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Synthesis, characterization and biological activity of ring-substituted 6-benzylamino-9-tetrahydropyran-2-yl and 9-tetrahydrofuran-2-ylpurine derivatives

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ABSTRACT

In an attempt to improve specific biological functions of cytokinins routinely used in plant micropropagation, 33 6-benzylamino-9-tetrahydropyran-2-ylpurine (THPP) and 9-tetrahydrofuran-2-ylpurine (THFP) derivatives, with variously positioned hydroxy and methoxy functional groups on the benzyl ring, were prepared. The new derivatives were prepared by condensation of 6-chloropurine with 3,4-dihydro-2*H*-pyran or 2,3-dihydrofuran and then by the condensation of these intermediates with the corresponding benzylamines. The prepared compounds were characterized by elemental analyses, TLC, HPLC, melting point determinations, CI+ MS and ^{1}H NMR spectroscopy. The cytokinin activity of all the prepared derivatives was assessed in three classical cytokinin bioassays (tobacco callus, wheat leaf senescence and *Amaranthus* bioassay). The derivatives 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (**3**) and 6-(3-hydroxybenzylamino)-9-tetrahydrofuran-2-ylpurine (**23**) were selected, because of the high affinity of their parent compound *meta*-topolin (*m*T, 6-(3-hydroxybenzylamino)purine) to cytokinin receptors, as model compounds for studying their perception by the receptors CRE1/AHK4 and AHK3 in a bacterial assay. Both receptors perceived these two derivatives less well than they perceived the parent compound. Subsequently, the susceptibility of several new derivatives to enzyme degradation by cytokinin oxidase/dehydrogenase was studied. Substitution of tetrahydropyran-2-yl (THP) at the N^9 position decreased the turnover rates of all new derivatives to some extent. To provide a practical perspective, the cytotoxicity of the prepared compounds against human diploid fibroblasts (BJ) and the human cancer cell lines K-562 and MCF-7 was also assayed in vitro. The prepared compounds showed none or marginal cytotoxicity compared to the corresponding N^9-ribosides. Finally, the pH stability of the two model compounds was assessed in acidic and neutral water solutions (pH 3–7) by high-performance liquid chromatography (HPLC).

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1. Introduction

Cytokinins belong to a group of plant growth hormones involved in the regulation of almost all stages of plant growth and development.[1] Naturally occurring cytokinins are based structurally on N^6-substituted adenine. Members of this group are classified as isoprenoid (ISCK) and aromatic (ARCK) according to the substituent on the N^6-atom of adenine.[1,2] Although ISCKs such as isopentenyladenine (iP), *trans*-zeatin (tZ) or dihydrozeatin have attracted the most attention,[3] ARCKs may also occur naturally and have been isolated, for example, from poplar leaves.[4–7] Two representatives of ARCKs, 6-benzylaminopurine (BAP) and 6-(3-hydroxybenzylamino)purine (*meta*-topolin, *m*T) have been increasingly used in plant micropropagation; in particular, BAP is currently regarded as one of the most effective and affordable cytokinins for routine use.[8–16] Nevertheless, the application of BAP has some disadvantages in the propagation of many crop species, including problems with heterogeneity of growth and rooting inhibition.[16,17] One possible way to eliminate the undesirable side effects of BAP whilst simultaneously retaining its beneficial organogenic activity is the development of new BAP derivatives.[16,17]

Various modifications of BAP moiety by substitution, especially at the N^9 atom of the adenine, directly influence the invigorating effects of cytokinins on plant growth and are associated with the reinforcement of targeted cytokinin effects.[17,18] The most effective N^9-substituted derivatives developed so far are 6-benzylamino-9-tetrahydropyran-2-ylpurine (PBA) and 6-benzylamino-9-tetrahydrofuran-2-ylpurine (FBA), which have both proved to be considerably more active than BAP in evoking of several growth

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responses.[18] Skoog et al. observed that PBA is exceptionally potent in chlorophyll retention, while Arena et al. described the growth-promoting effect of PBA on adventitious shoot formation in comparison to kinetin or iP.[19,20] In addition, PBA has, for a long time, been used successfully to control flowering and inflorescence development in various plants.[21–23] It has been suggested, however, that the observed increased activity of these N^9-substituted derivatives could be a consequence of their ability to release free bases, thus supplying desirable concentrations over a prolonged period.[18] Thus, susceptibility to enzymatic cleavage is probably the critical factor determining the biological activity of N^9-substituted cytokinins. Hence, less active compounds are probably not susceptible to cleavage of N^9-substituents and exhibit low or zero activity because of their stability.[18,24,27–30] These data are somewhat controversial because of the fast release of free aglycone after the application of much less active 9-methyl-BAP that was observed by Fox et al.[24] These authors studied the metabolism of 9-methyl-BAP, considered to exhibit low activity, in tobacco and soybean callus tissue and demonstrated rapid conversion to several products. The metabolites were not identified definitively, although it was proposed that conversion to free BAP occurred.[24] Pietraface and Blaydes examined the metabolism of 9-methyl-BAP in germinating lettuce seed and, based on chromatographic data, suggested that BAP riboside and nucleotide formation was occurring.[25] Nevertheless, free BAP was not detected. Although free BAP has not been found as a metabolite, the conversion to BAP 9-glucoside and 7-glucoside (inactive metabolites) has been established unequivocally by mass spectrometry.[25] The formation of these glucosides presumably requires them to be converted to BAP.

Zhang and Letham studied the metabolism of several 9-substituted BAP derivatives and suggested that another reason for their enhanced antisenescence properties might originate from differences in the catabolism of BAP,[18] PBA and possibly FBA. Their study established that PBA and FBA were subject to both N^6-debenzylation and N^9-substituent breakage in soybean leaves. However, debenzylation was more prominent in the metabolism of PBA than in the metabolism of BAP. Besides, these compounds were less likely to form inactive or toxic metabolites.[26] With respect to the different types of cytokinin derivatives mentioned here, the most remarkable seemed to be 9-tetrahydropyranyl and 9-tetrahydrofuranyl BAPs. We, therefore, prepared new series of 6-(hydroxy- and 6-(methoxy-benzylamino)purines substituted by tetrahydropyran-2-yl (THP) and tetrahydrofuran-2-yl (THF) groups at the N^9 position of purine, in order to improve their biological functions and maintain or even decrease their already low cytoxicity. Both these groups are cyclic ethers in which the ether oxygen is attached to the carbon linked to the N^9 atom of the purine moiety. THP and THF were originally described as protective groups in organic chemistry that are easily removed under acidic conditions.[31,32] Hence, we examined the stability of selected model derivatives, specifically 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (**3**) and 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (**23**), in acidic or neutral water solutions (pH 3–7) to be sure that breakage of THP or THF did not occur in the media used for the biological and receptor assays. Furthermore, using the model compounds in a bacterial assay, we studied the effect of the THP and THF groups on the ability of *Arabidopsis* cytokinin receptors AHK3 and CRE1/AHK4 to detect the compounds.[33]

Several papers about ARCK derivatives have mentioned the relationship between benzyl ring substitutions and cytokinin activity.[33–36] Therefore, we also studied the differences in biological activity of 6-(hydroxy- and 6-(methoxybenzylamino)-9-tetrahydropyran-2-ylpurine derivatives in classical cytokinin bioassays. Cytokinin activity was compared in three tests—the stimulation of tobacco callus growth, the retention of chlorophyll in excised wheat leaves and the dark induction of betacyanin synthesis in *Amaranthus* cotyledons. To investigate possible differences in in vivo stability, the newly prepared derivatives were also tested as substrates for cytokinin oxidase/dehydrogenase. This enzyme catalyzes irreversible cleavage of the N^6-substituent from the cytokinin molecule, leading to the total loss of biological activity, and seems to be one of the main mechanisms by which cytokinin homeostasis is maintained in plant tissues.[3]

It has been suggested recently that cytokinin derivatives substituted by ribose in the N^9 position exhibit significant cytotoxic activity.[36] In order to exclude cytotoxicity of newly prepared compounds with potential agricultural and cosmetic use their cytotoxic effect against human diploid fibroblasts (BG) and cancer cell lines (MCF-7 and K-532) was evaluated. The cytotoxic activity of selected THP and THF derivatives was then compared to the corresponding riboside analogues.

