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Amino Propynyl Benzoic Acid Building Block in Rigid Spacers of Divalent Ligands Binding to the Syk SH2 Domains with Equally High Affinity as the Natural Ligand

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Abstract—The construction of rigid spacers composed of amino propynyl benzoic acid building blocks is described. These spacers were used to link two phosphopeptide ligand sites towards obtaining divalent ligands with a high affinity for Syk tandem SH2 domains, which are important in signal transduction. The spacer containing two of those rigid building blocks led to a ligand which was as active as the natural ligand, indicating that this building block can be used in the design and synthesis of high affinity divalent constructs that can successfully interfere with crucial protein–protein interactions.

In eukaryotic cells, intracellular signaling proteins play a major role as biomolecular switches between receptor activation and ultimately changes in cell growth, metabolism or differentiation.[1,2] An intriguing example is the Syk protein tyrosine kinase ($p72^{syk}$), which comprises two tandemly arranged Src homology 2 (SH2) domains and a tyrosine kinase domain. This kinase plays an important role in the mast cell,[3,4] where it acts as a biomolecular switch between antigenic activation of the high affinity receptor for immunoglobulin E (FcεRI receptor) and mast cell degranulation, resulting in release of inflammatory mediators.[5,6] Mast cell degranulation may lead to excessive and undesired immune responses like asthma and hay fever.

A crucial step in mast cell activation is diphosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) in the γ-chain of the FcεRI receptor. This ITAM features the consensus sequence pTyr-Xxx-Xxx-Leu-$(Xxx)_{6-7}$-pTyr-Xxx-Xxx-Leu (pTyr = phosphotyrosine, Xxx = undefined amino acid). The ITAM binds upon phosphorylation to the tandem SH2 domains of Syk tyrosine kinase in a divalent mode in which the underlined tetrapeptides are mainly responsible for the interaction. This divalency results in a 5000-fold enhanced affinity compared to the monovalent interaction.[7–9] The divalent interaction is the simplest form of multivalency, which is one of nature's fascinating mechanisms to significantly increase affinity of individual weakly interacting modules.[10] Development of peptidomimetic molecular constructs that selectively interfere with this divalent interaction may provide valuable tools for biomolecular research and can ultimately lead to a new class of therapeutic compounds (Fig. 1).

Recently,[11] we described peptoid–peptide hybrids of the monophosphorylated Syk binding tetrapeptide Ac-pTyr-Glu-Thr-Leu-NH_2 **4**. However, the affinity of

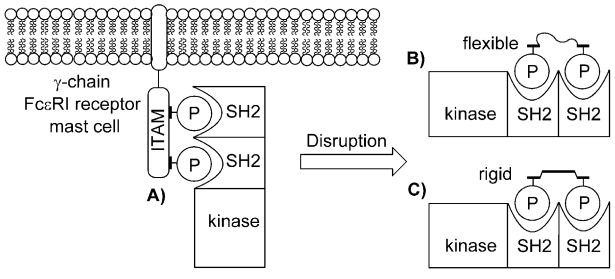

Figure 1. (A) Interaction of the Syk tandem SH2 domains with the double phosphorylated ITAM from the γ-chain of the FcεRI receptor. (B) Interference with this interaction by a divalent construct with a flexible spacer.[12] (C) Interference with this interaction by a molecular construct with a rigid spacer.

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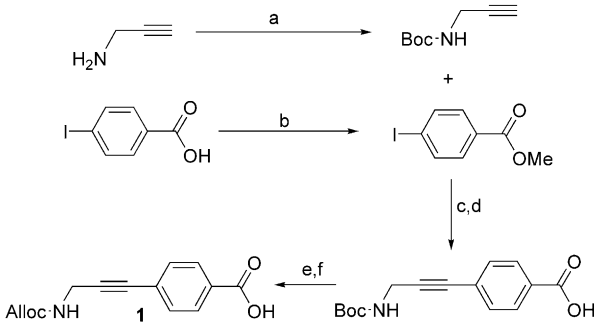
these monovalent peptidomimetic constructs remained modest. Thus, we introduced divalency by linking two monophosphorylated peptides with an oligoethylene glycol spacer resulting in a peptidomimetic construct **3** with a 500-fold increased affinity.[12] In order to improve the affinity of the Syk binding peptidomimetic constructs further, the rigidity of the non-peptide spacer has to be increased to avoid unfavorable entropy loss upon binding. For this purpose, a special rigid amino acid building block was synthesized (Scheme 1), which can easily be incorporated into peptides by solid phase peptide synthesis (vide infra) leading to a peptidomimetic construct with enhanced affinity for the Syk tandem SH2 domains.

