SH2-Directed Ligands of the Lck Tyrosine Kinase

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Two separate libraries, prepared via parallel synthesis, were employed to identify low-molecular-weight SH2-targeted ligands for the Lck tyrosine protein kinase. These libraries were constructed to furnish non-amino acid analogues of the (1) Glu-Glu and (2) Ile residues of the Lck SH2 domain peptide ligand Ac-pTyr-Glu-Glu-Ile-amide. The lead compound acquired in this study exhibits a dissociation constant for the Lck SH2 domain that is comparable to that displayed by Ac-pTyr-Glu-Glu-Ile-amide. These results demonstrate that the standard amino acid residues Glu-Glu-Ile can be completely replaced with non-amino acid moieties without loss of SH2 affinity.

Introduction

Tyrosine-specific protein kinases are composed of two subfamilies: (1) the receptor tyrosine kinases, which are integral membrane proteins, and (2) their nonreceptor cytoplasmic counterparts. The former, upon coordination of specific extracellular ligands, forms aggregates and subsequently suffers phosphorylation of key tyrosine residues. Cytoplasmic signaling proteins, including nonreceptor tyrosine kinases, coordinate to these phosphotyrosine (pTyr) residues through Src homology 2 (SH2) domains.[1] This binding event triggers the activation of specific intracellular signaling pathways ultimately leading to a cellular response in reaction to the extracellular stimulus. For example, antigen presentation to T cells results in the clustering of T cell receptors (TcR) and subsequent TcR tyrosine phosphorylation by the Src family member Lck (*L*ymphoid T *c*ell tyrosine *k*inase).[2] These TcR pTyr moieties serve as high-affinity binding sites for the SH2 domains of cytoplasmic signaling proteins, which generate complexes that are required for ensuing downstream events such as an increase in cytoplasmic Ca^{2+} levels and the ultimate production of interleukin-2. In short, SH2 domains play a critical role in organizing coherent signal transducing complexes that are essential for the appropriate cellular response to extracellular stimuli.

Constituitively active signal transduction pathways have been identified in a variety of disease states. Ligands that are able to disrupt these inappropriately hyperstimulated pathways, by blocking SH2 domain-dependent interactions, may ultimately find utility as therapeutic agents. For example, ligands directed against the Lck SH2 domain could serve in various capacities, such as for the treatment of autoimmune diseases and T cell-based leukemias and lymphomas. The Lck SH2 domain exhibits a marked preference for the sequence -pTyr-Glu-Glu-Ile-, and short peptides bearing this sequence exhibit a reasonably high affinity for the SH2 domain of Lck.[3] Dissociation constants for the Src family SH2 ligands, Ac-pTyr-Glu-Glu-Ile and Ac-pTyr-Glu-Glu-Ile-amide, have been reported to be in the range of $10^{-7}-10^{-6}$ M.[4] In addition, we have recently found that the latter affinities can be significantly enhanced by appending non-amino acid substituents off the N-terminus of peptide ligands (i.e. RCO-NH-pTyr-Glu-Glu-Ile-amide).[4a]

Although moderately high-affinity peptide-based ligands for SH2 domains have been reported, their ultimate utility as therapeutic agents is suspect. In general, peptide-based species exhibit poor bioavailability and are prone to hydrolysis by the presence of intra- and intercellular proteases. Furthermore, the pTyr moiety, which is essential for SH2 recognition, is hydrolytically unstable due to the presence of protein tyrosine phosphatases. The latter difficulty can be resolved via the introduction of nonhydrolyzable pTyr mimetics, such as the difluorophosphonate analogue of pTyr.[5] Peptide-based species that contain this nonhydrolyzable derivative exhibit affinities comparable to their pTyr-containing counterparts.[5] Nevertheless, the acquisition of nonpeptidic ligands that display high-affinity binding for SH2 domains has not yet been fully realized.[6,7] We report herein that the three C-terminal amino acids in the peptide-based SH2 ligand Ac-pTyr-Glu-Glu-Ile-amide can be replaced by non-amino acid moieties without loss of SH2 affinity.

Results and Discussion

The discovery of the role of SH2 domains in organizing coherent signaling pathways created an obvious target for the design of potential therapeutic agents.[8] An early report that small peptide ligands of SH2 domains exhibit dissociation constants that approach the picomolar range provided additional incentive to explore the possibility of creating nonpeptidic analogues.[9] Unfortunately, these K_D values overestimated true affinity by 2−3 orders of magnitude.[10] Nevertheless, the acquisition of nonpeptidic SH2 ligands has been abetted by several key recent contributions. First, a potent nonhydrolyzable pTyr mimetic not only has been described[5] but also has been rendered membrane permeable.[11] Second, SH2 domains that belong to different protein families exhibit significantly different amino acid sequence specificities, an observation that could lead to the eventual acquisition of nonpeptidic analogues

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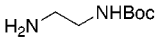
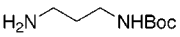
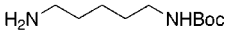
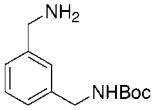
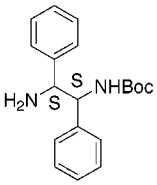
Table 1. Diamines (**A**−**G**) and Carboxylic Acids (**7**−**18**) Used in the Preparation of the 84-Member Library Outlined in Scheme 1

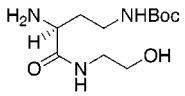
(A) 

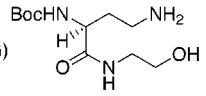
(B) 

(C) 

(D) 

(E) 

(F) 

(G) 

(7) –

(8) CH_3CO_2H

(9) $CH_3(CH_2)_4CO_2H$

(10) $(CH_3)_2CH(CH_2)_2CO_2H$

(11) (DL)-$CH_3CH_2(CH_3)CHCH_2CO_2H$

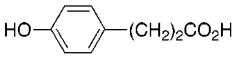
(12) $C_6H_5CO_2H$

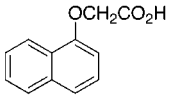
(13) $C_6H_5CH_2CO_2H$

(14) $C_6H_5CH_2CH_2CO_2H$

(15) $C_6H_5(CH_2)_3CO_2H$

(16) $C_6H_5(CH_2)_4CO_2H$

(17) 

