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A photoswitchable ITAM peptidomimetic: Synthesis and real time surface plasmon resonance (SPR) analysis of the effects of *cis–trans* isomerization on binding

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Abstract—The Syk protein plays an important role in immune receptor signaling. The Syk tandem SH2 domain (tSH2)–ITAM interaction is important for recruiting Syk to the receptor complex and for Syk kinase activation. A peptidomimetic ligand for tSH2 was synthesized in which a photoswitchable azobenzene moiety was incorporated. Such a photoswitchable moiety may regulate the distance between the two phosphotyrosine containing ITAM sequences, which bind to tSH2. Different affinities of the *cis* and *trans* isomer of the ligand were found by surface plasmon resonance (SPR). By in situ irradiation during SPR measurements the effect of the *cis–trans* isomerization on binding could be monitored in real time.
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1. Introduction

The Spleen tyrosine kinase (Syk) protein takes part in a number of receptor signaling cascades.[1–6] It is involved in early events after activation of, for example, the IgE-, T-cell-, and B-cell receptors. Furthermore, it is involved in IL-15-receptor and integrin signaling. The role of Syk in the high affinity IgE receptor (FcεRI) signaling in mast cells and basophils is shown in Figure 1. FcεRI consists of an α-, β- and two γ-chains. The β- and γ-chains contain a specific intracellular sequence called the Immunoreceptor Tyrosine based Activation Motif (ITAM). The ITAM sequence consists of *Tyr-Xxx-Xxx-(Leu/Ile)*-$(Xxx)_{n=6–8}$-*Tyr-Xxx- Xxx-(Leu/Ile)*, in which Xxx can be any amino acid. The residues in italics comprise the binding epitopes for the tandem SH2 (tSH2) domain of Syk, when tyrosine is phosphorylated. We have found that the intervening residues $(Xxx)_{n=6–8}$ (in FcεRI $n = 7$) were not essential for binding.[7,8]

Upon IgE stimulation of the receptor, the ITAM motif is diphosphorylated (γ-dpITAM). After this, Syk is recruited to the membrane by binding to γ-dpITAM (Fig. 1). This results in a conformational change and Syk activation.[5] Further events involved in activation of the kinase domain are still subject of investigation.

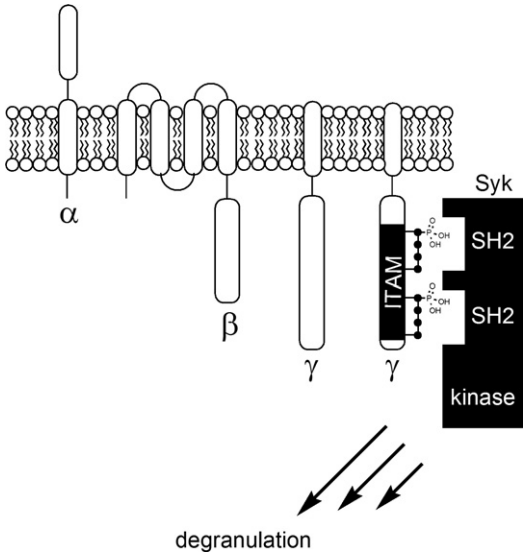

Figure 1. Recruitment of Syk to the diphosphorylated γ-ITAM of FcεRI results in activation of its kinase domain and ultimately degranulation.

Keywords: FcεRI; Syk; ITAM; SH2; Azobenzene; Conformational photoswitch; Surface plasmon resonance.
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They include phosphorylation of tyrosine residues in the linker region between the tSH2 and kinase domains of Syk, and in the kinase domain itself.[5] Syk activation eventually leads to cell degranulation and release of mediators. Overstimulation of this pathway leads to allergic responses and therefore Syk is an interesting target for potential anti-allergic therapy.

The relative orientation of the SH2 domains in tSH2 displays remarkable conformational flexibility. Recently, we have found that binding of Syk tSH2 to an ITAM peptide causes a significant change in the dynamics of tSH2.[1,9] This change could imply that a fixation in the inter SH2 distance is necessary for Syk activation by ITAM binding. To gain more insight into the functioning of Syk tSH2, we decided to take advantage of a photoswitchable building block and incorporate this into the ITAM sequence.

