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Preparation of an asymmetrically protected phosphoramidite and its application in solid-phase synthesis of phosphopeptides

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Abstract

O-tert-Butyl-*O'*-β-cyanoethyl-*N*,*N*-diisopropylphosphoramidite as a new global phosphorylation reagent and its application for solid-phase phosphopeptide synthesis via monoprotected phosphate-peptide ester during peptide synthesis are described.

Keywords: peptide; solid-phase synthesis; phosphorylation; phosphoramidite; phosphopeptide.

Protein phosphorylation has been recognized as one of the most important elements of cell regulation and signal transduction. For studying the role of the phosphorylation/dephosphorylation event in biological functions or investigating its conformational consequence, the isolation of the appropriate phosphorylated protein is not usually feasible. Therefore, the efficient chemical synthesis of the related phosphopeptide is a valuable alternative. There are two major strategies for the preparation of phosphopeptides: the synthon and the global approach. The synthon method[1–4] (both in liquid and solid-phase) requires appropriately protected phosphorylated derivatives—these derivatives are often not commercially available and their syntheses are multistep, complicated and expensive procedures. Sometimes the synthesis of the appropriate building block itself can be a challenge for the synthetic chemist. On the other hand, this approach is a safe and simple method to synthesize phosphate containing peptides. The global approach requires appropriate reagents for the phosphorylation (practically phosphitylation and subsequent oxidation to phosphate, or alternatively, reaction with phosphochloridate[5,6]) and experience in handling these reagents which are not routine chemicals for peptide chemists. For phosphitylation, usually symmetrical phosphoramidite derivatives[7–11] are used which produce symmetrically bis-protected phosphate derivatives. However, while this method can be applied with good yield in

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the case of phosphotyrosine containing peptides, for phosphoserine and phosphothreonine containing peptides the treatment with piperidine during the standard deprotection cycle leads to β-elimination with loss of the phosphate moiety and formation of the corresponding dehydropeptide.[12] Removal of the protecting groups from the phosphate moiety would hinder this reaction,[13] but the resulting free acidic hydroxyl functions can cause other side reactions such as pyrophosphate formation.[14] The monoprotected derivatives of these hydroxyl group-containing amino acid phosphate esters seem to be the optimal choice, but to date only the synthon method has been applied for this purpose utilizing Fmoc-Ser[PO(OBn)OH]-OH and Fmoc-Thr[PO(OBn)OH]-OH monomers.[13] The global approach, being applicable for any hydroxyl group-containing residue, would offer a more general method. However, the application of the unsymmetrically protected phosphoramidite reagent needed for this has not yet been reported. Here we describe the synthesis of a novel unsymmetric phosphoramidite reagent and its application for the synthesis of a phosphoserine containing peptide by the global approach.

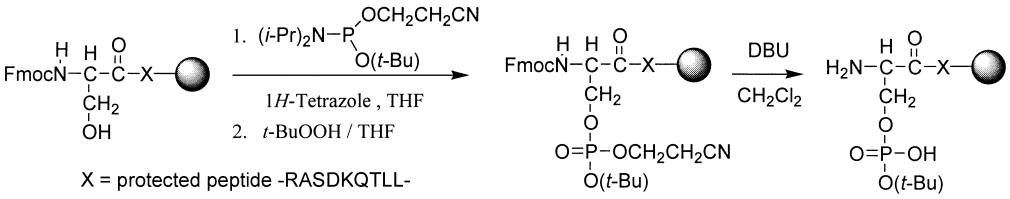
Our aim was to develop a phosphitylation reagent which can convert a hydroxyl group-containing peptide to the corresponding monoprotected phosphopeptide. The reason for this was the need for the phosphorylated derivative of the QDGVRQSRASDKQTLLPNDQ peptide. This peptide is a fragment of the CD3 γ-chain of the T-cell receptor complex, namely the 117–136 fragment phosphorylated on the Ser-126 residue. Phosphorylation of this serine residue has been implicated in the regulation of the TCR signalling by the involvement in the downmodulation of the receptor complex from the cell surface. The standard synthesis applying the routine amidite reaction resulted in a very complex crude product and the desired phosphopeptide could be isolated with a very low yield (below 1%).

