Pergamon

Tetrahedron Letters, Vol. 37, No. 45, pp. 8227-8230, 1996

0040-4039/96 $15.00 + 0.00

PII: S0040-4039(96)01874-6

Highly Efficient Solid-Phase Phosphopeptide Synthesis Using Bis-(Polyfluorophenyl) Chlorophosphates: Preparation of Serine-Threonine Protein Phosphatase Substrates.

Pantea Hormozdiari and David Gani *

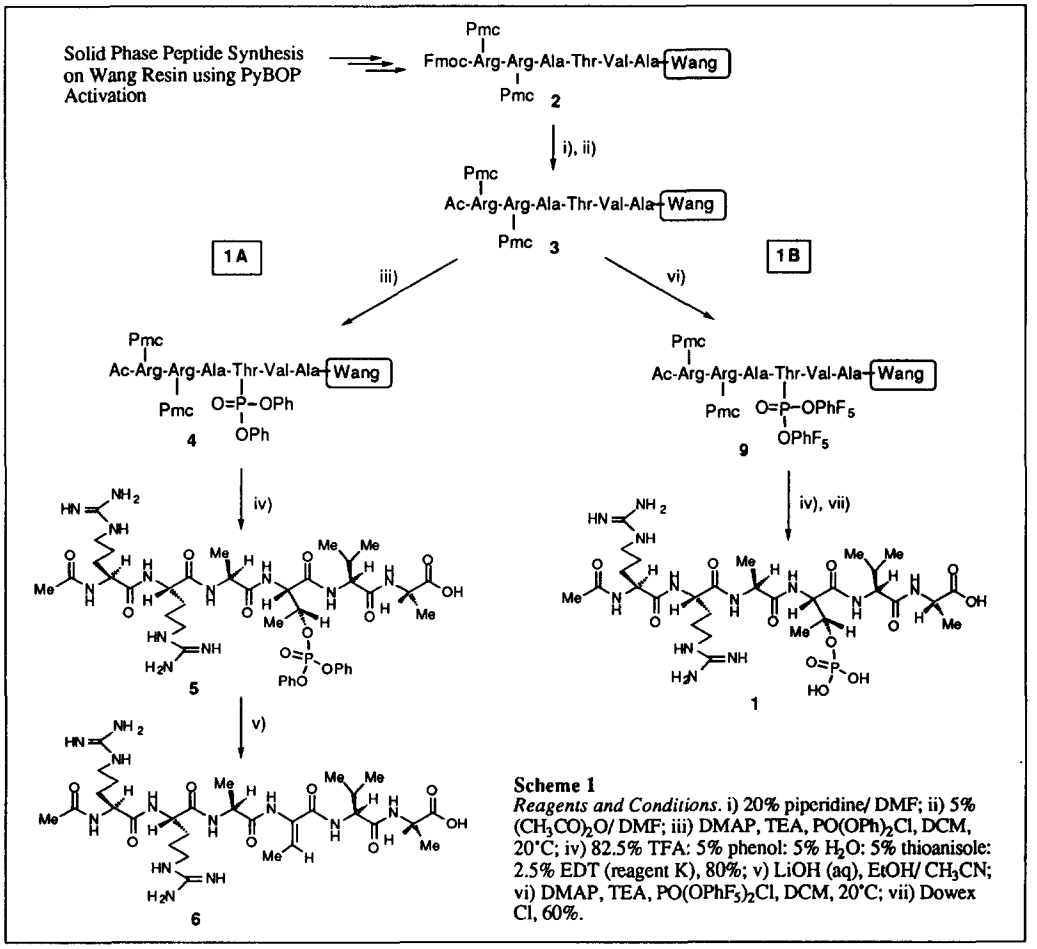
School of Chemistry and Centre for Biomolecular Sciences, The Purdie Building, The University, St. Andrews, Fife, KY16 9ST, UK.

