Synthetic Strategies to a Backbone-Side Chain Cyclic SHP-1 N-SH2 Ligand Containing N-Functionalized Alkyl Phosphotyrosine†

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Abstract: The cyclic peptide EGLNcΨ[CON($(CH_2)_3$NH)pYNleE(NHCH$_2$CO)]L-NH$_2$ (**1**) was designed and synthesized according to a native interaction partner of tyrosine phosphatase SHP-1. We introduced N-aminopropyl-phosphotyrosine to enable backbone-side chain cyclization with a glutamic acid derivative as counterpart for cyclization. Different approaches have been compared to find a strategy for the generation of backbone and backbone-side chain cyclic phosphopeptides.

Keywords: N-functionalized alkyl phosphotyrosine; backbone cyclization; SH2 domain, ligand; protein tyrosine phosphatase; SHP-1.

INTRODUCTION

Protein-protein interactions play a critical role in the regulation of signal transduction events during various stages of cell development [1]. Such interactions generally involve modular adaptor domains that function as docking or scaffolding components in proteins [2]. Phosphorylated tyrosine regions in proteins often exhibit potential binding affinity for other proteins containing the highly conserved Src homology 2 (SH2) domains. Binding studies for SH2 domains using phosphotyrosyl peptides with randomized sequences suggested that beside the primary interaction with the phosphotyrosyl (pTyr, pY) residue specificity is dictated by the surrounding peptide sequence [3,4]. In our previous work we have demonstrated that conformationally restricted linear and cyclic peptides represent high affinity binding partners of the N-terminal SH2 domain of SHP-1, a cytosolic protein tyrosine phosphatase (PTP) that is primarily involved in the regulation of hematopoietic cell signaling [5-7]. Our laboratory has synthesized derivatives of the sequence EGL-NpY^{2267}MVL derived from the epithelial receptor tyrosine kinase Ros which represents a high-affinity binding partner of SHP-1 [8]. Some of the synthetic variants showed more potent binding to SHP-1 N-SH2 than the native lead peptide, and in addition, cyclic peptides were found to partially inhibit SHP-1 phosphatase activity [5]. Within our series of N-SH2 peptide ligands we also observed that in general the cyclic peptides retained significant binding affinity relative to their linear counterparts. The fact that restriction of bioactive conformations through cyclization leads to an enhancement of binding affinity was also observed in other studies where cyclic peptides and, in particular, cyclic phosphopeptides were developed as potent inhibitors of a distinct binding event [9-11].

Considering the particular situation for ligand binding to the N-SH2 domain of SHP-1 our previously synthesized peptides were cyclized via side chains or modified side chains between positions -1 and +2 relative to the pY-residue in order not to interfere with protein-ligand contacts involving residues that determine high-affinity binding. Molecular modeling studies revealed that a shift of the cycle towards the C-terminus, in particular to the phosphotyrosine residue, may turn out advantageous with respect to binding affinity and at the same time may hamper the dissociation process of the N-SH2-PTP-complex that is responsible for the stimulation of the phosphatase activity. A predicted N-backbone-to-side-chain cyclic ligand is shown in Fig. (**1**). Due to the fact that we are interested in the development of inhibitors of PTP SHP-1 we first focused our work on the evaluation of synthetic strategies for the generation of such a cyclic phosphopeptide. Compared to the commonly used approach of introducing suitably protected phosphoamino acid building blocks into a peptide chain by standard solid phase methods we were faced with the incorporation of an N-functionalized alkyl phosphotyrosine residue.

In general, phosphorylation of the hydroxyl amino acids Ser, Thr and Tyr within a peptide sequence can be achieved by either postassembly phosphorylation ("global phosphorylation") or by insertion of a preformed phosphorylated amino acid derivative [12]. In our peptide, postassembly phosphorylation circumvents the preparation of the phosphotyrosine building block, but steric constraints, especially due to secondary structure formation, may hamper effective phos-

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phorylation [13]. However, the most effective and versatile phosphorylation procedure for this purpose described is based on P(III) chemistry converting the hydroxyl amino acid into phosphono derivative by treatment with either aryl- or alkyl phosphochloridite or *N*,*N*-dialkyl phosphoramidite [14-16]. Both methods require subsequent *in situ* oxidation of the phosphite triester intermediate into the phosphate triester. Global phosphorylation has been intensively studied and successfully used for the preparation of short- and medium-sized peptides. Still, the use of properly protected phosphorylated amino acids in general SPPS is certainly the preferred method, in particular if the peptide to be synthesized contains amino acids giving rise to side reactions during phosphorylation, e.g. Trp and Met in the oxidation step of the desired phosphorylation procedure [17]. However, the preparation and use of phosphorylated N-functionalized alkyl hydroxyl amino acids for the generation of backbone cyclic phosphopeptide ligands has not been reported yet. Herein, we describe the application of different synthetic approaches to generate the backbone-side chain cyclic peptide EGLNcΨ[CON($(CH_2)_3$NH)pYNleE(NHCH$_2$CO)]L-NH$_2$ (**1**) containing an N-aminopropyl-phosphotyrosine by using both a building block as well as the global phosphorylation approach (Fig. **1**). In compound (**1**), representing a progenitor for further N-SH2 ligands, Nle was used as an isostere for Met as described earlier, while Met was used in the model peptide LNYMVL (**2**) derived from the natural lead peptide Ros pY2267 (Fig. **1A**) [5-7, 18]. The model peptide was primarily used in order to evaluate different strategies for global phosphorylation and oxidation due to the occurrence of a Met residue within the native sequence, whereas the preparation and phosphorylation of the N-functionalized alkyl tyrosine and its use was investigated in parallel.

