Int. J. Peptide Protein Res. **33**, 1989, 468–476

Synthesis of phosphopeptides containing *O*-phosphoserine or *O*-phosphothreonine

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Received 23 August, accepted for publication 17 November 1988

Peptides containing phosphoserine or phosphothreonine were synthesized by solid phase methods. Phosphoserine and phosphothreonine were incorporated into peptides using Boc-diphenylphosphono esters of serine and threonine and standard DCC/HOBt coupling. The phenylphosphoesters were not removed when the peptides were cleaved from the resin by HF or by trifluoromethane sulfonic acid, but were subsequently removed by catalytic hydrogenation. Phosphopeptides were purified by HPLC and by Fe^{+3}-Chelex chromatography and their identity verified by mass spectrometry. Two peptides, Leu-Arg-Arg-Ala-Ser(P)-Leu-Gly and Leu-Arg-Arg-Ala-Thr(P)-Leu-Gly, were prepared by both enzymatic and chemical methods and had identical properties.

Key words: peptide phosphorylation; peptide synthesis; phosphopeptides; phosphopeptide synthesis; phosphoserine; phosphothreonine

Phosphorylation and dephosphorylation of proteins represents one of the most widespread and important reactions in the regulation of cellular processes (1). Specific serine, threonine, or tyrosine residues in substrate proteins become phosphorylated by the action of a protein kinase which catalyzes the transfer of phosphate from a high energy nucleoside triphosphate. Major proteins in bones, teeth, eggs, and milk are highly phosphorylated (e.g., casein, phosvitin). Enzymes, receptors and structural proteins become specifically and reversibly phosphorylated as part of mechanisms of cellular response to stimuli (2).

Preparation of phosphopeptides related to sequences of phosphoproteins is important for the study of their properties. Enzymatic methods for the synthesis of phosphopeptides can be useful but are limited to very specific peptide sequences (3). Due to the high specificity of enzymatic reactions, this approach is not suitable as a general approach to phosphopeptide synthesis.

Chemical phosphorylation of peptides/proteins offers an alternative way to prepare peptides with phosphorylated residues, but no general method has been developed which allows the introduction of specific phosphoserine or phosphothreonine residues (4). Dipeptides and tripeptides of phosphoserine have been obtained in 20–60% yield by chemical phosphorylation of free serine hydroxyl groups in protected peptides (5) and a modification of this method was recently used to synthesize the dipeptide Ser(P)Ser(P) (6) and a hexapeptide containing one phosphoserine (7). Applying this method to more complicated models has been less successful, e.g. one trial phosphorylation of an octapeptide with four serine residues gave a mixture of fully and partially phosphorylated peptides (8). An approach which should allow the preparation of synthetic peptides containing phosphorylated residues at specific sites was proposed in 1984 by P.F. Alewood and colleagues (9). Their strategy was to synthesize phosphoserine derivatives with protected phosphoryl and amino groups and to use these derivatives in stepwise liquid phase peptide synthesis. Unfortunately the *N*-(t-butoxycarbonyl)-*O*-(dibenzylphosphono)-L-serine which they employed was not sufficiently stable and reagents commonly used to remove Boc groups during their trial synthesis of GluSer(P)Leu caused considerable debenzylation of the phosphoserine derivative (10).

We have synthesized peptides by the solid phase method and employed the easily introduced and stable diphenylphosphono esters of serine and threonine (11). Suitable reagents were synthesized, [*N*-(t-butoxycarbonyl)-*O*-(diphenylphosphono)-L-serine and *N*-(t-butoxycarbonyl)-*O*-(diphenylphosphono)-L-threo-

nine], and used to prepare several analogs of bovine rhodopsin's carboxyl-terminal sequence. We found the solid phase methods suitable for the synthesis of diphenyl phosphopeptides and that the phosphoryl derivatives were sufficiently stable in our typical peptide-resin cleavage conditions using liquid HF. Initially, we deprotected the diphenyl phosphotriesters while still attached to the resin by reaction with tetrabutylammonium fluoride (TFAF) and then cleaved the peptide from the resin with HF (11). Subsequently, we found that TBAF often attacks the phosphoester bond (unpublished results) and can, therefore, yield a mixture of phosphorylated and unphosphorylated peptides. We have recently employed catalytic hydrogenolysis to remove the diphenyl protecting groups following some recent improvements (6, 7, 12) of an old method (13). In this report, we describe in detail the synthesis of the protected phosphoamino acid derivatives of both serine and threonine, their use in the solid phase synthesis of peptides, and the catalytic hydrogenolysis procedure used to remove the phosphate protecting groups. We have applied these techniques to the synthesis of four model peptides containing either phosphoserine or phosphothreonine residues and present the methods used for their purification and characterization.

