Cell permeable ITAM constructs for the modulation of mediator release in mast cells

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Spleen tyrosine kinase (Syk) is essential for high affinity IgE receptor (FcεRI) mediated mast cell degranulation. Once FcεRI is stimulated, intracellular ITAM motifs of the receptor are diphosphorylated (dpITAM) and Syk is recruited to the receptor by binding of the Syk tandem SH2 domain to dpITAM, resulting in activation of Syk and, eventually, degranulation. To investigate intracellular effects of ITAM mimics, constructs were synthesized with ITAM mimics conjugated to different cell penetrating peptides, *i.e.* Tat, TP10, octa-Arg and K(Myr)KKK, or a lipophilic C_{12}-chain. In most constructs the cargo and carrier were linked to each other through a disulfide bridge, which is convenient for combining different cargos with different carriers and has the advantage that the cargo and the carrier may be separated by reduction of the disulfide once it is intracellular. The ability of these ITAM constructs to label RBL-2H3 cells was assessed using flow cytometry. Fluorescence microscopy showed that the octa-Arg-SS-Flu-ITAM construct was present in various parts of the cells, although it was not homogeneously distributed. In addition, cell penetrating constructs without fluorescent labels were synthesized to examine degranulation in RBL-2H3 cells. Octa-Arg-SS-ITAM stimulated the mediator release up to 140%, indicating that ITAM mimics may have the ability to activate non-receptor bound Syk.

Introduction

The first event in IgE receptor signaling in mast cells is IgE and antigen binding to the multimeric ($\alpha\beta\gamma_2$) high affinity IgE receptor (FcεRI), resulting in aggregation and stimulation of the receptor.[1–5] The β- and γ-chains of this FcεRI receptor 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. Once FcεRI is stimulated, γ-ITAM is diphosphorylated and the bold residues become binding epitopes for SH2 domains. Spleen tyrosine kinase (Syk) is an SH2 domain containing protein that binds diphosphorylated γ-ITAM (γ-dpITAM). Syk consists of a kinase domain and two SH2 domains in tandem (tSH2). Syk is recruited to the cell membrane by binding to γ-dpITAM, resulting in activation of its kinase domain.[3] Then Syk transfers the signal further, which eventually leads to cell degranulation and mediator release. Syk is essential for degranulation. Overstimulation of this cascade leads to allergic responses, and therefore it is interesting to inhibit the ITAM–Syk interaction as a potential target for anti-allergic therapy (Fig. 1).

Although there is much knowledge about the functioning of Syk,[6,7] the process of kinase activation, which includes phosphorylation of Syk on multiple sites, is not fully understood. For example, the aggregation of FcεRI receptors in lipid rafts could also be important for ITAM phosphorylation and Syk activation.[8]

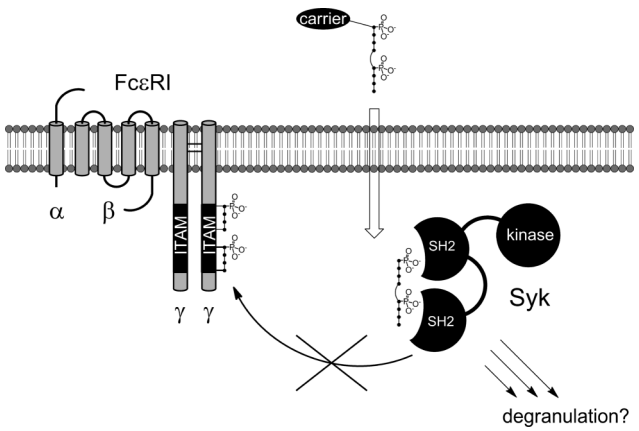

Fig. 1 Schematic overview of intervention in the FcεRIγ–Syk interaction, making use of a cell penetrating carrier coupled to an ITAM mimic.

In this study, the effect of a rigid, high affinity binding ITAM mimic on mast cell degranulation was evaluated. In this ITAM mimic, the seven intervening amino acid residues between the SH2 binding epitopes were replaced by a rigid linker without affecting the Syk binding affinity.[6,9] As a model for mast cells, rat basophilic leukemia (RBL-2H3) cells were used. These cells, having the IgE receptor signaling cascade, are widely studied as a model of secretory mucosal type mast cells.[10,11]

To allow penetration of the negatively charged dpITAM over the RBL-2H3 cell membrane, cell penetrating peptides (CPPs) or a lipophilic carrier were coupled to it. CPPs are often used to transport cargo into cells, allowing the study of cellular processes and protein-protein interactions in cells.[12–15] There is a large number

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of examples present in the literature of successful cell penetration, although it is still not possible to decide rationally which CPP to use, because cellular uptake is also dependent on the cargo, the cell type and the experimental settings.[15,16] For example, the translocation of negatively charged cargo (*e.g.* phosphopeptides) into cells is relatively difficult because of repulsion by the negative charges on cell membranes.[17–19]

Several cell penetrating carriers were selected to create a diverse set of constructs for studying cell penetration. Fluorescently labeled cell permeable constructs were synthesized and subjected to flow cytometry, to quantify the cell labeling. Then the cellular distribution of the most effective constructs was evaluated with fluorescence microscopy. Finally, constructs without a fluorescent label were also synthesized and their effect on exocytosis was evaluated in RBL-2H3 cells.

Results and discussion

Selection of different cargos and carriers

Several carriers were selected to evaluate which would be most suitable (Fig. 2). The Tat peptide was included, because it is the

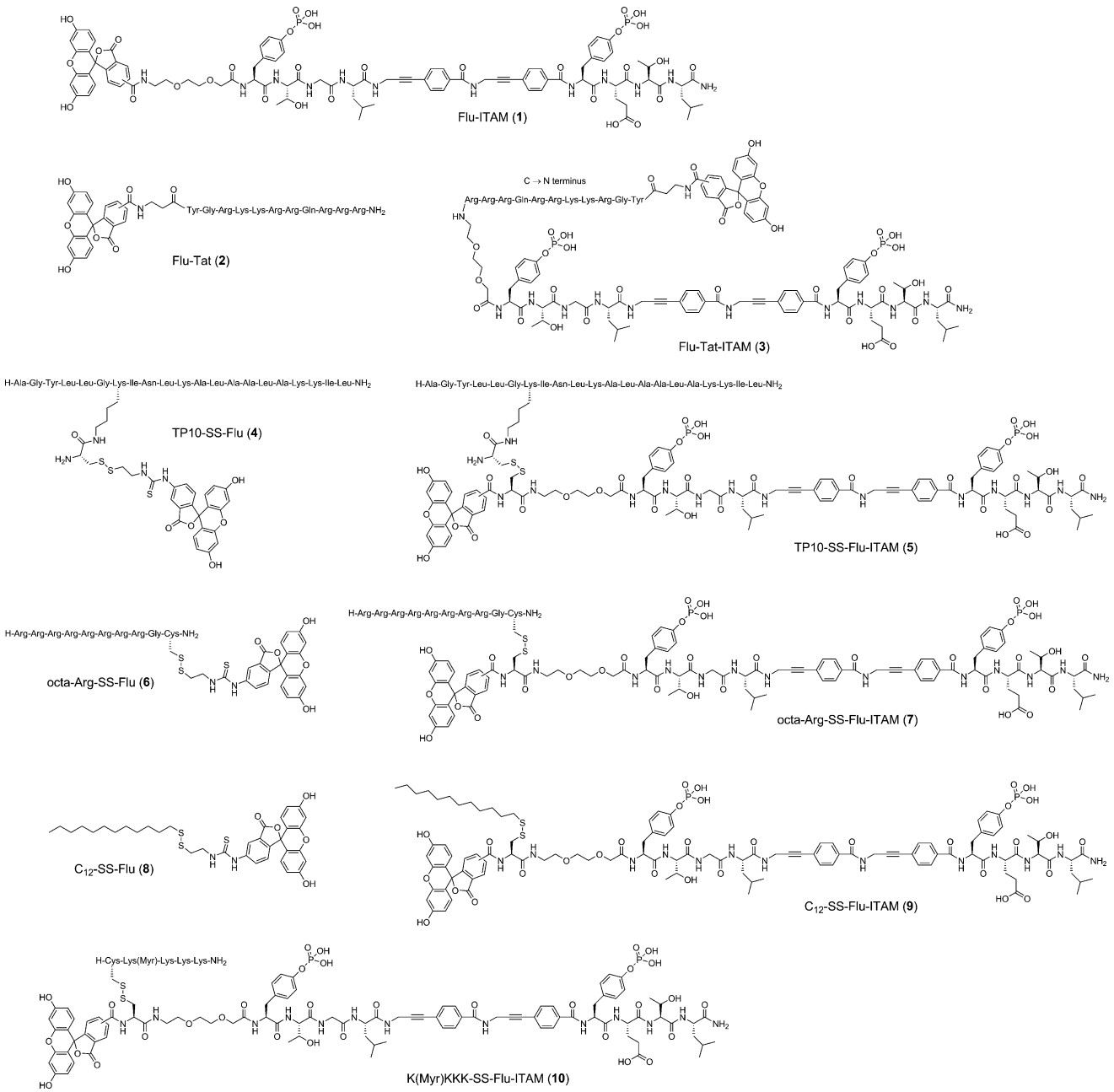


Fig. 2 Overview of all cell permeable constructs used in this study for flow cytometry analysis and fluorescence microscopy. Flu-Tat (**2**) and Flu-Tat-ITAM (**3**) were completely synthesized on the solid phase and therefore the cargo was attached to the carrier *via* an amide bond. TP10, octa-Arg, C_{12} and K(Myr)KKK were connected to the cargo *via* a disulfide bond. Abbreviations: Flu: fluorescein, SS: disulfide bond, Myr: myristate.

most frequently studied CPP.[20–23] Moreover, it has been reported that Tat is able to penetrate into RBL-2H3 cells.[24]

Transportan 10 (TP10), a deletion analog of transportan, is the only CPP which has been thoroughly studied for the delivery of peptides into RBL-2H3 cells.[25,26] In contrast to several other CPPs, TP10, consisting of 21 amino acid residues, possesses only 5 positive charges and no arginine residues.

Furthermore, a polyarginine containing peptide was selected. Octa-arginine was chosen as a polyarginine CPP, since it is a commonly used peptide which is able to penetrate many different cell types.[27–29]

A lipophilic alkyl chain was selected, because it has been shown that modification of peptides with a simple alkyl or fatty acid chain (*e.g.* myristoylation) allows cellular uptake.[30,31] Here, a C_{12} alkyl chain was chosen as an equivalent of a fatty acid chain.[32]

Finally, a combination of cationic residues and an alkyl chain was selected. For this, a peptide containing four lysine residues and a myristoyl group on one of the lysine side-chains was chosen (K(Myr)KKK).[24]

The cargo was composed of an ITAM mimic with two rigid amino propynyl benzoic acid building blocks replacing the seven intervening residues between the SH2 binding epitopes (Fig. 2).[6,9] This rigid ITAM mimic has similar affinity for Syk tSH2 as the native peptide[6,9] and is expected to be less prone to enzymatic degradation. The ITAM mimics were extended with an ethylene glycol spacer to avoid steric hindering. Fluorescein was attached to the N-terminus of the ITAM mimic to allow detection in flow cytometry and fluorescence microscopy.

