

# The synthesis of phosphopeptides *via* the Bpoc-based approach

Troy J. Attard,<sup>\*†a</sup> Eric C. Reynolds<sup>b</sup> and John W. Perich<sup>a</sup>

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The 2-(*p*-biphenyl)-2-propyloxycarbonyl (Bpoc) group was examined as an N<sup>α</sup>-protecting group in the stepwise assembly of the MAP Kinase ERK2 [178–188; Thr(*P*)<sup>183</sup>, Tyr(*P*)<sup>185</sup>] peptide. The mild acid deprotection of the Bpoc group permitted (i) incorporation of a fully protected phosphothreonyl derivative and (ii) a TFA-based final cleavage step. The first five C-terminal residues (184–188) were incorporated in the Fmoc mode of peptide synthesis, with all subsequent amino acids coupled as their Bpoc–Xxx–OH derivatives. The target product was obtained in high purity and yield, indicating that a Bpoc-based approach to phosphopeptide synthesis was compatible with both the acid-labile side chain protecting groups employed and Hmp–Wang resin.

## Introduction

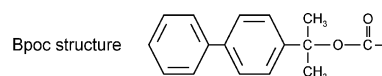
Protein phosphorylation is the most prevalent form of post-translational modification in eukaryotic organisms and plays important roles in processes such as protein trafficking,<sup>1</sup> enzyme activation<sup>2</sup> and extracellular signal transmission.<sup>3</sup> The study of systems involving protein phosphorylation/dephosphorylation has benefited significantly from the availability of synthetic phosphopeptides with which to probe consensus sequences or substrate specificities.<sup>4,5</sup> Recent developments in synthetic strategies for the preparation of biologically significant phosphopeptides have proved to be effective for simple or monophosphorylated peptides, though synthetic difficulties have been encountered in cases of more complex or multiphosphorylated peptides.<sup>6–8</sup>

Two general strategies are currently employed for solid phase phosphopeptide synthesis: (a) the post-synthetic ('global') phosphorylation of the peptidyl target site, or (b) the incorporation of a pre-phosphorylated amino acid derivative into the growing peptide chain. A particular limitation of the latter approach is the preclusion of fully protected phosphothreonyl and -seryl derivatives from Fmoc-based syntheses due to the propensity of the phosphorylated side chains to undergo base-catalysed β-elimination during peptide assembly. While the use of Fmoc–Xxx(PO<sub>3</sub>Bzl,H)–OH (Xxx = Ser, Thr) derivatives prevents β-elimination by ionization of the phosphoryl oxygen, this method is complicated by reports of incomplete acylation during the coupling step, particularly in the case of Fmoc–Thr(PO<sub>3</sub>Bzl,H)–OH.<sup>9</sup>

In view of these limitations, we have examined an alternative approach to phosphopeptide synthesis using the highly acid-labile

Bpoc group for N<sup>α</sup>-protection. Since this method enables mild acidolytic N<sup>α</sup>-deprotection, it was envisaged that (a) Hmp–Wang resin could be employed without hydrolysis of the peptide-resin linkage, and (b) fully protected phosphothreonyl derivatives could be incorporated into the growing peptide.

The synthesis of MAP Kinase ERK2 [178–188; Thr(*P*)<sup>183</sup>, Tyr(*P*)<sup>185</sup>], HTGFLT(*P*)EY(*P*)VAT (Scheme 4) (**14**) was chosen to examine this method since the generation of phosphothreonyl peptides have proven more difficult than for the corresponding seryl or tyrosyl substrates.<sup>10</sup> Furthermore, the application of standard protocols for the assembly and subsequent global phosphorylation of this particular sequence was reported to be problematic by Johnson *et al.*,<sup>8</sup> therefore providing an ideal substrate to evaluate the efficacy of the Bpoc-based approach to phosphopeptide preparation.



## Results

### Synthesis of MAP Kinase ERK2 [178–184; Thr(*P*)<sup>183</sup>], HTGFLT(*P*)E (Scheme 2) (**4**)

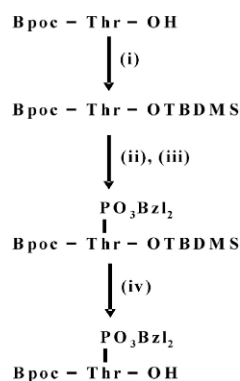
The first stage of this study was to determine the compatibility of the Bpoc method with the building block approach to phosphopeptide synthesis. The synthesis of the shorter MAP Kinase [178–184; Thr(*P*)<sup>183</sup>] peptide, HTGFLT(*P*)E (**4**), was selected to examine the incorporation of the Bpoc–Thr(PO<sub>3</sub>Bzl<sub>2</sub>)–OH derivative in order to ascertain the coupling efficiency and subsequent stability of the phosphothreonyl residue under these synthetic conditions.

The requisite Bpoc–Thr(PO<sub>3</sub>Bzl<sub>2</sub>)–OH derivative was prepared *via* the initial synthesis of Bpoc–Thr–OH followed by its 'one-pot' phosphorylation,<sup>11</sup> according to Scheme 1. Phosphorylation was effected using temporary *t*-butyldimethylsilyl (TBDMS) protection of the Bpoc–Thr–OH carboxyl group followed by phosphitylation with (BzlO)<sub>2</sub>PN<sup>i</sup>Pr<sub>2</sub>–1-*H*-tetrazole and subsequent oxidation of the dibenzyl phosphite triester with <sup>1</sup>BuOOH. Deprotection of the carboxyl group was achieved by treatment with 10% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>.

