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# A General Preparation of Protected Phosphoamino Acids

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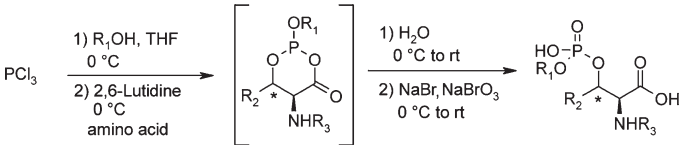
## ABSTRACT




**Fmoc-*O*-benzyl-L-phosphoserine is an important building block in the synthesis of Forigerimod, a phosphopeptide being investigated for Systemic Lupus Erythematosus (SLE). An efficient one-pot process was developed using inexpensive, readily available starting materials. This general procedure was used to prepare a variety of protected phosphoamino acids.**

Protein phosphorylation and dephosphorylation are ubiquitous reactions involved in many important cell processes, including cell signaling.[1] Accordingly, the preparation of phosphopeptides has received much attention in the chemical literature.[2] Phosphopeptides can be prepared either by phosphorylation of an existing peptide (global phosphorylation strategy) or by the use of individual phosphoamino acid building blocks in standard peptide synthesis (prephosphorylation strategy). As with other amino acids, the choice of the side chain protecting group is critical in order to minimize side reactions.

Forigerimod (Figure 1) is a spiceosomal 21 amino acid phosphopeptide currently being investigated for the treatment of Systemic Lupus Erythematosus (SLE).[3] It is synthesized via solid phase peptide synthesis using the Fmoc strategy. It contains two serine residues, only one of which is phosphorylated, making prephosphorylation an attractive option.

Fmoc-*O*-benzyl-phospho-L-serine (**1**) (Figure 2) is a commonly employed phosphoamino acid building block[4] used in the synthesis of forigerimod. It was introduced in 1994 by Wakamiya and co-workers for use in solid phase synthesis using the Fmoc strategy.[3a] The use of monobenzyl protection is necessary to avoid elimination of the phosphate group to form dehydroalanine under the basic Fmoc deprotection conditions.[4a] In addition, the single benzyl group can be removed under the same conditions as other acid-labile side-chain protecting groups.

Compound **1** has been used in the synthesis of several biologically important peptides, including a portion of heat shock protein 27,[4c] and the C-terminus of the c-fos protein.[4d] It is commercially available from several vendors; however it is expensive,[5] compared to other Fmoc-protected amino acids, and of inconsistent quality.

Although several syntheses of **1** (Figure 2) have been reported in the literature, all are multistep, requiring protection and deprotection of the carboxylate moiety, and use expensive and/or hazardous reagents.[4] For example, all published preparations involve the use of dialkyl *N,N*-phosphoramidite reagents, introduced by Beaucage and Caruthers for the synthesis of oligonucleotides.[6]

(1) Cohen, P. *Eur. J. Biochem.* **2001**, *268*, 5001.

(2) (a) Arendt, A.; Hargrave, P. A. In *Peptide Synthesis Protocols*; Pennington, M. W., Dunn, B. M., Eds.; Humana Press Inc.: Totowa, NJ, 1994; Vol. 35, Chapter 9, p 187. (b) Perich, J. W. In *Methods in Enzymology*; Fields, G. B., Ed.; Elsevier: 1997; Vol. 289, Chapter 12, p 245. (c) Attard, T. J.; O'Brien-Simpson, N.; Reynolds, E. C. *Int. J. Pept. Res. Ther.* **2007**, *13*, 447.

(3) (a) Monneaux, F.; Lozano, J. M.; Patarroyo, M. E.; Briand, J. P.; Muller, S. *Eur. J. Immunol.* **2003**, *33*, 287. (b) Muller, S.; Monneaux, F.; Schall, N.; Rashkov, R. K.; Oparanov, B. A.; Wiesel, P.; Geiger, J.-M.; Zimmer, R. *Arthritis Rheum.* **2008**, *58*, 3873.

(4) (a) Wakamiya, T.; Saruta, K.; Yasuoka, J.; Kusumoto, S. *Chem. Lett.* **1994**, 1099. (b) Wakamiya, T.; Nishida, T.; Togashi, R.; Saruta, K.; Yasuoka, J.; Kusumoto, S. *Bull. Chem. Soc. Jpn.* **1996**, *69*, 465. (c) Wakamiya, T.; Togashi, R.; Nishida, T.; Saruta, K.; Yasuoka, J.; Kusumoto, S.; Aimoto, S.; Kumagaye, K. Y.; Nakajima, K.; Nagata, K. *Bioorg. Med. Chem.* **1997**, *5*, 135. (d) Luo, S.; Li, Y.; Chen, Z.; Abe, H.; Cui, L.; Nakanishi, H.; Qin, X.; Zhao, Y. *Lett. Pept. Sci.* **2003**, *10*, 57.

