Acta Chemica Scandinavica, 1993: 47: 565–569
Printed in Belgium – all rights reserved

ACTA
CHEMICA SCANDINAVICA
ISSN 0904–213X

Synthesis and Function of an *O*-Phosphorylated Peptide Corresponding to the Cell Adhesion Sequence of Bone Sialoprotein (BSP)

Elisabeth Larsson,[a] Björn Lüning[a] and Dick Heinegård[b]

[a] Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, S-10691 Stockholm, Sweden and [b] Department of Medical Chemistry, University of Lund, Lund, Sweden

Larsson, E., Lüning, B. and Heinegård, D., 1993. Synthesis and Function of an *O*-Phosphorylated Peptide Corresponding to the Cell Adhesion Sequence of Bone Sialoprotein (BSP). – Acta Chem. Scand. 47: 565–569.

Bone sialoprotein contains a cell-binding RGD sequence followed by a threonine residue. Since the protein is extensively phosphorylated, this threonine may also be modified. To study whether such a phosphorylation may alter cell-binding properties, the hexapeptide Pro-Arg-Gly-Asp-Thr(*O*-phosphoryl)-Tyr has been synthesized by the Fmoc technique using benzyl protective groups for Tyr and phosphate, *tert*-butyl ester for Asp and Pmc for Arg. Removal of Fmoc groups was effected by treatment with 20% morpholine in DMF. The phospho-peptide inhibited binding of R1 cells to BSP-coated surfaces 10 times less efficiently compared with the non-phosphorylated peptide, as did, surprisingly, also the fibronectin-derived peptide Gly-Arg-Gly-Asp-Ser-Pro.

Bone sialoprotein (BSP) has been identified in bone only.[1,2] It is produced by osteoblasts and cDNA-clones have been isolated and sequenced from libraries prepared from such cells.[3] The sequence demonstrates several stretches of continuous glutamic acid residues, the longest comprising 10 amino acids. The protein contains an RGD-sequence characteristic of many cell-attachment proteins.[4] It has since been shown that BSP mediates attachment of a number of cells including osteoblasts.[5] This binding can be blocked by competition with RGD-containing peptides.[5] The sequence surrounding this tripeptide shows some homology with the corresponding sequence in vitronectin.[3] The sequence in BSP (PRGDTY) is, however, quite distinct and differs from corresponding sequences in another bone, cell-binding protein osteopontin (GRGDSL) and the classical cell-binding protein, fibronectin (GRGDSP). Although the functional implications of this variable sequence are not apparent, the proteins utilize different receptors. Thus, fibronectin binds to the fibronectin, VLA-5 receptor, while osteopontin and BSP appear to bind to integrins of the vitronectin receptor family.[5] Fibronectin too can bind to this receptor. The exact nature of the individual receptors for the two bone proteins is not known although data suggest the $\alpha_v\beta_3$-receptor.[5] Against this background it is interesting that the primary structure of the cell-binding domain in fibronectin and in osteopontin are rather similar, while that in BSP differs considerably.

A characteristic of both bone proteins is that they are highly phosphorylated, primarily at serine residues.[6] It cannot be ruled out, however, that single threonine residues are also phosphorylated. Thus, it is not known whether the threonine residues following the cell-binding tripeptide may be phosphorylated in the native protein. Phosphorylation of the hydroxyamino acid so close to the cell-binding sequence may serve a function in regulating cell binding. Thus, in model experiments, we have previously shown, using synthetic peptides, that phosphorylation of the serine residue in the GRGDSL-sequence will render a peptide much less efficient in preventing cell attachment.[7] Such a phosphorylation may therefore play a role in regulating cell interactions with the surrounding matrix.

To demonstrate whether phosphorylation of the threonine residues in BSP may alter cell binding, we present data on a study of cell binding activity of synthetic phosphorylated and non-phosphorylated peptides corresponding to the active domain of BSP.

Phosphopeptide synthesis has been performed previously by phosphorylating pre-synthesized peptides[8–11] or, more recently, by incorporating protected phosphono amino acids in liquid-phase[12] or solid-phase synthesis.[13] The latter approach was followed by us in the preparation of a hexapeptide phosphorylated at the serine following the RGD sequence of osteopontin.[7] The same general approach was followed in the synthesis of an analogous peptide derived from BSP. However, in this paper we show that the synthetic route had to be extensively modified to permit a reasonable yield of the correct peptide.

