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Synthesis and Biological Evaluation of N^2-Substituted 2,4-Diamino-6-cyclohexylmethoxy-5-nitrosopyrimidines and Related 5-Cyano-NNO-azoxy Derivatives as Cyclin-Dependent Kinase 2 (CDK2) Inhibitors

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The potent and selective cyclin-dependent kinase 2 (CDK2) inhibitor NU6027 (6-cyclohexylmethoxy-5-nitroso-2,4-diaminopyrimidine) was used as the lead for the synthesis of a series of analogues in order to provide further insight into the structure–activity relationships for 2,4-diaminopyrimidine CDK2 inhibitors. Aliphatic amino substituents were introduced at position 2. The use of linear or less sterically hindered amines gave rise to compounds endowed with slightly better activity than the lead; on the other hand, the compounds were less active if a bulkier amino substituent was used. Substitution of the 5-nitroso group with a 5-cyano-NNO-azoxy moiety afforded a new class of inhibitors, the activity of which against CDK2 was found to be similar to that of the nitroso series. The most active nitroso compound was **8b** ((2*S*)-2-[(4-amino-6-cyclohexylmethoxy-5-nitrosopyrimidin-2-yl)amino]propan-1-ol; IC_{50} = 0.16 μM), while in the 5-cyano-NNO-azoxy series the most active compound was **9b** (4-amino-5-[(*Z*)-cyano-NNO-azoxy]-2-{[(2*S*)-1-hydroxypropan-2-yl]amino}-6-cyclohexylmethoxypyrimidine; IC_{50} = 0.30 μM). Taken together, these new analogues of NU6027 enhance our understanding of the structure–activity relationships for 2,4-diaminopyrimidine CDK2 inhibitors.

Cyclin-dependent kinases (CDKs) are serine/threonine kinases that display abnormal activity in many kinds of tumors.[1] This family of kinases is represented by eleven members (CDK1–CDK11) and related cyclins.[2] Today, there is a great interest in small-molecule CDK inhibitors as potential anticancer drugs. Over the past decade, many such compounds belonging to different chemical classes have been developed.[3] Among these inhibitors, an interesting type is represented by 2,4-diamino-6-cyclohexylmethoxy-5-nitrosopyrimidine (**1**, NU6027) (Figure 1), a competitive inhibitor of CDK1 and CDK2 isoforms

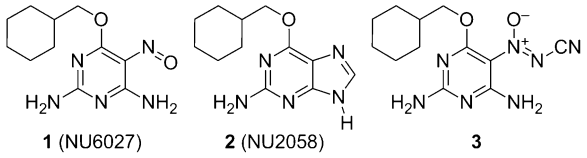

Figure 1. Reference compounds **1**–**3**.

with respect to ATP (CDK2 IC_{50} = 2.2 μM).[3–5] Owing to the intramolecular hydrogen bond between the adjacent 5-nitroso and 4-amino groups, this compound assumes a pseudo-purine geometry, which is reminiscent of the structure of 6-(cyclohexylmethoxy)-9*H*-purine (**2**, NU2058; Figure 1), an early relatively potent CDK1 and CDK2 inhibitor (CDK2 K_i = 12 μM).[6] Compound **1** can interact with the ATP binding site of the enzymes by a triplet of hydrogen bonds (for CDK2: 2-NH_2 to Leu83 (CO), N3 to Leu83 (NH), 4-NH_2 to Glu81 (CO)).[4] These interactions exactly reproduce those of **2**. An extended series of analogues of **1** modified at the 2-, 5- and 6-position(s) were synthesized in order to shed light on the structure–activity relationships (SARs) in this lead compound.[3,4] In a recent paper we described a new pyrimidine scaffold, the 2,4-diamino-5-(cyano-NNO-azoxy)-6-(cyclohexylmethoxy)pyrimidine (**3**; Figure 1), endowed with potent CDK2 inhibitory activity.[7] This substance can be formally obtained by substitution of the nitroso group of **1** with the cyano-NNO-azoxy moiety, which is present in the antibiotic "calvatic acid" (4-[(*Z*)-cyano-NNO-azoxy]benzoic acid) initially isolated from the culture broth of *Calvatia lilacina*.[8] This unusual functional group has been used to design several bioactive compounds, such as antimicrobial and antitumor agents, enzyme inhibitors, and calcium channel blockers.[9–13] The cyano-NNO-azoxy moiety displays an electron-withdrawing property very similar to that of the nitroso group (σ_{pNO} = 0.91, σ_{pONNCN} = 0.89), whereas it is endowed with different lipophilicity (π_{NO} = −1.20, π_{ONNCN} = −0.26) and steric properties.[14,15]