(5) **1** is available from Sigma-Aldrich for $252/g (2012−2014 catalog).

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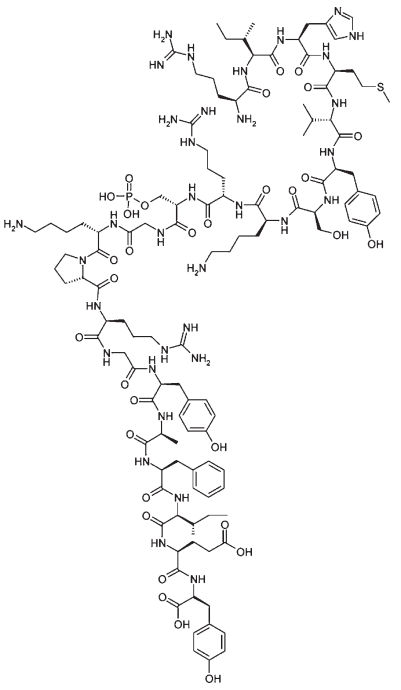
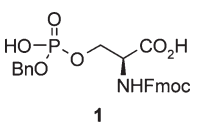


**Figure 1.** Forigerimod (Arg-Ile-His-Met-Val-Tyr-Ser-Lys-Arg-Sep-Gly-Lys-Pro-Arg-Gly-Tyr-Ala-Phe-Ile-Glu-Tyr).

Although such reagents are often readily available, they require the use of an activator, such as 1-*H*-tetrazole, which is no longer available due to its explosive nature. Although other heterocycles have been shown to promote phosphitylation, many are expensive or require synthesis.[7] In addition, most syntheses using phosphoramidites target a doubly protected phosphoamino acid as the penultimate intermediate, which requires an additional deprotection step to form the monobenzyl-protected species.

The original synthesis[4a,b] by Wakamiya and co-workers is shown in Scheme 1. In their synthesis, a dialkyl *N, N*-phosphoramidite (prepared in two steps) is reacted with a phenacyl-protected serine derivative in the presence of 3 equiv of 1-*H*-tetrazole. The resulting phosphite intermediate is oxidized with *m*-CPBA. Finally the phenacyl group and the trichloroethyl (TCE) group are removed via reduction with zinc in acetic acid.

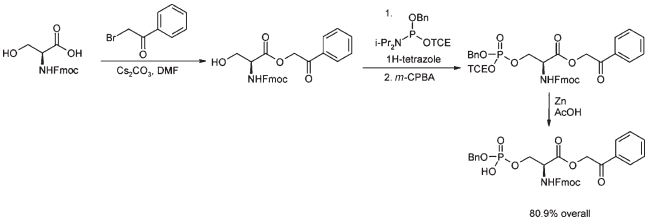


**1**

**Figure 2.** Fmoc-*O*-benzyl-phospho-L-serine.

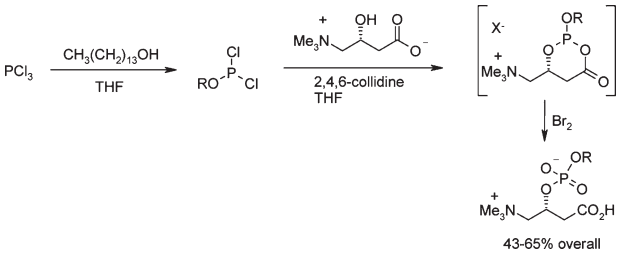
After an evaluation of this and other known synthetic routes[4] to **1**, we desired a more scalable, step-economical synthesis for the production of forigerimod. We initially envisioned the use of either $POCl_3$ or $PCl_3$ as an inexpensive, highly reactive source of phosphorus that would not require an activating agent. We also wished to access the monoprotected amino acid directly.

**Scheme 1.** Wakamiya Synthesis of **1**[4b]




Initial reactions employing Fmoc-L-serine, $POCl_3$, and benzyl alcohol led to complex mixtures, presumably due to activation of the carboxylate group by $POCl_3$. We then focused our attention on $PCl_3$ and a report by Prashad and co-workers, in which a cyclic mixed phosphite intermediate was oxidized to a phosphodiester (see Scheme 2).[8]

**Scheme 2.** One-Pot Preparation of Phosphodiester[8]




As shown in Scheme 3, treatment of $PCl_3$ (1.1 equiv) with BnOH (1.0 equiv) in the presence of 2,4,6-collidine (6.5 equiv) led to the presumed benzyl dichlorophosphite intermediate, as determined by $^{31}P$ NMR analysis (177 ppm in $CDCl_3$). Addition of Fmoc-L-serine as either the anion or the neutral species led to the presumed cyclic phosphite intermediate, as detected by $^{31}P$ NMR analysis (134 ppm in $CDCl_3$). Hydrolysis led to the acyclic phosphite, detected by $^{31}P$ NMR analysis and reversed-phase LC-MS. Oxidation of this species with $Br_2$ (1.3 equiv) led to **1**, albeit with numerous other impurities.

