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N9-Substituted N^6-[(3-methylbut-2-en-1-yl)amino]purine derivatives and their biological activity in selected cytokinin bioassays

Václav Mik [a], Lucie Szüčová [a,*], Lukáš Spíchal [a], Ondřej Plíhal [a], Jaroslav Nisler [b], Lenka Zahajská [c], Karel Doležal [a], Miroslav Strnad [a]

[a] Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University in Olomouc, Šlechtitelů 11, CZ 783 71 Olomouc, Czech Republic
[b] Laboratory of Growth Regulators, Institute of Experimental Botany AS CR, Šlechtitelů 11, CZ 783 71 Olomouc, Czech Republic
[c] Isotope Laboratory of Institute of Experimental Botany AS CR, Vídeňská 1083, CZ 142 20 Prague 4, Czech Republic

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ABSTRACT

Rational design is one of the latest ways how to evaluate particular activity of signal molecules, for example cytokinin derivatives. A series of N^6-[(3-methylbut-2-en-1-yl)amino]purine (iP) derivatives specifically substituted at the N9 atom of purine moiety by tetrahydropyran-2-yl, ethoxyethyl, and C2–C4 alkyl chains terminated by various functional groups were prepared. The reason for this rational design was to reveal the relationship between specific substitution at the N9 atom of purine moiety of iP and cytokinin activity of the prepared compounds. The synthesis was carried out either via 6-chloro-9-substituted intermediates prepared originally from 6-chloropurine, or by a direct alkylation of N9 atom of N^6-[(3-methylbut-2-en-1-yl)amino]purine. Selective reduction was implemented in the preparation of compound N^6-[(3-methylbut-2-en-1-yl)amino]-9-(2-aminoethyl-amino)purine (**12**) when 6-[(3-methylbut-2-en-1-yl)amino]-9-(2-azidoethyl)purine (**7**) was reduced by zinc powder in mild conditions. The prepared derivatives were characterized by C, H, N elemental analyses, thin layer chromatography (TLC), high performance liquid chromatography (HPLC), melting point determinations (mp), CI+ mass spectral measurement (CI+ MS), and by ^{1}H NMR spectroscopy. Biological activity of prepared compounds was assessed in three in vitro cytokinin bioassays (tobacco callus, wheat leaf senescence, and *Amaranthus* bioassay). Moreover, the perception of prepared derivatives by cytokinin-sensitive receptor CRE1/AHK4 from *Arabidopsis thaliana*, as well as by the receptors ZmHK1 and ZmHK3a from *Zea mays*, was studied in a bacterial assay where the response to the cytokinin treatment could be specifically quantified with the aim to reveal the way of the perception of the above mentioned derivatives in two different plant species, that is, *Arabidopsis*, a model dicot, and maize, a model monocot. The majority of cytokinin derivatives were significantly active in both *Amaranthus* as well as in tobacco callus bioassay and almost inactive in detached wheat leaf senescence assay. N9-Substituted iP derivatives remained active in both in vitro bioassays in a broad range of concentrations despite the fact that most of the derivatives were unable to trigger the cytokinin response in CRE1/AHK4 and ZmHK1 receptors. However, several derivatives induced low but detectable cytokinin-like activation in maize ZmHK3a receptor. Compound 6-[(3-methylbut-2-en-1-yl)amino]-9-(tetrahydropyran-2-yl)purine (**1**) was also recognized by CRE1/AHK4 at high concentration $\geqslant$50 μM.

1. Introduction

Isoprenoid cytokinins, among which N^6-[(3-methylbut-2-en-1-yl)amino]purine belongs, are a group of naturally occurring signaling molecules structurally based on purine that are able to influence physiological processes such as plant growth and development as well as cell differentiation.[1] N^6-[(3-Methylbut-2-en-1-yl)amino]-purine, also known as isopentenyladenine (iP), was originally synthesized by Leonard et al. in 1965 and it was discovered that the derivative has high cytokinin activity.[2,3] Despite of previous discoveries of iP hydroxylated analogs *cis*-zeatin (cZ), *trans*-zeatin (tZ), and dihydrozeatin (DHZ) in plants, a free base of iP was found in autonomous strains of tobacco tissue only in the year 1972.[4] Isoprenoid cytokinin derivatives were often modified with the aim to find a new, perspective plant growth regulator, especially after the finding that isoprenoid cytokinins were preferably recognized by cytokinin receptors AHK3 and CRE1/AHK4 present in a dicot plant *Arabidopsis thaliana* used currently as an experimental model.[5] Isoprenoid cytokinins also trigger signaling pathway in known *Zea mays* receptors ZmHK1, ZmHK2, and ZmHK3a/ZmHK3b.[6] The majority of synthetic derivatives of isoprenoid cytokinins prepared so far has been mainly

* Corresponding author. Tel.: +420 585634940; fax: +420 585634870.
E-mail address: lucie.szucova@upol.cz (L. Szüčová).

