Cyclic Peptides Incorporating 4-Carboxyphenylalanine and Phosphotyrosine Are Potent Inhibitors of pp60^{c-src}†

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ABSTRACT: The protein tyrosine kinase, pp60^{c-src}, is involved in cellular signaling and is activated during mitosis and in various tumors. We have been employing cyclic decapeptides to identify the determinants for substrate binding and phosphorylation to develop inhibitors competitive with protein substrates of Src. A structure−activity study [McMurray, J. S., Budde, R. J. A., Ke, S., Obeyesekere, O. U., Wang, W., Ramdas, L., and Lewis, C. A. (1998) *Arch. Biochem. Biophys. 355,* 124] revealed that, at the position 3 residues C-terminal to the phosphorylated tyrosine (Y + 3), both glutamic acid and phenylalanine gave identical K_i, K_m, and V_{max} values. We hypothesized that the area of Src that binds the Y + 3 residue contains either a positively charged lysine or an arginine, capable of ionic interactions with glutamic acid or cation−π interactions with phenylalanine. To test this hypothesis, a series of phenylalanine analogues were substituted at position 7 (the Y + 3 residue) in *cyclo*(Asp^1−Asn^2−Glu^3−Tyr^4−Ala^5−Phe^6−Phe^7−Gln^8−D-Phe^9−Pro^{10}). Of these, 4-carboxyphenylalanine (4-Cpa) and phosphotyrosine resulted in high affinity peptides exhibiting K_i values of 0.85 and 1.1 μM, respectively, 180- and 130-fold increases in potency over the parent cyclic peptide (K_i = 150 μM). These peptides were noncompetitive with respect to ATP and competitive against the phosphate-accepting substrate, polyGlu$_4$Tyr. The truncated cyclic peptide, *cyclo*(Phe−4-Cpa−Gln−D-Phe−Pro−Asp−Aca) (Aca = ϵ-aminocaproic acid), which did not contain tyrosine, was also a competitive inhibitor with a K_i value of 24 μM. We conclude that these cyclic peptides bind to a positively charged area that is near the phosphate transfer region of the active site of Src but does not necessarily include the tyrosine-binding pocket. Furthermore, the 4-Cpa-containing cyclic decapeptide shows remarkable selectivity in the inhibition of Src versus the *src* family members Yes and Lck, as well as other protein tyrosine kinases, Ser/Thr kinases, and other ATP-utilizing enzymes.

pp60^{c-src} (Src)[1] is the product of the *src* proto-oncogene and is the prototype enzyme of the Src family of cytosolic protein tyrosine kinases (PTKs). Src is composed of an N-terminal myristoyl site, a unique region of approximately 85 amino acids, an SH3 domain, an SH2 domain, and the catalytic or SH1 domain. Src participates in several signal transduction cascades such as those initiated by the binding of integrins to components of the extracellular matrix (*1*) and the binding of growth factors such as PDGF to their receptors (*2*). Src is activated during mitosis, and it and other Src family kinases are required for cell division (*3*). Its activity is highly regulated by phosphorylation (*4*). One of the better characterized mechanisms is phosphorylation of Tyr 527 by Csk, which deactivates the enzyme by an intramolecular association of this phosphorylated residue with the SH2 domain. This interaction leads to intramolecular contacts between the SH3 domain, the "linker region" (the amino acid sequence joining the SH2 domain and the catalytic domain), and the amino-terminal half of the catalytic domain, which displace catalytically important residues thereby inactivating the enzyme (*5*−*7*). Src is recruited to various signal transduction components such as PDGFR (*8*) and p125^{FAK} (*9*, *10*) by binding of its SH2 domain to phosphotyrosine (pTyr) residues on these molecules. These interactions increase the kinase activity by disrupting the intramolecular association of the SH2 domain with pTyr 527. With the exception of platelets (*11*), neurons (*12*), and osteoclasts (*13*), Src protein and activity levels are quite low in normal tissues (*14*, *15*). However, kinase activity is elevated in several tumors (*16*−*18*), and antisense oligonucleotides directed to *src* mRNA inhibit the growth of tumor cells both in culture and in nude mice implants (*19*−*21*). An inhibitor of Src would have the effect of uncoupling it from its signal transduction pathways, thus making this enzyme an attractive target for anti-cancer drug design (*22*, *23*).

Many studies used peptides to elucidate the determinants for substrate binding and phosphorylation (reviews include refs *22* and *24*; more recent works include refs *25*−*33*), with the long-term goal of developing inhibitors that compete with

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[1] Abbreviations: Aca, ϵ-amino caproic acid; ACN, acetonitrile; Bzl, benzyl; Cha, cyclohexylalanine; 3-Cpa, 3-carboxyphenylalanine; 4-Cpa, 4-carboxyphenylalanine; 4-Cpa(OMe), 4-carboxyphenylalanine methyl ester; 4-Cpa(NH$_2$), 4-carboxamidophenylalanine; DCC, dicyclohexylcarbodiimide; Gaba, γ-aminobutyric acid; HPLC, high-performance liquid chromatography; LSC, liquid scintillation counting; 3-Mpa, 3-methoxyphenylalanine; Nal, naphthylalanine; NBS, *N*-bromosuccinimide; Src, pp60^{c-src}; Tyr(Me), tyrosine methyl ether; Z, benzyloxycarbonyl; pTyr, phosphotyrosine.

