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Preparation, characterization and biological activity of C8-substituted cytokinins

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ABSTRACT

Naturally occurring cytokinins are adenine-based plant hormones. Although, the effect of various substituents at positions N1, C2, N3, N^6, N7, or N9 on the biological activity of cytokinins has been studied, the C8-substituted compounds have received little attention. Here, we report the synthesis and *in vitro* biological testing of thirty-one cytokinin derivatives substituted at the C8 position of the adenine skeleton and twenty-seven compounds which served as their N9-tetrahydropyranyl protected precursors. The cytokinin activity of all the compounds was determined in classical cytokinin biotests (wheat leaf senescence, *Amaranthus* and tobacco callus assays). With some exceptions, the compounds with a N9-tetrahydropyranyl group were generally less active than their de-protected analogs. The latter were further tested for their ability to activate the Arabidopsis cytokinin receptors AHK3 and CRE1/AHK4 in bacterial receptor activation assays. Using this approach, we identified derivatives bearing short aliphatic chains and retaining high cytokinin activity. Such compounds are suitable candidates for fluorescence labeling or as protein-affinity ligands. We further found that some C8-substituted cytokinins exhibited no or lower cytotoxicity toward tobacco cells when compared to their parent compound. Therefore, we also present and discuss the cytotoxicity of all the compounds against three normal human cell lines.
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1. Introduction

Cytokinins represent a class of naturally occurring or synthetically prepared phytohormones structurally based on adenine or phenylurea. These hormones influence, in cooperation with other groups of plant hormones, numerous aspects of plant development and physiology, including seed germination, development of all the plant organs, apical dominance, chloroplast differentiation, de-etiolation, plant-pathogen interactions, leaf senescence, etc. (reviewed by Mok and Mok, 2001). Adenine types of cytokinins are classified according to the nature of the N^6-substituent as aromatic (e.g., N^6-benzyladenine (BA) and N^6-furfuryladenine (kinetin, K)), or isoprenoid (e.g., *trans*-zeatin (*tZ*), *cis*-zeatin (*cZ*), dihydrozeatin and N^6-isopentenyladenine (iP)). Cytokinins and their derivatives are of use in plant biotechnology, horticulture and agriculture.

A fundamental survey on structure-activity relationships of adenine-type cytokinins was conducted by Matsubara (1990). This review outlined that an intact purine ring with suitable N^6-substituent was a necessary structural features for high cytokinin activity. Thus, most attempts of modification have targeted the N^6 side chain. Nevertheless, the effect of substitution at all variable positions of the purine core has been investigated with the conclusion that a substituent at any position, except for certain 2-chloro, 2-azido, and 8-methyl derivatives, decreases the cytokinin activity.

8-Substituted cytokinins have not received as much attention as derivatives modified at the positions N1, C2, N3, N^6, N7, or N9. Several authors have referred to 8-hydroxy derivatives of cytokinins as products of enzymatic oxidation by xanthine oxidase (Bergmann and Kwietny, 1958; Chen et al., 1975). Derivatives with short alkyl chain in the 8-position were synthesized in 1958 and 1968 (Craveri and Zoni, 1958; Cherkasov and Kurilenko, 1968; Kulaeva et al., 1968). Later, through an 8-halogen intermediate, an 8-azido group was introduced into cytokinin molecules to provide

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photo-affinity ligands (Mornet et al., 1979; Steklov et al., 2011; Sussman and Kende, 1977). There is only one publication from the twentieth century detailing a larger series of compounds to investigate the effect of 8-substitution of purine type cytokinins, focusing on iP derivatives (Dammann et al., 1974).

In general, 6,8-disubstituted purines can be obtained in two ways: first, by ring closure from a monocyclic precursor, and second, by transformation of already functionalized purine intermediates. The first approach, cyclization of 4,5-diamino-6-substituted pyrimidine with anhydrides (Koppel and Robins, 1958) or cyclization of 4-amino-5-acylamido-6-substituted pyrimidine with alcoholic alkali (Cherkasov and Kurilenko, 1968), has mainly been used to generate 8-alkyl cytokinin-like purine derivatives. The second approach involves introduction of a reactive substituent, usually a halogen atom, into position 8 of a 6- or 6,9-disubstituted purine. Three different synthetic strategies have been described. A) Direct bromination of the purine core of cytokinins. Owing to the nature of N^6 substituents, this method is only possible with BA (Steklov et al., 2011). However, not all attempts have been successful with this tactic (Moravec, 2004; Sussman and Kende, 1977). B) Bromination of adenosine is a well-established procedure (Holmes and Robins, 1964). 8-Bromoadenosine can be subjected to alkylation followed by Dimroth rearrangement to provide 8-bromocytokinin-9-riboside (Sussman and Kende, 1977). C) The most common approach starts with 6,8-dichloropurine and takes advantage of the difference in reactivity of chlorine atoms in the 6- and 8-position toward nucleophilic displacement (Dammann et al., 1974; Mornet et al., 1979). Nucleophilic attack on 6,8-dichloropurine occurs first at the 6-position (Robins, 1958). In contrast, in the case of 6,8-dichloropurine substituted at position 9 with a methyl, THP or ribofuranosyl group, strong bases attack both the 6- and 8-position and a mixture of isomers arises (Sutcliffe and Robins, 1963).

Current knowledge indicates that the 8-substituted cytokinins are usually less active than the 8-unsubstituted compounds but, in many cases, the cytokinin activity is retained. These findings suggest that these compounds may be suitable as linker molecules bearing a fluorescent label or as protein-affinity ligands. Thus, to better understand the biological activities of cytokinin-like compounds and identify possible applications, more detailed investigations of the structure-activity relationships of 8-substituted cytokinins are needed. Our study presented here aims to contribute to this goal.

2. Results and discussion

2.1. Synthesis

Thirty-one derivatives of *t*Z, *c*Z, iP, BA, and K substituted at position 8 were synthesized (Table 2) to investigate structure-activity relationships. Twenty-seven of their direct precursors in the synthesis, the N9-(tetrahydropyran-2-yl, THP) derivatives (Table 1), were also included in the investigation. Summaries of concise abbreviations used for the individual substances, their physical states, melting points, $[M+H]^+$ masses, and synthetic yields are presented in Tables 1 and 2.

Most of the compounds were prepared by the procedure outlined in Scheme 1. The position N9 of 6-chloropurine was protected with a THP group by using a conventional method previously described (Taddei et al., 2004). Reaction of 6-chloro-9-(tetrahydropyran-2-yl)purine with an alkenyl- or arylalkyl-amine in alcohol and a base (50 °C, several hours) gave the corresponding 9-THP-cytokinin (e.g., Robins et al., 1961). A halogen atom was introduced at position 8 of the 9-THP-cytokinin by lithiation and subsequent halogenation (1. lithium diisopropylamide (LDA) in THF, −78 °C, argon atmosphere; 2. hexachloroethane, 1 h; purification on a silica-gel column) as described by Nolsøe et al. (1998). The halogen atom at position 8 was able to be substituted with a nucleophile (amine or alcoholate) to afford an 8-substituted-9-THP-cytokinin. Deprotection of the N9 position was accomplished by acid-catalyzed hydrolysis of the THP group.

The synthesis of 8-amino derivatives (**6, 36**) was not possible using the steps described in Scheme 1 owing to the weak nucleophilicity of ammonia. The reactivity of halogens in 8-dihalogen-9-

Table 1
Basic characterization of the prepared compounds possessing a THP group (compounds **1**–**27**). - data not determined.

Compound number and designation		Physical state	Melting point (°C)	ESI–MS $[M+H]^+$	Yield (%)
1	*t*Z-Cl	beige crystals	130–134	338; 340	49
2	*t*Z-Br	beige crystals	138–142	382; 384	42
3	*t*Z-$N(CH_3)_2$	white crystals	110–114	347	90
4	iP-Cl	beige crystals	118–120	322; 324	66
5	iP-Br	beige crystals	125–128	368; 366	60
6	iP-NH_2	white needles	–	303	44
7	iP-$N(CH_3)_2$	yellowish crystals	76–79	331	91
8	iP-$NH(CH_2)_2NH_2$	white foam	–	346	93
9	iP-$NH(CH_2)_3NH_2$	viscous liquid	–	360	78
10	iP-$NH(CH_2)_4NH_2$	viscous liquid	–	374	78
11	iP-$NH(CH_2)_6NH_2$	glassy subst.	–	402	82
12	iP-$O(CH_2)_3NH_2$	viscous liquid	–	361	65
13	BA-Br	beige crystals	120–124	390; 388	76
14	BA-I	white crystals	153–155	436	32
15	BA-OCH_2Phe	white crystals	149–151	416	64
16	K-Cl	beige crystals	110–114	334; 336	69
17	K-Br	beige powder	108–112	380; 378	37
18	K-I	beige powder	131–134	426	66
19	K-OMe	white crystals	137–139	330	73
20	K-SMe	yellow crystals	153–156	346	96
21	K-$O(CH_2)_2$Me	viscous liquid	–	358	65
22	K-$O(CH_2)$Phe	white crystals	132–135	406	20
23	K-$N(CH_3)_2$	yellowish crystals	174–176	343	83
24	K-$NH(CH_2)_2OH$	yellowish foam	–	359	56
25	K-$NH(CH_2)_3OH$	yellowish foam	–	373	90
26	K-$NHCH_2CH{=}CH_2$	viscous liquid	–	355	58
27	K-$NH(CH_2)_2NH_2$	viscous liquid	–	358	77

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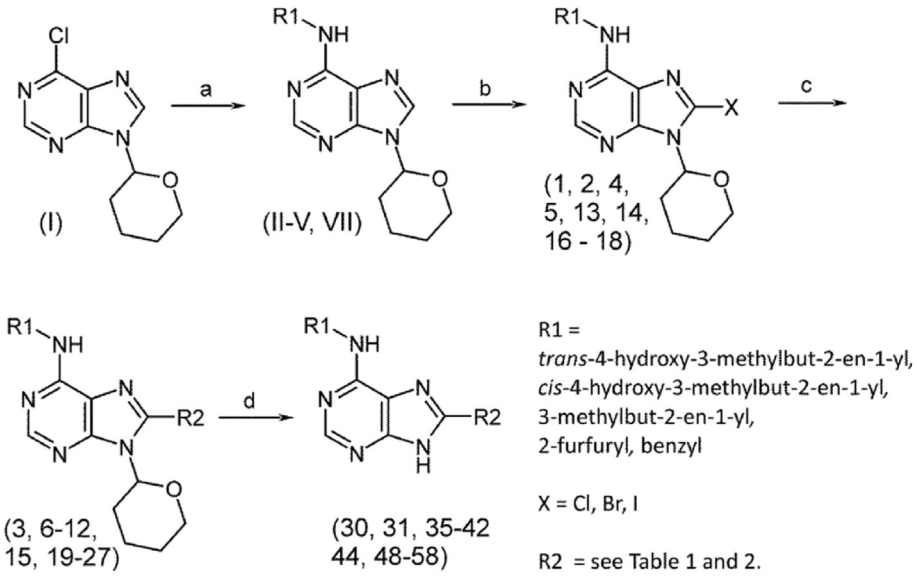
Table 2
Basic characterization of the prepared compounds without a THP group (compounds **28**–**58**). - data not determined.