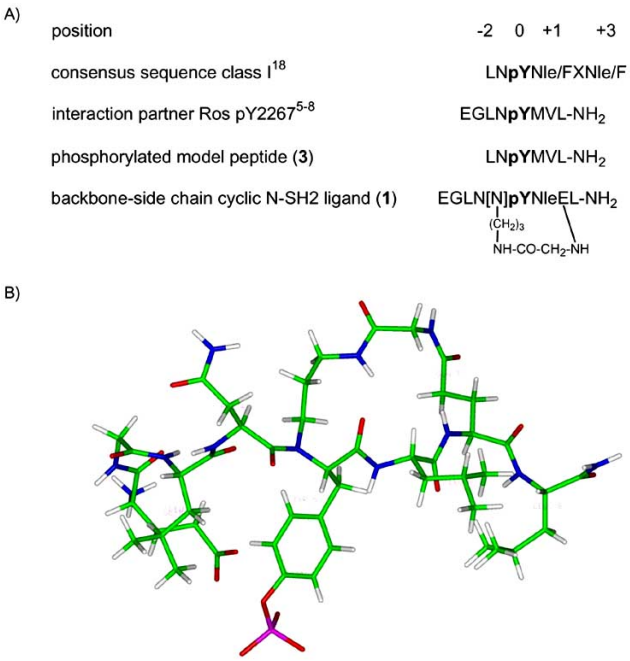


Figure 1. A) The backbone-side chain cyclic peptide EGLNcΨ[CON($(CH_2)_3$NH)pYNleE(NHCH$_2$CO)]L-NH$_2$ (**1**) was derived from consensus sequence class I for SHP-1 N-SH2 ligands as well as a natural high affinity binding partner, Ros pY2267. **B)** Stick illustration of a representative conformation of backbone-side chain cyclic peptide (**1**) as ligand for the N-SH2 domain of SHP-1.

MATERIALS AND METHODS

General

All amino acid derivatives and coupling reagents were purchased from Novabiochem (Merck KGaA, Darmstadt, Germany). Dibenzyl-*N*,*N*-diethylphosphoramidite, di-*tert*-butyl-*N*,*N*-diethylphosphoramidite and tetrakis(triphenylphosphine)palladium(0) were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Peptide synthesis reagents (DIEA, piperidine) and solvents (DMF, DCM) were of reagent grade from Fluka (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and Iris Biotech GmbH (Marktredwitz, Germany), respectively. Solvents for chromatography were of analytical grade obtained from VWR International (Dresden, Germany). Purifications of dipeptide building units by column chromatography were performed on Merck silica gel 60 (0.040-0.063 mm). NMR experiments were recorded at 30°C on a Bruker 400 MHz NMR spectrometer.

Building Block Synthesis

Fmoc-N[($CH_2)_3$NHAlloc]Tyr-OH

The generation of HN[($CH_2)_3$NHAlloc]Tyr-OH and subsequent N-terminal Fmoc-protection was performed according to earlier reports [19, 20]. NMR δ (C^{13}) ($CDCl_3$) 47.40 (Tyr Cβ), 65.57 (Tyr Cα), 73.3 (Fmoc CH_2), 115.4/117.78/119.9 (Tyr Ar C, Allyl CH_2), 127.25/127.78/130.1 (Fmoc Ar C, Allyl CH), 141.9/143.67 (Fmoc Ar C), 154.58 (Tyr Ar C4). MS m/z 545.0 $(M+H)^+$; HPLC t_R 30.7 min. R_f 0.89 (*n*-butanol/acetic acid/water 4:1:1). R_f 0.64 (chloroform/methanol 9:1).

