapeutic intervention in several diseases, including cancers and immune disorders.[1,2] Since in proteins, the phosphotyrosyl (pTyr) residue (**1**) provides a critical motif around which PTK-dependent signalling centers, analogues of pTyr have proven useful in the design of PTK inhibitors.[3] Azatyrosine (*L*-β-(5-hydroxy-2-pyridyl)-alanine) (**2**) is an antibiotic isolated from *Streptomyces chibanesis*[4] which has been shown to promote permanent reversion of *ras*-dependent transformed cells to the normal phenotype in culture[5] and to inhibit chemical induction of carcinogenesis in transgenic mice bearing oncogenic human *ras*.[6] Its phosphorylated version, phosphoazatyrosine (pAzaTyr, **3**), represents a pyridyl-containing variant of pTyr, which may be potentially interesting for the study of signal transduction.



		X	Y
1	pTyr	CH	$PO(OH)_2$
2	AzaTyr	N	H
3	pAzaTyr	N	$PO(OH)_2$

We have previously reported diethylphospho-*L*-azatyrosine bearing N^{α} Boc protection (**4**) as a reagent potentially useful for the synthesis of signal transduction-related peptides.[7] This latter synthesis was based on our approach for the preparation of AzaTyr itself.[8] However, as recently pointed out, the putative starting material which we indicated as 5-hydroxy-2-iodopyridine, was actually the isomeric 3-hydroxy-2-iodopyridine.[9] Accordingly, both in our original AzaTyr[8] and pAzaTyr papers,[7] compounds derived from this starting material have ring oxy-substituents at the 3-position rather than the 5-position as we reported. When this isomeric pAzaTyr analogue (**5**) was incorporated into peptides by solid-phase techniques, subsequent removal of the phosphate O,O-diethyl protection proved to be impractical, potentially indicating the unsuitability of the ethyl groups for solid-phase synthesis.[10] A need has therefore arisen for the synthesis of pAzaTyr possessing phospho and amino protection amenable to facile solid-phase synthesis. By analogy to pTyr itself,[11,12] such a derivative of pAzaTyr was therefore designed bearing *tert*-butyl phosphate and N^{α} Fmoc protection (**6**).

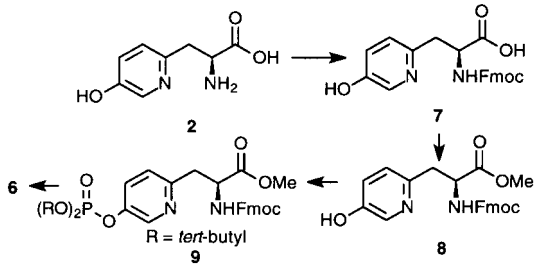




6

	X	Y
4	$O[PO(OEt)_2]$	H
5	H	$O[PO(OEt)_2]$

Utilizing unprotected AzaTyr (**2**) prepared by the enantioselective enzymatic route of Stevens,[13] the amino group was first derivatized in 95% yield using Fmoc N-hydroxysuccinimide active ester (Fmoc-OSu). The high water solubility induced by the zwitterionic pyridyl nitrogen resulted in workup difficulties not encountered with N^{α}-Fmoc Tyr itself, and efficient extraction of the N^{α} Fmoc AzaTyr (**7**) was only achieved using a H_2O:THF biphasic system. Next, protection of the carboxyl group was required prior to phosphorylation of the phenolic hydroxyl. Selective TBDMS esterification of the carboxyl group without silation of the 4'-hydroxyl is possible for N^{α} Fmoc Tyr when done in anhydrous THF.[11,12] However, the poor solubility of N^{α} Fmoc AzaTyr (**7**) in anhydrous THF or dioxane, necessitated the use of DMF, which resulted in loss of selectivity and silation of both the carboxyl and phenolic groups. Alternate esterification as the methyl ester was therefore employed, since this could be accomplished under aqueous conditions prior to phosphorylation, and subsequently removed post phosphorylation without cleaving the *tert*-butyl or Fmoc groups. Treatment of **7** with dimethoxypropane in aqueous HCl provided methyl ester **8** in 97% yield. Phosphorylation to provide **9** was then achieved in 88% yield using di-*tert*-butyl diisopropylphosphoramidite and 1H-tetrazole followed by MCPBA-mediated oxidation of the intermediate di-(*tert*-butyl)phosphite.[12] Hydrolysis of the methyl ester was accomplished using ice-cold 0.2 N LiOH without significant loss of the Fmoc group,[14] providing title compound **6** in 65% yield.