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Table 1: Kinetic Constants of Parent Cyclic Decapeptides[a,b]

peptide	K_i	K_m	V_{max}	V_{max}/K_m
1 c[DNEYAARQfP]	6300	2000	280	0.14
2 c[DNEYAAEQfP]	400	260	350	1.3
3 c[DNEYAAFQfP]	480	240	280	1.2
4 c[DNEYAFFQfP]	150	140	253	1.8

[a] K_i and K_m are in μM. V_{max} is in nmol (min)$^{-1}$ (mg of Src)$^{-1}$. [b] From ref *33*.

protein substrates of Src. Two independently synthesized combinatorial libraries produced the very potent substrates YIYGSFK (*26*) and AEEEIYGEFEAKKKK (*27*), which suggested the consensus sequence _ _IYG _F_. Phenylalanine at position Y + 3 appeared to be essential for recognition by Src and results from other laboratories (*30*, *31*) confirmed the importance of this aromatic residue.

In an earlier paper, we reported a structure−activity study on a cyclic decapeptide derived from the autophosphorylation site of Src: *cyclo*[DNEYAARQfP] (**1**, ref *33*). At positions 1−8, a series of amino acids representing various classes of side chains (hydrophobic, aromatic, negative charge, and positive charge) were substituted to determine which positions were important for affinity and phosphorylation and which amino acids produced the greatest activity. One of the most potent peptides resulting from this survey was *cyclo*[DNEYAFFQfP], **4**, as measured by both substrate and inhibitory activity. The net change of two amino acids (Phe at both positions 6 and 7) resulted in a 42-fold increase in affinity over that of **1**; the K_i values of **1** and **4** were 6300 and 150 μM, respectively. The K_m values also improved from 2000 μM for **1** to 140 μM for **4**, but V_{max} was not significantly changed (Table 1). In common with the consensus sequence for peptide substrates of Src, **4** had Phe at position 7 (Y + 3). Interestingly, during the amino acid survey, it was noted that at this position glutamic acid and phenylalanine both produced equivalent K_i, K_m, and V_{max} values (peptides **2** and **3**, Table 1). It therefore seemed likely that the site of Src that makes contact with the residue at position 7 contained a positively charged Lys or Arg, either of which is capable of forming an ionic interaction with the carboxyl group of Glu or a cation−π interaction with the benzene ring of Phe (*34*, *35*) such as that noted in the X-ray crystal structure of a phosphotyrosyl peptide bound to the SH2 domain of pp60^{v-src} (*36*). To test this hypothesis, a series of phenylalanine analogues bearing varying substituents on the side chain benzene ring were substituted at position 7 of **4**. We report here that incorporation of 4-Cpa and pTyr, bearing negatively charged groups at position 4 of the side chain aromatic ring, resulted in inhibitors with high affinity that are competitive against polyGlu$_4$Tyr (E$_4$Y), exhibiting K_i values of 0.85 and 1.1 μM, respectively. Furthermore, analysis with truncated cyclic peptides indicates that the determinant for inhibition does not contain tyrosine, suggesting that these cyclic peptide inhibitors may not bind to the tyrosyl-substrate binding pocket of the active site of Src.

EXPERIMENTAL PROCEDURES

N$^\alpha$-Fmoc-protected amino acids, aminomethylated polystyrene resin, and 4-hydroxymethyl-3-methoxyphenoxyphenoxybutyric acid were purchased from Advanced Chemtech, Novabiochem, or Chemimpex, Inc. Fmoc-4-Cpa(O-*t*-Bu)-OH was prepared as described in Wang et al. (*37*). PL-DMA peptide synthesis resin was purchased from Polymer Laboratories, Ltd., and the polyamide peptide synthesis resin SPAR50 was a gift from Dr. James T. Sparrow, Baylor College of Medicine. *p*-Methoxybenzyl bromide and *m*-methoxybenzyl bromide were from Aldrich Chemical Co. cAMP-dependent protein kinase A (PKA) catalytic subunit, hexokinase (HXK), lactate dehydrogenase (LDH), NAD, NADH, sodium pyruvate, glucose, and glucose-6-phosphate hydrogenase were obtained from Sigma Chemical Co. Analytical HPLC was performed on a Hewlett-Packard 1090 liquid chromatograph equipped with a diode array detector and a Vydac Peptide and Protein (C-18) column. The gradient was 10−50% ACN in H_2O/30 min at a flow rate of 1.5 mL/min; both ACN and H_2O contained 0.1% TFA. Elution was monitored at 230 and 275 nm. Preparative HPLC was carried out on a Rainin Rabbit HP system equipped with a 25 × 2.5 cm Vydac Peptide and Protein column using ACN/H_2O gradients containing 0.1% TFA.

