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Cyclic phosphopeptides for interference with Grb2 SH2 domain signal transduction prepared by ring-closing metathesis and phosphorylation

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Cyclic phosphopeptides were prepared using ring-closing metathesis followed by phosphorylation. These cyclic phosphopeptides were designed to interact with the SH2 domain of Grb2, which is a signal transduction protein of importance as a target for antiproliferative drug development. Binding of these peptides to the Grb2 SH2 domain was evaluated by a surface plasmon resonance assay. High affinity binding to the Grb2 SH2 domain was maintained upon macrocyclization, thus indicating that this method can be used to assemble high affinity cyclic phosphopeptides that interfere with signal transduction cascades.

Introduction

Signal transduction proteins play a major role in the complex dynamic networks that regulate cell function and are intensively studied now for their potential use as drug targets. Many signal transduction proteins contain small conserved modules, which recognize short defined peptide motifs within larger polypeptides thus enabling protein–protein interactions. An example of these small conserved modules is the family of Src homology-2 (SH2) domains that recognize tyrosine-phosphorylated sequences.[1,2] Peptides and peptidomimetics that interfere with signal transduction cascades are considered as powerful tools to unravel the complex processes involved. Ultimately, they might contribute to drug development for their respective targets.[3] Important factors for molecular recognition in peptide–protein and protein–protein interactions are the conformation and the conformational flexibility of the binding epitope.[4] Furthermore, covalent modifications, such as phosphorylation, are crucial because they often serve as an 'on/off switch' in signal transduction cascades.[5] To provide compounds that interfere with signal transduction, we present a method to assemble peptides with a conformationally controlled backbone as well as covalent modifications such as a phosphate moiety.

As a target the Grb2 SH2 domain was selected, which is part of the Grb2 adapter protein. The Grb2 adapter protein consists of an SH2 domain flanked by two SH3 domains and is a key component of the Ras signaling pathway,[6] which is an important regulator of cell growth and differentiation. Therefore, it has been recognized as a potential drug target for cancer therapy.[7] A crucial event in the Ras signaling pathway is recognition of the phosphorylated tail of the EGF receptor by the Grb2 SH2 domain. The Grb2 SH2 domain recognizes the consensus sequence –pTyr–Val–Asn–Val–, which binds in a beta-turn conformation.[8] To enhance binding affinity as well as selectivity it would be advantageous to assemble cyclic ligands for this binding site.

There has been considerable interest in ring closure of peptides, because it is expected to enhance the affinity due to a more favorable entropy of binding. However, few examples of cyclic phosphopeptides interacting with the Grb2 SH2 domain are available. These include the compounds assembled by Ettmayer *et al.*[9] employing head-to-tail cyclization leading to a compound with 10 times higher affinity as compared to the linear peptide. Furthermore, there are the phosphate mimic containing macrocyclic compounds of Burke *et al.*,[10–13] which bind with high affinity to the Grb2 SH2 domain. Another approach is application of cyclopropane-derived amino acid isosteres to assemble conformationally constrained peptide mimetics as demonstrated by Plake *et al.*[14] In general, there are only few methods for the assembly of cyclic phosphopeptide constructs.

We and others have shown that ring-closing metathesis is a powerful synthetic method to prepare cyclic peptides without the need for protection of connecting side chains.[11,15–26] Extending this method to the preparation of highly functionalized peptides such as phosphopeptides could advance functional studies and might facilitate drug development for signal transduction proteins. Here we describe the application of ring-closing metathesis for the synthesis of cyclic phosphopeptides, by subsequent ring closure and phosphorylation. We have approached this in an integrated manner combining design, synthesis and interaction analysis, resulting in high affinity ligands for the Grb2 SH2 domain.

Results and discussion

Modeling

In general, cyclic peptides lose less conformational entropy upon binding as compared to linear peptides resulting in higher Gibbs free energy of binding. To achieve this, the cyclic peptide should be able to adopt the proper conformation for optimal interaction with the SH2 domain. Thus, different bridges for ring-closing the peptide –pTyr–Val–Asn–Val– were designed and evaluated employing the program Sybyl 6.8. Construction of the peptide–protein complexes was based on the crystal structure of the Grb2 SH2 domain complexed with a heptapeptide inhibitor (PDB entry code 1TZE).[8] Cyclic peptides that are ring-closed by different bridges were constructed starting from the heptapeptide of the crystal structure. The constructed cyclic peptides were energy minimized in the presence of the Grb2 SH2 domain, the geometry of which was kept fixed. The resulting conformations of the cyclic peptides were superimposed on the conformation of the linear peptide by the α-carbon atoms of pTyr, Val, Asn and Val amino acids (Fig. 1).

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The modeled conformations of compounds **9** and **10** were very similar to the conformation of the linear peptide from the crystal structure. Considering these results, the cyclic peptides should be able to adopt the proper conformation for optimal interaction with the Grb2 SH2 domain thus giving high affinity binding.

