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Doctoral Thesis

Synthesis and study of biological activity of novel purine nucleosides

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Supervisor

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Abstrakt	Vývoj nových cytokininových derivátů je důležitý pro zemědělskou soběstačnost populace. Proto byly převážně jednokrokovou syntézou připraveny dvě série cytokininových derivátů: 33 N^6-substituovaných 2′-deoxyribosidů odvozených od benzylaminopurinu (BAP) a série 2′-deoxyribosidů a 2′,3′-dideoxyribosidů odvozených od isoprenoidních cytokininů isopentenyladeninu, *trans*-zeatinu a *cis*-zeatinu. Všechny tyto deriváty byly v biologických testech porovnány s příslušnými bázemi a ribosidy. Navzdory nezřetelné schopnosti aktivovat cytokininové receptory AHK2, AHK3 a CRE1/AHK4 vykazovaly zejména některé aromatické 2′-deoxyribosidy významnou anti-senescenční aktivitu a také odlišné chování v kalusovém a amarantovém biotestu v porovnání s BAP. Naopak isoprenoidní 2′-deoxyribosidy byly úspěšné v amarantovém a kalusovém biotestu, ale 2′,3′-dideoxyribosidy nedosahovaly významných výsledků v provedených testech. Nicméně nebyla prokázána toxicita jak aromatických 2′-deoxyribosisů, tak isoprenoidních 2′-deoxyribosidů a 2′,3′-dideoxyribosidů na nádorových i nenádorových buňkách. Dále byly vyselektovány dvě látky ze série aromatických 2′-deoxyribosidů, u kterých byl prokázán pozitivní vliv na zemědelský výnos jarního ječmene a ozimé pšenice.
Klíčová slova	cytokininy, nukleosidy, 2′-deoxyribosidy, 2′,3′-dideoxyribosidy, senescence, kalusový test, cytotoxicita
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Abstract	The development of new cytokinin derivatives is important for the agricultural self-sufficiency of the population. Therefore, two series of cytokinin derivatives were prepared mainly by one-step reaction: 33 N^6-substituted 2′-deoxyribosides derived from benzylaminopurine (BAP) and a series of 2′-deoxyribosides and 2′,3′-dideoxyribosides derived from the isoprenoid cytokinins isopentenyladenine, *trans*-zeatin and *cis*-zeatin. All derivatives were compared with the respective bases and ribosides in various biological assays. Despite the indistinct ability to activate cytokinin AHK2, AHK3 and CRE1/AHK4 receptors, several aromatic 2′-deoxyribosides showed significant anti-senescence activity as well as different behaviour in the callus and *Amaranthus* bioassays compared to BAP. On the contrary, isoprenoid 2′-deoxyribosides were successful in *Amaranthus* and tobacco callus bioassays, but 2′,3′-dideoxyribosides did not achieve significant results. However, the non-cytotoxicity of both aromatic and isoprenoid 2′-deoxyribosides and 2′,3′-dideoxyribosides on normal and cancer cells was demonstrated. Two substances from the series of aromatic 2′-deoxyribosides have shown a positive effect on agricultural yield of spring barley and winter wheat.
Keywords	cytokinins, nucleosides, 2′-deoxyribosides, 2′,3′-dideoxyribosides, senescence, tobacco callus, cytotoxicity
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Declaration of Authorship

I declare that this doctoral thesis is the result of my own work except where otherwise stated.

In Olomouc, 29th May 2023

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Content

Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid synthase
AHK2	*Arabidopsis* histidine kinase 2
AHK3	*Arabidopsis* histidine kinase 3
AHPs	authentic histidine phosphotransferases
AIBN	azobisisobutyronitrile
APX	ascorbate peroxidase
ARRs	*Arabidopsis* response regulators
BAP	N^6-benzylaminopurine
BOP	benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate
BTEA-NO_2	benzyltriethylammonium nitrite
Bu_4NI	tetrabutylammonium iodide
CAT	catalase
CDK	cyclin-dependent kinase
CK	cytokinin
CKX	cytokinin oxidase/dehydrogenase
CPE	cytopathic effect
CRE1/AHK4	cytokinin receptor 1/*Arabidopsis* histidine kinase 4
CRF6	cytokinin inducible transcription factor (cytokinin response factor 6)
CRFs	cytokinin response factors
CWINV	cell-wall invertase
*c*ZOG	*cis*-zeatin-*O*-glucoside
*c*ZROG	*cis*-zeatin riboside-O-glucoside
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCE	1,2-dichloroethane
DCM	dichloromethane
DEAD	diethyl azodicarboxylate
DHZ	dihydrozeatin
DHZ7G	dihydrozeatin-7-glucoside
DHZOG	dihydrozeatin-*O*-glucoside
DHZROG	dihydrozeatin riboside-*O*-glucoside
DIAD	diisopropyl azodicarboxylate

DIPEA	*N*,*N*-diisopropylethylamine
DMAPP	dimethylallyl pyrophosphate
DMEM	Dulbecco's Modified Eagle Medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
ENT	eqilibrative nucleotide traporter
ER	endoplasmic reticulum
EV71	enterovirus 71
FAD	flavin adenine dinucleotide
FAO	Food and Agriculture Organization of the United Nation
GCV	ganciclovir
HMBDP	hydroxymethylbutenyl diphosphate
CHASE	cyclases/histidine kinases-associated sensing extracellular domain
IAA	indole-3-acetic acid
IB-MECA	N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (Piclidenoson)
IFAD	International Fund for Agricultural Development
iP	isopentenyladenine
iPRDP	isopentenyladenosine diphosphate
iPRTP	isopentenyladenosine triphosphate
IPT	isopentenyl transferase
KR	kinetin riboside (6-furfurylaminopurine riboside)
LOG	cytokinin riboside 5′-monophosphate phosphoribohydrolase
LOX	lipoxygenase pathway
MEP	methylerythritol phosphate pathway
MHV-68	murine gammaherpesvirus-68
*m*T	*meta*-topolin 6-(3-hydroxybenzylamino)purine
MVA	mevalonate pathway
ORAC	oxygen radical absorbance capacity
oT	*ortho*-topolin 6-(2-hydroxybenzylamino)purine
*p*T	*para*-topolin 6-(4-hydroxybenzylamino)purine
PUP	purine permeases
SAG12	senescence associated gene 12

SAM	shoot apical meristem
SOD	superoxide dismutase
TBAA	*tert*-butyl acetoacetate
TBAF	tetra-*n*-butylammonium fluoride
TBDMS	*tert*-butyldimethylsilyl
TEA	triethylamine
TEAC	Trolox equivalent antioxidant capacity
THF group	tetrahydrofuranyl group
THP group	tetrahydropyranyl group
TMSCl	trimethylsilylchloride
TMSOTf	trimethylsilyl trifluoromethanesulfonate
TPC	total phenolic content
Type-A RRs	type-A response regulator proteins
Type-B RRs	type-B response regulator proteins
*t*Z	*trans*-zeatin
*t*ZOG	*trans*-zeatin-*O*-glucoside
*t*ZR	*trans*-zeatin riboside
*t*ZRMP	*trans*-zeatin monophosphate
*t*ZROG	*trans*-zeatin riboside-*O*-glucoside
UNICEF	United Nations International Children´s Emergency Fund
WLS	wheat leaf senescence
WLSA	wheat leaf senescence assay
ZmHK1	*Zea mays* histidine kinase 1
ZmHK2	*Zea mays* histidine kinase 2
ZmHK3a	*Zea mays* histidine kinase 3a

1. Introduction

The early beginnings of agriculture in the so-called Neolitic Revolution around 12 000 years ago triggered the changes in man´s lifestyle from hunter-gatherers to permanent settlements. The reliable food supply from crop and animal farming has increased the population from a several thousand to today´s eight billion and projections of United Nations (www.un.org) suggest the increase up to ten billion till 2050.

The current agroecosystems are already meeting urbanized landscapes and it will be necessary to provide additional areas for growing crops, especially the territories of Africa and South America (FAO, IFAD, UNICEF, 2022; Wright, 2022). Therefore, the pressure is placed, among other issues like water supply, on obtaining the largest possible harvest of crops on the smallest possible area.

Thus, the necessity to discover the plant signaling and how to affect the processes of plant growth and development in every detail is obvious. In addition to the study and development of novel ecological fertilizers and herbicides that would not harm the landscape and organisms but still be effective enough, the study of cytokinins and other plant hormones, as well as their novel synthetic derivatives, is also important factor to achieve success in future agricultural projects.

Furthermore, the structural features of cytokinins that mimic the structure of nucleobases or nucleosides suggests, and many times already proved (Voller *et al.*, 2019), the use of these substances and their derivatives in medicine for the treatment of proliferative diseases and viral infections.

Although this work is like a needle in a haystack on a global scale, hopefully, it will contribute to the current knowledge of cytokinin derivatives.

2. Aims

This thesis is aiming on the synthesis of the series of variously N^6-susbtituted analogues of aromatic cytokinin benzylaminopurine as well as kinetin with attached 2′-deoxyribosyl moiety at the position N9 to compare the biological activity with the ribosides and the influence of the missing 2′-hydroxygroup.

As well as the aromatic cytokinin 2′-deoxyriboside derivatives, another aim is focused on the synthesis and the biological activity study of analogous 2′-deoxyriboside and 2′,3′-dideoxyriboside series of isoprenoid cytokinins isopentenyladenine, *trans*-zeatin and *cis*-zeatin.

1. To review the field of N^6-substituted cytokinin analogues, their synthesis and biological activity

2. The synthesis of a series of aromatic N^6-substituted cytokinin 9-(β)-D-2′-deoxyribosides

3. The synthesis of a series of isoprenoid cytokinin 9-(β)-D-2′-deoxyribosides

4. The synthesis of a series of isoprenoid cytokinin 9-(β)-D-2′,3′-dideoxyribosides

5. Evaluation of the biological activity of all prepared drivatives with available assays and bioassays

6. Drawing conclusions based on structure-activity-relationship between the studied compounds and relative ribosides and bases

3. Plant hormones

Plant hormones are naturally occurring organic compounds produced by plants, regulating plant growth and development at low concentrations (Davies, 2010).

In 1935, auxin, particularly IAA (indole-3-acetic acid), was isolated from fungus *Rhizopus* (Thimann and Koepfli, 1935), in 1942 from cornmeal (Haagen-Smith, Leech and Bergren, 1942) and accepted as the first plant hormone (Jiang and Asami, 2018). Auxins stimulate differential growth as a response to light or gravity (Zhao, 2010) and the biosynthesis of IAA starts from indole or tryptophan in leaf primordia, young leaves and developing seeds (Davies, 2010).

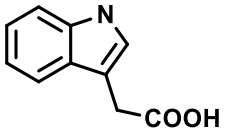


Fig. 1: The structure of IAA.

Gibberelins are tetracyclic diterpenoid substances essential for seed germination, stem elongation, trichome development, pollen maturation or flowering induction (Achard and Genschik, 2009; Davière and Achard, 2013), biosynthesized from glyceraldehyde-3-phosphate in the chloroplasts (Davies, 2010). Gibberellin was firstly identified in the pathogenic fungus *Giberrella fujikuroi*, causing excessive elongation of infected rice (Yabuta and Sumiki, 1938).

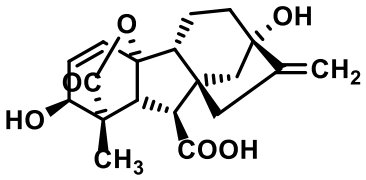


Fig. 2: The structure of gibberellic acid.

Abscisic acid is a single sesquiterpenoid hormone originally isolated from abscising fruits and maple dormant buds (Le Bris, 2003), inhibiting shoot growth and development.

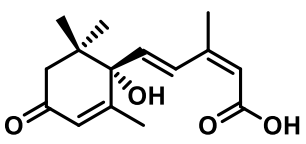


Fig. 3: The structure of abscisic acid.

Salicylic acid is a defense-related hormone involved in plant immunity, first reported by Raymond F. White who applied salicylic acid (or aspirin) on tobacco attacked by tobacco mosaic virus (White, 1979). Salicylic acid can be synthesized in plant by two pathways, the isochorismate and the phenylalanine ammonia-lyase pathway, both starting from chorismate obtained from shikimate pathway (Ding and Ding, 2020).

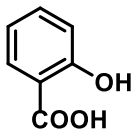


Fig. 4: The structure of salicylic acid.

Ethylene (ethene) is a gaseous hormone produced in all higher plants by enzymatic reaction from *S*-adenosyl-methionine catalyzed by 1-aminocyclopropane-1-carboxylic acid synthase (ACC). Ethylene influences maturation and senescence processes, flower bud desiccation and abscission, fruit ripening and affects root elongation and release of seed, bud dormancy, and opening of flowers (le Bris, 2003).

Jasmonic acid and its derivatives (jasmonates, especially methyl jasmonate) are compounds implicated in stress response regulating the gene expression involved in defense responses, and also development of land plants. The biosynthesis starts by oxygenation of α-linolenic acid (18:3) or 16:3 fatty acid of chloroplast membrane leading to 12-oxo-phytodienoic acid in lipoxygenase pathway (LOX), as an intermediate substance (Wasternack and Strnad, 2018). Jasmonic acid was firstly isolated from the culture of fungus *Lasiodiplodia theobromae* (Tsukada, Takahashi and Nabeta, 2019).

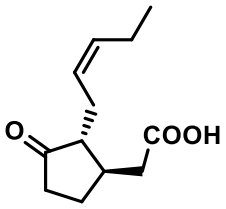


Fig. 5: The structure of jasmonic acid.

Brassinolide is the most active form of the growth-promoting plant steroids – brassinosteroids (Bishop and Koncz, 2002), plant hormones essential for the proper regulation of physiological processes needed for normal plant growth and development. Brassinosteroids are a class of polyhydroxylated sterol derivatives, structurally the most similar to animal steroid hormones. They regulate the expression of numerous genes, contribute to the cell division process and control morphogenesis (Clouse, 2011). Brassinosteroids were firstly isolated from

Brassica napus pollen with the effect on cell elongation and increasing yields when sprayed on young radishes or potatoes (Mandava, 1988).

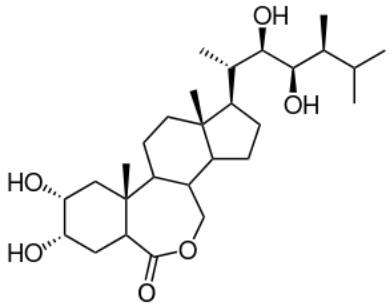


Fig. 6: The structure of brassinolide.

Strigolactones are carotenoid-derived signaling molecules that enable root-parasitic plants and symbiotic fungi to detect their host plants (Cook *et al.*, 1966) and regulate the process of adaptation of shoot and root growth to the environment (Wang, Wang and Li, 2017). The most common occurring strigolactones are tricyclic lactone structures composed of an ABC-ring and a D-ring butenolide group, connected by an enol-ether bridge.

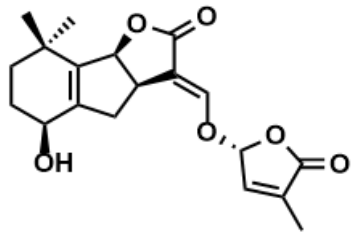


Fig. 7: The structure of strigol.

