Identification of Tricyclic Analogs Related to Ellagic Acid as Potent/Selective Tyrosine Protein Kinase Inhibitors

Robert L. Dow,*,† Thomas T. Chou,† Bruce M. Bechle,† Colin Goddard,‡ and Eric R. Larson†

Central Research Division, Pfizer Inc., Groton, Connecticut 06340, and Oncogene Science Inc., Manhasset, New York 11030

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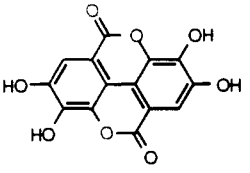
The plant-derived natural product ellagic acid (**1**) has recently been identified as a potent, though nonselective, inhibitor of the tyrosine-specific protein kinase pp60^{src}. This report details efforts directed toward the identification of tricyclic structures related to ellagic acid, with enhanced specificity for inhibition of pp60^{src} over other protein kinases. Phenanthridinone and carbazole core structures were selected for investigation, since N-functionalization allows for the synthesis of numerous analogs which can be utilized to probe enzyme–inhibitor interactions. These ring systems were prepared *via* a general sequence of biaryl bond formation followed by cyclization to form the desired tricyclic ring systems. N-Alkylation, -acylation, or -sulfonylation and deprotection with boron tribromide afford the target tetraphenolic phenanthridinones **5** and carbazoles **9**. Several analogs from both of these series have potencies comparable to that of **1** and exhibit substantially enhanced selectivities for inhibition of pp60^{src} relative to protein kinase A (PKA), a serine/threonine protein kinase. Carbazole-based analogs **9j,m,p** are submicromolar inhibitors of pp60^{src}, with potency for the target tyrosine kinase comparable to that of ellagic acid (**1**), however with 2 orders of magnitude greater selectivity versus that for PKA. As seen for ellagic acid, members of the phenanthridinone-based series (e.g., **5a**) exhibited inhibition of pp60^{src} in a manner which is partial mixed noncompetitive with respect to ATP, while analogs in the carbazole series (e.g., **9a**) inhibit pp60^{src} in an ATP competitive manner.

Since the initial discovery of retroviral tyrosine protein kinases, significant advances have been made in our understanding of the role this class of enzymes plays in transformation and regulation of cell proliferation.[1] Recently, the normal cellular variants, which include a number of growth factor receptors, have been shown to be involved in cellular regulation.[2,3] Clinical evidence now points to inappropriate expression of these normal cellular tyrosine-specific kinases being linked to human malignancies.[4-8] These findings suggest a role for selective inhibitors of tyrosine-specific protein kinases in the treatment of cancer and other hyperproliferative disorders.

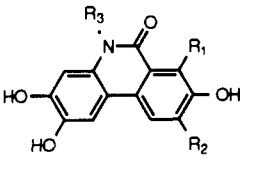
Natural product screening programs have identified a number of substances which inhibit tyrosine kinases or disrupt cellular processes mediated by this class of enzymes. By far, the majority of tyrosine kinase inhibitors reported are polyphenolic in nature. These include the fermentation isolates erbstatin[9] and lavendustin A,[10] members of the flavonoid family,[11,12] such as genistein,[13] and the plant-derived inhibitor piceatannol.[14] Nonphenolic natural products such as herbimycin A,[15] staurosporine,[16] epiderstatin,[17] and aeroplysinin-1[18] have also been reported. There has been significant follow-up of these leads through the preparation of synthetic analogs. Workers have utilized flavonoids[19,20] and erbstatin[21] as starting points for the preparation of derivatives with enhanced potency and selectivity toward tyrosine kinase enzymes over other protein kinases.

As part of a screening effort, we discovered ellagic acid (**1**) to be a potent (IC_{50} = 0.3 μM) inhibitor of the tyrosine protein kinase pp60^{src}.[22] Ellagic acid is present in a number of plant and fruit sources, including many in the human diet.[23] Diverse biological properties have been noted for ellagic acid, including antihemorrhagic,[24] antitumor,[25] antimutagenic,[26,27] and anticarcinogenic[28-30] activities. Though a relatively potent inhibitor of pp60^{src}, **1** exhibits a poor selectivity profile versus that for protein serine/threonine kinases, inhibiting c-AMP-dependent kinase (PKA) with an IC_{50} of 0.6 μM.

On the basis of structural considerations, direct modification of ellagic acid to improve selectivity/potency was not viewed as a viable option. Utilization of a backbone related to the ellagic acid nucleus, which could be readily derivatized, was an attractive starting point for improving the kinase profile of **1**. This would provide for the facile development of structure–activity relationships, in addition to probing binding interactions not readily achievable through direct modifications of ellagic acid. This paper details the identification of tricyclic-based ellagic acid analogs (**5** and **9**) which possess significantly enhanced selectivity profiles relative to that of the parent.



1 (ellagic acid)



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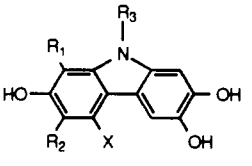

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Chemistry

A number of synthetic approaches to the carbazole/phenanthridinone nuclei have been described.[31-34] These

† Pfizer Inc.
‡ Oncogene Science Inc.
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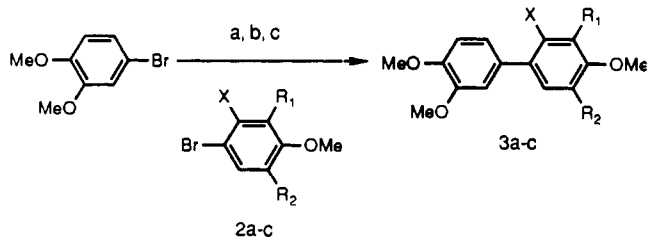
Scheme 1

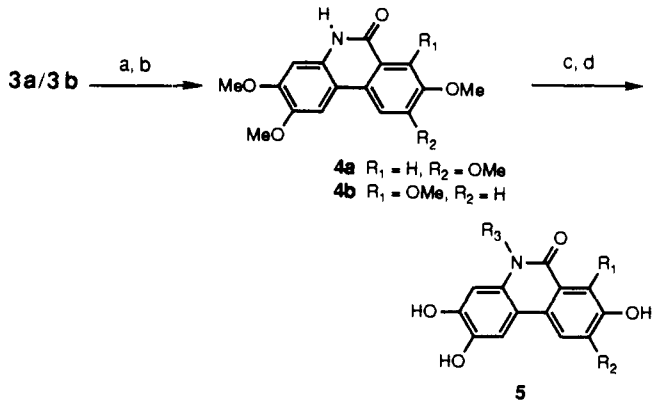


[a] (a) *n*-BuLi, Et_2O, −78 °C; (b) $ZnCl_2$, THF, 0 °C; (c) Pd(0), **2a–c**.

