Full Paper

Synthesis and Activity of Novel 5-Substituted Pyrrolo[2,3-*d*]pyrimidine Analogues as pp60$^{c\text{-}Src}$ Tyrosine Kinase Inhibitors

Süreyya Ölgen[1], Yasemin G Isgör[1], and Tülay Çoban[2]

[1] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Ankara, Tandogan-Ankara, Turkey
[2] Department of Toxicology, Faculty of Pharmacy, University of Ankara, Tandogan-Ankara, Turkey

Therapy with receptor tyrosine kinase inhibitors provides an improved treatment option in a number of diseases such as cancer, myocardial infection, osteoporosis, stroke, and neurodegeneration. We have designed, synthesized, and evaluated a series of novel 2-amino-5-[(benzyl)imino]methyl-3,7-dihydro-4*H*-pyrrolo[2,3-*d*]pyrimidine-4-one **7a** and 2-amino-5-[(substituted-benzyl)imino]methyl-3,7-dihydro-4*H*-pyrrolo[2,3-*d*]pyrimidine-4-one **7b-e** derivatives as potential tyrosine kinase inhibitors. These compounds were synthesized by condensation reaction using 2-tritylamino-4-oxo-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidine-5-carbaldehyde **5** and appropriate benzylamines followed by detritylation. Compounds were evaluated for their inhibitory activity toward tyrosine phosphorylation for the pp60$^{c\text{-}Src}$ tyrosine kinase. Compounds **7a, 7d**, and **7e** demonstrated potent inhibitory activities against pp60$^{c\text{-}Src}$ tyrosine kinase with IC_{50} values of 13.9, 34.5, and 78.4 μM, respectively. Dihalogenated compounds **7d** and **7e** have 3 to 7-times lower IC_{50} values than that of the parent compound **7a**.

Keywords: Cancer / Pyrrolopyrimidine derivatives / Schiff bases / Tyrosine kinase pp60$^{c\text{-}Src}$

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Introduction

Protein tyrosine kinases (PTKs) are enzymes that selectively phosphorylate tyrosine residues in different substrates and have been shown to be extensively involved in the formation and maintenance of cancer [1]. The Src family kinases (SFKs) are the largest family of non-receptor protein tyrosine kinases, which are responsible for signal transduction during many cellular activities, including differentiation, adhesion, and migration [2]. The Src family of tyrosine kinases comprise the highly homologous proteins Blk, Brk, Fgr, Frc, Fyn, Hck, Lck, Lyn, Src, Srm, and Yes [3]. To prevent constitutive Src family kinase activation, the catalytic activity of SFKs in normal mammalian cells is suppressed mainly by two inhibitors called C-terminal Src kinase (CSK) and CSK-homologous kinase (CHK), which inactivate SFKs by phosphorylating a consensus tyrosine near the C-terminus of SFKs. Src-modulated intracellular signal transduction through multiple pathways are heavily implicated in a number of diseases such as cancer, diabetes, rheumatoid arthritis, autoimmune diseases, stroke, myocardial infarction, osteoporosis, and numerous diseases of the eye [4]. Moreover, novel roles of various Src family members in the development of human leukemia and pancreatic adenocarcinomas have been recently reported [5]. New insights into downstream signaling mechanisms, including the activation of STAT3, PDK1, and Akt, further corroborate the importance of Src family kinases in tumorigenesis and chemoresistance [6]. All of these taken together explain how Src activation affects tumor progression through activation of several signaling molecules that are known to contribute to tumor-cell survival and increased metastatic potential. The normal cellular

Correspondence: Süreyya Ölgen, Associate Professor, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Ankara, Tandogan, Ankara 06100, Turkey.
E-mail: olgen@pharmacy.ankara.edu.tr
Fax: +90 312 213-1081

Abbreviations: Protein tyrosine kinases (PTKs); Src family kinases (SFKs)

homologue (c-src) of the rous sarcoma virus oncogene (v-src), the first molecularly defined proto-oncogene, encodes the pp60$^{c\text{-}Src}$ PTK (2ptk) [7]. The pp60$^{c\text{-}Src}$ is a non-receptor tyrosine kinase that is characterized by a N-terminal unique domain followed by two Src homology domains SH3 and SH2, a kinase domain, and a short C-terminal regulatory peptide segment. This protein is involved in oncogenic signal transduction by the receptor tyrosine kinases EGFR / HER1, HER2, and PDGFR [8]. The pp60$^{c\text{-}Src}$ has been implicated in the development of leukemia, breast, and colon cancer [9]. In addition, the inhibitors of pp60$^{c\text{-}Src}$ tyrosine kinase particularly have been identified as potential therapeutics for osteoporosis [10]. In the few past years, much progress has been made about different kinase inhibitors with utility in oncology; they often lack selectivity or show weak cellular potency.

