Identification of Efficient Pentapeptide Substrates for the Tyrosine Kinase pp60$^{c\text{-}src}$

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Received January 17, 1995[⊗]

The development of inhibitors of protein tyrosine kinases (PTKs) is a promising approach to obtaining new therapeutic agents for a variety of diseases, particularly cancer. However, the discovery of peptide-based inhibitors has been hindered by the lack of small peptide substrate sequences (i.e. five residues or less) with which a variety of inhibitor designs could be readily evaluated by replacing the Tyr with natural and unnatural amino acids. These prototypical small peptide inhibitors could then form the basis for designing analogous conformationally constrained, peptide-mimetic or non-peptide inhibitors with improved therapeutic potential. In this study we have identified the best known small peptide substrate for the PTK pp60$^{c\text{-}src}$, which is the parent of the src family of nonreceptor PTKs. This pentapeptide substrate, Ac-Ile-Tyr-Gly-Glu-Phe-NH_2, has a K_m of 368 μM and V_{max} of 1.02 μmol/min/mg when tested utilizing the assay methodology of Budde et al. (*Anal. Biochem.* **1992**, *200*, 347–351) after a series of modifications were made to more closely simulate the conditions inside a typical mammalian cell. This substrate was designed from information obtained by Songyang et al. (*Nature* **1995**, *373*, 536–539) with a 2.5 billion member combinatorial library of peptide substrates for pp60$^{c\text{-}src}$. A second pentapeptide substrate, Ac-Glu-Asp-Ala-Ile-Tyr-NH_2, with a weaker binding affinity (K_m = 880 μM) but improved V_{max} (1.86 μmol/min/mg), was also identified. This peptide was designed from the pp60$^{c\text{-}src}$ autophosphorylation sequence and information obtained by Songyang et al. (*Ibid.*) and Till et al. (*J. Biol. Chem.* **1994**, *269*, 7423–7428) with combinatorial libraries of peptide substrates. These new substrates provide sufficient binding affinities and rates of phosphorylation to be utilized for evaluating the relative effectiveness of various reversible and mechanism-based irreversible inhibitor designs for pp60$^{c\text{-}src}$ while appended to easily prepared small peptides.

Introduction

The transfer of the γ-phosphate from ATP to the hydroxyl-containing amino acids serine and threonine within proteins is catalyzed in the cell by protein serine kinases and analogously to tyrosine by protein tyrosine kinases (PTKs). This phosphorylation reaction enables the protein serine kinases[1] and the PTKs[2] to control various cell functions including signal transduction, differentiation, and proliferation through resulting effects on the structure and function of the protein substrates.[3] The overactivation or mutation of proto-oncogenes (thereby resulting in oncogenes) encoding protein kinases can result in constitutively active signalling pathways causing cell transformation.[4] Since it appears that inactivation of a single oncogene-encoded protein may be sufficient to reverse cell transformation,[5] the development of inhibitors of these protein kinases offers a new mechanism-based approach to the treatment of cancer. The potential utility of PTK inhibitors as anticancer agents and the status of their development has been extensively reviewed.[6]

The most actively pursued classes of PTK inhibitors thus far have been natural products and analogs, many of which compete with ATP for binding to the PTK.[6] For those inhibitors competing with ATP, high inhibitor binding affinity and specificity are required in order to compete with the intracellular millimolar levels of ATP and yet not inhibit the other ATP-utilizing enzymes present in cells. An alternate, and preferred,[6a,b] approach is to utilize peptide-based inhibitors which compete only with the protein and peptide PTK substrates. If short peptide inhibitors of this type became available, then they could serve as lead compounds for the design of non-peptide analogs better suited to drug developement. This peptide-based inhibitor approach has been relatively unexplored[6] partly because of the lack of good short peptide substrates (i.e. five residues or less) wherein the tyrosine can be replaced with nonphosphorylatable residues to generate inhibitors. In general, the previously investigated peptide substrates for PTKs were poor substrates (K_m's in the mM range and V_{max}'s in the nmol/min/mg range) and a strong preference for specific primary sequences was not observed.[6b,7] This situation is in contrast to that for the protein serine kinases wherein peptide substrates have been identified with binding affinities and maximal rates of phosphorylation 3 orders-of-magnitude better (K_m's in the μM range and V_{max}'s in the μmol/min/mg range) than those for the known PTK peptide substrates.[6b,7a,b]

In this report we present our work leading to the pentapeptide substrates Ac-Ile-Tyr-Gly-Glu-Phe-NH_2

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[⊗] Abstract published in *Advance ACS Abstracts*, October 1, 1995.

0022-2623/95/1838-4276$09.00/0

Table 1. Comparison of pp60$^{c\text{-}src}$ Peptide Substrates

pp60$^{c\text{-}src}$ substrates	CPM × 1000		K_m[b] (μM)	V_{max}[b] (μmol/min/mg)
	3 mM peptide[a]	1 mM peptide[a]		
		Series A		
1 RRLIEDAEYAARG (RR-src)	80.3	NT	NT	NT
2 Ac-EDAEYA-NH_2	28.5	NT	NT	NT
3 Ac-DAEYA-NH_2	23.4	NT	NT	NT
4 Ac-EDAIY-NH_2	NT	402.6	880(±350)	1.86(±0.32)
		Series B		
5 Ac-GEGTYGV-NH_2	119.6	NT	NT	NT
6 Ac-EGTYG-NH_2	89.5	63.5	NT	NT
		Series C		
7 AEEEIYGEFEAKKKK	NT	701.6	100(±18)	3.06(±0.31)
8 Ac-IYGEF-NH_2	NT	497.1	368(±124)	1.02(±0.14)
		Series D		
9 Ac-LPYA-$NHCH_3$	NT	1.6	NT	NT

[a] The counts per minute (cpm) are the average of triplicate experiments and indicate the amount of ^{32}P-labeled phosphopeptide product obtained in the pp60$^{c\text{-}src}$ phosphorylation assay as determined by Cerenkov counting after separation on a PEI–cellulose column and subtraction of the blank (no peptide). All the peptides were phosphorylated under identical assay conditions and at a 3 or a 1 mM concentration as listed. [b] K_m and V_{max} determinations utilized the experimental designs of Duggleby[31] and Cornish-Bowden direct linear plots.[30] Details are included in the experimental section. The standard deviation (in parentheses) is the square root of the variance and is given by SD = $[1/n\Sigma(x_i - x)^2]^{1/2}$, where SD = standard deviation, n = number of observations, and $(x_i - x)$ is the deviation of x_i from the mean x.

and Ac-Glu-Asp-Ala-Ile-Tyr-NH_2 for the PTK pp60$^{c\text{-}src}$ with sufficient binding affinities and maximal rates of phosphorylation (K_m = 368 and 880 μM with V_{max} = 1.02 and 1.86 μmol/min/mg, respectively) to be utilized as lead sequences for the development of short peptide inhibitors of pp60$^{c\text{-}src}$ and perhaps other members of the src family of PTKs.

