Development of Efficient Two-Step Deprotection Methodology for Dimethyl-Protected Phosphoamino Acid-Containing Peptide Resins and Its Application to the Practical Synthesis of Phosphopeptides[1]

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A protocol has been developed for the synthesis of peptides containing O-phosphorylated tyrosines, serines, and/or threonines. The procedure involves incorporation of dimethyl-protected O-phosphorylated amino acid derivatives (**1**–**3**) into peptides using standard Boc chemistry and subsequent removal of Me groups using a two-step deprotection method consisting of high-acidic and low-acidic treatments. Optimized deprotection conditions for the protected resins (**4**–**6**) were established, which consist of a combination of the first-step reagent (1 M TMSOTf–thioanisole in TFA (100), *m*-cresol (5), EDT (5), (v/v)) and the second-step reagent (first-step reagent (110) + DMS–TMSOTf (30:20 to 40:10), (v/v)). The two-step deprotection protocol can be conducted in one pot by appropriate modification of the first-step reagent. The second deprotection step proceeds by an S_N2 mechanism with little tendency to induce side reactions resulting from harsh acid treatment. A 19-residue MAP-kinase peptide **10** possessing not only two phosphoamino acids but also Met and Trp was subjected to this synthetic procedure and was obtained in 24% yield based on the protected resin. The present synthetic method afforded phosphoamino acid-containing peptides in high yield without significant accompanying side reactions (e.g., loss of phosphate groups, migration of phosphate groups, or alkylation of Met and Trp residues).

Introduction

Protein phosphorylation provides structural and functional changes for proteins involved in intracellular signal transduction pathways.[2] For example, the protein tyrosine kinase (PTK)-mediated generation of phosphotyrosyl (pTyr) residues within discrete amino acid sequences of signalling proteins allows for the recognition and binding of these proteins with *src* homology 2 (SH2) domains. These pTyr-dependent protein–protein associations with SH2 domains are known to be crucial for signal transduction triggered by PTKs.[3] Furthermore, phosphorylation/dephosphorylation of Ser residues in the NF-AT transcription factor regulates its translocation from the cytosol into the nucleus.[4]

Due to the important role of phosphorylation/dephosphorylation in biology, phosphopeptides have received much attention as useful biological and biochemical tools to elucidate various cellular processes including signal transduction and have been the object of intense synthetic activity. Two synthetic strategies, one being a preassembly phosphorylation method[5] and the other a postassembly phosphorylation method,[6] have been applied to the synthesis of phosphopeptides (Figure 1).

The postassembly phosphorylation method involves the preparation of protected peptide resins containing unprotected hydroxyl-bearing amino acids (Tyr, Ser, and/or Thr) followed by chemical phosphorylation of the free hydroxyl groups. The syntheses of relatively small and less complicated phosphopeptides have been achieved using this method; however, the postassembly phosphorylation method can be accompanied by difficulties. The use of side chain-unprotected Tyr, Ser, and Thr derivatives can result in O-acylation during chain elongation, and this is especially problematic for longer sequences having these amino acids at the C-terminal portions. For phosphorylation of the free hydroxyl groups, the phosphoramidate method, consisting of phosphitylation and oxidation, has been preferred due to its high reactivity. Nevertheless, phosphitylation proceeds incompletely when

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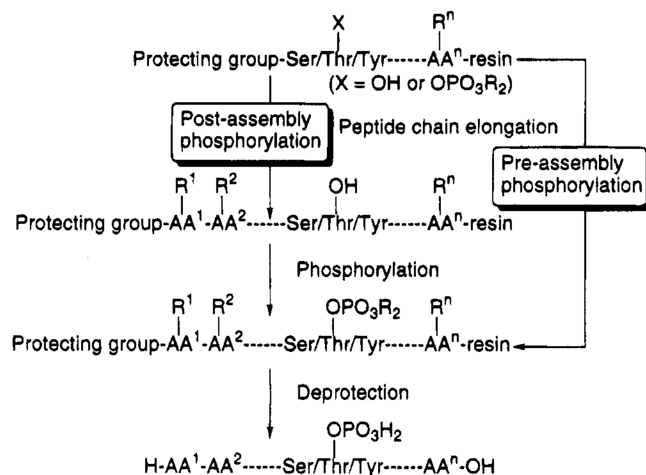

Figure 1. General synthetic routes for phosphopeptides. AA: amino acids. R: side chain protecting groups.

hydroxyl amino acids are located in the spatially hindered regions of peptides.[7] Additionally, the oxidation of P(III) species can cause side reactions to oxidant-sensitive amino acids such as Trp, Met, and Cys,[8] and the formation of H-phosphonate is known.[9] On the other hand, the preassembly phosphorylation method involves incorporation into the protected peptide resins of phosphoamino acid derivatives having dialkyl or diaryl phosphate protection followed by the final deprotection steps. A number of studies have been reported where this strategy has been utilized to synthesize phosphopeptides, especially pTyr-containing peptides. However, except for a few examples, efficient synthetic methodologies with wide applicability for phosphoserine (pSer)- and phosphothreonine (pThr)-containing peptides have not yet been reported. For example, the use of a preassembly phosphorylation strategy can involve laborious deprotections in two different modes[10] (e.g., acidolytic + hydrogenolytic deprotection), which may result in the partial splitting off of the phosphate groups during acidolytic deprotection.[11] The development by Wakamiya et al.[12] of N^{α}-(*tert*-butoxycarbonyl)-*O*-[bis(4-nitrobenzyl)phosphono]serine/threonine, Boc-Ser/Thr[$OPO_3(4\text{-}NO_2Bzl)_2$]-OH, and N^{α}-(*tert*-butoxycarbonyl)-*O*-(dicyclohexylphosphono)serine/threonine, Boc-Ser/Thr(OPO_3cHex_2)-OH, with practical applications to the synthesis of phosphopeptides may circumvent some of the phosphopeptides synthesis difficulties; however, its application has been restricted to short and less complicated peptides, and the protected amino acids have to be synthesized by researchers themselves. The same situation is encountered when using Wakamiya's efficient alternative method,[13] which utilizes a combination of monoalkyl phosphate protection and Fmoc-based solid-phase techniques. We have reported on the synthesis of 4-phosphono(difluoromethyl)-phenylalanine (F_2Pmp)-containing peptides[14] and 2-amino-4-(difluoromethyl)butanoic acid (F_2Pab)-containing peptides[15] as nonhydrolyzable pTyr- and pSer-peptide mimetics, respectively. In these syntheses, diethyl-protected phosphonate derivatives ($F_2Pmp(OEt)_2$ and $F_2Pab(OEt)_2$) were utilized as the protected amino acid species. We found that phosphonate ethyl protection was efficiently removed with 1 M TMSOTf−thioanisole in TFA + DMS or 1 M TMSOTf−2 M DMS in TFA.[16] On the other hand, the use of HF,[17] 1 M TMSOTf−thioanisole in TFA,[18] or 1 M TFMSA−thioanisole in TFA[19] systems, which are more acidic than the former systems, led to no deprotection or incomplete deprotection of the ethyl groups. This prompted us to examine whether the dimethyl phosphate protection of pSer, pThr, and pTyr would behave in a fashion similar to that of ethyl protection on F_2Pmp and F_2Pab.

