Backbone protection and its application to the synthesis of a difficult phosphopeptide sequence

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The *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) backbone amide-protecting group has been applied to the synthesis of phosphopeptides *via* post-assembly global phosphorylation. Reversible protection of the Hmb 2-hydroxy moiety was mandatory in order to prevent its phosphorylation and resultant irreversible stabilisation to acidolysis. This was achieved through the use of either the acetyl (Ac) or allyloxycarbonyl (Alloc) groups, introduced through their respective anhydrides in the presence of tertiary base. Following global phosphorylation, Ac or Alloc could be removed from resin-bound Hmb backbone-substituted fully protected phosphopeptide by either hydrazinolysis or palladium-catalysed cleavage to re-establish Hmb acid-lability. Similarly, hydrazinolysis in solution of otherwise deprotected, backbone-substituted phosphopeptide was found to be efficient and free from phosphoroamino acid β-elimination side-reactions. The optimised protocols were applied in the preparation of peptides from human Tau [390–406; Ser(PO_3H)^{396}] and the MAP kinase ERK 2 [178–188; Thr(PO_3H)^{183}; Tyr(PO_3H)^{185}].

Introduction

Phosphorylation of amino acids, particularly serine, threonine and tyrosine, within proteins is one of the most abundant and important post-translational modifications.[1] Many biologically important proteins, such as kinases, oncogenes, growth factor receptors and cytoskeletal proteins, are known to be phosphorylated.[2] Biochemical and/or structural investigation of these molecules is dependent upon the availability of appropriately phosphorylated peptides and proteins as model systems. Significant chemical effort has been expended in recent years on synthetic methods particularly for the preparation of phosphopeptides.[1,3–6] This has been necessary because of the small quantities of phosphoproteins produced by cells and their instability at extremes of pH, making isolation difficult.

Although synthesis of phosphopeptides in solution has been successful,[1] for other than short sequences the solid-phase synthesis strategy is to be preferred. Alternative routes for the chemical synthesis of phosphopeptides are available: (i) incorporation of suitably protected phosphoramino acid building blocks at the appropriate position during the stepwise assembly of the target peptide,[7] (ii) preparation of a peptide containing an unprotected hydroxy moiety followed by post assembly phosphorylation on the solid phase.[1,3–6] For the former method, the use of fluoren-9-ylmethoxycarbonyl (Fmoc)-based chemistry is precluded due to readily occurring β-elimination (for serine and threonine, to yield dehydroalanine-based peptides) during repetitive piperidine-mediated removal of Fmoc.

By far the most popular and practical method for preparing phosphorylated peptides is *via* post-assembly modification of resin-bound, fully protected (other than the free hydroxy group destined for phosphorylation) peptides. The use of phosphoramidite chemistry (phosphitylation followed by oxidation) appears to give the best results particularly as the phosphate triester approach gives incomplete phosphorylation and poor yields.[7] For effective post-assembly phosphorylation, reagents must be able to penetrate the peptide–resin assembly for reaction at all available sites. Steric constraints, especially due to secondary structure formation (peptide-chain association and aggregation) can hamper not only phosphorylation reactions but also the initial synthesis of the parent protected peptide.[3,8–10]

We have recently described a general strategy for overcoming the aggregation effects that are observed during the solid-phase synthesis of some peptides.[9,10] The protocol developed is based on reversible substitution of peptide amide bonds through use of the acid-labile *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) protecting group[11] that is compatible with the well established Fmoc/*tert*-butyl methodology.[12] The substitution of amide bonds during the course of a solid-phase synthesis prevents chain aggregation mediated through intra- and inter-molecular hydrogen bonding. The occurrence of such hydrogen-bonded association is believed to be the cause of secondary structure formation that leads to serious slowing of acylation and deprotection rates.[8,13,14] We have shown that introduction of the backbone-protecting group into known aggregating sequences prevents chain association and leads to much improved crude products when compared with standard syntheses.[10] We can anticipate that improvements in peptide purity will also be manifest in the synthesis of phosphopeptides where post-synthetic modification is attempted on aggregated peptides.

In this paper we describe the application of backbone protection to the synthesis of phosphopeptides *via* the phosphoramidite procedure.

Results

The chemical protocols required for the preparation of the difficult phosphopeptide sequence (Scheme 2, **17**) were initially optimised using the test pentadecapeptide (Scheme 1, **8**) from human Tau protein (residues 390–404). The test peptide **8** was smoothly assembled using standard Fmoc/*tert*-butyl/pentafluorophenyl (Pfp) active-ester chemistry[12] as shown in Scheme 1. Serine^{396} requiring post-assembly phosphorylation

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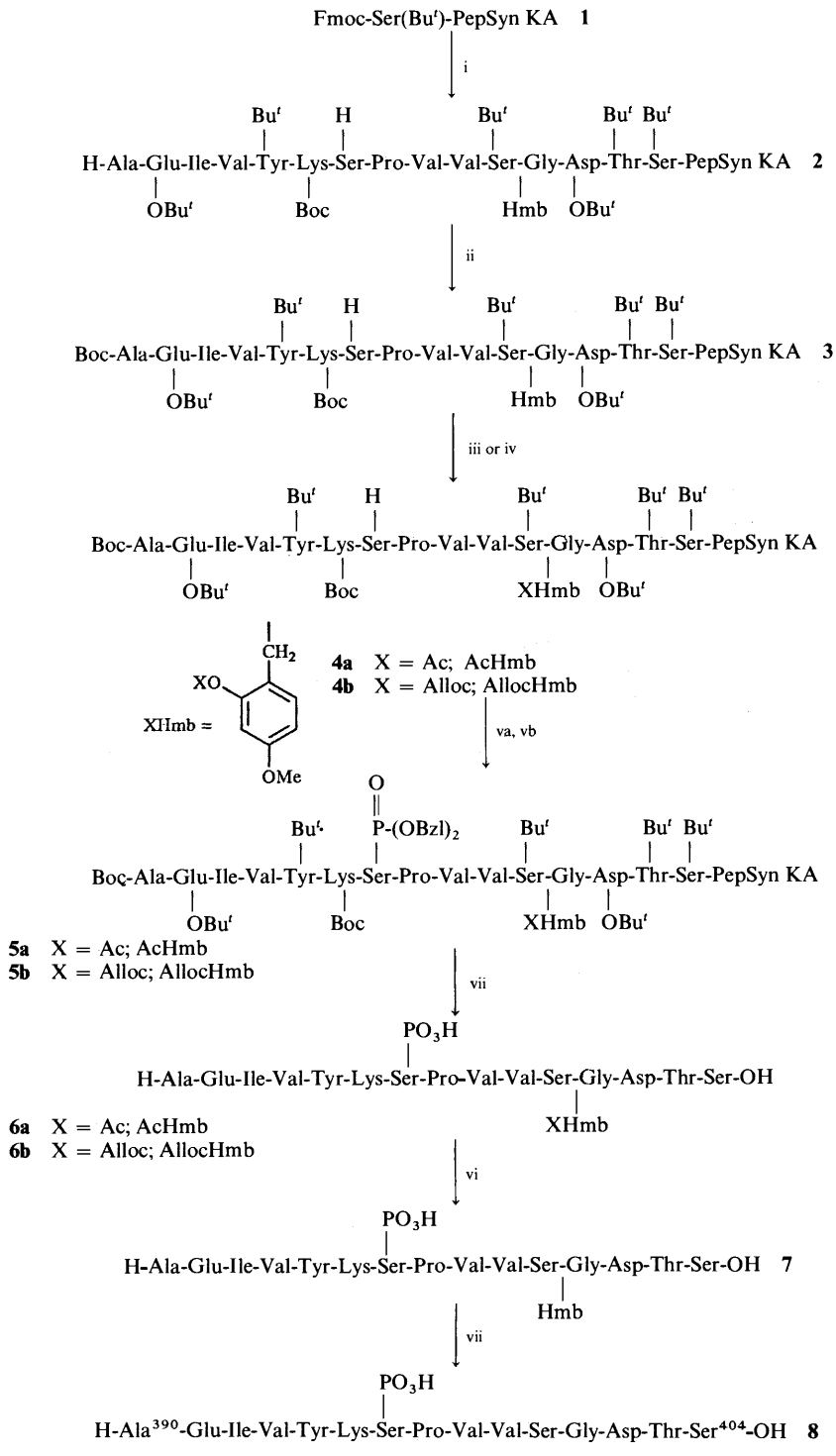


Scheme 1 *Reagents and conditions:* i, 14 cycles of Fmoc/*tert*-butyl solid-phase peptide synthesis; ii, Boc_2O/DMF; iii, Ac_2O/DIPEA/DMF; iv, diallyl pyrocarbonate/DIPEA/DMF; va, dibenzyl *N*,*N*-diisopropylphosphoramidite/tetrazole/acetonitrile; vb, I_2/water/THF/pyridine; vi, hydrazine hydrate/DMF; vii, TFA/scavengers

was introduced without side-chain protection, *via* activation of N^{α}-Fmoc-serine using, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP)[15]/1-hydroxybenzotriazole (HOBt) chemistry in the presence of diisopropylethylamine (DIPEA). Hmb backbone protection was introduced at Gly^{401} as previously described.[11] On completion of the assembly, the amino terminus was capped with di-*tert*-butyl dicarbonate (Boc_2O) to give assembly **3**.

