Cytokinin-Derived Cyclin-Dependent Kinase Inhibitors: Synthesis and cdc2 Inhibitory Activity of Olomoucine and Related Compounds

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Cyclin-dependent kinases (cdk) have recently raised considerable interest in view of their essential role in the regulation of the cell division cycle. The structure−activity relationships of cdk inhibition showed that the 1, 3, and 7 positions of the purine ring must remain free, probably for a direct interaction, in which it behaves as a hydrogen bond acceptor. Olomoucine (6-(benzylamino)-2-[(2-hydroxyethyl)amino]-9-methylpurine, OC), roscovitine (6-(benzylamino)-2(*R*)-[[1-(hydroxymethyl)propyl]amino]-9-isopropylpurine), and other N^6,2,9-trisubstituted adenines were found to exert a strong inhibitory effect on the $p34^{cdc2}$/cyclin B kinase. Removal or change of the side chain at position 2 or the hydrophobic group at position 9 dramatically decreased the inhibitory activity of olomoucine or roscovitine. Inhibition of cdk with OC and related compounds clearly arrests cell proliferation of many tumor cell lines at G_1/S and G_2/M transitions and also triggers apoptosis in the target tumor cells in vitro and in vivo. Thus, from a pharmacological point of view, OC may represent a model compound for a new class of antimitotic and antitumor drugs.

Introduction

Cytokinins represent a class of natural and artificial plant growth regulators. Since they are typical components of several tRNAs of plants, animals, and microorganisms, these compounds are likely to be present, at least in a bound form, in many organisms. Their major physiological effect is to induce cell division.[1]

The molecular mechanisms by which cytokinins mediate cell growth and division are still unknown. However, it has been reported that cytokinins may increase the accessibility of the DNA template, activate RNA polymerases, affect the polyadenylation and the secondary structure of mRNA, and stimulate the formation and activity of polyribosomes.[2] Given the prominent role of cytokinins in the control of cell division, an interesting question was whether these hormones interact with the known regulatory proteins of the cell cycle. Both cytokinins and cyclin-dependent kinases (cdks) act at multiple and similar control points of the division cycle, i.e., G_1/S and G_2/M transitions and S and M phases.[3] Recent reports have suggested that cytokinins affect reversible phosphorylation of ribosomal S_6 protein, RNA polymerase 1, and protein cofactors of translation in plant cells. They also have an effect on transcriptional regulation of the *cdc2a* gene, but the simultaneous presence of auxins and cytokinins is probably necessary for the induction of *cdc2a* expression.[4]

Natural cytokinins were found to be rather nonspecific inhibitors of various protein kinases.[5] Surprisingly, among adenine derivatives, we have discovered 6-(benzylamino)-2-[(2-hydroxyethyl)amino]-9-methylpurine (**13**), named "olomoucine" (OC), which specifically inhibits some cdks at micromolar concentration.[5] One of the inhibited kinases, the $p34^{cdc2}$/cyclin B kinase, is a key mitotic factor, which is highly conserved and strongly implicated in cell cycle transitions in all eucaryotic cells.[6] The total lack of inhibitory effect of olomoucine on major kinases, such as cAMP- and cGMP-dependent kinases, protein kinase C, and others, suggests that OC might be a useful tool for cell cycle studies.[5] The design and inhibitory activity of OC was further improved by modifications at positions 2, 6, and 9, i.e., the positions which control the binding to cdc2. This has recently led to the discovery of a novel specific cdk inhibitor named roscovitine (ROSC, 6-(benzylamino)-2(*R*)-[[1-(hydroxymethyl)propyl]amino]-9-isopropylpurine) (Meijer et al., submitted), which displays an enhanced inhibitory activity toward cdc2, a higher selectivity toward some cdks, an increased antimitotic activity at the G_1/S and G_2/M points of the cell cycle, and stronger and more selective antitumor effects (Hajdúch et al., submitted).

Here we report the synthesis of olomoucine, roscovitine, and structurally related compounds together with their structure−activity relationships in the cdc2 kinase assay.

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Table 1. IC_{50} Values for Various Olomoucine Derivatives Added to Purified cdc2 Kinase[a]

no.	compound	IC_{50}, μM
1	adenine	200
2	2,6-diaminopurine	>100[b]
3	6-amino-2-chloropurine	>1000[b]
4	6-amino-2-methylpurine	320
5	6-amino-2-[(2-(hydroxyethyl)amino]purine	200
6	6-amino-9-methylpurine	>350[b]
7	6-amino-2-chloro-9-methylpurine	70
8	6-amino-2-[(2-(hydroxyethyl)amino]-9-methylpurine	50
9	6-(benzylamino)purine (BAP)	200
10	6-(benzylamino)-9-methylpurine	40
11	2-amino-6-(benzylamino)purine	90
12	6-(benzylamino)-2-[(hydroxyethyl)amino]purine	25
13	6-(benzylamino)-2-[(2-(hydroxyethyl)amino]-9-methylpurine (olomoucine)	6
14	2-amino-6-(benzylamino)-9-methylpurine	40
15	2-chloro-6-(benzylamino)-9-methylpurine	12
16	2-chloro-6-(isopent-2-enylamino)-9-methylpurine	40
17	6-(isopent-2-enylamino)-2-[(2-hydroxyethyl)amino]-9-methylpurine	65
18	2-chloro-6-[(cyclohexylmethyl)amino]-9-methylpurine	130
19	6-[(cyclohexylmethyl)amino]-2-(2-[(hydroxyethyl)amino]-9-methylpurine	6
20	6-(benzylamino)-2-chloro-9-isopropylpurine	17
21	6-(benzylamino)-2-[(2-hydroxyethyl)amino]-9-(2-hydroxyethyl)purine	8
22	6-(benzylamino)-2-[(2-hydroxyethyl)amino]-9-isopropylpurine	2
23	6-(benzylamino)-(*R*/*S*)-[(2-hydroxypropyl)amino]-9-isopropylpurine	0.9
24	6-(benzylamino)-2-(*R*/*S*)-[[1-(hydroxymethyl)propyl]amino]-9-isopropylpurine	0.65
25	6-(benzylamino)-2(*S*)-[[1-(hydroxymethyl)propyl]amino]-9-isopropylpurine	0.8
26	6-(benzylamino)-2(*R*)-[[1-(hydroxymethyl)propyl]amino]-9-isopropylpurine (roscovitine)	0.2
27	6-amino-1-methylpurine	>1000
28	6-amino-3-methylpurine	400
29	6-amino-7-methylpurine	>500
30	6-(benzylamino)-1-methylpurine	>1000
31	6-(benzylamino)-3-methylpurine	300
32	6-(benzylamino)-7-methylpurine	>1000

