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The *H*-Phosphonate Approach to the Synthesis of Phosphopeptides on Solid Phase

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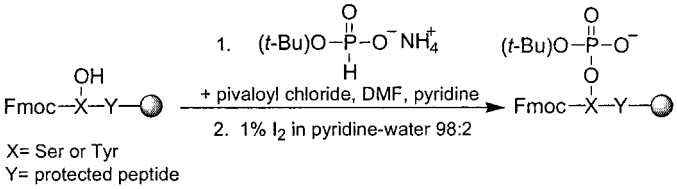
ABSTRACT



Ammonium *tert*-butyl *H*-phosphonate was used for the phosphorylation of Tyr- and Ser-containing peptides synthesized by an Fmoc strategy. This reaction, leading to a monoprotected peptide phosphate, was found to be highly efficient and generally applicable. Moreover, the method employed avoids undesired side reactions during chain elongation (pyrophosphate formation and β-elimination catalyzed by piperidine).

The phosphorylation of proteins is probably the most important reversible element of the cell regulation. The involvement of tyrosine phosphorylation/dephosphorylation in this process is well-known, and a similar controlling mechanism involving serine/threonine phosphorylation was recently discovered.[1] The isolation of phosphorylated peptides/proteins from biological sources for functional or conformational studies is usually not feasible, and there is therefore a need for efficient chemical phosphorylation methods. Although many papers on phosphopeptide synthesis were published in the past decade, a universal method which can be applied with high efficiency in all cases does not exist. From a chemical point of view, the problems arising during peptide phosphorylation can be divided into two groups:

1. In the case of tyrosine: the decreased nucleophilicity of the phenolic hydroxyl group inhibits the application of phosphoric acid-based phosphorylation reagents (e.g., phosphochloridates[2,3] or phosphoric anhydride[4]). Moreover, pyrophosphate formation may take place if an unprotected phosphate moiety is introduced.[5]

2. In the case of serine or threonine: after incorporation of the phosphate moiety, the molecule can undergo different side reactions. The most important of these is the β-elimination catalyzed by piperidine used for Fmoc deprotection during chain elongation, resulting in a loss of phosphate and formation of the corresponding dehydropeptide.[6]

To overcome these difficulties, many reagents have been tried and introduced into phosphopeptide chemistry.[3,7−13] The

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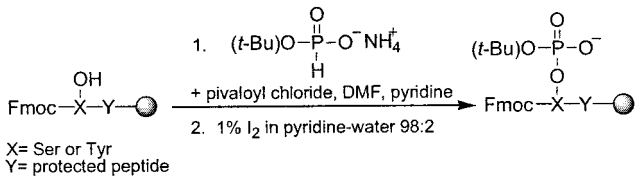
routinely used P(III) phosphoramidite derivatives[9−13] can be applied successfully in phosphotyrosine synthesis. However, for phosphoserine/phosphothreonine-containing peptides, application of these symmetrically protected derivatives may lead to the above-mentioned side reaction.

To eliminate these problems, one of the most widely used and favorable methods makes use of monoprotected phosphate-containing building blocks of Tyr, Ser, and Thr.[14] On the other hand, the synthesis of these monomers is multistep and tedious. In addition, only benzyl-protected building blocks of L-Ser, L-Thr, and L-Tyr are commercially available. The only reagent that can be applied universally to introduce a monoprotected phosphate into any hydroxyl group-containing peptide is an unsymmetrically protected phosphoramidite.[15] Unfortunately, phosphoramidites are difficult to synthesize, subject to decomposition and oxidation, and expensive to use.

Another alternative to prepare monoprotected peptide phosphates is the use of *H*-phosphonates (phosphonic acid monoesters) that have been applied successfully in oligonucleotide synthesis.[16,17] Subsequent oxidation after phosphonylation of a free hydroxyl function with the corresponding *H*-phosphonate yields the desired monoprotected peptide phosphate. Being P(III) compounds, *H*-phosphonates exhibit the same reactivity as phosphoramidites but are much less sensitive to oxidation and moisture. To date, the only case where the *H*-phosphonate method has been applied in solid-phase peptide synthesis is the phosphorylation of an *N*-terminal Ser residue with benzyl *H*-phosphonate.[18] We describe here a universally applicable phosphopeptide synthesis involving the *H*-phosphonate approach, with ammonium *tert*-butyl *H*-phosphonate as phosphorylating agent (Scheme 1). In our approach the phosphonylation was carried out right after the incorporation of the appropriate hydroxyamino acid. This method has several advantages of both the global and synthon procedures and seems to be applicable for multiphosphorylated peptides without the need of selective protection of the amino acid hydroxyls.

