Practical Synthesis of Phosphopeptides using Dimethyl-protected Phosphoamino Acid Derivatives

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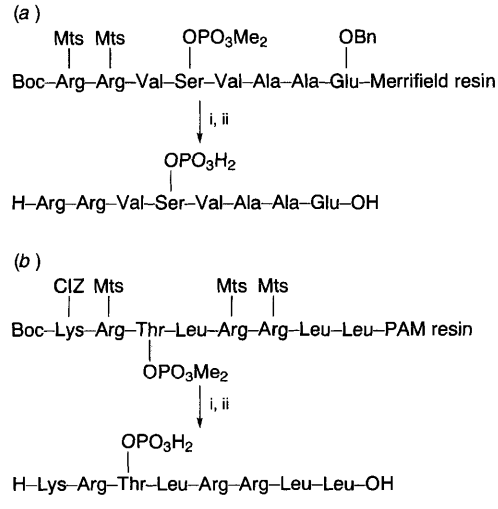
A practical synthesis of phosphopeptides was achieved using a combination of dimethyl-protected phosphoamino acids and two-step deprotection protocols consisting of high acidic (S_N1/S_N2) and low acidic (S_N2) reagent systems.

Protein phosphorylation provides structural and functional changes for proteins involved in intracellular signal transduction pathways.[1] The establishment of practical procedures for the synthesis of phosphopeptides is important to facilitate the elucidation of the function of proteins constituting signal transduction pathways. A number of methods for the synthesis of phosphopeptides have been published.[2] However, except for a few examples,[3] practical methodologies with wide applicability have not been developed yet. Previously, we reported the synthesis of 4-phosphono(difluoromethyl) phenylalanine (F_2Pmp)-containing peptides as nonhydrolysable phosphotyrosyl peptide mimetics.[4] In this synthesis, a diethyl-protected phosphonate derivative [F_2Pmp($OEt)_2$] was utilized for incorporation of F_2Pmp into peptide chains. Ethyl protection was revealed to be efficiently removed with a TMSOTf-sulfide (Me_2S or thioanisole Me_2S)† reagent–system which operates by an S_N2 mechanism.[5] This fact prompted us to apply the TMSOTf-sulfide reagent system to the synthesis of phosphopeptides using Boc–Ser(OPO_3Me_2)–OH‡ and Boc–Thr(OPO_3Me_2)–OH as protected phosphoamino acids. To our knowledge, Ser/Thr(OPO_3Me_2) derivatives have been rarely employed for the practical synthesis of phosphopeptides because harsh acid treatment, which results in side reactions, is required for complete removal of methyl groups.[6] Herein we report the evaluation of several deprotection methods for Ser(OPO_3Me_2) and Thr(OPO_3Me_2) residues in peptides with application of these methods to the practical synthesis of phosphopeptides.

To examine the efficiency of methods for deprotection and cleavage of Ser(OPO_3Me_2)-containing peptide resins, we synthesized a protected peptide resin [Boc-Arg(Mts)-Arg(Mts)-Val-Ser(OPO_3Me_2)-Val-Ala-Ala-Glu(OBn)-Merrifield resin (a partial sequence of the cAMP dependent protein kinase regulatory subunit)] by standard Boc-based solid-phase techniques. Mts and Bn groups were employed for protection of guanidino group of Arg and of carboxyl group of Glu,



Scheme 1 Deprotection for the synthesis of phosphopeptides: (*a*) Ser(OPO_3H_2)-containing peptide; (*b*) Thr(OPO_3H_2)-containing peptide. *Reagents and conditions*: (*a*): i, 1 mol dm^{-3} TMSOTf–thioanisole–TFA, *m*-cresol, EDT, room temp., 1 h; ii, Addition of Me_2S, room temp., 2 h. (*b*): i, 1 mol dm^{-3} TMSOTf–thioanisole–TFA, *m*-cresol, EDT, 4 °C, 1.5 h; ii, Addition of Me_2S–TMSOTf, 4 °C, 3 h, then TMSOTf, room temp., 1 h.

