L-Dopa: A Powerful Nonphosphorylatable Tyrosine Mimetic for pp60^{c-src}

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Tyrosine-specific protein kinases play a key role in transducing growth promoting signals from the cell surface to the nucleus. Consequently, it is not surprising that there has been, and continues to be, a profound interest in creating inhibitors that precisely target specific members of this enzyme family. The most powerful inhibitors described to date are targeted to the ATP binding site.[1] Unfortunately, the inhibitory effectiveness of these species is markedly impaired under physiological conditions due to the high intracellular concentrations of ATP.[2] Peptide-based inhibitors, entities competitive with protein substrate binding, have been described but are generally disappointing in terms of inhibitory potency with K_i values generally in the 1−2 mM range.[3] The latter species are typically generated by replacing the phosphorylatable tyrosine residue in an active site-directed peptide with a nonphosphorylatable phenylalanine moiety.[4] The discouraging inhibitory profiles of these phenylalanine-based peptides may be attributable to the missing aromatic hydroxyl functionality, which likely engages in productive active site interactions when present in the corresponding tyrosine-containing substrate. In addition, several recent studies have demonstrated that the K_m values exhibited by some peptide substrates of protein kinases exaggerate how well these peptides actually bind to the target kinase.[3a,5] Although these explanations may provide a rationale for the weak activity of phenylalanine-based peptides, the poor inhibitory behavior of these species nonetheless demonstrate the need for nonphosphorylatable tyrosine analogs that dramatically enhance enzyme affinity.

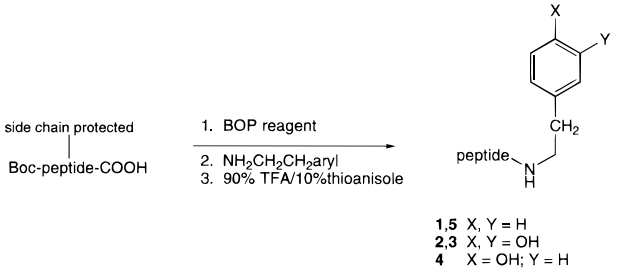

Figure 1. C-terminus-derivatized peptides **1**−**5** were prepared by activating the side chain-protected peptide (previously synthesized on the 2-methoxy-4-alkoxybenzyl alcohol resin[7]) with benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate ("BOP reagent") followed by condensation with phenethylamine (peptides **1** and **5**), dopamine (peptides **2** and **3**), and tyramine (peptide **4**) and subsequent side chain deprotection (90% trifluoroacetic acid/10% thioanisole).

The synthesis of peptides containing unnatural and potentially inhibitory residues can be time-consuming and, in many instances, challenging.[6] Fortunately, these synthetic obstacles can be circumvented by directly attaching the residues of interest to the N- or C-terminus of an active site-directed peptide.[6] The latter approach provides a rapid and efficient means to acquire peptides containing a structurally diverse ensemble of functionality specifically targeted for active site insertion. The general synthetic approach is illustrated in Figure 1. We recently constructed a peptide-based library containing an array of C-terminus-linked phenethylamine analogs in which the phosphorylatable para-substituted hydroxyl of tyramine was replaced with an assortment of nonphosphorylatable functionality.[7] Although a few functionality, such as sulfonamide, do enhance enzyme affinity without concomitant phosphorylation, the overall inhibitory potency of this family of peptides is disappointingly modest. 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 also 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 now report that dopamine, and its corresponding amino acid, L-Dopa, serve as potent nonphosphorylatable mimetics of tyrosine in pp60^{c-src}-targeted peptides.

A dopamine-substituted peptide is a significantly more potent inhibitor of pp60^{c-src} than the corresponding phenethylamine derivative. The phenethylamine-based peptide (**1**) and its dihydroxy-counterpart (**2**) were prepared according to the synthetic scheme outlined in Figure 1.[8] The dopamine-derivative **2** is 31-fold more effective as an inhibitor of pp60^{c-src} than peptide **1**.

