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1*H*-Pyrazolo[3,4-*b*]pyridine Inhibitors of Cyclin-Dependent Kinases

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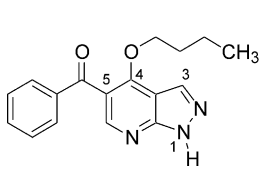
Abstract—1*H*-Pyrazolo[3,4-*b*]pyridine **3** (SQ-67563) has been shown to be a potent, selective inhibitor of CDK1/CDK2 in vitro. In cells **3** acts as a cytotoxic agent with the ability to block cell cycle progression and/or induce apoptosis. The solid state structure of **3** bound to CDK2 shows **3** resides coincident with the ATP purine binding site and forms important H-bonding interactions with Leu83 on the protein backbone.

Cyclin-dependent kinases (CDKs) are a family of protein kinases which play a key role in the growth, development, proliferation and death of eukaryotic cells.[1] In particular, CDKs along with their regulatory subunit cyclins are responsible for coordinating events which progress cells through the cell cycle and insure the genetic integrity of daughter cells. Due to their role as drivers of cell growth and division, combined with their hyperactivation in a number of cancers, oncology drug discovery programs have directed a major effort towards the identification of small molecule inhibitors of CDKs as potential therapeutic agents.[2] Olomoucine (**1**)[3] is a purine-based ATP-competitive inhibitor of CDKs with moderate potency which was identified a number of years ago. More recently, flavopiridol (**2**), a synthetic flavone, was described as a broad-spectrum ATP-competitive CDK inhibitor and has progressed into Phase 2 clinical trials as an anti-tumor agent.[4] (Fig. 1)

Our screening efforts recently revealed pyrazolo[3,4-*b*]pyridines SQ-67563 (**3**) and SQ-67454 (**4**)[5] were CDK inhibitors with potent in vitro activity (Table 1). Described herein are the synthesis and preliminary SAR of CDK inhibitors based on this chemotype. In addition, cellular effects and an X-ray crystal structure of a representative from this class bound to human CDK2 are described.[6]

Our initial focus in this series was on examining the effect on inhibitory potency of substituents at the 4-position of the pyrazolopyridine ring. The 4-substituted analogues in Table 1 were prepared from the 4-chloro intermediate **10** shown in Scheme 1. Thus, PMB-protected aminopyrazole **7** was prepared by condensation of acrylonitrile and *p*-methoxybenzaldehyde followed by based-induced rearrangement of the crude imine to give the pyrazole which was isolated as the hydrochloride salt.[7,8]



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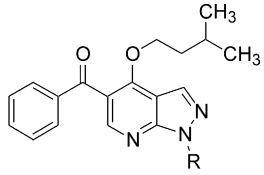

4: R=H
5: R=Et

Neutralization of salt **7** and condensation of the free amine with acrylate **8**[9,10] followed by pyrolysis (240–260 °C) in diphenyl ether afforded fused bicyclic intermediate **9**. Heating of **9** with phosphorous oxychloride gave 4–chloropyrazolopyridine **10**. Treatment of **10** with the appropriate anion followed by removal of the PMB protecting group in neat, hot TFA afforded the desired analogues **11**.

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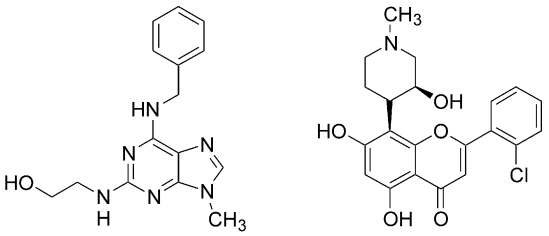


Figure 1. Structures of ATP-competitive inhibitors olomucine (**1**) and flavopiridol (**2**).

