Nonphosphorylatable Tyrosine Surrogates

IMPLICATIONS FOR PROTEIN KINASE INHIBITOR DESIGN*

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Tyrosine-specific protein kinases are known to utilize short synthetic tyrosine-containing peptides as substrates and, as a consequence, a number of inhibitory peptides have been prepared by replacing the tyrosine moiety in these peptides with a nonphosphorylatable phenylalanine residue. Unfortunately, the inhibitory efficacy of these phenylalanine-based peptides is often disappointing. These results demonstrate the need for nonphosphorylatable tyrosine surrogates that enhance enzyme affinity. As a consequence, we prepared nearly two dozen different phenethylamine derivatives, attached them to the C terminus of an active site-directed peptide (Glu-Glu-Leu-Leu), and examined their effectiveness as inhibitors of pp60c-src. Three derivatives exhibit enhanced inhibitory activity (relative to phenethylamine), including para-substituted sulfonamide and guanidino analogs as well as a pentafluoro-containing species. The para-sulfonamide derivative was selected for further study and was found to function as a competitive inhibitor versus variable peptide substrate and as a noncompetitive inhibitor versus variable ATP. In short, the enhanced inhibitory activity of the sulfonamide derivative is not due to the association of this moiety with the ATP binding site. Furthermore, peptides containing the para-guanidino and pentafluoro derivatives of phenylalanine were prepared. These species also display enhanced inhibitory activity toward pp60c-src relative to the corresponding phenylalanine-based peptide.

More than 2 decades ago, the cAMP-dependent protein kinase was shown to phosphorylate short synthetic peptides containing sequences that correspond to phosphorylated sites in intact proteins (1–4). This key observation, which has been demonstrated innumerable times for other protein kinases in the intervening years, has had a profound impact on the ability to examine the chemistry and biochemistry of this important family of enzymes. Synthetic peptides are readily available and, unlike common protein kinase substrates such as histones, can be prepared containing only a single site of phosphorylation. As a consequence, peptidic substrates have proven to be an indispensable tool in the many detailed enzymological studies that have been described for protein kinases.

In addition to their clear enzymological utility, peptide-based substrates immediately suggest the likelihood that structurally analogous inhibitors can be prepared. Indeed, the incorporation of an alanine residue, in place of the phosphorylatable serine moiety in a cAMP-dependent protein kinase-directed peptide, generates a species that serves as a competitive inhibitor versus both peptide and protein substrates (4). Feramisco and Krebs (5) subsequently introduced other residues in place of serine, including glycine, valine, aspartic acid, and asparagine. However, none of these substitutions provides an inhibitor as powerful as the alanine-based peptide. Interestingly, a naturally occurring inhibitor of the cAMP-dependent protein kinase also contains an alanine residue at the position typically reserved for serine (6). Based upon these observations, the design of protein kinase inhibitors appears to be relatively straightforward. Inhibitors for the serine/threonine kinases should contain an alanine at the appropriate position in an active site-directed peptide. In contrast, for the tyrosine-specific kinases, a phenylalanine-for-tyrosine exchange should generate the desired inhibitory species. Unfortunately, the potency of the peptide-based inhibitors created using the alanine-for-serine or phenylalanine-for-tyrosine strategy is often disappointing. For example, whereas kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) is an excellent cAMP-dependent protein kinase substrate \( K_m = 16 \mu M \), the corresponding alanine-containing peptide (Leu-Arg-Arg-Ala-Ala-Leu-Gly) is a modest inhibitor \( (K_i = 490 \mu M) \). One explanation offered for this discrepancy is the absence of the serine alcohol moiety in the inhibitor, a functional group that could potentially enhance enzyme affinity via hydrogen bonding to an active site residue. Indeed, analogous poor inhibitory performances have been recorded for phenylalanine-based peptides directed against tyrosine kinases (for recent examples see Refs. 7–9). However, Adams and Taylor (10) have recently uncovered an additional molecular mechanism that rationalizes the dismal inhibitory performances exhibited by nonphosphorylatable peptides. These investigators demonstrated that the \( K_m \), associated with the cAMP-dependent protein kinase-catalyzed phosphorylation of kemptide vastly overestimates the affinity of this peptide substrate for the enzyme active site \( (i.e. K_m < K_i) \). (10). Although these results explain why alanine- and phenylalanine-based peptides often serve as unexpectedly weak inhibitors, they also demonstrate the need for nonphosphorylatable serine/threonine and tyrosine analogs that enhance enzyme affinity. We describe herein phenethylamine derivatives that serve in this capacity for the tyrosine-specific protein kinase pp60c-src.