[a] Enzyme activities were assayed as described in the Experimental Section, in the presence of increasing concentrations of tested compounds. IC_{50} values were subtracted from the dose–response curves as shown in Figure 1. [b] Precipitation at the higher concentration tested.

Chemistry

Two series of substituted purine derivatives were synthesized or purchased. Series 1 (**1**–**26**) has substituents in positions 2, 6, and/or 9. Series 2 (**27**–**32**) combines substituents in positions 6–1, 6–3, and 6–7 (Table 1).

6-(Benzylamino)-9-methylpurine (**10**)[7] was prepared by methylation of 6-chloropurine according to the method described for adenine.[13a] The crude reaction mixture, after reaction with benzylamine, yielded the 9-methyl isomer **10** and the 7-methyl isomer **32,** which were readily separated by column chromatography.

6-Amino-2-chloropurine (**3**), when reacted with ethanolamine, afforded 6-amino-2-[(2-hydroxyethyl)amino]-purine (**5**), the structure of which was confirmed by ^1H and ^{13}C NMR (including DEPT 135 exp), MS, and elemental analysis, although our melting point and UV maxima differed from those reported in the literature.[8] Compound **3** was also used for the preparation of 6-amino-2-chloro-9-methylpurine (**7**) using the same methylation method described above for **10**. The aminolysis of compound **7** with ethanolamine gave a new purine derivative, 6-amino-2-[(2-hydroxyethyl)amino]-9-methylpurine (**8**).

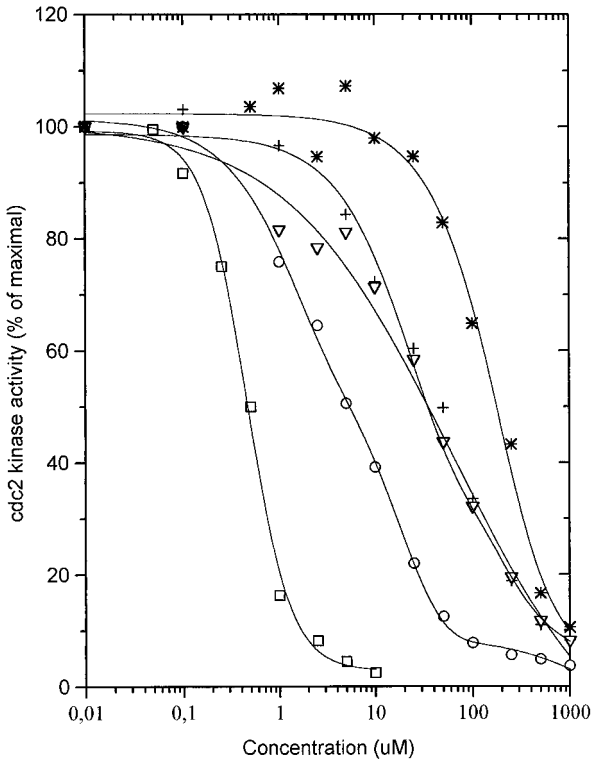

Figure 1. Effect of 6-(benzylamino)purine and structurally related compounds on the $p34^{cdc2}$/cyclin B kinase activity. Enzyme activity was assayed in triplicates as described in the Experimental Section: (*) 6-(benzylamino)purine, (+) 6-amino-2-[(2-hydroxyethyl)amino]-9-methylpurine (**8**), (○) 6-(benzylamino)-2-[(2-hydroxyethyl)amino]-9-methylpurine (**13**), (▽) 6-(benzylamino)-9-methylpurine (**10**), and (□) 6-(benzylamino)-2(*R*)-[[1-(hydroxymethyl)propyl]amino]-9-isopropylpurine (**26**).

Treatment of 2-amino-6-chlorpurine with benzylamine led to **11**, which after methylation yielded the new purine derivative 2-amino-6-(benzylamino)-9-methylpurine (**14**), the structure of which was confirmed by MS and NMR spectral analyses. The proof that compounds **8** and **14** are 9-methyl isomers was derived from UV data which were in agreement with literature reports.[10] The series of trisubstituted purines **16**–**26** was prepared from 2,6-dichloropurine similarly as described for olomoucine; the tetrahydropyranyl group was used for protection of the OH group during the synthesis of compound **21**.

$p34^{cdc2}$/Cyclin B Kinase Inhibition Assay

Thirty-two compounds were tested for $p34^{cdc2}$ inhibitory activity to determine basic relationships between their chemical structure and inhibitory activity (Table 1). Starting with the weakly active adenine (**1**), detectable at $IC_{50} = 200$ μmol, the inhibitory activity was not affected by a substitution in position 2 or 9. However, 2, 9-disubstituted adenines such as **7** and **8** were more active than the corresponding monosubstituted adenine derivatives **2**–**6**, when tested in the cdc2 kinase assay. Addition of a methyl group to position 9 of **3** and **5** caused at least a 3-fold increase in inhibitory activity.

