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Cytokinin receptor antagonists derived from 6-benzylaminopurine

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ABSTRACT

Recently we reported 6-(2-hydroxy-3-methylbenzylamino)purine (PI-55) as the first molecule to antagonize cytokinin activity at the receptor level. Here we report the synthesis and *in vitro* biological testing of eleven BAP derivatives substituted in the benzyl ring and in the C2, N7 and N9 positions of the purine moiety. The ability of the compounds to interact with Arabidopsis cytokinin receptors AHK3 and CRE1/AHK4 was tested in bacterial receptor and in live-cell binding assays, and in an Arabidopsis ARR5:GUS (Arabidopsis response regulator 5) reporter gene assay. Cytokinin activity of the compounds was determined in classical cytokinin biotests (tobacco callus, wheat leaf senescence and *Amaranthus* bioassays). 6-(2,5-Dihydroxybenzylamino)purine (LGR-991) was identified as a cytokinin receptor antagonist. At the molecular level LGR-991 blocks the cytokinin receptor CRE1/AHK4 with the same potency as PI-55. Moreover, LGR-991 acts as a competitive inhibitor of AHK3, and importantly shows reduced agonistic effects in comparison to PI-55 in the ARR5:GUS reporter gene assay and in cytokinin bioassays. LGR-991 causes more rapid germination of Arabidopsis seeds and increases hypocotyl length of dark-grown seedlings, which are characteristics of plants with a reduced cytokinin status. LGR-991 exhibits a structural motive that might lead to preparation of cytokinin antagonists with a broader specificity and reduced agonistic properties.

1. Introduction

Cytokinins are plant hormones with many essential signalling roles in plant growth and development. They are involved in the regulation of processes such as cell division, shoot and root development, apical dominance and growth of lateral buds, seed germination, and delay of senescence of plant organs (reviewed by Mok and Mok, 1994). The natural cytokinins are entirely adenine derivatives with an isoprenoid or aromatic side chain at the N6-position. Perception of cytokinins by receptors is the first step in the cytokinin signalling pathway which leads to a biological response triggered by the hormone. In *Arabidopsis thaliana* three membrane hybrid histidine kinases AHK2, AHK3 and CRE1/AHK4 have been identified as cytokinin receptors (Suzuki et al., 2001; Inoue et al., 2001; Yamada et al., 2001) and their function was reported by expression of AHK3 and CRE1/AHK4 proteins in heterologous systems (Suzuki et al., 2001; Inoue et al., 2001; Yamada et al., 2001). The coupling of the eukaryotic receptor to the bacterial (*Escherichia coli*) signalling pathway enabled studies of the cytokinin activity of various compounds to be carried out in an easy and rapid way (Suzuki et al., 2001; Yamada et al., 2001; Spíchal et al., 2004). Such a method was also employed for the determination of essential parameters of cytokinin-binding to CRE1/AHK4 and AHK3 receptors in a direct binding assay (Romanov et al., 2005, 2006).

The classical approach to investigations of cytokinin function has been based on analysis of the consequences of the exogenous cytokinin application (reviewed by Mok and Mok, 1994). These experiments gave important insights into the roles of cytokinins in plant physiology; however, the direct involvement of (endogenous) cytokinins in the control of important aspects of plant growth and development was analysed in studies of plants with receptor loss-of-function mutations (Riefler et al., 2006), mutations in signalling proteins (Hutchison et al., 2006) and cytokinin-synthesizing genes (Miyawaki et al., 2006) or plants with cytokinin-deficiency caused by enhanced cytokinin degradation (Werner et al., 2001, 2003). A chemical substance which would act as an inhibitor of cytokinin action when applied exogenously thus would be a very useful tool for further studies of cytokinin perception and signal transduction and might also find a practical use in agriculture.

Since the discovery of cytokinins, many compounds with agonistic and antagonistic effect have been synthesized. In the 1970's different heterocyclic compounds, such as pyrazolo[4,3-d]pyrimidines (Hecht et al., 1971), pyrrolo[2,3-d]pyrimidines

Abbreviations: BAP, 6-benzylaminopurine; ARR5, Arabidopsis response regulator 5; AHK2, Arabidopsis histidine kinase 2; AHK3, Arabidopsis histidine kinase 3; CRE1/AHK4, cytokinin response 1/Arabidopsis histidine kinase 4.

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(Iwamura et al., 1974, 1975), and 7-deaza analogues of 2-methylthioadenine cytokinins (Skoog et al., 1975) have been classified as potent anticytokinins based on their structural similarity to cytokinins and their antagonistic effects in various cytokinin bioassays. From structure–activity relationships and inhibitor-like effects, it was hypothesized that these compounds act as competitive inhibitors at the receptor level (Iwamura, 1994; Hecht et al., 1971; Skoog et al., 1973). Recent findings in cytokinin signalling have necessitated a re-examination of the classical anticytokinin mode of action. It was shown that these compounds do not act as competitors of cytokinins at the receptor level, but rather as inhibitors of cyclin-dependent kinases (Spíchal et al., 2007). Very recently the first inhibitor of the CRE1/AHK4 receptor that blocks cytokinin action *in vivo*, PI-55, a derivative of the aromatic cytokinin BAP, was reported (Spíchal et al., 2009). PI-55, however, does not effectively block the AHK3 receptor, which together with AHK2 play prominent roles in the control of leaf development and organ growth in Arabidopsis (Riefler et al., 2006). A cytokinin antagonist with broader specificity might thus represent a stronger tool for the regulation of cytokinin effects.

Here we describe the synthesis and *in vitro* biological testing of eleven BAP derivatives with various substitutions in the C2, N6, N7 and N9 positions (for structures see Table 1). We examined their interaction with the Arabidopsis cytokinin receptors CRE1/AHK4 and AHK3. These aromatic cytokinin analogues were further tested in three classical cytokinins bioassays (tobacco callus, wheat leaf senescence and *Amaranthus* bioassays) and in an ARR5:GUS reporter gene assay. The effect on germination of Arabidopsis seeds was also investigated.

