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Synthesis of linear and cyclic phosphopeptides as ligands for the *N*-terminal SH2-domain of protein tyrosine phosphatase SHP-1

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Abstract: Linear and cyclic phosphopeptides related to the pY2267 binding site of the epithelial receptor tyrosine kinase Ros have been synthesized as ligands for the amino-terminal SH2 (src homology) domain of protein tyrosine phosphatase SHP-1. The synthesis was accomplished by Fmoc-based solid-phase methodology using side-chain unprotected phosphotyrosine for the linear and mono-benzyl protected phosphotyrosine for the cyclic peptides. According to molecular modelling, the incorporation of a glycine residue between Lys (position pY−1 relative to phosphotyrosine) and Asp or Glu (position pY+2) was recommended for the cyclic candidates. The preparation of these peptides was successfully performed by the incorporation of a Fmoc-Xxx(Gly-OAll)-OH (Xxx = Asp, Glu) dipeptide building block that was prepared in solution prior to SPPS. The cyclization was achieved with PyBOP following Alloc/OAll-deprotection. This study demonstrates the usefulness of allyl-type protecting groups for the generation of side-chain cyclized phosphopeptides. Alloc/OAll-deprotection and cyclization are compatible with phosphorylated tyrosine.

Keywords: SHP-1; SH2 domain ligands; phosphopeptide synthesis; cyclic peptides

INTRODUCTION

The study of tyrosine phosphorylation- and dephosphorylation-states in signalling proteins is the focus of considerable attention today due to their importance in the regulation of cellular processes, such as cell growth and differentiation [1–3]. A tyrosine residue phosphorylated by the action of a protein tyrosine kinase (PTK) may serve as a substrate for protein tyrosine phosphatases (PTPs) or as a specific recognition feature within a sequence associating with small protein modules, such as SH2 (src homology 2) or PTB (phosphotyrosine binding) domains [4,5].

The cytoplasmic protein tyrosine phosphatase SHP-1 is present in haematopoietic and epithelial cells, and has been shown to interact downstream of, for example, receptor tyrosine kinases, antigen receptors and cytokine receptors [6,7]. The enzyme contains two tandem SH2 domains at the *N*-terminus and a phosphatase domain followed by a small tail in the *C*-terminus. The catalytic activity of SHP-1 is dependent on the occupancy of the amino-terminal SH2 (N-SH2) domain by phosphotyrosine-containing proteins. In the autoinhibitory state, the N-SH2 domain blocks the catalytic cleft of the phosphatase domain and renders the enzyme catalytically inactive. A conformational change caused by the association of a ligand to the N-SH2 domain releases the active site of the enzyme that is accompanied by an increase in phosphatase activity [7,8].

Binding studies with tyrosyl phosphopeptides of randomized sequences suggested that the specificity of SH2 domains results from distinct residues adjacent to the phosphotyrosine (pY), and in particular those on the *C*-terminal side of it. As with a variety of SH2 domains, the most critical interactions of the SHP-1 SH2 domains are in the binding pockets for the pY + 1 and pY + 3 residue of the ligand [9]. However, a systematic identification of the sequence specificity of the SHP-1 SH2 domains employing the phosphopeptide library Ac-DEXXpYXXXIBBRM-resin (B = β-alanine) revealed

Abbreviations: Standard abbreviations for amino acid derivatives and peptides are according to the suggestions of the IUPAC-IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984; **138**: 9–37). Abbreviations for protecting groups and peptide synthesis reagents were used as recommended in the guide published in *J. Peptide Sci.* 2003; **9**:1–8. Other abbreviations: Abu(βPh), 2-amino-3-phenyl-butyric acid; Ado, 8-amino-3,6-dioxaoctanoic acid; Btn, biotin; Dmab, 4-{N-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino}benzyl; EpoR, erythropoietin receptor; FcγRIIBl, receptor for the Fc region of immunoglobulin G; Hfe, homophenylalanine; PILR$_\alpha$, paired immunglobulin-like receptor α; Pd(PPh$_3$)$_4$, tetrakis(triphenylphosphine)palladium(0); PTB, phosphotyrosine binding domain; Ser(βPh), 2-amino-3-hydroxy-3-phenyl-propionic acid; N-SH2, *N*-terminal src homology 2 domain; SHP-1, SH2 domain protein tyrosine phosphatase-1.

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that the pY-2 position is another important specificity determinant [10].

The development of biologically active phosphopeptide analogues derived from physiologically relevant interaction partners of SH2 domain-containing proteins is of great interest today because of their potential as therapeutic drugs in the modulation of cell signalling. However, to obtain specific inhibitors for an individual SH2 domain, a detailed understanding of the binding interactions is necessary [11]. Although peptide libraries gave significant information about the preferred binding motif of the SHP-1 SH2 domains, there is still a lack of a detailed topographical and conformational characterization of the allowed modifications in the corresponding positions. The sequence surrounding phosphotyrosine pY2267 of the epithelial receptor tyrosine kinase Ros (Ros pY2267) was used as a lead structure to further characterize the structural requirements of effective ligands associating with the *N*-terminal SH2 (N-SH2) domain of SHP-1. Ros pY2267 has previously been identified as a high affinity ligand for the SHP-1 N-SH2 domain, exceeding known effective interaction partners, such as EpoR pY429, Fc_{γ}RIIB1 pY309 and PILRα pY269 [12]. The binding of the Ros native ligand to the N-SH2 domain is represented schematically in Figure 1. The ligand is bound in an extended conformation with intensive contacts of the residues Leu (pY-2), pTyr (0), Met (pY + 1) and Leu (pY + 3) with the protein domain. Besides linear structures containing modifications in different positions on the carboxy-terminal side of phosphotyrosine (pY + 1 to pY + 3), cyclic peptides were also synthesized as conformationally constrained analogues which may provide improvements in binding affinity and facilitate better understanding of the structural requirements of the SHP-1 N-SH2 domain. The selection of the peptide structures was supported by molecular modelling studies, which are described in detail together with the results of the biological testing elsewhere [13]. The present report is focused on the synthesis of the designed peptide structures with emphasis on the preparation of side-chain cyclized phosphopeptides.

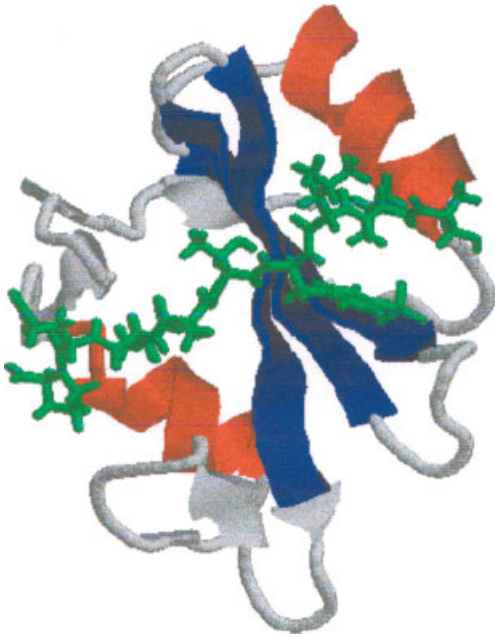


Figure 1 Schematic ribbon diagram of the SHP-1 N-SH2 domain in complex with Ros pY2267 (EGLNpYMVL) [9,13].