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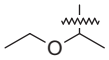
mimetic compounds with serious changes of N^6-side chain or heterocyclic moiety such as 6-alkenyl and 6-alkynylpurines,[7] N^6-alkylaminopurines,[8] 7-acylaminoimidazo[4,5-*b*]pyridines or 4-acylaminoimidazo[4,5-*c*]pyrimidines,[9] 5-amino substituted 7-chloro-imidazo[1,2-*c*]pyrimidines,[10] or N^6-ethoxyaminopurines.[11] Unfortunately, their cytokinin activity has never exceeded the activity of parent iP. In the last 15 years, after the discovery of benzylaminopurine (BA) derivatives in plants,[12] the majority of interest has been paid to synthesis and biological activity of fully or partly aromatic cytokinin derivatives, such as to (*Z*)- and (*E*)-β-substituted 6-styrylpurines,[13] benzyl ring substituted benzylaminopurines,[14] aromatic *N*9-(tetrahydropyran-2-yl)purines and *N*9-(tetrahydrofuran-2-yl)purines,[15] N9-substituted kinetin derivatives,[16] and aromatic N9-ribosides.[17] Even though many of the above mentioned aromatic cytokinin derivatives were highly active in various cytokinin bioassays, with the exception of *meta*-topolin, they usually did not trigger cytokinin signaling pathway of the above mentioned AHK3 and CRE1/AHK4 receptors.[4] Therefore, we have attempted to prepare molecules that are not mimetic but direct derivatives of the isoprenoid cytokinin with the aim to obtain active derivatives that are able to trigger cytokinin signaling pathway. Today, combinatorial organic chemistry allows us to prepare precisely substituted molecules, for example, N^6-[(3-methylbut-2-en-1-yl)amino]purine substituted in C2, N1, N3, N7, C8 as well as at N9 position of purine moiety. Therefore, we specifically focused on N9 position of iP as the position that is, due to 9-β-D-glucopyranylation, naturally occupied in the cytokinin metabolism in plants.[1,18] Generally, N9-glucosides are considered as nonactive or weakly active cytokinin forms.[1,19] For that reason, the preparation of N9-substituted cytokinin derivatives as the compounds prospective for plant growth is rather controversial. Isopentenyladenine derivatives substituted at N9 by 2-carboxyethyl, 2-carbo-*t*-butoxyethyl, 2-nitriloethyl were prepared by Corse et al. in 1989 and they were tested for growth response in the soybean callus assay. They all reduced the biological activity of the parent compound even though they all still remained considerably active.[20] Corse's finding contributed to the widely spread assumption that N9-substituted derivatives are less active than their free bases. On the other hand, the N9 analogs of aromatic cytokinins with certain substituents very often maintained cytokinin activity, for example, *N*9-(tetrahydropyran-2-yl) derivatives[15,21,22] or *N*9-methyl derivatives[23] and sometimes were even more effective than their free bases. Conversely, cytokinins substituted by 9-methoxymethyl, 9-cyclohexyl or 9-(3-hydroxypropyl) groups were less active in tobacco callus assay than the unsubstituted ones.[24] 9-(4-Chlorobutyl) derivative of benzylaminopurine was substantially more effective in the ability to cause chlorophyll retention in intact soybean leaves.[25] N9-Halogenoalkyl and other N9-substituted derivatives of kinetin showed strong anti-senescent activities under both dark and light conditions and in addition, the compounds were able to protect membrane lipids against the negative action of reactive oxygen species that accumulate in tissues during leaf senescence.[16] Young and Letham modified iP with the substitution in N9 by the addition of tetrahydropyran-2-yl (THP) group and the substitution markedly enhanced the activity of iP in tobacco leaf senescence assay.[19] Tetrahydropyran-2-yl derivative of iP can serve as a good example of how controversial can be the results from the testing of various N9 cytokinin derivatives in available cytokinin assays – substituent in N9 may probably play as important role as the chosen testing system. In addition, N9 substitution of cytokinins offers the easiest way how to prepare direct, not mimetic analogs of free cytokinin bases. Therefore, we prepared a small library of variously alkylated N9-iP derivatives with a number of terminal groups such as hydroxy-, chloro-, bromo-, amino-, azido-, cyano-, carboxy-, etc. These functional groups were placed at the end of the carbon or carbon/oxygen spacers with modifiable length. We tested their biological properties in cytokinin bioassays such as tobacco callus, detached wheat leaf senescence, and *Amaranthus* bioassay in order to clarify ambiguous situation that persists in the structure and activity relationship of N9-substituted cytokinins. In addition, using these compounds we also studied the cytokinin perception in *A. thaliana* receptor CRE1/AHK4 and maize ZmHK1 and ZmHK3a receptors to compare the results in two different model plants.

Table 1
Structures of the prepared compounds and their abbreviations. Wavy line represents a position of connection to the N9 atom of the purine moiety

Compound	R
1	
2	
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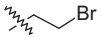
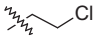
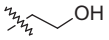
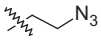
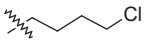
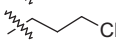
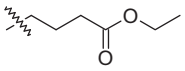
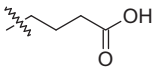
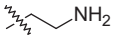
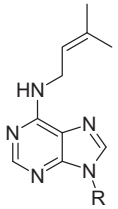
4	
5	
6	
7	
8	
9	
10	
11	
12	