A fluorescein moiety without ITAM was also used as cargo, as a control for the ability of the carriers to achieve the desired transport.

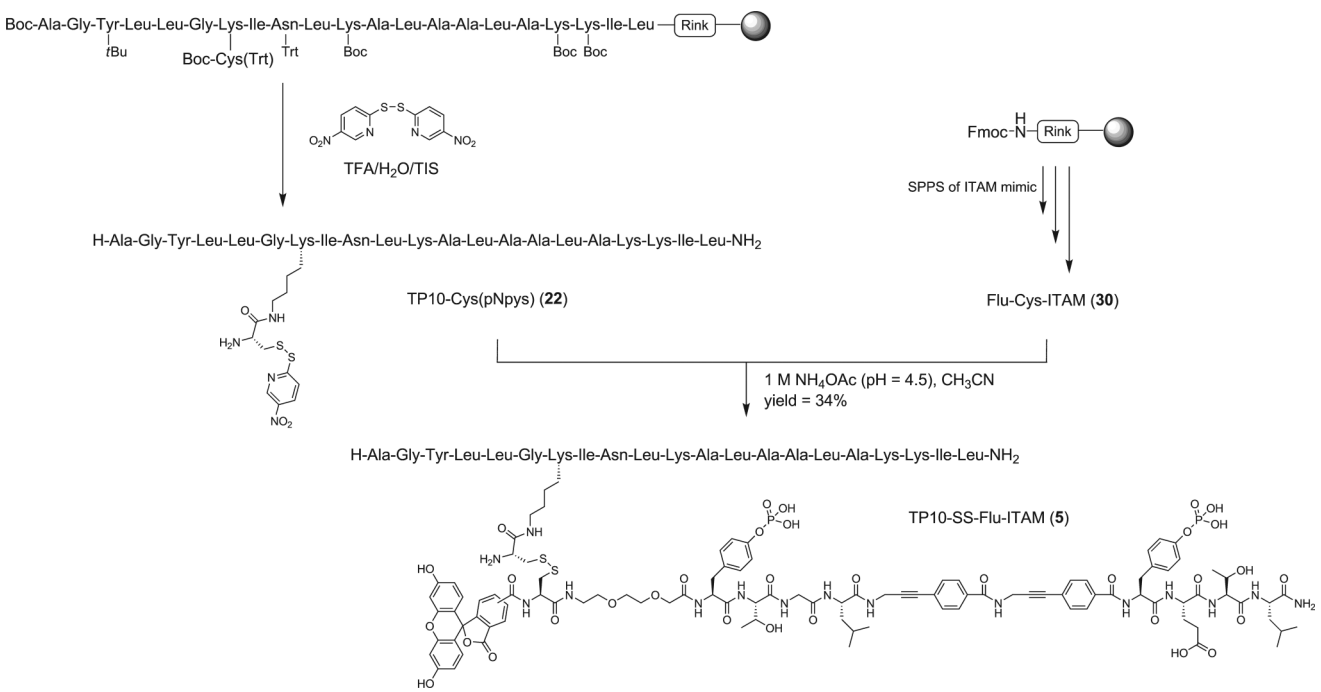
Synthesis of the fluorescein-labeled constructs

ITAM constructs **1**, **3**, **5**, **7**, **9** and **10** and the fluorescein constructs **2**, **4**, **6** and **8**, which were synthesized for flow cytometry and fluorescence microscopy, are shown in Fig. 2. Fluorescein labeled ITAM (**1**) as well as the two modified Tat peptides, Flu-Tat (**2**) and Flu-Tat-ITAM (**3**), were completely synthesized on the solid phase. In both these Tat containing peptides a β-alanine residue was attached to the N-terminus prior to the coupling of 5(6)-carboxyfluorescein (Fig. 2).

The other carriers were attached to the cargos *via* a disulfide bond leading to **4**–**10**. The advantage of a disulfide bond is that it easily allows combination of different carriers with different cargos. Another advantage is that a disulfide bond may be gradually cleaved inside cells and after that the carrier can no longer interfere with the bioactivity of the cargo.[33]

For mixed disulfide formation the thiol group of either the carrier or the cargo had to be activated using 2,2′-dithiobis(5-nitropyridine), which yields a *para*-nitro-2-pyridinesulfenyl activated thiol moiety (Scheme 1). The activation was performed during peptide cleavage from the resin[34] or after purification of the peptide.[35–37]

For the preparation of constructs in Fig. 2 several (peptide) building blocks had to be synthesized. The synthesis of the required compounds (**19**–**20** and **22**–**31**) is described in detail in the Experimental section. Since the synthetic strategy was similar for constructs **4**–**10**, only the synthesis of TP10-SS-Flu-ITAM (**5**) *via* **22** is shown as an illustrative example (Scheme 1). For optimal intracellular delivery TP10 should be connected *via* the side-chain of Lys7 to the cargo.[38] A known strategy for the attachment of the cargo to TP10 *via* a disulfide bond, is the introduction of a cysteine



Scheme 1 Synthesis of TP10-SS-Flu-ITAM (**5**). The TP10-carrier was first prepared on the resin with the Fmoc-Lys(Boc-Cys(Trt))-OH (**20**) building block incorporated into the sequence (seventh residue from the N-terminus). During cleavage and deprotection the cysteine was activated, yielding TP10-Cys(pNpys) (**22**). This carrier was conjugated to cargo Flu-ITAM (**30**) to yield the construct **5**.

residue attached to Lys7.[25,26] The Boc-Cys(Npys)-OH building block is frequently used for yielding directly the cysteine activated TP10. However, the 3-nitro-2-pyridinesulfenyl group is base labile and will be cleaved during Fmoc deprotection. Therefore, an activated cysteine residue as in **22** had to be introduced after completion of the assembly of the peptide. Because this strategy can be troublesome, instead the Fmoc-Lys(Boc-Cys(Trt))-OH (**20**) building block was incorporated during the solid phase peptide synthesis, which was prepared from Fmoc-Lys-OH and Boc-Cys(Trt)-OSu (**19**). Thus, after cleavage from the resin the thiol of the cysteine residue was directly activated using 2,2′-dithiobis(5-nitropyridine) (Scheme 1)[34] and the resulting activated TP10 carrier (TP10-Cys(pNpys), **22**) was purified by RP-HPLC.

The ITAM cargo **30** (Scheme 1) containing a cysteine residue, the ethyleneglycol spacer and an N-terminal fluorescein was synthesized on the solid phase (see Experimental section). The synthesis of the acetylene-containing rigid building block used has been described previously (see Experimental section).[9]

ITAM peptide **30** was reacted with the thiol activated TP10 (TP10-Cys(pNpys), **22**) under slightly acidic conditions to prevent homodimer formation of the free thiol (Scheme 1).[37] Within a few minutes a yellow color of the released 5-nitropyridine-2-thiol appeared. After the conjugation, the construct was purified by RP-HPLC and analyzed by analytical RP-HPLC and mass spectrometry. Similar strategies were applied for the synthesis of the other carrier-SS-Flu and carrier-SS-Flu-ITAM constructs.

Flow cytometry

Flow cytometry analysis was performed to quantify the cell labeling of the different constructs. First, the incubation conditions were established. The RBL-2H3 cells, which are adherent cells, were incubated with the constructs before or after detachment with trypsin. The histograms, displaying the amount of cell labeling, were comparable for both incubation conditions. However, the percentage of non-viable cells was lower when cells were incubated before detachment, as observed from the Forward Scatter and Side Scatter (data not shown). Therefore, incubation of the RBL-2H3 cells with the constructs was carried out when they were still attached to a 12 well plate. After incubation, the cells were washed, detached with trypsin, washed and analyzed with flow cytometry (Fig. 3). The trypsinization step after the incubation could also prevent over-estimation of the amount of construct present in the cells, since it has been shown that treatment of cells with trypsin reduces the amount of plasma membrane bound constructs by digestion of the CPP and/or membrane proteins.[15] For flow cytometry analysis the cells were gated on Forward Scatter and Side Scatter and approximately 10 000 viable cells were analyzed by making histograms with the amount of fluorescence on the *x*-axis and the number of cells on the *y*-axis. Untreated cells are in each histogram included as a reference (Fig. 3).

Cells incubated with the negative controls carboxyfluorescein and the fluorescein-containing ITAM mimic without a carrier (Flu-ITAM, **1**) showed no noticeable increase in fluorescence compared to the untreated cells even at 25 μM (data shown for 3 and 10 μM). With Flu-Tat (**2**) and, especially, Tat-Flu-ITAM (**3**) it seems that two populations of cells were present: one population with almost no cell labeling and one population with some labeling (Fig. 3).

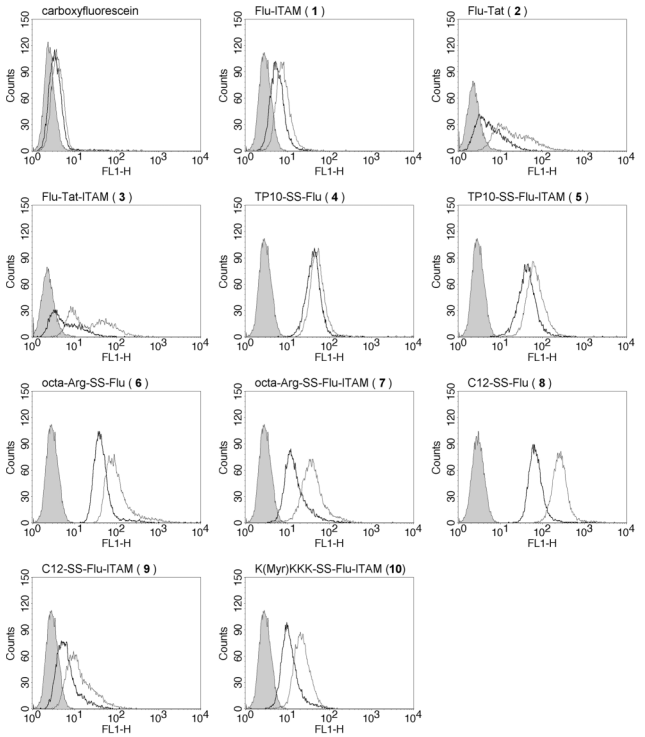


Fig. 3 Flow cytometry analysis of the cellular uptake of the fluorescein labeled constructs into RBL-2H3 cells. Each graph presents untreated cells (trace filled with grey), 3 μM compound (bold trace) and 10 μM compound (plain trace). The *x*-axis represents the fluorescence intensity and the *y*-axis the number of cells.