Using these conditions as a starting point, the process was rapidly optimized. Using 1.3 equiv of $PCl_3$ and 1.5 equiv of BnOH led to a 62% HPLC assay yield of **1**, based on an external standard. Using a greater excess of both reagents led to lower yields.

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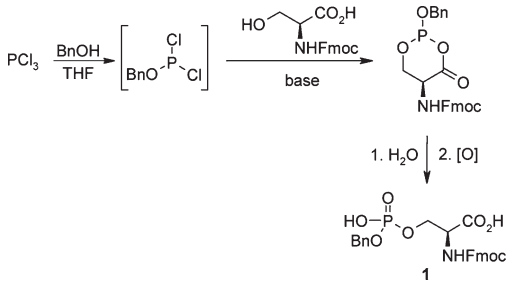
(7) Maung, J.; Mallari, J. P.; Choy, C. J.; Berkman, C. E. *Tetrahedron Lett.* **2004**, *45*, 6497.

(8) Prashad, M.; Amedio, J. C.; Ciszewski, L.; Lee, G.; Villa, C.; Chen, K.-M.; Prasad, K.; Repic, O. *Org. Process Res. Dev.* **2002**, *6*, 773.

**Scheme 3.** One-Pot Preparation of **1**




Reducing the amount of base to 4.0 equiv (sufficient to neutralize the HCl generated from the $PCl_3$) resulted in an improved yield (65%). At this stage, the conditions reliably generated a 60−70% HPLC assay yield of the desired compound on a multigram scale.

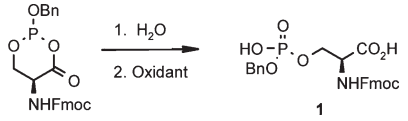
Although bromine is inexpensive and performed well in the oxidation, a more user-friendly oxidant was desired for handling reasons. Therefore, a screen of oxidants was performed (see Table 1). Many reagents are known to oxidize phosphites to phosphates; however many, such as *t*-BuOOH, employ anhydrous conditions, which are not suitable for our system.

In our hands, iodine (entry 2) resulted in a similar yield, while aqueous NaOCl, Oxone (potassium monoperoxysulfate), and diacetoxyiodobenzene (DAIB) all provided < 50% HPLC assay yield of **1**. Bromine-related oxidants gave the highest yield, with pyridinium tribromide providing an equivalent yield to bromine. The most suitable conditions proved to be an aqueous mixture of sodium bromide and sodium bromate, known to form molecular bromine at low pH. To our knowledge this is the first use of these reagents for the oxidation of phosphorus in either phosphoamino acids or nucleotides.

Other bases were also screened (see Table 2). The study was limited to organic bases due to the insolubility of inorganic bases in the reaction medium (THF). In addition, secondary amines were ruled out due to their ability to remove the Fmoc protecting group. The highest yield was obtained with 2,6-lutidine (entry 3), although other pyridine-related bases also performed well (entries 1,4,5). Hünig's base (entry 2) gave a slightly lower yield, while DBU resulted in an insoluble precipitate that inhibited stirring of the mixture.

The unique properties of **1** made the isolation difficult initially. Most literature preparations involve direct precipitation of **1**; however this was not feasible in the presence of other related impurities. The use of 2-MeTHF proved critical to extract **1** from the biphasic reaction mixture present at the end of the reaction. Fortuitously, upon concentration of the solvent, **1** crystallized as a 1:1 complex with 2-MeTHF, as determined by $^1$H NMR, in > 95% purity in 55−60% yield. The pure, desolvated compound could be obtained by stirring **1** in the presence of solvents such as EtOAc or $CH_2Cl_2$.[9] Gratifyingly, analysis of the isolated product by chiral HPLC indicated that no racemization occurred.

**Table 1.** Effect of Oxidant on the Yield of **1**



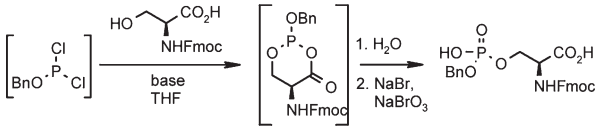

| entry | oxidant | yield[a] |
|---|---|---|
| 1 | $Br_2$ | 67% |
| 2 | $I_2$ | 66% |
| 3 | $PyrHBr_3$ | 66% |
| 4 | $NaBrO_3$/NaBr | 71% |
| 5 | NaOCl | 18% |
| 6 | Oxone | 34% |
| 7 | DAIB | 37% |

[a] HPLC assay yield using an external standard.