Table 1. Structure of CDK screening hits SQ-67563 (**3**) and SQ-67454 (**4**) and inhibition of CDKs[a]

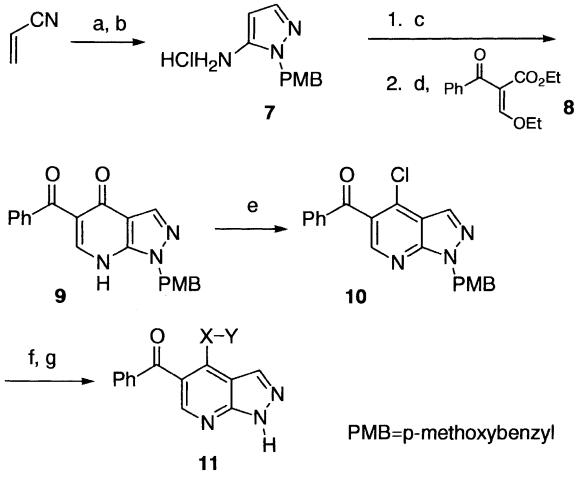
Compd	CDK1/cycB IC_{50}, μM	CDK2/cycE IC_{50}, μM	CDK4/cycD IC_{50}, μM	CDK7/cycH IC_{50}, μM
2	0.03	0.17	0.10	0.40
3	0.15	0.11	>25	—
4	0.24	0.18	>25	1.1
5	—	>25	—	—

[a]See ref 11 for description of biological assays.

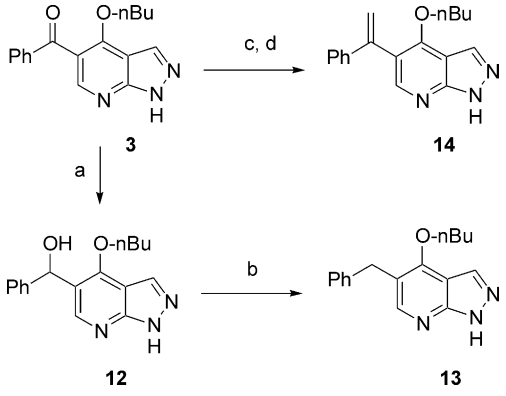
In order to determine the importance of the carbonyl functionality in the 5-position, we prepared the corresponding alcohol, *des*-keto and olefin analogues of **3** as shown in Scheme 2. Thus, carbinol analogue **12** was prepared by sodium borohydride reduction of **3**. Ionic reduction of **12** provided benzyl analogue **13** while methylene analogue **14** was obtained by addition of methyllithium to ketone **3** followed by acid-catalyzed dehydration of the crude tertiary carbinol intermediate.

Pyrazolopyridines **3**–**5** and **11**–**13** were examined for their ability to inhibit CDKs in vitro.[11] The results from cell-free enzyme assays are summarized in Tables 1 and 2. Thus, **3** and **5** were found to be selective inhibitors of CDK1/CDK2 within the CDK family. This is in contrast with flavopiridol which also shows potent inhibitory activity towards CDK4. Among other representative kinases pyrazolopyridine **4** did not show inhibition of PKA, PKC or LCK (IC_{50} > 50 μM, data not shown). The *N*-ethyl analogue **5** did not inhibit CDK2 indicating that an N–H at this position was either critical to binding to the enzyme or an alkyl group was not sterically tolerated in this position. Crystallographic studies confirmed that in this class of compounds the NH formed an important H-bonding interaction with the enzyme (vide infra). Examination of the 4-position (Table 2) showed that replacement of the oxygen with either nitrogen or sulfur (**3** vs **11a**,**b**) results in a significant loss in CDK2 inhibitory activity. Straight-chain, branched or cyclic alkoxy groups (**3**, **5**, and **11c**–**f**) were all tolerated in the 4-position. Benzyloxy was also tolerated although unexpectedly phenoxy shows a 50-fold loss in CDK2 inhibitory potency (**11g** vs **11h**). Finally, introduction of polar substituents, hydroxy and dimethylamino, in the 4-position resulted in a significant loss in potency (**11i** and **11j**). Examination of the 5-keto group indicated that it was also essential for potent CDK inhibitory activity. Thus, reduction to the alcohol **12** or further to a methylene analogue **13** afforded compounds devoid of CDK2 inhibitory activity (IC_{50} >25 μM). Removal of the ketone while retaining an sp^2 carbon at the 5-position to afford *exo* methylene analogue **14** also resulted in a loss of CDK2 inhibitory activity (IC_{50} > 25 μM).