MATERIALS AND METHODS

General

Fmoc- and Boc-amino acids, D- and L-amino acids, coupling reagents (TBTU, HOBt, PyBOP, DCC) and Rink-amide MBHA-resin were purchased from Orpegen (Heidelberg, Germany) and Novabiochem (Merck Biosciences AG, Läufelfingen, Switzerland), respectively. D/L-Abu(βPh) was obtained as a mixture containing 67% of the *threo*- and 33% of the *erythro*-form from Aldrich (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and as D/L-erythro-Abu(βPh) from Acros Organics (Geel, Belgium). Fmoc-D/L-*erythro*/*threo*-Abu(βPh) and Fmoc-D/L-*threo*-Ser(βPh) were prepared using known procedures for Fmoc-introduction described in the literature [14,15]. 1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) was from Novabiochem. Peptide synthesis reagents (DIEA, piperidine) and solvents were of reagent grade from Fluka (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Solvents for chromatography were of analytical grade obtained from Acros Organics (Geel, Belgium). Purifications of dipeptide building units by column chromatography were performed on Merck silica gel 60 (0.040–0.063 mm).

Building Block Syntheses

H-Gly-OAll (1). Boc-Gly-OH (10 g, 1 eq.) was treated with DCC (1.2 eq.) in allyl alcohol (9.2 eq.) as described by Sabatino *et al.* [16]. The product was crystallized from diethyl ether/hexane affording Boc-Gly-OAll as a white solid (11.5 g, 94%). m.p. 87 °C. R_{f1} 0.45 (hexane : ethyl acetate 8 : 2), R_{f2} 0.78 (benzene : acetone : acetic acid 27 : 10 : 0.5). ^{1}H-NMR σ 1.36 (m, 9H), 3.18 (m, 2H), 4.53 (m, 2H), 5.17–5.30 (m, 2H), 5.86–5.93 (m, 1H), 6.82 (1H). ESI MS $[MH]^+$ 216. Cleavage of the Boc-protecting group was performed using 50% TFA/DCM for 30 min. TFA and DCM were removed by evaporation and the residue was washed several times with diethyl ether. The product was obtained as a white solid in 96% yield. m.p. 101 °C. R_f 0.34 (benzene : acetone : acetic acid 27 : 10 : 0.5). ^{1}H-NMR σ 2.21 (2H), 3.41 (m, 2H), 4.57–4.60 (m, 2H), 5.21–5.34 (m, 2H), 5.85–5.98 (m, 2H). ESI MS $[MH]^+$ 116.1.

Fmoc-Asp(Gly-OAll)-OH (2). Coupling of compound **1** to the side chain of Fmoc-Asp-OBu^t was performed with the mixed anhydride method described in the literature [15,17]. The crude product was obtained as a yellow oil which was subsequently treated with 50% TFA/DCM for 1 h to remove the tert-butyl ester protecting group. After evaporation of TFA and DCM the crude product was

purified by column chromatography on silica gel eluted with chloroform : methanol (9 : 1) giving **2** as a white solid (65% yield). t_R 27.6 min (20%–80% 0.1% TFA/acetonitrile). R_f 0.25 (chloroform : methanol 9 : 1). ^{1}H-NMR σ 2.02–2.25 (m, 2H), 4.09 (s, 2H), 4.16 (s, 2H), 4.46 (s, 2H), 4.70 (s, 2H), 5.23–5.30 (m, 2H), 5.89 (m, 1H), 6.02 (s, 1H), 6.79 (s, 1H), 7.28–7.84 (m, 8H). FAB MS $[MH]^+$ 453.5.

Fmoc-Glu(Gly-OAll)-OH (3). Compound **3** was obtained from the reaction of Fmoc-Glu-OBu^t with **1** as described for building block **2** in 77% yield. t_R 27.8 min (20%–80% 0.1% TFA/acetonitrile). R_f 0.27 (chloroform : methanol 9 : 1). ^{1}H-NMR σ 2.05–2.63 (m, 4H), 4.04 (s, 2H), 4.18 (s, 2H), 4.36 (s, 2H), 4.60 (s, 2H), 5.22–5.32 (m, 2H), 5.82–5.89 (m, 1H), 5.99 (s, 1H), 6.70 (s, 1H), 7.23–7.74 (m, 8H). FAB MS $[MH]^+$ 467.2.

Peptide Synthesis

The linear peptides were synthesized manually or by using an automated peptide synthesizer MPS 396 from Advanced ChemTech (Cambridge, UK). The cyclic peptides were only assembled manually using syringes (Abimed, Langenfeld, Germany) to ensure monitoring of each synthetic step. Coupling reactions were performed using Fmoc amino acids (4 eq.) activated with TBTU (4 eq.) or PyBOP (4 eq.), in the presence of DIEA (8 eq.) for 0.5–1 h (double couplings). Fmoc-phosphotyrosine (4 eq.) was coupled with 12 eq. of DIEA. Fmoc removal was effected by treating the resin twice with 20% piperidine in DMF for a) 5 min and b) 15 min. The side chains of trifunctional amino acids were protected as follows: Asn(Trt), Glu(OBu^t), Tyr(PO_3H_2) (for linear peptides) and Tyr[PO(OBzl)OH] (for cyclic peptides). Protecting groups for the side chains to be cyclized were Alloc or OAll as appropriate. Rink amide MBHA resin (0.54 mmol/g) was used for all peptides described. If necessary, remaining unreacted amino groups were capped with 2 eq. of Z(2Cl)-OSu and DIEA in DCM/DMF (1 : 1) for 20 min. All deprotection and coupling steps were followed by intensive washings using DMF and DCM. After complete assembly of the Fmoc-protected sequences the resins were split in two portions in order to obtain *N*-terminally free and biotinylated peptides. Prior to biotinylation Fmoc-Ado was introduced in the same way as the other amino acids described above. Biotinylation was achieved with biotin (4 eq.), PyBOP (4 eq.) and DIEA (8 eq.) for 2–4 h. In the case of the *N*-terminally free cyclic peptides the Boc-group was introduced after final Fmoc-deprotection using $(Boc)_2O$ (5 eq.) and DIEA (10 eq.) in DMF for 1–2 h. Alloc/OAll-groups were removed with a mixture of DMF : THF:0.5N HCl : morpholine (2 : 2 : 1 : 0.9) and $Pd(PPh_3)_4$ as the catalyst. Cyclization was achieved using PyBOP (6 eq.) and DIEA (12 eq.) in DMF for 3 h. Cleavage of peptides from the resin with concomitant side-chain deprotection was achieved by treating the resins with TFA/water/triisopropylsilane (95 : 2.5 : 2.5) for 3 h (peptides containing Tyr(PO_3H_2)-OH or 5–6 h (peptides containing Tyr[PO(OBzl)OH]. The crude peptides were precipitated in diethyl ether, centrifuged and washed three times with diethyl ether. The yields of crude peptides ranged between 70%–80% (linear peptides) and 50%–60% (cyclic peptides).