For the pp60$^{c\text{-}Src}$ inhibitor discovery and development, several approaches have been adopted to inhibit or decrease kinase activity. In general, inhibitors of kinase activity fall into two broad classes: (i) inhibitors of the tyrosine kinase activity (adenosine triphosphate [ATP] binding domain-mediated) and (ii) inhibitors of protein–protein (SH2-, SH3-, or substrate binding domain-mediated) interactions. Inhibiting the activity of tyrosine kinases by low molecular weight compounds (small molecule inhibitors) that are capable of interfering with either ligand binding (in the case of receptor tyrosine kinases) [11] or with protein substrate (in case of non-receptor tyrosine kinase) has proved to be difficult [12]. Therefore, the ATP competitive inhibitors appear to be the target of choice [13] to discover and develop novel inhibitors of Src family kinases. The ATP binding site, though evolutionarily conserved, can be selectively targeted by taking advantage of the minor difference in the kinase domain. The minor difference leads to changes in hydrogen bonding and hydrophobic interactions resulting in differences of affinity [14]. Compounds that are found to be ATP mimics possess at least two aromatic rings.

The most potent and selective compounds with cellular activity belong to the related classes of heterocyclic ATP analogs, namely pyrazolo-, pyrido- and pyrrolopyrimidines. Among these classes of inhibitors, anilinoquinazolines [15], pyridopyrimidines [16], pyrrolopyrimidines [17–20], and pyrazolopyrimidines [21] have been identified and exhibited promising *in-vitro* and *in-vivo* potency toward several kinases. Several natural and synthetic pyrazolopyrimidine and pyrrolopyrimidines compounds have been studied as selective SFK inhibitors [22]. Toward the development of more effective SFK inhibitors, it is important to draw attention to the pyrazolopyrimidines PP1[1-*tert*-butyl-3-*p*-tolyl-1*H*-pyrazolo[3,4-*d*]pyrimidine-4-yl-amine] and PP2[1-*tert*-butyl-3-*p*-chloro-1*H*-pyrazolo [3,4-*d*]pyrimidine-4-yl-amine], which were found as selective inhibitors of Src family member pp60$^{c\text{-}Src}$ (Fig. 1) [23]. Although these compounds inhibit SFK with IC_{50} in nanomolar range with high selectivity for SFKs, usually PP1 and PP2 have been used to delineate SFK-dependent signal transduction pathways *in vitro* and *in vivo*. This is due to their potency to inhibit "off-target" kinases *in vitro* at sufficient concentrations to achieve complete SFK inhibition. More recently, several pyrrolopyrimidine compounds (7-pyrrolidinyl- and 7-piperidinyl-5-pyrrolo[2,3-*d*]-pyrimidines and 7-alkyl- and 7-cycloalkyl-5-aryl-pyrrolo[2,3-*d*]pyrimidines) were reported as potent tyrosine kinase pp60$^{c\text{-}Src}$ inhibitors, which are useful in the treatment of osteoporosis [24, 25]. 2-Amino-4-oxo-pyrrolo[2,3-*d*]pyrimidine (LY231514, pemetrexed) (Fig. 1) is also a pyrrolopyrimidine compound, which was reported as a strong anti-cancer agent [26]. Pyrrolopyrimidine and furopyrimidine derivatives have been also developed as inhibitors of folate metabolism. Folate metabolism has long been recognized as an effective target for chemotherapy because of its crucial role in the biosynthesis of nucleic acid precursors [17]. Inhibitors of folate-dependent enzymes have been found as clinically useful antitumor, antimicrobial, and antiprotozoal agents.

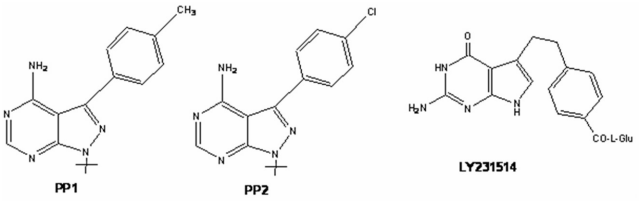
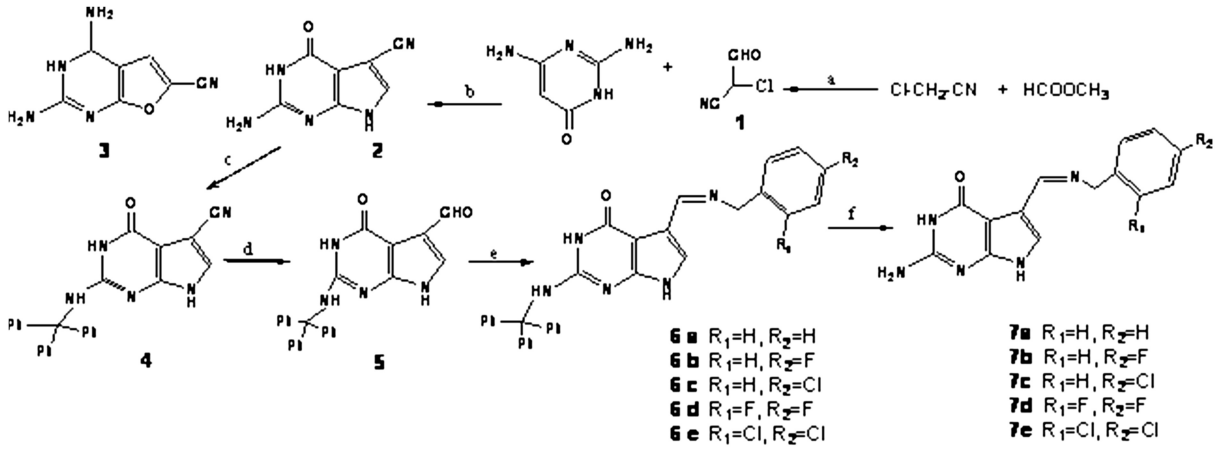


Figure 1. The structures of PP1, PP2, and LY231514 as TKIs inhibitors.