Materials and methods

Materials. 9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, *t*-butoxycarbonyl (Boc)-protected

amino acids and Fmoc-Tyr(Bzl)-Sasrin®-Resin were purchased from Bachem Feinchemikalien, Switzerland. 1,3-Diisopropylcarbodiimide (DIPCDI), 1-hydroxybenzotriazole (HOBT) and dimethyl sulfide were from Fluka, tetrazole, *m*-chloroperbenzoic acid and anisole were from Aldrich, dibenzyl *N,N*-diisopropylphosphoramidite was a gift from Roger Strömberg (Dept. of Org. Chem., Stockholm University). Dichloromethane (DCM) was run through an alumina column and then dried over 4 Å molecular sieves, *N,N*-dimethylformamide (DMF) was dried over 4 Å molecular sieves, *N,N*-diisopropylethylamine (DIPEA) was distilled over KOH and ninhydrin successively, anhydrous hydrogen fluoride (HF) was from AGA Gas, Sweden and was dried over CoF_3, trifluoroacetic acid (TFA), morpholine, ethyl acetate and chloroform were distilled before use.

Bovine bone sialoprotein (BSP) was prepared from bone as described elsewhere.[1]

Fast atom bombardment mass spectrometry (FAB-MS) and nuclear magnetic resonance (NMR). FAB-MS spectra were recorded on a JEOL SX102 double-focussing mass spectrometer with a FAB ion source, and a JMA DA6000 data system. The non-phosphorylated peptide was treated as follows. An aliquot of the sample was dissolved in 30% aqueous acetic acid and evaporated on the FAB-target, after which a mixture of 70% aqueous formic acid and glycerol (1 : 1) as the matrix was added, and the sample was bombarded with 10 keV xenon atoms. Spectra of positive ions were recorded in the range *m/z* 50–2550 with a resolution of 3000.

The phosphorylated peptide was treated as follows. An aliquot of the sample was dissolved in triethanolamine (TEA) and evaporated on the FAB target, and the sample was bombarded with 10 keV xenon atoms. Spectra of negative ions were recorded in the range *m/z* 100–1200 with a resolution of 1000.

Carbon (^{13}C) and phosphorus (^{31}P) NMR spectra were recorded on a JEOL GSX 270 spectrometer. Chemical shifts are given in parts per million (ppm) relative to tetramethylsilane (TMS $\delta_C = 0.00$) in C^2HCl_3 or dioxane ($\delta_C = 67.4$) in ^2H_2O for ^{13}C and 85% H_3PO_4 ($\delta_P = 0.00$) for ^{31}P.

Synthesis of N-*(fluorenylmethoxycarbonyl)-O-(dibenzylphosphono)*-L-*threonine* **1**. Treatment of L-threonine with fluorenylmethoxycarbonyl chloride gave *N*-fluorenylmethoxycarbonyl-L-threonine.[14] Fmoc-L-Thr-OH (500 mg, 1.47 mmol) and tetrazole (308 mg, 4.40 mmol) were dissolved in dry DCM (20 ml) and the solution was cooled to 0°C. Dibenzyl *N,N*-diisopropylphosphoramidite (0.759 ml, 2.20 mmol) was added to the stirred solution. The mixture was stirred for 2 h at room temperature and then cooled to −40°C. A solution of *m*-chloroperbenzoic acid (506 mg, 2.93 mmol) in DCM (10 ml) was added and the mixture was stirred 1 h at 0°C and was then extracted with 10% sodium bisulfite (3 × 30 ml). The organic layer was dried with sodium sulfate and the solvent was evaporated off to give the crude product which was purified by column chromatography on silica gel [heptane–ethyl acetate–acetic acid (30 : 30 : 5)] to give **1** as an oil (0.61 g, 67%), $[\alpha]_D = +18.6$. ^{13}C NMR (270 MHz, $CDCl_3$): δ 18.4 (γ-CH_3), 47.1 ($CHAr_2$), 58.3 (α-CH, d, $J_{POCC} = 7.3$ Hz), 67.3 ($NHCOOCH_2$), 69.9 and 70.0 ($PhCH_2O$, dd, $J_{POC} = 5.5$ Hz), 76.4 (β-CH, d, $J_{POC} = 5.5$ Hz), 119–143 (Ar–C), 156.7 (NHCOO), 171.2 (COOH). ^{31}P NMR (270 MHz, $CDCl_3$): δ −2.7.