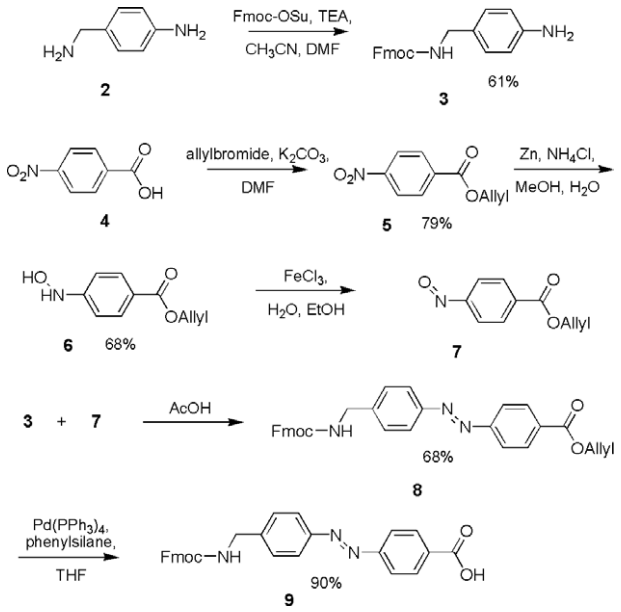
Azobenzene as part of (4-aminomethyl)phenylazobenzoic acid (AMPB) is the most widely used photoswitch because of its fast and fully reversible photoisomerization.[10–12] Moreover, the synthesis of AMPB is well described and the *cis* isomer is relatively stable in solution, when not exposed to light. AMPB is compatible with solid phase peptide synthesis and can easily be incorporated into a peptide sequence.

Binding of the *cis* and *trans* isomers of the photoswitch containing ITAM mimic was determined using surface plasmon resonance (SPR). In a convenient approach, the *cis–trans* transition and its effect on binding could be monitored in real time using in situ irradiation in the SPR measuring cell.

2. Results and discussion

The length of the seven intervening amino acids in γ-ITAM, which are not essential for binding, is 14.1–16.4 Å, according to the X-ray structure.[13] The photoswitch AMPB is only 6.2 Å in the *cis*- and 12.0 Å in the *trans*-conformation.[14,15] Therefore, an extra glycine residue was added to each side of AMPB and in this way a linker of 18.6 Å in the *trans* conformation was obtained.[16] The linker length, including the two glycine residues, is only 7.2 Å in the *cis* conformation.

For synthesis of the Fmoc protected azobenzene containing building block **9** (Scheme 1) a literature procedure was adapted.[12,17,18] First, (4-amino)benzylamine was selectively protected using Fmoc-OSu to yield the Fmoc protected amine **3**. Initially, it was attempted to synthesize AMPB from 4-nitrobenzoic acid *tert*-butyl protected as is described in the literature.[12,18] After the *tert*-butyl protection, the nitro group was converted into a hydroxylamine using activated zinc dust. However, in our hands under these conditions also the *tert*-butyl ester was removed, possibly due to traces of HCl left in the zinc after activation, resulting in the undesired 4-hydroxylaminebenzoic acid. Therefore, 4-nitrobenzoic acid (**4**) was instead allyl protected and now the preparation of hydroxylamine allylester **6** went smoothly.



Scheme 1. Synthesis of the photoswitchable building block **9** (Fmoc-AMPB-OH).

Next, hydroxylamine **6** was treated with $FeCl_3$ to produce the corresponding nitroso compound **7**. Due to the instability of this compound, it was immediately used in the condensation reaction with Fmoc-protected amine **3**. The allyl group was easily removed afterwards with $Pd(PPh_3)_4$ and phenylsilane as scavenger, which gave no undesired reduction of the azo group.

NMR spectra of 100% *trans* azobenzene moiety **9** and mixtures with *cis* and *trans* conformers revealed that a maximum *cis* content of 78% could be obtained for **9** upon irradiation with 366 nm light.

