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Selective Enzymatic Removal of Protecting Groups: The Phenylacetamide as Amino Protecting Group in Phosphopeptide Synthesis

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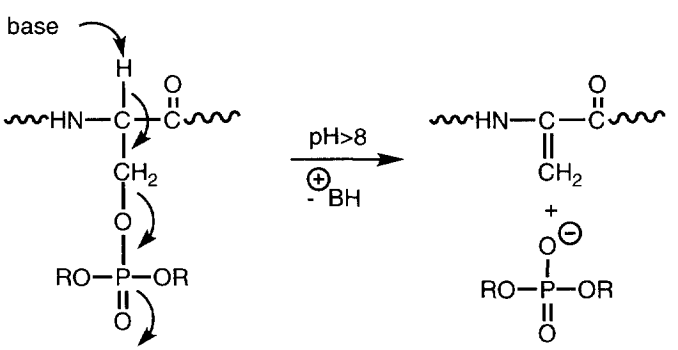
Abstract: The phenylacetamido (PhAc) amino protecting group can be removed from sensitive phosphopeptides by means of penicillin G acylase under mild conditions (pH 6.5, room temperature) and without attack on the peptide bonds, the C-terminal esters or the phosphates. In particular, under the conditions of the enzymatic reactions a β-elimination of the phosphate does not occur.

The phosphorylation and dephosphorylation of proteins on serine, threonine and tyrosine residues are among the most important regulatory processes in biology. These covalent protein modifications are employed by all organisms for numerous purposes, e.g. the control of the transduction of extracellular chemical signals to the nucleus, the regulation of the transcription of the genetic code by transcription factors, the control of the cell cycle and the regulation of cell growth and proliferation.[1] If the sensitive network of protein kinases and phosphatases involved in these processes is disturbed or interrupted, disease may result. This is particularly the case in the manifestation of cancer,[2] but also Alzheimer's disease and various other illnesses are associated with an aberrant phosphorylation state of different proteins.[1-3] For the study of the underlying biological phenomena on a molecular level and for the development of therapeutic agents which target intracellular phosphorylation and dephosphorylation processes characteristic peptides which embody the phosphorylation sites of their parent naturally occurring phosphoproteins serve as invaluable tools.[3] The importance of such phosphopeptides has stimulated an intense interest in the development of synthetic methods for their efficient synthesis both in solution and on solid supports.[4-6]

Scheme 1



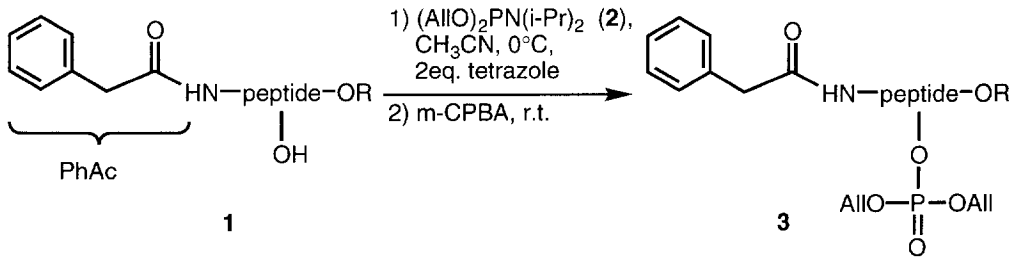
A major problem which has to be solved in one of the most flexible and reliable strategies for phosphopeptide synthesis, the stepwise assembly of the target compound from prephosphorylated building blocks (as opposed to global phosphorylation of a preassembled peptide) is the selective removal of N- or C-terminal protecting groups from fully protected serine- or threonine phosphopeptides. In the course of this transformation even under weakly basic conditions a rapidly occurring β-elimination (Scheme 1) of the phosphate moiety has to be feared,[4] thus making the use of blocking groups necessary which can be cleaved under very mild conditions. The problem of the β-elimination has alternatively been approached by using partially protected[5] (Ser-OP(O)(OR)(OH)) or unprotected[6] (Ser-OP(O)(OH)$_2$) building blocks, however, in these cases an undesired participation of the phosphates in the peptide coupling step may occur.[6]

Enzymatic protecting group techniques[7] offer viable alternatives to the established classical-chemical methods. In particular, the characteristically mild reaction conditions (pH 6-8, room temperature) under which these enzymatic transformations usually can be carried out has allowed for the construction of sensitive and multifunctional lipo-[8] and glycopeptides.[9] In the light of these findings we have investigated the application of enzymatically removable blocking functions in phosphopeptide chemistry.

In this paper we report that the phenylacetamido(PhAc) group which can be removed from the respective PhAc amides by means of penicillin G acylase at pH 7-8 and room temperature[10] can advantageously be applied as an enzyme-labile protecting group for the construction of serine/threonine phosphopeptides.

As model compounds for the investigation of the use of the PhAc group in phosphopeptide chemistry, the phosphorylated di- and tripeptides **3** were synthesized (Scheme 2).

