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Chemoenzymatic Synthesis of a Characteristic Phosphopeptide Fragment of the Raf-1 Kinase

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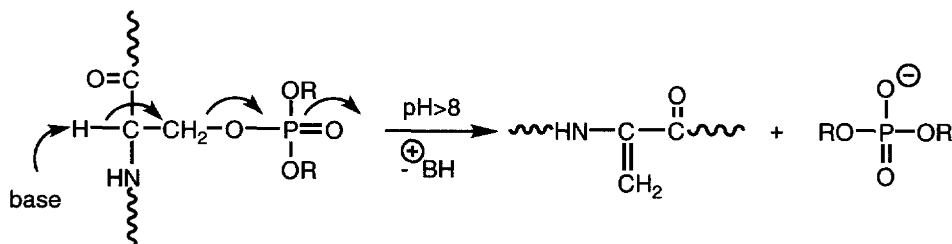
Abstract: Base-labile biologically relevant phosphopeptides can advantageously be synthesized under very mild conditions (pH 7, 37°C) by employing the heptyl (Hep) ester as enzyme-labile protecting group. This method was used in the construction of a characteristic, selectively phosphorylated hydroxyamino acid fragment of the Raf-1 kinase, an important constituent of the *Ras* pathway of signal transduction. © 1997 Elsevier Science Ltd.

The phosphorylation and dephosphorylation of proteins on serine, threonine and tyrosine residues belongs to 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] An illustrative and particularly relevant example is the *Raf*-1 serine/threonine kinase, product of the c-*raf* gene, the normal cellular counterpart of v-*raf*, the transforming gene of murine sarcoma virus[1,2]. It is ubiquitously expressed in numerous cell-types and serves as downstream effector of the *Ras* protein, the central molecular switch in the so-called *Ras* pathway, which transduces signals initiated by growth factors across the plasma membrane to the cell nucleus.[3] Activation of the *Ras* pathway by mitogens and growth factors results in the rapid hyperphosphorylation of *Raf* and the activation of its kinase activity.[1,4] *Raf* then phosphorylates MAP kinase kinase (MEK) and thereby passes the signal further on towards the nucleus. Upon dephosphorylation by a phosphatase the kinase activity of *Raf* and consequently its ability to transduce signals is greatly reduced.

If the sensitive network of protein kinases and phosphatases involved in such processes is disturbed or interrupted, disease may result. This is particularly the case in the manifestation of cancer (vide supra; the *raf* gene is an oncogene),[2] but also Alzheimer's disease and various other illnesses are associated with an aberrant phosphorylation state of different proteins.[1-4] 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.[5] This biological relevance of phosphopeptides has stimulated an intense interest in the development of synthetic methods for their efficient synthesis both in solution and on solid supports.[6-8]

One of the most flexible and reliable strategies for phosphopeptide synthesis, which is particularly important for the construction of peptides embodying several different phosphorylated and unphosphorylated serine/threonine residues, is the stepwise assembly of the target compound from prephosphorylated building blocks. A major problem which has to be solved in its application 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 (i.e. at pH >8) a rapidly occurring ß-elimination (Scheme 1) of the phosphate moiety has to be feared,[6] consequently, classical base-labile ester protecting groups cannot be applied for the synthesis of completely masked phosphopeptides, and new blocking functions which can be removed selectively under particularly mild conditions are needed.

Scheme 1



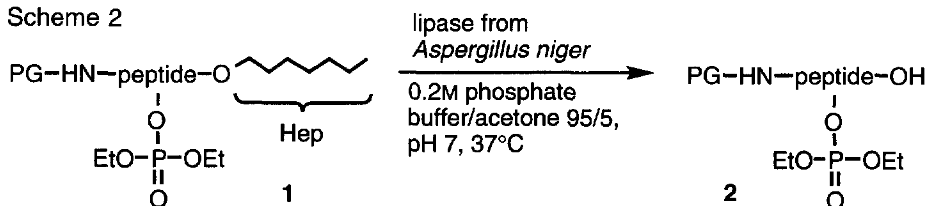
Enzymatic protecting group techniques[9] offer viable alternatives to the established classical-chemical methods. In particular, the characteristically mild reaction conditions (pH 6-8, room temperature) under which enzymatic transformations can be carried out has allowed for the chemoenzymatic synthesis of sensitive and multifunctional lipo-[10] and glycopeptides.[11] In the light of these findings we have investigated the application of enzymatically removable blocking functions in phosphopeptide chemistry.[12]

The purpose of this paper is to report that the enzymatic removal of the heptyl (Hep) ester protecting group under mildest conditions (pH 7, 37°C), is an efficient technique for the construction of sensitive base-labile biologically relevant serine/threonine phosphopeptides.