Compound number and designation		Physical state	Melting point (°C)	ESI–MS $[M+H]^+$	Yield (%)
28	*t*Z-Cl	white crystals	236–238	254; 256	75
29	*t*Z-Br	beige ppt.	224–226	300; 298	74
30	*t*Z-$N(CH_3)_2$	white crystals	246–248	263	85
31	*c*Z=O	white crystals	243–247	236	17
32	iP-Cl	white powder	262–265	238; 240	85
33	iP-Br	white powder	233–235	282; 284	84
34	iP=O	beige crystals	250–254	220	20
35	iP-OMe	white powder	205–210	234	33
36	iP-NH_2	white crystals	254–262	219	41
37	iP-$N(CH_3)_2$	cotton-like crystals	246–249	247	68
38	iP-$NH(CH_2)_2NH_2$	yellow amorphous	–	262	79
39	iP-$NH(CH_2)_3NH_2$	yellow amorphous	–	276	98
40	iP-$NH(CH_2)_4NH_2$	yellow amorphous	–	290	75
41	iP-$NH(CH_2)_2OH$	white powder	228–230	263	59
42	iP-$O(CH_2)_3NH_2$	yellow amorphous	–	277	75
43	BA-Cl	Lit.	–	–	–
44	BA=O	white crystals	255–258	242	8
45	K-Cl	beige powder	276–278	250; 252	19
46	K-Br	beige powder	239–240	296; 294	66
47	K-I	beige powder	220–222	342	97
48	K=O	yellow crystals	264–266	232	16
49	K-OMe	beige crystals	196–198	246	60
50	K-SMe	beige needle	226–228	262	42 + 36
51	K-NH_2	Lit.	–	–	–
52	K-$N(CH_3)_2$	white crystals	245–246	259	70
53	K-$NH(CH_2)_2NH_2$	yellowish glassy	105–110	274	88
54	K-$NH(CH_2)_2OH$	white plaster	230–233	275	78
55	K-$NH(CH_2)_3OH$	white powder	201–203	289	25 + 22
56	K-$NHCH_2CH=CH_2$	white solid	242–244	271	42
57	K-$O(CH_2)_2Me$	beige crystals	196–198	274	31
58	K-$O(CH_2)_2OH$	rusty crystals	182–184	276	31



Scheme 1. General reaction scheme for the syntheses of 8-substituted cytokinin derivatives. a) appropriate amine, Et_3N, PrOH, 65 °C, 14 h; b) 1. LDA, 2. source of halogen, e.g., CBr_4, THF, −78 °C, 1 + 1 h; c) nucleophile, e.g., alcoholate in alcohol or amine in alcohol, 65 °C, 2–4 d; d) AcOH: H_2O 4:1, rt, 2–4 d.

THP-purines is sufficiently high to be replaced by ammonia. However, the replacement is not selective, as mentioned in the introduction. Therefore, isolation of 8-amino-6-halogen-9-THP-purines was necessary before nucleophilic substitution of the halogen with an appropriate N^6 side chain precursor. Surprisingly, unambiguous direction of an ammonia nucleophile to the 8-position has been observed in 6,8-dihalogenpurin-9-ribosides (Szekeres et al., 1975). We applied this approach to synthesize compound **51**.

2.2. Biological activity in cytokinin bioassays

Cytokinins influence a wide array of important biological processes throughout the life of plants. Some of these have been employed in bioassays, in which cytokinin stimulatory effects occur in a concentration dependent manner. We employed three cytokinin bioassays (wheat leaf senescence, *Amaranthus* and tobacco callus bioassays, Holub et al., 1998) to investigate the complex cytokinin properties of the synthesized compounds. The activity of

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the compounds was compared with the activity of their unsubstituted parent compound and adjusted to the activity of BA at optimal concentration. All data obtained are described below and summarized in Tables 3 and 4.

2.2.1. Wheat leaf senescence assay performed in the dark

This assay is based on the ability of compounds to retard chlorophyll degradation in excised wheat (*Triticum aestivum* cv. Hereward) leaves in the dark. All compounds reached some biological activity at 100 μM concentration, and thus were easily compared. In this assay, *tZ* was the most active compound, reaching ca. 155% of BA activity. K possessed the same activity as BA. iP was not active in this assay, and therefore it was not surprising that none of its derivatives were active in this assay.

Regarding *tZ* derivatives bearing a THP group, we observed decreased activity of compounds with halogen substitutions (8-chloro- and 8-bromo-*tZ*, **1** and **2**) to ca. 50**%** when compared to *tZ*. The same compounds (**28**, **29**) without a THP group exhibited only a slight decrease in activity. A complete loss of activity was observed for *tZ* bearing a dimethylamino group, regardless of the presence/absence of a THP group (**3**, **30**).

Among BA derivatives having a THP group, only 8-iodo-BA (**14**) was active, but it reached only 25% of the BA activity. Among BA derivatives without a THP group, the presence of chlorine in the C8-position (**43**) decreased the activity to half when compared to BA, but interestingly, 8-oxo-BA (**44**) exhibited higher activity than BA by 34%.

K derivatives with a THP group were usually inactive in this assay. The exceptions were compounds with a 8-methylthio (**20**) and 8-allylamino (**26**) moiety, which reached 55**%** and 42**%** of BA activity, respectively. Among the K derivatives without a THP group, methoxy (**49**) and 2-hydroxyethyloxy (**58**) substituents did not decrease the activity of K. Lower activities were exhibited by 8-oxo- (**48**, 66**%**), 8-(2-hydroxyethylamino)- (**54**, 42**%**), 8-(3-hydroxypropylamino)- (**55**, 33**%**) and 8-methylthio-K (**50**, 30**%**). Interestingly, whereas derivatives of *tZ* with a halogen at C8 (**28, 29**) exhibited only a slight decrease in activity, the corresponding C8 halogen derivatives of K (**45, 46**) were inactive in this assay. Similarly, K derivatives with an amino (**51**), dimethylamino (**52**), 2-hydroxyethylamino (**54**), allylamino (**56**) and propoxy (**57**) group were inactive in this assay.

2.2.2. Amaranthus assay

This assay is based on the dark induction of betacyanin synthesis in *Amaranthus* (*Amaranthus caudatus* var. *atropurpurea*) cotyledons in the presence of cytokinin (Bamberger and Mayer, 1960). In this assay, *tZ*, BA, K and iP exhibited very similar activity, with EC_{50} values between 0.75 and 1.73 μM.

Among the *tZ* derivatives, 8-bromo-*tZ* (**29**, EC_{50} = 0.15 μM) was ca. 5 times more active than *tZ* (EC_{50} = 0.85 μM) and it was the most active compound among all the derivatives tested in this assay. 8-Chloro-*tZ* (**28**, EC_{50} = 0.72 μM) exhibited similar activity to that of *tZ*. The analogs of 8-chloro-*tZ* and 8-bromo-*tZ* bearing a THP group (**1**, **2**) exhibited activities ca. 10 times lower than *tZ*. 8-Dimethylamino-*tZ* (**30**) was inactive and its THP analog (**3**) exhibited very low activity with EC_{50} close to 100 μM.

Interestingly and contrary to the *tZ* derivatives, 8-halogen derivatives of iP with a THP group were also very active in this assay. 8-Chloro-9-THP-iP (**4**) exhibited similar activity (EC_{50} = 0.22 μM) to that of 8-bromo-*tZ* (**29**). 8-Bromo-9-THP-iP (**5**) was also very active

Table 3
Cytokinin activity of the C8-substituted cytokinins with N9-THP group in classical cytokinin bioassays. For the senescence and tobacco callus assays, the activity of the compounds was compared with the activity of BA (100%) at optimal concentrations of 100 μM and 1 μM, respectively. Errors show SD of at least two parallel assays, each comprising at least three replicates. NA refers to: not active; – data not determined. The compounds for which the variation of activity with concentration in the tobacco callus assay is shown in Fig. 1 are marked with bold letters and numbers.

Compound number and designation		Senescence bioassay	*Amaranthus* bioassay	Tobacco callus bioassay	
		(%)	(EC_{50}, μM)	Optimal c. (μM)	Relative activity (%)
	BA	100	0.75 ± 0.10	**1**	**100**
	K	100 ± 4	0.81 ± 0.08	**1**	**105 ± 7**
	tZ	155 ± 10	0.85 ± 0.11	**1**	**102 ± 7**
	iP	NA	1.73 ± 0.13	**1**	**104 ± 6**
1	*tZ*-Cl	78 ± 5	8.3 ± 1.2	0.1–100	112 ± 8
2	*tZ*-Br	85 ± 4	8.0 ± 1.6	0.1–100	108 ± 7
3	*tZ*-$N(CH_3)_2$	NA	100 ± 5	1	95 ± 6
4	iP-Cl	NA	0.22 ± 0.03	1–100	117 ± 10
5	iP-Br	NA	0.75 ± 0.13	1–100	109 ± 4
6	iP-NH_2	NA	7.8 ± 1.4	1–100	100 ± 6
7	iP-$N(CH_3)_2$	NA	9.5 ± 2.1	10–100	98 ± 3
8	iP-$NH(CH_2)_2NH_2$	NA	42 ± 4	10–100	105 ± 8
9	iP-$NH(CH_2)_3NH_2$	NA	>100	100	102 ± 5
10	iP-$NH(CH_2)_4NH_2$	NA	>100	100	102 ± 4
11	iP-$NH(CH_2)_6NH_2$	NA	100 ± 8	100	105 ± 7
12	iP-$O(CH_2)_3NH_2$	NA	–	–	–
13	BA-Br	NA	>100	10–100	101 ± 3
14	BA-I	25 ± 4	6.3 ± 1.3	100	102 ± 4
15	BA-OCH_2Phe	NA	NA	NA	–
16	K-Cl	32 ± 3	100 ± 11	10	88 ± 6
17	**K-Br**	–	–	**10**	**95 ± 2**
18	K-I	–	32 ± 5	10	85 ± 5
19	K-OMe	–	–	10	104 ± 2
20	K-SMe	55 ± 2	>100	10	72 ± 6
21	K-$O(CH_2)_2$Me	NA	NA	100	78 ± 8
22	K-$O(CH_2)$Phe	NA	NA	10	44 ± 6
23	K-$N(CH_3)_2$	NA	>100	100	57 ± 6
24	K-$NH(CH_2)_2OH$	NA	NA	100	57 ± 4
25	**K-$NH(CH_2)_3OH$**	–	–	**100**	**102 ± 3**
26	K-$NHCH_2CH{=}CH_2$	42 ± 3	>100	10	98 ± 2
27	K-$NH(CH_2)_2NH_2$	NA	NA	100	90 ± 3

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Table 4
Cytokinin activity of the C8-substituted cytokinins in classical cytokinin bioassays. For the senescence and tobacco callus assays, the activity of the compounds was compared with the activity of BA (100%) at optimal concentrations of 100 μM and 1 μM, respectively. Errors show SD of at least two parallel assays, each comprising at least three replicates. NA refers to: not active; – data not determined. The compounds for which the variation of activity with concentration in the tobacco callus assay is shown in Fig. 1 are marked with bold letters and numbers.

