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

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Abstract

A series of peptide analogs of Ac-CIYKYY (**1**) were synthesized by functional group modifications in peptide side chains or introducing conformational constraints, to improve the inhibitory potency against active Src kinase. Ac-CIYKF(4-NO₂)Y (**2**, IC₅₀ = 0.53 μM) and the conformationally constrained peptide **31** (IC₅₀ = 0.28 μM) exhibited 750- and 1400-fold higher inhibitory activities, respectively, versus **1** (IC₅₀ = 400 μM). Compound **2** exhibited a partial competitive inhibition pattern against ATP.

Keywords

c-Src; peptides; conformationally constrained peptides; structure-activity relationships

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 non-receptor 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 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 terminus: 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 attention has been on designing the 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 protein substrate (substrate binding site inhibitors).^{12–14} Lam et al.^{14,15} identified YIYGSKF (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 YIYGSK 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 the c-Src inhibition by CIYKYY depends on the substrate used in the kinase assay. PolyE₄Y 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**, $\text{IC}_{50} = 400 \mu\text{M}$) was a weak inhibitor of polyE₄Y phosphorylation by active c-Src.

We describe the synthesis and inhibitory activities of Ac-CIYKYY peptide analogs 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 polyE₄Y 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 analogs 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).

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. Due to 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 head to tail, C-terminal to a side chain, N-terminal to a side chain, and a side chain to a side chain of the amino acids (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 polyE₄Y as the substrate.

Results and Discussion

Chemistry

All linear peptides were synthesized on solid phase employing *N*-(9-fluorenyl) methoxycarbonyl (Fmoc) based 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} Peptides were N-terminally acetylated with acetic anhydride. As representative examples 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 4-nitrophenylalanine residue at position 5 from N-terminal underwent 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/H₂O/ethanedithiol (EDT) at room temperature to afford **2**, **4**, and **20**, respectively.

Scheme 2 shows the synthesis of the conformationally constrained peptide **22** by head to tail cyclization. Polymer-bound Fmoc protected methyl ester tyrosine (**38**) was synthesized from Wang resin (**37**) using Mitsunobu reaction.¹⁹ *N*-(9-Fluorenylmethoxycarbonyl)-tyrosine methyl ester (Fmoc-Tyr-OCH₃) and triphenylphosphine (PPh₃) 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 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, respectively] 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 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/H₂O/EDT/phenol and HPLC purification afforded the conformationally constrained peptide **22**.

The synthesis of the conformational constrained peptides **30** and **31** is shown in Scheme 3 as representative examples of a side chain-side chain cyclization. The assembly of the 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-NO₂)-OH, Fmoc-Ile-OH, and Fmoc-Cys(Trt)-OH, respectively, to give **41**. The reduction of nitro group in **41** to amino group was carried out in the presence of SnCl₂·2H₂O 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 chain of lysine with 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/H₂O/EDT/phenol and HPLC purification afforded the conformationally constrained peptides **30** and **31**.

Structure-Activity Relationships

Table 1 shows the inhibitory activities of the linear peptide analogs of Ac-C₁I₂Y₃K₄Y₅Y₆ (**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-NO₂)Y (**2**), in which 4-nitrophenylalanine is located at position 5 from N-terminal, demonstrated approximately 750-fold higher inhibitory potency (IC₅₀ = 0.53 μM) versus **1**. The incorporation of 4-nitrophenylalanine at positions 3 or 6 in peptides Ac-CIF(4-NO₂)KYY (**5**, IC₅₀ = 1.5 μM) or Ac-CIYKYF(4-NO₂) (**6**, IC₅₀ = 10.8 μM), respectively, showed lower inhibitory potency when compared to peptide Ac-CIYKF(4-NO₂)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-NH₂)Y (**4**), in which the 4-aminophenylalanine is at position 5, exhibited a significantly reduced inhibitory potency (IC₅₀ = 93 μM) when compared to the peptide **2**. Similarly, the peptides Ac-CIF(4-NH₂)KYY (**7**), and Ac-CIYKYF(4-NH₂) (**8**), in which the 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**) (IC₅₀ > 700 μM) (Table 1). These results indicate the importance of the nitro functional group for generating inhibitory potency.

