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13. ABSTRACT (Maximum 200) Our objectives have been to develop synthetic modulators of tyrosine-specific protein kinases (TPKs) so that the biochemical consequences of their deregulation in breast cancer can be studied. These enzymes play a crucial, albeit still enigmatic role in the signal transduction pathways responsible for cell growth and differentiation. Upon the loss of key regulatory controls, either through the over-expression or mutation of specific PTKs, the oncogenic potential of these enzymes is unleashed. Regulatory agents, which disturb or otherwise alter the myriad of protein-protein recognition events that drive signal transduction pathways, will be extraordinarily useful in helping to decipher the role of specific enzymes in these pathways. In addition, these regulatory agents may ultimately provide the foundation upon which therapeutically useful compounds can be devised. We have (1) compared and contrasted the kinetics of TPK and serine-specific protein kinase-catalyzed phosphoryl transfer, (2) created selective TPK substrates, (3) developed a new methodology for the synthesis of TPK inhibitors, (4) identified L-Dopa as an extraordinarily potent nonphosphorylatable tyrosine mimetic, (5) synthesized a high molecular diversity library targeted against SH2 domains and identified the most potent SH2-targeted agents described to date, and (6) developed the first examples of bivalent inhibitors of TPKs.				
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FOREWORD

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Introduction

Protein kinases, and the signal transduction pathways that they constitute, are now recognized to be medicinally attractive targets of opportunity. The simple notion that noncytotoxic agents can be employed to harness uncontrolled cell growth offers exciting possibilities in the battle against breast cancer. Unfortunately, the obstacles that impede the acquisition of specific and potent inhibitors for this family of enzymes are truly daunting. The mammalian genome codes for an estimated 2000 different protein kinases, raising serious concerns about inhibitor selectivity. Furthermore, all protein kinases utilize ATP as a common substrate, yet the vast majority of protein kinase inhibitors prepared to date target the ATP binding site. As a consequence, many of these so-called selective inhibitors are now being found to exhibit inhibitory activity in a profoundly non-selective fashion. Any ATP-utilizing process is a potential unintended target. For example, several well known protein tyrosine kinase (PTK) inhibitors have recently been shown to interfere with cellular respiration, an ATP-dependent process, but one which has nothing to do with cancer and everything to do with normal cell function.

Instead of targeting the ATP binding site, we have focused our efforts on those regions of PTKs that participate in protein-protein interactions, be it the active site (which catalyzes the phosphorylation of protein substrates), or the SH2 and SH3 domains (which control proper subcellular localization). The preparation of compounds that block these key protein-protein interactions (by binding to the active site, SH2, and/or SH3 domains themselves) frees us from the need to compete with ATP, the universal energy currency of the cell. The pharmaceutical industry has shied away from the development of such inhibitors since they are generally medicinally unattractive peptides. However, we have developed a new approach for the acquisition of compounds that bind tightly to these protein-protein interaction sites, one which first generates peptide/nonpeptide hybrids, and then which ultimately provides purely nonpeptidic species. Our work has emphasized mechanistic issues, the acquisition of PTK-selective substrates, and finally the identification of inhibitory agents.

Body

There are two major classes of protein kinases that are distinguished by their ability to phosphorylate serine versus tyrosine residues. Both kinetic and structural characterization of this latter class has lagged behind the former. This is due, no doubt, to the greater difficulty of obtaining pure and active forms of the TPKs. In general, the serine protein kinases ("SPKs") are cytosolic and the TPKs are either integral or peripheral membrane proteins which offer problems for standard bacterial expression. In contrast, these enzymes exhibit a substantial difference in their phosphoryl transfer rates. Interestingly, this difference has little effect on the maximum rate of substrate turnover, but rather its influence is primarily conveyed through K_{peptide} . Recent advances in protein fusion technology, though, has allowed the expression and purification of several nonreceptor TPKs in *E. coli* including c-src and v-abl. Adams and his colleagues recently expressed and purified the kinase domain of v-fps as an N-terminal GST-fusion protein ("GST-kin") from *E. coli*.¹ This enzyme is autophosphorylated at Tyr-1073 in the activation loop and phosphorylates exogenous peptide substrates with high catalytic efficiency. These advances set the stage for elaborate mechanistic studies designed to elucidate the key kinetic differences between SPKs and TPKs. To understand why these two classes of protein kinases phosphorylate different amino acid side chains, it is first important to determine complete kinetic mechanisms for both so that structure-function studies can be carried out. We have demonstrated that the kinetic mechanisms for these two enzyme classes are surprisingly similar. The major difference lies in the more than 5-fold slower rate constant for phosphoryl transfer from ATP to tyrosine relative to that of serine. Substrate binding and the rate of product release are similar in both PKA and v-fps.

The steady-state kinetic parameters for the phosphorylation of peptides **1**, **2**, **3**, and **4** are listed in Table 1. These data were obtained under fixed concentrations of ATP (3 mM) and MgCl_2 (13 mM) and

varied substrate. Previous studies have shown that the K_m for ATP is 250 μM under conditions of 10 mM free MgCl_2 so that no free enzyme is present in these experiments.¹

Peptide	Sequence	k_{cat} (sec^{-1})	K_{peptide} (μM)	$k_{\text{cat}}/K_{\text{peptide}}$ ($\text{mM}^{-1} \text{sec}^{-1}$)
1	EEEEIEEIE	13 ± 1	400 ± 50	32 ± 4
2	EAEIYEAEI	14 ± 1	400 ± 80	35 ± 7
3	DADIYDAID	14 ± 1	500 ± 80	28 ± 5
4	R ₅ AENLEYamide	0.35 ± 0.01	100 ± 20	3.4 ± 0.6

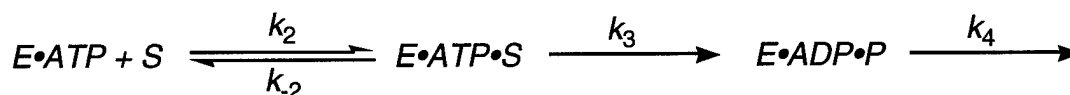
Table 1. Steady-State Kinetic Parameters for the Phosphorylation of Peptide Substrates by v-fps.

The effect of solvent viscosity on the rate parameters for enzyme-catalyzed reactions has been given a substantial amount of theoretical and experimental consideration.² The effect of solvent viscosity on the steady-state kinetic parameters of the catalytic subunit of the cAMP-dependent protein kinase ("PKA") has shown that the rate-determining step in catalysis under conditions of high substrate and ATP concentrations is the release of one of the products.³ For this SPK, the rate of phosphoryl transfer is fast relative to the release of ADP.

For a simple bimolecular process in solution, the association (k_1) and dissociation (k_{-1}) rate constants are inversely related to the intrinsic solvent viscosity at constant temperature,

$$\frac{k_1^o}{k_1} = \frac{k_{-1}^o}{k_{-1}} = \eta^{\text{rel}}$$

where η^{rel} is the ratio of the intrinsic solution viscosity in the presence and the absence of viscosogen and k_1^o (k_{-1}^o) and k_1 (k_{-1}) are the rate constants in the absence and presence of viscosogen, respectively. Scheme I describes the minimal kinetic mechanism for the phosphorylation of peptide substrates by GST-kin under saturating concentrations of ATP.



Scheme I

In this mechanism, substrate binds the $E \cdot \text{ATP}$ binary complex by the association and dissociation rate constants, k_1 and k_{-1} , respectively. The catalytic step, k_3 , describes the irreversible, unimolecular rate constant for the transfer of the phosphoryl group of ATP to the hydroxyl moiety of tyrosine. This step is presumed to be favorable since the reverse reaction for many protein kinases (i.e., phosphorylation of ADP) is very slow. The overall equilibrium constant for the phosphorylation reaction catalyzed by PKA was determined by ^{31}P NMR to be approximately 3000 at pH 7.2.⁴ This places an internal equilibrium constant of approximately 100 on the phosphorylation of Leu-Arg-Arg-Ala-Ser-Leu-Gly ("kemptide"). The final step in Scheme I (k_4) describes the net bimolecular rate constant for the release of both products. Since the viscosity measurements cannot distinguish between the dissociation rate constants for the

phosphorylated peptide and ADP, it is possible that k_4 may be limited by either product or partially limited by both.

By applying the steady-state assumption, a rate law describing Scheme I can be written and the relevant steady-state kinetic parameters extracted. The equations below represent the expressions for k_{cat} and $k_{cat}/K_{peptide}$ for Scheme I.

$$k_{cat} = \frac{k_3 k_4}{k_3 + k_4}$$

$$\frac{k_{cat}}{K_{peptide}} = \frac{k_2 k_3}{k_{-2} + k_3}$$

The equations for the linear functions of the ratios of k_{cat} and $k_{cat}/K_{peptide}$ in the absence and presence of viscosogen versus η^{rel} are shown below:

$$(k_{cat})^\eta = \frac{k_3}{k_3 + k_4^o}$$

$$\left(\frac{k_{cat}}{K_{peptide}}\right)^\eta = \frac{k_3}{k_{-2}^o + k_3}$$

where $(k_{cat})^\eta$ and $(k_{cat}/K_{peptide})^\eta$ are the slopes of $(k_{cat})/k_{cat}$ and $(k_{cat}/K_{peptide})/(k_{cat}/K_{peptide})$ versus η^{rel} , respectively, and k_2 and k_4^o are the rate constants in the absence of viscosogen. These two slope values lie between the theoretical limits of 0 and 1 where the former indicates that the enzyme-catalyzed reaction is not diffusion-controlled and the latter indicates that the reaction is completely diffusion-controlled.

Peptide	k_{cat}^η	$(k_{cat}/K_m)^\eta$	K_d (μM)	k_3 (sec^{-1})	k_4 (sec^{-1})
1	0.74 ± 0.08	0	1500 ± 500	50 ± 8	18 ± 2
2	0.63 ± 0.07	0	1100 ± 300	38 ± 5	22 ± 3
3	0.51 ± 0.09	0	1200 ± 300	32 ± 7	25 ± 4
4	0	0	100	0.35	> 3.5

Table 2. Effects of Solvent Viscosity on the Steady-State Kinetic Parameters.

Since $(k_{cat}/K_{peptide})$ is zero (Table 2), all four peptides dissociate faster than they are phosphorylated by GST-kin (i.e., $k_3 \ll k_{-2}$). This implies that the substrates are in rapid equilibrium with the enzyme and no definitive value can be placed on their dissociation rate constants. Since intermediate values for $(k_{cat})^\eta$ are obtained for peptides 1, 2, and 3, the rate constant for phosphoryl transfer must be partially rate-determining at high substrate concentration. Table 2 lists the rate constants for phosphoryl transfer and net product release for peptides 1, 2, and 3. For these peptides, the rate constants of phosphoryl transfer and product release are close in value indicating that GST-kin does not discriminate between these three substrates. Although the exact value of k_{-2} in Scheme I cannot be determined, the K_d for substrate binding

to the binary complex, $E \cdot ATP$, can be estimated. In Scheme I, $K_{peptide}$ can be related to the individual rate constants when $k_2 \gg k_3$ by the following equation:

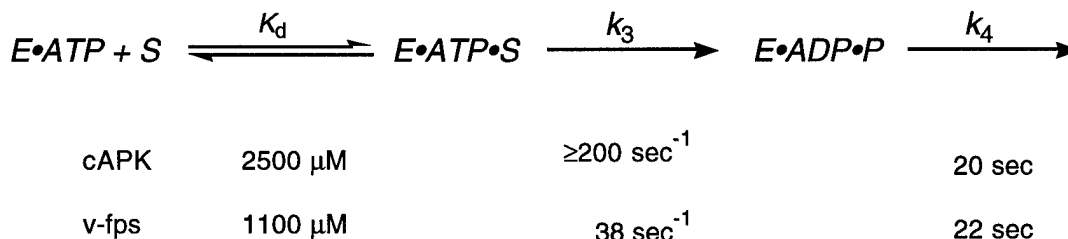
$$K_{peptide} = K_d \times \frac{k_4}{k_3 + k_4}$$

where $K_d = k_2/k_1$. The thermodynamic dissociation constants for peptides **1** - **4** can be estimated from the values of $K_{peptide}$, k_3 , and k_4 . These values are listed in Table 2. Peptides **1**, **2**, and **3** bind with similar affinity while peptide **4** binds 10-fold more tightly.

