Palacký University Olomouc

Faculty of Science

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Doctoral Thesis

Preparation and biological activity of the new cytokinin derivatives

P1527 Biology

1501V019 Experimental biology

Supervisor

Mgr. Karel Doležal, Dr., DSc.

Olomouc

2020

Bibliografická identifikace

Jméno a příjmení autora	Mgr. Magdaléna Bryksová
Název práce	Příprava a biologická aktivita nových cytokininových derivátů
Typ práce	Disertační
Pracoviště	Oddělení chemické biologie a genetiky, CRH
Vedoucí práce	Mgr. Karel Doležal, Dr., DSc.
Rok obhajoby práce	2020
Abstrakt	Předložená disertační práce se zabývá chemickou syntézou a biologickou aktivitou nově připravených cytokininových derivátů. Série 6-substituovaných purin-9-β-D-arabinofuranosidů byla připravena modifikovanou syntézou vycházející z hypoxanthin arabinosidu. Obdobným způsobem byla nasyntetizována i skupina 6-substituovaných purin-9-β-L-ribofuranosidů a 2′-deoxy-2′-fluoro-9-β-D-arabinofuranosidů. Všechny nově připravené deriváty byly charakterizovány pomocí fyzikálně-chemických metod, testovány v cytokininových biotestech a dále byla hodnocena jejich cytotoxicita jak proti nádorovým, tak i nenádorovým buněčným liniím. U dvou derivátů ze skupiny arabinofuranosidů byla prokázána schopnost podporovat rostlinnou imunitu a pozitivně ovlivňovat životnost listů. Rovněž jejich analoga s fluorovaným cukerným skeletem byly vyhodnoceny jako sloučeniny se schopností podporovat rostlinný růst za kontrolovaných podmínek a zároveň snižovat stres způsobený nadměrným zasolením či osmotickým tlakem.
Klíčová slova	cytokininy, biologická aktivita, senescence, BAP
Počet stran	85
Počet příloh	5
Jazyk	Anglický

Bibliographical identification

Author's first name and surname	Mgr. Magdaléna Bryksová
Title of thesis	Preparation and biological activity of the new cytokinin derivatives
Type of thesis	Doctoral
Department	Department of Chemical Biology and Genetics, CRH
Supervisor	Mgr. Karel Doležal, Dr., DSc.
The year of presentation	2020
Abstract	The presented Ph.D. thesis deals with the chemical synthesis and biological activity of newly prepared cytokinin derivatives. A series of 6-substituted purine-9-β-D-arabinofuranosides was prepared by a slightly modified synthesis, starting from hypoxanthine arabinoside. A group of 6-substituted purine-9-β-L-ribofuranosides and 2′-deoxy-2′-fluoro-9-β-D-arabinofuranosides were synthesized in a similar way. All newly prepared derivatives were characterized by physico-chemical methods and tested in cytokinin bioassays, their cytotoxicity against tumor and non-tumor cell lines was also evaluated. Two members of arabinosides family have been characterized to promote plant immunity and positively regulate leaf longevity. Also, their analogues with fluorinated carbohydrate moiety have been shown as hormopriming agents with ability to promote plant growth under controlled conditions and reduce salt and osmotic stress.
Keywords	cytokinins, biological activity, senescence, BAP
Number of pages	85
Number of appendices	5
Language	English

Declaration of Authorship

Hereby I declare that the presented Ph.D. thesis and the work is my original work except otherwise indicated.

In Olomouc, 5th October 2020

Magdaléna Bryksová

Acknowledgements

I would like to express my gratitude to my supervisor, Mgr. Karel Doležal, Dr., DSc. for his patience and effort invested in our collaboration, which provided me the best conditions for my research work and education. My second biggest thank belongs to prof. Nasri Nesnas from Florida Institute of Technology for opportunity to visit his laboratory and leadership during my research fellowship. I would like to also thank to RNDr. Marek Zatloukal, Ph.D., who gave me useful advices regarding the organic synthesis, to all collaborators who performed bioassays such as Jarmila Balonová and Mgr. Zuzana Pěkná for cytokinin bioassays, doc. RNDr. Vladimír Kryštof, Ph.D and his team, as well as Mgr. Jiří Voller, Ph.D. and his co-worker for cytotoxicity testing. My gratitude also belongs to all my colleagues from Department of Chemical Biology and Genetics, and Laboratory of Growth Regulators for creating friendly and supportive environment. Finally, I would like to thanks to everybody else, who support me during my doctoral study.

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List of abbreviations

ABC	ATP-binding cassette transporters
AHK	*Arabidopsis* histidine kinase
AHP	*Arabidopsis* histidine phototransfer protein
Ara-A	aminopurine-9-arabinoside, vidarabine
A-ARR	*Arabidopsis* response regulators type-A
B-ARR	*Arabidopsis* response regulators type-B
BAP	6-benzylaminopurine
BAP9G	6-benzylaminopurine-9-glucoside
BAP9RG	6-benzylaminopurine-9-riboside glucoside
BAPR	6-benzylaminopurine-9-β-D-riboside
β-L-BAPR	6-benzylaminopurine-9-β-L-riboside
BOP	benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate
BSA	bis(trimethylsilyl)acetamide
CK	cytokinin
CKX	cytokinin dehydrogenase
CRF6	cytokinin response factor
*c*Z	*cis*-zeatin, *(Z)*-6-(4-hydroxy-3-methylbut-2-enylamino)purine
*c*ZR	*cis*-zeatin-9-riboside
DAST	diethylaminosulfur trifluoride
DHZ	dihydrozeatin
DHZR	dihydrozeatin riboside
DIPEA	*N*,*N*-diisopropylethylamine
DMAPP	dimethylallyl pyrophosphate
DMF	dimethylformamide
DPU	*N*,*N*′-diphenylurea
ENT	equilibrative nucleoside transporter
ER	endoplasmatic reticulum

F-ara-A	9-(2′-deoxy-2′-fluoro-β-D-arabinosyl)adenine
F-ara-H	9-(2′-deoxy-2′-fluoro-β-D-arabinosyl)hypoxanthine
HMBDP	4-hydroxy-3-methyl-2-*(E)*-butenyl diphosphate
IAA	indole-3-acetic acid
iP	isopentenyladenine
iPR	isopentenyladenine-9-riboside
iPRMP	isopentenyladenine-9-riboside monophosphate
iPRDP	isopentenyladenine-9-riboside diphosphate
iPRTP	isopentenyladenine-9-riboside triphosphate
IPT	isopentenyl transferase
Kin	6-furfurylaminopurine
KinR	kinetin-9-riboside
MEP	methylerythritol phosphate pathway
MVA	mevalonate pathway
*m*T	*meta*-topolin, 6-(3-hydroxybenzylamino)purine
*m*TR	*meta*-topolin riboside,
*m*TROG	*meta*-topolin riboside-*O*-glucoside
*o*TR	*ortho*-topolin riboside, 6-(2-hydroxybenzylamino)purine riboside
*o*TROG	*ortho*-topolin riboside-*O*-glucoside
*p*TR	*para*-topolin riboside, 6-(4-hydroxybenzylamino)purine riboside
*p*TROG	*para*-topolin riboside-*O*-glucoside
PUP	purine permease
SAG	senescence-associated gene
SDG	senescence-downregulated gene
TDZ	thidiazuron, 1-phenyl-3- (1,2,3-thiadiazol-5-yl) urea
TMSOTf	trimethylsilyl trifluoromethanesulfonate
*t*Z	*trans*-zeatin, *(E)*-6-(4-hydroxy-3-methylbut-2-enylamino)purine
*t*ZR	*trans*-zeatin riboside
*t*ZOG	*trans*-zeatin-*O*-glucoside
*t*ZROG	*trans*-zeatin riboside-*O*-glucoside

Aims and scope

A wide spectrum of CK derivatives, structurally modified at adenine moiety, has been synthesized to this date and their biological activity has been evaluated in plants, as well as in mammalian cell lines. It was proved, that even small structural modification can completely change CK-like activity and therefore it is necessary to well-describe structure-activity relationship. In the presented thesis, the series of new CK derivatives was synthesized in order to modify their properties and reveal new active derivatives with potential practical application.

The aims of the presented doctoral thesis are:

- Synthesis of a wide range of aromatic N^6-substituted aminopurine-9-β-D-arabinofuranosides
- Synthesis of new N^6-substituted aminopurine-9-β-L-ribofuranosides
- Synthesis of N^6-substituted aminopurine-9-β-D-2′-deoxy-2′-fluoroarabinofuranosides
- Description of the structure-activity relationship
- Evaluation of biological activity in plants and animal cells

1. Introduction

Originally, the term *hormone* comes from the Greek language and it means *to stimulate*, as well as *to set in motion*. Firstly, this term was used in medicine about 100 years ago for a stimulatory factor with the ability to transport a chemical signal (Davies, 2010). In 1930´s, Went and Thimann defined plant hormones (phytohormones) very briefly, as substances which are able to migrate from one part of an organism to another (Went and Thimann, 1937). Nowadays, phytohormones are characterized as a group of naturally occurring, organic compounds with the ability to influence physiological processes at extremely low concentrations (Davies, 2010).

The first observation of phytohormone-related physiological processes is presumably dated between 1880 and 1893 and it was based on morphogenic and developmental correlations. Sachs postulated that *"Morphological differences between plant organs are due to differences in their material composition"*, which predicted the existence of root-forming, flower-forming and other substances, which are transported through the plant in different directions (Went and Thimann, 1937). Concurrently, Charles Darwin made the original observation of phototropism of oat coleoptiles and postulated the theory about a signal and its transport from the tips of the coleoptile to the lower regions of the plant. In 1934, the isolation of the first phytohormone from fresh plant material was published and the compound was identified as indol-3-acetic acid (IAA), which became known as auxin (Wildman, 1997).

Since the discovery of IAA, many endogenous signaling and regulatory molecules have been discovered, and more are to be discovered yet (Smith et al., 2017). The detailed investigation led to the discovery of the other hormones: research in plant pathogenesis led to gibberellins, efforts to culture tissues led to cytokinins, the control of abscission and dormancy led to abscisic acid and the effects of illuminating gas and smoke led to ethylene. Subsequently, the other compounds, such as brassinosteroids, jasmonates, salicylic acid, strigolactones, peptides and polyamines have been added to the group of phytohormones (El-Esawi, 2017).

The long story of cytokinin research started by the discovery of the first member of cytokinin group, followed by confirmation of their natural occurrence and clarification of their metabolism, biosynthesis pathway or signaling in plants. Nowadays, the cytokinin research is focused on a wide spectrum of applications, such as plant biotechnology and agriculture utilization, as well as pharmacology and cosmetic industry (Kamínek, 2015).

2. Cytokinins

2.1. Historical development and function

Purine bases play significant roles in many biological processes. These structurally modified bases, nucleotides and nucleosides, led to the discovery of several biologically active compounds. One group of them, naturally occurring N^6 and N9-substituted aminopurines are important members of the nucleoside family (Suhadolnik, 1979). Naturally occurring cytokinins (CKs) are included in this group, because their chemical structure is derived from adenine moiety (Davies, 2010).

CKs play an important role in various processes connected with plant growth and development, including promotion of cell division, regulation of apical dominance, chloroplast development, antisenescence effect or transmission of nutritional signals (Davies, 2010). As early as in 1913, the existence of chemical compounds that stimulate cell division was proved in potato parenchyma cells. It was suggested that this material causes induction of cell division localized in phloem (Haberlandt 1913). Lately, the same effect was observed in milk endosperm isolated from coconut and subsequently in many other plant species (Van Overbeek et al., 1941). The first CK was isolated by Miller and his co-workers from autoclaved herring sperm DNA in 1955 and it was named kinetin because of its ability to stimulate cytokinesis and its origin from degradation products of DNA was clarified immediately (Miller et al., 1956; Miller et al., 1955). Afterwards, several other growth-promoting factors similar to kinetin have been identified from plants, but their natural occurrence has been proved by isolation of *trans*-zeatin (*t*Z) from corn kernels (*Zea mays* L.) endosperm (Miller, 1961). Nowadays, it is known, that CK play an important role nearly in all important plant developmental processes usually together with other plant hormones and signaling molecules. Free CKs occur in all types of higher plants. Apart from plant species, they have been also identified in algae, fungi or bacteria and they also exist in animal cells as modified bases in tRNA (Takahashi, 1986).

CKs are described as plant hormones promoting cell division and bud differentiation in plant tissue cultures (Feng et al., 2017), but they are able to control many other metabolic, physiological and developmental processes in plants **(Fig.1)**, including seed germination, chlorophyll and starch production, apical dominance and leaf senescence (Strnad, 1997). They also have antioxidant properties, such as leaf protection from stress-induced oxidation (Zhang and Ervin, 2008). Additionally, they are able to control many other processes in plants

by interaction together with auxins (Gaspar et al., 1996) or by the crosstalk between them and other groups of plant hormones, such as abscisic acid and ethylene (Liu et al., 2017). In the plant species, they are present as free bases, glucosides, nucleosides and nucleotides in the low concentrations (pmol/g fresh weight) and the relative ratio of their different metabolites depends on the cell cycle stages, developmental phase and response to environmental cues (Redig et al., 1996).

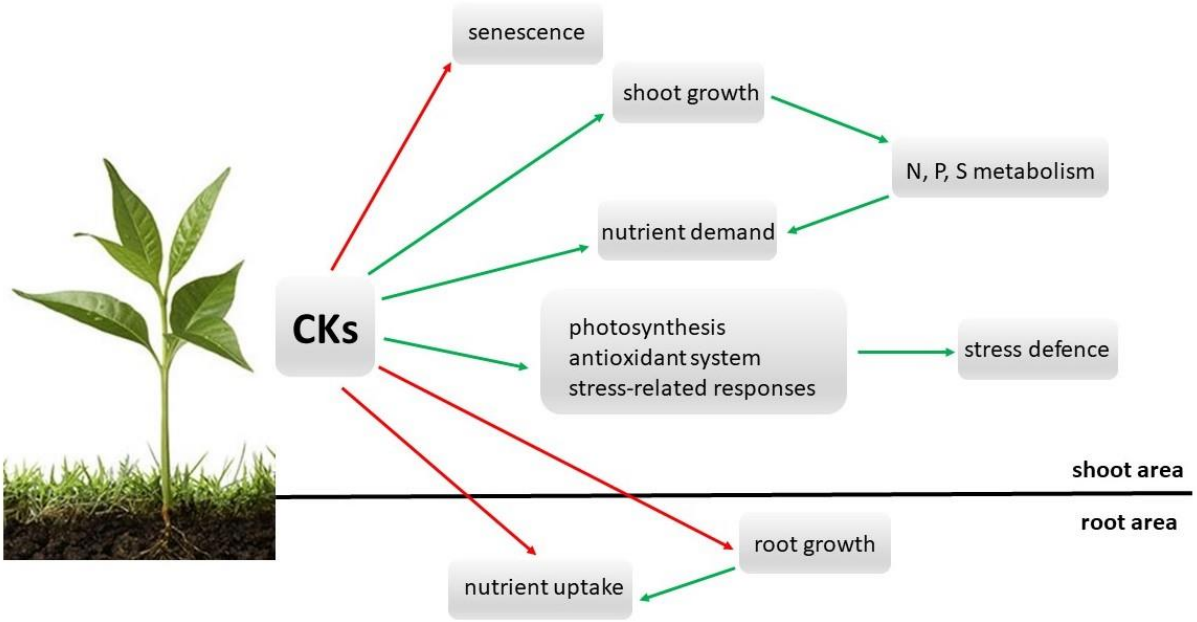


Fig.1: *The CK effect in shoot and root areas in plants. The processes induced by CKs are demonstrated by green arrows, red arrows represent the inhibition effects (edited) (Pavlů et al., 2018).*

Generally, CKs can be classified as isoprenoid or aromatic, according to their N^6-side chain configuration **(Fig.2)** (Mok and Mok, 2001). Since their discovery, the research has focused mainly on the isoprenoid group, because it was believed, that only CKs with this type of the side chain exist as endogenous compounds. This group is containing isopentyladenine (iP), having the isopentenyl side chain, as well as zeatin-type CKs with the presence of the hydroxylated isopentenyl chain. These hydroxylated forms occur as *trans* (*trans*-zeatin, *t*Z) or *cis* (*cis*-zeatin, *c*Z) isomers, and dihydrozeatin (DHZ) is formed by reduction of the double bond of the side chain (Mok and Mok, 2001). Their conjugates have also been proved as naturally occurring – for example iPR has been identified as a part of tRNA in many organisms, such as yeast, *Escherichia coli*, *Lactobacillus* or rat liver (Kamínek, 2015).

On the other hand, aromatic CKs were considered as synthetic compounds for a long time. The first Kin analog (6-benzylaminopurine, BAP) was synthesized immediately

after elucidation of Kin structure in the same laboratory (Okumura et al., 1957), however identification of 6-(2-hydroxybenzylamino)-9-β-D-ribofuranosylpurine (*o*TR) from poplar leaves proved their natural occurrence (Horgan et al., 1973). In 1997, its *meta*-derivative (*m*TR) was isolated from the same plant material (Strnad, 1997). The natural origin of aromatic CKs has been confirmed lately in many plant species, such as *Zantedeschia* (Chaves das Neves and Pais, 1980), tomato (Nandi et al., 1989), potato (Baroja-Fernández et al., 2002), *Cocos nucifera* (Sáenz et al., 2003) or *Arabidopsis thaliana* (Tarkowská et al., 2003).

Phenyl-urea type CKs, such as diphenylurea (DPU) or thidiazuron (TDZ), are structurally unrelated compounds classified as synthetic derivatives. Although, they were identified as highly active in various bioassays, their natural occurrence is not proved (Mok and Mok, 2001).

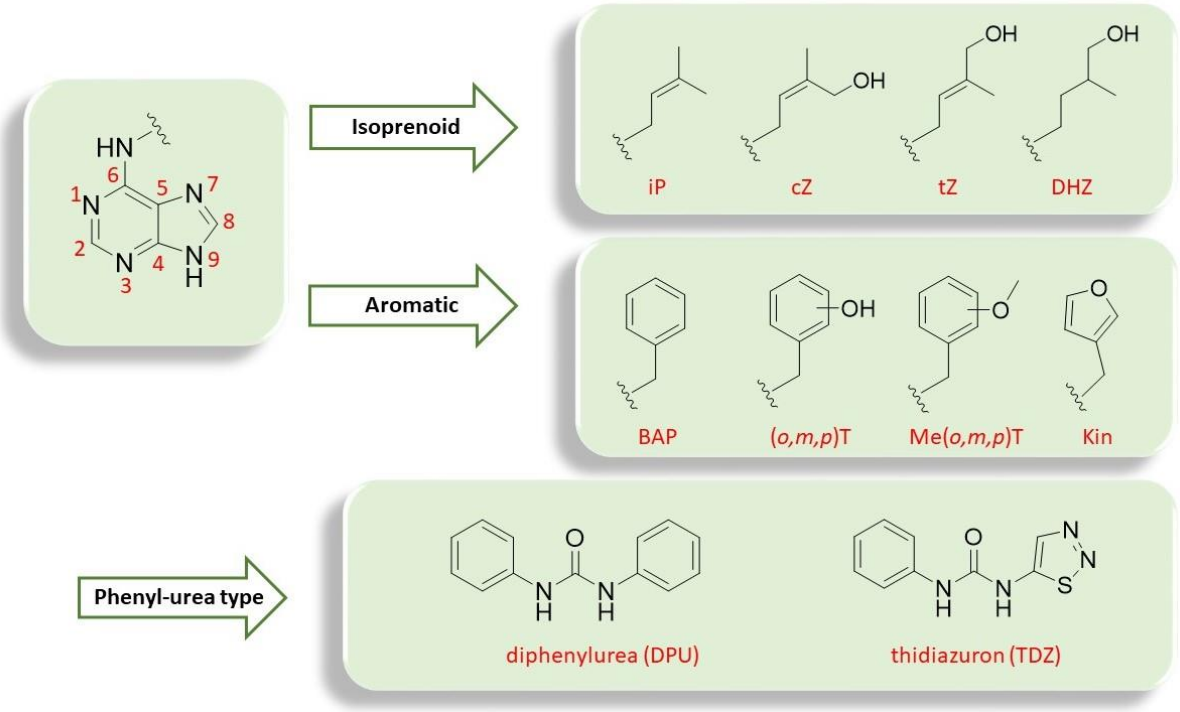


Fig.2: *General structures of isoprenoid, aromatic and phenyl-urea type CKs.*

2.2. Biosynthesis pathway

The CK biosynthesis pathway **(Fig.3)** is fully confirmed only for the isoprenoid CKs. Even though the aromatic CKs have been isolated from several plant species, their biosynthetic pathway has not been clarified yet. It is assumed that some parts of their metabolic pathway are shared with isoprenoid CKs. Also, catalytic enzymes used for their specific metabolic reaction and interconversion still have not been described yet (Davies, 2010). Previously it was thought that biosynthesis takes place primary in the roots, but lately its presence has been proved throughout the plant. For example, *t*Z riboside is predominantly occurring in xylem sap, whereas iP and *c*Z ribosides were identified mainly in phloem sap, but the CYP735A gene, responsible for hydroxylation of the iP side chain is mainly present in roots (Feng et al., 2017). CK biosynthesis can be proposed either from tRNA degradation and/or derived from the isopentenylation of free adenine nucleotides (Davies, 2010). Natural isoprenoid CKs such as iP and *t*Z are classified as N^6-prenylated adenine derivatives. Originally, the prenyl side chain can be obtained from the mevalonate pathway (MVA) identified in the cytosol of eukaryotes or the methylerythritol phosphate pathway (MEP), which is commonly established in plastids of plants or in bacteria. *Arabidopsis* seedling studies demonstrated that prenyl chains of iP and *t*Z are primarily formed through the MEP pathway. On the other hand, the prenyl chain of *c*Z is mainly synthesized through the MVA pathway (Kasahara et al., 2004). In the first step, the isoprenoid precursors dimethylallyl pyrophosphate (DMAPP) for the MVA pathway and 4-hydroxy-3-methyl-2-*(E)*-butenyl diphosphate (HMBDP) for the MEP pathway are attached to AMP, ADP or ATP by IPT (adenylate-isopentenyltransferase), which was firstly discovered in slime mold *Dictyostelium discoideum* (Sakakibara et al., 2005; Taya et al., 1978). Kakimoto et al. proved, that IPT plant genes are used for producing isopentenyladenosine-5´-phosphates, in minor for AMP, but mostly for ATP and ADP (Kakimoto, 2001). Firstly, identification of IPT genes in higher plants have been published for *Arabidopsis* (Kakimoto, 2001; Takei et al., 2001), hop (Sakano et al., 2004), *Petunia hybrida* (Zubko et al., 2002), mulberry (Abe et al., 2007) or rice (Sakamoto et al., 2006). In *Arabidopsis,* seven *At*-IPT genes (IPT1, IPT3, IPT4, IPT5, IPT6, IPT7 and IPT8) were identified and also two tRNA-IPTs genes were found (IPT2 and IPT9) (Kakimoto, 2001; Takei et al., 2001). Five members of *At*-IPT family were found in the plastids (IPT1, IPT3, IPT5 and IPT8), but IPT4 and IPT7 were localized in mitochondria and cytosol (Kasahara et al., 2004). Subsequently, the *tmr* (tumour morphology root) gene with ability to encode the enzyme with comparable activity was identified in *Agrobacterium tumefaciens*

(Akiyoshi et al., 1984; Barry et al., 1984). The iP was identified as a primary product of the biosynthetic pathway starting with DMAPP. Also, *t*Z nucleotide can be formed by direct action of iPT on HMBDP (Sakakibara, 2006). The next step is based on hydroxylation of isoprenoid chains of iPRMP, iPRDP or iPRTR respectively in the presence of cytochrome P450. In *Arabidopsis*, two types of cytochrome P450 monooxygenases – CYP735A1 and CYP735A2, which form corresponding nucleotides were characterized (Takei et al., 2004). There are two pathways for conversion from nucleotides to free bases – two-step activation, which is catalysed by 5´ribonucleotidephosphohydrolase to form ribonucleosides and by adenoside nucleosidase to form the active free bases. Previously, N-ribohydrolase (NRH) was identified as an enzyme to catalyze the hydrolysis from iPR to iP. The gene for the direct metabolic step was found in 2007 and it is also known as lonely guy (LOG).

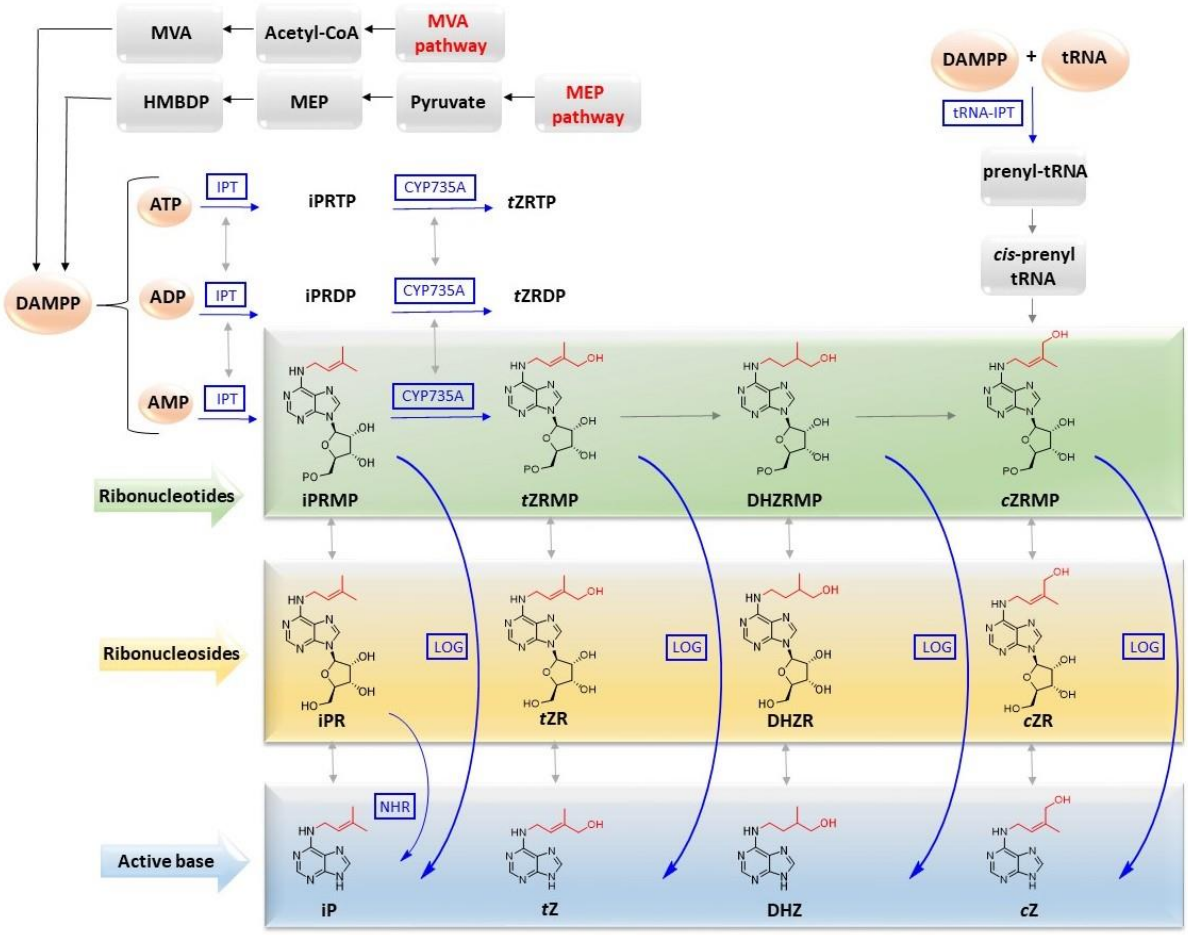


Fig.3: *Model for the isoprenoid CKs biosynthesis pathway in plants. The reactions with known genes encoding the enzyme are described by blue arrows and the genes, which have not been identified are shown by grey arrows (edited) (Márquez-López et al., 2019).*

The presence of other plant hormones and macronutrients indicate their function as factors, which can regulate CK biosynthesis. In *Arabidopsis*, CKs promote cell division by antagonizing with auxins. Whereas, auxins cause the expression of AtIPT5 and AtIPT7, CK inhibit the expression of AtIPT1, AtIPT3, AtIPT5 and AtIPT7 in shoots. Nitrate macronutrients cause the accumulation of *t*Z-type CKs in *Arabidopsis* roots, which is probably connected with AtIPT3 expression, but the other macronutrients like sulphate of phosphate can also regulate its expression (Feng et al., 2017).

2.3. Cytokinin transport

The previous studies showed, that CK may be transported in various ways – whereas iP is predominantly transported from shoots to roots, the transport pathway of *t*Z goes in the opposite direction (Bishopp et al., 2011; Kudo et al., 2010). Up to these days, three types of proteins with ability to transport CKs were identified: purine permeases (PUP), nucleoside transporters (ENT) and ATP-binding cassette transporters (ABC). The family of *Arabidopsis* permease genes (AtPUPs) have more than 20 members, which are mainly expressed in phloem, but two of them, AtPUP1 and AtPUP2, are localized in plasmatic membrane and they play a role as transfer intermediates for CK derivatives in *Arabidopsis* cells and yeast (Bürkle et al., 2003; Gillissen et al., 2000). Additionally, ENTs mediate the translocation of CKs in higher plants and some of them were identified as transporters in yeast or rice (Hirose et al., 2005; Kun et al., 2006). The subfamily of *Arabidopsis* ABC transporters controls the transport of *t*Z-type CKs from roots to shoots and the inhibition of its function has a negative effect on plant growth and development (Ko et al., 2014; Zhang et al., 2014).

2.4. Cytokinin signaling pathway

CK signaling system **(Fig.4)** is analogical to the previously described two-component system widely observed in bacteria, and it is based on the transport of phosphoryl group from receptors to subsequent cell components (Stock et al., 2000). In various parts of *Arabidopsis*, the receptor family including three histidine kinases (AHKs) has been localized in endoplasmatic reticulum (ER), as well as in plasmatic membrane (Lomin et al., 2018). They were named AHK2, AHK3 and CRE1/AHK4 and their influence on many factors in CK development was proved.

The prominent role of AHK2 and AHK3 is to control organ growth and regulate the functions in shoots and roots, such as leaf longevity regulated by mainly AHK3 itself (Kang et al., 2012). The CRE1/AHK4 receptor has been identified to have a specific role in regulation of sensitivity to CKs in root elongation, as well as regulated function in root patterning (Stolz et al., 2011). These receptors are activated after binding on CK-binding sites, commonly known as CHASE domains, and they are lately transferred to aspartate residue situated on the active domain of the same protein (Inoue et al., 2001). The next step is based on the transport of phosphoryl groups from the receptor to the histidine residue located in *Arabidopsis* histidine phototransfer protein (AHP). Then, phosphorylated AHPs are relocated from cytosol to the nucleus, but this mechanism remains unknown. The phosphorylated groups from AHPs are subsequently transferred to downstream cell component, such as *Arabidopsis* response regulators type-A (A-ARR), which negatively regulate CK response. On the other hand, type-B (B-ARR) response regulators became active after receiving the phosphoryl group and they directly cause the expression of A-ARR proteins (Feng et al., 2017). Up to these days, 3 AHKs, 6 AHPs, 10 A-ARRs and 11 B-ARRs genes are known as occurring in *Arabidopsis* (Ishida et al., 2008).

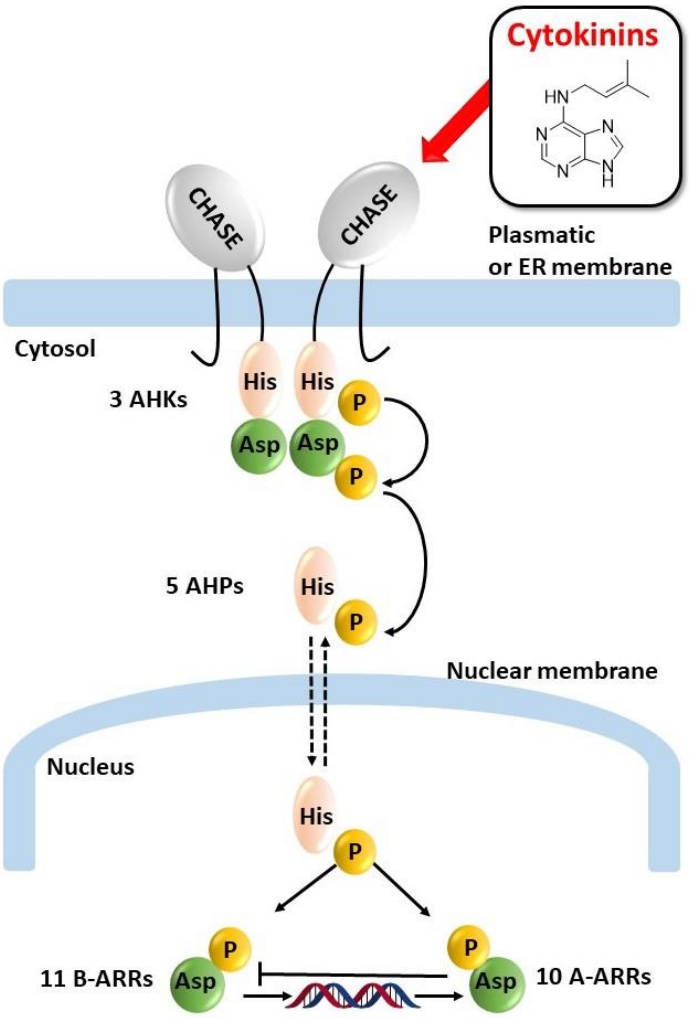
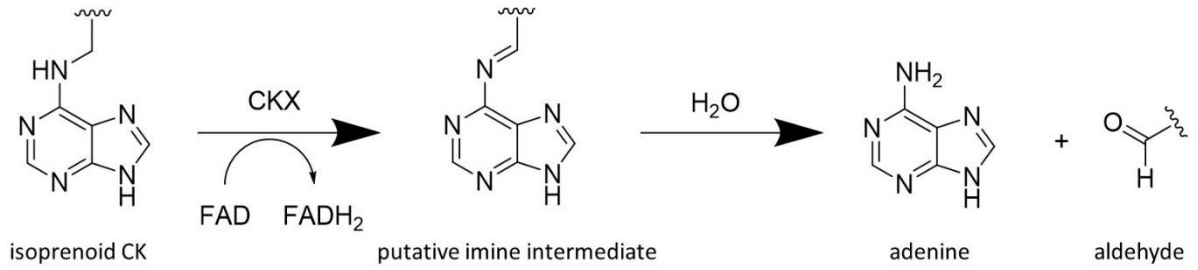


Fig.4: *General scheme of the CK signaling pathway. The CK is binding to the AHK receptors located in plasmatic membrane or ER membrane, which are activated through AHPs and they subsequently cause the phosphorylation of both ARR types. Firstly, the phosphoryl group (P) is transferred from His to Asp residue and then to AHP proteins. The AHPs continuously translocate between the cytosol and the nucleus, where the ARRs are phosphorylated. The activated B-ARRs promote transcription of A-ARRs, however A-ARRs are characterized as negative regulators (edited) (Cheng and Kieber, 2014).*

2.5. Cytokinin degradation

CKs can be inactivated and subsequently irreversibly degraded by oxidative cleavage of their N^6-side chain **(scheme 1)**. Firstly, the enzymatic process with ability to cleave a side chain was described in 1971 in *Nicotiana tabacum* extract (Pačes et al., 1971) and lately in various plant species, such as wheat, maize, poplar and beans (Hare and Van Staden, 1994). In 2001, the monomeric CK dehydrogenase (CKX), containing flavinadenindinucleotide (FAD)

as a cofactor, has been classified as an enzyme catalyzing their degradation (Galuszka et al., 2001). In *Arabidopsis*, the seven CKX genes were described (AtCKX1-AtCKX7) and lately, some other genes were discovered in various plant species, such as rice, maize, orchid or barley (Schmülling et al., 2003). CKX is able to degrade free bases and nucleoside conjugates of CKs with an unsaturated isoprenoid side chain like iP, iPR or both zeatin isomers and their ribosides. For example, the final products of degradation of iP are adenine and 3-methyl-2-butenal. On the other hand, CKX from *Arabidopsis* are also able to cleave CKs with a saturated side chain, such as DHZ and DHZR, as well as aromatic, but with less efficiency than unsaturated isoprenoid CKs (Galuszka et al., 2007; Kowalska et al., 2010). Also, *O*-glucosylation or *O*-xylosylation of the side chain causes their resistance (Davies, 2010).



Scheme 1: *The reaction mechanism of degradation for isoprenoid CKs (edited) (Popelková et al., 2006).*

3. Cytokinin conjugates

The theory about reversible conjugates of CKs suggests their role as the source of free active hormones under favourable developmental, physiological and environmental conditions. In plants, natural CKs are present in equilibrium as free bases, nucleosides or mono-, di- and tri- nucleotides, *O*-glucosides and *N*-glycosides (Bajguz and Piotrowska, 2009; Mok, 2019). The total level of the active CK form is controlled by many metabolic processes, such as biosynthesis, transport, (de)conjugation and degradation (Auer, 1997). CK free bases can be reversibly or irreversibly conjugated with sugar moiety or amino acids. Although, the free bases are highly active in many bioassays, the conjugation can regulate or reduce their activity. It is assumed, that CK conjugates play a storage, transporting and/or deactivation role, because of their resistance to CKX degradation (Bajguz and Piotrowska, 2009). Also, externally supplied CKs can create various types of conjugates as a storage form for later

use (Frankenberger and Arshad, 2020). Currently, two general types of conjugation are known – with a side chain and with adenine moiety **(Fig.5)**.

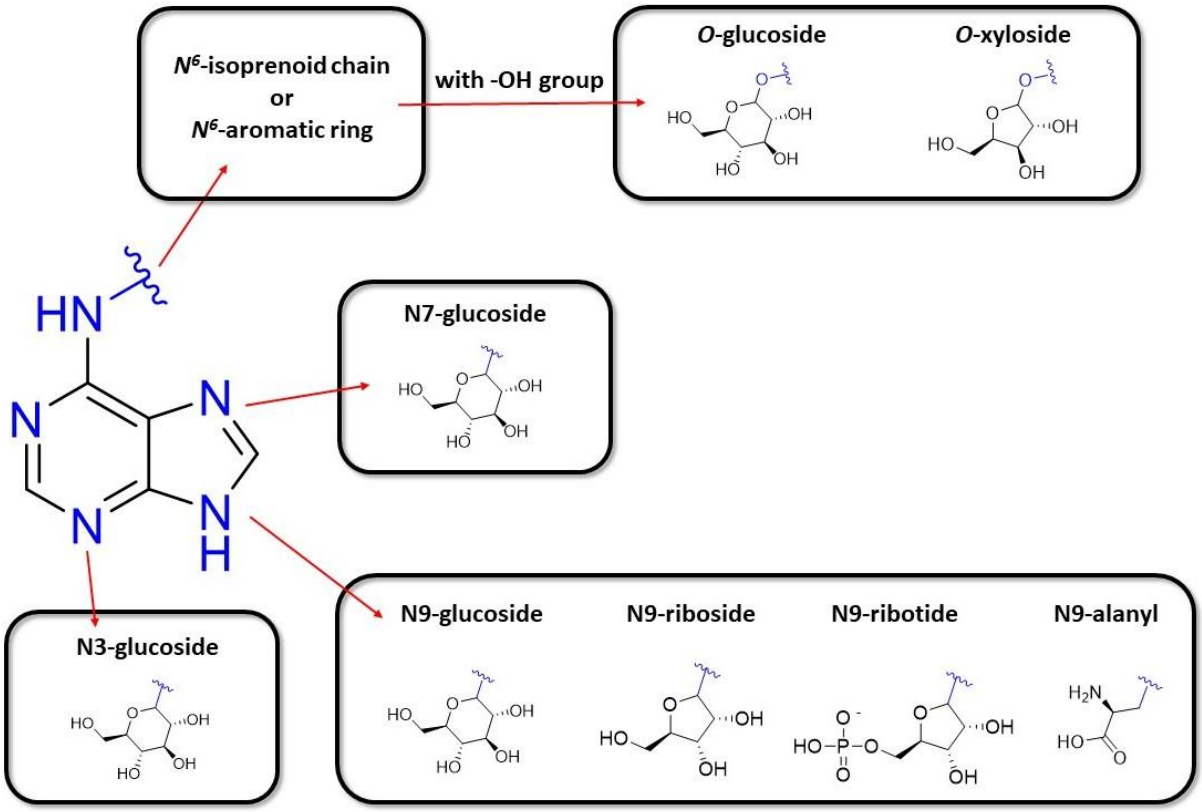


Fig.5: *Structures of CK naturally occurring conjugates.*

3.1. O-glucosides, N-glucosides and O-xylosides

The conjugation of CKs with glucose makes the molecule almost inactive, but the products play an important role in homeostasis of hormones (Hou et al., 2004). Glucose conjugates also have significant roles in CK transport, protection against degradation and reversible or irreversible inactivation (Werner et al., 2001). CKs can be glucosylated to form *N*-glucosides and *O*-glucosides. While, *O*-glucosylation and *N3*-glucosylation is reversible, the conjugates with glucose at *N7* a *N9* position are irreversible and they decrease the level of active CK forms in plant tissues (Blagoeva et al., 2004). Very recently, this theory about irreversible glucosylation has been discussed due to the study of kinetic metabolism of two isoprenoid CKs. Distinct metabolic cleavage of iP and *t*Z has been clarified and it was proved, that *N7*- and *N9*-glucosides of *t*Z were converted back to the active free base (Hošek et al., 2020). *O*-glucosides are formed by interaction of hydroxyl group of the side chain

with a molecule of glucose. These conjugates derived from isoprenoid CKs have been identified as naturally occurring in many higher plant species (Mok and Mok, 2001). For example, *trans*-zeatin-*O*-glucoside (*t*ZOG) and its riboside counterpart *trans*-zeatin riboside-*O*-glucoside (*t*ZROG) were isolated from immature seeds of *Dolichos lablab* (Yokota et al., 1980), as well as from shoots of chestnut tree (Yokota and Takahashi, 1980). Especially, zeatin-type *O*-glucosides have been characterized as important for transport of CKs, as well as the storage form (Kiran et al., 2012). Also, the aromatic CKs have been detected in *O*-glucoside form, for example topolins-*O*-glucosides or methoxy-topolins-*O*-glucosides and their 9-ribosides. *Meta*-topolin riboside-*O*-glucoside (*m*TROG) was isolated as a major metabolite of *meta*-topolin (*m*T) in all parts of *Spathiphyllum floribundum* (Werbrouck et al., 1996). Subsequently, two derivatives of topolins, *ortho*-topolin riboside-*O*-glucoside (*o*TROG) and *m*TROG, were detected in microalgae (Ördög et al., 2004). In the same year, *m*TROG and *para*-topolin riboside-*O*-glucoside (*p*TROG) were characterized in shoots of *Aloe polyphylla* (Ivanova et al., 2006). These aromatic CKs with hydroxyl group are cleaved by *O*-glucosyltranferases (Mok et al., 2005). *N*-glucosides are biologically stable and the most abundant naturally occurring CK metabolites, which are formed through the uridine diphosphate glycosyltransferase (UGT) enzyme family (Yang et al., 2018). They can form 80% or more of total CKs pool in plants - they are assumed as an irreversible inactive form. These CK derivatives are thought to be presented in the vacuole (Osugi and Sakakibara, 2015). Even though, they have been generally characterized as biologically inactive (Kamínek et al., 1997), the reduced biological activity of some *N*-glucosides was described in the radish leaf senescence assay, where the similar activity of BAP9G and BAP was observed (Letham et al., 1983). Lately, BAP and its 3-,7- and 9-glucosides were detected as active in the soybean callus assay in order 3G > 9G > 7G (Van Staden and Drewes, 1991). The side chain of isoprenoid CKs can be also glycosylated by xylose to form *O*-xyloside. As enzyme responsible for formation of *O*-xylosylzeatin from *trans*-zeatin was identified zeatin *O*-xylosyltransferase from *Phaseolus vulgaris* (Martin et al., 1993).

3.2. N9-substituted aromatic cytokinin derivatives

The structure diversity of CKs enables the development of large scale of their derivatives with significant biological properties. They can be substituted at various positions of purine moiety, such as *N1*, C2, *N3*, *N7*, C8 and *N9* (Plíhalová et al., 2016). It was proved, that modification at every position has an important influence on bioactivity, but especially for some *N9*-substituted derivatives no negative effect on root elongation was described with prevention to form irreversible *N9*-glucosides (Plíhal et al., 2013). Recently, the complete review including synthesis, endogenous occurrence and biological activity of numerous *N9*-substituted sugar as well as non-sugar CK derivatives were published and their structural aspects/biological activity relationship was discussed (Vylíčilová et al., 2020). Currently, BAP is the most frequently used aromatic CK in plant biotechnologies, but its utilization is connected with many disadvantages for some crops (Werbrouck et al., 1996), such as problematic plant acclimatization in greenhouses, lateral root inhibition (Podlešáková et al., 2012) and shoot-tip necrosis (Bairu et al., 2009). It is also assumed, that root inhibition is connected with accumulation of *N9*-glucosides as an inactive form in shoots or it can activate production of ethylene (Cary et al., 1995; Werbrouck et al., 1995). Until now, the simplest way to regulate the negative properties seems to be the substitution on the benzyl ring (Doležal et al., 2007; Vylíčilová et al., 2016).

Some non-sugar *N9*-substituted derivatives have been identified as naturally occurring in plant tissues, but many of them were prepared synthetically as a CK mimetics. The group of naturally occurring 9-alanyl derivatives isolated from lupin seeds and immature apple seeds is commonly known as lupinic and dihydrolupinic acids (Duke et al., 1978; Entsch et al., 1983). Although the lupinic acid is almost inactive in many bioassays, it can serve as potential storage form (Mok, 2019). Subsequently, many synthetic *N9*-substituted derivatives were prepared and their biological activity was observed in various bioassays. For example, a series of BAP derivatives with alkyl or cycloalkyl group were prepared, but none of them was more active than free base (Zhang and Letham, 1989). Lately, Mik et al. prepared kinetin derivatives including *N9*-halogenalkyl, carboxy chains and aliphatic or cyclic ether. The significant activity in tobacco callus was observed for derivatives with a short halogenalkyl chain and with aliphatic or cyclic ether at *N9*-position. The dependence on the length of the alkyl chain was also confirmed in the senescence assay, where the shorter halogenalkyls are able to significantly delay leaf senescence (Mik et al., 2011). These derivatives can be used as intermediates

for fluorescently labelled CKs, which can be subsequently used to study of their perception and receptors (Antoniadi et al., 2020; Kubiasová et al., 2020). The effect to delay senescence and promote chlorophyll retention with higher efficiency than BAP itself was published for BAP derivatives with tetrahydropyran-2-yl and tetrahydrofuran-2-yl at *N9* position (Skoog et al., 1967; Zhang and Letham, 1989). Moreover, the important anti-senescence properties were also observed for their kinetin counterparts (Pietraface and Blaydes, 1981). Based on these results, a group of 9-THP and 9-THF derivatives was synthesized and it was confirmed, that their activity improves the activity of corresponding free bases in CK bioassays (Szüčová et al., 2009). Subsequently, the profitability of these derivatives in plant tissue cultures has been proved by many authors. For example, no inhibition effect on primary root growth has been reported for 3-methoxyBAP-9-THP comparing with its corresponding free base (Podlešáková et al., 2012). Also, the positive influence of 3-methoxyBAP-9-THF on root elongation was described (Plíhal et al., 2013) and many of these analogs were successfully used in micropropagation techniques as well, e.g. acclimatization of micropropagated Williams banana (Aremu et al., 2012; Aremu et al. 2014).

CK *N9*-sugar conjugates are a significant group of CK metabolites. Whereas, some *N9*-sugar conjugates with ribose or glucose have been identified as naturally occurring, many of them were prepared in a synthetic way. The natural origin of CK ribosides was firstly confirmed by isolation of 6-(2-hydroxybenzylamino)-9-β-D-ribofuranosylpurine (*o*TR) from poplar leaves (Horgan et al., 1975) and lately, they have been identified in other plant species - BAPR has been found in coconut palm (Sáenz et al., 2003) and KinR was isolated from coconut water (Ge et al., 2005). Conjugation of ribose moiety is restricted at *N9*-position of purine ring and the biological importance of these compounds was confirmed by many authors. For example, culture medium enriched by *m*TR has a positive influence on micropropagation of potato (Baroja-Fernández et al., 2002) and this compound also suppressed the occurrence of shoot-tip necrosis in *Harpagophytum procumbens* (Bairu et al., 2009) or increase regeneration rates of orchid explants (Vasudevan and Van Staden, 2011). Based on these results, a series of BAPR derivatives were synthesized and the position-specific steric effect of substituents at benzyl ring was evaluated in following order: *meta* > *ortho* > *para.* Moreover, it was observed, that majority of BAPR analogues are twice as active as BAP in the senescence bioassay (Doležal et al., 2007). Subsequently, the strong anti-senescence activity was also proved for group of 2-chloro-6-(halogenbenzylamino)purine ribosides (Vylíčilová et al., 2016), 6-benzylaminopurine arabinosides and 6-benzylamino-9-(2′-deoxy-β-D-ribofuranosyl)purine

derivatives (Matušková et al., 2020). On the contrary, the change of sugar stereochemistry causes a significant loss of biological activity – the activity of β-L-BAPR derivatives has been evaluated as significantly weaker than their corresponding D-counterparts (Vylíčilová et al., 2020). CK disaccharide conjugates, ribose and glucose moiety at *N9* position, have been also identified as naturally occurring and they have been isolated from *Pinus radiata* (Taylor et al., 1984). It is believed, that BAP9RG can influence metabolic regulation of active CK pool (Auer and Cohen, 1993).

3.3. Role of N9-substituted aromatic cytokinin nucleosides in human disease therapy

Except the fact, that CKs play an important role in growth and differentiation of plants, their utility for treating of mammalian diseases required cell proliferation and differentiation has been proved by many authors. The cytotoxic mechanism depends on the type of treated CK, as well as on the type of the cell line. It has been studied mainly on three classical naturally occurring CKs – iPR, KinR and BAPR. These derivatives have been found to prevent ATP depletion (Cabello et al., 2009; Mlejnek and Kuglík, 2000) and they cause caspase-3-activation in CK treated HL-60 cell lines (Ishii et al., 2002).The significant pro-apoptotic effect was described especially for BAPR and its derivatives – these compounds cause decreasing in ATP production and apoptosis induction because of intracellular formation of mononucleotides from appropriate BAPR derivatives (Mlejnek and Kuglík, 2000; Mlejnek and Doležel, 2005). Other effects of CK ribosides including genotoxic stress induction (Cabello et al., 2009), reduction of farnesyl-protein transferase activity (Laezza et al., 2006), c-jun N-terminal kinase activation (Laezza et al., 2009) or inhibition of epidermal growth factor receptor signalling (Ciaglia et al., 2016). For some of them, the ability to limit angiogenesis and increase tumor immunogenicity was also reported (Ciaglia et al., 2018; Pisanti et al., 2014). The complete summary of molecular effects of CK nucleosides in cancer cell lines has been recently published by Voller et al. (Voller et al., 2019).

The growth inhibitory effect for BAPR analogues in leukemia cells were proved in 1975 (Dutta et al., 1975). Lately, *in vivo* and *in vitro* anticancer activity of CKs ribosides was tested on wide panel of cancer cell lines and *o*TR was identified as the most potent compound against human cancer cell lines with specific mechanism of activity depending on intracellular phosphorylation by adenosine kinase (ADK) (Mlejnek and Doležel, 2005; Voller et al., 2010).

The therapeutic potential of *para*-topoln-9-riboside (*p*TR) for treating neurotoxicity and inhibition of cell apoptosis in rat phechromocytoma cells was proved (Chen et al., 2011; Huang et al., 2007) and the same derivative was lately characterized as the most effective CK riboside of *in vitro* platelet aggregation (Vistoli et al., 2014). Also, the potent antiviral activity of BAPR analogues against enterovirus 71 was evaluated and it was shown, that the activity greatly depends on the size of linker between benzyl ring and adenine moiety - the linkers containing 2-3 atoms have been classified as most effective antiviral agents (Drenichev et al., 2016a). Moreover, a group of BAPR analogues were investigated as a potential drug against *Trypanosoma brunei* (Bressi et al., 2000), as anti-toxoplasma agents (Kim et al., 2007), as well as for their neuroprotective effect (Wang et al., 2019). The significant toxic effect of BAPR on human microvascular endothelial cells was described in 2018 (Castiglioni et al., 2018). Recently, the high efficiency of the same compound against human glioma cell lines was reported (Ciaglia et al., 2017) and subsequently, this theory was confirmed for a series of its derivatives (Grimaldi et al., 2020). The complete summary of unique biological properties of CK nucleosides was published by Drenichev et al. in 2016 (Drenichev et al., 2016b) and the effects of adenosine-related drugs are illustrated in **Fig.6**. However, not all CK ribosides have anti-cancer activity – *t*ZR, DHZR or *m*TR have been evaluated as inactive, as well as *O*- and *N*-glucosides. Also, the activity of CK ribotides was detected as lower or similar to their riboside counterparts (Voller et al., 2019).

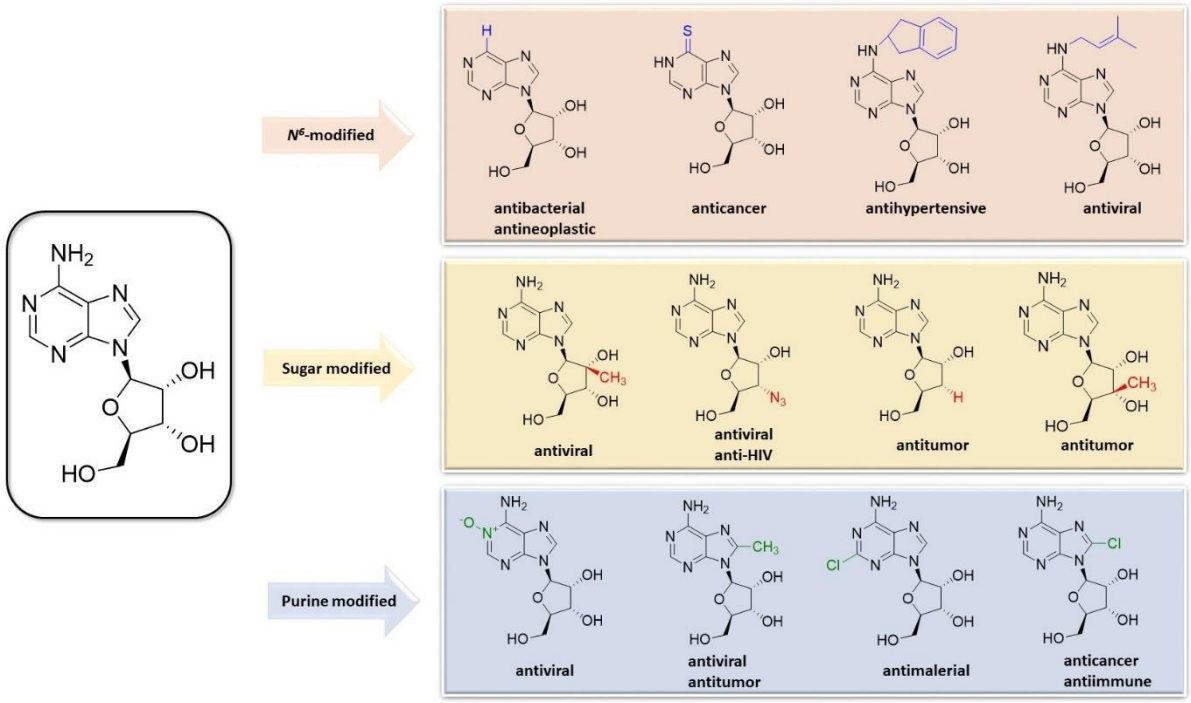


Fig.6: *The overview of adenosine-related drugs (Aerschot et al., 1993; Biesele et al., 1954; Bohr, 1978; Carlson et al., 2000; Coomber et al., 1994; De Clercq, 1980; Ehrenberg et al., 1946; Eldrup et al., 2004; Franchetti et al., 2005; Gadthula et al., 2005; Goldin et al., 1968; Kane and Shuman, 1995; Kusachi et al., 1986; Macer-Wright et al., 2020; Smee and Sidwell, 2004; Tararov et al., 2015; Wang et al., 2016).*

Also, adenine L-nucleoside analogues, characterized by opposite configuration compared to their naturally occurring D-analogues, have been investigated as new effective agents in antiviral therapy. The preferable features of these conjugates are especially high metabolic stability and low cellular toxicity, which predict their use in drug design. For example, L-3′-amino-3′-deoxy-N^6-dimethyladenosine was proved to enhance functional recovery from ischemia as cardioprotective agent (Kasiganesan et al., 2009). The antimalarial effect was proved for 6-thio-L-inosine with no toxic side effect comparing with its D-counterpart (Gero and Weis, 2001). Two L-nucleoside analogues, L-adenosine and 2,6-diamino-9-(L-ribofuranosyl)purine were targeted for development of novel parasite-specific antimalarials (Brown et al., 1999). Some L-nucleosides derivatives of adenine were also identified as effective against HIV and hepatitis-B viruses or as antitumor agents with no toxicity for normal human cells (Gumina et al., 2001). Hence, several L-nucleosides are currently tested as potential antiviral agents in human disease therapy.

