ITAM-derived phosphopeptide-containing dendrimers as multivalent ligands for Syk tandem SH2 domain

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Received 25th March 2009, Accepted 2nd July 2009
First published as an Advance Article on the web 7th August 2009
DOI: 10.1039/b905938e

Spleen tyrosine kinase (Syk) is activated when its tandem SH2 domain (tSH2) binds to a diphosphorylated ITAM motif of *e.g.* the FcεRI receptor. In this divalent interaction each SH2 domain binds to a phosphotyrosine-containing tetrapeptide motif in ITAM. One of those tetrapeptide sequences was synthesized and conjugated to dendrimers *via* 'click' chemistry to create a series of functional phosphopeptide-containing dendrimers ranging from a monovalent to an octavalent dendrimer. The affinity of the functionalized dendrimers for Syk tSH2 has been assayed in SPR competition experiments. Both the tetra- and octavalent dendrimer had an affinity in the high nanomolar range, which is approximately 100-fold enhanced compared to the monovalent tetrapeptide, indicating a multivalency effect.

Introduction

Multivalency can enhance binding affinity in peptide–protein and in carbohydrate–protein interactions to a great extent.[1–8] The simplest form of a multivalent interaction is a divalent interaction, as is exemplified by the interaction between ITAM (Immunoreceptor Tyrosine based Activation Motif) and the tandem SH2 domain (tSH2) of the Syk kinase (Fig. 1A).[9–13] This interaction is essential in the IgE receptor signaling pathway.[14–17] γ-ITAM is part of the intracellular domain of the γ-chain of the multimeric ($\alpha\beta\gamma_2$) high affinity IgE receptor (FcεRI). Once this receptor is stimulated by IgE binding, γ-ITAM is diphosphorylated at two tyrosine residues leading to 'γ-dpITAM'. The tSH2 domain of Syk can then bind to γ-dpITAM, which results in activation of the Syk kinase domain. The resulting kinase activity initiates an intracellular cascade which ends with mast cell degranulation.[15] Overstimulation of this cascade leads to allergic responses and therefore interference with this peptide–protein interaction can be relevant for therapeutic purposes.

In the divalent ITAM-Syk tSH2 interaction, the two monovalent interactions are not identical. Both the binding epitopes of γ-dpITAM as well as the two SH2 domains of Syk are slightly different. In the ITAM sequence, *pTyr-Thr-Gly-Leu*-Asn-Thr-Arg-Ser-Gln-Glu-Thr-*pTyr-Glu-Thr-Leu* (the SH2 binding epitopes are italicised), two out of four residues of both SH2 epitopes are not identical. The phosphorylated tyrosine and the leucine residue, which are identical, are most important for binding. Likewise, the two SH2 domains in Syk tSH2 are not identical, but there is a considerable amount of sequence similarity: 35% of the residues are identical, 63% are identical or conserved, and 75% are identical, conserved or semi-conserved (Fig. 1B).[18]

The affinity of a monovalent phosphorylated ITAM-tetrapeptide for Syk tSH2 is approximately 1000-fold lower than that of dpITAM.[10–12] Thus, for high affinity both phosphotyrosine epitopes are needed and they should be connected to each other as in native ITAM. Several linkers have been designed to create high-affinity ITAM mimics, of which two (**5** and **6**) are depicted in Fig. 2.[11,12] Both compounds showed distinct multivalency effects and compound **5** had even similar affinity for Syk tSH2 as native ITAM. The fact that Syk tSH2 binds ITAM-based ligands with

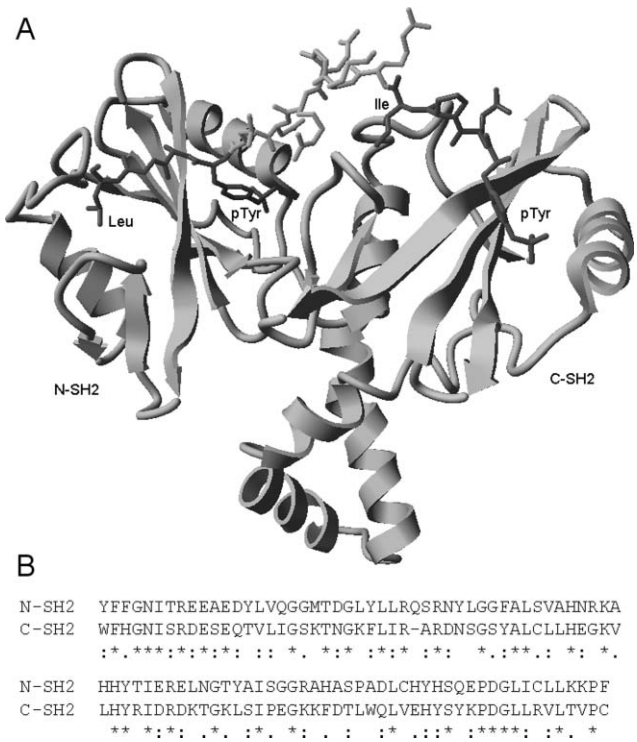

Fig. 1 Syk tSH2. **A**: Crystal structure of Syk tSH2 complexed with ITAM, derived from the CD3ε chain of the T cell receptor.[9] The two tetrapeptidic epitopes of ITAM are shown in black. The N-terminal ITAM epitope binds Syk C-SH2 and the C-terminal ITAM epitope binds Syk N-SH2. **B**: Alignment of the murine SH2 domains of Syk. * identical residues; : conserved residues; . semi-conserved residues.