Another group of agents acting as cell-to-cell signal mediators, or membrane-receptor kinases and thus considered as hormones in plants are peptides (Matsubayashi and Sakagami, 2006). The first plant peptide of novel plant hormone group with signaling activity was systemin, isolated from tomato (*Lycopersicon esculentum*) as the peptide agent (protease inhibitor) of systemic wound response following mechanical damage on leaves or stems (Pearce *et al.*, 1991; Bergey, Howe and Ryan, 1996; Lindsey *et al.*, 2002). Together with systemins, other peptide groups belong to this plant hormone family, such as sulfokine, or phytosulfokine, a sulfated peptide composed of five amino acids, playing a role as the "conditioning factor" that induces cellular proliferation and differentiation at nanomolar concentrations and promotes callus formation of low-density cell cultures (*Asparagus officinalis L.*) (Matsubayashi and Sakagami, 1996, 2006).

Last but not least, very important group of plant hormones are cytokinins, plant growth promoting signal substances, that cooperates with other plant hormones to support and regulate the proper development of the plant and which name was derived from the word “cytokinesis”, division of cells (Mok and Mok, 1994). These derivatives are the fundamental topic of this work and a separate chapter will be devoted to them.

4. Cytokinins

The first cytokinin named kinetin was isolated from autoclaved herring sperm DNA as the product of the DNA degradation in 1955 by Miller (Miller, Folke Skoog, *et al.*, 1955), who followed up on the research and discoveries of cell division stimulating substance, occurring in phloem or coconut milk endosperm, conducted by Haberlandt (Haberlandt, 1913), Johannes van Overbeek (Van Overbeek, Conklin and Blakeslee, 1941) or Jablonski and Skoog (Jablonski and Skoog, 1954). In 1955, chemical characterization of kinetin, in cold water slightly soluble substance but with great solubility in aqueous HCl or NaOH, as 6-furfurylaminopurine (Miller, F. Skoog, *et al.*, 1955) was reported. In 1961, Miller and Letham (Letham and Bollard, 1961; Miller, 1961) simultaneously described cell division factor, extracted from immature milky corn kernels and observed also in apple fruitlets, which supported the growth of callus tissue from cotyledons of sterile plants in the same manner as kinetin (in the presence of 3-indoleacetic acid), and was named zeatin (after corn *Zea mays*). Zeatin is the most common cytokinin in plants (Davies, 2010) and, as cytokinins in general, is transported by xylem from roots to shoots.

Cytokinins play important roles in plant development including cell division, responses to biotic and abiotic stress or nutrient concentration, vascular and flower development, leaf expansion, seed germination, branching, nodulation, lateral rooting, regulation of auxin transport, shoot apical meristem development, inducing shoot formation in callus, or leaf senescence delay (Patton and Meinke, 1988; Mok and Mok, 1994, 2001; Gan and Amasino, 1996; Mok, Martin and Mok, 2000; Kieber, 2002; Sakakibara, 2006; Davies, 2010; Wybouw and De Rybel, 2019).

In chemical terms, naturally occurring cytokinins are N^6-substituted adenines, according to the N^6 substituents divided into two main groups: isoprenoid and aromatic (Mok and Mok, 2001). Generally, in plants more abundant isoprenoid cytokinins are N^6-(Δ^2-isopentenyl)-adenine (iP) and *trans*-zeatin (*t*Z); *cis*-zeatin (*c*Z) and dihydrozeatin (DHZ) are presented with lower abundance (Sakakibara, 2006). However, the cytokinin composition of the plant is dependent on the species and the stage of development, as well as on the type of the tissue or circadian rhythm. For instance, chickpea (Emery *et al.*, 1998), maize (Veach *et al.*, 2003) or rice (Izumi *et al.*, 1988) differ from e.g. *Arabidopsis thaliana* with prevalent presence of *c*Z and its conjugates.

Aromatic cytokinins are adenines bearing aromatic substituent attached to the N^6 position of the adenine scaffold and the first cytokinin discovered, kinetin, belongs to them (Miller, Folke Skoog, *et al.*, 1955). Synthetic N^6-benzylaminopurine and its derivative (N^6-benzylaminopurine

riboside), used in agriculture and micropropagation, were believed to be artificial cytokinins for a long time. After further investigation of the recorded high cytokinin activity of mature poplar leaves (*Populus* x *robusta* Schneid), the structure of the first isolated, naturally occurring aromatic cytokinin, N^6-(2-hydroxy)-benzylaminopurine riboside (6-(*o*-hydroxybenzylamino)-9-(*β*)-D-ribofuranosylpurine), was discovered (Horgan *et al.*, 1973; Horgan, 1975). Several years later, the presence of - on the benzyl ring non-hydroxylated base - N^6-benzylaminopurine (BAP) and its riboside, nucleotide (ribotide) and glucosides was reported in natural plant sources other than poplar, such as *Pimpinella anisum* L., or *Agrobacterium tumefaciens* causing crown gall tumours of tomato *Lycopersicon esculentum* L. (Ernst, Schäfer and Oesterhelt, 1983; Nandi, Letham, *et al.*, 1989; Nandi, Palni, *et al.*, 1989). Since then, BAP and its metabolites are considered as naturally occurring cytokinins.

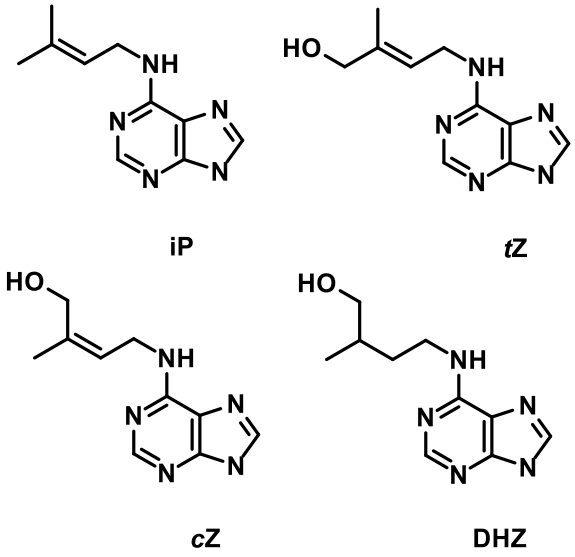


Fig. 8: The structures of isoprenoid cytokinin bases (iP, *t*Z, *c*Z, and DHZ).

Discovery of N^6-(2-hydroxy)-benzylaminopurine riboside in natural source and the evidence of preferential affinity of cytokinin-binding proteins to BAP and its analogues over zeatin led to detailed investigation of poplar leaves extract to obtain more information and naturally occurring analogues with using high-performance liquid chromatography combined with enzyme-linked immunosorbent assay. The examination of *Populus* x *canadensis* Moench., *cv. Robusta* leaves extracts led to identification of N^6-(2-hydroxy)-benzylaminopurine and its 9-*O*-glucoside (Strnad *et al.*, 1994), N^6-benzylaminopurine riboside and novel, highly active aromatic cytokinin, N^6-(3-hydroxy)-benzylaminopurine riboside (Strnad, 1997; Strnad *et al.*, 1997) which was named *meta*-topolin, and the existence of the aromatic cytokinin class, consisting of BAP, *o*T, *m*T, and *p*T (topolins) and their conjugates with ribose or glucose, was

conclusive. Furthermore in 2003, newly identified methoxyderivatives of topolins and their ribosides were examined for their cytokinin activity and great anti-senescence abilities (Tarkowská *et al.*, 2003).

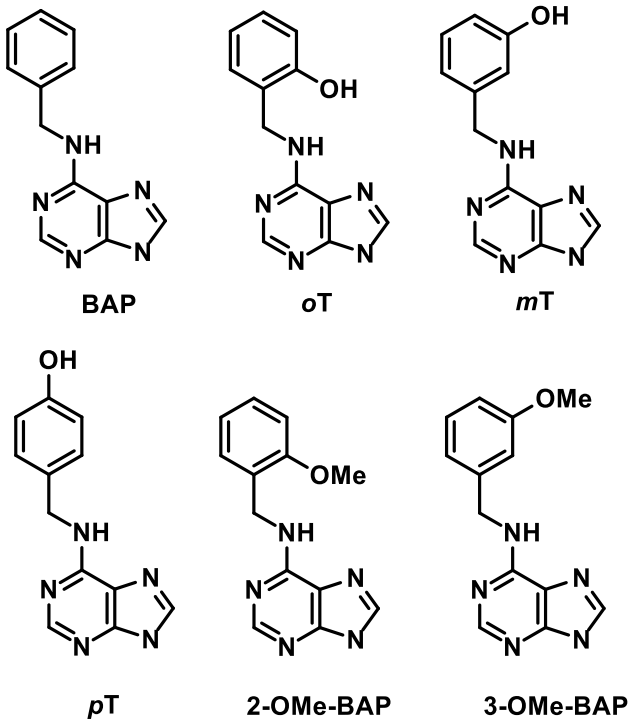


Fig. 9: The structures of aromatic cytokinins (benzylaminopurine, *ortho*-topolin, *meta*-topolin, *para*-topolin, 2-methoxy-BAP and 3-methoxy-BAP).

4.1. Cytokinin biosynthesis

4.1.1. Isoprenoid cytokinin biosynthesis

Due to the presence of isopentenyladenosine residue adjacent to 3′-end of the anticodon on some tRNA, obtained by prenylation of the adenine using tRNA isopentenyltransferase (IPT) (identified e.g. in *Escherichia coli* or *Homo sapiens*), the tRNA degradation pathway was assumed as the main source of isoprenoid cytokinin (Caillet and Droogmans, 1988; Golovko *et al.*, 2000; Kamada-Nobusada and Sakakibara, 2009). The prenylated tRNA contains a *cis*-hydroxyl group, therefore its degradation leads to *cis*-zeatin (Kamada-Nobusada and Sakakibara, 2009). In higher plants, such as *Arabidopsis thaliana*, seven genes (*AtIPT1*, *AtIPT3* - *AtIPT8)* encoding IPT enzyme and two genes encoding tRNA-IPT enzyme (*AtIPT2* and *AtIPT9*) were found (Takei, Sakakibara and Sugiyama, 2001). The IPT enzymes utilize ATD, ADP, or AMP as their preffered substrates to produce iP-type and *t*Z-type cytokinins and tRNA-type IPT enzymes transfer isopentenyl groups to the N^6 position of adenines in tRNAs to produce *c*Z-type cytokinins (Takei, Sakakibara and Sugiyama, 2001). Study of both tRNA-IPT

(*AtIPT2* and *AtIPT9*) defficient mutant plant proved *cis*-zeatin formation as a result of tRNA-IPT degradation in *Arabidopsis* where the cytokinin content remained unaffected for iP and *t*Z whereas *c*Z was barely detectable (Miyawaki, Matsumoto-Kitano and Kakimoto, 2004). On the other hand, *E. coli* cultures expressing *AtIPT1*, *3*, *4*, *5*, *6*, *7* or *8* secreted iP and *t*Z into the medium and exhibited DMAPP:AMP isopentenyltransferase activity. However, due to the rapid phosphorylation of radiolabeled AMP to ADP or ATP in *E. coli*, the real substrate affinities were needed to be examined on purified proteins. For example, *AtIPT1* and *AtIPT4* has been reported to use ADP and ATP more efficiently than AMP, suggesting the ATP/ADP content as a rate-limiting factor for cytokinin biosynthesis (Kakimoto, 2001, 2003; Takei, Sakakibara and Sugiyama, 2001).

Isopentenyltransferase (IPT) gene was firstly characterized in a neoplastic crown-gall forming bacterium *Agrobacterium tumefaciens* which is carrying two IPT genes (plasmid *Tmr and Tzs*) effectively cooperating after infection and integration into the host genome (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984; Hwang *et al.*, 2010). These enzymes utilize DMAPP (dimethylallyl diphosphate) and HMBDP (hydroxymethylbutenyl diphosphate) as a prenyl donor and present much higher affinity towards adenosine 5′- monophosphate (AMP) than those of ADP and ATP (Chu, Ko and Wang, 2010; Subramoni *et al.*, 2014). The *N*-prenylation of AMP (and ADP or ATP - preferentially utilized in higher plants (Lichtenthaler, 1999; Kakimoto, 2001; Sakakibara, 2006) at the N^6-terminus with DMAPP or HMBDP is catalyzed by IPT as the first step to the isoprenoid cytokinin formation (Sakakibara, 2006). The substrates for the isoprenoid cytokinin biosynthesis were firstly discovered in the slime mold *Dictyostelium discoidenum*, producing spore germination inhibitor discadenine [3-(3-amino-3-carboxypropyl)- N^6-(Δ^2-isopentenyl)adenine] (Abe, Hashimoto and Uchiyama, 1981).

DMAPP is synthesized via the methylerythritol phosphate (MEP) pathway and the mevalonate (MVA) pathway (commonly found in the eucaryotic cytosol) (Hecht *et al.*, 2001). On the other hand, HMBDP is commonly occuring in bacteria and plastids, hence, the HMBDP origin in higher plants is assumed to be in plastids (Lichtenthaler, 1999; Rohmer, 1999; Sakakibara, 2006). Isopentenyl nucleotide is the primary product of biosynthetic path using DMAPP whereas *t*Z nucleotide can be formed in the IPT reaction with HMBDP (Sakakibara, 2006).

Isoprenoid cytokinin biosynthetic pathway model in *Arabidopsis* utilizes iP and *t*Z side chains originating from the MEP pathway while the main part of *c*Z side chain originates from the MVA pathway (Kasahara *et al.*, 2004). The reaction of ADP or ATP catalyzed with plant adenosine phosphate-IPTs forms iPRTP and iPRDP and their subsequent dephosphorylation by

phosphatase, phosphorylation of iPR by adenosine kinase or phosphoribosyl residue conjugation to iP (or othe CK nucleobases) by adenine phosphoribosyltransferase maintain the metabolic pool of iPRMP and iPRDP (Kakimoto, 2001; Sakakibara, 2006). The iP-nucleotides are converted to the *t*Z-nucleotides by CYP735A and *cis-trans* isomerase posses the ability to interconvert *c*Z and *t*Z (Takei, Yamaya and Sakakibara, 2004; Sakakibara, 2006).