CK arabinosides and their precursors were also determined for potential therapeutic usage. In 1980´s, adenine arabinoside (Ara-A, vidarabine) was abundantly tested as a drug against many human diseases, however the arabinose moiety is also presented in other common anticancer drugs, such as fluodarabine, cytarabine and clofarabine **(Fig.7)** (Bonate et al., 2006). Ara-A was specifically synthesized for treatment of human herpes virus infection, like herpex simplex and varicella-zoster viruses, but its sensitivity to Epstein Barr virus and cytomegalovirus was also proved (Whitley et al., 1980). Additionally, its cytotoxic effect for cancer cell lines, such as mouse mammary tumor, confirmed its therapeutic assumption (Smee and Sidwell, 2004). In 1977, the group of N^6- or C8- substituted Ara-A derivatives were prepared and their *in vitro* antiviral activity was confirmed (Kaneko et al., 1977). Lately, the biological activity of 2-halogen-2´-substituted Ara-A derivatives were evaluated on mouse leukemia, mouse fibroblasts laryngeal carcinoma cells and some derivatives showed a high effect (Secrist et al., 1988). These results may predict the future prospects of purine arabinosides in many therapeutic applications.

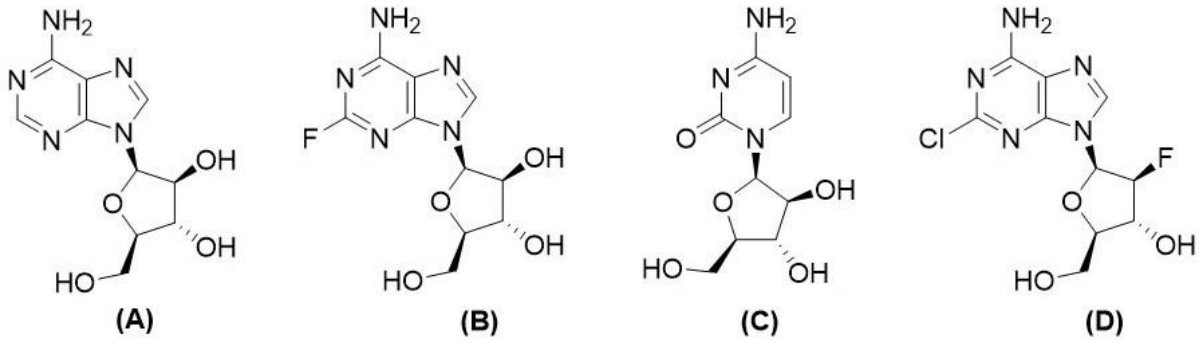


Fig.7: *Chemical structures of nucleosides containing β-D-arabinofuranose at N9 position of base, commercially used as anticancer drugs:* ***(A)*** *vidarabine,* ***(B)*** *fluodarabine,* ***(C)*** *cytarabine and* ***(D)*** *clofarabine.*

3.4. The cytokinin role in plant tissue culture and micropropagation techniques

The term tissue culture refers to *in vitro* cell culture, organs or whole plant under specifically controlled environmental and nutritional conditions with ability to produce plant clones. The tissue culture technology applied on plants is commonly used for variety of plant multiplications and these techniques are widely applicable in the area of plant propagation, plant improvement and disease elimination. The small pieces of tissue, also known as explants,

can be used for producing up to thousands of new plants in a relatively short period under controlled conditions without season and weather influences (Hussain et al., 2012). Micropropagation is a commercially used technique, which starts by selection of explants from a healthy mother plant. Any part of the healthy plant can be used as explant – shoots, roots, leaves, apical meristems, cotyledons, nodes or embryos (Hill and Schaller, 2013). Shortly after Kin discovery, the influence on CK/auxin ratio in growth medium on differentiation of cultured tobacco callus tissue has been observed. High auxin/low CK ratio induces the root formation, low auxin/high CK ratio stimulates the regeneration of shoots and the balance of high concentration of both phytohormones forms undifferentiated callus (Skoog and Miller, 1957). Generally, the decreasing ratio of CK to auxin causes the shift from shoot to root organogenesis however the exact ratio of CK/auxin in cultured medium mainly depends on plant species (Hussain et al., 2012).

In *Arabidopsis*, the organ regeneration is classified as a two-step process **(Fig.8)**. In the first step, explants are incubated in auxin-rich callus medium (CIM) to form the callus. Subsequently, the callus is treated by shoot-inducing medium (SIM) or root-inducing medium (RIM) with the specific CK/auxin ratio to prefer stimulation of shoots or roots, respectively (Hill and Schaller, 2013).

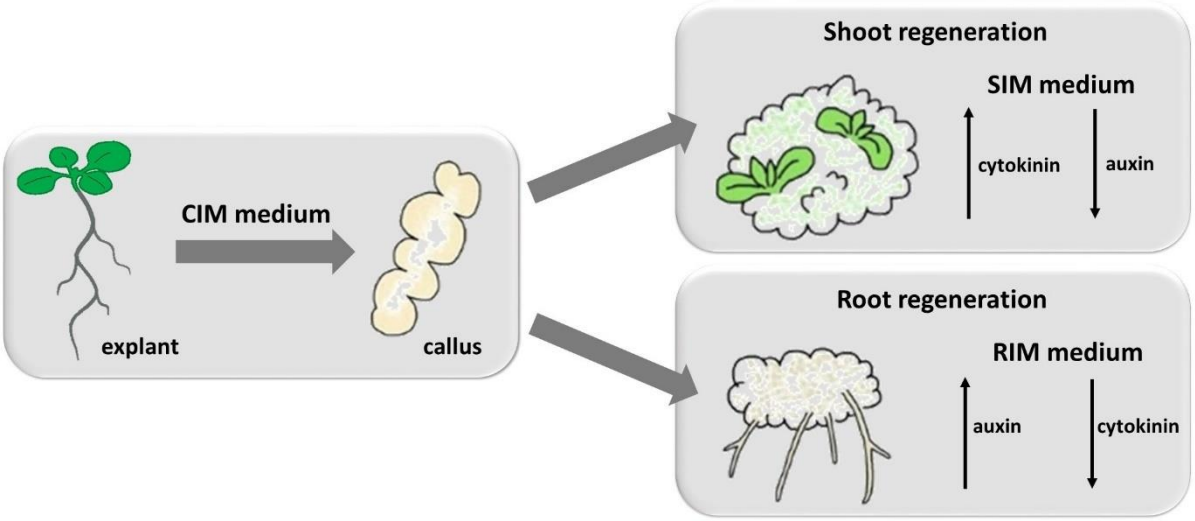


Fig.8: *The scheme of in vitro shoot and root regeneration for Arabidopsis (edited) (Su and Zhang, 2014).*

Nowadays, the number of research studies is targeted on hormonal regulation of regeneration in many plant species, where BAP and Kin are mainly used, because of their high stimulating effect and low price. The positive influence of BAP enriched medium on micropropagation of more than hundreds plant species, such as trees, fruits, vegetables or flowers was published by many authors. Recently, its effect was confirmed for vanilla, philodendron tree or kiwifruit (Alawaadh et al., 2020; Erawati et al., 2020; Saeiahagh et al., 2019) and its mixture with Kin was newly used for ginger regeneration (Miri, 2020). Moreover, the efficiency of some BAP derivatives was reported – for example *m*T improved regeneration of gooseberry and its fluorinated derivative was used for orchid micropropagation (Kucharska et al., 2020; Murvanidze et al., 2019).

3.5. The practical role of aromatic cytokinins in agriculture

Depending on previous *in vitro* studies, some CKs, especially BAP, became interesting compounds in biotechnological and agricultural research as potentially active agrochemicals, because of their effect to stimulate branching, control of flowering and seedlings and delay of leaf senescence. They are also known to regulate cell cycle, senescence, seed dormancy and germination, apical dominance, as well as *de novo* bud formation, they are able to control the growth and development of plant organs, responses on exogenous factors, such as availability of water and nutrients and responses to biotic or abiotic stresses, which focus their exogenous application in field conditions (Koprna et al., 2016).

For example, the BAP was tested to increase crop yield of some cereals as wheat, where its ability to improve the size and grain formation was observed (Criado et al., 2009), or in barley, where the seedling raised by 20% (Woodward and Marshall, 1989). The similar trend was also confirmed in soybean (Yashima et al., 2005) and *Vicia faba* beans, where the application of BAP led to significant changes in parameters such as the number of leaves, leaf area, plant height etc. (Ibrahim et al., 2007). The effect of BAP was also studied on various species of fruits and vegetables – BAP application significantly increases the various root and leaf parameters in tomato (Haroun et al., 2011) or in spinach, where BAP reduced the time of flowering or significant increase of yield (Durrani et al., 2010). Also, the positive effect of BAP sprayed of apple and pear trees, especially on the size and weight of fruit was published. Wismer et al. proved the positive effect of BAP on ´Empire´ apple trees fruit mass (Wismer et al., 1995) and also the significantly increases of “Spadona,, and “Coscia,, pear size

with no influence on their shape (Stern and Flaishman, 2003). However, the resulting effect of exogenous applied CKs on crops depends on many other factors like the type of used CK, combination with other phytohormones, the type of plant species, growth conditions or application time (Koprna et al., 2016).

4. The testing of cytokinin-like activity

The bioassays play a crucial role in detection of CK activity. After discovery of CKs, various types of bioassays have been developed to evaluate the activity of these biologically important molecules. The effect of CKs to influence some aspects of plant development can be evaluated using various bioassays with characteristic parameters, such as high sensitivity and specificity for CKs. Using the bioassay can also clarify the structure-activity relationship, as well as the effect of aromatic ring modification or optimal length of the side chain (Mok, 2019). Soon after Kin discovery, the first bioassay to evaluate the CK effect on lettuce seed germination after red light exposure was reported by Miller (Miller, 1957). The effect to stimulate leaf growth of *Raphanus sativus* was evaluated in the radish bioassay performed by Kuraishi et al. in 1956. After CK treatment, the leaf discs were incubated in light as well as in the dark conditions and the parameters, such as fresh weight of leaf discs area were evaluated (Kuraishi and Okumura, 1956). Subsequently, the prime assay based on the ability of CKs to stimulate callus growth from *Nicotiana tabacum* have been developed by Murashige and Skoog and resulting biological activity was determined as the difference of callus increment between non-treated and CK treated growth medium (Murashige and Skoog, 1962). Lately, the tobacco callus growth assay has been repeatedly modified and nowadays it is widely used as a specific and sensitive test for evaluating CK activity. The CK activity is detected after four-week cultivation from the increase of fresh callus mass (Holub et al., 1998). Similarly, stimulation of cell division on plant tissue can be tested on soybean incubated in the dark, commonly known as the soybean hypocotyl section bioassay (Manos and Goldthwaite, 1976; Newton et al., 1980), as well as on carrot tissue (Letham, 1967). Lately, a series of bioassays based on the activity of tested CK inducing cotyledon expansion were developed in various modifications. This promoting effect was observed in many plant tissues, such as etiolated bean leaf disc (Miller, 1963) or duckweed (Letham, 1967). A wide group of assay was utilized to investigate the ability of CK to promote expansion of cotyledon in plant species as pumpkin

(Banerji and Laloraya, 1965), *Xanthium pensylvanicum* (Esashi and Leopold, 1969), cucumber (Narain and Laloraya, 1974) and radish (Letham, 1971). The test can be evaluated either after dark incubation or after light exposure. A number of assays have been also developed with the ability to stimulate formation of new buds and apical buds as well. The pea lateral buds bioassay depends on the ability of CK to form new buds from apical dominance (Thimann and Sachs, 1966) and high specificity and sensitivity *Funaria hygrometrica* bioassay is based on formation of new buds on caulonema filaments (Hahn and Bopp, 1968). The cucumber cotyledon greening bioassay is based on increment of fresh weight, area and formation of chlorophyll after dark incubation followed by light exposure. The resulting amount of chlorophyll depends on cotyledon age, the time of dark incubation and the exact ratio of CK and KCl in tested solution (Fletcher et al., 1982; Tahbaz, 1977). The *Amaranthus* bioassay is still used for testing of CK activity and it is based on synthesis of betacyanin in *Amaranthus caudatus* hypocotyl and cotyledon in the dark (Biddington and Thomas, 1973). Hypocotyl and cotyledon of *A. caudatus* seedling are incubated in medium enriched by the tested CK and the amount of contained betacyanin is detected by the absorbance measurement at 537 nm and 620 nm (Holub et al., 1998). The senescence delay bioassay is also still used for determination of CK ability to delay leaf yellowing. It was utilized for CK treated tobacco, cabbage, radish and oat leaves (Letham, 1967; Varga and Bruinsma, 1973), but today wheat is mainly used as a model plant. The tip sections of wheat leaves are incubated four days in the dark and the absorbance of extracted chlorophyll is detected at 665 nm (Holub et al., 1998).

Despite the number of known CK bioassays, none of them is able to distinguish between the direct CK action and indirect action caused by their metabolic conversion typical for naturally occurring CKs, which are rapidly metabolized or cleaved in plant tissues. Therefore, these assays are now combined with modern techniques of the CK analysis and modern assays have been also developed (Mok, 2019).

In 1992, Boerjan et al. published a new bioassay with ability to detect the presence of CKs, as well as auxins in cultured medium. The mesophyll protoplasts of the chimer gene from *Nicotiana tabacum*, which are consisting from upstream sequences of *A. tumefaciens* are expressed and coupled to coding β-glucuronidase (Boerjan et al., 1992). The lately reported receptor bioassay is based on heterologous complementation of the receptor AHK3 and CRE1/AHK4 of bacterial cells with the *Escherichia coli* signaling pathway. In general, the receptors are activated in the presence of CK and phosphorelay cascade is triggered, which

stimulates the expression of cps::lacZ gene coding β-galactosidase. Its activity is subsequently measured after chromogenic or fluorogenic substrate application. The ligand preference of each receptor is evaluated via ability of tested compounds to activate the CK signaling pathway (Suzuki et al., 2001; Yamada et al., 2001). Despite the fact, that individual *Arabidopsis* receptors are highly sensitive to free bases of isoprenoid CKs, their sensitivity to distinguish CK metabolites is specific. The AHK2 and CRE1/AHK4 receptors effectively differ between CK free bases and conjugates, but AHK3 is able to identify also CK ribosides and nucleosides. In contrast to high activity in most bioassays, the aromatic CKs have significantly lower ability to activate these receptors – the difference can be caused by their high stability in plant tissue (Spíchal et al., 2004). The *Arabidopsis* ARR5:GUS reported gene assay uses ARR5 CK-induced primary response regulator gene as a promoter. The exogenously applied CK switch on the CK signaling pathway, the ARR5 gene is activated, β-glucuronidase is produced and its activity is spectrophotometrically measured after fluorogenic substrate application (Romanov et al., 2002). Lately, Lomin et al. described a new assay system, which allows the study of every receptor located in plant membrane separately. In this assay, *Arabidopsis* receptors (AHK2, AHK3 and AHK4) and maize receptor (ZmHK1) were used (Lomin et al., 2015).

5. Leaf senescence

Plant senescence is defined as the last step of coordinated degenerative developmental process in plants (Woo et al., 2018). The senescence can be dependent on many external and internal signals. During this program, the macromolecules (lipids, proteins, nucleic acids) are degraded and nutrients produced by catabolism of degraded macromolecules are recycled to form young leaves, new buds, and fruit and seeds. Hence, this process is also known as nutrient mining and recycling program different from other developmental processes (Ali et al., 2018). The senescence timing is affected by stress responses and plant development a phytohormones can be evaluated as regulating factors on this program (Jibran et al., 2013).Moreover, the group of senescence regulatory factors also includes receptors and signaling components affecting stress responses and hormones, regulators of metabolism as well as transcription regulators (Ali et al., 2018). Two main types of senescence are presented in plant - mitotic typical in shoot

apical meristem and/or leaves and fruits at the beginning of development and post-mitotic senescence occurs in floral petals and mature leaves (Gan, 2003).

Leaf senescence can be regulated by internal factors, such as age and phytohormones. These plant hormones have a key role in many developmental processes and also their role in regulation of leaf senescence has been proved. It is believed, that this passive aging can be stimulated by developmental age (Ali et al., 2018). One of the main visual features of leaf senescence is the change of the color from green to yellow/brown, but the leaf yellowing is not only accompanied with aging – mechanical damage, it is also caused by biotic or environmental stress and harvesting (Koyama, 2018; Toscano et al., 2018). The leaf yellowing is also associated with the loss of chlorophyll, proteins and RNA degradation or reduction of photosynthetic activity and these processes are controlled by specific genes. Molecular mechanism of senescence is based on reduction of photosynthetic genes expression and other genes decresion during this process - the group of senescence-downregulated genes (SDGs), including genes encoding photosynthesis and genes expressed in young leaves, occur at the beginning of senescence program. On the other hand, the regulation of senescence-associated genes (SAGs) takes place during senescence and their role is crucial for biochemical and physiological steps of leaf senescence. The complex of processes is triggered at the onset of senescence and the various signal pathways are involved in this mechanism **(Fig.9)** (Li and Guo, 2019). The primary processes activated during senescence, such as signalization of senescence-promoting hormones, upregulation of transcription factors and downregulation of chlorophyll biosynthesis and they are followed by degradative processes and inhibition of photosynthetic activity. The yellowing progression causes activation of catabolism accompanied with enrichment of transport-related activities, which indicate nutrient remobilization (Breeze et al., 2011).

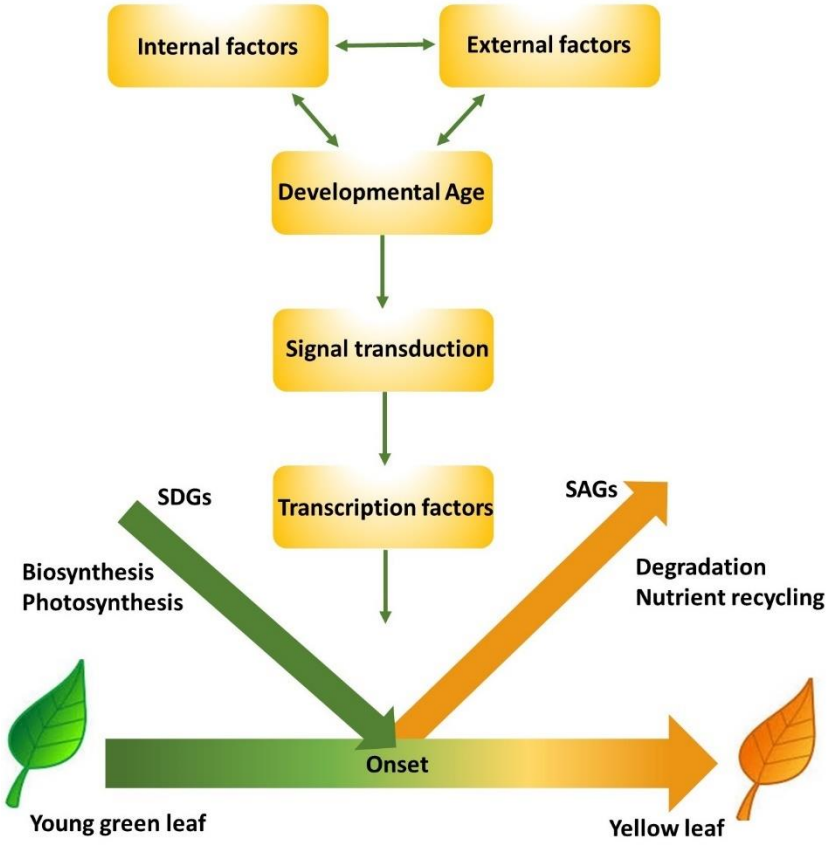


Fig.9: *The leaf senescence regulation pathway (edited) (Li and Guo, 2019).*

5.1. Role of cytokinins in senescence inhibition

CKs are known as phytohormones with a significant effect to delay leaf senescence. The first experiment to clarify the influence of CKs on this process was performed by Richmond and Lang in 1957, who described kinetin as a compound with ability to delay senescence in cocklebur leaves. It is generally known, that CKs are able to regulate the maintenance of chlorophyll, RNA and protein level, which decrease during senescence, thus confirmed the theory about the connection between the CK level and senescence progress (Van Staden et al., 1988). The three approaches are known to support the theory about control function of CK during senescence process. Firstly, the exogenous application also known as „spray and pray". The second approach is based on the measurement of endogenous CK level during senescence and the third approach is including transgenic plant strategy (Gan and Amasino, 1996). The senescence inhibition after exogenous application of CKs was observed by many authors in various species of monocotyledonous and dicotyledonous plants, such as barley, wheat, soybean or rice. However, the external application of CK is not always

effective against leaves yellowing. For example, BAP is able to delay senescence in *Arabidopsis* leaves in the dark, but it induces the chlorophyll degradation in light conditions (Gan and Amasino, 1996). The evidence of the role of CKs as negative endogenous regulators of leaf senescence is based on strong correlation between the reduced level of CKs and senescence progress. Synthetized CK is transported from roots to the leaves and it has been found that the CK level in xylem rapidly declines at onset of senescence as it has been observed in sorghum cultivar. CK biosynthesis can be also particularly localized in leaves, which suggests that the effect of CKs in senescence inhibition can be caused by exogenous applications, as well as by endogenous developmental program (Zwack and Rashotte, 2013).

In *Arabidopsis*, some specific components were identified to regulate the leaf senescence, including AHK3 receptor, B-ARR response regulators and CK response factor (CRF6). The CK signaling pathway is described as two-component system related to bacteria. AHK3, B-ARR and CRF6 are expressed in vascular tissue of leaves and all of them play a crucial role in regulation of senescence process. The function of AHK3 in this system is to mediate the response of delay senescence in plants, which is partially dependent on phosphorylation or activation of B-ARR genes. The delay of dark induced senescence was observed in plants with resistant B-ARR gene and with extremely induced CRF6 factor. Thus, it suggested that B-ARR and CRF6 can have a potential function as response transcriptional regulators during senescence program (Zwack and Rashotte, 2013).

Recently, the effect of naturally occurring, as well as synthetically modified CKs in senescence and their role in antioxidant defense and photosynthesis was reviewed (Hönig et al., 2018).

6. Chemical synthesis of purine nucleosides

The origin of chemical synthesis aimed at preparation of nucleosides was dated at the beginning of 20th century, when the method of silver salt attached to tetra-*O*-acetyl-α-D-glucopyranosyl bromide to yield 9-(β-D-glucopyranosyl)adenine and 9-(β-D-glucopyranosyl)guanine was reported (Fischer and Helferich, 1914). Subsequently, the synthesis based on condensation of chloromercury-6-benzamidopurine with 2′,3′,5′-tri-*O*-acetyl-D-ribofuranosyl chloride to form adenosine were described (Davoll and Lowy, 1951). Since, various strategies for purine nucleosides have been reported to create wide spectrum of affective, specific, nontoxic antitumor or antiviral agents formed by modification of naturally occurring analogues.

The most common pathways are presented in **Fig.10** with β-D-ribose as a representative example.

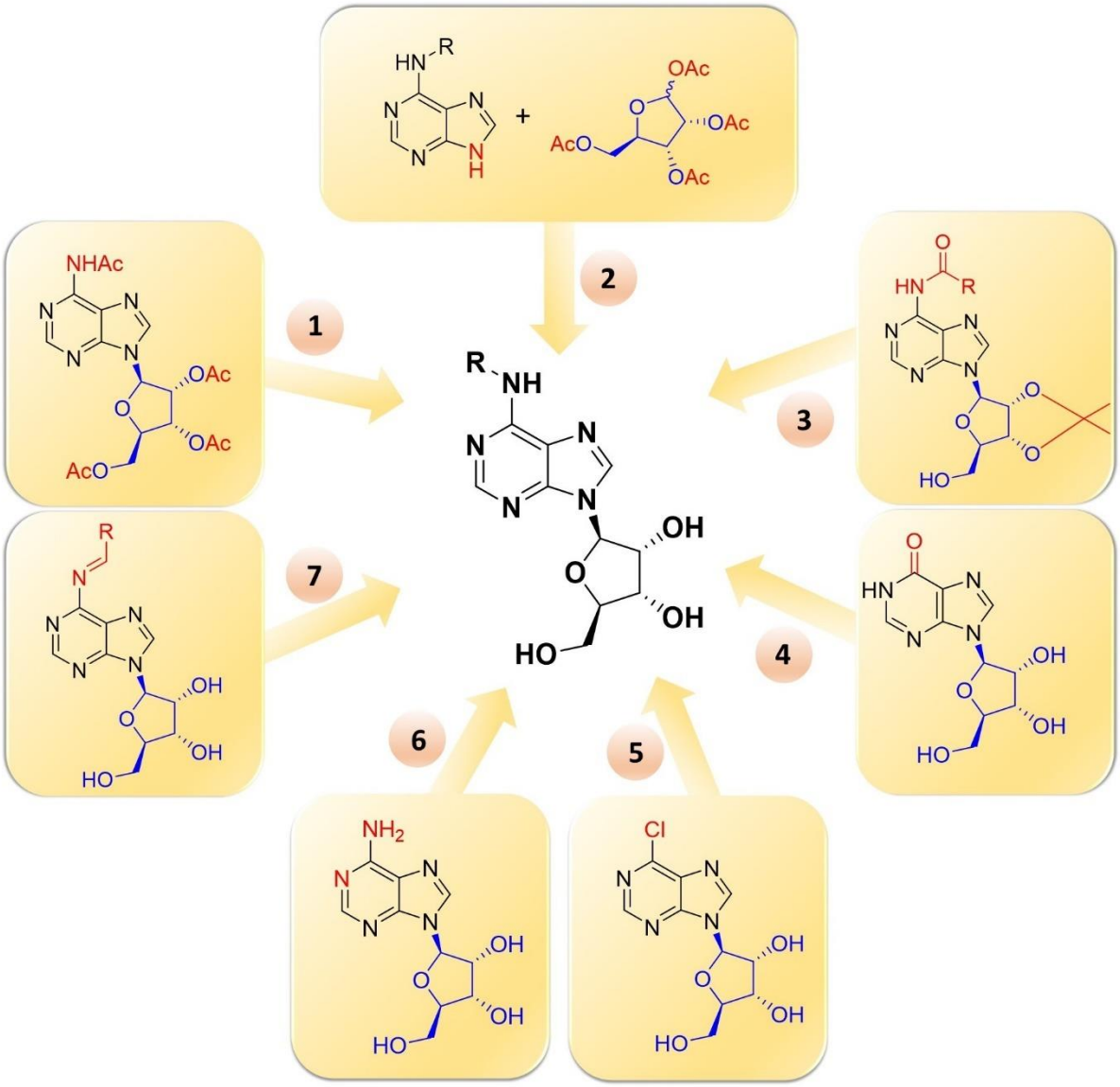
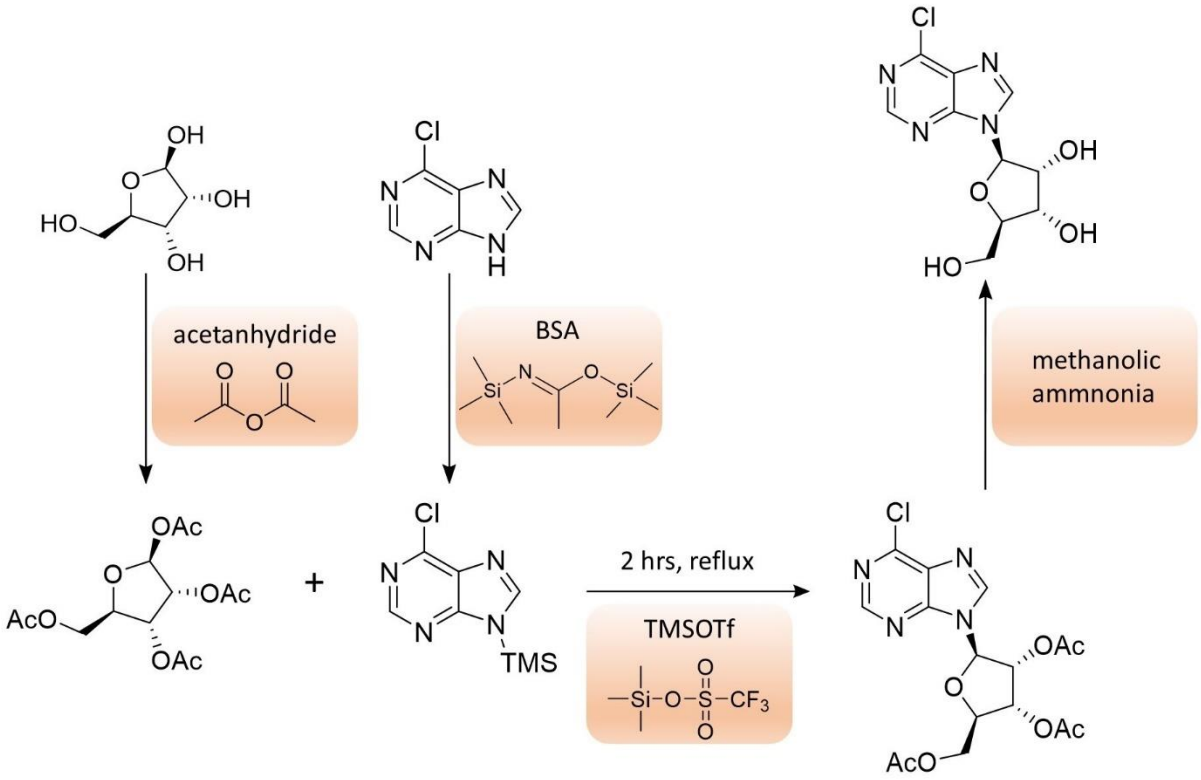


Fig.10: *The most common pathways for synthesis of N^6-substituted purine nucleosides, with β-D-ribofuranoside as representative example. Pathway* ***(1)*** *radioselective alkylation of N^6-acetyl-2′,3′,5′-tri-O-adenosine,* ***(2)*** *N-glycosylation of N^6-substituted purine,* ***(3)*** *the reduction of N^6-acyladenosines,* ***(4)*** *condensation of inosine with appropriate amines,* ***(5)*** *nucleophilic substitution of appropriate amines with 6-chloropurine riboside,* ***(6)*** *Dimroth rearrangement of 1-N-alkylated adenosine and* ***(7)*** *reduction of adenosine aldimines (edited) (Drenichev et al., 2016b).*

The simple and effective regioselective N^6-alkylation **(1)**, using N^6-acetyl-2′,3′,5′-tri-*O*-acetyladenosine under base-promoted or Mitsunobu conditions was published as an alternative method for synthesis of CK nucleosides. Especially, Mitsunobu conditions are preferable for synthesis of hydroxy derivatives without protection of their –OH group. Generally, the method is based on the easy conversion of N^6-acetyl-2′,3′,5′-tri-*O*-acetyladenosine to corresponding N^6-analogues, subsequently treated by alkyl halides in presence of 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU) or K_2CO_3 as catalyst with total yield over 80% (Tararov et al., 2011).

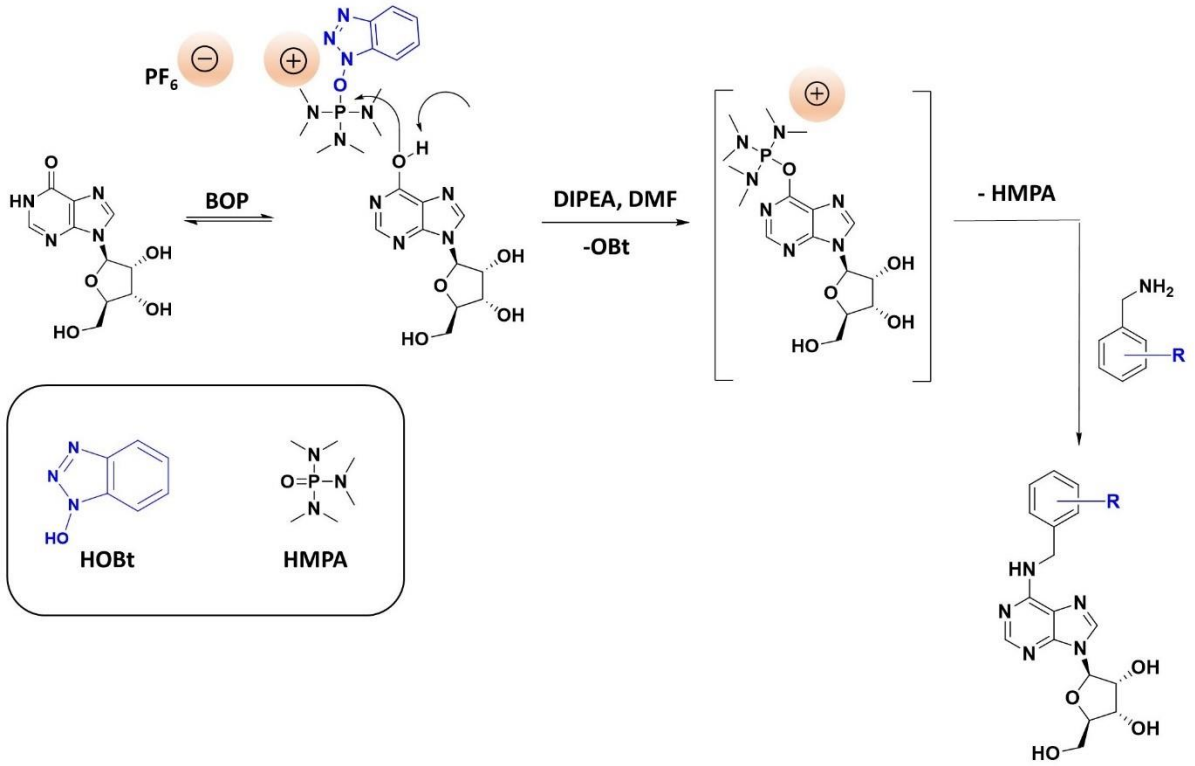
N-glycosylation **(2)** is based on the direct coupling of nucleobase with sugar moiety a and it has been divided into three types: acid-catalysed fusion of nucleobases with peracetylated sugar, the reaction of metal salt of heterocyclic moiety with protected sugar halides and Vorbrüggen method of silylation also known as Hilbert-Johnson silylation (Liu et al., 2015). The Vorbrüggen glycosylation was first reported by Vorbrüggen and Niedballa in 1970. It is usually performed in acetonitrile or dichlorethane in presence of TMSOTf or SnCl4 as acidic component to give corresponding nucleosides as a mixture of regioisomers in low yield, but it can be increased by carrying out the reaction at high temperature (Wang, 2010). Although, the Vorbrüggen method is widely used as a predominant route for synthesis of nucleosides, it is still keen to develop new strategies for various purine acceptors and sugar donors under mild conditions. For example, the modified facile synthesis to form adenine nucleosides using peracetylated sugars **(scheme 2)** was published by Ando et al. (Ando et al., 2007).



Scheme 2: *The general scheme of glycosylation of acetylated β-D-ribose with silylated 6-chloropurine to form β-anomer of acetylated ribosyl 6-chloropurine derivative exclusively and followed by deacetylation of desired product (edited) (Ando et al., 2007).*

The three- step procedure depending on reduction of N^6-acyladenosines was published in 1996 **(3)**. The starting N^6-acylated-2′, 3′-O-isopropylidene adenosine is reduced in presence $LiAlH_4$ by N^6-alkylated adenosine, followed by cleavage of the isopropylidene protecting group and the final product is crystalized from methanol in yield about 30% (Lescrinier et al., 1996). The method of reduction of N^6-substituted nucleosides by aldimines with sodium borohydride **(7)** seems to be a good alternative to the increase of the yield. The unprotected nucleobases react with aryl or alkyl aldehydes to give corresponding Schiff bases and they are subsequently reduced to desired compounds with high yield (Adamska et al., 2012). The reaction of fully silylated inosine with amines **(4)** in the presence of Lewis acid was firstly published by Vorbrüggen et al. (Vorbruggen and Krolikiewicz, 1976). Lately, this method has been modified to lead the activation of protected inosine in the presence of triphenyl phosphine, I_2 and DIPEA to form phosphonium salt which react with amines in good yield (Lin and Robins, 2000). A high facile one-step synthesis depending on the treatment of inosine with alkyl or arylamines was developed for N^6-substituted adenosines and their 2′-deoxy derivatives **(scheme 3)**. The use of nucleosides with unprotected hydroxyl

groups of sugars is the biggest benefit of this way. The reaction is enhanced by the presence of BOP and DIPEA in DMF and after purification the final product is obtained in very good yield (Wan et al., 2005).

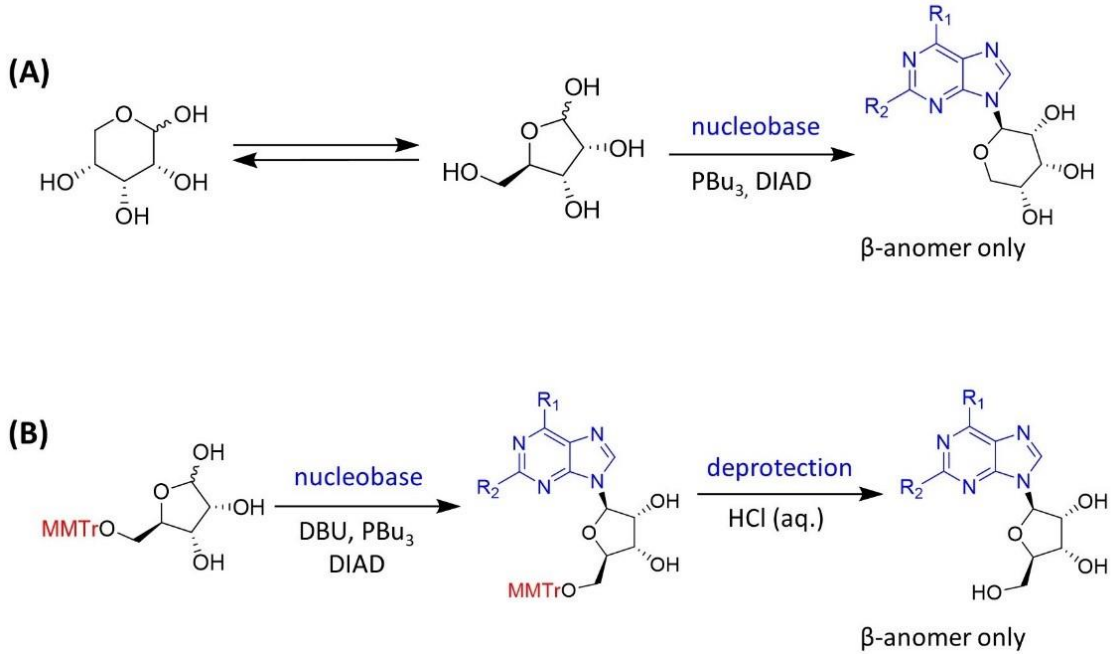


Scheme 3: *The mechanism of one-step synthesis based on the treatment of inosine with various substituted benzylamines (edited) (Matušková et al., 2020).*

The various N^6-substituted nucleosides can be also prepared by nucleophilic substitution of 6-chloropurine nucleoside with appropriate alkyl or aryl amines in the presence of triethylamine **(5)** and the targeted product is obtained by crystallization. Depending on the amine reactivity, the reaction yield is above 70% (Fleysher et al., 1968). For the reaction with low-reactive aryl amines, the slightly modified way with *O*-acetyl-protected 6-bromopurine nucleoside can be used, as well as the method using palladium acetate as a catalyst (Thomson et al., 2010; Véliz and Beal, 2001). The 1-N-alkylation of adenosines by Dimroth rearrangement under basic conditions was published as one of the first ways to prepare N^6-substituted sugar conjugates **(6)**. The mechanism is based on the alkylation of starting nucleoside with methyl iodide to form 1-N-methylnucleoside and manage N^6-methylnucleoside in high yield. Alternatively, 1-N-alkylation of 2′,3′,5′-tri-*O*-acetyladenosine with alkyl halides in presence of potassium iodide and barium

carbonate also afford desired product, but using of alkyl halides can limit this method (Jones and Robins, 1963; Oslovsky et al., 2015).

Despite the number of existing strategies to synthesized nucleosides, new approaches are still investigated. For example, the novel method of glycosylation for nucleoside synthesis using Mitsunobu conditions and unprotected or 5-*O*-monoprotected ribose **(scheme 4)** was presented, to give preferably β-ribopyranosyl nucleosides in good yield. The method is applicable for purine, as well as for pyrimidine based heterocycles, and the β-anomer is exclusively formed (Downey et al., 2015). In the last decade, the synthesis using microwave radiation becomes popular, especially to reduce reaction time and solvent consumption and recently the non-solvent method has been also reported (Grimaldi et al., 2020). The strategies using microwave-assisted organic synthesis under control conditions were summarized by Elgemeie et al. in 2019 (Elgemeie and Mohamed, 2019).



Scheme 4: *The mechanism of novel direct one-pot synthesis of purine nucleosides with possible substitution at C2 and N^6 position of purine moiety under Mitsunobu conditions. The reaction was performed with unprotected D-ribose* ***(A)*** *or with 5-O-monoprotected D-ribose* ***(B)****, where 4-methoxytrityl (MMTr) was used as the protecting group (edited) (Downey et al., 2015).*

7. Cytokinin nucleosides with fluorinated carbohydrate moiety

Fluorinated analogues of natural biomolecules are interesting substances for many research fields, because the replacement of hydrogen or hydroxyl group often increases the biological activity compared to their non-fluorinated counterparts and therefore these derivatives are investigated for their unique biological properties (Wójtowicz-Rajchel, 2012). The incorporation of fluorine into biological active molecules is bases on its physico-chemical properties such as very similar size of fluorine and hydrogen atom. As the most electronegative elements, fluorine can serve as isosteric or isopolar analogue of hydroxyl group – the length of C-F bond is close to the C-O bond. Also, the strength of C-F bond comparing with C-H bond increases biological and chemical stability of these derivatives (Liu et al., 2008). Hence, fluorine atom incorporation to sugar structure of nucleoside causes only negligible shape changes or structure modification. Because of biological activity and unique properties of fluorinated nucleosides in many research areas, the variety of these molecules rapidly grew. The chemistry of fluorinated compounds is currently connected with many fields, such as pharmacy, biomedical applications, medicine or agrochemistry, which represent the importance advances in this topic (Tressaud, 2019).

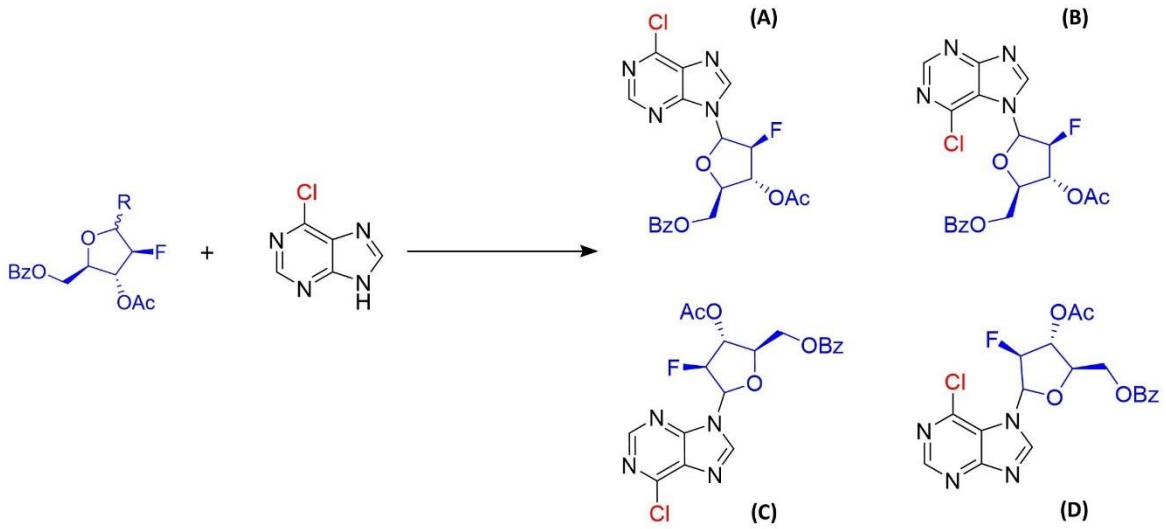
7.1. Fluorinated purine nucleosides in human diseases therapy

The nucleosides with fluorinated substituents or bearing fluorine within sugar moiety are commonly used in many therapeutical treatments, especially as antiviral or anticancer drugs. The significant biological effect was demonstrated for 2-deoxy-2-fluoropurine riboside and its derivatives, these compounds were characterized as antiviral (Maruyama et al., 1994), anticancer (Tisdale et al., 1993), antileukemic (Thomas et al., 1994) and antiinfluenza agents (Tuttle et al., 1993). The fluorinated arabinoside derivatives were also proved as effective agents against a wide range of human diseases. As the most potent compound from this family seems to be 9-(2-deoxy-2′-fluoro-β-D-arabinofuranosyl)adenine, which was evaluated as antiviral (Ma et al., 1997), antileukemic (Shokar et al., 2012), antiprotozoal (Ranjbarian et al., 2017; Shokar et al., 2012), as well as antimicrobial agent (Gao et al., 2015). The detailed summary of biological properties of fluorinated nucleosides was published by many authors (Elzagheid, 2016; Liu et al., 2008; Wójtowicz-Rajchel, 2012). Despite the wide range of their applications described above, their biological effect in plant species was

first investigated in the presented work (Bryksová 2020, supplementary material III in attachment).

7.2. Synthesis of fluorinated purine nucleosides

The unique chemical, as well as biological properties of fluorinated carbohydrates started a new era of developing synthetic procedure in order to prepare new analogues with chemotherapeutical potential. The origin of fluorination in nucleoside chemistry is dated at the beginning of 1960s, when 2´-fluoro-2´-deoxyuridine was synthesized as the first nucleoside with fluorinated carbohydrate moiety (Codington et al., 1961), but the first method for synthesis of 2-fluoropentose from pentoside precursor following reaction with 2,6-dichloropurine to form 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyI)adenine was reported lately (Wright et al., 1969). In order to simplify this method, the direct condensation 3-*O*-acetyl-5-*O*-benzoyl-2-deoxy-2-fluoro-β-D-arabinofuranosyl bromide with N^6-benzoylaminopurine to form F-ara-A as the desired product were described. The same authors also developed the strategy for preparation of its hypoxanthine analogue by direct deamination of F-ara-A (Chu et al., 1989). Concurrently, the efficient conversion of aristeromycin into its 2´-fluoro-arabino-analogue in presence of DAST it was reported. Starting aristeromycin is easily available from fermentation of bacteria (*Streptomyces citricolor*) and is converted to target product by four-step synthesis (Biggadike et al., 1988). Lately, the new strategies focused especially at the preparation of nucleosides with fluorinated sugar moiety have been reported. For example, the condensation of 6-chlorpurine with 2´-deoxy-2´-fluoro-β-D-arabinofuranosyl bromide and follow conversion of purine to adenine was used for preparation of F-ara-A, but the presence of four isomers mixture decreased the yield of the desired product **(scheme 5)** (Marquez et al., 1990).



Scheme 5: *The reaction scheme reported by Marquez et al. Instead of desired 9β isomer* ***(A)****, but also the other isomers are presented - 7β* ***(B)****, 9α* ***(C)*** *and 7α* ***(D)*** *(edited) (Marquez et al., 1990).*

The practical three-step synthesis of 2′-fluoro-2′-deoxyarabinofuranosyl purines (F-ara-A and F-ara-H) was reported via displacement of 2′-OH group of $O^{3'}$, $O^{5'}$-ditrytilinosine and $O^{3'}$, $O^{5'}$, N^6-tritrityladenosine with diethylaminosulfur trifluoride (DAST) followed by deprotection to give the target product in good yield (Pankiewicz et al., 1992). The slightly modified strategy was used for synthesis of 6-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)purine. The desired product was obtained from 2′-deoxy-2′-fluoroarabinoside by hydrolysis with HCl in the mixture of dichloromethane and dioxane (Maruyama et al., 1996). In the last decade, some authors have also focused on chemoenzymatic methods for synthesis of 2′-fluoro-2′-deoxyarabinosnucleosides. For example, the method developed especially for synthesis of F-ara-A was published by Yamada et al. This stereoselective process is based on the hydrolysis of 1-bromo derivative and subsequent phosphorylation of 2-deoxy-2-fluoro-α-D-arabinofuranose to form corresponding α-1-phospate utilized for preparation of the desired product by enzymatic glycosylation reaction. In this step, purine nucleoside phosphorylase (PNPase) obtained from *Bacillus stearothermophilus* was used as a catalyst (Yamada et al., 2009). The newest approach for synthesis of F-ara-A accomplished in good yield was reported in 2016. Commercially available N^6-pivaloyladenine was glycosylated in acetonitrile in the presence of calcium hydride and the mixture of α/β anomers was formed in the 1:14 ratio. On the other hand, by a similar reaction with N^6-benzoyladenine as an alternative, the α/β mixture in ratio 1:10 is obtained (Sivets, 2016). Despite the number

of synthetic strategies mentioned above, the new approaches are still investigated in order to develop a new generation of fluorinated drugs with therapeutic potential.

Material and methods

The material and method summary including detailed description of all experimental approaches used in this work is located in the attached publications.

General procedures

The chromatographic purity and mass spectra of the synthesized compounds were obtained using the HPLC-PDA-MS method. Samples were dissolved in methanol (10 µl of 3.10^{-5} M in 1% methanol) and injected onto a reverse-phased column (Symmetry C18, 5 µm, 150 mm × 2.1 mm; Waters, Milford, MA, USA) incubated at 40 °C. Solvent A contained of 15 mM ammonium formate ($HCOONH_4$) adjusted to pH 4.0, solvent B contained of methanol and flow-rate was set to 0.2 mL/min. The samples were analysed using gradient: 0 min, 10 % of B; 25 min; 90 % of B; 35 min; 90 % of B; 45 min; 10 % of B in *Waters* Alliance *2695* Separations *Module* (Waters, Manchester, UK). Subsequently, the effluent was introduced to the Waters 2996 PDA detector (Waters, Manchester, UK) (scanning range 210–400 nm with 1.2 nm resolution) and *QDa Mass* Spectrometer (Waters MS Technologies, Manchester, UK) with an ESI system. The cone voltage was set to 15 V, ion source was heated to 120°C and the measurement was performed in the positive mode (ESI+) or negative mode (ESI-) therefore molecular ions were recorded as $[M + H]^+$, $[M-H]^-$ or ESI adduct ions. ^1H NMR spectra were measured on the Jeol 500 SS spectrometer operating at the temperature of 300 K and the frequency of 500.13 MHz. The samples were prepared by dissolving in DMSO-d_6. Tetramethylsilane (TMS) was used as an internal standard. Thin-layer chromatography (TLC) was carried out using silica gel 60 WF_{254} plates (Merck) and $CHCl_3$/MeOH (4:1, v/v) were used as the mobile phase. Purification *via* column chromatography was obtained using silica gel Davisil R LC60A 40-63 micron.

HPLC-MS purification

Preparative HPLC-MS chromatography machine (Agilent 1290 Infinity II) was used coupled with UV-VIS detector with a mass LC/MSD detector (Agilent InfinityLab) and Agilent Prep-C189 column (5 µm, 21.2 mm x 50 mm, Waters, Milford, MA, USA) to obtain the final product. The analyzed sample was dissolved in 50% MeOH before injection. The mobile phase MeOH (A):H_2O (B) with a flow rate of 20 $mL.min^{-1}$ and linear gradients (0 min, 10% B; 0–12 min; 90% B) was used.

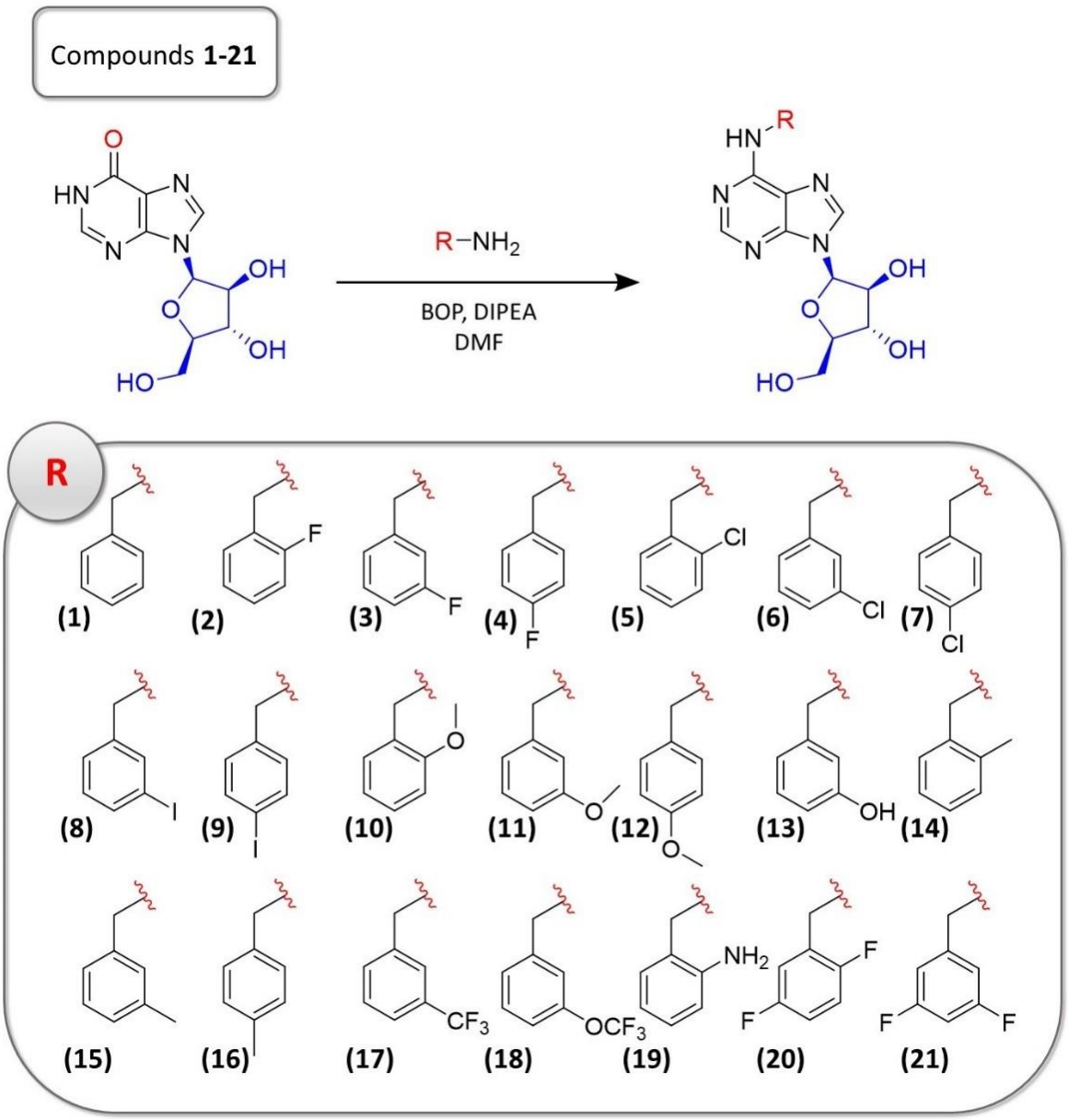
HRMS conditions

The prepared samples (5 μL) were characterized using the HPLC-PDA-MS method. The samples were injected onto a reversed-phase column (Symmetry C18, 5 μm, 150 mm × 2.1 mm; Waters, Milford, MA, USA) incubated at 40°C. Solvent A was 15 mM ammonium formate adjusted to pH 4.0. Solvent B was methanol. The following linear gradient was used at a flow rate of 250 μL min^{-1}: 0 min, 10% B; 0–15 min, 90% B. The effluent was introduced to a DAD detector (scanning range 210–400 nm with 1.2 nm resolution) and then to an electrospray source (source temperature 150°C, desolvation temperature 550°C, capillary voltage 1 kV, cone voltage 25 V). Nitrogen was used as the cone gas (50 L h^{-1}) and the desolvation gas (1000 L h^{-1}). Data acquisition was performed in the full-scan mode (50–1000 Da) with a scan time of 0.5 s and collision energy of 4 eV; argon was used as the collision gas (optimized pressure of 5×10^{-3} mbar). Analyses were performed in the positive mode (ESI^+) and protonated molecules $[M+H]^+$ were collected in each MS spectrum. For exact mass determination experiments, external calibration was performed using lock spray technology and the mixture of leucine/encephalin (50 pg μL^{-1}) in an acetonitrile and water (1:1) solution with 0.1% formic acid as a reference sample. Accurate masses were calculated and used to determine the elemental composition of the analytes with fidelity better than 1.0 ppm.

