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Acquisition of Fyn-Selective SH3 Domain Ligands via a Combinatorial Library Strategy

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Summary

A stepwise library-based strategy has been employed to acquire a potent ligand for the SH3 domain of Fyn, a Src kinase family member that plays a key role in T cell activation. The easily automated methodology is designed to identify potential interaction sites that circumscribe the protein/peptide binding region on the SH3 domain. The library protocol creates peptide/nonpeptide chimeras that are able to bind to these interaction sites that are otherwise inaccessible to natural amino acid residues. The peptide-derived lead and the Fyn-SH3 domain form a complex that exhibits a K_D of 25 ± 5 nM, approximately 1000-fold more potent than that displayed by the corresponding conventional peptide ligand. Furthermore, the lead ligand exhibits selectivity against SH3 domains derived from other Src kinases, in spite of a sequence identity of approximately 80%.

Introduction

The Src homology 3 (SH3) domain is one of many protein recognition modules that play an essential role in the operation of signaling pathways [1–3]. Like all protein recognition units, the SH3 domain exhibits a marked preference for a particular consensus sequence of amino acid residues, namely, a proline-rich motif of the general form Pro-Xaa-Xaa-Pro [4]. The molecular basis for the proline-based sequence preference has been resolved via the structural elucidation of several SH3 domain/peptide ligand complexes. The peptide ligand is bound as a type II polyproline helix. Side chains on every third residue of the helix are oriented in the same direction. Two of these side chains are positioned into hydrophobic pockets of the SH3 domain. In addition, other residues of the peptide ligand are also able to productively interact with the SH3 domain. One of the interesting aspects of the polyproline helix structure is that the ligand can associate with the binding pocket in an N-to-C orientation or in the opposite sense [5]. The relative bound orientation is dictated by the presence of a positively charged residue on either the N terminus of the peptide ligand ("Type I") or at the corresponding C terminus ("Type II").

More than 400 different SH3 domains are encoded within the human genome. Consequently, even at this early stage in the analysis of the human proteome, it is not surprising that these domains have been implicated in a variety of normal and abnormal physiological processes [6]. At the biochemical level, SH3 domains have been shown to regulate enzymatic activity as well as promote the assembly of signaling complexes. For example, all nine members of the Src family of protein kinases contain the general structure (N terminus)-SH3-SH2-SH1-(C terminus) [7]. The SH3 domain not only serves as an intramolecular on/off switch that controls kinase activity, but also acts as a targeting moiety that directs Src kinase family members to the proper intracellular sites and substrates. SH3 domains are present in a variety of different proteins, including other tyrosine kinases, such as Abl and Crk; protein phosphatases, such as SHP-1; and adaptor proteins, such as Grb2 and Sos.

Many studies that seek to correlate the activity of a given signaling protein with cellular phenotype typically resort to the tools of molecular biology to create constitutively active, dominant-negative, or other analogs of the corresponding wild-type protein. Although this general strategy is a powerful one, deletion of the natural protein or expression of mutated analogs can result in complications arising from compensation by closely related proteins, inappropriate localization of the protein analog, or unintended modulation of other signaling pathways. In contrast to genetically expressed protein analogs, inhibitors or synthetic ligands rapidly perturb the wild-type protein, which is present in its natural state in terms of quantity, activity, and location. In this regard, SH3 domain-selective ligands are a much sought-after commodity. Unfortunately, the widespread presence of SH3 domains throughout the human proteome and their generally similar ligand recognition profiles renders this task a formidable one. In addition, several studies have demonstrated that conventional peptide ligands, consisting of standard amino acid (>10) residues, generally exhibit modest affinities (low μM) and low selectivities for their intended SH3 domain targets [8–11]. Indeed, protein interaction domains, in general, exhibit only moderately robust affinities for their endogenous ligands, a not too surprising observation given the fact that the protein-protein interactions that drive signaling cascades are transient by necessity.

We report, in this study, the synthesis and identification of a peptide-derived ligand that selectively targets the Fyn-SH3 domain. Fyn, a member of the Src tyrosine protein kinase family, is known to play an important role in the biochemical cascade responsible for T cell activation [12]. Both Fyn and its closely related counterpart, Lck, exhibit similar substrate specificities, and the SH2/SH3 domains of both proteins play key roles in thymic development and T cell proliferation in response to antigenic challenges. In particular, the SH3 domains of both have been shown to interact with the Wiskott Aldrich Syndrome protein (WASp) in vitro [13]. To the best of our knowledge, this is the first report of a ligand that is able to discriminate between SH3 domains of the members of the Src kinase family.

Results and Discussion

The consensus sequences for a number of SH3 domains have been reported. However, peptides (deca-

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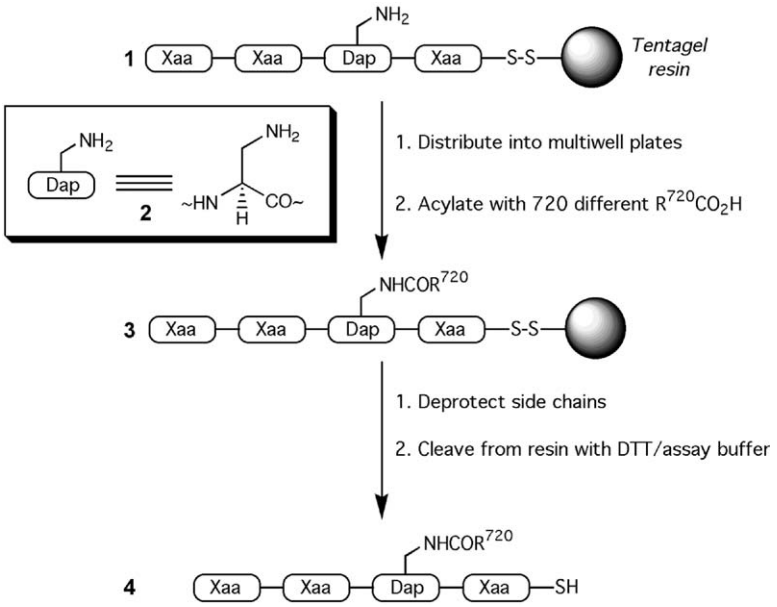

Figure 1. Outline of the Iterative Library-Based Strategy

The protocol seeks to identify potential interaction sites that circumscribe the peptide binding on the SH3 domain that are otherwise inaccessible to conventional amino acid residues. The final synthetic step, namely, cleavage of the modified peptide from the resin with assay buffer, delivers the library in an assay-ready form.