Scheme 1. Phosphorylation of Ser/Tyr-Containing Peptides with *tert*-Butyl *H*-Phosphonate on Solid Phase



The peptides listed in Table 1 were synthesized by the Fmoc protocol, applying Tentagel SRAM and HMP resins for Tyr- and Ser-containing peptides, respectively. Phosphonylation was performed on the resin directly after incorporation of the free hydroxyl-containing Ser/Tyr residue through the use of 10 equiv of ammonium *tert*-butyl *H*-phosphonate[19] and 10 equiv of pivaloyl chloride in 1:1 DMF/pyridine for 2 h at room temperature. Subsequent oxidation was carried out with 1% I_2 in 98:2 pyridine/water at room temperature for 2 h. After phosphorylation, chain elongation was performed in the usual manner (the carboxamide functions of Gln and Asn were protected with the trityl group). Final cleavage and deprotection (removing of *tert*-butyl, *tert*-butyloxycarbonyl, trityl, and 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl protecting groups from the final product) were carried out with a 95:2.5:2.5 (v/w/v) TFA/DTT/water mixture at room temperature for 2 h.

Table 1. Phosphopeptide Sequences Synthesized[a]

entry	sequence
1a	S(*P*)ALPG-OH
1b	RKKRIS(*P*)ALPG-OH
2a	Y(*P*)VPMTGP−NH_2
2b	IQEANY(*P*)VPMTGP−NH_2

[a] *(P)* denotes side-chain phosphorylation.

To monitor the phosphate incorporation and possible side reactions, after phosphonylation followed by oxidation an aliquot of each peptide (**1a** and **2a**) was cleaved off the resin. LC-MS analyses of the resulting mixtures revealed >90% phosphate incorporation for both Tyr and Ser (illustrated for Tyr in Figure 1).

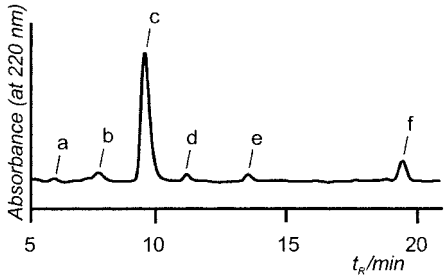


Figure 1. HPLC chromatogram of crude peptide **2a**. Peaks were analyzed by ESI-MS: (a) m/z^+ 859.6 [M + H^+] of the Met-oxidized byproduct of peptide **2a**; (b), d), (e) unidentified peaks; (c) m/z^+ 843.1 [M + H^+] of peptide **2a** (M_{calcd} 842.35); (f) m/z^+ 763.4 [M + H^+] of nonphosphorylated peptide **2a**.

Surprisingly, iodination of the Tyr aromatic ring could not be detected (although some small peaks (Figure 1, peaks b, d, and e) could not be identified, the *m*/*z* values for the monoiodinated or diiodinated peptides were not observed by mass screening), while oxidation of the Met side chain was

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negligible (<1%, Figure 1, peak a). The desired peptides **1b** and **2b** were obtained in high yield, as determined by HPLC (Figures 2 and 3). Therefore, significant β-elimination

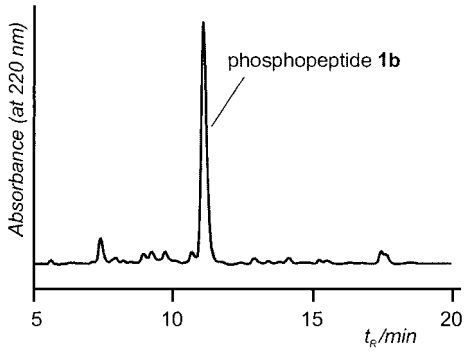

Figure 2. HPLC chromatogram of crude peptide **1b**. Major peak identified by ESI-MS (M_{calcd} 1204.69, found m/z^+ 1205.1 [M + H]$^+$).

did not take place during chain elongation for the Ser-containing peptide.

In summary, we have successfully applied the *H*-phosphonate method to the phosphorylation of Ser- and Tyr-containing peptides on solid-phase with high efficiency and without considerable side reactions during oxidation following phosphonylation and chain elongation. Ammonium *tert*-butyl *H*-phosphonate is inexpensive, easy to synthesize and purify, and less sensitive to oxidation than the phosphoramidite reagents. Furthermore, final removal of the *tert*-butyl group can be performed under milder conditions than those for the benzyl group in the previously described benzyl *H*-phosphonate. The method published here offers a cheaper and more convenient alternative over the phosphoramidite approach for phosphorylation of any hydroxyl group-containing peptide.

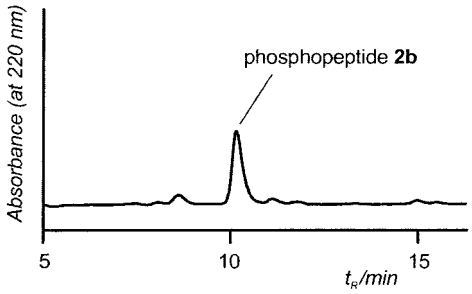

Figure 3. HPLC chromatogram of crude peptide 2**b**. Major peak identified by ESI-MS (M_{calcd} 1398.49, found m/z^+ 1399.2 [M + H]$^+$).

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