TP10 showed a large degree of cell labeling, irrespective of the cargo (**4** and **5** in Fig. 3). Also octa-Arg-SS-Flu (**6**) was capable of efficiently labeling the RBL-2H3 cells. The labeling with octa-Arg-SS-Flu-ITAM (**7**) was only slightly less than that of octa-Arg-SS-Flu. The lipid C_{12}-SS-Flu construct **8** displayed the highest levels of cell labeling with a geometric mean of 264.03 for 10 μM of **8** (for the untreated cells it was 2.87). In C_{12}-SS-Flu-ITAM (**9**), the relative hydrophilic ITAM mimic had a dramatic diminishing effect on cell labeling, showing approximately the same amount of fluorescent loading as for the negative control Flu-ITAM (**1**). The combination of a small cationic peptide and an alkyl chain (K(Myr)KKK, **10**) was moderately effective in cell labeling.

Fluorescence microscopy

Fluorescence microscopy was performed to evaluate the intracellular distribution of constructs in the cells. Because of the promising flow cytometry results, the distribution of TP10-SS-Flu-ITAM (**5**), octa-Arg-SS-Flu-ITAM (**7**) and C_{12}-SS-Flu (**8**) inside RBL-2H3 cells was assessed. The cells were incubated with 10 μM of a construct followed by washing steps. The viable cells were directly analyzed while still adherent.

Unexpectedly, cells incubated with TP10-SS-Flu-ITAM (**5**) showed almost no intracellular fluorescence under the microscope (Fig. 4), although the incubation conditions were very similar compared to those used for the flow cytometry experiments, except that in these experiments cells were detached by incubation with trypsin. However, it should be noted that a few bright spots were present in the cells, which might explain the positive

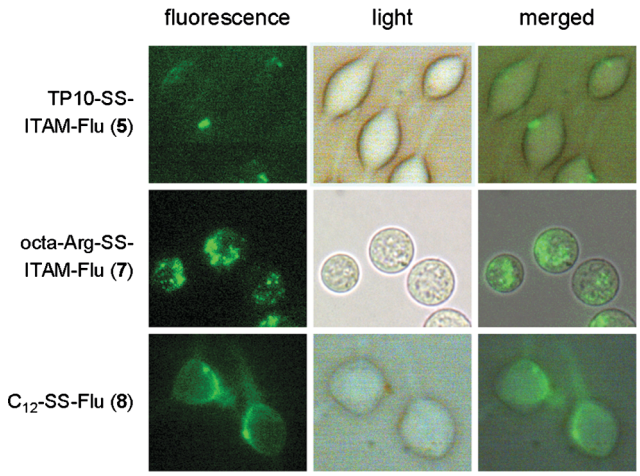


Fig. 4 The uptake and distribution of, TP10-SS-ITAM-Flu (**5**), octa-Arg-SS-ITAM-Flu (**7**) and C_{12}-SS-Flu (**8**) in RBL-2H3 cells. The cells were incubated with 10 μM compound for 1 h at 37 °C. After washing steps the viable cells were immediately analyzed with fluorescence microscopy.

flow cytometry results. The incubation was also performed with medium without fetal calf serum, but also then no change in intracellular fluorescence was detected.

Octa-Arg-SS-Flu-ITAM (**7**) could be visualized inside the RBL-2H3 cells with fluorescence microscopy. The octa-Arg construct was not homogeneously distributed and present in various parts of the cells.

Fluorescence microscopy showed that C_{12}-SS-Flu (**8**) was retained in the cell membrane (Fig. 4), being unable to penetrate into the cytosol. Apparently, the C_{12}-chain could not deliver the fluorescein cargo intracellularly.

Synthesis of non-fluorescent labeled constructs

After establishing the cellular uptake and distribution using the fluorescently labeled derivatives, the bioactivity of the most promising penetrating molecular constructs had to be evaluated. For this purpose the fluorescent label required for visualization in flow cytometry or fluorescent microscopy was not needed anymore, and therefore, was omitted. ITAM constructs with carriers Tat (**13**), TP10 (**14**), octa-Arg (**16**) and C_{12} (**17**) were synthesized (Fig. 5). Furthermore, a TP10 and a C_{12} construct with a nonphosphorylated ITAM mimic ("npITAM", compounds **15** and **18**) were synthesized as negative controls. The used synthetic strategy was analogous to that used for the fluorescently labeled constructs above. Peptide building blocks **21–22**, **24–25** and **32–34** were used for the preparation of constructs **14–18** as is described in detail in the Experimental section.

Affinity of cell penetrating ITAM constructs for Syk tSH2

Before assessing the effect of the constructs on IgE receptor mediated signaling in RBL-2H3 cells, first the affinity of the constructs for Syk tSH2 as well as the cytotoxicity was determined. The binding affinity was established with surface plasmon resonance (SPR) competition experiments, as described earlier.[39,40] The K_D values of native ITAM (**11**) and rigid ITAM mimic (**12**) were virtually identical to the previously reported values (Table 1 and Fig. 6).[6,9] The affinities of TP10-SS-ITAM (**14**), octa-Arg-SS-ITAM (**16**) and C_{12}-SS-ITAM (**17**) for Syk tSH2 were close to the affinity of the rigid ITAM mimic without a carrier (**12**). The only diphosphorylated construct with a much lower affinity was Tat-ITAM (**13**). Possibly, the positively charged residues in the Tat sequence bind to the two phosphate groups of ITAM, which reduces the accessibility of the phosphotyrosines for SH2 binding. The nonphosphorylated C_{12}-SS-npITAM (**18**) control construct showed no binding.

Table 1 Affinities of native ITAM, the rigid ITAM mimic and the cell permeable constructs for Syk tSH2 from SPR competition experiments

Compound	K_D/nM
native ITAM (**11**)	8.3 ± 0.7
rigid ITAM mimic (**12**)	5.3 ± 0.5
Tat-ITAM (**13**)	283 ± 21
TP10-SS-ITAM (**14**)	11.7 ± 3.2
octa-Arg-SS-ITAM (**16**)	29.7 ± 5.4
C_{12}-SS-ITAM (**17**)	26.6 ± 2.4
C_{12}-SS-npITAM (**18**)	>100 000

From the SPR data it can be concluded that all diphosphorylated constructs, except Tat-ITAM (**13**), bind Syk tSH2 without significant interference by the carrier part.

Viability assay

The cytotoxicity of the constructs was determined using an XTT viability assay[41] to exclude any artifacts in the bioactivity assay (vide infra) due to toxicity. The XTT assay determines the viability of living cells from their mitochondrial dehydrogenases, which convert XTT into the corresponding UV active orange formazan. Cells in 96-well plates were incubated with different concentrations of the ITAM constructs **13–17** for 1 h, which is the maximum incubation time used in all other cell experiments. After incubation the amount of viable cells was determined with an XTT assay (Fig. 7). It was found that concentrations of 10 μM and lower of all constructs are not toxic. At 20 μM all constructs showed some toxicity and therefore 10 μM was the highest concentration used in the degranulation assay.

Effect of cell penetrating ITAM constructs on degranulation

The effect of the cell penetrating constructs on RBL-2H3 degranulation was established by a β-hexosaminidase release assay.[42] In a typical experiment, first anti-DNP-IgE in medium was added to RBL-2H3 cells in a 96-well plate. After 1 h of incubation, the medium was aspirated and compounds were added in different concentrations. After 20 min of incubation, DNP_{30}-HSA was added to cross-link and stimulate the FcεRI receptors. Then the cells were allowed to degranulate for 30 min. Next the supernatant was collected, the cells were lyzed and the lysate was also collected. The amount of β-hexosaminidase present in the supernatant and the lysate was determined by the addition of the substrate 4-methyl umbelliferyl-*N*-acetyl-β-D-glucosaminide. This substrate can be cleaved by β-hexosaminidase, liberating the fluorescent 4-methyl umbelliferone.

First, the amount of β-hexosaminidase release was established without addition of constructs. The percentage of this control release compared to the total amount of β-hexosaminidase was 41%, which is indicative for viable, competent cells.[43] This amount was approximately obtained from all release assays and for every

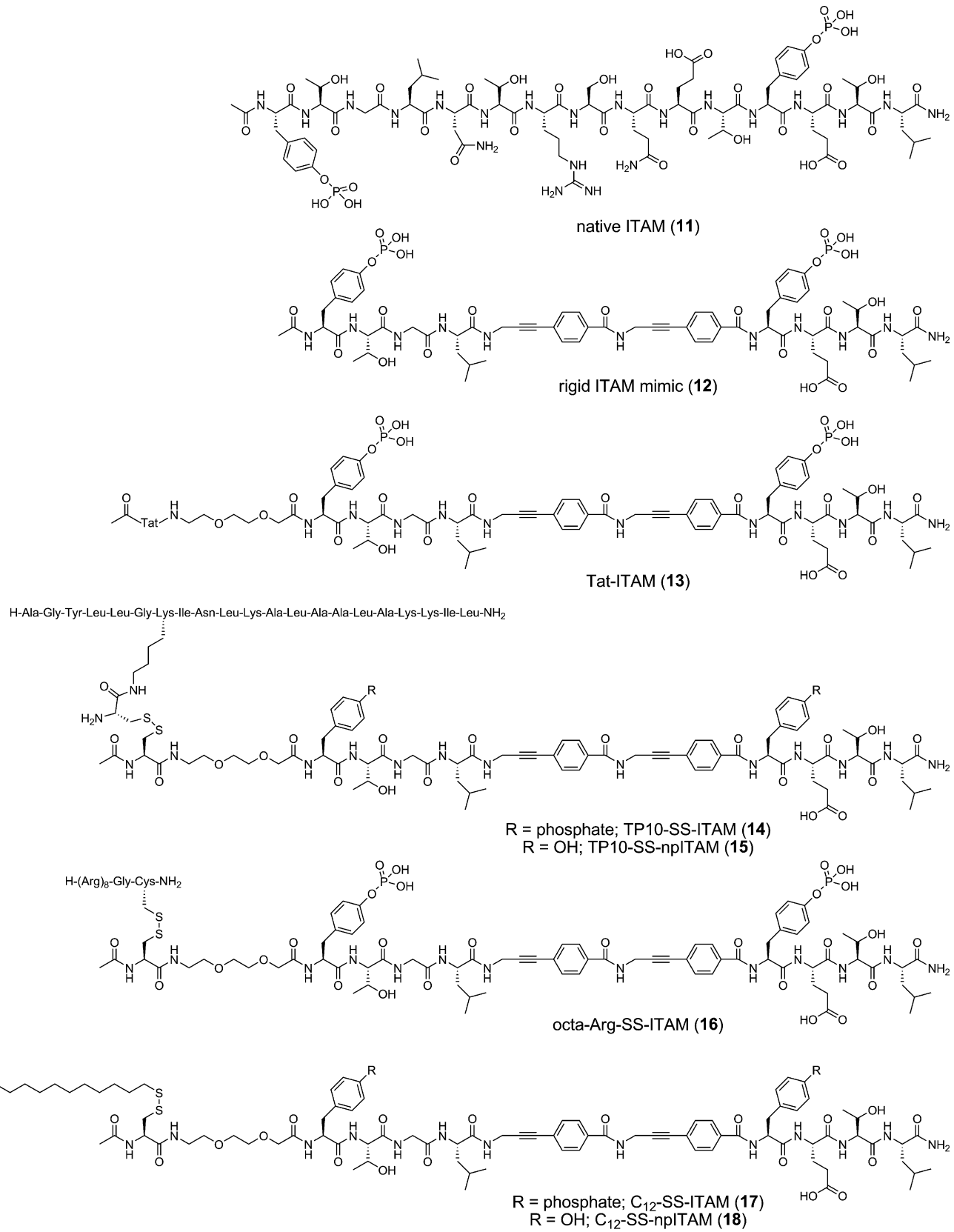


Fig. 5 Native ITAM (**11**), the rigid ITAM mimic **12** and cell penetrating constructs **13–18** used to study exocytosis.