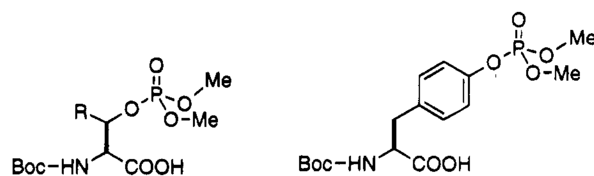

Figure 2. Structures of dimethyl-protected phosphoamino acid derivatives.

N^{α}-(*tert*-Butoxycarbonyl)-*O*-(dimethylphosphono)serine/threonine, Boc-Ser/Thr(OPO_3Me_2)-OH (**1** and **2**, respectively), are commercially available derivatives (Figure 2). Nevertheless, these derivatives have rarely been employed for the practical synthesis of phosphopeptides. This may be attributed to the fact that harsh acid treatment resulting in side reactions is thought to be required for complete removal of these methyl groups.[20] N^{α}-(*tert*-Butoxycarbonyl)-*O*-(dimethylphosphono)tyrosine, Boc-Tyr(OPO_3Me_2)-OH (**3**), has been successfully used in the synthesis of pTyr-containing peptides.[21,22] However, practical deprotection protocols with general applicability toward Me groups and other protecting

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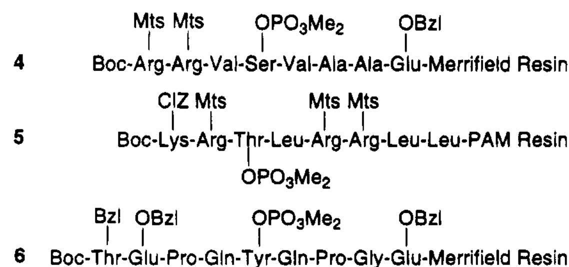

Figure 3. Protected peptide sequences of three model peptides.

groups employed in the solid-phase synthesis of peptides also containing pSer and pThr have not yet been developed. Herein, we report on the evaluation of several deprotection methods for Ser/Thr/Tyr(OPO_3Me_2) (**1**, **2**, and **3**) residues in peptides with application of these methods to the practical synthesis of phosphopeptides.

Results and Discussion

Initially, in order to evaluate the general applicability of several acidic reagent systems for the final deprotection of dimethyl-protected phosphoamino acid-containing peptide resins, three model peptide resins (**4**, **5**, and **6** corresponding to the partial sequences of the cAMP-dependent protein kinase regulatory domain,[23] the EGF receptor,[24] and a Src protein,[25] respectively) were prepared using standard Boc-based solid-phase techniques[26] because Fmoc techniques are incompatible with the synthetic method using dimethyl-protected phosphoamino acids (**1** and **2**) due to the instability of the phosphate group resulting from β-elimination during piperidine treatment for Fmoc deprotection[13] (Figure 3). Starting from appropriate Boc-protected amino acid resins (Boc-Glu(OBzl)-Merrifield resin for **4** and **6** and Boc-Leu-PAM resin[27] for **5**), the protected peptide resins were synthesized using manual Boc methodology with a combination of TFA-mediated Boc deprotection followed by neutralization with DIPEA and DIPCDI/HOBt-mediated coupling of Boc amino acids. The following side chain protecting groups were utilized: Mts for Arg,[28] Bzl for Glu, ClZ for Lys,[29] and Me for the phosphoamino acids. In our preliminary work,[1] we showed that the Me groups on a pSer residue were efficiently removed by a two-step deprotection involving treatment with 1 M TMSOTf–thioanisole (molar ratio of 1:1) in TFA system followed by the addition of DMS. Although this system worked efficiently at room temperature, it was incompatible with the Thr(OPO_3Me_2)-containing peptide resin due to its low ability to remove Me groups on the pThr residue. We found that the addition of DMS along with TMSOTf was more effective for the removal of Me groups on pThr residues than was the addition of DMS itself. Therefore, we attempted to establish the best conditions which were compatible with all three dimethyl-protected phosphoamino acid derivatives and then compare the TMSOTf-mediated method with other acidolytic deprotection systems.

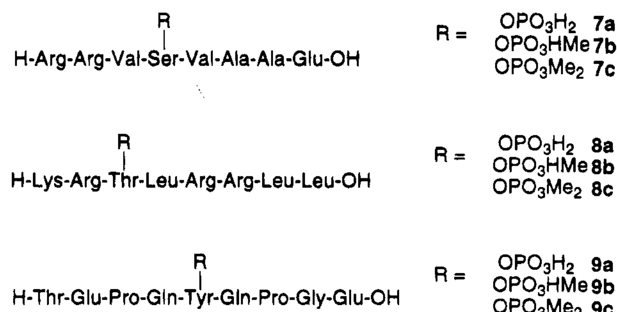

Figure 4. Possible phosphopeptide products obtained by acidolytic deprotections.