The sequence of peptide **1** is based on the optimum substrate for v-fps derived from a random peptide library.⁵ As a control we prepared peptide **2**, which we expected to serve as a relatively ineffective substrate. Since the glutamic acid residues at the P-3 and P-2 positions are important for substrate recognition, we replaced each of these negatively charged residues with an alanine moiety. Much to our surprise, we found that GST-kin phosphorylates peptide **2** as efficiently as peptide **1**. To further probe the substrate specificity of GST-kin, all of the glutamate residues in peptide **2** were replaced by aspartate residues (peptide **3**). Again, GST-kin phosphorylates this peptide as effectively as peptides **1** and **2**. Thrombin cleavage studies have previously shown that removal of GST does not influence the steady-state kinetic parameters for the phosphorylation of peptide **1** so that these similarities are not an artifact of the fusion system.¹

The effect of solvent viscosity on the phosphorylation of peptide **4** provides a good control for the interpretation of viscosometric effects and offers new insight into the substrate specificity of the kinase domain of v-fps. Elevated solvent viscosity ($\eta^{rel} = 1-4$) is not expected to adversely affect the structure of protein kinases. Viscous solutions of 36% glycerol have no effect on the circular dichroism spectra or the stability of the catalytic subunit of PKA indicating that both the structure of the enzyme is intact in this viscosogen.³ The lack of an effect on the steady-state kinetic parameters for peptide **4** (Table 2) indicate that viscosogens do not influence greatly the structure of the kinase domain or act as inhibitors of the enzyme's activity.

Comparison of v-fps and PKA: Our data provides, for the first time, a detailed comparison of the catalytic properties of SPKs and TPKs by evaluating the individual steps in the mechanism. In depth viscosity studies of the catalytic subunit of PKA illustrate that the substrate kemptide is in rapid equilibrium with the enzyme and that the rate of phosphoryl transfer from ATP to serine is approximately 10-fold higher than the release of the products (200 versus 20 sec⁻¹) under conditions of 10 mM free Mg²⁺.³ A comparison of this kinetic mechanism with that for the kinase domain of v-fps shows many similarities. Scheme II lists the rate constants for the phosphorylation of peptide **2** and kemptide by the kinase domain of v-fps and the catalytic subunit of PKA, respectively.



Scheme II

Two similarities are apparent in Scheme II. First, peptide **2** and kemptide bind to the active sites of their respective enzymes with similar affinities. Second, the net rate constant for the release of the products is the same. The most significant difference between the two kinetic mechanisms lies in the 5-fold or more slower rate of phosphoryl transfer in GST-kin compared to PKA. This difference has only a

small effect on the maximal rate constant, k_{cat} (20 sec^{-1} in PKA and 14 sec^{-1} in GST-kin) but a very large effect on $k_{\text{cat}}/K_{\text{peptide}}$. The $k_{\text{cat}}/K_{\text{peptide}}$ value for PKA is 1000 $\text{mM}^{-1} \text{sec}^{-1}$ while that for GST-kin is 35 $\text{mM}^{-1} \text{sec}^{-1}$. This 30-fold difference does not originate from tighter binding of the substrate. We consider the 2-fold difference in K_d values in Scheme II to be insignificant compared to the 30-fold difference in $k_{\text{cat}}/K_{\text{peptide}}$ for the two enzymes. Furthermore, this difference in $k_{\text{cat}}/K_{\text{peptide}}$ does not stem from a larger maximal rate constant, k_{cat} but rather from a smaller K_{peptide} for PKA. The catalytic efficiency of PKA as measured by the apparent second order rate constant, $k_{\text{cat}}/K_{\text{peptide}}$, is higher than GST-kin because it maintains a high rate of phosphoryl transfer relative to the net release rate of the products ($k_3 \gg k_4$), thereby, lowering the apparent dissociation constant, K_{peptide} . This finding stresses that the source of catalytic efficiency for protein kinases cannot be established based on cursory steady-state kinetic analysis alone but must be weighed carefully in light of other steps in a detailed kinetic mechanism.

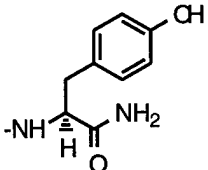
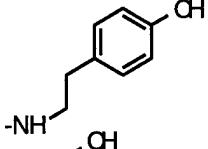
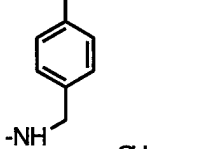
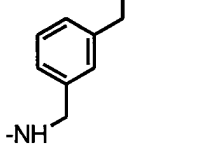
We have previously demonstrated that SPKs will catalyze the phosphorylation of a wide variety of synthetic alcohol-containing residues, including achiral species.⁶ This observation is particularly germane to inhibitor design since it implies that these enzymes are relatively accommodating with respect to incorporating a multitude of structural motifs within their active site regions. In addition, we have found that the active site substrate specificity of SPKs is remarkably dissimilar, even for those enzymes that are closely related.⁷ This observation suggests that subtle differences in active site structure among kinase family members may ultimately lead to the creation of highly specific protein kinase inhibitors. Consequently, our initial goals were to explore the ability of TPKs to recognize residues other than the standard tyrosine moiety and to determine if these enzymes exhibit a difference in specificity for abiotic alcohol-containing compounds.

Our initial efforts focused on c-Src. The vast majority of the compounds illustrated in Tables 3 - 5 serve as substrates for c-Src. Nevertheless, there are some intriguing facets of the active site substrate specificity of this tyrosine-specific protein kinase:

A. *Achiral aromatic and benzylic alcohols serve as c-Src substrates.* c-Src will not only phosphorylate the aromatic alcohol of tyrosine, but the hydroxyl moiety of achiral residues as well. For example, the tyramine-containing peptide **6** exhibits a somewhat better K_m than that of the parent peptide **5** (Table 3). This behavior is in marked contrast to that displayed by PKA and PKC, where peptide substrates containing achiral residues exhibit elevated K_m values relative to their chiral amino acid counterparts.⁷ We have also previously shown that PKA will phosphorylate both aliphatic and aromatic alcohols.^{6,8} Consequently, we investigated the ability of c-Src to catalyze the phosphorylation of the benzylic alcohol-containing peptides **7** and **8**. Both peptides serve as substrates, with species **7** exhibiting a relatively impressive K_m but a modest V_{max} . There are several noteworthy features concerning these results with respect to inhibitor design. Inhibitors, particularly transition state analogs or suicide substrates, often contain elaborate functionality whose preparation can be time-consuming. The latter is especially true if the key functionality of interest must be incorporated onto a chiral residue. The fact that c-Src will recognize and catalyze the phosphorylation of structurally simple achiral alcohols should simplify the synthesis of novel inhibitory species. In addition, the active site of c-Src appears to be particularly accommodating since the enzyme phosphorylates both aromatic and aliphatic alcohols, including the two structurally dissimilar benzylic alcohols **7** and **8**.

B. *Straight chain achiral aliphatic alcohols serve as c-Src substrates.* PKA will not catalyze the phosphorylation of tyrosine.⁹ However, this "serine/threonine"-specific kinase will phosphorylate aromatic alcohols as long as the hydroxyl moiety on these residues can be positioned in the active site in a manner comparable to that of serine or threonine. In an analogous fashion, one might predict that if an aliphatic side chain can properly orient a hydroxyl functionality into the active site of a tyrosine-specific protein kinase, then phosphoryl transfer should ensue as well. To a rough approximation, this corresponds to a residue containing a side chain length of six carbon atoms. Therefore, one might expect that chain lengths much smaller or larger than this ideal would not be able to optimally position the

Table 3. K_m (μM) and V_{max} (nmol/min-mg) values for the aromatic and benzylic alcohol-containing peptides 5 - 8. The values are given as the average $\pm$ S.D.

RRRRRLEELL-amino alcohol	c-Src		v-Abl	
	K_m	V_{max}	K_m	V_{max}
5 	789 $\pm$ 202	2190 $\pm$ 325	524 $\pm$ 73	34.6 $\pm$ 2.0
6 	396 $\pm$ 61	309 $\pm$ 31	982 $\pm$ 200	5.0 $\pm$ 0.6
7 	182 $\pm$ 34	53 $\pm$ 3	764 $\pm$ 136	4.9 $\pm$ 0.3
8 	645 $\pm$ 78	111 $\pm$ 6	889 $\pm$ 120	5.3 $\pm$ 0.2

phosphoryl acceptor hydroxyl moiety in the appropriate region of the enzyme active site. In spite of this eminently reasonable assumption, it is clear that c-Src does not discriminate in the expected fashion on the basis of chain length (Table 4). For example, c-Src phosphorylates peptide **9**, in spite of the fact that the distance from the hydroxyl moiety to the peptide backbone is identical to that present in serine, and significantly shorter than that in tyrosine. Furthermore, there is little variance in k_{cat}/K_m from the two carbon-bearing alcohol (i.e. **9**) up to the ten carbon-containing species (i.e. **16**). Indeed, only with peptide **17**, which possess a side chain of twelve carbon atoms, is there a significant loss in substrate effectiveness. These observations are certainly not in accord with those from our earlier analysis of the substrate specificity of PKA and PKC. Clearly, c-Src does not conform to the limitations displayed by these related enzymatic species. What is the structural basis for this behavior? In addition, if the tyrosine-specific c-Src is able to phosphorylate **9**, shouldn't it be able to phosphorylate serine as well?

C. An L-serine-containing peptide serves as a substrate for c-Src. Since the ethanolamine-bearing peptide **9** is a substrate for c-Src, we investigated the possibility that the corresponding serine-containing peptide **18** could also serve as a substrate for this "tyrosine-specific" protein kinase. Indeed, the latter is a substrate (Table 5), albeit a significantly poorer one than the former. Nevertheless, this observation is clearly reminiscent of the behavior of a small group of protein kinases; namely those that are apparently able to phosphorylate serine, threonine, and tyrosine.¹⁰ What is the structural basis for this dual specificity? One possibility is that the active site is inherently flexible and is able to assume a conformation that structurally complements that of the active site-bound residue. However, this does not account for the difference in k_{cat}/K_m values between peptides **9** and **18**. An alternative explanation for this "dual-specificity" assumes a more conventional conformationally-fixed active site. In the model illustrated in

Table 4. K_m (μM) and $V_{\max}$ (nmol/min-mg) values for the alcohol-containing peptides 9 - 17. The values are given as the average $\pm$ S.D.