Preparation of D,L-Substituted Phenylalanines. These noncoded amino acids were prepared as outlined for Fmoc-D,L-4-Cpa(O-*t*-Bu)-OH (*37*). Briefly, *N*-diphenylmethylene glycine methyl ester (*38*) in THF was treated with potassium *tert*-butoxide and was alkylated with *tert*-butyl α-bromo-*p*-toluate (prepared by treatment of *p*-toluyl chloride with potassium *tert*-butoxide followed by bromination with NBS). Acidolytic removal of the diphenylmethylene protecting group [1 M HCl (aq)] and saponification of the methyl ester (1 M NaOH) were followed by protection of the α-amino group with Fmoc-OSu to give Fmoc-D,L-4-Cpa(O-*t*-Bu)-OH. The other noncoded residues were prepared by alkylation of *N*-diphenyl methylene glycine methyl ester with *p*-methoxybenzyl bromide, *m*-methoxybenzyl bromide, and *tert*-butyl α-bromo-*m*-toluate, followed by acidolyis, saponification, and N$^\alpha$-Fmoc protection.

Preparation of Fmoc-4-Cpa(OMe)-OH. Z-4-Cpa-OBzl, an intermediate in the synthesis of Fmoc-4-Cpa(O-*t*-Bu)-OH (*37*) was treated with methanol and DCC. Hydrogenolysis of the Z and Bzl groups followed by N$^\alpha$-Fmoc protection afforded Fmoc-4-Cpa(OMe)-OH.

Preparation of Fmoc-4-Cpa(NH$_2$)-OH. This amino acid derivative was prepared as described in Wang and McMurray (*38*).

Synthesis of Cyclic Peptides. Cyclic peptides were synthesized using solid-phase/solution-phase procedures essentially as described in McMurray et al. (*40*, *41*). Briefly, peptides were synthesized using Fmoc chemistry on aminomethylated polystyrene resin or on either of the polydimethylacrylamide supports developed by the Sheppard (PL-DMA) (*42*) or Sparrow (SPAR50) (*43*) groups, functionalized with the dilute-acid labile handle, 4-hydroxymethyl-3-methoxyphenoxybutyric acid (*44*). Peptides were cleaved from the support with 1% TFA in CH_2Cl_2 with the N-terminal and all side chain protecting groups intact. The Fmoc group was removed with diethylamine, and the peptides were cyclized at a 1 mM concentration in DMF using 3 mM BOP/HOBt/DIPEA. After all solvents and by-products were removed, side chains were deprotected with TFA/phenol (95:5). All peptides were purified to >98% purity by reverse-phase HPLC and gave the correct molecular mass as measured by fast atom bombardment or electrospray mass spectrometry.

To determine the difference in inhibition of the diastereomers of peptide **11** (Table 2), peptide was separated on the same preparative column as above, but the mobile phase was a gradient of acetonitrile in 10 mM ammonium acetate, pH 6.0. The peaks were collected and exhaustively lyophilized to remove the ammonium acetate. IC_{50} values were determined as described in Ramdas et al. (*45*).

Protein Tyrosine Kinases. Recombinant, full-length Src was purified from baculovirus-infected insect cells as described previously (*46*). Csk, Yes, Lck, Abl, and FGF-receptor (FGFr) were expressed in *Escherchia coli* (DH5α) as glutathione S-transferase (GST)-fusion proteins (*47*). Yes was expressed and purified as described by Sun and Budde (*48*). *E. coli* (DH5α) expressing the catalytic domain (residues 398−822) of the FGFr was obtained from X. Zhan (St. Francis Hospital and Medical Center, Hartford, CT).

Protein Serine/Threonine Kinases. The activity of PKA was determined in a reaction medium of 0.02 M MES−NaOH buffer (pH 6.5). Substrates used were 12 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP, and kemptide (Leu−Arg−Arg−Ala−Ser−Leu−Gly). After 30 min incubation, 35 μL of the reaction mixture was loaded onto a 1-mL DEAE-sephadex column equilibrated with the above buffer, and the column was washed with 1 mL of this solution. The eluant was analyzed by LSC for the incorporation of ^{32}P.

Other Enzymes. LDH and HXK were assayed spectrophotometrically by monitoring the absorbance change at 340 nm (*49*). For LDH, the reaction velocity was measured by the decrease in absorbance at 340 nm resulting from the oxidation of NADH. HXK activity was measured as an increase in absorbance at 340 nm resulting from the reduction of NAD^+ through a coupled reaction with glucose-6-phosphate dehydrogenase.

Assay of Peptides as Substrates and Inhibitors of Src. As substrates, peptides were assayed using the polyethyleneimine column technique described by Budde et al. (*50*). K_m and V_{max} values were obtained from the intercepts of Lineweaver−Burke plots ($1/V$ vs $1/[S]$). As inhibitors, peptides were assayed for the inhibition of the phosphorylation of E_4Y, a random copolymer of glutamic acid and tyrosine in a molar ratio of 4:1, using procedures described in Ramdas et al. (*31*). In our hands, this substrate has a K_m of 40 μg/mL, and V_{max} is 3.9 μmol min^{-1} (mg of Src)$^{-1}$. For IC_{50} determination, the E_4Y concentration was 50 μg/mL. IC_{50} values were determined first, and these values were used to set inhibitor concentration ranges for K_i determination. Competitive inhibition constants (K_i) and modes of inhibition were obtained from Lineweaver−Burke plots or secondary plots of the slope of the Lineweaver−Burke plots vs [I] or from plots of $1/V$ vs [I]. For IC_{50} and K_i plots, each point was the average of two determinations, which were within 5% of each other.