Design

Different spacers were designed in modeling studies[12] based on the crystal structure of the Syk tandem SH2 domains complexed with the ITAM peptide.[13] Modeling showed that the intervening amino acids between the interacting tetrapeptides can be replaced by two rigid amino acid building blocks **1** leading to a rigid spacer as in molecular construct **5** (Scheme 2). However, for binding of rigid molecules small spatial mismatches between ligand and receptor are very likely to occur thus resulting in low affinity. This problem might be circumvented by a combination of flexible and rigid building blocks as in molecular construct **6** containing two glycine residues flanking the rigid amino acid derivative.

Chemistry

For the synthesis of rigid building block **1** 4-iodobenzoic acid was converted to the methyl ester and propargylamine was *tert*-butyloxycarbonyl (Boc) protected using Boc_2O and sodium hydroxide (Scheme 1). The resulting 4-iodobenzoic acid methyl ester and Boc-protected propargylamine were coupled by Sonogashira coupling[14] (Scheme 1) using tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$), cupper(I)iodide and triethylamine followed by saponification of the methyl ester. Finally the Boc-group was removed and the allyloxycarbonyl-group (Alloc) was introduced to give 4-(3-allyloxycarbonylamino-prop-1-ynyl)-benzoic acid **1**.[15] Alloc protection was used instead of Fmoc protection, because the Fmoc protected building block was poorly soluble in organic solvents. The Alloc-group is nicely compatible with Fmoc-based peptide chemistry.



4-(3-allyloxycarbonylamino-prop-1-ynyl)-benzoic acid

Scheme 1. Synthesis of the rigid amino acid building block; (a) Boc_2O, NaOH, dioxane/H_2O; (b) H_2SO_4, MeOH; (c) $Pd(PPh_3)_4$, CuI, Et_3N, CH_3CN; (d) NaOH, dioxane/H_2O; (e) HCl in Et_2O, ethylacetate; (f) allylchloroformate, Et_3N, dioxane/H_2O.

This rigid building block was now used for the assembly of the designed peptide molecular constructs using Fmoc based peptide chemistry on Rink (amide) resin, followed by purification as described previously (Scheme 2).[11,12] The pure compounds were characterized by high resolution mass spectroscopy and ^1H 500 MHz NMR.[16] Removal of the Alloc-group was effected with 20 mol% $Pd(PPh_3)_4$ and 8 equiv sodium paratoluene sulfinate in methanol/tetrahydrofuran.[17]