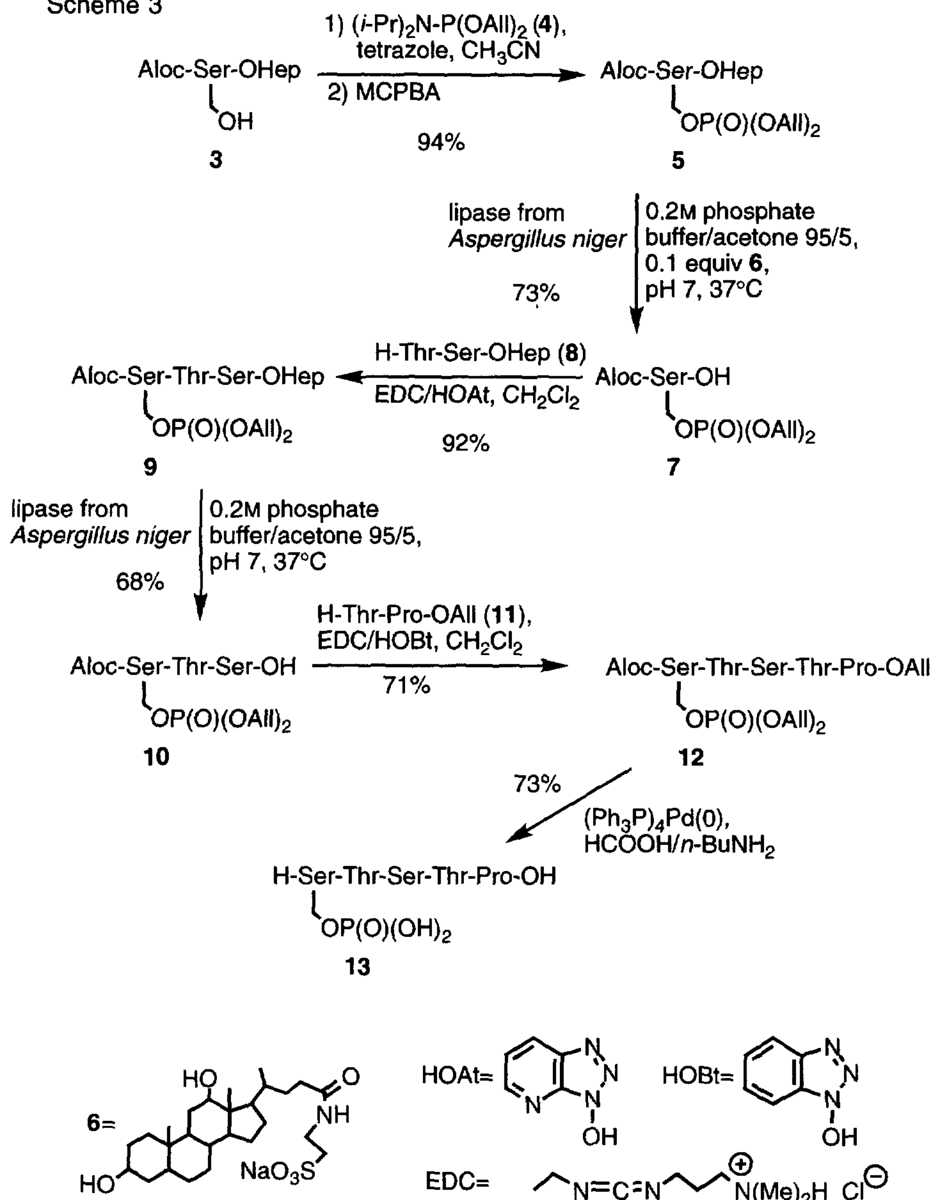
In order to investigate if the lipase-mediated saponification of heptyl esters[13] can be employed successfully in phosphopeptide chemistry, the model phosphopeptides **1** (Scheme 2) were built up and investigated as substrates for several lipases stemming from different biological sources. Most of the biocatalysts saponified the heptyl esters, but in the presence of lipase from *Aspergillus niger* the C-terminal deprotection proceeded with the highest velocity. This enzyme displays a broad tolerance towards the phosphopeptide esters **1**. It accepts variations in the structure of the N-terminal blocking group the side chains of the amino acids the position of the phosphate in the peptide and the length of the peptide chain (Scheme 2). By means of this enzymatic deprotection technique the selectively unmasked phosphopeptides **2** are available in high yields. In all cases the enzymatic transformations proceed without any unwanted side reaction, i.e. neither the N-terminal blocking groups and the peptide bonds nor the phosphoric acid esters are attacked. In particular, the reaction conditions are so mild (pH 7, 37°C) that a ß-elimination of the phosphate resulting in the formation of dehydroalanyl-peptides is not observed at all.