Recently, Src has emerged as an attractive candidate molecule for targeted therapies, with development of several small molecule inhibitors of Src family kinases that may be of use in targeting tumor growth and metastases. With an emphasis on combination therapies with standard chemotherapeutic agents [27], Src kinase family enzymes, and especially the pp60$^{c\text{-}Src}$ inhibition, may exhibit the dual function by virtue of increasing the sensitivity of tumors to established chemotherapeutic agents and improve disease prognosis by preventing tumors to metastasize.

Tyrosine kinase pp60$^{c\text{-}Src}$ has been, and still is, one of the most-studied cellular protein tyrosine kinases. Several SFK inhibitors, both of natural and synthetic origin, have been reported with improved selectivity and potency, and some of them have reached preclinical and clinical investigation, yet none of them has reached the



Reagents. a: NaOMe, anhydrous toluene, 0 °C; 5N HCl, 0 °C b: 100 °C, H_2O; 6N KOH, 30%HCl, pH=6. c: trityl chloride, dry pyridine, 90 °C. d: DIBAL-H, CH_2Cl_2, 0 °C. e: corresponding amines/Na_2CO_3, ethanol, 50-60 °C. f: CF_3COOH, absolute ethanol, RT.

Scheme 1. The synthesis of 5-substituted pyrrolo[2,3-*d*]pyrimidine derivatives.

market either for osteoporosis or for cancer by targeting tumor growth, cell adhesion, and mobility. Herein, we report the studies on new promising compounds, 2-amino-5-[(benzyl)imino]methyl-3,7-dihydro-4*H*-pyrrolo-[2,3-*d*]pyrimidine-4-one **7a** and 2-amino-5-[(substituted-benzyl)imino]methyl-3,7-dihydro-4*H*-pyrrolo[2,3-*d*]pyrimidine-4-one **7b – e**.

Results and discussion

In this study, a practical synthesis of 2-amino-5-[(benzyl)imino]methyl-3,7-dihydro-4*H*-pyrrolo[2,3-*d*]pyrimidine-4-one **7a** and 2-amino-5-[(substituted-benzyl)imino]methyl-3,7-dihydro-4*H*-pyrrolo[2,3-*d*]pyrimidine-4-one **7b – e** derivatives has been developed (Scheme 1). We required a convenient method for the preparation of 2-tritylamino-4-oxo-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidine-5-carbaldehyde **5** as an intermediate. Information regarding the synthesis of compound **5**, which is the key intermediate throughout the whole synthesis procedure, and also its Schiff' base derivatives, is not available in the literature. Here, we describe the synthesis method for compound **5** and its Schiff' bases

A previously reported procedure was used for the synthesis of 2-amino-4-oxo-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidine-5-carbonitrile (PreQ$_0$) **2** [18, 28]. In the reaction of methyl formate with chloroacetonitrile in toluene, the use of $NaOCH_3$ afforded the chloroaldehyde **1** in quantitative yield. Compound **1** was cyclo-condensed with 2,4-diamino-6-hydroxypyrimidine and sodium acetate in water at 100°C. ^{1}H-NMR indicated the regiospecific formation of the PreQ$_0$ with no detectable formation of the corresponding 6-cyano-furo[2,3-*d*]pyrimidine **3**. The ^{1}H-NMR spectrum showed the characteristic downfield lactam NH signal at 11.96 ppm and the pyrrole NH signal at 10.67 ppm. In addition, the presence of only one amino group at 6.36 ppm and the absence of the pyrimidine 5-proton between 5 and 6 ppm confirmed the structure of the desired intermediate **2**. Although a report on acetyl protection of PreQ$_0$, and the trityl protection of intermediate compound **5** exists [29, 30], trityl protection was chosen as the most suitable one to exploit a condensation process leading to fully acid-labile compounds. The presence of a trityl group in the resulting product provided us with the most soluble intermediate in a good yield. In addition to improving the solubility of the resulting intermediate compound, the trityl protection of the 2-amino group has several other important advantages such as, the protection can easily be removed by CF_3COOH at room temperature under mild conditions [31], and the tritylated intermediate compound facilitates the following reaction step in a reasonably good yield. Whereas no yield was obtained with the unprotected intermediate compound.

Although several methods are available for reducing a cyano group to aldehyde [32, 33], in this study, the cyano group was converted to aldehyde with DIBAL reduction with some modifications compared to the method reported in the literature [34]. In brief, among the tested conditions, the best one was the reaction carried out in methylene chloride at 0°C; it resulted in the intermediate

Table 1. Physicochemical properties and spectral data of 5-substituted-pyrrolo[2,3-*d*]pyrimidine derivatives.