HPLC Analysis

The crude peptides were purified by semipreparative reversed-phase HPLC using a Shimadzu LC-8A system equipped with a C18 column (Knauer Eurospher 100, Berlin, Germany). The gradient elution system was 0.1% TFA in water (buffer A) and 0.1% TFA in acetonitrile/water (90 : 10) (buffer B). The peptides were eluted with a gradient from 15% to 65% of buffer B in 120 min at a flow rate of 10 ml/min. The peaks were detected at 220 nm. The collected fractions were evaporated until the acetonitrile was removed and lyophilized from the aqueous solution. The purity of the peptides was established by analytical reversed-phase HPLC on a Shimadzu LC-10AT chromatograph (Duisburg, Germany) with a Vydac 218TP column (5 µm particle size, 300 Å pore size, 4.6 × 25 mm). Building blocks were eluted with the gradient 20%–80% B in 60 min and peptides with 10%–60% in 50 min at a flow rate of 1.0 ml/min, where A was 0.1% TFA in water and B 0.1% TFA in acetonitrile; detection was at 220 nm.

Mass Spectrometry

The molecular weights of the peptides were determined by MALDI-TOF mass spectrometry on a Laser Tec Research mass spectrometer (Perseptive Biosystems, Weiterstadt, Germany) using α-cyano-4-hydroxycinnamic acid as matrix.

Amino Acid Analysis

The amino acid composition of all *N*-terminally free peptides was verified by amino acid analysis using a LC 3000 system from Eppendorf-Biotronik (Hamburg, Germany). Solutions of peptides were prepared in the buffer used for the SHP-1 phosphatase assay [13] and an aliquot was used for hydrolysis in order simultaneously to determine the peptide content of the solutions that was necessary for the enzymatic assay. The peptide solutions were freeze-dried prior to hydrolysis which was then performed

Table 1 Amino Acid Sequences of Ros pY2267 and Analogues

Peptide	Sequence
Class I[a]	Leu-Xxx-pTyr-Met-Xxx-Phe Phe Met[b]
Ros pY2267	H-Glu-Gly-Leu-Asn-pTyr-Met-Val-Leu-NH_2
1	H-Glu-Gly-Leu-Asn-pTyr-Phe-Val-Leu-NH_2
2	H-Glu-Gly-Leu-Asn-pTyr-Met-Val-Phe-NH_2
3	H-Glu-Gly-Leu-Asn-pTyr-Hfe-Val-Leu-NH_2
4	H-Glu-Gly-Leu-Asn-pTyr-Met-Val-Hfe-NH_2
5	H-Glu-Gly-Leu-Asn-pTyr-Abu(βPh)-Val-Leu-NH_2
6	H-Glu-Gly-Leu-Asn-pTyr-Met-Val-Abu(βPh)-NH_2
7	H-Glu-Gly-Leu-Asn-pTyr-Ser(βPh)-Val-Leu-NH_2
8	H-Glu-Gly-Leu-Asn-pTyr-Met-Val-Ser(βPh)-NH_2
9	H-Glu-Gly-Leu-Asn-pTyr-Nle-Val-Phe-NH_2
10	H-Glu-Gly-Leu-Asn-pTyr-Phe-Val-Phe-NH_2
11	H-Glu-Gly-Leu-Asn-pTyr-Met-Tyr-Phe-NH_2
12	H-Glu-Gly-Leu-Asn-pTyr-Tic-Ile-Tic-NH_2
13	H-Glu-Gly-Leu-Lys-pTyr-Met-Asp-Leu-NH_2 └─$COCH_2NH$─┘
14	H-Glu-Gly-Leu-Lys-pTyr-Met-Glu-Leu-NH_2 └─$COCH_2NH$─┘
15	H-Glu-Gly-Leu-Lys-pTyr-Met-Glu-Leu-NH_2 └──────┘

[a] Class I consensus sequence according to the literature [11].
[b] Xxx ∂←any amino acid; Met can also be exchanged by the isostere Nle as described in the literature [11].

using 6N HCl in sealed tubes at 110 °C for 24 h. The obtained analyses gave the expected quantitative results for all peptides.

Racemization Study

Derivatization of the standard amino acids. The racemization study was performed according to the method of Marfey [18–20]. 10 μmol of each amino acid was dissolved in 100 μl of water. Then, 200 μl of 1% Marfey's reagent in acetone and 40 μl of 1N $NaHCO_3$ were added. The mixture was left at 40 °C for 1.5 h. The reaction was stopped by adding 20 μl of 2N HCl. The samples were degassed and diluted (1 : 10) for injection.

Derivatization of the hydrolysed peptides. From all peptides 2 mM solutions were prepared in order to carry out the phosphatase assay [13]. 100 μl of these solutions was used which were freeze-dried prior to acidic hydrolysis. Hydrolysis was performed as described for amino acid analysis. After evaporation of HCl and water the remaining residue was washed several times with water and used for derivatization after final evaporation. The hydrolysis product was dissolved in 100 μl of water. A neutralization with saturated $NaHCO_3$-solution and concentration of the mixture to 100 μl is necessary prior to the addition of 200 μl of 1% Marfey's reagent in acetone and 40 μl of 1N $NaHCO_3$. The mixture was left at 40 °C for 1.5 h. The reaction was then stopped by adding 20 μl of 2N HCl. The samples were degassed and appropriately diluted for injection.

