Bioorganic & Medicinal Chemistry 16 (2008) 9268–9275

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Novel potent inhibitors of *A. thaliana* cytokinin oxidase/dehydrogenase

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ARTICLE INFO

Article history:
Received 23 April 2008
Revised 28 August 2008
Accepted 4 September 2008
Available online 7 September 2008

Keywords:
Cytokinins
6-Anilinopurines
Cytokinin oxidase/dehydrogenase

ABSTRACT

The synthesis of a new group of 2-X-6-anilinopurines, including compounds with potential cytokinin-like activities, with various substitutions (X = H, halogen, amino, methylthio or nitro) on the phenyl ring is described. The prepared compounds have been characterized using standard physico-chemical methods, and the influence of individual substituents on biological activity has been compared in three different bioassays, based on the stimulation of tobacco callus growth, retention of chlorophyll in excised wheat leaves and the dark induction of betacyanin synthesis in *Amaranthus* cotyledons. The biological activity of the prepared compounds was also assessed in receptor assays, in which the ability of the compounds to activate the cytokinin receptors AHK3 and AHK4/CRE1 was studied. Finally, the interactions of the compounds with the *Arabidopsis* cytokinin oxidase/dehydrogenase AtCKX2 (heterologously expressed) were investigated. Systematic testing led to the identification of two very potent inhibitors of AtCKX2: 2-chloro-6-(3-methoxyphenyl)aminopurine and 2-fluoro-6-(3-methoxyphenyl)aminopurine.

1. Introduction

Adenine derivatives, bearing various substituents at N^6-position, are an important group of biologically active molecules which can be used in both medicine and plant biotechnology.[1,2]

Systematic testing of purines with different substituents in various assays, including receptor tests, has recently led to several general conclusions regarding structure/activity relationships of the plant hormones cytokinins.[1,2] The chemical nature of the substituents and their positions influence their recognition as ligands by specific receptors and/or as substrates of degradative enzymes (histidine kinase receptors and cytokinin oxidase/dehydrogenase, respectively, in this context). Cytokinin dehydrogenase (CKX) is involved in the regulation of endogenous cytokinin contents in plants by oxidative removal of the side chain and seven distinct genes, AtCKX1 to AtCKX7, encode the enzyme in *Arabidopsis thaliana*.[3] Isolation of the pure protein is difficult because of its extremely low concentration in plant tissues. However, the CKX2 gene from *A. thaliana*[4] has recently been cloned in yeast *S. cerevisiae*, allowing us to obtain large amounts of homogenous, active protein from the yeast culture media, and to test the ability of a library of novel 6-anilinopurine derivatives to inhibit the activity of the enzyme.

Here, we describe the preparation of a series of new 2-X-6-anilinopurines (X = H, halogen, amino, methylthio or nitro) with various substitutions on the phenyl ring. Both their AtCKX2 inhibition and other potential cytokinin-like activities were examined. These compounds were prepared via condensation of 2-substituted-6-chloropurines with corresponding anilines (S_N2 mechanism) and characterized by standard physico-chemical methods. The synthesis and subsequent purification of the 6-anilinopurines was sometimes tedious, in comparison to purifying corresponding 6-benzylaminopurines, due to the relatively low nucleophilicity of the anilines and large amounts of by-products arising from side reactions. Due to the structure similarity to aromatic cytokinins, the prepared derivatives were tested for their biological activity in classical cytokinin bioassays. Subsequently, the influence of individual substituents on the activation of cytokinin receptors (AHK3, CRE1/AHK4) and on the interaction with the key enzyme involved in cytokinin degradation in *A. thaliana* (cytokinin oxidase/dehydrogenase 2, AtCKX2) was also examined. This screening led to the discovery that 2-chloro-6-(3-methoxyphenyl)aminopurine and 2-fluoro-6-(3-methoxyphenyl)aminopurine are highly potent AtCKX2 inhibitors with decreased ability to activate cytokinin receptors.

2. Results and discussion

2.1. Chemistry

Nineteen substituted 6-anilinopurines (Table 1) were prepared according to the synthetic procedures outlined above. The identity and purity of the prepared compounds were verified by ^{1}H NMR, mass spectrometry (chemical ionization), HPLC-UV and elemental analysis (Table 2b). Synthesis of some of the substituted 6-anilinopurines has been previously described.[5–9] The parent molecule,

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doi:10.1016/j.bmc.2008.09.008

Table 1
Structure and abbreviations of prepared compounds

Compound	R	R1	R2	R3	R4	R5	R6
1	H	H	H	H	H	H	H
2	Cl	H	H	H	H	H	H
3	Cl	H	Cl	H	H	H	H
4	Cl	H	H	Cl	H	H	H
5	Cl	H	F	H	H	H	H
6	Cl	H	H	F	H	H	H
7	Cl	OH	H	H	H	H	H
8	Cl	H	OH	H	H	H	H
9	Cl	H	H	OH	H	H	H
10	Cl	OCH_3	H	H	H	H	H
11	Cl	H	OCH_3	H	H	H	H
12	Cl	H	H	OCH_3	H	H	H
13	H	H	OCH_3	H	H	H	H
14	F	H	OCH_3	H	H	H	H
15	NO_2	H	OCH_3	H	H	H	H
16	NH_2	H	OCH_3	H	H	H	H
17	CH_3S	H	OCH_3	H	H	H	H
18	Cl	H	OCH_3	H	H	H	CH_3
19	Cl	H	OCH_3	H	H	H	Isopropyl

2-chloro-6-anilinopurine (**2**), previously prepared by Thompson et al. by condensation of 2,6-dichloropurine with aniline in *n*-propanol at 100 °C in the presence of *N*,*N*-diisopropylethylamine as an auxiliary base,[5] is an important intermediate for the synthesis of 2,6,9-trisubstituted purines, which have been found to be potent CDK inhibitors.[10] Unsubstituted 6-anilinopurine (**1**) was first synthesized (by Elion et al.[7]) by direct reaction of 6-methylsulfanylpurine and aniline at a higher temperature and later (by Daly et al.[6]) by condensation of 6-chloropurine with aniline without either solvent or auxiliary base. In our study, both anilinopurines were prepared using a slightly different method, by reacting 6-chloro or 2,6-dichloropurine with aniline in *n*-propanol in the presence of *N*,*N*-diisopropylethylamine at a somewhat lower temperature (90 °C), for a prolonged time (2 days) with very good yield (84.9%) and satisfactory purity for testing biological activity. Both of these anilinopurines were tested for CKX inhibitory activity, but were found to be almost inactive (Table 3).

