Tetrahedron Letters,Vol.30,No.45,pp 6229-6232,1989 0040-4039/89 $3.00 + .00
Printed in Great Britain Pergamon Press plc

FMOC-POLYAMIDE SOLID PHASE SYNTHESIS OF AN O-PHOSPHOTYROSINE-CONTAINING TRIDECAPEPTIDE

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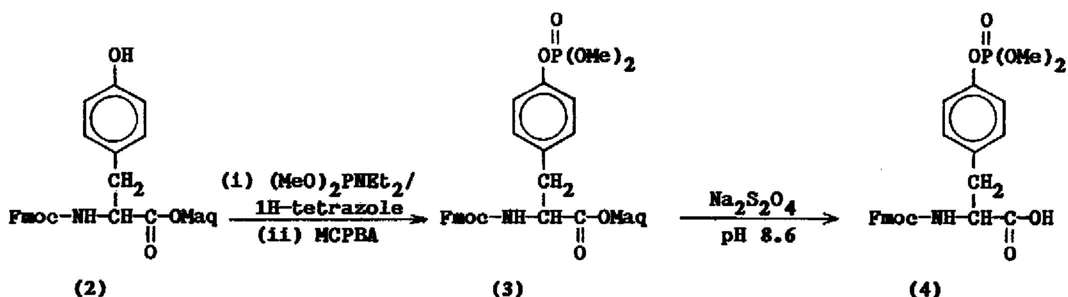
Abstract: *The incorporation of O-phosphotyrosine into synthetic peptides using Fmoc-Tyr(PO_3Me_2)-OH in the Fmoc-polyamide procedure is described with the preparation of the model PTyr-tridecapeptide sequence H-Arg-Leu-Ile-Glu-Asp-Asn-Glu-PTyr-Thr-Ala-Arg-Gln-Gly-OH.*

Protein tyrosine phosphorylation is now implicated in many cell regulatory processes,[1] and synthetic PTyr peptides may serve as suitable model substrates for obtaining a better understanding of tyrosine kinase phosphorylation and its mechanism of action. Previously, we have reported the preparation and use of Boc-Tyr(PO_3Me_2)-OH (1) in the solution[2] and solid phase[3] synthesis of small PTyr peptides. HF deprotection was found to be incompatible with PTyr peptides as it caused significant O-dephosphorylation.[4,5] This side reaction, however, can be avoided by the use of TFMSA or TMSBr for the final deprotection which also permits the ready cleavage of the methyl phosphate groups.[6] The TMSBr reagent has also been shown to be valuable in the final deprotection step in the preparation of peptide amides by the Fmoc method of peptide synthesis.[7] In this letter, we report on the utility of Fmoc-Tyr(PO_3Me_2)-OH (4) in the Fmoc-polyamide solid phase method[8] for the incorporation of the O-phosphotyrosine residue, using as a test sequence the tridecapeptide H-Arg-Leu-Ile-Glu-Asp-Asn-Glu-PTyr-Thr-Ala-Arg-Gln-Gly-OH. This sequence occurs in the viral proteins pp60^{v-src} and p90$^{gag-yes}$ where the tyrosine residue is autophosphorylated.[9]

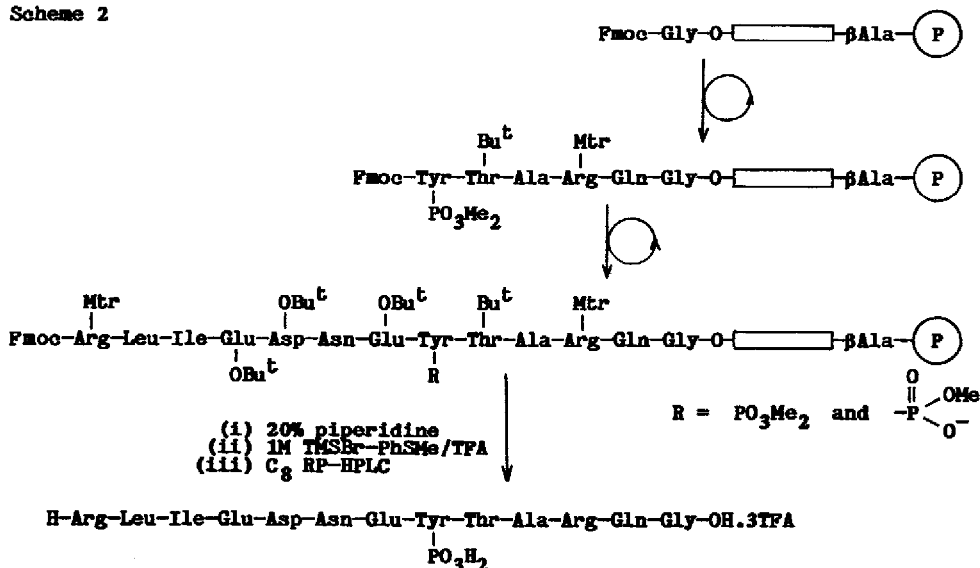
The protected phosphotyrosine derivative, Fmoc-Tyr(PO_3Me_2)-OH (4) was prepared by two independent routes. In the first route, the Boc group was cleaved from (1) with 4 M HCl/1,4-dioxan and the resultant H-Tyr(PO_3Me_2)-OH.HCl salt was treated with Fmoc-Cl under basic conditions to give (4) in 75 % yield. Alternatively, a 'phosphite-triester' phosphorylation procedure[10] was used to prepare the dimethyl phosphono-triester (4) (Scheme 1). In this approach, Fmoc-Tyr(PO_3Me_2)-OMaq (3) was prepared in 85 % yield by the phosphorylation of Fmoc-Tyr-OMaq[11] using dimethyl N,N-diethylphosphoramidite/1H-tetrazole followed by in situ MCPBA oxidation of the dimethyl phosphite-triester intermediate. Mild sodium dithionite reduction of the Maq group[12] yielded (4) in 70 % yield. A feature of this latter approach is the compatability of the phosphoramidite reagent with the preparation of protected, base sensitive, Fmoc-PTyr derivatives. Previously reported procedures[3,4] required the presence of base to generate in situ the phenoxide ion prior to phosphorylation. The structure of Fmoc-Tyr(PO_3Me_2)-OH (4) was confirmed by ^{13}C nmr spectroscopy and FAB-MS[13] and its purity was established by rp-hplc (Fig. 1A).