Comp.	Isomers (ratio)	Mp. (°C)	Yield[a)] (%)	Molecular Formula	IR/λ_{max} (cm^{-1})	Mass	^{1}H-NMR[b)] DMSO-d_6
2		>300	36	$C_7H_5N_5O$			6.36 (s, 2H, NH_2-2), 7.59 (s, 1H, H-6), 10.67 (s, 1H, NH-7), 11.96 (s, 1H, NH-3)
4		65	79	$C_{26}H_{19}N_5O$	2227 (CN) 3380 (indole NH)	M^++Na=440.2 (100%)	7.20-7.33 (m, 15H, a.p.), 7.43 (s, 1H, NH-2), 7.59 (s, 1H, H-6), 10.66 (s, 1H, NH-7), 11.96 (s, 1H, NH-3)
5		165	33	$C_{26}H_{20}N_4O$	1641 (CH=O) 3431 (indole NH)	M^++1=421.19 (100%)	7.19-7.29 (m, 15H, a.p.), 7.39 (s, 1H, NH-2), 7.55 (s, 1H, H-6), 9.99 (s, 1H, CHO), 10.64 (s, 1H, NH-7), 11.84 (s, 1H, NH-3)
6a	E/Z 5/1	105	74.8	$C_{33}H_{27}N_5O$	1604 (CH=N) 3386 (indole NH)	M^++1=510.28 (100%)	4.02 (s, 2H, CH_2-Ph), 4.87 (s, 2H, CH_2-Ph), 7.21-7.40 (m, 22 H a.p., H-6, NH-2), 8.85 (s, 1H, CH=N), 8.73 (s, 1H, CH=N)
6b	E	142	76.6	$C_{33}H_{26}FN_5O$	1552 (CH=N) 3449 (indole NH)	M^++1=528.30 (100%)	4.69 (s, 2H, CH_2-Ph), 7.13-7.34 (m, 20 H, a.p., NH-2), 7.57 (s, 1H, H-6), 8.59 (s, 1H, CH=N)
6c	E/Z 2/1	216	57.2	$C_{33}H_{26}ClN_5O$	1576 (CH=N) 3432 (indole NH)	M^++1=544.25 (100%)	3.94 (s, 2H, CH_2-Ph), 4.65 (s, 2H, CH_2-Ph), 7.19-7.40 (m, 20 H, a.p., NH-2), 7.46 (s, 1H, H-6), 8.58 (s, 1H, CH=N), 8.63 (s, 1H, CH=N)
6d	E/Z 20/1	172	61.4	$C_{33}H_{25}F_2N_5O$	1612, 1596 (CH=N) 3431 (indole NH)	M^++1=546.35 (100%)	4.04 (s, 2H, CH_2-Ph), 4.58 (s, 2H, CH_2-Ph), 6.94 (s, 1H, NH-2), 7.17-7.39 (m, 18 H, a.p.), 7.46 (s, 1H, H-6), 8.43 (s, 1H, CH=N), 8.66 (s, 1H, CH=N)
6e	E/Z 1/1	268	80.5	$C_{33}H_{25}Cl_2N_5O$	1609 (CH=N) 3432 (indole NH)	M^++1=578.16 (100%)	4.37 (s, 2H, CH_2-Ph), 4.68 (s, 2H, CH_2-Ph), 7.19-7.62 (m, 20 H, a.p., H-6, NH-2), 8.32 (s, 1H, CH=N), 8.63 (s, 1H, CH=N
7a	E	>171 Decomp.	66.7	$C_{14}H_{13}N_5O$	1590 (CH=N) 3381 (indole NH)	M^++1=268.14 (100%)	Z: 4.68 (s, 2H, CH_2-Ph), 6.53 (s, 2H, NH_2-2), 6.75 (s, 1H, H-6), 7.24-7.50 (m, 5 H a.p.), 8.58 (s, 1H, CH=N), 10.40 (s, 1H, NH-7), 11.86 (s, 1H, NH-3) E: 4.81 (s, 2H, CH_2-Ph), 6.59 (s, 2H, NH_2-2), 7.38-7.50 (m, 6 H a.p., H-6), 8.70 (s, 1H, CH=N), 10.44 (s, 1H, NH-7), 11.88 (s, 1H, NH-3)
7b	E	>199 Decomp.	60.4	$C_{14}H_{12}FN_5O$	1562 (CH=N) 3446 (indole NH)	M^++1=286.14 (100%)	E: 5.10 (s, 2H, CH_2-Ph), 6.70 (s, 2H, NH_2-2), 7.15-7.19 (m, 3 H-3',5', H-6), 7.33-7.37 (m, 2H, H-2',6'), 8.60 (s, 1H, CH=N), 10.60 (s, 1H, NH-7), 11.08 (s, 1H, NH-3)
7c	E/Z 1/1	>200.6 Decomp.	37.4	$C_{14}H_{12}ClN_5O$	1590 (CH=N) 3431 (indole NH)	M^++1=302.17 (100%)	4.99 (s, 2H, CH_2-Ph), 5.10 (s, 2H, CH_2-Ph), 6.92 (s, 2H, NH_2-2), 7.08-7.51 (m, 5 H a.p., H-6), 8.14 (s, 1H, CH=N), 8.82 (s, 1H, CH=N), 10.65 (s, 1H, NH-7), 11.99 (s, 1H, NH-3)
7d	E	197	65.5	$C_{14}H_{11}F_2N_5O$	1596 (CH=N) 3468 (indole NH)	M^++1=304.08 (100%)	5.07 (s, 2H, CH_2-Ph), 7.05 (s, 2H, NH_2-2), 7.19-7.67 (m, 4H a.p.), 8.88 (s, 1H, CH=N), 10.66 (s, 1H, NH-7), 11.62 (s, 1H, NH-3)
7e	E/Z 1/1	>163 Decomp.	43.9	$C_{14}H_{11}Cl_2N_5O$	1555 (CH=N) 3493 (indole NH)	M^++1=336.16 (100%)	4.15 (s, 2H, CH_2-Ph), 5.12 (s, 2H, CH_2-Ph), 7.13 (s, 2H, NH_2-2), 7.20-7.77 (m, 4H a.p., H-6), 8.24 (s, 1H, CH=N), 8.89 (s, 1H, CH=N), 10.72 (s, 1H, NH-7), 11.74 (s, 1H, NH-3)