2-Chloro-6-(x-halogenoanilino)purines (**3**–**6**) were prepared using a similar method to that described by Imbach et al.,[9] reacting corresponding halogenoanilines with 6-chloropurine in dimethylformamide at 80–100 °C in the presence of triethylamine as a base. 2-Chloro-6-(*x*-halogenoanilino)purines have been previously used as intermediates for preparing very potent CDK inhibitors of the purvalanol type, with strong anti-proliferative properties.[9,11] Using this approach we successfully prepared chloro- and fluoro-anilinopurines in *meta*- and *para*-positions, while attempts to prepare *ortho*-derivatives failed (these compounds have not yet been described in the literature to date). The synthesis, which was carried out in *n*-propanol or *n*-butanol at 90 °C under a nitrogen atmosphere, was complicated by the large amounts of impurities that arose from side reactions of halogenoanilines. Furthermore, purification by crystallization or flash chromatography of the crude products, especially 6-(3-fluoroanilino) and 6-(3-chloroanilino)-purines, was also essential in these cases.

Two methoxyanilinopurines have also been synthesised and used as precursors for the synthesis of CDK inhibitors. Imbach et al.[9] synthesised 2-chloro-6-(3-methoxyanilino)purine (**11**) in *n*-pentanol without use of tertiary amine, while Qu et al.[12] very recently prepared the corresponding *para*-isomer (**12**), by reacting 2,6-dichloropurine with *para*-anisidine without use of either solvent or auxiliary base. Preparation of 2-chloro-6-(3-methoxyanilino)purine using dimethylformamide as solvent and triethylamine as a base, at reaction temperatures of 80–100 °C, has also been previously described,[8] but the yield was low (56%). On the other hand, synthesis of the corresponding *ortho*-isomer has not been previously reported. We synthesised all three isomers as hydrochlorides by reacting 2,6-dichloropurine and the corresponding anisidine in refluxing *n*-propanol with high yields (80–90%) and high purity (>98% HPLC). Free bases were obtained from hydrochlorides by treatment with diluted aqueous ammonia. The same method was used to prepare all three isomers of hydroxyanilinopurines (**7**–**9**), which have not been previously reported in the literature; although the yields were somewhat lower compared with the previous case.

Compounds **14**–**19** (see Table 1) have also not been previously described. We prepared them using a conventional method (see

Table 2a
Detailed reaction conditions

Compound	Starting purine	Base	Solvent	Temperature (°C)	Time (h)	Auxiliary base	Yield (%)/HPLC purity (%)
1	*A*	*a*	*n*-Propanol	90	20	DIPEA	
2	*B*	*a*	*n*-Propanol	98–100	4	DIPEA	84.9/98.0+
3	*B*	*b*	*n*-Butanol	110	3	Triethylamine	65.8/98.0+*
4	*B*	*c*	*n*-Propanol	98–100	4	DIPEA	65.0/98.0+
5	*B*	*d*	*n*-Butanol	100–110	3	Triethylamine	69.6/98.0+*
6	*B*	*e*	*n*-Butanol	100–110	3	Triethylamine	19.0/98.0+**
7	*B*	*f*	*n*-Butanol	90	3	Triethylamine	75.3/98.0+
8	*B*	*g*	*n*-Butanol	90	4	DIPEA	84.1/98.0+
9	*B*	*h*	*n*-Butanol	90	4	DIPEA	83.0/99.0+
10	*B*	*i*	*n*-Butanol	90	4	DIPEA	64.0/98.0+
11	*B*	*j*	*n*-Pentanol	100	4	Triethylamine	77.8/98.0+
12	*B*	*k*	*n*-Butanol	100	4	Triethylamine	88.8/99.0+
13	*A*	*j*	*n*-Butanol	110	3	Triethylamine	82.9/98.0+
14	*C*	*j*	*n*-Propanol	90	4	Triethylamine	61.0/99.0+
15	*D*	*j*	(1) *n*-Propanol(2) Methanolic HCl(3) 3% NH_4OH	(1) -5–0; then rt(2) 50(3) rt	(1) 12(2) 12(3) 1	—	34.9/99.0+
16	*E*	*j*	*n*-Propanol	98–100	3	—	85.0/99.0+ (as HCl salt)
17	*F*	*j*	*n*-Propanol	98–100	4	—	69.0/99.0+ (as HCl salt)
18	*G*	*j*	*n*-Propanol	98–100	4	—	46.0/99.0 (as HCl salt)
19	*H*	*j*	*n*-Propanol	90	4	—	80.0/98.7 (as HCl salt)

A, 6-chloropurine; *B*, 2,6-dichloropurine; *C*, 2-fluoro-6-chloropurine; *D*, 2-nitro-6-chloropurine; *E*, 2-amino-6-chloropurine; *F*, 2-methylthio-6-chloropurine; *G*, 2,6-dichloro-9-methylpurine; *H*, 2,6-dichloro-9-isopropylpurine; *a*, aniline; *b*, 3-chloroaniline; *c*, 4-chloroaniline; *d*, 3-fluoroaniline; *e*, 4-fluoroaniline; *f*, 2-aminophenol; *g*, 3-aminophenol; *h*, 4-aminophenol; *i*, 2-anisidine; *j*, 3-anisidine; *k*, 4-anisidine; DIPEA, *N*,*N*-diisopropyl-*N*-ethyl amine; rt, room temperature.

* Purified by flash chromatography.