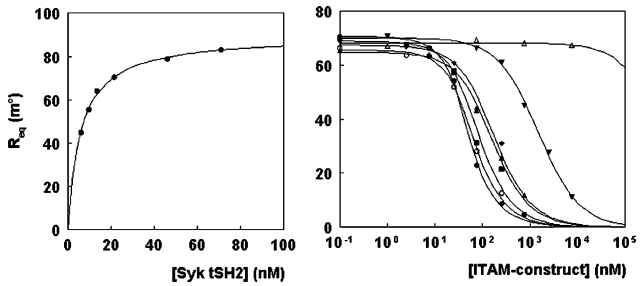


Fig. 6 A: SPR determination of the affinity of Syk tSH2 for immobilized γ-dpITAM phosphopeptide on the sensor surface. Data of equilibrium signals are fitted with a Langmuir binding isotherm. B: SPR competition experiments in the presence of 25 nM Syk tSH2. Data are fitted with a competition model yielding the affinity in solution (K_D) as described.[40] The inhibition curves represent native ITAM **11** (○), rigid ITAM mimic **12** (●), Tat-ITAM **13** (▼), TP10-SS-ITAM **14** (■), octa-Arg-SS-ITAM **16** (◆), C_{12}-SS-ITAM **17** (▲) and C_{12}-SS-npITAM **18** (△).

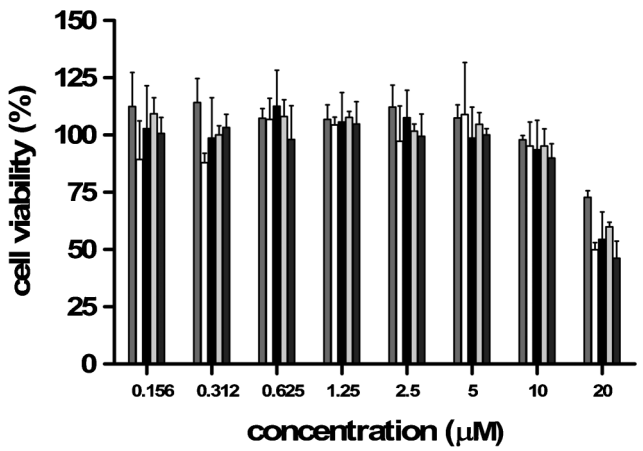


Fig. 7 Cell viability of RBL-2H3 cells in the presence of different concentrations of cell penetrating constructs. RBL-2H3 cells were incubated with 0–20 μM construct for 1 h under cell culture conditions prior to performing the XTT assays. Cell viability is expressed as the percentage of viable cells relative to untreated controls. Data are from experiments performed in quadruplicate and represent one of two independent experiments. The bars represent average ± SD of Tat-ITAM **13** (grey) TP10-SS-ITAM **14** (white), TP10-SS-npITAM **15** (black), octa-Arg-SS-ITAM **16** (light grey) and C_{12}-SS-ITAM **17** (dark grey).

separate experiment the obtained control release was defined as 100%. Furthermore, the basal release of non-stimulated cells, which were treated with plain medium and plain buffer instead of, respectively, anti-DNP-IgE and DNP_{30}-HSA, was defined as 0%.

The effect of Tat-ITAM (**13**) on β-hexosaminidase release could not be established, because too much DMSO was needed the keep the construct in solution. As shown in Fig. 6 this compound has also a low affinity for Syk tSH2. This might be due to formation of intramolecular salt-bridges by the positively charged amino acid residues in the Tat sequence with the negatively charged phosphotyrosine residues, leading to an intramolecular collapse.

TP10-SS-ITAM (**14**), as well as the nonphosphorylated negative control TP10-SS-npITAM (**15**), showed no effect on β-hexosaminidase release up to 10 μM (Fig. 8). This result is in line with the low cellular uptake of these constructs as appeared from fluorescence microscopy (Fig. 4). The C_{12}-SS-ITAM construct **17**

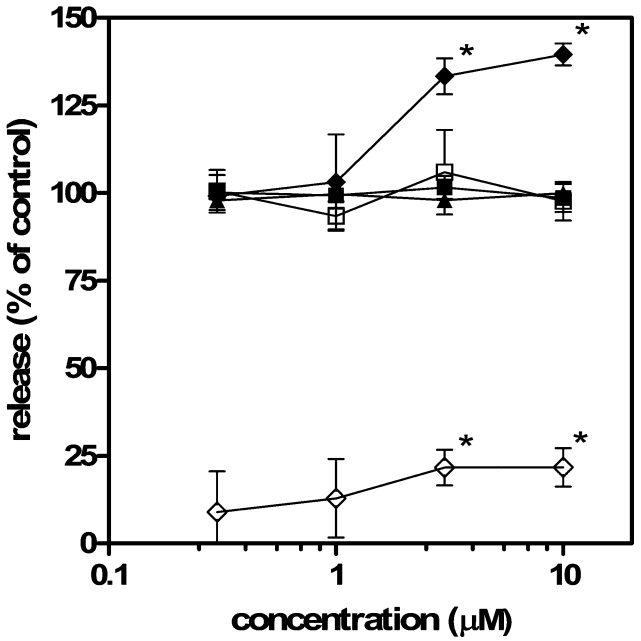


Fig. 8 Effect of cell penetrating ITAM constructs on β-hexosaminidase release upon FcεRI induced stimulation of RBL-2H3 cells. The β-hexosaminidase release was expressed as percentage of the controls, which were not treated with a construct. Data are from experiments performed in quadruplicate and represent one of at least two independent experiments. The bars represent average ± SD. The curves represent TP10-SS-ITAM **14** (■), TP10-SS-npITAM **15** (□), octa-Arg-SS-ITAM **16** (◆) and C_{12}-SS-ITAM **17** (▲). The lower curve (◇) represents the effect of octa-Arg-SS-ITAM **16** on non-stimulated cells. Significance with respect to the controls (*i.e.* 0% and 100%) is indicated by * ($p < 0.05$).

also displayed no effect on β-hexosaminidase release (Fig. 8), in line with the low degree of labeling of the cells with **9** (Fig. 3).

In contrast, the octa-Arg-SS-ITAM construct **16** stimulated release in a concentration dependent manner. The increase in mediator release relative to the control release of untreated stimulated cells was significant for both 3 and 10 μM **16**, with *p*-values for both of $p < 0.05$. The increase of the degranulation by 10 μM octa-Arg-SS-ITAM (**16**) in stimulated cells was 40% compared with untreated stimulated cells. This result suggests that binding of the soluble ITAM mimic activates Syk kinase, like also its binding to receptor-bound γ-dpITAM does.[3] We assume that the majority of Syk activated by the ITAM mimic is outside the IgE-receptor environment (Fig. 1). Apparently this intracellularly activated Syk contributes to exocytosis.

Since construct **16** was the only ITAM mimic that had an effect on exocytosis, it was also investigated whether construct **16** could initiate release in non-stimulated cells. For the non-stimulated cells a slight increase was observed, but the effect was less pronounced. The increase in degranulation of the non-stimulated cells compared to the control release in non-stimulated cells was significant for 3 and 10 μM (for both $p < 0.05$).

The full-length crystal structure of the closely related Zap-70 kinase might give a clue how binding of tSH2 to ITAM may affect kinase activity.[44] In this structure the inactive form of Zap-70 shows a close contact between the SH2-SH2 linker of tSH2 and the catalytic domain. This interaction helps to keep the kinase in the inactive state with a larger distance between the SH2 domains

than when bound to γ-ITAM. ITAM engagement leads to a conformational change involving the distance and position of the SH2 domains and the SH2-SH2 linker between them. Although the structure of full-length Syk kinase is not yet known in such detail,[45] we propose that a similar mechanism is also involved in the activation of the Syk kinase.

In the regulation of Syk activity, next to the conformational change in the Syk tSH2 domain, also other processes are involved, such as phosphorylation of the tSH2-kinase linker. These other processes only take place in stimulated cells, *i.e.* cells treated with anti-DNP-IgE and DNP_{30}-HSA. This might explain why the octa-Arg-SS-ITAM peptidomimetic construct **16** was capable of stimulating kinase activity in stimulated RBL-2H3 cells, and has a much smaller effect in non-stimulated cells.

Our previous work demonstrated that the Syk tSH2 domain adapts to a large variation in the ITAM-linker length.[9,39,46–48] Recently, we discovered in an extracellularly Syk kinase assay that an ITAM mimic with a longer rigid linker between the SH2 binding epitopes is able to inhibit the kinase activity (to be published). Getting such longer ITAM mimics into the cell, as described in the present study, might be a strategy to obtain inhibitors of mediator release.

Conclusions

This is the first report on the intracellular effect of ITAM mimics on exocytosis. The effect on exocytosis of construct octa-Arg-SS-ITAM (**16**) agrees with the firm experimental evidence that it penetrates into RBL-2H3 cells from flow cytometry and fluorescence microscopy. From a viability assay it appears that the construct is not cytotoxic under conditions of the exocytosis assay. Furthermore, the construct has a high affinity for Syk-tSH2. Binding of soluble ITAM apparently activates Syk and contributes to the signaling leading to exocytosis.

Experimental section

General remarks concerning the syntheses

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, except for MeOH, which was stored over 3 Å molecular sieves. Reactions were performed at room temperature unless stated otherwise. Monitoring took place and R_f values were determined by thin layer chromatography (TLC). The TLC plates were obtained from Merck and were coated with silica gel 60 F254 (0.25 mm). Spots were visualized by UV light and by ninhydrin 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 with Silicycle UltraPure silica gels, SiliaFlash (pore size 60 Å, particle size distribution 40–63 μm).

^{1}H NMR spectra were measured on a Varian Mercury plus 300 MHz spectrometer and 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 chemical shifts are given in ppm (δ) relative to $CDCl_3$, DMSO-d_6, or CD_3OD. The ^{13}C NMR spectra were measured using the attached proton test (APT).