A molecular modeling study has suggested a role for a conserved water molecule in stabilizing the bioactive pose of **3** in its interaction with the ATP binding site of the enzyme. Preliminary SARs showed that the substitution of the cyano group of

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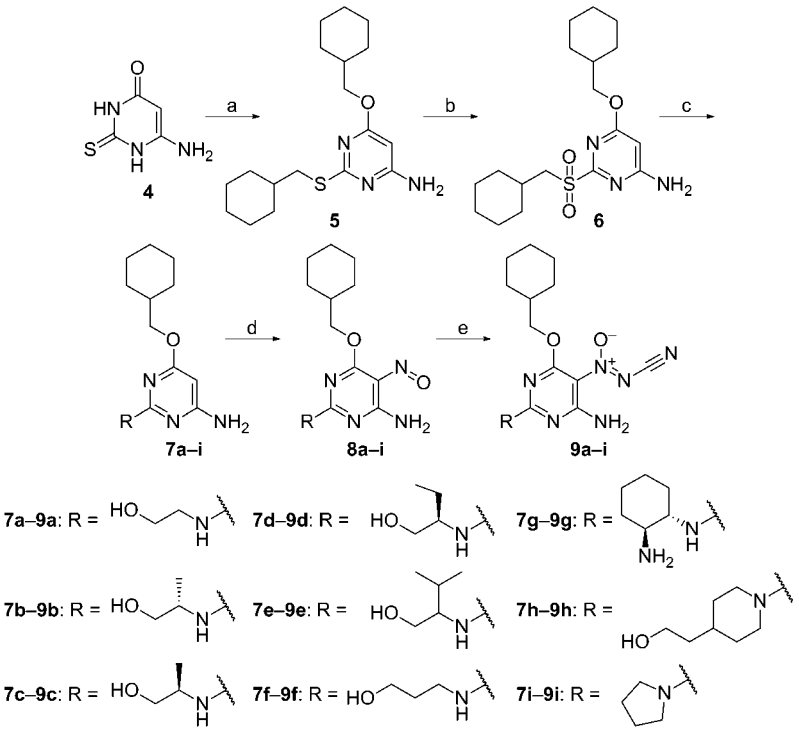
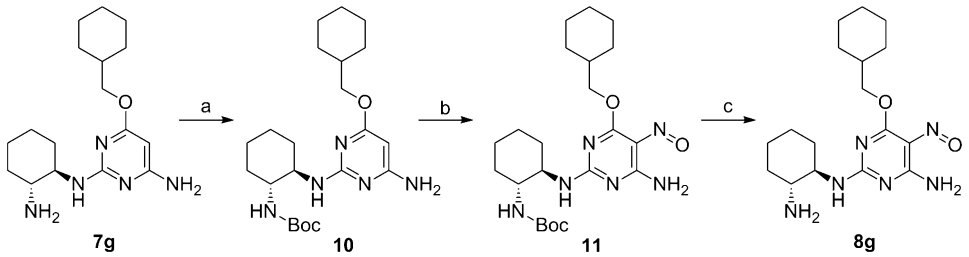
3 with other electron-withdrawing moieties induced a significant decrease in activity, whilst introduction of a *p*-methylaminosulfonyl-substituted phenyl ring at the 2-amino group gave rise to a product with potency in the nanomolar range.[7] In this study a detailed description of the synthetic routes to a series of new N^2-substituted examples of this structural class (**9a–i**) and of their 5-nitroso precursors (**8a–i**) is reported, and the influence of the lateral chain at this position on the their CDK2 inhibitory activity is discussed.