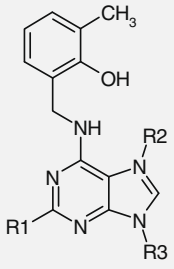
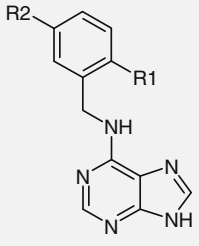
2. Results and discussion

2.1. Synthesis

Two groups of compound – six PI-55 derivatives with substitutions in the C2, N7 and N9, and five BAP analogues with various substituents attached to the phenyl ring – were synthesized (Table 1) in order to determine their structure–activity relationships. The prepared compounds were characterised by HPLC, TLC, melting point, ES(+/−)-MS (Table 2) and ^{1}H NMR (Supplementary data). Compounds **3**, **4** and **5** were prepared from the corresponding starting 2-X-6-chloropurines (X = CH_3S, Cl, F, respectively) by conventional methods previously described (Corse and Kuhnle, 1972; Tarkowská et al., 2003). Compound **2** was prepared from 2-amino-6-chloropurine and the corresponding amine by long term heating, owing to the low reactivity of starting compounds. N9-methyl purines are usually prepared by alkylation with methyl iodide in suitable solvent (DMF, DMSO) in the presence of base (potassium carbonate, sodium hydride) (Tarkowská et al., 2003; de Ligt et al., 2004). Our method was based on Mitsunobu alkylation of 6-chloropurine with methanol. Although this method is more simple, less time consuming and does not involve the use of toxic alkyl halogenides, it did not lead to higher regioselectivity (Weibing et al., 2007). N9 and N7 derivatives were obtained in the ratio 7 to 3.

Preparation of 6-(2-hydroxy-5-methylbenzylamino)purine and a large variety of mono- and disubstituted benzylaminopurines was described by Doležal et al. (2006, 2007).

Table 1
Structures of the prepared compounds.

Compound	R1	R2	R3	Structure
PI-55	H	H	H	
1	NH_2	H	H	
2	CH_3S	H	H	
3	Cl	H	H	
4	F	H	H	
5	H	CH_3	H	
6	H	H	CH_3	
7	OH	CH_3	–	
8 (LGR-991)	OH	OH	–	
9	OH	Cl	–	
10	OH	F	–	
11	F	F	–	

2.2. Interaction with Arabidopsis receptors AHK3 and CRE1/AHK4

Transformed *E. coli* expressing the Arabidopsis cytokinin receptors AHK3 or CRE1/AHK4 and the cytokinin-activated reporter gene cps::lacZ (Suzuki et al., 2001; Yamada et al., 2001; Spíchal et al., 2004) were employed to separate 11 compounds into those which activate cytokinin signalling pathway via these receptors and those which do not. The EC_{50} values (half maximal effective concentration for receptor activation) were determined and the ability of the compounds to activate the receptors was compared to the classical cytokinins tZ and BAP. The EC_{50} in CRE1/AHK4 bacterial assay for tZ was 0.2 μM and for BAP 7 μM, respectively. The receptor was activated by compounds **3**, **4**, **7**, **9**, **10** and **11**, but none of the compounds was as active as BAP. The overall activities of the tested compounds were very low, the most active compound was **4** with an EC_{50} of 14 μM, the next active compounds were **10** (EC_{50} 35 μM) and **11** (EC_{50} 50 μM). The EC_{50} of compounds **3**, **7** and **9** was higher than 50 μM (Fig. 1A and C). The same receptor-expressing bacteria were also used in a "live-cell hormone-binding assay" (Romanov et al., 2005) to observe whether any of inactive compounds (**1**, **2**, **5**, **6** and **8**) was able to decrease the binding of radiolabelled natural ligand (tZ) to the receptors. Hence, PI-55 and these compounds were tested for their capability to block the binding of 2 nM [2-^{3}H]tZ to the cytokinin receptors. PI-55 and LGR-991 (**8**) reduced the binding of radiolabelled tZ to 34% and to 35%, respectively (Fig. 2A). Compound **5** did not affect the binding of radiolabelled tZ and compound **3** even increased the binding to 124%. Importantly, only PI-55 and LGR-991 (**8**) significantly blocked the receptor CRE1/AHK4 without activating it (Fig. 2A).

The receptor AHK3 is known to have broader ligand specificity than CRE1/AHK4 (Spíchal et al., 2004). Furthermore, in this study it was generally more sensitive to the newly-prepared cytokinin derivatives than was CRE1/AHK4. The EC_{50} for tZ was 10 nM and for BAP was 4 μM. Compounds **3**, **4**, **9** and **11** had very similar activities with EC_{50} values of 10, 8, 17 and 8 μM, respectively. Compound **7** was much less active (EC_{50} 34 μM). The recently reported cytokinin antagonist PI-55 slightly activates AHK3 (EC_{50} 35 μM), whereas LGR-991 (**8**) had an EC_{50} higher than 50 μM and activated AHK3 to only 20% at the highest concentration (Fig. 1B and D). In a competitive binding assay with bacteria expressing AHK3 receptor, compounds **1**, **5** and **6** had little or no effect on the binding of radiolabelled tZ to the receptor. Interestingly, compound **2** increased the binding of the radiolabelled tZ to 247% by increasing the non-specific binding of radiolabelled ligand (not shown). Again, PI-55 and LGR-991 (**8**) were the only compounds that reduced binding of radiolabelled ligand to 45%, and to 54%, respectively. LGR-991 (**8**) activated receptor AHK3 with about two-fold lower efficacy than PI-55, whereas both compounds showed similar ability to block the binding of the natural ligand

Table 2
Basic characterisation of the tested compounds using exact mass spectrometry (QqTOF) and melting point analysis.