Treatment of assembly **3** with acetic anhydride/DIPEA for 1 h effected Hmb 2-hydroxy group acetylation.[16] Acidolytic cleavage [mediated by trifluoroacetic acid (TFA)/ethane-1,2-dithiol/triethylsilane/water; 91:3:3:3, v/v/v/v] of compound **4a** [Fig. 1(*a*)] gave the AcHmb backbone-protected free peptide with MALDITOF-MS at 1732 Da (theoretical molecular mass = 1731 Da). Assembly **4a** was phosphitylated at Ser^{396} by using dibenzyl *N*,*N*-diisopropylphosphoramidite (25 mol equiv.) in the presence of tetrazole (50 mol equiv.) with dry acetonitrile as solvent under argon. On completion of the phosphitylation reaction, oxidation was performed by washing the peptide–resin with I_2/tetrahydrofuran (thf)/pyridine/water.[3] Acidolytic cleavage of assembly **5a** gave crude phosphopeptide **6a** of good quality [Fig. 1(*b*)] with MALDITOF-MS at 1812 Da (theoretical molecular mass = 1811 Da), and which was purified by preparative HPLC and re-analysed [Fig. 1(*c*)] to show a main species, at retention time (t_R) = 14.88 min (> 98%).

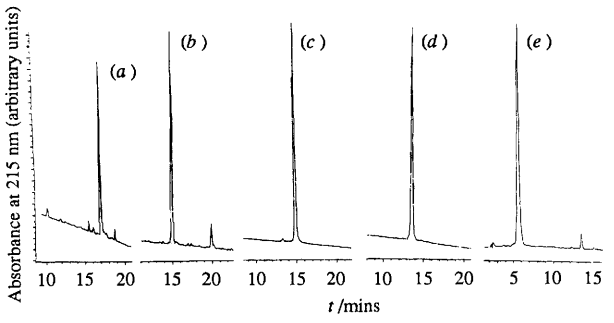


Fig. 1 Analytical HPLC intermediates in the synthesis of the model phosphopentadecapeptide **8** (Scheme 1). (*a*) Small-scale cleavage product from peptide–resin **4a**; (*b*) crude AcHmb backbone-protected phosphopeptide **6a**; (*c*) purified phosphopeptide **6a**; (*d*) Hmb backbone-protected phosphopeptide **7** from hydrazine treatment of compound **6a** in solution; (*e*) fully deprotected phosphopeptide **8** from treatment of compound **7** with TFA/scavenger mixture. HPLC conditions: Aquapore RP-300 C_8 column (250 × 4.6 mm), 15–40% B in A, linear gradient over a period of 25 min (flow rate 1.5 cm^3 min^{-1}; 215 nm UV detection) where buffer A = 0.1% aq. TFA and buffer B = 90% acetonitrile/10% buffer A.

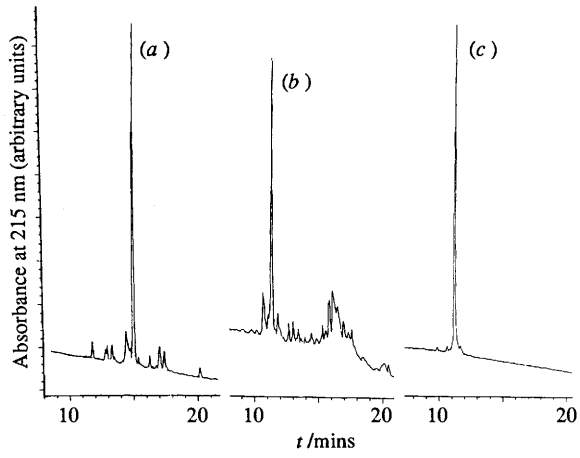


Fig. 2 Analytical HPLC intermediates in the synthesis of the MAP ERK 2 peptide **17** (Scheme 2). (*a*) Small-scale cleavage product from peptide–resin **10**; (*b*) crude and (*c*) purified bisphosphopeptide **17**. HPLC conditions: Aquapore RP-300 C_8 column (250 × 4.6 mm), 15–65% B in A, linear gradient over a period of 25 min (flow rate 1.5 cm^3 min^{-1}; 215 nm UV detection) where buffer A = 0.1% aq. TFA and buffer B = 90% acetonitrile/10% buffer A.

Removal of AcHmb backbone protection required an initial de-O-acetylation step followed by acidolytic removal of Hmb. De-O-acetylation was achieved, without significant β-elimination from phosphoserine, by using hydrazine hydrate (10 mol equiv.) in dimethylformamide (DMF) for 2 h. Hmb-phosphopeptide **7** was of excellent quality [Fig. 1(*d*)], giving a main species at t_R = 13.70 min (>98%) with MALDITOF-MS at 1769 Da (theoretical molecular mass = 1769 Da). Final acidolytic cleavage gave peptide **8** as a main species with t_R = 5.54 min (>95%) [Fig. 1(*e*)] with MALDITOF-MS at 1634 Da (theoretical molecular mass = 1633 Da), isolated in 30% yield.

The protocols developed in the preparation of the test peptide **8** were applied to the preparation of a difficult bisphosphopeptide (Scheme 2, **17**) encountered during routine synthesis (see Discussion section). Assembly of peptide–resin **11** proceeded smoothly. Both Fmoc-Tyr(All)-OH and Fmoc-Thr(H)-OH were introduced *via* BOP/HOBt/DIPEA as described above. Bis-Fmoc(Hmb)Leu^{182} was coupled as the Pfp ester (5 mol. equiv.) for 3 h. The Phe^{181} residue was coupled to (Hmb)Leu *via* the *N*-carboxyanhydride (10 mol equiv.; 6 h) in dichloromethane as solvent.[11]

The parent peptide [from undecapeptide–resin **10**; Fig. 2(*a*)] and products from small-scale acidolytic cleavage of peptide–resins **11**–**14** were analysed for completion of reaction by HPLC and MALDITOF-MS analysis (summarised in Table 1).

Peptide–resin **11**, containing a free hydroxy group on threonine, was phosphitylated and oxidised using the standard protocol described above. Owing to the hindered nature of the side-chain, in comparison with serine, the phosphorylation method was repeated to give assembly **12**. Peptide–resin **12** was subjected to palladium(0)-catalysed cleavage of the Tyr side-chain allyl protecting group as described by Kates *et al.*[17] The dried monophosphorylated peptide–resin **13** now containing a free side-chain hydroxy group on Tyr, was phosphorylated using the standard protocol to give compound **14**. Cleavage and analysis of crude product **15** indicated the phosphorylation to be essentially complete (Table 1).

Peptide **15** was suspended in DMF and hydrazine hydrate (10 mol equiv.) was added, as described above, to effect de-O-acetylation of the AcHmb function. Analytical HPLC monitoring (Table 1) during the reaction indicated that 2 h was sufficient for its completion to give compound **16**.

Finally, peptide **16** was dissolved in a TFA/scavenger cocktail to remove the Hmb backbone-protecting group and bis-phosphate **17** was isolated by ethereal precipitation. Analytical HPLC analysis of the crude product [Fig. 2(*b*)] showed a main species with MALDITOF-MS at 1398 Da (theoretical molecular mass = 1398 Da). The target bisphosphopeptide was isolated (15% yield) by preparative HPLC and re-analysed by analytical HPLC to confirm its purity [Fig. 2(*c*)] (>98%).