Synthetic procedure I.

<u>*N*^{6}-substituted purine-9-β-D-arabinofuranoside derivatives **(1-21) (scheme 6)**</u>

The starting hypoxanthine-9-β-D-arabinofuranoside and BOP (1.2 equiv.) were dissolved in dry DMF following by DIPEA (1.5 equiv.) and appropriate benzylamine (1.2 equiv.) as the final component. The reaction mixture was heated at 55-60°C under argon atmosphere and stirred for 24 hours as summarized in the reaction scheme. The crude mixture was evaporated on the vacuum rotary evaporator and the final gel was formed. The distillation residue was mixed with MeOH and a drop of $CHCl_3$ and mixture was sonificated. The resulting white solid powder was recrystallized using EtOH. The process is described in detail in Bryksová et al. 2020 (Bryksová et al., 2020).



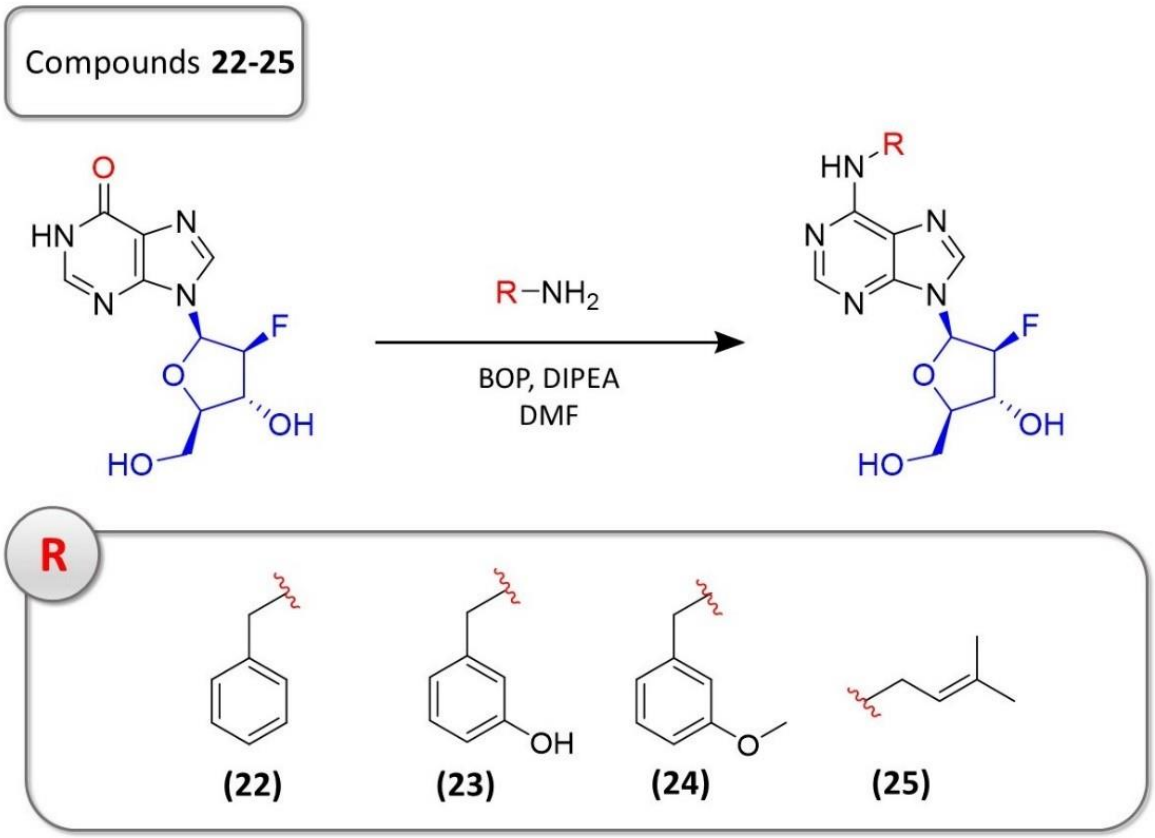
***Scheme 6**: Synthesis of N^6-substituted purine-9-β-D-arabinofuranoside derivatives. The synthesis was performed in DMF with the presence of BOP and DIPEA to form the desired product (55-60°C, 24 hrs).*

Synthetic procedure II. – fluoro derivatives

<u>N^6-substituted purine-9-(2′-deoxy-2′-fluoroarabinofuranoside) derivatives **(22-25) (scheme 7)**</u>

The 9-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl) hypoxanthine and BOP (1.2 equiv.) were mixed and dissolved in dry DMF under argon atmosphere and subsequently DIPEA (1.5 equiv.) and appropriate amine (1.2 equiv.) were added to the reaction mixture. The mixture was heated in oil bath at the temperature of 55-60°C for 24 hours. After evaporation on the vacuum rotary

evaporator, the specifically colored gel was formed. The crude residue was purified by column chromatography (**22** and **24**) or preparative HPLC (**23** and **25**) to give the required product. After column chromatography, compounds **22** and **24** were recrystallized in the mixture of various solvents (Bryksová 2020, supplementary material III in attachment).



Scheme 7: *Synthesis of N^6-substituted purine-9-(2′-deoxy-2′-fluoro-β-D-arabinofuranoside) derivatives. The synthesis was performed in dry DMF with the presence of BOP and DIPEA to form the required product (55-60°C, 24 hrs).*

Synthetic procedure III. – L-ribosides

N^6-substituted purine-9-β-L-ribofuranoside derivatives **(26-31)**

The starting β-L-inosine and BOP (1.2 equiv.) were dissolved in dry DMF and DIPEA (2 equiv.) and appropriate amine (1.2 equiv.) followed. The reaction mixture was heated at 60°C in oil bath and stirred under argon atmosphere for 24 hours. The reaction mixture was evaporated on vacuum rotary evaporator to give specifically colored gel. The resulting residue was precipitated or purified by column chromatography. The reaction procedure is described and summarized in detail in the section of unpublished results.

Biological activity

The biological activity of prepared compounds was evaluated in three classical cytokinin bioassays previously reported by Holub et al. (Holub et al., 1998) and BAP was used as positive control for all three bioassays. The results were recorded as the highest activities of tested derivatives for the tested concentration range from 10^{-8} to 10^{-4} M solution in DMSO.

Amaranthus bioassay

Amaranthus caudatus var. *atropurpurea* seeds were sterilized on their surface (10 min with 10% sodium hypochloride, washed with 0.5 L water, 10 min with 70% ethanol, washed with 0.5 L water), placed on a Petri dish containing paper tissue soaked with deionized water and cultivated at 24°C for 72 hours in the dark. Under green safe light in a dark room, roots were removed from the seedlings and clean residues, consisting of two cotyledons and a hypocotyl, were placed on a Petri dish (25 explants per dish) containing filter paper soaked with 1 mL of incubation medium consisting of 10 mM Na_2HPO_4/KH_2PO_4 (pH 6.8), 5 mM tyrosine, and the test compound (from 10^{-8} to 10^{-4} M solution in DMSO). The dishes were cultivated at 24°C for 48 h in the dark, followed by extraction of the resulting betacyanin by repeated freezing and thawing (three times) of the plant material in 4 mL of 3.33 mM acetic acid. The concentration of betacyanin was determined from the difference between absorbances at 537 and 625 nm.

Wheat leaf senescence bioassay

Wheat seeds (*Triticum aestivum* L. cv. Aranka) were washed in 96% ethanol, 1 h under running water, sown in verticulite soaked with Hoagland solution and then grown in a cultivation chamber (light/dark period = 16 h/8 h; 7000 lx) at 22 °C for 7 days. Tip cuttings of fully developed first leaves (3.5 cm long) were taken (four pieces were combined to give a total weight of 0.1 g (± 1 mg) per well), immersed by the basal part in a well containing test compound (150 µL/well), and cultivated in a closed plastic box containing moist paper tissue at 24°C for 96 h in the dark. Residual chlorophyll was extracted by heating the leaf material in 5 mL of 80% (v/v) ethanol at 80°C for 10 min. The absorbance at 665 nm was measured and the values were compared with values from the extracts of fresh leaves (stored at -80 °C after detachment) and the extracts of leaves cultivated in deionized water.

Tobacco callus bioassay

Cytokinin-dependent tobacco callus cells (*Nicotiana tabacum* L. cv. Wisconsin 38) were cultivated on solid MS medium (3 mL/well) containing different concentrations of the test compound (from 10^{-8} to 10^{-4} M solution in DMSO) in six-well plates (0.1 g of callus divided into 3 pieces per well) at 24 °C for 4 weeks in the dark. The biological activity of each test compound was determined as an increase in the callus fresh weight compared to a positive control (BAP).

Cell viability assay

The cytotoxicity measurement using the resazurin reduction assay on cancer cell lines, as well as human skin cells was proved as previously described (Jorda et al., 2019).

The bioassay was performed by doc. RNDr. Vladimír Kryštof, Ph.D. and his co-workers at Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany, The Czech Academy of Sciences, Olomouc, Czech Republic.

Photosynthetic activity of detached wheat and *Arabidopsis* leaves

The experiment used spring wheat and *Arabidopsis* plants grown under 16-hour-light/8-hour-dark cycle for 7 days on artificial medium containing perlite and Hoagland´s solution. The leaf segments were placed in 100 nM, 1 μM or 10 μM solution of BAP, 3-methoxyBAPA or 3-hydroxyBAPA dissolved in 0.1% DMSO, segments were kept in the dark at 24°C for 6 days and subsequently, the chlorophyll parameters were measured.

The bioassay was performed by doc. RNDr. Martina Špundová, Ph.D. and Mgr. Zuzana Kučerová at Department of Biophysics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Olomouc, Czech Republic.

RNA-seq analysis

The standard protocol for Illumina TruSeq Stranded mRNA Sample Preparation Kit (Illumina) was used for generation of two independent libraries. Prepared replicates were validated by DNA 1000 Chip (2100 Bionalayzer, Agilent Technology). Libraries were subsequently

pooled to a final concentration of 12 pM for cluster regeneration a sequencing. For mapping and quantification, the *Arabidopsis* reference genome was used.

RNA-seq analysis was carried out by RNDr. Ondřej Plíhal, Ph.D. at Department of Molecular Biology, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science and Laboratory of Growth Regulators, Institute of Experimental Botany of the Czech Academy of Sciences, Faculty of Science, Palacký University, Olomouc, Czech Republic.

Plant phenotyping – rosette growth of seedling from *Arabidopsis* hormoprimed seeds

The prepared compounds were tested as priming agents under optimal and two different stress conditions including salt and osmotic stress. The *Arabidopsis* seeds were sterilized and germinated. During germination, the tested compounds were added to the medium at four different concentrations from 10^{-7} to 10^{-4} M. After three days germination, the seeds were tested by using various conditions (MS medium, with 100mM NaCl or 100mM mannitol) in 7-day light/dark cycles. By imaging of all plates, the traits among seeds were determined and PBC index was calculated.

The plant phenotyping was performed by Dr. Nuria de Diego and her co-workers at Department of Chemical Biology, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Olomouc, Czech Republic.

Determination of *Arabidopsis* rosette colour indices

The greenness of *Arabidopsis* seeds and leaf colour changes were evaluated by three vegetation indices. The pictures were captured after 7 days of the *Arabidopsis* rosette growth assay and vegetation indices were calculated. Subsequently, indices representing particular seedlings were calculated for each plant mask and the standard error was determined and represented in graph.

The bioassay was performed by Dr. Nuria de Diego and her co-workers at Department of Chemical Biology, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Olomouc, Czech Republic.

Plant hormone quantification and statistical analysis

Four independent biological replicates were quantified, and their concentrations were calculated by using standard isotope dilution method. The plant tissue samples were purified by micro solid-phase extraction. Cytokinins were quantified by using ultra-high performance liquid chromatography-electrospay tandem mass spectroscopy. Auxins and abscisic acid were performed, and their concentrations were calculated using standard isotope dilution method on Infinity II system coupled with Triple Quadrupole LC/MS system. The statistical data were evaluated using the packages *multcomp, FSA* and *agricolae* in RStudio.

The plant hormone quantification and statistical analysis were carried out by doc. Mgr. Ondřej Novák, Ph.D. and Mgr. Aleš Pěnčík, Ph.D. at Laboratory of Growth Regulators, Centre of the Region Haná, for Biotechnological and Agricultural Research, Faculty of Science, Palacký University & Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Olomouc, Czech Republic.

Survey of published results

Publication I.

<u>Bryksová, M.</u>, Dabravolski, S., Kučerová, Z., Zavadil Kokáš, F., Špundová, M., Plíhalová, L., Takáč, T., Grúz, J., Hudeček, M., Hloušková, V., Koprna, R., Novák, O., Strnad, M., Plíhal, O., Doležal, K.: Aromatic cytokinin arabinosides promote PAMP-like responses and positively regulate leaf longevity. ACS Chem Biol, 2020, 15, 1949-1963.

CKs belong to the group of plant growth regulators with ability to control biotic and abiotic stress-related responses and senescence. A series of CK arabinoside derivatives was synthesized and compounds were characterized by physico-chemical methods and their CK-like activity was evaluated in three classical CK bioassays such as the *Amaranthus*, senescence and tobacco callus assay. The significant anti-senescence activity was observed for variety of them. Two derivatives, 3-hydroxy-BAPA and 3-methoxy-BAPA have been proved to be the most effective in delaying senescence while having low interactions with the CK pathway. Moreover, RNA-seq profiling study showed that by 3-methoxy-BAPA treatment on *Arabidopsis* leaves, the transcriptional response is markedly shifted toward defense. This treatment causes the upregulation of genes associated with plant immunity as well as cell wall remodeling and upregulation of specific mitogen-activated protein (MAP) kinases, especially MPK11, which is connected with stress-related signaling during pathogen-associated molecular patterns (PAMP) response. In addition, the increasing level of jasmonic acid and its metabolites, elevated level of plant defensins and the temporarily increased level of reactive oxygen forms have been observed after 3-methoxy-BAPA treatment. On the other hand, the plants treated by 3-hydroxy-BAPA together with flagellin-derived bacterial PAMP peptide showed the enhanced expression of pattern –triggered immunity (PTI) marker gene FRK1. These results show some BAPA derivatives as sensitive prime PTI responses in a low concentration range with no negative effect on the overall plant fitness.

Publication II.

Vylíčilová, H., Bryksová, M., Matušková, V., Doležal, K., Plíhalová, L., Strnad, M.: Naturally occurring and artificial N9-cytokinin conjugates: From synthesis to biological activity and back. Biomolecules, 2020, 10, 1-29.

Sugar and non-sugar CK conjugates are very active factors in plants. Although they occur at very low concentrations, their ability to promote plant growth was repeatedly proved. The role of N9-substituted aminopurine- based CK conjugates in plants has been summarized and their biological activity in various bioassays was evaluated. Despite the fact, that N9-substituted CKs are endogenously occurring, the majority of them have been synthetically prepared to clarify the interaction between their chemical structure and biological properties. Due to current advances in synthesis of phytohormones, it is possible to prepare CK derivatives with targeted biological properties, such as the antisenescence effect, shoot formation or cell division stimulation. Additionally, many of them improve rooting or stimulate mass production higher than naturally occurring CKs. Hence, the knowledge about the CK role in plant species extends their field of use in plant biotechnology, agriculture and tissue cultures.

Publication III. (submitted manuscript)

Bryksová, M., Hybenová, A., Esteban Hernandiz, A., Novák, O., Pěnčík, A., Spíchal, L., De Diego, N., Doležal, K.: Hormopriming for mitigating abiotic stress affects: A case study of N9-substituted cytokinin derivatives with fluorinated carbohydrate moiety, 2020, submitted manuscript.

The drought and salinity lands are factors depending on global climate change with the influence on already-stressed agricultural ecosystems. The stressed plants reduce seed germination, seedling establishment to affect plant metabolism, which reduce crop yield and therefore new biotechnological approaches to increase plant stress tolerance are studied. One of them, CK activated seed priming has an ability to reduce the negative factors of environmental stresses. BAP is the most widely applied CK in biotechnology for senescence delay and stress factors reducing, however its application is connected with some negative effects, especially fast N9-glucosylation, which makes the molecule almost inactive and it can be suppressed by appropriate N9-substitution. Also, the replacement of hydroxyl groups of N9-substituted carbohydrate with a fluorine enhanced biological activity, but the effect of exogenous application of these fluorinated derivatives on plant fitness never been

investigated. In this study, four derivatives have been characterized as significant hormopriming agents due to their antisenescence properties.

Publication IV. (book chapter)

Doležal, K., Bryksová, M.: Topolin metabolism and its implications for in vitro plant micropropagation.

The BAP is highly active and affordable CK used in a wide range of plant biotechnology applications, but its disadvantages in certain crops were observed. Hence, naturally occurring *m*T has been discovered, developed and thoroughly tested as BAP alternative.

Publication V. (patent and patent application)

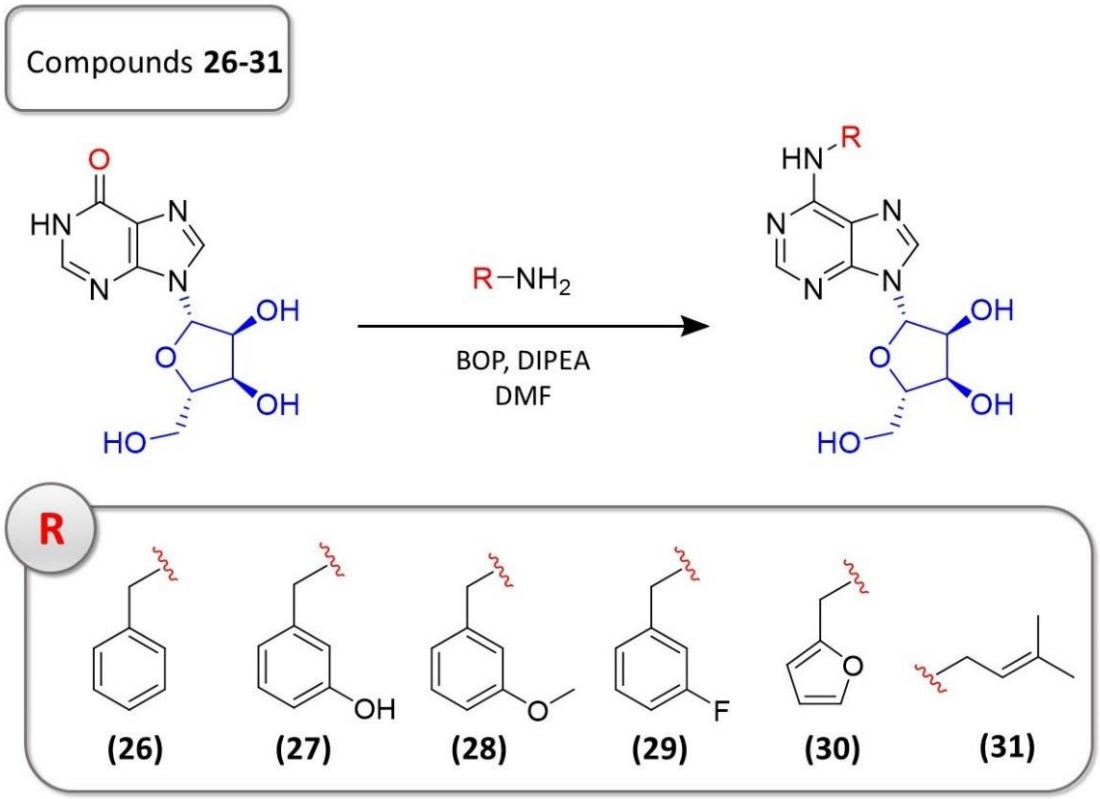
Doležal, K., Plíhalová, L., Vylíčilová, H., Zatloukal, M., Plíhal, O., Voller, J., Strnad, M., Bryksová, M., Vostálová, J., Rajnochová Svobodová, A., Ulrichová, J., Spíchal, L.: 6-aryl-9-glycosylpurines and use thereof. US patent US 10,100,077 B2, 2018.

A group of N9-substituted 6-benzylaminopurine arabinoside derivatives published in Bryksová et al. (Bryksova et al., 2020) is a part of the granted Czech patent CZ2014875A3, Singaporean patent SG11201704019TA, US patent US10100077B2 and PCT application WO2016091235A1 as compounds possessing antisenescence and UV protective properties in animal cells.

Survey of unpublished results

N^6-substituted purine-9-β-L-ribofuranoside derivatives

A series of non-toxic N^6-substituted benzylaminopurines-9-β-L-ribosides, as analogues of their highly active and cytotoxic β-D-counterparts, was synthesized by slightly modified synthesis **(scheme 8)** previously published by Wan et al. (Wan et al., 2005) and their physico-chemical properties, as well as their biological activity have been evaluated.



***Scheme 8**: Reaction scheme for synthesis of N^6-substituted purine-9-β-L-ribofuranoside derivatives (BOP, DIPEA, DMF, 60°C, 24 hrs).*

Preparation procedure

6-(benzylamino)purine-9-β-L-ribofuranoside **(26)**

β-L-inosine (100 mg, 1 equiv.) and BOP (198 mg, 1.2 equiv.) were dissolved in DMF (2 ml) followed by DIPEA (130 μL, 2 equiv.) and benzylamine (49 μL, 1.2 equiv.) as the last component were added. The reaction mixture was heated on 60°C in oil bath and stirred under argon atmosphere for 24 hours and controlled by TLC ($CHCl_3$/MeOH 4:1). The reaction

mixture was evaporated on vacuum rotary evaporator to give yellow gel. The resulting residue was precipitated by DCM and light yellow powder was filtrated and washed by Et_2O. The liquid filtrate was evaporated to gel and distilled water was added to precipitate white crystals of resulting products overnight. Yield: 29 %. HPLC purity 99.3 %. MS ESI+ m/z: 358 $[M+H]^+$.

^{1}H NMR (500 MHz, DMSO-d6) δ ppm: 3.80-3.99 (m, 2H), 4.12 (q, J=4.5Hz, 1H), 4.32 (q, J=5Hz, 1H), 4.57 (q, J=5.5Hz, 1H), 4.69 (bs, 1H), 5.28 (d, J=4Hz, 1H), 5.49-5.57 (m, 2H), 5.85 (d, J=6.5Hz, 1H), 7.17-7.32 (m, 6H), 8.17 (s, 1H), 8.33 (s, 1H), 8.39 (bs, 1H)

6-(3-hydroxybenzylamino)purine-9-β-L-ribofuranoside **(27)**

β-L-inosine (100 mg, 1 equiv.) and BOP (198 mg, 1.2 equiv.) were dissolved in DMF (2 ml) following by DIPEA (130 μL, 2 equiv.) and 3-hydroxybenzylamine (56 mg, 1.2 equiv.) at the last component were added. The reaction mixture was heated at 60°C in oil bath and stirred under argon atmosphere for 24 hours and controlled by TLC ($CHCl_3$/MeOH 4:1). The reaction mixture was evaporated on vacuum rotary evaporator to form dark brown gel residue. The residue was purified by column chromatography with mobile phase $CHCl_3$:MeOH and gradient 29:1 → 9:1 to give light yellow gel. The gel was mixed with distilled water and sonificated and white crystals of the desired product were precipitated and filtered. Yield: 23 %. HPLC purity: 98.8 %. MS ESI+ m/z: 374 $[M+H]^+$.

^{1}H NMR (500 MHz, DMSO-d6) δ ppm: 3.82-3.98 (m,2H), 4.12 (bs, 1H), 4.32 (q, J=4.5Hz, 1H), 4.51-4.68 (m, 3H), 5.29 (d, J=4.5Hz, 1H), 5.51 (d, J=6Hz, 1H), 5.56 (t, J=5Hz, 1H), 5.85 (d, J=6Hz, 1H), 6.57 (d, J=7.5Hz, 1H), 6.65-6.76 (m, 2H), 7.05 (t, J=8Hz, 1H), 8.17 (s, 1H), 8.33 (s, 2H), 9.39 (s, 1H)

6-(3-methoxybenzylamino)purine-9-β-L-ribofuranoside **(28)**

β-L-inosine (100 mg, 1 equiv.) and BOP (198 mg, 1.2 equiv.) were dissolved in DMF (2 ml) followed by DIPEA (130 μL, 2 equiv.) and 3-methoxybenzylamine (58 μL, 1.2 equiv.) at the last component were added. The reaction mixture was heated at 60°C in oil bath and stirred under argon atmosphere for 24 hours and controlled by TLC ($CHCl_3$/MeOH 4:1). The mixture was evaporated on vacuum rotary evaporator to form light yellow gel. The residue was purified by column chromatography with mobile phase $CHCl_3$:MeOH and gradient

29:1 → 9:1 to white powder. The white powder was recrystallized in EtOH to precipitate the desired product. Yield: 27%. HPLC purity: 99.7 %. %. MS ESI+ m/z: 388 $[M+H]^+$.

^{1}H NMR (500 MHz, DMSO-d6) δ ppm: 3.14 (d, J=5Hz, 3H), 3.82-3.98 (m, 2H), 4.12 (q, j=4.5Hz, 1H), 4.32 (q, j=5Hz, 1H), 4.57 (q, j=5.5Hz, 1H), 4.65 (bs, 1H), 5.28 (d, J=4.5Hz, 1H), 5.48-5.58 (m, 3H), 5.85 (d, J=5Hz, 1H), 6.73-6.90 (m, 3H), 7.18 (t, J=8Hz, 1H), 8.17 (s, 1H), 8.34 (s, 1H), 8.37 (bs, 1H)

6-(3-fluorobenzylamino)purine-9-β-L-ribofuranoside **(29)**

β-L-inosine (100 mg, 1 equiv.) and BOP (198 mg, 1.2 equiv.) were dissolved in DMF (2 ml) followed by DIPEA (130 µL, 2 equiv.) and 3-fluorobenzylamine (51 µL, 1.2 equiv.) as the last component were added. The reaction mixture was heated at 60°C in oil bath and stirred under argon atmosphere for 24 hours and controlled by TLC ($CHCl_3$/MeOH 4:1). The mixture was evaporated on vacuum rotary evaporator to form yellow gel. The gel was mixed with acetone and diethylether was added dropwise. The white crystal of the desired product was precipitated in the mixture, filtered and washed by distilled water and diethylether. After recrystallization overnight, the white crystals with high purity were filtered from solution. Yield: 29%. HPLC purity: 100%. MS ESI+ m/z: 376 $[M+H]^+$.

^{1}H NMR (500 MHz, DMSO-d6) δ ppm: 3.95-4.16 (m, 2H), 4.32 (q, J=5Hz, 3H), 4.58 (q, J=6Hz, 1H), 4.70 (bs, 1h), 5.29 (d, J=4.5Hz, 1H), 5.52 (d, J=6Hz, 2H), 5.86 (d, J=6.5Hz, 1H), 6.97-7.17 (m, 3H), 7.32 (q, J=8Hz, 1H), 8.18 (s, 1H), 8.36 (s, 1H), 8.45 (bs, 1H)

6-(furfurylamino)purine-9-β-L-ribofuranoside **(30)**

β-L-inosine (100 mg, 1 equiv.) and BOP (198 mg, 1.2 equiv.) were dissolved in DMF (2 ml) followed by DIPEA (130 µL, 2 equiv.) and furfurylamine (40 µL, 1.2 equiv.) as the last component were added. The reaction mixture was heated at 60°C in oil bath and stirred under argon atmosphere for 24 hours and controlled by TLC ($CHCl_3$/MeOH 4:1). The reaction mixture was evaporated on rotary evaporator to give brown gel. The gel was purified by column chromatography with mobile phase $CHCl_3$:MeOH (29:1→ 9:1) to give yellow foam. The foam was dissolved in MeOH and white powder was precipitated after sonification, filtered and washed by cold MeOH. Yield: 29%. HPLC purity: 98.3 %. MS ESI+ m/z: 348 $[M+H]^+$.

^{1}H NMR (500 MHz, DMSO-d6) δ ppm: 3.81-3.97 (m, 2H), 4.12 (q, J=4.5Hz, 1H), 4.32 (q, J=5Hz, 2H), 4.56 (q, J=6Hz, 1H), 4.67 (bs, 1H), 5.28 (d, J=4.5Hz, 1H), 5.52 (d, J=6Hz,2H), 5.85 (d, J=6Hz,1H), 6.22 (d, J=3Hz, 1H), 6.31-6.36 (m, 1H), 7.49 (bs, 1H), 8.12-8.30 (m, 2H), 8.34 (s, 1H)

6-(isopentenylamino)purine-9-β-L-ribofuranoside **(31)**

β-L-inosine (100 mg, 1 equiv.) and BOP (198 mg, 1.2 equiv.) were dissolved in DMF (2 ml) followed by DIPEA (130 μL, 2 equiv.) and isopentenylamine hydrochloride (58 mg, 1.2 equiv.) as the last component were added. The reaction mixture was heated at 60°C in oil bath and stirred under argon atmosphere for 24 hours and controlled by TLC ($CHCl_3$/MeOH 4:1). The reaction mixture was evaporated on vacuum rotary evaporator and form yellow gel. The gel was purified by column chromatography with mobile phase $CHCl_3$:MeOH (29:1→ 9:1) to give colorless gel. The gel was mixed with distilled water and the solution was recrystallized overnight in the refrigerator. The white crystals of desired products were filtered and washed by cooled water. Yield: 30%. HPLC purity: 100 %. MS ESI+ m/z: 336 $[M+H]^+$.

^{1}H NMR (500 MHz, DMSO-d6) δ ppm: 1.63 (s, 3H), 1.67 (s, 3H), 3.81 (s, 2H), 3.86-4.15 (m, 3H), 4.32 (q, j=5.5Hz, 1H), 4.56 (d, J=6Hz, 1H), 5.22-5.30 (m, 2H), 5.51 (d, J=6Hz, 1H), 5.58 (q, J=4.5Hz, 1H), 5.84 (d, J=6Hz, 1H), 7.85 (bs, 1H), 8.18 (bs, 1H), 8.30 (s, 1H)

The biological activity of prepared N^6-substituted purine-9-β-L-ribofuranoside derivatives was evaluated in three classical CK bioassays (*Amaranthus*, tobacco callus and senescence) and the results are summarized in **Table 1**.

Table 1: *Relative CK activities of prepared compounds in three classical CK bioassays. The optimal concentration for compounds was compared with the activity of BAP, where 100 % means 10^{-5} M BAP in the Amaranthus bioassay, 10^{-4} M BAP in the senescence bioassay and 10^{-5} M BAP in the tobacco callus bioassay.*

	Amaranthus		**Senescence**		**Tobacco callus**	
Compound	**Optimal concentration (mol/L)**	**Relative activity (%)**	**Optimal concentration (mol/L)**	**Relative activity (%)**	**Optimal concentration (mol/L)**	**Relative activity (%)**
26	10^{-5}	21(±4)	10^{-4}	9(±2)	10^{-5}	80(±3)
27	10^{-4}	13(±1)	10^{-8}	29(±2)	10^{-4}	66(±4)
28	10^{-4}	9(±2)	10^{-8}	56(±2)	10^{-4}	70(±3)
29	10^{-4}	18(±4)	10^{-4}	64(±5)	10^{-4}	55(±4)
30	10^{-4}	14(±3)	10^{-5}	15(±4)	10^{-4}	72(±4)
31	10^{-4}	52(±6)	10^{-7}	14(±4)	10^{-4}	79(±7)

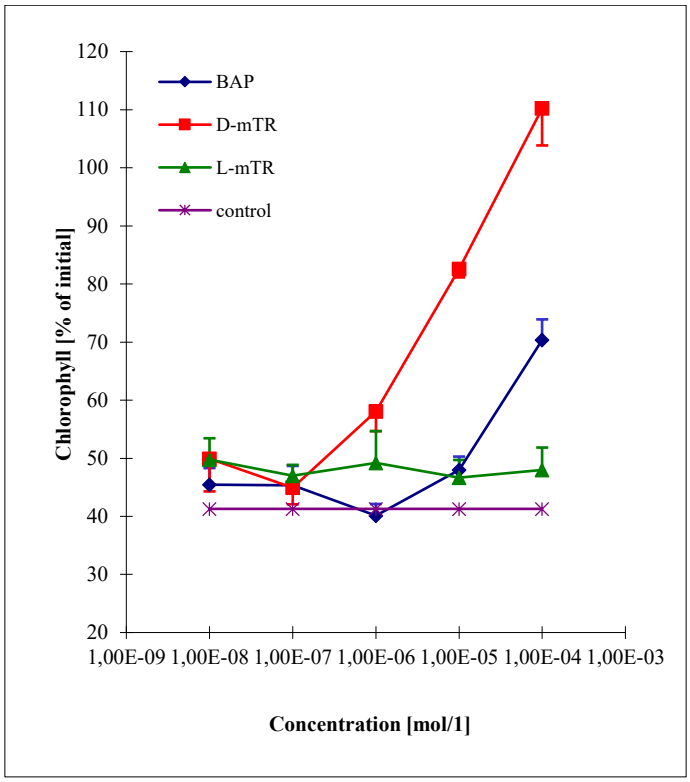
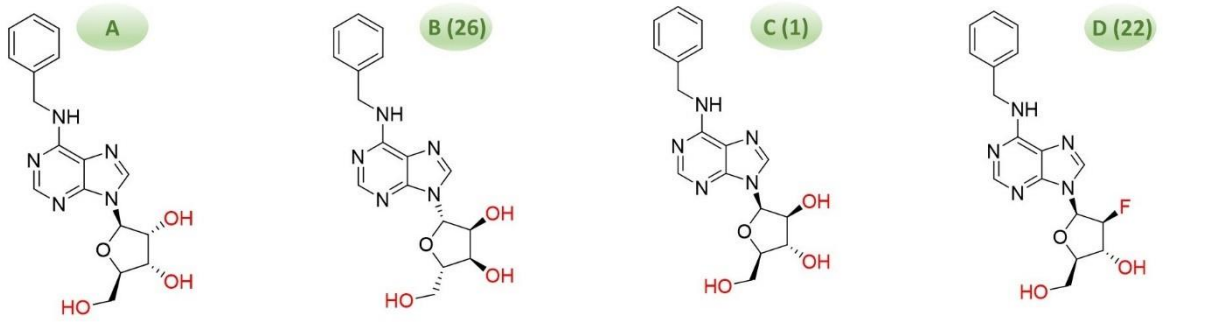


Fig 11*: Comparison of biological activity of the two riboside enantiomers (D-mTR and L-mTR) and BAP in the senescence bioassay: Dose-response curves for CK-induced delay of chlorophyll degradation in excised wheat leaves. The horizontal line indicates values under the control treatment with no CK application.*



	Amaranthus		Senescence		Tobacco callus		Cytotoxicity	
	Optimal concentration (mol/L)	Relative activity (%)	Optimal concentration (mol/L)	Relative activity (%)	Optimal concentration (mol/L)	Relative activity (%)	MCF7 IC_{50} (µmol/L)	NH3T3 IC_{50} (µmol/L)
A	10^{-5}	83(±5)	10^{-4}	148(±5)	10^{-5}	112(±7)	5.4	39
B (26)	10^{-4}	21(±4)	10^{-4}	9(±2)	10^{-5}	80(±3)	> 100	> 100
C (1)	10^{-4}	22(±3)	10^{-4}	138(±9)	10^{-4}	34(±5)	> 100	> 100
D (22)	10^{-4}	72(±11)	10^{-4}	277(±9)	10^{-4}	45(±2)	> 100	> 100

Fig. 12: *The summary of biological activity of four structurally related 6-benzylaminopurine nucleosides. The compound A was previously synthesized by Doležal et al. (Doležal et al., 2007) and derivatives B, C and D were prepared in the presented work. The optimal concentration was compared with activity of BAP, where 100% means 10^{-5} M in Amaranthus, 10^{-4} M in senescence and 10^{-5} M in tobacco callus bioassay.*

Conclusions and perspectives

A series of new CK nucleosides with various substitution at N^6-position of aminopurine was synthesized in combination with N9-substitution, including β-D-arabinofuranose, β-L-ribofuranose and 2′-deoxy-2′-fluoroarabinofuranose. For the synthesis, the high step facile one-step method using non-protected starting purine nucleosides was used.

The synthesis of CK conjugates with N9-substituted β-D-arabinofuranose starting with hypoxanthine arabinofuranoside, β-L-inosine was used as starting precursor for preparation of N9-substituted β-L-ribofuranoside analogues and hypoxanthine-2′-deoxy-2′-fluoroarabinofuranoside obtained corresponding N9-substituted 2′-deoxy-2′-fluoroarabinofuranoside derivatives. The reaction conditions have been slightly modified, depending on the type of the side chain or carbohydrate.

The prepared compounds were tested in three classical CK bioassays, such as *Amaranthus*, senescence and tobacco callus to clarify structure-activity relationship related to their biological activity.

In arabinosides family, the significant anti-senescence effect was evaluated in 3-methoxyBAPA and 3-hydroxyBAPA and therefore the mechanism of action of these two CK nucleosides was examined in detail – both of them have been characterized to promote plant immunity and positively regulate leaf longevity.

The group of β-L-ribofuranosides have been evaluated as almost non-active in CK bioassays, but interestingly also non-cytotoxic besides of their β-D counterparts.

The arabinofuranoside analogues with fluorinated carbohydrates have been shown as hormopriming agents due to their high anti-senescent effect. One of them, 6-(3-hydroxybenzylamino)-2′-deoxy-2′-fluoro-9-β-D-arabinofuranosylpurine has been described as a compound with ability to promote plant growth under controlled conditions and reduce negative effects, such as salt and osmotic stress.

Some of the newly prepared CK nucleoside analogs are potential candidates for using in biotechnology as well as agriculture. Many of them will be investigated for their potential antiviral activity.

List of publications

I. **Bryksová, M.***, Dabravolski, S.*, Kučerová, Z.*, Zavadil Kokáš, F., Špundová, M., Plíhalová, L., Takáč, T., Grúz, J., Hudeček, M., Hloušková, V., Koprna, R., Novák, O., Strnad, M., Plíhal, O., Doležal, K.: Aromatic cytokinin arabinosides promote PAMP-like responses and positively regulate leaf longevity. *ACS Chem Biol*, 2020, vol, pp. * These authors are contributed equally to this work.

II. Vylíčilová, H.*, **Bryksová, M.***, Matušková, V., Doležal, K., Plíhalová, L., Strnad, M.: Naturally occurring and artificial N9-cytokinin conjugates: From synthesis to biological activity and back. *Biomolecules*, 2020, 10, 1-29. * These authors are contributed equally to this work.

III. **Bryksová, M.**, Hybenová, A., Esteban Hernandiz, A., Novák, O., Pěnčík, A., Spíchal, L., De Diego, N., Doležal, K.: Hormopriming for mitigating abiotic stress affects: A case study of N9-substituted cytokinin derivatives with fluorinated carbohydrate moiety. Submitted 2020

IV. Doležal, K., **Bryksová, M.**: Topolin metabolism and its implications for in vitro plant micropropagation. book chapter

V. 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. US patent US 10,100,077 B2, 2018.

Contribution report

I. Join first author – synthesis, compounds characterization, interpretation of results, tables and scheme creation, cooperation in manuscript writing

II. Join first author – writing of chapters 2.2., 2.4., 3.1., 3.2 and 3.3., cooperation in creation of table, figures creation, editing

III. First author – synthesis, compounds characterization, scheme and tables creation, cooperation in manuscript writing

IV. Co-author – writing of subchapter: Future perspectives – application of fluorinated compounds, figures creation

V. Co-author – synthesis and characterization (arabinosides), table and scheme creation

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Supplementary material I.

Bryksová, M., Dabravolski, S., Kučerová, Z., Zavadil Kokáš, F., Špundová, M., Plíhalová, L., Takáč, T., Grúz, J., Hudeček, M., Hloušková, V., Koprna, R., Novák. O., Strnad, M., Plíhal, O., and Doležal, K. (2020). Aromatic cytokinin arabinosides promote PAMP-like responses and positively regulate leaf longevity. *ACS Chemical Biology*, *15*, 1949-1963.

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Articles

Aromatic Cytokinin Arabinosides Promote PAMP-like Responses and Positively Regulate Leaf Longevity

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Cite This: *ACS Chem. Biol.* 2020, 15, 1949−1963

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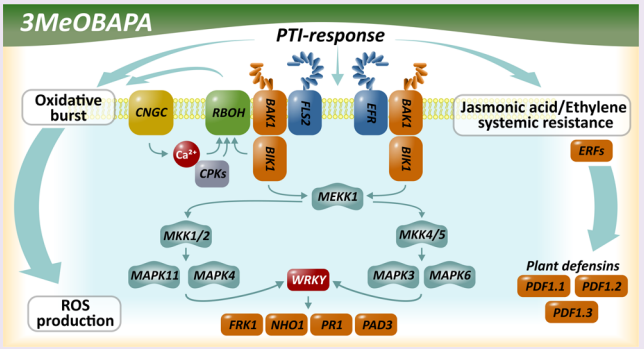
ABSTRACT: Cytokinins are plant hormones with biological functions ranging from coordination of plant growth to the regulation of biotic and abiotic stress-related responses and senescence. The components of the plant immune system can learn from past elicitations by microbial pathogens and herbivores and adapt to new threats. It is known that plants can enter the primed state of enhanced defense induced by either natural or synthetic compounds. While the involvement of cytokinins in defense priming has been documented, no comprehensive model of their action has been provided to date. Here, we report the functional characterization of two aromatic cytokinin derivatives, 6-benzylaminopurine-9-arabinosides (BAPAs), 3-methoxy-BAPA and 3-hydroxy-BAPA, that proved to be effective in delaying senescence in detached leaves while having low interactions with the cytokinin pathway. An RNA-seq profiling study on *Arabidopsis* leaves treated with 3-methoxy-BAPA revealed that short and extended treatments with this compound shifted the transcriptional response markedly toward defense. Both treatments revealed upregulation of genes involved in processes associated with plant innate immunity such as cell wall remodeling and upregulation of specific MAP kinases, most importantly *MPK11*, which is a MAPK module involved in stress-related signaling during the pathogen-associated molecular patterns (PAMPs) response. In addition, elevated levels of JA and its metabolites, jasmonate/ethylene-driven upregulation of *PLANT DEFENSIN 1.2* (*PDF1.2*) and other defensins, and also temporarily elevated levels of reactive oxygen species marked the plant response to 3-methoxy-BAPA treatment. Synergistic interactions were observed when plants were cotreated with 3-hydroxy-BAPA and the flagellin-derived bacterial PAMP peptide (flg22), leading to the enhanced expression of the PAMP-triggered immunity (PTI) marker gene *FRK1*. Our data collectively show that some BAPAs can sensitively prime the PTI responses in a low micromolar range of concentrations while having no observable negative effects on the overall fitness of the plant.



INTRODUCTION

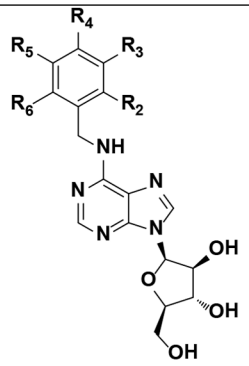
Naturally occurring cytokinins (CKs) are purine-based molecules that promote cell division, regulate growth and differentiation, and orchestrate many physiological processes in plants.[1] They also help suppress leaf senescence[2,3] and are involved in complex phytohormone-regulated plant response mechanisms to various pathogens.[4] In plants, CKs can exist as free bases but are more commonly found in the form of glucosides, nucleosides, or nucleotides depending on the cell type, developmental phase, and environmental conditions. CK content and activity are tightly controlled by enzymatic degradation or glucosylation of free bases. Glucose conjugation to CKs may occur at the purine *N*3, *N*7, or *N*9 atoms (*N*-glucosylation), generating terminal CK metabolites, or at the oxygen of the zeatin/dihydrozeatin side chain (*O*-glucosylation). Whereas CK *N*-glucosides are considered to be irreversibly deactivated, *O*-glucosides can be converted back into active free bases by β-glucosidases.[5] Another way to selectively regulate plant physiology is to modulate plants CK status using cytokinin analogues or inhibitors that affect specific aspects of CK sensing and metabolism.[6−8]

N^6-benzylaminopurine (BAP) is an aromatic cytokinin (ArCK) that is widely used in micropropagation because of its low cost and high biological activity, which is largely due to its resistance to degradation.[9] For a relatively long period, most ArCKs were regarded as synthetic compounds with limited usefulness. However, a detailed characterization of plant

Received: April 20, 2020
Accepted: June 10, 2020
Published: June 10, 2020

https://dx.doi.org/10.1021/acschembio.0c00306
ACS Chem. Biol. 2020, 15, 1949−1963

Table 1. Structure−Activity Relationship Studies of 6-Benzylaminopurine-9-β-D-arabinosides Tested in This Work[a]

			*Amaranthus* assay		Senescence assay		Tobacco callus assay	
			Optimal concentration (mol/L)	Relative activity (%)	Optimal concentration (mol/L)	Relative activity (%)	Optimal concentration (mol/L)	Relative activity (%)
# ID	MW	R_x =						
1	357.37	R_{2-6} = H	10^{-4}	19(±1)	10^{-4}	138(±5)	10^{-4}	34(±5)
2	375.36	R_2 = F	10^{-4}	13(±2)	10^{-4}	138(±12)	10^{-4}	32(±9)
3	375.36	R_3 = F	10^{-4}	31(±7)	10^{-4}	140(±9)	10^{-7}	15(±4)
4	375.36	R_4 = F	10^{-4}	9(±1)	10^{-4}	134(±11)	10^{-4}	73(±9)
5	391.81	R_2 = Cl	10^{-4}	15(±2)	10^{-4}	99(±6)	10^{-4}	20(±2)
6	391.81	R_3 = Cl	10^{-4}	13(±4)	10^{-4}	121(±6)	10^{-4}	40(±1)
7	391.81	R_4 = Cl	10^{-5}	10(±3)	10^{-8}	13(±1)	10^{-8}	12(±2)
8	483.27	R_3 = I	10^{-4}	5(±2)	10^{-8}	29(±4)	10^{-7}	8(±1)
9	483.27	R_4 = I	10^{-4}	12(±4)	10^{-4}	53(±4)	10^{-5}	12(±2)
10	387.40	R_2 = CH_3O	10^{-4}	4(±1)	10^{-4}	174(±11)	10^{-4}	30(±4)
11	387.40	R_3 = CH_3O	10^{-4}	7(±2)	10^{-4}	119(±8)	10^{-4}	26(±3)
12	387.40	R_4 = CH_3O	10^{-5}	0	10^{-4}	40(±3)	10^{-8}	23(±5)
13	373.37	R_3 = OH	10^{-4}	8(±1)	10^{-4}	180(±7)	10^{-4}	32(±2)
14	371.40	R_2 = CH_3	10^{-4}	5(±1)	10^{-7}	49(±5)	10^{-6}	22(±3)
15	371.40	R_3 = CH_3	10^{-4}	4(±1)	10^{-4}	152(±7)	10^{-4}	36(±5)
16	371.40	R_4 = CH_3	10^{-4}	0	10^{-7}	62(±7)	10^{-6}	23(±6)
17	372.39	R_2 = NH_2	10^{-5}	2(±1)	10^{-7}	91(±6)	10^{-4}	22(±2)
18	425.37	R_3 = CF_3		n.t.		n.t.		n.t.
19	441.37	R_3 = OCF_3		n.t.		n.t.		n.t.
20	393.35	$R_{2,5}$ = F	10^{-4}	27(±6)	10^{-4}	113(±12)	10^{-9}	6(±1)
21	393.35	$R_{3,5}$ = F	10^{-4}	15(±3)	10^{-4}	156(±11)	10^{-8}	29(±5)

[a]All compounds except of **1** were modified at the benzyl ring with a single or double substitution (remaining R's are hydrogens). Relative activity of the newly synthesized cytokinin derivatives was assessed in standardized cytokinin bioassays: the *Amaranthus* assay, wheat leaf senescence, and tobacco callus assays. The reported values are activities expressed as percentages of the activity of the control compound (BAP), measured at the concentration yielding the maximum positive effect. Thus, a relative activity of 100% indicates that the given compound has the same activity as 10 μM BAP in the case of the *Amaranthus* betacyanin bioassay, 100 μM BAP in the case of the senescence bioassay, and 1 μM BAP in the case of the tobacco callus bioassay. n.t.: not tested.

regulators including the ArCKs 6-(2-hydroxybenzylamino)-purine (*ortho*-topolin), 6-(3-hydroxybenzylamino)purine (*meta*-topolin), and their ribosides revealed them to have strong activity in classical cytokinin bioassays.[10] Structure−activity relationship analyses established that the biological activity of these ArCKs depends on the position of the substituent on the benzyl ring and is commonly increased by the presence of a hydroxy or methoxy group in the *meta* position.[9,11]

Over the past decade, we have synthesized and evaluated several CK derivatives with different modifications at the *N*9-, C2-, C8-, and C6-positions of the purine moiety by exploiting combinatorial synthetic strategies.[3,9,12−15] In most of these derivatives, *N*9 substitution plays a central role because it prevents the unfavorable glucosylation that renders the final CK-*N*9-glucoside biologically inactive. To identify active derivatives resistant to *N*-glucosylation, *N*9-ribosylated BAP derivatives bearing variously substituted benzyl rings were prepared and tested.[16] A series of kinetin-*N*9-substituted derivatives was prepared with various attached alkyl halides at *N*9 atom of purine exhibiting promising biological activities including the ability to reduce membrane lipid peroxidation, which is a typical symptom of senescence.[13] 2,6-Disubstituted BAPR derivatives with halogen substituents at the *meta* position of the benzyl ring exhibited significant activity in a detached wheat leaf senescence bioassay that correlated with their ability to reprogram the expression of many photosynthesis-related genes.[3] Several *N*9-tetrahydropyran-2-ylated and *N*9-tetrahydrofuran-2-ylated ArCKs were also synthesized and shown to exhibit strong antisenescent effects as well as significantly lower cytotoxicity in breast carcinoma cells (MCF-7) and human foreskin fibroblasts (BJ) than previously prepared *N*9-ribosides.[12] The metabolism of two ArCKs bearing a tetrahydropyranyl substituent at *N*9 atom, 6-benzylamino-9-(tetrahydropyran-2-yl)purine (BA9THPP) and 6-(3-methoxybenzylamino)-9-(tetrahydropyran-2-yl)-

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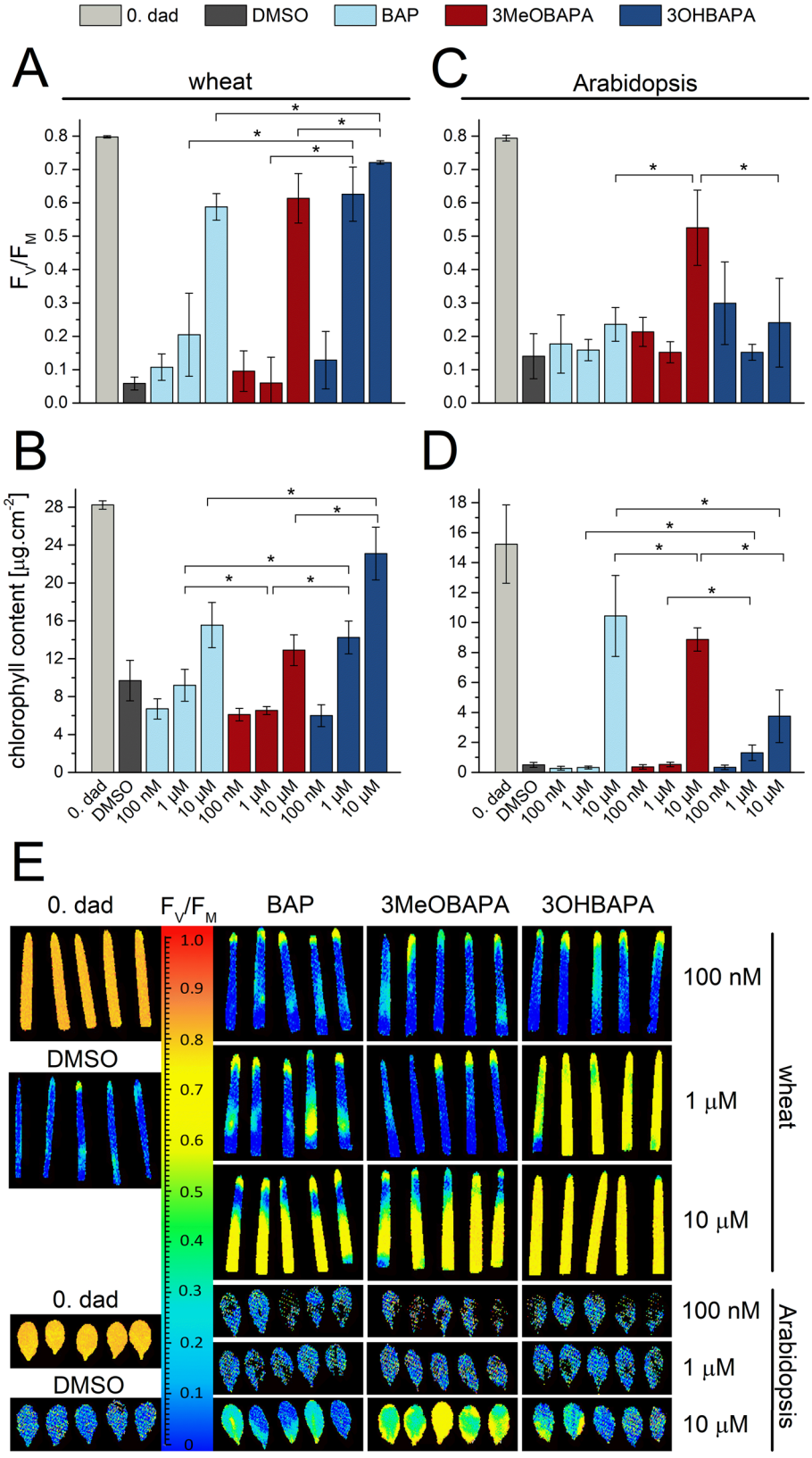

Figure 1. Effect of two 6-benzylaminopurine-9-β-D-arabinosides on detached wheat and *Arabidopsis* leaves. The maximum quantum yield of photosystem II photochemistry (F_V/F_M; A, C and E) and chlorophyll content (B, D) in leaves of wheat and *Arabidopsis* measured immediately after detachment (0. dad) and after 6 days' dark incubation in aqueous DMSO (0.1%) or aqueous DMSO solutions of BAP, 3-methoxy-BAPA (3MeOBAPA), or 3-hydroxy-BAPA (3OHBAPA). Concentration-dependent effect of BAP, 3-methoxy-BAPA, and 3-hydroxy-BAPA treatments is depicted as F_V/F_M in wheat leaves (E, top) and *Arabidopsis* leaves (E, bottom) using color-coding shown in the figure. The bar charts show means ± SD estimated from measurements of the individual leaves (n = 5) imaged in panel (E). Asterisks indicate statistically significant differences at the tested concentrations according to Student's unpaired t test ($P < 0.05$).

