# Synthesis of a Phosphotyrosine-Containing Peptide Fragment of the Regulatory Domain of pp60$^{c\text{-}src}$

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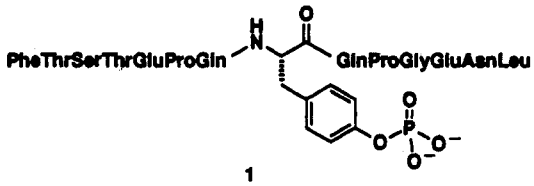
*t*-Boc-Tyr($PO_3Me_2$)-OH (4) was ulitized in the synthesis of PheThrSerThrGluProGlnTyr($PO_3H_2$)-GlnProGlyGluAsnLeu (1), a phosphotyrosine-containing peptide fragment of the regulatory domain of pp60$^{c\text{-}src}$. The protected amino acid 4 was employed during the synthesis of PheThrSer-ThrGluProGlnTyr($PO_3Me_2$)GlnProGlyGluAsnLeu (5), during which problems involving incomplete incorporation of the Tyr($PO_3Me_2$) residue, as well as partial demethylation of the dimethylphosphono group and dephosphorylation during peptide synthesis, were encountered. In addition, an acid-catalyzed backbone rearrangement was encountered involving migration of the phenylalanine acyl group from the nitrogen to the hydroxyl group of the adjacent threonine residue during the deprotection of the phosphate group of 5 with a mixture of trifluoromethanesulfonic acid, trifluoroacetic acid, dimethyl sulfide, and *m*-cresol (10:50:30:10).

The SH2 domains of proteins, which bind to phosphotyrosine-containing sequences in either the same or different protein molecules, are emerging as important targets for drug design.[1-5] The ligand-activated protein-tyrosine phosphorylation of "receptor type" protein-tyrosine kinases (PTK's) creates binding sites for the SH2 domains of certain enzymes which play critical roles in signal transduction pathways, including phosphatidylinositol 3-kinase (PI3K),[1,6-8] phospholipase C-γ (PLC-γ),[9-12] and p21$^{ras}$ GTPase-activating protein (GAP).[13-15] In addition to mediating protein–protein interactions between different protein molecules, the binding of phosphotyrosine-containing sequences to SH2 domains within the same PTK molecule are important in regulation of PTK activity. For example, phosphorylation of Tyr 527 located in the regulatory domain near the carboxy terminal in pp60$^{c\text{-}src}$ results in the binding of the resulting phosphotyrosine-containing sequence to the SH2 domain in the same macromolecule.[16-19] This sterically blocks the active site of pp60$^{c\text{-}src}$ and inhibits its PTK activity. The synthesis of ligands for the SH2 domains of PTK's and PTK substrate proteins is therefore of considerable current interest, both because it offers a strategy to control protein-protein binding in signal transduction pathways and also because it provides a way to influence regulation of PTK enzymatic activity.[7,18,20-28]

The goal of the present study was to investigate the preparation of phosphotyrosine-containing peptide 1, in which the phosphotyrosine residue is embedded in a sequence surrounding Tyr 527 in *c-src*. Various methods for phosphotyrosine peptide synthesis were considered for the preparation of 1. Synthesis and direct phosphorylation of the tyrosine analog of 1 with phosphorus oxychloride and pyridine is obviously not suitable because of the presence of the phosphorylatable threonine and

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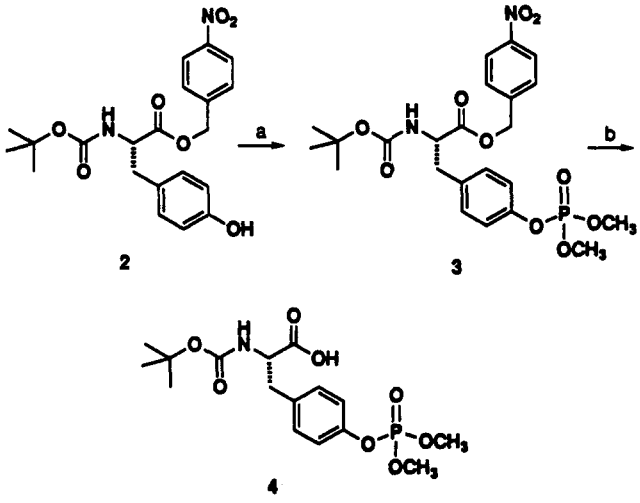
**Scheme 1**[a]




[a] Reagents: (a) $(CH_3O)_2PH$, $CCl_4$, EtOAc, 0 °C (1 h), 23 °C (24 h); (b) $H_2$, Pd/C, MeOH, AcOH, 23 °C (3 h).

serine residues.[29] Although the direct incorporation of an *O*-phosphonotyrosyl residue at the *N*-terminal position of peptide sequences has been accomplished with Boc-*O*-phosphonotyrosine *p*-nitrophenyl ester, this method would most likely present problems with peptides having internal phosphotyrosyl residues due to the unprotected phosphate side chain.[30] On the other hand, incorporation of Boc-Tyr($PO_3Bzl_2$)-OH into peptides and deprotection with HF during peptide-resin cleavage has been accomplished,[18,31] but the reported yields were low due to extensive decomposition.[31] We therefore decided to perform a detailed investigation of methodology employing dimethyl protection for the phosphate of phosphotyrosine during the synthesis of 1 and deprotection under acidic conditions. This strategy has previously been used successfully during the synthesis of phosphotyrosine-containing peptides, although the products were generally shorter and less complicated than 1.[32-38] During these studies, we have isolated and determined the structures of all of the major side products resulting during the incorporation of Boc-Tyr($PO_3Me_2$)-OH into the required sequence. In addition, the isolation and structure determination of a rearrangement product formed during deprotection of the phosphate has been accomplished.

