Journal of Peptide Science, Vol. 3, 186–192 (1997)

Solid-phase Synthesis of Tyrosyl H-Phosphonopeptides and Methylphosphonopeptides

ANDREAS THOLEY[1] and RALF HOFFMANN[2]

[1]German Cancer Research Center, Department of Pathochemistry, Heidelberg, Germany
[2]The Wistar Institute, Philadelphia, PA, USA

Received 25 June 1996
Accepted 14 August 1996

Abstract: Phosphopeptides are a useful tool for the investigation of phosphorylation as a reversible post-translational modification. There is a growing interest in using mimics of phosphoamino acids involved in phosphorylation in order to study the enzymes concerned in these processes. These mimics should contain a non-hydrolysable or isoelectrically modified phosphate moiety to be used as a specific inhibitor of phosphatases and kinases. We introduce solid-phase synthesis of H- and methylphosphonopeptides as a new class of mimics of phosphotyrosyl peptides. The peptides were synthesized on solid phase using the standard fluorenyl-methyloxycarbonyl (Fmoc) strategy. Tyrosine residues were incorporated as allyl-protected derivatives, which were selectively deprotected on the resin by treatment with $Pd(PPh_3)_4$. The peptide resin carrying the side-chain unprotected tyrosine of the model peptide Gly-Gly-Tyr-Ala was phosphonylated with di-*tert*-butyl-*N*,*N*-diethyl-phosphoramidite in the presence of ^{1}H-tetrazole, yielding H-phosphonopeptides after trifluoroacetic acid (TFA) cleavage. Alternatively, phosphonylation of the unprotected tyrosine with O-*tert*-butyl-*N*,*N*-diethyl-P-methylphosphonamidite catalysed by ^{1}H-tetrazole and followed by oxidation led to the methylphosphonopeptides after TFA cleavage. We obtained both the H-phosphonopeptides and the methylphosphonopeptides of the tetrapeptide in high yields and purities above 90%, according to reversed-phase high-performance liquid chromatography (RP-HPLC). To investigate the general applicability of our new methodology, we synthesized phosphonopeptides up to 13 amino acids long, corresponding to recognition sequences of tyrosine kinases. After cleavage and deprotection, all phosphonopeptides were obtained in high yields and purities of about 90%, as shown by mass spectrometry. The only by-product found was the unmodified peptide. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

J. Pep. Sci. 3: 186–192, 1997

(No. of Figures: 3. No. of Tables: 2. No. of Refs: 25.)

Keywords: H-phosphonopeptides; mass spectrometry; methylphosphonopeptides; peptide synthesis; phosphopeptides

INTRODUCTION

Phosphorylation and dephosphorylation of proteins are key steps in the control of many biological processes, catalysed by kinases and phosphatases. Recently, major advances have been achieved in the investigation of cellular signalling pathways which involve the regulation of protein activities by phosphorylation and dephosphorylation of tyrosine residues [1–4]. The generation of mitogenic signals from abnormally expressed or deregulated protein tyrosine kinases (PTK) has been associated with a number of proliferative diseases [5–7]. Therefore, it seems necessary to develop mimics which will allow a detailed investigation of these regulatory processes. Peptides containing phosphotyrosine are synthesized by direct incorporation of a phosphotyrosine building block [8–10]. The search for

Abbreviations: All, O-allyl; HBTU, *O*-benzotriazol-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HP, H-phosphono; MP, methylphosphono; PTK, protein tyrosine kinases; RP-HPLC, reversed-phase high-performance liquid chromatography; TBTU, *O*–benzotriazol-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate.

Address for correspondence: Dr Ralf Hoffmann, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA. Tel. (215) 898-4412; Fax (215) 898-5821;
email: OTVOSLAB@wist.wistar.upenn.edu

CCC 1075-2617/97/030186-07

mimics of phosphotyrosine peptides which contain a non-hydrolysable or isoelectrically modified phosphate moiety has led to three classes of mimics. The most investigated feature of the phosphate group has been the ester linkage to the amino acid, which was replaced by a non-hydrolysable methylene group or a fluorinated methylene group (CH_2, CHF, CF_2) [11–15]. The difluorinated phosphonotyrosine best mimics the pK_a-values, the steric orientation and the hydrogen bond formation of the natural phosphotyrosine in peptides [15]. The second type of modification is the replacement of the double bonded oxygen of phosphate by sulphur [16].

