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Heterocyclic derivatives of natural compounds with antisenescence properties

Ph.D. thesis

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Statement of authorship:

I hereby declare that the submitted Ph.D. thesis is entirely my own original work. All sources used in this work are listed in the bibliography.

In Olomouc on May 25, 2021

Hana Vylíčilová

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Abstrakt

Tato disertační práce je zaměřena na syntézu a biologickou aktivitu nových aromatických *N*9-cukerných derivátů cytokininů. Teoretická část se zabývá obecnou charakteristikou cytokininů, jejich biosyntézou, regulací a buněčnou signalizací. Velká pozornost byla věnována jejich antisenescenčním, antioxidačním účinkům a jejich úloze během procesu stárnutí rostlin. Nezbytnou součástí práce je shrnutí o již publikovaných aromatických *N*9 cukerných konjugátech a jim podobných látkám, které významně oddalující degradaci chlorofylu v rostlinách.

Experimentální část se zabývá přípravou syntetických 2,6-disubstituovaných purin-*N*9-glykosidů ve většině případů substituovaných na C2 atomu purinu chlorem a na *N*9 purinovém atomu glykopyranosou nebo glykofuranosou, odvozených od aromatických cytokininů. V první části práce jsou popsány syntetické postupy použité pro jejich přípravu. Kromě klasických metod byly cytokininové deriváty připraveny s využitím mikrovlnného či průtokového reaktoru ve snaze optimalizovat reakční podmínky. Nedílnou součástí práce je charakterizace nových látek dostupnými fyzikálně-chemickými metodami (HPLC/MS, NMR, TLC a HRMS). Ve druhé části je u těchto derivátů zkoumána biologická aktivita v různých cytokinových biotestech se zaměřením na antisenescenční aktivitu, aktivaci cytokininové signální dráhy, cytotoxicitu a architekturu kořene.

Klíčová slova	cytokininy, senescence, syntéza, glykosidy, ribosidy, arabinosidy, xylosidy, furanosidy, pyranosidy
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Abstract

This doctoral thesis focuses on the synthesis and biological activity of new aromatic *N*9 sugar derivatives of cytokinins. The theoretical part deals with the general characteristics of cytokinins, their biosynthesis, regulation, and cell signalling. Attention was also paid to their influence on senescence, antioxidant effects and their role during the senescence process in plants. We also focused on already prepared aromatic cytokinin *N*9-glycosyl conjugates and similar analogues delaying chlorophyll degradation.

The experimental part shows the preparation of 2,6-disubstituted purine-*N*9-glycosides substituted on the C2 atom of the purine by chlorine and on the *N*9 atom of the purine by various glycopyranose or glycofuranose derived from aromatic cytokinins. The first part describes the synthetic procedures used for their preparation. In addition, the preparation of purine derivatives was performed using innovative techniques such as a microwave or a flow reactor to optimize the reaction conditions. An integral part of the work is also the characterization of new substances by available physicochemical methods (HPLC/MS, NMR, TLC and HRMS). The second part of the thesis examines in detail the biological activity of prepared compounds in various bioassays focused on antisenescence activity, activation of the cytokinin signalling pathway, cytotoxicity, and root architecture.

Keywords	cytokinin, senescence, synthesis, glycosides, ribosides, arabinosides, xylosides, firanosides, pyranosides
Number of pages	106
Number of appendices	4
Language	English

Contents

Aim of work

1. Summary of knowledge of cytokinin and its antisenescence properties, search for new effective structural motifs.
2. Synthesis of purine derivatives and other cytokinin analogues with significant biological properties.
3. Implementation of a continuous flow reactor and a microwave reactor into the synthesis of the purine derivatives.
4. Characterization of prepared derivatives using available physico-chemical methods.
5. Study of biological activity of prepared cytokinin derivatives (antioxidant and antisenescence activity, cytotoxicity, cytokinin receptor assays and others).

THEORETICAL PART

1 Heterocyclic compound

Heterocyclic compounds are widely distributed in nature and it is fascinating how often they are found as a key component in numerous biomolecules (Arora *et al.*, 2012). Compounds with aromatic heterocyclic nuclei have been known since the earliest study of organic chemistry. At the end of the 19th century, Emil Fisher synthesized naturally occurring purine from uric acid for the first time (Fechete, 2016). Among heterocyclic compounds, substituted purines are the most abundant naturally occurring nitrogen heterocycles. They were identified in nucleic acids and many purine derivatives are involved in crucial biological processes. These biologically active compounds are represented by energy associated adenosine triphosphate (ATP), the second messengers including cyclic adenosine monophosphate (cAMP), coenzymes that are required in cellular reduction-oxidation processes demonstrated by nicotinamide adenine dinucleotide (NAD) or flavin adenine dinucleotide (FAD) etc. (Rosemeyer, 2004).

Many purine derivatives were isolated long before their structures were clarified. In general, they have trivial names such as hypoxanthine, guanine, isoguanine, xanthine, caffeine, theobromine, or uric acid (Figure 1); (Rosemeyer, 2004). The structural features of their derivatives are beneficial and many purine nucleosides have a profound impact on human health. They are extensively studied for their valuable biological properties which can be used as a structural template in medicinal chemistry and pharmaceutical industry (Dinesh *et al.*, 2012, Kerru *et al.*). To date, a wide range of naturally occurring heterocyclic compounds have been identified, synthesized and their biological properties carefully tested (Davies, 2010).

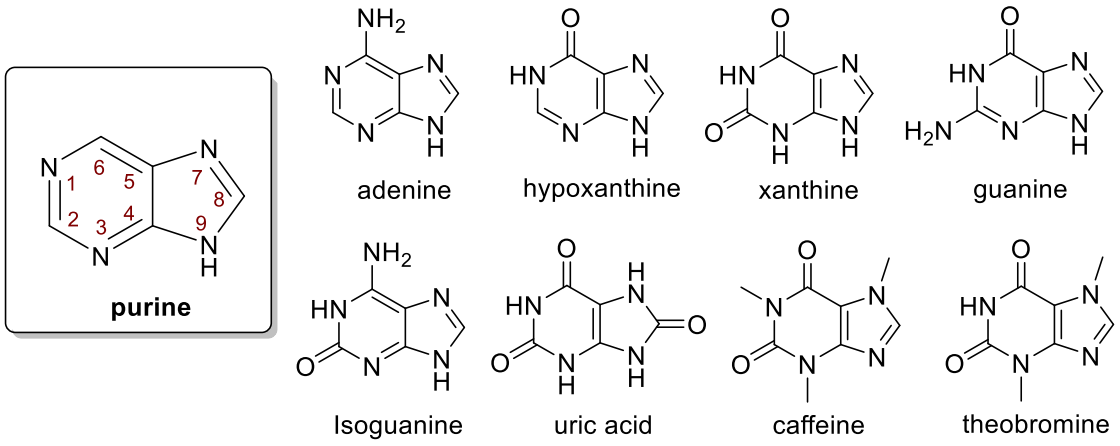


Figure 1. *The numbering of purine and chemical structures of selected purine biomolecules (Huang et al., 2014).*

2 Cytokinin

Cytokinins (CKs) are small signalling molecules based on the purine heterocycle. CKs represent an important group of phytohormones affecting various essential processes of plant growth and development (Casati *et al.*, 2011). The current development of analytical methods allows CK quantification at the organ level where they are present at very low concentrations (0.1 to 10 pmol g^{-1} of fresh weight) which may vary between different organs and during growth conditions (Osugi and Sakakibara, 2015). Notably, CKs are not only plant-specific compounds but they are also produced by other microorganism such as bacteria, fungi, microalgae, and insects *(*Akhtar *et al.*, 2020).

CKs promote cell division and differentiation, therefore Folke Skoog and his colleagues discovered the first aromatic cytokinin from autoclaved DNA named kinetin (Kin, 6-(furfurylamino)purine) as a growth factor. A crosstalk of CKs with other signalling molecules (auxin) plays a significant role in embryogenesis, shoot/root balance, bud formation, leaf senescence, photosynthesis and, last but not least, its mediates response to environmental stimuli and stress (Osugi and Sakakibara, 2015; Romanov, 2009).

Generally, naturally occurring CKs are purine-based derivatives carrying either the isoprenoid or the aromatic side chain attached the 6 position of the purine ring (Figure 2); (Sakakibara, 2006). Regarding the structure of the side chain, the isoprenoid group of CKs includes 6-(2-isopentenylamino)purine (iP) and zeatin type, which was first CK identified in a plant tissue. Moreover, zeatin exists in two geometrical isomers: *trans*-zeatin (6-(*E*)-(4-hydroxy-3-methylbut-2-enylamino)purine, *t*Z) and *cis*-zeatin (6-(*Z*)-(4-hydroxy-3-methylbut-2-enylamino)purine, *c*Z). Also, it can also exist as a derivative with a saturated side chain reported as dihydrozeatin (6-(4-hydroxy-3-methylbutylamino)purine, DZ); (Galuszka *et al.*, 2008; Sáenz *et al.*, 2003).

The classic aromatic cytokinin (ArCK) represents 6-benzylaminopurine (BAP) and Kin and they have been for a long time considered unnatural compounds but they were later detected together with their *N*9-conjugates in *Cocos nucifera* (L.); (Ge *et al.*, 2005; Sáenz *et al.*, 2003; Sakakibara, 2006). The hydroxylated analogues of BAP demonstrate biologically active phytohormones called topolins referred to as *orto*-topolin (*o*T), *meta*-topolin (*m*T) and *para*-topolin (*p*T), which were firstly discovered in poplar leaves (topol in the Czech language). Lately, methoxy derivatives of BAP (methoxytopolins) have been identified in *Arabidopsis. thaliana* (Strnad, 1997; Tarkowská *et al.*, 2003).

Although the occurrence of ArCKs is rare in nature, they have a greater stability and biological activity. Therefore, they are prepared synthetically and widely used in many tissue cultures (Schmülling, 2004). However, the application of BAP shows a negative effect on root growth and development causing problematic acclimatization of micropropagated plants. It has been proven repeatedly that it could be due to the formation of inactive *N*9-glucosides or through the activation of ethylene production (Plíhal *et al.*, 2013).

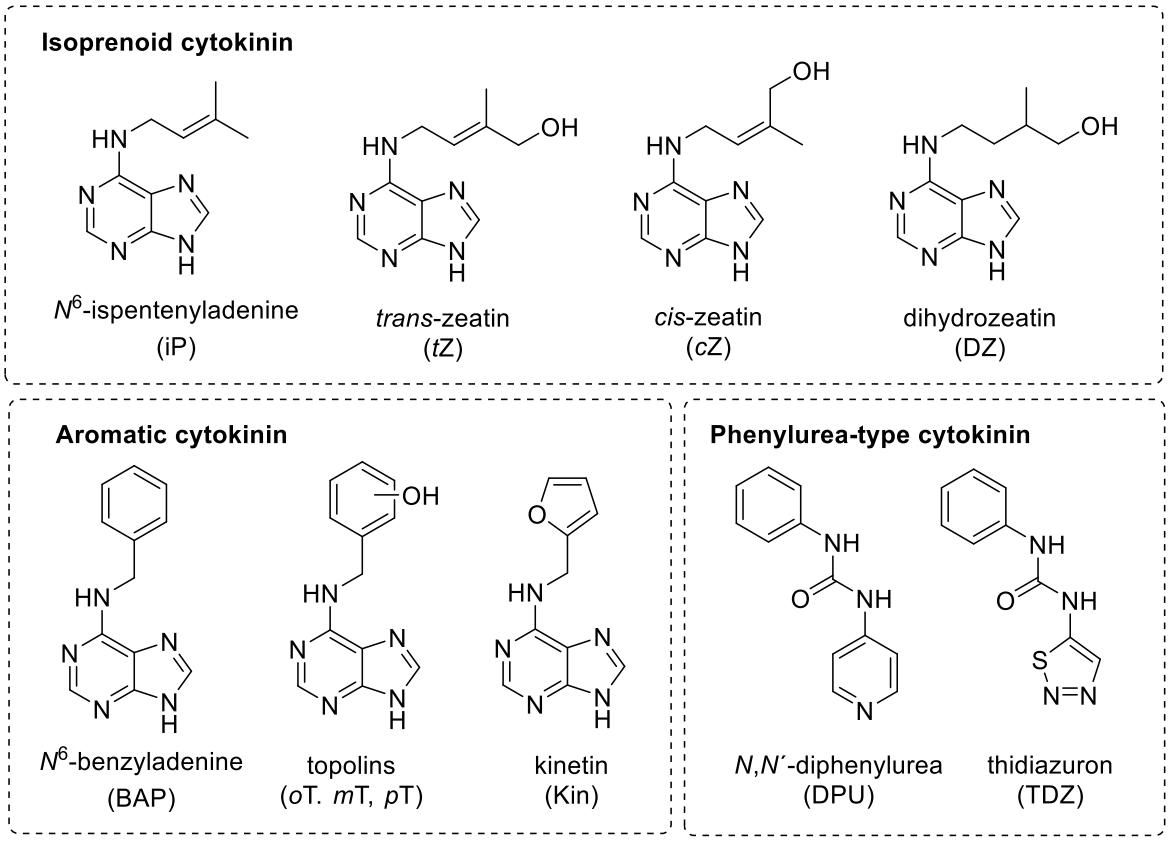


Figure 2. *Chemical structure of representative cytokinins of isoprenoid, aromatic and phenylurea-type. Commonly used abbreviations are given in parentheses (Mok and Mok, 2001; Sakakibara, 2006).*

Many compounds with similar CK activity have been identified so far. Synthetic urea derivatives, such as *N*,*N´*-diphenylurea (DPU), thidiazuron (1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea, TDZ) have been proved to be positive growth regulators of cell division and differentiation in the replacement of purine-based CK (Ricci and Bertoletti, 2009; Yip and Yang, 1986). Although CKs of phenylurea-type have been showed to be more stable than *t*Z, they have not been found in plant tissues (Mok and Mok, 2001).

Moreover, the *N*9 atom of the purine can be conjugated with the amino acid L-alanine to form less common *N*9-alanylzeatin and *N*9-alanyldihydrozeatin which were

first identified in lupin seeds (*Lupinus luteus* L.); (Frébort *et al.*, 2011). Alanyl CK conjugates showed a low activity in CK bioassays due to the lack of hydrolysis enzymes responsible to produce active forms of CK and therefore these conjugates serve as potential storage forms (Bajguz and Piotrowska, 2009). Furthermore, the degradation of tRNAs of many bacteria produces 2-methylthio CK derivatives (2MeSCKs) which are substituted with hydrophobic methylthiol group at the C2 position of the purine (Tarkowski *et al.*, 2010; Žižková *et al.*, 2017). The current knowledge of 2MeSCK was summarized in Gibb *et al.* (Gibb *et al.*, 2020).

Plant tissues metabolize exogenous application of CK easily, changes in CK metabolites of coniferous' bud have revealed an interesting case of disaccharide conjugates, where a glucose molecule is attached directly to the ribosyl group joining the purine at the *N*9 position (Taylor *et al.*, 1984; Zhang *et al.*, 2010). These conjugates probably play an important role in the shoot formation in tissue culture (Zhang *et al.*, 2010).

3 Cytokinin biosynthesis and regulation

Similarly, to other plant hormones, the distribution of bioactive CK levels *in planta* is strictly controlled by the balance between synthesis, catabolism and inactivation during plant development, growth, and environmental responses (Kurakawa *et al.*, 2007). CKs do not exist only as free bases but often as sugar conjugates represented by ribosides, ribotides or glucosides (Casati *et al.*, 2011; Sakakibara, 2006). Generally, a free base is thought to be active CK form. On the other hand, CK ribosides are perhaps transport form regarding their less biological activity and predominant presence in xylem (*t*Z riboside) and floem (iP) sap (Galuszka *et al.*, 2008).

3.1 Biosynthesis of cytokinin

Biosynthesis of CKs proceeds generally through two different biopathways, either *de novo* synthesis or by the tRNA degradation pathway (Figure 3). The combination of both ways is also possible. Generally, *de novo* synthesis is a direct route and the most common source of predominantly iP- and *t*Z-type of CKs (Hrtyan *et al.*, 2015). In both pathways, adenosine phosphate-isopentenyltransferase (IPT; EC 2.5.1.27), first found in the slime mould *Dictyostelium discoideum,* facilitated the initial step of biosynthesis (Sakakibara, 2006). It involves the addition of the isoprenoid side chain donors such as dimethylallyl diphosphate (DMAPP) or hydroxymethyl-butenyl diphosphate (HMBDP) to the N^6

terminus of adenosine tri-, di- or monophosphates (ATP, ADP or AMP); (Hirose *et al.*, 2008).

In the case of DMAPP attachment and formation of iP-type CK, the side chain can be further hydroxylated by cytochrome P450 monooxygenase (P450; EC 1.14.14.1) to produce *t*Z (Frébort *et al.*, 2011; Takei *et al.*, 2004). The double bond in *t*Z can also be reduced by a zeatin reductase (EC 1.3.1.69) to generate DZ (Spíchal, 2012). A recently published work revealed that zeatin *cis-trans* interconversion *in vitro* is responsible for a non-enzymatic flavin induced photoisomerization (Hluska *et al.*, 2017).

The other known alternative of CK biosynthesis pathway plays a minor role in the CK production. It involves the addition of DMAPP to adenine at the position of 37 on tRNA (prenylation of tRNA) with tRNA-isopentenyltransferase (tRNA-IPT; EC 2.5.1.8) and upon tRNA degradation *c*Z-type of CKs is released (Hrtyan *et al.*, 2015; Kasahara *et al.*, 2004).

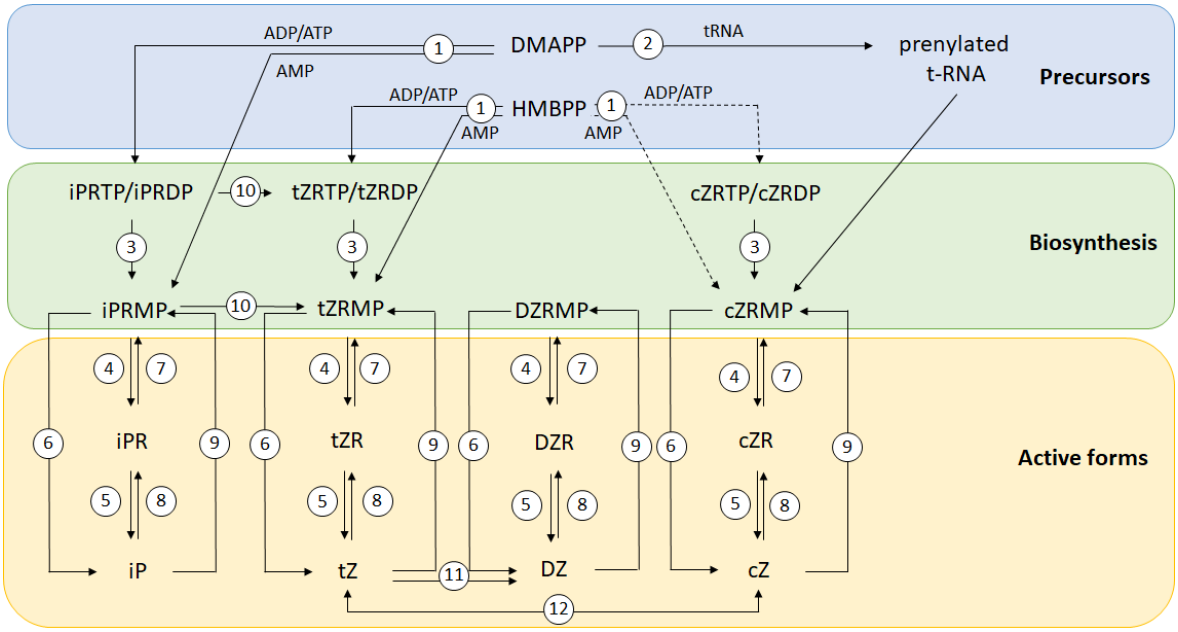


Figure 3. *Scheme of cytokinin biosynthesis and interconversion that was modified according to Spíchal, 2012. Dashed lines show pathways that have not yet been enough proven, (1) adenosine phosphate-isopentenyltransferase (EC 2.5.1.27); (2) tRNA isopentenyltransferase (EC 2.5.1.8); (3) phosphatase (EC 3.1.3.1); (4) 5´-ribonucleotide phosphohydrolase (EC 3.1.3.5); (5) adenosine nucleosidase (EC 3.2.2.7); (6) CK phosphoribohydrolase (EC 3.2.2.n1.); (7) purine nucleoside phosphorylase (EC 2.4.2.1); (8) adenosine kinase (EC 2.7.1.20); (9) adenine phosphoribosyltransferase; (10) cytochrome P450 mono-oxygenase; (11) zeatin reductase (EC 1.3.1.69); (12) flavin induced photoisomerization.*

The last step in this pathway is catalysed by the specific enzyme lonely guy (LOG; EC 3.2.2.n1). It directly converts inactive CK nucleotides to the active CK free base, by CK specific phosphoribohydrolase activity with CK nucleoside 5´-monophosphates, but

not with the di- or triphosphate (Kurakawa *et al.*, 2007). The free base, nucleotide or nucleoside can be easily interconverted in plant tissue (Mok and Mok, 2001). Five enzymes of general purine metabolism are responsible for their interconversion. Associated enzymes are purine nucleoside phosporylase (EC 2.4.2.1), adenosine kinase (EC 2.7.1.20), phosporibosyltransferase (EC 2.4.2.7), 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5) and adenosine nucleotidase (EC 3.2.2.7); (Hwang and Sakakibara, 2006; Spíchal, 2012).

CK biosynthesis focuses exclusively on the isoprenoid side chain, thus our understanding of ArCK biosynthesis is still poor. Due to the differences between the N^6 isoprenoid and the aromatic side chain, they are unlikely to have the same biosynthetic origin (Taylor *et al.*, 2003). It has been suggested, that the ArCKs precursors in *Arabidopsis* are theoretically able to synthesize trough the shikimate, polyketide and phenylpropanoid pathways (Jaworek *et al.*, 2019).

3.2 Glycosylation of cytokinin

Glycosidic CK conjugates play an important role in the regulation of the amount of the endogenous CK pool (Werner *et al.*, 2001). The level of active CKs can be modulated conjugating with the glucose moiety which can be attached to the *N*3, *N*7 and *N*9 atoms of the purine moiety or to the oxygen on the side chain of zeatin, DZ or *ortho*- and *meta*-topolin catalysed by uridine diphosphate glycosyltransferase (UGT; EC 2.4.1.); (Taylor *et al.*, 2003). The hydroxyl group of the side chain can also be more rarely attached to the xylose moiety by O-xylosyltransferase (EC 2.4.1.204); (Kieber, 2002; Kuroha *et al.*, 2009). CK O-glycosides and *N*3-glucosides play an important storage role in CK homeostasis since they can be easily converted back to the active CK by specific *β*-glucosidase (Bajguz and Piotrowska, 2009). On the other hand, *N*7- and *N*9-glucosides represent irreversible inactivated CK metabolites.

However, a recent study of short-term CK metabolism in *Arabidopsis* with exogenously applied CKs contradicts the role of *t*Z (DZ) N-glucosides as the terminal products of CK metabolism. While exogenous treatment of *t*Z *N*7/*N*9-glucoside has been shown to be metabolically reactivated and converted back to the free *t*Z base by yet unidentified enzyme *in vitro* which is responsible for the biological activities, N-glucosides of iP are not metabolized and accumulated as terminal products. The authors suggest that differences between the N-glucosides may be due to dissimilar metabolism of iP and *t*Z (Hošek *et al.*, 2020; Hoyerová and Hošek, 2020).

The concentration of CKs is irreversibly altered when the isoprenoid side chain is cleaved after the reaction is catalysed by CK oxygenase/dehydrogenase (CKX; EC 1.5.99.12); (Galuszka *et al.*, 2007). This FAD-containing enzyme mediates the formation of an unstable CK-derived imine intermediate that is hydrolysed to the adenine and the corresponding unsaturated aldehyde (Figure 4); (Avalbaev *et al.*, 2012). The CKX enzyme operates with unsaturated side chains, thus DZ and ArCKs are rather resistant to CKX activity (Zürcher and Müller, 2016)

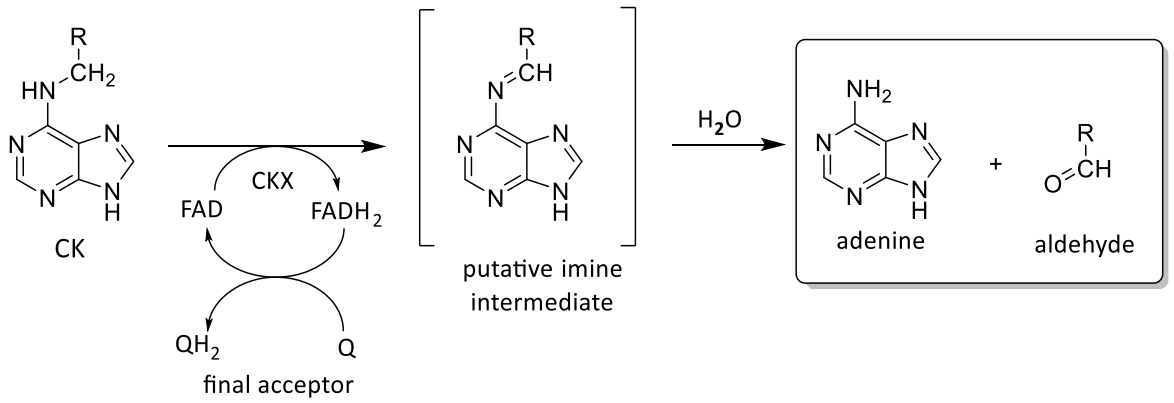


Figure 4. *Reaction mechanisms of cytokinin degradation by cytokinin oxygenase/dehydrogenase (CKX) that produce adenine and corresponding aldehyde (Popelka et al., 2006).*

In *Arabidopsis* genome, a family of seven homologues CKX-encoding genes (*AtCKX1-AtCKX7*) have been identified. Individual AtCKX isoenzymes differ in cellular localisation found either in vacuoles (AtCKX1 and AtCKX3), or in the apoplast (AtCKX2, AtCKX4, AtCKX5 and AtCKX6) and even in the cytosol (AtCKX7); (Frébort *et al.*, 2011). In addition, plants overexpressing revealed that individual AtCKXs differ in their substrate specificity (Galuszka *et al.*, 2007).

4 Cytokinin signalling pathway

Signalling components have contributed to understanding the basic mechanism of biosynthesis and CK perception throughout the plant body. The primary targets of CKs are cytokinin-sensitive genes in the nucleus (Romanov *et al.*, 2018). A model for CK signal transduction has emerged as a simple two-component signalling system involving a phosphotransfer cascade in *Arabidopsis* (To and Kieber, 2008). Firstly, CK is perceived by receptors of *Arabidopsis* Histidine Kinase (AHK) located mainly at endoplasmic reticulum

(ER) membrane or plasma membrane (PM). The CK signalling process is shown in Figure 5 (Wulfetange *et al.*, 2011).

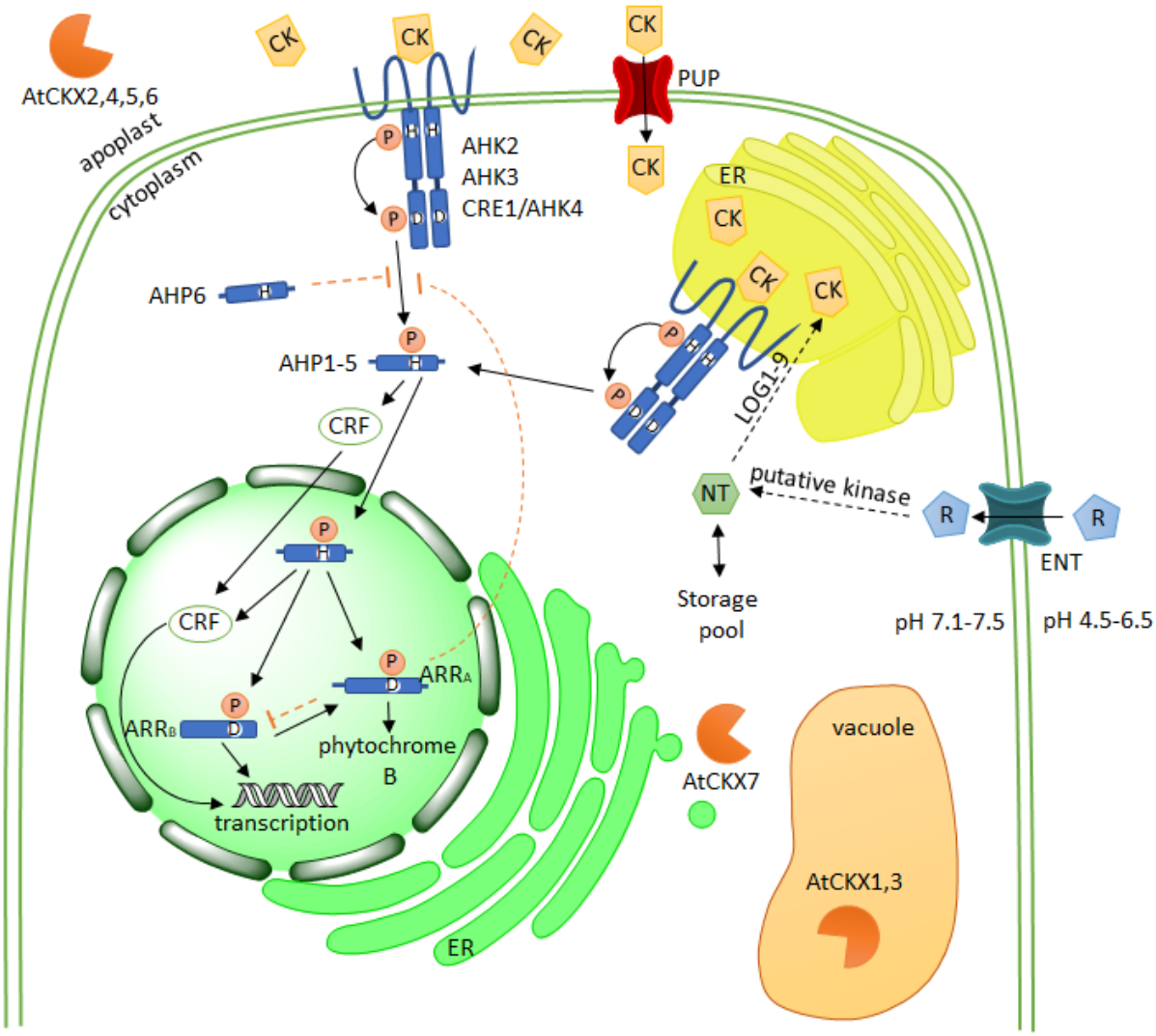


Figure 5. *Cytokinin signal transduction pathway and alternative model of signalling with a part of cytokinin metabolism. The figure is modified according to Lomin et al., 2018 and Shi and Rashotte, 2012. Black dashed lines show pathways that have not yet been enough proven, R-cytokinin riboside, CK-cytokinin, NT-cytokinin nucleotide ENT-equilibrative nucleoside transporter, PUP-purine permease, P-phosphate group.*

Three types of transmembrane receptors with great similarity have been identified: AHK2 (*Arabidopsis* Histidine Kinase 2), AHK3 (*Arabidopsis* Histidine Kinase 3) and CRE1/AHK4 (Cytokinin Response 1/*Arabidopsis* Histidine Kinase 4); (Shi and Rashotte, 2012). The CK ligand is bound through the extracellular CHASE (Cyclase/His kinase-Associated Sensing Extracellular) domain which is thought to elicit a conformational change in the receptors that induces dimerization of hybrid histidine kinase and autophosphorylation of a conserved His (histidine) residue in their kinase domain

(Plückthun *et al.*, 2001). This phosphate group is then transferred to a conserved Asp (aspartate) residue on the receiver domain of these AHK proteins, and subsequently transferred to *Arabidopsis* histidine-containing phosphotransfer protein (AHP). This AHP shuttles between the CK receptor and nuclear *Arabidopsis* response regulator (ARR); (Argueso *et al.*, 2010; Hutchison *et al.*, 2006).

The *Arabidopsis* genome encodes five AHP proteins (AHP1-AHP5) with phosphorelay activity and one protein (AHP6) acting as the inhibitor of the CK signalling (Figure 5); (El-Showk *et al.*, 2013; Rashotte *et al.*, 2006). The *Arabidopsis* genome encodes 23 ARRs which are classified based on their C-terminal domain (Zürcher and Müller, 2016). The type-A (ARR_A) contains the conserved receiver domain and a short C-terminal region, while type-B (ARR_B) has a longer C terminal region that contains DNA binding and transactivating domains that regulate transcription of cytokinin-activated targets, including the ARR_A group (To and Kieber, 2008).

Generally, the group of ARR_A has only the receiver domain and acts as a negative regulator of CK signal transduction pathway with the exception of ARR4 which has shown to upregulate phytochrome B. The group of ARR_B has additional domain and directly mediate the transcription of CK-responsive genes and positively regulates the CK signalling (Rashotte *et al.*, 2006). Moreover, the transcription of the ARR_A is partially regulated by the ARR_B and the rates of the transcription of most of the ARR_A, but not the ARR_B, are rapidly and specifically induced in response to exogenous CK (To *et al.*, 2004). The expression of the ARR_A, in particular ARR5 or ARR6, has been used to monitor transcriptional activity in response to the CK signalling (Zürcher and Müller, 2016) where *ARR5::GUS* fusion gene was used as a CK response reporter to study the general response to different CK metabolites (Galuszka *et al.*, 2007).

Microarray experiments of CK-treated seedlings have discovered a potential parallel branch of the CK signalling pathway mediated by CRFs (Cytokinin Response Factors) which transferred a phosphate group and overlapped the ARR_B. The *Arabidopsis* CRFs are a family of six genes (*CRF1–CRF6*) which belong to the AP2/ERF superfamily that is related to the ethylene response factor (ERF) involved in the controlling chloroplast division (Rashotte *et al.*, 2006). For example, the level of the plastid division protein (PDV2) was increased by CK treatment in *Arabidopsis* that is downstream of CRFs (Okazaki *et al.*, 2009). Currently, it was shown that each of the CRF proteins can interact directly with almost all the AHPs (AHP1-AHP5) and a few ARRs (Cutcliffe *et al.*, 2011). An analysis of *Arabidopsis* lines with an altered CRF function and mutants has

demonstrated that CRF1, CRF2, CRF3, CRF5, and/or CRF6 positively regulate the primary and lateral root growth, embryonic development, leaf senescence, and hypocotyl elongation, although they regulate negatively the rosette size and the shoot growth (Kim, 2016).

4.1 Cytokinin receptors and membrane transporters

The naturally occurring CKs and their conjugates have different affinities for CK receptors in *Arabidopsis*. Their differences have been demonstrated by using a bacterial heterologous system which expresses receptor proteins (Suzuki *et al.*, 2001). IsCK free bases (*t*Z, iP) have been reported to exhibit high activity with AHK3 and CRE1/AHK4 receptors in *E. coli* bacterial assay. Generally, ribosides have been shown to be more active in AHK3 than in the CRE1/AHK4 receptor (Spíchal *et al.*, 2004). In many cases, the functional overlap is particularly high for the AHK2 and AHK3 receptor as neither the *ahk2* nor the *ahk3* mutation alone has a major effect on plant growth. Whereas the *ahk2ahk3* double mutants show marked developmental disorders, suggesting that the two receptors act together (Riefler *et al.*, 2006). Remarkably, promoter swap experiments have showed that CRE1/AHK4 receptor can functionally replace AHK2 but not the AHK3 receptor (Stolz *et al.*, 2011).

According to the molecular modelling and docking studies, *N*9-ribosylation alters the binding pattern in the CK-receptor. Due to the presence of the *N*9-riboside residue, CK ribosides cannot form the same hydrogen bond between *N*9 of the purine moiety and leucine residue in the binding site and form a tight binding interaction compared to the corresponding CK free base (Hothorn *et al.*, 2011). In addition, CK binding to receptors is thought to have a pH-dependent character which prefers neutral-alkaline pH that is characteristic for the inner endomembrane system, than for PM whose outer side is in contact with an acidic apoplast (Lomin *et al.*, 2015). Thus, the ability of receptors to transmit CK signals is drastically decreased at acidic pH (Romanov *et al.*, 2018).

Subcellular localization of fluorescently labelled natural CK with CRE1/AHK4 receptor fused to GFP reporter demonstrated multiple perceptions of the CK signal both on the ER and PM. CK receptors located in ER entered into the secretory pathway and reached PM in the cells of root apical meristems and the cell plate of dividing meristematic cells (Kubiasová *et al.*, 2019). It is possible that CK signalling occurs from more than one site and that both ER and PM are used in different tissues or developmental stages (Romanov *et al.*, 2018).

Based on structural CK and purine similarity, CK translocation outside the sensing domain of receptors located on the PM causing a decrease in CK signalling have been postulated. The existence of two classes of transmembrane transporters with influx activity of apoplastic CKs into the cytosol has been identified, including purine permeases (PUPs) and equilibrative nucleoside transporters (ENTs); (Kang *et al.*, 2017). The subset of PUPs can transport purines, such as adenine and CK free base. While ENTs can transport nucleosides (CK *N*9-ribosides) from the apoplast into the cell (Kieber and Schaller, 2018).

Eight members of ENTs have been found in *Arabidop*sis (AtENT1-8) and localized at the PM. A further characterization has been demonstrated by nucleoside transport. For example, proteins AtENT3, AtENT6, and AtENT8 exhibit transport ability for iPR and *t*ZR (Durán-Medina *et al.*, 2017). Recently, a new model of signalling pathway has been proposed, in which ENTs are involved in *t*ZR transport into the cytosol. Thereafter, *t*ZR is successive converted through phosphorylation by putative kinase (*t*ZMP) and then LOG into the active free base (tZ) which trigger signalling from ER-located CK receptors (Lomin *et al.*, 2018) An alternative model of the CK signalling pathway with a part of the metabolism is in Figure 5 shown above.

The PUP family has 23 members and some PUPs could mediate CK free base (*t*Z, *c*Z, iP, BAP, Kin) transport (Durán-Medina *et al.*, 2017). Among them, PUP14 has been found to be involved in the CK signal modulation in early stages of plant development in *Arabidopsis*. It is assumed that PUP14 protein imports bioactive CKs from the apoplast to the cytosol, away from the sensing domains of AHK receptors localized on the PM, thus depleting apoplastic CK pools, causing an inhibition of CK signalling perception. In addition, a portion of PUP14 appears to exist in the ER (Romanov *et al.*, 2018; Zürcher *et al.*, 2016). Nevertheless, there is no clear and definitive evidence that PUPs or ENTs play a key role in the CK transport in plants (Kieber and Schaller, 2014). Moreover, ATP-binding cassette (ABC) transporters, mainly AtABCG14, are involved in the root-shoot CK translocation in *Arabidopsis* (Kang *et al.*, 2017).

5 Antioxidant defence

The progression of senescence process is influenced by the accumulation of reactive oxygen species (ROS) and the decrease of antioxidant capacity. ROS, such as hydroxyl radicals, superoxide anions, hydrogen peroxide and singlet oxygen are extremely reactive and toxic compounds causing oxidative stress and progressive damage to organisms

(Andersson, 2013; Huang *et al.*, 2019). Plant organelles with highly oxidative metabolic activity or intense electron flux, such as chloroplasts, mitochondria, and peroxisomes, are the main sources of ROS production. In addition, cell walls peroxidases, amine oxidases and plasma membrane NADPH oxidase produce ROS, usually in response to stressful conditions (Tripathy and Oelmüller, 2012). Hydrogen peroxide (H_2O_2) is the most stable ROS and the increase of H_2O_2 level most likely induces the expression of transcription factors (WRKY, NAC) and senescence-associated genes (SAGs); (Bieker *et al.*, 2012). However, in the last decade, H_2O_2 has been shown to act as a signal molecule and to participate in multiple physiological functions, including e.g. cell division (de Souza *et al.*, 2019).

Plants have developed sophisticated antioxidant defence mechanisms that can regulate the ROS accumulation to minimize the harmful effects of ROS. The enzymes superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), and peroxidase (POD; 1.11.1.7) contribute to the scavenging and reduce the excessive amount of harmful ROS (Poór *et al.*, 2018). The decrease of the activity of antioxidative enzymes is accompanied with the oxidative damage caused by the disruption of the PM with carbohydrate deoxidation, lipid peroxidation, protein denaturation and the destruction of nucleic acids, enzymes, and pigments (Xie *et al.*, 2019).

Changes in antioxidant enzyme activities are dependent on plant tissues and organs, plant developmental stages, stressors intensity, and also on light conditions (Poór *et al.*, 2018). It has been shown that shading plants or leaves and shortening the photoperiod generally accelerates senescence including the decreased chlorophyll content and photosynthetic activity and increased oxidative stress and lipid peroxidation which is easily manifested by an increased amount of malondialdehyde (MDA), which is the end product and marker of oxidative stress (Janečková *et al.*, 2018). It is known that individually darkened leaves initiate senescence much more rapidly than the leaves from the whole darkened plant which try to adopt a metabolic strategy to survive if possible (Law *et al.*, 2018).

CKs may play an indirect role in maintaining antioxidant systems that have led to the inhibition of senescence-promoting enzymes, such as lipoxygenases, to slow down ROS production by anabolic processes, or by stress. The inhibitory effect of CKs on lipid peroxidation could be due to direct scavenging of free radicals or increased activity of antioxidant enzymes such as APX, SOD and CAT (Wu *et al.*, 2012). Moreover, CKs

inhibit the activity of xanthine oxidase (EC 1.1.3.22), an enzyme which is one of the generators of ROS in the cell (Stoparic and Maksimovic, 2008). The molecular structure of CKs allows them to react directly (non-enzymatic antioxidant defence) with superoxide anion, but also with the other ROS, thus removing them from the cell metabolism (Stoparic and Maksimovic, 2008).

There is a great similarity in the chemical structure between phenolic and aniline compounds and the aromatic side chain of CKs as the simplest phenolic compounds also contain only one aromatic ring with a different type of functional groups (Świetek *et al.*, 2019). The antioxidant activity related to the compound structure depends on the number of the involved active group such as OH or NH_2 (Bendary *et al.*, 2013). A recently published comprehensive study of various substituted ArCK derivatives has revealed that the antioxidant capacity probably depends on the chemical structure of the substituent at the C6 atom of the purine. The most effective antioxidants are CK derivatives containing hydroxy-, methoxy- group or their combination binding on the benzyl aromatic ring (Matušková *et al.*, 2020). It has also been also reported that Kin scavenge free oxygen radicals by direct neutralization of ROS with hydrogen from the α-carbon of the amine bond in Kin. Nevertheless, the mechanism of ArCK action in alleviating oxidative stress has not been fully elucidated. It has been also been suggested that Kin stimulates the biosynthesis of phenolic and alkaloid enzymes through an appropriate transcription factor (Acidri *et al.*, 2020).

A recent study of CK receptor double mutants revealed that CRE1/AHK4 receptor has the main responsibility for the inhibition of CK-induced lipid peroxidation, followed by the AHK2 receptor (Janečková *et al.*, 2018). In response to CK, CRF6 has been shown to play a role in dark-induced senescence. CRF6 has recently been proposed as part of the regulatory network for ROS and CK crosstalk, which suppresses CK signalling during oxidative stress. Increased *CRF6* expression mediates repression of a set of genes involved in the CK signalling (ARR6, ARR9, ARR11, AHP1), CK biosynthesis (LOG7) and CK long-distance translocation (ABCG14); (Zwack *et al.*, 2016). Thus, an exogenous CK supplementation may be useful in maintaining normal cellular metabolism during oxidative stress, by preventing a reduction in endogenous CK content and may delay senescence (Acidri *et al.*, 2020).

6 Leaf senescence

Leaf senescence represents controlled and dynamic process mobilization of nitrogen, carbon, and minerals from the mature leaf. It initiates coordinated changes in cell structure, metabolism and gene expression leading to remobilization of resulting products of other plant organs (Buchanan-Wollaston, 1997). Physiological senescence, if not subject to suspension or reversal, will eventually be superseded by terminal cell death. Cell death must be prevented until all relocating nutrients have been translocated from the leaf to other organs that may be critical to the plants' fitness (Jansson and Thomas, 2008).

The first visible sign of leaf senescence is their yellowing caused by the destruction of chloroplast pigment-protein complexes and the conversion of chlorophyll components to catabolic non-green derivatives after the opening of the chlorine ring system (Mayta *et al.*, 2019). The analysis of gene expression of *Arabidopsis* leaf development has demonstrated chronology and changes in genes, including CK signalling during early senescence, which are shown in Figure 6 (Breeze *et al.*, 2011).

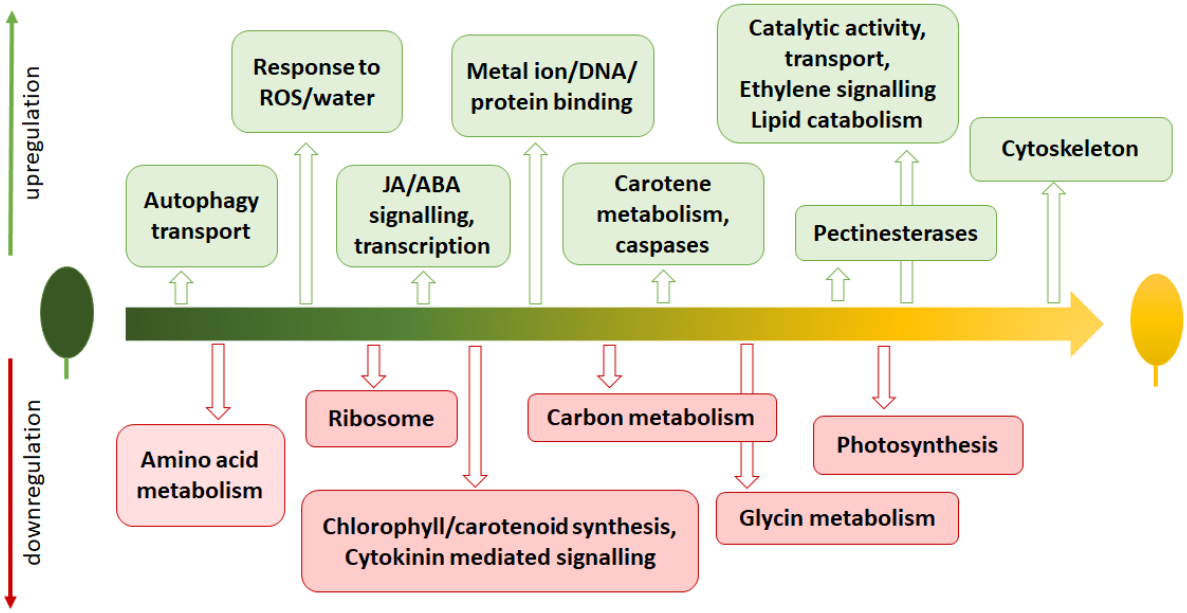


Figure 6. *Metabolic processes at different time points during leaf development (Breeze et al., 2011).*

The onset of leaf senescence is influenced by many internal and external stimuli, including environmental stress, nutrient deficiencies, pathogenic infections, reproductive age and phytohormonal level (Munné-Bosch and Alegre, 2004). All plant hormones are involved in the regulation of senescence. While ethylene, abscisic acid, jasmonic acid and

polyamines promote senescence, CKs inhibit it. Senescence regulation by auxins and sugars is supposed to play a dual inhibiting/stimulating role (Wojciechowska *et al.*, 2018).

In addition, CK treatment of decapitated senescence leaves can even cause re-greening. This reversibility has been observed in many plant species e.g. in *Nicotiana rustica* L. (Zavaleta-Mancera *et al.*, 1999). At the cellular level, an exogenous CK application has been reported to stimulate chloroplast gene transcription (Zubo *et al.*, 2008). CK biosynthesis occurs in chloroplasts since they contain four of the seven IPT enzymes (IPT1, IPT3, IPT5, IPT8) that catalyse the limiting step of the iP and *t*Z-type of CKs found in *Arabidopsis* plastids (Kasahara *et al.*, 2004).

Exogenous CK treatment has been showed to maintain both RuBisCo and plastid content (chlorophyll a and b, carotenoids), increase photochemical efficiency and the rate of oxygen-evolving complex (OEC). Moreover, CKs keep reaction centres (RCII) active, stabilize light-harvesting complex II (LHCII) and xanthophyll cycle during dark-induced senescence (Janečková *et al.*, 2019; Vlčková *et al.*, 2006; Vylíčilová *et al.*, 2016, Talla *et al.*, 2016).

Some authors suggest that the delaying of the senescence may relate to the CK signalling pathway machinery. Perception of CK mainly by the AHK3 receptor and subsequent phosphorylation of the ARR_B group, specifically ARR2, seem to be necessary for mediating CK-dependent chlorophyll retention in leaves (Kim *et al.*, 2006). Both AHK2 and AHK3 receptors are considered to play a major role in controlling CK mediated leaf longevity in contrast to the CRE1/AHK4 receptor. A recent study by Danilova *et al.* showed the opposite role of the AHK2 and AHK3 receptors in the expression of plastid genes and genes for the plastid transcriptional machinery during senescence. Although the AHK3 receptor retards senescence, the AHK2 receptor supposedly mediates CK-induced leaf senescence (Danilova *et al.*, 2017).

Moreover, the increased *CRF6* expression acts downstream of CK perception trough the AHK3 receptor and directly interacts with most of the AHPs (AHP1-AHP5) and with the group of ARR_B during dark induced senescence (Cutcliffe *et al.*, 2011; Zwack and Rashotte, 2013). It has been suggested that ARR2 and CRF6 proteins may interact directly with each other and then they regulate extracellular invertase which has a crucial role in sink-source regulation (Figure 7). This enzyme catalyses the irreversible cleavage of sucrose into hexose monomers and thus regulates the apoplasmic phloem unloading and CKs inhibit expression of the invertase inhibitor (Balibrea Lara *et al.*, 2004).

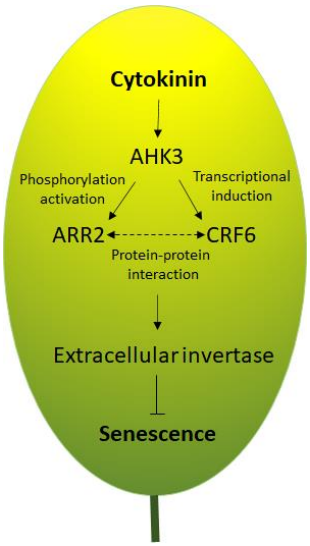


Figure 7. *Model of cytokinin regulation in Arabidopsis during leaf senescence (Zwack and Rashotte, 2013).*

However, the molecular mechanism of leaf senescence process is still not very well understood. In some cases, the application of CK, when added in high doses to plants, accelerates senescence and can induce programmed cell death in plant cell cultures (Carimi *et al.*, 2004). Remarkably, the accelerated senescence has been reported in experiments where entire plants are supplemented with sugars, which may be due to a change in the sink-source relationships in the opposite direction (Zwack and Rashotte, 2013).

The regulation of senescence is accompanied by decreased expression of genes related to photosynthesis (e.g., chlorophyll A/B-binding protein 2), protein synthesis (e.g., ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBisCo) and by increased expression of senescence-associated genes (SAGs); (Lim *et al.*, 2007). The expression of *SAG12* and *SAG13* is the most extensively used SAG markers, reflecting late and early status of senescence. *SAG12* has been closely associated with naturally induced senescence. *SAG12* encodes a putative cysteine protease, located predominantly in senescence-associated vacuoles involved in protein degradation and is highly up-regulated at the mRNA level only in senescent tissues. *SAG13* encodes a protein related to a short-chain alcohol dehydrogenase and/or oxidoreductase. Many transcription factors modulate SAG expression, genetic and molecular studies revealed that mainly NAC, WRKY and TCP are members of the major plant-specific transcription family during leaf senescence (Li *et al.*, 2018).

The crosstalk between auxin and CK signalling indicates that auxin suppresses the expression of *SAG12* through ARF2-ANT pathways, thus delaying leaf senescence (Woo

et al., 2019). During the senescence, the *CKX* expression is upregulated, *IPT* expression is downregulated, as it also increases the activity of N- and O-glucosyltransferases leading to a decrease in the endogenous CKs. Maintaining a high level of CKs in the leaf prevents and retards senescence (Buchanan-Wollaston, 1997; Buchanan-Wollaston *et al.*, 2005; Hwang *et al.*, 2012).

The regulatory role of CKs during the onset of senescence has been supported using transgenic tobacco plants. The senescence specific *SAG12* promoter from *Arabidopsis* was fused to CK biosynthesizing enzyme *IPT*. P_{SAG12}-*IPT* expression led to an increased level of endogenous CKs and supressed senescence. In return, to prevent CKs overproduction, increased level of CKs attenuated P_{SAG12}-*IPT* gene activity (Gan and Amasino, 1995). This autoregulatory CK production system does not cause any abnormalities in growth and development of stay-green transgenic plants and has been successfully used on other plant species in agricultural crops (Guo and Gan, 2014).

6.1 Cytokinin *N*9-glycosides with antisenescence effect

Natural and synthetic CKs have been extensively studied, since a small change in the structure of the purine moiety and/or various substitution on the benzyl ring can affect the CK biological activity (Plíhalová *et al.*, 2016). For example, a substitution with hydroxyl on the benzyl ring demonstrates a structural advantage as it allows the production of O-glucosides which can be activated by *β*-glucosidases. On the other hand, unsubstituted BAP is converted to inactive and stable *N*9-glucoside. This unfavourable effect can be blocked by appropriate substitution at the *N*9 position of the purine (Werbrouck *et al.*, 1996; Werbrouck *et al.*, 1995).

It has been demonstrated that derivatives of 6-benzylaminopurine-9-*β*-D-ribofuranosides (BAPR) were more active in delaying chlorophyll degradation in WLSA compared to their free bases (Holub *et al.*, 1998). The results showed that the most efficient substitution on the benzyl ring appears to be the *meta* position followed by the *ortho* and *para* position (Doležal *et al.*, 2007). Lately, other similar BAPR derivatives (Figure 8) with chlorine at the C2 atom of the purine have been prepared and halogenated derivatives with equivalent *meta*-substitution on the benzyl ring possess highly antisenescence properties (Vylíčilová *et al.*, 2016).

Although these compounds showed low interaction with the AHK3 and CRE1/AHK4 receptors, they specifically activated subsequent component of the CK signalling pathway (ARR_A) studied in transgenic CK-responsive *ARR5::GUS* reporter line.

Moreover, a genome-wide expression profiling revealed that the most potent compounds upregulated genes coding components reaction centre of PSII, LHCII, and OEC, as well as several stress factors responsible for regulating photosynthesis and chlorophyll degradation (Vylíčilová *et al.*, 2016).

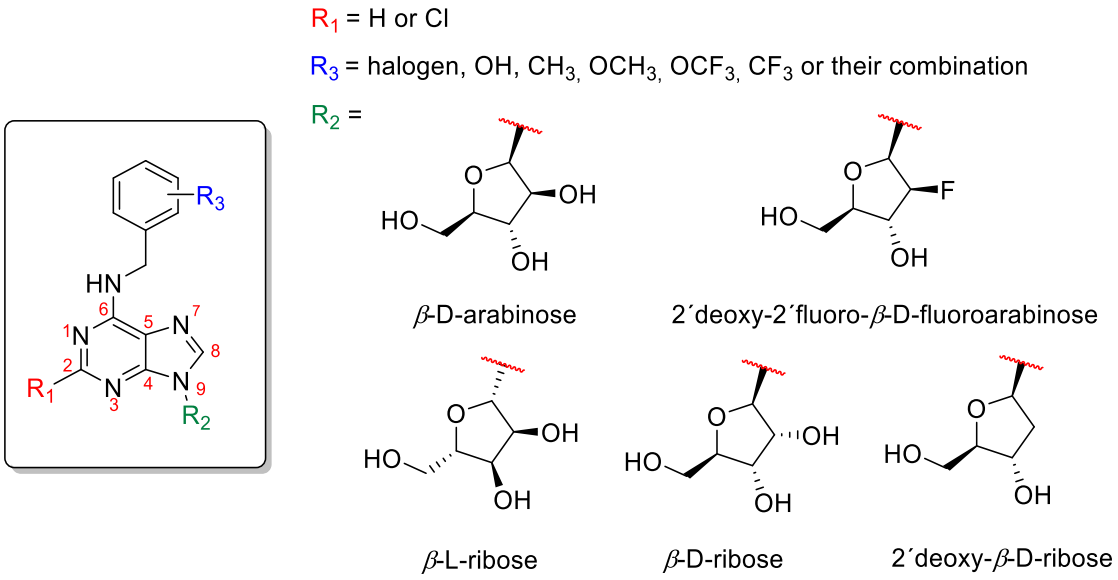


Figure 8. *Chemical structure of prepared aromatic 2,6-disubstituted/6-substitued-N9-glycosylpurines with high antisenescence effect.*

Recently, a large group of various 6-benzylaminopurine-2′-deoxy-9-*β*-D-ribofuranosides (Figure 8) have been synthesized and the removal of oxygen from the 2´-position of the D-ribofuranose ring has led to the remarkable increase suppression of leaf senescence compared to appropriate *N*9-ribofuransides. Although 2'-deoxyribosides even exceeded 3-fold the activity of BAP in WLSA, they were also unable to interact with AHK4 or AHK3 receptors (Doležal *et al.*, 2018; Matušková *et al.*, 2020). These aromatic 6-substituted purine-*N*9-glycosides and similar sugar-modified compounds which exhibit a strong antisenescence effect were patented in 2018 (Patent No. EP 3229772); (Doležal *et al.*, 2018).

Subsequent a study of 6-benzylaminopurine-9-*β*-D-arabinofuranosides (BAPA) bearing variously substituted benzyl rings was published. The authors hypothesized that the antisenescence effect of BAPA derivatives may be species-specific. Whereas *meta*-hydroxyl BAPA were highly effective in wheat leaves, *meta*-methoxy BAPA specifically delayed the onset of senescence in *Arabidopsis* leaves (Bryksová; *et al.*, 2020a). According to RNA-sequencing analysis, the application of BAPA derivatives on *Arabidopsis* leaves

sensitively promoted the plant-pathogen response or activated repair and defence mechanisms without adversely affecting plant condition (Bryksová; *et al.*, 2020a). This defence response was observed by downregulation of several photosynthetic genes simultaneously with unmeasurable reduction of chlorophyll content or photosynthetic function in leaves. The authors suggested that antistress/antisenescent activity related to the existence of a parallel mechanism different from the standard CK pathway (Bryksová; *et al.*, 2020a).

The replacement of a 2′-hydroxyl group of the sugar moiety with fluorine considerably affects stereoeletronic properties and increases the chemical and metabolic stability of nucleosides. Thus, newly prepared aromatic derivatives of 2′-deoxy-2′-fluoro-9-*β*-D-arabinofuranosides improved the CK antisenescence properties (Bryksová *et al.*, 2020b). The stereochemistry of sugars (Figure 8) at the *N*9 position of the purine appears to be very important as aromatic 6-substituted purine-9-*β*-L-ribofuranosides show only slight biological activity in WLSA compared to their D-counterparts (Vylíčilová *et al.*, 2020).

6.2 Synthetic cytokinin *N*9-analogues with antisenescence effect

Based on structural similarities, numerous aromatic 6-substituted-*N*9-(tetrahydropyran-2-yl) (THP) or *N*9-(tetrahydrofuran-2-yl) (THF) purines with CK-like activity have been reported (Skoog *et al.*, 1967; Zhang and Letham, 1989). The group of THP and THF mimics the structure of a five/six-membered sugar ring. Generally, tetrahydropyranyl and tetrahydrofuranyl ethers are commonly used in the organic chemistry as protecting groups and therefore can be easily removed under the acidic conditions. Synthetic THP/THF derivatives substituted at the *N*9 position of the purine represent a storage pool, where active CKs can be gradually released (Falck *et al.*, 2006; Zhang and Letham, 1989). The hydroxyl group attached to the benzyl ring in *meta* position of aromatic 6-substituted purine-9*N*-THP/THF derivatives have slightly improved the antisenescence effects (Szüčová *et al.*, 2009).

Derivatives of CK *N*9-THP/THF can gradually release the corresponding free base and can thus serve as a prevention against shortening of the main root, which is typical for exogenous treatment of CK (Plíhal *et al.*, 2013; Podlešáková *et al.*, 2012). Applied synthetic CK analogues with stimulation effect on root development have proved to be an alternative to the commonly used BAP and Kin in the micropropagation of many plant species (Aremu *et al.*, 2014; Aremu *et al.*, 2012). Recently, different Kin-9*N*-THF

analogues with modified furfuryl ring (Figure 9) have been synthesized, but they have not exceeded the antisenescence activity of the parent compound (Kin-*N*9-THF) in WLSA (Hönig *et al.*, 2018).

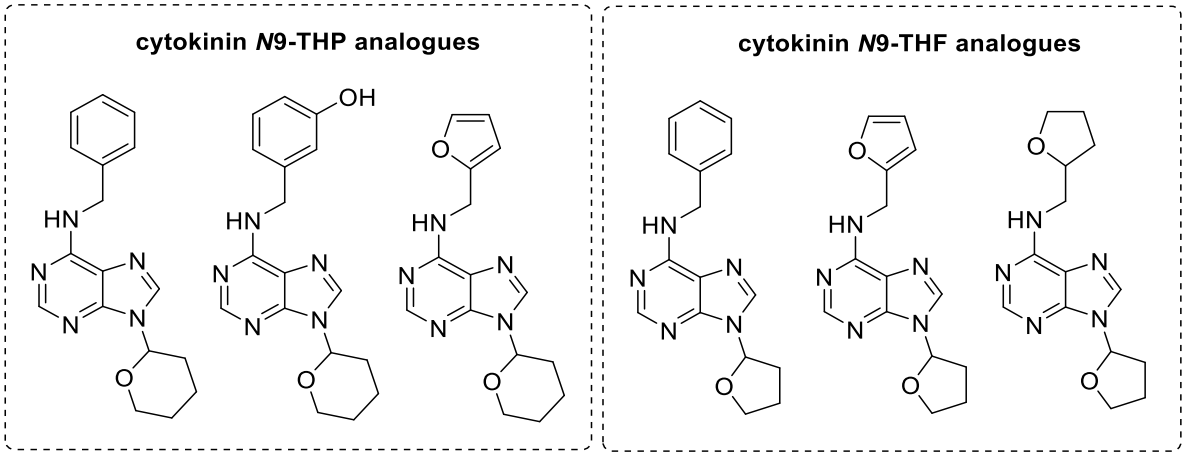


Figure 9. *Chemical structures of synthetic* 6*-subtituted purine-N9-THP/THF derivatives (Hönig et al., 2018).*

On the other hand, the treatment of Kin-*N*9-THP derivative possesses extraordinary anti-aging activity on human skin cells which leads to their application in cosmetics (McCullough *et al.*, 2008; Szüčova *et al.*, 2011). Others possess remodelling UV photoprotective activity and are protected by a patent (Patent No. CZ 2016050029); (Hönig *et al.*, 2020; Hönig *et al.*, 2018).

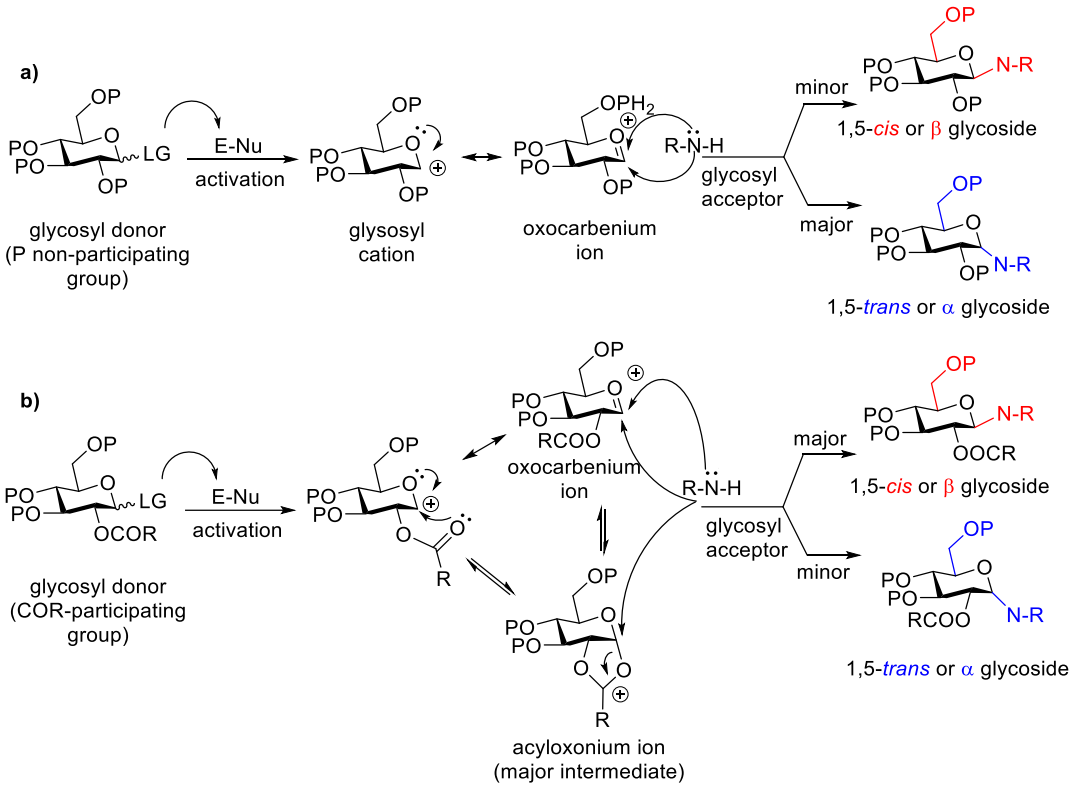
7 Synthetic approaches to purine glycosylation

During N-glycosylation, a molecule of sugar is attached to the nitrogen atom of purine moiety that usually provides an anomeric mixture. While *β*-nucleoside typically exists in nature, *α*-nucleoside is very rare (Ni *et al.*, 2019). Nucleoside synthesis can be performed by several pathways including non-enzymatic and chemoenzymatic strategies involving glycosyltransferases, which are regioselective for the *N*9 atom of the purine (Downey and Hocek, 2017).

The glycosylation reaction is considered to follow S_N1 most often, although S_N2 mechanism is also possible. The best-known non-enzymatic glycosylation involves preparing a glycosyl donor at C1 glycosyl anomeric centre by converting an anomeric substituent (e.g. hydroxyl group) into a leaving group. In most cases, the electrophilic promoter activates the departure of the anomeric leaving group which leads to the formation of a flattened glycosyl cation that can be stabilized in a sugar oxacarbenium ion

or acyloxonium intermediate. The stereoselectivity correlates with protecting groups situated especially at the adjacent C2 position (Scheme 1); (Demchenko, 2008). Unprotected or partially protected glycosyl donors are less common (Ranade and Demchenko, 2013).

Subsequently, a glycosyl bond is formed by the reaction between an activated glycosyl donor and the nucleophilic glycosyl acceptor. Thus, the anomeric carbon C1 is hybridized to sp^2 and nucleophilic attack is possible either from the top or bottom of the glycosyl acceptor which generates *β*-glycoside or *α*-glycoside (Scheme 1); (Das and Mukhopadhyay, 2016; Demchenko, 2008). The glycosylation reaction is strictly controlled since it is easily influenced by many factors, including the reactivity of glycosyl donors, temperature, solvents, and other additives (Hoang *et al.*, 2017).



Scheme 1. *General mechanism of the glycosylation reaction. The scheme was modified according to Demchenko, 2008; LG, leaving group; Nu, nucleophile; E, electrophile.*

Classic non-enzymatic glycosylation strategies dominated in several preparation methods in nucleoside synthesis. First, the Fischer and Helferich method utilized metal salt of heterocycle bases that condensed with protected glycosyl halide to provide a nucleoside (Davoll *et al.*, 1948). Afterward, the fusion coupling process of peracylated sugar with a nucleobase was performed (Christensen *et al.*, 1972). However, the most popular

glycosylation is Vorbrüggen variant of the Hilbert-Johnson reaction where protected sugar is coupled to silylated nucleobase with a presence of Lewis acids ($SnCl_4$, trimethylsilyl trfluoromethanesulfonate-TMSOTf); (Henschke *et al.*, 2013; Niedballa and Vorbrüggen, 1970). Glycosylation of the heterocyclic base by Vorbrüggen method with microwave irradiation has been applied to get higher yields in a shorter time compared to the standard organic method (Elgemeie and Mohamed, 2019; Nikolaus *et al.*, 2007).

In general, glycosylation is a coupling reaction with fully protected sugar gave a mixture of anomers which is difficult to separate. Several years ago, an improved glycosylation strategy was described using Mitsunobu conditions for nucleoside synthesis with unprotected/5-*O*-monoprotected D-ribose which favours the formation of only the *β*-anomeric configuration of pyranoside/furanoside (Downey *et al.*, 2015). Later, a modified strategy using monoprotected sugar trough anhydrase intermediate was used. However, the poor regiochemical control was observed and a mixture of purine *N*9/*N*3 regioisomers was isolated (Downey and Hocek, 2017).

EXPERIMENTAL PART

8 Material and Methods

8.1 General synthesis procedures

Most chemicals (reagents, substances, and solvents) were purchased from commercial sources (Sigma Aldrich ®, TCI Chemicals®, VWR®, Jena Bioscience® or OlChemIm®). Solvents were dried by distillation over calcium hydride.

All reactions were monitored by the thin-layer chromatography (TLC) on Silica Gel 60 F_{254} aluminium plates (Merck, US) using $CHCl_3$/MeOH (9:1 v/v or 4:1 v/v) as the mobile phase. The TLC plates were visualized by using a UV lamp (Camag, Switzerland) at a wavelength of 254 nm, and the detection of sugar units were proofed by the vanillin solution (3.2 g vanillin, 200 mL EtOH, 1 mL conc. acetic acid, 2 mL conc. sulfuric acid).

The purification of the prepared compounds was performed using a column liquid chromatography on Davisil LC60A 40-63-micron silica gel (Grace Davison Discovery Sciences, UK). The used mobile phase is mentioned in the individual experimental procedures.

The final products were separated by preparative HPLC-MS chromatography Agilent 1290 Infinity II coupled to the UV-VIS detector with the mass detector Agilent InfinityLab LC/MSD. The dissolves samples (10 mg/1 mL in MeOH) were injected into the reverse phase column (Agilent 5Prep-C18 10x21.2 mm). The flow rate of the mobile phase (H_2O, A/ACN, B) was set at 20 mL/min with the following linear gradients: 0 min (70% A; 30%B); 5 min (30% A; 70% B); 6 min (30% A, 70% B); 7 min (70% A, 30%B).

The chromatographic purity of the prepared substances was determined by HPLC-PDA-MS assembly. An Alliance 2695 separation module (Waters, UK) and *Q-Tof micro* mass spectrometer (Waters, UK) with an electrospray was used. The samples (10 µl of 3.10^{-5} M in 1% MeOH) were injected onto the reverse-phased column (Symmetry C18, 5 µm, 150×2.1 mm; Waters, USA) and incubated at 25 °C. The separation was performed using a binary gradient (0 '- 90% A; 25' - 10% A; 35 '- 10% A; 36' - 90% A; 45 '- 10% A) and the flow rate was set at 0.25 mL/min. Solvent (A) consisted of 15 mM formic acid adjusted to pH 4.0 with ammonium hydroxide. MeOH was used as the organic modifier (solvent B). The effluent was introduced into the diode array UV-VIS detector (PDA 2996, Waters®, UK) scan in a range 210-400 nm with a resolution of 1.2 nm and the detector was equipped with an electrospray source heated to 120 °C. The capillary voltage was set to +3 kV, cone voltage to +20 V and desolvation temperature to 300°C. Nitrogen was used as

desolvation gas (500 l/h) as well as cone gas (50 l/h). The mass spectrometer was operated in the positive ion detection mode (ESI+) in the mass range: 50-1000 *m/z*.

Nuclear magnetic resonance (NMR) spectra were recorded on ECA-500 spectrometer (Jeol, Japan) operating at frequency of 500 MHz (^{1}H) and 125 MHz (^{13}C). The samples were dissolved in deuterated dimethyl sulfoxide (DMSO-d_6), and tetramethylsilane was used as an internal reference.

Microwave experiments were performed in the Discover SP microwave reactor (CEM, US). The reactor was used in the standard configuration containing the Synergy software. The reactions were performed in 10 mL borosilicate glass vessels, which were sealed with a silicone cap. The temperature was measured by an infrared sensor. The continuous flow reaction was performed on the X-cube instrument (ThalesNano, Hungary).

High-resolution mass spectrometry (HRMS) experiments were kindly provided by Mgr. Hana Omamiková (Laboratory of Growth Regulators). HRMS equipped with Agilent 1290 Infinity II liquid chromatography (Agilent, USA) with the UV-VIS detector followed by Agilent 6230 MS-TOF (Agilent, USA) was performed to confirm the molecular formula. Dissolved samples in MeOH (1 mg/mL) was diluted with the initial mobile phase and 20 µl of applied samples was injected onto the column (Agilent 5Prep-C18 10x21.2 mm). The separation was performed using a linear gradient (0 '- 30% ACN; 5'-70% ACN) with the flow rate 0.5 mL/min. The solvent consisted of 15 mM formic acid: ACN (50% ACN) and the split ratio was set to 5000:1. The analytes were introduced into the ion source includes Dual AJS ESI and scanning in a range of 100-1700 *m/z* (gas temperature 260°C, capillary voltage 3500V, nozzle voltage 300V).

8.2 General bioassays procedures

Standard cytokinin bioassays

Two standard CK bioassays were used to determined CK activity. The tobacco callus bioassay is based on the stimulation growth of the cytokinin-dependent tobacco callus cells (*Nicotiana tabacum* L. cv. Wisconsin 38) The wheat leaf senescence assay (WLSA) is based on the chlorophyll degradation delay in excised wheat leaves (*Triticum aestivum* L. cv. Aranka) in the dark condition. WLSA were performed according to the literature (Holub *et al.*, 1998). The protocols for both CK tests are described in detail and available on Aesculab (aesculab.upol.cz).

In all cases, BAP solutions used as a positive control and test compounds were dissolved in DMSO. The final concentration of DMSO in the medium did not exceed

0.2%. The results were determinate as the strongest biological response and the relative activity at this concentration of each compound was calculated.