## Results and Discussion

Phosphorylation of *N*-*tert*-Boc-L-tyrosine-*p*-nitrobenzyl ester (**2**)[31] with dimethyl phosphite in carbon tetrachloride and triethylamine afforded the desired phosphate **3**.[39] The $^1H$ NMR spectrum of **3** displayed the expected three-bond P–H coupling constant of 11.4 Hz. Catalytic debenzylation of **3** then provided the protected phosphotyrosine derivative **4** in 53% overall yield.

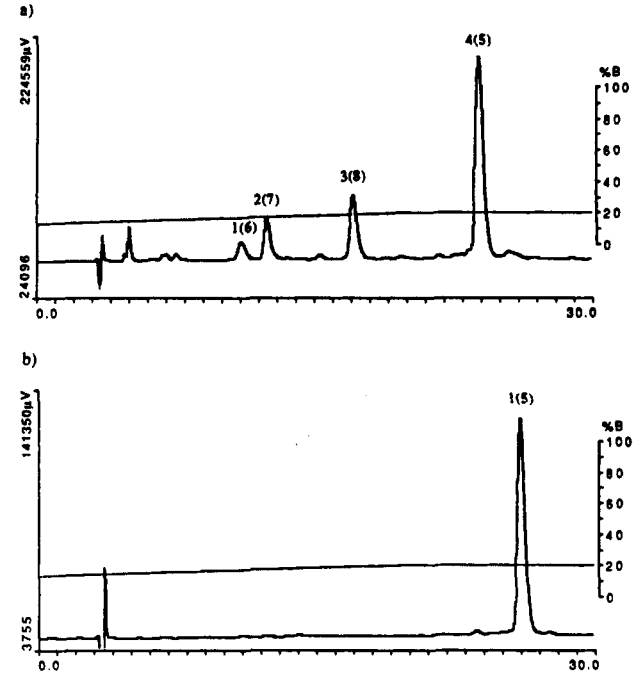



**Figure 1.** HPLC analysis of (a) the crude material obtained after cleavage of the peptide-resin during the synthesis of **5**, and (b) purified **5**. Column: Vydac 218TP104, C-18, 10 μm, 4.6 × 250 mm. A gradient elution of 13–20% aqueous acetonitrile in 0.1% TFA in 20 min followed by 20% aqueous acetonitrile in 0.1% TFA for 10 min at a flow rate of 1 mL/min was used. The detector was at 230 nm. Chromatogram a, peak 1: peptide **6**, retention time 11.068 min, relative peak area 5.1%; peak 2: peptide **7**, retention time 12.440 min, relative peak area 9.8%; peak 3: peptide **8**, retention time 17.115 min, relative peak area 18.3%; peak 4: peptide **5**, retention time 23.866 min, relative peak area 66.8%. Peptide **5** had a longer retention time in chromatogram b because a 3-mL injection loop was used instead of a 200-μL loop, which was used for chromatogram a.

The protected amino acid **4** was then utilized in an automated solid-phase peptide synthesis using standard *t*-Boc methodology. The HPLC analysis of the crude material obtained after cleavage of the peptide-resin indicated the presence of four major products (Figure 1a). The FABMS analysis showed that the compound corresponding to the peak of longest retention time (23.866 min, relative peak area 66.8%) was the desired peptide **5** (MW 1717). Products of retention time 11.068 min (relative peak area 5.1%), 12.440 min (relative peak area 9.8%), and 17.115 min (relative peak area 18.3%) were the tyrosine-deletion peptide **6** (MW 1445), resulting from incomplete coupling, the monomethylphosphopeptide **7** (MW 1703), and the dephosphorylated peptide **8** (MW 1609), respectively. It is therefore clear that dephosphorylation and demethylation of the dimethyl phosphate residue do occur when **4** is used for the synthesis of dimethylphosphotyrosine-containing peptides under the conditions described here. It is likely that the dephosphorylation product **8** forms during HF cleavage of the peptide-resin.[33] The desired product **5** was obtained by preparative HPLC. The analytical HPLC trace obtained