General procedure for solid phase peptide synthesis

All peptides were manually assembled on Tentagel®-Rink-NH-Fmoc resin using standard Fmoc/*t*Bu chemistry. The Fmoc protecting group was removed using 20% piperidine in NMP (3 × 10 mL for each gram of resin, each 8 min) followed by washing steps with NMP (3 × 10 mL for each gram of resin, each 2 min), CH_2Cl_2 (3 × 10 mL for each gram of resin, each 2 min) and NMP (3 × 10 mL for each gram of resin, each 2 min). The Alloc protecting group was removed using 0.25 equivalents of $Pd(PPh_3)_4$ and 10 equivalents of anilinium *para*-toluene sulfinate in MeOH–THF (1 : 1) (2 × 10 mL for each gram of resin, each 60 min). After Alloc deprotection the resin was washed with 0.1% sodium diethyldithiocarbamate in NMP (1 × 10 mL for each gram of resin for 2 min), NMP (3 × 10 mL for each gram of resin, each 2 min), CH_2Cl_2 (3 × 10 mL for each gram of resin, each 3 min) and NMP (3 × 10 mL for each gram of resin, each 2 min). The amino acid coupling mixtures were prepared by dissolving 4 equivalents of amino acid, 4 equivalents of HOBt and HBTU and 8 equivalents of DiPEA in NMP and coupled during a coupling time of 60 min. Using the same coupling conditions 5(6)-carboxyfluorescein was coupled overnight. The resin was washed with NMP (3 × 10 mL for each gram of resin, each 1 min) and CH_2Cl_2 (3 × 10 mL for each gram of resin, each 1 min) after every coupling step. The coupling steps and deprotection steps were monitored using the Kaisertest.[49] When the Fmoc deprotection was not complete, the deprotection step was repeated. Fmoc-Tyr(OP(OBn)OH)-OH was coupled overnight using 2 equivalents of amino acid, 2 equivalents of the coupling reagents HOBt and HBTU and 5 equivalents of DiPEA. Alloc-4-(3-aminoprop-1-ynyl)benzoic acid was also coupled overnight using 2 equivalents of amino acid and 2 equivalents of the coupling reagents HOBt and HBTU and 4 equivalents of DiPEA. After the first Fmoc-Tyr(OP(OBn)OH)-OH coupling an additional washing step (2 × 10 mL for each gram of resin, each 10 min) with a mixture of 1 M TFA–1.1 M DiPEA in NMP was performed after each Fmoc deprotection step in order to replace the piperidinium counter ion of Tyr(OP(OBn)O^-) by protonated DiPEA. Peptides containing an N-terminal carboxyfluorescein were subjected to the standard Fmoc deprotection protocol (3 × 10 mL of 20% piperidine in NMP for each gram of resin, each 8 min) after carboxyfluorescein coupling. This was necessary to remove any carboxyfluorescein residues, which were coupled to the phenolic hydroxy groups of the carboxyfluorescein residue already attached to the peptide chain.[50] When all coupling steps were completed acetylation was carried out 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 NMP (100 mL) for 2 × 30 min to give acetylated peptides **13** and **32–34**. The peptides were cleaved from the resin and the side chains were deprotected with various cleavage cocktails, as indicated for each peptide, for 3 h. The resin was removed from the solution by filtration and the peptides were precipitated with MTBE–hexane 1 : 1 v/v at –20 °C and lyophilized from CH_3CN–H_2O 1 : 1 v/v yielding the crude peptides. The peptides were purified by preparative HPLC and analyzed by analytical HPLC and mass spectrometry. For the preparative HPLC a Gilson system

with a UV detector operating at 220 and 254 nm or an Applied Biosystems workstation with a UV detector operating at 214 nm were employed with different gradients, which are indicated for each compound below. Analytical HPLC was measured on a Shimadzu HPLC system with a UV detector operating at 220 and 254 nm and for some peptides also an evaporative light scattering detector (PL-ELS 1000, Polymer Laboratories) was used with a gradient from 100% buffer A to 100% buffer B in 20 min. Two buffer combinations were used for all HPLC runs. The buffer combination for all preparative HPLC runs and for some analytical HPLC runs, is indicated for each compound, was the 'standard TFA buffers': buffer A (0.1% TFA in H_2O–CH_3CN 95 : 5) and buffer B (0.1% TFA in H_2O–CH_3CN 5 : 95). The buffer combination for the analytical HPLC of most of the phosphotyrosine containing compounds, is indicated for each compound, was 'the TEAP buffers': buffer A (15 mM TEA in H_2O titrated at pH 6 with 85% H_3PO_4) and buffer B (buffer A–CH_3CN 1 : 9).

Alloc-4-(3-aminoprop-1-ynyl)benzoic acid

The synthesis of the rigid building block Alloc-4-(3-aminoprop-1-ynyl)benzoic acid has been described earlier by us.[9]

Synthesis of the carriers

Boc-Cys(Trt)-OSu (19). *N*-Hydroxysuccinimide (1.38 g, 12 mmol) was added to Boc-Cys(Trt)-OH (11.4 mmol, 5.27 g) in CH_3CN (200 mL). The mixture was cooled to 0 °C and DCC (2.35 g, 11.4 mmol) was added. The mixture was stirred overnight at r.t. and it was filtered and concentrated. The product was crystallized from 2-propanol yielding 4.97 g (8.86 mmol, 78%) of **19** as white crystals. R_f 0.61 (EtOAc–hexane 1 : 1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 1.43 (s, 9H, *t*Bu), 2.66 (d, 2H, β CH_2), 2.79 (s, 4H, 2 CH_2), 4.33 (t, 1H, CH), 4.87 (d, 1H, NH), 7.20–7.46 (m, 15H, trityl). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 25.5 (2 CH_2), 28.2 (3 CH_3), 33.6 (β CH_2), 51.1 (CH), 67.4 (C trityl), 80.6 (C *t*Bu), 127.0, 128.2, 129.5, 144.1 (Ar), 154.5 (CO Boc), 166.8 (COO), 168.3 (2 CO).

Fmoc-Lys(Boc-Cys(Trt))-OH (20). TFA (20 mL) and CH_2Cl_2 (20 mL) were added to Fmoc-Lys(Boc)-OH (2.34 g, 5.0 mmol) and the mixture was stirred for 1 h and concentrated. DMF (20 mL) and CH_3CN (100 mL) were added to the resulting TFA salt. DiPEA (2.97 mL, 18 mmol) and Boc-Cys(Trt)-OSu (**19**) (2.52 g, 4.5 mmol) were added and the mixture was stirred overnight. The solvents were evaporated and the residue was dissolved in EtOAc. The solution was washed with 5% citric acid (3 ×) and brine (3 ×), dried over Na_2SO_4, filtered and concentrated. The product was purified by silica gel column chromatography (0.5% CH_3COOH in EtOAc–hexane 2 : 1) yielding 2.50 g (3.07 mmol, 68%) of Fmoc-Lys(Boc-Cys(Trt))-OH (**20**) as a white foam. R_f 0.34 (0.5% CH_3COOH in EtOAc–hexane 2 : 1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 1.39 (s, 9H, *t*Bu), 1.41–1.47 (m, 4H, Lys γ δ CH_2), 2.29–2.68 (m, 2H, Cys β CH_2), 3.17 (bs, 2H, Lys ε CH_2), 3.80 (bs, 1H, Cys α CH), 4.18 (t, 1H, Lys α CH), 4.31–4.42 (m, 3H, CH, CH_2 Fmoc), 5.06 (d, 1H, Cys NH), 5.72 (d, 1H, Lys NH), 6.21 (d, 1H, Lys εCH_2–N*H*), 7.16–7.39 (m, 19H, 15 trityl, 4 Ar Fmoc), 7.58, 7.74 (2d, 4H, Ar Fmoc). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 21.9 (Lys γ CH_2), 28.3 (3 CH_3), 28.6 (Lys δ CH_2), 31.4 (Lys β CH_2), 33.8 (Cys β CH_2), 38.7 (Lys ε CH_2), 47.1 (CH Fmoc), 53.6 (2 α CH), 67.1, 67.2 (C trityl, CH_2 Fmoc), 80.3 (C *t*Bu), 119.9, 125.2, 127.1, 127.7, 141.3, 143.8 (Ar Fmoc), 126.9, 128.0, 129.6, 144.4 (Ar trityl), 155.7, 156.2 (CO Boc, Fmoc), 170.9 (CO Cys), 174.8 (COOH).

TP10-Cys (21) and TP10-Cys(pNpys) (22). These peptides were assembled on Tentagel®-Rink-NH-Fmoc resin. For **21** 641 mg of resin (0.17 mmol, loading 0.26 mmol g^{-1}) and for **22** 321 mg of resin, (0.08 mmol, loading 0.26 mmol g^{-1}) was used. The appropriate Fmoc-amino acid building blocks including dipeptide **20** were included in the solid phase peptide synthesis (see general procedure). TFA–H_2O–TIS–EDT (90 : 5 : 2.5 : 2.5) was used for cleavage and deprotection yielding **21** and 2,2′-dithiobis(5-nitropyridine) (1298 mg, 0.42 mmol) in TFA–H_2O–TIS (92.5 : 5 : 2.5) was used for cleavage and deprotection yielding **22**. Lyophilization from CH_3CN–H_2O 1 : 1 v/v gave 338 mg of crude **21**, of which 250 mg was purified, and 335 mg of crude **22**. Purification was achieved by preparative HPLC using an Alltech Alltima C8 100 Å 10 μm (250 × 22 mm) column. Gradients of 100% buffer A to 100% buffer B in 90 min for **21** and in 80 min for **22** were used. The fractions were analyzed by analytical HPLC using an Alltech Alltima C8 5 μm (250 × 4.6 mm) column and the standard TFA buffers. 111.2 mg of pure **21** and 77.7 mg of pure **22** were obtained after pooling and lyophilization as white fluffy solids.

HRMS (ESI) of **21**: $[M + 2H]^{2+}$ calculated 1142.7193, found 1142.7195; $[M + 3H]^{3+}$ calculated 762.1488, found 762.1577.

MS (MALDI-TOF) of **22**: $[M + H]^+$ calculated 2438.415, found 2438.500; $[M + Na]^+$ calculated 2460.397, found 2460.321.

Octa-Arg-Cys (23). This peptide was assembled on Tentagel®-Rink-NH-Fmoc resin (962 mg, 0.25 mmol, loading 0.26 mmol g^{-1}). The amino acid building blocks Fmoc-Cys(Trt)-OH, Fmoc-Gly-OH and Fmoc-Arg(Pbf)-OH (8 ×) were subsequently coupled. TFA–H_2O–TIS–EDT (90 : 5 : 2.5 : 2.5) was used for cleavage and deprotection. After lyophilization from CH_3CN–H_2O 1 : 1 v/v 371 mg of the crude peptide was obtained, of which 200 mg was purified by preparative HPLC using an Alltech Alltima C8 100 Å 10 μm (250 × 22 mm) column. A gradient of 100% buffer A to 100% buffer B in 60 min was used. The fractions were analyzed by analytical HPLC using an Alltech Alltima C8 5 μm (250 × 4.6 mm) column and the standard TFA buffers. 113.3 mg of the pure compound was obtained after pooling and lyophilization as a white fluffy solid.

HRMS (ESI): $[M + 2H]^{2+}$ calculated 713.9409, found 713.9344; $[M + 3H]^{3+}$ calculated 476.2965, found 476.3069.