Table 1. Biphenyl Intermediates

no.	R_1	R_2	X	yield (%)
3a	H	OMe	CO_2Et	45
3b	OMe	H	CO_2Et	40
3c	H	OMe	NO_2	53

Scheme 2



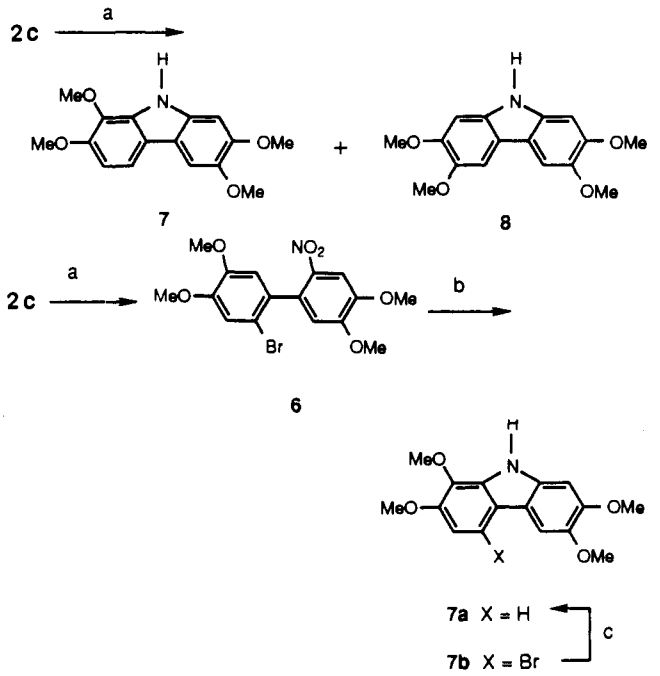
[a] (a) HNO_3, AcOH, room temperature; (b) Fe dust, AcOH, 100 °C; (c) KO*t*-Bu, DMSO, alkyl halide; (d) BBr_3, CH_2Cl_2, room temperature.

routes can be grouped into two categories: those in which the tricyclic backbone is generated during biaryl formation or through modification/cyclization of a biaryl precursor. Both strategies were investigated during the early stages of our ellagic acid analog synthetic program, but the latter approach proved to be more efficient.

Scheme 1 outlines the syntheses of the biaryl intermediates used in the construction of carbazole- and phenanthridinone-based ellagic acid analogs. In analogy to the work of Negishi and co-workers,[35] palladium-catalyzed coupling of the organozinc derived from 4-bromoveratrole with the appropriate aryl bromide (**2a–c**) generates the desired biaryls (Table 1). Due to steric encumbrance of the reacting centers in **2a–c**, elevated temperatures and extended reaction times (relative to substrates lacking ortho substitution[35,36]) were required to effect coupling. Side reactions, such as arylzinc addition to the ester functionality, become competitive under these conditions. Nevertheless, this straightforward approach to the construction of the biaryl bond readily provided multigram quantities of **3a–c**.

Construction of the key phenanthridinone-based intermediates (**4a,b**) is initiated through nitration of the more electron-rich aromatic ring in near quantitative yields (Scheme 2). Reduction affords the corresponding amines, which spontaneously cyclize to provide the requisite phenanthridinone backbone. Incorporation of substituents at the 5-position of **4a,b** is accomplished *via* alkylation of the potassium salts, generated with potassium *tert*-butoxide in dimethyl sulfoxide. In addition to the desired

Scheme 3



[a] (a) $(EtO)_3P$, 160 °C. [b] (a) Br_2, NaOAc, AcOH, $MeNO_2$; (b) $(EtO)_3P$, 160 °C; (c) Pd/C, 50 psi H_2, THF, 45 °C.

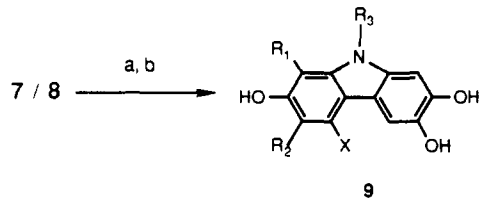
N-alkylated products (60–80%), the corresponding O-alkylated derivatives (10–20%) are also obtained. The two products are readily separated by flash chromatography,[37] with the N-alkylated product being more polar in all cases. Structural assignment of these regioisomers was confirmed by an X-ray analysis of the major product from the alkylation of **4b** with benzyl bromide. Treatment of the tetramethylated phenanthridinones with boron tribromide affords tricyclic, ellagic acid analogs **5a–l**.

Synthesis of the carbazole-based intermediates is achieved in a straightforward manner from **2c** (Scheme 3, top). Treatment of **2c** with excess triethyl phosphite at reflux affords equal proportions of **7a** and **8** in a 62% combined yield.[38] Symmetrical isomer **8** is selectively crystallized from ethyl acetate, and chromatography of the filtrate affords **7a**. During the course of our studies, it became apparent that tetraphenolic analogs derived from **7a** were more potent/selective tyrosine kinase inhibitors than the symmetrical-based isomers (*vide infra*). This finding stimulated us to develop a more efficient synthesis of **7a**. A regioselective synthesis of **7a** was developed (Scheme 3, bottom), utilizing bromine as a temporary blocking group to direct the course of cyclization. Bromination of **2c** in a mixed solvent system of acetic acid and nitromethane afforded **6**, which is cyclized to afford **7b** in 68% overall yield. Hydrogenolysis of **7b** affords **7a** in a 98% recrystallized yield. This latter route more than doubles the yield of **7a**, relative to that obtained from the direct cyclization of **2c**. Alkylation, acylation, and sulfonylation of the sodium anions (NaH/DMSO) of **7a,b** and **8** proceed smoothly (Scheme 4). Demethylation with boron tribromide affords tetrahydroxylated carbazoles **9a–ff**.

Biology

The tyrosine kinase chosen for these studies was $pp60^{c\text{-}src}$. There are two variants of the *src* gene: the retroviral form, which encodes the abrogated tyrosine

Scheme 4

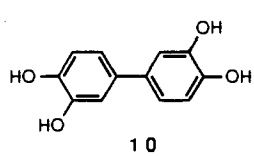


[a] (a) NaH, DMSO, R_3-halide; (b) BBr_3, CH_2Cl_2.

kinase pp60$^{v\text{-}src}$ lacking a negative regulatory sequence, and the normal cellular homolog, which produces pp60$^{c\text{-}src}$.[1] While there is no evidence that the viral form of pp60 is associated with human malignancies, pp60$^{c\text{-}src}$ has been implicated in human colon carcinoma.[4,5] More detailed studies have revealed that pp60$^{c\text{-}src}$ is overexpressed in colon adenocarcinomas and possesses higher specific activity than the corresponding enzyme derived from adjoining normal colonic tissue.[39-42] Tumor progression studies in colon carcinoma point toward altered kinase activity being present in the early stages of tumorigenesis.[43] These findings suggest that the tyrosine kinase pp60$^{c\text{-}src}$ is a worthwhile target for antitumor drug design. The pp60$^{c\text{-}src}$ utilized in these studies was isolated from human platelets and assayed by measuring the degree of phosphorylation of a synthetic substrate (gastrin analog). The purified catalytic subunit of the protein serine/threonine kinase, PKA, was employed as a specificity control.