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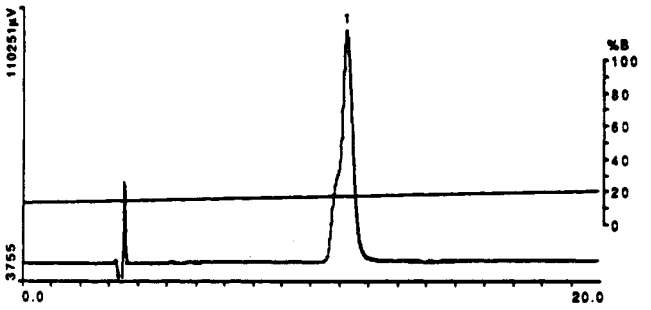
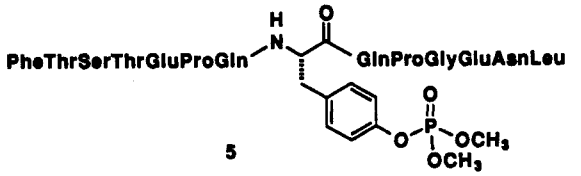



**Figure 2.** HPLC analysis of the crude product mixture obtained after demethylation of **5**. Column: Vydac 218TP104, C-18, 10 μm, 4.6 × 250 mm. A gradient elution of 13–20% aqueous acetonitrile in 0.1% TFA in 20 min at a flow rate of 1 mL/min was used. The detector was at 230 nm.

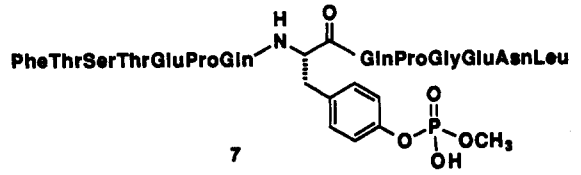
after injection of the isolated material showed only one peak (Figure 1b).



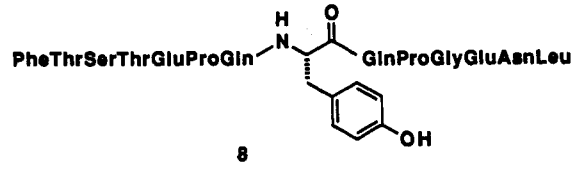

**5**

**PheThrSerThrGluProGlnGlnProGlyGluAsnLeu**

**6**




**7**




**8**

In order to demethylate the phosphate group, peptide **5** was treated with a mixture of trifluoromethanesulfonic acid, trifluoroacetic acid, dimethyl sulfide, and *m*-cresol (10:50:30:10) for 1 h at room temperature.[33,35] Analysis of the crude product by HPLC on a C-18 reverse-phase column using a gradient elution of 13–20% aqueous acetonitrile in 0.1% TFA in 20 min showed one major peak, but it had a shoulder, indicating that the crude material was a mixture (Figure 2). After considerable experimentation to find a suitable HPLC system for resolution of the mixture, it was discovered that complete separation (Figure 3) could be achieved using a 20-min gradient elution of 20–60% solvent B in solvent A where solvent A was 0.25 N triethylamine-phosphate buffer solution (pH 2.25) and solvent B was 40% solvent A in acetonitrile. The two products were present in approximately 1:2 ratio and could be isolated on a semipreparative column using a gradient elution of 20–42.5% solvent B in solvent A in 15 min where the solvent system was the same as described above. Both of the products were desalted by a further purification on a semipreparative column using a gradient elution of 13–20% aqueous acetonitrile containing 0.1% TFA in 20 min. The high-resolution FABMS, $^1$H NMR analysis, and amino acid analysis of the major product having the longer retention time were consistent with structure **1**.

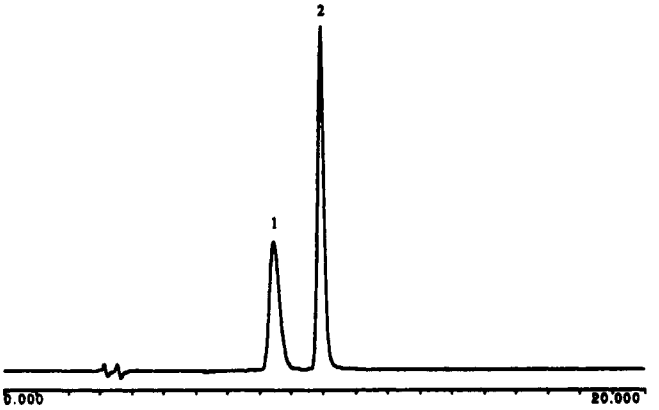



**Figure 3.** HPLC analysis of the crude product mixture obtained after demethylation of **5**. Column: Vydac 218TP104, C-18, 10 μm, 4.6 × 250 mm. A gradient elution was used of 20–60% solvent B in solvent A in 20 min where solvent A was 0.25 N triethylamine phosphate buffer solution (pH 2.25) and solvent B was 40% solvent A in acetonitrile. The detector was set at 230 nm.

A variety of analytical methods were used for the assignment of the structures of the major and minor products resulting from demethylation of **5**. The FABMS analysis of both products indicated that they had the same molecular weights of 1689. The major product was stable in water, but the minor product was unstable in water and slowly converted to the major product. In pH 7.2 buffer solution, more than 95% of the minor product was converted into the major product within 1 h.

The initial considerations suggested six structures, represented by **9**, **10**, **11**, and **12** for the minor product. Peptide **9** is an epimer of the desired product **1**. The three peptides represented by structure **10** are possible products resulting from the migration of the phosphono group from the tyrosine residue to the serine residue or to one of the two threonine residues. In peptide **11**, the *N*-terminal phenylalanine residue has migrated from the nitrogen to the oxygen of the adjacent threonine residue. Compound **12** is a related rearrangement product in which the serine has migrated from the nitrogen to the oxygen of the adjacent threonine residue.