Compound number and designation		Senescence bioassay	*Amaranthus* bioassay	Tobacco callus bioassay	
		(%)	(EC_{50}, μM)	Optimal c. (μM)	Relative activity (%)
	BA	100	0.75 ± 0.10	**1**	**100**
	K	100 ± 4	0.81 ± 0.08	**1**	**105 ± 7**
	***t*Z**	155 ± 10	0.85 ± 0.11	**1**	**102 ± 7**
	iP	NA	1.73 ± 0.13	**1**	**104 ± 6**
28	*t*Z-Cl	124 ± 6	0.72 ± 0.07	0.1–100	115 ± 4
29	***t*Z-Br**	122 ± 8	0.15 ± 0.03	**0.1–100**	**114 ± 6**
30	***t*Z-N(CH$_3$)$_2$**	NA	>100	**100**	**102 ± 5**
31	*c*Z=O	36 ± 5	>100	10	51 ± 4
32	iP-Cl	NA	0.72 ± 0.21	1–100	104 ± 7
33	iP-Br	NA	0.61 ± 0.20	1–100	112 ± 8
34	**iP=O**	NA	5.4 ± 0.8	**10**	**82 ± 3**
35	iP-OMe	NA	1.02 ± 0.17	1–10	127 ± 10
36	iP-NH$_2$	NA	2.5 ± 0.2	10–100	92 ± 4
37	iP-N(CH$_3$)$_2$	NA	10.3 ± 0.6	10–100	98 ± 5
38	iP-NH(CH$_2$)$_2$NH$_2$	NA	>100	1–100	103 ± 5
39	iP-NH(CH$_2$)$_3$NH$_2$	NA	>100	100	102 ± 6
40	iP-NH(CH$_2$)$_4$NH$_2$	NA	>100	100	101 ± 4
41	iP-NH(CH$_2$)$_2$OH	NA	9.8 ± 1	1–100	104 ± 7
42	**iP-O(CH$_2$)$_3$NH$_2$**	NA	22 ± 4	**1–100**	**110 ± 8**
43	BA-Cl	53 ± 5	92 ± 3	10	77 ± 5
44	BA=O	134 ± 10	NA	10	55 ± 2
45	K-Cl	NA	91 ± 7	10	71 ± 5
46	K-Br	NA	82 ± 4	1–100	94 ± 6
47	K-I	NA	>100	10	100 ± 4
48	K=O	66 ± 4	NA	10	61 ± 4
49	K-OMe	105 ± 7	0.44 ± 0.03	1	105 ± 7
50	K-SMe	30 ± 5	3.6 ± 0.3	10	85 ± 3
51	K-NH$_2$	NA	–	10	82 ± 7
52	K-N(CH$_3$)$_2$	NA	62 ± 17	100	41 ± 2
53	K-NH(CH$_2$)$_2$NH$_2$	NA	9.5 ± 2	100	98 ± 1
54	K-NH(CH$_2$)$_2$OH	42 ± 7	>100	10	65 ± 6
55	K-NH(CH$_2$)$_3$OH	33 ± 4	100 ± 5	100	96 ± 5
56	K-NHCH$_2$CH=CH$_2$	NA	10.5 ± 1	1	96 ± 4
57	K-O(CH$_2$)$_2$Me	NA	14 ± 4	10	81 ± 7
58	K-O(CH$_2$)$_2$OH	98 ± 8	7.4 ± 2.8	1	95 ± 7

(EC_{50} = 0.75 μM). 8-Halogen-iPs without a THP group (**32**, **33**) showed the same activity, both with EC_{50} lower than iP itself. Other iP derivatives with a THP group were also active in this assay, i.e., 8-amino-9-THP-iP (**6**), 8-dimethylamino-9-THP-iP (**7**) and 8-aminoethylamino-9-THP-iP (**8**) with EC_{50} 7.8 μM, 9.5 μM and 42 μM, respectively. Among the iP derivatives without a THP group, compound 8-methoxy-iP (**34**, EC_{50} = 1.02 μM) was more active than iP itself and then the activity decreased in the following order: 8-amino-iP (**36**, EC_{50} = 2.52 μM), 8-oxo-iP (**34**, EC_{50} = 5.36 μM), 8-(2-hydroxyethylamino)-iP (**41**) and 8-dimethylamino-iP (**37**, both EC_{50} close to 10 μM). Less active was the derivative with a 3-aminopropoxy group (**42**, EC_{50} = 22 μM) and compounds with longer moieties, such as 2-aminoethylamino (**38**) 3-aminopropylamino (**39**) and 4-aminobutylamino (**40**), which were only active at the highest concentration tested.

Regarding BA derivatives with a THP group, 8-iodo-9-THP-BA (**14**, EC_{50} = 6.3 μM) was more active than 8-bromo-9-THP-BA (**13**, EC_{50} over 100 μM). The derivatives of BA without a THP group were also less active compared to BA. 8-Oxo-BA (**44**) was totally inactive in this assay and 8-chloro-BA exhibited at least 100 times lower activity than BA (EC_{50} = 0.75 μM).

Similar trends in activities were exhibited by 9-THP derivatives of K, which in most cases were inactive or with EC_{50} over 100 μM (compounds **20**–**27**). Similarly, the 8-halogeno K derivatives without a THP group (**45**, **46**) possessed very low activity with EC_{50} over 80 μM. 8-Oxo-K (**48**) was also not active in this assay. Very low activities were exhibited by the derivatives with 2-hydroxyethylamino (**54**, EC_{50} > 100 μM), 3-hydroxypropylamino (**55**, EC_{50} = 100 μM) and dimethylamino (EC_{50} = 62 μM) moieties. Reduced activity was also exhibited by 8-propoxy K (**57**, EC_{50} = 14 μM). Compounds 8-allylamino-K (**56**) and 8-(2-aminoethylamino)-K (**53**) had EC_{50} close to 10 μM and 8-(2-hydroxyethyloxy)-K (**54**) had EC_{50} = 7.4 μM. 8-Methylthio-K (**50**) also exhibited a lower activity (EC_{50} = 3.57 μM) than K and only 8-methoxy-K (**49**, EC_{50} = 0.44 μM) exceeded twice the K activity in this assay.

From the results obtained in the *Amaranthus* assay, we observed specific differences in the activity between 8-substituted isoprenoid (aliphatic) and 8-substituted aromatic cytokinins. Whereas halogen substitution in the 8-position increased the activity of isoprenoid cytokinins (*t*Z, iP), it caused a large decrease in activity in aromatic cytokinins. Even the 8-halogeno-9-THP derivatives of iP exhibited higher activities than iP. Similarly, 8-oxo-K (**48**) and 8-oxo-BA (**44**) were inactive and 8-oxo-iP (**34**) had a slightly lower activity than iP in this assay. Given that none of the mentioned 8-oxo derivatives were able to activate Arabidopsis cytokinin receptors AHK3 and CRE1/AHK4 (Figs. 2 and 3), it seems that isoprenoid and aromatic cytokinins induce betacyanin synthesis in *Amaranthus via* a different mechanism or at least *via* activation of cytokinin receptor(s) with different ligand specificities. We do not consider that AHK2 plays a role in this effect because it has been shown that the AHK2 receptor has a similar ligand binding spectrum to that of the highly conserved CRE1/AHK4 receptor (Stolz et al., 2011), which is also not activated by 8-oxo cytokinins, as

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shown here.

2.2.3. Tobacco callus assay

This assay is based on the ability of cytokinins to promote cell division in the presence of auxin. Cytokinin-dependent tobacco callus *Nicotiana tabacum* L. cv. Wisconsin 38 was used in the experiments. The activity of compounds was compared to the activity of BA (100%) at an optimal concentration of 1 μM. The most active compounds in this assay were 8-bromo-*tZ* and 8-chloro-*tZ*, independent of whether they had a THP group. All those compounds (**1, 2, 28, 29**) exhibited very high activity over a wide concentration range from 0.1 μM to 100 μM. Within this range, the compounds exceeded the activity of BA at a concentration of 1 μM by 8%–15% (Fig. 1). Interestingly, 8-dimethyl amino-9-THP-*tZ* (**3**) had a comparable activity to that of BA, whereas its 9-THP deprotected analog (**30**) only reached the BA activity at a concentration of 100 μM.

The 8-halogen-iP derivatives (**32, 33**) and their THP analogs (**4, 5**) possessed activities comparable to those of BA and their parent compound (iP), albeit lower than analogous *tZ* derivatives. This result is in accordance with Dammann's observations (Dammann et al., 1974), who reported the same result for 8-chloro-iP. The other derivatives of iP with a THP group and bearing 8-aminoalkylamino chains (**7**–**12**) were also active in this assay, although they only reached the BA activity at concentrations from 10 μM to 100 μM. Their analogs without a THP group exhibited better activities. 8-(3-Aminopropoxy)-iP (**42**) exhibited exceptionally high cell growth promoting activity over a very large concentration range (Fig. 1). This is an interesting result as it is very unusual that compounds exceed the *tZ* activity in the callus assay. There is only one example reported in the literature - the synthetic cytokinin thidiazuron (Thomas and Katterman, 1986).

8-(2-Aminoethylamino)-iP (**38**) and 8-(2-hydroxyethylamino)-iP (**41**) also slightly exceeded the BA activity. Less active were compounds bearing 8-dimethylamino (**37**), 8-oxo (**34**) and 8-amino (**36**) moieties, which reached the BA activity at a concentration of 10 μM. Dammann et al. (1974) have reported reduced activity for 8-mercapto-iP, consistent with the activity of 8-oxo-iP (**34**) measured by us. This result is also in good agreement with another work (Chen et al., 1975), which showed that 8-hydroxy-zeatin and 8-hydroxy-iP have lower activity than the unsubstituted cytokinin. IP derivatives bearing 3-aminopropylamino (**39**) and 4-aminobutylamino (**40**) moieties reached the BA activity at a concentration of 100 μM.

Regarding aromatic cytokinins, BA and K exhibited the same activity in this assay. All the BA (**13, 14, 15**) and K (**16**–**27**) derivatives with a THP group exhibited lower cell growth promoting activity than the parent free bases. None of the compounds reached the maximum activity at a concentration of 1 μM. The K analogs without a THP group, such as 8-bromo- (**46**) and 8-methoxy-K (**48**), exhibited activities comparable with those of BA and K. Interestingly, K bearing 2-hydoxyethyloxo (**58**) and 2-allylamino (**56**) reached the activity of K at a concentration of 1 μM but were less active at lower concentrations (Fig. 1). Other substitutions decreased the activity and most of the K derivatives were only active at concentrations of 10 μM or higher, e.g., 8-iodo- (**47**), 8-chloro- (**45**), 8-(2-hydroxyethylamino)- (**54**), 8-methylthio- (**50**), 8-propoxy- (**57**) and 8-oxo- (**48**) K. Other **derivatives, i.e.,** 8-(2-aminoethylamino)- (**53**), 8-(3-hydroxypropylamino)- (**55**) and 8-dimethylamino- (**51**), K were only active at a concentration of 100 μM.

It is noteworthy that both groups of compounds, C8 substituted cytokinins and their N9-THP analogs, exhibited lower toxicity against tobacco cells than their parent compounds. Whereas *tZ*, iP, BA and K caused inhibition of tobacco cell growth at concentrations of 10–100 μM, compounds 8-bromo-*tZ* (**29**), 8-dimethylamino-*tZ* (**30**), 8-(3-aminopropoxy)-iP (**42**), 8-(1-hydroxypropylamino)-K (**25**), etc. did not show such an effect. These examples are shown in Fig. 1 and can be distinguished in Tables 3 and 4 by the wide optimal concentration range. These new compounds thus have great potential for use in the micropropagation industry because, unlike cytokinins, they do not exhibit a detrimental effect at high concentrations.

2.2.4. Activation of Arabidopsis cytokinin receptors AHK3 and CRE1/AHK4

Transformed *Escherichia coli* expressing the Arabidopsis cytokinin receptors AHK3 or CRE1/AHK4 and the cytokinin-activated reporter gene cps::lacZ (Spíchal et al., 2004; Suzuki et al., 2001) were employed to examine the ability of the synthesized compounds to activate these receptors. Only compounds without the THP substitution were tested.