[a)] Yield is given as *Z*/*E* mixture.

[b)] The ^{1}H-NMR data of *Z* and *E* isomers are given together in mixture samples; a. p. = aromatic protons

5 [35]. Many compounds and nearly all important functional groups have been tested as adducts for highly reactive alkylaluminium hydrides [36]. Their popularity has risen considerably due to several reasons, such as, diisobutylaluminium hydride has proved to be safe and easy-to-handle in toluene or hexane. The target 2-amino-5-[(benzyl)imino]methyl-3,7-dihydro-4*H*-pyrrolo[2,3-*d*]pyrimidine-4-one **7a** and 2-amino-5-[(substituted-benzyl)imino]-methyl-3,7-dihydro-4*H*-pyrrolo[2,3-*d*]pyrimidine-4-one **7b–e** compounds were obtained by the reaction of aldehyde **5,** with appropriate amines (Scheme 1) followed by the removal of trityl protection [37]. All resulted products

Table 2. Inhibitory concentrations (IC_{50}; μM) of 5-substituted pyrrolo[2,3-*d*]pyrimidine derivatives against pp60$^{c\text{-}Src}$.

Compound	Isomers	IC_{50} (μM) $\overline{X}$ ± S.D.
7a	*E*	13.98 ± 4.06
7b	*E*	> 200
7c	*E*/*Z*	174.30 ± 86.5
7d	*E*	34.50 ± 3.75
7e	*E*/*Z*	78.36 ± 4.81

were purified by recrystallization from the ethanol. Purification on a silica-gel column was performed before recrystallization, only if it was found necessary. The structure of all synthesized compounds was confirmed by ^{1}H-NMR, IR, and MS spectral data as shown in Table 1. Both protected and deprotected 5-substituted pyrrolo[2,3-*d*]pyrimidine analogues **6a – e** and **7a – e** were obtained in good yield. Under the standard conditions, compounds were obtained as a mixture of Z/E-isomers. In general, attempts to separate the *Z*/*E* mixtures were extremely tedious. In some cases, the repeated flash chromatographic separations, followed by recrystallization, resulted in partial separation of the pure *E*-isomer. The *Z*-isomer could not be obtained either free or in good yield (<10%). In one case, compound **7a** was obtained in analytic amounts only and characterized as *Z*-isomer. The data of the isomers are reported in Table 1. Configuration of the separated isomers was determined using NOE analysis. The *E*-configured compounds have a NOE effect between the proton at the imine (CH=N) and the proton(s) at the C-6 substitution of the 5-substituted pyrrolo[2,3-*d*]pyrimidine compounds. These assignments were also confirmed by ^{1}H-NMR spectral data, where the imine protons displayed a slight down-field shift in the trans-(*E*) isomers than cis-(*Z*) isomers. Their infrared spectra provided good evidence for their structure, showing clearly both CH=N (1552 – 1612 cm^{-1}) and indole NH peaks (3381 – 3493 cm^{-1}) in the expected regions. The CH=N stretching values of *Z* isomers were found higher then *E* isomers. For compound **2,** the CN stretching was detected at 2227 cm^{-1}.

The biological response of the compounds was evaluated for their inhibitory activity towards tyrosine phosphorylation for the pp60$^{c\text{-}Src}$ tyrosine kinase [38]. Briefly, the biological activity was monitored by the kinase-catalyzed transfer of γ-phosphate residue from ATP to immobilized peptide substrates, and the results were expressed as% inhibition of the c-Src activity in the presence of varying concentrations of compounds (dose-response curves) **7a – e.** The inhibitory effect of compounds was reported as IC_{50} of the compounds and is summarized in Table 2. The IC_{50} values were defined as the concentration of a compound required to achieve 50% inhibition of pp60$^{c\text{-}Src}$ tyrosine kinase activity with respect to the activity measured in the presence of vehicle alone (dimethyl sulfoxide). The dose response curves of the most active compounds **7a** and **7d** were shown in Figs. 2 and 3, respectively.