RRRRRLEELL-amino alcohol		c-Src		v-Abl	
		K_m	$V_{\max}$	K_m	$V_{\max}$
9	-NH-(CH ₂) ₂ OH	83 $\pm$ 32	38 $\pm$ 4	-	-
10	-NH-(CH ₂) ₃ OH	128 $\pm$ 17	43 $\pm$ 1	-	-
11	-NH-(CH ₂) ₄ OH	142 $\pm$ 5	44 $\pm$ 1	2641 $\pm$ 311	1.0 $\pm$ 0.1
12	-NH-(CH ₂) ₅ OH	170 $\pm$ 40	54 $\pm$ 5	962 $\pm$ 64	1.1 $\pm$ 0.1
13	-NH-(CH ₂) ₆ OH	208 $\pm$ 24	80 $\pm$ 3	1735 $\pm$ 112	2.7 $\pm$ 0.1
14	-NH-(CH ₂) ₇ OH	149 $\pm$ 5	80 $\pm$ 1	1056 $\pm$ 68	2.6 $\pm$ 0.1
15	-NH-(CH ₂) ₈ OH	166 $\pm$ 8	98 $\pm$ 2	547 $\pm$ 101	2.3 $\pm$ 0.2
16	-NH-(CH ₂) ₁₀ OH	300 $\pm$ 62	70 $\pm$ 8	434 $\pm$ 57	1.6 $\pm$ 0.1
17	-NH-(CH ₂) ₁₂ OH		< 25	443 $\pm$ 118	0.9 $\pm$ 0.1

Figure I, the peptide is able to occupy any of several closely positioned loci on the enzyme surface, each of which lies a characteristic distance from the phosphorylation site. In this scenario, an alcohol moiety positioned on a "shorter-than-ideal" side chain (e.g. 9) can assume the proper location in the active site if

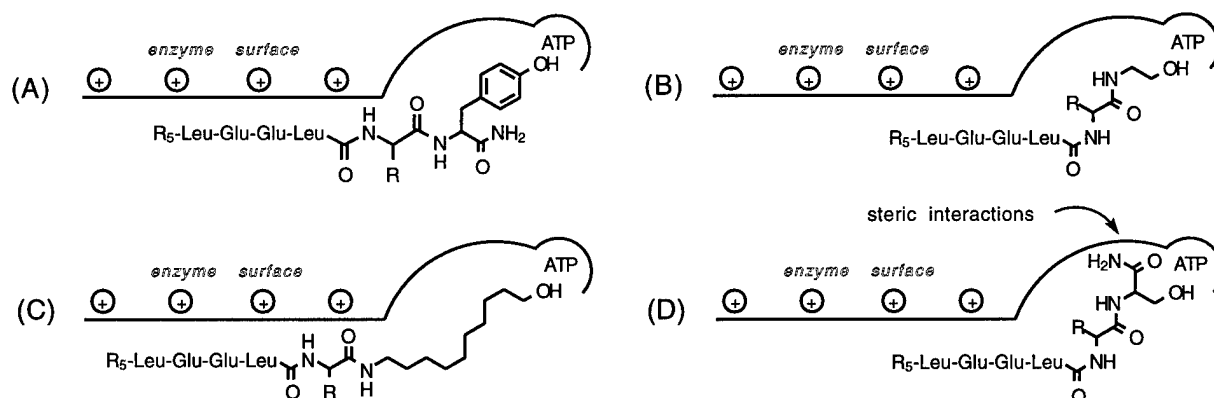
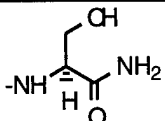
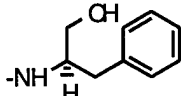
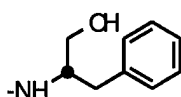
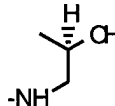
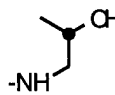


Fig. I. Interactions of alcohol-containing peptides with c-Src. (A) The tyrosine-bearing peptide 5 is aligned on the enzyme surface to properly position the aromatic alcohol in the active site. (B) Since the ethyl side chain of 9 is short relative to that in 5, the P-1 residue must be partially inserted into the active site in order for the hydroxyl functionality to be correctly situated in the active site. (C) The side chain of 17 is of sufficient length so that the peptide backbone may be located at a greater distance from the active site than that in (B). (D) Unfavorable steric interactions between active site residues and the α -amide moiety of L-serine may arise upon interaction of 18 with c-Src.

Table 5. K_m (μM) and V_{max} (nmol/min-mg) values for the alcohol-containing peptides 18 - 23. The values are given as the average $\pm$ S.D.

RRRRRLEELL-amino alcohol		c-Src		v-Abl	
		K_m	V_{max}	K_m	V_{max}
18		452 ± 65	46 ± 2	-	-
19		-	< 25	-	-
20		-	<25	-	-
21		-	<25	-	-
22		-	<25	-	-
23	D-Tyr-amide	328 ± 27	79 ± 2	1639 ± 336	1.6 ± 0.5

a portion of the peptide backbone can be accommodated in the active site region. For an alcohol-functionality located on a "longer-than-ideal" side chain (e.g. 17), the peptide backbone would be positioned at a greater distance from the active site. *Implicit within this model is the assumption that the interaction between the peptide substrate and the enzyme surface is not fixed to a single prescribed site, but instead fluctuates according to the structural nature of the substrate.* For example, this behavior might be observed if the enzyme surface is characterized by an extended region of positive charge density. Under these circumstances, the peptide could favorably associate with the enzyme at a multitude of sites. In addition, this model may offer an explanation for the disparate K_m values associated with the phosphorylation of peptides 9 and 18. While incorporation of the alcohol moiety of 9 into the active site should not be structurally demanding, insertion of the hydroxyl functionality of serine (i.e. 18) by necessity, positions a structurally obtrusive amide group in the activesite. If this notion is correct, then a residue that contains a substituent even larger than the amide moiety should produce a poorer substrate. Indeed, peptide 19, which possesses a benzyl substituent at the α -position, is such a poor substrate that we were unable to obtain accurate kinetic constants. We also examined the ability of peptide 21, which contains a methyl substituent at the β position, to serve as a substrate for c-Src. This peptide proved to be an exceedingly poor substrate as well. Finally, we prepared the diastereomers of 19 (i.e. 20) and 21 (i.e.22). These peptides also failed to serve as efficient substrates. In short, it is clear that substituents at the α or β positions of the phosphorylatable residue dramatically interfere with the ability of short chain alcohols to serve as a substrates for c-Src. While these observations are certainly consistent with the model depicted in Figure I, it remains to be seen whether this type of analysis is applicable to the several dual-specificity protein kinases that have recently been identified.

D. c-Src phosphorylates a D-tyrosine residue in an active site-directed peptide. Although it is clear that substituents at either the α or β positions in the ethylalcohol-based species (9) markedly lower

substrate efficacy (cf. 18-22), this is obviously not the case with the aromatic alcohol-based peptides **5** and **6**. Indeed, the α -amide substituent in **5** is actually beneficial in terms of k_{cat}/K_m . Evidently, α -substituents can be accommodated near the active site as long as these substituents are positioned some distance from the alcohol that suffers phosphorylation. This is also consistent with the model depicted in Figure I. While it is clear that an α -substituent can have favorable consequences in terms of substrate efficacy for aromatic alcohols (e.g. **5**), we wondered whether the configuration at the α -center played any role in substrate recognition. Indeed, the D-tyrosine-containing peptide **23** does serve as a substrate for c-Src, although not as efficiently as its' isomeric counterpart **9**. We have not yet conclusively resolved how a D-amino acid residue can be recognized by the active sites of PKC and c-Src. In particular, if the active site regions of these enzymes are analogous to that of PKA, then the Michaelis complex of a D-amino acid residue should experience severe steric interactions that would preclude the requisite alignment of the hydroxyl moiety in the active site. The alcohol functionality in a D-residue could be presented to the active site of PKC in a structurally acceptable fashion if the peptide is able to bind to the enzyme surface in a retrograde C- to N-terminus sense. An analogous binding mode may be in operation for c-Src as well.

The oncogenic form of *abl* was first discovered as the transforming gene of Abelson murine leukemia virus.¹¹ In humans, the normal cellular homolog, *c-abl*, is involved in chronic myelogenous leukemias and some acute lymphocytic leukemias.¹² In these leukemias, a chromosomal translocation takes place in which 5' sequences of the *bcr* gene become fused to *abl*, generating an oncogenic Bcr-Abl fusion protein. In all cases, there is a strong correlation between oncogenic transformation and tyrosine kinase activity. v-Abl and c-Src are members of the nonreceptor tyrosine kinase family and, as such, share many common features. Both contain SH2 and SH3 domains,¹³ and both src and several forms of abl undergo myristylation,¹⁴ which promotes membrane association. These enzymes also share a strong sequence homology within their respective active site regions.¹⁵ Finally, both kinases exhibit overlapping substrate specificities *in vitro*; a feature that can render the design of kinase-specific inhibitors problematic. In spite of these similarities, we have found that these enzymes can be distinguished from one another by their active site substrate specificities.

Both v-Abl and c-Src phosphorylate aromatic and benzylic alcohols (Table 3). For example, peptide **6**, which contains an tyramine residue, is a substrate for both enzymes. While it is significant that these tyrosine-specific kinases will phosphorylate an achiral residue, it is equally noteworthy that **6** displays an enhanced specificity for c-Src versus v-Abl relative to that of the tyrosine-containing peptide **5** (Table 3). In contrast, the aminomethylbenzyl alcohol **7** is almost identical to L-tyrosine in its ability to discriminate between these enzymes. Finally, the benzylic alcohol in **8** is less efficient than L-tyrosine in distinguishing between c-Src and v-Abl. The decrease in c-Src/v-Abl specificity as one moves from compound **6** to **7** to **8** is largely due to changes in activity of c-Src toward these substrates. All three peptides are virtually identical as substrates for v-Abl. In short, while c-Src does discriminate between aromatic and aliphatic alcohols (i.e. **6** versus **7** or **8**), v-Abl does not.

The results in Table 3 clearly demonstrate that both c-Src and v-Abl will phosphorylate aromatic and benzylic (i.e. aliphatic) alcohols. However, each of these substrates possesses an aromatic moiety. Furthermore, the hydroxyl functionality in species **6** - **8** is oriented, relative to the peptide backbone, in a manner reminiscent of that found in the tyrosine-containing peptide **5**. Is this structural alignment of functionality a requirement for recognition by tyrosine-specific protein kinases? In order to address this question, we prepared a series of peptides containing straight chain aliphatic alcohols. In a broad sense, both v-Abl and c-Src utilize these species as substrates (Table 4). Therefore, it is evident that an aromatic moiety is not required for substrate recognition by either of these enzymes. Furthermore, the distance between the aromatic alcohol in tyrosine and the α -amino group is roughly equivalent to a chain containing 5-7 methylene groups. Consequently, one might predict that compounds **12** - **14** should serve as the most efficient substrates for v-Abl and c-Src, and that the remainder of the compounds listed in Table 4 would be extraordinarily inefficient substrates (if they are even phosphorylated at all). As we noted earlier, the k_{cat}/K_m values for peptides **9** - **16** do not differ to any significant extent with this enzyme. Only at a chain length of 12 carbon atoms is a drop-off in substrate efficacy apparent. In this particular case, we

were only able to obtain a lower limit of the maximal velocity. This is due to the fact that substrate saturation appears to occur at a relatively low concentration ($<150\ \mu\text{M}$) and that at these concentrations the rate of phosphorylation of **17** is exceedingly weak. This implies that the concentrations of **17** employed may exceed the K_m . As a consequence, we are able to provide only an estimate of $V_{\max}$ for this peptide, as well for peptides **19-22** in Table 4.

In contrast to the behavior displayed by c-Src toward peptides **9-17**, v-Abl exhibits a definite dependence upon chain length. Short chains, such as the ethanolamine (**9**) and propanolamine (**10**) derivatives, are extremely poor v-Abl substrates. In the case of v-Abl, although high concentrations (1 - 2 mM) of peptides **9** and **10** provide weak rates of phosphorylation, we did not detect any evidence of substrate saturation. This implies that the K_m values for peptides **9** and **10** are quite large, a notion consistent with the results obtained for peptide **11**, the next higher homolog. At substrate concentrations significantly below the K_m , the Michaelis-Menten equation reduces to $v = (k_{\text{cat}}/K_m)[E][S]$, which allows the ready acquisition of k_{cat}/K_m . However, since we are uncertain of the actual K_m , the k_{cat}/K_m values indicated for peptides **9 - 10** and **18** in Table 4 should be viewed as approximations. Finally, the v-Abl-catalyzed rates of phosphorylation for peptides **19 - 22** are so weak that we could only provide an estimate of k_{cat}/K_m in these cases.