2.2.4.1. Activation of Arabidopsis cytokinin receptor AHK3. In the AHK3 receptor activation assay (Fig. 2), *tZ* was used as a standard and its activity at 1 μM concentration was set as 100%. All *tZ* derivatives (compounds **28**–**30**) possessed lower activity than *tZ*. However, 8-chloro- and 8-bromo-*tZ* remained highly active, reaching ca. 65–70% and 90% of *tZ* activity at 1 and 10 μM concentration, respectively. *TZ* with a dimethylamino group (**30**) was poorly active and exhibited only 35% of *tZ* activity at 10 μM

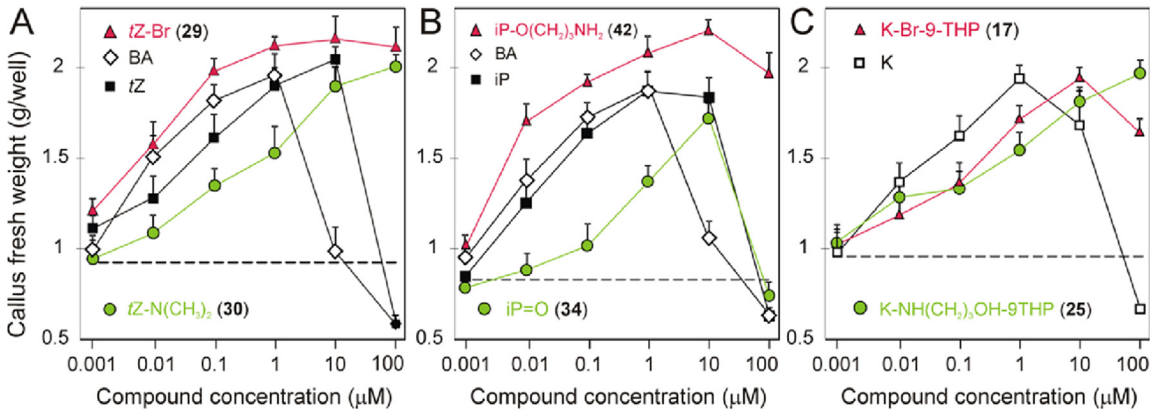

Fig. 1. Effect of selected derivatives of *tZ* (A), iP (B) and K (C) on the growth of cytokinin-dependent tobacco callus compared to their mother compound and cytokinin BA. Dashed lines indicate values obtained for the control treatment with no added compound. Error bars show SD ($n = 5$). The whole experiment was repeated at least twice and the graphs presented are representative examples.

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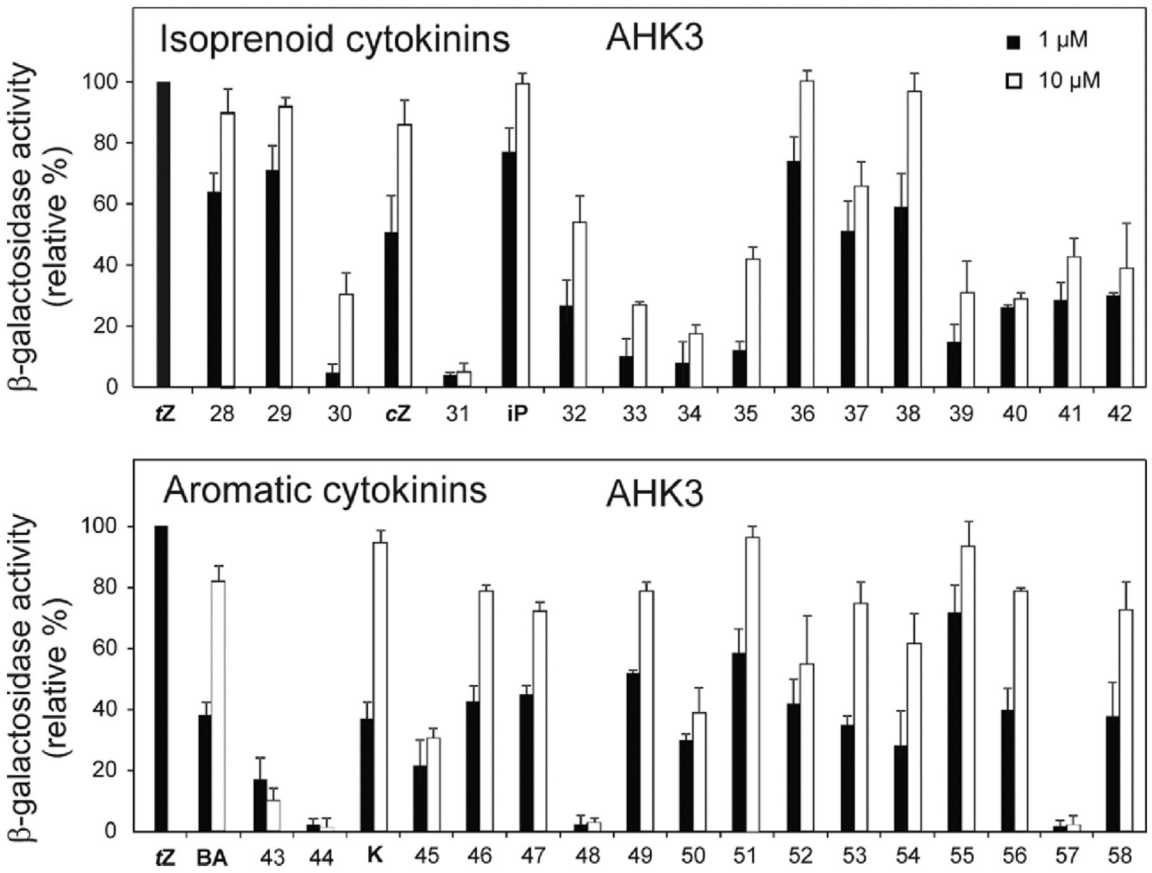

Fig. 2. Ability of selected compounds to activate the cytokinin receptor AHK3 in a receptor activation assay. The β-galactosidase activity correlates with the activation of the receptor by the tested compound. Activities of the compounds are shown relative to that of *t*Z activity at concentration 1 μM and compared with the activity of the parent compound. Error bars show SD ($n = 3$).

concentration. In the case of 8-oxo-*c*Z (**31**), this substitution led to **a** loss of activity of the compound at this receptor. Regarding the iP derivatives, none of them were more active than an iP free base, but the activity of compounds **36** (8-amino-iP) and **38** (8-aminoethylamino-iP) remained almost unaffected. Compound **37** (8-dimethylamino-iP) was ca. 25% less active than iP at both 1 and 10 μM concentration. Compounds **39**–**42** had significantly decreased activity to ca. 35–40% when compared to iP but still remained active even at 1 μM concentration. This result indicates that the activity decreased with increasing length of side chain. Similar trends were observed when iP was substituted by a halogen (**32**, **33**), oxo- (**34**) or methoxy-group (**35**). These compounds had much lower activities than unsubstituted iP. Compounds **33** and **34** were the least active of the prepared iP derivatives on this receptor.

BA and K were weaker activators of AHK3 than *t*Z, reaching ca. 40% and 70**%** of *t*Z activity at 1 and 10 μM concentration, respectively. These results are in accordance with another study (Spíchal et al., 2004). Substitution of BA or K by chlorine (**43**, **45**) led to a dramatic decrease of the compound activity. A complete loss of AHK3 receptor activity was obtained by substituting oxygen at the C-8 position of BA (**44**) and K (**48**). Interestingly, neither the bromine (**46**) nor iodine (**47**) derivative decreased the K activity. Both compounds possessed the same activity as K at 1 μM concentration and only a slightly lower activity at 10 μM concentration. Similar trends were observed for the other derivatives of K (**49**–**58**), except for compound **57,** which was inactive. Interestingly, and in contrast to the iP derivatives, compounds **49**–**58** had comparable activity to that of K at 1 μM concentration or even exceeded it (**49, 51, 55**). The most active of these was compound **55** bearing a 3-hydroxypropylamino moiety. This result indicates that this compound may be suitable for fluorescent labeling or as an affinity probe for K binding proteins. However, all of the compounds **53, 54, 55, 56** and **58** would also be suitable.

2.2.4.2. Activation of Arabidopsis receptor CRE1/AHK4. The receptor CRE1/AHK4 is known to be generally more selective and less sensitive than the receptor AHK3 (Spíchal et al., 2004). This was apparent from our results as only six compounds were able to activate this receptor (Fig. 3). Moreover, the active compounds were all derivatives of isoprenoid cytokinins. Aromatic cytokinins possessed generally lower activity at this receptor (Spíchal et al., 2004). In the AHK4 receptor activation assay, *t*Z was used as a standard and its activity at 10 μM concentration was set as 100%. 8-Chloro-*t*Z (**28**) activated the receptor to the same extent as *t*Z at a concentration of 10 μM but only showed an activity of 35% at 1 μM concentration. 8-Bromo-*t*Z (**29**) was even less active. Its activity reached only 48% of *t*Z activity at a concentration of 10 μM. Therefore, the activity of 8-bromo-*t*Z was ca. ten times weaker than 8-chloro-*t*Z at the AHK4 receptor, whereas at the receptor AHK3, these two compounds possessed similar activity. Among the iP derivatives, compounds **35, 36, 38** and **41** were able to activate the AHK4 receptor. 8-Amino-iP (**36**) was the most active derivative at the AHK4 receptor, exceeding the activity of 10 μM *t*Z by 10%. However, at 1 μM concentration, it was slightly less active than iP itself. Interestingly, the activity of compound **35** (8-methoxy-iP) only reached 46% of *t*Z activity at 10 μM concentration. This compound was only poorly active at the AHK3 receptor. Compound **38**, bearing a 8-aminoethylamino moiety, reached 24% of *t*Z activity. In

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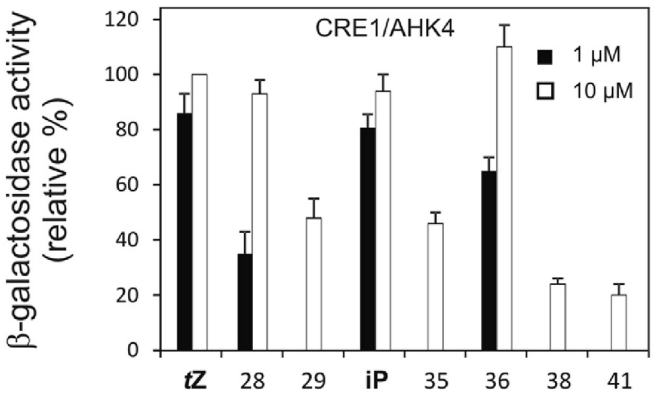

Fig. 3. Ability of selected compounds to activate the receptor CRE1/AHK4 in a receptor activation assay. The β-galactosidase activity correlates with the activation of the receptor by the tested compound. Activities of the compounds are shown relative to that of *t*Z activity at concentration 10 μM and compared with the activity of the parent compound. Error bars show SD ($n = 3$).

contrast with the results obtained for AHK3, compounds **38** and **41** (8-(2-hydroxyethylamino)-iP) were the only two compounds with a short C8 side chain that were able to activate the CRE1/AHK4 receptor (Fig. 3).

2.2.5. Cytotoxicity on human non-cancer cell lines

Some aromatic and isoprenoid cytokinin ribosides are known to be toxic for human cells with IC_{50} values in the sub-micromolar or micromolar range (Doležal et al., 2006; Voller et al., 2010). Therefore, we evaluated the effect of all the compounds on rapidly proliferating human non-tumor cells in sub-confluent cultures after 3 days of treatment. The assay used was based on the ability of cells to reduce resazurin into fluorescent resofurin. A toxic effect results in a reduced conversion of resazurine into fluoresent resofurine. It was assumed that the measured signal correlated with the number of cells. The cell panel comprised skin fibroblast (BJ) and two spontaneously immortalized cell lines - keratinocytes (HaCaT) and retinal pigment epithelial cells (ARPE-19). The data are summarized in Supplementary Tables 1 and 2.