We first established the reaction conditions of the first deprotection step (1 M TMSOTf–thioanisole in TFA, *m*-cresol, EDT) needed to cleave the peptides from the resins (**4–6**) with concomitant removal of the Mts, ClZ, and Bzl groups. This method has been reported to be effective for the final acidolytic deprotection of other protected peptide resins.[30] After treating **4–6** with the first-step reagent for 1.5 h at 4 °C, we added composite additives (DMS + TMSOTf) in different mixing ratios. Herein, additives at 50% (**4–6**) or 25% (**4**) volume of the first-step reagent system except for *m*-cresol and EDT were added. The progress of deprotection was monitored by HPLC and IS-MS analyses of the crude deprotected peptides. The ratios of components (**7a**:**7b**:**7c** for **4**, **8a**:**8b**:**8c** for **5**, and **9a**:**9b**:**9c** for **6**, Figure 4) in the crude deprotected peptides were estimated by comparison of the relative HPLC peak areas. In the case of **4** and **6**, removal of the pSer and the pTyr residue Me groups was completed by a second-step treatment (4 °C, 3 h) involving the addition of composite additives (DMS:TMSOTf = 30:20 to 40:10, v/v for **4**, Figure 5; DMS:TMSOTf = 30:20 to 50:0, v/v for **6**, Figure 6).[31]

Although the peptide resin **5** was less susceptible than **4** and **6** to the second stage deprotection reagents, the Me groups on the pThr residue were completely deprotected using the second-step reagent containing DMS–TMSOTf (30:20 to 40:10, v/v) as additives (4 °C, 2 h + room temperature, 1 h) (Figure 7). IS-MS analysis of partly deprotected pSer or pThr peptides indicated that the remaining methyl groups were located on the phosphate groups, since the observed β-elimination fragment gave the same m/z value as was found with the completely deprotected peptides.[32]

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(31) In the TMSOTf system, neither **7c** nor **9c** was detected on HPLC since one Me group on the Ser(OPO_3Me_2) and Tyr(OPO_3Me_2) residues was completely removed. The half-lives for the cleavage with the TMSOTf–thioanisole system of the first Me group on Tyr(OPO_3Me_2) residues was reported to be 7 min in the literature.[21]

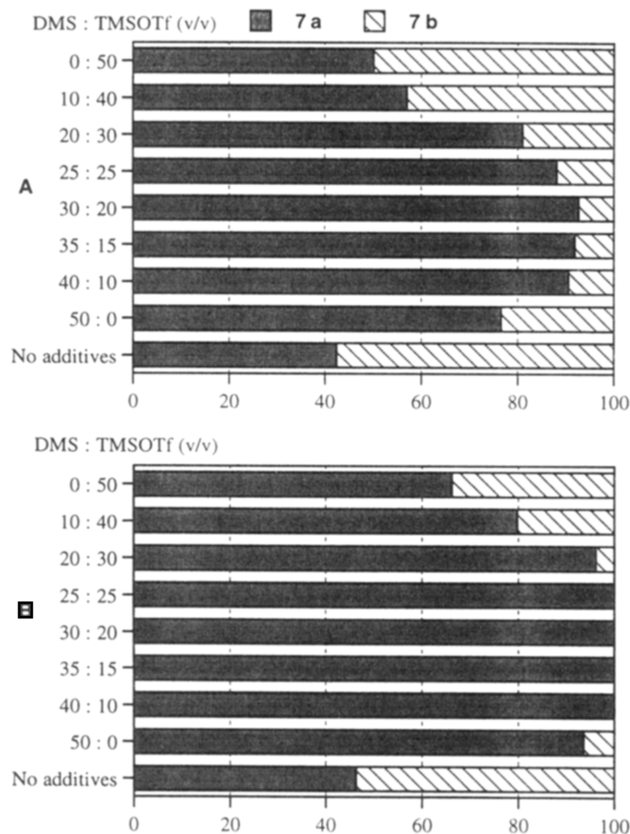

Figure 5. Ratio of components in crude deprotected peptide obtained by treatment of **4** with 1 M TMSOTf−thioanisole in TFA (4 °C, 1.5 h) followed by addition of additives (DMS + TMSOTf) with additional stirring for 1 h (A) and 3 h (B) at 4 °C.

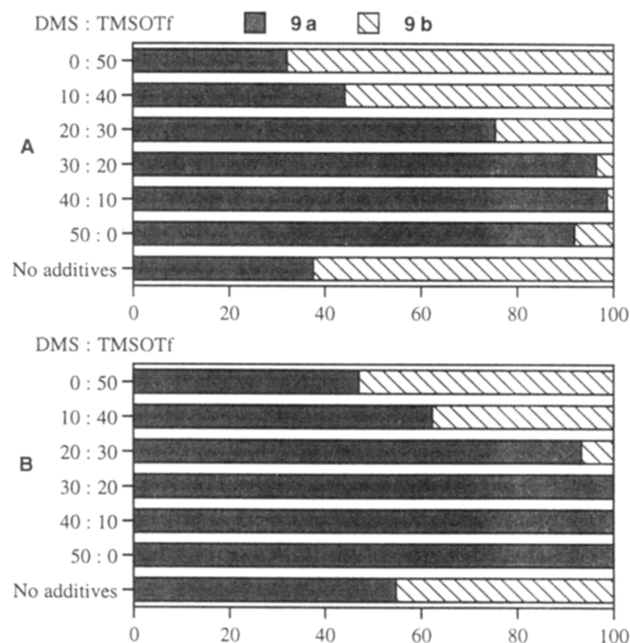

Figure 6. Ratio of components in crude deprotected peptide obtained by treatment of **6** with 1 M TMSOTf−thioanisole in TFA (4 °C, 1.5 h) followed by addition of additives (DMS + TMSOTf) with additional stirring for 1 h (A) and 3 h (B) at 4 °C.

The HPLC analysis of crude peptides revealed no significant side reactions under the above deprotection conditions. Furthermore, the addition of reagents at 50% volume of the first-step reagent was shown to be more effective than addition at 25% volume (the best results were **7a**:**7b** = 94:6 and DMS:TMSOTf = 20:5, v/v). These results indicate that the addition of composite additives with ratios (v/v) of 30(DMS):20(TMSOTf) to 40(DMS):10-(TMSOTf) to the 1 M TMSOTf−thioanisole in TFA system is critically important for removal of the Me groups on dimethyl-protected phosphoamino acids. Generally, the addition of DMS is known to change the reaction mode of acid-catalyzed dealkylations from high-acidic (S_N1 or S_N1/S_N2 (which represents an intermediate between S_N1 and S_N2)) to low-acidic (S_N2) conditions.[33] In this case, since we added DMS along with TMSOTf, we examined the acidity of the second-step deprotection. Using protected amino acids and protected amino acid-linked resins, the effectiveness of the second-step reagent mixture (1 M TMSOTf−thioanisole in TFA (100), *m*-cresol (5), EDT (5) + DMS (30)−TMSOTf (20) (v/v)) for effecting the removal of the side chain protecting groups and the release of amino acids from the resins was examined (Table 1).