The effects of substitution in positions 2 and 9 were further tested with 6-(benzylamino)purine (**9**), an aromatic cytokinin, which was as active as adenine (**1**)

alone. The addition of a second substituent (**10**−**12**) to the little active 6-(benzylamino)purine (**9**) increased its inhibitory activity. Finally, the substitution of **9** by a side chain at C2 and by a hydrophobic group at N9 resulted in compounds **13** and **26**, the potent cdc2 inhibitors olomoucine and roscovitine. These compounds have been shown to act as competitive inhibitors, exhibiting a higher binding affinity than ATP itself.[5]

The inhibitory activity of purine derivatives in the cdc2 assay varied depending on the C2 substituent. The (2-hydroxyethyl)amino, (2-hydroxypropyl)amino, and [1-(hydroxymethyl)propyl]amino substituents at C2 increased the inhibitory effect, while amino or chlorine substituents in position 2 had a lower effect. 6-(Benzylamino)-2-[(2-hydroxyethyl)amino]purine (**12**) was also much more active (ca. 10 times) than 6-(benzylamino)purine. Thus, a polar side chain at position 2 appears to be essential since it has a positive binding effect (compare **18** and **19**, **20** and **21**, **13** and **15**) and also causes an increased solubility of the compounds. Furthermore, it binds to an area of the ATP binding pocket occupied by ribose in the cdk2/ATP complex.[10]

The N^6-substitutions (**16**−**19**) were of special interest because of the natural occurrence of cytokinins and their extremely high effect on cell division.[1,11] The superiority of N^6-benzyl in OC and ROSC over **16** and **17** bearing an isopentenyl side chain of the natural cytokinins is evident from comparing their inhibitory effects in Table 1. No improvement of inhibitory activity has as yet been obtained by modifications of the N^6-side chain by a hydrophobic residue (isopentenyl, cyclohexylmethyl). Since the benzyl group binds outside the conserved binding pocket, it is most likely to be responsible for the specifity of OC and ROSC for cdks.

N^9-Substitution by a hydrophobic residue is probably important for a positive binding, and isopropyl appears to be the most active group at position 9. Addition of a methyl substituent at positions 1, 3, and 7 of adenine (**27**−**29**) drastically reduced or eliminated the cdc2 inhibitory activity. The effect of these substitutions was further tested on BAP (**9**). A methyl group at N1 (**30**) and N7 (**32**) practically removed the inhibitory effect, while a 3-methyl group (**31**) was somewhat less effective. It can therefore be suggested that positions 1 plus 7 and possibly 3 must be free for hydrogen bonding.

2,9-Disubstituted-N^6-alkyladenines described in this study are of special interest because of the strong inhibitory action of olomoucine and roscovitine in several human tumor cell lines.[5,12] It is even possible that through its specificity to cdks, olomoucine might lead to the development of a new generation of selective anticancer compounds which will preferentially inhibit the proliferation of certain tumor cells (Hajdúch et al., submitted).

Experimental Section

Evaporations were carried out under water pump vacuum or rotary oil pump in the case of ethanolamine, dimethylformamide, dimethyl sulfoxide, and benzylamine. UV spectra were determined in 0.1 N HCl or 0.1 N NaOH solution in 90% MeOH, and the solutions are referred to as pH 1 and pH 13. The ^{1}H NMR spectra were measured on a Jeol GX 270 spectrometer or on a Varian VXR-400 instrument. All J values are given in hertz. In the case of compounds **5**, **8**, **17**, and **21** ^{13}C NMR spectra were also measured (data not shown) and were in accordance with the proposed structure. Electron impact mass spectra were recorded on instruments specified further in the text for each compound (ionizing energy 70 eV, source temperature 250 °C, direct inlet). Optical rotation values were determined on a Perkin-Elmer 141 MCA polarimeter at 20 °C. All compounds gave satisfactory elemental analyses (±0.4%). Merck silica gel Kieselgel 60 (230−400 mesh) was used for column chromatography. TLC was carried out on Merck DC Alufolien Kieselgel 60 F_{254} plates. Compounds **1**, **2**, **4**, **9**, and **27**−**29** were purchased from Aldrich. The following compounds were prepared as described in the literature:[13,14] 6-amino-9-methylpurine (**6**), 6-(benzylamino)-2-[(2-hydroxyethyl)amino]purine (**12**), 6-(benzylamino)-2-[(2-hydroxyethyl)amino]-9-methylpurine (**13**), 6-(benzylamino)-2-chloro-9-methylpurine (**15**), 6-(benzylamino)-1-methylpurine (**30**), 6-(benzylamino)-3-methylpurine (**31**), and 6-amino-2-chloropurine (**3**).

6-(Benzylamino)-9-methylpurine (10) and 6-(Benzylamino)-7-methylpurine (32). A mixture of 6-chloropurine (0.6 g, 3.88 mmol), aqueous 40% tetrabutylammonium hydroxide (2.75 mL), dichloromethane (12 mL), and iodomethane (0.26 mL, 4.27 mmol) was vigorously shaken at room temperature for 6 h. The organic layer was concentrated in vacuo, and the crude product was dissolved in *n*-butanol (25 mL). Triethylamine (3 mL) and benzylamine (2.7 mL) were added, and the reaction mixture was heated under reflux for 3 h and then evaporated. Column chromatography with 2% MeOH in $CHCl_3$ afforded the desired product **10** with mp (after crystallization from $CHCl_3$−Et_2O, 215 mg, 23% yield) and UV spectra (pH 1, pH 13) corresponding with literature.[7] The 7-methyl isomer **32** was eluted with 10% MeOH in $CHCl_3$ and crystallized from $CHCl_3$−Et_2O (270 mg, 29%); mp and UV spectra (pH 1, pH 13) corresponded with the literature data.[7]