Dinitropeptides, Ac-CIF(4-NO₂)KF(4-NO₂)Y (**9**, IC₅₀ = 1.4 μM) and Ac-CIYKF(4-NO₂)F(4-NO₂) (**10**, IC₅₀ = 5.6 μM), in which the 4-nitrophenylalanine was incorporated at position 5 from the N-terminal, partially improved the inhibitory potency compared to the corresponding mononitropeptides, **5** and **6**, respectively; this may be due to the importance of the additive inhibitory effect of the 4-nitrophenylalanine at position 5. Similarly, the dinitropeptide Ac-CIF(4-NO₂)KYF(4-NO₂) (**11**, IC₅₀ = 3.4 μM) exhibited the inhibitory potency between mononitropeptides Ac-CIF(4-NO₂)KYY (**5**, IC₅₀ = 1.5 μM) and Ac-CIYKYF(4-NO₂) (**6**, IC₅₀ = 10.8 μM) (Table 1). All peptides containing nitro group (**2**, **5**, **6**, **9–12**) exhibited higher

inhibitory potency than **1**, suggesting that the binding mode of the nitropeptides for interaction with c-Src is probably different from **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 *para* position of the phenyl in phenylalanine at position 5 completely eliminated the inhibitory potency, as shown in **18** and **21**. On the other hand, compounds with substituted halogens (**13–15**) exhibited inhibitory potency in the order of $I > Cl > F$, possibly due to the importance of the hydrophobic interaction with the kinase domain. For example, Ac-CIYKF(4-I)Y (**15**) exhibited ($IC_{50} = 0.78 \mu M$) 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 electronic effects of the functional groups, conformation of 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*-NO₂ and *p*-iodo groups, respectively, appear to be different based on electronic effect of the functional groups at *para* position. On the other hand, these functional groups may interact with other functional groups within the peptide causing the change of the conformation of the peptide. Molecular modeling studies of energetically minimized structures of **1**, **4**, **18**, **20**, and **21** showed the presence of an intramolecular hydrogen bonding of the amino group of the K4 with the functional group in the side chain of amino acid residue at position 5 in these peptides. The hydrogen bonding was disrupted by replacement of hydroxyl group with nitro (**2**), halogens (**13–15**), cyano (**16**), or azide (**17**), led to 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 the 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 C₁I₂Y₃K₄Y₅Y₆ (**22–32**) were synthesized (Figure 1) to determine whether the presence of the constrained ring in the peptide has any effect in 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** reduced the inhibitory potency significantly, suggesting that the side chain of amino acid residue at position 5 is probably involved in interaction with the kinase domain. On the other hand, introducing conformationally constraints using the side chain of the residue at position 3 (see compounds **25**, **30**, **31**) significantly improved the inhibitory potency. For example the 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 ($IC_{50} = 0.28 \mu M$) compared to the linear peptide **1**. The size of linker appears to be important since the conformationally constrained peptide **30** with shorter linker showed reduced inhibitory activity ($IC_{50} = 1.9 \mu M$)

when compared to **31**. These results suggest that it is possible to convert a weak peptide inhibitor of active Src kinase to potent inhibitors with reduced peptidic nature by introducing conformational constraints. The binding mode of conformationally constrained peptides appears to be different from the linear peptides. For example compound **27** containing a 4-nitrophenylalanine at position 4 showed approximately 1.8 fold lower inhibitory potency than that of the 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 the functional group modifications in the side chains of amino acids or designing of conformationally constrained peptide mimics. The nature of the peptide plays an important role in generating inhibitory activity. Replacing the hydroxyl group of Y5 with nitro group or halogens in linear peptides, or introducing conformationally constraints at Y3 increased inhibitory potency significantly. These peptides may offer novel templates for further design of the Src inhibitors and in turn provide a conceptual approach toward the design of peptidomimetics. Taken together, these results suggest that exploring further 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 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 Company (Milwaukee, WI, USA). All the six amino acids in Ac-C₁I₂Y₃K₄Y₅Y₆ (**1**) were numbered based on 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 addition of cold diethyl ether (25 mL, Et₂O) and purified by HPLC (Shimadzu LC-8A preparative liquid chromatograph; Shimadzu fraction collector 10A) on a Phenomenex® Prodigy 10 µm ODS reversed-phase column. 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 were lyophilized. The purity of 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 ¹H 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-NO₂)-Tyr (**2**, Ac-CIYKF(4-NO₂)Y) (Scheme 1)