Results and Discussion

Choice of a Representative PTK. The PTKs can be classified into two categories, the membrane receptor PTKs (e.g. the epidermal growth factor receptor PTK) and the nonreceptor PTKs (e.g. the src family of proto-oncogene products).[8] There are at least eight members of the src family of non-receptor PTK's, namely the src, yes, fgr, lyn, lck, hck, fyn, and blk proto-oncogene products, with pp60$^{c\text{-}src}$ being the prototype PTK of the family wherein the ca. 300 amino acid catalytic domains are highly conserved.[8] The overexpression or hyper-activation of pp60$^{c\text{-}src}$ has been reported in a human cancers of the colon,[9] breast,[10] lung,[11] bladder,[12] and skin,[13] as well as in gastric cancer,[14] hairy cell leukemia,[15] and neuroblastoma.[16] Since pp60$^{c\text{-}src}$ is the parent of the src family of nonreceptor PTKs and is commercially available in soluble form (facilitating the interpretation of substrate and inhibitor kinetic constants) and inhibitors of pp60$^{c\text{-}src}$ may be effective treatments for a variety of cancers, we have chosen this representative PTK for our initial peptide substrate and inhibitor studies.

Previous pp60$^{c\text{-}src}$ Peptide Substrate Results and Design of New Peptide Substrates. Early studies with peptide substrates for pp60$^{c\text{-}src}$, or the analogous enzyme pp60$^{v\text{-}src}$ derived from the Rous sarcoma virus, have focused largely on analogs of the pp60$^{c/v\text{-}src}$ autophosphorylation sequence and angiotensin I.[6b,7] These sequences resulted in relatively poor pp60$^{v\text{-}src}$ substrates as exemplified by the autophosphorylation sequence peptide EDNEYTARQG with a K_m of 6250 μM and a V_{max} of 0.0005 μmol/min/mg. The commercially available "RR-src" peptide 1 (Table 1) was originally designed by Casnellie et al.[17] from the pp60$^{c/v\text{-}src}$ autophosphorylation sequence by substituting alanines for the P+1 Thr and the P−2 Asn (P refers to the position of the phosphorylatable Tyr, where residues N-terminal are numbered negatively and C-terminal positively, beginning with the P position as 0). In addition, arginines were added to the N-terminus, and the glutamine was removed from the C-terminus, giving a basic PTK peptide substrate which can be isolated, after phosphorylation with [γ-^{32}P]ATP as the second substrate, by binding to phosphocellulose cation exchange paper, and the extent of phosphorylation can be measured by liquid scintillation counting of the paper.

Peptide **1** has since become the most commonly used substrate for a wide variety of PTK assays and therefore we included it in our series of substrates based upon the pp60$^{c\text{-}src}$ autophosphorylation sequence (series A, Table 1) as a standard against which our shorter peptide substrates can be compared. We investigated the effect of shortening the RR-src (**1**) peptide to six (**2**) and five (**3**) residues in length with *N*-acetyl and *C*-amide capping to mimic a longer peptide (Table 1). The truncation to **2** and **3** was done so as to maintain at least one residue C-terminal to the Tyr (on the assumption that the presence of a residue adjacent to the reaction site would be important) and also to preserve as many N-terminal acidic residues as possible (because they are generally thought to be important for substrate recognition by the PTKs[7b]).

Till et al.[19] prepared a library of 19 peptides, the sequence of which RRLIEDAXYAARG (wherein X is the variable P−1 residue) was derived from peptide 1. The optimal residue for the P−1 position was identified as Ile when analyzed with the nonreceptor tyrosine kinase v-Abl giving a K_m of 670 μM and a V_{max} of 0.073 μmol/min/mg. On the basis of this result we designed peptide **4** as the last member of series A wherein an Ile is analogously positioned at the P−1 site, both acidic N-terminal residues are present, and the length is only five residues. This combination of design criteria necessitated that the Tyr be positioned as the C-terminal residue.

Previous studies with peptide fragments of the protein substrate p34^{cdc2} produced a 15 amino acid pp60$^{c\text{-}src}$ substrate, cdc2(6−20)NH_2 (KVEKIGEGTYGVVYK-NH_2),

with a K_m of 102 μM,[18] which is the parent sequence of our series B substrates. The peptide **6** (Table 1) was designed from this sequence so as to include the P−3 Glu, be five amino acids in length, and not leave the Tyr as the C-terminal residue. Peptide **5** was designed to analyze the effect of extending **6** by one residue at each terminus.

Songyang et al[20] have developed a new combinatorial peptide library technique for determining the best substrates for protein kinases and have applied this technique to identifying optimal substrates for PTKs.[21] On the basis of the optimal substrate information obtained for pp60$^{c\text{-}src}$ from a library of more than 2.5 billion peptides with the sequence MAXXXXYXXXX-AKKK, the peptide AEEEIYGEFEAKKKK (peptide **7**, Table 1) was prepared and found to have a K_m of 33 μM and V_{max} of 0.8 μmol/min/mg[21] and is currently the best known peptide substrate for this PTK. Sequencing of the phosphorylated peptides from this library showed that the selectivity for the individual amino acids present within this optimal sequence was highest for the P−1 Ile and the P+3 Phe. We therefore designed pentapeptide **8** (Table 1) from this optimal sequence to span the P−1 to P+3 positions with end capping. Since the assay conditions and technology we utilized in our current investigation are different from those used by Songyang et al[21] in determining the reported K_m and V_{max} for **7**, we included the parent peptide **7** in our series C for direct comparison to our shorter peptides.

We were also attracted to the report of Tinker et al.[22] wherein the C-capped tetrapeptide LPYA-NHCH$_3$ was analyzed with the PTK present in LSTRA membrane flakes and reported to have a K_m of 190 μM and V_{max} of 1600 μmol/min/mg (98 mmol/h/mg). The most abundant PTK present in LSTRA membranes is p56^{lck}, a member of the src family of PTK's. However, a subsequent study carried out with cloned and purified p56^{lck} gave a K_m of 1700 μM and V_{max} of 0.0014 μmol/min/mg for this peptide and a K_m of 700 μM and V_{max} of 0.0019 μmol/min/mg for the capped analog Ac-LPYA-NHCH$_3$.[23] Since this is the shortest PTK substrate with a reported submillimolar K_m, we also included the capped analog **9** (series D, Table 1) in our current investigation with pp60$^{c\text{-}src}$.

Assay Technology Used for Testing the pp60$^{c\text{-}src}$ Peptide Substrates. Of the nine peptide substrates listed in Table 1 only the parent peptides 1 and **7** from previous studies contain multiple basic residues, allowing them to be tested utilizing the standard phosphocellulose ion exchange paper assay technology. We had intentionally not included additional basic residues in our new peptide substrates because we did not want to introduce ambiguities involving potential contributions of these additional residues into the interpretation of our kinetic results. Also our goal was to identify a good substrate containing five or less residues. The absence of mutliple basic residues in the peptides we had designed required that we utilize an alternate assay technology.

After considering the various PTK assay methods which had been published, we chose the method of Budde et al[24] to further develop and apply to testing of our peptide substrates. As in the phosphocellulose filter paper binding assay, [γ-^{32}P]ATP is used as the second substrate to generate γ-^{32}P-labeled phosphorylated peptide product, which is then quantitated by liquid scintillation counting. Whereas the phosphocellulose filter paper binding assay method involves selective binding of the cationic peptide product/substrate to the paper and removal of the other radioactive materials by washing of the paper, the method of Budde et al. utilizes molybdate chelation/precipitation of ^{32}P-labeled inorganic phosphate followed by polyethylenimine (PEI)−cellulose anion exchange chromatography to separate the ^{32}P-labeled phosphopeptide from [γ-^{32}P]ATP and other radioactive materials. The Budde et al. method has been shown[24] to be applicable to acidic and basic peptides and therefore was expected to be suitable for testing the peptides listed in Table 1.