Discussion

During the routine synthesis of a peptide required in a phosphorylated form (Scheme 2, **17**) we encountered significant difficulty not only in assembling the parent peptide but also with the subsequent phosphorylation reaction (see later). Having already developed a successful strategy for the solid-phase synthesis of peptides that undergo chain aggregation,[9,10,18] we investigated the procedures required to extend the backbone protection methodology to the synthesis of phosphopeptides. It was anticipated that not only would the parent non-phosphorylated peptide exhibit greater crude purity, but that the presence of the Hmb group would allow on-resin phosphorylation to proceed efficiently on an unstructured resin-bound peptide. Before tackling such a difficult sequence, reaction conditions were optimised by using a phosphopeptide sequence that had previously been synthesized without difficulty.

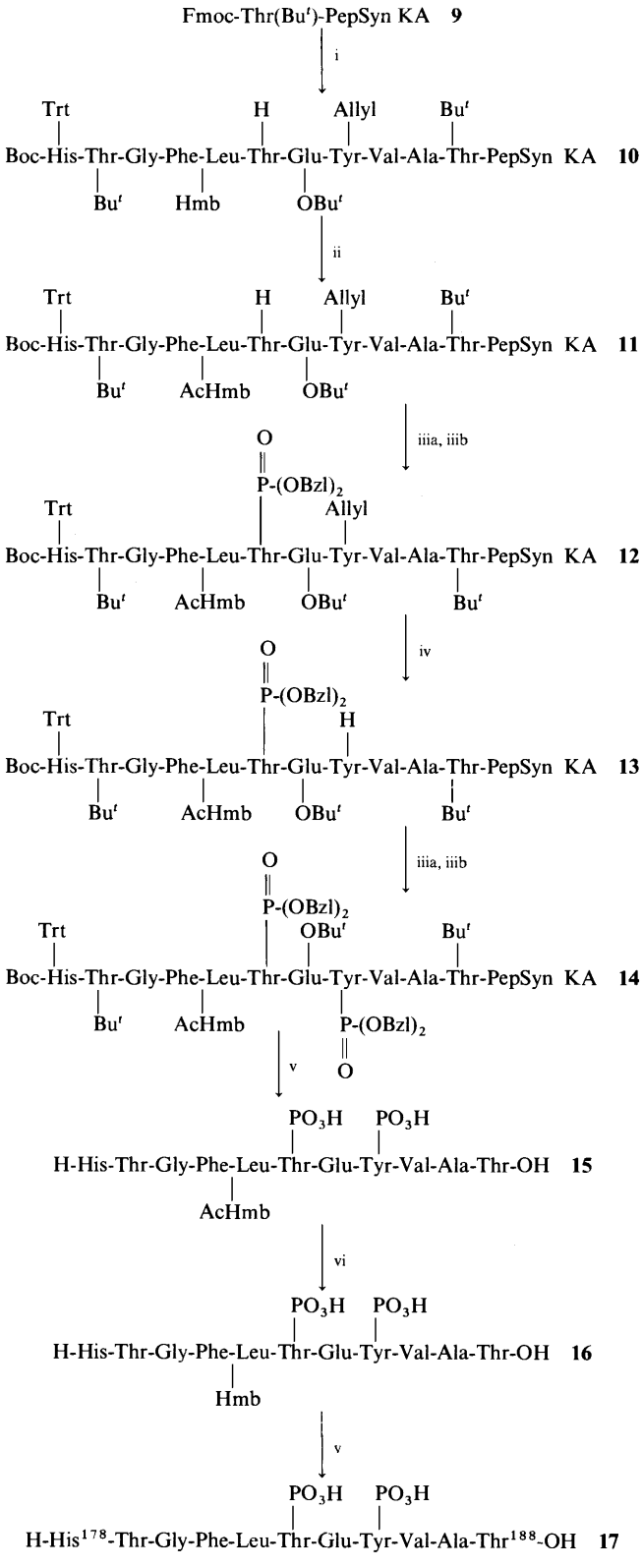
Choice of model phosphopeptide

Sequence:-H-A^{390}-E-I-V-Y-K-S^{396}-P-V-V-S-G^{401}-D-T-S-OH; Human Tau 390–404.

The main criteria for choosing the human Tau (390–404) sequence were (a) ease of synthesis of the parent non-phosphorylated peptide, (b) simple introduction of the Hmb amide substitution at a glycine residue, enabling a convenient machine-driven assembly to be used[11] and (c) complete resolution of phosphorylated and non-phosphorylated peptides by reversed-phase HPLC. The latter consideration was given the greatest weight as this would provide an experimentally convenient procedure to judge the efficacy of various reactions performed either on the solid phase or in solution. In our experience of other peptide sequences, phosphorylation does not always result in significant changes in HPLC elution behaviour.

Comparison of phosphorylation protocols

Post-assembly (global) phosphorylation proceeds *via* an initial



Scheme 2 *Reagents and conditions:* i, 10 cycles of Fmoc/*tert*-butyl solid-phase peptide synthesis; ii, Ac_2O/DIPEA/DMF; iiia, dibenzyl, *N*,*N*-diisopropylphosphoramidite/tetrazole/acetonitrile; iiib, I_2/water/THF/pyridine; iv, $Pd(PPh_3)_4$/DMF/chloroform/AcOH/NMM; v, TFA/scavengers; vi, hydrazine hydrate/DMF

phosphitylation reaction in the presence of tetrazole followed by an oxidation step. From the literature, two approaches to this procedure have been described. The first involves phosphoramidite coupling in dry DMF followed by *tert*-butyl hydroperoxide-mediated oxidation.[6] Alternatively, phosphitylation is performed in dry acetonitrile followed by oxidation with an I_2 solution.[3] These latter reagents are based on experience gained from nucleotide chemistry. Prior to investigating the use of backbone protection, the standard protocols referred to above were evaluated for their reactivity and ease of use. From the work of Andrews *et al.*[6] we chose the commercially available dibenzyl *N*,*N*-diisopropylphosphoramidite as phosphitylating reagent in our studies. In our hands (data not shown), both methods behaved in an identical fashion and gave >90% phosphorylation of the model peptide (see above). The ready availability of synthesis-quality reagents from suppliers of

DNA/RNA chemicals made the latter procedure experimentally convenient and this procedure was used for the remainder of the investigation.

Reversible protection of the Hmb 2-hydroxy function

(i) Optimisation of substitution/removal of 2-hydroxy protection. In order to achieve phosphorylation of specific hydroxy amino acid residues, all functional residues not to be modified require suitable orthogonal protection. Where Hmb backbone amide bond protection is used during the course of a synthesis, the 2-hydroxy moiety must also be protected on completion of peptide assembly. Selective, reversible protection in a manner compatible with the sensitive nature of the phosphoroamino acids (particularly serine) is necessary to prevent its unwanted phosphorylation. If unchecked, this substitution renders the Hmb group acid-stable and permanently attached to the phosphopeptide.

During the course of our investigations into the application of backbone protection in solid-phase peptide synthesis, we have reported on a simple method for substituting the 2-hydroxy position.[16,18] Acetylation was used to render the Hmb group stable to the acidolysis conditions used simultaneously to effect both peptide deprotection and cleavage from a resin support. This was achieved using Ac_2O in the presence of DIPEA and could be reversed (de-O-acetylation) upon brief treatment with hydrazine hydrate in DMF. There is a potential danger of β-elimination from phosphoroamino acid residues under basic conditions,[7] *i.e.* in the presence of hydrazine. However, our recent experience[19] with direct hydrazine treatment of base-sensitive Fmoc-protected amino acid Pfp esters (leading exclusively to Fmoc amino acid acyl hydrazides) led us to believe that conditions could be found that would promote de-O-acetylation without concomitant de-phosphorylation. As an alternative, we also investigated the use of allyl-based protection.[20,21] Allyl-based groups can be readily removed using palladium reagents and have been used as protecting groups for phosphoramidite reagents.[3]

The model peptide was synthesized as described in Scheme 1. The residue destined to become phosphorylated (in this case serine) was introduced without side-chain protection *via in situ* activation using BOP reagent.[15] Hmb backbone protection was introduced at the glycine residue, allowing the following residue (side-chain-protected serine) to be introduced through double coupling *via* its dihydrooxobenzotriazine (ODHBt) ester.[11] On completion of the synthesis, the amino terminus was capped with the acid-labile Boc group to prevent unwanted reaction with either phosphitylating agents or 2-hydroxy capping groups.