Results and Discussion

Following the discovery that ellagic acid is a potent, though nonselective, tyrosine kinase inhibitor, our efforts were directed toward defining the key structural elements required for activity. A number of structurally simplified analogs were prepared and screened for inhibitory activity.[44] Removal of the lactone bridges present in 1 affords biphenyl **10** but eliminates (IC_{50} >1000 μM) pp60$^{c\text{-}src}$ inhibitory activity. Reincorporation of one of the bridging elements (**11**) restores inhibitory activity. A series of dihydroxylated analogs, exemplified by **12**, were prepared to determine the degree and orientation of hydroxylation required for inhibitory activity. All of these derivatives are at least 2 orders of magnitude less potent than ellagic acid.[44]



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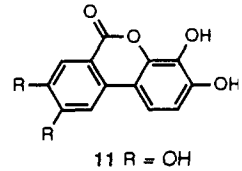


11 R = OH
12 R = H

Unpublished work in our laboratories[44] has shown that a "catechol" arrangement of the hydroxyl groups is a necessary requirement for significant inhibition of pp60^{src} by a variety of phenolic-based inhibitors; the studies described here focus exclusively on targets containing this hydroxyl substitution pattern. It is of interest to note that the inhibition profile of this enzyme is clearly distinguished from other tyrosine-specific protein kinases. For instance, a number of phenolic-based epidermal growth factor receptor inhibitors,[9,21] lacking this "catechol-like" substitution pattern, are devoid of significant activity against pp60^{src}.[45]

Though modification of ellagic acid to yield a tricyclic-based analog, such as **11**, represents a fairly minor structural modification, this finding proved to be a critical aspect of this work. Adopting a tricyclic framework not only simplifies the synthetic effort required but also greatly expands the range of readily accessible targets. In addition, replacement of the ester linker unit present in **11** with a moiety to which substituents could be appended was key to a rapid SAR development, with a goal of identifying analogs possessing enhanced selectivity for tyrosine protein kinases. With this in mind, the phenanthridinone backbone was selected as an initial building block.

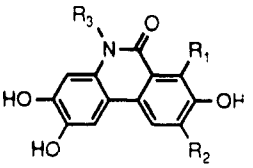
Table 2 details the parental and N-derivatized phenanthridinone-based analogs prepared in this study. Phenolic substitution patterns were selected mainly on the basis of synthetic accessibility; earlier work in the tricyclic lactone series had shown that repositioning of the "catechol" functionality produced only minor perturbations in pp60$^{c\text{-}src}$ inhibitory activity. The parental analogs **5a,b** were slightly less potent than ellagic acid; however, there had been a significant diminution in inhibitory activity versus that for PKA, a member of the serine/threonine protein kinase family. Whereas ellagic acid possesses only a 2-fold selectivity for pp60$^{c\text{-}src}$ versus that for PKA, **5a,b** had greatly enhanced (800- and 100-fold, respectively) specificities for tyrosine kinase inhibition. These results prompted us to prepare a series of N-alkylated derivatives of **5a,b**. Several conclusions can be drawn from the kinase inhibitory activities associated with these analogs. For all of the phenanthridinone-based inhibitors prepared to date, those derived from **5a** were consistently more potent than those derived from **5b** (e.g., **5e** versus **5f**). Analogs (**5c,e**, etc.) from the former series were also significantly more selective protein tyrosine kinase inhibitors than those derived from **5b**. These results point to a strong dependence of potency and selectivity on the hydroxyl substitution pattern within this phenanthridinone-based series of tyrosine kinase inhibitors. Other regioisomers related to **5a,b** may possess enhanced potency/selectivity; however, an unrelated issue (*vide infra*) redirected our efforts at this juncture.

In an attempt to expand our understanding of the structure–activity relationships within the phenanthridinone series, synthesis of the corresponding N-acylated series was attempted. However, O-acylation was the exclusive course from the reaction of the potassium salt of **4b** (Scheme 2) and benzoyl chloride. We were also unsuccessful in attempts to prepare N-sulfonylated analogs of this phenanthridinone-based series. Because of these limitations, our attention was directed toward identification of an alternative tricyclic core structure which would allow for a broader SAR development.

The carbazole ring system was selected for pursuit because it met the design criterion of being readily functionalized on nitrogen with a range of substituents. This series was also of interest because the nitrogen substituents could occupy spatial orientations, relative to the phenolic backbone, which were not accessible in the phenanthridinone-based series. Table 3 details those analogs prepared utilizing the carbazole backbone, including N-alkylated, -acylated, and -sulfonylated derivatives.

Two phenolic substitution patterns were investigated during the course of this study: one in which the phenolic functionality is symmetrically disposed (e.g., **9a**) and another in which it is unsymmetric (e.g., **9b**). The latter

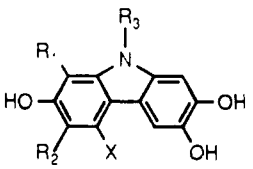
Table 2. *In Vitro* Kinase Activities of Phenanthridinones **5**