Compound	Calculated mass $[M+H]^+/[M-H]^-$	HR mass $[M+H]^+/[M-H]^-$	Mass difference (ppm)	Elemental composition	Melting point (°C)
PI-55	256.1198	256.1200	0.6	$C_{13}H_{14}N_5O^+$	278.5–279.5
	254.1042	254.1048	2.4	$C_{13}H_{13}N_5O^-$	
1	271.1307	271.1306	−0.5	$C_{13}H_{13}N_6O^-$	226–227.5
	269.1151	269.1155	1.5	$C_{13}H_{15}N_6O^+$	
2	302.1076	302.1075	−0.2	$C_{14}H_{16}N_5OS^+$	259–260
	300.0919	300.0921	0.6	$C_{14}H_{14}N_5OS^-$	
3	290.0809	290.0810	0.5	$C_{13}H_{13}ClN_5O^+$	244.5–245
	288.0652	288.0645	−2.5	$C_{13}H_{11}ClN_5O^-$	
4	274.1104	274.1111	2.5	$C_{13}H_{13}FN_5O^+$	259–260.5
	272.0948	272.0945	−1.0	$C_{13}H_{11}FN_5O^-$	
5	270.1355	270.1356	0.4	$C_{14}H_{16}N_5O^+$	229.5–230
	268.1198	268.1197	−0.5	$C_{14}H_{14}N_5O^-$	
6	270.1355	270.1357	0.8	$C_{14}H_{16}N_5O^+$	212–213.5
	268.1198	268.1193	−2.0	$C_{14}H_{14}N_5O^-$	
7	256.1198	256.1197	−0.5	$C_{13}H_{14}N_5O^+$	252–256
	254.1042	254.1043	0.5	$C_{13}H_{13}N_5O^-$	
8	258.0991	258.0985	−2.3	$C_{12}H_{12}N_5O_2^+$	281.5–283
	256.0834	256.0835	0.2	$C_{12}H_{10}N_5O_2^-$	
9	276.0652	276.0651	−0.4	$C_{12}H_{11}ClN_5O^+$	258–260.5
	274.0496	274.0487	−3.1	$C_{12}H_9ClN_5O^-$	
10	260.0948	260.0948	0.1	$C_{12}H_{11}FN_5O^+$	255–258
	258.0791	258.0789	−0.8	$C_{12}H_9FN_5O^-$	
11	262.0904	262.0904	−0.1	$C_{12}H_{10}F_2N_5^+$	263.5–266.0
	260.0748	260.0748	0.1	$C_{12}H_8F_2N_5^-$	

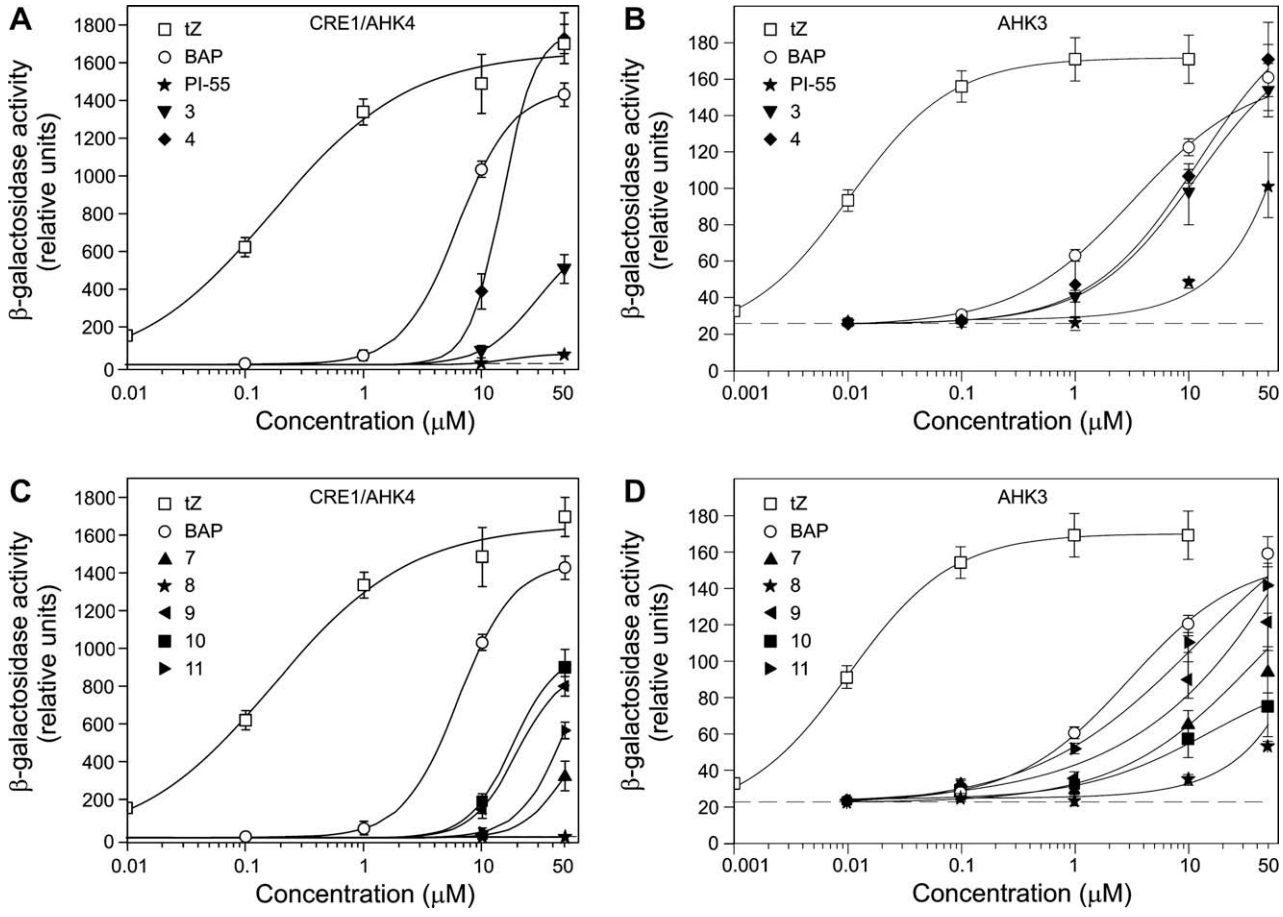


Fig. 1. Activation of the cytokinin receptors CRE1/AHK4 (A and C) and AHK3 (B and D) by the studied compounds in the *E. coli* receptor assay. The activities of PI-55 derivatives (A and B) and 2,5-disubstituted BAP derivatives (C and D) are compared with the cytokinins tZ and BAP. Dashed lines show β-galactosidase activity in non-induced strains (control). Error bars show s.d. ($n = 3$).