RESULTS

To probe the binding site for the side chain of position 7 of **4**, a series of phenylalanine analogues were substituted for Phe^7. If cation−π interactions between a positively charged group on Src, such as the side chain of Lys or Arg, and the side chain of Phe^7 contribute to the binding of **4**, then modifications to the aromatic ring that affect its electron density would be expected to alter the affinity of the peptide. Additionally, incorporation of a negative charge would also be expected to increase binding.

Substitution of cyclohexylalanine (Cha) (peptide **5**) for Phe^7 resulted in a K_i value of 1200 μM, an 8-fold lower affinity, indicating the need for aromaticity at this position. 1-Naphthylalanine (1-Nal) and 2-naphthylalanine (2-Nal) (peptides **6** and **7**, respectively) resulted in decreased inhibition, suggesting that the large size of these aromatic side chains interferes with association of the peptide with the binding site, probably due to steric crowding. The orientation of these rings is important as 2-Nal produced higher affinity than 1-Nal.

Tyrosine (peptide **8**), bearing a hydroxyl group at position 4 of the phenyl group, resulted in 2-fold less inhibition than Phe in **4**. This is surprising since the proposed positively charged group in this subsite of Src would be expected to form a formal hydrogen bond to the phenolic oxygen thereby increasing affinity. Incorporation of tyrosine methyl ether [Tyr(Me)] (peptide **9**) resulted in a K_i value of 100 μM, and placing the methoxy group on position 3 of the aromatic ring (peptide **10**) resulted in a K_i value of 260 μM. Hydroxyl and methyl ether groups might be expected to increase the electron density of the side chain benzene ring through resonance effects, possibly increasing the strength of the proposed amino-aromatic interaction. However, theoretical calculations show that the electron density of the phenyl ring of phenol is about the same as that of benzene and that the reactivity usually associated with the phenolic oxygen does not contribute to cation−π interactions (*51*). The affinities of Na^+ (*51*) and Li^+ (*52*) for the phenyl ring moiety of phenol were calculated to be nearly equivalent to those of benzene. In keeping with these findings, the oxygen substituents on the benzene ring of Phe^7 had no major impact on the affinity of the peptide for Src.

To study ionic interactions between the putative positive charge on Src and the residue Y + 3 of **4**, carboxyl groups were appended to the side chain ring of phenylalanine. Because of the aromatic ring, the negative charge on these Glu−Phe hybrid amino acids resides farther from the peptide backbone and would experience reduced conformational flexibility than that of Glu. Of the carboxylated phenylalanines assayed, D,L-3-carboxyphenylalanine (D,L-3-Cpa) (peptide **12**) produced a 5-fold increase in affinity as compared to **4**, suggesting that ionic interactions contribute to affinity. However, placing the carboxyl group on position 4 of the aromatic ring (peptide **11**) dramatically increased the affinity of the peptide for Src, producing a submicromolar K_i. Peptide **11** was a competitive inhibitor of the phosphorylation of polyE_4Y with an inhibition constant of 850 nM (Figure 1A). This represents an increase of more than 2 orders of magnitude over **4** (K_i = 150 μM, and nearly a 10 000-fold increase over the parent peptide, **1**, which possesses a positively charged arginine residue at position 7 (K_i = 6300 μM).

Tyr(Me), 3-Mpa, 3-Cpa, and 4-Cpa were synthesized as racemic mixtures of D- and L-epimers, and incorporation of the racemates into peptides **9**, **10**, **11**, and **12** essentially allowed the testing of two inhibitors simultaneously. In the case of the 4-Cpa peptide, it is important to determine which of the two isomers is responsible for the observed inhibition. Therefore, the diastereomers of **11** were separated by reverse-phase HPLC and were tested for inhibition of Src. The IC_{50}

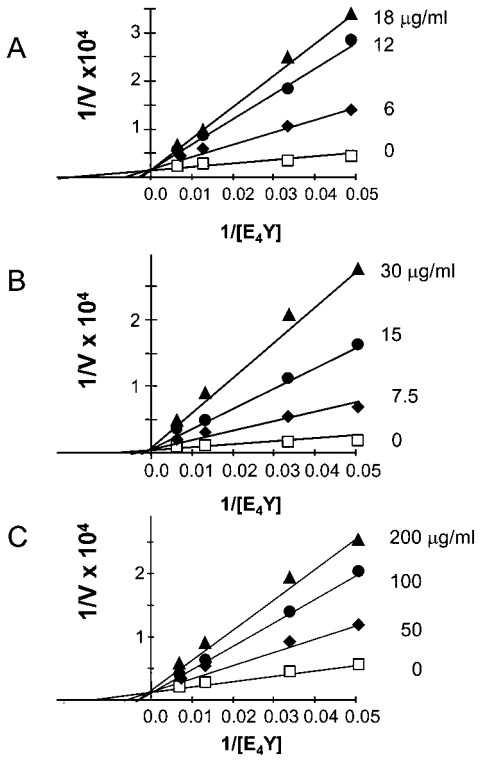


FIGURE 1: Lineweaver−Burke plots of inhibition of Src phosphorylation of E_4Y by cyclic peptides. The units of $1/[E_4Y]$ are $(mg/mL)^{-1}$, and the units of $1/V$ are $(dpm/min)^{-1}$. (A) peptide **11**; (B) peptide **15**; (C) peptide **22**.