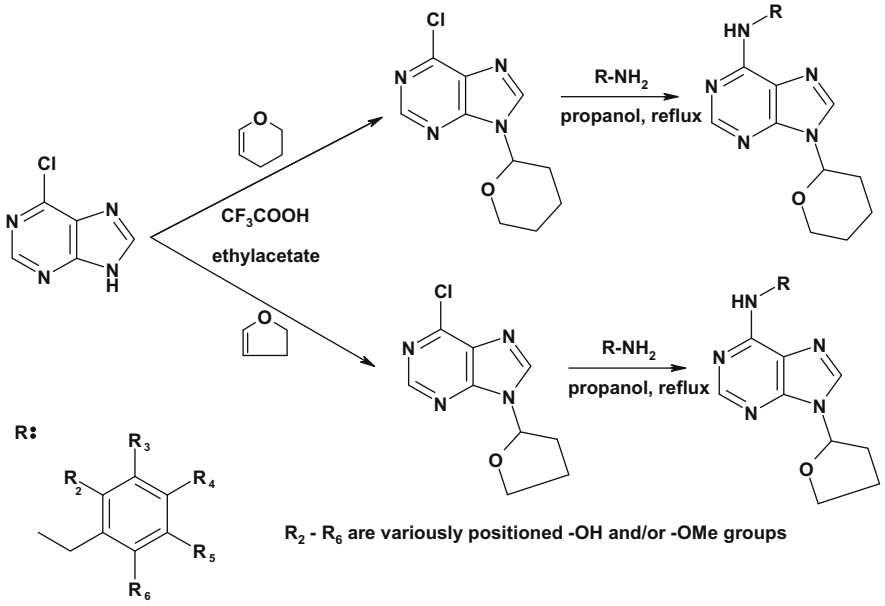
2. Results and discussion

2.1. Synthesis

Thirty-three ring-substituted derivatives of benzylamino-9-tetrahydropyran-2-ylpurine and 9-tetrahydrofuran-2-ylpurine were synthesized according to Scheme 1 (Table 1, Scheme 2). Prepared compounds were characterized by elemental analyses, thin layer chromatography (TLC), melting point determinations, CI+ MS and ^{1}H NMR spectroscopy. For better lucidity, Scheme 2 shows the schematic representation of newly prepared tetrahydropyran-2-yl and tetrahydrofuran-2-yl derivatives. The purity of prepared derivatives was confirmed by high-performance liquid chromatography (HPLC). Table 2 shows C, H, N elemental analysis data, mp, CI+ MS and HPLC purity data, whilst ^{1}H NMR spectral data are given in Supplementary data. Compounds PBA (**1**) and FBA (**21**) were prepared using a slightly modified version of a method previously published in the literature.[18] The melting points and ^{1}H NMR spectral data for compounds **1** and **21** prepared in our laboratory were consistent with the data found in the literature.[18] The preparation of new derivatives is described in greater detail in Section 4.3.

2.2. Stability in acidic solutions

Tetrahydropyran-2-yl (THP) and tetrahydrofuran-2-yl (THF) groups have been commonly used in organic chemistry as protective groups, readily removable under acidic conditions.[31] Therefore we verified that THP or THF groups did not break off under our bioassay conditions, since their removal would turn them into the corresponding free bases thereby influencing the cytokinin activity. 6-(3-Hydroxybenzylamino)-9-(tetrahydropyran-2-yl)purine (**3**) and 6-(3-hydroxybenzylamino)-9-(tetrahydrofuran-2-yl)-purine (**23**) were chosen as the model compounds. We performed HPLC stability measurements in 10^{-4} M stock solutions with pH decreasing from 7 to 3. The percentage of the THPP and THFP derivatives **3** and **23** together with the pH- and time-dependent release of the free base (*m*-topolin), as determined by HPLC, is given in Table 3. Both tested compounds were stable at pH 7 and 6 even 24 h after sample preparation. Compound **23** started to decompose after 24 h at pH 5 (9% of the free base released). A significant decomposition of **3** and **23** occurred after 24 h at pH 4 and increased significantly at pH 3 (Table 3). It can, therefore, be concluded that the prepared THPP and THFP cytokinin derivatives are not entirely stable at pH < 4 and under more acidic conditions they decompose to their free bases. Since the pH of the media in the assays used within the framework of this study varied between 6 and 7, we could be sure that the original compounds were those being tested.



Scheme 1. Schematic representation of the syntythetic pathway of THPP and THFP derivatives described in the study.

2.3. Cytokinin activity in bioassays

Cytokinin activity of all the prepared compounds was determined by three classical cytokinin bioassays (tobacco callus, wheat senescence and *Amaranthus* bioassays) and the results are presented in Table 4. The activities of the compounds were compared to those of BAP, which represents a highly active, commonly used cytokinin. Figure 1A–C compares the activity of 6-(*ortho*-, 6-(*meta*- and 6-(*para*-hydroxybenzylamino)tetrahydropyran-2-ylpurines in all three bioasays, showing a decrease in cytokinin activity between the substituents, as follows: *meta* > *ortho* ≫ *para*. These results are in agreement with those previously published in the literature related to *ortho*-, *meta*-, and *para*-topolin.[7,34,35]

For 6-(methoxybenzylamino)tetrahydropyran-2-ylpurine derivatives, the cytokinin activity decreased in the substituent order *ortho* > *meta* ≫ *para*; this is also consistent with the data found in the literature (Fig. 1D–F).[35,36] Moreover, it was found that substitution in the *para*-position caused the loss of activity in senescence as well as in tobacco callus bioassay. This finding has been reported recently for similar compounds,[36] differing in their N^9-substitution by ribose, and supported by the fact that compounds **4**, **7**, **18**, **24**, **27**, **30**, **32**, **34** and **35**, which are substituted in the *para* position by a methoxy group, were inactive in all three bioassays (Table 4, Fig. 1). We also compared the activity of THPP and THFP derivatives in tobacco callus, senescence and *Amaranthus* biotests with the corresponding free base. This test used compounds **3** and **23** and their parent compound 6-(3-hydroxybenzylamino)purine (*meta*-topolin, *m*T). The results presented in Figure 2 show that the antisenescence activity of the THPP derivative was slightly higher than that of the free base and the THFP derivative (Fig. 2A). The activities of the THPP and THFP derivatives were comparable in the tobacco callus assay and did not differ significantly from the activity exhibited by the corresponding base (Fig. 2B). A different result was found in the *Amaranthus* bioassay, where both THPP and THFP derivatives were less active, although their activities were of a similar order to that in the senescence assay (Fig. 2C). Moreover, the results of *Amaranthus* and tobacco callus bioassay are similar to the values obtained for the corresponding ribotides.[7,34,36] However, the activity of methoxybenzyladenosines in senescence bioassay is twice higher in comparison with those for THPP and THFP derivatives. This, again, suggests that different receptor and/or signaling systems are involved in transduction of different cytokinin-dependent physiological responses.