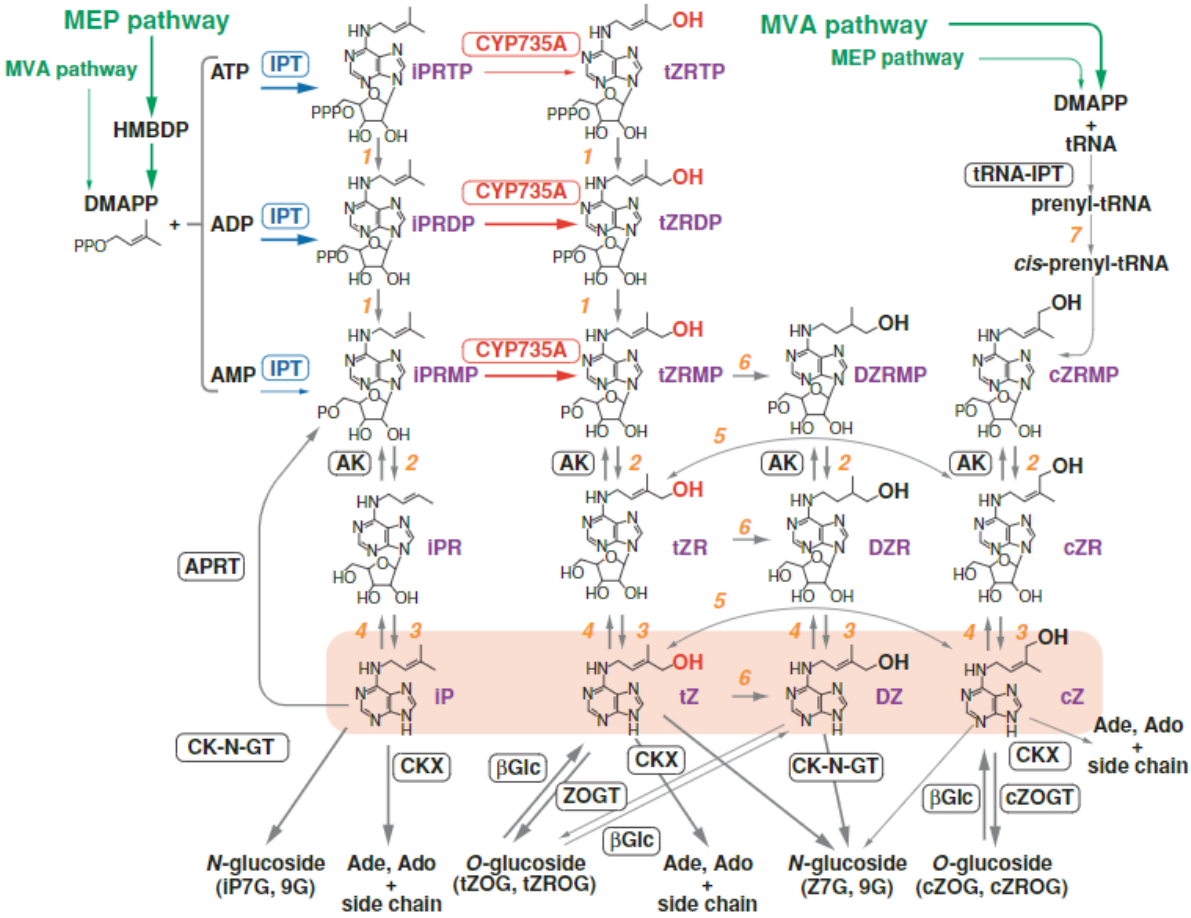


Fig. 10: The pathway of cytokinin biosynthesis (Takei, Yamaya and Sakakibara, 2004; Sakakibara, 2006). *Trans*-zeatin can be reversibly converted to *t*Z-*O*-glucoside by zeatin-*O*-glucosyltransferase (ZOGT) and *β*-glucosidase and cytokinin nucleobases can be converted to the *N*-glucoside by *N*-glucosyltransferase (Takei, Yamaya and Sakakibara, 2004; Sakakibara, 2006).

In higher plants, the iP-nucleotide-dependent pathway of *t*Z synthesis utilizes cytochrome P450 monooxygenase, namely CYP735A1 and CYP735A2 (identified in *Arabidopsis*) capable of *trans*-hydroxylation of iP nucleotides only (preferably iPRMP and iPRDP over iPRTP but not nucleoside or free base forms) (Takei, Yamaya and Sakakibara, 2004; Sakakibara, 2006). In the iP-independent pathway the *t*Z nucleotide is presumably produced by IPT with an unknown hydroxylated precursor, probably derived from the MVA pathway as the MVA

pathway inhibitor mevastatin causes *t*Z biosynthesis reduction (Astot *et al.*, 2000; Sakakibara, 2006).

The roots were assumed as the main site of cytokinin production, however it was revealed that the cytokinins are produced in various organs throughout the plant and the biosynthesis depends on the species as well as developmental and environmental conditions (Mok and Mok, 1994; Miyawaki, Matsumoto-Kitano and Kakimoto, 2004).

4.1.2. Aromatic cytokinin biosynthesis (?)

Although aromatic cytokinins and their metabolites have been found as naturally occurring in plants (Horgan, 1975; Horgan *et al.*, 1975; Strnad, 1997), their biosynthesis remains unclear (Sakakibara, 2006). Oslovsky et al. (Oslovsky *et al.*, 2020) prepared a series of isoprenoid and aromatic ribosides, 2′-deoxyribosides and 5′-deoxyribosides, derived from natural cytokinins iP, kinetin, benzylaminopurine and N^6-phenylethyladenine (Savelieva *et al.*, 2018), to compare the final steps of biosynthetic processes in two bioassays using *Arabidopsis thaliana* AHK2, AHK3 or AHK4 mutants and *Amaranthus caudatus* seedlings with the conclusion that, in *Arabidopsis*, the isoprenoid cytokinin derivatives and aromatic ribosides were metabolically activated to bases, while the aromatic 2′-deoxyriboside and 5′-deoxyriboside analogues were not. These results are suggesting differences in the biosynthesis of isoprenoid and aromatic cytokinins in plants as a possible result of the different substrate specificity of adenosine nucleosidase towards aromatic nucleosides, the lack of 5′-phosphorylation of 2′-deoxyriboside or the inability of phosphoribohydrolase (LOG) to cleave 2′-deoxyribo-5′-monophosphate in this model. However, only two bioassays were used and the results were not uniform for both of them, e.g., in comparison with the *Arabidopsis* bioassay, the iP and aromatic deoxyribo-derivatives were similarly low active in *Amaranthus* bioassay (Oslovsky *et al.*, 2020).

4.2. Cytokinin signaling

Cytokinin activity is regulated by diverse processes including biosynthesis, activation, degradation and conjugation together with the transmembrane transport and long-distance translocation. *Arabidopsis thaliana* possesses three cytokinin receptors (AHK2, AHK3, and CRE1/AHK4); membrane located sensor histidine kinases (Sakakibara, 2006). The relative cytokinin activities of four isoprenoid cytokinins were measured by their affinity to *Arabidopsis* cytokinin receptors AHK3 and CRE1/AHK4, showing that *t*Z and iP utilize more activity than *c*Z or DHZ, caused by the differences in chemical structure and polarity (Schmulling, Schafer and Romanov, 1997; Inoue *et al.*, 2001; Yamada *et al.*, 2001; Spíchal *et al.*, 2004; Romanov *et*

al., 2006). It was shown that AHK3 receptor is connected primarily with the development of shoots and leaves, as well as chloroplasts, leaf senescence and chlorophyll retention, on the other hand, CRE1/AHK4 receptor fulfills its duty primarily in root development and tissue culture (Inoue *et al.*, 2001; Riefler *et al.*, 2006; Romanov, Lomin and Schmülling, 2006). Also, the preference of *Arabidopsis* receptors towards cytokinin isoforms differs; CRE1/AHK4 and AHK2 have similar affinity to *t*Z and iP and low affinity to *c*Z and DHZ. AHK2 receptor, on the contrary, preferentially binds DHZ and less iP (Romanov, Lomin and Schmülling, 2006; Lomin *et al.*, 2011; Stolz *et al.*, 2011). Additionally, a cytokinin receptor from maize (ZmHK1) showed similar sensitivity to *t*Z and *c*Z, supporting the varying relative activities related to the different species (Yonekura-Sakakibara *et al.*, 2004).

Cytokinin signaling is conducted through two-component system, similar to that found in bacteria, involving membrane anchored sensor kinase His-Asp phosphorelay. Signal transduction proceeds via transfer of phosphate between His residue of the sensor kinase and an Asp residue of the receiver domain of the response regulator (Kieber and Schaller, 2014, 2018). The histidine-kinase cytokinin receptors, localized primarily in the endoplasmatic reticulum (ER) membrane, consist of cytokinin-binding extracytosolic CHASE (cyclases/histidine kinases-associated sensing extracellular) domain, oriented to the ER, transmembrane domains, cytosolic histidine kinase domain, canonical receiver domain and diverged receiver domain (Inoue *et al.*, 2001; Higuchi *et al.*, 2004; Kieber and Schaller, 2018). After binding of cytokinin to the CHASE domain, the cytosolic histidine-kinase domain is activated and phosphate group is transferred from autophosphorylated His resiude to Asp residue within the residue domain (Yamada *et al.*, 2001; Kieber and Schaller, 2018). The phosphate is then transferred to AHPs (authentic histidine phosphotransferases), possessing a conserved cysteine residue, and type-B response regulator proteins (RRs), with Asp residue, resulting in the transcriptional activity of cytokinin-responsive genes in the nucleus, such as genes encoding the cytokinin signaling repressors A-type ARRs or cytokinin response factors (CRFs) (Hwang and Sheen, 2001; Kieber and Schaller, 2018; Wybouw and De Rybel, 2019).

Type-B RRs contain a receiver domain and C-terminal with Myb-like DNA-binding domain and bind to their target by cytokinin-dependent manner activated by phosphorylation of the receiver domain (Zubo *et al.*, 2017). Type-A RRs contain a receiver domain lacking an output domain for transcriptional regulation; they are negative regulators transcriptionally proceeded in response to cytokinin after activation of type-B RRs. Their expression, regulated by other signaling pathways, could moderate cytokinin sensitivity of different cell type under different environmental conditions (To *et al.*, 2007). Cytokinins promote cell proliferation preferentially

in the shoots but, on the other hand, they inhibit root growth at the expense of promoted cell differentiation in the apical meristem and regulated lateral root branching (Chang, Ramireddy and Schmülling, 2013).

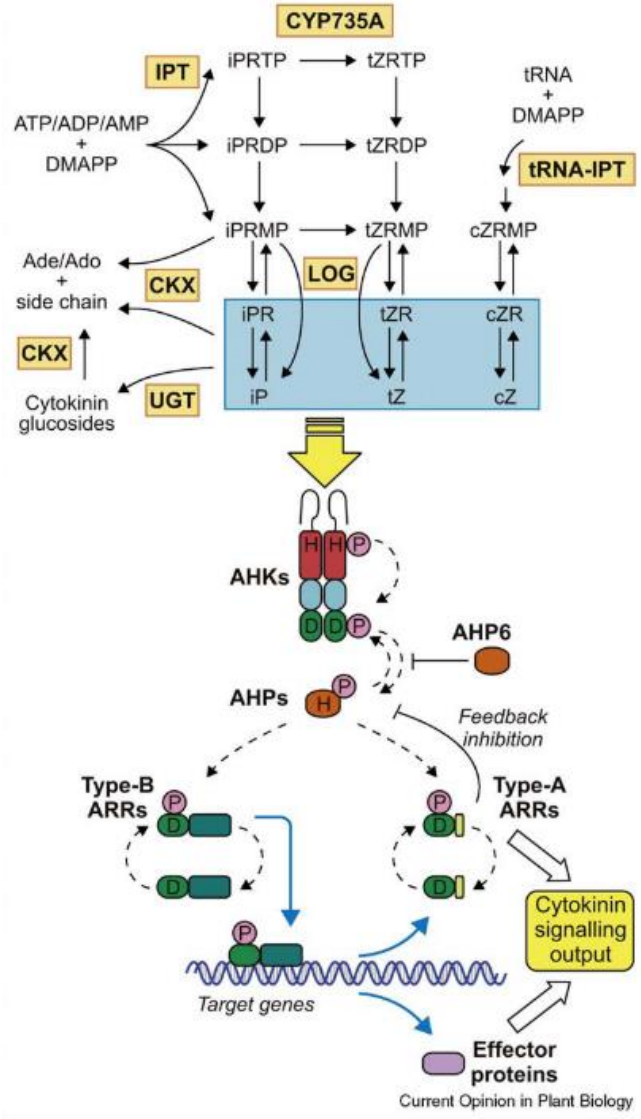


Fig. 11: Biosynthesis of isoprenoid cytokinins and cytokinin signaling scheme (Werner and Schmülling, 2009).

4.3. Cytokinin transport

The cytokinin transport across the biological membrane is enabled through simple diffusion, facilitated diffusion, primary and secondary active transport (Nedvěd *et al.*, 2021). The primary active transport is connected to the ATP hydrolysis which is catalysed by multi-domain membrane carriers of the ABC family (ATP-Binding Cassette) (Wilkens, 2015; Kang *et al.*, 2017). The most employed secondary active transport by ENT (eqilibrative nucleotide traporters) and PUP (purine permeases) families utilizes the energy from translocation of

another substrate – often connected with the proton gradient and/or pH gradient between two compartments (Girke *et al.*, 2014).

Passive simple diffusion is allowed to small, non-charged and hydrophobic molecules, in case of weak acids and bases (e.g. auxins) to non-dissociated compounds (Kramer, 2006). The free cytokinin bases have up to 100 times higher affinity to hydrophobic environment in comparison with the hydrophilic environment, according to the log(*P*) prediction using Fick´s law, which positively contributes to the simple diffusion consideration (El-Showk, Ruonala and Helariutta, 2013; Moore *et al.*, 2015; Nedvěd *et al.*, 2021). Interestingly, plant pathogen *Rhodococcus fascians* produces mono- and dimethylated iP derivatives able to easily diffuse through membrane and thus support the bacterial infection (Radhika *et al.*, 2015).

Facilitated diffusion is employing membrane-bound carriers, e.g. *AtENT7* for CK transport (Wormit *et al.*, 2004; Girke *et al.*, 2014) and can be described using Michaelis-Menten kinetics (Michaelis and Maud Menten, 1913; Nedvěd *et al.*, 2021).

The ENTs recognize adenine nucleosides and nitrogenous bases (cytokinins) as non-specific substrates and mediate secondary active transport coupled to the proton gradient dependent on pH (Girke *et al.*, 2014). The ENT genes were characterized in *Arabidopsis thaliana* (*AtENT1*, *3*, *6*, and *7*) and in rice (*Oryza sativa*) (Möhlmann *et al.*, 2001; Sun *et al.*, 2005; Hirose *et al.*, 2008; Girke *et al.*, 2014; Nedvěd *et al.*, 2021). The transporters' function in plant cytokinin homeostasis differs among the proteins, e.g. *AtENT1*, localized in plasma membrane and tonoplast, recognizes nucleosides but not the corresponding bases. Furthermore, the *atent1* gene mutation did not changed CK response or uptake in *Arabidopsis* hypocotyl explants. On the other hand, *atent3* mutation caused *t*ZR and iPR accumulation in these explants (Li and Wang, 2000; Möhlmann *et al.*, 2001; Sun *et al.*, 2005; Bernard *et al.*, 2011).

Most of the purine permeases (PUPs) also recognize cytokinins as non-specific substrates, nevertheless, unlike ENTs, PUPs recognize adenine derivatives as free bases (Girke *et al.*, 2014). In *Arabidopsis thaliana*, rice, and tobacco 23, 13, and 2 PUP genes have been found, respectively (Cedzich *et al.*, 2008; Girke *et al.*, 2014; Nedvěd *et al.*, 2021).

More importantly, the cytokinin specific transporters linked to distinct CK physiology have been recognized – AtPUP14 (plasma membrane localized transporter dependent on ATP causing *t*Z uptake increasement inhibited by iP, BAP and adenine but not *t*ZR) (Zürcher and Müller, 2016), AtABCG14 (responsible for long-distance CK transport from roots to xylem as the *Arabidopsis* cotyledon mutants manifested CK deficiency in shoots and an accumulation in roots) (Kang *et al.*, 2011; Ko *et al.*, 2014), AtAZG1 (*Arabidopis* root cells plasma membrane localized transporter; in yeast expression system with particular affinity towards adenine which

was strongly inhibited by kinetin, *t*Z, BAP, and also iP) (Tessi *et al.*, 2023), and AtAZG2 (localized to plasma membrane and endoplasmic reticulum in root primordia; the gene expression is stimulated by auxins and the transporter is responsible for *t*Z uptake, in yeast expression system the adenine uptake was strongly inhibited by iP, kinetin, BAP, and *t*Z) (Tessi *et al.*, 2021).