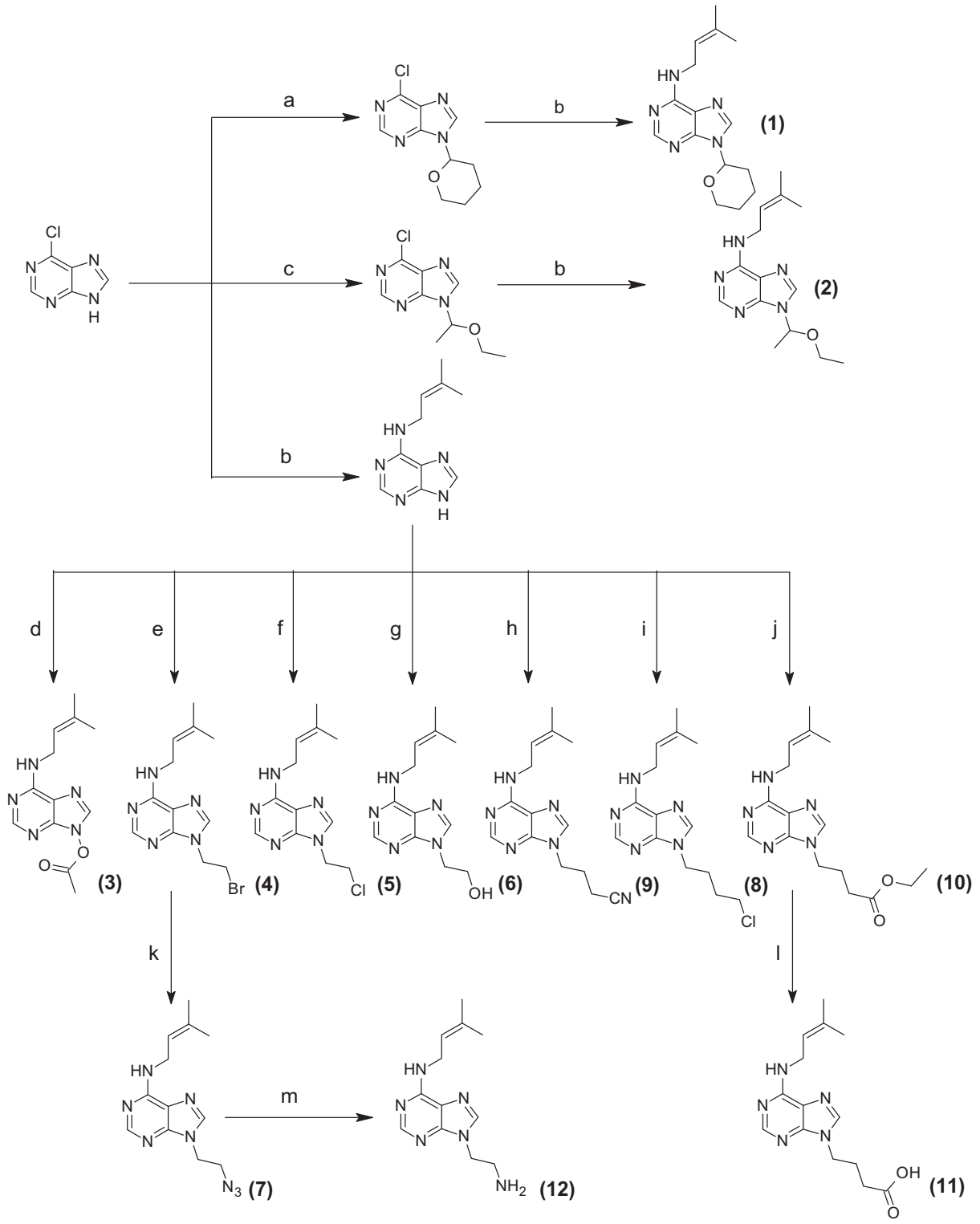
2. Results and discussion

2.1. Synthesis

The compounds described in this paper are shown in the Scheme 1 while the substituents in N9 position of the purine moiety are



Scheme 1. Schematic representation of *N*6-isopentenyladenine derivatives.



Scheme 2. Reaction scheme for the synthesis of N9-substituted cytokinin derivatives. (a) 3,4-Dihydropyran, EtOAc, TFA, rt, 2 h; (b) 3-methylbut-2-en-1-amine HCl, Et_3N, PrOH, 100 °C, 4 h; (c) ethyl vinyl ether, EtOAc, TFA, rt, 2 h; (d) sodium acetate, DMF, 100 °C, 3 h; (e) 1,2-dibromoethane, K_2CO_3, DMSO, rt, 12 h; (f) 1-bromo-2-chloroethane, K_2CO_3, DMSO, rt, 12 h; (g) ethylene carbonate, NaOH, DMF, 154 °C, 2 h; (h) 4-chlorobutyronitrile, K_2CO_3, DMF rt, 12 h; (i) 1-bromo-4-chlorobutane, K_2CO_3, DMSO, rt, 12 h; (j) ethyl 4-bromobutyrate, K_2CO_3, DMF, rt, 12 h; (k) 0.5 M NaN_3 in DMSO, 80 °C, 1 h; (l) NaOH, 50% ethanol, rt, 12 h; (m) Zn, NH_4Cl, EtOH, H_2O, rt, 2 h.

listed in Table 1. From Scheme 2 it is obvious that the compounds were prepared either via the appropriate 6-chloro-9-substituted derivative (**1** and **2**) made of 6-chloropurine that afterward reacted with 3-methylbut-2-en-1-yl)amine, or by a direct alkylation of 6-[(3-methylbut-2-en-1-yl)amino]purine (**3**–**12**) in the presence of potassium carbonate in DMSO or DMF followed by purification from arising N7 derivative using flash chromatography. 6-[(3-Methylbut-2-en-1-yl)amino]purine was prepared according to slightly modified procedure described by Robins et al. in the literature.[26] Compound **2** was prepared according to a paper previously

published in literature.[27] We used one of the suggested methods that was reaction of 6-chloropurine with ethyl vinyl ether and subsequently with 3-methylbut-2-en-1-ylamine as given in Scheme 2. For the compound **6**, we adopted preparation published by Rosengren et al. for 9-(2-hydroxyethyl)adenine synthesis[28] but the yield of **6** was not as high as for 9-(2-hydroxyethyl)adenine. Particular syntheses are given in greater detail in Section 4.3. Prepared compounds were characterized by elemental analyses, thin layer chromatography (TLC), melting point determinations (mp), CI+ MS, and ^{1}H NMR spectroscopy. The purity of the prepared substances was confirmed by high-performance liquid chromatography (HPLC). Table 2 shows C, H, N elemental analysis data, melting points, MS and HPLC purity data. ^{1}H NMR spectral data are given in Supplementary data.

2.2. Cytokinin bioassays

The preparation of N9-substituted cytokinins as the compounds prospective for plants is rather controversial due to unlike results obtained for N9-substituted derivatives in some cytokinin bioassays[29] as well as for the known fact that glucosylation on N9 position of the purine based cytokinins may turn active free cytokinin into their inactive forms: N9-glucosides. On the other hand, some N9 analogs of aromatic cytokinins very often maintained cytokinin activity[15,22,23,25] while some others were reported to be less effective than free bases[24] or reduced the biological activity of parent compound even when still maintained considerable activity.[20] However, Corse emphasis the fact that the activity is probably launched with the polarity and steric effect of particular substituents.[20] The compounds prepared within the frame of this study were subjected to three cytokinin bioassays (tobacco callus, wheat senescence, and *Amaranthus* bioassay)[17] and the results are summarized in Table 3. The achieved activities of new compounds were compared to those of iP that represents free base without any N9 substitution. The effects of novel compounds on the proliferation of cytokinin-dependent tobacco callus were tested in the callus bioassay. Derivatives **2**, **3**, and **7**–9 showed very high activity exceeding 110% of the activity of parent compound (iP), when used at their optimal concentrations (Table 3). The activity of compounds **5**, 10, and **12** almost equaled the activity of iP and, interestingly, also the other derivatives **1**, **4**, **6**, 11 reached only slightly lower activity then their parent compound. Cytokinins are known for their stimulatory effects on cell proliferation in their optimal concentrations, however typically in the concentrations exceeding 10 μM their effect on cell division turns into inhibitory.[31] The compound **1**, behaved as classical cytokinin and, in a manner similar to iP, inhibited cell proliferation at concentrations exceeding 10 μM (Fig. 1A). In contrast, many other compounds, for example, **2**–**4** and **6**–8 showed no such activity, instead maintaining a strong stimulatory effect on cell division, even at the highest concentration tested (100 μM, Fig. 1A). In *Amaranthus* bioassay, some of new compounds were found to be highly active again. The most active substances (compounds **2** and **4**) exceeded 120% of iP activity (Table 3; Fig. 1B) followed by compounds **1**, 5, and **10** that were still more active than iP. Other compounds were less active and their activities fluctuated from approximately 80% to 30% (Table 3). In