Our experience with the Hmb group has shown that for O-acylation to occur, anhydride derivatives possess the required reactivity for quantitative reaction. Thus, acetylation of Hmb-substituted peptide-resin **3** was performed using Ac_2O in the presence of the tertiary base DIPEA. As shown from analytical HPLC [Fig. 1(*a*)] and mass analysis, acetylation proceeds selectively (no acetylation of the serine hydroxy group) in almost quantitative fashion. Substitution of the 2-hydroxy position renders the Hmb backbone-protecting group stable to acidolysis. Therefore, cleaved AcHmb-peptide from resin **4a** was a suitable substrate to determine the minimum hydrazinolysis conditions necessary to effect cleavage of the Ac moiety and to re-establish Hmb acid-lability. Small-scale hydrazinolyses (data not shown) indicated that 2 h was sufficient to cleave the acetyl group without formation of other side-products. The AllocHmb peptide (see below) (from cleavage of peptide **4b**) behaved in an identical manner.

In an effort to design an acid-labile protecting group for the 2-hydroxy moiety, we also investigated the use of di-*tert*-butyl pyrocarbonate to introduce an acid-labile Boc group. Experiments have met with little success thus far.

(ii) Optimised removal of 2-hydroxy protection in the presence of phosphoserine. (*a*) *Solution procedure.* Having established the minimum hydrazinolysis conditions necessary to remove Hmb 2-hydroxy protection, these were repeated on the phosphorylated test peptide containing the base-sensitive Ser(PO_3H) residue (peptide **6a**). Phosphorylation of compound **4a** (and **4b**) proceeded smoothly as shown by analytical HPLC [Fig. 1(*b*)] and mass analysis. Careful examination of the HPLC data indicated that complete phosphorylation had occurred. Treatment of the crude AcHmb-phosphopeptide **6a** with hydrazine hydrate in DMF quantitatively de-O-acetylated the AcHmb function without side-reactions. This is quite clearly seen on hydrazinolysis of purified peptide **6a** [Fig. 1(*d*)]. Even after reaction for 24 h, no change in HPLC profile was observed (data not shown). Acidolytic cleavage of the Hmb group from compound **7** occurred smoothly to give peptide **8** of excellent quality [Fig. 1(*e*)] with MALDITOF-MS at 1634 Da (theoretical molecular mass = 1633 Da) in 30% yield. Hydrazinolysis of the AllocHmb-substituted phosphopeptide **6b** behaved in an identical manner.

(*b*) *On-resin procedure.* As an alternative to the solution procedure described above, the hydrazinolysis protocol can of course be applied to the resin-bound protected phosphopeptide. This has the advantage that reagents are removed by simple washing and only one acidolysis treatment is required at the end of the synthesis to remove *all* protecting groups and effect peptide–resin cleavage. Protected phosphopeptide–resin **5a** (Scheme 1) was treated with hydrazine hydrate/DMF for 2 h followed by TFA-mediated acidolytic cleavage of the target phosphopeptide from the support. HPLC analysis (data not shown) of the gel-filtered product showed a single main species (80%) that co-eluted with phosphopeptide **8** prepared above. Although this procedure is more convenient than the solution protocol, it would appear from HPLC analysis that unidentified side-products arise during the on-resin procedures.

Whilst conducting these initial trial experiments we anticipated that the potential danger of base-catalysed β-elimination from phosphoro amino acids would be more problematic (although as shown above this was not the case). Therefore, we also investigated the use of allyl-based protection. Palladium reagents can be used to effect removal of allyl protecting groups in a mild, specific manner and have previously been used as phosphoramidite-protecting groups without problem.[3] Substitution of the Hmb 2-hydroxy position by using diallyl–pyrocarbonate ($Alloc_2O$) in the presence of base (DIPEA) was achieved in an analogous manner to acetylation (Scheme 1). Analytical HPLC and mass analysis indicating the reaction to be quantitative, likewise the following phosphorylation step (data not shown). Cleavage of the Alloc group was effected using palladium(0) by the method of Kates *et al.*[17] and the residual palladium products were removed by washing with the metal chelator sodium diethyldithiocarbamate. HPLC analysis of the crude final product **8** from the AllocHmb-protected intermediate **5b** revealed that other, unidentified side-products (total of 30%) were also present (data not shown). The target phosphopeptide **8** was isolated in 30% yield after preparative HPLC.

From the initial sets of experiments it was concluded that substitution of the Hmb 2-hydroxy function could be readily and selectively accomplished in the presence of a serine side-chain free hydroxy function. Subsequent serine phosphitylation and oxidation occurred without formation of side-products for acetyl protection of Hmb. Hydrazine hydrate-mediated de-O-acetylation of AcHmb could be performed selectively in solution without significant β-elimination from phosphoserine. Subsequent acidolytic cleavage of the Hmb group was also trouble-free. Hydrazinolysis of resin-bound AcHmb-phosphopeptide followed by acidolysis also released the target

Table 1 Analytical HPLC and MALDITOF-MS data for intermediate stages in the preparation of bis-phosphopeptide **17** (see Scheme 2)

Peptide (see Scheme 2)	Substitution[a]	HPLC retention time t/min (gradient)[b]	HPLC purity (%)[c]	MALDITOF-MS data (M/Da)[d]	
				Obtained	Expected
From peptide–resin **10**	Tyr(All)	14.9 (10–90), 16.4 (15–65)	80	1279	1279
From peptide–resin **11**	Tyr(All) (AcHmb)Leu	17.2 (10–90), 20.0 (15–65)	80	1458	1459
From peptide–resin **12**	Thr(PO_3H) Tyr(All) (AcHmb)Leu	17.5 (10–90), 20.3 (15–65)	75 70	1537	1538
From peptide–resin **13**	Thr(PO_3H) (AcHmb)Leu	17.0 (15–65)	70	1498	1499
Peptide **15**	Thr(PO_3H) Tyr(PO_3H) (AcHmb)Leu	16.5 (15–65)	60	1579	1579
Peptide **16**	Thr(PO_3H) Tyr(PO_3H) (Hmb)Leu	16.1 (15–65)	55	1535	1536
Peptide **17**	Thr(PO_3H) Tyr(PO_3H)	11.2 (15–65)	50	1398	1398

[a] Refers to side-chain substitution at Thr^{183} and Tyr^{185} and backbone amide substitution between Phe^{181}–Leu^{182} (see Scheme 2), post-TFA-treated peptides. [b] HPLC conditions: Brownlee Aquapore RP300 C_8 column (250 × 4.6 mm). A gradient of %B in A over a period of 25 min (at 1.5 cm^3 min^{-1}; 215 nm UV detection) was used (see Table), where A = 0.1% aq. TFA and B = 90% acetonitrile/10% A. [c] Crude peptide as judged by integrated areas of main peak compared with the remainder of the peptide-related material. [d] MALDITOF-MS conditions are fully described in the Experimental section.

peptide but HPLC analysis revealed that other minor side-products were also formed. Use of Alloc protection revealed a weakness in the phosphitylation/oxidation step, probably partial removal of the Alloc carbonate protection of Hmb, that resulted in formation of side-products, and overall crude peptides from this protocol were not as pure.

Application of protocols to the synthesis of a difficult phosphopeptide

During an attempted preparation of a bis-phosphorylated peptide, residues 178–188; $PO_3H^{183,185}$ from the MAP kinase ERK 2 (Scheme 2, **17**) we encountered significant difficulty in synthesizing the parent peptide, as well as in performing the phosphorylation/oxidation protocol. Solubility problems were also encountered during purification and analysis of the final crude compound, making synthesis by standard protocols intractable (unpublished data).

