Synthesis and Structure−Activity Relationships of Linear and Conformationally Constrained Peptide Analogues of CIYKYY as Src Tyrosine Kinase Inhibitors

Anil Kumar,[†] Guofeng Ye,[†] Yuehao Wang,[‡] Xiaofeng Lin,[‡] Gongqin Sun,[‡] and Keykavous Parang*[,†]

Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, Rhode Island 02881, and Department of Cell and Molecular Biology, University of Rhode Island, Kingston, Rhode Island 02881

Received March 22, 2006

A series of peptide analogues of Ac-CIYKYY (**1**) were synthesized by functional group modifications in peptide side chains or by introducing conformational constraints, to improve the inhibitory potency against active Src kinase. Ac-CIYKF(4-NO_2)Y (**2**, IC_{50} = 0.53 μM) and conformationally constrained peptide **31** (IC_{50} = 0.28 μM) exhibited 750- and 1400-fold higher inhibitory activities, respectively, versus that of **1** (IC_{50} = 400 μM). Compound **2** exhibited a partial competitive inhibition pattern against ATP.

Introduction

Protein tyrosine kinases (PTKs) are enzymes that catalyze the transfer of the γ-phosphoryl group from ATP to the hydroxyl groups of specific tyrosine residues in proteins.[1] Src, a nonreceptor tyrosine kinase, is involved in proliferation and migration responses in many cell types, cell activation, adhesion, motility, and survival, growth factor receptor signaling, and osteoclast activation.[2,3] Src has been implicated in the development of osteoporosis and inflammation-mediated bone loss,[4,5] and several different cancers[2,6] for which the transformed phenotypes have been correlated with Src mutations and/or overexpression. Therefore, Src has become an intriguing target for drug discovery.[7,8] Src family kinases share five distinct functional domains from the *N* to *C* termini: the fatty acid acylation domain, the Src homology 3 (SH3) and Src homology 2 (SH2) domains, the kinase domain (including ATP and substrate binding sites), and a *C*-terminal regulatory domain.[9]

Most of the attention has been on designing Src kinase inhibitors through blocking Src-dependent phosphorylation of substrate proteins. The kinase-domain inhibitors are designed to inhibit the binding of ATP (ATP-binding-site inhibitors)[10,11] or the protein substrate (substrate-binding-site inhibitors).[12−14] Lam et al.[14,15] identified YIYGSFK (K_m = 55 μM) as a peptide substrate for c-Src. The peptide CIYKYY was reported to be a potent inhibitor of Src phosphorylation of YIYGSFK in a kinase assay, when the substrate was used at a concentration of 55 μM.[13,14] The mechanism of c-Src inhibition by CIYKYY remains unknown. Furthermore, it appears that c-Src inhibition by CIYKYY depends on the substrate used in the kinase assay. PolyE$_4$Y is a nonspecific substrate that is commonly used in kinase assays and is easily phosphorylated on its multiple tyrosine residues by all eukaryotic protein tyrosine kinases. Our radioactive kinase assay showed that Ac-CIYKYY (**1**, IC_{50} = 400 μM) was a weak inhibitor of polyE$_4$Y phosphorylation by active c-Src.

We describe the synthesis and inhibitory activities of Ac-CIYKYY peptide analogues as part of our ongoing research to develop Src-kinase inhibitors. The purpose of this study was to determine whether it is possible to convert **1**, a weak inhibitor of polyE$_4$Y phosphorylation by active c-Src, to more potent inhibitors by structural modifications and to establish the structure−activity relationships of these compounds. Two classes of peptide analogues of **1** were synthesized. Class I included linear peptides (**1**−**21**) that were synthesized by replacing tyrosine residues with tyrosine mimics containing other functional groups or by modifying the side chains of tyrosine residues (Table 1).

Table 1. Chemical Structures and Inhibitory Potency Values (IC_{50}) for Modified Linear Peptide Analogues (**1**−**21**) against Active Src Kinase

compd	IC_{50} (μM)[a]	compd	IC_{50} (μM)
Ac-CIYKYY (**1**)	400	Ac-CIYKF(4-NO_2)YF (**12**)	1.0
Ac-CIYKF(4-NO_2)Y (**2**)	0.53	Ac-CIYKF(4-F)Y (**13**)	26
Ac-CIYKFY (**3**)	>700	Ac-CIYKF(4-Cl)Y (**14**)	7.3
Ac-CIYKF(4-NH_2)Y (**4**)	93	Ac-CIYKF(4-I)Y (**15**)	0.78
Ac-CIF(4-NO_2)KYY (**5**)	1.5	Ac-CIYKF(4-CN)Y (**16**)	6.0
Ac-CIYKYF(4-NO_2) (**6**)	10.8	Ac-CIYKF(4-N_3)Y (**17**)	2.0
Ac-CIF(4-NH_2)KYY (**7**)	760	Ac-CIYKpYY (**18**)	>700
Ac-CIYKYF(4-NH_2) (**8**)	>700	Ac-CYKpYY (**19**)	>700
Ac-CIF(4-NO_2)KF(4-NO_2)Y (**9**)	1.4	Ac-CIYKF(4-$NHSO_2CH_3$)Y (**20**)	>700
Ac-CIYKF(4-NO_2)F(4-NO_2) (**10**)	5.6	Ac-CIYKF(4-guanidine)Y (**21**)	>700
Ac-CIF(4-NO_2)KYF(4-NO_2) (**11**)	3.4		

[a] IC_{50} is the concentration required to produce 50% inhibition in the phosphorylation of polyE$_4$Y by active c-Src (average of triplicate experiments). The upper limit of the standard error of the mean (SEM) was ±10%.

Class II included conformationally constrained peptides (**22**−**32**) (Figure 1) that were synthesized by linking the side chains of amino acids together. These compounds were designed to determine whether the presence of the constrained ring has any effect in the inhibitory potency. Because of the presence of three tyrosine residues and one lysine in Ac-CIYKYY (**1**), there were several opportunities for linking the side chains of amino acids. Four types of conformational constraints were introduced by linking the amino acids head to tail, *C*-terminal to a side chain, *N*-terminal to a side chain, and a side chain to a side chain (Figure 1).

All final compounds were purified (>95%) using preparative HPLC and characterized by a high-resolution time-of-flight mass spectrometer. The inhibitory potencies of the synthesized linear and constrained peptides against active c-Src were examined using a radioactive kinase assay with polyE$_4$Y as the substrate.