Solid-phase synthesis of H-Pro-Arg-Gly-Asp-Thr(P)-Tyr-OH **2**. The peptide was synthesized manually using an orthogonal technique with Fmoc-protected amino acids[15–18] starting with Fmoc-Tyr(Bzl)-Sasrin®-resin (0.5 mequiv. g^{-1} substitution). The additional side-chain functional groups were protected as follows. *t*-butyl ester for aspartic acid, 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc)[19] for the guanidyl group of arginine and dibenzyl phosphotriester for threonine. Coupling of the amino acids to the *N*-terminus of the growing peptide was achieved using a threefold excess of the appropriate *N*-Fmoc protected amino acids in the presence of three equivalents of DIPCDI and 4.5 equivalents of HOBT. The coupling reaction was monitored for completion with a ninhydrin test.[20] The synthesis was performed on a 0.25 mmol scale in a 100 ml fritted glass bottommed reaction vessel with dry nitrogen for stirring and filtration. A general protocol for each step of the synthesis of **2** was as follows: 1, wash with DMF 2 × 1 min; 2, deblock with 20% morpholine in DMF 1 × 2 min; 3, wash with DMF 3 × 1 min; 4, deblock with 20% morpholine in DMF 1 × 10 min; 5, wash with DMF 10 + 1 min; 6, add Fmoc-amino acid 3 × molar excess; 7, couple in DMF; 8, wash with DMF 5 × 1 min; 9, check for free amino groups using ninhydrin. Fmoc-Thr(Bzl_2PO_3) and Fmoc-Asp(*t*-Bu)-OH were coupled 24 h for completion.

TFA cleavage of **2** *from resin.* The protected resin–peptide was treated first with 20% morpholine in DMF to cleave off the Fmoc-protecting group (see synthesis protocol for deblock procedures), washed with 1, DMF 5 × 1 min; 2, DCM 10 × 1 min and then treated 7 × 3 min with 1% TFA in DCM in order to cleave the peptide from the resin. The acidic organic phase was evaporated together with toluene to give the crude protected peptide.

Removal of side chain and benzyl blocking groups from the peptide phosphotriester. The peptide (247 mg) was dissolved in 5 ml TFA and 402 mg *p*-cresol were added as a scavenger. The mixture was stirred for 2 h at room temperature and then the peptide was precipitated from the acid with diethyl ether at −70°C and taken up in 10% aqueous acetic acid. The aqueous phase was evaporated to dryness and the residue desalted on Sephadex® G15 in water and lyophilized to yield the peptide as a white fluffy powder. Analysis and further purification was achieved by reversed-phase liquid chromatography on a Waters Bondapark C18 10μ column in a water–acetonitrile

0–40% gradient in 0.05% TFA, detection at 280 nm, resulting in a chromatographically homogeneous product (yield 51.7 mg, 29%).

The amino acid analyses were satisfactory. FAB-MS $(M-1)^-$ m/z 786.8, calc. m/z 787.3. ^{13}C NMR (270 MHz, ^2H_2O): δ Pro: 60.4 (C-α), 30.5 (C-β), 24.6 (C-γ), 47.4 (C-δ); Arg: 55.0 (C-α), 28.8 (C-β), 25.1 (C-γ), 41.4 (C-δ); Gly: 43.2 (C-α); Asp: 51.1 (C-α), 36.8 (C-β); Thr: 60.2 (C-α J_{CCOP} = 9.1 Hz), 72.0 (C-β J_{COP} = 3.7 Hz); Tyr: 56.2 (C-α), 37.0 (C-β), 116.2, 129.5, 131.5 and 155.2 (Ar–C). ^{31}P NMR (270 MHz, ^2H_2O): δ + 0.6.