Octa-Arg-Cys(pNpys) (24). Octa-Arg-Cys **23** (23.5 mg, 10 μmol) in H_2O (3 mL) was added to 2,2′-dithiobis(5-nitropyridine) (15.5 mg, 50 μmol) dissolved in TFA (1 mL). The mixture was stirred for 3 h and then evaporated to dryness. The product was obtained by preparative HPLC using an Alltech Alltima C8 100 Å 10 μm (250 × 22 mm) column. A gradient of 100% buffer A to 100% buffer B in 60 min was used. The fractions were analyzed by analytical HPLC using an Alltech Alltima C8 5 μm (250 × 4.6 mm) column and the standard TFA buffers. Pure **24** was obtained after pooling and lyophilization (14.1 mg, 54%) as a white fluffy solid.

C_{12}-S-pNpys (25). To a solution of 2,2′-dithiobis(5-nitropyridine) (776 mg, 2.5 mmol) in TFA–DCM 1 : 1 (15 mL), 1-dodecanethiol (120 μL, 0.5 mmol) was added dropwise and the mixture was stirred for 1 h. The solvents were evaporated and **25** was obtained after silica gel column chromatography (hexane, then EtOAc–hexane 1 : 3) in quantitative yield as a yellow solid. R_f 0.86 (EtOAc–hexane 1 : 3). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 0.82 (t, 3H, CH_3), 120 (bs, 16H, CH_2), 1.35 (bs, 2H, CH_2CH_3),1.63–1.72 (m, 2H, SCH_2CH_2) 2.79 (t, 2H, SCH_2), 7.88 (d, 1H, Ar), 8.36 (d, 1H, Ar), 9.20 (s, 1H, Ar). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 14.0 (CH_3), 22.6 (CH_2CH_3), 28.4–29.5 (8 CH_2), 31.8 (SCH_2CH_2), 39.3 (SCH_2), 118.9, 131.4, 141.8, 144.9, 169.3 (Ar).

Fmoc-Lys(Myr)-OH (26). To a solution of myristic acid (1.60 g, 7.0 mmol) in DMF (10 mL) and CH_3CN (100 mL), *N*-hydroxysuccinimide (863 mg, 7.5 mmol) was added. After cooling to 0 °C DCC (1.44 g, 7.0 mmol) was added. The mixture was then stirred overnight at r.t. Filtration and concentration afforded Myr-OSu.

Fmoc-Lys(Boc)-OH (2.34 g, 5.0 mmol) was dissolved in TFA (20 mL) and CH_2Cl_2 (20 mL). The mixture was stirred for 3 h followed by concentration and DMF (25 mL) and CH_3CN (100 mL) were added to the TFA salt. After neutralization with TEA, additional TEA (826 μL, 5.0 mmol) and Myr-OSu were added and stirring was continued for 3 h. The solvents were evaporated and the product was dissolved in EtOAc. The solution was washed with 1 M $KHSO_4$ (3 ×) and brine (1 ×), dried over Na_2SO_4, filtered and concentrated. The product was purified by silica gel column chromatography (0.5% CH_3COOH in EtOAc–hexane 2 : 1) yielding 2.01 g (3.65 mmol, 73%) of **26** as a white foam. R_f 0.16 (0.5% CH_3COOH in EtOAc–hexane 2 : 1). ^{1}H NMR (DMSO, 300 MHz) δ = 0.85 (t, 3H, CH_3), 1.14–1.28 (m, 22H, 10 CH_2 myristoyl and Lys γ CH_2), 1.36 (m, 2H, Lys δ CH_2), 1.46 (t, 2H, myristoyl β CH_2), 1.56–1.77 (m, 2H, Lys β CH_2), 2.02 (t, 2H, myristoyl α CH_2), 3.03 (t, 2H, Lys ε CH_2), 3.87–3.91 (m, 1H, CH Fmoc), 4.22–4.28 (m, 3H, Lys α CH, CH_2 Fmoc), 7.30–7.44 (m, 4H, Ar Fmoc), 7.58–7.61 (m, 2H, 2 NH), 7.73, 7.89 (2d, 4H, Ar Fmoc). ^{13}C NMR (DMSO, 75 MHz) δ = 19.4 (CH_3), 27.5 (Lys δ CH_2), 28.5 (CH_2CH_3), 30.8 (myristoyl β CH_2), 34.1–34.4 (myristoyl 9 CH_2), 35.9 (Lys δ CH_2), 36.7 (Lys β CH_2), 40.9 (myristoyl α CH_2), 43.5 (Lys ε CH_2), 52.1 (CH Fmoc), 59.2 (Lys α CH), 71.1 (CH_2 Fmoc), 125.5, 130.7, 132.5, 133.1, 146.2, 149.3 (Ar Fmoc), 161.6 (CO Fmoc), 177.4 (myristoyl CO), 179.4 (COOH).

Cys-K(Myr)KKK (27). This peptide was assembled on Tentagel®-Rink-NH-Fmoc resin (1.02 g, 0.25 mmol, loading 0.24 mmol g^{-1}). The amino acid building blocks Fmoc-Lys(Boc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Boc)-OH, **26**, and Fmoc-Cys(Trt)-OH were subsequently coupled. TFA–H_2O–TIS–EDT (90 : 5 : 2.5 : 2.5) was used for cleavage and deprotection of the peptide. After lyophilization from CH_3CN–H_2O 1 : 1 v/v 239 mg of the crude peptide was obtained, of which 100 mg was purified by preparative HPLC using an Alltech Alltima C8 100 Å 10 μm (250 × 22 mm) column. A gradient of 100% buffer A to 100% buffer B in 60 min was used. The fractions were analyzed by analytical HPLC using an Alltech Alltima C8 5 μm (250 × 4.6 mm) column and the standard TFA buffers. 70.0 mg of the pure compound was obtained after pooling and lyophilization as a white fluffy solid.

MS (ESI): $[M + H]^+$ calculated 843.62, found 843.65; $[M + 2H]^{2+}$ calculated 422.32, found 422.35.

Synthesis of the cargos

2-(tritylthio)ethanamine (28)[51]. Tritrylchloride (3.63 g, 13.0 mmol) was added to a solution of cysteamine.HCl (1.00 g, 8.80 mmol) in DMF–CH_2Cl_2 1 : 1 (50 mL) and the mixture was stirred for 2 h. The solvents were evaporated and the product was purified by silica gel column chromatography (CH_2Cl_2–EtOAc 9 : 1, then CH_2Cl_2–MeOH 4 : 1) yielding 2.35 g (7.36 mmol, 84%) of **28** as a pale yellow solid. R_f 0.68 (CH_2Cl_2–MeOH 4 : 1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 1.50 (s, 2H, NH_2), 2.28 (t, 2H, SCH_2), 2.50 (t, 2H, $NHCH_2$), 7.11–7.24, 7.39–7.44 (2 m, 15H, trityl). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 35.6 (CH_2S), 40.5 ($NHCH_2$), 66.2 (C trityl), 126.3, 127.5, 129.2 144.5 (Ar).

Fluorescein-CH_2-CH_2-S(Trt) (29). Cysteamine derivative **28** (160 mg, 0.50 mmol) in DMF (1 mL) was added to fluorescein isothiocyanate isomer 1 (195 mg, 0.50 mmol) in DMF (6 mL) and the mixture was stirred for 2 h. Then the DMF was evaporated and the product was purified by silica gel column chromatography (CH_2Cl_2–MeOH 95 : 5) yielding 319 mg (0.45 mmol, 90%) of thiocarbamate **29** as an orange solid. R_f 0.13 (CH_2Cl_2–MeOH 95 : 5). ^{1}H NMR (DMSO, 300 MHz) δ = 2.42 (t, 2H, SCH_2), 3.50 (d, 2H, $NHCH_2$), 6.54–6.68 (m, 6H, Ar fluorescein), 7.17 (d, 1H, Ar fluorescein), 7.22–7.36 (m, 15H, trityl), 7.71 (d, 1H, Ar fluorescein), 8.23 (s, 1H, Ar fluorescein), 10.01 (bs, 2H, OH). ^{13}C NMR (DMSO, 75 MHz) δ = 30.8 (SCH_2), 42.3 ($NHCH_2$), 66.0 (C trityl), 102.2, 109.7, 112.6, 116.7, 124.1, 126.4, 127.7, 128.6, 141.1, 147.2, 151.9, 159.5 (Ar fluorescein), 126.8, 128.1, 129.1, 144.4 (Ar trityl), 168.5 (CO), 180.4 (CS).

Flu-Cys-ITAM (30) and Flu-Cys(pNpys)-ITAM (31). These peptides were assembled on Tentagel®-Rink-NH-Fmoc resin (each 1.02 g, 0.25 mmol, loading 0.24 mmol g^{-1}). The following amino acid building blocks were subsequently coupled: Fmoc-Leu-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Tyr(OP(OBn)OH)-OH, Alloc-4-(3-aminoprop-1-ynyl)benzoic acid, Alloc-4-(3-aminoprop-1-ynyl)benzoic acid, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Tyr(OP(OBn)OH)-OH, Fmoc-2-(2-(2-aminoethoxy)ethoxy)acetic acid, Fmoc-Cys(Trt)-OH. After coupling of 5(6)-carboxyfluorescein, TFA–H_2O–TIS–EDT (90 : 5 : 2.5 : 2.5) was used for cleavage and deprotection of **30**. 2,2′-dithiobis(5-nitropyridine) (388 mg, 1.25 mmol) in TFA–H_2O–TIS (92.5 : 5 : 2.5) was used for cleavage and deprotection of **31**. After lyophilization from CH_3CN–H_2O 1 : 1 v/v 298 mg of crude **30** and 457 mg of crude **31** were obtained. The peptides were purified by preparative HPLC using an Alltech Alltima C8 100 Å 10 μm (250 × 22 mm) column. A gradient of 100% buffer A to 100% buffer B in 100 min was used for both peptides. The fractions were analyzed by analytical HPLC using an Alltech Alltima C8 5 μm (250 × 4.6 mm) column and the TEAP buffers. 40.8 mg of pure **30** was obtained and 14.2 mg of pure **31** was obtained, starting from 228 mg crude peptide **31**, both as yellow fluffy solids after pooling and lyophilization.

HRMS (ESI) of **30**: $[M + H]^+$ calculated 2038.6576, found 2038.6373.

MS (MALDI-TOF, negative mode) of **31**: $[M - H]^-$ calculated 2192.101, found 2192.105.