Results and Discussion

Chemistry. All linear peptides were synthesized on solid phase employing *N*-(9-fluorenyl)methoxycarbonyl (Fmoc)-based

* To whom correspondence should be addressed. Tel: +1-401-874-4471. Fax: +1-401-874-5787. E-mail: kparang@uri.edu.
[†] Department of Biomedical and Pharmaceutical Sciences.
[‡] Department of Cell and Molecular Biology.

10.1021/jm060334k CCC: $33.50
Published on Web 04/29/2006

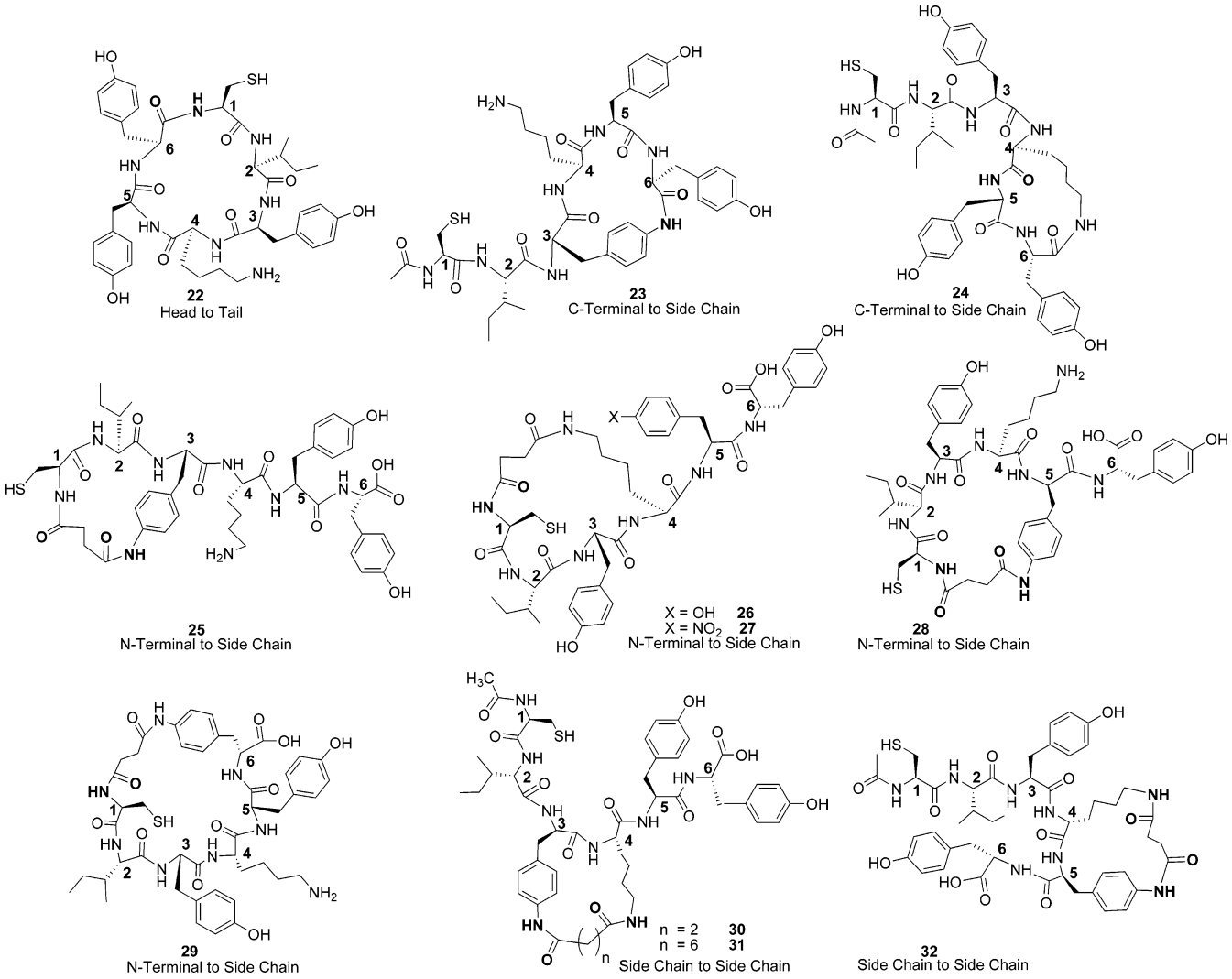


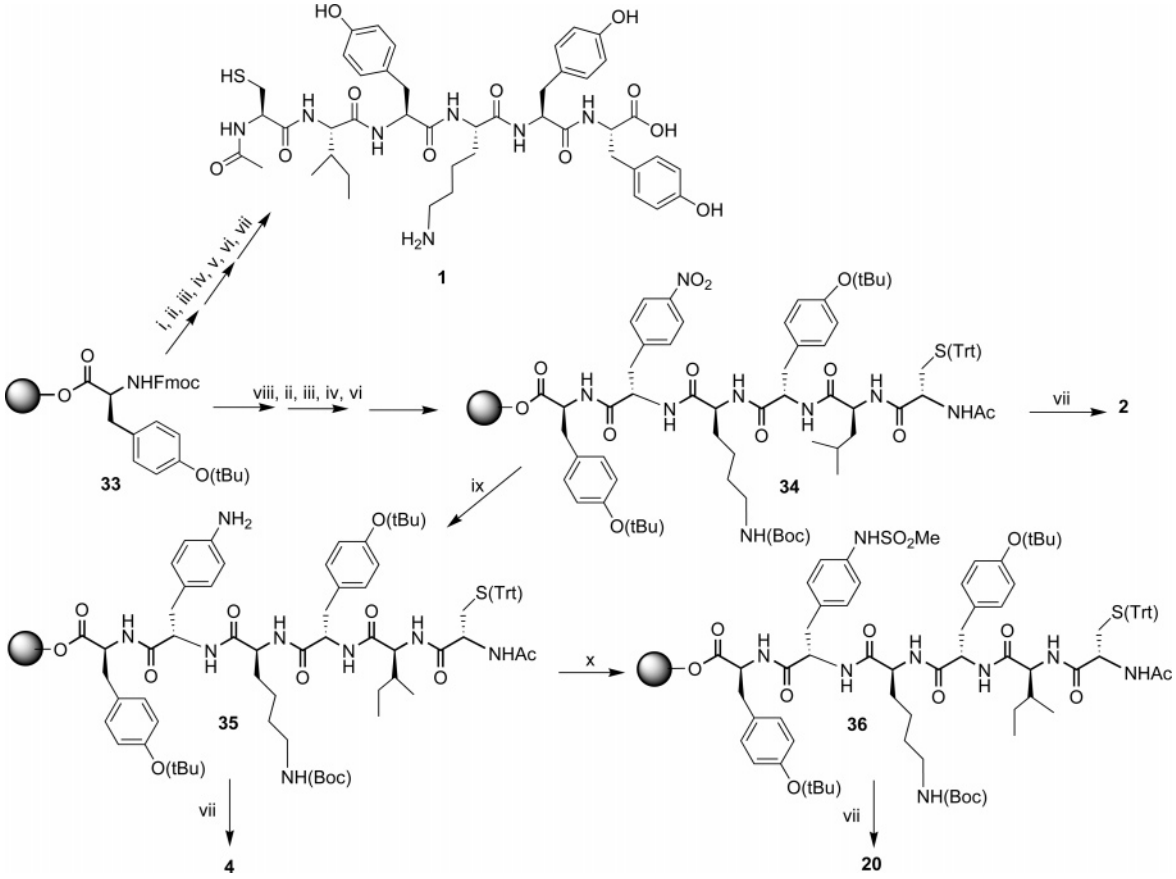
Figure 1. The chemical structures of conformationally constrained peptides (**22**−**32**).