Fmoc-N[($CH_2)_3$NHAlloc]Tyr($PO_3Bu^t_2$)-OH

The phosphorylation of the Fmoc-N-aminopropyl-tyrosine was modified from the literature [21]. In brief, to a solution of Fmoc-N[($CH_2)_3$NHAlloc]Tyr-OH (0.5 g, 0.92 mmol) in dry THF was added DIEA (0.16 ml, 0.92 mmol) followed by TBDMS-Cl (0.17 g, 1.10 mmol). The solution was stirred at room temperature for 4 h. To this solution 1*H*-tetrazole (0.19 g, 2.76 mmol) and di-*tert*-butyl-*N*,*N*-diethylphosphoramidite (0.28 g, 1.10 mmol) in dry THF was added. The mixture was stirred at room temperature for 3 h, then *t*-BuOOH (0.50 ml, 3 M solution in toluene) in 3 ml dichloromethane was added. After 20 min, the solvents were removed, and the residue was dissolved in EtOAc (150 ml) and washed with 5% $KHSO_4$ (3-times) and NaCl (3-times), then dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography with chloroform/methanol to afford Fmoc-N[($CH_2)_3$NHAlloc]Tyr($PO_3Bu^t_2$)-OH (0.48 g, 85%) as a white powder. NMR δ (C^{13}) ($CDCl_3$) 29.67 (PBu^t CH_3), 47.35 (Tyr Cβ), 65.57 (Tyr Cα), 76.48/76.98/77.49/78.67 (Fmoc CH_2, PBu^t C), 115.37/117.77/119.91 (Tyr Ar C, Allyl CH_2), 127.20/127.74/130.21 (Fmoc Ar C, Allyl CH), 141.6/143.67 (Fmoc Ar C), 154.58 (Tyr Ar C4). MS m/z 737.02 $(M+H)^+$; HPLC t_R 44.37 min. R_f 0.54 (chloroform/methanol 9:1). After cleavage of the *tert*-butyl protection of the phosphate group using 50% TFA/dichloromethane Fmoc-N[($CH_2)_3$NHAlloc]Tyr(PO_3H_2)-OH was ob-

tained and lyophilized from 80% *tert*-butanol to yield a white solid compound (0.39 g, 95%). MS m/z 647.1 $(M+Na)^+$; HPLC t_R 21.9 min; R_f 0.19 (chloroform/methanol 9:1).

Peptide Syntheses

All peptides were prepared manually by the solid-phase method using Fmoc chemistry on Rink amide MBHA resin (0.54 mmol g^{-1}). In general, coupling reactions were performed using Fmoc amino acids (4 eq.) activated by HBTU (4 eq.) in the presence of DIEA (8 eq.) for 0.5 – 1 h (double couplings). Selected couplings were carried out with TFFH (2 eq.) as the coupling reagent in the presence of Fmoc-amino acid (2 eq.) and DIEA (4 eq.), preactivation time of this mixture was 12 min, coupling was performed for 30 min (double couplings). Fmoc removal was effected by treating the resin twice with 20% piperidine in DMF (1x 5 min and 1x 15 min). The side chains of trifunctional amino acids were protected as follows: Asn(Trt) and Glu(OBu^t). Protecting groups for the side chains to be cyclized were Alloc- or -OAll as appropriate. All deprotection and coupling steps were followed by intensive washings using DMF and DCM, alternately. Capping reactions, if necessary, were performed using acetic anhydride/N-methylimidazole/DMF (1:2:3) for 30 min. Alloc/OAll-groups were removed with a mixture of DMF/THF/0.5N HCl/morpholine (2:2:1:0.9) and approximately 10 mg of $Pd(PPh_3)_4$ as the catalyst. Alloc/OAll deprotection was followed by washing with DMF/THF (1:1, 3x) and DMF (3x). Cyclization was achieved using PyBOP (6 eq.) and DIEA (12 eq.) in DMF for at least 3 h. Cleavage of peptides from the resin with concomitant side-chain deprotection was achieved by treating the resins with TFA/water/TIS (95:2.5:2.5) for 3 h. The crude peptide was precipitated in diethyl ether, centrifuged and washed three times with diethyl ether. After lyophilization peptides were purified by either semipreparative or analytical HPLC.

Peptide Phosphorylations

The phosphorylation of the peptides was performed on the solid phase as well as in solution. In case of the phosphorylation in solution the linear peptide (46 mg, 0.06 mmol) and the phosphorylation reagent di-*tert*-butyl-*N,N*-diethylphosphoramidite (20 µl, 0.07 mmol) were dissolved in 1 ml THF and 1*H*-tetrazole (15.1 mg, 0.216 mmol) was added. After 15 min (20°C) the solution was cooled down to -40°C. A mixture of *m*CPBA* (18.1 mg, 0.11 mmol) in DCM (150 µl) was added. The reaction batch was agitated for 15 min at -20 °C. Afterwards 10% $Na_2S_2O_5$ (602 µl) and diethyl ether (3.0 ml) were added. The solution was taken up in diethyl ether and washed twice with 10% $NaHCO_3$ and saturated aqueous NaCl solution, dried over Na_2SO_4 and evaporated to dryness. The product, a yellow oil was taken up in 80% *tert*-butanol and lyophilized. *In case *t*-BuOOH was used instead of *m*CPBA, then a mixture of *t*-BuOOH (25 µl, 3 M solution in toluene) in DCM (150 µl) was added. The reconversion of oxidized Met was performed according to Andrews *et al.* [22].