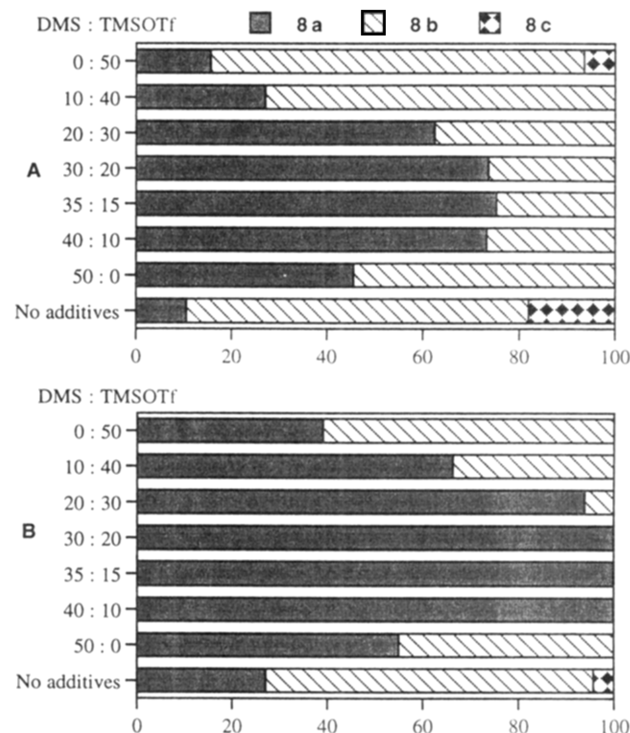

Figure 7. Ratio of components in crude deprotected peptide obtained by treatment of **5** with 1 M TMSOTf−thioanisole in TFA (4 °C, 1.5 h) followed by addition of additives (DMS + TMSOTf) with additional stirring for 2 h at 4 °C (A) and 2 h at 4 °C + 1 h at room temperature (B).

These results indicate that the second-step reagent mixture operates at milder conditions than the first-step one. Judging from the S_N2 deprotection data,[33] the second-step deprotection reagent mixture may also operate via an S_N2 mechanism. Under such S_N2 conditions, attack of a soft nucleophile such as DMS on the methyl

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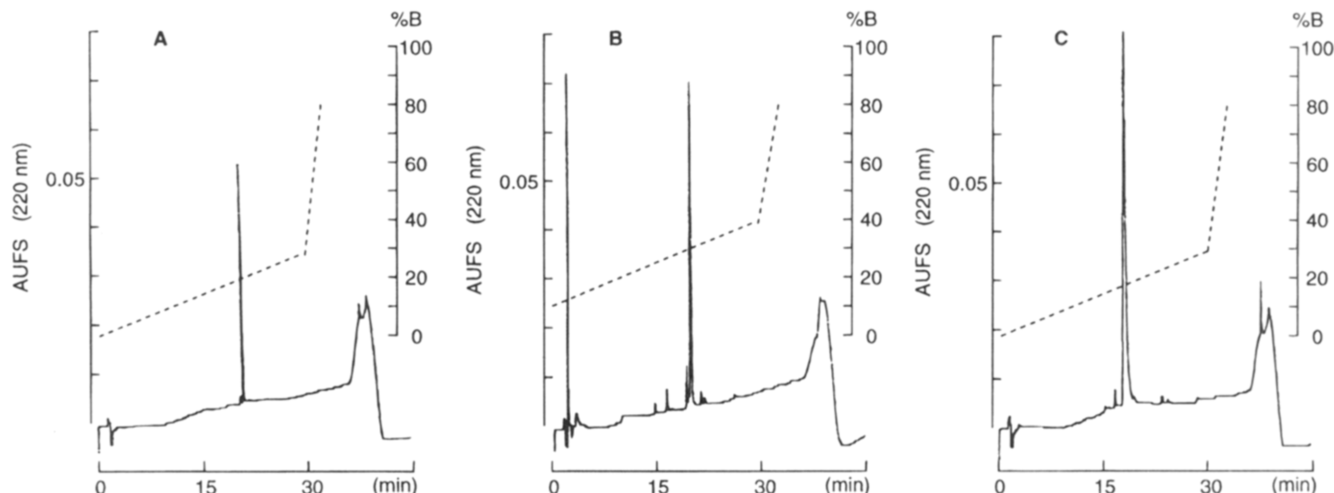

Figure 8. HPLC profiles of crude **7a** (A), **8a** (B), and **9a** (C). (A and C): column, μBondasphere 5μC$_{18}$-100 Å (3.9 × 150 mm); buffer A, 0.1% aqueous TFA; B, MeCN (0.1% TFA); linear gradient, 0–30% B over 30 min; flow rate, 1.0 mL/min; detector, 220 nm. (B): buffer A, 0.1% aqueous TFA; B, MeCN (0.1% TFA); linear gradient, 10–40% B over 30 min; otherwise, the same conditions as in parts A and C.

Table 1. Examination of the Acidity of the Second-Step Reagent System[a]

	regeneration (%) of free amino acids[b]		
	30 min	1 h	2 h
Boc-Arg(Mts)-OH	15.2	24.3	42.7
Boc-Trp(Mts)-OH	23.8	36.8	60.2
Boc-Lys(ClZ)-OH	84.4	91.2	95.9
Boc-Tyr(Cl_2Bzl)-OH	91.3	89.8	93.0
Boc-Asp(OBzl)-OH	100	100	100
Boc-Glu(OBzl)-Merrifield resin	24.1	34.6	48.9
Boc-Leu-PAM resin	4.0	6.6	10.9

[a] 1 M TMSOTf–thioanisole in TFA (100), *m*-cresol (5), EDT (5) + DMS (30)–TMSOTf (20) (v/v) = TMSOTf (25) + thioanisole (7) + DMS (19) + TFA (43) + *m*-cresol (3) + EDT (3) (v/v). [b] From the corresponding protected derivatives (4 °C).

carbon in a $(CH_3O)_2PO$ moiety along with silylation on the O atom by a hard acid (TMSOTf) would facilitate removal of the Me groups.[18,34] Because S_N2 reagent systems have insufficient acidity for the removal of some protecting groups and linkers employed in Boc methodology, this two-step deprotection protocol employing the S_N1/S_N2 (high acidity)–S_N2 (low acidity) combination is reasonable and crucial for deprotection and cleavage of dimethyl-protected phosphoamino acid-containing peptide resins prepared by Boc methodology. Of note is the fact that this two-step deprotection can be conducted as a one-pot reaction. Since treatment of protected phosphoamino acid derivatives with acid sometimes results in the loss of phosphate groups,[11] we examined the stability of the phosphate groups during the two-step deprotection. The amino acid derivatives **1**–**3** were treated with the above two-step reagent system. Aliquots from the reaction mixture were subjected to amino acid analysis to detect Ser, Thr, and Tyr resulting from the loss of phosphate groups. No loss of phosphate was encountered during the two-step deprotection. Using these optimized conditions, we obtained HPLC-purified phosphopeptides **7a**, **8a**, and **9a** in 81, 69, and 81% yields, respectively, based on the corresponding protected peptide resins (Figure 8).