Since the goal of our work was to obtain a small peptide pp60$^{c\text{-}src}$ substrate as a lead for inhibitor design with potential therapeutic applications, we put the additional requirement on our study that it be done under conditions that approximate the overall physical chemical conditions existing inside cells. Therefore, we made the following series of changes from the assay conditions used by Budde et al. to those present inside a typical mammalian cell:[25] (1) pH changed from 8.0 to 7.1, (2) free Mg^{2+} (i.e. beyond that bound to ATP/ADP, etc.) changed from ca. 6.0 to 0.5 mM, (3) ADP was added at 10% of the concentration of ATP used (reflecting the ratio present in cells; this modification was made mainly in anticipation of our subsequent inhibitor studies wherein binding to the enzyme:ADP complex may be important). We also switched the buffer from HEPES to MOPS (does not significantly chelate Mg^{2+} [26]) in order to be consistent with our ongoing assay work with protein serine kinases[26] and to provide greater buffering capacity at the lower pH, we added BSA (to reduce peptide and protein binding to the assay vessels), and we raised the specific activity of the [γ-^{32}P]ATP from 0.36 to 1.35 mCi/μM to obtain a stronger phosphopeptide product signal.

We evaluated our final assay conditions with the computer program "Bound and Determined"[27] (BAD, calculates equilibrium concentrations of individual species involved in multiple interacting equilibria) after updating the stability constants to those recommended most recently.[28] The free Mg^{2+} concentration was calculated to be 0.44 mM (we have found good agreement between our calculated and experimentally determined free Mg^{2+} levels in other protein kinase assays[26]) and the ionic strength 72 mM.

Having made a number of changes to the literature assay, we then proceeded to analyze peptide **5**, as a representative substrate, under these modified conditions to determine if the assay was still working properly and to investigate further improvements. We found that increasing the volume of the PEI−cellulose column from 250 to 570 μL and the quenched reaction mixture loaded onto the column from 20 μL to 60 μL improved the signal to noise ratio and that the phosphopeptide was still eluted within the first 1.5 mL of 1 M LiCl eluent, as reported by Budde et al. (see Figure 1). Using PEI−cellulose TLC, with 1 M LiCl containing 10% acid molybdate [i.e. 27 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in 1.2 M H_2SO_4] as the mobile phase, and autoradiography for ^{32}P detection, we compared the 0.25 mL fractions shown in Figure 1 obtained at 0.25, 0.5, 1.5, and 2.25 mL total LiCl eluent to ^{32}P-labeled inorganic phosphate

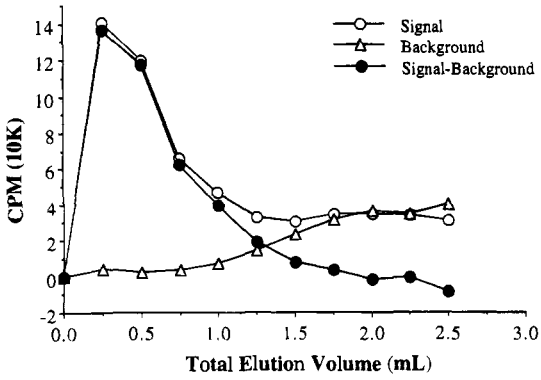

Figure 1. Separation of ^{32}P-labeled phosphopeptide obtained with **5** from other radioactive materials in the pp60$^{c\text{-}src}$ assay mixture using a 570 μL PEI–cellulose anion exchange column and 1 M LiCl as the mobile phase. Each 0.25 mL fraction was analyzed for ^{32}P content by Cerenkov counting as indicated by the counts per minute (cpm) obtained. Signal indicates the cpm obtained when **5** is included in the assay. Background indicates the cpm obtained from a separate experiment wherein **5** is not included in the assay. Signal–background was obtained by mathematical subtraction. Details are included in the Experimental Section.

and [γ-^{32}P]ATP. This TLC analysis confirmed that the phosphorylated peptide **5** is eluted in the first 1.5 mL followed mainly by [γ-^{32}P]ATP as background thereafter.

We also attempted to increase the ionic strength in our assay to the intracellular ca. 150 mM level[25] from the current 72 mM by reducing the MOPS concentration to 50 mM and adding 125 mM KCl, but the ability of the ion exchange column to separate the phosphopeptide product from other radioactive materials was lost.[29]

Rank Order Analysis of New pp60$^{c\text{-}src}$ Peptide Substrates. In order to quickly focus on the best substrates we chose to conduct initial rank order experiments with the nine peptide substrates listed in Table 1. The series A autophosphorylation analogs (except for pentapeptide **4**) and the series B p34^{cdc2} analogs were analyzed first. On the basis of the literature results described above, it was expected that the K_m's for these substrates would range from about 1 mM or better for the p34^{cdc2} heptapeptide analog **5** to greater than 6 mM for the autophosphorylation pentapeptide analog **3**. Consequently, an intermediate peptide concentration of 3 mM was chosen for rank order comparison of series A and B substrates. Since we expected heptapeptide **5** to be the best substrate of these two series (except for **4**) we first confirmed that the reaction velocity with **5** at 3 mM was linear for the duration of the assay.

The peptide substrates from series A and B (except for **4**) were then compared for the amount of phosphopeptide product produced (as determined by the ^{32}P cpm in the first 1.5 mL eluted from the PEI–cellulose columns) when all were assayed under identical conditions at a 3 mM concentration. The results shown in Table 1 verified that p34^{cdc2} heptapeptide **5** is indeed the best substrate followed by its pentapeptide analog **6**, both of which are better than the standard RR-src peptide 1 and shorter analogs thereof.

From these initial results we selected pentapeptide **6** to be compared to the remaining pentapeptide **4** (from autophosphorylation series A) and to the peptides in series C and D. Since we expected these peptides to be better substrates, we reduced the concentration used for the comparison from 3 to 1 mM, confirmed that the reaction velocity with **7** at 1 mM was linear for the duration of the assay, and obtained the results shown in Table 1. These peptides indeed proved to be much better substrates (except for **9**), as indicated by the increase in cpm for the phosphopeptide products even though the peptide concentrations were reduced 3-fold. Three peptides, **4**, **7**, and **8**, all containing isoleucine at the P–1 sites, were found to be significantly better substrates than pentapeptide **6** chosen from the previous rank order comparison, and were therefore selected for the more detailed analysis described below.

K_m and V_{max} Comparison of the Best pp60$^{c\text{-}src}$ Peptide Substrates. The K_m and V_{max} values for peptides **4**, **7**, and **8** shown in Table 1 were determined utilizing our modified PEI–cellulose column assay described above and the direct linear plot method of Cornish-Bowden,[30] wherein the low and high peptide substrate concentrations to be multiply tested were chosen using the procedure of Duggleby.[31]

The K_m and V_{max} values we obtained for the 15mer **7** differ somewhat from that obtained by Songyang et al.[21] (33 μM and 0.8 μmol/min/mg, respectively) for this substrate with pp60$^{c\text{-}src}$. However, we have found that changes in the assay parameters such as the ionic strength and the free Mg^{2+} level can affect the measured K_m values by as much as an order of magnitude for the cAMP-dependent protein kinase[26] and suspect that these assay parameters may also affect the measured K_m values for peptide substrates of pp60$^{c\text{-}src}$. Some of the major differences in our assay conditions vs those under which Songyang et al. obtained their K_m and V_{max} values for **7** are PEI–cellulose column assay vs phosphocellulose filter paper binding assay, ca. 0.5 mM free Mg^{2+} vs 10 mM Mn^{2+}, and 138 mM MOPS at pH 7.1 vs 50 mM Tris at pH 7.5, respectively. Since the nature[32] and concentration (see below) of the divalent metal ion used is known to significantly affect the kinetics of protein kinases, these differences are a likely source of the discrepancy in the measured K_m's for **7** and suggests that all of the K_m and V_{max} values in Table 1 would be lower under the assay conditions used by Songyang et al.