6-(Benzylamino)-2-chloro-9-[2-[(2-tetrahydropyranyl)-oxy]ethyl]purine. Crude 2-[(2-iodoethyl)oxy]tetrahydropyran (8.8 mmol) (prepared from 2-iodoethanol, 3,4-dihydro-2*H*-dihydropyran, and Amberlyst 15, 2 h, ambient temperature) was added at −20 °C to the sodium salt of 2,6-dichloropurine (prepared from 5.8 mmol of 2,6-dichloropurine and NaH) dissolved in 10 mL of absolute DMF. The reaction mixture was warmed for 3 h to 60 °C, and then evaporated, and the residue was partitioned between water and ethyl acetate. Column chromatography ($CHCl_3$−MeOH, 20:1) afforded 2,6-dichloro-9-[2-[(2-tetrahydropyranyl)oxy]ethyl]purine as a viscous oil which crystallized from EtOH−pentane; yield 10% (calculated on dichloropurine); mp 73−76 °C. The product, after reaction with benzylamine (triethylamine, *n*-butanol, 2 h, 105 °C) afforded 6-(benzylamino)-2-chloro-9-[2-[(2-(tetrahydropyranyl)oxy]ethyl]purine: crystals from *n*-butanol; yield 65%; mp 121−125 °C; ^{1}H NMR (400 MHz, DMSO) 1.27−1.65 (6H, m, THP), 3.305 (1H, m, THP), 3.425 (1H, m, THP), 3.712 (1H, m, part of CH_2N), 3.897 (1H, m, part of CH_2N), 4.300 (2H, m, C H_2O), 4.561 (1H, m, CH in THP), 4.646 (2H, d, J = 6.1, NHC**H**$_2$Ph), 7.221 (1H, tt, J = 6.9, 1.9, Ph), 7.302 (2H, dd, J = 6.9, 6.9, Ph), 7.336 (2H, dd, J = 6.9, 1.9, Ph), 8.136 (1H, s, H-C^8), 8.792 (1H, t, J = 6.1, N**H**CH_2Ph).

Preparation of 6-(Alkylamino)-2-chloro- or 6-(Alkylamino)-2-aminopurines. 2,6-Dichloropurine or 2-amino-6-chloropurine (1 mmol), the appropriate alkylamine (1.5 mmol), and triethylamine (2.5 mmol) were heated with stirring in 3 mL of *n*-butanol (3 h, 110 °C). After cooling the product was filtered and washed with *n*-propanol. 2-Chloro-6-(isopent-2-enylamino)purine: yield 83%; mp over 270 °C; TLC $CHCl_3$−MeOH−concentrated NH_4OH (8:2:0.2), single spot. 2-Chloro-6-[(cyclohexylmethyl)amino]purine: yield 64%; mp 267 °C. 2-Amino-6-(benzylamino)purine (**11**): yield 96%; mp 228−230 °C (corresponds with the literature value[16]).

Preparation of 6-(Alkylamino)-2-chloro-9-alkylpurines. 6-(Alkylamino)-2-chloropurine (1 mmol), powdered potassium carbonate (5 mmol), and alkyl halogenide were vigorously stirred in 3 mL of DMF (DMSO) overnight. After evaporation of the solvent the product was extracted (water/EtOAc) and purified either by column chromatography or by crystallization.

16: 1.1 mmol of methyl iodide, DMF; column chromatography stepwise 0, 1, 2% MeOH in $CHCl_3$; crystallization $CHCl_3-Et_2O$; yield 79%; mp 197−200 °C. Anal. ($C_{11}H_{14}N_5Cl$) C, H, N.

18: 1.1 mmol of methyl iodide, DMF; column chromatography $CHCl_3$−MeOH (100:4); yield 85%; mp 209−210 °C. Anal. ($C_{13}H_{18}N_5Cl$) C, H, N.

20: 10 mmol of isopropyl bromide, DMSO; crystallization from hot MeOH; yield 85%; mp 181−182 °C; ^{1}H NMR (400 MHz, DMSO) 1.48 (6H, d, J = 6.8, CH_3), 4.57 (1H, m, CH), 4.66 (2H, d, J = 6.1, CH_2), 7.19 (1H, tt, J = 7.2, 1.7, H-p), 7.27 (2H, dd, J = 7.2, H-m), 7.34 (2H, dd, J = 7.2, 1.7, H-o), 7.69 (1H, s, H-C^8). Anal. ($C_{15}H_{16}N_5Cl$) C, H, N.

Reaction of 2-Chloro Derivatives of Purine with Alkylamines: Preparation of 2,6-Bis(alkylamino)-9-alkylpurines, 6-Amino-2-(alkylamino)-9-alkylpurines, and 6-Amino-2-(alkylamino)purines. 6-(Alkylamino)- or 6-amino-2-chloro-9-alkylpurine or 6-amino-2-chloropurine (0.5 mmol) and 3 mL of the appropriate amine were heated for 3 h to 155−160 °C (sealed ampule). Excess of the amine was evaporated at a temperature below 60 °C, and the residue was purified by column chromatography or (and) crystallized.

5: decolorized and crystallized from water; yield 51%; mp 259−260 °C (differs from that given in the literature, 247−248 °C or over 300 °C[8]); likewise UV λ_{max} (pH 1) 245 (sh), 289, (pH 13) 290 disagree with previously described results,[8] i.e., water (pH 1.1) 249, 298, water (pH 12.4) 263 (sh), 286; MS and NMR spectra are in accordance with the proposed structure; MS (AEI-MS 902) 194 (M^+, 21),163 (100), 150 (26), 134 (42), 121 (11); ^{1}H NMR (270 MHz, DMSO) 3.30 (2H, t, J = 6.0, CH_2), 3.48 (2H, t, J = 6.0, CH_2), 4.67 (1H, br s, exch with D_2O, OH), 6.02 (1H, t, J = 5.0, exch with D_2O, NH), 6.64 (2H, s, exch with D_2O, NH_2), 7.67 (1H, s, H-8), 12.14 (1H, br s, exch with D_2O, H-9). Anal. ($C_7H_{10}N_6O\cdot0.5H_2O$) C, H, N.