Chlorophyll retention in the receptor mutants

Approximately the seventh leaf of the 24-day-old seedling was detached and floated on distilled H_2O supplemented with 0.1 μM BAP or CK in six-well plates for 10 days in the dark. Alternatively, the basal part of the detached leaf was immersed in a well containing test compound (150 μL/well) and cultured in dark for 5 days. Each genotype was measured in triplicate and samples were weighed into 100 mg of fresh mass. Chlorophyll was extracted with MeOH/ for 24 hours in the dark or heated with 80% EtOH at 80°C for 10 min. The light absorption at 665 nm was determined spectrophotometrically (Synergy H4 Hybrid Multi-Mode Microplate Reader, Biotek, USA) and normalized to fresh weight. The chlorophyll content at the beginning of the experiments was taken as a reference.

Turbidimetric solubility assay

The solubility of the prepared compounds in aqueous solution with a final concertation of 0.1 mM was measured by turbiditimetry (TurbiCheck WL).

Arabidopsis ARR5::GUS reporter gene assay

This assay was carried out according to the protocol (Romanov *et al.*, 2002) with little modification. After the stratification, transgenic *Arabidopsis ARR5::GUS* seeds were grown in six-well plates on an orbital shaker in a growth chamber (21°C) under long-day conditions (16 h light/8 h dark) for 3 days. The seedlings were added to the liquid growth solution contained tested compound, a standard (BAP) or pure DMSO (solvent, final conc. 0.05%) and cultured for additional 16 hours. The specific activity of *β*-glucuronidase in the plant extract was spectrophotometric measured using a Fluoroscan Ascent microplate reader (Labsysems, Helsinki, Finland) at excitation/emission wavelengths of 365/450 nm.

Oxygen radical absorbance capacity (ORAC) assay

The oxygen radical absorbance capacity (ORAC) was determined as described before (Ou *et al.*, 2001). In short, 100 μL of a 500 mM fluorescein stock solution (2,7-dichlorofluorescein) and solutions (10 mM) of test compounds were diluted in phosphate buffer and then pipetted into each 96-well microplate. The reaction was initiated by the addition 25 μL of 250 mM 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), the microplate was shaken for 5s and the fluorescence was monitored kinetically with data taken every 3 min over 90 min. Excitation and emission were performed at 485 nm and 510 nm using Infinite M200 Pro (Tecan, Switzerland). The net area under the curve was used to calculate the antioxidant capacity expressed as Trolox equivalents (TE). This assay was performed by the team of Mgr. Jiří Grúz, Ph.D. in the Laboratory of Growth Regulators.

Bacterial receptor bioassay

All receptors bioassays used transformed *Escherichia coli* KMI001 strains harbouring the plasmids pIN-III-AHK4, pSTV28-AHK3 which express the CRE1/AHK4 and AHK3 receptor. Bacterial CK assays were carried out as previous described procedure (Spíchal *et al.*, 2004) and the protocol is available on the Aesculab website (aesculab.upol.cz).

Competitive binding assay

The assay was performed according to a published protocol (Nisler *et al.*, 2010) and the experimental procedure is also described on the Aesculab website (aesculab.upol.cz). The same bacterial strains were used as described above. The competition reaction of test compounds was performed with ^{3}H *t*Z at 2 nM and incubated for 30 min. at 4°C. This assay was kindly made by Mgr. Zuzana Pěkná in the Department of Chemical Biology and Genetics, Centre of Region Haná for Biotechnological and Agricultural Research.

System architecture of Arabidopsis seedling roots

The root test was performed on *A. thaliana* seedlings of the Columbia wild type (Col-0). The solution of test compounds (0.1 μM in DMSO) were added to AM^{+} medium and poured into 12×12 cm square Petri dishes. The corresponding free bases and BAP were used as positive controls and DMSO as the negative control. *A. thaliana* sterile seeds were applied to the culture plates in a row of approx. ten pieces and four dishes were prepared for each substance. After the stratification, seeds were grown on vertically oriented plates

in a growth chamber under a photoperiod of 16 h light/8 h dark at 18°C. The seedlings were scanned using a horizontal scanner and the root length was measured with ImageJ software.

Cytotoxicity

In vitro toxicity of the prepared compounds was screened on selected non-tumor human cell lines: HaCaT (immortalized human keratinocyte), BJ (skin fibroblasts) and ARPE (immortalized retinal epithelial cells). The effect of test compounds was measured at 6 concentrations (max. conc. 100 μmol/l) 72 hours after the addition of test compound into the medium. DMSO was used as the negative control. The measurement of fluorescence was performed at 544 nm and 590 nm using Resazurin (Tecan) reduction assay. The cytotoxicity of test compounds was kindly performed by Mgr. Jiří Voller, Ph.D. and his team in the Laboratory of Growth Regulators.

SUMMARY OF PUBLISHED RESULTS

Publication I

Vylíčilová, H., Husičková, A., Spíchal, L., Srovnal, J., Doležal, K., Plíhal, O., Plíhalová, L.: C2 substituted aromatic cytokinin sugar conjugates delay the onset of senescence by maintaining the activity of the photosynthetic apparatus, *Phytochemistry*, 2016, 122, 22-33.

Fourteen 2-chloro-6-(halogenbenzylamino)purine-9-ribofuranosides were prepared and properly characterized by NMR (^{13}C, ^{1}H), HPLC purity and elemental analysis. Their biological activity was studied in three standard CK bioassays such as tobacco callus, WLSA and *Amaranthus* bioassay. The prepared synthetic ArCK analogues generally showed similar biological activity as standard (BAP) in tobacco and *Amaranthus* bioassays. In contrast, they significantly delayed the degradation of chlorophyll in detached wheat leaves and in several cases even reached almost double activity of BAP. Compounds bearing a halogen in the *meta* or *para* position of the benzyl ring were determined as the most active.

Moreover, all the prepared derivatives were tested in the bacterial CK receptor assay performed both monocot and dicot members of the CK receptor family, specifically for the ZmHK1 and ZmHK3 receptors of *Zea mays* and for the AHK3 and CRE1/AHK4 receptors of *Arabidopsis*. Although the prepared compounds did not trigger the CK signalling pathway trough *Arabidopsis* receptors, some compounds specifically activated

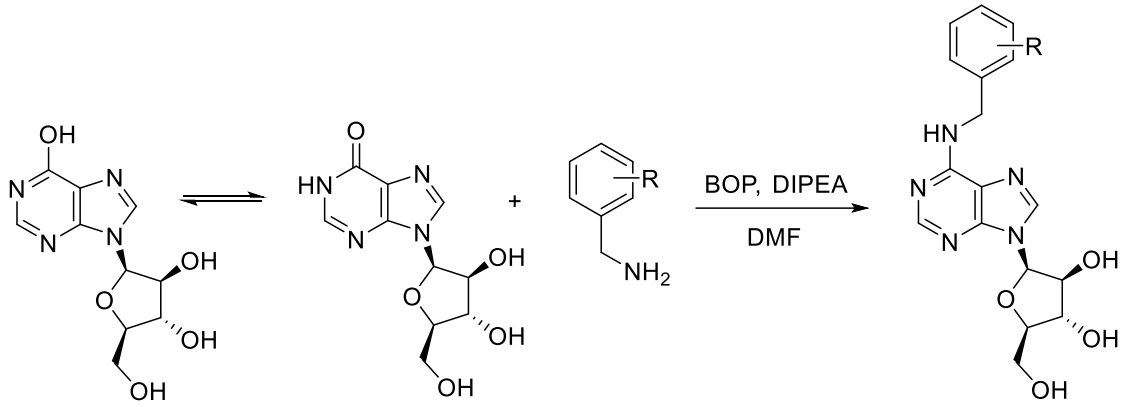
the ZmHK1 receptor and were also found to be able to activate the CK pathway comparable as BAP in *ARR5::GUS* reporter gene assay using transgenic seedlings of *Arabidopsis* plants.

A genome-wide expression profiling (Affymetrix) revealed that treatment of the plant with two tested halogenated ArCK derivatives induced gene expression associated with photosystems II reaction centre (PSII), light harvesting complex (LHCII), and the OEC as well as several stress factors responsible for regulating photosynthesis and chlorophyll degradation, thereby specifically protected from leaves senescence. Both compounds maintained the level of photosynthetic pigments (chlorophyll a/b and carotenoids content) and increased abundance of LHCII in detached *Arabidopsis* leaves under the light condition, thereby protecting photosystem II activity even more effectively than BAP during senescence (Vylíčilová *et al.,* 2016). The research article is listed in Appendix I.

Publication II (patent)

Doležal, K., Plíhalová, L., **Vylíčilová, H**., Zatloukal, M., Plíhal, O., Voller, J., Strnad, M., Bryksová, M., Vostálová, J., Rajnochová Svobodová, A., Ulrichová, J., Spíchal, L.: *6-aryl-9-glycosylpurines and use thereof* (Palacký University Olomouc) granted as CZ 2014875, EP 3229772, SG 11201704019TA and US 10550144 patents, 2014.

A group of aromatic unsubstituted or substituted (by one or more substituents) 6-(benzylamino/furfurylaminopurine)-9-*β*-D-arabinofuranosides was prepared with one-step condensation of hypoxanthine-9-*β*-D-arabinofuranoside with appropriate benzylamine in the presence of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and *N*,*N*-diisopropylethylamine (DIPEA) in DMF according to the reaction scheme (Scheme 2); (Wan *et al.,* 2005). The synthesis was also performed using a microwave reactor. The chemical structures were confirmed using NMR (^{1}H,13C), HPLC/MS, and elemental analysis. The prepared compounds were screened for biological activity and they significantly delayed senescence in plants *in vivo/vitro*. Moreover, the regulation of plant growth and development *in vivo,* and UV photoprotectivity in animal cell *in vitro* are published in the patent (Appendix II.).



Scheme 2. *Synthesis of 6-(benzylamino/furfurylamino)purine-9-β-D-arabinofuranosides from hypoxanthin-9-β-D-arabinofuranoside that is in an equilibrium mixture.*

Publication III

Vylíčilová, H., Bryksová, M., Matušková, V., Doležal, K., Plíhalová, L., Strnad, M.: Naturally occurring and artificial *N*9-cytokinin conjugates: From synthesis to biological activity and back, *Biomolecules*, 2020, 10, 1-29.

So far, various isoprenoid or aromatic CKs and their metabolites have been isolated in plants species. The review is focused on active *N*9-substituted CKs derivatives both sugar and non-sugar conjugates. Their natural occurrence, historical progress, synthesis, and biological activity mainly their potential application in various plant biotechnology, tissue culture and agriculture are described here. The review summarized the knowledge of the CK structural motifs able to increase the biological activity in various CK bioassays (Vylíčilová *et al.*, 2020). The publication is listed in Appendix III.

Publication IV

Plíhalová, L., **Vylíčilová, H.,** Doležal, K., Zahajská, L., Zatloukal, M., Strnad, M.: Synthesis of aromatic cytokinins for plant biotechnology, *New Biotechnology*, 2016, 33(5), 614-624.

The review presented brief information about ArCKs derivatives. The historical development of their synthesis, various substitution possibilities on the purine moiety and their promising application in practice were examined. Current monosubstituted and disubstituted CK derivatives and simplify preparation to design more effective structural motives were summarized here (Plíhalová *et al.*, 2016). The publication is listed in Appendix IV.

UNPUBLISHED RESULTS

Introduction

The synthetic approach of this part of work was to prepare other 2-chloro-6-substituted purine-9-glycosides with potential antisenescence effect. A chemical library of monosaccharides with diversity of stereochemistry substituted at the *N*9 position of the purine can provide a detailed assessment of CK structure-activity relationship. We were inspirited by ArCKs/plant growth regulators as many of them successfully delayed the chlorophyll breakdown, especially various *N*9-glycosides (Bryksová; *et al.*, 2020a; Doležal *et al.*, 2007; Matušková *et al.*, 2020).

The aim was to obtain more effective biologically active CK analogues. A series of 2-chloro-6-substituted purine-9-glycosides derived from BAP, 3FBAP and Kin substituted with chlorine at the C2 position and various glycopyranosides or glycofuranosides at the *N*9 position of the purine have been synthesised (Figure 10).

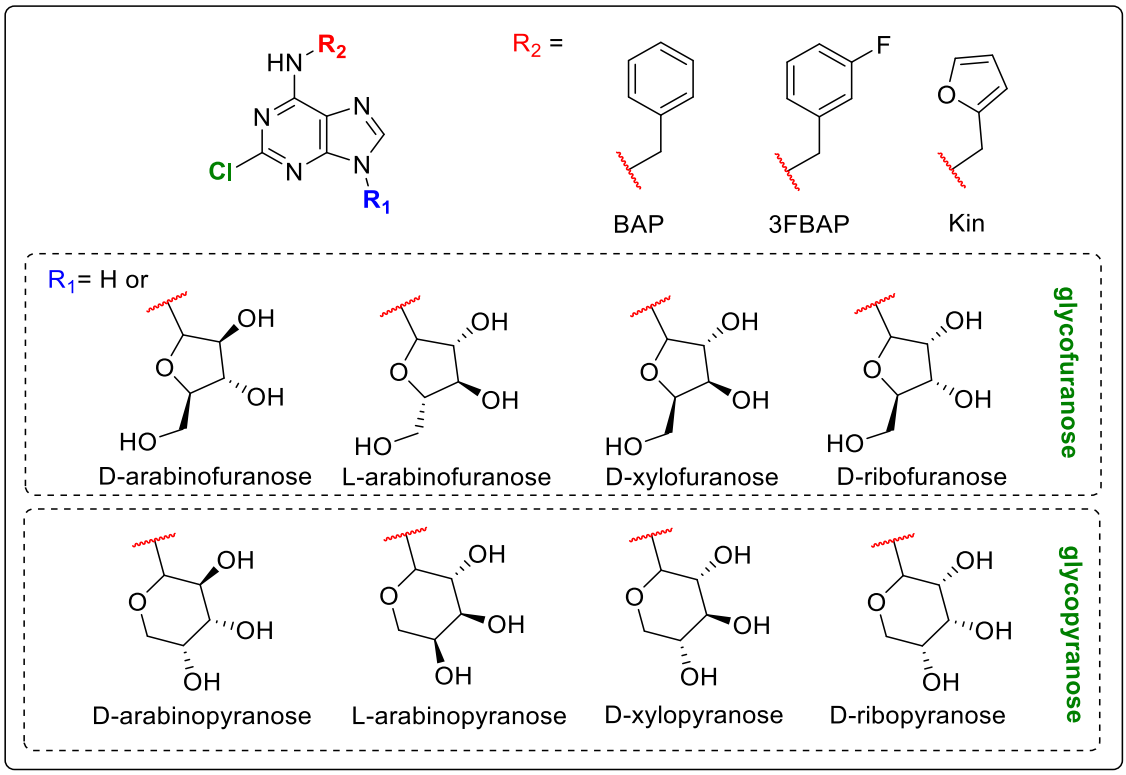


Figure 10. *Chemical structures of 2-chloro-6-substituted purine-9-glycosides with different pyranose and furanose forms, derived from aromatic cytokinins.*

Different pentoses such as D, L-arabinosa, D-xylose and D-ribose were selected as they are widely distributed in plant glycosylated compounds (Lojkova *et al.*, 2020). All the prepared compounds were investigated for their biological activity. Another approach of

the thesis was to find new synthetic methodologies for the preparation of 6-benzylaminopurine-9-*β*-D-arabinofuranosides from other precursors such as adenine-9-*β*-D-arabinofuranoside (vidarabine). Apart from that, we attempted to use a microwave-assisted reactor or continuous flow reactor to find better reaction conditions that could be used in CK synthesis.

9 Result and discussion

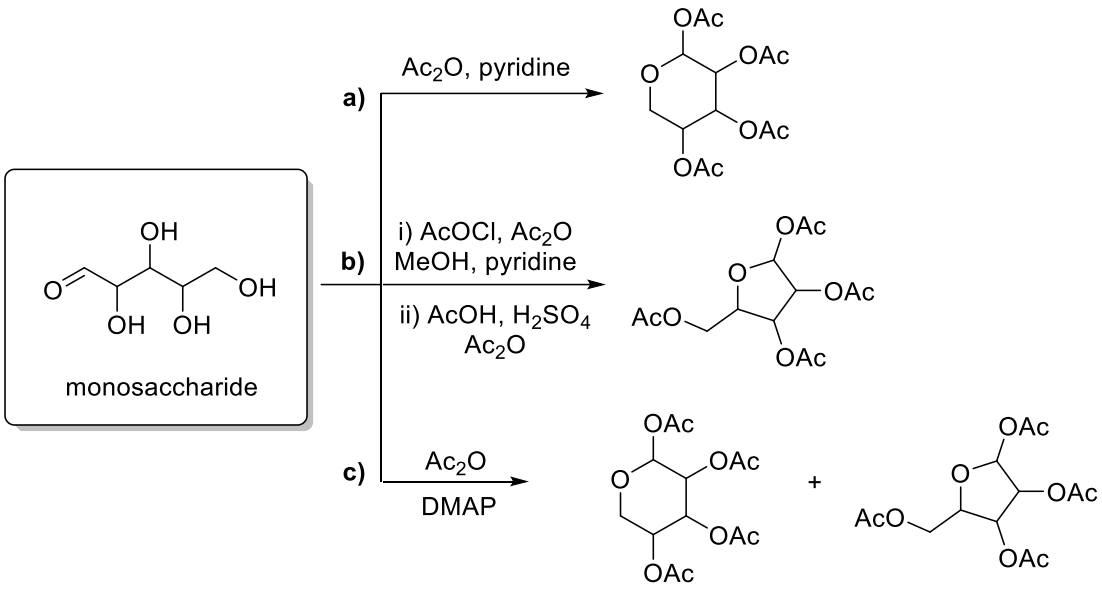
9.1 Synthesis

9.1.1 Aromatic 2-chloro-6-substituted purine-9-glycosides

Synthesis of peracetylated glycosyl donors

The first step of *de novo* synthesis involves cyclization of linear monosaccharides and protection of hydroxyl group trough acetylation. The cyclization of open-chain sugars stabilizes the molecule through hemiacetal and forms a new stereocentre. The implementation of suitable reaction conditions may provide preferably a 5-membered cyclic form of sugar called furanose and those with a 6-membered ring called pyranose. Three synthetic approaches were tried leading to acetylated sugar in the desired ring conformation, which was used in the next reaction step. The experimental protocols are described in Supplementary information I.

First, acetylated pyranose was prepared according to the protocol published in literature (Zhang and Vasella, 2007). Incorporating acetyl groups protects hydroxyl groups of sugar and controls chemoselectively in subsequent reaction step. The monosaccharide was acetylated with acetanhydride in pyridine that serves as a solvent and base. The reaction provided fully protected sugar in the form of syrup in an excellent yield (96% and more). According to the ^{1}H NMR data, the crude product was an inseparable mixture of acetylated pyranose/furanose with the formation of predominantly pyranose (Scheme 3, a) in *α*/*β* anomeric configuration. The crude reaction mixture was used in the next step without further separation/purification.



Scheme 3. *Synthetic procedures for cyclization and protection of monosaccharides.*

Secondly, protected furanose was prepared as described in published protocol with some modifications (Crich *et al.*, 2007). The monosaccharide was treated with acetyl chloride and acetic anhydride in pyridine and cooled at 0°C overnight. The ^{1}H NMR data for this synthetic pathway indicated specific formation of the peracetyl-arabinofuranose (Scheme 3, b) and only approx. 10% of peracetyl-arabinopyranose in the reaction mixture. In the case of D-xylose, protected xylofuranose/xylopyranose in 1/1 mixing ratio in high yield (89%) was prepared which was used in the next reaction step without separation process.

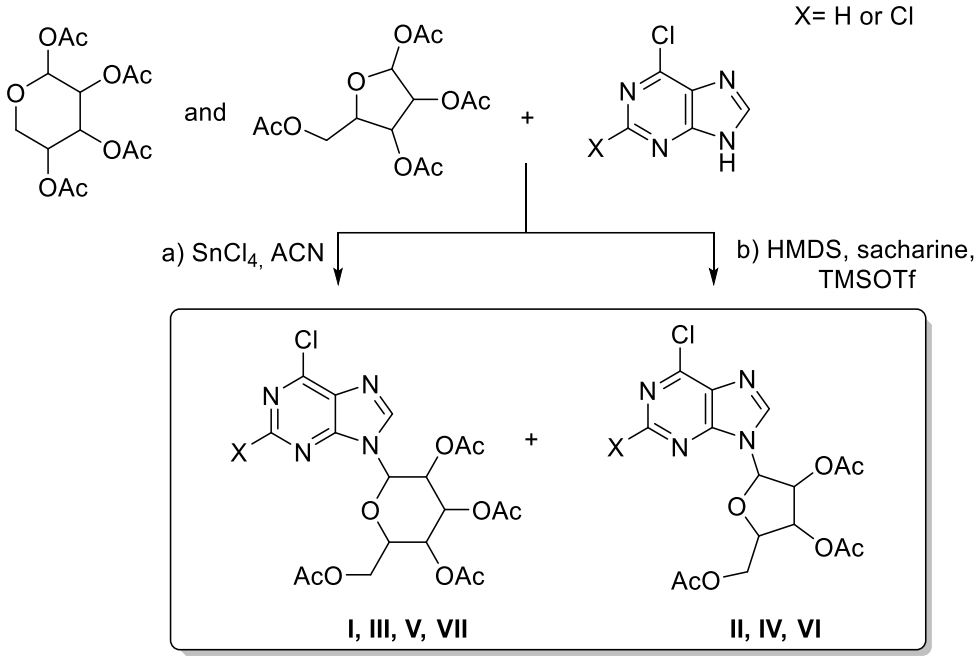
Finally, the modified synthesis for cyclization and protection of monosaccharides was tried (Fuertes *et al.*, 1975). The sugar cyclization and acetylation were performed using acetic anhydride and (4-dimethylamino)pyridine cooled at 0°C for 24 hours. In the case of arabinose, this synthetic strategy provided a larger amount of protected arabinopyranose than arabinofuranose. At the same time, the cyclization reaction of D-ribose provided peracetylated ribofuranose/ribopyranose in the same ratio (Scheme 3, c).

Glycosylation of 2,6-dichloropurine

The next reaction step involved the conjugation of acetylated sugar with 6-chloropurine/2,6-dichloropurine, which generated purine-*O*-acetylated nucleoside. First, peracetylated arabinosides were carried out through Vorbrüggen glycosylation with $SnCl_4$, as previously reported. (Hocek *et al.*, 2006). This synthetic pathway performed a partial

conversion, yielding 47% even with an extension of the reaction time. We decided to find another synthetic pathway due to the low conversion.

As an alternative to this coupling reaction, a modified Vorbrüggen variant of the Hilbert-Johnson reaction, was employed according to the reaction scheme published in literature (Dimopoulou *et al.*, 2015). 2,6-Dichloropurine/6-chloropurine was refluxed in dry acetonitrile (ACN) with hexamethyldisilazane (HMDS) to provide silylated purine. Purine silylated intermediate was then coupled to the acetylated sugar in the presence of TMSOTf (Lewis acid) to synthesize acetylated 2,6-dichloropurine nucleosides (Scheme 4). Under these conditions, a mixture of furanoside/pyranoside **(I**-**VII**) and *α*/*β* anomers was obtained.



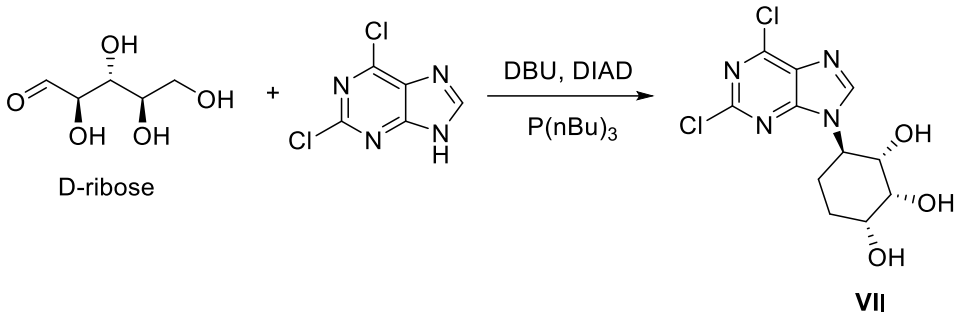
Scheme 4. *N-glycosylation of 2,6-dichloropurine/6-chloropurine with peracetylated glycosides.*

Glycosylation usually provides a combination of products due to the formation of a new chiral stereocentre through a planar oxocarbenium ion, which allows an axial/equatorial coupling reaction to form a mixture of the two anomers. Moreover, the stereochemical outcome is generally dictated by adjacent 2′-hydroxyl group depending on stereochemical assignments of glycosyl (Downey *et al.*, 2015).

The separation techniques, such as liquid column chromatography, allowed the separation of the individual sugar cyclic forms unlike anomeric products. Thin layer chromatography (TLC) together with NMR analysis identified 2,6-dichloropurine-*O*-acetyl-pyranoside with R_f = 0.19 and 2,6-dichloropurine-*O*-acetyl-furanoside with R_f =

0.16 on vanilla-stained TLC using PE/EtOAc (1/1) as a mobile phase. After purification by column chromatography, the white solids were obtained from precipitation from PE and Et_2O. A series 2,6-dichloropurine-*O*-acetyl-furanoside/pyranoside of D, L-arabinose, D-xylose and D-ribopyranose were prepared in the yield ranging 18.5%-75% depending on the previous reaction step.

Moreover, the direct one-pot synthesis from unprotected D-ribose using modified Mitsunobu condition with DBU, DIAD and P(*n*-Bu)$_3$ was performed according to the literature (Downey *et al.*, 2015). This synthetic procedure led to an exclusive form of *β*-configurated purine ribopyranoside in very poor yield (5%); (Scheme 5). The structure of 2,6-dichloropurine-9-*β*-D-ribopyranoside was verified by NMR as indicated in a previously published article (Downey *et al.*, 2015).



Scheme 5. *One-pot synthesis from unprotected monosaccharide and 2,6-dichloropurine to 2,6-dichloropurine-9-β-D-ribopyranoside.*

Synthesis of aromatic 2-chloro-6-substituted purine-9-glycosides

The final reaction step was performed by a simple nucleophilic substitution at the C6 position of 2,6-dichloropurine-*O*-acetylated glycosides with the appropriate benzylamine with an excess of TEA (Tarkowská *et al.*, 2003). Acetylated groups of protected glycosides were removed in NH_3/MeOH. After the deprotection, preparative HPLC-MS chromatography with UV-VIS and LC/MS detector was used to separate individual anomeric products. The gel-like product was lyophilized to a white solid. The only purine D-ribofuranose analogues were prepared from commonly available precursor 2,6-dichloropurine-9-*β*-D-ribofuranoside.

CK 2-chloro-6-substituted purines, specifically 2-chloro-6-benzylaminopurine (2ClBAP, **25**), 2-chloro-6-(3-fluorobenzylamino)purine (2Cl(3FBAP), **26**) and 2-chloro-(6-furfurylamino)purine (2ClKin, **27**) was prepared according to the protocol published previously (Vylíčilová *et al.*, 2016). The final product was purified by crystallization. These 2-chloro-6-substituted purines (Figure 11) were used as standards to compare their

biological activity with newly prepared 2-chloro-6-substituted purine-*N*9-glycosides in different CK biological assays.

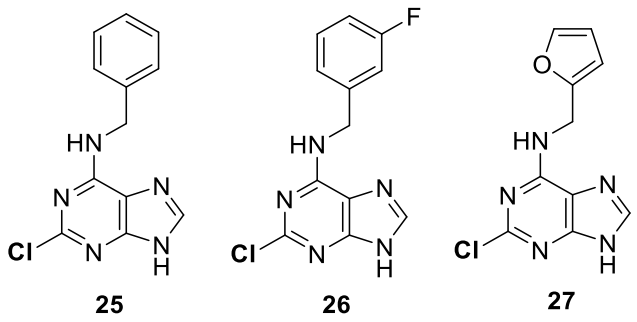


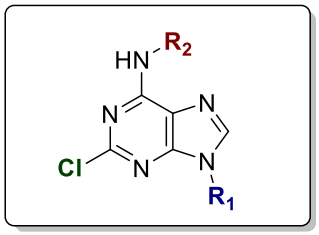
Figure 11. *Chemical structure of 2-chloro-6-substituted purines: 2-chloro-6-benzylaminopurine (2ClBAP, **25**), 2-chloro-6-(3-fluorobenzylamino)purine, (2Cl(3FBAP), **26**) and 2-chloro-(6-furfurylamino)purine (2ClKin, **27**).*

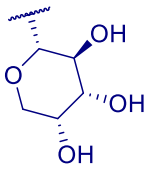
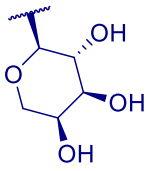
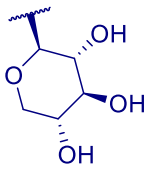
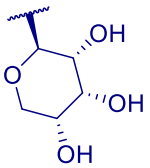
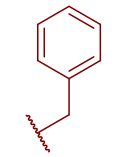
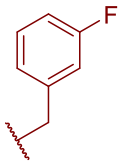
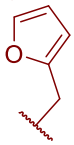
Active CKs are usually present in the form of a free base. Depending on the structure of the molecule, the free base of CK may have limited solubility, which is important for biological activity (Doležal *et al.*, 2007). In addition, the solubility with substitution at the C2 position of the purine by chlorine reduced the aqueous solubility to form a precipitate. On the other hand, an improvement in the solubility was achieved by N9 substitution of the purine, as in our case by substitutions with a glycosyl group. The solubility of the prepared aromatic 2-chloro-6-substituted purine-*N*9-glycosides in aqueous solution (0.1 mM) was fully verified by turbidimetry measures.

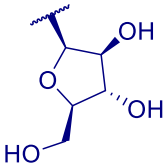
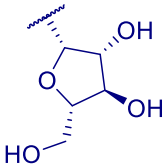
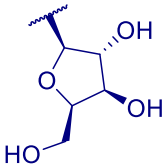
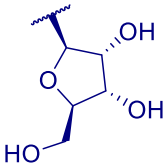
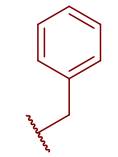
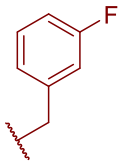
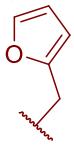
All new 2-chloro-6-substituted purine-*N*9-glycosides (**1**-**24**); (Table 1) were characterized by the available chemical-analytical methods. The complete ^{1}H and ^{13}C NMR spectra are given in Supplementary information II, which includes the yield, HPLC retention time, purity, MS of positively charged molecular ion, and HRMS analysis. The structural analysis of the compounds was performed according 2D NMR experiments (COSY, HMBC, and HMQC). The determination of anomeric configuration (α/β) of sugar moiety was obtained as deduced from ^{1}H HMR vicinal coupling data and compared with the previously reported data (Crich *et al.*, 2007; Konstantinova *et al.*, 2011; Mizutani *et al.*, 1989).

To conclude, α-anomeric configuration was identified in aromatic 2-chloropurine-9-α-D/L-arabinopyranosides (**1**-**3**, **7**-**9**) and the remaining CK nucleosides were prepared as the β-anomeric product. The synthetic procedures are specified in Supplementary information I.

Table 1. *Summary, chemical structures and the numbering of prepared 2-chloro-6-substituted purine-9-α/β-D/L-glycofuranosides/glycopyranosides.*



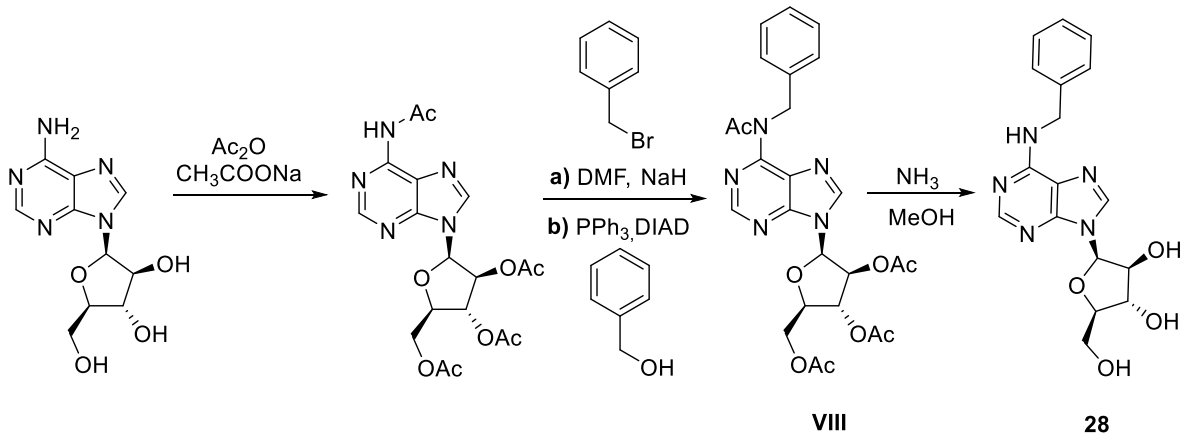
R_2 / R_1	 α-D-arabinopyranose	 α-L-arabinopyranose	 β-D-xylopyranose	 β-D-ribopyranose
	1	7	13	19
	2	8	14	20
	3	9	15	21

R_2 / R_1	 β-D-arabinofuranose	 β-L-arabinofuranose	 β-D-xylofuranose	 β-D-ribofuranose
	4	10	16	22
	5	11	17	23
	6	12	18	24

9.1.2 New synthetic strategies for aromatic arabinoside

In this part of the thesis, we tried to develop alternative synthetic procedures for the preparation of 6-benzylaminopurine-9-*β*-D-arabinofuranoside (BAPA) which is included in the patent mentioned before (Patent No. EP 3229772) in Appendix II. To address this challenge, we used another commercially available precursor, adenine 9-*β*-D-arabinofuranoside (vidarabine), which allows cheaper BAPA preparation.

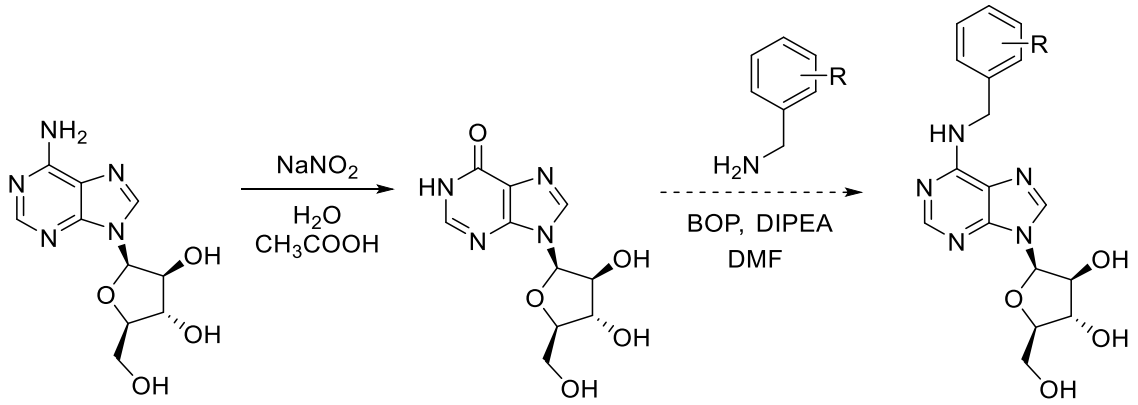
The alternative pathway of BAPA synthesis from vidarabine was prepared according to the published protocol (Ingall *et al.*, 1999). The Mitsunobu reaction of 6-acetamidopurine-2′,3′,5′-tri-*O*-acetyl-9-ribofuranoside with benzyl halides or benzyl alcohols was applied (Scheme 6). The first step involved the acetylation of vidarabine which led to the simultaneous protection hydroxyls of the ribose and activated the exocyclic amino group in a yield of 61%. During Mitsunobu condition, this intermediate was trapped by electrophiles, such as benzyl bromide to produce 6-alkylated purine-tri-*O*-acetyl-9-arabinofuranoside (**VIII**) formed in a good yield (58%); (Scheme 6, a). The subsequent treatment with NH_3/MeOH removed the protecting groups provided BAPA (**28**) in a yield of 24% and the chemical structure was confirmed by NMR analyses.



Scheme 6. *Synthesis of 6-benzylaminpurine-9-β-D-arabinofuranoside from adenine-9-β-D-arabinofuranoside using Mitsunobu reaction.*

Afterwards, we decided to try path B strategy using Mitsunobu condition. The reaction of fully protected vidarabine with benzyl alcohol mediated with triphenylphospine (PPh_3) and DIAD, which upon the deprotection provided BAPA (**28**); (Scheme 6, b). The progress of the Mitsunobu alkylation was monitored by TLC and vanillin staining solution (PE/EtOAc 1/1), which indicated very low reactivity leading to BAPA in a small yield (17%).

Another synthetic method was the deamination of vidarabine from hypoxanthine-9-*β*-D-arabinofuranoside. Afterwards, hypoxanthine-9-*β*-D-arabinofuranoside reacted with benzylamine using BOP and DIPEA in DMF to produced BAPA according to the already known reaction scheme (Scheme 7); (Patent, Doležal *et al.*,, 2014).



Scheme 7. *Deamination of adenine-9-β-D-arabinofuranoside (vidarabine) to hypoxanthine arabinofuranoside, a suitable method for the preparation of substituted 6-benzylaminopurine-9-β-D-arabinofuranosides using BOP and DIPEA. The dashed arrow hypothetically indicates the next reaction step.*

Deamination of vidarabine to hypoxanthine-9-*β*-D arabinofuranoside was prepared with sodium nitrate ($NaNO_2$) and acetic acid (Kaminskii *et al.*, 1987). The reaction temperature was necessary to raise to 60 °C for 24 hours due to low reactivity achieved at room temperature (RT). Finally, hypoxanthine-9-*β*-D-arabinofuranoside was obtained in moderate-good yield (50-80%) as white solid. Furthermore, this efficient method can be used in organic synthesis as it avoids protection/deprotection steps (Scheme 7).

In short, an alternative strategy for BAPA preparation, which usually led through several reaction steps, which are included in Supplementary information I. The chemical structure of BAPA was determined by ^1H NMR analysis listed in Supplementary information II. We performed two different synthetic procedures. First, the synthesis of fully protected vidarabine which was further subjected to Mitsunobu reaction. Second, the conversion of vidarabine to hypoxanthine arabinofuranoside to produce the desired product using couplings agents (BOP DIPEA). It should be noted that the exclusion of protection of the hydroxyl groups of nucleobases greatly facilitates the reaction steps. The methods described above may be useful in the search an alternative method for the BAPA synthesis

9.1.3 Synthetic application of modern methods

Microwave-assisted synthesis

During the last decades, microwave-assisted organic synthesis has been extensively applied in preparation of nucleosides (Bordoni *et al.*, 2019; Elgemeie and Mohamed, 2019). A one-pot microwave-assisted synthesis offers advantages in the possibility of setting different reaction conditions such as temperature, pressure, the power of microwave energy, shortening the reaction time or solvent consumption regarding the green chemistry principles. Herein, we focused particularly on the final step of CK synthesis: nucleophile substitution at the C6 position of 2-chloropurine-9-ribofuranoside with various benzylamines.

The dynamic method was chosen as the best condition for the synthesis. The reaction conditions were set as follows: 90-100 °C, 50 watts (power of microwave), 100 psi (pressure), 7-40 min (hold time). Using this innovative method, the reaction time was significantly reduced from 4 h to 7 min with an excellent yield in the case of aromatic 2-chloropurine ribosides. On the other hand, aromatic 2-aminopurine ribosides were provided in good to excellent yields. Nevertheless, it was necessary to extend the reaction times by approx. 1 h probably due to the presence of an amino group at the C2 position of the purine which reduced the molecule reactivity. Nevertheless, the reaction time was shortened. More information about synthesis is described in Supplementary information I

Not surprisingly, the reaction time depends on the molecular structure and reactivity of starting material. The use of the microwave reactor facilitates the search for ideal reaction conditions.

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Continuous flow synthesis

Flow chemistry is a widely explored technology. It involves the chemical reaction performing into tubes or pipes which can be pressured. Separately, reagents are pumped at known rates, mixed, and flowed into the well-controlled reaction zone equipped with one-pot catalysts/reagent cartridge. The flow chemistry reactor allows a quick exchange of reaction parameters and can be used in a large-scale production over a standard batch (de Souza *et al.*, 2018; Newman and Jensen, 2013).

The continuous flow reactor (X-cube instrument) equipped with quartz sand filled cartridges was performed, reaction conditions such as flow rate, temperature, and pressure were individually changed. We used TLC to monitor the conversion of the starting

material. The reaction mixture was prepared a concertation of 0.035 M, 0.67 M and 0.1 M. It was found that increasing molarity is ineffective for reaction rates, on the other hand, it caused a decrease in the solubility of reagents. A continuous flow reactor has been used to nucleophilic substitution of 6-chloropurine ribosides with amines, a popular synthetic procedure used in the synthesis of CK ribosides (iPR, 3MeOBAPR); (Doležal *et al.*, 2007).

Table 2. *Reaction conditions for the synthesis of 6-(2-isopentenylamino)purine riboside (iPR) using the continuous flow reactor.*

	reaction conditions	*p = 50 bar, t = 90 °C*					
A.	flow rate (mL/min)	0.2	0.5	0.7	1		
	conversion	decrease					
	reaction conditions	*p = 50 bar, flow rate = 0.5 mL/min*					
B.	temperature (°C)	90	100	120	140	160	180
	conversion	increase					
	reaction conditions	*200°C, flow rate 0.2 mL/min*					
C.	pressure (bar)	40		50		70	
	conversion	const.					

Table 3. *Reaction progress for the synthesis of 6-(3-methoxybenzylamino)purine riboside (3MeOBAPR) using the continuous flow reactor.*

reaction conditions	*t = 170°C, flow rate = 0.5 mL/min, p = 50 bar*			
Number of cycles	1	2	3	4
Conversion (HPLC)	51.2 %	64.8 %	73.3%	81%

Based on the changing conditions, the yield increased with the decreasing flow rate (Table 2, A), the increasing number of performed cycles (Table 3) and the rising temperature (Table 2, B). On the other hand, the change in pressure did not affect the yield of the reaction (Table 2, C). Based on HPLC measurements, the best conversion was estimated to be approx. 85% using the conditions as 0.2 flow rate, 50 bar, and 170°C with inert catalyst (quartz sand). No improvement was observed when Cu (Raney copper) catalyst was used for synthesis known as Ullmann reaction published in the literature (Cortes-Salva *et al.*, 2011). In this case a Cu complex was probably formed, or a slow dissolution of Cu catalysts appeared during the reaction.

Although a continuous flow reactor may be practical for automated technology, it is not suitable for the tried synthesis due to poorer solubility of the starting material by precipitation of the product during the reaction, which caused frequent blockage of the dual pump system in the instrument. Despite all efforts, no suitable reaction conditions were

found for the above-mentioned preparation of CK nucleosides with the continuous flow reactor in comparison with a classical batch reaction performed in a round bottom flask.

9.2 Biological activity

9.2.1 Aromatic purine-9-arabinoside

In this part of the work, the main emphasis is put on the development of other synthetic methods leading to 6-benzylaminopurine-9-*β*-D-arabinofuranoside (BAPA). The biological activity of BAPA with similar structures with high antisenescence properties are described in the above-mentioned patent (Patent, Doležal *et al.*, 2014); (Appendix II.). Recently, new BAPA derivatives with different substituents on the benzyl ring were synthetized and physiological processes related to delaying senescence are detailed in Bryksová *et al.* (Bryksová *et al.*, 2020a)

9.2.2 Aromatic 2-chloro-6-substituted-9-glycosides

Senescence bioassay

The structure of the prepared compounds was chosen with the respect to finding a correlation between the biological activity and the stereochemistry of the monosaccharide unit. The senescence assay (WLSA) is based on the ability of the prepared compound to retard chlorophyll degradation in detached wheat leaves during the dark condition determined after 5 days. Solutions of the prepared compounds at the final concentration of 0.1 mM were tested. Aromatic 2-chloropurines (**25**-**27**) have not been tested in WLSA due to their limited aqueous solubility.

The results indicated the ability to delay chlorophyll degradation of mostly aromatic 2-chloro-6-substituted purine-9-glycosides compared to BAP. No apparent correlations were observed between different substituents on C6 atom of the purine or individual *α*/*β* anomers and antisenescence properties. It was shown that, 2-chloropurine-9-D-arabinopyranosides (**1**-**3**) were more active than 2-chloropurine-9-L-arabinopyranosides (**7**-**9**). 2-Chloropurine-9-L-arabinofuranosides (**10**-**12**) exhibited the highest anti-senescence activity compared to their D-counterparts: 2-chloropurine-9-D-arabinofuranosides (**4**-**6**).

Additionally, 2-chloropurine-9-D-xylofuranosides (**16**-**18**) and 2-chloropurine-9-D-ribofuranosides (**22**-**24**) showed better chlorophyll retention in senescence leaves than appropriate xylopyranosides (**13**-**15**) and ribopyranosides (**19**-**21**). The most effective compounds appeared to be 2-chloropurine-9-L-arabinofuranosides (**10**-**12**), which retain

approximately 90% of the amount of residual chlorophyll and 2-chloropurine-9-D-arabinopyranosides (**1**, **2**) followed by 2-chloropurine-9-D-xylofuranosides (**16**, **17**) and 2-chloropurine-9-D-ribofuranosides (**22**-**24**). In general, 2-chloropurine-9-furanosides were more active than 2-chloropurine-9-pyranosides except for 2-chloropurine-9-D-arabinopyranosides (**1**,**2**,**8**). The results are shown in Figure 12.

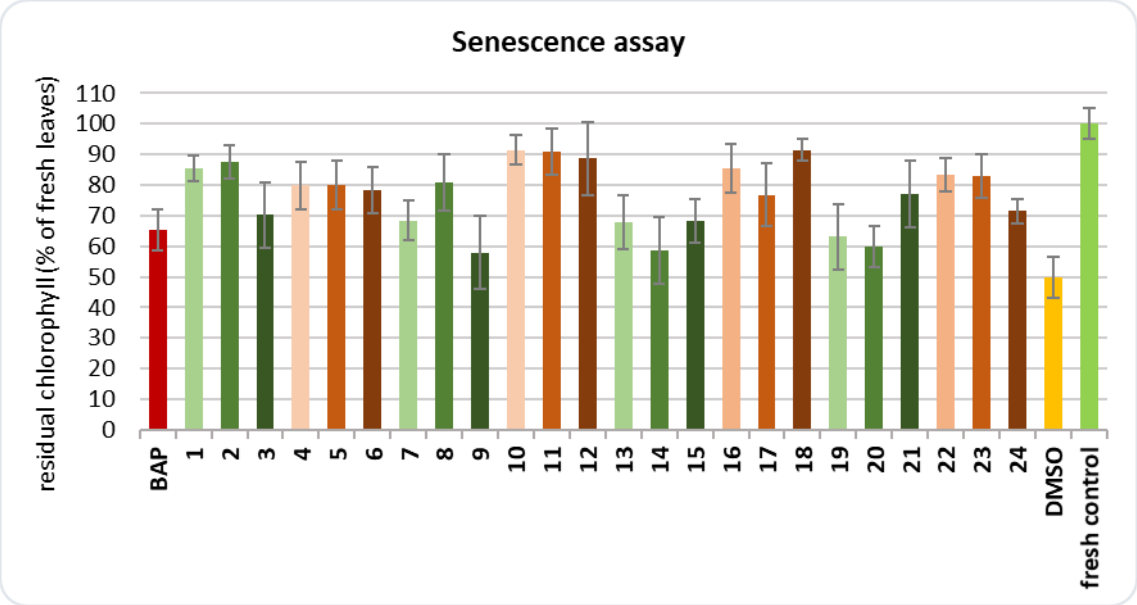

Figure 12. *Anti-senescence activity of aromatic 2-chloropurine-9-glycosides (**1**-**21**) and appropriate 2-chloropurines (**22**-**24**). The chlorophyll content at the beginning of the experiment was taken a reference and set at 100% (fresh control). The values represent at least two independent assays each performed in six replicates. DMSO was used as a solvent control and BAP as a positive control. Shades of green characterize pyranosides and shades of orange represent furanosides.*

These findings correlated with the previous study which reported that aromatic 2-chloropurine ribosides (Vylíčilová *et al.*, 2016), arabinosides (Bryksová *et al.*, 2020a) and 2′-deoxyribosides (Matušková *et al.*, 2020) which delayed senescence in WLSA. The prepared aromatic 2-chloropurine-9-glycosides could be involved in an affiliated metabolic/signalling pathway. Thus, their anti-senescence effect can act through a different mechanism than classical CKs as is mentioned in the literature (Bryksová; *et al.*, 2020a).

Considered together, these results confirm that ArCKs bearing a glycosyl at the *N*9 position of the purine activates mechanisms in the plant that prevent chlorophyll degradation and significantly delay leaves senescence in the dark condition but their molecular mechanism of action during senescence requires a further study.

Tobacco callus bioassay

The tobacco callus assay is based on the ability of hormone treatment to stimulate cell division and proliferation in CK-dependent tobacco callus in the dark. The selected compounds derived from BAP, 3FBAP were tested in two different concentrations (0.1 mM. 0.01 mM) to promote callus growth. Solid media supplemented of all tested CK analogues were less effective to induce callus than BAP at 0.01 mM. On the other hand, it was observed, that at higher concentration of 0.1 mM, they increased the production of callus in contrast to callus tissue supplied with BAP (0.1 mM) where the weight of callus dropped sharply. The growth inhibition by BAP is due to its high concentration applied in the solid medium which has a cytotoxic effect on callus cell division (Doležal *et al.*, 2007).

At the concertation of 0.01 mM, the most effective 2-chloro-6-(benzylamino)purine-9-*β*-L-arabinofuranoside (**10**) following 2-chloro-6-(3-fluorobenzylamino)purine-9-*α*-D-arabinopyranoside (**2**) were identified, albeit less potent than BAP. An advantage of test compounds was the ability to maintain a partial cell proliferation even at higher concertation (0.1 mM). The results of aromatic 2-chloropurine-9-glycosides from the tobacco callus assay are compiled in Figure 13. These findings are consistent with those previously reported (Matušková *et al.*, 2020a; Vylíčilová *et al.*, 2016).

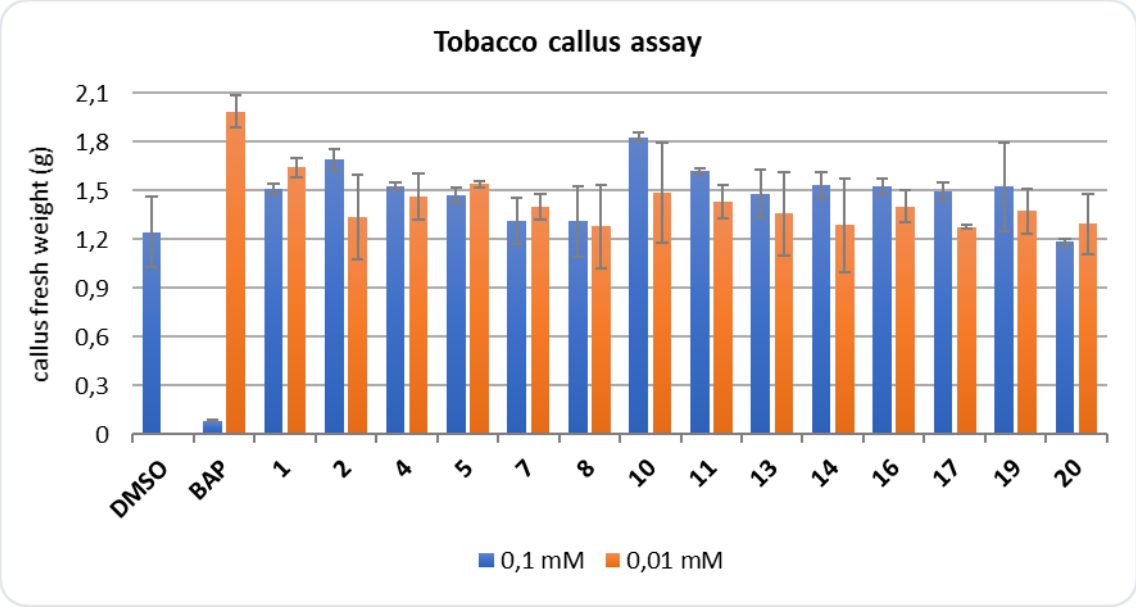

Figure 13. *Biological activity of 2-chloropurine-9-glycosides derived from BAP and 3FBAP in tobacco callus assay. The values represent at least two independent assays each performed in six replicates. DMSO was used as a solvent control.*

Chlorophyll retention assay

We used the *ahk3ahk4* mutant plants expressing only the AHK2 receptor and measured chlorophyll retention after the application of selected compounds compared with the *Arabidopsis* wild type (Col-0) in the modified dark-induced senescence assay (Holub *et al.*, 1998; Riefler *et al.*, 2006). Two selected CK analogues, 2-chloro-6-(3-fluorobenzylamino)purine-9-*β*-D-arabinofuranoside (**5**) and similar 2-chloro-6-(3-fluorobenzylamino)purine-9-*β*-D-ribofuranoside (**23**) with an equivalent antisenescence activity in WLSA were nominated for this assay. Moreover, tested compounds did not initiate the CK signalling cascade trough AHK3 and CRE1/AHK4 receptors in the bacterial receptor assay as shown below. The purpose of this experiment was to elucidate the antisenescence effect of selected compounds *in vivo* studies trough the AHK2 receptor which is difficult to express in *E. coli* system due to protein toxicity for bacteria (Lomin *et al.*, 2015).

A comparison of the amount of residual chlorophyll from detached leaves of the *ahk3-7ahk4* mutant and wild type in response to hormone treatment showed that the AHK2 receptor seem to be ineffective in the maintaining chlorophyll content. In addition, test compounds (BAP, **23**) in a dicotyledonous system of *Arabidopsis* wild plant effectively retarded leaf senescence similarly as reported for monocots used in WLSA (wheat). The individual experiments of this chlorophyll retention assay were performed in two different arrangement (Figure 14, 15).

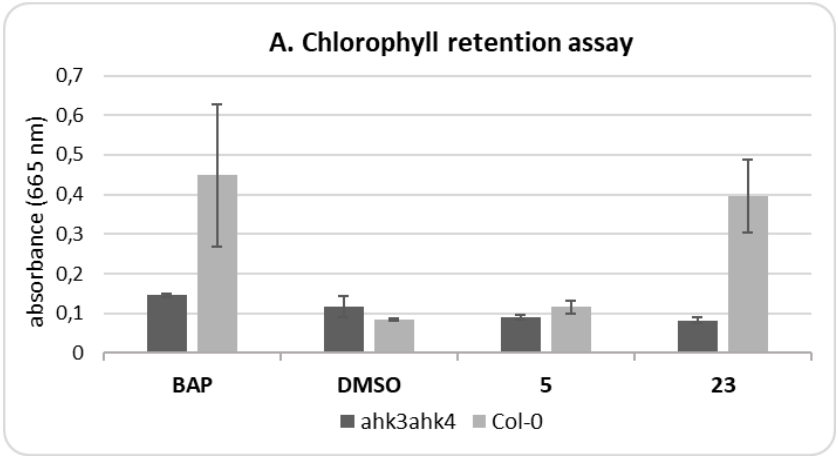

Figure 14. *Quantitative evaluation of chlorophyll content in the dark induced senescence in detached ahk3-7ahk4 leaves treated with 2-chloro-6-(3-fluorobenzylamino)purine-9-β-D-arabinofuranoside (5) and 2-chloro-6-(3-fluorobenzylamino)purine-9-β-D-ribofuranoside (23) The values represent at least three replicates. DMSO was used as the solvent control. The detached Arabidopsis leaves were floated on distilled water supplemented with 0.1mM test compound in Petri dishes for 10 days in the dark. The experiment was performed according to Riefler et al., 2006.*

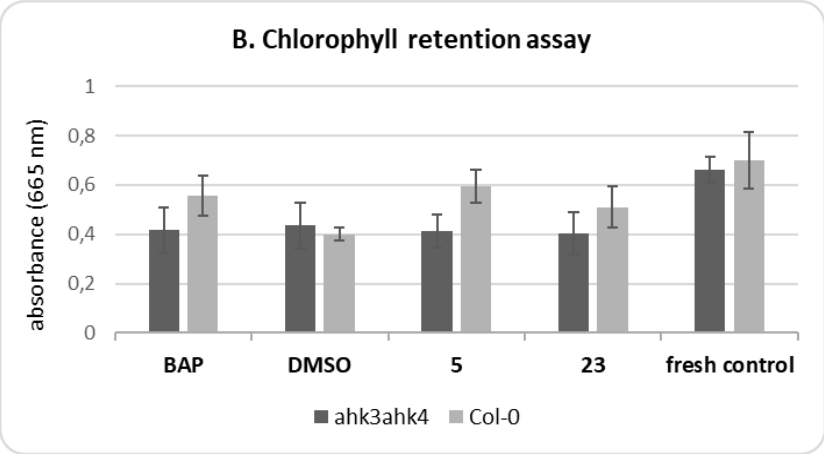

Figure 15. *Quantitative evaluation of chlorophyll content in the dark induced senescence in detached ahk3-7ahk4 leaves treated with 2-chloro-6-(3-fluorobenzylamino)purine-9-β-D-arabinofuranoside (5) and 2-chloro-6-(3-fluorobenzylamino)purine-9-β-D-ribofuranoside (23). The values represent at least three replicates. DMSO was used as the solvent control. The detached Arabidopsis leaves were placed with basal part into distilled water supplemented with 0.1mM test compound and kept in the dark for 5 days.*

ARR5::GUS reporter gene assay

The group of aromatic 2-chloropurine-9-glycosides and 2-chloropurines (**25**-**27**) were tested in the *Arabidopsis ARR5::GUS* assay for their ability to induce the expression of the promoter of the primary response gene (ARR5) that is upregulated by CK (Romanov *et al.*, 2002). This assay participates in the responses of several putative signalling pathways and it should not discriminate responses to individual CK receptors (Spíchal *et al.*, 2004). Quantitative fluorometric evaluation of *β*-glucuronidase (GUS) activity in extract of 3-day old *ARR5::GUS* seedlings was measured after incubation with aromatic 2-chloropurine-9-glycosides with a final concentration at 5 μM and 10 μM together with corresponding 2-chloropurines (1 μM).

Generally, the GUS activity was directly proportional to the concentration at 5 μM and 10 μM treatment. All prepared aromatic 2-chloropurines (**25**-**27**) induced completely *ARR5::GUS* response as applied standard BAP (1μM) and were considered to be the most effective together with 2-chloropurine-9-ribofuranosides (**22**, **24**). This data is comparable with the previously published paper (Vylíčilová *et al.*, 2016). The remaining tested 2-chloropurine-9-furanosides (**5**, **11**, **16**, **17**) and 2-chloropurine-9-pyranosides (**2**, **8**, **14**, **19**, **20**) fluctuated in a range of 40% BAP activity and they hardly promoted gene expression of *ARR5::GUS* (Figure 16).

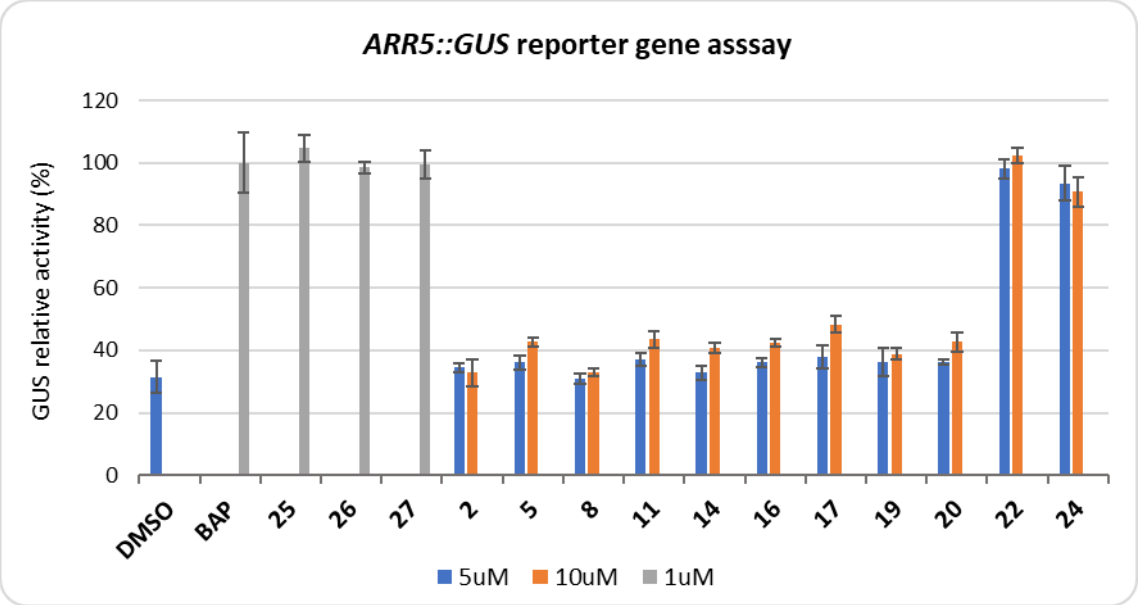

Figure 16. *Effect of test compounds on GUS activity in transgenic ARR5::GUS Arabidopsis plant seedlings in different concentration ranges. The incubation with 1 μM BAP was taken a reference and set at 100%. DMSO (0.1%) was used as a control solvent. The values represent at least two independent assays performed in triplicates.*

Indeed, only aromatic 2-chloropurine-9-ribofuranosides (**22**, **24**) performed different mechanisms of the CK signalling action demonstrated via bacterial receptors assay and *ARR5::GUS* assay. Other tested sugar motifs of the prepared 2-chloropurine-9-glycosides were ineffective. Of the chlorinated glycosides as CK analogues, only ribofuranosides are structurally close to naturally occurring CKs in plant tissue, which may lead to different biological activity between 2-chloropurine-9-ribofuranosides (**22**, **24**) with other prepared 2-chloropurine-*N*9-glycosides. Hence, it is possible that high biological response of 2-chloropurine-9-ribofuranosides (**22**, **24**) in *ARR5::GUS* test is enhanced by specific enzymatic activation commonly used in metabolism to regulate endogenous CK levels.

Bacterial receptor bioassay

The group of aromatic 2-chloro-6-substituted purine-9-glycosides derived from FBAP was examined for the activation CRE1/AHK4 and AHK3 receptors in *E. coli* bacterial assay. In general, the majority of tested sugar conjugates did not activate the CRE1/AHK4 and the AHK3 receptor mediated the CK signalling pathway even at 50μM compared to the appropriate 2-chloro-6-(3-fluorobenzylamino)purine (**26**) and the activity required for *t*Z (Figure 17, 18). However, it was observed that 2-chloro-6-(3-fluorobenzylamino)purine-9-

β-D-ribofuranoside (**23**) at the highest concentration (up to 10 µM) induced at least a weak response to the AHK3 receptor than other aromatic 2-chloropurine-9-glycosides (Figure 18). These findings are consistent with the results of a previous report on the bacterial CK receptor assay (Spíchal *et al.*, 2004; Vylíčilová *et al.*, 2016).

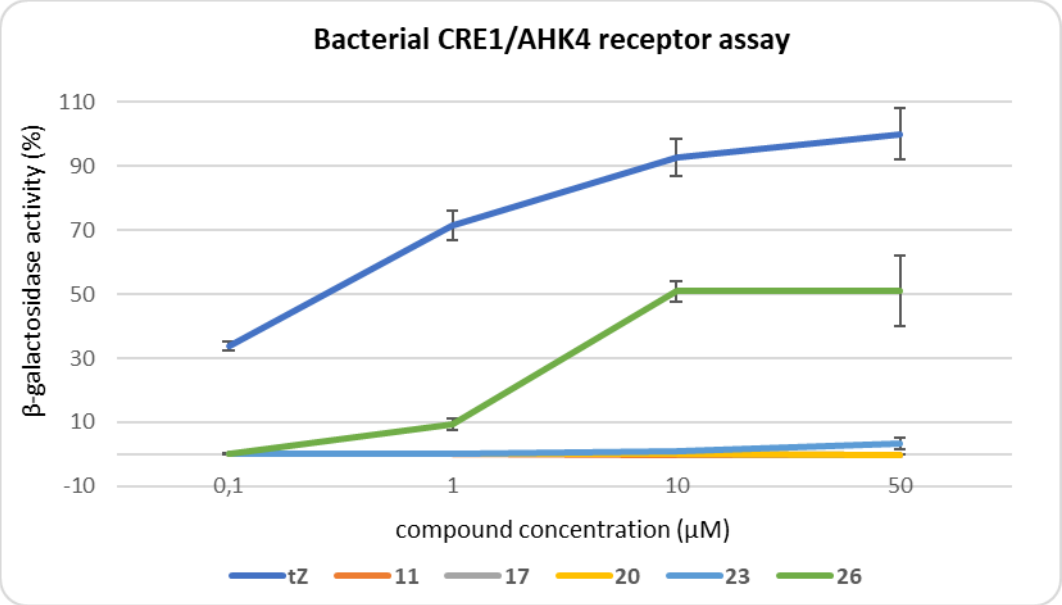

Figure 17. *Relative activities of test compounds (**11**, **17**, **20**, **23**, **26** and tZ) to activate the CK signalling pathway through the CRE1/AHK4 receptors in E. coli bacterial test. Standard tZ was used as a positive control and the activity at 50 µM tZ was taken as a reference and set to 100%.*

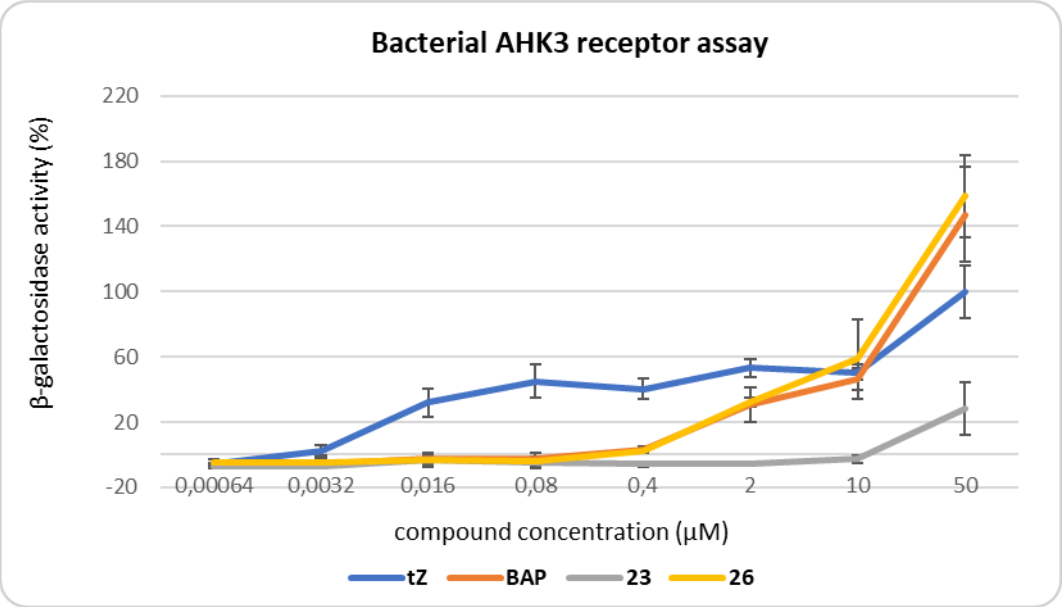

Figure 18. *Relative activities of test compounds (**23**, **26**, BAP and tZ) to activate the CK signalling pathway through the AHK3 receptors in E. coli bacterial test. Standard tZ was used as a positive control and the activity at 50 µM tZ was taken as a reference and set to 100%.*

Competitive binding assay

The competitive binding assay provides an important information about the ability of test compounds to compete with radiolabelled ^{3}H *t*Z for the receptor binding side in a live cell-based assay using transgenic bacteria expressing *Arabidopsis* CK receptors. The advantage of this assay is the shorter incubation time, which can suppress the metabolic conversion of CK (Romanov *et al.*, 2005).

Generally, a series of 2-chloro-6-(3-fluorobenzylamino)purine-9-glycosides (**2**,**5**,**8**,**11**,**14**,**17**,**20**) showed no competitive activity in this assay. The 2-chloropurine-9-ribofuranosides (**22**-**24**) performed little binding activity in competition with radiolabelled *t*Z for AHK3 receptor binding sites (Figure 19). Nerveless, the mother compound: 2-chloro-6-(3-fluorobenzylamino)purine (**26**) showed partial effectiveness determined as 40% residual radioactivity (Figure 19) that contrasts with the high activity in the bacterial AHK3 receptor assay (Figure 18 above).

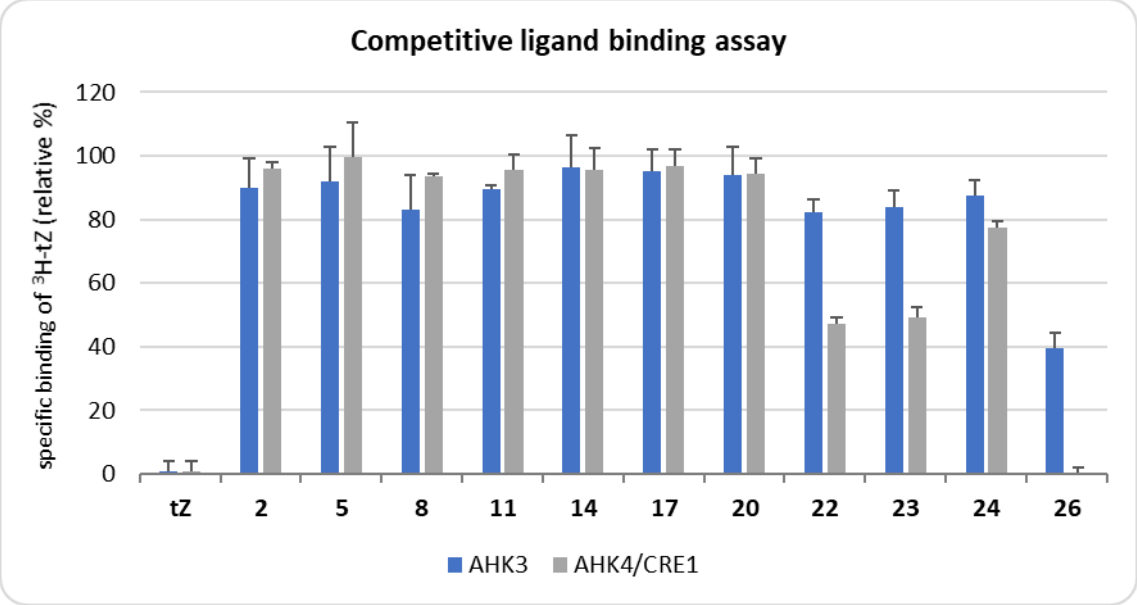

Figure 19. *Effect of aromatic 2-chloropurine-9-glycosides and 2-chloropurine (26) on specific binding of ^{3}H tZ to the hormone-receptor E. coli system expressing AHK3 and CRE1/AHK4. The selected compounds were tested at the 20 µM and positive control tZ at 10µM concertation. The values obtained with ^{3}H tZ (3nM) were set to 100% activation.*

Among chlorinated ribofuranosides, 2-chloro-6-(3-fluorobenzylamino)purine-9-ribofuranoside (**22**) and 2-chloro-6-(benzylamino)purine-9-ribofuranoside (**23**) were found to bind preferentially to the AHK4/CRE1 receptor where they markedly reduced the binding activity of radiolabelled *t*Z. In general, other selected purine glycosides were inactive in the tested CK receptors as their ability to block *t*Z binding sites into the

CRE1/AHK4 receptor was very poor. In addition, compounds **22**, **23** derived from aromatic BAP and 3FBAP were not able to induce the CK signalling cascade trough the bacterial CRE1/AHK4 receptor assay mentioned earlier. On the other hand, 2-chloro-6-(3-fluorobenzylamino)purine (**26**) strongly bound the CRE1/AHK4 receptor in the competitive binding assay (Figure 19) but showed only half activity of *t*Z in the CRE1/AHK4 bacterial receptor assay (Figure 17 above).

To conclude, a comparison of ligand preferences for studied *Arabidopsis* receptors showed differences in ligand preference of 2-chloropurine-9-ribofuranosides (**22**, **23**) for the CRE1/AHK4 rather than for the AHK3 receptor. Another modification on sugar ring showed low binding properties for both AHK3 and CRE1/AHK4 receptor. Tested 2-chloro-6-(3-fluorobenzylamino)purine-9-glycosides were less effective than the parent compound 2-chloro-6-(3-fluorobenzylamino)purine (**26**) which appeared to be a favourable competitor of the CRE1/AHK4 receptor. However, it has previously been reported that sensitivity of *Arabidopsis* receptors towards different ligand is not identical (Savelieva *et al.*, 2018; Spíchal *et al.*, 2004).

Taking everything into consideration, prepared aromatic 2-chloropurine-9-glycosides (**1**-**21**) are not well recognized by studied CK receptors in both bacterial assay systems and were not able to activate transcription in *ARR5::GUS* assay unlike appropriate 2-chloropurines. In contrast, 2-chloropurine-9-ribofuranosides (**22**-**24**) displayed a reduced activity for CK receptors and strong expression of *ARR5::GUS*. These results may suggest the possibility of e.g. ENTs involvement, which could transport glycosylated CK analogues into the cytosol. 2-Chloropurine-9-ribofuranosides (**22**-**24**) are structurally close to naturally occurring ribosides with a transport function and they were only partially perceived trough CRE1/AHK4 and AHK3 receptor in both receptor assays (competitive ligand binding assay and bacterial receptor assay).

In addition, the difference in CK receptors activities between the CRE1/AHK4 and AHK3 receptor may be due to the different ligand specificity of the test compounds for each receptor and may also be affected by other transporters, such as ENT. Recently, Lomin *et al*. proposed an alternative signalling intracellular pathway mediated by ENTs, where the CK riboside achieves a receptor activation through the PM into the cytosol (Lomin *et al.*, 2018).

Root assay

It is generally known that ArCKs negatively affect root growth due to the reduction of primary root length and lateral roots density (Laplaze *et al.*, 2007). CKs inhibit lateral root development which is mediated through the blocking of the pericycle founder cells enabled by mainly the CRE1/AHK4 receptor while AHK2 and AHK3 receptors are redundant in the root (Li *et al.*, 2006). It was observed that exogenous treatment of CK controls the expression of genes encoding *PIN-FORMED* auxin transporters in lateral root founder cells and prevents the formation of auxin gradient which is important for lateral root development (Laplaze *et al.*, 2007). CK ribofuranosides, mostly in the form of *t*Z, transport long distance CK signal from the root to the shoot where they release biologically active CKs, but the regulation of this process can be distinct for ArCKs. Moreover, an analysis of CK endogenous content showed that CK ribosides are more abundant in potato roots than in shoots (Raspor *et al.*, 2020).

