Preparation and Use of N^{α}-Fluorenylmethoxycarbonyl-*O*-dibenzylphosphono-L-tyrosine in Continuous Flow Solid Phase Peptide Synthesis

Eric A. Kitas,[a,b] John D. Wade,*[a] R. B. Johns,[b] John W. Perich[b] and Geoffrey W. Tregear[a]

[a] *Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, Victoria 3052, Australia*

[b] *Department of Organic Chemistry, University of Melbourne, Parkville, Victoria 3052, Australia*

N^{α}-Fluorenylmethoxycarbonyl-*O*-dibenzylphosphono-L-tyrosine is a useful derivative for the solid phase synthesis of *O*-phosphotyrosine peptides.

The phosphorylation of tyrosine residues in proteins is a fundamental mechanism for regulating diverse cellular processes.[1,2] This has led to a need for an efficient and facile procedure for the preparation of *O*-phosphotyrosine peptides. We have described recently the preparation and use of N^{α}-fluorenylmethoxycarbonyl-*O*-dimethylphosphono-L-tyrosine, Fmoc–Tyr(PO_3Me_2)–OH, in Fmoc–polyamide solid phase peptide synthesis.[3] Simultaneous *O*-phosphate demethylation and peptide-resin cleavage is afforded by treatment with trimethylsilyl bromide (TMSBr) in trifluoroacetic acid (TFA).[4] More recent work in our laboratory has focused on the development of a derivative which permits the generation of *O*-phosphotyrosine peptides using short deprotection times and preferably with mild acids such as TFA.

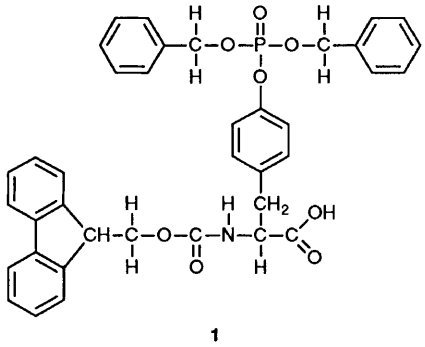
The preparation of *tert*-butoxycarbonyl-*O*-dibenzylphosphono-L-tyrosine, Boc–Tyr(PO_3Bzl_2)–OH, and its use in the Boc–polystyrene method of solid phase synthesis has recently been reported.[5,6] It was observed that the final treatment with liquid hydrogen fluoride gave rise to a high level of dephosphorylated product. In addition, the use of 40% TFA–CH_2Cl_2 for N^{α}-deprotection caused significant *O*-debenzylation thus rendering it an inappropriate derivative for this mode of synthesis. However, such lability to TFA suggested that the derivative may be suitable for use in the Fmoc–polyamide synthesis procedure.

Fmoc–Tyr(PO_3Bzl_2)–OH **1** was prepared in modest yield (64%) by one pot phosphite triester phosphorylation of Fmoc–Tyr–OTBDMS (TBDMS, *tert*-butyldimethylsilyl) using dibenzyl *N,N*-diethylphosphoramidite[7] followed by *tert*-butyl hydroperoxide oxidation of the dibenzyl phosphite triester intermediate. Removal of the silyl ester was by aqueous acetic acid treatment. The structure of **1** was confirmed by ^{13}C NMR spectroscopy, elemental analysis and positive ion FAB-mass spectroscopy [*m/z* 686 $(M + Na)^+$], and its purity by reversed-phase HPLC; $[\alpha]_D^{18}$ +53.8° (*c* 1.2, $CHCl_3$).

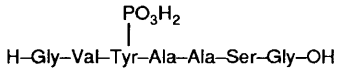
Use of the new tyrosine derivative in solid phase synthesis is illustrated by assembly of the heptapeptide **2** which consists of a sequence[8] of the viral protein $p85^{gag-fes}$. The continuous flow variant[9] of the Fmoc–polyamide method was used. Synthesis was carried out manually on low-loading (0.1 mmol g^{-1}) poly(dimethylacrylamide)–kieselguhr (Pepsyn K, CRB) which had been functionalized with an internal reference amino acid, acid labile handle and *C*-terminal glycine residue as previously described.[10] Peptide bond forming reactions utilized the appropriate N^{α}-Fmoc–amino acid (3 equiv.) in dimethylformamide (DMF) together with benzotriazol-1-oxytris(dimethylamino)phosphonium hexfluorophosphate (BOP), 1-hydroxybenzotriazole (HOBt) and *N*-methylmorpholine (3:3:4.5 equiv.) and 25 minute acylation times. Asparagine was incorporated as its *o*-nitrophenyl ester (3 equiv.) in the presence of an equivalent amount of catalyst, HOBt. A one hour coupling time was used. N^{α}-Fmoc deprotection was carried out by treatment with 20% piperidine in DMF for 10 min.