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purine (3MeOBA9THPP), was studied in maize. The *N*9-THP substitution of the parental ArCK significantly enhanced the acropetal transport of these derivatives, enabling a slow gradual release of the active base from the synthetic precursor.[9]

Arabinosyl nucleosides such as fludarabine (9-β-D-arabinosyl-2-fluoroadenine, F-ara-A) and its derivatives are biologically active compounds with important antitumor activity.[17] In humans, several ArCK derivatives[16] and F-ara-A[18] seem to affect the differentiation of certain cancer cells by interfering with cell cycle regulation. Moreover, some of these adenine derivatives such as 6-chloropurine arabinoside possess potent activity against varicella-zoster virus (VZV) and other viruses.[19] Other arabinosyl nucleosides including (2'S)-2'-deoxy-2'-fluoro-5-ethynyluridine, F-ara-EdU exhibit selective DNA labeling but have negligible effects on genomic stability and function.[20] The activity of these compounds in plants has not yet been explored, but arabinose is a vital component of various lipopolysaccharides and oligosaccharides and is also found in antimicrobial flavonoids.[21]

One of the aims of this work was to create a new class of CK derivatives that retain the antisenescence activity of ArCKs without the negative effects on root/shoot development typically associated with CK use in plants.[12,22] The biological activity of the newly prepared CK derivatives was tested in standard CK bioassays and bacterial receptor assays. Two derivatives with strong senescence-delaying activity were further tested by performing chlorophyll fluorescence measurements to assess their ability to maintain photosynthetic function in detached wheat and *Arabidopsis* leaves. RNA-seq analysis of detached dark-incubated *Arabidopsis* leaves treated with one of these derivatives revealed significant reprogramming of gene expression leading to the primed state of enhanced defense with several marker genes of PAMP-triggered immunity (PTI) response being affected by either short or extended treatment with the compound. Our previous results showed that several ArCKs and their derivatives interact only weakly with CK receptors, suggesting that their antistress/antisenescent activity may be at least partly due to a parallel mechanism that is distinct from the standard CK pathway.[23] The results presented here show that ArCK arabinosides can significantly promote plant protective and defense mechanisms and that these actions are not significantly linked to the standard CK signaling pathway.

RESULTS AND DISCUSSION

6-Benzylaminopurines Substituted with β-D-Arabinose at the *N*9-Position (BAPAs) Show Antisenescence Activity in Plants. We prepared 21 novel BAPA derivatives with various substituents at different positions on the benzyl ring (Table 1) using established synthetic protocols (Supplementary Methods and Supplementary Table 1). Their activity was assessed using three classical cytokinin bioassays. The measured activities are reported relative to that of the natural aromatic cytokinin BAP (Table 1). None of the compounds exceeded 31% of the activity observed for BAP in the *Amaranthus* bioassay, suggesting they have only weak cytokinin-like activity. A similar pattern emerged in the tobacco callus bioassay: aside from compound **4**, whose relative activity was 73% of that of the BAP control, the compounds exhibited low or modest relative activities in the range of 6−40%. In stark contrast, several of the new BAPAs exhibited activity similar to (compounds **5**, **17**, and **20**) or greater than (compounds **1**, **2**, **3**, **4**, **6**, **10**, **11**, **13**, **15**, and **21**) that of BAP in the wheat leaf senescence bioassay, in which the degree of senescence is evaluated based on the chlorophyll (Chl) content in dark-incubated detached wheat leaves. This indicated that the new BAPAs specifically affect physiological processes primarily related to senescence and/or stress without being active in the cytokinin pathway.

We also examined the new compounds' ability to activate cytokinin sensor histidine kinase (HK) receptor-linked signaling cascade by using a previously reported protocol[24] in which the *Arabidopsis* AHK3 and CRE1/AHK4 receptors[25,26] are overexpressed in the *Escherichia coli* KMI001 strain (Δ*rcsC*, *cps::lacZ*), using β-galactosidase as a reporter gene. In line with the *Amaranthus* bioassay and tobacco callus bioassay results, none of the BAPAs triggered a cytokinin response from the AHK3 and CRE1/AHK4 receptors, demonstrating that BAPAs did not initiate the cytokinin signaling *in vitro*.

Thus, several of our new compounds, especially those bearing a substituent (*i.e.*, a fluorine/chlorine atom or a hydroxy/methoxy group) in the *meta* position on the benzyl ring, effectively delayed the onset of senescence but had negligible standard hormonal activity. This is particularly interesting because it is well-known that CK treatment can have adverse developmental effects.[27] While many of the new compounds exhibited promising activity in the senescence bioassay, some of them are unsuitable for use *in planta*. For example, the metabolic deactivation of compound **1** might be expected to proceed similarly to that of BAP, which is converted into inactive CK 9-glucosides that are probably responsible for the aberrant root formation mentioned above. Conversely, *meta*-topolin is metabolized via a different pathway, which results in its reversible conversion into a storage form that does not interfere with efficient root development.[11,22] We therefore selected two derivatives for further mechanistic investigation *in planta*: compounds **11** and **13**, which bear CH_3O- and −OH groups, respectively, in the *meta* position (Table 1). Despite having low activity in standard cytokinin activity tests (the *Amaranthus* and tobacco callus bioassays), these compounds exhibited either above average (**11**) or high (**13**) activity in the senescence assay (Table 1). Compound **13** (hereafter referred to as 3-hydroxy-BAPA) is structurally related to the naturally occurring ArCK *meta*-topolin,[28] while compound **11** (hereafter referred to as 3-methoxy-BAPA) is its methoxy analogue.

Influence of 3-Hydroxy-BAPA and 3-Methoxy-BAPA on the Maintenance of Photosynthetic Activity in Detached Wheat and *Arabidopsis* Leaves. The senescence bioassay results demonstrated that the chosen compounds suppress the Chl degradation in detached wheat leaves incubated in darkness (Table 1). To determine whether they also preserve photosynthetic activity (specifically, the activity of photosystem II (PSII), which decreases markedly during dark-induced senescence of detached leaves[3,29]), we measured the maximum quantum yield of PSII photochemistry (F_V/F_M) in wheat and *Arabidopsis* leaves kept in darkness for 6 days, together with the leaf Chl content. Wheat leaves treated with dimethyl sulfoxide (DMSO; negative control) exhibited low F_V/F_M values (Figure 1A and E), indicating only minimal PSII activity. However, wheat leaves treated with 3-methoxy-BAPA (10 μM) and 3-hydroxy-BAPA (1 and 10 μM) exhibited F_V/F_M values above 0.6, indicating effective preservation of PSII function. In *Arabidopsis*, a comparable effect was only found in leaves treated with 3-methoxy-BAPA at 10 μM, which generated a stronger protective response than the control

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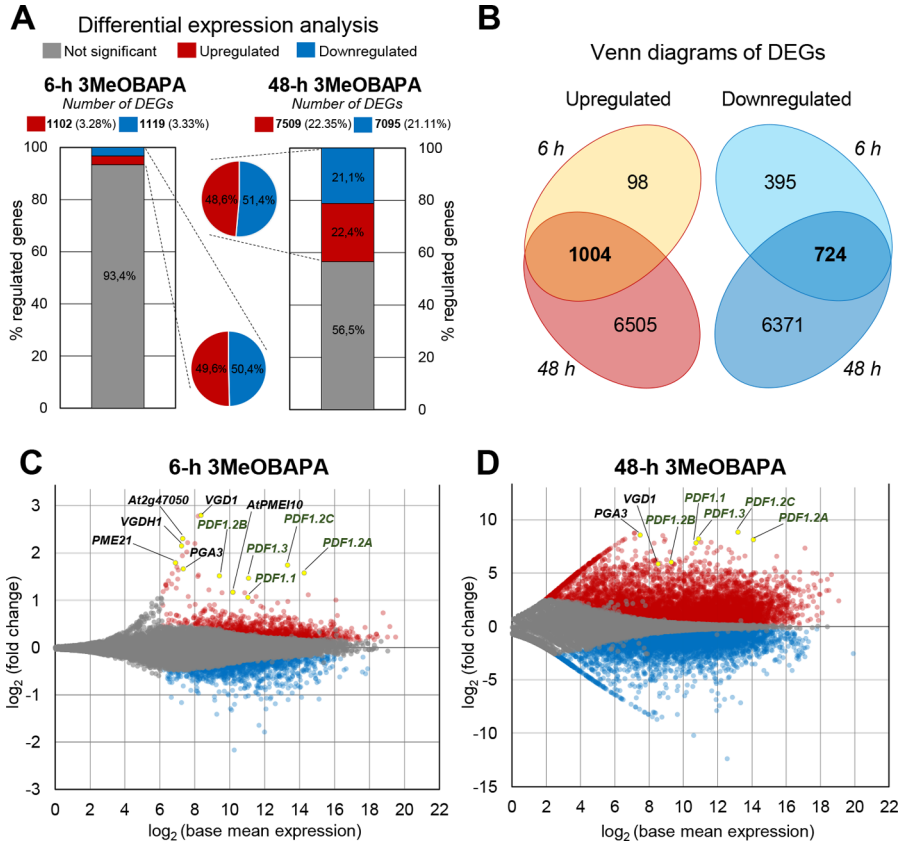

Figure 2. 3-Methoxy-BAPA treatment in *Arabidopsis* leaves shows a massive transcriptional reprogramming that significantly affects defense-related genes. (A, C, D) Differential expression analysis of genes affected by 3-methoxy-BAPA treatment (adjusted *P*-value < 0.05; data normalization and statistical evaluation was performed in the DESeq2 program). (B) Venn diagrams comparing gene expression in detached *Arabidopsis* leaves after short-term (6 h) and long-term treatment (48 h) with 10 μM 3-methoxy-BAPA. Both upregulated DEGs (left diagram) and downregulated DEGs (right diagram) are shown.

BAP treatment (Figure 1C and E). In wheat leaves, 3-hydroxy-BAPA had a significantly stronger protective effect on both Chl content and PSII function than 3-methoxy-BAPA (Figure 1A, B, and E top), which is consistent with its higher relative activity in the standard senescence bioassay (Table 1). In *Arabidopsis* leaves, 3-methoxy-BAPA was more effective than 3-hydroxy-BAPA in both Chl retention and maintenance of PSII function (Figure 1C, D, and E bottom). Whereas the effects of 3-methoxy-BAPA treatment at concentrations of 1 and 10 μM were similar in wheat, the minimum 3-methoxy-BAPA concentration needed to produce a protective effect in *Arabidopsis* was 10 μM.

Collectively, these results show that both compounds have significant senescence-delaying effects and that these effects are (quantitatively) species-specific. Whereas 3-hydroxy-BAPA exhibited stronger antisenescence activity in wheat, 3-methoxy-BAPA's protective effect proved to be more specific for *Arabidopsis* leaves, and the positive effect on the photosynthetic function in *Arabidopsis* was found to be superior to that exhibited by highly active aromatic cytokinin BAP. Therefore, 3-methoxy-BAPA was selected for use in a whole transcriptome differential expression study using *Arabidopsis* as a model system.

Whole Transcriptome Expression Changes in 3-Methoxy-BAPA-Treated *Arabidopsis* Leaves and GO Distribution Analysis. A high-throughput mRNA sequencing analysis (RNA-seq) of dark-incubated *Arabidopsis* leaves was performed to characterize the reprogramming of gene transcription induced by treating detached leaves with 3-methoxy-BAPA. We analyzed gene expression after short (6 h) and long (48 h) treatments with 10 μM 3-methoxy-BAPA (*i.e.*, the lowest concentration found to elicit a significant response in the preceding experiments) to clarify the molecular mechanisms of adaptation to short and extended treatment with the compound.

Overall, the transcriptome of 3-methoxy-BAPA-treated *Arabidopsis* leaves in the short term treatment was significantly different from that of DMSO treated control, with 1102 upregulated and 1119 downregulated genes based on an adjusted *P*-value of < 0.05 (Figure 2A). Extended treatment (*i.e.*, 48 h) led to a massive transcriptional reprogramming, with a much higher number of differentially expressed genes (DEGs) being affected: 7509 were upregulated and 7095 were downregulated. A comparison of DEGs obtained from the short-term treatment with genes coming from the extended treatment revealed a high overlap: more than 91% among upregulated DEGs and 65% among downregulated DEGs (Figure 2B).

Among the genes with the highest upregulation in both 3-methoxy-BAPA treatment were genes of defense regulons, namely, antifungal plant defensins (*PDF*; Figure 2C, D and Supplementary Table 2): *At5g44430* coding for defensin-like protein 1.2C (*PDF1.2C*), *At5g44420* coding for defensin-like protein 1.2A (*PDF1.2A*), *At2g26020* coding for defensin-like protein 1.2B (*PDF1.2B*), and *At2g26010* coding for defensin-like protein 1.3 (*PDF1.3*), all of which are important antifungal

https://dx.doi.org/10.1021/acschembio.0c00306
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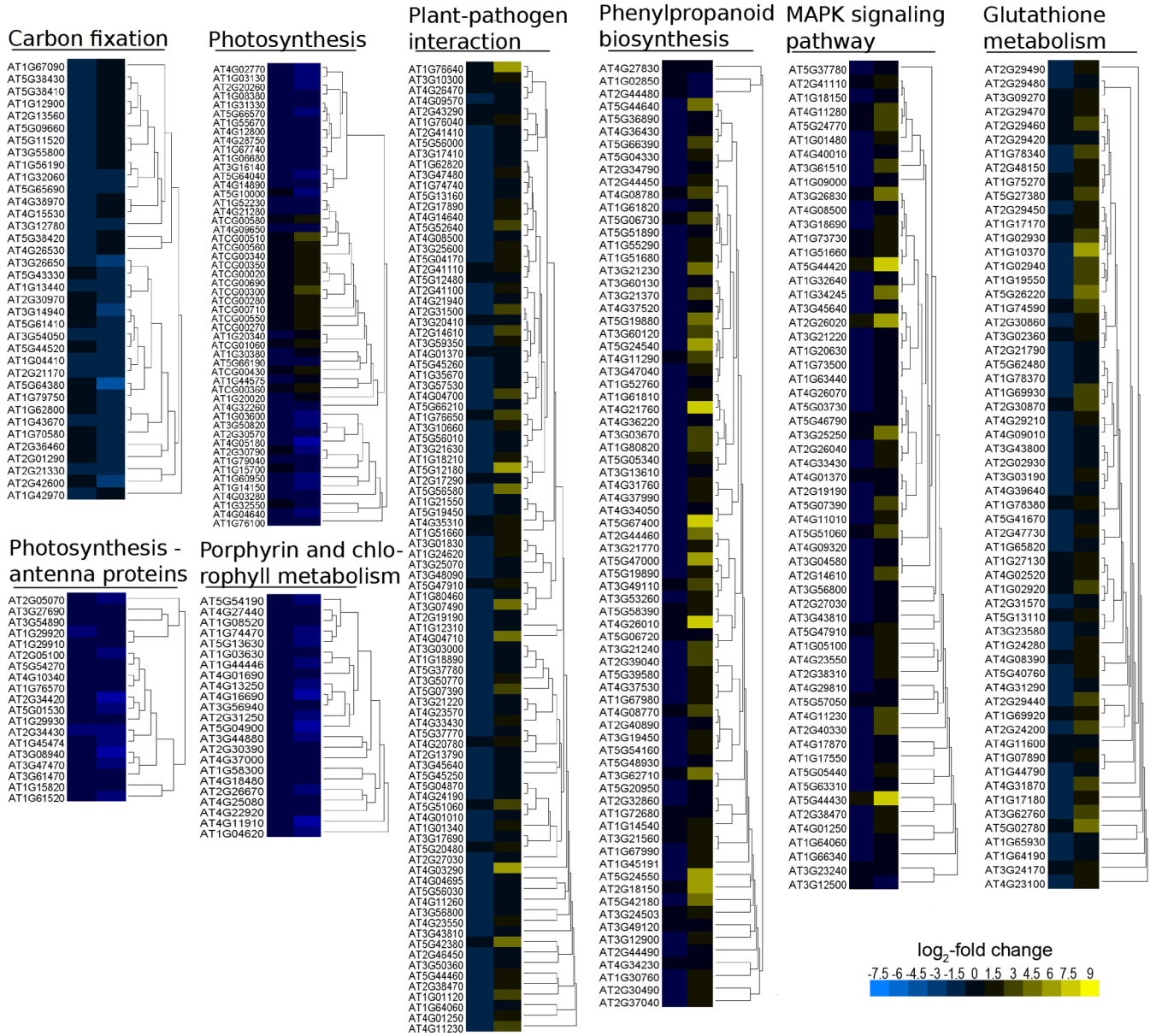

Figure 3. KEGG pathways over-representation analysis. Fold changes (log_2 scale) in expression of selected metabolic pathway genes are shown for the short-term (left columns) and long-term (right columns) treatment with 3-methoxy-BAPA. Genes were clustered using average-linkage hierarchical clustering. The metabolic pathways shown in the figure represent the most significantly affected group of genes related to carbon fixation (map00710), photosynthetic machinery (map00195 "Photosynthesis," map00196 "Photosynthesis - antenna proteins," map00860 "Porphyrin and chlorophyll metabolism," plant-pathogen interactions (map04626), MAPK signaling (map04016), phenylpropanoid biosynthesis (map00940), and glutathione metabolism (map00480) according to KEGG database. The log_2 fold change values are expressed as negative or positive numbers on the bar, and the color scale indicates which genes are up- (yellow) and downregulated (blue).

and antistress factors that comprise part of the innate immunity arsenal in plants.[30,31] We also detected strong transcription of several genes encoding inhibitors of pectin methylesterases (PMEI), especially in the short term treatment, including *At1g62760*, which encodes *AtPMEI10*, and *At2g47050* coding for a previously uncharacterized PMEI. Several PMEIs, together with their target pectin methylesterases (PME), were shown to play protective function by controlling the status of methylesterification of pectin during infection by necrotrophic pathogens.[32]

In addition, some tissue-specific PMEs and polygalacturonases (pectinases) were also upregulated including *At2g47040*, which encodes pectin methylesterase Vanguard1 (VGD1),[33] *At3g05610* coding for pathogen-induced PME21,[32] and *At3g07830*, which codes for pectin lyase/polygalacturonase 3 (PGA3) (Supplementary Table 2). The unique expression pattern of specific PMEs and their inhibitory PMEIs together with the plant defensins strongly suggests that 3-methoxy-BAPA in the early stages of transcriptional reprogramming specifically regulates processes necessary for cell wall remodeling and consequent enhanced resistance to stresses and fungal pathogens in plants. It seems that the action of 3-methoxy-BAPA might be related to jasmonic acid (JA) and ethylene (ET) signaling as the expression of both PMEIs and defensins is regulated by JA/ET stress signaling pathway.[34]

On the other hand, over 30% of the 50 most strongly downregulated DEGs were related to photosynthesis, including 10 genes encoding subunits of the light-harvesting chlorophyll-

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protein complexes (LHC) of PSII (*At1g29920*, *At2g34430*, *At3g27690*, *At2g05100*, *At2g05070*, *At1g29930*, *At5g54270*, *At3g08940*, *At2g34420*, and *At4g10340*; Supplementary Tables 2 and 3). These results are consistent with the hypothesis that 3-methoxy-BAPA activates defense responses because downregulation of photosynthetic genes is a hallmark of plant responses to pathogens during the first few hours or days after a pathogen attack.[35]

To characterize the functions of the genes exhibiting differential expression following 3-methoxy-BAPA treatment, all genes were analyzed for enriched biological processes (BPs) or molecular functions (MFs) according to the Gene Ontology (GO) project and the distribution of GO terms was analyzed at level 6 and above. The results suggested that 3-methoxy-BAPA indeed affected JA/ET signaling pathways, as DEGs related to JA/ET-dependent systemic resistance[34] (*e.g.*, *ORA59*, *PDF1.2*, and *MPK4*) as well as to various cell wall modifications including pectin catabolic processes (*e.g.*, *VGD1*, *VGDH1*, *At3g01270*, *PME21*, *PME50*, and *CYP79B3*) were upregulated after the short-term treatment (Supplementary Tables 2 and 3). Conversely, in the fast responding group of downregulated DEGs, we observed a generally higher number of genes in the most affected GO categories, with clear enrichment of GO terms related to photosynthesis (Supplementary Table 3). Notably, over 50% of the DEGs were contained within one of annotated GO categories: light-harvesting complex, photosystem I, nonphotochemical quenching, and reductive pentose-phosphate cycle.

To look into complex pattern of DEGs coming particularly from the long-term treatment with 3-methoxy-BAPA, both GO and KEGG databases were used; the KEGG database, however, proved to be more useful in the enrichment analysis of biological processes and mapping of signaling pathways.[36] A hierarchical clustering analysis was performed to decipher the biological consequences of the observed upregulation of pathogen defense-related genes under both short- and long-term treatments. Consistent with the previous enrichment analysis, we observed eight major distinct clusters of transcripts with similar expression profiles (Figure 3). Genes encoding stromal proteins ("Carbon fixation") and thylakoid proteins ("Photosynthesis-antenna proteins," "Photosynthesis") were downregulated by both treatments (particularly after 48 h treatment, with a few exceptions; Figure 3), while plant defense response-related elements (*e.g.*, "Plant-pathogen interaction," "Phenylpropanoid biosynthesis," and "Glutathione metabolism") were significantly upregulated by the long-term treatment with 3-methoxy-BAPA. "MAPK-signaling pathway" stands out as a relevant category with several gene products annotated also within "Plant-pathogen interaction" category that reflects plant immunity response triggered by pathogen-associated molecular patterns (PAMPs), *i.e.*, PAMP-triggered imunity (PTI) (Figure 3). In line with the previous results, such reprogramming of the leaf transcriptome from photosynthesis toward defense suggests that 3-methoxy-BAPA induces plant responses similar to those triggered by pathogens or PAMPs (see *e.g.*, refs 37 and 38) and promotes PTI.

As has been known for some time, both exogenous and endogenous CKs can enhance plant resistance to pathogens[4,39,40] but the mechanism of their action in plant immunity remains unclear. CKs have been reported to promote PTI in *Arabidopsis*.[4,41] A model proposed by Choi *et al.*[39] suggests that CKs enhance defense responses (and consequently plant resistance to pathogens) via AHK2/AHK3 and ARR2 activation. We suggest that the activation of defense responses by 3-methoxy-BAPA is most likely not significantly related to the standard cytokinin pathway because 3-methoxy-BAPA was not recognized by AHK3 or CRE1/AHK4 *in vitro*, and neither short- nor long-term treatment with 3-methoxy-BAPA induced any significant upregulation of *ARR5* or most other cytokinin response regulators (with the exception of *ARR3* and *ARR7* upregulation in the long term treatment only) that are usually upregulated by other cytokinin derivatives.[3] This suggestion is also in line with the low CK activity of 3-methoxy-BAPA in the *Amaranthus* and tobacco callus bioassays.

It is generally accepted that the PTI responses in plants rely on MAPK modules that are activated by diverse biotic and abiotic stress conditions.[42] The activation of plasma membrane-localized pattern recognition receptors (PRRs) triggers defense response in affected cells. Genetic studies revealed that MEKK1-MKK1/2-MPK4 is the main module involved in PTI-mediated defense responses (reviewed in ref 43). Other MPKs that respond to elicitation in PTI include MPK3 and MPK6, possibly independently of MEKK1.[43] MPK11 was recently characterized as a fourth PAMP-activated MAPK that is involved in the stress signaling including a very sensitive response to flg22 (the PAMP derived from the bacterial motor protein flagellin) and other elicitors, acting in synergy with MPK4.[44,45]

Notably, in the "Plant-pathogen interaction" and "MAPK signaling pathway" groups of genes, we observed upregulation of several mitogen-activated protein-kinase (MAPK) signaling modules (*MPK3*, *MPK4*, *MPK6*, and *MPK11*) and their upstream MAPKKs (*MKK1*, *MKK2*, *MKK4*, *MKK5*, and *MKK6*) and MAPKKKs (*MEKK1*, *MAPKKK21*, *MAPKKK20*, and *MAPKKK19*; Figure 3). Further, typical PTI marker genes, which act downstream of MEKK1, were upregulated including several *RBOH*s (coding for respiratory burst oxidase homologues), *FLG22-INDUCED RECEPTOR-LIKE KINASE 1* (*FRK1*), pathogen-related WRKY transcription factors (*WRKY22*, *WRKY29*, *WRKY33*, and *WRKY53*) or pathogenesis-related protein 1 (*PR1*) (see, *e.g.*, ref 42).

These results corroborate our view that the PTI response was provoked by 3-methoxy-BAPA treatment through the standard MAPK cascade including also MPK11, an important MAPK module activated in the PTI signaling. Interestingly, *MPK11* was the single most upregulated MAPK module in our transcriptomic screenings, with a $\log_2$ fold change value of 2.57 after long-term 3-methoxy-BAPA treatment. In addition, *MPK4* was upregulated by both short- and long-term elicitation with 3-methoxy-BAPA. Long-term incubation with 3-methoxy-BAPA also induced significant upregulation of several other MPKs and their upstream MAPK regulators as well as their downstream elements.

3-Methoxy-BAPA Affects PTI through MAPK Signaling Cascades. In an attempt to verify that MAPK signaling pathways are involved in promoting PTI induced by 3-methoxy-BAPA, we further investigated the possible involvement of MAPK signaling modules in detached *Arabidopsis* leaves. Specifically, we examined the expression profiles of *MPK3*, *MPK4*, *MPK6*, and *MPK11* after the short- (6 h) and long-term (48 h) treatments, and also after 30 min treatment to evaluate earlier changes in their expression. While none of 3-methoxy-BAPA treatments induced statistically significant changes in *MPK6* expression profiles (data not shown), we observed a concentration-dependent effect on the expression patterns of *MPK3*, *MPK4*, and *MPK11* in detached *Arabidopsis*

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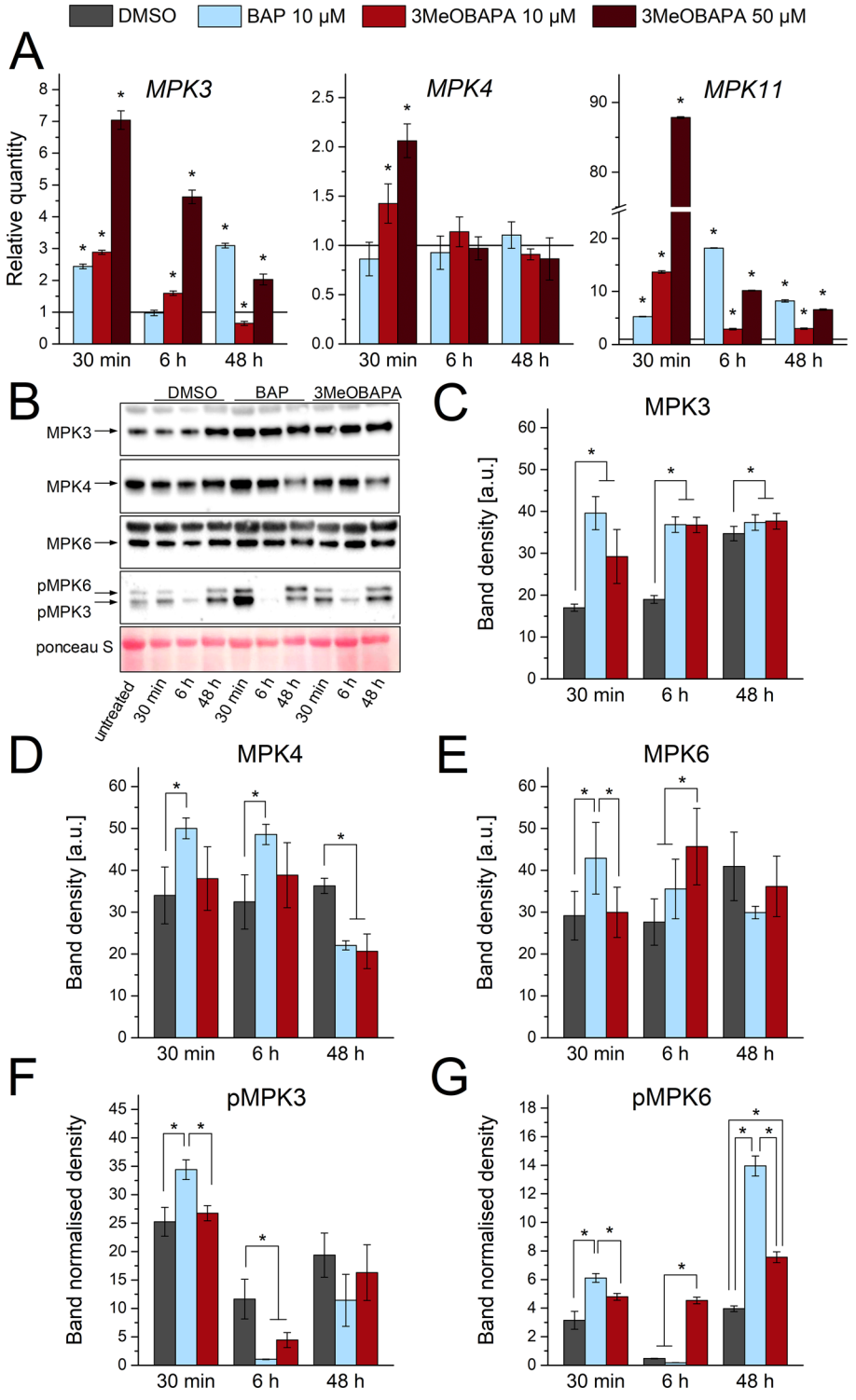

Figure 4. Expression profiles of selected *MPK* genes and MPK protein immunoblots following treatment. (A) Two 3-methoxy-BAPA concentrations were used: 10 and 50 μM; BAP was applied only at 10 μM. Changes in the expression versus the DMSO-treated control were followed by qPCR after time intervals ranging from 30 min to 48 h in detached *Arabidopsis* leaves (n = 4 biological replicates; at least two technical replicates were performed for each biological sample). (B) Immunoblots of *Arabidopsis* leaves incubated in aqueous DMSO (0.1%) or aqueous DMSO solutions of 10 μM BAP and 3-methoxy-BAPA (3MeOBAPA). Detached leaves were incubated with the above compounds for designated time periods, probed with anti-MPK3, anti-MPK4, and anti-MPK6, as well as anti-pERK phosphospecific antibodies recognizing the phosphorylated forms of MPK3 and MPK6 (pMPK3, pMPK6). Ponceau S staining was used for loading control to verify that equal quantities of all studied proteins were loaded onto each probed membrane. (C−G) Quantification of band densities from immunoblots (B) for MPK3 (C), MPK4 (D), MPK6 (E), and phosphorylated MPK3 (F) and MPK6 (G). In (F) and (G), the densities are normalized to the abundance of the corresponding MPKs. In all charts, means ± SD are presented, and Student's unpaired t test was used for statistical analyses ($P < 0.05$).

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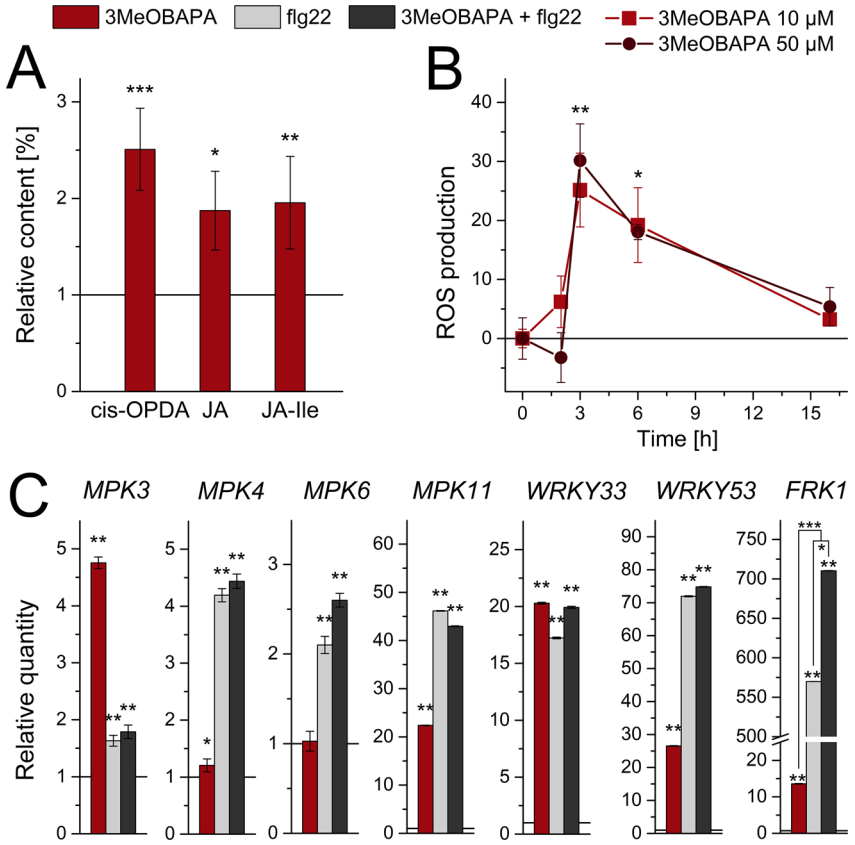

Figure 5. 3-Methoxy-BAPA treatment leads to changes in the intracellular content of jasmonic acid (JA) forms, ROS production and expression profiles of PTI-responsive genes. (A) Content of *cis*-(+)-12-oxo-phytodienoic acid (*cis*-OPDA), jasmonic acid (JA) and jasmonoyl-L-isoleucin (JA-Ile) in *Arabidopsis* leaves treated with 10 μM 3-methoxy-BAPA for 48 h versus the DMSO-treated leaves (n = 4). (B) ROS production in *Arabidopsis* cells treated with 10 μM or 50 μM 3-methoxy-BAPA as determined by DCFH-DA assay (n = 5); asterisks indicate significant difference between untreated (0 h) and treated cells with either 10 or 50 μM 3-methoxy-BAPA. (C) Effect of 3-methoxy-BAPA and bacterial elicitor peptide flg22 on the expression of PTI-responsive genes. Seven days old (DAG) *A. thaliana* Col-0 seedlings were treated with 10 μM 3-methoxy-BAPA and/or 1 μM flg22 for 2 h. The expression levels of *MPK3*, *MPK4*, *MPK6*, *MPK11*, *WRKY33*, *WRKY53*, and *FRK1* were analyzed in 3-methoxy-BAPA, flg22, and 3-methoxy-BAPA + flg22 treated plants versus the DMSO-treated control. Genes for elongation factor 1 alpha and actin 7 were used as reference genes. Each bar represents a biological pool of approximately 50 seedlings ($n \geq 4$). In all charts, means ± SD are presented, and Student's unpaired t test was used (*P < 0.05, **P < 0.01, ***P < 0.001).

leaves (Figure 4A). The effect of 3-methoxy-BAPA was generally strongest 30 min after its application, and it declined significantly after 6 h, suggesting a fast response mechanism. Strikingly, a dramatic increase of *MPK11* transcript levels was achieved by treatment with 50 μM 3-methoxy-BAPA; after 30 min incubation, we could observe almost 90-fold increase in *MPK11* expression levels followed by a modest decline at 6 h, but the levels stayed significantly elevated after 48 h treatment in line with our transcriptomic profiling results. Further, treatment with 3-methoxy-BAPA for 30 min increased the transcript accumulation of *MPK11* almost three times more strongly than treatment with BAP at the same concentration (Figure 4A).

Immunoblotting experiments showed that the abundance of MPK3 in BAP- as well as 3-methoxy-BAPA-treated leaves increased after treatments of both 30 min and 6 h (Figure 4B and C). The abundance of MPK4 increased significantly after BAP treatment but was only slightly elevated after treatment with 3-methoxy-BAPA for 30 min or 6 h (Figure 4B and D). The abundance of MPK6 slightly increased after 30 min of treatment with BAP and after 6 h of treatment with 3-methoxy-BAPA (Figure 4B and E). The abundance of the protein product of the highly responsive *MPK11* could not be assessed due to unavailability of the respective antibody.

The phosphorylation of MPK3 and MPK6 was monitored using pTEpY, a specific anti phospho-p44/42 antibody.[46] The phosphorylation of MPK3 peaked after 30 min treatment with BAP or 3-methoxy-BAPA and decreased sharply after 6 h treatment. The level of MPK3 phosphorylation after 48 h treatment with BAP or 3-methoxy-BAPA did not differ significantly from that in the negative (DMSO) control (Figure 4F). This suggests that MPK3 status in response to 3-methoxy-BAPA changes rapidly and that there was a possible correlation between changes on the gene level (Figure 4A) to those on the corresponding (phospho-)protein level (Figures 4C and F). As with MPK3, MPK6 phosphorylation was observable after 30 min BAP treatment. However, unlike MPK3, MPK6 remained strongly phosphorylated after 48 h of incubation with either BAP or 3-methoxy-BAPA (Figure 4G) indicating possible involvement of MPK6 in long-term 3-methoxy-BAPA-induced responses.

Together, these results demonstrate that like BAP, 3-methoxy-BAPA can enhance the expression of several *MPK* genes, especially when applied in short (30 min) treatment. Treatment with this compound increased the protein-level

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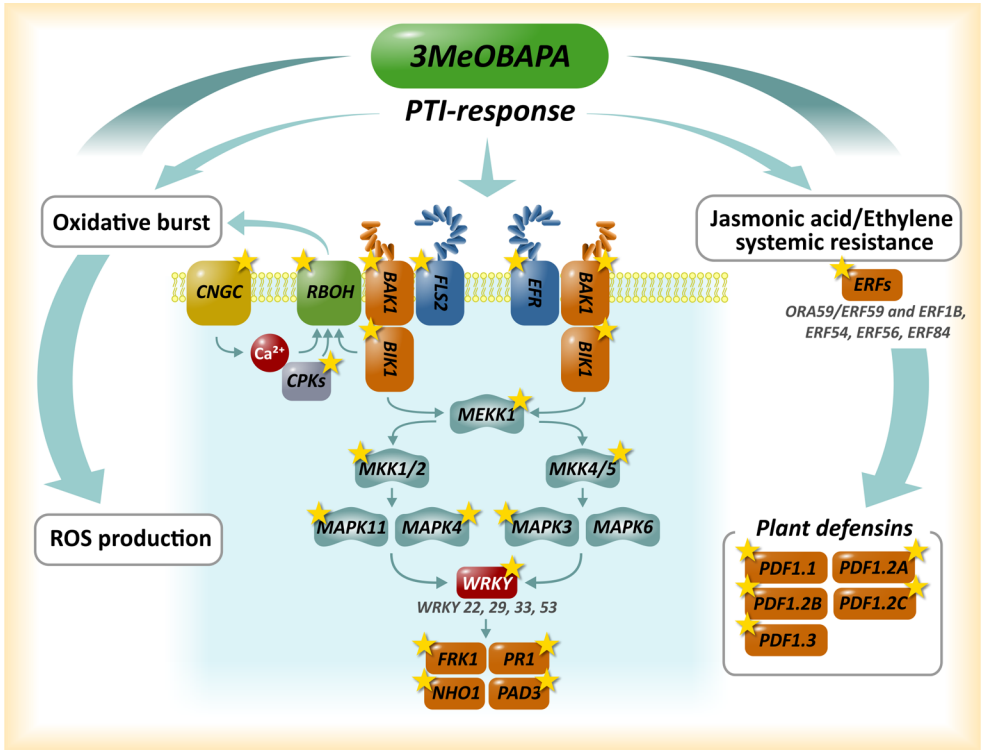

Figure 6. Hypothetical model of 3-methoxy-BAPA-mediated priming in *Arabidopsis* plants. The model is based on data from our RNA-seq transcriptomics and gene expression studies (significantly upregulated genes are marked with a star), data from the KEGG pathway database, and references cited throughout the text. Priming (either short or extended treatment with 3-methoxy-BAPA) leads to JA/ET stress signaling and expression of plant defensins, to the oxidative burst and production of ROS, and the backbone of the PTI responses is characterized by activation of the MAPK signaling pathway, leading to the expression of *FLG22-INDUCED RECEPTOR-LIKE KINASE 1* (*FRK1*), *PATHOGENESIS RELATED 1* (*PR1*), *NONHOST RESISTANCE TO P. S. PHASEOLICOLA 1* (*NHO1*), and *PHYTOALEXIN DEFICIENT 3* (*PAD3*).

expression of MPK3 and (to lesser degrees) MPK4 and MPK6. However, the effect of 3-methoxy-BAPA is better understood by considering the phosphorylation status of these MAPKs. Elicitation with 3-methoxy-BAPA had a considerably stronger effect than BAP treatment on the expression profile of *MPK11*, a stress signaling MAPK module belonging to the PAMP-triggered pathway.

Toward an Integrative Model of 3-methoxy-BAPA Action in Plants. Our data suggest that treatment of *Arabidopsis* leaves with 3-methoxy-BAPA promotes PTI defense pathways in several ways. In the short term response, the plant utilizes defense-related JA/ET pathway through *ORA59/ERF59* and *ERF1B* transcription factors,[34] and subsequent upregulation of downstream plant defensins *PDF1.1*, *PDF1.2*, and *PDF1.3* and others (Figure 2C). Notably, PDF1.2 (with isoforms 1.2A, 1.2B, and 1.2C) seems to be of particular importance, because it is primarily induced in leaves upon pathogen attack, probably through MPK3/6-EIN3-ERF1-PDF1.2 pathway.[47]

To corroborate our transcriptomic results suggesting the activation of JA/ET pathways by 3-methoxy-BAPA, we estimated the content of three JA forms (JA, its precursor *cis*-(+)-12-oxo-phytodienoic acid (*cis*-OPDA), and the major biologically active form, jasmonoyl-L-isoleucine (JA-Ile)). The content of all three forms was significantly higher in leaves treated with 3-methoxy-BAPA (10 μM) for 48 h compared to the negative control (DMSO) (Figure 5A), which in turn may lead to enhanced JA biosynthesis and signaling. All JA forms might have contributed to the 3-methoxy-BAPA-induced PTI response including *cis*-OPDA that was found to be an active player in defense responses.[48]

The formation of active JA forms within the PAMP-response is typically preceded by an accumulation of reactive oxygen species (ROS) that also help to induce the cell wall integrity/remodeling processes (as observed in the GO enrichment analysis; see Supplementary Table 3) and protect the plant from biotic or abiotic stresses. NADPH oxidase (mostly known as respiratory burst oxidase homologue, RBOH), whose expression was also stimulated by 3-methoxy-BAPA, is thought to be a key player in the PAMP-induced ROS accumulation (for a review, see *e.g.*, ref 49).

To see whether the redox state of plant cells responds to 3-methoxy-BAPA treatment, we took advantage of an available *A. thaliana* suspension cell culture. Using a standard 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay, we quantified the production of ROS in 3-methoxy-BAPA-treated *Arabidopsis* cells with either a high (50 μM) or low (10 μM) concentration of the compound (Figure 5B). Interestingly, both treatments resulted in a comparably significant increase in the ROS production 3 h after the elicitation, which was followed by a slow decrease back to the pretreatment levels after 16 h of treatment. Thus, in the early stages of the response to 3-methoxy-BAPA, temporally elevated levels of ROS seem to be involved in signaling defense responses in concert with other pathways.

It is well-known that plant defense against pathogens can be enhanced by both synthetic and natural compounds including PAMPs and also by CKs (for a recent review, see *e.g.*, ref 50). This effect is collectively referred to as defense priming. In the

https://dx.doi.org/10.1021/acschembio.0c00306
ACS Chem. Biol. 2020, 15, 1949−1963

primed state, the plant defense response to pathogen attack, often initiated by PAMP receptors including FLS2 (the receptor specific for bacterial flg22) and EFR (the bacterial EF-Tu receptor), is faster and stronger as compared to nonprimed plants. The complex molecular mechanism of priming comprises upregulation of the mentioned PAMP receptors and FLS2 co-receptor BAK1,[51] activation of a hierarchical MPK signal cascade including MEKK1, MKK4/5, MPK3/6 and MPK 11,[44,45] and upregulation of WRKY.[42] It is suggested that defense priming induces an "open chromatin" configuration at promoter regions of PTI-responsive genes[52] enabling a more effective defense gene response to a subsequent triggering stimulus (such as PAMP).

The transcriptomic changes induced by 3-methoxy-BAPA treatment in detached *Arabidopsis* leaves suggested that 3-methoxy-BAPA acts as a priming agent for the PTI response. To further confirm whether 3-methoxy-BAPA primes the PTI response in *Arabidopsis* plants, we evaluated expression levels of *MPK3*, *MPK4*, *MPK6*, and *MPK11* and other typical PTI-responsive genes including flg22-induced *FRK1* and transcription factors *WRKY33* and *WRKY53*.[45] As expected, priming of the expression of these PTI-responsive genes was also observed after treatment with flg22 (Figure 5C). In line with our results obtained with 3-methoxy-BAPA-treated detached leaves (Figure 4A), we could observe relatively strong elevation of *MPK11* expression levels and somewhat weaker effects on the expression pattern of *MPK3* and *MPK4*. Importantly, the effect of 3-methoxy-BAPA treatment proved to be significant with other examined PTI-response marker genes, although flg22, unsurprisingly, provoked a much stronger effect in the case of *FRK1*. While combined treatment of 3-methoxy-BAPA/flg22 showed little effect in the cases of MPKs and WRKYs, their downstream *FRK1* proved to be very sensitive to the combined treatment: a strong synergistic effect between the elicitor peptide flg22 and 3-methoxy-BAPA was observed in the FLS2-initiated MAPK defense pathway (Figure 5C). This leads us to the conclusion that the PTI response appears to have a dominant role in 3-methoxy-BAPA-induced defense response alongside JA/ET defense signaling cascade-mediated expression of plant defensins (Figure 6). We thus propose a model in which 3-methoxy-BAPA primes and upregulates expression of pattern recognition receptors (PRRs), such as FLS2 and EFR1, and downstream components of this signaling cascades as explained above, in agreement with our transcriptomic and gene expression analyses (Figure 6). Priming of this signaling pathway then leads to a sensitive response to bacterial peptide flg22 through *FRK1* in combined 3-methoxy-BAPA/flg22 cotreatments (Figure 5C).

The predicted ability of 3-methoxy-BAPA (and BAPAs generally) to prime plants should enhance plant resistance to pathogens. Indeed, a foliar application of both 3-methoxy-BAPA and 3-hydroxy-BAPA on field-grown wheat and barley plants in various growth stages (tillering, beginning of stem elongation, and start of flag leaf extending) significantly suppressed infection by several fungal pathogens (Figure 7). This protective effect in primed plants does not necessarily lead to impaired plant fitness and growth (unlike typical defense responses), as evidenced by a significantly increased number of tillers per plant observed for barley plants, and slightly increased (but statistically nonsignificant) number of spikes per square meter and grain yield (Supplementary Table 4).

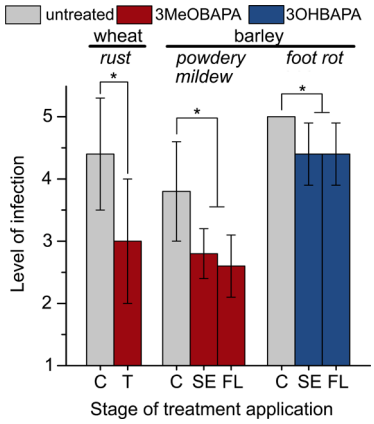

Figure 7. Effect of 3-methoxy-BAPA (3MeOBAPA) and 3-hydroxy-BAPA (3OHBAPA) treatment on the level of pathogen infection in winter wheat and spring barley plants in field-plot experiments. Plants were evaluated at the beginning of heading (wheat leaf rusts *Puccinia striiformis* syn. *Puccinia glumarum*, *Puccinia recondita* f. sp. *tritici*, *Puccinia graminis*; barley powdery mildew *Blumeria graminis* syn. *Erysiphe graminis*) or early milk ripeness (barley foot rot diseases *Gaeumannomyces graminis* syn. *Ophiobolus graminis*, *Ramulispora herpotrichoides* syn. *Pseudocercosporella herpotrichoides*, teleomorpha *Tapesia yallundae*, *Fusarium* spp., *Rhizoctonia* spp.). The 5 μM solutions were applied as a foliar spray at different plant growth stages: tillering (T), at the beginning of stem elongation (SE), and at the beginning of flag leaf extending (FL). The level of infection versus the untreated control (C) was evaluated according to the standardized methodology; for details, see Supplementary Methods. Means ± SD are presented (n = 5); asterisks indicate statistically significant difference according to Student's unpaired t test ($P < 0.05$).

While activation of early defense response by PAMPs can protect plants from pathogen attacks, prolonged PAMP exposure can worsen plant fitness because of the persistent stimulation of high-cost defense mechanisms and simultaneous inhibition of photosynthesis. For example, treating *Arabidopsis* seedlings with flg22 for 7 days reduced growth and inhibited PSII photochemistry.[38] However, the treatment of detached *Arabidopsis* leaves with 3-methoxy-BAPA had no negative effect on PSII function after 6 h, 48 h (data not shown), or even after 6 days (Figure 1C and E). On the contrary, 6 day treatment with BAPAs maintained high PSII function in both *Arabidopsis* and wheat leaves as compared with the negative control.

CONCLUSIONS

Our results show that BAPAs can emerge as a new type of priming agents in plants with a good potential for manipulating plant PAMP responses through specific elicitation of MAPK modules and series of repair and defense response mechanisms. The promotion of these defense mechanisms is linked with JA/ET stress signaling that may be directly related to enhanced levels of JA and its metabolites in response to 3-methoxy-BAPA treatment. Further, temporarily elevated levels of ROS seem to play a role in the early stages of priming with the compound. The downregulation of several photosynthetic genes, particularly in the immediate aftermath of 3-methoxy-BAPA treatment, was observed, but the prolonged elicitation with 3-methoxy-BAPA resulted in a good preservation of chlorophyll levels and significantly improved photosynthetic function. These beneficial effects also delayed the onset of senescence in BAPAs treated *Arabidopsis* and wheat leaves. In

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ACS Chem. Biol. 2020, 15, 1949−1963

addition, our field trial experiments with 3-methoxy-BAPA and 3-hydroxy-BAPA suggested that application of this type of compounds provides positive effects that may occur through the above mechanisms and protects the plant against some fungal pathogens. We believe that our approach will help integrate existing results on the role of ArCKs with a new methodology aimed at characterization of their lesser-known functions, such as the promotion of plant resistance to pathogens.

METHODS

Plant Material and Sample Preparation. *Arabidopsis thaliana* (L.) Heynh (*Arabidopsis*) and *Triticum aestivum* L. cv. Aranka (wheat) plants were used. For isolation of total RNA or total protein from detached leaves, *Arabidopsis* plants (ecotype Columbia, Col-0) were grown in soil for 6 weeks in a controlled growth chamber at 22/20 °C under short-day conditions: 8 h light (120 μmol photons m^{-2} s^{-1})/16 h dark cycle. Subsequently, leaves of similar size and chlorophyll content were cut and immediately subjected to the treatment. At least 20 detached leaves were submerged in 1 x MS medium supplemented with DMSO (0.1%) or DMSO solutions of BAP or 3-methoxy-BAPA and left incubated in given MS medium for either 30 min, 6 h or 48 h in darkness. After incubation, detached leaves were frozen in liquid nitrogen and used for RNA or protein isolation; 150 mg of liquid nitrogen-ground material was used per isolation.

For isolation of total RNA from seedlings, *Arabidopsis* Col-0 plants were grown on 1/2 × MS agar plates in a controlled growth chamber at 22/20 °C under long-day conditions: 16 h light (120 μmol photons m^{-2} s^{-1})/8 h dark cycle. Seedlings (5 DAG) were transferred into the 1/2 × liquid MS medium for another 3 days under constant shaking (60 rpm). Seedlings were then treated with 10 μM 3-methoxy-BAPA, 1 μM flg22 or both and 0.1% DMSO (negative control) for 2 h. Seedlings were frozen in liquid nitrogen and immediately used for RNA isolation.

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 natural cytokinin BAP. The bioassays were performed as described[7] 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.

Photosynthetic Activity of Detached Wheat and *Arabidopsis* Leaves. Spring wheat plants used in these experiments were grown on an artificial medium composed of perlite and Hoagland's solution in a growth chamber at 25 °C under a 16 h light (120 μmol photons m^{-2} s^{-1})/8 h dark cycle for 7 days. Then segments were cut off from the primary leaves 4 cm from the leaf tip. The basal ends of the leaf segments were placed in 100 nM, 1 μM, or 10 μM solutions of BAP, 3-methoxy-BAPA, or 3-hydroxy-BAPA dissolved in 0.1% DMSO, or in a 0.1% DMSO solution in distilled water (negative control). *A. thaliana* (Col-0) plants were grown in soil in a growth chamber at 22/20 °C under an 8 h light (120 μmol photons m^{-2} s^{-1})/16 h dark cycle for 6 weeks, after which the sixth and seventh rosette leaves were detached from the plants and placed in the above-mentioned solutions. The wheat leaf segments as well as *Arabidopsis* leaves were then kept in darkness at 24 °C for 6 days. The Chl content was estimated analytically as described previously.[29] Chl fluorescence parameters were measured on the adaxial side of the leaf segments at RT using a FluorCam 700MF imaging system (PSI). Before the Chl fluorescence measurement, the control leaves were dark-adapted for 30 min. The minimal fluorescence of the dark-adapted leaf sample (F_0) was determined by applying several microseconds-long measuring flashes (red light, 0.1 μmol photons m^{-2} s^{-1}) at the beginning of the procedure. The maximal fluorescence of the dark-adapted sample (F_M) was measured using a 1.6 s saturating pulse (white light, 850 μmol photons m^{-2} s^{-1}). The maximum quantum yield of PSII photochemistry was calculated as $F_V/F_M = (F_M - F_0)/F_M$.

RNA-seq Analysis. Plant cDNA sequencing libraries were prepared using 3.5 μg of total RNA per sample with the Illumina TruSeq Stranded mRNA Sample Preparation Kit (Illumina) in accordance with the standard procedure. Two independent replicate samples per condition were used to generate cDNA libraries. Prepared libraries were validated using a DNA 1000 chip with a 2100 Bioanalyzer (Agilent Technologies) instrument, and all concentrations were assessed using a Kapa Library Quantification Kit (Kapa Biosystems, Roche). Prepared libraries were pooled to a final concentration of 12 pM for cluster generation and sequencing. The clusters were generated using an Illumina TruSeq SR Cluster Kit v3 cBot HS and sequenced on a HiSeq SR Flow Cell v3 (50 bp reads) with a HiSeq 2500 Sequencing System (Illumina). Two independent libraries were prepared for each condition at each time point from two biological replicates. The quality control of reads generated by sequencing was performed with FASTQC v.0.11.5,[53] and reads were mapped to the reference genome of *A. thaliana* v.25 obtained from Ensembl[54] using a TopHat v.2.0.12 splice-read mapper[55] with default parameters. The reads mapped to the transcripts annotated in the reference genome were quantified using HTSeq v.0.6.0[53] with respect to the stranded library. The tests for differential gene expression were performed using the DESeq2 package.[56] Gene ontology (GO) analysis was performed using the Blas2GO v.3.0 program.[57] HemI software was used to generate heat maps.[58]

Quantitative PCR Analysis. Expression profiling of genes coding for MPKs and PTI-marker genes was done as described previously.[6] Briefly, 5 μg of total RNA isolated using RNAqueousH kit and treated twice with TURBO DNase-freeTM kit (Life Technologies) was used for first strand cDNA synthesis by RevertAidTM H Minus M-MuLV RT and oligo-dT (Fermentas). Diluted cDNA samples were used as templates in real-time PCR reactions containing gb SG PCR Master Mix (Generi Biotech) and 300 nM of each primers. RNA samples were isolated from four biological replicates and transcribed in four independent reactions and each cDNA sample was run in at least two technical replications on StepOnePlusTM Real-Time PCR System in a default program (Life Technologies). Ct values were normalized with respect to actin 2 and elongation factor 1 genes. Expression values were determined and statistically evaluated with DataAssist v3.0 Software (Life Technologies). The Benjamini and Hochberg false discovery rate was used to obtain adjusted *p*-values for unpaired *t* test.