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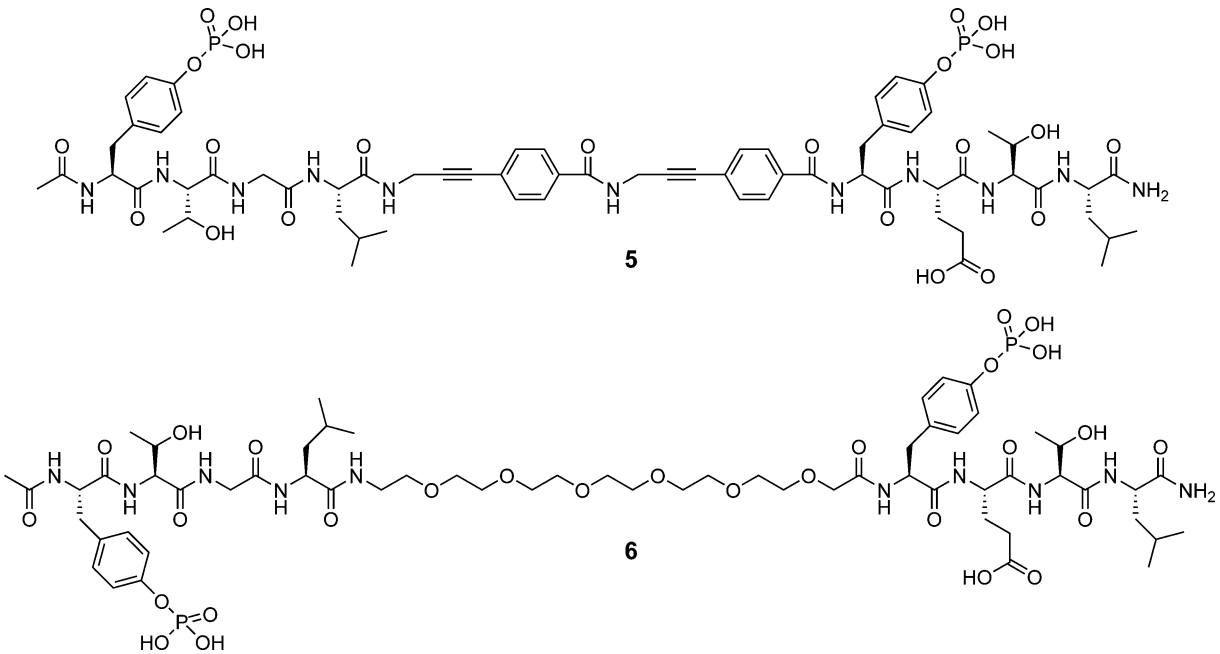


Fig. 2 Previously prepared Syk tSH2 ligands containing C-ITAM.[12] The affinities for Syk tSH2 of compounds **5** and **6** are, respectively, 1000-fold and 100-fold higher than C-ITAM.

varying distances between the SH2 binding epitopes with high affinity indicates that this protein possesses a considerable degree of flexibility. With this in mind, we wanted to explore the possibility of incorporating completely different linkers between the two binding epitopes, while still retaining acceptable binding affinity. For this, multivalent dendrimeric scaffolds were very appropriate.

Dendrimers are often successfully applied as multivalent ligands for proteins.[19–23] In this study our versatile amino acid based dendrimers were used (Scheme 1) outfitted with alkyne moieties suitable for ligand attachment by 'click' chemistry.[24,25] Monovalent up to octavalent dendrimers were functionalized with ITAM-phosphotetrapeptide **7**, yielding ligands with various valencies and various lengths between the SH2 binding epitopes. The affinity for Syk tSH2 of the functionalized dendrimers has been assayed in SPR competition experiments.

Results and discussion

Design

The design of multivalent ligands for Syk tSH2 started with the selection of one of the two tetrapeptidic epitopes of ITAM. The C-terminal epitope (C-ITAM: pTyr-Glu-Thr-Leu) binds Syk N-SH2 and the N-terminal epitope (N-ITAM: pTyr-Thr-Gly-Leu) binds Syk C-SH2 (Fig. 1A).[9] The full-length ITAM phosphopeptide with only phosphorylation of the C-terminal tyrosine residue binds Syk tSH2 with a slightly higher affinity than when the N-terminal tyrosine residue is phosphorylated (K_D = 1.3 μM and 7.2 μM, respectively).[26] Furthermore, C-ITAM binds Syk C-SH2 with somewhat higher affinity than N-ITAM does (IC_{50} = 25 μM and 48 μM, respectively).[27] Therefore, the C-ITAM phosphopeptide was chosen for attachment to the dendrimers. In addition, the C-ITAM phosphopeptide incorporated in *e.g.* diphosphopeptide divalent molecular constructs **5** and **6** has been used in previous studies to improve the monovalent interaction.[10–12]