chemistry and Fmoc-L-amino acid building blocks. 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and NMM in *N*,*N*-dimethylformamide (DMF) were used as coupling and activating reagents, respectively.[16−18] The peptides were *N*-terminally acetylated with acetic anhydride. As a representative example, the synthesis of linear peptides **1**, **2**, **4**, and **20** from Fmoc-Tyr(tBu)-Wang resin (**33**) is shown in Scheme 1. Polymer-bound peptide **34**, containing the 4-nitrophenylalanine residue at position 5 from the *N*-terminal, underwent a reduction reaction with stannous chloride under acidic conditions to yield **35** containing 4-aminophenylalanine. The reaction of polymer-bound peptide **35** with methylsulfonyl chloride gave **36**. Polymer-bound peptides **34**−**36** were cleaved with TFA/anisole/H_2O/ethanedithiol (EDT) at room temperature to afford **2**, **4**, and **20**, respectively.

Scheme 2 shows the synthesis of conformationally constrained peptide **22** by head to tail cyclization. Polymer-bound Fmoc-protected methyl ester tyrosine (**38**) was synthesized from Wang resin (**37**) using the Mitsunobu reaction.[19] *N*-(9-Fluorenylmethoxycarbonyl)-tyrosine methyl ester (Fmoc-Tyr-OCH_3) and triphenylphosphine (PPh_3) were dissolved in dichloromethane (DCM) and added to **37**. Diisopropylazodicarboxylate (DIAD) was added dropwise at 0 °C, and the mixture was shaken overnight. The resin was collected by filtration and washed successively with DMF, MeOH, and DCM to yield **38**. The linear peptide CIYKY was assembled on **38** by the Fmoc solid-phase peptide synthesis strategy using Fmoc-protected amino acids (Fmoc-Tyr(tBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Tyr-(tBu)-OH, Fmoc-Ile-OH, and Fmoc-Cys(Trt)-OH) to yield **39**. The hydrolysis of the methyl ester was performed using sodium hydroxide in methanol to afford **40**. The cyclization of **40** by linking the *N*-terminal amino group with the *C*-terminal carboxylic acid group in the presence of a mixture of HBTU, HOBt, and *N*,*N*-diisopropylethylamine (DIPEA) in dry DMF for 48 h, followed by the cleavage of the peptide from the resin with TFA/thioanisole/H_2O/EDT/phenol and HPLC purification afforded conformationally constrained peptide **22**.

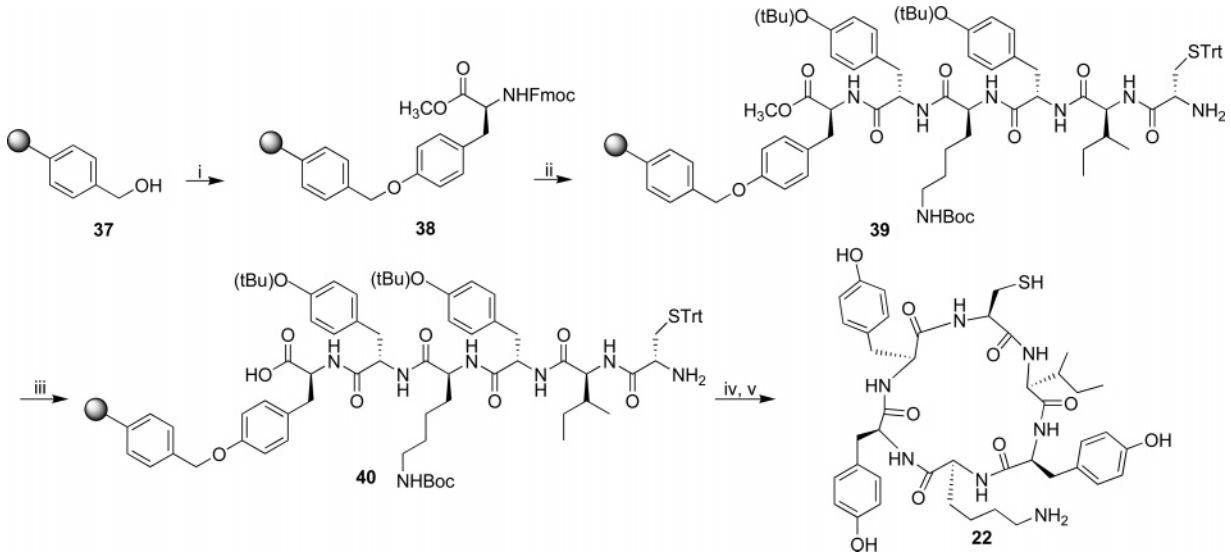
The synthesis of conformational constrained peptides **30** and **31** is shown in Scheme 3 as a representative example of a side chain−side chain cyclization. The assembly of linear peptide **41** was performed by Fmoc solid-phase peptide synthesis strategy on Fmoc-Tyr(tBu)-Wang resin (**33**), using Fmoc-Tyr-(tBu)-OH, Fmoc-Lys(Dde)-OH, Fmoc-Phe(4-NO_2)-OH, Fmoc-Ile-OH, and Fmoc-Cys(Trt)-OH to give **41**. The reduction of the nitro group in **41** to the amino group was carried out in the presence of $SnCl_2 \cdot 2H_2O$ in DMF at room temperature for 24 h to yield **42**. The free amino group in **42** was reacted with succinic anhydride or suberic acid/HBTU in DMF for 6 h followed by filtration and washing to yield **43**. The Dde-protecting group was selectively removed with 2% hydrazine monohydrate in DMF to afford **44**. The cyclization of the peptides was carried out by linking the amino group of the side