Solid-phase synthesis of H-Pro-Arg-Gly-Asp-Thr-Tyr-OH **3**. The peptide was synthesized manually using Merrifield's solid-phase technique with Boc-protected amino acids,[21] side-chain protective groups: 2,6-dichlorobenzyl for tyrosine, benzyl for threonine and aspartic acid, and mesitylenesulfonyl for the guanidyl group of arginine. The first Boc-amino acid was covalently linked to a Biorad® chloromethylated polystyrene resin (1.25 equiv. g^{-1} substitution, 1% crosslink) according to the KF method[22] in DMF. Subsequent amino acids were incorporated using a fourfold excess of the appropriate *N*-Boc protected amino acids in the presence of 2 equiv. DIPCDI as preformed symmetric anhydrides. Boc-glycine and Boc-arginine were coupled as active esters produced *in situ* in the presence of six equiv. HOBT and four equivalents DIPCDI. The coupling reaction was monitored for completion with a ninhydrin test and repeated if necessary. The synthesis was performed on a 0.6 mmol scale in a 100 ml fritted glass-bottomed reaction vessel, using nitrogen for stirring and filtration.

HF cleavage of **3** *from resin.* Protected peptide–resin (0.42 g) was treated with Low-High HF.[23] After evaporation of the reagents the crude mixture was dissolved in 10% aqueous HOAc and extracted three times with ether. The aqueous phase was evaporated to dryness and desalted on Sephadex® G15 in 0.1 M ammonium hydrogencarbonate, and lyophilized to yield the peptide as a white fluffy powder (yield 107.4 mg, 51%). Verification of homogeneity was done by reversed-phase liquid chromatography on a Waters Bondapak C18 10μ column in a water–acetonitrile 0–100% gradient in 0.05% TFA, detection at 214 nm.

Amino acid analyses were within 0.04 of the expected values. FAB-MS $(M+1)^+$ m/z 708.2, calc. m/z 707.3. ^{13}C NMR (270 MHz, ^2H_2O) δ Pro: 60.5 (C-α), 30.5 (C-β), 24.6 (C-γ), 47.4 (C-δ); Arg: 55.0 (C-α), 28.8 (C-β), 25.1 (C-γ), 41.4 (C-δ); Gly: 43.2 (C-α); Asp: 51.5 (C-α), 37.6 (C-β); Thr: 59.8 (C-α), 67.9 (C-β), 19.5 (C-γ); Tyr: 56.5 (C-α), 37.1 (C-β), 116.2, 129.8, 131.5, 155.1 (Ar–C).

Cell-binding experiments. Polystyrene 96-well microtiter plates (NUNC immunolon) were coated overnight at room temperature with 50 μl of bovine BSP (bone sialoprotein) (5 μg ml^{-1} in 0.15 M NaCl, 5 mM sodium phosphate, pH 7.4).

Samples (50 μl) of the peptides to be tested for capacity to inhibit cell binding to fibronectin were added to the microtiter plate in triplicate at the dilutions indicated.

Rat R1 fibroblasts were released from monolayer cultures on tissue culture plates by trypsin digestion. Cells (500000) were suspended in 2.5 ml of Ham's F12 medium, containing 0.1 mg ml^{-1} bovine serum albumin. A 50 μl sample of the cell suspension was added to each peptide-containing well in the microtiter plate. After incubation for 2 h in a CO_2-incubator to allow cells to attach, non-bound cells were carefully removed by rinsing. Bound cells were quantified by measuring their *N*-acetylglucosaminidase content. This lysosomal enzyme was liberated from the cells by treatment with detergent, and enzyme activity was measured using *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside as the substrate. Bound cells are expressed as absorbance of released *p*-nitrophenol.

Results

Synthesis of phosphopeptides. Easily removable benzyl groups for the protection of the phosphate group and peptide synthesis according to an Fmoc-based method were used. However, the phosphate group is easily lost by elimination during the base treatment with piperidine[24] so that a weaker base, morpholine, had to be used. With these precautions a reasonable yield of the desired product was obtained.