Table 2
Elemental analyses, melting points, CI+ MS spectral data and HPLC purity of the prepared compounds

Compound	Elemental analyses calculated/found			Mp (°C)	CI+ MS [M+H$^+$]	HPLC (%)
	%C	%H	%N			
1	62.7/62.5	7.37/7.5	24.4/24.4	85.7–89.5	288.3	98.5
2	61.1/60.6	7.7/7.8	25.4/25.6	44.1–45.5	276.3	99.4
3	62.7/53.9	7.4/6.5	24.4/22.8	55.7–57.1	290.3	98.8
4	46.5/46.3	5.2/5.2	22.6/22.5	120.2–121.5	311.2	99.5
5	54.2/54.6	6.1/6.2	26.4/26.2	130.2–132.3	266.1	99.8
6	58.3/57.7	6.9/6.7	28.3/24.2	73.1–76.2	248.3	98.5
7	52.9/52.2	5.9/5.9	41.2/43.3	92.4–94.9	273.1	99.9
8	57.2/56.4	6.9/6.9	23.8/22.7	141.1–142.7	294.8	99.0
9	62.2/59.1	6.7/7.0	31.1/28.1	59.5–61.3	271.2	98.8
10	60.6/60.4	7.3/7.4	22.1/20.7	55.5–57.8	318.2	99.9
11	58.1/56.1	6.6/6.5	24.2/26.0	152.6–154.7	290.2	99.5
12	58.5/58.5	7.4/7.5	34.1/35.2	164–169	247.5	98.5

Table 3
Relative cytokinin activity of the prepared *N*6-(2-isopentenyl)-adenine (iP) derivatives in classical cytokinin bioassays. The effect in optimal concentration is compared with the activity of parental compound iP (100% means 10^{-5} M iP for the *Amaranthus* betacyanin bioassay, 10^{-4} M iP for the senescence bioassay and 10^{-5} M iP for the tobacco callus bioassay). The values represent means ± SD of at least two independent assays each performed in five technical replicates

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 (%)
1	10^{-4}	109.3 ± 24	10^{-4}	53.0 ± 3.9	10^{-5}	79.3 ± 6.1
2	10^{-4}	122.8 ± 0.1	10^{-4}	11.0 ± 1.8	10^{-5}	118.0 ± 11
3	10^{-4}	82.1 ± 32	10^{-5}	66.5 ± 0.8	10^{-5}	110.5 ± 7.8
4	10^{-4}	120.8 ± 4.1	10^{-4}	37.7 ± 14.6	10^{-5}	80.9 ± 30
5	10^{-4}	115.0 ± 19	10^{-4}	23.2 ± 6.6	10^{-5}	98.7 ± 19
6	10^{-4}	29.0 ± 1.8	10^{-4}	47.0 ± 14	10^{-5}	82.7 ± 1.0
7	10^{-4}	82.3 ± 7.3	10^{-4}	n.a.	10^{-4}	122.5 ± 6.3
8	10^{-4}	59.9 ± 7.0	10^{-4}	39.5 ± 5.9	10^{-4}	115.15 ± 10
9	10^{-4}	61.2 ± 3.2	10^{-4}	15.5 ± 7.8	10^{-4}	119.5 ± 0.1
10	10^{-4}	102.2 ± 21	10^{-4}	53.2 ± 4.3	10^{-5}	92.6 ± 23
11	10^{-4}	47.8 ± 5.4	10^{-4}	n.a.	10^{-5}	79.7 ± 10
12	10^{-4}	33.7 ± 8.3	10^{-4}	n.a.	10^{-5}	95.0 ± 18

n.a. means not active.

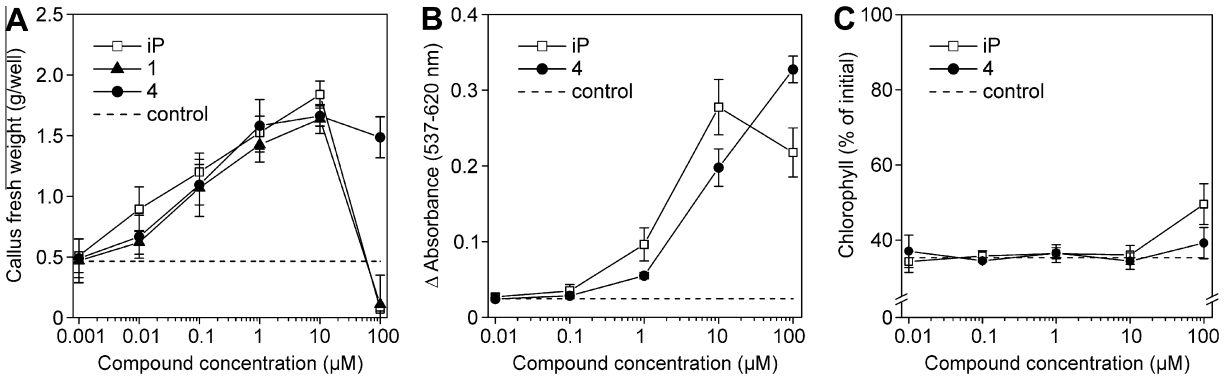


Figure 1. The influence of the N9-substituents of iP, particularly tetrahydropyran-2-yl (**1**) and 2-bromoethyl (**4**), on biological activity in cytokinin bioassays, particularly on the stimulation of cytokinin-dependent tobacco callus growth (A), the dark induction of betacyanin synthesis in *Amaranthus* cotyledons (B) and on the retention of chlorophyll in excised wheat leaves (C).