The substitution of one hydroxyl group of phosphate is a third way to mimic the natural phosphoamino acids and is expected to alter their chemical behavior. To our knowledge only three publications deal with this third class of mimics [17–19]. First Wijkmans *et al.* [17] described the synthesis of short methylphosphonopeptides. Although H-phosphonopeptides were described in the literature as by-products of the amidite approach [20], their synthesis was only recently described by Ruzza *et al.* [18] for tyrosyl H-phosphonopeptides and simultaneously for seryl and threonyl H-phosphonopeptides by Hoffmann *et al.* [19]. Whereas both Wijkmans *et al.* and Ruzza *et al.* [17, 18] used solution-phase syntheses, Hoffmann *et al.* [19] introduced solid-phase synthesis of these mimics of serine and threonine as an easy and fast access to both H- and methylphosphonopeptides [19].

For the first time, we report in this paper the synthesis of both H- and methylphosphonotyrosyl peptides on solid phase by the global amidite approach. As a model peptide we choose GGYA, which is generally regarded as a random coil sequence, as part of an ongoing project [19, 20]. Furthermore, we synthesized two longer peptides; ENDYINASL (a recognition sequence highly conserved in many protein tyrosine phosphates) [21] and RRLIEDAEYAARG (raytide, a substrate of PTPases and $pp60^{c\text{-}src}$) [22], both modified at tyrosine, to show the general applicability of the described method for longer peptides, as typically used for studies of kinases and phosphatases. To circumvent *O*-acylation of side-chain unprotected tyrosine, we used the allyl-protected derivative Fmoc-Tyr(All)-OH [23]. After selective cleavage of the allyl-group, tyrosine was globally phosphonylated, yielding either H- or methylphosphonopeptides.

MATERIAL AND METHODS

Reagents

Amino acid derivatives were purchased from Novabiochem (Bad Soden, Germany) or Perseptive Biosystems (Framingham, MA). All other reagents and solvents were obtained from Fluka, Aldrich or Sigma. Solvents were stored over molecular sieve and used without further purification.

Peptide Synthesis and Tyrosine Deprotection

The peptides were synthesized with standard Fmoc-chemistry [24] on a 431A peptide synthesizer (Applied Biosystems, Weiterstadt, Germany) employing HBTU/HOBt-activation or a 9050 peptide synthesizer (Milligen, Burlington, MA) employing TBTU/HOBt-activation. The model peptide GGYA was synthesized on a preloaded Fmoc-Ala-HMP resin (0.55 mmol/g, Novabiochem). The N-terminal glycine was incorporated either as the Boc- or Fmoc-derivative, yielding either a protected or free N-terminus after peptide synthesis. The C-terminal amino acids glycine and leucine of the longer peptides were activated with diisopropyl carbodiimide and attached to HMP resin in the presence of dimethyl aminopyridine.

Loading capacities were about 0.5 mmol/g, as determined by a quantitative Fmoc-test [25]. Phosphopeptides were synthesized by incorporation of Fmoc-Tyr(PO_3H_2)-OH, whereas Fmoc-Tyr(All)-OH was used to synthesize the corresponding unmodified peptides. The N-terminal Fmoc-groups were cleaved at the end of the synthesis on the synthesizer yielding an unprotected N-terminal amino group. Cleavage of the allyl group from tyrosine was achieved on the resin using a three-fold molar excess of $Pd(PPh_3)_4$, two-fold molar excess of tributyltinhydride and three-fold molar excess of acetic acid in chloroform. This mixture was stirred under nitrogen for 4 h. The cleavage procedure was repeated once again for 6 h. Afterwards, the resin was washed with chloroform, methanol and diethylether, and dried in a vacuum.