Almost all THPP and THFP derivatives containing two or more hydro or methoxy groups on the benzyl ring were inactive in the tobacco callus bioassay; the exceptions were compounds **11**, **12** and **29**, although these exhibited only half the activity of BAP (Table 4). None of di- or tri- substituted compounds exceeded the activity of BAP either in the senescence or *Amaranthus* bioassays (Table 4). This fact supports the recently published fact that di- and trihydroxy (methoxy) BAPs are not strongly active in cytokinin assays.[35]

2.4. Recognition by cytokinin receptors

In our previous work we have shown that, in contrast to activity in the classical cytokinin bioassays, the position of ARCK benzyl ring substituents do not improve the recognition of a compound by cytokinin receptors in a bacterial assay.[35,36] Here we used a bioassay employing transformed *E. coli* strains expressing the cytokinin receptors AHK3 and CRE1/AHK4 with the cytokinin-activated reporter gene *cps::lacZ* to investigate the effect of THP and THF groups on the ability of *meta*-topolin, as a model compound, to activate the cytokinin signaling pathway through these receptors.[33,37,38] As shown in Figure 3, *m*T itself was recognized well by both receptors, although to a lesser extent than the *trans*-zeatin used as a control. In contrast, the THPP and THFP derivatives were not detected by CRE1/AHK4 at all (Fig. 4A). Partial receptor activation occurred only with AHK3 at the highest concentration, corroborating the sensitivity of AHK3 to N^9-substituted cytokinins (Fig. 3).[33] The experiment showed that THP and THF ARCK derivatives are only weak ligands of the cytokinin receptors. This fact means that, like other aromatic cytokinins, a different recognition system that is able to interact with BAP derivatives may exist in plants.[35]

2.5. Enzymatic degradation in vitro

Cytokinin oxidase/dehydrogenase in higher plants is encoded by small gene families, and the enzymes are distinguished by diverse subcellular compartmentalization as well as by different spa-

Table 1
Structures of the prepared compounds and their abbreviations

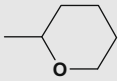
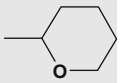
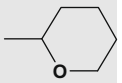
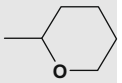
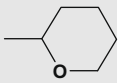
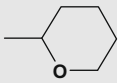
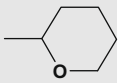
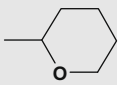
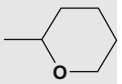
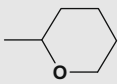
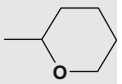
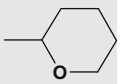
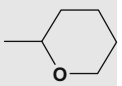
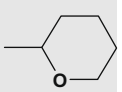
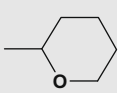
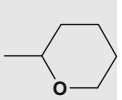
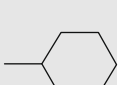
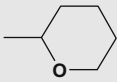
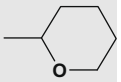
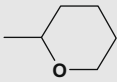
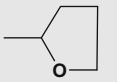
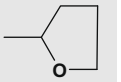
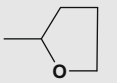
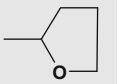
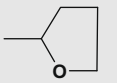
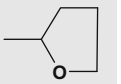
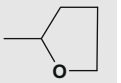
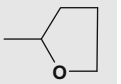
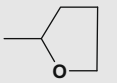
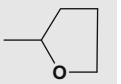
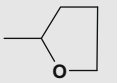
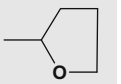
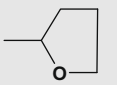
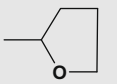
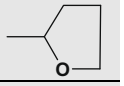
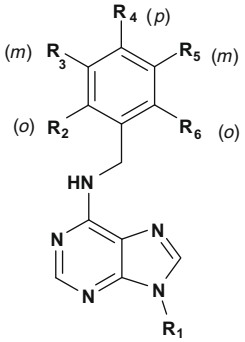
Compound	R_2 (*o*)	R_3 (*m*)	R_4 (*p*)	R_5 (*m*)	R_6 (*o*)	R_1
PBA (**1**)	H	H	H	H	H	
2	OH	H	H	H	H	
3	H	OH	H	H	H	
4	H	H	OH	H	H	
5	OCH_3	H	H	H	H	
6	H	OCH_3	H	H	H	
7	H	H	OCH_3	H	H	
8	OH	OCH_3	H	H	H	
9	H	OCH_3	OH	H	H	
10	OCH_3	H	OCH_3	H	H	
11	OCH_3	H	H	OCH_3	H	
12	OCH_3	H	H	H	OCH_3	
13	H	OCH_3	OCH_3	H	H	
14	H	OCH_3	H	OCH_3	H	
15	OH	OH	H	H	H	
16	H	OH	H	OH	H	
17	OCH_3	OCH_3	OCH_3	H	H	

Table 1 (*continued*)

Compound	R_2 (*o*)	R_3 (*m*)	R_4 (*p*)	R_5 (*m*)	R_6 (*o*)	R_1
18	OCH_3	H	OCH_3	OCH_3	H	
19	OCH_3	H	OCH_3	H	OCH_3	
20	H	OCH_3	OCH_3	OCH_3	H	
FBA (**21**)	H	H	H	H	H	
22	OH	H	H	H	H	
23	H	OH	H	H	H	
24	H	H	OH	H	H	
25	OCH_3	H	H	H	H	
26	H	OCH_3	H	H	H	
27	H	H	OCH_3	H	H	
28	H	OCH_3	OCH_3	H	H	
29	H	OCH_3	H	OCH_3	H	
30	OCH_3	H	OCH_3	H	H	
31	OCH_3	H	H	OCH_3	H	
32	H	OCH_3	OH	H	H	
33	OCH_3	OCH_3	OCH_3	H	H	
34	OCH_3	H	OCH_3	OCH_3	H	
35	H	OCH_3	OCH_3	OCH_3	H	



Scheme 2. Schematic representation of tetrahydropyran-2-yl and tetrahydrofuran-2-yl derivatives; (*o*) indicates *ortho*-, (*m*) *meta*- and (*p*) *para*- position in the benzyl ring.

tial and temporal expression patterns.[39] In this study, the recombinant maize enzyme *Zea mays* cytokinin oxidase/dehydrogenase 1 (ZmCKX1) was used to study the ability of selected compounds, especially those that bioassays showed to be more active than their corresponding free bases, to be cleaved as substrates by this enzyme. This protein has previously been localized in the apoplast of vascular bundles, and exhibits its peak expression there in germinated seeds and young roots.[40,41] Thus, it can contribute to the degradation of exogenous cytokinins in micropropagation systems where these, and other additives, are absorbed from the growing medium through the developing roots and are then distributed via the xylem bundles.