all cases, the maximal activities were reached in 10-times higher concentration (100 μM) than in the case of free iP control. The newly prepared compounds were further tested in detached wheat leaves senescence bioassay where the effects of the compounds on the retention of chlorophyll in detached wheat leaves in the dark was observed. Despite of the recently published results on N9-substituted kinetin derivatives[16] N9-iP derivatives did not show significant antisenescence activity as obvious from Table 3. The overall low activity of iP derivatives in the senescence assay is not surprising due to the fact that iP itself is only weakly active in this assay compared to other cytokinins such as BA or tZ. Although we claim that the structural motifs for senescence delay are rather localized in the N6 part of the purine molecule, we showed again that short alkylhalogen substitutions (2-chloroethyl, 2-bromoethyl) in N9 part of the molecule can also significantly improve cytokinin properties of the compound and, additionally, can prevent negative effects on cell proliferation, which are commonly caused by high concentrations of exogenously applied cytokinins.

2.3. CRE/AHK4, ZmHK1, and ZmHK3a receptor bioassays

Previous tests of various N9-substituted cytokinins showed that majority of aromatic cytokinins are probably not very good substrates for CRE1/AHK4 receptor.[5,15,16] Therefore, we chose isoprenoid cytokinin isopentenyladenine (iP) as a joint structural motif of newly prepared derivatives, because iP triggers signaling pathway in all the intended receptors very effectively.[5,6] We compared the response of CRE1/AHK4 receptor of a dicot model plant *Arabidopsis* with the response in the receptors of a model monocot maize, particularly in ZmHK1 and ZmHK3a, using a bacterial assay we have adapted previously,[5] to reveal if N9 isopentenyladenine derivatives perception may differ among different plants. We were also interested in the fact if the response is dependent on structural motif of the substituent in N9 position and/or polarity of terminal group, and if the perception in one plant differs from one receptor to another. We therefore tested the interactions between our new iP derivatives and the *Arabidopsis* cytokinin receptor CRE1/AHK4, using a transformed *Escherichia coli* strain expressing the receptors and the cytokinin-activated reporter gene *cps*::*lacZ* (fusion gene of capsular polysaccharide synthesis operon and reporter gene coding for β-galactosidase.[5,32] The risk of metabolic conversion of the tested cytokinin derivative was eliminated due to the short incubation time in this assay. With the exception of compound **1** which activated the CRE1/AHK4 receptor to approximately 25% in 50 μM concentration, all other N9-substituted iP derivatives that were otherwise also highly active in bioassays (i.e., compounds **2**, **7**, and **8**) were not able to activate CRE1/AHK4 (Fig. 2A. Therefore, we performed receptor tests also in maize receptors ZmHK1 ad

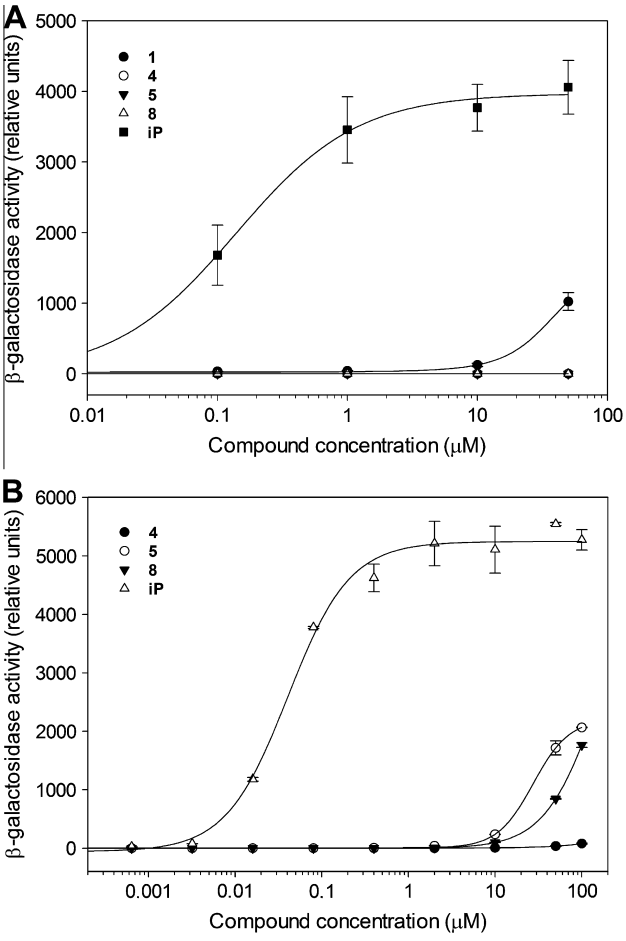


Figure 2. The perception of N9-substituted compounds **1**, **4**, **5**, and **8** by cytokinin recognizing receptor CRE1/AHK4 (A) and of the compounds **4**, **5**, and **8** by cytokinin recognizing receptor ZmHK3a (B), both by cytokinin-induced β-galactosidase assay. iP was used as a positive control. Error bars show SEM for three to six replicates.