The photoswitchable peptidomimetics **1a**–**b** (Fig. 2) were synthesized using standard Fmoc/tBu chemistry including **9** as a building block. A small amount of TIS could be used in the cleavage cocktail without reducing the azobenzene. This was also recently observed by Hilvert et al.[19]

After preparative RP-HPLC, a small amount of the peptide was dissolved in water to record UV-VIS spectra. First, the sample was irradiated with visible light to obtain *trans* isomer **1a**. NMR spectra of compounds **8** and **9** showed that the amount of *trans* isomer after irradiation with visible light was nearly 100%. This 100% *trans* isomer cannot be obtained by irradiation alone, due to partial overlap of the n-π^* and π–π^* transitions.[11] To reach 100% *trans* also thermal isomerization has to occur. After the spectrum of **1a** was measured, the sample was irradiated with 366 nm light for several time intervals and spectra were recorded (Fig. 3). This was repeated until no further change in the spectrum was observed after 120 s. The spectra showed three isosbestic points at 240, 286, and 403 nm.

Integration of the signals from NMR spectra of *trans* isomer **1a** (8.61 and 8.95 ppm) and *cis* isomer **1b** (8.49

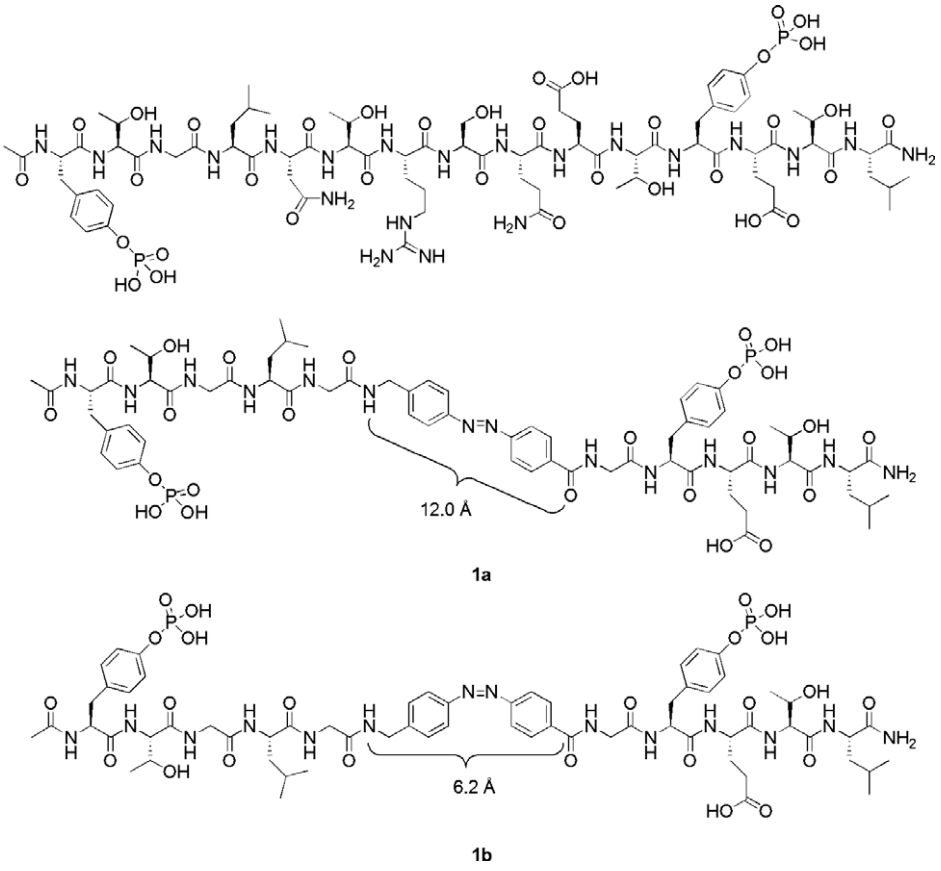


Figure 2. The native γ-ITAM peptide (derived from FcεRI) and the photoswitchable ITAM mimics **1a** and **1b**. The indicated distances are between the SH2 binding epitopes.[14,15]

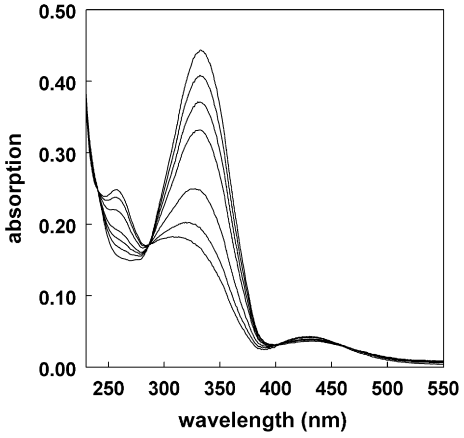


Figure 3. UV-absorption spectra of *trans* isomer **1a** ($t = 0$) upon increasing time of irradiation with 366 nm light. From top (335 nm) spectrum: $t = 0$ s, then $t = 10$, 20, 30, 60, 90, and 120 s.