The K_m and V_{max} values we report in Table 1 are more precisely defined as "apparent" values, i.e. K_m^{app} and V_{max}^{app}, and whereas their relative values give a good assessment of which is the best substrate under our assay conditions, their absolute values may differ somewhat from the "true" K_m and V_{max} values. The true K_m and V_{max} values could be obtained by correcting the apparent values using the appropriate equations for the particular kinetic mechanism involved with the peptide substrates along with the K_m for MgATP and the K_i's and concentrations for MgATP and MgADP under the assay conditions used. Using [Val^5]angiotensin II as the peptide substrate, ca. 10 mM free Mn^{2+}, and low ionic strength, Wong and Goldberg[33] concluded that pp60$^{v\text{-}src}$ utilizes a steady-state ordered Bi Bi mechanism with ATP (K_m = 7.0 μM, K_i = 11.7 μM) as the first substrate to bind and ADP (K_i = 4.0 μM) as the last product released. In an analogous study with the more extensively investigated prototypical[34] protein kinase, cAMP-dependent protein kinase (PKA, a serine kinase), using a small peptide substrate (Kemptide), ca. 12 mM free Mg^{2+}, and low ionic strength, Whitehouse et al.[35] concluded that PKA also utilizes a steady-state ordered

Bi Bi mechanism with ATP (K_m = 7.6 μM, K_i = 4.4 μM) as the first substrate to bind and ADP (K_i = 7.3 μM) as the last product released. In a separate study, using the same peptide substrate (Kemptide), but at ca. 0.5 mM free Mg^{2+} and high ionic strength, Kong and Cooke[36] concluded that PKA utilizes a steady-state random mechanism with an ATP K_m = 177 μM and K_i = 160 μM and an ADP K_i = 227 μM,[37] all of which are significantly higher than those obtained[35,37] under high free [Mg^{2+}] and low ionic strength conditions as indicated above. Since the kinetic mechansim of pp60$^{c\text{-}src}$ and binding constants for MgATP and MgADP have not been determined under ca. 0.5 mM free Mg^{2+} and high ionic strength conditions, we can only estimate the true K_m and V_{max} values by assuming that the binding constants, which are similar to those determined for PKA under high divalent metal ion concentration and low ionic strength, will increase under our assay conditions to those measured for PKA under ca. 0.5 mM free Mg^{2+} and high ionic strength. Given this assumption and using the appropriate equations[38] for a steady-state ordered Bi Bi mechanism (more kinetic values are needed to solve the appropriate equations for a steady-state random mechanism), the "true" K_m's and V_{max}'s for **4**, **7**, and **8** are calculated to be 926, 105, 387 μM and 3.83, 6.30, 2.10 μmol/min/mg, respectively. These calculations indicate that the presence of nonsaturating MgATP (172 μM under our assay conditions via BAD calculations; increasing the concentration to saturating levels is impractical due to the excessive radioactivity which would be required) and [ADP] at 10% of [ATP] results in K_m^{app}'s which are within experimental error of the true K_m's but that the V_{max}^{app}'s underestimate the true V_{max}'s by a factor of 2.

The data in Table 1 shows that deleting 10 residues from **7** to give pentapeptide **8** only reduces the binding affinity (as estimated by the K_m's) by a factor of 3.7. This result confirms that the five residues present in **8** form the major recognition region for pp60$^{c\text{-}src}$ as predicted when **8** was designed. Pentapeptide **4** contains a P−1 Ile which is also present in **8** and was found to be the most important recognition residue in the combinatorial library results of Songyang et al.[21] However, extending the pentapeptide sequence on the N-terminus, to include the P−3 and P−4 acidic residues from the autophosphorylation sequence instead of the P+3 Phe, results in a 2.4-fold reduction in binding affinity as indicated by the K_m's of **4** vs **8**. This finding is also consistent with the combinatorial library results of Songyang et al.,[21] wherein Glu (best) or Asp (second best) were the preferred residues at the P−3 and P−4 positions but were less important than Phe at the P+3 position. Comparison of the V_{max} results for **4** vs **8** shows that, whereas the C-terminal residues are more important for binding affinity, the N-terminal residues are more important for the V_{max}. This finding indicates that the intrinsic binding energy available from the C-terminal residues is utilized to a greater extent for initial substrate recognition whereas that from the N-terminal residues is utilized to a greater extent for lowering the transition state energy of the reaction. The inversion of contributions of the N- and C-terminal residues to the K_m and V_{max} values results in a V_{max}/K_m for **8** which is only 1.3-fold better than that for **4** even though the K_m is 2.4-fold better.

Conclusions and Future Implications

We have identified two pentapeptide substrates, **4** and **8**, which provide lead peptide sequences for the design of peptide-based pp60$^{c\text{-}src}$ inhibitors. Since **8** has the better K_m it may be the preferred lead sequence for evaluating reversible inhibitor designs wherein the Tyr is replaced with other natural or unnatural amino acids (e.g. see Kim et al[23]). On the other hand, **4** may be the preferred lead sequence for evaluating mechanism-based irreversible peptide inhibitor designs (see Cushman et al.[39] and Lee et al.[40]) since it provides the better maximal rate of reaction. Although the K_m's for these pentapeptide PTK substrates are not as good as has been achieved with peptide substrates of protein serine kinases which are heptapeptides or larger[7a] (i.e. K_m's < 10 μM), the V_{max}'s are in the same range. If desired, the sequences of **4** and **8** could be merged into the single octapeptide sequence Ac-EDAIYGEF-NH_2 which would contain the residues identified as most important for the K_m as well as those for the V_{max} and is therefore predicted to be a very good substrate. However, the binding affinity and V_{max} of **4** and **8** with pp60$^{c\text{-}src}$ is sufficient for evaluating the *relative* effectiveness of various inhibitor designs while appended to a *small* peptide. This reduced size is particularly helpful for the facile synthesis, purification, and characterization of inhibitor analogs wherein the Tyr is replaced with a series of difficult to obtain unnatural amino acids. A convergent synthetic route can then be utilized wherein the constant peptide fragment (Ac-EDAI or GEF-NH_2 for **4** or **8**, respectively) is prepared and purified in bulk (suitably protected) as a feedstock for coupling with the various unnatural amino acid-containing fragments. We have now initiated such inhibitor studies based upon these lead pentapeptide sequences and will report our results in due course. Another potential advantage for utilizing these pentapeptide sequences is that good inhibitors identified in this fashion can form the foundation for developing related conformationally constrained peptides, peptide mimetics, or non-peptides in order to increase binding affinity and selectivity and improve their ability to penetrate cells.