Our attention turned next to the preparation of other phosphorylated serine derivatives (Table 3). Fmoc-*O*-methylphospho-L-serine **2** (entry 1) was prepared in 69% HPLC assay yield and could be isolated by preparative reversed-phase HPLC chromatography in 39% yield (not optimized). A previous synthesis of this molecule involved the use of an additional phosphate protecting group.[10]

The use of the (±)-1-(2-nitrophenyl)ethyl (NPE) protecting group (entry 2) was also investigated. Imperiali and co-workers have shown that phosphoserine derivatives employing this protecting group can be incorporated into phosphopeptides.[11] The NPE group can be removed in the presence of far UV light (> 350 nm), unmasking the free phosphate group. A related reagent had previously been prepared as a doubly protected phosphate in several steps. In our hands, the one-pot procedure gave a 51% isolated yield of **3** as a mixture of diastereomers after preparative chromatography.

**Table 2.** Effect of Base on the Yield of **1**

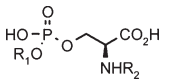



| entry | base | yield[a] |
|---|---|---|
| 1 | 2,4,6-collidine | 63% |
| 2 | *i*-$Pr_2$EtN | 59% |
| 3 | 2,6-lutidine | 71% |
| 4 | 2-picoline | 68% |
| 5 | pyridine | 67% |

[a] HPLC assay yield using an external standard.

The use of the CBz protecting group (entry 3) resulted in a 63% HPLC assay yield of **4**. The use of Boc-L-serine resulted in a 45% HPLC assay yield; however, chromatography resulted in decomposition.

**Table 3.** Preparation of Phosphoserine Derivatives



| entry | $R_1$ | $R_2$ | product | yield[a] |
|---|---|---|---|---|
| 1 | Me | Fmoc | **2** | 69% (39%) |
| 2 | 1-(2-$NO_2$-Ph)Et | Fmoc | **3** | 79% (51%[b]) |
| 3 | Bn | Boc | **4** | 45% (nd) |
| 4 | Bn | Cbz | **5** | 63% (16%) |

[a] HPLC assay yield using an external standard; isolated yields (not optimized) are in parentheses. [b] 93% pure by HPLC.

Finally, the phosphorylation of other Fmoc-hydroxyamino acids was performed. Fmoc-*O*-benzyl-phospho-L-threonine (**6**) was prepared in 72% HPLC assay yield and crystallized in 51% yield (Figure 3). This important building block had previously been prepared via a multistep synthesis, using phosphoramidite chemistry.[4b] The isomeric Fmoc-L-allo-threonine (**7**) was also prepared in 88% HPLC assay yield, 55% isolated yield. In addition, the racemic phenyl substituted derivative **9** was prepared in 96% assay yield, 48% isolated yield.

The L-homoserine derivative **8** was also prepared in 70% HPLC assay yield. Interestingly, Fmoc-L-tyrosine (**10**), which is not capable of forming a cyclic phosphite, also resulted in a high HPLC assay yield (86%) and good isolated yield (69%). This intermediate was previously prepared in a multistep procedure involving protection of the carboxylate group as well as phosphorylation with a phosphoramidite reagent.[12]

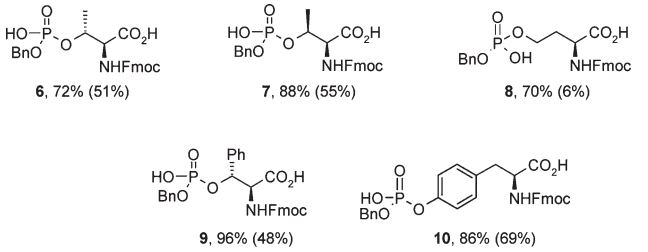



**Figure 3.** Additional phosphoamino acids prepared with HPLC assay yields (nonoptimized isolated yields are in parentheses).

In summary, a highly efficient, one-pot synthesis of protected phosphoamino acids was developed using inexpensive, readily available reagents. This method allows the rapid preparation of these useful building for use in the synthesis of phosphopeptides.

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**Supporting Information Available.** Experimental procedures, spectral characterization, and copies of $^1H$, $^{13}C$, and $^{31}P$ NMR spectra for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

(9) The details of the preparation and isolation of **1** on a kilogram scale will be detailed. Mowrey, D. R. Petrillo, D. E.; Allwein, S. P.; Graf, S.; Bakale, R. P. Manuscript in preparation.

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