Scheme 2

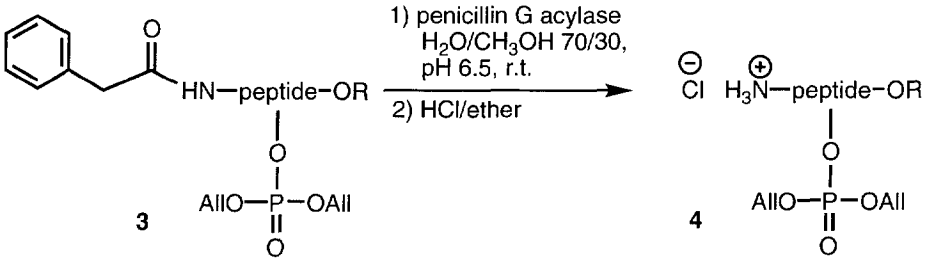


No.	peptide-OR	yield [%]
3a	Ala-Ser-OtBu	70
3b	Ala-Thr-OtBu	90
3c	Leu-Ala-Thr-OtBu	89
3d	Leu-Ala-Ser-OtBu	72
3e	Ala-Ala-Ser-OtBu	63

To this end, the PhAc-protected peptides **1** were built up from PhAc amino acids and N-terminally deblocked dipeptide esters by employing well established procedures of peptide chemistry.[10a] The OH-groups of the serine and threonine residues embodied in **1** were then converted to the diallyl phosphates by phosphitylation of the peptides **1** with the phosphoramidite **2**[11] followed by oxidation of the resulting phosphites to phosphates by means of m-chloroperbenzoic acid (MCPBA). By this procedure the phosphopeptides **3** were obtained in overall yields of 63-90%.

The phenylacetyl protected substrates **3** were then subjected to the penicillin G acylase mediated hydrolysis of the N-terminal amide bond at pH 6.5 and room temperature in a water/methanol mixture (70:30) (Scheme 3). Gratifyingly, under these mild conditions penicillin acylase accepted the phosphodi- and tripeptides **3** as substrates. In the course of the ensuing enzymatic transformations only the phenylacetamido blocking group was attacked, the peptide bonds, the C-terminal ester and the phosphates remained intact. In particular, the reaction conditions are so mild that no trace of β-elimination was observed. Reaction control by t.l.c. revealed that the enzymatic deprotections proceeded virtually to completion within several hours, the isolated yields of 40 to 72% of the N-terminally deprotected phosphopeptides **4** are due to losses upon isolation of the hydrophilic products from the aqueous reaction mixtures.[12] The presence of 30 vol % of methanol as solubilizing cosolvent is recommendable to guarantee that the phosphopeptides **3** are well solubilized in the aqueous reaction medium and thereby readily accessible to the biocatalyst.

Scheme 3



No.	peptide-OR	yield [%]
4a	Ala-Ser-OtBu	55
4b	Ala-Thr-OtBu	60
4c	Leu-Ala-Thr-OtBu	62
4d	Leu-Ala-Ser-OtBu	40
4e	Ala-Ala-Ser-OtBu	72

The results depicted in Scheme 3 demonstrate that the enzymatic method is compatible with different ester groups and amino acid combinations, which is in accord with the findings recorded for the use of penicillin acylase in the construction of simple peptides.[10a] Thus, by employing this method various selectively deblocked phosphopeptides are readily accessible under mild conditions. They may be applied further in the construction of biologically relevant peptide conjugates. Work along these lines is currently in progress in our laboratory.

Acknowledgement:
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12) *General Procedure for the enzymatic deprotection of the phosphopeptides 3*:
To a solution of 1 mmol of the PhAc-protected peptide **3** in a mixture of water and methanol 70:30 (v/v); 300 ml; pH 6.5) 3000 units of penicillin G acylase (immobilized on Eupergit C) is added. The reaction mixture is stirred at room temperature until tlc control indicates that the substrate has been consumed completely (6-20h). The immobilized biocatalyst is filtered off, washed with water and methanol and from the filtrate the methanol is distilled off in vacuo. The pH of the remaining aqueous solution is adjusted to 1-2 and the solution is extracted three times with 50 ml of ether. After adjustment of the pH to 9 the aqueous phase is extracted five times with 80 ml of CH_2Cl_2, the combined organic layers are dried with $MgSO_4$ and 30 ml of a saturated solution of HCl in ether is added. The solvent if distilled off in vacuo and the desired N-deprotected phosphopeptide hydrochlorides are isolated as colourless or lightly yellow oils which can be further purified by chromatography on silica gel employing ethyl acetate: methanol 2:1 (v/v) as eluent. **4a**: 55%, $[\alpha]_D^{23}$= -17.2 (c=1, CH_3OH); **4b**: 60%, $[\alpha]_D^{23}$=4 (c=1, CH_3OH); **4c**: 62%, $[\alpha]_D^{23}$= -12.9 (c=1, CH_3OH); **4d**: 40%, $[\alpha]_D^{23}$=5.9 (c=2, CH_3OH); **4e**: 72%, $[\alpha]_D^{23}$= -31,8 (c=1, CH_3OH).

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