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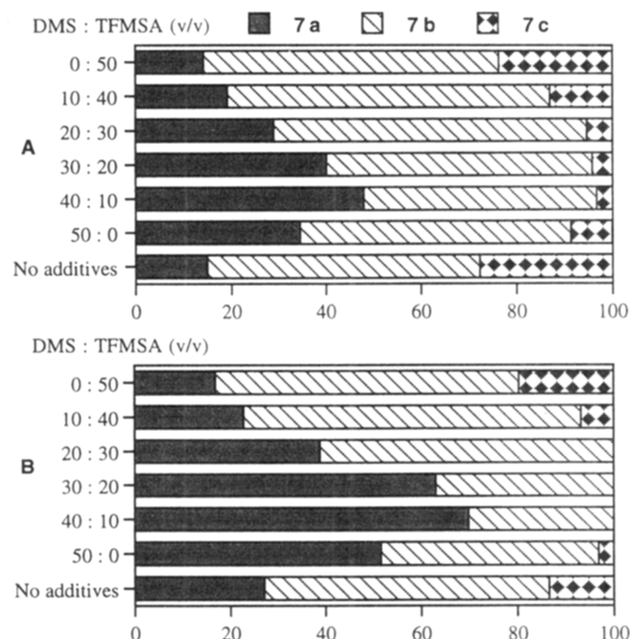

Figure 9. Ratio of components in crude deprotected peptide obtained by treatment of **4** with 1 M TFMSA–thioanisole in TFA (4 °C, 1.5 h) followed by addition of additives (DMS + TFMSA) with additional stirring for 1 h (A) and 3 h (B) at 4 °C.

Next, we examined the efficiency of other reagent systems for deprotection of the Me groups. We carried out the acidolytic deprotection of **4** using TFMSA in a manner similar to that described for the above TMSOTf-mediated deprotection. In this case, complete removal of the Me groups cannot be achieved (Figure 9).

The “Low-TFMSA” (TFMSA–DMS–TFA–*m*-cresol (10:30:50:10, v/v)),[33] which was reported by Tam et al. to operate by an S_N2 mechanism, was successfully applied to the deprotection of Tyr(OPO_3Me_2)-containing peptides.[21,22] However, application of this methodology to the deprotection of Ser(OPO_3Me_2)- and Thr(OPO_3Me_2)-containing peptide resins has not been shown. Therefore, we attempted a two-step deprotection involving a Low-TFMSA procedure as the second-step treatment. For the first-step deprotection, a “High-TFMSA” system[33]

(TFMSA–TFA–*m*-cresol (5:50:10, v/v)) was utilized. We conducted this high–low method in one pot. The treatment of protected peptide resin **4** with High-TFMSA (4 °C, 1.5 h) followed by the addition of DMS + TFMSA (Low-TFMSA, 4 °C, 3 h) gave crude peptide in a ratio of **7a**:**7b**:**7c** (56:41:3). From these results, we concluded that the two-step deprotection protocol involving treatment with 1 M TMSOTf–thioanisole in TFA, *m*-cresol, and EDT followed by the addition of additives (DMS: TMSOTf = 30:20 to 40:10, v/v) was the most preferable for deprotection of the dimethyl-protected phosphoamino acid-containing peptide resins examined so far. Among the acidic systems examined here, the deprotection system having combinations of TMSOTf (hard acid) and DMS or DMS + thioanisole (soft nucleophile) in the ratio described above most efficiently accelerated the removal of Me groups under the S_N2 or "push–pull" mechanism.[34]

In order to verify the general applicability of the two-step deprotection protocol, we attempted the synthesis of H-Phe-Leu-pThr-Glu-pTyr-Val-Ala-Thr-Arg-Trp-Tyr-Arg-Ala-Pro-Glu-Ile-Met-Leu-Asn-NH_2 (**10**, a partial sequence of the MAP-kinase[35]). Starting from MBHA resin,[36] the protected peptide chain was elongated using standard Boc methodology. For side chain protection, the following protecting groups were utilized: Bzl for Thr and Glu, Cl_2Bzl for Tyr,[37] Mts for Arg and Trp,[38] and Me for pTyr and pThr. This peptide sequence contained not only two different phosphoamino acids but also Met and Trp, which are problematic when postassembly phosphorylation procedures are being used. The treatment of the completed resin with 1 M TMSOTf–thioanisole in TFA, *m*-cresol, and EDT (4 °C, 1.5 h) followed by the addition of DMS–TMSOTf (35:15, v/v) with additional stirring for 4 h at 4 °C resulted in the release of the peptide from the resin with concomitant removal of all protecting groups, including Me groups on the pTyr and pThr residues. The analyses of crude deprotected peptide by HPLC and IS-MS showed that no significant side reactions were encountered (Figure 10).

After HPLC purification, the purified peptide was obtained in 24% yield based on the protected peptide resin. Purified peptide was characterized by amino acid analysis of the 6 N HCl–hydrolysate and by IS-MS. In order to determine the position of the phosphate groups, we subjected the purified peptide to enzymatic digestion using trypsin to obtain a phosphopeptide fragment (H-Trp-Tyr-Arg-OH, **11a**, H-Phe-Leu-Thr(OPO_3H_2)-Glu-Tyr-(OPO_3H_2)-Val-Ala-Thr-Arg-OH, **11b**, and H-Ala-Pro-Glu-Ile-Met-Leu-Asn-NH_2, **11c**). The phosphopeptide fragment **11b** was characterized by IS-MS/MS.[39] These results indicated that the two-step deprotection protocol has wide applicability to the synthesis of phosphopeptides using multiple dimethyl-protected phosphoamino acids.