In the light of the original, unsuccessful syntheses, the target peptide was synthesized as outlined in Scheme 2. The residues to be phosphorylated, Tyr^{185} and Thr^{183}, were introduced as the allyl ether and free hydroxy group respectively. This would enable individual phosphorylation reactions to be monitored by analytical HPLC and mass spectrometry. Allyl protection of Tyr^{185} was chosen as it is orthogonal with the Fmoc/*tert*-butyl solid-phase peptide synthesis protocols and its palladium-catalysed removal can be performed in the presence of phosphorylated residues as shown earlier. The Hmb backbone protecting group was positioned on the phenylalanyl^{181}–leucyl^{182} amide bond as earlier syntheses had indicated that onset of peptide-chain aggregation on the solid support occurred upon deprotection at Phe^{181}. Acetylation of the Hmb function, required to prevent its unwanted phosphorylation, renders it acid-stable and thus preserved upon cleavage of the peptide from the support. Solubility of Hmb backbone-protected peptides, devoid of other protecting groups, is considerably enhanced in aqueous solutions and enables standard chromatographic procedures to be used to obtain the target peptide.[16,18] This was an important factor, considering our earlier solubility problems with the target peptide **17**. The final residue, His^{178}, was introduced as the N^{α}-Boc-protected derivative, using BOP/HOBt coupling, to prevent substitution of the amino terminus during subsequent on-resin post-assembly reactions.

Unlike earlier attempts at synthesis of this peptide, no problems were encountered with peptide-chain aggregation during solid-phase peptide synthesis with the introduction of Hmb protection of Leu^{182}. Analytical HPLC and mass analysis of the base peptide from acidolytic cleavage of peptide–resin **10** was of good quality [Fig. 2(*a*) and Table 1] with only small quantities of unidentified side-products. On completion of the synthesis, the Hmb 2-hydroxy function was quantitatively acetylated, prior to subsequent phosphorylations.

Owing to the hindered nature of the threonine side-chain, in comparison with that of serine, the phosphorylation protocols were repeated to ensure near quantitative reaction (Table 1). It is interesting to note that, unlike phosphorylation of the model peptide, Thr phosphorylation of the MAP kinase ERK 2 peptide results in an increased HPLC retention time (Table 1), *i.e.* the peptide behaves as though it has increased its hydrophobicity. Presumably this must be related to a change in structure initiated by phosphorylation.[22]

Following threonine phosphorylation, the allyl ether protecting group of Tyr^{185} was removed from peptide–resin **12** by palladium-catalysed cleavage as described earlier. A small change in retention time by HPLC resulted (Table 1). The resultant AcHmb-monophosphorylated peptide containing a free hydroxy group on tyrosine was then subjected to on-resin phosphorylation as described earlier. Peptide–resin **14** was cleaved with TFA/scavengers, and remaining reactions were performed in solution, this being the optimal route identified during the preparation of test peptide **8**. Peptide **15** was completely soluble in aqueous solvents, unlike the product from earlier unsuccessful syntheses (assembled without backbone protection), which was barely soluble.

Small-scale orienting experiments indicated that purification of the bis-phosphopeptide could be performed after removal of the backbone-protecting group, *i.e.* as the very last step. Therefore, optimised hydrazine-mediated de-O-acetylation of the AcHmb function was performed on crude compound **15**, the reaction being judged as complete in 2 h (Table 1). The Hmb group was smoothly removed from compound **16** by TFA-mediated acidolysis. At this stage crude product **17** contained small quantities of many impurities [Fig. 2(*b*)] but was easily purified by preparative HPLC, to give the target bis-phosphopeptide, isolated in 15% overall yield. Re-analysis by analytical HPLC gave a single species [Fig. 2(*c*)] with MALDITOF-MS at 1398 Da (theoretical molecular mass = 1398 Da).

Conclusions

We have developed a series of protocols designed to facilitate the synthesis of phosphopeptides requiring backbone protection for their assembly. Standard phosphitylation/oxidation procedures are compatible with Hmb backbone protection provided the 2-hydroxy function is capped with a reversible protecting group. The acetyl group has been thoroughly investigated for this purpose, optimal conditions for its removal being hydrazinolysis in solution. Based on our results these mild and specific reactions lead to minimal side-reactions even in the presence of sensitive phosphoroamino acids.‡

Experimental

Equipment, materials and methods

Continuous-flow Fmoc-polyamide methods reviewed by Atherton and Sheppard [12] were used exclusively. Fmoc amino acid Pfp activated esters (Novabiochem, Beeston, Nottingham, UK, NG9 2JR) were used exclusively except for Ser(OBu^t) and Thr(Bu^t) which were coupled as the dihydrooxobenzotriazine (ODHBt) esters (Novabiochem, UK). Ser(H), Thr(H) and Tyr(All) were coupled through the free acid activated by BOP reagent in the presence of HOBt and DIPEA (see below). *N,O*-BisFmoc-*N*-(2-hydroxy-4-methoxybenzyl) (Hmb) glycine and leucine Pfp esters were prepared as previously detailed.[11] Phenylalanine coupling to (Hmb)Leu utilised the *N*-carboxyanhydride (Propeptide, BP12, 91710 Vert-le-Petit, France) (see below). Pepsyn KA resin (containing the acid-labile linker) was purchased from Novabiochem, UK. All solvents were purified as previously described.[12]

Solid-phase peptide synthesis was performed on an LKB 'Biolynx' 4170 automated synthesizer programmed to perform acylation reactions (in DMF) for 45 min unless otherwise stated, and Fmoc-deprotection reactions (in 20% piperidine/DMF v/v) for 10 min. All chiral amino acids used were of the L-configuration. Amino acid side-chain protection was as follows: aspartic and glutamic acid (*tert*-butyl ester, OBu^t), histidine (trityl, Trt), lysine (N^{ε}-*tert*-butoxycarbonyl, Boc), serine (*tert*-butyl ether, Bu^t; otherwise free hydroxy group, H), threonine (*tert*-butyl ether, Bu^t; otherwise free hydroxy group, H), tyrosine (*tert*-butyl ether, Bu^t; allyl ether, All). Peptide hydrolyses were performed at 110 °C for 24 h in aq. HCl (6 mol dm^{-3}), containing a trace of phenol, in evacuated, sealed tubes. Analysis of hydrolysis products was performed on a Beckman 7300 analyser. Amino acid separation was obtained using ion-exchange resin with manufacturer's buffer solutions and post-column detection by ninhydrin. Analytical HPLC was performed using a Brownlee Aquapore RP300 C_8 column (250 × 4.6 mm). A gradient of %B in A over a period of 25 min at (1.5 cm^3 min^{-1}) was used (see individual compounds for gradient data), where A = 0.1% aq. TFA and B = 90% acetonitrile/10% A. Preparative HPLC was performed using a Vydac 208TP1022, protein C_8 column (22 × 250 mm) at 10 cm^3 min^{-1} flow rate and UV detection at 215 nm, solvents A and B as above. MALDITOF-MS data were obtained on a Kratos MALDI III bench-top linear/reflectron mass spectrometer. Data were acquired in linear mode with external calibration giving a mass with standard deviation of 0.1%, *i.e.* 1 in 1000. Anhydrous acetonitrile and oxidising solution (I_2/THF/pyridine/water) (DNA Synthesis Grade) were used as purchased from Cruachem (Glasgow, G20 0UA, Scotland). Tetrazole (sublimed) was purchased from Aldrich (Gillingham, Dorset, SP8 4BR, UK); dibenzyl *N,N*-diisopropylphosphoramidite was obtained from Novabiochem Ltd, UK.

Preparation of Boc-Ala-Glu(OBu^t)-Ile-Val-Tyr(Bu^t)-Lys(Boc)-Ser(H)-Pro-Val-Val-Ser(Bu^t)-(Hmb)Gly-Asp(OBu^t)-Thr(Bu^t)-Ser(Bu^t)-PepSyn KA (peptide–resin 3; Scheme 1)

Peptide–resin **2** was synthesized by standard Fmoc/*tert*-butyl solid-phase methods on Pepsyn KA resin (Scheme 1). Fmoc-Ser(Bu^t)-Pepsyn KA resin **1** (1.0 g, 0.1 mmol) was suspended in DMF for 10 min. The supernatant, containing any fine particles, was removed by decantation and the resin was loaded onto the Biolynx synthesizer. The peptide sequence was synthesized in a stepwise manner with activated residues coupled (0.5 mmol vials) (amino acid Pfp esters, HOBt catalyst) under standard conditions except that *N,O*-bisFmoc-*N*-(2-hydroxy-4-methoxybenzyl)glycine Pfp ester (0.5 mmol) was coupled for 3 h. The subsequent serine residue was double coupled (2 × 2 h). Fmoc-Ser^7 (H)-OH was coupled using BOP (0.5 mmol, 221 mg), HOBt (0.5 mmol, 76.5 mg) and DIPEA (0.5 mmol, 64.6 mg) for 2 h.

