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4-Alkoxy-2,6-diaminopyrimidine Derivatives: Inhibitors of Cyclin Dependent Kinases 1 and 2

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Abstract—The cyclin dependent kinase (cdk) inhibitor NU6027, 4-cyclohexylmethoxy-5-nitroso-pyrimidine-2,6-diamine (IC_{50} vs cdk1/cyclinB1 = 2.9 ± 0.1 μM and IC_{50} vs cdk2/cyclinA3 = 2.2 ± 0.6 μM), was used as the basis for the design of a series of 4-alkoxy-2,6-diamino-5-nitrosopyrimidine derivatives. The synthesis and evaluation of 21 compounds as potential inhibitors of cyclin-dependent kinases 1 and 2 is described and the structure–activity relationships relating to NU6027 have been probed. Simple alkoxy- or cycloalkoxy-groups at the O^4-position were tolerated, with the 4-(2-methylbutoxy)-derivative (IC_{50} vs cdk1/cyclinB1 = 12 ± 2 μM and cdk2/cyclinA3 = 13 ± 4 μM) retaining significant activity. Substitutions at the N^6 position were not tolerated. Replacement of the 5-nitroso substituent with ketone, oxime and semicarbazone groups essentially abolished activity. However, the derivative bearing an isosteric 5-formyl group, 2,6-diamino-4-cyclohexylmethoxy-pyrimidine-5-carbaldehyde, showed modest activity (IC_{50} vs cdk1/cyclinB1 = 35 ± 3 μM and cdk2/cyclinA3 = 43 ± 3 μM). The X-ray crystal structure of the 5-formyl compound bound to cdk2 has been determined to 2.3 Å resolution. The intramolecular H-bond deduced from the structure with NU6027 bound to cdk2 is not evident in the structure with the corresponding formyl compound. Thus the parent compound, 4-cyclohexylmethoxy-5-nitrosopyrimidine-2,6-diamine (NU6027), remains the optimal basis for future structure–activity studies for cyclin-dependent kinase inhibitors in this series.

The progression of cells through the cell cycle is tightly controlled by the sequential activation and inactivation of a family of serine-threonine kinases, the cyclin-dependent kinases (cdks). In particular, cdk1 controls progression from S phase through G2 and into the M phase. Similarly, progression from G1 to S phase is controlled sequentially by cdk 4/6 and cdk2. Cdk activity is regulated by binding to cyclin partners, as well as by phosphorylation and dephosphorylation events, and the action of endogenous inhibitory peptides.[1,2] Loss of cell cycle control leading to uncontrolled proliferation is common in cancer and so the identification of potent and selective cyclin dependent kinase inhibitors is a priority for anti-cancer drug discovery.

A large number of ATP-competitive cdk inhibitory pharmacophores have been identified.[3–10] First generation representatives of these compounds are in clinical trials.[11] We have identified compounds based on purine (e.g., NU2058) and pyrimidine scaffolds which are competitive inhibitors of both cdk1 and cdk2 with respect to ATP.[12,13] The 5-nitrosopyrimidine (NU6027, **1a**) is an inhibitor of cdk1/cyclin B1 and cdk2/cyclinA3 with IC_{50} values of 2.9 ± 0.1 μM and 2.2 ± 0.6 μM, respectively. Interestingly, the crystal structure of **1a** bound to both non-phosphorylated cdk2, in the absence of the cyclin partner, and also to the fully

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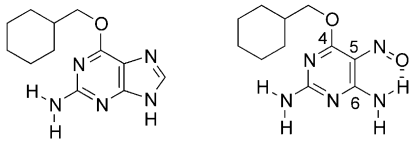
active phospho cdk2-cyclinA complex, shows the inhibitor binding in an orientation distinct from that of the majority of the 6-aminopurine based inhibitors such as olomoucine, roscovitine and purvalanol,[14] but in a nearly identical binding mode to that of the 6-alkoxyguanine derivative NU2058.[12] The key interactions within the ATP-binding site are a triplet of H-bonds (2-NH_2 to Leu 83, N-1 to Leu 83 and 6-NH_2 to Glu 81). Preliminary structure–activity studies suggested that the presence of an intramolecular hydrogen bond between the C^5-nitroso group and the amino group at C^6 of **1a** was necessary to maintain the activity in the pyrimidine series. The intramolecular H-bond orientates **1a** into a purine-like shape, enabling an optimal interaction between the 6-amino group and Glu 81.