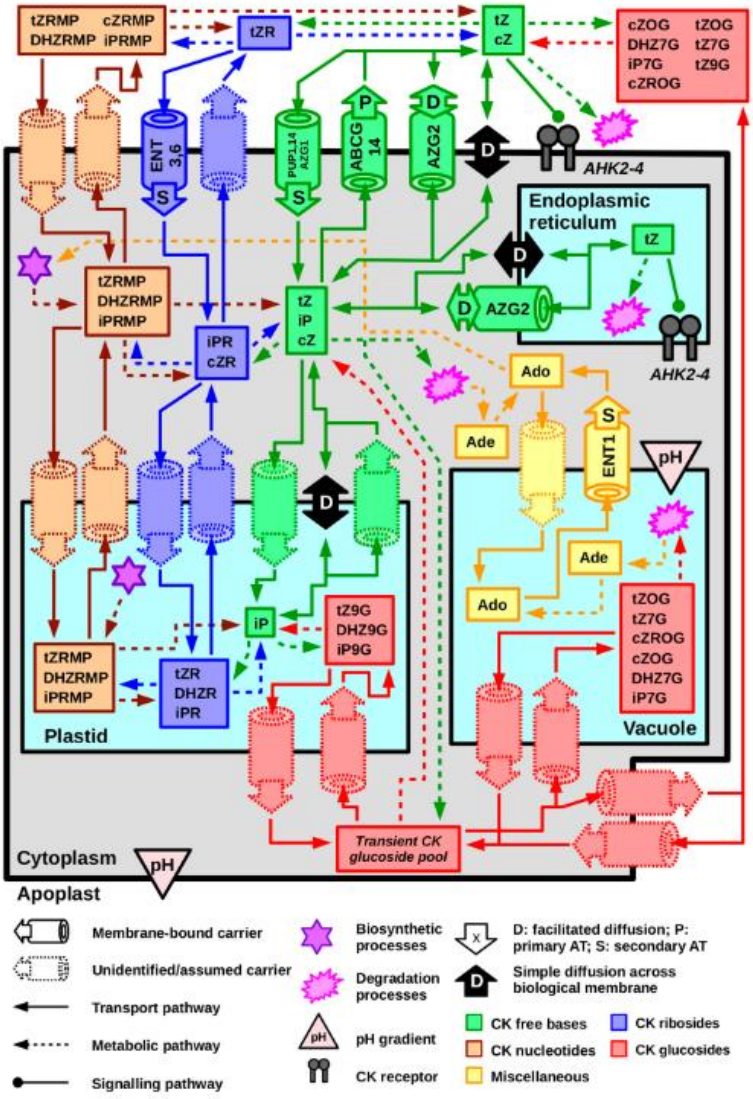


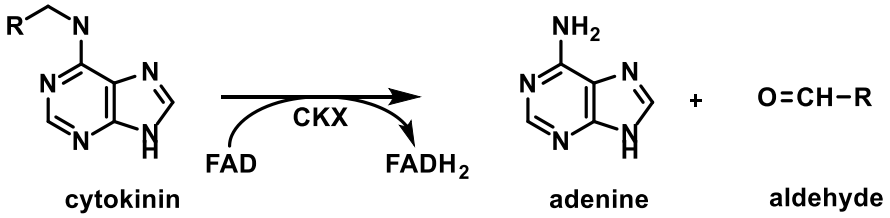
Fig. 12: The scheme illustrating cytokinin cellular homeostasis in *Arabidopsis* plant cell model divided into a plastid, a vacuole, endoplasmic reticulum, cytoplasm, and apoplast compartments. Cytokinin bases, ribosides, nucleotides and glucosides are grouped according to their pools in the cell. Membrane-bound transporters are depicted with the symbolic indication of the transport type (Nedvěd *et al.*, 2021).

Not only at the level of the plant cells but also at the level of the whole plants the paracrine hormonal signalization is connected to the membrane transport and metabolism of CK forms and their distribution (Kudoyarova *et al.*, 2014; Nedvěd *et al.*, 2021). For example, in *Arabidopsis* (a dicot) leaf study, the *t*Z was mostly found as extracellular with a small

cytoplasmic fraction, similarly to the *t*ZR and *t*Z-type glucosides, the *t*ZRMP was found in the cytoplasm (Jiskrová *et al.*, 2016; Nedvěd *et al.*, 2021). The iP and iPR were predominatly found in cytoplasm, while most of iPRMP in the extracellular space; iP-type glucosides were localized in the extracellular space and vacuoles. DHZ7G was detected in extracellular space and vacuoles (Jiskrová *et al.*, 2016; Nedvěd *et al.*, 2021).

4.4. Cytokinin degradation

The cytokinin irreversible degradation is proceeded *via* cytokinin oxidase/dehydrogenase (CKX) enzymes which cleave the cytokinin unsaturated N^6-side chain of iP, *t*Z, *c*Z and their ribosides. Correctly, the enzyme was classified as a dehydrogenase due to the unnecessity of the molecular oxygen for electron acceptation. The quinone type molecules function more efficiently than oxygen (Galuszka *et al.*, 2001) and also the CKX enzyme contains covalently bound flavin cofactor (FAD) as the electron acceptor resulting in an imine intermediate in the reaction that produces side-chain-derived aldehyde and adenine derivative as the degradation products of cytokinins (Frébort *et al.*, 2011).



Scheme 1: Cytokinin degradation (Frébortová *et al.*, 2010).

The CKX enzyme was firstly described in a crude tobacco culture (Pačes, Werstiuk and Hall, 1971; Whitty and Hall, 1974) and was isolated from corn kernels in 1999 (Houba-Hérin *et al.*, 1999; Morris *et al.*, 1999; Feng *et al.*, 2017). In *Arabidopsis thaliana*, seven *CKX* genes (*AtCKX1-7*) have been found (Morris *et al.*, 1999) and their expression occurs specifically in various plant cells and organelles affectting the enzyme function in different plant tissues and plant phenotypes (Werner *et al.*, 2003). The overexpression of *CKX*s results in cytokinin-deficient phenotypes depending on the specific *CKX* gene (Werner *et al.*, 2003). Furthermore, targeted *CKX* genes overexpression can increase inflorescence meristems and thus the seed sizes and crop yields and can also be used to alter the adaptability of plants to drought (Werner *et al.*, 2003; Ashikari *et al.*, 2005; Bartrina *et al.*, 2011; Köllmer *et al.*, 2014; Feng *et al.*, 2017).

The enzyme is not able to degrade cytokinins with saturated side chain (DHZ) and cytokinins with *O*-glycosylated or *O*-xylosylated side chains, or with side-chain containing an aromatic

ring (BAP, kinetin) (Galuszka *et al.*, 2007). However, CKX from *Arabidopsis thaliana* is able to cleave aromatic cytokinins but with lower reaction rates than unsaturated isoprenoid cytokinins (Galuszka *et al.*, 2007; Kowalska *et al.*, 2010).

5. Biological activity of cytokinins and cytokinin conjugates

Cytokinins fulfill an important role during plant development (Mok and Mok, 2001). They can be naturally found not only as the free bases but also mainly N9-ribosides, N9-ribotides, N3-glucosides, N9-glucosides, N7-glucosides, *O*-glucosides or N9-substituted with alanine (Sakakibara, 2006). However, the main role of cytokinins used in agriculture is to facilitate crop production with enhanced yields, increased longevity of plants and more resistant toward pathogens. Therefore, the preparation and the study of synthetically modified novel cytokinin analogues, summarized in (Vylíčilová *et al.*, 2020), is a crucial step to obtain the important knowledge.

The main active form of cytokinins are free bases, although they exist in plants in form of ribosides with attached ribosyl moiety at position N9 of purine scaffold, or ribotides. The level of active cytokinin concentration in plant is controlled through the sugar conjugation, commonly glucose (glycosylation), leading to the formation of inactive, either reversibly conjugated *O*-glucosides, occurring at the oxygen of the side chain of isoprenoid cytokinins, or irreversibly conjugated N3, N7- or N9-glucosyl derivatives. *O*-glycosylation is catalyzed by glucosyltransferases and reversed by *β*-glucosidases (Brzobohaty *et al.*, 1993), *N*-glycosides can be irreversibly decomposed by cytokinin oxidases/dehydrogenases (CKX) which cleave N^6-side chain of free bases and also ribosides (Werner *et al.*, 2006). Developmental defects of the plant can occur in case of CKX overexpression, due to deficient levels of cytokinins (Werner *et al.*, 2003). On the other hand, kinetin, dihydrozeatin and N^6-benzylaminopurin are resistant towards CKX enzyme (Galuszka *et al.*, 2007; Zalabák *et al.*, 2014).

5.1.1. Isoprenoid cytokinins and their N9-conjugates

The naturally occurring isoprenoid cytokinins, comprising of isopentenyladenine, *trans*-zeatin, *cis*-zeatin and dihydrozeatin, are more abundant in plants in comparison with aromatic cytokinins (Kieber, 2002). The bases, forming only a small part of total cytokinin pool in plants, are thought to be the most active forms (Spíchal *et al.*, 2004; Lomin *et al.*, 2015; Oshchepkov *et al.*, 2020) responsible for the biological outcomes.

The study of isoprenoid cytokinins started after the first identification in *Zea mays* (Letham, 1963), however, they were identified not only in plants but also in culture filtrates of bacterium *Pseudomonas savastanoi* pv. *savastanoi* (Psv) (Gardan *et al.*, 1992; Rodríguez-Moreno, Jiménez and Ramos, 2009) which affect e.g. olive trees (*Olea europaea* L.), jasmine and oleander (*Nerium oleander* L.) and cause knots and galls (Iacobellis *et al.*, 1994; Sisto, Cipriani

and Morea, 2004). The presence of *hrp*/*hrc* genes in plant-pathogenic bacteria also contributes to plant necrotic disease development (Sisto, Cipriani and Morea, 2004; Xie, Shao and Deng, 2019). The study of cytokinins accumulated in the culture medium filtrate extracts of pathogenic isolates of *Pseudomonas syringae* pv. *savastanoi* (Surico *et al.*, 1985; Evidente *et al.*, 1991) revealed the presence of *t*Z and *t*ZR as well as other naturally occuring isoprenoid cytokinins further characterized as 1′-methylzeatin (1′Me*t*Z) with its 9-(*β*)-D-riboside (1′′Me*t*ZR) and 2′-deoxyzeatin riboside (2′deO*t*ZR; ZdR). The 1′Me*t*Z derivatives showed higher chlorophyll synthesis stimulating activity in etiolated cucumber cotyledons than 2′-deoxyzeatin riboside (Evidente *et al.*, 1991). The reduction of *trans* double bond in 1′Me*t*Z and its riboside, as well as deoxygenation at C4 or acylation of C4 hydroxy group of the isoprenoid side chain or acetylation of the hydroxyl groups of the ribose moiety did cause reduction or complete loss of activity (Evidente *et al.*, 1991). Due to the presence of asymmetric centre adjacent to N^6 of 1′Me*t*Z and 1′′Me*t*ZR the absolute stereochemistry was studied on the basis of the chiral syntheses starting from D- and L-alanine (Itaya *et al.*, 1986) resulting in the confirmation of *R* stereochemistry of the chiral centre of (*1′R*)-1′Me*t*Z and its riboside. It was also revealed that the natural 1′*R* form of 1′Me*t*Z showed similar activity in the tobacco callus and the lettuce seed germination bioassays as the unsubstituted *t*Z while the unnatural enantiomer (*1′S*) showed lower activity (Fujii *et al.*, 1989; Fujii, Itaya and Matsubara, 1989). The cytokinin activity of the *trans*-isomers of (*1′R*)-1′-methylated zeatin derivatives ((*1′R*)-1′Me*t*Z and (*1′R*)-1′Me*t*ZR) was also compared with (*1′R*)-1′-methylated *cis*-zeatin isomers (base and riboside) that were synthesized also for analytical purposes (Fujii *et al.*, 1990). The 1′-methylated *cis*-zeatin isomers ((*1′R*)-1′Me*c*Z and (*1′R*)-1′Me*c*ZR) were less active in tobacco callus and lettuce seed germination assays than 1′-unsubstituted *cis*-zeatin and (*1′R*)-1′-methylated-*trans*-zeatin derivatives ((*1′R*)-1′Me*t*Z and (*1′R*)-1′Me*t*ZR) (Fujii *et al.*, 1990) which correlated with the lower cytokinin activity in tobacco callus assay of the *cis*-zeatin and its riboside compared to the *trans*-zeatin and its riboside (Matsubara, 1980). Furthermore, the introduction of 1′-methyl group to the *trans*-zeatin did not alter the cytokinin activity compared to 1′-unsusbtituted *trans*-zeatin while 1′-methylation decreased the *cis*-zeatin cytokinin activity (Fujii *et al.*, 1989; Fujii, Itaya and Matsubara, 1989). As well as the *1′S* isomer of 1′-methyl-*trans*-zeatin was examined (Fujii *et al.*, 1989; Fujii, Itaya and Matsubara, 1989), the *1′S* isomer of 1′-methyl-*cis*-zeatin and its 9-(*β*)-D-riboside were synthesized (Fujii *et al.*, 1993) and tested in tobacco callus assay together with (*1′S*)-, (*1′R*)-, and racemic mixture of 2-hydroxy-1′-methyl-*trans*-zeatin (isolated from extracts of marine green alga (Fujii *et al.*, 1993)) with the result showing higher cytokinin activity of (*1′S*)-1′-methyl-*cis*-zeatin in comparison with its

riboside, which was completely inactive, however, the positive effect of the presence of methyl group at *1´R* configuration and *trans* isomerism of the side chain double bond of (*1´R*)-2-hydroxy-1´-methyl-*trans*-zeatin was rewarded with the great activity at 1 μM (Fujii *et al.*, 1993). On the other hand, the *1´S* configured 2-hydroxy-1´-methyl-*trans*-zeatin was inactive (Fujii *et al.*, 1993). Another isoprenoid side chain modifications (introduction of N^6-(3-methyl-2-butenyl)- or N^6-(1,3-dimethyl-2-butenyl)-side chain) performed by Fujii et al. (Fujii *et al.*, 1994) lead to tobacco callus assay results confirming higher activity of *trans*-zeatin derivatives compared to *cis*-zeatin derivatives and the advantageous features were also the presence of 1´-methyl group at *1´R* configuration. However, the highest cytokinin activity belonged to *trans*-zeatin (Fujii *et al.*, 1994).

Furthermore, the introduction of hydroxyl group to the isopentyl or isopentenyl N^6-side chain can alter the cytokinin activity – the hydroxylation at the position 4- increases while the hydroxylation at the positions 2- and 3- reduces the cytokinin activity (Leonard *et al.*, 1969).