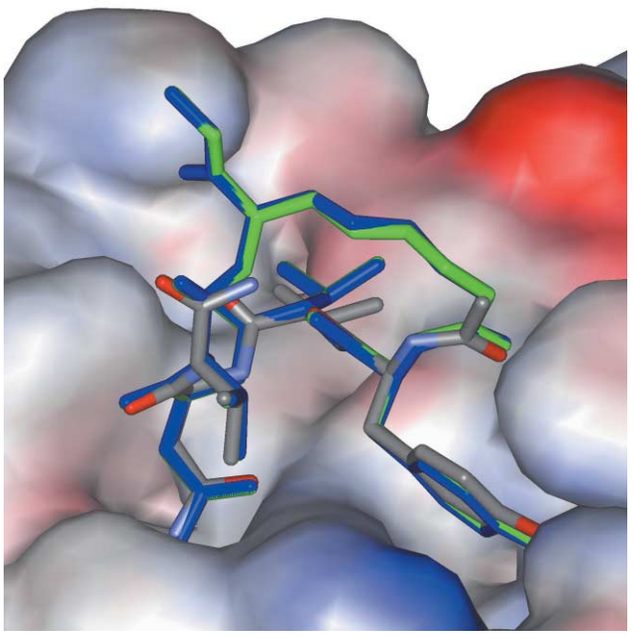
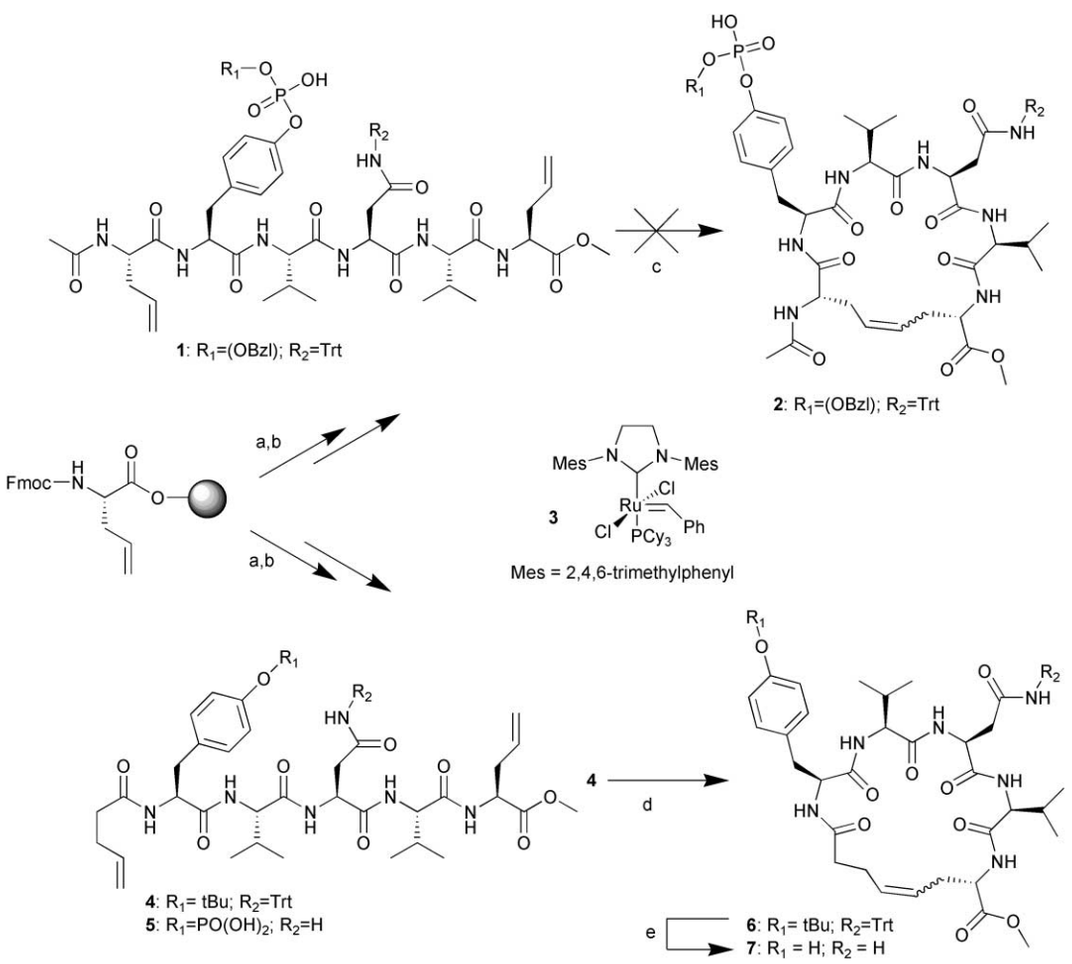


Fig. 1 Superimposition of the crystal structure conformation of –pTyr–Val–Asn–Val– (Protein Databank entry code 1TZE)[8] (coloured by atom) and the energy minimized conformations of compound **9** (green) and **10** (blue) in the Grb2 SH2 domain.

Synthesis

Ring-closing metathesis was first attempted on the protected linear peptide **1** (Scheme 1) containing a monobenzyl-protected phosphotyrosine residue. Ring closure experiments were carried out using the second-generation Grubbs catalyst **3**[27] in the presence of 2,6-dichlorotoluene in three different solvents: 1,1,2-trichloroethane (1,1,2-TCE), *N*,*N*-dimethylformamide (DMF) and an ionic liquid; 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF_6). After the ring closure experiment, the product was deprotected and analyzed by mass spectroscopy. Since the starting material **1** did not dissolve in 1,1,2-TCE, refluxing overnight did not result in conversion. In contrast, peptide **1** dissolved in DMF and disappeared completely after overnight heating (50 °C). However, subsequent workup did not indicate formation of the desired product. Although it was expected that peptide **1** would dissolve in an ionic liquid it just gave a suspension and overnight heating resulted in (unidentified) degradation products in addition to the starting material. The starting material **1** was poorly soluble in organic solvents such as ethyl acetate, methanol, dichloromethane and 1,4-dioxane, probably due to the monoprotected phosphate moiety. To obtain a better soluble starting material, it was decided to use a fully protected peptide **4** without a phosphate moiety for ring-closing metathesis, which is followed by phosphorylation resulting in the desired end products **9** and **10**.

