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AUTOMATIC SYNTHESIS OF PHOSPHOPEPTIDES BY PHOSPHORYLATION ON THE SOLID PHASE

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Abstract: An efficient automatic synthesis of phosphopeptides on the solid phase is described. Following completion of their synthesis on the solid support, peptides were phosphorylated using N,N-diisopropyl-bis(4-chlorobenzyl)phosphoramidite. After deprotection, pure phosphopeptides containing a phosphorylated serine or threonine were obtained in high yield.

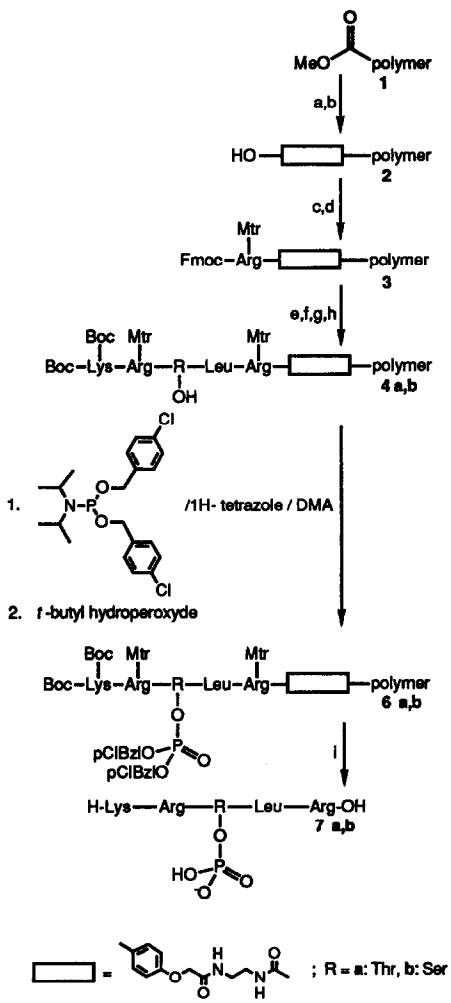
Protein phosphorylation is recognized as a major regulatory process mediated by protein kinases[1,2]. Despite the importance of protein phosphorylation, the molecular basis of regulation induced by phosphorylation is virtually unknown. Therefore we are engaged in the development of synthetic methods for the preparation of phosphoamino acids and phosphopeptides[3-7] with the ultimate goal to investigate the influence of phosphorylation on the structure of a peptide.

Synthesis in solution has been applied successfully for the preparation of a number of phosphopeptides[8]. However this method is rather time-consuming and seems only practical for the synthesis of relatively small phosphopeptides[9,10]. A disadvantage of the presently available solid phase methods is that the base labile 9-fluorenylmethoxycarbonyl (Fmoc) group cannot be employed for the solid phase synthesis of peptides containing phosphorylated serine or threonine residues[11]. Furthermore, excess of the relatively difficult accessible phosphorylated amino acid is necessary when it is introduced **in the course** of the synthesis of the peptide attached to the solid support[12].

These disadvantages can be easily circumvented when phosphorylation is carried out **after** completion of the solid phase synthesis. Recently dibenzylphosphochloridate was used for the phosphorylation of a heptapeptide on the solid phase leading to a phosphopeptide in low yield as well as an unknown byproduct[10]. In addition, a phosphoramidite was used for the phosphorylation of a tripeptide on the solid phase which was only partially deprotected and not cleaved from the resin[13]. These results prompted us to communicate our work on the synthesis of phosphopeptides by phosphorylation on the solid phase using the versatile phosphitylating agent N,N-diisopropyl-bis(4-chlorobenzyl)phosphoramidite[7]. We decided to prepare the phosphopeptide **7a** (Scheme) which comprises the phosphorylation site of the EGF receptor[4,14]. The phosphopeptide **7b** (Scheme) was synthesized to demonstrate that the method is equally well suitable for the preparation of serine containing phosphopeptides.

For the solid phase synthesis of the phosphopeptide commercially available kieselguhr-polydimethylacrylamide methyl ester resin **1**[15] was used. Aminolysis with ethylene diamine and subsequent introduction of the Wang linker[16] afforded resin **2**. Attachment (1 g of resin) of the first amino acid, employing its benzotriazole ester[17], was carried out analogous to van Nispen *et al.*[18] to yield **3**. Capping, deprotection, coupling steps as well as the phosphorylation step were carried out on an automatic peptide synthesizer. Remaining hydroxyl groups on the resin were capped with acetic anhydride (10 eq.) in the presence of DMAP (1 eq.). After each coupling step the Fmoc group was removed using a 20% solution of piperidine in DMA[19]. Appropriate amino acids were introduced as their pentafluorophenyl esters[20] in the presence of 1-hydroxybenzotriazole. Each acylation and deprotection step was monitored by U.V. The guanidinium side chain functionality of arginine was protected with the 4-methoxy-2,3,6-trimethylbenzene (Mtr) protecting group[21]. The N-terminal lysine was introduced as its α,ε-amino diBoc protected pentafluorophenyl ester derivative instead of the α-Fmoc-ε-Boc derivative, thus avoiding one additional step otherwise necessary for the removal of the N-terminal Fmoc group. As is known from the literature[9] and our own