8: crystallized from water; yield 87%; mp 206−208 °C; UV λ_{max} (pH 1) 216, 254, 297, (pH 13) 221, 255, 287; MS (AEI-MS 902): 208 (M^+, 21), 177 (100), 164 (26), 148 (44), 42 (32); ^{1}H NMR (270 MHz, DMSO) 3.33 (5H, m, $CH_2 + NCH_3$), 3.51 (2H, t, J = 5.9, CH_2), 4.734 (1H, br s, exch with D_2O, OH), 6.11 (1H, t, J = 5.6, exch with D_2O, NH), 6,69 (2H, s, exch with D_2O, NH_2), 7.66 (1H, s, H-8). Anal. ($C_8H_{12}N_6O\cdot1H_2O$) C, H, N.

17: column chromatography stepwise 0, 2, 4% MeOH in $CHCl_3$ with a trace of concentrated NH_4OH; crystallized from $CHCl_3$−cyclohexane; yield 91%; mp 130−131 °C; UV λ_{max} (pH 1) 251, 294, (pH 13) 260, 288.5; MS (AEI-MS 902) 276 (M^+, 39), 261 (13), 245 (27), 233 (22), 189 (26), 178 (18), 177 (100), 148 (32), 69 (12), 41 (43); ^{1}H NMR (400 MHz, $CDCl_3$) 1.71 (3H, d, J = 1.2, CH_3), 1.73 (3H, dt, J = 1.2, 1.2, CH_3), 2.01 (1H, br s, OH), 3.58 (2H, m, NC**H**$_2$$CH_2O$), 3.64 (3H, s, N^9-$CH_3$), 3.83 (2H, m, OC**H**$_2$$CH_2$N), 4.10 (2H, br s, CH_2), 5.29 (1H, t, J = 5.4, N**H**CH_2), 5.33 (1H, dqq, J = 7.2, 1.2, 1.2, -CH=), 5.55 (1H, br s, N-H), 7.40 (1H, s, -CH=). Anal. ($C_{13}H_{20}N_6O$) C, H, N.

19: column chromatography stepwise 0, 2, 3, 4% MeOH in $CHCl_3$ with a trace of concentrated NH_4OH; crystallized from benzene; yield 75%; mp 130−31 °C. UV λ_{max} (pH 1) 254, 294.5, (pH 13) 288; MS (Jeol DX 303, direct inlet) 304 (M^+, 56), 273 (64), 260 (7), 221 (57), 208 (24), 203 (25), 191 (29), 177 (80), 164 (39), 148 (57), 133 (20), 121 (21), 106 (14), 79 (12), 67 (15), 55 (100); ^{1}H NMR (200 MHz, $CDCl_3$) 0.95−1.30 (5H, m, C_6H_{11}), 1.50−1.90 (6H, m, C_6H_{11}), 3.32−3.45 (2H, br s, $CH_2C_6H_{11}$), 3.55 (2H, m, CH_2CH_2NH), 3.64 (3H, s, CH_3N^9), 3.828 (3H, J = 4.2, CH_2OH), 5.25 (1H, br t, NHC^2), 5.65 (1H, br s, NH), 7.403 (1H, HC^8). Anal. ($C_{15}H_{24}N_6O$) C, H, N.

21: column chromatography toluene−MeOH (85:15): after hydrolysis of tetrahydropyranyl group (MeOH−2 N HCl, 10:1, 8 h, ambient temperature) crystals from reaction mixture; yield 65%; mp 180−190 °C; MS (EEI-MS 902) 328 (22, M^+), 310 (10, M − H_2O), 298 (26, M − CH_2O), 284 (18, M − C_2H_4O), 254 (7, 298 − C_2H_4O), 240 (5, 284 − C_2H_4O), 106 (10, pHCH=NH_2^+), 91 (100, C_7H_7), 65 (7, C_5H_5); ^{1}H NMR (400 MHz, D_2O) COSY [3.694 (2H, br s, C**H**$_2$NHC^2), 3.561 (2H, t, J = 5.4, C**H**$_2$$CH_2$NHC^2)], COSY [3.912 (2H, t, J = 5.0, CH_2N^9), 4.257 (2H, t, J = 5.0, C**H**$_2$$CH_2$N^9)], 4.735 (2H, br s, C**H**$_2$Ph), COSY [7.316 (1H, m, Ph), 7.356−7.368 (4H, m, Ph)], 8.008 (1H, br s, H-C^8). Anal. ($C_{14}H_{14}N_5ClO\cdot0.25H_2O$) C, H, N.

22: column chromatography stepwise 0, 1, 2, 4% MeOH in $CHCl_3$; crystallized from $CHCl_3-Et_2O$; yield 71%; mp 142−143 °C; ^{1}H NMR (200 MHz, $CDCl_3$) 1.535 (6H, d, J = 7.0 Hz, (C**H**$_3$)$_2$CH), 3.53−3.60 (2H, m, C**H**$_2$NHC^2), 3.83 (2H, t, J = 4.2, CH_2O), 3.61 (1H, hept, J = 7.0, C**H**$(CH_3)_2$), 3.75 (2H, br t, CH_2Ph), 5.20 (1H, br s, OH or NH), 5.26 (1H, br t, NHC^2), 6.00 (1H, br s, NH or OH), 7.23−7.36 (5H, m, Ph), 7.48 (1H, s, HC^8). Anal. ($C_{17}H_{22}N_6O$) C, H, N.