Immunoblotting Analyses of MAPKs. Detached *Arabidopsis* leaves were homogenized in liquid nitrogen to a fine powder. Proteins were extracted by adding ice-cold 50 mM HEPES (pH 7.5) containing 75 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM DTT, and Complete EDTA-free protease inhibitor cocktail (Roche). After 15 min incubation on ice, the extracts were centrifuged at 13000*g* at 4 °C for 15 min and the protein amount was quantitated in supernatants. Extracts were proportionally mixed to give a protein concentration of 1.5 mg protein/mL with 4-fold concentrated Laemmli buffer (final concentration 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 300 mM 2-mercaptoethanol), heat-denatured at 95 °C for 5 min, and centrifuged to remove undissolved components. An amount of 15 μg of each protein was loaded on 12% PAGE gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes using the TransBlot Turbo (BioRad) semidry transfer system. To validate the protein transfer, membranes were stained with Ponceau S. Afterward, they were blocked overnight in 15% (v/v) commercial blocking solution (Western blocking reagent; Roche) diluted in Tris-HCl buffered saline with 0.1% (v/v) Tween-20 (TBS-T) and then incubated overnight with anti-MPK3 (1:3000), anti-MPK4 (1:1000), and anti-MPK6 (1:10000) antibodies (all from Merck) and anti-phospho p44/42 MAPK antibody (Erk1/2,Thr202/Tyr204) (Cell SignalingTechnology), all prepared in TBS-T with 4% (v/v) Western blocking reagent (Roche). After repeated washing in TBS-T, membranes were incubated with a HRP-conjugated secondary antibody (F(ab′)2-goat anti-rabbit IgG (H+L) secondary antibody, HRP; Thermo Fisher Scientific) diluted to 1:5000 in 4% (v/v) Western blocking reagent (Roche). The signal was developed after washing with TBS-T using Clarity ECL Western Blotting Substrate

https://dx.doi.org/10.1021/acschembio.0c00306
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(BioRad) and recorded with the ChemiDoc documentation system (BioRad). Band densities were quantified using ImageLab (BioRad). The densities of phosphorylated MPK3 and MPK6 were normalized to the abundance of the corresponding MPKs (see Figure 4F and G). All immunoblot analyses were performed using at least three biological replicates. Student's *t* test was used to evaluate the statistical significance of differences.

Quantification of Jasmonic Acid Forms. Detached seventh or eighth leaves of *Arabidopsis* (Col-0) plants (grown for 6 weeks under the same conditions as plants used for measurement of photosynthetic activity) were put into 0.1% DMSO or 10 μM 3-methoxy-BAPA solutions by a petiole for 48 h. Subsequently, the samples were frozen in liquid nitrogen and stored at −80 °C before measurement. Endogenous levels of jasmonates (jasmonic acid, JA; jasmonoyl-L-isoleucine, JA-Ile; and *cis*-12-oxo-phytodienoic acid, *cis*-OPDA) were determined in 30−40 mg of plant material according to the method described previously.[59] All experiments were repeated in five biological replicates. Briefly, the phytohormones were extracted using 10% methanol with a cocktail of stable isotope-labeled standards added as follows: 10 pmol of [$^2H_6$]JA and [$^2H_2$]JA-Ile, and 20 pmol of [$^2H_5$]OPDA (all from Olchemim) per sample. The extracts were purified using Oasis HLB columns (30 mg/1 mL, Waters) and then evaporated to dryness under a stream of nitrogen. Jasmonate levels were quantified by ultrahigh performance liquid chromatography-electrospray tandem mass spectrometry (an Acquity UPLC I-Class System coupled to a Xevo TQ-S MS, all from Waters) using stable isotope-labeled internal standards as a reference.

ROS Measurements. *Arabidopsis* cells of PSB-D suspension culture in conical flasks were cultivated in the dark at 25 °C, under constant shaking (130 rpm), and subcultured every 7 days (1:5) in a cell suspension medium (1 × MS with 3% sucrose, 1 mg mL^{-1} NAA, 50 ug/mL kinetin). The generation of ROS in PSB-D culture was determined with DCFH-DA (2′,7′-dichlorofluorescin diacetate) (Merck). Cells were treated with either 10 μM or 50 μM 3-methoxy-BAPA, or 0.1% DMSO (negative control) for designated time periods. Briefly, cells were loaded with 10 μM DCFH-DA and the fluorescence was detected after 10 min using a microplate reader (Tecan Infinite M200) operating at excitation and emission wavelengths of 485 and 525 nm, respectively.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00306.

> Physicochemical properties and chemical purity of the BAPA series; most strongly upregulated and downregulated differentially expressed genes regulated by 3-methoxy-BAPA; most strongly affected gene ontology (GO) terms assigned to upregulated or downregulated differentially expressed genes; effects of 3-methoxy-BAPA and 3-hydroxy-BAPA application on field-grown wheat and barley plants in terms of grain yield and other growth parameters; information about primer design; and references (PDF)

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https://dx.doi.org/10.1021/acschembio.0c00306
ACS Chem. Biol. 2020, 15, 1949−1963

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https://pubs.acs.org/10.1021/acschembio.0c00306

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Funding

The work was supported by ERDF projects "Development of Pre-Applied Research in Nanotechnology and Biotechnology" (No. CZ.02.1.01/0.0/0.0/17_048/0007323) and "Plants as a tool for sustainable global development" (No. CZ.02.1.01/0.0/0.0/16_019/0000827), Czech Science Foundation Grant 16-04184S (O.P., K.D., S.D., and L.P.), IGA_PrF_2018_022 (Z.K. and M.S.), and IGA_PrF_2020_010 (K.D.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to thank P. Ocvirková for her skillful technical assistance and T. Pospíšil for measurements of ^{1}H NMR and analysis of 2D NMR spectral data. The authors thank to H. Martínková for her help with phytohormone analyses and J. Balonová for performing cytokinin bioassays.

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https://dx.doi.org/10.1021/acschembio.0c00306
ACS Chem. Biol. 2020, 15, 1949−1963

Aromatic cytokinin arabinosides promote PAMP-like responses and positively regulate leaf longevity

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Supporting Information

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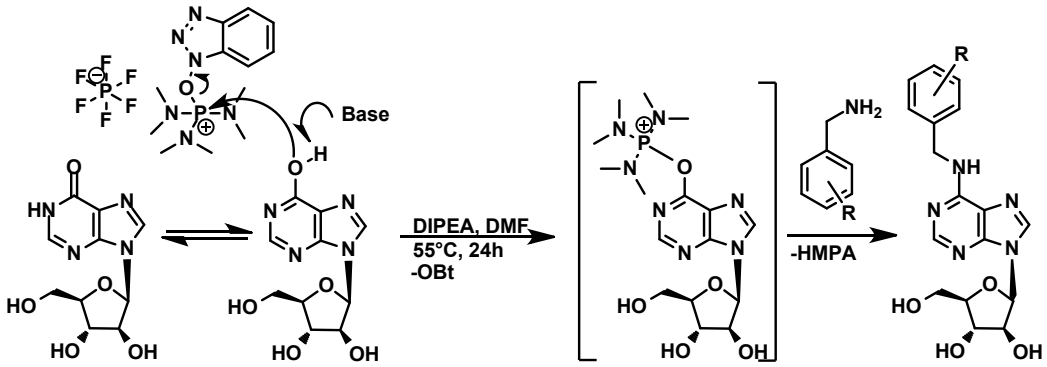
Supplementary Methods

Synthesis of prepared derivatives

The synthesis of BAPAs was achieved according to the following general reaction scheme given here:



9-(β-D-arabinofuranosyl)hypoxantine (100 mg, 0.37 mmol) and BOP (196 mg, 0.37 mmol) were mixed together in DMF (2 ml). Subsequently, the appropriate benzylamine (0.37 mmol) and DIPEA (97 µl, 0.44 mmol) were added. The reaction mixture was stirred under an argon atmosphere in an oil bath at 60 °C for 24 h then evaporated using a rotary evaporator. MeOH with a drop of chloroform was added to the distillation residue and the mixture was sonicated. The resulted white solid was isolated by filtration and re-crystallized from EtOH. The identity and purity of the synthesized compounds was confirmed by elemental and melting point analysis, analytical thin layer chromatography, high performance liquid chromatography, ES+MS spectrometry, and ^{1}H NMR (Supplementary Table 1).

^{1}H NMR data for the new compounds (DMSO-d6, 500 MHz) δ ppm:

Compound **1**:

3.55-3.75 (m,3H), 4.09 (t,J=4,5Hz,2H), 4.66 (bs,2H), 5.05 (t,J=5,5Hz,1H), 5.48 (d,J=4,5Hz,1H), 5.57 (d,J=5Hz,1H), 6.21 (d,J=4Hz,1H), 7.12-7.32 (m,5H), 8.14 (s,1H), 8.16 (s,1H), 8.32 (bs,1H)

Compound **2**:

3.55-3.68 (m,2H), 3.74 (d,J=3,5Hz,1H), 4.10 (s,2H), 4.71 (bs,2H), 5.05 (d,J=4,5Hz,1H), 5.49 (s,1H), 5.58 (d,J=3,5Hz,1H), 6.23 (d,J=3,5Hz,1H), 7.03-7.32 (m,4H), 8.15 (s,1H), 8.18 (s,1H), 8.30 (bs,1H)

Compound **3**:

3.55-3.67 (m,2H), 3.74 (d,J=4Hz,1H), 4.10 (s,2H), 4.67 (bs,2H), 5.05 (t,J=4,5Hz,1H), 5.48 (d,J=4Hz,1H), 5.57 (d,J=3,5Hz,1H), 6.22 (d,J=4Hz,1H), 6.98 (t,J=6Hz,1H), 7.06-7.16 (m,2H), 7.25-7.33 (m,1H), 8.15 (s,1H), 8.18 (s,1H), 8.37 (bs,1H)

Compound **4**:

3.55-3.66 (m,2H), 3.73 (d,J=4,5 Hz,1H), 4.07-4.11 (m,2H), 4.62 (bs,2H), 5.06 (t,J=5,5 Hz,1H), 5.49 (d,J=4,5 Hz,1H), 5.57 (d,J=5 Jz,1H), 6.22 (d,J=5 Hz,1H), 7.07 (t,J=8,5 Hz,2H), 7.33 (t,J=5,5 Hz,2H), 8.16 (s,2H), 8.34 (bs,1H)

Compound **5**:

3.54-3.80 (m,3H), 4.10 (bs,2H), 4.71 (bs,2H), 5.06 (bs,1H), 5.49 (d,J=3,5Hz,1H), 5.60 (d,J=4Hz,1H), 6.23 (d,J=4Hz,1H) 7.21 (d,J=4Hz,3H), 7.37-7.44 (m,1H), 8.13 (s,1H), 8.20 (s,1H), 8.34 (bs,1H)

Compound **6**:

3.54-3.65 (m,2H), 3.74 (bs,1H), 4.09 (bs,2H), 4.65 (bs,2H), 5.11 (bs,1H), 5.56 (bs,2H), 6.21 (d, J=4,5 Hz,1H), 7.21-7.35 (m,4H), 8.15 (s,1H), 8.17 (s,1H), 8.37 (s,1H)

Compound **7**:

3.56-3.67 (m,2H), 3.73 (bs,1H), 4.09 (bs,2H), 4.63 (bs,2H), 5.05 (t,J=4,5Hz,1H), 5.48 (d,J=3Hz,1H), 5.57 (d,J=4,5Hz,1H), 6.22 (d,J=4Hz,1H), 7.30 (s,4H), 8.14 (s,1H), 8.17 (s,1H), 8.36 (bs,1H)

Compound **8**:

3.75-3.79 (m,2H), 4.13 (t,J=5 Hz,3H), 4.64 (bs,2H), 5.23 (bs, 1H), 5.66 (bs,2H), 6.25 (d,J=5,5 Hz,1H), 7.09 (t,J=7,5 Hz,1H), 7.33 (d,J=7,5 Hz,1H), 7.55 (d,J=8 Hz,1H), 7.68 (s,1H), 8.18 (s,1H), 8.21 (s,1H), 8.36 (bs,1H)

Compound **9**:

3.54-3.65 (m,2H), 3.72 (d,J=4Hz,1H), 4.09 (t,J=4,5Hz,2H), 4.58 (bs,2H), 5.04 (t,J=5Hz,1H), 5.48 (d,J=4Hz,1H), 5.56 (d,J=4,5Hz,1H), 6.21 (d,J=4Hz,1H), 7.09 (d,J=8,5Hz,2H), 7.59 (d,J=8Hz,2H), 8.13 (s,1H), 8.16 (s,1H), 8.34 (bs,1H)

Compound **10**:

3.55-3.68 (m,2H), 3.74 (d,J=4Hz,1H), 3.79 (s,3H), 4.10 (s,2H), 4.62 (bs,2H), 5.07 (bs,1H), 5.50 (s,1H), 5.59 (s,1H), 6.23 (d,J=4Hz,1H), 6.79 (t,J=8Hz,1H), 6.93 (d,J=7,5Hz,1H), 7.05 (d,J=6,5Hz,1H), 7.16 (t,J=8Hz,1H), 8.06 (s,1H), 8.11 (s,1H), 8.17 (bs,1H)

Compound **11**:

3.56-3.79 (m,6H), 4.10 (s,2H), 4.63 (bs,2H), 5.06 (s,1H), 5.49 (s,1H), 5.58 (s,1H), 6.22 (d,J=2,5Hz,1H), 6.72 (d,J=6Hz,1H), 6.86 (d,J=8Hz,2H), 7.15 (t,J=8Hz,1H), 8.15 (s,1H), 8.17 (s,1H), 8.30 (bs,1H)

Compound **12**:

3.52-3.75 (m,6H), 4.03-4.12 (m,2H), 4.56 (bs,2H), 5.00-5.09 (m,1H), 5.44-5.59 (m,2H), 6.15-6.24 (m,1H), 6.79 (d,J=8,5Hz,2H), 7.16-7.26 (m,2H), 8.12 (s,1H), 8.14 (s,1H), 8.24 (bs,1H)

Compound **13**:

3.54-3.67 (m,2H), 3.74 (d,J=3,5Hz,1H), 4.10 (s,2H), 4.57 (bs,2H), 5.06 (s,1H), 5.49 (s,1H), 5.58 (d,J=3,5Hz,1H), 6.22 (d,J=3Hz,1H), 6.54 (d, J=7,5Hz,1H), 6.65-6.74 (m,2H), 7.02 (t,J=7,5Hz,1H), 8.14 (s,1H), 8.16 (s,1H), 8.26 (bs,1H), 9.20 (s,1H)

Compound **14**:

3.54-3.76 (m,6H), 4.03-4.15 (m,2H), 4.62 (bs,1H), 5.04 (t,J=5,5Hz,1H), 5.49 (d,J=4,5Hz,2H), 5.59 (d,J=6Hz,2H), 6.12-6.25 (m,1H), 7.01-7.18 (m,2H), 7.99 (s,1H), 8.10 (s,1H), 8.12 (s,1H), 8.23 (bs,1H)

Compound **15**:

3.55-3.78 (m,6H), 4.06-4.15 (m,2H), 4.62 (bs,2H), 5.14 (t,J=5,5HZ,1H), 5.56 (d,J=4Hz,1H), 5.62 (d,J=5Hz,1H), 6.22 (d,J=4Hz,1H), 6.96 (d,J=7Hz,1H), 7.04-7.15 (m,3H), 8.14 (s,1H), 8.16 (s,1H), 8.26 (bs,1H)

Compound **16**:

3.54-3.77 (m,6H), 4.09 (s,2H), 4.61 (bs,2H), 5.05(t,J=5,5Hz,1H), 5.48 (d,J=3,5Hz,1H), 5.57 (d,J=4,5Hz,1H), 6.21 (d,J=4Hz,1H), 7.04 (d,J=7,5Hz,2H), 7.17 (d,J=7,5Hz,2H), 8.13 (s,1H), 8.15 (s,1H), 8.26 (bs,1H)

Compound **17**:

3.57-3.70 (m,2H), 3.76 (d,J=4 Hz,1H), 4.12 (s,2H), 4.51 (bs,2H), 5.09 (t,J=5 Hz,1H), 5.19 (s,2H), 5.52 (d,J=4 Hz,1H), 5.59 (d,J=5 Hz,1H), 6.25 (d,J=4 Hz,1H), 6.45 (t,J=7 Hz,1H), 6.59 (d,J=8 Hz,1H), 6.90 (t,J=7 Hz,1H), 7.07 (d,J=7,5 Hz,1H), 8.20 (s,3H)

Compound **18**:

3.55-3.76 (m,3H), 4.06-4.13 (m,2H), 4.73 (bs,2H), 5.05 (t,J=5Hz,1H), 5.48 (d,J=4,5Hz,1H), 5.56(d,J=5,5Hz,1H), 6.22 (d,J=5Hz,1H), 7.46-7.68 (m,4H), 8.15 (s,1H), 8.18 (s,1H), 8.44 (bs,1H)

Compound **19**:

3.56-3.76 (m,3H), 4.05-4.14 (m,2H), 4.68 (bs,2H), 5.05 (t,J=5,5Hz,1H), 5.48 (d,J=4,5Hz,1H), 5.57 (d,J=5,5Hz,1H), 6.22 (d,J=4,5Hz,1H), 7.15 (d,J=8Hz,1H), 7.25-7.42 (m,3H), 8.15 (s,1H), 8.18 (s,1H), 8.40 (bs, 1H)

Compound **20**:

3.54-3.76 (m,3H), 4.03-4.15 (m,2H), 4.68 (bs,2H), 5.05 (t,J=5,5Hz,1H), 5.48 (d,J=4Hz,1H), 5.56 (d,J=5Hz,1H), 6.23 (d,J=4,5Hz,1H), 7.02-7.22 (m,3H), 8.16 (s,1H), 8.19 (s,1H), 8.34 (bs,1H)

Compound **21**:

3.40-3.53 (m,2H), 3.60 (d,J=2,5 Hz,1H), 3.95 (s,2H), 4.52 (bs,2H), 4.91 t,J=5Hz,1h), 5.35 (d,J=2Hz,1H), 5.42 (d,J=4Hz,1H), 6.09 (d,J=3Hz,1H), 6.79-6.92 (m,3H), 8.02 (s,1H), 8.05 (s,1H), 8.26 (bs,1H)

General procedures

Elemental analyses were performed using an EA1112 CHN analyzer (Thermo Finnigan, Thermofisher Scientific). Melting points were determined on a Buchi Melting Point B-540 apparatus and are uncorrected. Analytical thin-layer chromatography (TLC) was carried out using silica gel 60 WF_{254} plates (Merck) with $CHCl_3$/MeOH (4:1, v/v) as the solvent. The purity of the synthesized compounds was determined as described previously [1]. Briefly, samples were dissolved in methanol (1 mg ml^{-1}) and then diluted to a concentration of 10 µg ml^{-1} in the initial mobile phase. 10 µl of the resulting solution was injected onto a thermostated (25 °C) RP column (150 mm × 2.1 mm, 5 µm C18 Symmetry, Waters) and analyzed using an Alliance 2695 Separations Module high-performance liquid chromatograph coupled to a PDA 2996 detector (Waters) 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). HPLC elution was performed at a flow rate of 0.3 ml min^{-1} using a linear gradient of 15 mM ammonium formate at pH 4.0 (mobile phase A) and pure 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 120 °C with a capillary voltage of +3.0 kV, a cone voltage of +20 V, and a desolvation temperature of 300 °C. Nitrogen was used as both the desolvation gas (500 l h^{-1}) and the cone gas (50 l h^{-1}). The mass spectrometer was operated in positive (ESI+) ionization mode and data were acquired in the 50–1000 *m/z* range. The mass spectrometer was directly coupled to a MassLynx 4.1 data system. ^{1}H NMR spectra were measured on a JEOL 500 ECA instrument at 500 MHz. Samples were prepared by dissolving the compounds in DMSO-d_6. Tetramethylsilane (TMS) was used as the internal standard.

KEGG pathway over-representation analysis

For the visual interpretation of transcriptomic data from 3-methoxy-BAPA treatments, the KEGG database was employed (https://www.kegg.jp). KEGG Mapper was used for processing of KO annotation data [2].

Bacterial cytokinin assay

E. coli strain KMI001 (*ΔrcsC, cps::lacZ*), harboring either the plasmid pIN-III-AHK4 or pSTV28-AHK3, which express the Arabidopsis histidine kinases CRE1/AHK4 or AHK3 [3, 4] was used in the experiments. Activity of β-galactosidase was measured using 4-methylumbelliferyl β-D-galactopyranoside as a substrate after overnight growth in the presence of CK [5]. In addition, live-cell cytokinin-binding assay with radiolabeled *tZ* as a competitor was performed as previously described [6]. Homogenous bacterial suspensions with OD_{600} values of 0.8 and 1.2 were found to be optimal for CRE1/AHK4 and AHK3 cultures, respectively. The competition reaction was allowed to proceed with 2 nM [2-^{3}H]*tZ* and various concentrations of the tested compounds for 30 min at 4 °C. When binding equilibrium was reached, the suspension was centrifuged (6000 x g), the supernatant was removed, and the bacterial pellet was resuspended in the scintillation cocktail (Beckman).

Effect of 3-methoxy-BAPA and 3-hydroxy-BAPA on infection level in field trial experiments

An effect of 3-methoxy-BAPA and 3-hydroxy-BAPA was tested in field trials on winter wheat (cv. Turandot; Selgen, Czech Republic) and spring barley (cv. Francin; Selgen, Czech Republic) performed near Olomouc, Czech Republic (GPS: 49.5748542N, 17.2851261E; altitude 218 m) in a growth season 2017/2018. The 5 μM solution of 3-methoxy-BAPA or 3-hydroxy-BAPA was applied as a foliar spray on wheat at a growth stage of tillering (BBCH 25 [7]) and on barley at stage of beginning of stem elongation (BBCH 30-33 [7]) or start of flag leaf extending (BBCH 39-41 [7]) in amount of 300 ml per 10 m^2. A level of infection was evaluated at a growth stage of beginning of heading (BBCH 51 [7]) in the case of wheat and leaf rusts and barley and powdery mildew or at growth stage of early milk ripeness (BBCH 73 [7]) in the case of barley and foot rot diseases. The evaluation of each variant was carried out on 5 plots (n = 5), 20 plants were evaluated from each plot. The level of infection was evaluated according the following scales:

i) for wheat and leaf rusts (namely *Puccinia striiformis* syn. *Puccinia glumarum*, *Puccinia recondita* f. sp. *tritici*, *Puccinia graminis*): 1 – without infection; 2 – a few clusters of chlorosis; 3 – clusters to 1 % of leaf area; 4 – clusters from 1 to 3 % of leaf area; 5 - clusters from 3 to 5 % of leaf area, beginning of strips; 6 – clusters and strips from 5 to 15 % of leaf area;

ii) for barley and powdery mildew (*Blumeria graminis* syn. *Erysiphe graminis*): 1 – without symptoms; 2 – mycelium at max. 1 % of leaf area; 3 – mycelium at 1 – 5 % of leaf area; 4 – mycelium at 5 – 10 % of leaf area; 5 - mycelium at 10 – 30 % of leaf area;

iii) for barley and foot rot diseases (*Gaeumannomyces graminis* syn. *Ophiobolus graminis*, *Ramulispora herpotrichoides* syn. *Pseudocercosporella herpotrichoides*, teleomorpha *Tapesia yallundae*, *Fusarium* spp., *Rhizoctonia* spp.): 1 – without symptoms; 3 – brown spots to 5 % of stem perimeter; 5 – brown spots from 5 to 25 % of stem perimeter.

In the end of each experiment, a number of tillers per plant was determined with 50 randomized plants of the variant (10 plants from each plot). A number of spikes per 0.25 m^2 was counted and converted to 1 m^2. After harvest, grain yield was determined.

Supplementary Tables

Supplementary Table 1. Elemental analysis, ES+ mass spectrometry analysis, melting points and HPLC purity of prepared BAPA derivatives.

Compound	Elemental analysis calculated/found			Mp (°C)	ES-MS	HPLC (%)
	% of C	% of H	% of N		[M+H$^+$]	
1	57.1/55.9	5.3/4.9	19.6/19.0	191-193	358	>98
2	54.4/52.6	4.8/4.7	18.7/18.0	191-193	376	>98
3	54.4/54.0	4.8/4.7	18.7/18.8	224-225	376	>98
4	54.4/52.9	4.8/4.9	18.7/18.0	190-192	376	>98
5	52.1/51.7	4.6/4.7	17.9/17.6	226-229	393	>99
6	52.1/51.9	4.6/4.6	17.9/17.4	240-242	393	>98
7	52.1/52.3	4.6/4.4	17.9/17.2	181-183	393	>99
8	42.3/41.8	3.8/3.6	14.5/14.1	221-223	484	>98
9	42.3/42.2	3.8/3.8	14.5/13.8	182-184	484	>98
10	55.8/55.6	5.5/5.5	18.1/17.9	224-225	388	>98
11	55.8/55.4	5.5/5.4	18.1/17.9	236-238	388	>99
12	55.8/55.7	5.5/5.5	18.1/17.9	182-183	388	>99
13	54.7/53.9	5.1/5.0	18.8/18.3	235-238	374	>98
14	58.2/57.9	5.7/5.4	18.9/18.8	224-227	372	>98
15	58.2/57.4	5.7/5.3	18.9/18.9	216-218	372	>99
16	58.2/58.0	5.7/5.4	18.9/18.6	173-177	372	>98
17	54.8/54.2	5.4/5.3	22.6/21.8	193-196	373	>98
18	50.8/49.9	4.3/3.9	16.5/15.8	182-184	426	>98
19	49.0/48.6	4.1/3.9	15.9/15.4	175-176	442	>98
20	51.9/51.2	4.4/4.3	17.8/17.6	214-216	394	>98
21	51.9/51.4	4.4/3.7	17.8/17.3	236-237	394	>98

Supplementary Table 2. Overview of DEGs regulated by 3-methoxy-BAPA. The table lists the 50 most strongly upregulated and downregulated genes (adjusted P-value < 0.05), respectively, that showed the highest absolute $\log_2$ fold change ($\log_2$FC) after 6 h dark-incubation of detached Arabidopsis leaves with 10 µM 3-methoxy-BAPA.

UPREGULATED DEGs		
AGI number	**Description**	**$\log_2$ FC**
At2g47040	Vanguard 1/Pectinesterase 5 [Source:Uniprot/SWISSPROT 3BAcc:Q5MFV8]	**2.80**
At3g01270	Probable pectate lyase 7 [Source:Uniprot/SWISSPROT 3BAcc:Q9SRH4]	**2.79**
At2g47050	Plant invertase/pectin methylesterase inhibitor superfamily protein [Source:TAIR 3BAcc:47050]	**2.31**
At3g07820	Pectin lyase-like superfamily protein [Source:TAIR 3BAcc:07820]	**2.22**
At3g28750	Hypothetical protein	**2.21**
At2g47030	Plant invertase/pectin methylesterase inhibitor superfamily protein (VGDH1) [Source:Uniprot/SWISSPROT 3BAcc:O80722]	**2.16**
At4g35010	Beta-galactosidase 11 (BGAL11) [Source:Uniprot/SWISSPROT 3BAcc:Q9SCV1]	**2.03**
At3g62230	F-box protein At3g62230/DUO1-ACTIVATED F-BOX 1 [Source:Uniprot/SWISSPROT 3BAcc:Q9M1Q1]	**1.95**
At3g05610	Probable pectinesterase/pectinesterase inhibitor 21 [Source:Uniprot/SWISSPROT 3BAcc:Q8GX86]	**1.80**
At5g44430	PLANT DEFENSIN 1.2C (PDF1.2C)/Defensin-like protein 17 [Source:Uniprot/SWISSPROT 3BAcc:Q9FI22]	**1.75**
At1g55560	SKU5 similar 14/SKS14 [Source:TAIR 3BAcc:55560]	**1.71**
At1g02790	Exopolygalacturonase 4 (PGA4) [Source:Uniprot/SWISSPROT 3BAcc:P49062]	**1.67**

At5g39880	Transmembrane protein [Source:TAIR 3BAcc:39880]	**1.64**
At5g44420	PLANT DEFENSIN 1.2A/Defensin-like protein 16 [Source:Uniprot/SWISSPROT 3BAcc:Q9FI23]	**1.58**
At5g07430	Pectin lyase-like superfamily protein/Probable pectinesterase 50 [Source:Uniprot/SWISSPROT 3BAcc:Q9LY17]	**1.57**
At1g07260	UDP-glycosyltransferase 71C3 (UGT71C3) [Source:Uniprot/SWISSPROT 3BAcc:Q9LML7]	**1.54**
At2g26020	PLANT DEFENSIN 1.2B/Putative defensin-like protein 15 [Source:Uniprot/SWISSPROT 3BAcc:O80994]	**1.52**
At5g19580	Glyoxal oxidase-related protein [Source:TAIR 3BAcc:19580]	**1.51**
At2g26010	PLANT DEFENSIN 1.3/Defensin-like protein 14 [Source:Uniprot/SWISSPROT 3BAcc:O80995]	**1.47**
At5g45880	Pollen Ole e 1 allergen and extensin family protein [Source:TAIR 3BAcc:45880]	**1.45**
At5g50030	Plant invertase/pectin methylesterase inhibitor superfamily protein [Source:TAIR 3BAcc:50030]	**1.42**
At3g01240	Splicing regulatory glutamine/lysine-rich-like protein	**1.35**
At3g62710	Glycosyl hydrolase family protein [Source:TAIR 3BAcc:62710]	**1.35**
At5g04180	Alpha carbonic anhydrase 3 (ATACA3) [Source:Uniprot/SWISSPROT 3BAcc:Q9FYE3]	**1.33**
At5g12960	Proline-tRNA ligase (DUF1680) [Source:TAIR 3BAcc:12960]	**1.33**
At5g26700	RmlC-like cupins superfamily protein/Probable germin-like protein subfamily 2 member 5 [Source:Uniprot/SWISSPROT 3BAcc:O65252]	**1.26**
At1g05580	Cation/H(+) antiporter 23 2C chloroplastic (CHX23) [Source:Uniprot/SWISSPROT 3BAcc:Q8VYD4]	**1.26**
At5g58390	Peroxidase superfamily protein [Source:Uniprot/SWISSPROT 3BAcc:Q9LVL2]	**1.23**
At1g61566	Protein RALF-like 9 (RALFL9) [Source:Uniprot/SWISSPROT 3BAcc:Q3ECL0]	**1.19**
At1g62760	Pectin methylesterase inhibitor (ATPMEI10) [Source:TAIR 3BAcc:AT1G62760]	**1.18**
At1g06160	Ethylene-responsive transcription factor ERF094/Ethylene responsive factor 59 [Source:Uniprot/SWISSPROT 3BAcc:Q9LND1]	**1.16**
At2g04460	Transposable element gene [Source:TAIR 3BAcc:04460]	**1.16**
At5g61160	Agmatine coumaroyltransferase/Anthocyanine 5-aromatic acyltransferase 1 [Source:Uniprot/SWISSPROT 3BAcc:Q9FNP9]	**1.13**
At3g20470	Glycine-rich protein 5 [Source:TAIR 3BAcc:AT3G20470]	**1.09**
At1g59950	NAD(P)-linked oxidoreductase superfamily protein [Source:TAIR 3BAcc:59950]	**1.08**
At1g75830	PLANT DEFENSIN 1.1 (PDF1.1)/Defensin-like protein 13 [Source:Uniprot/SWISSPROT 3BAcc:P30224]	**1.06**
At4g16260	Putative beta-1,3-endoglucanase [Source:TAIR 3BAcc:16260]	**1.01**
At2g41180	Sigma factor binding protein 2 2C chloroplastic (SIB2) [Source:Uniprot/SWISSPROT 3BAcc:O80669]	**0.98**
At3g49110	Peroxidase 33 (PRX33) [Source:Uniprot/SWISSPROT 3BAcc:P24101]	**0.98**
At2g43590	Chitinase family protein [Source:TAIR 3BAcc:43590]	**0.95**
At3g28153	Transposable element gene [Source:TAIR 3BAcc:28153]	**0.92**
At2g43580	Chitinase family protein [Source:TAIR 3BAcc:43580]	**0.91**
At1g61563	Protein RALF-like 8 (RALFL8) [Source:Uniprot/SWISSPROT 3BAcc:Q1ECR9]	**0.90**
At3g43828	Transposable element gene [Source:TAIR 3BAcc:43828]	**0.90**
At1g15415	Protein phosphatase 2A (PP2A) B'gamma subunit [Source:TAIR 3BAcc:15415]	**0.88**
At2g31141	Hypothetical protein	**0.88**
At2g28210	Alpha carbonic anhydrase 2 (ATACA2) [Source:TAIR 3BAcc:28210]	**0.85**
At3g13400	SKU5 similar 13/SKS13 [Source:TAIR 3BAcc:13400]	**0.82**
At2g22320	Hypothetical protein	**0.82**
At1g66390	Transcription factor MYB90 [Source:Uniprot/SWISSPROT 3BAcc:Q9ZTC3]	**0.81**
DOWNREGULATED DEGs		
At4g36850	PQ-loop repeat family protein / transmembrane family protein [Source:TAIR 3BAcc:At4g36850]	**-2.16**
At1g29920	Chlorophyll a/b binding protein 2 (LHCB1.2) [Source:Uniprot/SWISSPROT 3BAcc:Q8VZ87]	**-1.78**

At2g34430	Light-harvesting chlorophyll-protein complex II subunit B1 [Source:TAIR 3BAcc:At2g34430]	**-1.64**
At3g27690	Photosystem II light harvesting complex gene 2.3 [Source:TAIR 3BAcc:At3g27690]	**-1.40**
At2g05100	Photosystem II light harvesting complex gene 2.1 [Source:TAIR 3BAcc:At2g05100]	**-1.32**
At1g29910	Light-harvesting chlorophyll a/b binding protein 1.2 [Source:TAIR 3BAcc:At1g29910]	**-1.29**
At2g05070	Photosystem II light harvesting complex gene 2.2 [Source:TAIR 3BAcc:At2g05070]	**-1.29**
At3g19390	Granulin repeat cysteine protease family protein [Source:TAIR 3BAcc:At3g19390]	**-1.20**
At3g19710	Methionine aminotransferase BCAT4 [Source:Uniprot/SWISSPROT 3BAcc:Q9LE06]	**-1.18**
At1g62510	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein [Source:TAIR 3BAcc:At1g62510]	**-1.16**
At4g21650	Subtilase 3. 13 (SBT3.13) [Source:TAIR 3BAcc:At4g21650]	**-1.11**
At5g65690	Phosphoenolpyruvate carboxykinase 2 (PCK2) [Source:TAIR 3BAcc:At5g65690]	**-1.09**
At5g24490	30S ribosomal protein 2C putative [Source:TAIR 3BAcc:At5g24490]	**-1.08**
At1g29930	Chlorophyll a/b binding protein 1 2C chloroplastic/Light-harvesting chlorophyll a/b protein 1.3 [Source:Uniprot/SWISSPROT 3BAcc:P04778]	**-1.08**
At5g54270	Light-harvesting chlorophyll B-binding protein 3 [Source:TAIR 3BAcc:At5g54270]	**-1.08**
At5g38420	Ribulose bisphosphate carboxylase small chain 2B 2C chloroplastic (RBCS2B) [Source:Uniprot/SWISSPROT 3BAcc:P10797]	**-1.06**
At4g25580	CAP160 protein [Source:TAIR 3BAcc:At4g25580]	**-1.05**
At1g16410	Dihomomethionine N-hydroxylase/Cytochrome P450 79F1 (CYP79F1) [Source:Uniprot/SWISSPROT 3BAcc:Q949U1]	**-1.05**
At4g26260	Myo-inositol oxygenase 4 (MIOX4) [Source:Uniprot/SWISSPROT 3BAcc:Q8H1S0]	**-1.05**
At3g26060	Peroxiredoxin Q 2C chloroplastic [Source:Uniprot/SWISSPROT 3BAcc:Q9LU86]	**-1.03**
At5g02160	FTSH5 interacting protein [Source:TAIR 3BAcc:At5g02160]	**-1.03**
At5g64040	Photosystem I reaction center subunit N 2C chloroplastic [Source:Uniprot/SWISSPROT 3BAcc:P49107]	**-1.02**
At3g22740	Homocysteine S-methyltransferase 3 [Source:Uniprot/SWISSPROT 3BAcc:Q8LAX0]	**-1.01**
At2g10940	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein [Source:TAIR 3BAcc:AT2G10940]	**-0.99**
At3g08940	Chlorophyll a-b binding protein CP29.2 2C chloroplastic/LHCB4.2 [Source:Uniprot/SWISSPROT 3BAcc:Q9XF88]	**-0.98**
At4g01330	Protein kinase superfamily protein [Source:TAIR 3BAcc:At4g01330]	**-0.97**
At1g30280	Chaperone DnaJ-domain superfamily protein/Auxilin-like 7 [Source:TAIR 3BAcc:At1g30280]	**-0.96**
At2g20670	Putative sugar phosphate exchanger (DUF506) [Source:TAIR 3BAcc:At2g20670]	**-0.96**
At2g34420	Photosystem II light harvesting complex gene B1B2 [Source:TAIR 3BAcc:At2g34420]	**-0.95**
At1g68560	Alpha-xylosidase 1 (ATXYL1)/Altered xyloglucan 3 (AXY3) [Source:Uniprot/SWISSPROT 3BAcc:Q9S7Y7]	**-0.94**
At4g08870	Arginine amidohydrolase 2 2C mitochondrial [Source:Uniprot/SWISSPROT 3BAcc:Q9ZPF5]	**-0.93**
At5g23010	Methylthioalkylmalate synthase 1 2C chloroplastic [Source:Uniprot/SWISSPROT 3BAcc:Q9FG67]	**-0.93**
At3g50800	Hypothetical protein	**-0.93**
At2g46740	L-gulonolactone oxidase 5 [Source:Uniprot/SWISSPROT 3BAcc:O81030]	**-0.92**
At5g63450	Cytochrome P450 94B1 [Source:Uniprot/SWISSPROT 3BAcc:Q9FMV7]	**-0.92**
At4g03060	Alkenyl hydroxalkyl producing 2 (AOP2) [Source:TAIR 3BAcc:At4g03060]	**-0.91**
At5g55150	F-box SKIP23-like protein (DUF295) [Source:Uniprot/SWISSPROT 3BAcc:Q9FLP7]	**-0.90**
At1g12900	Glyceraldehyde-3-phosphate dehydrogenase GAPA2 2C chloroplastic [Source:Uniprot/SWISSPROT 3BAcc:Q9LPW0]	**-0.89**
At3g02020	Aspartokinase 3 2C chloroplastic [Source:Uniprot/SWISSPROT 3BAcc:Q9S702]	**-0.88**
At1g68010	Glycerate dehydrogenase HPR 2C peroxisomal [Source:Uniprot/SWISSPROT 3BAcc:Q9C9W5]	**-0.86**
At3g46780	Plastid transcriptionally active 16 [Source:TAIR 3BAcc:AT3G46780]	**-0.86**

At5g14740	Beta carbonic anhydrase 2 2C chloroplastic [Source:Uniprot/SWISSPROT 3BAcc:P42737]	**-0.85**
At1g32060	Phosphoribulokinase 2C chloroplastic [Source:Uniprot/SWISSPROT 3BAcc:P25697]	**-0.85**
At4g10340	Light-harvesting complex of Photosystem II 5 (LHCB5) [Source:Uniprot/SWISSPROT 3BAcc:Q9XF89]	**-0.85**
At4g28040	Nodulin MtN21-like transporter family protein/WAT1-related protein [Source:Uniprot/SWISSPROT 3BAcc:Q9SUD5]	**-0.85**
At1g01620	Aquaporin PIP1;3 [Source:Uniprot/SWISSPROT 3BAcc:Q08733]	**-0.84**
At5g57550	Probable xyloglucan endotransglucosylase/hydrolase protein 25 [Source:Uniprot/SWISSPROT 3BAcc:Q38907]	**-0.84**
At2g13360	L-Serine: glyoxylate aminotransferase (SGAT) [Source:Uniprot/SWISSPROT 3BAcc:Q56YA5]	**-0.84**
At2g46820	Curvature thylakoid 1B 2C chloroplastic (CURT1B) [Source:Uniprot/SWISSPROT 3BAcc:Q8LCA1]	**-0.84**
At5g13630	Magnesium-chelatase subunit ChlH 2C chloroplastic/ABA-binding protein (ABAR) [Source:Uniprot/SWISSPROT 3BAcc:Q9FNB0]	**-0.84**

Supplementary Table 3. The most strongly affected gene ontology (GO) terms assigned to upregulated or downregulated differentially expressed genes (adjusted *P*-value < 0.05) in Arabidopsis leaves treated with 3-methoxy-BAPA for 6 h.

GO number	Domain	Description	Total #	Affected genes (%)
Up-regulated				
GO:0009861	BP	jasmonic acid and ethylene-dependent systemic resistance	13	23.1
GO:0016653	MF	oxidoreductase activity, acting on NAD(P)H, heme protein as acceptor	9	22.2
GO:0047893	MF	flavonol 3-O-glucosyltransferase activity	11	18.2
GO:0004089	MF	carbonate dehydratase activity	14	14.3
GO:0006026	BP	aminoglycan catabolic process	24	12.5
GO:0046348	BP	amino sugar catabolic process	24	12.5
GO:1901072	BP	glucosamine-containing compound catabolic process	24	12.5
GO:0006032	BP	chitin catabolic process	24	12.5
GO:0080043	MF	quercetin 3-O-glucosyltransferase activity	24	12.5
GO:0016998	BP	cell wall macromolecule catabolic process	27	11.1
GO:0071456	BP	cellular response to hypoxia	26	7.7
GO:0071804	BP	cellular potassium ion transport	43	7.0
GO:0009817	BP	defense response to fungus, incompatible interaction	44	6.8
GO:0004364	MF	glutathione transferase activity	54	5.6
GO:0019722	BP	calcium-mediated signaling	55	5.5
Down-regulated				
GO:0030076	CC	light-harvesting complex	26	61.5
GO:0009522	CC	photosystem I	42	59.5
GO:0010196	BP	nonphotochemical quenching	7	57.1
GO:0019253	BP	reductive pentose-phosphate cycle	20	55.0
GO:0009765	BP	photosynthesis, light harvesting	39	53.9
GO:0016168	MF	chlorophyll binding	38	52.6
GO:0019685	BP	photosynthesis, dark reaction	21	52.4
GO:0015977	BP	carbon fixation	22	50.0
GO:0042549	BP	photosystem II stabilization	6	50.0
GO:0009533	CC	chloroplast stromal thylakoid	8	50.0
GO:0008187	MF	poly-pyrimidine tract binding	18	44.4
GO:0008266	MF	poly(U) RNA binding	18	44.4
GO:0009773	BP	photosynthetic electron transport in photosystem I	48	41.7
GO:0018298	BP	protein-chromophore linkage	41	41.5

GO:0009523	CC	photosystem II	68	39.7

GO, gene ontology; BP, biological process; CC, cellular component; MF, molecular function.
Detached Arabidopsis leaves were treated with 10 μM 3-methoxy-BAPA or an equivalent volume of buffer containing 0.1% DMSO (negative control) for 6 h in darkness. The most strongly affected GO terms at level 6 or higher are shown. Percentages of DEGs are calculated based on the total number of genes in *Arabidopsis thaliana* genome annotated with the same GO number (total #).

Supplementary Table 4. Effect of the treatment with 3-methoxy-BAPA (3MeOBAPA) and 3-hydroxy-BAPA (3OHBAPA) on the grain yield, number of tillers and number of spikes in winter wheat / spring barley plants. The same plants were used in field-plot experiments evaluating the level of plant infection by fungal pathogens (see Figure 7). The 5 μM solutions were applied as a foliar spray at different plant growth stages (tillering, the beginning of stem elongation, the beginning of flag leaf extending). The values in bold italics show statistically significant difference between treated and non-treated plants ($P < 0.01$). n.d., not determined.

	Compound (growth stage during application)	**Grain yield**		**Number of tillers per plant**		**Number of spikes per m²**	
		[t ha^{-1}]	[%]		[%]		[%]
wheat	non-treated	6.72	100	5.9	100	432	100
	3MeOBAPA (tillering)	6.48	96	6.3	107	470	109
barley	non-treated	8.53	100	3.8	100	698	100
	3MeOBAPA (stem elongation)	8.78	103	***4.7***	***124***	748	107
	3MeOBAPA (flag leaf extending)	8.67	102	n.d.	n.d.	721	103
	3OHBAPA (stem elongation)	8.62	101	4.2	111	688	99
	3OHBAPA (flag leaf extending)	8.86	104	n.d.	n.d.	686	98

Supplementary Table 5. Primers for expression profiling of *A. thaliana* MAPK genes.

Gene	**AGI No.**	**Primers 5′ - 3′**
Housekeeping genes (Endogenous controls)		
Act2	*At3g18780*	TGGTCGTACAACCGGTATTGTG ATCAGTAAGGTCACGTCCAGCAA
Act7	*At5g09810*	CTAGAGACAGCCAAGAGCAGTTC GTTTCATGGATTCCAGGAGCTTC
EF1α	*At5g60390*	TGAGCACGCTCTTCTTGCTTTCA GGTGGTGGCATCCATCTTGTTACA
MAPK/PTI marker genes		
MPK3	*At3g45640*	GACAGAGTTGCTTGGCACACCGA GGCTGACGTGGGAAGTTGGGA
MPK4	*At4g01370*	TGTCGGCTGGTGCAGTCGATTT TGGCACAACGCCTCATCAACTGT
MPK6	*At2g43790*	ACAGCTTCCACCTTATCCTCGCCA TGGGCCAATGCGTCTAAAACTGTG
MPK11	*At1g01560*	TTCTTAAGAAGCGACAACGCTAG ATTGACCGACATGTTTGGGAATC
FRK1	*At2g19190*	GAGACTATTTGGCAGGTAAAAGGT AGGAGGCTTACAACCATTGTG
WRKY33	*At2g38470*	GGGAAACCCAAATCCAAGA GTTTCCCTTCGTAGGTTGTGA
WRKY53	*At4g23810*	CGGAAGTCCGAGAAGTGAAG TCTGACCACTTTGGTAACATCTTT

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Supplementary material II.

Vylíčilová, H., Bryksová, M., Matušková, V., Doležal, K., Plíhalová, L., and Strnad, M. (2020). Naturally occurring and artificial N9-cytokinin conjugates: From synthesis to biological activity and back. *Biomolecules*, *10*, 1-29.

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Review

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

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Received: 7 April 2020; Accepted: 26 May 2020; Published: 29 May 2020

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].

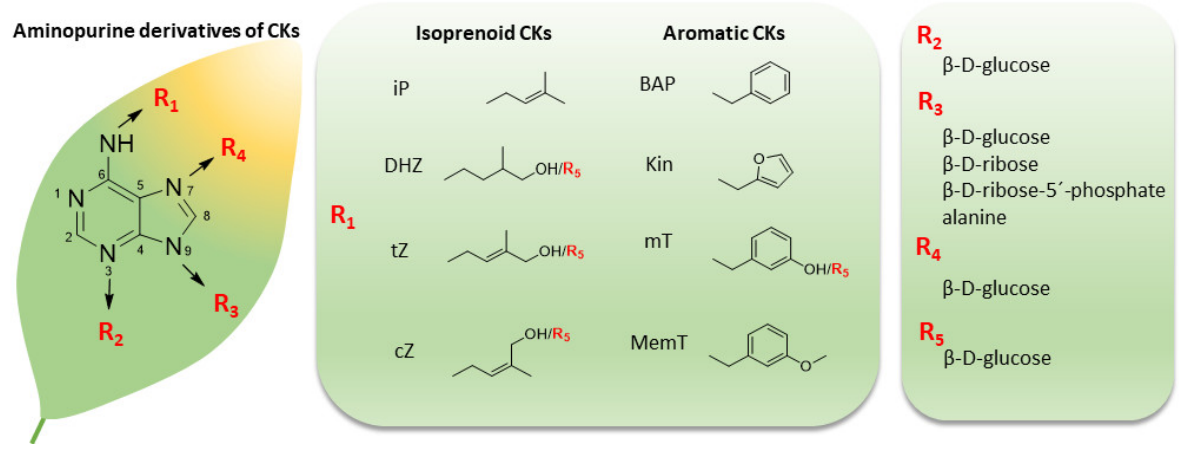

Figure 1. Structure of naturally occurring cytokinin (CK) aminopurine derivatives. The R_1 determines the type of side chain, R_2-R_5 specify the type of CK conjugate.

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].

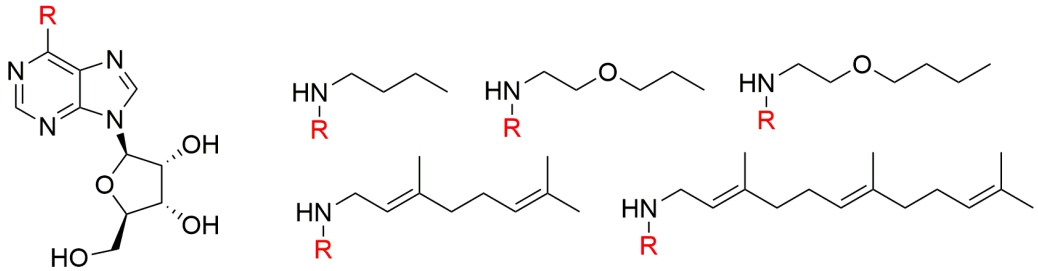

Figure 2. General structure of N6-substituted adenosines and their N6-substituents [68].

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].

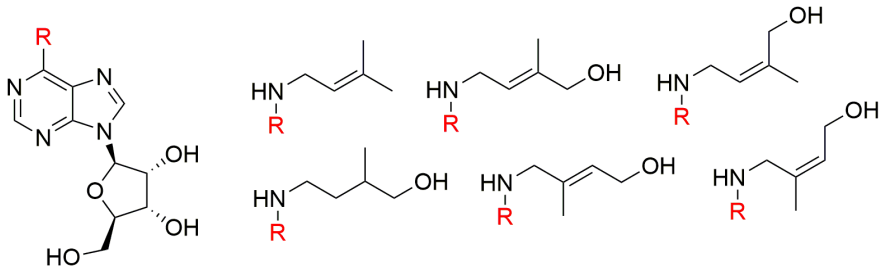

Figure 3. Compounds prepared and tested by Schmitz et al. in 1972 [69].

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, *tZ*-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].

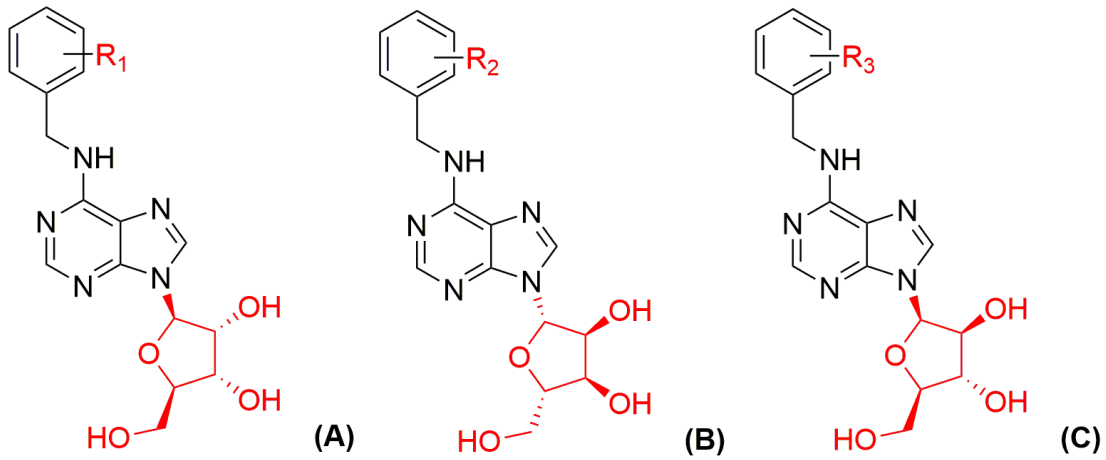

Figure 4. General structures of aromatic (**A**) 6-benzylaminopurine-9-β-D-riboside derivatives (R_1 = X, CH_3, OCH_3, OH, $OCHF_2$, OCF_3, CF_3 or a combination of these groups), (**B**) 6-benzylaminopurine-9-β-L-riboside derivatives (R_2 = H, F, Cl, OCH_3 or OH) and (**C**) 6-benzylaminopurine-9-β-D-arabinoside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

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].

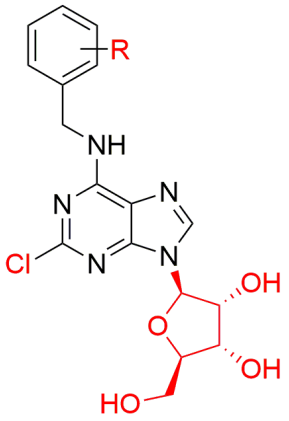

Figure 5. General structure of 2-chloro-6-disubstituted benzylaminopurine riboside derivatives, where R = halogens [46].

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].

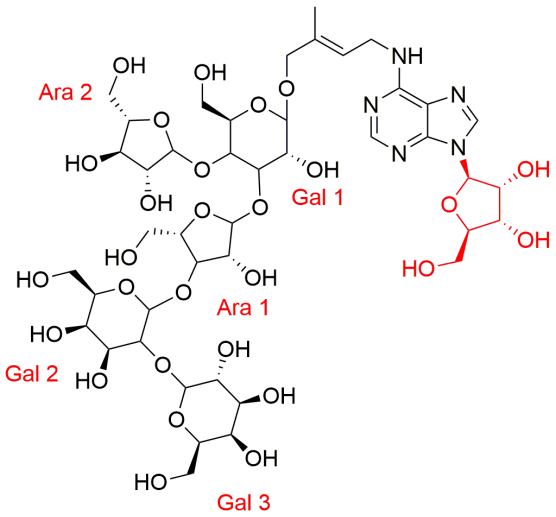

Figure 6. Structure of 14-*O*-{3-*O*-[β-D-galactopyranosyl-(1→2)-α-D-galactopyranosyl-(1→3)-α- L-arabinofuranosyl]-4-*O*-(α-L-arabinofuranosyl)-β-D-galactopyranosyl}-trans-zeatin riboside (G_3A_2-ZR) [113].

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].

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].

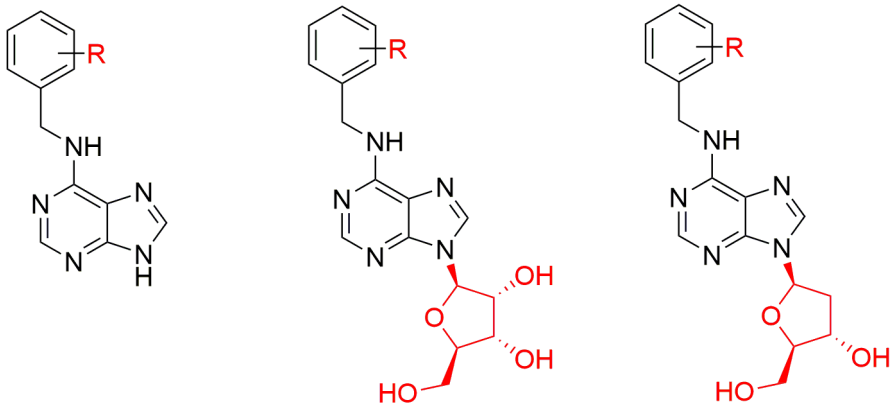

Figure 7. Comparison and general structures of free base, riboside and 2′-deoxyriboside derivatives, where R = X, OH, OCH_3, CH_3, OCF_3, CF_3 or a combination of these groups.

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].

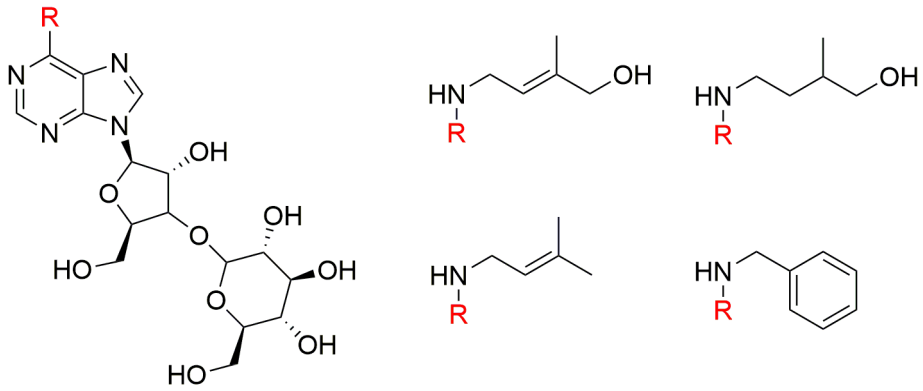

Figure 8. CK disaccharides (Z9RG, DHZ9RG, iP9RG) reported by Zhang et al. (2001) and BAP9RG reported by Auer and Cohen (1993) [138,140].