We have designed and synthesised derivatives of NU6027 (**1a**) in order to gain a better understanding of the structural basis of inhibition by this new class of ATP-competitive cdk inhibitor. In this paper we report the synthesis of several O^4-, N^6- and C^5-substituted pyrimidines and their biological evaluation as inhibitors of cdk1 and cdk2.



NU2058 NU6027 (**1a**)

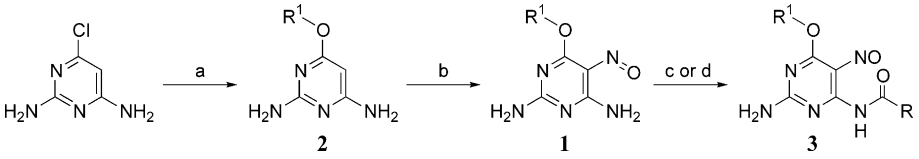
Variation of the substituent at the 4-position of the pyrimidine was achieved by reaction of 4-chloro-2,6-diaminopyrimidine with the appropriate sodium alkoxide (ROH, NaH, DMSO, 80 °C, or ROH, Na, 150 °C) to give the corresponding O^4-substituted pyrimidines (**2a**–**g**) in 43–84% yield. Nitrosation of compound **2a**–**f** using sodium nitrite in 30% acetic acid/H_2O gave the required 5-nitroso derivative (**1a**–**g**) in 50–86% yield (Scheme 1).

A series of N^6-substituted derivatives (**3a**–**c**) was prepared to determine the effect of substitution at this position (Scheme 1). The cyclohexylmethyl group was retained as the O^4 substituent for this series thereby allowing a direct comparison with NU6027 (**1a**). The *N*-acyl and carbamate derivatives (**3a**–**c**; 30, 44 and 25% yields, respectively) were prepared from **1a** by treatment with acetic anhydride or the appropriate chloroformate, respectively.

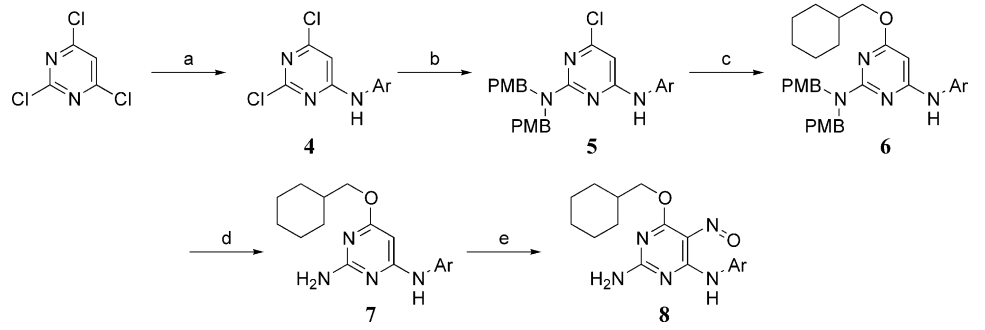
The introduction of hydrophobic arylamino substituents at the 6-position required a different synthetic route (Scheme 2). Reaction of 2,4,6-trichloropyrimidine with aniline or 4-methoxyaniline in 30% aq EtOH gave the 6-arylaminopyrimidines (**4a**,**b**; 46 and 20%, respectively), accompanied by the 2,6-disubstituted products which were separable by chromatography. Substitution at the 2-position of compounds **4a**,**b** with the masked ammonia equivalent, bis-*p*-methoxybenzylamine (Et_3N, *n*-butanol, reflux), gave the disubstituted pyrimidines (**5a**,**b**) in 30–40% yield. Bis-(4-methoxybenzyl) protection of the 2-amino group allowed the alkoxide substitution at the 4-position to proceed smoothly with sodium cyclohexylmethoxide to give the products (**6a**,**b**) in 32 and 74% yield, respectively. Deprotection was effected with TFA at 60 °C to give the 2-aminopyrimidines (**7a**,**b**) in good yields. The 5-nitroso compounds (**8a**,**b**) were prepared as described above (48 and 21%, respectively).