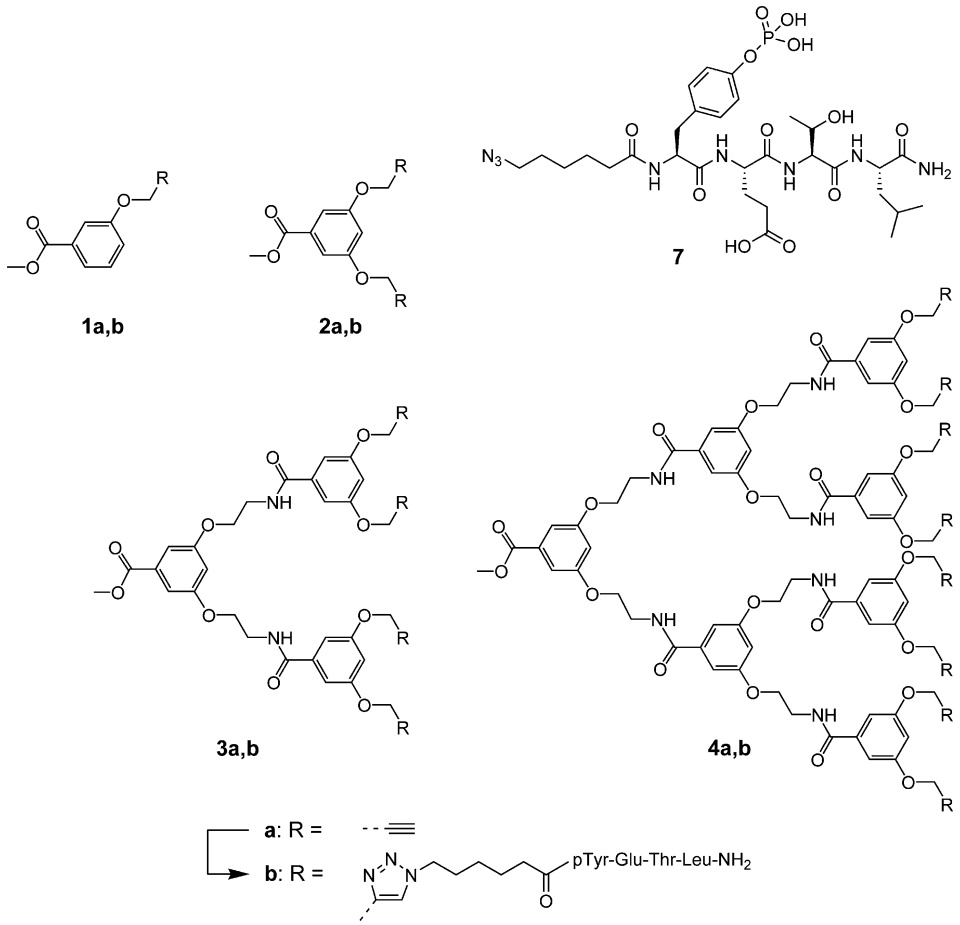
Synthesis

The tetrapeptide (pTyr-Glu-Thr-Leu) was attached to dendrimers with a different number of alkyne endgroups *via* 'click' chemistry, that is the copper(I) catalyzed [3 + 2] cycloaddition between azides and alkynes.[21,25,28–30] Consequently, an azide functionality had to be introduced into the peptide. Furthermore, a spacer between the peptide and the dendrimers was introduced to provide sufficient space for bridging both SH2 domains in Syk tSH2 and therefore the 'clickable' phosphopeptide **7** (Scheme 1) containing a 6-azidohexanoic acid spacer was prepared.

Phosphopeptide **7** was assembled on the solid phase, and the synthesis of the dendrimers has been described previously.[25] The dendrimers were subsequently functionalized with the azidophosphopeptide using 'click' chemistry (Scheme 1). To ensure completion of the reaction, 1.5 equivalent of phosphopeptide per arm was added. All reactions were complete after 20 min using microwave heating. The yield of monovalent structure **1b**, after RP-HPLC purification, was very good (94%). The yields, after purification of the other dendrimers **2b**, **3b** and especially **4b**, were very satisfactory.

Binding studies

The binding of the dendrimers to Syk tSH2 was examined using surface plasmon resonance (SPR) as was described previously.[11] Native γ-dpITAM containing an N-terminal 6-aminohexanoic acid spacer was immobilized on a SPR sensor chip. First, the affinity of Syk tSH2 for the immobilized native ITAM peptide was determined by addition of different concentrations of Syk tSH2 and the equilibrium signal was fitted to a Langmuir binding isotherm



Scheme 1 Synthesis of the dendrimers functionalized with phosphopeptide **7**. General reaction conditions: 1.5 equiv **7** per arm, 0.5 equiv $CuSO_4 \cdot 5H_2O$ and 0.5 equiv sodium ascorbate per arm in DMF/H_2O, 80 °C, microwave heating, 20 min. Yields: **1b** 94%, **2b** 53%, **3b** 50%, **4b** 56%.