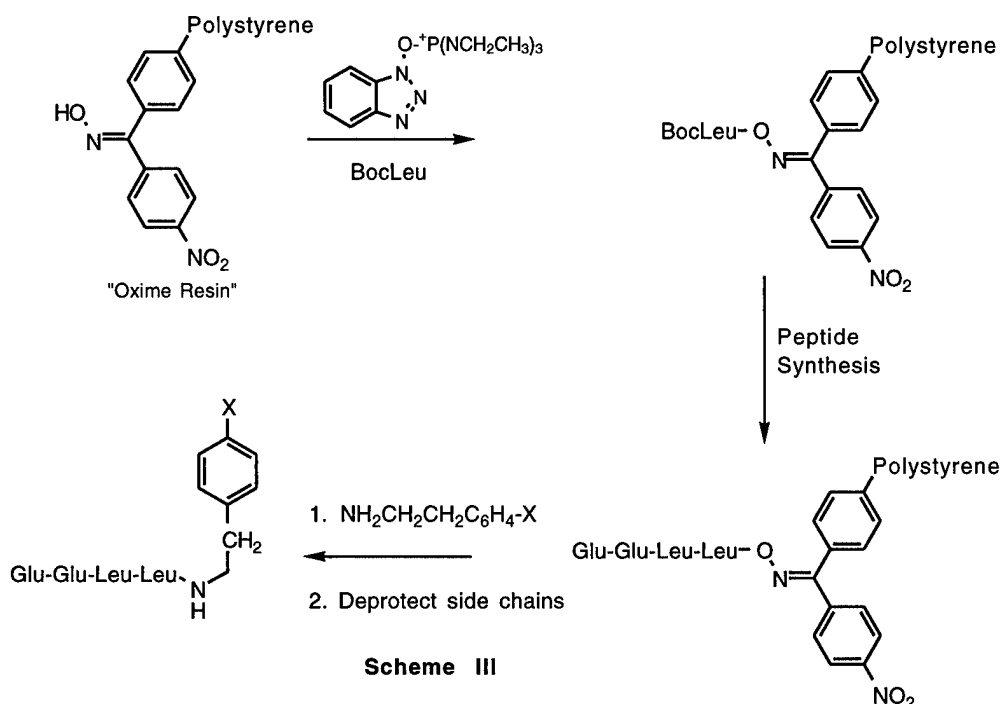
Based upon the k_{cat}/K_m value extracted for peptide **9**, it is evident that this species is a significantly more potent substrate ($>2,000$ -fold) for c-Src than for v-Abl. As such, peptide **9** is the most accurate c-Src-targeted substrate that we have evaluated to date. However, this bias in favor of c-Src decreases in a dramatic fashion with increasing chain length. This decrease is a consequence of an improvement in k_{cat}/K_m for v-Abl with the longer aliphatics, which in turn is due to a decrease in K_m . Indeed, the K_m for peptide **20** is even smaller than that obtained for the L-tyrosine peptide **5**.

Protein kinases are commonly classified by their ability to phosphorylate aliphatic (i.e. serine/threonine) or aromatic (i.e. tyrosine) hydroxyl groups in naturally occurring proteins. However, as is apparent from this active site substrate specificity study, these categories break down with synthetic substrates. Nonetheless, a new pattern is beginning to emerge with respect to the classification of protein kinases. We have now found that peptide **23**, which contains a D-tyrosine residue, serves as a substrate for both c-Src and v-Abl. The following features concerning this activity are notable: First, although v-Abl will phosphorylate peptides containing either D- or L-tyrosine, the latter is a significantly more efficient (65-fold) substrate than the former. Second, the D-tyrosine-containing peptide (**23**) is a 5-fold poorer substrate than the corresponding achiral species (**6**). Consequently, it is clear that the inverted configuration associated with D-tyrosine, in conjunction with the amide substituent, *actively* interferes with the ability of this species to be recognized as a substrate for v-Abl. Third, peptide **23** serves as a 230-fold more efficient substrate for c-Src than for v-Abl, which may indicate that the former is able to accommodate D-amino acid residues more readily than the latter.

Given the key role played by tyrosine kinases in transducing growth promoting signals from the cell surface to the nucleus, it is not surprising that there has been intense interest in developing potent inhibitors for individual members of this enzyme family. The majority of successful inhibitors described to date are targeted to the ATP binding site.¹⁶ In marked contrast, the few peptide-based species designed to impede protein substrate binding have been, in general, disappointing. These peptides often contain a nonphosphorylatable phenylalanine moiety in place of the tyrosine residue. Their poor inhibitory efficacy may be due to the fact that the aromatic alcohol of tyrosine has been replaced by a single hydrogen atom, and the latter is incapable of participating in any productive interactions with active site residues. Is it possible to replace the tyrosyl hydroxyl with a functional group that simultaneously blocks phosphoryl transfer yet promotes enzyme affinity? The obvious way to address this question is to prepare phenylalanine derivatives containing a variety of functional groups positioned at the *para* position. These derivatives can then be inserted into active site-directed peptides, via solid phase peptide synthesis, and subsequently assayed for inhibitory potency. Unfortunately, the synthetic obstacles associated with this approach are impressive. First, although some phenylalanine analogs are commercially available, many structurally interesting derivatives are not. Consequently, a labor intensive synthetic research effort will be

required to generate a reasonable variety of phenylalanine derivatives for testing purposes. Second, for each inhibitor candidate to be examined, a complete peptidic species must be synthesized. Finally, some functional groups may simply not survive the harsh conditions of solid phase peptide synthesis, thereby limiting the range of compounds that can be investigated as nonphosphorylatable replacements for tyrosine.

We have previously demonstrated that protein kinases will catalyze the phosphorylation of alcohol-bearing residues at the C-terminus of active site-directed peptides. For example, c-Src utilizes Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Leu-Tyr-amide as a substrate.¹⁶ This observation provides a means to circumvent the synthetic disadvantages (enumerated above) associated with the preparation of peptides containing an assortment of internally-positioned hypermodified amino acid residues. The general approach is illustrated in Scheme III. First, the active site-directed peptide is synthesized employing a



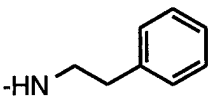
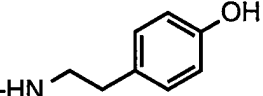
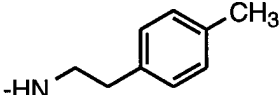
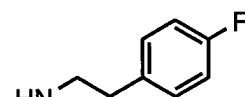
standard Boc protocol on a modified polystyrene support.⁴ The peptide can subsequently be simultaneously displaced from the solid support and condensed with an appropriate amine. This double displacement/condensation reaction is possible due to the labile nature of the oximate ester linkage between the peptide and the polystyrene bead. Glu-Glu-Leu-Leu-oxime•resin was treated with a variety of *para*-substituted *phenethylamine* derivatives ($\text{H}_2\text{NCH}_2\text{CH}_2\text{C}_6\text{H}_4\text{-X}$) to produce active site-directed peptides of the general structure $\text{Glu-Glu-Leu-Leu-HNCH}_2\text{CH}_2\text{C}_6\text{H}_4\text{-X}$. We note that, with this methodology it is not necessary to synthesize an entirely new peptide for each inhibitor candidate. Indeed, the oxime resin-attached Glu-Glu-Leu-Leu peptide was synthesized only once and then subsequently employed to generate the inhibitory species listed in Table 6. In addition, these phenethylamine analogs were not exposed to the conditions of solid phase peptide synthesis since these derivatives are attached to the peptide after solid phase peptide synthesis is completed. Finally, although some of the phenethylamine derivatives employed are not commercially available they are nonetheless more synthetically accessible than their phenylalanine homologs, since the former lack both a chiral center as well as a reactive carboxylate moiety.

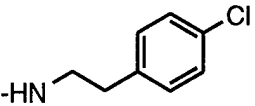
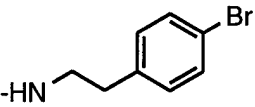
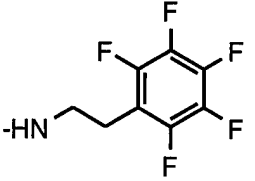
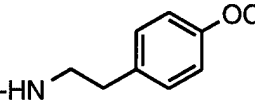
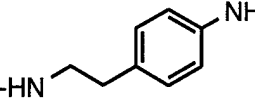
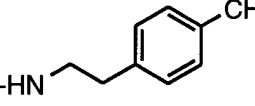
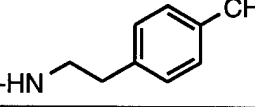
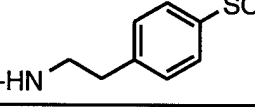
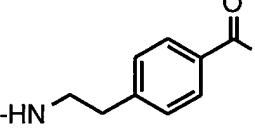
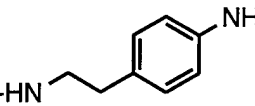
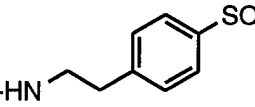
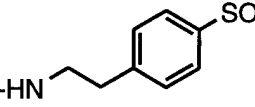
We examined a total of 20 different nonphosphorylatable phenethylamine derivatives (Table 6). Our initial survey of the inhibitory efficacy of these compounds focused on the acquisition of IC_{50} values at fixed ATP (100 μM) and peptide substrate (Arg-Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Leu-Tyr-amide, at

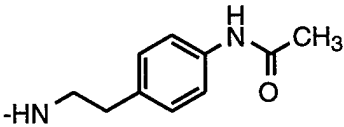
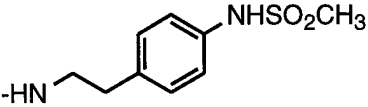
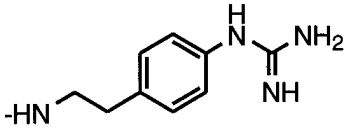
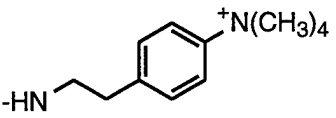
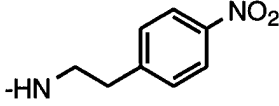
its K_m of 750 μM) since significantly larger quantities of inhibitor and, in particular, enzyme, are required to obtain K_i values. Compound **24**, which contains the parent phenethylamine itself, is structurally analogous to that of phenylalanine. The nearly 2 mM IC_{50} value associated with this species is in keeping with the poor inhibitory performances exhibited by other, previously described, phenylalanine-based tyrosine kinase inhibitors.¹⁷ For comparative purposes, we also investigated the inhibitory activity of the tyramine-containing analog **25**. This alcohol-bearing residue is phosphorylated by c-Src when attached to the C-terminal position of Arg-Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Leu- (**6**). However, since we attached tyramine to the tetrapeptide Glu-Glu-Leu-Leu-, any phosphorylation of the aromatic alcohol will be "invisible" to the phosphocellulose paper disc detection method. As an inhibitor of the c-Src-catalyzed phosphorylation of Arg-Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Leu-Tyr-amide, peptide **25** exhibits an IC_{50} of $300 \pm 10 \mu\text{M}$. In short, there is an apparent 7-fold difference in inhibitory efficacy between **24** and **25**. Clearly, it is tempting to attribute this difference in inhibitory activity to the presence of the hydroxyl group on **25**, a known hydrogen bond donor and acceptor. Is it possible to replace the hydroxyl moiety with a nonphosphorylatable functional group that retains (or even exceeds) the apparent enhancement in affinity for c-Src afforded by the aromatic alcohol?