Phosphonylation Procedure

The dry allyl-deprotected resin was suspended in THF at 0 °C and a 25 molar excess of both ^{1}H-tetrazole and *O,O′*-di-*tert*-butyl-*N,N*-diethyl-phosphoramidite, dissolved in THF, was added [19, 20]. After 4 h stirring under nitrogen, the resin was

washed (THF) and the procedure was repeated once with stirring overnight. After washing, the peptide was cleaved from the resin with TFA, yielding H-phosphonopeptides.

Methylphosphonopeptides were synthesized in the same way as described for H-phosphonopeptides, but using *O*-*tert*-butyl-*N*,*N*-diethyl-P-methylphosphonoamidite [19] (50-fold molar excess) and ^{1}H-tetrazole (25-fold molar excess) as catalyst. Oxidation was carried out with a 20-fold molar excess of *tert*-butylhydroperoxide or *m*-chloroperbenzoic acid in THF (two times for 4 h).

Cleavage

The peptides or phosphonopeptides were cleaved from the resin either by treatment with 9% triisopropylsilane and 1% water in TFA or 5% thioanisol and 4% water in TFA (by volume) at room temperature for 2.5 h. The peptides were precipitated with cold diethylether and washed three times with diethylether.

Chromatography

Analytical RP-HPLC was performed on a Jasco-HPLC (PU 980 intelligent pump, LG-980-02 ternary gradient unit, DG-980-50 3-line degasser; Jasco, Gross-Umstadt, Germany) equipped with a Waters 991 photodiode array detector (Waters, Eschborn, Germany) using either an analytical (250 mm × 4 mm) or a semi-preparative (250 mm × 8 mm) Kromasil C_{18}-column (5 μm, 100 Å). After isocratic elution with 0.1% aqueous TFA for 1 min we used a linear gradient to 60% acetonitrile (0.09% TFA) in 40 min.

Mass Spectroscopy

Electrospray mass spectroscopy was carried out on an MAT TSQ 7000 triple quadrupole mass spectrometer (Finnigan, Bremen, Germany). The samples were dissolved in methanol/water (2 : 1; v : v) in a concentration of about 0.5 mg/ml). The solution was continuously infused with a medical infusion pump (model 22, Harvard Apparatus, Southmatick, MA) at a flow rate of 5 μl/min. The potential of the spray was held at 4.9 kV. Spectra were recorded in positive and negative ion mode; values are given as the average of both modes. Matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) was performed on the samples using the PerSeptive Biosystems Voyager Biospectrometry Workstation in the linear mode. The instrument is equipped with a 1.3 m flight tube and a variable two-stage ion source set at 30 kV. Samples were mixed with an ultraviolet light-absorbing matrix, in this case α-cyano (α-cyano-4-hdroxycinnamic acid at 10 mg/ml in 33% acetonitrile, 0.1% TFA) from Sigma. Masses were determined using external calibration.

RESULTS AND DISCUSSION

Peptide Synthesis

Although direct incorporation of side-chain unprotected tyrosine is described in the literature, we sometimes observed a high percentage of a by-product, most probably *O*-acylated peptides. Therefore we used Fmoc-Tyr(All)-OH for solid-phase peptide synthesis. Experiments with dissolved Fmoc-Tyr (All)-OH showed that the allyl group was cleaved quantitatively by treatment with tetrakis (triphenylphosphine) palladium ($Pd[PPh_3]_4$), tributyltin hydride and acetic acid within 1 h. Because of expected steric hindrance on the resin, we increased the deprotection time to 4 h. The allyl group was cleaved almost quantitatively, which was shown by comparison of the areas of both peaks on RP-HPLC of the allyl-protected and deprotected peptide (Tables 1 and 2). The deprotected peptides were either cleaved with TFA, yielding the unmodified peptides in high yields and purities (Table 2), or phosphonylated, as discussed in the following section.