and 8.79 ppm) revealed that a maximum *cis* isomer percentage of 60% could be reached upon irradiation with 366 nm light. The lower maximal *cis* percentage for the peptide as compared to its building block **9** has also been observed in other peptides and might be due to a higher degree of steric hindrance.[20]

The interaction between the two isomers and the Syk tSH2 protein was measured with SPR. Competition experiments were performed with the native ITAM peptide immobilized on the SPR chip as previously described.[1,7,8] Prior to addition, the competing ligand **1** was irradiated with UV light (366 nm) or visible light to give 60% *cis* isomer or 100% isomer, respectively. The tSH2 and the irradiated *cis* or *trans* isomer in different concentrations were injected into the SPR cuvette and the binding of the tSH2 to the sensor chip was measured. From these measurements the dissociation constants (K_D) could be calculated as described previously

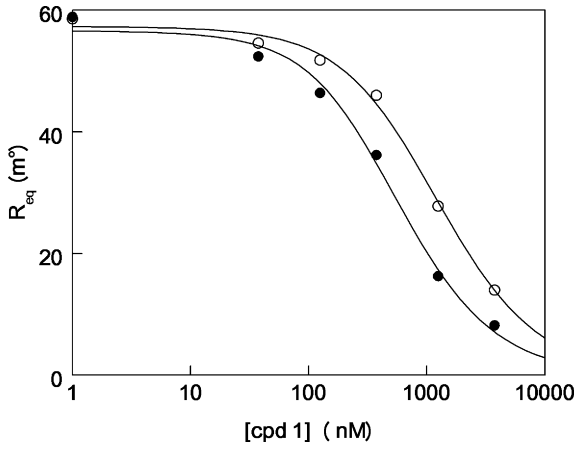


Figure 4. SPR competition experiments with Syk tSH2 (50 nM) in the presence of various concentraties of peptide **1** in *cis* (○) or *trans* (•) conformation. The data are fitted and K_D derived as described.[21]

(Fig. 4).[21] The K_D value of the *trans* isomer was 65 ± 8 nM and the observed K_D of the mixture containing 60% *cis* isomer was 146 ± 11 nM. Since the mixture still contained 40% *trans* isomer **1a**, the actual $K_{D,cis}$ can be calculated with Eq. 1 and amounts to 860 nM.

Although the difference in affinity is more than 10-fold, these numbers show that the *cis* isomer, in which the SH2 domains are forced to be much closer to each other than in the complex with native ITAM, still has an affinity that is significantly higher than for a monovalent interaction, which has a K_D of 27 μM.[22] Syk tSH2 has a very flexible coiled-coil linker which connects the two SH2 domains.[1] Therefore, it accommodates fairly easy ITAM sequences containing different linker lengths. Furthermore, the glycine residues also have a moderate degree of flexibility, which can result in a somewhat larger linker length in the *cis* isomer **1b**, when bound to tSH2 compared to the free linker, which was used for initial modeling.

Now that different affinities for *cis* isomer **1b** and *trans* isomer **1a** have been established, it is interesting to monitor the conversion from *cis* to *trans*, and the effect on binding in real time with SPR. For this, an experiment was performed, making use of the convenient cuvette design of the IBISII SPR instrument, which makes it possible to irradiate the sample during the SPR binding assay. First, the sensograms of only tSH2 and tSH2 with **1a** were recorded (Fig. 5, top - and bottom curve). Then the mixture containing 60% **1b** with tSH2 was injected into the cuvette and after 800 s a steady state situation was almost reached (Fig. 5, middle curve). Then the sample in the SPR cuvette was irradiated with visible light with the SPR instrument in the measuring mode. This resulted in a decrease of SPR signal, as the weaker binding **1b** was transformed to the stronger binding **1a**, displacing more Syk tSH2 from the sensor surface in the competition experiment. Within 500 s the signal reached the level of binding of only the trans isomer (bottom curve), showing that a 100% *trans* state could be obtained in reasonably short time. The *cis–trans* isomerization could be perfectly fitted by a mono-exponential function (not shown), showing pseudo first order kinetics, as was expected for a photochemical process with constant irradiation intensity.