no	R_1	R_2	R_3	mp (°C)[a]	formula	IC_{50} (μM) *c-src*	IC_{50} (μM) PKA
1		ellagic acid dihydrate				0.3	0.6
5a	H	OH	H	>250	$C_{13}H_9NO_5 \cdot 1.3H_2O$	1.0	>800
5b	OH	H	H	>250	$C_{13}H_9NO_5 \cdot 0.8H_2O$	0.9	92
5c	H	OH	Et	309–310	$C_{15}H_{13}NO_5 \cdot 0.5H_2O$[b]	4.1	>2000
5d	OH	H	Et	275	$C_{15}H_{13}NO_5 \cdot 1H_2O$	11	541
5e	H	OH	CH_2Ph	261–263	$C_{20}H_{15}NO_5 \cdot 0.5H_2O$[c]	5.4	390
5f	OH	H	CH_2Ph	224–225	$C_{20}H_{15}NO_5 \cdot 0.75H_2O$	21	284
5g	H	OH	$CH_2(4\text{-}NO_2Ph)$	325	$C_{20}H_{14}N_2O \cdot 0.75H_2O$[d]	0.9	8.4
5h	OH	H	$CH_2(4\text{-}NO_2Ph)$	258–263	$C_{20}H_{14}N_2O \cdot 0.75H_2O$	61	309
5i	H	OH	$CH_2(3,4\text{-}Cl_2Ph)$	318	$C_{20}H_{13}Cl_2NO_5 \cdot 0.5H_2O$	1.3	20
5j	OH	H	$CH_2(3,4\text{-}Cl_2Ph)$	246	$C_{20}H_{13}Cl_2NO_5 \cdot 0.5H_2O$	197	410
5k	H	OH	$CH_2(4\text{-}(SO_2Ph)Ph)$	245–251	$C_{26}H_{19}NO\text{-}S \cdot 0.45CCl_4$	2.0	51
5l	OH	H	$CH_2(4\text{-}(SO_2Ph)Ph)$	305–307	$C_{26}H_{19}NO\text{-}S \cdot 0.5H_2O$	7.2	634

[a] Recrystallized from methanol/carbon tetrachloride, *except* for **5a,b,c**. **5c** was recrystallized from acetone/hexanes. [b] N: calcd, 4.73; found, 4.17. [c] H: calcd, 4.50; found, 3.91. [d] H: calcd, 3.83; found, 3.32.

Table 3. *In Vitro* Kinase Activities of Carbazoles **9**



no.	R_1	R_2	R_3	X	mp (°C)[a]	formula	IC_{50} (μM) *c-src*	IC_{50} (μM) PKA
1		ellagic acid dihydrate					0.3	0.6
9a	H	OH	H	H	>300	$C_{12}H_9NO_4 \cdot 0.5H_2O$	1.5	58
9b	OH	H	H	H	>300	$C_{12}H_9NO_4$	0.8	47
9c	OH	H	H	Br	>250	$C_{12}H_8BrNO_4$	0.6	17
9d	H	OH	CH_2Ph	H	266–271	$C_{19}H_{15}NO_4 \cdot 0.25H_2O$	7.0	115
9e	OH	H	CH_2Ph	H	241–246	$C_{19}H_{15}NO_4 \cdot 0.25H_2O$	2.0	210
9f	H	OH	COPh	H	287–292	$C_{19}H_{13}NO_5 \cdot H_2O$	4.0	415
9g	OH	H	COPh	H	242–243	$C_{19}H_{13}NO_5$	0.9	240
9h	H	OH	CH_2(3-pyridyl)	H	290–294 dec	$C_{18}H_{14}N_2O_4 \cdot HBr \cdot 0.5H_2O$	8.0	108
9i	OH	H	$(CH_2)_3Ph$	H	246–252	$C_{21}H_{19}NO_4$	0.5	0.5
9j	OH	H	$CH_2(4\text{-}CNPh)$	H	225–227	$C_{20}H_{14}N_2O_4 \cdot 0.25H_2O$	0.4	275
9k	OH	H	$CH_2(4\text{-}CNPh)$	Br	>250	$C_{20}H_{13}BrN_2O_4$	0.9	6.6
9l	OH	H	$CH_2(4\text{-}(CF_3)Ph)$	H	266–270	$C_{20}H_{14}F_3NO_4 \cdot 0.25H_2O$	9.0	50
9m	OH	H	$CH_2(2,6\text{-}Cl_2Ph)$	H	262–265 dec	$C_{19}H_{13}Cl_2NO_4$	0.9	435
9n	OH	H	$CH_2(4\text{-}BrPh)$	H	249–252	$C_{19}H_{14}BrNO_4 \cdot 0.25H_2O$	3.4	374
9o	OH	H	$CH_2(3\text{-}MePh)$	H	250–255 dec	$C_{20}H_{17}NO_4$	1.7	23
9p	OH	H	$CH_2((4\text{-}PhSO_2)Ph)$	H	>300	$C_{25}H_{19}NO_6S \cdot 0.8H_2O$	0.6	317
9q	OH	H	$CO(4\text{-}BrPh)$	H	272–278	$C_{19}H_{12}BrNO_5$	1.4	4.2
9r	OH	H	$CO((4\text{-}Ph)Ph)$	H	291–294	$C_{25}H_{17}NO_5 \cdot 0.25H_2O$	0.6	3.2
9s	OH	H	2-naphthoyl	H	266–270	$C_{23}H_{15}NO_5 \cdot 0.15H_2O$	0.5	1.0
9t	OH	H	$CO(3\text{-}(NO_2)Ph)$	H	275–279 dec	$C_{19}H_{12}N_2O_7$	0.6	1.5
9u	OH	H	$CO(4\text{-}(CF_3)Ph)$	H	229–230	$C_{20}H_{12}F_3NO_5 \cdot 0.4H_2O$	0.7	10
9v	OH	H	$CO(4\text{-}(CF_3)Ph)$	Br	228–230	$C_{20}H_{11}BrF_3NO_5$	2.0	7
9w	OH	H	$CO(4\text{-}t\text{-}BuPh)$	H	234–236	$C_{23}H_{21}NO_5$	0.7	50
9x	OH	H	$CO(4\text{-}t\text{-}BuPh)$	Br	>280	$C_{23}H_{20}BrNO_5$	12	40
9y	OH	H	$CO(4\text{-}(MeSO_2)Ph)$	H	286–288	$C_{20}H_{15}NO_7S$	0.4	40
9z	OH	H	$CO(4\text{-}(MeSO_2)Ph)$	Br	>280	$C_{20}H_{14}BrNO_7S$	9	4
9aa	OH	H	$CO(CH_2)_2Ph$	H	223–227	$C_{21}H_{17}NO_5$	0.4	2.3
9bb	H	OH	SO_2Ph	H	240–241	$C_{18}H_{13}NO_6S \cdot H_2O$	24	>600
9cc	OH	H	SO_2Ph	H	176–178	$C_{18}H_{13}NO_6S \cdot 0.4H_2O$	15	250
9dd	OH	H	$SO_2(2,5\text{-}Cl_2Ph)$	H	>300	$C_{18}H_{11}Cl_2NO_6S \cdot 0.25H_2O$	7.7	223
9ee	OH	H	SO_2CH_2Ph	H	216–218	$C_{19}H_{15}NO_6S$	14	78
9ff	OH	H	$SO_2(4\text{-}(NO_2)Ph)$	H	233–234	$C_{18}H_{12}N_2O_8S \cdot 0.25H_2O$	12	168