The methyl-substituted derivative **26** is a slightly poorer inhibitor than **24**. The halogenated derivatives **27**, **28**, and **29** are also somewhat weaker as inhibitors compared to **24**. However, the pentafluoro derivative **30** is significantly more effective as an inhibitor than its monohalogenated counterparts. One possible explanation for this behavior is that the electron deficient aromatic ring in **30** may interact with an electron rich moiety in the active site of c-Src, thereby enhancing enzyme affinity. Interestingly, the methoxy-substituted species (**31**) contains a significantly more electron rich aromatic system than its counterpart in **24**, however, the inhibitory potency of **31** is nearly identical to that of **24**. This may imply that the electron density associated with the π system has little influence on active site affinity or, at the very least, that only profoundly electron deficient aromatic systems (i. e. as in **30**) interact with the active site in a unique fashion. An alternative explanation for the unusual behavior of **30** is based upon recent work by Whitesides and his colleagues.¹⁸ These investigators demonstrated that the apparent greater lipophilicity of fluorocarbons, relative to their hydrocarbon counterparts, is due to the larger hydrophobic surface area associated with the former. In short, the enhanced inhibitory potency of **30**, compared to that of **24**, may be due to this difference in relative hydrophobic surface area.

Table 6. IC_{50} values of c-Src inhibitory peptides.

Glu-Glu-Leu-Leu-NH(CH ₂) ₂ C ₆ H ₄ -X		IC_{50} (μM)
	24	1975 ± 170
	25	300 ± 10
	26	2470 ± 185
	27	2160 ± 30

	28	2940 ± 250
	29	2350 ± 285
	30	490 ± 40
	31	1730 ± 30
	32	2670 ± 110
	33	1600 ± 75
	34	1325 ± 140
	35	325 ± 15
	36	835 ± 30
	37	2050 ± 20
	38	2680 ± 230
	39	4820 ± 140

	40	4700 ± 240
	41	6300 ± 345
	42	410 ± 20
	43	2650 ± 160
	44	3140 ± 195

Although the functional groups in compounds **24** and **26-31** are structurally and/or electronically dissimilar, they do share two common chemical features. First, they all lack the ability to serve as hydrogen bond donors (such as the tyrosine hydroxyl). Second, they cannot participate in electrostatic interactions (as can the tyrosine alkoxide) with an oppositely charged species. Functionality that can participate in these types of interactions could markedly promote inhibitor efficacy by enhancing active site affinity. Of all the phenethylamine derivatives examined, perhaps the one that exhibits the greatest structural similarity to the aromatic alcohol of tyrosine is **32**. The alcohol in tyrosine and the amine in **32** are not only similar in size but are also electronically analogous in that they both can serve as hydrogen bond donors and acceptors. Furthermore, since the amine is directly positioned on the aromatic ring in **32**, it is not strongly basic and therefore will not be protonated under physiological conditions. Most importantly, the aromatic amine, unlike the corresponding alcohol, is not particularly acidic and therefore should not be deprotonated by the active site base. Rather, it could conceivably hydrogen bond to the latter without promoting phosphoryl transfer. Given these promising characteristics, the inhibitory potency of **32** is surprisingly poor. In fact, **32** is an even poorer inhibitor of c-Src than **24**. In contrast, and somewhat unexpectedly, the aliphatic amines **33** and **34** are slightly better inhibitors than **24**. The amine in **32** exhibits profoundly different structural properties than those in **33** and **34**. First, the latter two will be protonated under physiological conditions (of course, it is not clear if they bind to the active site in the positively charged form). In addition, **33** and **34** both enjoy a considerably greater degree of conformational mobility than that of the aromatic amine in **32**. In addition, the amines in **33** and **34** likely penetrate somewhat deeper into the active site than their counterpart in **32**. Is conformational mobility and/or the degree of active site penetration responsible for the enhanced inhibitory potencies associated with **33** and **34**? We prepared several compounds to address this question.

The sulfonamide **35** displays an IC_{50} of 325 μ M, which is significantly better than that exhibited by the benzylamine **33**. Indeed, the inhibitory potency of **35** is essentially identical to that displayed by the "substrate" **25**. The nitrogen atom in **35** is positioned, to a first approximation, somewhat analogously to that in **33**. However, the amine in **35** is clearly more conformationally restricted than its counterpart in **28**. In addition, the sulfonamide nitrogen is not basic enough to be protonated at physiological pH. In short, the sulfonamide functionality in **35** is a neutral conformationally-restricted version of the methylamine moiety in **33**. This may imply that it is the position of the nitrogen relative to

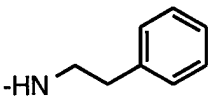
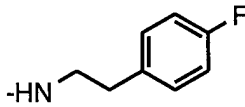
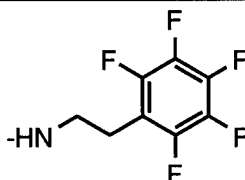
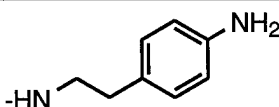
the aromatic ring, and not conformational mobility, that is crucial for inhibitory potency. Interestingly, **36**, which contains a carboxamide moiety at the *para* position, is not as potent an inhibitor as **35**. Clearly, additional structural factors must influence enzyme affinity (*vide infra*). We also prepared the derivative **37**, in which a nonbasic amine is now positioned further from the aromatic ring system than in **35**. Unfortunately, **37** is disappointing as an inhibitor.

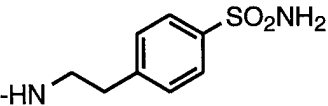
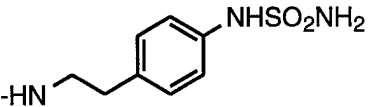
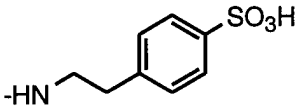
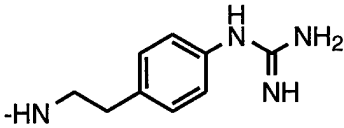
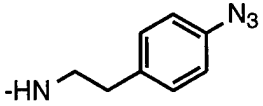
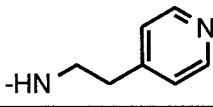
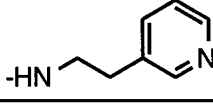
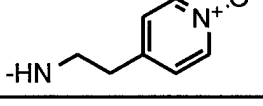
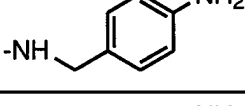
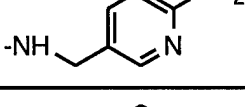
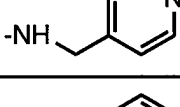
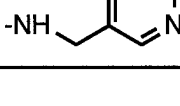
Is the sulfonyl group, and not the amine, responsible for the inhibitory properties of **35**? Although we viewed this as being unlikely, we did prepare the methyl sulfone (**38**) as well as the sulfonic acid (**39**). Both **38** and **39** lack the amine moiety present in **35**. In addition, the sulfonic acid moiety in **39** is negatively charged at neutral pH. Neither **38** nor **39** are effective inhibitors of c-Src.

Given the apparent requirement for a nitrogen atom, one (i.e. **33**) or two (i.e. **34**) atoms removed from the aromatic nucleus on the *para*-positioned side chain, we synthesized the guanidino-derivatized analog **42**. Indeed, this compound is nearly as effective as **35** in its inhibitory potency toward c-Src. In contrast, the bulky positively charged trimethylammonium derivative **43** is a poor inhibitor as well as its neutral, but polar, nitro-containing counterpart **44**.

Since **35** serves as the most potent inhibitor in this study, we decided to examine its mode of action in somewhat greater detail. In particular, the aromatic sulfonamide moiety in **35** is structurally reminiscent of various isoquinoline sulfonamides that have been previously shown to serve as protein kinase inhibitors by coordinating to the ATP binding site. Consequently, we were concerned that the phenylsulfonamide in **35** may actually be functioning as an ATP analog. However, peptide **35** serves as a competitive inhibitor versus variable peptide substrate and as a noncompetitive inhibitor versus variable ATP (data not shown). These results confirm that **35** functions as a Src kinase inhibitor in the intended fashion, namely, by coordinating exclusively to the protein, and not the ATP, binding site of c-Src. The K_i ($300 \pm 20 \mu\text{M}$) value obtained from the variable substrate experiment is analogous to the IC_{50} ($325 \pm 14 \mu\text{M}$) value exhibited by **35**.

Table 7. IC_{50} values of insulin receptor inhibitory peptides.

Glu-Glu-Glu-Glu-NH(CH ₂) ₂ C ₆ H ₄ -X		IC_{50} (μM)
	24	2230
	27	2585
	30	1765
	32	1130

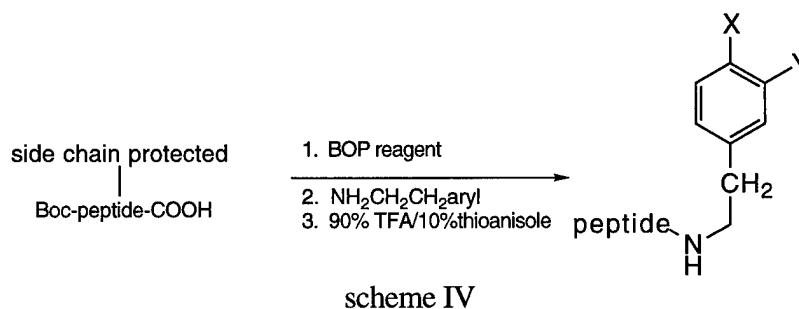
	35	2600
	37	1730
	39	1725
	42	1610
	49	1100
	50	1060
	51	685
	52	1310
	53	1100
	54	1100
	55	1080
	56	730

Do tyrosine-specific protein kinases exhibit the same active site specificity toward the inhibitory functionality of Table 6? We addressed this question by comparing the results described above for c-Src with those obtained for the insulin receptor ("IR") (Table 7). Interestingly, the very best inhibitors of c-Src proved to be, for the most part, ineffective as inhibitors of the IR. The lead sulfonamide-containing species **35** is a surprisingly impotent toward the IR. Indeed, the vast majority of compounds that we

previously tested as inhibitors of c-Src display little inhibitory potency toward the insulin receptor. One interesting exception is the *para*-amino substituted derivative **32**. This compound is a poorer inhibitor of the parent phenethylamine **24** for c-Src, but does display a somewhat better inhibitory profile against the IR. Although the later improvement is minor, it is notable in that the other species (**24**, **27**, **30**, **35**, **37**, **39**, and **42**) display nearly identical (and unimpressive) IC_{50} values. However, we've expanded the range of derivatives in this study to include heteroaromatics. In this regard, the *meta*-substituted pyridine moiety **51** is our best IR inhibitor to date. Most importantly, the results obtained with the pyridine-based species suggest that interactions at sites other than the *para*-position of the aromatic ring, can enhance enzyme affinity.

Clearly, tyrosine-specific protein kinases have evolved to coordinate an aromatic hydroxyl moiety within the active site region. Is it possible to retain the alcohol of tyrosine, yet affix additional functionality to the aromatic nucleus that not only precludes phosphoryl transfer but actually enhances enzyme affinity? We decided at the outset not to employ sterically demanding substituents *ortho* to the alcohol moiety since we were concerned that these types of substitution patterns might interfere with the ability of the aromatic hydroxyl group to engage in productive hydrogen bonding interactions with active site residues. We have found that dopamine, and its corresponding amino acid, L-Dopa, serve as potent nonphosphorylatable mimetics of tyrosine in c-Src-targeted peptides.

A dopamine-substituted peptide is a significantly more potent inhibitor of c-Src than the corresponding phenethylamine derivative. The phenethylamine-based peptide (**57**; X=Y=H) and its dihydroxy-counterpart (**58**; X=Y=OH) were prepared according to the synthetic scheme outlined in scheme IV.¹⁹ The dopamine-derivative **58** is 31-fold more effective as an inhibitor of c-Src than peptide **57**.



A dopamine-substituted peptide is not a c-Src substrate. Protein kinases are commonly assayed for activity by quantitating the incorporation of ^{32}P (from ^{32}P - γ [ATP]) into serine, threonine, and/or tyrosine residues positioned within positively charged active site-directed peptides. The latter coordinate to a negatively charged phosphocellulose disk, which can be subsequently counted for radioactivity. Since **58** lacks the primary sequence necessary for binding to phosphocellulose disks, we prepared an arginine-substituted derivative, Arg-Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Glu-dopamine, (**59**). The substrate efficacy of **59** was then compared with that of the tyramine-based analog Arg-Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Glu-tyramine (**60**), a c-Src substrate.²⁰ In addition, we prepared the nonphosphorylatable phenethylamine-based peptide, Arg-Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Glu-phenethylamine (**61**), as a control. As is apparent from Fig. II, peptide **59** is inert under conditions in which **60** suffers phosphorylation.