The major product **1** as well as the minor product and the *src* peptide **8** obtained as a side product during preparation of **5** were analyzed by 500-MHz $^1$H NMR spectroscopy. The chemical shifts of the methyl groups of the threonine residues had almost the same values in **1** and **8**, but one of the methyl groups on a threonine residue was shifted downfield approximately 0.2 ppm in the minor, unstable product (Figure 4). This indicates that one of the two threonine residues may have been altered by attachment of an electron-withdrawing group on the hydroxyl, consistent with structures **10**–**12**. However, it rules out structure **9** for the minor product, since the chemical shifts of the threonine methyl groups would be expected to be the same in **9** and **1**. Structure **9** would also seem unlikely for the minor product on the grounds that it is converted to the major product **1** under mild basic

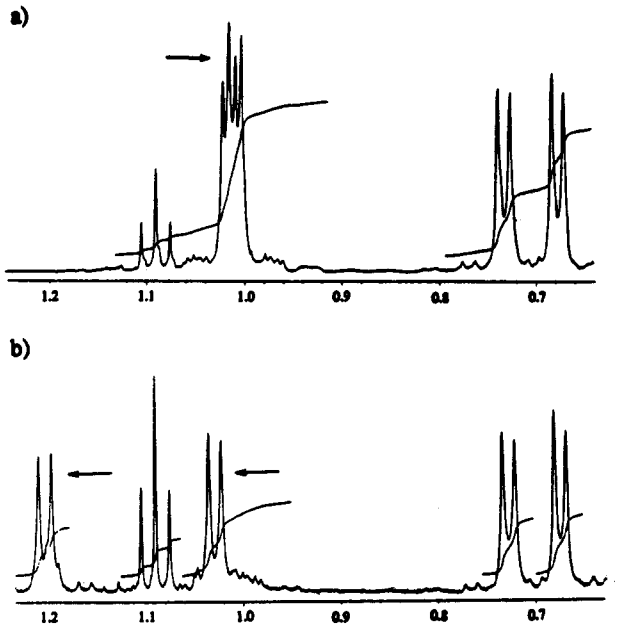
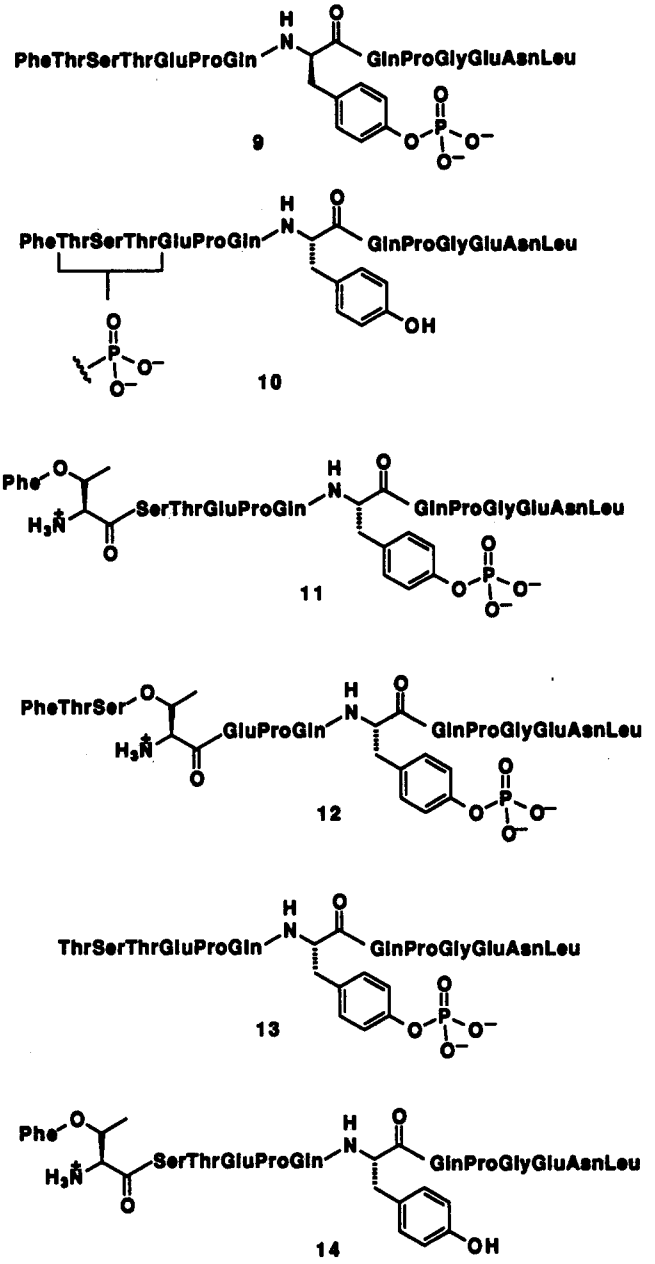