MATERIALS AND METHODS

Reagents
t-Butoxycarbonyl (Boc)-protected amino acids were purchased from Bachem Inc. Pam-amino acid resins were from Applied Biosystems Inc., trifluoroacetic acid (TFA), diisopropylethylamine (DIEA), 1-hydroxybenzotriazole (HOBt), dichloromethane (DCM), dimethylformamide (DMF), trichloroacetic acid (TCA) were from Fisher Scientific. Hydrogen fluoride (HF) was from Matheson and 1,3-diisopropylcarbodiimide (DIC), 4,4′-dimethoxytrityl chloride (DMT-Cl), anisole, diphenyl chlorophosphate, dicyclohexylamine (DCHA), trifluoromethanesulfonic acid (TFMSA), methyl sulfide (DMS), p-cresol and platinum oxide were from Aldrich. The catalytic subunit of protein kinase was from Sigma. $\gamma[^{32}P]$-ATP was purchased from New England Nuclear.

Solid phase peptide synthesis
Peptides were synthesized from Boc-amino acids using an automated synthesizer (model PSS80, Applied Protein Technologies, Cambridge, MA). The side-chain functional groups were protected as follows: 2-chlorobenzyloxycarbonyl for lysine, tosyl for arginine, benzyl for serine and threonine and diphenyl phosphotriester for phosphoserine and phosphothreonine. Syntheses were performed at the 0.3 mmol scale in a 24 mL reaction vessel using Pam resin and the DIC/HOBt coupling strategy. A typical protocol of synthesis is as follows: 1) wash with DCM 3 × 1 min, 2) deblock with TFA 1 × 1 min, then 1 × 19 min, 3) wash with DCM 6 × 1 min, 4) neutralize with 10% DIEA 2 × 1 min, 5) wash with DCM 6 × 1 min, 6) add Boc amino acid 4 × molar excess, 7) couple in DCM-DMF (4:1) for 30 min, then couple in DCM-DMF (1:1) for 15 min, 8) wash with DCM 3 × 1 min, 9) monitor free amino groups using DMT-Cl (14). If the efficiency of the reaction was less then 99.5%, steps 4–9 were repeated.

Peptides were cleaved from resin using HF/anisole (9:1 v/v) for 45 min at 0°.

Fast atom bombardment mass spectrometry (FABMS)
Dried sample aliquots were dissolved in water and 1 μL of the solution containing approximately 2 nmol peptide was mixed with 1.5 μL of 90% glycerol 40 mM oxalic acid on the copper target of a sample probe. The probe was introduced into a Kratos MS50RF mass spectrometer equipped with an Ion Tech B11NF saddle field gun operated at 8 kV and 40 μa of current using Xe to create energetic atoms. The spectrometer was generally operated at a resolving power of 1500 and an accelerating voltage of 8 kV. The instrument was set to scan the mass range from 1200 or 1600 to 500 amu and data were collected with multichannel analyzer programs available on the DS90 data system. CsI was used to calibrate the instrument.

Synthesis of protected O-phosphoserine
Boc-L-SerOBzl (1 g, 3.385 mmol) was dissolved in dry pyridine (8.5 mL) and the solution was cooled to 0°. To the stirred solution on ice, diphenyl chlorophosphate (0.77 mL, 3.72 mmol) was added. The mixture was stirred overnight at room temperature, diluted with chloroform (30 mL), and washed successively with H_2O, 1 N HCl, and H_2O (2 × 20 mL each). After drying (Na_2SO_4) and solvent evaporation, the solid was crystallized from ethyl acetate-hexane and gave white needles of *N*-(t-butoxycarbonyl)-*O*-(diphenylphosphono)-L-serine benzyl ester (*1*) (1.55 g, 86%). In the same manner, from 6.8 g Boc-L-SerONBzl 10.2 g (89%) of (*2*) was obtained.

N-(t-Butoxycarbonyl)-O-(diphenylphosphono)-L-serine (3). *a) from* (1): Hydrogen was bubbled 3 h through a solution of 1.5 g (2.8 mmol) of (*1*) in 40 mL of a mixture of methanol and ethyl acetate (1:1 v/v) containing 170 mg of suspended catalyst (5% Pd/C). Catalyst was removed by filtration, washed with methanol and the solvents evaporated. The oily residue crystallized after a few hours at room temperature. After recrystallization from isopropanol-water, 1.18 g (95%) of (*3*) was obtained.

b) from (*2*): Hydrogenolysis of (*2*) was performed under conditions similar to that of (*1*) but the oily

residue obtained after evaporation of solvent was dissolved in ethyl acetate and washed with 1 N HCl (6 times) and H_2O (3 times) in order to remove *p*-toluidine. The organic layer was dried (Na_2SO_4), evaporated, and the residue was crystallized from isopropanol-water. From 4 g (7 mmol) of (*2*), 2.86 g (93.5%) of (*3*) was obtained. Physical properties were identical to the compound obtained from (*1*).

DCHA salt of N-*(t-butoxycarbonyl)*-O-*(diphenylphosphono)*-L-*serine (4)*. To a solution of 218.7 mg (0.5 mmol) of (*3*) in 6 mL ethyl ether, 0.1 mL (0.5 mmol) dicyclohexylamine was added. White crystals of (*4*) were collected. The yield was 294 mg (95%).