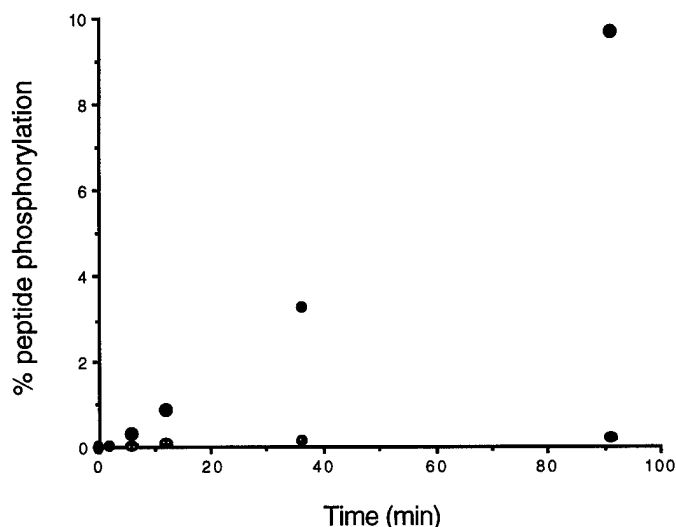


Figure II. c-Src-catalyzed phosphorylation of **59** (+), **60** (•), and **61** (◐) as a function of time. After 16 h, 50% of **60** underwent phosphorylation, whereas less than 1% of **59** or **61** is ^{32}P -labeled under identical conditions (the 16 h data points are not shown). Since **61** lacks a hydroxyl moiety, it is unlikely that the trace levels of radioactivity associated with **59** and **61** are due to phosphorylation.

An *L*-Dopa-substituted peptide is a significantly more potent inhibitor of c-Src than the corresponding phenylalanine derivative. Do the inhibitory trends observed with C-terminus-substituted peptides (i.e. **57** and **58**) hold in more conventional peptidic environments? In order to address this question we prepared the peptides Glu-Glu-Leu-Leu-**Phe**-Gly-Glu-Ile (**62**) and Glu-Glu-Leu-Leu-**Dopa**-Gly-Glu-Ile (**63**). The primary sequence encompassing the Phe and Dopa residues was chosen, in part, from the results of a previous study using a combinatorial peptide library to assess c-Src-specificity.²¹ As is evident from Table 8, the inhibitory trend between **57** and **58** holds for the conventional peptide dyad

Table 8. Alcohol-containing nonphosphorylatable inhibitors of c-Src.

Inhibitor	IC ₅₀ (μM)	K _i (μM)
Glu-Glu-Glu-Glu- phenethylamine (57)	1650 ± 10	—
Glu-Glu-Glu-Glu- dopamine (58)	53 ± 2	—
Glu-Glu-Leu-Leu- Phe -Gly-Glu-Ile (62)	950 ± 25	860 ± 20
Glu-Glu-Leu-Leu- Dopa -Gly-Glu-Ile (63)	29 ± 2	16 ± 3

62 and **63**. In the latter case, **63** is a 33-fold more effective inhibitor than **62**. Furthermore, both peptides are competitive inhibitors versus peptide substrate (Fig. III). Indeed, the difference in K_i values

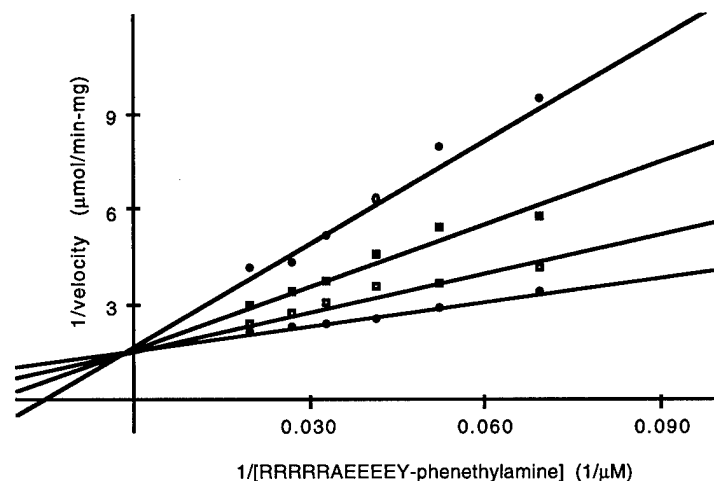


Figure III. The Lineweaver-Burk double reciprocal plot of the L-Dopa-containing peptide **63** as a function of varied peptide substrate.

exhibited by **62** and **63** is even more substantial (55-fold) than that observed for the corresponding IC_{50} s. Finally, since the Dopa-containing peptide serves as a *noncompetitive* inhibitor versus variable ATP ($K_i =$

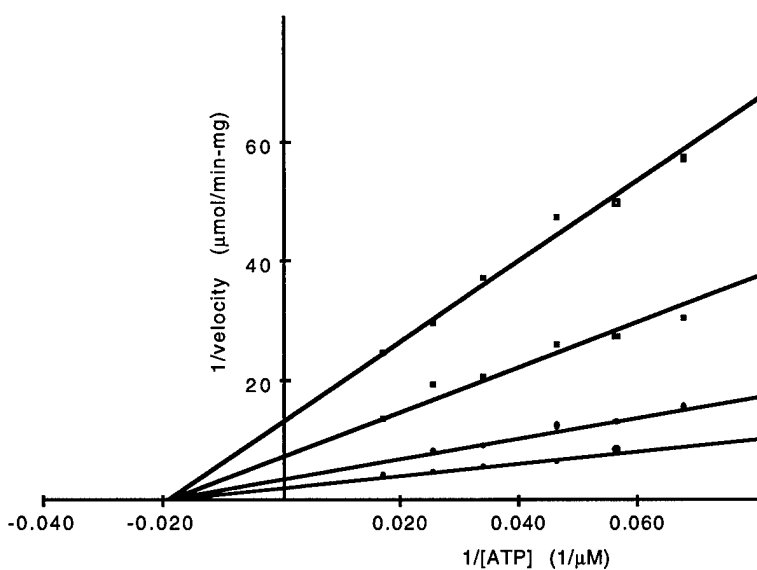


Figure IV. The Lineweaver-Burk double reciprocal plot of the L-Dopa-containing peptide **63** as a function of varied ATP.

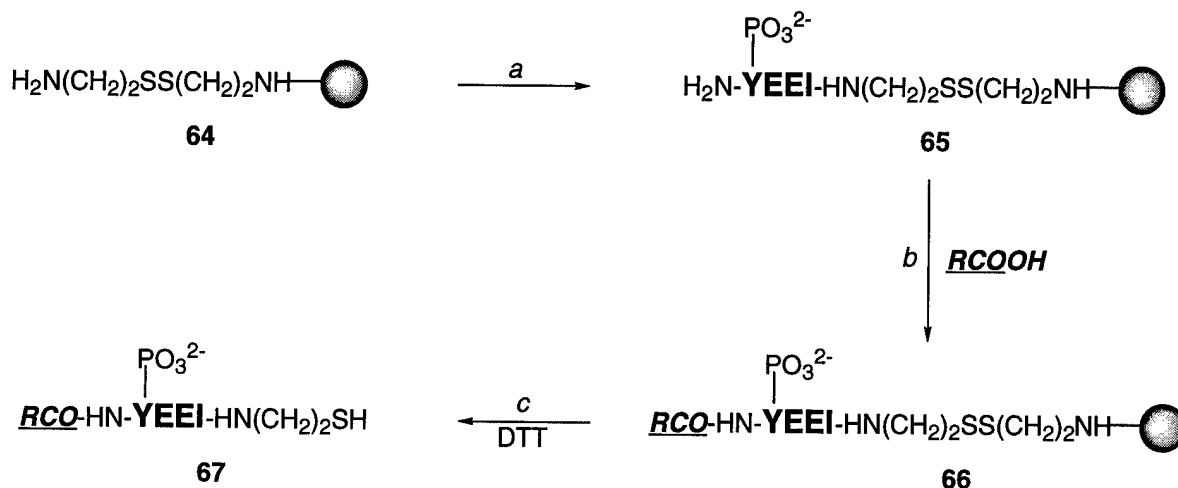
14 $\pm$ 2 μ M; see Fig. IV), it is evident that this inhibitory species does not coordinate to the ATP binding site.

The L-Dopa peptide 63 only serves as a reversible inhibitor of c-Src. L-Dopa is best known as a medicinal agent for the treatment of Parkinson's disease.²² However, this amino acid has also been found in proteins. Dopa plays a key role in protein cross-linking in invertebrates (e.g. byssal adhesion of marine mussels) via oxidation to the corresponding *ortho* quinone.²³ As a result, we were somewhat concerned that fortuitous oxidation of the L-Dopa residue might be responsible for the impressive inhibitory profile of **63**. However, we failed to detect a time-dependent inactivation of c-Src in the presence of **63** that is any more substantial than in the absence of the peptide (i.e. a slight, yet identical, loss in tyrosine kinase activity as a function of time is observed in both the presence and absence of **63**). Consequently, we conclude that the Dopa-containing peptide only serves as a simple reversible inhibitor of c-Src.

The most potent tyrosine kinase-specific inhibitors reported to date are species that coordinate to the ATP-binding site. Although the K_i values associated with these inhibitory species are in the nanomolar range, micromolar concentrations will be required to overcome the high intracellular concentrations of ATP present in most cell types. Furthermore, it is important to be cognizant of the fact that there are a large number of ATP utilizing enzymes present in mammalian cells. Although peptide-based inhibitors do suffer from the bioavailability point of view, their peptidomimetic counterparts may ultimately offer greater opportunities for creating inhibitors that can exquisitely discriminate between closely related kinases. Until recently, the inhibitory potency of tyrosine kinase-targeted peptides has been, in general, extremely disappointing. With the advent of nonphosphorylatable tyrosine mimetics, such as L-Dopa, peptide-based species and their cognates can now be given serious consideration as potentially useful inhibitors for members of the tyrosine-specific subfamily of protein kinases.

The Src homology 2 (SH2) domain, a component of many signal transducing proteins, plays a critical role in organizing coherent signaling cascades.²⁴ The latter transpires when primary sequences encompassing phosphotyrosine in one protein are recognized by and coordinate to the SH2 domain of a second. The design of SH2-targeted agents has received considerable attention since such species could disrupt signaling pathways known to be responsible for a variety of disease states.²⁵ In general, the affinity (i.e. K_D) of SH2 domains for phosphotyrosine-containing peptides lies in the range of 200 - 800 nM (for an exception *vide infra*).²⁶ Nonpeptidic SH2-targeted compounds have also been described, but the affinity of these species for SH2 domains is typically 1 - 3 orders of magnitude less than that displayed by their peptidic counterparts.²⁷ We have shown that unnatural motifs, when appended to active site-directed peptides, can dramatically enhance both the affinity and selectivity for elements as protein kinases.