The peptide sequence (**1**) was assembled on Fmoc-Tyr(tBu)-Wang resin (250.0 mg, 0.4 mmol/g) by Fmoc solid-phase peptide synthesis strategy on a PS3 automated peptide synthesizer at room temperature using Fmoc protected amino acids [Fmoc-Phe(4-NO₂)-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 of the resin with a mixture of TFA:anisole:H₂O:ethanedithiol (EDT) (95:2.0:2.0:1.0 v/v, 5.0 mL) for 1 h. The crude peptide was precipitated by addition of cold diethyl ether (25 mL, Et₂O) 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): C₄₄H₅₈N₈O₁₂S calcd. 922.3895; found 922.7380 [M]⁺.

¹H NMR (DMSO-d₆, 400 MHz, δ ppm): 12.78 (1H, COOH), 9.26 (s, 1H, NH), 9.16 (s, 1H, NH), 8.40–7.82 (m, 6H, -CONH), 8.09 (d, 2H, F₅, *ortho*-H, *J* = 8.6 Hz), 7.50 (d, 2H, F₅, *meta*-H, *J* = 8.6 Hz), 7.01 (d, 2H, Y₆, *meta*-H, *J* = 8.4 Hz), 6.96 (d, 2H, 6.70, Y₃, *meta*-H, *J* = 8.4 Hz), 6.66 (d, 2H, Y₆, *ortho*-H, *J* = 8.4 Hz), 6.58 (d, 2H, Y₃, *ortho*-H, *J* = 8.4 Hz), 4.70–4.60 (m, 1H, F₅, NHCHCO), 4.50–4.30 (m, 3H, C₁, Y₃, Y₆, NHCHCO), 4.24–4.10 (m, 1H, K₄, NHCHCO), 4.10–4.00 (m, 1H, I₂, NHCHCO), 3.20–3.10 (m, 2H, C₁, CH₂SH), 2.92–2.54 (m, 8H, Y₃, F₅, Y₆, COCHCH₂; K₄, CH₂NH₂), 2.23–2.18 (m, 1H, I₂, COCHCH), 1.86 (s, 3H, COCH₃), 1.64–0.92 (m, 8H, I₂ CHCH₂CH₃; K₄, CH₂CH₂CH₂), 0.74 (t, 3H, I₂, CH₂CH₃, *J* = 7.4 Hz), 0.67 (d, 3H, I₂, CHCH₃, *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 the supporting information section.

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 Fmoc solid-phase peptide synthesis strategy and assembling Fmoc-Tyr(tBu)-OH, Fmoc-Lys(Dde)-OH, Fmoc-Phe(4-NO₂)-OH, Fmoc-Ile-OH and Fmoc-Cys(Trt)-OH, respectively, 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 amino group with SnCl₂·2H₂O (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 by 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 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 μ L, 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/H₂O/EDT/phenol (82.5:5:5:2.5:5 v/v) as described in the general information. The 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): C₄₈H₆₂N₈O₁₂S calcd. 974.4208, found 976.5308 [M⁺ + 2H].