Earlier, we successfully used the symmetrical di-*tert*-butyl-*N*,*N*-diisopropylphosphoramidite for synthesis of phosphotyrosine containing peptides[15] which affords an efficient, fast, convenient and symmetrical phosphitylation. The *tert*-butyl group is easily removable at the end of the peptide synthesis.[12] Based on these data, we designed a novel unsymmetric compound in which one of the *tert*-butyl groups is replaced by a β-cyanoethyl group. This latter group, well known in oligonucleotide chemistry,[16] is removable with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)[17,18] by β-elimination to afford the desired monoprotected phosphopeptide. In addition, DBU also removes the Fmoc *N*-protecting group[19] of the Ser/Thr residue; therefore, a subsequent deprotection step is not necessary.

O-*tert*-Butyl-*O*′-β-cyanoethyl-*N*,*N*-diisopropylphosphoramidite was prepared from *O*-β-cyanoethyl-*N*,*N*-diisopropylchlorophosphite,[20] by a literature method.[†] Peptide synthesis was carried out on Rink amide resin using standard Fmoc protocol. Phosphitylation was carried out in tetrahydrofuran (THF) at room temperature for 30 min using 1*H*-tetrazole as activator and the subsequent

[†] To a stirred solution of *tert*-butanol (0.1 mol) and *N*,*N*-diisopropylethylamine (0.24 mol, 2.4 equiv.) in dry DCM (50 ml) a solution of *O*-β-cyanoethyl-*N*,*N*-diisopropylchlorophosphite (0.12 mol, 1.2 equiv.) in dry DCM (10 ml) was added at 0°C. After 1 h, the reaction mixture was extracted with 5% aq. $NaHCO_3$ (2×50 ml) and the organic layer was dried and concentrated in vacuo. The residue was distilled at 100–114°C/2–4 mmHg to give the desired product as a colourless oil (6.0 g, 55%). ^{1}H NMR ($CDCl_3$, 500 MHz, δ, ppm): 1.16–1.19 (m, 12H, $CH(CH_3)_2$), 1.36 (s, 9H, $(CH_3)_3$), 2.61 (t, 2H, 6.6 Hz, CH_2CN), 3.61 (m, 2H, CH), 3.68–3.81 (m, 2H, CH_2O). ^{13}C NMR ($CDCl_3$, 125 MHz, δ, ppm, assignment based on *J*-modulated spin–echo and HMQC experiments): 21.05 (d, $^3J_{CP}=7.0$ Hz, CH_2CN), 24.84 (d, $^3J_{CP}=7.7$ Hz, $CH(CH_3)_2$), 25.30 (d, $^3J_{CP}=7.2$ Hz, $CH(CH_3)_2$), 31.53 (d, $^3J_{CP}=8.1$ Hz, $C(CH_3)_3$), 43.85 (d, $^3J_{CP}=12.7$ Hz, CH); 58.44 (d, $^2J_{CP}=17.6$ Hz, CH_2O), 76.09 ($C(CH_3)_3$), 118.52 (CN). ^{31}P NMR ($CDCl_3$, 200 MHz, δ, ppm, proton-decoupled spectrum): 139.84. The compound is too unstable to be characterized by elemental analysis or high resolution mass spectrometry.

oxidation was performed by 15% *tert*-butyl hydroperoxide in THF. Simultaneous cleavage of β-cyanoethyl and Fmoc groups was performed by 20% DBU in dichloromethane (DCM) (Scheme 1).



Scheme 1.

DBU, being a strong base, could also promote the β-elimination resulting in the loss of the phosphate group. To investigate this possible side reaction, an aliquot of the peptide-resin after phosphorylation followed by DBU deprotection was cleaved from the resin with trifluoroacetic acid (TFA).‡ As can be seen in Fig. 1, the crude cleavage mixture contained one main product. According to ESI-MS analysis, this product has the M_W of the desired phosphopeptide.§ All these data suggest that the β-cyanoethyl group is a better leaving group than the phosphate moiety and the resulting monoprotected phosphate is not subject to further elimination.