ARCKs are, in general, poor substrates for cytokinin oxidase/dehydrogenase activity; this probably increases their stability in vivo compared to isoprenoid cytokinins. None of the seven isoenzymes of cytokinin oxidase/dehydrogenase from *Arabidopsis thaliana* prefer aromatic cytokinins.[42] It has been shown that aromatic rings act as spherical obstacles to the formation of substrate/product intermediates. Thus, the catalytic reaction cannot be speeded up even in the presence of appropriate electron acceptors within ISCK, which should transfer electrons from the reduced FAD cofactor of the enzyme and thus dramatically increase the rate of degradation.[43] Kinetic constants of maize cytokinin oxidase/dehydrogenase for selected THPP derivatives compared to their free bases, BAP, BAPR and PBA are given in Table 5. N^9-substitution by THP decreases turnover rates of all aromatic cytokinins to some extent. Furthermore, the lower susceptibility to degradation by ZmCKX1 of these derivatives is sustained by higher K_m values, decreasing the total efficiency (k_{cat}/K_m) by a factor of 7–33. The higher activity of THPP derivatives in bioassays may therefore be explained by their greater resistance to enzymatic breakdown by CKX, which increases their stability and prolongs their lifespan in plant tissues. Previous studies of the metabolism of several 9-substituted BAP derivatives have suggested that the reason for their enhanced cytokinin activity might originate from differences in the catabolism of BAP, PBA and possibly FBA.[18] This attribute can even overcome the poorer recognition of THPP and THFP derivatives by the known cytokinin receptors.

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

Compound	Elemental analyses calculated/found			Mp (°C)	CI+ MS [M+H$^+$]	HPLC (%)
	%C	%H	%N			
PBA,1	66.0/66.1	6.2/6.1	22.6/22.5	110–112	310	>98
2	62.8/62.7	5.9/5.8	21.5/21.6	172–175	326	>98
3	62.8/62.4	5.9/5.9	21.5/21.3	120–121	326	>99
4	62.8/62.8	5.9/5.9	21.5/21.3	173–174	326	>99
5	63.7/63.9	6.2/6.3	20.6/20.7	106–108	340	>98
6	63.7/63.9	6.2/6.3	20.6/20.6	134–135	340	>98
7	63.7/63.9	6.2/6.2	20.6/20.6	137–138	340	>99
8	60.8/60.7	5.9/5.9	19.7/19.8	187–188	356	>98
9	60.8/60.6	5.9/6.0	19.7/19.8	189–190	356	>98
10	61.8/61.8	6.3/6.3	18.9/18.7	479–180	370	>98
11	61.8/61.7	6.3/6.4	18.9/18.9	150–151	370	>98
12	61.8/61.8	6.3/6.3	18.9/18.9	170–171	370	>98
13	61.8/61.9	6.3/6.3	18.9/18.8	156–157	370	>98
14	61.8/61.9	6.3/6.4	18.9/18.9	145–146	370	>98
15	59.8/59.9	5.6/5.6	20.5/20.5	174–175	342	>98
16	59.8/59.9	5.6/5.7	20.5/20.4	212–214	342	>99
17	60.1/60.2	6.3/6.2	17.5/17.5	142–143	400	>98
18	60.1/60.2	6.3/6.4	17.5/17.6	128–129	400	>98
19	60.1/60.0	6.3/6.4	17.5/17.4	178–179	400	>98
20	60.1/59.9	6.3/6.3	17.5/17.8	127–128	400	>98
FBA,21	65.1/65.1	5.8/5.8	23.7/23.8	100–102	296	>98
22	61.7/61.9	5.5/5.5	22.5/22.3	135–136	312	>98
23	61.7/61.7	5.5/5.5	22.5/22.6	124–126	312	>98
24	61.7/61.8	5.5/5.6	22.5/22.5	182–183	312	>98
25	62.8/62.9	5.9/5.8	21.5/21.5	97–99	326	>98
26	62.8/62.9	5.9/6.0	21.5/21.5	87–88	326	>98
27	62.8/62.9	5.9/5.8	21.5/21.5	182–183	326	>98
28	60.8/60.9	5.9/5.9	19.7/19.8	131–132	356	>98
29	60.8/60.7	5.9/5.7	19.7/19.6	99–100	356	>98
30	60.8/60.7	5.9/5.9	19.7/19.6	125–126	356	>98
31	60.8/60.6	5.9/5.8	19.7/19.7	103–104	356	>98
32	59.8/60.0	5.6/5.7	20.5/20.5	140–142	342	>98
33	59.2/59.1	6.0/6.0	18.2/18.2	140–141	386	>98
34	59.2/59.2	6.0/5.9	18.2/18.2	99–100	386	>98
35	59.2/59.3	6.0/6.0	18.2/18.3	118–119	386	>98

2.6. Cytotoxicity

The substitution of certain BAP derivatives with ribose moiety in the N^9 position is known to significantly increase their cytotoxicity against human cells.[35,36] Therefore, we compared the cytotoxic effects of corresponding ribosyl and tetrahydropyran-2-yl derivatives in vitro against cancer human cell lines (breast carcinoma, MCF-7, and chronic myelogenous leukaemia, K-562) by Calcein AM viability assay. The results summarizing the IC_{50} values for all the tested compounds are presented in Table 6.

We discovered that the alternation of ribose by a THP or THF group in the N^9 position can markedly decrease cytotoxicity. Figure 4 shows a comparison of dose–response curves for 6-(2-hydroxy-3-methoxybenzylamino)-9-tetrahydropyran-2-ylpurine (**8**) and 6-(2,3-dihydroxy-benzylamino)-9-tetrahydropyran-2-ylpurine (**15**) and their analogues with ribose at N^9. While cytotoxic effects of both THPP derivatives against the MCF-7 cell line were negligible, their riboside analogues 6-(2-hydroxy-3-methoxybenzylamino)-9-β-D-ribofuranosylpurine and 6-(2,3-dihydroxybenzylamino)-9-β-D-ribofuranosylpurine exhibited significant cytotoxicity with IC_{50} values of 20.2 μmol/L and 5.2 μmol/L, respectively. Similar results were obtained for K-562 cells where the replacement of ribose moiety with a THP group led to an increase in the IC_{50} value from 27.9 μmol/L to >100 μmol/L (compound **8**) and from 27.9 to 92 μmol/L (compound **15**). IC_{50} values for the corresponding free bases were above 100 μmol/L.[35]

The cytotoxicity of the prepared compounds was also evaluated in human diploid fibroblasts (BJ) by MTT viability assay. Almost all the tested THPP and THFP compounds were only marginally toxic or were non-toxic, even at the highest concentration tested (100 μmol/L or solubility limit). Their toxicity profile was comparable or better than the profile of the corresponding cytokinin bases under the assay conditions.[35] We, therefore, conclude that tetrahyropyranylation or tetrahydrofuranylation of cytokinins can lead to a general reduction in cytotoxicity, a useful property in agricultural applications and cosmetics.