ZmHK3a for selected derivatives with *N*9-(2-bromoethyl) (compound 4), *N*9-(2-chloroethyl) (compound 5), and *N*9-(4-chlorobutyl) (compound 8) side chains. While neither of the compounds was able to activate ZmHK1 receptor, both of them (**5** and **8**) triggered signaling pathway via ZmHK3a receptor approximately at the concentration above 10 μM and higher (given in Figure 2B). Despite the fact that the perception via ZmHK3a receptor was lower than in the case of iP control, it contributed the fact

that N9-halogenoalkyls of cytokinins maintain their cytokinin activity at high concentrations.[16] The length of the spacer did not make a difference in the cytokinin perception, but the terminal substitution proved to be critically important in the subsequent receptor activation. While *N*9-(2-chloroethyl) and N9-(2-chlorobutyl) showed measurable activation of the receptor, 2-bromoethyl derivative did not show any cytokinin-like activity (Fig. 2B) This can be caused by the fact that bromine atom is markedly bigger than chlorine and that dimensions of the terminal group might thus play role in the interaction with the receptor domain.

3. Conclusions

We prepared twelve N9-substituted isopentenyladenine derivatives with the aim to bring more light on structure and activity relationship of N9-substituted cytokinin derivatives. The substitution of iP in N9 position of the purine with ethoxyethyl-, acetoxy-, azido-, 4-chlorobutyl-, and 3-cyanopropyl-groups significantly improved their biological activity in proliferation of plant cells in tobacco callus assay in comparison to iP, although it negatively influenced the recognition of these compounds by cytokinin receptors CRE1/AHK4, ZmHK1, and ZmHK3a. Due to the fact that all the above mentioned groups have high polarity and the substituents consist of at least three spacer atoms, we can state that polarity and length of the N9-substituent chain has significant impact on compound activity in tobacco callus assay. Majority of prepared compounds also showed high activity in *Amaranthus* bioassay. Isopentenyladenine derivatives substituted on N9 position are not, despite of their similarity to the kinetin derivatives that possess significant activities, effective retardants of plant leaves senescence as showed in detached wheat leaves senescence assay. Almost all the compounds were active cytokinins even at high concentrations where free cytokinins are generally no longer effective. Receptor assay showed that these N9 derivatives are not recognized via *Arabidopsis* CRE1/AHK4 receptor and only slightly via maize ZmHK3a receptor at high concentrations. The result why these derivatives still show significant cytokinin activity in tobacco callus and *Amaranthus* bioassay can rather contribute to the previously published observations that other ways for N9 cytokinins may exist that enable them to effectively transmit the signal into the cell and to control the cell growth and development.

4. Experimental

4.1. Chemicals

(3-methylbut-2-en-1-yl)amine hydrochloride, 6-[(3-methylbut-2-en-1-yl)amino]purine and 6-chloropurine were purchased from Olchemim, triethylamine, ethylacetate, *n*-propanol, *n*-butanol, diethyl ether, petrolether, hexane, dimethylsulfoxide (DMSO), *N,N′*-dimethylformamide (DMF), and other common organic solvents used for synthesis were purchased from Litolab and Lachema. 1-bromo-2-chloroethane, 1-bromo-4-chlorobutane, 1,2-dibromoethane, and ethyl vinyl ether were purchased from Sigma–Aldrich. 3,4-dihydro-*2H*-pyran, 4-chlorobutyronitrile, ethyl-4-bromobutyrate, sodium iodide were purchased from Fluka. NaOH, sodium azide, $CaCl_2$, acetic acid, Zn powder, NH_4Cl, NH_3, K_2CO_3, and ethylene carbonate were purchased from Litolab.

4.2. General procedures

Elemental analyses (C, H, and N) were determined of an EA1112 Flash analyzer (Thermo-Finnigan). The melting points were determined on Büchi Melting Point B-540 apparatus and are uncorrected. Thin layer chromatography (TLC) was carried out using silica gel 60 WF_{254} plates (Merck Co.). $CHCl_3$:MeOH (4:1, v:v) or ethyl acetate/toluene (2:1, v:v) were used as mobile phase. CI+ MS were recorded using 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 am, and spectra were collected using 1.0 s cyclical scans, applying 70 eV electron energy. In the CI+ ionization mode, isobutane was used as the reagent gas at a flow-rate of 2 L/h. The mass spectrometer was directly coupled to an Xcalibur data system. The purity of synthesized compounds was confirmed by high performance liquid chromatography (Beckman Gold system). To determine their HPLC purity, the samples were dissolved in HPLC mobile phase (initial conditions), applied to an RP-column (150 mm × 4.6 mm, 5 mL, Microsorb C18, Varian) and the separated constituents were eluted with a linear methanolic gradient (10–90% over 30 min, pH adjusted to four 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. ^{1}H NMR spectra were recorded on a Bruker Avance 300 spectrometer operating at a temperature of 300 K and a frequency of 300.13 MHz. Samples were prepared by dissolving the substances in DMSO-d_6. Tetramethylsilane (TIR) was used as the internal reference standard.

4.3. Syntheses

Reaction procedures of all the below mentioned substances are given in Scheme 2, particular steps are marked with *a–m* letters and explained under the scheme.

4.3.1. Synthesis of 6-[(3-methylbut-2-en-1-yl)amino]-9-(tetrahydropyran-2-yl)purine (1)

The substance was prepared according to the slightly modified procedure described previously in the literature.[21] In the first step, 6-chloro-9-(tetrahydropyran-2-yl)purine (*a*) was prepared from 6-chloropurine.[15,26] The intermediate (1 g, 4.2 mmol) was then refluxed with the appropriate amount of (3-methylbut-2-en-1-yl)amine hydrochloride (0.61 g, 5 mmol) in *n*-propanol (30 mL) in the presence of triethylamine (1.06 g, 10.5 mmol, 1.46 mL) for 4 h (*b*). The reaction mixture was evaporated to dryness. The residue was dissolved in water (50 mL) and extracted into EtOAc (3 × 10 mL). The combined ethyl acetate phases were dried (Na_2SO_4) and concentrated under reduced pressure. The pure product was obtained using flash chromatography ($CHCl_3$:MeOH, 9:1). C, H, N elemental analyses, CI+ MS and HPLC purity data as well as mp are given in Table 2 while ^{1}H NMR data are given in Supplementary data.