(Fig. 3A). The K_D value for binding to the chip (K_C) was found to be 7.8 nM, which is comparable to the 5.6 nM affinity found earlier.[31] Then SPR competition experiments were performed by adding different concentrations of the phosphopeptide-containing dendrimers in the presence of 25 nM Syk tSH2 to evaluate the affinity of the compounds for Syk tSH2. From the obtained inhibition curves the dissociation constants were calculated by fitting to a competition model yielding thermodynamic binding constants in solution (K_S) (Fig. 3B and Table 1).[32]

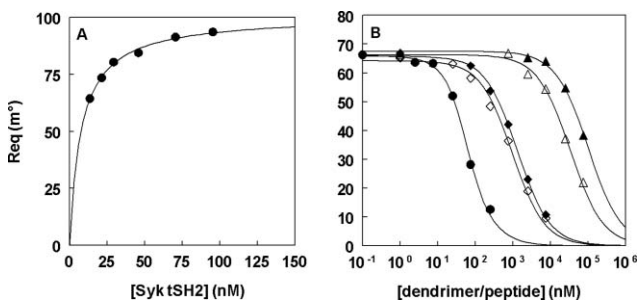


Fig. 3 **A**: SPR determination of the affinity of Syk tSH2 for immobilized γ-dpITAM phosphopeptide on the sensor surface (K_C). Data of equilibrium signals are fitted with a Langmuir binding isotherm. **B**: SPR competition experiments in the presence of 25 nM Syk tSH2. Data are fitted with a competition model yielding the affinity in solution (K_S) as described.[32] The inhibition curves represent γ-dpITAM (●), **1b** (▲), **2b** (△), **3b** (◆) and **4b** (◇).

Table 1 Affinities of the phosphopeptide-containing dendrimers and two references peptides for Syk tSH2 from SPR competition experiments (K_S values)

Compound	Valency	K_S (nM)	Relative potency (per peptide)
Ac-pTyr-Glu-Thr-Leu-NH_2 (ref. 10)	1	27000	—
Native ITAM	2	8.3 ± 0.7	—
1b	1	19810 ± 1168	1 (1)
2b	2	6518 ± 565	3.0 (1.5)
3b	4	252 ± 16	79 (20)
4b	8	183 ± 17	108 (14)

The affinity of monovalent phosphopeptide construct **1b** for Syk tSH2 is 20 μM. This is very similar to the K_D value of Ac-pTyr-Glu-Thr-Leu-NH_2, which is 27 μM.[10] Divalent compound **2b** shows a very modest increase in affinity (K_S = 6.5 μM), which is explained by the two-fold increase in binding sites and is still indicative of a monovalent interaction. The fact that **2b** is still monovalently bound can be rationalized by the fact that the orientation of the two epitopes with respect to each other is inverted in **2b** compared to ITAM. This also explains

why this divalent construct is significantly less active than the earlier reported divalent constructs **5** and **6**. Thus, compound **2b** might be considered as H_2N-Leu-Thr-Glu-pTyr-dendrimer-pTyr-Glu-Thr-Leu-NH_2, whereas the ITAM sequence is pTyr-Thr-Gly-Leu-$(Xxx)_7$-pTyr-Glu-Thr-Leu. The space between both phosphotyrosine-containing epitopes is apparently not enough to allow repositioning and truly bivalent binding.

The tetravalent phosphopeptide-containing dendrimer **3b** did bind with a significantly higher affinity than **1b**, showing a distinct multivalency effect. The K_S value was 252 nM, which is 79 times lower than the value for the monovalent dendrimer **1b**. This means that the relative potency of **3b** per peptide is 20. Octavalent phosphopeptide-containing **4b** displayed a similar affinity as phosphopeptide-containing **3b**. Because of this, the relative potency per peptide is lower, although the affinity for tSH2 is slightly higher than of **3b**. The similar potency of dendrimer **3b** and **4b** might indicate that upon binding of one Syk tSH2 protein, there is no space left to bind a second Syk tSH2.

The tetra- and octavalent phosphopeptide-containing dendrimers **3b** and **4b** had similar affinities for Syk tSH2 as previously synthesized ITAM mimic **6**.[12] However, none of these dendrimers had a similar affinity to the native γ-dpITAM peptide or ITAM mimic **5**. A possible explanation for this lower affinity might be that the orientation of the two SH2 epitopes in the dendrimers is not optimal for binding, as was discussed above for **2b**. All phosphopeptide epitopes were N-terminally conjugated to the alkyne-containing dendrimers, whereas in native ITAM and in the mimics **5** and **6** the N-terminus of one epitope (C-ITAM) was connected to the C-terminus of the other epitope (N-ITAM) (Fig. 4). Hence, to mimic ITAM better, ultimately it might be preferable to functionalize the dendrimers with a mixture of epitopes possessing N-terminal and C-terminal azides. However, this would yield mixtures of phosphopeptide-containing dendrimers, which are difficult to characterize. Furthermore, the aim of this study was to prepare relative simple phosphodendrimers with high affinity for Syk tSH2. Therefore, we chose to attach all peptidic epitopes N-terminally, which resulted in pure compounds that could be fully characterized and displayed considerable multivalency effects.

In addition to divalent binding *via* a chelation mechanism,[2] addressed above, also other mechanisms of multivalent binding may take place, such as statistical rebinding.[5,33,34] This effect is caused by an overall slower off-rate of the multivalent ligand due to the close proximity of, in this case, other phospho-epitopes, which can replace the bound epitope when released from one of the SH2 domains. Comparing **3b** with **4b**, in **4b** more phospho-epitopes are available (Fig. 4), which could lead to enhanced rebinding. However, no marked increase in affinity is observed, which might indicate that these additional epitopes are not within reach of an SH2 domain from which a bound epitope is released.