<sup>a</sup>Dept of Chemistry, University of Melbourne, Victoria, Australia 3010; Fax: +61 (03) 8344 5104; Tel: +61 (03) 8344 4000

<sup>b</sup>Cooperative Research Centre for Oral Health Science, School of Dental Science and the Bio21 Institute of Molecular Science and Biotechnology, The University of Melbourne, 30 Flemington Rd, Melbourne, Australia, 3010; Fax: +61 (03) 83442545; Tel: +61 (03) 83442562

<sup>†</sup>Current address: Cooperative Research Centre for Oral Health Science, School of Dental Science and the Bio21 Institute of Molecular Science and Biotechnology, The University of Melbourne, 30 Flemington Rd, Melbourne, Australia 3010; Fax: +61 (03) 83442545; Tel: +61 (03) 83442562; tjattard@unimelb.edu.au

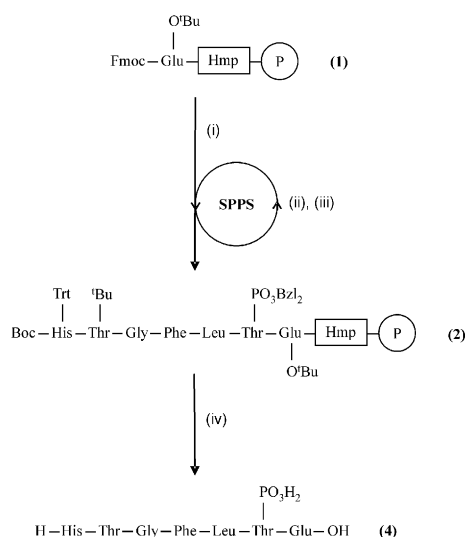


**Scheme 1** Reagents and conditions: (i) TBDMSCl (0.9 eq.)–NMM (1 eq.) in THF (3 min), (ii) (BzlO)<sub>2</sub>PN<sup>i</sup>Pr<sub>2</sub> (1.2 eq.)–1*H*-tetrazole (3 eq.) (2 h), (iii) 14% <sup>t</sup>BuOOH (30 min), (iv) 10% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> (30 min).

(pH 4.0) and the product extracted using 5% NaHCO<sub>3</sub> (pH 9.0) to give the pure Bpoc-Thr(PO<sub>3</sub>Bzl<sub>2</sub>)–ONa salt in 63% yield.

The <sup>13</sup>C NMR profile of the final product gave signals for Thr(*P*) C<sub>α</sub>, Thr(*P*) C<sub>β</sub> and benzyl CH<sub>2</sub> as broad singlets at 59.9, 69.4 and 80.2 ppm respectively. The aromatic C1-carbon of the benzyl phosphate group was detected as a phosphorous-coupled doublet at 135.7 ppm with a coupling constant of *J*<sub>PC</sub> 6.5 Hz. The spectrum showed no evidence of any Bpoc-Thr–ONa compound in the final product since the characteristic peaks for the Thr–C<sub>α</sub> and Thr–C<sub>β</sub> carbons at 58.9 and 67.7 ppm, respectively, were absent. The phosphorylation of the protected threonyl synthon was confirmed by its <sup>31</sup>P NMR spectra which gave a major signal at –2.10 ppm in ~98% purity. The remaining Bpoc-protected amino acids were synthesized according to the procedure outlined by Carey *et al.*<sup>12</sup> in 51–74% yields.

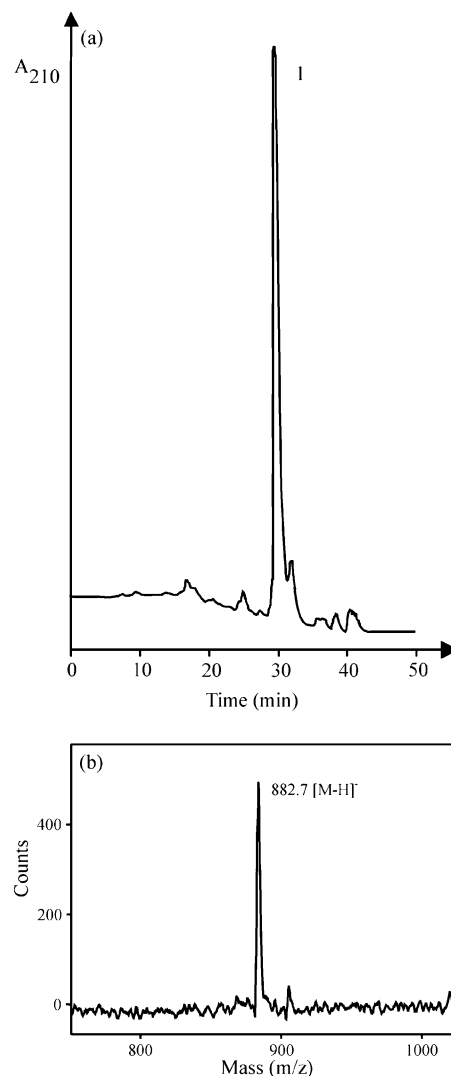
The synthesis of HTGFLT(*P*)E (Scheme 2) (4) was accomplished by peptide extension from H–Glu(O<sup>t</sup>Bu)–Wang with all Bpoc amino acids and Boc–His(Trt)–OH incorporated as the free acids, using a 1% TFA–DCM solution for N-terminal deprotection. Upon completion of chain assembly, the peptide-



**Scheme 2** Reagents and conditions: (i) 25% piperidine–DMF (20 min), (ii) Bpoc-amino acid (3 eq.)–HBTU (3 eq.)–NMM (9 eq.) (1 h) [Final coupling: Boc–His(Trt)–OH], (iii) 1% TFA–DCM (20 min), (iv) TFA–TES–H<sub>2</sub>O (95 : 2.5 : 2.5, v/v/v, 6 h).

resin (2) was cleaved by the addition of TFA–TES–H<sub>2</sub>O (95 : 2.5 : 2.5, v/v/v) for 6 h and the crude peptide (3) isolated by ethereal precipitation.