The phosphorylation on the solid phase was performed using 50 mg resin-bound peptide, 1*H*-tetrazole (32.5 mg, 0.46 mmol) and di-*tert*-butyl-*N,N*-diethylphosphoramidite (43 µl, 0.15 mmol) in 2 ml DCM. The mixture was taken up in a syringe and incubated and agitated for 30 min at room temperature. Afterwards the solution was removed and *m*CPBA* (39.7 mg, 0.23 mmol) in 1 ml DCM was added. After an incubation time of 15 min at room temperature, the resin was washed for three times with DCM (2 min) and lyophilized. *Instead of *m*CPBA, also a mixture of *t*-BuOOH (53 µl, 3 M solution in toluene) in DCM (320 µl) was added in selected experiments. The reconversion of oxidized Met was performed according to Andrews *et al.* [22].

Peptide Purification and Characterization

Analytical HPLC was carried out on a Shimadzu LC 10A chromatograph equipped with a UV/VIS detector (λ = 220 nm) and a Vydac 215TP C_{18} column (5 µm, 4.6 x 250 mm). The following solvent systems were used: A) 0.1% TFA in water, B) 0.1% TFA in acetonitrile. Building blocks were eluted with the gradient 20% to 80% of eluent B in 60 min at a flow rate of 1 ml/min. Peptides were eluted with the gradient 10% to 60% of eluent B in 50 min at a flow rate of 1 ml/min. Semipreparative HPLC was carried out on a Shimadzu LC 8A instrument equipped with a Knauer Eurospher 100 C_{18} column (5 µm, 25 x 250 mm) with a gradient from 15% to 65% of eluent B in 120 min at a flow rate of 10 ml/min (λ = 220 nm). Mass spectra were recorded either on a Laser Tec Research MALDI TOF mass spectrometer (Perseptive Biosystems) using α-cyano-4-hydroxycinnamic acid as matrix or a TSQ Ultra AM mass spectrometer (Finnigan). The amino acid composition of all peptides was verified by amino acid analysis using a LC 3000 system from Eppendorf-Biotronik (Hamburg, Germany). The peptides were hydrolyzed using 6N HCl in sealed tubes at 110°C for 24 h. The obtained analyses gave the expected quantitative results for all peptides.

EGLNcΨ[CON($(CH_2)_3$NH)pYNleE(NHCH$_2$CO)]L-NH_2 (**1**). MALDI-MS 1147.5 $(M+Na)^+$; HPLC t_R 33.05 min; R_f 0.54 (*n*-butanol/acetic acid/water 48:18:24). *LNYMVL-NH_2* (**2**). MALDI-MS 722.9 $(M+H)^+$; HPLC t_R 20.58 min; R_f 0.63 (*n*-butanol/acetic acid/water 48:18:24). *LNpYMVL-NH_2* (**3**). MALDI-MS 801.8 $(M+H)^+$; HPLC t_R 16.38 min; R_f 0.45 (*n*-butanol/acetic acid/water 48:18:24).