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(2) Intracellular concentrations of ATP can be as high as 5 mM (Traut T W. *Mol Cell. Biochem.* **1994**, *140*, 1−22) and tend to be higher in transformed versus untransformed cells. These high concentrations interfere with the inhibitory effectiveness of compounds that are competitive with respect to ATP. Percent inhibition at a given concentration of a competitive inhibitor (i.e., v/v) is given by eq 1. The K_m for ATP (∼5 μM) in a protein kinase-catalyzed reaction is significantly less than the intracellular concentration of ATP [eq 2]. In order to achieve 50% inhibition under physiological conditions, the concentration of competitive inhibitor needs to be approximately 1000-fold greater than its experimentally derived K_i value. Although the affinities of the most potent PTK inhibitors are in the nanomolar range, their effectiveness under physiological conditions should be decidedly micromolar. In contrast, the concentration that generates a 50% reduction in activity for inhibitors that are noncompetitive with respect to ATP [$v_i/v = K_i/(K_i + [I])$] is equivalent to K_i.

$$\frac{v_i}{v} = \frac{K_m + [\mathrm{ATP}]}{K_m\left(1 + \frac{[\mathrm{I}]}{K_i}\right) + [\mathrm{ATP}]} \quad (1)$$

$$\frac{v_i}{v} = \frac{5}{0.005\left(1 + \frac{[\mathrm{I}]}{K_i}\right) + 5} \quad (2)$$

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(4) The only notable exception to this are tetrafluorotyrosine-containing peptides, which have been reported to serve as an potent inhibitors of the insulin receptor kinase (Yuan, C. J.; Jakes, S.; Elliott, S.; Graves, D. J. *J. Biol. Chem.* **1990**, *265*, 16205−16209) and of the epidermal growth factor receptor kinase (Fry, D. J.; McMichael, A.; Singh, J.; McNamara, D. J. *Peptides* **1994**, *15*, 951−957). Interestingly, a trifluorotyrosine-based peptide has been found to serve as a substrate for the C-terminal pp60^{c-src} kinase, see: Cole, P. A.; Grace, M. R.; Phillips, R. S.; Burn, P.; Walsh, C. T. *J. Biol. Chem.* **1995**, *270*, 22105−22108.

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(6) For a detailed discussion, see: Kwon, Y.-G.; Mendelow, M.; Srinivasan, J.; Lee, T. R.; Pluskey, S.; Salerno, A.; Lawrence, D. S. *J. Biol. Chem.* **1993**, *268*, 10713−10716.

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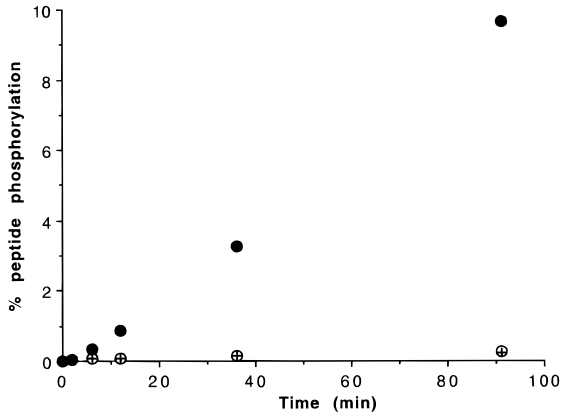

Figure 2. pp60^{c-src}-catalyzed phosphorylation of Arg-Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Glu-dopamine **3** (+), Arg-Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Glu-tyramine **4** (●), and Arg-Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Glu-phenethylamine **5** (○) as a function of time. Assays were performed at pH 7.5 (20 mM Hepes) and 30 °C in a solution containing 20 mM $MgCl_2$, 100 μM Na_3VO_4, 0.125 mg/mL bovine serum albumin, 250 μM [γ-^{32}P]ATP, 2.22 nM pp60^{c-src}, and 60 μM inhibitor/substrate peptides. After 16 h, 50% of **4** underwent phosphorylation, whereas less than 1% of **3** or **5** is ^{32}P-labeled under identical conditions (the 16 h data points are not shown). Since **5** lacks a hydroxyl moiety, it is unlikely that the trace levels of radioactivity associated with **3** and **5** are due to phosphorylation.