[a] Recrystallization solvents: **9d–g,i,j,l,n,p,w,x,bb–ff**—methanol/water; **9a,b**—water; **9m,r–t**—ethyl acetate; **9h,k**—methanol/dichloromethane; **9o,q,u,v,aa**—ethyl acetate/hexanes; **9c,y**—acetone/dichloromethane.

substitution pattern is most closely aligned with that observed in ellagic acid. Protein tyrosine kinase inhibitory activities associated with the parental compounds (**9a–c**) suggested that those analogs with the unsymmetric hydroxylation pattern should be the more potent of the two regioisomeric series. To confirm this, the correspond-

Table 4. Mode of Inhibition Models versus Substrate ATP

compound	model with best fit	RMS	parameters	V_M (pmol/min)	K_M (μM)	K_I (μM)	$K_{I'}$ (μM)
ellagic acid 1	partial mixed	0.1016	value	2.19	78.78	0.081	0.161
	noncompetitive		% error	5.99	18.88	35.87	33.06
	pure	0.2848	value	1.65	58.95	0.307	
	competitive		% error	11.48	43.98	64.91	
	partial	0.2429	value	1.66	43.90	0.037	0.447
	competitive		% error	10.36	46.71	106.8	124.4
phenanthridinone 5a	partial mixed	0.0543	value	1.54	78.31	0.99	3.44
	noncompetitive		% error	5.78	14.25	39.00	52.12
	pure	0.0704	value	1.49	85.82	4.41	
	competitive		% error	5.79	14.86	24.67	
	partial	0.0524	value	1.50	74.85	1.05	4.68
	competitive		% error	4.09	12.02	36.73	49.07
carbazole 9a	pure	0.1778	value	2.21	68.06	1.06	
	competitive		% error	7.85	29.23	0.53	

ing *N*-benzyl (**9d,e**), *N*-benzoyl (**9f,g**), and *N*-phenylsulfonyl (**9bb,cc**) derivatives were prepared. As for the parental compounds, the unsymmetrically substituted isomers were more potent tyrosine kinase inhibitors. On the basis of these results, effort was focused on this regioisomeric series (see Table 3).

Because ellagic acid is a nonselective kinase inhibitor (2-fold separation between c-*src* and PKA), one of the major goals of this study was to identify ellagic acid-based analogs with enhanced selectivity for pp60$^{c\text{-}src}$. Of the initial set of compounds discussed above, all are substantially more selective for pp60$^{c\text{-}src}$ than ellagic acid, with the *N*-benzoyl analog (**9g**) greater than 100-fold more selective than **1**. This enhanced selectivity arises from significantly diminished inhibition of PKA rather than from increased potency versus that of the tyrosine kinase.

To expand these initial findings, a series of *N*-benzyl/-benzoyl analogs were prepared, focusing on the unsymmetrical hydroxyl substitution pattern. On the basis of side-by-side comparisons (**9g/e**, **9u/l**, and **9q/n**), the benzoyl analogs are from 2- to 10-fold more potent protein tyrosine kinase inhibitors than the corresponding benzyl congeners. However, there is not a clear trend for these two series in their ability to selectivity inhibit pp60$^{c\text{-}src}$.

From this study, three *N*-benzyl analogs (**9j,m,p**) stand out as being potent and selective inhibitors of pp60$^{c\text{-}src}$. These analogs are submicromolar inhibitors of pp60$^{c\text{-}src}$, but more importantly, they possess substantially reduced PKA inhibitory activities. These agents show a 500–700-fold separation between tyrosine kinase and serine/threonine kinase inhibitory activities, representing an improvement of greater than 2 orders of magnitude relative to that by ellagic acid. Since several of the most potent pp60$^{c\text{-}src}$ inhibitors possess bulky benzyl/benzoyl substituents, the corresponding 3-phenylpropyl/-propionyl analogs (**9i/aa**, respectively) were prepared. Both of these compounds are potent inhibitors of pp60$^{c\text{-}src}$, but unlike the parental *N*-benzyl/benzoyl derivatives, these homologs are potent inhibitors of PKA. A more detailed SAR study will be required to sort out the reason(s) for subtle structural modifications leading to substantial differences in PKA activity.

On the basis of the excellent potency observed for **9c**, several 4-bromocarbazole-based analogs were prepared, including the homolog (**9k**) of **9j**. Though all of these compounds were potent inhibitors of pp60$^{c\text{-}src}$, all had substantially reduced selectivity profiles relative to those of their desbromo counterparts (Table 3). Tyrosine kinase inhibition by a set of *N*-sulfonyl-based carbazoles (**9bb–ff**) suggests this series has significantly reduced potency relative to that of the corresponding *N*-benzyl/-benzoyl derivatives.

Studies on phenolic-based tyrosine kinase inhibitors such as the bioflavones (e.g., quercetin) have demonstrated a mode of inhibition which is best described as being purely competitive with respect to substrate ATP. We were interested in exploring the nature of the inhibition elicited by ellagic acid and representatives of each of the structural series in this study. To this end, the inhibition of ellagic acid, compound **5a** from the phenanthridinone series, and compound **9a** from the carbazole series was investigated at varying concentrations of ATP, and the data were analyzed using the weighted nonlinear least-squares curve-fitting program Enzyme PC. The results of these studies are summarized in Table 4. To our surprise, data for both the parent compound and phenanthridinone **5a** indicate a significant ($P > 0.95$) and opposite effect of the inhibitor on both the apparent K_M and V_M, consistent with either pure or partial mixed noncompetitive models. The curved nature of Dixon plots (not shown) indicated partial inhibition, and the fit obtained for this model (partial mixed noncompetitive inhibition) was clearly the best for the parent compound ellagic acid and apparently so for the phenanthridinone **5a**. Studies on the carbazole **9a** provided; however, a very good fit to a purely competitive model. Significant effects were only seen on the apparent K_M obtained at different ATP concentrations in the presence of inhibitor, and good fits to the pure competitive model were also obtained for other compounds in this series. Since there is evidently no direct correlation between the mode of inhibition observed and the selectivity versus those for PKA, this difference in apparent modes of inhibition was not pursued further. In addition, because of the difference in models, the overall data have been presented as IC_{50}'s to facilitate a broad comparison of trends. Data are also presented for model fitting of compounds **1** and **5a** to pure and partial competitive models in order to provide a comparison.

In summary, a series of *N*-substituted carbazole analogs have been identified which potently inhibit the tyrosine protein kinase activity associated with pp60$^{c\text{-}src}$. By varying the nitrogen substituent, it is possible to substantially alter the associated kinase inhibition profile, leading to agents with greater than 500-fold specificities for pp60$^{c\text{-}src}$ tyrosine kinase over a representative serine/threonine kinase. Separation of kinase inhibitory activities is a critical first step toward the development of selective agents for the treatment of diseases dependent on tyrosine kinases.