to the receptor. LGR-991 (**8**) showed a similar competitive activity with both cytokinin receptors as PI-55, but in comparison to PI-55 had only a limited effect on the activation of AHK3 (Figs. 1 and 2). This indicates that LGR-991 (**8**) posses a new structural motive that can lead to preparation of cytokinin antagonists with broader specificity but reduced agonistic properties. To determine the IC_{50} for each receptor, LGR-991 (**8**) was tested over a larger concentration range. It reduced the binding of radiolabelled tZ to both receptors

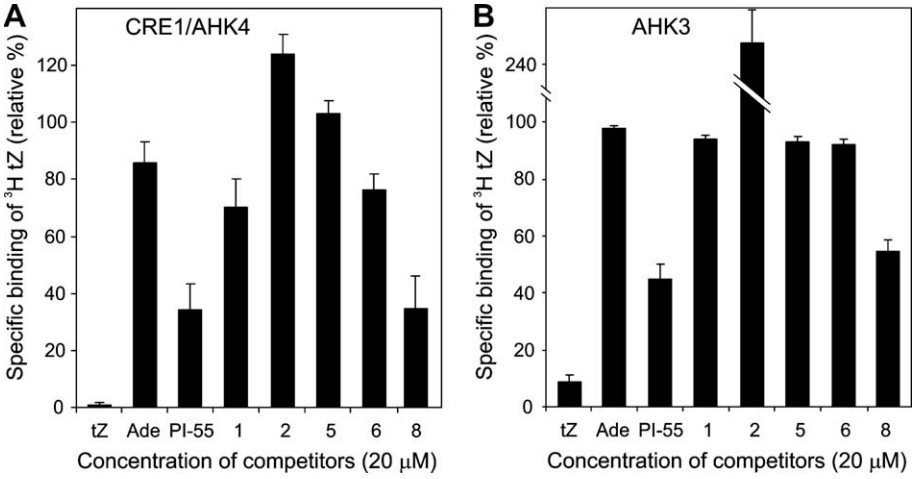


Fig. 2. Effect of selected compounds on specific binding of 2 nM [2−^{3}H]tZ in a live-cell binding assay employing *E. coli* expressing cytokinin receptors CRE1/AHK4 (A) and AHK3 (B). The concentration of the positive control tZ was 1 μM in the case of CRE1/AHK4 and 0.1 μM in the case of the AHK3 assay. To discriminate the specific and non-specific binding of [2−^{3}H]tZ on bacteria membrane 10 μM tZ was used and this residual value was subtracted from all the data. Adenine (Ade) was used as a negative control.

in a dose-dependent manner, reaching IC_{50} values 4 μM and 20 μM in the cases of CRE1/AHK4 and AHK3, respectively (Fig. 3). These values are very close to those published by Spíchal (Spíchal et al., 2009), where Ki values of 2.9 and 18 μM were reached with CRE1/AHK4 and AHK3, respectively.

It can be concluded that fluorine and chlorine in the C2 position of PI-55 enhanced its ability to activate both receptors, while methylthio and amino groups had the opposite effect. Unfortunately, these two substitutions did not enhance the antagonistic properties of PI-55. A methyl group in the N7 and N9 positions prevented binding and/or activation of the receptors. In the case of 2-hydroxy-5-X-benzylaminopurine analogues (X = OH, CH_3, Cl, F), the sequence of substituents that increased receptor activation was F > Cl > CH_3 > OH in case of CRE1/AHK4, and Cl > CH_3 > F > OH in the case of AHK3. In the terms of the enhancement of antagonistic activity without simultaneous activation of the receptors, only the hydroxyl group seemed to be a potential substituent for preparation of cytokinin antagonist with broader specificity.

2.3. The impact on the activation of cytokinin response gene

To gain more complete information about the cytokinin activities of studied compounds at the molecular level, their effect on cytokinin-induced expression of the response regulator gene ARR5 (D'Agostino et al., 2000) was investigated *in planta* using transgenic Arabidopsis seedlings expressing cytokinin reporter ARR5:GUS (Romanov et al., 2002). The intrinsic cytokinin activities of the prepared compounds, as well as their ability to repress the effect of 1 μM BAP in a competition assay, were tested. When applied alone, compounds **5**, **6**, LGR-991 (**8**) did not induced the expression of ARR5:GUS, compounds PI-55 and **1** triggered only weak expression, and compounds **3**, **4** and **11** induced expression of cytokinin reporter to the same level as the cytokinin BAP (Fig. 4). Of the compounds that did not activate ARR5:GUS, only LGR-991 (**8**) worked as an inhibitor in the competition assay, in which it significantly repressed the effect of 1 μM BAP to 79% and to 52% at concentrations of 1 and 10 μM, respectively (Fig. 4). Compounds **9** and **10** were found to antagonize the effect of 1 μM BAP by reducing its effect to 62% and 82%, and to 87% and 52% at concentrations of 1 and 10 μM, respectively. These compounds probably have a mode of action similar to that of PI-55, which works rather as partial cytokinin antagonist, reducing expression of cytokinin reporter to 67% and 32%, in 1 and 10 μM concentrations, respectively (Fig. 4). The fact that compounds **9** and **10** have partial antagonistic activity in the ARR5:GUS reporter gene assay further indicates that in the case of cytokinin-derived compounds, there is probably a very narrow dividing line between structural requirements for the activation and blocking of cytokinin receptors.