Generally, various assays including e.g., tobacco callus or cucumber cotyledon expansion assay confirmed the less activity of isoprenoid CK ribosides then that of the free bases and among the free bases the favourable effects of *t*Z over *c*Z assay (Skoog *et al.*, 1967; Schmitz *et al.*, 1972; Laloue, Terrine and Guern, 1977; Kamínek *et al.*, 1979; Mok and Mok, 2001; Spíchal *et al.*, 2004). Furthermore, among cytokinin ribosides similarly the greater cytokinin activity in tobacco callus or oat leaf senescence assay belongs to the *t*ZR over *c*ZR (Gajdošová *et al.*, 2011). The activity of isoprenoid CK bases and ribosides varies also in the cytokinin receptor perception when *A. thaliana* AHK3 and CRE1/AHK4 receptors are more sensitive to *t*Z and iP over their riboside, however, AHK3 receptor is more sensitive towards CK ribosides than CRE1/AHK4 *in vitro* (Spíchal *et al.*, 2004). Additionally, also *Zea mays* L. receptor ZmHK2 presented increased sensitivity towards ribosides than ZmHK1 and ZmHK3a (Yonekura-Sakakibara *et al.*, 2004).

Cytokinin bases' and ribosides' side chain conjugation with glucosyl residue via hydroxyl-group forms endogenous derivatives (Turner, Mok and Mok, 1985) in vascular (Duke *et al.*, 1979; Scott *et al.*, 1982; Singh, Letham and Palni, 1992; Aremu *et al.*, 2015) and also non-vascular (von Schwartzenberg *et al.*, 2007) plants (*t*ZOG, *c*ZOG and DHZOG, *t*ZROG, *c*ZROG and DHZROG) serving as an important storage and transport forms, predominantly accumulated in the roots (Fusseder and Ziegler, 1988; von Schwartzenberg *et al.*, 2007), which are resistant to CKX degradation and possess the adventitious ability of facile conversion to the active forms via *β*-glucosidases (Kiran *et al.*, 2012). The performed high cytokinin activity of *t*ZROG in *A. thaliana* reporter gene test together with the inability of this glucoside to trigger

the CRE1/AHK4 or AHK3 receptor response have suggested the rapid conversion to the cytokinin base (Spíchal *et al.*, 2004).

5.1.2. Aromatic cytokinins and their N9-conjugates

The free base, BAP, is the most affordable and efficient derivative used worldwide in micropropagation systems and agriculture (Werbrouck *et al.*, 1996). The riboside of BAP, firstly found in coconut water (Sáenz *et al.*, 2003; Ge *et al.*, 2005), emphasizely possess high cytokinin activity in *Amaranthus* bioassay and tobacco callus bioassay, which is comparable to that of *trans*-zeatin riboside, with exception of senescence assay where the BAPR derivative has weaker activity than *t*ZR (Holub *et al.*, 1998). Obviously, the corresponding free bases were more potent in these assays. Unfortunately, the use of BAP in plant production can in some crops cause problematic growth and inhibition of root formation (Werbrouck *et al.*, 1995) which can be associated with the accumulation of inactive but highly stable BAP-9-glucoside (BAP9G) metabolite in explants, mainly roots (Bairu *et al.*, 2010, 2011). Furthermore, higher dosage of BAP induces senescence processes, inhibits plant cyclin-dependent kinases and the toxic effect results in programmed cell death (Carimi *et al.*, 2004; Doležal *et al.*, 2006). The disadvantage of BAP treatment has been the trigger of searching for new possibilities how to overcome the plant growth heterogenity. Therefore, e.g., riboside or tetrahydropyranoside, where the N9 position is already occupied, led to the lower BAP9G formation (Werbrouck *et al.*, 1995; Podlešáková *et al.*, 2012). Another option how to increase the desired effect is the treatment with on the benzyl ring substituted analogues of BAP or BAPR (Werbrouck *et al.*, 1996; Doležal *et al.*, 2006; Bairu *et al.*, 2011; Aremu *et al.*, 2016). The introduction of hydroxyl group to the *ortho*-, *meta*- or *para*- position of the aromatic ring forms topolins (*o*-, *m*- or *p*-) in which the cytokinin activity varies, depending on the hydroxyl position. Plants treated with *m*T produce significantly richer and longer roots than plants treated with BAP (Werbrouck *et al.*, 1996). The *meta*-substitution enhances the cytokinin base activity, whereas the *para*-position in monosubstituted BAP analogues leads to the activity reduction (Doležal *et al.*, 2006) and the general CK activity pattern is *meta* > *ortho* > *para*. In case of ribosides of variously substituted BAP derivatives the cytokinin activity was similarly influenced by the substituent (function group) position on the benzyl ring and, emphasizely, the *meta*-position substitution generally enhanced the bioassays outcome too (Doležal *et al.*, 2007). The hydroxyl group on the benzyl ring in *m*T or *m*TR (or *o*T and *o*TR) is allowing the *O*-glucosylation leading to the glucoside formation (which is not possible for BAP derivatives) that can be easily cleaved by *β*-glucosidases to the active forms (Werbrouck *et al.*, 1996).

In 1975, the discovery of 6-(2-hydroxybenzylamino)-9-(*β*)-D-ribofuranosylpurine (*ortho*-topolin riboside, *o*TR) in poplar leaves (Horgan, 1975) and kinetin in coconut (Ge *et al.*, 2005) have ended the era of assertion that aromatic cytokinins are only synthetic artificial compounds. Since then, the natural occurrence studies revealed more evidence of the presence of aromatic CKs in plant materials (Chaves Das Neves and Pais, 1980; Sáenz *et al.*, 2003; Ge *et al.*, 2005). The glucosides *m*TROG and *o*TROG were isolated from microalgae (Ördög *et al.*, 2004) and *m*TROG and *p*TROG from cultured aloe plants treated with BAP (Ivanova *et al.*, 2006). The presence of the methoxy group in ribosides Me*m*TR and Me*o*TR, found in *A. thaliana* and *Populus x canadensis* Moench cv. Robusta, enhanced the cytokinin activity of these compounds above that of BAP and *t*Z (Tarkowská *et al.*, 2003) and thus the replacement of BAP and *t*Z, classically used in micropropagation, has begun with the gain of convenient results and greater potencial in agriculture future (Bogaert *et al.*, 2006; Bairu *et al.*, 2007; Amoo, Finnie and Van Staden, 2011; Aremu *et al.*, 2012).

Therefore, the numbers of publications studying the biological activities of extensive panels of variously synthetically modified novel analogues of aromatic cytokinin BAP have emerged (Doležal *et al.*, 2007; Vylíčilová *et al.*, 2016). The general requirement for enhanced purine aromatic cytokinin activity is the presence of appositely substituted benzylamine in the position N^6: the position of substituent on the benzyl ring and its nature. The *meta*- position of the substituent and its increased electronegativity enhance the hydrogen bond formation with electron donors of the cytokinin receptor, thus the presence of fluorine significantly increases the CK activity (Doležal *et al.*, 2007; Vylíčilová *et al.*, 2016). Furthermore, the combination of substituent positions of on the benzyl ring disubstituted analogues (Doležal *et al.*, 2007; Vylíčilová *et al.*, 2016) has also a great impact on the resulting activities, e.g., the difference between 6-(2,4-dichlorobenzylamino)purine-9-riboside and 6-(3,4-dichlorobenzylamino)purine-9-riboside (Doležal *et al.*, 2007).

The metabolic interconversion of exogenously applied CKs to their ribosides and ribotides, demonstrated by radiolabelled kinetin (Miernyk and Blaydes, 1977; Pietraface and Blaydes, 1981), supports the study of N9 substituent influence on the final activity. The study of several compounds containing in the position N9 L-ribosyl enantiomer reported significantly weaker biological outcome in comparison with D-ribosides (Bryksová *et al.*, 2020). The biological activity of cytokinin ribosides is with the great certainty affected by their conversion to the free bases (Mok and Mok, 2001; Vylíčilová *et al.*, 2016), however, in the biological cytokinin assays greatly successful BAP riboside analogues did not significantly trigger the CRE1/AHK4 or AHK3 recptors (Doležal *et al.*, 2007). Therefore, there may be a different sensing mechanism

or extracellular perception system aromatic cytokinins are using in plants (Doležal *et al.*, 2007; Durán-Medina, Díaz-Ramírez and Marsch-Martínez, 2017).

Among studying N9-sugar conjugates the *β*-D-arabinosides should be also mentioned (Reist *et al.*, 1962; Dutta *et al.*, 1975; Hansske, Madej and Robins, 1984; Secrist, Shortnacy and Montgomery, 1988; Bryksová *et al.*, 2020). The ability to activate the cytokinin receptors was not observed (Bryksová *et al.*, 2020), however, some derivatives showed higher efficacy to delay senescence in WLS assay than BAP which suggests different physiological effect of arabinosides in contrast to cytokinins sensed by their receptors.

Furthermore, study of activity of cytokinin purine analogues with alkyl-substituted position N9 proved the importance of the structure, polarity and the size of such N9 substituent to maintain the biological effect in cytokinin assays (Fox *et al.*, 1971; Corse *et al.*, 1989; Zhang and Letham, 1989; Mik, Szüčová, Šmehilová, *et al.*, 2011; Mik, Szüčová, Spíchal, *et al.*, 2011). Therefore, the presence of N9-residues such as 9-(4-chlorobutyl) (Zhang and Letham, 1989) or 9-(2-chloroethyl) (Robins, Hall and Thedford, 1967) could enhance the obtained activity of aromatic cytokinin analogues.

Additionally, several cytokinin analogues N9-susbtituted with tetrahydropyran or tetrahydrofuran cyclic ether residue (Pietraface and Blaydes, 1981; Zhang and Letham, 1989; Szüčová *et al.*, 2009; Zahajská *et al.*, 2017; Hönig *et al.*, 2018) showed enhanced activity in cytokinin assays, compared to BAP. The THP or THF groups are labile to the acidic conditions, hence, the cleavage to the active form is highly convenient but still with the great advantage of preventing N9-glucoside formation associated with unwanted root inhibition (Podlešáková *et al.*, 2012; Plíhal, Szüčová and Galuszka, 2013).

6. Short history of plant cytokinin assays and bioassays

The most widely used methods for evaluation of cytokinin activity were based either on the cytokinin promoting physiological cell division and expansion or delaying the leaf senescence. One of them, the 3-weeks long soybean callus assay (Miller, 1963), showed cytokinin specificity and accurate linearity over the $\log_{10}$ concentration range 0.02 – 50 μM (of kinetin) at that time. This bioassay was then improved to remove certain limitations (variations between replicates at the same concentration, callus growth failure, and the callus growth independent of cytokinins) and therefore the soybean hypocotyl bioassay was reported (Manos and Goldthwaite, 1976) with the response over the concentrations 0.0005 – 10 μM (of zeatin).

Comparable results in study of the cytokinin activity of kinetin analogues were obtained from the soybean and tobacco tissue assays as well as the radish leaf disk assay (Kuraishi and Muir, 1963) using the *Raphanus sativus* L. var. acanthiformis Makino (Riso-daikon) seeds planted in soil to first foliage leafs of certain areas from which leaf disks were then cut and used for the assay with cytokinin solution. The bean leaf disk test was also reported using the similar principle with *Phaseolus vulgaris* seedlings, however, the resulting response – the increased cell enlargement – was in the both above mentioned methods affected by microorganisms, nevertheless, provided a shorter evaluation time than the callus bioassays (Miller, 1963).

In the 1950´s, also germination tests were used to study the cell enlargement of the embryo promoted by cytokinin action (Skinner and Shive, 1957; Skinner *et al.*, 1957; Skinner, Talbert and Shive, 1958; Miller, 1963). These tests using the lettuce seeds (*Lactuca sativa*) were useful for screening synthetic compounds, however, the need of high concentrations of the compounds and the germination affecting treatments (red light, gibberelins, thiourea) or non-sterile environment contributed to the assays' disadvantage.

The discovery of kinetin's ability to retard breakdown of protein in detached yellowing *Xanthium pennsylvanicum* (Richmond and Lang, 1957) leaves led to the developing of the method studying the amount of extracted chlorophyll from leaf discs incubated on filter paper with the cytokinin substances in the darkness for 48 h (linear kinetin concentration range 0.1 – 10 mg/L) (Osborne and McCalla, 1961; Miller, 1963).

In 1957, the cytokinin bioassays were described where the *Tortella caespilosa* moss (from spores grown protonema) was used to study the increased budding caused by 6-substituted purines (Gorton, Skinner and Eakin, 1957) or the tobacco stem slabs were planted on the medium and the bud formation was evaluated (Skoog and Miller, 1957). Furthermore, the inhibitory effect of kinetin at concentration of 0.86 mg/L on the callus growth was reported,

nevertheless, it was overcome by inclusion of 54 mg/L adenine (Skoog and Miller, 1957; Miller, 1963).

In 1987, the aromatic cytokinin ribosides' activities were tested in three bioassays (Kamínek, Vaněk and Motyka, 1987). In the tobacco callus bioassay, which was modified from the tobacco wound tissue system (Miller, 1963), cytokinin-dependent callus tissues derived from *Nicotiana tabacum* L. cv. Wisconsin 38 were cultivated on RM-1965 medium (Murashige and Skoog, 1962; Linsmaier and Skoog, 1965) supplemented with 2 mg/L IAA and cytokinin activity was expressed as average fresh weight of tissue per flask after 5 weeks of cultivation in darkness at 26°C. In the chlorophyll retention assay (wheat leaf senescence assay, WLSA) the seeds of *Triticum aestivum* L. cv. Jara were planted into vermiculite saturated with Knop's solution, germinated for 48 h in the dark at 26°C, and then transferred into an illuminated growth chamber (7000 lx, day/night period 18 h/6 h, 20°C). After 10 day cultivation, when the first wheat leaf was fully developed and the second was partially developed, the leaves were cut off 7 cm from their apical tips. Four leaf pieces were placed with their basal ends down in the test tube containing 0.5 mL test solution (cytokinin dissolved in 0.1 M HCl and neutralized with NaOH to final NaCl concentration 0.01 M). Test tubes without stoppers were incubated for 4 days at 26°C in the dark and then 4 leaf pieces were transferred into calibrated test tubes containing 10 mL 80% EtOH (v/v) and a boiling chip – the chlorophyll was extracted by heating the test tubes in a water bath at 80 °C for 8 min and the optical absorbance was measured at 665 nm. In the *Amaranthus bioassay*, modified from the previously published methods (Biddington and Thomas, 1973; Elliott, 1979a, 1979b), the seeds of *Amaranthus caudatus* L. var. *atropurpurea* were surface-sterilized in 10% aqueous chloramine B solution (w/v) for 10 min, washed 6-times in distilled water and germinated aseptically on filter papers moistened with 20 mL distilled water in the dark at 37°C for 46 h. Explants consisting of the upper portion of the hypocotyl plus cotyledons were kept in distilled water for 3 h at 26°C in darkness and then transferred on two layers of filter paper in a Petri dish containing 1 mL incubation medium (10 mM Na_2HPO_4-KH_2PO_4 pH 6.8, 5 mM tyrosine, and the cytokinin tested) and after incubation in the dark at 26 °C for 46 h the betacyanin was extracted with 3.33 mM acetic acid using freezing and thawing procedure repeated twice – the concentration of betacyanin was obtained from the difference spectra at 537 and 620 nm. These three bioassays were modified in 1998 (Holub *et al.*, 1998) and are considered as classical assays since then.