Experimental Section

Cerenkov counting was carried out with a Packard Tri-Carb 1900 TR liquid scintillation analyzer. Analytical and semi-preparative reversed phase (RP)-HPLC results were obtained with Microsorb C-18 columns, Dynamax, 4.6 × 250 mm, 5 μm particle size, and Dynamax, 10.0 × 250 mm, 5 μm particle size, respectively, both purchased from Rainin Instrument Co. Inc., Woburn, MA, while the eluant was monitored at 254 nm using a variable wavelength UV detector. ^{1}H-NMR spectra were obtained with a Varian Gemini 300 MHz spectrometer. Amino acid analyses were performed at the Biopolymer facility of Roswell Park Cancer Institute, Buffalo, NY. Positive and negative ion fast atom bombardment [FAB(+) and (−)] mass spectra were acquired with a VG 70SEQ mass spectrometer at the Instrument Center, Chemistry Department, SUNY at Buffalo, Buffalo, NY.

Partially purified human src kinase (pp60$^{c\text{-}src}$) was purchased from Upstate Biotechnology Inc., Lake Placid, NY. This pp60$^{c\text{-}src}$ is obtained from a recombinant baculovirus containing the human c-src gene in SF9 insect cells and is purified by sequential column chromatography using hydroxyapatite and affinity columns. The partially purified pp60$^{c\text{-}src}$ is reported to be essentially free of other protein kinases and is supplied as 75 units [1 unit transfers 1 pmol of phosphate/min/mg of kinase to a saturating concentration of cdc2(6−20) peptide

substrate] of specific activity 900 000 units/mg, in 25 μL of 25 mM HEPES, pH 7.0, containing 50% glycerol, 0.1% NP-40, and 1 mM DTT. Peptide **1** (RR-src) was purchased from Peninsula Laboratories, Inc., Belmont, CA. Peptide **7** was kindly provided by Lewis C. Cantley and Zhou Songyang of the Department of Cell Biology, Harvard Medical School, Boston, MA. Peptides **4** and **8** were custom synthesized and purified via semipreparative RP-HPLC to >95% purity (by analytical RP-HPLC and analysis at 215 nm with a SYNCHROM C18 column using a CH_3CN/H_2O gradient containing 0.1% TFA) by Quality Controlled Biochemicals, Inc. Hopkinton, MA. Purified **4** and **8**, as received, gave the following amino acid analyses (calculated). **4**: Asp 1.05 (1.0), Glu 0.99 (1.0), Ala 1.05 (1.0), Tyr 0.93 (1.0), Ile 0.99 (1.0). **8**: Ile 1.05 (1.0), Tyr 0.93 (1.0), Gly 0.99 (1.0), Glu 1.09 (1.0), Phe 0.94 (1.0). The structures of **4** and **8** were also confirmed by FAB MS: **4** FAB(+) *m/e* calcd [M + H$^+$] 651, found 651; **8** FAB(−) *m/e* calcd [M − H$^+$] 667, found 667. Peptide **9** was prepared as described in Kim et al.[23] [γ-^{32}P]ATP was purchased from ICN Biomedicals, Inc., Costa Mesa, CA. PEI−cellulose (fine mesh grade), ATP, ADP, Triton-X-100, glycerol, β-mercaptoethanol, $MgCl_2$, and ammonium molybdate were purchased from Sigma Chemical Co., St. Louis, MO. PEI−cellulose TLC plates were purchased from EM-Separations Technology, Gibbstown, NJ, and autoradiography (described in the text) was performed with Kodak X-Omat AR film.

Synthesis of Peptides 2, 3, 5, and 6. These peptides were synthesized using a p-methylbenzhydrylamine (pMBHA) resin and t-Boc-protected amino acids on an automated peptide synthesizer (Biosearch Model 9500) using the coupling protocols and side chain protection recommended by the Biosearch software supplied with the synthesizer. The peptides were then cleaved from the resin with anhydrous HF (10 mL/g resin), using anisole as the scavenger (1 mL/g resin), for 60 min at 0 °C. The deprotected peptides were purified by semipreparative RP-HPLC to at least 95% purity (as determined by analytical RP-HPLC) using a gradient of 0−100% (70% acetonitrile in water containing 0.1% TFA)/water containing 0.1% TFA over 45 min. All purified peptides were characterized by FAB(+) MS, amino acid analysis, and 300 MHz ^{1}H-NMR. All of the peptides gave FAB(+) MS strong molecular ion peaks (M + H$^+$) which agreed with the (calculated) m/e values as follows: **2**, 738 (738); **3**, 609 (609); **5**, 723 (723); and **6**, 567 (567). These peptides gave the following amino acid analyses (calculated). **2**: Ala 2.00 (2.0), Asp 1.01 (1.0), Glu 1.97 (2.0), Tyr 1.02 (1.0). **3**: Ala 2.01 (2.0), Asp 1.06 (1.0), Glu 0.98 (1.0), Tyr 0.95 (1.0). **5**: Gly 3.04 (3.0), Glu 0.97 (1.0), Thr 1.01 (1.0), Tyr 0.99 (1.0), Val 0.99 (1.0). **6**: Gly 2.01 (2.0), Glu 0.98 (1.0), Thr 0.97 (1.0), Tyr 1.04 (1.0). All of the peptides gave a ^{1}H-NMR peak (in CD_3OD) at ca. δ 1.9 ppm (relative to Me_4Si) for the *N*-acetyl methyl group in addition to peaks consistent with the expected amino acids in the sequences.

pp60$^{c\text{-}src}$ Peptide Phosphorylation Assay. Phosphorylation of the peptide substrates were carried out using the assay procedure of Budde et al.[24] after a variety of modifications to the procedure and assay conditions had been made, some of which were mentioned in the text. Due to these changes, a detailed description of the assay we used is given below.

The phosphorylation reactions were performed in 0.5 mL microcentrifuge tubes in a final volume of 40 μL containing 138 mM MOPS and 2.08 mM HEPES at pH 7.1, 0.64 mM $MgCl_2$, 2.75 mM β-mercaptoethanol, 0.083 mM DTT, 13% glycerol, 0.01% Triton-X-100, 0.0083% NP-40, BSA (0.37 mg/mL), 200 μM [γ-^{32}P]ATP (1200 cpm/pmol), 20 μM ADP, 10 units of pp60$^{c\text{-}src}$, and peptide substrate. The HEPES, DTT, and NP-40 are present in the commercial preparation of pp60$^{c\text{-}src}$ along with glycerol.

All of the final concentrations listed above, except that of ATP and ADP, are reduced slightly (typically about 2%) by the required amount of concentrated commercial [γ-^{32}P]ATP which is added to the cold ATP/ADP solution as described below. A stock solution of 150 mM MOPS at pH 7.1, 0.7 mM $MgCl_2$, 3 mM β-mercaptoethanol, 10% glycerol, 0.01% Triton-X-100, and BSA (0.4 mg/mL) was prepared, hereafter referred to as the "buffer solution". This buffer solution was then used to prepare 370 μL (this volume is adjusted such that when the commmercial [γ-^{32}P]ATP solution is added a final volume of 400 μL is obtained) of an 884 μM ATP, 88.4 μM ADP stock solution. This stock ATP/ADP solution was then spiked with the required amount of concentrated commercial [γ-^{32}P]ATP (typically about 30 μL) to obtain a final specific activity of 1200 cpm/pmol ATP in the assay. The pp60$^{c\text{-}src}$, as obtained commercially, was diluted with twice as much of the buffer solution to give a final stock enzyme solution containing 1 unit/μL. The peptide substrates were dissolved in the buffer solution at 4 times the needed final concentration. These stock solutions were added to the reaction vessels in the following order and in the indicated amounts: (1) the enzyme stock solution (10 μL), (2) the ^{32}P-spiked ATP/ADP stock solution (10 μL), (3) the buffer solution (10 μL) and (4) the peptide solution (10 μL). After incubating these reaction mixtures for 30 min with orbital stirring at 30 °C in a constant temperature bath, the reactions were quenched by adding 40 μL of acid molybdate (27 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in 1.2 M H_2SO_4). The reaction vessels were then centrifuged on a Beckman microfuge B for about 3 min to remove the precipitated protein and $^{32}P_i$ as a blue molybdate complex. A 60 μL portion of the supernatants was loaded immediately onto disposable PEI−cellulose columns prepared as described below.