Synthesis of protected O-*phosphothreonine*

Using conditions described for the synthesis of (*1*), 10.86 g (35.1 mmol) of Boc-L-ThrOBzl yielded a colorless oil of *N*-(t-butoxycarbonyl)-*O*-(diphenylphosphono)-L-threonine benzyl ester (*5*) (17.68 g, 93%). T.l.c. of the oil was performed using three solvent systems on silica gel sheets (Kodak #13179). After chromatography, the sheets were exposed for 5 min to HCl vapors to remove Boc groups and the derivative was visualized with a ninhydrin spray. The homogeneous product was used for the synthesis of (*7*) without further purification. Following the same procedure, 4.94 g (13.9 mmol) of Boc-L-ThrONBzl yielded 7.74 g (95%) of noncrystalline (*6*) which was used for synthesis of (*7*) without purification.

DCHA salt of N-*(t-butoxycarbonyl)*-O-*(diphenylphosphono)*-L-*threonine* (7). *a) from* (*5*): 17.68 g (32.6 mmol) of (*5*) were submitted to hydrogenolysis under conditions similar to those used to convert (*1*) to (*3*). The product was dissolved in 400 mL of ethyl ether-hexane (1:1 v/v) and *DCHA* (6.4 mL, 32 mmol) was added. After 12 h at 0°, 18.51 g (90%) of white needles of (*7*) were obtained.

b) from (*6*): 7.74 (13.9 mmol) of (*6*) was submitted to hydrogenolysis as described for the conversion of (*5*) to (*7*) and 7.74 g (88%) of (*7*) was obtained. The physical properties were identical to those of (*7*) obtained from (*5*).

Peptide purification

Peptides were purified by preparative HPLC on a Whatman Partisil 10 ODS-3 column (22 × 250 mm) using a Beckman/Altex HPLC. Elution was performed with a linear gradient from 0.1% aqueous TFA to 30–40% 0.1% TFA in acetonitrile-isopropanol (2:1) in 40 min at 14 mL/min with the eluent monitored at 220 nm, AUFS = 0.3 (system A). Alternatively, peptides were purified using a Waters cation exchange cartridge in an RCM 100 module, Partisil SCX 8 mm × 10 cm, and eluting isocratically at 1 mL/min with 5% acetonitrile in 10 mM formate-triethylamine, pH 3.0 (System B). The eluent was monitored at 230 nm (0.3 AUFS).

Analytical HPLC was performed on a reverse phase Whatman Partisil 5 ODS-3 column (4.6 × 250 mm) using a linear gradient of aqueous 0.1% H_3PO_4 to 0.1% H_3PO_4 in acetonitrile in 40 min at 1 mL/min with the eluent monitored at 210 nm with AUFS = 0.25 (system C). System D used the above Waters cation exchange cartridge (Partisil SCS 8 mm × 10 cm) with a gradient from 10% acetonitrile in 20 mM sodium phosphate, pH 2.35 to 10% acetonitrile in 0.5 M NaCl, 20 mM sodium phosphate, pH 7.0, over 40 min and monitoring at 210 nm.

Synthesis of hexapeptide Leu-Gln-Thr-Val-Lys-Ala (8), *Leu-Gln-Thr(P-Ph$_2$)-Val-Lys-Ala* (9), *and Leu-Gln-Thr(P)-Val-Lys-Ala*-(10)

Peptide (*8*) was synthesized using solid phase methodology starting with Boc-Ala-Pam resin (0.3 mmol, 0.76 mmol/g). The synthetic protected peptide was deprotected and cleaved from the resin and purified by HPLC in system A, 168 mg (85%) of (*8*) was obtained.

Phenyl-blocked phosphopeptide was synthesized in the same way using Boc-ThrO(P-Ph_2) (obtained by treating the DCHA salt (*7*) with a mixture of 1 M H_2SO_4 and ethyl acetate) instead of Boc-ThrOBzl. Peptide (*9*) was cleaved from the resin by two different procedures.

a) *HF cleavage of phosphopeptide from resin.* Protected peptide-resin (301 mg; 0.15 mmol) was treated with HF as described earlier. Evaporation of the reagents and extraction of the residue with 10% acetic acid (200 mL) gave a solution of the crude peptide (*9*) which retained the phenyl blocking groups on phosphothreonine (129.6 mg; 97.1%). Peptide (*9*) was homogeneous by analytical HPLC (systems C and D).

b) *TFMSA cleavage of phosphopeptide from resin.* Peptide-resin (301 mg; 0.15 mmol) was stirred with 5 mL of cleavage mixture (10% TFMSA, 50% TFA, 34.7% DMS, 5.3% p-cresol v/v) 4 h at 0°. The mixture was lyophilized under high vacuum overnight. Peptide (*9*) was extracted from the resin with water and was purified by preparative HPLC using system A (84.4 mg, 63.2%).

Removal of phenyl blocking groups from the peptide phosphotriester

133.5 mg (0.15 mmol) of phenyl-protected peptide (*9*) was dissolved in 2 mL of 40% TFA/AcOH, and 72 mg (1 equiv.) of amorphous platinum (IV) oxide was added. Hydrogenolysis was performed for 24 h at room temperature under 4.05 bar (4 atm) of hydrogen pressure. The mixture was evaporated to dryness under vacuum, suspended in water, and the catalyst removed by filtration. Pure peptide (*10*) was obtained by preparative HPLC in system B; (81.9 mg; 74%).

