Acquisition of High-Affinity, SH2-Targeted Ligands via a Spatially Focused Library

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Introduction. The Src[1] homology 2 (SH2) domain, a component of many signal-transducing proteins, plays a critical role in organizing coherent signaling cascades.[2] The latter transpires when primary sequences encompassing phosphotyrosine in one protein are recognized by and coordinate to the SH2 domain of a second. The design of SH2-targeted agents has received considerable attention since such species could disrupt signaling pathways known to be responsible for a variety of disease states.[3] In general, the affinity (i.e., K_D) of SH2 domains for phosphotyrosine-containing peptides lies in the range of 200−800 nM (for an exception vide infra).[4] Nonpeptidic SH2-targeted compounds have also been described, but the affinity of these species for SH2 domains is typically 1−3 orders of magnitude less than that displayed by their peptidic counterparts.[5] Recently, several studies have shown that unnatural motifs, when appended to active site-directed peptides, can dramatically enhance both the affinity and selectivity for such signal-transducing elements as protein kinases[6] and SH3 domains.[7] We describe herein the acquisition of peptide/nonpeptide conjugates that are among the tightest binding and most selective SH2-targeted agents reported to date.[8,9]

Our initial studies have focused on the acquisition of compounds that target the SH2 domains of Lck[1] and Fyn[1], Src tyrosine kinase family members known to participate in T cell activation.[10] Like other Src tyrosine kinases, the SH2 domains of Lck and Fyn exhibit an identical preference for the sequence -**phosphoTyr**-Glu-Glu-**Ile**-,[11] where SH2 affinity is strongly dependent upon the phosphoTyr and Ile side chains.[2b] Interestingly, structural and biochemical studies indicate that conventional amino acid residues positioned to the N-terminus of phosphoTyr have little influence on either SH2 selectivity or affinity.[2b] Despite the latter observation, a hydrophobic indentation in the SH2 surface does lie adjacent to the amine of phosphoTyr in an SH2-bound peptide (Figure 1). Consequently, this invagination could serve as a potential binding site for a structurally compatible ligand appended off the N-terminus of phosphoTyr-Glu-Glu-Ile-amide. Since the size of the putative binding region is modest, there is little opportunity to create extensive molecular diversity via a combinatorial amalgamation of an array of subunits connected in series. Instead, we developed a parallel synthesis strategy (Scheme 1) that achieves a high degree of molecular diversity within a spatially focused region: 900 different carboxylic acids have been affixed to the N-terminus of phosphoTyr-Glu-Glu-Ile-amide, creating a structural diversity within a narrow spatial region that is significantly greater than what is possible using a conventional combinatorial library.

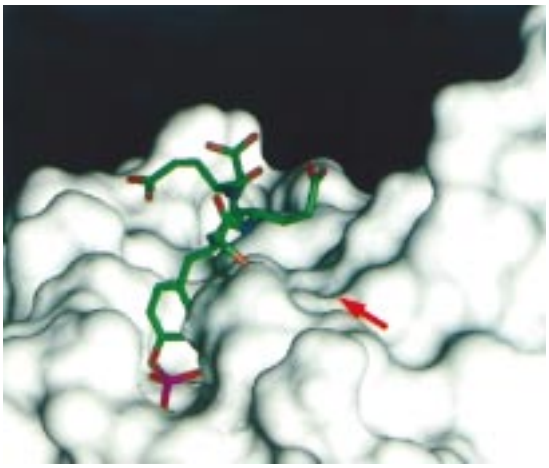

Figure 1. Acetyl-pYEEI-amide bound to the Lck SH2 domain.[15] The acetylated amide is oriented into the region of the SH2 domain targeted by the spatially focused library.

Results and Discussion. Solid-phase peptide synthesis using the Fmoc protocol was performed on the novel disulfide-containing Tentagel-based resin **1** (for the synthetic protocol, see Supporting Information). The Fmoc protecting group at the N-terminus was subsequently removed, and the Tentagel-bound peptide **2** was distributed in 5-mg quantities to individual wells of 96-well microfiltration plates. The free N-terminus of the peptide was condensed with a different carboxylic acid in each well to furnish the substituted species **3**. A total of 10 microfiltration plates were employed to accommodate the 900 different carboxylic acids employed in this study. The latter array of carboxylic acids included aliphatic, aromatic, multiring, hydrophobic, and hydrophilic species, as well as negatively and positively charged species. All resin-washing steps (including removal of excess activated carboxylic acid) were conducted by vacuum filtration using a 96-well filter plate vacuum manifold. After deprotection of the Glu side chains with 50% trifluoroacetic acid in CH_2Cl_2, the peptide/nonpeptide conjugates were cleaved from the solid support with three washings of 10 mM dithiothreitol (DTT) in 50 mM Tris buffer. Each washing was directly filtered into a receiving set of 96-well plates using the vacuum manifold. *The disulfide link between peptide and resin permits the application of cleavage conditions that are virtually identical to the conditions employed in the subsequent assay. As a consequence, the peptide/nonpeptide conjugates* ***4*** *are delivered to the receiving plates in a solution that is assay-ready.* The efficiency of amine acylation and DTT cleavage, as well as purity of the peptide/nonpeptide conjugate, were assessed with four of the synthesized species. No free N-terminus was detected in these compounds, and over 90% of the total peptide/nonpeptide conjugate was cleaved from the resin with the first DTT washing step. The final two DTT