3. Conclusions

We prepared and characterized thirty-three hydroxy and/or methoxy ring-substituted 6-benzylamino-9-tetrahydropyran-2-ylpurine and 6-benzylamino-9-tetrahydrofuran-2-yl derivatives. The prepared compounds were subjected to three cytokinin bioassays. The N^9 substitution by a THP or THF group either did not change or improved the biological activity of free bases recorded in classical cytokinin bioassays, although it negatively influenced the recognition of these compounds by cytokinin receptors. The improved activity of the THP cytokinins could be explained by their higher resistance to enzymatic breakdown by CKX. The resistance to degradation, and thus probably their prolonged lifespan in vivo, can be enhanced by the presence of hydroxy- or methoxy groups in the *meta* position of the benzyl ring. The specificity constant (k_{cat}/K_m) of 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine toward ZmCKX1 is 25-fold and 530-fold lower than that for 6-benzylaminopurine and 6-(3-hydroxybenzylamino)purine, respectively. However, in vivo metabolic studies of the newly prepared compounds are required to explain in more detail the mode of action of THPP and THFP derivatives in plants. Such a study is underway in our laboratory.

The hydroxy- and methoxybenzyl THFPs and THPPs with high levels of cytokinin activity, along with the majority of newly prepared compounds, were found to be non-toxic in the cytotoxicity tests performed in vitro on different cancer and normal human cell lines. HPLC assaying of the stability of *meta*-topolin THPP and THFP derivatives at various pHs (3–7) showed that the compounds were stable in the pH range of the media used for the cytokinin bioassays. The fact that almost all the substances exhibited no or only slight cytotoxicity even at high concentrations is very encouraging and may mean that these cytokinins can be used not only in the

Table 3
Stability of 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (3) and 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (23) in acidic solutions

pH	Compound peak area (%)			
	3	Free base	**23**	Free base
(A) 1 h After sample preparation				
7	99.9	0.1	99.7	0.3
6	99.8	0.2	99.6	0.4
5	99.5	0.5	99.5	0.5
4	99.5	0.5	97.2	2.8
3	98.8	1.2	63.8	36.2
(B) 24 h After sample preparation				
7	99.8	0.2	99.7	0.3
6	99.3	0.7	99.4	0.6
5	99.2	0.8	90.8	9.2
4	93.9	6.1	37.5	62.5
3	39.6	60.4	0.3	99.7

The pH stability of compounds **3** and **23** in 10^{-4} M water solutions with pHs decreasing from 7 to 3 measured 1 h (A) and 24 h (B) after sample preparation. The percentage of the released free base 6-(3-hydroxybenzylamino)purine (*m*T) was determined by HPLC.

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

Compound	Tobacco callus bioassay		*Amaranthus* bioassay		Senescence bioassay	
	Optimal concentration (mol l^{-1})	Relative activity(%)	Optimal concentration (mol l^{-1})	Relative activity (%)	Optimal concentration (mol l^{-1})	Relative activity (%)
PBA (1)	10^{-6}	102 (±2)	10^{-4}	114 (±7)	10^{-4}	104 (±1)
2	10^{-5}	37 (±2)	10^{-4}	46 (±9)	10^{-4}	31 (±10)
3	10^{-6}	95 (±8)	10^{-4}	100 (±6)	10^{-4}	140 (±10)
4	10^{-5}	20 (±9)	10^{-4}	34 (±9)	10^{-4}	4 (±8)
5	10^{-6}	93 (±9)	10^{-4}	84 (±2)	10^{-4}	104 (±5)
6	10^{-6}	90 (±1)	10^{-4}	76 (±2)	10^{-4}	72 (±5)
7	10^{-6}	10 (±6)	10^{-4}	18 (±5)	n.a.	
8	10^{-4}	12 (±4)	10^{-4}	18 (±7)	n.a.	
9	10^{-4}	8.7 (±2)	10^{-4}	26 (±0.2)	10^{-4}	7 (±3)
10	n.a.		10^{-4}	9 (±4)	10^{-4}	25 (±1)
11	10^{-5}	42 (±10)	10^{-4}	28 (±5)	10^{-4}	19 (±3)
12	10^{-4}	57 (±5)	10^{-4}	13 (±1)	10^{-4}	14 (±3)
13	n.a.		10^{-4}	8 (±3)	10^{-4}	8 (±0.3)
14	n.a.		10^{-4}	9 (±2)	10^{-4}	7 (±4)
15	10^{-4}	3,2 (±0.1)	10^{-4}	8 (±3)	n.a.	
16	10^{-5}	36 (±1)	10^{-4}	33 (±6)	10^{-4}	15 (±8)
17	n.a.		10^{-4}	4 (±3)	10^{-4}	7 (±4)
18	10^{-4}	11 (±3)	10^{-4}	3 (±2)	n.a.	
19	10^{-4}	2 (±1)	10^{-4}	14 (±2)	10^{-4}	3 (±2)
20	10^{-4}	17 (±9)	10^{-4}	19 (±6)	10^{-4}	7 (±4)
FBA (21)	10^{-6}	95 (±3)	10^{-4}	109 (±9)	10^{-4}	107 (±8)
22	10^{-4}	58 (±8)	10^{-4}	41 (±7)	10^{-4}	25 (±8)
23	10^{-6}	87 (±11)	10^{-4}	82 (±5)	10^{-4}	131 (±7)
24	10^{-5}	19 (±1)	10^{-4}	10 (±5)	10^{-4}	8 (±6)
25	10^{-5}	91 (±7)	10^{-4}	107 (±10)	10^{-4}	72 (±1)
26	10^{-6}	96 (±2)	10^{-4}	123 (±8)	10^{-4}	82 (±11)
27	10^{-6}	7 (±3)	10^{-4}	25 (±6)	10^{-4}	16 (±4)
28	10^{-6}	3 (±2)	10^{-4}	5 (±2)	10^{-4}	7 (±1)
29	10^{-4}	14 (±6)	10^{-4}	18 (±3)	10^{-4}	8 (±0.1)
30	10^{-6}	3 (±0.1)	10^{-4}	14 (±2)	10^{-4}	18 (±9)
31	10^{-5}	61 (±7)	10^{-4}	41 (±9)	10^{-4}	41 (±2)
32	10^{-4}	13 (±2)	10^{-4}	17 (±6)	10^{-4}	4 (±5)
33	10^{-4}	7 (±1)	10^{-4}	15 (±2)	10^{-4}	9 (±4)
34	10^{-4}	14 (±4)	10^{-4}	14 (±2)	10^{-4}	9 (±1)
36	10^{-4}	4 (±2)	10^{-4}	17 (±3)	10^{-4}	10 (±4)

n.a. Means not active.

