Chapter 10

Solid-Phase Synthesis of Phosphorylated Tyr-Peptides by "Phosphite Triester" Phosphorylation

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1. Introduction

Phosphorylation of tyrosine, serine, and threonine residues by protein kinases has been shown to be a key step in the regulation of many cellular events *(1)*. A better understanding of the molecular basis of this regulatory step is needed in order to gain insight into the specificity of the kinases that control these events.

Chemical methodology for preparing the phosphate esters of Tyr, Ser, and Thr has recently improved making it possible to synthesize peptides containing one or more of these residues routinely *(2–6)*. Some of these methods require the preparation of diphenyl-phosphono esters of Thr and Ser, which are reported to be stable to HF treatment (*see* Chapter 4) making a Boc-synthesis strategy possible. These derivatives are ultimately deblocked by hydrogenation *(7)*. Use of this strategy for preparing Tyr-P peptides is hampered by reduction of the aromatic ring of Tyr. However, a newer strategy has recently been developed that allows the convenient synthesis of phosphotyrosine. This method, developed by Perich and coworkers *(8)*, utilizes an Fmoc strategy where the Tyr residue to be phosphorylated is incorporated with an unprotected side chain. Following completion of synthesis of the remaining sequence, the peptide resin is subjected to phosphitylation and subsequent oxidation. Early reports have shown that Ser, Thr, and Tyr could be successfully incorporated

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with unprotected side chains into the peptide in Fmoc strategies either by carbodiimide/HOBt or BOP reagent *(9–11)*. Automated syntheses of phosphopeptides using this type of procedure have also recently been reported *(12,13)*. Additionally, peptides containing multiple phosphorylated residues have been prepared using this method as a "global" phosphorylation method *(14)*. Although these procedures were all developed in other laboratories and are all well documented in the literature, since we routinely prepare our phosphopeptides by this method, it is our desire to include a chapter describing this methodology to make our volume as complete and up to date as possible.

2. Materials

1. An Fmoc strategy is necessary to use this procedure. All Fmoc derivatives are available from Bachem Bioscience (King of Prussia, PA). The special Fmoc amino acids with unprotected side chains (Fmoc-Ser-OH, Fmoc-Thr-OH and Fmoc-Tyr-OH) are also available from the same vendor.
2. The phosphitylation reagents di-*tert*-butyl-*N,N* diethyl phosphoramidite and 1H-tetrazole are available from Aldrich (Milwaukee, WI). These reagents must be kept frozen and dry.
3. The oxidation reagents used to convert the phosphite triester to the phosphate most commonly used are *m*-chloroperbenzoic acid and *t*-butyl hydroperoxide. These reagents are also available from Aldrich.
4. All solvents required from peptide synthesis are available from standard commercial sources.
5. Commercially prepared protected Boc-diphenylphosphonate derivatives of Ser, Thr, and dimethylphosphonate-Tyr are available from Bachem Bioscience. Additionally, the Fmoc-Tyr dimethylphosphonate derivative is also available.

3. Methods

This short section describes the standard procedure for preparing phosphorylated peptides containing Tyr using an Fmoc-based synthesis strategy. An excellent review of Fmoc-strategy solid-phase synthesis may be found in Atherton and Sheppard *(15)*. This phosphorylation procedure will work well on peptides that do not contain oxidation-prone residues, such as Met, Cys, and Trp. In cases where these residues are present, a Boc strategy, such as that described by Kitas et al. *(5)*, may be more appropriate. The incorporation of the Boc-protected dimethylphospho derivative instead of the derivative containing the unprotected side-chain

hydroxyl group is suggested. This will eliminate the postphosphitylation oxidation step and help to preserve these susceptible residues.

3.1. Preparation of the Protected Phosphorylated Peptide

Initially, the phosphite triester is formed on the unprotected phenolic hydroxyl group of Tyr. Subsequently, the phosphitylated derivative is oxidized to the phosphonate derivative by treatment with an oxidant.