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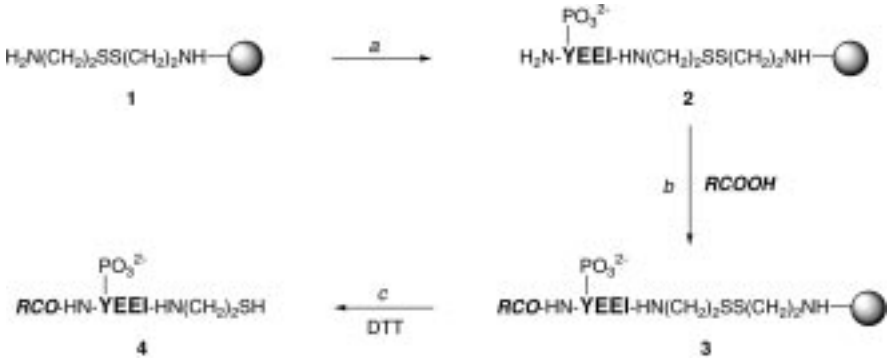
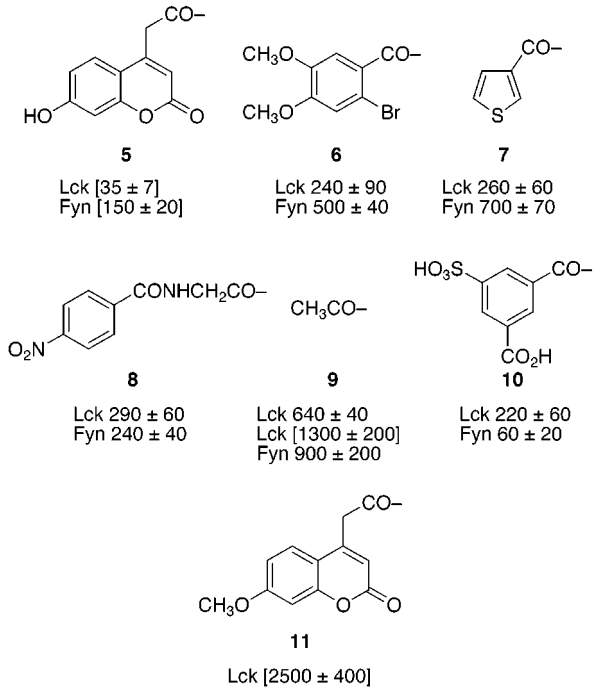
Scheme 1. Chemical Synthesis of a Spatially Focused Library[a]



[a] (a) (i) Fmoc-based solid-phase peptide synthesis, (ii) piperidine/DMF; (b) (i) RCOOH (400 equiv based on resin-bound peptide), benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (200 equiv), 1-hydroxybenzotriazole (200 equiv), *N*-methylmorpholine (1000 equiv), 100 μL of DMF, 18 h, rt, (ii) 50% trifluoroacetic acid/50% CH_2Cl_2, 2 h, rt; (c) 10 mM dithiothreitol (DTT) in 50 mM Tris (pH 7.5) (1 × 200 μL, 2 × 150 μL; 1 h each at rt).

Chart 1. Structures and K_D Values (nM) for Peptide/Nonpeptide Conjugates **5**−**11**[a]



[a] K_D's obtained via the equilibrium dialysis method (Supporting Information) are provided in brackets; all other K_D values were acquired using a competition assay versus the AANS-labeled peptide as described in ref 13.

washings removed the residual resin-bound material. The purity of these four compounds was greater than 90% as assessed by HPLC. These representative members of the 900 compound library were HPLC-purified (i.e., removal of Tris buffer and DTT) and subsequently characterized by electrospray mass spectrometry.

With the 900 member library in hand, high-affinity SH2-targeted ligands were identified via an enzyme-linked immunosorbent assay (ELISA).[12] Under the stringent conditions employed ([peptide/nonpeptide conjugate] = 50 nM), more than 65% of the compounds failed to display any activity in the ELISA assay. We selected a few representative compounds from the assay screen and resynthesized them on the Rink resin, without the mercaptan tail (Chart 1). The absence of the latter eliminates the formation of any possible side products (e.g., disulfide bond formation or other forms of oxidation) when working with isolated pure material. K_D values were initially determined by a previously reported spectrofluorometric method using the acetamidoanilino-naphthyl-sulfonic acid (AANS)-labeled peptide Phe-Thr-Ala-Thr-Glu-Cys(AANS)-Gln-phosphoTyr-Glu-Glu-Ile-Pro.[13] The latter exhibits an 8.7-fold enhancement in fluorescence intensity upon SH2 coordination and has a reported K_D for the Lck GST-SH2 fusion protein of 40 nM. In our hands, we obtained a value of 78 ± 14 nM. The K_D values for compounds **6**−**9** were obtained by the competitive displacement of the AANS-labeled peptide. Unfortunately, we were unable to acquire a binding constant for **5** by this method, since the highly fluorescent coumarin nucleus interferes with the AANS fluorophore. However, since coumarin fluorescence is not altered when **5** is coordinated to the SH2 domain, we were able to obtain a K_D via equilibrium dialysis using slide cassettes (see Supporting Information). Although the equilibrium dialysis and spectrofluorometric assays were performed under nearly identical conditions, we felt that it was prudent to determine K_D values for at least one compound using both assays. The K_D furnished by equilibrium dialysis for compound **9** (by competitive displacement of **5**) is twice as large as that obtained via the spectrofluorometric method (i.e., compound **5** displays a 36-fold higher affinity for the Lck SH2 domain than **9**, whereas the AANS-derivatized peptide is only 8-fold more potent than **9**). We note that the K_D values reported herein are not simply the concentrations required to displace one-half of the fluorescent ligand but take into account the concentration and K_D of the probe fluorescent ligand itself (see Supporting Information).