In conclusion, the use of dimethyl-protected phosphoamino acid derivatives in conjunction with two-step deprotection protocols involving the combination of high-acidic and low-acidic deprotection provides a general and efficient procedure for the practical synthesis of phosphopeptides. The methodology described here features the use of commercially available dimethylphosphono amino acid derivatives, which are compatible with standard Boc-based solid-phase techniques, and one-pot deprotection with an appropriately modified TMSOTf deprotection system. Furthermore, the second-step deprotection operates via an S_N2 mechanism with little tendency to induce side reactions which could result from harsh acid treatment. Using this methodology, we achieved the synthesis of the MAP-kinase peptide possessing two phosphoamino acids as well as Met and Trp. Finally, the present methodology allows for the synthesis of phosphopeptides in a manner similar to that used for nonmodified peptides.

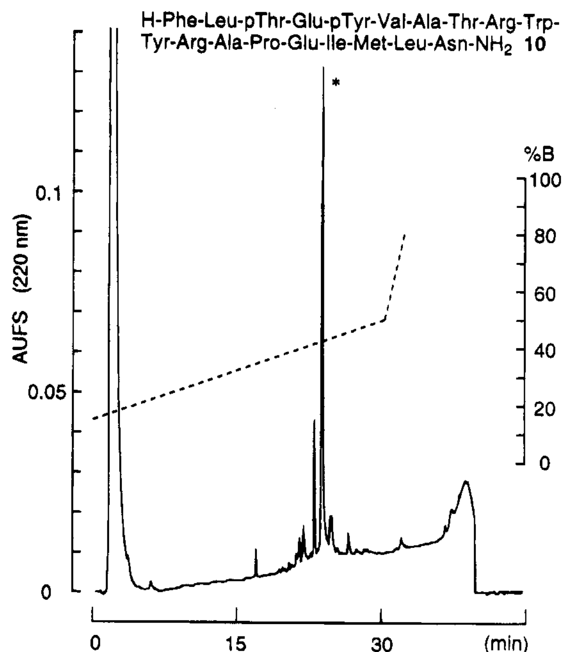

Figure 10. HPLC profile of crude **10**. An asterisk denotes the desired peptide: column, μBondasphere 5μC_{18}-100 Å (3.9 × 150 mm); buffer A, 0.1% aqueous TFA; B, MeCN (0.1% TFA); linear gradient, 15–50% B over 30 min; flow rate, 1.0 mL/min; detector, 220 nm.

Experimental Section

General Procedure. HPLC separations were carried out using either a μBondasphere 5μ C_{18}-100 Å analytical (3.9 × 150 mm) or a Cosmosil 5C_{18}-AR preparative column (20 × 250 mm). Eluting products were detected by UV at 220 nm. Solvent A was 0.1% TFA (v/v) in water, and solvent B was 0.1% TFA in acetonitrile. A flow rate of 1 mL/min was used for all analytical separations and a 7 mL/min flow rate for preparative runs. In all cases, linear gradient programs were utilized. Ion spray and tandem mass spectra were obtained with a Sciex API *III*E triple quadrupole mass spectrometer. In the triple quadrupole system, argon was used as the collision gas. Protected amino acids, Merrifield and PAM resin-coupled amino acids, MBHA resin, and other peptide synthesis chemicals were purchased from Peninsula Laboratories, Inc., Peptide Institute, Inc. or Watanabe Chemical Inc. All the other chemicals were purchased from either Nakarai Tesque Inc. or Wako Pure Chemical Industries, Ltd.

Synthesis of Protected Peptide Resins (4–6). Protected peptide resins were manually constructed using the Boc-based solid-phase method on appropriate Boc-amino acid resins (0.2 mmol scale each, Boc-Glu(OBzl)-Merrifield resin (0.67 mmol Glu/g) for **4** and **6**, Boc-Leu-PAM resin (0.5 mmol of Leu/g) for **5**). Boc deprotection was achieved using 50% TFA–2% anisole

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in CH_2Cl_2 (1 × 1 min, 1 × 15 min). DIPEA (5%) in CH_2Cl_2 (2 × 1 min) was used for neutralization of the TFA salts. The Boc-protected amino acids (2.5-fold molar excess, 0.5 mmol) were sequentially condensed using DIPCDI (0.5 mmol)–HOBt (0.5 mmol) in DMF.

Treatment of 4, 5, or 6 with the Two-Step Deprotection System. (a) TMSOTf System. To each protected resin (10 mg each of **4** (3.7 μmol), **5** (2.7 μmol), or **6** (3.7 μmol)) were added thioanisole (117 μL), *m*-cresol (50 μL), and EDT (50 μL). After stirring for 5 min at room temperature, each reaction mixture was cooled in an ice-chilled bath, and then precooled TFA (0.69 mL) and TMSOTf (194 μL) were successively added. After stirring for 1.5 h at 4 °C, the reaction mixture was divided into several portions (110 μL each). To these divided reaction mixtures were successively added either DMS and TMSOTf (50:0 to 0:50, v/v (50 μL) for **4–6** or 25:0 to 0:25, v/v (25 μL) for **4**) at 4 °C or no additives. In the case of **4** and **6**, the reactions were carried out at 4 °C, and aliquots (60 μL each) were withdrawn at specified intervals (1 and 3 h). To each reaction mixture aliquot was added ether (1 mL) to afford a powder. The resulting powders were washed with ether (three times) and dissolved in H_2O (500 μL). After filtration, the crude peptide-containing solutions were subjected to HPLC analysis using analytical HPLC (B, 0 → 30% over 30 min for **4** and **6**; B, 10 → 40% over 30 min for **5**). Each component, corresponding to **7a,b** and **9a,b** obtained by analytical HPLC purification, was identified using IS-MS analysis. The ratios of components were determined by comparison of peak areas. In all cases, no significant side reactions were observed which could prevent the comparison of the peak areas. In the case of **5**, reactions were carried out at 4 °C for 2 h and allowed to proceed at room temperature for 1 h. Aliquots (60 μL each) were withdrawn from the reaction mixtures at specified intervals (2 and 3 h). A workup similar to that previously described was utilized to determine the ratio of components (**8a–8c**) in the crude peptides and to characterize the products. IS-MS (reconstructed): *m/z* 966.24 (966.99 calcd for $C_{36}H_{67}N_{14}O_{15}P$ (**7a**)), 868.98 (868.70 calcd for $C_{36}H_{64}N_{14}O_{11}$ (**7a**–H_3PO_4)), 980.24 (981.02 calcd for $C_{37}H_{69}N_{14}O_{15}P$ (**7b**)), 868.98 (868.70 calcd for $C_{36}H_{64}N_{14}O_{11}$ (**7b**–H_2PO_4Me)), 1134.74 (1135.33 calcd for $C_{46}H_{91}N_{18}O_{13}P$ (**8a**)), 1037.0 (1037.33 calcd for $C_{46}H_{88}N_{18}O_9$ (**8a**–H_3PO_4)), 1148.24 (1149.35 calcd for $C_{47}H_{93}N_{18}O_{13}P$ (**8b**)), 1036.24 (1037.33 calcd for $C_{46}H_{88}N_{18}O_9$ (**8b**–H_2PO_4Me)), 1162.49 (1163.38 calcd for $C_{48}H_{95}N_{18}O_{13}P$ (**8c**)), 1036.49 (1037.33 calcd for $C_{46}H_{88}N_{18}O_9$ (**8c**–HPO_4Me_2)), 1127.24 (1128.06 calcd for $C_{45}H_{66}N_{11}O_{21}P$ (**9a**)), 1141.24 (1142.09 calcd for $C_{46}H_{68}N_{11}O_{21}P$ (**9b**)).