Synthesis of kemptide (11) *and [Thr⁵]kemptide* (12)

Kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly (*11*), was synthesized from Boc-Gly Pam resin (0.3 mmol, 0.78 mmol/g) using solid phase methodology. Following preparative HPLC purification in system A, 195 mg (84.3%) of pure peptide was obtained.

[Thr^5]kemptide, Leu-Arg-Arg-Ala-Thr-Leu-Gly (*12*) was synthesized by the same procedure. Following HPLC purification, 180 mg (76.2%) of pure peptide was obtained.

Enzymatic preparation of phosphokemptide (13)

The enzymatic phosphorylation of kemptide was performed following the procedure of Kemp *et al.* (16).

36 μmol of kemptide (*11*) were dissolved in 4 mL of 60 mM potassium phosphate buffer, pH 7.5, containing 30 mM γ[-^{32}P]-ATP (35 000 cpm/nmol) and 25 mM Mg $(OAc)_2$. The catalytic subunit of protein kinase A (8 μg) was added and the reaction mixture was maintained at room temperature. The progress of the reaction was monitored by measuring incorporation of radioactive phosphate into the peptide. At various times 50 μL of the mixture were withdrawn and mixed with 200 μL of 30% acetic acid. Aliquots of the above mixture (150 μL) were passed through ca. 1.5 mL Dowex 1 × 8 columns (in Pasteur pipettes) that had been equilibrated with 30% acetic acid. ATP binds to the column but neither phosphorylated nor unphosphorylated peptides bind. Counting the unbound fraction, therefore, yielded the amount of phosphate incorporated into the peptide. After 300 min, the whole mixture was passed through a 1 × 10 cm Dowex 1 × 8 column in 30% acetic acid. Fractions (1 mL) were collected and counted and the fractions containing the phosphorylated kemptide were combined and lyophilized. The phosphokemptide was then dissolved in 3 mL of 1% dicyclohexylamine and 5 mL ethanol, lyophilized, and stored desiccated at $-20°$. The yield of the above procedure was near 100%.

Enzymatic preparation of [Thr(P)⁵]kemptide (14)

When the threonine analog of kemptide was treated essentially as described above for kemptide (*11*), the yield of [Thr(P)^5]kemptide obtained was only 1%. Since threonine kemptide is a poor substrate for protein kinase A (16), some changes were introduced to maximize the amount of [Thr(P)^5]kemptide produced. The amount of catalytic subunit was increased to 400 μg, the time of phosphorylation was extended to 24 h, pH was increased to 8.2 and [Mg^{++}] to 50 mM, and the amount of [Thr^5]kemptide was reduced to 2 μmol. Under these conditions the yield of the reaction was 71%. Separation of the phosphorylated from the unphosphorylated [Thr^5]kemptide was achieved using either cation-exchange or Fe^{+3}-Chelex chromatography as described below.

Chemical synthesis of phosphokemptide (13) *and phospho[Thr⁵]kemptide* (14)

Phosphokemptide (*13*) was synthesized using solid phase methodology in the same manner as kemptide (*11*) except that Boc-SerO(P-Ph_2)(*5*) was used instead of Boc-SerOBzl. After HF cleavage, the crude protected-phosphoserine peptide was dissolved in 1.5 mL of 40% TFA/AcOH, 145 mg PtO_2 was added and the mixture submitted to hydrogenolysis in the same manner as peptide (*9*). Phosphokemptide (*13*) was purified by HPLC in system B. 161.9 mg (63.4%) of pure peptide was obtained.

Phospho[Thr^5]kemptide (*14*) was synthesized by the same method as for (Thr^5)-kemptide (*12*) using Boc-ThrO(P-Ph_2) instead of Boc-ThrOBzl. The peptide was submitted to hydrogenolysis and purified under the conditions used to prepare phosphopeptide (*13*) and yielded 103 mg (41.1%).

Affinity chromatography of phosphopeptide on Chelex-column

After enzymatic synthesis of phosphokemptide (*13*) or [Thr(P)^5]kemptide (*14*) and separation of excess ATP from peptides on Dowex 1X8 resin, the phosphopeptides were separated from the unphosphorylated peptide substrate by affinity chromatography on a Fe^{+3}-Chelex column (17, 18).

To prepare the column, Chelex gel (iminodiacetic acid epoxy activated Sepharose 6B, Sigma No. I-8507) was suspended in 0.1% acetic acid, degassed and packed into a Pasteur pipet (about 1 mL gel). Then 1 mL 0.1 M $FeCl_3$ was passed through the column. The gel was washed with 20 mL of 0.1% acetic acid in order to remove excess Fe^{+3} ions.

Phosphopeptide (typically 1 to 1000 nmol) was dissolved in 1 mL 0.1% acetic acid and loaded on the above Chelex column. The column was washed with 10 mL of 0.1% acetic acid to elute all unphosphorylated peptide, and then washed with 10 mL H_2O. The phosphopeptide was eluted with 0.1% dicyclohexylamine adjusted to pH 8.2 with acetic acid and then lyophilized. Recovery of radioactive peptide was about 80%. The phosphopeptide was free of unphosphorylated substrate as shown by ion exchange chromatography. The phosphopeptide is stable for at least 1 year when stored dry as the cyclohexylammonium salt at $-20°$.