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].

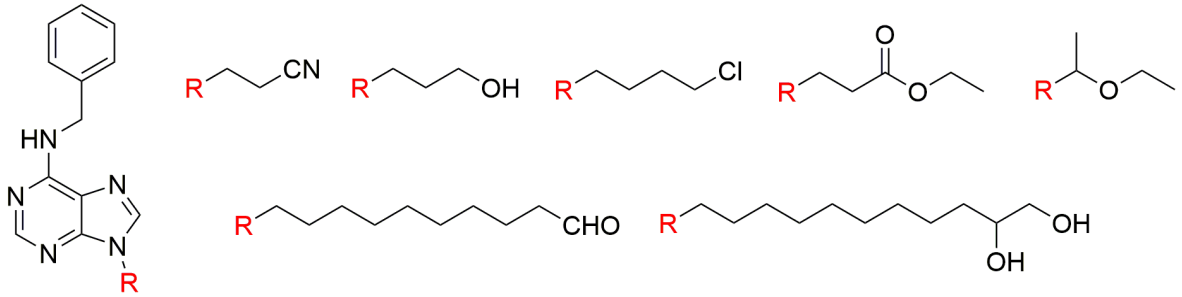

Figure 9. Structures of aliphatic chains substituted at the N9 atom of BAP [145].

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].

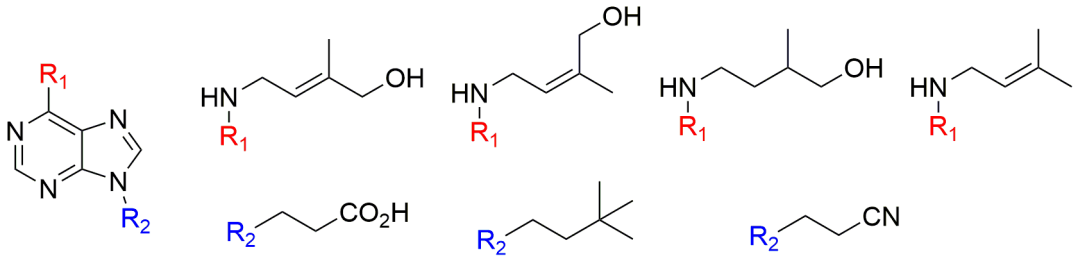

Figure 10. Structures of compounds prepared by Corse et al. (1989). On N6 (R1): tZ, cZ, DHZ, and iP side chains. On N9 (R2): 2-carboxyethyl, 2-carbo-*t*-butoxyethyl and 2-nitriloethyl) [146].

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].

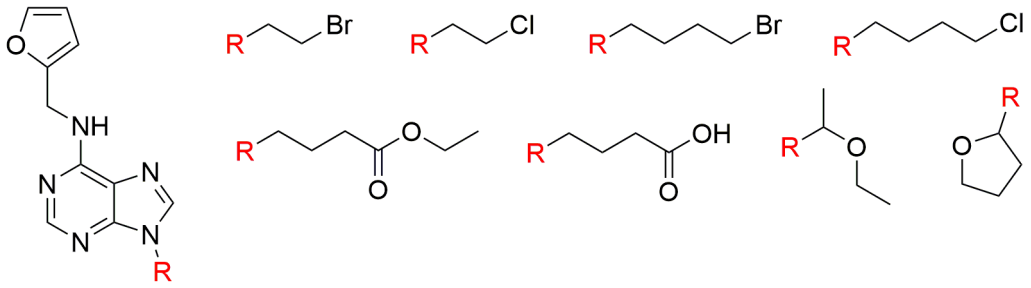

Figure 11. Structures of Kin derivatives prepared by Mik et al. (2011) [157].

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.

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].

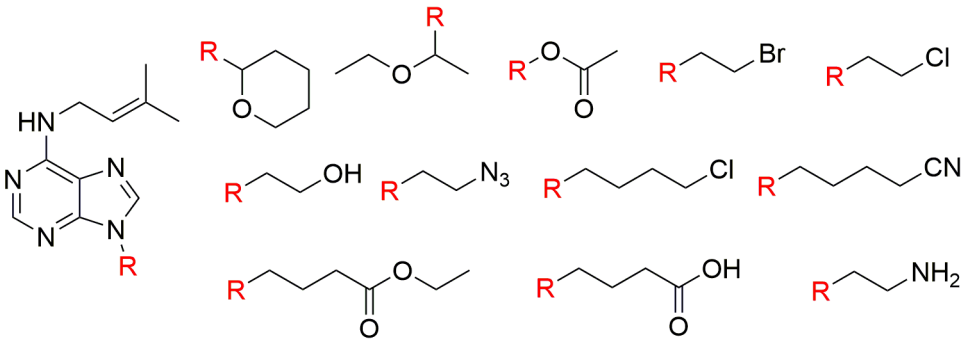

Figure 12. Structures of iP derivatives prepared by Mik et al. 2011 [158].

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].

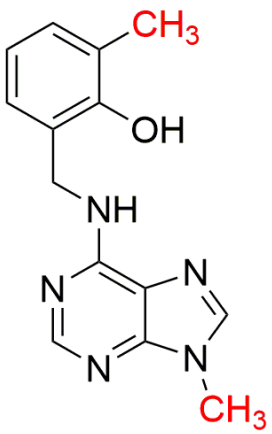

Figure 13. Structure of N9-methylated PI-55 [160].

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].

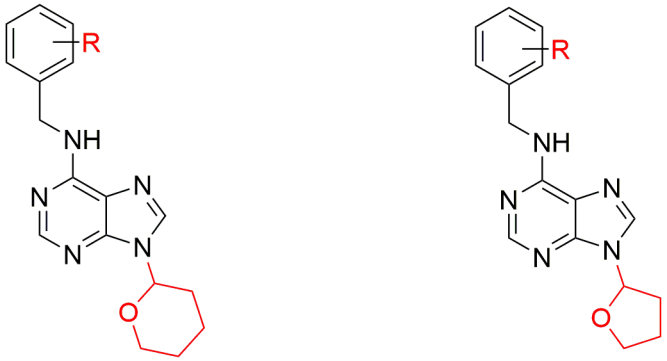

Figure 14. General structures of 9THP and 9THF ArCK derivatives (R = OH, OCH_3 or their combination) [147].

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]
Senescence (WLS)	**BAP**	β-D-ribofuranosyl-	2-fluorobenzyl-, 3-fluorobenzyl-, 4-fluorobenzyl-, 2-chlorobenzyl-, 4-chlorobenzyl-, 2-methylbenzyl-,	H	H	[45]

			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-			
			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]
Tobacco callus	**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.

Author Contributions: Conceptualization, L.P.; feedback providing and final proofreading, L.P. and K.D..; writing—original draft preparation and literature review, L.P., H.V., M.B. and V.M..; writing—review and editing L.P., M.S., K.D., supervision, L.P.; project administration, L.P. and K.D.; funding acquisition, L.P. and K.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by an ERDF project entitled ''Development of Pre-Applied Research in Nanotechnology and Biotechnology'' (No. CZ.02.1.01/0.0/0.0/17_048/0007323) and IGA_PrF_2020_010.

Acknowledgments: We would like to thank Jarmila Balonová for performing biological tests and Seed editing—for careful reading of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of manuscript or decision to publish the results. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Supplementary material III.

Bryksová, M., Hybenová, A., Esteban Hernandiz, A., Novák, O., Pěnčík, A., Spíchal, L., De Diego, N., and Doležal, K. (2020). Hormopriming for mitigating abiotic stress affects: A case study of N9-substituted cytokinin derivatives with fluorinated carbohydrate moiety. Submitted manuscript.

Hormopriming to mitigate abiotic stress effects: a case study of N9 -substituted cytokinin derivatives with a fluorinated carbohydrate moiety

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Submitted to Journal:
Frontiers in Plant Science

Specialty Section:
Plant Physiology

Article type:
Original Research Article

Manuscript ID:
599228

Received on:
26 Aug 2020

Frontiers website link:
www.frontiersin.org

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

MK and KD synthesized the compounds. AH, AEH, LS and NDD designed and performed the phenotyping experiments. AH, AP and ON carried out the metabolite quantification. AH and NDD performed the data analysis. All authors discussed the results. MK, AH, LS, KD and NDD wrote the manuscript.

Keywords

abiotic stress, Antisenescence, Arabidopsis, cytokinin analogs, hormopriming, plant biostimulant characterization index

Abstract

Word count: 339

Drought and salinity reduce seed germination, seedling emergence, and early seedling establishment, affect plant metabolism, and hence reduce crop yield. Development of technologies that can increase plant tolerance of these challenging growth conditions is a major current interest among plant scientists and breeders. Seed priming has become established as one of the practical approaches that can alleviate the negative impact of many environmental stresses and improve the germination and overall performance of crop plants. Hormopriming using different plant growth regulators has been widely demonstrated as effective, but information about using cytokinins (CKs) as priming agents is limited to only a few studies using kinetin or 6-benzylaminopurine (BAP). Moreover, the mode of action of these compounds in improving seed and plant fitness through priming has not yet been studied. For many years, BAP has been one of the CKs most commonly applied exogenously to plants to delay senescence and reduce the impact of stress. However, rapid endogenous N9-glucosylation of BAP can result in negative effects. These can be suppressed by hydroxylation of the benzyl ring or by appropriate N9 purine substitution. Replacement of the 2′ or 3′ hydroxyl groups of a nucleoside with a fluorine atom has shown promising results in drug research and biochemistry as a means of enhancing biological activity and increasing chemical or metabolic stability. Here, we show that the application of this chemical modification in four new N9-substituted CK derivatives with a fluorinated carbohydrate moiety improved the antisenescence properties of CKs. Detailed phenotypical analysis of the growth and development of Arabidopsis plants primed with the new CK analogues over a broad concentration range and under various environmental conditions revealed that they showed improved growth regulation and antistress activity. Seed priming with, for example, 6-(3-hydroxybenzylamino)-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurine promoted plant growth under control conditions and alleviated the negative effects of the salt and osmotic stress. The mode of action of this hormopriming and its effect on plant metabolism were further analyzed through quantification of the endogenous levels of phytohormones such as CKs, auxins and ABA, and the results are discussed.

Contribution to the field

In this work, we presented a complex study that covers from the synthesis to the characterization of new chemicals candidates to be used as priming agents for improving plant growth promotion and/or stress alleviation. As example, we present a case study of N9-substituted cytokinin derivatives with a fluorinated carbohydrate moiety, compounds that have shown highly promising results in pharmacology, but never tested in plants. As relevant results, we demonstrate that these compounds, synthetized using very simple chemistry, have improved antisenescence properties and are more efficiently inducing plant growth and reducing the negative effects of two different stresses (salinity and osmotic stress), compared to a classical cytokinin. Additionally, the use of a complex multi-trait high-throughput screening approach allowed us simultaneous testing of the newly prepared chemicals over broad concentration ranges and under different growth conditions to gain a complex picture for better selection and characterization of their mode of action. Overall, we show that hormopriming with cytokinins and especially with their N9-substituted derivatives with a fluorinated carbohydrate moiety is an effective biotechnological approach to improve plant growth and stress tolerance by delaying stress induced senescence.

Ethics statements

Studies involving animal subjects
Generated Statement: No animal studies are presented in this manuscript.

Studies involving human subjects
Generated Statement: No human studies are presented in this manuscript.

Inclusion of identifiable human data
Generated Statement: No potentially identifiable human images or data is presented in this study.

Data availability statement

Generated Statement: All datasets presented in this study are included in the article/ supplementary material.

Hormopriming to mitigate abiotic stress effects: a case study of N^9-substituted cytokinin derivatives with a fluorinated carbohydrate moiety

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Keywords: abiotic stress, antisenescence, Arabidopsis, cytokinin analogs, hormopriming, Plant Biostimulant Characterization index.

Abbreviations; 6-benzylaminopurine (BAP), abscisic acid (ABA), cytokinins (CKs), indole-3-acetic acid (IAA), N^6-isopentenyladenine (iP), *cis*-zeatin (*c*Z), dihydrozeatin (DHZ), *trans*-zeatin (*t*Z), high-throughput screening (HTS), Plant Biostimulant Characterization index (PBC).

ABSTRACT

Drought and salinity reduce seed germination, seedling emergence, and early seedling establishment, affect plant metabolism, and hence reduce crop yield. Development of technologies that can increase plant tolerance of these challenging growth conditions is a major current interest among plant scientists and breeders. Seed priming has become established as one of the practical approaches that can alleviate the negative impact of many environmental stresses and improve the germination and overall performance of crop plants. Hormopriming using different plant growth regulators has been widely demonstrated as effective, but information about using cytokinins (CKs) as priming agents is limited to only a few studies using kinetin or 6-benzylaminopurine (BAP). Moreover, the mode of action of these compounds in improving seed and plant fitness through priming has not yet been studied. For many years, BAP has been one of the CKs most commonly applied exogenously to plants to delay senescence and reduce the impact of stress. However, rapid endogenous N^9-glucosylation of BAP can result in negative effects. These can be suppressed by hydroxylation of the benzyl ring or by appropriate N^9 purine substitution. Replacement of the 2′ or 3′ hydroxyl groups of a nucleoside with a fluorine atom has shown promising results in drug research and biochemistry as a means of enhancing biological activity and increasing chemical or metabolic stability. Here, we show that the application of this chemical modification in four new N^9-substituted CK derivatives with a fluorinated carbohydrate moiety improved the antisenescence properties of CKs. Detailed phenotypical analysis of the growth and development of Arabidopsis plants primed with the new CK analogues over a broad concentration range and under various environmental conditions revealed that they showed improved growth regulation and antistress activity. Seed priming with, for example, 6-(3-hydroxybenzylamino)-2´-deoxy-2´-fluoro-9-(β)-D-arabinofuranosylpurine promoted plant growth under control conditions and alleviated the negative effects of the salt and osmotic stress. The mode of action of this hormopriming and its effect on plant metabolism were further analyzed through quantification of the endogenous levels of phytohormones such as CKs, auxins and ABA, and the results are discussed.

Introduction

Global climate change is increasing the severity of drought and soil salinity, with deleterious effects on already-stressed agricultural ecosystems. Moreover, predictions for the future indicate that the areas affected by these two types of stress are going to expand and as a consequence the productivity of many plant species will be reduced (Pavlů et al., 2018; Savvides et al., 2016; Uddin et al., 2016). The development of biotechnological approaches that increase plant tolerance and assure the maintenance of yield under these challenging growth conditions is therefore one of the main aims of plant scientists and breeders.

One of the technologies that attracts a high level of interest nowadays is "seed priming" (Savvides et al., 2016). Seed priming is an effective pre-sowing technology in which seeds are treated with small doses of certain agents just prior to germination. Unlike un-primed seeds, primed seeds are able to respond to very low levels of specific stimuli, which helps plants to prepare their metabolism for better defence responses to stress factors (Conrath, 2011; Paparella et al., 2015). Thus, priming can improve seed performance, ensure higher uniformity among the seeds, result in faster and better synchronized germination, and enhance plant growth (Gamir et al., 2014; Ibrahim, 2016; Lutts et al., 2016). Several methods of seed priming, including hydropriming, osmopriming, hormopriming, biopriming and chemical priming, have been developed (Jisha et al., 2013; Paparella et al., 2015). Hormopriming consists in the exogenous application of plant growth regulators or phytohormones that can stimulate seed imbibition and modify seed metabolism. The plant growth regulators most often used in this way are abscisic acid (ABA), gibberellins, cytokinins (CKs), auxins, ethylene and polyamines (reviewed by De Diego and Spíchal, 2020).

In plants, CKs are involved in many biological processes: regulating sink/source relationships (Roitsch and Ehneß, 2000; Werner et al., 2008), nutrient uptake (Criado et al., 2009; Sakakibara, 2006), leaf senescence (Jordi et al., 2000; Marchetti et al., 2018) and responses to abiotic stress (Bielach et al., 2017). Since the discovery of the first CK, kinetin, by Skoog, Miller, and associates in 1955 (Miller et al., 1955), the number of chemicals fitting the definition of CK has grown to include a large array of natural and synthetic compounds, among which are adenine and phenylurea derivatives (Mok and Mok, 2001). Depending on their chemical structure, natural CKs are adenine derivatives with an isoprenoid or aromatic N^6-side chain (Mok and Mok, 2001). CKs are present in plants in the forms of free bases, glucosides, nucleosides and nucleotides, at very low concentrations [pmol g^{-1} fresh weight (FW)] (Strnad, 1997). The precursor nucleotides, namely

N^9-riboside-5'-mono-, di- and tri- phosphates, are endogenously synthesized *de-novo* and converted to active free bases. The bases can be subsequently conjugated with glucose at positions N^3, N^7, and N^9 of the purine ring and at the hydroxyl group of the side chain, which can be also conjugated with xylose (Frébort et al., 2011). Addition of sugar moieties to the N^9 position of the purine ring can also form N^9-riboside-glucoside (George et al., 2008). While the O-glycosylated forms can be converted back into active CKs, *N*-glycosylation occurs primarily at positions N^7 or N^9 of the purine ring, and is thought to be irreversible (Brzobohatý et al., 1993), except in the case of the *t*Z forms (Hošek et al., 2020). Furthermore, it has been demonstrated that some of these conjugates may have significant CK activity, especially in the case of N^9-riboside analogues (Doležal et al., 2007).

The aromatic CK BAP is considered to be the most effective and the cheapest CK, which has led to its widespread use in biotechnology. However, many disadvantages associated with its applications have been reported (Bairu et al., 2009; Werbrouck et al., 1995). Negative effects can be caused by natural N^9-glucosylation of the applied purine based CK, leading to extensive accumulation of non-active CK glucosides (Werbrouck et al., 1995). Moreover, N^9-glucosides can activate ethylene production and the ethylene signaling pathway causing inhibition of root elongation (Podlešáková et al., 2012). One way of avoiding the negative effects of N^9-glucosylation is to suppress it by appropriate N^9 purine substitution in BAP or by hydroxylation of its benzyl ring (Plíhal et al., 2013).

Fluorination has a long tradition in nucleoside chemistry and the replacement of the 2′ or 3′ hydroxyl group of a nucleoside with a fluorine atom causes only a minor change in the overall structure, but significantly affects the stereoelectronic properties of the sugar moiety (Thibaudeau et al., 1998). It has been reported that important factors in the substitution of fluorine for hydrogen are the comparable size of the two atoms and the powerful electron withdrawing properties of fluorine relative to hydrogen, as well as the increased stability of the carbon-fluorine bond relative to the carbon-hydrogen bond. Hence, replacement of hydrogen by fluorine in a bioactive molecule is expected to cause minimal steric perturbations with respect to the molecule's mode of binding to receptors or enzymes (Pitzer, 1960). Moreover, replacement of the hydrogen by fluorine causes not only changes in biological activity, but also increases the chemical and metabolic stability of nucleosides. The conformation of the sugar moiety of these analogues is strongly affected by the

presence of the fluorine substituent and is different from that of natural deoxynucleosides (Pankiewicz et al., 1992).

Nucleosides bearing fluorine or fluorinated substituents within the carbohydrate moiety have been widely used in biochemical research and therapeutic treatment (Hagmann, 2008; Kirk, 2008; Meng and Qing, 2006). However, to date only few fluorinated CK derivatives have been prepared and their biological activity tested in plants (Clemenceau et al., 1996; Doležal et al., 2007). Only recently, several 6-benzylaminopurines substituted with *β*-D-arabinose at the N^9-position with similar structures were synthesized in our laboratory, and subsequently patented as powerful antisenescence compounds compared with BAP, after we had tested their activity in a detached wheat leaves senescence bioassay (Patent No. US 10,100,077 B2, 2018). Here, we present a new class of N^6-substituted-2´-deoxy-2´-fluoro-9-(*β*)-D-arabinofuranosylpurine derivatives which show not only high levels of antisenescence activity but also promise as seed priming agents due to their high efficiency as plant growth promotors and plant stress alleviators. Their mode of action as priming agents is also discussed.

Material and Methods

General synthesis of N^6-substituted-2´-deoxy-2´-fluoro-9-(β)-D-arabinofuranosylpurine derivatives

All the compounds presented here were prepared by a slightly modified one-step synthesis (Wan, Binnun, Wilson, & Lee, 2005) of 9-(2´-deoxy-2´-fluoro-*β*-D-arabinofuranosyl)hypoxanthine with benzylamine or isopentenylamine hydrochloride as appropriate in the presence of BOP and DIPEA in DMF (**Figure 1**). Firstly 9-(2´-deoxy-2´-fluoro-*β*-D-arabinofuranosyl)hypoxanthine (200 mg, 1 equiv.) and BOP (396 mg, 1.2 equiv) were mixed together in DMF (4 mL) and subsequently DIPEA (194 µL, 1.5 equiv) and benzylamine (1-3) or isopentenylamine hydrochloride (4) (1.2 equiv.) as the last component were added. Each reaction mixture was stirred under an argon atmosphere in an oil bath at a temperature of 55-60°C for 24 h and the effectiveness of the reaction was checked by TLC ($CHCl_3$/MeOH 4:1). The reaction mixture was evaporated using a vacuum rotary evaporator to give a specifically colored gel. The resulting residue was carefully purified by column chromatography (1 and 3) or by preparative HPLC (2 and 4) to give the desired product, which in some cases (1 and 3) could be crystallized from various solvents.

General procedures

The chromatographic purity and mass spectra of the compounds described were characterized using the HPLC-PDA-MS method. Samples (10 µL of 3 x 10^{-5} M in 1% methanol) were injected onto a reverse-phased column (Symmetry C18, 5 µm, 150 mm × 2.1 mm; Waters, Milford, MA, USA) tempered at 25°C. Solvent (A) consisted of 15 mM ammonium formate adjusted to pH 4.0 and solvent (B) consisted of methanol. The flow-rate was set to 200 µL min^{-1}. A binary gradient was used: 0 min, 10 % of B; 24 min; 90 % of B; 34 min; 90 % of B; 45 min; 10 % of B using a Waters Alliance 2695 Separations Module (Waters, Manchester, UK). Then the effluent was introduced to a Waters 2996 PDA detector (Waters, Manchester, UK) (scanning range 210–700 nm with 1.2 nm resolution) and a tandem mass analyser Q-Tof micro Mass Spectrometer (Waters, Manchester, UK) with an electrospray. The cone voltage was set to 20 V. Exact mass was determined by QTOF-MS (Synapt G2-Si, Waters, UK) operating in positive ion mode and recorded as $[M+H]^+$. Melting points were determined on a Büchi Melting Point B-540 apparatus and are uncorrected. ^{1}H NMR spectra were analyzed on a Jeol 500 SS spectrometer operating at a temperature of 300 K and a frequency of 500.13 MHz. The samples were prepared by dissolving in DMSO-d_6. Tetramethylsilane (TMS) was used as an internal standard. Thin-layer chromatography (TLC) was carried out using silica gel 60 WF_{254} plates (Merck). Purification *via* column chromatography was performed using silica gel Davisil R LC60A 40-63 micron.

HPLC-MS purification

A preparative HPLC-MS chromatography machine (Agilent 1290 Infinity II) was used coupled to a UV-VIS detector with a mass LC/MSD detector (Agilent InfinityLab) and an Agilent Prep-C18 column (5 µm, 21.2 mm x 50 mm, Waters, Milford, MA, USA) to obtain the final products. Analyzed samples were dissolved in 50% MeOH before injection. The mobile phase was methanol (A):H_2O (B) with a flow rate of 20 mL min^{-1} and linear gradients (0 min, 10% B; 0–12 min; 90% B) were used.

HRMS conditions

Samples (5 µL) were characterized using the HPLC-PDA-MS method. They were injected onto a reversed-phase column (Symmetry C18, 5 µm, 150 mm × 2.1 mm; Waters, Milford, MA, USA) incubated at 40°C. Solvent A was 15 mM ammonium formate adjusted to pH 4.0. Solvent B was

methanol. The following linear gradient was used at a flow rate of 250 µL min^{-1}: 0 min, 10% B; 0–15 min, 90% B. The effluent was introduced to a DAD detector (scanning range 210–400 nm with 1.2 nm resolution) and then to an electrospray source (source temperature 150°C, desolvation temperature 550°C, capillary voltage 1 kV, cone voltage 25 V). Nitrogen was used as the cone gas (50 L h^{-1}) and the desolvation gas (1000 L h^{-1}). Data acquisition was performed in full-scan mode (50–1000 Da) with a scan time of 0.5 s and collision energy of 4 eV; argon was used as the collision gas (optimized pressure of 5×10^{-3} mbar). Analyses were performed in positive mode (ESI^+), therefore protonated molecules $[M+H]^+$ were collected in each MS spectrum. For exact mass determination experiments, external calibration was performed using lock spray technology and a mixture of leucine/encephalin (50 pg μL^{-1}) in an acetonitrile and water (1:1) solution with 0.1% formic acid as a reference. Accurate masses were calculated and used to determine the elemental composition of the analytes with a fidelity better than 1.0 ppm.

Cytokinin bioassays

CK bioassays, including Amaranthus, tobacco callus and senescence bioassays, were carried out as previously described by Holub et al. (1998), using BAP as a positive control for all three of these classical CK bioassays. Results were recorded to define the highest activities of the four compounds prepared. All of them were dissolved in 0.5% DMSO and tested at five concentrations (from 10^{-8} to 10^{-4} M).

Plant phenotyping - rosette growth of seedlings from Arabidopsis hormoprimed seeds

The four compounds synthesized were tested as priming agents under optimal and two different stress conditions. Arabidopsis seeds (*Arabidopis thaliana* accesion Col-0) were sterilized and germinated as described by Ugena et al. (2018). During germination the compounds were added at four different concentrations (from 10^{-7} to 10^{-4}) to germination medium containing 0.5x MS (pH 5.7) supplemented with a gelling agent (0.6% Phytagel; Sigma-Aldrich, Germany). Three days after germination, seedlings of similar size were transferred under sterile conditions into 48-well plates (Jetbiofil, Guangzhou, China). One seedling was transferred to each well filled with 850 mL 1x MS medium (pH 5.7; supplemented with 0.6% Phytagel), without stress treatment (optimal conditions) or containing 100 mM NaCl (as salt stress) or 100 mM Mannitol (as osmotic stress), and the plates were sealed with perforated transparent foil allowing gas and water exchange. Hormopriming of 10^{-5} M BAP was also used as positive control for all tested growth conditions.

The 48-well plates containing the transferred Arabidopsis seedlings were placed in an OloPhen platform (http://www.plant-phenotyping.org/db_infrastructure#/tool/57), which uses the PlantScreen™ XYZ system installed in a growth chamber with a controlled environment, and cool-white LED and far-red LED lighting (Photon Systems Instruments, Brno, Czechia). The conditions were set to simulate a long day with a temperature regime of of 22°C/20°C in a 16/8 h light/dark cycle, an irradiance of 120 µmol photons of PAR m^2 s^{-1} and a relative humidity of 60%. The PlantScreen™ XYZ system consists of a robotically driven arm holding an RGB camera with customized lighting panel and growing tables. The XYZ robotic arm was automatically moved above the plates to take RGB images of single plates from the top. The imaging of each 48 well plate was performed twice per day (at 10 a.m. and 4 p.m.) for 7 days as described in Ugena et al. (2018).

Different traits were determined from the RGB images: Arabidopsis rosette growth curves [as changes in the green area (Pixels)], relative (RGR) and absolute (AGR) growth rate and final rosette size. All these traits were then used to define the mode of action of the compound under test. Using the traits, the plant biostimulant characterization (PBC) index was determined as described by Ugena et al. (2018). The PBC index was calculated as the sum of the values obtained from each phenotyping trait calculated as the differences (as the log2 of the ratio in each case) between the controls and treatment variants (compound and concentration) under the same growth conditions.

Determination of Arabidopsis rosette color indices

To estimate the greenness of the Arabidopsis seedlings, and changes in leaf color, three vegetation indices (NGRDI, VARI and GLI), which have been shown to be correlated with plant biomass, nutrient status or tolerance to abiotic stress (Gitelson et al., 2002; Hunt et al., 2013; Perry and Roberts, 2008), were used. The images captured on the seventh day of an Arabidopsis rosette growth assay subjected to HTS were segmented for the extraction of leaf rosettes using software described in our previous report (De Diego et al., 2017). The values corresponding to particular color channels (red = R, green = G, and blue = B) were then extracted for each pixel within the plant mask, and the vegetation indices were calculated as described by Ugena et al. (2018). Subsequently, indices representing particular seedlings were determined by calculating the mean

values for each plant mask. The mean and the standard error (s.e.) values for each 48-well plate were then calculated and represented in a graph.

Plant hormone quantification

Four independent biological replicates were performed and the concentration of each analyte was calculated using the standard isotope dilution method (Rittenberg and Foster, 1940). As the first step, a micro solid-phase extraction (µSPE) based on StageTip (STop And Go Extraction Tip) technology was used to purify the plant tissue samples. The µSPE protocol used in CK extraction and purification was applied as described by Svačinová et al. (2012), whereas auxins and ABA were isolated as described by Pěnčík et al. (2018). Cytokinins were determined using ultra-high performance liquid chromatography-electrospray tandem mass spectrometry (an Acquity UPLC I-Class System coupled with a Xevo TQ-S MS, Waters). Quantification of auxins and ABA was performed and the concentration of each analyte was calculated using the standard isotope dilution method **on** a 1260 Infinity II system coupled with a 6495B Triple Quadrupole LC/MS system (Agilent Technologies).

Statistical analysis

To assess differences in treatment (compound and concentration) values for each non-invasive trait extracted by means of image analysis, a non-parameric (Dunn's test after Kruskal-Wallis'test parametric) method and a parametric method (Tukey's HSD test after two-way ANOVA) were applied using the packages *multcomp, FSA* and *agricolae* in RStudio (Version 1.1.463 – © 2009-2018 RStudio, Inc.). Multivariate statistical analyses**,** including heatmap and principal component analysis**,** were also performed in RStudio using the packages *gplots*, *cluster*, *tidyverse*, *factoextra*, *heatmap.plus*, *ggpubr*, *factoextra*, *FactoMineR* and *corrplot*.

RESULTS

Synthesis of four N^9-substituted CK derivatives with a fluorinated carbohydrate moiety

In this work, a group of four N^9-substituted aromatic or isoprenoid CK derivatives each with a fluorinated carbohydrate moiety were synthesized (**Figure 1**) and their biological activity was investigated. The compounds prepared were characterized by ^{1}H NMR, elemental analysis,

melting points, TLC and ESI+ MS. The purity of the prepared derivatives was confirmed by high-performance liquid chromatography (HPLC-UV) (**Table 1**).

First, the synthesis of a 2-fluoropentose from a pentoside precursor followed by its conversion into 9-(2-deoxy-2-fluoro-*β*-D-arabinofuranosyl) adenine was performed as reported in 1969 (Wright et al., 1969). Subsequently, 3-deoxy-3-fluoro-D-glucose was synthesized and converted into 2-deoxy-2-fluoro-D-arabinose via oxidation by sodium metaperiodate as described by Reichman et al. (1975). The compound 9-(2-deoxy-2-fluoro-*β*-D-arabinofuranosyl) adenine (F-ara-A) has previously been prepared by condensation of 6-chloropurine with 2-deoxy-2-fluoro-D-arabinofuranosyl bromide followed by conversion of the purine into adenine, but the reaction produced a mixture of four isomers and only a very low yield of the desired isomer could be isolated (Marquez et al., 1990). Later, a three-step synthesis of 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine was carried out via displacement of the 2'-hydroxyl group of 03',05',N^6-tritrityladenosine and 03',05'-ditritylinosine with diethylaminosulfur trifluoride, as published by Pankiewicz et al. (1992). The importance of introducing a fluorine at the 2´(S)(ara) site of purine deoxynucleosides has been highlighted, since 2´-deoxy-2´-fluoroarabinosides have been found to be biologically active and chemically stable against hydrolysis catalyzed both chemically and by purine nucleoside phosphorylase (Chu et al., 1989).

In the present study, all the aforementioned steps were followed and finally the synthesis of new compounds was performed as previously reported by Wan et al. (2005) with some modifications. Typically, the synthesis of purine nucleosides is based on the protection of hydroxyl groups, which prolongs this method to a four-step process with low yield. This transformation usually causes cleavage of the glycosyl bond, therefore only acid-labile protecting groups must be used. In our new simple one-step unprotected synthesis, BOP was used to activate the formation of a C-N bond. Subsequently, substitution by appropriate amines led to the formation of final products, after elimination of hexamethylphosphoramide (HMPA). However, the nucleophilic substitution of unprotected purine nucleosides with amines required longer reaction times compared with their protected counterparts (Wan et al., 2005).

CK-like activity of the new N^9-substituted CK derivatives with a fluorinated carbohydrate moiety in cytokinin bioassays

To evaluate the CK activities of the newly synthesized compounds, three classical *in vitro* CK bioassays were used. Despite the fact that all four of the new compounds are derived from CKs with known high levels of activity in all three bioassays, their 2´-deoxy-2´-fluoro-9-(*β*)-D-arabinofuranosylpurine derivatives showed decreases in activity in the Amaranthus and tobacco callus bioassays (**Supplementary Table S1**). On the other hand, high antisenescence activity was recorded in the bioassay based on evaluating the effect of the compound on retention of chlorophyll in excised wheat leaves kept in the dark (**Table 2**). The greatest ability to prevent chlorophyll degradation was shown by compounds 1 and 2, which reached, respectively, 277% and 267% of the values for the positive control BAP at concentrations of 10^{-4} M, followed by compound 3 which showed 179% of the BAP activity. Compound 4 had comparable activity to BAP (**Table 2**). Overall, these results showed that substitution at the N^9 position with a fluorinated carbohydrate moiety selectively influences the CK-like activity, specifically improving the antisenescence properties of CKs modified in this way. This suggests that such CK analogues could activate plant processes related to stress responses and would therefore have antistress properties when applied to plants.

Priming with N^9-substituted CK derivatives with a fluorinated carbohydrate moiety improves the growth of Arabidopsis under both optimal and stress conditions

To corroborate the involvement of these compounds in plant stress tolerance and better define the mode of action of our four newly synthesized compounds, we tested their effects on Arabidopsis growth and development under optimal and stress conditions using a complex multi-trait high-throughput screening approach (Ugena et al., 2018). The four compounds were used as seed priming agents at four concentrations (from 10^{-7} to 10^{-4} M). Non-primed and primed seeds were germinated under optimal conditions and then the seedlings were transferred into 48 well plates with 1xMS alone, or supplemented with 100 mM NaCl or 100 mM mannitol to induce salt or osmotic stress respectively. First, we evaluated how the priming affected early seedling establishment. To do so, the rosette area of the seedlings transferred to control conditions (1x MS) at day 1 was determined. Here, we saw a clear interaction between compound and concentration affecting early seedling establishment (**Figure 3** and **Supplementary Figure S1**). The seedlings developed from seeds primed with all the compounds except compound 1 had increased rosette area. The largest rosettes were observed after priming with the highest concentrations of compound

2 and 3, or lower concentrations of compound 4. Interestingly, priming with the highest concentration (10^{-4} M) of compound 4 caused strong growth inhibition, leading to seedlings reaching only half the size of the control (MOCK) seedlings (**Figure 3** and **Supplementary Figure S1**).

The rosette areas of the seedlings were further analyzed twice a day for an additional 6 days to record a growth curve (**Supplementary Figure S2**). All four compounds improved Arabidopsis seedling growth under control and stress conditions at some of the concentrations tested and there was significant interaction between compound concentration and growth conditions according to ANOVA. On the other hand, the highest concentration of compound 4 (10^{-4} M) showed inhibitory activity under all three of the conditions tested and the rosette areas were significantly reduced to 20%, 60% and 48% of those in the non-primed control (MOCK) seedlings under, respectively, control, salt and osmotic stress (**Supplementary Figure S2**).

Other traits such as relative growth rate (RGR) and absolute growth rate (AGR) were also calculated. For better visualization, these traits together with early seedling establishment and final rosette area (at Day 8, **Supplementary Figure S3**) are presented in a parallel coordinate plot shown in Figure 3. To construct this, the differences between the controls and variants (compound and concentration) under the same growth conditions were calculated as the log2 of the ratio. The value obtained for each trait is shown in three independent parallel coordinate plots, for optimal conditions, salt stress and osmotic stress (**Figure 3**). Additionally, the priming effect of 10^{-5} BAP was evaluated as a positive control. Under optimal growth conditions, priming with the new CK analogs improved the development and growth related traits analyzed (early seedling establishment, and the slope of the RGR and AGR curve), and at the assay end-point the primed plants had larger rosettes compared to the non-primed seedlings (MOCK) or those primed with the positive control (BAP) (**Figure 3**). The best results were obtained with compound 2 at almost all its concentrations and with a 10^{-6} M concentration of compound 4.

Importantly, hormopriming improved the tolerance of the Arabidopsis seedlings to salt and mannitol induced stress by increasing the values of the slope of the curve, RGR, ARG and final rosette size compared to the negative and positive controls (**Figure 3**). In both cases, low concentrations of compound 4 (10^{-7} M for salt stress and 10^{-6} M for osmotic stress) resulted in the highest increases in the traits analyzed, whereas a concentration of 10^{-4} M inhibited plant growth

under all growth conditions (**Figure 3**, **Supplementary Figures S1 and S2**). Conversely, plants primed with compound 2 showed improvements in all traits under both control and stress conditions. All these results were then combined to calculate the plant biostimulant characterization (PBC) index, which helps to simplify and sum up the overall outcomes in order to define the mode of action of a biostimulant (Ugena et al., 2018). As listed in Table 3, all compounds worked as plant growth promotors and stress alleviators at some of the concentrations tested, all with higher efficiency than the control CK BAP. The most efficient plant growth promotor was compound 4 followed by compound 2. However, whereas compound 2 improved growth at all concentrations tested, compound 4 was highly toxic at the highest concentration (10^{-4} M), at which it showed a growth inhibitory effect (Table 3). Overall, we conclude that priming with the newly prepared N^6-substituted-2´-deoxy-2´-fluoro-9-(β)-D-arabinofuranosylpurines had positive effects on Arabidopsis growth and, importantly, improved tolerance to salt and osmotic stress, with a stronger effect in the latter case (**Figure 3; Table 3**).

Hormopriming with N^6-substituted-2´-deoxy-2´-fluoro-9-(β)-D-arabinofuranosylpurines maintains seedling greenness

To gain a further understanding of priming with compound 2 (N^6-substituted-2´-deoxy-2´-fluoro-9-(β)-D-arabinofuranosylpurines), changes in seedling color after 7 days under different growth conditions (optimal, salt or osmotic stress) were determined. The degradation of chlorophyll, manifested as a change in Arabidopsis rosette color, represents one of the most important symptoms of stress (Ugena et al., 2018). Three different indices (NGRDI, VARI and GLI) were calculated and are presented in **Figure 4**. Significant differences were observed between seedlings from non-primed and primed seeds, especially regarding NGRDI and VARI indices under all growth conditions (Figure 4). Under optimal conditions, the highest values were obtained when the compound was applied at 10^{-4} M, a concentration that also resulted in the highest PBC index (**Table 3**). However, under salt and osmotic stress, the highest NGRDI and VARI indices were observed when 10^{-5} M and 10^{-6} M were used (**Figure 4**). Taken together, these results corroborated the aforementioned antisenescence effect of this compound observed in the CK-like bioassays (**Table 2**).

Hormopriming with N^6-substituted-2´-deoxy-2´-fluoro-9-(β)-D-arabinofuranosylpurines improves Arabidopsis growth and stress tolerance by altering the hormonal profile.

To understand the molecular nature of the mode of action of priming by the N^6-substituted-2´-deoxy-2´-fluoro-9-(*β*)-D-arabinofuranosylpurines, the hormonal profile of Arabidopsis seedlings sampled at the end of the phenotyping experiment was analyzed. The endogenous levels of CKs, some auxins and ABA were quantified using LC/MS (**Supplementary Table S2 and S3**). For better visualization and interpretation, all metabolites were analyzed together using a heatmap (**Figure 5A**). The results separated the variants (treatments and growth conditions) into two clusters using the Spearman correlation as the distance method: one for the plants grown under salt stress and the control for osmotic stress, and a second cluster for the rest. Additionally, the first group was separated into two subclusters in which all plants grown under salt stress showed, in general, a reduction in content of the CK nucleotides, total auxin and ABA (**Figure 5A**). On the other hand, the variants represented in the second cluster showed increased levels of these metabolites and of some *N*- and *O*- glucosides such as DHZ7G, DHZ9G, *t*Z7G and tZ9G, and IAA conjugated with glucose (IAAGlu), but reduced content of total CKs, and bases and ribosides, especially in the case of the hormoprimed seedlings under optimal and osmotic stress conditions (**Figure 5A**). Similar results were obtained when the distance among variants was determined (**Supplementary Figure S4**), in which the hormopriming seedlings grown under optimal and osmotic stress were separated by a short distance (close to 0; similar behavior), but the distance was longer for the primed plants grown under salt stress conditions.

To extend the analysis, a principal component (PC) analysis was also performed (**Figure 5B**). The components PC1 and PC2 accounted for 60.5% of the total variance of the model. In PC1, there was clear evidence of contrasting behavior between all plants grown under optimal conditions and hormoprimed seedlings under salinity stress (blue ellipses). Thus, whereas the first group was positively correlated with the phenotyping traits and the CK nucleotides (synthesized *de novo*), which also showed a strong relationship (**Supplementary Figure S5**), the second group had a higher content of total CKs due to an increase in ribosides and *N*- glucosides (iP7G and iP9G) and *O*-Glucosides (*c*ZOG and *c*ZROG) (**Figure 5B**). Interestingly, hormopriming with the highest concentration (10^{-4} M) of compound 2 (as shown by PC2, red ellipses) induced similar contents of total auxins and the degradation form 2-oxindole-3-acetic acid (oxIAA) independent of growth conditions (**Figure 5B**), a pattern opposite to that in the MOCK variant under salt and osmotic stress. Overall, we demonstrated that in general hormopriming with the N^6-substituted-2´-deoxy-2´-fluoro-9-(*β*)-D-arabinofuranosylpurines prepared here induced changes in the hormonal content

of Arabidopsis seedlings, thus conditioning the final phenotype, with the changes depending on the concentration of the compound and on growth conditions.

DISCUSSION

In this work, a group of four N^9-substituted CK derivatives with a fluorinated carbohydrate moiety with an aromatic or isoprenoid N^6 side chain were synthesized (Wan et al., 2005) by a slightly modified one-step reaction of 9-(2´-deoxy-2´-fluoro-*β*-D-arabinofuranosyl)hypoxanthine with the appropriate amine or amine hydrochloride in the presence of BOP and DIPEA in DMF (**Figure 1** and **Table 1**). Nucleosides bearing fluorine or fluorinated substituents within the carbohydrate moiety have been used successfully in many biochemical research studies and therapeutic treatments. As an example, the ability of 9-(2-deoxy-β-D-arabinofuranosyl)adenine to completely inhibit the protozoan parasite *Trichomonas vaginalis* (Shokar et al., 2012), as well as its antibacterial (Gao et al., 2015) and antitrypanosomal (Ranjbarian et al., 2017) effect, have been reported. Significant antiviral activity was also confirmed for their dideoxy analogues. Montgomery et al. proved that 2-fluoro-9-(2,3-dideoxy-2-fluoro-*β*-D-arabinofuranosyl)adenine had anti-HIV properties (Montgomery et al., 1992), and 9-(2,3-dideoxy-2-fluoro-*β*-D-arabinofuranosyl)adenine was identified as an anti-HBV agent (Maruyama et al., 1999). However, their effects on plant species have never been investigated. Only recently have positive bioassay results for kinetin and isopentenyladenine analogs with N^9 substituted with short aliphatic chains been published (Mik et al., 2011b, 2011a). Similar to these results, our newly synthesized compounds (especially compound 2) also showed high levels of antisenescence activity. Moreover, the low activity of the N^6-substituted-2´-deoxy-2´-fluoro-9-(*β*)-D-arabinofuranosylpurine derivatives in the callus bioassay confirms that fluorination of the sugar moiety prevents its hydrolysis to free bases and makes these compounds metabolically stable. Due to the strong effect of the CK analogues with a fluorinated carbohydrate moiety on the retention of chlorophyll in excised wheat leaves in the dark, we hypothesized the that the compounds would have antistress properties and analyzed their mode of action and their potential use as priming agents.

For many years, it has been shown that seed priming with certain hormones or other compounds improves seed germination and fitness in many plants (Hussain et al., 2016; Van Hulten et al., 2006). Seed priming improves seed stress tolerance through “priming memory“, which is

established during priming and can be recruited later when seeds are exposed to stresses during germination (Chen and Arora, 2013). The beneficial effect of seed priming with CKs has been previously described under a range of growth conditions for many plant species, such as spring wheat (*Triticum aestivum* L.) (Iqbal et al., 2006; Iqbal and Ashraf, 2005) or basil (*Ocimum basilicum* L) (Bagheri et al., 2014). Despite this, there is not always a clear positive effect of priming, and it may also have a negative effect (Miyoshi and Sato, 1997; Sneideris et al., 2015; Willams et al., 2016), depending on the type of compound, the concentration used for priming, or the plant species and cultivars tested (reviewed by De Diego and Spíchal, 2020). In this work, hormopriming with the new N^9-substituted CK derivatives improved early seed establishment and plant growth in *Arabidopsis thaliana* under optimal and stress growth conditions (**Figure 3**), mainly by maintaining plant greenness (less chlorophyll degradation) and better nutrient status as defined by higher color indices (**Figure 4**). However, this response was concentration dependent. The best-performing compound was 6-(3-hydroxybenzylamino)-2´-deoxy-2´-fluoro-9-(*β*)-D-arabinofuranosylpurine, which was a good growth promotor under optimal growth conditions and a stress alleviator under both salt and osmotic stress at almost all concentrations tested, according to the PBC index (**Table 3**). As an exception, compound 4 at 10^{-4} M showed a strong growth inhibitory (toxic) effect. However, lower concentrations (10^{-7} or 10^{-6} M) improved plant growth under different growth conditions (**Table 3**). This underlines the importance of testing chemicals over broad concentration ranges and under different growth conditions. This is possible through initial high-throughput approaches using model plants such as *Arabidopsis*, followed by studies in the targeted species and specific growth conditions (Rouphael et al., 2018).

To understand better how these new compounds modify plant metabolism when they are used as priming agents, the endogenous levels of some plant hormones (CK, auxins and ABA) were quantified. It was clear that hormopriming with compound 2 disrupted the plants' hormonal homeostasis (**Figure 5B**). However, the changes varied depending on the conditions under which the plants were grown. Thus, different behaviors were observed between hormoprimed seedlings under optimal and osmotic stress conditions, and those under salt stress (Supplementary Figure S4). For example, under optimal conditions, primed Arabidopsis seedlings accumulated higher levels of ribotides (precursors), which were positively correlated with phenotypic traits such as AGR, RGR, slope of the growing curve and final rosette area (**Supplementary Figure S5A**). However, primed plants grown under salt stress conditions elevated their total CK content by

increasing the amounts of conjugated forms including ribosides (iPR. DHZR and *c*ZR), O-glucosides (*c*ZOG and *c*ZROG) and N-glucosides (iP7G and iP9G). It has been reported that riboside accumulation under stress conditions can be a defense mechanism, helping plants to deal with stress (De Diego et al., 2015; Man et al., 2011; Veerasamy et al., 2007). This may be because they play a crucial role in CK-mediated leaf longevity, and hence senescence, through phosphorylation of the CK response regulator ARR2 (reviewed by Hönig et al., 2018). For several years the *c*Z-type CKs and the base *c*Z were considered to be low-activity forms. However, in recent years, it has been proved that *c*Z-type CKs play important roles during plant development and in environmental interactions (Lacuesta et al., 2018; Schäfer et al., 2015). Thus, in primed Arabidopsis plants high levels of accumulation of *c*ZOG and *c*ZROG could be a strategy for maintaining plant growth under salt stress conditions. In support of this, it has been reported that the content of *c*Z-type CKs changes rapidly during maize seedling growth, and that *c*Z catabolism and glycosylation by *c*Z O-glucosyl transferases work synergistically to fine-tune *c*Z levels during plant development (Zalabák et al., 2014). Finally, in these primed plants there was also considerable accumulation of iP7G and iP9G. These two iP derivates are the terminal products of iP metabolism (Hošek et al., 2020). The iP metabolites including iP-N9G are the least active CKs, which seem not to be hydrolyzed and simply accumulate in the tissue (if not degraded by CKX) with no physiological effects (Hoyerová and Hošek, 2020). Overall, it is clear that priming with CK analogs modifies CK metabolism, but these changes are dependent on plant growth conditions. The results also pointed to the iP-type and *c*Z-type CKs as the main metabolites regulating the alleviation of salt stress in primed Arabidopsis seedlings.

Regarding auxins, levels of oxIAA mainly increased when compound 2 was applied at a high concentration (10^{-4} M) (Figure 5 and Supplementary Table S3). In recent years it has been proved that oxidizing IAA into oxIAA is of major physiological significance in the regulation of plant growth and development (Stepanova and Alonso, 2016). However, changes in other auxin-related metabolites did not show any correlation with the phenotypical changes in plants primed with compound 2.

In summary, in this case study we showed that hormopriming with N^9-substituted CK derivatives with a fluorinated carbohydrate moiety seems to be a promising biotechnological approach for improving early seedling establishment and plant growth under both control and stress conditions. This is due to changes in plant hormone metabolism (especially of CKs and

auxins) that differ according to growth conditions. Moreover, we believe that we have shown here that a complex approach is needed for selection of suitable compounds, by employing strategies allowing simultaneous testing of a broad range of concentrations and different growth conditions to define the conditions in which they are most efficient as priming agents.

Author contributions

MK and KD synthesized the compounds. AH, AEH, LS and NDD designed and performed the phenotyping experiments. AH, AP and ON carried out the metabolite quantification. AH and NDD performed the data analysis. All authors discussed the results. MK, AH, LS, KD and NDD wrote the manuscript.

Acknowledgments

This work was funded by the project "Plants as a tool for sustainable global development" (registration number: CZ.02.1.01/0.0/0.0/16_019 /0000827) within the program Research, Development and Education (OP RDE) and the Internal Grant Agency of Palacký University (IGA_PrF_ 2019_018).

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Table 1| Physico-chemical properties of prepared compounds: HRMS mass analysis and mass of positively charged molecular ions analyzes by HPLC-MS.

Compound	HRMS				ES-MS
	Exact mass	Theoretical monoisotopic mass	Difference (ppm)	Elementary analysis $[M+H^+]$	$[M+H^+]$
1	359.1393	359.1394	-0.23	$C_{17}H_{19}FN_5O_3$	360
2	375.1341	375.1343	-0.48	$C_{17}H_{19}FN_5O_4$	376
3	389.1502	389.1499	0.74	$C_{18}H_{21}FN_5O_4$	390
4	337.1552	337.155	0.4	$C_{15}H_{21}FN_5O_3$	338

Table 2| Relative CK activities of prepared compounds in the senescence bioassay. The optimal concentration for compounds **1-3** was compared with the activity of benzylaminopurine (BAP), where 100% means 10^{-4} M BAP. The optimal concentration for compound **4** was compared with the activity of isopentenyladenine (iP), where 100% means 10^{-4} M iP.

Compounds	Senescence bioassay	
	Optimal concentration (M)	Relative activity (%)
1	10^{-4}	277(±9)
2	10^{-4}	267(±17)
3	10^{-4}	179(±3)
4	10^{-4}	95(±6)

Table 3| Plant Biostimulant Characterization (PBC) index calculated by summing the relative changes (log2) obtained for the parallel coordinate plot (**Figure 4**) for each synthetized compound (four different N^9-substituted CK derivatives with a fluorinated carbohydrate moiety) at four concentration (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M) and growth condition; optimal, salt stress (100 mM NaCl), or osmotic stress (100 mM Mannitol).

	OPTIMAL GROWTH CONDITION				**100 mM NaCl**				**100 mM Mannitol**			
	Concentration (M)				**Concentration (M)**				**Concentration (M)**			
	10e-7	**10e-6**	**10e-5**	**10e-4**	**10e-7**	**10e-6**	**10e-5**	**10e-4**	**10e-7**	**10e-6**	**10e-5**	**10e-4**
Comp_1	2.00	1.73	1.95	1.11	0.53	-0.40	-0.16	0.52	1.16	0.77	0.86	0.06
Comp_2	2.73	1.73	2.64	2.90	1.04	1.31	1.13	0.15	1.43	1.40	0.62	1.22
Comp_3	1.40	2.14	2.29	2.54	0.12	0.62	-0.25	1.35	1.09	0.98	1.33	1.19
Comp_ 4	0.74	3.22	0.75	-5.77	1.74	0.26	0.58	-2.22	0.97	2.07	0.26	-4.04
BAP			0.41				0.10				-0.37	
Comp_1	**Strong growth promotor**				Weak stress alleviator				Medium stress alleviator			
Comp_2	**Strong growth promotor**				**Strong stress alleviator**				**Strong stress alleviator**			
Comp_3	**Strong growth promotor**				Medium growth promotor				**Strong stress alleviator**			
Comp_4	Growth promotor and inhibitor				Stress alleviator and inhibitor				Stress alleviator and inhibitor			
BAP	Weak growth promotor				Weak stress alleviator				Stressor			

Figure 1| Scheme for the preparation of substituted 6-benzylamino-2´-deoxy-2-´fluoro-9-(*β*)-D-arabinofuranosylpurine derivatives by a method using Castro´s reagent and Hunig base.

Figure 2| Structures of the newly synthesized 6-benzylamino-2´-deoxy-2-´fluoro-9-(*β*)-D-arabinofuranosylpurine derivatives.

Figure 3| Parallel coordinate plot of the traits (germination, seedling establishment, plant growth capacity, and leaf color index) obtained from multi-trait high-throughput screening of Arabidopsis seedlings non-primed (MOCK) or primed with four different N^9-substituted CK derivatives with a fluorinated carbohydrate moiety at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) and grown under optimal (upper panel), or salt (100 mM NaCl, middle panel) or osmotic (100 mM mannitol, bottom panel) stress conditions.

Figure 4| Color indices [NGRDI (A), VARI (B) or GLI (C)] used as greenness parameters for Arabidopsis seedlings from non-primed (MOCK) seeds or seeds primed with compound 2 at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) grown under optimal (upper panel), or salt (100 mM NaCl, middle panel) or osmotic (100 mM mannitol, bottom panel) stress conditions for 7 days. Different letters mean significant differences among treatments (priming effect) for each growth conditions according to Dunn's test after Kruskal-Wallis' test.

Figure 5| Heatmap (A) representing the changes in CKs, auxins and ABA for Arabidopsis seedlings from non-primed (MOCK) seeds or seeds primed with compound 2 at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) grown under optimal (black line), or salt (100 mM NaCl, red line) or osmotic (100 mM mannitol, blue line) stress conditions for 7 days. Principal component analysis (Dimension, Dim) (B) of the same Arabidopsis seedlings.