In this experiment the *Arabidopsis* seedlings (Col-0) were grown on agar medium supplied with selected sugar conjugates derived from 3FBAP at a final concentration of 0.1 μM. 2-Chloro-6-(3-fluorobenzylamino)purine-9-glycosides demonstrated positive effect on root growth, in contrast to BAP and 2-chloro-6-(3-fluorobenzylamino)purine (**26**), which significantly inhibited root system (Figure 20).

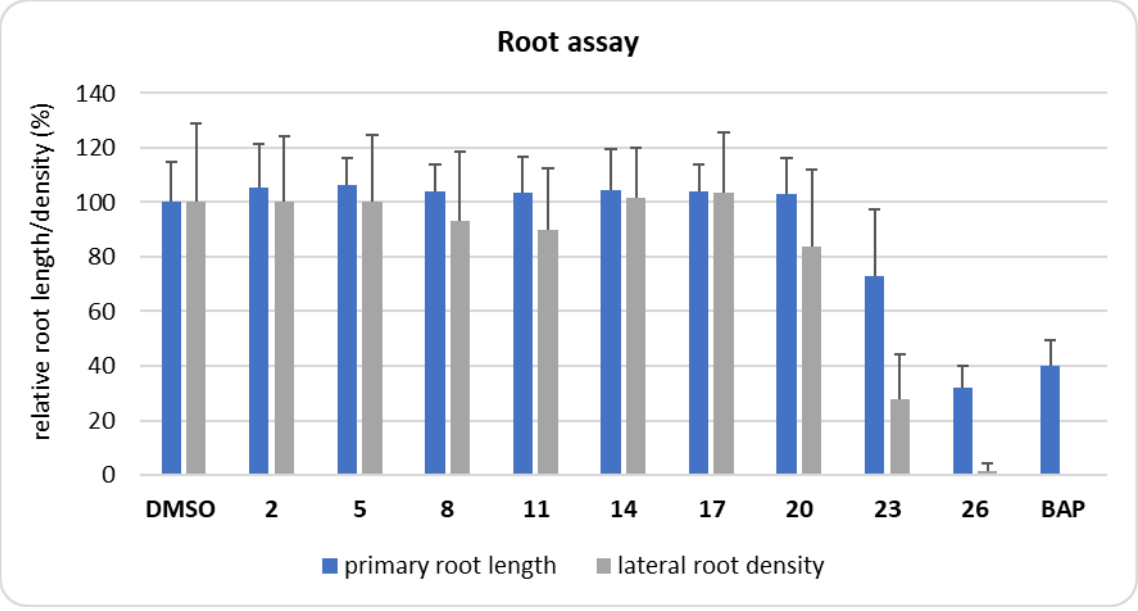

Figure 20. *Effect of test compounds on primary root length and lateral root density in Arabidopsis seedlings grown on media supplemented with 0.1 μM treatment. The root length and the number of emerged lateral roots was determined in 14-days-old seedlings. Each value is the average of the measurements of at least 24 plants grown on 4 independent Petri dishes. DMSO was used as a solvent control.*

The results showed that sugar conjugates promoted effective primary root elongation and lateral root branching as untreated plants (DMSO) except for 2-chloro-6-(3-fluorobenzylamino)purine-9-ribofuranoside (**23**). A supplement of compound **23** caused morphological root disbalance in the form of a partial inhibitory effect on the main root length, uneven distribution of hairy roots and reduced lateral roots (Figure 20).

2-Chloro-6-(3-fluorobenzylamino)purine-9-ribofuranoside (**23**) showed a different effect compared to other 2-chloro-6-(3-fluorobenzylamino)purine-9-glycosides which may be related to its interconversion by enzymes of general adenine metabolism as only **23** occurred as natural CK *N*9-riboside in the plant (Podlešáková *et al.*, 2012). In addition, sugar substitution of the purine at the *N*9 position clearly demonstrated benefits for root growth, consistent with the previous study of CK analogues with *N*9-THP or *N*9-(4-chlorobutyl) substituents. The authors hypothesized that this positive effect on the root elongation, which is important during acclimatization, was due to the *N*9 substituent which prevents the formation of biologically inactive the *N*9-glucosides or ethylene biosynthesis. Moreover, the *N*9 substituent can be slowly released to form active CK without an inhibitory effect and thus serve as a CK reservoir (Plíhal *et al.*, 2013; Podlešáková *et al.*, 2012).

Cytotoxicity

The cytotoxic effect of ArCK ribosides has been published against various human, especially cancer cell lines, useful as potential anticancer drugs (Doležal *et al.*, 2007, Voller *et al.*, 2010). Prepared 2-chloro-6-substituted purine-9-glycosides showed no or limited toxicity when tested on non-cancer skin fibroblasts BJ and immortalized keratinocytes HaCaT after 72-hour incubation in resazurin reduction assay. At 33.3 μM, the reduction of viability did not exceed 10% exception for treatment of HaCaT with **26** and **27** (data is not shown).

Derivatives substituted at the C2 atom with chlorine were probably not toxic due to the decreasing activity of adenosine kinase for intracellular phosphorylation marked as ATP depletion compared to unsubstituted purine CK ribosides which showed anti-cancer activity (Voller *et al.*, 2010).

Antioxidant capacity

The total antioxidant capacity expressed as Trolox equivalents (TE) was measured using the Oxygen Radical Absorbing Capacity (ORAC) assay that is based on the ability of the antioxidant measures the quenching of fluorescein (Brizzolari *et al.*, 2016). It was shown that antioxidant activity significantly depends on the chemical structure of the group substituted at the C6 position. Most of the prepared 2-chloro-6-substitued purine-9-glycosides (10 mM) were almost inactive, except for the Kin series.

All Kin derivatives (**3**, **6**, **9**, **12**, **15**, **18**, **21**, and **24**) exhibited higher ORAC values than BAP and FBAP derivatives, the results are presented in Table 4. These findings are in general in agreement with the trapping capability of peroxyl radicals in the previously published study (Brizzolari *et al.*, 2016) and structural requirement using the hydrogen from the *α*-carbon of the amine bond of Kin to neutralize ROS (Acidri *et al.*, 2020).

Table 4. *Oxygen Radical Absorbing Capacity expressed as Trolox equivalents (TE) on equimolar basis of prepared 2-chloro-6-substituted purine-9-glycosides (**1-24**) and 2-chloro-6-substituted purines (**25-27**).*

comp.	*N*9-sugar substituent	C6-substituent	TE (mean ± SD)
1		BAP	0.004 ± 0.000
2	*β*-D-arabinofuranoside	3FBAP	0.007 ± 0.001
3		Kin	0.723 ± 0.035
4		BAP	0.006 ± 0.001
5	*β*-L-arabinofuranoside	3FBAP	0.000
6		Kin	1.031 ± 0.048
7		BAP	0.005 ± 0.000
8	*β*-D-xylofuranoside	3FBAP	0.004 ± 0.000
9		Kin	0.639 ± 0.086
10		BAP	0.012 ± 0.001
11	*β*-D-ribofuranoside	3FBAP	0.011 ± 0.000
12		Kin	1.117 ± 0.085
13		BAP	0.006 ± 0.001
14	*α*-D-arabinopyranoside	3FBAP	0.005 ± 0.000
15		Kin	0.828 ± 0.081
16		BAP	0.004 ± 0.000
17	*α*-L-arabinopyranoside	3FBAP	0.003 ± 0.000
18		Kin	0.970 ± 0.078
19		BAP	0.002 ± 0.000
20	*β*-D-xylopyranoside	3FBAP	0.002 ± 0.001
21		Kin	1.099 ± 0.109
22		BAP	0.005 ± 0.001
23	*β*-D-ribopyranoside	3FBAP	0.005 ± 0.000
24		Kin	1.072 ± 0.047
25		BAP	0.003 ± 0.000
26	purine	3FBAP	0.003 ± 0.000
27		Kin	0.32 ± 0.007

10 Conclusion

A series of new aromatic CKs analogues with different *N*9-glycosyl substituents were prepared by several synthetic procedures and their biological activity in various assays was determined. The implementation of modern synthetic approaches such as the continuous flow reactor and the microwave reactor were used for the optimization of reaction conditions. An alternative strategy for BAPA preparation was described.

Moreover, a new group of 2-chloro-6-substituted purine-9-glycosides derived from BAP, 3FBAP and Kin with D-arabinose, L-arabinose, D-xylose and D-ribose in the form of glycopyranose and glycofuranose substituted at *N*9 atom and with chlorine on the C2 atom of the purine was prepared by *de novo* synthesis. The individual reaction steps were optimized, and various synthetic procedures were also applied. The structures of the new chlorinated CK *N*9-glycosyl analogues were characterized by available physico-chemical methods (HPLC/MS, NMR, TLC and HRMS). It was verified that the *N*9 glycosylation of the 2-chloropurine significantly increase aqueous solubility.

The prepared aromatic 2-chloropurine-9-glycosides were tested in various bioassays to screen their CK properties and activation of the CK signalling cascade. The majority of 2-chloro-6-substituted purine-*N*9-glycosides effectively delayed senescence on decapitated wheat leaves in comparison with BAP. On the other hand, they were ineffective in the promotion of callus growth in tobacco callus assay. Test compounds were not recognized by the AHK3 and CRE1/AHK4 receptors in both receptors assays and did not induce the gene expression in *ARR5::GUS* assay with the exception of 2-chloropurine-9-ribofuranosides, that showed a different mode of action. The prepared 2-chloro-6-substitued purine-*N*9-glycosides perhaps activate the alternative signalling pathway that could serve as an explanation of their powerful antisenescence activity.

Test compounds showed a positive effect on primary root length and density of lateral roots in *A. thaliana*. The cytotoxicity of the prepared derivatives was not confirmed, and only 2-chloropurine-9-glycosides with Kin substituent at the C6 position of the purine showed mild antioxidant effects against ROS. All these findings showed an interesting relationship between the structure and biological activity of the prepared aromatic 2,6-disubstituted purine-9-glycofuranosides/glycopyranosides and their action in the CK signalling pathway but further molecular studies are needed.

11 Abbreviations

3FBAP	6-(3-fluorobenzylamino)purine
ABC	ATP-binding cassette transporter
AHK	*Arabidopsis* histidine kinase
AHP	*Arabidopsis* phosphotransfer protein
ArCK	aromatic cytokinin
AtCKX	*A. thaliana* cytokinin dehydrogenase
ARR	*Arabidopsis* response regulator
BAP	6-benzylamino-9*H*-purine, 6-benzylaminopurine
BAP9THF	6-benzylamino-9-(tetrahydrofuran-2-yl)purine
BAP9THP	6-benzylamino-9-(2-tetrahydropyranyl)purine
BAPA	6-benzylaminopurine-9-(*β*)-D-arabinofuranoside
BAPR	6-benzylaminopurine-9-(*β*)-D-ribofuranoside
BOP	benzotriazole-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate
CHASE	Cyclase/His kinase-Associated Sensing Extracellular
$CHCl_3$	chloroform
CK	cytokinin
CKX	cytokinin oxygenase/dehydrogenase
CRE1/AHK4	Cytokinin Response 1/*Arabidopsis* histidine kinase 4
CRF	Cytokinin Response Factors
*c*Z	*cis*-zeatin, 6-(*Z*)-(4-hydroxy-3-methylbut2-enylamino)purine
DIPEA	*N*,*N*-iisopropylethylamine
DMAPP	dimethylallyl diphosphate
DMSO	dimetylsulfoxide
DZ	dihydrozeatin
ENT	equilibrative nucleoside transporter
ER	endoplasmic reticulum
Et_2O	diethylether
EtOAc	ethyl acetate
EtOH	ethanol
GUS	*β*-glucuronidase
HLPC-MS	high-performance liquid chromatography/mass spectroscopy

HMBDP	hydroxymethyl-butenyl diphosphate
HRMS	high resolution mass spectroscopy
iP	N^6-isopentenyladenine, 6-(2-isopentenylamino)purine
IsCK	isoprenoid cytokinin
iPR	6-(2-isopentenylamino)purine riboside
Kin	kinetin, 6-furfurylaminopurine
LHCII	light-harvesting complex II
LOG	cytokinin riboside 5'-monophosphate phosporibohydrolase,
MeCN	acetonitril
MeOH	methanol
*m*T	*meta*-topolin, 6-(3-hydroxybenzylamino)purine
NMR	nuclear magnetic resonance
OEC	oxygen-evolving complex
*o*T	*ortho*-topolin, 6-(2-hydroxybenzylamino)purine
PE	petrolether
PM	plasma membrane
PSII	photosystem II
PUP	purine permease
ROS	reactive oxygen species
RT	room temperature
SAGs	senescence associated gene
TEA	triethylamine
THF	tetrahyrofuran-2-yl
THP	tetrahyropyran-2-yl
TLC	thin layer chromatography
TMSOTf	trimethylsilyl trifluoromethanesulfonate
*t*Z	*trans*-zeatin, 6-(E)-(4-hydroxy-3-methylbut-2-enylamino)purine
WLSA	wheat leaves senescence assay
ZmHK	*Zea mays* histidine kinase
ZR	zeatin riboside

12 List of author´s papers

Papers in journals with an impact factor

Plíhalová, L., Vylíčilová, H., Doležal, K., Zahajská, L., Zatloukal, M., Strnad, M. (2016): Synthesis of aromatic cytokinins for plant biotechnology, *New Biotechnology*, **33**(5), 614–624, doi: 10.1016/j.nbt.2015.11.009.

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Doležal, K., Plíhalová, L., Vylíčilová, H., Zatloukal, M., Plíhal, O., Voller, J., Strnad, M., Bryksová, M., Vostálová, J., Rajnochová Svobodová, A., Ulrichová, J., Spíchal, L. (2014): *6-aryl*-9-*glycosylpurines and use thereof* (Palacký University in Olomouc), CZ 2014875, EP 3229772, SG 11201704019TA and US 10550144 patents.

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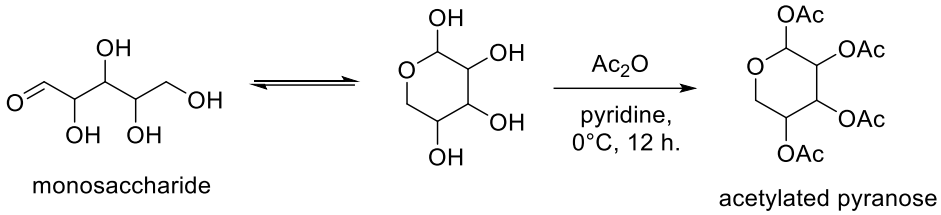
Supplementary information I

General methods of synthesis

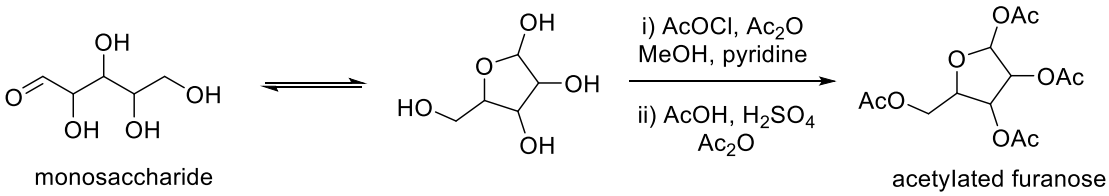
(Unpublished results)

Synthesis of tetra-*O*-acetyl-pyranoses



Generally, an unprotected sugar (2.5 g, 16.65 mmol) was suspended in pyridine (18.5 mL) cooled at 0°C, and acetic anhydride (14 mL, 146.5 mmol) was dropwise added under argon atmosphere. The reaction mixture was stirred at RT overnight and monitored by TLC (DCM/MeOH, 4:1). The reaction mixture was treated with DCM (100 mL) and organic layers were extracted with H_2O (6 x 100 mL), 1M HCl (3 x 100 mL), saturated $NaHCO_3$ (3 x 50 mL) and H_2O (3 x 50 mL). The organic layers were washed with brine and dried (Na_2SO_4), filtrated and evaporated under the reduce pressure to give a colourless gel.

Synthesis of tetra-*O*-acetyl-furanoses

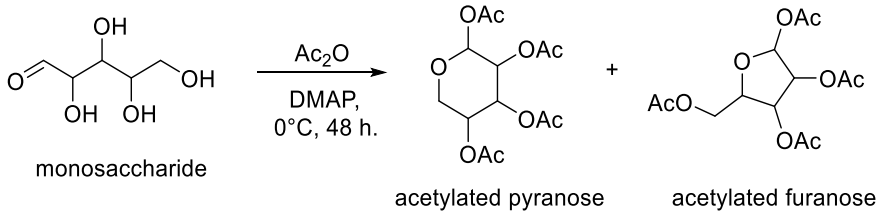


In the first step, the suspension of unprotected sugar (5 g, 33.3 mmol, 1.15 eq.) in MeOH (100 mL) was carefully treated with the solution of acetyl chloride (2.5 mL, 1 eq.) in MeOH (30 mL) and stirred at RT for 2.5 h until the reaction mixture was completely clear. The disappearance of starting white material was monitored by TLC (DCM/MeOH 4/1). The reaction mixture was quenched with pyridine and evaporated. The crude product was dissolved in pyridine (40 mL), cooled to 0°C and acetic anhydride (20 mL) was dropwise added. The reaction mixture was stirred overnight at RT and then diluted with DCM (300mL), washed with H_2O (2 x 50 ml), 1M HCl (50 mL), sat. $NaHCO_3$ (2 x 50 ml) and brine. The combined organics layers were dried (Na_2SO_4) and evaporated (Scheme, i).

The crude product was dissolved in acetic anhydride (80 mL) and cooled at 0 °C in the ice bath then AcOH (20 mL) and H_2SO_4 was dropwise added. After 1.5-hour the reaction mixture was warm up to RT and neutralize by slowly pour into the mixture of sat. $NaHCO_3$ (250 mL), ice (300g) and DCM (200 mL). The organics layers were separated,

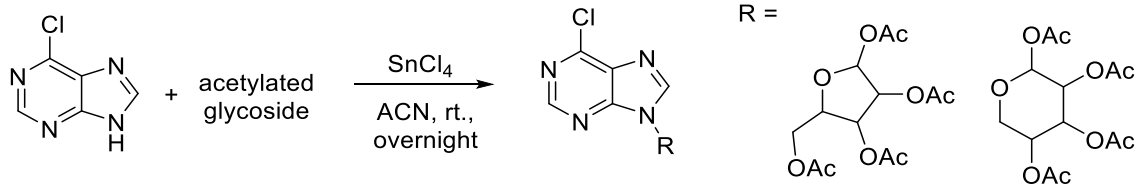
and H_2O phase re-extracted with DCM (6 x 30 mL). The combine organics layers were washed with additional sat. $NaHCO_3$, brine, dried (Na_2SO_4) and evaporated to give the colourless gel. (TLC PE/EtOAc 4:1).

Synthesis of tetra-*O*-acetyl glycosylfuranose and glycosylpyranose



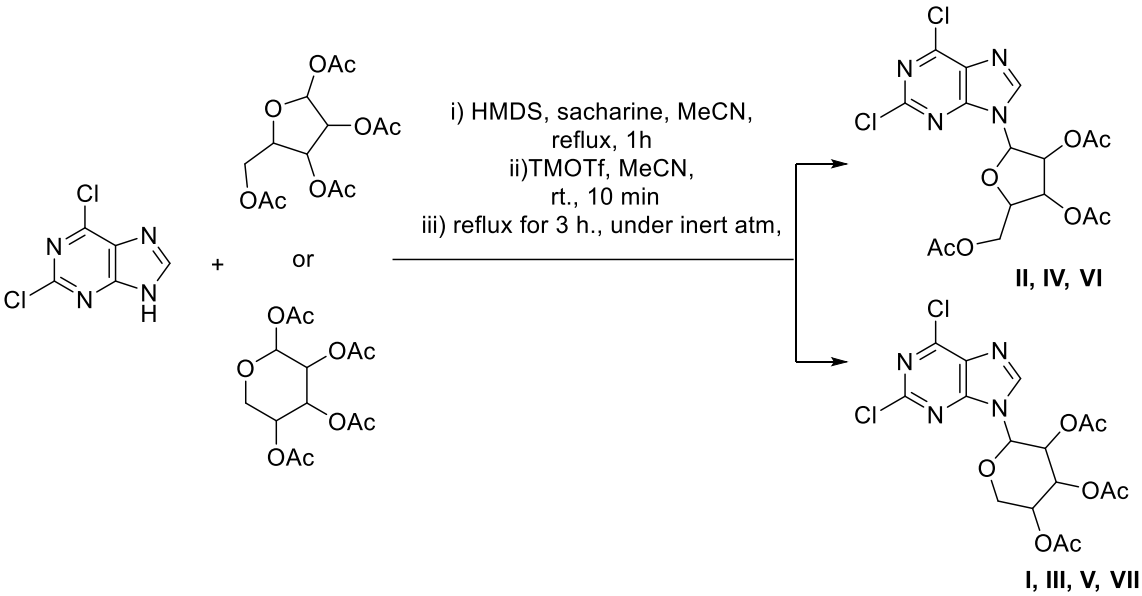
The suspension of unprotected sugar (2 g, 13.321 mmol, 1 eq.), acetyl anhydride (12.6 mL, 133.21 mmol, 10 eq.) and DMAP (0.85g, 6.95 mmol, 0.52 eq.) was kept in the fridge for 48 hours until the sugar was completely dissolved, which was monitored by TLC (DCM/MeOH 4/1). The reaction mixture poured into H_2O (20 mL) and extracted with DCM (4 x 20 mL). The organic phases were washed with brine, dried Na_2SO_4 and evaporated to give a yellow gel which was dissolved with Et_2O and stirred at RT for 1 day. The solid crystals of tetra-*O*-acetyl-furanose from the solution was removed by filtration and the filtrate was evaporated. The NMR spectra indicated the presence products of both tetra-*O*-acetyl-furanose and tetra-*O*-acetyl-pyranose in the ration 1/1.

Synthesis of 6-chloro-9-(tetra-*O*-acetyl-glycosyl)purines



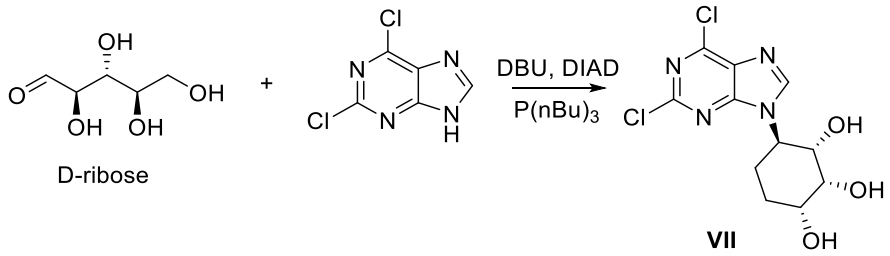
$SnCl_4$ (2.1 eq. 0.6 mL) was dropwise added to the mixture of 6-chloropurine (412 mg, 1.1 eq.) in dry ACN (10 mL) and penta-*O*-acetyl saccharide (1 eq.) in dry ACN (5 mL), stirred overnight at RT under the inert atmosphere. The crude was evaporated, dissolved in EtOAc (25 mL), washed with sat. $NaHCO_3$ (30 mL), H_2O (2 x 30 mL), brine and dried with Na_2SO_4. The crude was purified by the column chromatography (DCM/aceton, 9/1) to give a white solid.

Synthesis of 2,6-dichloro-9-(tri-*O*-acetyl-glycosyl)purines



The mixture of 2,6-dichloropurine (1g, 5.29 mmol. 1 eq.) hexamethyldisilazane (HMDS) (1.11 mL, 5.29 mmol, 1 eq.) and saccharine (34 mg, 0.185 mmol, 0.035 eq. in anhydrous MeCN (20 mL) was refluxed for 1 h under the inert atmosphere. Trimethylsilyl trifluoromethanesulfonate (TMSOTf) (1.84 mL, 10.58 mmol, 2 eq.) was dropwise added to the solution of tetra-*O*-acetyl-saccharide (2.52 g, 7.93 mmol. 1.5 eq.) in MeCN (9 mL), stirred for 10 min under the inert atmosphere and then were dropwise added. The reaction mixture was stirring under reflux for more than 3 h under an inert atmosphere, cooled, neutralized sat. $NaHCO_3$ and extracted with EtOAc. The organic layers were washed with brine, dried (Na_2SO_4) and evaporated. The crude product was purified by silica column chromatography (PE/EtOAc 4/1 or Et_2O) and precipitated in Et_2O and PE to give the product as a white solid.

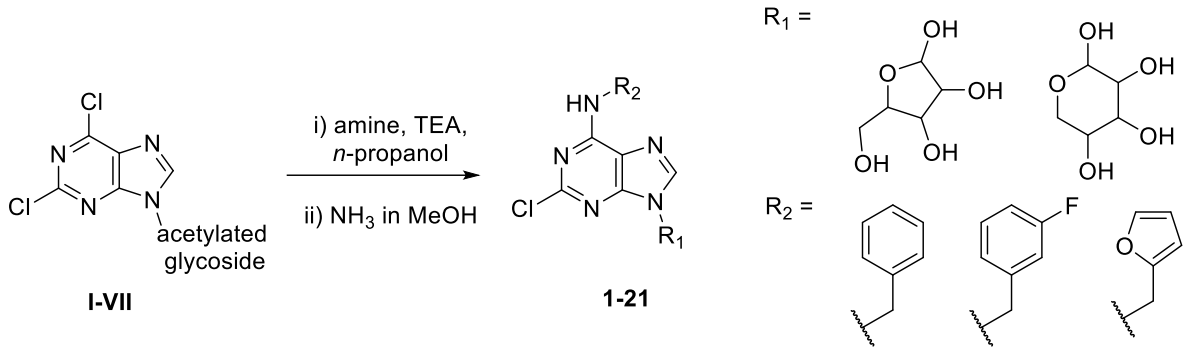
Direct glycosylation *via* Mitsunobu condition



1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was dropwise added to the mixture of 2,6-dichloropurine (188mg, 1mmol, 1eq.) in MeCN (10 mL) and stirred at RT for 1 hour. After

cooling to 0°C diisopropyl azodicarboxylate (DIAD) (420 ul, 2.1 mmol, 2.1 eq.) was added followed by P(*n*-Bu)$_3$ (530ul, 2 mmol, 2eq.) dropwise over 5 min, then D-ribose (300 mg, 2 mmol, 2 eq.) was added at once and stirred overnight. The reaction mixture was neutralized with 1 M HCl and evaporated. The crude was dissolved in MeOH/DCM (1:1) (2 mL), triturated PE/Et$_2$O (6:1), stirred at RT for 20 min and filtrated. The oil product was evaporated, purified by column chromatography (DCM/MeOH) with 0-10% MeOH gradient to give a syrup. The white product was obtained by precipitation in Et$_2$O

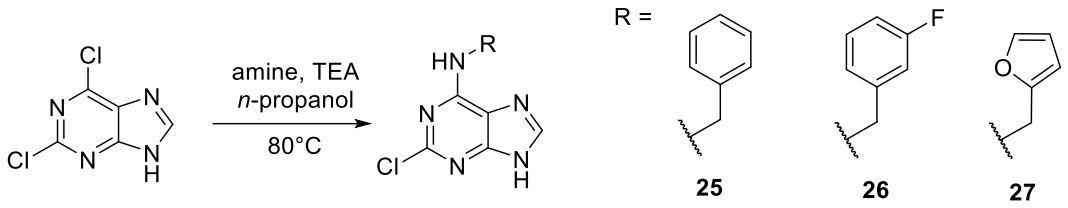
Synthesis of aromatic 2,6-disubstituted-purine-9-glycosides



The mixture of tetra-*O*-acetyl-2,6-dichloropurine glycoside (155 mg, 0.347, 1 eq.), appropriate benzylamine (0.417 mmol, 1.2 eq.) and trimethylamine (TEA) (121 µl, 0.868 mmol, 2.5 eq.) in *n*-propanol (3.5 mL) was refluxed at 90 °C more than 3 hour usually in an pressure tube. The disappearance of starting material was monitored by TLC ($CHCl_3$/MeOH or PE/EtOAc 1/1). The reaction mixture was evaporated, diluted in H_2O (20 mL), extracted with EtOAc (4 x 15 mL), brine, dried Na_2SO_4 and evaporated *in vacuo*.

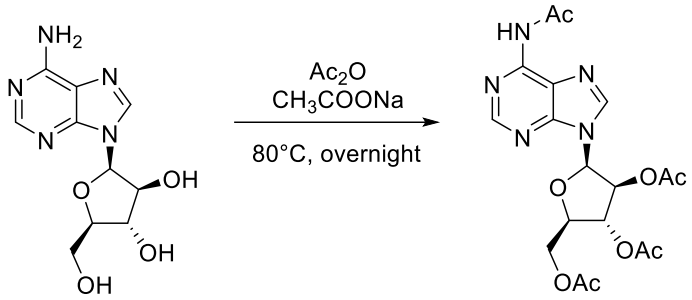
The crude was purified by silica column chromatography ($CHCl_3$/MeOH 100/1-90/1) and evaporated. The residue was dissolved in MeOH (5 mL) and NH_3/MeOH (5 mL), stirred at RT overnight and evaporated. The final product was separated by preparative HPLC-MS chromatography which was coupled to UV-VIS and LC/MSD detector using H_2O/ACN as eluent (in detail described in the chapter material and method), concentrated *in vacuo* and lyophilized from MeOH/H_2O solution to give a white product.

Synthesis of 2-chloro-6-subsituted purines



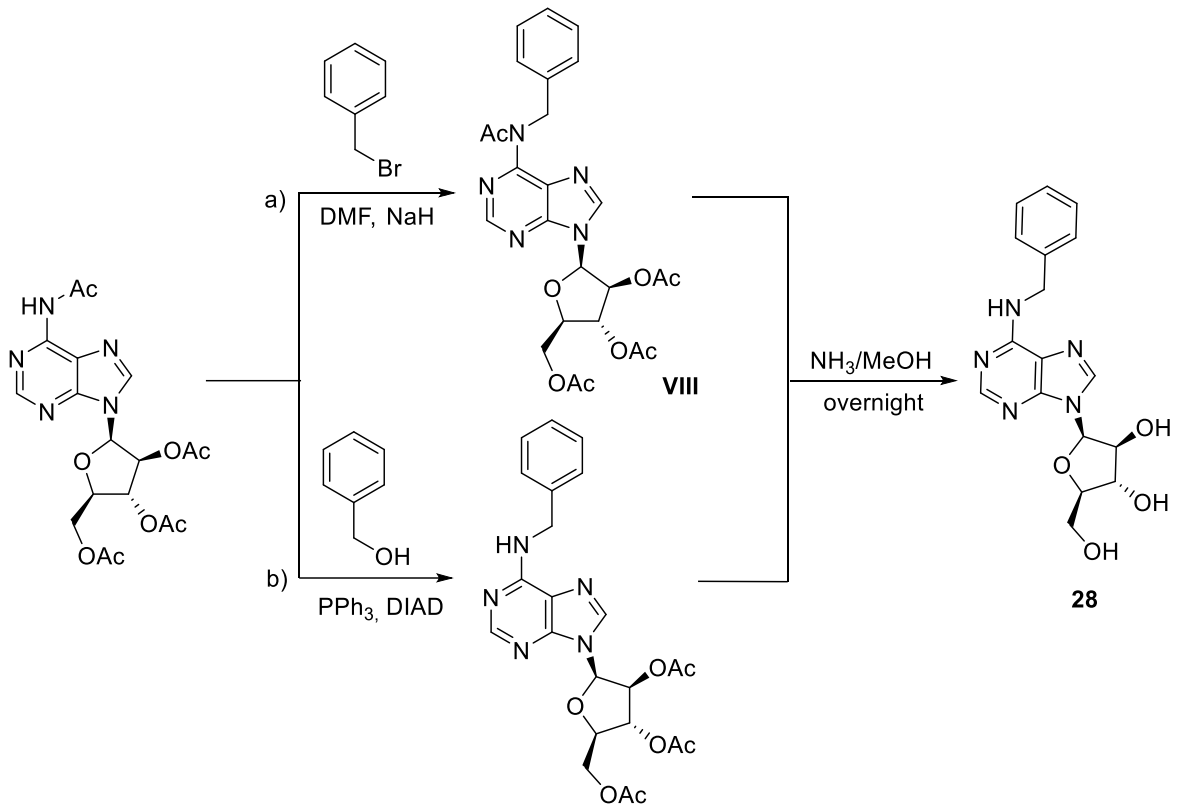
2,6-dichloro-9*H*-purine (300 mg, 1.587 mmol, 1 eq.) was refluxed with the corresponding amine (1.904 mmol, 1.2 eq.), TEA (552 µl, 3.968 mmol, 2.5 eq.) in *n*-propanol for 3 h. The reaction mixture was monitored by TLC ($CHCl_3$/MeOH 9/1) and cooled to RT. The white product was precipitated, filtrated, washed with *n*-propanol and ice H_2O. The resulting white solid was recrystallization in MeOH, washed with cold MeOH, cold Et_2O, and dried *in vacuo*.

Acetylation of adenine-9-*β*-D-arabinofuranoside



The solution of adenine-9-*β*-D-arabinofuranoside (0.5 g, 1.8mmol) in anhydrous sodium acetate (0.1g, 1.22 mmol) and acetic anhydride (5.85 mL, 62 mmol) were heated at 80°C overnight and monitored by TLC ($CHCl_3$/MeOH 9/1). The reaction mixture was diluted with ice H_2O (20 mL) and stirred at RT and then extracted into DCM (4x 20 mL). The organic extracts were combined, washed with brine, dried Na_2SO_4 and evaporated. The crude product was purified by silica column chromatography ($CHCl_3$/MeOH 50/1 to 15/1) to give a gel-like product.

Alkylation of peracetylated adenine-9-*β*-D-arabinofuranoside



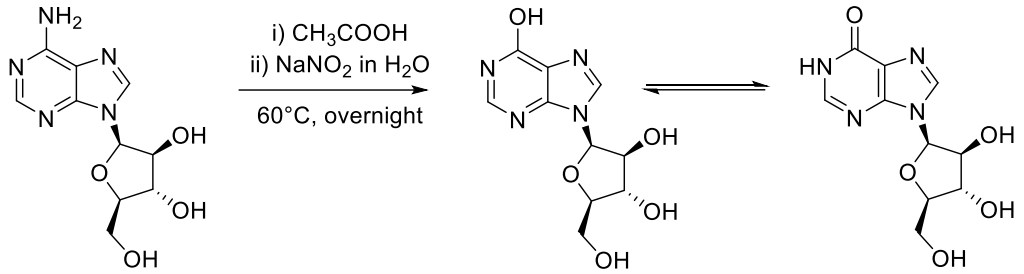
The solution of peracetylated vidarabine (239 mg, 0.548 mmol) in dry DMF (3 mL) was dropwise added to the suspension of sodium hydride (60%, in mineral oil, 26.3 mg and 0.657 mmol) in dry DMF (3 mL) and benzylbromide (281.3 mg, 1.644 mmol) was slowly added. The reaction mixture was stirred under an inert atmosphere at 60°C overnight with colour changes from white to orange. The disappearance of the starting material was monitored by TLC ($CHCl_3$/MeOH, 9/1). The reaction mixture was diluted with cold H_2O (20 mL), stirred, extracted in EtOAc (4x15 mL), brine, dried Na_2SO_4 and evaporated. The crude product was purified by silica column chromatography ($CHCl_3$/MeOH, 92/1 to 10/1) to give a yellow gel

Another method of preparation used was as follows: diisopropyl azodicarboxylate (DIAD) (98µl, 0.490 mmol, 1.2 eq.) was dropwise added to the mixture of peracetylated vidarabine (178 mg, 0.408 mmol, 1 eq.), benzylalcohol (50 µl, 0.490 mmol, 1.2 eq.) and triphenylphosphin (PPh_3) (129 mg, 0.490 mmol, 1.2 eq.) in THF. The reaction mixture was stirred at RT under an inert atmosphere for 3 hour and controlled by TLC (PE/EOAc 1/1). After the evaporation the crude product was diluted in toluene, cooled in a fridge overnight

and filtered. The filtrate was evaporated and purified by silica column chromatography (PE/EtOAc 100/1-10/1)

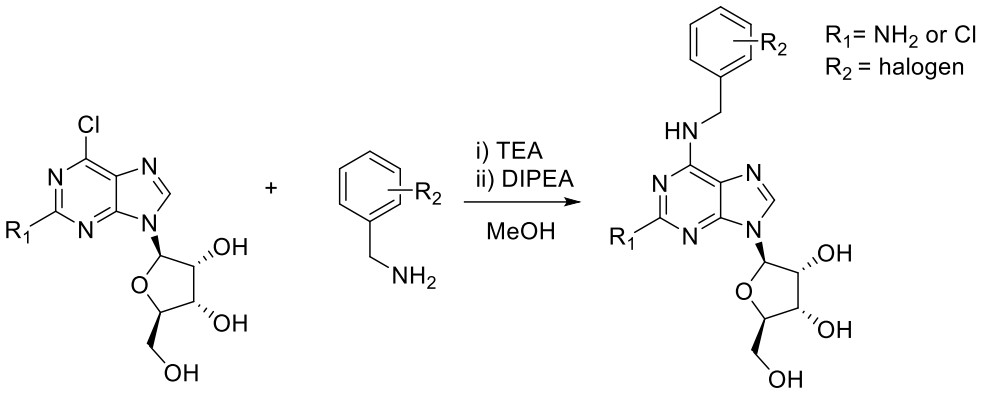
Finally, the alkylated intermediate (168 mg, 0.32 mmol) was dissolved in the solution of NH_3/MeOH (5 mL), MeOH (5 mL) and stirred overnight. The final product was separated by preparative HPLC-MS chromatography which was coupled to UV-VIS and LC/MSD detector using H_2O/CAN, subsequent it was concentrated and lyophilized to give the product (**28**).

Deamination of adenine arabinofuranoside



Into the solution of adenine arabinofuranoside (100 mg, 1eq.) in glacial acetic acid (1.28 mL, 60 eq.) was dropwise added $NaNO_2$ (258 mg, 10 eq.) in H_2O (1 mL) and heated at 60 °C overnight. The crude was evaporated with *n*-hepane (30 mL) and MeOH (10 mL). The residue was recrystallized in H_2O, filtrated, and washed with H_2O/Et_2O to give a white solid.

Microwave-assisted synthesis



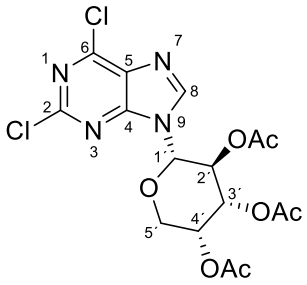
The mixture of substituted 2,6-chloropurine riboside (100 mg, 1 eq.) with the appropriate benzylamine (1.2 eq.-1.5 eq.), and TEA (110 µl, 1.5 eq.) or DIPEA (2 eq. 115µl) in MeOH (3 mL) was introduced into the microwave vessel. The reaction mixture was sonicated until dissolved and inserted into the microwave reactor (CEM discovery) and the heating program was applied. After the cooling the reaction mixture was transferred to the round bottom flask and evaporated. It was extracted with H_2O (10 mL), EtOAc (2x10 mL), brine, dried Na_2SO_4 and purified by column chromatography ($CHCl_3$/MeOH, 9/1). The white solid was precipitated in DCM/Et_2O, evaporated, and dried in an oven at 50 °C.

Supplementary information II

Chemical-analytical properties of newly prepared compounds

(Unpublished results)

Acetylated 2,6-dichloro-*N*9-glycofuranyl/glycopyranyl purines



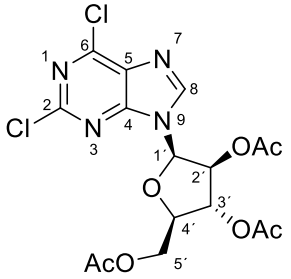
(2*S*,3*S*,4*R*,5*R*)-2-(2,6-dichloro-9*H*-purin-9-yl)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (**I**)

White solid, chemical formula: $C_{16}H_{16}Cl_2N_4O$.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 1.75 (s, 3H, OAc2' C$\underline{H}_3$), 1.97 (s, 3H, OAc4' C$\underline{H}_3$), 2.20 (s, 3H, OAc3' C$\underline{H}_3$), 4.06 (d, *J* = 12.8 Hz, 1H, H5'), 4.22 (d, *J* = 13.1 Hz, 1H, H5'), 5.29 (s, 1H, H4'), 5.50 (dd, *J* = 9.6, 2.3 Hz, 1H, H3'), 5.71 (t, *J* = 9.6 Hz, 1H, H2'), 6.11 (d, *J* = 8.9 Hz, 1H, H1'), 8.97 (s, 1H, H8).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 19.9 (OAc2' $\underline{C}H_3$), 20.4 (OAc4' $\underline{C}H_3$), 20.7 (OAc3' $\underline{C}H_3$), 66.3 (C5'), 67.8 (C4'), 68.0 (C2'), 70.0 (C3'), 80.9 (C1'), 130.5 (C5), 146.4 (C8), 150.2 (C6), 151.5 (C2), 153.1 (C4), 169.2 (OAc2' $\underline{C}O$), 169.54 (OAc4' $\underline{C}O$), 169.97 (OAc3' $\underline{C}O$).

(2*R*,3*R*,4*S*,5*R*)-2-(acetoxymethyl)-5-(2,6-dichloro-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate (**II**)

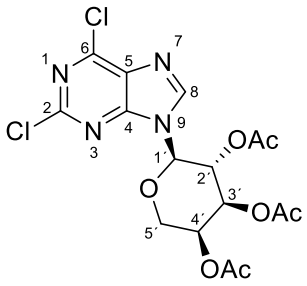


White solid, chemical formula: $C_{16}H_{16}Cl_2N_4O_7$

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 1.77 (s, 3H, OAc2' C$\underline{H}_3$), 2.03 (s, 3H, OAc5' C$\underline{H}_3$), 2.11 (s, 3H, OAc3' C$\underline{H}_3$), 4.33-4.40 (m, 3H, 2 × H5', H4'), 5.59 (t, *J* = 5.5 Hz, 1H, H3'), 5.63 (t, J = 5.5 Hz, 1H, H2'), 6.63 (d, *J* = 5.5 Hz, 1H, H1'), 8.85 (s, 1H, H8).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 19.9 (OAc2' C$\underline{H}_3$), 20.5 (OAc5' C$\underline{H}_3$), 20.6 (OAc3' C$\underline{H}_3$), 63.1 (C5'), 74.5 (C3'), 74.5 (C2'), 78.0 (C4'), 82.1 (C1'), 130.4 (C5), 147.2 (C8), 150.2 (C6), 151.3 (C2), 152.8 (C4), 169.0 (OAc2' $\underline{C}O$), 169.7 (OAc3' $\underline{C}O$), 170.1 (OAc5' $\underline{C}O$).

(2*R*,3*R*,4*S*,5*S*)-2-(2,6-dichloro-9*H*-purin-9-yl)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (**III**)

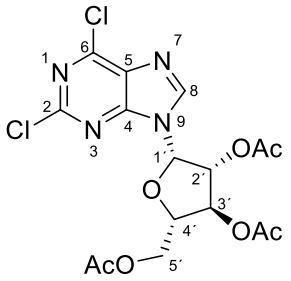


White solid, chemical formula: $C_{16}H_{16}Cl_2N_4O_7$

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 1.75 (s, 3H, OAc2' C$\underline{H}_3$), 1.97 (s, 3H, OAc4' C$\underline{H}_3$), 2.20 (s, 3H, OAc3' C$\underline{H}_3$), 4.06 (dd, *J* = 13.1, 1.8 Hz, 1H, H5'), 4.23 (d, *J* = 13.1 Hz, 1H, H5'), 5.29 (s, 1H, H4'), 5.50 (dd, *J* = 10.0, 3.6 Hz, 1H, H3'), 5.71 (t, *J* = 9.5 Hz, 1H, H2'), 6.11 (d, *J* = 9.2 Hz, 1H, H1'), 8.97 (s, 1H, H8).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 19.9 (OAc2' $\underline{C}H_3$), 20.4 (OAc4' $\underline{C}H_3$), 20.7 (OAc3' $\underline{C}H_3$), 66.3 (C5'), 67.8 (C4'), 68.0 (C2'), 70.0 (C3'), 80.9 (C1'), 130.5 (C5), 146.4 (C8), 150.2 (C6), 151.5 (C2), 153.1 (C4), 169.2 (OAc2' $\underline{C}O$), 169.54 (OAc4' $\underline{C}O$), 169.97 (OAc3' $\underline{C}O$).

(2*S*,3*S*,4*R*,5*S*)-2-(acetoxymethyl)-5-(2,6-dichloro-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate (**IV**)

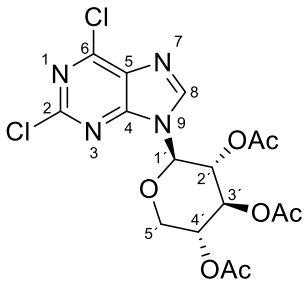


White solid, chemical formula: $C_{16}H_{16}Cl_2N_4O_7$

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 1.77 (s, 3H, OAc2' C$\underline{H}_3$), 2.03 (s, 3H, OAc5' C$\underline{H}_3$), 2.11 (s, 3H, OAc3' C$\underline{H}_3$), 4.31-4.41 (m, 3H, 2 × H5', H4'), 5.59 (t, *J* = 5.5 Hz, 1H, H3'), 5.63 (t, J = 5.5 Hz, 1H, H2'), 6.63 (d, *J* = 5.5 Hz, 1H, H1'), 8.86 (s, 1H, H8).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 19.9 (OAc2' $\underline{C}H_3$), 20.5 (OAc5' $\underline{C}H_3$), 20.6 (OAc3' $\underline{C}H_3$), 63.1 (C5'), 74.50 (C3'), 74.56 (C2'), 78.0 (C4'), 82.1 (C1'), 130.4 (C5), 147.2 (C8), 150.1 (C6), 151.3 (C2), 152.8 (C4), 169.0 (OAc2' $\underline{C}O$), 169.7 (OAc3' $\underline{C}O$), 170.1 (OAc5' $\underline{C}O$).

(2*R*,3*R*,4*S*,5*R*)-2-(2,6-dichloro-9*H*-purin-9-yl)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (**V**)



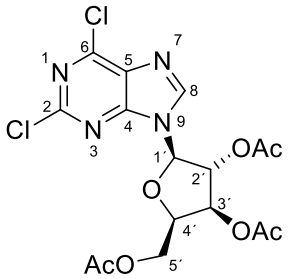
White solid, chemical formula: $C_{16}H_{16}Cl_2N_4O_7$

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 1.72 (s, 3H, OAc2' C$\underline{H}_3$), 2.00 (s, 3H, OAc3' C$\underline{H}_3$), 2.02 (s, 3H, OAc4' C$\underline{H}_3$), 3.91 (t, *J* = 11.0 Hz, 1H, H5'), 4.07 (dd, *J* = 11.0, 5.5 Hz, 1H, H5'), 5.16 (td, *J* = 10.2, 5.3 Hz, 1H, H4'), 5.61 (t, *J* = 9.5 Hz, 1H, H3'), 5.75 (t, *J* = 9.2 Hz, 1H, H2'), 6.22 (d, *J* = 9.2 Hz, 1H, H1'),

9.07 (s, 1H, H8).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 19.9 (OAc2' $\underline{C}H_3$), 20.3 (OAc3' $\underline{C}H_3$), 20.5 (OAc4' $\underline{C}H_3$), 64.0 (C5'), 67.8 (C4'), 70.4 (C2'), 71.5 (C3'), 80.0 (C1'), 130.3 (C5), 146.4 (C8), 150.1 (C6), 151.6 (C2), 153.1 (C4), 169.0 (OAc2' $\underline{C}O$), 169.5 (OAc3' $\underline{C}O$), 169.6 (OAc4' $\underline{C}O$).

(2*R*,3*S*,4*R*,5*R*)-2-(acetoxymethyl)-5-(2,6-dichloro-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate (**VI**)

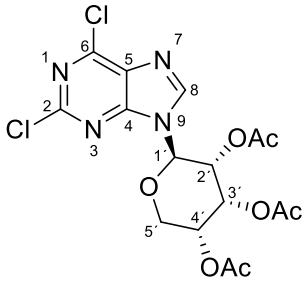


White solid, chemical formula: $C_{16}H_{16}Cl_2N_4O_7$

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 2.02 (s, 3H, OAc5' C$\underline{H}_3$), 2.05 (s, 3H, OAc2' C$\underline{H}_3$), 2.10 (s, 3H, OAc3' C$\underline{H}_3$), 4.33 (ABX, Δ_{AB} = 20.8 Hz, J_{AB} = 12.07 Hz, J_{AX} = 6.1 Hz, J_{BX} = 4.3 Hz 2H, 2 × H5'), 4.61 (dt, *J* = 7.0, 3.9 Hz, 1H, H4'), 5.49 (dd, *J* = 4.3, 1.8 Hz, 1H, H2'), 5.70 (dd, *J* = 2.4, 2.1 Hz, 1H, H2'), 6.28 (d, *J* = 2.8 Hz, 1H, H1'), 8.84 (s, 1H, H8).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 20.5 (3× C, OAc2' C$\underline{H}_3$, OAc3' C$\underline{H}_3$, OAc5' C$\underline{H}_3$), 61.3 (C5'), 74.0 (C3'), 78.3 (C2'), 78.5 (C4'), 87.0 (C1'), 130.8 (C5), 146.3 (C8), 149.9 (C6), 151.3 (C2), 152.8 (C4), 169.2 (OAc2' $\underline{C}O$), 169.3 (OAc3' $\underline{C}O$), 170.0 (OAc5' $\underline{C}O$).

(2*R*,3*R*,4*R*,5*R*)-2-(2,6-dichloro-9*H*-purin-9-yl)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (**VII**)

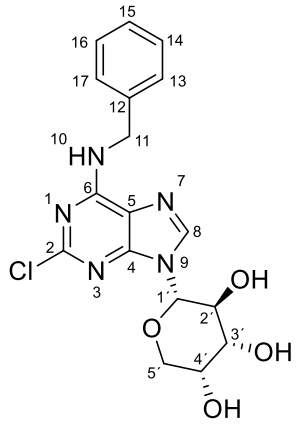


White solid, chemical formula: $C_{16}H_{16}Cl_2N_4O_7$

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 1.75 (s, 3H, OAc C$\underline{H}_3$), 2.00 (s, 3H, OAc C$\underline{H}_3$), 2.23 (s, 3H, OAc C$\underline{H}_3$), 4.07-4.00 (m, 2H, H5'), 5.20 (ddd, *J* = 10.2, 6.3, 2.4 Hz, 1H, H4'), 5.74 (t, *J* = 2.8 Hz, 1H, H3'), 5.86 (dd, *J* = 9.5, 3.1 Hz, 1H, H2'), 6.02 (d, *J* = 9.5 Hz, 1H, H1'), 9.01 (s, 1H, H8).

CK 2-chloro-6-(benzylamino/furfurylamino)purine-9-(*α*)-D-arabinopyranosides

(2*S*,3*S*,4*R*,5*R*)-2-(6-(benzylamino)-2-chloro-9*H*-purin-9-yl)tetrahydro-2*H*-pyran-3,4,5-triol (**1**)

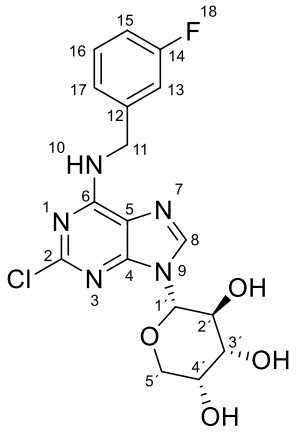


White solid, chemical formula: $C_{17}H_{18}ClN_5O_4$, yield: 70%, EI^+-MS m/z: 392.2, HPLC retention time (min.): 18.43, purity: 98%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.56 (ddd, *J* = 9.2, 5.8, 3.4 Hz, 1H, H3‘), 3.74-3.81 (m, 3H, H4‘, H5‘), 4.12 (td, *J* = 9.2, 6.1 Hz, 1H, H2‘), 4.64 (ABX, Δ_{AB} = 16.5 Hz, *J* = 15.3, 6.1 Hz, 2H, H11), 4.77 (d, *J* = 5.2 Hz, 1H, OH4‘), 5.03 (d, *J* = 5.8 Hz, 1H, OH3‘), 5.24 (d, *J* = 8.9 Hz, 1H, H1‘), 5.25 (d, *J* = 6.1 Hz, 1H, OH1‘), 7.22 (tt, *J* = 6.6, 2.1 Hz, 1H, H15), 7.29-7.33 (m, 4H, H13, H14, H16, H17), 8.31 (s, 1H, H8), 8.88 (*t*, *J* = 6.1 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 43.1 (C11), 68.5 (C4‘), 68.9 (C2‘), 69.3 (C5‘), 73.3 (C3‘), 83.3 (C1‘), 117.9 (C5), 126.8 (C15), 127.2 (2× C, C13, C17), 128.3 (2× C, C14, C16), 139.2 (C12), 139.8 (C8), 150.2 (C4), 153.2 (C2), 154.9 (C6).

(2*S*,3*S*,4*R*,5*R*)-2-(2-chloro-6-((3-fluorobenzyl)amino)-9*H*-purin-9 -yl)tetrahydro-2*H*-pyran-3,4,5-triol (**2**)

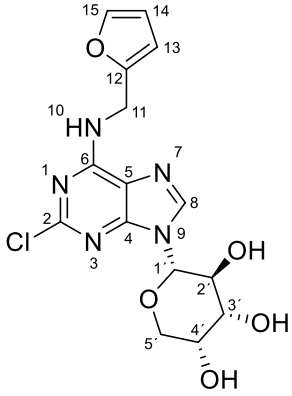


White solid, chemical formula: $C_{17}H_{17}ClFN_5O_4$, yield: 30%, EI^+-MS m/z: 410.3, HPLC retention time (min.): 19.10, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-D_6) δ (ppm): 3.58 (ddd, *J* = 9.3, 5.7, 3.4 Hz, 1H, H3’), 3.75-3.82 (m, 3H, H4‘, H5‘), 4.14 (td, *J* = 9.2, 5.8 Hz, 1H, H2’), 4.66 (ABX, Δ_{AB} = 13.4 Hz, *J* = 15.9, 6.4 Hz, 2H, H11), 4.81 (d, *J* = 5.2 Hz, 1H, OH4’), 5.06 (d, *J* = 5.8 Hz, 1H, OH3’), 5.25 (d, *J* = 9.5 Hz, 1H, H1’), 5.27 (d, *J* = 6.1 Hz, 1H, OH2’), 7.05 (td, *J* = 8.6, 2.1 Hz, 1H, H15), 7.13 (d, *J* = 9.8 Hz, 1H, H13), 7.16 (d, *J* = 7.9 Hz, 1H, H17), 7.34 (td, *J* = 7.9, 6.1 Hz, 1H, H16), 8.33 (s, 1H, H8), 8.92 (t, *J* = 6.1 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 42.7 (C11), 68.5 (C4‘), 69.0 (C2‘), 69.4 (C5‘), 73.3 (C3‘), 83.4 (C1), 113.7 (d, $^2J_{CF}$ = 21.6 Hz, C15), 113.9 (d, $^2J_{CF}$ = 21.6 Hz, C13), 118.0 (C5), 123.2 (C17), 130.3 (d, $^3J_{CF}$ = 7.2 Hz, C16), 140.0 (C8), 142.3 (d, $^3J_{CF}$ = 7.2 Hz, C12), 150.3 (C4), 153.3 (C2), 154.9 (C6), 162.2 (d, *J* = 243.5 Hz, C14).

(2*S*,3*S*,4*R*,5*R*)-2-(2-chloro-6-((furan-2-ylmethyl)amino)-9*H*-purin-9-yl)tetrahydro-2*H* pyran-3,4,5-triol (**3**)



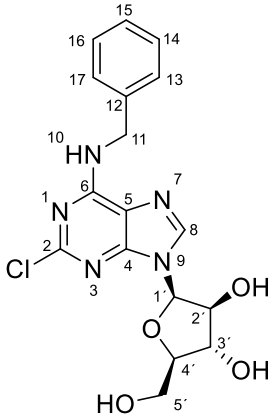
White solid, chemical formula: $C_{15}H_{16}ClN_5O_5$, yield: 67%, EI^+-MS m/z: 382.3, HPLC retention time (min.): 15.67, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.56 (ddd, *J* = 9.0, 5.5, 3.4 Hz, 1H, H3'), 3.74-3.81 (m, 3H, H4', H5'), 4.12 (td, *J* = 9.3, 5.9 Hz, 1H, H2'), 4.62 (ABX, Δ_{AB} = 13.2 Hz, *J* = 15.3, 6.1 Hz, 2H, H11), 4.77 (d, *J* = 4.9 Hz, 1H, OH4'), 5.03 (d, *J* = 5.8 Hz, 1H, OH3'), 5.23-5.25 (m, 2H, H1', OH2'), 6.26 (d, *J* = 2.8 Hz, 1H, H13), 6.37 (s, 1H, H14), 7.56 (s, 1H, H15), 8.31 (s, 1H, H8), 8.78 (t, *J* = 5.5 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 36.7 (C11), 68.5 (C4'), 68.9 (C2'), 69.3 (C5'), 73.3 (C3'), 83.3 (C1'), 107.1 (C13), 110.5 (C14), 118.0 (5), 140.0 (C8), 142.06 (C15), 150.3 (C4), 152.0 (C12), 153.1 (C2), 154.7 (C6).

CK 2-chloro-6-(benzylamino/furfurylamino)purine-9-(*β*)-D-arabinofuranosides

(2*R*,3*S*,4*S*,5*R*)-2-(6-(benzylamino)-2-chloro-9*H*-purin-9-yl)-5-(hydroxymethyl) tetrahydrofuran-3,4-diol (**4**)

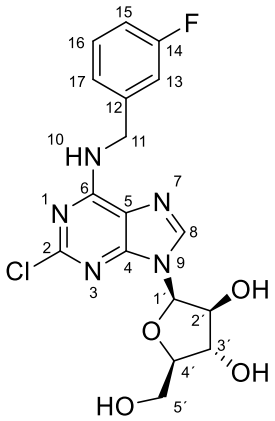


White solid, chemical formula**:** $C_{17}H_{18}ClN_5O_4$, yield: 35%, EI^+-MS m/z: 392.2, HPLC retention time (min.): 18.98, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.46-3.51 (m, 1H, H5'), 3.59-3.61 (m, 1H, H5'), 3.99 (td, *J* = 5.5, 5.2 Hz, 1H, H3'), 4.08-4.16 (m, 1H, H4'), 4.57 (q, *J* = 4.6 Hz, 1H, H2'), 4.60-4.68 (m, 2H, H11), 4.92 (t, *J* = 5.5 Hz, 1H, OH5'), 5.60 (d, *J* = 4.6 Hz, 1H, OH3'), 5.79 (d, *J* = 4.6 Hz, 2H, H1', OH2'), 7.22 (t, *J* = 6.4 Hz, 1H, H15), 7.28-7.31 (m, 4H, H13, H14, H16, H17), 8.39 (s, 1H, H8), 8.91 (t, *J* = 5.8 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 43.1 (C11), 61.1 (C5'), 74.9 (C3'), 79.3 (C2'), 85.4 (C4'), 88.2 (C1'), 118.5 (C5), 126.8 (C15), 127.28 (2× C, C13, C17), 128.3 (2× C, C14, C16), 139.2 (C12), 140.4 (C8), 149.6 (C4), 153.2 (C2), 154.9 (C6).

(2*R*,3*S*,4*S*,5*R*)-2-(2-chloro-6-((3-fluorobenzyl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl) tetrahydrofuran-3,4-diol (**5**)

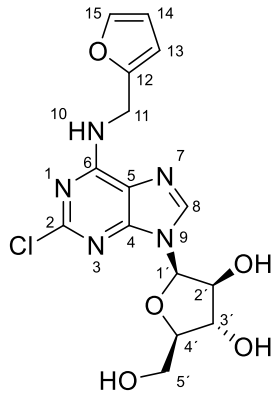


White solid, chemical formula: $C_{17}H_{17}ClFN_5O_4$, yield: 32%, EI^+-MS m/z: 410.2, HPLC retention time (min.): 19.31, purity: 99.7%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.46 (dt, *J* = 11.9, 5.5 Hz, 1H, H5'), 3.56 (ddd, *J* = 11.8, 5.0, 3.7 Hz, 1H, H5'), 3.99 (dt, *J* = 5.5, 5.2 Hz, 1H, H3'), 4.10 (td, *J* = 5.2, 3.9 Hz, 1H, H4'), 4.53 (q, *J* = 5.2 Hz, 1H, H2'), 4.62 (ABX, Δ_{AB} = 5.7 Hz, *J* = 14.5, 6.4 Hz, 2H, H11), 4.92 (t, *J* = 5.7 Hz, 1H, OH5'), 5.59 (d, *J* = 4.6 Hz, 1H, OH3'), 5.78 (d, *J* = 5.7 Hz, 1H, OH2'), 5.80 (d, *J* = 4.9 Hz, 1H, H1'), 7.06 (td, *J* = 8.4, 2.1 Hz, 1H, H15), 7.11 (d, *J* = 9.5 Hz, 1H, H13), 7.13 (d, *J* = 8.25 Hz, 1H, H17), 7.35 (td, *J* = 7.8, 6.1 Hz, 1H, H16), 8.40 (s, 1H, H8), 8.94 (t, *J* = 6.1 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 42.7 (C11), 61.0 (C5'), 74.8 (C3'), 79.3 (C2'), 85.4 (C4'), 88.2 (C1'), 113.7 (d, $^2J_{CF}$ = 21.6 Hz, C15), 113.9 (d, $^2J_{CF}$ = 21.6 Hz, C13), 118.5 (C5), 123.2 (C17), 130.3 (d, $^3J_{CF}$ = 8.4 Hz, C16), 140.5 (C8), 142.2 (d, $^3J_{CF}$ = 7.2 Hz, C12), 149.7 (C4), 153.1 (C2), 154.8 (C6), 162.1 (d, $^1J_{CF}$ = 243.5 Hz, C14).

(2*R*,3*S*,4*S*,5*R*)-2-(2-chloro-6-((furan-2-ylmethyl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (**6**)



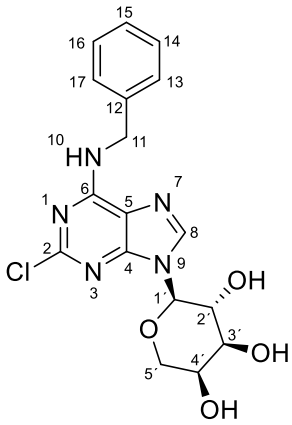
White solid, chemical formula: $C_{15}H_{16}ClN_5O_5$, yield: 58%, EI^+-MS m/z: 382.1, HPLC retention time (min.): 16.40, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.46-3.51 (m, 1H, H5'), 3.59 (ddd, *J* = 11.9, 5.3, 3.7 Hz, 1H, H5'), 3.99 (td, *J* = 5.5, 5.2 Hz, 1H, H3'), 4.13 (ddd, *J* = 6.1, 5.2, 3.7 Hz, 1H, H4'), 4.56 (q, *J* = 5.2 Hz, 1H, H2'), 4.62 (ABX, Δ_{AB} = Hz, *J* = 15.8, 6.4 Hz, 2H, H11), 4.92 (t, *J* = 5.7 Hz, 1H, OH5'), 5.59 (d, *J* = 4.6 Hz, 1H, OH3'), 5.79-5.80 (m, 2H, H1', OH2'), 6.26 (d, *J* = 3.0 Hz, 1H, H13), 6.37 (s, 1H, H14), 7.56 (dd, *J* = 1.7, 0.6 Hz, 1H, H15), 8.39 (s, 1H, H8), 8.81 (t, *J* = 5.8 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 36.6 (C11), 61.0 (C5'), 74.8 (C3'), 79.3 (C2'), 85.4 (C4'), 88.2 (C1'), 107.1 (C13), 110.5 (C14), 118.5 (C5), 140.5 (C8), 142.0 (C15), 149.7 (C4), 152.0 (C12), 153.0 (C2), 154.7 (C6).

CK 2-chloro-6-(benzylamino/furfurylamino)purine-9-(*α*)-L-arabinopyranosides

(2*R*,3*R*,4*S*,5*S*)-2-(6-(benzylamino)-2-chloro-9*H*-purin-9-yl)tetrahydro-2*H*-pyran-3,4,5-triol (**7**)

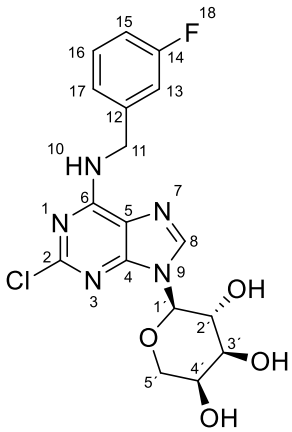


White solid, chemical formula: $C_{15}H_{16}ClN_5O_5$ yield: 67%, EI^+-MS m/z: 392.2, HPLC retention time (min.): 18.66, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.56 (ddd, *J* = 9.17, 5.8, 3.4 Hz, 1H, H3'), 3.73-3.80 (m, 3H, H4', H5'), 4.13 (td, J = 9.0, 6.1 Hz, 1H, H2'), 4.64 (ABX, Δ_{AB} = 16.3 Hz, *J* = 15.7, 6.4 Hz, 2H, H11), 4.77 (d, *J* = 4.9 Hz, 1H, OH4'), 5.04 (d, *J* = 5.8 Hz, 1H, OH3'), 5.23 (d, *J* = 9.48, 1H, H1'), 5.25 (d, *J* = 6.1 Hz, 1H, OH2'), 7.22 (tt, *J* = 6.4, 2.1 Hz, 1H, H15), 7.29-7.33 (m, 4H, H13, H14, H16, H17), 8.30 (s, 1H, H8), 8.88 (t, *J* = 6.1 Hz, 1H, H10)

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 43.6 (C11), 69.0 (C4'), 69.4 (C2'), 69.9 (C5', 73.8 (C3'), 83.8 (C1'), 118.4 (C5), 127.3 (C15), 127.7 (2× C, C13, C17), 128.8 (2× C, C14, C16), 139.7 (C12), 140.3 (C8), 150.7 (C4), 153.8 (C2), 155.4 (C6).

(2*R*,3*R*,4*S*,5*S*)-2-(2-chloro-6-((3-fluorobenzyl)amino)-9*H*-purin-9-yl)tetrahydro-2*H*-pyran-3,4,5-triol (**8**)

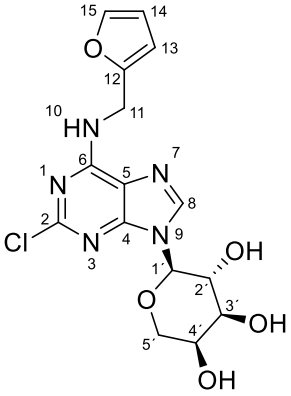


White solid, chemical formula: $C_{17}H_{17}ClFN_5O_4$, yield: 57%, EI^+-MS m/z: 410.2, HPLC retention time (min.): 19.07, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.56 (ddd, *J* = 9.2, 5.8, 3.4 Hz, 1H, H3'), 3.74-81 (m, 3H, H4', H5'), 4.13 (td, *J* = 9., 6.1 Hz, 1H, H2'), 4.65 (ABX, Δ_{AB} = 14.6 Hz, *J* = 15.2, 6.4 Hz, 2H, H11), 4.79 (d, *J* = 4.9 Hz, 1H, OH4'), 5.05 (d, *J* = 5.8 Hz, 1H, OH3'), 5.24 (d, *J* = 9.5 Hz, 1H, H1'), 5.26 (d, *J* = 6.7 Hz, 1H, OH2'), 7.05 (td, *J* = 8.4, 2.4 Hz, 1H, H15), 7.13 (d, *J* = 10.0 Hz, 1H, H13), 7.16 (d, *J* = 8.3 Hz, 1H, H17), 7.35 (td, *J* = 7.8, 6.1 Hz, 1H, H16), 8.33 (s, 1H, C8), 8.92 (t, *J* = 5.8 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 42.7 (C11), 68.5 (C4'), 68.9 (C2'), 69.4 (C5'), 73.3 (C3), 83.4 (C1), 113.7 (d, $^2J_{CF}$ = 20.4 Hz, C15), 113.9 (d, $^2J_{CF}$ = 21.5 Hz, C13), 118.0 (C5), 123.2 (C17), 130.3 (d, $^3J_{CF}$ = 8.4 Hz, C16), 140.0 (C8), 142.3 (d, $^3J_{CF}$ = 7.2 Hz, C12), 150.3 (C4), 153.2 (C2), 154.8 (C6), 162.2 (d, $^1J_{CF}$ = 243.5 Hz, C14).

(2*R*,3*R*,4*S*,5*S*)-2-(2-chloro-6-((furan-2-ylmethyl)amino)-9*H*-purin-9-yl)tetrahydro-2*H*-pyran-3,4,5-triol (**9**)



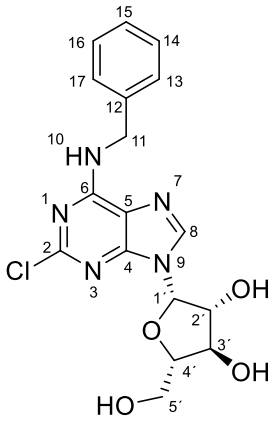
White solid, chemical formula: $C_{15}H_{16}ClN_5O_5$, yield: 56%, EI^+-MS m/z: 382.3, HPLC retention time (min.): 15.75, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.56 (ddd, *J* = 9.2, 5.5, 3.4 Hz, 1H, H3‘), 3.73-3.81 (m, 3H, H4‘, H5‘), 4.12 (td, *J* = 9.2, 5.8 Hz, 1H), 4.62 (ABX, Δ_{AB} = 13.2 Hz, *J* = 15.9, 5.8 Hz, 2H, H11), 4.77 (d, *J* = 4.9 Hz, 1H, OH4‘), 5.03 (d, *J* = 5.8 Hz, 1H, OH3‘), 5.24 (d, *J* = 9.2 Hz, 1H, H1‘), 5.25 (d, *J* = 4.6 Hz, 1H, OH2‘), 6.26 (d, *J* = 2.5 Hz, 1H, H13), 6.37 (s, 1H, H14), 7.55 (d, *J* = 0.9 Hz, 1H, H15), 8.31 (s, 1H, H8), 8.78 (t, *J* = 5.5 Hz, 1H, H11).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 36.7 (C11), 68.5 (C4‘), 68.9 (C2‘), 69.3 (C5‘), 73.3 (C3‘), 83.3 (C1‘), 107.1 (C13), 110.5 (C14), 118.0 (C5), 140.0 (C8), 142.0 (C15), 150.3 (C4), 152.0 (C12), 153.1 (C2), 154.7 (C6).

CK 2-chloro-6-(benzylamino/furfurylamino)purine-9-(*β*)-L-arabinofuranosides

(2*S*,3*R*,4*R*,5*S*)-2-(6-(benzylamino)-2-chloro-9*H*-purin-9-yl)-5-(hydroxymethyl) tetrahydrofuran-3,4-diol (**10**)

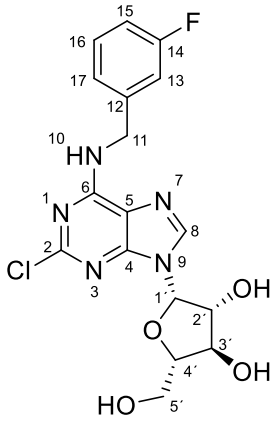


White solid, chemical formula: $C_{17}H_{18}ClN_5O_4$, yield: 61%, EI^+-MS m/z: 392.2, HPLC retention time (min.): 19.01, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.54 (ABX_2, Δ_{AB} = 48.5 Hz, J_{AB} = 12.0 Hz, $J_{AX2,BX2}$ = 5.5 Hz, 2H, H5‘), 3.99 (appear dt, *J* = 10.8, 5.4 Hz, 1H, H3’), 4.13 (appear dt, *J* = 9.1, 5.5 Hz, 1H, H4’), 4.57 (dt, *J* = 5.5, 5.2 Hz, 1H), 4.64 (ABX, Δ_{AB} = 10.1 Hz, *J* = 15.9, 6.4 Hz, 2H, H11), 4.93 (t, *J* = 5.5 Hz, 1H, OH5’), 5.60 (d, *J* = 4.6 Hz, 1H, OH3’), 5.79 (d, *J* = 5.5 Hz, 1H, H1’), 5.80 (d, *J* = 4.9 Hz, 1H, OH2’), 7.22 (t, *J* = 6.7 Hz, 1H), 7.29-7.33 (m, 4H, H13, H14, H16, H17), 8.39 (s, 1H, H8), 8.91 (t, *J* = 6.1 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 43.1 (C11), 61.1 (C5’), 74.9 (C3’), 79.3 (C2’), 85.4 (C4’), 88.2 (C1’), 118.5 (C5), 126.8 (C15), 127.2 (2× C, C13, C17), 128.3 (2× C, C14, C16), 139.2 (C12), 140.4 (C8), 149.6 (C4), 153.2 (C2), 154.9 (C6).