** Purified by crystallization from 2-propanol.

Table 2b
Chemical characteristics of prepared compounds

Compound	Melting points (°C)	Melting points (°C)—literature	MS-CI	Calculated%			Found%		
			$(M+H)^+$	C	H	N	C	H	N
1	279	279–282[6]	211(EI)	62.5	4.3	33.2	62.1	4.2	32.9
2	320	320[19]	246	53.8	3.3	28.5	53.4	3.3	28.3
3	>350	Not given[9]	280	47.0	2.9	24.9	46.8	2.9	24.6
4	>350	—	280	47.0	2.9	24.9	46.9	3.0	24.7
5	>350	Not given[8,9]	264	50.1	2.7	26.6	49.9	3.0	26.5
6	>350	Not given[9]	264	50.1	2.7	26.6	49.9	2.9	26.0
7	>350	—	262	50.5	3.1	26.8	49.9	3.1	26.7
8	>350	—	262	50.5	3.1	6.8	49.8	3.1	26.8
9	>350	—	262	50.5	3.1	26.8	50.5	3.1	26.8
10	258	—	276	52.3	3.7	25.4	51.9	3.8	25.5
11	238	Not given[8,9]	276	52.3	3.7	25.4	51.8	3.5	25.7
12	>300	Not given[20]	276	52.3	3.7	25.4	52.0	3.7	25.2
13	226	—	242	59.7	3.7	29.0	59.4	3.5	29.0
14	195	—	260	55.6	3.9	27.0	55.2	3.9	26.8
15	280	—	287	50.3	3.5	29.4	50.0	3.6	29.1
16	>300	—	257	56.2	4.7	32.8	56.1	4.7	32.4
17	261	—	288	54.3	4.6	24.4	54.1	4.6	24.2
18	225	—	290	53.9	4.2	24.2	53.3	4.3	23.9
19	>300	—	318	56.7	5.1	22.0	56.2	5.0	21.8

Table 3
Biological activity of novel 6-anilinopurines: activation of cytokinin receptors AHK4/CRE1 and AHK3 expressed as EC_{50} values, and inhibition of recombinant AtCKX2 as IC_{50} values

Compound	EC_{50} (μM)		IC_{50} (μM)
	CRE1/AHK4	AHK3	AtCKX2
1	na	>50	ni
2	na	>50	80
3	7.5	10.7	3.9
4	>50	15.1	25.4
5	24.8	6	7.5
6	na	50	50
7	>50	19	>100
8	3.75	13	3.75
9	4.6	16.5	>100
10	na	33	>100
11	7	21	1.9
12	na	33.1	>100
13	31	2.3	12.4
14	19	19	1
15	na	na	14
16	na	na	27
17	na	na	>100
18	na	na	61
19	na	na	>100
tZ	1.1	0.7	ni
TDZ	1	0.95	29

The natural cytokinin *trans*-zeatin and synthetic cytokinin thidiazuron were used as controls. na, no activation; ni, no inhibition.

text above) from corresponding precursors (2-fluoro-, 2-nitro-, 2-amino and 2-methylthio-6-chloropurines). Substances **18** and **19** were prepared from *m*-anisidine and 2,6-dichloro-9-methylpurine or 2,6-dichloro-9-isopropylpurine, respectively. These precursors were prepared via alkylation of 2,6-dichloropurine by methyl iodide and isopropylbromide, respectively, in alkaline media in a polar aprotic solvent (dimethylformamide or dimethylsulfoxide) as previously described.[25,26]

2-Fluoro-6-chloropurine was synthesised from 2-amino-6-chloropurine by diazotization using *t*-butyl nitrite and subsequent Schiemann reaction of the diazonium salt with tetrafluoroboric acid in dimethylformamide at −15 °C (modified method according to Ref. [22,23]) with satisfactory yield (65%). Our method eliminates the previous requirement for tedious separation of the desired product from large amounts of inorganic salts,[22] and purification of the crude product by column chromatography. Using this method we prepared one of the strongest CKX inhibitors obtained in this study; 2-fluoro-6-(3-methoxyanilino)purine (compound **14**) (Table 3). This compound is also a very important precursor for the solid phase synthesis of 2,6,9-trisubstituted purine CDK inhibitors,[23] because of the high reactivity of the C2-position, activated by the fluorine atom.

2-Nitro-6-chloropurine is a compound that has been described very recently by Rodenko et al.[24] It was synthesised using 6-chloro-9-*t*-butoxycarbonylpurine (prepared from 6-chloropurine and *t*-butyl bicarbonate[24]) via nitration by trifluoroacetyl nitrate, generated in situ from trifluoroacetic anhydride and tetra-*n*-butylammonium nitrate, at the temperature −5 °C. The authors explored the reaction mechanism, which is rather complex, using detailed ^{1}H, ^{13}C, ^{15}N, and ^{19}F NMR analyses.[24] The presence of the strongly electron-withdrawing nitro group enables easy substitution by nucleophiles, which can be introduced at the C6 position by displacement of the chlorine atom at low temperatures (below 0 °C), and thus a large number of biologically active compounds can be prepared from this intermediate.[24] Finally, the protecting *t*-butoxycarbonyl (*t*-Boc) group can be removed in acidic media either before or after condensation of this precursor with various amines.[24]

It could be very interesting for future preparation of 6-arylaminopurines to use Hartwig–Buchwald coupling of electron deficient halopurines and anilines. This method requires usage of special catalysts—Pd complexes with phosphine derivatives, and 1,3-dialkyl or 1,3-diarylimidazolium halogenides. The reported yields are relatively high, but purification of raw product by column chromatography is required (removal of traces of heavy metal catalysts and other impurities).[21]

Our method for the preparation of 6-arylaminopurines—aromatic nucleophilic substitution—is cheap and without presence of expensive Pd catalysts, therefore it can be used for large scale preparations. The raw products can be purified by simple crystallization, if needed. The yields are relatively high in most cases, as well.

2.2. Biological activities

Biological effects of prepared compounds were compared to those of thidiazuron, a well known synthetic cytokinin with high biological activities mediated by strong activation of cytokinin

receptors,[1,28] and CKX inhibition,[13] as well as classical isoprenoid and aromatic cytokinins *trans*-zeatin (tZ) and 6-benzylaminopurine (BAP), respectively. To explore the cytokinin-like effects of the prepared anilinopurines *E. coli* strains with *Δrcs* and *cps::lacZ* mutant backgrounds that express AHK3 and AHK4 cytokinin receptors were used.[1,28,29] Interactions of the 6-anilinopurines with AtCKX2 were investigated using an assay based on the coupled redox reaction of PMS and MTT resulting in the formation of a formazan dye.[27] The effects of substitution position on the phenyl ring and various C2 and N9 substitutions on receptor activation and CKX inhibition were assessed by determining and comparing EC_{50} and IC_{50} values, respectively, of the prepared compounds.