Experimental Section

All melting points were taken on a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were carried out by the Analytical Department of Pfizer Central Research, and results obtained for specified elements are within +/−0.4% of the theoretical values unless otherwise denoted. ^{1}H NMR spectra of deuteriochloroform or DMSO-d_6 solutions (solvent utilized as an internal standard and deuterium lock) were recorded on a Varian XL-300 spectrometer. Ellagic acid dihydrate was purchased from Aldrich Chemical Co.

General Procedure for Palladium-Catalyzed Biphenyl Formation. Ethyl 2-(3,4-dimethoxyphenyl)-4,5-dimethoxybenzoate (3a). To a cooled (−78 °C), stirred solution of 4-bromoveratrole (3.60 g, 16.6 mmol) in diethyl ether (40 mL) was added dropwise a 2.5 M solution of *n*-butyllithium (6.60 mL, 16.6 mmol) in hexanes. The resulting slurry was allowed to warm to 0 °C and maintained at this temperature for 30 min. The resulting mixture was diluted with THF (20 mL) and added, *via* a cannula, to a cooled (0 °C), stirred solution of fused zinc chloride (2.71 g, 19.9 mmol) in THF (40 mL). This solution was maintained at 0 °C for 30 min. In a separate flask, a slurry of bis(triphenylphosphine)palladium(II) chloride (0.39 g, 0.55 mmol) in THF (20 mL) was treated with a 1.0 M solution of diisobutylaluminum hydride (1.1 mL, 1.1 mmol) in dichloromethane for 20 min at ambient temperature. Ethyl 2-bromo-4,5-dimethoxybenzoate (4.00 g, 13.8 mmol) was added to this catalyst solution, and the resulting dark solution was added to the organozinc reagent prepared above. This solution was refuxed for 18 h. The reaction solution was cooled, poured into water, and extracted with EtOAc. The organic layer was washed with brine, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was purified by flash chromatography over silica gel (20% EtOAc/hexanes) to afford the title compound (2.15 g, 45%) as an off-white solid: mp 91–94 °C; ^{1}H NMR (DMSO-d_6) δ 7.28 (s, 1H), 6.99 (d, J = 8 Hz, 1H), 6.94 (s, 1H), 6.86 (d, J = 2 Hz, 1H), 6.80 (dd, J = 2, 8 Hz, 1H), 4.03 (q, 2H), 3.87 (s, 3H), 3.83 (s, 3H), 3.78 (s, 3H), 3.74 (s, 3H), 0.98 (t, 3H). Anal. ($C_{19}H_{22}O_6$) C, H.

Ethyl 2-(3,4-dimethoxyphenyl)-5,6-dimethoxybenzoate (3b): prepared using the above general procedure; mp 76–77 °C; ^{1}H NMR (DMSO-d_6) δ 7.18 (d, J = 8 Hz, 1H), 7.11 (d, J = 8 Hz, 1H), 6.95 (d, J = 8 Hz, 1H), 6.85 (d, J = 2 Hz, 1H), 6.82 (dd, J = 2, 8 Hz, 1H), 4.11 (q, 2H), 3.97 (s, 3H), 3.78 (s, 3H), 3.77 (s, 3H), 3.75 (s, 3H), 1.07 (t, 3H). Anal. ($C_{19}H_{22}O_6$) C, H.

2-(3,4-Dimethoxyphenyl)-4,5-dimethoxynitrobenzene (3c): prepared using the above general procedure; mp 148.5–150.5 °C; ^{1}H NMR (DMSO-d_6) δ 7.50 (s, 1H), 6.93 (d, J = 9 Hz, 1H), 6.92 (s, 1H), 6.85 (d, J = 2 Hz, 1H), 6.75 (dd, J= 2, 9 Hz, 1H), 3.86 (s, 3H), 3.83 (s, 3H), 3.74 (s, 3H), 3.72 (s, 3H). Anal. ($C_{16}H_{17}NO_6$) C, H, N.

General Procedure for Preparation of Tetramethoxy-Substituted Phenanthridinone Intermediates. 2,3,8,9-Tetramethoxy-6(5*H*)-phenanthridinone (4a). To a stirred solution of **3a** (2.00 g, 5.77 mmol) in glacial acetic acid (30 mL) was added concentrated nitric acid (0.72 mL, 11.5 mmol) dropwise. After 10 min, the reaction mixture was poured onto ice (150 g) and the solids were extracted into EtOAc. The organic phase was washed with water, 1 N NaOH, and brine, dried (Na_2SO_4), filtered, and concentrated *in vacuo* to afford ethyl 4,5-dimethoxy-2-(4,5-dimethoxy-2-nitrophenyl)benzoate as a yellow solid (2.25 g, 100%): mp 126–128 °C; ^{1}H NMR (DMSO-d_6) δ 7.66 (s, 1H), 7.44 (s, 1H), 6.82 (s, 1H), 6.80 (s, 1H), 3.93 (q, 2H), 3.88 (s, 3H), 3.82 (s, 6H), 3.79 (s, 3H), 0.92 (t, 3H).

To a stirred, heated (100 °C) slurry of iron dust (325 mesh, 2.8 g) in glacial acetic acid (30 mL) was added a solution of ethyl 4,5-dimethoxy-2-(4,5-dimethoxy-2-nitrophenyl)benzoate (1.42 g, 3.63 mmol) in glacial acetic acid (40 mL) over a 5-min period. After 1.5 h, the residual iron fillings were removed with a magnetic stir bar and the reaction slurry was poured onto ice/water (150 mL). The solids were filtered, washed with water, air-dried, and dried *in vacuo* at 80 °C to afford the title compound as a gray solid (1.00 g, 88%): mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 7.75 (s, 1H), 7.74 (s, 1H), 7.67 (s, 1H), 6.91 (s, 1H), 4.04 (s, 3H), 3.92 (s, 3H), 3.89 (s, 3H), 3.82 (s, 3H).

2,3,7,8-Tetramethoxy-6(5*H*)-phenanthridinone: prepared from **3b** using the above general procedure; mp 264–266 °C; ^{1}H NMR (DMSO-d_6) δ 8.10 (d, 1H), 7.62 (s, 1H), 7.47 (d, 1H), 6.81 (s, 1H), 3.86 (s, 3H), 3.82 (s, 3H), 3.75 (s, 1H), 3.73 (s, 3H).