The PEI−cellulose disposable columns were prepared from 1 mL pipet tips (they hold 1.6 mL). Glass wool was placed into the pipet tips and tapped down with a wooden pin. Holes were bored in the center of the caps of 5-mL polypropylene scintillation vials. The narrow ends of the pipet tips were then inserted through the holes in the caps on the scintillation vials after small vinyl tubing support rings had been slipped over the narrow end of the pipet tips to prevent them from extending too far into the vials. The scintillation vials were then placed inside 20 mL glass vials (to stabilize the scintillation vial while in the centrifuge) to give the "centrifuge assemblies". A 10 g portion of PEI−cellulose powder was suspended in 60 mL of distilled water and stirred vigorously to form a homogenous gel. While stirring, 1.5 mL of this suspension was transferred into each pipet tip and then the centrifuge assemblies were spun in a International Clinical Centrifuge (a standard bench top centrifuge) at a very low speed (so that the gel is compacted but not run dry) to prepare a uniform column having a volume of ca. 570 μL.

After loading the columns with 60 μL of the quenched reaction mixtures, the centrifuge assemblies were spun at very low speed for about 15 s (until the reaction mixtures were just absorbed on the top of the columns). Then 0.75 mL of 1 M LiCl was added to the top of the columns and the centrifuge assemblies were spun at full speed for about 1 min such that the entire solution is pushed through the columns. Another 0.75 mL of 1 M LiCl was then added to the top of the columns and the centrifuge assemblies were spun at full speed for about 2 min until this second solution was pushed through the columns. The combined eluants (which are directly collected in the scintillation vials) are then counted using Cerenkov counting.

Validation of the Modified pp60$^{c\text{-}src}$ Assay. Reaction mixtures (prepared as above) were incubated in the presence (*signal*) and absence (*background*) of a 5 mM concentration of **5**. A 60 μL sample of the quenched reaction mixtures were loaded onto PEI−cellulose columns as described above and the [^{32}P]phosphopeptide was eluted in steps by adding 0.25 mL portions of 1 M LiCl to the top of the columns and then sequentially eluting them into a supporting scintillation vial by centrifuging at high speed for 3 min. This elution procedure was repeated until a total of 2.5 mL of LiCl eluent had been collected in 0.25 mL increments. Each 0.25 mL fraction was then analyzed separately by Cerenkov counting. The results shown in Figure 2 are the average of duplicate experiments. In this experiment the PEI−cellulose columns (570 μL) were prepared by dry packing the pipet tips with PEI−cellulose powder as described by Budde et al.[24] However, we later found that wet packing the columns (as described above) provided more uniform columns and therefore switched to wet packing for all subsequent experiments.

Rank Order Comparison of the pp60$^{c\text{-}src}$ Substrates. All the peptides were phosphorylated under identical conditions as described above at a concentration of either 1 or 3 mM. Each peptide phosphorylation reaction was run in triplicate and the values listed in Table 1 are the average of these values. The blank was also run in triplicate and was obtained by substituting buffer solution for the peptide solution in the assay.

Determination of the K_m and V_{max} for 4, 7, and 8. Two different peptide concentrations, one near the estimated K_m and the other at ca. 10 times the estimated K_m, were used, as recommended by Duggleby.[31] The peptide concentrations used were 200 μM and 2 mM for **4**, 25 μM and 300 μM for **7**, and 50 μM and 1 mM for **8**. Initial velocities, calculated after subtracting the blank velocities (wherein the reaction was quenched immediately after the peptide solution was added), were obtained six times at the low peptide concentrations and six times at the high peptide concentrations for **4** and **8** and four times at each concentration for **7**, all after incubating for 30 min at 30 °C. Direct linear plots of the velocity vs concentration were drawn and the median estimates of the K_m and V_{max} given in Table 1 were obtained from the 36 (with **4** and **8**) or 16 (with **7**) values obtained for each as described by Cornish-Bowden.[30]

Acknowledgment. This work was supported by grants from the National Cancer Institute (R01 CA52800 to D.G.H.), Lederle Laboratories, and the Center for Biotechnology at Stony Brook, funded by the New York State Science and Technology Foundation (to D.G.H.). We thank W. Todd Miller and Walter D. Conway for helpful discussions and W.D.C. for help in preparing the "centrifuge assembly". We thank S. P. J. Brooks for kindly providing version 4.35 of his computer program "Bound and Determined" and for helpful discussions regarding the same.

References

(1) Edelman, A. M.; Blumenthal, D. K.; Krebs, E. G. Protein Serine/threonine Kinases. *Ann. Rev. Biochem.* **1987**, *56*, 567–613.

(2) (a) Hunter, T. Protein Modification: Phosphorylation on Tyrosine Residues. *Curr. Opin. Cell Biol.* **1989**, *1*, 1168–81. (b) Fantl, W. J.; Johnson, D. E.; Williams, L. T. Signalling by receptor tyrosine kinases. *Ann. Rev. Biochem.* **1993**, *62*, 453–81. (c) Cantley, L. C.; Auger, K. R.; Carpenter, C.; Duckworth, B.; Granziani, A.; Kapeller, R.; Soltoff, S. Oncogenes and Signal Transduction. *Cell* **1991**, *64*, 281–302.

(3) Johnson, L. N.; Barford, D. The Effects of Phosphorylation on the Structure and Function of Proteins. *Ann. Rev. Biophys. Biomol. Struct.* **1993**, *22*, 199–232.

(4) Powis, G. Signalling Targets for Anticancer Drug Development. *TIPS* **1991**, 188–194.

(5) (a) Klein, G. Multistep emancipation of tumors from growth control: can it be curbed in a single step? *BioEssays* **1990**, *12*, 347–350. (b) Marx, J. Oncogenes evoke new cancer therapies. *Science* **1990**, *249*, 1376–1378.

(6) (a) Levitzki, A.; Gazit, A. Tyrosine Kinase Inhibition: An Approach to Drug Development. *Science* **1995**, *267*, 1782–1788. (b) Boutin, J. A. Tyrosine Protein Kinase Inhibition and Cancer. *Int. J. Biochem.* **1994**, *26*, 1203–1226. (c) Burke, T. R. Jr. Protein-Tyrosine Kinases: Potential Targets for Anticancer Drug Development. *Stem Cells* **1994**, *12*, 1–6. (b) Levitzki, A. Tyrphostins: tyrosine kinase blockers as novel antiproliferative agents and dissectors of signal transduction. *FASEB Journal* **1992**, *6* (14), 3275–82. (c) Workman, P.; Brunton, V. G.; Robins, D. J. Tyrosine kinase inhibitors. *Seminars in Cancer Biology* **1992**, *3* (6), 369–81. (d) Burke, T. R., Jr. Protein-tyrosine kinase inhibitors. *Drugs Future* **1992**, *17* (2), 119–131. (e) Dobrusin, E. M.; Fry, D. W. Chapter 18. Protein tyrosine kinases and cancer. *Ann. Rep. Med. Chem.* **1992**, *27*, 169–178. (f) Chang, C. J.; Geahlen, R. L. Protein-tyrosine kinase inhibition: Mechanism-based discovery of antitumor agents. *J. Nat. Prod.* **1992**, *55* (11), 1529–60.