A series of N^2-substituted 2,4-diamino-6-cyclohexylmethoxy-5-nitrosopyrimidines (**8a–i**) was prepared by using a synthetic strategy (Scheme 1) similar to that described previously by Marchetti et al.[3,4] Some modifications improved the reported reaction conditions. Alkylation of 6-amino-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-one (**4**) was performed using (bromomethyl)-cyclohexane. At variance with the previous synthetic strategy, (bromomethyl)cyclohexane was used to enable the concomitant alkylation at the 2- and 4-positions. In addition, the use of microwave heating (μW) afforded the desired product **5** in 16 min at 140 °C. Oxidation of **5** with *m*CPBA gave the corresponding cyclohexylmethylsulfone **6**. The nucleophilic displacement of the cyclohexylsulfonyl group in **6** with diverse aliphatic amines was performed in an organic solvent (diglyme or THF) using microwave heating, and in some cases a Lewis acid such as $Yb(OSO_2CF_3)_3$ was added to improve the yield. The N^2-substituted 4-amino-6-cyclohexylmethoxypyrimidines **7a–i** were thus obtained in moderate yield. Subsequent nitrosation at the 5-position with alkyl nitrites (menthyl nitrite or amyl nitrite) gave the desired nitroso compounds **8a–i**. The 5-nitroso derivative **8g** was obtained after $(Boc)_2O$ protection of compound **7g**, followed by nitrosation and subsequent deprotection of the amino group (Scheme 2). The final products **9a–i** were prepared by treating the nitroso derivatives **8a–i** with (diacetoxyiodo)benzene (IBA) and cyanamide (NH_2CN) in dry CH_3CN.[16]



Scheme 1. *Reagents and conditions*: a) (bromomethyl)cyclohexane, 140 °C μW heating, 16 min; b) *m*CPBA, CH_2Cl_2, RT, 18 h; c) RNH_2, 120 °C μW heating; d) R^2ONO, DMSO, RT; e) NH_2CN, (diacetoxyiodo)benzene (IBA), CH_3CN, RT, 2 h.

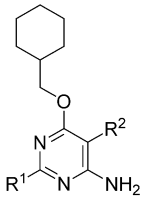


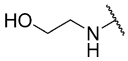
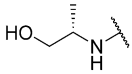
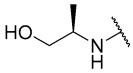
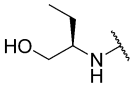
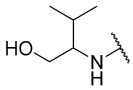
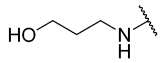
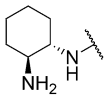
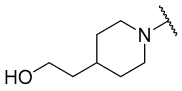
Scheme 2. *Reagents and conditions*: a) $(Boc)_2O$, THF, RT, 2 h; b) menthyl nitrite, DMSO, RT, 18 h; c) TFA, CH_2Cl_2, RT, 2 h.

In the ^{1}H NMR experiments reported for the 5-nitroso derivatives (**8a–i**), the resonance of the 4-NH_2 group was split into two neat signals, which were observed at ~8 and 10 ppm, respectively. This observation was previously reported,[7] and confirms the formation of an intramolecular hydrogen bond between the nitroso oxygen atom and one hydrogen atom of the NH_2 group at the 4-position. In contrast, for the cyano-NNO-azoxy derivatives **9a–i**, the signal of the 4-NH_2 group was reported as a broad singlet. In addition, ^{1}H and ^{13}C NMR spectra of both nitroso and cyano-NNO-azoxy derivatives presented two sets of resonances. This can be ascribed to the significant π component of the C2–N bond, as a consequence of strong conjugation between the electron-donating N groups (e.g., $NHCH_3$ $\sigma_p = -0.84$)[14] and the strong electron-withdrawing groups, the cyano-NNO-azoxy and the nitroso group, respectively, with consequent restricted rotation around the C2–N bond at room temperature (Figure 2). This hypothesis was confirmed by a variable-temperature NMR experiment (VT-NMR) performed on compound **8a** for which coalescence of the two sets of signals was observed at 125 °C.

The new series of the 5-(cyano-NNO-azoxy)-substituted compounds and of the related 5-nitroso precursors were evaluated for their CDK2 inhibitory activity using published procedures.[18] The results, expressed as IC_{50} values, are listed in Table 1 together with the inhibitory potency of compounds **1** and **3** as references. The potency in the 5-nitroso series exhibits the order **8b** > **8f** ≥ **8a** > **8c** > **1** > **8d** > **8e** > **8i** ≥ **8g** > **8h**. Analysis of the data shows that introduction of a hydroxyethyl group at the 2-NH_2 of **1** (**8a**) increases the inhibitory potency by about threefold, whilst the introduction of a hydroxypropyl substructure gives rise to **8f**, which is about fourfold more potent than the reference. Also, the presence of the 2-hydroxy-1-methylethyl moiety (as in **8b**, **8c**) induces a potency increase that is particularly evident in the *S* stereoisomer (**8b**). As for compounds **8d**,**e**,**g**, the activity decreases with growth of the substituent at the 2-position. This is probably due to the steric-based weakening of the hydrogen bond between the 2-NH group of the compounds and the CO group of the Leu83 residue in the enzyme, and to the fact that given the partially hindered rotation

Table 1. Inhibitory activity of target compounds against CDK2.