RESULTS

In order to develop methods for the synthesis of peptides containing phosphoserine or phosphothreonine, we have first prepared protected phosphoesters of serine and threonine which are stable under the conditions of solid phase peptide synthesis. We have prepared and characterized the diphenylphosphono-derivatives of serine- and threonine-*O*-benzyl (and

TABLE 1

Physicochemical properties of protected phosphoserine and phosphothreonine derivatives

Derviative	Formula	M.p. (°C)	Yield %	Formula	Elemental analysis calc/found C	H	N	P	^{31}P-n.m.r. $CDCl_3/P(O\ CH_3)_3$[a] δ[ppm]
1	Boc-L-SerO(P-Ph_2)OBzl	109–109.5	87	$C_{27}H_{30}NO_8P$	61.6	5.73	2.66	5.87	
					61.7	5.70	2.72	5.78	
2	Boc-L-SerO(P-Ph_2)ONBzl	118–118.5[b]	89	$C_{27}H_{29}N_2O_{10}P$	56.6	5.11	4.89	5.41	
					56.4	5.13	4.66	5.29	
3	Boc-L-SerO(P-Ph_2)OH	74.5–75.5[c]	95[d]	$C_{20}H_{24}NO_8P$	54.9	5.53	3.20	7.08	− 13.55[f]
			93.5[e]		54.8	5.56	3.09	7.07	
4	Boc-L-SerO-(P-Ph_2)-OH × DCHA	110–112(dec)	95	$C_{32}H_{47}N_2O_8P$	62.1	7.66	4.53	5.01	
					61.9	7.72	4.46	4.81	
5	Boc-L-ThrO(P-Ph_2)OBzl	oil[g]	93						
6	Boc-L-ThrO(P-Ph_2)ONBzl	oil[h]	95						
7	Boc-L-ThrO(P-Ph_2)OH × DCHA	187–184(dec)	90[i]	$C_{33}H_{49}N_2O_8P$	62.7	7.80	4.43	4.90	− 13.932
					62.6	7.60	4.35	5.06	

[a]General Electric NT-300, ref $P(OCH_3)_3$ at − 140 ppm.
[b]Lit. 113–114.5° (15).
[c]Lit. 62–64° (15).
[d]From *1*.
[e]From *2*.
[f]Lit. 12.5 ppm $CDCl_3$/95% P_3PO_4 (15).
[g]Homogeneous by t.l.c. on silica gel with Rf 0.50 in $CHCl_3$; 0.66 in benzene-acetone (3:1), and 0.92 in $CHCl_3$-AcOH (95:5).
[h]Homogeneous by t.l.c. on silica gel with Rf 0.56 in $CHCl_3$; 0.70 in benzene-acetone (3:1), and 0.91 in CH_3Cl-AcOH (95:5).
[i]From *5*.
[j]From *6*.

O-nitrobenzyl) esters (see Methods). The physicochemical properties of these derivatives are presented in Table 1. Using these compounds we have synthesized the model peptide *10* (Leu-Gln-Thr(P)-Val-Lys-Ala) by the solid phase approach. Peptide *8*, an analogue containing threonine (Leu-Gln-Thr-Val-Lys-Ala), was synthesized for purposes of comparison. For synthesis of peptide *10*, we employed the threonine derivative *N*-(Boc)-*O*-diphenylphosphono-L-threonine. The protected amino acid was incorporated using the standard DCC/HOBt chemistry with yields of >99.5% per cycle as verified by the automated monitoring reaction (14). In the present study, we have removed the peptide from the resin using liquid HF/anisole. Analysis of the crude product by HPLC shows that there is a single major product and that no dephosphorylated peptide is present (Fig. 1a).

Although most blocking groups typically used in synthesis of peptides by methods employing Boc amino acids are removed by HF or TFMSA, we find that the phenyl phosphoester group is not removed. Mass spectral analysis of the HF-cleaved peptide shows that the dominant product has m/z = 891.1; i.e., it is the diphenylphosphonopeptide (*9*) in which the threonine retains both phenyl groups (Fig. 2a). A small amount of peptide containing one phenyl group is observed (m/z = 814.9) but there is no product in which both phenyl groups have been removed (m/z = 739.4). Similar results are obtained when the peptide is removed from the resin by TFMSA.

Once the peptide has been cleaved from the resin, removal of phenyl groups was accomplished by catalytic hydrogenation. The reaction is conveniently monitored by HPLC and is complete after 24 h. A single major product is formed (Fig. 1b). The phenyl blocking groups have been removed (loss of peptide *9*) and there is no evidence of dephosphorylation to produce the nonphosphorylated peptide (peptide *8*). Identity of the product as the phosphopeptide (peptide *10*, Leu-Gln-Thr(P)-Val-Lys-Ala) is provided by mass spectral analysis, m/z = 739.4 (Fig. 2b). For comparison, the mass spectrum of the synthetic nonphosphorylated peptide is presented in Fig. 2c (m/z = 659.1).