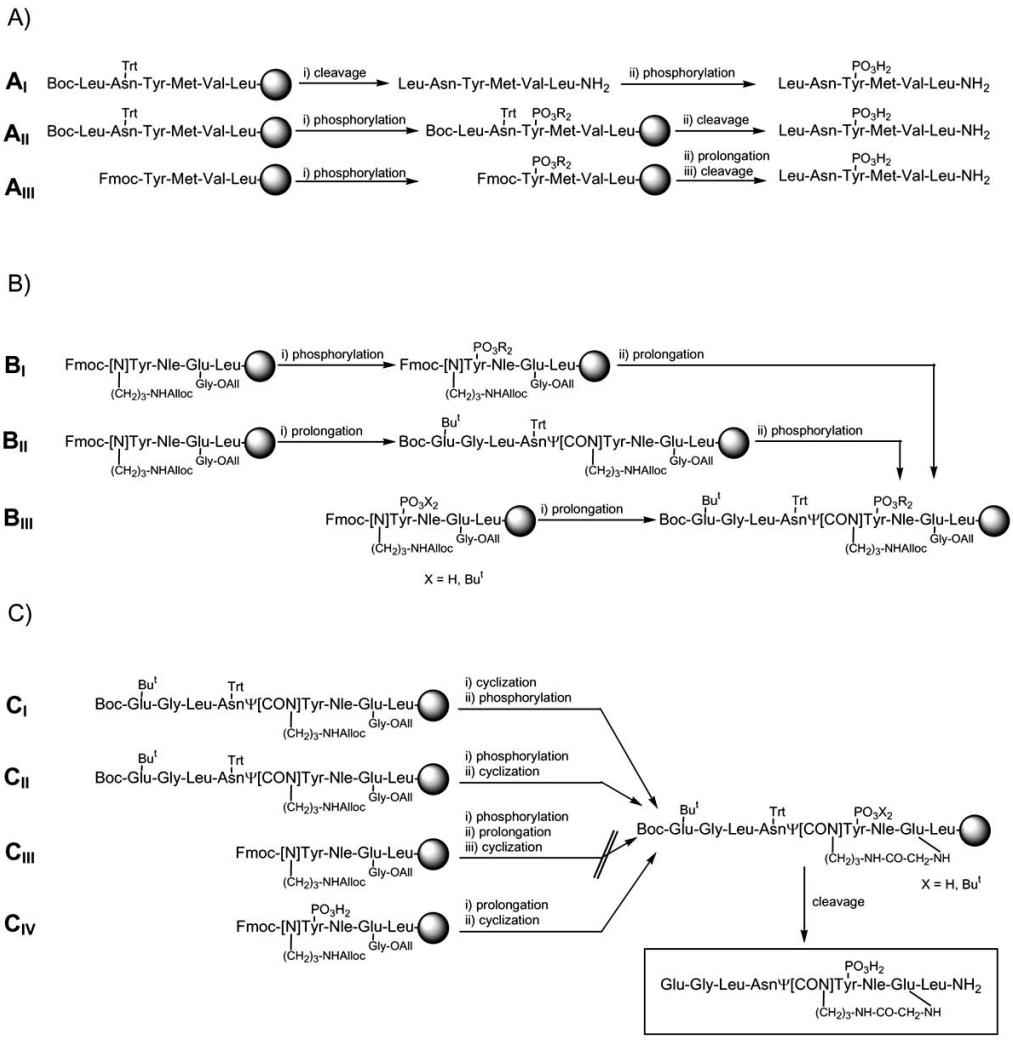
Molecular Modeling

Structural models for the complex of SHP-1 N-SH2 with phosphopeptide ligand were generated using the molecular modeling package InsightII (Accelrys Inc., San Diego, CA) based on homology to the SHP-2 N-SH2 domain in complex with a peptide (SVLpYTAVQP) for which a structure is available (pdb1aya) [23]. The molecular modeling method used has been presented in more detail earlier [5]. In order to test if the peptide cyclization is compatible with the known linear peptide binding geometry the necessary amino acid substitutions were introduced *in silico* assuming a peptide backbone structure identical to the template structure (pdb1aya). After introducing additional chemical bonds between side chains (cyclization) the resulting structural model was energy minimized in complex with the SH2-domain. The process was repeated for several different starting geometries of the cyclic structure and the lowest energy model was retained.

RESULTS AND DISCUSSION

A major consideration in our work on N-SH2 ligands for protein tyrosine phosphatase SHP-1 was the availability of different conformationally constrained peptides that allow for a more extensive biochemical examination of SH2 domain ligand interactions. In previous studies we employed constrained amino acids as well as side chain to side chain cyclization to study the topographical and conformational determinants for peptide ligands belonging to the different binding modes known for SHP-1 N-SH2 association [5-7]. These binding modes dictate whether a peptide is able to efficiently stimulate SHP-1 activity or inhibit the phosphatase due to an imperfect fit at position pY+3 [7]. Prior to the synthesis, a molecular modeling approach was used to suggest modifications with increased binding affinities compared to a natural lead peptide (Ros pY2267) by docking of the structures into the N-SH2 domain of SHP-1. In the course of these investigations it was also suggested that cyclization involving the phosphotyrosine residue might be advantageous with respect to induce a conformation possessing high binding affinity, yet reduced capacity to stimulate SHP-1. One such ligand (**1**) containing N-aminopropyl phosphotyrosine at position 0 is shown in Fig. (**1**). However, considering accessibility of this backbone-side chain cyclic peptide a variety of synthetic approaches were conceivable keeping in mind that a suitably protected N-functionalized alkyl phosphotyrosine building block was not available at the time we started with our investigations. In Scheme **1**, different strategies that can be applied in order to generate peptide (**1**) are summarized. Starting from the evaluation of several variants for the introduction of the phosphate group in the model peptide (**2**) (Scheme **1A**) and concomitant attempts to introduce the N-aminopropyl tyrosine (nonphosphorylated/phosphorylated) in the suggested backbone-side chain cyclic peptide (Scheme **1B**), the approaches finally used to synthesize peptide (**1**) are represented in Scheme **1C**.

The chemical phosphorylation itself can be either performed on the level of a suitably protected peptide having the side chain of phosphorylation still unprotected or by using properly protected phosphorylated amino acids in the



Scheme 1. Different synthetic pathways to backbone-side chain cyclic peptide (**1**). **A)** model peptide LNYMVL-NH_2 was used in order to test different global phosphorylation strategies (R = *tert*-butyl), **B)** introduction of N-functionalized alkyl tyrosine in the phosphorylated and non-phosphorylated form (R = *tert*-butyl, X = H, *tert*-butyl), and **C)** selected strategies for generation of the backbone-side chain cyclic peptide (**1**) resulting from investigations performed according to A and B (R = *tert*-butyl, X = H, *tert*-butyl).