**Figure 4.** Partial (0.6–1.3 ppm) 500-MHz NMR spectra of (a) peptide 1 and (b) the minor product obtained during demethylation of 5. The arrows indicate the positions of the two threonine methyl groups.

conditions (pH 7.2), as described above. On the other hand, the chemical shifts of the tyrosyl aromatic protons had almost the same values in 1 and the minor product of demethylation, but they differed from those of 8, indicating that the tyrosine was phosphorylated in the minor product, not the serine or threonine residues. This evidence argues against the three compounds indicated by structure 10 for the minor product. Linked scan FABMS analysis of the major and minor products indicated that the peptide sequence and the location of the phosphate group were the same in the major and minor products, confirming that the three compounds indicated by structure 10 are not reasonable candidates for the structure of the minor compound.

Enzymatic reactions were also investigated in order to obtain additional evidence about the structures of the demethylation products. As expected, enzymatic hydrolysis of the major product 1 with alkaline phosphatase in pH 7.1 buffer solution converted it completely to compound 8. Enzymatic reaction of the minor product with acid phosphatase was performed in pH 4.8 buffer solution, since at that pH the further conversion of the peptide was not so fast as to interfere with the analysis. When the reaction was monitored by HPLC, the peak at retention time 7.035 min of the minor product was replaced by a new peak at 8.905 min. If the solution was then buffered at pH 7.1, the peak at retention time 8.905 min was replaced by one at retention time 10.105 min, which is identical with that of peptide 8. This result indicated that the difference between the major product 1 and the minor product of demethylation was not due to the location of the phosphate group. Hence, the compounds represented by structure 10 are also excluded on the basis of the enzyme reaction data, since the enzymatic hydrolysis products initially formed should be identical if structure 10 is assumed for the minor product formed from demethylation of 5.

The results described above, including $^1$H NMR, linked scan FABMS, enzymatic reactions using phosphatases,




and the properties of the minor product, are consistent with either of the two peptides 11 and 12. Rearranged peptides such as 11 and 12 can be formed by the slow intramolecular migration of an acyl group from the $\alpha$-amino group to the hydroxyl group on serine or threonine residues under acidic conditions.[40,41] The reverse migration of the rearranged peptides is expected to occur at pH above 3.5.[40,41] It was assumed that either 11 or 12 could form from rearrangement of 1 under the acidic demethylation conditions (trifluoromethanesulfonic acid, trifluoroacetic acid, dimethyl sulfide, and *m*-cresol). In order to test this hypothesis, peptide 1 was subjected to the demethylation conditions and the reaction followed by HPLC. This resulted in an HPLC trace similar to that shown in Figure 3, indicating that the rearranged peptide had indeed formed.

To determine the structure of the minor product as either 11 or 12, the major product 1 was treated with

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chymotrypsin, followed by subjection of the enzyme reaction product 13 to the demethylation reaction conditions (trifluoromethanesulfonic acid, trifluoroacetic acid, dimethyl sulfide, and *m*-cresol). As expected, chymotrypsin only cleaved the phenylalanine residue in peptide 1 to form 13 as indicated by FABMS analysis. Treatment of 13 with trifluoromethanesulfonic acid, trifluoroacetic acid, dimethyl sulfide, and *m*-cresol should determine whether the structure of the minor product is 11 or 12, since if it is 11, in which the *N*-terminal phenylalanine has migrated, then peptide 13 should not rearrange since it lacks the *N*-terminal phenylalanine present in 1. On the other hand, if the structure of the minor product is 12, then the rearrangement could still occur in 13. Accordingly, the chymotrypsin cleavage product 13 was subjected to trifluoromethanesulfonic acid, trifluoroacetic acid, dimethyl sulfide, and *m*-cresol (10:50:30:10) for 1 h at room temperature and the reaction mixture analyzed by HPLC. No rearrangement product was detected. It was therefore concluded that the structure of the minor product is 11, and that the product of phosphatase treatment of the minor product is 14, which rearranges to 8 under basic conditions. In conclusion, the utilization of N-(*tert*-butoxycarbonyl)-*O*-(dimethylphosphono)-L-tyrosine (4) appears to be one of the best available methods for the synthesis of phosphotyrosine-containing peptides. However, as a result of the present work, problems involving incomplete incorporation, partial demethylation of the dimethylphosphono group, and dephosphorylation during peptide synthesis, as well as acid-catalyzed backbone rearrangements during final phosphate deprotection, have been identified.

## Experimental Section

All reactions were performed under nitrogen or argon, unless stated otherwise, or unless an aqueous reaction medium was employed. Melting points were uncorrected. Analytical thin-layer chromatography was performed on Merck Kieselgel 60 $F_{254}$ plates, 0.25 mm thickness, or Aldrich precoated TLC sheets, silica gel on polyester, 0.25 mm thickness. Column chromatography was carried out on silica gel, 60–200 or 230–400 mesh. $^1$H NMR spectra were recorded at 200 or 500 MHz. The low-resolution electron impact (EI) and chemical ionization (CI) mass spectra were determined using an ionization potential of 70 eV. The CI mass spectra were obtained using isobutane as a reagent gas. Fast atom bombardment (FAB) mass spectra were recorded using a glycerol/thioglycerol or dithiothreitol/dithioerythritol (DTT/DTE) matrix. HPLC was performed on a Rainin HPLC system using a Dynamax program.