Because chelation was probably the predominant factor of the enhanced binding affinity, it is important that the dendrimers are large enough to bridge the distance between the two SH2 domains of Syk tSH2. When no ligand is present, tSH2 probably has an extended conformation in which the two SH2 domains are more apart from each other (left cartoon in Fig. 4).[31] The SH2 domains move closer to one another upon binding of the native ITAM sequence.[31] It was expected that the distance between the alkyne endgroups in the dendrimers was not sufficiently large to bridge the two SH2 domains of Syk tSH2. Therefore, a flexible 6-azidohexanoic acid spacer was introduced between the dendrimeric scaffold and the SH2-binding epitopes for providing enough length and flexibility to adapt the construct to the correct shape for binding. Clearly, this did not work for divalent compound **2b**. In the tetravalent and octavalent dendrimers **3b** and **4b**, however, there is sufficient space for bridging the two SH2 domains, indicated by the strongly increased affinity. The fact that **3b** and **4b** had similar affinities for Syk tSH2 indicates that in the larger **4b** inter-SH2 binding distances are not better than in **3b**.

The lower affinities of the dendrimers compared to native ITAM showed that the dendrimeric phosphopeptide ligands cannot fully adapt their orientation for optimal binding to Syk tSH2 and *vice versa*. Hence, despite the fact that Syk tSH2 is a relatively flexible protein,[31] complete adjustment of its conformation for a proper alignment with the tetravalent and octavalent compounds seems not to be possible.

The dendrimeric scaffolds are possibly not the only reason for the fact that the dendrimers cannot fully adapt their orientation for Syk tSH2 binding. Another reason for the lower affinity than native ITAM might be that in ITAM both SH2 domains bind their preferred optimal sequence of four amino acid residues. In the case of the phosphodendrimers, C-SH2 has to bind to a non-native sequence, as was discussed in the Design section. Although

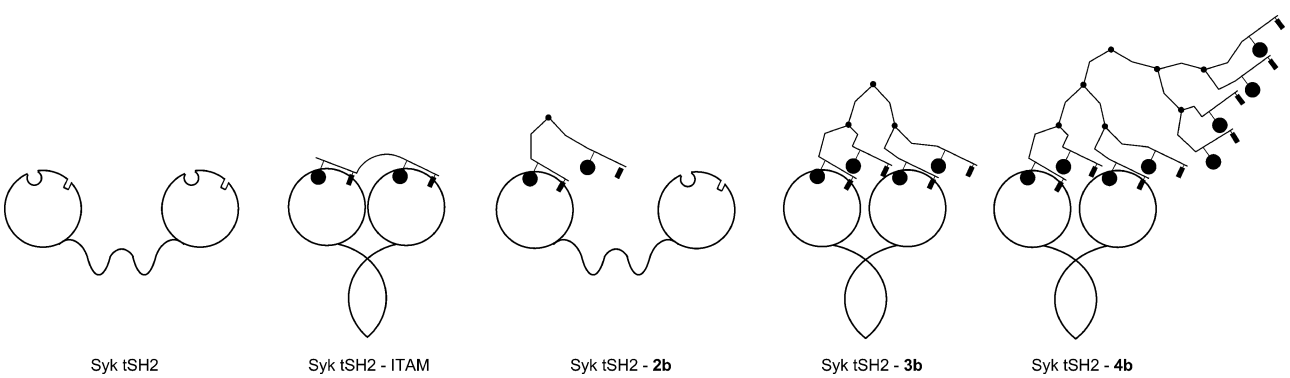


Fig. 4 Models of possible binding to Syk tSH2. When Syk tSH2 is unbound, then the SH2 domains are more apart from each other (left cartoon).[31] Ligands, which can bind divalently, such as ITAM, **3b** and **4b**, are likely capable to bring the SH2 domains closer to one another,[31] which could be important for Syk kinase activation, as has been suggested for the closely related Zap-70 kinase.[36,37] The black circles represent the side-chains of pTyr and the black rectangles represent the side-chains of Leu.

the effect of the absence of the native C-SH2 ligand is probably minor, it is possible that dendrimers containing both C-ITAM and N-ITAM epitopes will bind Syk tSH2 with higher affinity.

Conclusions

An azide-containing phosphopeptide ligand for binding to Syk N-SH2 was synthesized and efficiently coupled by a copper(I) catalyzed [3 + 2] cycloaddition ('click') reaction to different generations of alkyne-containing dendrimers. The affinity of the resulting phosphopeptide-containing dendrimers for Syk tSH2 was determined by SPR in competition assays. The affinity of the monovalent construct **1b** and the divalent phosphopeptide-containing dendrimer **2b** was in the same order of magnitude as the phosphopeptide itself. The tetravalent and octavalent phosphopeptide-containing dendrimers **3b** and **4b** showed indeed a multivalency effect and they both had K_D values in a range comparable to previously synthesized ITAM mimics.[11–13] The affinity might be improved further by using multivalent phosphopeptide dendrimeric constructs containing both C-terminally and N-terminally linked phosphopeptides to dendrimers, which is under present investigation.