peptides and longer) bearing these sequences serve as relatively modest SH3 domain ligands (μM range). A number of attempts have been made to enhance the affinities of SH3 domain-directed peptides, either by searching for optimized sequences via library methods or by the introduction of nonnatural substituents at the N or C termini [8–11]. However, these strategies typically do not furnish ligands that exhibit affinities significantly beyond the μM/nM border. By contrast, Lim and his colleagues have demonstrated that N-substituted peptides ("peptoids") exhibit high affinities and good selectivities for specific classes of SH3 domains [14, 15]. The critical design element in these studies is the notion that N-substituted glycine residues serve as proline mimetics and, given the diversity inherent within the former, can be screened to identify substituents that dramatically enhance SH3 domain affinity. Peptoid ligands that exhibit nM affinities were identified for the SH3 domains of Grb2 (N terminus; K_D = 30 nM), Src (K_D = 140 nM), and Crk (K_D = 8 nM) [14, 15]. Furthermore, these ligands exhibit a high selectivity in favor of their targeted SH3 domain in spite of the fact that these domains are structurally homologous (e.g., Crk and Src SH3 domains display a 33% sequence similarity) [16]. These results dispel the previously held notion that it is not possible to obtain tight binding ligands for SH3 domains due to the small size (60 amino acids).

We have previously described an iterative library-based strategy that converts low-affinity consensus sequence peptides into high-affinity species that display pronounced selectivities for their intended protein targets [17–20]. The methodology, as illustrated in general form in Figure 1, has been successfully applied to SH2 domains, protein tyrosine phosphatases, and protein kinases, including specific isoforms of the highly conserved protein kinase C subfamily. However, the strategy relies upon identifying a series of unique and widely interspersed interaction sites on the protein surface. Given the small size of SH3 domains, it was far from clear whether proteins of this size would be amenable to such an approach. We decided to explore this question by employing the SH3 domain of the Fyn tyrosine kinase as our target. Fyn is a member of the Src kinase family, a group of highly homologous tyrosine kinases. The extremely high sequence homology displayed by the SH3 domains from these proteins represents a serious challenge in terms of acquiring selective inhibitors (e.g., the SH3 domains of Src and Fyn are 78% identical [21]). To the best of our knowledge, there are no examples of ligands that distinguish between the SH3 domains of the Src kinase family.

A good portion of the binding energy that drives SH3 domain/consensus sequence complex formation results from the interaction of key residues on the consensus sequence peptide with hydrophobic pockets of the SH3 domain. However, adjacent residues can also contribute binding energy by engaging in noncovalent interactions with subsites located proximal to these lipophilic pockets. The methodology described in Figure 1 allows one to screen for substituents positioned on the peptide ligand that can engage in interactions with the SH3 domain, which are simply not accessible to naturally occurring amino acid residues. The consensus sequence peptide **1** was prepared on a disulfide-linked Tentagel resin. One of the residues in **1** contains (L)-2,3-diaminopropionic acid **2** (Dap), which possesses an amine handle on the side chain that can be readily modified with an array of activated carboxylic acid moieties. Peptide-resin **1** was then distributed, in equal amounts, into individual wells of eight 96-well synthesis plates. Each well was subsequently charged with a single carboxylic acid from a total library of 720 different commercially available carboxylic acids that vary in size, shape, polarity, and charge. Following acylation of the amine moiety on Dap (**3**), the side chains of the peptide were deprotected, and the peptide was subse-

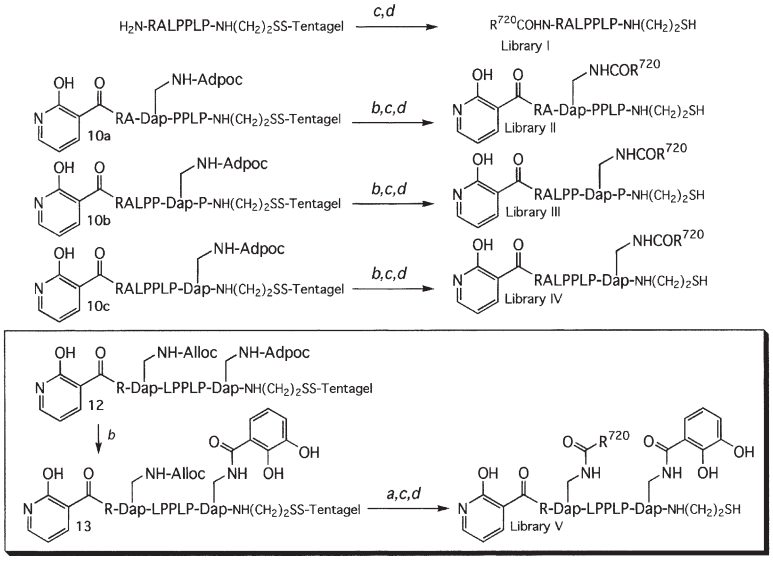

Figure 2. Preparation of Libraries I–V

In Libraries I–V, *a* = Pd, *b* = 3% CF_3CO_2H, *c* = 720 RCO_2H, and *d* = assay buffer containing dithiothreitol.

quently cleaved from the resin (**4**) by using assay buffer that contains dithiothreitol (DTT). The peptides were then filtered from the synthesis plate into a receiving plate and the 720-member library was subsequently screened.

We employed Arg-Ala-Leu-Pro-Pro-Leu-Pro as the starting point for Fyn-SH3 ligand optimization, and this sequence is consistent with most known SH3 domain binding elements. We initially introduced diversity elements on the N terminus of this peptide to furnish Library I (Figure 2). The 720 individual members of this library were subsequently assessed for SH3 binding potency via an ELISA-based screen. The latter was performed in parallel by using strepavidin-coated 96-well plates. A biotinylated peptide SH3-directed ligand was appended to the individual wells, and a SH3 domain GST fusion protein was subsequently introduced. Competition between members of the library and the microwell bound biotinylated peptide ligand determined the extent to which the SH3 domain GST fusion protein was retained by the individual wells after washing. The latter was quantified by using an anti-GST-peroxidase fusion construct. The assay is dependent upon a strong binding interaction between the SH3 domain GST fusion protein and the biotinylated peptide ligand associated with the strepavidin-coated well. Unfortunately, as noted above, conventional peptide ligands bind modestly to SH3 domains. Consequently, we decided to prepare, for screening purposes, a variant of the peptoid series reported by Lim and his colleagues [14, 15]. Although the 13 residue-containing peptoid **5** does not discriminate between the SH3 domains of Src, and Grb2, it does exhibit a moderately good affinity for these domains (K_D = ~150 nM). We prepared construct **6**, which contains an aminohexanoic acid (Ahx) dyad between the biotin substituent and the N-benzylated consensus sequence (Figure 3). The Ahx_2 linker serves to ensure that the SH3 domain has spatial access to the microwell bound peptoid moiety. We also prepared the fluorescein-labeled analog **7**, which exhibits a K_D of 230 ± 30 nM for the Fyn-SH3 domain, but with little selectivity versus other members of the Src kinase family (vide infra and Table 1).