Most derivatives had no or only a limited detrimental effect on the proliferation (resofurin fluorescence > 85% of control) at 50 μM, the maximal concentration tested. A decrease of over 20% for one or more of the cell lines was observed in the case of 8-oxoderivatives of *c*Z (**31**), iP (**34**) and K (**48**), as well as for 8-(3-aminopropoxy)-iP (**42**). In the group of compounds with THP at position 9, a similar effect was **observed for** the derivatives of iP with longer 8-side chains terminated by an amino group (**9**, **10** and **12**).

Overall, these results are in accordance with previous studies showing that cytokinin bases and their THP derivatives usually have favorable toxicity profiles (Doležal et al., 2006; Szüčová et al., 2009a; Voller et al., 2010).

3. Conclusion

In summary, two groups of C8 substituted cytokinins were prepared, characterized and screened for their cytokinin activity. Some of the synthesized compounds were found to be very active in different cytokinin bioassays, especially C8-halogenated isoprenoid cytokinins. However, representatives of both the aromatic and isoprenoid cytokinins bearing short alkyl-amino side chains at the C8 position were shown to possess high to satisfactory cytokinin activity. Two of them (compounds **38** and **41**) were also able to activate both cytokinins receptors. These compounds are very promising candidates for further labeling or conjugation for application as protein-affinity ligands or fluorescent labeled probes.

4. Experimental procedures

4.1. Chemicals

6-Chloropurine and 3-methylbut-2-en-1-amine hydrochloride were purchased from Olchemim. EtOAc, MeOH and $CHCl_3$ were purchased from Penta. HOAc, PrOH and Me_2CO were purchased from Lach-Ner. TEA (stored over KOH), THF (pre-dried with KOH, dried with $LiAlH_4$ and distilled under Ar) and 1,6-diaminohexane were purchased from Fluka. LDA (1.8 M soln. in THF/heptane/ethylbenzene) and 1,3-diaminopropane were purchased from Sigma-Aldrich. CBr_4, 1,2-diaminoethane and 1,4-diaminobutane were purchased from Koch-Light Laboratories. 3,4-dihydro-2*H*-pyran (stored over KOH) was purchased from Janssen Chimica. Hexane (mixture of isomers, *puriss*) and *iso*-PrOH were purchased from Riedel-de Haën. All solvents were *pro analysis* purity.

4.2. General procedures

Melting points were determined on Kofler apparatus and are reported uncorrected. TLC was carried out using silica gel 60 F_{254} plates (Merck Co.). $CHCl_3$-MeOH-NH_4OH (95:5:0.5, 9:1:0.1, 8:2:0.2 or 7:3:0.3) and $CHCl_3$-Me_2CO (4:1 or 3:2) were used as mobile phases.

Crude products were purified by middle pressure column chromatography (CC) using silica gel PharmPrep 60 CC (40–63 μm, Merck) as a stationary phase packed in a glass column (Kronlab 15 mm diameter column or Pharmacia Fine Chemicals 25 mm diameter column). As a mobile phase, hexanes-EtOAc (3:2) or $CHCl_3$-MeOH-NH_4OH (95:5 0.5, 9:1:0.1 or 3:1:2.5) were used. The flow rate of the mobile phase through the system was 7.5 ml/min.

Samples for HPLC and UV characterization were dissolved in MeOH, applied to a LiChroCARD 250 × 4 mm column filled with Purospher RP-18e, 5 μm (Merck) and analyzed using a Beckman Gold system. The separated constituents were eluted by isocratic elution using a mixture of MeOH (HPLC grade) and HOAc/$AcONH_4$ buffer (pH = 3,4; 40 mM; with addition of 5% MeOH) at a flow rate of 0.5 ml/min. Eluting compounds were detected by scanning the UV absorbance of the eluate between 200 and 300 nm. The HPLC purity (230 nm) of the presented compounds was always higher than 97%, unless otherwise stated.

Elemental analyses were performed on a CHN-O analyzer (Thermo Finnigan Flash, EA 1112 series); measured values (C, H, N) agreed with calculated values within acceptable limits. Mass spectra were measured on a Waters Micromass mass spectrometer equipped with a ZMD detector, direct inlet, electrospray ionization (ESI) and a coin voltage (CV).

^{1}H NMR spectra were recorded on a Bruker Avance 300 spectrometer operating at a temp. of 300 K and frequencies of 300.13 MHz (^{1}H) and 75 MHz (^{13}C) or a JEOL ECA-500 spectrometer operating at a temp. of 298 K and frequencies of 500.16 MHz (^{1}H) and 125.76 MHz (^{13}C). Samples were dissolved in DMSO-d_6 or $CDCl_3$. TMS was used as the internal reference standard. HPLC, UV, ^{1}H NMR, ^{13}C NMR and MS data are presented in full in the Supplementary Information. For compounds **31**, **34**, **44** and **48**, IR data are also presented.

4.3. Syntheses

4.3.1. Syntheses of described compounds

Preparation and characterization of the following compounds have been described in the lit. We applied analogical methods and

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obtained substances with similar properties to those mentioned in the listed references.

6-chloro-9-(tetrahydropyran-2-yl)-9*H*-purine (I, Taddei et al., 2004),

(*E*)-6-(4-hydroxy-3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9*H*-purine (II, Szüčová et al., 2009b),

6-(3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9*H*-purine (III, Mik et al., 2011),

6-benzylamino-9-(tetrahydropyran-2-yl)-9*H*-purine (IV, Szüčová et al., 2009b),

6-furfurylamino-9-(tetrahydropyran-2-yl)-9*H*-purine (V). We modified the method of preparation described in the lit. Robins et al. (1961) by using PrOH as a solvent and TEA as a basic additive. Consequently, only two equivalents of furfurylamine were needed.

6,8-dichloro-9-(tetrahydropyran-2-yl)-9*H*-purine (VI, Nolsøe et al., 1998).

6-benzylamino-8-chloropurine (43, Moravec, 2004).

8-amino-6-furfurylaminopurine (51, Zahajská et al., 2013).

4.3.2. Synthesis of (Z)-6-(4-hydroxy-3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9H-purine (VII)

(*Z*)-4-Amino-1-methylbut-2-en-1-ol (0.87 g, 8.6 mmol, Tolman et al., 1999) was dissolved in PrOH (14 ml). TEA (0.95 ml, 6.9 mmol) and 6-chloro-9-(tetrahydropyran-2-yl)purine (I, 1.64 g, 6.9 mmol) were added and the mixture was heated at 45 °C for 6 h. After cooling, the mixture was filtered and the filtrate was partly evaporated and purified by CC (60.3 g of silica gel, mobile phase $CHCl_3$-Me_2CO 4:1, flow rate 7.5 ml/min). The product was a light yellow viscous substance that started to crystallize after two weeks of drying over P_2O_5 in a vacuum desiccator. Yield 1.50 g (72%). M. p.: 127–131 °C. Elem. anal. (Found: C, 59.0; H, 6.8; N, 23.0. $C_{15}H_{21}N_5O_2$ requires: C, 59.4; H, 7.0; N, 23.1%).

*4.3.3. General procedure for syntheses of C8-halogenated 9-(tetrahydropyran-2-yl)cytokinines (compounds **1, 2**, **4**, **5**, **13**, **14**, **16**–**18**)*

9-(Tetrahydropyran-2-yl)cytokinines (compounds **II**–**V**, **VII**, **IX**; 0.9–8.0 mmol) was dissolved under Ar in dry THF (1 mmol/3–5 ml). The soln. was treated dropwise with LDA soln. during 15–60 min (15–45 ml, 30–90 mmol). The reaction mixture was stirred at −78 °C for 1–1.5 h. Afterwards, a soln. of halogen donor (9.0–24.2 mmol) in dry THF (6–20 ml) was added dropwise during 12–35 min to the reaction mixture, which was then stirred for 1 h. Next, 20% NH_4Cl (20–70 ml) was added dropwise. After spontaneous warming to room temp., the mixture separated into different layers. The aq. layer was extracted with EtOAc (3 × 10–25 ml), whereas the collected organic layers were dried with Na_2SO_4 and evaporated. The crude product was purified by CC. Details of the syntheses are summarized in Supplementary Table 3. This procedure was applied to synthesize the following compounds:

(*E*)-8-chloro-6-(4-hydroxy-3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9*H*-purine (1). Beige crystals. Yield 0.51 g (49%). M. p.: 130–134 °C (cryst. from *iso*-PrOH). Elem. anal. (Found: C, 53.2; H, 6.1; N, 21.0. $C_{15}H_{20}ClN_5O_2$ requires: C, 53.3; H, 6.0; N, 20.7%).

(*E*)-8-bromo-6-(4-hydroxy-3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9*H*-purine (2). Light beige crystals. Yield 1.19 g (45%). M. p.: 138–142 °C (cryst. from EtOAc). Elem. anal. (Found: C, 47.3; H, 5.1; N, 18.5. $C_{15}H_{20}BrN_5O_2$ requires: C, 47.1; H, 5.3; N, 18.3%).

8-chloro-6-(3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9*H*-purine (4). Light beige crystals. Yield 1.53 g (66%). M. p.: 118–120 °C (cryst. from hexanes-EtOAc 3:2). Elem. anal. (Found: C, 55.7; H, 6.2; N, 21.5. $C_{15}H_{20}ClN_5O$ requires: C, 56.0; H, 6.3; N, 21.8%).

8-bromo-6-(3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9*H*-purine (5). Light beige crystals. Yield 2.28 g (60%). M. p.: 125–128 °C. Elem. anal. (Found: C, 49.4; H, 5.6; N, 19.0. $C_{15}H_{20}BrN_5O$ requires: C, 49.2; H, 5.5; N, 19.1%).

6-benzylamino-8-bromo-9-(tetrahydropyran-2-yl)-9*H*-purine (13). Light beige crystals. Yield 2.05 g (76%). M. p.: 120–124 °C. Elem. anal. (Found: C, 60.4; H, 7.5; N, 23.6. $C_{17}H_{18}BrN_5O$ requires: C, 52.6; H, 4.7; N, 18.0%).

6-benzylamino-8-iodo-9-(tetrahydropyran-2-yl)-9*H*-purine (14). White crystals. Yield 134 mg (32%). M. p.: 153–155 °C. Elem. anal. (Found: C, 46.8; H, 4.2; N, 16.1. $C_{17}H_{18}IN_5O$ requires: C, 46.9; H, 4.2; N, 16.1%).

8-chloro-6-furfurylamino-9-(tetrahydropyran-2-yl)-9*H*-purine (16). Light beige crystals. Yield 2.80 g (69%). M. p.: 110–114 °C. Elem. anal. (Found: C, 53.7; H, 4.7; N, 20.8. $C_{15}H_{16}ClN_5O_2$ requires: C, 54.0; H, 4.8; N, 21.0%).

8-bromo-6-furfurylamino-9-(tetrahydropyran-2-yl)-9*H*-purine (17). Light beige-brown powder. Yield 0.44 g (37%). M. p.: 108–112 °C (cryst. from EtOAc). Elem. anal. (Found: C, 47.2; H, 4.3; N, 18.5. $C_{15}H_{16}BrN_5O_2$ requires: C, 47.3; H, 4.3; N, 18.5%).

6-furfurylamino-8-iodo-9-(tetrahydropyran-2-yl)-9*H*-purine (18). Light beige powder. Yield 2.00 g (66%). M. p.: 130–134 °C (cryst. from EtOAc). Elem. anal. (Found: C, 42.2; H, 3.8; N, 16.6. $C_{15}H_{16}IN_5O_2$ requires: C, 42.4; H, 3.8; N, 16.5%).