(7) (a) Kemp, B. E.; Pearson, R. B. Design and use of peptide substrates for protein kinases. *Methods Enzymol.* **1991**, *200*, 121–34. (b) Geahlen, R. L.; Harrison, M. L. In *Peptides and Protein Phosphorylation*; Kemp, B. E., Ed.; CRC Press, Inc.; Boca Raton, Florida, 1990; Chapter 10, pp 239–253. (c) Casnellie, J. E.; Krebs, E. G. The use of synthetic peptides for defining the specificity of tyrosine protein kinases. *Adv. Enzyme Regul.* **1984**, *22*, 501–515.

(8) Rudd, C. E.; Janssen, O.; Prasad, K. V. S.; Raab, M.; da Silva, A.; Telfer, J.C.; Yamamoto, M. src-related protein tyrosine kinases and their surface receptors. *Biochim. Biophys. Acta* **1993**, *1155*, 239–266.

(9) (a) Cartwright, C. A.; Coad, C. A.; Egbert, B. M. Elevated c-Src tyrosine kinase activity in premalignant epithelia of ulcerative colitis. *J. Clin. Invest.* **1994**, *93*, 509–515. (b) Talamonti, M. S.; Roh, M. S.; Curley, S. A.; Gallick, G. E. Increase in activity and level of pp60$^{c\text{-}src}$ in progressive stages of human colorectal cancer. *J. Clin. Invest.* **1993**, *91*, 53–60. (c) Termuhlen, P. M.; Curley, S. A.; Talamonti, M. S.; Saboorian, M. H.; Gallick, G. E. Site-specific differences in pp60$^{c\text{-}src}$ activity in human colorectal metastases. *J. Surg. Res.* **1993**, *54*, 293-298. (d) Novotny-Smith, C. L.; Gallick, G. E. Growth inhibition of human colorectal carcinoma cell lines by tumor necrosis factor-alpha correlates with reduced activity of pp60$^{c\text{-}src}$. *J. Immunother.* **1992**, *11*, 159–168. (e) Talamonti, M. S.; Curley, S. A.; Gallick, G. E. Development and progression of human colon cancer. *Cancer Bull* **1992**, *44*, 321–326. (f) Garcia, R.; Parikh, N. U.; Saya, H.; Gallick, G. E. Effect of herbimycin A on growth and pp60$^{c\text{-}src}$ activity in human colon tumor cell lines. *Oncogene* **1991**, *6*, 1983–1989. (g) Cartwright, C. A.; Meisler, A. I; Eckhart, W. Activation of the pp60$^{c\text{-}src}$ protein kinase is an early event in colonic carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 558–562. (h) Cartwright, C. A.; Kamps, M. P.; Meisler, A. I.; Pipas, J. M.; Eckhart, W. pp60$^{c\text{-}src}$ activation in human colon carcinoma. *J. Clin. Invest.* **1989**, *83*, 2025–2033. (i) Bolen, J. B.; Veillette, A.; Schwartz, A. M.; Deseau, V.; Rosen, N. Activation of pp60$^{c\text{-}src}$ protein kinase activity in human colon carcinoma. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 2251–2255. (j) Bolen, J. B.; Veillette, A.; Schwartz, A. M.; Deseau, V.; Rosen, N. Analysis of pp60$^{c\text{-}src}$ in human colon carcinoma and normal human mucosal cells. *Oncogene Res.* **1987**, *1*, 149–168. (k) Rosen, N.; Bolen, J. B.; Schwartz, A. M.; Cohen, P.; Deseau, V.; Israel, M. Analysis of pp60c-src activity in human tumor cell lines and tissues. *J. Biol. Chem.* **1986**, *261*, 13754–13759.

(10) (a) Guy, C. T.; Muthuswamy, S. K.; Cardiff, R. D.; Soriano, P.; Muller, W. J. Activation of the c-Src tyrosine kinase is required for the induction of mammary tumors in transgenic mice. *Genes Dev.* **1994**, *8*, 23–32. (b) Luttrell, D. K.; Lee, A.; Lansing, T. J.; Crosby, R. M.; Jung, K. D.; Willard, D.; Luther, M.; Rodriguez, M.; Berman, J.; Gilmer, T. M. Involvement of pp60$^{c\text{-}src}$ with two major signaling pathways in human breast cancer. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 83–87. (c) Muthuswamy, S. K.; Siegel, P. M.; Dankort, D. L.; Webster, M. A.; Muller, W. J. Mammary tumors expressing the neu proto-oncogene possess elevated c-Src tyrosine kinase activity. *Mol. Cell. Biol.* **1994**, *14* (1), 735–743. (d) Ottenhoff-Kalff, A. E.; Rijksen, G.; van Beurden, E. A.; Hennipman, A.; Michels, A. A.; Stall, G. E. Characterization of protein tyrosine kinases from human breast cancer: involvement of the c-src oncogene product. *Cancer Res.* **1992**, *52*, 4773–4778.

(11) Mazurenko, N. N.; Kogen, E. A.; Zborovskaya, I. B.; Kisseljov, F. L. Expression of pp60$^{c\text{-}src}$ in human small cell and non-small cell lung carcinomas. *Eur. J. Cancer* **1992**, *28*, 372–377.

(12) Fanning, P.; Bulovas, K.; Saini, K. S.; Libertino, J. A.; Joyce, A. D.; Summerhayes, I. C. Elevated expression of pp60$^{c\text{-}src}$ in low grade human bladder carcinoma. *Cancer Res.* **1992**, *52*, 1457–1462.

(13) Barnekow, A.; Paul, E.; Schartl, M. Expression of the c-src protooncogene in human skin tumors. *Cancer Res.* **1987**, *47*, 235–240.

(14) Takeshima, E.; Hamaguchi, M.; Watanbe, T.; Akiyama, S.; Kataoka, M.; Ohnishi, Y.; Xiao, H.; Hagai, Y., Taka, H. Aberrant elevation of tyrosine-specific phosphorylation in human gastric cancer cells. *Jpn. J. Cancer Res.* **1991**, *82*, 1428–1435.

(15) Lynch, S. A.; Brugge, J. S.; Fromowitz, F.; Glantz, L.; Wang, P.; Caruso, R.; Viola, M. V. Increased expression of the src proto-oncogene in hairy cell leukemia and a subgroup of B-cell lymphomas. *Leukemia* **1993**, *7*, 1416–1422.

(16) (a) Bjelfman, C.; Hedborg, F.; Johansson, I.; Nordenskjold, M.; Pahlman, S. Expression of the neuronal for of pp60c-src in neuroblastoma in relation to clinical stage and prognosis. *Cancer Res.* **1990**, *50*, 6908–6914. (b) O'Shoughnessy, J.; Deseau, V.; Amini, S.; Rosen, N.; Bolen, J.B. Analysis of the c-src gene product structure, abundance, and protein kinase activity in human neuroblastoma and glioblastoma cells. *Oncogene Res.* **1987**, *2*, 1–18.

(17) Casnellie, J. E.; Harrison, M. L.; Pike, L. J.; Hellstrom, K. E.; Krebs, E. G. Phosphorylation of synthetic peptides by a tryosine protein kinase from the particulate fraction of a lymphoma cell line. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 282–286.