4.3.2. Synthesis of 6-[(3-methylbut-2-en-1-yl)amino]-9-(ethoxyethyl)purine (2)

In the first step, 6-chloro-9-(ethoxyethyl)purine was prepared according to the data found in the literature (*c*).[30] The intermediate (1 g, 4.4 mmol) was refluxed with 3-methylbut-2-en-1-yl)amine hydrochloride (0.64 g, 5.3 mmol) in *n*-propanol (25 mL) in the presence of triethylamine (1.34 g, 13.5 mmol, 1.84) for 4 h (*b*). After the evaporation of the solvent, the crude product was treated with H_2O (30 mL) and extracted into EtOAc (20 mL). The organic phase was dried (Na_2SO_4) and concentrated under reduced pressure. Pure white waxy substance was obtained by flash chromatography (mobile phase EtOAc:hexane, 1:2). C, H, N elemental analyses, CI+ MS and HPLC purity data as well as mp are given in Table 2 while ^{1}H NMR data are given in Supplementary data.

4.3.3. 6-[(3-Methylbut-2-en-1-yl)amino]-9-(acetoxy)purine (3)

6-[(3-Methylbut-2-en-1-yl)amino]purine (0.5 g, 2.5 mmol) was mixed with sodium acetate (0.32 g, 3.9 mmol) in DMF (20 mL) and stirred for 3 h at 95–100 °C and subsequently, 12 h at 25 °C (*d*). A

crude product obtained after the evaporation of the solvent was poured onto trash ice and extracted to EtOAc (20 mL). The organic phase was dried (Na_2SO_4) and concentrated under reduced pressure. Pure white crystalline product was obtained after the crystallization from methanol. C, H, N elemental analyses, CI+ MS, and HPLC purity data as well as mp are given in Table 2 while ^{1}H NMR data are given in Supplementary data.

4.3.4. Synthesis of 6-[(3-methylbut-2-en-1-yl)amino]-9-(2-bromoethyl)purine (4)

The mixture of 6-[(3-methylbut-2-en-1-yl)amino]purine (1 g, 4.9 mmol), 1,2-dibromoethane (2.31 g, 12.3 mmol, 1.06 mL) and potassium carbonate (1.7 g, 12.3 mmol) was stirred in DMF (30 mL) under the $CaCl_2$ drying tube at room temperature for 12 h (*e*). The reaction mixture was subsequently concentrated in vaccuo, treated with ice water (50 mL) and extracted into ethyl acetate (4 × 10 mL). The organic phases were combined, washed with water (2 × 10 mL), brine (2 × 10 mL), dried over Na_2SO_4, and finally concentrated under reduced pressure. The pure product was obtained after the purification *via* flash chromatography using ($CHCl_3$:MeOH, 19:1) as eluent. C, H, N elemental analyses, CI+ MS, and HPLC purity data as well as mp are given in Table 2 while ^{1}H NMR data are given in Supplementary data.

4.3.5. Synthesis of 6-[(3-methylbut-2-en-1-yl)amino]-9-(2-chloroethyl)purine (5)

6-[(3-Methylbut-2-en-1-yl)amino]purine (2 g, 9.8 mmol) was stirred with 1-bromo-2-chloroethane (1.4 g, 9.8 mmol, 0.82 mL), and with K_2CO_3 (3 g, 21.7 mmol) in DMSO (50 mL) under $CaCl_2$ drying tube at room temperature for 12 h (*f*). The reaction mixture was then poured on trash ice (200 mL) and extracted into EtOAc (50 mL). The organic layer was dried (Na_2SO_4) a concentrated under reduced pressure. The crude product was purified using flash chromatography (EtOAc:hexane, 1:2). White crystals were obtained after the crystallization from hexane. C, H, N elemental analyses, CI+ MS, and HPLC purity data as well as mp are given in Table 2 while ^{1}H NMR data are given in Supplementary data.

4.3.6. Synthesis of 6-[(3-methylbut-2-en-1-yl)amino]-9-(2-hydroxyethyl)purine (6)

The substance was prepared according to the procedure, found for 9-(2-hydroxyethyl)adenine in the literature.[28] 6-[(3-methylbut-2-en-1-yl)amino]purine (1 g, 4.9 mmol) was stirred with ethylene carbonate (0.44 g, 5 mmol) and NaOH (0.1 g, 2.5 mmol) in DMF (20 mL) (*g*). The reaction lasted for 2 h at 154 °C. The reaction mixture was poured onto trash ice and extracted into EtOAc (20 mL). After drying (Na_2SO_4) and evaporation of the solvent, the crude product was dissolved in mobile phase and purified by flash chromatography ($CHCl_3$:MeOH, 95:5). White, flocky substance was obtained after the crystallization with petroleum ether. C, H, N elemental analyses, CI+ MS and HPLC purity data as well as mp are given in Table 2 while ^{1}H NMR data are given in Supplementary data.

4.3.7. 6-[(3-Methylbut-2-en-1-yl)amino]-9-(2-azidoethyl) purine (7)

Compound **4** (2.82 g, 9.1 mmol) was heated with sodium iodide (0.14 g, 0.9 mmol) in 0.5 M solution of sodium azide in DMSO (37 mL) at 80 °C for 1 h and after that stirred at room temperature overnight (*k*). The mixture was poured onto trash ice (300 mL) and white precipitate was obtained. The solid was filtered off, washed with cold water and dried at room temperature. Yield 92%: C, H, N elemental analyses, CI+ MS, and HPLC purity data as well as mp are given in Table 2 while ^{1}H NMR data are given in Supplementary data.

4.3.8. Synthesis of 6-[(3-methylbut-2-en-1-yl)amino]-9-(4-chlorobutyl)purine (8)

6-[(3-Methylbut-2-en-1-yl)amino]purine (2 g, 9.8 mmol) was stirred with 1-bromo-4-chlorobutane (2.02 g, 11.8 mmol, 1.36 mL), and with potassium carbonate (3 g, 21.7 mmol) in DMSO (50 mL) under $CaCl_2$ drying tube at room temperature for 12 h (*i*). The reaction mixture was then poured onto trash ice (200 mL) and extracted into EtOAc (50 mL). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was purified using flash chromatography (EtOAc:hexane, 1:2). White crystals were obtained after the crystallization from diethyl ether. C, H, N elemental analyses, CI+ MS, and HPLC purity data as well as mp are given in Table 2 while ^{1}H NMR data are given in Supplementary data.