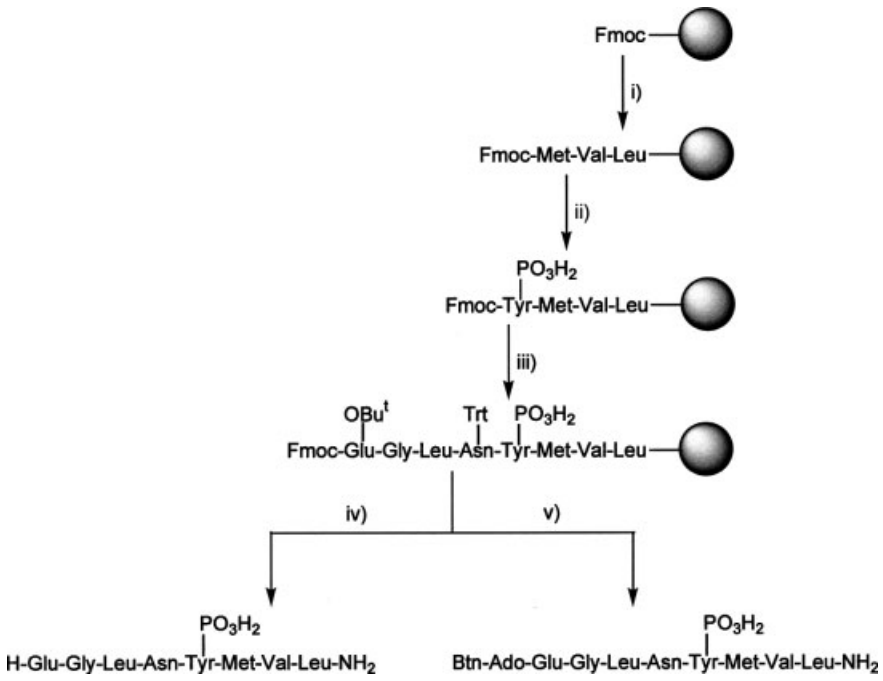
Chromatographic conditions. Separations were performed on a Shimadzu LC-10AT chromatograph with a Vydac 218TP column (5 μm particle size, 300 Å pore size, 4.6 × 25 mm). Derivatized samples were eluted with the gradient 10%–70% B in 60 min at a flow rate of 1.0 ml/min, A was 0.1% TFA in water and B 0.1% TFA in acetonitrile. Detection was at 340 nm.

RESULTS AND DISCUSSION

Peptide Synthesis

H-Glu-Gly-Leu-Asn-pTyr^{2267}-Met-Val-Leu-NH_2, a phosphorylated octapeptide derived from the cytoplasmic region of the transmembrane receptor tyrosine kinase Ros was used as the lead structure for all the peptides included in this study (Table 1). In order to meet the specificity determinants for binding to the *N*-terminal SH2 domain of protein tyrosine phosphatase SHP-1, only positions on the *C*-terminal side of phosphotyrosine (pY + 1 to pY + 3) have been modified within the linear sequences. The selection of the unnatural amino acids introduced in the relevant positions and the design of the peptides using a molecular modelling approach is described in more detail elsewhere [13].

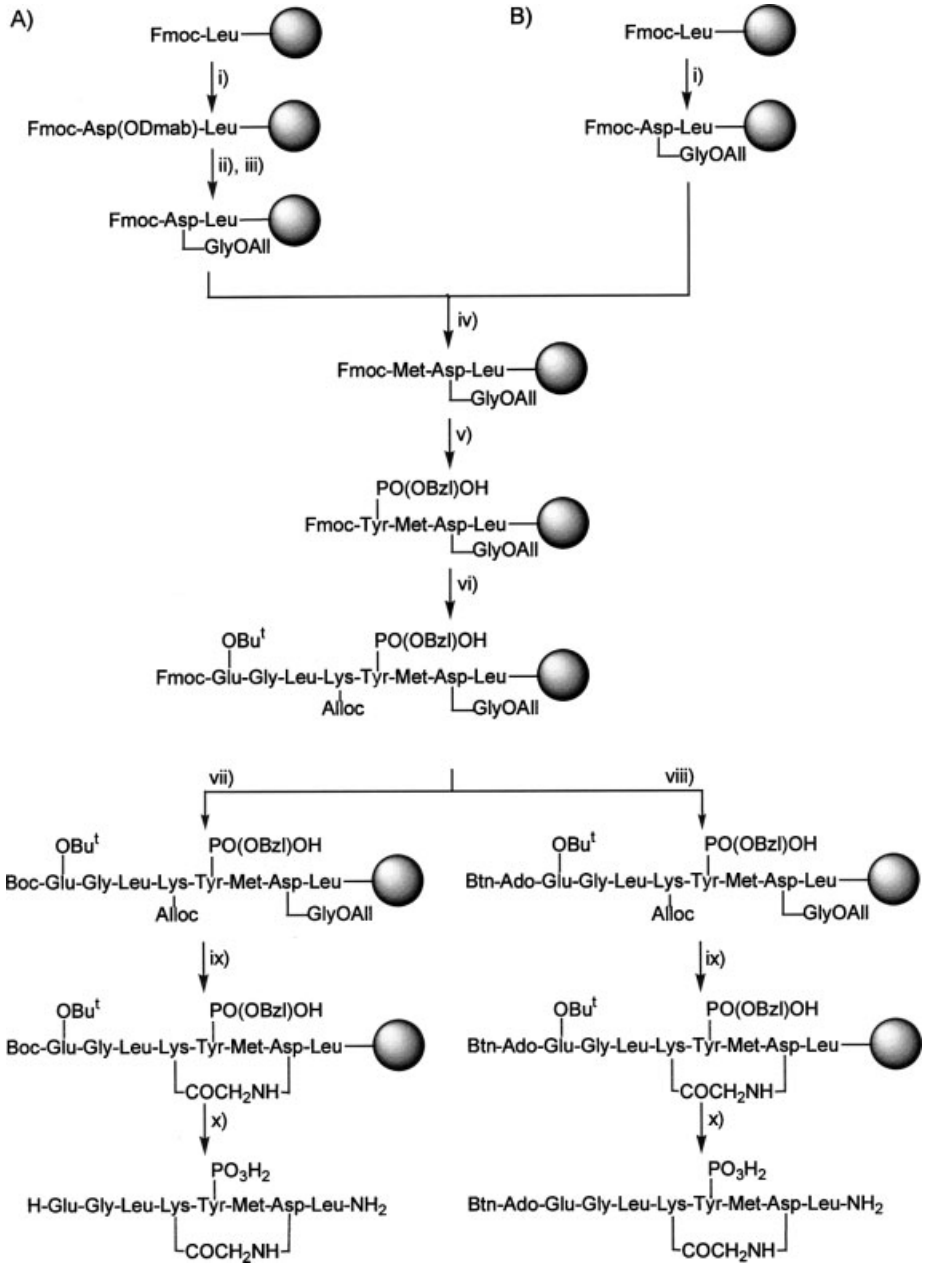
In recent years there have been substantial efforts in the development of methods for the chemical synthesis of phosphopeptides. The phosphorylation can be performed either on the level of the protected peptide with the side-chain to be phosphorylated still