(18) Cheng, H-C.; Nishio, H.; Hatase, O.; Ralph, S.; Wang, J. H. A synthetic peptide derived from p34^{cdc2} is a specific and efficient substrate of src-family tyrosine kinases. *J. Biol. Chem.* **1992**, *267*, 9248–9256.

(19) Till, J. H.; Annan, R. S.; Carr, S. A.; Miller, W. T. Use of synthetic peptide libraries and phosphopeptide-selective mass spectrometry to probe protein kinase substrate specificity. *J. Biol. Chem.* **1994**, *269*, 7423–7428.

(20) Songyang, Z.; Blechner, S.; Hoagland, N.; Hoekstra, M. F.; Piwnica-Worms, H.; Cantley, L. C. Use of an oriented peptide library to determine the optimal substrates of protein kinases. *Current Biol.* **1994**, *4*, 973–982.
(21) Songyang, Z.; Carraway III, K. L.; Eck, M. J.; Harrison, S. C.; Feldman, R. A.; Mohammodi, M.; Schlessinger, J.; Hubbard, S. R.; Mayer, B. J.; Cantley, L. C. Protein tyrosine kinases and SH2 domains have overlapping specificities. *Nature* **1995**, *373*, 536–539.
(22) Tinker, D. A.; Krebs, E. A.; Feltham, I. C.; Attah-Poku, S. K.; Ananthanarayanan, V. S. Synthetic β-Turn Peptides as Substrates for a Tyrosine Protein Kinase. *J. Biol. Chem.* **1988**, *263*, 5024–5026.
(23) Kim, M. H.; Lai, J. H.; Hangauer, D. G. Tetrapeptide tyrosine kinase inhibitors: Enantioselective synthesis of p-hydroxymethyl-L-phenylalanine, incorporation into a tetrapeptide, and subsequent elaboration into p-(R,S-hydroxyphosphonomethyl)-L-phenylalanine. *Int. J. Pept. Protein Res.* **1994**, *44*, 457–465.
(24) Budde, R. J. A.; McMurray, J. S.; Tinker, D. A. An assay for acidic peptide substrates of protein kinases. *Anal. Biochem.* **1992**, *200*, 347–351.
(25) Alberts, B.; Bray, D.; Lewis, J.; Roberts, K.; Watson, J. D. *Molecular Biology of the Cell*, 2nd ed.; Garland Publishing Inc., New York, 1989; pp 301 & 353.
(26) Warren, S. D. Design, synthesis, enzyme binding evaluation, and analysis of preferred enzyme-complexes for cAMP-dependent protein kinase inhibitors. Ph.D. thesis, SUNY at Buffalo, 1995.
(27) Brooks, S. P. J.; Storey, K. B. Bound and Determined: a computer program for making buffers of defined ion concentrations. *Anal. Biochem.* **1992**, *201*, 119-126.
(28) Smith, R. M.; Martell, A. E.; Chen, Y. Critical evaluation of stability constants for nucleotide complexes with protons and metal ions and the accompanying enthalpy changes. *Pure Appl. Chem.* **1991**, *63*, 1015–1080.
(29) Nair. S. A. Part I: Design, Synthesis and biological evaluation of sulfur-based inhibitors for PKA and PKC; Part II: Design and evaluation of substrates and inhibitors for protein tyrosine kinase. Ph.D. thesis, SUNY at Buffalo, 1995.
(30) Eisenthal, R.; Cornish-Bowden, A. The direct linear plot: a new graphical procedure for estimating enzyme kinetic parameters. *Biochem. J.* **1974**, *139*, 715–720.
(31) Duggleby, R. G. Experimental designs for estimating the kinetic parameters for enzyme-catalysed reactions. *J. Theor. Biol.* **1979**, *81*, 671–684.
(32) Adams, J. A.; Taylor, S. S. Divalent metal ions influence catalysis and active-site accessibility in the cAMP-dependent protein kinase. *Protein Sci.* **1993**, *2*, 2177–2186.
(33) Wong, T. W.; Goldberg, A. R. Kinetics and mechanism of angiotensin phosphorylation by the transforming gene product of Rous Sarcoma Virus. *J. Biol. Chem.* **1984**, *259*, 3127–3131.
(34) Taylor, S. S.; Knighton, D. R.; Zheng, J.; Sowadski, J. M.; Gibbs, C. S.; Zoller, M. J. A template for the protein kinase family. *Trends Biol. Sci.* **1993**, 84–89.
(35) Whitehouse, S.; Feramisco, J. R.; Casnellie, J. E.; Krebs, E. G.; Walsh, D. A. Studies on the kinetic mechanism of the catalytic subunit of the cAMP-dependent protein kinase. *J. Biol. Chem.* **1983**, *258*, 3693–3701.
(36) Kong, C-T.; Cook, P. F. Isotope partitioning in the adenosine 3′,5′-monophosphate dependent protein kinase reaction indicates a steady-state random kinetic mechanism. *Biochemistry* **1988**, *27*, 4795–4799.
(37) Cook, P. F.; Neville, M. E.; Vrana, K. E., Hartl, F. T.; Roskoski, R., Jr. Adenosine 3′,5′-monophosphate dependent protein kinase: Kinetic mechanism for the bovine skeletal muscle catalytic subunit. *Biochemistry* **1982**, *21*, 5794–5799.
(38) The equations I and II used, and shown below, were obtained from the graphical analysis given in Figure IX-20(a), p 584, of the text *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems* by I. H. Segel, 1993 edition, John Wiley & Sons, Inc., New York, NY. This graphical analysis is applicable to a steady-state ordered Bi Bi mechanism wherein the first binding substrate is MgATP (which is present at a constant and unsaturating concentration of 172 μM as calculated via BAD[27,28]), a fixed concentration of the end product inhibitor MgADP is present (8.29 μM as calulcated via BAD[27,28]), and the concentration of phosphopeptide product is essentially zero (i.e. initial velocity conditions).

$$K_{m(peptide)} = K_{m(peptide)}{}^{app}\left(\frac{1+\frac{K_{m(MgATP)}}{[MgATP]}+\frac{K_{m(MgATP)}[MgADP]}{K_{i(MgADP)}[MgATP]}}{1+\frac{K_{i(MgATP)}}{[MgATP]}+\frac{K_{i(MgATP)}[MgADP]}{K_{i(MgADP)}[MgATP]}}\right) \quad \text{(I)}$$

$$V_{max} = V_{max}{}^{app}\left(1+\frac{K_{m(MgATP)}}{[MgATP]}+\frac{K_{m(MgATP)}[MgADP]}{K_{i(MgADP)}[MgATP]}\right) \quad \text{(II)}$$

(39) Cushman, M.; Chinnasamy, P.; Chakraborti, A. K.; Jurayi, J.; Geahlen, R. L., Haugwitz, R. D. Synthesis of [beta-(4-pyridyl-1-oxide)-L-alanine[4]]-angiotensin I as a potential suicide substrate for protein-tyrosine kinases. *Int. J. Pept. Protein Res.* **1990**, *36*, 538–543.
(40) Lee, E-S.; Jurayj, J.; Cushman, M. Synthesis of [L-3-deoxymimosine[4]]angiotensin I as an approach to the preparation of selective protein-tyrosine kinase (PTK) inhibitors. *Tetrahedron* **1994**, *33*, 9873–9882.

JM950025M