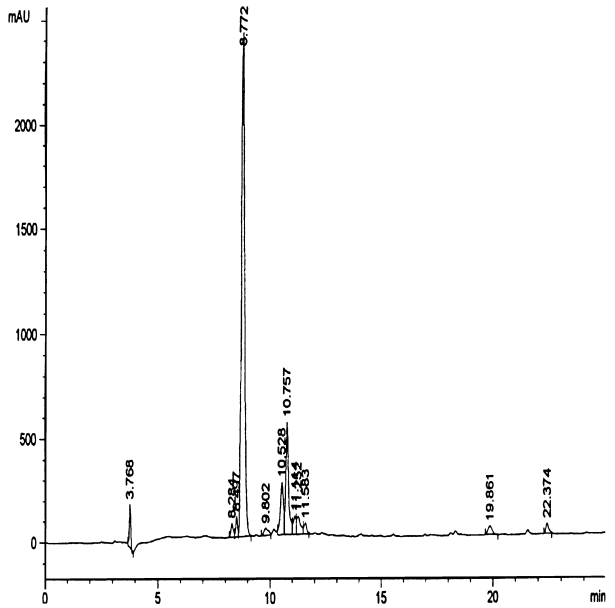


Figure 1. HPLC chromatogram of the crude S(PO_3H_2)DKQTLLPNDQ peptide

‡ Conditions of the final deprotection and the cleavage of the peptide from the resin: 87.5% TFA, 5% H_2O, 2.5% 1,4-DL-dithiothreitol, 5% phenol, 2 h at O°C.

§ M_W of the S(PO_3H_2)DKQTLLPNDQ peptide: calcd 1337.3; found 1337.1.

After completion of the elongation of the peptide chain, the protecting groups were removed and the peptide was detached from the resin using TFA.‡ The mass spectrum of the final crude product can be seen in Fig. 2.

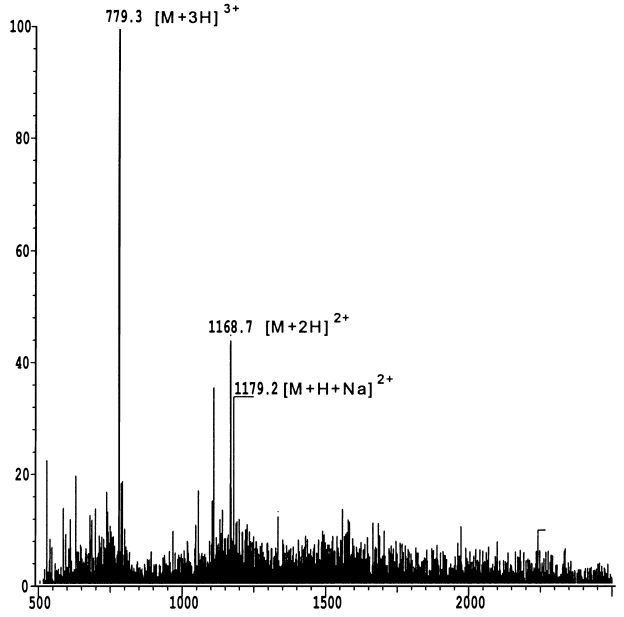


Figure 2. ESI-MS spectrum of the crude QDGVRQSRAS(PO_3H_2)DKQTLLPNDQ peptide M_W (calcd): 2335.4

In conclusion, we have introduced a new phosphitylation reagent which affords the synthesis of Ser/Thr containing phosphopeptides by the global approach without the undesired phosphate loss. Application of this new reagent has the following advantages: the phosphite incorporation is practically quantititive and removal of the β-cyanoethyl group does not require an additional step if DBU is used for the Fmoc deprotection. Furthermore, final removal of the *tert*-butyl group from the phosphate can be performed under milder conditions compared to those required for the previously described OBn containing synthons. TFA, normally used for final cleavage in Fmoc peptide synthesis, also removes the *tert*-butyl group and, therefore, an additional deprotection step is not necessary. The major advantage of this method is that the reagent is universally applicable for the phosphorylation of any hydroxyl group-containing compound without the same time-consuming multistep synthesis of the corresponding synthons.

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