H-Phosphonopeptides

After ^{1}H-tetrazole catalysed phosphitilation with *O*,*O*′-di-*tert*-butyl-*N*,*N*-diethylphosphoramidite [19] the resulting Boc-GGY($PO_2{}^tBu_2$)A-HMP-resin was cleaved with TFA without previous oxidation (Figure 1). The resulting H-phosphonopeptide is the dominant species in a tautomeric equilibrium with the 3-coordinated P(III)-tautomer (Figure 1) [18, 19]. Protection of the N-terminus did not influence the yields and purities of the phosphonylated model peptide GGY(HP)A. These results were confirmed by the yields and purities of all longer H-phosphonopeptides, which were phoshonylated after Fmoc-deprotection. The H-phosphonopeptide eluted in the main fraction (Y[HP]2, Figure 2) of the crude product. Performing the reaction under atmospheric conditions led to about 15% phosphopeptide (fraction Y[HP]1, Figure 2) because of oxidation by air oxygen. This oxidation was completely prevented by

Table 1 Analytical Data for the GGYA Model Peptide in its Various Modified Forms

Peptide	Retention time RP-HPLC (min)	Content on RP-HPLC (%)	Calculated mass $[M+H]^+$	Observed mass $[M+H]^+$
GGYA	17.8	>98	367.2	366.0
GGY(OAll)A	26.1	>98	407.2	407.3
GGY(P)A	13.9	>98	447.2	446.0
GGY(HP)A	15.4	92 (N_2) 76 (air)	431.2	430.2
GGY(MP)A	16.5	94	445.2	444.1

Table 2 Analytical Data of the Longer H-Phosphono- and Methylphosphonopeptides

Peptide	Retention time RP-HPLC (min)	Content on RP-HPLC (%)	Calculated mass $[M+H]^+$	Observed mass $[M+H]^+$
RRLIE DAEYA ARG	18.6	>97	1519.0	1519.8
RRLIE DAEY(P)A ARG	18.3		1599.0	1599.2
RRLIE DAEY(HP)A ARG	18.4	91	1583.0	1583.4
RRLIE DAEY(MP)A ARG	18.5	88	1597.0	1597.3
ENDYI NASL	20.2	>97	1038.4	1037.4
ENDY(P)I NASL	18.2		1118.4	1117.2
ENDY(HP)I NASL	18.6	89	1102.4	1101.5
ENDY(MP)I NASL	18.9	91	1116.4	1115.1

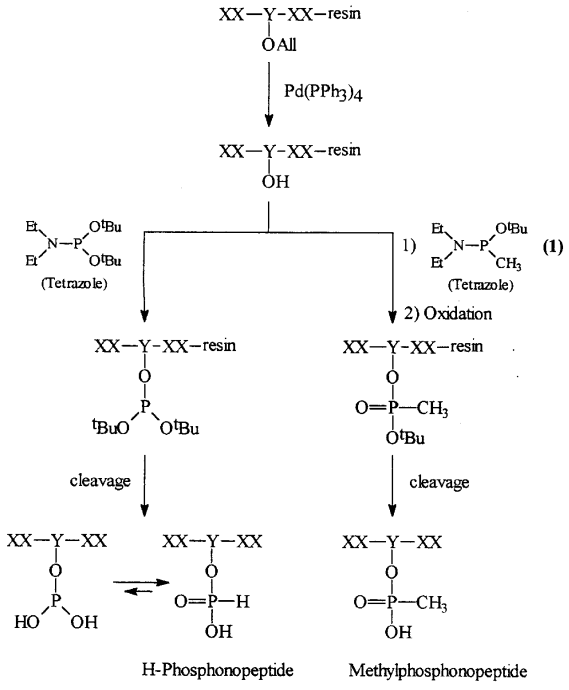

Figure 1 H- and methylphosphonopeptides were synthesized on solid phase using the amidite approach in the presence of ^{1}H-tetrazole, followed by oxidation (*m*-chloroperbenzoic acid MCPBA) and TFA cleavage.