Scheme 1. Synthesis of Linear Peptides **1**, **2**, **4**, and **20**[a]



[a] Reagents: (i) (a) Piperidine, DMF, (b) Fmoc-Tyr(tBu)-OH, HBTU, NMM, DMF; (ii) (a) Piperidine, DMF, (b) Fmoc-Lys(Boc)-OH, HBTU, NMM, DMF; (iii) (a) Piperidine, DMF, (b) Fmoc-Tyr(tBu)-OH, HBTU, NMM, DMF; (iv) (a) Piperidine, DMF, (b) Fmoc-Ile-OH, HBTU, NMM, DMF; (v) (a) Piperidine, DMF, (b) Fmoc-Cys(Trt)-OH, HBTU, NMM, DMF; (vi) (a) Piperidine, DMF, (b) Acetic anhydride, DMF; (vii) TFA/H_2O/anisole/EDT (95: 2.0:2.0:1.0 v/v); (viii) (a) Fmoc-Phe(4-NO_2)-OH, HBTU, NMM, DMF, (b) Piperidine, DMF; (ix) $SnCl_2 \cdot 2H_2O$/DMF; (x) Methylsulfonyl chloride.

Scheme 2. Synthesis of Conformationally Constrained Peptide **22**[a]

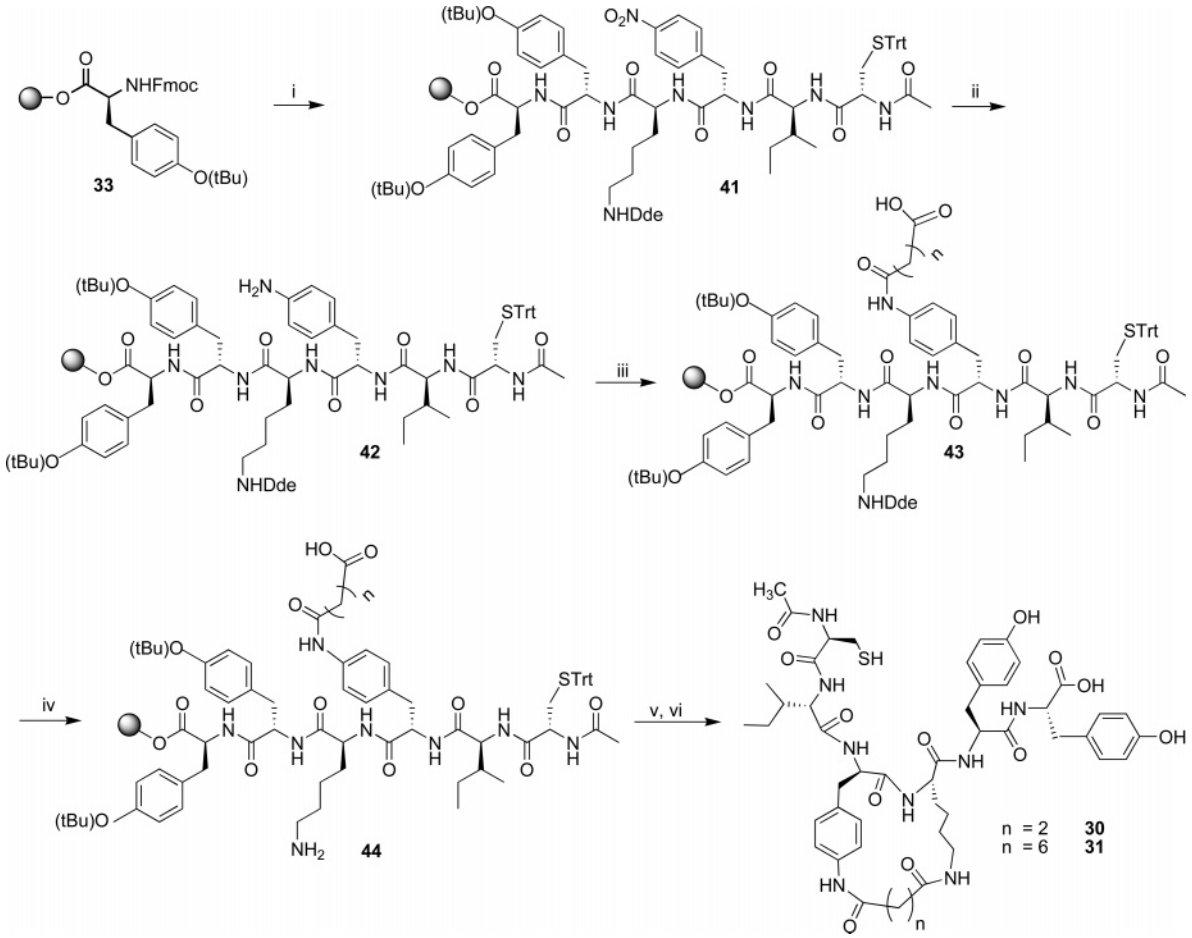


[a] Reagents: (i) Fmoc-Tyr-OCH_3, PPh_3, DIAD, DCM, 24 h; (ii) Fmoc solid-phase peptide synthesis; (iii) NaOH/MeOH (0.4 M), 3 days; (iv) HBTU, HOBt, DIPEA, DMF, 2 days; (v) TFA/thioanisole/H_2O/EDT/phenol (82.5:5:5:2.5:5 v/v).

chain of lysine with the free carboxylic acid group in the presence of a mixture of HBTU, HOBt, and DIPEA in dry DMF for 48 h. The cleavage of the peptides from the resin with TFA/thioanisole/H_2O/EDT/phenol and HPLC purification afforded conformationally constrained peptides **30** and **31**.

Structure−Activity Relationships. Table 1 shows the inhibitory activities of the linear peptide analogues of Ac-$C_1I_2Y_3K_4Y_5Y_6$ (**1**−**21**). The presence of the specific amino acids proved to be critical for generating the inhibitory potency. Among the linear peptides, peptide Ac-CIYKF(4-NO_2)Y (**2**), in which 4-nitrophenylalanine is located at position 5 from the *N*-terminal, demonstrated an approximately 750-fold higher inhibitory potency (IC_{50} = 0.53 μM) versus that of **1**. The incorporation of 4-nitrophenylalanine at positions 3 or 6 in

Scheme 3. Synthesis of Conformationally Constrained Peptides **30** and **31**[a]



[a] Reagents: (i) Fmoc solid-phase peptide synthesis; (ii) $SnCl_2 \cdot 2H_2O$/DMF, 24 h; (iii) Succinic anhydride, DMF, 6 h or suberic acid, HBTU, DMF, 6 h; (iv) $NH_2NH_2 \cdot H_2O$/DMF (2%), 40 min; (v) HBTU, HOBt, *N*,*N*-DIPEA, DMF, 48 h; (vi) TFA/thioanisole/H_2O/EDT/phenol (82.5:5:5:2.5:5).

peptides Ac-CIF(4-NO_2)KYY (**5**, IC_{50} = 1.5 μM) or Ac-CIYKYF(4-NO_2) (**6**, IC_{50} = 10.8 μM), respectively, showed lower inhibitory potencies when compared to that of peptide Ac-CIYKF(4-NO_2)Y (**2**), suggesting that the incorporation of 4-nitrophenylalanine at position 5 is optimal for generating the maximal inhibitory potency.