(2*S*,3*R*,4*R*,5*S*)-2-(2-chloro-6-((3-fluorobenzyl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (**11**)

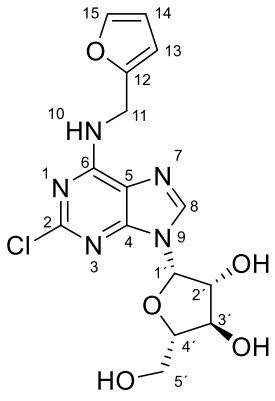


White solid, chemical formula: $C_{17}H_{17}ClFN_5O_4$, yield: 61%, EI^+-MS m/z: 410.1, HPLC retention time (min.): 19.44, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.47-3.51 (m, 1H, H5'), 3.54 (dt, *J* = 11.9, 4.6 Hz, 1H, H5'), 3.99 (appear dt, *J* = 10.2, 5.5 Hz, 1H, H3'), 4.13 (appear dt, *J* = 9.2, 5.5 Hz, 1H, H4'), 4.56 (appear dt, *J* = 10.2, 5.1 Hz, 1H, H2'), 4.64 (ABX, Δ_{AB} = 5.7 Hz, *J* = 15.8, 6.7 Hz, 2H, H11), 4.93 (t, *J* = 5.5 Hz, 1H, OH5'), 5.60 (d, *J* = 4.6 Hz, 1H, OH3'), 5.79 (d, *J* = 4.6 Hz, 1H, H1'), 5.80 (d, *J* = 4.9 Hz, 1H, OH2'), 7.06 (td, *J* = 8.6, 1.8 Hz, 1H, H15), 7.13 (d, *J* = 9.5 Hz, 1H, H13), 7.16 (d, *J* = 8.3 Hz, 1H, H17)), 7.35 (td, *J* = 7.9, 6.4 Hz, 1H, H16), 8.40 (s, 1H, H8), 8.93 (t, *J* = 6.3 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 42.7 (C11), 61.0 (C5'), 74.8 (C3'), 79.3 (C2'), 85.4 (C4'), 88.2 (C1'), 113.6 (d, $^2J_{CF}$ = 21.5 Hz, C15), 113.9 (d, $^2J_{CF}$ = 21.5 Hz, C13), 118.5 (C5), 123.2 (C17), 130.3 (d, $^3J_{CF}$ = 7.2 Hz, C16), 140.5 (C8), 142.2 (d, $^3J_{CF}$ = 4.8 Hz, C12), 149.7 (C4), 153.1 (C2), 154.8 (C6), 162.2 (d, $^1J_{CF}$ = 243.5 Hz, C14).

(2*S*,3*R*,4*R*,5*S*)-2-(2-chloro-6-((furan-2-ylmethyl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (**12**)



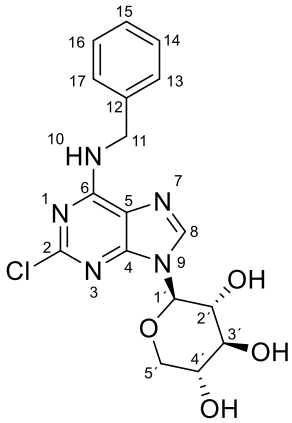
White solid, chemical formula: $C_{15}H_{16}ClN_5O_5$, yield: 38%, EI^+-MS m/z: 382.2, HPLC retention time (min.): 16.47, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.49 (dd, *J* = 11.7, 5.2 Hz, 1H, H5'), 3.59 (dd, *J* = 11.7, 3.3 Hz, 1H, H5'), 3.99 (bs, 1H, H3'), 4.13 (appear td, *J* = 5.5, 3.7 Hz, 1H, H4'), 4.56 (td, *J* = 9.0, 4.5 Hz, 1H, H2'), 4.62 (bd, *J* = 5.5 Hz, 2H, H11), 4.92 (bs, 1H, OH5'), 5.59 (bs, 1H, OH3'), 5.78-5.80 (m, 2H, H1', OH2'), 6.26 (d, *J* = 2.8 Hz, 1H, H13), 6.37 (bs, 1H, H14), 7.55 (d, *J* = 0.9 Hz, 1H, H15), 8.38 (s, 1H, H8), 8.79 (t, *J* = 5.2 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 36.7 (C11), 61.1 (C5'), 74.9 (C3'), 79.3 (C2'), 85.5 (C4'), 88.2 (C1'), 107.1 (C13), 110.5 (C14), 118.5 (C5), 140.5 (C8), 142.0 (C15), 149.7 (C4), 152.0 (C12), 153.0 (C2), 154.7 C6).

CK 2-chloro-6-(benzylamino/furfurylamino)purine-9-(*β*)-D-xylopyranosides

(2*R*,3*R*,4*S*,5*R*)-2-(6-(benzylamino)-2-chloro-9*H*-purin-9-yl)tetrahydro-2*H*-pyran-3,4,5-triol (**13**)

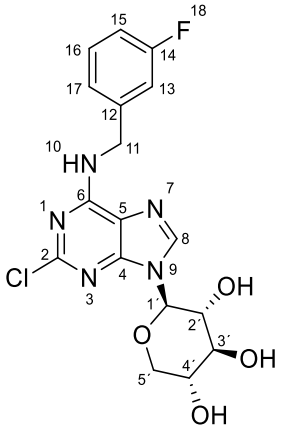


White solid, chemical formula: $C_{17}H_{18}ClN_5O_4$, yield: 45%, EI^+-MS m/z: 392.2, HPLC retention time (min.): 19.02, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.30-3.34 (m, 2H, H3', H5'), 3.46-3.52 (m, 1H, H4'), 3.83 (dd, *J* = 11.6, 5.5 Hz, 1H, H5'), 3.93 (td, *J* = 8.8, 5.5 Hz, 1H, H2'), 4.64 (ABX, Δ_{AB} = 9.9 Hz, *J* = 15.2, 6.4 Hz, 2H, H11), 5.19 (d, *J* = 5.2 Hz, 1H, OH4'), 5.27 (d, *J* = 9.2 Hz, 1H, H1'), 5.35 (d, *J* = 4.6 Hz, 1H, OH3'), 5.38 (d, *J* = 5.8 Hz, 1H, OH2'), 7.22 (tt, *J* = 6.7, 2.1 Hz, 1H, H15), 7.28-7.33 (m, 4H, H13, H14, H16, H17), 8.37 (s, 1H, H8), 8.88 (t, *J* = 6.1 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 43.1 (C11), 68.4 (C4', 69.2 (C2'), 71.2 (C5'), 77.1 (C3'), 83.7 (C1'), 118.1 (C5), 126.8 (C15), 127.2 (2× C, C13, C17), 128.3 (2× C, C14, C16), 139.2 (C12), 140.4 (C8), 150.1 (C4), 153.2 (C2), 154.9 (C6).

(2*R* 3*R*,4*S*,5*R*)-2-(2-chloro-6-((3-fluorobenzyl)amino)-9*H*-purin-9-yl)tetrahydro-2*H*-pyran-3,4,5-triol (**14**)

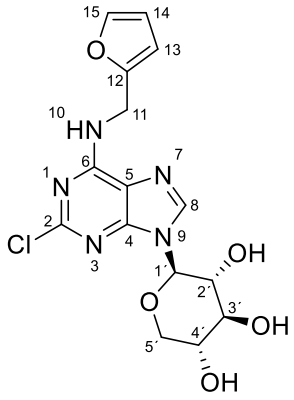


White solid, chemical formula: $C_{17}H_{17}ClFN_5O_4$, yield: 21%, EI^+-MS m/z: 410.3, HPLC retention time (min.): 19.47, purity: 99.5%.

^{1}H-NMR (500 MHz, DMSO-*d*6) δ (ppm): 3.29-3.38 (m, 2H, H3', H5'), 3.45-3.51 (m, 1H, H4'), 3.82 (dd, *J* = 11.2, 5.5 Hz, 1H, H4'), 3.92 (td, *J* = 8.8, 6.4 Hz, 1H, H2'), 4.64 (bd, *J* = 6.1 Hz, 2H, H11), 5.17 (d, *J* = 4.9 Hz, 1H, OH4'), 5.27 (d, *J* = 9.2 Hz, 1H, H1'), 5.34 (d, *J* = 4.6 Hz, 1H, OH3'), 5.36 (d, *J* = 5.8 Hz, 1H, OH2'), 7.06 (td, *J* = 8.5, 2.1 Hz, 1H, H15), 7.13 (d, *J* = 9.8 Hz, 1H, H13), 7.16 (d, *J* = 7.9 Hz, 1H, H17), 7.35 (td, *J* = 7.9, 6.1 Hz, 1H, H16), 8.37 (s, 1H, H8), 8.90 (t, *J* = 5.8 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-*d*6) δ (ppm): 42.7 (C11), 68.4 (C5'), 69.1 (C4'), 71.1 (C2'), 77.0 (C3'), 83.7 (C1'), 113.6 (d, $^2J_{CF}$ = 21.5 Hz, C15), 113.9 (d, $^2J_{CF}$ = 21.5 Hz, C13), 118.2 (C5), 123.2 (C17), 130.3 (d, $^3J_{CF}$ = 8.4 Hz, C16), 140.5 (C8), 142.2 (d, $^3J_{CF}$ = 7.2 Hz, C13), 150.1 (C4), 153.1 (C2), 154.8 (C6), 162.2 (d, $^1J_{CF}$ = 243.5 Hz, C14).

(2*R* 3*R*,4*S*,5*R*)-2-(2-chloro-6-((furan-2-ylmethyl)amino)-9*H*-purin-9-yl)tetrahydro-2*H*-pyran-3,4,5-triol (**15**)



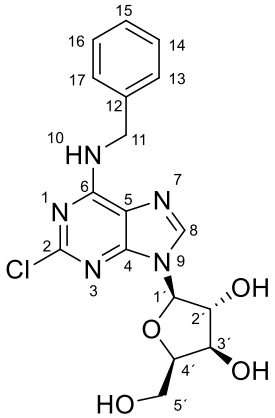
White solid, chemical formula: $C_{15}H_{16}ClN_5O_5$, yield: 67%, EI^+-MS m/z: 382.1, HPLC retention time (min.): 16.52, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-*d6*) δ (ppm): 3.30-3.33 (m, 2H, H3', H5'), 3.45-3.51 (m, 1H, H4'), 3.83 (dd, *J* = 11.3, 5.5 Hz, 1H, H5'), 3.92 (td, *J* = 8.8, 6.1 Hz, 1H, H2'), 4.62 (bd, *J* = 5.5 Hz, 2H, H11), 5.19 (d, *J* = 4.9 Hz, 1H, OH4'), 5.27 (d, *J* = 9.2 Hz, 1H, H1'), 5.34 (d, *J* = 4.3 Hz, 1H, OH3'), 5.37 (d, *J* = 5.5 Hz, 1H, OH2'), 6.26 (d, *J* = 3.0 Hz, 1H, H13), 6.37 (bs, 1H, H14), 7.55 (d, *J* = 0.9 Hz, 1H, H15), 8.36 (s, 1H, H8), 8.78 (t, *J* = 5.5 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 36.7 (C11), 68.4 (C5'), 69.2 (C4'), 71.1 (C2'), 77.1 (C3'), 83.7 (C1'), 107.1 (C13), 110.5 (C14), 118.2 (C5), 140.5 (C8), 142.0 (C15), 150.2 (C4), 152.0 (C12), 153.1 (C2), 154.7(C6).

CK 2-chloro-6-(benzylamino/furfurylamino)purine-9-(*β*)-D-xylofuransides

(2*R*,3*R*,4*R*,5*R*)-2-(6-(benzylamino)-2-chloro-9*H*-purin-9-yl)-5-(hydroxymethyl) tetrahydrofuran-3,4-diol (**16**)

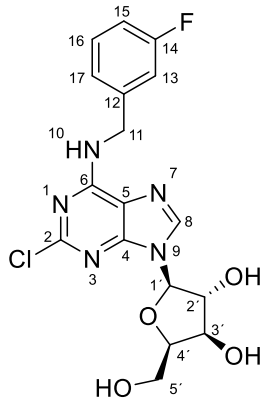


White solid, chemical formula: $C_{17}H_{18}ClN_5O_4$, yield: 42%, EI^+-MS m/z: 392.2, HPLC retention time (min.): 19.25, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.70 (ABX_2, Δ_{AB} = 39.2 Hz, J_{AB} = 11.6 Hz, $J_{AX2,BX2}$ = 5.8 Hz, 2H, H5'), 4.04 (bs, 1H, H3'), 4.17 (appear dt, *J* = 9.0, 5.1 Hz, 1H, H4'), 4.29 (bs, 1H, H2'), 4.63 (ABX, Δ_{AB} = 13.2 Hz, *J* = 15.2, 6.4 Hz, 2H, H11), 4.76 (t, *J* = 5.5 Hz, 1H, OH5'), 5.55 (d, *J* = 4.2 Hz, 1H, OH3'), 5.80 (d, *J* = 1.2 Hz, 1H, H1'), 5.93 (d, *J* = 4.0 Hz, 1H, OH2'), 7.22 (t, *J* = 6.7 Hz, 1H, H15), 7.28-7.33 (m, 4H, H13, H14, H16, H17), 8.26 (s, 1H, H8), 8.91 (t, *J* = 6.2 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 43.1 (C11), 59.4 (C5'), 74.8 (C3'), 80.7 (C2'), 83.9 (C4'), 89.2 (C1'), 118.1 (C5), 126.8 (C15), 127.3 (2×C, C13, C17), 128.3 (2× C, C14, C16), 139.2 (C12), 140.0 (C8), 149.3 (C4), 153.1 (C2), 154.9 (C6).

(2*R*,3*R*,4*R*,5*R*)-2-(2-chloro-6-((3-fluorobenzyl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (**17**)

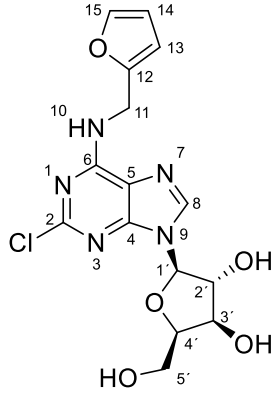


White solid, chemical formula: $C_{17}H_{17}ClFN_5O_4$, yield: 51%, EI^+-MS m/z: 410.2, HPLC retention time (min.): 19.44, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.70 (ABX_2, Δ_{AB} = 39.5 Hz, J_{AB} = 11.3 Hz, $J_{AX2,BX2}$ = 6.11 Hz, 2H, H5‘), 4.04 (bs, 1H, H3‘), 4.17 (appear dt, *J* = 9.7, 5.0 Hz, 1H, H4‘), 4.28 (bs, 1H, H2‘), 4.64 (ABX, Δ_{AB} = 14.7 Hz, *J* = 15.2, 6.1 Hz, 2H, H11), 4.74 (t, *J* = 5.8 Hz, 1H, OH5‘), 5.52 (d, J = 4.3 Hz, 1H, OH3‘), 5.81 (d, J = 1.5 Hz, 1H, H1‘), 5.91 (d, J = 3.9 Hz, 1H, OH2‘), 7.05 (td, *J* = 8.6, 2.1 Hz, 1H, H15), 7.13 (d, *J* = 10.1 Hz, 1H, H17) 7.16 (d, *J* = 7.6 Hz, 1H, H13), 7.35 (td, *J* = 7.8, 6.4 Hz, 1H, H16), 8.28 (s, 1H, H8), 8.94 (t, *J* = 6.1 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-D6) δ (ppm): 42.72 (C11), 59.4 (C5‘), 74.7 (C3‘), 80.6 (C2‘), 83.9 (C4‘), 89.2(C1‘), 113.6 (d, $^2J_{CF}$ = 21.5 Hz, C13), 113.9 (d, $^2J_{CF}$ = 21.5 Hz, C15), 118.1 (C5), 123.2 (C17), 130.2 (d, $^3J_{CF}$ = 7.2 Hz, C16), 140.0 (C8), 142.20 (d, $^3J_{CF}$ = 7.2 Hz, C12), 149.4 (C4), 153.0 (C2), 154.8 (C6), 161.2 (d, $^1J_{CF}$ = 243.5 Hz, C14).

(2*R*,3*R*,4*R*,5*R*)-2-(2-chloro-6-((furan-2-ylmethyl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (**18**)



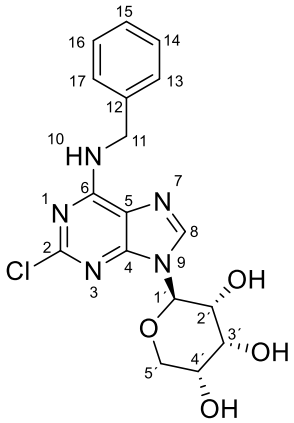
White solid, chemical formula: $C_{15}H_{16}ClN_5O_5$, yield: 51%, EI^+-MS m/z: 382.2, HPLC retention time (min.): 16.48, purity: 97.2%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.70 (ABX_2, Δ_{AB} = 39.5 Hz, J_{AB} = 11.6 Hz, $J_{AX2,BX2}$ = 5.8 Hz, 2H, H5‘), 4.04 (td, *J* = 4.0, 1.5 Hz, 1H, H3’), 4.17 (appear dt, *J* = 9.7, 5.0 Hz, 1H, H4’), 4.28 (dt, *J* = 3.7, 2.1 Hz, 1H, H2’), 4.61 (ABX, Δ_{AB} = 13.4 Hz, *J* = 15.8, 5.8 Hz, 2H, H11), 4.74 (t, *J* = 5.4 Hz, 1H, OH5’), 5.52 (d, *J* = 4.0 Hz, 1H, OH3’), 5.81 (bs, 1H, H1’), 5.91 (d, *J* = 4.3 Hz, 1H, OH2’), 6.26 (d, *J* = 2.8 Hz, 1H, H13), 6.37 (bs, 1H, H14), 7.55 (dd, *J* = 1.5, 0.6 Hz, 1H, H15), 8.25 (s, 1H, H8), 8.81 (t, *J* = 5.6 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 36.6 (C11), 59.4 (C5’), 74.7 (C3’), 80.6 (C2’), 83.9 (C4’), 89.2 (C1’), 107.1 (C13), 110.5 (C14), 118.1 (C5), 140.0 (C8), 142.0 (C15), 149.4 (C4), 152.0 (C12), 153.0 (C2), 154.6 (C6).

CK 2-chloro-6-(benzylamino/furfurylamino)purine-9-(*β*)-D-ribopyranosides

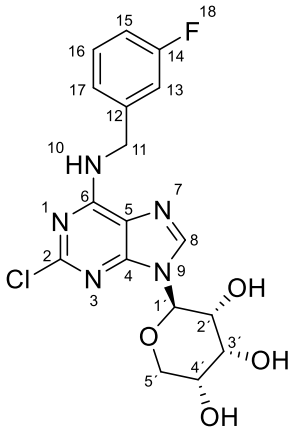
(2*R*,3*R*,4*R*,5*R*)-2-(6-(benzylamino)-2-chloro-9*H*-purin-9-yl)tetrahydro-2*H*-pyran-3,4,5-triol (**19**)



White solid, chemical formula: $C_{17}H_{18}ClN_5O_4$, yield: 51%, EI^+-MS m/z: 392.2, HPLC retention time (min.): 18.67, purity: 99.0%. **^{1}H-NMR** (500 MHz, DMSO-d_6) δ (ppm): 3.59 (dd, *J* = 9.6, 4.6 Hz, 1H, H5'), 3.66 (t, *J* = 10.4 Hz, 1H, H4'), 3.70-3.73 (m, 1H, H5'), 4.02 (bs, 1H, H3'), 4.14 (td, *J* = 9.5, 2.1 Hz, 1H, H2'), 4.64 (ABX, Δ_{AB} = 9.9 Hz, *J* = 15.0, 6.4 Hz, 2H, H11), 4.92 (d, *J* = 6.1 Hz, 1H, OH4'), 5.13 (d, *J* = 7.3 Hz, 1H, OH2'), 5.17 (d, *J* = 3.7 Hz, 1H, OH3'), 5.54 (d, *J* = 9.5 Hz, 1H, H1'), 7.22 (t, *J* = 6.4 Hz, 1H, H15), 7.28-7.33 (m, 4H, H13, H14, H16, H17), 8.36 (s, 1H, H8), 8.87 (t, *J* = 6.1 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 43.1 (C11), 65.2 (C5'), 66.6 (C4'), 68.3 (C2'), 71.1 (C3'), 79.7 (C1'), 118.1 (C5), 126.8 (C15), 127.2 (2× C, C13, C17), 128.3 (2× C, C14, C16), 139.2 (C12), 140.3 (C8), 150.3 (C4), 153.2 (C2), 154.9 (C6).

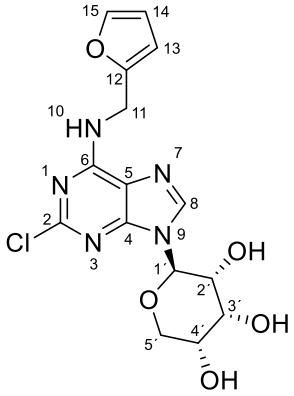
(2*R*,3*R*,4*R*,5*R*)-2-(2-chloro-6-((3-fluorobenzyl)amino)-9*H*-purin-9-yl)tetrahydro-2*H*-pyran-3,4,5-triol (**20**)



White solid, chemical formula: $C_{17}H_{17}ClFN_5O_4$, yield: 35%, EI^+-MS m/z: 410.2, HPLC retention time (min.): 19.14, purity: 99.2%. **^{1}H-NMR** (500 MHz, DMSO-d_6) δ (ppm): 3.59 (dd, *J* = 9.5, 4.8 Hz, 1H, H5'), 3.66 (t, *J* = 11.0 Hz, 1H, H4'), 3.68-3.73 (m, 1H, H5'), 4.02 (bs, 1H, H3'), 4.13 (td, *J* = 9.0, 2.1 Hz, 1H, H2'), 4.64 (bd, *J* = 5.8 Hz, 2H, C11), 4.92 (d, *J* = 6.4 Hz, 1H, OH4'), 5.13 (d, *J* = 7.0 Hz, 1H, OH2'), 5.16 (d, *J* = 3.7 Hz, 1H, OH3'), 5.55 (d, *J* = 9.5 Hz, 1H, H1'), 7.05 (td, *J* = 8.7, 2.1 Hz, 1H, H15), 7.13 (d, *J* = 9.8 Hz, 1H, H17), 7.16 (d, *J* = 8.3 Hz, 1H, H13), 7.35 (td, *J* = 7.8, 6.1 Hz, 1H, H16), 8.37 (s, 1H, H8), 8.89 (t, *J* = 6.1 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 42.7 (C11), 65.2 (C5'), 66.5 (C4'), 68.3 (C2'), 71.1 (C3'), 79.8 (C1'), 113.6 (d, $^2J_{CF}$ = 21.5 Hz, C13), 113.9 ($^2J_{CF}$ = 21.5 Hz, C15), 118.1 (C5), 123.2 (C17), 130.3 (d, $^3J_{CF}$ = 8.4 Hz, C16), 140.4 (C8), 142.2 (d, $^3J_{CF}$ = 8.4 Hz, C12), 150.4 (C4), 153.1(C2), 154.8(C6), 162.1 (d, $^1J_{CF}$ = 243.5 Hz, C14).

(2*R*,3*R*,4*R*,5*R*)-2-(2-chloro-6-((furan-2-ylmethyl)amino)-9*H*-purin-9-yl)tetrahydro-2*H*-pyran-3,4,5-triol (**21**)



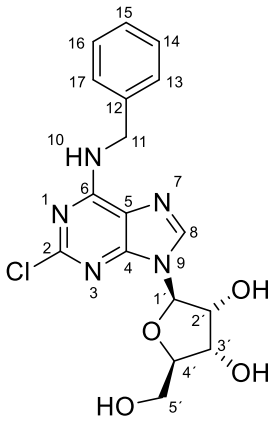
White solid, chemical formula: $C_{15}H_{16}ClN_5O_5$ yield: 40%, EI^+-MS m/z: 382.1, HPLC retention time (min.): 15.98, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.59 (dd, *J* = 9.6, 4.6 Hz, 1H, H5'), 3.66 (t, *J* = 10.4 Hz, 1H, H4'), 3.68-3.73 (m, 1H, H5'), 4.01 (bs, 1H, H3'), 4.13 (td, *J* = 8.3, 2.5, 1H, H2'), 4.62 (bd, *J* = 4.6 Hz, 2H, H11), 4.92 (d, *J* = 6.1 Hz, 1H, OH4'), 5.14 (d, *J* = 7.0 Hz, 1H, OH2'), 5.17 (d, *J* = 3.4 Hz, 1H, OH3'), 5.54 (d, *J* = 9.2 Hz, 1H, H1'), 6.26 (d, *J* = 3.1 Hz, 1H, H13), 6.37 (bs, 1H, H14), 7.55 (dd, *J* = 1.5, 0.6 Hz, 1H, H15), 8.36 (s, 1H, H8), 8.77 (t, *J* = 5.5 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 36.6 (C11), 65.2 (C5'), 66.6 (C4'), 68.3 (C2'), 71.1 (C3'), 79.8 (C1'), 107.1 (C13), 110.5 (C14), 118.2 (C5), 140.4 (C8), 142.0 (C15), 150.4 (C4), 152.0 (C12), 153.1 (C2), 154.7 (C6).

CK 2-chloro-6-(benzylamino/furfurylamino)purine-9-(*β*)-D-ribofuranosides

(2*R*,3*R*,4*S*,5*R*)-2-(6-(benzylamino)-2-chloro-9*H*-purin-9-yl)-5-(hydroxymethyl) tetrahydrofuran-3,4-diol (**22**)

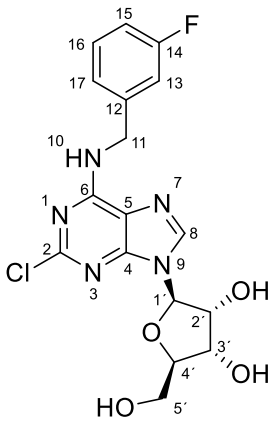


White solid, chemical formula: $C_{17}H_{18}ClN_5O_4$, yield: 56%, EI^+-MS m/z: 392.2, HPLC retention time (min.): 19.36, purity: 99.4%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.54 (dt, *J* = 11.6, 4.3 Hz 1H, H5'), 3.65 (dt, *J* = 11.9, 4.6 Hz, 1H, H5'), 3.93 (appear dt, *J* = 3.7, 3.4 Hz, 1H, H4'), 4.12 (appear dt, *J* = 4.6, 3.7 Hz, 1H, H3'), 4.51 (dt, *J* = 5.8, 5.5 Hz, 1H, H2'), 4.64 (ABX, Δ_{AB} = 13.2 Hz, *J* = 15.6, 6.7 Hz, 2H, H11), 5.05 (t, *J* = 5.8 Hz, 1H, OH5'), 5.22 (d, *J* = 4.9 Hz, 1H, OH3'), 5.49 (d, *J* = 6.1 Hz, 1H, OH2'), 5.82 (d, *J* = 5.8 Hz, 1H, H1'), 7.22 (t, *J* = 6.7 Hz, 1H, H15), 7.28-7.33 (m, 4H, H13, H14, H16, H17), 8.41 (s, 1H, H8), 8.93 (t, *J* = 6.1 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 43.1 (C11), 61.3 (C5'), 70.3 (C3'), 73.6 (C2'), 85.7 (C4'), 87.4 (C1'), 118.6 (C5), 126.8 (C15), 127.2 (2× C, C13, C17), 128.3 (2× C, C14, C16), 139.1 (C12), 140.1 (C8), 149.6 (C4), 153.1 (C2), 154.9 (C6).

(2*R*,3*R*,4*S*,5*R*)-2-(2-chloro-6-((3-fluorobenzyl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (**23**)

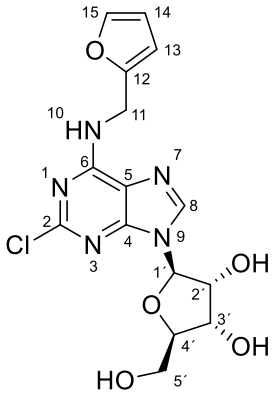


White solid, chemical formula: $C_{17}H_{17}ClFN_5O_4$, yield: 44%, EI^+-MS m/z: 410.2, HPLC retention time (min.): 19.80, purity: 96.8%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.54-3.57 (m, 1H, H5'), 3.65-3.68 (m, 1H, H5'), 3.95 (bs, 1H, H4'), 4.14 (bs, 1H, H3'), 4.52 (bd, J = 4.5 Hz, 1H, H2'), 4.66 (bs, 2H, H11), 5.07 (t, J = 4.6 Hz, 1H, OH5'), 5.22 (d, J = 4.6 Hz, 1H, OH3'), 5.50 (d, J = 5.8 Hz, 1H, OH2'), 5.84 (d, J = 5.8 Hz, 1H, H1'), 7.04 (t, J = 7.5 Hz, 1H, H15), 7.14 (d, J = 9.2 Hz, 1H, H13), 7.16 (d, J = 8.0 Hz, 1H, H17), 7.34 (td, J = 7.0, 6.7 Hz, 1H, H16), 8.43 (s, 1H, H8), 8.95 (t, J = 5.8 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 42.7 (C11), 61.3 (C5'), 70.4 (C3'), 73.7 (C2'), 85.7 (C4'), 87.5 (C1'), 113.61 (d, $^2J_{CF}$ = 20.3 Hz, C15), 114.0 (d, $^2J_{CF}$ = 21.5 Hz, C13) 118.6 (C5), 123.2 (C17), 130.3 (d, $^3J_{CF}$ = 7.2 Hz, C16), 140.2 (C8), 142.2 (d, $^3J_{CF}$ = 6.0 Hz, C12), 149.7 (C4), 153.1 (C2), 154.9 (C6), 161.2 (d, $^1J_{CF}$ = 243.5 Hz, C14).

(2*R*,3*R*,4*S*,5*R*)-2-(2-chloro-6-((furan-2-ylmethyl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (**24**)



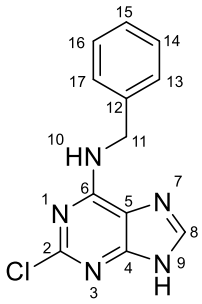
White solid, chemical formula: $C_{15}H_{16}ClN_5O_5$, yield: 65%, EI^+-MS m/z: 382.1, HPLC retention time (min.): 16.96, purity: 99.7%.

^{1}H-NMR (500 MHz, DMSO-*d6*) δ (ppm): 3.55 (dd, J = 12.1 Hz, 3.4 Hz, 1H, H5'), 3.66 (dd, J = 11.5, 3.1 Hz, 1H, H5'), 3.94 (appear dt, J = 7.3, 3.7 Hz, 1H, H4'), 4.13 (bs, 1H, H3'), 4.52 (bs, 1H, H2'), 4.63 (bd, J = 4.9 Hz, 2H, H11), 5.07 (bs, 1H, OH5'), 5.22 (bs, 1H, OH3'), 5.50 (bs, 1H, OH2'), 5.83 (d, J = 5.5 Hz, 1H, H1'), 6.26 (d, J = 2.7 Hz, 1H, H13), 6.37 (bs, 1H, H14), 7.55 (d, J = 0.9 Hz, 1H, H15), 8.42 (s, 1H, H8), 8.83 (t, J = 5.5 Hz, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 36.7 (C11), 61.3 (C5'), 70.3 (C3'), 73.7 (C2'), 85.7 (C4'), 87.4 (C1'), 107.1 (C13), 110.5 (C14), 118.7 (C5), 140.2 (C8), 142.0 (C15), 149.8 (C4), 151.9 (C12), 153.0 (C2), 154.7 (C6).

2-chloro-6-(benzylamino/furfurylamino)purines

N-benzyl-2-chloro-9*H*-purin-6-amine (**25**)

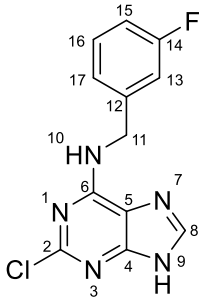


White solid, chemical formula: $C_{12}H_{10}ClN_5$, yield: 75%, EI^+-MS m/z: 260.2, HPLC retention time (min.): 23.9, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 4.63 (d, J = 5.5 Hz, 2H, H11), 7.22 (bs, 1H, H15), 7.31 (t, J = 7.0 Hz, 2H, H14, H16), 7.33 (d, J = 6.7 Hz, 2H, H13, H17), 8.13 (s, 1H, H8), 8.74 (bs, 1H, H10), 13.11 (bs, 1H, H9).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 43.0 (C11), 117.8 (C5), 126.8 (C15), 127.3 (2× C, C13, C17), 128.3 (2× C, C14, C16), 139.4 (C12), 139.6 (C8), 150.5 (C4), 152.9 (C2), 154.7 (C6).

2-chloro-*N*-(3-fluorobenzyl)-9*H*-purin-6-amine (**26**)

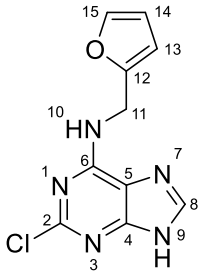


White solid, chemical formula: $C_{12}H_9ClFN_5$, yield: 73%, EI^+-MS m/z: 278.1, HPLC retention time (min.): 20.12, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 4.64 (d, J = 3.1 Hz, 2H, H11), 7.06 (t, J = 7.6 Hz, 1H, H15), 7.14 (d, J = 10.1 Hz, 1H, H13), 7.17 (d, J = 7.6 Hz, 1H, H17), 7.35 (q, J = 7.2 Hz, 1H, H16), 8.15 (s, 1H, H8), 8.69 (bs, 1H, H10), 13.06 (bs, 1H, H9).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 42.6 (C11), 113.6 (d, $^2J_{CF}$ = 20.3 Hz, C15), 114.0 (d, $^2J_{CF}$ = 21.5 Hz, C13), 117.9 (C5), 123.3 (C17), 130.3 (d, $^3J_{CF}$ = 8.4 Hz, C16), 139.7 (C8), 142.4 (d, $^3J_{CF}$ = 6.0 Hz, C12), 150.6 (C4), 152.8 (C2), 154.7 (C6), 162.1 (d, $^1J_{CF}$ = 243.5 Hz, C14).

2-chloro-*N*-(furan-2-ylmethyl)-9*H*-purin-6-amine (**27**)



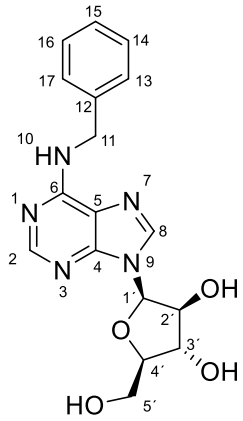
White solid, chemical formula: $C_{10}H_8ClN_5O$, yield: 89%, EI^+-MS m/z: 250.2, HPLC retention time (min.): 16.94, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 4.62 (bs, 2H, H11), 6.27 (s, 1H, H13), 6.38 (s, 1H, H14), 7.56 (s, 1H, H15), 8.14 (s, 1H, H8), 8.55 (bs, 1H, H10), 13.02 (s, 1H, H9).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 36.6 (C11), 107.0 (C13), 110.4 (C14), 117.8 (C5), 139.5 (C8), 141.9 (C15), 150.7 (C4), 152.1 (C2), 152.6 (C12), 154.5 (C6).

CK 6-benzylaminopurine-9-(*β*)-D-arabinofuranoside and its intermediate

(2*R*,3*S*,4*S*,5*R*)-2-(6-(benzylamino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (**28**)

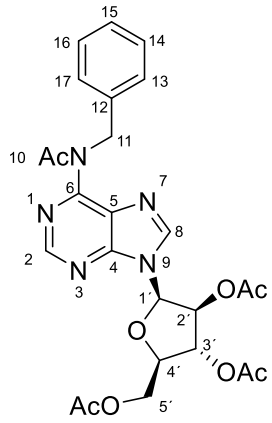


White solid, chemical formula: $C_{17}H_{19}N_5O_4$, yield: 17.2%, EI^+-MS m/z: 358.3, HPLC retention time (min.):15.24 , purity: 99.07%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 3.64 (ABX_2, Δ_{AB} = 21.9 Hz, J_{AB} = 11.9 Hz, J_{AX2} = 5.2 Hz, J_{BX2} = 4.8 Hz, 2H, H5‘), 3.76 (td, *J* = 4.5, 4.2 Hz, 1H, H4‘), 4.11-4.15 (m, 2H, H2‘, H3‘), 4.69 (bd, *J* = 15.9 Hz, 2H, H11), 5.10 (t, *J* = 5.5 Hz, 1H, OH5‘), 5.53 (d, *J* = 4.6 Hz, 1H, OH3‘), 5.62 (d, *J* = 4.9 Hz, 1H, OH2‘), 6.25 (d, *J* = 4.3 Hz, 1H, H1‘), 7.17-7.21 (m, 1H, H15), 7.27 (t, *J* = 7.6 Hz, 2H, H14, H16), 7.32 (d, *J* = 7.3 Hz, 2H, H13, H17), 8.18 (bs, 1H, H2), 8.20 (s, 1H, H8), 8.38 (bs, 1H, H10).

^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 42.8 (C11), 60.8 (C5‘), 74.8 (C3‘), 75.6 (C2‘), 83.5 (C4‘), 84.0 (C1‘), 118.6 (C5), 126.5 (C15), 127.1 (2× C, C13, C17), 128.2 (2× C, C14, C16), 140.2 (2× C, C8, C12), 148.9 (C4), 152.3 (C2), 154.2 (C6).

(2*R*,3*R*,4*S*,5*R*)-2-(acetoxymethyl)-5-(6-(*N*-benzylacetamido)-9*H*-purin-9-yl) tetrahydrofuran-3,4-diyl diacetate (**VIII**)



White solid, chemical formula: $C_{25}H_{27}N_5O_8$, yield 25%: EI^+-MS m/z: 358.24, HPLC retention time (min.):15.24, purity: 100%.

^{1}H-NMR (500 MHz, DMSO-d_6) δ (ppm): 1.55 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.21 (s, 3H, OAc), 4.30-4.39 (m, 3H, H4‘, H5‘), 5.39 (s, 2H, H11), 5.60 (dd, *J* = 5.8, 5.2 Hz, 1H, H3‘), 5.67 (dd, *J* = 5.8, 5.5, 1H, H2‘), 6.68 (d, *J* = 5.8 Hz, 1H, H1‘), 7.09-7.15 (m, 1H, H15), 7.17-7.18 (m, 4H, H13, H14, H16, H17), 8.72 (s, 1H, H2/8), 8.82 (s, 1H, H2/8).

Results of physicochemical properties of the prepared 2-chloro-6-substituted purine-*N*9-glycosides and appropriate 2-chloro-6-substituted purines. HPLC-retention time of the substance (min.) and its spectrophotometric purity (%), MS-positively charged molecular ion, HRMS analysis

comp.	monosaccharide	C6 substituent	HPLC [min, %]	MS $[M+H]^+$	yield [%]	HRMS			
						calculated mass $[M+H]^+$	measured mass $[M+H]^+$	molecular formula	fidelity (ppm)
1	*α*-D-arabinopyranose	BAP	18.43, 98	392.2	70	391.1047	391.1046	$C_{17}H_{18}ClN_5O_4$	-0.24
2	*α*-D-arabinopyranose	3FBAP	19.10,100	410.3	33	409.0953	409.0955	$C_{17}H_{17}ClFN_5O_4$	0.36
3	*α*-D-arabinopyranose	Kin	15.67, 100	382.3	67	381.0840	381.0843	$C_{15}H_{16}ClN_5O_5$	0.75
7	*α*-L-arabinopyranose	BAP	18.66, 100	392.2	67	391.1047	391.1050	$C_{17}H_{18}ClN_5O_4$	0.67
8	*α*-L-arabinopyranose	3FBAP	19.07, 100	410.2	57	409.0953	409.0956	$C_{17}H_{17}ClFN_5O_4$	0.8
9	*α*-L-arabinopyranose	Kin	15.75, 100	382.3	56	381.0840	381.0844	$C_{15}H_{16}ClN_5O_5$	0.94
13	*β*-D-xylopyranose	BAP	19.02, 100	392.2	45	391.1047	391.1051	$C_{17}H_{18}ClN_5O_4$	1.01
14	*β*-D-xylopyranose	3FBAP	19.47, 99.5	410.3	21	409.0953	409.0958	$C_{17}H_{17}ClFN_5O_4$	1.18
15	*β*-D-xylopyranose	Kin	16.52, 100	382.1	67	381.0840	381.0845	$C_{15}H_{16}ClN_5O_5$	1.27
19	*β*-D-ribopyranose	BAP	18.67, 99	392.2	51	391.1047	391.1051	$C_{17}H_{18}ClN_5O_4$	0.83
20	*β*-D-ribopyranose	3FBAP	19.14, 99.2	410.2	35	409.0953	409.0957	$C_{17}H_{17}ClFN_5O_4$	0.85
21	*β*-D-ribopyranose	Kin	15.98, 100	382.1	40	381.0840	381.0844	$C_{15}H_{16}ClN_5O_5$	0.93
4	*β*-D-arabinofuranose	BAP	18.98, 100	392.2	35	391.1047	391.1050	$C_{17}H_{18}ClN_5O_4$	0.77
5	*β*-D-arabinofuranose	3FBAP	19.31, 99.7	410.2	32	409.0953	409.0958	$C_{17}H_{17}ClFN_5O_4$	1.09
6	*β*-D-arabinofuranose	Kin	16.40, 100	382.1	58	381.0840	381.0845	$C_{15}H_{16}ClN_5O_5$	1.09
10	*β*-L-arabinofuranose	BAP	19.01, 100	392.2	61	391.1047	391.1049	$C_{17}H_{18}ClN_5O_4$	0.38
11	*β*-L-arabinofuranose	3FBAP	19.44, 100	410.1	61	409.0953	409.0958	$C_{17}H_{17}ClFN_5O_4$	1.27
12	*β*-L-arabinofuranose	Kin	16.47, 100	382.2	38	381.0840	381.0846	$C_{15}H_{16}ClN_5O_5$	1.64
16	*β*-D-xylofuranose	BAP	19.25, 100	392.2	42	391.1047	391.1052	$C_{17}H_{18}ClN_5O_4$	1.28
17	*β*-D-xylofuranose	3FBAP	19.44, 100	410.2	51	409.0953	409.0954	$C_{17}H_{17}ClFN_5O_4$	0.12
18	*β*-D-xylofuranose	Kin	16.48, 97.2	382.2	51	381.0840	381.0844	$C_{15}H_{16}ClN_5O_5$	1,1
22	*β*-D-ribofuranose	BAP	19.36, 99.4	392.2	56	391.1047	391.1048	$C_{17}H_{18}ClN_5O_4$	0.23
23	*β*-D-ribofuranose	3FBAP	19.80, 96.8	410.2	44	409.0953	409.0957	$C_{17}H_{17}ClFN_5O_4$	0.93
24	*β*-D-ribofuranose	Kin	16.96, 99.7	382.1	65	381.0840	381.0844	$C_{15}H_{16}ClN_5O_5$	1.1
25	H	BAP	23.9, 100	260.2	75	259.0625	259.0623	$C_{12}H_{10}ClN_5$	-0.6
26	H	3FBAP	20.12, 100	278.1	73	277.0530	277.0531	$C_{12}H_9ClFN_5$	-0.06
27	H	Kin	16.94, 100	250.2	89	249.0419	249.0417	$C_{10}H_8ClN_5O$	0.55

Appendix I

Research article

C2-substituted aromatic cytokinin sugar conjugates delay the onset of senescence by maintaining the activity of the photosynthetic apparatus

Vylíčilová, H., Husičková, A., Spíchal, L., Srovnal, J., Doležal, K., Plíhal, O., Plíhalová, L.

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C2-substituted aromatic cytokinin sugar conjugates delay the onset of senescence by maintaining the activity of the photosynthetic apparatus

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ABSTRACT

Cytokinins are plant hormones with biological functions ranging from coordination of plant growth and development to the regulation of senescence. A series of 2-chloro-N^6-(halogenobenzylamino)purine ribosides was prepared and tested for cytokinin activity in detached wheat leaf senescence, tobacco callus and *Amaranthus* bioassays. The synthetic compounds showed significant activity, especially in delaying senescence in detached wheat leaves. They were also tested in bacterial receptor bioassays using both monocot and dicot members of the cytokinin receptor family. Most of the derivatives did not trigger cytokinin signaling *via* the AHK3 and AHK4 receptors from *Arabidopsis thaliana* in the bacterial assay, but some of them specifically activated the ZmHK1 receptor from *Zea mays* and were also more active than the aromatic cytokinin BAP in an *ARR5::GUS* cytokinin bioassay using transgenic *Arabidopsis* plants. Whole transcript expression analysis was performed using an *Arabidopsis* model to gather information about the reprogramming of gene transcription when senescent leaves were treated with selected C2-substituted aromatic cytokinin ribosides. Genome-wide expression profiling revealed that the synthetic halogenated derivatives induced the expression of genes related to cytokinin signaling and metabolism. They also prompted both up- and down-regulation of a unique combination of genes coding for components of the photosystem II (PSII) reaction center, light-harvesting complex II (LHCII), and the oxygen-evolving complex, as well as several stress factors responsible for regulating photosynthesis and chlorophyll degradation. Chlorophyll content and fluorescence analyses demonstrated that treatment with the halogenated derivatives increased the efficiency of PSII photochemistry and the abundance of LHCII relative to DMSO- and BAP-treated controls. These findings demonstrate that it is possible to manipulate and fine-tune leaf longevity using synthetic aromatic cytokinin analogs.

1. Introduction

Cytokinins (CKs) are an important group of plant hormones that control many physiological, metabolic and developmental processes in plants (for a review, see e.g. Spichal, 2012). They stimulate cell division and differentiation in the presence of other phytohormones, such as auxins. In addition, they promote the formation and activity of shoot apical meristems, participate in *de novo* bud formation, control apical dominance, inhibit root growth and branching, delay leaf senescence, and stimulate seed germination (Mok et al., 2000; Riefler et al., 2006). It has also been shown that CKs can modulate plants' responses to various pathogenic infections (Bari and Jones, 2009). Naturally occurring CKs are

Abbreviations: EA, elemental analysis; ESI–MS, electrospray ionization–mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin layer chromatography; BAP, 6-benzylaminopurine; MS medium, Murashige–Skoog medium; CKs, cytokinins; iP, N^6-isopentenyladenine; BAPR, 6-benzylaminopurine riboside; ArCKs, aromatic cytokinins; tZ, 6-(4-hydroxy-3-methylbut-2-enylamino)purine; *meta*-topolin, 6-(3-hydroxybenzylamino)purine; AHK, *Arabidopsis* histidine kinase; ARR, *Arabidopsis* response regulator; GUS, β-glucuronidase; RMA, robust multi-array average; RuBisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; CKX, cytokinin dehydrogenase; LHCII, light harvesting complex II; PSII, photosystem II; kinetin, 6-furfurylaminopurine; P_N, net photosynthetic rate; F_M, maximal fluorescence; F_V, variable fluorescence; ZmHK, *Zea mays* histidine kinases; qP, photochemical quenching.

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0031-9422/

purine derivatives with an isoprenoid or aromatic side chain bound to the N6 atom of the adenine moiety. N^6-isopentenyladenine (iP) and *trans*-zeatin (tZ) are typical isoprenoid CKs; notable aromatic CKs include 6-benzylaminopurine (BAP), the topolins (hydroxylated BAP derivatives), and kinetin (6-furfurylaminopurine) (Mok and Mok, 2001). In plant tissues, CKs may be present in the form of free bases but they occur more frequently as nucleosides or nucleotides. In addition, they can be conjugated to glucose or xylose at various positions of the adenine ring (Auer, 1997; Frebort et al., 2011).

N^6-(2-hydroxybenzylamino)purine-β-D-riboside, the first known aromatic CK, was initially isolated from fully expanded poplar (*populus* × *robusta* Schneid) leaves (Horgan et al., 1975). For a long time, it was believed that aromatic CKs (ArCKs) were merely biologically inactive degradation products (Strnad et al., 1997). However, Strnad and co-workers isolated and identified various natural ArCKs from mature poplar leaves, including 6-(3-hydroxybenzylamino)purine and its 9-β-D-ribofuranosyl or 9-β-D-glucopyranosyl conjugates (Strnad et al., 1992; Strnad, 1997). Hydroxylated ArCK derivatives were subsequently prepared and their activity was tested in three classical cytokinin bioassays: the tobacco callus, *Amaranthus*, and wheat leaf senescence bioassays (Holub et al., 1998). Structure–activity relationship analyses revealed that the biological activity of these aromatic CKs was heavily dependent on the presence and position of the hydroxyl groups on the benzyl ring, and on their ribosylation or glucosylation at the N9 position of the purine moiety (Holub et al., 1998). A large family of BAP and BAP riboside (BAPR) analogs with various substituents on the benzyl ring was also prepared and screened in the standard cytokinin bioassays. Some of these compounds exhibited particularly high activities in the senescence bioassay, being up to twofold more efficient than BAP itself at delaying the onset of senescence (Dolezal et al., 2006, 2007).

Leaf senescence is one of the final stages of plant development. It is a highly regulated degradation process that involves changes in cell structure, metabolism, gene expression, and also environmental signals. In particular, it is associated with substantial changes in the expression of genes involved in degrading proteins into amino acids and membrane lipids into fatty acids and sugars (Sarwat et al., 2013). Plant senescence involves enhanced chlorophyll catabolism activity and the degradation of leaf proteins, membrane lipids and RNA; these processes are largely responsible for the color changes of autumn leaves. There is extensive crosstalk between plant hormones such as CKs, auxins, ethylene and abscisic acid, which establishes an endogenous regulatory pathway for age-controlled senescence (Lim et al., 2007). CKs play an essential role in this process by delaying the onset of senescence. In *Arabidopsis*, the AHK3 receptor seems to play a major role in regulating cytokinin-mediated leaf longevity because it promotes the phosphorylation of the cytokinin response regulator ARR2 (Kim et al., 2006). In this context it is interesting that bacterial receptor assays have shown that the AHK3 and AHK4 receptors have important differences in their ligand preferences (Lomin et al., 2015; Spichal et al., 2004). While iP and tZ are preferred ligands for both receptors, only AHK3 is activated by CK ribosides and ribotides. In addition, AHK3 also has a higher affinity for BAP and other ArCKs, which generally exhibit low activity in bacterial receptor tests despite their significant biological activity *in planta* (Spichal et al., 2004).

The anti-senescent activity of CKs has been recognized since 1957, when Richmond and Lang showed that treatment with exogenous kinetin delayed chlorophyll breakdown and extended the lifespans of detached cocklebur leaves and cut carnation flowers (Eisinger, 1977; Richmond and Lang, 1957). These effects were subsequently found to be heavily dependent on the light conditions: CKs delayed leaf senescence under dark conditions but may have accelerated chlorophyll degradation under strong illumination (Gan and Amasino, 1996; Vlckova et al., 2006). The onset of leaf senescence may be related to the quantity of reactive oxygen species in the plant cells, which increases as the cells age (BuchananWollaston, 1997; Prochazkova and Wilhelmova, 2009). It was reported that CKs may retard senescence by scavenging or interfering with free radicals (Synkova et al., 2006). In keeping with this hypothesis, treatment with exogenous BAP reduced superoxide radical production, improved the quenching of hydrogen peroxide, protected the photosynthetic system and supported carbohydrate production (Xiaotao et al., 2013). It was also shown that treatment with synthetic kinetin derivatives significantly protected lipid membranes against the negative influence of accumulated reactive oxygen species in the dark phase (Mik et al., 2011). Recently, Li et al. developed a database of genes potentially associated with leaf senescence (the Leaf Senescence Database, a gene network for identifying common regulators of leaf senescence in *Arabidopsis thaliana*). It is now widely accepted that phytohormones such as CKs play a critical role in regulating senescence (Li et al., 2012; Sarwat et al., 2013).

As mentioned above, CKs are also involved in the control of photosynthesis. Treatment with exogenous CKs usually delays senescence-induced changes such as the decline in chlorophyll content, and reduces the values of photosynthetic parameters such as the net photosynthetic rate (P_N) (Čatský et al., 1996; Gan and Amasino, 1995; Rulcová and Pospíšilová, 2001), the photochemical chlorophyll fluorescence quenching (qP), and the maximal photochemical efficiency of photosystem II (PSII) as measured by the variable-to-maximal fluorescence ratio (F_V/F_M) (for a review, see e.g. Synková et al., 1997). Moreover, a recent study showed that adding aromatic cytokinins (BAP, BAPR, or *meta*-topolin) to culture media induced changes in the chlorophyll a and b contents of apple leaves *in vitro* and also affected the capacity of the photosynthetic apparatus as determined by fluorescence measurements conducted after three weeks of cultivation (Dobránszki and Mendler-Drienyovszki, 2014). Importantly, Cortleven et al. showed that plants with reduced cytokinin levels are more susceptible to photodamage due to the malfunctioning of photosystem II and deregulation of the associated photoprotective mechanisms (Cortleven et al., 2014).

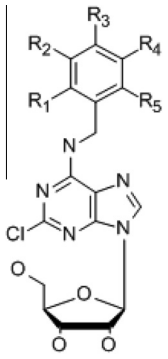
Here we report the synthesis of a library of 2-chloro-N^6-(halogenobenzylamino)purine riboside ArCK sugar conjugates, tests of their activity in standardized cytokinin bioassays, and analyses of their effects on the photosynthetic apparatus. To clarify the molecular mechanisms underpinning the observed physiological changes in senescent *Arabidopsis* leaves, we performed a gene expression study using the two most active of these compounds. The results revealed that treatment with active ArCKs alters the expression of several genes that are closely related to photosynthesis and which encode important anti-stress factors.

2. Results and discussion

2.1. Chemistry

A series of 2,6-disubstituted ArCK derivatives (see Table 1) was prepared by the reaction of 2,6-dichloropurine riboside or 2,6-dichloropurine with suitable benzylamines in the presence of triethylamine in *n*-propanol. The yields of these reactions along with the purities of the prepared substances and their ESI–MS and NMR data are given in the supplementary data section. All of the tested compounds were dissolved in DMSO and subsequently diluted in water or buffer for use in the biological experiments. Consequently, a 0.05% solution of DMSO in water was used as a negative control in all of the assays.

Table 1
Structures and abbreviations of the synthesised compounds.



Compound	R_1	R_2	R_3	R_4	R_5
1	**F**	H	H	H	H
2	H	**F**	H	H	H
3	H	H	**F**	H	H
4	**Cl**	H	H	H	H
5	H	**Cl**	H	H	H
6	H	H	**Cl**	H	H
7	**Br**	H	H	H	H
8	H	**Br**	H	H	H
9	H	H	**Br**	H	H
10	H	**I**	H	H	H
11	H	H	**I**	H	H
12	H	**Cl**	**Cl**	H	H
13	**Cl**	H	**Cl**	H	H
14	**Cl**	H	H	H	**F**

2.2. Cytokinin bioassays

The biological effects of the newly prepared compounds were tested in three classical cytokinin bioassays based on cytokinin-sensitive processes in plant tissues: the tobacco callus bioassay, the *Amaranthus* bioassay, and the detached wheat leaf senescence assay (Table 2). In the detached wheat leaf senescence bioassay, the treatment-induced delay of senescence is measured by monitoring the retardation of chlorophyll degradation in detached leaves that are kept under constant dark conditions for 4 days. The results compiled in Table 2 show the activities of the prepared compounds relative to that of the natural aromatic cytokinin BAP. Derivatives **1**–**3**, **5**, **6**, **8**, and **9** all caused significantly greater retardation of chlorophyll degradation than was achieved with BAP. In particular, compounds **2**, **3**, and **6** were almost twice as active as BAP. The most active derivatives were those bearing a fluorine substituent anywhere on the benzyl ring (**1**–**3**), derivatives with a chlorine atom in the *meta* or *para* positions (**5**, **6**), and those with a bromine atom in the *meta* or *para* positions (**8**, **9**). Derivatives containing an iodine atom in the *meta* or *para* positions (**10**, **11**) and those bearing multiple halogen substituents on the benzyl ring (**12**, **13**) were generally less active than BAP.

These findings are consistent with previous studies from our laboratories, in which we showed that various 6-substituted BAPR derivatives and fluorinated derivatives were more active in the detached wheat leaf senescence bioassay than other halogenated compounds, regardless of the location of the fluorine substituent on the benzyl ring (Dolezal et al., 2007). Halogenated ArCKs may exhibit enhanced biological activity because derivatives bearing appropriately sized electron-withdrawing substituents (fluoro and chloro but not iodo) in suitable positions on the benzyl ring can enter the binding pocket of the CHASE receptor domain and form more favorable hydrogen bonds with electron donors in the binding site (Dolezal et al., 2006, 2007). Steric hindrance may also be responsible for the relatively low biological activity of disubstituted halogenated adenosine derivatives **12**–**14** (Table 2): it is possible that ArCKs whose benzyl rings carry two or more substituents encounter severe steric barriers when binding to the CK receptor.

In summary, these results confirm that ArCK derivatives bearing substituents at the N9 position of the purine moiety tend to exhibit high biological activity, preventing chlorophyll degradation and delaying the onset of senescence. This is consistent with our previous reports (Dolezal et al., 2007; Mik et al., 2011; Szucova et al., 2009). To gain insight into the molecular mechanism through which ArCKs exert their anti-senescent effects on senescent leaves, the compounds showing the greatest activity in the senescence assay, i.e. derivatives **3** and **6**, were selected for further screening *in planta*. The selection of these compounds was motivated by their high activity and the fact that their halogen substituents are located in the same position on the benzyl ring, making it possible to directly compare the effects of substitution with different halogens on bioactivity.

2.3. Cytokinin signaling

The precise biological effects of individual CK derivatives are probably determined by differences in their perception (and potentially their metabolic activation) in the tissues of different plants. CKs exert their effects by interacting with cytokinin receptors. Three membrane-bound cytokinin receptors have been identified in *Arabidopsis*: AHK2, AHK3 and AHK4. All three are sensor histidine kinases containing a ligand-binding CHASE (cyclases/histidine kinases-associated sensory extracellular) domain (Heyl and Schmülling, 2003). The three analogous cytokinin receptors in maize are designated ZmHK1, ZmHK2 and ZmHK3a (Yonekura-Sakakibara et al., 2004). To assess the activity of our newly synthesized ArCK derivatives toward individual CK receptors, we used a bacterial receptor assay in which the *Arabidopsis* cytokinin receptors AHK3 and AHK4 and the maize receptors ZmHK1 and ZMHK3a were functionally expressed in *Escherichia coli* strain KMI001

Table 2
The activity of the synthetic cytokinin derivatives in the tobacco callus and *Amaranthus* bioassays and the detached wheat leaf senescence bioassay relative to that of the control compound (BAP) at the optimal concentration (i.e. the concentration where the maximum positive effect of the tested compound was observed). All values were determined from two independent assays each performed in six technical replicates.

Comp.	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 (%)
1	10^{-5}	103.7 ± 0.8	10^{-4}	110.7 ± 3.1	10^{-4}	152.8 ± 23.9
2	10^{-5}	102.7 ± 1.2	10^{-4}	107.3 ± 1.6	10^{-4}	181.5 ± 18.3
3	10^{-5}	102. 4 ± 3.7	10^{-4}	102.8 ± 8.8	10^{-4}	195.9 ± 30.3
4	10^{-5}	102.6 ± 1.7	10^{-4}	126.7 ± 2.5	10^{-4}	105.8 ± 9.7
5	10^{-5}	103.7 ± 7.0	10^{-4}	105.51 ± 4.2	10^{-4}	176.6 ± 5.9
6	10^{-5}	94.8 ± 0.6	10^{-4}	75.4 ± 6.8	10^{-4}	183.9 ± 50.4
7	10^{-5}	100.5 ± 1.8	10^{-4}	127.8 ± 8.2	10^{-4}	64.4 ± 7.1
8	10^{-6}	77.6 ± 11.9	10^{-4}	134.8 ± 3.4	10^{-4}	153.2 ± 33.8
9	10^{-5}	103.7 ± 0.8	10^{-4}	110.7 ± 3.1	10^{-4}	152.8 ± 23.9
10	10^{-5}	87.9 ± 10.2	10^{-4}	137.7 ± 2.6	10^{-4}	87.1 ± 47.1
11	10^{-4}	63.7 ± 5.5	10^{-4}	41.8 ± 5.6	10^{-4}	27.1 ± 12.2
12	10^{-5}	39.1 ± 6.0	10^{-4}	94.1 ± 13.8	10^{-4}	71.7 ± 10.9
13	10^{-4}	84.1 ± 7.3	10^{-4}	43.9 ± 4.9	10^{-4}	9.2 ± 6.6
14	10^{-4}	81.5 ± 16.1	10^{-4}	63.6 ± 6.3	10^{-4}	56.9 ± 18.9

(Podlesakova et al., 2012; Spichal et al., 2004). This assay enables precise quantification of the receptors' responses to CK treatment, using β-galactosidase as a reporter gene (Yamada et al., 2001).

We initially examined the activation of the *Arabidopsis* receptors AHK3 and AHK4. These receptors together with maize receptors discussed below were selected because they show good responses to ArCK treatments in our bacterial assays which is in agreement with previously published results (Spichal et al., 2004; Yonekura-Sakakibara et al., 2004). Most of the prepared compounds, including **3** and **6**, did not activate cytokinin signaling *via* the AHK4 receptor and only weakly activated the AHK3 receptor at higher concentrations (Fig. S1, Supplementary data). This finding is consistent with the results of an earlier study from our laboratories on BAP, Kin and *ortho*-topolin (Spichal et al., 2004), in which we found that BAP and (to a lesser extent) BAPR were capable of activating AHK3 but had negligible activity toward the AHK4 receptor. Thus, CK ribosides seem to be biologically active forms whose activity can be further enhanced *in planta* by metabolic activation. In keeping with these conclusions, compounds **3** and **6** showed high biological activity in all of the cytokinin assays performed in this work but especially in the senescence assay, as discussed above.

We also tested the new compounds' ability to trigger CK signaling in monocots using the ZmHK1 and ZmHK3a receptors as models. The maize cytokinin receptors were more sensitive to our ArCKs in the bacterial assay than their *Arabidopsis* analogs. ZmHK3a was weakly activated by some of the tested ligands at higher concentrations, but ZmHK1 was strongly activated by several compounds at concentrations as low as 0.01 μM (Fig. S1, Supplementary data). As shown in Figs. 1, 3 and 6 were not active toward ZmHK3a at any tested concentration, but did activate ZmHK1 (with EC50 for **3**, **6** and tZ being 5.82, 1.77 and 0.20 μM, respectively). This was expected because previous studies have shown that maize cytokinin receptors are generally more sensitive to ArCKs than those from *Arabidopsis*; the best known activators of ZmHK1 are BAP and iP (Lomin et al., 2011, 2015).

Compounds **3** and **6** were also evaluated *in planta* using the P_{ARR5}*::GUS* reporter assay in *Arabidopsis* plants, which assesses ligands' ability to induce the expression of a receptor-controlled reporter gene (Romanov et al., 2002). Both compounds were found to be capable of inducing cytokinin pathway activity *in planta*; their activity exceeded that of BAP by 40–70% (Fig. 1C).

In conclusion, the halogenated ArCK riboside derivatives were shown to interact with cytokinin receptors *in vitro*. However, we could not exclude the possibility that they might be metabolically activated by conversion into the free base in plant tissues, particularly given that ribosides are considered to be the transport forms of CKs (for a review, see Kudo et al., 2010). It is thus possible that sensing and signaling mediated by these ArCKs may be regulated by transport processes and the gradual release of the active free base. To further confirm that the tested compounds can induce cytokinin responses, their activity was verified *in planta* using the P_{ARR5}*::GUS* reporter assay.

2.4. Regulation of gene expression in senescent Arabidopsis leaves

Using BAP and DMSO treatments as references, we performed genome-wide expression profiling of senescent *Arabidopsis* leaves treated with compounds **3** and **6** in order to better understand the regulation of senescence by ArCKs at the molecular level. Compounds **3** and **6** were selected because they exhibited the highest anti-senescence activity in the detached wheat leaf bioassay (Table 2). Expression changes were monitored in detached senescent *Arabidopsis* leaves after 48 h of incubation in darkness with a 10 μM solution of one of the tested compounds or with DMSO alone; a complete list of DE genes from the three treatments (i.e. treatment with **3**, **6** and BAP) is shown in the Supplementary Table S1. Transcriptome profiling was performed using a standardized procedure developed for the detached leaf assay, which has previously been used to investigate the genetic effects of treatment with Kin derivatives that exhibit anti-senescent activity (Mik et al., 2011). While these Kin derivatives were effective anti-senescence agents under both light and constant dark conditions, their senescence-delaying activity was greatest in darkness.

Hierarchical clustering analysis of the resulting data sets revealed that the samples treated with specific cytokinins clustered together and exhibited low variability in their responses, as did the mock-treated wild type *Arabidopsis* leaf samples (Fig. 2A). Interestingly, the BAP-treated samples formed the most distinct group and had a gene expression profile that differed significantly from those for all of the other groups. In contrast, the gene expression profiles for the groups treated with **3** and **6** were quite similar. This is readily apparent in the heat map shown in Fig. 2B, which presents data for 8659 genes whose expression changed significantly after treatment with **3**, **6** or BAP (P value $\leqslant$0.01 in at least one treatment). To limit the number of target genes, we adopted more stringent statistical criteria for identifying genes whose expression had changed significantly. Specifically, the data were RMA normalized and genes were required to have a signal ratio change $\log_2$ of $\geqslant$0.5 or $\leqslant$−0.5 in addition to a P-value of <0.01; see the Methods section for details. In this way we defined a group of 1128 genes whose expression changed after treatment with **3** (of which 510 were upregulated and 618 downregulated), and 944

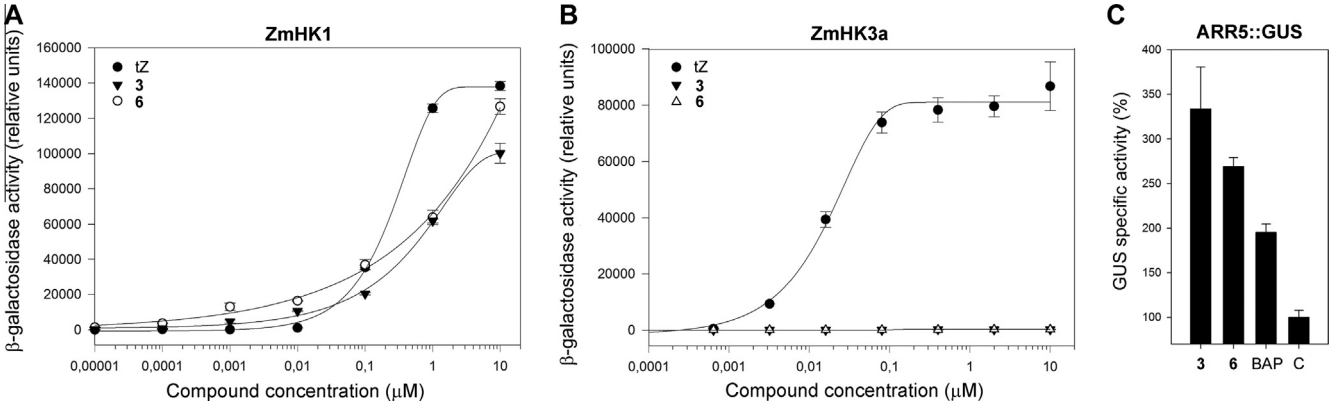


Fig. 1. Cytokinin-dependent expression of β-galactosidase in *E. coli* (ΔrcsC, *cps::lacZ*) expressing ZmHK1 (A) and ZmHK3a (B). tZ was used as a positive control; error bars show SD values for three replicates. Relative upregulation of GUS expression is shown in comparison to a DMSO-treated control (C); error bars show SD values for three replicates. Three-day-old transgenic seedlings were incubated with the tested compounds at a concentration of 10 μM.

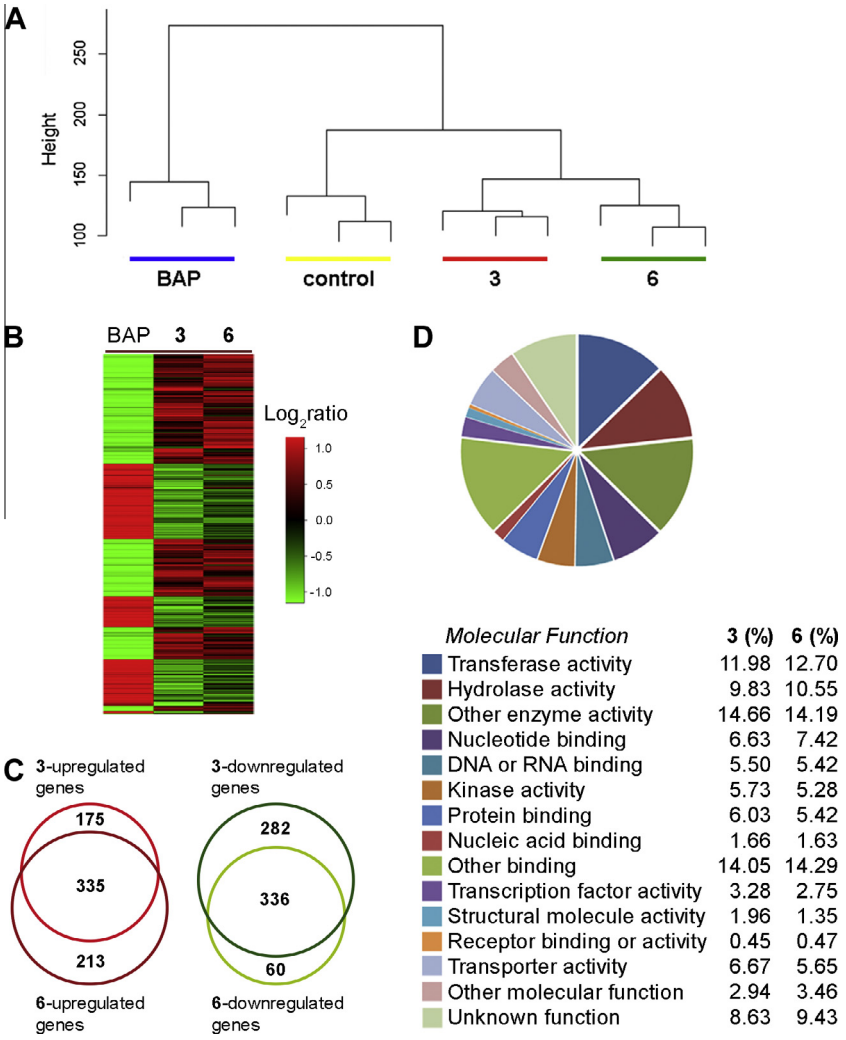


Fig. 2. Transcriptome reprogramming mediated by cytokinin derivatives as revealed by DNA microarray analysis (Affymetrix). Hierarchical clustering was performed for all data sets, i.e. *Arabidopsis* leaves treated with BAP, compound 3, compound 6, and DMSO alone (A). Clustering analysis of genes exhibiting differential expression after treatment with BAP, compound 3 or compound 6 (B). Venn diagrams of genes regulated by 3 and genes regulated by 6, showing the substantial overlap of the two sets (C). GO classification of genes regulated by 3 and 6 (D); genes were categorized into groups according to their GO terms, based on their predicted or putative functions.

genes whose expression changed upon treatment with **6** (548 upregulated, 396 downregulated). These two groups overlapped extensively: there were 671 genes common to both (Fig. 2C; Table S2, Supplementary data). To better understand the molecular functions of these genes, we categorized the transcripts in both groups according to their GO terms (Ashburner et al., 2000) using categories such as 'transcription factor activity', 'DNA or RNA binding', and 'protein binding' (Fig. 2D). This analysis indicated that the affected genes were rather evenly distributed over the defined categories.

We then examined the genes whose expression changed significantly in the three datasets (i.e. in senescent leaves treated with **3**, **6** or BAP) in more detail. This analysis revealed that the cytokinin derivatives had distinct modes of action to those observed for the parent free bases. In keeping with previous reports (Brandstatter and Kieber, 1998; Rashotte et al., 2003), many cytokinin-related genes were upregulated in the BAP-treated leaves (Fig. 3; Table S2, Supplementary data). Importantly, these included the cytokinin response regulators *ARR7*, *ARR9*, *ARR5*, *ARR6* and *ARR4*. Exposure to high concentrations of BAP also prompted the induction of several cytokinin dehydrogenase genes including *CKX1*, *CKX2*, *CKX3* and *CKX4*. Upregulation of response regulators, several *CKX* genes, and other cytokinin response genes was also observed in senescent leaves treated with **3** or **6**.

It is interesting to compare our results to those of a recent meta-analysis of microarray data reported by various laboratories, which identified a core list of cytokinin response genes (Brenner et al., 2012). The results of both this meta-analysis and a search of the Genevestigator database (https://genevestigator.com/gv/) conducted by ourselves indicate that tZ treatment leads to rapid reprogramming of gene expression in *Arabidopsis*. Specifically, cytokinin response regulators such as *ARR15*, *ARR5*, *ARR16*, *ARR7*, *ARR4*, *ARR6* and *ARR9* were strongly upregulated in response to tZ treatment. Other cytokinin-responsive genes identified in the meta-analysis and database search include *CKX4* and *CKX5* (which code for two cytokinin dehydrogenase isoforms), *AHK4* and *AHK1*, *CRF5* or *CYP735A2*, and *CYP82F1*. This group of core cytokinin-responsive genes clearly overlaps extensively with the list of genes whose expression was altered significantly following treatment with **3**, **6**, or BAP (Fig. 3). It is also consistent with the cytokinin bioassay results presented in the preceding section and thus confirms that the halogenated aromatic cytokinin derivatives considered in this work are indeed active cytokinins whose signaling effects partially mirror those of BAP and tZ.