(b) TFMSA System. The two-step deprotection of **4** with the TFMSA system was conducted in a manner similar to that previously described for the TMSOTf system. The protected peptide resin **4** (10 mg, 3.7 μmol) was treated with 1 M TFMSA–thioanisole in TFA (1 mL) in the presence of *m*-cresol (50 μL) and EDT (50 μL) at 4 °C for 1.5 h. After the mixture was divided into several portions (110 μL), additional treatment (4 °C, 3 h) with or without DMS–TFMSA (50 μL, 50:0 to 0:50, v/v) afforded the crude deprotected peptides. The ratio of components was determined as already described. IS-MS (reconstructed): *m/z* 996.24 (995.05 calcd for $C_{38}H_{71}N_{14}O_{15}P$ (**7c**)), 868.98 (868.70 calcd for $C_{36}H_{64}N_{14}O_{11}$ (**7c**–HPO_4Me_2)).

(c) High-TFMSA + Low-TFMSA. Protected peptide resin **4** (1.5 mg, 0.55 μmol) was initially treated with High-TFMSA (130 μL, TFMSA:TFA:*m*-cresol = 5:50:10, v/v) for 1.5 h at 4 °C. Subsequently, DMS (60 μL) and TFMSA (10 μL) were added to the reaction mixture to change the reaction mode from High-TFMSA to Low-TFMSA, and the reaction was continued for 3 h at 4 °C. A procedure identical to that already described was utilized to obtained the crude peptides and to determine the ratio of products.

Examination of Acidity of the Second-Step Reagent. A mixture of Boc-Arg(Mts)-OH (10.0 μmol), Boc-Lys(ClZ)-OH (10.2 μmol), Boc-Trp(Mts)-OH (9.9 μmol), Boc-Tyr(Cl_2Bzl)-OH (8.4 μmol), Boc-Asp(OBzl)-OH (12.1 μmol), Boc-Glu(OBzl)-Merrifield resin (9.5 μmol), Boc-Leu-PAM resin (9.2 μmol), and H-Ala-OH (8.2 μmol, internal standard) was treated with the second-step reagent mixture (1 M TMSOTf–thioanisole in TFA (2 mL), *m*-cresol (100 μL), and EDT (100 μL) + DMS–TMSOTf (1 mL, 30:20, v/v)) at 4 °C. At specified intervals (30 min, 1 h, 2 h), aliquots (30 μL) were sampled and diluted with H_2O (1 mL). The amount of each amino acid in solution (50 μL) was quantified using an amino acid analyzer.

Stability of Phosphate Groups during the Two-Step Deprotection. The protected phosphoamino acids **1–3** (15 μmol each) were treated with 1 M TMSOTf–thioanisole in TFA (1 mL), *m*-cresol (50 μL), and EDT (50 μL) for 1.5 h at 4 °C. Following the addition of DMS–TMSOTf (0.5 mL, 30:20, v/v) with additional stirring for 3 h at 4 °C, aliquots (20 μL) were sampled and diluted with H_2O (1 mL). The amounts of Ser, Thr, and Tyr resulting from the loss of phosphate groups was quantified using an amino acid analyzer. The results of these studies indicated that no detectable Ser, Thr, or Tyr was present.

Synthesis of H-Arg-Arg-Val-Ser(OPO_3H_2)-Val-Ala-Ala-Glu-OH (7a). The protected peptide resin **4** (20 mg, 7.4 μmol) was treated with 1 M TMSOTf–thioanisole in TFA (1 mL), *m*-cresol (50 μL), and EDT (50 μL) at 4 °C. After 1.5 h, DMS (300 μL) and TMSOTf (200 μL) were successively added to the reaction mixture, and the reaction was continued for 2 h at 4 °C. After removal of the resin by filtration, ether (12 mL) was added to the filtrate to precipitate the crude product. The precipitate was collected by centrifugation and washed with ether (12 mL, three times) to remove scavengers. HPLC purification (linear gradient of B: 11–15% over 30 min) of the crude peptide gave the pure **7a** as a white powder with a yield of 7.8 mg (81% based on the protected peptide resin **4**). Amino acid ratios after 6 N HCl–0.1% phenol hydrolysis (values in parentheses are theoretical) were as follows: Ser, 0.82 (1); Glu, 1.02 (1); Ala, 2.0 (2); Val, 2.06 (2); and Arg, 2.19 (2). IS-MS/MS analysis was utilized for further characterization of the synthetic peptide. The fragment ion nomenclature as previously reported[39] was utilized. Positive-ion mode: 966.8 (M + H), 887.2 (M + H – HPO_3), 869.2 (M + H – H_3PO_4), 156.8 (b_1), 313.2 (b_2), 412.0 (b_3), 579.6 (b_4), 678.2 (b_5), 384.4 (a_3), 551.4 (a_4), 219.0 (y_2), 290.6 (y_3).