In order to understand the molecular basis of the observed relationship between structure and biological activity of this series, the three-dimensional structure of **3** in complex with CDK2 was determined by X-ray crystallography. Crystals were obtained by incubating inhibitor **3** (72 h) with crystalline protein in the absence of cyclin.[11] The crystal structure (Fig. 2) revealed that **3** binds in the ATP-binding site with the pyrazolopyridine ring adjacent to Leu83 in the space occupied by the ATP purine ring. In this configuration the pyridine nitrogen acts as an acceptor for the amide NH of Leu83. The hydrogen on N1 of the pyrazolopyridine ring serves as a H-bond donor to the carbonyl oxygen of Leu83. This is consistent with the SAR which indicates that the alkylation of N1 is not tolerated (**4** vs **5**) and substituents in

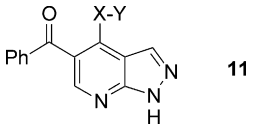


Scheme 1. Synthesis of 4-substituted pyrazolopyridine analogues **11**: (a) $NH_2NH_2H_2O$ (1 equiv), THF, 0–25 °C, 2 h; then *p*-methoxybenzaldehyde, (1 equiv), 25 °C, 2 h; (b) *n*BuONa (1 equiv)/*n*BuOH, 25–120 °C, 3 h then HCl, 42% from acrylonitrile; (c) desalt, 10% K_2CO_3; (d) **8** (1 equiv), 120 °C, 1.5 h then Ph_2O, 250 °C, 1.5 h, 40%; (e) $POCl_3$, 110 °C, 60%; (f) NaX–Y, CH_2Cl_2, 25 °C, 6 h; (g) TFA, 65 °C, 2.5 h, 50–80% from **10**.



Scheme 2. Synthesis of 5-keto analogues **12**–**14**: (a) $NaBH_4$/EtOH, 25 °C, 84%; (b) TFA/Et_3SiH (1:4), 25 °C, 69%; (c) MeLi (3 equiv)/ether, THF, 0 °C; (d) TFA/CH_2Cl_2, 25 °C, 54%.

Table 2. SAR of 4-substituent of pyrazolopyridines **11**[a]



11

Compd	X	Y	CDK1/cycB IC_{50}, μM	CDK2/cycE IC_{50}, μM
11a	S	*n*-Butyl	—	4.4
11b	NMe	*n*-Butyl	—	>25
11c	O	*n*-Propyl	0.22	0.18
3	O	*n*-Butyl	0.15	0.11
11d	O	*n*-Pentyl	0.31	0.24
11e	O	(1-Methyl)butyl	0.31	0.52
11f	O	Cyclohexyl	0.60	0.66
11g	O	Benzyl	0.35	0.28
11h	O	Phenyl	—	15
11i	O	(2-Hydroxy)ethyl	—	3.7
11j	O	(2-Dimethylamino)ethyl	—	17

[a]See ref 11 for description of biological assays.

the 4-position which effect the basicity of the pyridine nitrogen also have a large effect on activity. The 4-alkoxy substituent extends into the space occupied by the ribose of ATP and does not appear to form any specific contacts with the protein. Although this position tolerated a wide number of lipophilic substituents it was surprising that we were unable to successfully introduce simple polar substituents (e.g., **11i** and **11j**) into the ribose binding region. It was also surprising that the SAR indicates that the oxygen at the 4-position cannot be replaced by a sulfur or nitrogen since the structure did not show specific interactions with the protein. It is possible that the 4-oxo substituent may simply provide the optimal electronic characteristics to maximize hydrogen bonding of the pyrazolopyridine to Leu83 and/or be sufficiently small in comparison to a alkylthio or dialkylamino group not to interfere with the orientation of the 5-phenacyl side chain. The pendent phenyl group was found to lie deep within the ATP-pocket stacking neatly with the side chain of Phe80. The SAR indicates the importance of the 5-keto oxygen which may serve as a hydrogen bond acceptor although we do not consistently see a hydrogen bonding interaction with the ketone and Lys33. Finally, we were unable to explain the observed CDK1/CDK2 selectivity of this series of inhibitors based on the solid-state structure since there are few interactions with either Ile10 or Lys89 which are the only residues in the binding site that differs between CDK1, CDK2, and CDK4.