Function	Affymetrix ID	AGI	Description	3	6	BAP
Photosystem II / Photosystem I related	13407918	At2g05070	Photosystem II light harvesting complex protein 2.2 (LHCB2.2)	2.84	2.88	
	13514872	At5g54270	Light harvesting chlorophyll B-binding protein 3 (LHCB3)	2.02	2.23	-1.31
	13467069	At4g10340	Photosystem II light harvesting complex protein 5 (LHCB5)	1.35	1.36	
	13368591	At1g15820	Light harvesting complex photosystem II subunit 6 (LHCB6)	1.27	1.58	-1.50
	13348790	At1g44575	Non-photochemical quenching 4 (NPQ4)	1.43	2.30	
	13422843	At3g01440	Photosystem II oxygen-evolving enhancer protein (PQL1)	1.81	2.25	
	13460363	At3g55330	Photosystem II reaction center PsbP family protein (PPL1)	1.80	2.01	
	13402772	At2g39470	Photosystem II reaction center PsbP family protein 2 (PPL2)	2.42	2.02	0.96
	13336710	At1g06680	Oxygen-evolving enhancer protein 2-1 (PSBP-1)	1.20	1.34	
	13346044	At1g29930	Chlorophyll A/B binding protein 1 (CAB1)	1.38	1.41	
	13496423	At5g02120	One helix protein (OHP)	1.29	1.62	
	13460163	At3g54890	Photosystem I light harvesting complex protein 1 (LHCA1)		1.72	
	13351098	At1g52230	Photosystem I subunit H-2 (PSAH2)	1.13	1.36	-1.33
	13379948	At1g55670	Photosystem I subunit G (PSAG)	1.03	1.03	-0.97
	13346202	At1g30380	Photosystem I subunit K (PSAK)	1.24	1.42	
Calvin cycle	13533250	At5g38430	RuBisCO small chain 1B (RBCS1B)	2.97	3.59	
	13425558	At3g07670	RuBisCO methyltransferase-like protein	1.01	1.35	
	13516095	At5g57180	Chloroplast import apparatus 2 (CIA2)	1.99	2.13	
	13346956	At1g32060	Phosphoribulokinase (PRK)	1.90	1.79	
	13367225	At1g12900	Glyceraldehyde-3-phosphate dehydrogenase (GAPA-2)	1.50	1.69	
	13348538	At1g42970	Glyceraldehyde-3-phosphate dehydrogenase B subunit (GAPB)	3.34	3.59	1.67
	13411334	At2g21330	Fructose-bisphosphate aldolase (FBA1)	1.91	2.14	
	13495533	At4g38970	Fructose-bisphosphate aldolase (FBA2)	1.85	2.05	
Chlorophyll / LHCII degradation	13435643	At3g44880	Pheophorbide a oxygenase / Accelerated cell death (PAO/ACD1)	-1.41	-1.02	-0.98
	13488234	At4g22920	Non-yellowing protein 1 (NYE1)	-2.07	-1.84	
	13467648	At4g11910	Non-yellowing protein 2 (NYE2)	-3.52	-2.74	-3.25
	13483892	At4g13250	Non-yellow coloring protein 1 (NYC1)	-1.53	-1.25	
	13497670	At5g04900	Chlorophyll b reductase (NOL)	-1.63	-1.23	-2.39
	13525779	At5g13800	Pheophytinase (PPH)	-1.39	-1.21	
Cytokinin signaling and metabolism	13369913	At1g19050	Cytokinin response regulator 7 (ARR7)	5.83	4.68	4.86
	13440390	At3g57040	Cytokinin response regulator 9 (ARR9)	4.05	3.49	3.08
	13387433	At1g74890	Cytokinin response regulator 15 (ARR15)	4.86	3.05	2.19
	13542701	At5g62920	Cytokinin response regulator 6 (ARR6)	3.82	3.53	2.53
	13403683	At2g41310	Cytokinin response regulator 8 (ARR8)	2.87	2.44	1.41
	13366187	At1g10470	Cytokinin response regulator 4 (ARR4)	2.62	2.24	2.01
	13457365	At3g48100	Cytokinin response regulator 5 (ARR5)	2.98	2.41	2.84
	13469404	At4g16110	Cytokinin response regulator 2 (ARR2)	-1.94	-1.77	-1.20
	13472529	At4g23750	Cytokinin response factor 2 (CRF2)	2.43	1.84	
	13405743	At2g46310	Cytokinin response factor 5 (CRF5)	1.77		
	13406995	At2g01830	Cytokinin receptor CRE1/AHK4	1.46	1.12	
	13403813	At2g41510	Cytokinin dehydrogenase 1 (CKX1)	-1.39		-1.97
	13540259	At5g56970	Cytokinin dehydrogenase 3 (CKX3)	2.13		3.12
	13475311	At4g29740	Cytokinin dehydrogenase 4 (CKX4)	2.93	2.12	1.80
	13498118	At5g05860	Cytokinin-N-glucosyltransferase 2 (UGT76C2)	2.29	1.61	1.99
Other plant hormones related	13405141	At2g44990	Carotenoid cleavage dioxygenase 7 (CCD7)	-3.59	-3.95	-3.39
	13476816	At4g32810	Carotenoid cleavage dioxygenase 8 (CCD8)	-5.86	-4.24	-5.12
	13541306	At5g59220	Senescence-associated gene 113 (SAG113)	-1.36	-1.72	-1.11
	13363859	At1g05560	UDP-glucosyltransferase 1 (UGT1)	-2.39	-2.17	
	13357654	At1g70940	PIN-formed 3 (PIN3)	1.42	2.10	-1.45
	13502287	At5g16530	PIN-formed 5 (PIN5)	-2.08	-1.65	-1.45
	13474175	At4g27260	Indole-3-acetic acid amido synthetase (GH3.5/WES1)	-2.71	-2.71	-1.10
	13345472	At1g28130	Indole-3-acetic acid amido synthetase (GH3.17)	-3.27	-3.14	-3.22
	13433059	At3g25290	Auxin-responsive family protein	-2.68	-2.18	-1.19
	13539385	At5g54510	Indole-3-acetic acid amido synthetase (DFL1)	-2.41	-2.11	-1.88
	13405267	At2g45210	Senescence-associated gene 201 (SAG201)	-1.66	-1.50	-1.42

Fig. 3. Selected genes identified in differential expression analyses of detached *Arabidopsis* leaves treated with cytokinin derivatives. Senescent leaves were incubated in darkness for 48 h in MS medium containing 10 μM **3**, **6**, or BAP and their gene expression profiles were compared to those for DMSO-treated control leaves of the same age. Gene expression values were calculated as $\log_2$-ratios of the signals for the treated samples relative to those for the control leaves. The heat map is color-coded using the scheme presented in the figure; genes shown in gray were not significantly affected. Only genes related to photosynthesis (specifically those related to photosystem I/II, the Calvin cycle, and chlorophyll/LHCII catabolism) and cytokinin/other plant hormone signaling, with expression changes significant at the $P < 0.01$ level and $\log_2$ fold change values of $\geqslant 1$ or $\leqslant -1$ for at least one treatment are shown.

Our analysis also revealed some genes that were only affected by treatment with **3** and **6**, most of which were directly or indirectly linked to photosynthesis. Some of these genes are listed in Fig. 3, in which the most significant hits are categorized according to their function in cytokinin signaling and metabolism or photosynthesis and related categories. Importantly, genes encoding components of the photosystem II light harvesting complex (LHCII), namely At2g05070, At5g54270, At1g44575, At3g01440, At3g55330 and At2g39470, were upregulated by treatment with **3** and **6**. In contrast, BAP treatment had mostly negligible effects on these genes. In addition, our *in silico* analyses using the Genevestigator database confirmed that these genes are probably not affected by tZ treatment in *Arabidopsis* seedlings: treatments with 1 μm tZ solution for 30 min, 1 h, or 3 h had almost no measurable effects on the expression of any photosystem II-related gene. As mentioned above, the different modes of action of **3**, **6** and BAP may not be directly attributable to differential activation of cytokinin response regulators because all three of these ligands upregulated most of the *ARR* genes to a similar degree. However, *ARR8* and *ARR15* were found to be less upregulated in samples treated with BAP than those treated with **3** or **6** (Fig. 3). This may be important because *ARR15* is a negative regulator of AHK4-mediated cytokinin signal transduction whose expression is particularly strong in roots (Kiba et al., 2003). Therefore, negative regulation of the cytokinin signaling machinery may diminish some of the negative effects associated with exogenous cytokinin treatment at higher concentrations. However, we cannot exclude the possibility that the tested cytokinin derivatives may activate multiple signaling

pathway(s) simultaneously, some of which may be closely related to the cytokinin pathway. One of these may be the auxin signaling pathway: we found several auxin-related genes that were differentially regulated by treatment with **3** and **6,** including *PIN3* and *PIN5* (Fig. 3). Another example is downregulation of the auxin-responsive gene At2g45210, which codes for senescence-associated gene 201 (SAG201), a positive regulator of senescence.

We also found a group of genes involved in chlorophyll degradation that were downregulated in response to treatment with **3** and **6** (Fig. 3; Table S2, Supplementary data). Several of these genes were previously described as leaf anti-senescent markers that respond to auxin, cytokinin and some other molecules that regulate leaf senescence (Li et al., 2012). This group included At4g13250, which codes for a protein that is involved in LHCII degradation in rice, non-yellow coloring protein 1 (NYC1) (Kusaba et al., 2007). It also included genes encoding other chlorophyll/LHCII catabolic reductases such as At4g22920, which codes for non-yellowing protein 1 (NYE1), and At4g11910, which codes for non-yellowing protein 2 (NYE2). The latter gene was also downregulated in response to BAP treatment together with At3g44880, which codes for Pheophorbide A oxygenase/accelerated cell death 1 (PAO/ACD1).

In conclusion, we have collected evidence that selected cytokinin derivatives have similar signaling outputs to their parent free bases in general but also exhibit selectively modulated anti-senescence activity. This modulation is primarily due to upregulation of genes coding for the subunits of LHCII and LHCI and downregulation of genes that are responsible for LHCII and chlorophyll degradation.

2.5. Photosynthetic pigments levels and efficiency of photosystem II

Having determined that the tested ligands (**3**, **6**, and BAP) had different effects on the expression of photosynthetic genes, we investigated their effects on the functioning of the photosynthetic apparatus. Detached *Arabidopsis* leaves kept in darkness were treated with the three compounds separately (and with DMSO as a control). The control leaves exhibited a gradual decrease in their chlorophyll content and the maximum quantum efficiency of PSII photochemistry (F_V/F_M) but this decrease was retarded for leaves treated with the newly prepared derivatives (Fig. 4), particularly those treated with **6**.

The initial rate of variable fluorescence rise (dV/dt_0) for leaves treated with **6** was significantly greater than that for the other leaves (Fig. 4). This parameter reflects the maximal rate of accumulation of closed PSII reaction centers (Strasser et al., 2000) and may be linked to a greater supply of excitations to the PSII reaction centers due to an increase in the relative abundance of the components of its antenna system. The results of the chlorophyll analysis were thus consistent with the gene expression analysis, which suggested that treatment with either **3** or **6** increased the abundance of LHCII components, with compound **6** being the more effective of the two (Fig. 3).

We repeated the experiment using detached *Arabidopsis* leaves that were maintained under growing light conditions (150 µmol photons m^{-2} s^{-1}). Whereas BAP retarded the decrease in the leaves' chlorophyll content in darkness (Fig. 4), it strongly accelerated this decrease when applied at concentrations of 5 or 10 µM under illumination (Fig. 5). Light is known to invert the senescence-delaying effects of cytokinins in this way (Prokopova et al., 2010; Rulcová and Pospíšilová, 2001; Vlckova et al., 2006). Remarkably, however, light exposure did not change the senescence-retarding effects of treatment with compound **6**. Moreover, **6** was more effective than **3** or BAP at retarding the senescence-induced decrease in the leaves' carotenoid contents (Fig. 5).

Further confirmation of the increased abundance of LHCII in leaves treated with compound **6** was obtained by analytical

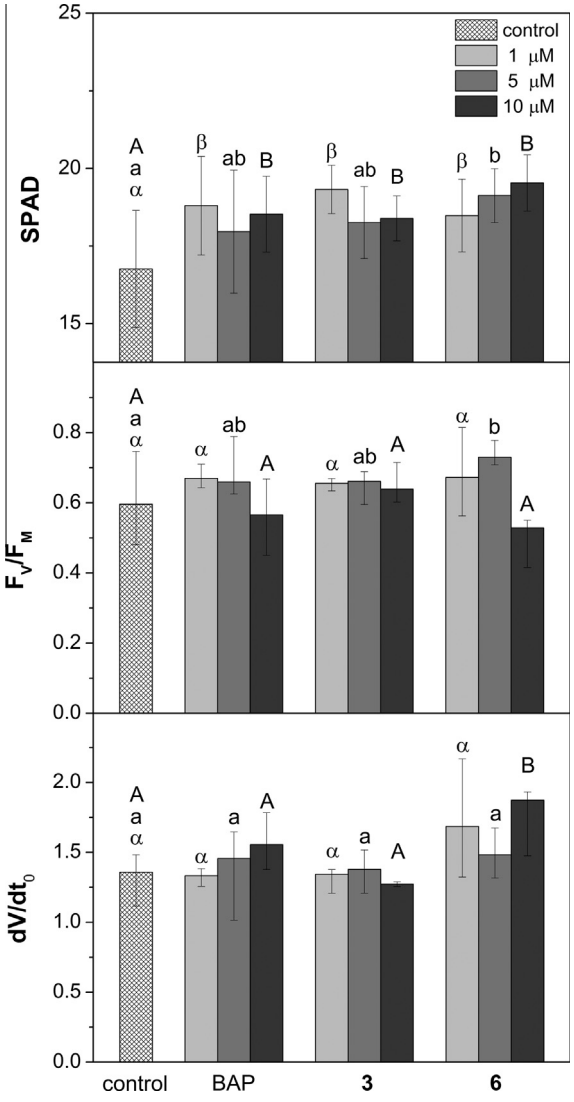


Fig. 4. Overall content of chlorophylls (SPAD), the maximum quantum efficiency of PSII photochemistry (F_V/F_M), and the initial slope of the variable fluorescence rise (dV/dt_0) in detached *Arabidopsis* leaves kept in darkness for 2 days in the DMSO control solution or in 1, 5 or 10 µM solutions of BAP, **3** or **6**. ANOVA tests were performed to compare the effects of the different compounds at each of the three tested concentrations; lower case, upper case, and Greek letters indicate results for the 1 µM, 5 µM, and 10 µM concentrations, respectively. Results labeled with different letters of the same case or alphabet differ significantly.

determination of the chlorophyll a and chlorophyll b contents of such leaves, since LHCII is known to have a higher relative content of chlorophyll b. The ratio of chlorophyll a to b decreased in leaves treated with compound **6**, confirming the expected increase in the relative abundance of LHCII (Fig. 5). Moreover, an analysis of the kinetics of chlorophyll fluorescence revealed that it was not just the antenna system of photosystem II (PSII) that was protected and maintained by treatment with compound **6**. Fig. 6 shows that leaves treated with 1 and 5 µM solutions of **3** or **6** exhibited greater photochemical quenching (qP) than those treated with BAP during the period of light induction until the photosynthetic apparatus reached a steady state. In particular, all of the tested concentrations of compounds **3** and **6** caused significant increases in the rate of qP increase during the first phase of the transition from darkness to actinic illumination.

Finally, we analyzed the allocation of excitations captured by the PSII antenna system to the photosynthetic flux of electrons via PSII (which is reflected in the quantum yield of PSII electron

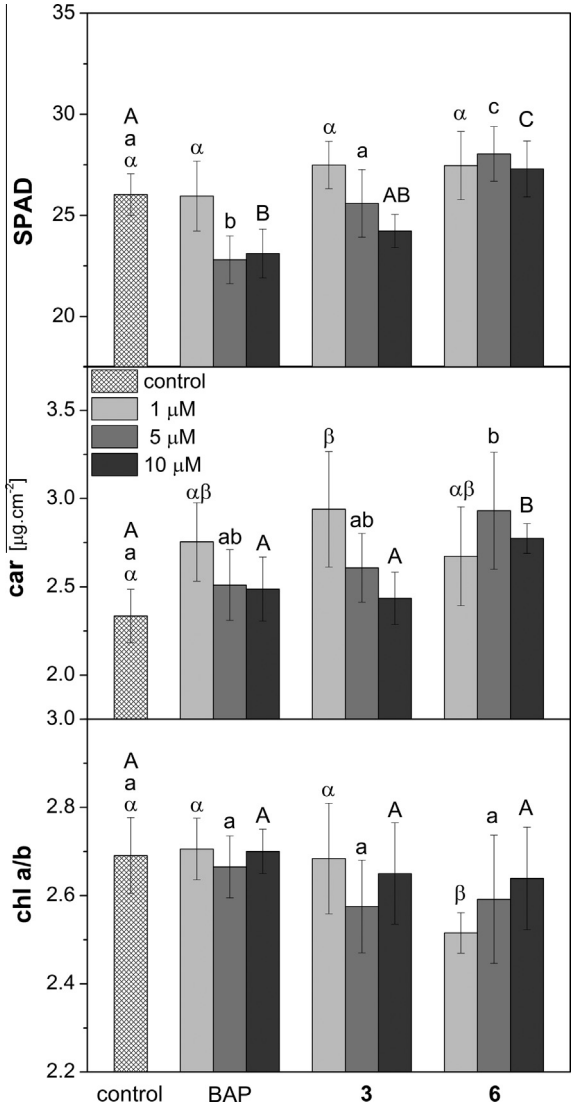


Fig. 5. Overall contents of chlorophylls and carotenoids and ratios of the contents of chlorophyll *a* and *b* in detached *Arabidopsis* leaves kept for 4 days under growing light conditions in a DMSO control solution or in 1, 5 or 10 μM solutions of BAP, **3** or **6**. ANOVA tests were performed to compare the effects of the different compounds at each of the three tested concentrations; lower case, upper case, and Greek letters indicate results for the 1 μM, 5 μM, and 10 μM concentrations, respectively. Results labeled with different letters of the same case or alphabet differ significantly.

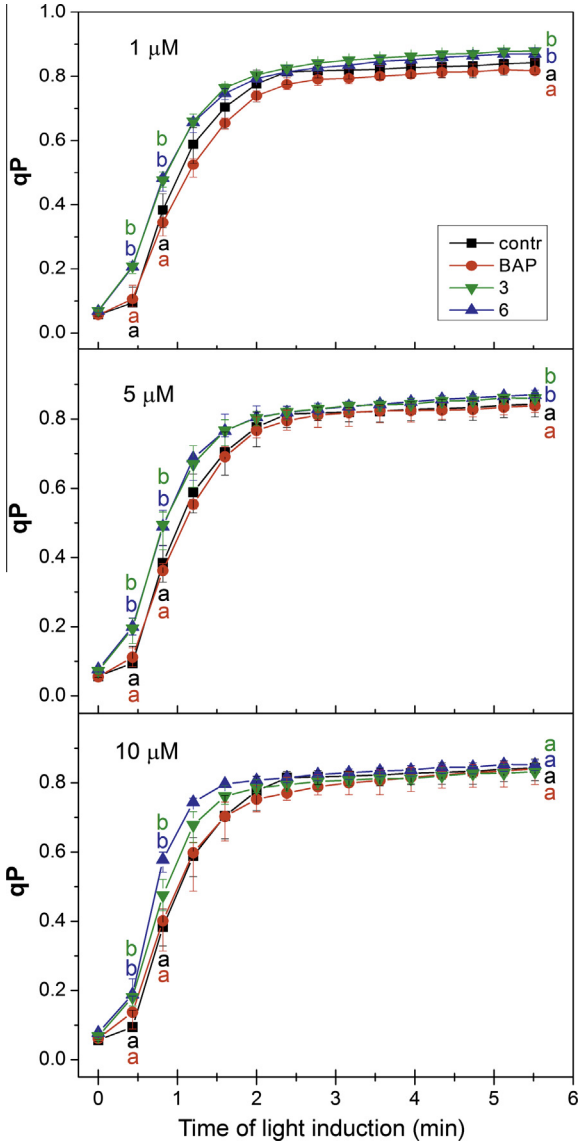


Fig. 6. The photochemical chlorophyll fluorescence quenching (qP) during chlorophyll fluorescence induction in detached *Arabidopsis* leaves kept for 4 days under growing light conditions in a DMSO control solution (black) or in 1 (top), 5 (middle) or 10 μM (bottom) solutions of BAP (red), compound **3** (green) or compound **6** (blue). Results of statistical analyses are shown for 2nd, 3rd and last saturating pulses. Different letters indicate statistically significant differences.

transport, Φ_{PSII}) and regulated thermal dissipation (reflected in the quantum yield of regulated non-photochemical quenching, Φ_{NPQ}) after 1 min of light induction (Fig. 7). The proportion of excitations allocated to PSII photochemistry was considerably greater for leaves kept in solutions of either **3** or **6** (Fig. 7E and H) than for leaves treated with BAP or DMSO alone (Fig. 7B). Consequently, these leaves had a markedly lower quantum yield of regulated thermal dissipation (cf. Fig. 7C, F, and I).

Our results clearly demonstrate that compounds **3** and **6** are both considerably more effective than BAP at maintaining the photosynthetic apparatus during senescence, with compound **6** being the more potent of the two.

3. Conclusions

A series of 2-chloro-N^6-(halogenobenzylamino)purine ribosides was prepared and tested in three cytokinin bioassays. Some of these compounds exhibited enhanced cytokinin activity in these assays, particularly the detached wheat leaf senescence bioassay, in which compounds bearing fluorine or chlorine monosubstitution on the benzyl ring almost doubled the delay of chlorophyll decay. The most potent compounds were those bearing a fluorine or chlorine atom in the *meta* or *para* position of the benzyl ring. All of the halogenated compounds that were tested in the bacterial receptor assay exhibited low affinities for the AHK3, AHK4 and ZmHK3a cytokinin receptors but were selectively recognized by ZmHK1. However, the tested compounds also exhibited high activity relative to BAP in the *Arabidopsis ARR5::GUS* bioassay. This suggests that these aromatic cytokinins have a separate mode of action to other cytokinins, or that they act as negative regulators of cytokinin perception by upregulating certain genes.

A high throughput comparative gene expression analysis was conducted to probe the mechanisms by which compounds **3** and **6** exert their antisenescence activity in *Arabidopsis*. Both of these cytokinin derivatives reprogrammed gene transcription and

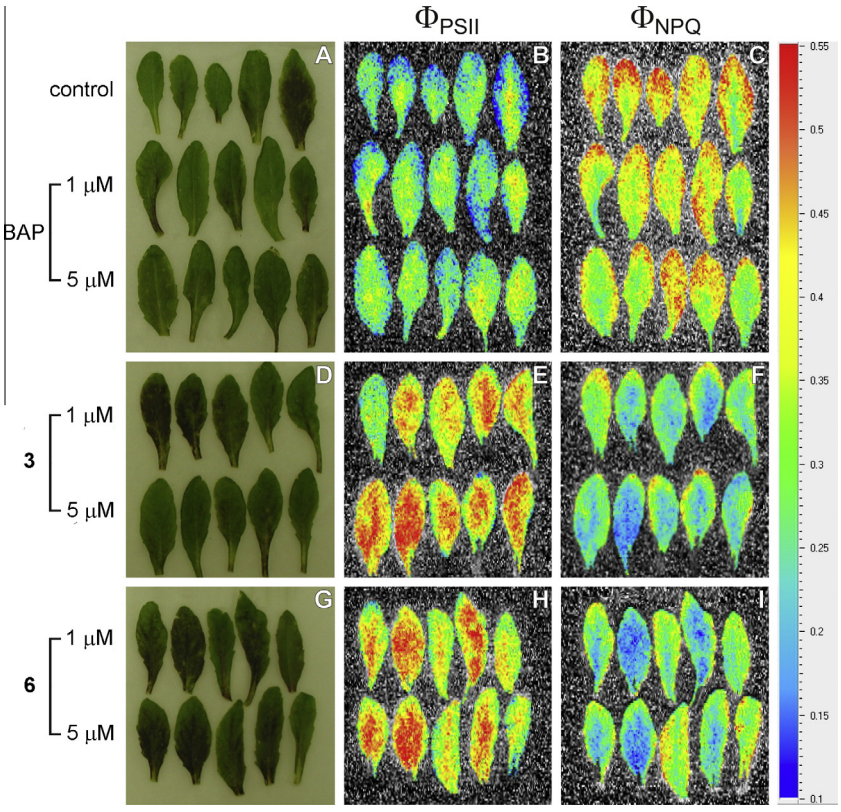


Fig. 7. Photographs of detached *Arabidopsis* leaves kept for 4 days under growing light conditions in a DMSO control solution or in 1 or 5 μM solutions of BAP (A), compound **3** (D), or compound **6** (G). The spatial distribution of the quantum yields of PSII electron transport (Φ_{PSII}) is shown for leaves treated with BAP, **3** and **6** (B, E, H). Regulated non-photochemical quenching of the chlorophyll fluorescence (Φ_{NPQ}) for the same samples (C, F, I) was measured after 70 seconds' exposure to actinic light.

specifically protected leaves from senescence by inducing the upregulation of several genes associated with photosystems I and II as well as other components of the photosynthetic machinery. We also investigated the influence of compounds **3** and **6** on photosynthetic pigments levels and the efficiency of photosystem II, revealing that both compounds had positive effects on the functioning of the photosynthetic apparatus during senescence: they protected the activity of PSII by maintaining the levels of chlorophylls and carotenoids, and by increasing the relative abundance of LHCII. Interestingly, even when these compounds were applied at relatively high concentrations there was no sign of the negative effects on photosynthetic activity observed when natural aromatic cytokinins such as BAP are applied to senescent *Arabidopsis* leaves at high concentrations under intense illumination.

4. Experimental

4.1. General experimental procedures

Thin layer chromatography (TLC) was performed on silica gel 60 WF_{254} plates (Merck Co, Darmstadt, Germany). Column chromatography was carried out using silica gel Davisil LC60A 40–63 μm (Grace Davison Discovery Sciences, Deerfield, IL) and compounds were eluted with $CHCl_3$:MeOH (9:1, v/v).

The purity of the synthesized compounds was determined by HPLC as described previously (Gucky et al., 2013). Briefly, the samples were dissolved in methanol (1 mg/ml) and then diluted to a concentration of 10 μg/ml in the initial mobile phase. 10 μl of the resulting solution was injected onto an thermostated (25 °C) RP column (150 mm × 2.1 mm, 3.5 μm C18 Symmetry, Waters, Inc., Milford, MA) and analyzed using an Alliance 2695 Separations Module high-performance liquid chromatograph (Waters, Inc.) coupled to a PDA 2996 detector (Waters, Inc.) with detection at wavelengths of 210–400 nm. The chromatograph's effluent was directed into the ion source of a benchtop quadrupole orthogonal acceleration time-of-flight Q-TOF Micro tandem mass spectrometer (Waters, Inc.). HPLC elution was performed at a flow rate of 0.2 ml/min using a linear gradient of 15 mM ammonium formate at pH 4.0 (mobile phase A) and methanol (mobile phase B). The proportion of B in the mobile phase was initially 10% and was increased linearly to 90% over 24 min, after which isocratic elution was performed with 90% B. The column was then re-equilibrated under the initial conditions (10% B) for 10 min. The electrospray source was maintained at 110 °C with a capillary voltage of +3.0 kV, a cone voltage of +20 V, and a desolvation temperature of 250 °C. Nitrogen was used as both the desolvation gas (500 l/h) and the cone gas (50 l/h). The mass spectrometer was operated in positive (ESI+) ionization mode and data were acquired in the 50–1000 *m*/*z* range.

Elemental analyses (C, H and N) were conducted using an EA1112 Flash analyzer (Thermo-Finnigan, Bellefonte, PA). ^1H NMR spectra were recorded on a Bruker Avance 300 spectrometer operating at a frequency of 300.13 MHz while ^{13}C spectra were acquired on a JEOL 500 ECA spectrometer at a frequency of 125 MHz. Samples were prepared by dissolving the relevant purified material in DMSO-d_6 (99.9% D).

4.2. Chemicals

3-Chlorobenzylamine, 4-chlorobenzylamine, 3-bromobenzylamine hydrochloride, 4-bromobenzylamine hydrochloride, 2-bromobenzylamine hydrochloride, 4-idobenzylamine hydrochloride, 3-fluorobenzylamine, 4-fluorobenzylamine, 2-fluorobenzylamine,

3,4-dichlorobenzylamine, 2,4-dichlorobenzylamine, ampicillin, casein hydrolysate, glycine, 4-methylumbelliferyl-β-D-galactoside, glucose, and isopropyl-β-D-thiogalactopyranoside were purchased from Sigma–Aldrich. 3-Iodobenzylamine hydrochloride was purchased from Acros Organics. 2-Chlorobenzylamine was purchased from Fluka and 2-chloro-6-fluorobenzylamine was obtained from Fluorochem. 2,6-Dichloropurine riboside, 2,6-dichloropurine, and 6-(3-metylbut-2-en-1-ylamino)-9*H*-purine were purchased from Olchemim. 3-Methylbut-2-en-1-amine and 4-amino-2-methylbut-2-en-1-ol hemioxalate were prepared in the Laboratory of Growth Regulators (Olomouc, Czech Republic). Common chemicals and other solvents were obtained from Lach-Ner s.r.o.

4.3. Organic synthesis

A series of 2,6-disubstituted derivatives (Table 1) was prepared according to Scheme 1. 2,6-dichloropurine ribosides (1.25 mmol) or 2,6-dichloropurine (2.12 mmol) were refluxed with the appropriate substituted benzylamine (1 equiv.) and triethylamine (10 equiv.) in *n*-propanol (30 ml) for 4 h. The mixture was then evaporated under reduced pressure and partitioned between water (15 ml) and ethyl acetate (3 × 20 ml). The organic phases were washed with brine (20 ml), dried over Na_2SO_4, and concentrated *in vacuo*. The pure products (white powders) were obtained after silica column chromatography purification using $CHCl_3$/MeOH (9:1, v/v) as the mobile phase. The purity of the prepared substances was verified by high performance liquid chromatography (HPLC-UV) and the compounds were further characterized by elemental analysis, ESI + MS, and NMR. NMR data are presented in the supplementary material.

4.4. Bioassays

4.4.1. Cytokinin bioassays

The prepared compounds were tested in three classical cytokinin bioassays: the tobacco callus assay, *Amaranthus* assay, and detached wheat leaf senescence assay. In all cases, their biological activity was compared to that of BAP. The bioassays were performed as described (Holub et al., 1998) using 6-well microtiter plates with each well containing 3 ml of MS medium and 0.1 g of callus tissue in the case of the tobacco callus bioassay.

4.4.2. Receptor bioassays

All receptor bioassays were conducted using *Escherichia coli* strain KMI001 harboring the plasmid pIN-III, which contained a coding sequence for cytokinin receptors ZmHK1, ZmHK3a and AHK4 (Yonekura-Sakakibara et al., 2004), or the plasmid pSTV28, which contained a coding sequence for AHK3 (Suzuki et al., 2001; Yamada et al., 2001). Bacterial strains were kindly provided by Dr. T. Mizuno (Nagoya, Japan). This bioassay was performed according to the published procedure (Spichal et al., 2004).

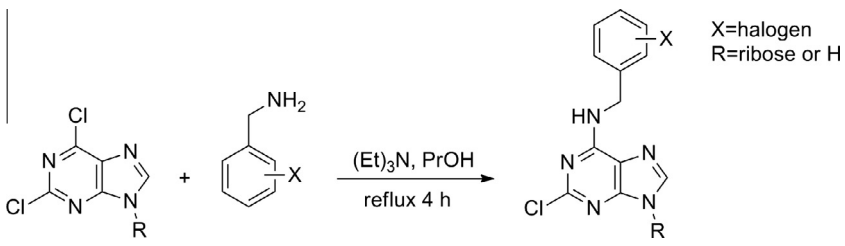
4.4.3. ARR5::GUS cytokinin bioassay

Transgenic *Arabidopsis ARR5::GUS* seeds were sterilized and placed in the wells of 6-well plates (THPP, Switzerland) containing half-strength MS medium. Plates with seeds were cultivated for vernalization in darkness (4 °C) for 4 days and then transferred to an orbital shaker in a growth chamber (21 °C) under long-day conditions (16 h light/8 h dark) for 3 days. The seedlings were then treated with a DMSO solution of one of the compounds to be tested, the standard (BAP), or with neat DMSO (solvent, final concentration 0.05%) and cultivated for another 16 h. Histochemical GUS activity measurements were performed according to the published assay procedure (Romanov et al., 2002). Fluorescence was measured using a Fluoroscan Ascent microplate reader (Labsystems, Helsinki, Finland) at excitation and emission wavelengths of 365 and 450 nm. GUS specific activity was determined in units of nmol 4-methylumbelliferone (MU)/h/mg protein. The protein content of the plant extracts was estimated using the bicinchoninic acid method (Smith et al., 1985).

4.4.4. Microarray analysis

4.4.4.1. Preparation of RNA. For gene chip analysis, total RNA was isolated from *Arabidopsis* leaves using the RNAqueous total RNA isolation kit (Ambion, Life Technologies Ltd., UK) according to the manufacturer's recommendations. In addition, samples were treated with DNase I to remove contaminant genomic DNA.

4.4.4.2. DNA microarray analysis. Total RNA was isolated from wild-type *Arabidopsis* plants (Col-0) that had been treated with **3**, **6** or BAP, or left untreated. The plants were grown in soil for 3 weeks in a controlled growth chamber maintained under 16/8-h day/night cycle (150 μmol photons m^{-2} s^{-1}) at 21/18 °C and a relative humidity of 60/75% (day/night). Subsequently, leaves of similar size and chlorophyll content were cut and immediately treated with the appropriate cytokinin. 10–15 detached leaves were immersed in 1 × MS medium supplemented with 10 μM of the chosen cytokinin; this amount represented one sample. Three biological replicates were performed for each cytokinin treatment and the control group. Control samples were treated with 0.05% DMSO in MS medium. After incubation for 48 h in the dark, detached leaves were frozen in liquid nitrogen and used for RNA isolation. All plant material from cytokinin-treated and control samples was ground in liquid nitrogen and 150 mg of homogenized material from each sample was used for RNA isolation. Double-stranded cDNA was prepared and amplified using the WT expression kit (Ambion, Life Technologies Ltd., UK). The resulting cDNA was then fragmented by the combined action of uracil DNA glycosylase and apurinic/apyrimidinic endonuclease 1 with the WT terminal labeling kit (Affymetrix, Inc., Santa Clara, CA) to yield biotin-tagged cDNA, which was subsequently used for hybridization with the Arabidopsis Gene 1.0 ST Array (Affymetrix, Inc., Santa Clara, CA). A standard post-hybridization wash procedure was performed using the Affymetrix GeneChip Fluidics Station 450 and the arrays were scanned on a GC Scanner 3000 7G. Three independent replicates were conducted for each of the cytokinin-treated materials



Scheme 1. Synthesis of the 2,6-disubstituted cytokinin derivatives described in this study.

and the DMSO-treated controls. Statistical analyses were performed using the R/Bioconductor packages. Raw data were RMA-normalized and the obtained expression levels were further analyzed using the lmFit function of the limma R-package. In addition, the eBayes function was used for variance shrinkage. Genes were considered specifically de-regulated if the change in their expression was found to be statistically significant by a Welch's *t*-test with the Benjamini–Hochberg correction and they had a log2 fold change of $\geqslant 0.5$ or $\leqslant -0.5$. The original Affymetrix data were submitted to the GEO repository (http://www.ncbi.nlm.nih.gov/geo/) with the GEO accession number GSE75875.

4.4.5. Chlorophyll fluorescence analysis

Chlorophyll fluorescence was monitored using the FluorCam 700 MF imaging system (Photon Systems Instruments, Czech Republic). All measurements were performed on 10–15 replicates. To measure fluorescence signals, microseconds-long measuring flashes of red light were applied in 20 ms intervals. The overall integral light intensity of the measuring flashes was low enough to measure fluorescence only without closing the reaction centers of photosystem II (PSII). The minimum chlorophyll fluorescence (F_0) was determined in leaves after 30 min of dark adaptation; a pulse of strong white light (2500 μmol m^{-2} s^{-1}) was applied for 1.6 s to determine the maximum chlorophyll fluorescence in the dark-adapted state (F_M). This was followed by a further 2 min of dark adaptation and then 5.5 min of exposure to red actinic light (200 μmol m^{-2} s^{-1}). To estimate the maximum fluorescence during light adaptation (F_M'), after the first 2 seconds' exposure to the actinic light, the samples were subjected to 15 pulses of strong light applied at 25 s intervals. The maximum quantum efficiency of PSII photochemistry (F_V/F_M) was calculated as $(F_M–F_0)/F_M$; the regulated non-photochemical quenching of chlorophyll fluorescence (Φ_{NPQ}) was calculated as $(F_M–F_M')/F_M'$; and the coefficient of photochemical quenching (qP) was calculated as $(F_M'–F_t)/(F_M'–F_0')$, where F_t is the fluorescence measured just prior the application of a pulse and F_0' is the minimal fluorescence for the light-adapted state, which is calculated as $F_0/(F_V/F_M + F_0/F_M')$ (Oxborough and Baker, 1997). The quantum yield of PSII electron transport (Φ_{PSII}) was calculated as qP × $(F_M'–F_0')/F_M'$. A Plant Efficiency Analyzer (Hansatech Instruments, UK) was used to analyze the very fast induction of chlorophyll fluorescence in *Arabidopsis* leaves kept in darkness. One leaf was exposed to red light at an intensity of 2100 μmol m^{-2} s^{-1}. F_V/F_M was then calculated as described above and the initial slope of the variable fluorescence rise (dV/dt_0) was calculated as $4 \times (F_{300\mu s}–F_{50\mu s})/(F_M–F_{50\mu s})$, where $F_{300\mu s}$ and $F_{50\mu s}$ are the fluorescence yields obtained at the indicated times. The results were analyzed using ANOVA (Tukey and Bonferroni tests) with a significance threshold of 0.05; these calculations were performed using OriginPro 8.5.1 (OriginLab Corporation, MA, USA).

4.4.6. Photosynthetic pigment content

The overall chlorophyll content was estimated in 7–8 replicates using a SPAD-502 chlorophyll meter (Konica Minolta Sensing, Japan). Samples' contents of chlorophyll a and b were measured separately in 5 replicates after extraction with 80% acetone, and their total carotenoid contents were determined in the same way as reported in the literature (Lichtenthaler, 1987).

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.12.001.

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Appendix II

Patent

6-aryl-9-glycosylpurines and use thereof (Palacký University Olomouc)

CZ 2014875, EP 3229772, SG 11201704019TA and US 10550144 patents

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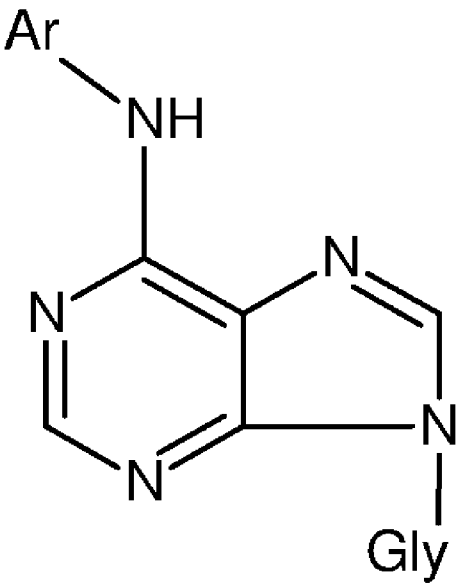
[Continued on nextpage]

(54) Title: 6-ARYL-9-GLYCOSYLPURINES AND USE THEREOF



(I)

(57) Abstract: The present invention provides 6-aryl-9-glycosidpurines of general formula I and pharmaceutically acceptable salts thereof with alkali metals, ammonia, amines, or addition salts with acids, wherein Gly represents β-D-arabinofuranosyl or β-D-2'-deoxyribofuranosyl, Ar represents benzyl or furfuryl, each of which can be unsubstituted or substituted by one or more, preferably one to three, substituents selected from the group comprising hydroxyl, alkyl, halogen, alkoxy, amino, mercapto, carboxyl, cyano, amido, sulfo, sulfamido, acyl, acylamino, acyloxy, alkylamino, dialkylamino, alkylmercapto, trifluoromethyl, trifluoromethoxy, for use as antisenescent and UV protective compounds in animals.

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LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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6-aryl-9-glycosylpurines and use thereof

Field of Art

The invention relates to 6-(benzylamino/furfurylamino)-9 -P-D-arabinofuranosylpurine or -β-D-2´-deoxyribofuranosylpurine derivatives which slow down the aging of animal and human cells and which show UV photoprotective properties.

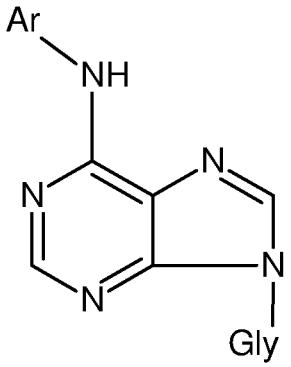
Background Art

Substituted adenine derivatives are known as phytohormones. The range of their properties is rather broad, especially known is their anti-tumor and pro-apoptotic activity, i.e., activities relating to inhibition of tumor cell growth. Several glycosylated derivatives were prepared, in particular ribosylated derivatives. An arabinosylated derivative prepared so far was 6-benzylamino-9-arabinosylpurine which was published as a compound participating in inhibition of replication of tobacco mosaic virus in extirpated leaves *Nicotiana glutinosa* (Barai el al. Vestsi Akademii Nauk Belarusi 1: 18-22, 1992). 6-chloropurine arabinoside was prepared from 6-chloropurine riboside and its antiviral activity was explored (Maruyama et al. Chem. Pharm. Bull. 44: 2331-2334, 1996). Several methylated derivatives of 6-(benzylamino)-9 -P-D-arabinofuranosylpurine were prepared for antiviral activity testing, and their activity in killing *Vaccinia* virus and *Herpes simplex* virus was tested. No effect was observed for benzylamino derivative, neither for 2-methylbenzyl, 3-methylbenzyl, 2,3-dimethylbenzyl, 2,6-dimethylbenzyl, 3,4-dimethylbenzyl derivatives. A low activity against *Vaccinia* virus, strain IHD, was observed for 2,4-dimethylbenzyl and 2,5-dimethylbenzyl substituents (Masakatsu et al. Chem. Pharm. Bull. 25: 2482-2489, 1977). Some N6-substituted derivatives of adenine arabinoside were prepared as selective inhibitors of varicella- zoster virus, the substituents were 6-methylamino, 6-dimethylamino-, 6-ethylamino-, 6-N-ethylmethylamino-, NN- diethylamino-, 6-n-propylamino-, 6-isopropylamino-, 6-n-hexylamino-, 6-cyclohexylamino-, 6-anilino (Koszalka et al. Antimicrob. Agents Chemother. 35: 1437-1443, 1991).

The object of the present invention are glycosylated derivatives of adenine with antisenescent and photoprotective properties which show extremely low or no toxicity and high activity in aging, cell division and differentiation processes and photoprotection.

Disclosure of the Invention

Object of the invention is use of 6-aryl-9-glycosylpurines of general formula I



I

and pharmaceutically acceptable salts thereof with alkali metals, ammonia, amines, or addition salts with acids, wherein

Gly represents β-D-arabinofuranosyl or β-D-2´-deoxyribofuranosyl,

Ar represents benzyl or furfuryl, each of which can be unsubstituted or substituted by one or more, preferably one to three, substituents selected from the group comprising hydroxyl, alkyl, halogen, alkoxy, amino, mercapto, carboxyl, cyano, amido, sulfo, sulfamido, acyl, acylamino, acyloxy, alkylamino, dialkylamino, alkylmercapto, trifluoromethyl, trifluoromethoxy,

for regulation, in particular inhibition, of aging in animals, in particular mammals, for cosmetic purposes, and/or for UV photoprotection of animals, in particular mammals, for cosmetic purposes.

The invention further encompasses the 6-aryl-9-glycosylpurines of general formula I for use in a method of regulation, in particular inhibition, of aging in animals, in particular

mammals, for therapeutic purposes, and/or for UV photoprotection of animals, in particular mammals, for therapeutic purposes.

Another object of the invention is a method for regulating aging and/or UV photodamage of microorganisms, and animal cells *in vitro,* in which at least one compound of general formula I is applied to the plant or cells or microorganisms.

If not stated otherwise, then:
alkyl represents a linear or branched C1-C6, preferably C1-C4, alkyl chain,
acyl represents an acyl group having 2 to 6 carbon atoms,
halogen represents a halogen atom selected from the group consisting of fluorine, bromine, chlorine and iodine atom,
sulfo represents -S0 $_3R_c$, wherein R_c represents hydrogen atom, linear or branched alkyl, alkenyl or alkynyl group containing 1 to 6 carbon atoms,
sulfoamido represents -NHS0 $_3R_d$, wherein R_d represents hydrogen atom, linear or branched alkyl group containing 1 to 6 carbon atoms.

Particularly preferred compounds of the invention are the compounds of formula I selected from the group consisting of: 6-furfurylamino-9 -P-D-arabinofuranosylpurine, 6-(3-methylfurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(4-methylfurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(5-methylfurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(3-fluorofurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(4-fluorofurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(5-fluorofurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(3-chlorofurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(4-chlorofurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(5-chlorofurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(3-bromofurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(4-bromofurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(5-bromofurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(3-hydroxyfurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(4-hydroxyfurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(5-hydroxyfurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(3-methoxyfurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(4-methoxyfurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(5-methoxyfurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(2-aminofurfurylamino)-9 -P-D-arabinofuranosylpurine, 6-(3-aminofurfurylamino)-9 -P-D-

arabinofuranosylpurine, 6-(4-aminofurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dihydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3,5-dihydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dihydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4-dihydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2,5-dihydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2,6-dihydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dimethoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dimethoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3,5-dimethoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2,3-dimethoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4-dimethoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2,5-dimethoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2,6--dimethoxyfurfurylamino)--9-p-D-arabinofuranosylpurine, 6-(2--hydroxy-3-methoxyfurfurylamino)- -9-p-D-arabinofuranosylpurine, 6-(2--hydroxy-4-methoxyfurfurylamino)- -9-p-D-arabinofuranosylpurine, 6-(2--hydroxy-5-methoxyfurfurylamino)- -9-p-D-arabinofuranosylpurine, 6-(2 -hydroxy-6-methoxyfurfurylamino)- -9-p-D-arabinofuranosylpurine, 6-(3--hydroxy-2-methoxyfurfurylamino)- -9-p-D-arabinofuranosylpurine, 6-(3--hydroxy-4-methoxyfurfurylamino)- -9-p-D-arabinofuranosylpurine, 6-(3--hydroxy-5-methoxyfurfurylamino)- -9-p-D-arabinofuranosylpurine, 6-(3--hydroxy-6-methoxyfurfurylamino)- -9-p-D-arabinofuranosylpurine, 6-(4--hydroxy-2-methoxyfurfurylamino)- -9-p-D-arabinofuranosylpurine, 6-(4--hydroxy-3-methoxyfurfurylamino)- -9-p-D-arabinofuranosylpurine, 6-(4--hydroxy-5-methoxyfurfurylamino)- -9-p-D-arabinofuranosylpurine, 6-(4--hydroxy-6-methoxyfurfurylamino)- -9-p-D-arabinofuranosylpurine, 6-(2-fluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-fluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-fluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-

methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-hydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-hydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-hexylbenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-fluoro-6-(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-chloro-2,6-difluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-(trifluoromethylthio)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-chloro-3,6-difluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-(trifluoromethylthio)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-fluoro-5-(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-chloro-4-fluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-(trifluoromethoxy)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-chloro-3-(trifluoromethyl) benzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-fluoro-3-(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,5-bis(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-(trifluoromethoxy)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-(trifluoromethoxy)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-aminobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3-aminobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-aminobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-diethylaminobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,5-dihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,5-dihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,6-dihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3,5-dimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,3-dimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4-dimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,5-

dimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,6-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-3-methoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-4-methoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-5-methoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-6-methoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3-hydroxy-2-methoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3-hydroxy-4-methoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3-hydroxy-5-methoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3-hydroxy-6-methoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(4-hydroxy-2-methoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(4-hydroxy-3-methoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(4-hydroxy-5-methoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(4-hydroxy-6-methoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,3,4-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4,5-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4,6-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4,5-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3,4,5-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3,4,6-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4,5,6-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4,6-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,3,4-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4,6-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,3,4-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4,5-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4,6-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-5-chlorobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-6-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-5-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-6-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-bromobenzylamino)-9-P-D-

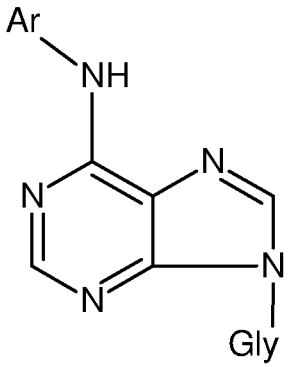
arabinofuranosylpurine, 6-(2-hydroxy-5-bromobenzylamino)-9 -P-D-arabinofuranosylpurine, 6-(2-hydroxy-6-bromobenzylamino)-9 -P-D-arabinofuranosylpurine, 6-(2-hydroxy-3-fluorobenzylamino)-9 -P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-fluorobenzylamino)-9 -P-D-arabinofuranosylpurine, 6-(2-hydroxy-5-fluorobenzylamino)-9 -P-D-arabinofuranosylpurine, 6-(3-methylfurfurylamino)-9-p-D-2'-deoxyribofuranosylpurine, 6-(4-methylfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(5-methylfurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-fluorofurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(4-fluoiOfurfurylamino)-9-p-D-2'-deoxyribofuranosylpurine, 6-(5-fluorofurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(3-chlorofurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-chlorofurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(5-chlorofurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(3-bromo-furfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(4-bromofurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(5-bromofurfurylamino)-9 -β-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxyfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxyfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(5-hydroxyfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(3-methoxyfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(4-methoxyfurfurylamino)-9-p-D-2'-deoxyribofuranosylpurine, 6-(5-methoxyfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(2-aminofurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(3-aminofurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(4-aminofurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3,4-dihydroxyfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(3,5-dihydroxyfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(3,4-dihydroxyfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(2,4-dihydroxyfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(2,5-dihydroxyfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(2,6-dihydroxyfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(3,4-dimethoxyfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(3,4-dimethoxyfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(3,5-dimethoxyfurfurylamino)-9 -P-D-2'-deoxyribofuranosylpurine, 6-(2,3-dimethoxyfurfurylamino)-9 -P-D-2'-

deoxyribofuranosylpurine, 6-(2,4-dimethoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2,5-dimethoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2,6-dimethoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxy-3-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxy-4-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxy-5-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxy-6-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxy-2-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxy-4-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxy-5-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxy-6-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxy-2-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxy-3-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxy-5-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxy-6-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2-fluorobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-fluorobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-fluorobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-bromobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-bromobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-bromobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-iodobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-iodobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-iodobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-chlorobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-chlorobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-chlorobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-chlorobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-aminobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-aminobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-aminobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxybenzylamino)- 9-P-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine,

6-(3,4-dihydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3,5-dihydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3,4-dihydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2,4-dihydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2,5-dihydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2,6-dihydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3,4-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3,4-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3,5-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2,3-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2,4-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2,5-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2,6-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxy-3-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxy-4-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxy-5-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxy-6-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxy-2-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxy-4-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxy-5-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxy-6-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxy-2-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxy-3-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxy-5-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxy-6-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine.

Object of the invention are further compositions for inhibiting aging and/or for UV photoprotection of mammals, or for inhibiting aging and/or for UV photoprotection of mammalian cells, such as keratinocytes and fibroblasts, containing at least one 6-aryl-9-glycosidpurine of general formula I.

Object of the invention are also 6-aryl-9-glycosidpurines of general formula Ia



la

and pharmaceutically acceptable salts thereof with alkali metals, ammonia, amines, or addition salts with acids, wherein

Gly represents β-D-arabinofuranosyl or β-D-2´-deoxyribofuranosyl,

Ar represents benzyl or furfuryl, each of which is substituted by one or more, preferably one to three, substituents selected from the group comprising hydroxyl, halogen, alkoxy, amino, mercapto, carboxyl, cyano, amido, sulfo, sulfamido, acyl, acylamino, acyloxy, alkylamino, dialkylamino, alkylmercapto, trifluoromethyl, trifluoromethoxy, or Ar is unsubstituted furfuryl,

whereas, if Gly is β-D-arabinofuranosyl, Ar is not methyl-substituted benzyl.

The invention further encompasses cosmetic and/or tissue compositions containing as an active ingredient at least one compound of general formula la. Tissue compositions are especially suitable for use in biotechnologies.

Compositions

Suitable administration for cosmetic application is local, topical. The cosmetic composition typically contains from 0.1 to 95 wt. % of the active ingredient, whereas single-dose forms contain preferably 10 to 90 wt. % of the active ingredient and administration forms which are not single-dose preferably comprise 1 wt. % to lOwt. % of the active ingredient. The application forms include, e.g., ointments, creams, pastes, foams, tinctures, lipsticks, drops, sprays, dispersions and the like. The compositions are prepared in a known manner, for example by means of conventional mixing, dissolving or lyophilizing processes.

Solutions of the active ingredients, suspensions or dispersions, especially isotonic aqueous solutions, dispersions and suspensions, can be prepared before use, for example in the case of lyophilised compositions which comprise the active substance alone or together with a carrier, for example mannitol.

Suspensions in oil comprise, as the oily component, vegetable, synthetic or semi-synthetic oils. Oils which may be mentioned are, in particular, liquid fatty acid esters which contain, as the acid component, a long-chain fatty acid having 8-22, in particular 12-22, carbon atoms, for example lauric acid, tridecylic acid, myristic acid, pentadecylic acid, palmitic acid, margaric acid, stearic acid, arachidonic acid, behenic acid or corresponding unsaturated acids, for example oleic acid, elaidic acid, erucic acid, brasidic acid or linoleic acid, if appropriate with the addition of antioxidants, for example vitamin E, β-carotene or 3,5-di-ie/t-butyl-4-hydroxytoluene. The alcohol component of these fatty acid esters has not more than 6 carbon atoms and is mono- or polyhydric, for example mono-, di- or trihydric alcohol, for example methanol, ethanol, propanol, butanol, or pentanol, or isomers thereof, but in particular glycol and glycerol. Fatty acid esters are, for example: ethyl oleate, isopropyl myristate, isopropyl palmitate, "Labrafil M 2375" (polyoxyethylene glycerol trioleate from Gattefosee, Paris), "Labrafil M 1944 CS" (unsaturated polyglycolated glycerides prepared by an alcoholysis of apricot kernel oil and composed of glycerides and polyethylene glycol esters; from Gattefosee, Paris), "Labrasol" (saturated polyglycolated glycerides prepared by an alcoholysis of TCM and composed of glycerides and polyethylene glycol esters; from Gattefosee, Paris) and/or "Miglyol 812" (triglyceride of saturated fatty acids of chain length C_8 to C_{12} from Hiils AG, Germany), and in particular vegetable oils, such as cottonseed oil, almond oil, olive oil, castor oil, sesame oil, soybean oil and, in particular, groundnut oil.

Ointments are oil-in-water emulsions which comprise not more than 70 %, preferably 20 to 50 % of water or aqueous phase. The fatty phase consists, in particular, of hydrocarbons, for example vaseline, paraffin oil or hard paraffins, which preferably comprise suitable hydroxy compounds, such as fatty alcohols or esters thereof, for example cetyl alcohol, or wool wax alcohols, such as wool wax, to improve the water-binding capacity. Emulsifiers are corresponding lipophilic substances, such as sorbitan fatty acid esters (Spans), for example sorbitan oleate and/or sorbitan isostearate. Additives to the aqueous phase are, for

example, humectants, such as polyalcohols, for example glycerol, propylene glycol, sorbitol and/or polyethylene glycol, or preservatives and odoriferous substances.

Fatty ointments are non-aqueous and are in particular hydrocarbon-based, e.g. paraffin, vaseline or paraffin oil, and natural or semi-synthetic lipids, such as hydrogenated coconut fatty acid triglycerides or hydrogenated oils, such as hydrogenated castor or groundnut oil, and partially fatty acid glycerol esters, e.g. glycerol mono- and distearate. They further contain, e.g., fatty alcohols, emulsifiers and additives mentioned above in connection with ointments which increase water binding.

Creams are oil-in-water emulsions containing more than 50 % of water. The oil bases used include fatty alcohols, e.g., isopropyl myristate, lanolin, bees wax or hydrocarbons, preferably vaseline (petrolatum) and paraffine oil. Emulsifiers are surface active compounds with predominantly hydrophilic characteristics, such as corresponding non-ionic emulsifiers, e.g., fatty acid polyalcohol esters or ethyleneoxy adducts thereof, e.g., polyglyceridic fatty acids or polyethylene sorbitan esters or acidic polyflyceridic fatty acid esters (Tween), polyoxyethylene fatty acid ethers or polyoxyethylene fatty acid esters; or corresponding ionic emulsifiers, such as alkali sulfate salts of fatty alcohols, such as sodium laurylsulfate, sodium cetylsulfate, or sodium stearylsulfate, which are typically used in the presence of fatty alcohols, e.g., cetyl stearyl alcohol or stearyl alcohol. The aqueous phase additives include agents preventing drying out of the creams, e.g., polyalcohols such as glycerol, sorbitol, propylene glycol and polyethylene glycol, and preservatives and fragrances.

Pastes are creams or ointments containing powdered secretion-absorbing components such as metal oxides, e.g., titanium oxides or zinc oxide, further talc or aluminium silicates for binding humidity or secretion.

Foams are applied from pressurized containers and include liquid oil-in-water emulsions in aerosol form, whereas the propellant gases include halogenated hydrocarbons such as chloro-fluoro-lower alkanes, e.g., dichlorofluoromethane and dichlorotetrafluoroethane, or preferably non-halogenated gaseous hydrocarbons, air, N_20 or carbon dioxide. The oily phases used are the same as for ointments and the additives mentioned for ointments are used.

Tinctures and solutions usually comprise an aqueous-ethanolic base, to which humectants for reducing evaporation, such as polyalcohols, for example glycerol, glycols and/or

polyethylene glycol, and re-oiling substances, such as fatty acid esters with lower polyethylene glycols, i.e. lipophilic substances soluble in the aqueous mixture to substitute the fatty substances removed from the skin with ethanol, and, if necessary, other excipients and additives, are admixed.

The invention is further illustrated by the following examples which should not be construed as further limiting.

Brief Description of Drawings

Fig. 1: Growth curve for the compound 6-benzylamino-9 -P-D-arabinofuranosylpurine (Example 15).

Fig. 2: Effect of 6-furfurylamino-9 -P-D-arabinofuranosylpurine (2212) and 3-fluorobenzylamino-9 -P-D-arabinofuranosylpurine (2213) on the retention of chlorophyl in extirpated wheat leaf segments (Example 18).

Fig. 3. UVA-induced effects of test compounds on NHDF viability of 6(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurine (Example 20).

Fig. 4. UVA-induced effect of chlorpromazine on NHDF viability (Example 20).

Fig. 5. Effect of test compounds on UVA-induced damage to NHDF. (A) 6(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurine (Example 21).

Fig. 6. Effect of test compounds on UVB-induced damage to NHDF. (A) 6(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurine (Example 21).

Fig. 7. Venn diagrams of 3MeOBAPA-responsive genes as revealed in the short-term (6 h) and long-term (48 h) treatments (Example 22).

Examples of carrying out the Invention

EXAMPLE 1: Synthesis of 6-(3-methoxybenzylamino)-9-y5-D-arabinofuranosylpurine

9-(/?-D-arabinofuranosyl) hypoxantine (100 mg, 0.37 mmol), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 196 mg, 0.44 mmol) and *N,N*-diisopropylethylamine (DIPEA, 97 μï, 0.56 mmol) were mixed in

dimethylformamide (DMF, 1.86 ml). Subsequently, 3-methoxybenzylamine (56 μï, 0.56 mmol) was added. 9-(/?-D-arabinofuranosyl) hypoxantine is commercially available (Jena Bioscience, N-1002) or can be prepared from 9-(/?-D-arabinofuranosyl) adenine. Reaction mixture was mixed at laboratory temperature (25 °C) under argon overnight (16 hrs). After that period, reaction mixture was evaporated on vacuum rotary evaporator and one of the following procedures was used to obtain the crude product: First, the reaction mixture was purified by column liquid chromatography (mobile phase chloroform- methanol 19:1) or cold water was slowly added (15 ml) and the reaction mixture was vortexed: a yellowish substance started to occur after a few minutes. Reaction mixture was than placed into the fridge and left overnight. Arising product was filtrated and once recrystallized from isopropanol and twice from ethanol. Final product is a white crystalline solid. Both ways of isolation of the product described above gave 40 % yield, TLC (40% chlorform:metanol (90:10, v:v): one spot; HPLC purity > 98%. $[M+H^+]$388, ^{1}H(DMSO-J $_6$, 300 MHz) δ ppm: 3.65-3.66 (m, 2H), 3.70 (s, 3H), 3.78 (d, *J* = 3,7 Hz), 4.14 (s, 2H), 4.67 (bs, 2H), 5.09 (t, *J* = 5,3 Hz), 5.52 (d, *J* = 3,8 Hz), 5.61 (d, *J* = 4,5 Hz), 6.27 (d, *J* = 3,9 Hz), 6.77 (d, *J* = 7,1 Hz), 6.89 (s, 1H), 6.91 (s, 1H), 7.20 (t, *J* = 7,6 Hz), 8.19 (s, 1H), 8.21 (s, 1H), 8.34 (bs, 1H).

EXAMPLE 2: Synthesis of 6-(3-hydroxybenzylamino)-9-y5-D-arabinofuranosylpurine

9-(/?-D-arabinofuranosyl) hypoxantine (100 mg, 0.37 mmol), BOP (196 mg, 0.44 mmol) and DIPEA (97 μï, 0.56 mmol) were mixed in v DMF (1.86 ml). Subsequently, 3-hydroxybenzylamine (50 μï, 0.50 mmol) was added. 9-(/?-D-arabinofuranosyl) hypoxantine is commercially available (Jena Bioscience, N-1002). Reaction mixture was mixed under argon atmosphere at laboratory temperature (25 °C) overnight (10 hrs). Reaction mixture was evaporated using vacuum rotary evaporator and purified by column liquid chromatography (mobile phase chloroform: methanol 19/1). Product is a white crystalline solid, yield 5%, TLC (chlorform:metanol (90:10, v:v): one spot; HPLC purity > 98%, $[M+H^+]$374, NMR: ^{1}H(DMSO-J $_6$, 300 MHz) δ ppm: 3.66 (s, 2H), 3.78 (s, 1H), 4.15 (s, 2H), 4.63 (bs 2H), 5.12 (s, 1H), 5.55 (s, 1H), 5.64 (s, 1H), 6.28 (s, 1H), 6.58 (d, *J* = 7,5 Hz), 6.73 (s, 1H), 6.76 (s, 1H), 7.07 (t, *J* = 7,5 Hz), 8.19 (s, 1H), 8.21 (s, 1H), 9.27 (bs , 1H).

EXAMPLE 3: Synthesis of 6-(3-fluorobenzylamino)-9-y5-D-arabinofuranosylpurine

9-(/?-D-arabinofuranosyl) hypoxantine (100 mg, 0.37 mmol),BOP (196 mg, 0.44 mmol) and DIPEA (97 μï, 0.56 mmol) were mixed together in DMF (1.86 ml) and subsequently, 3-fluorbenzylamine (70 μï, 0.76 mmol) was added. 9-(/?-D-arabinofuranosyl) hypoxantine was prepared from commercially available 9-(/?-D-arabinofuranosyl)adenine. Reaction mixture was mixed under argon at laboratory temperature of 25 °C for 24 h. Reaction mixture was evaporated on vacuum rotary evaporator and cold water was added after small portions of 15 ml. After several minutes of vortexing, yellowish substance started to occur. Reaction mixture was then refridgerated for 10 hrs. A product was filtered off and once recrystallized using isopropanol and twice using ethanol. Produkt is a white crystalline solid, in both cases of isolation, the yield 70%, TLC (chlorform:metanol (90:10, v:v): one spot; HPLC purity > 98%. [M+H$^+$] 376, NMR: ^{1}H(DMSO-J $_6$, 300 MHz) δ ppm: 3.66 (s, 2H), 3.79 (s, 1H), 4.14 (s, 2H), 4.74 (bs, 2H), 5.09 (s, 1H), 5.50 (s, 1H), 5.60 (s, 1H), 6.29 (s, 1H), 7.17 (s, 4H), 8.22 (s, 2H), 8.37 (bs, 1H).

EXAMPLE 4: Synthesis of 6-(3-iodobenzylamino)-9- β-D-arabinofuranosylpurine

9-(/?-D-arabinofuranosyl) hypoxantine (100 mg, 0.37 mmol),BOP (196 mg, 0.44 mmol) were mixed together in DMF (2 ml) and subsequently, 3-iodbenzylamine (60 μï) and DIPEA (97 μï) was added. 9-(/?-D-arabinofuranosyl) hypoxantine was prepared from commercially available 9-(/?-D-arabinofuranosyl)adenine. Reaction mixture was mixed under argon atmosphere in oil bath at the temperature of 60 °C for 24 h. Reaction mixture was evaporated on vacuum rotary evaporator. Arising gel was absorbed to silicagel and chromatography columns was used for sample purification using chloroform:methanol mobile phase with the gradient 99:1 to 9:1. TLC (chlorform:metanol (90:10, v:v): one spot; HPLC purity > 98%. [M+H$^+$] 484, NMR: ^{1}H(DMSO-J $_6$, 300 MHz) δ ppm: ^{1}H(DMSO-J $_6$, 500 MHz) δ ppm: 3.6 (s,lH), 3.7 (s,lH), 3.76-3.81 (m,2H), 4.09-4.17 (m,2H), 4.64 (bs,lH), 5.23 (bs,lH), 5.66 (bs,2H), 6.24 (d, J=5 Hz, 1H), 7.09 (t, J=7,5 Hz, 1H), 7.34 (d, J=7,5 Hz, 1H), 7.55 (d, J=8 Hz, 1H), 7.69 (s, 1H), 8.18 (s, 1H), 8.21 (s, 1H), 8.37 (bs, 1H)

EXAMPLE 5: Synthesis of 6-furfurylamino-9-y5-D-arabinofuranosylpurine

6-chloropurine tetraacetylarabinopyranoside (100 mg, 0.242 mmol) dispersed in methanol (3 ml) was placed into microwave reactor CEM SP reaction vessel (10 ml). Subsequently, furfurylamine (26.8 μï, 0.291 mmol) with triethylamine (151 μï, 1.09 mmol) were added. Reaction conditions were adjusted as follows: dynamic method, it means: reaction time 3 hrs, temperature: 100 °C, pressure 100 psi and performance: 50 watt. Reaction mixture was evaporated using vacuum rotary evaporator and purified using column liquid chromatography (mobile phase: chloroform:methanol 9/1). The product is a white solid: kinetin arabinopyranoside, mixture of a and β anomers in ration 5/1. The anomers were separated from each other: yield: 30% of a anomer and 10% of β anomer. Starting compound 6-chloropurine tetraacetylarabinopyranoside was prepared as follows: 6-chloropurine (0.412 g, 2.66 mmol) was placed into a dry flask and a tetraacetylarabinose (0.77 l g 2.42 mmol) dissolved in dry acetonitrile was added through the septum by a needle (15ml). Subsequently, tin tetrachloride was slowly added by a needle (5.6 mmol 0.6 ml). Reaction mixture was mixed at laboratory temperature under argon overnight. After that period, a mixture was evaporated using vacuum evaporater and ethylacetate (25 ml) was added. Organic phase was extracted by sodium carbonate solution (30 ml) and water(2 x 30 ml) and after that dried over sodium sulphate and again evaporated using vacuum evaporator. A product was purified by column liquid chromatography, mobile dichlormethane-aceton 9/1. As the result after the evaporation of organic solvents, there occurred clear gel like residue and this residue was mixed with diethyl ether and changed into a white solid 6-chloropurine tetraacetylarabinopyranosid, a mixture of a and β anomers. Yield: 50 %, HPLC purity: 98%, [M+H$^+$] 348, NMR: ^{1}H(DMSO-J $_6$, 300 MHz) δ ppm: 3.61-3.72 (m, 2H), 3.78 (d, *J* = 3,9 Hz), 4.14 (s, 2H), 4.69 (bs, 2H), 5.11 (t, *J* = 5,4 Hz), 5.54 (d, *J* = 3,9 Hz), 5.62 (d, *J* = 4,8 Hz), 6.22 (d, *J* = 2,7 Hz), 6.27 (d, *J* = 4,2 Hz), 6.36 (t, *J* = 3,0 Hz), 7.54 (s, 1H), 8.21 (s, 3H).

EXAMPLE 6: Synthesis of 6-(2-chlorobenzylamino)-9-y5-D-arabinofuranosylpurine

9-(/?-D-arabinofuranosyl) hypoxantine (100 mg, 0.37 mmol),BOP (196 mg, 0.44 mmol) were mixed together in DMF (2 ml) and subsequently, 2-chlorobenzylamine (55 μï) and

DIPEA (97 μï) was added. Reaction mixture was mixed under argon atmosphere in oil bath at the temperature of 60 °C for 24 h. Reaction mixture was evaporated on vacuum rotary evaporator. MeOH with a drop of chloroform was added to distillation residue and the mixture was ultrasonised. The process formed arising of a white paste solid that was filtrated. The resulted white solid was re-crystallized from EtOH and left in refndgerator overnight. Result was a white solid. TLC (chlorform:metanol (90:10, v:v): one spot; HPLC purity > 98%. [M+H$^+$] 392, NMR: ^{1}H(DMSO-J $_6$, 300 MHz) δ ppm: ^{1}H(DMSO-J $_6$, 500 MHz) δ ppm: 3.60-3.71 (m,2H), 3.77 (q,J=4 Hζ,IH), 4.11-4.15 (m,2H), 4.69 (bs,2H), 5.14 (bs,lH), 5.57 (bs,2H), 6.25 (d,J=4,5 Hζ,IH), 7.25-7.33 (m,3H), 7.36 (s,lH), 8.18 (s,lH), 8.21 (s,lH), 8.40 (bs,lH).

EXAMPLE 7: The synthesis of 6-(2-aminobenzylamino)-9-y5-D-arabinofuranosylpurine

9-(/?-D-arabinofuranosyl) hypoxantine (100 mg, 0.37 mmol),BOP (196 mg, 0.44 mmol) were mixed together in DMF (2 ml) and subsequently, 2-aminobenzylamine (55 μï) and DIPEA (97 μï) was added. Reaction mixture was mixed under argon atmosphere in oil bath at the temperature of 60 °C for 24 h. Reaction mixture was evaporated on vacuum rotary evaporator. The resulted distillation residue was re-crystallized from EtOH and left in refndgerator overnight. Result was a white solid that was filtrated off and dried. TLC (chlorform:metanol (90:10, v:v): one spot; HPLC purity > 98%, NMR: ^{1}H(DMSO-J $_6$, 300 MHz) δ ppm: ^{1}H(DMSO-J $_6$, 500 MHz) δ ppm: 3.59-3.70(m,2H), 3.77(q,J=4Hz,lH), 4.11-4.15(m,2H), 4.51(bs,2H), 5.10(t,J=5.5Hz,lH), 5.20(s,2H), 5.52(d,J=4Hz,lH), 5.60(d,J=5Hz,lH), 6.26(d,J=4hz,lH), 6.46(t,J=7.5Hz,lH), 6.59(d,J=8Hz,lH), 6.91(t,J=7.5Hz,lH), 7.07(d,J=7.5Hz,lH), 8.19(s,3H)

Table 1: 6-substituted-9-y5-D-arabinofuranosylpurines prepared by the method according to Examples 1-7, elemental analyses and ES-MS measurement results of these compouds

Substituent in position 6 (-NH-Ar)	Elemental analysis calculated/found			
	%C	%H	%N	ES-MS [M+H$^+$]
furfurylamino	51.9/51.6	4.9/4.8	20.2/20.2	348
2-fluorobenzylamino	54.4/54.1	4.8/4.8	18.7/18.4	376
3-fluorobenzylamino	54.4/53.9	4.8/4.7	18.7/18.2	376
4-fluorobenzylamino	54.4/54.3	4.8/4.8	18.7/18.3	376

2-chlorobenzylamino	52.1/52.0	4.6/4.7	17.9/17.5	392
3-chlorobenzylamino	52.1/51.9	4.6/4.6	17.9/17.3	392
4-chlorobenzylamino	52.1/51.8	4.6/4.5	17.9/17.1	392
2-bromobenzylamino	46.8/46.3	4.2/4.1	16.1/15.5	437
3-bromobenzylamino	46.8/47.8	4.2/4.5	16.1/15.6	437
4-bromobenzylamino	46.8/46.9	4.2/4.3	16.1/15.4	437
3-iodobenzylamino	42.3/42.4	3.8/3.9	14.5/14.6	484
2-methoxybenzylamino	55.8/55.9	5.5/5.3	18.1/17.9	388
3-methoxybenzylamino	55.8/55.5	5.5/5.7	18.1/18.0	388
4-methoxybenzylamino	55.8/55.6	5.5/5.5	18.1/18.1	388
2-hydroxybenzylamino	54.7/54.6	5.1/5.0	18.8/18.8	374
3-hydroxybenzylamino	54.7/54.5	5.1/5.1	18.8/18.5	374
4-hydroxybenzylamino	54.7/54.6	5.1/4.9	18.8/18.6	374
2,4-dichlorobenzylamino	47.9/47.8	4.0/4.1	16.4/16.5	427
3,4-dichlorobenzylamino	47.9/47.9	4.0/4.2	16.4/16.5	427
2,3-dihydroxybenzylamino	52.4/52.5	4.9/4.8	18.0/18.1	390
3,5-dihydroxybenzylamino	52.4/52.6	4.9/4.9	18.0/18.3	390
2-hydroxy-3-methoxybenzylamino	53.6/53.4	5.2/5.1	17.4/17.5	404
3-hydroxy-4-methoxybenzylamino	53.6/53.5	5.2/5.0	17.4/17.6	404
2,3-dimethoxybenzylamino	54.7/54.8	5.6/5.7	16.8/16.7	418
2,4-dimethoxybenzylamino	54.7/54.6	5.6/5.5	16.8/16.6	418
3,4-dimethoxybenzylamino	54.7/54.8	5.6/5.6	16.8/16.5	418
3,5-dimethoxybenzylamino	54.7/54.6	5.6/5.7	16.8/16.9	418

EXAMPLE 8: The synthesis of 6-(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurine in bench scale

Feedstock: 9-P-D-Arabinofuranosyl-hypoxanthine (1072 g, 4 mol), (Benzotriazol-l-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 2124 g (4.8 mol), dimethylformamide (DMF, 20 L, N,N-Diisopropyl-N-ethylamine (DIPEA, 1045 mL (6 mol), 3-methoxybenzylamine (658 g,4.8 mol), demi water 40 1, 2-propanol 30 L.

Procedure:

Dimethylformamide (20 1) was placed into a glass duplicated reactor A l equiped with thermometer (PT100) and reflux condenser. Reactor was filled in with inert atmosphere (nitrogen). Stirring with hopper opening was switched on. 9-|3-D-arabinofuranosyl hypoxanthine and BOP (2124 g) were poured to the reactor using the respirator. As soon as

the solid is dissolved, DIPEA (1045 mL) and 3-methoxybenzylamine (658 g) were added. The reactor stayed under continuous mild nitrogen flow.Reaction mixture was heated (using duplication) to 50 °C, and was stirred for 20 hrs. Reaction course control: after 12 hrs of reaction, sample for TLC: 1 ml aliquot was dilluted by 4 ml of methanol and the solution was applied next to the other starting compounds and standard product on TLC plate. TLC plate was evolved in the following mobile phase: chloroform:methanol:ammonium water solution; 4:1:0.05). If the reaction was still not finished, the mixture was further stirred at 50 °C, but next portion of BOP could be also added (200 g). If the reaction was finished (> 90 %), reaction mixture was cooled *(via* duplication) to the temperature of 20-25 °C and after that was reaction mixture drained into the transport vessel. Reactor was subsequently splashed with a small amount of methanol (3 x 1 L), and methanolic portions were mixed with reaction mixture). Reaction mixture was evaporated on rotary evaporator - a vacuum was secured by water ring vacuum pump - parameters such as pressure and temperature were established according to technolog instructions. Distillation residue was (hot) drained into transport vessel. Evaporator was splashed with hot methanol - and this portion was evaporated using vacuum evaporater separately from the main portion. Reactor A l was filled with demi water (40 L), stirring and cooling in duplication was switched on. Reactor was cooled to 10 °C and distillation residue was slowly added. Transport vessel was splashed with methanol (3 x 250 mL) and methanolic solution was also poured into the reactor. The reactor content was stirred for three hours at 10 - 15 °C. Emerging precipitate was filtered off on great Buchner channel and washed first with cold water (+5 °C) than only by water (4 x 1 L). Crude product was dried in a convection oven at 80 °C. *Yield:* 1200 - 1250 g.