In 2001, the breakthrough in cytokinin activity testing was started with the discovery of three cytokinin receptor histidine kinases in *Arabidopsis* (CRE1/AHK4, AHK2 and AHK3) (Inoue *et al.*, 2001; Suzuki *et al.*, 2001; Yamada *et al.*, 2001) and with the introduction of the cytokinin

reporter proteins *via* expressing the receptor gene coupled with the reporter gene *cps::lacZ* in *E. coli* (Suzuki *et al.*, 2001; Yamada *et al.*, 2001). This *in vitro* assay enabled a rapid test of cytokinin activity without the influence of the plant complexity. Based on the same principle, more assays were developed, e.g. cytokinin-induced *β*-galactosidase activity in cytokinin receptor expressing *E.coli* (Spíchal *et al.*, 2004) or competitive ligand binding assay (Romanov *et al.*, 2005; Romanov, Lomin and Schmülling, 2006) using *E. coli* expressing cytokinin receptor and the radiolabelled standard ^{3}H-*trans*-zeatin compete with the tested compounds to evaluate the affinity towards the receptor.

To approximate the real conditions and compartmentation in plants the plant membrane assay system was developed (Lomin *et al.*, 2015). The assay is using microsomes isolated from tobacco leaves expressing specific cytokinin receptor and therefore mimics the obstacles that the substance must overcome in the real plant environment. Additionally, when compared cytokinin bases and ribosides in *E. coli* system vs. plant membrane system, the presence of ribose and other substituents at the N9-position of adenine scaffold dramatically decreases the ligand affinity to receptors and the active substances in *E. coli* system turns into inactive compounds (Savelieva *et al.*, 2018).

The important discovery facilitating the study of the cytokinin receptor activaton was the description of the cytokinin CRE1/AHK4 receptor 3D structure (Hothorn, Dabi and Chory, 2011) which can predict suitable substitutions of novel derivatives, nevertheless, the discrepancy between the base vs. riboside activities is still subject of study and should be viewed from a broad perspective including *in vitro* and *in vivo* assays and bioassays as well as agriculture results.

7. Anti-senescence and anti-oxidative activity of cytokinins in plants

Cytokinins are essential regulators of plant growth involved in all stages of leaf development from the formation of leaf primordia at the peripheral zone of the shoot apical meristem (SAM) to the polarity establishment, the leaf morphology and the senescence (Mok and Mok, 2001; Sakakibara, 2006).

In 1957, the ability of cytokinin treatment to prolong the chlorophyll retention in excised leaves of cocklebur plants (*Xanthium pennsylvanicum*) was shown for the first time (Richmond and Lang, 1957). Cytokinins are synthesized in roots and are transported to leaves through the transpiration stream or may be produced by local synthesis in leaves (Noodén, Singh and Letham, 1990; Breeze *et al.*, 2011). The amount of cytokinin in xylem (*Glycine max*) highly decrease at the onset of senescence as well as expression of cytokinin biosynthetic genes in leaves (*Arabidopsis*) (Ambler, Morgan and Jordan, 1992; Zwack and Rashotte, 2013). Thus, cytokinins delay leaf senescence as part of endogenous development and not only as a result of exogenous treatment (Zwack and Rashotte, 2013). It was proved that cytokinin overproducing plants could show increased leaf longevity (Li, Hagen and Guilfoyle, 1992; Gan and Amasino, 1996). These results were probably due to a cytokinin imposed shift in sink and source identities in organs (Roitsch and Ehneß, 2000). To resolve this findings, the auto-regulatory loop system targeted cytokinin production to senescing cells with prevented over-accmulation where the IPT gene was expressed in tobacco plants under the promoter of SAG12 (Senescence Associated Gene 12) to increase cytokinin production limited to leaves at the beginning of senescence was designed (Gan and Amasino, 1995). This proSAG::IPT system has already been implemented in number of important crop species which demonstrated delayed leaf senescence (Calderini *et al.*, 2007).

The senescence delayed plant phenotype was also observed in plants expressing a proteolytic resistant version of ARR2 discovered after the characterization of *Arabidopsis* mutant with delayed senescence phenotype, *ore12*, the gain of function allele of the cytokinin receptor AHK3 (Kim *et al.*, 2006, 2012). A similar phenotype was described in plants overexpressing the cytokinin inducible transcription factor CRF6 and *crf6* mutants were found to have decreased sensitivity to the anti-senescence effect of cytokinins (Zwack *et al.*, 2013). Interestingly, ARR2, CRF6 and AHK3 are expressed in leaf vascular tissues which is crucial in regulating senescence process (Zwack and Rashotte, 2013).

Furthermore, it was shown that cytokinins accelerate nutrient mobilization within source-sink relationships (Mothes and Engelbrecht, 1963). The cytokinin induced CWINV (cell-wall

invertase) gene expression was shown to be linked with delayed leaf senescence (Godt and Roitsch, 1997; Zwack and Rashotte, 2013). The CWINV enzyme is localized in cell walls where it catalyzes the cleavage of sucrose into hexose monomers which allows sucrose unloaded from the phloem into the apoplasm of sink organs to be metabolized and taken up by adjacent cells with hexose transporters (Eschrich, 1980; Zwack and Rashotte, 2013). The sucrose can passively defund through the phloem and therefore, the rate of its metabolism at the site of unloading determines the sink strength (Roitsch and Tanner, 1996; Jin, Ni and Ruan, 2009; Zwack and Rashotte, 2013). In tobacco proSAG12::IPT lines the long-lived leaves with unusually high levels of CWINV activity were reported (Balibrea Lara *et al.*, 2004).

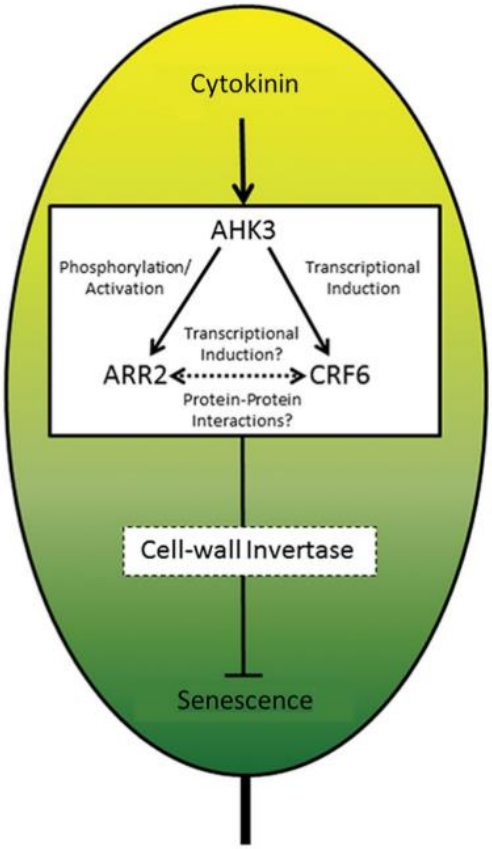


Fig. 13: The cytokinin perception by AHK activates ARR2 and induced expression of CRF6. ARR2 and CRF6 can regulate downstream genes such as cell-wall invertase, necessary for delay senescence (Zwack and Rashotte, 2013).

It was also reported that in the *Arabidopsis ahk2,3* double mutant the expression levels of chlorophyll synthesis genes *HEMA1*, *GUN4*, *GUN5*, and *CHLM* are reduced, explaining the ability of cytokinins to increase chlorophyll content and delay senescence (Cortleven *et al.*, 2016; Wu *et al.*, 2021).

Additionally, the leaf senescence is accompanied by increased levels of H_2O_2 that could lead to the lipid peroxidation and membrane leakiness (Dhindsa, Plumb-Dhindsa and Reid, 1982;

del Río *et al.*, 1998; Cabello, Agüera and de la Haba, 2006; Jajic, Sarna and Strzalka, 2015). When BAP was externally applied, the H_2O_2 level was reduced per increased activity of antioxidant enzymes SOD (superoxide dismutase), CAT (catalase) and APX (ascorbate peroxidase) enzymes in dark-induced senescent wheat leaves (Zavaleta-Mancera *et al.*, 2007; Wilson-García *et al.*, 2008; Mik, Szüčová, Spíchal, *et al.*, 2011). Therefore, externally applied cytokinins may protect the cells and photosynthetic system from oxidative damage. Nevertheless, in dexamethasone-inducible IPT transgenic tobacco plants where the cytokinin levels are increased endogenously the reduced H_2O_2 level was not observed in chloroplasts (Novák *et al.*, 2013) and, furthermore, the role of antioxidant enzymes varies between natural and dark-induced plant senescence (Poór *et al.*, 2018).

8. Action of cytokinins and cytokinin analogues on non-plant cells

The discovery of cytokinins' abilities to promote and control growth and development of plant cells led to the suggestion of the potential effects on human cells. Therefore, the cytokinin bases were applied to keratinocytes (Berge, 2006) and leukaemia cell lines HL-60 and K-562 (Mlejnek and Kuglík, 2000; Ishii, Sakai and Honma, 2003) nevertheless, their concentrations needed to be relatively high (25-100 μM) to induce differentiation.

A different situation applies for cytokinin ribosides where the depletion of adenosine triphosphate, mitochondrial depolarization and caspase-3 activation proceeds at low micro-molar concentrations (Mlejnek, 2001; Mlejnek and Doležel, 2005). The requirement for the cytotoxic mechanism of cytokinin ribosides is intracellular phosphorylation by adenosine kinase and this step is necessary for both animal as well as plant cells (Mlejnek and Procházka, 2002; Mlejnek, Doležel and Procházka, 2005). Therefore, the affinity of adenosine kinase towards some cytokinin ribosides and/or cytokinin analogues explains the lacking toxicity (Mlejnek and Doležel, 2005). Furthermore, the cytokinin ribosides are predominantly metabolized to their respective 5′-monophosphates while other nucleoside analogues are converted to the triphosphates and then interfere with the synthesis of nucleic acid (Mlejnek and Doležel, 2005).

However, the cytotoxic activity of BAPR and iPR was studied in a limited clinical trial already in 1975 (Mittelman, Evans and Chheda, 1975). The panel of known cytokinin ribosides and novel analogues was later prepared for their anticancer *in vitro* activity evaluation on diverse human cancer cell lines (Voller *et al.*, 2010). The antiproliferative activity of iPR, KR, BAPR as well as hydroxylated *o*TR, *p*TR and *m*TR or *c*Z was confirmed with the significant *in vitro* and also *in vivo* anticancer properties showing *ortho*-topolin as the best substance from the study (Voller *et al.*, 2010). On the other hand, the toxicity of *t*Z is very limited (Ishii *et al.*, 2002; Rattan and Sodagam, 2005) as well as of iPR analogues where saturation of the side chain or replacement of ribose with acyclic moieties caused a significant cytotoxicity drop (Colombo *et al.*, 2009; Ottria *et al.*, 2009).

In multiple myeloma, KR caused suppression of cyclin D1 and D2 transcription and following cell apoptosis (Tiedemann *et al.*, 2008). Additionally, known inhibitors of cyclin-dependent kinases olomoucine, bohemine, roscovitine and analogues derived from them were structurally inspired by cytokinins (Veselý *et al.*, 1994; Havlíček *et al.*, 1997; Kryštof *et al.*, 2002; Vermeulen *et al.*, 2002). Unlike cytokinin ribosides, the roscovitine-type CDK inhibitors cannot be converted to the respective 5′-phosphates.

The cytotoxic activity of cytokinin analogue derivative IB-MECA (1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-*N*-methyl-*β*-D-ribofuranuronamide) at micromolar concentrations is connected to the interaction with adenosine A3 receptor (Mlejnek and Doležel, 2010).

The activity of BAPR towards the replication of human enterovirus 71 was reported in 1952 (Davoll and Lowy, 1952). The antivirotic abilities of iPR and KR were also examined on EV71 in 2015 (Tararov *et al.*, 2015; Drenichev *et al.*, 2016). In 2017, the increased antiviral effect of BAPR analogues on EV71 was connected with the presence of fluorine atom/s on the benzyl ring. Antiviral efficiency of cytokinin derivatives (BAPR and BAPdR) against Semliki Forest virus and Sindbis virus alphaviruses was described in 2008 (Pohjala *et al.*, 2008). Generally, the purine nucleoside pattern is important for inhibitory activity toward viruses and could be more or less observed by several inhibitors of nucleoside reverse transcriptase used for HIV treatment; this area was the life's work of prof. Holý (De Clercq, 2013). The antiviral and antimicrobial action of nucleosides is reviewed e.g., in (Kataev and Garifullin, 2021; Zenchenko *et al.*, 2021).

From another point of view, the anti-senescence activity of cytokinins observed in plants inspired the study of ability of cytokinins and their analogues to retard aging of mammalian and also human cells. The one of the first evidences of the *in vitro* anti-aging activity of non-toxic cytokinin base kinetin on fibrolasts was reported in 1994 (Rattan and Clark, 1994) and that of *t*Z in 2005 (Rattan and Sodagam, 2005). The efficient kinetin analogous substance 6-(furfurylamino)-9-(tetrahydropyran-2-yl)purine (Pyratine), used in cosmetics, proved the ability to improve roughness, hyperpigmentation or fine wrinkles as reported in the clinical study of topical use from 2008 (McCullough, Garcia and Reece, 2008). Along with the anti-senescence properties, the question of antioxidant capacity arose. In 1997, the first proposal of kinetin formation mechanism in DNA, based on the analyses of mass spectra, was described. The hydroxyl radical attacs at the 5′ carbon of the deoxyribose residue to form furfural which reacts with amino group of adenine and, after intramolecular rearrangement, the Shiff base is reduced to form kinetin (Barciszewski *et al.*, 1997). The antioxidant activity of kinetin, BAP, *p*T and iP were also evaluated: in oxygen radical absorbance capacity (ORAC) assay with the significant activity of all compounds tested except for BAP; and in the Trolox equivalence antioxidant capacity (TEAC) assay where only topolin isomer was active, probably due to the presence of hydroxyl, and in the 2-deoxyribose degradation assay where all tested cytokinins were able to react with generated hydroxyl radicals (Brizzolari *et al.*, 2016). Additionally, the DNA damage protective activity of low doses of kinetin (up to 100 nM) was conferred in

mammalian cells using the dihydroethidium staining method while high doses (500 nM and higher) mediated DNA damage *in vitro* (Othman *et al.*, 2016).

9. Practical use of cytokinins and cytokinin derivatives in crop production

In vitro micropropagation is a method used for large expansion of clonal explant numbers in short period of time and sterile conditions (Bhatia and Sharma, 2015). The method is consisting of initiation of culture, shoot multiplication, rooting of the developed shoots and transfer plantlets to the *ex vitro* conditions (Bhatia and Sharma, 2015). This biotechnological method is used worldwide for commercial production of many important crops such as banana (Muhammad *et al.*, 2007), apple (Dobránszki and Teixeira da Silva, 2010), strawberry (Borkowska, 2001), potatoes (Baroja-Fernández *et al.*, 2002), rose (Pati *et al.*, 2006), and orchid (Chugh, Guha and Rao, 2009) as well as for the preservation of endangered species (e.g., *Harpagophytum procumbens* or *Aloe polyphylla* (Bairu *et al.*, 2009, 2010)).