Scheme 1



The continuous flow kieselguhr-polydimethylacrylamide resin (1.0 g, 1.0 mmol) was functionalized with a β-alanine internal reference amino acid and the acid labile p-hydroxymethylphenoxyacetic acid linkage agent. Esterification of the C-terminal Fmoc-amino acid, glycine, utilized the preformed symmetric anhydride in the presence of 4-dimethylaminopyridine catalyst.[14] Subsequent peptide bond forming reactions utilized the appropriate Fmoc-amino acid (3 equiv.) in DMF together with benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) and hydroxybenzotriazole (HOBt) (3 equiv. each) in the presence of N-methylmorpholine (4.5 equiv.).[15,16] Side chain protection was as t-butyl esters and ethers for Asp, Glu and Thr and with the 4-methoxy-2,3,6-trimethylbenzenesulphonyl group for Arg.[16] Each acylation was monitored by the 2,4,6-trinitrobenzenesulphonic acid test for free amine and was found to be complete after the allotted reaction time (25 min).[17] Fmoc group deprotection was carried out with 20 % piperidine in DMF[18] (Scheme 2).

Scheme 2



At the completion of the synthesis, 0.15 g of the resin-bound peptide (1.11 g) was treated with 1 M TMSBr-thioanisole/TFA (m-cresol, 10 mmeq Tyr) (15 ml) for 16 h at 0-4°C.

C_8 rp-hplc of the isolated crude peptide showed one major product (Fig. 1B) whose amino acid analysis gave the constituent amino acids in their expected ratios.[19] Subsequent semi-preparative C_8 rp-hplc purification gave the PTyr-tridecapeptide (21 mg) in 70 % yield.

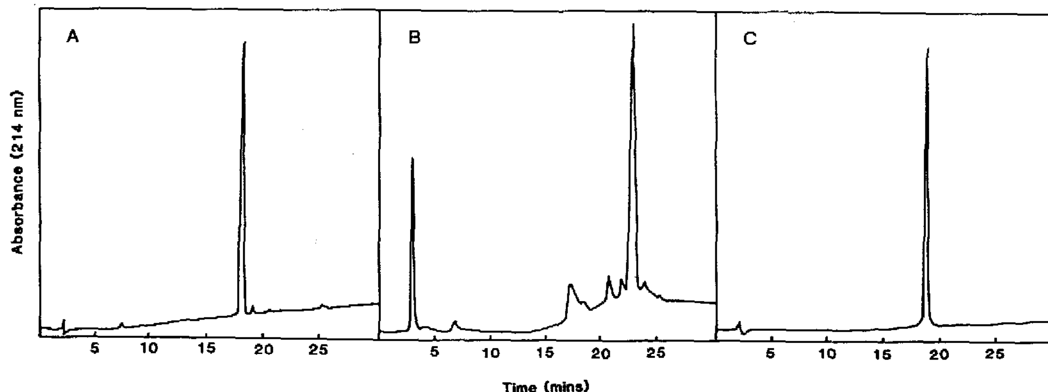


Figure 1: Analytical hplc (Brownlee RP-300), Buffer A, 0.1% aq. TFA, Buffer B, 0.1% TFA/AcCN, linear gradient. **(A)** derivative (4), 20-60%B in 30 min, **(B)** crude peptide, 0-40%B in 30 min, **(C)** purified peptide, 0-45%B in 30 min.