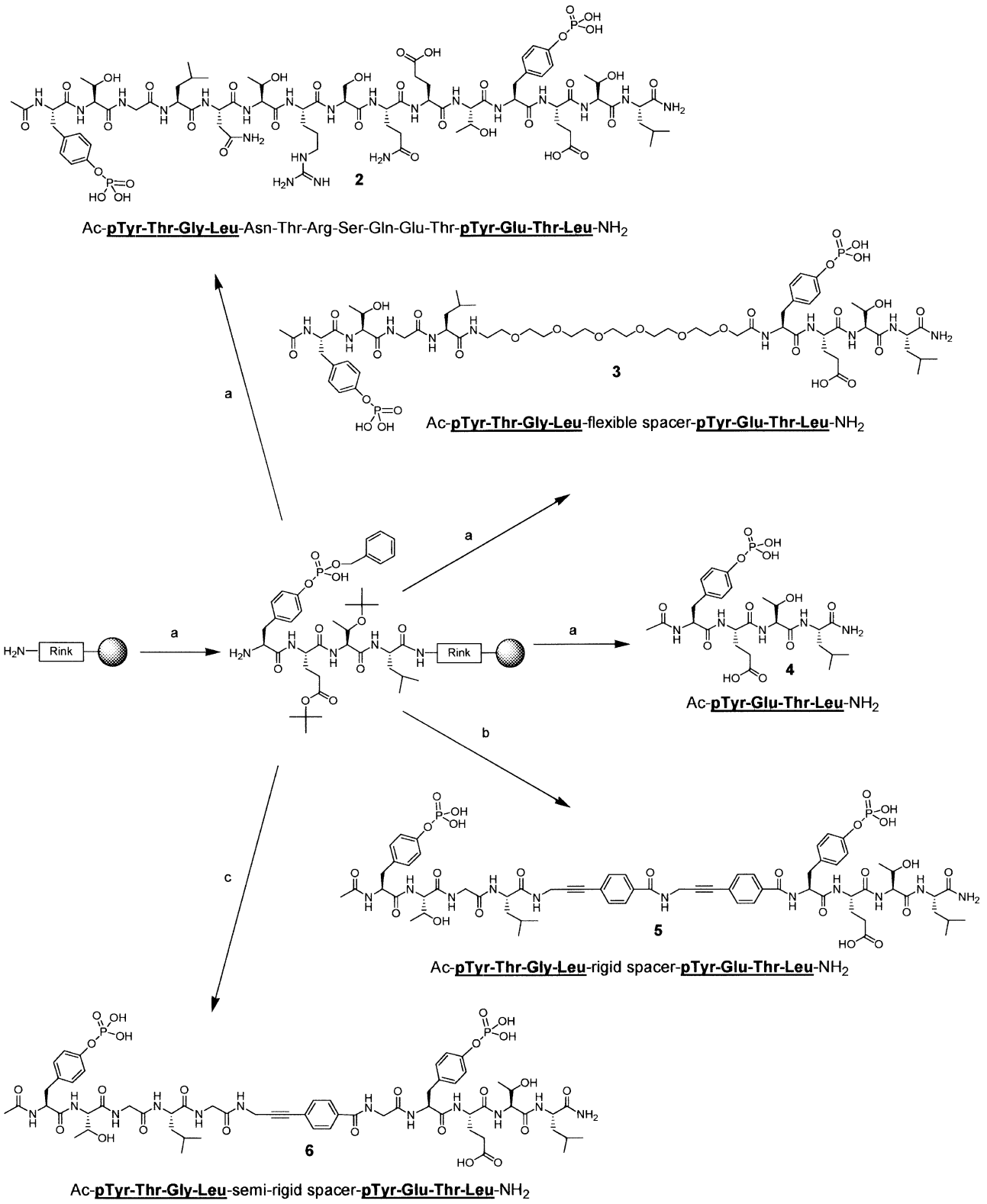
Affinity Measurement

The rigid and semi-rigid peptide constructs **5** and **6** were evaluated for their binding to the Syk tandem SH2 domains using a surface plasmon resonance assay (SPR) as was described previously.[11,12] In this competition assay a Syk binding ITAM peptide was immobilized on a sensor-chip surface on which competition experiments were performed in triplicate (Fig. 2). From these experiments the IC_{50} values were obtained and shown in Table 1 ([Syk tandem SH2] was 100 nM). The dissociation constant for the interaction of the Syk tandem SH2 domains with the ITAM on the sensor-chip surface was 40 nM ($n = 2$, measured at equilibrium), which was used to calculate dissociation binding constants (K_d) of the different peptides and peptide-hybrids in solution.[18] For comparison the monovalent and divalent Syk binding ligands **4** and **2** and peptide-oligoethylene glycol hybrid **3** were included in this study.[12]

