Pergamon

Tetrahedron Letters, Vol. 35, No. 17, pp. 2737-2738, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4039/94 $6.00+0.00

0040-4039(94)E0366-6

Solid Phase Phosphorylation of a Peptide by the H-Phosphonate Method

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Abstract: The partially protected peptide SerValSerGluAla was selectively phosphorylated at Ser3 by benzyl H-phosphonate activated by pivaloyl chloride in pyridine, followed by in situ oxidation with iodine.

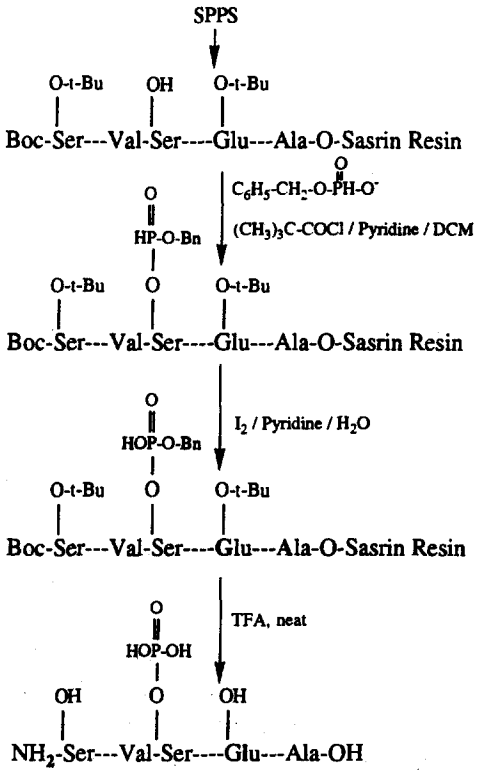
Phosphorylation of proteins is an important reversible process in cellular regulation[1]. Therefore synthetic phosphopetides offer possibilities for model studies of protein phosphorylation/dephosphorylation in biological systems[1].

Two principal ways are known for the chemical synthesis of phosphorylated peptides. 1: The phosphate group can be introduced to a properly protected peptide. 2: A phosphorylated amino acid can be used in solution or solid phase peptide synthesis.

Phosphorylation of resin-bound peptides has been promoted as an alternative method[2] for the preparation of selectively phosphorylated peptides in solid phase synthesis. The phosphites used for this purpose are, however, sensitive to moisture and oxidation and do not lend themselves to any lengthy storage[2]

In this paper we have applied the stable H-phosphonates, not previously employed for this purpose, for the reaction. H-phosphonate monoesters are stable and resistant to oxidation in solution but upon activation with various condensing agents, they become at least as reactive as tri-coordinated P(III)[3-5]. This phosphorylating agent is compatible with the Fmoc/t-butyl solid phase synthesis strategy for production of phosphoserine containing peptides. For a target peptide we chose SerValSerGluAla that is phosphorylated at Ser3 by the calmodulin dependent kinase. The peptide was synthesized manually by Fmoc stragety starting from Fmoc-Ala-Sasrin® (0.73 g, 0.68 mmol/g loading). Glu4 was protected by the t-butyl group, Ser1 was coupled as a Boc-Ser(O-tBu)-OH and Ser3 was left unprotected. It was found advisable to introduce the N-terminal amino acid as a Boc derivative rather than using an Fmoc amino acid and substitute a Boc group for the Fmoc group. Coupling of the amino acids was achieved with TBTU, 2-(1H-benzotriazol-yl)-1,1,3,3,-tetrametyluronium tetrafluoroborate, and the coupling reactions were monitored by a ninhydrin test. The Fmoc group was removed using a 20% solution of piperidine in DMF after each coupling step. Phosphorylation of the unprotected hydroxyl of serine was carried out on part of the resin-bound peptide using 5 equiv.benzyl H-phosphonate[6] activated by 5 equiv. pivaloyl chloride in DCM/pyridine (5.25/1), followed by oxidation with 1% iodine (w/v) in pyridine/H_2O (98/2). Cleavage from the resin was achieved with 2% TFA in DCM. The protecting groups were all removed with neat TFA, with anisol and p-cresol added as scavengers. The peptide was precipitated from the acid with diethyl ether at -70^0 C, taken up in 10% aqueos acetic acid, desalted (Sephadex®G-15 in water) and lyophilized. Yield of phosphopeptide[7] 83 %. FAB/MS, $(M+1)^+$=572.2.

In conclusion, we have demonstrated the utility of a new type of phosphorylating agent for production of phosphoserine containing peptides and also expect that H-phosphonates should be suitable for making



phosphothreonine and phosphotyrosine containing peptides using the post assembly approach. The peptide is best synthesized using Fmoc startegy, although the N-terminal amino acid is best introduced as a Boc derivative. The use of a Sasrin® resin permits the easy removal ot the peptide from the resin in the protected form that permits a safe NMR analysis of the product.

Acknowledgements: Benzyl H-phosphonate was a gift from Tomas Szabó this department, and TBTU was a gift from Peboc Limited. This work was supported by a grant from NUTEK.

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7. The peptide gave satisfactory values upon amino acid analysis. ^{13}C-n.m.r. (67.94 MHz in D_2O) δ 55.3 (J_{POCC} 6.5 Hz) Ser 3 α, δ 64.7 (J_{POC} 3.7 Hz) ser 3 β, all other carbon signals as expected. ^{31}P-n.m.r. (27.02 MHz in D_2O, pH 5.0, external standard H_3PO_4) δ 0.47.

(*Received in UK* 27 *December* 1993; *revised* 14 *February* 1994; *accepted* 18 *February* 1994)