(18) 

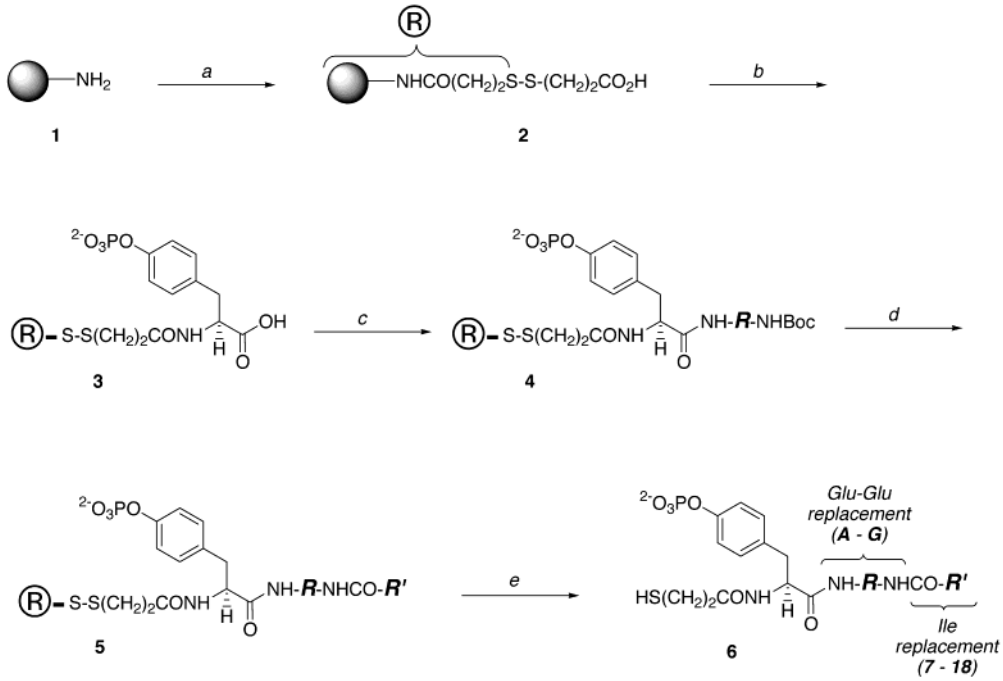
displaying analogous specificities.[3] Finally, nonpeptide/peptide conjugates have recently been reported whose SH2 affinities dramatically exceed those displayed by their peptidic counterparts.[4a] These relatively recent innovations suggest that there is good cause for optimism in using SH2 domains as possible therapeutic targets. Given these facts, we set out to determine whether the three C-terminal amino acids in the peptide-based species Ac-pTyr-Glu-Glu-Ile-amide can be replaced without loss of affinity for the Lck SH2 domain.

Our strategy is based on previously reported structural and biochemical studies on the interaction of peptide ligands with the SH2 domains of the Src family of protein kinases, including the Src family member Lck.[1] The 3-dimensional structure of Ac-pTyr-Glu-Glu-Ile bound to the Lck SH2 domain has been solved.[12] Two residues on the peptide ligand are engaged in key interactions with the SH2 domain. This includes the pTyr moiety, which is embedded within a cavity on the SH2 surface. The negatively charged phosphate group participates in an electrostatic interaction with an Arg moiety. The other key peptide residue is the P+4 Ile, whose hydrophobic side chain is buried within a second cavity located on the SH2 surface. The double-cavity motif has been described as a "two-holed socket" that engages the "two-pronged plug" (i.e. pTyr and Ile) of the peptide-based ligand.[1b] In contrast, the two bridging **glutamic acid** residues of the Ac-pTyr-**Glu-Glu**-Ile-amide ligand do not appear to be strongly bound.[13] With these features in mind, we developed a two-step strategy to identify analogues of the SH2 tetrapeptide ligand by posing the following questions. Can the Glu-Glu dyad, which serves to link the pTyr and Ile prongs of the "two-pronged plug", be replaced by a simple non-amino acid moiety that functions in an analogous capacity? Can the C-terminal Ile "prong", which is embedded within a lipophilic cavity of the SH2 domain, be replaced with a non-amino acid analogue? We addressed these questions, in a stepwise fashion, by first preparing an 84-member library to identify a Glu-Glu dyad surrogate. We subsequently synthesized a 900-member library to acquire non-amino acid mimetics for the P+4 Ile moiety.

We examined the ability of seven different diamines (**A**−**G**) to serve as Glu-Glu dyad replacements in the context of twelve (**7**−**18**) different aliphatic and aromatic acyl moieties (i.e. Ile replacements) (Table 1). The 84 members of this initial library have the general structure **6** (Scheme 1). The seven diamine tethers (**A**−**G**) were chosen using the known 3-dimensional structure of the Lck SH2/Ac-pTyr-Glu-Glu-Ile complex as a guide.[12] Molecular modeling studies suggested that diamines of the general structure $H_2N(CH_2)_nNH_2$ (where $n = 2-5$) would be ideal as Glu-Glu analogues, depending upon the nature of the appended acyl moiety. Consequently, **A**−**C** were examined as possible Glu-Glu surrogates. Modeling also suggested that hydrophobic substituents on the diamine chain could augment SH2 affinity. Therefore, we included **D** and **E** as part of this preliminary evaluation of Glu-Glu dyad replacements. Finally, substituents that could hydrogen bond with residues on the SH2 domain might also promote SH2 affinity. We employed **F** and **G** to address the latter issue.

The disulfide-containing Tentagel-based resin **2** was prepared via modification of commercially available Tentagel resin (**1**) with 3,3′-dithiodipropionic acid in the presence of benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP), hydroxybenzotriazole (HOBt), and *N*-methymorpholine (NMM) in DMF (Scheme 1). The Kaiser ninhydrin test was used to monitor completion of the reaction. The free carboxylate moiety in **2** was activated with *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU)

Scheme 1. Preparation of the 84-Member Library **6**[a]



[a] (a) Tentagel resin (**1**), dithiodipropionic acid, BOP, HOBt, NMM, DMF, 2 h at rt; (b) TSTU, NMM, pTyr, DMF/H_2O; (c) BOP, HOBt, NMM, CH_2Cl_2/DMF, Boc-protected diamine (**A**−**G**), 2 h at rt; (d) i. TFA/CH_2Cl_2, 1 h at rt, ii. BOP, HOBt, NMM, DMF, RCO_2H (**7**−**18**); (e) 5 washes of 10 mM dithiothreitol, 50 mM Tris, pH 7.5, 1 h at rt.