4.3.9. 6-[(3-Methylbut-2-en-1-yl)amino]-9-(3-cyanopropyl) purine (9)

6-[(3-Methylbut-2-en-1-yl)amino]purine (0.5 g, 2.5 mmol) was mixed with potassium carbonate (0.87 g, 6.25 mmol) in DMF (15 mL). 4-chlorobutyronitrile (0.31 g, 270 μL, 3 mmol) was slowly added and the reaction mixture was vigorously stirred at room temperature under $CaCl_2$ drying tube (*h*). Another four portions of 4-chlorobutyronitrile were added during 26 h. The reaction mixture was subsequently evaporated to dryness, treated with trash ice (50 mL) and extracted into EtOAc (4 × 10 mL). The combined organic layers were washed with water (2 × 10 mL), brine (2 × 10 mL), dried (Na_2SO_4) and finally concentrated under reduced pressure. Pure product was obtained by flash chromatography using $CHCl_3$:MeOH (19:1) as eluent. Yield 94%: C, H, N elemental analyses, CI+ MS, and HPLC purity data as well as mp are given in Table 2 while ^{1}H NMR data are given in Supplementary data.

4.3.10. 6-[(3-Methylbut-2-en-1-yl)amino]-9-(4-ethoxy-4-oxobutyl)purine (10)

6-[(3-Methylbut-2-en-1-yl)amino]purine (1 g, 4.9 mmol) was mixed with potassium carbonate (1.7 g, 12.3 mmol), and DMF (30 mL) and stirred vigorously at room temperature. Ethyl 4-bromobutyrate (1.06 g, 784 μL, 5.4 mmol) was then dropwise added and the reaction mixture was stirred under $CaCl_2$ drying tube at room temperature overnight (*j*). The mixture was evaporated to dryness and treated with trash ice (150 mL). The white precipitant was filtered, washed with cold water and dried at room temperature. The pure product was obtained by flash chromatography purification using ($CHCl_3$:MeOH, 19:1) as eluent. Yield 74%: C, H, N elemental analyses, CI+ MS, and HPLC purity data as well as mp are given in Table 2 while ^{1}H NMR data are given in Supplementary data.

4.3.11. 6-[(3-Methylbut-2-en-1-yl)amino]-9-(3-carboxypropyl) purine (11)

Compound **10** (1 g, 3.2 mmol) was stirred in the solution of NaOH (0.63 g, 15.8 mmol) in 50% ethanol (50 mL) at room temperature overnight (*l*). The mixture was cooled in the ice bath and 1.1 mL of concentrated acetic acid was added (pH approximately 5–6). Ethanol was distilled off and the solution was extracted with ethyl acetate and water. The organic phase was evaporated to ½.of total volume and stored 2 days at 4 °C. White precipitant that appeared was filtered off, washed with cold ethyl acetate and dried at room temperature. Pure product was obtained by flash chromatography purification using ($CHCl_3$:MeOH, 9:1) as eluent. Yield 82%: C, H, N elemental analyses, CI+ MS, and HPLC purity data as well as mp are given in Table 2 while ^{1}H NMR data are given in Supplementary data.

4.3.12. 6-[(3-Methylbut-2-en-1-yl)amino]-9-(2-aminoethyl) purine (12)

6-[(3-Methylbut-2-en-1-yl)amino]-9-(2-azidoethyl)purine (0.9 g, 3.35 mmol) was dissolved in EtOH (9 mL) and water (3 mL). NH_4Cl (0.41 g, 7.7 mmol) and Zn powder (0.29 g, 4.4 mmol) were then added to the solution and resulting mixture was vigorously stirred at room temperature. The reaction was accompanied by the formation of a white precipitate and by releasing gaseous nitrogen and it was completed within 2 h (monitored by TLC). The white precipitate was dissolved by the addition of concd. aqueous ammonia (7.5 mL) and residual Zn powder was filtered off. The volume of the filtrate was reduced by half by concentration in vacuum. A white solid formed during concentration was filtered off a clear filtrate was evaporated in reduced pressure. Yield 92%: C, H, N elemental analyses, CI+ MS, and HPLC purity data as well as mp are given in Table 2 while ^{1}H NMR data are given in Supplementary data.

4.4. Cytokinin bioassays

All the prepared compounds were tested in three cytokinin bioassays—a tobacco callus, an *Amaranthus* and a senescence bioassay with excised wheat leaves—and their activity was compared with parent iP and performed according to the literature.[15,31] Tested cytokinin derivatives were dissolved in dimethylsulfoxide (DMSO) and diluted with distilled water to 5×10^{-2} M solutions. The stock solution was further diluted in the media appropriate to each biotest to concentrations from 10^{-8} to 10^{-4} M. The final concentration of DMSO in the media did not exceed 0.2% and thus did not affect the biological activity of the substance tested in the assay. Five replicates were prepared for each compound concentration and the entire tests were repeated in triplicate.

4.5. AHK3, CRE1/AHK4, ZmHK1, and ZmHK3a receptor bioassay

E. coli strain KMI001, harboring a plasmid pIN-III-AHK4encoding for the *Arabidopsis* histidine kinases CRE1/AHK4,[32] and respective plasmids for *ZmHK*1 and *ZmHK*3a were used in the bacterial receptor assay. Bacterial strains were kindly provided by Dr. T. Mizuno (Nagoya, Japan) and the whole assay was performed as previously described in Spíchal et al.[5] The assay was optimized to 96-well microtiter plates according to the literature.[14] The pre-culture was diluted 1:10 and the incubation time was 16 h. Relative activation of the cytokinin receptors was determined by measuring β-galactosidase activity using the fluorescent substrate 4-methylumbelliferyl-β-D-galactoside (Sigma–Aldrich) and by monitoring the culture density at OD_{600}. The test was performed in triplicate and the entire test was repeated at least twice.

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Supplementary data

Supplementary data associated (^{1}H NMR data for iP derivatives **1**–**12**) with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.09.052.

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