The X-ray structure of NU6027 bound to cdk2 shows evidence of an intramolecular H-bond between the 5-nitroso group and the 6-NH. This H-bond effectively locks the orientation of the 6-amino group in a conformation that facilitates the donation of an H-bond to Glu 81 of the enzyme.[12] In order to explore the scope of this interaction, the synthesis of a variety of derivatives bearing potential H-bond acceptors at the 5-position was carried out. The 5-formyl derivative (**12**) was prepared from 2-amino-4,6-dichloro-5-formylpyrimidine (**9**)[15] by sequential displacement of chloride at the 6- and 4-positions with a masked amine equivalent and cyclohexylmethoxide, respectively, followed by deprotection. The oximes (**13a**–**c**) were prepared by the reaction of **12** with hydroxylamine, methoxylamine, and benzylhydroxylamine, respectively, in glacial acetic acid. The semicarbazone (**13d**) was prepared in 62% yield by the reaction of **12** with semicarbazide hydrochloride (pyridine, EtOH, reflux). Reaction of formyl compound (**12**) with three equivalents of phenyl magnesium bromide gave the alcohol (**14**) in 74% yield. Oxidation of **14** to the ketone (**15**) was effected with Dess–Martin periodinane (82% yield) (Scheme 3).

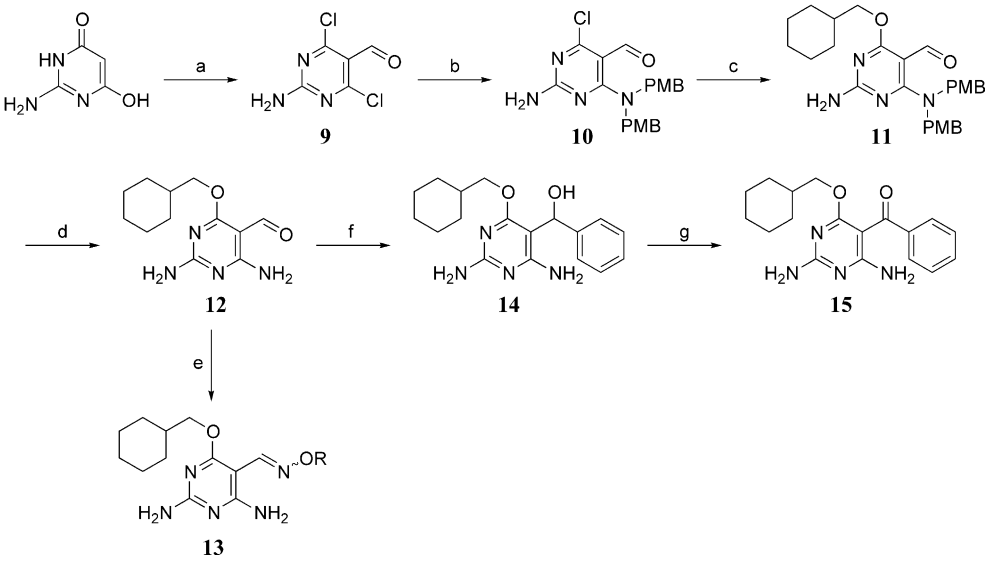
Compounds were assayed for inhibitory activity against cdk1/cyclinB1 and cdk2/cyclinA3 using the methods previously described.[12] The final ATP-concentration in both cdk assays was 12.5 μM. Some of the compounds assayed were insufficiently soluble for an IC_{50} determination. In these cases the activity at 10 μM is given. Values for inhibition at 100 μM concentration are given for inactive compounds. The results are summarised in Table 1.