The peptide Ac-CIYKF(4-NH_2)Y (**4**), in which 4-aminophenylalanine is at position 5, exhibited a significantly reduced inhibitory potency (IC_{50} = 93 μM) when compared to that of peptide **2**. Similarly, peptides Ac-CIF(4-NH_2)KYY (**7**) and Ac-CIYKYF(4-NH_2) (**8**), where 4-aminophenylalanine is incorporated at positions 3 and 6, respectively, were not active when compared to the corresponding nitropeptides **5** and **6**. Furthermore, all of the inhibitory potency disappeared when the 4-nitrophenylalanine in **2** was replaced with the phenylalanine residue in peptide Ac-CIYKFY (**3**) (IC_{50} > 700 μM) (Table 1). These results indicate the importance of the nitro functional group for generating inhibitory potency.

Dinitropeptides Ac-CIF(4-NO_2)KF(4-NO_2)Y (**9**, IC_{50} = 1.4 μM) and Ac-CIYKF(4-NO_2)F(4-NO_2) (**10**, IC_{50} = 5.6 μM), in which the 4-nitrophenylalanine was incorporated at position 5 from the *N*-terminal, partially improved the inhibitory potency compared to that of corresponding mononitropeptides **5** and **6**, respectively; this may be because of the importance of the additive inhibitory effect of the 4-nitrophenylalanine at position 5. Similarly, dinitropeptide Ac-CIF(4-NO_2)KYF(4-NO_2) (**11**, IC_{50} = 3.4 μM) exhibited the inhibitory potency between mononitropeptides Ac-CIF(4-NO_2)KYY (**5**, IC_{50} = 1.5 μM) and Ac-CIYKYF(4-NO_2) (**6**, IC_{50} = 10.8 μM) (Table 1). All peptides containing the nitro group (**2**, **5**, **6**, **9**−**12**) exhibited higher inhibitory potencies than that of **1**, suggesting that the binding mode of the nitropeptides for interaction with c-Src is probably different from that of **1**.

The effect of substitution on the phenyl group at position 5 of Ac-CIYKYY with other substituents, such as phosphate, guanidine, or halogens, was investigated. The presence of negatively or positively charged groups, such as phosphate and guanidine, at the para position of the phenyl in phenylalanine at position 5 completely eliminated the inhibitory potency, as shown in **18** and **21**. However, compounds with substituted halogens (**13**−**15**) exhibited inhibitory potencies in the order of I > Cl > F, possibly because of the importance of the hydrophobic interaction with the kinase domain. For example, Ac-CIYKF(4-I)Y (**15**) exhibited (IC_{50} = 0.78 μM) an approximately 510-fold higher inhibitory potency than **1**.

Therefore, the presence of the 4-nitrophenylalanine or 4-iodophenylalanine at position 5 appears to be optimal for generating the maximal inhibitory potency. Several factors, such as the electronic effects of the functional groups, conformation of the peptides, and hydrogen bonding, may be involved in generating an optimal inhibition pattern by the peptides. The binding sites of peptides **2** and **15** containing *p*-NO_2 and *p*-iodo groups, respectively, appear to be different on the basis of the electronic effect of the functional groups at the para position. However, these functional groups may interact with other functional groups within the peptide causing a change in the conformation of the peptide. Molecular modeling studies of energetically minimized structures of **1**, **4**, **18**, **20**, and **21** showed

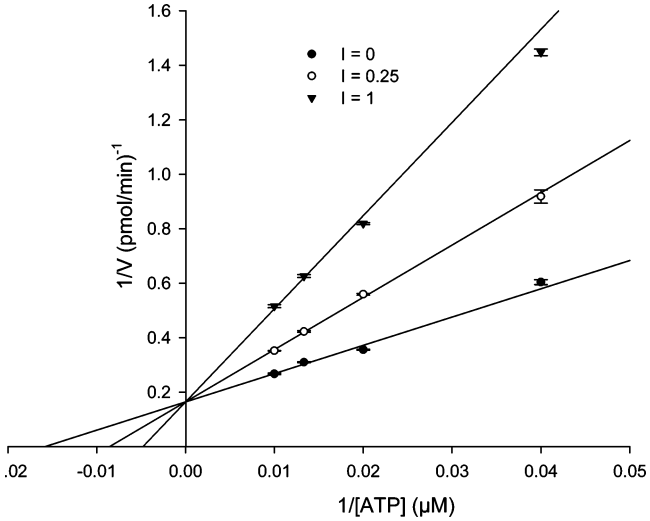


Figure 2. Pattern of inhibition of c-Src by compound **2**. Lineweaver−Burk plot of 1/*V* vs 1/ATP with varying concentrations of **2** shows partial competitive inhibition ($V_m = 6.1 \pm 0.2$ pmol/min; $K_m = 63.1 \pm 4.2$ μM; $K_i = 0.21 \pm 0.02$ μM; $R^2 = 0.996$; AIC value = −125.1).

Table 2. Inhibitory Potency Values for the Constrained Peptide Analogues of Ac-CIYKYY (**22**−**32**) against Active Src

compd	IC_{50} (μM)[a]	compd	IC_{50} (μM)[a]
22	6.4	**28**	>400
23	33.6	**29**	16
24	54.5	**30**	1.9
25	17	**31**	0.28
26	75	**32**	>400
27	134		

[a] Average of triplicate experiments; The upper limit of the standard error of the mean (SEM) was ±10%.

the presence of an intramolecular hydrogen bonding of the amino group of K4 with the functional group in the side chain of the amino acid residue at position 5 in these peptides. The hydrogen bonding was disrupted by the replacement of the hydroxyl group with nitro (**2**), halogens (**13**−**15**), cyano (**16**), or azide (**17**) groups, led to the change in the conformation of the peptide, and allowed the K4 side chain amino group to remain free. Therefore, modified conformations and/or bonding interactions with the active site may explain the enhanced inhibitory potency for peptides **2** and **15**. Additionally, c-Src is able to undergo conformational changes during the binding process to differentiate distinct topographies on the interacting side chains of the peptide. X-ray crystallographic studies of peptides **2** and **15** with active c-Src are required to correctly determine the binding modes of these compounds.