2 **7** **8** **9** R = *tert*-butyl

In order to demonstrate the utility of **6** for the synthesis of pAzaTyr-containing peptides, preparation of Ac-Asp-Ala-Asp-Glu-[pAzaTyr]-Leu-amide (**10**) was achieved using the acid-labile Rink amide resin[15] and standard Fmoc-based protocols. Cleavage of the peptide from the

resin with concomitant global deprotection was achieved using aqueous TFA with triethylsilane (TES) scavenger. Finally, purification by HPLC provided desired peptide **10** in 20% yield based on resin substitution.

Experimental Section

Elemental analyses were obtained from Atlantic Microlab Inc., Norcross, GA and fast atom bombardment mass spectra (FABMS) were acquired with a VG Analytical 7070E mass spectrometer under the control of a VG 2035 data system. Amino acid analysis was obtained from Peptide Technologies, Corp., Gaithersburg, MD. ^{1}H NMR data were obtained on a Bruker AC250 (250 MHz) instrument. Solvent was removed by rotary evaporation under reduced pressure and silica gel chromatography was performed using Merck silica gel 60 with a particle size of 40 - 63 µ. Anhydrous solvents were obtained commercially and used without further drying.

2(*S*)-2-((Fluorenyl-9-ylmethoxy)carbonylamino)-3-(5-hydroxy(2-pyridyl))propanoic acid (7): To a suspension of *L*-azatyrosine (**2**) (420 mg, 2.30 mmol) and $NaHCO_3$ (966 mg, 11.5 mmol) in dioxane (8 mL) with water (8 mL) was added Fmoc-OSu (775 mg, 2.30 mmol) at rt. The reaction was stirred at rt (6 hr), then 1 N HCl was added dropwise at 0 °C until the pH was adjusted to 4.5~5.0. The solution was then extracted with THF (3 x 40 mL), the combined organic solutions washed with brine (50 mL), dried over Na_2SO_4 and concentrated. The residue was dried in vacuo to provide **7** as a white foam (868 mg, 95%), which was pure enough for the next step. ^{1}H NMR (DMSO-d_6) δ 9.72 (1H, brs), 8.04 (1H, s), 7.87 (2H, d, *J* = 7.5 Hz), 7.62 (3H, m), 7.40 (2H, t, *J* = 7.3 Hz), 7.29 (2H, dd, *J* = 7.2 Hz, 11.6 Hz), 7.08 (2H, s), 4.34 (1H, m), 4.18 (3H, m), 3.07 (1H, dd, *J* = 4.6 Hz, 14.0 Hz), 2.91 (1H, dd, *J* = 9.9 Hz, 13.9 Hz). FABMS (*m/z*): 405 (MH^+, 25).

2(*S*)-2-((Fluorenyl-9-ylmethoxy)carbonylamino)-3-(5-hydroxy(2-pyridyl))propanoic acid methyl ester (8): To a suspension of **8** (261 mg, 0.646 mmol) in 2,2-dimethoxypropane (10 mL) was added 36% HCl (0.6 mL) and the resulting homogeneous solution was stirred at rt overnight. The reaction mixture was quenched by addition of saturated aqueous $NaHCO_3$ until the pH was adjusted to 5, then it was extracted with EtOAc (40 mL). The organic layer was washed with saturated aqueous NH_4Cl (2 x 20 mL), dried (Na_2SO_4) and concentrated. Residue was purified by silica gel chromatography (MeOH : $CHCl_3$, 1:40) to provide **8** as a white solid (262 mg, 97%), Mp 109.5-111.0 °C. ^{1}H NMR (DMSO-d_6) δ 9.73 (1H, s), 8.04 (1H, s), 7.89 (2H, d, *J* = 7.4 Hz), 7.81 (1H, d, *J* = 8.1 Hz), 7.63 (2H, dd, *J* = 3.6 Hz, 7.3 Hz), 7.42 (2H, t, *J* = 7.3 Hz), 7.32 (2H, dd, *J* = 3.8 Hz, 7.2 Hz), 7.08 (2H, s), 4.44 (1H, m), 4.22 (3H, m), 3.60 (3H, s), 3.00 (2H, m). FABMS (*m/z*): 419 (MH^+, 74). Analysis ($C_{24}H_{22}N_2O_5$): C, 68.89, H, 5.30, N, 6.69; Found: C 68.84, H, 5.38, N, 6.65.