Scheme 1. Preparation of O^4- and N^6- substituted pyrimidines. Reagents and conditions: (a) NaH, R^1OH, DMSO, 80 °C or Na, R^1OH, 150 °C; (b) $NaNO_2$, 30% AcOH H_2O, 80 °C; (c) $(CH_3CO)_2O$, 80 °C; (d) K_2CO_3/RCOCl, acetone, reflux. R = Me, MeO, BnO.



Scheme 2. Synthesis of N^6-anilino-substituted pyrimidines. Reagents and conditions: (a) $ArNH_2$, 30% EtOH H_2O, reflux; (b) $HN(PMB)_2$, Et_3N, *n*-BuOH, reflux; (c) Na, cyclohexylmethanol, 150 °C; (d) TFA, 60 °C; (e) $NaNO_2$, 30% AcOH, H_2O, 80 °C.

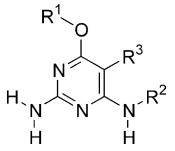


Scheme 3. Synthesis of C^5- substituted derivatives. Reagents and conditions: (a) $POCl_3$, DMF; (b) $HN(PMB)_2$, Et_3N, DCM; (c) NaH, cyclohexylmethanol, DMSO, 80 °C; (d) TFA, 60 °C; (e) $H_2NOR.HCl$, AcOH, EtOH; (f) PhMgBr, THF; (g) Dess–Martin periodinane, DCM. R = H, Me, Bn.

The series of O^4-substituted pyrimidines revealed that the nature of the O^4-substituent can have a major impact on cdk inhibitory activity. Substitution at O^4 with *n*-pentyl and *n*-heptyl, **1b** and **1c**, produced a large decrease in activity compared with **1a** and a significant loss of solubility, suggesting that the increased length and conformational flexibility of these groups is detrimental to cdk binding. Interestingly, the *iso*-pentyl derivative **1d**, retained solubility and displayed modest inhibitory activity against both cdk1 and cdk2 ($IC_{50} = 12 \pm 2$, $13 \pm 4\,\mu M$, respectively), suggesting that a shorter more compact alkyl chain is preferred. The oxopyrrolidinylmethyl substituent introduces H-bonding possibilities within the ribose pocket, analogous to those predicted in the O^6-alkylguanine series.[13] However, as in the corresponding O^6-guanine derivative any such interactions are not observed to be favourable and potency is lost (**1e**; $IC_{50} = 57 \pm 14$, $65 \pm 26\,\mu M$, vs cdk1 and cdk2, respectively). The O^4-cyclohexenyl derivative **1f** retained potency against cdk1 and cdk2 ($IC_{50} = 4.0 \pm 0.3\,\mu M$ and 5.0 ± 0.1, respectively). However, in comparison with the parent compound NU6027 (**1a**; $IC_{50} = 2.9 \pm 0.1$, $2.2 \pm 0.6\,\mu M$, vs cdk1 and cdk2, respectively) the introduction of unsaturation into the ring is seen to have a small negative effect on potency. The X-ray structure of **1a** bound to cdk2 shows the cyclohexylmethyl group bound within the ATP ribose binding pocket of the enzyme.[12] The cyclohexane ring exists in a chair conformation and makes favourable hydrophobic interactions with a non-polar patch on the glycine loop formed principally by the sidechain of Val 18. The moderate loss of potency observed with the unsaturated compound **1f** can be explained by flattening of the cyclohexene ring, which may have a negative effect on packing complementarity between the inhibitor and kinase. This effect is even more dramatic in the case of the O^4-benzyl compound (**1g**; $IC_{50} = 27 \pm 2$, $27 \pm 3\,\mu M$, vs cdk1 and cdk2, respectively),[16] suggesting that the planar benzene ring packs poorly within the ribose-binding site.

The necessity for the 5-nitroso group, believed to maintain an intramolecular H-bond with the 6-amino-group, has been demonstrated previously with NU6034 (**2a**).[12] This requirement was also found in the case of the O^6-cyclohexenylmethyl compound (**2f**) which lacks the 5-nitroso group and showed no significant activity against cdk1 and cdk2.