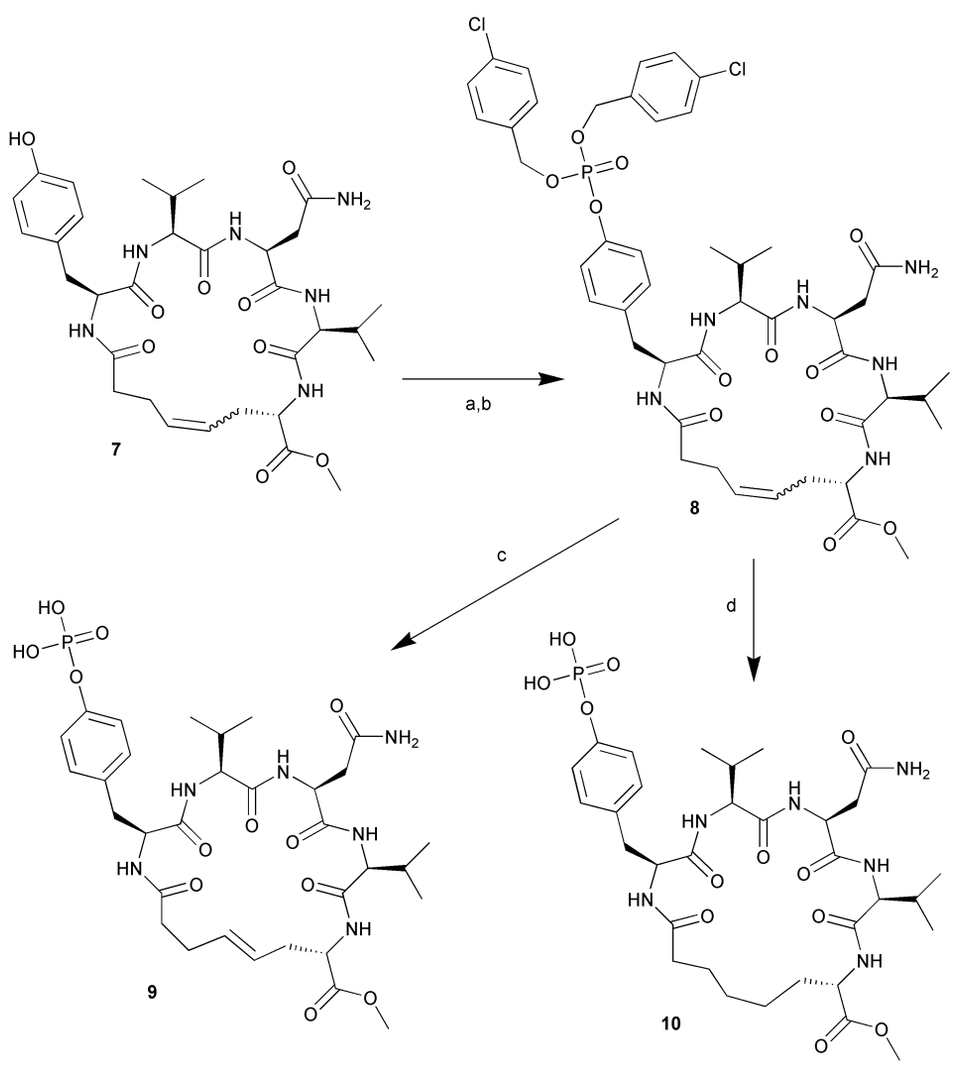


Scheme 1 (a) Peptide synthesis with subsequently Fmoc–Val–OH, Fmoc–Asn(Trt)–OH, 4-pentenoic acid and Fmoc–Tyr(tBu)–OH or Fmoc–Tyr-(PO(OBzl)OH)–OH, (b) KCN (cat.), MeOH, (c) 20 mol% second generation Grubbs catalyst **3**, 2,6-dichlorotoluene, in 1,1,2-TCE, DMF or 1-butyl-3-methylimidazolium hexafluorophosphate [bmim]PF_6, (d) 20mol% second generation Grubbs catalyst **3**, 2,6-dichlorotoluene, in 1,1,2-TCE, (e) TFA, 1,2-ethanedithiol (EDT), triisopropylsilane (TIS), H_2O, 90/2.5/2.5/5.

Thus, fully protected peptide **4** was assembled on Argogel®–OH using commercially available amino acid building blocks (Scheme 1). The peptide was cleaved from the resin by treatment with a catalytic amount of KCN in MeOH, which gave the fully protected linear peptide in high yields. This precursor **4** was soluble in 1,1,2-TCE and in this solvent subjected to ring-closing metathesis. Ring closure was monitored by thin layer chromatography (TLC), which showed separated product spots, probably the *cis* and *trans* isomer. After refluxing for 4 h, some starting material was still present and workup yielded 30% of the cyclic compounds **6**. However, refluxing overnight (16 h) increased the yield of compounds **6** to 50%. After deprotection of cyclic peptides **6** the *trans* and the *cis* isomer of compound **7** can be separated by preparative HPLC and were obtained in a ratio *trans* : *cis* of 3 : 1. The *cis* and *trans* product could be unambiguously assigned by ^{1}H 500 MHz NMR spectroscopy. At 5 °C the coupling patterns were not very well resolved, however spectra recorded at 25 °C showed the *trans* coupling patterns for compounds **7** and **9**. A double-triplet was observed at 5.56 ppm –C*H*=C*H*– with a *J*-coupling of 15.6 Hz, which was also observed for comparable compounds by others.[12,18] No clear separation of the peaks was observed for the *cis* isomer of compound **7**, due to the smaller *J*-coupling constant in case of the *cis* double bond. The NOE of the alkene hydrogens of the *cis* isomer was stronger than the NOE of the *trans* isomer, which confirmed the *cis*/*trans* assignment.