actual synthesis [17]. It is generally accepted that the use of the preformed phosphorylated residues in solid phase synthesis is preferred towards the global phosphorylation approach. However, in which way a phosphorylated N-functionalized alkyl amino acid can be introduced in a peptide sequence has not been described yet, though recently backbone cyclic peptides containing phosphoserine residues were reported to inhibit NF-κB [24]. Nevertheless, these peptides did not contain an N-modified phosphoserine, and backbone cyclization was achieved via N-carboxyalkyl and N-aminoalkyl amino acids, e.g. derived from Asp and Gly, respectively. Using the model peptide LNYMVL derived from the lead compound Ros pY2267, three different strategies of post-assembly phosphorylation were evaluated that were extracted from literature reports in this field. While in strategy A_I phosphorylation was performed in solution after completion of peptide synthesis according to Nomizu *et al.* [25], in A_{II} and A_{III} phosphorylation was achieved on the solid phase, yet at different stages of the synthesis protocol [26-30]. Certainly, optimal phosphorylation can be achieved by using differentially protected phosphoramidites as efficient phosphorylation reagents [12,13,26-30]. For the subsequent oxidation of the tyrosinyl phosphotriester derivative (PIII to PV) several reagents were successfully used in the past, and *m*-chloroperoxybenzoic acid (*m*CPBA) turned out the most effective in this respect. However, our lead peptide contains a Met residue at position pY+1 and future backbone cyclic peptides will be more related to the natural interaction partner Ros pY2267 rather than to the consensus sequence class I containing bulky amino acids at pY+1 (Phe, Nle) due to sterical hindrance introduced by the building blocks required for cyclization. Andrews *et al.* have demonstrated that the use of *tert*-butylhydroperoxide (*t*-BuOOH) for oxidation and subsequent treatment with N-methyl mercaptoacetamide is suitable for regeneration of methionine residues, while this amino acid is irreversibly oxidized if using *m*-chloroperoxybenzoic acid [22]. Both, the resin-bound peptide (**2)** as well as the purified peptide underwent phosphorylation according to Perich *et al.* by using di-*tert*-butyl-*N,N*-diethylphosphoramidite and dibenzyl-*N,N*-diethylphosphoramidite, respectively. In all cases, independent on solution or solid phase global phosphorylation, the yields achieved in the literature [26-30], 96% for dibenzyl-*N,N*-diethylphosphoramidite and 95% for di-*tert*-butyl-*N,N*-diethylphosphoramidite, could be confirmed. In further experiments we only used the *tert*-butyl protection due to prolonged cleavage times required for benzyl-protected phosphopeptides as reported earlier [5-7]. The same was found for the oxidation step of the phosphotriester intermediate that could successfully be performed with *m*-chloroperoxybenzoic acid as well as with *t*-BuOOH, while the latter is more suitable with respect to the required reconversion of Met. However, because of the reduced number of purification steps for the peptide prepared by solid phase synthesis and the higher overall yield we finally decided to only incorporate the solid phase post-assembly phosphorylation strategy for the generation of the backbone-side chain cyclic peptide (Scheme **1C**, strategies C_I and C_{II}).

In order to demonstrate that the application of a suitably protected N-functionalized alkyl phosphotyrosine derivative is equally well or even better suitable for the preparation of the desired peptide (**1**), we performed several experiments according to the strategies shown in Scheme 1B. Thus, it was necessary to seek methods for the preparation of N-aminopropyl phosphotyrosine which would be applicable to the Fmoc-chemistry in solid phase synthesis, while the generation of nonphosphorylated N-functionalized alkyl amino acids has been reported earlier by our lab and others [19,20,24,31]. Already in 1989 Perich and Johns described a versatile one-pot synthesis of a Boc-protected dialkyl phosphotyrosine derivative using dialkyl-*N,N*-diethylphosphoramidites which was initiated by a temporary *tert*-butyldimethylsilyl protection [32]. This method has later been applied to Fmoc-protected tyrosines, too [29,33]. The effective use of DIEA as the base during phosphorylation instead of N-methyl morpholine was later demonstrated by Mathe *et al.* for the introduction of S-acyl-2-thioethyl-enzyme-labile phosphate protection [21]. This synthetic approach was used herein and has allowed us to prepare in a suitable and easy way the N-functionalized alkyl phosphotyrosine derivative as demonstrated in Fig. (**2**), while the omission of the temporary *tert*-butyldimethylsilyl protection did not yield the desired product.

