Communications

Synthesis and Application of Fmoc-*O*-[bis(dimethylamino)phosphono]-tyrosine, a Versatile Protected Phosphotyrosine Equivalent

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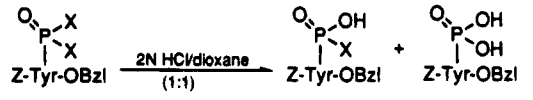
The discovery of SH2 domains in proteins and their interactions with phosphotyrosine motifs in signal transduction pathways presents an opportunity to develop new anticancer therapeutic agents.[1,2] To understand protein–protein interactions that propagate signal transduction at the molecular level, the use of phosphotyrosine-containing peptides has become the favored approach owing to their well-defined structures and their increasing ease of synthesis. Recently, we reported the synthesis of the (methyldiphenylsilyl)ethyl (MDPSE)-protected Fmoc-phosphotyrosine derivative and its use to make phosphopeptides, all in good yields and high purity.[3] In this paper, we describe the development of a different and perhaps more versatile building block, Fmoc-*O*-[bis-(dimethylamino)phosphono]tyrosine (**8**), for the preparation of peptides containing phosphotyrosine or one of its analogs. The chemistry underlying the *O*-[bis(dimethylamino)phosphono]tyrosine protection strategy was modeled upon hydrolytic lability of the P–N bond under acidic conditions.

The phosphorus–nitrogen bond in a phosphoramidate group is stable under alkaline conditions but unstable in acids, hydrolyzing readily to the corresponding substituted phosphate compound.[4,5] The rate of acid-catalyzed hydrolysis depends on both electronic and steric effects of the substituents on the amide nitrogen as well as acid strength.[6–8] Little is known about acid-catalyzed hydrolysis of phosphodiamidate derivatives, but we anticipated that phosphodiamidate derivatives would behave similarly. To define the scope and limitations for the use of phosphodiamidate derivatives as phosphotyrosine building blocks in Fmoc-based solid phase synthesis, we prepared five phosphordiamidate derivatives (**1a–5a**, Table 1) using a literature procedure.[9] When compounds **1a** or **4a** were dissolved in 1 N HCl solution, hydrolysis to the corresponding phosphate was observed reaching 95% completion after 6 h as determined by HPLC (Table 1). By comparison, after 24 h of hydrolysis the piperidine derivative **3a** resulted in 40% of the desired phosphate **3c** and 24% of the phosphoramidate **3b**, the hydrolytic intermediate. Surprisingly, the diethylamino derivative **2a** gave only 1% of the desired phosphate **2b** and 30% of the phosphoramidate **2c** after 24 h. We view these results as evidence that steric hindrance of the substituents on nitrogen profoundly influences the rate of P–N bond hydrolysis. In the case of the anilino derivative **5a**, the rate of hydrolysis was also slow, giving 23% of the desired phosphate **5c** and 14% of the intermediate **5b** after 24 h. Here, electron-withdrawing effects of the aromatic ring probably govern the slow hydrolysis by reducing protonation of the amide nitrogen and consequently decreasing the rate of hydrolysis. This is consistent with the slow rates of acid hydrolysis of phosphinanilide derivatives.[10] Although the rate of hydrolysis can be increased by using more concentrated acids, we prefer 1 N HCl because it offered acceptable hydrolysis rates without compromising the peptide backbone. In addition to the HCl-catalyzed hydrolysis reaction, several acids were also examined using the dimethylamino derivative **1a** as a model. Among them, formic acid and acetic acid were ineffective, but *p*-toluenesulfonic acid (1 N aqueous solution) and TFA/H_2O (9:1) give complete hydrolysis in 20 and 12 h, respectively. To further investigate the hydrolysis reaction in aqueous TFA, compound **1a** was dissolved in TFA/H_2O (9:1) and the progress of hydrolysis was monitored by ^{31}P NMR. These results were in agreement with HPLC. After considering all of these results, aqueous TFA was chosen because it had been used successfully in Fmoc-based peptide synthesis for the final step of deprotection and cleavage of the peptide from the resin. Furthermore, it is also an excellent solvent for dissolving peptides.

Table 1. HCl-Catalyzed Hydrolysis of Phosphodiamidate Derivatives[a]



a b c

Amines	Time (h)	Hydrolysis Products (%)	
1. X= NMe_2	6		95
2. X= NEt_2	24	30	1
3. X= 	24	24	40
4. X= $HNCH_2CH_2CH_3$	6		95
5. X= HNPh	24	23	14

[a] 2 mg of the phosphodiamidate derivatives was dissolved in 2 mL of 2 N HCl/dioxane solution (1:1), and hydrolysis was monitored by HPLC at 215 nm.