2.2.1. Receptor binding assay

The affinity of the cytokinin receptors for the prepared 6-anilinopurines was compared to their affinity for the natural ligand *trans*-zeatin (tZ) and synthetic cytokinin thidiazuron (TDZ). tZ and TDZ were recognized by both receptors with high affinity (EC_{50} ca. 1 μM in each case), while the non-substituted 6-anilinopurine (**1**) was not recognized by either of the receptors, and substituted 6-anilinopurines were recognized to varying degrees (Table 3). Histidine-kinase CRE1/AHK4 recognized only *meta*-substituted derivatives, with preferences for hydroxy (**8**) and methoxy (**11**) groups, followed by the halogens chlorine (**3**) and fluorine (**5**), reaching EC_{50} values from 3.75 to 19 μM (Table 3). An interesting exception was compound **9**, which harbours a hydroxy group at the *para*-position of aniline and showed strong activation of CRE1/AHK4, equalling that of the *meta*-analog. It is clear from Table 3 that the strongest binding was shown by the compounds bearing a halogen group at C2 of the purine ring, while the presence of other groups at C2, and substitution of the N9 led to loss of affinity. Receptor AHK3 bound the tested compounds with higher affinity than CRE1/AHK4, again with highest preference for *meta*-substituted derivatives, but their EC_{50} values were not much lower than those of derivatives with substituents at the *para*- and *ortho*-positions (Table 3). The findings that there were no strict relationships between either the types or positions of substituents and the ability of the 6-anilinopurine to activate the AHK3 signalling pathway confirms previous findings indicating that AHK3 has broader ligand specificity than AHK4/CRE1.[1,2]

2.2.2. Cytokinin bioassays

Most of the tested compounds retained high cytokinin activity in bioassays, based on the dark induction of betacyanin synthesis in *Amaranthus* cotyledons and stimulation of cytokinin-dependent tobacco callus growth, comparable to the activity of corresponding, previously described 6-benzylaminopurines[2] (Table 4). The ring-substituted aminopurine with the strongest cytokinin activity is 6-benzylaminopurine, according to various reports.[14,15] Either removal of the methylene group between the ring and the amino group at the 6-position or lengthening of the bridge reduces the compound's cytokinin activity.[14,15] Thus, 6-anilinopurine (**2**), 6-(2-phenylethylamino)purine and 6-[(2-furan-2-yl-ethyl)amino]-purine have been reported to be either as active or less active than BAP and kinetin. However, our results from comparison of biological activity of BAP and prepared 6-anilinopurine derivatives in the tobacco and *Amaranthus* bioassays show that the benzyl group can be replaced by a phenyl ring without loss of cytokinin activity (Table 4). In good agreement with results of the receptor binding assays, *meta*-substituted derivatives (compounds **3**, **5**, **11**, **13** and **14**) showed high activity in both tests. On the other hand, in contrast to the AHK assay, non-substituted 6-anilinopurine (**1**), as well as compounds **2** (2-chloro derivative) and **4**, which also carry a chlorine atom in the *para*-position of the phenyl ring, also highly actively induced betacyanin synthesis and stimulated tobacco callus growth.

The position of the hydroxyl group on the side of the cytokinin ring usually has a significant effect on the biological activity of hydoxylated BAP derivatives. Hydroxylation of the benzyl group in *meta*-position has been found to increase cytokinin activity while *ortho*- and *para*-hydroxylation strongly reduces it.[30,16] However, surprisingly, both *meta*- and *para*-hydroxy derivatives of 2-chloro-6-anilinopurine (**8**,**9**) were much less active in both the tobacco and *Amaranthus* bioassays, compared with unsubstituted 2-chloro-6-anilinopurine (Table 4).

In contrast with the results of the two bioassays described above, and the activity of the corresponding 6-benzylaminopurines,[2] all tested 6-anilinopurine derivatives were virtually inactive in the senescence bioassay (Table 4).

Similar biological activity was found with *meta*- and *para*-fluorine derivatives (**5**,**6**). Interestingly, in the callus bioassay the *meta*-derivative (**6**) embodied maximum of biological activity in broad concentration range and, in contrary to control cytokinin BAP, even the highest concentration applied (100 μM) was not toxic and did not inhibited callus growth (Fig. 1A). The same activity of the highest concentration of compound **6** was observed also in *Amaranthus* assay (Fig. 1B).

Interestingly, in contrast with the results of the two bioassays described above, and the activity of the corresponding 6-benzylaminopurines,[2] all tested 6-anilinopurine derivatives were virtually inactive in the senescence bioassay (Table 4 and Fig. 1C).

Finally, we compared biological activity of compounds **11** and **14**, which are interesting due to their interaction with CKX (as described bellow), to the activity of highly active synthetic cytokinin TDZ. As shown in Figure 2A EC_{50} of the compound **11** was about 1000-fold higher compared to TDZ in the callus assay. In the *Amaranthus* assay, EC_{50} of TDZ was again much lower than of the compound **11** (about 100-fold), however the compound **11** showed