MATERIALS AND METHODS

All chemicals were obtained from Aldrich, except for \( [\gamma-^{32}P]ATP \) (DuPont NEN), benzhydrylamine and Rink resins, and piperidine (Advanced ChemTech), protected amino acid derivatives and para-amino-phenylalanine (Advanced ChemTech and Bachem), and Liquiscint (National Diagnostics). The oxime resin was prepared as described previously (11). Phosphocellulose P-81 paper disks were purchased

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from Whatman. 1H NMR experiments were performed at 400 MHz (Varian VXR-400S). Fast atom bombardment (peptides) mass spectral analyses were conducted with a VG-70SE mass spectrometer.

**Human Recombinant pp60src**

Human pp60src was purchased from Upstate Biotechnology Inc. The enzyme was expressed by recombinant baculovirus containing the human c-src gene in SF9 insect cells.

**Preparation of Phenethylamine Derivatives**

The phenethylamine analogs contained in peptides 1, 2, 3, 4, 5, 6, 8, 9, 12, and 21 (see Tables I and II) were purchased from Aldrich. All others were synthesized as described below. All compounds gave satisfactory NMR data.

**Protocol for the Synthesis of Fmoc-protected Intermediates—Fmoc-N-hydroxysuccinimide was added portonwise (in a 1:1 molar ratio) to para-aminophenethanolamine (dissolved in 1,4-dioxane) and to para-aminophenethanolamine (dissolved in 30% 1,4-dioxane, 70% water, and 1 equivalent of triethylamine) maintained at 0°C. The respective solutions were stirred at room temperature for 2 h, and then water was added to produce precipitates. The heterogeneous mixtures were separately extracted with CHCl3, and the organic extracts were removed under reduced pressure. Both compounds were separately purified via silica gel chromatography using CHCl3 as the eluting solvent. The Fmoc-protected phenethylamine was obtained in 91% yield, whereas the corresponding phenethylamine derivative was furnished in 90% yield. Note that in both cases the Fmoc moiety is exclusively attached to the aliphatic amine.

**Protocol for the Synthesis of Phenethylamine Derivative in Peptide 7—**Borane-THF (5 ml, 5 mmol) was added dropwise over a 30-min period to phenethylamine (0.25 g, 1 mmol) in 4 ml of anhydrous THF. The solution was then stirred for an additional 45 min to a stirred solution of the benzyl cyanide (0.25 g, 1.09 g, 5 mmol) in dioxane (15 ml) maintained at 0°C. The mixture was allowed to warm to room temperature and then stirred for an additional 30 min at room temperature and 4 h. Methanol was added to decompose unreacted borane, and the resulting crude product was suspended in 20 ml of CHCl3 and subsequently washed with water. The organic layer was separated, and the solvent was then removed in vacuo. The crude product was subsequently purified by silica gel chromatography (CHCl3:CH3OH:NH3 in 10:90:1) to furnish the desired compound in 75% yield.

**Protocol for the Synthesis of para-Aminomethylphenethylamine (Phenethylamine Derivative in Peptide 10—)**Di-t-butyl-dicarbonate (1.09 g, 5 mmol) was added to a solution of para-aminomethylbenzyl alcohol (0.69 g, 5 mmol) (12) in dioxane (15 ml) maintained at 0°C. The mixture was warmed to room temperature and stirred for an additional 4 h. Methanol was slowly added to decompose unreacted borane, and the residual solution was then removed under reduced pressure. The residue was dissolved in 20 ml of CHCl3 and subsequently washed with water. The organic layer was separated, and the solvent was then removed in vacuo. Methanol was added to extract the desired product from the residual organic phase. The residue was then added to a solution of triethylamine (0.34 g, 3.4 mmol) in 2 ml of CH2Cl2 (argon, 0°C). The solution was warmed to room temperature and stirred for an additional 4 h. Methanol was slowly added to decompose unreacted borane, and the residual solution was then removed under reduced pressure. The residue was dissolved in 20 ml of CHCl3 and subsequently washed with water. The organic layer was separated, and the solvent was then removed in vacuo. Methanol was added to decompose unreacted borane, and the resulting solution was collected by silica gel chromatography (CHCl3:CH3OH:NH3 in 10:90:1) to furnish the desired compound in 75% yield.