Ac-Cys-ITAM (32), Ac-Cys(pNpys)-ITAM (33) and Ac-Cys-npITAM (34). These peptides were assembled on Tentagel®-Rink-NH-Fmoc resin (**32**: 865 mg, 0.225 mmol, loading

0.26 mmol g^{-1}; **33**: 417 mg, 0.10 mmol, loading 0.24 mmol g^{-1}; **34**: 769 mg, 0.20 mmol, loading 0.26 mmol g^{-1}). The amino acid building blocks were identical to those used for the synthesis of **30** and **31**. After removal of the last Fmoc group, the N-terminus was acetylated with capping solution. TFA–H_2O–TIS–EDT (90 : 5 : 2.5 : 2.5) was used for cleavage and deprotection of peptides **32** and **34**. For **33** 2,2′-dithiobis(5-nitropyridine) (155 mg, 0.50 mmol) in TFA–H_2O–TIS (95.5 : 5 : 2.5) was used. After lyophilization from CH_3CN–H_2O 1 : 1 v/v 163 mg of crude **32**, 177 mg of crude **33** and 135 mg of crude **34** were obtained. The peptides were purified by preparative HPLC using an Alltech Adsorbosphere XL C8 90 Å 10 μm (250 × 22 mm) column for **32** and **34** and an Alltech Alltima C8 100 Å 10 μm (250 × 22 mm) column for **33**. A gradient of 100% buffer A to 100% buffer B in 40 min was used for **32** and **34** and a gradient of 100 min was used for **33**. The fractions were analyzed by analytical HPLC using an Alltech Adsorbosphere XL C8 90 Å 5 μm (250 × 4.6 mm) column and the standard TFA buffers for **32** and **34** and an Alltech Alltima C8 5 μm (250 × 4.6 mm) column and the TEAP buffers for **33**. 40.1 mg of pure **32**, 4.8 mg of pure **33** (only 80 mg was purified of **33**) and 15.5 mg of pure **34** were obtained after pooling and lyophilization as white fluffy solids.

HRMS (ESI) of **32**: $[M + Na]^+$ calculated 1744.6019, found 1744.9742; $[M + 2Na]^{2+}$ calculated 883.7955, found 884.0009.

MS (ESI, negative mode) of **33**: $[M - H]^-$ calculated 1875.84, found 1876.25.

HRMS (ESI) of **34**: $[M + H]^+$ calculated 1562.6877, found 1563.0153; $[M + Na]^+$ calculated 1664.6361, found 1664.9752; $[M + 2H]^{2+}$ calculated 821.831, found 822.0479; $[M + H + Na]^{2+}$ calculated 832.8214, found 833.0208; $[M + 2Na]^{2+}$ calculated 843.8123, found 844.0172.

Flu-ITAM (1). This peptide was assembled on Tentagel®-Rink-NH-Fmoc resin (417 mg, 0.10 mmol, loading 0.24 mmol g^{-1}). The amino acid building blocks were identical to those used for the synthesis of **30** and **31** excepting Fmoc-Cys(Trt)OH. After coupling of 5(6)-carboxyfluorescein TFA–H_2O–TIS–EDT (90 : 5 : 2.5 : 2.5) was used for cleavage and deprotection of the peptide. Lyophilization from CH_3CN–H_2O 1 : 1 v/v gave 148 mg of the crude peptide, of which 50 mg was purified by preparative HPLC using an Alltech Alltima C8 100 Å 10 μm (250 × 22 mm) column. A gradient of 100% buffer A to 100% buffer B in 100 min was used. The fractions were analyzed by analytical HPLC using an Alltech Alltima C8 5 μm (250 × 4.6 mm) column and the TEAP buffers and 9.4 mg of the pure compound was obtained after pooling and lyophilization as a yellow fluffy solid.

HRMS (ESI): $[M + H]^+$ calculated 1935.6484, found 1935.6533; $[M + 2H]^{2+}$ calculated 968.3281, found 968.2862.

Native ITAM (11), rigid ITAM mimic (12). The synthesis of the native ITAM peptide (**11**) and the rigid ITAM mimic (**12**) has been described earlier by us.[9]

Synthesis of carrier-fluorescein constructs

Flu-Tat (2). This peptide was assembled on Tentagel®-Rink-NH-Fmoc resin (1.04 g, 0.25 mmol, loading 0.24 mmol g^{-1}). The amino acid building blocks Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-βalanine-OH and 5(6)-carboxyfluorescein were subsequently coupled. TFA–H_2O–TIS (92.5 : 5 : 2.5) was used for cleavage and deprotection of the peptide. After lyophilization from CH_3CN–H_2O 1 : 1 v/v 394 mg of the crude peptide was obtained, of which 51 mg was purified by preparative HPLC using an Alltech Adsorbosphere XL C8 90 Å 10 μm (250 × 22 mm) column. A gradient of 100% buffer A to 100% buffer B in 40 min was used. The fractions were analyzed by analytical HPLC using an Alltech Adsorbosphere XL C8 90 Å 5 μm (250 × 4.6 mm) column and the standard TFA buffers and 9.6 mg of the pure peptide was obtained as a yellow fluffy solid after pooling and lyophilization.

HRMS (ESI): $[M + 2H]^{2+}$ calculated 995.0343, found 995.2886; $[M + 3H]^{3+}$ calculated 663.6328, found 663.4452; $[M + 4H]^{4+}$ calculated 497.9844, found 497.8207.

TP10-SS-Flu (4). TFA (2 mL), TIS (5 μL) and CH_2Cl_2 (1 mL) were added to **29** (5.4 mg, 7.6 μmol) and the mixture was stirred for 2 h. After evaporation of the volatiles, TP10-Cys(pNpys) (**22**) (23 mg, 7.6 μmol) dissolved in CH_3CN (1 mL) and 1 M NH_4OAc (pH 4.5, 2 mL) was added. Stirring was continued overnight and then the solvents were evaporated followed by purification of the product by preparative HPLC using an Alltech Alltima C8 100 Å 10 μm (250 × 22 mm) column. A gradient of 100% buffer A to 100% buffer B in 90 min was used. The fractions were analyzed by analytical HPLC using an Alltech Alltima C8 5 μm (250 × 4.6 mm) column and the standard TFA buffers. The product was obtained as a yellow fluffy solid (4.2 mg, 17%) after pooling and lyophilization.

HRMS (ESI): $[M + 2H]^{2+}$ calculated 1375.7322, found 1375.2452; $[M + 3H]^{3+}$ calculated 917.4908, found 917.0551.

Octa-Arg-SS-Flu (6). A mixture of TFA (2 mL), TIS (5 μL) and CH_2Cl_2 (1 mL) was added to **29** (3.8 mg, 5.4 μmol), the resulting solution was stirred for 2 h and after that the volatiles were evaporated. Octa-Arg-Cys(pNpys) (**24**) (14.1 mg, 5.4 μmol) in 1 M NH_4OAc (pH 4.5, 4 mL) was added and the mixture was stirred overnight. The solvents were evaporated and the product was purified by preparative HPLC using an Alltech Alltima C8 100 Å 10 μm (250 × 22 mm) column. A gradient of 100% buffer A to 100% buffer B in 100 min was used. The fractions were analyzed by analytical HPLC using an Alltech Alltima C8 5 μm (250 × 4.6 mm) column and the standard TFA buffers. After pooling and lyophilization of the appropriate fractions the product was obtained as a yellow fluffy solid (7.7 mg, 55%).

HRMS (ESI): $[M + 3H]^{3+}$ calculated 630.9799, found 630.9855; $[M + 4H]^{4+}$ calculated 473.4869, found 473.5040.

C_{12}-SS-Flu (8). A mixture of TFA (2 mL), TIS (40 μL) and H_2O (40 μL) was added to **29** (35 mg, 50 μmol), the mixture was stirred for 4 h and the solvents were evaporated. C_{12}-S-pNpys (**25**) (18 mg, 50 μmol) in THF (2 mL) and 1 M NH_4OAc (pH 4.5, 2 mL) was added and the mixture was stirred overnight. The solvents were evaporated and the product was purified by silica gel column chromatography (CH_2Cl_2–MeOH 92.5 : 7.5) and lyophilized from CH_3CN–H_2O 1 : 1 yielding 14.1 mg (21 μmol, 42%) of the product as an orange powder. R_f 0.49 (CH_2Cl_2–MeOH 9 : 1). ^1H NMR (CD_3OD, 300 MHz) δ = 0.88 (t, 3H, CH_3), 1.27 (bs, 16H, CH_2), 1.37–1.40 (m, 2H, CH_3CH_2), 1.67–1.72 (m, 2H, SCH_2CH_2), 2.74 (t, 3H, SCH_2), 2.99 (t, 2H, $NHCH_2CH_2$), 3.93 (t, 2H, $NHCH_2$),

6.52 (d, 2H, Ar), 6.55–6.68 (m, 4H, Ar), 7.14 (d, 1H, Ar), 7.78 (dd, 1H, Ar), 8.17 (d, 1H, Ar). ^{13}C NMR (CD_3OD, 75 MHz) δ = 14.5 (CH_3), 23.7 (CH_3CH_2), 29.5 (SH_3CH_2), 30.2–30.7 (7 CH_2), 33.1 ($CH_3CH_2CH_2$), 37.8 ($NHCH_2CH_2$), 39.8 (SCH_2), 44.6 ($NHCH_2$), 79.1 (C fluorescein), 103.5, 111.5, 113.6, 120.0, 125.7, 129.1, 130.3, 131.9, 142.3, 150.1 154.2, 161.5 (Ar), 171.1 (CO), 183.0 (CS).

Synthesis of carrier-(fluorescein-)ITAM constructs

Flu-Tat-ITAM (3). This peptide was assembled on Tentagel®-Rink-NH-Fmoc resin (739 mg, 0.17 mmol, loading 0.23 mmol g^{-1}). The appropriate amino acid building blocks were subsequently coupled. TFA–H_2O–TIS–EDT (90 : 5 : 2.5 : 2.5) was used for cleavage and deprotection of the peptide. After lyophilization from CH_3CN–H_2O 1 : 1 v/v 133 mg of the crude peptide was obtained. 17 mg of the peptide was purified by preparative HPLC using an Alltech Prosphere C4 300 Å 10 µm (250 × 22 mm) column. A gradient of 100% buffer A to 100% buffer B in 40 min was used. The fractions were analyzed by analytical HPLC using an Alltech Prosphere C4 300 Å 5 µm (250 × 4.6 mm) column and the standard TFA buffers. 4.3 mg of the pure compound was obtained after pooling and lyophilization as a yellow fluffy solid.

HRMS (ESI): $[M + 2H]^{2+}$ calculated 1775.3047, found 1775.4950; $[M + 3H]^{3+}$ calculated 1184.4453, found 1184.0286.

Tat-ITAM (13). This peptide was assembled on Tentagel®-Rink-NH-Fmoc resin (626 mg, 0.144 mmol, loading 0.23 mmol g^{-1}). The amino acid building blocks were identical to those used for the synthesis of **3**, except the last two building blocks, *i.e.* Fmoc-β-alanine-OH and 5(6)-carboxyfluorescein, which were not coupled. After deprotection of the last Fmoc group, the N-terminus was acetylated with capping solution. TFA–H_2O–TIS–EDT (90 : 5 : 2.5 : 2.5) was used for cleavage and deprotection of the peptide. After lyophilization from CH_3CN–H_2O 1 : 1 v/v 183 mg of the crude peptide was obtained. 26 mg of the peptide was purified by preparative HPLC using an Alltech Prosphere C4 300 Å 10 µm (250 × 22 mm) column. A gradient of 100% buffer A to 100% buffer B in 40 min was used. The fractions were analyzed by analytical HPLC using an Alltech Prosphere C4 300 Å 5 µm (250 × 4.6 mm) column and the standard TFA buffers. 4.3 mg of the pure compound was obtained after pooling and lyophilization as a white fluffy solid.