*4.3.4. General procedure for syntheses of C8-dimethylamino substituted 9-(tetrahydropyran-2-yl)cytokinines (compounds **3**, **7**, **23**)*

8-Halogeno-9-(tetrahydropyran-2-yl)cytokinin (compounds **1**, **5**, **16**; 0.56–0.69 mmol) was dissolved in 50% soln. of Me_2NH in MeOH (2.5–3 ml). The soln. was left at room temp. overnight or for 2 days, evaporated and purified by extraction and crystallization or by CC. Details of the syntheses are summarized in Supplementary Table 4. This procedure was applied to synthesize the following compounds:

(*E*)-6-(4-hydroxy-3-methylbut-2-en-1-ylamino)-8-dimethylamino-9-(tetrahydropyran-2-yl)-9*H*-purine (3). White crystals. Yield 217 mg (90%). M. p.: 110–114 °C. Elem. anal. (Found: C, 60.0; H, 7.6; N, 24.5. $C_{17}H_{26}N_6O_2$ requires: C, 58.9; H, 7.6; N, 24.3%).

8-dimethylamino-6-(3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9*H*-purine (7). Yellowish crystals. Yield 169 mg (91%). M. p.: 76–79 °C. Elem. anal. (Found: C, 61.9; H, 7.9; N, 25.6. $C_{17}H_{26}N_6O$ requires: C, 61.8; H, 7.9; N, 25.4%).

6-furfurylamino-8-dimethylamino-9-(tetrahydropyran-2-yl)-9*H*-purine (23). Yellowish crystals (crystallization from EtOAc). Yield 195 mg (83%). M. p.: 174–176 °C. Elem. anal. (Found: C, 59.7; H, 6.6; N, 24.6. $C_{17}H_{22}N_6O_2$ requires: C, 59.6; H, 6.5; N, 24.5%).

*4.3.5. Syntheses leading to 8-amino-6-(3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9H-purine (**6**)*

*4.3.5.1. Synthesis of 8-amino-6-chloro-9-(tetrahydropyran-2-yl)-9H-purine (**VIII**).* Compound VI (0.22 g, 0.81 mmol) was dissolved in *iso*-PrOH satd. with NH_3 at 0 °C (3 ml). The reaction was carried out at room temp. for 1 day. The resulting crystals were filtered off, washed with cooled *iso*-PrOH and dried in a desiccator with P_2O_5. White needles. Yield 0.09 g (44%). M. p. = 198 °C (decomp.). Elem. anal. (Found: C, 47.3; H, 4.8; N, 27.6. $C_{10}H_{12}ClN_5O$ requires: C, 47.4; H, 4.8; N, 27.6%).

*4.3.5.2. Synthesis of 8-amino-6-(3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9H-purine (**6**).* Compound VIII (72 mg, 0.28 mmol) and 3-methylbut-2-en-1-amine hydrochloride (60 mg, 0.55 mmol) were dissolved in PrOH (5 ml) and DIPEA (194 µl,

1.13 mmol). The mixture was heated at 110 °C for 90 h, then it was evaporated and the residue mixed with the mobile phase EtOAc-Me_2CO-NH_4OH (9:1:0.1). The resulting solid was filtered off, the filtrate evaporated and the residue purified by CC (mobile phase EtOAc-Me_2CO-NH_4OH 9:1:0.1). White solid. Yield 21 mg (24%). Elem. anal. (Found: C, 59.3; H, 7.2; N, 27.6. $C_{15}H_{22}N_6O$ requires: C, 59.6; H, 7.3; N, 27.8%).

*4.3.6. General procedure for syntheses of C8-alkylamino substituted 9-(tetrahydropyran-2-yl)cytokinines (compounds **8**–**11**, **24**–**27**, **IX**)*

8-Halogeno-9-(tetrahydropyran-2-yl)cytokinines (0.64–1.05 mmol) were dissolved in an appropriate alkylamine (2.0 ml). In some cases, *iso*-PrOH (4.0 ml) was used as a solvent and TEA (0.15 ml) as an additional basic reagent. The reaction mixture was heated under Ar at 50–80 °C for 75 min–4 days. Afterwards, the reaction mixture was evaporated and the residue purified by CC. The product was dried in a desiccator with P_2O_5. Details of the syntheses are summarized in Supplementary Table 5. This procedure was applied to synthesize the following compounds:

8-(2-aminoethylamino)-6-(3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9*H*-purine (8). White foam. Yield 0.26 g (93%). Elem. anal. (Found: C, 58.8; H, 8.1; N, 28.7. $C_{17}H_{27}N_7O$ requires: C, 59.1; H, 7.9; N, 28.4%).

8-(3-aminopropylamino)-6-(3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9*H*-purine (9). Yellowish, viscous liquid. Yield 0.25 g (78%). Elem. anal. (Found: C, 60.4; H, 8.0; N, 27.6. $C_{18}H_{29}N_7O$ requires: C, 60.1; H, 8.1; N, 27.3%).

8-(4-aminobutylamino)-6-(3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9*H*-purine (10). Yellowish, viscous liquid. Yield 264 mg (78%). Elem. anal. (Found: C, 61.4; H, 8.5; N, 26.6. $C_{19}H_{31}N_7O$ requires: C, 61.1; H, 8.4; N, 26.3%).

8-(6-aminohexylamino)-6-(3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9*H*-purine (11). Light yellowish, glassy substance. Yield 289 mg (82%). Elem. anal. (Found: C, 62.4; H, 8.5; N, 24.6. $C_{21}H_{35}N_7O$ requires: C, 62.8; H, 8.8; N, 24.4%).

6-furfurylamino-8-(2-hydroxyethylamino)-9-(tetrahydropyran-2-yl)-9*H*-purine (24). Yellowish foam. Yield 0.18 g (56%). Elem. anal. (Found: C, 56.7; H, 6.5; N, 23.8. $C_{17}H_{22}N_6O_3$ requires: C, 57.0; H, 6.2; N, 23.5%).

6-furfurylamino-8-(3-hydroxypropylamino)-9-(tetrahydropyran-2-yl)-9*H*-purine (25). Yellowish foam. Yield 0.28 g (90%). Elem. anal. (Found: C, 58.4; H, 6.5; N, 22.4. $C_{18}H_{24}N_6O_3$ requires: C, 58.1; H, 6.5; N, 22.6%).

8-allylamino-6-furfurylamino-9-(tetrahydropyran-2-yl)-9*H*-purine (26). Rust-colored, viscous liquid. Yield 0.13 g (58%). Elem. anal. (Found: C, 61.4; H, 6.5; N, 23.4. $C_{18}H_{22}N_6O_2$ requires: C, 61.0; H, 6.3; N, 23.7%).

8-(2-aminoethylamino)-6-furfurylamino-9-(tetrahydropyran-2-yl)-9*H*-purine (27). Yellowish, viscous substance. Yield 0.36 g (77%). Elem. anal. (Found: C, 56.7; H, 6.6; N, 27.7. $C_{17}H_{23}N_7O_2$ requires: C, 57.1; H, 6.5; N, 27.4%).

8-(2-hydroxyethylamino)-6-(3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9*H*-purine (IX). Rusty, viscous substance. Yield 180 mg (78%). Elem. anal. (Found: C, 58.7; H, 7.6; N, 24.5. $C_{17}H_{26}N_6O_2$ requires: C, 58.9; H, 7.6; N, 24.3%). This compound was used to synthesize compound **41**.

*4.3.7. Synthesis of 8-(3-aminopropyloxy)-6-(3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9H-purine (**12**)*

NaH in mineral oil (34%, 67 mg, 0.95 mmol) was dissolved under Ar in a mixture of 3-amino-1-propanol (66 μl, 0.86 mmol) and DMF (5 ml) for 1.5 h. Compound **4** (214 mg, 0.66 mmol) was added and the reaction mixture was stirred under Ar at room temp. for 4 days, then evaporated and the residue purified by CC (gradient elution, starting with $CHCl_3$ as a mobile phase, ending with mobile phase $CHCl_3$-CH_3OH-NH_4OH 9:1:0.1). The product was dried in a desiccator with P_2O_5. Yellow, viscous substance. Yield 156 mg (65%). Elem. anal. (Found: C, 60.4; H, 7.5; N, 23.6. $C_{18}H_{28}N_6O_2$ requires: C, 60.0; H, 7.8; N, 23.3%).

*4.3.8. Synthesis of 6-benzylamino-8-benzyloxy-9-(tetrahydropyran-2-yl)-9H-purine (**15**)*

Sodium (40 mg, 1.7 mmol) was dissolved in dry benzyl alcohol (1.0 ml). Compound **13** (387 mg, 1.00 mmol) was dissolved in DMF (3.0 ml) and the soln. was added dropwise to the sodium benzyl alcoholate soln. The reaction mixture was heated at room temp. under an Ar atmosphere for 4.5 h. Then, the mixture was neutralized with 2 drops of glacial HOAc and evaporated. The crude product, a white solid, was triturated with EtOAc (10 ml), heated in a boiling water bath and filtered. The filtrate was left in a refrigerator for 2 days. The resulting crystals were filtered off and dried in a desiccator with P_2O_5. White crystals. Yield 267 mg (64%). M. p.: 149–151 °C. Elem. anal. (Found: C, 69.8; H, 6.3; N, 16.8. $C_{24}H_{25}N_5O_2$ requires: C, 69.4; H, 6.1; N, 16.9%).

*4.3.9. Synthesis of 6-furfurylamino-8-methoxy-9-(tetrahydropyran-2-yl)-9H-purine (**19**)*

KOtBu (107 mg, 0.53 mmol) was added to a soln. of compound **16** (264 mg, 0.79 mmol) in dry MeOH (7 ml) and the soln. was heated at 60 °C for 10 h. After filtration, the mixture was evaporated and purified by CC (mobile phase $CHCl_3$-Me_2CO 9:1). The resulting product was crystallized from EtOAc. Yield 201 mg (73%). White crystals. M. p.: 137–139 °C. Elem. anal. (Found: C, 58.8; H, 6.0; N, 21.0. $C_{16}H_{19}N_5O_3$ requires: C, 58.4; H, 5.8; N, 21.3%).

*4.3.10. Synthesis of 6-furfurylamino-8-propyloxy-9-(tetrahydropyran-2-yl)-9H-purine (**21**)*

Compound **17** (303 mg, 0.80 mmol) was dissolved in PrOH (5 ml). A soln. of NaOH (0.41 g, 10.25 mmol) in H_2O (13 ml) was added. The reaction mixture was heated under vigorous stirring at 60 °C overnight and then evaporated. The residue was dissolved in EtOAc (10 ml), washed with H_2O (3 × 10 ml), dried with $MgSO_4$, filtered off and evaporated. The crude product was purified by CC (mobile phase hexanes-EtOAc 3:2, on TLC $R_f = 0.14$). After CC, the collected fractions were concentrated *in vacuo*. The product was dried in a desiccator with P_2O_5. Yellow viscous liquid. Yield: 187 mg (65%). Elem. anal. (Found: C, 60.3; H, 6.6; N, 19.2. $C_{18}H_{23}N_5O_3$ requires: C, 60.5; H, 6.5; N, 19.6%).

*4.3.11. Synthesis of 8-benzyloxy-6-furfurylamino-9-(tetrahydropyran-2-yl)-9H-purine (**22**)*

Sodium (25 mg, 1.1 mmol) was dissolved in dry benzyl alcohol (3 ml). Compound **17** (206 mg, 0.54 mmol) was added to the sodium benzyl alcoholate soln. and the reaction mixture was heated at 65 °C overnight. After cooling, the mixture was diluted with Et_2O (8 ml). The resulting precipitate was filtered off and the filtrate purified by CC (mobile phase hexanes-EtOAc 1:1, on TLC $R_f = 0.09$). After CC, the collected fractions were concentrated *in vacuo*. The product was precipitated from hexanes, crystallized from MeOH and dried in a desiccator with P_2O_5. White crystals. Yield 43 mg (20%). M. p.: 132–135 °C. Elem. anal. (Found: C, 65.5; H, 5.6; N, 17.2. $C_{22}H_{23}N_5O_3$ requires: C, 65.2; H, 5.7; N, 17.3%).