Ring-closing metathesis of peptides may be favored by the introduction of a proline residue, which allows *cis*/*trans* isomerization of the amide bond and can induce a turn in the peptide backbone.[16,26] Another strategy is reversible protection of a backbone amide nitrogen, thereby facilitating *cis*/*trans* isomerization of the amide bond.[28] A nice example combining both strategies using a pseudoproline residue is found in the cyclic peptides prepared by ring-closing metathesis of Schmiedeberg and Kessler.[21] These consist of four amino acid residues flanked by allylglycine residues, which is comparable to peptide **1**. However, it turned out that in our case these approaches were not needed, possibly because of the use of the second-generation Grubb's catalyst and/or the use of a higher reflux temperature.

Deprotected cyclic peptides **7** were phosphorylated using our earlier developed bis(4-chlorobenzyl)-*N*,*N*-diisopropylphosphoramidite reagent (Scheme 2).[29] Phosphitylation of the peptide by this reagent can be easily monitored using mass spectroscopy showing the typical isotope pattern of the chlorine atoms. After oxidation with 3-chloroperoxybenzoic acid, the phosphate moiety can be deprotected by TFA or by catalytic hydrogenation on Pd/C. TFA cleavage suffered from demethylation of the C-terminal methylester (approximately 10–20%), which lowered the yields. Only the *trans* isomer of compound **9** was obtained in a sufficiently high yield and purity (>95%) for characterization and binding studies. Demethylation by TFA cleavage can be avoided by catalytic hydrogenation on Pd/C, which proved to be a very effective method for simultaneous deprotection of the phosphate triester and reduction of the double bond resulting in compound **10**.



Scheme 2 Phosphorylation and deprotection of the phosphate moiety. (a) Bis(4-chlorobenzyl)-*N*,*N*-diisopropylphosphoramidite, 1*H*-tetrazole, CH_3CN/1,4-dioxane, (b) 3-chloroperoxybenzoic acid (mCPBA) in H_2O/CH_3CN, (c) TFA/EDT/TIS/H_2O 90/2.5/2.5/5, (d) H_2 (3–4 atm) Pd/C tBu–OH/H_2O.

Table 1 Affinity for the Grb2 SH2 domain measured by a surface plasmon resonance assay

Compound	K_d/μM
5	0.44 ± 0.04
9	0.60 ± 0.05
10	0.48 ± 0.04

Affinity for the Grb2 SH2 domain

The interaction of linear peptide **5**, cyclic peptide **9** (*trans* isomer) and reduced cyclic peptide **10** with the Grb2 SH2 domain was studied by a surface plasmon resonance assay (SPR). A competition assay was performed similar to procedures described earlier for the Lck SH2 domain.[30,31] Here, the Grb2 binding peptide Ahx–Pro–Ser–pTyr–Val–Asn–Val–Gln–Asn–NH_2 was immobilized on the sensor surface instead of an Lck binding peptide. Binding constants for the interaction in solution from SPR competition experiments are included in Table 1. The *trans* isomer of the cyclic peptide **9** is slightly less active than the linear peptide **5** whereas the reduced cyclic peptide **10** is almost equally active as the linear peptide **5**. It should be noted that the differences are relatively small (Fig. 2, Table 1).

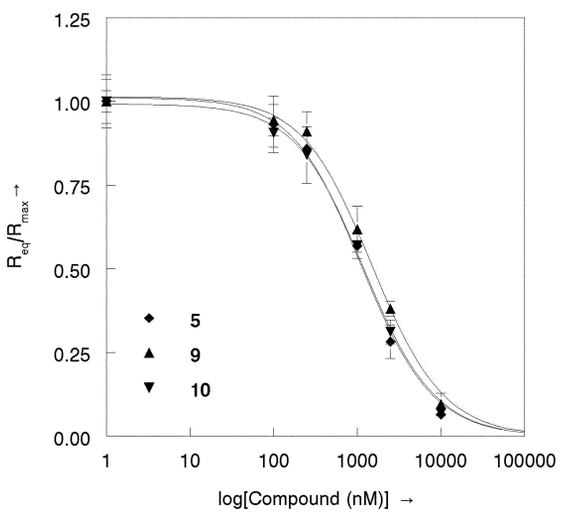


Fig. 2 Affinities of the linear compound **5** and the cyclic compounds **9** and **10** for the Grb2 SH2 domain measured in a surface plasmon resonance assay ($n = 3$).

Our modeling studies show that both compounds **9** and **10** can adopt the proper conformation for interaction, as appears from the similarity with the ligand conformation in the crystal structure (Fig. 1). Thus, we do not think that the slightly lower affinity of the cyclized compounds compared to the linear compound is due to a not fully optimal conformation for binding to the SH2 domain.

Our results are in line with reports describing that conformational constraints do not enhance affinity to SH2 domains in all cases[14,32] and that high affinity binding is due to subtle effects of the closed ring on the conformation of the cyclic peptide.[9] A possible cause for the small affinity differences between the linear peptide **5** and the cyclic peptides **9** and **10** might be enthalpy–entropy compensation, which means that favorable entropy changes ($\Delta S°$) are compensated for by unfavorable enthalpy changes ($\Delta H°$) resulting in minimal Gibbs free energy changes ($\Delta G°$) and affinity differences. This phenomenon is observed for peptides interacting with the Lck and Src SH2 domain[31,32] and many other peptide–protein interactions. The origin of enthalpy–entropy compensation is not yet well understood. There appears to emerge a common opinion that the compensation is caused by solvent reorganization,[33,34] but how this counteracts entropy gain due to rigidisation remains unclear.