Our initial screen of the heptapeptide-based Library I revealed three lead derivatives. These leads were resynthesized on the Rink resin as the C-terminal amide-capped derivatives (compounds **8a–8c**; Figure 4), purified, and their IC_{50} values subsequently determined by using the ELISA assay described in the previous paragraph. Their relative affinities ranged from 15 μM for the 2-hydroxynicotinic acid derivative **8a** up to 45 μM for the cyclopropyl derivative **8c**. K_D values were obtained via equilibrium dialysis by using the fluorescent intensity inherent within the nicotinic acid moiety. The K_D for the lead derivative from Library I, compound **8a**, is 1.2 ± 0.2 μM, a nearly 15-fold improvement relative to the corresponding acetylated derivative **9** (K_D = 15 ± 4 μM). As expected, the parent peptide **9** is unable to discriminate between the various SH3 domains of the Src family members (Table 1). By contrast, some selectivity in favor of Fyn is apparent with **8a**, particularly with respect to the SH3 domains from Lck and Hck.

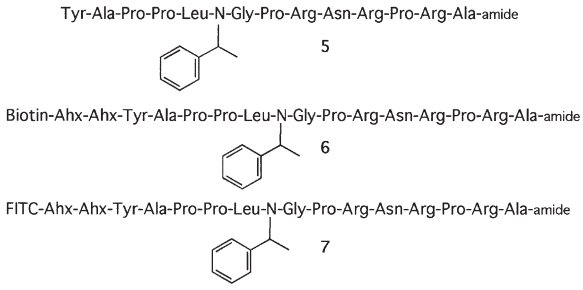

Figure 3. Peptoides 5, 6, and 7

Peptoid **6**, which was based on the previously described peptoid **5** [15], was used for the ELISA screen. The fluorescently labeled peptoid **7** exhibits a K_D of 230 ± 30 nM for the Fyn-SH3 domain.

Table 1. Dissociation Constants of Ligands for Various Src Family SH3 Domains

Ligand	Fyn	Lck	Src	Yes	Hck
7	0.23 ± 0.03	0.29 ± 0.05	0.21 ± 0.04	N/A	N/A
8a	1.2 ± 0.2	5.3 ± 0.4	2.5 ± 0.2	2.7 ± 0.3	5.1 ± 0.7
9	15 ± 4	19 ± 8	21 ± 4	17 ± 6	31 ± 8
11a	0.11 ± 0.03	0.59 ± 0.08	0.37 ± 0.05	0.38 ± 0.14	0.62 ± 0.07
14	0.025 ± 0.005	0.27 ± 0.03	0.23 ± 0.04	0.19 ± 0.02	0.26 ± 0.03

Dissociation constants are given in μM.

However, compound **8a** does not distinguish between the SH3 domains of Fyn, Yes, and Src. Although the molecular basis for this emerging selectivity remains to be resolved, we do note that, on the basis of sequence identity, Lck and Hck (Group B Src kinases) lie on a separate branch of the evolutionary tree from Fyn, Yes, and Src (Group A Src kinases) [22]. This might account for the modest selectivity that compound **8a** exhibits for the Fyn-SH3 domain versus those from Lck and Hck.

With a biasing element at the N terminus of Arg-Ala-Leu-Pro-Pro-Leu-Pro in place, we subsequently examined the effect of nonnatural substituents at various internal sites of the consensus sequence peptide. Specifically, three sublibraries of the peptide **8a** lead containing Dap replacements at Leu-3 (Library II), Leu-6 (Library III), and at the C terminus (Library IV) were prepared. These libraries were prepared as depicted in Figure 2. In all three cases (**10a–10c**), the Dap-containing 2-hydroxynicotinic acid-derivatized peptides were synthesized via an Fmoc protocol on the disulfide Tentagel resin. Following completion of the peptide framework, the Adpoc protecting group on the Dap side chain was selectively removed under mildly acidic conditions, the peptide-resin distributed to individual microwells, and the Dap amino group subsequently acylated with 720 different carboxylic acid derivatives (Libraries II–IV). As with Library I, each of the three sublibraries contained 720 distinct and physically separate members. The 2160 compounds that constitute Libraries II–IV were screened by using the ELISA assay described above. The lead compounds from this screen were obtained exclusively from Library IV. These compounds were resynthesized on the Rink resin as the C-terminal amide-capped derivatives (**11a–11d**, Figure 4), purified, and subsequently assessed for Fyn-SH3 affinity. Compound **11a** exhibits a K_D of 110 ± 30 nM, more than a 100-fold enhancement in affinity relative to the parent compound **9** (Table 1). In addition, a slight improvement in selectivity for the Fyn-SH3 domain relative to Lck and Hck is apparent, as is the emergence of discriminatory behavior versus that of Yes and Src.