As well as in micropropagation also in agriculture the plant growth regulators help to obtain the desired yields of plants/products. Exogenous application of CKs, due to the financial availability predominantly used are BAP and kinetin, is mainly performed on crop plants such as cereals (wheat (Iqbal, Ashraf and Jamil, 2006; Yasmeen and Muhammad, 2013), barley (Sharif and Dale, 1980; Williams and Cartwright, 1980), oat (Harrison and Kaufman, 1980; Peltonen-Sainio, 1997), rice (Yang, 2002; Gurmani, Bano and Salim, 2006) or maize (Pan *et al.*, 1999; Kaya, Tuna and Okant, 2010)), legumes (soybean (Cho *et al.*, 2001; Nonokawa *et al.*, 2007), pea (Schroeder, 1984), chickpea (Zarrin *et al.*, 2008), beans (Ibrahim *et al.*, 2007)), vegetables (eggplant (Wu *et al.*, 2012), tomato (Jackson and Campbell, 1979; Haroun *et al.*, 2011), lettuce (Prokopová *et al.*, 2010), spinach (Durrani *et al.*, 2010) or watermelon (Hayata, Niimi and Iwasaki, 1995)), fruit trees (Stern and Flaishman, 2003; Moghaddam, Moghaddam and Piri, 2013) or olive tree (Aziz *et al.*, 2011). Generally, the cytokinins effectively improve the size and yield of grains (Koprna *et al.*, 2016), the flower abortion problems (soybean) (Yashima *et al.*, 2005), quality of seeds and fiber (cotton) (Mehasen, G. Gebaly and Seoudi, 2012), increasement of fruit weight in apple and pear trees (Stern and Flaishman, 2003), branching in young trees in nurseries for better tree architecture (Moghaddam, Moghaddam and Piri, 2013) or accelerated flowering in tomatoes (Haroun *et al.*, 2011). The detailed effects of cytokinins on agricultural plants were greatly reviewed in 2020 (Aremu *et al.*, 2020).

Usually, solutions for exogenous applications are prepared by solvatation of cytokinin/cytokinin analogue in dimethylsulfoxide (DMSO), e.g., 2-Cl-3-MeoBAP (Koprna *et al.*, 2016), which is then dissolved in water to the desired concentration. The formation of cytokinin salt (mesylate) with methanesulfonic acid (Klos *et al.*, 2022) was advantageously used for the preparation of water solutions without the need of DMSO addition. Furthermore,

the salt solution application of BAP-mesylate and *m*T-mesylate significantly reduced the chlorophyll degradation in barley leaf segments and sustained the high concentrations of functional endogenous CK pools in leaves compared to the parent cytokinin which led to an increase of the harvest yield (Klos *et al.*, 2022).

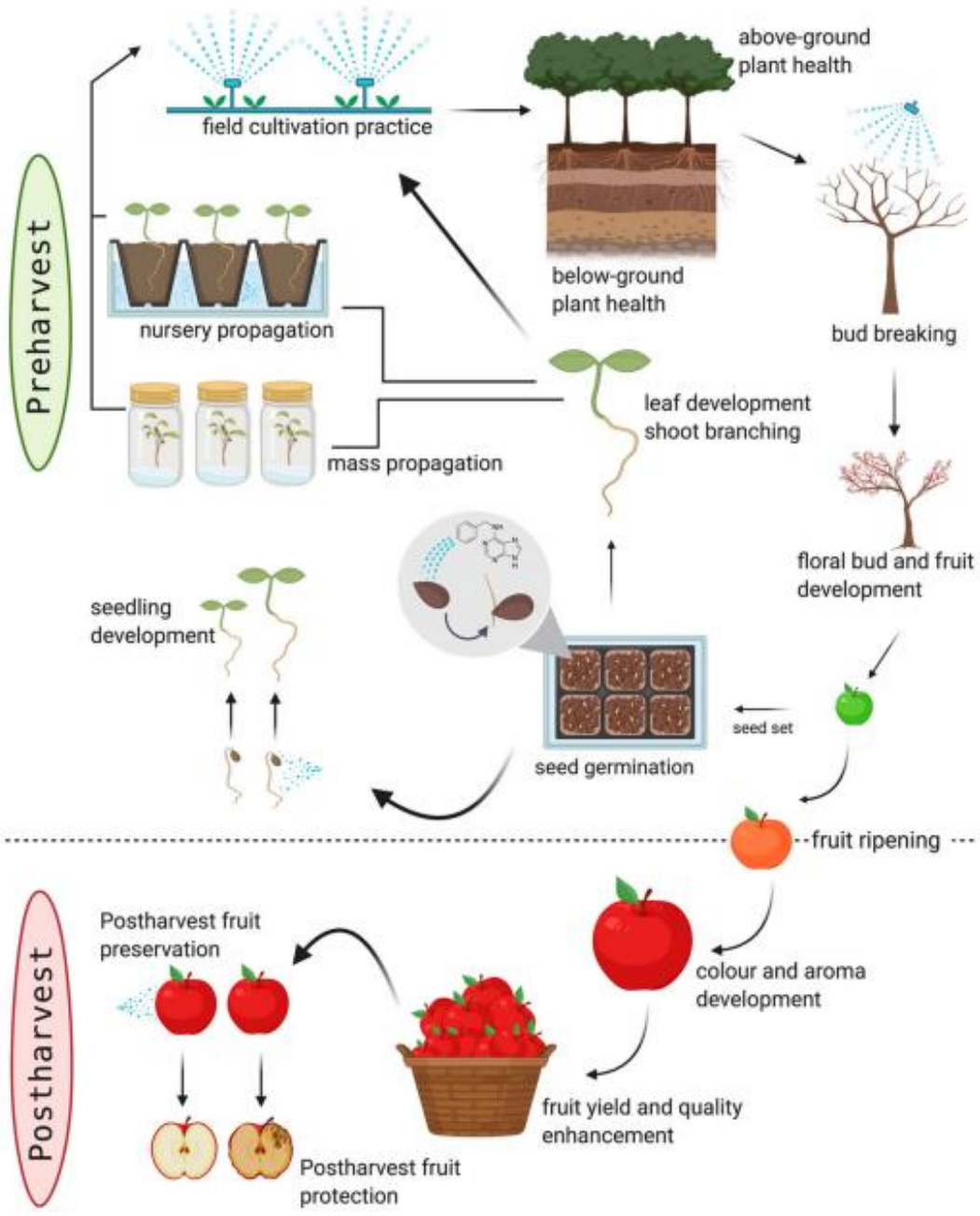
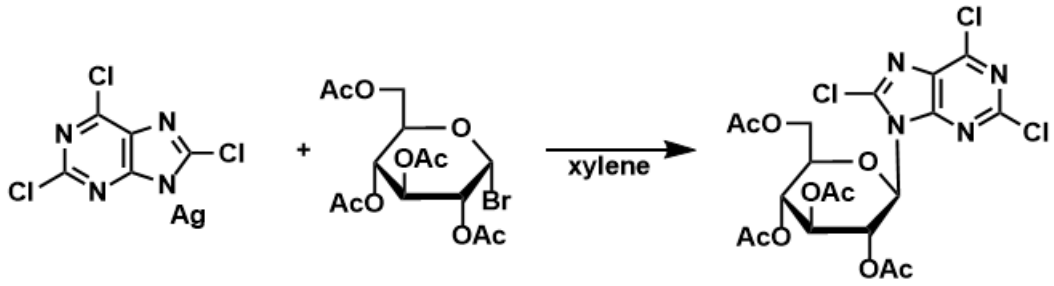


Fig. 14: Cytokinin application in horticulture (Aremu *et al.*, 2020).

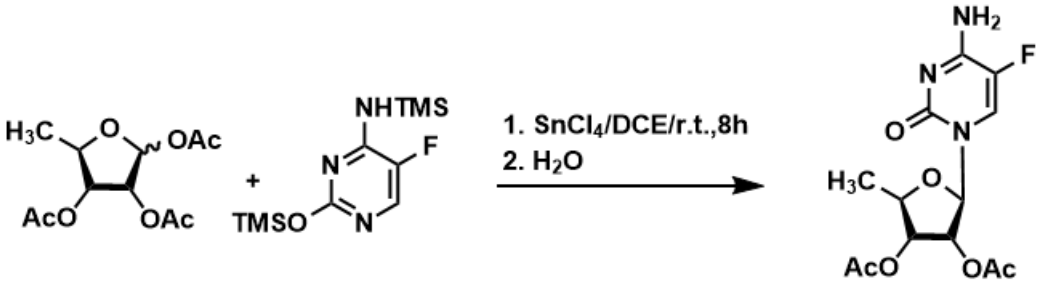
10. Approaches to the synthesis of purine nucleosides

The first attempts to obtain ribonucleoside/2´-deoxyribonucleoside molecule were held in 1914 by Fischer and Helferich (Fischer and Helferich, 1914) by condensation of silver 2,8-dichloroadenine with tetraacetylglucosyl bromide with following deacetylation of the product to give 2,8-dichloro-9-glucopyranosyladenine (**Scheme 2**) that could be further dehalogenated with nitrous acid and then aquous ethanolic ammonia, or e.g., by heating of chloromercuri-6-acetamidopurine with tetraacetylglucosyl bromide in xylene to obtain glucopyranosyladenine after deacetylation. Davoll and Lowy (Davoll, Lythgoe and Todd, 1946; Davoll and Lowy, 1951, 1952) were further improving Fischer and Helferich method.



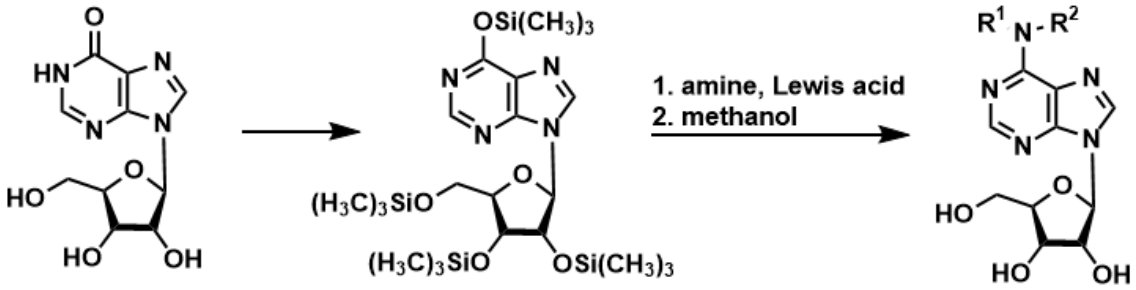
Scheme 2: The Fischer-Helferich (Fischer and Helferich, 1914) original procedure – the silver salt of 2,6,8-trichloropurine was heated with acetobromoglucose in xylene to obtain glucopyranoside.

Hilbert and Johnson reported a method for nucleoside synthesis applied to quinolones, pyridines or pyrazolones where the *O*, *N*-silylated heterocycle is coupled to sugar halide or acetate with the Lewis acid catalyst (Johnson and Hilbert, 1929; Hilbert and Johnson, 1930). The reaction is also known as the Vorbrüggen reaction. TMSOTf (trimethylsilyl trifluoromethanesulfonate) can be advantageously used to transform an unreactive and unsoluble nucleobase into highly reactive nucleophilic silylated analogue reacting then with electrophile in the presence of Lewis acid (usually Sn^{4+} or Sn^{2+}) in organic solvent (Niedballa and Vorbrüggen, 1970, 1974; Vorbrüggen, Krolikiewicz and Bennua, 1981; Vorbrüggen and Ruh-Pohlenz, 1999).



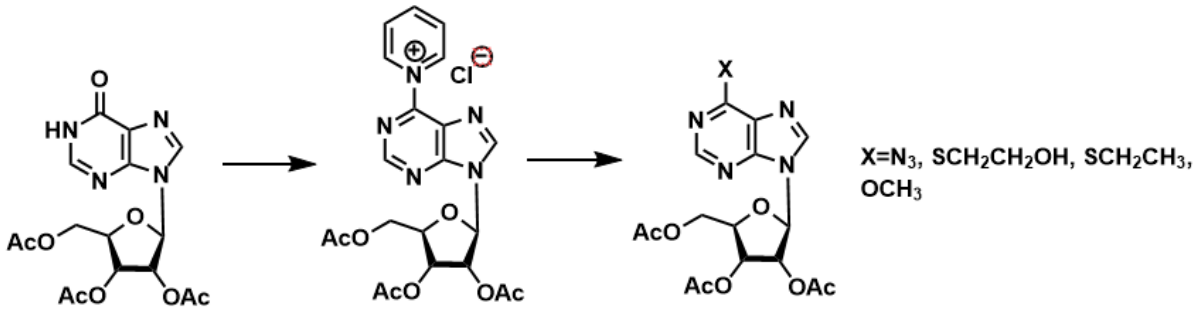
Scheme 3: The original Hilbert-Johnson method for synthesis of nucleoside employing acylated sugar and silylated base in the presence of Lewis acid (Johnson and Hilbert, 1929).

The important starting material for synthesis of various adenosine analogues is commercially available inosine (and analogously 2′-deoxyinosine and 2′,3′-dideoxyinosine). To modify the position 6 of the purine core, the reaction of inosine or guanosine and their 5′-phosphates with hexamethyldisilazane and ammonia or primary/secondary amines in the presence of Lewis acid ($(NH_4)_2SO_4$, $(CH_3)_3Si\text{-}O\text{-}SO_2CF_3$, *p*-toluensulfonic acid hydrate or Hg^{2+} salts) leading to corresponding nucleoside (**Scheme 4**) was reported multiple times (Gerster, Jones and Robins, 1963; Robins and Basom, 1973; Vorbrüggen and Krolikiewicz, 1976; Reese and Ubasawa, 1980; Robins and Uznański, 1981; Adamiak, Biala and Skalski, 1985; Vorbrüggen and Ruh-Pohlenz, 1999).



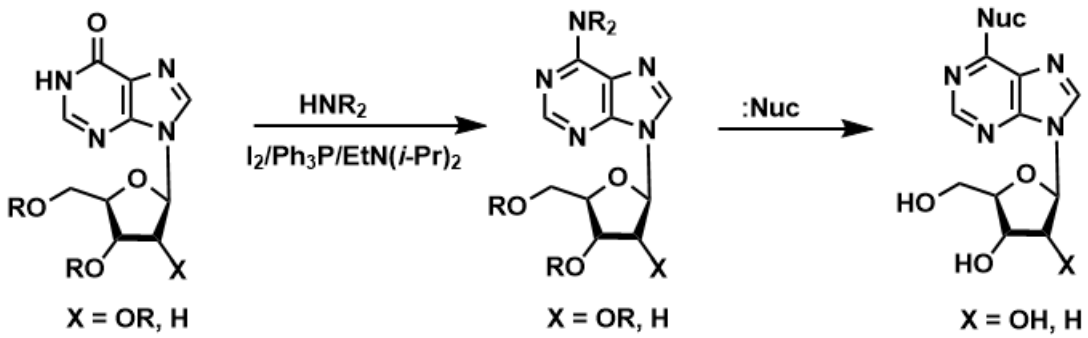
Scheme 4: Transformation of inosine into N^6-substituted adenosine.

Another approach utilizes more reactive trialkyl(6-purinyl)ammonium salts, or 6-alkylsulfonylpurines derived from them, furthermore, the reaction product of pyridine assisted phosphorylation of 2′,3′,5′-tri-*O*-acetylinosine or 9-metyl-hypoxanthine with 4-chlorophenyl phosphorodichloridate led to the purine analogue (**Scheme 5**) equipped with a good leaving group towards nuclephiles (Adamiak, Biala and Skalski, 1985; Adamiak, Biata and Skalski, 1985).