The mechanism of inhibition by **2** was studied using variable concentrations of ATP and the inhibitor. The lineweaver plot (Figure 2) showed that compound **2** follows a partial competitive inhibition pattern against ATP.

We have previously shown that the introduction of conformational constraints improves the binding affinities of pTyr-Glu-Glu-Ile (pYEEI), a conformationally flexible molecule, to the Src SH2 domain.[18] Conformationally constrained derivatives of $C_1I_2Y_3K_4Y_5Y_6$ (**22**−**32**) were synthesized (Figure 1) to determine whether the presence of the constrained ring in the peptide has any effect on improving the inhibitory potency of **1** (Table 2).

Head to tail cyclization (see compound **22**) improved the inhibitory potency more than other *N*-terminal or *C*-terminal cyclizations (see compounds **23**−**28**). Furthermore, introducing constraints using the side chain of the residue at position 5 in compounds **28** and **32** significantly reduced the inhibitory potency, suggesting that the side chain of amino acid residue at position 5 is probably involved in the interaction with the kinase domain. However, introducing conformational constraints using the side chain of the residue at position 3 (see compounds **25**, **30**, and **31**) significantly improved inhibitory potency. For example, conformationally constrained peptide **31** synthesized by linking side chains of the 4-aminophenylalanine at position 3 and K4 with suberic acid as a linker exhibited an approximately 1400-fold increase in inhibitory potency ($IC_{50} = 0.28$ μM) compared to that of linear peptide **1**. The size of the linker appears to be important because conformationally constrained peptide **30** with a shorter linker showed reduced inhibitory activity ($IC_{50} = 1.9$ μM) when compared to that of **31**. These results suggest that it is possible to convert a weak peptide inhibitor of active Src kinase into a potent inhibitor with reduced peptidic nature by introducing conformational constraints. The binding mode of conformationally constrained peptides appears to be different from those of the linear peptides. For example, compound **27** containing a 4-nitrophenylalaine at position 4 showed approximately 1.8-fold lower inhibitory potency than that of corresponding compound **26** containing a tyrosine residue at the same position. The introduction of conformational constraints at an appropriate position results in a higher inhibitory potency against active Src, possibly by reducing unfavorable entropic effects and/or creating novel bonding interactions.

Conclusions

In conclusion, this study revealed a number of important features regarding functional group modifications in the side chains of amino acids and designing conformationally constrained peptide mimics. The nature of the peptide plays an important role in generating inhibitory activity. Replacing the hydroxyl group of Y5 with the nitro group or halogens in linear peptides or introducing conformational constraints at Y3 significantly increased inhibitory potency. These peptides may offer novel templates for further design of Src inhibitors and in turn provide a conceptual approach toward the design of peptidomimetics. Taken together, these results suggest that further exploring the sequence diversity of functional groups of peptide side chains or introducing conformational constraints may lead to more potent peptide inhibitors.

Experimental Section

General. All reactions were carried out in Bio-Rad polypropylene columns by shaking and mixing using a Glass-Col small tube rotator in dry conditions or on a PS3 automated peptide synthesizer (Rainin Instrument Co., Inc.) at room temperature unless otherwise stated. In general, all peptides were synthesized by the solid-phase synthesis strategy employing *N*-(9-fluorenyl)methoxycarbonyl (Fmoc)-based chemistry and Fmoc-L-amino acid building blocks. 2-(1H−Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and NMM in *N*,*N*-dimethylformamide (DMF) were used as coupling and activating reagents, respectively. Wang resin, Fmoc-amino acid Wang resins, coupling reagents, and Fmoc-amino acid building blocks were purchased from Novabiochem. Other chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI). All of the six amino acids in Ac-$C_1I_2Y_3K_4Y_5Y_6$ (**1**) were numbered on the basis of their positions relative to the *N*-terminal cysteine residue (C1). Fmoc deprotection at each step was carried out using piperidine in DMF (20%). The crude peptide was precipitated by the addition of cold diethyl ether (25 mL, Et_2O) and purified by HPLC (Shimadzu LC-8A preparative liquid chromatograph; Shimadzu fraction collector 10A) on a

Phenomenex Prodigy 10 μm ODS reversed-phase column. The peptides were separated by eluting the crude peptide at 4.0 mL/min using a gradient of 0−100% acetonitrile (0.1% TFA) and water (0.1% TFA) over 85 min, and then, they were lyophilized. The purity of the final products (>95%) was confirmed by analytical HPLC. The analytical HPLC was performed on the Hitachi analytical HPLC system on C18 Shimadzu Premier 3 μm column (150 × 4.6 mm) using two different gradient systems and a flow rate of 1 mL/min with UV detection at 250 nm. The chemical structures of compounds were confirmed by a high-resolution PE Biosystems Mariner API time-of-flight mass spectrometer and/or ^{1}H NMR. Details of procedures and spectroscopic data of the key representative compounds are presented below. The percentage yields for the linear peptides (**1**−**22**) and the conformationally constrained peptides (**22**−**32**) were 70−80% and 20−30%, respectively.

Synthesis of Linear Peptides (1−21). All linear peptides were synthesized by the solid-phase synthesis strategy employing Fmoc-based chemistry as described in the general information section. The synthesis of nitropeptide **2** is explained here as a representative example. The syntheses of other linear peptides are given in the Supporting Information section.

Ac-Cys-Ile-Tyr-Lys-Phe(4-NO_2)-Tyr (2, Ac-CIYKF(4-NO_2)Y) (Scheme 1). The peptide sequence (**1**) was assembled on Fmoc-Tyr(tBu)-Wang resin (250.0 mg, 0.4 mmol/g) by the Fmoc solid-phase peptide synthesis strategy on a PS3 automated peptide synthesizer at room temperature using Fmoc-protected amino acids (Fmoc-Phe(4-NO_2)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ile-OH, and Fmoc-Cys(Trt)-OH). The *N*-terminal was acetylated using acetic anhydride (2.0 mL). The side chain deprotection of amino acids and cleavage of the synthesized peptide from the resin was carried out by shaking the resin with a mixture of TFA/anisole/H_2O/ethanedithiol (EDT) (95:2.0:2.0:1.0 v/v, 5.0 mL) for 1 h. The crude peptide was precipitated by the addition of cold diethyl ether (25 mL, Et_2O) and purified by preparative HPLC as described above. The chemical structure of **2** was determined by a high-resolution electrospray time-of-flight mass spectrometer. HR-MS (ESI-TOF) (*m*/*z*): $C_{44}H_{58}N_8O_{12}S$: calcd, 922.3895; found, 922.7380 $[M]^+$.