**Protocol for the Synthesis of para-Hydroxysuccinimide (Phenethylamine Derivative in Peptide 11—)**—Borane-THF (5 ml, 5 mmol) was added dropwise over a 30-min period to para-hydroxysuccinimide (1.09 g, 5 mmol) (12) in dioxane (15 ml) maintained at 0°C. The mixture was allowed to warm to room temperature and then stirred for an additional 3 h. Water (30 ml) was added, and the aqueous solution was subsequently extracted with CHCl3 (2 x 30 ml). The combined organic phases were removed under reduced pressure, and the residue was subjected to silica gel column chromatography (CHCl3:CH3OH:NH3:H2O, 79:20:1) to furnish the desired product in 80% yield. The Boc protecting group was removed after this phenethylamine derivative was coupled to the peptide (see below).

**Protocol for the Synthesis of para-Aminooctanoylphenethylamine (Phenethylamine Derivative in Peptide 12—)**—This was synthesized from 1,4-phenylenediacetonitrile via LiAlH4 reduction as described previously (12) in 67% yield.

**Protocol for the Synthesis of para-Butamidophenethylamine (Phenethylamine Derivative in Peptide 13—)**—KCN (0.4 g, 6.2 mmol) in 2 ml of water was added, portonwise, to a solution of 3-bromo-para-toluic acid (0.86 g, 4 mmol) in ethanol (15 ml). The solution was heated to reflux for 10 h and then allowed to cool to room temperature. The solvent was evaporated under reduced pressure. The product was dissolved in 10 ml of water and subsequently precipitated upon addition of a few drops of 1 N HCl. The aqueous phase was extracted with ethyl acetate (3 x 50 ml), and the organic layers were combined and evaporated to give 570 mg (88% yield) of para-cyano-para-toluic acid. Borane-THF (1 ml, 5 mmol) was added dropwise over a 30-min period to 95 mg of para-cyano-para-toluamide (0.59 mmol) dissolved in 2 ml of THF (argon, 0°C). The solution was warmed to room temperature and stirred for an additional 4 h. Methanol was slowly added to decompose unreacted borane, and the resulting solution was then removed under reduced pressure. The residue was dissolved in 20 ml of CHCl3 and subsequently washed with water. The organic layer was separated, and the solvent was then removed in vacuo. The crude product was subsequently purified by silica gel chromatography (CHCl3:CH3OH:NH3:H2O, 79:20:1) to furnish the desired compound in 75% yield.

**Protocol for the Synthesis of para-Sulfamidophenethylamine (Phenethylamine Derivative in Peptide 14—)**—Butylamine (30 mmol) was added portionwise to para-sulfamidophenethylamine (8 ml, 0.1 mol) in 15 ml of acetonitrile. The solution was heated to reflux with stirring for 24 h, after which time an additional 8 ml of sulfonyl chloride was added. After heating at reflux for an additional 2 h, the reaction mixture was cooled to room temperature, and the solvent was evaporated under reduced pressure. The residue was extracted with ethyl ether (3 x 30 ml), and the organic extracts were combined and removed in vacuo. The resulting crude product was washed with 10 ml of CHCl3 and subsequently washed with water. The organic layer was removed under reduced pressure, and 5 ml of 10% piperidine in CHCl3 was introduced to deprotect the Fmoc-protected amine (the solution was allowed to stand for 15 min). The solvent was removed in vacuo, and the residue was subjected to silica gel column chromatography (CHCl3:CH3OH:NH3:H2O, 79:20:1) to furnish the desired product in 75% yield.

**Protocol for the Synthesis of para-Methylsulfonylphenethylamine (Phenethylamine Derivative in Peptide 15—)**—Borane-THF (5 ml, 5 mmol) was slowly added over 20 min at −10°C to a stirred solution of para-methylsulfonylbenzoic acid (1.0 g, 5 mmol) in 5 ml of THF. After the addition was complete, the solution was allowed to warm to room temperature and then stirred for 4 h. Water (30 ml) was added, and the solution was then extracted with ethyl ether (3 x 30 ml). Evaporation of the combined organic phases furnished para-methylsulfonylbenzyl alcohol in 75% yield. The benzyl alcohol was subsequently converted to the corresponding bromide (85% yield), cyano (75% yield), and desired phenethylamine (73% yield) derivatives via the protocol described above for 10.