The same strategy was effective in producing analogous peptides containing phosphoserine. *N*-(Boc)-*O*-(diphenylphosphono)-L-serine (*3*) was prepared and used to synthesize the blocked serine-containing peptide analogue of peptide *9*. This peptide was successfully cleaved from the resin and converted to the corresponding phosphopeptide by catalytic hydrogenation. HPLC, mass spectral and amino acid analyses confirmed the identity of the peptides (data not shown).

In order to further extend this work, we sought to prepare phosphopeptides by both chemical and enzymatic methods and to establish by multiple criteria that the products were identical. The heptapeptide substrate of protein kinase A, Leu-Arg-Arg-Ala-Ser-Leu-Gly [peptide *11*; known as kemptide (16)] was synthesized and submitted to enzymatic phosphorylation. Conversion of substrate to product was followed by incorporation of ^{32}P from [^{32}P]ATP yielding phosphokemptide, peptide *13*. The reaction followed a linear time course, reaching 100% completion in 220 min. The reaction was also monitored by HPLC (Fig. 3). Kemptide (peptide *11*) and phosphokemptide (peptide *13*) are well separated by ion exchange chromatography (Fig. 3) but their elution times by reverse phase chromatography are too close to achieve com-

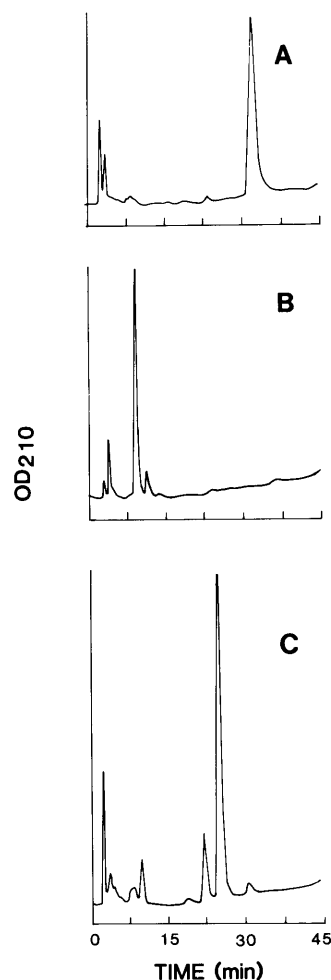

FIGURE 1
Cation exchange HPLC using a Waters Partisil SCX cartridge in a RCM 100 module and a linear gradient from 100% A (10% acetonitrile in 20 mM $NaPO_4$, pH 2.35) to 100% B (10% acetonitrile in 0.5 M NaCl, 20 mM $NaPO_4$, pH 7.0) in 40 min. Panel a = peptide *9* the diphenyl protected peptide Leu-Gln-Thr(P-Ph_2)-Val-Lys-Ala after cleavage from the resin. Panel b = peptide *10* [Leu-Gln-Thr(P)-Val-Lys-Ala], which is produced from peptide *9* by hydrogenolysis. Panel c = peptide *8*, the nonphosphorylated peptide Leu-Gln-Thr-Val-Lys-Ala.

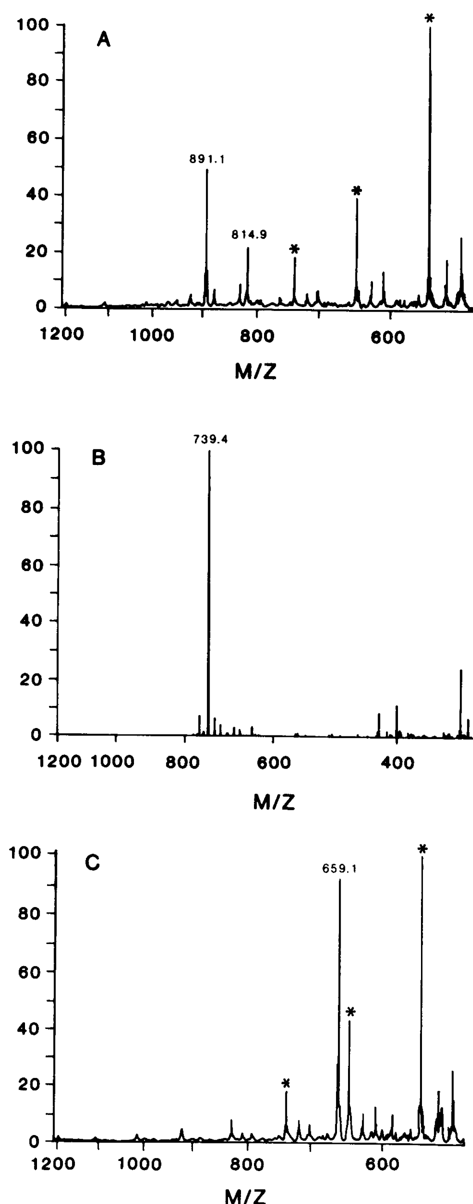

FIGURE 2

Fast atom bombardment mass spectra of peptides. Panel a = peptide *9* the diphenylphosphopeptide Leu-Gln-Thr(P-Ph_2)-Val-Lys-Ala. Panel b = peptide *10*, the phosphopeptide Leu-Gln-Thr(P)-Val-Lys-Ala. Panel c = peptide *8*, the nonphosphorylated peptide Leu-Gln-Thr-Val-Lys-Ala. Ions marked with an asterisk result from the glycerol matrix used in the analyses.