^{1}H NMR (DMSO-d_6, 400 MHz, δ ppm): 12.78 (1H, COO*H*), 9.26 (s, 1H, N*H*), 9.16 (s, 1H, N*H*), 8.40−7.82 (m, 6H, −CON*H*), 8.09 (d, 2H, F_5, *ortho*-H, J = 8.6 Hz), 7.50 (d, 2H, F_5, *meta*-H, J = 8.6 Hz), 7.01 (d, 2H, Y_6, *meta*-H, J = 8.4 Hz), 6.96 (d, 2H, 6.70, Y_3, *meta*-H, J = 8.4 Hz), 6.66 (d, 2H, Y_6, *ortho*-H, J = 8.4 Hz), 6.58 (d, 2H, Y_3, *ortho*-H, J = 8.4 Hz), 4.70−4.60 (m, 1H, F_5, NHC*H*CO), 4.50−4.30 (m, 3H, C_1, Y_3, Y_6, NHC*H*CO), 4.24−4.10 (m, 1H, K_4, NHC*H*CO), 4.10−4.00 (m, 1H, I_2, NHC*H*CO), 3.20−3.10 (m, 2H, C_1, CH_2SH), 2.92−2.54 (m, 8H, Y_3, F_5, Y_6, COCHCH_2; K_4, CH_2NH_2), 2.23−2.18 (m, 1H, I_2, COCHC*H*), 1.86 (s, 3H, COCH_3), 1.64−0.92 (m, 8H, I_2 CHCH_2CH_3; K_4, CH_2CH_2-CH_2), 0.74 (t, 3H, I_2, CH_2CH_3, J = 7.4 Hz), 0.67 (d, 3H, I_2, CHCH_3, J = 6.7 Hz).

Synthesis of Conformationally Constrained Peptides (22−32). All conformationally constrained peptides were synthesized by the solid-phase synthesis strategy employing Fmoc-based chemistry as described in the general information section. The synthesis of conformationally constrained peptide **30** is explained here as a representative example. The syntheses of other conformationally constrained peptides are given in Supporting Information.

Cys-Ile-cyclo[Phe-Lys]-Tyr-Tyr (Peptide 30): Linking the Side Chain of 4-Aminophenylalanine at Position 3 to the Side Chain of K4 Using a Succinic Acid Linker (Scheme 3). The assembly of the linear peptide was performed by using the Fmoc solid-phase peptide synthesis strategy and assembling Fmoc-Tyr-(tBu)-OH, Fmoc-Lys(Dde)-OH, Fmoc-Phe(4-NO_2)-OH, Fmoc-Ile-OH, and Fmoc-Cys(Trt)-OH on Fmoc-Tyr(tBu)-Wang resin (100.0 mg, 1.1 mmol/g) to yield **41**. After capping with acetic anhydride, the nitro group in **41** was reduced to the amino group with $SnCl_2 \cdot 2H_2O$ (2.70 g, 14.2 mmol) in DMF (6 mL) at room temperature by shaking for 24 h. The resin was collected by filtration and washed successively with DMF (50 mL), MeOH (50 mL), and DCM (50 mL) to afford **42**. The free amino group of the side chain of phenylalanine at position 3 was conjugated with the linker using the reaction with succinic anhydride (400 mg, 4.00 mmol) by shaking for 6 h. The resin was collected by filtration and washed successively with DMF (50 mL), MeOH (50 mL), and DCM (50 mL). The Dde-protecting group was selectively removed with the addition of hydrazine monohydrate in DMF (twice, 2%, 5 mL) and shaking for 20 min at room temperature. The cyclization of the peptide was carried out in the presence of a mixture of HBTU (379.3 mg, 1.0 mmol), HOBt (135.1 mg, 1.0 mmol), and DIPEA (99%, 300 μL, 1.7 mmol) in dry DMF (5 mL) by shaking for 48 h. The resin was collected and washed successively with DMF (50 mL), MeOH (50 mL), and DCM (50 mL). The cleavage was carried out with TFA/thioanisole/H_2O/EDT/phenol (82.5:5:5:2.5:5 v/v) as described in the general information. Compound **30** was purified by preparative HPLC, and the chemical structure was determined by a high-resolution electrospray time-of-flight mass spectrometer. HR-MS (ESI-TOF) (*m*/*z*): $C_{48}H_{62}N_8O_{12}S$: calcd, 974.4208; found, 976.5308 $[M^+ + 2H]$.

^{1}H NMR (DMSO-d_6, 400 MHz, δ ppm): 8.28−7.65 (m, 8H, −CON*H*), 7.19 (d, 2H, F_3, *ortho*-H, J = 7.5 Hz), 7.00−6.80 (m, 6H, F_3, *meta*-H; Y_5, *ortho*-H; Y_6, *ortho*-H), 6.66 (d, 2H, Y_5, *meta*-H, J = 8.4 Hz), 6.61 (d, 2H, Y_6, *meta*-H, J = 8.4 Hz), 5.80 (br s, 2H, phenolic O*H*), 4.52−4.07 (m, 6H, NHC*H*CO, C_1, I_2, F_3, K_4, Y_5, Y_6, NHC*H*CO), 2.97−2.61 (m, 14H, C_1, CH_2SH; COCH_2CH_2-CO; F_3, Y_5, Y_6, COCH$_2$CH_2; K_4 CH_2NH_2), 2.22−2.18 (m, 1H, I_2 COCHC*H*), 1.87 (s, 3H, COCH_3), 1.59−1.48 (m, 4H, K_4, CO-CHCH_2; K_4, CH_2CH$_2$NH_2), 1.30−1.10 (m, 4H, I_2, CHCH_2CH$_3$; K_4, CHCH$_2$CH_2), 0.74 (t, 3H, I_2, CH_2CH_3, J = 7.2 Hz), 0.69 (d, 3H, I_2 CHCH_3, J = 6.7 Hz).