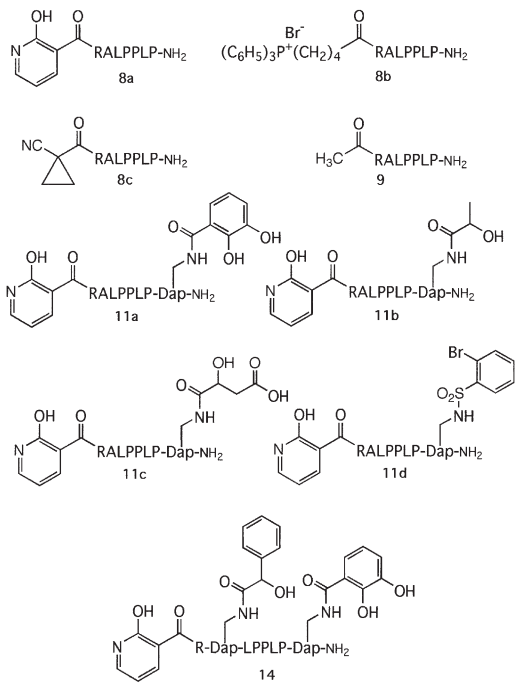

Figure 4. Leads Derived from Libraries I–V

Based on the optimized sequence contained in **11a**, we prepared a second sublibrary containing molecular diversity introduced at the Ala-2 position. Library V contains two Dap residues, each of which must be selectively modified. A number of synthetic strategies are possible. However, given the possible reactivity of the catechol hydroxyl moieties of the Dap substituent in **11a**, we decided to introduce the 2,3-dihydroxybenzoic acid moiety after completion of the peptide framework (Figure 2). This required the use of two different Dap residues that were differentially side chain protected. The peptide-Tentagel resin **12** was prepared via Fmoc chemistry. The Adpoc group on the C-terminal Dap residue was selectively removed with 3% CF_3CO_2H, and 2,3-dihydroxybenzoic acid was subsequently coupled to the free amine side chain (**13**). The Alloc-protected Dap moiety at position 2 was then removed with Pd^O, and the resultant peptide-resin containing the free amine was distributed in equal amounts to 720 individual microwells for coupling with activated carboxylic acids (Library V). The peptide library was then side chain deprotected, extensively washed, and subsequently released from the Tentagel resin by using assay buffer. Screening of Library V furnished a single lead compound, which was resynthesized, purified, and evaluated as a ligand (**14**) for the SH3 domain of Fyn. As depicted in Table 1, peptide **14** displays a K_D of 25 ± 5 nM for the SH3 domain of Fyn, a nearly 1,000-fold enhanced affinity relative to the starting parent peptide **9**. In addition, the former exhibits a 10-fold selective affinity for Fyn versus SH3 domains from other Src kinase family members. To the best of our knowledge, this is the first example of a ligand that is able to distin-

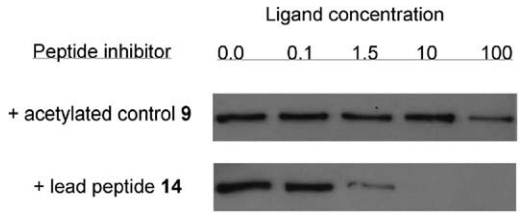

Figure 5. Inhibition of WASp/Fyn-SH3 Interaction with the Fyn SH3 Domain Ligand **14**

Glutathione sepharose beads saturated with Fyn SH3 were incubated with U937 cell lysates in the presence of inhibitory peptide **14** or acetylated control **9** at the appropriate concentrations (μM).

guish between the SH3 domains of this closely related group of protein kinases.

As noted in the Introduction, Fyn is known to play an important role in mediating the activation and proliferation of T cells via a signaling pathway that emanates from the T cell receptor. Recent studies have demonstrated that WASp is a participant in this pathway by directly interacting with and undergoing phosphorylation by Fyn [13, 23]. The association of a proline-rich region on WASp with the SH3 domain of Fyn drives the formation of the transient WASp/Fyn complex. Fyn-mediated WASp phosphorylation at Tyr-291 releases WASp from an autoinhibitory conformation. Upon activation, WASp induces Arp2/3 activity, which, in turn, promotes actin polymerization. In an apparently analogous fashion, the B cell lineage Src kinase, Hck, likewise interacts with WASp in an SH3 domain-dependent manner [24]. For our initial studies, we examined the ability of compound **14** to block the interaction of WASp with the Fyn-SH3 domain. We employed the human leukemia monocyte U937 cell line that was previously used to identify WASp as a Fyn-SH3 domain interacting protein [13]. Lysates from both undifferentiated U937 cells and phorbol 12-myristate 13-acetate-treated cells were employed [25]. Both protocols furnished similar results. The lysates were separately added to a mixture of glutathione-sepharose beads saturated with Fyn-SH3 GST fusion protein and varied amounts of Fyn-SH3 ligand **14** or the corresponding acetylated parent peptide **9**. The sepharose beads were collected, the bound proteins released and fractionated by SDS-PAGE, and WASp visualized via chemiluminescence with an anti-WASp monoclonal antibody. As is evident from Figure 5, ligand **14** disrupts formation of the WASp/Fyn complex with a greater than 100-fold enhanced potency relative to peptide **9**.

Conclusions

We have acquired a Fyn-SH3 domain-selective ligand via an iterative library-based approach. This strategy relies on the identification of widely distributed interaction sites on the surface of the target protein. Our results demonstrate that, even for a protein as small as an SH3 domain, potent ligands can be prepared by using the stepwise protocol outlined in Figure 1. To the best of our knowledge, ligand **14** displays the highest affinity ever reported for an SH3 domain from the Src kinase family. Furthermore, we have observed for the first time the emergence of a ligand that distinguishes between the SH3 domains (~80% sequence identity) of the highly conserved Src family of proteins. Clearly, it would be extremely useful to have an assortment of relatively low-molecular weight ligands that selectively target individual members of the Src kinase family. These studies are in progress, as is an examination of the molecular basis for the potency and selectivity associated with ligand **14**.

Significance

Signal transduction is primarily driven by protein-protein interactions. Given the important role that cell signaling plays in both normal and abnormal cellular events, there has been intense interest in the acquisition of inhibitory agents that block signaling pathways by interfering with the ability of proteins to recognize their appropriate binding partners. SH3 domains are amongst the smallest of all known protein binding modules and thus represent a significant challenge in terms of acquiring high-affinity ligands. We have developed a straightforward stepwise strategy that has furnished an extremely potent ligand for the Fyn-SH3 domain. The latter exhibits a sequence identity of 80% with other SH3 domains from the Src family of protein kinases. The Fyn ligand identified in this study represents the first example of a small nonprotein molecule that is able to distinguish amongst the Src SH3 domain proteins.