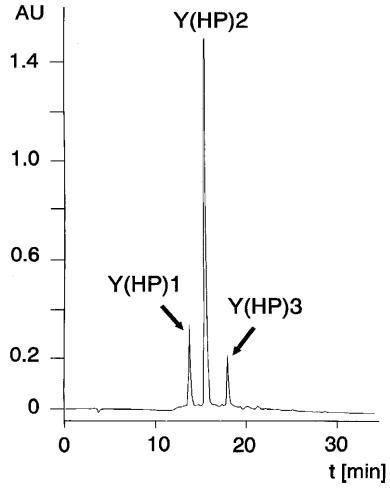

Figure 2 RP-HPLC of the crude product after TFA-cleavage of the H-phosphonopeptide GGY(HP)A, using a linear acetonitrile gradient. The main fraction Y(HP)2 contained only the H-phosphonopeptide, whereas the unmodified GGYA-peptide eluted in fraction Y(HP)3 and the phosphopeptide eluted in fraction Y(HP)1.

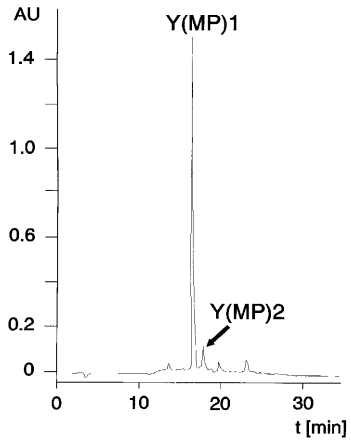

Figure 3 RP-HPLC of the crude product after TFA cleavage of the methylphosphonopeptide GGY(MP)A, using a linear acetonitrile gradient. Fraction Y(MP)1 contained only the methylphosphonopeptide, whereas the unmodified GGYA-peptide eluted in fraction Y(MP)2.

performing the phosphitilation procedure under nitrogen (Table 1). After cleavage from the resin, the purified H-phosphonopeptides were stable against air oxidation and basic conditions of pH 7.5 and 8.5 in Tris-buffer for 48 h. This indicated their applicability for biological tests. Besides the phosphorylated peptide there was only one further by-product observed in this approach, which was identified as the unmodified peptide GGYA (fraction Y[HP]3, Figure 2). This was not an effect of incomplete deprotection of tyrosine, because the allyl group is not cleaved by TFA, and the retention time of this fraction on RP-HPLC was identical to the unmodified peptide (Table 1). The observed by-product resulted from an incomplete phosphitilation of the resin-bound peptide, as often observed for the global amidite-approach, especially in longer peptides. The high purity of the crude H-phosphonopeptide allowed a simple one-step purification based on RP-HPLC.

Methylphosphonopeptides

The ^{1}H-tetrazole catalyzed phosphoramidite approach offers an easy access to phosphorylated peptides [20]. For this reason we used the phosphonylating reagent ^{t}BuOMePNEt$_2$ (Figure 1), which was synthesized in a one-pot procedure [19], to synthesize the methylphosphonopeptides. The resulting Boc-Gly-Gly-Tyr(PO^{t}BuMe)-Ala-HMP resin was oxidized and cleaved. The methylphosphonopeptide GGY(MP)A eluted in the main fraction Y(MP)1 of the crude product (Figure 3). GGY(MP)A eluted before the corresponding unmodified peptide GGYA but after the corresponding phosphonopeptide GGY(P)A (Table 1). The retention times were in accordance with the lower polarity of the methylphosphonopeptide compared with the phospho- and H-phosphonopeptides. This elution order is reversed for the corresponding seryl- and threonyl-methylphosphonopeptides (GGS[MP]A, GGT[MP]A) [20], which eluted later than the unmodified peptides. This reflected the influence on the elution times of the more hydrophobic aromatic ring system of tyrosine, compared with the polar aliphatic hydroxy amino acids. The differences in the retention times of the methylphosphonopeptides and unmodified peptides as by-products allowed an easy purification by RP-HPLC as mentioned above for H-phosphonopeptides. These elution times indicated that it should be possible to separate both products, even for longer peptides.

For oxidation we used two reagents, *m*-chloroperbenzoic acid (MCPBA) and *tert*-butylhydroperoxide, dissolved in THF. Both yielded the methylphosphonopeptides after TFA cleavage. No difference in the resulting crude peptides was observed on the chromatograms of RP-HPLC. This means that the less reactive *tert*-butylhydroxyperoxide can be used for peptides containing sensitive amino acids, especially methionine and tryptophan.