Table 1. Inhibition of cdk1 and cdk2 by pyrimidine derivatives[19]



	R^1	R^2	R^3	IC_{50} (μM)[a] or % inhibition	
				cdk1	cdk2
1a	Cyclohexylmethyl	H	NO	2.9±0.1	2.2±0.6
1b	$CH_3(CH_2)_4$	H	NO	35±6%[b]	36±2%[b]
1c	$CH_3(CH_2)_6$	H	NO	17±8%[b]	22±7%[b]
1d	$(CH_3)_2CH(CH_2)_2$	H	NO	12±2	13±4
1e	(*S*)-3-Oxopyrrolidin-2-yl)methyl	H	NO	57±14	65±26
1f	(3-Cyclohexenyl)methyl	H	NO	4.0±0.3	5.0±0.1
1g	CH_2Ph	H	NO	27±2	27±3
2a	Cyclohexylmethyl	H	H	4±5%[b]	7±3%[b]
2f	(3-Cyclohexenyl)methyl	H	H	16±14%[b]	8±1%[b]
3a	Cyclohexylmethyl	$COCH_3$	NO	36±4%[c]	28±2c
3b	Cyclohexylmethyl	CO_2CH_3	NO	8±2%[c]	6±5%[c]
3c	Cyclohexylmethyl	CO_2Bn	NO	14±6%[b]	11, 24%[b]
8a	Cyclohexylmethyl	C_6H_5	NO	23±7%[c]	47±14%[c]
8b	Cyclohexylmethyl	*p*-Methoxyphenyl	NO	7%[c]	8%[c]
12	Cyclohexylmethyl	H	CHO	35±3%[b]	43±3%[b]
13a	Cyclohexylmethyl	H	CH=NOH	25±5%[c]	37±23%[c]
13b	Cyclohexylmethyl	H	$CH=NOCH_3$	13±13%[b]	13±6%[b]
13c	Cyclohexylmethyl	H	CH=NOBn	5±6%[b]	3±1%[b]
13d	Cyclohexylmethyl	H	$CH=NNH(CO)NH_2$	61±23%[c]	44±28%[c]
14	Cyclohexylmethyl	H	CH(Ph)OH	4±5%[c]	12±4%[c]
15	Cyclohexylmethyl	H	C(Ph)O	21±23%[b]	6±6%[b]
16	Cyclohexylmethyl	H	NH_2	40±3%[c]	52±6%[c]

[a]12.5 μM ATP concentration.
[b]% Inhibition at 10 μM.
[c]% Inhibition at 100 μM.

Substitution at the 6-amino group resulted in a loss of cdk inhibitory activity (**3a**–**c**, **8a**, **8c**). This is in accordance with the binding mode determined from the crystal structure of **1a** bound to cdk2. The disruption of either the H-bond between the 6-amino NH and Glu 81 or the intramolecular H-bond to the 5-nitroso group would be predicted to result in a significant loss of activity.

The reduction in inhibitory activity resulting from replacement of the 5-nitroso group with an isosteric formyl group in **12** (% inhibition vs cdk and cdk2 at 10 μM = 35±3 and 43±3%, respectively) was surprising. The formyl group was expected to be able to form an intramolecular H-bond with the 6-amino group and so restrict the 6-amino group in the active conformation. In order to investigate this further, the crystal structure of **12** bound to the phosphorylated cdk2-cyclinA complex was determined at 2.3 Å (Fig. 1).[17] Examination of this complex showed that the formyl group was not coplanar with the 6-amino group. The orientation of the formyl group could not be attributed to an intermolecular H-bond with the enzyme or any other structural feature and so is unexpected. This apparent absence of the anticipated intramolecular H-bond may explain the significant loss in activity observed for **12** compared with the nitroso-compound **1a**. The 5-phenylmethanol derivative (**14**) and the 5-phenylketone derivative (**15**) showed a significant decrease in activity compared with **1**, suggesting a lack of steric tolerance at this position. Interestingly, the 5-amino compound (**16**) displayed significant activity at 10 μM against both cdks, but was sparingly soluble.

The 5-oxime derivatives (**13a**–**c**) and the 5-semicarbazone derivative (**13d**) were expected to retain the intramolecular H-bond and allow new interactions with the enzyme binding pocket. However, none of these derivatives displayed good activity against the cdks and their solubility was poor.