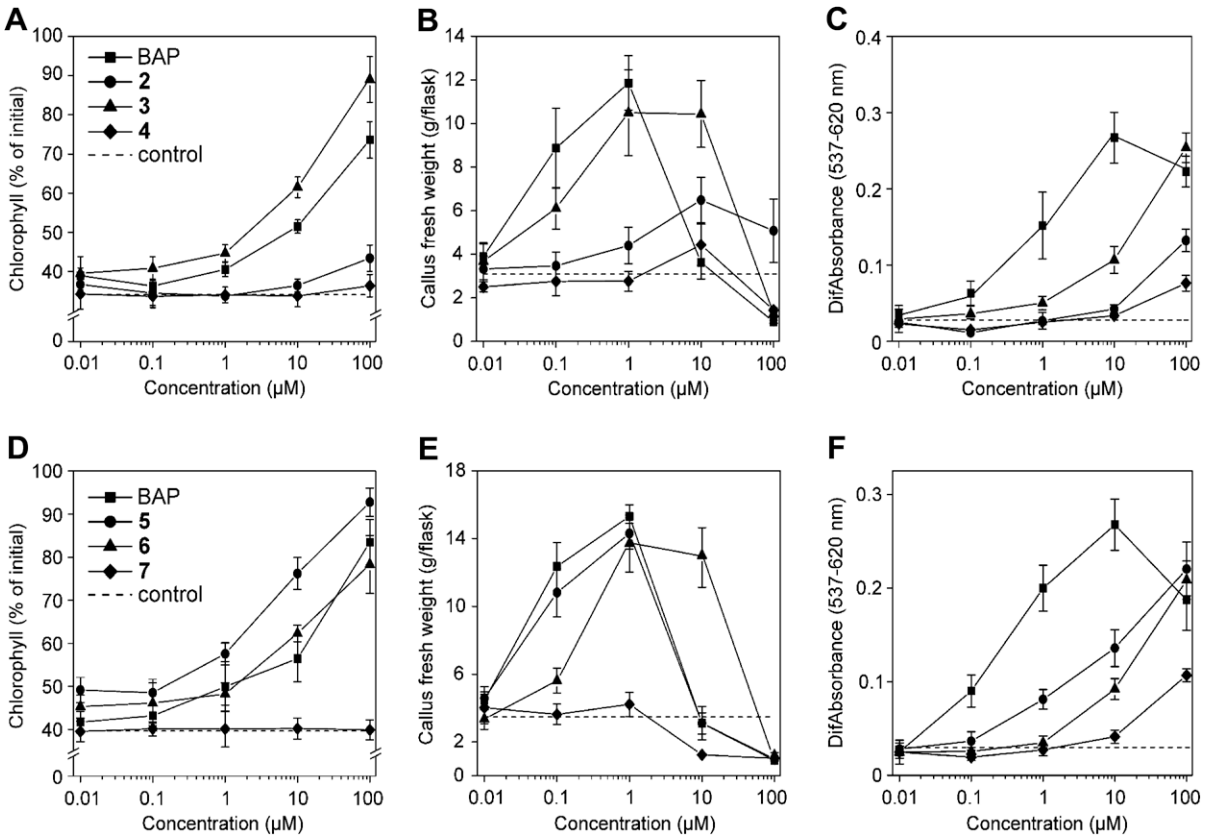


Figure 1. The influence of hydroxy or methoxy group position in the benzyl ring of 6-benzyl-9-tetrahydropyran-2-yl-purines on their biological activity in classical cytokinin bioassays. Comparison of the impact of hydroxy (A–C) and methoxy (D–F) groups substituted in *ortho*- (circles), *meta*- (triangles), and *para*- (diamonds) positions, respectively, on: the retention of chlorophyll in excised wheat leaves (A, D); the stimulation of cytokinin-dependent tobacco callus growth (B, E); and the dark induction of betacyanin synthesis in *Amaranthus* cotyledons (C, F). The graphs show representative examples of results obtained in individual bioassays. BAP was used as a positive control (squares), the dashed line shows the solvent control (0.2% DMSO). Error bars represent SD ($n = 5$).

regulation of plant growth and development, agriculture and plant biotechnology, but also, potentially, in cosmetics, where the use of cytotoxic substances is prohibited.

4. Experimental

4.1. Chemicals

6-Chloropurine was purchased from Olchemim, triethylamine (TEA), ethyl acetate (EtOAc), *n*-propanol, *n*-butanol, isopropanol, diethyl ether, hexane, *N*,*N*′-dimethylsulfoxide and other common organic solvents used for syntheses and subsequent crystallization of the products were purchased from Sigma–Aldrich and Lachema, and used as received. 2,3-Dihydrofuran (98%) was purchased from Across Organics; 3,4-dihydro-2*H*-pyran and trifluoracetic acid were obtained from Fluka; furfurylamine, 2-methoxybenzylamine, 3-methoxybenzylamine, 4-methoxy-benzylamine, 2,4-dimethoxybenzylamine, 2,5-dimethoxybenzylamine, 2,6-dime-thoxybenzylamine, 3,4-dimethoxybenzylamine, 3,5-dimethoxybenzylamine, 2,4,6-trimethoxybenzylamine, 3,4,5-trimethoxybenzylamine, aniline, 4-methoxyaniline, 4-aminophenol were obtained from Aldrich; 2-hydroxybenzylamine, 3-hydroxybenzylamine, 4-hydroxy-

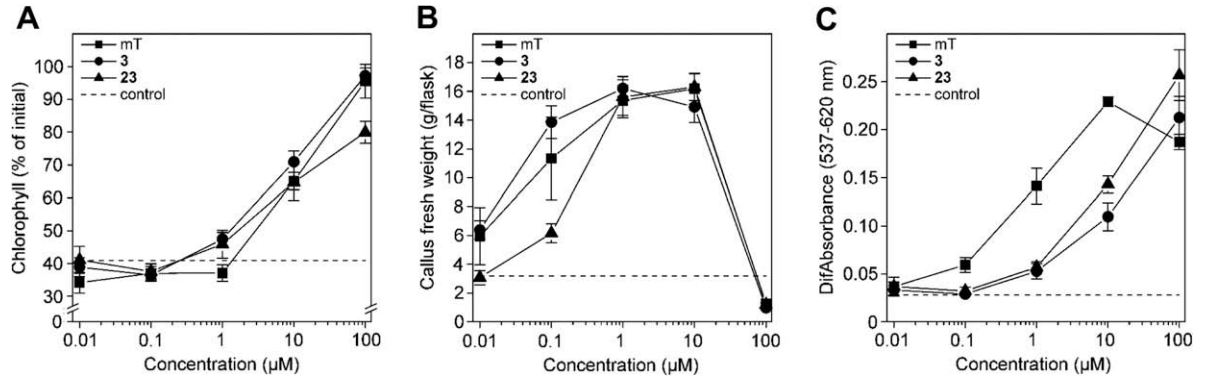


Figure 2. The influence of 9-tetrahydropyran-2-yl and 9-tetrahydrofuran-2-yl groups on the biological activity of 6-benzyl-(3-hydroxybenzylamino)purine in classical cytokinin bioassays. (A) The effect on chlorophyll retention in excised wheat leaf tips; (B) the growth of cytokinin-dependent tobacco callus; (C) the effect on dark betacyanin synthesis in *Amaranthus caudatus* cotyledon-hypocotyl explants. The dashed line shows the solvent control (0.2% DMSO). Error bars represent SD ($n = 5$).