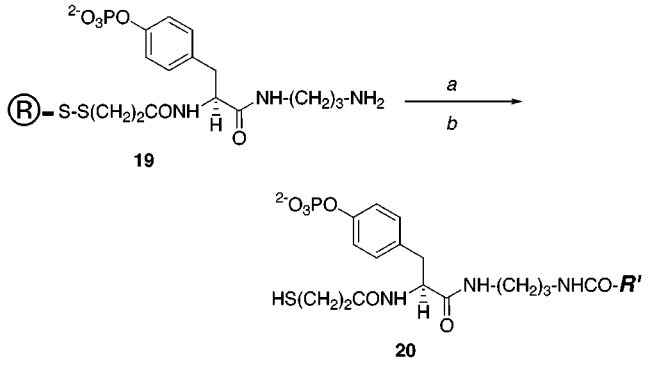
and NMM in DMF and subsequently condensed with pTyr to furnish **3**. The free C-terminus of the latter was activated, split into seven equal portions, and condensed with the array of mono-Boc-protected diamines displayed in Table 1. The resultant products **4** were deprotected with 25% trifluoroacetic acid (TFA) in CH_2Cl_2 and then further split into twelve equal portions and condensed with a variety of representative aliphatic and aromatic carboxylic acids, to yield the library of 84 nonpeptidic compounds **5** in a 96-well microfiltration plate format. Each member of this library was individually treated with five washings (1 h duration/washing) of a 10 mM dithiothreitol (DTT)-based buffer (pH 7.6, 50 mM Tris). We found that the first two of these five DTT washings cleave greater than 90% of the disulfide-appended compounds from the resin. The final three washings were employed to remove any residual resin-bound material. Three members of this library were assessed for purity by HPLC and characterized by mass spectrometry following removal of the Tris buffer and DTT by HPLC (see Experimental Section). The resulting individual solutions of **6** were then vacuum-filtered into a 96-well receiving plate using a vacuum filter manifold.

The final synthetic step (e) depicted in Scheme 1 also represents the first step of the subsequent library assay since cleavage from the resin is effected by treatment with assay buffer. This furnishes a library of compounds in an assay-ready form. The highest-affinity SH2-targeted ligands in this library were identified via an enzyme-linked immunosorbent assay (ELISA).[14] ELISAs were performed using a 96-well strepavidin-coated plate that had been pretreated with the biotinylated SH2-directed ligand, biotinyl-ϵ-aminocaproyl-EPQpYEEIPIYL. The GST-SH2 fusion protein of Lck was introduced and the structural integrity of the GST-SH2/biotinyl-ϵ-aminocaproyl-EPQpYEEIPIYL complex separately challenged with each component (@ 100 μM) of the 84-member nonpeptide SH2 library. The presence of GST-SH2 bound to the strepavidin-coated plate was assessed via the standard sequential treatment of each well with rabbit anti-GST antibody followed by horseradish peroxidase-conjugated mouse anti-rabbit antibody.

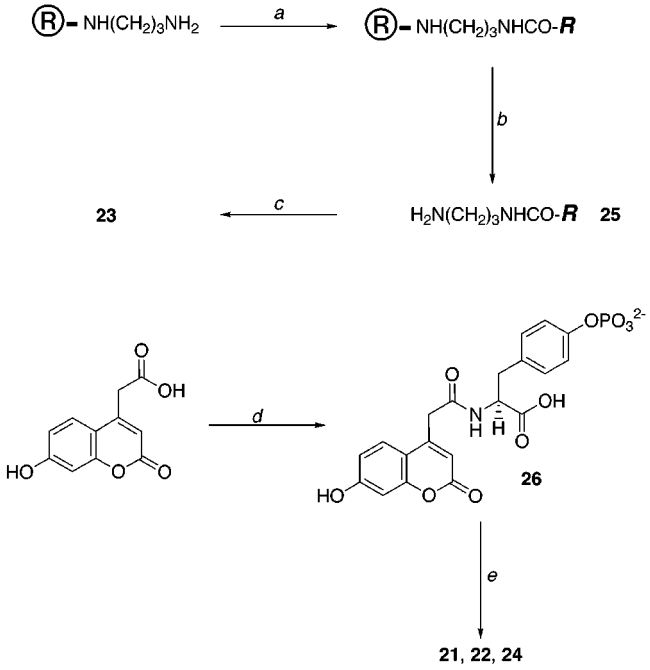
In general, all seven of the diamine tethers furnish effective SH2 ligands (data not shown). Although some differences in SH2 affinity between the seven different diamine tethers do exist, this limited survey revealed that it is the appended acyl moiety, and not the diamine, that has the most pronounced influence on SH2 affinity. For example, all of the best SH2 ligands from this library contain an aromatic acyl group, with the naphthalene-containing species **18** exerting the greatest beneficial influence on SH2 affinity, an effect that is almost independent of the actual diamine to which it is appended. The roughly comparable influence that **A**−**G** have on SH2 affinity is not too surprising given the fact that we employed the Lck SH2 crystal structure to assist in the selection of the diamines (Table 1). We decided to use diamine **B** as the Glu-Glu replacement in the subsequent 900-member library due to its structural simplicity and the fact that two of the twelve diamine **B**-containing derivatives are among the most potent SH2 ligands in the 84-member library.