In order to probe the capacity of this enzymatic technique and to construct a biologically relevant phosphopeptide the synthesis of the phosphopentapeptide **13** was carried out. **13** represents a consensus sequence of the *Raf*-1

Scheme 2



No.	PG-peptide-OH	yield [%]
2a	Aloc-Ser(P)-Gly-OH	90
2b	Aloc-Ser(P)-Val-OH	88
2c	Aloc-Ser(P)-Leu-OH	90
2d	Aloc-Ser(P)-Tyr-OH	56
2e	Aloc-Ser(P)-Phe-OH	78
2f	Boc-Ser(P)-Ala-OH	96
2g	Boc-Ala-Ser(P)-OH	78
2h	Boc-Ser(P)-Ala-Ser-Ala-OH	50



kinase whose phosphorylation alters the activity of *Raf*-1 and therefore plays a role in the regulation of this signal transducing protein.[14] Since **13** embodies several hydroxyamino acids of which only one should be phosphorylated, for its synthesis a stepwise approach (as opposed to the global phosphorylation of a preformed peptide) making use of selectively phosphorylated and unphosphorylated amino acid building blocks together with selective deprotections in the presence of a sensitive serine phosphate is indicated.

To this end, the Aloc-protected serine heptyl ester **3** was converted to the diallyl phosphate **5** in high yield by phosphitylation with the phosphoramidite **4**[15] followed by oxidation of the resulting phosphite to the phosphate by means of m-chloroperbenzoic acid (MCPBA; Scheme 3). From **5** the C-terminal heptyl ester was

removed with complete selectivity and without any unwanted side reaction by means of enzymatic deprotection with lipase from *Aspergillus niger*. In order to obtain the phosphorylated amino acid **7** in consistently high yield, in this transformation the addition of 0.1 equiv. of deoxytaurocholic acid sodium salt **6** proved to be particularly useful. **6** obviously serves as emulsifying agent which guarantees that under the reaction conditions an emulsion of **5** is formed in which the lipase works particularly well. Coupling of **7** with the dipeptide **8** then delivered the selectively phosphorylated tripeptide ester **9** in high yield. From **9** once more the lipase from *Aspergillus niger* removed exclusively the C-terminal heptyl ester with complete selectivity and without any detectable side reaction. In this case the addition of deoxytaurocholic acid was not necessary to obtain a high yield. The peptide chain of the selectively unmasked phosphotripeptide **10** obtained thereby was then elongated to give the phosphopentapeptide **12**. From **12** finally all allyl-based protecting groups were removed in one final step by Pd(0)-mediated deprotection in the presence of formic acid/*n*-butylamine.[16]

These results demonstrate that by means of enzymatic protecting group techniques sensitive biologically relevant phosphopeptides are accessible under mildest conditions and with complete selectivity. Together with the recently developed classical-chemical methods[6-8] enzymatic transformations therefore should open up new and advantageous alternatives for the study of biological phenomena by means of a bioorganic approach.

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