Conclusion

A versatile method for the preparation of cyclic phosphopeptides for modulation of signal transduction *via* the Grb2 SH2 domain has been described. This method features ring-closing metathesis followed by phosphorylation. It is expected that this method can be applied to a variety of cyclic phosphopeptides. Although our expectation of increased affinity was not borne out in truth, the results show that covalent control of peptide conformation is a subtle process and/or that other factors may play dominant roles in the binding process.

Material and methods

General

Unless stated otherwise chemicals were obtained from commercial sources and used without previous purification, except for *N*,*N*-diisopropylethylamine (DiPEA) and triethylamine (TEA), which were distilled from ninhydrin and KOH, respectively as well as *N*,*N*-diisopropylamine which was distilled from CaH_2 and stored on CaH_2. Solvents were stored on molsieves (Merck 0.4 nm) whenever anhydrous solvents were required. Reactions were carried out at room temperature unless stated otherwise. Column chromatography was performed using Merck silica gel 60 and thin layer chromatography (TLC) was performed on Merck precoated silicagel 60 F-254 plates and detection with UV or Cl_2–TDM (4,4′-tetramethyldiamino-diphenylmethane).[35] (300 MHz) ^{1}H NMR spectra were recorded on a Varian G-300 spectrometer and are reported in chemicals shifts (ppm) relative to tetramethylsilane (TMS). (500 MHz) ^{1}H NMR spectra were recorded on a Varian Inova 500 MHz spectrometer at a concentration of 3–4 mM peptide in H_2O/D_2O (9 : 1) or D_2O with 20 mM phosphate buffer (pH = 6.5) and are reported in chemical shifts (ppm) relative to TMS. TOCSY, NOESY and ROESY experiments were performed for assignment of all protons. Electrospray ionization mass spectrometry (ESI-MS) was carried out using a Shimadzu LCMS QP-8000 mass spectrometer. High-resolution masses were measured on a Micromass Q-TOF hybrid mass spectrometer, with pentaphenylalanine as reference. Analytical HPLC was performed on a Shimadzu HPLC system with an Alltech adsorbosphere C8 (5 μm, 250 × 4.6 mm) column with UV detection at 220 and 254 nm using a gradient from 100% buffer A (15 mM triethylamine/phosphoric acid, pH = 6) to 100% buffer B (Buffer A/acetonitrile 1 : 9) in 20 minutes. Preparative HPLC was performed using an Alltech adsorbosphere C8 (10 μm, 250 × 22 mm) column with a gradient from 100% Buffer A (0.1% TFA in H_2O) to 100% buffer B (0.085% TFA in CH_3CN/H_2O 95 : 5) in 50 min and detection at 220 nm.

Synthesis

Protected linear phosphopeptide 1. Fmoc–AllylGly–OH (2.0 mmol, 675 mg) was coupled to ArgoGel®–OH (0.5 mmol, 1.04 g) according to the method of Sieber.[36] The loading was determined by measuring the UV-absorbance of the piperidin–dibenzofulvene adduct (λ_{max} = 301 nm) in a resin sample and was approximately 0.35 mmol g^{-1}. The peptide was assembled by Fmoc solid-phase peptide chemistry synthesis in a reaction vessel through which nitrogen was bubbled for mixing. A typical cycle for the coupling of an individual amino acid by the Fmoc strategy was: (I) Fmoc deprotection with 20% piperidine in NMP (2 times 2.5 mL, each 8 min); (II) wash with NMP (3 times 2.5 mL, each 2 min); (III) wash with CH_2Cl_2 (3 times 2.5 mL, each 2 min); (IV) coupling for 1 h of the Fmoc amino acid by addition of a freshly prepared mixture of an amino acid

(0.5 mmol), BOP (0.5 mmol, 221 mg) and DiPEA (1 mmol, 0.18 mL) in NMP (2.5 mL); (V) repeating steps (II) and (III). This procedure was subsequently performed with the amino acid building blocks: Fmoc–AllylGly–OH, Fmoc–Val–OH, Fmoc–Asn(Trt)–OH, Fmoc–Val–OH, Fmoc–Tyr(PO(OBzl)OH)–OH and Fmoc–AllylGly–OH. Finally, the N-terminus of the peptide was acetylated by a mixture of acetic acid anhydride (0.5 M), DiPEA (0.125 M), and HOBt (0.015 M) in NMP. The protected peptide was cleaved from the resin in 10 mL MeOH with a catalytic amount of KCN at room temperature for 16 h and used for ring closure by the second generation Grubbs catalyst **3**[27] (20 mol%) in the presence of 2,6-dichlorotoluene (2 equiv.) at elevated temperatures in three different solvents: 1,1,2-TCE (reflux), DMF (50 °C) and an ionic liquid; 1-butyl-3-methylimidazolium hexafluorophosphate [bmim]PF_6 (50 °C). After metathesis the protective groups were removed from the peptide by treatment with a mixture of TFA/EDT/TIS/H_2O (3 mL/0.08 mL/0.08 mL/0.16 mL) for 3 h. Finally, the peptide was precipitated by an ice-cold mixture of methyl *tert*-butylether (MTBE) and hexane (1 : 1), washed 3 times with diethyl ether and lyophilized from a *tert*-butanol/H_2O mixture (1 : 3). The resulting product was analyzed by ESI-MS.