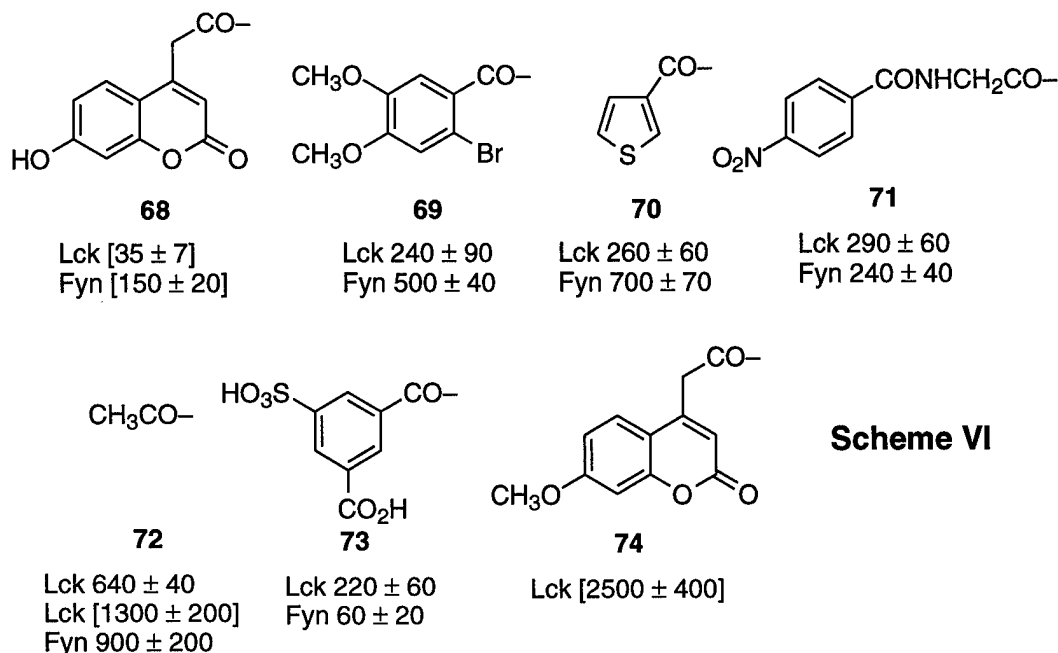
Our initial studies focused on the acquisition of compounds that target the SH2 domains of Lck and Fyn, Src tyrosine kinase family members known to participate in T cell activation.²⁸ Like other Src tyrosine kinases, the SH2 domains of Lck and Fyn exhibit an identical preference for the sequence **-phosphoTyr-Glu-Glu-Ile-**,²⁹ where SH2 affinity is strongly dependent upon the **phosphoTyr** and **Ile** side chains. Interestingly, structural and biochemical studies indicate that conventional amino acid residues positioned to the N-terminus of phosphoTyr have little influence on either SH2 selectivity or affinity. In spite of the latter observation, a hydrophobic indentation in the SH2 surface does lie adjacent to the amine of phosphoTyr in an SH2-bound peptide. Consequently, this invagination could serve as a potential binding site for a structurally compatible ligand appended off the N-terminus of phosphoTyr-Glu-Glu-Ile-amide. Since the size of the putative binding region is modest, there is little opportunity to create extensive molecular diversity via a combinatorial amalgamation of an array of subunits connected in series. Instead, we developed a parallel synthesis strategy (scheme V) that achieves a high degree of molecular diversity within a spatially-focused region.



Scheme V. Chemical synthesis of a spatially-focused library. (a) (i) Fmoc-based solid phase peptide synthesis; (ii) piperidine/DMF; (b) (i) RCOOH (400 eq. based on resin-bound peptide), benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (200 eq.), 1-hydroxybenztriazole (200 eq.), N-methylmorpholine (1,000 eq.), 100 μL DMF, 18 hr, RT; (ii) 50% trifluoroacetic acid/50% CH_2Cl_2 , 2 hr, RT; (c) 10 mM dithiothreitol (DTT) in 50 mM Tris (pH 7.5) (1 x 200 μL ; 2 x 150 μL ; 1 hr each at RT).

Solid phase peptide synthesis using the Fmoc protocol was performed on the novel disulfide-containing Tentagel-based resin **64**. The Fmoc protecting group at the N-terminus was subsequently removed and the Tentagel-bound peptide **65** was distributed in 5 mg quantities to individual wells of 96-well microfiltration plates. The free N-terminus of the peptide was condensed with a different carboxylic acid in each well to furnish the substituted species **66**. A total of 10 microfiltration plates were employed to accommodate the 900 different carboxylic acids employed in this study. The latter array of carboxylic acids included aliphatic, aromatic, multiring, hydrophobic, hydrophilic, as well as negatively and positively charged species. All resin washing steps (including removal of excess acylating agent) were conducted by vacuum filtration using a 96-well filter plate vacuum manifold. After deprotection of the Glu side chains with 50% trifluoroacetic acid in CH_2Cl_2 , the peptide/nonpeptide conjugates were cleaved from the solid support with 3 washings of 10 mM dithiothreitol (DTT) in 50 mM Tris buffer. Each washing was directly filtered into a receiving set of 96-well plates using the vacuum manifold. *The disulfide link between peptide and resin permits the application of cleavage conditions that are virtually identical to the conditions employed in the subsequent assay. As a consequence, the peptide/nonpeptide conjugates 67 are delivered to the receiving plates in a solution that is assay-ready.* The efficiency of amine acylation and DTT cleavage, as well as purity of the peptide/nonpeptide conjugate, were assessed with four of the synthesized species. No free N-terminus was detected and over 90% of the total conjugate was cleaved from the resin with the first DTT washing step. The final two DTT washings removed the residual resin-bound material. Compound purity was greater than 90% as assessed by HPLC and the HPLC-purified compounds (i.e. removal of Tris buffer and DTT) were characterized by electrospray mass spectrometry.

With the 900 member library in hand, high affinity SH2-targeted ligands were identified via an enzyme-linked immunosorbent assay (ELISA).³⁰ Under the stringent conditions employed ([peptide/nonpeptide conjugate] = 50 nM), more than 65% of the compounds failed to display any activity in the ELISA assay. We selected a few representative compounds from the assay screen and resynthesized them on the Rink resin, without the mercaptan tail (scheme VI). The absence of the latter eliminates the formation of any possible side products (e.g. disulfide bond formation or other forms of oxidation) when working with isolated pure material. K_D values were initially determined by a previously reported



spectrofluorometric method using the acetamido-anilino-naphthyl sulfonic acid (AANS)-labeled peptide Phe-Thr-Ala-Thr-Glu-Cys(AANS)-Gln-phosphoTyr-Glu-Glu-Ile-Pro.³¹ The latter exhibits an 8.7-fold enhancement in fluorescence intensity upon SH2 coordination and has a reported K_D for the Lck GST-SH2 fusion protein of 40 nM. In our hands, we obtained a value of 78 ± 14 nM. The K_D values for compounds **69** - **72** were obtained by the competitive displacement of the AANS-labeled peptide. Unfortunately, we were unable to acquire a binding constant for **68** by this method, since the highly fluorescent coumarin nucleus interferes with the AANS fluorophore. However, since coumarin fluorescence is not altered when **68** is coordinated to the SH2 domain, we were able to obtain a K_D via equilibrium dialysis using slide cassettes. Although the equilibrium dialysis and spectrofluorometric assays were performed under nearly identical conditions, we felt that it was prudent to determine K_D values for at least one compound using both assays. The K_D furnished by equilibrium dialysis for compound **72** (by competitive displacement of **69**) is twice as large as that obtained via the spectrofluorometric method (i.e. compound **68** displays a 36-fold higher affinity for the Lck SH2 domain than **72**, whereas the AANS-derivatized peptide is only 8-fold more potent than **72**).

The affinity of compound **68** (35 ± 7 nM) for the Lck SH2 domain is the highest reported for any species to date, even exceeding the affinities displayed by much longer peptides, such as the AANS-labeled species. In addition, **68** is a significantly weaker ligand for the SH2 domains of PLC γ 1 ($K_D = 4.9 \pm 0.7$ μ M), the p85 α subunit of PI3 kinase ($K_D = 9.3 \pm 0.9$ μ M), and Grb2 (11.3 ± 3.1 μ M). A recent combinatorial library study demonstrated that the SH2 domains of Src kinase family members exhibit identical sequence specificities.²⁹ Indeed, this observation is borne out by the virtually identical K_D values displayed by the SH2 domains of Lck (78 ± 14 nM) and Fyn (66 ± 13 nM) for the AANS-labeled peptide. In contrast, **68** exhibits a nearly 5-fold preference for the Lck SH2 domain versus that of Fyn, which represents the first example of selectivity between any members of the Src kinase family. We also screened our library of compounds against the Fyn SH2 domain, and identified compound **73** as the most potent Fyn-targeting agent. The latter displays a discriminatory preference in favor of Fyn versus that of Lck. This selectivity, although modest, is promising given the fact that the N-terminus acyl appendages are likely directed into a region of the SH2 domain that is highly conserved in the Src enzyme family. In contrast, there is less sequence homology in the region that coordinates the Ile moiety of Acyl-phosphoTyr-Glu-Glu-Ile. Consequently, application of the methodology described herein to replace the

C-terminal region of Acyl-phosphoTyr-Glu-Glu-Ile may produce species that exhibit even greater selectivities than those displayed by **68** and **73**.

An energy minimization analysis suggests that the coumarin ring system of **68** lies over a hydrophobic patch on the SH2 surface and that the coumarin hydroxyl is engaged in a hydrogen bonding interaction with the carboxyl side chain of Glu138. The importance of the free coumarin alcohol was confirmed by the behavior of the methylated analog **74** which, like the majority of compounds in the library, failed to display any activity in the initial ELISA assay screen. The K_D for the Lck SH2 domain/**74** complex (2.5 ± 0.4 mM) is 70-fold larger than that for the complex containing **68**.

In summary, we have employed a spatially-focused high diversity library to identify SH2-targeted agents. The disulfide linker used to connect the peptide/nonpeptide conjugates to the solid support allows delivery of the conjugates to a receiving plate in an assay-ready form. The coumarin-based analog **68** and the sulfonated derivative **73** are among the highest affinity SH2-targeted agents ever reported. Furthermore, since the intense coumarin fluorescence is not affected by SH2 coordination, competitive displacement of the coumarin analog under equilibrium dialysis conditions offers a sensitive method for the determination of dissociation constants of nonfluorescent SH2 ligands. Finally, compounds **68** and **73** exhibit the heretofore unprecedented ability to discriminate between the SH2 domains of two members of the Src family of protein kinases.

The SH2 and SH3 domains present in the Src family of PTKs are protein modules that have evolved for the unique purpose of promoting protein-protein interactions. SH2 domains are composed of approximately 100 amino acids and specifically recognize and bind to protein segments containing phosphotyrosine. In contrast, SH3 domains are approximately 60 amino acids in length and coordinate proline rich regions that are present in a variety of signaling molecules. Peptides directed against the SH2 domain of a kinase can exhibit as much as four orders of magnitude enhanced affinity compared to active site-directed peptides. Is it possible to circumvent the problem of overlapping substrate specificity and exploit the SH2 and/or SH3 molecular recognition motifs to develop selective, high affinity inhibitors for tyrosine kinases? Or, more simply stated, can SH2 and SH3 recognition sequences be utilized to "deliver" otherwise low affinity peptides to the active site of PTKs?

In an effort to address these questions, we prepared a series of bivalent peptides that contain an SH2 recognition sequence linked, through a flexible tether, to an active site-directed inhibitory peptide. The SH2 recognition sequence employed, acetyl-pTyr-Glu-Glu-Ile-Glu, is based on the SH2 recognition motif found in the hamster polyoma virus middle-T antigen. The x-ray crystal and NMR solution structures of this peptide bound to the SH2 domain of Src have been determined.³² Acetyl-pTyr-Glu-Glu-Ile-Glu binds moderately well to the SH2 domain of Src with a K_d value of under 1 μ M. The active site-directed portion of the inhibitor, Glu-Glu-Leu-Leu-(F₅)Phe-amide, is based on previous work from our laboratory which identified **pentafluorophenylalanine** as a nonphosphorylatable tyrosine surrogate (*vide supra*). Although the inhibitory potency of this peptide is, at best, only a modest improvement over its simpler Phe-containing counterpart (approximately a 4-fold decrease in K_i), we decided to incorporate this moiety into the active site-directed sequence for our preliminary studies to assess the effect that a potent SH2-directed sequence linked to a poor active site-targeted peptide has on inhibitory activity.