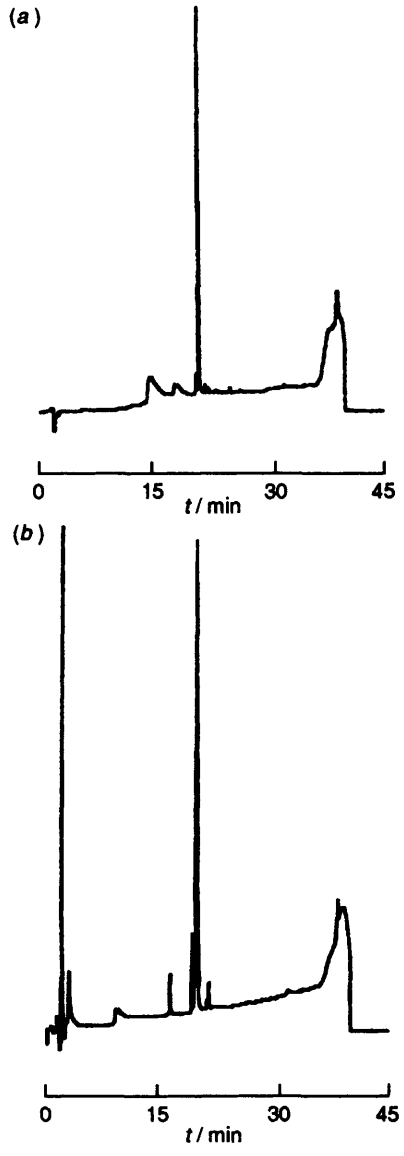


Fig. 1 Analytical HPLC of the crude products: (*a*) H-Arg-Arg-Val-Ser(OPO_3H_2)-Val-Ala-Ala-Glu-OH: column, μBondasphere $5C_{18}$ (3.9 mm × 150 mm); buffer A, 0.1% aq. TFA; B, MeCN (0.1% TFA); linear gradient 0–30% B over 30 min; flow rate 1.0 cm^{-3} min^{-1}; detection 220 nm. (*b*) H-Lys-Arg-Thr(OPO_3H_2)-Leu-Arg-Arg-Leu-Leu-OH: buffer A, 0.1% aq. TFA; B, MeCN (0.1% TFA); linear gradient 10–40% B over 30 min; otherwise the same conditions as in (*a*).

Table 1 Examination of the acidity of deprotecting reagent mixtures[a]

Reagent mixture	Regeneration (%) of Arg from Arg(Mts) (4 °C)		
	30 min	1 h	2 h
1 mol dm^{-3} TMSOTf–thioanisole–TFA, *m*-cresol, EDT (first step)	98.5	99.3	100
1 mol dm^{-3} TMSOTf–thioanisole–TFA, *m*-cresol, EDT, TMSOTf, Me_2S (second step)	25.5	34.6	54.1

[a] Boc–Arg(Mts)–OH (5 μmol) and H–Ala–OH (internal standard, *ca.* 5 μmol) were treated with above reagent mixtures [1 mol dm^{-3} TMSOTf– thioanisole–TFA (500 mm^3), *m*-cresol (25 mm^3), EDT (25 mm^3) or 1 mol dm^{-3} TMSOTf–thioanisole–TFA (500 mm^3), *m*-cresol (25 mm^3), EDT (25 mm^3), Me_2S (150 mm^3), TMSOTf (100 mm^3)]. At intervals (30 min, 1 h, 2 h), an aliquot was removed and diluted with H_2O, and then regeneration (%) of Arg from Arg(Mts) was quantitated by an amino acid analyser.

respectively, because Mts and Bn groups can be deprotected with the 1 mol dm^{-3} TMSOTf–thioanisole–TFA reagent system.[7] One-pot treatment of the completed peptide resin with 1 mol dm^{-3} TMSOTf–thioanisole–TFA, *m*-cresol, EDT (room temp., 1 h),§ followed by addition of Me_2S with additional stirring 2 h at room temp., resulted in cleavage of the peptide from the resin with removal of all protecting groups including the Me groups on the phosphoserine (*P*Ser) residue (Scheme 1).¶ Examination by HPLC showed no incompletely deprotected peptide side products (Fig. 1). After HPLC purification, pure peptide‖ was obtained in 54% yield calculated from the protected peptide resin. In this deprotection procedure, the initial reagent mixture (1 mol dm^{-3} TMSOTf–thioanisole–TFA) may be operating by a mechanism which is intermediate between S_N1 and S_N2 (S_N1/S_N2) and is effective for deprotection of Mts groups on Arg residues and Bn groups on Glu residues. Addition of Me_2S to the above reagent mixture changed the reaction mechanism of strong acid catalysed dealkylation from S_N1 (high acidity) to S_N2 (low acidity).[8] This S_N2-type reaction condition is critically important for deprotection of Me groups on *P*Ser residues. Since S_N2-type reagent systems do not have sufficient acidity for removal of some protecting groups employed in Boc-based solid-phase methods, this two-step deprotection protocol employing the S_N1/S_N2 (high acidity)–S_N2 (low acidity) combination is crucial for deprotection and cleavage of Ser(OPO_3Me_2)-containing protected peptide resins.