Protected linear peptide 4. A procedure similar to that for peptide **1** was used to assemble peptide **4** on a scale of 0.2 mmol (0.57 g resin) with the building blocks: Fmoc–AllylGly–OH, Fmoc–Val–OH, Fmoc–Asn(Trt)–OH, Fmoc–Val–OH, Fmoc–Tyr(tBu)–OH and, subsequently, 4-pentenoic acid. The crude protected peptide was purified by column chromatography with 4% MeOH/CH_2Cl_2 to give the product (0.2 mmol, 0.20 g) as a white solid. R_f (10% MeOH/CH_2Cl_2) = 0.44. ESI-MS [M + H^+] *m*/*z* 986.5, [M + Na^+] 1008.1.

Deprotected linear phosphopeptide 5. A procedure similar to that for peptide **1** was used to assemble peptide **5** on a scale of 0.1 mmol (0.29 g resin) with building blocks: Fmoc–AllylGly–OH, Fmoc–Val–OH, Fmoc–Asn(Trt)–OH, Fmoc–Val–OH, Fmoc–Tyr(PO(OBzl)OH)–OH and, subsequently, 4-pentenoic acid. After cleavage from the resin, the protected peptide was stirred in a mixture of TFA/EDT/TIS/H_2O (3 mL/0.08 mL/0.08 mL/0.16 mL) for 3 h. Next, the peptide was precipitated by an ice-cold mixture of MTBE and hexane (1 :1) and washed 3 times with diethyl ether. The residue was lyophilized and purified by preparative HPLC to give the desired product (20 mg, 0.026 mmol) as a white fluffy solid in a yield of 26%. The purity according to HPLC was greater than 95%. ESI-MS [M + H^+] *m*/*z* 767.7, [M + Na^+] 789.5. HR-MS [M + H^+] calculated *m*/*z* 767.338, found 767.345. ^{1}H NMR (500 MHz, D_2O) δ = 7.19–7.10 (4H, m, H_{ar}–Tyr), 5.80–5.60 (2H, m, CH=CH_2), 5.20–5.10 (2H, m, CH=CH_2), 4.85–4.80 (1H, m, αH Asn), 4.65–4.60 (1H, m, αH Tyr), 4.45–4.50 (1H, m, αH AllylGly), 4.20–4.05 (2H, m, αH Val), 3.10–2.88 (2H, m, βH Tyr), 2.82–2.51 (4H, m, βH Asn/Alkyl), 2.31–2.26 (4H, m, Alkyl), 2.10–1.98 (2H, βH Val), 0.94–0.90 (12H, m, γH Val); (H_2O/D_2O) δ = 8.61–8.59 (2H, m, NH Asn/AllylGly), 8.31–8.30 (2H, m, NH Tyr/Val), 8.17 (1H, m, NH Val), 7.81(1H, s, NH_2Asn), 7.00 (1H, s, NH_2Asn).

Protected cyclic peptides 6. Linear peptide **4** (0.11 mmol, 115 mg) was dissolved in 1 mL dry MeOH and 20 mL dry 1,1,2 trichloroethane (1,1,2-TCE) followed by bubbling with N_2 and heating the mixture for 30 min to 110 °C to remove most of the MeOH thus yielding a clear solution of the peptide in mostly 1,1,2-TCE. After addition of 2,6-dichlorotoluene (0.4 mmol, 52 µl), the mixture was refluxed for 10 min. Subsequently, second generation Grubbs catalyst **3**[27] (0.02 mmol, 17 mg) was added followed by refluxing the mixture for 16 h under a nitrogen flow. Finally, volatiles were evaporated and the residue was subjected to column chromatography with 3% MeOH/CH_2Cl_2 to give the desired product (0.05 mmol, 47 mg) as a yellowish solid in a yield of 45%. R_f (10% MeOH/CH_2Cl_2) = 0.38–0.41 (*cis*/*trans* visible). ESI-MS [M + H^+] *m*/*z* 957.7.

Deprotected cyclic peptide 7. Protected peptide **6** (0.21 mmol, 204 mg) was deprotected with a mixture of TFA/EDT/TIS/H_2O (3 mL/0.08 mL/0.08 mL/0.16 mL) for 2.5 h followed by precipitation in ice-cold MTBE and hexane (1 : 1) and washing the residue 3 times with diethyl ether. The residue was lyophilized yielding the product as a yellowish fluffy solid (125 mg, 0.19 mmol) in a yield of 90%. ESI-MS [M + H^+] *m*/*z* 659.7, [M + Na^+] 681.7. HR-MS [M + H^+] calculated *m*/*z* 659.340, found 659.341. The *cis* and *trans* ratio was discernible by HPLC as *trans* : *cis* = 3 : 1. These isomers could be separated by preparative HPLC yielding 37% of the *trans* and 9% of the *cis* isomer. The purity of both separated isomers was greater than 95% by HPLC.

Trans isomer. ^{1}H NMR (500 MHz, D_2O) δ = 7.25 (2H, d, H_{ar}Tyr, d, *J* = 8.2 Hz), 6.89 (2H, d, H_{ar}Tyr, *J* = 8.5 Hz), 5.60–5.52 (1H, apparent d CH=CH, *J* = 15.6 Hz), 5.36–5.29 (1H, m, CH=CH), 4.69–4.60 (3H, m, αH Asn, αH Tyr, αH AllylGly), 4.15–4.02 (2H, m, αH Val), 3.30–3.27 (1H, m, βH Tyr), 3.01–2.84 (3H, m, βH Asn, βH Tyr), 2.49 (2H, m, Alkyl), 2.38–2.16 (6H, m, H Alkyl, βH Val), 0.96–1.03 (12H, m, γH Val); (H_2O/D_2O) additional peaks at δ = 8.51 (1H, m, NH–Val), 8.27 (1H, d, NH–AllylGly, d, *J* = 7.4 Hz), 8.19 (1H, m, NH–Tyr), 8.15 (1H, m, NH–Asn), 8.10 (1H, d, NH Val,d, *J* = 9.7 Hz), 7.74 (1H, s, NH_2Asn), 6.96 (1H, s, NH_2Asn).