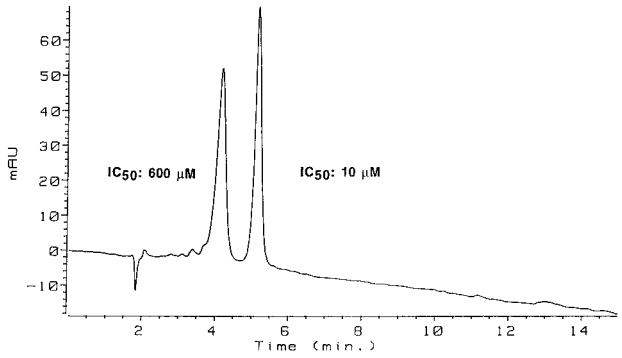


FIGURE 2: HPLC separation of the diastereomers of *cyclo*(Asp[1]−Asn[2]−Glu[3]−Tyr[4]−Ala[5]−Phe[6]−D,L-4-Cpa[7]−Gln[8]−D-Phe[9]−Pro[10]), **11**, and inhibition of Src. Analytical HPLC separation of **11** was achieved using a gradient of 10−40% ACN in 10 mM NH_4OAc, pH 6.0, for 30 min, flow rate 1.5 mL/min, monitored at 230 nm. After preparative separation of the diastereomers, IC_{50} values were determined and are indicated next to the corresponding peak. See Experimental Procedures.

of the first eluting isomer was 600 μM, and that of the second was 10 μM (Figure 2). To determine whether the D- or L-enantiomer of 4-Cpa was responsible for the increase in inhibition, we incorporated Fmoc-L-4-Cpa(O-*t*-Bu)-OH (*37*) into our amino acid sequence, which gave a diastereomer that coeluted with the second eluting isomer in reverse-phase HPLC (data not shown). Therefore, we conclude that the L enantiomer of 4-Cpa is responsible for the large increase in affinity of our cyclic peptide.

To probe further the ionic nature of the binding interaction between Src and **11**, L-4-Cpa was replaced by L-4-Cpa(OMe) (13), L-4-Cpa(NH_2) (**14**), and phosphotyrosine (**15**). The first two removed the negative charge of 4-Cpa while still retaining the ability to form hydrogen bonds with Src. These substitutions resulted in dramatic increases in the K_i to 300 (**13**) and 32 μM (**14**), 350- and 37-fold decreases in affinity, respectively. On the other hand, substitution of 4-Cpa with pTyr retained negative charge on the side chain ring to interact with the proposed positive charge on Src. This substitution had very little effect on the K_i (1.1 μM). Peptide **15** was also a competitive inhibitor with respect to E_4Y phosphorylation (Figure 1B). These results demonstrate the critical importance of negatively charged substituents on the side chain of the amino acid at position 7. We conclude that the site of Src that interacts with the residue at Y + 3 of our cyclic decapeptide possesses a positively charged group.

Interestingly, 4-Cpa appears to be detrimental to the ability of this peptide to serve as a substrate. Peptide **11** is phosphorylated with a K_m of 14 μM and a V_{max} of 0.2 nmol min^{-1} (mg of protein)$^{-1}$ (data not shown). In comparison, **4** had K_m and V_{max} values of 140 μM and 253 nmol min^{-1} mg^{-1}, respectively (Table 1).

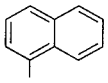
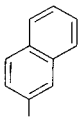
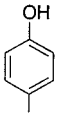
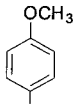
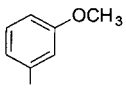
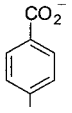
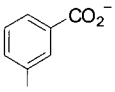
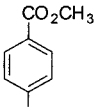
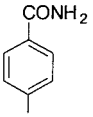
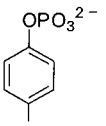
In addition to the amino acid sequence, knowledge of the bioactive conformation, both the backbone and the side chains, is essential for complete understanding of the interactions between Src and our cyclic peptide inhibitors. In lieu of an X-ray crystal structure of Src co-crystallized with **11**, structure determination of the unliganded cyclic peptide can provide useful insight into possible binding conformations. Preliminary molecular dynamics simulations (M. Pettitt, personal communication) and NMR studies suggest that cyclic decapeptides **4** and **11**, despite their cyclic structure, likely exist in several low-energy conformations. Reducing the size of the peptide is expected to reduce the number of conformations available, facilitating structure determination, both by NMR and ab initio modeling. To determine the effect of reduced size on inhibition, three sets of smaller cyclic peptides in which tetrapeptide portions were replaced by various sized spacers were synthesized and tested (Table 3). Although structural studies are outside the scope of this paper, these truncated peptides have provided important information on which residues of **11** are important for binding.