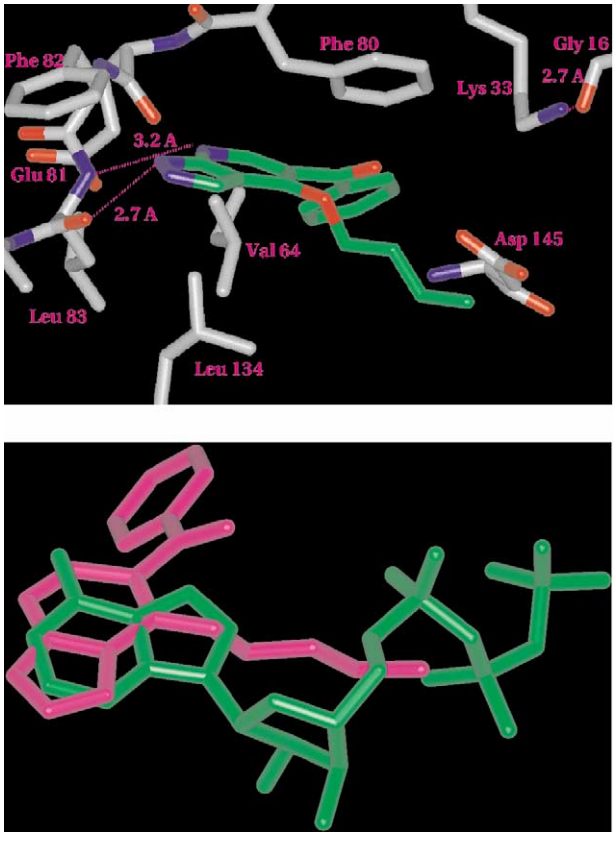


Figure 2. Top: Solid-state structure of pyrazolopyridine **3** bound in the ATP-pocket of CDK2 (no cyclin). The inhibitor carbon atoms are in green, the nitrogen atoms are in blue and oxygen atoms are in red. The protein carbons are in gray. Hydrogen bonds are shown by the magenta dotted lines. Bottom: Overlap showing relative binding modes of compound **3** (magenta) bound to CDK2 with the that of ATP (green) bound to CDK2.[11,12]

In an ovarian cancer cell line (A2780) inhibitor **3** was found to act as a cytotoxic agent with an $IC_{50} = 5.8$ μM. FACS analysis demonstrated **3** blocked cell cycle progression and/or induced apoptosis. Specifically, A2780 and HCT116 cells treated with inhibitor **3** exhibited a significant increase in the G2/M population, while CEM cells predominantly showed an increase in the apoptotic cell fraction versus untreated cells. In addition, examination of A2780 cells treated with inhibitor **3** (IC_{90} concentration for 4 h) showed inhibition of phosporylation of the CDK substrates Rb protein, histone H1 and DNA polα consistent with a mechanism of action involving CDK inhibition.[11]

In summary, pyrazolopyridine **3** has been been shown to be a potent, selective inhibitor of CDK1/CDK2 in vitro. In cells **3** acts as a cytotoxic agent with the ability to block cell progression and/or induce apoptosis and

has been shown to act by a mechanism of action consistent with CDK inhibition. The solid-state structure of **3** bound to CDK2 shows that **3** resides in the ATP purine pocket with important hydrogen bonding interactions between the pyridine nitrogen and N1-hydrogen of the heterocyclic ring and Leu83 of the enzyme.

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9. Prepared in 40% yield by heating ethylbenzoyl acetate (1 equiv), triethylorthoformate (1.5 equiv) and acetic anhydride (2.5 equiv) at 145 °C for 6 h. Compound **8** was isolated by distillation as a brown oil (bp 165–169 °C, 3 mm).
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