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., phosphochloridates2,3 or phosphoric anhydride4). 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 $\beta$-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

out right after the incorporation of the appropriate hydroxamino 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.

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. Phosphorylation 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 and 10 equiv of pivaloyl chloride in 1:1 DMF/pyridine for 2 h at room temperature. Subsequent oxidation was carried out with 1% I2 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-butyloxy carbonyl, trityl, and 2,2,4,6,7-pentamethyldihydrobenzofuran-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.

To monitor the phosphate incorporation and possible side reactions, after phosphorylation 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).

### Table 1. Phosphopeptide Sequences Synthesized

<table>
<thead>
<tr>
<th>entry</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>S(P)ALPG-OH</td>
</tr>
<tr>
<td>1b</td>
<td>RKKRIS(P)ALPG-OH</td>
</tr>
<tr>
<td>2a</td>
<td>Y(P)VPMTGP-NH2</td>
</tr>
<tr>
<td>2b</td>
<td>IQEANY(P)VPMTGP-NH2</td>
</tr>
</tbody>
</table>

*(P) denotes side-chain phosphorylation.

![Figure 1](image-url)

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<sub>calc</sub> 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 moniodinated or diiodinated peptides were not observed by mass screening), while oxidation of the Met side chain was

### References

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 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 phosphorylation 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.

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

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