Synthesis of Longer H- and Methylphosphonopeptides

To demonstrate the effectiveness of our new methodology for synthesizing tyrosyl H- and methylphosphonopeptides on solid phase, we synthesized the phosphonopeptides of two longer peptides, RRLIE DAEYAARG and ENDYINASL. The allyl groups used for protection of tyrosine were almost quantitatively cleaved, yielding the unmodified peptides in purities above 95% after TFA cleavage. These results were almost the same, as described above for the model peptide GGYA. The corresponding H- and methylphosphonopeptides eluted in the main fractions of the chromatograms of the crude peptides. The yields for all four phosphonopeptides were about 90%, which corresponded very well to the results for the model peptide GGYA, as did their chromatographic behavior (Tables 1 and 2). These results indicate that our approach is compatible with longer peptides and, even more important, that the yields and purities were almost the same as obtained for the tetrapeptide. Moreover, we observed

only the unmodified peptides as by-products, which were separated on RP-HPLC. Since we phosphitilated the peptides under nitrogen, we obtained no phosphopeptides after TFA cleavage.

CONCLUSIONS

The data presented indicate that short and medium-sized H- and methylphosphonopeptides can be synthesized in high yields and purities on solid phase. Using both methods it will be possible to screen protein tyrosine phosphatases for specific inhibitor sequences containing either H-phosphonopeptides or methylphosphonopeptides. Enzymatic tests of both sequences containing recognition motifs of tyrosine phosphatases are presently underway and will be published elsewhere.

Acknowledgements

We thank Drs L. Otvos Jr, V. Kinzel, J. Reed, W. Wunner and R. Berger-Hoffmann for reading the manuscript and helpful discussions. Furthermore we thank Dr W. Lehrmann for assisting the electrospray mass spectrometry measurements.