We subsequently focused our efforts on obtaining a non-amino acid replacement for the **Ile** moiety of Ac-pTyr-Glu-Glu-**Ile**-amide. A secondary library of the general structure **20** was prepared using a protocol analogous to that depicted in Scheme 1. Intermediate **19** was separately condensed with 900 different carboxylic acids and then cleaved from the resin with DTT, as described above, to furnish the library of compounds **20** (Scheme 2). Two lead SH2 ligands were identified from the ELISA screen, and these were subsequently resynthesized (Scheme 3) as the coumarin derivatives **21** and

Scheme 2. Preparation of the 900-Member Library **20**[a]



Scheme 3. Preparation of Compounds **21**−**24**[a]

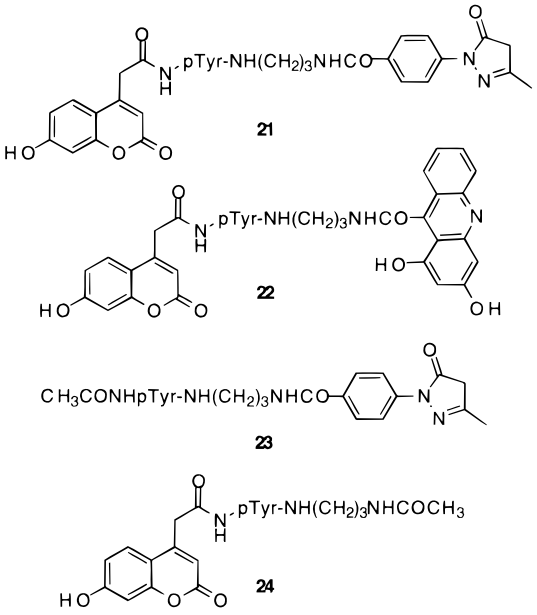


[a] (a) RCO_2H, BOP, HOBt, NMM, DMF added to the diaminopropane trityl resin, overnight at rt; (b) 10% TFA/CH_2Cl_2; (c) Ac-pTyr, BOP, HOBt, NMM, DMF, 2 h at rt; (d) TSTU, NMM, DMF and added to pTyr/H_2O; (e) **25**, BOP, HOBt, NMM, DMF, 2 h at rt.

22. The coumarin analogue that is appended to the amine of pTyr in **21** and **22** has been previously shown to enhance the SH2 affinity of pTyr-Glu-Glu-Ile-amide.[4a] In addition to **21** and **22**, we also prepared the acetylated compounds **23** and **24**, which allowed us to assess the influence that the coumarin- and pyrazoline-derivatized benzoyl substituents in **21** have on SH2 affinity. For comparison, the Ac-pTyr-Glu-Glu-Ile-amide tetrapeptide was synthesized as well.

IC_{50} values for the five compounds listed in Table 2 were obtained using the ELISA assay. These values were acquired (in duplicate) for all five ligands using a single 96-well plate. Compound **21** is nearly as potent an Lck SH2 ligand as its tetrapeptide counterpart. The acridine-containing species **22** is a likewise proficient SH2 ligand. The coumarin-containing species **21** is a 37-fold more potent SH2 ligand than the corresponding acetylated derivative **23**. Furthermore, the pyrazoline-derivatized benzoyl substituent furnishes a 16-fold enhancement relative to a simple acetyl moiety (**24**). Unfortunately, in our hands, the absolute values of these IC_{50}s vary considerably from plate-to-plate and from day-to-day. Given the uncertainty of these values, we employed an alternate method to assess absolute ligand affinity for the SH2 domain. The fluorescence associated with the coumarin moiety in **21** is not altered when the ligand coordinates to the SH2 domain. Consequently, we were able to obtain a K_D for **21** via equilibrium dialysis using 10K cutoff Slide-A-Lyzer cassettes (Table 2).[4a] The dissociation constant for the tetrapeptide Ac-pTyr-Glu-Glu-Ile-amide was obtained via competitive displacement of a coumarin-substituted peptide ligand. The equilibrium dialysis assay confirms that **21** exhibits an affinity for the Lck SH2 domain comparable to that displayed by Ac-pTyr-Glu-Glu-Ile-amide.

Table 2. IC_{50} and K_D Values for the Lck SH2 Domain Complexed with Ligands **21**−**24** and Ac-pTyr-Glu-Glu-Ile-amide[a]



SH2 ligand	IC_{50} (μM)	K_D (μM)
Ac-PYEEI-NH_2	0.66 ± 0.02	1.3 ± 0.2
21	1.4 ± 0.1	2.9 ± 1.0
22	2.4 ± 0.1	−
23	53 ± 5	−
24	22 ± 4	−

[a] IC_{50} values were obtained via the ELISA assay, and K_D values were acquired by equilibrium dialysis (see Experimental Section).

The aromatic substituents identified from the 900-member library **20** display surprisingly little structural homology with the P+4 Ile side chain (cf. **21**−**22**). As noted above, the side chain of Ile is embedded within a narrow hydrophobic pocket of the Lck SH2 domain. One possible explanation for the selection of the aromatic substituents in **21** and **22** is that the P+4 Ile binding pocket is conformationally flexible and can therefore accommodate an array of structurally diverse, yet compatible, moieties. Alternatively, these substituents may be simply interacting with sites other than the Ile binding pocket. This particular issue awaits future resolution.

We have identified two species that exhibit affinities for the Lck SH2 domain that are comparable to that displayed by the standard peptide ligand for this protein. Recently, a Boehringer Ingelheim group has

achieved similar success with dipeptide derivatives.[7] Nevertheless, from the therapeutic point of view, it is clearly desirable to identify compounds that exhibit significantly greater affinities for SH2 domains than conventional peptides. Naturally occurring amino acids are limited to only 20 different side chains. In contrast, the array of structural motifs available with non-amino acid substituents is virtually limitless, allowing access to interactions with a target protein that are simply not possible using common peptides. The high-throughput parallel synthesis strategy described herein takes advantage of the ready commercial availability of the wide structural array of carboxylic acid derivatives. Analogous studies are now underway to improve SH2 affinity via the identification of Glu-Glu dyad analogues that are both structurally less flexible than the diamine tether **B** yet able to productively interact with adjacent subsites on the SH2 domain.