Abstract. A new fast and efficient solid phase phosphorylation protocol suitable for the preparation of phosphoserine- and phosphothreonine-containing peptides is described. The method allows the facile conversion of primary and secondary alcohol groups to the corresponding *bis*-(pentafluorophenyl) phosphate triesters which are cleanly converted to the phosphate monoesters under acidic conditions.

The reversible phosphorylation of proteins on serine, threonine and tyrosine residues, as catalysed by protein kinases and phosphatases, is the principal mechanism by which eukaryotic cells respond to external stimuli.[1,2] Ser-Thr protein phosphatases are collectively responsible for the dephosphorylation of phosphoserine and phosphothreonine residues within phosphoproteins and several different types exist (*eg.* PP1, PP2A, PP2B and PP2C) most of which appear to be associated with regulatory proteins. Nevertheless, over the past several months excellent progress has been made in gaining information on the active-site structure of the catalytic subunit of PP1, much of which is derived from X-ray crystal data.[3,4] Structure-activity studies for the phosphorylated peptide substrates of Ser-Thr protein phosphatases have been limited by the availability of structurally diverse substrates because, to date, almost all of these have been prepared by enzymic phosphorylation using adenosine triphosphate and appropriate protein kinase enzymes which are specific for certain sequences.[5] Moreover, the non-enzymic synthesis of phosphopeptides, in particular phosphothreonine peptides is severely hampered by the β-elimination of phosphoric acid diester which occurs in synthetic intermediates to give the corresponding dehydroamino acid moieties.[6-8]

Phosphothreonine peptide syntheses typically employ large excesses of highly electrophilic phosphorus(III) reagents to introduce phosphorus into the preformed peptide and then an oxidant (*eg.* *t*-BuOOH) to convert the phosphite triester to the phosphate triester prior to deprotection of the ester groups. While the peptide exists as its phosphate triester, it is particularly vulnerable to β-elimination. Our goal was, therefore, to prepare a phosphorylating agent that would be electrophilic enough to react directly and rapidly with primary and secondary alcohol groups within resin-bound peptides, thereby obviating the need for an oxidant, and which possessed labile phosphate ester protecting groups that would be compatible with solid-phase peptide synthesis.

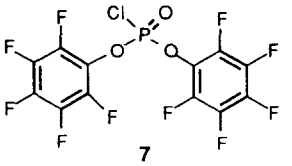
Diphenyl chlorophosphate had been successfully employed by ourselves and others to phosphorylate the secondary alcohol groups of *myo*-inositol and its analogues.[9] Using an N-acetyl capped analogue of a known consensus sequence for a PP2A substrate as the target, AcNH-Arg-Arg-Ala-Thr(PO_3H_2)-Val-Ala-OH (**1**), a series of solid-phase phosphorylation reactions were examined. Accordingly, using Wang resin, standard Fmoc chemistry with PyBOP activation, and arginine residue precursors containing 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) protected guanidino groups, the peptide Fmoc-NH-Arg-Arg-Ala-Thr-Val-Ala-O-Wang (**2**) was prepared. The N-terminal Fmoc group was removed with 20% piperidine in DMF and the free amino group was capped with 5% acetic anhydride in DMF to give compound (**3**), Scheme 1. Treatment of the resin-bound peptide (**3**) with diphenyl chlorophosphate gave some of the required diphenyl threonine phosphate triester (**4**), and under optimised conditions (repeated treatments with 20 equivalents of diphenyl chlorophosphate in the presence of DMAP and TEA for 6-8 hours at ambient temperature) essentially quantitative conversion to the triester (**4**) could be achieved, as determined by NMR-spectroscopic analysis of the products after cleavage from the resin, Scheme 1A.



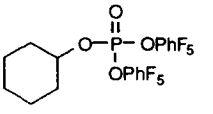
Scheme 1
Reagents and Conditions. i) 20% piperidine/ DMF; ii) 5% $(CH_3CO)_2O$/ DMF; iii) DMAP, TEA, $PO(OPh)_2Cl$, DCM, 20°C; iv) 82.5% TFA: 5% phenol: 5% H_2O: 5% thioanisole: 2.5% EDT (reagent K), 80%; v) LiOH (aq), EtOH/ CH_3CN; vi) DMAP, TEA, $PO(OPhF_5)_2Cl$, DCM, 20°C; vii) Dowex Cl, 60%.