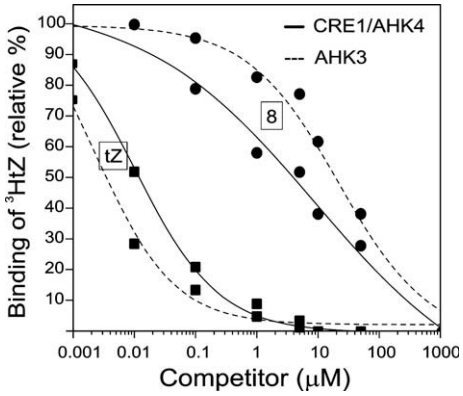


Fig. 3. Competitive binding assay with *Escherichia coli* expressing *A. thaliana* histidine kinases AHK3 and CRE1/AHK4. Binding of 2 nM [2−^{3}H]tZ was assayed together with increasing concentrations of compound **8** (circles); unlabelled tZ (squares) was used as positive control.

2.4. Activity in classical cytokinin bioassays

Cytokinin bioassays are based on various biological effects of cytokinins and reflect cytokinin activity in discrete biological processes. We chose three classical cytokinin bioassays (the tobacco callus, the *Amaranthus*, and excised wheat leaf senescence bioassay) to investigate the effect of different substitutions on the biological activity of the newly synthesized compounds. For comparison, BAP, a highly active and widely used cytokinin, was employed as a standard compound (all data are summarized in Table 3). Derivatives of PI-55 with a chlorine, a fluorine or an amino group in the C2 position are more biologically active than PI-55, exceeding BAP activity in the *Amaranthus* bioassay by 65%, 88% and 82%, respectively. In contrast a methylthio group in the C2 position caused a decrease of activity. Methylation in the N7 and N9 positions caused entire loss of activity in this bioassay. Compound **11** had the highest activity, which is in agreement with Doležal (Doležal et al., 2006). In contrast, LGR-991 (**8**) reached only 25% of BAP activity in this bioassay. In the senescence bioassay the only active compounds were **7**, **10** and **11**, whilst none of the PI-55

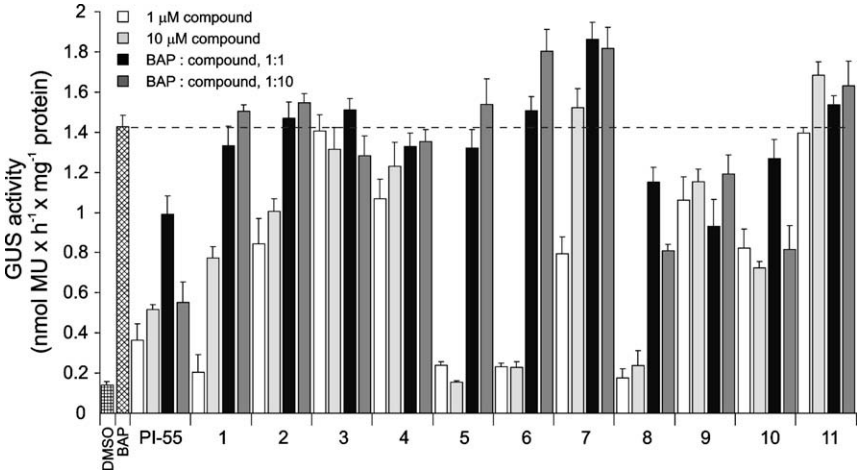


Fig. 4. Quantitative evaluation of GUS activity in *ARR5:GUS* transgenic Arabidopsis plants 3 days after germination. Compounds were tested alone in 1 and 10 μM concentrations or in a competitive assay at the same concentrations together with 1 μM BAP. Concentration of BAP was 1 μM; DMSO (0.1%) was used as solvent control. Error bars show s.d. ($n = 3$). MU, 4-methylumbelliferone.

Table 3
Relative cytokinin activity of the prepared 6-benzylaminopurine derivatives in classical cytokinin bioassays. The effect in optimal concentration is compared with the activity of 6-benzylaminopurine (BAP) (100% means 10^{-5} M BAP for the *Amaranthus* betacyanin bioassay, 10^{-4} M BAP in the case of the senescence bioassay and 10^{-6} M BAP for the tobacco callus bioassay).

Compound	*Amaranthus* bioassay		Senescence bioassay		Tobacco callus bioassay	
	Optimal concentration (mol l^{-1})	Relative activity (%)	Optimal concentration (mol l^{-1})	Relative activity (%)	Optimal concentration (mol l^{-1})	Relative activity (%)
PI-55	10^{-4}	55 (±12)	10^{-4}	2 (±2)	10^{-5}	9 (±6)
1	10^{-4}	82 (±8)	10^{-4}	1 (±1)	10^{-4}	29 (±7)
2	10^{-4}	37(±2)	10^{-4}	2 (±2)	10^{-5}	52 (±9)
3	10^{-4}	65 (±3)	10^{-4}	14 (±5)	10^{-5}	66 (±2)
4	10^{-4}	88 (±7)	10^{-4}	11 (±6)	10^{-5}	23 (±2)
5	10^{-4}	8 (±5)	10^{-4}	6 (±2)	10^{-4}	36 (±8)
6	10^{-4}	6 (±7)	10^{-4}	4 (±6)	10^{-4}	18 (±8)
7	10^{-4}	58 (±11)	10^{-4}	79 (±13)	10^{-4}	111 (±2)
8	10^{-4}	25 (±9)	10^{-4}	4 (±5)	10^{-4}	0
9	10^{-4}	39 (±3)	10^{-4}	22 (±9)	10^{-4}	48 (±9)
10	10^{-4}	69 (±12)	10^{-4}	48 (±12)	10^{-5}	43 (±6)
11	10^{-4}	107 (±16)	10^{-4}	56 (±8)	10^{-5}	99 (±10)

derivatives reached even 20% of the activity of BAP. Completely different results were obtained in the callus bioassay, where all substitutions on the PI-55 molecule increased the proliferation of callus in contrast to PI-55. Compound **11** had the same activity as BAP and the activity of **7** even exceeded that of the BAP. The only inactive compound in this assay was LGR-991 (**8**).