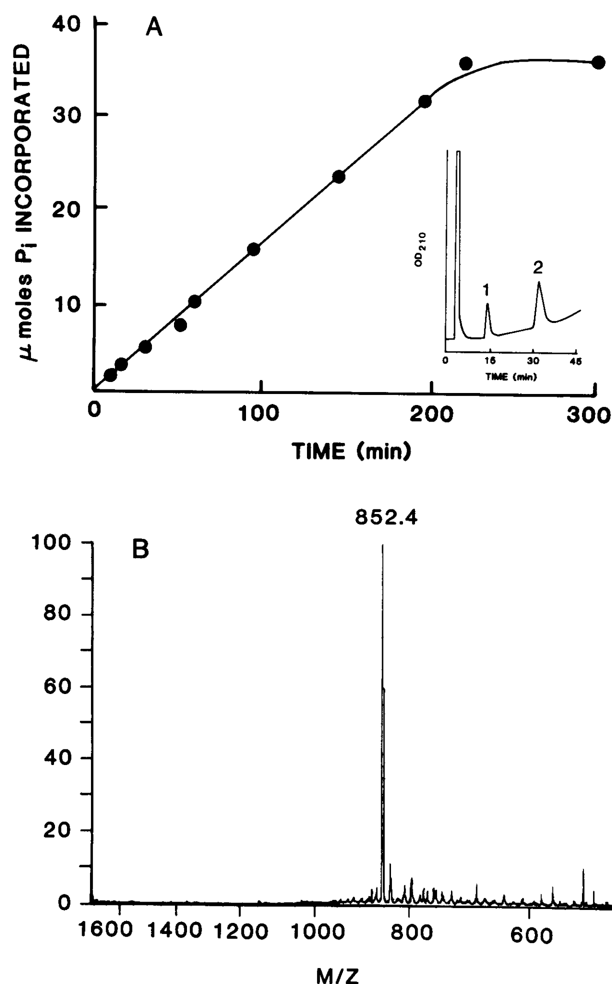

FIGURE 3

(A) Time course of phosphorylation of kemptide (peptide *11*, Leu-Arg-Arg-Ala-Ser-Leu-Gly). Inset: Cation exchange HPLC separation of phosphorylated and unphosphorylated kemptide at 75 min. Peak 1 is phosphorylated kemptide and peak 2 is the unphosphorylated kemptide substrate. (B) Fast atom bombardment mass spectrum of chemically synthesized Leu-Arg-Arg-Ala-Ser(P)-Leu-Gly, the principle m/z = 852.4 shows that the desired product has been synthesized.

plete separation. Peptide *13* was separated from excess [^{32}P]ATP by Fe^{+3}-Chelex chromatography and was further purified by ion exchange chromatography.

Peptide *13* was also synthesized by solid phase methods using the Boc derivative of diphenylphosphono-serine (*3*). The crude peptide, following removal of phenyl groups by hydrogenolysis, showed an identical retention time by HPLC by comparison with the phosphopeptide prepared by the enzymatic method. Mass spectral analysis (Fig. 3B) and the nature of the peptide verified that the two peptides, synthesized by enzymatic and chemical methods, were identical.

In order to make the same comparison of enzymatic vs. chemical synthesis of phosphopeptides containing phosphothreonine, the analogue of kemptide (peptide *11*) was synthesized replacing serine with threonine. This peptide (Leu-Arg-Arg-Ala-Thr-Leu-Gly; peptide *12*) was known to be a poor substrate for protein

TABLE 2
Amino acid and mass spectral analyses of synthetic peptides

Peptide	Sequence	Yield of synthesis	Leu	Glx	Thr	Val	Lys	Ala	Arg	Ser	Gly	FABMS (MH^+) m/z
8	LQTVKA	85	1.00	0.98	0.84	1.03	1.06	1.06				calc 659.4
			(1)	(1)	(1)	(1)	(1)	(1)				found 659.1
9	LQT(P-Ph_2)VKA	97.1[a]	1.01	1.00	0.80	0.94	1.02	0.97				calc 891.5
		63.2[b]	(1)	(1)	(1)	(1)	(1)	(1)				found 891.1[c]
10	LQT(P)VKA	74	1.04	1.00	0.68	0.85	1.08	0.97				calc 739.4
			(1)	(1)	(1)	(1)	(1)	(1)				
11	LRRASLG	84.3	2.06					0.97	1.96	0.91	1.00	
			(2)					(1)	(2)	(1)	(1)	
12	LRRATLG	76.2	2.04		0.87			0.98	1.89		1.00	
			(2)		(1)			(1)	(2)		(1)	
13	LRRAS(P)LG	63.4	2.07					1.03	1.89	0.82	1.00	calc 852.5
			(2)					(1)	(2)	(1)	(1)	found 852.4
14	LRRAT(P)LG	41.1	2.04		0.87			1.06	1.84		1.00	calc 866.5
			(2)		(1)			91)	(2)		(1)	found 866.5

[a]HF cleavage from resin.
[b]TFMSA cleavage from resin.
[c]A small amount of the monophenyl ester was observed at m/z 814.7 compared to the theoretical 815.4.

kinase A, and the amount of peptide *14* produced was only 1% under conditions used for phosphorylation of kemptide (as measured by recovery of ^{32}P product recovered from HPLC ion exchange chromatography). When the synthesis was repeated under more optimal conditions, including an increased kinase concentration (see Materials and Methods), 71% of phosphopeptide product was obtained. The chemically and enzymatically synthesized phosphopeptides proved to be identical when subjected to HPLC (ion exchange and reverse phase) and to mass spectral analysis.