The RP-HPLC profile of the crude peptide (3) (Fig 1a) showed one major signal (peak 1) at 29.4 min, which gave a single peak by MALDI-MS corresponding to the target phosphopeptide (4) at *m/z* 882.7 [M–H]<sup>–</sup> (Fig 1b). Importantly, the mass spectrum of the crude peptide (3) showed no evidence of a phosphothreonyl deletion at *m/z* 700.8 [M–H]<sup>–</sup>, indicating quantitative incorporation of the Bpoc–Thr(PO<sub>3</sub>Bzl<sub>2</sub>)–OH derivative. As expected, the high purity of the crude product also indicates that the benzyl phosphate protecting group is stable to the mild acidic conditions employed for Bpoc group removal.

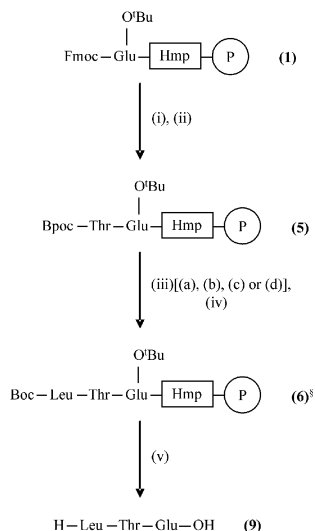


**Fig. 1** Crude MAP Kinase ERK2 [178–184; Thr(*P*)<sup>183</sup>] (3), (a) RP-HPLC, (b) MALDI-MS (peak 1).

### Examination of Bpoc group acid lability

The next stage of this study was the determination of the minimum acid requirement for the removal of the Bpoc group. While Kemp *et al.*<sup>13</sup> has shown that 0.5% TFA–DCM is sufficient for deprotection within 10 min, Bpoc removal by an acid with a lower

$pK_a$  is preferable for the stability of the resin linkage and side-chain protecting groups. Formic acid–DCM was examined for suitability as a deprotection solution in the synthesis of the MAP Kinase ERK2 (182–184) peptide, H–Leu–Thr–Glu–OH (Scheme 3) (9), in which the Thr(183) residue was coupled as the Bpoc–Thr–OH derivative. Fmoc–Glu(O<sup>t</sup>Bu)–Wang resin (1) was treated with 20% piperidine–DMF for 20 min and coupled to Bpoc–Thr–OH for 1 h. Deprotection trials were then performed on the Bpoc–Thr–Glu(O<sup>t</sup>Bu)–Wang peptide-resin (5) using 5%, 10%, 15% and 20% formic acid–DCM solutions (20 min). Bpoc–Leu–OH was subsequently coupled to each of the four deprotected dipeptides and the protected peptide-resins (6)<sup>‡</sup> cleaved with TFA–TES–H<sub>2</sub>O (95 : 2.5 : 2.5, v/v/v) for 1 h. RP-HPLC and MALDI-MS analysis of the resultant crude peptide (7)<sup>‡</sup> from each trial showed that the H–Thr–Glu–OH dipeptide (8) eluted at 8.6 min {peak 1 ( $m/z$  247.3, [M–H]<sup>–</sup>)} followed by the target tripeptide (9) at 19.3 min {peak 2 ( $m/z$  360.6 [M–H]<sup>–</sup>)} (Fig 2). Notably, the dipeptide (8) resulting from incomplete Bpoc removal is absent in the 15% and 20% formic acid trials, indicating that formic acid concentrations of 15% or greater are sufficient for the removal of the Bpoc protecting group within 20 min.

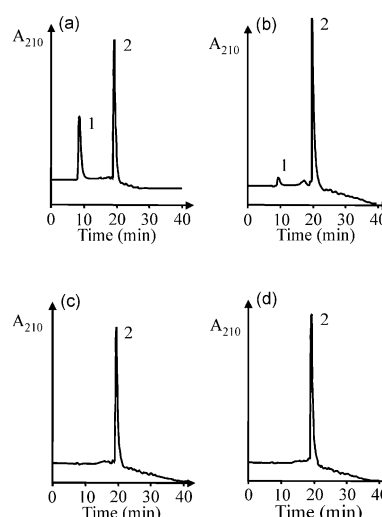


**Scheme 3** Reagents and conditions: (i) 25% piperidine–DMF (20 min), (ii) Bpoc–Thr–OH (3 eq.)–HBTU (3 eq.)–NMM (9 eq.) (1 h), (iii) formic acid–DCM [(a) 5%, (b) 10%, (c) 15% or (d) 20%; 20 min], (iv) Boc–Leu–OH (3 eq.)–HBTU (3 eq.)–NMM (9 eq.) (1 h), (v) TFA–TES–H<sub>2</sub>O (95 : 2.5 : 2.5, v/v/v; 1 h).