Based on the above findings, we attempted to apply the two-step deprotection method consisting of S_N1/S_N2 (1 mol dm^{-3} TMSOTf–thioanisole–TFA)- and S_N2 (1 mol dm^{-3} TMSOTf–thioanisole–TFA–Me_2S)-type reagent system to the deprotection of Thr(OPO_3Me_2)-containing protected peptide resins. We prepared a protected peptide resin using standard Boc methodology [Boc-Lys(ClZ)-Arg(Mts)-Thr(OPO_3Me_2)-Leu-Arg(Mts)-Arg(Mts)-Leu-Leu-PAM resin]. Treatment of the completed resin with a reagent system identical to that utilized in deprotection of the Ser(OPO_3Me_2)-containing resin, resulted in incomplete deprotection of the Thr(OPO_3Me_2)-peptide. Therefore, we sought more effective conditions for deprotection of Me groups on the phosphothreonine (*P*Thr) residue. We established the reaction conditions of the first deprotection step (1 mol dm^{-3} TMSOTf–thioanisole–TFA, *m*-cresol, EDT) to cleave the peptide from the resin with concomitant removal of Mts and ClZ groups. Next, we changed the ratio of additives in the reagent mixture of the second deprotection step [1 mol dm^{-3} TMSOTf–thioanisole–TFA, *m*-cresol, EDT plus additives]. We found that addition of TMSOTf and Me_2S to the first step reagent mixture was effective for removal of Me groups on the *P*Thr residue. One-pot treatment of the protected peptide resin with 1 mol dm^{-3} TMSOTf–thioanisole–TFA, *m*-cresol, EDT (4 °C, 1.5 h),** followed by addition of Me_2S and TMSOTf with additional stirring (4 °C, 3 h, room temp.,1 h) (Scheme 1),†† gave completely deprotected crude peptide as indicated by HPLC (Fig. 1). After HPLC purification, pure peptide‡‡ was obtained in 71% yield based on the protected peptide resin. Of note is the fact that this two-step deprotection methodology is applicable to deprotection of both Ser(OPO_3Me_2)- and Tyr-(OPO_3Me_2)-containing peptide resins.

The acidity of reagent mixture in the second step [1 mol dm^{-3} TMSOTf–thioanisole–TFA, *m*-cresol, EDT, Me_2S, TMSOTf] was examined from the yield of Arg regenerated from Arg(Mts) as compared with that of reagent mixture in the first step [1 mol dm^{-3} TMSOTf–thioanisole–TFA, *m*-cresol, EDT] (Table 1). These results indicate that the second step reagent mixture operates at milder acidic conditions than the first step. That is, the second step reagent mixture removes dimethyl phosphate protection with little tendency to induce side reactions resulting from harsh acid treatment.

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Footnotes

† Abbreviations used: TMSOTf = trimethylsilyl trifluoromethanesulfonate, Mts = mesitylene-2-sulfonyl, ClZ = 2-chlorobenzyloxycarbonyl, EDT = ethanedithiol, PAM = 4-(oxymethyl)phenylacetamidomethyl.

‡ Boc–Ser(OPO_3Me_2)–OH was purchased from Peninsula Lab. Inc. (USA). Boc–Thr(OPO_3Me_2)–OH was prepared by a phosphoramidite method, but is also available from Peninsula Lab. Inc.

§ One Me group on the Ser(OPO_3Me_2) residue was removed; however, complete removal of the Me groups could not be achieved, mono–Me *P*Ser-:*P*Ser-peptide = 31:69. Prolonged treatment with the first step reagent (4 °C, 4.5 h) also gave incompletely deprotected peptide (mono–Me *P*Ser-:*P*Ser–peptide = 54:46). Ion-spray MS (reconstructed), *m/z* 980.24: [981.02 calc. for $C_{37}H_{69}N_{14}O_{15}P$ (mono–Me*P*Ser-peptide)].

¶ Peptide resin (0.01 mmol) was treated with 1 mol dm^{-3} TMSOTf–thioanisole (molar ratio, 1:1)–TFA (1 cm^3) in the presence of EDT (50 mm^3) and *m*-cresol (50 mm^3) at room temp. 1 h, and then Me_2S (200 mm^3) was added to this reaction mixture and the treatment was continued for 2 h at room temp. Dry diethyl ether was then added to precipitate the crude peptides. The precipitate was washed with diethyl ether (3 times) and crude deprotected peptides were analysed and purified using HPLC.

‖ Ion-spray MS (reconstructed), *m/z* 966.24: [966.99 calc. for $C_{36}H_{67}N_{14}O_{15}P$ (*P*Ser-peptide)].

** Treatment with the first step reagent gave incompletely deprotected peptides, di-Me *P*Thr-:mono–Me *P*Thr-:*P*Thr–peptide = 52:46:2 (4 °C, 1.5 h), 4:69:27 (4 °C, 3.5 h, room temp. 1 h). Ion-spray MS (reconstructed), *m/z* 1148.24: [1149.35 calc. for $C_{47}H_{93}N_{18}O_{13}P$ (mono–Me *P*Thr–peptide)], *m/z* 1162.49: [1163.38 calc. for $C_{48}H_{95}N_{18}O_{13}P$ (di–Me *P*Thr–peptide)].

†† Peptide resin (0.01 mmol) was treated with 1 mol dm^{-3} TMSOTf–thioanisole (molar ratio, 1:1)–TFA (1 cm^3) in the presence of EDT (50 mm^3) and *m*-cresol (50 mm^3) at 4 °C, 1.5 h, and then Me_2S (300 mm^3) and TMSOTf (200 mm^3) were added to this reaction mixture and the treatment was continued for 3 h at 4 °C, 1 h at room temp. Work up similar to that described above was utilized to obtain and to characterize the product.

‡‡ Ion-spray MS (reconstructed), *m/z* 1134.74: [1135.33 calc. for $C_{46}H_{91}N_{18}O_{13}P$ (*P*Thr–peptide)].

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