Peptide-resin was treated with 95% TFA–5% thioanisole for 2 hours at room temperature. Reversed-phase HPLC [Fig. 1(*a*)] indicated the presence of a minor impurity corresponding to approximately 9% by weight of the total product. Peptide from each of the two peaks gave similar and excellent amino acid analysis results. However their positive ion FAB-mass spectra differed. The major product displayed a molecular ion at *m/z* 704 which corresponded to the calculated molecular weight of the *O*-phosphotyrosine containing peptide. The spectrum of the minor product showed a molecular ion at *m/z* 624 which is indicative of dephosphorylated peptide. A similar result was obtained when the peptide-resin was treated with 1 mol dm^{-3} TMSBr–PhSMe–TFA for 1 hour at 0 °C. This suggests that the tyrosine–phosphate ester bond may not be completely stable to TFA. Curiously, this lability is not observed when *O*-dimethylphospho protection is used under similar cleavage conditions.[3,11]

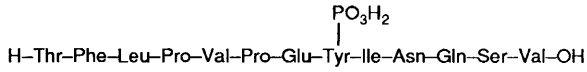
Previous work in our laboratories showed that one of the two methyl protecting groups of Tyr(PO_3Me_2) was removed by the strong nucleophile, piperidine, during Fmoc-solid phase synthesis.[3] The exposed monomethyl phosphate group theoretically could take part in a side reaction, for example, the formation of a mixed anydride at this site with the incoming amino acid.[12] Although this side product is labile to further base treatment, its presence should nevertheless be avoided as it may consume activated Fmoc–amino acid, therefore decreasing the effective molar excess of amino acid for peptide bond formation. It was therefore necessary to determine if a similar problem occurred with Tyr(PO_3Bzl_2). Using Boc–Tyr(PO_3Bzl_2)–OH and ^{31}P NMR spectroscopy, it was observed that 20% piperidine in DMF caused rapid mono-debenzylation with a $t_{\frac{1}{2}}$ of 12 minutes. In contrast use of the non-nucleophilic base, 1,8-diazabicylo[5.4.0]undec-7-ene (DBU), at a concentration of 1 mol dm^{-3} (15.4%) in DMF,



1



2



3

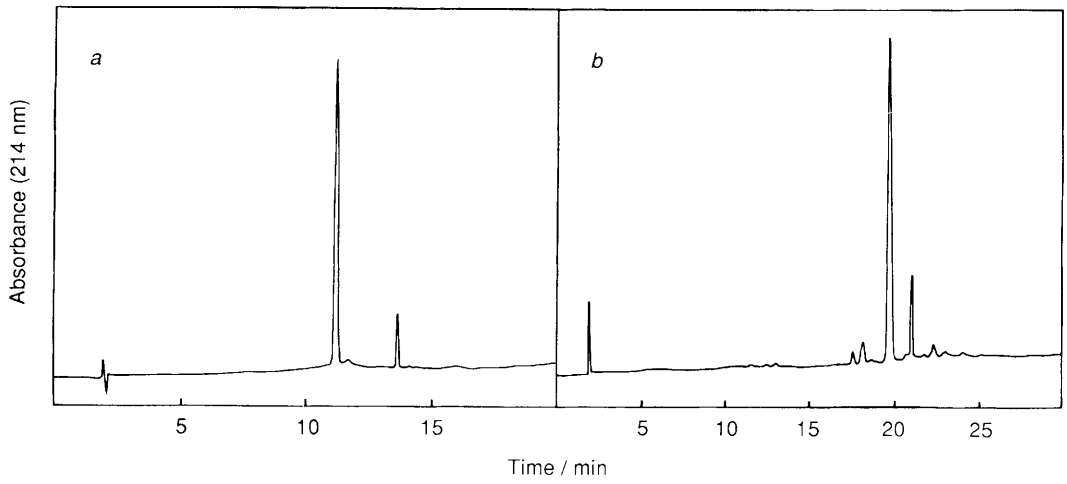


Fig. 1 HPLC of (*a*) crude *O*-phosphotyrosine heptapeptide **2** and (*b*) crude *O*-phosphotyrosine tridecapeptide **3**. *Conditions*: Brownlee RP-300 column; solvent A, 0.1% aq. TFA; solvent B, 0.1% TFA in acetonitrile. Flow rate, 1.5 ml min^{-1}. Gradient in (*a*) was 0–40% B in 20 min; in (*b*), 5–50% B in 30 min.

caused only slow deprotection with a $t_{\frac{1}{2}}$ of 14 hours which augurs well for its use with **1**. The results of our preliminary studies have indicated that DBU in a concentration of 2% in DMF causes very rapid N^{α}-Fmoc deprotection with no apparent side reaction in solid phase peptide synthesis.