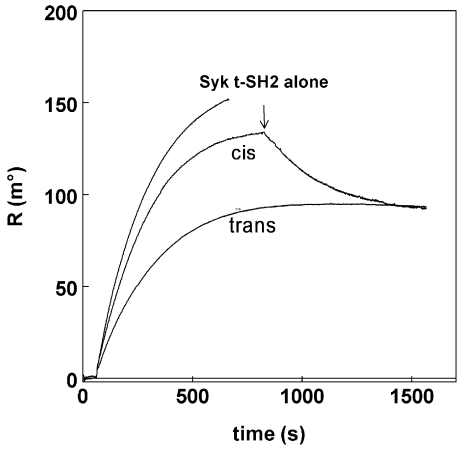


Figure 5. Effect of conversion of *cis* isomer **1b** to *trans* isomer **1a** in an SPR competition experiment within situ irradiation with visible light. Top curve: Real time SPR signal for binding of 50 nM Syk tSH2 to immobilized native diphosphorylated γ-ITAM peptide on the SPR sensor surface. Bottom curve: SPR signal of 50 nM Syk tSH2 in the presence of 375 nM **1a**. Middle curve: 50 nM Syk tSH2 in the presence of 375 nM 60% **1b**, after 800 s the sample in the SPR cuvette was irradiated with visible light as indicated by the arrow.

3. Conclusions

In conclusion, we have shown that we can design and synthesize an ITAM peptidomimetic with significant difference in binding affinity between the *cis* and *trans* isomer for Syk tSH2. Using SPR the change in binding affinity due to photoisomerization could be studied in real time. To our knowledge, this is the first report on the effect of isomerization of a photoswitchable ligand on binding to a protein that is monitored during an SPR binding assay. In the near future we aim to design a photoswitchable Syk tSH2 ligand, which can enter cells, having a larger difference in affinity between *cis* and *trans* isomers. Such a ligand might be an interesting tool to study the possibility of instantaneously switching intracellular processes on and off by irradiation.

4. Experimental

All chemicals were obtained from commercial sources and used without further purification. Solvents, which were used for the solid phase peptide synthesis, were stored at 4 Å molsieves, except for MeOH, which was stored at 3 Å molsieves. Prior to use, zinc was activated by stirring in 2% HCl for 30 min. The zinc was filtered, washed with water, ethanol, and diethyl ether, and dried under reduced pressure. The reactions were performed at room temperature unless stated otherwise. The reactions were monitored and the R_f values were determined by thin layer chromatography (TLC). The TLC plates were obtained from Merck and were coated with silica gel 60 F-254 (0.25). The spots were visualized by UV light and ninhydrin staining. Solvents were removed under reduced pressure at a temperature of 40 °C. Column chromatography was performed on silica gel. Photoisomerization was performed using a visible light emitting lamp (Schott/Paes KL-1500) in combination with a glass plate to filter the UV light or a 6 W 366 nm handheld TLC lamp (Konrad Benda).[14]

^{1}H NMR spectra were measured on a Varian Mercury plus 300 MHz spectrometer and the chemical shifts are given in ppm (δ) relative to TMS. ^{13}C NMR spectra were measured on a Varian Mercury plus 75 MHz spectrometer and the chemical shifts are given in ppm (δ) relative to $CDCl_3$ or DMSO-d_6. The ^{13}C NMR spectra were measured using the attached proton test (APT). NMR spectra of peptide **1** were recorded on a Varian Inova 500 MHz spectrometer in H_2O/D_2O 9:1 and were calibrated on dioxane. UV-VIS spectra were recorded with a UV1 spectrophotometer (Thermo Electron Cor-

poration). Analytical HPLC was measured on a Shimadzu HPLC system with a UV detector operating at 220 and 254 nm using an Alltech Adsorbosphere XL C8 90Å 5 μm (250 × 4.6 mm) column. For the analytical HPLC a gradient from 100% buffer A (15 mM TEA in H_2O titrated at pH 6 with 85% H_3PO_4) to 100% buffer B (buffer A/acetonitrile 1:9) in 20 min was used. For the preparative HPLC a Gilson system with a UV detector operating at 220 and 254 nm equipped with an Alltech Adsorbosphere XL C8 90 Å 10 μm (250 × 22 mm) column was used. A gradient of 100% buffer A (0.1% TFA in H_2O/acetonitrile 95:5) to 100% buffer B (0.1% TFA in H_2O/acetonitrile 5:95) was used. For the use in HPLC buffers TEA was distilled over ninhydrin and KOH.