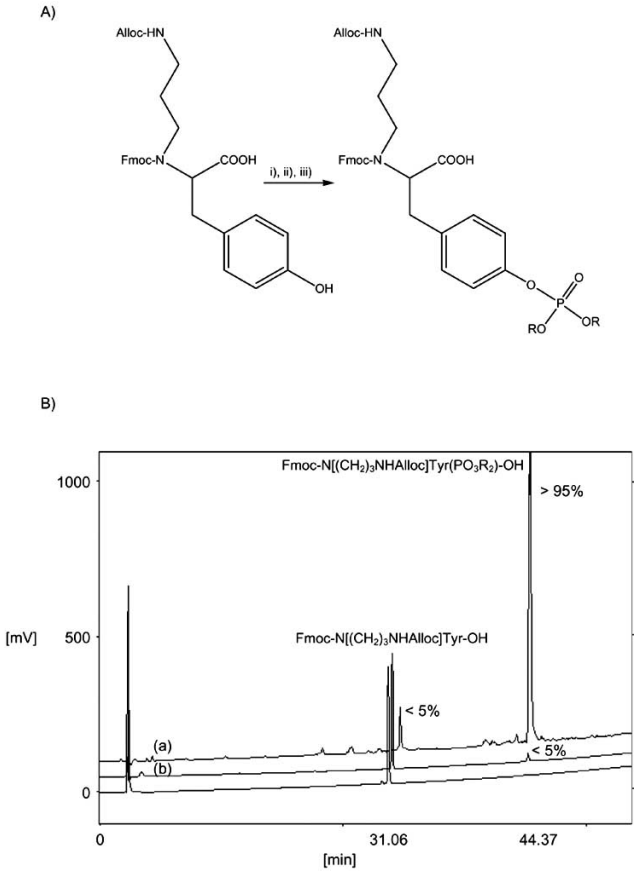


Figure 2. A) Synthetic route to Fmoc-N[$(CH_2)_3$NHAlloc]Tyr($PO_3Bu^t_2$)-OH, reagents: i) TBDMS-Cl (1.2 eq.)/DIEA/THF [19], ii) 1*H*-tetrazole (3 eq.)/$(Et)_2NP[OBu^t]_2$ (1.2 eq.) [26-30], iii) *t*-BuOOH (70%) [22]; and **B)** HPLC-analysis of the conversion of the starting compound to the phosphorylated analogue with (a) and without (b) TBDMS-Cl; R = *tert*-butyl. Gradient: 20–80% eluent B in 60 min with A: 0.1% TFA in water and B: 0.1% TFA in acetonitrile; flow rate at 1 ml/min, 220 nm.

The principles of the strategies selected according to the results obtained from the experiments in A_I-B_{III} or in combination with these experiments are demonstrated for cyclic octapeptide (**1**) in Scheme 1C. We decided to use the following approaches: C_I) solid phase assembly of peptide - cyclization - phosphorylation, C_{II}) solid phase assembly of peptide - phosphorylation - cyclization, C_{III}) solid phase assembly up to N-aminopropyl-tyrosine (position 0) - phosphorylation - prolongation - cyclization, and C_{IV}) solid phase assembly using N-aminopropyl-phosphotyrosine - cyclization. As reported earlier, introduction of N-functionalized alkyl amino acids for N-backbone cyclization is difficult in solid phase synthesis due to sterical hindrance for the subsequent acylation step. Therefore, the preparation of dipeptide building blocks has been suggested in several publications in the past [19,20,31]. However, we also found herein that if using TFFH (tetramethylfluoroformamidinium hexafluorophosphate) for the *in situ* generation of amino acid fluorides coupling onto the building block can be achieved in acceptable yields (40-50%) and time-consuming additional deprotection and purification steps for the dipeptide building block can be omitted. Fig. (**3**) demonstrates the coupling yields for each step according to the synthetic pathways C_I-C_{IV}. Though yields in strategy C_I and C_{II} are reduced after coupling of Fmoc-Asn(Trt) to N-aminopropyl-tyrosine, the complete sequence could be prepared. In contrast, introduction of the di-*tert*-butyl-protected N-aminopropyl-phosphotyrosine derivative led to sequence termination at the stage of Asn(Trt) coupling. Following consideration of the reaction, it was concluded that a possible reason for the not feasible coupling onto the building unit could be the sterical hindrance of the additional protection at the phosphate group of tyrosine. We therefore tried to introduce the deprotected N-aminopropyl-phosphotyrosine instead (C_{IV}). As demonstrated in Fig. (**3**), we were indeed able to prolongate the sequence by Asn at position pY-1 relative to phosphotyrosine and beyond. The coupling yield (43%) was only slightly reduced in comparison to the same reaction in strategies C_I and C_{II} (48%). We can therefore conclude that both, post-assembly as well as a building block approach led to the desired linear precursor peptide. Cyclization of the linear peptide has been performed as described earlier [31]. In addition, according to the results obtained for peptide (**1**) it is recommended to perform cyclization after phosphorylation of the complete linear peptide (strategies C_I and C_{IV}), as was shown to be successful for cyclic N-SH2-ligands containing a phosphotyrosine residue [5-7]. However, in contrast to previous preparations of backbone cyclic peptides it was necessary to repeat cyclization at least two times in order to increase the yields. Nevertheless, side-products, e.g. linear peptide, linear Alloc-/OAll-protected peptide and partially protected linear peptide were still identified in the crude material (Fig. **4A**). Fig. (**4B**) represents the purified peptide (**1**) obtained from the crude material. Thus, continuing work is focused on the optimization of the Alloc-/OAll-deprotection and the cyclization reaction.