Since no x-ray crystal structure of Src in its biologically active conformation has been reported, optimum tether length was determined empirically utilizing alkyl chains of varying length. We chose tethers that are structurally simple, reasonably flexible, and compatible with standard solid phase peptide synthesis protocols. On the basis of these criteria, γ -aminobutyric acid (Abu) was employed as the monomeric building block for tether construction. Thus, a series of bivalent inhibitors of the general structure Ac-pTyr-Glu-Glu-Ile-Glu-(Abu)_n-Glu-Glu-Leu-Leu-(F₅)Phe-amide were prepared where n varied, in multiples of two, from 4 to 12. These inhibitors, along with the active site-directed control peptide, Ac-Glu-Glu-Leu-Leu-(F₅)Phe-amide (**75**), were evaluated as inhibitors of c-Src.

The results obtained with the bivalent inhibitors are summarized in Table 9. An obvious trend emerges from the data. The active site-directed control peptide **75** is clearly a poor inhibitor with an IC_{50} of nearly 1.6 mM. However, as can be seen from the results obtained with peptide **76**, inhibitory potency is enhanced roughly 8-fold by tethering the active site-directed peptide to an SH2 recognition sequence. The inhibitory efficacy of the bivalent inhibitors improves as a function of increasing chain length until a maximum is reached at 8 aminobutyric acid residues (18.5 μ M). This represents a nearly 86-fold enhancement in inhibitory activity relative to peptide **75**. Furthermore, **78** fails to show any inhibition against the Src family member Lck at concentrations up to 270 μ M. At a tether length of 10 γ -Abu residues, inhibitory potency sharply decreases and continues to decline with peptide **80** being the poorest inhibitor of those sampled.

Table 9. Bivalent inhibitors of c-Src.

Peptide	Structure	IC_{50} (μ M)
75	Ac-EELL-(F ₅)Phe-amide	1590 $\pm$ 170
76	Ac-pYEEIE-(Abu) ₄ -EELL-(F ₅)Phe-amide	195 $\pm$ 26
77	Ac-pYEEIE-(Abu) ₆ -EELL-(F ₅)Phe-amide	35 $\pm$ 5
78	Ac-pYEEIE-(Abu) ₈ -EELL-(F ₅)Phe-amide	18.5 $\pm$ 1
79	Ac-pYEEIE-(Abu) ₁₀ -EELL-(F ₅)Phe-amide	250 $\pm$ 25
80	Ac-pYEEIE-(Abu) ₁₂ -EELL-(F ₅)Phe-amide	400 $\pm$ 50
81	Ac-(Abu) ₈ -EELL-(F ₅)Phe-amide	860 $\pm$ 90
82	coumarin-pYEEIE-(Abu) ₈ -EELL-(F ₅)Phe-amide	6.9 $\pm$ 0.4

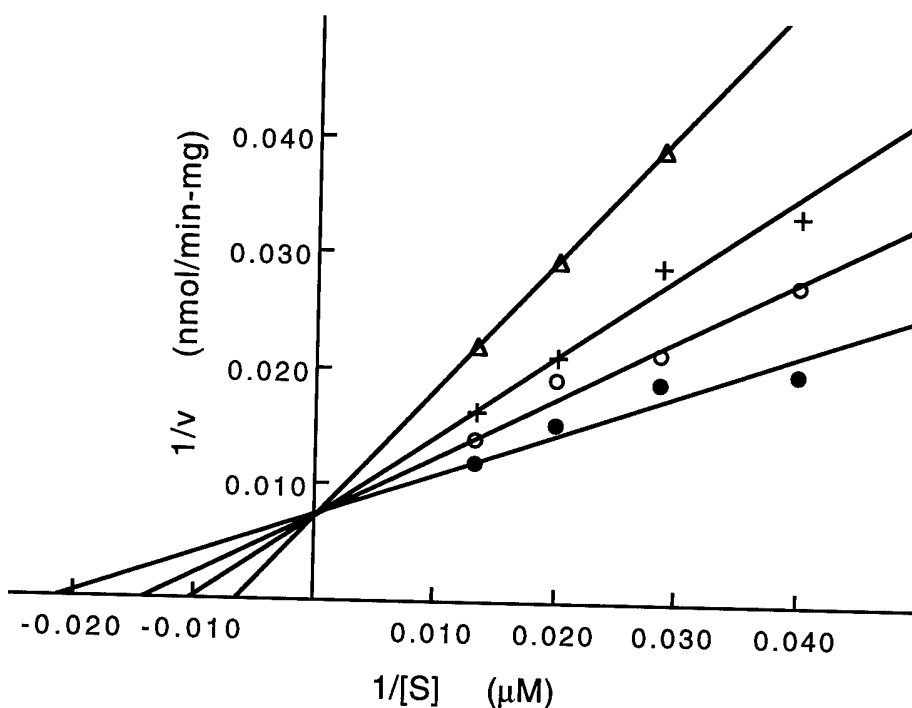
Is the dramatic difference in IC_{50} values between peptides **75** and **78** due to the simultaneous occupancy of the SH2 and active site regions of Src by peptide **78**? We performed two experiments to address this question. First, we prepared peptide **81**, which is identical to **78** except that it lacks the SH2 recognition sequence. If the SH2 recognition motif is responsible for the enhanced potency exhibited by **78** then **81** should be a much poorer inhibitor than **78**. As can be seen in Table 9, this is indeed the case. In addition, we also assessed the inhibitory activity of **78** in the presence of 300 μ M Ac-pTyr-Glu-Glu-Ile-amide, which at this concentration should saturate the SH2 domain of Src. Under these conditions, the ability of peptide **78** to serve as an inhibitor of Src kinase activity should be significantly impaired since the SH2 targeting motif contained within **78** must compete with the overwhelming presence of Ac-pTyr-Glu-Glu-Ile-amide. Indeed, **78** exhibits an IC_{50} of 240 $\pm$ 70 μ M under these circumstances. Both experiments provide strong evidence that the inhibitors prepared in this study coordinate to Src in a bivalent fashion.

As noted above, we have demonstrated that 7-hydroxy-coumarin-4-acetic acid linked to the N-terminus of pTyr-Glu-Glu-Ile-amide significantly enhances the affinity of this peptide for the SH2 domain of Lck. Although this effect appears to be quite specific for Lck, we do observe a modest enhancement for the SH2 domain of Fyn as well. Since both Lck and Fyn are members of the Src kinase family, we prepared the coumarin derivative **82** and found that it serves as an approximately 2.5-fold better inhibitor than **78** (Table 9). The coumarin derivatized peptide **82** is a 230-fold more potent inhibitor of Src than the monovalent active site-directed peptide **75**.

Since **82** is the best inhibitory agent in this study, we decided to examine its mode of action in greater detail. We previously demonstrated that the simple active site-directed peptide, Glu-Glu-Leu-Leu-(pentafluoro)Phe-Gly-Glu-Ile, is a competitive inhibitor with respect to peptide substrate (14). In contrast, peptide **82** exhibits a complex inhibition pattern versus the variable substrate [(Arg)₅-Ala-(Glu)₄-Tryphenethylamine] (data not shown). Since binding to the higher affinity SH2 site is the primary driving force for the complexation of the bivalent inhibitors with Src, one might envision the formation of several discrete intermediates. For example, it is possible that some fraction of the peptide **82**/Src complex contains the SH2 recognition sequence coordinated to the SH2 domain with the active site portion free in solution. Competition with a substrate that has only one binding modality available to it (i.e. only active site-directed) may lead to mechanistic complexities that preclude the observation of a clean competitive inhibition pattern. In an effort to address this matter, we prepared the bivalent substrate (biotin-aminohexanoic acid)-pTyr-Glu-Glu-Ile-Glu-(Abu)₆-Glu-Glu-Leu-Leu-Tyr-amide (**83**). An avidin-impregnated membrane was employed for the assays utilizing this substrate. The bivalent nature of substrate **83** suggests that it should form the same discrete intermediates upon binding to Src as **82**. Furthermore, as with all the bivalent inhibitors in this study, binding of the substrate peptide to the SH2 domain should be the dominant driving force for complexation.

Peptide **83** is a reasonably effective substrate for Src, exhibiting a K_m of $47 \pm 9 \mu\text{M}$ and a V_{max} of $130 \pm 20 \text{ nmol/min-mg}$. Peptide **82** displays an IC_{50} of $19 \pm 3 \mu\text{M}$ with this substrate. Most importantly, **8** serves as a competitive inhibitor versus variable peptide substrate **82** and exhibits a K_i of $9 \pm 2 \mu\text{M}$ (Fig. V).

Figure V



In this study, we have taken a poor active site-directed inhibitory peptide and tethered it to an SH2 recognition sequence. The resultant bivalent peptide **82** exhibits two orders of magnitude greater inhibitory efficacy against Src than the corresponding monovalent active site directed peptide **75**. Clearly, several additional issues need to be addressed in order to optimize the potency of this new family of PTK inhibitors. The incorporation of more potent active site-directed peptides (e.g. those containing L-dopa)

into these compounds should markedly improve their inhibitory efficacy. More ridged tethers, with fewer degrees of rotational freedom, could likewise enhance the effectiveness of these species. Finally, the relative orientation of the active site-directed and SH2-targeted segments in **82** may not be optimal. By varying the N- to C-terminal orientation of each subligand, it may ultimately be possible to dramatically minimize the current (Abu)₈ tether length.

There are many possible applications and variations of the bivalent approach. Cowburn *et al.* prepared a series of "consolidated" ligands containing SH2 and SH3 binding subligands.³³ These species bind to the regulatory apparatus (SH2 and SH3 domains) of the Abelson protein tyrosine kinase with enhanced affinity. Likewise, Pluskey and coworkers tethered two phosphotyrosyl peptides together *via* aminohexanoic acid linkages to produce bivalent ligands that bind to the two SH2 domains of the phosphatase SH-PTP2, which results in enzyme activation.³⁴ The bivalent ligands were found to stimulate catalytic activity in a more pronounced fashion than their monovalent phosphopeptide counterparts. As in the SH-PTP2 example, the interaction of an SH2 or SH3 ligand with members of the Src family of tyrosine kinases often results in enhanced enzymatic activity. In contrast, our bivalent Src kinase inhibitors behave in a dramatically different fashion. These species are both bivalent and bifunctional. Not only do these compounds coordinate to the SH2 domain of Src, which should block the assembly of Src-based signaling complexes, but they shut down the catalytic activity of the enzyme as well.

Conclusions

In the last decade, research in the general area of signal transduction has exploded with activity. From vision to cell division, protein kinases serve as the predominant component of signal transduction pathways. As a consequence, protein kinase inhibitors and substrates are valuable tools for assessing the physiological consequences of protein kinase activation. Furthermore, since these enzymes are medicinally attractive targets of opportunity, potent and selective kinase inhibitors are an intensely sought after commodity. The vast majority of inhibitors described to date coordinate to the ATP binding site. Unfortunately, the impressive K_i values reported for a few of these competitive inhibitors of ATP vastly overstate their *in vivo* effectiveness, since their potency is at the mercy of high intracellular ATP levels and low protein kinase Michaelis constants. On the other hand, peptide-based inhibitors suffer from susceptibility to proteolysis, poor bioavailability, and disappointing inhibitory efficacy. In our research program, we have examined the enzymological differences between SPKs and TPKs, created TPK selective artificial substrates, and devised TPK inhibitors that are a composite of peptide and nonpeptide elements. The latter structural motif represents a significant departure from the inhibitor design strategies that have been employed to date. This was achieved by employing a strategy built around a parallel synthesis approach that rapidly creates moderately large libraries of active site-directed compounds. We have also acquired peptide/nonpeptide conjugates that are targeted to the SH2 domains of TPKs. Perhaps most significantly, we have combined the active site- and SH2-directed strategies to create "bivalent" ligands that simultaneously associate with two binding sites of the target TPK.

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