SPR measurements were performed on a double channel IBISII SPR instrument (Eco Chemie, Utrecht, The Netherlands) equipped with a CM 5 sensor chip (BIAcore AB, Uppsala, Sweden).

4.1. N-Fmoc(4-amino)benzylamine (3)

Compound **3** was synthesized according to a procedure described in the literature.[17] Yield: 10.47 g (30.42 mmol, 61%) of a white solid.

4.2. Allyl 4-nitrobenzoate (5)

Allylbromide (2.50 mL, 30 mmol) and K_2CO_3 (4.15 g, 30 mmol) were added to a solution of 4-nitrobenzoic acid (3.36 g, 20 mmol) in DMF (100 mL). After 5 min of stirring the solvent was removed under reduced pressure. The resulting solid was dissolved in CH_2Cl_2 and water. The organic layer was washed with 5% $NaHCO_3$ (2×), 1 M $KHSO_4$, and brine, dried over Na_2SO_4, filtered, and concentrated yielding 3.30 g (15.9 mmol, 79%) of **5** as a brown solid. $R_f = 0.61$ (hexane/EtOAc 5:1) ^{1}H NMR ($CDCl_3$, 300 MHz) $\delta = 4.87$–4.89 (d, 2H, OCH_2), 5.32–5.48 (2dd, 2H, CH_2), 6.00–6.12 (m, 1H, CH), 8.22-8.30 (m, 4H, ar.) ^{13}C NMR ($CDCl_3$, 75 MHz) $\delta = 66.6$ (OCH_2), 119.3 ($CHCH_2$), 123.7, 130.9, 135.7 and 150.7 (C_6H_4), 131.70 ($CHCH_2$), 164.5 (COO).

4.3. Allyl 4-hydroxylaminebenzoate (6)

Compound **6** was synthesized according to a procedure described in the literature.[23] Yield: 524 mg (2.71 mmol, 68%) of **6** as an orange solid. $R_f = 0.35$ (CH_2Cl_2/MeOH 98:2) ^{1}H NMR ($CDCl_3$, 300 MHz) $\delta = 4.74$–4.76 (d, 2H, OCH_2), 5.22–5.39 (2dd, 2H, CH_2), 5.92–6.05 (m, 1H, CH), 6.92 + 7.20 (2 bs, 2H, NH + OH) 6.87–6.89 (m, 2H, ar) 7.90–7.92 (d, H, ar.) ^{13}C NMR ($CDCl_3$, 75 MHz) $\delta = 65.9$ (OCH_2), 113.4, 123.1, 131.5 and 154.9 (C_6H_4), 118.6 ($CHCH_2$), 132.8 ($CHCH_2$), 167.3 (COO).

4.4. Allyl 4-nitrosobenzoate (7)

Compound **7** was synthesized according to a procedure described in the literature.[24] The product was used immediately without further purification.