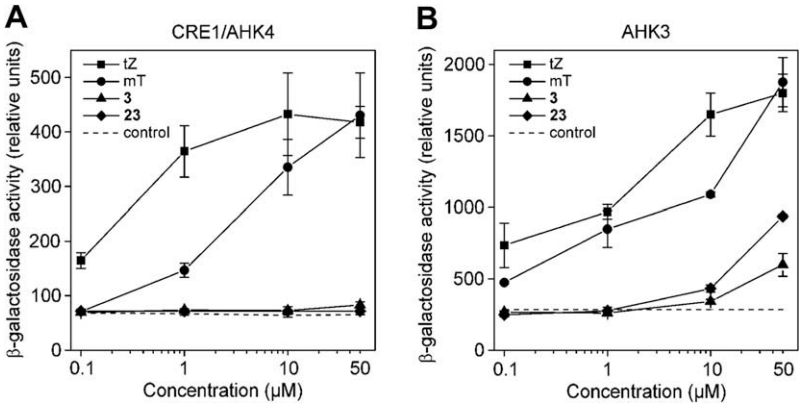


Figure 3. Comparison of the perception of THP and THF substituted 6-(3-hydroxybenzylamino)purine (*meta*-topolin, *m*T) and *m*T by selected cytokinin receptors. The effect of 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (6(3OHBA)9THPP) and 6-(3-hydroxybenzylamino)-9-tetrahydrofuran-2-ylpurine (6(3OHBA)9THFP) on the sensing of *m*T by the cytokinin receptors CRE1/AHK4 (A) and AHK3 (B). *trans*-Zeatin (tZ) was used as the positive control. The dashed line shows the solvent control (0.5% DMSO). Error bars show SD ($n = 3$).

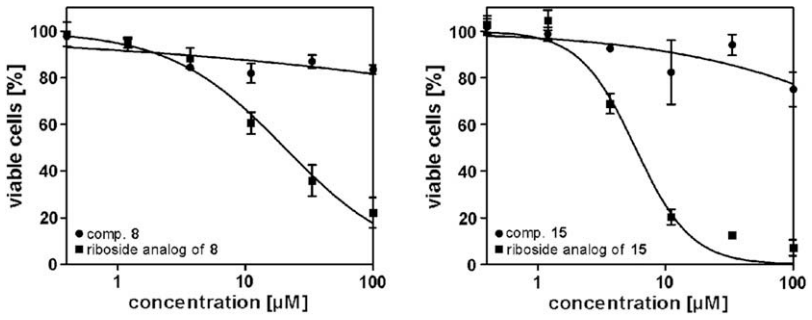


Figure 4. The effect of THP substitution on the cytotoxicity of MCF-7 cells compared to the corresponding ribosides. The compounds 6-(2-hydroxy-3-methoxybenzylamino)-9-tetrahydropyran-2-ylpurine (**8**) and 6-(2,3-dihydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (**15**) did not reduce the number of viable MCF-7 cells. The MCF-7 human breast cancer cell line was treated for 72 h with increasing concentrations of **8**, **15** or their riboside analogues. Then, the number of viable cells was determined by a Calcein AM assay. Results represent the average ± SD in three independent experiments.

benzylamine, 4-hydroxy-3-methoxybenzylamine, 2,3-dihydroxybenzylamine, 2,3,4-trimethoxybenzylamine, 2,4,5-trimethoxybenzylamine were supplied by Olchemim, The preparation and characterization of the commercially unavailable 2-hydroxy-3-methoxybenzylamine hydrobromide and 3,5-dihydroxybenzylamine hydrobromide have been described previously.[35] Mili-Q water was used throughout. The other solvents and chemicals used were all of standard *pa* quality. The substances PBA (**1**) and (**21**) were prepared according to slightly modified versions of standard methods described in the literature.[18]

Table 5
Kinetic constants of maize cytokinin oxidase/dehydrogenase for selected cytokinins and their THPP derivatives; enzyme activity was determined by measuring the amount of aldehyde produced by oxidative cleavage of the side chain of the measured cytokinins, using the 4-aminophenol assay[42]

Compound	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m
iP	0.208	1.8	0.1156
BAP	0.225	29.6	0.0076
BAPR	0.218	n.d.	n.d.
PBA, **1**	0.069	98.9	0.0007
*m*T	0.244	24.4	0.0100
3	0.032	125.0	0.0003
6-*fb*	0.145	n.d.	n.d.
6	0.006	n.d.	n.d.
4-*fb*	0.871	5.5	0.1583
4	0.195	33.8	0.0058
7-*fb*	0.476	n.d.	n.d.
7	0.256	n.d.	n.d.

All data represent mean values of at least two replicates. Deviations between replicates did not exceed 10%; n.d.—not determined.
iP: N^6-(2-isopentenyl)adenine, BAP: 6-benzylaminopurine, BAPR: 6-benzylaminopurine-9-ribosylpurine, *fb*: free base.

4.2. General procedures

Evaporations were carried out under vacuum rotary oil pump for *n*-propanol, *n*-butanol and ethyl acetate. Elemental analyses (C, H, N) were determined on 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 (9:1, v:v) or ethyl acetate/toluene (8:2, v:v) were used as solvents. CI+ mass spectra were recorded using a Polaris Q (Finnigan) mass spectrometer equipped with a Direct Insertion Probe (DIP). The compounds were heated in an ion source with a 40–450 °C temperature gradient, the mass monitoring interval was 50–1000 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 a reagent gas at a flow-rate of 2 L/h. The mass spectrometer was directly coupled to an Xcalibur data system. ^{1}H spectra were recorded on a Bruker Avance 300 spectrometer operating at a temperature of 300 K and a frequency of

Table 6
Cytotoxicity IC_{50} values of the prepared compounds in Calcein AM (K-562 and MCF-7) and in MTT (BJ) assays

Compound	Cell line/IC_{50} (μmol/L)		
	K-562	MCF-7	BJ
1	>100	>100	>100
2	>100	>100	>100
3	81	>100	>100
4	>100	>100	>100
5	97	>100	>100
6	61	97	>100
7	>100	>100	>100
8	>100	>100	>100
9	>100	>100	>100
10	>25	>25	>25
11	16.5	25.4	>100
12	10.3	>100	>100
13	>100	>100	>100
14	>100	>100	>100
15	92	>100	63.3
16	>100	>100	>100
17	>37.5	>37.5	>37.5
18	>100	>100	>100
19	>50	>50	>50
20	>100	>100	>100
21	>100	>100	>100
22	>100	>100	>100
23	>100	>100	>100
24	>100	>100	>100
25	>100	>100	>100
26	>100	>100	>100
27	>100	>100	>100
28	>100	>100	>100
29	>100	>100	>100
30	>100	>100	>100
31	>50	>50	>50
32	>100	>100	>100
33	>100	>100	>100
34	>100	>100	>100
35	>100	>100	>100

The maximum concentration tested was 100 μmol/L, except for compounds **11** and **17,** where lower concentrations (25 and 37 μmol/L, respectively) were used due to their limited solubility in the culture media.

300.13 Hz. Samples were prepared by dissolving the substances in DMSO-d_6. Tetramethylsilane (TMS) was used as the internal reference standard.