On completion of the synthesis, de-Fmoc resin **2** was removed from the synthesizer, washed successively with DMF and diethyl ether, and dried *in vacuo* (over KOH pellets). The amino terminus of peptide–resin **2** was temporarily blocked by reaction with Boc_2O (10 mol equiv., 1.0 mmol, 220 mg) in DMF for 1 h. Completion of the reaction was confirmed by the ninhydrin reaction. Resin-bound amino acid analysis [Found (expected): Asp/Asn, 1.12 (1); Thr, 1.00 (1); Ser, 2.72 (3); Glu, 1.04 (1); Pro, 0.94 (1); Gly, 1.10 (1); Ala, 0.96 (1); Val, 2.88 (3); Ile, 0.84 (1); Tyr, 0.99 (1); Lys, 0.95 (1)].

In order to check on the progress of the assembly, a small-scale cleavage was performed and the crude peptide product was examined by analytical HPLC and MALDITOF-MS. Peptide–resin **3** (30 mg) was cleaved using TFA/water/triethylsilane/ethane-1,2-dithiol (91:3:3:3: v/v/v/v; 2 cm^3) for 2 h. The cleaved resin was removed by filtration, washed with a little neat TFA, and the combined filtrates were sparged with N_2 to remove the bulk of the TFA. Ice-cooled diethyl ether (20 cm^3) was added, precipitating the peptide; the suspension was cooled in acetone/solid CO_2 for 5 min and centrifuged at 3000 rpm for 5 min. The ethereal solution was decanted from the peptide and further diethyl ether extractions (5 × 20 cm^3) were performed. The residue was dried *in vacuo* over KOH pellets and dissolved in 0.1% aq. TFA (1 cm^3). Crude peptide gave the following ratios upon amino acid analysis [Found (expected): Asp/Asn, 1.10 (1); Thr, 1.02 (1); Ser, 2.82 (3); Glu, 1.02 (1); Pro, 1.05 (1); Gly, 1.09 (1); Ala, 0.93 (1); Val, 2.79 (3); Ile, 0.82 (1); Tyr, 1.0 (1); Lys, 0.97 (1)]. Analytical HPLC (Gradient: 10–30% B) showed the presence of a single major peak of retention time 16.94 min (95%) (alternative gradient: 15–40% B; retention time 9.88 min; 95%) with MALDITOF-MS at 1553 Da (theoretical molecular mass = 1553 Da).

‡ Since the completion of this work, a new derivative, Fmoc-Ser[PO(OBzl)OH]-OH (available from Novabiochem Ltd., UK) suitable for incorporation during routine solid-phase synthesis has been introduced. This does not suffer from the disadavantage of requiring an oxidation step and is reportedly stable to base conditions (no β-elimination). However, in our hands, incorporation of this derivative into a sequence was sensitive to steric factors, being 85% complete after 2 couplings.

Preparation of Boc-Ala-Glu(OBu^t)-Ile-Val-Tyr(Bu^t)-Lys(Boc)-Ser(H)-Pro-Val-Val-Ser(Bu^t)-(AcHmb)Gly-Asp(OBu^t)-Thr(Bu^t)-Ser(Bu^t)-PepSyn KA (peptide–resin 4a; Scheme 1)

Peptide–resin **3** (500 mg, 0.05 mmol) was suspended in DMF (1.5 cm^3) and Ac_2O (51 mg, 0.5 mmol, 10 mol equiv.) added followed by DIPEA (26 mg, 0.2 mmol, 4 mol equiv.) and reaction was allowed to proceed for 2 h. The peptide–resin, **4a**, was washed successively with DMF and diethyl ether, and dried *in vacuo* over KOH pellets.

Peptide–resin **4a** (50 mg, 0.005 mmol) was treated with TFA/water/triethylsilane/ethane-1,2-dithiol (91:3:3:3, v/v/v/v; 2 cm^3) for 2 h and the crude AcHmb-peptide was isolated as described above.

The crude peptide was dissolved in 0.1% aq. TFA (1 cm^3). Amino acid analysis [Found (expected): Asp/Asn, 1.13 (1); Thr, 0.98 (1); Ser, 2.62 (3); Glu, 1.03 (1); Pro, 0.98 (1); Gly, 1.10 (1); Ala, 0.96 (1); Val, 2.85 (3); Ile, 0.84 (1); Tyr, 0.98 (1); Lys, 0.96 (1); 4 μmol peptide content]. Analytical HPLC [gradient: 15–40% B; Fig. 1(*a*)] showed the presence of a single major peak of retention time 17.05 min with MALDITOF-MS at 1732 Da (theoretical molecular mass = 1731 Da).

Preparation of Boc-Ala-Glu(OBu^t)-Ile-Val-Tyr(Bu^t)-Lys(Boc)-Ser[P(O)(OBzl)$_2$]-Pro-Val-Val-Ser(Bu^t)-(AcHmb)Gly-Asp(OBu^t)-Thr(Bu^t)-Ser(Bu^t)-PepSyn KA (peptide–resin 5a; Scheme 1)

The peptide–resin support **4a** (450 mg, 0.045 mmol), containing side-chain unprotected serine, under argon, was placed on a frit in a syringe barrel and capped with a rubber septum. The peptide–resin was suspended in anhydrous acetonitrile (4 cm^3) containing tetrazole (157.5 mg, 2.25 mmol, 50 mol equiv.), added *via* syringe, for 5 min following which dibenzyl *N*,*N*-diisopropylphosphoramidite (360 mm^3, 1.125 mmol, 25 mol equiv.) was added also *via* syringe. The peptide–resin suspension was gently agitated and reaction allowed to proceed for 2 h. The phosphitylating mixture was removed by suction and the peptide–resin was washed with oxidising solution (5 cm^3) (0.2 mol dm^{-3} I_2 in THF/pyridine/water) added *via* syringe and after 30 s was removed by suction. The process was repeated five times. The resin support was finally washed successively in anhydrous acetonitrile, DMF, and diethyl ether, and dried *in vacuo* over KOH pellets to yield peptide–resin **5a**.

Preparation of H-Ala-Glu-Ile-Val-Tyr-Lys-Ser(PO$_3$H)-Pro-Val-Val-Ser-(AcHmb)Gly-Asp-Thr-Ser-OH (peptide 6a; Scheme 1)

Peptide–resin **5a** (AcHmb) (400 mg, 0.04 mmol) was treated with TFA/water/triethylsilane/ethane-1,2-dithiol (91:3:3:3, v/v/v/v; 10 cm^3) for 2 h and the crude phosphopeptide, **6a**, isolated as described above. The crude peptide was dissolved in 0.1% aq. TFA (10 cm^3). Amino acid analysis [Found (expected): Asp/Asn, 1.16(1); Thr, 1.05 (1); Ser, 2.83 (3); Glu, 1.07 (1); Pro, 1.09 (1); Gly, 1.11 (1); Ala, 1.05 (1); Val, 2.74 (3); Ile, 0.76 (1); Tyr, 1.02 (1); Lys, 1.00 (1); 23 μmol peptide content]. Analytical HPLC [gradient: 15–40% B; Fig. 1(*b*)] showed the presence of a single major peak of retention time 14.97 min with MALDITOF-MS at 1812 Da (theoretical molecular mass = 1811 Da). Analytical HPLC indicated that the phosphorylation reaction had proceeded in >98% yield.

An aliquot of the peptide **6a**-solution (250 mm^3, 0.8 μmol) was removed and the solvent was removed by freeze drying. The peptide thus obtained was suspended in DMF (40 mm^3), treated with hydrazine hydrate/DMF (10 mm^3) (46.6 mg hydrazine hydrate/1 cm^3 DMF; 10 mol equiv.), and the reaction progress was monitored at 15, 120 min and 24 h by HPLC analysis: t = 15 min, analytical HPLC (gradient: 15–40% B) showed the presence of a single major peak of retention time 13.34 min (82%) and a peak at retention time 14.92 min (AcHmb-phosphopeptide, 18%); t = 120 min, analytical HPLC (gradient: 15–40% B) showed the presence of a single major peak of retention time 13.37 min (>95%) with MALDITOF-MS at 1770 Da (theoretical molecular mass = 1769 Da); t = 24 h, analytical HPLC (gradient: 15–40% B) showed the presence of a single major peak of retention time 13.39 min (>95%) and no additional peaks compared with the HPLC profile obtained at t = 120 min.