4.5. Allyl N-Fmoc-(4-aminomethyl)phenylazobenzoate (8)

Compound **8** was synthesized according to a procedure described in literature,[18] except for the following. The reaction time was 16 h. The solvent was removed under reduced pressure and the resulting orange solid was dissolved in EtOAc and water. The organic layer was removed and the water layer was extracted with EtOAc (2×). During the extraction a slightly orange precipitate occurred. This precipitate was removed by filtration and the organic layers were combined, washed twice with brine, dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure and the resulting orange solid was purified by silica gel chromatography with CH_2Cl_2/MeOH 198:1 yielding 220 mg (0.42 mmol, 65%) of diazocompound **8** as an orange solid. $R_f = 0.83$ (CH_2Cl_2/MeOH 98:2). ^{1}H NMR ($CDCl_3$, 300 MHz) $\delta = 4.20$–4.22 (t, 1H, CH Fmoc), 4.43–4.51 (2d, 4H, $CH_2NH + CH_2$ Fmoc), 4.85–4.87 (d, 2H, $COOCH_2CHCH_2$), 5.25 (bs, 1H, NH), 5.29–5.46 (2dd, 2H, $COOCH_2CHCH_2$), 6.00–6.12 (m, 1H, $COOCH_2CHCH_2$), 7.24–7.60 (m, 6H, 2 ar + 4 ar Fmoc), 7.74–7.77 (d, 2H, ar Fmoc), 7.88–7.90 (d, 2H, ar Fmoc), 7.92–7.95 (m, 4H, ar), 8.19–8.22 (d, 2H, ar). ^{13}C NMR ($CDCl_3$, 75 MHz) $\delta = 45.0$ (CH_2NH), 47.6 (CH Fmoc), 66.1 ($COOCH_2CHCH_2$), 66.9 (CH_2 Fmoc), 118.8 ($COOCH_2CHCH_2$), 120.2, 125.2, 127.3, 127.9, 141.6 and 144.1 (ar Fmoc), 122.9, 128.4, 142.5 and 152.2 ($C_6H_4CH_2NH$), 123.7, 132.1, 132.3 and 155.4 (C_6H_4 COO), 130.9 ($COOCH_2CHCH_2$), 156.4 (NHCOO), 165.9 ($COOCH_2CHCH_2$).

4.6. N-Fmoc-(4-aminomethyl)phenylazobenzoic acid (Fmoc-AMPB-OH, 9)

Phenylsilane (56 μL, 0.46 mmol) was added to a solution of **8** (220 mg, 0.42 mmol) in THF (20 mL) and stirred under a nitrogen atmosphere. $Pd(PPh_3)_4$ (49.8 mg, 0.008 mmol) was added under an argon atmosphere and the reaction mixture was stirred for 2 h under a nitrogen atmosphere. The solvent was removed under reduced pressure and the resulting solid was dissolved in DMF and purified by silica gel chromatography with CH_2Cl_2/MeOH/CH_3COOH 97:2:1 yielding 180 mg (0.37 mmol, 90%) of Fmoc-AMPB-OH **9** as an orange solid. $R_f = 0.65$ (CH_2Cl_2/MeOH 95:5) ^{1}H NMR (DMSO, 300 MHz) $\delta = 4.25$ (d, 2H, CH_2NH), 4.28–4.30 (d, 1H, CH Fmoc), 4.38–4.40 (d, 2H, CH_2 Fmoc), 7.31–7.36 (t, 2H, ar), 7.40–7.46 (t, 4H, ar Fmoc), 7.70–7.72 (d, 2H, ar Fmoc), 7.89–7.97 (m, 6H, 4 ar + 2 ar Fmoc), 8.12–8.15 (d, 2H, ar). ^{13}C NMR (DMSO, 75 MHz) $\delta = 43.5$ (CH_2NH), 46.8 (CH Fmoc), 65.3 (CH_2 Fmoc), 120.1, 125.1, 127.0, 127.6, 140.8 and 144.3 (ar Fmoc), 122.5, 127.4, 143.8 and 155.9 ($C_6H_4CH_2NH$), 122.9, 130.6, 132.7 and 154.3 (C_6H_4COOH), 156.4 (NHCOO), 166.7 (COOH).

4.7. Solid phase peptide synthesis

Peptide **1** was assembled on Tentagel[®]-Rink-NH-Fmoc resin (0.65 g, 0.15 mmol, loading 0.23 mmol/g) using standard Fmoc/tBu chemistry. The Fmoc protecting group was removed using 20% piperidine in NMP