4.3. Synthesis of 9-tetrahydropyran-2-ylpurine (2–20) and 9-tetrahydrofuran-2-ylpurine (22–35) cytokinin derivatives

The synthesis of the derivatives consisted of two independent steps, as presented in Scheme 1. In the first step, we employed a modified version of a method found in the literature.[44,45] 6-chloropurine (10 g, 64.7 mmol) was stirred with 3,4-dihydro-2*H*-pyrane (12.4 g, 147 mmol, 14 mL) for 6-chloro-9-tetrahydropyran-2-ylpurine (**1a**) or with 2,3-dihydrofurane (11.3 g, 162 mmol, 12 mL) for 6-chloro-9-tetrahydrofuran-2-ylpurine (**1b**), for 10 min in ethyl acetate (100 mL) and subsequently, trifluoroacetic acid (10.9 g, 84.2 mmol for **1a** and 9.6 g, 95.5 mmol for **1b**), was added dropwise. The reaction mixture was stirred at room temperature for 1 h and then neutralized by the appropriate amount of a mixture of ammonia and water (2:3). The ethyl acetate phase was separated and purified using charcoal and SiO_2. Yellowish waxy products were obtained after vacuum evaporation of the solvents. The products were washed with cyclohexane (**1a**) or hexane (**1b**) and dried in the air. The yields, HPLC purity, MS (CI+) and ^{1}H NMR data, along with the melting points (mp) are presented in Supplementary data section. In the second step, **1a** (1 g, 4.2 mmol) or **1b** (1 g, 4.4 mmol) was coupled with the appropriate benzylamine in 1:1.2 ratio in *n*-propanol or *n*-butanol (30 mL) in the presence of triethylamine (4 g, 39.5 mmol, 5 mL) at about 100 °C. The reaction lasted for 3 h. The reaction mixture was then evaporated to dryness and the residue was treated with 50 mL of ethyl acetate and 50 mL of distilled water. The ethyl acetate phase was separated and purified using SiO_2 and charcoal and then dried over Na_2SO_4. Final products were isolated by crystallization using diethyl ether or hexane and dried in air. C, H, N elemental analyses, CI+ MS and HPLC purity data and mp for compounds **2**–**35** are given in Table 2 while the yields and ^{1}H NMR are given in Supplementary data.

4.4. pH Stability testing of 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine (3) and 6-(3-hydroxybenzylamino)-9-tetrahydrofuran-2-ylpurine (23)

The pH stability of compounds **3** and **23** was analyzed by HPLC-PDA (System Gold; Beckman Instruments, Fullerton, CA, USA); analytes were monitored at 270 nm. 10^{-2} M solutions of compounds **3** or **23** in methanol were prepared and diluted to 10^{-4} M using McIlvaine buffer solution for the appropriate pH (3, 4, 5, 6 or 7).[46] One hour after incubation at 25 °C, 5 μL of the prepared solution was directly injected onto a reversed phase column (Symmetry C18; 5 μm, 150 × 2.1 mm; Waters, Milford, USA). At flow-rate of 0.3 mL/min, the following binary gradient was used: 0 min, 10% A; 0–25 min, a linear gradient to 90% A; followed by 5 min isocratic elution of 90% A, where A was 100% methanol and B was 15 mM formic acid adjusted to pH 4 with ammonium. The HPLC measurement of the solutions was repeated after a 24 h incubation at 25 °C. The analyses were repeated at least three times.

4.5. Cytokinin bioassays

All the prepared compounds were tested in three cytokinin bioassays—the tobacco callus, *Amaranthus* and senescence bioassays—and their activity was compared with BAP. The biological activities of THP and THF derivatives of 6(3-hydroxybenzylamino)purine (**3**, **23**) and 6(3-methoxybenzylamino)purine (**6, 26**) were compared to those of free bases. Tested cytokinin derivatives were dissolved in dimethylsulfoxide (DMSO) and diluted with distilled water to 5×10^{-2} M solutions. This 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 at least twice. Detailed descriptions of the conditions and performance of the tobacco callus bioassay, *Amaranthus* bioassay and senescence bioassay are given in Zatloukal et al.[47]

4.6. Bacterial receptor assay

Escherichia coli KMI001 strains harboring plasmids pIN-III-AHK4 and pSTV28-AHK3 (Suzuki 2001, Yamada 2001,) were provided by Dr. T. Mizuno. The strains were used in the bacterial receptor assays as described, but with a slight modification.[33] The assay was optimized to 96-well microtiter plates according to the literature.[35] The preculture was diluted 1:10 and the incubation time was 6 h. Relative activation of cytokinin receptors was determined by measuring β-galactosidase activity using the fluorescent substrate 4-methylumbelliferyl-β-D-galactoside (Sigma) and monitoring the culture density at OD_{600}. The test was performed in triplicate and the entire test was repeated at least twice.

4.7. Enzymatic cytokinin degradation assay

The enzyme, recombinant ZmCKX1 produced by the yeast *Pichia pastoris* and purified as described previously,[48] was obtained from Dr. Kristin Bilyeu from USDA/ARS Plant Genetics Research Unit, University of Missouri, Columbia, USA. The end-point assay was performed according to the literature.[49] The reaction mixture for measuring dehydrogenase activity contained 100 mM McIlvaine buffer pH 6.0, an aliquot of 10 mM cytokinin substrate stock dissolved in DMSO and 0.1 mM 2,6-dichlorophenolindophenol. The final concentration of the substrate, when the k_{cat} parameter was determined, was 0.2 mM. The Michaelis constants were estimated using a double reciprocal plot with the substrate concentration in the range 0.05–0.25 mM.

4.8. Cytotoxicity testing

4.8.1. Calcein AM assay

The cytotoxicity of the prepared compounds against human chronic myelogenous leukaemia (K-562) and human breast carcinoma (MCF-7) cell lines was determined by standard Calcein AM assay.[35] The cells were maintained in plastic tissue culture flasks and grown in Dulbecco's modified Eagle's cell culture medium (DMEM) at 37 °C in a 5% CO_2 atmosphere and 100% humidity. The cells were seeded into 96-well microtitre plates (Nunc, Denmark) and after 12 h of preincubation, the tested compounds were added to give a final concentration in the range 0–100 μM. The concentration was adjusted in the case of compounds with limited solubility. The cells were incubated for another 72 h. At the end of the incubation period Calcein AM in PBS was added to a final concentration of 1 μg/mL. After another 1 h of incubation, fluorescence at 485/538 nm (ex/em) was measured with a Fluoroskan Ascent reader (Labsystems, Finland). IC_{50} values, the drug concentration causing a 50% reduction in Calcein AM conversion, were calculated from the dose–response curves. All experiments were repeated in quadruplicate with a maximum deviation of 15%. Because of their limited solubility in water, all the compounds tested were dissolved in DMSO and then diluted with water to a final DMSO concentration of 0.6%.

4.8.2. MTT test on human fibroblasts

Human diploid fibroblasts BJ (passage 15–20) were seeded in a 96-well plate (5000 cells per well). After 6 h, the cultivation medium (DMEM containing 5 g/l glucose, 2 mM glutamin, 100 U/mL penicillin, 100 μg/ml streptomycin and 10% fetal calf serum) was removed and fresh medium containing a test compound in concentration range 0–100 μM was added. The concentration was adjusted in the case of compounds with limited solubility. Each concentration was tested five times. MTT was added to the cells after 72 h to a final concentration of 0.5 mg/ml. The incubation time was 3 h. The resulting MTT was dissolved in DMSO and absorbance at 570 nm with a reference wavelength of 650 nm was measured. The IC_{50} value, that is, the compound concentration causing a 50% reduction in mitochondrial activity, was calculated from the dose–response curves.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.01.041.

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