This indicates that in the case of 2-hydroxy-5-X-benzylaminopurine analogues (X = CH_3, Cl, F), both chlorine and fluorine enhanced the cytokinin activity, although not to the same extent as the methyl group. To conclude, 6-(2-hydroxy-5-methylbenzylamino)purine and 6-(2,5-difluorobenzylamino)purine were the most active compounds in the senescence and tobacco bioassays. The different activities in the bioassays and the receptor assay may indicate that either the cytokinin receptor AHK2 was involved in the biological response in bioassays, or there is another specific receptor for aromatic cytokinins, as suggested by previous studies of the cytokinin activity of BAP derivatives (Spíchal et al., 2004; Doležal et al., 2006).

2.5. Germination assay

Recent work by Riefler et al. (2006) showed that cytokinins inhibit the early stages of germination and that seeds of the Arabidopsis *ahk2 ahk3* receptor double mutant with decreased ability to detect cytokinins, germinate faster than wild-type seeds. For this reason we tested whether germination is affected when LGR-991 (**8**) is applied in the growth medium. As shown in Fig. 5 only 40% of all seeds germinated on the control medium containing 0.1% DMSO, whereas more than 60% of seeds germinated after 16 h on the medium which contained 1 nM LGR-991 (**8**) and almost 75% of seeds germinated on the medium which contained ten-times higher concentration. After 32 h 75% of the control seeds germinated, while all seeds had germinated in the presence of 10 nM LGR-991 (**8**). Similarly to PI-55 (Spíchal et al., 2009) LGR-991 (**8**) causes more rapid germination of Arabidopsis seeds, which is a characteristic of seeds from plants with a reduced cytokinin perception. These data thus confirm the *in vivo* anticytokinin activity of the novel cytokinin derivative LGR-991 (**8**).

2.6. Hypocotyl elongation of dark-grown seedlings

High cytokinin concentrations induce inhibition of hypocotyl elongation in dark-grown wild-type seedlings (Chory et al., 1994). Analyses of cytokinin receptor loss-of function mutants showed that hypocotyls of *ahk2 ahk3* and *ahk3 ahk4* seedlings were most resistant to cytokinin-induced hypocotyl shortening in the dark (Riefler et al., 2006). This suggested that AHK3 in combination with either AHK2 or CRE1/AHK4 is important to mediate cytokinin dependent deetoliation (Riefler et al., 2006). As shown in Fig. 6A, BAP (3 μM) reduced hypocotyl length of dark-grown wild-type

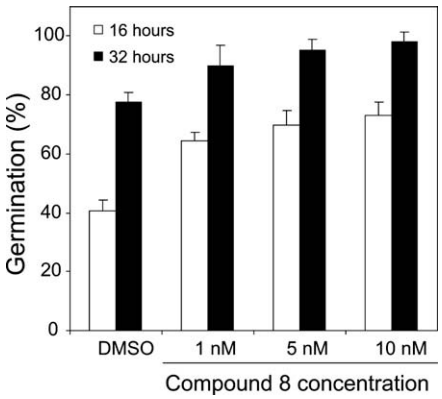


Fig. 5. Effect of compound **8** on germination of Arabidopsis seeds. The seeds were incubated on the medium containing indicated concentrations of compound **8**. The experiment was done in triplicate with 50 seeds for each experiment. Error bars show s.d. ($n = 3$).

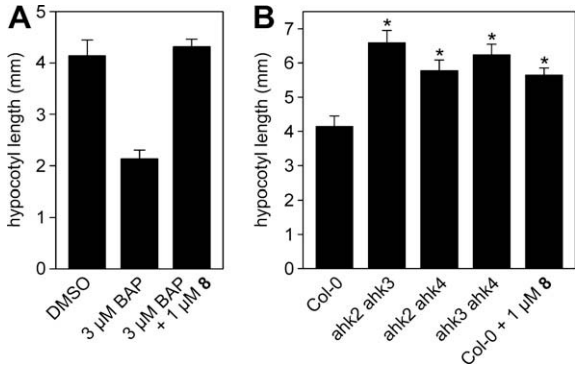


Fig. 6. Effect of compound **8** on hypocotyl elongation of dark-grown Arabidopsis seedlings. (A) Hypocotyl elongation of dark-grown wild-type (Col-0) plants on MS medium containing 3 μM BAP or in a competitive assay together with 1 μM LGR-991 (**8**) 2 days after transfer from 4 °C. (B) Comparison of hypocotyl elongation of Arabidopsis receptor double-mutants and wild-type (Col-0) plants (2 days after transfer from 4 °C) grown in the dark in the absence or presence of 1 μM LGR-991 (**8**). DMSO (0.026%) was used as solvent control in both experiments. Error bars represent s.e. ($n = 10$). Asterisks indicate statistically significant differences from the control wild-type plants (Student's t test, $P < 0.002$).

seedlings to almost 50%. LGR-991 (1 μM) completely reversed the hypocotyl length in the competition assay (Fig. 6A). This suggests that LGR-991 (**8**) effectively antagonized the deetoliation effect of exogenous cytokinin. Interestingly, LGR-991 (**8**) had a positive effect on hypocotyl growth when applied alone and significantly increased the length of dark-grown wild-type hypocotyls, thus mimicking the phenotype of cytokinin receptor double-mutants (Fig. 6B). This finding is in accordance with increase in the length of hypocotyl of the receptor triple mutants observed by Riefler et al. (2006) and supports the idea that LGR-991 (**8**) blocks perception of endogenous cytokinins *in planta*.