(3 × 8 min) followed by washing steps with NMP (3 × 2 min), CH_2Cl_2 (3 × 2 min) and NMP (3 × 2 min). The amino acid coupling mixtures were prepared by dissolving 4 equivalents of amino acid (0.60 mmol), 4 equivalents of HOBt and HBTU, and 8 equivalents of DiPEA in NMP (10 mL) and coupled during a coupling time of 60 min. The resin was washed with NMP (3 × 1 min) and CH_2Cl_2 (3 × 1 min) after every coupling step, followed by Fmoc deprotection. The coupling steps and deprotection steps were monitored using the Kaisertest.[25] When the Fmoc deprotection was not complete, the deprotection steps were repeated. The amino acid building blocks Fmoc-Leu-OH, Fmoc-Thr(tBu)-OH, Fmoc-Glu (OtBu)-OH, Fmoc-Tyr(OP(OBn)OH)-OH, Fmoc-Gly-OH, Fmoc-AMPB-OH (**9**), Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Thr(tBu)-OH, and Fmoc-Tyr(O-P(OBn)OH)-OH were subsequently coupled. For the coupling of Fmoc-Tyr(OP(OBn)OH)-OH, 2 equivalents of amino acid, 2 equivalents of the coupling reagents HATU and HOAt, and 5 equivalents of DiPEA were used. For the coupling of Fmoc-AMPB-OH also 2 equivalents of amino acid and 2 equivalents of the coupling reagents HATU and HOAt were used and 4 equivalents of DiPEA. After the first Fmoc-Tyr(OP(OBn)OH)-OH coupling an extra washing step (2 × 10 min) with a mixture of 1 M TFA/1.1 M DiPEA in NMP was performed after each Fmoc deprotection step. This was done to replace the piperidine counterion of Tyr(OP(OBn)OH) for DiPEA. When all the coupling steps were completed the peptide was acetylated using a capping solution of Ac_2O (4.72 mL, 42.7 mmol), DiPEA (2.18 mL, 22.8 mmol), and HOBt (0.23 g, 1.7 mmol) in 100 mL NMP for 2 × 20 min. The peptide was cleaved from the resin and the side chains were deprotected with a solution of TFA/H_2O/TIS (95/2.5/2.5) for 3 h. The resin was removed from the solution by filtration. The peptide was precipitated with MTBE/hexane 1:1 v/v at −20 °C and lyophilized from CH_3CN/H_2O 1:1 v/v yielding 141 mg (0.093 mmol, 62%) of the crude peptide. The peptide was purified by preparative HPLC. About half (62.4 mg) of the crude peptide was purified yielding 9.7 mg of **1**. If all of the crude peptide was purified with the same yield, the overall yield would have been 21.3 mg (16%).

HPLC retention times: **1b** 9.8 min, **1a** 10.4 min. HRMS (ESI): $[M + Na]^+$ calculated 1533.547, found 1533.664; $[M + 2Na]^{2+}$ calculated 778.268, found 778.366.

4.8. Surface plasmon resonance

Peptide **1** (1.02 mg, 0.675 μmol) was dissolved in HBS buffer (675 μL) to obtain a stock solution with a concentration of 1 mM. A series of samples was prepared in HBS buffer containing 50 nM Syk tSH2 and **1** with concentrations ranging from 0 to 3750 nM. The sensor chip was immobilized with the native ITAM peptide as described.[7] Competition experiments were performed with the *trans* isomer **1a** and with the isomer mixture containing the maximum percentage of *cis* isomer **1b**. For this 240 μL samples were irradiated with visible light for complete conversion to the *trans* isomer **1a**. Then, half of the sample was injected into the SPR apparatus. The other half of the sample was irradiated with UV light of 366 nm to obtain the maximum percentage of *cis* conformer **1b** and subjected to the SPR assay under exclusion of light. K_D values have been derived from the competition experiments as was earlier described.[21] The intrinsic affinity of **1b** ($K_{A,cis} = 1/K_{D,cis}$) is derived by applying Eq. 1, in which K_{obs} is the observed association constant, f is the fraction of **1** in the *cis* isomer, and $K_{A,trans}$ is the affinity assayed for the pure *trans* conformer.

$$K_{obs} = f.K_{A,cis} + (1 - f)K_{A,trans} \quad (1)$$

To monitor the effect of conversion of **1a** to **1b** on binding in real time, experiments were performed with in situ irradiation in the SPR instrument. The isomer mixture **1ab** was first irradiated with 366 nm light to obtain a maximum percentage of *cis*-isomer. Then the sample was injected into the SPR cuvette under exclusion of light. On reaching binding equilibrium the sample in the SPR cuvette was irradiated with visible light for conversion to *trans* isomer **1a**, and the change in SPR signal was recorded.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.10.049.

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