Recently, 4-amino-6-(4′-sulfanilyl)pyrimidine has been described as an ATP-competitive cdk2/cyclinE inhibitor with a K_i of 2 μM.[18] When soaked into cdk2 crystals, like **1a**, this 4-aminopyrimidine formed H-bonds with Glu 81 and Leu 83, confirming the utility of the aminopyrimidine pharmacophore in structure based cdk inhibitor design.

In summary, a series of 4-alkoxy-2,6-diaminopyrimidine derivatives has been prepared and evaluated as inhibitors of cdk1 and cdk2. The ATP-ribose binding pocket of the enzyme is occupied by the 4-alkoxy substituent and the structural requirements are rigorous. The cyclohexylmethoxy group is optimal, with minor modifications to the ring resulting in loss of potency. The 5-nitroso group is required for activity, with the isosteric 5-formyl derivative surprisingly lacking potency. Modifications at the 6-amino group were not tolerated. In conclusion, the parent compound NU6027 (**1a**) is the

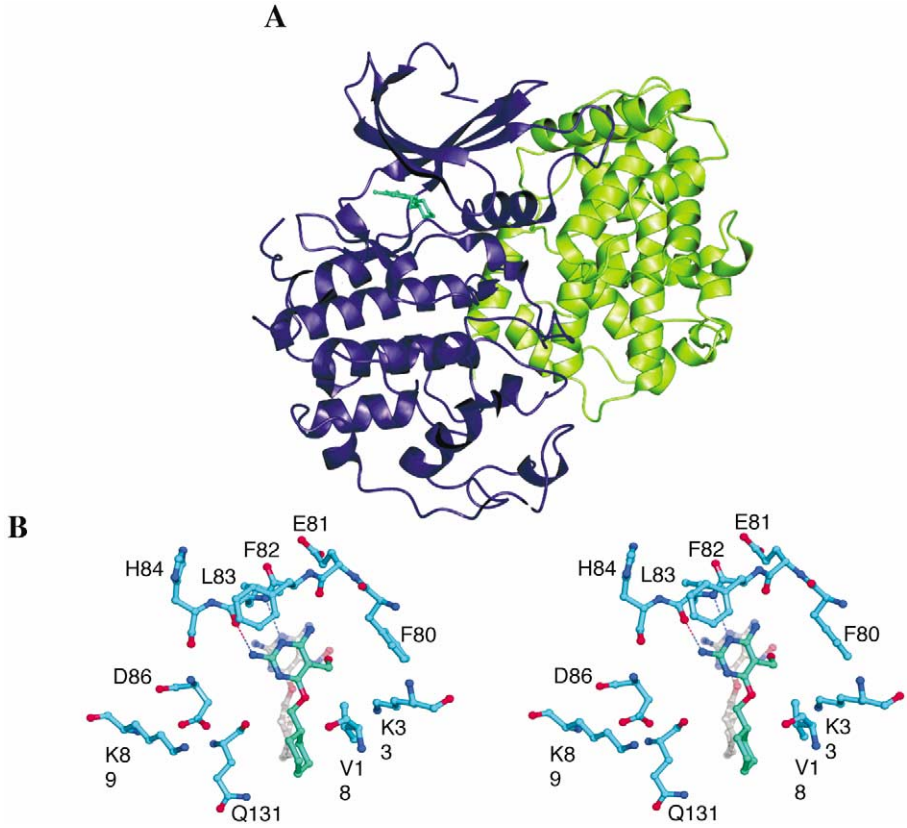


Figure 1. (A) Crystal structure of compound **12** bound to the phospho-cdk2-cyclinA complex. Compound **12** shown in cyan, phospho-cdk2 in purple, and cyclinA in lime. (B) Stereoview of the active site showing two H-bonds between compound **12** (green) and cdk2 with the structure of NU6027 (grey) superimposed.

most potent inhibitor of cdk1 and cdk2 discovered so far in this series and represents the optimal starting point for cdk inhibitory structure activity studies in the future.

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