Tetrahydrofuran was distilled from sodium metal and benzophenone or from lithium aluminum hydride. Diethyl ether was distilled from lithium aluminum hydride. Methylene chloride and *N*,*N*-dimethylformamide were distilled from calcium hydride. Pyridine was distilled from potassium hydroxide. For all analytical and semipreparative HPLC, double distilled water and HPLC grade acetonitrile, methanol, 2-isopropanol, and *n*-hexane were used.

All of the enzymes utilized were purchased from Sigma Chemical Co. α-Chymotypsin was TLCK-treated type VII, obtained from bovine pancreas, dialyzed, lyophilized and essentially salt-free powder. The activity was 40–60 units/mg protein. Phosphatase, alkaline was type VII-NTA, obtained from bovine intestinal mucosa, affinity purified, and used as a solution in 3.0 M NaCl containing 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 30 mM triethanolamine, pH 7.6. The activity was 2000–3000 DEA units (1000–1500 glycine units)/mg of enzyme protein. Phosphatase, acid was obtained from human semen and was a crude lyophilized powder containing approximately 25% protein, the balance being primarily buffer salts, pH 4.8. The activity was 15 units/mg of protein.

***N*-(*tert*-Butoxycarbonyl)-*O*-(dimethylphosphono)-L-tyrosyl *p*-Nitrobenzyl Ester (3).** To a round-bottomed flask containing a mixture of *N*-(*tert*-butoxycarbonyl)-L-tyrosyl *p*-nitrobenzyl ester (2, 10.0 g, 23.6 mmol), dimethyl phosphite (4.0 g, 3.4 mL, 36.0 mmol), and $CCl_4$ (62 mL) in 62 mL of ethyl acetate at 0 °C was added dropwise triethylamine (5.0 mL, 36.0 mmol), and the mixture was stirred for 1 h at 0 °C. After stirring for 2 h at room temperature, more dimethyl phosphite (4.0 g, 3.4 mL, 36.0 mmol) and triethylamine (5.0 mL, 36.0 mmol) were added, and the stirring was continued overnight at room temperature. The mixture was diluted with ethyl acetate (100 mL), filtered to remove triethylamine·HCl, washed with cold 1 N hydrochloric acid (2 × 50 mL) and 5% aqueous $NaHCO_3$ solution (2 × 50 mL), dried with magnesium sulfate, and evaporated to give an oil. To this oil were added $CCl_4$ (62 mL) dimethyl phosphite (1.6 mL, 2.0 g, 18.0 mmol) and triethylamine (2.6 mL, 18.0 mmol). The reaction mixture was stirred overnight until no trace of starting material remained. The mixture was again diluted with ethyl acetate, filtered, washed, dried, and evaporated to yield a light yellow oil[34] (7.67 g, 60.9%) which was homogeneous in TLC and $^1$H NMR: TLC (silica gel, EtOAc/hexane = 1:1, v:v), $R_f$ 0.20; CIMS *m/e* 525 ($MH^+$); $^1$H NMR (200 MHz, $CDCl_3$) δ 8.21 (d, 2 H), 7.40 (d, 2 H), 7.09 (s, 4 H), 5.20 (s, 2 H), 5.02 (d, 1 H, exchangeable with $D_2O$), 4.71 (q, 1 H), 3.87 (d, 6 H), 3.07 (d, 2 H), 1.42 (s, 9 H).

***N*-(*tert*-Butoxycarbonyl)-*O*-(dimethylphosphono)-L-tyrosine (4).[34]** *N*-(*tert*-Butoxycarbonyl)-*O*-(dimethylphosphono)-L-tyrosyl *p*-nitrobenzyl ester (3, 7.0 g, 13.35 mmol) was dissolved in methanol (250 mL) containing acetic acid (18 mL) and hydrogenolyzed at room temperature using 10% Pd/C (0.95 g, 40 psi) for 3 h. After the residual solid was filtered off and the solvent was removed by evaporation at reduced pressure, the crude product was dissolved in ethyl acetate (200 mL). The solution was washed with 1 N hydrochloric acid (2 × 100 mL) and water (2 × 100 mL) and then extracted with 5% aqueous $NaHCO_3$ solution (3 × 100 mL). The combined aqueous extract was washed with diethyl ether (100 mL) and ethyl acetate (50 mL). The aqueous extract was acidified to pH 3 at 0 °C with 1 N hydrochloric acid and extracted with ethyl acetate (3 × 100 mL). The combined extract was dried with magnesium sulfate and evaporated at reduced pressure to give a colorless oil[34] (4.85 g, 93.4%) which was homogeneous in TLC and $^1$H NMR: TLC (silica gel, EtOAc/hexane = 1:1, v:v), $R_f$ 0.14; CIMS *m/e* 390 ($MH^+$); $^1$H NMR (200 MHz, $CDCl_3$) δ 7.14 (q, 4 H), 6.67 (bs, 1 H), 5.15 (d, 1 H, exchangeable with $D_2O$), 4.58 (m, 1 H), 3.83 (d, 6 H, $J$ = 11.4 Hz), 3.11 (m, 2 H), 1.42 (s, 9 H).