Experimental section

General

All chemicals were obtained from commercial sources and used without further purification. Solvents, which were used for the solid-phase peptide synthesis, were stored over 4 Å molecular sieves. 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 Cl_2-TDM (*N*,*N*,*N*′,*N*′-tetramethyl-4,4′-diaminodiphenylmethane) staining. Solvents were removed under reduced pressure at a temperature of 40 °C. Column chromatography was performed on silica gel.

^{1}H NMR spectra were measured on a Varian Mercury plus 300 MHz spectrometer or a Varian Inova 500 MHz spectrometer and the chemical shifts are given in ppm (δ) relative to TMS, except for **7** (MeOH) and for **1b** and **2b** (dioxane). ^{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 $CDCl_3$. The ^{13}C NMR spectra were measured using the attached proton test (APT). Microwave reactions were carried out in a Biotage Initiator microwave oven. The microwave power was limited by temperature control once the desired temperature was reached. A sealed vessel of 0.5–2 mL was used. The crude peptide and dendrimers were analyzed with an analytical Shimadzu HPLC system with a UV detector operating at 220 and 254 nm using an Alltech Alltima C8 90 Å 5 μm (250 × 4.6 mm) column. For analytical HPLC a gradient of 100% buffer A (15 mM TEA in H_2O titrated at pH 6 with 85% H_3PO_4) to 100% buffer B (buffer A/CH_3CN 1:9) in 20 min was used. For preparative HPLC a Gilson system with a UV detector operating at 220 and 254 nm equipped with an Alltech Alltima C8 100 Å 10 μm (250 × 22 mm) column was used. A gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5) to 100% buffer B (0.1% TFA in H_2O/CH_3CN 5:95) in 100 min was used unless otherwise stated. The purity of the preparative HPLC fractions was assessed with analytical HLPC using the same conditions as stated above.

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).

6-Azidohexanoic acid

6-Bromohexanoic acid (1.95 g, 10 mmol) and NaN_3 (3.25 g, 50 mmol) were added to 100 mL DMF and the mixture was stirred overnight at 100 °C. DMF was evaporated and EtOAc was added. The organic phase was washed with 1 M $KHSO_4$ (3×) and brine, dried with Na_2SO_4, filtered and concentrated yielding 1.57 g of 6-azidohexanoic acid as a brown oil in quantitative yield. The product was used without further purification. R_f = 0.44 (0.5% CH_3COOH in CH_2Cl_2/MeOH 95:5). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 1.41–1.49 (m, 2H, CH_2), 1.58–1.73 (m, 4H, 2 CH_2), 2.34–2.41 (m, 2H, C*H*$_2$COOH), 3.29 (t, 2H, N_3CH_2), 11.28 (bs, 1H, COOH). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 23.8, 27.6, 32.3, 33.4, 33.8, (5 CH_2), 179.6 (COOH).

Azido phosphopeptide 7

The peptide was manually assembled on Tentagel®-Rink-NH-Fmoc resin (1.92 g, 0.50 mmol, loading 0.26 mmol/g) using standard Fmoc/tBu chemistry. The Fmoc protecting group was removed using 20% piperidine in NMP (3 × 8 min) followed by washing 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 (2.0 mmol), 4 equivalents of HOBt and HBTU and 8 equivalents of DiPEA in NMP (10 mL) and coupled during a coupling time of 60 minutes. The resin was washed with NMP (3 × 1 min) and CH_2Cl_2 (3 × 1 min) after every coupling step. The coupling steps and deprotection steps were monitored using the Kaisertest.[35] The amino acid building blocks Fmoc-Leu-OH, Fmoc-Thr(tBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Tyr(OP(OBn)OH)-OH and 6-azidohexanoic acid were subsequently coupled. Fmoc-Tyr(OP(OBn)OH)-OH was coupled overnight using 2 equivalents of amino acid, 2 equivalents of the coupling reagents HBTU and HOBt and 5 equivalents of DiPEA. After the 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 the Fmoc deprotection step. This was done to replace the piperidine counterion of Tyr(OP(OBn)OH) for DiPEA. When all the coupling steps and deprotection steps were completed 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 260 mg of the crude peptide. 150 mg of the peptide was purified by preparative HPLC. A gradient of 100% buffer A to 100% buffer B in 40 minutes was used. The product was obtained after lyophilization as a fluffy white solid (71.2 mg).

HRMS (ESI): $[M + H]^+$ calculated 743.3129, found 743.3163.

^{1}H NMR (D_2O, 300 MHz) δ = 0.91, 0.97 (2d, 6H, 2 CH_3 Leu), 1.25 (d, 3H, Thr CH_3), 1.26–1.30 (m, 2H, Azhx CH_2), 1.50–1.62

(m, 4H, Azhx 2 CH_2), 1.66–1.76 (m, 3H, Leu β CH_2 and δ CH), 1.97–2.14 (m, 2H, Glu β CH_2), 2.27 (t, 2H, Azhx CH_2), 2.44 (dd, 2H, Glu δ CH_2), 2.94–3.17 (m, 2H, pTyr β CH_2), 3.30 (t, 2H, Azhx CH_2), 4.19–4.23 (m, 1H, Thr β CH), 4.34 (d, 1H, Thr α CH), 4.38–4.44 (m, 2H, Leu α CH, Glu α CH), 4.59 (dd, 1H, pTyr α CH), 7.16, 7.25 (2d, 4H, ar pTyr).