23: column chromatography stepwise 0, 1, 2, 4% MeOH in $CHCl_3$; crystallized from $CHCl_3$−heptane; yield 73%; mp 144−146 °C; MS (AEI-MS 902) 340 (20, $M^+ + 1$), 295 (60), 282 (15), 253 (10), 106 (11), 91 (100); ^{1}H NMR (200 MHz, $CDCl_3$) 1.213 (3H, d, J = 6.4, C**H**$_3$,CHOH), 1.535 (6H, d, J = 6.8, (C**H**$_3$)$_2$-CH), 3.4 (2H, m, CH_2NHC^2), 4.03 (1H, m, CHO), 4.62 (1H, hept, J = 6.8, C**H**$(CH_3)_2$), 4.75 (2H, br d, CH_2Ph), 5.20 (1H, NHC^2), 5.95 (1H, NH). Anal. ($C_{18}H_{24}N_6O$) C, H, N.

24: column chromatography stepwise 0, 1, 2, 4% MeOH in $CHCl_3$; crystallized from $CHCl_3-Et_2O$; yield 69%; mp 137−139 °C; MS(Finnigan MAT 90) 354.2167 (M^+, $C_{19}H_{26}N_6O$, calcd 354.2168, 27), 325 (7), 324 (29), 323 (100), 295 (3), 282 (7), 281 (3), 217 (6), 185 (5), 134 (3), 106 (3), 91 (34); ^{1}H NMR (200 MHz, $CDCl_3$) 1.018 (3H, t, J = 6.8, C**H**$_3$$CH_2$), 1.522 (6H, d, J = 6.6, (C**H**$_3$)$_2$CH), 1.58 (2H, m, C**H**$_2$$CH_3$), 3.62 (1H, m, C**H**NH), 3.83 (2H, m, CH_2OH), 4.59 (1H, hept, J = 6.8, C**H**$(CH_3)_2$), 4.75 (2H, br s, CH_2Ph), 4.9 (1H, br d, J = 6.0, NHC^2), 5.15 (1H, br s, OH or NH), 6.07 (1H, br s, NH or OH), 7.22−7.36 (5H, m, Ph), 7.23 (1H, s, HC^8). Anal. ($C_{19}H_{26}N_6O$) C, H, N.

25: for purification see **24**; yield 64%; mp 118−120 °C; $[\alpha]_D$ = +35.3 (c = 0.57, $CHCl_3$). Anal. ($C_{19}H_{26}N_6O$) C, H, N.

26: for purification see **24**; yield 62%; mp 102−104 °C; $[\alpha]_D$ = −34.6 (c = 0.43, $CHCl_3$). Anal. ($C_{19}H_{26}N_6O$) C, H, N.

Alkylation of 2,6-Substituted Purines at Position 9 ((*n*-Bu)$_4$NOH method). 6-Amino-2-chloropurine or 2-amino-6-(benzylamino)purine (1 mmol), 40% aqueous tetrabutylammonium hydroxide (1 mL), methyl iodide (1.2 mmol), and 6 mL of dichloromethane were vigorously shaken for 1 h. After 10 min all the components had dissolved and the product started to precipitate. It was filtered and washed with dichloromethane. Crystallization from 1 N HCl afforded the appropriate hydrochloride, or after alkalization with NH_4OH the free base was prepared.

7: hydrochloride; yield 46%; mp over 330 °C; UV λ_{max} (pH 1) 267, (pH 13) 265; TLC 1 M solution of NH_3 in $CHCl_3$−MeOH (95:5), one spot R_f 0.3 identical with the sample prepared from 2,6-dichloro-9-methylpurine according to ref 8. Anal. ($C_6H_6N_5Cl\cdot HCl\cdot0.5H_2O$) C, H, N.

14: free base; yield 30%; mp 214−216 °C; UV λ_{max} (pH 1) 252, 288, (pH 13) 260 (sh), 282; MS (AEI-MS 902) 254 (M^+, 100), 149 (65), 106 (64), 91 (46), 65 (17), 42 (30); ^{1}H NMR (270 MHz, DMSO) 3.36 (3H, br s, N-CH_3), 4.66 (2H, br s, CH_2), 5.91 (2H, br s, NH_2), 7.17−7.36 (5H, mt, Ar), 7.68 (1H, s, H-8), 8.00 (1H, t, NH). Anal. ($C_{13}H_{14}N_6\cdot1H_2O$) C, H, N.

p34^{cdc2}/Histone H$_1$ Kinase Assay. p34^{cdc2}/cyclin B was purified from M phase oocytes of the starfish *Marthasterias glacialis* by affinity chromatography on p^{9CKhs1}-Sepharose beads,[17] from which it was eluted by free p^{9CKhs1}. The cdc2 assay mixture contained 10 μL of histone H_1 (1 mg/mL), 10 μL of [γ-^{32}P]ATP (15 μmol, 3000 Ci/mmol, 1 mCi/mL), and 10 μL of the inhibitor (0.1−1000 μmol), all in reaction buffer C (60 mmol of β-glycerolphosphate, 15 mmol of p-nitrophenyl phosphate, 25 mmol of MOPS, pH 7.2, 5 mmol of EGTA, 15 mmol of $MgCl_2$, 1 mmol of dithiothreitol, 1 mmol of sodium vanadate, 1 mmol of phenyl phosphate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, 100 μmol of benzamidine). For determination of maximum phosphate incorporation, buffer C was used instead of inhibitor. Nonspecific binding was determined in the absence of histone H_1 in the reaction mixture and substracted from each volume. The assays were started by addition of radioactive ATP, and after 10 min incubation at 30 °C, 25 μL aliquots of the supernatant were spotted onto 2.5 × 3.0 cm pieces of Whatman P81 phosphocellulose paper. After 20 s, the filters were washed five times (for at least 5 min each time) in 0.1% phosphoric acid. The wet filters were transferred into 5 mL of ACS scintillation cocktail (Amersham), and after mixing, ^{32}P radioactivity was determined using a Packard Tri-Carb

counter. Control analyses were also performed with appropriate dilutions of DMSO because the inhibitors were dissolved in DMSO as 100 mM stock solutions. However, the final DMSO concentration in the reaction mixture never exceeded 1%. The kinase activity is expressed as pmol of phosphate groups incorporated in histone H_1 during a 10 min incubation. Dose−response curves were drawn for every compound tested and used for calculating IC_{50}'s (for example, see Figure 1). All assays were carried out in triplicate.