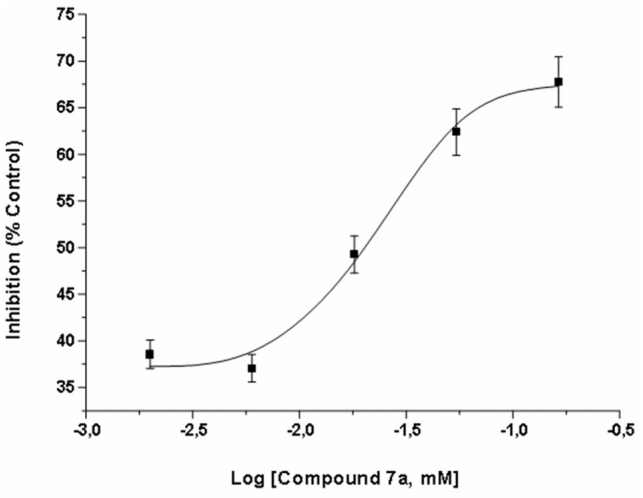


Figure 2. The dose-response curves for compound **7a** with IC_{50} 0.0139 mM, in the ELISA-based *in-vitro* pp60$^{c\text{-}Src}$ tyrosine kinase assay with an initial concentration of 2 μM to a final concentration of 400 μM. The IC_{50} values obtained by the dose-response curves for each compound (n = 3 – 4, two independent experiments each in duplicates) were obtained by non-linear regression analysis using Bio Data Fit 1.02 and Origin 6.0.

The most potent inhibition was observed with compound **7a** (IC_{50} = 13.9 μM), which has no substituents in the benzyl ring. In addition, compounds **7d** and **7e** showed good *in-vitro* efficacies at IC_{50} values of 34.5 and 78.4 μM, respectively. The benzyl-ring substitutions with an electron-withdrawing fluorine and chlorine atoms at the 2- and/or 4-positions result in a 3 to 20-fold decrease for the inhibition of pp60$^{c\text{-}Src}$ tyrosine kinase. The better inhibitory effect of dihalogenated over monohalogenated compounds can be explained with the molecule's 3D conformation and flexibility to compete with ATP. Here, we can hypothesize that the mono-halogen substitution on the aromatic ring, possibly results in more flexible compounds, which may not easily fit into the ATP-binding site of Src, and hence, can not compete with ATP in the medium. Introducing conformational constraints into rigid dihalogenated compounds **7d** and **7e** generate better interaction with the binding pocket and provide more activity. In the process of binding, different conformers of the target and a given ligand may fit in one of the most favorable energetic states. The dynamic nature of both proteins and ligands can determine the interactions and biological activity.

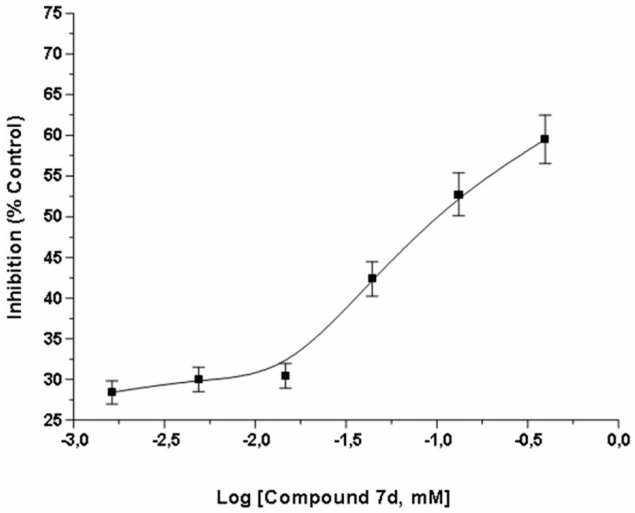


Figure 3. The dose-response curves for the compound **7d** with IC_{50} 0.0345 mM, in the ELISA-based *in-vitro* $pp60^{c\text{-}Src}$ tyrosine kinase assay with an initial concentration of 2 μM to a final concentration of 400 μM. The IC_{50} values obtained by the dose-response curves for each compound (n = 3–4, two independent experiments each in duplicates) were obtained by non-linear regression analysis using Bio Data Fit 1.02 and Origin 6.0.

In this context, considering that the geometric orientation of isomers can cause the diverse activity pattern, the inhibitory potency of *E*- and *Z*-isomers may also be different. Perhaps, the reason for the two-fold lower activity of compound **7e** compared to that of **7d** may be due to the presence of the *E*- and *Z*-isomers as a mixture. Since the most active compound **7a** has no substituent in the aromatic ring, this indicates that here, the electron-withdrawing groups are not favorable for the biological activity. In the view of these results, it appeared necessary to investigate more substitutions particularly more hydrophilic substituent replacement to better understand the structure-activity relationship. Taking into consideration the receptor-ligand interactions on biological activity, it can be concluded that hydrophobic interactions are non-directional for our 5-substituted pyrrolo[2,3-*d*]pyrimidine compounds. H-bonds are more directional and are needed within the protein to enhance the c-Src inhibition.

The most active pyrrolo- and pyrazolopyrimidine compounds have a N^5-substituted phenyl ring and N^7-alkyl- or -heterocyclic substituents. It was assumed that N^7-substituents of pyrrolopyrimidine occupies the pocket which is usually utilized by the ribose moiety of ATP. Both pyrrolo and pyrazolopyrimidine compounds bearing substitute 5-phenyl ring were reported as favorable selective c-Src inhibitors [24, 25]. The structural differences of our compounds at N^5 and N^7 positions compared to active pyrrolopyrimidine compounds may be responsible for the activity loss. In the view of these findings, our future goal should be oriented to investigate the effects of new substituents at N^5 and N^7 positions.