Dendrimers 1a, 2a, 3a, 4a

The synthesis of these dendrimers was described earlier.[25,30]

Phosphopeptide-containing dendrimer 1b

A solution of **1a** (2.85 mg, 15 μmol), **7** (16.71 mg, 22.5 μmol), $CuSO_4 \cdot 5H_2O$ (1.87 mg, 7.5 μmol) and sodium ascorbate (1.49 mg, 7.5 μmol) in 1 mL DMF and 100 μL H_2O was heated under microwave irradiation at 80 °C for 20 min. Analytical HPLC showed complete consumption of **1a**. The mixture was concentrated and subjected to purification by preparative HPLC. The product was obtained after lyophilization as a fluffy white solid (13.1 mg, 94%).

HRMS (ESI): $[M + Na]^+$ calculated 955.3657, found 955.3717.

^{1}H NMR (D_2O, 500 MHz) δ = 0.84, 0.90 (2d, 6H, 2 CH_3 Leu), 0.93–0.98 (m, 2H, Azhx CH_2), 1.20 (d, 3H, Thr CH_3), 1.43 (bs, 2H, Azhx CH_2), 1.56–1.67 (m, 3H, Leu β CH_2 and δ CH), 1.80 (bs, 2H, Azhx CH_2), 1.89–2.08 (m, 2H, Glu β CH_2), 2.14 (bs, 2H, Azhx CH_2), 2.37 (bs, 2H, Glu δ CH_2), 2.90–3.05 (m, 2H, pTyr β CH_2), 3.90 (s, 3H, CH_3OOC), 4.17 (m, 1H, Thr β CH), 4.29 (m, 1H, Thr α CH), 4.38 (m, 1H, Leu α CH), 4.55 (m, 1H, Glu α CH), 4.66 (bd, 1H, pTyr α CH), 4.78 (bs, 4H, Azhx CH_2 and CH_2CCH), 7.07, 7.14 (2d, 4H, ar pTyr), 7.29, 7.45, 7.63 (3 m, 4H, ar), 8.08 (s, 1H, $CH_{triazole}$), 8.25, 8.29, 8.36, 8.39 (4d, 4H, 4 NH).

Phosphopeptide-containing dendrimer 2b

A solution of **2a** (1.83 mg, 7.5 μmol), **7** (16.71 mg, 22.5 μmol), $CuSO_4 \cdot 5H_2O$ (1.87 mg, 7.5 μmol) and sodium ascorbate (1.49 mg, 7.5 μmol) in 1 mL DMF and 100 μL H_2O was heated under microwave irradiation at 80 °C for 20 min. Analytical HPLC showed complete consumption of **2a**. The mixture was concentrated and subjected to purification by preparative HPLC. The product was obtained after lyophilization as a fluffy white solid (6.9 mg, 53%).

HRMS (ESI): $[M + H]^+$ calculated 1729.6916, found 1729.5875; $[M + Na]^+$ calculated 1751.6735, found 1751.6201; $[M + 2H]^{2+}$ calculated 865.3497, found 865.324; $[M + H + Na]^{2+}$ calculated 876.3407, found 876.2612; $[M + 2Na]^{2+}$ calculated 887.3317, found 887.2672.

^{1}H NMR (D_2O, 500 MHz) δ = 0.83, 0.88 (2d, 12H, 4 CH_3 Leu), 0.94 (m, 4H, 2 Azhx CH_2), 1.20 (s, 6H, 2 Thr CH_3), 1.42 (bs, 4H, 2 Azhx CH_2), 1.55–1.66 (m, 6H, 2 Leu β CH_2 and δ CH), 1.78 (bs, 4H, 2 Azhx CH_2), 1.89–2.06 (m, 4H, 2 Glu β CH_2), 2.13 (bs, 4H, 2 Azhx CH_2), 2.36 (bs, 4H, 2 Glu δ CH_2), 2.89–3.01 (m, 4H, 2 pTyr β CH_2), 3.89 (s, 3H, CH_3OOC), 4.17 (m, 2H, 2 Thr β CH), 4.29 (m, 2H, 2 Thr α CH), 4.37 (m, 2H, 2 Leu α CH), 4.56 (m, 2H, 2 Glu α CH), 4.68 (bd, 2H, 2 pTyr α CH), 4.81 (bs, 8H, 2 Azhx CH_2 and 2 CH_2CCH), 6.89 (s, 4H, 2 NH_2), 7.06, 7.09 (2d, 8H, 2 ar pTyr), 7.16, 7.27, 7.64 (3 m, 3H, ar), 8.06 (s, 2H, 2 $CH_{triazole}$), 8.22, 8.27, 8.36, 8.37 (4d, 8H, 8 NH).

Phosphopeptide-containing dendrimer 3b

A solution of **3a** (2.55 mg, 3.75 μmol), **7** (16.71 mg, 22.5 μmol), $CuSO_4 \cdot 5H_2O$ (1.87 mg, 7.5 μmol) and sodium ascorbate (1.49 mg, 7.5 μmol) in 1 mL DMF and 100 μL H_2O was heated under microwave irradiation at 80 °C for 20 min. Analytical HPLC showed complete consumption of **3a**. The mixture was concentrated and subjected to purification by preparative HPLC. The product was obtained after lyophilization as a fluffy white solid (6.8 mg, 50%).