**Preparation of Dimethylphospho-*src* peptide (5) and Byproducts 6–8.** The crude peptide was prepared on an automated Applied Biosystems 430A peptide synthesizer using standard *t*-Boc methodology. The final peptide-resin cleavage and deprotection of the benzyl protecting groups were performed by treatment with HF containing 5% anisole and 5% DMS at 0 °C for 1 h. The crude peptide 5 (100 mg) obtained from the resin was analyzed by HPLC on a Vydac column (218TP104, C-18, 10 μm, 4.6 × 250 mm) and FABMS. A gradient elution of 13–20% aqueous acetonitrile in 0.1% TFA for 20 min and retaining 20% aqueous acetonitrile in 0.1% TFA in 10 min at a flow rate of 1 mL/min was used. Separation was monitored by UV detection (230 nm). There were four major peaks (Figure 1) and fractions corresponding to each peak were collected and lyophilized for FABMS analysis. The peptide 5 having the longest retention time (23.866 min), which was identified by FABMS, was separated by semipreparative HPLC on a Rainin column (Dynamax-300A, C-18, 12 μm, 10 × 250 mm). A gradient elution of 13–25% aqueous acetonitrile in 0.1% TFA in 30 min at a flow rate of 4.5 mL/min was used. Fractions corresponding to the peak (UV detector at 230 nm) of retention time 21.490 min were collected. The combined fractions were lyophilized to afford the pure peptide 5 as a white solid (60.2 mg, 60.2%), HPLC retention time 23.866 min: FABMS *m/e* 1718 ($MH^+$). Also obtained from HPLC as white solids were peptide 6 (2.5 mg, 2.5%), HPLC retention time 11.068 min: FABMS *m/e* 1446 ($MH^+$); peptide 7 (8.1 mg, 8.1%), HPLC retention time 12.440 min: FABMS *m/e* 1704 ($MH^+$); and peptide 8 (8.1 mg, 8.1%), HPLC retention time 17.115 min: FABMS *m/e* 1610 ($MH^+$).

**Preparation of Peptides 1 and 11.** To the dimethylphospho-*src* peptide **5** (40 mg) was added a mixture of TFMSA/TFA/DMS/*m*-cresol (2 mL, 10:50:30:10, v:v) and the mixture was stirred for 1 h at room temperature. Diethyl ether (10 mL) was added to induce precipitation. The precipitate was filtered and washed with diethyl ether several times (3 × 10 mL) and dissolved in water (4 mL). Crude peptides 1 and 11 were analyzed by HPLC on a Vydac column (218TP104, C-18, 10 μm, 4.6 × 250 mm) with a gradient elution of 20–60% solvent B in solvent A in 20 min at a flow rate of 1 mL/min, where solvent A was 0.25 N triethylamine phosphate buffer (pH 2.25) solution and solvent B was 40% solvent A in acetonitrile. There were two major peaks having retention times of 8.415 min (peptide 11) and 9.890 min (peptide 1) which were identified by $^1$H NMR, FABMS, enzymatic reactions, and chemical reactions. The peptides 1 and 11 were isolated by semipreparative HPLC on a Rainin column (Dynamax-300A, C-18, 12 μm, 10 × 250 mm). A gradient elution of 20–42.5% solvent B in solvent A in 15 min at a flow rate of 4.5 mL/min was used where the solvent system was the same as above. Fractions corresponding to each peak (UV detector at 230 nm) of retention time 8.2 min (peptide 11) and 9.5 min (peptide 1) were collected. The combined fractions were lyophilized to afford peptides 1 and 11. Each peptide 1 and 11 contained triethylamine phosphate salt obtained from the mobile phase, and they were desalted by semipreparative HPLC on a Rainin column (Dynamax-300A, C-18, 12 μm, 10 × 250 mm) with a gradient elution of 13–20% aqueous acetonitrile in 0.1% TFA in 20 min at a flow rate of 4.5 mL/min. Fractions corresponding to the peak (UV detector at 230 nm) of retention time 11.9 min (both peptides had the same retention time in this solvent system) were collected. The combined fractions were lyophilized to give the pure peptide 1 (16.0 mg, 47.0%) and 11 (8.7 mg, 20.7%) as white solids. Peptide 1, FABMS *m/e* 1690 ($MH^+$); amino acid analysis, (expected/found) Asp (1.0/1.0), Phe (1.0/1.0), Thr (2.0/1.9), Ser (1.0/0.9), Glu (4.0/4.0), Pro (2.0/2.1), Tyr (1.0/1.0), Gly (1.0/1.0), Leu (1.0/1.0); $^1$H NMR (500 MHz, $D_2O$) δ 1.011 (d, 3 H), 1.017 (d, 3 H), 6.919 (d, 2 H), 6.989 (d, 2 H). Peptide 11, FABMS *m/e* 1690 ($MH^+$); $^1$H NMR (500 MHz, $D_2O$) δ 1.030 (d, 3 H), 1.204 (d, 3 H), 6.914 (d, 2 H), 6.982 (d, 2 H).