**Protocol for the Synthesis of para-Sulfonoxophenethylamine (Phenethylamine Derivative in Peptide 16—)**—Phenethylamine (2.4 g, 20 mmol) was added to chlorosulfonic acid (5 g, 43 mmol) at −10°C. The mixture was stirred overnight. Wet ice was then added, the solution was neutralized with 6 N NaOH, and the solvent was removed under reduced pressure. Methanol was added to extract the desired product from the residual
salt, the mixture was filtered, and the solvent was removed under reduced pressure to furnish para-sulfonophenethylamine in 51% yield. Protocol for the Synthesis of para-Acetamidophenethylamine (Phenethylamine Derivative in 17)—Prepared as described below for para-methanesulfonylaminophenethylamine with the exception that acetic anhydride was used in place of methanesulfonyl chloride. para-acetamidophenethylamine was obtained in 90% yield.

Protocol for the Synthesis of para-Methanesulfonylaminophenethylamine (Phenethylamine Derivative in 18)—Methanesulfonyl chloride (69 mg, 0.6 mmol) was added dropwise over a period of 10 min to an ice-cold solution of the mono-Fmoc derivative of para-aminophenethylamine (0.18 g, 0.55 mmol) dissolved in 4 ml of anhydrous CH₂Cl₂ containing triethylamine (76 mg, 0.76 mmol). The solution was then stirred at room temperature for 2 h. CH₂Cl₂ (20 ml) was added to the reaction mixture, and the solution was then washed with water. The organic phase was evaporated under reduced pressure, and 5 ml of 10% piperidine in CH₂Cl₂ was added to deprotect the Fmoc-protected amine (15-min reaction time). The solvent was removed in vacuo, and the residue was subjected to silica gel column chromatography (CH₂Cl₂: CH₃OH:NH₃⋅H₂O, 79:20:1) to furnish the desired product in 81% yield.

Protocol for the Synthesis of para-Guanidinophenethylamine (Phenethylamine Derivative in 19)—HgCl₂ (105 mg, 0.39 mmol) was added to a solution containing the mono-Fmoc derivative of para-aminophenethylamine (i.e. Fmoc-NHCH₂(CH₂)₄NH₂) (125 mg, 0.35 mmol), bis-Boc-thioiuorea (102 mg, 0.37 mmol) (13), and pyridine (92 mg, 1.2 mmol) in 2 ml of N,N-dimethylformamide maintained at 0°C. The resulting solution was stirred at room temperature for 4 h. The solution was then filtered, and the solvent was removed under reduced pressure. 5 ml of 10% piperidine in CH₂Cl₂ was added to remove the Fmoc protecting group (15-min reaction time). The solvent was removed in vacuo, and the residue was subjected to silica gel column chromatography (CH₂Cl₂: CH₃OH:NH₃⋅H₂O, 79:20:1) to furnish para-bis-Boc-guanidinophenethylamine in 65% yield. The Boc protecting groups were removed after this phenethylamine derivative was coupled to the peptide (see below).

Protocol for the Synthesis of Peptide 20—The side-chain-protected crude peptide 9 (from 200 mg of Glu(O-t-butyl)-Glu(O-t-butyl)-Leu-Leu-oxime resin; see below) was suspended in 2 ml of water. 100 mg of NaHCO₃ was added to this mixture, followed by 126 mg of dimethylamino resin; see below) was suspended in 2 ml of water. 100 mg of Glu(O-t-butyl)-Glu(O-t-butyl)-Leu-Leu-oxime resin in 65% yield. The Boc protecting groups were removed as described above under “Preparation of the Glu-Glu-Leu-phenethylamine Conjugates.” Protocol for the Synthesis of N-Fmoc-L-para-bis-Boc-guanidinophenylalanine (the Protected Analog of 24 (see Fig. 3))—The mono-Fmoc derivative of para-aminophenylalanine was prepared as described above and subsequently converted to the bis-Boc derivative (92% yield) employing the same protocol as described for para-guanidinophenethylamine.