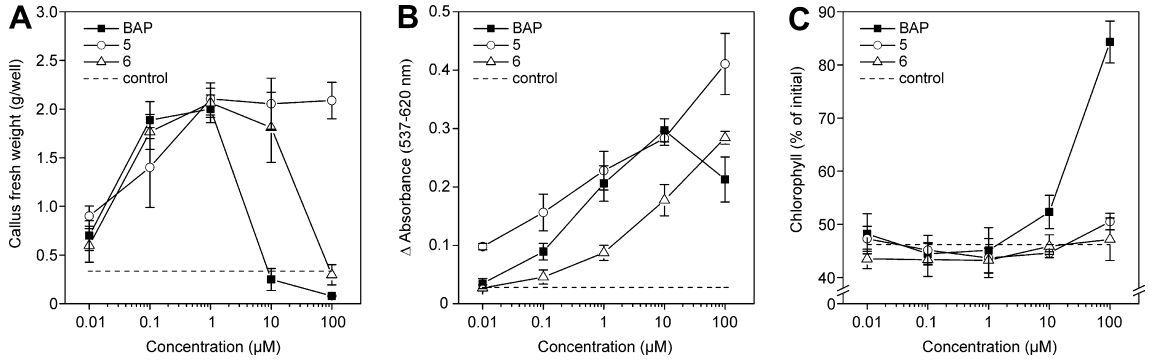


Figure 1. Dose–response curves for: cytokinin-induced dark betacyanin synthesis in *Amaranthus caudatus* cotyledon-hypocotyl explants (A), chlorophyll retention in excised wheat leaf tips (B), and growth of cytokinin-dependent tobacco callus (C). Error bars show standard deviations of the mean for five replicate determinations. Dashed lines indicate values obtained for the control treatment without any cytokinin.

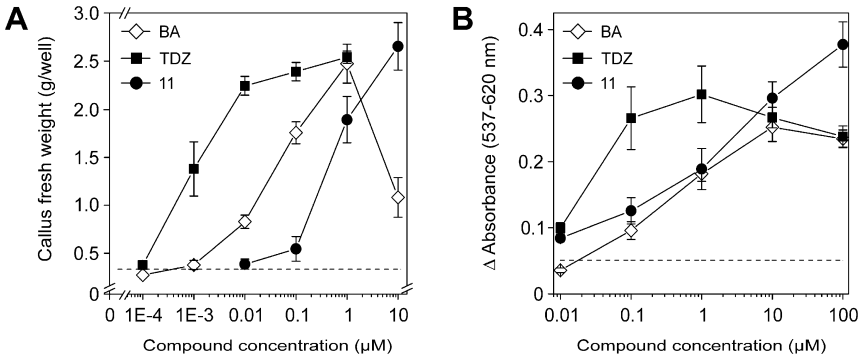


Figure 2. Biological activity of CKX inhibitor 2-chloro-6-(3-methoxyphenyl)aminopurine (compound **11**) in classical cytokinin bioassays compared to synthetic cytokinins thidiazuron (TDZ) and 6-benzylaminopurine (BAP). (A) Effect on growth of cytokinin-dependent tobacco callus; (B) effect on dark betacyanin synthesis in *Amaranthus caudatus* cotyledon-hypocotyl explants. Error bars show standard deviations of the mean for five replicate determinations. Dashed lines indicate values obtained for the control treatment without any cytokinin.

very high maximal activity in the concentration that is toxic for TDZ (Fig. 2B). Similar results were obtained with compound **14** (Table 4, graphs not shown).

2.2.3. Inhibition of cytokinin oxidase/dehydrogenase

The ability of the novel 6-anilinopurines to inhibit the activity of CKX was compared with that of thidiazuron, a well known inhibitor of CKX. As shown in Figure 3A, substitution of the phenyl side chain and/or C2 substitution at the purine ring were found to be essential for the inhibition of AtCKX2. The combination of these two substitutions led to the discovery of the most potent compounds; 2-chloro-6-(3-methoxyphenylamino)purine (**11**) and 2-fluoro-6-(3-methoxyphenylamino)purine (**14**). While monosubstituted derivatives **2** and **13** had similar IC_{50} values to TDZ (20–30 μM), that of the disubstituted anilinopurine **11** was much lower, submicromolar (Fig. 3A, Table 3). With the methoxy group placed on the phenyl ring, the ability of the compound to inhibit CKX activity decreased in the order *meta* > >*para* ⩾ *ortho* (Fig. 3B). C2 substituents influenced the strength of inhibition in the order F > Cl > NO_2 > NH_2 > SCH_3, indicating that electron-withdrawing substituents enhance the inhibitory activity of these compounds (Fig. 3C). Similar effects have been described amongst fluorine-containing derivatives of natural cytokinins, for which it has been proposed that the fluorine substituents form hydrogen bonds with electron donors of cytokinin receptors.[17,18] The presence of the third substituent at the N9 position of the purine ring, i.e. methyl or isopropyl, significantly decreased the compounds' potency to inhibit AtCKX2 (Fig. 3D and Table 3).

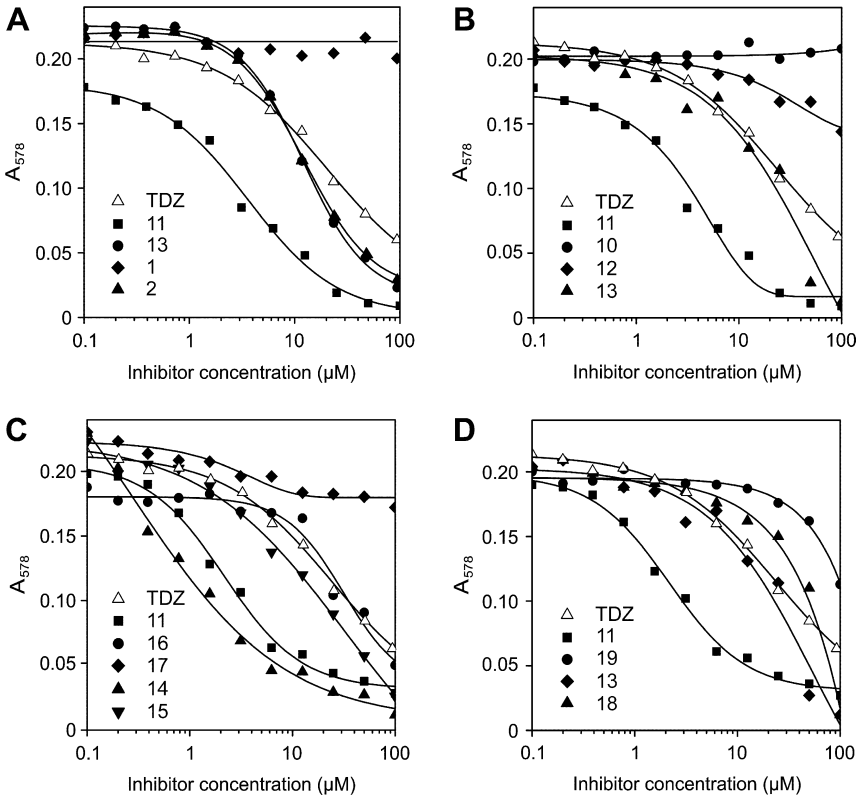


Figure 3. Effects of substituents on the purine ring on inhibition of AtCKX2 activity. (A) Key substitutions of the 6-anilinopurine moiety leading to the potent inhibitor 2-chloro-6-(3-methoxyphenylamino)purine (**11**). (B) Effect of the methoxy group's position on the phenyl side chain. (C) Effects of substitutions at the C2 position. (D) Effects of substitutions at N9.