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References

(1) (a) Letham, D. S.; Palni, L. M. S. The Biosynthesis and Metabolism of Cytokinins. *Annu. Rev. Plant. Physiol.* **1983**, *34*, 163−167. (b) Skoog, F.; Hamzi, H. G.; Szweykowska, A. M.; Leonard, N. J.; Carraway, K. L.; Fujii, T.; Helgeson, J. P.; Loeppky, R. N. Cytokinins: Structure/Activity Relationships. *Phytochemistry* **1967**, *6*, 1169.

(2) (a) Waliszewska-Wojtkowiak, B.; Schneider, J.; Sweykowska, A. Effect of Kinetin on the Transcription Activity of Chromatin from Cucumber Cotyledons. *Biochem. Physiol. Pflanz.* **1985**, *180*, 413−422. (b) Selivankina, S. Y.; Romanko, E. G.; Ovhcarov, A. K.; Kharchenko, V. I. Involvement of Cytokinin-Binding Proteins from Barley Leaves in Action by Cytokinin of Chromatin Bound RNA-polymerase. *Plant Physiol.* (*Moscow*) **1985**, *29*, 274−281. (c) Jackowski, G.; Jarmolowski, A.; Sweykowska, A. Kinetin modifies the secondary structure of poly(A)RNA in cucumber cotyledons. *Plant Sci.* **1987**, *52*, 67−72. (d) Gwózdz, E. A.; Wozny, A. Cytokinin-controlled polyribosome formation and protein synthesis in cucumber cotyledons. *Physiol. Plant.* **1983**, *59*, 103−110

(3) (a) Fosket, D. The Regulation of the Plant Cell Cycle by Cytokinins. In Mechanism and Control of Cell Division; Ross, T. L., Gifford, E. M., Eds.; Dowden Hutchinson and Ross: Strasbourg, PA, 1977; pp 66−87. (b) Housa, C.; Jacqmard, A.; Bernier, G. Activation of replication origins as a possible target for cytokinins in shoot meristems of Sinapis. *Planta* **1990**, *181*, 324−332. (c) Ferreira, P. C. G.; Hemerly, A. S.; Villarroel, R.; Van Montagu, M.; Inzé, D. The Arabidopsis functional homolog of the $p34^{cdc2}$ protein kinase. *Plant Cell* **1991**, *3*, 1531−540.

(4) (a) Hemerly, A. S.; Ferreira, P.; Engler, J. A.; Van Montagu, M.; Engler, G.; Inzé, D. cdc2a Expression in Arabidopsis is linked with competence for cell division. *Plant Cell* **1993**, *5*, 1711−1723. (b) Yakovleva, L. A.; Kulaeva, O. N. The effect of phytohormones on phosphorylation of ribosomal proteins in detached pumpkin cotyledons. *Biochem. Physiol. Pflanz.* **1987**, *189*, 359−365. (c) Selivankina, S. Y.; Romanko, E. G.; Novikova, G. V.; Murontseva, D. G.; Kulaeva, O. N. Effect of cytokinins and other phytohormones on protein kinases associated with chromatin and RNA polymerase I in barley leaves. *Plant Physiol.* (*Moscow*) **1988**, *35*, 205−212. (d) Rialet, V.; Meijer, L. A new Screening Test for Antimitotic Compounds Using the Universal M Phase-Specific Protein Kinase, $p34^{cdc2}$/cyclin B^{cdc13}, Affinity-Immobilized on $p13^{suc1}$-coated microtitration plates. *Anticancer Res.* **1991**, *11*, 1581−1590.

(5) Veselý, J.; Havlíček, L.; Strnad, M.; Blow, J. J.; Donella-Deana, A.; Pinna, L.; Letham, D. S.; Kato, J. Y.; Detivaud, L.; Leclerc, S.; Meijer, L. Inhibition of cyclin-dependent kinases by purine analogues. *Eur. J. Biochem.* **1994**, *224*, 771−786.

(6) (a) Nurse, P. Universal Control Mechanism Regulating onset of M-phase. *Nature* **1990**, *344*, 503. (b) Pines, J.; Hunter, T. $p34^{cdc2}$: the S and M kinase? *New Biol.* **1990**, *2*, 389. (c) McGowan, C. H.; Russell, P.; Reed, S. I. Periodic biosynthesis of the human M-phase promoting factor catalytic subunit p34 during the cell cycle. *Mol. Cell. Biol.* **1990**, *10*, 3847−3851. (d) Draetta, G. Cell cycle control in eukaryotes: molecular mechanisms of cdc2 activation. *Trends Biochem. Sci.* **1990**, *15*, 378−379. (e) Solomon, M. J.; Glotzer, M.; Lee, T. H.; Philippe, M.; Kirschner, M. W. Cyclin activation of $p34^{cdc2}$. *Cell* **1990**, *63*, 1013−1017. (f) Reed, S. I. The Role of p34 kinases in the G_1 to S-phase transition. *Annu. Rev. Cell Biol.* **1992**, *8*, 529−543.