Conclusion

SH2 domains have been identified as potential therapeutic targets for a variety of medical disorders. Consequently, ligands that are able to interfere with the biochemical role of SH2 domains, such as peptidomimetics of the Lck SH2 domain ligand Ac-pTyr-Glu-Glu-Ile-amide, are a much sought after commodity. Key residues of the tetrapeptide include the pTyr and P+4 Ile moieties, whose side chains interact with cavities that are embedded within the SH2 surface. In contrast, the Glu-Glu component appears to play a less critical role in terms of promoting SH2 affinity. We initially examined the ability of seven structurally simple diamines to serve as Glu-Glu dyad mimetics in the context of an 84-member library. We found that diamines **A**−**G** (Table 1) are all able to furnish effective SH2 ligands. The diamine **B**-based 900-member library (**20**) was subsequently prepared and assayed using an ELISA screen. Two lead compounds were identified and resynthesized as the coumarin derivatives **21** and **22**. Both compounds exhibit affinities for the target SH2 protein that are comparable to that of the tetrapeptide Ac-pTyr-Glu-Glu-Ile-amide. *These results demonstrate that the three C-terminal amino acid residues of the standard SH2 ligands can be replaced with non-amino acid substituents without loss of affinity*. Future studies will focus on the acquisition of SH2-targeted species that exhibit significantly better affinity for SH2 domains than conventional peptide ligands.

Experimental Section

General. Chemicals were obtained from Aldrich, except for piperidine (Advanced Chemtech); protected amino acids, amino acid derivatives, HOBt, BOP, and TSTU (Advanced Chemtech and Bachem California); diaminopropane trityl resin (Nova biochem); and Rink resin, Wang resin, and Tentagel resin (Advanced Chemtech). Biotinyl-ϵ-aminocaproyl-EPQpYEEIPIYL was purchased from Bachem California. Lck SH2-GST fusion proteins and polyclonal rabbit anti-GST antibody were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated mouse anti-rabbit antibody, peroxidase substrate (1-Step Turbo TMB-ELISA, trimethylbenzidine), steptavidin-coated 96-well plates, and Slide-A-Lyzer 10K MWCO dialysis slide cassettes were purchased from Pierce. Solvent-resistant multiscreen 96-well filter plates and the multiscreen 96-well filter plate vacuum manifold were purchased from Millipore. All final products were purified via preparative HPLC using three Waters radial compression C18 reversed-phased column modules (25 × 10 cm) connected in series. A linear gradient of 100% solvent A (0.1% TFA in water) to 50% solvent A/50% solvent B (0.1% TFA in CH_3CN) was applied over the course of 50 min. Purity was assessed by HPLC using an analytical C18 column and two different solvent systems: a 100% A to 50%A/50%B linear gradient over 25 min, and a 100% solution of 20 mM aqueous Na_2HPO_4 (pH 7.4) to 75% (20 mM aqueous Na_2HPO_4)/25% CH_3CN linear gradient over 25 min. ^1H NMR spectra were obtained on a Bruker spectrometer (300 MHz) using DMSO-d_6 as solvent. Data are reported as follows: chemical shift (ppm relative to the solvent), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, qn = quintet, br = broad, m = multiplet), coupling constant (*J*, reported to the nearest 0.5 Hz), and integration. Compounds were further characterized by electrospray mass spectrometry.

Peptide Synthesis. All peptides, except biotinyl-ϵ-aminocaproyl-EPQpYEEIPIYL, were synthesized on an automated peptide synthesizer (Advanced Chemtech model ACT90) using a standard Fmoc solid-phase peptide synthesis protocol. Rink amide and Wang acid resins were employed for the preparation of peptides containing amide and free acid C-termini, respectively. Crude peptides were purified on a preparative HPLC using three Waters radial compression modules (25 × 10 cm) connected in series. Purified peptides were further characterized by ESI mass spectrometry.

Synthesis of the 84-Member Combinatorial Ligand Library. 3,3′-Dithiodipropionic acid (15 mmol, 3.15 g) was added to a mixture of Tentagel S NH_2 resin (90 μm, 5 g, 0.3 mmol/g), BOP (30 mmol, 13.2 g), HOBt (30 mmol, 4.59 g), and NMM (45 mmol, 4.5 g) in 60 mL DMF and subsequently shaken for 2 h at room temperature (Scheme 1). Completion of the reaction was monitored using the Kaiser ninhydrin test. After a series of wash steps (3 × 50 mL DMF, 3 × 50 mL MeOH, 3 × 50 mL CH_2Cl_2), the free carboxylate moiety in **2** was activated with TSTU (3 mmol, 903 mg) and NMM (4.5 mmol, 455 mg) in 60 mL DMF for 10 min, washed with DMF (2 × 50 mL), and then condensed with pTyr (3 mmol, 784 mg) in NMM (15 mmol, 1.52 g) and 60 mL DMF/water (1:1) to furnish **3**. The resin (**3**) was divided into 7 equal portions (200 mg each) and activated with BOP (0.15 mmol, 67 mg), HOBt (0.15, 23 mg) and NMM (1 mmol, 101 mg) in CH_2Cl_2/DMF - (1:1) and condensed with the array of mono-Boc-protected diamines (displayed in Table 1) for 2 h at room temperature. After a series of wash steps (3 × 10 mL DMF, 3 × 10 mL MeOH, 3 × 10 mL CH_2Cl_2), the Boc protecting group was removed via treatment with 10 mL TFA/CH_2Cl_2 (1:3) for 1 h. The products were extensively washed as described above and subsequently dried in vacuum. The substitution level for the free amine, determined via the Kaiser ninhydrin test, was 0.05 mmol/g. The 7 free amine-containing resins were further split into 12 equal portions (4-mg quantities into each well of solvent-resistant 96-well filter plates) and condensed with 12 representative alipatic and aromatic acids (40 μmol) using BOP (20 μmol), HOBt (20 μmol), and NMM (100 μmol) in 100 μL DMF. The plates were shaken overnight and then each well was subjected to a series of wash steps (3 × 200 μL DMF, 3 × 200 μL water, 3 × 200 μL DMF, 3 × 200 μL CH_2Cl_2, 200 μL 10% NMM in CH_2Cl_2, 2 × 200 μL 50 mM Tris, pH 7.5) using a 96-well filter plate vacuum manifold. The products were cleaved from the disulfide-containing resin with 10 mM DTT in Tris buffer (5 × 200 μL for 1 h each) and filtered into a receiving set of 96-well plates using the vacuum manifold (final volume: 1 mL). The efficiency of acid coupling, product cleavage from the resin with DTT solution, and purity of the products were assessed with several ligands (**B-18**, **D-10**, and **F-12** from Table 1). No free N-terminus peptide was detected and over 90% of total ligand was cleaved from resin with first two DTT wash steps. The final three DTT washings removed the residual resin-bound material. Compound purity was greater than 60% as assessed by HPLC and the HPLC-purified compounds (i.e. removal of Tris buffer and DTT) were characterized by ESI mass spectrometry.