3. Conclusion

In summary, a group of 6-anilinopurine derivatives with different substitutions on the phenyl moiety, as well as with C2 and N9 substitutions, have been prepared. Biological testing showed that these compounds can be classified as a new generation of purine-derived CKX inhibitors. Compared to thidiazuron, a synthetic cytokinin with strong biological activity resulting from strong activation of cytokinin receptors and CKX inhibition, most of the 6-anilinopurines inhibited CKX much more strongly, but their sensing by cytokinin receptors was much weaker.

4. Materials and methods

4.1. General procedures

The elemental contents of the prepared compounds were determined using an EA1108 CHN analyser (Thermo Finnigan). Their melting points were determined using a Büchi Melting Point B-540 apparatus (uncorrected values are presented here), and they were separated from reaction by-products and reactants by analytical thin layer chromatography (TLC) using silica gel 60 WF_{254} plates (Merck) with a mobile phase of $CHCl_3$/MEOH/conc. NH_4OH (8:2:0.2, v/v/v).

Flash chromatography was performed using a VersaFlash purification station (Supelco) coupled to a 2110 Fraction Collector (Bio-Rad). Compounds were separated on VersaPak Cartridges (25 × 100 mm, Supelco) containing 23 g of spherical silica and eluted with a mobile phase containing $CHCl_3$/MeOH (90:10, v/v).

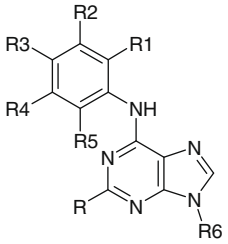
To determine their HPLC purity, samples were dissolved in HPLC mobile phase (initial conditions), applied to an RP-column (150 mm × 4.6 mm, 5 μm, Microsorb C18; Varian) and the separated constituents were eluted with a linear methanolic gradient (10–90% over 30 min, pH adjusted to 4 using ammonium formate) at a flow rate of 0.6 ml/min. Eluting compounds were detected by scanning the UV absorbance of the eluate between 240 and 300 nm.

CI+ and EI+ mass spectra were recorded using a Polaris Q (Finnigan) mass spectrometer equipped with a Direct Insertion Probe (DIP). The compounds were heated in an ion source with a 40–450 °C temperature gradient, the mass monitoring interval was 50–1000 amu, and spectra were collected using 1.0 s cyclical scans, applying 70 eV electron energy. In the CI+ ionization mode, isobutane was used as a reagent gas at a flow rate of 2L/h. The mass spectrometer was directly coupled to an Xcalibur data system.

NMR spectra were acquired using a Bruker Avance AV 300 spectrometer operating at a temperature of 300 K and a frequency of 300.13 MHz (^{1}H). Samples were prepared by dissolving the compounds in DMSO-d_6, and tetramethylsilane (TMS) was used as the internal standard.

4.2. Chemicals

2,6-Dichloropurine, 2-amino-6-chloropurine, and 2-methylthio-6-chloropurine were obtained from Olchemim, aniline, *o*-,*m*-,*p*-anisidine, 3-chloroaniline, 4-chloroaniline, diisopropylethylamine, 3-fluoroaniline, 4-fluoroaniline and tetrafluoroboric acid from Fluka, *o*-, *m*- and *p*-aminophenol from Baker. 2-fluoro-6-chloropurine was synthesised from 2-amino-6-chloropurine by a previously published method.[22,23] Lach-Ner supplied *n*-butanol, *n*-propanol, diethyl ether, dimethylformamide, ethyl acetate, *n*-propanol and anhydrous sodium sulphate. Milli-Q water was used throughout. The other solvents and chemicals used were all of standard p.a. quality.



Scheme 1. General structure of 6-anilinopurines.

4.3. Synthesis of N^6-anilinopurines

4.3.1. General procedure

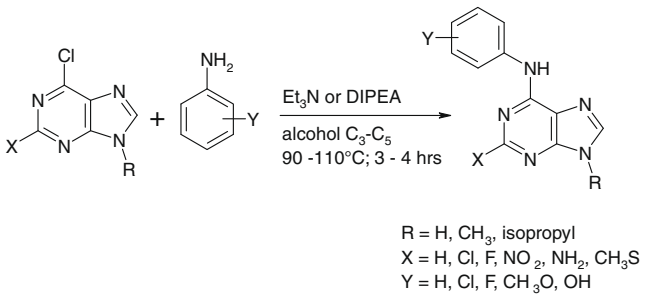
Variously substituted 6-anilinopurines (Scheme 1) were prepared by reacting corresponding 2-substituted 6-chloropurines or 6-methylthiopurines with appropriate anilines in various solvents, preferably alcohols (C2–C5), or dimethylformamide, in the presence (or absence, depending on the basicity of the aniline used) of base (tertiary amine, potassium carbonate, sodium hydride, and sodium methoxide) (Scheme 2 and Table 2a). The reaction temperatures varied from sub-zero to 110 °C, depending on the reactivity of the substituted 6-chloro- or 6-methylthiopurine used and the basicity of the selected substituted aniline (Table 2a). Basic chemical characteristics of the synthesised compounds are presented in Table 2b. NMR data are available in the supplementary material.