Figure 1.TIF

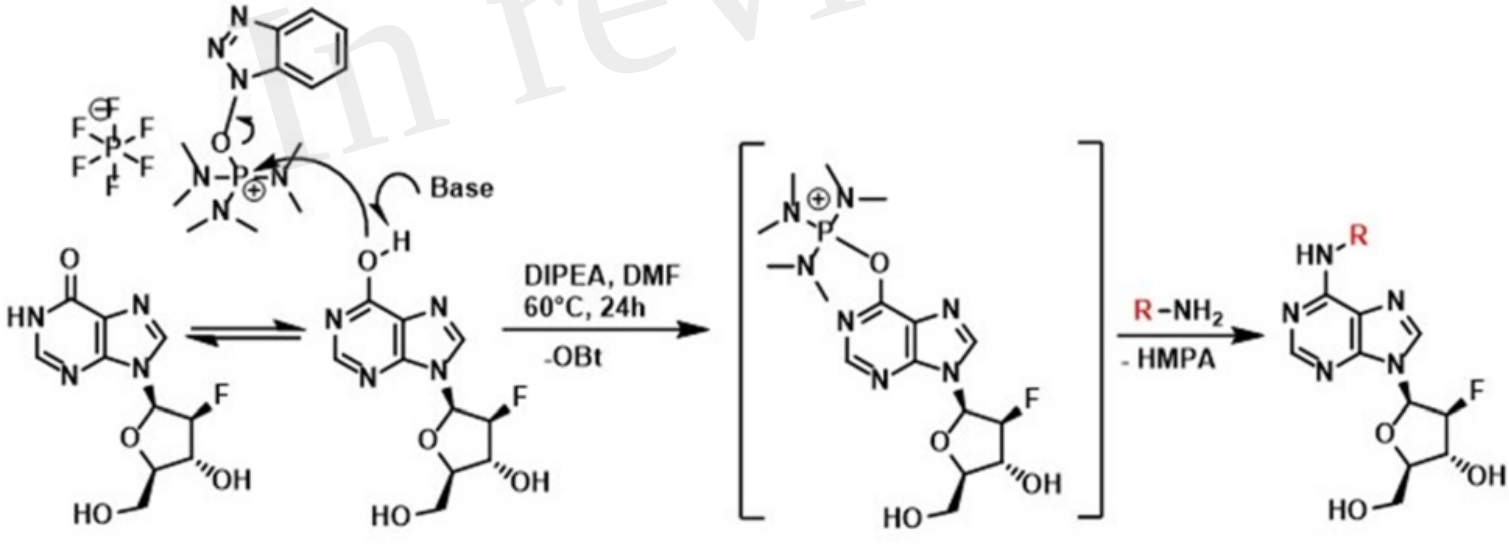
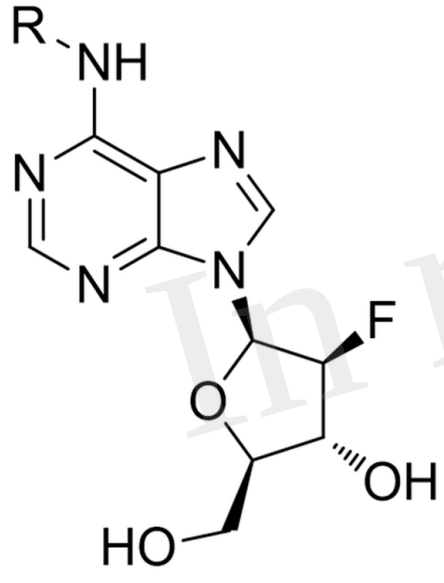


Figure 2.TIF



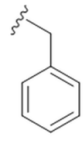
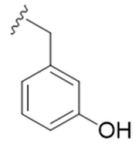
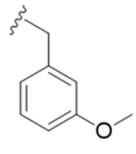
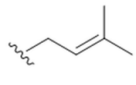
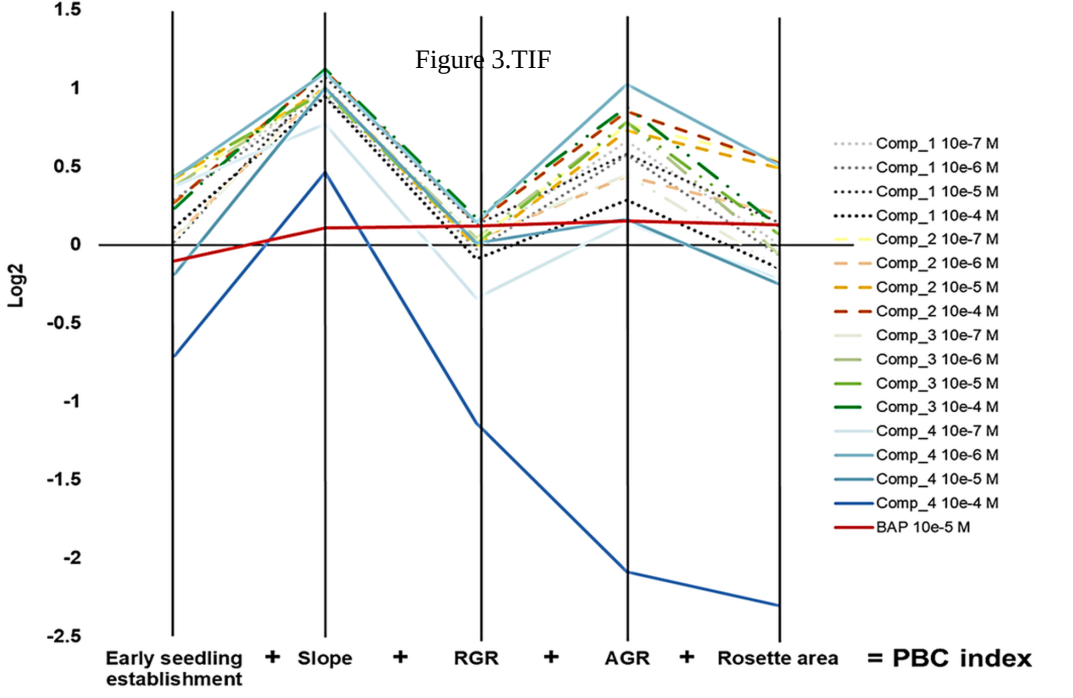
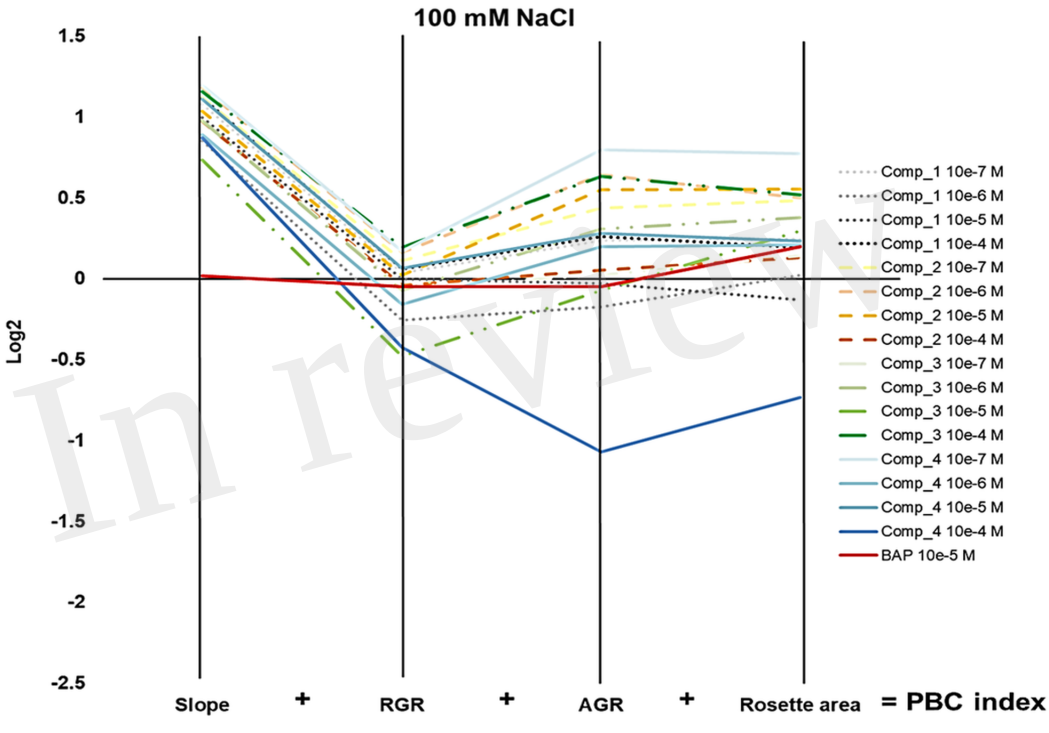
Compound	N^6-substituent
1	
2	
3	
4	

Figure 3.TIF





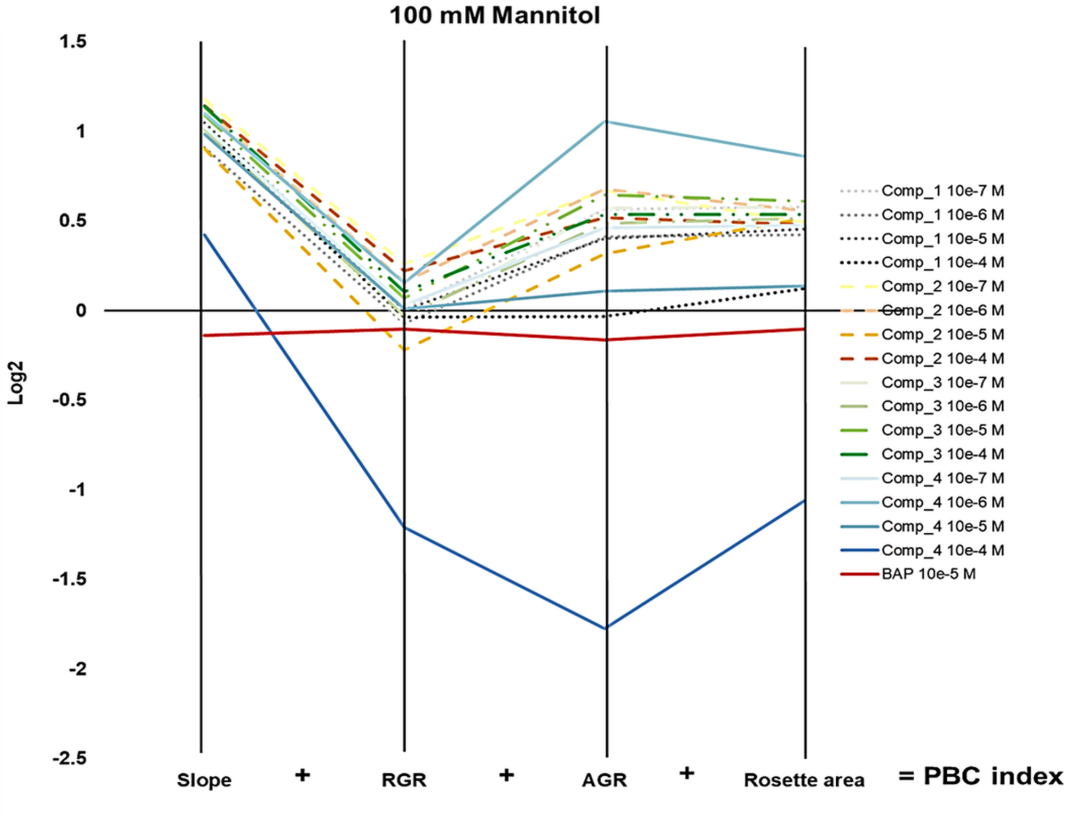


Figure 4.TIF

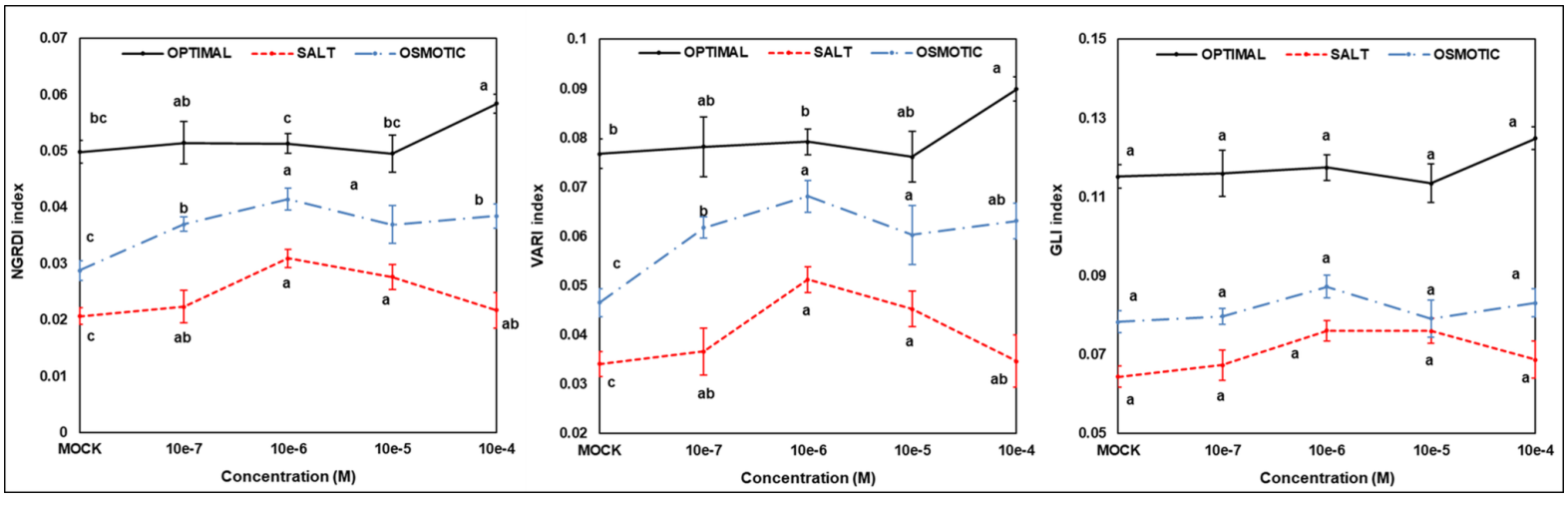
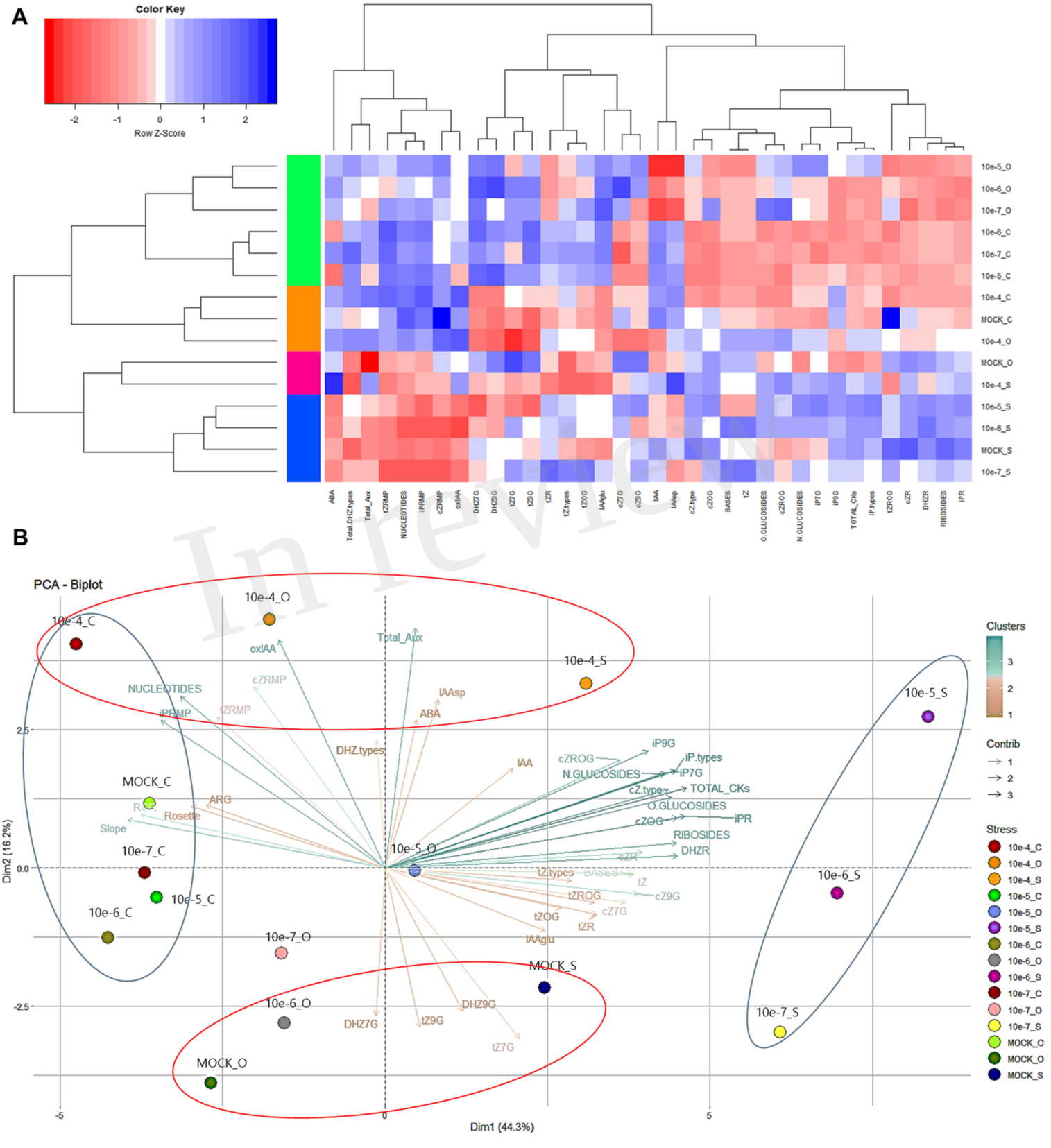


Figure 5.TIF



Supplementary material IV. (book chapter)

Doležal, K., and Bryksová, M. Topolin metabolism and its implications for *in vitro* plant micropropagation. In *Meta-topolin: A plant growth regulator in vitro*. Book in preparation.

Chapter title: Topolin metabolism and its implications for *in vitro* plant micropropagation

Karel Doležal*, Magdalena Bryksová

Abstract

Topolins are a relatively recent discovery, following search for viable alternatives to 6-benzylaminopurine (BAP) which while effective and affordable has important disadvantages for certain crops. This chapter reviews some biochemical and technical aspects of topolin metabolism in relation to *in vitro* plant micropropagation.

Keywords: *meta*-topolin, topolin, cytokinin, metabolism, plant tissue culture

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6-Benzylaminopurine (BAP, also known as N^6-benzyladenine: BA) was long the most widely used cytokinin in micropropagation systems, due to its efficacy and affordability (Holub et al. 1998). However, its adverse affects on the growth, rooting and acclimatization of some recalcitrant species and induction of other physiological disorders (Aremu et al. 2012) prompted the search for and subsequent discovery of viable alternatives: the choice of CK remains critical to the success or failure of any newly developed micropropagation protocol (Werbrouck 2010, Aremu et al., 2012, Valero-Aracama et al., 2010). The increasing importance of hydroxylated derivatives commonly referred to as topolins (Strnad et al., 1997) in micropropagation has been critically reviewed more recently (Aremu et al., 2012, Aremu et al., 2017).

Following description of the basic aspects of cytokinin activity of topolin ribosides (Kaminek et al. 1987) and unambiguous identification of 6-(3-hydroxybenzylamino)purine (meta-topolin, mT) and its derivatives as naturally occurring cytokinins *in planta* (Strnad et al., 1996, Strnad et al., 1997, Strnad 1997), pioneering work was published on the metabolism and *in vitro* effects of *m*T in micropropagated *Spathiphyllum floribundum* (Werbrouck et al. 1996). mT was compared to BAP, 6-benzylamino-9-(2-tetrahydropyranyl)purine (BAP9THP) and 6-benzylamino-9-riboside (BAPR) using an HPLC separation system, coupled to a tandem

quadrupole mass spectrometer (MS/MS) equipped with an electrospray interface (ESI). *In vitro*, BAP as well as its 9-substituted derivatives BAP9THP and BAPR, were mainly converted into the stable and inactive metabolite, 6-benzylamino-9- β-D-glucopyranosylpurine (BAP9G) (Fig. 1), located mostly in the basal part of the micropropagated plant. In contrast, 6-(3-O-β-D-glucopyranosyl)benzylaminopurine-9-riboside (*m*TROG) (Fig. 1) was identified as the main metabolite of *m*T. This new cytokinin-O-glucoside, which was present in all plant parts, was formed much faster than BAP9G during acclimatization process. The effect of BAP and *m*T on *in vitro* shoot and root production *and ex vitro* rooting was then compared (Werbrouck et al. 1996). Only *m*T combined sufficient shoot production with acceptable *in vitro* root formation. The plants developed on medium with 10 µM or more mT, rooted better also during acclimatization in comparison with those grown on medium with comparable BA concentrations.

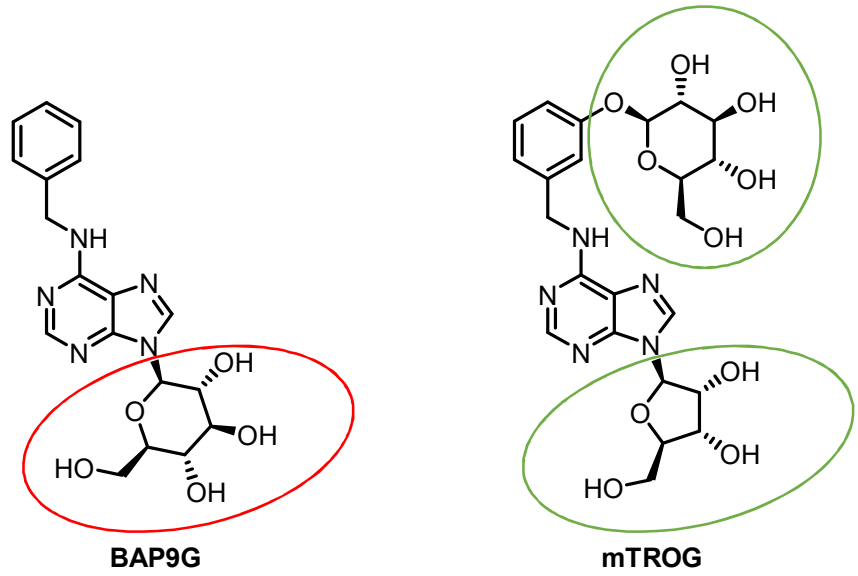

Fig.1: Chemical structure of 6-benzylaminopurine-9-β-D-glucopyranoside (BAP9G) and 6-(3-hydroxybenzylamino)purine-9-β-D-ribofuranoside-O-glucopyranoside (*m*TROG).

Very similar results were later obtained by Bairu et al. (2009) in micropropagated *Harpagophytum procumbens* tissues, where changes in endogenous CK profiles and the physiological implications of this in relation to shoot-tip necrosis (STN) and CK treatments were also studied. Generally, necrotic shoots contained more total CKs compared to normal shoots and cytokinin accumulation was higher in the basal section. More importantly, further analysis of structural and functional CK forms revealed the inability of BAP to form O-glucosides as well as excessive accumulation of 9-glucosides (irreversible deactivation product) in necrotic and basal callus like tissues of BAP-treated shoots (Bairu et al 2009). The addition of IAA enhanced the formation of 9-glucosides in BAP-treated cultures. The symptom of STN could therefore be attributed to conversion of active cytokinin to other forms such as

9-glucoside (Bairu et al 2009). On the other hand, the presence of a hydroxyl group in their molecule gives topolins a structural advantage over BAP. This was reflected in the presence of a generous amount of O-glucosides in topolin-treated samples and hence little or no CK shortage (Bairu et al 2009). Moreover, the level of irreversible inactivation (9-glucoside formation) of *m*T was found to be even lower when exogenously applied in the form of 9-riboside, compared with free base application.

As the most efficient plant growth regulator (PGR), 10 µM 6-(3-hydroxybenzylamino)purine-9-β-D-ribofuranoside (*m*TR, fig. 2) treatment also produced the highest number of shoots (approximately five shoots per explant) during clonal propagation of *Lachenalia montana*, a species endemic to southern Africa, and extensively traded as ornamental plants in the international floriculture industry (Aremu et al., 2017b). Based on the concentrations of endogenous CKs subsequently determined, 10 µM *m*TR regenerants also had the highest CK levels which were mainly of the aromatic-type (98%). In terms of the functional role of the CKs, O-glucosides were again the dominant CK metabolites in the regenerants of the 10 µM *m*TR treatment. On the other hand, the insufficient rooting, predominantly in regenerants of the BA treatments, was closely related to the high accumulation of N9-glucosides compared to regenerants from other treatments. These findings provided further evidence of the interrelationship between exogenous topolin application, positive phenotypic responses as well as endogenous CK levels in the *in vitro* regenerants (Aremu et al., 2017b).

Based on these studies, a series of attempts was made to prepare other topolin derivatives substituted at the 9-position using various protective groups to improve the specific biological functions of the cytokinins already routinely used in the plant micropropagation industry. For example, inspired by discovery of the protective role of ribose in the 9-position (Bairu et al 2009), Szučová et al. (2009) prepared several substituted 6-benzylamino-9-tetrahydropyran-2-ylpurine (THPP) and 9-tetrahydrofuran-2-ylpurine (THFP) derivatives, with hydroxy and methoxy functional groups at various positions on the benzyl ring. The new compoudss were synthesised by condensation of 6-chloropurine with 3,4-dihydro-2H-pyran or 2,3-dihydrofuran and then by the reaction of these intermediates with the corresponding benzylamines (in *n*-propanol or *n*-butanol, in the presence of trimethylamine). The identity and purity prepared compounds was confirmed by CHN analysis, TLC, HPLC, melting point determinations, CI+ MS and 1H NMR spectroscopy. The cytokinin activity of the prepared derivatives was determined in three classical cytokinin bioassays (tobacco callus, wheat leaf senescence and Amaranthus bioassay).

In another study (Podlešáková et al. 2012), in contrast to canonical cytokinins, the 9-tetrahydropyranyl derivative of *m*T (Fig. 2) and its methoxy-counterpart showed negative effects on root development at only three orders of magnitude higher concentration. The methoxy-derivative also demonstrated a positive effect on lateral root branching and leaf emergence in nanomolar concentration range in comparison with untreated plants. Tetrahydropyranyl substitution at the N9-position of cytokinin purine ring was also found to

significantly enhanced acropetal transport of a given cytokinin. Together with the methoxy-substitution, inhibition of the formation of non-active cytokinin glucosides in roots, allows gradual release of the active base, and has a significant effect on the distribution and amount of endogenous isoprenoid cytokinins in different plant parts (Podlešáková et al. 2012). These results provided a basis for anticipating that the use of novel aromatic cytokinin derivatives could improve the expected hormonal effects in plant propagation methodology in the future.

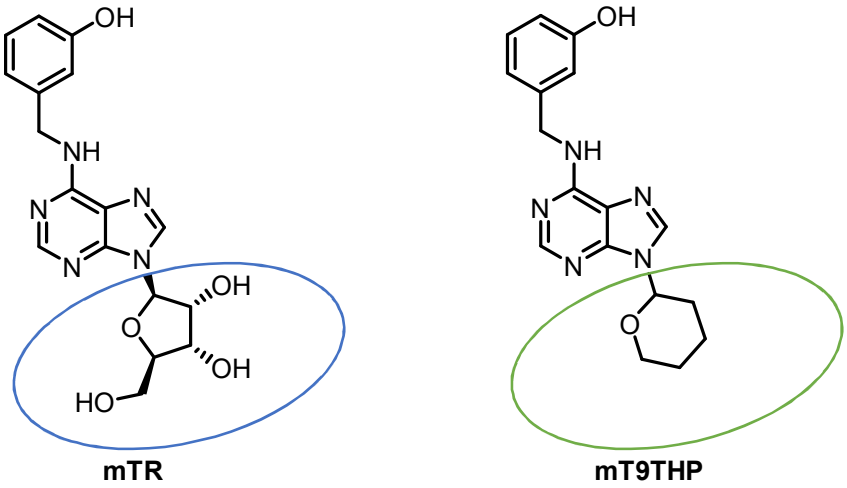

Fig.2: Chemical structure of 6-(3-hydroxybenzylamino)purine-9-β-D-ribofuranoside (*m*TR) and 6-(3-hydroxybenzylamino)purine-9-(tetrahydropyran-2-yl) (*m*T9THP)

This led to 9-THP topolin derivative (Fig. 2) being successfully used in various micropropagation systems, for example, two widely used medicinal plants, *Aloe arborescens* and *Harpagophytum procumbens* (Amoo et al., 2014). In terms of *A. arborescens* shoot multiplication, *m*TTHP and *m*T (at equimolar level) showed similar effects and both were comparably better than the control and 6-benzylaminopurine riboside (BAPR). In *H. procumbens*, *m*T treated cultures were the most responsive to treatment at 2.5 μM compared to the control. At 5.0 μM concentration, *m*T9THP and *meta*-topolin riboside (*m*TR) demonstrated a similar activity on shoot proliferation. Particularly at low concentrations, *m*T9THP had a better rooting stimulatory activity than the other CKs in both plant species. It is conceivable that *m*T9THP is another viable alternative topolin with the added advantage of inducing rooting at low concentrations (Amoo et al., 2014). Later (Amoo et al., 2015), this compound was also successfully used to improve micropropagated *Merwilla plumbea* shoot production without rooting inhibition as well as its positive carry-over effect on *ex vitro* growth. Unlike *m*TTHP treatments, an increase in concentration of *m*TR or TDZ, other tested cytokinins, beyond 0.5 μM resulted in a significant decrease in the concentrations of all the photosynthetic pigments quantified. Even after 6 months of *ex vitro* growth, regenerated

plants of the 0.5 μM *m*TTHP treatment had the significantly higher total leaf area, total leaf fresh weight and bulb size compared to all *m*TR and TDZ-treated plants (Amoo et al., 2015).

In a similar study on *Merwilla plumbea* (Lindl.) Speta, a popular and highly sought after South African medicinal plant with diverse therapeutic uses (Aremu et al. 2014), the effect of another *meta*-topolin derivative, 6-(3-methoxybenzylamino)-9-tetrahydropyran-2-ylpurine (Me*m*TTHP), was evaluated on the growth and level of endogenous CKs during micropropagation and acclimatization stages. A total of 37 (22 isoprenoid and 15 aromatic) CKs were determined in both *in vitro* and *ex vitro* acclimatized plants. Based on their metabolic function, these were seperated into five different groups, including free bases, ribosides, ribotides, O- and 9-glucosides. In addition to enhancing our understanding of the hormone physiology in *M. plumbea*, the current findings were discussed in line with the effect of exogenously applied CK on the observed differences in growth before and after the important stage of acclimatization. The observed dynamics in endogenous CK can provide an avenue to optimize the *in vitro* growth and development of investigated species.

Another successful attempt to enhance the anti-senescence properties of topolins was described by Doležal et al. (2017), by preparing their 9-β-D-arabinofuranosyl or β-D-2´-deoxyribofuranosyl derivatives (Fig. 3) via a one-step reaction. The starting material, optically pure unprotected 9-(2´-deoxy-(β)-D-ribofuranosyl)hypoxanthine (2´-deoxy-9-(β)-D-inosine) or 9-(β-D-arabinofuranosyl)hypoxantine and BOP [(benzotriazol-1yloxy)tris(dimethylamino) phosphonium hexafluorophosphate] were dissolved in dry DMF (3 mL) under nitrogen or argon atmosphere at 55-60 °C and DIPEA was added, followed by appropriate substituted benzylamine (1.2 eq.) as the last component. The resulting white solid was isolated by filtration and re-crystallized from EtOH. The synthesized compounds were characterized by CHN and melting point analysis, analytical thin layer chromatography, high performance liquid chromatography, ES^+ MS spectrometry, and ^1H NMR. The positive effect of selected derivatives on shoot proliferation in *Harpagophytum procumbens* and *Amelanchier alnifolia*, as well as control of shoot-tip necrosis in *in vitro* cultures of the medicinal plant *Gymnosporia buxfolia* was then demonstrated (Doležal et al., 2017).

Modulating the CK status with inhibitors of CK perception and/or degradation may affect the general physiology of the plant (Dwivedi et al., 2010, Motte et al., 2013, Zatloukal et al., 2008). For this reason, regulation of phytohormone metabolism may be another potential way to improve plant growth and development during micropropagation.

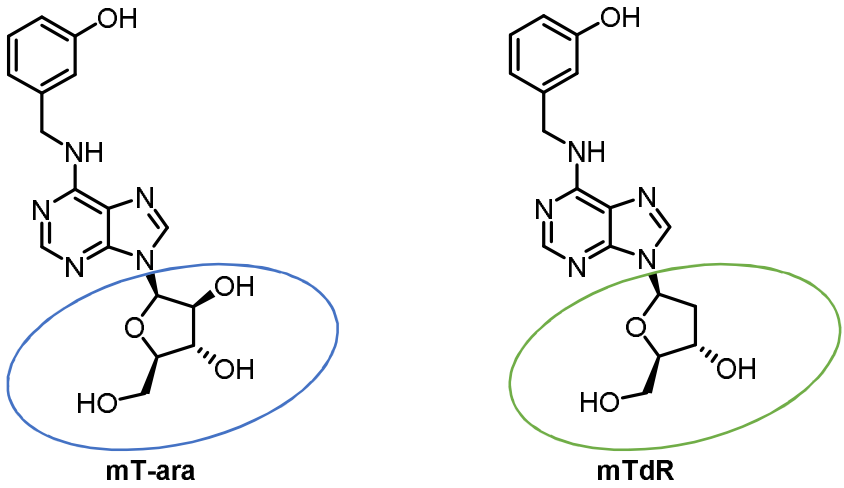

Fig.3: Chemical structure of 6-(3-hydroxybenzylamino)purine-9-β-D-arabinofuranoside (*m*T-ara) and 6-(3-hydroxybenzylamino)purine-9-(2´-deoxy-β-D-ribofuranoside) (*m*TdR).

The effect of supplementing either *m*T or BAP requiring cultures with INCYDE (2-chloro-6-(3-methoxyphenyl)aminopurine) (Fig. 4), an inhibitor of cytokinin (CK) degradation (Spíchal et al., 2012), on the endogenous CK profiles and physiology of banana *in vitro* was hence investigated (Aremu et al., 2012b).

Another interesting alternative approach for decreasing levels of irreversible topolin deactivation in banana tissue cultures was developed by Aremu et al. (2012b). An inhibitor of 9-glucosylation, roscovitine (6-benzylamino-2-(R)-[1-(hydroxymethyl) propylamino]-9-isopropylpurine) (Fig. 4), which was previously discovered among a number of 2,6,9-trisubstituted purines, tested as potential N-glucosylation inhibitors (Blagoeva et al., 2004, Letham et al., 1977, Dwivedi et al., 2010). Aremu et al. (2012b) demonstrated that its application simultaneously with exogenous CK(s) in the cultivation media has the potential to change endogenous CK pools thereby influencing the rooting and *ex vitro* acclimatization of *in vitro*-derived *Musa* spp. It was observed that plantlets regenerated from *m*T + roscovitine media produced the most shoots. They also had the highest total CK content (661 pmol/g FW) with the rootsts having approximately a 68-fold more than the shoots (Aremu et al., 2012b).

A general trend observed was that the addition of roscovitine and/or INCYDE with mT improved the total CK pool in both roots and shoots of the tissue-cultured 'Williams' banana regenerants (Aremu et al., 2012b). A similar pattern was determineded in the shoots when BAP was supplemented with roscovitine and INCYDE; however, both compounds reduced the total CK pool in the roots as well as the sum total in the plantlets. It is noteworthy to highlight that the reduction in total CK pool was mainly due to the decrease in the level of 9-glucosides, which are generally detrimental to plant growth (Aremu et al., 2012b).

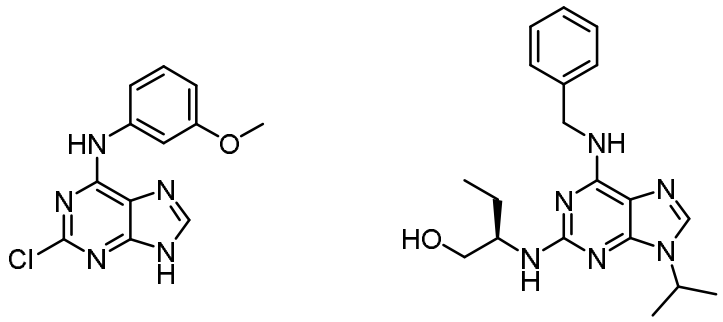

Fig. 4: Chemical structure of 2-chloro-6-(3-methoxyphenyl)aminopurine (INCYDE) and 2-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (roscovitine).

Future perspectives – application of fluorinated compounds

Fluorination has a long tradition in nucleoside chemistry. It was demonstrated that replacement of the 2' or 3' hydroxyl groups of a nucleoside with a fluorine atom causes only a minor change in the total structure, but significantly affects the stereoelectronic properties of the sugar moiety (Thibaudeau et al., 1998). Fluorine substitution has been extensively investigated in drug research and biochemistry as a means of increasing biological activity and inhancing chemical or metabolic stability. However, to date only a few fluorinated cytokinin derivatives have been prepared and their biological activity tested (Clemenceau et al., 1996, Doležal et al. 2006; Doležal et al. 2007). Later, Murvanidze et al. (2019) evaluated the impact of 6-(3-fluorobenzylamino)purine (*m*F-BAP) and its 9-β-D-riboside (mF-BAPR) (Fig. 5) on *in vitro* cloning of *Phalaenopsis* hybrids, which are usually characterized by slow growth and low multiplication rates. The plantlets formed significantly more, but smaller new shoots when treated with *m*F-BAPR (25.3) compared to *m*F-BAP (14.6) and BAP (7.0). The results suggest the following strategy: massive micropropagation of small shoots for a number of cycles on *m*F-BAPR in closed containers, followed by a final step with BAP in filter vessels to produce large shoots with roots. For this reason, the use of fluorinated topolins might present a breakthrough in the *in vitro* micropropagation of *Phalaenopsis* (Murvanidze et al., 2019). Another class of N^6-substituted-2´-deoxy-2´-fluoro-9-(β)-D-arabinofuranosylpurine derivatives (Fig. 5) was very recently prepared and their biological activity is currently being evaluated (Bryksová et al., manuscript in preparation).

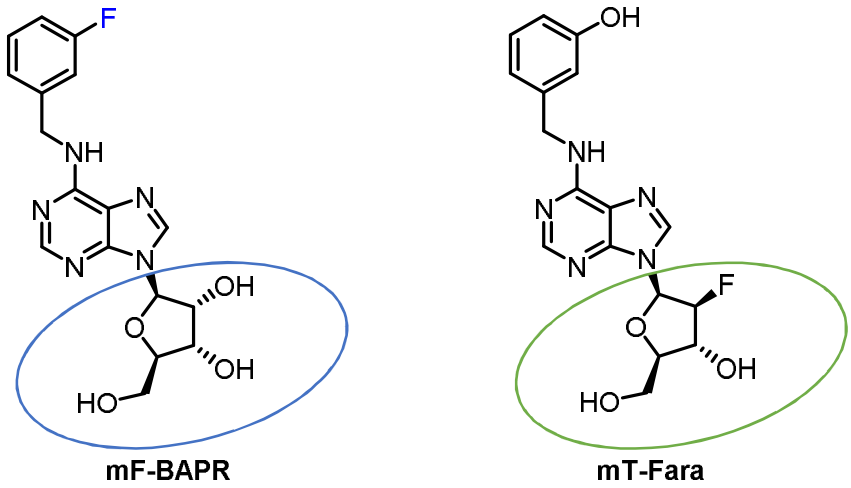

Fig. 5: Chemical structure of 6-(3-fluorobenzylamino)purine-9-β-D-ribofuranoside (*m*F-BAPR) and 6-(3-hydroxybenzylamino)purine-9-(2´-fluoro-2´-deoxy-β-D-arabinofuranoside) (*m*T-Fara).

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US010100077B2

(12) **United States Patent**
Dolezal et al.

(10) **Patent No.: US 10,100,077 B2**
(45) **Date of Patent: Oct. 16, 2018**

(54) **6-ARYL-9-GLYCOSYLPURINES AND USE THEREOF**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **15/527,613**

(22) PCT Filed: **Sep. 14, 2015**

(86) PCT No.: **PCT/CZ2015/050005**
§ 371 (c)(1),
(2) Date: **May 17, 2017**

(87) PCT Pub. No.: **WO2016/091235**
PCT Pub. Date: **Jun. 16, 2016**

(65) **Prior Publication Data**

US 2017/0334943 A1 Nov. 23, 2017

(30) **Foreign Application Priority Data**

Dec. 9, 2014 (CZ) 2014-875

(51) **Int. Cl.**

C07H 19/19	(2006.01)
C07H 19/173	(2006.01)
A61K 8/60	(2006.01)
A61K 31/7076	(2006.01)
A61Q 17/04	(2006.01)
A61Q 19/08	(2006.01)
C07H 19/16	(2006.01)

(52) **U.S. Cl.**
CPC ***C07H 19/16*** (2013.01); ***A61K 8/606*** (2013.01); ***A61K 31/7076*** (2013.01); ***A61Q 17/04*** (2013.01); ***A61Q 19/08*** (2013.01); ***C07H 19/173*** (2013.01); ***C07H 19/19*** (2013.01)

(58) **Field of Classification Search**
CPC C07H 19/16; C07H 19/19; C07H 19/173; A61K 8/60; A61K 31/7076; A61Q 17/04; A61Q 19/08
USPC 544/264; 514/263.2; 424/70.9; 536/27.2, 27.4
See application file for complete search history.

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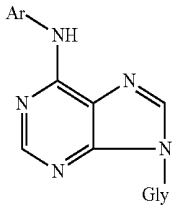
Primary Examiner — Venkataraman Balasubramanian
(74) *Attorney, Agent, or Firm* — Notaro, Michalos & Zaccaria P.C.

(57) **ABSTRACT**

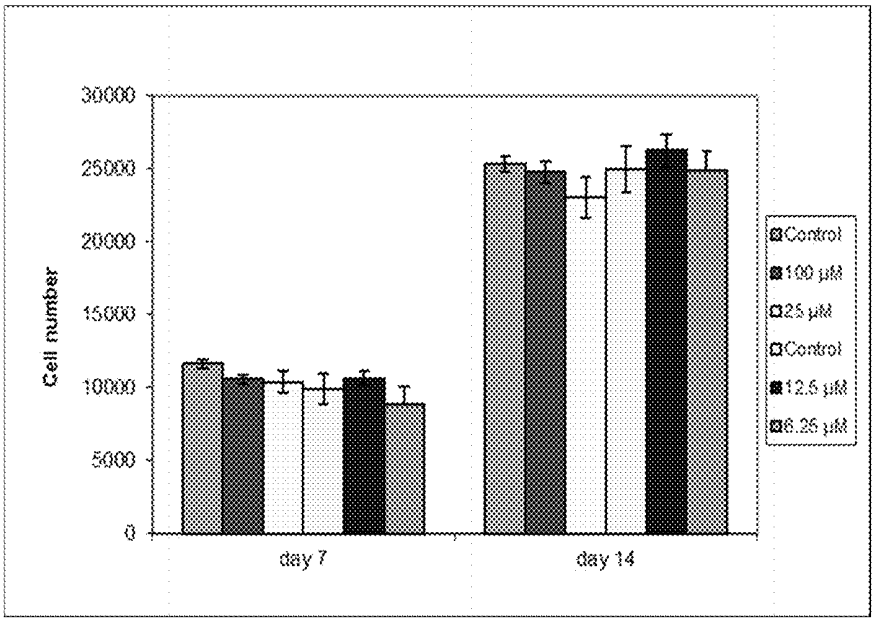
6-aryl-9-glycosidpurines of general formula I

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 use as antisenescent and UV protective compounds in animals.

10 Claims, 3 Drawing Sheets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100 µM
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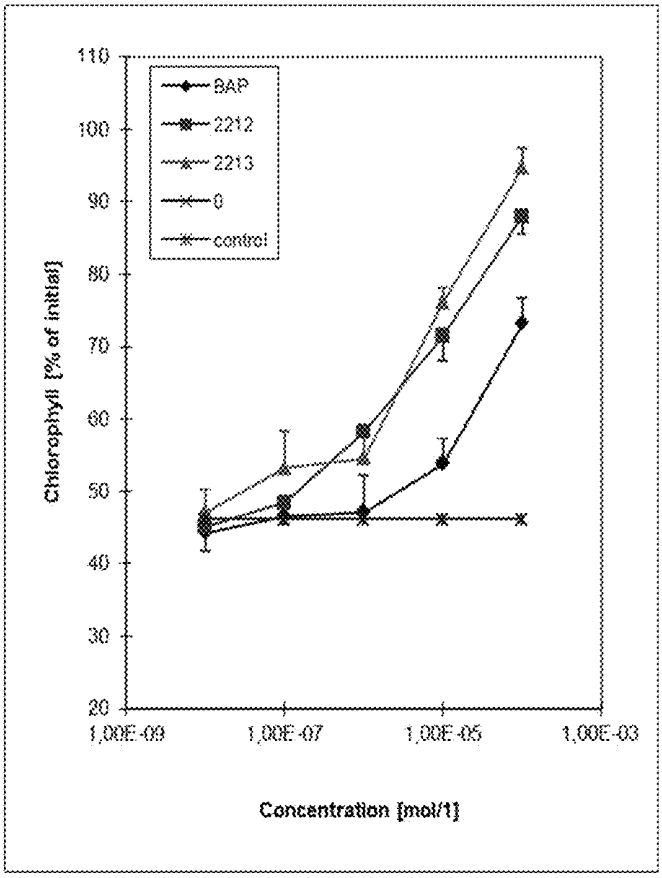
Fig. 1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Chlorophyll [% of initial]
BAP
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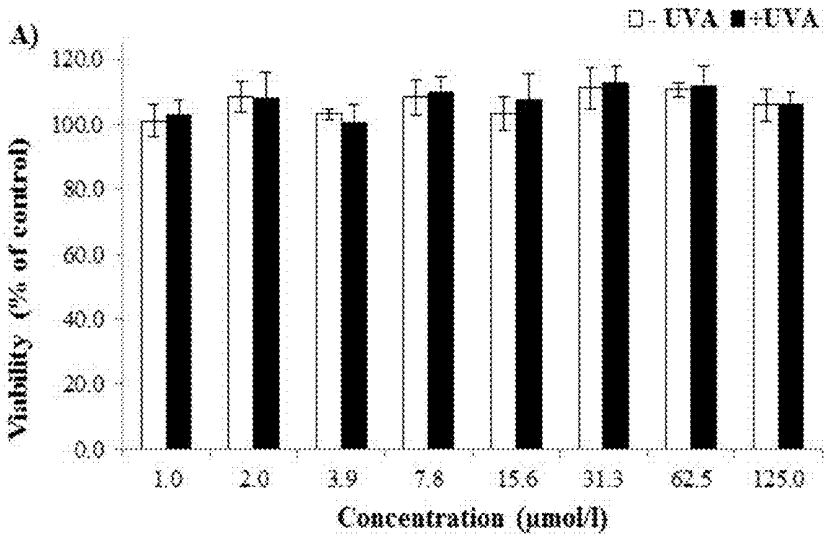
Concentration [mol/l]

Fig. 2


A)
□- UVA ■+UVA
Viability (% of control)
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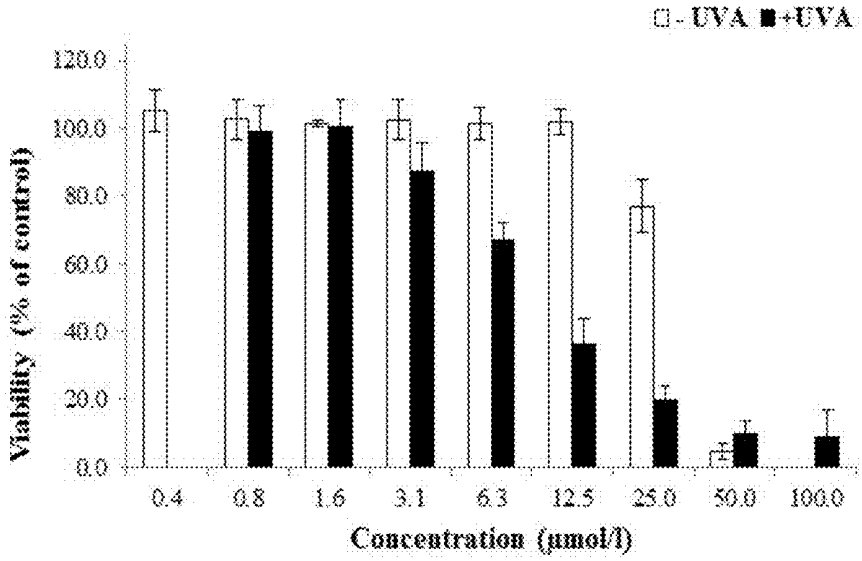
15.6
31.3
62.5
125.0
Concentration (µmol/l)

Fig. 3


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

Fig. 4

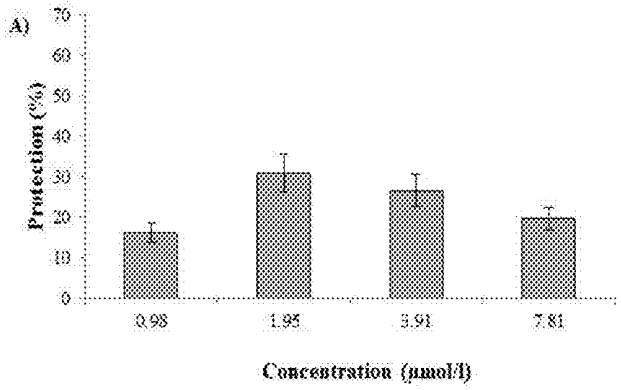


Fig. 5

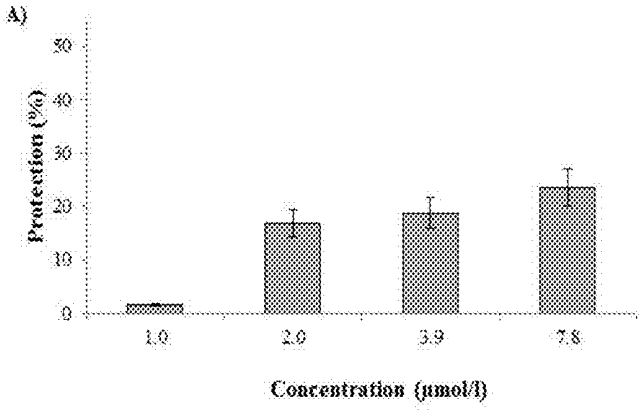


Fig. 6

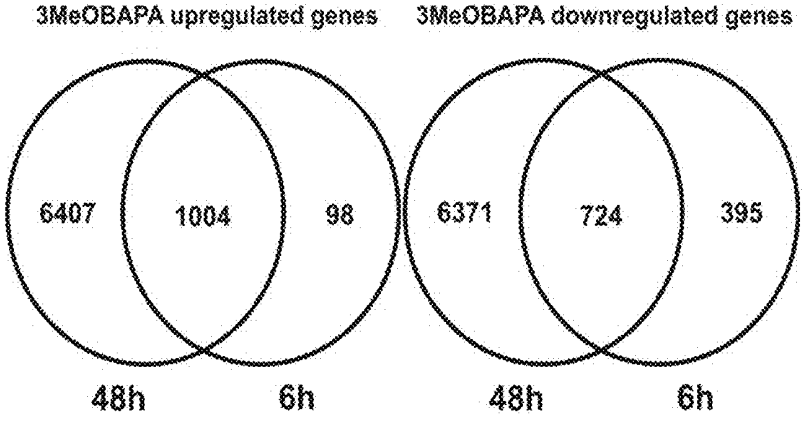


Fig. 7

6-ARYL-9-GLYCOSYLPURINES AND USE THEREOF

FIELD OF ART

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

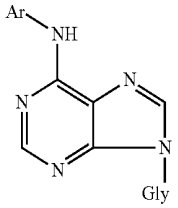
BACKGROUND ART

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 et 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-β-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 —SO_3R_c, wherein R_c represents hydrogen atom, linear or branched alkyl, alkenyl or alkynyl group containing 1 to 6 carbon atoms,

sulfoamido represents —$NHSO_3R_d$, 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-β-D-arabinofuranosylpurine, 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-β-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-β-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-(3-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-β-D-arabinofuranosylpurine, 6-(3-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine, 6-(4-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine, 6-(4-chloro-3-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine, 6-(4-fluoro-3-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine, 6-(3,5-bis(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine, 6-(3-(trifluoromethoxy)benzylamino)-9-β-D-arabinofuranosylpurine, 6-(4-(trifluoromethoxy)benzylamino)-9-β-D-arabinofuranosylpurine, 6-(4-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-aminobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3-aminobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(4-aminobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(4-diethylaminobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3,4-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3,5-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3,4-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,4-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,5-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,6-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3,4-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3,4-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3,5-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,3-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,4-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,5-dimethoxybenzylamino)-9-β-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-β-D-arabinofuranosylpurine, 6-(2,4,5-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,4,6-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3,4,5-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-3,4,5-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-3,4,6-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-4,5,6-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,4,6-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,3,4-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,4,6-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,3,4-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3,4,5-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2,4,6-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-3-chlorobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-4-chlorobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-5-chlorobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-6-chlorobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-3-iodobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-4-iodobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-5-iodobenzylamino)-9-β-D-

arabinofuranosylpurine, 6-(2-hydroxy-6-iodobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-3-bromobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-4-bromobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-5-bromobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-6-bromobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-3-fluorobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-4-fluorobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(2-hydroxy-5-fluorobenzylamino)-9-β-D-arabinofuranosylpurine, 6-(3-methylfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-methylfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(5-methylfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-fluorofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-fluorofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(5-fluorofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-chlorofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-chlorofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(5-chlorofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-bromo-furfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-bromofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(5-bromofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(5-hydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(5-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2-aminofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-aminofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-aminofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3,4-dihydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3,5-dihydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3,4-dihydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2,4-dihydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2,5-dihydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2,6-dihydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3,4-dimethoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3,4-dimethoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3,5-dimethoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2,3-dimethoxyfurfurylamino)-9-β-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, 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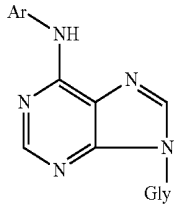
6-(2-iodobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-iodobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-iodobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2-chlorobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2-chlorobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-chlorobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-chlorobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2-aminobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-aminobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-aminobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2-methoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-methoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-methoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3-hydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(4-hydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3,4-dihydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3,5-dihydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3,4-dihydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2,4-dihydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2,5-dihydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2,6-dihydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3,4-dimethoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3,4-dimethoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(3,5-dimethoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2,3-dimethoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2,4-dimethoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2,5-dimethoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2,6-dimethoxybenzylamino)-9-β-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.

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



Ia

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 Ia. 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 10 wt. % 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-tert-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 Gattefoseé, 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 Gattefoseé, Paris), "Labrasol" (saturated polyglycolated glycerides prepared by an alcoholysis of TCM and composed of glycerides and polyethylene glycol esters; from Gattefoseé, Paris) and/or "Miglyol 812" (triglyceride of saturated fatty acids of chain length C_8 to C_{12} from Hills A G, 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_2O 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-β-D-arabinofuranosylpurine (Example 15).

FIG. **2**: Effect of 6-furfurylamino-9-β-D-arabinofuranosylpurine (2212) and 3-fluorobenzylamino-9-β-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-β-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-β-D-arabinofuranosylpurine (Example 21).

FIG. **6**. Effect of test compounds on UVB-induced damage to NHDF. (A) 6(3-methoxybenzylamino)-9-β-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-β-D-arabinofuranosylpurine

9-(β-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 μl, 0.56 mmol) were mixed in dimethylformamide (DMF, 1.86 ml). Subsequently, 3-methoxybenzylamine (56 μl, 0.56 mmol) was added. 9-(β-D-arabinofuranosyl) hypoxantine is commercially available (Jena Bioscience, N-1002) or can be prepared from 9-(β-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, ^1H (DMSO-d_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-β-D-arabinofuranosylpurine

9-(β-D-arabinofuranosyl) hypoxantine (100 mg, 0.37 mmol), BOP (196 mg, 0.44 mmol) and DIPEA (97 μl, 0.56 mmol) were mixed in v DMF (1.86 ml). Subsequently, 3-hydroxybenzylamine (50 μl, 0.50 mmol) was added. 9-(β-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: ^1H (DMSO-d_6, 300 MHz) β 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-β-D-arabinofuranosylpurine

9-(β-D-arabinofuranosyl) hypoxantine (100 mg, 0.37 mmol), BOP (196 mg, 0.44 mmol) and DIPEA (97 μl, 0.56 mmol) were mixed together in DMF (1.86 ml) and subsequently, 3-fluorbenzylamine (70 μl, 0.76 mmol) was added. 9-(β-D-arabinofuranosyl) hypoxantine was prepared from commercially available 9-(β-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: ^1H (DMSO-d_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-(β-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 μl) and DIPEA (97 μl) was added. 9-(β-D-arabinofuranosyl) hypoxantine was prepared from commercially available 9-(β-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-d$_6$, 300 MHz) δ ppm: ^{1}H (DMSO-d$_6$, 500 MHz) δ ppm: 3.6 (s, 1H), 3.7 (s, 1H), 3.76-3.81 (m, 2H), 4.09-4.17 (m, 2H), 4.64 (bs, 1H), 5.23 (bs, 1H), 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-β-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 μl, 0.291 mmol) with triethylamine (151 μl, 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 α 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 (0.771 g 2.42 mmol) dissolved in dry acetonitrile was added through the septum by a needle (15 ml). 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 (2×30 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 α and β anomers. Yield: 50%, HPLC purity: 98%, [M+H$^+$] 348, NMR: ^{1}H (DMSO-d$_6$, 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-β-D-arabinofuranosylpurine

9-(β-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 μl) and DIPEA (97 μl) 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 refridgerator 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-d$_6$, 300 MHz) δ ppm: ^{1}H (DMSO-d$_6$, 500 MHz) δ ppm: 3.60-3.71 (m, 2H), 3.77 (q, J=4 Hz, 1H), 4.11-4.15 (m, 2H), 4.69 (bs, 2H), 5.14 (bs, 1H), 5.57 (bs, 2H), 6.25 (d, J=4.5 Hz, 1H), 7.25-7.33 (m, 3H), 7.36 (s, 1H), 8.18 (s, 1H), 8.21 (s, 1H), 8.40 (bs, 1H).