Synthesis of the 900-Member Ligand Library. The 900-member ligand library (**20**) was prepared as described above except for the following modifications: After **2** was condensed with pTyr, **3** was activated with BOP (3 mmol, 1.33 g), HOBt (3 mmol, 459 mg), and NMM (15 mmol, 1.52 g) in CH_2Cl_2/DMF (1:1) and condensed with mono-Boc-protected diaminopropane (7.5 mmol, 1.31 g) for 2 h at room temperature. After deprotection of Boc group, the free amine-containing resin (**19**) was distributed in 4-mg quantities into each well of solvent-resistant 96-well filter plates. In addition, each well contained a carboxylic acid-containing compound (40 μmol), BOP (20 μmol), HOBt (20 μmol), and NMM (100 μmol) in 100 μL DMF. A total of 900 different carboxylic acids were employed.

Screening of the Ligand Library. An ELISA assay was employed to screen the library for SH2 affinity. 100 μL biotinyl-ϵ-aminocaproyl-EPQpYEEIPIYL (10 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, 2 × 200 μL) and then rinsed with 2 × 200 μL of a standard BSA-T-TBS solution (0.2% BSA, 0.1% Tween 20, TBS). A 50-μL solution of the nonpeptidic compounds (200 μM, in BSA-T-TBS) from the library and a 50-μL solution of the Lck SH2-GST fusion protein (6.4 ng/mL, in BSA-T-TBS) were added to each well of a 96-well plate and the plate was then shaken for 1 h at room temperature. The solutions were removed and each well was rinsed with 4 × 200 μL BSA-T-TBS. 100 μL polyclonal rabbit anti-GST antibody (100 ng/mL in BSA-T-TBS) was then added to each well and incubated for 1 h at room temperature. Following subsequent washing steps with BSA-T-TBS (4 × 200 μL), 100 μL horseradish peroxidase-conjugated mouse anti-rabbit antibody (200 ng/mL in BSA-T-TBS) was added to each well and subsequently incubated for 1 h at room temperature. After a series of final wash steps (4 × 200 μL BSA-T-TBS, 2 × 300 μL TBS), 100 μL peroxidase substrate (1-Step Turbo TMB-ELISA, trimethylbenzidine) was added to each well and incubated for 5−15 min. 100 μL 1 M sulfuric acid solution was introduced to stop the peroxidase reaction and the absorbance was measured at 450 nm with a plate reader.

Determination of IC_{50} Values. IC_{50} values were determined using the ELISA screening method described above except that, instead of a fixed 100 μM concentration for each library member, a 200-fold range of concentrations were employed around the apparent IC_{50}. Since the IC_{50} values determined in this way appear to be extraordinarily sensitive to even subtle changes in experimental conditions, the IC_{50} values for all compounds were determined at the same time using a single 96-well plate.

Determination of K_D Values: Equilibrium Dialysis Method. The K_D values were determined using a previously described equilibrium dialysis assay,[4a] except that the concentrations employed for Lck GST-SH2 fusion protein and compound **21** were 2 μM each. The final volume in the Slide-A-Lyzer dialysis slide cassettes was 200 μL.

Synthesis of NH_2-$(CH_2)_3$-NHCOR (25). A mixture of RCOOH (2 mmol), BOP (2 mmol, 885 mg), HOBt (2 mmol, 306 mg), and NMM (6 mmol, 607 mg) in 10 mL DMF was added to a diaminopropane trityl resin (0.5 g, 0.7 mmol/g) and shaken overnight at room temperature. After a series of wash steps (3 × 10 mL DMF, 3 × 10 mL MeOH, 3 × 10 mL CH_2Cl_2), the compounds were cleaved from the resin with 10% TFA in CH_2Cl_2 (3 × 10 mL × 20 min). After evaporation of solvent, crude compounds were purified via preparative HPLC and subsequently found to be over 98% pure based on two different analytical HPLC solvent systems. Yields were over 70% based on substitution level of diaminopropane trityl resin. *N*-(3-Aminopropyl)-4-(3-methyl-5-oxo-2-pyrazolin-1-yl)benzamide (**25a**): ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.59 (t, J = 5.5 Hz, 1H), 7.89 (d, J = 8.5 Hz, 2H), 7.83 (d, J = 8.5 Hz, 2H), 7.69 (br, 3H), 5.37 (s, 1H), 3.5−3.3 (m, 2H), 2.83 (br, 2H), 2.11 (s, 3H), 1.79 (qn, J = 7 Hz, 2H); ESIMS m/z 275.2 (MH^+). *N*-(3-Aminopropyl)-1,3-dihydroxy-9-acridinecarboxamide (**25b**): ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.85 (t, J = 5.5 Hz, 1H), 8.06 (s, 3H), 7.98 (d, J = 8.5 Hz, 1H), 7.82 (br, 3H), 7.70 (br, 1H), 6.91 (d, J = 2 Hz, 1H), 6.67 (d, J = 2 Hz, 1H), 3.5−3.3 (m, 2H), 2.94 (br, 2H), 1.91 (qn, J = 7 Hz, 2H); ESIMS m/z 312.2 (MH^+).

Synthesis of *N*-(7-Hydroxycoumarin-4-acetyl)-*O*-phospho-L-tyrosine (26). 7-Hydroxycoumarin-4-acetic acid (10 mmol, 2.2 g), TSTU (10 mmol, 3.0 g), and NMM (30 mmol, 3 g) were added to 3 mL DMF and the mixture was shaken for 5 min at room temperature and subsequently added to a solution of *O*-phospho-L-tyrosine (2 mmol, 522 mg) in 3 mL water. The mixture was shaken for 2 h at room temperature and directly purified using preparative HPLC without further manipulation: yield 840 mg, 91% based on *O*-phospho-L-tyrosine; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.66 (d, J = 8 Hz, 1H), 7.42 (d, J = 8 Hz, 1H), 7.19 (d, J = 8.5 Hz, 2H), 7.06 (d, J = 8.5 Hz, 2H), 6.72−6.67 (m, 2H), 6.15 (s, 1H), 4.41 (br, 1H), 3.65 (s, 2H), 3.1−3.0 (m, 1H), 2.9−2.7 (m, 1H); ESIMS m/z 464.1 (MH^+).