^{1}H-, ^{13}C- and ^{31}P-NMR spectra showed the expected signals, chemical shift changes and P-C and P-H couplings for the required triester (**5**). All attempts to hydrolyse the pure triester (**5**) under mild basic conditions resulted in the formation of significant quantities of the β-elimination product, dehydrobutyrine peptide (**6**), as judged by ^{1}H- and ^{31}P-NMR spectroscopy.

In order to increase the electrophilicity at phosphorus in the phosphorylating species (to decrease reaction times) and also in the required peptidic phosphate triester (to facilitate deprotection), the preparation and use of *bis*-(pentafluorophenyl) chlorophosphate (**7**) was investigated. The reagent was prepared by treating phosphorus oxychloride with 1.8 equivalents of pentafluorophenol at 140 °C for 16-24 h and was purified by removing the unreacted starting materials by distillation. The resulting reagent (**7**) was 85-90% pure as judged by ^{19}F- and ^{31}P-NMR spectroscopy and could be further purified by fractional distillation.[10] In model reactions using cyclohexanol, the *bis*-(pentafluorophenyl) chlorophosphate (**7**) reacted at least 30-fold more rapidly than diphenyl chlorophosphate to give the required triester (**8**) which was fully characterised. Interestingly the C-1 and H-1 signals for the cyclohexane moiety were shifted downfield by 3.5 and 0.18 ppm respectively, in the fluorinated triester (**8**) compared to the non-fluorinated diphenyl triester (δ_C 79.15 ppm; δ_H 4.62 ppm) in NMR spectra run in deuterochloroform.



7



8

In solution the pentafluorophenyl groups of triester (**8**) could be removed rapidly under acidic conditions, in accord with expectations,[10] (full details will be disclosed elsewhere) thus, attention was turned to the solid-phase properties of the reagent. Note that the *bis*-(2,3,5,6-tetrafluorophenyl) chlorophosphate analogue of reagent (**7**), which was more useful for mechanistic studies and for product characterisation (due to the presence of an integratable proton resonance in ^{1}H-NMR spectra), behaved similarly in effecting rapid phosphorylation. The resulting triester (*cf.* (**8**)) was also acid labile.[10]

Treatment of the Pmc protected resin-bound peptide, Ac-NH-Arg-Arg-Ala-Thr-Val-Ala-O-Wang (**3**), with 10 equivalents of *bis*-(pentafluorophenyl) chlorophosphate under optimised conditions (overnight at 20°C in the presence of excess TEA and 0.1 equivalent of DMAP) gave the resin-bound phosphate triester (**9**) in excellent yield, Scheme 1B. Immediate deprotection of the two Pmc groups, the two pentafluorophenyl groups, and simultaneous cleavage from the resin occurred upon treatment with reagent K[11] to give the almost pure N-capped phosphorylated threonine peptide (**1**) in almost quantitative conversion. There was no evidence whatsoever for β-elimination products and purification on Dowex 1 chloride gave the pure phosphopeptide (**1**) in 60% overall yield (over 14 solid-phase steps). This material was fully characterised by ^{1}H-, ^{13}C- and ^{31}P-NMR spectroscopy and by ES mass spectrometry and served as a substrate for protein phosphatases λ and 2A, as judged by directly monitoring the course of phosphopeptide hydrolysis by ^{1}H-NMR spectroscopy. The radiolabelled ^{14}C-acetyl capped substrate is now being developed for use in a new assay to determine the inhibition constants of synthetic nodularin and microcystin analogues.[12]