In the first set, the tetrapeptide Gln[8]−D-Phe[9]−Pro[10]−Asp[1] of **4** was replaced with γ-aminobutyric acid (Gaba) or ϵ-aminocaproic acid (Aca). This tetrapeptide is distal from the tyrosine and its deletion kept intact the region from Y − 2 to Y + 3, which was found to be important for binding interactions (*31*, *33*). These size reductions gave peptides, **16** and **17**, both of which inhibited Src 7-fold less than **4** (K_i = 900 μM). Thus, there was no apparent affect of the size of the spacer on affinity for Src.

In a second set, analogous substitutions were made to **11**, and in this case the size of the spacer did have an effect. Replacement of Gln[8]−D-Phe[9]−Pro[10]−Asp[1] with Gaba (**18**) produced a K_i value of 200 μM, a 300-fold loss of activity. The mode of inhibition of **19** was not a common type (e.g., competitive, mixed, noncompetitive, etc.); therefore, no K_i value could be determined. Comparison of **16** and **18** shows again that 4-Cpa is capable of increasing the affinity of these cyclic peptides for Src.

The third set (peptides **20**−**22**) was synthesized to determine if the region from Asn[2]−Glu[3]−Tyr[4]−Ala[5] of **11** plays a role in inhibition. This tetrapeptide was replaced with Gaba and Aca as in the other truncated peptide series.

Table 2: Effect of Unnatural Amino Acids at Position 7 of *cyclo*-(Asp^1−Asn^2−Glu^3−Tyr^4−Ala^5−Phe^6−Xxx^7−Gln^8−D-Phe^9−Pro^{10})

Peptide #	Amino acid at position 7	Side chain at position 7	*Ki* (μM)
4	Phe		150
5	Cha		1,200
6	1-Nal		2,100
7	2-Nal		700
8	Tyr		300
9	D,L-Tyr(Me)[a]		100
10	D,L-3-Mpa[a]		260
11	D,L-4-Cpa[a]		0.85[b]
12	D,L-3-Cpa[a]		32
13	4-Cpa(OMe)		300
14	4-Cpa(NH_2)		32[b]
15	Tyr(OPO_3H_2)		1.1[b]

[a] These amino acids were synthesized by us as D,L racemates, and peptides were assayed as 50:50 mixtures of diastereomers. Other amino acids were of the L configuration. [b] These peptides were competitive inhibitors. All others displayed mixed inhibition.

However, the system was expanded in that glycine was added to further test the effect of spacer size on inhibition. It was found that the degree of inhibition correlated with increasing size of the spacer. Gly produced a peptide (**20**) with very weak, mixed inhibition (K_i = 566 μM). Gaba produced a mixed inhibitor with intermediate affinity (**21**, K_i = 42 μM), and Aca gave the most potent inhibitor in this series, **22** (K_i = 24 μM). Like **11**, **22** was found to be noncompetitive with respect to ATP and competitive with respect to E_4Y (Figure 1C), although it exhibited 40-fold less affinity than the parent peptide.

Table 3: Inhibition Constants of Truncated Cyclic Peptides

peptide	sequence	K_i (μM)[a]
4	c[NEYAFFQfPD]	150
16	c[NEYAFF Gaba]	900
17	c[NEYAFF Aca]	900
11	c[NEYAF (D,L-4-Cpa) QfPD]	0.85[b]
18	c[NEYAF (D,L-4-Cpa) Gaba]	200
19	c[NEYAF (D,L-4-Cpa) Aca]	not determinable
20	c[F (D,L-4-Cpa) QfPD Gly]	566
21	c[F (D,L-4-Cpa) QfPD Gaba]	42
22	c[F (D,L-4-Cpa) QfPD Aca]	24[b]

[a] Unless otherwise noted, these peptides displayed mixed inhibition. [b] Mode of inhibition: competitive.

Table 4: Inhibition of PTKs and ATP-Utilizing Enzymes by Peptide **11**

enzyme	IC_{50} (μM)	K_i (μM)
Src	6	0.85
Yes	>100	330
Lck	320	ND[a]
Csk	>2000	4000
Abl	1800	ND
FGFr	1200	680
PKA	1070	2000
HXK	NI[b]	NI
LDH	NI	NI

[a] ND = not determined. [b] NI = no inhibition up to 4 mM.

To serve as a model or template for peptidomimetic design, it is important that a peptide not only possess high affinity for its target but also be selective and not bind to related enzymes. To measure its specificity for Src, peptide **11** was tested as an inhibitor of several PTKs, protein kinase A (a Ser/Thr kinase), and the ATP-utilizing enzymes hexokinase and lactate dehydrogenase. IC_{50} and K_i values are shown in Table 4. Peptide **11** showed excellent selectivity for Src over the other kinases and ATP-utilizing enzymes. Furthermore, this cyclic decapeptide shows remarkable selectivity for Src over the Src family PTKs Yes and Lck, despite the high sequence homology of these two enzymes.