Experimental Procedures

The resins and reagents used for solid phase peptide synthesis, including Tentagel resin, Rink resin, N-9-fluorenylmethyloxycarbonyl (Fmoc)-L-amino acids, N,N,N′,N′-tetramethyl-(succinimido)uranium tetrafluoroborate (TSTU), benzotriazole-1-yloxytrispyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt), were purchased from Advanced ChemTech. Peptide synthesis grade dichloromethane (DCM), N,N-diisopropylethylamine (DIPEA), isopropyl alcohol (IPA), dimethylformamide (DMF), and trifluoroacetic acid (TFA) were purchased from Fisher, and piperidine was obtained from Lancaster. 2-Fmoc-3-[1-(1′-Adamantyl)-1-methylethoxycarbonyl]-diaminopropionic acid (Fmoc-Dap(Adpoc)-OH) was obtained from Bachem. Triisopropylsilane (TIS) was purchased from Acros. The 720 carboxylic acids used for the preparation of the peptide libraries were purchased from Aldrich. The SH3 domain GST fusion proteins, Fyn (85–139) and Lck (54–120), and polyclonal rabbit anti-GST HRP conjugate antibody were purchased from Santa Cruz Biotechnology. Peroxidase substrate (1-step Turbo trimethylbenzidine ELISA), streptavidin-coated 96-well plates, and Slide-A-Lyzer dialysis slide cassettes (M_r 10,000 cutoff) were purchased from Pierce. Solvent-resistant MultiScreen 96-well filter plates and the MultiScreen 96-well filter plate vacuum manifold were purchased from Millipore Corp.

One-dimesional and two-dimensional ^{1}H NMR spectra of the peptide inhibitors were recorded on a DRx600 MHz Spectrometer in H_2O/D_2O (90:10), and chemical shifts are reported in parts per million (ppm) downfield from $(CH_3)_4Si$. The molecular weights of the peptides were analyzed with MALDI mass spectrometry on an Applied Biosystems Voyager DE STR and ESI-MS on an Applied Biosystems MDS SCIEX API Qstar Pulsar I. Reverse-phase high-performance liquid chromatography (RP-HPLC) was performed on a Waters SD-200 solvent delivery system equipped with a 500 UV/Vis-absorbance detector and recorded on an Apple Macintosh computer with model 600 software (Applied Biosystems, Inc.). Chromatographic separations were achieved by using linear gradi-

ents of buffer B in A (A = 0.1% aqueous TFA; B = 0.1% TFA in CH_3CN) over 50 min at a flow rate of 12 ml/min with a detection wavelength of 218 nm on Delta-Pak C18 (300 Å, 15 μm, 3 × 15 cm) column.

Peptide Synthesis

Peptides were synthesized by using a standard Fmoc solid phase peptide synthesis (SPPS) protocol on an Innova 2000 platform shaker, or with an Advanced Chemtech Model 90 Tabletop Peptide Synthesizer.

Synthesis of Library I

A total of 5 g Tentagel S COOH (90 μm, 0.2 mmol/g) and 1.94 g (15 mmol) DIPEA were successively added to a solution of 1.5 g (5 mmol) TSTU in 20 ml DMF. The mixture was shaken for 2 hr at ambient temperature. Subsequently, a mixture of 2.25 g (10 mmol) cystamine dihydrochloride and 2.02 g (20 mmol) N-methylmorpholine (NMM) in 20 ml water was slowly added to the Tentagel reaction mixture. Heat was evolved upon addition. Upon cooling to room temperature, the reaction vessel was sealed and shaken overnight. The resin was then drained and washed successively with H_2O (3 × 20 ml), DMF (3 × 20 ml), and CH_2Cl_2 (3 × 20 ml). The free amine substitution level on linker-coupled resin was found to be 0.05 mmol/g. The linker-coupled resin (5 g) was successively submitted to coupling reactions with the required amino acids, followed by removal of the Fmoc protecting group via standard conditions (vide infra). Each residue was coupled for 3 hr, and coupling efficiencies were determined by a quantitative ninhydrin reaction. The standard coupling conditions employed 5 equiv. of Fmoc amino acid, 5 equiv. of HOBt, 5 equiv. of PyBOP, and 10 equiv. of NMM in 30 ml DMF with shaking for 3 hr. After each coupling step, the resin was successively washed with DMF (3 × 20 ml), IPA (3 × 20 ml), and CH_2Cl_2 (3 × 20 ml). The Fmoc protecting group was removed with 20% piperidine in DMF (shaking for 20 min). After the assembly of the consensus sequence of Fmoc-Arg(Pbf)-Ala-Leu-Pro-Pro-Leu-Pro-S-S-Tentagel-Resin, the Fmoc group at the amino terminus was removed, and the resin was extensively washed and subsequently dried in vacuo. The peptide bound resin was distributed in 5 mg quantities into individual wells of solvent-resistant MultiScreenTM 96-well filter plates (8 plates total). To each well was added a solution of a carboxylic acid (200 equiv.) in 100 μl DMF and a second solution containing PyBOP (200 equiv.), HOBt (200 equiv.), and NMM (400 equiv.) in 100 μl DMF. A total of 720 different carboxylic acids were employed. The plates were gently shaken overnight, and then each well was subjected to a series of washing steps (3 × 200 μl DMF, 3 × 200 μl IPA, and 3 × 200 μl CH_2Cl_2). The side chain protecting group Pbf was removed via treatment with TFA:H_2O:TIS (v/v 95:2.5:2.5) for 2 × 3 hr at ambient temperature. The resin was washed with DMF (3 × 20 ml), IPA (3 × 20 ml), and CH_2Cl_2 (3 × 20 ml), and the peptide/nonpeptide conjugates were subsequently cleaved from the disulfide-containing resin with 10 mM dithiothreitol (DTT) in 50 mM Tris (pH 7.5) (1 × 200 μl for 3 hr and 2 × 150 μl for 3 hr each) and filtered into a receiving set of 96-well plates by using a vacuum manifold (final volume of 500 μl). The coupling efficiency of the acylation reaction and the purity of peptide/nonpeptide conjugates were assessed via the ninhydrin test and RP-HPLC, respectively. No free N-terminal peptide was detected, and >90% of total ligand was cleaved from the resin with the first DTT cleaving step. The final two DTT washings removed the residual resin bound peptide. Compound purity was >90%, as assessed by HPLC, and the HPLC-purified compounds (i.e., removal of Tris buffer and DTT) were characterized by MALDI-MS. These peptides, containing 720 different groups at the N terminus of consensus sequence in 8 plates, comprise Library I.