N-(Fmoc)-*O*-dibenzylphosphono-L-threonine **1** was prepared using a convenient one-pot synthesis. The ^{31}P NMR spectrum of **1** gave a single resonance at −2.7 ppm, which is consistent with the phosphoric triester structure and the ^{13}C NMR spectrum showed resonances and coupling constants typical of the phosphorylated derivative (see materials and methods). This derivative was then used in the synthesis of the peptide **2** [Pro-Arg-Gly-Asp-Thr(P)-Tyr] by the Fmoc solid-phase approach. The unphosphorylated analogue **3** (Pro-Arg-Gly-Asp-Thr-Tyr), was synthesized for comparison.

Coupling of the amino acids was achieved using standard DIPCDI/HOBT chemistry in DMF with yields of >99.5% per cycle as verified by ninhydrin. The peptide was removed from the resin using 1% TFA in CH_2Cl_2. Analysis of the crude product by ^{31}P NMR spectroscopy showed that the protected phosphate group is stable in 20% morpholine. Removal of amino acid protecting groups and benzyl protecting groups from the peptide phosphotriester was accomplished by treatment with TFA. The phosphorylated peptide was then desalted on Sephadex® G15 and purified by reversed-phase liquid chromatography to give a homogeneous product. Verification of the product as the phosphopeptide **2** [Pro-Arg-Gly-Asp-Thr(P)-Tyr] was established by negative-ion FAB mass spectrum analysis [$(M-1)^-$ m/z 786.8], ^{13}C and ^{31}P NMR spectroscopy and amino acid analysis (see Materials and methods). These data show that the benzyl blocking groups had been quantitatively removed

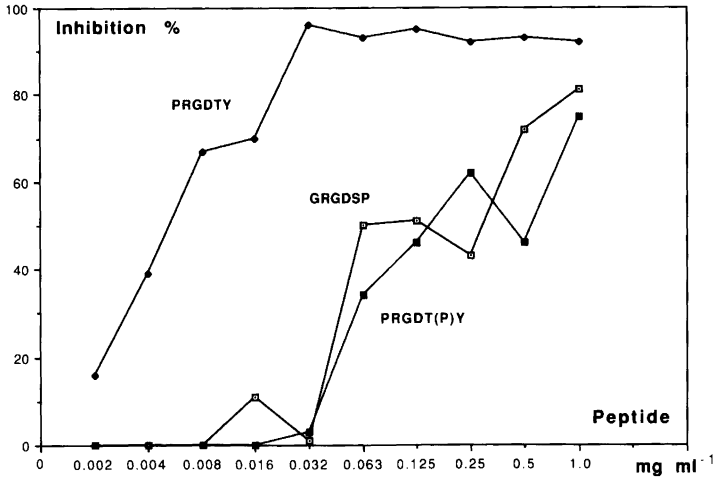

Fig. 1. Inhibition of attachment of R1-cells to BSP by the peptides PRGDTY, GRGDSP and PRGT(P)Y. Inhibition is given as a percentage of binding relative to the total binding in the absence of peptide.

and gave no evidence of dephosphorylation. In the ^{13}C NMR spectrum the coupling of the α- and β-carbon with the phosphorus atom is discernible.

The mass spectrum analysis $[(M+1)^+ m/z\ 708.2]$, ^{13}C NMR spectrum and amino acid analysis of the non-phosphorylated peptide **3** all showed the expected values (see Materials and methods).

Cell-binding results. The R1-cells showed good binding to surfaces coated with BSP, Fig. 1. Increased competition for binding to the coated surface caused by added increasing amounts of GRGDSP, i.e., the fibronectin sequence, showed progressive inhibition of cell binding to BSP, Fig. 1. It is of particular interest that the peptide, PRGDTY, representing the relevant sequence in BSP, showed a manifold higher degree of inhibition than the corresponding hexapeptide from fibronectin. As shown previously[7] the corresponding osteopontin (GRGDSL) peptide showed a very similar degree of inhibition as the BSP-peptide (data not shown).

The phosphorylated peptide, PRGDT(P)Y, showed a similar, albeit lower, degree of inhibition as the fibronectin peptide, Fig. 1. The binding was, in both cases, some 10 times less efficient compared with the non-phosphorylated peptide from BSP.

In further support, the cell-binding profile of the phosphorylated peptide showed that the modification of the threonine residue was complete, with an altered slope of the inhibition curve.