Synthesis of H-Lys-Arg-Thr(OPO_3H_2)-Leu-Arg-Arg-Leu-Leu-OH (8a). The protected peptide resin **5** (31 mg, 8.3 μmol) was treated with 1 M TMSOTf–thioanisole in TFA (2.5 mL), *m*-cresol (125 μL), and EDT (125 μL) at 4 °C for 1.5 h, followed by the addition of DMS (0.75 mL) and TMSOTf (0.5 mL). After additional stirring for 3 h at 4 °C followed by 1 h at room temperature, a work up similar to that previously mentioned was utilized to obtain the crude peptide. HPLC purification (linear gradient of B: 20–24% over 30 min) of the crude peptide gave pure **8a** as a white powder with a yield of 9.7 mg (69% based on the protected peptide resin **5**). The amino acid ratios after 6 N HCl–0.1% phenol hydrolysis (values in parentheses are theoretical) were as follows: Thr, 0.53 (1); Leu, 3.00 (3); Lys, 0.92 (1); Arg, 2.82 (3). IS-MS/MS analysis (positive-ion mode): 1136.0 (M + H), 1056.0 (M + H – HPO_3), 1037.8 (M + H – H_3PO_4), 129.2 (b_1), 285.2 (b_2), 466.0 (b_3), 735.4 (b_5), 401.4 (y_3), 557.2 (y_4), 670.4 (y_5).

Synthesis of H-Thr-Glu-Pro-Gln-Tyr(OPO_3H_2)-Gln-Pro-Gly-Glu-OH (9a). The protected peptide resin **6** (29 mg, 11 μmol) was treated with 1 M TMSOTf–thioanisole in TFA (2.0 mL), *m*-cresol (100 μL), and EDT (100 μL) at 4 °C for 1.5 h, followed by the addition of DMS (0.6 mL) and TMSOTf (0.4 mL) with additional stirring for 2 h at 4 °C. A workup similar to that already mentioned was utilized to obtain the crude peptide. HPLC purification (linear gradient of B: 5–15% over 30 min) of the crude peptide gave pure **9a** as a white powder with a yield of 10.9 mg (81% based on the protected peptide resin **6**). Amino acid ratios after 6 N HCl–0.1% phenol hydrolysis (values in parentheses are theoretical) were as follows: Thr, 0.93 (1); Glu, 4.21 (4); Pro, 1.85 (2); Gly, 1.00 (1); Tyr, 0.95 (1). IS-MS/MS analysis (positive-ion mode): 1128.2 (M + H), 231.0 (b_2), 328.2 (b_3), 456.2 (b_4), 699.0 (b_5), 827.2 (b_6), 302.0 (y_3), 430.2 (y_4), 673.0 (y_5), 898.4 (y_7).

Synthesis of H-Phe-Leu-Thr(OPO_3H_2)-Glu-Tyr(OPO_3H_2)-Val-Ala-Thr-Arg-Trp-Tyr-Arg-Ala-Pro-Glu-Ile-Met-Leu-Asn-NH_2 (10). A protected peptide resin corresponding to the MAP-kinase peptide **10** was prepared by standard Boc-based solid-phase techniques on MBHA resin (0.05 mmol, NH_2 0.24 mmol/g) as previously described. The completed resin (32 mg,

4.1 μmol) was treated with 1 M TMSOTf–thioanisole in TFA (2.0 mL), *m*-cresol (100 μL), and EDT (100 μL) at 4 °C for 1.5 h, followed by the addition of DMS (0.7 mL) and TMSOTf (0.3 mL) with additional stirring for 4 h at 4 °C. A workup similar to that already mentioned was utilized to obtain the crude peptide. HPLC purification (linear gradient of B: 30–40% over 30 min) of the crude product gave the pure peptide **10** as a white powder with a yield of 2.8 mg (24% based on the protected peptide resin). IS-MS (reconstructed): *m/z* 2531.98 (2532.75 calcd for $C_{111}H_{168}N_{28}O_{34}SP_2$ (**10**)). Amino acid ratios after 6 N HCl–0.1% phenol hydrolysis (values in parentheses are theoretical) were as follows: Asp, 1.05 (1); Thr, 1.60 (2); Glu, 2.10 (2); Pro, 1.00 (1); Ala, 2.00 (2); Val, 0.98 (1); Met, 0.98 (1); Leu, 2.07 (2); Ile, 1.00 (1); Phe, 1.02 (1); Tyr, 2.07 (2); Trp, ND (1); Arg, 2.00 (2). The position of the phosphate groups in **10** was determined from IS-MS/MS measurement of the phosphopeptide fragment derived from the tryptic digest of the intact peptide. An HPLC-purified peptide **10** (300 μg) was digested with trypsin for 20 h at 37 °C using an enzyme-to-substrate ratio of 1:100 (w/w) in 600 μL of 0.2 M $AcONH_4$ (pH 8.4). The digest was separated using an analytical HPLC (linear gradient of B: 0–30% over 30 min), followed by characterization of each peptide fragment using IS-MS measurement. IS-MS (reconstructed): *m/z* 522.99 (523.60 calcd for $C_{26}H_{33}N_7O_5$ (**11a**, retention time 25.3 min)), 1259.48 (1259.22 calcd for $C_{57}H_{80}N_{12}O_{21}P_2$ (**11b**, retention time 28.8 min)), 785.24 (785.96 calcd for $C_{34}H_{59}N_9O_{10}S$ (**11c**, retention time 29.2 min)). Phosphopeptide fragment **11b** was further characterized using IS-MS/MS. Characteristic fragment ions are from MS/MS analysis of **11b**. Positive-ion mode: 629.8 (M + 2H), 261.0 (b_2), 442.2 (b_3), 175.0 (y_1), 276.4 (y_2), 347.2 (y_3), 446.2 (y_4), 689.0 (y_5), 818.2 (y_6), 999.2 (y_7).

Abbreviations. All amino acids are of the L-configuration. TMSOTf = trimethylsilyl trifluoromethanesulfonate, TFMSA = trifluoromethanesulfonic acid, TFA = trifluoroacetic acid, DMS = dimethyl sulfide, EDT = 1,2-ethanedithiol, DMF = *N,N*-dimethylformamide, IS-MS = ion-spray mass spectrometry, MS/MS = tandem mass spectrometry, Mts = mesitylene-2-sulfonyl, Tos = *p*-toluenesulfonyl, ClZ = 2-chlorobenzyloxycarbonyl, Cl_2Bzl = 2,6-dichlorobenzyl, BrZ = 2-bromobenzyloxycarbonyl, MBzl = *p*-methoxybenzyl, cHex = cyclohexyl, PAM = 4-(oxymethyl)phenylacetamidomethyl, MBHA = 4-methylbenzhydrylamine, DIPCDI = *N,N*-diisopropylcarbodiimide, HOBt = 1-hydroxybenzotriazole, DIPEA = *N,N*-diisopropylethylamine, NF-AT = nuclear factor of activated T cells, and MAP = mitogen-activated protein.

Supporting Information Available: HPLC charts of the progress of the deprotection reactions (9 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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