HRMS (ESI): $[M + H + Na]^{2+}$ calculated 1837.298, found 1837.514.

^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.82, 0.86 (2d, 24H, 8 CH_3 Leu), 1.04 (d, 12H, 4 Thr CH_3), 1.07–1.18 (m, 8H, 4 Azhx CH_2), 1.39–1.53 (m, 16H, 8 Azhx CH_2), 1.56–1.65 (m, 4H, 4 Leu δ CH), 1.73–1.83 (m, 8H, 4 Leu β CH_2), 1.91–2.09 (m, 16H, 4 Glu β CH_2 and 4 Azhx CH_2), 2.24–2.32 (m, 8H, 4 Glu δ CH_2), 2.69–2.73, 2.95–2.99 (2 m, 8H, 4 pTyr β CH_2), 3.85 (s, 3H, CH_3OOC), 4.00 (t, 4H, 2 OCH_2CH_2NH), 4.18–4.33 (m, 28H, 4 Thr β CH and 4 Thr α CH and 4 Leu α CH and 4 Glu α CH and 4 Azhx CH_2 and 2 OCH_2CH_2NH), 4.51 (m, 4H, 4 pTyr α CH), 5.17 (s, 8H, 4 CH_2CCH), 6.92 (s, 8H, 4 NH_2), 7.04 (d, 8H, ar pTyr), 7.18–7.27 (m, 17H, ar pTyr and ar), 7.72 (d, 4H, 4 Thr NH), 7.75 (d, 4H, 4 Leu NH), 8.04 (d, 4H, 4 pTyr NH), 8.22 (s, 4H, 4 $CH_{triazole}$), 8.24 (d, 4H, 4 Glu NH), 9.01 (bs, 2H, 2 OCH_2CH_2NH).

Phosphopeptide-containing dendrimer 4b

A solution of **4a** (2.90 mg, 1.875 μmol), **7** (16.71 mg, 22.5 μmol), $CuSO_4 \cdot 5H_2O$ (1.87 mg, 7.5 μmol) and sodium ascorbate (1.49 mg, 7.5 μmol) in 1 mL DMF and 100 μL H_2O was heated under microwave irradiation at 80 °C for 20 min. Analytical HPLC showed complete consumption of **4a**. The mixture was concentrated and subjected to purification by preparative HPLC. The product was obtained after lyophilization as a fluffy white solid (7.9 mg, 56%).

HRMS (ESI): $[M + 4H]^{4+}$ calculated 1873.353, found 1873.312; $[M + 3H + Na]^{4+}$ calculated 1879.101, found 1879.177; $[M + 4H + Na]^{5+}$ calculated 1503.482, found 1503.581.

^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.81, 0.86 (2d, 48H, 16 CH_3 Leu), 1.03 (d, 24H, 8 Thr CH_3), 1.07–1.12 (m, 16H, 8 Azhx CH_2), 1.39–1.49 (m, 32H, 16 Azhx CH_2), 1.55–1.62 (m, 8H, 8 Leu δ CH), 1.72–1.77 (m, 16H, 8 Leu β CH_2), 1.91–2.03 (m, 32H, 8 Glu β CH_2 and 8 Azhx CH_2), 2.24–2.29 (m, 16H, 8 Glu δ CH_2), 2.69–2.73, 2.95–2.99 (2 m, 16H, 8 pTyr β CH_2), 3.79 (s, 3H, CH_3OOC), 4.00–4.02 (m, 12H, 6 OCH_2CH_2NH), 4.14–4.36 (m, 60H, 8 Thr β CH and 8 Thr α CH and 8 Leu α CH and 8 Glu α CH and 8 Azhx CH_2 and 6 OCH_2CH_2NH), 4.51 (m, 8H, 8 pTyr α CH), 5.15 (s, 16H, 8 CH_2CCH), 6.90 (s, 16H, 8 NH_2), 7.04 (d, 16H, ar pTyr), 7.15–7.27 (m, 37H, ar pTyr and ar), 7.75 (d, 8H, 8 Thr NH), 7.83 (d, 8H, 8 Leu NH), 8.04 (d, 8H, 8 pTyr NH), 8.21 (s, 8H, 8 $CH_{triazole}$), 8.24 (d, 8H, 8 Glu NH), 8.68 (bs, 6H, 6 OCH_2CH_2NH).

SPR binding studies

Stock solutions of dendrimers **1b**, **2b**, **3b** and **4b** with a concentration of 1 mM in HEPES-buffered saline (HBS) buffer were prepared. For **3b** and **4b** 17% DMSO was present in this stock solution to keep the compounds dissolved. The sensor chip was immobilized with the native γ-dpITAM peptide as was described earlier.[11] The affinity of (murine) Syk tSH2 for the immobilized ITAM peptide was determined by addition of Syk tSH2 in a

concentration range of 0 to 100 nM in HBS buffer. The K_C value was calculated by fitting the equilibrium signals to a Langmuir binding isotherm.

Competition experiments were performed with different concentrations of the phosphopeptide-containing dendrimers in the presence of 25 nM Syk tSH2 in HBS buffer. K_S values were calculated according to described procedures.[32]

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