Discussion

Choice of cell lines. In the present study we used a fibroblast cell line for studies of cell binding to the bone matrix protein, BSP. This cell line was used since, in a previous study, results indicated that osteoblasts of various origin produced sufficient alkaline phosphatase to dephosphorylate proteins and peptides used, while the R1-cells appeared to have no such capacity.

Differential inhibition of adhesion by hexapeptides. Two observations in the present study were of great relevance. Firstly, we could show that there was a very distinct difference in the inhibition capacity on comparison of the peptide corresponding to the sequence in BSP with the one corresponding to the fibronectin sequence. This finding clearly demonstrates that although the tripeptide, RGD, appears sufficient for the inhibition of cell binding to the proteins, the specificity depends on a larger surrounding sequence. This finding has implications for studies of individual matrix proteins and integrin, cell surface receptors, regarding their role in cell–matrix interaction. By selecting an appropriate peptide it should become possible selectively to block one specific interaction, while intervening little or not at all with other interactions depending on the RGD-sequence.

Possible regulation of adhesion through phosphorylation. A second finding in the present study was that phosphorylation of the threonine residue following the active tripeptide, although not precluding cell binding, exerted a major effect that decreased this binding. This observation also demonstrates the importance of the structures of amino acids surrounding the active tripeptide. Phosphorylation of the hydroxyamino acid following the cell-binding sequence is particularly interesting in view of the capacity of both osteoblasts and osteoclasts, the two cells with major roles in bone turnover, to produce phosphatases. We found previously that osteoblasts and osteoblastic cells, known to produce alkaline phosphatase, caused apparent dephosphorylation of the phosphorylated GRGDS(P)L peptide, thereby causing a major improvement in its cell-binding capacity.[7] Thus, phosphorylation of this hydroxyamino acid residue, next to the cell binding sequence, may provide the cell with a protein that is secreted in an inactive form, but that can be activated in the matrix by enzymes produced by the cell. Future experiments are necessary to demonstrate whether

the threonine residue actually occurs phosphorylated in BSP isolated from bone.

New achievements in the synthesis of phosphopeptides. The initial attempts to synthesize PRGDT(P)Y using Boc-technique with phenyl as the protecting group of the phosphate resulted in total hydrogenation of the tyrosine during attempts to remove the phenyl protecting groups hydrogenolytically. Deprotection of the diphenyl phosphotriester while still attached to the resin by reaction with tetrabutylammonium fluoride (TBAF)[25] followed by cleavage of the peptide from the resin with 10% trifluoromethanesulfonic acid gave a mixture of several peptides still containing one or two phenyls and also some dephosphorylated material. In early syntheses of phosphorylated peptides, benzyl esters were used as protection of the phosphate group, but these esters are not stable under the acidic conditions[12] used in synthesis according to a Boc-based method. For these reasons it was necessary to find another approach. Thus synthesis according to an Fmoc-based method with benzyl-protected phosphate proved to be the method of choice.

Previous syntheses of phosphate group esterified, *N*-protected phospho-threonine involved either a three-step procedure starting with *N*-protected threonine, carboxy-group protection, *O*-phosphorylation and deprotection,[26] or a one-pot synthesis[27] using unstable dialkyl and diaryl phosphorochloridites as phosphorylating agents. We now report a one-pot synthesis of Fmoc-Thr(Bzl_2PO_3)-OH using the more stable dibenzyl phosphoramidite as the phosphorylating agent giving a product without impurities discernible by NMR spectroscopy.

The protected *O*-phosphono-threonine derivative was then used in a synthesis using the Fmoc technique. Thus, the desired PRGDT(P)Y was synthesized via incorporation of Fmoc-Thr(Bzl_2PO_3)-OH. A recent publication mentions that benzyl-protected *O*-phosphotyrosine derivatives are substantially debenzylated by secondary amines[28] such as piperidine and are therefore not suitable for use in an Fmoc-based method. To reduce the possibility of elimination of the phosphate or benzyl protecting groups, morpholine was used for deprotection of the Fmoc group. According to both NMR and mass spectrometry the derivative is sufficiently stable in 20% morpholine in DMF. It was also successfully used in the solid-phase peptide synthesis of PRGDT(P)Y.

Acknowledgments. Support was obtained from the Swedish National Board for Technical Development and the Swedish Medical Research Council.