2(*S*)-2-((Fluorenyl-9-ylmethoxy)carbonylamino)-3-(5-(di-*tert*-butyl phosphoryloxy)(2-pyridyl))propanoic acid methyl ester (9) To a solution of **8** (132 mg, 0.316 mmol) in anhydrous DMF (1.5 mL) was added 1H-tetrazole (72 mg, 0.950 mmol) followed by di-*tert*-butyl diisopropylphosphoramidite (90 mg, 0.395 mmol) under argon. The reaction mixture was stirred at rt (2 hr), then cooled to -40 °C and a solution of 80% MCPBA (81 mg, 0.411 mmol) in anhydrous dichloromethane (2 mL) was added. The reaction mixture was stirred on ice (30 min) then diluted with EtOAc (15 mL), washed with saturated aqueous $NaHCO_3$ (5 mL x 3) and dried (Na_2SO_4). Concentration and purification by silica gel column chromatography (MeOH : $CHCl_3$, from 1:100 to 1:40) provided **9** as a clear oil (170 mg, 88%). ^{1}H NMR (DMSO-d_6) δ 8.35 (1H, s), 7.88 (3H, m), 7.63 (2H, d, *J* =7.4 Hz), 7.53 (1H, dd, *J* =8.5 Hz), 7.41 (2H, t, *J* =7.3 Hz), 7.32 (3H, d, *J* =8.6 Hz), 4.51 (1H, m), 4.22 (3H, m), 3.61 (3H, s), 3.11 (2H, m), 1.42 (18H, s). FABMS (*m/z*): 611 (MH^+, 79).

2(*S*)-2-((Fluorenyl-9-ylmethoxy)carbonylamino)-3-(5-(di-*tert*-butyl phosphoryloxy)(2-pyridyl))propanoic acid [N^α Fmoc 4-O-(di-*tert*-butyl)phospho-*L*-azatyrosine] (6): To a cooled solution of **9** (200 mg, 0.327 mg) in THF (7 mL) was added 0.2 N LiOH (3.5 mL, 0.70 mmol) slowly at 0 °C. The reaction mixture was stirred at 0 °C (25 min) then quenched by addition of 0.2 N HCl (3.5 mL, 0.700 mmol) and adjusted to pH 5. The mixture was extracted with EtOAc (3 x 10 mL), washed with brine (10 mL), dried (Na_2SO_4) and concentrated. Purification by silica gel column chromatography (MeOH : $CHCl_3$, from 1:10 to 1:3) provided **6** as a white foam (126 mg, 65%),mp 172 °C (dec.). ^{1}H NMR (DMSO-d_6) δ 8.30 (1H, s), 7.88 (2H, d, *J* =7.4 Hz), 7.63 (2H, d, *J* = 6.3 Hz), 7.40 (3H, m), 7.31 (3H, m), 7.03 (1H, m), 4.14 (4H, m), 2.99 (2H, m), 1.40 (18H, s) ppm. HR FABMS calcd. For $C_{31}H_{36}N_2O_9P$: 595.2209 [$(M-H)^-$]. Found: 595.2205.

Solid Phase Synthesis of Ac-Asp-Ala-Asp-Glu-[pAzaTyr]-Leu-amide (10): Solid-phase peptide synthesis of **10** was accomplished on Fmoc-protected Rink amide resin[15] using 20% piperidine deblock with 1-hydroxybenzotriazole (HOBt) active ester coupling of amino acids in the order: N^α-Fmoc-*L*-Leu-OH, N^α-Fmoc-*L*-Azatyr(PO_3(*tert*-butyl)$_2$)-OH (**6**), N^α-Fmoc-*L*-Glu(O-*tert*-butyl)-OH, N^α-Fmoc-*L*-Asp(O-*tert*-butyl)-OH, Fmoc-*L*-Ala-OH and Fmoc-*L*-Asp(O-*tert*-butyl)-OH followed by 1-actylimidazole mediated N-terminal acetylation. The finished resin was cleaved using a mixture of TFA-water-TES (92.5 : 5 : 2.5, 1.5 mL) (2 h). The crude peptide was subjected to HPLC purification (peak retention time of 14.3 min) using a Vydac Protein & Peptide C_{18} column (218TP1022) using an elution of B = 0% (from 0 - 5 min) with a linear gradient of from B = 0% to 50% over 20 min. Solvent A = 0.1% TFA in H_2O and B = 0.1% TFA in acetonitrile. Product **10** was obtained as a white solid (8.3 mg, 20% overall based on resin substitution). ^{1}H NMR (D_2O) δ 8.55 (1H, s), 8.18 (1H, m), 7.81 (1H, d, *J* = 8.8 Hz), 4.69 (3H, m), 4.31 (3H, m), 3.54 (1H, m), 3.43 (1H, m), 2.88 (4H, m), 2.49 (2H, m), 2.19 (1H, m), 2.04 (4H, s), 1.63 (3H, m), 1.41 (3H, d, *J* = 7.2 Hz), 0.94 (3H, d, *J* = 5.0 Hz), 0.87 (3H, d, *J* = 5.0 Hz). FABMS (*m/z*) [$(M-H)^-$, 14]. Amino acid analysis: Asp 2.04 (2), Glu 1.00 (1), Ala 0.92 (1), Leu 1.05 (1).

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