1. Synthesis of the desired peptide sequence is performed using a standard Fmoc strategy, except that at the position where the phosphorylated residue is located, an Fmoc amino acid derivative of Tyr is incorporated with the side chain unprotected (*see* Note 1). This coupling may be mediated by either standard carbodiimide/HOBT methods *(16)* or with BOP or similar uronium-type reagents *(17)*.
2. Synthesis then resumes until the entire peptide chain is assembled. We recommend incorporating the final amino acid as the appropriate Boc-*t*Bu derivative. These groups are cleaved when the entire peptide resin is deprotected and cleaved. Maintaining the blocked N-terminus prevents undesired reactions at this position by incorporating acid-labile-protecting groups. This eliminates exposure to a final treatment of piperidine to remove the N-terminal Fmoc group.
3. Prepare a fresh solution of di-*tert*-Butyl *N,N* diethyl-phosphoramidite in fourfold excess over the total theoretical peptide amount to be phosphorylated, and dilute this into 1–2 mL/g of resin of dry DCM. The amount of reagent to be prepared is determined by the relative substitution of the solid-phase resin times the amount of resin used for the synthesis. For example, 2 g of resin with an initial substitution of 0.65 mmol/g would result in 1.3 mmol of peptide, thus requiring 5.2 mmol of the phosphoramidite. **Always remove a portion of resin in case a problem occurs during the phosphorylation procedure or cleavage.**
4. This solution is added to the peptide resin preswollen in dry DCM.
5. 1H-Tetrazole is added directly to the reaction vessel in a tenfold excess over the total amount of the phosphoramidite.
6. The reaction vessel is mixed for 1–1.5 h at room temperature. Following this reaction time, the vessel is drained and the peptide resin washed with three DCM washes.
7. To complete the phosphorylation procedure:
   a. The phosphitylated derivative is oxidized *in situ* by treatment with a solution of 85% *m*-chloroperbenzoic acid (3–5 Eq based on the amount of peptide) in 5 mL of DCM/g of resin for 10–30 min at room temperature.

b. Alternatively, oxidation can be performed with a solution of *t*-butyl hydroperoxide in DMF at a ratio of 6–20 Eq/Eq of peptide resin for 30–60 min at room temperature (*see* Note 2). This step may be used successfully with Met peptides if the temperature is lowered to 4°C.

8. Following the oxidation, the reaction vessel is drained and washed with 5 × 5 mL/g DCM, and dried.

3.2. Cleavage of the Final Phosphopeptide

Following the completion of the phosphorylation reaction, the peptide resin is ready for final cleavage and deprotection. As with all Fmoc-based peptides, the scavenger cocktail is dictated by the types of protecting groups present. We have found the "Reagent K" cocktail to be the most satisfactory for phosphopeptides *(18)*. The cleavage of Fmoc-synthesized peptides is covered in greater detail elsewhere in this volume (*see* Chapter 5).

1. Prepare a solution of 82.5% trifluoroacetic acid, 5% thioanisole, 5% phenol, 5% H_2O, and 2.5% 1,2-ethanedithiol at a ratio of 10 mL of cleavage cocktail/g of resin.
2. Cool this solution on ice for 1 h.
3. Initiate the cleavage by adding this cold solution to a flask containing the peptide resin and a magnetic stir bar. Actuate the stir bar, and allow the cleavage vessel to warm up to room temperature.
4. Stir this solution for 1–2.5 h depending on the number of Arg(Pmc) residues present. Longer cleavage time is sometimes necessary for peptides containing more than three or four Arg residues.
5. Filter the peptide through a fritted funnel, and wash the resin fines with TFA.
6. Precipitate the peptide into ice-cold ether.
7. Analyze the crude peptide by RP-HPLC (*see* Chapter 3, *PAP*), and compare the retention time vs the unphosphorylated form. Phosphopeptides generally elute significantly earlier than the nonphosphorylated peptide on a C_{18}-column.
8. FAB/MS analysis (*see* Chapter 7, *PAP*) of the product (*see* Notes 3 and 4) should result in a mol-wt increase of 79.5 mass units/phosphate incorporated. If the mass is 16 mass units low, the oxidation reaction was unsuccessful and should be repeated for a longer period with a greater excess of *m*-chloroperbenzoic acid or *t*-butyl hydroperoxide (*see* Note 5).

4. Notes

1. An Fmoc strategy must be employed when using this methodology.
2. It has been reported that use of *t*-butyl hydroperoxide was successfully employed in peptides containing Met and Cys *(19)*. In this scenario, the

Cys(Trt) was resistant to oxidation, and the Met was only partially oxidized.

3. FAB/MS analysis is required for all peptides containing Tyr-P. Also, the UV spectrum of Tyr-P is shifted with a λ_{max} at 266 nm compared to Tyr with a λ_{max} at 275 nm *(20)*.
4. Standard amino acid hydrolysis procedures destroy the phospho-ester linkage, and the amino acid derivative will be detected as the standard underivatized form with the minor amount of destruction associated with hydrolysis of these hydroxy residues.
5. Peptides containing any of oxidation-sensitive residues, such as Met, Cys, and Trp, may be synthesized with a Boc strategy using the appropriate protected diphenylphosphonate derivative of Ser or Thr and dimethylphosphonate for Tyr. These derivatives are commercially available. The phenyl-protecting groups are stable to the cleavage reaction and removed by catalytic hydrogenation as described in the preceding chapter. The Tyr derivative may be incorporated as the dimethylphosphonate, and the protecting groups for the entire peptide as well as the dimethylphosphonate-Tyr are cleaved by TFMSA/TFA/thioanisole *(5)*. Peptides containing phosphoserine or phosphothreonine can also be prepared with this type of strategy, but the phosphorylation proceeds much slower than with the phosphotyrosine residue. Peptides containing these residues may be best prepared by the method described in Chapter 9.

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