work, a serine as well as a threonine residue could be introduced with an unprotected hydroxyl function. As a consequence the immobilized pentapeptide could be phosphorylated immediately after the last coupling step. The phosphorylation protocol was programmed as part of the automatic synthesis of the phosphopeptides and consisted of the following steps: 1: washing (DMA, 8 min., 2 mL/min.) of the completed pentapeptide.; 2: phosphitylation using N,N-diisopropyl-bis(4-chlorobenzyl)phosphoramidite **5**[7] (10 eq.) and 1-H-tetrazole (50 eq.) for 1 hr.; 3: washing (DMA, 8 min., 2 mL/min.).; 4: oxidation with *t*-butylhydroperoxyde (20 eq., 1 hr).; 5: washing (DMA, 8 min., 2 mL/min.). Before deprotection the immobilized phosphopentapeptide was washed with *t*-amyl alcohol, acetic acid, *t*-amyl alcohol, ether and dried *in vacuo* over P_2O_5[19]. Deprotection of the phosphopeptide was carried out for 1 h at 0^0 C^{22} using trifluoromethanesulfonic acid (10 eq.) as a 0.5 M solution in trifluoroacetic acid in the presence of thioanisole (20 eq.) and metacresol (20 eq.)[4]. Gel filtration (Sephadex G-15) afforded the phosphopeptides **7a** and **7b** respectively, both of which showed only one major peak according to reverse phase FPLC (fig. 1a). Both phosphopeptides **7a** and **7b** were purified by preparative reverse phase chromatography using a PepRPC HR16/10



a: ethylene diamine, b: 4-hydroxymethylphenoxyacetic acid pentafluorophenyl ester; c: Fmoc-Arg(Mtr)-OBt, DMAP, HOBt; d: Ac_2O, DMAP; e-h: deprotection (piperidine), coupling (e: Fmoc-Leu-Opfp, f: Fmoc-Thr-Opfp (**a**) or Fmoc-Ser-Opfp (b), g: Fmoc-Arg(Mtr)-Opfp, h= Boc-Lys(Boc)-Opfp) cycles; i= TFA/TFMSA/mcresol/thioanisole.

column and were obtained in 75% and 84% overall yield, respectively. The thus obtained pure phosphopeptides (fig. 1b) were characterized[23] by two-dimensional ^{13}C-^{1}H and ^{1}H-^{1}H NMR and $^{1}H(^{31}P)$ selective echo difference NMR[24] as well as FAB mass spectroscopy[25].

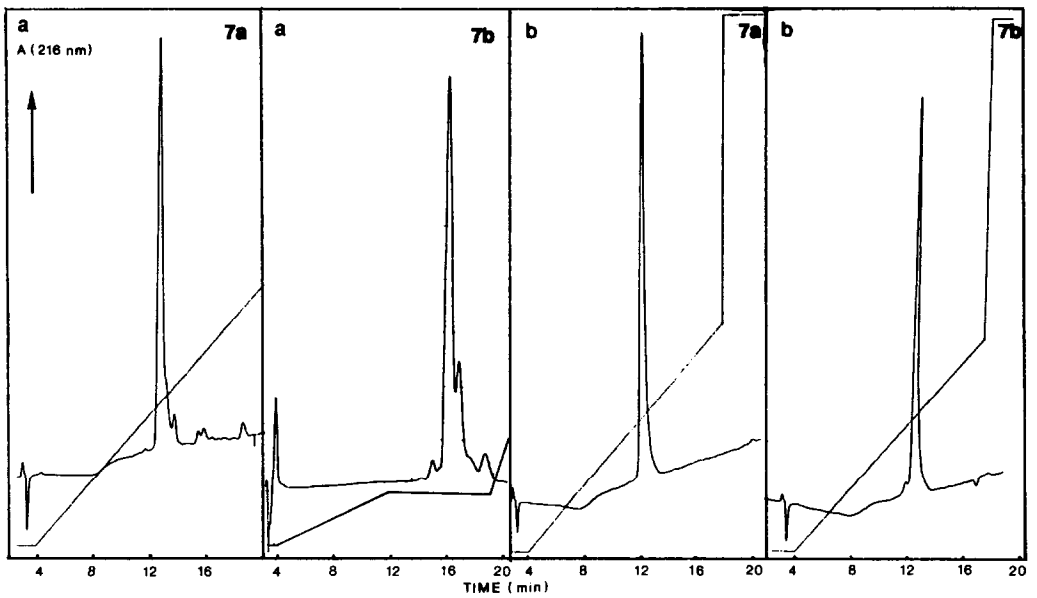


figure 1: phosphopeptides **7a** and **7b** before (a) and after preparative FPLC (b)

In conclusion, we have described a solid phase synthetic procedure for an efficient automatic synthesis of phosphopeptides containing a phosphorylated serine or threonine. Using this methodology based on the phosphite triester method for the introduction of a phosphate[7] onto a hydroxy amino acid residue it is most likely that it will be possible to synthesize large and complex phosphopeptides of high purity. In addition, the possibility of introducing both unprotected hydroxy amino acids as well as protected hydroxy amino acids in the same peptide will enable us to synthesize phosphopeptides having a phosphate moiety attached to a desired hydroxy amino acid among other - non phosphorylated- hydroxy amino acids present in a peptide. Under present investigation is synthesis of part of the subunit interface around the phosphorylated serine 14 residue present in phosphorylase a[26].

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