3. Conclusions

Eleven 6-benzylaminopurine analogues were synthesized and their cytokinin and anticytokinin properties were investigated. Modification of PI-55 did not lead to its enhanced antagonistic activity. It was found, however, that the newly synthesized BAP analogue, 6-(2,5-dihydroxybenzylamino)purine, inhibited CRE1/AHK4 at the molecular level to the same extent as PI-55 and, in addition, also blocked the AHK3 receptor. Thus this compound shows a new structural motive that can lead to the preparation of cytokinin antagonists with broader specificity. The *in vivo* experiments with transgenic ARR5:GUS Arabidopsis plants confirmed the antagonistic action of 6-(2,5-dihydroxybenzylamino)purine. Classical cytokinin bioassays proved that the compound does not have intrinsic cytokinin activity. The findings that 6-(2,5-dihydroxybenzylamino)purine accelerated germination of Arabidopsis seeds and blocked cytokinin-mediated inhibition of hypocotyl growth in the dark point to the ability of the compound to inhibit cytokinin action. Thus, this compound might find potential use as inhibitor of some cytokinin effects *in planta*.

4. Experimental procedures

4.1. General experimental procedures

The purity of synthesized compounds was confirmed by high performance liquid chromatography (Beckman Gold System). The melting points were determined on a Boetius stage apparatus and are uncorrected. Analytical thin-layer chromatography (TLC) was carried out using silica gel 60 WF_{254} plates (Merck). Flash chromatography was performed using a VersaFlash purification station (Supelco) coupled to a 2110 Fraction Collector (Bio-Rad). Compounds were separated on VersaPak Cartridges (23 × 110 mm, Supelco) containing 23 g of spherical silica and eluted with a mobile phase containing $CHCl_3$/MeOH (90:10, v/v). Elemental composition of prepared compounds was confirmed by cap-LC-HR-(ESI^+) MS (Q-Tof *micro*™ Waters MS Technologies, Manchester, UK). Accurate masses were calculated and used for the determination of the elemental composition of the analytes with fidelity better than 3 ppm. For the MS/MS experiments, the fragmentation was done in argon gas-filled collision cell with collision energies of 20, 25, 30 and 35 eV. Data are summarized in Table 2. Structure confirmation of the newly synthesized compounds is supported by the list of product ions in Table 4, Supplementary data). NMR spectra were measured on a Bruker Avance AV 300 spectrometer operating at a temperature of 300 K and a frequency of 300.13 MHz (^1H). Samples were prepared by dissolving the compounds in DMSO-d_6. Tetramethylsilane (TMS) was used as the internal standard.

4.2. Chemicals

2-Methylthio-6-chloropurine, 2-amino-6-chloropurine, 2,6-dichloropurine, 6-chloropurine, 6-benzylaminopurine, 6-(4-hydroxy-3-methylbut-2-enylamino)purine (tZ) were purchased from Olchemim (Olomouc, Czech Republic). Sigma Aldrich supplied triphenylphosphine, diisopropyl azodicarboxylate, 2,5-dimethoxybenzylamine, *N*,*N*-diisopropyl-*N*-ethylamine, 2,5-difluorobenzylamine, 2-hydroxy-5-fluorobenzaldehyde, 2-hydroxy-5-chlorobenzaldehyde, 2-hydroxy-5-methybenzaldehyde, 2-hydroxy-3-methylbenzaldehyde, hydroxylamine hydrochloride, tetrafluoroboric acid, 4-methyl umbelliferyl galactoside, 4-methylumbelliferone and DMSO. Sodium nitrite, triethylamine, hydroiodide acid and acetic anhydride were obtained from Lachner. Merck supplied casamino acids. Radiolabelled *trans*-zeatin ([2-^3H]zeatin) was synthesized by J. Hanuš (Isotope Laboratory, Institute of Experimental Botany, AS CR, Prague, Czech Republic). The other solvents and chemicals used were all of standard p.a. quality.

4.3. Synthesis of 6-benzylaminopurines

The general procedures for preparation of 6-benzylaminopurines were described earlier (Okumura et al., 1959; Tarkowská et al., 2003). In brief, 6-chloropurine was heated with the appropriate primary amine at 90 °C for 4 h in *n*-butanol containing an

excessive amount of triethylamine. After cooling, the precipitated product was filtered off, washed with cold water and *n*-butanol and crystallized from ethanol. The synthesis of 2-fluoro-6-chloropurine was previously described by Beach et al. (1992). 6-(2-Hydroxy-3-methylbenzylamino)-9-methylpurine and 6-(2-hydroxy-3-methylbenzylamino)-7-methylpurine were prepared by a common procedure from appropriate starting compounds.

4.3.1. Preparation of 6-chloro-9-methylpurine and 6-chloro-7-methylpurine

The 6-chloropurine (1.54 g, 10 mmol) was added to a solution of methanol (3.5 ml, 100 mmol) and triphenylphosphine (3.14 g, 12 mmol) in anhydrous tetrahydrofuran (50 ml) under a nitrogen atmosphere. Then diisopropyl azodicarboxylate (2.42 g, 12 mmol) was added dropwise and the reaction mixture was stirred at room temperature for 1 h and evaporated to the yellow semisolid residue, which was purified by flash chromatography on silica gel (as described above) to give 6-chloro-9-methylpurine and 6-chloro-7-methylpurine in ratio 7 to 3. These isomers were then used for synthesis of compounds **5** and **6**.

4.3.2. Preparation of substituted aminomethylphenoles

The synthesis of 2-aminomethyl-4-methylphenol and 2-aminomethyl-6-methylphenol was described elsewhere (Raiford and Clark, 1923; Weinstein and Holm, 1972). Generally, they are derived from the appropriate benzaldehydes which are converted to corresponding aldoximes and are consequently reduced by sodium amalgam in aqueous lower alcohol to give desired primary amines. 2-Aminomethyl-4-fluorophenol and 2-aminomethyl-4-chlorophenol were prepared according to Stokker et al. (1980), by a similar procedure as mentioned above using 5% Rh/C as a catalyst.