Judging from the model hydrolysis studies, we suspected that tyrosine phosphordiamidate derivative would be a useful building block for preparing phosphotyrosine containing peptides and therefore optimized the synthesis of Fmoc-*O*-[bis(dimethylamino)phosphono]tyrosine (**8**) as shown in Scheme 1. This compound was prepared in a three-step synthesis starting from commercially available

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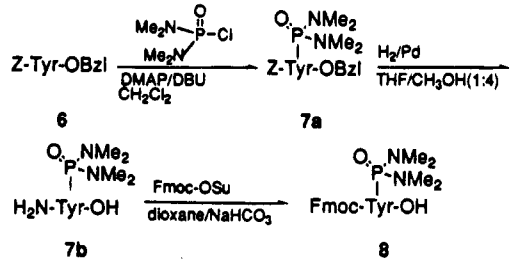
Scheme 1



and inexpensive Z-Tyr-OBzl. Reaction of Z-Tyr-OBzl (10 mmol) with the bis(dimethylamino)phosphoryl chloride (20 mmol) in the presence DMAP and DBU in CH_2Cl_2 gave crystalline Z-protected **7a** in 85% yield. The subsequent hydrogenolysis step quantitatively removed the Z and OBzl protecting groups, and introduction of the Fmoc protecting group proceeded in 82% yield to give the Fmoc-*O*-[bis(dimethylamino)phosphono]tyrosine (**8**) as a white powder in overall 70% yield.[11,12]

To demonstrate the utility, we synthesized several peptides containing phosphotyrosine (Table 2).[13] Phosphopeptides **9**–**11** were used for binding studies to the mBlk SH2 domain by calorimetric analysis. In this study, the nonphosphorylated peptide **9** and the analog peptide **11** showed no binding to the mBlK SH2 domain, but the phosphorylated peptide **10** binds to the mBlk SH2 with micromolar affinity.[14] It is noteworthy that phosphopeptide **11** with the bis(dimethylamino) moiety is obtainable when the hydrolysis step is omitted.[15] Phosphopeptides **12**–**19** represent the B cell immunoreceptor tyrosine activation motifs (ITAMs) which play a critical role in B cell signal transduction events.[16] These peptides were used to investigate whether binding Syk protein tyrosine kinase to the phosphopeptides could stimulate the catalytic activity of the enzyme. Results indicated that association of Syk tandem SH2 domains with the doubly phosphorylated ITAM peptides (**15** and **19**) lead to Syk enzyme activation.[17]

Table 2. Phosphotyrosine Peptides[a]

9	Ac-SM Y EDISGRL-$CONH_2$
10	Ac-sM pY EDISGRL-$CONH_2$
11	Ac-SM pY*EDISGRL-$CONH_2$
12	DHT YEGLNIDQATAT YEDIVT-$CONH_2$
13	DHTpYEGLNIDQATAT YEDIVT-$CONH_2$
14	DHT YEGLNIDQATATpYEDIVT-$CONH_2$
15	DHTpYEGLNIDQATATpYEDIVT-$CONH_2$
16	ENL YEGLNLDDCSM YEDISR-$CONH_2$
17	ENLpYEGLNLDDCSM YEDISR-$CONH_2$
18	ENL YEGLNLDDCSMpYEDISR-$CONH_2$
19	ENLpYEGLNLDDCSMpYEDISR-$CONH_2$

[a] pY = phosphotyrosine. pY* = bis(dimethylamino)phosphonotyrosine.

We have optimized the synthesis and demonstrated the usefulness of the Fmoc-*O*-[bis(dimethylamino)phosphono]-tyrosine (**8**) as a building block for the synthesis of peptides containing phosphotyrosine or [bis(dimethylamino)phosphono]tyrosine. Furthermore, this derivative can now be used for automated synthesis of peptides containing phosphotyrosine in discreet or combinatorial fashion.

Supporting Information Available: Copies of NMR spectra and HPLC chromatograms of peptides **9**–**11** (11 pages).

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(11) All new compounds gave satisfactory C, H, N analysis (±0.3%).

(12) To a solution of Z-Tyr-OBzl (**6**) (4.03 g, 10 mmol) in 25 mL of CH_2Cl_2 at 0 °C were added DBU (1.5 equiv), DMAP (2 equiv), and bis-(dimethylamino)phosphoryl chloride (20 mmol). The resulting mixture was stirred at 0 °C for 1 h and at room temperature for an additional 1 h. The organic solution was washed with 5% $KHSO_4$ solution, 10% $NaHCO_3$ solution, and brine. After drying, 4.6 g of **7a** was obtained as white crystals, mp 112–3 °C . Hydrogenolysis in THF/MeOH (1:4) with 10% Pd/C for 4–5 h gave phosphotyrosine **7b** in quantitative yield. Reaction with Fmoc- OSu in dioxane/$NaHCO_3$ yielded 3.7g of the Fmoc-protected phosphotyrosine derivative **8** as a white powder.

(13) See ref 3 for protocols used in peptide synthesis. General procedure for deprotection and cleavage peptide resin containing the [bis(dimethylamino)phosphono]tyrosine residue: 0.1 mmol of peptide polystyrene resin was treated with 10 mL of TFA/Phenol/Et_3SiH (95: 3:2) solution for 2 h at room temperature and then 1 mL of H_2O was added, and the resulting mixture was allowed to stand overnight. The crude phosphopeptides were purified to homogeneity by C_{18}-RP HPLC using 50 mM $(NH_4)HCO_3$ buffer 6–64% in 30 min. The identity of phosphopeptides was established by AAA and MS/ES.

(14) Data of the binding studies will be published elsewhere.

(15) After cleavage from the resin, a small amount of the corresponding phosphotyrosine-containing peptides was always present in the crude product but was easily separated by HPLC.

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