DISCUSSION

Starting with a cyclic version of the sequence surrounding Tyr 416, **1**, the autophosphorylation site of Src, we showed previously that the arginine at position Y + 3 was detrimental for binding affinity (*33*). Whereas reports have shown that Phe or His is preferred at this position (*26*, *27*, *31*, *53*), our amino acid survey of cyclic peptide **1** showed that Glu gave equal activity to Phe, both as a substrate and as an inhibitor (*33*). We hypothesized that a positively charged residue on Src makes contact with the Y + 3 residue and is involved in a cation−π interaction with the benzene ring of Phe or an ionic interaction with the carboxyl group of Glu. By substituting a series of Phe analogues at the Y + 3 position of peptide **4**, we found that aromaticity is important for Phe

derivatives as evidenced by reduced affinity of a cyclohexyl group at this position. This supports the presence of a cation−π interaction. Adding an acidic group to the side chain aromatic ring of Phe^7, the carboxyl group of 4-Cpa, or the phosphate of pTyr greatly enhanced the affinity of the cyclic peptide for Src, suggesting that ionic interactions do indeed play a large role in binding of these peptides. Placement of the carboxyl group at position 3 resulted in a decrease in affinity relative to the 4-carboxyl group, indicating that proper position of this substituent is important. Converting the carboxyl group of 4-Cpa to a methyl ester or an amide reduced inhibition. We therefore conclude that there is indeed a positively charged group on Src that interacts with the Y + 3 residue of our cyclic peptide either in an ionic interaction (4-Cpa or pTyr) or through hydrogen bonding (4-Cpa amide).

Although the lack of an increase in affinity of the 4-hydroxy, 3-methoxy, and 4-methoxy substituted analogues, relative to Phe in peptide **4**, apparently does not support the presence of positively charged residues in the binding pocket of Src, it does not exclude this possibility. It could be that these substituents are not positioned for optimal interaction with the proposed Lys or Arg of the enzyme.

The inhibition exhibited by the truncated peptides **21** and **22** suggests that the determinant for binding resides in the hexapeptide Phe^6−4-Cpa^7−Gln^8−D-Phe^9−Pro^{10}−Asp^1 and that tyrosine is not necessary for inhibition of Src. Thus, the poor substrate activity of the 4-Cpa-containing cyclic decapeptide **11** is likely to be the result of binding to the enzyme in a mode in which Tyr^4 is not in a position to become phosphorylated.

The majority of the peptides in Table 2 exhibited mixed inhibition, suggesting multiple modes of binding. The reported competitive inhibition constants (K_i) are a measure of the ability of a compound to compete with the protein substrate. For the mixed inhibitors, these values were extracted from secondary plots of the slope of Lineweaver−Burke plots vs [I] or from plots of 1/V vs [I]. Cyclic peptides **1**−**4**, from which the peptides in this study were derived, were all mixed inhibitors (*33*). However, peptides **11**, **14**, **15**, and **22** were strictly competitive inhibitors, suggesting a single binding mode. These peptides possessed Phe analogues that incorporated either the negatively charged carboxyl or phosphate groups or the carboxamide group in the *para* position of the side chain benzene ring. Note that peptide **12** has a carboxyl group in the *meta* position and displays mixed inhibition. Thus, the *para*-substituted aromatic residue directs the peptide into a binding mode leading to competition with the protein substrate, E_4Y.

Mode of inhibition is also dependent on the sequence surrounding 4-Cpa. In Table 3, peptides **18** and **19** displayed either mixed or an unusual type of inhibition. Of the peptides based on Phe^6−4-Cpa^7−Gln^8−D-Phe^9−Pro^{10}−Asp^1 (**20**−**22**), the size of the spacer had an influence on the mode of inhibition, in addition to affinity. The largest, aminocaproic acid, produced a strictly competitive inhibitor, **22**. The others were mixed.

Ruzza et al. (*54*) found that a similar peptide possessing a pTyr, EDNEpYTA, inhibited the Src family kinase Lyn. It was more effective at inhibiting autophosphorylation than the phosphorylation of the exogenous peptide substrate, angiotensin II. Lai et al. (*32*) reported that acetyl-IXGEF-amide, in which X was L-4-Cpa or 4-(*R*,*S*-hydroxyphosphonomethyl)-L-phenylalanine, inhibited purified, recombinant human Src. Of the two negatively charged Phe analogues, 4-Cpa provided greater inhibition. No K_i values were reported, but the 4-Cpa-containing peptide at 1 mM concentration inhibited the phosphorylation of 2 mM RRLIEDNEYAAR, a commonly used peptidic Src substrate, by 68%. Therefore, the inhibition of Src family kinases by peptides possessing phenylalanine substituted by negatively charged groups appears to be a general phenomenon that may be useful in the design of peptidomimetic antagonists.

Peptide **11** shows remarkable selectivity for Src over the Src family members Yes and Lck, as well as receptor PTKs and other kinases. These results demonstrate that it is indeed possible to program selectivity in compounds that compete with protein substrates, as has been so elegantly described for ATP-directed inhibitors (reviewed in refs *55* and *56*).