We describe here a few examples to illustrate key synthetic procedures used throughout the study:

4.3.1.1. 2-Chloro-6-anilinopurine (compound 2). *N*,*N*-Ethyldiisopropylamine (6.44 g; 0.05 mol) and aniline (4.66 g; 0.05 mol) were added to a suspension of 2,6-dichloropurine (5.67 g; 0.03 mol) in *n*-propanol (60 ml). The reaction mixture was stirred at 100 °C for 4 h. After cooling to room temperature, the precipitate was filtered off, washed with cold *n*-propanol (2× 10 ml) and water (3× 10 ml) then dried at 60 °C to constant weight. Yield: 6.26 g yellowish substance (84.9 %). TLC (chloroform/methanol 85:15): one single spot; free of the starting material. HPLC purity: 98+%.

4.3.1.2. 2-Chloro-6-(4-chloroanilino)purine (compound 4). *N*,*N*-Ethyldiisopropylamine (6.44 g; 0.05 mol) and 4-chloroaniline (6.38 g; 0.05 mol) was added to a suspension of 2,6-dichloropurine (5.67 g; 0.03 mol) in *n*-propanol (60 ml). The reaction mixture was stirred at 100 °C for 4 h. After cooling to room temperature, the precipitate was filtered off, washed with cold *n*-propanol (2× 10 ml) and water (3× 10 ml) then dried in an oven at 60 °C to constant weight. Yield: 5.46 g yellowish substance (65%). TLC (chloroform/methanol 85:15): one single spot; free of the starting material. HPLC purity: 98+%.

4.3.1.3. 2-Chloro-6-(3-methoxyanilino)purine (compound 11). To a suspension of 2,6-dichloropurine (3.78 g; 0.02 mol) and *m*-anisi-



Scheme 2. Reaction scheme for the preparation of 6-arylaminopurines.

Table 4
Relative cytokinin bioassay activity of prepared 6-anilinopurine derivatives at optimal concentration compared with activity of 6-benzylaminopurine (BAP) (100% means 10^{-6} M BAP for the tobacco callus bioassay, 10^{-5} M BAP for the *Amaranthus* betacyanin bioassay, and 10^{-4} M BAP in the case of the senescence bioassay)

Compound	Tobacco callus bioassay		*Amaranthus* bioassay		Senescence bioassay	
	Optimal concentration ($mol\ l^{-1}$)	Relative activity (%)	Optimal concentration ($mol\ l^{-1}$)	Relative activity (%)	Optimal concentration ($mol\ l^{-1}$)	Relative activity (%)
tZ	10^{-5}	104.6 (±7)	10^{-4}	129.6 (±10)	10^{-4}	122 (±8)
TDZ	10^{-7}	102.3 (±11)	10^{-7}	121.1 (±9)	10^{-4}	108.7 (±5)
1	10^{-4}	47 (±2)	10^{-4}	52 (±2)	10^{-4}	14 (±5)
2	10^{-6}	103 (±4)	10^{-4}	139 (±16)	10^{-4}	23 (±1)
3	10^{-5}	95 (±23)	10^{-5}	119 (±15)	10^{-4}	11.5 (±1)
4	10^{-5}	77 (±11)	10^{-4}	139 (±6)	10^{-4}	10 (±3)
5	10^{-6}	101 (±7)	10^{-4}	147 (±14)	10^{-4}	15 (±5)
6	10^{-6}	99 (±6)	10^{-4}	92 (±27)	10^{-4}	5 (±3)
7	nt		nt		nt	
8	10^{-5}	111 (±5)	10^{-4}	52 (±12)	10^{-4}	6 (±3)
9	10^{-4}	60 (±8)	10^{-4}	47.5 (±2)	10^{-4}	14 (±8)
10	10^{-5}	100 (±10)	10^{-4}	63 (±19)	10^{-4}	29 (±4)
11	10^{-5}	103 (±1)	10^{-4}	149 (±17)	10^{-4}	11 (±1)
12	10^{-5}	76 (±7)	10^{-4}	60 (±29)	10^{-4}	19 (±14)
13	10^{-5}	42 (±18)	10^{-4}	124.5 (±31)	10^{-4}	13 (±2)
14	10^{-5}	74 (±17)	10^{-4}	107 (±31)	10^{-4}	13.5 (±1)
15	10^{-4}	35.5 (±4)	10^{-4}	43 (±25)	10^{-4}	15 (±2)
16	10^{-5}	71 (±22)	10^{-4}	116 (±3)	10^{-4}	16 (±1)
17	10^{-5}	91 (±10)	10^{-4}	101 (±35)	10^{-4}	2 (±3)
18	10^{-5}	76 (±10)	10^{-4}	69 (±9)	10^{-4}	30 (±16)
19	10^{-5}	38 (±23)	10^{-4}	44 (±14)	10^{-4}	5 (±16)

dine (3.69 g; 0.03 mol) in *n*-pentanol (40 ml), triethylamine (7 ml; 0.05 mol) was added. The reaction mixture was stirred at 100 °C for 4 h and subsequently allowed to cool to room temperature. The white precipitate was collected, washed with cold isopropanol (2× 10 ml) and water (3× 10 ml). The crude product was purified by crystallization from methanol in the presence of activated charcoal to give almost white crystals. Yield: 4.36 g (77.8%). TLC (chloroform/methanol 85:15): one single spot, free of starting material. HPLC purity: 98+%.

4.3.1.4. 2-Fluoro-6-(3-methoxyanilino)purine (compound 14). This compound was prepared by reacting 2-fluoro-6-chloropurine (1.7 g; 0.01 mol), *m*-anisidine (1.23 g; 0.01 mol) and triethylamine (3.5 ml; 0.025 mol) in *n*-propanol (20 ml) at 90 °C for 4 h. The reaction mixture was then cooled to room temperature. The white precipitate was filtered off, rinsed with *n*-butanol (3× 10 ml) and water (3× 10 ml) then dried to constant weight. Yield: 5.55 g (61%) of yellowish crystalline powder. TLC (chloroform/methanol 85:15): one single spot, free of starting material. HPLC purity: 99+%.