*4.3.12. Synthesis of 6-furfurylamino-8-methylsulfanyl-9-(tetrahydropyran-2-yl)-9H-purine (**20**)*

MeSNa (0.28 g, 4.00 mmol) was added to a soln. of compound **17** (0.76 g, 2.01 mmol) in *iso*-PrOH (8 ml) and the soln. was heated at 45 °C for 2.5 h. The mixture was evaporated and diluted with H_2O (6 ml) and EtOAc (7 ml). After extraction, the water layer was washed with EtOAc (1 × 7 ml). Collected organic layers were dried

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with Na_2SO_2, filtered and evaporated. The crude crystalline product (0.67 g, 96%) was crystallized from *iso*-PrOH (9 ml) and EtOH (8 ml). Light yellow-brownish crystals. M. p.: 153–156 °C. Elem. anal. (Found: C, 55.8; H, 5.6; N, 23.0. $C_{16}H_{19}N_5O_2S$ requires: C, 55.6; H, 5.5; N, 23.3%).

4.3.13. Synthesis of 8-methoxy-6-(3-methylbut-2-en-1-ylamino)-9-(tetrahydropyran-2-yl)-9H-purine (X)

Compound **5** (248 mg, 0.68 mmol) was added to a soln. of KOtBu (103 mg, 0.98 mmol) in dry MeOH (7 ml). The soln. was heated at 45 °C for 13 h and after cooling, placed in a refrigerator overnight, then filtered and evaporated. The crude product was purified by CC (mobile phase $CHCl_3$-Me_2CO 9:1. Viscous, yellow substance. Yield 170 mg (79%). The product contained ca. 30% of impurities. Therefore, elemental analysis was not performed and the product was used directly in the next synthesis (compound **35**).

4.3.14. Synthesis of 6-furfurylamino-8-(2-hydroxyethyloxy)-9-(tetrahydropyran-2-yl)-9H-purine (XI)

KOtBu (122 mg, 1.08 mmol) was added to a suspension of compound **16** (261 mg, 0.78 mmol) in dry ethylene glycol (2.4 ml) and THF (0.4 ml). The suspension was heated at 45 °C overnight and the resulting rusty soln. was heated for another 8.5 h at 60 °C. The soln. was evaporated and purified by CC (mobile phase $CHCl_3$-Me_2CO 3:1). Rusty, viscous substance. Yield 307 mg (>100% theory). According to TLC, the product contained impurities and was used without detailed characterization for the synthesis of compound **58**.

*4.3.15. General procedures for the cleavage of a THP group (synthesis of compounds **28**–**33**, **35**–**42**, **45**–**47**, **49**–**58**)*

*4.3.15.1. Procedure A (synthesis of compounds **28**, **30**, **35**–**41**, **45**–**47**, **49**, **50**, **52**–**58**).*

8-Substituted-9-(tetrahydropyran-2-yl)cytokinin (0.07–0.78 mmol) was dissolved in HOAc-H_2O (4:1 or 5:3, 1.6–2.0 ml) and stirred at room temp. overnight for 2 days. The mixture was evaporated to dryness. The residuum was dissolved in H_2O-EtOH 2:1, and evaporated. This procedure was repeated ×3 to facilitate removal of HOAc. The crude product was crystallized from alcohol and/or purified by CC (details are given separately for each substance) and dried in a desiccator with P_2O_5. This procedure was applied to synthesize the following compounds:

(*E*)-8-chloro-6-(4-hydroxy-3-methylbut-2-en-1-ylamino)purine (28). CC (mobile phase $CHCl_3$, on TLC $R_f = 0.16$). White crystals. Yield 96 mg (75%). M. p.: 236–238 °C. Elem. anal. (Found: C, 47.5; H, 4.8; N, 27.7. $C_{10}H_{12}ClN_5O$ requires: C, 47.4; H, 4.8; N, 27.6%).

(*E*)-6-(4-hydroxy-3-methylbut-2-en-1-ylamino)-8-dimethylaminopurine (30). CC (mobile phase $CHCl_3$, on TLC $R_f = 0.05$). White crystals. Yield 64 mg (85%). M. p.: 245–249 °C. Elem. anal. (Found: C, 54.7; H, 6.8; N, 31.6. $C_{12}H_{18}N_6O$ requires: C, 55.0; H, 6.9; N, 32.0%).

8-methoxy-6-(3-methylbut-2-en-1-ylamino)purine (35). Crystallized from *iso*-PrOH (3 ml) to give **a** white powder. Yield 34 mg (33%). M. p.: 205–210 °C. Elem. anal. (Found: C, 56.8 H, 6.4; N, 30.1. $C_{11}H_{15}N_5O$ requires: C, 56.6; H, 6.5; N, 30.0%).

8-amino-6-(3-methylbut-2-en-1-ylamino)purine (36). CC (mobile phase $CHCl_3$-MeOH-NH_4OH 9:1:0.1). White crystals. Yield 6 mg (41%). M. p.: 254–262 °C (decomp.). Elem. anal. (Found: C, 54.8 H, 6.4; N, 38.4. $C_{10}H_{14}N_6$ requires: C, 55.0; H, 6.5; N, 38.5%).

8-dimethylamino-6-(3-methylbut-2-en-1-ylamino)purine (37). Crude product was crystallized from MeOH. White, cotton-like crystals. Yield 46 mg (68%). M. p.: 246–249 °C. Elem. anal. (Found: C, 58.6 H, 7.2; N, 34.4. $C_{12}H_{18}N_6$ requires: C, 58.5; H, 7.4; N, 34.1%).

8-(2-aminoethylamino)-6-(3-methylbut-2-en-1-ylamino)purine (38). CC (mobile phase $CHCl_3$-MeOH-NH_4OH 3:1:2.5). Yellowish, amorphous product. Yield: 84 mg (79%). Elem. anal. (Found: C, 55.6 H, 7.5; N, 38.0. $C_{12}H_{19}N_7$ requires: C, 55.2; H, 7.3; N, 37.5%).

8-(3-aminopropylamino)-6-(3-methylbut-2-en-1-ylamino) purine (39). CC (mobile phase $CHCl_3$-MeOH-NH_4OH 7:3:0.3). Yellowish, amorphous product. Yield 126 mg (98%). Elem. anal. (Found: C, 57.1 H, 7.2; N, 35.1. $C_{13}H_{21}N_7$ requires: C, 56.7; H, 7.7; N, 35.6%).

8-(4-aminobutylamino)-6-(3-methylbut-2-en-1-ylamino) purine (40). CC (mobile phase $CHCl_3$-MeOH-NH_4OH 3:1:2.5). Yellowish, amorphous product. Yield 108 mg (75%). Elem. anal. (Found: C, 58.5 H, 8.3; N, 34.4. $C_{14}H_{23}N_7$ requires: C, 58.1; H, 8.0; N, 33.9%).

8-(2-hydroxyethylamino)-6-(3-methylbut-2-en-1-ylamino) purine (41). Crystallized from MeOH. White powder. Yield 41 mg (59%). M. p.: 228–230 °C. Elem. anal. (Found: C, 54.5 H, 6.6; N, 31.5. $C_{12}H_{18}N_6O$ requires: C, 55.0; H, 6.9; N, 32.0%).

8-chloro-6-furfurylaminopurine (45). Crude product was dissolved in MeOH satd. with NH_3 at 0 °C (3 ml), diluted with MeOH (1 ml), partly evaporated and left to crystallize in a refrigerator to give a beige powder. Yield 23 mg (19%). M. p.: 276–278 °C. Elem. anal. (Found: C, 48.0 H, 3.2; N, 28.0. $C_{10}H_8ClN_5O$ requires: C, 48.1; H, 3.2; N, 28.1%).

8-bromo-6-furfurylaminopurine (46). Crystallized from MeOH to give a beige powder. Yield 97 mg (66%). M. p.: 239–240 °C. Elem. anal. (Found: C, 40.9 H, 2.6; N, 23.5. $C_{10}H_8BrN_5O$ requires: C, 40.8; H, 2.7; N, 23.8%).

6-furfurylamino-8-iodopurine (47). Beige powder. Yield 172 mg (97%). M. p.: 220–222 °C. Elem. anal. (Found: C, 35.0 H, 2.5; N, 20.7. $C_{10}H_8IN_5O$ requires: C, 35.2; H, 2.4; N, 20.5%).

Synthesis of 6-furfurylamino-8-methoxypurine (49). The residuum was crystallized from MeOH (2 ml) with filtration. The soln. was placed into a refrigerator for 2 days and then solid impurities were removed by decantation. The mother liquor was diluted with MeOH and H_2O and partly evaporated until cotton wool-like crystals appeared. The mother liquor was placed into a refrigerator overnight and then resulting crystals were filtered off and dried. Light beige crystals. Yield 50 mg (60%). M. p.: 196–198 °C. Elem. anal. (Found: C, 53.7 H, 4.4; N, 28.7. $C_{11}H_{11}N_5O_2$ requires: C, 53.9; H, 4.5; N, 28.6%).

6-furfurylamino-8-methylsulfanylpurine (50). Light beige needle crystals. Yield (I) 64 mg (42%). M. p.: 226–228 °C. A second portion of the product was obtained from the concentrated mother liquor. Yield (II) 54 mg (36%). Elem. anal. (Found: C, 50.3 H, 4.4; N, 26.6. $C_{11}H_{11}N_5OS$ requires: C, 50.6; H, 4.2; N, 26.8%).

6-furfurylamino-8-dimethylaminopurine (52). After deprotection, the mixture was neutralized with satd. aq. NH_3 (2.0 ml). The suspension was placed into a refrigerator overnight, the solid precipitate was filtered off and crystallized from MeOH (5 ml, with filtration). To initiate crystallization, partial evaporation of MeOH was necessary. Recrystallization from a mixture of conc. NH_4OH-MeOH-H_2O gave white, fibrous crystals. Yield 52 mg (70%). M. p.: 245–246 °C. Elem. anal. (Found: C, 55.9 H, 5.5; N, 32.5. $C_{12}H_{14}N_6O$ requires: C, 55.8; H, 5.5; N, 32.5%).

8-(2-aminoethylamino)-6-furfurylaminopurine (53). CC (mobile phase $CHCl_3$-MeOH-NH_4OH 7:3:0.3). Yellowish, glassy substance. Yield 24 mg (88%). M. p.: 105–110 °C. Elem. anal. (Found: C, 52.2 H, 5.6; N, 35.7. $C_{12}H_{15}N_7O$ requires: C, 52.7; H, 5.5; N, 35.9%).

6-furfurylamino-8-(2-hydroxyethylamino)purine (54). White plaster solid. Yield 52 mg (78%). M. p.: 230–233 °C. Elem. anal. (Found: C, 52.1 H, 4.8; N, 30.7. $C_{12}H_{14}N_6O_2$ requires: C, 52.6; H, 5.1; N, 30.6%).

6-furfurylamino-8-(3-hydroxypropylamino)purine (55). The product (110 mg) was crystallized from $CHCl_3$-MeOH (95:5, 3 ml) to give a white powder. Yield (I) 25 mg (25%). M. p.: 201–203 °C. A

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second portion of the product was obtained from the mother liquor placed in a refrigerator. Yield (II) 22 mg (22%). Elem. anal. (Found: C, 54.6 H, 5.5; N, 29.0. $C_{13}H_{16}N_6O_2$ requires: C, 54.2; H, 5.6; N, 29.2%).