### Synthesis of MAP Kinase ERK2 [178–188; Thr(P)<sup>183</sup>, Tyr(P)<sup>185</sup>], HTGFLT(P)EY(P)VAT (Scheme 4) (14)

Following the successful synthesis of HTGFLT(P)E (4) *via* Bpoc–Thr(PO<sub>3</sub>Bzl<sub>2</sub>)–OH incorporation, this method was applied to the extended bis-phosphorylated peptide from the MAP Kinase ERK2 protein, HTGFLT(P)EY(P)VAT (14) for all residues subsequent to the glutamyl residue. Since fully protected phosphotyrosyl derivatives are not susceptible to  $\beta$ -elimination by piperidine, the first five residues in the sequence were incorporated as their Fmoc derivatives.

<sup>‡</sup> Fully assembled peptide-resins resulting from each of the four formic acid trials were all designated (6) for simplicity of discussion. Similarly, the corresponding crude peptides were all designated (7).

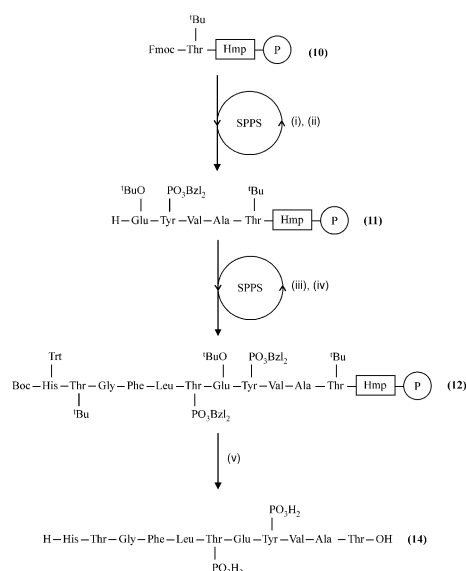


**Fig. 2** Crude MAP Kinase ERK2 [182–184] (7) RP-HPLC profile from formic acid–DCM deprotections of Bpoc–Thr–Glu(O<sup>t</sup>Bu)–Wang (5): (a) 5% formic acid–DCM, (b) 10% formic acid–DCM, (c) 15% formic acid–DCM, (d) 20% formic acid–DCM.

The Fmoc–Tyr(PO<sub>3</sub>Bzl<sub>2</sub>)–OH synthon was prepared using temporary phenacyl carboxyl protection as described by Valerio *et al.*<sup>14</sup> Fmoc–Tyr–OPac was phosphorylated by treatment with (BzlO)<sub>2</sub>PN<sup>i</sup>Pr<sub>2</sub>–1*H*-tetrazole followed by oxidation with mCPBA. The phenacyl group was removed by addition of Fmoc–Tyr(PO<sub>3</sub>Bzl<sub>2</sub>)–OPac to a solution of Zn–EtOAc–AcOH–H<sub>2</sub>O (0.3 : 2 : 5 : 1, w/v/v/v) for 2 h and the Fmoc–Tyr(PO<sub>3</sub>Bzl<sub>2</sub>)–ONa salt extracted with 5% NaHCO<sub>3</sub> (pH 8.5). The combined base extracts were acidified to pH 2.5 using solid citric acid, the product extracted with DCM, and subsequent solvent evaporation under reduced pressure gave Fmoc–Tyr(PO<sub>3</sub>Bzl<sub>2</sub>)–OH as a crispy white foam in 73% yield.

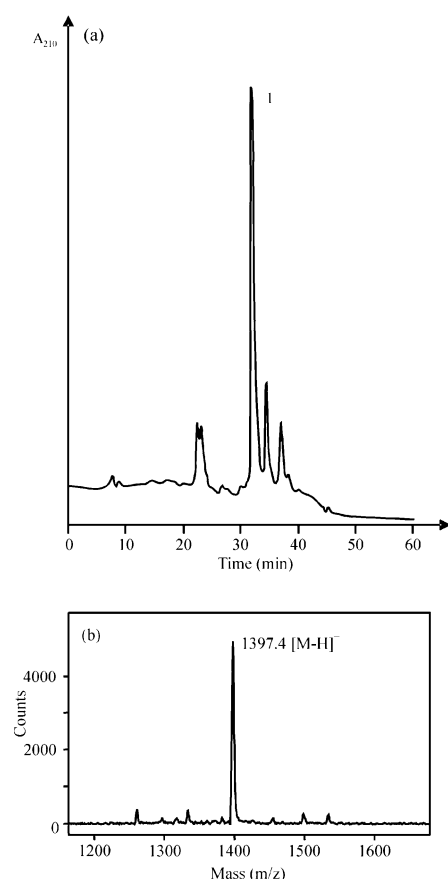
The MAP Kinase ERK2 [178–188; Thr(P)<sup>183</sup>, Tyr(P)<sup>185</sup>] peptide (Scheme 4) (14) was prepared by extension from Fmoc–Thr(<sup>t</sup>Bu)–Wang Resin (10) in the Fmoc mode of peptide synthesis for the first five C-terminal residues. Following coupling of Bpoc–Thr(PO<sub>3</sub>Bzl<sub>2</sub>)–OH to H–Glu(O<sup>t</sup>Bu)–Tyr(PO<sub>3</sub>Bzl<sub>2</sub>)–Val–Ala–Thr(<sup>t</sup>Bu)–Wang, the deprotection of all intermediate N-terminal residues was achieved using 20% formic acid–DCM for 20 min. At the completion of peptide synthesis, peptide-resin (12) was cleaved by the addition of TFA–TES (95 : 5, v/v) for 6.5 h and the crude peptide (13) isolated by ethereal precipitation.