1,2,6,7-Tetramethoxy-9*H*-carbazole (7a) and 2,3,6,7-Tetramethoxy-9*H*-carbazole (8). A stirred solution of **3c** (6.5 g, 20 mmol) in triethyl phosphite (10.5 mL, 61.1 mmol) was heated at 160 °C under a nitrogen atmosphere for 10 h. The excess triethyl phosphite was removed *in vacuo* and the residue slurried in chloroform. The resulting solids were filtered and recrystallized from EtOAc to afford 2,3,6,7-tetramethoxy-9*H*-carbazole (**8**) (1.9 g, 33%): R_f = 0.17 (25% EtOAc/CCl_4); mp 232–233 °C; ^{1}H NMR (DMSO-d_6) δ 7.49 (s, 2H), 6.91 (s, 2H), 3.78 (s, 12H). The chloroform filtrate from above was concentrated *in vacuo* and flash chromatographed (silica gel, 20% EtOAc/CCl_4) to afford 1,2,6,7-tetramethoxy-9*H*-carbazole (**7a**) (1.7 g, 29%): R_f = 0.35 (25% EtOAc/CCl_4); mp 170–171 °C (MeOH); ^{1}H NMR (DMSO-d_6) δ 7.53 (d, J = 8 Hz, 1H), 7.46 (s, 1H), 6.86 (s, 1H), 6.76 (d, J = 8 Hz, 1H), 3.81 (s, 3H), 3.79 (s, 3H), 3.75 (s, 6H). Anal. ($C_{16}H_{17}NO_4$) C, H, N.

2-(2-Bromo-4,5-dimethoxyphenyl)-4,5-dimethoxynitrobenzene (6). To a stirred solution of **3c** (9.0 g, 28 mmol) and sodium acetate (5.8 g, 70 mmol) in nitromethane (100 mL) and acetic acid (70 mL) was added a solution of bromine (9.0 g, 56 mmol) in glacial acetic acid (8 mL). After 1 h, the reaction solution was poured into saturated aqueous sodium bicarbonate and extracted with EtOAc. The organic layer was washed with water and brine, dried (Na_2SO_4), filtered, and concentrated *in vacuo* to afford the title compound (11.2 g, 99%): mp 175–176.5 °C (MeOH); ^{1}H NMR (DMSO-d_6) δ 7.66 (s, 1H), 7.16 (s, 1H), 6.93 (s, 1H), 6.87 (s, 1H), 3.89 (s, 3H), 3.86 (s, 3H), 3.79 (s, 3H), 3.73 (s, 3H). Anal. ($C_{16}H_{16}BrNO_6$) C, H, N.

4-Bromo-1,2,6,7-tetramethoxy-9*H*-carbazole (7b). A stirred solution of **6** (11 g, 28 mmol) in triethyl phosphite (14 mL) was heated at 160 °C for 10 h. The excess triethyl phosphite was removed *in vacuo* and the residue recrystallized from EtOAc to afford the title compound (6.9 g, 69%): mp 206–207 °C; ^{1}H NMR (DMSO-d_6) δ 7.88 (s, 1H), 7.03 (s, 1H), 6.97 (s, 1H), 3.87 (s, 6H), 3.84 (s, 3H), 3.81 (s, 3H). Anal. ($C_{16}H_{16}BrNO_4$) C, H, N.

1,2,6,7-Tetramethoxy-9*H*-carbazole (7a) *via* Reduction of 7b. To a solution of **7b** (6.5 g, 18 mmol) in THF (130 mL) was added 10% palladium-on-carbon (8 g), and the resulting slurry was hydrogenated in a Parr shaker at 45 °C and 50 psi hydrogen pressure for 70 h. The reaction mixture was filtered, and the solids were washed with THF and EtOAc; the combined filtrates were concentrated *in vacuo*. The residue was purified by flash chromatography over silica gel (10% acetone/toluene) to afford the title compound (5.0 g, 98%) which was identical with the previously synthesized material.

General Procedure for Preparation of N-Alkylated, -Acylated, and -Sulfonylated Carbazoles. 9-(Phenylmethyl)-2,3,6,7-tetramethoxycarbazole. To a stirred solution of **8** (0.6 g, 2.0 mmol) in anhydrous dimethyl sulfoxide (5 mL) was added sodium hydride (60% in oil, 0.16 g, 4.0 mmol). After 0.5 h, benzyl bromide (2.0 g, 12 mmol) was added and the reaction mixture was stirred for 1 h, diluted into water, and extracted into EtOAc. The organic layer was washed with water and brine, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was flash chromatographed (silica gel, 14% EtOAc/CCl_4) to afford the title compound (0.7 g, 93%): mp 174–176 °C; ^{1}H NMR (DMSO-d_6) δ 7.58 (s, 2H), 7.23–7.06 (m, 7H), 5.54 (s, 2H), 3.79 (s, 6H), 3.75 (s, 6H). Anal. ($C_{23}H_{23}NO_4$) C, H, N.

General Procedure for N-Alkylation of Lactams. 5-(Phenylmethyl)-2,3,8,9-tetramethoxy-6(5*H*)-phenanthridinone. To a slurry of **4a** (0.6 g, 1.9 mmol) in anhydrous dimethyl sulfoxide (10 mL) was added potassium *tert*-butoxide (0.32 g, 2.85 mmol), and the resulting brown solution was stirred at room temperature for 15 min. After the addition of benzyl bromide (0.6 g, 3.8 mmol), the reaction mixture was allowed to stir for 1 h. The reaction mixture was poured into 1 N HCl and extracted with EtOAc. The organic phase was washed with water and brine, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was flash chromatographed (silica gel, 30% EtOAc/hexanes) to afford the title compound (0.54 g, 70%): mp 214–215 °C ($CHCl_3$/hexanes); ^{1}H NMR (DMSO-d_6) δ 7.85 (s, 1H), 7.82 (s, 1H), 7.80 (s, 1H), 7.38–7.23 (m, 5H), 6.95 (s, 1H), 5.70 (br s, 2H), 4.10 (s, 3H), 3.97 (s, 3H), 3.96 (s, 3H), 3.74 (s, 3H). Anal. ($C_{24}H_{23}NO_5$) C, H, N.

General Procedure for the Demethylation of Tetramethoxy Intermediates. 5-(Phenylmethyl)-2,3,8,9-tetrahydroxy-6(5*H*)-phenanthridinone (5e). To a cooled (0 °C), stirred solution of 5-(phenylmethyl)-2,3,8,9-tetramethoxy-6(5*H*)-phenanthridinone (0.50 g, 1.23 mmol) in dichloromethane (12 mL) was added boron tribromide (0.58 mL, 6.17 mmol) dropwise. The reaction mixture was allowed to stir at room temperature for 2 h, poured into ice water, and extracted with EtOAc. The organic phase was washed with water and brine, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was recrystallized from MeOH/CCl_4 to afford the title compound (0.30 g, 70%): mp 261–263 °C; ^{1}H NMR (DMSO-d_6) δ 10.04 (s, 1H, exchanges with D_2O), 9.72 (s, 1H, exchanges with D_2O), 9.45 (s, 1H, exchanges with D_2O), 9.14 (s, 1H, exchanges with D_2O), 7.71 (s, 1H), 7.47–7.17 (m, 7H), 6.73 (s, 1H), 5.48 (br s, 2H). Anal. ($C_{20}H_{15}NO_5 \cdot 0.5H_2O$) C, H, N.