Synthesis of Compounds 21 and 22. 25 (0.05 mmol) was added to a mixture of **26** (0.25 mmol, 116 mg), BOP (0.25 mmol, 111 mg), HOBt (0.25 mmol, 38 mg), and NMM (1 mmol, 101 mg) in 1 mL DMF and subsequently shaken for 2 h at room temperature. The mixture was purified on preparative HPLC without further manipulation. Compound **21**: analytical HPLC >95% pure; yield 10 mg, 28% based on **25a**; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.65 (br, 1H), 8.45 (br, 1H), 8.11 (br, 1H), 7.99−7.26 (m, 5H), 7.13 (br, 4H), 6.70 (br, 2H), 6.09 (s, 1H) 5.37 (s, 1H), 4.39 (br, 1H), 3.94−2.72 (m, 8H), 2.11 (s, 3H), 1.62 (br, 2H); ESIMS m/z 720.2 (MH^+). Compound **22**: analytical HPLC >90% pure; yield 14 mg, 37% based on **25b**; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.71 (br, 2H), 8.20 (br, 1H), 8.02−7.30 (m, 6H), 7.20−7.10 (m, 4H), 6.88 (s, 1H), 6.70−6.63 (m, 3H), 6.08 (s, 1H), 4.43 (br, 1H), 3.94−2.64 (m, 8H), 1.74 (br, 2H); ESIMS m/z 757.2 (MH^+).

Synthesis of Compound 23. 25a (0.07 mmol, 20 mg) was added to a mixture of *N*-acetyl-*O*-phospho-L-tyrosine (0.35 mmol, 106 mg), BOP (0.35 mmol, 155 mg), HOBt (0.35 mmol, 54 mg), and NMM (1 mmol, 101 mg) in 1 mL DMF and subsequently shaken for 2 h. The mixture was purified on preparative HPLC without further manipulation and analyzed via analytical HPLC (>90% pure): yield 25 mg, 61% based on **25a**; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.41 (br, 1H), 8.10 (d, J = 8.5 Hz, 1H), 8.00 (br, 1H), 7.89−7.79 (m, 4H), 7.19−7.02 (m 4H), 6.52 (br, 3H), 5.36 (s, 1H), 4.37 (br, 1H), 3.72−3.09 (m, 4H), 2.95−2.67 (m, 2H), 2.11 (s, 3H), 1.77 (s, 3H), 1.60 (br, 2H); ESIMS m/z 560.0 (MH^+).

Synthesis of Compound 24. 3-Acetamido-1-propylamine (0.1 mmol, 11 mg) was added to a mixture of **26** (0.5 mmol, 232 mg), BOP (0.5 mmol, 221 mg), HOBt (0.5 mmol, 77 mg), and NMM (2 mmol, 202 mg) in 1 mL DMF and subsequently shaken for 2 h. The mixture was purified on preparative HPLC without further manipulation and analyzed on analytical HPLC (>95% pure): yield 27 mg, 48% based on 3-acetamido-1-propylamine; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.65 (d, J = 7.5 Hz, 1H), 8.06 (br, 1H), 7.80 (br, 1H), 7.18 (d, J = 8 Hz, 2H), 7.06 (d, J = 8 Hz, 2H), 6.66−6.52 (m, 3H), 6.08 (s, 1H), 4.41 (br, 1H), 3.72−2.91 (m, 5H), 2.77−2.68 (m, 1H), 1.78 (s, 3H), 1.48 (br, 2H); ESIMS m/z 561.8 (MH^+).

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References

(1) (a) Mayer, B. J.; Gupta, R. Functions of SH2 and SH3 Domains. *Curr. Top. Microbiol. Immunol.* **1998**, *222*, 1−22. (b) Kuriyan, J.; Cowburn, D. Modular Peptide Recognition Domains in Eukaryotic Signaling. *Annu. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 259−88. (c) Schaffhausen B. SH2 domain structure and function. *Biochim. Biophys. Acta* **1995**, *1242*, 61−75.

(2) Lewis, L. A.; Chung, C. D.; Chen, J.-y.; Parnes, J. R.; Moran, M.; Patel, V. P.; Miceli, M. C. The Lck SH2 Phosphotyrosine Binding Site is Critical for Efficient TCR−Induced Processive Tyrosine Phosphorylation of the Zeta-Chain and IL-2 Production. *J. Immunol.* **1997**, *159*, 2292−300 and references therein.

(3) (a) Songyang, Z.; Shoelson, S. E.; McGlade, J.; Olivier, P.; Pawson, T.; Bustelo, X. R.; Barbacid, M.; Sabe, H.; Hanafusa, H.; Yi, T.; et al. Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol.*

Cell. Biol. **1994**, *14*, 2777−85. (b) Songyang, Z.; Shoelson, S. E.; Chaudhuri, M.; Gish, G.; Pawson, T.; Haser, W. G.; King, F.; Roberts, T.; Ratnofsky, S.; Lechleider, R. J.; Neel, B. G.; Birge, R. B.; Fajardo, J. E.; Chou, M. M.; Hanafusa, H.; Schaffhausen, B.; Cantley, L. C. SH2 Domains Recognize Specific Phosphopeptide Sequences. *Cell* **1993**, *72*, 767−78.

(4) (a) Lee, T. R.; Lawrence, D. S. Aquisition of High-Affinity, SH2−Targeted Ligands via a Spatially Focused Library. *J. Med. Chem.* **1999**, *42*, 784−7. (b) Lunney, E. A.; Para, K. S.; Rubin, J. R.; Humblet, C.; Fergus, J. H.; Marks, J. S.; Sawyer, T. K. Structure-Based Design of a Novel Series of Nonpeptide Ligands that Bind to the pp60^{src} SH2 Domain. *J. Am. Chem. Soc.* **1997**, *119*, 12471−6. (c) Morelock, M. M.; Ingraham, R. H.; Betageri, R.; Jakes, S. Determination of receptor−ligand kinetic and equilibrium binding constants using surface plasmon resonance: application to the lck SH2 domain and phosphotyrosyl peptides. *J. Med. Chem.* **1995**, *38*, 1309−18.