(7) (a) Fujii, T.; Itaya, T.; Saito, T. Purines. XVII. Kinetic Studies of the Base-catalyzed Conversion of 1-Alkyladenosines into N-Alkyladenosines: Effect of Substituents on the Rearrangement Rate. *Chem. Pharm. Bull.* **1975**, *23*, 54−61. (b) Kunieda, T.; Witkop, B. Selective N-Methylations of Heterocycles with Dimethyloxosulfonium Methylide. *J. Org. Chem.* **1970**, *35*, 3981−3982. (c) Fujii, T.; Saito, T.; Inoue, I.; Kumazawa, Y.; Tamura, K. Purines xxx. Ring Fission of 3,7-Dialkyladenines by Alkaline Hydrolysis. *Chem. Pharm. Bull.* **1988**, *36*, 107−117.

(8) (a) Roitshtein, L. M.; Muravich-Aleksander, Kh. L.; El'tsov, A. V. Synthetic Analogs of Kinetin. *Zh. Obshch. Khim.* **1969**, *39*, 2125−2129. (b) Cresswel, R. M.; Strauss, T.; Brown, G. B. Some 2-Substituted Aminopurines and Purine Analogs. *J. Med. Chem.* **1963**, *6*, 817−819.

(9) *CRC Handbook of Biochemistry*, 2nd ed.; The Chemical Rubber Co.: Boca Raton, FL, 1970; pp G23, G36.

(10) Schulze-Gahmen, U.; Brandsen, J.; Jones, H. D.; The Chemical Rubber Co.: Boca Raton, FL, 1970; pp G23, G36.

(11) Skoog, F.; Strong, F. M.; Miller, C. O. Cytokinins. *Science* **1965**, *148*, 532−536.

(12) Abraham, R. T.; Acquarone, M.; Andersen, A.; Asensi, A.; Bellé, R.; Berger, F.; Bergounioux, C.; Brunn, G.; Buquet-Fagot, C.; Fagot, D.; Glab, N.; Goudeau, H.; Goudeau, M.; Guerrier, P.; Houghton, P. J.; Hendriks, H.; Kloareg, B.; Lippa, M.; Marie, D.; Maro, B.; Meijer, L.; Mester, J.; Mulner-Lorillon, O.; Poulet, S. A.; Shierenberg, E.; Schutte, B.; Vaulot, D.; Verlhac, M. H. Cellular effects of olomoucine, an inhibitor of cyclin dependent kinases. *Biol. Cell* **1995**, *83*, 105−120.

(13) (a) Hedayatullah, M. Les systèmes biophasiques. 3. Alkylation des purines en catalyse par transfert de phase. *J. Heterocycl. Chem.* **1982**, *19*, 249−251. (b) Letham, D. S.; Summons, R. E.; Entsch, B.; Gollnow, B. I.; Parker, Ch. W.; MacLeod, J. K. Glucosylation of Cytokinin Analogues. *Phytochemistry* **1978**, *17*, 2053−2057. (c) Parker, Ch. W.; Entsch, B.; Letham, D. S. Inhibitors of Two Enzymes which Metabolize Cytokinins. *Phytochemistry* **1986**, *25*; 303−310. (d) Hocart, C. H.; Letham, D. S.; Parker, C. W. Substituted Xanthines and Cytokinin Analogues as Inhibitors of Cytokinin N-Glucosylation. *Phytochemistry* **1991**, *30*, 2477−2486. (e) Monsees, T.; Meijer, L.; Jastorff, B. 8-Dimethylamino-1-methyladenine, a Novel Potent Antagonist of the 1-Methyladenine Receptor in Starfish Oocytes. *Eur. J. Biochem.* **1993**, *213*, 155−165. (f) Skoog, F.; Hamzi, H. Q.; Szweykowska, A. M.; Leonard, N. J.; Carraway, K. L.; Fujii, T.; Helgeson, J. P.; Loeppky, R. N. Cytokinins: Structure/Activity Relationship. *Phytochemistry* **1967**, *6*, 1169−1192.

(14) Brown, G. B.; Weliky, V. S. 2-Chloradenine and 2-Chloradenosine. *J. Org. Chem.* **1958**, *23*, 125−126.

(15) Falconer, R.; Gulland, J. M.; Story, L. F. The Constitution of Purine Nucleosides. Part IX. Carotonoside. *J. Chem. Soc.* **1939**, 1784−1787.

(16) Leonard, E. O.; Skinner, Ch. G.; Lansford, E. M., Jr.; Shive, W. Synthesis and Biological Activity of Some 2-Amino-6-(substituted)-purines. *J. Am. Chem. Soc.* **1959**, *81*, 907−909.

(17) (a) Baratte, B.; Meijer, L.; Galaktionov, K.; Beach, D. Screening for antimitotic compounds using the cdc 25 tyrosine phosphatase, an activator of the mitosis-inducing $p34^{cdc2}$/cyclin B^{cdc13} protein kinase. *Anticancer Res.* **1992**, *12*, 873−880. (b) Azzi, L.; Meijer, L.; Reed, S. I.; Pidikiti, R.; Tung, H. Y. L. Interaction between the cell-cycle-control proteins $p34^{cdc2}$ and $p9^{CKShs2}$. Evidence for two cooperative binding domains in $p9^{CKShs2}$. *Eur. J. Biochem.* **1992**, *203*, 353−360. (c) Rialet, V.; Meijer, L. A new Screening Test for Antimitotic Compounds Using the Universal M Phase-Specific Protein Kinase, $p34^{cdc2}$/cyclin B^{cdc13}, Affinity-Immobilized on $p13^{suc1}$-coated microtitration plates. *Anticancer Res.* **1991**, *11*, 1581−1590.

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