Cis isomer. ^{1}H NMR (500 MHz, D_2O) δ = 7.18 (2H, d, H_{ar}-Tyr, d, *J* = 8.5 Hz), 6.85 (2H, d, H_{ar}Tyr, d, *J* = 8.5 Hz), 5.60–5.50 (1H, apparent d CH=CH, *J* = 10.7 Hz), 5.35–5.28 (1H, m, CH=CH), 4.62–4.54 (3H, m, αH Tyr/Asn/AllylGly), 4.10–4.01 (2H, m, αH Val), 3.22–3.18 (1H, m, βH Tyr), 3.01–2.87 (3H, m, βH Tyr/Alkyl), 2.68–2.12 (8H, m, βH Asn/Val/Alkyl), 0.96–0.90 (12H, m, γH Val); (H_2O/D_2O) additional peaks at δ = 8.31–8.25 (3H, m, NH Asn/Tyr/Alkyl), 7.93 (1H, br. NH Val), 7.83 (1H, d, NHVal, *J* = 7.9 Hz), 7.73 (1H, s, NH_2Asn), 7.00 (1H, s, NH_2Asn).

Protected cyclic phosphopeptide 8. After coevaporation of cyclic peptide **7** (100 mg, 0.15 mmol) and bis(4-chlorobenzyl)-*N*,*N*-diisopropylphoshoramidite[29] (0.35 g, 0.9 mmol) with 1,4-dioxane (1.8 mL) the reaction flask was flushed with nitrogen followed by addition of a 0.5 M solution of 1*H*-tetrazole in CH_3CN (1.8 mL, 0.9 mmol) and 3 mL 1,4-dioxane. The mixture was stirred for 5 h after which a 0.5 M solution of 3-chloroperoxybenzoic acid (70–75%) in CH_3CN (2.0 mL, 1.0 mmol) was added and stirred for 30 min followed by addition of a 1 M solution of Na_2SO_3 in H_2O (2.0 mL, 1.0 mmol). After 30 min, the solvent was evaporated and the residue was dissolved in CH_2Cl_2 and extracted with aqueous 5% $NaHCO_3$ solution. The organic layer was evaporated and the residue purified by column chromatography with 4% MeOH/CH_2Cl_2 to give the product (104 mg, 0.1 mmol) as a white solid in a yield of 66%. R_f (10% MeOH/CH_2Cl_2) = 0.48. ESI-MS [M + H^+] *m*/*z* 987.5, [M + Na^+] 1009.2.

Deprotected cyclic phosphopeptide 9. Protected cyclic phosphopeptides **8** (20 mg, 0.02 mmol) was treated with TFA/EDT/TIS/H_2O (3 mL/0.08 mL/0.08 mL/0.16 mL) for 3 h, followed by precipitation in ice-cold MTBE/hexane and washing the residue 3 times with diethyl ether. The residue was lyophilized yielding the product, which was purified by preparative HPLC to give the *trans* isomer in a yield of 32% (4.8 mg, 0.0065 mmol) as a white fluffy solid. The purity according to HPLC was greater than 95%. ESI-MS [M + H^+] *m*/*z* 739.7, [M + Na^+] 761.7. HR-MS [M + H^+] calculated *m*/*z* 739.307, found 739.308.

Trans isomer. ^{1}H NMR (500 MHz, D_2O) δ = 7.27 (2H, d, H_{ar}Tyr, *J* = 8.2 Hz), 7.15 (2H, d, H_{ar}Tyr, *J* = 7.9 Hz), 5.60–5.52 (1H, dt, *J* = 15.9 Hz, CH=CH), 5.36–5.29 (1H, m, CH=CH),

4.75–4.61 (3H, m, αH Tyr/Asn/AllylGly), 4.13–4.03 (2H, m, αH Val), 3.42–3.38 (1H, m, βH Tyr), 3.03–2.98 (1H, m, βH Asn), 2.85–2.81 (2H, m, βH Asn/Tyr), 2.49–2.17 (8H, m, βH Val/Asn/Alkyl), 1.05–0.98 (12H, m, γH Val); (H_2O/D_2O) additional peaks at δ = 8.73 (1H, apparent s, NH Val), 8.30–8.29 (2H, m, NH AllylGly/Asn), 8.20 (1H, br, NHTyr), 8.09 (1H, br, NHVal), 7.75 (1H, s, NH_2Asn), 6.96 (1H, s, NH_2Asn).

Cis isomer. The *cis* isomer was obtained in a yield of about 10% (1.6 mg, 0.0022 mmol), however it was contaminated with about 15% of the *trans* isomer.