Analysis by RP-HPLC gave a crude peptide spectrum of high purity with a major peak at 32.3 min (Fig 3a). MALDI-MS analysis of the main signal showed a single peak corresponding to the target bis-phosphorylated peptide (14) ( $m/z$  1397.4, [M–H]<sup>–</sup>) (Fig 3b). Analysis of the crude peptide by MALDI-MS showed no signal for either Thr(P) or Tyr(P) deletions, suggesting full incorporation of the phosphorylated derivatives. Interestingly, examination of both the crude peptide (13) and the major peak from its RP-HPLC profile showed no signal for any contaminating H-phosphonate peptide. This byproduct has been observed for various global phosphorylations,<sup>6,15–18</sup> and arises from the acid-catalysed rearrangement of the intermediate phosphite triester under concentrated reaction conditions during the phosphitylation step. The absence of this undesired byproduct in the current study was attributed to the lower concentration of 1*H*-tetrazole in the



**Scheme 4** Reagents and conditions: (i) 25% piperidine-DMF (20 min), (ii) Fmoc-aa (3 eq.)-HBTU (3 eq.)-NMM (9 eq.) (1 h), (iii) Bpoc-aa (3 eq.)-HBTU (3 eq.)-NMM (9 eq.) (1 h) [final coupling: Boc-His(Trt)-OH], (iv) 20% formic acid-DCM (20 min), (v) TFA-TES (95 : 5, v/v, 6.5 h).

phosphitylation step during synthesis of the Bpoc-Thr( $\text{PO}_3\text{Bzl}_2$ )-OH and Fmoc-Tyr( $\text{PO}_3\text{Bzl}_2$ )-OH derivatives, in comparison with that required for solid phase phosphorylations.



**Fig. 3** Crude MAP Kinase ERK2 [178–188; Thr( $P$ )<sup>183</sup>, Tyr( $P$ )<sup>185</sup>] (**13**), (a) RP-HPLC, (b) MALDI-MS (peak 1).

## Discussion

An important feature of the Bpoc approach is that fully protected phosphorylated threonyl derivatives may be directly incorporated into the growing peptide chain, with  $N^{\alpha}$ -deprotections effected using a 20% formic acid-DCM solution. This deprotection strategy circumvents complications associated with base-catalysed  $\beta$ -elimination of the protected phosphate group. Another major advantage of this deprotection method is that TFA-labile side chain protecting groups and resin linkages may be employed, allowing the same mild acidic conditions that are utilized in Fmoc solid phase syntheses for the final cleavage step. Formic acid was chosen for Bpoc removal due to its lower  $pK_a$  compared with TFA, and is therefore expected to be more compatible for syntheses of longer peptide chains.

For single Ser( $P$ )- or Thr( $P$ )-containing peptides, the global phosphorylation method provides a convenient route to the preparation of the monophosphorylated peptide, however long or ‘difficult’ peptide sequences may render this approach ineffective due to the inaccessibility of phosphorylation reagents to the target site. It has also been established that increasing phosphorylation reagent concentrations to overcome this limitation is directly related to an increase in H-phosphonate generation.<sup>18,19</sup> In such cases, a Bpoc-based approach would improve the purity of the final product with respect to non-phosphorylated or H-phosphonylated peptide byproducts and allow easier purification of the crude sample in the absence of these contaminants.

The high purity of the crude MAP Kinase ERK2 [178–188; Thr( $P$ )<sup>183</sup>, Tyr( $P$ )<sup>185</sup>] peptide (**13**) (Fig 3a) indicates that the Bpoc methodology is also compatible with an Fmoc strategy when used in combination for the same synthesis. In this case, the phosphotyrosyl residue is located to the C-terminal side of the phosphothreonyl residue, enabling the Fmoc approach for the first five residues. While Fmoc-Tyr( $\text{PO}_3\text{R}_2$ )-OH derivatives have been successfully used in the Fmoc mode of synthesis,<sup>14,20–23</sup> a Bpoc protected phosphotyrosyl synthon may also conceivably be utilized if the phosphotyrosyl residue occurs on the N-terminal side of a phosphothreonyl or -seryl residue.

We anticipate that a Bpoc-based approach to phosphopeptide synthesis would also facilitate the coupling of phosphorylated derivatives in multiphosphorylated peptide chains. While the development of Fmoc-Xxx( $\text{PO}_3\text{Bzl}_2$ )-OH (Xxx = Tyr, Thr, Ser) derivatives has allowed the stepwise incorporation of phosphothreonyl and -seryl derivatives in the Fmoc mode, this approach is limited by the incomplete incorporation of the partially protected derivative, caused by ionization of the phosphorylated moiety during the coupling step.<sup>9</sup> This problem may therefore be avoided through the use of Bpoc-Xxx( $\text{PO}_3\text{Bzl}_2$ )-OH synthons in the Bpoc mode of peptide synthesis using 20% formic acid for  $N^{\alpha}$ -deprotection on account of the stability of the benzyl phosphate groups to such mild acid treatment. In regard to storage, it is recommended that the phosphorylated Bpoc derivatives be stored as their sodium salts at  $-20^\circ\text{C}$  or used immediately to avoid loss of the highly labile Bpoc group over extended periods of time.

## Conclusions

The Bpoc approach to phosphopeptide synthesis provides an efficient method for the generation of phosphopeptides that are free



from non-phosphorylated or H-phosphonylated contaminants. The mild acidolytic N<sup>α</sup>-deprotection conditions permit the use of TFA-labile side chain protection and resin linkages, allowing a final cleavage step using TFA solutions.