The affinity of compound **5** (35 ± 7 nM) for the Lck SH2 domain is the highest reported for any species to date, even exceeding the affinities displayed by much longer peptides, such as the AANS-labeled species. In addition, **5** is a significantly weaker ligand for the SH2 domains of PLCγ1[1] (K_D = 4.9 ± 0.7 μM), the p85α subunit of PI3 kinase[1] (K_D = 9.3 ± 0.9 μM), and Grb2[1] (11.3 ± 3.1 μM). A recent combinatorial library study demonstrated that the SH2 domains of Src kinase family members exhibit identical sequence specificities.[11] This observation is borne out by the virtually identical K_D values displayed by the SH2 domains of Lck (78 ± 14 nM) and Fyn (66 ± 13 nM) for the AANS-labeled peptide. In contrast, **5** exhibits a nearly 5-fold

preference for the Lck SH2 domain versus that of Fyn, which represents the first example of selectivity between any members of the Src kinase family. We also screened our library of compounds against the Fyn SH2 domain and identified compound **10** as the most potent Fyn-targeting agent. The latter displays a discriminatory preference in favor of Fyn versus that of Lck. This selectivity, although modest, is promising given the fact that the N-terminus acyl appendages are likely directed into a region of the SH2 domain that is highly conserved in the Src enzyme family. In contrast, there is less sequence homology in other regions of the SH2 domain that coordinate acyl-phosphoTyr-Glu-Glu-Ile. Consequently, application of the methodology described herein to identify nonpeptidic substituents for other sites on acyl-phosphoTyr-Glu-Glu-Ile may produce species that exhibit even greater selectivities than those displayed by **5** and **10**.

Perhaps one of the most intriguing results obtained with the compounds illustrated in Chart 1 is the 70-fold higher affinity that compound **5** exhibits for the Lck SH2 domain versus that of the closely related derivative **11** ($K_D = 2.5 \pm 0.4\ \mu M$). The methyl moiety of the latter may either preclude a key hydrogen-bonding interaction that might transpire between **5** and some residue on the SH2 surface or sterically impede optimal formation of a tight Lck SH2 domain/**11** complex.

Finally, we note that despite the high affinity that several of the compounds in this study exhibit for the SH2 domains of Lck and Fyn, the key phosphotyrosine moiety contained within these species presents a number of difficulties that could impede the facile intracellular application of these compounds. The aromatic phosphate moiety is not only prone to hydrolysis by the presence of adventitious intracellular phosphatases, but its negatively charged nature (along with that of the attendant glutamic acid residues) will likely preclude ready membrane permeability. However, a number of hydrolytically resistant analogues of phosphotyrosine have been reported, including a difluorophosphonate derivative that has been successfully employed in SH2-directed peptides.[14] Furthermore, negatively charged moieties, such as carboxylates, can be readily derivatized as neutral esters, which can render these species membrane permeable. Upon intracellular entry, the ester is then hydrolyzed by nonspecific esterases. The preparation of hydrolytically resistant, membrane permeable analogues of **5** and **10**, along with the acquisition of even more potent and selective SH2-targeted ligands, are goals that are actively under investigation.

In summary, we have prepared a spatially focused high-diversity library to identify SH2-targeted agents. The disulfide linker used to connect the peptide/nonpeptide conjugates to the solid support allows delivery of the conjugates to a receiving plate in an assay-ready form. The coumarin-based analogue **5** and the sulfonated derivative **10** are among the highest-affinity SH2-targeted agents ever reported. Furthermore, since the intense coumarin fluorescence is not affected by SH2 coordination, competitive displacement of the coumarin analogue under equilibrium dialysis conditions (see Supporting Information) offers a sensitive method for the determination of dissociation constants of nonfluorescent SH2 ligands. Finally, compounds **5** and **10** exhibit the heretofore unprecedented ability to discriminate between the SH2 domains of two members of the Src family of protein kinases.

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Supporting Information Available: Full experimental details and ^{1}H NMR spectra for compounds **5** and **9**. This information is available free of charge via the Internet at http://pubs.acs.org.

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