The presence of the important positive charge prompts the question: which Arg or Lys near the substrate binding site of Src is interacting with 4-Cpa or pTyr in these cyclic peptides? X-ray crystal structures of Src (*5*−*7*) and the Src family kinase, Hck (*57*), have been reported, but these are inactive enzymes and do not have peptide substrates bound to their active sites. In studies of the crystal structure of activated Lck, no electron density was found for a peptide substrate soaked into the crystal (*58*). Therefore, no direct models of peptides bound to the catalytic domain of Src family PTKs are available. However, the crystal structure of the catalytic domain of the insulin receptor kinase cocrystallized with a non-hydrolyzable ATP analogue and KKKLPATDGYMNMSPVGD, a peptide substrate, provides a model for substrate binding to PTKs (*59*). In this structure, there is a hydrophobic pocket consisting of the residues Val 1173, Leu 1219, and aliphatic portions of Asn 1215 and Glu 1216 that binds the side chain of Met at position Y + 1 in the peptide. In Src, the corresponding residues are Ile 426, Leu 472, Asn 468, and Arg 469 (*60*). In IRK, Glu 1216 forms the base of the pocket, whereas in Src the corresponding residue is Arg 469. It is conceivable that the side chain of 4-Cpa of **11** and **22** or pTyr in **15** sits in a similar pocket such that these acidic groups interact with Arg 469. If so, then the aromatic group of Phe^6 might be placed in the tyrosine-binding pocket. If this were the major binding orientation, then other modes in which the Tyr^4 of the cyclic decapeptide is positioned to accept a phosphate would play a minor role, which could explain why **11** was a poor phosphate acceptor.

A second positive charge in the catalytic domain of Src that might bind 4-Cpa or pTyr resides in the tyrosine-binding pocket. In the catalytic loop of the Src family of kinases, there is an arginine (Arg 388 in Src) that is two residues C-terminal to the conserved catalytic aspartic acid (Asp 386 in Src). In other PTK families, this arginine is four residues to the C-terminal side of the catalytic Asp (*60*). Therefore, the topology of the phosphoryl transfer region of Src family members likely differs from other PTKs. It is possible that the 4-Cpa residue or the pTyr residue of our cyclic peptides docks in the tyrosine-binding pocket of Src, making contact with Arg 388. This would result in a nonproductive enzyme−peptide complex, which would explain the low V_{max} value exhibited by **11** when it was tested as a substrate. Considering the high homology between Src family members, this model

may not explain the specificity of this cyclic decapeptide for Src over Yes or Lck. However, the combinatorial library of Songyang et al. (*27*) revealed that despite high sequence homology, Src and Lck had different substrate specificities, which suggests slightly different architecture in the tyrosine-binding pockets of these Src family PTKs.

A third positively charged area in the catalytic domain consists of Arg 385 and/or Arg 409 of Src. In the crystal structure of activated Lck, the analogous residues (Arg 363 and Arg 387) were shown to bind the autophosphorylated tyrosine (Tyr 416 in Src and 394 in Lck) of the activation loop, presumably positioning this segment so that protein substrates can bind efficiently to the phosphate transfer region (*58*). Analogous interactions were observed in the X-ray crystal structure of IRK and peptide discussed above (*59*). If the 4-Cpa or pTyr residues of our cyclic peptides engage these arginines, then other amino acids of these inhibitors, or possibly residues of the activation loop, may prevent the substrate from binding to the active site.

A fourth possible binding site for the negatively charged cyclic peptides is the SH2 domain, which specifically binds phosphotyrosine-containing peptides. Although this domain is not at the phosphate transfer site, Garcia et al. (*61*) showed that phosphopeptides derived from the region surrounding Tyr 527 of Src [Glu−Pro−Gln−Tyr(OPO_3H_2)−Gln−Pro−Gly−Glu−Asn−Leu] and the middle T antigen [Glu−Pro−Gln−Tyr(OPO_3H_2)−Glu−Glu−Ile−Pro−Ile−Tyr−Leu] were able to inhibit v-Src. The latter peptide is a high affinity ligand for Src family SH2 domains (*62*) and was shown to activate Src that was repressed by phosphorylation on Tyr 527 (*63*). Weiland et al. (*64*) showed that this peptide did not inhibit the catalytic domain of Src. Thus, SH2 domain-targeted phosphopeptides, containing a negatively charged aromatic residue, are able to inhibit the catalytic activity of Src. The precise determination of the site of binding of the peptides possessing the negatively charged Phe residues, as well as the bound conformation and enzyme−inhibitor interactions, awaits the X-ray crystallographic structure of Src−inhibitor cocrystals.

As opposed to screening thousands of compounds in combinatorial libraries (*26*, *27*, *65*), in this paper we demonstrated that by using a rational approach we developed a high-affinity peptide inhibitor with excellent selectivity for Src. We designed eight peptides (**5**−**12**) to test the hypothesis of a positive charge on the enzyme surface, which was based on an observation made in testing another series of 48 rationally designed peptides (*33*). Of the new peptides, **11** was found to be an excellent inhibitor, both in terms of selectivity and affinity. The remaining 10 new peptides (**13**−**22**) provided information pertaining to the interaction of **11** and Src. Structural studies of **11** and **22** are in progress and will be reported on completion.

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