8-allylamino-6-furfurylaminopurine (56). Crystallized from MeOH (11 ml, with filtration). White solid. Yield 32 mg (42%). M. p.: 242–244 °C. Elem. anal. (Found: C, 57.4 H, 5.0; N, 31.6. $C_{13}H_{14}N_6O$ requires: C, 57.8; H, 5.2; N, 31.1%).

6-furfurylamino-8-(3-propyloxy)purine (57). Crystallized from EtOH. Light beige crystals. Yield 42 mg (55%). M. p.: 196–198 °C. Elem. anal. (Found: C, 56.8 H, 5.3; N, 25.7. $C_{13}H_{15}N_5O_2$ requires: C, 57.1; H, 5.5; N, 25.6%).

6-furfurylamino-8-(2-hydroxyethyloxy)purine (58). Rusty crystals. Yield 67 mg (31%). M. p.: 182–184 °C. Elem. anal. (Found: C, 52.0 H, 4.5; N, 25.7. $C_{12}H_{13}N_5O_3$ requires: C, 52.4; H, 4.8; N, 25.4%).

*4.3.15.2. Procedure B (synthesis of compounds **29**, **32**, **33**, **42**).* 8-Substituted-9-(tetrahydropyran-2-yl)cytokinin (1.2 mmol) was dissolved in MeOH (8 ml) with heating. H_2O (0.8 ml) and 1 drop of 10% HCl were added. The reaction mixture was stirred at 45 °C for 24 h. The suspension was then placed in a refrigerator overnight. This procedure was applied to synthesize the following compounds:

(*E*)-8-bromo-6-(4-hydroxy-3-methylbut-2-en-1-ylamino)purine (29). Beige **precipitate**. Yield 274 mg (74%). M. p.: 224–226 °C. Elem. anal. (Found: C, 40.5; H, 3.8; N, 23.5. $C_{10}H_{12}BrN_5O$ requires: C, 40.3; H, 4.1; N, 23.5%).

8-chloro-6-(3-methylbut-2-en-1-ylamino)purine (32). White powder. Yield 142 mg (85%). M. p.: 262–265 °C. Elem. anal. (Found: C, 50.2 H, 5.1; N, 29.6. $C_{10}H_{12}ClN_5$ requires: C, 50.5; H, 5.1; N, 29.5%).

8-bromo-6-(3-methylbut-2-en-1-ylamino)purine (33). White powder. Yield 137 mg (84%). M. p.: 233–235 °C. Elem. anal. (Found: C, 43.0 H, 4.5; N, 25.0. $C_{10}H_{12}BrN_5$ requires: C, 42.6; H, 4.3; N, 24.8%).

8-(3-aminopropyloxy)-6-(3-methylbut-2-en-1-ylamino)purine (42). After de-protection, the soln. was cooled and the pH adjusted with 1 M NaOH (30 drops) to pH 7–8. The soln. was evaporated to dryness. The residuum was purified by CC (mobile phase $CHCl_3$-MeOH-NH_4OH 8:2:0.2). Yellowish, amorphous product. Yield 83 mg (75%). Elem. anal. (Found: C, 56.5 H, 7.0; N, 30.0. $C_{13}H_{20}N_6O$ requires: C, 56.5; H, 7.3; N, 30.4%).

*4.3.16. Synthesis of 6-benzylamino-7,9-dihydro-8H-purin-8-one (**44**)*

Compound **13** (405 mg, 1.04 mmol) and NaOAc (870 mg, 10.6 mmol) were added to glacial HOAc (5.0 ml) and then heated at 120 °C for 2 days. After cooling, H_2O (11.0 ml) was added and the pH of the mixture was adjusted to pH = 6 with 30% NaOH (1.8 ml). The resulting suspension was placed into a refrigerator for 4 h, and then the precipitate was filtered off. The solid substance on the filter was an un-pure substance of unknown structure analogical to those obtained for compound **34** (8-oxo iP) and compound **31** (8-oxo-*cis*-zeatin). The desired 8-oxo-BA derivative was obtained by diluting the filtrate with H_2O and placing it into a refrigerator for 9 days. Yield after crystallization from PrOH: 21 mg (8%) of white crystals. M. p.: 255–258 °C; Elem. anal. (Found: C, 59.5 H, 4.8; N, 29.3. $C_{12}H_{11}N_5O$ requires: C, 59.7; H, 4.6; N, 29.0%).

*4.3.17. Synthesis of 6-furfurylamino-7,9-dihydro-8H-purin-8-one (**48**)*

Compound **46** (720 mg, 2.45 mmol) was dissolved in glacial HOAc (17 ml), NaOAc (2.0 g, 24.4 mmol) was added and the suspension heated at 120 °C for 1.5 days. Two main products were generated. The desired 8-oxoderivative had on TLC R_f = 0.61 (CHCl3-MeOH-NH_4OH 8:2:0.2). The other substance was unwanted and had R_f = 0.83 (CHCl3-MeOH-NH_4OH 8:2:0.2). After cooling, the mixture was diluted with H_2O (10.0 ml) and 30% NaOH (7 ml) was added. The resulting suspension was placed into a refrigerator for 2 h. The solid, which contained the unwanted substance, was filtered off. The filtrate was neutralized with 36% NaOH (8 ml), a second portion of the unwanted substance was filtered off and the filtrate further neutralized with solid NaOH until the pH = 6. The resulting opaque soln. was placed into a refrigerator for 4 days, then the solid precipitate was filtered off and crystallized from MeOH. Yellow crystals. Yield 90 mg (16%). M. p.: 264–266 °C. Elem. anal. (Found: C, 52.0 H, 4.1; N, 30.7. $C_{10}H_9N_5O_2$ requires: C, 52.0; H, 3.9; N, 30.3%).

*4.3.18. Synthesis of 6-(3-methylbut-2-en-1-ylamino)-7,9-dihydro-8H-purin-8-one (**34**)*

NaOAc (694 mg, 8.5 mmol) was added to a soln. of compound **33** (225 mg, 0.8 mmol) in glacial HOAc (8.0 ml) and the suspension was stirred at 120 °C for 20 h. After cooling, H_2O (4.0 ml) was added and the pH of the soln. adjusted to pH = 5 with 30% NaOH (1 ml). The mixture was placed into a refrigerator overnight and then the resulting crystals filtered off. The crystals were composed of two substances. The desired 8-oxoderivative had on TLC R_f = 0.52 ($CHCl_3$-MeOH-NH_4OH 8:2:0.2). The other unwanted substance had R_f = 0.74 ($CHCl_3$-MeOH-NH_4OH 8:2:0.2) and m. p. = 261–262 °C. The two substances were separated by multiple crystallization from MeOH. The unwanted substance crystallized more easily than the 8-oxoderivative. 6-(3-Methylbut-2-en-1-ylamino)-7,9-dihyrdo-8*H*-purin-8-one: yield 35 mg (20%); light beige crystals, m. p.: 250–254 °C. Elem. anal. (Found: C, 55.1 H, 5.8; N, 32.3. $C_{10}H_{13}N_5O$ requires: C, 54.8; H, 6.0; N, 31.9%).

*4.3.19. Syntheses leading to (Z)-6-(4-hydroxy-3-methylbut-2-en-1-ylamino)-7,9-dihydro-8H-purin-8-one (**31**)*

4.3.19.1. Synthesis of (Z)-6-(4-tert-butyldimethylsilyloxy-3-methylbut-2-en-1-ylamino)-9H-(tetrahydropyran-2-yl)purine (XI). Compound VII (0.3 g, 0.99 mmol), *tert*-butyldimethylchlorosilane (0.29 g, 1.92 mmol) and imidazole (0.16 g, 2.35 mmol) were dissolved in DMF (5.0 ml). After stirring for 20 h at room temp., the soln. was evaporated *in vacuo* (p = 1 mbar, *t* = 50 °C). The residuum was mixed with EtOAc (7 ml) and extracted once with H_2O (7 ml) and with 10% NaCl soln. (2 × 5 ml). The organic layer was dried with Na_2SO_4 overnight, filtered and evaporated. The product was a light yellow, amorphous substance. Yield 0.40 g (97%).

4.3.19.2. Synthesis of (Z)-8-chloro-6-(4-tert-butyldimethylsilyloxy-3-methylbut-2-en-1-ylamino)-9H-(tetrahydropyran-2-yl)purine (XII). Compound XI was halogenated according to the procedure described in 4.3.3. Details of the synthesis are in Supplementary Table 1. Rust-colored, viscous substance. Yield 0.28 g (64%).

4.3.19.3. Synthesis of (Z)-8-benzyloxy-6-(4-tert-butyldimethylsilyloxy-3-methylbut-2-en-1-ylamino)-9H-(tetrahydropyran-2-yl)purine (XIII). Sodium (24 mg, 1.01 mmol) was dissolved in benzyl alcohol (0.7 ml). A soln. of compound XII (250 mg, 0.55 mmol) in dry DMF (2.5 ml) was added dropwise and the mixture stirred under Ar overnight. The next day, the mixture was evaporated and the residuum purified by CC (mobile phase $CHCl_3$-Me_2CO 9:1, flow rate 7.5 ml/min). Rust-colored, transparent liquid. Yield 0.28 g (97%).

*4.3.19.4. Synthesis of (Z)-6-(4-hydroxy-3-methylbut-2-en-1-ylamino)-7,9-dihydro-8H-purin-8-one (**31**).* Compound XIII (0.26 g, 0.50 mmol) was dissolved in MeOH (4.0 ml), H_2O (0.4 ml) and 1 M HCl (6 drops) were added and the mixture stirred at 65 °C for 3 days. Afterwards, the mixture was neutralized with 16 drops of 0.1 M NaOH, evaporated, mixed with $CHCl_3$ and filtered. The filtrate was injected on the column and subjected to CC (mobile phase $CHCl_3$, flow rate 7.5 ml/min). White crystals. Yield 20 mg (17%). M.

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p.: 243–247 °C; Elem. anal. (Found: C, 51.4 H, 5.8; N, 29.6. $C_{10}H_{13}N_5O_2$ requires: C, 51.1; H, 5.6; N, 29.8%).

4.4. Cytokinin bioassays

Classical cytokinin bioassays were performed, and the same plant material was used as described in Holub et al. (1998). Tobacco callus assay was modified according to Nisler et al. (2016).

4.5. Bacterial cytokinin receptor assay

Escherichia coli strain KMI001, harboring either the plasmid pIN-IIIAHK4 or pSTV28-AHK3, which express the Arabidopsis histidine kinases CRE1/AHK4 or AHK3 (Spíchal et al., 2004), was used in the experiments. Bacterial strains were kindly provided by T. Mizuno (Nagoya, Japan). The receptor activation assays were performed as previously described in Spíchal et al. (2004).

4.6. Cytotoxicity on human cell lines

Skin fibroblasts (BJ), retinal epithelial cells (ARPE-19, obtained from American Type culture collection) and keratinocytes HaCaT (from Cell Line Services) were maintained in DMEM medium containing 5 g/l glucose, 2 mM glutamine, 100 U/mL penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (Sigma) under standard conditions (5.5% CO2, 37 °C, 100% relative humidity). The cells were sub-cultured twice a week. For the cytotoxicity evaluation, the cells were trypsinized and dispensed into 96 well plates (5000 cells in 80 μL of medium per well). After 24 h, a 5× concentrated soln. of the test compounds (maximum concentration of 50 μM and 5 two-fold dilutions) in the medium was added. DMSO vehiculum served as a negative control. After 72 h, a 5× concentrated soln. of resazurin (Sigma) in the culture medium was added to the cells to give a final concentration of 100 μM. Fluorescence (ex = 570 nm, em = 610 nm) was measured after 1 h (ARPE-19) or 3 h (HaCaT and BJ). Incubation was carried out using an Enspire plate reader (PerkinElmer). Each experiment was repeated at least three times.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2016.12.005.

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