4.3.1.5. 2-Nitro-6-(3-methoxyanilino)purine (compound 15). 2-Nitro-6-chloro-9-*t*-butoxycarbonyl-purine, prepared according to a previously described method[24] (300 mg; 1 mmol), was suspended in *n*-propanol (10 ml). The suspension was cooled to −5 °C and *m*-anisidine (132 mg; 1 mmol) was slowly added while keeping the temperature of the reaction mixture at ⩽0 °C. The reaction mixture was then stirred at room temperature for 12 h. The crystalline mass was filtered off, washed with cold *n*-propanol (2× 5 ml), and dried in a desiccator (in the presence of phosphorus pentoxide) to constant weight, yielding a yellow solid (290 mg). This product was found to be a mixture of 2-nitro-6-(3-methoxyanilino)-9-*t*-butoxycarbonyl-purine, 2-nitro-6-(3-methoxyanilino)purine and unreacted starting compound, 2-nitro-6-chloro-9-*t*-butoxycarbonyl-purine. Complete deprotection of *t*-butoxycarbonyl group was achieved by stirring the crude product in methanolic HCl (2 ml conc. HCl and 8 ml methanol) at 50 °C for 12 h to give the hydrochloride, which was transformed to the free base using diluted aqueous ammonia (3%; 10 ml) and simultaneously purified from traces of unreacted starting compound, 2-nitro-6-chloro-9-*t*-butoxycarbonyl-purine, which stayed in solution. The yellow precipitate was filtered off, rinsed with water (3× 10 ml), and dried to constant weight. Yield: 100 mg (34.9%) of yellow crystalline powder. HPLC purity: 99+%.

4.3.1.6. 2-Amino-6-(3-methoxyanilino)purine (compound 16). To a suspension of 2-amino-6-chloropurine (1.69 g; 0.01 mol) in *n*-propanol (20 ml) *m*-anisidine (1.23 g; 0.01 mol) was added. The thick resulting suspension was stirred at 100 °C for 3 h. TLC analysis showed the absence of the starting material and the presence of the desired product. The reaction mixture was then cooled to room temperature. The white precipitate was filtered off, rinsed with *n*-butanol (3 × 10 ml) and water (3 × 10 ml), then dried in an oven to constant weight. Yield: 2.19 g (85 %). TLC (chloroform-methanol/NH_4OH 4:1:0.05): single spot, free of starting material. HPLC purity: 99+%.

4.3.1.7. 2-Methylthio-6-(3-methoxyanilino)purine (compound 17). This substance was prepared by reacting 2-methylthio-6-chloropurine (200 mg; 1 mmol) and *m*-anisidine (132 mg; 1 mmol) in refluxing *n*-propanol (100 °C; 4 h). Yield: 220 mg as hydrochloride (69%); HPLC purity: 99+%.

4.3.1.8. 2-Chloro-6-(3-methoxyanilino)-9-methylpurine (compound 18) and 2-chloro-6-(3-methoxyanilino)-9-isopropylpurine (compound 19). These compounds were prepared from previously prepared 2,6-dichloro-9-methylpurine[25] and 2,6-dichloro-9-isopropylpurine,[26] respectively, and *m*-anisidine by previously described methods. Yields: 46% and 80%, respectively. Purity: 99% and 98.7%, respectively.

4.4. CKX inhibition measurements

The ability of each of the prepared compounds to inhibit CKX was evaluated by determining their IC_{50} values in an assay that had been previously adapted for screening such activity in ELISA microtitre plates.[27] Each well contained 100 μL of a reaction mixture consisting of 0.1 M KH_2PO_4 (pH 7.4), 1 mM phenazine methosulfate (MTT) and 0.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), final concentrations, the tested compound (at various concentrations) and 30 μM of iP as

a substrate. Cell-free growth medium of *S. cerevisiae* strain 23344c *ura*⁻ harbouring the plasmid pYES2-AtCKX2 (100 μL) was used directly as a source of AtCKX2.[4] Plates were incubated in the dark for 30 min at 37 °C and the enzymatic reaction was stopped by adding 25 μL of 35% acetic acid per well. The absorbance of the mixture in each well at 578 nm was then measured using a Tecan spectrophotometer (Sunrise, Canada), and the absorbance of samples with no iP was subtracted. Compounds were tested in two repetitions and the entire test was repeated at least twice.

4.4.1. Bacterial receptor assay

Transformed *Escherichia coli* KMI001 strains harbouring the plasmids pIN-III-AHK4 and pSTV28-AHK3, which, respectively, express the cytokinin receptors CRE1/AHK4 and AHK3[28,29] were used in bacterial cytokinin assays as described elsewhere.[2] Relative activation of cytokinin receptors was determined by measuring β-galactosidase activity using the fluorescent substrate 4-methylumbelliferyl-β-D-galactoside and monitoring the culture density at OD_{600}. The test was performed in three repetitions and the entire test was repeated at least twice.

4.4.2. Cytokinin bioassays

Standard bioassays based on stimulation of cytokinin-dependent tobacco callus growth, the retention of chlorophyll in excised wheat leaves and the dark induction of betacyanin synthesis in *Amaranthus* cotyledons were carried out as previously described.[30] The only exception to the published protocols was that the tobacco callus bioassay was performed in 6-well microtiter plates (3 mL of MS medium in each well). Prior to testing, stock solutions of 6-benzylaminopurine (BAP) and tested compounds in DMSO were prepared and further diluted as required in the media used for each biotest. The final concentration of DMSO in the media did not exceed 0.2%. Five replicates were prepared for each cytokinin concentration and the entire tests were repeated at least three times. From the data acquired in these tests the concentration inducing the strongest biological response and the relative activity at this concentration of each compound were calculated (Table 4). The activity of BAP at the optimal concentration was set at 100 and the activities of the tested compounds were related to it. The optimal BAP concentrations used for these calculations were 10^{-5}, 10^{-4} and 10^{-6} M for the *Amaranthus* betacyanin, senescence and tobacco callus bioassays, respectively.

Acknowledgements

This work was supported by the Grant Agency of the Czech Republic (GA 206/07/0570, GA 522/06/0108) and Czech Ministry of Education (MSM 6198959216, 1M0630, LC06034).

We thank Jarmila Balonová, Jarmila Greplová, and Miloslava Šubová for skilful technical assistance.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.09.008.

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