Scheme 1 Reagents and conditions; (i) 20% piperidine/DMF, Fmoc-aa/TBTU/HOBt/DIEA/DMF, alternately; (ii) 20% piperidine/DMF, Fmoc-Tyr(PO_3H_2)-OH or Fmoc-Tyr[PO(OBzl)OH]-OH/TBTU/HOBt/DIEA/DMF; (iii) 20% piperidine/DMF, Fmoc-aa/TBTU/HOBt/DIEA/DMF, alternately; resin split; (iv) 20% piperidine/DMF, cleavage: 95% TFA/2.5% triisopropyl silane/2.5% water; (v) 20% piperidine/DMF, Fmoc-Ado/TBTU/HOBt/DIEA/DMF, 20% piperidine/DMF, biotin/PyBOP/DIEA/DMF, cleavage: 95% TFA/2.5% triisopropyl-silane/2.5% water.

unprotected (solution or solid phase post-assembly phosphorylation) or by using preformed phosphorylated amino acids (building block approach). However, for the preparation of short-to medium-sized monophosphorylated peptides the second is the commonly preferred method if the peptide of interest contains amino acids that may cause side reactions (Trp, Met) during the oxidation step of P^{III} phosphorylation techniques, or if steric constraints, for example, due to secondary structure formation, hamper effective phosphorylation [21–23].

The synthetic strategy used herein was based on the building block approach using commercially available monobenzyl-protected or phosphate-unprotected Fmoc-phosphotyrosine. It has been reported that the use of unprotected Fmoc-phosphotyrosine gave better results with respect to yield and purity than the monobenzyl-protected building block in the preparation



Scheme 2 Reagents and conditions; strategy A: (i) 20% piperidine/DMF, Fmoc-Asp(ODmab)/TBTU/HOBt/DIEA/DMF; (ii) 2% hydrazine/DMF (v/v), 5% DIEA/DMF (v/v); (iii) PyBOP/HOBt/DIEA/DMF, H-Gly-OAll; (iv) 20% piperidine/DMF, Fmoc-aa/PyBOP/HOBt/DIEA/DMF; (v) 20% piperidine/DMF, Fmoc-Tyr[PO(OBzl)OH]-OH/TBTU/HOBt/DIEA/DMF; (vi) 20% piperidine/DMF, Fmoc-aa/TBTU/HOBt/DIEA/DMF, alternately; (vii) 20% piperidine/DMF, $(Boc)_2O$/DIEA/DMF; (ix) $Pd(PPh_3)_4$, DMF/THF/0.5N HCl/morpholine (2 : 2 : 1 : 0.9), PyBOP/DIEA/DMF; (x) cleavage: 95% TFA/2.5% triisopropyl silane/2.5% water; strategy B (steps differing from strategy A): (i) 20% piperidine/DMF, Fmoc-Asp(Gly-OAll)-OH/PyBOP/HOBt/DIEA/DMF; (viii) 20% piperidine/DMF, Fmoc-Ado/TBTU/HOBt/DIEA/DMF, 20% piperidine/DMF, biotin/PyBOP/DIEA/DMF.

of the mono- and bisphosphorylated forms of H-Glu-Asp-Tyr-Glu-Tyr-Thr-Ala-Arg-Phe-NH_2 [24]. However, the use of Fmoc-Tyr[PO(OBzl)OH]-OH is generally preferred for multiply phosphorylated peptides [21]. Both building blocks were tested for the synthesis of the parent linear Ros sequences, and no differences were found in yield and purity of the obtained crude product. However, the cleavage of the benzyl-protecting group which is performed simultaneously with the removal of the peptide from the resin was not complete after a 3 h cleavage time, which had to be prolonged to at least 5–6 h for completion. Therefore the unprotected Fmoc-phosphotyrosine was used for the preparation of the linear phosphopeptides.

Beside the evaluation of different protecting groups on the phosphate moiety, coupling methods also were intensively investigated for the differentially protected Fmoc-phosphotyrosine building blocks. Commonly used coupling reagents such as DCI, BOP, HBTU and HATU were tested and revealed that HBTU/HOBt or similar combinations of so-called uronium-based coupling reagents were the most efficient for incorporation of phosphorylated amino acids [25–27]. As a result of these investigations optimized procedures for the introduction of these building blocks in solid phase synthesis are available and led to the synthesis of the linear N-SH2 ligands according to Scheme 1. However, for the cyclic peptides additional synthetic steps were considered, i.e. removal of protecting groups from the moieties involved in cyclization, and the cyclization reaction itself which had to be carried out in the presence of a phosphorylated tyrosine residue.

Candidates suitable for binding to the SHP-1 N-SH2 domain were also selected computationally [13]. The positions pY-1 and pY + 2 that are not relevant for high-affinity binding to the N-SH2 domain were used to achieve ring closure by introducing Lys in pY-1 and Asp/Glu in pY + 2. A ring size of 12 to 13 atoms was predicted to be essential with respect to binding to the protein domain. According to molecular modelling, a smaller ring size leads to an increased ring strain forcing the peptide backbone into a turn-like structure around residues pY and pY + 1. This backbone structure is not in agreement with the extended conformation of the SH2 complexed natural ligand (Figure 1). Therefore, the introduction of a glycine residue in the bridging unit was necessary to maintain and stabilize the favourable binding conformation (peptides **13** and **14**). Peptide **15**, which was predicted to be unsuitable as an N-SH2 ligand, was introduced in the study to check the molecular modelling approach [13].

Most cyclic phosphopeptides hitherto described are head-to-tail [28–30] or thioether-bridged [31,32] structures. In contrast, the cyclic peptides reported herein are side-chain cyclic phosphopeptides. To achieve this, a third protecting group had to be introduced for the side-chain moieties to be connected during the cyclization reaction. Furthermore, with respect to the cyclic structures different synthetic strategies seemed applicable. Due to the usefulness of allyl type protecting groups for the preparation of side-chain and backbone cyclic peptides [33] it was decided to use this type of protection for the moieties to be cyclized, i.e. Lys(Alloc) and Asp(Gly-OAll) or Glu(Gly-OAll). The strategies for the incorporation of these dipeptide units are shown in Scheme 2. On the one hand, it is possible to generate such a dipeptide unit on the solid phase by coupling H-Gly-OAll to resin-bound side-chain unprotected Fmoc-Asp and Fmoc-Glu, respectively (Scheme 2, strategy A). Different coupling reagents, such as HBTU and PyBOP have been tested to achieve the condensation, but the yield was lower than 20%. In the second procedure (Scheme 2,