Synthesis of Libraries II, III, and IV

Construction of Libraries II, III, and IV is depicted in Figure 2. The side chain-protected consensus sequence Fmoc-Arg(Pbf)-Ala-Leu-Pro-Pro-Leu-Pro-S-S-Tentagel-Resin was assembled on the Tentagel resin as described in the previous paragraph with the following substitutions: the N terminus leucine was replaced with a Dap(Adpoc)-OH to afford Library II, the C terminus leucine was replaced with a Dap(Adpoc)-OH to afford Library III, a Dap(Adpoc)-OH residue was inserted into the C terminus of the sequence to afford Library IV. The N terminus Fmoc group (5 g resin) was then removed with 20% piperidine in DMF, and the resin was thoroughly washed with DMF, IPA, and CH_2Cl_2. A solution containing 2-hydroxynicotinic acid (695.5 mg, 8 equiv.), PyBOP (2.6 g, 8 equiv.), HOBt (765 mg, 8 equiv.), and NMM (1.26 g, 20 equiv.) in 30 ml DMF was added, and the slurry was shaken overnight at room temperature. The solvent was removed from the resin, and the resin was subsequently washed with DMF (3 × 20 ml), IPA (3 × 20 ml), and CH_2Cl_2 (3 × 20 ml). The Adpoc group in these three sequences (5 g resin) was selectively removed with 40 ml 3% TFA in CH_2Cl_2 (3 × 5 min), and the resin bearing free amine on the Dap residue was washed, dried, and then added in 5 mg quantities to the individual wells of eight solvent-resistant MultiScreenTM 96-well filter plates. The following procedures, as described for Library I, were employed: the resin in each well was coupled with 1 of 720 different carboxylic acids, the side chain protecting groups were removed, and the peptides were cleaved from the resin to furnish Libraries II, III, and IV.

Synthesis of Library V

Construction of Library V was depicted in Figure 2. The side chain-protected consensus sequence Fmoc-Arg(Pbf)-Dap(Alloc)-Leu-Pro-Pro-Leu-Pro-Dap(Adpoc)-S-S-Tentagel-Resin was assembled on the Tentagel resin as previously described. The N terminus Fmoc group (5 g resin) was removed with 20% piperidine in DMF, and the resin was washed and coupled to 2-hydroxynicotinic acid in the same fashion described in the previous paragraph. The Adpoc group in the sequence (5 g resin) was selectively removed with 40 ml 3% TFA in CH_2Cl_2 (3 × 5 min), and the resulting free amine on the side chain of the Dap residue was coupled with 770 mg (5 mmol) 2,3-dihydroxybenzoic acid in the presence of 1.9 g (5 mmol) HATU, 0.765 g (5 mmol) HOAt, and 1.29 g (10 mmol) DIPEA in 40 ml DMF. The reaction mixture was shaken overnight at room temperature. After the solvent was removed and the resin was subsequently washed, a solution of 58 mg (0.05 mmol, 0.2 equiv.) $Pd(PPh_3)_4$ in 30 ml solvent of THF:DMSO:(0.5 M HCl):morpholine (v/v 20:20:10:1) was added to the resin and shaken for 24 hr at room temperature [26] to selectively deprotect the Alloc group protecting the N terminus Dap residue. The doubly modified peptide resin of (2-hydroxynicotinic)-HN-Arg(Pbf)-Dap-(NH_2)-Leu-Pro-Pro-Leu-Pro-Dap-(NH-2,3-dihydroxybenzoic)-Phe-S-S-Tentagel-Resin at this stage was thoroughly washed, dried in vacuum, and added in 5 mg quantities to the individual wells of eight solvent-resistant MultiScreenTM 96-well filter plates. The resin in each well was then coupled with 1 of 720 different carboxylic acids, the side chain protecting groups were removed, and the peptides were cleaved from the resin to furnish Library V.

Peptide 8a. The peptide-resin Fmoc-Arg(Pbf)-Ala-Leu-Pro-Pro-Leu-Pro-NH-Rink-resin was prepared by using the protocol described above for Library I, but by using the Rink SS resin instead of TentaGel S COOH. After the Fmoc group was removed on the N terminus and the resin was thoroughly washed, a solution containing 2-hydroxynicotinic acid (5 equiv.), PyBOP (5 equiv.), HOBt (5 equiv.), and NMM (15 equiv.) in DMF was added, and the slurry was shaken overnight at room temperature to afford the peptide modified at the N terminus. The peptide was subsequently deprotected and released from the resin in one step by using a TFA/TIS/H_2O (v/v 95:2.5:2.5) cocktail for 2–3 hr. ESIMS *m/z* calculated for $C_{42}H_{65}N_{11}O_{10}$ 884.03 (MH^+), Found *m/z* 884.8.

Peptide 11a. The peptide-resin (2-hydroxynicotinic-NH)-Arg(Pbf)-Ala-Leu-Pro-Pro-Leu-Pro-Dap(Adpoc)-NH-Rink-resin was prepared by using the protocol described above for Library IV, but by using Rink SS resin instead of TentaGel S COOH. After Adpoc deprotection with 3% TFA in DCM and resin washing, a solution of 2,3-dihydroxybenzoic acid (5 equiv.), HATU (5 equiv.), HOAt (5 equiv.), and DIPEA (10 equiv.) in DMF was added, and the slurry was shaken for 3 hr at room temperature to afford the peptide modified at the C terminus. The peptide was subsequently deprotected and released from the resin in one step by using a TFA/TIS/H_2O (v/v 95:2.5:2.5) cocktail for 2–3 hr. ^1H NMR (600 MHz, H_2O, ppm) 7.81 (1H), 6.75 (1H), 8.49 (1H) for N terminus nicotinic acid aromatic hydrogens; 10.31 (1H, s, CONH), 4.55 (1H, $C_\alpha H$), 1.95 (1H, $C_\beta H$), 1.88 (1H, $C_\beta H$), 1.69 (2H, $C_\gamma H_2$), 3.23 (2H, $C_\delta H_2$), 7.24 ($N^\varepsilon H$) for Arg-1; 8.58 (1H, s, CONH), 4.37 (1H, $C_\alpha H$), 1.38 (2H, $C_\beta H_2$) for Ala-2; 8.31 (1H, s, CONH), 4.62 (1H, $C_\alpha H$), 1.56 (2H, $C_\beta H_2$), 1.67 (2H, $C_\gamma H_2$), 0.91 (2H, $C_\delta H_2$) for Leu-3; 4.70 (1H, $C_\alpha H$), 2.34 (1H, $C_\beta H$), 1.90 (1H, $C_\beta H$), 2.03 (2H, $C_\gamma H_2$), 3.83 (1H, $C_\delta H$), 3.67 (1H, $C_\delta H$) for Pro-4; 4.42 (1H, $C_\alpha H$),