Crystallization of 6-(3-methoxybenzylamino)-9-β-D-arabinofuranosylpurine

Crude product: 1000 g, 2-propanol: 18 l, active carbon CXV 50 g

Procedure: 2-propanol (15 l) was poured into A l reactor and stirring was switched on. Crude 6-(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurin (1000 g) was added. The content or reactor was heated via duplication to 80 °C - the solid of crude product should be dissolved. If the product was not dissolved, it is necessary to continue stirring at 80 °C, or add next portion of 2-propanol. As soon as was all solid dissolved, active carbon was added and stirring is continued at 80 °C for 15 minutes. After that, the solution was

filtrated off using preheated Biichner channel (preheated in convection oven, 110 °C). Glass reactor was rinsed by 2-propanol (2 x 1 L) and filtration cake was washed with this portion of 2-propanol Biichner chanell. Filtrate and flushing were merged together and placed into transport vessel for crystallization. Product crystallized at the temperature of +5 - +10 °C for 12 hrs. After the solid appeared, the product was filtrated off, rinsed with cold (+5 °C) 2-propanol (3 x 500 mL) and dried in convection oven at 70 °C to constant weight. *Yield:* 750 - 800 g, HPLC purity: > 98 %.

EXAMPLE 9: Synthesis of 6-(3-methoxybenzylamino)-9 -β-D-2´-deoxyribofuranosylpurine

2'-deoxyinosine (252 mg) and BOP (531 mg) were dissolved in dry DMF (5 ml) and stirred at laboratory temperature under argon atmosphere. After five minutes, DIPEA (261 μï) and 3-methoxybenzylamine (167 μï) were added. Reaction mixture was stirred at laboratory temperature for 16 hrs. Reaction process was controlled via TLC (mobile phase: chloroform-methanol-25% aqueous ammonia, 4 : 1 : 0.05). As soon as the conversion was not complete, next portion BOP (354 mg; 0.8 mmol) was added and reaction mixture was heated to 60 °C and stirred for 6 hrs. As soon as there were not detected a spot of starting 2'-deoxyinosine, reaction mixture was evaporated using vacuum evaporator (maximal temperature 55 °C). A residue (cca 1.5 g) was chromatographically purified using silica gel (150 g); mobile phase: 0-20 % methanol in dichlormethane. *Yield:* 270 mg (79 %), *HPLC-MS purity:* 98+%, [M+H $^{+}$] 372, mp 165-170 °C, C/H/N: 58,2/58,1; 5,7/5,7; 18,9/18,8; ^{1}H(DMSO-J $_{6}$, 300 MHz) δ ppm: 2.25-2.37 (m, 1H), 2.45-2.64 (m, 1H), 3.50-3.68 (m, 2H), 3.70 (s, 3H), 3.85-3.90 (m, 1H), 4.33-4.45(m, 1H), 4.67 (bs, 2H), 4.85 (t, 1H), 5.10 (d, 7 = 4.0 Hz), 6.30 (t, 7 = 6.9 Hz), 6.77 (d, 7 = 7.1 Hz), 6.89 (s, 1H), 6.91 (s, 1H), 7.20 (t, 7 = 7.6 Hz), 8.19 (s, 1H), 8.21 (s, 1H), 8.34 (bs, 1H).

EXAMPLE 10: Synthesis of 6-(2-hydroxybenzylamino)-9-p-D-2'-deoxyribofurano sylpurine

2'-deoxyinosine (252 mg) and BOP (664 mg) were dissolved in dry DMF (8 ml) and stirred at laboratory temperature under argon atmosphere. After five minutes, DIPEA (348 μï) and 2-hydroxybenzylamine (131 μï) were added. Reaction mixture was stirred at 50 °C for 20

hrs. Reaction process was controlled via TLC (mobile phase: chloroform- methanol-25% aqueous ammonia, 4 : 1 : 0.05). As soon as the conversion was not complete, next portion BOP (354 mg; 0.8 mmol) was added and reaction mixture was heated to 60 °C and stirred for 10 hrs. As soon as there were not detected a spot of starting 2'-deoxyinosine, reaction mixture was evaporated using vacuum evaporator (maximal temperature 55 °C). A residue (cca 1.5 g) was chromatographically purified using silica gel (150 g); mobile phase: 0-20 % methanol in dichlormethane. *Yield:* 250 mg (70 %), *HPLC-MS purity:* 98+%, [M+H $^+$] 358, mp 172-175 °C, C/H/N: 57,1/57,2; 5,3/5,4; 19,6/19,2; 1H(DMSO-i¾, 300 MHz) δ ppm: 3.66 (s, 2H), 3.78 (s, 1H), 4.15 (s, 2H), 4.63 (bs 2H), 5.12 (s, 1H), 5.55 (s, 1H), 5.64 (s, 1H), 6.28 (s, 1H), 6.58 (d, *J* = 7.5 Hz), 6.73 (s, 1H), 6.76 (s, 1H), 7.07 (t, *J* = 7.5 Hz), 8.19 (s, 1H), 8.21 (s, 1H), 9.27 (bs , 1H).

EXAMPLE 11: Synthesis of 6-(2-hydroxy-3-methoxybenzylamino)-9-p-D-2'-deoxyribofurano sylpurine

2'-deoxyinosine (252 mg) and BOP (664 mg) were dissolved in dry DMF (10 ml) and stirred at 50 °C. After five minutes, DIPEA (348 µï) and 2-hydroxy-3-methoxybenzylamine (165 µï) were added. Reaction mixture was stirred at laboratory temperature for at least 20 hrs. Reaction process was controlled via TLC (mobile phase: chloroform-methanol-25% aqueous ammonia, 4 : 1 : 0.05). As soon as the conversion was not complete, next portion BOP (354 mg; 0.8 mmol) was added and reaction mixture was heated to 70 °C and stirred for 6 hrs. As soon as there were not detected a spot of starting 2'-deoxyinosine, reaction mixture was evaporated using vacuum evaporator (maximal temperature 55 °C). A residue (cca 1.5 g) was chromatographically purified using silicagel (150 g); mobile phase: 0-20 % methanol in dichlormethane. *Yield:* 270 mg (79 %), *HPLC-MS purity:* 98+%, mp 174-178 °C, [M+H $^+$] 388, C/H/N: 55,8/55,3; 5,5/5,6; 18,1/18,2; ^{1}H(DMSO-J $_6$, 300 MHz) δ ppm: 2.25-2.37 (m, 1H), 2.45-2.64 (m, 1H), 3.50-3.68 (m, 2H), 3.77 (s, 3H), 3.85-3.90 (m, 1H), 4.33-4.45(m, 1H), 4.67 (bs, 2H), 4.85 (t, 1H), 5.10 (d, *J* = 4.0 Hz), 5.39 (d, *J* = 6.0 Hz), 6.30 (t, *J* = 6.9 Hz), 6.77 (d, *J* = 7.1 Hz), 6.89 (s, 1H), 7.20 (t, *J* = 7.6 Hz), 8.19 (s, 1H), 8.21 (s, 1H), 8.34 (bs, 1H).

EXAMPLE 12: Synthesis of 6-(furfurylamino)-9 -β-D-2´-deoxyribofuranosylpurine

2'-deoxyinosine (252 mg) and BOP (670 mg) were dissolved in dry DMF (10 ml) and stirred at laboratory temperature under argon atmosphere. After five minutes, DIPEA (350 μï) and furfurylamine (150 μï) were added. Reaction mixture was stirred at 60 °C for at least 10 hrs. Reaction process was controlled via TLC (mobile phase: chloroform-methanol-25% aqueous ammonia, 4 : 1 : 0.05). As soon as the conversion was not complete, next portion BOP (354 mg; 0.8 mmol) was added and reaction mixture was heated to 70 °C and stirred for 6 hrs. As soon as there were not detected a spot of starting 2'-deoxyinosine, reaction mixture was evaporated using vacuum evaporator (maximal temperature 55 °C). A residue (cca 1.5 g) was chromatographically purified using silica gel (150 g); mobile phase: 0-20 % methanol in dichlormethane. *Yield:* 300 mg (82 %), *HPLC-MS purity:* 98+%, [M+H $^+$] 332, C/H/N: 54,4/54,3; 5,2/5,2; 19,3/19,5 ^{1}H(DMSO-J $_6$, 300 MHz) δ ppm: 3.61-3.72 (m, 2H), 3.78 (d, J = 3.9 Hz), 4.14 (s, 2H), 4.69 (bs, 2H), 5.11 (t, J = 5.4 Hz), 5.54 (d, J = 3.9 Hz), 5.62 (d, J = 4.8 Hz), 6.22 (d, J = 2.7 Hz), 6.27 (d, J = 4.2 Hz), 6.36 (t, J = 3.0 Hz), 7.54 (s, 1H), 8.21 (s, 3H).

Table 2: 6-substituted-9 -β-D-2´-deoxyribofuranosylpurines prepared according to examples 9-12

Substituent in position 6 (-NH-Ar)	Elemental analysis calculated/found			
	%C	%H	%N	ES-MS [M+H $^+$]
4-methylfurfurylamino	55.6/55.5	5.5/5.6	18.5/18.4	346
5-methylfurfurylamino	55.6/55.4	5.5/5.6	18.5/18.3	346
4-hydroxyfurfurylamino	51.9/51.8	4.9/5.0	20.2/20.1	348
5-hydroxyfurfurylamino	51.9/52.0	4.9/4.8	20.2/20.2	348
3-chlorobenzylamino	54.3/54.2	4.8/4.8	18.6/18.6	376
4-chlorobenzylamino	54.3/54.3	4.8/4.9	18.6/18.5	376
2-bromobenzylamino	48.6/48.7	4.3/4.4	16.7/16.7	421
3-bromobenzylamino	48.6/48.6	4.3/4.5	16.7/16.8	421
4-bromobenzylamino	48.6/48.3	4.3/4.3	16.7/16.9	421
2-methoxybenzylamino	58.2/58.3	5.7/5.5	18.9/18.6	372
3-methoxybenzylamino	58.2/58.1	5.7/5.9	18.9/18.7	372
2-hydroxybenzylamino	57.1/57.2	5.4/5.3	19.6/19.8	358
3-hydroxybenzylamino	57.1/57.3	5.4/5.3	19.6/19.5	358
4-hydroxybenzylamino	57.1/57.1	5.4/5.2	19.6/19.5	358
2,3-dihydroxybenzylamino	54.7/54.5	5.1/5.2	18.8/18.7	374

3,5-dihydroxybenzylamino	54.7/54.8	5.1/5.3	18.8/18.6	374
2-hydroxy-3-methoxybenzylamino	55.8/55.7	5.5/5.6	18.1/18.2	388
3-hydroxy-4-methoxybenzylamino	55.8/55.6	5.5/5.7	18.1/18.3	388
2,3-dimethoxybenzylamino	56.9/56.8	5.8/5.7	17.5/17.4	402
2,4-dimethoxybenzylamino	56.9/56.9	5.8/5.6	17.5/17.5	402
3,4-dimethoxybenzylamino	56.9/56.8	5.8/5.9	17.5/17.7	402
3,5-dimethoxybenzylamino	56.9/56.9	5.8/5.9	17.5/17.8	402

EXAMPLE 13: Evaluation of cytotoxicity of novel derivatives for skin cell by MTT in vitro test

MTT assay is a standard test of toxicity based on photometric measurement of the ability of metabolically active cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Using the assay, the effects of 72 hour treatments with several concentrations of the compounds (sixfold dilution, maximal concentration = 50 microM) on viability of skin fibroblasts BJ and keratinocytes HaCaT were evaluated. About 5,000 cells were seeded per well of a 96-well plate 24 hours before the treatment. DMSO vehiculum was used as a negative control. After 72 hour treatment, new medium with MTT (Sigma, M2128) was added to a final concentration of 0.5 mg/ml. After 3 hours, medium was removed and resulting formazan in the cells was dissolved in DMSO. The absorbance was measured at 570 nm (640 nm reference wavelength). The IC50 values were calculated from the dose-response curves. 6-Benzylaminopurine riboside and 6-furfurylaminopurine riboside were used as positive controls - they were toxic in the MTT test. The following results were obtained.

	IC50 (μM)
dimethylsulfoxide	>50
6-benzylamino-9-P-D-arabinofuranosylpurine	>50
6-furfurylamino-9-P-D-arabinofuranosylpurine	>50
6-(3-methoxybenzylamino)-9-P-D-arabinofuranosylpurine	>50
6-(2-chlorobenzylamino)-9-P-D-arabinofuranosylpurine	>50
6-(3-hydroxybenzylamino)-9-P-D-arabinofuranosylpurine	>50
6-(3-chlorobenzylamino)-9-P-D-arabinofuranosylpurine	>50

6-(2,3-dimethoxybenzylamino)-9 -P-D-arabinofuranosylpurine	**49**
6-(2,3,4-trimethoxybenzylamino)-9 -P-D-arabinofuranosylpurine	48
6-(3-iodobenzylamino)-9 -P-D-arabinofuranosylpurine	>50
6-(3-aminobenzylamino)-9 -P-D-arabinofuranosylpurine	>50
6-(furfurylamino)-9 -β-D-2´-deoxyribofuranosylpuri ne	>50
6-(2-hydroxybenzylamino)-9 -β-D-2´-deoxyribofuranosylpuri ne	>50
6-benzylaminopurin9-ribosylpurine (comparative example)	≤ 3
6-furfurylamino-9-ribosylpurine (comparative example)	≤ 3

EXAMPLE 14: SRB in vitro toxicity test

SRB (sulforhodamine B) assay is a standard toxicity test based on a photometric measurement of the cellular protein content after the staining with sulphorhodamine B. Using the assay, the effects of 72 hour treatments with several concentrations of the compounds (sixfold dilution, maximal concentration = 50 microM) on viability of skin fibroblasts BJ and keratinocytes HaCaT were evaluated.About 5,000 cells were seeded per well of a 96-well plate 24 hours before the treatment. DMSO vehiculum was used as a negative non-toxic control. 6-benzylaminopurine riboside a 6-furfurylaminopurin riboside were used as positive toxic controls. After three days the medium was removed and the cells fixed with 10% (wt/vol) trichloroacetic acid. After the extensive washing in distilled water, 0.4% (wt/vol) solution of SRB in acetic acid was added and the fixed cells were stained for 30 minutes. The unbound stain was washed away by distilled water and the bound SRB was solubilized in unbuffered 10 mM Tris base. Absorbance was measured at 564 nm. IC50 values were calculated from dose-response curves.

The following results were obtained:

	IC 50 (μM)
dimethylsulfoxide	>50
6-benzylaminopurine-9-P-D-arabinofuranosylpurine	>50
6-furfurylamino-9-P-D-arabinofuranosylpurine	>50
6-(3-methoxybenzylamino)-9-P-D-arabinofuranosylpurine	>50
6-(2-chlorobenzylamino)-9-P-D-arabinofuranosylpurine	>50

6-(3-hydroxybenzylamino)-9-P-D-arabinofuranosylpurine	>50
6-(3-chlorobenzylamino)-9-P-D-arabinofuranosylpurine	48
6-(3-iodobenzylamino)-9-P-D-arabinofuranosylpurine	>50
6-(3-aminobenzylamino)-9-P-D-arabinofuranosylpurine	>50
6-(furfurylamino)-9-P-D-2'-deoxyriboside	>50
6-(2-hydroxybenzylamino)-9-P-D-2'-deoxyriboside	>50
6-(2,3-dimethoxybenzylamino)-9-P-D-arabinofuranosylpurine	>50
6-(2,3,4-trimethoxybenzylamino)-9-P-D-arabinofurano sylpurine	>50
6-furfurylamino-9-ribosylpurine (comparative example)	≤ 3

EXAMPLE 15: One-step growth curve for the compound 6-(benzylamino)purine-9 -P-D-arabinofurano sylpurine

The experiments were performed with BJ skin fibroblasts in 24-well tissue culture plates. About 10,000 cells in culture medium comprising DMEM with 10 % FBS were seeded into the individual wells. The cells were allowed to attach for 24 hourse. The test compound was added to final concentrations in a range from 12.5 to 100 μM. DMSO vehiculum was also tested. In order to control for variability, two plate columns (A,D) were treated with DMSO vehiculum. The culture medium with the test chemicals or DMSO vehiculum was changed twice a week. Following trypsinization, the numbers of cells in 4 wells for each concentration were counted using Coulter counter on 7th and 14th day. The obtained results are showed in Fig. 1. The tested compound did not have a negative influence on cell viability.

EXAMPLE 16: In vitro test of skin irritation in EpiDERM™

EpiDERM™ is a 3D model of epidermis manufactured by Mattek company. The effects of tested substances are evaluated by MTT. 2 solutions (2 application forms) of the compounds 6-furfurylamino-9 -P-D-arabinofuranosylpurine and 6-(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurine in concentrations 1 mM a 200 microM in 0,5% DMSO/99,5% PBS were evaluated according to a standard protocol "INVrrPvO EpiDermTM SKIN IRRITATION TEST". After a preincubation of the tissues,

30 microliters of the application form - solution were applied on the individual tissues. The exposition time was 60 minutes. Three tissues were used for each tested concentration as well as for controls. Following washing out of the applied solution, the tissues were incubated for 42 hour in order to allow a reparation of possible damage. In the next step, the tissue was incubated with MTT for 3 hours. The resulting formazan was extracted to isopropanol. Relative viability of the individual tissues was calculated as a percentage of viability of the average of the negative controls. Average viability of the tissues treated with 1 mM solution of the test substance was 99.3 % for the compound 6-(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurine and 98.0 % for the compound 6-(furfurylamino)-9 -P-D-arabinofuranosylpurine where 100 % is an average viability of the tissues treated with the solvent only. None of the tested compounds in any of the concentrations caused any tissue damage or irritation.. The compounds were evaluated as non-irritant in the tested concentrations. This result is favorable for the intended use of the compounds in cosmetics.

EXAMPLE 17: Evaluation of eye irritation in EpiOcular™

EpiOcular is a model of corneal epithelium manufactured by Mattek company. The effect of the tested substances is evaluated by MTT. A substance is considered irritant/corrosive if the absorbance of the formazane created by reduction of MTT is lower than 60 percent of value for negative control. Solutions (250 a 500 microM) of compounds 6-benzylamino-9-β-D-arabinofuranosylpurine, 6-furfurylamino-9 -P-D-arabinofuranosylpurine and 6-(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurin in the medium from the EpiOcular kit were tested in duplicates. The medium served as a negative control. The test was carried out according the manufacturer's manual - the protocol for testing of liquid application forms. Only incubation time was increased from 30 min to 18 hours. The procedure is summarized below. After the delivery, the tissues were left in laboratory temperature for 15 min. After the integrity control and removal of agarose, the tissues were transferred into the wells of the 6-well plates containing 1 ml of cultivation medium. After 1 hour, the medium was exchanged for fresh one and the tissues were cultivated for 18 hours. Subsequently 20 microliters of DPBS without Ca2+ and Mg2+ (a component of the kit) was applied on the tissues, followed by 50 microliters of test solutions. After 18 hour incubation time, the

tissues were removed from cultivation plate and washed out repeatedly in an excess of DPBS without Ca2+/Mg2+ (3 beakers with 100 ml). Subsequently the tissues were transferred to the wells of 12-well plates with 5 ml temperated medium and incubated for 12 minutes. After that, the tissues were transferred into the wells of 6-well plate with 1 ml of medium and incubated for 2 hours. Three hour incubation with MTT solution (1 mg/ml) was carried out in 24-well plates (0.3 ml of medium per well). After the incubation, the tissues were transferred to a new 24-well plate with 2 ml isopropanol per well. The plate was placed on a shaker and the resulting formazan was extracted for 3 hours. 200 microliters of the extracts were transferred to 96-well plate. Absorbance was measured at 570 nm and related to that of the negative control. All the tissue manipulation before the isopropanol extraction was carried out in sterile conditions. The cultivation was done in the standard cultivation conditions (C02 5,5 procent, 37 °C), the cultivation medium and DPBS without Ca^{2+} and Mg^{2+} ions.

Conclusion: The relative viability of the tissues treated with the test compounds was 98-103 %. The compounds in the tested concentration range do not cause irritation and can be favorably used in cosmetics including preparations that could be applied on face and the area around eyes.

EXAMPLE 18: Anti-senescent activity of novel compounds tested in senescent bioassay on wheat leaf segments

Seeds of winter wheat, *Triticum aestivum* cv. Hereward, were washed under running water for 24 hours and then sown on vermiculite soaked with Knop's solution. They were placed in the growth chamber at 25°C with a 16/8 h light period at 50 μηοϊ.ιη^{-2}.s^{-1}. After 7 days, the first leaf was fully developed and the second leaf had started to grow. A tip section of the first leaf, approximately 35 mm long, was removed from 5 seedlings and trimmed slightly to a combined weight of 100 mg. The basal ends of the five leaf tips were placed in the wells of a microtiter polystyrene plate containing 150 μL of the tested derivative solution each. The entire plate was inserted into a plastic box lined with paper tissues soaked in distilled water to prevent leaf sections from drying out. After 96 h incubation in the dark at 25°C, the leaves were removed and chlorophyll extracted by heating at 80°C for 10 min in 5 mL of 80% ethanol (v/v). The sample volume was then restored to 5 mL by the

addition of 80% ethanol (v/v). The absorbance of the extract was recorded at 665 nm. In addition, chlorophyll extracts from fresh leaves and leaf tips incubated in deionised water were measured. The results are means of five replicates and the entire test was repeated twice. In each experiment activities of the novel compounds were tested and compared with activity of BAP, which is known to be highly active cytokinin.

The compounds to be tested were dissolved in dimethylsulfoxide (DMSO) and the solution brought up to 10^{-3} M with distilled water. This stock solution was further diluted with the respective media used for the biotest to a concentration ranging from 10^{-8} M to 10^{-4} M. The final concentration of DMSO did not exceed 0.2 % and therefore did not affect the biological activity in the assay system used. The activity obtained for 10^{-4} M of BAP was postulated as 100 %.

Newly developed compounds possess very strong antisenescent properties. Some of them cause 200% increase of chlorophyll content in detached wheat leaves in comparison to BAP.

Tab. 3: The effect of novel compounds on delaying senescence in detached leaf segments of Triticum aestivum cv. Hereward. The results are expressed in % of initial content of chlorophyll in fresh leaves before incubation.

Compound	maximum effective concentration (moll $^{-1}$)	activity (%) [Iθ^{-4}ηοΠ^{-1} BAP = 100%]
6-furfurylamino-9-P-D-arabinofuranosylpurine	10^{-4}	193+1
6-(3-methoxybenzylamino)-9-P-D-arabinofuranosylpurine	10^{-4}	118+2
6-benzylaminopurine-9 β-D-deoxyribosylpurine	10^{-4}	178+9
6-(3-chlorobenzylamino)-9-P-D-arabinofuranosylpurine	10^{-4}	172+8
6-(3-fluorobenzylamino)-9-P-D-arabinofuranosylpurine	10^{-4}	195+6
6-(2-bromobenzylamino)-9-P-D-arabinofuranosylpurine	10^{-5}	186+19
6-(3-bromobenzylamino)-9-P-D-arabinofuranosylpurine	10^{-4}	198+10
6-(4-bromobenzylamino)-9-P-D-arabinofuranosylpurine	10^{-4}	176+1 1

6-(3-iodobenzylamino)-9-P-D-arabinofuranosylpurine	10^{-4}	198+4
6-(3,4-dimethoxybenzylamino)-9-P-D-arabinofuranosylpurine	10^{-4}	147+6
6-(3-chlorobenzylamino)-9-riboside (comparative example)	10^{-4}	72+8
6-(3-iodobenzylamino)-9-riboside (comparative example)	10^{-4}	58+19
6-(3-bromobenzylamino)-9-riboside (comparative example)	10^{-4}	89+10
6-(3,4-dimethoxybenzylamino)-9-riboside (comparative example)	10^{-4}	47+6
6-(2,4-dichlorbenzylamino)-9-riboside (comparative example)	10^{-4}	5+1

EXAMPLE 19: *In vitro* cytotoxic activity of new derivatives on cancer cell lines

One of the parameters used as the base for cytotoxic analysis is metabolic activity of viable cells, such as microtiter assay, which uses the Calcein AM, is now widely used to quantitate cell proliferation and cytotoxicity. The quantity of reduced Calcein AM corresponds to the number of viable cells in culture. The cell lines of breast cancer (MCF-7), mousse fibroblasts (NIH3T3), human erythromleukemia (K562) were used for routine screening of cytotoxicity of the compounds. The cells were maintened in Nunc/Corning 80 cm^2 plastic bottles and grown in media for cell culture (DMEM containing 5g/l of glucose, 2mM of glutamin, 100 U/ml of penicilin, 100 μg/ml of streptomycin, 10% of fetal bovine serum and sodium hydrogencarbonate). Cell suspensions were diluted according to cell types and according to expected final cell density (10^4 of cells per well according to characteristics of cell growth), pipetted 80 μï of cell suspension on 96-well microtiter plates.. Innoculates were stabilized by 24 hrs preincubation at 37°C in $C0_2$. Particular concentrations of tested compounds were added in time zero as 20 μï aliquotto wells of microtiter plates. Usually, the compounds were diluted into six concentrations in four-fold dilution series. In routine testing, the highest well concentration was 166.7 μM, of change dependent on the substance. All drag concentrations were examined in duplicates .The incubation of cells with tested derivatives lasted 72 hrs at 37°C, 100 % humidity and in the atmosphere of $C0_2$. At the end of the incubation period, the cells were tested and analysed according to the addition of Calcein AM (Molecular probes) solution and the incubation

lasted for next 1 hour. Fluorescence (FD) was measured using Labsystem FIA reader Fluorskan Ascent (Microsystems). The survival of tumor cells (The tumor cell survival-TCS) was counted according to equation: $GI_5o=(FD_{we}u_{with\ derivative}/FD_{con\ troi\ well}) \times 100\ \%$. The value of GI_{50}, that is equal to the concentration of compound at which 50 % of tumour cells are terminated. To evaluate the antitumor activity was tested toxicity of new derivatives on panel of cell lines of different histogenetic and species origin (Tab. 7 , GI50 concentration given in μM) . It turned out that new compounds showed to be non toxic for neither of all tested tumor lines nor for nonmalignant cell line NIH3T3. Effective derivatives killed tumor cells in concentrations close to 0.1 to 50. None of the newly prepared compounds only reached the value.

Tab. 4. Cytotoxicity of newly prepared compouds for various tumour cell lines and NIH3T3

Compound	MCF-7	K562	NIH3T3
6-benzylamino-9-ribosylpurine (comparative example)	5.4	5.5	39
6-benzyl-9 -P-D-arabinofuranosylpurine	>100	>100	>100
6-furfuryl-9-p-D-arabinofuranosylpurine	>100	>100	>100
6(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurine	>100	>100	>100
6-benzylamino-9 -P-D-deoxyribosylpurine	>100	>100	>100
3-methylbenzylamino-9 -P-D-arabinofuranosylpurine	>100	>100	>100
4-methylbenzylamino-9 -P-D-arabinofuranosylpurine	95	>100	>100
2-methylbenzylamino-9 -P-D-arabinofuranosylpurine	>100	>100	>100
3-fluorobenzylamino-9 -P-D-arabinofuranosylpurine	>100	>100	>100
4-chlorobenzylamino-9 -P-D-arabinofuranosylpurine	>100	>100	>100
2-fluorobenzylamino-9 -P-D-arabinofuranosylpurine	87	>100	>100
3-chlorobenzylamino-9 -P-D-arabinofuranosylpurine	>100	>100	>100
4-hydroxybenzylamino-9 -P-D-arabinofuranosylpurine	>100	>100	87
3-fluorobenzylamino-9- β-D-2'-	>100	>100	>100

deoxyribosylpurine			
3-chlorobenzylamino-9-P-D-2'-deoxyribosylpurine	>100	>100	>100
3-hydroxybenzylamino-9-P-D-2'-deoxyribosylpurine	>100	>100	>100
2,4-dimethoxybenzylamino-9-P-D-arabinofuranosylpurine	98	>100	>100
2-chloro-4-fluorobenzylamino-9-P-D-arabinofuranosylpurine	>100	>100	>100
3-chloro-4-fluorobenzylamino-9-P-D-arabinofuranosylpurine	>100	>100	>100

EXAMPLE 20. *In vitro* test of phototoxic effects of 6-(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurine on normal human dermal fibroblasts

Phototoxic potential of test compound was determined by modified *in vitro* test validated phototoxicity evaluation (Spielmann H, Balls M, Dupuis J, Pape WJ, Pechovitch G, de Silva O, Holzhiitter HG, Clothier R, Desolle P, Gerberick F, Liebsch M, Lovell WW, Maurer T, Pfannenbecker U, Potthast JM, Csato M, Sladowski D, Steiling W, Brantom P., Toxicol In Vitro. **1998** Jun l;12(3):305-27). Normal human dermal fibroblasts (NHDF) were used as an *in vitro* model. Cells were isolated from tissue specimens obtained from healthy patients undergoing plastic surgery at the Department of Plastic and Aesthetic Surgery (University Hospital Olomouc). The use of skin tissue was in accordance with the Ethics Committee of the University Hospital and Faculty of Medicine and Dentistry, Palacky University, Olomouc and all patients signed written informed consent. Fibroblasts were used between the 2nd and 4th passage. For all experiments the fibroblasts were seeded onto 96-well plates at a density of 0.8×10^5 cells/ml (0.2 ml per well) of cultivation medium (DMEM supplemented with foetal calf serum (10%, v/v), penicillin (100 mg/ml) and streptomycin (100 U/ml)).

Test compound was 6-(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurine. Compound was dissolved in DMSO and then diluted in serum free medium (DMEM supplemented with penicillin (100 mg/ml) and streptomycin (100 U/ml)). The final applied concentrations of the compound 0.39-125 μηοΐ/ΐ. As a control, serum free medium supplemented with appropriate concentration of DMSO (0.5 %, v/v) was used. In parallel with test compounds, chlorpromazine (CPZ; 0.39-100 μηοΐ/ΐ) was used as a known

phototoxic compound. The test compound was in parallel applied on two 96-well plates with NHDF. After 60 minutes incubation with test compound medium was discarded, cells were washed two-times with PBS and PBS supplemented with glucose (1 mg/ml) was applied. A plate was then exposed to a non-cytotoxic dose of UVA radiation (5.0 J/cm^2) using a solar simulator SOL 500 (Dr. Hoenle Technology, Germany) equipped with a HI filter transmitting wavelengths of 320-400 nm. Intensity of UVA radiation was evaluated before each irradiation by UVA-meter. A control (non-irradiated) plate was for the period of irradiation incubated in dark. After UVA exposure PBS with glucose was discarded and serum free medium was applied. After 24 hours (37 °C, 5 % $C0_2$) cell damage was evaluated by neutral red (NR) incorporation into viable cells. Medium was discarded and NR solution (0.03% w/v, PBS) was applied. After 60 minutes NR solution was discarded, cells were fixed with a mixture of formaldehyde (0.5%, v/v) and $CaCl_2$ (1 %, m/v) in ratio 1:1 and then NR was dissolved in methanol (50%, v/v) with acetic acid (1%, v/v). After 5 minutes of intensive shaking absorbance was measured at 540 nm. Experiments were performed in four independent repetition with use of cells from four donors to minimize individual sensitivity of donor cells. Phototoxic effect was evaluated as % of viability of control cells that was calculated from experimental data (absorbance) according to the following equation:

$$\text{Viability (\% of control)} = \left(\frac{(A_S - A_B)}{(A_C - A_B)} \right) \cdot 100$$

As … absorbance of sample (cells pre-incubated with test compound in serum free medium and irradiated)

A_C ... absorbance of control (cells pre-incubated with DMSO in serum free medium and irradiated)

A_B … absorbance of background (extraction solution)

Result: Treatment with test compounds and following exposure to non-toxic UVA dose did not cause decrease in cell viability ~ incorporation of NR and thus test compound can be considered as non-phototoxic in the used concentration range (0.9-125 μηοϊ/ϊ or 3.9-500 μηοϊ/ϊ). Results are given in Fig. 3. Effect of a well-known phototoxic compound chlorpromazine (Spielmann H, Balls M, Dupuis J, Pape WJ, Pechovitch G, de Silva O, Holzhiitter HG, Clothier R, Desolle P, Gerberick F, Liebsch M, Lovell WW, Maurer T,

Pfannenbecker U, Potthast JM, Csato M, Sladowski D, Steiling W, Brantom P., Toxicol In Vitro. **1998** Jun l;12(3):305-27), used as positive control is demonstrated in Fig. 4. Above data indicate that test compounds are safe for cosmetic and dermatological application including use with following exposure of treated skin with solar radiation.

EXAMPLE 21: *In vitro* test of photoprotective effects of 6(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurine on dermal fibroblasts

Normal human dermal fibroblasts (NHDF) were used as an *in vitro* model. Cells were isolated from tissue specimens obtained from healthy patients undergoing plastic surgery at the Department of Plastic and Aesthetic Surgery (University Hospital Olomouc). The use of skin tissue was in accordance with the Ethics Committee of the University Hospital and Faculty of Medicine and Dentistry, Palacky University, Olomouc and all patients signed written informed consent. Fibroblasts were used between the 2nd and 4th passage. For all experiments the fibroblasts were seeded onto 96-well plates at a density of 0.8×10^5 cells/ml (0.2 ml per well) of cultivation medium (DMEM supplemented with foetal calf serum (10%, v/v), penicillin (100 mg/ml) and streptomycin (100 U/ml)).

Test compounds included 6-(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurine. Compounds were dissolved in DMSO and then diluted in serum free medium (DMEM supplemented with penicillin (100 mg/ml) and streptomycin (100 U/ml)). The final applied concentrations of 6(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurine were 0,9; 1,8; 3,9 a 7,8 μηοϊ/ϊ. As a control serum free medium supplemented with appropriate concentration of DMSO (0.5 %, v/v) was used. Each test compound was in parallel applied on two 96-well plates with NHDF. After 60 minutes incubation medium with test compound was discarded, cells were washed two-times with PBS and PBS supplemented with glucose (1 mg/ml) was applied. To study UVA photoprotection, a plate was exposed to a cytotoxic dose of UVA radiation (7.5 J/cm^2) using a solar simulator SOL 500 (Dr. Hoenle Technology, Germany) equipped with a H I filter transmitting wavelengths of 320-400 nm. To study UVB photoprotection, a plate was exposed to a cytotoxic dose of UVB radiation (400 mJ/cm^2) using the solar simulator equipped with a H2 filter transmitting wavelengths of 295-320 nm. Intensity of UVA or UVB radiation was evaluated before each irradiation by UVA- or UVB -meter. Control (non-irradiated) plates were for the period of

irradiation incubated in dark. After UVA or UVB exposure PBS with glucose was discarded and serum free medium was applied. After 24 hours (37 °C, 5 % $C0_2$) cell damage was evaluated by neutral red (NR) incorporation into viable cells. Medium was discarded and NR solution (0.03% w/v, PBS) was applied. After 60 minutes NR solution was discarded, cells were fixed with a mixture of formaldehyde (0.5%, v/v) and $CaCl_2$ (1 %, m/v) in ratio 1:1 and then NR was dissolved in methanol (50%, v/v) with acetic acid (1%, v/v). After 5 minutes of intensive shaking absorbance was measured at 540 nm. Experiments were performed in four independent repetition with use of cells from four donors to minimize individual sensitivity of donor cells. Photoprotective effect was evaluated by comparison of experimental data (absorbance) of test compounds with a positive control and a negative control (according to the following equation:

$$\text{Protection (\%)} = 100 - \left| \frac{As - Anc}{Apc - Anc} \right| \cdot 100$$

As ... absorbance of sample (cells pre-incubated with test compounds in serum free medium and irradiated)

Anc ... absorbance of negative control (cells pre-incubated with s DMSO in serum free medium and non-irradiated = incubated in dark)

Ape ... absorbance of positive control (cells pre-incubated with s DMSO in serum free medium and irradiated)

Results: Cells pre-incubated with test compounds and exposed to UVA or UVB radiation showed higher viability (ability to incorporate NR) compared to those pre-incubated with DMSO (control) and UVA or UVB irradiated (Fig. 5 and 6). Therefore test compound has high photoprotective potential.

EXAMPLE 19: Differential gene expression study

Comparative gene expression analysis in Arabidopsis model was performed to gain information about the reprogramming of gene transcription when senescent leaves were treated with 6-(3-methoxybenzylamino)-9 -P-D-arabinofuranosylpurine (3MeOBAPA).

For isolation of total RNA, Arabidopsis wild-type plants (Col-0) were used, which were either treated with 3MeOBAPA or left untreated. Wild-type plants were grown in soil for 30 days, and then leaves of similar size and chlorophyll content were cut and immediately used for the treatment. At least 20 detached leaves were submerged in 1 x MS medium

supplemented with 10 μM 3MeOBAPA. Control samples were mock treated with DMSO only. After incubation for 6 h or 48 h in the dark, detached leaves were frozen in liquid nitrogen and used for RNA isolation; 150 mg of liquid nitrogen-ground material was used per isolation. cDNA sequencing libraries were prepared with the Illumina TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA) according to standard Illumina' s protocols and sequenced on HiSeq 2500 apparatus (50 bp single-end reads).

Data were subjected to differential transcriptomic analysis with the aim to reveal significantly regulated genes and their expression levels. To gain insight into the molecular mechanism of 3MeOBAPA action in Arabidopsis we decided to analyze the gene expression patterns *via* comparison of mock (DMSO)-treated plants with those obtained after i) short time treatment with 3MeOBAPA (6 h) and ii) long time treatment (48 h). This comparison leads to identification of group of genes with similar kinetic of expression and helps to understand possible mechanism of regulation. For data analysis, we performed *ab initio* method where sequencing reads were mapped to the reference genome. The short time treatment resulted in reprogramming of the gene transcription compared to the mock-treated control with 1119 downregulated and 1102 upregulated genes ($P \leq 0.05$). Further treatment with 3MeOBAPA (i.e. 48 h) led to alterations in the expression profiles and, thus, we could observe more profound changes in the numbers of affected genes: 7 095 genes were downregulated and 7509 genes were upregulated ($P \leq 0.05$). Analysis of regulated genes in both groups (short time treatment vs. long time treatment) showed a substantial overlap in the two categories. Indeed, we could detect 1102 genes that were upregulated in response to 3MeOBAPA treatment and 724 genes that were downregulated in both groups (Fig. 7). The overlap is particularly visible in the group of upregulated genes suggesting a rapid response to the elicitation after 6 h treatment that reaches maximal values after 48 h incubation with 3MeOBAPA. This trend is well documented in the list of top 50 genes upregulated in response to 3MeOBAP treatment (Tab. 1). As evident, all genes that were upregulated after 6h of the treatment probably remain activated over a period of 48 h and their expression levels in the latter time point are one or two orders of magnitude higher than those recorded in 6h.

A closer inspection of the top 50 3MeOBAPA upregulated genes reveals that several most abundant gene transcripts present in both groups are related directly to plant defense mechanisms (Tab. 5). This was the case of plant defensins family proteins including

At5g44430 coding for defensin-like protein 1.2C, At5g44420 coding for defensin-like protein 1.2A, At2g26020 coding for defensin-like protein 1.2B or At2g26010 coding for defensin-like protein 1.3 which are important anti-stress factors upregulated in response to pathogen or stress elicitation and, importantly, also during plant senescence. Moreover, we also detected high expression levels of several enzymes involved in modifications and in remodeling of cell wall that are also important for pollen tube growth. These enzymes belong either to pectin methylesterase or pectin lyase families, such as At2g47040 coding for pectin methylesterase Vanguardl and At3g07820 coding for pectin lyase-like superfamily protein, or, interestingly, there were also genes coding for enzymes with combined pectin methylesterase/pectin methylesterase inhibitor activity such as At2g47050 or At3g05610. Other enzymes of cell wall synthesis were also detected such as products of gene At4g35010 coding for β-galactosidase 11 (BGALll), Atlg02790 coding for polygalacturonase 4 (PGA4) or At3g62710 coding for glycosyl hydrolase family protein. This strongly suggests that in plants 3MeOBAP specifically regulates processes that are necessary for cell wall remodeling and consequent enhanced resistance to stresses and fungal pathogens.

Table 5.

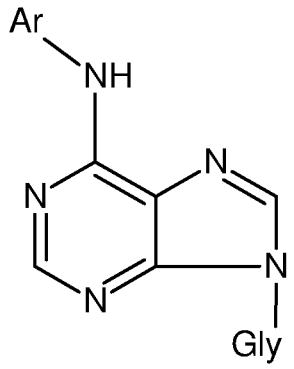
Top 50 genes upregulated in response to 3MeOBAPA treatment in two selected time points. Genes with $P \leq 0.05$ that are changed both after 6h and after 48h of treatment with 10 μM 3MeOBAP are shown.

AGI code	Description	logFC	
		6h	48h
AT2G47040	Pectin methylesterase Vanguardl (VGD1)	2.80	5.89
AT2G47050	Plant invertase/pectin methylesterase inhibitor superfamily protein	2.31	5.82
AT3G07820	Pectin lyase-like superfamily protein	2.22	6.45
AT4G35010	Beta-galactosidase 11 (BGALl 1)	2.03	5.33
AT3G05610	Pectinesterase/pectinesterase inhibitor 21 (PME21)	1.80	5.58
AT5G44430	Defensin-like protein 1.2C (PDF1 .2C)	1.75	8.87
AT1G55560	SKU5 similar 14 (SKS 14)	1.71	8.75
AT1G02790	Polygalacturonase 4 (PGA4)	1.67	8.59
AT5G44420	Defensin-like protein 1.2A (PDF1 .2A)	1.58	8.15
AT2G26020	Defensin-like protein 1.2B (PDF1 .2B)	1.52	6.04
AT2G26010	Defensin-like protein 1.3 (PDF1 .3)	1.47	8.23

	AT5G45880	Pollen Ole e 1 allergen and extensin family protein	1.45	5.52
	AT3G62710	Glycosyl hydrolase family protein	1.35	5.26
	AT5G12960	Putative glycosyl hydrolase	1.33	5.26
	AT1G05580	Cation/H(+) exchanger 23 (CHX23)	1.26	5.46
	AT2G04460	Transposable element gene	1.16	5.43
	AT5G61160	Agmatine coumaroyltransferase (ACT)	1.13	5.05
	AT1G59950	NAD(P)-linked oxidoreductase superfamily protein	1.08	5.72
	AT1G75830	Defensin-like protein 1.1 (PDF1.1)	1.06	7.85
	AT3G28153	Transposable element gene	0.92	5.70
	AT2G28210	Alpha carbonic anhydrase 2 (ATACA2)	0.85	5.86
	AT3G13400	SKU5 similar 13 (SKS13)	0.82	5.57
	AT4G01390	TRAF-like family protein	0.79	6.24
	AT1G76640	Calcium-binding EF-hand family protein (CML39)	0.78	7.06
	AT2G18150	Peroxidase 15 (PER15)	0.68	7.22
	AT4G24350	Phosphorylase superfamily protein	0.64	6.45
	AT1G19670	Chlorophyllase-1 (CLH1)	0.63	6.42
	AT3G28155	ARM repeat superfamily protein	0.59	6.06
	AT1G15540	2-oxoglutarate and Fe(II)-dependent oxygenase superfamily protein	0.57	5.63
	AT5G52670	Copper transport family protein	0.57	6.03
	AT5G63270	RPM1-interacting protein 4 (RIN4) family protein	0.56	7.11
	AT2G39030	L-ornithine N5-acetyltransferase (NATA1)	0.56	5.48
	AT4G21830	Peptide methionine sulfoxide reductase B7 (MSRB7)	0.55	5.95
	AT3G09340	Transmembrane amino acid transporter family protein	0.55	8.54
	AT2G02010	Glutamate decarboxylase 4 (GAD4)	0.55	5.09
	AT2G21900	WRKY transcription factor 59 (WRKY59)	0.54	5.81
	AT4G26010	Peroxidase 44 (PER44)	0.53	7.92
	AT3G11340	UDP-Glycosyltransferase superfamily protein	0.51	6.60
	AT2G26695	Ran BP2/NZF zinc finger-like superfamily protein	0.50	5.90
	AT1G59860	17.6 kDa class I heat shock protein 1 (HSP17.6A)	0.48	5.47
	AT4G22620	SAUR-like auxin-responsive family protein	0.46	5.05
	AT4G39320	Microtubule-associated protein-related	0.45	5.09
	AT5G62720	Integral membrane HPP family protein	0.44	5.31
	AT2G37430	Zinc finger protein ZAT11	0.42	4.96
	AT5G03610	GDSL esterase/lipase	0.42	4.61
	AT4G37780	Myb domain protein 87 (MYB87)	0.41	6.88
	AT4G22030	F-box domain, cyclin-like, F-box domain, Skp2-like protein	0.41	5.02
	AT1G10585	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein	0.38	4.85
	AT3G44830	Putative phospholipid:diacylglycerol acyltransferase 2 (PDAT2)	0.38	4.91
	AT4G31950	Cytochrome P450 82C3 (CYP82C3)	0.37	5.29

CLAIMS

1. Use of 6-aryl-9-glycosidpurine of general formula I



I

and pharmaceutically acceptable salts thereof with alkali metals, ammonia, amines, or addition salts with acids, wherein

Gly represents β-D-arabinofuranosyl or P-D-2'-deoxyribofuranosyl,

Ar represents benzyl or furfuryl, each of which can be unsubstituted or substituted by one or more, preferably one to three, substituents selected from the group comprising hydroxyl, alkyl, halogen, alkoxy, amino, mercapto, carboxyl, cyano, amido, sulfo, sulfamido, acyl, acylamino, acyloxy, alkylamino, dialkylamino, alkylmercapto, trifluoromethyl, trifluoromethoxy,

for regulation, in particular inhibition, of aging in animals, in particular mammals, for cosmetic purposes, and/or for UV photoprotection of animals, in particular mammals, for cosmetic purposes.

2. 6-aryl-9-glycosylpurines of general formula I as defined in claim 1, for use in a method of regulation, in particular inhibition, of aging in animals, in particular mammals, for therapeutic purposes, and/or for UV photoprotection of animals, in particular mammals, for therapeutic purposes.

3. A method for regulating aging and/or UV photodamage wherein at least one compound of general formula I according to claim 1 is applied to cells or microorganisms.

4. Use of 6-aryl-9-glycosidpurine according to claim 1, 6-aryl-9-glycosidpurine for use according to claim 2, or the method according to claim 3, wherein the compound of general formula I is selected from the group consisting of: 6-furfurylamino-9-P-D-arabinofuranosylpurine, 6-(3-methylfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(4-methylfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(5-methylfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3-fluorofurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(4-fluorofurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(5-fluorofurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3-chlorofurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(4-chlorofurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(5-chlorofurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3-bromofurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(4-bromofurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(5-bromofurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3-hydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(4-hydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(5-hydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(4-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(5-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2-aminofurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3-aminofurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(4-aminofurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dihydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3,5-dihydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dihydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4-dihydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2,5-dihydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2,6-dihydroxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dimethoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dimethoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3,5-dimethoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2,3-dimethoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4-dimethoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2,5-dimethoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2,6-dimethoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-

methoxyfurfurylamino)-9--β-D--arabinofuranosylpurine, 6-(2-hydroxy-5-methoxyfurfurylamino)-9--β-D--arabinofuranosylpurine, 6-(2-hydroxy-6-methoxyfurfurylamino)-9--β-D--arabinofuranosylpurine, 6-(3-hydroxy-2-methoxyfurfurylamino)-9--β-D--arabinofuranosylpurine, 6-(3-hydroxy-4-methoxyfurfurylamino)-9--β-D--arabinofuranosylpurine, 6-(3-hydroxy-5-methoxyfurfurylamino)-9--β-D--arabinofuranosylpurine, 6-(3-hydroxy-6-methoxyfurfurylamino)-9--β-D--arabinofuranosylpurine, 6-(4-hydroxy-2-methoxyfurfurylamino)-9--β-D--arabinofuranosylpurine, 6-(4-hydroxy-3-methoxyfurfurylamino)-9--β-D--arabinofuranosylpurine, 6-(4-hydroxy-5-methoxyfurfurylamino)-9--β-D--arabinofuranosylpurine, 6-(4-hydroxy-6-methoxyfurfurylamino)-9--β-D--arabinofuranosylpurine, 6-(2-fluorobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(3-fluorobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(4-fluorobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(2-bromobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(3-bromobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(4-bromobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(2-iodobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(3-iodobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(4-iodobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(2-chlorobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(2-chlorobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(3-chlorobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(4-chlorobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(2-methoxybenzylamino)-9- β-D-arabinofuranosylpurine, 6-(3-methoxybenzylamino)-9- β-D-arabinofuranosylpurine, 6-(4-methoxybenzylamino)-9- β-D-arabinofuranosylpurine, 6-(2-hydroxybenzylamino)-9- β-D-arabinofuranosylpurine, 6-(3-hydroxybenzylamino)-9- β-D-arabinofuranosylpurine, 6-(4-hydroxybenzylamino)-9- β-D-arabinofuranosylpurine, 6-(4-hexylbenzylamino)-9- β-D-arabinofuranosylpurine, 6-(2-fluoro-6-(trifluoromethyl)benzylamino)-9- β-D-arabinofuranosylpurine, 6-(3-chloro-2,6-difluorobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(3-(trifluoromethylthio)benzylamino)-9- β-D-arabinofuranosylpurine, 6-(2-chloro-3,6-difluorobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(4-(trifluoromethylthio)benzylamino)-9- β-D-arabinofuranosylpurine, 6-(3-fluoro-5-(trifluoromethyl)benzylamino)-9- β-D-arabinofuranosylpurine, 6-(2-chloro-4-fluorobenzylamino)-9- β-D-arabinofuranosylpurine, 6-(2-(trifluoromethoxy)benzylamino)-9^-D-arabinofuranosylpurine, 6-(3-(trifluoromethyl)benzylamino)-9- β-D-

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arabinofuranosylpunne, 6-(2,3,4-trimethoxybenzylarriino)-9-P-D-arabinofuranosylpurine, 6-(2,4,5-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4,6-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4,5-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3,4,5-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3,4,6-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4,5,6-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4,6-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,3,4-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4,6-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,3,4-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4,5-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4,6-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-5-chlorobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-6-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-5-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-6-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-5-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-6-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3-fluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-fluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-5-fluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-methylfurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-methylfurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(5-methylfurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-fluorofurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-fluorofurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(5-fluorofurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-chlorofurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-chlorofurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(5-chlorofurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-bromo-furfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-

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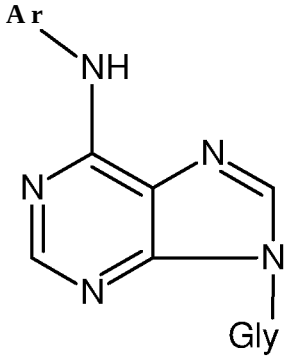
deoxyribofuranosylpurine, 6-(4-hydroxy-5-methoxyfurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxy-6-methoxyfurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-fluorobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-fluorobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-fluorobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-bromobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-bromobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-bromobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-iodobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-iodobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-iodobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-chlorobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-chlorobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-chlorobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-chlorobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-aminobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-aminobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-aminobenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxybenzylamino)- 9-P-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3,4-dihydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3,5-dihydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3,4-dihydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2,4-dihydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2,5-dihydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2,6-dihydroxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3,4-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3,4-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3,5-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2,3-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2,4-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2,5-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2,6-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxy-3-

methoxybenzylamino)-9-β-D-2´-deoxyribofuranosylpurine, 6-(2-hydroxy-4-methoxybenzylamino)-9-β-D-2´-deoxyribofuranosylpurine, 6-(2-hydroxy-5-methoxybenzylamino)-9-β-D-2´-deoxyribofuranosylpurine, 6-(2-hydroxy-6-methoxybenzylamino)-9-β-D-2´-deoxyribofuranosylpurine, 6-(3-hydroxy-2-methoxybenzylamino)-9-β-D-2´-deoxyribofuranosylpurine, 6-(3-hydroxy-4-methoxybenzylamino)-9-β-D-2´-deoxyribofuranosylpurine, 6-(3-hydroxy-5-methoxybenzylamino)-9-β-D-2´-deoxyribofuranosylpurine, 6-(3-hydroxy-6-methoxybenzylamino)-9-β-D-2´-deoxyribofuranosylpurine, 6-(4-hydroxy-2-methoxybenzylamino)-9-β-D-2´-deoxyribofuranosylpurine, 6-(4-hydroxy-3-methoxybenzylamino)-9-β-D-2´-deoxyribofuranosylpurine, 6-(4-hydroxy-5-methoxybenzylamino)-9-β-D-2´-deoxyribofuranosylpurine, 6-(4-hydroxy-6-methoxybenzylamino)-9-β-D-2´-deoxyribofuranosylpurine.

5. Compositions for inhibiting aging and/or for UV photoprotection of mammals, or for inhibiting aging and/or for UV photoprotection of mammalian cells, such as keratinocytes and fibroblasts, containing at least one 6-aryl-9-glycosidpurine of general formula I.

6. 6-aryl-9-glycosidpurine of general formula **Ia**



Ia

and pharmaceutically acceptable salts thereof with alkali metals, ammonia, amines, or addition salts with acids, wherein

Gly represents β-D-arabinofuranosyl or P-D-2'-deoxyribofuranosyl,

Ar represents benzyl or furfuryl, each of which is substituted by one or more, preferably one to three, substituents selected from the group comprising hydroxyl, halogen, alkoxy, amino, mercapto, carboxyl, cyano, amido, sulfo, sulfamido, acyl, acylamino, acyloxy, alkylamino, dialkylamino, alkylmercapto, trifluoromethyl, trifluoromethoxy, or Ar is unsubstituted furfuryl,

whereas, if Gly is β-**D**-arabinofuranosyl, Ar is not methyl-substituted benzyl.

7. 6-aryl-9-glycosidpurine according to claim 5, selected from the group comprising 6-(3-methylfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(4-methylfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(5-methylfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(3-fluorofurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(4-fluorofurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(5-fluorofurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(3-chlorofurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(4-chlorofurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(5-chlorofurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(3-bromofurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(4-bromofurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(5-bromofurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(3-hydroxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(4-hydroxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(5-hydroxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(3-methoxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(4-methoxyfurfurylamino)-9-P-**D**-arabinofuranosylpurine, 6-(5-methoxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(2-aminofurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(3-aminofurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(4-aminofurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(3,4-dihydroxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(3,5-dihydroxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(3,4-dihydroxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(2,4-dihydroxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(2,5-dihydroxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(2,6-dihydroxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(3,4-dimethoxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(3,4-dimethoxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(3,5-dimethoxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(2,3-dimethoxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(2,4-dimethoxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(2,5-dimethoxyfurfurylamino)-9-β-**D**-arabinofuranosylpurine, 6-(2,6-dimethoxyfurfurylamino)-9-β-**D**-

arabinofuranosylpurine, 6-(2-hydroxy-3-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-5-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-6-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3-hydroxy-2-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3-hydroxy-4-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3-hydroxy-5-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(3-hydroxy-6-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(4-hydroxy-2-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(4-hydroxy-3-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(4-hydroxy-5-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(4-hydroxy-6-methoxyfurfurylamino)-9-P-D-arabinofuranosylpurine, 6-(2-fluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-fluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-fluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-hydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-hydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-hexylbenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-fluoro-6-(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-chloro-2,6-difluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-(trifluoromethylthio)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-chloro-3,6-difluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-(trifluoromethylthio)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-fluoro-5-(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-chloro-4-

fluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-(trifluoromethoxy)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-chloro-3-(trifluoromethyl) benzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-fluoro-3-(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,5-bis(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-(trifluoromethoxy)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-(trifluoromethoxy)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-(trifluoromethyl)benzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-aminobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3-aminobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-aminobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-diethylaminobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,5-dihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,5-dihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,6-dihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3,5-dimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,3-dimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4-dimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,5-dimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,6-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-3-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-5-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-6-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-hydroxy-2-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-hydroxy-4-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-hydroxy-5-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-hydroxy-6-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-hydroxy-2-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-hydroxy-3-methoxybenzylamino)-9-P-D-

arabinofuranosylpunne, 6-(4-hydroxy-5-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(4-hydroxy-6-methoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,3,4-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4,5-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4,6-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4,5-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3,4,5-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3,4,6-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4,5,6-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4,6-trimethoxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,3,4-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4,6-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,3,4-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3,4,5-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2,4,6-trihydroxybenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-5-chlorobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-6-chlorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-5-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-6-iodobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-5-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-6-bromobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-3-fluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-4-fluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(2-hydroxy-5-fluorobenzylamino)-9-P-D-arabinofuranosylpurine, 6-(3-methylfurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-methylfurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(5-methylfurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-fluorofurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-fluorofurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(5-fluorofurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-chlorofurfurylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-chlorofurfurylamino)-9-P-D-2'-

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dimethoxybenzylamino)-9-P-D-2'-deoxynbofuranosylpurine, 6-(2,6-dimethoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxy-3-methoxybenzylamino)-9-β-D-2'-deoxynbofuranosylpurine, 6-(2-hydroxy-4-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxy-5-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxy-6-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxy-2-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxy-4-methoxybenzylamino)-9-β-D-2'-deoxynbofuranosylpurine, 6-(3-hydroxy-5-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxy-6-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxy-2-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxy-3-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxy-5-methoxybenzylamino)-9-P-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxy-6-methoxybenzylamino)-9-β-D-2'-deoxynbofuranosylpurine.

8. Cosmetic compositions containing as an active ingredient at least one compound of general formula Ia according to claim 5 or 6.

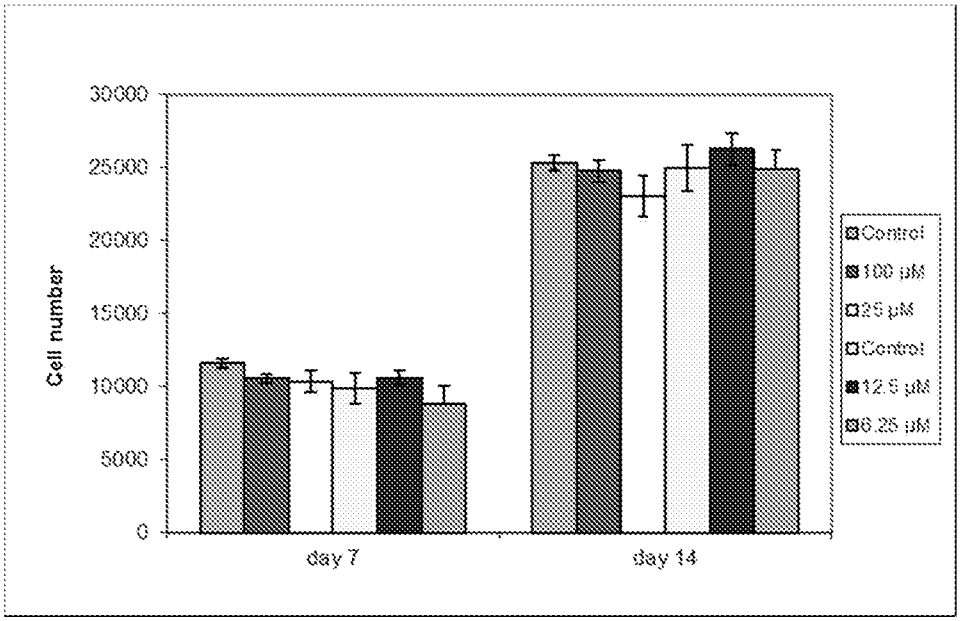
30000
25000
20000
15000
10000
5000
0
Cell number
day 7
day 14
Control
100 µM
25 µM
Control
12.5 µM
6.25 µM

Fig. 1

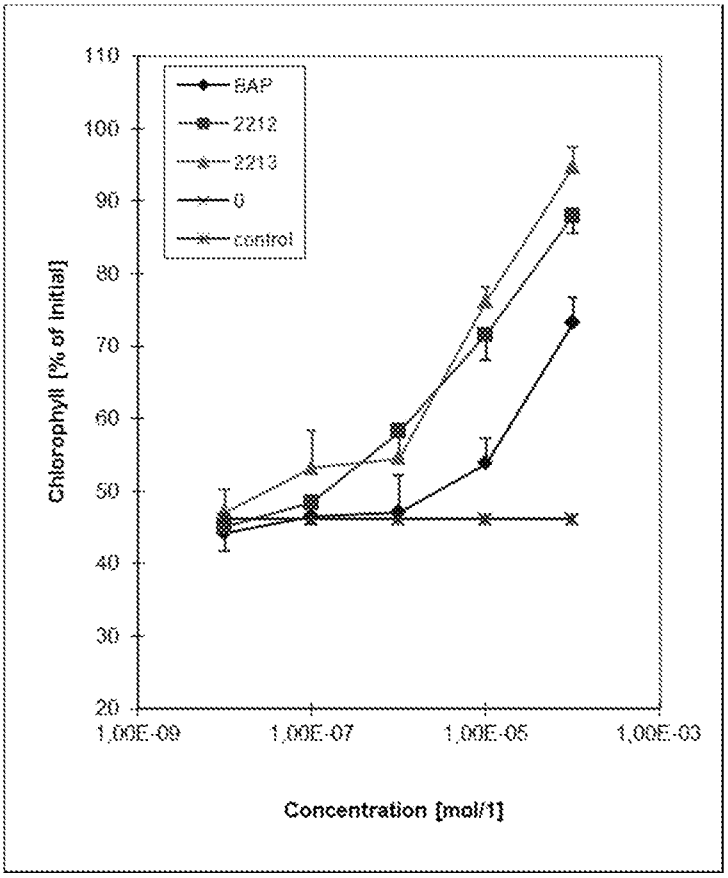
110
100
90
80
70
60
50
40
30
20
Chlorophyll [% of initial]
BAP
2212
2213
0
control
1,00E-09
1,00E-07
1,00E-05
1,00E-03
Concentration [mol/l]

Fig. 2

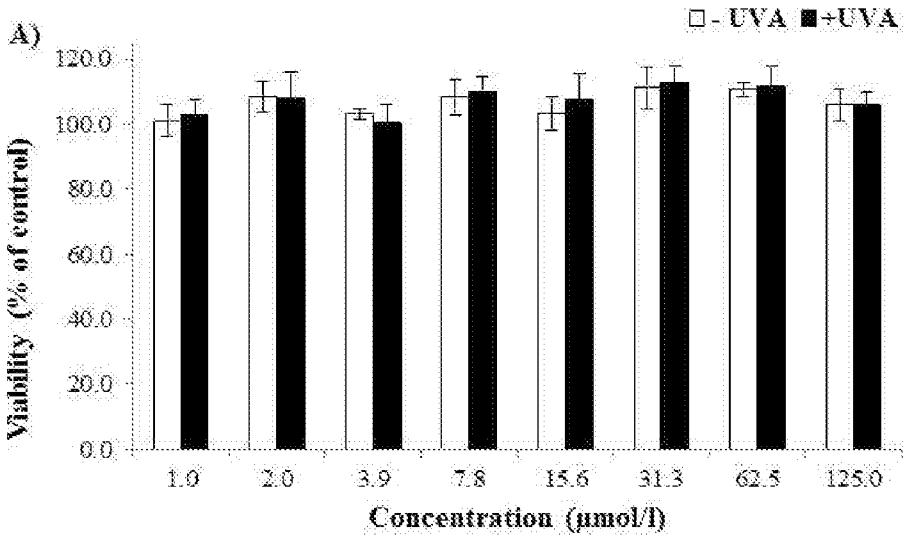
A)
□- UVA ■+UVA
Viability (% of control)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1.0
2.0
3.9
7.8
15.6
31.3
62.5
125.0
Concentration (µmol/l)

Fig. 3

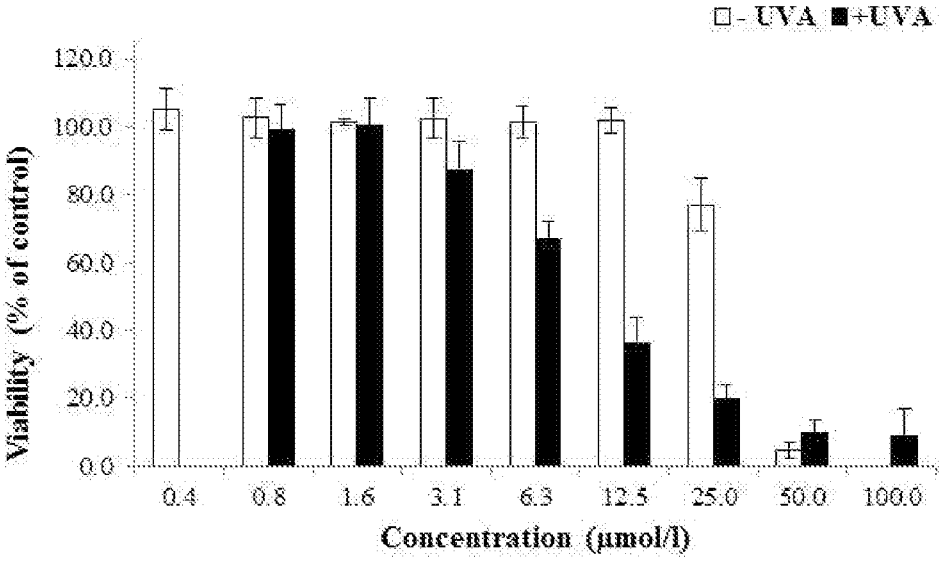
□- UVA ■+UVA
Viability (% of control)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0.4
0.8
1.6
3.1
6.3
12.5
25.0
50.0
100.0
Concentration (µmol/l)

Fig. 4

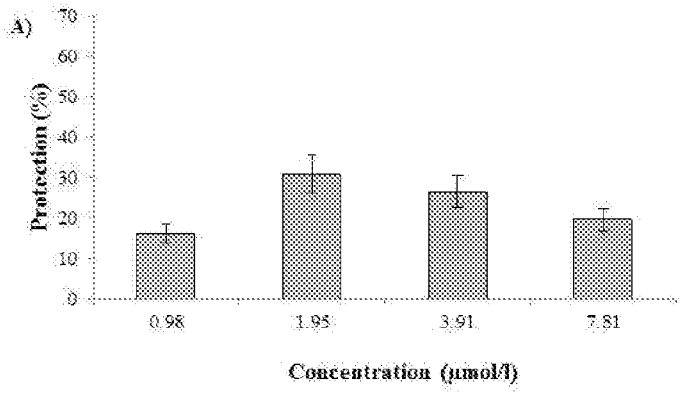

Fig. 5

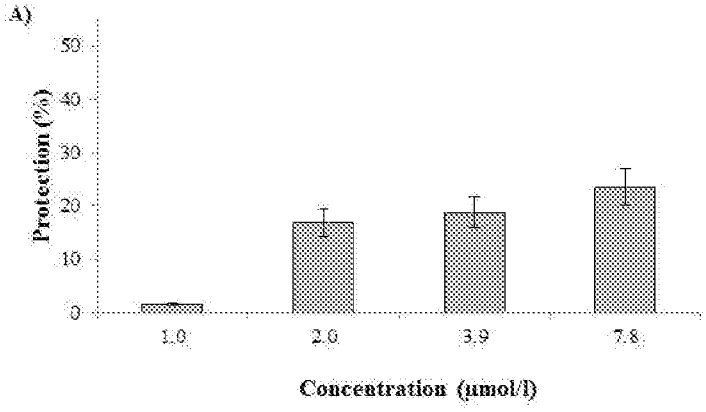

Fig. 6

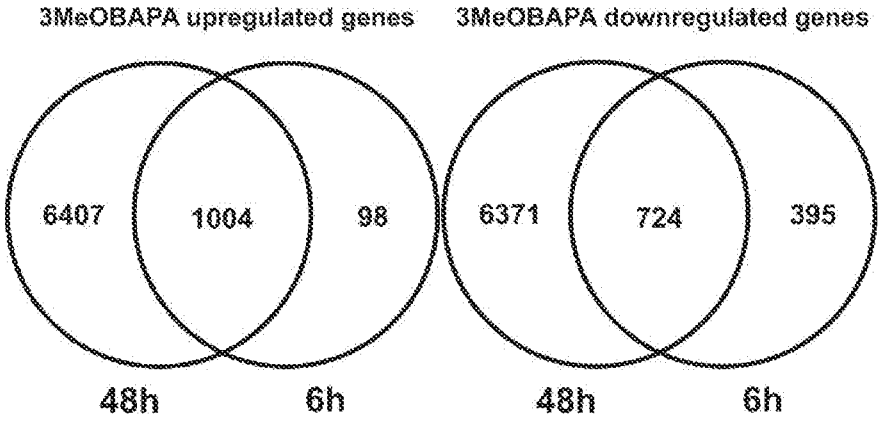

Fig. 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2015/050005

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K8/60 A61Q17/04 A61Q19/08 A61K31/7076 C07H19/173 C07H19/19
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61Q C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MICHAEL P. MCCRANE ET AL: "Oxidative Quenching of Quinone Methide Adducts Reveals Transient Products of Reversible Alkylation in Duplex DNA", CHEMICAL RESEARCH IN TOXICOLOGY, vol. 27, no. 7, 21 July 2014 (2014-07-21), pages 1282-1293, XP055224440, US ISSN: 0893-228X, DOI: 10.1021/tx500152d scheme 2 cpd 6; page 1285	5,6,8
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-/- .

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" documentwhich may throw doubts on priority claim(s) orwhich is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 October 2015	16/11/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Tullberg, Erik

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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Appendix III

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Naturally Occurring and Artificial *N*9-Cytokinin Conjugates: From Synthesis to Biological Activity and Back

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Review

Naturally Occurring and Artificial N9-Cytokinin Conjugates: From Synthesis to Biological Activity and Back

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Abstract: Cytokinins and their sugar or non-sugar conjugates are very active growth-promoting factors in plants, although they occur at very low concentrations. These compounds have been identified in numerous plant species. This review predominantly focuses on 9-substituted adenine-based cytokinin conjugates, both artificial and endogenous, sugar and non-sugar, and their roles in plants. Acquired information about their biological activities, interconversions, and metabolism improves understanding of their mechanisms of action and functions in planta. Although a number of 9-substituted cytokinins occur endogenously, many have also been prepared in laboratories to facilitate the clarification of their physiological roles and the determination of their biological properties. Here, we chart advances in knowledge of 9-substituted cytokinin conjugates from their discovery to current understanding and reciprocal interactions between biological properties and associated structural motifs. Current organic chemistry enables preparation of derivatives with better biological properties, such as improved anti-senescence, strong cell division stimulation, shoot forming, or more persistent stress tolerance compared to endogenous or canonical cytokinins. Many artificial cytokinin conjugates stimulate higher mass production than naturally occurring cytokinins, improve rooting, or simply have high stability or bioavailability. Thus, knowledge of the biosynthesis, metabolism, and activity of 9-substituted cytokinins in various plant species extends the scope for exploiting both natural and artificially prepared cytokinins in plant biotechnology, tissue culture, and agriculture.

Keywords: cytokinin sugar conjugates; glucoside; riboside; D-arabinoside; disaccharides; cytokinin nucleosides; plant biotechnology; *meta*-topolin; zeatin; plant tissue culture

1. Introduction

Plants must adapt to continuous changes in their environments, such as variations in temperature, light, water status, nutrient availability, and pathogen attacks [1]. Many of these responses, and developmental processes, are controlled by interactions or 'cross-talk' between phytohormones (small organic signaling molecules) that include cytokinins (CKs), auxins, abscisic acid, gibberellins, ethylene, jasmonates, strigolactones, and brassinosteroids [2]. The first discovery of a CK (6-furfurylaminopurine, also known as kinetin, Kin), and its identification as a compound that

strongly promotes cell division, in the mid-1950s [3,4], initiated intense investigations of CKs' action mechanisms. This was mainly due to the obvious utility of CKs in tissue culture, and subsequently in plant biotechnology, agriculture, and horticulture [5,6]. We can distinguish two types of adenine-based CKs according to the substitution at N6 atom of adenine moiety. While isoprenoid CKs (IsCKs) are substituted by isoprenoid chain, aromatic CKs (ArCKs) by aromatic ring that can be further substituted by another functional group (Figure 1) [7].

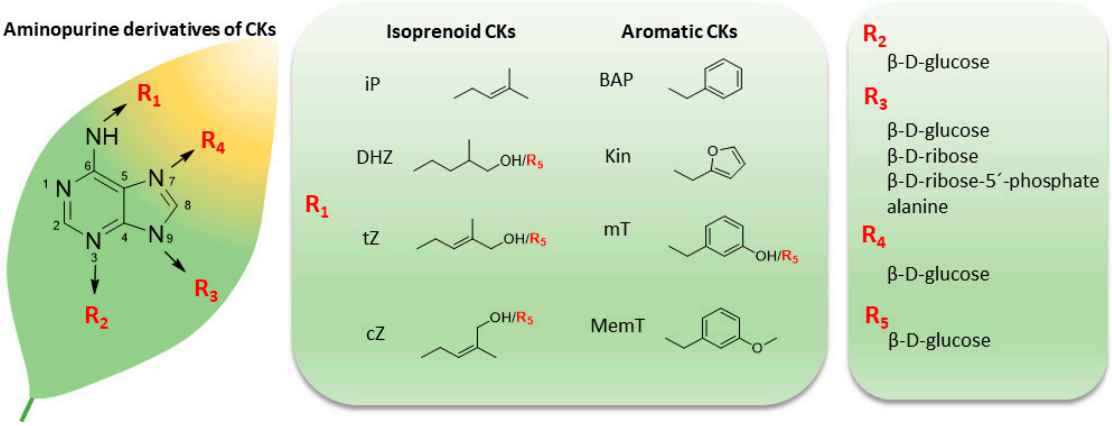

Figure 1. Structure of naturally occurring cytokinin (CK) aminopurine derivatives. The R_1 determines the type of side chain, R_2-R_5 specify the type of CK conjugate.