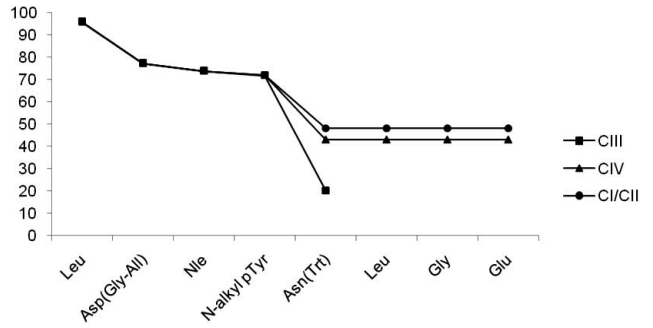


Figure 3. Monitoring of coupling yields (%) obtained for the assembly of the linear sequence of peptide **1** according to different synthetic strategies.

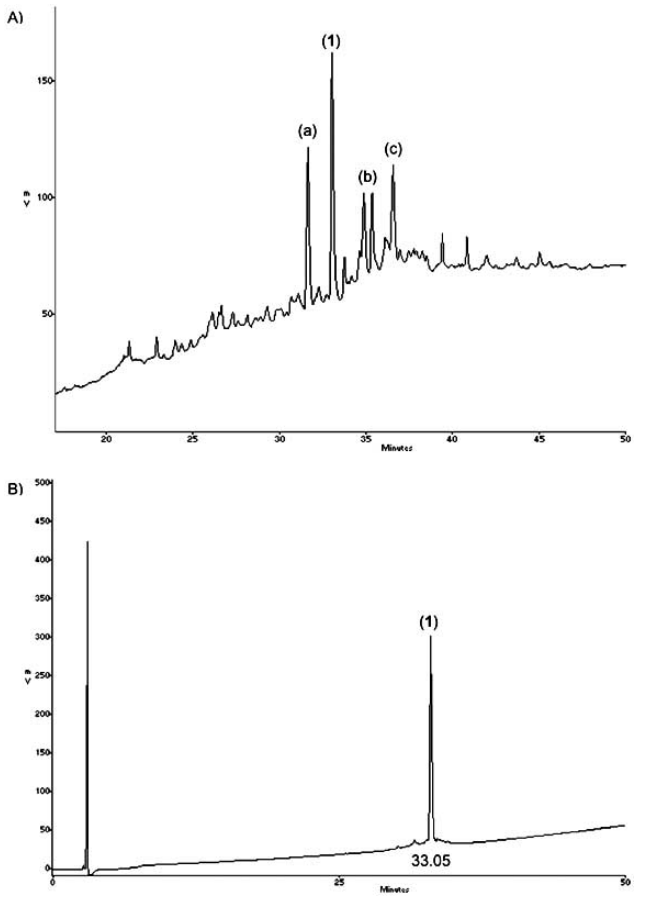


Figure 4. HPLC analysis of cyclic peptide (**1**) in the crude mixture obtained from strategy C_{IV} **(A),** and after purification **(B)**. Peak (a) represents the linear peptide, (b) the monoallyl-protected peptide, and (c) the diallyl-protected precursors. Peptides were eluted with a gradient of 10-60% in 50 min, where B was 0.1% TFA in acetonitrile.

CONCLUSIONS

In this study, the backbone-side chain cyclic peptide EGLNcΨ[CON($(CH_2)_3$NH)pYNleE(NHCH$_2$CO)]L-NH_2 has been prepared according to different synthetic ways including the post-assembly phosphorylation as well as a building block approach. The latter required the availability of Fmoc-N-aminopropyl-phosphotyrosine that was successfully obtained by phosphorylation of the nonphosphorylated precursor amino acid using dialkyl-*N,N*-diethylphosphoramidites following temporary *tert*-butyldimethylsilyl protection of the starting compound. Coupling of this building unit to the

resin-bound peptide and coupling of the subsequent amino acid (Asn) to N-aminopropyl-phosphotyrosine was demonstrated to proceed most efficiently if using TFFH as the coupling reagent. In contrast, the acylation of the phosphate-diprotected N-aminopropyl-phosphotyrosine was not at all possible for obvious sterical hindrance through the additional protecting groups. However, further work dealing with the optimization of the successful strategies, e.g. optimization of the cleavage of allyl-type protecting groups and the cyclization reaction, are currently in progress.

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ABBREVIATIONS

Standard abbreviations for amino acids, peptides, protecting groups and peptide synthesis reagents were used as recommended in the guide published in *J. Peptide Sci.*, **2006**; *12*, 1-12.

OTHER ABBREVIATIONS USED

t-BuOOH	=	*tert*-butylhydroperoxide
mCPBA	=	Meta-chloroperoxybenzoic acid
$Pd(PPh_3)_4$	=	Tetrakis(triphenylphosphine)palladium(0)
N-SH2	=	*N*-terminal Src homology 2 domain
SHP-1	=	SH2 domain protein tyrosine phosphatase-1
TBDMS-Cl	=	*tert*-butyldimethylsilyl chloride
TFFH	=	Tetramethylfluoroformamidinium hexafluorophosphate
TIS	=	Triisopropyl silane

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