Radioactive Kinase Assay. Active p60-c-Src was purchased from Upstate Cell Signaling (Cat. No. 14-117). An artificial substrate polyE_4Y (average MW: 35) was used for routine kinase activity. The inhibitory potency of the compounds was determined using a standard radiometric PTK activity assay. This assay contains polyE_4Y (1 mg/mL), as the phosphate-accepting substrate, [γ-^{32}P]-ATP, ATP (200 μM), and $MgCl_2$ (12 mM). After a reaction time of 30 min at 30 °C, 35 μL of the reaction mixture was removed and spotted onto a filter paper and placed into warm 5% trichloroacetic acid (TCA). The TCA stops the kinase reaction, precipitates the proteins and polyE4Y onto the filter paper, and washes the unreacted ATP and others away. After three TCA washes for 10 min each, the radioactivity remaining on the filter paper was determined by liquid scintillation counting. The assays were done in duplicate and repeated at least three times. Control reactions lacking polyE_4Y were included for each enzyme concentration to correct for any nonpolyE_4Y-specific phosphorylation. The percentage of inhibition was plotted as a function of the compound. The concentration and IC_{50} values (the concentration of a compound that caused 50% inhibition) were obtained from such a plot.

The steady-state kinetic assay with active Src was carried out using a radioactive assay to evaluate mechanisms of inhibition by peptide Ac-CIYKF(4-NO_2)Y (**2**) relative to natural substrate ATP. To determine the inhibitory mechanism with regard to ATP, the K_m and V_m values with ATP as the variable substrate was determined at various concentrations of **2**, while other components of the assay were at fixed concentrations using Lineweaver double reciprocal plots. The inhibitory mechanism was determined on the basis of the effect of the compound on the K_m and V_m values. The inhibitory constant (K_i) was determined by using the SigmaPlot 8.0 enzyme kinetics module.

Acknowledgment. Anil Kumar and Guofeng Ye contributed equally. We acknowledge the financial support from the American Cancer Society (RSG CDD-106966) and NIH (1 R01 CA111687 and 1 P20 RR16457).

Supporting Information Available: Synthetic procedures for all compounds, spectral data, HPLC profile for compounds, and molecular modeling. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hubbard, S. R.; Till, J. H. Protein tyrosine kinase structure and function. *Annu. Rev. Biochem.* **2000**, *69*, 373−398.

(2) (a) Biscardi, J. S.; Tice, D. A.; Parsons, S. J. c-Src, receptor tyrosine kinases, and human cancer. *Adv. Cancer Res.* **1999**, *76*, 61−119. (b) Yeatman, T. J. A renaissance for Src. *Nat. Rev. Cancer* **2004**, *4*, 470−480.

(3) Owens, D. W.; McLean, G. W.; Wyke, A. W.; Paraskeva, C.; Parkinson, E. K.; Frame, M. C.; Brunton, V. G. The catalytic activity of the Src family kinases is required to disrupt cadherin-dependent cell-cell contacts. *Mol. Biol. Cell* **2000**, *11*, 51−64.

(4) Susa, M.; Teti, A. Tyrosine kinase Src inhibitors: potential therapeutic application. *Drug News & Perspect.* **2000**, *13*, 169−175.

(5) Sawyer, T.; Boyce, B.; Dalgarno, D.; Luliucci, J. Src inhibitors: genomics to therapeutics. *Expert. Opin. Invest. Drugs* **2001**, *10*, 1327−1344.

(6) Warmuth, M.; Damoiseaux, R.; Liu, Y.; Fabbro, D.; Gray, N. Src family kinases: potential targets for the treatment of human cancer and leukemia. *Curr. Pharm. Des.* **2003**, *9*, 2043−2059.

(7) Parang, K.; Sun, G. Recent advances in the discovery of Src kinases inhibitors. *Expert Opin. Ther. Pat.* **2005**, *15*, 1183−1207.

(8) Parang, K.; Sun, G. Design strategies for protein kinases inhibitors. *Curr. Opin. Drug Discovery Dev.* **2004**, *7*, 630−638.

(9) Sicheri, F.; Moarefi, I.; Kuriyan, J. Crystal structure of the Src family tyrosine kinase Hck. *Nature* **1997**, *385*, 602−610.

(10) Toledo, L. M.; Lydon, N. B.; Elbaum, D. The structure-based design of ATP-site directed protein kinase inhibitors. *Curr. Med. Chem.* **1999**, *6*, 775−805.

(11) Schenone, S.; Bruno, O.; Ranise, A.; Bondavalli, F.; Brullo, C.; Fossa, P.; Mosti, L.; Menozzi, G.; Carraro, F.; Naldini, A.; Bernini, C.; Manetti, F.; Botta, M. New pyrazolo[3,4-*d*]pyrimidines endowed with A431 antiproliferative activity and inhibitory properties of Src phosphorylation. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2511−2517.

(12) Budde, R. J.; Obeyesekere, N. U.; Ke, S.; McMurray, J. S. Use of synthetic peptides and copolymers to study the substrate specificity and inhibition of the protein tyrosine kinase pp60c-src. *Biochim. Biophys. Acta* **1995**, *1248*, 50−56.

(13) Kamath, J. R.; Liu, R.; Enstrom, A. M.; Lou, Q.; Lam, K. S. Development and characterization of potent and specific peptide inhibitors of p60c-src protein tyrosine kinase using pseudosubstrate-based inhibitor design approach. *J. Pept. Res.* **2003**, *62*, 260−268.

(14) Lam, K. S.; Wu, J.; Lou, Q. Identification and characterization of a novel synthetic peptide substrate specific for Src-family protein tyrosine kinases. *Int. J. Pept. Protein Res.* **1995**, *45*, 587−592.

(15) Alfaro-Lopez, J.; Yuan, W.; Phan, B. C.; Kamath, J.; Lou, Q.; Lam, K. S.; Hruby, V. J. Discovery of a novel series of potent and selective substrate-based inhibitors of $p60^{c-Src}$ protein tyrosine kinase: Conformational and topographical constraints in peptide design. *J. Med. Chem.* **1998**, *41*, 2252−2260.

(16) Nam, N. H.; Pitts, R.; Sun, G.; Sardari, S.; Tiemo, A.; Xie, M.; Yan, B.; Parang, K. Design of tetrapeptide ligands as inhibitors of the Src SH2 domain. *Bioorg. Med. Chem.* **2004**, *12*, 779−787.

(17) Nam, N. H.; Ye, G.; Sun, G.; Parang, K. Conformationally constrained peptide analogues of pTyr-Glu-Glu-Ile as inhibitors of the Src SH2 domain binding. *J. Med. Chem.* **2004**, *47*, 3131−3141.

(18) Nam, N. H.; Lee, S.; Ye, G.; Sun, G.; Parang, K. ATP-phosphopeptide conjugates as inhibitors of Src tyrosine kinase. *Bioorg. Med. Chem.* **2004**, *12*, 5753−5766.

(19) Cabrele, C.; Langer, M.; Beck-Sickinger, A. G. Amino acid side chain attachment approach and its application to the synthesis of tyrosine-containing cyclic peptides. *J. Org. Chem.* **1999**, *64*, 4353−4361.

JM060334K