References

1. Franzén, A. and Heinegård, D. *Biochem. J. 232* (1985) 715.
2. Fisher, L., Whitson, S., Avioli, L. and Termine, J. *J. Biol. Chem. 258* (1983) 12723.
3. Oldberg, Å., Franzén, A. and Heinegård, D. *Proc. Natl. Acad. Sci. USA 83* (1986) 8819.
4. Ruoslahti, E. and Pierschbacher, M. *Science 238* (1987) 491.
5. Oldberg, Å., Franzén, A., Heinegård, D., Pierschbacher, M. and Ruoslahti, E. *J. Biol. Chem. 263* (1988) 19433.
6. Heinegård, D., Hultenby, K., Oldberg, Å., Reinholt, F. and Wendel, M. *Connect. Tissue Res. 21* (1989) 333.
7. Pettersson, E., Lüning, B., Mickos, H. and Heinegård, D. *Acta Chem. Scand. 45* (1991) 604.
8. Fölsch, G. *Svensk Kem. Tidskr. 79* (1967) 38.
9. Ferrel, R. E., Olcott, H. S. and Fraenkel-Conrat, H. J. *J. Am. Chem. Soc. 70* (1948) 2101.
10. Kemp, B. E., Graves, D. J., Benjamin, E. and Krebs, E. G. *J. Biol. Chem. 252* (1977) 4888.
11. Blumenthal, D. K. In: Kemp, B. E., Ed., *Peptides and Protein Phosphorylation: Use of Synthetic Peptides to Study Substrate Specificity of Protein Phosphatases*, CRC Press, Cleveland (1990) 135.
12. Perich, J. W., Alewood, P. F. and Johns, R. B. *Tetrahedron Lett. 27* (1986) 1373.
13. Perich, J. W., Valerio, R. M. and Johns, R. B. *Tetrahedron Lett. 27* (1986) 1377.
14. Chang, C.-D., Waki, M., Ahmad, M., Meinhofer, J., Lundell, E. O. and Haug, J. *Int. J. Peptide Protein Res. 15* (1980) 59.
15. Atherton, E., Gait, M. J., Sheppard, R. C. and Williams, B. J. *Bioorg. Chem. 8* (1979) 351.
16. Atherton, E. and Sheppard, R. C. In: Eberle, A., Geiger, R. and Wieland, T., Eds., *Perspectives in Peptide Chemistry: The Solid Phase in Solid Phase Synthesis*, Karger, Basel, 1981, p. 101.
17. Carpino, L. A. *Acc. Chem. Res. 20* (1987) 401.
18. Atherton, E., Logan, C. J. and Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1* (1981) 1538.
19. Ramage, R. and Green, J. *Tetrahedron Lett. 28* (1987) 2287.
20. Sarin, V. K., Kent, S. B. H., Tam, J. P. and Merrifield, R. B. *Anal. Biochem. 117* (1981) 147.
21. Barany, G. and Merrifield, R. B. In: Gross, E. and Meienhofer, J., Eds., *The Peptides*, Academic Press, 1980, Vol. 2, Chap. 1.
22. Clark, J. H. and Miller, J. M. *Tetrahedron Lett. 7* (1977) 599.
23. Tam, J. P., Heath, W. F. and Merrifield, R. B. *J. Am. Chem. Soc. 105* (1983) 6442.
24. Otvos, L., Elekes, I. and Lee, V. M.-Y. *Int. J. Peptide Protein Res. 34* (1989) 129.
25. Arendt, A. and Hargrave, P. A. In: Deber, C. M., Hruby, V. J. and Kopple, K. P., Eds., *Peptides. Structure and Function: Synthesis of Phosphopeptides from the Carboxyl-Terminus of Rhodopsin*, Pierce Chemical Co., Rockford, IL 1981, p. 237.
26. Perich, J. W. and Johns, R. B. *Tetrahedron Lett. 29* (1988) 2369.
27. Perich, J. W., Alewood, P. F. and Johns, R. B. *Synthesis* (1986) 572.
28. Valerio, R. M., Alewood, P. F., Johns, R. B. and Kemp, B. E. *Int. J. Peptide Protein Res. 33* (1989) 428.

Received May 26, 1992.