A portion of the remaining crude peptide **6a** (8.5 cm^3, 19.3 μmol) was purified by preparative HPLC using a gradient of 15–40% B in A and 10 × 850 mm^3 injections. The purified product was lyophilised (11.2 mg, 6.2 μmol, 31%). Analytical HPLC (gradient: 15–40% B) [Fig. 1(*c*)] gave a single species with retention time 14.88 min (99%). Amino acid analysis [Found (expected): Asp/Asn, 1.07 (1); Thr, 0.97 (1); Ser, 2.47 (3); Glu, 1.16 (1); Pro, 0.97 (1); Gly, 1.06 (1); Ala, 1.06 (1); Val, 2.84 (3); Ile, 0.88 (1); Tyr, 0.98 (1); Lys, 0.97 (1); 6.2 μmol peptide content].

Preparation of H-Ala-Glu-Ile-Val-Tyr-Lys-Ser(PO$_3$H)-Pro-Val-Val-Ser-(Hmb)Gly-Asp-Thr-Ser-OH (peptide 7; Scheme 1)

Purified AcHmb-peptide **6a** was de-O-acetylated by being suspended in DMF (1.8 cm^3) and treated with hydrazine hydrate/DMF (100 mm^3) (19.9 mgs hydrazine hydrate/1 cm^3 DMF; 10 mol equiv.) for 2 h. Reaction was quenched by dropwise addition of the peptide–DMF solution to stirred, ice-cold diethyl ether (200 cm^3). The precipitated peptide was isolated by centrifugation and was washed with further volumes of ice-cold diethyl ether (5 × 45 cm^3) (11.4 mg, 100%). Analytical HPLC [gradient: 15–40% B; Fig. 1(*d*)] showed a single species (peptide **7**) with retention time 13.70 min and MALDITOF-MS at 1769 Da (theoretical molecular mass = 1769 Da).

Preparation of H-Ala-Glu-Ile-Val-Tyr-Lys-Ser(PO$_3$H)-Pro-Val-Val-Ser-Gly-Asp-Thr-Ser-OH (peptide 8; Scheme 1)

Backbone Hmb substitution was removed from peptide **7** by TFA/triethylsilane (95:5, v/v; 5 cm^3) for 90 min and the peptide was isolated as described above. Amino acid analysis [Found (expected): Asp/Asn, 1.06 (1); Thr, 0.98 (1); Ser, 2.59 (3); Glu, 1.10 (1); Pro, 1.08 (1); Gly, 1.09 (1); Ala, 1.09 (1); Val, 2.65 (3); Ile, 0.82 (1); Tyr, 1.03 (1); Lys, 1.04 (1); 6.2 μmol peptide content (100%)]. Analytical HPLC [gradient: 15–40% B; Fig. 1(*e*)] showed the presence of a single major peak of retention time 5.54 min (>95%) with MALDITOF-MS at 1634 Da (theoretical molecular mass = 1633 Da).

Preparation of Boc-His(Trt)-Thr(OBu^t)-Gly-Phe-(Hmb)Leu-Thr(H)-Glu(OBu^t)-Tyr-(Allyl)-Val-Ala-Thr(Bu^t)-PepSyn KA (peptide–resin 10; Scheme 2)

Peptide–resin **10** was synthesized by standard Fmoc/*tert*-butyl solid-phase methods on PepSyn KA resin. Fmoc-Thr(Bu^t)-PepSyn KA resin **9** (1.0 g, 0.08 mmol) was suspended in DMF for 10 min. The supernatant, containing any fine particles, was removed by decantation and the resin was loaded onto the Biolynx synthesizer. The peptide sequence was synthesized in a stepwise manner with activated residues coupled (0.5 mmol vials) (amino acid Pfp esters, HOBt catalyst) under standard conditions except that *N*,*O*-bisFmoc-*N*-(2-hydroxy-4-methoxybenzyl)leucine pentafluorophenyl ester (0.5 mmol) was coupled for 3 h. The subsequent phenylalanine residue was coupled as its *N*-carboxyanhydride (NCA). The de-Fmoc N-terminal Hmb-resin was suspended in a solution of Fmoc-Phe-NCA (400 mg, 1.0 mmol) in the minimum volume of dichloromethane. After 16 h reaction, the resin was washed successively with DMF and diethyl ether, re-suspended in DMF, and reloaded back onto the synthesizer to continue the synthesis. Boc-His(Trt)^1-OH, Fmoc-Thr(H)^{183}-OH and Fmoc-Tyr(All)^{185}-OH were coupled using BOP (221 mg, 0.5 mmol), HOBt (76.5 mg, 0.5 mmol) and DIPEA (64.6 mg, 0.5 mmol) for 2 h as described above. Amino acid analysis [Found (expected): Thr, 2.89 (3); Glu, 1.10 (1); Gly, 0.96 (1); Ala, 1.01 (1); Val, 1.00 (1); Leu, 1.01 (1); Tyr, 0.99 (1); Phe, 0.99 (1); His, 1.29 (1)].

In order to check on the progress of the assembly, a small-scale cleavage was performed and the crude peptide product examined by amino acid analysis, analytical HPLC and MALDITOF-MS. Peptide–resin **10** (30 mg) was cleaved using TFA/water/triethylsilane/ethane-1,2-dithiol (91:3:3:3, v/v/v/v; 2 cm^3) for 2 h and the peptide was isolated as described above. Analytical HPLC [gradient: 15–65% **B**; Fig. 2(*a*)] showed the presence of a single major peak with the expected mass analysis (Table 1). Amino acid analysis [Found (expected): Thr, 2.94 (3); Glu, 1.10 (1); Gly, 1.07 (1); Ala, 1.03 (1); Val, 1.00 (1); Leu, 0.97 (1); Tyr, 0.90 (1); Phe, 0.89 (1); His, 1.26 (1)].

Preparation of Boc-His(Trt)-Thr(OBu^t)-Gly-Phe-(AcHmb)-Leu-Thr(H)-Glu(OBu^t)-Tyr(Allyl)-Val-Ala-Thr(Bu^t)-PepSyn KA (peptide–resin 11; Scheme 2)

Peptide–resin **10** (750 mg, 0.06 mmol) was suspended in DMF (5 cm^3), Ac_2O (61 mg, 0.6 mmol, 10 mol equiv.) and DIPEA (39 mg, 0.3 mmol, 5 mol equiv.) were added, and reaction was allowed to proceed for 1 h. The peptide–resin **11** was washed thoroughly and successively with DMF and diethyl ether and dried *in vacuo* (over KOH pellets). Peptide–resin **11** (30 mg) was cleaved using TFA/water/triethylsilane/ethane-1,2-dithiol (91:3:3:3, v/v/v/v; 2 cm^3) for 2 h and the peptide was isolated as described above. Analytical HPLC showed the presence of a single major peak with the expected mass analysis (Table 1). No evidence for non-Hmb peptide was evident.

Preparation of Boc-His(Trt)-Thr(OBu^t)-Gly-Phe-(AcHmb)Leu-Thr[P(O)(OBzl)$_2$]-Glu(OBu^t)-Tyr(Allyl)-Val-Ala-Thr(Bu^t)-PepSyn KA (peptide–resin 12; Scheme 2)

Peptide–resin **11** (600 mg, 0.048 mmol) was phosphorylated as described above using tetrazole (168 mg, 2.40 mmol, 50 mol equiv.) and dibenzyl *N*,*N*-diisopropylphosphoramidite (384 mm^3, 1.20 mmol, 25 mol equiv.) in acetonitrile (4 cm^3). The peptide–resin suspension was gently agitated and reaction was allowed to proceed for 2 h. The resin was washed with dry acetonitrile after which the phosphitylation was repeated for a further 2 h. The phosphitylating mixture was removed by suction and the peptide–resin was washed with oxidising solution (as described above), then was washed thoroughly and successively with DMF and diethyl ether, and dried *in vacuo* (over KOH pellets).

Peptide–resin **12** (30 mg) was cleaved using TFA/water/triethylsilane/ethane-1,2-dithiol (91:3:3:3, v/v/v/v; 2 cm^3) for 2 h and the peptide was isolated as described above. Analytical HPLC showed the presence of a single major peak with the expected mass analysis (Table 1).