In Table 1 the 5000-fold difference between the monophosphorylated peptide **4** and the diphosphorylated peptide **2** is shown,[7–9] which clearly demonstrates the magnitude of the divalency effect in this interaction. The construct with the ethylene glycol spacer **3** showed that a complete different spacer can replace the seven intervening amino acids between the interacting phosphotetrapeptides of the ITAM peptide.[12] Since this is a molecular construct containing a flexible spacer, it provided a starting point to evaluate the effect of rigid spacers. If a more rigid construct has the proper geometry for interaction with the Syk SH2 domains, the affinity is expected to be higher due to a more favorable

Table 1. Affinities of the peptide and peptide-hybrids for the Syk tandem SH2 domains

Compd	IC_{50}, μM	K_d, μM
2	0.13 (±0.01)	0.037
3	1.2 (±0.2)	0.34
4	570 (±50)	166
5	0.10 (±0.007)	0.029
6	62 (±12)	4.0



Scheme 2. Synthesis of the peptide molecular constructs in three main steps: i = Coupling cycle : Fmoc-Xxx-OH, BOP, DiPEA, NMP; 20% piperidine/NMP (Fmoc deprotection) or $Pd(PPh_3)_4$ with sodium paratoluene sulfinate in MeOH/THF (Alloc deprotection);[17] ii = Acetylation : Ac_2O, DiPEA, HOBt, NMP; iii = Final cleavage and deprotection: TFA, EDT, Tis, H_2O (90/2.5/2.5/5); (a) reference[12] (b) (i) **1**, **1**, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(PO(OBzl)OH)-OH; (ii) and (iii) (c) (i) Fmoc-Gly-OH, **1**, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(PO(OBzl)OH)-OH; (ii) and (iii)

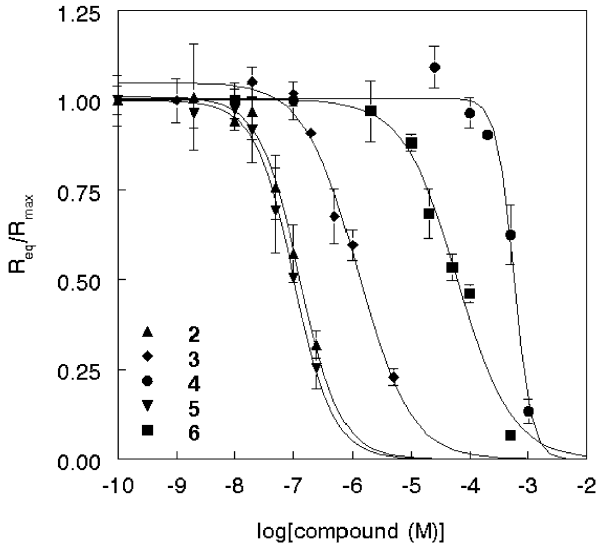


Figure 2. Affinities of the peptide and peptide-hybrids for the Syk tandem SH2 domain as measured by SPR in competition experiments.

entropic contribution. Molecular construct **6** containing the semi-rigid spacer shows a 10-fold reduced affinity compared to the oligoethylene glycol spaced construct **3**. The combination of flexible and rigid building blocks in compound **6** was not effective. Apparently, the length of the spacer was too small and the proper geometry for optimal positioning of the interacting tetrapeptides could not be obtained. Molecular construct **5** with the two rigid spacing building blocks showed a 10-fold increased affinity compared to the molecular construct containing the flexible oligoethylene glycol spacer, thereby reaching an affinity that is equal to the affinity of the native diphosphorylated ITAM peptide **2**. Clearly, this construct has a more optimal geometry for interaction with the Syk tandem SH2 domains. The interaction is expected to be facilitated by the rigidity of the spacer. Finally, the entropic contribution related to flexibility is only one of the factors playing a role in binding thermodynamics. A more detailed thermodynamic study will be subject of further research, as well as the ability of these constructs to interfere with cellular processes.

To our knowledge this is the first time that two interacting phosphopeptides have been linked by a rigid spacer to give rise to a divalent interaction with an affinity that is both better than a molecular construct containing a flexible linker and equally high to that of the natural ligand. We think that this and other rigid amino acid derivatives can be more generally applied as versatile building blocks connecting ligands, which can be used for binding to- widely- separated multivalent interaction sites prevalent in many protein–protein interactions.

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