Generally, CKs participate in control of cell growth and differentiation, and numerous physiological processes in plants. They increase antioxidant activity in plant tissues, which (inter alia) limits peroxidative damage of lipid membranes [8], and participate in chloroplast development [9], regulation of photosynthesis and senescence delay [8]. Other CK roles include participation in shoot and root growth [10], flowering [11], lateral bud formation [12], nitrogen accumulation in roots and translocation to leaves [13], carbohydrate supply [14], and responses to diverse environmental signals [15].

Numerous compounds with CK activity have been identified and structural requirements for CK activity have been formulated [16]. Naturally occurring CK free bases can be converted into the corresponding nucleosides, nucleotides, and glucosides. CKs also often occur as N9-alanine derivatives, but only free bases and ribosides seem to be biologically active [16]. Isoprenoid N6-isopentenyl aminopurine riboside (iPR) and *trans*-zeatin riboside (*t*ZR) are commonly present in plants and considered to be CK transport forms [17] while nucleotides are the key biosynthetic form. Conversion of CK free bases to their N-glucosides usually leads to their inactivation [18]. Moreover, 6-benzylaminopurine (BAP) and Kin, which are widely used in many commercial tissue culture techniques [19,20], due to their low cost and high efficacy, are probably the most well-known ArCKs [21]. Benzyalminopurine is used for the micropropagation of vast numbers of plant species [22–27]. Kin has been usually used in mixtures with α-naphthalene acetic acid (NAA) in tissue culture of many plants as well [28–32]. However, combinations of BAP and Kin in growth medium have often been used for micropropagation [33–38]. In addition, both Kin and BAP are more stable in vivo than naturally occurring IsCKs, which are more susceptible to fast degradation by CK oxidase/dehydrogenase, a key CK degrading enzyme [39]. Although BAP is currently the most affordable and widely used ArCK in tissue culture-based micropropagation, its utilization is associated with several disadvantages [40], mainly lateral root inhibition, growth heterogeneity, problematic acclimatization of plants in the greenhouse [41] and shoot tip necrosis [42]. Some authors attribute the inhibition of root initiation and growth to extensive accumulation of non-active CK N9-glucosides in the shoot base [43] or activation of ethylene production [44].

Hence, increasing research efforts have been geared toward enhancing the efficiency, and avoiding negative effects, of the commonly used CKs on root development. Generally, the easiest way to change the BAP properties is by a substitution on the benzyl ring [45,46]. However, CKs can also be substituted

at several other positions of the purine ring, such as N1, C2, N3, N7, C8, and N9 [47]. All substitutions significantly influenced CK activity, but several N9-substituted CKs had no negative effects on root elongation, which was attributed to prevention of irreversible formation of 9-glucosides [48]. Here, we review current knowledge on O-, N7-, and N9-glucosides, L- and D-ribosides, D-arabinosides, deoxy-D-ribosides and other sugar CK conjugates. We also included some purely artificial mimetic derivatives, such as 9-tetrahydropyran-2-yl, 9-tetrahydrofuran-2-yl, 9-halogenalkyls, and other CK derivatives that are biologically active and could find potential applications in many important sectors, such as agriculture, tissue culture, the cosmetic industry, and medicine.

2. N7- and N9-Sugar Cytokinin Conjugates

2.1. Cytokinin 7- and 9-Glucosides

Cytokinins can form N-glucosides, in which glucose may be attached to the N3, N7, or N9 atoms of the purine moiety. CKs also form *O*-glucosides, in which glucose is linked via an oxygen atom bound to the benzene ring or N6-side chain attached to N6 atom of adenine-based CKs. *N*-glucosides are biologically stable and one of the most abundant naturally occurring CK forms. At certain circumstances, they may account for approximately 80% of the total CK content in plants [17]. Different glucose conjugates play different roles in CK transport, protection of CKs from degradation and reversible or irreversible CK inactivation [10]. Conjugation to the N3 atom has been described rather rarely. It has been assumed that both 7-glucoside and 9-glucoside formation is irreversible and inactivates CKs [49]. For example, it has long been known that BAP-9-glucoside (BAP9G) has weak activity in CK bioassays and does not release appreciable amounts of free active BAP [50]. Both aromatic and isoprenoid 9-glucosides have been synthesized via condensation of 6-chloropurine-9-glucoside with appropriate amines and found to be inactive in *Amaranthus*, tobacco callus, and senescence bioassays [51]. Moreover, tobacco callus grew more slowly on media supplemented with CK 9-glucosides than controls that received no CK treatment, and generally, 9-glucosylation dramatically reduced activities of all CKs tested in these assays [52]. The 7- and 9-glucosylation generally almost reduce CK activity completely in nearly all CK bioassays, including the radish cotyledon, *Amaranthus* betacyanin, oat leaf senescence, and tobacco pith callus bioassays [52]. This is because 7- and 9-conjugates are usually resistant to α-glucosidases, and thus cannot be hydrolytically converted into active CKs, unlike *O*-glucosides, which are conjugated via an oxygen atom [21]. Moreover, none of the *N*-glucosides tested reportedly triggered any response of *Arabidopsis thaliana* (L.) Heynh. CK receptors of *Arabidopsis* histidine kinase (CRE1/AHK4, AHK3) in a bacterial assay [53]. Very recently, distinct metabolisms of *N*-glucosides of N6-isopentenyladenine (iP) and *trans*-zeatin (*t*Z) were described. Despite of iP, both N9 and N7-*t*Z glucosides were cleaved to *t*Z free base [54]. Subsequently, constructed mathematical model provides estimation of the metabolic conversion rates. However, supplementary experiments using tritiated standards did not fully confirmed the findings. Therefore, in our opinion, because this study is in contradiction with many observations published before, it needs to be confirmed by detailed biochemical experiments before being fully accepted.

In the late 1980s, a novel zeatin-*O*-glucoside-9-glucoside was identified in young wheat spikes in [55]. This diglucoside was subsequently detected in transgenic *A. thaliana* plants overexpressing an *IPT* gene (encoding the key CK biosynthesis enzyme isopentenyl transferase) as dihydrozeatin-*O*-glucoside-9-glucoside [56]. A phosphorylated form of zeatin-9-glucoside was also identified. We can thus conclude that 9-glucosides are probably involved in homeostatic mechanisms that control endogenous CK levels, and biological activities of the mentioned forms in three CK bioassays are reportedly low.

Natural formation of *N*-glucosides has attracted significant interest over many years, because it was considered to be a major barrier to the successful use of CKs in field applications [57]. Two enzymes that catalyze 7- and 9-glucopyranosylation of BAP were found in soluble extracts of expanded cotyledons of radish (*Raphanus sativus* L. cv. Long Scarlet) and purified more than 40 years ago [58]. In recent years,

molecular approaches have been used to elucidate functions of various CK-specific glycosyltransferases and CKs have been shown to be deactivated by uridine diphosphate glycosyltransferases (UGTs) [59]. Uridine diphosphate glycosyltransferases, also called 1-glycosyltransferases, are the most common plant enzymes that catalyze transfers of sugar moieties from activated donor molecules to specific acceptor molecules such as phytohormones, secondary metabolites, and amino acids [60,61]. Two closely related *A. thaliana* genes encoding cytosolic enzymes with ability to catalyze CK *N*-glucosylation (UGT76C1 and UGT76C2) in vitro have been identified. Both recognize classical CKs such as *t*Z, dihydrozeatin (DHZ), BAP, iP or Kin, and glucosylate them mainly at the N7 and N9 atoms, but not N3 atom [57]. However, the 7-H tautomer is the favored state, so the N7 is most available for glucosylation by UGTs, and accordingly the two UGTs reportedly generate higher levels of 7-glucoside in vitro [57]. Subsequent experiments with transgenic plants confirmed that both glucosyltransferases can finely modulate CK responses via *N*-glucosylation, but UGT76C2 seems to have stronger effects [62,63].

2.2. Cytokinin 9-Ribosides

2.2.1. IsCK 9-Ribosides

Isoprenoid CKs are ubiquitous in the plant kingdom [64] and regarded as the predominant type of CKs [65]. More than 50 years ago, 9-ribosides of Kin and iP were found to be 2- to 5-fold less active than their free bases in the tobacco callus assay [66], and effects of side alkyl chain hydroxylation on CKs' growth-promoting activity in this bioassay were described [67]. Generally, the most striking effects observed are that hydroxylation of the isopentyl or isopentenyl chains at the 4-position increases this CK activity while hydroxylation at the 2- or 3- positions, reduces it. The same bioassay was also used to test a series of N6-substituted (N6-butyl-, N6-*N*-2-propoxylethyl-, N6-*n*-2-butoxyethyl-, N6-geranyl- and N6-farnesyl-) adenine ribosides (Figure 2). The N6-butyl and propoxyethyl adenosines showed CK activity, although they were less potent than *t*ZR. In contrast, the other compounds showed only marginal or none CK activity [68].

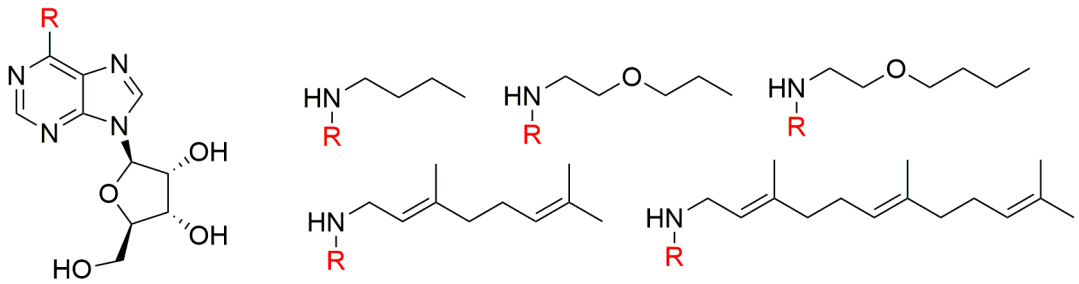

Figure 2. General structure of N6-substituted adenosines and their N6-substituents [68].

In tobacco bioassays, none of the geometric or position isomers of ZR and other compounds closely related to zeatin (Figure 3) was found to be more active than zeatin [69]. The 9-ribosyl derivatives of *t*Z, *cis*-zeatin (*c*Z), *trans*-isozeatin, and *cis*-isozeatin were also prepared and found to be less active than the original free bases [69].

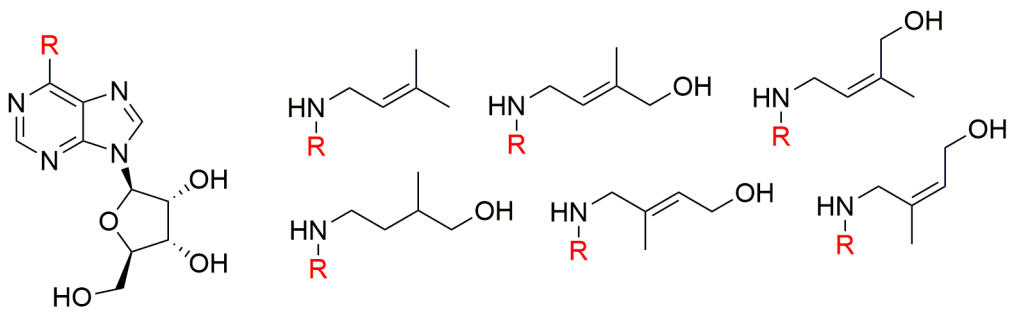

Figure 3. Compounds prepared and tested by Schmitz et al. in 1972 [69].

Comparison of the cell-division stimulatory activity of iP and iPR in tobacco callus bioassay more than 40 years ago [70], and numerous subsequent experiments have shown that free bases generally have higher biological activity than corresponding ribosides [21]. Differences in relative activities could be explained by differences in the perception and transmission of the CK signals by various CK receptors. For example, the two *A. thaliana* CK receptors AHK3 and CRE/AHK4 are more sensitive to the IsCK bases *trans*-zeatin and iP than their ribosides, but AHK3 is more sensitive to ribosides than CRE1/AHK4 in vitro and the ability of *t*ZR to activate CRE1/AHK4 does not increase even after prolonged incubation [53]. Therefore, it was assumed that ribosides have genuine biological activity, with specificity for AHK3. Variations in ligand preference of three *Zea mays* L. histidine kinase receptors (ZmHK1, ZmHK2, and ZmHK3a) have also been detected, with indications that ZmHK2 is most sensitive to ribosides [71].

Comparison of the activities of *cis*-zeatin riboside (*c*ZR) and *t*ZR isomers and iP, has also shown that *t*ZR is more active than *c*ZR in stimulation of cucumber cotyledon expansion, retention of chlorophyll in detached leaf pieces, induction and stimulation of chlorophyll synthesis in cucumber cotyledons, and betacyanin synthesis in *Amaranthus caudatus* L. seedlings grown in the dark [72]. In addition, iP was less active than *t*ZR in all these bioassays, but more active than *c*ZR in the induction and stimulation of betacyanin and chlorophyll synthesis. Moreover, the ability of another IsCK, dihydrozeatin riboside (DHZR), to delay senescence of carnation flowers is similar to that of the free base [73].

Cytokinins are also synthesized by some phytopathogens to disrupt the hormonal balance and to facilitate niche establishment in their hosts. In pathology of *Rhodococus fascians* and related microorganisms, methylated CKs, have been repeatedly shown to play an important role [74–76]. Cytokinin ribosides can be methylated on side-chain or purine moiety. Moreover, 6-(4-hydroxy-1,3-dimethylbut-*trans*-2-enylamino)-9-β-D-ribofuranosylpurine (1-methylzeatin riboside), CK methylated on side chains, has been identified endogenously in *Pseunomonas syringae* pv *savastanoi.* Tests with the naturally occurring CK 1′-methylzeatin, its riboside and various derivatives have shown that they have stronger ability to stimulate synthesis of chlorophyll in etiolated cucumber cotyledons than *t*Z and *t*ZR, respectively [77]. In contrast, dihydro-4′-deoxy-1′-methylzeatin riboside proved to be inactive, mainly due to absence of the hydroxyl group at C4 of the side chain, and iPR was slightly active. Generally, the length of the alkyl side chain and *cis/trans* isomerism reportedly influence CK activity, and the presence of a hydroxyl group at the C4 atom seems to strongly promote it [77]. The same bioassay was used to test CK activity of *t*ZR and *c*ZR, and the ribosides were found to be less active than corresponding free bases [78]. Fas operon of *R. fascians* is essential for the enhanced production of CK mix including 2-methylthio derivatives of the zeatin ribosides, which are also important part of the pathogenicity mechanism [75,76].

Cytokinins are synthesized in many cell types, in both roots and shoots, and act both short and long distances [79]. Generally, *t*Z-type CKs, mainly *t*ZR, are transported from roots to shoots via xylem, whereas IsCKs are transported from shoots to roots via phloem [80]. While *t*Z is an active CK, *c*Z shows only limited CK activity [53]. In response to nitrogen availability, plants are thought to be able to modulate the relative ratio of *t*Z / *t*ZR in xylem sap and allows them to fine-tune the manner of shoot growth to adapt to changing environmental condition [81]. In addition, the ratio of *c*Z/*t*Z and their ribosides changes in behalf of *c*Z type needed for root hair elongation and phosphate allocation in the root during phosphate starvation [82]. Moreover, both *t*ZR and *c*ZR can reportedly suppress chlorophyll degradation in an oat leaf senescence assay and maize leaf segments in a drop bioassay but *t*ZR more effective than *c*ZR as well as in tobacco callus bioassay [64].

Cytokinin ribosides may also contain glucosyl conjugated via oxygen in the hydroxyl group of the side chain of IsCKs [83]. These CK-riboside-*O*-glucosides, namely *trans*-zeatin riboside-*O*-glucoside (*t*ZROG), *cis*-zeatin riboside-*O*-glucoside (*c*ZROG), dihydrozeatin riboside-*O*-glucoside (DHZROG), and the corresponding o-glucosides of free bases, are endogenous CKs that have been recorded in many species of vascular plants [84], for example *Nicotiana rustica* L. [85], *Vinca rosea* L. [86], *Populus alba* L. [87],

and *Tulbaghia* L. [88]. They have also been detected in non-vascular plants, particularly in the moss *Physcomitrella patens* (Hedw.) Bruch and Schimp., in which analysis of CK profiles revealed that *c*ZROG and *t*ZROG were the most abundant intracellular conjugates of CKs [89]. Generally, *O*-glucosides of zeatin-type CKs are considered important for storage and transport because they are resistant to CK oxidase/dehydrogenase-mediated breakdown, and easily converted into the active form by the action of β-glucosidases [90]. Moreover, findings that *t*ZROG is biologically active in an *A. thaliana* reporter gene test but does not trigger responses by either CRE1/AHK4 or AHK3 receptors of *A. thaliana* [53], could be due to rapid breakdown of this metabolite, yielding biologically active free base in *A. thaliana*. Evaluations of endogenous CKs' distributions indicate that *O*-glucosides accumulate most strongly in roots [88,91].

2.2.2. ArCK 9-Ribosides

Neither ArCK ribosides nor free ArCKs were identified as naturally occurring compounds for many years after the discovery of CKs in plants, although many were prepared in the laboratory and used widely in tissue culture almost immediately after their discovery. Their natural origin was only confirmed with the reported isolation of 6-(2-hydroxybenzylamino)-9-β-D-ribofuranosylpurine (*ortho*-topolin riboside, *o*TR) from poplar leaves in 1975 [92] and from *Zantedeschia aethiopica* (L.) Spreng. fruits in 1980 [93]. Kinetin riboside (KinR) was initially identified as a naturally occurring conjugate in coconut water [94] and BAP 9-β-ribofuranoside (BAPR) has been identified in natural plant (*Cocos nucifera* L.) material [95]. Benzylaminopurine 9-β-ribofuranoside is reportedly more active than zeatin-9-riboside (*t*ZR) in the tobacco callus bioassay, both BAPR and *t*ZR have high activity in the *Amaranthus* bioassay (but lower than that of the corresponding free bases), and BAPR has weaker anti-senescence activity than *t*ZR [51]. Benzylaminopurine 9-β-ribofuranoside is also a putative precursor of hydroxybenzylaminopurines (topolins) in plant tissues, and hydroxylation of the benzyl ring at *meta*- and *ortho*- positions, yielding *meta*-topolin-9-riboside (*m*TR) and *o*TR, putatively promote CK activity and/or deactivate BAPR [96]. Thus, for example, *m*TR and *o*TR reportedly have higher and lower activity in CK bioassays than corresponding free bases *meta*-topolin (*m*T) and *ortho*-topolin (*o*T), respectively [51]. Since their discovery, highly active *m*T and its riboside have been employed for culture initiation, protocol optimization and for counteracting various in vitro induced physiological disorders in many species. Evidence from various studies indicate the rising popularity and advantages (although not universal for all species) of topolins compared to other CKs [97]. For example, adding *m*TR to the culture medium during in vitro propagation of potato can significantly improve survival rates [98]. Further, treatments including *m*TR provision can overcome the problematic occurrence of necrotic shoot-tips associated with use of BAPR and its free base in micropropagated *Harpagophytum procumbens* (Burch.) DC. ex Meisn. [42]. Moreover, in vitro regeneration rates of explants of the orchid *Ansellia africana* Lindl. are significantly higher in *m*TR-containing media than in other tested media [99], and it has proposed utility as an alternative to other commonly used CKs in micropropagation of smoke bush (*Cotinus coggygria* Scop.) [100]. However, it should be noted that there are plant species that respond better to other CKs than topolins; hence topolins should not be taken as a panacea and must pass through the routine process of selection and optimization of tissue culture protocol [97].

It should be emphasized that the hydroxyl group on the benzyl ring in *m*T allows reversible *O*-glucosylation. Before or after *O*-glucosylation, the N9 position can be conjugated with ribose, forming *meta*-topolin riboside-*O*-glucoside (*m*TROG), which has been detected as a main metabolite of *m*T in all parts of micropropagated *Spathiphyllum floribundum* (Linden & André) N.E.Br. However, *m*TROG can be easily cleaved in plant tissues by β-glucosidases, and thus it penetrates plant tissue with biologically active *m*T or its riboside. On the other hand, major metabolite of widely used BAP is the highly stable and biologically inactive BAP9G, which accumulates in plant basal parts and might be responsible for undesirable inhibition of root development. Plants treated with *m*T reportedly produce significantly more, and longer, roots than counterparts treated with BAP during acclimatization [40].

Clearly, the presence of a hydroxyl group gives topolins a structural advantage over BAP, since it allows formation of O-glucosides, which cannot be formed from BAP [5].

Recently, two endogenous ArCK isomers of topolins, *ortho*-topolin-9-riboside-*O*-glucoside (*o*TROG), and *meta*-topolin-9-riboside-*O*-glucoside (*m*TROG) were detected in microalgae [101]. Roughly concurrently, two *O*-glucosides *m*TROG and *para*-topolin-9-riboside-*O*-glucoside (*p*TROG) were detected in shoots of tissue-cultured *Aloe polyphylla* Pillans plants treated with BAP, at levels that depended on the type of gelling agent used to solidify the medium [102].

Targeted searches for naturally occurring ArCKs in *A. thaliana* plants and *Populus x canadensis* Moench cv. Robusta leaves led to the identification of two methoxy ArCK ribosides: 6-(2-methoxybenzylamino)purine-9-riboside (*ortho*-methoxytopolin riboside) and 6-(3-methoxybenzylamino) purine (*meta*-methoxytopolin-9-riboside, Me*m*TR). In the same study, these compounds were found to have higher CK activity in tobacco callus, *Amaranthus*, and detached wheat leaf senescence bioassays than BAP and *t*Z [103]. Recently, Me*m*TR was also found to have stronger anti-senescence effects during early senescence than BAP in micropropagation of rose [104]. The high potential utility of *m*TR and Me*m*TR was subsequently studied to replace BAP and zeatin in micropropagation of *A. polyphylla* [105]. Additionally, Me*m*TR has shown high potential for promoting adventitious shoot production in micropropagation of the endangered endemic shrub *Barleria greenii* M. Balkwill and K. Balkwill [106]. In further recent studies of the effects of *m*T, *m*TR, *meta*-methoxytopolin (Me*m*T), and Me*m*TR, micropropagated banana plantlets regenerated with Me*m*TR had significantly longer roots and higher shoot/root ratios than controls and BAP-treated plants. Me*m*TR and *m*TR also induced higher chlorophyll a/b ratios than BAP treatments, which were closer to the optimum for photosynthesis during acclimatization [107].

Based on some of the findings described above, numerous BAPR analogues with various substituents on the benzyl ring (Figure 4) were synthesized and their biological activities were studied [45]. The results suggested that position-specific steric effects of the benzyl ring substituents decrease CK activity, with strength declining in the following order: *meta* > *ortho* > *para* [51]. The highest activities were observed in the wheat leaf senescence bioassay (WLS), in which some compounds delayed senescence up to 2.2 times more efficiently than BAP, and almost 50% of the prepared compounds were more active than BAP. It was assumed that substituents with high electronegativity enhance the activity of aromatic ribosides, probably through hydrogen bond formation with electron donors of a CK receptor [108]. This assumption was supported by the findings that fluoro derivatives are the most active compounds [45]. Important variations in the selectivity of disubstituted derivatives were also reported. For example, 6-(2,4-dichlorobenzylamino)purine-9-riboside was active in the tobacco callus bioassay, but not in other CK bioassays, while 6-(3,4-dichlorobenzylamino)purine-9-riboside was active in WLS and *Amaranthus* assays. Therefore, small changes in benzyl ring substitution can clearly lead to significant changes in specificity of compound biological activity [45]. Interestingly, none of the prepared BAPR derivatives significantly activated either of the *A. thaliana* CRE1/AHK4 or AHK3 CK receptors [45]. Thus, it was assumed that their biological activities involve other mechanisms. Furthermore, two of these compounds, 6-(2-hydroxy-3-methoxybenzylamino)purine-9-β-D-ribofuranoside and 6-(2,4-dimethoxybenzylamino)purine-9-β-D-ribofuranoside, were isolated from *A. thaliana* and *Agrobacterium tumefaciens* extracts, and identified as new plant growth substances [45].

Another derivative, 6-(3-fluorobenzylamino)purine-9-riboside (FBAPR), was found to promote shoot multiplication significantly more strongly than BAP in rose micropropagation [104]. Similarly, FBAPR treatment resulted in formation of significantly more, but smaller, new shoots during in vitro cloning of *Phalaenopsis amabilis* (L.) Blume hybrids (which is generally characterized by slow growth and low multiplication rates), than treatment with either 6-(3-fluorobenzylamino)purine (FBAP) or BAP [109]. The results suggested that use of fluorinated BAPRs could substantially improve in vitro micropropagation of *P. amabilis* [109].

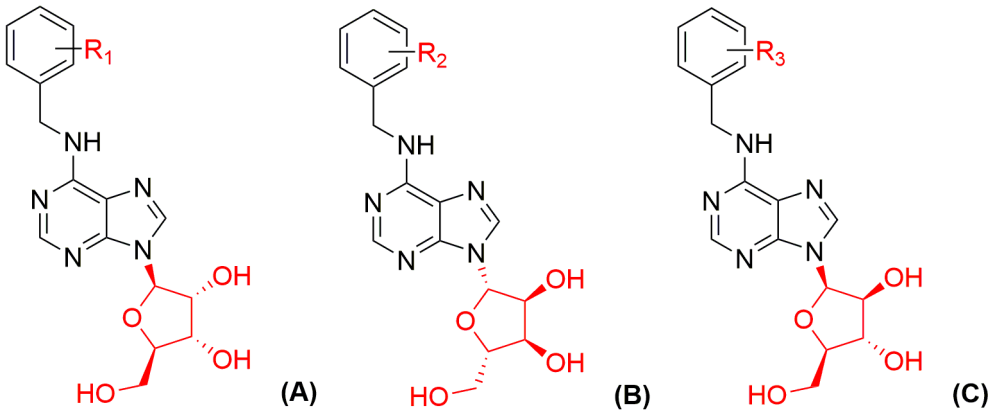

Figure 4. General structures of aromatic (**A**) 6-benzylaminopurine-9-β-D-riboside derivatives (R_1 = X, CH_3, OCH_3, OH, $OCHF_2$, OCF_3, CF_3 or a combination of these groups), (**B**) 6-benzylaminopurine-9-β-L-riboside derivatives (R_2 = H, F, Cl, OCH_3 or OH) and (**C**) 6-benzylaminopurine-9-β-D-arabinoside derivatives (R_3 = X, CH_3, OCH_3, OH, OCF_3, CF_3 or NH_2).

In our opinion, there is enough evidence to conclude that 6-benzylaminopurine-9-β-D-ribosides, bearing appropriate substituent on the phenyl ring, have a great potential to be a solution to many problems afflicting current tissue culture industry and agriculture in general (such as drought and other abiotic stress tolerance).

Recently, the number of available N9-conjugates of ArCK sugars with halogen atoms on benzyl ring has been extended by the preparation of new aromatic 2-chloro-6-(halogenobenzylamino)purine ribosides and their biological activity was studied [46]. A group of 2,6-disubstituted CK derivatives was also prepared by reacting 2,6-dichloropurine riboside with the appropriate benzylamines in the presence of triethylamine in *n*-propanol [45] and their structures are shown in Figure 5.

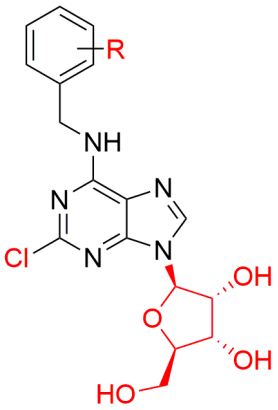

Figure 5. General structure of 2-chloro-6-disubstituted benzylaminopurine riboside derivatives, where R = halogens [46].

Derivatives bearing a fluorine atom on the benzyl ring have generally strong activity in the WLS bioassay; 2-chloro-6-(4-fluorobenzylamino)purine-9-riboside, the most potent compound, delayed loss of 50% chlorophyll 1.96-fold longer than BAP [45,46]. The most active compounds are always found among the derivatives bearing a halogen in the *meta* or *para* position of the N6-benzyl ring. Moreover, high-throughput comparative gene expression analysis revealed that two tested halogenated ArCK derivatives upregulated several genes associated with photosystems I and II, as well as other components of the photosynthetic apparatus. Both compounds delayed the onset of senescence by maintaining chlorophyll and carotenoid levels and increasing the relative abundance of light harvesting complex II, thereby protecting photosystem II activity. Prepared compounds showed similar biological activity to standard BAP in tobacco callus and A*maranthus* bioassays. Most of the derivatives did not

trigger CK signaling via the AHK3 and CRE1/AHK4 receptors from *A. thaliana*, but some of them specifically activated the ZmHK1 receptor from *Zea mays* and were more active than BAP in the ARR5::GUS CK bioassay using transgenic *A. thaliana* plants [46].

It should be noted that halogenated ArCK ribosides can induce CK responses that could be caused by their conversion to the free bases [21,46]. There may also be a different sensing mechanism for ArCKs in plants [45] and there is strong evidence of the presence of another extracellular CK perception system involving plasma-membrane-bound receptors [110].

Recently, several derivatives of 6-benzylamino-9-β-L-ribofuranosylpurines were synthesized (Figure 4) and their CK activities were measured [111]. These were prepared by one-step nucleophilic substitution, starting with reaction of β-L-inosine with corresponding benzyl amines in the presence of Castro reagent and Hünig base, largely following previously published procedures [112]. CK activity of the newly prepared derivatives was tested in *Amaranthus*, tobacco callus, and WLS bioassays. Generally, the L-enantiomers had significantly weaker biological activity in WLS bioassays than corresponding D-enantiomers [111]. For example, classical *meta*-topolin-9-β-D-riboside (D-*m*TR) had 2.37-fold higher and its L-enantiomer had 3.44-fold lower activity than BAP, respectively. The D-ribosides were also significantly more active in the tobacco callus bioassay [45].

A remarkable compound, detected in coconut milk, was 14-*O*-{3-*O*-[*β*-D-galactopyranosyl-(1→2)-*α*-D-galactopyranosyl-(1→3)-*α*-L-arabinofuranosyl]-4-*O*-(*α*-L-arabinofuranosyl)-*β*-D-galactopyranosyl}-*trans*-zeatin riboside (G_3A_2-ZR) (Figure 6). The discoverers found that at least 20% of the CK activity of coconut milk could be attributed to G_3A_2-ZR [113]. Thus, G_3A_2-ZR is an order of magnitude more potent than 1,3-diphenylurea and an order of magnitude less potent than *t*ZR. Its CK activity in tobacco callus could be mediated by hydrolysis to zeatin and, in addition, this conjugate could be preferentially accepted because it is water soluble, while zeatin and ZR are more lipophilic and have lower solubility in water. Production of a highly water-soluble CK (or precursor) such as G_3A_2-ZR and its accumulation in coconut milk could be beneficial for nourishment of the immature coconut embryo [113].

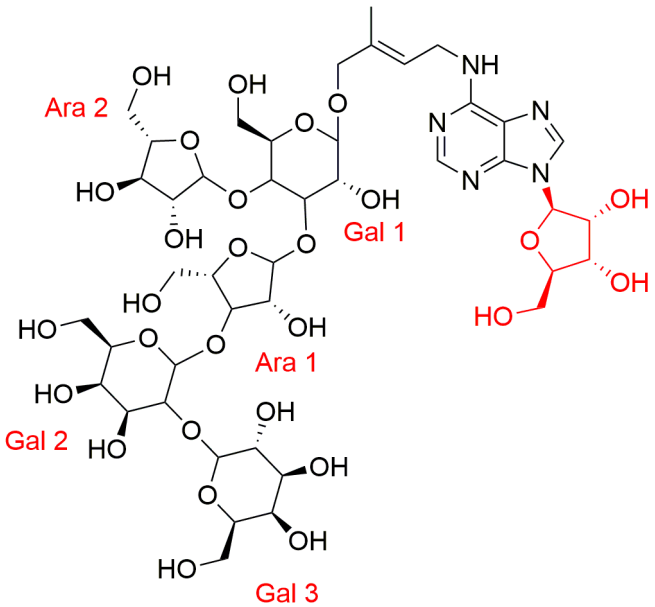

Figure 6. Structure of 14-*O*-{3-*O*-[β-D-galactopyranosyl-(1→2)-α-D-galactopyranosyl-(1→3)-α-L-arabinofuranosyl]-4-*O*-(α-L-arabinofuranosyl)-β-D-galactopyranosyl}-trans-zeatin riboside (G_3A_2-ZR) [113].

Cytokinin ribosides and riboside monophosphates (ribotides) were commonly reported as metabolites of exogenously applied CKs, and their interconversion was demonstrated by radiolabeling in a study of lettuce seed germination. The results showed that exogenously applied [^{14}C]Kin is rapidly metabolized in lettuce seeds to the corresponding nucleoside and nucleotide [114,115].

Another endogenous ArCK ribotide (BAPR-5′-monophosphate - BAPRMP) and isoprenoid CKs (isopentenyladenosine-5′-monophosphate, dihydrozeatin- riboside-5′-monophosphate, and zeatin riboside-5′-monophosphate) have been found in aerial parts of the coconut palm [95]. In tests of *trans*-zeatin riboside-5′-monophosphate in CK receptor bacterial assays, it activated the CRE1/AHK4 but not the AHK3 receptor. The ribotide was also active in the ARR5::GUS CK bioassay. Recently described BAPRMP derivatives have potential medical uses because they have anticancer, antimitotic, and pro-apoptotic activities in animal and human cells [116]. Furthermore, a group of BAPR-5′-*O*-di- and tri-phosphate derivatives have similar activities against selected cell lines to the parent ribosides [117]. The activity of such ribotides has also been recently patented [116].

2.3. Purine 9-(2′-Deoxyribosides) Cytokinin Conjugates

Purines substituted at N9 atom with 2′-deoxyribose are important components of various biomolecules that are essential for physiological processes, e.g. DNA, and various signaling molecules [118]. Test results ca. 30 years ago showed that zeatin-9-(2′-deoxyriboside) (*t*Z2′dR) and its monoacetyl and triacetyl derivatives were able to stimulate chlorophyll synthesis in etiolated cucumber cotyledons but very weakly [77]. On the other hand, *t*Z2′dR inhibited the DNA-polymerizing reaction catalyzed by DNA-polymerase I of *Escherichia coli* [119]. In addition, *cis*-zeatin-2′-deoxyriboside reportedly had no CK activity in the tobacco callus bioassay [78]. Recently, benzyl ring-substituted 6-benzylamino-9-(2′-deoxy-*β*-D-ribofuranosyl)purine derivatives (Figure 7) have been prepared [112,120] and tested in various classical CK bioassays. The results showed that attachment of a 2′-deoxyribosyl moiety to the N9 atom significantly enhanced the prepared derivatives anti-senescence activity in the WLS bioassay, relative to activities of both corresponding free bases and ribosides [120].

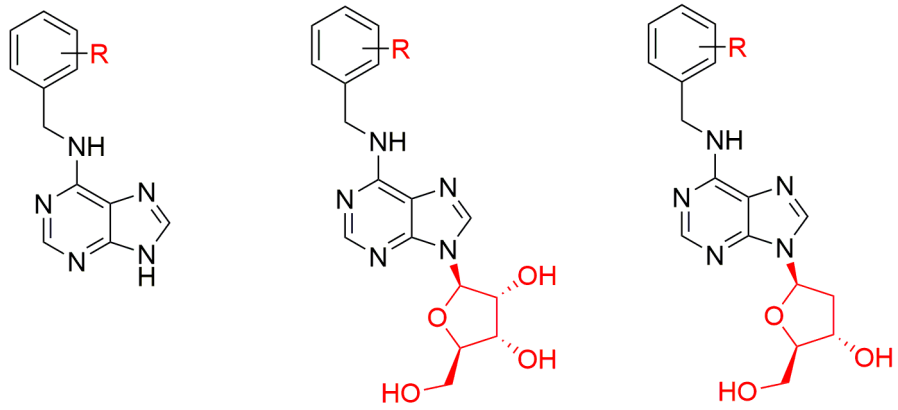

Figure 7. Comparison and general structures of free base, riboside and 2′-deoxyriboside derivatives, where R = X, OH, OCH_3, CH_3, OCF_3, CF_3 or a combination of these groups.

In the *Amaranthus* bioassay, replacement of ribose by a 2′-deoxyribose sugar moiety did not significantly affect activity of most of the prepared derivatives, but the activity of some of them reached the maximum level at ca. 10-fold higher concentration (100 μM) than BAP [120]. Furthermore, 6-(3-hydroxybenzylamino)-9-(2′-deoxyribofuranosyl)purine and 6-(3-methoxybenzylamino)-9-(2′-deoxyribofuranosyl)purine reportedly have significantly higher anti-senescence and chlorophyll maintenance activities than BAP in WLS assays. In contrast, most tested compounds had lower activity than BAP in tobacco callus bioassay [120].

Purine 2′deoxy-nucleoside analogues have been reported to have antiviral potency. Benzylaminopurine 9-β-ribofuranoside and N6-benzyl-2′-deoxyadenosine are active against alphaviruses (Semliki Forest and Sindbis viruses) [121] and Human enterovirus 71 [122,123].

Generally, despite the fact that 2′-deoxyadenosines do not bind the CK receptor, they possess an incredible anti-senescent activity in plant bioassays [120]. Thus, a simple synthetic exchange of

the pentose sugar group on the N9 atom led to the preparation of substances, which are no longer apparently CKs, but which have a high added value due to the preservation (and improvement) of influencing leaf senescence.

2.4. Purine N9-Arabinosides and Their Precursors

More than 40 years ago, a small library of iP analogues substituted at N9 by a ribose or arabinose with the side chain containing acetylenic, dimethylaminoethyl, pyridylmethyl, cyclopropylbenzyl, or cyclopropylmethyl functional groups was synthesized and tested for CK activity in the tobacco callus bioassay [124]. Most of them showed moderate or strong activity. Replacement of D-ribose by D-arabinose or replacement of the isopentenyl side chain also lowered CK activity [124].

A group of 6-alkylaminopurine arabinosides was also prepared in the 1980s [125–128], by transferring the arabinosyl moiety from a pyrimidine arabinoside to the purine aglycone [129]. These compounds were found to be selectively active against varicella-zoster virus [130]. This was not surprising because the β-anomer of 9-(D-arabinofuranosyl)adenine (Ara-A), and a series of N6- or C8-substituted variants of Ara-A had been previously synthesized and found to have in vitro antiviral activities against herpes simplex and vaccinia viruses as well [131]. Some other derivatives 8-amino-9-(β-D-arabinofuranosyl)adenine and 8,5′-anhydro-8-oxy-9-(β-D-arabinofuranosyl)adenine were also tested against vaccinium and herpes simplex [132,133]. However, the results showed that the substitution of Ara-A's C8-atom caused loss of antiviral activity against both tested viruses. Finally, the tested derivatives carrying substituents at the N6 atom of the adenine moiety also had lower antiviral activity than their parent compounds, except for N6-(β-naphthylmethyl)-Ara-A [131].

Recently, a new class of non-toxic CK 9-(β-D-arabinosides) (Figure 4) was prepared according to a previously published protocol with a slight modification [112]. It is based on by reaction 9-(β-D-arabinofuranosyl)hypoxanthine with the corresponding benzylamines in the presence of Castro's reagent and Hünig's base [134]. In the *Amaranthus* bioassay, none of the derivatives had stronger activity than BAP, and, in addition, they exhibited low or modest activity (6–40% of BAP activity) also in the callus bioassay. These data suggested that the CK 9-(β-D-arabinosides) have only weak CK activity. However, it is interesting to note that several of the new derivatives had similar or higher activity in the WLS bioassay than BAP. These findings indicate that the new compounds can specifically affect the physiological processes associated with senescence and/or stress without being active CKs in receptor assays. Metabolic conversion of 6-benzyladenine arabinoside (BAPA) appears to be similar to that shown by BAP and is related to the formation of inactive CK 7- and 9-glucosides that are responsible for the aberrant root formation after BAP treatment [107].

2.5. Cytokinin Disaccharide Conjugates

In the early 1980s, a novel isoprenoid conjugate of *t*ZR with a hexose moiety was identified by analyses of MS spectra of *Pinus radiata* D. Don bud extracts, indicating that the hexose moiety, attached to the ribose moiety, was probably glucose [135]. The zeatin disaccharide conjugate was active in a soybean hypocotyl bioassay [135], and subsequently detected in buds of the conifer Douglas fir [136]. Structures of three novel endogenous CK ribosyl-linked glycosides—dihydrozeatin-9-glucopyranosyl riboside (DHZ9RG), 6-(2-isopentenylamino)purine-9-glucopyranosyl riboside (iP9RG) and *trans*-zeatin-9-glucopyranosyl riboside (*t*Z9RG)—were identified (Figure 8) some years later, together with their phosphorylated forms, in mature buds of *P. radiata* [137]. The cited authors suggested that synthesis of these CK glycosides and their phosphorylated forms in conifers must involve enzymes that do not participate in formation of glucosides and nucleotides of traditional CKs [137]. Moreover, levels of phosphorylated CK disaccharides (*t*Z9RG and DHZ9RG) in *P. radiata* positively correlated with numbers of fascicle needle primordia in the adult buds [138]. Disaccharides have been found to be the major BAP metabolites formed during organogenesis in *Gerbera jamesonii* Bolus [139] and were detected in *Petunia hybrida* Vilm. [140].

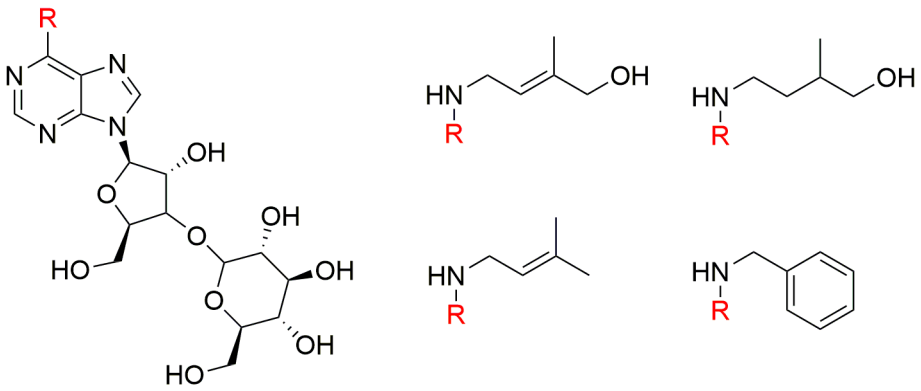

Figure 8. CK disaccharides (Z9RG, DHZ9RG, iP9RG) reported by Zhang et al. (2001) and BAP9RG reported by Auer and Cohen (1993) [138,140].

In both of these cases, the culture media were supplemented with BAP, so it appeared that in the angiosperms new glycosides were synthesized from the aglycone present in the medium [137]. Moreover, 6-benzylaminopurine-9-glucopyranosyl riboside (BAP9RG, Figure 8) may be an important component of the metabolic regulation of the pool of active CKs, which is responsible for shoot organogenesis in culture [140]; it was also recently identified in tissues of the conifer *Pinus pinea* L. during adventitious bud formation in vitro after BAP treatment [141].

The phosphorylated form of BAP9RG was subsequently identified in metabolic profiling of mature *P. radiata* bud fragments cultured on BAP containing medium. In conclusion, BAP induces reinvigoration of the mature buds, in which BAP is converted into metabolites including BAP9RG and 6-benzylaminopurine-9-glucopyranosyl riboside phosphate (BAP9RGP). Anatomical examinations revealed that BAP inhibited development of secondary needle primordia and the reactivated meristem regained the ability to produce green primary needles with juvenile characteristics [142]. Understanding maturation of *P. radiata*, and other trees, is very important in clonal forestry, i.e., production of genetically identical trees from the same parental material [138,143]. Due to the frequent isolation of these disaccharides in conifers, it seems that these disaccharides have an irreplaceable function in their development, which is related to further improvement that might follow in tissue culture of tree species.

3. Non-Sugar N9-Substituted Cytokinins

A number of non-sugar 9-substituted derivatives of CKs have been described, several of which occur naturally and were discovered in plant tissues, such as 9-alanyl derivatives [7]. Most non-sugar 9-substituted CKs have been prepared as mimetics of CK sugar conjugates in the laboratory conditions [66,144–147]. We summarize current knowledge of these non-sugar 9-substituted CK derivatives and their biological activity in the following sections.

3.1. 9-Alanyl Derivatives

Attachment of the amino acid alanine to the N9 atom of the purine moiety in zeatin results in formation of 9-alanylzeatin and 9-alanyldihydrozeatin. These two naturally occurring isoprenoid CKs were named lupinic and dihydrolupinic acid, respectively, because they were initially identified in *Lupinus angustifolius* L. seeds [148]. Later, a novel transferase, which catalyzes conversion of zeatin to lupinic acid, was isolated and purified from *Lupinus luteus* L. cv. Weiko III seeds [19,149]. Lupinic acid is metabolically stable, but inactive or much less active than zeatin in CK bioassays, such as tobacco callus and radish cotyledon assay [149,150]. In contrast, lupinic acid has appreciable activity in soybean callus and *Amaranthus* bioassays [150], so its apparent CK activity acid depends on the assay. Release of free zeatin from lupinic acid has been observed, indicating that alanine conjugates may serve as potential storage rather than deactivation forms [151].

3.2. Synthetic 9-Substituted Alkyl, Cycloalkyl, and Halogenoalkyl CK Derivatives

Substitution at the purine N9 atom with alkyl or cycloalkyl groups significantly affects CK activity. Almost 50 years ago, it was reported that 9-methoxymethyl, 9-propyl, and 9-cyclohexyl derivatives of BAP (Figure 9) are less active than the free base in tobacco and soybean bioassays [144]. Several 9-substituted halogenoalkyl derivatives of BAP were also tested in the soybean senescence assay. The most potent, 9-(4-chlorobutyl), had slightly more ability than BAP to promote chlorophyll retention in intact soybean leaves. Its activity is probably due to easy dealkylation and release of free BAP, but in general, the mode of action of such 9-alkyl CK remains unclear [145].

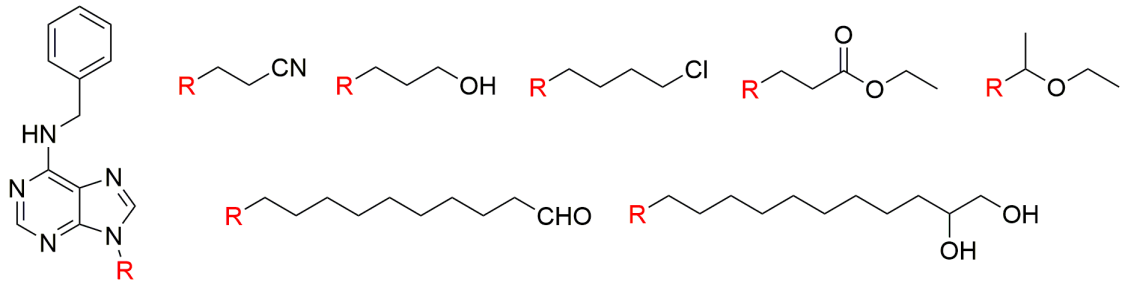

Figure 9. Structures of aliphatic chains substituted at the N9 atom of BAP [145].

Subsequently, series of 9-substituted ethyl derivatives of four naturally occurring CKs (*t*Z, *c*Z, DHZ, iP Figure 10) were synthesized [146]. All of these derivatives were less active than the parent CKs in the soybean callus bioassay and their relative activities were more dependent on the structure of the isoprenoid side chain. The results clearly showed the importance of the structure and size of N9 substitutions and indicated that steric effects cause much lower losses of biological activity than the polarity of the substituent functional groups [146].

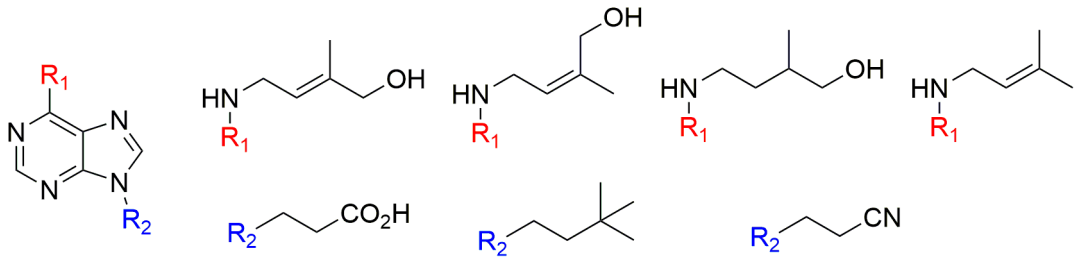

Figure 10. Structures of compounds prepared by Corse et al. (1989). On N6 (R1): tZ, cZ, DHZ, and iP side chains. On N9 (R2): 2-carboxyethyl, 2-carbo-*t*-butoxyethyl and 2-nitriloethyl) [146].

Early experiments showed that many CKs (for example, Kin, *t*Z, and BAP) can delay senescence in detached leaves of various plant species, such as wheat, soybean [145,152], barley [153], rice [154], and oat [155]. These studies also showed that CKs can prolong the lifespan of cut carnation flowers [156]. Many CK derivatives have been prepared and tested, including various alkyls, halogenoalkyls and cycloalkyls, inter alia a group of 9-substituted Kin derivatives with halogenoalkyl, aliphatic or cyclic ether, and carboxylic chains (Figure 11) [157]. Derivatives substituted at the N9 atom with a short halogenoalkyl (chloroethyl, bromoethyl) have higher activity in tobacco callus bioassay than Kin, and derivatives with an aliphatic group and/or cyclic ether at the N9 atom have significant activity in this assay [157].

In the *Amaranthus* bioassay, these halogenoalkyl Kin derivatives were found to be only slightly active. Generally, the aliphatic and cyclic ethers were the most active, followed by halogenoethyl and halogenobutyl derivatives, while substances bearing 9-carboxylic chains were totally inactive. Halogenoalkyl derivatives also significantly delayed senescence, and their activity seems to depend on

both the length of the alkyl chain and the halogen atom. CKs with short N9-halogenoalkyls (chloroethyl or bromoethyl) had the highest activity [157].

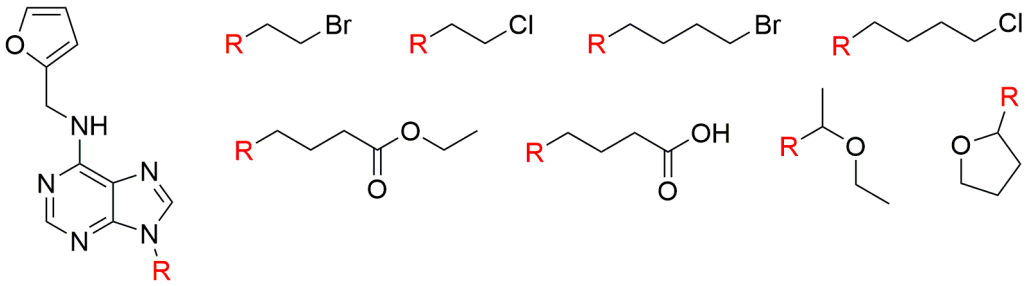

Figure 11. Structures of Kin derivatives prepared by Mik et al. (2011) [157].

Subsequently, a series of iP derivatives specifically substituted at the purine N9 atom by ethoxyethyl and C2-C4 alkyl chains terminated by various functional groups (Figure 12) were prepared [158]. These compounds were synthesized using a previously described procedure [159] with slight modification.

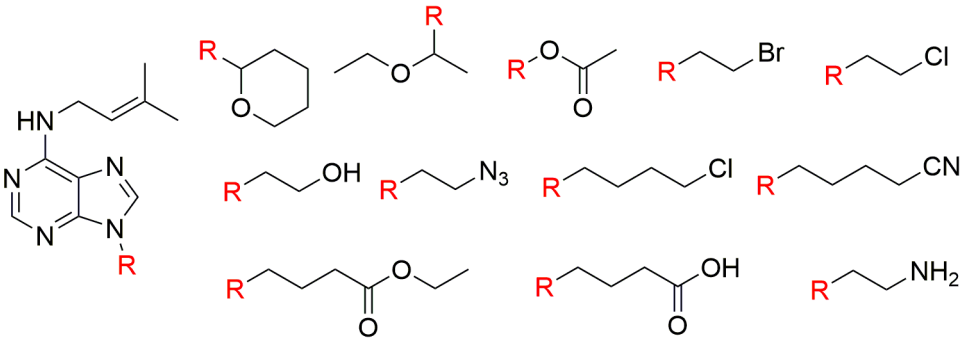

Figure 12. Structures of iP derivatives prepared by Mik et al. 2011 [158].

Substitution at the purine N9 atom with ethoxyethyl-, acetoxy-, azido-, 4-chlorobutyl-, and 3-cyanopropyl groups significantly improved iP cell-proliferation activity in tobacco callus bioassay. Generally, most of the derivatives showed high activity in the *Amaranthus* bioassay. However, the prepared derivatives did not show antisenescence activity in the WLS assay [157], probably because iP itself has much weaker activity in this bioassay than other CKs, such as BAP, Kin, and *t*Z [158].

N9-methylated CK antagonist 6-(2-hydroxy-3-methylbenzylamino)purine (PI-55, Figure 13), has also been synthesized and tested in CK bioassays [160]. PI-55 was the first identified CK receptor antagonist. This 'anti-cytokinin' has strong inhibitory effects on CK-induced responses in various bioassays, but also accelerates germination of *A. thaliana* seeds and promotes both root growth and formation of lateral roots [161]. However, methylation of the N9 atom caused complete loss of CK activity in all of three CK bioassays used to test them [160].

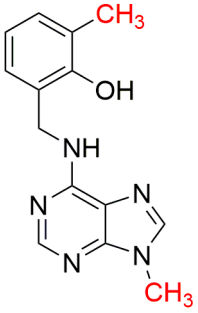

Figure 13. Structure of N9-methylated PI-55 [160].

It seems that alkyl substituent at N9 atom does not have exclusive effect on CK activity itself. It is rather the combination of appropriate 9-alkyl and functional 6-substitution of adenine, often containing hydrocarbon residue with suitably located oxo or hydroxyl groups.

3.3. 9-(Tetrahydropyran-2-yl) and 9-(Tetrahydrofuran-2-yl)ated CKs

In a study reported in 1967, 6-benzylamino-9-(tetrahydropyran-2-yl)purine (BAP9THP) was prepared and found to promote chlorophyll retention (and senescence delay) in plant tissues exceptionally strongly, and growth of tobacco callus almost as strongly as BAP. Its high activity was attributed to the lability of the 9-substituent [66]. Other early studies showed that some synthetic BAP9THP derivatives could stimulate tiller bud elongation in cereals [162] and increase numbers of apple and grape fruits [163,164]. A comparative study published in 1981 demonstrated that activities of BAP and various 9-substituted derivatives in the promotion of lettuce seed germination declined in the following order: BAP = 9-BAP9THP > 9-methyl BAP > 9-methoxymethyl BAP > 9-cyclopentyl BAP > 9-cyclohexyl BAP [115]. Later, 6-benzylamino-9-(tetrahydrofuran-2-yl)purine (BAP9THF) was prepared, its impact on leaf senescence was studied, and both BAP9THP and BAP9THF were found to delay senescence and induce several growth responses more strongly than BAP. The increased senescence-retarding activity of these compounds was at least partially attributed to the gradual cleavage of pyranyl or furanyl and release of free base there form [145]. 6-benzylaminopurine and BAP9THP have been reported to induce adventitious shoot formation significantly more strongly than iP or Kin [165]. Generally, 2-tetrahydropyranyl and 2-tetrahydrofuranyl cyclic ether groups are widely used in organic chemistry as protective groups and can be easily removed in acidic conditions [166]. The 9THP- or 9THF-substituted Kin and other 9THP and 9THF ArCKs have significant anti-senescence effects, as previously described for BAP [115].

In attempt to improve specific biological properties of CKs reported in 2009, a number of new hydroxyl and/or methoxy benzene ring-substituted 9THP and 9THF CKs (Figure 14) were synthesized and tested [147]. They were prepared via the condensation of 6-chloropurine with 3,4-dihydro-2H-pyran or 2,3-dihydrofuran, catalyzed by trifluoroacetic acid, followed by coupling of the intermediates with corresponding benzylamines [147].

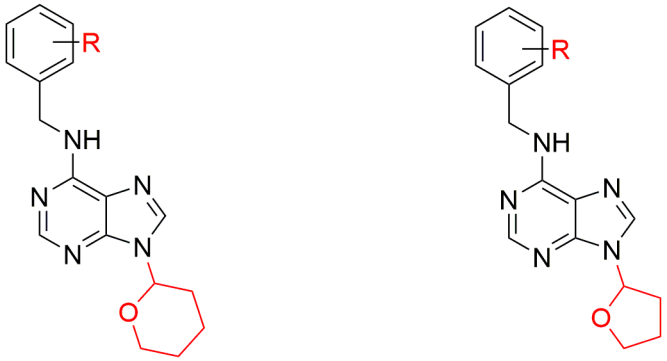

Figure 14. General structures of 9THP and 9THF ArCK derivatives (R = OH, OCH_3 or their combination) [147].

The 9THP and 9THF ArCKs were found to have higher activities than corresponding free bases in tobacco callus, WLS, and *Amaranthus* bioassays. Not all the prepared 9THP and 9THF derivatives are entirely stable at pH < 4, because they slowly decompose to their free bases [147].

In 2012, 6-(3-methoxybenzylamino)-9-(tetrahydropyran-2-yl)purine (3MeOBAP9THP) derivative showed that it does not inhibit the primary root growth compared to the parent CK 6-(3-methoxybenzylamino)purine (3MeOBAP) [41]. Besides, the compound showed a positive impact on the growth of the aerial part compared to free base, all in the nanomolar (8 to 40 nM) concentration range [41]. This may be due to stimulation of ethylene biosynthesis, which correlated

with observed root phenotypes and the strength of inhibition of root cell elongation. Root inhibition is probably caused by the formation of 9-glucosides, as explained above. An appropriate substituent at the purine N9 atom blocks its fast glucosylation and can thus protect the active CK from deactivation and prevent the primary root inhibition. Metabolic analysis with radioactively labelled 3MeOBAP9THP has revealed that the THP group can be slowly eliminated in vivo and its application indeed results in a significantly lower total content of inactive glucosides than treatments with unprotected 3MeoBAP [41]. In a study published the following year, the effects of 3MeOBAP9THP, 6-(3-methoxybenzylamino)-9-(tetrahydrofuran-2-yl)purine (3MeOBAP9THF), and 3MeOBAP on root elongation were compared [48]. 6-(3-methoxybenzylamino)-9-(tetrahydrofuran-2-yl)purine had a much weaker inhibitory effect than 3MeOBAP, but its ability to compete with tritium-labelled *tZ* for the activation site of the *A. thaliana* CRE1/AHK4 receptor in competitive receptor tests was comparable to that of 3MeOBAP [48]. Subsequently, physiological effects of these compounds have been tested in a number of micropropagation protocols, inter alia for horticultural and medicinal plants [167–169]. The results of their testing showed effects in diverse processes, e.g. acclimatization of micropropagated 'Williams' banana [170,171]. It indicates that these second-generation ArCKs have great potential for improving in vitro regeneration techniques for various economically important and endangered plants.

Recently, a large collection of 8-substituted 9THP CK derivatives was also synthesized [172]. Most were generated via multiple synthesis following previously published protocols [173–175] and substitution of the halogen atom at position C8 with a nucleophile (amine or alcoholate) to afford 8-substituted-9-THP-CK. The CK activity of all the compounds was determined in classical CK bioassays In the WLS assay, prepared compounds with a THP group generally had lower CK activity than the parent compounds. However, 8-chloro-9THP-iP and 8-bromo-9THP-iP exhibited very high activity over a wide concentration range, from 0.1 to 100 μM, in the tobacco callus bioassay (8–15% stronger activity than 1 μM BAP). Furthermore, all 8-substituted CKs with a 9THP group had comparable activities to their parent compounds (*tZ*, iP, and BAP) [172].

In view of the above structure-activity relationships resulting from the synthesis and testing of various 9-substituted ArCKs, new 9-substituted Kin derivatives were prepared and their antisenescence activity in WLS assays was investigated [176]. Seven Kin derivatives and analogues were prepared via nucleophilic substitution of 6-chloro-9-(tetrahydrofuran-2-yl)purine or 2,6-dichloro-9-(tetrahydrofuran-2-yl)purine with the appropriate amines. The most potent derivatives had slightly higher activity than BAP in WLS bioassays, similar to that of a previously synthesized compound, 6-furfurylamino-9-(tetrahydrofuran-2-yl)purine (Kin9THF).

Tetrahydropyranylation/tetrafuranylation of known CKs started a new era in the implementation of novel class of CK derivatives in tissue culture—their potential lies mainly in a small design change—a suitable easily removable substitution on N9 atom of purine, which prevents the formation of unwanted 9-glucoside associated with root inhibition. Besides, selected known CKs or newly developed mimetics of BAP and Kin substituted by these THP or THF groups retain very special antisenescent properties of CKs.

In Table 1, we list derivatives with such combinations of N9, N6, C2 and C8 substitutions that were significantly more active than the widely applied classical CK compounds BAP, Kin and iP in three basic CK bioassays in the last 15 years.

Table 1. Summary of substitutions at N9, N6, C2, and C8 atoms, and their combinations, that resulted in compounds with significantly higher activity than appropriate standards in indicated CK bioassays.

Bioassay	Std.	Position of the Substituent on the Purine Ring				Ref.
		N9	N6	C2	C8	
***Amaranthus caudatus* betacyanin**	**BAP**	β-D-ribofuranosyl-	2-chlorobenzyl-, 3-chlorobenzyl-, 2-bromobenzyl-, 3-bromobenzyl-, 3-iodobenzyl-, 3,5-difluorobenzyl-, 2,4,5-trifluorobenzyl-, 2-chloro-4-fluorobenzyl-, 2-trifluoromethylbenzyl-, 3-trifluoromethoxybenzyl-	H	H	[45]
			2-fluorobenzyl-, 3-fluorobenzyl-, 4-fluorobenzyl-, 2-chlorobenzyl-, 3-chlorobenzyl-, 2-bromobenzyl-, 3-bromobenzyl-, 4-bromobenzyl-, 3-iodobenzyl-	Cl	H	[46]
		2′-deoxy-β-D-ribofuranosyl-	3-hydroxybenzyl-, 2-fluorobenzyl-, 4-fluorobenzyl-, 2-chlorobenzyl, 3-chlorobenzyl-, 2-bromobenzyl-, 3-brombenzyl-, 2-methybenzyl-, 2-trifluoromethylbenzyl-	H	H	[120]
		tetrahydropyran-2-yl	isopentenyl-, furfuryl-	H	3-aminopropyl-, 4-aminobutyl-, methylsulfanyl-, dimethyl-, allyl-	[172]
			benzyl-	H	H	[147]
		tetrahydrofuran-2-yl	benzyl-, 2-methoxybenzyl-, 3-methoxybenzyl-	H	H	[147]
			thiofen-2-yl-	Cl	H	[176]
	iP	tetrahydropyran-2-yl, ethoxyethyl-, 2-bromoethyl-, 2-chloroethyl-, 4-ethoxy-4-oxobutyl-	isopentenyl-	H	H	[158]

Table 1. *Cont.*

Bioassay	Std.	Position of the Substituent on the Purine Ring				Ref.
		N9	N6	C2	C8	
Senescence (WLS)	**BAP**	β-D-ribofuranosyl-	2-fluorobenzyl-, 3-fluorobenzyl-, 4-fluorobenzyl-, 2-chlorobenzyl-, 4-chlorobenzyl-, 2-methylbenzyl-, 3-methybenzyl-, 2-methoxybenzyl-, 3-methoxybenzyl-, 3,4-dichlorobenzyl-, 2,3-dimethoxybenzyl-, 2,4-difluorobenzyl-, 3,5-difluorobenzyl-, 2,3,4-trifluorobenzyl-, 2,3,6-trifluorobenzyl-, 2-chloro-4-fluorobenzyl-, 3-chloro-4-fluorobenzyl-, 2-hydroxy-5-methylbenzyl-, 2-difluoromethoxybenzyl-	H	H	[45]
			2-fluorobenzyl-, 3-fluorobenzyl-, 4-fluorobenzyl-, 2-chlorobenzyl-, 3-chlorobenzyl-,4-chlorobenzyl-, 3-bromobenzyl-, 4-bromobenzyl-	Cl	H	[46]
		β-D-arabinofuranosyl-	benzyl-, 2-fluorobenzyl-, 3-fluorobenzyl-, 4-fluorobenzyl-, 3-chlorobenzyl-, 2-methoxybenzyl-,3-methoxybenzyl-, 3-hydroxybenzyl-, 3-methylbenzyl-, 2,5-difluorobenzyl-, 3,5-difluorobenzyl-	H	H	[134]
		2′-deoxy-β-D-ribofuranosyl-	benzyl-, 2-hydroxybenzyl-, 3-hydroxybenzyl-, 4-hydroxybenzyl, 3-methoxybenzyl-, furfuryl- 2,5-dimethoxybenzyl-, 2-fluorobenzyl-, 3-fluorobenzyl-, 4-fluorobenzyl-, 2-chlorobenzyl-, 3-chlorobenzyl-, 4-chlorobenzyl-, 2-bromobenzyl-, 3-bromobenzyl-, 4-bromobenzyl-, 2-methylbenzyl-, 3-methylbenzyl-, 2-trifluoromethylbenzyl-, 3-trifluoromethylbenzyl-,	H	H	[120]
		tetrahydropyran-2-yl	benzyl-, 3-hydroxybenzyl-, 2-methoxybenzyl-,	H	H	[147]
		tetrahydrofuran-2-yl	benzyl-, 3-hydroxybenzyl-	H	H	[147]
			tetrahydrofuran-2-yl-, thiofen-2-yl-, 5-methylthiofen-2-yl-	H	H	[176]
			tetrahydrofurfuryl-	Cl	H	[176]
	Kin	2-bromoethyl-, 2-chloroethyl-, 4-chlorobutyl-, 1-ethoxyethyl-, tetrahydrofuran-2-yl	furfuryl-	H	H	[157]

Table 1. *Cont.*

Bioassay	Std.	Position of the Substituent on the Purine Ring				Ref.
		N9	N6	C2	C8	
Tobaccocallus	**BAP**	β-D-ribofuranosyl-	2-fluorobenzyl-, 4-fluorobenzyl-, 2-bromobenzyl-, 2-methoxybenzyl-	H	H	[45]
			2-flourobenzyl-, 3-fluorobenzyl-, 4-fluorobenzyl-, 2-chlorobenzyl-, 3-chlorobenzyl-, 4-bromobenzyl-	Cl	H	[46]
		2′-deoxy-β-D-ribofuranosyl-	benzyl-, 4-fluorobenzyl-, furfuryl-	H	H	[120]
		tetrahydropyran-2-yl	isopentenyl-, furfuryl-	H	2-aminoethyl-, 3-aminopropyl-, 4-aminobutyl-, 6-aminohexyl-, methoxy-, 2-hydroxyethyl-	[172]
			benzyl-	H	H	[147]
		tetrahydrofuran-2-yl	furfuryl-, thiofen-2-yl, 5-hydroxymethylfuran-2-yl-	H	H	[176]
			furfuryl-, tetrahydrofurfuryl-, thiofen-2-yl-	Cl	H	[176]
	iP	ethoxyethyl-, acetoxy-, 2-azidoethyl-, 4-chlorobutyl-, 3-cyanopropyl-	isopentenyl-	H	H	[158]
	Kin	2-bromoethyl, 2-chloroethyl-, 1-ethoxyethyl-, tetrahydrofuran-2-yl	furfuryl-	H	H	[157]

4. Conclusions

The objective of this review was to describe, as far as possible, the endogenous occurrence, synthesis, and biological activity of numerous sugar and non-sugar 9-substituted CK derivatives. We have also covered their natural occurrence in plants in relation to their biological properties, toxicity and effects on plant growth and development, especially root and shoot development. We have summarized knowledge regarding natural disaccharide conjugates that are soluble in water, and thus particularly attractive for use in tissue culture. We have also summarized historical progress in their discovery and synthesis of these derivatives and highlighted several structural aspects of 9-substituted CKs and CK-like compounds, as well as their relationships to biological activities. Active derivatives and conjugates are summarized in Table 1, together with references.

We analyzed the inactivation (reversible and reversible) of ArCKs and IsCKs through the formation of various forms of ribosides and glucosides and discussed the effect of their isomerism on CK activity. The discovery of new 9-substituted CKs and their potent developmental effects on plants has induced a boom in synthesis and testing of 9-substituted CK derivatives and their analogues useful in plant and human biotechnologies. Recently, the generation and testing of a number of new compounds has provided unexpected information on the biological properties of various 9-substituted CKs, whose research has historically been halted due to early conclusions by scientists about 9-glucoside inactivity in plants, since these compounds were considered to be the metabolic end-products of functional CKs. The development of new CK derivatives with knowledge of efficient structural motifs allows for an increase in their biological activities and thus provides interesting new molecules with various potential effects and metabolic advantages.

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Appendix IV

Rewiev

Synthesis of aromatic cytokinins for plant biotechnology

Plíhalová, L., **Vylíčilová, H**., Doležal, K., Zahajská, L., Zatloukal, M. Strnad, M.

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Review

Synthesis of aromatic cytokinins for plant biotechnology

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Cytokinins represent an important group of plant growth regulators that can modulate several biotechnological processes owing to their ability to influence almost all stages of plant development and growth. In addition, the use of purine based cytokinins with aromatic substituent in C6 position of the purine moiety in tissue culture techniques is currently experiencing a surge in interest, made possible by the ongoing systematic synthesis and study of these compounds. This review article outlines progress in the synthesis of aromatic cytokinins, the *in vitro* and *in vivo* effects of these substances and insights gleaned from their synthesis. As the purine moiety in these compounds can be substituted at several positions, we examine each of the substitution possibilities in relation to the derivatives prepared so far. The discussion highlights the gradual simplification of their preparation in relation to their application in practice and summarizes the relevant organic chemistry literature and published patents.

Contents

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Introduction

Purine based cytokinins (CKs) are naturally occurring plant growth hormones. Their homeostasis *in planta* influence almost all stages of plant development and directly affect processes of plant growth, often in concert with other plant hormones [1]. Although the first discovered naturally occurring CK was the isoprenoid derivative *trans*-zeatin isolated from *Zea mays* in 1963 [2], the first aromatic cytokinin (ARCK) was synthesized and tested as a specific cell-division factor much earlier. A postdoctoral fellow, Carlos Miller, experimented with coconut milk and used UV spectrum analysis in combination with other techniques to show that the prepared concentrates contained purines. In 1955, Miller tested coconut milk concentrates and discovered that they were highly active in tobacco pith tissue culture. After several experiments, Miller *et al.* isolated a white crystalline solid from aged DNA by chromatographic procedures and identified its structure as N^6-furfuryladenine. The authors suggested that the compound promoted cell division (cytokinesis) and named it kinetin (KIN) [3,4]. In fact, discovery of this compound was largely driven by the need to enhance cytokinesis in *in vitro* plant regeneration [5].

The first attempt to use CKs in plant tissue culture appeared immediately after the discovery of KIN [6,7]. In 1956, the term 'kinin' was coined by Miller for all substances able to promote cytokinesis and *permit continuous growth of various plant tissues in vitro* [5]. In the same laboratory, another aromatic purine derivative, 6-benzylaminopurine (BAP), was synthesized and shown to stimulate cell division in cultured tissues [5]. The above mentioned compounds formed the cornerstone of the first generation of synthetic ARCKs able to elicit plant growth and stimulate cell division. Since that time, micropropagation techniques have been used in conjunction with ARCKs in culture media to provide a rapidly developing biotechnological strategy for the commercialization of many important plant species. The main achievements so far are in the micropropagation of bananas [8,9], strawberries [10], apples [11], roses [12], melons [13] and many other plants, especially ornamental and/or medicinal [14,15]. For traditional medicinal plants, tissue culture is often the best way to reduce the cost of seedling/plant production whilst enhancing their chance of survival in nature [16]. One of the most widely used and reasonably cheap CK 6-benzylaminopurine (BAP), also known as N^6-benzyladenine (BA), unfortunately causes side effects, such as shoot-tip necrosis [17], problematic acclimatization in the greenhouse [18] and inhibition of rooting [19], that complicate micropropagation processes, especially in rare and susceptible medicinal plants [16,20].

Therefore, several CK derivatives have been prepared to avoid the unfavorable properties of BAP. Although the most obvious solution was to use KIN or coconut water containing KIN and other CK derivatives in the media [21], many laboratories attempted to design more sophisticated and effective ARCKs. Notably, the adenine moiety can be substituted at several positions, such as N1, C2, N3, N6, N7, C8 and N9, as shown in Fig. 1. This opens up possibilities for preparing a wide range of derivatives with interesting biological properties thanks to modern combinatorial chemistry. In addition, if the N6-position is substituted with phenyl or benzyl, further substitutions can be made on the phenyl/benzyl ring, extending the range of interesting derivatives available. Further, hydroxylated and methoxylated BAP derivatives were discovered in plants as natural phytohormones [22–24].

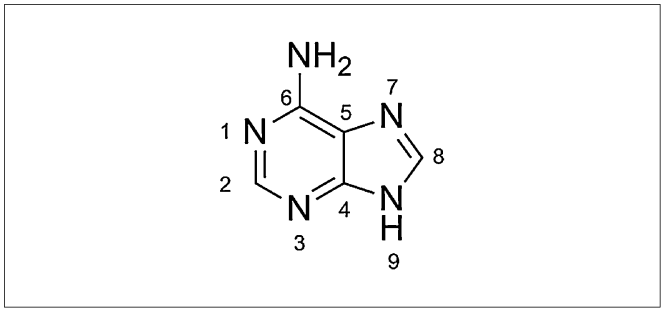


FIGURE 1
Structure of adenine with numbered atom positions.