¹H NMR (DMSO-d₆, 400 MHz, δ ppm): 8.28-7.65 (m, 8H, -CONH), 7.19 (d, 2H, F₃, *Ortho*-H, *J* = 7.5 Hz), 7.00-6.80 (m, 6H, F₃, *meta*-H; Y₅, *Ortho*-H; Y₆, *Ortho*-H), 6.66 (d, 2H, Y₅, *meta*-H, *J* = 8.4 Hz), 6.61 (d, 2H, Y₆, *meta*-H, *J* = 8.4 Hz), 5.80 (br s, 2H, phenolic OH), 4.52-4.07 (m, 6H, NHCHCO, C₁, I₂, F₃, K₄, Y₅, Y₆, NHCHCO), 2.97-2.61 (m, 14H, C₁, CH₂SH; COCH₂CH₂CO; F₃, Y₅, Y₆, COCH₂CH₂; K₄ CH₂NH₂), 2.22-2.18 (m, 1H, I₂ COCHCH), 1.87 (s, 3H, COCH₃), 1.59-1.48 (m, 4H, K₄, COCHCH₂; K₄, CH₂CH₂NH₂), 1.30-1.10 (m, 4H, I₂, CHCH₂CH₃; K₄, CHCH₂CH₂), 0.74 (t, 3H, I₂, CH₂CH₃, *J* = 7.2 Hz), 0.69 (d, 3H, I₂ CHCH₃, *J* = 6.7 Hz).

Radioactive kinase assay

Active p60-c-Src was purchased from Upstate Cell Signaling (Cat. No. 14–117). Artificial substrate polyE₄Y (average MW: 35kD) was used for routine kinase activity. The inhibitory potency of compounds was determined using a standard radiometric PTK activity assay. This assay contains poly(E₄Y) (1 mg/mL) as the phosphate accepting substrate, [γ -³²P]-ATP, ATP (200 μ M) and MgCl₂ (12 mM). After a reaction time of 30 min at 30 °C, 35 μ L 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 poly-E₄Y 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 duplicates and repeated at least three times. Control reactions lacking polyE₄Y were included for each enzyme concentration to correct for any nonpolyE₄Y specific phosphorylation. Percentage of inhibition was plotted as a function of the compound. Concentration and the IC₅₀ value (the concentration of a compound that caused 50% inhibition) were obtained from such a plot.

Steady-state kinetic assay with active Src was carried out using a radioactive assay to evaluate mechanisms of inhibition by peptide Ac-CIYKF(4-NO₂)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 based on the effect of the compound on the K_m and V_m values. Inhibitory constant (K_i) was determined by using SigmaPlot 8.0 Enzyme Kinetics Module.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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

Figure 2. Pattern of inhibition of c-Src by compound **2**; Lineweaver–Burk plot of $1/V$ versus $1/ATP$ with varying concentration 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).

Scheme 1.

The 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/H₂O/anisole/EDT (95:2.0:2.0:1.0 v/v); (viii) (a) Fmoc-Phe(4-NO₂)-OH, HBTU, NMM, DMF, (b) Piperidine, DMF; (ix) SnCl₂·2H₂O/DMF; (x) Methylsulfonyl chloride.

Scheme 2.

The synthesis of conformationally constrained peptide **22**.^a

^a Reagents: (i) Fmoc-Tyr-OCH₃, PPh₃, 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/H₂O/EDT/phenol (82.5:5:5:2.5:5 v/v).

Scheme 3.

The synthesis of conformationally constrained peptides **30** and **31**.^a

^a Reagents: (i) Fmoc solid-phase peptide synthesis; (ii) $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}/\text{DMF}$, 24 h; (iii) Succinic anhydride, DMF, 6 h or suberic acid, HBTU, DMF, 6 h; (iv) $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}/\text{DMF}$ (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).

Table 1

The chemical structures and inhibitory potency values (IC_{50}) for modified linear peptide analogs (**1–21**) against active Src kinase.

Compound	$IC_{50}(\mu M)^a$	Compound	$IC_{50}(\mu M)$
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 ₂ CH 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 phosphorylation of polyE4Y by active c-Src (average of triplicate experiments). The upper limit of Standard Error of the Mean (SEM) was $\pm 10\%$.

Table 2Inhibitory potency values for the constrained peptide analogs of Ac-CIYKYY (**22–32**) against active Src.

Compound	IC ₅₀ (μM) ^a	Compound	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 Standard Error of the Mean (SEM) was ± 10%.