The purified PTyr-tridecapeptide (Fig. 1C) was characterized by amino acid analysis,[19] FAB-MS and amino acid sequencing. Amino acid analysis, gave the correct amino acid composition following acid hydrolysis (the hydrolysis product, Tyr, was observed for PTyr) and the FAB mass spectrum of the target peptide (Fig. 2) contained a distinct molecular ion at m/z 1644 for a calculated molecular weight of 1643.75. Phenylthiohydantoin (PTH) gas-phase sequencing of the PTyr-tridecapeptide, successively gave the PTH forms of ^{1}Arg, ^{2}Leu, ^{3}Ile, ^{4}Glu, ^{5}Asp, ^{6}Asn, ^{7}Glu, a blank eighth cycle for PTyr, and then the PTH forms of ^{9}Thr, ^{10}Ala, ^{11}Arg, ^{12}Gln and ^{13}Gly. The observation of a blank cycle for PTH-PTyr is consistent with earlier observations made by us and others[4] and has been attributed to its irreversible binding to the solid support.

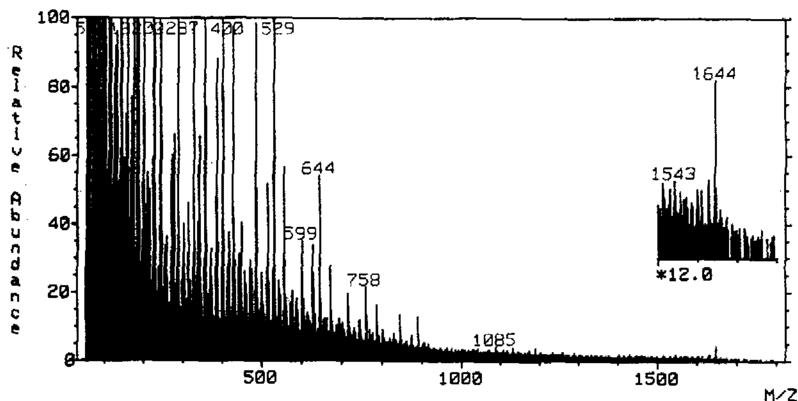


Figure 2 : FAB mass spectrum (Ar, +ve mode) of the PTyr tridecapeptide.

In conclusion, the synthon, Fmoc-Tyr(PO_3Me_2)-OH (4), has been demonstrated to be useful

in practice in the Fmoc solid phase synthesis of PTyr-containing peptides. Continual piperidine-mediated monodemethylation of the Tyr(PO_3Me_2)-residue in each Fmoc-cleavage cycle was found not to be a hindrance to the assembly of the peptide chain with the use of the BOP/HOBt coupling procedure.[18] The use of non-nucleophilic bases (e.g. DBU) for Fmoc group removal, in the Fmoc synthesis of PTyr peptides is currently being investigated.

Acknowledgements E.A.K. acknowledges a Commonwealth Postgraduate Award and J.W.P. the financial support of the Australian Wool Corporation. We thank Mr. W. Rule for performing the amino acid analyses, Mrs. M. John for the sequencing and Mr. B. Shirriffs for the FAB-MS.

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11. Prepared by first treating L-tyrosine with 10 % Na_2CO_3 and Fmoc-Cl at 0°C and further stirring for 5 h at 20°C. Formation of Fmoc-dipeptide was not observed. The cesium salt of Fmoc-Tyr-OH was treated with Maq-Br in DMF for 27 h to give (2) in 61 % yield.

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13. ^{13}C nmr δ($CDCl_3$): 37.1, 47.2, 54.4, 55.0 (d, J_{PC} 5.49 Hz), 67.0, 119.9, 125.0, 127.0, 127.7, 130.9, 133.2, 141.3, 143.7, 149.4 (d, J_{PC} 6.62 Hz) 155.7, 172.91, ^{31}P nmr δ($CHCl_3$): -4.6, FAB-MS (Ar, +ve mode) m/z 512 (MH^+, 35 %), 490 (5), 412 (8), 354 (12), 334 (53), 316 (55), 290 (45), 270 (18).

14. Wu, C-R., Wade, J. D. and Tregear, G. W., Int. J. Peptide Protein Res., 31, 47, (1988). The solid phase synthesis was performed semi-manually using a Cambridge Research Biochemicals Ltd. Pepsynthesizer.

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16. Fmoc-Asn was coupled as its pentafluorophenyl ester.

17. Fmoc-Ile required a double couple with BOP/HOBt activation.

18. Although a model study whereby treatment of (1) with 20 % piperidine/DMF and monitoring the rate of reaction by ^{31}P nmr indicated that monodemethylation was taking place ($t_{1/2}$ 7 min), this was found not to retard the elongation of the peptide chain when BOP was used.

19. Amino acid analysis (6M HCl/0.1% phenol, 110°C, 24h): (i) crude peptide, Asx 1.86 (2), Thr 1.06 (1), Glx 3.16 (3), Gly 1.13 (1), Ala 1.05 (1), Ile 0.95 (1), Leu 0.81 (1), Tyr 1.06 (1), Arg 1.92 (2), (ii) purified peptide, Asx 1.92 (2), Thr 1.00 (1), Glx 3.12 (3), Gly 1.05 (1), Ala 1.01 (1), Ile 0.97 (1), Leu 1.01 (1), Tyr 0.99 (1), Arg 1.95 (2).

(Received in UK 31 August 1989)