HRMS (ESI): $[M + 2H]^{2+}$ calculated 1581.3125, found 1581.4844; $[M + 3H]^{3+}$ calculated 1054.6566, found 1054.6653; $[M + 4H]^{4+}$ calculated 791.2578, found 791.4642; $[M + 5H]^{5+}$ calculated 633.2344, found 633.3635.

General procedure for hetero disulfide formation[37]

CH_3CN and 1 M NH_4OAc (pH 4.5) were added (approximately 1 : 1 v/v) to the free thiol (1 eq) and the pNpys activated thiol (1 eq). The mixture was stirred overnight and lyophilized. The product was purified and preparative, Alltech Alltima C8 100 Å 10 µm (250 × 22 mm or 250 × 10 mm), HPLC columns were used unless otherwise stated. A gradient of 100% buffer A to 100% buffer B in 100 min was applied. The fractions were analyzed by analytical HPLC using an Alltech Alltima C8 5 µm (250 × 4.6 mm) column and the appropriate fractions were pooled and lyophilized.

TP10-SS-Flu-ITAM (5). The general disulfide formation procedure was applied at 3 µmol scale using TP10-Cys(pNpys) (**22**) and Flu-Cys-ITAM (**30**). For analytical HPLC the standard TFA buffers were used. The product was obtained as a yellow fluffy solid (4.4 mg, 34%). HRMS (ESI): $[M + 2H]^{2+}$ calculated 2161.9592, found 2161.8464; $[M + 3H]^{3+}$ calculated 1440.6935, found 1440.8491; $[M + 4H]^{4+}$ calculated 1080.7721, found 1080.9844.

Octa-Arg-SS-Flu-ITAM (7). The general disulfide formation procedure was applied at 1.3 µmol scale using octa-Arg-Cys (**23**) and Flu-Cys(pNpys)-ITAM (**31**). For analytical HPLC the standard TFA buffers were used. The product was obtained as a yellow fluffy solid (1.9 mg, 32%). MS (MALDI-TOF): $[M + H]^+$ calculated 3462.508, found 3462.239.

C_{12}-SS-Flu-ITAM (9). The general disulfide formation procedure was applied at 3 µmol scale using C_{12}-S-pNpys (**25**) and Flu-Cys-ITAM (**30**). For analytical HPLC the TEAP buffers were used. The product was obtained as a yellow fluffy solid (1.3 mg, 27%). HRMS (ESI): $[M + 2H]^{2+}$ calculated 1119.9126, found 1119.8400; $[M + H + 2Na]^{3+}$ calculated 762.1228, found 762.2044.

K(Myr)KKK-SS-Flu-ITAM (10). The general disulfide formation procedure was applied at 1.6 µmol scale using K(Myr)KKK-Cys (**27**) and Flu-Cys(pNpys)-ITAM (**31**). For analytical HPLC the standard TFA buffers were used. The product was obtained as a yellow fluffy solid (0.3 mg, 6%). MS (MALDI-TOF): $[M + H]^+$ calculated 2879.256, found 2879.723.

TP10-SS-ITAM (14). The general disulfide formation procedure was applied at 2.5 µmol scale using TP10-Cys (**21**) and Ac-Cys(pNpys)-ITAM (**33**), except that the reaction time was 1 h. For analytical HPLC the standard TFA buffers were used. The product was obtained as a white fluffy solid (2.1 mg, 21%). MS (MALDI-TOF): $[M + H]^+$ calculated 4004.028, found 4004.219.

TP10-SS-npITAM (15). The general disulfide formation procedure was applied at 2.4 µmol scale using TP10-Cys(pNpys) (**22**) and Ac-Cys-npITAM (**34**). For analytical HPLC the standard TFA buffers were used. The product was obtained as a white fluffy solid (2.8 mg, 30%). HRMS (ESI): $[M + 2H]^{2+}$ calculated 1923.8445, found 1923.6176; $[M + 3H]^{3+}$ calculated 1282.8990, found 1282.6471; $[M + 4H]^{4+}$ calculated 962.4262, found 962.2748.

Octa-Arg-SS-ITAM (16). The general disulfide formation procedure was applied at 1.4 µmol scale using octa-Arg-Cys(pNpys) (**24**) and Ac-Cys-ITAM (**32**). For preparative HPLC an Alltech Prosphere C4 300 Å 5 µm (250 × 10 mm) column was used and for analytical HPLC the standard TFA buffers were used. The product was obtained as a white fluffy solid (1.6 mg, 27%). MS (MALDI-TOF): $[M + H]^+$ calculated 3146.471, found 3146.305.

C_{12}-SS-ITAM (17). The general disulfide formation procedure was applied at 3.3 µmol scale using C_{12}-S-pNpys (**25**) and Ac-Cys-ITAM (**32**). For analytical HPLC the TEAP buffers were used. The product was obtained as a white fluffy solid (1.1 mg, 17%). HRMS (ESI): $[M + H]^+$ calculated 1922.7803, found 1922.8661; $[M + Na]^+$ calculated 1944.7622, found 1944.8240; $[M + 2H]^{2+}$ calculated 961.8941, found 961.8193; $[M + H + Na]^{2+}$ calculated 972.885, found 972.8207; $[M + 2Na]^{2+}$ calculated 983.8760, found 983.8316; $[M + H + K]^{2+}$ calculated 980.8720, found 980.8080.

C_{12}-SS-npITAM (18). The general disulfide formation procedure was applied at 2.6 µmol scale using C_{12}-S-pNpys (**25**)

and Ac-Cys-npITAM (**34**). For analytical HPLC the standard TFA buffers were used. The product was obtained as a white fluffy solid (1.4 mg, 31%). HRMS (ESI): $[M + H]^+$ calculated 1762.8476, found 1762.6885, $[M + Na]^+$ calculated 1784.8296, found 1784.6439, $[M + 2H]^{2+}$ calculated 881.9277, found 881.8311; $[M + H + Na]^{2+}$ calculated 892.9187, found 892.8214; $[M + 2Na]^{2+}$ calculated 903.9097, found 903.8288, $[M + H + K]^{2+}$ calculated 900.9057, found 900.7785.

Cell culture and stock solutions

RBL-2H3 cells were cultured in 25, 75 and 225 cm^2 cell culture flasks in RPMI 1640 with L-glutamine, supplemented with 10% heat-inactivated fetal calf serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. For serial passage and experiments, the cells were detached with trypsin (0.05%) and ethylenediaminetetraacetic acid (EDTA) (0.02%) for 10 min at 37 °C. After trypsinization cells were resuspended in medium for further use.

Stock solutions of the compounds with a concentration of 1 mM in H_2O–DMSO 9 : 1 were prepared and used for all experiments, except for Tat-ITAM (**13**), of which a stock solution of 1 mM in H_2O–DMSO 1 : 1 was prepared.

Flow cytometry

RBL-2H3 cells were plated in 12-well plates overnight resulting in a confluency of ± 50%. The medium was aspirated and compounds **1–10** and 5(6)-carboxyfluorescein were added in different concentrations in complete RPMI 1640 medium. The cells were incubated for 1 h at 37 °C. The cells were washed with PBS, detached with trypsin–EDTA and resuspended in complete medium. The cells were transferred into tubes and centrifuged for 5 min at 300 rpm at 4 °C. The supernatant was removed and the cells were washed with ice-cold PBS. Finally the cells were resuspended in ice-cold PBS (250 μL) and fluorescence was measured using a BD (Becton Dickinson) FACSCalibur flow cytometer. Live cells were gated on Forward Scatter and Side Scatter and approximately 10 000 viable cells were analyzed.

Fluorescence microscopy

RBL-2H3 cells were plated on 30 mm glass-bottom culture dishes (MatTek Corp.) and cultured overnight in complete medium to allow the cells to adhere. The medium was removed and the cells were incubated for 1 h at 37 °C with complete medium (1 mL) containing 10 μM compound. The medium was discarded, and the cells were washed 2 × 2 min with PBS (2 mL) and 1 × 2 min with D-MEM/F-12 (2 mL) without phenol red, antibiotics or serum (imaging medium). Imaging medium was added and the cells were immediately analyzed with fluorescence microscopy using a Nikon Eclipse TE2000-U microscope.

SPR competition experiments

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). Native γ-dpITAM peptide containing a 6-aminohexanoic acid spacer was immobilized on the sensor chip as was described earlier.[39] Competition experiments were performed with different concentrations of compound in the presence of 25 nM Syk tSH2 in HBS buffer. K_D values for the affinity in solution were calculated according to described procedures.[40]

Cell viability assay

RBL-2H3 cells were plated in 96-well plates in complete RPMI 1640 medium and cultured overnight. The cells were incubated with different concentrations of construct in complete RPMI 1640 medium (100 μL in each well) for 1 h under tissue culture conditions. Cell viability was assessed using XTT assays.[41] Briefly, the medium was discarded and complete medium (160 μL) was added to each well. Then 1 mg mL^{-1} XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) and 25 μM PMS (phenazine methosulfate) in plain RPMI 1640 (40 μL) was added and the cells were incubated for 2 h under cell tissue conditions. The absorbance was measured at 450 nm with a reference wavelength of 690 nm using a BioTek μQuant microplate spectrophotometer.

β-Hexosaminidase release assay

The assay was essentially identical to the assay described earlier.[42] Briefly, RBL-2H3 cells were plated in 96-well plates in complete RPMI 1640 medium and cultured overnight. The cells were sensitized with a monoclonal IgE directed against the dinitrophenyl hapten (anti-DNP-IgE, 0.2 μg mL^{-1}) for 1 h in complete RPMI 1640 medium. After sensitization, the cells were washed (2 × 50 μL) with a Tyrode's salt buffer (137 mM NaCl, 2.7 mM KCl, 0.31 mM NaH_2PO_4, 12 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Hepes, 5.6 mM glucose, 0.1% bovine serum albumin, pH 7.2) and the Tyrode's salt buffer (50 μL) was added. After 10 min, constructs in Tyrode's salt buffer (50 μL) were added in different concentrations and the cells were incubated for 20 min. Exocytosis was triggered by the addition of DNP-albumin conjugate (DNP_{30}-HSA, 25 μL, 0.1 μg mL^{-1}) followed by incubation for 30 min. Supernatant (50 μL of each well) was collected. The remaining buffer was removed and the cells were treated with 1% Triton-X-100 in Tyrode's salt buffer (125 μL) for 5 min and the lysate (50 μL) was also collected.

To the collected samples 4-methyl umbelliferyl-*N*-acetyl-β-D-glucosaminide (160 μM) in citrate buffer (0.1 M, pH 4.5, 50 μL) was added and the samples were incubated for one hour at 37 °C. The enzymatic reaction was stopped by adding ice-cold glycine buffer (0.2 M, pH 10.7, 100 μL). The fluorescence was measured in a well plate reader at excitation and emission wavelengths of 360 nm and 452 nm, respectively (BMG LABTECH FLUOstar OPTIMA), and was quantitated using 4-methyl umbelliferone as a standard.

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