4.3.3. Preparation of 2-aminomethyl-benzene-1,4-diol

2,5-Dimethoxybenzylamine (5.5 g, 33.2 mmol) was converted to 2,5-dihydroxybenzylamine as previously described (Ikeda et al., 1977; Lin and Driscoll, 1981). Acetic anhydride (40 ml) was added, by syringe, to 2,5-dimethoxybenzylamine solution in conc. hydroiodic acid (60 ml) under nitrogen. The reaction mixture was refluxed at 107 °C for 4 h. After evaporation of the acid *in vacuo*, ethanol (30 ml) was added to the residue, and the solvent was again evaporated. The crude product was crystallized from ethanol to give the amine as hydroiodide.

4.3.4. Preparation of 2-amino-6-(2-hydroxy-3-methylbenzylamino)purine

To a suspension of 2-amino-6-chloropurine (170 mg, 1 mmol) and 2-aminomethyl-6-methylphenol (137 mg, 1 mmol) in methanol (20 ml), *N*,*N*-diisopropyl-*N*-ethylamin (1.29 g, 10 mmol) was added. The reaction mixture was heated in a glass Ace pressure tube (Sigma–Aldrich, 35 ml) at 100 °C for 5 days. The solvent was then removed by vacuum distillation and the white residue was briefly refluxed in isopropanol (15 ml) to remove traces of starting material. The white precipitate was filtered off, washed with isopropanol (4 ml) three times and dried in a vacuum oven at 60 °C. The yield was 35 mg (12%).

4.3.5. Preparation of 2-Fluoro-6-(2-hydroxy-3-methylbenzylamino)purine

2-Fluoro-6-chloropurine (173 mg, 1 mmol) and 2-aminomethyl-6-methylphenol (137 mg, 1 mmol) were refluxed (78 °C) in ethyl acetate (4 ml) in the presence of triethylamine (506 mg, 5 mmol) for 5 h. After solvent evaporation the residue was treated with water (5 ml) to give a crude product which was re-crystallized from ethanol (3 ml). The yield was 220 mg (80%).

4.4. Bacterial cytokinin assay

E. coli strain KMI001, harbouring either the plasmid pIN-III-AHK4 or pSTV28-AHK3, which express the Arabidopsis histidine kinases CRE1/AHK4 or AHK3 (Suzuki et al., 2001; Yamada et al., 2001) was used in the experiments. Bacterial strains were kindly provided by T. Mizuno (Nagoya, Japan) and the whole assay was performed as previously described in (Spíchal et al., 2004).

4.5. Live-cell cytokinin-binding assay

The same bacterial strains and growing conditions were used as described above. The live-cell cytokinin-binding assay was performed essentially as described in Romanov et al. (2005). Homogenous bacterial suspension with an OD_{600} of 0.8 and 1.2 were found to be optimal for CRE1/AHK4 and AHK3, respectively. The competition reaction was allowed to proceed with 2 nM [2-^{3}H]tZ and various concentrations of tested compounds for 30 min at 4 °C. When binding equilibrium was reached, the suspension was centrifuged (6000×*g*), the supernatant was removed and the bacterial pellet was resuspended in scintillation cocktail (Beckman, Ramsey, MN, USA). Radioactivity was measured by scintillation counting on a Beckman LS 6500 scintillation counter (Beckman, Ramsey, MN, USA).

4.6. The cytokinin bioassays

Classical cytokinin bioassays (tobacco callus, *Amaranthus* assay and senescence assay with excised wheat leaves) were performed as described in Holub et al. (1998).

4.7. Arabidopsis ARR5:GUS reporter gene assay

Arabidopsis ARR5:GUS transgenic seeds were surface-sterilized and sown on half-strength MS medium including vitamins (Duchefa, Haarlem, The Netherlands) supplemented with 0.1% (w/v) sucrose and 0.05% (w/v) MES–KOH (pH 5.7) in 6-well plates (TPP, Switzerland). After pre-treatment at 4 °C for 3 days in darkness, the seedlings were grown under long-day conditions (16 h light/8 h dark) at 22 °C in a growth chamber. To the wells containing 3-day-old seedlings, BAP and/or tested compound or DMSO (solvent control, final concentration 0.1%) was added and the seedlings were grown for an additional 16 h. Quantitative determination of GUS activity was performed according to a published protocol (Romanov et al., 2002) by measuring fluorescence on a Fluoroskan Ascent microplate reader (Labsystems, Helsinki, Finland) at excitation and emission wavelengths of 365 and 450 nm, respectively. GUS specific activity was expressed as nmol 4-methylumbelliferone (MU)/h/mg protein. Determination of protein content was done by bicinchonin acid reagent (Smith et al., 1985).

4.8. Germination assay

A. thaliana ecotype Columbia (Col-0) seeds were sown on MS medium lacking sucrose, vernalized at 4 °C and then transferred to white light (~75 μE) under long-day conditions at 22 °C. The germination rate was determined at 16 and 32 h after sowing. Experiments were done in triplicate with 50 seeds for each experiment.

4.9. Hypocotyl elongation assay

Seeds of *A. thaliana* wild-type (Col-0), and receptor double mutants (*ahk2 ahk3*, *ahk2 ahk4* and *ahk3 ahk4*; kindly provided by Prof. Thomas Schmülling) were surface sterilized and sown on vertical plates on half-strength Murashige-Skoog medium (supplied

with 0.1% sucrose) containing different concentrations of BAP and/or LGR-991 (**8**), or 0.06% DMSO (solvent control). After vernalization (2 days in the dark, 4 °C) plates were transferred into the growth chamber (24 °C, dark). Three days after transfer, seedlings were photographed and hypocotyl elongation was scored using Scion image software (Scion Corp., Frederick, MD, USA). At least 10 hypocotyls were measured for each genotype.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.01.018.

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