Other peptides containing serine residues were also successfully phosphorylated with *bis*-(pentafluorophenyl) chlorophosphate (**7**) using similar protocols. The method is of considerable potential in

the solid-phase synthesis of a whole range of organic phosphates and its application to the preparation of sugar and cyclitol phosphates is presently underway in our laboratory. In the area of peptide chemistry the method offers very significant advantages over the previously used two step phosphitylation-oxidation strategies. Furthermore, the use of *bis*-(pentafluorophenyl) chlorophosphate is likely to be of particular utility in the preparation of peptides containing two or more phosphorylated residues *via* a "global phosphorylation" strategy which involves introducing all phosphoryl groups in one step after the synthesis of the required peptide. Existing useful methods for avoiding β-elimination in the synthesis of phosphoserine and phosphothreonine peptides involve introducing each of the phosphorylated amino acid residues as their protected phosphate diester monoanions[8] which are tedious to prepare.

Acknowledgements: We thank the BBSRC and EPSRC for BMS grant GR/J55243, the Wellcome Trust for grant 04033, Dr. W. Ward for useful discussion and Zeneca Pharmaceuticals and the EPSRC for a CASE studentship to P.H.

Abbreviations

DCM, dichloromethane; DMF, N,N-dimethylformamide; DMAP, 4-dimethylaminopyridine; EDT, 1,2-ethanedithiol; PyBOP, Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; TEA, triethylamine; TFA, trifluoroacetic acid.

References and Notes

1. Hunter, T. *Cell*, 1995, **80**, 225.
2. a) Cohen, P. *Annu. Rev. Biochem.*, 1989, **58**, 453; b) Cohen, P. T. W.; Brewis, N. D.; Hughes, V.; Mann, D. J. *FEBS Lett.*, 1990, **268**, 355.
3. Goldberg, J.; Huang, H-B.; Kwon, Y-G.; Greengard, P.; Nairn, A. C.; Kuriyan, J. *Nature*, 1995, **376**, 745.
4. Egloff, M-P.; Cohen, P. T. W.; Reinemer, P.; Barford, D. *J. Mol. Biol.*, 1995, **254**, 942.
5. a) Donella-Deana, A.; Mac Gowan, C. H.; Cohen, P.; Marchiori, F.; Meyer, H. E.; Pinna, L. A. *Biochim. Biophys. Acta*, 1990, **1051**, 199; b) Pinna, L. A.; Agostinis, P.; Donella-Deana, A.; Marchiori, F. *Adv. Prot. Phosphatases*, 1989, **5**, 51.
6. Paquet, A.; Johns, M. *Int. J. Peptide Protein Res.*, 1990, **36**, 97.
7. Otvos, L.; Elekens, J.; Lee, V. M.-Y. *Int. J. Peptide Protein Res.*, 1989, **34**, 129.
8. Vorherr, T.; Bannwarth, W. *Bioorg. Med. Chem. Lett.*, 1995, **5**, 2661.
9. Schulz, J.; Wilkie, J.; Lightfoot, P.; Rutherford, T.; Gani, D. *J. Chem. Soc. Chem. Commun.*, 1995, 2353.
10. The large increase in the rates of acid-catalysed hydrolysis for the pentafluorophenyl and tetrafluorophenyl phosphate triesters compared to those for the non-fluorinated analogues, may be accounted for by their abilities to chelate a proton, possibly through an *ortho*-fluorine atom and the phenol phosphate O-atom, to enhance the electrophilicity of the phosphorus atom. The acid lability of *bis*-perfluorophenyl phosphate triesters [prepared from reagent (7)] has been noted previously. a) Boschan, R. H.; Holder, J. P. *U.S. Patent Office 3,341,630*, 1967; b) Podolskii, A. V.; Bulatov, M. A. *Chem Abs.*, 1976, **85**, 32557h. Details on the kinetics of the phosphorylation and deprotection chemistry will be reported elsewhere.
11. King, D. S. ; Fields, C. G.; Fields, G. B. *Int. J. Peptide Protein Res.*, 1990, **36**, 255.
12. Mehrotra, A. P.; Gani, D. *Tet. Lett.*, 1996, **37**, 6915.

(*Received in UK* 2 *August* 1996; *revised* 18 *September* 1996; *accepted* 20 *September* 1996)