2.27 (1H, $C_\beta H$), 1.90 (1H, $C_\beta H$), 2.03 (2H, $C_\gamma H_2$), 3.79 (1H, $C_\delta H$), 3.65 (1H, $C_\delta H$) for Pro-5; 8.16 (1H, s, CONH), 4.57 (1H, $C_\alpha H$), 1.45 (2H, $C_\beta H_2$), 1.65 (2H, $C_\gamma H_2$), 0.86 (2H, $C_\delta H_2$) for Leu-6; 4.39 (1H, $C_\alpha H$), 2.27 (1H, $C_\beta H$), 1.90 (1H, $C_\beta H$), 2.03 (2H, $C_\gamma H_2$), 3.84 (1H, $C_\delta H$), 3.66 (1H, $C_\delta H$) for Pro-7; 8.34 (1H, s, CONH), 4.66 (1H, $C_\alpha H$), 3.86 (1H, $C_\beta H$), 3.77 (1H, $C_\beta H$), 7.61 (amide NH of C terminus), 7.25 (amide NH of C terminus) for Dap-8; 8.74 (1H, s, CONH); 7.10 (1H), 6.88 (1H), 7.25 (1H) for C terminus 2,3-dihydroxyl benzoic acid. ESIMS *m/z* calculated for $C_{52}H_{76}N_{14}O_{13}$ 1105.25 (MH^+), Found *m/z* 1104.6.

Peptide 14. The peptide-resin (2-hydroxynicotinic-NH)-Arg(Pbf)-Dap(alloc)-Leu-Pro-Pro-Leu-Pro-Dap(NH-2,3-dihydroxybenzoic)-NH-Rink-resin was prepared by using the protocol described above for Library V using Rink SS resin. After alloc deprotection with Pd(0) and resin washing, a solution of (*R*)-(-)-Mandelic acid (5 equiv.), PyBOP (5 equiv.), HOBt (5 equiv.), and NMM (15 equiv.) in DMF was added, and the slurry was shaken for 3 hr at room temperature to afford the second Dap residue modification. The peptide was subsequently released from the resin in one step by using a TFA/TIS/H_2O (v/v 95:2.5:2.5) cocktail for 2–3 hr. ^1H NMR (600 MHz, H_2O, ppm) 7.78 (1H), 6.70 (1H), 8.32 (1H) for N terminus nicotinic acid aromatic hydrogens; 10.30 (1H, s, CONH), 4.48 (1H, $C_\alpha H$), 1.88 (1H, $C_\beta H$), 1.80 (1H, $C_\beta H$), 1.65 (2H, $C_\gamma H_2$), 3.17 (2H, $C_\delta H_2$), 7.19 ($N^\varepsilon H$) for Arg-1; 8.57 (1H, s, CONH), 4.60 (1H, $C_\alpha H$), 3.82 (1H, $C_\beta H$), 3.57 (1H, $C_\beta H$) 8.49 (CONH, s, CONH of Dap-2-hydroxyl benzoic acid) for Dap-2; 8.40 (1H, s, CONH), 4.62 (1H, $C_\alpha H$), 1.59 (2H, $C_\beta H_2$), 1.64 (2H, $C_\gamma H_2$), 0.90 (2H, $C_\delta H_2$) for Leu-3; 4.65 (1H, $C_\alpha H$), 2.32 (1H, $C_\beta H$), 1.89 (1H, $C_\beta H$), 2.04 (2H, $C_\gamma H_2$), 3.86 (1H, $C_\delta H$), 3.62 (1H, $C_\delta H$) for Pro-4; 4.42 (1H, $C_\alpha H$), 2.27 (1H, $C_\beta H$), 1.89 (1H, $C_\beta H$), 2.0 (2H, $C_\gamma H_2$), 3.73 (1H, $C_\delta H$), 3.60 (1H, $C_\delta H$) for Pro-5; 8.15 (1H, s, CONH), 4.57 (1H, $C_\alpha H$), 1.45 (2H, $C_\beta H_2$), 1.64 (2H, $C_\gamma H_2$), 0.85 (2H, $C_\delta H_2$) for Leu-6; 4.42 (1H, $C_\alpha H$), 2.27 (1H, $C_\beta H$), 1.89 (1H, $C_\beta H$), 2.0 (2H, $C_\gamma H_2$), 3.82 (1H, $C_\delta H$), 3.65 (1H, $C_\delta H$) for Pro-7; 8.34 (1H, s, CONH), 4.64 (1H, $C_\alpha H$), 3.86 (1H, $C_\beta H$), 3.76 (1H, $C_\beta H$), for Dap-8; 8.73 (1H, s, CONH); 7.09 (1H), 6.87 (1H), 7.22 (1H) for C terminus 2,3-dihydroxyl benzoic acid. ESIMS *m/z* calculated for $C_{60}H_{82}N_{14}O_{16}$ 1255.38 (MH^+), Found *m/z* 1255.9.

Synthesis of Peptoid Ligands 5–7

Peptoid ligands **5–7** were synthesized on the Rink resin by using a standard solid phase peptide synthesis protocol with the following exception to introduce the N-glycine-substituted peptoid moiety: after the assembly of the Pro-Arg-Asn-Arg-Pro-Arg-Ala sequence on the Rink resin, the N terminus of the peptide was acylated by reaction with equal volumes of 1 M bromoacetic acid in DCM (10 equiv.) and 1 M diisocarbodiimide in DMF (10 equiv.) twice for 1 hr each; nucleophilic displacement was effected with 2 M N-(*S*)-phenylethyl amine in dimethyl sulfoxide (15 molar equiv.) twice for 2 hr each. The subsequent Fmoc amino acid (10 equiv.) was appended by using the coupling reagent HATU (10 equiv.) and DIPEA (18 equiv., twice for 2 hr each). Two Fmoc-Aminohexanoic acid (Fmoc-Ahx) residues were coupled to the N terminus as spacers between the peptoid ligand and the appended moiety (biotin, **6**; fluorescein, **7**). Biotinylation was effected on the resin with 4 equiv. of biotin in DMSO/DMF (v/v 50:50) in the presence of coupling reagents (PyBOP, 4 equiv.; HOBt, 4 equiv.; NMM, 4 equiv.) at room temperature for 3 hr. FITC labeling was likewise conducted on the resin with 2 equiv. of FITC dissolved in pyridine/DMF/DCM (v/v 12:7:5) at room temperature in the dark overnight. Peptoid ligands **5–7** were subsequently deprotected and released from the Rink resin by using a TFA/TIS/H_2O cocktail (v/v 95:2.5:2.5) for 3 hr. ESIMS of **6** calculated for $C_{96}H_{150}N_{26}O_{20}S$ *m/z* 2020.45 (MH^+), Found *m/z* 2020.78. ESIMS of **7** calculated for $C_{107}H_{147}N_{25}O_{23}S$ *m/z* 2183.53 (MH^+), Found *m/z* 2184.13.