Protein Tyrosine Kinase Purification and Assays. 1. Purification of pp60$^{c\text{-}src}$. The pp60$^{c\text{-}src}$ used in these studies was purified from outdated human platelets obtained from Long Island Blood Services Inc. (Oncogene Science Product No. PK03). Briefly, platelets from the enriched plasma of 50 units of blood were pelleted by centrifugation at 13000*g* (Sorvall GSA rotor; 9 000 rpm, 10 min, 4 °C) and washed three times in PBS/1 mM EDTA. Washed pellets were then lysed at 4 °C in 750 mL of a hypotonic lysis buffer (5 mM HEPES, pH 7.4, 1 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 10 μg/mL aprotinin, 25 μg/mL soybean trypsin inhibitor (STI)), using a sonicator probe (Heat Systems Probe; 3 × 30 s, set 7). Membrane vesicles were pelleted in a Beckman 70 Ti rotor at 100 000 rpm for 30 min (4 °C) and the supernatants discarded. Vesicles were then resuspended in a small volume of lysis buffer and layered onto a 27% sucrose cushion in 13-mL Beckman Ultraclear tubes. The tubes were then centrifuged in a SW40.1 rotor (15 000 rpm, 90 min, 4 °C) and the enriched plasma membraned vesicles collected from the interface. pp60$^{c\text{-}src}$ was purified from the vesicles by immunoaffinity chromatography over a column prepared by cross-linking Oncogene Science's pp60$^{v\text{-}src}$ (AB-1) antibody to agarose. Membrane vesicles were resuspended in lysate buffer (10 mM Tris/HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 30 mM NaPPi, 50 mM NaF, 500 μM orthovanadate, 2 mM PMSF, 25 μg/mL STI, 1% Triton X-100, 4 °C) and loaded. The column was washed with a high-salt (1 M NaCl, 4 °C) buffer and then the purified pp60$^{c\text{-}src}$ eluted in a high-pH elution buffer (20 mM borate, pH 10.0, 0.05% Triton X-100, 0.125 M sucrose, 4 °C). The eluate was immediately mixed with 500 mM HEPES (pH 7.4) plus 20 μg/mL STI (as a carrier protein). The protein was stored at −20 °C in 50% ethylene glycol prior to use. Analysis by 4 °C autophosphorylation, SDS–PAGE, and silver staining indicated essentially a single band and a single protein tyrosine kinase.

2. pp60$^{c\text{-}src}$ Assay Conditions. Activity of the pp60$^{c\text{-}src}$ enzyme was measured by the transfer of a ^{32}P label from the γ-position of ATP to a synthetic gastrin-based peptide substrate (KKKGPWLEEEEEAYGWLDF; MW 2352) in an assay buffer consisting of 50 mM HEPES, pH 7.4, 0.015% Brij 35, 0.1 mM EDTA. Under these conditions and at 30°C, the K_M's for the pp60$^{c\text{-}src}$ were determined to be approximately 75 μM for ATP and 85 μM (0.2 mg/mL) for the peptide substrate. Standard assay conditions were set at 50 μM ATP and 0.2 mg/mL peptide substrate. One unit of enzyme activity was defined as 1 pmol/min of phosphate transferred under these conditions. Assays were run in 96-well microtiter plates on a 40-μL scale. Reactions were initiated by the addition of γ-^{32}P-labeled ATP (0.002 light unit/pmol specific activity) to 0.8 unit of pp60$^{c\text{-}src}$ and terminated 45 min later by the addition of 5% phosphoric acid. Reactants were then transferred to a white microtiter plate containing affixed nitrocellulose filters and washed in a microtiter plate washer (5% acetic acid). Radioactivity determinations were performed using a Dynatech microplate luminometer. IC_{50}'s were derived from the mean of at least two independent determinations for each compound against each enzyme. Six concentrations of inhibitor were tested in triplicate (a total of 18 data points) in order to generate an inhibition curve. Inhibitors were dissolved in 25% DMSO/3.75 mM NaOH within 1 h of the experiment and added immediately prior to the initiation of the enzyme reactions (final DMSO concentration = 3.125%). Percent of inhibition values were obtained by taking the mean value at each concentration and comparing the data to the data of a solvent only control. Positive controls (ellagic acid) were employed on every microplate used, and control data were required to meet quality control criteria prior to processing the inhibitor data.

3. Mode of Inhibition Studies. The mode of inhibition of selected analogs with respect to ATP was examined using essentially the same basic assay protocol. Inhibition was studied in the presence of four concentrations of ATP (5, 50, and 400 μM and 2 mM). Six concentrations of inhibitor were tested in triplicate at each ATP concentration. Data for initial reaction rates (V_o) were determined as pmol/min of phosphate transferred and then the data set analyzed using the weighted nonlinear least-squares curve-fitting program Enzyme PC.[46] The program assumes the nomenclature and classification guidelines employed by Wong.[47] The program utilizes an Eadie plot and regression analysis to provide initial estimates of K_M and V_{Max}. The effects of the test compound on apparent K_M and V_{Max} parameters are used to suggest the most likely model of inhibition to which the data are then fit and the parameters (K_M, V_{Max}, and K_I) determined and errors evaluated. The root mean square (RMS, Table 4) is a measure of the deviation of points from the predicted curve relative to the predicted error. Data presented for each compound are from a representative experiment which included independent substrate only controls. Data were essentially reproducible for repeated experiments on each compound. Values for the parameters in the substrate only control experiments ranged between 71 and 86 μM for K_M and 1.62 and 2.33 pmol/min for V_M in this series of experiments.

Protein Serine/Threonine Kinase Assays. Protein serine/threonine kinase assays were performed in a manner analogous to that described for the protein tyrosine kinase assays. The synthetic peptide Kemptide (LRRASLG) was employed in these assays at a concentration of 10 μg/mL, and the catalytic subunit of cyclic AMP-dependent protein kinase (PKA; a kind gift from Dr. J. Corbin, Vanderbilt University, TN) was used at 0.8 unit/assay. All other buffers, etc., were identical to those utilized in the pp60$^{c\text{-}src}$ assay.

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