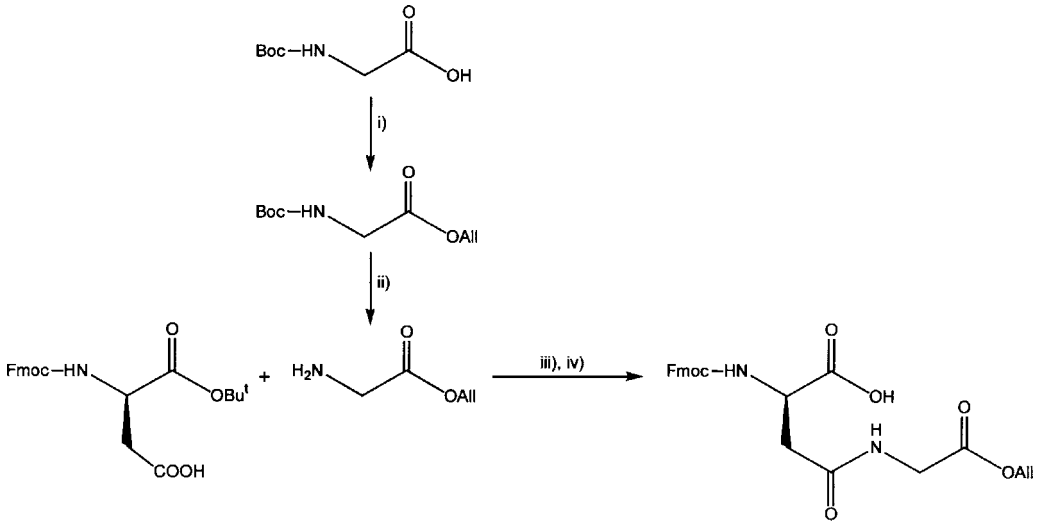


Figure 2 Synthetic route to dipeptide building block Fmoc-Xxx(Gly-OAll)-OH (Xxx = Asp, Glu). Reagents; (i) allyl alcohol/DCC; (ii) 50% TFA/DCM; (iii) isobutyl chloroformate/NMM/THF; (iv) 50% TFA/DCM.

strategy B), a preformed dipeptide unit was employed that was prepared in solution prior to solid phase synthesis (Figure 2). Due to the fact that the coupling of H-Gly-OAll with Fmoc-Asp-OBu^t or Fmoc-Glu-OBu^t can be performed in high yield and purity according to the mixed anhydride method [15,17], whereas strategy A leads to a high amount of incomplete sequence, it was decided to incorporate these dipeptide building units in all the cyclic peptides synthesized in this study. Beside the introduction of the Fmoc-Xxx(Gly-OAll) (Xxx = Asp, Glu) building block, it is also possible to generate the cyclic structures by incorporation of Fmoc-Lys(Gly-Alloc) or by coupling of Alloc-Gly-OH onto resin-bound Fmoc-Lys containing peptide. In general, the introduction of dipeptide building blocks [14,15,33] was preferred rather than the solid phase assembly of such dipeptide structures. Thus, due to the fact that the number of synthetic steps for the preparation of Fmoc-Asp(Gly-OAll) and Fmoc-Lys(Gly-Alloc) is identical and due to the successful and easy accessibility of Fmoc-Asp(Gly-OAll) alternative synthetic pathways were not explored further.

The use of phosphate-protected tyrosine building blocks, especially Fmoc-Tyr[PO(OBzl)OH]-OH is recommended for the preparation of cyclic phosphopeptides [28–32]. Therefore it was used for the synthesis of our cyclic N-SH2 domain ligands. In contrast to the linear peptides, where TBTU was used throughout the peptide assembly, for the cyclic peptides different reagents were used to achieve complete couplings. From our experience with the condensation of dipeptide building units onto resin-bound amino acids it was known that successful couplings can be performed with PyBOP [14,15,33]. Also, the condensation of the adjacent Met to polymer-bound Asp(Gly-OAll) or Glu(Gly-OAll) was carried out using PyBOP, since couplings with HBTU or TBTU were incomplete. All other couplings could efficiently be done with HBTU or TBTU, each in combination with HOBt. After completion of the linear sequence the resin was split in order to obtain *N*-terminally free and biotinylated cyclic peptides as necessary for the enzymatic assay and the binding study, respectively [13]. Cyclization of the Boc-protected or biotinylated peptides was achieved using PyBOP following Alloc/OAll-deprotection as described in the literature [33]. It was realized that neither the Alloc/OAll-deprotection nor the cyclization reaction affected the quality of the phosphotyrosine containing

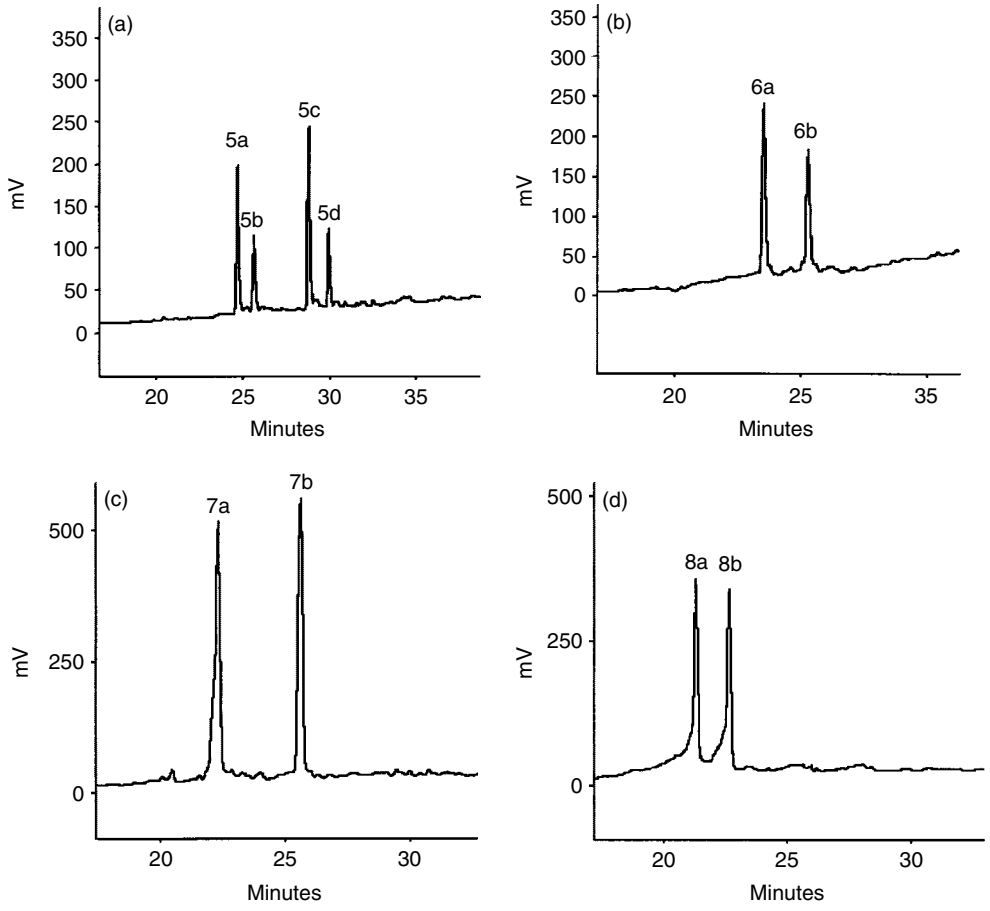


Figure 3 Analytical RP-HPLC-elution pattern of crude peptides **5**–**8** containing D/L-*erythro*/*threo*-Abu(β-Ph) and D/L-*threo*-Ser(β-Ph), respectively, indicating different diastereomers of (a) peptide **5** (Abu(β-Ph)6), (b) peptide **6** (Abu(β-Ph)8), (c) peptide **7** (Ser(β-Ph)6), and (d) peptide **8** (Ser(β-Ph)8).

peptides. However, the cyclization was not completed after a 2–3 h reaction time and therefore had to be repeated to convert completely the linear precursor. All together, such side-chain cyclized phosphopeptides can be efficiently prepared according to the pathway shown in Scheme 2.