**Conversion of Peptide 11 to Peptide 1.** 0.1 M Sodium phosphate buffer solution (1 mL, pH 7.2) was added to peptide 11 (5 mg), and the mixture was allowed to stand for 1 h at room temperature. The product was analyzed by HPLC on a Vydac column (218TP54, C-18, 5 μm, 4.6 × 250 mm) with a gradient elution of 20–60% solvent B in solvent A in 20 min at a flow rate of 1 mL/min where solvent A was 0.25 N triethylamine phosphate buffer (pH 2.25) solution and solvent B was 40% solvent A in acetonitrile. Coinjection of peptide 1 and the reaction product showed one peak of retention time 8.6 min. After collection of fractions corresponding to the peak of the product, they were combined and lyophilized, and the residue was desalted by semipreparative HPLC on a Rainin column (Dynamax-300A, C-18, 12 μm, 10 × 250 mm) with a gradient elution of 13–20% aqueous acetonitrile in 0.1% TFA in 20 min at a flow rate of 4.5 mL/min. Fractions corresponding to the peak (UV detector at 230 nm) of retention time 11.9 min were collected. The combined fractions were lyophilized to give peptide 1 as a white solid (5.0 mg, quantitative).

**Enzymatic Reaction of Peptide 1 with Phosphatase, Alkaline to Form Peptide 8.** A solution of phosphatase, alkaline (5 μL, 1000 units/0.12 mL) was added to a solution of peptide 1 (0.1 mg) in 0.1 M tris buffer solution (0.1 mL, pH 7.1) at room temperature. After 5 min, the reaction mixture was analyzed by HPLC on a Vydac column (218TP54, C-18, 5 μm, 4.6 × 250 mm) with a gradient elution of 20–60% solvent B in solvent A in 20 min at a flow rate of 1 mL/min where solvent A was 0.25 N triethylamine phosphate buffer (pH 2.25) solution and solvent B was 40% solvent A in acetonitrile. There was one peak of retention time 10.480 min. Coinjection of the product and authentic peptide 8 (obtained as a side product during formation of peptide 5 during peptide synthesis) showed one peak with the same retention time: FABMS *m/e* 1610 ($MH^+$).

**Enzymatic Reaction of Peptide 1 with Chymotrypsin To Form Peptide 13.** Chymotrypsin (0.2 mg, 40–60 units/mg) was added to a solution of peptide 1 (0.5 mg) in 0.1 M tris buffer solution (0.2 mL, pH 7.1) and the mixture was stirred for 6 h at room temperature. The reaction mixture was analyzed by HPLC as described above. Fractions corresponding to the peak of retention time 7.185 min were collected and lyophilized to give a white solid. The FABMS analysis indicated that the phenylalanine residue located on the *N*-terminus was cleaved by chymotrypsin: FABMS *m/e* 1542 ($MH^+$).

**Treatment of Peptide 1 with TFMSA/TFA/DMS/*m*-Cresol.** A mixture of TFMSA/TFA/DMS/*m*-cresol (0.1 mL, 10:50:30:10, v:v) was added to peptide 1 (0.2 mg) and the mixture was stirred for 1 h at room temperature. Diethyl ether (1 mL) was added to induce precipitation. The precipitate was washed with diethyl ether several times (3 × 1 mL) and dissolved in water (0.1 mL). The solution was analyzed by HPLC on a Vydac column as described above. From the HPLC analysis, the products were composed of 25.2% of peptide 11 and 74.8% of peptide 1.

**Treatment of Peptide 13 with TFMSA/TFA/DMS/*m*-cresol.** A mixture of TFMSA/TFA/DMS/*m*-cresol (0.1 mL, 10:50:30:10, v:v) was added to peptide 13 (0.1 mg) and the mixture was stirred for 1 h at room temperature. Diethyl ether (1 mL) was added to induce precipitation. The precipitate was washed with diethyl ether several times (3 × 1 mL) and dissolved in water (0.1 mL). The solution was analyzed by HPLC on a Vydac column as described above. From the HPLC analysis, peptide 13 remained unreacted.

**Enzymatic Reaction of Peptide 11 with Phosphatase, Acid To Form Peptide 14.** A solution of phosphatase, acid (7 μL, 3.5 units) was added to a solution of peptide 11 (0.1 mg) in 0.1 M AcOH/NaOAc buffer solution (20 μL, pH 4.8) and the mixture was stirred for 15 min at room temperature. The reaction mixture was analyzed by HPLC as described above. The peak corresponding to peptide 11 (7.035 min) was replaced by one corresponding to peptide 14 (8.905 min) after completion of the reaction: FABMS *m/e* 1610 ($MH^+$).

**Conversion of Peptide 14 to Peptide 8.** 0.1 M Tris buffer solution (0.5 mL, pH 7.1) was added to peptide 14 (0.1 mg) and the mixture was allowed to stand for 30 min at room temperature. The reaction mixture was analyzed by HPLC as described above. The retention time of the product 8 was 10.105 min as opposed to 8.905 min for the starting material 14. Coinjection of peptide 8 and the product showed one peak having the same retention time as peptide 8.

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