REFERENCES

1. K. M. Walton and J. E. Dixon (1993). Protein tyrosine phosphatases. *Annu. Rev. Biochem. 62*, 101–120.
2. L. J. Mauro and J. E. Dixon (1994). 'Zip codes' direct intracellular protein tyrosine phosphatases to the correct cellular 'address'. *Trends Biochem. Sci. 19*, 151–155.
3. K. Marakey, C. M. Belham, A. Paul, A. Graham, A. McLees, P. H. Scott and R. Plevin (1995). The regulation of tyrosine kinase signalling pathways by growth factor and G-protein-coupled receptors. *Biochem. J. 309*, 361–375.
4. R. L. Stone and J. E. Dixon (1994). Protein-tyrosine phosphatases. *J. Biol. Chem. 269*, 31323–31326.
5. G. Carpenter (1987). Receptors for epidermal growth factor and other polypeptide mitogens. *Annu. Rev. Biochem. 56*, 881–914.
6. A. Ullrich and J. Schlessinger (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell 61*, 203–212.
7. S. A. Aaronson (1991). Growth factors and cancer. *Science 254*, 1146–1153.
8. E. A. Kitas, J. W. Perich, R. B. Johns and G. W. Tregear (1988). The efficient synthesis of an *O*-phosphotyrosine-containing peptide using modern deprotection methods. *Tetrahedron Lett. 29*, 3591–3592.
9. E. A. Kitas, J. W. Perich, J. D. Wade, R. B. Johns and G. W. Tregear (1989). Fmoc-polyamide solid phase synthesis of an *O*-phosphotyrosine-containing tridecapeptide. *Tetrahedron Lett. 30*, 6229–6232.
10. R. M. Valerio, P. F. Alewood, R. B. Johns and B. E. Kemp (1989). Synthesis of *O*-phosphonotyrosyl peptides. *Int. J. Peptide Protein Res. 33*, 428–433.
11. G. Tong, J. W. Perich and R. B. Jones (1992). The improved synthesis of Boc-Abu(PO_3Me_2)-OH and its use for the facile synthesis of Glu-Abu(P)-Leu. *Aust. J. Chem. 45*, 1225–1240.
12. D. B. Berkowitz, Q. Shen and J. H. Maeng (1994). Synthesis of the (α,α-difluorolkyl) phosphonate analogue of phosphoserine. *Tetrahedron Lett. 35*, 6445–6448.
13. T. R. Burke, M. S. Smyth, M. Nomizu, A. Otaka and P. P. Roller (1993). Preparation of fluoro- and hydroxy-4-(phosphonomethyl)-*D-L*-phenylalanine suitably protected for solid-phase synthesis of peptides containing hydrolytically stable analogues of *O*-phosphotyrosine. *J. Org. Chem. 58*, 1336–1340.
14. A. Otaka, K. Miyoshi, T. R. Burke, P. P. Roller, H. Kubato, H. Tamamura and N. Fujii (1995). Synthesis and application of *N*-Boc-L-2-amino-(diethylphosphono)-4,4-difluorobutanoic acid for solid phase synthesis of nonhydrolyzable phosphoserine peptide analogues. *Tetrahedron Lett. 36*, 927–930.
15. L. Chen, L. Wu, A. Otaka, M. S. Smyth, P. P. Roller, T. R. Burke, J. den Hertog and Z. Y. Zhang (1995). Why is phosphonodifluoromethyl phenylalanine a more potent inhibitory moiety than phosphonomethyl phenylalanine toward protein-tyrosine phosphatases? *Biochem. Biophys. Res. Commun. 216*, 976–984.
16. D. B. A. de Bont, J. W. Moree, J. H. van Boom and R. M. J. Liskamp (1993). Solid phase synthesis of *O*-phosphonothioylserine- and -threonine containing peptides as well as of *O*-phosphoserine- and threonine containing peptides. *J. Org. Chem. 58*, 1309–1317.
17. J. C. H. M. Wijkmans, W. Ottenheim, E. Kuyl-Yeheskiely, G. A. van der Marel, W. Bloemhoff and J. H. van Boom (1995). Benzyloxy (diispropylamine)/methylphosphine: A powerful reagent for the synthesis of methylphosphonopeptides. *Synthesis*, 97–100.
18. P. Ruzza, A. D. Deana, A. Calderan, M. Pavanetto, L. Cesaro, L. A. Pinna and G. Borin (1995). Synthetic Tyr-phospho and non-hydrolyzable phosphonopeptides as PTKs and dTC-PTP inhibitors. *Int. J. Peptide Protein Res. 46*, 535–546.
19. R. Hoffmann, A. Tholey, T. Hoffmann and H. R. Kalbitzer (1996). Solid-phase synthesis of H- and methylphosphonopeptides. *Int. J. Peptide Protein Res. 47*, 245–253.
20. R. Hoffmann, W. O. Wachs, R. G. Berger, H. R. Kalbitzer, D. Waidelich, E. Bayer, W. Wagner-Redeker and M. Zeppezauer (1995). Chemical phosphorylation of the peptides GGXA (X = S, T, Y): an evaluation of different chemical approaches. *Int. J. Peptide Protein Res. 45*, 26–34.

21. G. Daum, F. Solca, C. D. Diltz, Z. Zhao, D. E. Cool and E. H. Fischer (1993). A general peptide substrate for protein tyrosine phosphatases. *Anal. Biochem.*, 50–54.
22. Y. Kambayashi, K. Takahashi, S. Bardhan and T. Inagami (1995). Cloning and expression of protein tyrosine phosphatase-like protein derived from a rat pheochromocytoma cell line. *Biochem. J. 306*, 331–335.
23. A. Loffet and H. X. Xhang (1993). Allyl-based groups for side-chain protection of amino-acids. *Int. J. Peptide Protein Res. 42*, 346–351.
24. G. B. Fields and R. L. Noble (1990). Solid-phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Peptide Protein Res. 35*, 161–214.
25. V. K. Sarin, S. B. H. Kent, J. P. Tam and R. B. Merrifield (1981). Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. *Anal. Biochem. 117*, 147–157.