Compd	R^1	R^2	IC_{50} [μM][a]
1	NH_2	NO	2.2
3		–N(O)=N–CN	0.94
8a		NO	0.61
9a		–N(O)=N–CN	0.45
8b		NO	0.16
9b		–N(O)=N–CN	0.30
8c		NO	0.84
9c		–N(O)=N–CN	0.55
8d		NO	4.02
9d		–N(O)=N–CN	2.19
8e		NO	24.5
9e		–N(O)=N–CN	12.9
8f		NO	0.59
9f		–N(O)=N–CN	0.42
8g		NO	35% at 100 μM
9g		–N(O)=N–CN	66
8h		NO	9% at 100 μM
9h		–N(O)=N–CN	65.5
8i		NO	49% at 100 μM
9i		–N(O)=N–CN	54% at 100 μM

[a] All IC_{50} or percent inhibition values are results obtained from $n = 2$ determinations. CDK inhibition was determined at a minimum of five different inhibitor concentrations; CDK assays were conducted at 12.5 μM ATP; full details are provided in reference [17].

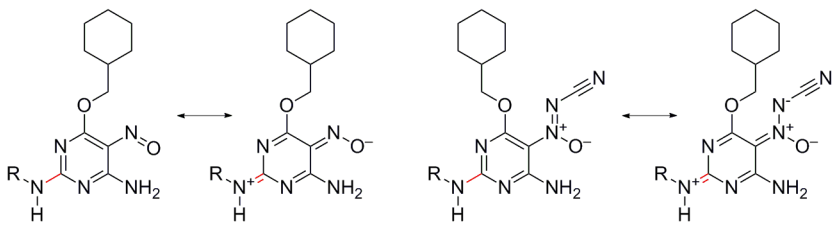


Figure 2. Electronic conjugation between the electron-releasing N group and the electron-withdrawing NO or cyano-NNO-azoxy groups

around C2–NH bond, one of the two conformers is unable to give this interaction. As expected, compounds **8h,i** displayed very weak activity due to the absence of NH. The potencies and SAR in the 5-(cyano-NNO-azoxy) series **9a–i** closely paralleled what was found in the 5-nitroso series. Again, the most active substance **9b** bears the 2-hydroxy-1-methylethyl moiety at the 2-position and is about threefold more active than the reference compound **3**.

The study reported herein extends the findings of the previous investigation.[7] A series of N^2-substituted derivatives of the reference compound **1** was synthesized in order to explore structure–activity relationships concerning the substitution at this nitrogen position with aliphatic amino substituents present in relevant CDK inhibitors. Although no significant improvements were achieved in terms of biological activity, the SARs and understanding of the criteria for achieving CDK2 inhibitory activity were enhanced. Introduction of the 5-(cyano-NNO-azoxy) function gave no significant improvement over the corresponding 5-nitroso derivatives. However, the cyano-NNO-azoxy group is a suitable replacement for the nitroso group at the 5-position, able to maintain CDK2 inhibitory activity. Because these two moieties are endowed with different chemical and physicochemical properties, they should give rise to two different classes of inhibitors, which should display different ADMET profiles worthy of additional investigation.

Acknowledgements

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Keywords: antitumor agents · cyclin-dependent kinases · inhibitors · nitrosation · substituted pyrimidines

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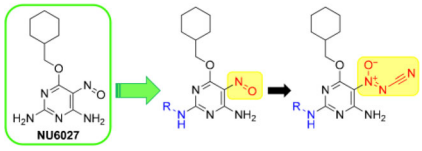
Say yes to NNO! Starting from compound NU6027, a series of 2,4-diamino-5-nitrosopyrimidines were synthesized. Structure–activity relationship studies of this compound class led to an improved understanding of the criteria for inhibitory activity toward cyclin-dependent kinase 2. The cyano-NNO-azoxy substituent was confirmed to be a valuable alternative to a 5-nitroso group.



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Synthesis and Biological Evaluation of N^2-Substituted 2,4-Diamino-6-cyclohexylmethoxy-5-nitrosopyrimidines and Related 5-Cyano-NNO-azoxy Derivatives as Cyclin-Dependent Kinase 2 (CDK2) Inhibitors

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