Screening of the Peptide/Nonpeptide Conjugate Library

An ELISA assay was employed to screen the library for SH3 affinity. A total of 100 μl biotinyl-(ε-aminocaproyl)$_2$-Tyr-Ala-Pro-Pro-Leu-[N-(*S*)-phenylethyl]Gly-Pro-Arg-Asn-Arg-Pro-Arg-Ala-amide **6** (333 ng/ml in 50 mM Tris, 150 mM NaCl [pH 7.5]) was added to each well of streptavidin-coated 96-well microtiter plates. The plates were shaken overnight at 4°C and rinsed with TBS (50 mM Tris, 150 mM NaCl [pH 7.5], 3 × 200 μl). Each well was then blocked with 100 μl of a solution containing 2% BSA and 0.2% Tween 20 in TBS (2 hr at room temperature). The wells were rinsed with 4 × 200 μl of a standard "BSA-T-TBS" solution (0.2% BSA, 0.1% Tween 20, TBS). A 50 μl solution of the peptide/nonpeptide conjugate (100 nM, in BSA-T-TBS) from the library and a 50 μl solution of the Fyn-SH3 GST fusion protein (32 ng/ml, in BSA-T-TBS) were added in each well, and the plate was shaken for 2.5 hr at room temperature. The solutions were removed, and each well was rinsed with 4 × 200 μl BSA-T-TBS. 100 μl of horse radish peroxidase-conjugated rabbit polyclonal anti-GST antibody (100 ng/ml in BSA-T-TBS) was then added to each well and incubated for 2 hr at room temperatrure. After a series of final wash steps (4 × 200 μl BSA-T-TBS; 4 × 250 μl TBS), 100 μl peroxidase substrate (1-step Turbo TMB-ELISA, trimethylbenzidine) was added to each well and incubated for 5–15 min. A total of 100 μl 1 M sulfuric acid solution was introduced to stop the peroxidase reaction, and absorbance was measured at 450 nm with a plate reader. IC_{50} values were determined by using the ELISA screening method around a 200-fold range of ligand concentrations.

WASp/Fyn Binding Assay

U937 cells were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum. To induce differentiation toward a monocytic lineage, exponentially growing U937 cells were subcultured at a density of 5 × 10^5 cells ml^{-1} and treated with PMA (Sigma) at a final concentration of 10 ng ml^{-1} for 24 hr. Cells were then harvested and washed in ice-cold phosphate-buffer saline and lysed in 10 mM Tris-HCl (pH 7.5), 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl chloride, 5 mM benzamidine, 1 mM $NaVO_4$, and protease inhibitors (5 μg/ml aprotinin, 5 mg/ml leupeptin, 5 μg/ml pepstatin). Protein concentrations were measured by using the Bradford assay after preclearing the lysate with glutathione-sepharose beads. Aliquots of cell lysate were added to 20 μl glutathione-sepharose beads saturated with Fyn-SH3 GST fusion protein in the presence of peptide **14** or acetylated control **9**. Beads and lysate were gently shaken for 2 hr at 4°C and then washed five times with lysis buffer. Bound proteins were eluted into gel loading buffer by boiling, were fractionated by SDS-PAGE under reducing conditions, and were then electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked via overnight exposure to PBS buffer with 5% nonfat milk. The amount of WASp associated with Fyn SH3 was detected by sequential incubation with anti-WASp monoclonal antibody (Pharmingen, CA) and secondary HRP-conjugated antibody (Pharmingen, CA), and were then visualized with an enhanced chemiluminescent (ECL) Western blotting reagent according to the manufacturer's instructions (Amersham). The same PVDF membrane was stripped in 0.2 N NaOH solution for 10 min and was blotted against anti-GST HRP antibody (Santa Cruz Biotech, CA) to verify that an equal amount of Fyn SH3 protein was loaded into each lane.

Determination of K_d Values

Peptides containing a nicotinic acid substituent at their N terminus are highly fluorescent and exhibit little or no change in fluorescence upon coordination to the Fyn-SH3 domain. Therefore, the K_D values for the SH3 complexes of these species were determined via equilibrium dialysis. All samples were prepared in buffer containing TBS and 1 mM DTT at pH 7.5. A Slide-A-Lyzer dialysis slide cassette (0.1–0.5 ml capacity) was employed and contained 30 nM or 500 nM Fyn-SH3 GST fusion proteins. The cassettes (400 μl final volume) were placed in a beaker containing a volume of buffer solution (TBS and 1 mM DTT [pH 7.5]) that was at least 400-fold greater than that of the sample volume in the dialysis cassette. As a consequence, concentrations of non-SH3 bound peptide were held constant in the dialysis slide cassette over the course of the experiment. Equilibrium dialysis experiments were performed over a period of at least 16 hr and were maintained at 4°C. Differences in the fluorescence between the solution in the slide cassette and that in the beaker were measured. The excitation wavelengths employed for the peptides were 328 nm. Emission was monitored at 378 nm. The K_d values were calculated from the following equation for fluorescent peptides **8a**, **11a**, **14**, and **7**, where $[E]_t$ = total en-

zyme concentration, [E · L] = enzyme-peptide complex, and [L] = free peptide concentration.

$$K_d = \frac{\{[E]_t - [E \cdot L]\}[L]}{[E \cdot L]}$$

For nonfluorescent peptide **9**, the following equation was used for the determination of K_d':

$$K_d = \frac{K_d \cdot [L']}{\frac{[E]_t \cdot [L]}{[E \cdot L]} - (K_d + [L])}$$

where K_d = the dissociation constant of competition fluorescent peptide, [L′] = total nonfluorescent peptide concentration, [L] = total competitive fluorescent peptide concentration, $[E]_t$ = total SH3 domain concentration, [E · L] = enzyme-peptide (fluorescent competitive peptide) complex concentration.

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