Racemization Study

All amino acids were introduced as L-configured residues with the exception of Abu(βPh) und Ser(βPh). The commercially available amino acid Abu(βPh) was incorporated into peptides **5** and **6** as a racemic D/L-mixture containing ~67% of the *threo*- and ~33% of the *erythro*-form. The presence of the two chiral centers allows four possible diastereomers. RP-HPLC-analysis of the crude peptide **5** showed that four different isomers were produced (Figure 3a). We were able to separate each peak by semipreparative RP-HPLC, thus, biological evaluation of these isomers could be performed with the purified fractions a–d [13]. In the case of peptide **6**, only two peaks were obtained instead of four as expected for the different diastereomers (Figure 3b). In peptides **7** and **8**, Ser(βPh) was introduced as racemic D/L-*threo*-form [13] which was prepared as described in the literature [34]. For both peptides two peaks were found in the crude product (Figure 3c,d). For the purpose of structure elucidation pre-column derivatization was used with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDNP-L-Ala-NH_2), known as Marfey's reagent [18]. The D- and L-configuration of all proteinogenic amino acids occurring in the sequences of peptides **5**–**8** were derivatized and analysed with RP-HPLC.

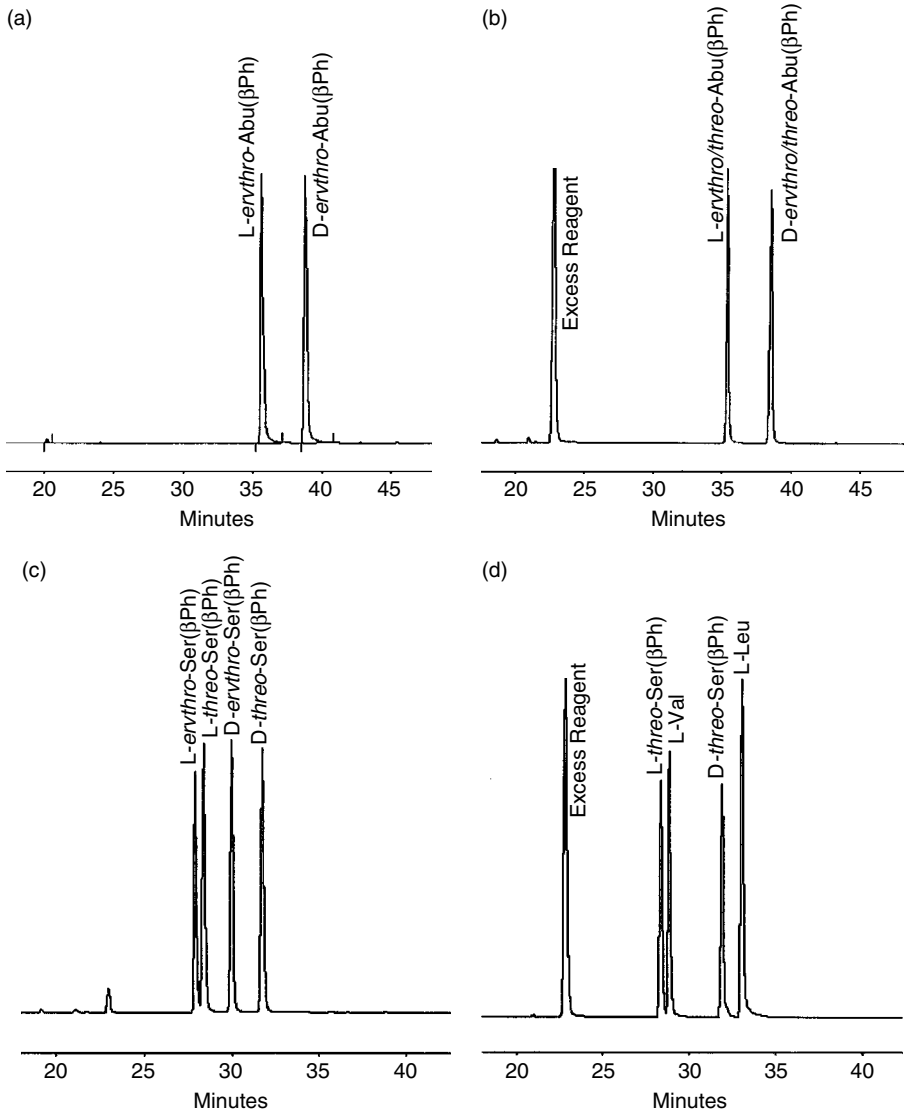


Figure 4 Separation of diastereomers obtained by reaction of Marfey's reagent with the D/L-amino acids. (a) D/L-*erythro*-Abu(βPh), (b) D/L-*erythro*/*threo*-Abu(βPh), (c) D/L-*erythro*-Ser(βPh), and (d) D/L-*threo*-Ser(βPh). Conditions: Vydac 218TP column, elution gradient: 10%–70% B in 60 min (A: 0.1% TFA/water, B: 0.1% TFA/acetonitrile), flow rate 1.0 ml/min, detection: 340 nm.

The D-form eluted later than the L-form, in agreement with the literature [19,20]. To determine the identity of Abu(βPh) both commercially available D/L-*erythro*-Abu(βPh) and D/L-*threo*/*erythro*-Abu(βPh) were derivatized. The chromatographic analysis revealed that only the D- and L-configuration, but not the *threo*- and *erythro*-form can be distinguished (Figure 4a–b). However, due to the fact that the mixture contained ~67% of *threo*- and ~33% of *erythro*-Abu(βPh) the peaks found for peptide **5** were assigned as shown in Table 2, whereas for peptide **6** the peaks turned out to represent mixtures of L-*threo*/*erythro*- and D-*threo*/*erythro*-Abu(βPh) (Table 2). Stereochemistry for Ser(βPh) in peptides **7** and **8** could be easily determined, since all four diastereomers can be separated using derivatization with Marfey's reagent (Figure 4c, Table 2). However, since peptides **7** and **8** only contained *threo*-Ser(βPh) the possibility was examined whether the natural amino acids occurring in the sequences can be separated from *threo*-Ser(βPh). In Figure 4d it can be seen that the amino acids Val and Leu can be distinguished from D- and L-*threo*-Ser(βPh) using pre-column derivatization with FDNP-L-Ala-NH_2. All fractions of peptides **5**–**8** were hydrolysed, derivatized and analysed by RP-HPLC as exemplified for peptide **5a** in Figure 5.