In conclusion, noteworthy activity results of our new, strategic 5-substituted pyrrolo[2,3-*d*]pyrimidine derivatives, which exhibit potent inhibition of $pp60^{c\text{-}Src}$ tyrosine kinase, are reported here.

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The authors have declared no conflict of interest.

Experimental

Chemistry

Melting points were measured with a capillary melting point apparatus (Electrothermal 910, Essex, UK) and are uncorrected. The Nuclear Magnetic Resonance (^{1}H-NMR) spectra were recorded on a Varian Mercury 400 NMR spectrometer for 400 MHz (Varian Inc., Palo Alto, CA, USA). The chemicals-shift values were expressed in parts per million (ppm) relative to tetramethylsilane as an internal standard and signals are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Mass spectra were recorded on a Waters ZQ Micromass LC-MS spectrometer (Waters Corporation, Milford, MA, USA) Electrospray Ionization (ESI) method. Infrared (IR) spectra were measured on Jasco FT/IR-420 (Jasco, Tokyo, Japan). Elemental analysis was taken on a Leco-932 CHNS-O analyzer (Leco, St. Joseph, MI, USA). Element compositions are within ± 0.4% of the calculated values. Molecular Devices Spectra MAX 190 (from Molecular Devices Corporation, Sunnyvale, CA, USA) was used to measure of absorbance of the phosphorylation reaction. Analytical TLC was carried out on Merck 0.2 mm pre-coated silica gel (^{60}F 254) aluminium sheets (Merck, Darmstadt, Germany), with visualization by irradiation with a UV lamp. The flash-column chromatography was accomplished on silica gel 60 (230–400 mesh, Merck).

Anhydrous magnesium sulphate, sodium sulphate, sodium acetate, hexane, ethyl acetate, anhydrous toluene, trifluoroacetic acid, sodium dihydrogen phosphate, potassium hydrogen phosphate, sodium chloride, dimethylsulfoxide, sulfuric acid, mercaptoethanol, celite (Merck, Darmstadt, Germany); deutero dimethylsulfoxide, 2,6-diamino-6-hydroxy pyrimidine, sodium methoxide, benzylamine, 4-chlorobenzylamine, 4-fluorobenzylamine, 2,4-dichlorobenzylamine, 2,4-difluorobenzylamine, methyl formate, dry pyridine, chloroacetonitrile, diisobutylaluminium bromide, trityl chloride (Acros Organics, Geel, Belgium); methanol, hydrochloric acid, dichloromethane, potassium hydroxide, sodium carbonate, anhydrous ethanol, ethanol, toluene, anhydrous toluene (Riedel-de Häen, Sigma-Aldrich, St. Louis, MO, USA) were purchased. Takara Universal Tyrosine Assay Kit (from Takara-Bio Inc., Shiga, Japan) was used to test our synthesized compounds. The contents of a kit: PTK substrate immobilized microplate (8 wells × 12 wells), kinase reacting solution (11.0 mL), 40 mM ATP-2Na (0.55 mL), extraction buffer (11.0 mL), PTK control (0.50 mL), anti-phospho-tyrosine (PY20-HRP, for 5.5 mL/H_2O), blocking solution (11.0 mL), HRP coloring solution (TMBZ = tetra methyl benzidine, 12.0 mL).

*2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile **2***

Methyl formate (4.76 mL, 0.077 mol) was added to a stirred mixture of 3.57 g (0.066 mol) $NaOCH_3$ in anhydrous toluene at 0°C. Chloroacetonitrile (4.18 mL, 0.066 mol) was added dropwise over 30 min at 0°C and the reaction mixture was stirred at 0°C for 3 h. Water (100 mL) was added and extracted with toluene (2×100 mL). The aqueous phase was cooled at 0°C and acidified to pH 4 using 5 N HCl; then, it was extracted with 3×100 mL EtOAc. The organic phase was dried over Na_2SO_4. Concentrated to dryness, it gave a crude compound, which was used without further purification. Chloro(formyl)acetonitrile (3.98 g) **1** was obtained as an oily compound. Sodium acetate (5.36 g, 0.065 mol) was dissolved in 118 mL of distilled water and 2,6-diamino-6-hydroxy pyrimidine (4.12 g, 0.033 mol) was added. The mixture was heated at 100°C and compound **1** in 62 mL of water was added. The reaction mixture was refluxed for 5 h and it was cooled to room temperature. The precipitate was collected by filtration and washed with copious amounts of water and acetone. The compound was dissolved in 30 mL of 6 N KOH, boiled with charcoal, and then filtered over celite. The pH was adjusted to 6 at 0°C with 30% HCl. The pure yellow solid (2.21 g, yield 58.7%) was collected and dried in a vacuum oven at 50°C for 24 h.

*4-Oxo-2-tritylamino-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile **4***

Compound **2** (1.05 g, 5.99 mmol) was suspended in dry pyridine (65 mL) and trityl chloride (2.51 g, 8.99 mmol) was added to this suspension. The reaction was warmed to 90°C and stirred for 48 h. Water (3.2 mL) was added and the mixture was stirred for 20 min. The solvent was removed under reduced pressure and the crude product was co-evaporated with toluene and ethanol, providing a solid residue, which was purified by flash chromatography (hexanes/EtOAc, 1 : 1).