## Experimental

### General

Fmoc- and Boc-amino acids, HBTU, NMM and TFA were purchased from Auspep and stored at 4 °C. Solvents were of AnalaR grade with THF distilled from the potassium ketyl of benzophenone prior to use. DMF, DCM, dibenzyl *N,N*-diisopropylphosphoramidite, 1*H*-tetrazole, triethylsilane and phenacyl bromide were obtained from Sigma–Aldrich Inc.

Solid phase peptide synthesis was performed manually on Hmp–Wang resin using a solid phase reaction vessel attached to a rotation instrument. The resin was suspended in a DMF–DCM (1 : 1, v/v) solution for 30 min prior to the commencement of peptide synthesis. Acylation was achieved by the addition of a mixture of amino acid (3 eq.), HBTU (3 eq.) and NMM (9 eq.) in a minimum volume§ of DMF–DCM (1 : 1, v/v) to the N<sup>α</sup>-deprotected peptide-resin and rotated for 1 h (see below for specific N<sup>α</sup>-deprotection protocols). The peptide-resin was then filtered by suction and rinsed with DCM (2 ml, 3 × 2 min). Cleavage of peptide-resins was performed in a solid phase reaction vessel, the combined cleavage filtrates evaporated to dryness by rotary evaporation and the crude product isolated by ethereal precipitation (4 × 30 ml).

Analytical RP-HPLC was performed using a Waters liquid chromatograph instrument (model 510) equipped with a UV detector (model 420.AC) and a Zorbax 300 SB-C<sub>18</sub> reversed phase column (4.6 mm × 25 cm). Peptide separation was achieved using a linear acetonitrile gradient in 0.1% TFA (aq) at a flow rate of 1 ml min<sup>−1</sup>. Semi-preparative RP-HPLC was performed on a Perkin Elmer 200 Series liquid chromatograph instrument with a Zorbax 300 SB-C<sub>18</sub> reversed phase column (9.4 mm × 25 cm) linked to a fluorescence detector (model ABI 783A) and a chart recorder (model SE 120). Peptide separation was accomplished at a flow rate of 2 ml min<sup>−1</sup>. MALDITOF-MS data were obtained on a Voyager-DE MALDI bench top linear mass spectrometer. Samples were prepared in a saturated α-cyano hydroxycinnamic acid solution [solvent: MeCN–H<sub>2</sub>O–CHOOH (33 : 66 : 1, v/v/v)]. <sup>13</sup>C NMR spectra were recorded on a Unity-300 fourier transform instrument operating at 300 and 75 MHz respectively with chemical shifts in ppm relative to internal CDCl<sub>3</sub> adjusted to 77.0 ppm (central peak). <sup>31</sup>P NMR data were obtained relative to internal H<sub>3</sub>PO<sub>4</sub> (85%) adjusted to 0.0 ppm.

### General procedure for the synthesis of Bpoc–Xxx–OH [Xxx = Thr('Bu), Gly, Phe, Leu]

(*p*-Biphenyl)-2-propyloxycarbonylphenyl carbonate (BpocOPh) was prepared in 98.3% yield using the procedure described by Kemp *et al.*<sup>13</sup> The preparation of all Bpoc-amino acids was performed according to the procedure outlined by Carey *et al.*<sup>12</sup>

with the following exceptions: a solution of Bpoc–OPh (5.0 mmol), L-amino acid (5.0 mmol) and tetramethylguanidine (15 mmol) in DMF (3.5 ml) was stirred at 50 °C for 48 h. The solvent was evaporated under reduced pressure and the resultant oil dissolved in 5% NaHCO<sub>3</sub> (15 ml), transferred to a separation funnel and washed with ether (2 × 30 ml). The aqueous layer was collected and acidified to pH 6.5 (20% NaHSO<sub>4</sub>), washed with ether (2 × 40 ml), acidified to pH 2.5 and the product extracted with DCM (3 × 35 ml). Evaporation of the solvent gave the Bpoc–Xxx–OH products in 51%–74% yields.

### Synthesis of Bpoc–Thr(PO<sub>3</sub>Bzl<sub>2</sub>)–OH

*N*-Methylmorpholine (101 mg, 1.0 mmol) in anhydrous THF (0.8 ml) and *t*-butyldimethylsilyl chloride (136 mg, 0.9 mmol) in THF (0.8 ml) were added successively to a solution of Bpoc–Thr–OH (356 mg, 1.0 mmol) in THF (1 ml) at room temperature. After 3 min, 1*H*-tetrazole (105 mg, 1.5 mmol) was added to the mixture, followed by the addition of (BzlO)<sub>2</sub>PN<sup>i</sup>Pr<sub>2</sub> (415 mg, 1.2 mmol). After 2 h, the mixture was cooled to −20 °C and 14% <sup>t</sup>BuOOH (0.9 ml) added for 30 min. The THF was evaporated under reduced pressure and the reaction mixture transferred to a separation funnel by the addition of ether (8 ml). An aqueous solution of 10% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> (pH 4.5, 6 ml) was added at 10–15 °C and the mixture stirred rapidly for 20 min. The organic layer was collected, washed with 10% Na<sub>2</sub>SO<sub>4</sub> (2 × 6 ml) and the product extracted with 5% NaHCO<sub>3</sub> (pH 8.5; 3 × 4 ml). The combined aqueous extract was evaporated under reduced pressure and the resultant oil dissolved in DCM, filtered and the solvent evaporated to give Bpoc–Thr(PO<sub>3</sub>Bzl<sub>2</sub>)–ONa in 402 mg (62.9% yield). The final product, Bpoc–Thr(PO<sub>3</sub>Bzl<sub>2</sub>)–OH was generated prior to peptide synthesis by dissolution in EtOAc (6 ml), washing with 10% citric acid (10 ml) and solvent evaporation. <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 18.8, 27.9, 30.2, 59.9 (br s, Thr C<sub>α</sub>), 69.4 (br s, Thr C<sub>β</sub>), 80.2 (br s, Bzl CH<sub>2</sub>), 124.7, 126.7, 127.0, 127.6, 127.9, 128.1, 128.4, 128.5, 135.7 (d, *J*<sub>PC</sub> 6.5 Hz, Bzl C1), 139.2, 140.9, 145.9, 155.7, 176.0 ppm. <sup>31</sup>P NMR (85% H<sub>3</sub>PO<sub>4</sub>): δ −2.1 ppm.