CONCLUSIONS

Linear and cyclic phosphopeptide ligands of the *N*-terminal SH2 domain of SHP-1 preselected by a molecular modeling approach were synthesized on the solid phase using Fmoc-Tyr(PO_3H_2)-OH and Fmoc-Tyr[PO(OBzl)OH]-OH building blocks, respectively. To achieve side chain-to-side chain on-resin cyclization suitably protected amino acid residues (Lys, Asp/Glu) had to be assembled in the linear precursor, i.e. three levels of orthogonal protection were necessary. Furthermore, the designed cyclic peptides required the introduction of a glycine residue in the bridging unit. With respect to yield and purity we recommend the use of preformed dipeptide building blocks, in this case Fmoc-Xxx(Gly-OAll)-OH (Xxx = Asp, Glu), for the assembly of such cyclic phosphopeptides. The allyl type protecting group has proven useful for the moieties to be connected during cyclization reaction, and the conditions, in particular for Alloc/OAll-deprotection as well as the cyclization reaction did not affect the phosphotyrosine contained in the sequence. Also, the quality and yield of the cyclic peptides derived from the Boc-protected or the biotinylated precursor was comparable. However, as far as the cyclization efficiency

Table 2 Characterization of the Synthesized Linear and Cyclic Phosphopeptides

Peptide	Isomer	Molecular Weight[a]		HPLC[d]	R_{f1}[e]	R_{f2}[f]
		Calcd.	Found	t_R (min)		
Ros pY[2267]	—	1016.4	1016.8	20.62	0.48	0.48
1	—	1033.1	1055.9[b]	24.08	0.42	0.48
2	—	1051.1	1089.7[c]	23.02	0.34	0.34
3	—	1046.5	1070.3[b]	26.14	0.54	0.53
4	—	1064.4	1088.5[b]	24.04	0.52	0.51
5a	L-*threo*-Abu(βPh)	1046.5	1070.3[b]	24.77	0.39	0.48
5b	L-*erythro*-Abu(βPh)	1046.5	1070.1[b]	25.73	0.39	0.52
5c	D-*threo*-Abu(βPh)	1046.5	1070.4[b]	28.87	0.41	0.57
5d	D-*erythro*-Abu(βPh)	1046.5	1084.3[c]	30.03	0.40	0.59
6a	L-*threo*/*erythro*-Abu(βPh)	1064.4	1088.3[b]	23.57	0.55	0.52
6b	D-*threo*/*erythro*-Abu(βPh)	1064.4	1088.5[b]	25.33	0.54	0.52
7a	L-*threo*-Ser(βPh)	1049.1	1050.2	22.15	0.33	0.47
7b	D-*threo*-Ser(βPh)	1049.1	1050.1	25.89	0.34	0.55
8a	L-*threo*-Ser(βPh)	1066.4	1067.9	21.32	0.46	0.51
8b	D-*threo*-Ser(βPh)	1066.4	1090.2[b]	22.70	0.50	0.64
9	—	1032.5	1055.8[b]	24.58	0.36	0.56
10	—	1067.1	1068.2	25.83	0.42	0.49
11	—	1113.1	1138.1[b]	21.74	0.40	0.44
12	—	1104.5	1128.6[b]	27.52	0.35	0.59
13	—	1086.2	1109.5[b]	18.8	0.45	0.57
14	—	1100.2	1101.4	19.4	0.47	0.59
15	—	1043.1	1066.3[b]	18.3	0.50	0.65

[a] $[M + H]^+$; [b] $[M + Na]^+$; [c] $[M + K]^+$; [d] conditions: 10%–60% eluent B in 50 min (A: 0.1% TFA/water, B: 0.1% TFA/acetonitrile), flow rate 1.0 ml/min, detection: 220 nm. [e] system 1: *n*-butanol/acetic acid/water 48 : 18 : 24; [f] system 2: pyridine/ethyl acetate/acetic acid/water 5 : 5 : 1:3.

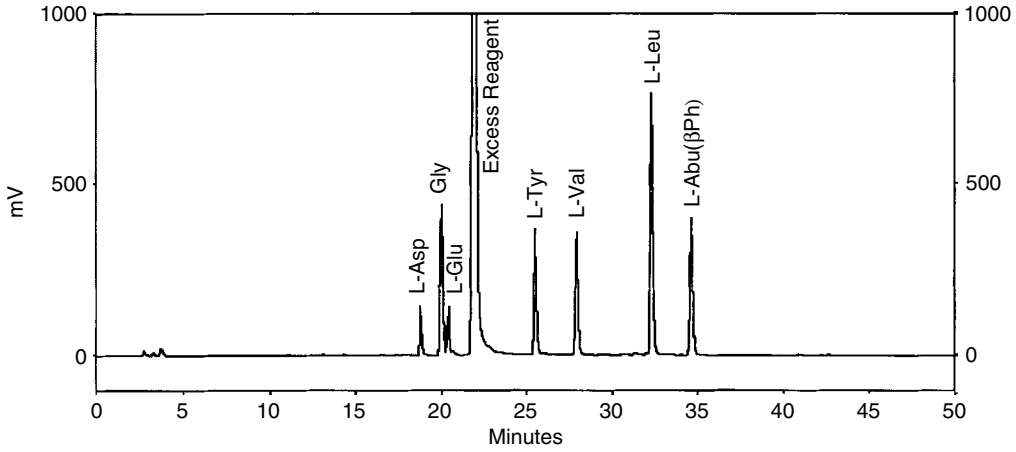


Figure 5 RP-HPLC chromatogram of the FDNP-derivatized hydrolysis product of peptide **5a**.

is concerned double coupling protocol is recommended resulting in a higher yield of the corresponding cyclic peptide.

The amino acids *erythro*/*threo*-Abu(βPh) and *threo*-Ser(βPh) were introduced in some linear sequences as structurally related analogues of Phe. Both residues were used as racemic D/L-mixtures leading to the formation of different isomers in the crude peptides. Using pre-column derivatization with FDNP-L-Ala-NH_2, the diastereomers of the amino acids were separable chromatographically. The stereochemistry of these residues in each peptide fraction was determined by RP-HPLC-analysis of the corresponding derivatized hydrolysis product.

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