Preparation of Glu(O-t-butyl)-Glu(O-t-butyl)-Leu-Leu-oxime Resin

The tetrapeptide-oxime resin was prepared according to a previously described Boc protocol using an Advanced ChemTech Act 90 peptide synthesizer (14). Preparation of the Glu-Glu-Leu-phenethylamine Conjugates

The tetrapeptide-phenethylamine conjugates were synthesized by treating Glu(O-t-butyl)-Glu(O-t-butyl)-Leu-Leu-oxime resin with the phenethylamine derivatives described above. These reactions were performed with CHCl₃ as the solvent (except for 12 and 16, for which N,N-dimethylformamide was employed). The side chain protecting groups were then removed via treatment of the peptide with 1 ml of 90% trifluoroacetic acid, 10% thioanisole for 6 h and that both 23 and 25 were purified on a Dowex 1 × 8–200 (chloride form) resin using a 0.5% NaCl solution. Peptide 23 was purified by HPLC using the protocol described for peptides 16–20, and peptide 25 was purified employing the HPLC protocol described for peptides 1–8.

Preparation of Glu-Glu-Leu-(para-guanidine)-Phe-Gly-Glu-Ile (23) and Glu-Glu-Leu-Phe-Gly-Glu-Ile (25)

These peptides were prepared as described above for Arg-Arg-Arg-Leu-Glu-Leu-Leu-Tyr-amide with the exception that 23 was cleaved from the resin with 90% trifluoroacetic acid, 10% thioanisole for 20 h and that both 23 and 25 were purified on a Dowex 1 × 8–200 (chloride form) resin using a 0.5% NaCl solution. Peptide 23 was purified by HPLC using the protocol described for peptides 16–20, and peptide 25 was purified employing the HPLC protocol described for peptides 1–8.

Kinase Assay

Assays were performed in triplicate at pH 7.5 and thermostatted in a water bath maintained at 30°C. For determination of the IC₅₀ values, the following protocol was employed. Phosphorylation reactions were initiated by the addition of 10 μl of pp60⁰⁺⁺ diluted from a concentrated stock solution (3.97 nm in 1 mM dithiothreitol and 20 mM HEPES, pH 7.5) to a final 50-μl solution containing peptide inhibitor concentrations that varied about their respective IC₅₀ values, 100 μM (γ⁻³²P)ATP (1000 cpm/pmol), 750 μM Arg-Arg-Arg-Glu-Glu-Leu-Leu-Leu-Tyr-amide substrate, 20 mM HEPES, 20 mM MgCl₂, 0.125 mM/μl bovine serum albumin, 100 μM Na₃VO₄, and 0.79 nM pp60⁰⁺⁺. Reactions were terminated after 30 min by spotting 25-μl aliquots onto 2.1-cm-diameter phosphocellulose paper disks. After 10 s, the disks were immersed in 10% glacial acetic acid and allowed to soak with occasional stirring for 1 h. The acetic acid was removed, and the disks were subsequently washed with 4 volumes of 0.5% H₂O₂, 1 volume of water, followed by a final acetone rinse. The disks were air-dried and placed in plastic scintillation vials containing 3 ml of Liquiscint prior to scintillation counting for radioactivity. The following conditions were employed for the determination of the Kᵢ values. The [γ⁻³²P]ATP (1000 cpm/pmol) was fixed at 100 μM for experiments with peptide substrate (600–1800 μM). The peptide substrate was fixed at 750 μM for experiments with variable peptide substrate (γ⁻³²P)ATP (1800 cpm/pmol, 15–60 μM). Inhibitor concentrations were varied about their respective Kᵢ values in both sets of experiments. The reactions were initiated and terminated as described above.

RESULTS AND DISCUSSION

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 (for recent examples, see Refs. 7–9). 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. In addition, Wang et al. (7) have demonstrated that, in some cases, the \( K_{\text{m}} \) values exhibited by tyrosine-based peptide substrates are gross overestimates of how well these peptides bind to the target enzyme. 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 \( \text{C} \) terminus of active site-directed peptides. For example, \( \text{pp}60^{\text{src}} \) utilizes Arg-Arg-Arg-Arg-Leu-Glu-Leu-Leu-Tyr-amide as a substrate (12). 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 Fig. 1. First, the active site-directed peptide is synthesized by employing a standard Boc protocol on a modified polystyrene support (17). 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. In the study described herein, Glu-Glu-Leu-Leu-oxime-resin was treated with a variety of \( \text{para} \)-substituted phenylmethane derivatives \( (H_3\text{NCH}_2\text{CH}_2\text{C}_6\text{H}_4\text{X}) \) to produce active site-directed peptides of the general structure Glu-Glu-Leu-Leu-HNCH_2\text{CH}_2\text{C}_6\text{H}_4\text{X}.