When the phosphopeptides were prepared and stored dry without conversion to the DCHA salt, dephosphorylated peptide was observed after prolonged storage even at $-20°$. However, storing the DCHA salt of the phosphopeptide resulted in no observable dephosphorylation for at least 6 months of storage at $-20°$.

All peptides were subjected to amino acid analysis after HPLC purification. The analyses as well as overall yield of the syntheses and peaks observed on FABMS are presented in Table 2.

CONCLUSION

Previous attempts to prepare phosphopeptides have had several shortcomings. In chemically phosphorylating peptides, the yields were not high. While this presents little difficulty for small peptides and/or peptides with only one serine or threonine residue, it presents a major problem with peptides containing multiple threonine and serine residues. Secondly, when multiple serine and threonine residues are present, there is no control over where the phosphates will be introduced. Previously, techniques were developed to synthesize protected phosphopeptides essentially as presented here (11), though deprotection and storage resulted in some dephosphorylation (unpublished data) and, therefore, a heterogeneous product. In the current work, these problems have been surmounted. We have been able to produce four phosphopeptides and store them for prolonged periods as a dry salt with no decomposition. While the peptides described here contain only one phosphate group, these techniques should be directly applicable to preparing multiply-phosphorylated peptides. Their synthesis and characterization is in progress. The objective is to be able to introduce phosphoserine or phosphothreonine into any desired position of any synthetic peptide. Our current results indicate that these techniques should be able to accomplish this goal.

ACKNOWLEDGMENT

This work was supported by grants EY 06225 and EY 06226 from the National Eye Institute and by an unrestricted departmental grant from Research to Prevent Blindness, Inc. P.A.H. was supported by a Jules and Doris Stein Professorship from Research to Prevent Blindness, Inc.

REFERENCES

1. Weller, M. (1979) *Protein Phosphorylation*, Pion Ltd., London
2. Edelman, A.M., Blumenthal, D.K. & Krebs, E.G. (1987) *Ann. Rev. Biochem.* **56**, 567–613

3. Blumenthal, D.K. (1988) in *Peptides and Protein Phosphorylation* (Kemp, B.E. & Alewood, P.F., eds.), CRC Press, Cleveland, in press
4. Frank, A.W. (1984) *CRC Crit. Rev. Biochem.* **16**, 51–101
5. Folsch, G. (1967) *Svensk Kem. Tidskr.* **79**, 38–66
6. Schlesinger, D.H., Buku, A., Wyssbrod, H.R. & Hay, D. (1987) *Int. J. Peptide Protein Res.* **30**, 257–262
7. Grehn, L., Fransson, B. & Ragnarsson, U. (1987) *J. Chem. Soc. Perkin Trans.* **1**, 529–535
8. Alewood, P.F., Johns, R.B. & Perich, J.W. (1981) in *Peptides: Proceedings of the Seventh American Peptide Symposium* (Rich, D.H. & Gross, E., eds.), pp. 65–67, Pierce Chemical Co., Rockford, IL
9. Alewood, P.F., Perich, J.W. & Johns, R.B. (1984) *Aust. J. Chem.* **37**, 429–433
10. Alewood, P.F., Perich, J.W. & Johns, R.B. (1984) *Tetrahedron Lett.* **25**, 987–990
11. Arendt, A. & Hargrave, P.A. (1985) in *Peptides: Structure and Function* (Deber, C.M., Hruby, V.J. & Kopple, K.P., eds.), pp. 237–240, Pierce Chemical Co., Rockford, IL
12. Perich, J.W., Alewood, P.F. & Johns, R.B. (1986) *Tetrahedron Lett.* **27**, 1373–1376
13. Sciarini, L.J. & Fruton, J.S. (1949) *J. Am. Chem. Soc.* **71**, 2940
14. Horn, M. & Novak, C. (1987) *Am. Biotechnol. Lab.* **5**, 12–21
15. Perich, J.R., Alewood, P.A. & Johns, R.B. (1986) *Synthesis*, 572–573
16. Kemp, B.E., Graves, D.J., Benjamin, E. & Krebs, E.G. (1977) *J. Biol. Chem.* **252**, 4888–4894
17. Muszynska, G., Anderson, L. & Porath, J. (1986) *Biochemistry* **25**, 6850–6853
18. Michel, H., Hunt, D.F., Shabanowitz, J. & Bennet, J. (1988) *J. Biol. Chem.* **263**, 1123–1130

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