### Preparation of MAP Kinase ERK2 [178–184; Thr(*P*)<sup>183</sup>; H–His–Thr–Gly–Phe–Leu–Thr(*P*)–Glu–OH (4)]

Fmoc–Glu(O<sup>t</sup>Bu)–Wang (1) (36 mg, 0.02 mmol) was suspended in 25% piperidine–DMF (1 ml) for 20 min and rinsed with DCM (5 × 1 ml). Subsequent amino acid couplings were performed by addition of Bpoc-amino acid (3 eq.)–HBTU (3 eq.)–NMM (9 eq.) in DCM–DMF (1 : 1, v/v) (1 h) with deprotection of the Bpoc N<sup>α</sup>-protecting group achieved by suspension of the peptide-resin in 1% TFA–DCM (1 ml) for 20 min (*N.B.* the phosphothreonyl residue was coupled as the Bpoc–Thr(PO<sub>3</sub>Bzl<sub>2</sub>)–OH acid). The N-terminal amino acid was incorporated as the Boc–His(Trt)–OH derivative. The resultant peptide-resin [Boc–His(Trt)–Thr('Bu)–Gly–Phe–Leu–Thr(PO<sub>3</sub>Bzl<sub>2</sub>)–Glu(O<sup>t</sup>Bu)–Wang] (2) was treated with TFA–TES–H<sub>2</sub>O (95 : 2.5 : 2.5, v/v/v; 1 ml) for 6 h and the crude peptide (3) (18 mg) isolated as described under General procedure (above). Analytical RP-HPLC [gradient: 0–50% MeCN in 50 min] gave one major peak (~90%) at 29.4 min. MALDI-MS (negative mode): *m/z* 882.7 [M–H]<sup>−</sup> (4).

§ Minimum volume is defined as the minimum amount of solvent required for adequate peptide-resin solvation during the coupling step. An increase in the minimum volume required was observed during progression of peptide assembly.

### Preparation of MAP Kinase ERK2 [182–184]; H–Leu–Thr–Glu–OH (9)

Fmoc–Glu(O<sup>t</sup>Bu)–Wang (1) (144 mg, 0.08 mmol) was suspended in 25% piperidine–DMF (1 ml) for 20 min and rinsed with DCM (5 × 1 ml). A mixture containing Bpoc–Thr–OH (3 eq.)–HBTU (3 eq.) and NMM (9 eq.) in DCM–DMF (1 : 1, v/v) was added to the H–Glu(O<sup>t</sup>Bu)–Wang peptide-resin for 1 h. The resultant Bpoc–Thr–Glu(O<sup>t</sup>Bu)–Wang peptide-resin (5) was divided into four equal portions (0.02 mmol each), and one of four solutions containing formic acid–DCM (5%, 10%, 15% or 20%, v/v; 1 ml) was added to each for 20 min. Each peptide-resin was rinsed with DCM (3 × 1 ml) and Boc–Leu–OH coupled as above. The resultant peptide-resins (6)† were suspended in a TFA–TES–H<sub>2</sub>O (95 : 2.5 : 2.5, v/v/v, 1 ml) solution (1 h) and the corresponding crude peptides (7)‡ isolated according to the general procedure (above). Analytical RP–HPLC gave peaks for H–Leu–Thr–Glu–OH (9) at 19.3 min and H–Thr–Glu–OH (8) at 8.6 min. MALDI–MS (negative mode): *m/z* 360.6, [M–H]<sup>–</sup> (9), *m/z* 247.3 [M–H]<sup>–</sup> (8).