We examined a total of 20 different nonphosphorylatable phenethylamine derivatives (Table I). Our initial survey of the inhibitory efficacy of these compounds focused on the acquisition of \( IC_{50} \) values at fixed ATP (100 \( \mu \text{M} \)) and peptide substrate (Arg-Arg-Arg-Arg-Leu-Glu-Leu-Leu-Tyr-amide, at its \( K_{\text{m}} \) of 750 \( \mu \text{M} \)), since significantly larger quantities of inhibitor and, in particular, enzyme, are required to obtain \( K_{\text{m}} \) values. Compound 1, which contains the parent phenethylamine itself, is structurally analogous to that of phenylalanine. The nearly 2 \text{mM} \( IC_{50} \) value associated with this species is in keeping with the poor inhibitory performances exhibited by other, previously described, phenethylamine-based tyrosine kinase inhibitors (7–9). For comparative purposes, we also investigated the inhibitory activity of the tyramine-containing analog, 2. We have previously shown that this alcohol-bearing residue is phosphorylated by \( \text{pp}60^{\text{src}} \) when attached to the C-terminal position of Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Leu- (12). However, since we attached tyramine to Glu-Glu-Leu-Leu- in this study, any phosphorylation of the aromatic alcohol will be “invisible” to the phosphocellulose paper disc detection method. As an inhibitor of the \( \text{pp}60^{\text{src}} \)-catalyzed phosphorylation of Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Leu-Tyr-amide, peptide 2 exhibits an \( IC_{50} \) of 300 ± 10 \( \mu \text{M} \). In short, there is an apparent 7-fold difference in inhibitory efficacy between 1 and 2. Clearly, it is tempting to attribute this difference to inhibitory activity to the presence of the hydroxyl group on 2, 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 \( \text{pp}60^{\text{src}} \) afforded by the alcoholic alcohol?

The methyl-substituted derivative 3 is a slightly poorer inhibitor than 1. The halogenated derivatives 4, 5, and 6 are also somewhat weaker as inhibitors compared with 1. However, the pentfluoro derivative 7 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 7 may interact with an electron-rich moiety in the active site of \( \text{pp}60^{\text{src}} \), thereby enhancing enzyme affinity. Interestingly, the methoxy-substituted species 8 contains a significantly more electron-rich aromatic system than its counterpart in 1; however, the inhibitory potency of 8 is nearly identical to that of 1. This may imply that the electron density associated with the \( \pi \) system has little influence on active site affinity or, at the very least, that only profoundly electron deficient aromatic systems (i.e. as in 7) interact with the active site in a unique fashion. An alternative explanation for the unusual behavior of 7 is based upon recent work by Whitesides and 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 7, compared with that of 1, may be due to this difference in relative hydrophobic surface area. Indeed, the recently elucidated three-dimensional structure of the insulin receptor reveals that the tyrosine binding site is in a particularly lipophilic region of the active site (19). Sequence align-
ments suggest that a structurally analogous situation should be present in pp60c-src (20).

The alcohol in tyrosine and the amine in 9 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 9, 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 9 is surprisingly poor. In fact, 9 is an even poorer inhibitor of pp60c-src than 1. In contrast, and somewhat unexpectedly, the aliphatic amines 10 and 11 are slightly better inhibitors than 1. The aromatic amine of 9 exhibits profoundly different structural properties than those in 10 and 11. 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, 10 and 11 both enjoy a considerably greater degree of conformational mobility than that of the aromatic amine in 9. In addition, the amines in 10 and 11 may penetrate somewhat deeper into the active site than their counterpart in 9. Is conformational mobility and/or the degree of active site penetration responsible for the enhanced inhibitory potencies associated with 10 and 11? We prepared several compounds to address this question.