Reduced cyclic phosphopeptide 10. Protected cyclic phosphopeptides **8** (27 mg, 0.027 mmol) was dissolved in 20 mL of a mixture of tBuOH/H_2O (3 : 1). To this mixture, NaOAc (50 mg, 0.6 mmol) and a catalytic amount of Pd/C were added. The mixture was treated overnight with H_2 (3–4 atm) in a Parr apparatus. Subsequently, the catalyst was removed by filtration over hyflo after which the volatiles were evaporated. Finally, the peptide was purified by preparative HPLC to afford the pure product (9 mg, 0.012 mmol) as a white fluffy solid in a yield of 45%. The purity according to HPLC was greater than 95%. ESI-MS [M + H^+] *m*/*z* 741.7, [M + Na^+] 763.8. HR-MS [M + H^+] calculated *m*/*z* 741.323, found 741.323. ^{1}H NMR (500 MHz, D_2O) δ = 7.26 (2H, d, H_{ar}Tyr, *J* = 7.9 Hz), 7.15 (2H, d, H_{ar}Tyr, *J* = 7.6 Hz), 4.80–4.75 (1H, m, αH Tyr), 4.70–4.65 (1H, m, αH Asn), 4.52–4.48 (1H, m, αH Alkyl), 4.16–4.08 (2H, m, αH Val), 3.37 (1H, m, βH Tyr), 3.00–2.80 (3H, m, βH Asn/Tyr), 2.21 (2H, m, βH Val), 1.90–1.80 (2H, m, CH_2Alkyl), 1.70–1.65 (4H, m, CH_2Alkyl), 1.64–1.54 (2H, m, CH_2Alkyl), 1.28–1.18 (2H, m, CH_2Alkyl), 0.89–1.02 (12H, m, γH Val); (H_2O/D_2O) δ = 8.58 (1H, apparent s, NHVal), 8.39–8.38 (2H, m, NH Asn/Tyr), 8.24 (1H, d, NHAllylGly, *J* = 6.7 Hz), 7.93 (1H, d, NHVal, *J* = 9.5 Hz), 7.76 (1H s, NH_2Asn), 6.98 (1H, s, NH_2Asn).

Modeling

Molecular modeling of the ligand–protein complex was carried out using Sybyl 6.8 (Tripos, Inc., St. Louis, MO) on a Silicon Graphics workstation. Construction of the peptide–protein complexes was based on the crystal structure of the Grb2 SH2 domain complexed with a heptapeptide inhibitor (PDB entry code 1TZE).[8] The water molecules were removed from the protein and hydrogens were added to the protein using the biopolymer module in Sybyl. Cyclic peptides that are ring-closed by different bridges were constructed starting from the heptapeptide of the crystal structure. The constructed cyclic peptides were energy minimized in the presence of the Grb2 SH2 domain, the geometry of which was kept fixed. The energy minimization was performed using a Powell gradient minimization with the MMFF94s forcefield in 100 steps. The minimized conformations of the cyclic peptides were compared to the conformation of the linear peptide that was not energy minimized. The conformations were compared after superimposing the α-carbon atoms of the pTyr, Val, Asn and Val residues (Fig. 1).

Protein expression

Grb2 SH2 domain was expressed as a Glutathione S-Transferase (GST) fusion construct in *E. coli* (strain DH5α), which was kindly provided by Dr A. S. Shaw.[37] The expression vector (pGEX-KG) in *E. Coli* was grown in LB-medium with ampicillin (50 μg mL^{-1}) and induced with 1 mM isopropyl-thiogalactoside for 3 h. Harvested cells were lysed and the crude cell extracts, containing appropriate protease inhibitors, were centrifuged and loaded onto an affinity GSTrap column (Pharmacia Biotech). After removal of unbound material by thorough washing; the Grb2 SH2 GST fusion construct was eluted using glutathione and characterized by polyacrylamide gel electrophoresis and SPR. Mass was verified by mass spectrometry analysis (M_w = 41,711 Da). The protein concentration was measured using the Micro-BCA kit of BioRad.

SPR binding studies

Experiments were performed on a double channel IBIS II SPR instrument (IBIS Technologies, Enschede, The Netherlands) that was equipped with a CM5 sensor chip (BIAcore AB, Uppsala, Sweden). These chips contain a carboxymethylated dextran surface to which the primary amine of Ahx in the peptide Ahx–Pro–Ser–pTyr–Val–Asn–Val–Gln–Asn–NH_2 was coupled with *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) using the Amine Coupling Kit (BIAcore AB, Uppsala, Sweden) according to the manufacturer's instructions. Thus, the sensor surface of the sample cell was treated with EDC and NHS for 5 min, after which 2 mM of the Ahx-peptide in 100 mM borate buffer with 1 M NaCl (pH 8.3) was coupled to the chip for 10 min. After that the sensor surface was treated with 1 M ethanolamine solution in H_2O (pH 8.5) for 7 min. The reference cell was treated identically except that no peptide was added. The net SPR signal was obtained by subtracting the signal in the reference cell from that in the sample cell. In a typical experiment 35 μl of a sample in HBS-buffer was added by an autosampler into the sample cell as well as to the reference cell. The composition of the HBS-buffer was; 10 mM Hepes, 3.4 mM EDTA, 150 mM NaCl and 0.005% Tween-20, pH was titrated with NaOH to 7.4. The chip was regenerated with 0.2% SDS in 50 mM HCl. Competition experiments were performed, in triplicate, by addition of 50 nM of SH2 domain premixed with various concentrations of phosphopeptide to the cells at 25 °C. The association constant for binding of the Grb2 SH2 domain to the chip surface was determined in duplicate and was 35 ± 3 nM. The dissociation binding constants of the peptides in solution (K_d) was calculated as described previously,[30] by non-linear curve fitting of the averaged triplicates. The K_d for the Ahx–Pro–Ser–pTyr–Val–Asn–Val–Gln–Asn–NH_2 peptide was 0.30 ± 0.03 μM.

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