The earliest syntheses usually started from substituted pyrimidine or imidazole precursors and generated the purine by sophisticated building-block synthesis. This type of method also allowed the preparation of N1-, N3-, N7-derivatives of purine [25]. Over the past 15 years, an extensive library has been constructed containing thousands of compounds derived from naturally occurring ARCKs, mostly obtained by the reaction of 6-halogenopurines with the appropriate aromatic amines. The first artificial representatives were prepared in the laboratory by substitution of the benzyl ring, for example, 6-(3-methoxybenzylamino)purine and 6-(3-hydroxybenzylamino)purine [26], currently called topolins [22]. Substitution of one or more hydrogen atoms by -hydroxy, -methoxy, -halogen, -mercapto or -alkyl groups or their mutual combinations has been proposed as an effective strategy for CK effect improvement [27]. New CK derivatives have been prepared not only by substitution of the benzyl ring but also by various substitutions in the purine moiety, especially at the C2, C8 and N9 atoms. These compounds are often called second generation CKs because they exhibit CK properties but do not usually exist in nature [28–36]. ARCKs of the second generation, successfully tested for biotechnological applications, usually possess a combination of these features: they contain substitution at the C2 and/or N9, eventually C8-positions of the purine moiety and concurrently substitution of the benzyl ring attached to the N6-atom of adenine moiety. In this review, we summarize developments in the synthetic procedures of ARCKs currently attracting interest for their possible use in biotechnologies, especially plant tissue culture. Our main aim was to map the continual process of searching for new, more effective structural motifs that are straightforward to synthesize for widespread biotechnological use.

Monosubstituted ARCKs: N6 adenine atom substitutions

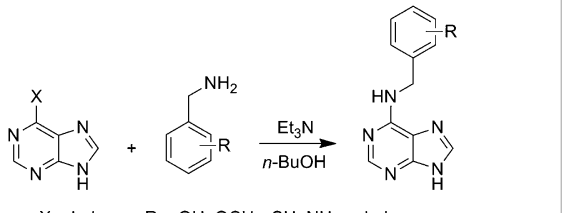
Kinetin discovery and synthesis

Kinetin was the first known ARCK discovered in 1955 by Miller and his collaborators [3,5]. The synthesis of KIN was reported in the same paper [5]. Although the first attempt at the condensation of adenine with furfural was unsuccessful, the authors managed to perform furfuryl chloride and adenine condensation under alkaline conditions established with sodium bicarbonate [5]. The improved synthesis was based on 6-methylmercaptopurine and freshly redistilled furfurylamine as the starting materials. KIN was obtained after heating the abovementioned mixture at 115–120°C for nine hours [5,37]. This method with minor or major modifications is still used

today. The stereochemistry and hydrogen bonding of kinetin were first described by Soriano-Garcia and Parthasarathy [38]. The authors reported molecular packing based on extramolecular hydrogen bonding between N6H–N3H and intermolecular N7H–N6H [38]. Sattsangi *et al.* successfully prepared KIN using furfural and adenine as starting compounds in the presence of hydrogen chloride and sodium cyanoborohydride in methanol [39]. KIN was also prepared from guanine after reaction with furfurylamine in *N,N?*-dimethylcyclohexylamine in the presence of phosphorus pentoxide [40]. Another published synthesis was based on the reaction of furfurylamine with 2,6-dichloro-7-benzylpurine in acetonitrile under alkaline conditions established with NaOH solution [41]. At present, KIN is usually prepared by nucleophilic substitution of 6-chloropurine by furfurylamine under alkaline conditions [42] and its preparation time has been shortened to ~3 hours.

N^6-Benzyladenines

Currently, the most frequently used ARCK in tissue culture is BA. BAs can be prepared by the method previously described by Strong and Miller for KIN using 6-methylthiopurine and an appropriate benzylamine [5]. However, this reaction has been exploited less frequently as it requires quite a long reaction time (16 hours) and high temperature (about 140°C). Unfortunately, despite the demanding synthetic conditions, the reaction provides only moderate yields of approximately 40–60%. Daly and Christensen prepared BAP by reaction of 6-chloropurine with benzylamine in *n*-butanol [43]. This type of S_N2 reaction, in which a 6-halogenopurine (or its nucleoside) reacts with an appropriate benzylamine, can generally be used for the preparation of ARCKs derivatives (Scheme 1). The reaction depends on the nucleophilicity of the amine used but usually provides good yields of >80% [26]. BAP can also be prepared from adenine and benzylamine hydrochloride by heating at 165–170°C for 8 h, as described by Calvert and Traverso [37]. The reaction of adenine with benzylaldehyde in the presence of formic acid was patented in 1965 [44], but the procedure was rather slow as it took approximately four days to obtain the product. In 1975, a labeled benzylamino-[$^{15}N_4$]-purine was prepared from 6-chloro-[$^{15}N_4$]-purine and benzylamine hydrochloride [45] in a 1:1 molar ratio after refluxing the mixture in 1-butanol for 3 h. BAP was also prepared by the reaction of hypoxanthine with benzylamine in the presence of *N,N?*-dimethyl cyclohexylamine and phosphorus pentoxide at 170°C for 24 h [40]. One year later in 1983, the crystal structure of BAP was solved by Raghunathan *et al.*, which revealed several important structural characteristics, such as molecular packing, intramolecular hydrogen bonding between N3H–N9H and N6H–N7H atoms, indicating that BAP can have a strong influence on CK receptor domain behavior [46]. In 1989, the nucleophilic substitution of 6-chloropurine by benzylamine was improved by the addition of excess of triethylamine as a strong base and the reaction was performed in a 1:1.2:5 ratio by refluxing in absolute ethanol [47]. BAP derivatives substituted in the phenyl ring have been prepared by many groups for different application purposes since the introduction of this procedure. The reaction can be accelerated by employing a microwave reactor, which allows 95% yield of 6-benzyl or 6-phenyl derivatives within 5 min [48]. The preparation of BAP and its utilization in plant tissue culture has been the subject of at least two patents [44,49]. Unfortunately disadvantages have been found during the use of BAP in some plant species, especially related to acclimatization, heterogeneity in growth and inhibition of rooting [9,50]. One way to eliminate these side effects is the development of new BAP derivatives [26]. The easiest route toward changing BAP is substitution of the benzyl ring, especially with CH_3, NH_2, NO_2, OH, CH_3O, SH and halogenides. Derivatives were initially prepared by reaction of 6-(methylmercapto)purine with the corresponding amine in a 1:2 (1:3) molar ratio, but this method gave low yields [51]. The method currently in use is based on reaction of 6-chloropurine with relevant benzylamines [52,53]. This improved version of the method employs the reaction between 6-chloropurine and an appropriate amine in the presence of an excess amount of triethylamine at 90°C and has so far led to the preparation of several benzyl mono-, di- and tri-substituted derivatives containing OH, Cl, Br, F, I, CH_3, CH_3O and their mutual combinations [26,54]. Many of these derivatives were shown to possess comparable or better activity than BAP in three classical CK bioassays based on the stimulation of tobacco callus growth, retention of chlorophyll in excised wheat leaves and dark induction of betacyanin synthesis in Amaranthus cotyledons [26,54]. In addition, the methoxy and hydroxy derivatives prepared by Doležal *et al.* overcome some of the problems related to BAP use in micropropagation techniques, such as problems with explant rooting [17,18]. 6-(3-Hydroxybenzylamino)purine, also called *meta*-topolin, has been effectively employed in many plant tissue culture production protocols [9,15,50,55].



X = halogen; R = OH, OCH_3, SH, NH_2 or halogen

SCHEME 1

Synthesis of BAP through reaction of 6-halogenopurine with benzylamines, suggested by Daly and Christensen [43].

In summary, KIN and BA are ARCKs of the first generation, very widely used (together with isoprenoid cytokinins isopentenyladenine and more expensive *trans*-zeatin) as supplement of plant tissue culture media, basic tool for *in vitro* micropropagation practice. First such a medium was invented by plant scientists Toshio Murashige and Folke K. Skoog in 1962 [56] during Murashige's search for a new plant growth regulator. Since then, many modifications have been developed to optimalize micropropagation process, which has been developed for nearly all agriculturally important plant species, such as for example, bananas [8,9,57], sugarcane [58], *Stevia rebaudiana Bertoni* [59], potatoes [60], strawberries [10], and apples [11]. KIN and BA are also often used for the tissue culture of many ornamental plants such as *Gerbera* species [61], Lily, Tobacco and Chrystanthemum [62], roses [12] and many others [63,64]. Besides, KIN and BA are currently used in tissue culture media for many medicinal plants such as *Aloe vera* L. [65], Fabaceae [66] or Rutaceae [67].

Moreover, availability of labeled as well unlabeled topolin standards was indispensable in their discovery as naturally occurring compounds [68,69] as well during subsequent development of

mass spectrometry-based quantification methods [70] used nowadays for extremely sensitive and precise monitoring of their endogenous levels [71].

N^6-Phenyladenines

The preparation and biological activity of BAP derivatives inspired the preparation of N^6-phenyladenines and its ring substituted derivatives. Unsubstituted N^6-phenyladenine was synthesized for the first time by direct reaction of 6-methylsulfanylpurine and aniline at high temperature [72]. Later, it was synthesized by reaction of 6-chloropurine with excess phenylamine without any other solvent [49]. Imbach *et al.* implemented a method based on the reaction of halogenophenylamines with 6-chloropurine in dimethylformamide in the presence of triethylamine at approximately 100°C [73]. More recently, new synthetic routes for these derivatives were invented during the preparation of intermediates for the synthesis of 6-phenylamino-2,9-disubstituted purines as cyclin-dependent kinase inhibitors [74]. The procedure was utilized and improved for the preparation of novel potent 2-halogeno-N^6-phenyladenine inhibitors of *Arabidopsis thaliana* CK oxidase/dehydrogenase [75]. As an addition to existing state-of-the-art syntheses, the authors used an alcohol (C2–C5) or dimethylformamide as the medium for the reaction and additional auxiliary base to establish alkaline conditions.

Disubstituted ARCKs based on adenines

1-Substituted derivatives of N^6-benzyladenines

Numerous procedures for performing the N-alkylation or N-glycosylation of purines have been published. Nevertheless, 1, 6-disubstituted adenines with an aromatic substituent at the N6 atom have rarely been prepared, mainly due to the unfavorable low electron density at N1 in such a substituted purine moiety. Although 1-substituted derivatives of ARCKs have not yet been used in tissue culture, we here describe how to obtain them because these derivatives are very often mentioned in relation to protonization at the N1 atom of the purine moiety, which can have a significant impact on the compound biological activity [76,77]. Moreover, specific 1-alkylation of 9-benzylhypoxanthine has been described *via* reaction of 9-benzylhypoxanthine with a potassium salt and/or potassium hydroxide and phenacyl bromide in ethanol. The reaction generated 1-phenacyl-9-benzylhypoxanthine in 82% yield [78]. To achieve chemical simulation of the 'ATP-imidazole' cycle, reaction of 9-benzyladenine with phenacyl bromide in dry DMF at room temperature was performed [79]. The reaction generated a bis-salt that, when treated with hot water, hydrolyzed to the neutral 6-imino-1,9-dibenzyladenine. Latter compound was refluxed in xylene with benzylamine for 4 h and yielded 1-benzyl-5-phenylimidazole [79]. Leonard and Swaringen published a paper describing the intramolecular cyclization of 1,6- disubstituted purines to form an unsaturated carbonyl system [80]. The cyclization process was mainly observed in isoprenoid 6-(3-methyl-2-butenylamino)purine and its corresponding riboside, when treated with strong acids, such as HCl or trifluoroacetic acid. A similar intramolecular cyclization process was found to accompany the N1 atom substitution of 6-(2-methoxybenzylamino)purine, 6-(2,4-dimethoxybenzylamino)purine, 6-(2-methoxy-1,4-dihydroxybenzylamino)purine and 6-(2-methoxy-2,5-dihydroxybenzylamino)purine [81]. This may explain the absence or low cytokinin activity of such derivatives.

2-Substituted derivatives of 6-anilinopurines and 6-benzylaminopurines

The compound 2-methyl-N^6-benzyladenine was first mentioned in a Shell Oil Company patent among compounds able to increase resistance to deterioration and protect plants from spoilage [82]. 2-Methyladenine was first prepared by hydrogenating 4,6-diamino-5-benzenediazo-2-methylpyrimidine hydrochloride in the presence of formic acid using palladium as catalyst. The 2-methyladenine was then treated with benzyl alcohol in the presence of sodium hydroxide to yield 2-methyl-N^6-benzyladenine [82]. Currently, the starting material for the preparation of 2,6-disubstituted purines is usually 2,6-dichloropurine. It is well known that selective substitution at the C6 purine atom can be easily achieved by reacting 2,6-dichloropurine with oxygen, nitrogen and halogen nucleophiles [83]. Usually, 2,6-dichloropurine is reacted directly with an appropriate nucleophile and the reaction is performed in the presence of an appropriate amine under alkaline conditions for several hours to generate the product with 6-chloro group replaced in quantitative amounts. 2-X-6-anilinopurines (X = H, halogen, amino, methylthio or nitro) with various substitutions on the phenyl ring were prepared by Zatloukal *et al.* [75]. The compounds were prepared by condensation of 2-substituted-6-chloropurines with corresponding anilines, and the compounds were tested for their biological activity in classical CK bioassays. In addition, the compounds were tested for their activation of receptors (AHK3, CRE1/AHK4) and interaction with the key enzyme involved in CK degradation in *Arabidopsis thaliana* (cytokinin oxidase/dehydrogenase 2, AtCKX2). The study showed that 2-chloro-N^6-(3-methoxyphenyl)adenine and 2-fluoro-N^6-(3-methoxyphenyl)adenine are highly potent AtCKX2 inhibitors [35,75,84].

CK homeostasis and signaling has emerged as interesting target for enabling plant adaptation to adverse conditions and development of stress-tolerant plants. Thus, modulating the CK status with inhibitors of CK perception and/or degradation may be useful in protecting plants against adverse abiotic effects [85]. A novel inhibitor of cytokinin degradation (INCYDE), even at 10 nM concentration significantly influenced various biochemical parameters and photosynthetic apparatus in NaCl stressed tomato plants. From an agricultural perspective, obtained findings indicate the potential of INCYDE in protecting plants against NaCl stress and the possibility of enhanced productivity [84]. The effects of these substances on seedling growth in the medicinal plant *Bulbine natalensis* Baker (Asphodelaceae) and the metal tolerant *Rumex crispus* L. (Polygonaceae) under abiotic stress caused by cadmium (Cd) were subsequently tested [85]. Treatment had positive effects on seedling shoot and root growth and the fresh weight of treated seedlings grown in the presence of Cd. Even a single application of either compound at sub-micromolar concentrations was sufficient to reverse the inhibitory effects of Cd. Our results demonstrate that modulating cytokinin status with inhibitors of cytokinin perception and/or degradation may be useful in protecting plants against the adverse effects of high Cd levels [85].

These inhibitors were also tested on *in vitro* organogenesis, phytohormone accumulation, phytochemical content and antioxidant activity during micropropagation of several plant species, for example banana [50], or medicinally important *Eucomis autumnalis* [86], and a beneficial effect on these plants was observed [50,86]. 2-Substituted derivatives of BAP were prepared by Nisler

et al. [87]. In particular, 2-amino-6-(2-hydroxy-3-methylbenzylamino)purine and 2-fluoro-6-(2-hydroxy-3-methylbenzylamino)-purine were prepared and tested in tobacco callus, Amaranthus, detached wheat leaves senescence, germination and hypocotyl elongation assays. It was suggested that these compounds might find potential use as inhibitors of some CK effects in planta.

3-Substituted derivatives of 6-benzyl or N^6-furfuryladenines

Skoog and Leonard published the first detailed paper about the relationship between chemical structure and CK activity [88]. They revealed that substitution on the N3 atom of the purine moiety leads to inactivation of the growth of tobacco callus, although 3-(γ,γ-dimethylallyl)adenine seems to possess some CK activity. Further, the addition of a second substituent on the N3 atom of N^6-substituted adenines was shown to dramatically reduce or eliminate the CK activity. As 3-allyl, 3-furfuryl-, 3-phytyl-, 3-benzyladenine were found to be inactive in CK bioassays, it was suggested that a prerequisite for the CK effects was that the N3-atom should remain free of substituents [88]. Electrophilic substitution of adenine by alkylation and glycosylation has been shown to proceed mainly at the N3 and partly at the N9 and N7 atoms [89]. Reaction of adenine with benzyl, allyl or alkenyl halide in *N*,*N*-dimethylformamide produced the 3-alkyl-N^6-adenine accompanied by the 9-alkyl isomer. Likewise, glycosylation of adenine afforded the 3-glycosyl and 9-glycosyl derivatives. Later in 1977, the synthesis and CK activity of 3-methyl-N^6-benzyladenine and 3-methyl-N^6-furfuryladenine were published. The compounds were prepared by heating 3-methyl-6-(methylthio)purine with an appropriate amount of aromatic benzylamine in dry methanol. The resulting compounds were evaluated in the tobacco callus bioassay. It was confirmed that replacement of a methyl group at the N3-position lowered the CK activity in comparison with unsubstituted analogues [90]. However, compared to the 7- and 9-glucopyranoside isomers of BAP, the 3-glucopyranoside isomers surprisingly still exhibited low CK activity, perhaps due to hydrolyzation and formation of active free bases [91]. Reaction of commercially available BAP with methyl iodide and sodium hydroxide in ethanol resulted in the formation of 3-methyl-N^6-benzyladenine and N^6-benzyl-9-methyladenine. The isomers were separated by preparative centrifugal thin-layer chromatography to give microcrystalline products in a yield of up to 30%. Moreover, the crystal structure of the N3-isomer was characterized by single-crystal-X-ray diffraction analyses [92].

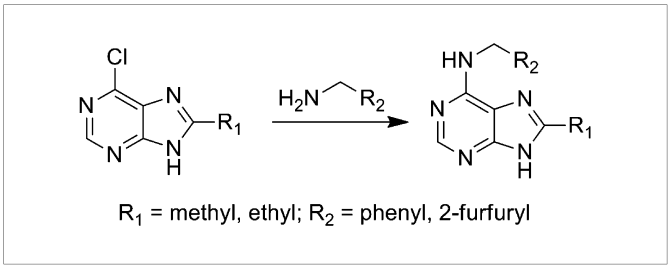
7-Substituted derivatives of N^6-benzyladenines

Some naturally occurring compounds, such as caffeine, are probably the most well known 7-substituted purine derivatives. However, other important 7-substituted purine derivatives include raphanatin and 6-(benzylamino)-7-(β-D-glucopyranosyl)purine [93,94]. 7-Derivatives of aromatic purine based CKs were originally prepared by *de novo* synthesis from diaminopyrimidine derivatives by cyclization [95]. Skoog *et al.* prepared 7-(γ,γ-dimethylallyl)adenine in 1967 and concluded that the compound was not active in CK biotests and they suggested that previous data that showed the apparent activity might be due to contamination with traces of active 6-isomers [88]. 7-Substituted purines were later prepared by an approach similar to that for the N-arylation of 7-deazapurines. Two protocols were developed: the first (Lam–Chan protocol) involved the introduction of a substituent to the *meta* and *para* positions of the aryl ring [96], whereas the second (Buchwald protocol) involved the introduction of a substituent to the *ortho* position of the benzyl ring [97]. Direct alkylation of purines has also been described, but it usually leads to a mixture of 7- and 9-regioisomers. A few reports have been published on the preparation of 7,8-dihydropurine derivatives using $NaBH_4$, BH_3.THF or $NaBH_4$/AcOH [98–100].

8-Derivatives of N^6-benzyl and N^6-furfuryladenines

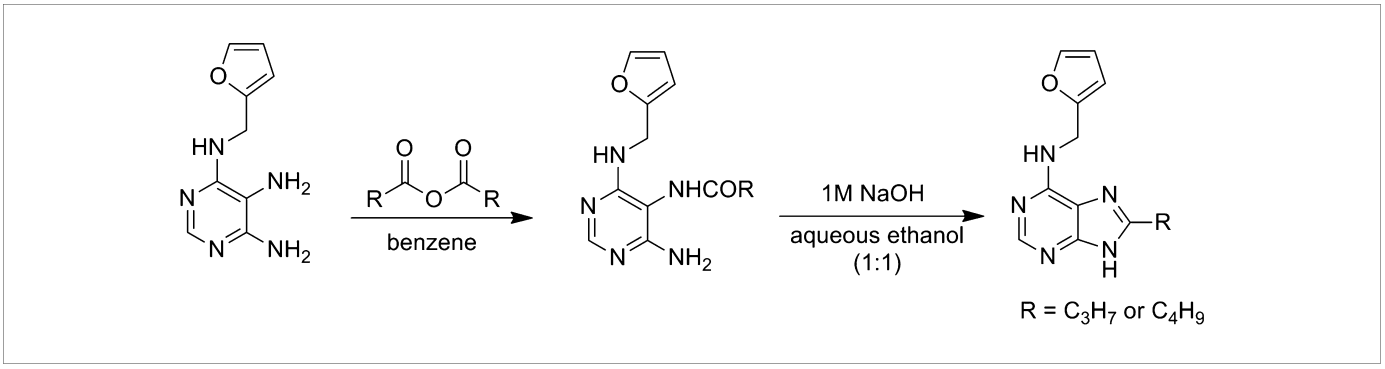
8-Substituted purine CKs have not received as much attention as derivatives modified at the positions 6 or 9. They were first described in studies of the enzymatic conversion of CKs: several authors reported that the enzymatic oxidation of CKs by xanthine oxidase proceeds through 8-hydroxy intermediates to 2,8-dihydroxy derivatives [101]. The first synthesized 8-substituted ARCKs were 8-alkyl derivatives. 8-Methyl/ethylkinetin and BAP were prepared by the substitution of chlorine at the C6 atom of 6-chloro-8-methyl/ethylpurine (Scheme 2) [102].

8-Propyl and 8-butyl derivatives of KIN were prepared by the cyclization of 4-amino-5-butyramido/valeramido-6-furfurylaminopyrimidine in alcoholic alkali (Scheme 3) [103]. The CK activity of 8-substituted purine CKs has mainly been assessed by their ability to retard the etiolation of barley leaves cuttings. In comparison with KIN, the presence of a methyl group at the C8 position was found to significantly increase CK activity, whereas 8-butyl-KIN and 8-propyl-KIN were less active [104]. Several authors have inserted an azido group at the C8 position of CKs for photoaffinity labeling: 8-azido-BAP was prepared from 8-bromoadenosine by a four-step synthesis using 8-azidoadenosine, 8-azido-1-benzyladenosine and 8-azido-BAP as intermediates (Scheme 4) [105], by a four-step synthesis starting from 6,8-dichloropurine using 8-chlorobenzyladenine and 6-benzylamino-8-chloro-9-(1-ethoxyethyl)purine as intermediates (Scheme 5) [106] or, more recently, by direct bromination of BAP and subsequent substitution of a bromine atom with sodium azide [107]. 8-Azido-BAP was found to be as active as BAP in eliciting buds in moss protonemata of *Funaria hygrometrica* and significantly more active than BAP in the tobacco callus bioassay [105]. No significant reduction in the sensitivity to CKs was observed during treatment of protonemata with 8-azido-BAP after irradiation with a UV lamp. This indicates that 8-azido-BAP has a low propensity to form covalent bonds at the active site of proteins [105]. Mornet *et al.* tested the CK activity of a photoproduct mixture resulting from irradiation of 8-azido-BAP [106]. The mixture was only 1/10 as active as 8-azido-BAP in the tobacco bioassay. Mass



SCHEME 2

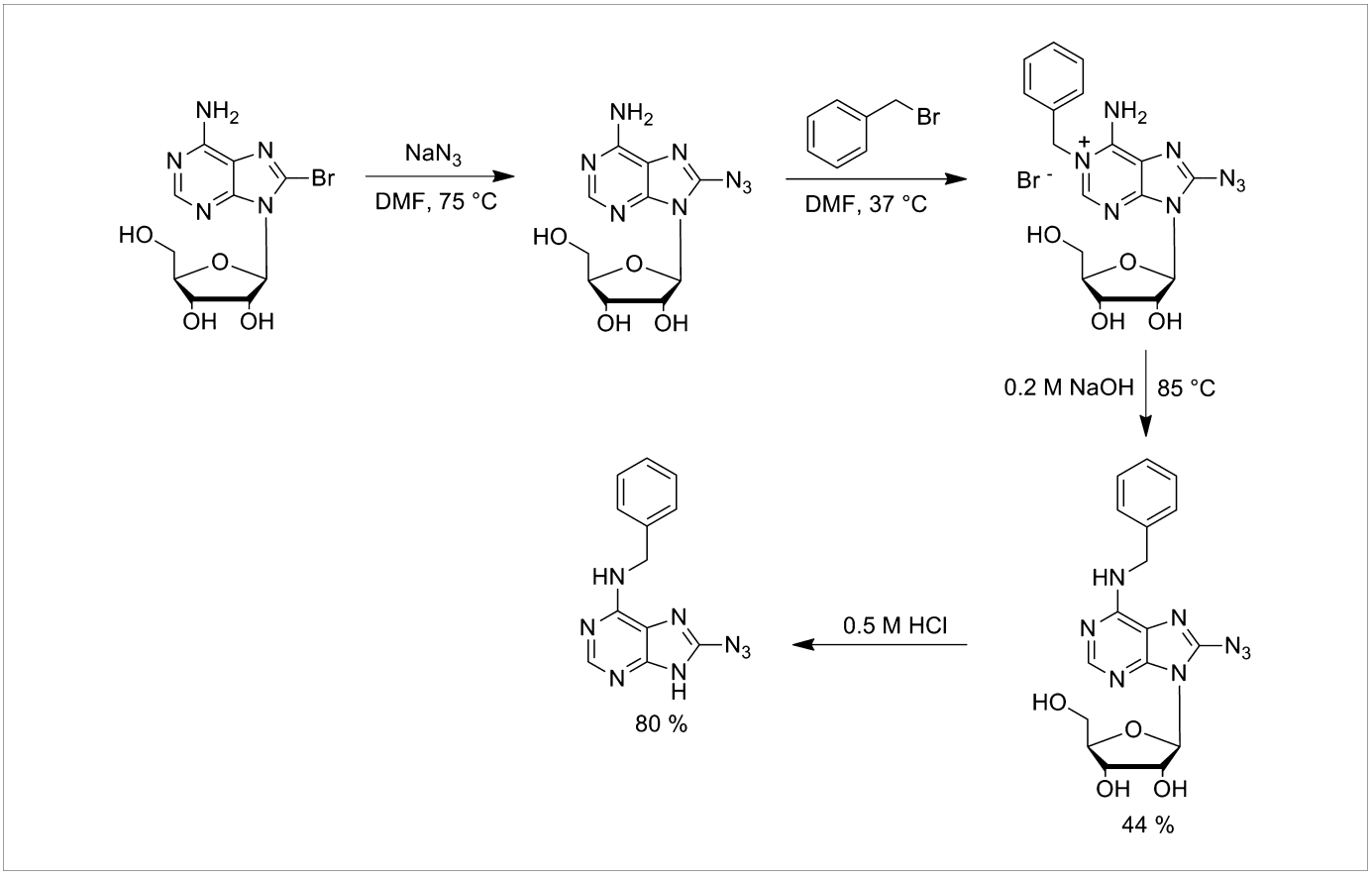
8-Methyl/ethyl aromatic cytokinins prepared by the reaction of 6-chloro-8-methyl/ethylpurine with the appropriate phenyl or 2-furfuryl amines.



SCHEME 3

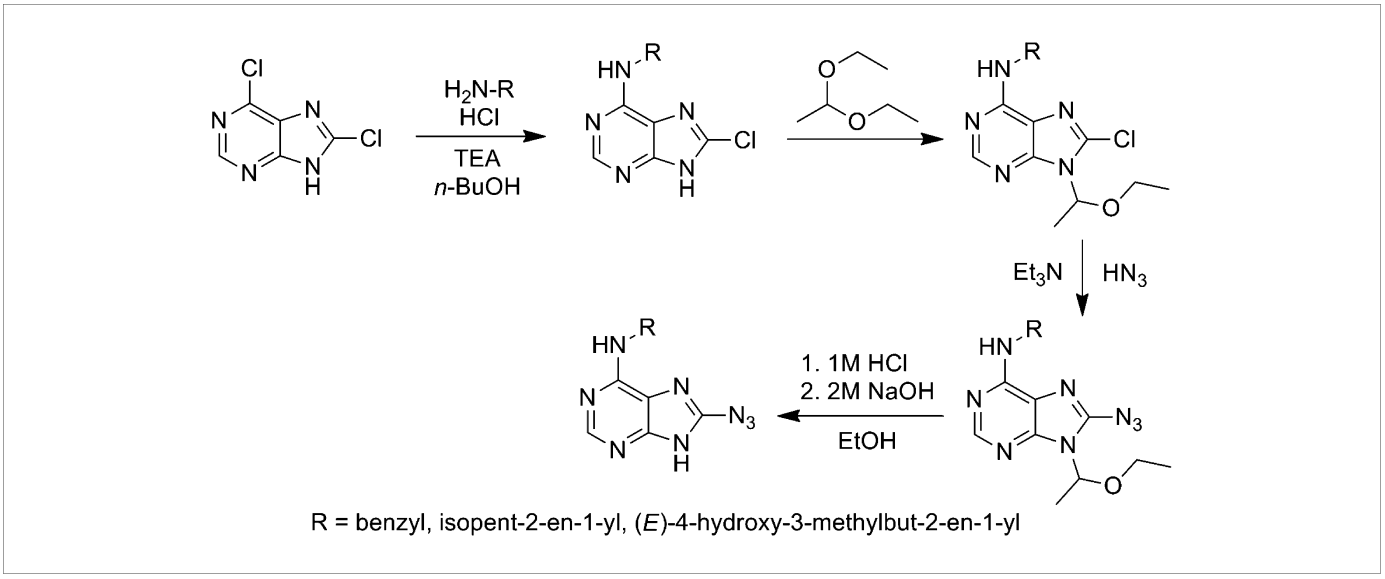
Cyclization of 4,5-diamino-6-furfurylaminopyrimidine with butyric or valeric anhydride.

spectrometry revealed that the 8-azido group transforms into NH_2, NHOH, $NHOC_2H_5$ or NHC_2H_5. 8-Nonafluorobutyl-BAP and 8-nonafluorobutyl-KIN were prepared to investigate application of the electrochemical monomolecular aromatic nucleophile substitution ($S_{RN}1$) methodology for synthesizing F-alkylated CK and auxin derivatives. 8-Nonafluorobutyl-BAP was tested and showed low CK activity [108]. After years of neglect, a library of new BAP derivatives possessing simple groups at the C8 position was synthesized as a part of PhD study by Moravec [109]. The author studied the synthetic availability of 8-halogenated CKs and the reactivity of halogens in the C8 position toward nucleophiles. 8-Substituted 9-(β-D-ribofuranosides) of BAP containing bromo, chloro, dimethylamino, amino, methoxy, sulfanyl, methylsulfanyl and hydroxy groups, 8-amino-9-(β-D-ribofuranoside)iP and 8-amino-9-(β-D-ribofuranoside)kinetin were subjected to the tobacco callus bioassay. Except for 8-amino CK ribosides and 8-methylsulfanyl-BAP, the 8-substituted CK ribosides were tested at concentrations of 0.01–1 μM and found to be less active in the tobacco bioassay than reference BAP. However, their activity increased with higher concentrations, in contrast to BAP, which inhibits plant cell growth at concentrations >10 μM. Some of the substances and 8-chloro-BAP were also tested in Amaranthus and senescence bioassays. Similar or higher activity compared to BAP was observed, especially by 8-amino, 8-methoxy and 8-bromo derivatives. Recently, a large group



SCHEME 4

Synthesis of 8-azido-N^6-benzyladenine from 8-bromoadenosine.



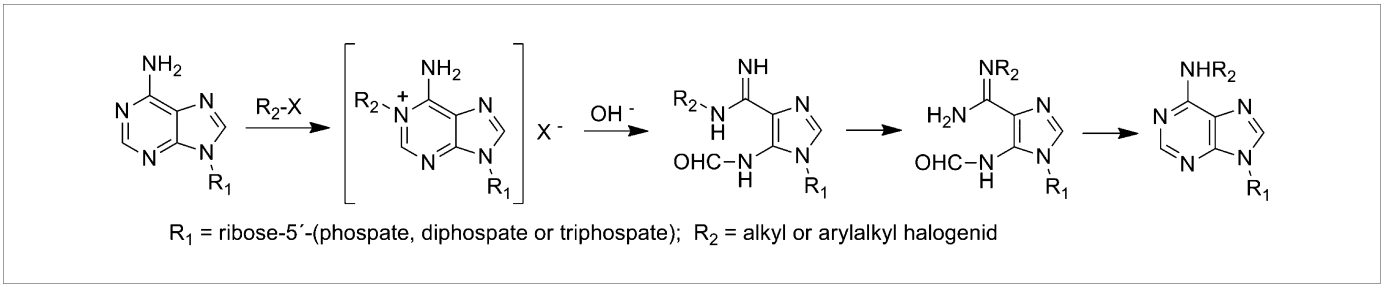
SCHEME 5

Synthesis of 8-azido-cytokinines from 6,8-dichloropurine.

of 6,8-disubstituted purines have been claimed in a US patent [110]. The invention provided purines with an alkyl or arylalkyl group at the N^6-position of the adenine moiety and a variety of substituents at the C8 atom, such as hydroxyl, amino, methyl, acyloxy, amido, carbamoyl, carboxyl, alkoxycarbonyl, cyano, hydrazino, hydroxylamino, aminoalkylamino and hydroxyalkylamino. The most preferred substance was 8-aminokinetin. 6,8-Disubstituted purines were prepared using two methods. The first method involved reacting 6,8-dihalogen-9-(2′,3′,5′-tri-O-acetyl-β-D-ribofuranosyl)purines with an nucleophile to afford a mixture of 6,9-disubstituted-8-halogen purines and 8,9-disubstituted-6-halogenpurines subsequently separated by column chromatography. After hydrolysis, the desired isomer gave the final compound or was subjected to further substitution. The second method was based on direct halogenation of 6,9-disubstituted purines at the C8-position. The resulting 6,8-disubstituted purines showed a wide range of biological activities and were able to interact with cytokinin receptors, making them ideal candidates as growth regulators in tissue cultures. They also displayed anti-inflammatory properties and were also able to inhibit cell senescence and aging in mammals or plants with improved selectivity and efficiency and lower toxicities than analogues known so far [110].

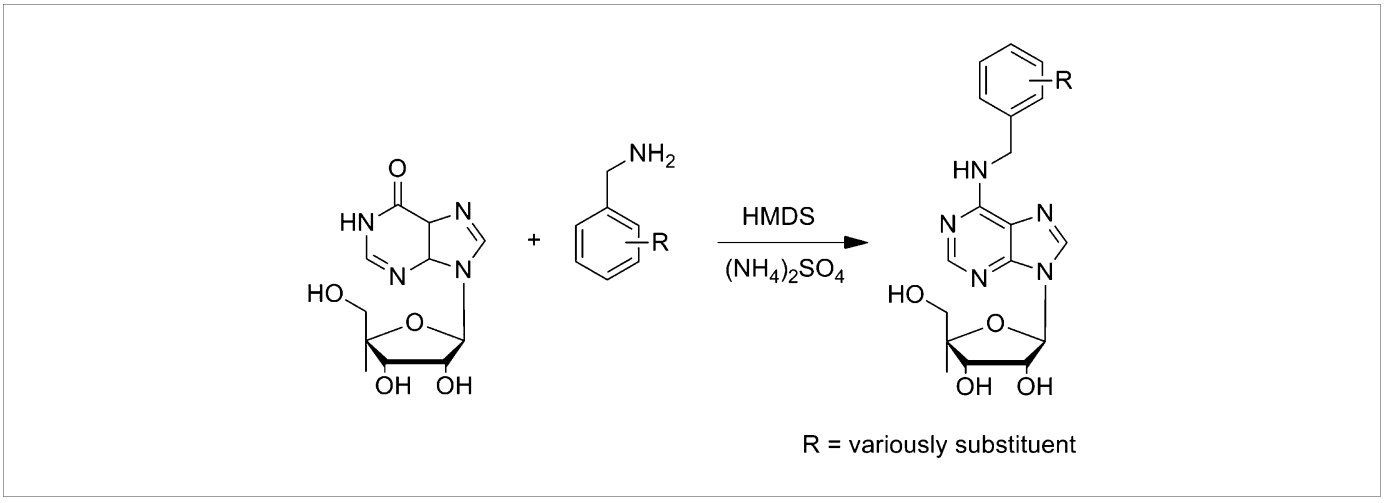
9-Derivatives of N^6-benzyladenines

ARCKs in plant tissue naturally occur as 9-derivatives in the form of nucleotides, nucleosides and other sugar conjugates, as well as L-alanine conjugates. Although glucosylation of CKs can occur at other positions in the purine moiety, 9-glucosides showed very low activity in bioassays, suggesting that they very probably represent an inactive storage form of CKs in plants because of the irreversible nature of 9-glucosylation [93,94,111,112]. The first natural aromatic 9-ribosides were identified in mature poplar leaves by Horgan and his co-workers in the early 1980s [113,114]. Whereas 9-substituted BAPs have for a long time been considered as less active (ribosides) or inactive (glucosides) cytokinins, several publications have suggested that some derivatives (especially ribosides) may possess significant plant beneficial regulating properties [115–119]. The first approach to prepare these derivatives involved reacting benzylamine with 6-chloropurine riboside in butanol in the presence of triethylamine [120]. Ribose was introduced as a protecting group at the N9-position of adenine. However, the reaction took a long time and produced low yields. In 1975, a new method was introduced based on N1 atom alkylation of an adenosine derivative and subsequent Dimroth rearrangement (Scheme 6) [121]. The reaction generated the



SCHEME 6

N1 atom alkylation of adenosine derivatives and subsequent Dimroth rearrangement to form the 6-isomer of the appropriate riboside.



SCHEME 7

Reaction of inosine and benzylamine in HMDS.

corresponding 6-isomer and offered advantages of easy availability of the starting materials and better yield. The method required the presence of a sugar and/or sugar phosphate at the N9 atom of purine and consisted of several steps. The first step was the alkylation of adenosine or its 9-nucleotide at the N1-position by the corresponding alkyl, alkenyl or arylalkyl halogenide, followed by Dimroth rearrangement in alkaline media to the appropriate 6-derivative. As the reaction was rather complicated and gave rise to a lot of impurities, the final product had to be purified by column chromatography. Nevertheless, the method was successfully used for the preparation of, for example, N^6-benzyl-cAMP [121]. A few years later, a different method with higher yield was implemented for ribosides (Scheme 7) [122]. The reaction was based on the amination of inosine in hexamethylendisilazane (HMDS). Inosine and an appropriately substituted benzylamine were heated in HMDS and ammonium sulfate at 145°C for 20 h. The final benzyladenosine derivative was purified by cellulose column chromatography and the yields were approx. 70%. Generally, the 9-alkylation of purines usually resulted in the formation of regioisomeric mixtures of 7- and 9-alkylpurines, although the 9-isomer was usually the major product [25]. This problem was solved by making improvements to the synthesis [123]. The synthesis of BAP ribosides was also improved through the use of BOP and DIPEA catalysts [124]. Ding and co-workers performed the 9-arylation of purines using boronic acid/cupric acetate/NEt_3 in dichloromethane [125], whereas Bakkestuen described the regioselective 9-arylation of purines using arylboronic acids in the presence of Cu(II) and organic base [126]. In 1991, Mitsunobu alkylation was introduced for electron-rich purine moiety 9-substitutions [127]. This method was used to synthesize derivatives from 6-chloropurine and appropriate alcohols [128–131]. Selected ARCKs ribosides prepared using the improved Leonard method from 1969 were shown to display anticancer activity both *in vitro* and *in vivo* [132]: some derivatives induced apoptosis and/or blocked the cell cycle of human cancer cells. The cytotoxic activity was dependent on the structure of the derivative and mostly differed from the activity displayed in plant bioassays. Several of the derivatives also showed high activity in three classical CK bioassays: 6-benzyladenosines showed very high CK activity in Amaranthus, senescence and tobacco callus bioassays [132]. Ribosides and ribotides are generally considered as transport forms that are very quickly metabolised into more active free bases [133]. Studies have shown that the majority of ARCK N9- derivatives are not able to activate CK receptors but induce a biological response [19,42,133]. The preparation of N9-substituted ARCKs for use in plant tissue culture is therefore slightly controversial because substitution at the N9-position very often, but not always, decreases the CK activity in bioassays [27]. By contrast, it was recently shown [134] that use cytokinin in nucleoside form in the micropropagation media can partially protect the purine moiety from *in vivo* formation of 9-glucosides. These metabolites are neither active nor reversibly sequestrated to active forms and they are known to have a detrimental effect in plant tissue culture [134]. The compound 6-benzylamino-9-(tetrahydropyran-2-yl)purine has been also reported to be a better plant growth regulator in comparison with BAP [135]. Subsequently, number of benzyl ring substituted tetrahydropyran-2-yl and tetrahydrofuran-2-yl derivatives were prepared by Szüčová *et al. via* reaction of an appropriately ring substituted BAP with 2,3-dihydropyran catalyzed by trifluoroacetic acid [27]. All the derivatives were either non-toxic or had only marginal cytotoxicity and possessed very high CK activity [27]. Generally, CKs have a negative effect on root growth and development. It was found that two of the prepared tetrahydropyran-2-yl derivatives prevented the root growth inhibition that limits CK application *in vivo* culturing [17]. Moreover, the tested compounds showed enhanced acropetal transport [136]. It has been suggested that the increased CK and especially antisenescent biological activity of these derivatives could stem from protection of the N9-position against glucosylation and slow release of the active free base from this conjugated form, thereby avoiding the stress generated by the induction of CK degradation mechanisms and decreasing the endogenous biosynthesis of isoprenoid CK [19,27,136]. Tetrahydropyran-2-yl was originally used as a protective group, but it was shown that the prepared intermediates were also active plant growth regulators. The metabolism of 9-tetrahydropyran-2-yl-BAPs has been studied in detail and another reason

for their enhanced anti-senescent activity proposed, that is, differences in their catabolism [27,137]. The increased biological activity accompanying 9-tetrahydropyranylation is probably also due to increased resistance to enzymatic degradation by cytokinin oxidase/dehydrogenase (CKX), thus prolonging the derivatives' persistence *in vivo* [27]. These second generation cytokinins were successfully used to replace BAP in several micropropagation systems, for example of *Merwilla plumbea* and [138,139], 'Williams' bananas [140]. Subsequent quantification of endogenous cytokinins confirmed decreased levels of N9-glucosides, which are regarded as terminal products of their irreversible deactivation or a detoxification pathway, not only for ARCKs, but also for their naturally occurring isoprenoid counterparts. Moreover, it was shown that these metabolic differences are stable also in greenhouse-acclimatized plants [138], even several months after plant transfer to *ex vitro* conditions [139]. In 2011, several N9-substituted derivatives of KIN were prepared by alkylation of 6-chloropurine intermediates in an alkaline environment using an appropriate alkylation agent [42]. Although a small amount of the N7-isomer was also formed, the 9-derivatives could be purified by *VersaFlash* chromatography. Mitsunobu alkylation according to Fletcher *et al.* was also used to obtain several derivatives [141]. The prepared derivatives were found to be active CKs and were significantly active toward a senescence bioassay. The most active prepared compounds were 9-halogenoethyl kinetin derivatives, mainly 6-furfurylamino-9-(2-chloroethyl)purine, which was shown to significantly protect membrane lipids against reactive oxygen species. Moreover, these compounds caused no inhibition effect on root growth in Arabidopsis and maize seedlings [42].

In conclusion, a broad spectrum of new ARCK derivatives has been prepared not only by substitution in the benzyl ring but also in the purine moiety, especially at the C2, C8 and N9-positions. Such compounds have shown promising properties for applications in plant tissue culture and biotechnology [9,16].

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Biochemistry

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Summary of the Doctoral Thesis

Heterocyclic derivatives of natural compounds with antisenescence properties

P1416 Biochemistry

Supervisor

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This PhD. thesis was realized in the Department of Chemical Biology and Genetics, Centre of the Region Haná for Biotechnological and Agricultural Research & Department of Chemical Biology, Faculty of Science, Palacký University in Olomouc within the framework of internal Ph.D. studies, guaranteed by the Department of Biochemistry, Faculty of Science, Palacký University in Olomouc, in period 2012-2021.

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The PhD. thesis and expert reviews will be available 14 days before the defence in the Study Department of Faculty of Science (Mgr. Karásková), Palacký University, 17. listopadu 12, Olomouc.

After the defense, the Ph.D. thesis will be stored in the library of the Biochemistry Departments of Faculty of Science, Palacký University, Šlechtitelů 27, Olomouc – Holice.

Content

Introduction

To date, a wide range of naturally occurring heterocyclic compounds have been identified and synthesized and their biological properties have been extensively studied. Cytokinins (CKs) are an important group of phytohormones which regulate various aspects of plant growth and development. Depends on the crosstalk with other hormones, they promote cell division and differentiation, chloroplast development, lateral shoot formation, seed germination etc. (Davies, 2010). In addition, the exogenous application of CK can delay plant senescence but the details remain still unresolved (Zwack and Rashotte, 2013).

Naturally occurring CKs are heterocycles, substituted at the C6 position of the purine moiety with the aromatic or the isoprenoid side chain (Mok and Mok, 2001). In planta, CKs exist as free bases but also as sugar conjugates represented by ribosides, ribotides or glucosides (Kurakawa *et al.*, 2007). Multiple hormonal interactions combined with other signalling networks lead to a pleotropic effect in plants (Koprna *et al.*, 2016).

Aromatic CKs are relatively rare in the plant kingdom, unlike isoprenoid CKs, and have long been considered as unnatural (Strnad, 1997). Compared to isoprenoid CKs, aromatic CKs show greater *in vivo* stability due to their significant resistance to CK degradation through oxidative side-chain removal mediated by CK oxygenase/dehydrogenase (CKX); (Amoo *et al.*, 2014). Benzylaminopurine (BAP) is widely used aromatic CK in plant biotechnology. However, its application is associated with several disadvantages, such as early necrosis of explants, root inhibition and subsequent problematic acclimatization of micropropagated plants (Plíhalová *et al.*, 2016). Many authors explain this negative effect on root development by accumulation of the inactive metabolite *N*9-glucoside or activating ethylene biosynthesis. (Podlešáková *et al.*, 2012).

Since the discovery of first CK in 1955, various CK derivatives have been isolated from natural resources or synthetized in the laboratory. The substitution at the *N*9 position of the purine influences the CK activity and many prepared aromatic CK *N*9-analogues showed significant biological activity, especially slowing chlorophyll degradation (Bryksová; *et al.*, 2020; Vylíčilová *et al.*, 2020; Doležal *et al.*, 2007). In addition, the *N*9 substituent can be slowly released to form active CK, so that artificial CK *N*9-analogues can serve as a CK reservoir (Podlešáková *et al.*, 2012).

Objectives

The biological activity of CKs depends on their chemical structure, and many CK derivatives have been prepared to date. Novel CK purine analogues substituted at the C2 position by chlorine and at the *N*9 position of the purine moiety by glycopyranose or glycofuranose derived from canonical aromatic CKs were synthetized. The biological activity of prepared compounds was studied in various CK bioassays.

In the presented Ph.D. thesis, the following research goals were set:

- Summary of knowledge about cytokinin and their antisenescence properties and search for new effective structural motifs.

- Synthesis of purine derivatives and other cytokinin analogues with significant biological properties.

- Introduction of a continuous flow reactor and a microwave reactor into the synthesis of purine derivatives.

- Characterization of prepared derivatives using available physico-chemical methods.

- Biological evaluation of prepared cytokinin derivatives (antioxidant capacity, antisenescence activity, cytotoxicity, cytokinin receptor assays and others).

Material and methods

General synthesis procedures

Reactions were monitored by thin-layer chromatography (TLC) on Silica Gel 60 F_{254} aluminium plates (Merck, US) using $CHCl_3$/MeOH (9:1 or 4:1 v/v) as a mobile phase. The purification was performed with column liquid chromatography on Davisil LC60A 40-63 micron silica gel (Grace Davison Discovery Sciences, UK) using a $CHCl_3$/MeOH with MeOH gradient, PE/EtOAc or Et_2O as s mobile phase. The solubility of compounds (0.1 mM) in H_2O were verified by turbidimetry (TurbiCheck WL).

Preparative HPLC-MS chromatography Agilent 1290 Infinity II coupled to UV-VIS detector with the mass detector Agilent InfinityLab LC/MSD was used for the separation of the final products. Dissolves samples (10 mg/1 mL in MeOH) were injected into the reverse phase column (Agilent 5Prep-C18 10x21.2 mm). The flow rate of a mobile phase (H_2O, A/ACN, B) was 20 mL/min and following linear gradients: 0 min (70% A; 30%B); 5 min (30% A; 70% B); 6 min (30% A, 70% B); 7 min (70% A, 30%B) were used.

Chromatographic purity of the prepared compounds was determined using a HPLC-PDA-MS assembly. An Alliance 2695 separation module (Waters, UK) and *Q-Tof micro* mass spectrometer (Waters, UK) with an electrospray was used. The samples (10 μL of 3.10^{-5} M in 1% MeOH) were injected onto the reverse-phased column (Symmetry C18, 5 μm, 150×2.1 mm; Waters, USA) and incubated at 25°C. The separation was performed using a binary gradient (0 '- 90% A; 25' - 10% A; 35 '- 10% A; 36' - 90% A; 45'- 10% A) and the flow rate was set to 0.25 mL/min. The solvent A consisted of 15 mM formic acid adjusted to pH 4.0 by ammonium hydroxide and MeOH was used as the solvent B. The flow rate was set to 200 μL/min. The effluent was introduced into the diode array UV-VIS detector (PDA 2996, Waters, UK) scan in a range 210-400 nm and 1.2 nm resolution. The detector was equipped with an electrospray source heated to 120 °C. The capillary voltage was set to +3 kV, cone voltage to +20 V and desolvation temperature to 300 °C. Nitrogen was used as the desolvation gas (500 l/h) and cone gas (50 l/h). The mass spectrometer was operated in the positive ion detection mode (ESI+) in the mass range: 50-1000 *m/z*.

Nuclear magnetic resonance (NMR) spectra were obtained on ECA-500 spectrometer (Jeol, Japan) operating at frequency of 500 MHz (^{1}H) and 125 MHz (^{13}C). The samples were dissolved in DMSO-d_6 and tetramethylsilane was used as an internal standard.

The microwave experiments were performed in the Discover SP microwave reactor (CEM, US). The continuous flow reaction was performed on the X-cube instrument (ThalesNano, Hungary)

High-resolution mass spectrometry (HRMS) equipped with Agilent 1290 Infinity II liquid chromatography (Agilent, USA) with the UV-VIS detector followed by Agilent 6230 MS-TOF (Agilent, USA) was performed to confirm the molecular formula. Dissolved samples in MeOH (1 mg/mL) was diluted with the initial mobile phase and 20 μL of applied samples was injected onto the column (Agilent 5Prep-C18 10x21.2 mm). The separation was performed using a linear gradient (0 '- 30% ACN; 5'-70% ACN) and the flow rate was set to 0.5 mL/min. The solvent consisted of 15 mM formic acid (50% ACN) and the split ratio was set to 5000:1. The analytes were introduced into the ion source includes Dual AJS ESI and scanning in a range of 100-1700 *m/z* (gas temperature 260°C, capillary voltage 3500V, nozzle voltage 300V).

General bioassays procedures

Standard cytokinin assays

Two standard CK bioassays were used to determined CK activity. The tobacco callus bioassay is based on the stimulation growth of the CK-dependent tobacco callus cells (*Nicotiana tabacum* L. cv. Wisconsin 38) The wheat leaf senescence assay (WLSA) is based on the chlorophyll degradation delay in excised wheat leaves (*Triticum aestivum* L. cv. Aranka) in the dark condition. WLSA were performed according to the literature (Holub *et al.*, 1998). The protocols are described in detail and available on the Aesculab webpage (aesculab.upol.cz).

In all cases, BAP was used as a positive control and test compounds were dissolved in DMSO. The final concentration of DMSO in the medium did not exceed 0.2%. The results were determinate as the strongest biological response and the relative activity at this concentration of each compound was calculated.

Chlorophyll retention in the receptor mutants

Approximately the seventh leaf of the 24-day-old seedling was detached and floated on distilled H_2O supplemented with 0.1 μM BAP or test compounds in six-well plates for 10 days in the dark. Alternatively, the basal part of the detached leaf was immersed in a well containing test compound (150 μL/well) and cultured in dark for 5 days. Each genotype was measured in triplicate and samples were weighed into 100 mg of fresh mass. Chlorophyll was

extracted with MeOH/ for 24 hours in the dark or heated with 80% EtOH at 80°C for 10 min. The light absorption at 665 nm was determined spectrophotometrically (Synergy H4 Hybrid Multi-Mode Microplate Reader, Biotek, USA) and normalized to fresh weight. The chlorophyll content at the beginning of the experiments was taken as a reference.

Turbidimetric solubility assay

The solubility of the prepared compounds in aqueous solution with a final concertation of 0.1 mM was measured by turbiditimetry (TurbiCheck WL).

Arabidopsis ARR5::GUS reporter gene assay

This assay was carried out according to the protocol (Romanov *et al.*, 2002) with little modification. After the stratification, transgenic *Arabidopsis ARR5::GUS* seeds were grown in six-well plates on an orbital shaker in a growth chamber (21°C) under long-day conditions (16 h light/8 h dark) for 3 days. The seedlings were added to the liquid growth solution contained test compound, a standard (BAP) or DMSO (solvent, final conc. 0.05%) and cultured for additional 16 hours. The specific activity of *β*-glucuronidase in the plant extract was spectrophotometric measured using a Fluoroscan Ascent microplate reader (Labsysems, Helsinki, Finland) at excitation/emission wavelengths of 365/450 nm.

Oxygen radical absorbance capacity (ORAC) assay

The oxygen radical absorbance capacity (ORAC) was determined as described before (Ou *et al.*, 2001). In short, 100 μL of a 500 mM fluorescein stock solution (2,7-dichlorofluorescein) and solutions (10 mM) of test compounds were diluted in phosphate buffer and then pipetted into each 96-well microplate. The reaction was initiated by the addition 25 μL of 250 mM 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), the microplate was shaken for 5s and the fluorescence was monitored kinetically with data taken every 3 min over 90 min. Excitation and emission were performed at 485 nm and 510 nm using Infinite M200 Pro (Tecan, Switzerland). The net area under the curve was used to calculate the antioxidant capacity expressed as Trolox equivalents (TE). This assay was performed by the team of Mgr. Jiří Grúz, Ph.D. in the Laboratory of Growth Regulators.

Bacterial receptor bioassay

All receptors bioassays used transformed *Escherichia coli* KMI001 strains harbouring the plasmids pIN-III-AHK4, pSTV28-AHK3 which express the CRE1/AHK4 and AHK3 receptor. Bacterial CK assays were carried out as previous described procedure (Spíchal *et al.*, 2004) and the protocol is available on the Aesculab website (aesculab.upol.cz).

Competitive binding assay

The assay was performed according to the published protocol (Nisler *et al.*, 2010) and the experimental procedure is also described on the Aesculab website (aesculab.upol.cz). The same bacterial strains were used as described above. The competition reaction of test compounds was performed with ^{3}H *t*Z at 2 nM and incubated for 30 min. at 4°C. This assay was kindly made by Mgr. Zuzana Pěkná in the Department of Chemical Biology and Genetics, Centre of Region Haná for Biotechnological and Agricultural Research.

Root assay

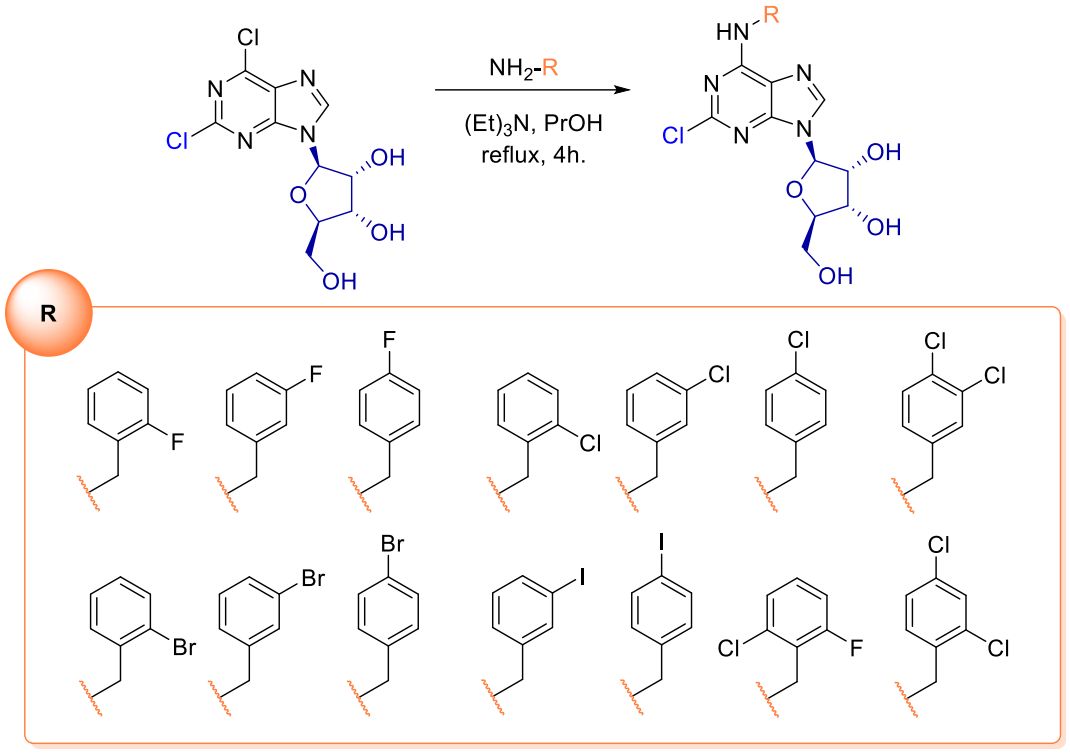
The root test was performed on *A. thaliana* seedlings of the Columbia wild type (Col-0). The test compounds at the final concentration of 0.1 μM in AM^{+} medium were poured into 12×12 cm square Petri dishes. The corresponding free bases and BAP were used as positive controls and DMSO as the negative control. *A. thaliana* sterile seeds were applied to the culture plates in a row of approx. ten pieces and four dishes were prepared for each substance. After the stratification, seeds were grown on vertically oriented plates in a growth chamber under a photoperiod of 16 h light/8 h dark at 18°C. The seedlings were scanned using a horizontal scanner and the root length was measured with ImageJ software.

Cytotoxicity

In vitro toxicity of the prepared compounds was screened on selected non-tumor human cell lines: HaCaT (immortalized human keratinocyte), BJ (skin fibroblasts) and ARPE (immortalized retinal epithelial cells). The effect of test compounds was measured at 6 concentrations (max. conc. 100 μmol/l) 72 hours after the addition of test compound into the medium. DMSO was used as the negative control. The measurement of fluorescence was performed at 544 nm and 590 nm using Resazurin (Tecan) reduction assay. The cytotoxicity of test compounds was kindly performed by Mgr. Jiří Voller, Ph.D. and his team in the Laboratory of Growth Regulators.

Survey of results

In the first part of thesis, a group of aromatic 2-chloro-6-(halogenbenzylamino)purine-9-ribofuranosides (Scheme 1) was synthesized and properly characterized by NMR (^{13}C, ^{1}H), HPLC purity and elemental analysis. Their biological activity was studied in three standard CK bioassays such as tobacco callus, WLSA and *Amaranthus* bioassay. The prepared artificial ArCK analogues generally showed similar biological activity as the standard (BAP) in tobacco and *Amaranthus* bioassays. In contrast, they significantly delayed the degradation of chlorophyll in detached wheat leaves and in several cases even reached almost double activity of BAP. Compounds bearing a halogen in the *meta* or *para* position of the benzyl ring were determined as the most active.



Scheme 1. *The synthesis and chemical structures of 2-chloro-6-(halogenbenzylamino)purine-9-ribofuranosides.*

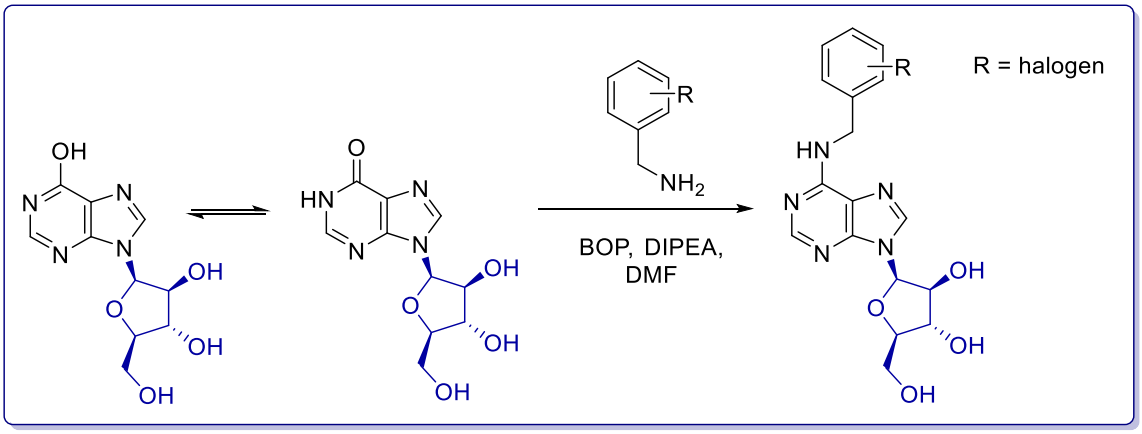
Moreover, all the prepared compounds were tested in the bacterial CK receptor assay performed both monocot and dicot members of the CK receptor family, specifically for the ZmHK1 and ZmHK3 receptors of *Zea mays* and for the AHK3 and CRE1/AHK4 receptors of *Arabidopsis*. Although the prepared compounds did not trigger the CK signalling pathway trough *Arabidopsis* receptors, some compounds specifically activated the ZmHK1 receptor

and were also found to be able to activate the CK pathway comparable as BAP in *ARR5::GUS* reporter gene assay using transgenic seedlings of *Arabidopsis* plants.

Methods of molecular biology and biophysics have further revealed a possible mechanism of action. DNA microarray analysis showed that two selected derivatives induced the expression of several genes associated with photosystem II reaction center (PSII), the light collection system (LHCII) and the oxygen-releasing complex (OEC). By determining chlorophyll fluorescence, it was found that both selected derivatives applied on detached *A. thaliana* leaves delayed senescence by keeping chlorophyll a/b and carotenoid levels active. In addition, both compounds increased the relative amount of LHCII, even at higher concentrations under intense illumination thereby protecting photosystem II activity even more effectively than BAP during senescence. These findings were published in research paper (Vylíčilová *et al.*, 2016).

Another aim of the work was the implementation of the continuous flow reactor and the microwave reactor to optimize the reaction condition for the synthesis of 2,6-disubstituted purine ribosides. Compared to conventional reactions placed in round-bottom flask, the microwave reactor significantly reduced the reaction time. Despite all efforts, the involvement of the continuous flow reactor X-cube was not successful, mainly due to poorer solubility of starting material and product precipitation during the reaction, which blocked the system of dual HPLC pumps in the instrument.

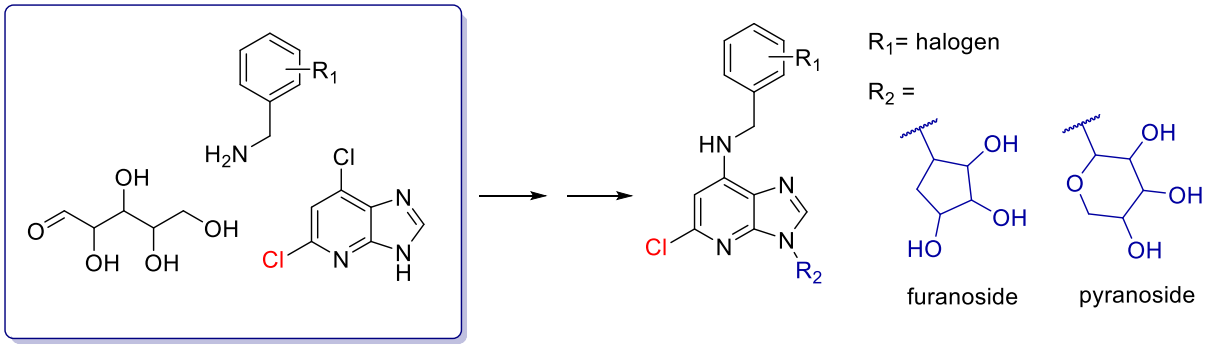
Aromatic 6-substituted purine-9-*β*-D-arabinofuranosides were prepared by a one-step synthesis from hypoxanthine 9-*β*-D-arabinofuranoside using the microwave reactor (Scheme 2). The prepared CK 9-*β*-D-arabinofuranosides showed interesting biological activity, especially high antisenescence properties, and are therefore protected by the patent (Patent, Doležal *et al.*, 2014).



Scheme 2. *The synthesis of 6-substituted purine-9-β-D-arabinofuranosides from hypoxanthine 9-β-D-arabinofuranoside that is in equilibrium mixture.*

In the second part, a new group of aromatic *N*9-glycosides substituted at the C2 position with chlorine and at the *N*9 position of the purine with various glyfuranoses and glycopyranoses was prepared. With respect to naturally occurring glycosides, we selected D/L-arabinose, D-xylose, and D-ribose to determine the structure activity relationship.

The synthesis of 2-chloro-6-substituted purine-9-glycosides (Scheme 3) proceeded in several reaction steps initiated by cyclization and acetylation of monosaccharides, followed by *N*9-glycosylation further nucleophilic substitution of the C6 attached chlorine by appropriate amine and final deprotection of hydroxyl groups. Various synthetic procedures were tried, each reaction step was optimized, individual pyranoses/furanoses and α/β anomers were separated from each other. The structure of prepared CK glycosides were characterized by available physico-chemical methods (HPLC/MS, NMR, TLC and HRMS). Moreover, *N*9-glycosilation of 2-chloro-6-substituted purines significantly increased aqueous solubility, as verified by turbidimetry.



Scheme 3. *General scheme for the synthesis of aromatic 2-chloro-6-substituted purine-9-glycosides.*

These aromatic 2-chloro-6-substituted purine-9-glycosides have been subsequently tested for biological activity in standard CK bioassays such as detached wheat leaves senescence assay (WLSA) and tobacco callus assay. Most of the prepared compounds delayed the degradation of chlorophyll in the dark condition compared to BAP. We modified dark-induced senescence assay using the *ahk3ahk4* mutants expressing only the AHK2 receptor and measured chlorophyll retention after the application of selected compounds in detached leaves of the *ahk3ahk4* mutants and the *Arabidopsis* wild type (Col-0). Further we screened compounds for the activation of the CK signalling pathway using *Arabidopsis ARR5::GUS* reporter gene test, CK bacterial receptor assays and competitive ligand binding assay for the CRE1/AHK4 and AHK3 receptor.

Test aromatic 2-chloro-6-substituted purine-9-glycosides clearly showed a positive effect on *Arabidopsis* root development which contrasted with BAP that strongly inhibits root

elongation. The antioxidant capacity of the prepared compounds was also studied, which significantly depends on the chemical structure of the group substituted at the C6 position of the purine.

Another aim of the dissertation was to summarize the existing knowledge about aromatic CKs in the form of reviews. The first review is focused on *N*9-substituted CKs derivatives both sugar and non-sugar conjugates their natural occurrence, historical progress, synthesis, biological activity mainly their potential application in various plant biotechnology, tissue culture and agriculture. This review provides a summary of CK structural motifs capable of increasing biological activity in various CK bioassays (Vylíčilová *et al.*, 2020)

The second review presents the current overview of aromatic CKs derivatives, their synthesis and various substitution possibilities on the purine ring and their promising application in practice. The role of monosubstituted and disubstituted CK derivatives with exhibited CK properties and their simply preparation is described here (Plíhalová *et al.*, 2016).

List of published papers and other contributions

Papers in journals with impact factor

Plíhalová, L., Vylíčilová, H.**,** Doležal, K., Zahajská, L., Zatloukal, M., Strnad, M. (2016): Synthesis of aromatic cytokinins for plant biotechnology, *New Biotechnology*, **33**(5), 614–624, doi: 10.1016/j.nbt.2015.11.009.

Vylíčilová, H., Husičková, A., Spíchal, L., Srovnal, J., Doležal, K., Plíhal, O., Plíhalová, L. (2016): C2-substituted aromatic cytokinin sugar conjugates delay the onset of senescence by maintaining the activity of the photosynthetic apparatus, *Phytochemistry*, **122**, 22–33, doi: 10.1016/j.phytochem.2015.12.001.

Vylíčilová, H.; Bryksová, M.; Matušková, V.; Doležal, K.; Plíhalová, L.; Strnad, M. (2020): Naturally occurring and artificial *N*9-cytokinin conjugates: from synthesis to biological activity and back. *Biomolecules*, **10**, 832, doi: 10.3390/biom10060832.

Granted patents

Doležal, K., Plíhalová, L., Vylíčilová, H., Zatloukal, M., Plíhal, O., Voller, J., Strnad, M., Bryksová, M., Vostálová, J., Rajnochová Svobodová, A., Ulrichová, J., Spíchal, L. (2014): *6-aryl-*9*-glycosylpurines and use thereof* (Palacký University in Olomouc), CZ 2014875, EP 3229772, SG 11201704019TA and US 10550144 patents.

Conference contributions

2012 Bucharest (Romania), Chemistry and target identification of natural products, poster presentation: 2,6-disubstituted aromatic cytokinin ribosides with anti-senescent properties.

2013 Paris (France), Biologically Relevant Molecular Diversity, poster presentation: 2,6-disubstituted aromatic cytokinin ribosides with anti-senescent properties.

2013 Versailles (France), 6th European Workshop on Leaf Senescence, poster presentation: Synthesis and biological activity of novel aromatic cytokinin ribosides.

2013 Karlov pod Pradědem, Conference of Chemical Biology and Genetics, oral presentation: Preparation of aromatic cytokinin derivatives with antisenescence activity.

2014 Kouty nad Desnou, Conference of Chemical Biology, oral presentation: Synthesis and biological activity of novel aromatic cytokinin ribosides.

2014 Prague, Auxins and Cytokinins in Plant Development, poster presentation: Synthesis and biological activity of novel aromatic cytokinin ribosides.

2014 Olomouc, 12th PhD Student Conference of Plant Experimental Biology, oral presentation: Synthesis and biological activity of novel aromatic cytokinin ribosides.

2015 Velké Karlovice, Biotechnology of Phytohormones and Substances, oral presentation: Synthesis and biological activity of novel aromatic cytokinin ribosides.

Conclusion and perspective

The presented Ph.D. thesis focuses on purine-type CKs that are involved in various physiological processes in plants, such as leaf senescence, chloroplast, root and shoot development, cell division, biotic and abiotic stress repsonse, etc. (Davies, 2010). This work deals with the synthesis of new artificial CK analogues derived from purine *N*9-glycosides substituted at the C2 position by chlorine and with various C6 aromatic substituents.

In addition, innovative techniques such as the microwave and continuous flow reactors have been implemented to optimize reaction conditions. Aromatic purine 9-*β*-D-arabinofuranosides have been prepared and are covered by a patent protection due to their significant antisenescence activity.

The new 2-chloro-6-substituted purine-9-glycosides derived from arabinose, xylose, and ribose in the form of pyranoses and furanoses were *de novo* synthetized. The structure of new compounds was characterized by available physicochemical methods (TLC, NMR, HPLC/MS and HRMS). It was found that *N*9-glycosilation significantly increased the solubility of chlorinated CK derivatives in water.

The biological activity of the prepared compounds was determined using standard CK bioassays (detached wheat leaf senescence assay and tobacco callus assay). Most of the prepared derivatives showed antisenescence properties in wheat leaves in the dark condition. The selected 2,6-disubstituted purine-9-glycosides were subsequently tested for the activation of the CK signalling pathway in *A. thaliana*. Furthermore, prepared compounds positively promoted the primary root length and increased the number of lateral roots of *A. thaliana*. The antioxidant capacity of the prepared derivatives was also studied.

The presented aromatic 2,6-disubstituted purine-9-glycofuranosides/glycopyranosides provide a new effective CK motif that could contribute to the rational design of new growth regulators and to a better understanding of the CK mechanism of action in plants.

Souhrn (in Czech)

Název disertační práce: Heterocyklické deriváty přírodních látek s antisenescenční aktivitou

Předložená disertační práce se věnuje cytokininům purinového typu, které se účastní rozmanitých fyziologických procesů v rostlinách, jako je ovlivnění listové senescence, vývoje chloroplastů, kořene a prýtu, buněčného dělení, odpovědi na biotický a abiotický stres atd. (Davies, 2010). Tato práce se zabývá syntézou nových umělých cytokininových analogů odvozených od purin *N*9-glykosidů substituovaných v poloze C2 chlorem a různými aromatickými C6 substituenty.

U připravené série aromatických 2,6-disubstituovaných purin-9-ribosidů byla blíže stanovena jejich biologická aktivita a rovněž byla optimalizována jejich syntéza s využitím průtokového a mikrovlnného reaktoru. Pomocí jednokrokové syntézy byly připraveny aromatické purin 9-β-D-arabinofuranosidy, které se pro svou významnou cytokininovou aktivitu v senescenčním biotestu, jež může být uplatnitelná v praxi, staly předmětem ochrany vynálezu patentem. Pro jejich přípravu byly navrženy a vyzkoušeny i jiné syntetické postupy.

Dalším cílem práce byla *de novo* syntéza nových 2-chloro-6-substituovaných purin-*N*9-glykosidů odvozených od arabinózy, xylózy a ribózy ve formě pyranóz a furanóz s využitím různých syntetických postupů. Bylo zjištěno, že substituce cukrem v poloze *N*9 významně zvýšila rozpustnost chlorovaných cytokininových derivátů ve vodě. Nově připravené látky byly charakterizovány dostupnými fyzikálně-chemickými metodami (HPLC/MS, NMR, TLC a HRMS).

Nedílnou součástí práce bylo studium jejich biologické aktivity a mechanismu působení. Pro určení cytokininové aktivity byly připravené látky otestovány ve standartních cytokininových biotestech (senescenční a kalusová test). Většina připravených látek vykazovala antisenescenční účinky na ustřižených listech pšenice inkubovaných ve tmě.

U vybraných 2,6-disubtituovaných purin-9-glykosidů byla následně zkoumána v několika experimentech schopnost aktivace cytokininové signální dráhy. Navíc, připravené deriváty pozitivně podporovaly vývoj hlavního kořene a laterárních kořínků u semenáčků *A thaliana*. Rovněž byla stanovena jejich antioxidační kapacita. Prezentované aromatické 2,6-disubtituované purin-9-glykofuranosidy/glykopyranosidy poskytují nový účinný cytokininový motiv, který by mohl přispět k dalšímu návrhu nových růstových regulátorů a rovněž k lepšímu porozumění mechanismu cytokininového účinku v rostlinách.

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