The sulfonamide 12 displays an IC50 of 325 μM, which is significantly better than those exhibited by the parent species 1 as well as the benzylamine 10. Indeed, the inhibitory potency of 12 is essentially identical to that displayed by the substrate 2. The nitrogen atom in 12 is positioned, to a first approximation, somewhat analogously to that in 10. However, the amine in 12 is clearly more conformationally restricted than its counterpart in 10. In addition, the sulfonamide nitrogen is not basic enough to be protonated at physiological pH. In short, the sulfonamide functionality in 12 is a neutral conformationally restricted version of the methylamine moiety in 10. 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, 13, which contains a carbamoyl moiety at the para position, is not as potent an inhibitor as 12. Clearly, additional structural factors must influence enzyme affinity (see below). We also prepared the derivative 14, in which a nonbasic amine is now positioned further from the aromatic ring system than in 12. Unfortunately, 14 is disappointing as an inhibitor.

Is the sulfonyl group, and not the amine, responsible for the inhibitory properties of 12? Although we viewed this possibility as unlikely, we did prepare the methyl sulfone (15) as well as the sulfonic acid (16). Both 15 and 16 lack the amine moiety present in 12. In addition, the sulfonic acid moiety in 16 is negatively charged at neutral pH. Neither 15 nor 16 are effective inhibitors of pp60c-src. The poor inhibitory performance of 16, in particular, is noteworthy in light of the previous work of Graves and colleagues (21). These investigators prepared a gastrin analog in which the phosphorylatable tyrosine is replaced with both L- and D-tetrafluorotyrosine residues. Neither of these peptides serve as substrates for the insulin receptor, although both act as potent inhibitors. The L-tetrafluorotyrosine-containing peptide is particularly intriguing, since it functions as a competitive inhibitor versus protein substrate as well as versus ATP. Tetrafluorotyrosine exists as the negatively charged phenoxone at physiological pH as a consequence of the electron withdrawing fluorine substituents. These investigators proposed that the ionized phenoxone may mimic the transition state of the kinase-catalyzed reaction, which in turn explains the “bisubstrate” inhibition patterns exhibited by this species. We simply note here that the negatively charged sulfonic acid-containing derivative (16) is a weak inhibitor compared with the majority of inhibitors listed in Table I. This may imply that negatively charged functionality must be properly positioned on the aromatic nucleus in order to ensure potent...
inhibition. Alternatively, it is possible that structural motifs that are effective against the insulin receptor may be spectac-
ularly ineffective against pp60-src. Finally, we also prepared two compounds in which the sulfonyl (carbonyl) and amine
groups are inverted relative to their positions in 12 (and 14). 17
and 18 are among the weakest inhibitors identified in this study.

Given the apparent requirement for a nitrogen atom, one (i.e.
10) or two (i.e. 11) atoms removed from the aromatic nucleus on the para-positioned side chain, we synthesized the guanidine-
derivatized analog 19. Indeed, this compound is nearly as effec-
tive as 12 in its inhibitory potency toward pp60-src. In con-
trast, the bulky positively charged trimethylammonium derivative 20 is a poor inhibitor as well as its neutral, but polar, nitro-containing counterpart 21.

Since 12 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 12 is
structurally reminiscent of various isouquinoline sulfonamides
that have been previously shown to serve as protein kinase
inhibitors by coordinating to the ATP binding site (22). Conse-
sequently, we were concerned that the phenylsulfonamide in 12
may actually be functioning as an ATP analog. However, pep-
tide 12 serves as a competitive inhibitor versus variable peptide
substrate and as a noncompetitive inhibitor versus variable
ATP (Fig. 2). These results confirm that 12 functions as a Src
kinase inhibitor in the intended fashion, namely, by coordinat-
ing exclusively to the protein and not to the ATP binding site of
pp60-src. The Ki (300 ± 20 μM) value obtained from the variable
substrate experiment is analogous to the IC50 (325 ± 14 μM)
value exhibited by 12.