Peptide **3** which represents the primary autophosphorylation site of the EGF receptor kinase[13] was assembled fully automatically on a MilliGen 9050 peptide synthesizer as described above with the exception that benzotriazole-l-yloxytrispyrrolidinophosphonium hexafluorophosphate (pyBOP)[14] was used in place of BOP and 2% DBU–DMF employed for N^{α}-Fmoc deprotection. Crude product obtained on treatment of dry peptide-resin with 95% TFA–5% phenol for 1 hour at room temperature gave the reversed phase HPLC profile shown in Fig. 1(*b*). The major product was isolated in 56% yield and gave the following amino acid analysis after acid hydrolysis: Asx (1), 1.04; Thr(1), 1.02; Ser (1), 1.11; Glx(2), 2.15; Pro(2), 2.02; Val(2), 1.88; Ile(1), 0.95; Leu(1), 1.01; Tyr(1), 0.90; Phe(1), 0.98. Positive ion FAB-mass spectroscopy confirmed the presence of the *O*-phosphate moiety. The principal trailing impurity on HPLC again corresponded to the dephosphorylated peptide (*ca.* 7% by weight).

Our results indicate that Fmoc–Tyr(PO_3Bzl_2)–OH can be employed for the introduction of *O*-phosphotyrosine residues in continuous flow solid phase peptide synthesis in which DBU in DMF is the Fmoc-deprotecting reagent of choice. Impurities arising from instability of the hydrophilic *O*-phosphate moiety to the final cleavage reagent can be separated adequately from the target product by reversed-phase HPLC or ion exchange chromatography. Further studies are in progress to devise alternative deprotection strategies which avoid this problem.

This work is supported by an Institute Block grant from the National Health and Medical Research Council of Australia.

Received, 17th September 1990; Com. 0/04219F

References

1 P. J. Blackshear, A. C. Nairn and J. F. Kuo, *FASEB J.*, 1988, **2**, 2957.
2 A. Ullrich and J. Schlessinger, *Cell*, 1990, **61**, 203.
3 E. A. Kitas, J. W. Perich, J. D. Wade, R. B. Johns and G. W. Tregear, *Tetrahedron Lett.*, 1989, **30**, 6229.
4 S. Funakoshi, E. Murayama, L. Guo, N. Fujii and H. J. Yajima, *J. Chem. Soc., Chem. Commun.*, 1988, 382.
5 B. W. Gibson, A. M. Falick, A. L. Burlingame, L. Nadasdi, A. C. Nguyen and G. L. Kenyon, *J. Am. Chem. Soc.*, 1987, **109**, 5343.
6 R. M. Valerio, P. F. Alewood, R. B. Johns and B. E. Kemp, *Int. J. Pept. Protein Res.*, 1989, **33**, 428.
7 J. W. Perich and R. B. Johns, *Aust. J. Chem.*, 1990, **43**, 1623.
8 M. Shibuya and H. Hanafusa, *Cell*, 1982, **30**, 757.
9 A. Dryland and R. C. Sheppard, *Tetrahedron*, 1988, **44**, 858.
10 C.-R. Wu, V. C. Stevens, G. W. Tregear and J. D. Wade, *J. Chem. Soc., Perkin Trans. 1*, 1989, 81.
11 E. A. Kitas, J. W. Perich, G. W. Tregear and R. B. Johns, *J. Org. Chem.*, 1990, **55**, 4181.
12 R. Ramage, D. Hopton, M. J. Parrot, R. S. Richardson, G. W. Kenner and G. A. Moore, *J. Chem. Soc., Perkin Trans. 1*, 1985, 461.
13 J. Downward, P. Parker and M. D. Waterfield, *Nature*, 1984, **311**, 483.
14 J. Coste, D. Le-Nguyen and B. Casto, *Tetrahedron Lett.*, 1990, **31**, 205.