*4-Oxo-2-tritylamino-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidine-5-carbaldehyde **5***

Compound **4** (0.59 g, 1.4 mmol) was dissolved in 14 mL anhydrous dichloromethane. DIBAL-H (1 M in dichloromethane, 4.24 mL, 4.24 mmol) was added at 0°C over period of 30 min. After 3 h, 4 mL of MeOH was added slowly at 0°C and stirred for 30 min. The precipitate was filtered off and washed with MeOH. The organic phase was dried over anhydrous $MgSO_4$ and evaporated to dryness to yield the crude compound **5**, which was purified by flash chromatography (CH_2Cl_2/MeOH, 9.8 : 0.2).

*2-Tritylamino-5-[(benzyl)imino]methyl-3,7-dihydro-4H-pyrrolo[2,3-d]pyrimidine-4-one **6a** and 2-Tritylamino-5-[(substituted-benzyl)imino]methyl-3,7-dihydro-4H-pyrrolo[2,3-d]pyrimidine-4-one derivatives **6b – e***

Compound **5** (1 eq.) was dissolved in ethanol (1 mmol, 2 mL). The appropriate benzylamine (1.5 eq.) and Na_2CO_3 (1 eq.) in water (1 mmol, 0.5 mL) were added at room temperature, respectively. The reaction mixture was stirred at 50–60°C for 2 h. The solvent was removed *in vacuo* and the crude compound was purified by silica gel chromatography (CH_2Cl_2/MeOH; 9.5 : 0.5).

*2-Amino-5-[(benzyl)imino]methyl-3,7-dihydro-4H-pyrrolo[2,3-d]pyrimidine-4-one **7a** and 2-Amino-5-[(substituted-benzyl)imino]methyl-3,7-dihydro-4H-pyrrolo[2,3-d]pyrimidine-4-one derivatives **7b – e***

Compounds **6a – e** were dissolved in anhydrous ethanol (1 mmol, 10 mL) and CF_3COOH (1 mmol, 10 mL, containing 15% anhydrous dichloromethane) was added and stirred for 3 h at room temperature. The solvent was removed *in vacuo* and 5% $NaHCO_3$ was added. The precipitate was collected, purified by silica gel column chromatography (CH_2Cl_2/MeOH; 8 : 2) and crystallized from ethanol.

Inhibition of pp60$^{c\text{-}Src}$ tyrosine kinase

The ELISA-based *in vitro* tyrosine kinase assay, here the pp60$^{c\text{-}Src}$ tyrosine kinase activity, was used to verify the inhibition of the compounds synthesized in our laboratory. Briefly, the assay is based on monitoring the phosphorylation of immobilized peptide (substrate) by virtue of classical ELISA sandwich method, where the phosphorylation of immobilized poly(Glu-Tyr) was probed with HRP-conjugated anti-phosphotyrosine (PY20) antibody. In the assay, the inhibitory activities of compounds against pp60$^{c\text{-}Src}$ tyrosine kinase were monitored as the diminished activity of kinase at 450 nm [38]. The kinase assay was performed at 37°C in a final assay volume of 50 µL. The concentrations of the PTKs used to construct the calibration curve were as follows: 1520, 760, 380, 190, 94.8, 47.4×10^{-7} units/µL for pp60$^{c\text{-}Src}$ PTK. The kinase reactions were initiated by the addition of 40 nM ATP (10 µL) into each well, and the plate was subsequently incubated at 37°C for 30 min. After the completion of the reaction, the remaining liquid was removed and the wells were washed with PBS-Tween (0.05%, v/v) four times. The blocking solution (100 µL) was added to the wells and incubated at 37°C for 30 min. After washing the plate with PBS-Tween, 50 µL of anti-phosphotyrosine was added to the wells and incubated at 37°C for 30 min. Then, the liquid in the wells was decanted and the remaining solution was removed by rinsing with PBS-Tween four times. 100 µL of HRP coloring agents was added to each well and incubated at 37°C for 15 min. Reaction was terminated by the addition of 100 µL 1 N sulfuric acid into each well. The absorbance of the reaction mixture was measured at 450 nm with a microplate reader.

The pp60$^{c\text{-}Src}$ tyrosine kinase activity is measured as the difference between the total activity of no vehicle (DMSO) and the activity of enzyme in the presence of DMSO. The IC_{50} value is defined as the concentration of a compound required to achieve 50% inhibition of pp60$^{c\text{-}Src}$ tyrosine kinase activity with respect to the activity measured in the presence of vehicle alone (dimethyl sulfoxide). The dose-response curves of the compounds were obtained by using dilutions at final concentrations of 400 to 2 µM in the ELISA-based *in vitro* protein kinase assay described above and IC_{50} values were determined by non-linear regression analysis. The dose-response curves of the compounds and non-linear regression analysis were performed using Origin 6.0, (for Windows, Microcal Software, Northampton, MA, USA) and BioDataFit 1.02 (Chang Bioscience, Inc., Castro Valley, CA, USA).

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