The sulfonamide moiety in 12 is the most potent tyrosine surro-
gate that we have identified to date. However, we were curious whether the results that we obtained with the phen-
ethylamine derivatives would apply to the more conventional phe-
nylalanine-based inhibitory peptides. For these studies we pre-
pared Glu-Glu-Leu-Leu-(pentafluorophenyl)glycine-glutam-
eine (22) and Glu-Glu-Leu-Leu-(para-guanidino)phenyl-
alanine (23). In the latter case, we employed the para-guanidine derivative 24 (Fig. 3), whose synthesis is described under “Materials and Methods.” As a control, we also prepared peptide Glu-Glu-Leu-Leu-
Phenylglycine-glutamic acid (25). One might expect that the completely peptidic environment would enhance enzyme affinity compared with the truncated peptides employed for the preparation of the inhibitors listed in Table I. Indeed, it does, but only to a modest extent with the primary sequence chosen. The pentafluorophenyl-
alanine-containing derivative (22) exhibits a Ki of 240 ± 10
μM, and the guanidine-containing derivative (23) displays a Ki
of 180 ± 10 μM. In contrast, Glu-Glu-Leu-Leu-(pentafluoro-
phenyl)glycine-glutamic acid (22) is a somewhat more modest inhibitor (Ki of 860 ± 20 μM). Furthermore, the phenethylamine/phenylalanine pairs exhibit the same relative difference in inhibitory potency. The Ki of the peptide containing para-guanidinophenylalanine (23) is nearly half (i.e. 0.46) of that displayed by its para-guanidinophen-
alanine analog (19). Similarly, the corresponding ratio for the pentafluorophenylalanine/pentafluorophenethylamine pair 22/27 is 0.44. In short, the IC50 and Ki values that we have obtained with the phenethylamine series of compounds listed
in Tables I and II appear to be excellent indicators of the

![Image](https://example.com/figure2.png)

**Fig. 2.** Inhibition pattern of peptide 12 versus variable Arg-
Arg-Arg-Arg-Leu-Glu-Glu-Leu-Leu-Tyr-amide at fixed ATP (100 μM) (A) and versus variable ATP at fixed Arg-Arg-Arg-
Arg-Leu-Glu-Glu-Leu-Leu-Tyr-amide (750 μM) (B). Peptide sub-
strate concentrations were varied from 600 to 1800 μM (12 = 0–330 μM), and ATP concentrations varied from 15 to 60 μM (12 = 0–512 μM). All experiments were performed in triplicate.

![Image](https://example.com/figure3.png)

**Fig. 3.** The structure of para-guanidinophenylalanine (24).

### Table II

| Inhibitor | Ki (μM) | Inhibition pattern (versus peptide substrate) |
|-----------|---------|---------------------------------------------|
| 7         | 540 ± 20 | Competitive                                  |
| 12        | 300 ± 20 | Competitive                                  |
| 19        | 390 ± 20 | Competitive                                  |
| 22        | 240 ± 10 | Competitive                                  |
| 23        | 180 ± 10 | Competitive                                  |
| 25        | 860 ± 20 | Competitive                                  |
inhibitory potency of the corresponding tyrosine analogs in conventional peptides.

To the best of our knowledge, the para-guanidino (19), para-sulfonamido (12), and pentafluorophenyl (7) derivatives are the first and only examples of nonphosphorylatable tyrosine surrogates\(^2\) with demonstrated enhanced inhibitory properties relative to both phenethylamine (1) and phenylalanine (24). The sulfonamide-based species is the best inhibitor identified to date. However, given the number of nonphosphorylatable tyrosine analogs investigated in this study, it is curious that the most potent species is equivalent to, but not better than, the corresponding aromatic alcohol substrate in terms of inhibitory potency. Although it would be somewhat rash to rule out the possibility that more potent analogs may be acquired in the future, it is conceivable that there is little additional binding energy to be gained within the immediate microenvironment of the alcohol binding region. However, based on the results described herein, it is clear that analogous strategies may lead to the acquisition even more powerful inhibitors. For example, functional groups can be positioned on the aromatic ring that extends into the ATP binding domain, a region where additional electrostatic and hydrophobic interactions can be appropriated. Indeed, “bisubstrate analogs” have been employed as inhibitors for a number of enzymes, including protein kinases. In addition, the comparatively good inhibitory profile of the pentafluoro derivative (7) suggests a second alternative for inhibitor development, namely modification of the phenolic aromatic ring. The latter approach, in combination with the results described herein, may ultimately provide inhibitors that exhibit both selectivity and high affinity for specific protein kinase targets.

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\(^2\) Hangauer and colleagues (23) found that a peptide containing phosphorylated tyrosine at the P position is a somewhat better inhibitor of p56\(^{\text{Lck}}\) than the corresponding phenylalanine-based peptide at low inhibitor concentrations. At higher concentrations, protein kinase activation was observed.

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**Nonphosphorylatable Tyrosine Surrogates**

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