(5) (a) Burke, T. R., Jr.; Smyth, M. S.; Otaka, A.; Nomizu, M.; Roller, P. P.; Wolf, G.; Case, R.; Shoelson, S. E. Nonhydrolyzable Phosphotyrosyl Mimetics for the Preparation of Phosphatase-Resistant SH2 Domain Inhibitors. *Biochemistry* **1994**, *33*, 6490−4. (b) Wange, R. L.; Isakov, N.; Burke, T. R., Jr.; Otaka, A.; Roller, P. P.; Watts, J. D.; Aebersold, R.; Samelson, L. E. $F_2(Pmp)_2$-TAM zeta 3, A Novel Competitive Inhibitor of the Binding of ZAP-70 to the T Cell Antigen Receptor, Blocks Early T cell Signaling. *J. Biol. Chem.* **1995**, *270*, 944−8.

(6) Nonpeptidic SH2-targeted compounds have 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. For an exception see ref 5b: (a) Burke, T. R., Jr.; Barchi, J. J., Jr.; George, C.; Wolf, G.; Shoelson, S. E.; Yan, X. Conformationally Constrained Phosphotyrosyl Mimetics Designed as Monomeric Src Homology 2 Domain Inhibitors. *J. Med. Chem.* **1995**, *38*, 1386−96. (b) Plummer, M. S.; Holland, D. R.; Shahripour, A.; Lunney, E. A.; Fergus, J. H.; Marks, J. S.; McConnell, P.; Mueller, W. T.; Sawyer, T. K. Design, Synthesis, and Cocrystal Structure of a Nonpeptide Src SH2 Domain Ligand. *J. Med. Chem.* **1997**, *40*, 3719−25. (c) Plummer, M. S.; Lunney, E. A.; Para, K. S.; Shahripour, A.; Stankovic, C. J.; Humblet, C.; Fergus, J. H.; Marks, J. S.; Herrera, R.; Hubbell, S.; Saltiel, A.; Sawyer, T. K. Design of Peptidomimetic Ligands for the pp60^{src} SH2 Domain. *Bioorg. Med. Chem.* **1997**, *5*, 41−7.

(7) A series of dipeptide ligands for the Lck SH2 domain have recently been described that exhibit K_D values comparable to that of Ac-pTyr-Glu-Glu-Ile: (a) Beaulieu, P. L.; Cameron, D. R.; Ferland, J. M.; Gauthier, J.; Ghiro, E.; Gillard, J.; Gorys, V.; Poirier, M.; Rancourt, J.; Wernic, D.; Llinas-Brunet, M.; Betageri, R.; Cardozo, M.; Hickey, E. R.; Ingraham, R.; Jakes, S.; Kabcenell, A.; Kirrane, T.; Lukas, S.; Patel, U.; Proudfoot, J.; Sharma, R.; Tong, L.; Moss, N. Ligands for the Tyrosine Kinase p56lck SH2 Domain: Discovery of Potent Dipeptide Derivatives with Monocharged, Nonhydrolyzable Phosphate Replacements. *J. Med. Chem.* **1999**, *42*, 1757−66. (b) Llinas-Brunet, M.; Beaulieu, P. L.; Cameron, D. R.; Ferland, J. M.; Gauthier, J.; Ghiro, E.; Gillard, J.; Gorys, V.; Poirier, M.; Rancourt, J.; Wernic, D.; Betageri, R.; Cardozo, M.; Jakes, S.; Lukas, S.; Patel, U.; Proudfoot, J.; Moss, N. Phosphotyrosine-Containing Dipeptides as High-Affinity Ligands for the p56^{lck} SH2 Domain. *J. Med. Chem.* **1999**, *42*, 722−9.

(8) Smithgall, T. E. SH2 and SH3 domains: Potential Targets for Anti-Cancer Drug Design. *J. Pharmacol. Toxicol. Methods* **1995**, *34*, 125−32.

(9) Payne, G.; Shoelson, S. E.; Gish, G. D.; Pawson, T.; Walsh, C. T. Kinetics of p56lck and p60src Src Homology 2 Domain Binding to Tyrosine-Phosphorylated Peptides Determined by a Competition Assay or Surface Plasmon Resonance. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 4902−6.

(10) Ladbury, J. E.; Lemmon, M. A.; Zhou, M.; Green, J.; Botfield, M. C.; Schlessinger, J. Measurement of the Binding of Tyrosyl Phosphopeptides to SH2 domains: A Reappraisal. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3199−203.

(11) Stankovic, C. J.; Surendran, N.; Lunney, E.; Plummer, M. S.; Para, K. S.; Shahripour, A.; Humblet, C.; Fergus, J. H.; Marks, J. S.; Herrera, R.; Hubbell, S. E.; Saltiel, A. R.; Stewart, B. H.; Sawyer, T. K. The Role of 4-Phosphonodifluoromethyl- and 4-phosphonophenylalanine in the Selectivity and Cellular Uptake of SH2 Domain Ligands. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1909−14.

(12) Tong, L.; Warren, T. C.; King, J.; Betageri, R.; Rose, J.; Jakes, S. Crystal Structures Of The Human p56Lck SH2 Domain In Complex With Two Short Phosphotyrosyl Peptides At 1.0 Å And 1.8 Å Resolution. *J. Mol. Biol.* **1996**, *256*, 601−10.

(13) A recent study that assessed the thermodynamic binding parameters of various pTyr-Xaa-Xaa-Xaa tetrapeptides with the SH2 domain of c-Src suggests that the Glu residue at +2 may play a role in promoting a high-affinity interaction with the Src SH2 domain; see: Bradshaw, J. M.; Waksman, G. Calorimetric Examination of High-Affinity Src SH2 Domain-Tyrosyl Phosphopeptide Binding: Dissection of the Phosphopeptide Sequence Specificity and Coupling Energetics. *Biochemistry* **1999**, *38*, 3147−54.

(14) Gay, B.; Furet, P.; Garcia-Echeverria, C.; Rahuel, J.; Chene, P.; Fretz, H.; Schoepfer, J.; Caravatti, G. Dual Specificity Of Src Homology 2 Domains For Phosphotyrosine Peptide Ligands. *Biochemistry* **1997**, *36*, 5712−18.

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