Preparation of Boc-His(Trt)-Thr(OBu^t)-Gly-Phe-(AcHmb)Leu-Thr[P(O)(OBzl)$_2$]-Glu(OBu^t)-Tyr(H)-Val-Ala-Thr(Bu^t)-PepSyn KA (peptide–resin 13; Scheme 2)

Peptide–resin **12** (550 mg, 0.044 mmol) was treated with tetrakistri(phenylphosphine)palladium(0) (154 mg, 0.132 mmol, 3 mol equiv.) in DMF/$CHCl_3$/AcOH/*N*-methylmorpholine (NMM) (18.5:18.5:2:1, v/v/v/v; 6 cm^3) for 3 h. The resin-bound peptide **13** was washed successively with DMF, DMF containing DIPEA (0.5% v/v) and sodium diethyldithiocarbamate (0.5% w/v), DMF and finally diethyl ether. Peptide–resin **13** was dried *in vacuo* (over KOH pellets) overnight.

Peptide–resin **13** (30 mg) was cleaved using TFA/water/triethylsilane/ethane-1,2-dithiol (91:3:3:3, v/v/v/v; 2 cm^3) for 2 h and the peptide was isolated as described above. Analytical HPLC showed the presence of a single major peak with expected mass analysis (Table 1).

Preparation of Boc-His(Trt)-Thr(OBu^t)-Gly-Phe-(AcHmb)Leu-Thr[P(O)(OBzl)$_2$]-Glu(OBu^t)-Tyr[P(O)(OBzl)$_2$]-Val-Ala-Thr(Bu^t)-PepSyn KA (peptide–resin 14; Scheme 3)

Peptide–resin **13** (400 mg, 0.032 mmol) was phosphorylated as described above using tetrazole (112 mg, 1.6 mmol, 50 mol equiv.) and dibenzyl *N*,*N*-diisopropylphosphoramidite (256 mm^3, 0.80 mmol, 25 mol equiv.) in acetonitrile (3 cm^3). The peptide–resin suspension was gently agitated and reaction was allowed to proceed for 2 h; the mixture was then washed with dry acetonitrile after which the phosphitylation was repeated for a further 2 h. The reaction mixture supernatant was removed by suction and the peptide–resin was washed with oxidising solution as described above. The peptide–resin **14** was washed thoroughly and successively with DMF and diethyl ether, and dried *in vacuo* (over KOH pellets).

Prepartion of H-His-Thr-Gly-Phe-(AcHmb)Leu-Thr(PO_3H)-Glu-Tyr(PO_3H)-Val-Ala-Thr-OH(peptide 15; Scheme 2)

Peptide–resin **14** (400 mg, 0.032 mmol) was cleaved using TFA/water/triethylsilane/ethane-1,2-dithiol (91:3:3:3, v/v/v/v; 15 cm^3) for 2 h and the peptide **15** was isolated as described above. Analytical HPLC showed the presence of a single major peak (peptide **15**) with expected mass analysis (Table 1). Amino acid analysis [Found (expected): Thr, 2.49 (3); Glu, 1.13 (1); Gly, 0.91 (1); Ala, 1.04 (1); Val, 1.04 (1); Leu, 0.90 (1); Tyr, 1.04 (1); Phe, 0.85 (1); His, 1.08 (1); 21.5 μmol peptide content].

Preparation of H-His-Thr-Gly-Phe-Leu-Thr(PO_3H)-Glu-Tyr(PO_3H)-Val-Ala-Thr-OH(peptide 17; Scheme 2)

Crude peptide **15** (10 μmol) was suspended in DMF (500 mm^3) and hydrazine hydrate (2.9 mg, 50 μmol, 10 mol equiv.) was added. Reaction was monitored by HPLC, which indicated reaction was complete in 2 h. The crude Hmb-bisphosphopeptide **16** was isolated by ethereal precipitation and centrifugation as described above (see Table 1 for HPLC and mass analysis). Crude peptide **16** was treated with TFA/water/triethylsilane/ethane-1,2-dithiol (91:3:3:3, v/v/v/v; 4 cm^3) for 2 h and the peptide **17** was isolated as described above. Crude peptide **17** was dissolved in 0.1% aq. TFA (10 cm^3); analytical HPLC [Fig. 2(*b*)] showed the presence of a single major peak with expected mass analysis (Table 1).

The crude peptide **17** was purified by preparative HPLC using a gradient of 18–40% B in A and 10 × 1 cm^3 injections. The purified product was lyophilised. Analytical HPLC [Fig. 2(*c*)] gave a single species with retention time 11.19 min (94%) with MALDITOF-MS = 1398 Da (theoretical molecular mass = 1398 Da). Amino acid analysis [Found (expected): Thr, 2.53 (3); Glu, 1.06 (1); Gly, 1.08 (1); Ala, 1.00 (1); Val, 0.96 (1); Leu, 0.98 (1); Tyr, 1.01 (1); Phe, 0.92 (1); His, 0.95 (1); 1.5 μmol peptide content].

References

1 D. B. A. De Bont, W. J. Moree, J. H. van Boom and R. M. J. Liskamp, *J. Org. Chem.*, 1993, **58**, 1309 and references cited therein.
2 *Protein Phosphorylation*, eds. O. M. Rosen and E. G. Krebs, Cold Spring Harbour Laboratory, Cold Spring Harbor, New York, 1981.
3 E. A. Kitas, R. Knorr, A. Trzeciak and W. Bannwarth, *Helv. Chim. Acta*, 1991, **74**, 1314.
4 W. Bannwarth and E. A. Kitas, *Helv. Chim. Acta*, 1992, **75**, 707.
5 J. W. Perich, D. L. Nguyen and E. C. Reynolds, *Tetrahedron Lett.*, 1991, **32**, 4033.
6 D. M. Andrews, J. Kitchen and P. W. Seale, *Int. J. Pept. Protein Res.*, 1991, **38**, 469.
7 L. Otvos, Jr., I. Elekes and V. M.-Y. Lee, *Int. J. Pept. Protein Res.*, 1989, **34**, 129.
8 J. Bedford, C. Hyde, T. Johnson, W. Jun, D. Owen, M. Quibell and R. C. Sheppard, *Int. J. Pept. Protein Res.*, 1992, **40**, 300.
9 T. Johnson, D. Owen, M. Quibell and R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1993, 369.
10 C. Hyde, T. Johnson, D. Owen, M. Quibell and R. C. Sheppard, *Int. J. Pept. Protein Res.*, 1994, **43**, 431.
11 T. Johnson, M. Quibell and R. C. Sheppard, *J. Pept. Sci.*, 1995, **1**, 11.
12 For an overall review of Fmoc chemistry see E. Atherton and

R. C. Sheppard, *Solid Phase Peptide Synthesis: A Practical Approach*, Oxford University Press, 1989.
13 S. B. H. Kent, D. Alewood, P. Alewood, M. Baca, A. Jones and M. Schnollzer, in Innovations and Perspectives in Solid Phase Synthesis, 2nd International Symposium, ed. R. Epton, Intercept, Andover, UK, 1992, p. 1.
14 R. C. de L Milton, S. C. F. Milton and P. A. Adams, *J. Am. Chem. Soc.*, 1990, **112**, 6039.
15 B. Castro, J. R. Domoy, G. Evin and C. Selve, *Tetrahedron Lett.*, 1975, 1219.
16 M. Quibell, W. G. Turnell and T. Johnson, *Tetrahedron Lett.*, 1994, **35**, 2237.
17 S. A. Kates, B. G. de la Torre, R. Eritja and F. Albericio, *Tetrahedron Lett.*, 1994, **35**, 1033.
18 M. Quibell, W. G. Turnell and T. Johnson, *J. Org. Chem.*, 1994, **59**, 1745.
19 M. Quibell, W. G. Turnell and T. Johnson, *J. Chem. Soc., Perkin Trans. 1*, 1993, 2843.
20 H. Kunz and B. Dombo, *Angew. Chem., Int. Ed. Engl.*, 1988, **27**, 711.
21 B. K. Handa and E. Keech, *Int. J. Pept. Protein Res.*, 1992, **40**, 66.
22 J. Zhang, F. Zhang, D. Ebert, M. H. Cobb and E. J. Goldsmith, *Structure*, 1995, **3**, 299.

Paper 5/05324B
Received 8*th August* 1995
Accepted 12*th October* 1995