A dopamine-substituted peptide is not a pp60^{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 positvely charged active site-directed peptides.[6] The latter coordinate to a negatively charged phosphocellulose disk, which can be subsequently counted for radioactivity. Since **2** lacks the primary sequence necessary for binding to phosphocellulose disks, we prepared an arginine-substituted derivative of **2** (Arg-Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Glu-dopamine; **3**). The substrate efficacy of **3** was then compared with that of the tyramine-based analog **4**, a known pp60^{c-src} substrate.[9] In addition, we prepared the nonphosphorylatable phenethylamine-based peptide, Arg-Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Glu-phenethylamine (**5**), as a control. As is apparent from Figure 2, peptide **3** is inert under conditions in which **4** suffers phosphorylation.

An L-Dopa-substituted peptide is a significantly more potent inhibitor of pp60^{c-src} than the corresponding phenylalanine derivative. Do the inhibitory trends observed with C-terminus-substituted peptides (i.e., **1** and **2**) hold in more conventional peptidic environments? In order to address this question we prepared the peptides Glu-Glu-Leu-Leu-**Phe**-Gly-Glu-Ile (**6**) and Glu-Glu-Leu-Leu-**Dopa**-Gly-Glu-Ile (**7**). 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 pp60^{c-src} specificity.[10] As is evident from Table 1, the inhibitory trend between **1** and **2** holds for the conventional peptide dyad **6** and **7**. In the latter case, **7** is a 33-fold more effective inhibitor than **6**. Furthermore, both peptides are competitive inhibitors versus peptide substrate (see Supporting Information). Indeed, the difference in K_i values exhibited by **6** and **7** is even more substantial (55-fold) than that observed for the corresponding IC_{50}s. Finally, since the Dopa-containing peptide serves as a *non*competitive inhibitor versus variable ATP ($K_i = 14 \pm 2$ μM; see Supporting Information), it is evident that this inhibitory species does not coordinate to the ATP binding site.

Table 1. The IC_{50} and K_i Values of Inhibitors **1**, **2**, **6**, and **7**

Inhibitor	IC_{50} (μM)	K_i (μM)
Glu-Glu-Glu-Glu-**phenethylamine** (**1**)	1650 ± 10	
Glu-Glu-Glu-Glu-**dopamine** (**2**)	53 ± 2	
Glu-Glu-Leu-Leu-**Phe**-Gly-Glu-Ile (**6**)	950 ± 25	860 ± 20
Glu-Glu-Leu-Leu-**Dopa**-Gly-Glu-Ile (**7**)	29 ± 2	16 ± 3

*The L-Dopa peptide **7** only serves as a reversible inhibitor of pp60^{c-src}.* L-Dopa is best known as a medicinal agent for the treatment of Parkinson's disease.[11] 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 *o*-quinone.[12] As a result, we were somewhat concerned that fortuitous oxidation of the L-Dopa residue might be responsible for the impressive inhibitory profile of **7**. However, we failed to detect a time-dependent inactivation of pp60^{c-src} in the presence of **7** 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 **7**; see Supporting Information). Consequently, we conclude that the Dopa-containing peptide only serves as a simple reversible inhibitor of pp60^{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 typically 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.

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Supporting Information Available: Figures of Lineweaver-Burk double reciprocal plot of the L-Dopa-containing peptide **7** as a function of varied peptide substrate and ATP and time-dependent activity of pp60^{c-src} (5 pages). See any current masthead page for ordering and Internet access instructions.

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