### Synthesis of Fmoc–Tyr(PO<sub>3</sub>Bzl<sub>2</sub>)–OH

Triethylamine (203 mg, 2.0 mmol) in anhydrous THF (3 ml) and phenacyl bromide (398 mg, 2.0 mmol) in THF (3 ml) were added successively to a solution containing Fmoc–Tyr(<sup>t</sup>Bu)–OH (965 mg, 2.1 mmol) in THF (3 ml) and the mixture stirred for 8 h. The solvent was evaporated, the resultant foam redissolved in EtOAc (20 ml) and the organic phase washed with NaCl (sat) (2 × 20 ml), 10% citric acid (2 × 20 ml) and 0.1 M NaOH (2 × 20 ml). The organic phase was collected, filtered and evaporated under reduced pressure to give Fmoc–Tyr(<sup>t</sup>Bu)–OPac as a white powder (930 mg, 80.5% yield). Fmoc–Tyr(<sup>t</sup>Bu)–OPac (930 mg, 1.61 mmol) was then treated with TFA–H<sub>2</sub>O (95 : 5, 3 ml) at 20 °C for 1 h. Following evaporation of TFA, the resultant residue was triturated with EtOEt–petroleum spirits (1 : 1, 50 ml) and the Fmoc–Tyr–OPac powder filtered and dried (8 h) under reduced pressure. Fmoc–Tyr–OPac (800 mg, 1.53 mmol) was dissolved in anhydrous THF (2 ml) and (BzlO)<sub>2</sub>PN<sup>i</sup>Pr<sub>2</sub> (795 mg, 2.3 mmol) in THF (2 ml) and 1*H*-tetrazole (193 mg, 2.76 mmol) added successively. After 1 h, the reaction mixture was cooled to 0 °C and mCPBA (609 mg, 3.0 mmol) added for 30 min. The mixture was then transferred to a separation funnel with the addition of EtOAc (40 ml) and water (35 ml), the aqueous phase discarded and the organic layer washed with 10% citric acid (2 × 30 ml), 0.1M NaOH (30 ml) and 5% NaHCO<sub>3</sub> (2 × 30 ml). The EtOAc layer was collected and the solvent evaporated under reduced pressure to give Fmoc–Tyr(PO<sub>3</sub>Bzl<sub>2</sub>)–OPac as a white crispy solid. A mixture containing zinc dust (690 mg) in EtOAc–CH<sub>3</sub>COOH–H<sub>2</sub>O (2 : 5 : 1, v/v/v; 19.2 ml) was added to Fmoc–Tyr(PO<sub>3</sub>Bzl<sub>2</sub>)–OPac and the suspension stirred vigorously for 2 h. The mixture was then transferred to a separation funnel with the addition of EtOEt (70 ml) and H<sub>2</sub>O (80 ml), the aqueous phase discarded and the organic layer washed with 10% citric acid (2 × 20 ml) and extracted with 5% NaHCO<sub>3</sub> (pH 8.5, 3 × 15 ml). The combined aqueous extracts were acidified to pH 2.5 with solid citric acid, extracted with DCM (3 × 25 ml) and the solvent evaporated under reduced pressure to give Fmoc–Tyr(PO<sub>3</sub>Bzl<sub>2</sub>)–OH as a white crispy foam (776 mg, 72.6% yield). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 47.0, 54.4,

66.9, 70.2 (d, *J*<sub>PC</sub> 6.0 Hz, Bzl CH<sub>2</sub>), 119.9 (d, *J*<sub>PC</sub> 5.0 Hz, Tyr Ar C3), 125.0, 127.0, 127.6, 127.9, 128.0, 128.5, 128.6, 130.7, 133.1, 135.0 (d, *J*<sub>PC</sub> 7.1 Hz, Bzl C1), 141.2, 143.6, 143.7, 149.2 (d, *J*<sub>PC</sub> 7.0 Hz, Tyr Ar C4), 155.7, 173.2 ppm. <sup>31</sup>P NMR (85% H<sub>3</sub>PO<sub>4</sub>): δ –7.6 ppm.

### Preparation of MAP Kinase ERK2 [178–188; Thr(<sup>P</sup>)<sup>183</sup>, Tyr(<sup>P</sup>)<sup>185</sup>]; H–His–Thr–Gly–Phe–Leu–Thr(<sup>P</sup>)–Glu–Tyr(<sup>P</sup>)–Val– Ala–Thr–OH (14)

Peptide-resin [Boc–His(Trt)–Thr(<sup>t</sup>Bu)–Gly–Phe–Leu–Thr(PO<sub>3</sub>Bzl<sub>2</sub>)–Glu(O<sup>t</sup>Bu)–Tyr(PO<sub>3</sub>Bzl<sub>2</sub>)–Val–Ala–Thr(<sup>t</sup>Bu)–Wang] (12) was assembled by (a) standard Fmoc–<sup>t</sup>Bu protocols for residues 184–188 from Fmoc–Thr(<sup>t</sup>Bu)–Wang resin (10) (38 mg, 0.025 mmol) including incorporation of Fmoc–Tyr(PO<sub>3</sub>Bzl<sub>2</sub>)–OH and (b) the procedure outlined for the synthesis of peptide (4) for residues 178–183 including incorporation of Bpoc–Thr(PO<sub>3</sub>Bzl<sub>2</sub>)–OH and Boc–His(Trt)–OH as the N-terminal residue. All couplings were effected as described under General procedure (above); Fmoc deprotection was performed by addition of 25% piperidine–DMF (1 ml) to the peptide-resin, while Bpoc removal was achieved by suspension in 20% formic acid–DCM (1 ml). Following completion of peptide assembly, the peptide-resin (12) was suspended in TFA–TES (95 : 5, v/v; 1 ml) for 6.5 h and the crude peptide (13) (32 mg) isolated according to general procedure (above). Semi-preparative RP–HPLC purification (gradient: 0–50% MeCN in 50 min) of crude peptide (13) (10 × 2 mg) and lyophilization of the major eluting fraction gave MAP Kinase ERK2 [178–188; Thr(<sup>P</sup>)<sup>183</sup>, Tyr(<sup>P</sup>)<sup>185</sup>]; H–His–Thr–Gly–Phe–Leu–Thr(<sup>P</sup>)–Glu–Tyr(<sup>P</sup>)–Val–Ala–Thr–OH (14) as a fluffy white powder (13 mg, 60% yield). MALDI–MS: (negative mode) (*m/z* 1397.4 [M–H]<sup>–</sup>).

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