Example 7: The synthesis of 6-(2-aminobenzylamino)-9-β-D-arabinofuranosylpurine

9-(β-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 μl) and DIPEA (97 μl) 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 refridgerator 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-d$_6$, 300 MHz) δ ppm: ^{1}H (DMSO-d$_6$, 500 MHz) δ ppm: 3.59-3.70 (m, 2H), 3.77 (q, J=4 Hz, 1H), 4.11-4.15 (m, 2H), 4.51 (bs, 2H), 5.10 (t, J=5.5 Hz, 1H), 5.20 (s, 2H), 5.52 (d, J=4 Hz, 1H), 5.60 (d, J=5 Hz, 1H), 6.26 (d, J=4 hz, 1H), 6.46 (0=7.5 Hz, 1H), 6.59 (d, J=8 Hz, 1H), 6.91 (t, J=7.5 Hz, 1H), 7.07 (d, J=7.5 Hz, 1H), 8.19 (s, 3H)

TABLE 1

6-substituted-9-β-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	Elemental analysis calculated/found			ES-MS
(—NH—Ar)	% C	% H	% N	[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

TABLE 1-continued

6-substituted-9-β-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	Elemental analysis calculated/found			ES-MS
(—NH—Ar)	% C	% H	% N	[M + H⁺]
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-β-D-arabinofuranosylpurine in Bench Scale

Feedstock: 9-β-D-Arabinofuranosyl-hypoxanthine (1072 g, 4 mol),

(Benzotriazol-1-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 l, 2-propanol 30 L.

Procedure:

Dimethylformamide (20 l) was placed into a glass duplicated reactor A1 equiped with thermometer (PT100) and reflux condenser. Reactor was filled in with inert atmosphere (nitrogen). Stirring with hopper opening was switched on. 9-β-D-arabinattranosyl 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 (3×1 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 A1 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 (3×250 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 Büchner channel and washed first with cold water (+5° C.) than only by water (4×1 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 A1 reactor and stirring was switched on.

Crude 6-(3-methoxybenzylamino)-9-β-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 Büchner channel (preheated in convection oven, 110° C.). Glass reactor was rinsed by 2-propanol (2×1 L) and filtration cake was washed with this portion of 2-propanol Búchner 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 (3×500 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 μl) and 3-methoxybenzylamine (167 μl) 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-d_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, J=4.0 Hz), 6.30 (t, J=6.9 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 10: Synthesis of 6-(2-hydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine

2'-deoxyinosine (252 mg) and BOP (664 mg) were dissolved in dry DMF (8 ml) and stirred at laboratory tempera-

ture under argon atmosphere. After five minutes, DIPEA (348 µl) and 2-hydroxybenzylamine (131 µl) 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; ^{1}H (DMSO-d$_6$, 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 11: Synthesis of 6-(2-hydroxy-3-methoxy-benzylamino)-9-β-D-2'-deoxyribofuranosylpurine

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 µl) and 2-hydroxy-3-methoxybenzylamine (165 µ1) 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$^+$]0 388, C/H/N: 55.8/55.3; 5.5/5.6; 18.1/18.2; ^{1}H (DMSO-d$_6$, 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 µl) and furfurylamine (150 µl) 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-d$_6$, 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-β-D-arabinofuranosylpurine	>50
6-furfurylamino-9-β-D-arabinofuranosylpurine	>50
6-(3-methoxybenzylamino)-9-β-D-arabinofuranosylpurine	>50
6-(2-chlorobenzylamino)-9-β-D-arabinofuranosylpurine	>50
6-(3-hydroxybenzylamino)-9-β-D-arabinofuranosylpurine	>50
6-(3-chlorobenzylamino)-9-β-D-arabinofuranosylpurine	>50

-continued

	IC50 (μM)
6-(2,3-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine	49
6-(2,3,4-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine	48
6-(3-iodobenzylamino)-9-β-D-arabinofuranosylpurine	>50
6-(3-aminobenzylamino)-9-β-D-arabinofuranosylpurine	>50
6-(furfurylamino)-9-β-D-2'-deoxyribofuranosylpurine	>50
6-(2-hydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine	>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-β-D-arabinofuranosylpurine	>50
6-furfurylamino-9-β-D-arabinofuranosylpurine	>50
6-(3-methoxybenzylamino)-9-β-D-arabinofuranosylpurine	>50
6-(2-chlorobenzylamino)-9-β-D-arabinofuranosylpurine	>50
6-(3-hydroxybenzylamino)-9-β-D-arabinofuranosylpurine	>50
6-(3-chlorobenzylamino)-9-β-D-arabinofuranosylpurine	48
6-(3-iodobenzylamino)-9-β-D-arabinofuranosylpurine	>50
6-(3-aminobenzylamino)-9-β-D-arabinofuranosylpurine	>50
6-(furfurylamino)-9-β-D-2'-deoxyriboside	>50
6-(2-hydroxybenzylamino)-9-β-D-2'-deoxyriboside	>50
6-(2,3-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine	>50
6-(2,3,4-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine	>50
6-furfurylamino-9-ribosylpurine (comparative example)	≤3

Example 15: One-Step Growth Curve for the Compound 6-(benzylamino)purine-9-β-D-arabinofuranosylpurine

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-β-D-arabinofuranosylpurine and 6-(3-methoxybenzylamino)-9-β-D-arabinofuranosylpurine in concentrations 1 mM a 200 microM in 0.5% DMSO/99.5% PBS were evaluated according to a standard protocol "INVITRO EpiDerm™ 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-β-D-arabinofuranosylpurine and 98.0% for the compound 6-(furfurylamino)-9-β-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-β-D-arabinofuranosylpurine and 6-(3-methoxybenzylamino)-9-β-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 (CO2 5.5 percent, 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 $\mu mol \cdot m^{-2} \cdot s^{-1}$. 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.

TABLE 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 ($mol \cdot l^{-1}$)	activity (%) [10^{-4} $mol \cdot l^{-1}$ BAP = 100%]
6-furfurylamino-9-β-D-arabinofuranosylpurine	10^{-4}	193 ± 1
6-(3-methoxybenzylamino)-9-β-D-arabinofuranosylpurine	10^{-4}	118 ± 2
6-benzylaminopurine-9 β-D-deoxyribosylpurine	10^{-4}	178 ± 9
6-(3-chlorobenzylamino)-9-β-D-arabinofuranosylpurine	10^{-4}	172 ± 8
6-(3-fluorobenzylamino)-9-β-D-arabinofuranosylpurine	10^{-4}	195 ± 6
6-(2-bromobenzylamino)-9-β-D-arabinofuranosylpurine	10^{-5}	186 ± 19
6-(3-bromobenzylamino)-9-β-D-arabinofuranosylpurine	10^{-4}	198 ± 10
6-(4-bromobenzylamino)-9-β-D-arabinofuranosylpurine	10^{-4}	176 ± 11
6-(3-iodobenzylamino)-9-β-D-arabinofuranosylpurine	10^{-4}	198 ± 4
6-(3,4-dimethoxybenzylamino)-9-β-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 5 g/l of glucose, 2 mM of glutamin, 100 U/ml of penicilin, 100 mg/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 μl of cell suspension on 96-well microtiter plates. Innoculates were stabilized by 24 hrs preincubation at 37° C. in CO_2. Particular concentrations of tested compounds were added in time zero as 20 μl 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 drug 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 CO_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: $GI_{50}=(FD_{well\ with\ derivative}/FD_{control\ well})\times 100\%$. 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, G150 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.

TABLE 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-β-D-arabinofuranosylpurine	>100	>100	>100
6-furfuryl-9-β-D-arabinofuranosylpurine	>100	>100	>100
6(3-methoxybenzylamino)-9-β-D-arabinofuranosylpurine	>100	>100	>100
6-benzylamino-9-β-D-deoxyribosylpurine	>100	>100	>100
3-methylbenzylamino-9-β-D-arabinofuranosylpurine	>100	>100	>100
4-methylbenzylamino-9-β-D-arabinofuranosylpurine	95	>100	>100
2-methylbenzylamino-9-β-D-arabinofuranosylpurine	>100	>100	>100
3-fluorobenzylamino-9-β-D-arabinofuranosylpurine	>100	>100	>100
4-chlorobenzylamino-9-β-D-arabinofuranosylpurine	>100	>100	>100
2-fluorobenzylamino-9-β-D-arabinofuranosylpurine	87	>100	>100
3-chlorobenzylamino-9-β-D-arabinofuranosylpurine	>100	>100	>100
4-hydroxybenzylamino-9-β-D-arabinofuranosylpurine	>100	>100	87
3-fluorobenzylamino-9-β-D-2'-deoxyribosylpurine	>100	>100	>100
3-chlorobenzylamino-9-β-D-2'-deoxyribosylpurine	>100	>100	>100
3-hydroxybenzylamino-9-β-D-2'-deoxyribosylpurine	>100	>100	>100
2,4-dimethoxybenzylamino-9-β-D-arabinofuranosylpurine	98	>100	>100
2-chloro-4-fluorobenzylamino-9-β-D-arabinofuranosylpurine	>100	>100	>100
3-chloro-4-fluorobenzylamino-9-β-D-arabinofuranosylpurine	>100	>100	>100

Example 20: In Vitro Test of Phototoxic Effects of 6-(3-methoxybenzylamino)-9-β-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 W J, Pechovitch G, de Silva O, Holzhater H G, Clothier R, Desolle P, Gerberick F, Liebsch M, Lovell W W, Maurer T, Pfannenbecker U, Potthast J M, Csato M, Sladowski D, Steiling W, Brantom P., Toxicol In Vitro. 1998 Jun. 1; 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, Palacký 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-β-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 μmol/l. 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 μmol/l) 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 H1 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% CO_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:

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

A_S . . . 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 μmol/l or 3.9-500 μmol/l). Results are given in FIG. **3**. Effect of a well-known phototoxic com-

pound chlorpromazine (Spielmann H, Balls M, Dupuis J, Pape W J, Pechovitch G, de Silva O, Holzhater H G, Clothier R, Desolle P, Gerberick F, Liebsch M, Lovell W W, Maurer T, Pfannenbecker U, Potthast J M, Csato M, Sladowski D, Steiling W, Brantom P., Toxicol In Vitro. 1998 Jun. 1; 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-β-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, Palacký 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-β-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-β-D-arabinofuranosylpurine were 0.9; 1.8; 3.9 a 7.8 μmol/l. 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 H1 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% CO_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)

Apc . . . 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 (FIGS. **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-β-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×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, Calif.) 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 ($P\leq0.05$). 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: 7095 genes were downregulated and 7509 genes were upregulated ($P\leq0.05$). 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 6 h 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 6 h.

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 (BGAL11), At1g02790 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 6 h and after 48 h of treatment with 10 μM 3MeOBAP are shown.

		logFC	
AGI code	Description	6 h	48 h
AT2G47040	Pectin methylesterase Vanguard1 (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 (BGAL11)	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 (SKS14)	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

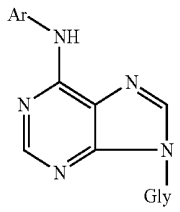
TABLE 5-continued

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

		logFC	
AGI code	Description	6 h	48 h
AT3G44830	Putative phospholipid: diacylglycerol acyltransferase 2 (PDAT2)	0.38	4.91
AT4G31950	Cytochrome P450 82C3 (CYP82C3)	0.37	5.29

The invention claimed is:

1. A Method for regulation of aging in animals and/or for photoprotection of animals for cosmetic or therapeutic purposes, comprising the step of administering to an animal, who is in need of cosmetic or therapeutic treatment, a therapeutically effective or cosmetically effective amount of a 6-aryl-9-glycosidpurine of formula I



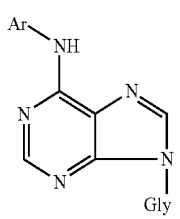
I

or a pharmaceutically acceptable salt thereof with alkali metal, ammonia, amine, or addition salt with acid, 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.

2. A method for regulating aging and/or UV photodamage in cells or microorganisms wherein an effective amount of at least one compound of formula I



I

or a pharmaceutically acceptable salt thereof with alkali metal, ammonia, amine, or addition salt with acid, 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 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,

is applied to cells or microorganisms in need of regulation of aging and/or UV photodamage.

3. The method according to claim **1**, wherein the compound of formula I is selected from the group consisting of:

6-furfurylamino-9-β-D-arabinofuranosylpurine,

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-β-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,

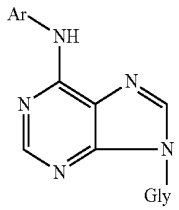
6-(3,4-dihydroxyfurfurylamino)-9-β-D-arabinofuranosylpurine,
6-(2,4-dihydroxyfurfurylamino)-9-β-D-arabinofuranosylpurine,
6-(2,5-dihydroxyfurfurylamino)-9-β-D-arabinofuranosylpurine,
6-(2,6-dihydroxyfurfurylamino)-9-β-D-arabinofuranosylpurine,
6-(3,4-dimethoxyfurfurylamino)-9-β-D-arabinofuranosylpurine,
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-β-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-β-D-arabinofuranosylpurine,
6-(3-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(4-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(4-chloro-3-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(4-fluoro-3-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,5-bis(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(3-(trifluoromethoxy)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(4-(trifluoromethoxy)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(4-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-aminobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3-aminobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(4-aminobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(4-diethylaminobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,4-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,5-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,4-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,4-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,5-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,6-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,4-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,4-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,5-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,3-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,4-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,

6-(2,5-dimethoxybenzylamino)-9-β-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-β-D-arabinofuranosylpurine,
6-(2,4,5-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,4,6-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,4,5-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-3,4,5-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-3,4,6-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-4,5,6-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,4,6-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,3,4-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,4,6-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,3,4-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,4,5-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,4,6-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-3-chlorobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-4-chlorobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-5-chlorobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-6-chlorobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-3-iodobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-4-iodobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-5-iodobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-6-iodobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-3-bromobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-4-bromobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-5-bromobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-6-bromobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-3-fluorobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-4-fluorobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-5-fluorobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3-methylfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(4-methylfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(5-methylfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3-fluorofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(4-fluorofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(5-fluorofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3-chlorofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(4-chlorofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(5-chlorofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3-bromo-furfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(4-bromofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(5-bromofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3-hydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(4-hydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(5-hydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(4-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(5-methoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2-aminofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3-aminofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(4-aminofurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3,4-dihydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3,5-dihydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3,4-dihydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2,4-dihydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,

6-(2,5-dihydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2,6-dihydroxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3,4-dimethoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3,4-dimethoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3,5-dimethoxyfurfurylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2,3-dimethoxyfurfurylamino)-9-β-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-β-D-2'-deoxyribofuranosylpurine,
6-(3-fluorobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(4-fluorobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2-bromobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3-bromobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(4-bromobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2-iodobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3-iodobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(4-iodobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2-chlorobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2-chlorobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3-chlorobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(4-chlorobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2-aminobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3-aminobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(4-aminobenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2-methoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3-methoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(4-methoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine, 6-(2-hydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3-hydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(4-hydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3,4-dihydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3,5-dihydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3,4-dihydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2,4-dihydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2,5-dihydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2,6-dihydroxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3,4-dimethoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3,4-dimethoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(3,5-dimethoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2,3-dimethoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2,4-dimethoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2,5-dimethoxybenzylamino)-9-β-D-2'-deoxyribofuranosylpurine,
6-(2,6-dimethoxybenzylamino)-9-β-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.

4. 6-aryl-9-glycosidpurine of formula Ia



Ia

or a pharmaceutically acceptable salt thereof with alkali metal, ammonia, amine, or addition salt with acid, wherein

Gly represents β-D-arabinofuranosyl,

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

5. 6-aryl-9-glycosidpurine according to claim **4**, selected from the group consisting of,

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-β-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-β-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-β-D-arabinofuranosylpurine,
6-(3-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(4-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(4-chloro-3-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(4-fluoro-3-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,5-bis(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(3-(trifluoromethoxy)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(4-(trifluoromethoxy)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(4-(trifluoromethyl)benzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-aminobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3-aminobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(4-aminobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(4-diethylaminobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,4-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,5-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,4-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,4-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,5-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,6-dihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,4-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,4-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,5-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,3-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,4-dimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,5-dimethoxybenzylamino)-9-β-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-β-D-arabinofuranosylpurine,
6-(2,4,5-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,4,6-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,4,5-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-3,4,5-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-3,4,6-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-4,5,6-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,4,6-trimethoxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,3,4-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,4,6-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,3,4-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(3,4,5-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2,4,6-trihydroxybenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-3-chlorobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-4-chlorobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-5-chlorobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-6-chlorobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-3-iodobenzylamino)-9-β-D-arabinofuranosylpurine,
6-(2-hydroxy-4-iodobenzylamino)-9-β-D-arabinofuranosylpurine,

6-(2-hydroxy-5-iodobenzylamino)-9-β-D-arabinofuranosylpurine,

6-(2-hydroxy-6-iodobenzylamino)-9-β-D-arabinofuranosylpurine,

6-(2-hydroxy-3-bromobenzylamino)-9-β-D-arabinofuranosylpurine,

6-(2-hydroxy-4-bromobenzylamino)-9-β-D-arabinofuranosylpurine,

6-(2-hydroxy-5-bromobenzylamino)-9-β-D-arabinofuranosylpurine,

6-(2-hydroxy-6-bromobenzylamino)-9-β-D-arabinofuranosylpurine,

6-(2-hydroxy-3-fluorobenzylamino)-9-β-D-arabinofuranosylpurine,

6-(2-hydroxy-4-fluorobenzylamino)-9-β-D-arabinofuranosylpurine,

6-(2-hydroxy-5-fluorobenzylamino)-9-β-D-arabinofuranosylpurine.

6. The method of claim **1**, wherein the regulation of aging is inhibition of aging.

7. The method of claim **1**, wherein the animal is a mammal.

8. The method of claim **1**, wherein Ar represents benzyl or furfuryl, each of which can be substituted by 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.

9. The method of claim **2**, wherein Ar represents benzyl or furfuryl, each of which can be substituted by 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.

10. 6-aryl-9-glycosidpurine of claim **4**, wherein Ar represents benzyl or furfuryl, each of which can be substituted by 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.

* * * * *

Palacký University Olomouc

Faculty of Science

Laboratory of Growth Regulators & Chemical Biology and Genetics Department

Magdaléna Bryksová

Summary of the Doctoral Thesis

Preparation and biological activity of the new cytokinin derivatives

P1527 Biology

1501V019 Experimental biology

Supervisor

Mgr. Karel Doležal, Dr., DSc.

Olomouc

2020

The presented PhD. thesis was realized in the Laboratory of Growth Regulators & Centre of the Region Haná for Biotechnological and Agricultural Research within the framework of internal Ph.D. The Ph.D program of Experimental Biology, guaranteed by the Laboratory of Growth Regulators, Faculty of Science, Palacký University in Olomouc, between the years 2016-2020.

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The oral defense will take place on …… before the Commission for the Ph.D. thesis of the Study Program Experimental Biology, room……., Šlechtitelů 27, Olomouc – Holice.

The PhD. thesis and expert reviews will be available 14 days before the defense in the Study Department of Faculty of Science (Mgr. M. Karásková), Palacký University, 17. listopadu 12, Olomouc.

After the defense, the Ph.D. thesis will be stored in the Library of the Biological Departments of Faculty of Science, Palacký University, Šlechtitelů 27, Olomouc – Holice.

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Aims and scope

A wide spectrum of CK derivatives, structurally modified at adenine moiety, has been synthesized to this date and their biological activity has been evaluated in plants, as well as in mammalian cells. It was proved, that even small structural modification can completely change CK-like activity and therefore it is necessary to well-describe structure-activity relationship. In presented thesis, the series of new CK derivatives was synthesized in order to modify their properties and reveal new active derivatives with potential practical applications.

The aims of presented doctoral thesis are:

- Synthesis of a wide range of aromatic N^6-substituted aminopurine-9-β-D-arabinofuranosides
- Synthesis of new N^6-substituted aminopurine-9-β-L-ribofuranosides
- Synthesis of N^6-substituted aminopurine-9-β-D-2′-deoxy-2′-fluoroarabinofuranosides
- Description of structure-activity relationship
- Evaluation of biological activity in plants and animal cells

Introduction

Originally, the term *hormone* comes from the Greek language and it means *to stimulate*, as well as *to set in motion*. Firstly, this term was used in medicine about 100 years ago for a stimulatory factor with the ability to transport a chemical signal (Davies, 2010). In 1930´s, Went and Thimann defined plant hormones (phytohormones) very briefly, as substances which are able to migrate from one part of an organism to another (Went and Thimann, 1937). Nowadays, phytohormones are characterized as a group of naturally occurring, organic compounds with the ability to influence physiological processes at extremely low concentrations (Davies, 2010).

The first observation of phytohormone-related physiological processes is presumably dated between 1880 and 1893 and it was based on morphogenic and developmental correlations. Sachs postulated that *"Morphological differences between plant organs are due to differences in their material composition"*, which predicted the existence of root-forming, flower-forming and other substances, which are transported through the plant in different directions (Went and Thimann, 1937). Concurrently, Charles Darwin made the original observation of phototropism of oat coleoptiles and postulated the theory about a signal and its transport from the tips of the coleoptile to the lower regions of the plant. In 1934, the isolation of the first phytohormone from fresh plant material was published and the compound was identified as indol-3-acetic acid (IAA), which became known as auxin (Wildman, 1997).

Since the discovery of IAA, many endogenous signaling and regulatory molecules have been discovered, and more are to be discovered yet (Smith et al., 2017). The detailed investigation led to the discovery of the other hormones: research in plant pathogenesis led to gibberellins, efforts to culture tissues led to cytokinins, the control of abscission and dormancy led to abscisic acid and the effects of illuminating gas and smoke led to ethylene. Subsequently, the other compounds, such as brassinosteroids, jasmonates, salicylic acid, strigolactones, peptides and polyamines have been added to the group of phytohormones (El-Esawi, 2017).

The long story of cytokinin research started by the discovery of the first member of cytokinin group, followed by confirmation of their natural occurrence and clarification of their metabolism, biosynthesis pathway or signaling in plants. Nowadays, the cytokinin research is focused on a wide spectrum of applications, such as plant biotechnology and agriculture utilization, as well as pharmacology and cosmetic industry (Kamínek, 2015).

Material and methods

General procedures

The chromatographic purity and mass spectra of the synthesized compounds were obtained using the HPLC-PDA-MS method. Samples were dissolved in methanol (10 µl of 3.10^{-5} M in 1% methanol) and injected onto a reverse-phased column (Symmetry C18, 5 µm, 150 mm × 2.1 mm; Waters, Milford, MA, USA) incubated at 40 °C. Solvent A contained of 15 mM ammonium formate ($HCOONH_4$) adjusted to pH 4.0, solvent B contained of methanol and flow-rate was set to 0.2 mL/min. The samples were analysed using gradient: 0 min, 10 % of B; 25 min; 90 % of B; 35 min; 90 % of B; 45 min; 10 % of B in *Waters* Alliance *2695* Separations *Module* (Waters, Manchester, UK). Subsequently, the effluent was introduced to the Waters 2996 PDA detector (Waters, Manchester, UK) (scanning range 210–400 nm with 1.2 nm resolution) and *QDa Mass* Spectrometer (Waters MS Technologies, Manchester, UK) with an ESI system. The cone voltage was set to 15 V, ion source was heated to 120°C and the measurement was performed in positive mode (ESI+) or negative mode (ESI-) therefore molecular ions were recorded as $[M + H]^+$, $[M-H]^-$ or ESI adduct ions. ^1H NMR spectra were measured on a Jeol 500 SS spectrometer operating at a temperature of 300 K and a frequency of 500.13 MHz. The samples were prepared by dissolving in DMSO-d_6. Tetramethylsilane (TMS) was used as an internal standard. Thin-layer chromatography (TLC) was carried out using silica gel 60 WF_{254} plates (Merck) and $CHCl_3$/MeOH (4:1, v/v) were used as the mobile phase. Purification *via* column chromatography was obtained using silica gel Davisil R LC60A 40-63 micron.

HPLC-MS purification

Preparative HPLC-MS chromatography machine (Agilent 1290 Infinity II) was used coupled with UV-VIS detector with a mass LC/MSD detector (Agilent InfinityLab) and Agilent Prep-C189 column (5 µm, 21.2 mm x 50 mm, Waters, Milford, MA, USA) to obtain the final product. The analyzed sample was dissolved in 50% MeOH before injection. The mobile phase MeOH (A):H_2O (B) with a flow rate of 20 $mL.min^{-1}$ and linear gradients (0 min, 10% B; 0–12 min; 90% B) was used.

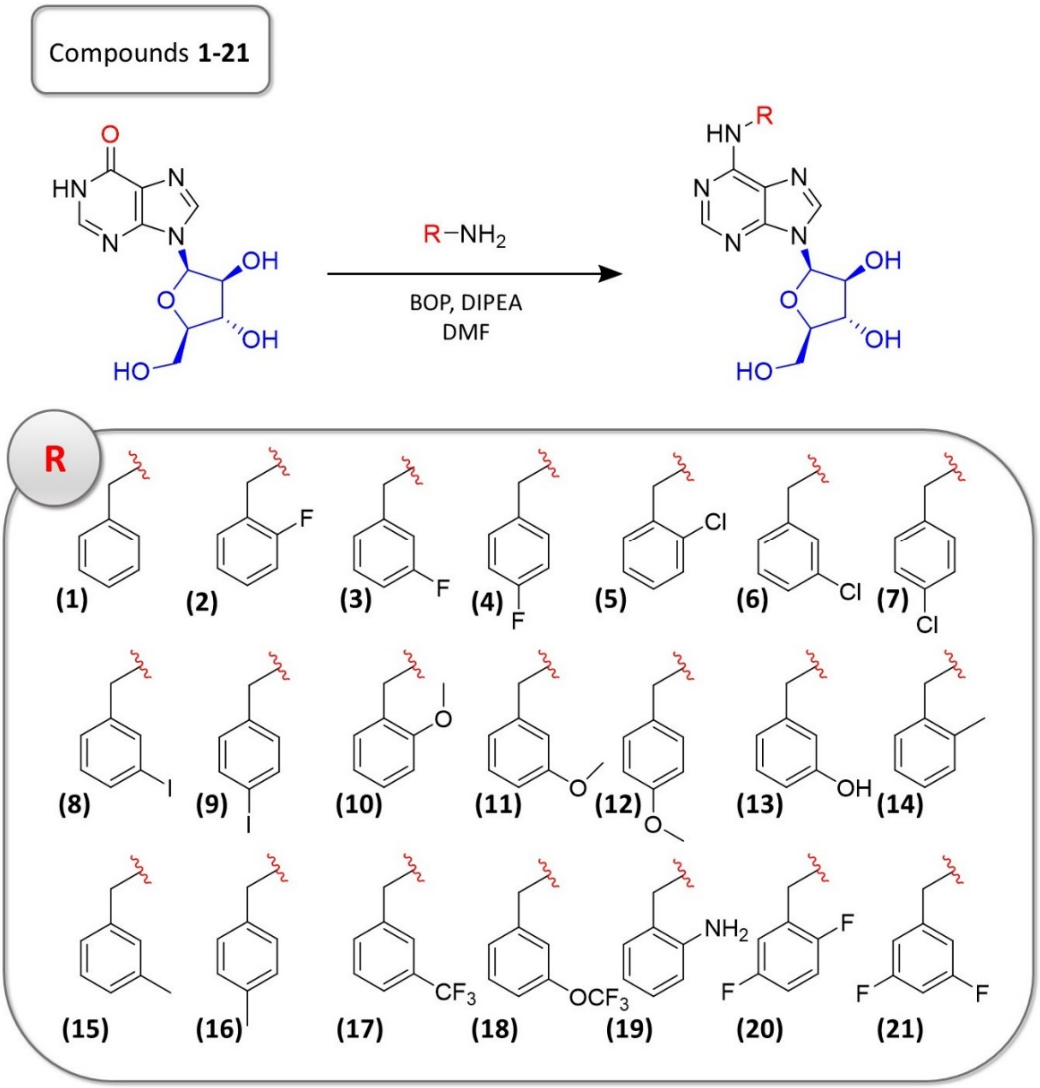
HRMS conditions

The prepared samples (5 μL) were characterized using the HPLC-PDA-MS method. The samples were injected onto a reversed-phase column (Symmetry C18, 5 μm, 150 mm × 2.1 mm; Waters, Milford, MA, USA) incubated at 40°C. Solvent A was 15 mM ammonium formate adjusted to pH 4.0. Solvent B was methanol. The following linear gradient was used at a flow rate of 250 μL min^{-1}: 0 min, 10% B; 0–15 min, 90% B. The effluent was introduced to a DAD detector (scanning range 210–400 nm with 1.2 nm resolution) and then to an electrospray source (source temperature 150°C, desolvation temperature 550°C, capillary voltage 1 kV, cone voltage 25 V). Nitrogen was used as the cone gas (50 L h^{-1}) and the desolvation gas (1000 L h^{-1}). Data acquisition was performed in the full-scan mode (50–1000 Da) with a scan time of 0.5 s and collision energy of 4 eV; argon was used as the collision gas (optimized pressure of 5×10^{-3} mbar). Analyses were performed in the positive mode (ESI^+) and protonated molecules $[M+H]^+$ were collected in each MS spectrum. For exact mass determination experiments, external calibration was performed using lock spray technology and the mixture of leucine/encephalin (50 pg μL^{-1}) in an acetonitrile and water (1:1) solution with 0.1% formic acid as a reference sample. Accurate masses were calculated and used to determine the elemental composition of the analytes with fidelity better than 1.0 ppm.

Synthetic procedure I.

N^6-substituted purine-9-β-D-arabinofuranoside derivatives **(1-21) (scheme 1)**

The starting hypoxanthine-9-β-D-arabinofuranoside and BOP (1.2 equiv.) were dissolved in dry DMF following by DIPEA (1.5 equiv.) and appropriate benzylamine (1.2 equiv.) as the final component. The reaction mixture was heated at 55-60°C under argon atmosphere and stirred for 24 hours as summarized in reaction scheme. The crude mixture was evaporated on vacuum rotary evaporator and final gel was formed. The distillation residue was mixed with MeOH and a drop of $CHCl_3$ and mixture was sonificated. The resulting white solid powder was recrystallized using EtOH. The process is described in detail in Bryksová et al. 2020 (Bryksová et al., 2020).



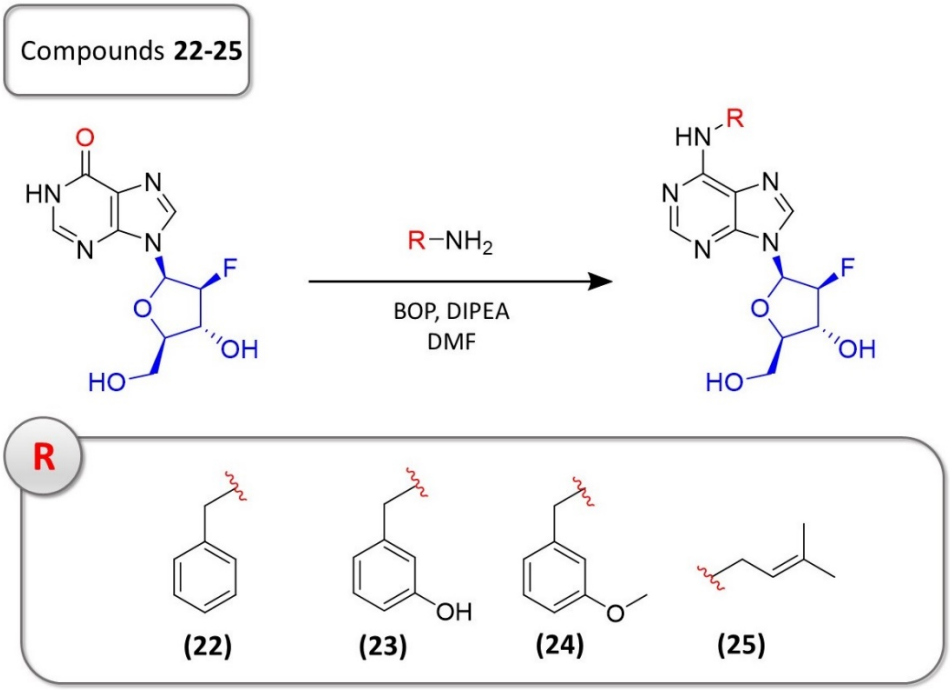
***Scheme 1**: Synthesis of N^6-substituted purine-9-β-D-arabinofuranoside derivatives. The synthesis was performed in DMF with presence of BOP and DIPEA to form the desired product (55-60°C, 24 hrs).*

Synthetic procedure II. – fluoro derivatives

N^6-substituted purine-9-(2′-deoxy-2′-fluoroarabinofuranoside) derivatives **(22-25) (scheme 2)**

The 9-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl) hypoxanthine and BOP (1.2 equiv.) were mixed and dissolved in dry DMF under argon atmosphere and subsequently DIPEA (1.5 equiv.) and appropriate amine (1.2 equiv.) were added to the reaction mixture. The mixture was heated in oil bath at the temperature of 55-60°C for 24 hours. After evaporation on the vacuum rotary evaporator, the specifically colored gel was formed. The crude residue was purified by column chromatography (**22** and **24**) or preparative HPLC (**23** and **25**) to give required product. After column chromatography, compounds **22** and **24**

were recrystallized in the mixture of various solvents (Bryksová 2020, supplementary material III in attachment).

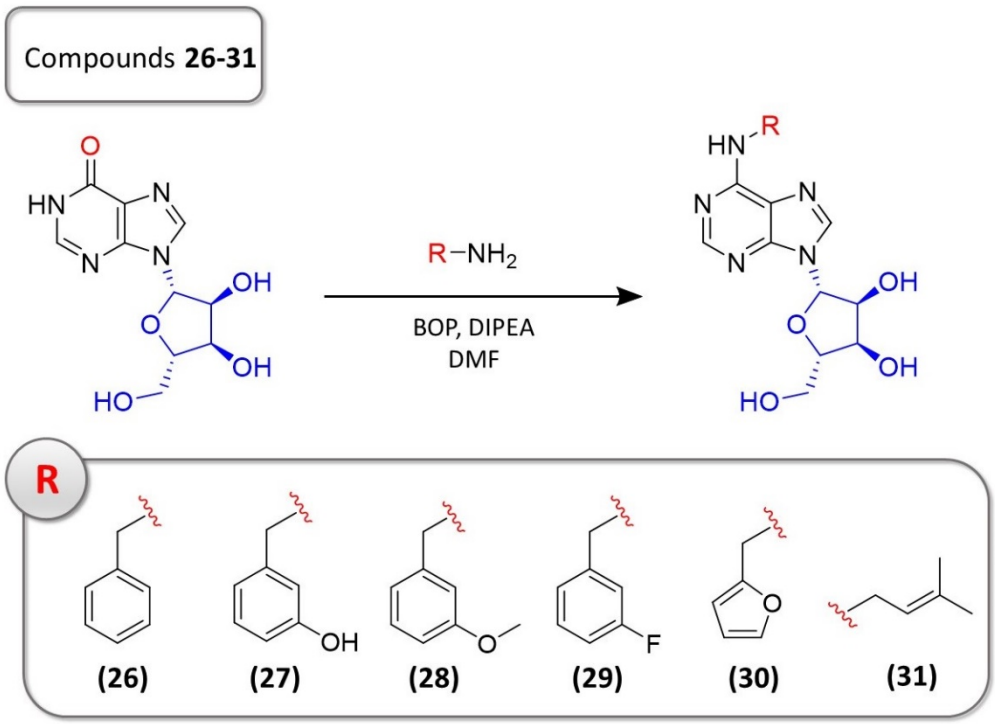


Scheme 2: *Synthesis of N^6-substituted purine-9-(2´-deoxy-2´-fluoro-β-D-arabinofuranoside) derivatives. The synthesis was performed in dry DMF with presence of BOP and DIPEA to form the required product (55-60°C, 24 hrs).*

Synthetic procedure III. – L-ribosides

N^6-substituted purine-9-β-L-ribofuranoside derivatives **(26-31) (scheme 3)**

The starting β-L-inosine and BOP (1.2 equiv.) were dissolved in dry DMF and DIPEA (2 equiv.) and appropriate amine (1.2 equiv.) followed. The reaction mixture was heated at 60°C in oil bath and stirred under argon atmosphere for 24 hours. The reaction mixture was evaporated on vacuum rotary evaporator to give specifically colored gel. The resulting residue was precipitated or purified by column chromatography. The reaction procedure is described and summarized in detail in the section of unpublished results.



***Scheme 3**: Reaction scheme for synthesis of N^6-substituted purine-9-β-L-ribofuranoside derivatives (BOP, DIPEA, DMF, 60°C, 24 hrs).*

Biological activity

The biological activity of prepared compounds was evaluated in three classical cytokinin bioassays previously reported by Holub et al. (Holub et al., 1998) and BAP was used as positive control for all three bioassays. The results were recorded as the highest activities of tested derivatives for the tested concentration range from 10^{-8} to 10^{-4} M solution in DMSO.

***Amaranthus* bioassay**

Amaranthus caudatus var. *atropurpurea* seeds were sterilized on their surface (10 min with 10% sodium hypochloride, washed with 0.5 L water, 10 min with 70% ethanol, washed with 0.5 L water), placed on a Petri dish containing paper tissue soaked with deionized water and cultivated at 24°C for 72 h in the dark. Under green safe light in a dark room, roots were removed from the seedlings and clean residues, consisting of two cotyledons and a hypocotyl, were placed on a Petri dish (25 explants per dish) containing filter paper soaked with 1 mL of incubation medium consisting of 10 mM Na_2HPO_4/KH_2PO_4 (pH 6.8), 5 mM tyrosine, and the test compound (from 10^{-8} to 10^{-4} M solution in DMSO). The dishes were cultivated at 24°C for 48 h in the dark, followed by extraction of the resulting betacyanin by repeated freezing and thawing (three times) of the plant material in 4 mL of 3.33 mM acetic acid.

The concentration of betacyanin was determined from the difference between absorbances at 537 and 625 nm.

Wheat leaf senescence bioassay

Wheat seeds (*Triticum aestivum* L. cv. Aranka) were washed with 96% ethanol, 1 h under running water, sown in verticulite soaked with Hoagland solution and then grown in a cultivation chamber (light/dark period = 16 h/8 h; 7000 lx) at 22 °C for 7 days. Tip cuttings of fully developed first leaves (3.5 cm long) were taken (four pieces were combined to give a total weight of 0.1 g (± 1 mg) per well), immersed by the basal part in a well containing test compound (150 µL/well), and cultivated in a closed plastic box containing moist paper tissue at 24°C for 96 h in the dark. Residual chlorophyll was extracted by heating the leaf material in 5 mL of 80% (v/v) ethanol at 80°C for 10 min. The absorbance at 665 nm was measured and the values were compared with values from extracts of fresh leaves (stored at -80 °C after detachment) and extracts of leaves cultivated in deionized water.

Tobacco callus bioassay

Cytokinin-dependent tobacco callus cells (*Nicotiana tabacum* L. cv. Wisconsin 38) were cultivated on solid MS medium (3 mL/well) containing different concentrations of the test compound (from 10^{-8} to 10^{-4} M solution in DMSO) in six-well plates (0.1 g of callus divided into 3 pieces per well) at 24 °C for 4 weeks in the dark. The biological activity of each test compound was determined as an increase in the callus fresh weight compared to a positive control (BAP).

Cell viability assay

The cytotoxicity measurement using the resazurin reduction assay on cancer cell lines, as well as human skin cells was proved as previously described (Jorda et al., 2019).

The bioassay was performed by doc. RNDr. Vladimír Kryštof, Ph.D. and his co-workers at Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany, The Czech Academy of Sciences, Olomouc, Czech Republic.

Photosynthetic activity of detached wheat and *Arabidopsis* leaves

The experiment used spring wheat and *Arabidopsis* plants grown under 16-hours-light/8-hours-dark cycle for 7 days on artificial medium containing perlite and Hoagland´s solution. The leaf segments were placed in 100 nM, 1 μM or 10 μM solution of BAP, 3-methoxyBAPA or 3-hydroxyBAPA dissolved in 0.1% DMSO, segments were kept in the dark at 24°C for 6 days and subsequently, the chlorophyll parameters were measured.

The bioassay was performed by doc. RNDr. Martina Špundová, Ph.D. and Mgr. Zuzana Kučerová at Department of Biophysics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Olomouc, Czech Republic.

RNA-seq analysis

The standard protocol for Illumina TruSeq Stranded mRNA Sample Preparation Kit (Illumina) was used for generation of two independent libraries. Prepared replicates were validated by DNA 1000 Chip (2100 Bionalayzer, Agilent Technology). Libraries were subsequently pooled to a final concentration of 12 pM for cluster regeneration a sequencing. For mapping and quantification, the *Arabidopsis* reference genome were used.

RNA-seq analysis was carried out by RNDr. Ondřej Plíhal, Ph.D. at Department of Molecular Biology, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science and Laboratory of Growth Regulators, Institute of Experimental Botany of the Czech Academy of Sciences, Faculty of Science, Palacký University, Olomouc, Czech Republic.

Plant phenotyping – rosette growth of seedling from *Arabidopsis* hormoprimed seeds

The prepared compounds were tested as priming agents under optimal and two different stress conditions including salt and osmotic stress. The *Arabidopsis* seeds were sterilized and germinated. During germination, the tested compounds were added to the medium at four different concentrations from 10^{-7} to 10^{-4} M. After three days germination, the seeds were tested by using various conditions (MS medium, with 100mM NaCl or 100mM mannitol) in 7-days light/dark cycles. By imaging of all plates, the traits among seeds were determined and PBC index was calculated.

The plant phenotyping was performed by Dr. Nuria de Diego and her co-workers at Department of Chemical Biology, Centre of the Region Haná for Biotechnological

and Agricultural Research, Faculty of Science, Palacký University, Olomouc, Czech Republic.

Determination of *Arabidopsis* rosette colour indices

The greenness of *Arabidopsis* seeds and leaf colour changes were evaluated by three vegetation indices. The pictures were captured after 7 days of the *Arabidopsis* rosette growth assay and vegetation indices were calculated. Subsequently, indices representing particular seedlings were calculated for each plant mask and the standard error were determined and represented in graph.

The bioassay was performed by Dr. Nuria de Diego and her co-workers at Department of Chemical Biology, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Olomouc, Czech Republic.

Plant hormone quantification and statistical analysis

Four independent biological replicates were quantified, and their concentrations were calculated by using standard isotope dilution method. The plant tissue samples were purified by micro solid-phase extraction. Cytokinins were quantified by using ultra-high performance liquid chromatography-electrospay tandem mass spectroscopy. Auxins and abscisic acid were performed, and their concentrations were calculated using standard isotope dilution method on Infinity II system coupled with Triple Quadrupole LC/MS system. The statistical data were evaluated using the packages *multcomp*, *FSA* and *agricolae* in RStudio.

The plant hormone quantification and statistical analysis were carried out by doc. Mgr. Ondřej Novák, Ph.D. and Mgr. Aleš Pěnčík, Ph.D. at Laboratory of Growth Regulators, Centre of the Region Haná, for Biotechnological and Agricultural Research, Faculty of Science, Palacký University & Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Olomouc, Czech Republic.

Survey of published results

Publication I.

Bryksová, M., Dabravolski, S., Kučerová, Z., Zavadil Kokáš, F., Špundová, M., Plíhalová, L., Takáč, T., Grúz, J., Hudeček, M., Hloušková, V., Koprna, R., Novák, O., Strnad, M., Plíhal, O., and Doležal, K. (2020). Aromatic cytokinin arabinosides promote PAMP-like responses and positively regulate leaf longevity. *ACS Chemical Biology*, *15*, 1949-1963.

CKs belong to the group of plant growth regulators with ability to control biotic and abiotic stress-related responses and senescence. A series of CK arabinoside derivatives was synthesized and compounds were characterized by physico-chemical methods and their CK-like activity was evaluated in three classical CK bioassays such as the *Amaranthus*, senescence and tobacco callus assay. The significant anti-senescence activity was observed for variety of them. Two derivatives, 3-hydroxy-BAPA and 3-methoxy-BAPA have been proved to be the most effective in delaying senescence while having low interactions with the CK pathway. Moreover, RNA-seq profiling study showed that by 3-methoxy-BAPA treatment on *Arabidopsis* leaves, the transcriptional response is markedly shifted toward defense. This treatment causes the upregulation of genes associated with plant immunity as well as cell wall remodeling and upregulation of specific mitogen-activated protein (MAP) kinases, especially MPK11, which is connected with stress-related signaling during pathogen-associated molecular patterns (PAMP) response. In addition, the increasing level of jasmonic acid and its metabolites, elevated level of plant defensins and the temporarily increased level of reactive oxygen forms have been observed after 3-methoxy-BAPA treatment. On the other hand, the plants treated by 3-hydroxy-BAPA together with flagellin-derived bacterial PAMP peptide showed the enhanced expression of pattern –triggered immunity (PTI) marker gene FRK1. These results show some BAPA derivatives as sensitive prime PTI responses in a low concentration range with no negative effect on the overall plant fitness.

Publication II.

Vylíčilová, H., Bryksová, M., Matušková, V., Doležal, K., Plíhalová, L., and Strnad, M. (2020). Naturally occurring and artificial N9-cytokinin conjugates: From synthesis to biological activity and back. *Biomolecules*, *10*, 1-29.

Sugar and non-sugar CK conjugates are very active factors in plants. Although they occur at very low concentrations, their ability to promote plant growth was repeatedly proved. The role of N9-substituted aminopurine- based CK conjugates in plants has been summarized and their biological activity in various bioassays was evaluated. Despite the fact, that N9-substituted CKs are endogenously occurring, the majority of them have been synthetically prepared to clarify the interaction between their chemical structure and biological properties. Due to current advances in synthesis of phytohormones, it is possible to prepare CK derivatives with targeted biological properties, such as the antisenescence effect, shoot formation or cell division stimulation. Additionally, many of them improve rooting or stimulate mass production higher than naturally occurring CKs. Hence, the knowledge about the CK role in plant species extends their field of use in plant biotechnology, agriculture and tissue cultures.

Publication III. (submitted manuscript)

Bryksová, M., Hybenová, A., Esteban Hernandiz, A., Novák, O., Pěnčík, A., Spíchal, L., De Diego, N., and Doležal, K. (2020) Hormopriming for mitigating abiotic stress affects: A case study of N9-substituted cytokinin derivatives with fluorinated carbohydrate moiety. Submitted manuscript.

The drought and salinity lands are factors depending on global climate change with the influence on already-stressed agricultural ecosystems. The stressed plants reduce seed germination, seedling establishment to affect plant metabolism, which reduce crop yield and therefore new biotechnological approaches to increase plant stress tolerance are studied. One of them, CK activated seed priming has an ability to reduce the negative factors of environmental stresses. BAP is the most widely applied CK in biotechnology for senescence delay and stress factors reducing, however its application is connected with some

negative effects, especially fast N9-glucosylation, which makes the molecule almost inactive and it can be suppressed by appropriate N9-substitution. Also, the replacement of hydroxyl groups of N9-substituted carbohydrate with a fluorine enhanced biological activity, but the effect of exogenous application of these fluorinated derivatives on plant fitness never been investigated. In this study, four derivatives have been characterized as significant hormopriming agents due to their antisenescence properties.

Publication IV. (book chapter)

Doležal, K., Bryksová, M.: Topolin metabolism and its implications for in vitro plant micropropagation. In *Meta-topolin: A plant growth regulator in vitro*. Book in preparation.

The BAP is highly active and affordable CK used in wide range of plant biotechnology applications, but its disadvantages in certain crops were observed. Hence, naturally occurring *m*T has been discovered, developed and thoroughly tested as BAP alternative.

Publication V. (patent and patent application)

Doležal, K., Plíhalová, L., Vylíčilová, H., Zatloukal, M., Plíhal, O., Voller, J., Strnad, M., Bryksová, M., Vostálová, J., Rajnochová Svobodová, A., Ulrichová, J., and Spíchal, L.: 6-aryl-9-glycosylpurines and use thereof. US patent US 10,100,077 B2, 2018.

A group of N9-substitutes 6-benzylaminopurine arabinoside derivatives published in Bryksová et al. (Bryksova et al., 2020) is part of the granted Czech patent CZ2014875A3, Singaporean patent SG11201704019TA, US patent US10100077B2 and PCT application WO2016091235A1 as compounds possessing antisenescence and UV protective properties in animal cells.

Conclusions and perspectives

A series of new CK nucleosides with various substitution at N^6-position of aminopurine was synthesized in combination with N9-substitution, including β-D-arabinofuranose, β-L-ribofuranose and 2′-deoxy-2′-fluoroarabinofuranose. For the synthesis, the high step facile one-step method using non-protected starting purine nucleosides was used.

The synthesis of CK conjugates with N9-substituted β-D-arabinofuranose starting with hypoxanthine arabinofuranoside, β-L-inosine was used as starting precursor for preparation of N9-substituted β-L-ribofuranoside analogues and hypoxanthine-2′-deoxy-2′-fluoroarabinofuranoside obtained corresponding N9-substituted 2′-deoxy-2′-fluoroarabinofuranoside derivatives. The reaction conditions have been slightly modified, depending on the type of the side chain or carbohydrate.

The prepared compounds were tested in three classical CK bioassays, such as *Amaranthus*, senescence and tobacco callus to clarify structure-activity relationship related to their biological activity.

In arabinosides family, the significant anti-senescence effect was evaluated in 3-methoxyBAPA and 3-hydroxyBAPA and therefore the mechanism of action of these two CK nucleosides was examined in detail – both of them have been characterized to promote plant immunity and positively regulate leaf longevity.

The group of β-L-ribofuranosides have been evaluated as almost non-active in CK bioassays, but interestingly also non-cytotoxic besides of their β-D counterparts.

The arabinofuranoside analogues with fluorinated carbohydrates have been shown as hormopriming agents due to their high anti-senescent effect. One of them, 6-(3-hydroxybenzylamino)-2′-deoxy-2′-fluoro-9-β-D-arabinofuranosylpurine has been described as a compound with ability to promote plant growth under controlled conditions and reduce negative effects, such as salt and osmotic stress.

Some of the newly prepared CK nucleoside analogs are potential candidates for using in biotechnology as well as agriculture. Many of them will be investigated for their potential antiviral activity.

List of publications

I. **Bryksová, M.***, Dabravolski, S.*, Kučerová, Z.*, Zavadil Kokáš, F., Špundová, M., Plíhalová, L., Takáč, T., Grúz, J., Hudeček, M., Hloušková, V., Koprna, R., Novák, O., Strnad, M., Plíhal, O., Doležal, K.: Aromatic cytokinin arabinosides promote PAMP-like responses and positively regulate leaf longevity. *ACS Chem Biol*, 2020, *15*, 1949-1963.

* These authors are contributed equally to this work.

II. Vylíčilová, H.*, **Bryksová, M.***, Matušková, V., Doležal, K., Plíhalová, L., Strnad, M.: Naturally occurring and artificial N9-cytokinin conjugates: From synthesis to biological activity and back. *Biomolecules*, 2020, 10, 1-29.

* These authors are contributed equally to this work.

III. **Bryksová, M.**, Hybenová, A., Esteban Hernandiz, A., Novák, O., Pěnčík, A., Spíchal, L., De Diego, N., Doležal, K.: Hormopriming for mitigating abiotic stress affects: A case study of N9-substituted cytokinin derivatives with fluorinated carbohydrate moiety. Submitted 2020

IV. Doležal, K., **Bryksová, M.**: Topolin metabolism and its implications for in vitro plant micropropagation. book chapter

V. 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. US patent US 10,100,077 B2, 2018.

Published abstracts

CBPRS, 3. - 5.3.2016, Malá Morávka, Czech Republic: Importance of N9-substituted cytokinins for biotechnological applications (oral presentation)

CBPRS, 21. – 23.5.2017, Kouty nad Desnou, Czech Republic: The influence of stereoisomerism N9-substituted BAP derivatives on bioactivity in various assays (oral resentation)

CBPRS, 24. – 26.5.2018, Luhačovice, Czech Republic: Synthesis and biological activity of new N9-substituted BAP derivatives and their UVA/UVB protectivity (oral presentation)

PBE, 18. – 21.6.2018, Copenhagen, Denmark: Cytokinin arabinosides and their biological properties in various bioassays (poster)

CBPRS, 19. – 21.5.2019, Luhačovice, Czech Republic: Anti-senescence activity of newly prepared N9-substituted BAP derivatives (oral presentation)

G4G, 10. – 13.6.2019, Olomouc, Czech Republic: Cytokinin arabinosides and their biological properties in various bioassays (poster)

Souhrn (in Czech)

Název disertační práce: Příprava a biologická aktivita nových cytokininových derivátů.

Předložená disertační práce se zaměřuje na přípravu nových cytokininových derivátů, modifikovaných na pozicích N^6 a N9 purinového kruhu, a dále pak na studium jejich biologických vlastností na rostlinných i živočišných buněčných liniích a mechanismu jejich působení. Cytokininy patří do skupiny rostlinných hormonů, jež jsou schopny ovlivňovat morfologii a jiné děje spojené s růstem a vývojem rostlin, jako například podporu buněčného dělení, regulaci apikální dominance, vývoj chloroplastů, antisenescenční efekt či přenos živin. Kromě jiného jsou také schopny regulovat odpověď rostlin na stresové faktory (Davies, 2010).

V rámci disertační práce byla syntetizována série cukerných cytokininových derivátů, zahrnující skupinu β-D-arabinofuranosidů, β-L-ribofuranosidů a 2′-deoxy-2′-fluoroarabinofuranosidových derivátů. V závislosti na typu postranního řetězce nebo použitého cukru byly reakční podmínky pro každou ze skupin mírně optimalizovány. Všechny nově připravené deriváty byly testovány ve třech klasických cytokininových testech (Amaranthový, kalusový a senescenční) za účelem stanovení jejich biologické aktivity.

Ve skupině β-D-arabinosidů byl pozorován významný anti-senescenční účinek zejména u 3-MeoBAPA a 3-OHBAPA, a proto byl u těchto dvou derivátů podrobně zkoumán mechanismus jejich účinku v rostlinných druzích. Oba byly vyhodnoceny jako sloučeniny podporující rostlinou imunitu, a také jako pozitivní regulátory dlouhověkosti listů.

Naproti tomu, skupina β-L-ribofuranosidů byla vyhodnocena jako téměř neaktivní ve všech cytokininových biostestech, avšak oproti jejich β-D-enantiomerům nevykazovala žádnou cytotoxicitu vůči nádorovým i normálním buněčným liniím.

Deriváty arabinofuranosidů s fluorovanou cukernou strukturou se ukázaly jako hormopromitující sloučeniny díky jejich vysokému antisenescenčnímu účinku. Jeden z nich, 6-(3-hydroxybenzylamino)-2'-deoxy-2'-fluoro-9-β-D-arabinofuranosylpurin, byl charakterizován jako sloučenina se schopností podporovat růst rostlin za kontrolovaných podmínek a snižovat negativní účinky spojené s rostlinným stresem, jako nadměrné zasolení a osmotický stres.

Některé z nově připravených cytokininových nukleosidových analogů jsou potenciálními kandidáty pro použití v biotechnologiích a polních experimentech. U mnoha z nich bude dále zkoumána potenciální antivirová aktivita.

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