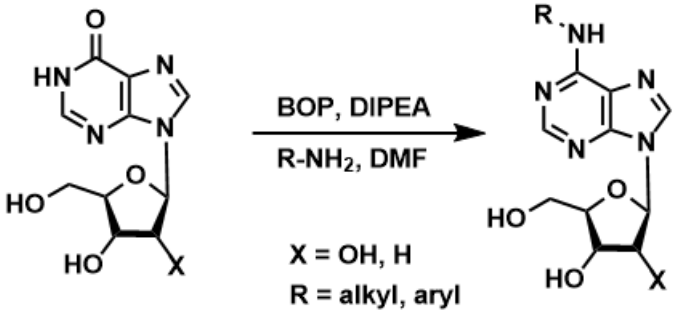
Scheme 5: The synthesis of 6-substituted purines and ribonucleosides with *N*-(6-purinyl)pyridinium salts (Adamiak, Biala and Skalski, 1985).

In 2000, Lin and Robins (Lin and Robins, 2000) reported a method (**Scheme 6**) where protected inosine or 2′-deoxyinosine is transformed into 6-substituted nucleoside with using $I_2/Ph_3P/EtN(i\text{-}Pr)_2$ in the presence of secondary amine (imidazole, piperidine or morpholine) in DCM or toluene.



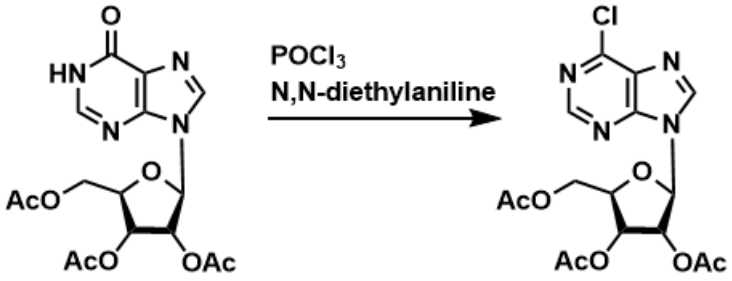
Scheme 6: The preparation of N^6-substituted adenosine/2′-deoxyadenosine analogues (Lin and Robins, 2000).

In this thesis highly employed one-step method (**Scheme 7**), described in 2005 (Wan *et al.*, 2005, 2007), is using the unprotected 2′-deoxyinosine and 1*H*-benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP, Castro′s reagent) where the O^6-(benzotriazole-1-yl) derivative is able to replace 1-hydroxybenzotriazolide (BtO^-) with a nuclephilic residue (amine) under mild conditions. The method is also suitable for syntheses of 2′,3′-dideoxynucleosides when 2′,3′-dideoxyinosine is used.



Scheme 7: The one-step preparation of N^6-substituted adenosines/2′-deoxyadenosines from inosine/2′-deoxyinosine (Wan *et al.*, 2005).

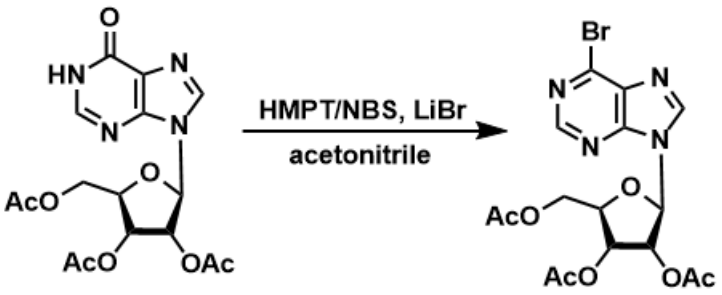
Advantageous for the syntheses of series of nucleosides substituted in the position 6 of the purine ring is to have a proper halogenderivative. This attempt was succesfull in early 60´s by base-sugar coupling (chloromercury (Iwamoto, Acton and Goodman, 1962) or fusion method (Robins and Robins, 1964)). To obtain 6-halogen-2′-deoxynucleoside the chlorination of *O*-acyl protected purine-2′-deoxynucleoside is not available due to its acidic and heat unstability which is more prominent there than for *O*-acylated 2′-deoxyinosine (Gerster, Jones and Robins, 1963; Ikehara and Uno, 1965; Žemlička and Šorm, 1965).



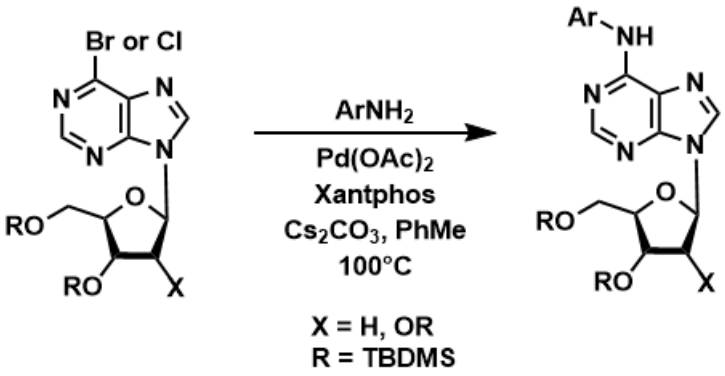
Scheme 8: The reaction of hypoxanthines (2′,3′,5′-tri-*O*-acetylinosine) with $POCl_3$ in the presence of *N*,*N*-diethylaniline (Robins and Christensen, 1952; Gerster, Jones and Robins, 1963; Adamiak, Biala and Skalski, 1985) leading to 6-chloro-9-(2′,3′,5′-tri-*O*-acetyl-(*β*)-D-ribofuranosyl)purine.

The nucleophilic displacement (S_NAr) of halogens with amine nucleophile in the presence of base (triethylamine TEA, diisopropylethylamine DIPEA) in alcohol is of great interest because of the possibility to obtain the series of e.g., N^6-substituted nucleoside analogues in good yields (Fleysher *et al.*, 1968, 1969). The most syntheses employed the 6-chloro derivative, however, the utilization of *O*-acetyl-protected 6-bromopurine nucleoside as a starting material enables the reaction with primary aliphatic alkylamines and the low-reactive arylamines or imidazoles (Véliz and Beal, 2001). In 2010, Thomson et al. modified this method to enable the palladium catalysed C-N bond forming reaction to the low-reactive arylamines and used the acetyl-protected 6-bromopurine nucleoside (or 6-chloropurine nucleoside) (Thomson *et al.*,

2010). The acyl-protected brominated nucleosides, prepared by the reaction (**Scheme 9**) of triacetylinosine and HMPT/NBS/LiBr in acetonitrile (Véliz and Beal, 2000), were firstly used as a substrates for nucleophilic substitutions with amines in 2001 (Véliz and Beal, 2001). Furthermore, N^6-aryl-2′-deoxadenosine analogues could be efficiently synthesized using suitable Pd-ligand complex (Lakshman *et al.*, 2001).



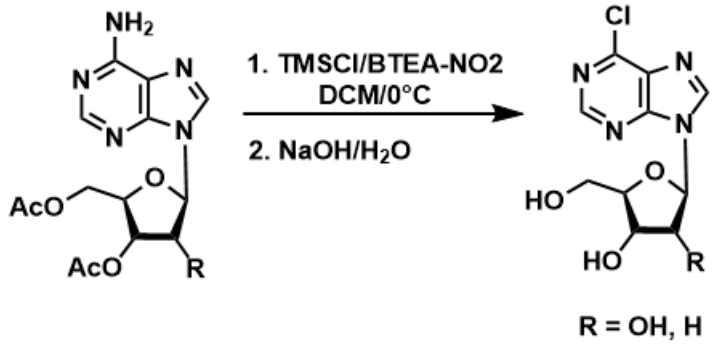
Scheme 9: The bromination of inosine derivative (Véliz and Beal, 2000, 2001).



Scheme 10: The palladium acetate assisted amination reaction of 6-bromoderivative (Thomson *et al.*, 2010).

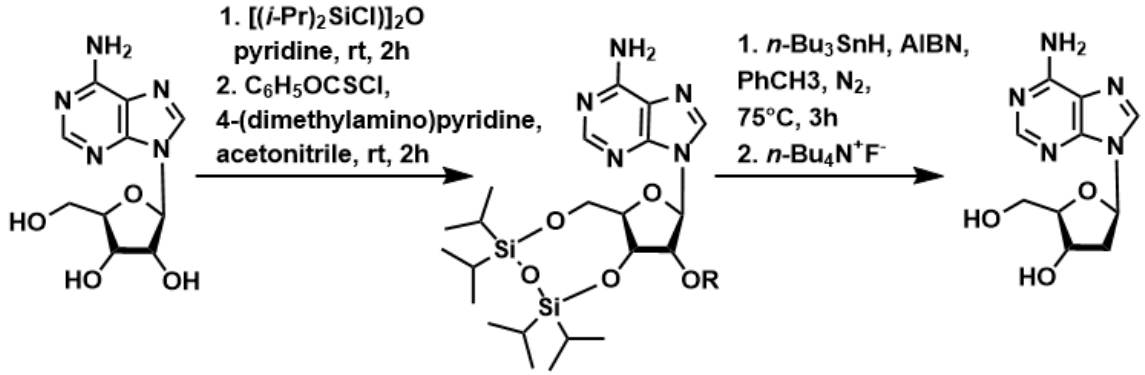
The 6-chloro-2′-deoxyadenosine was also prepared by the reaction using 6-chloropurine, sodium hydride in anhydrous acetonitrile (sodium salt of 6-chloropurine) and protected 1-chloro-2′-deoxy-3,5-di-*O*-*p*-toluoyl-(*α*)-D-*erythro*-pentofuranose resulting in the mixture of anomers in favour of *β*-anomer, that can be transformed into 2′-deoxyadenosine with ammonolysis (NH_3, MeOH) to obtain 2′-deoxyadenosine (Kazimierczuk *et al.*, 1984, 1990).

Francom and Robins (Francom and Robins, 2003) proceeded the nonaqueous diazotiation/halo-dediazoniation (**Scheme 11**) to replace the amino-group in the position 6 of purine with the halogen and thus followed Weiss and Wagner (Weiß and Wagner, 1984) who dealed with the *in-situ* generation of nitrosylhalides by reaction of trimethylsilyl halides with alkyl nitrites.



Scheme 11: The scheme of diazotiation/halo-dediazoniation reaction introducing the halogen atom in the purine position 6 (Francom and Robins, 2003).

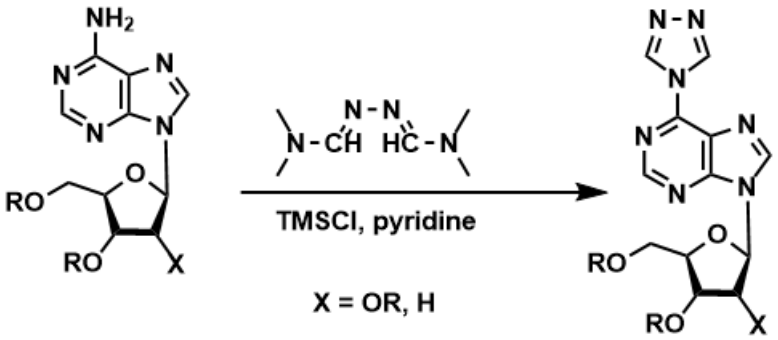
Using of protecting groups is mostly necessary in the chemistry of carbohydrates. Robins and Wilson (Robins and Wilson, 1981) selectively protected 3′ and 5′ hydroxy groups of ribonucleoside by 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane in pyridine and the resulting derivative was prepared for further modification reactions only at 2′-position (**Scheme 12**).



Scheme 12: Method utilizing the protecting group strategy to modify specific position (Robins and Wilson, 1981).

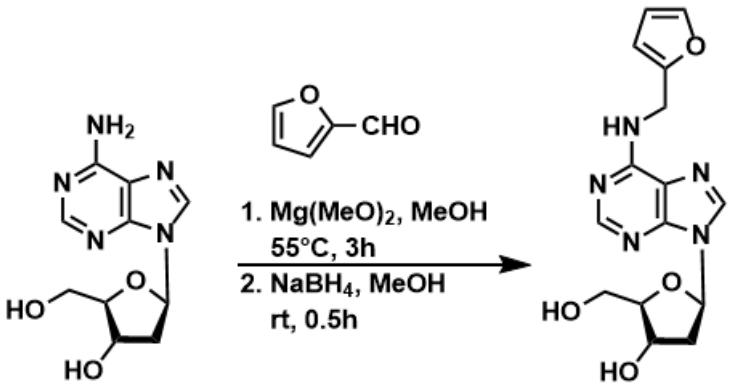
Another approach utilized the transformation of amino group of 2′-deoxyadenosine into the 1,2,4-triazole residue by the reaction with 1,2-bis[(dimethylamino)methylene]hydrazine (Miles, Samano and Robins, 1995). The requirements of the general methodology includes activation of NH_2 group by substituents diminishing its pKa and enable using mild conditions for the displacement, the intermediate can be as reactive as the NRR′ group displacement does not occur during generation or isolation and nuclephiles attack the carbon atom of the parent heterocycle and not the NRR′ part (Miles, Samano and Robins, 1995). The reaction aimed to unprotected adenosine led to a significant sugar cleavage while when tri-*O*-acetyl analogue was used the moderate yield of 9-(2,3,5-tri-*O*-acetyl-(*β*)-D-*erythro*-pentofuranosyl)-6-(1,2,4-triazol-4-yl)-purine was obtained after 20 h at 100°C in anhydrous pyridine. Also treatment of adenosine with 1,2-bis[(dimethylamino)methylene]hydrazine hydrochloride and TMSCl at

100°C for 24 h followed by deprotection with MeOH gave 9-((*β*)-D-*erythro*-pentofuranosyl)-6-(1,2,4-triazol-4-yl)purine in high yield. Furthermore, treatment of 2′-deoxyadenosine with TMSCl (for protection/glycosyl bond stabilization and HCl formation) and 4 eq. of azine (1,2-bis[(dimethylamino)methylene]hydrazine) in pyrimidine at 100°C for 24 h gave 9-(2-deoxy-(*β*)-D-*erythro*-pentofuranosyl)-6-(1,2,4-triazol-4-yl)-purine (Miles, Samano and Robins, 1995).



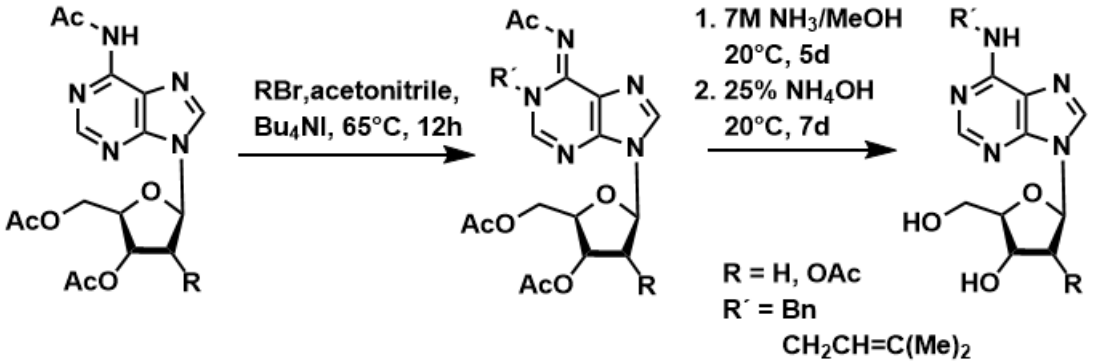
Scheme 13: The reaction using 1,2,4-triazol-4-yl substituent as the leaving group ready for displacement by nucleophile (Miles, Samano and Robins, 1995).

Adamska et al. (Adamska, Barciszewski and Markiewicz, 2012) then published the method employing unprotected 2′-deoxyadenosine and aromatic aldehyde in anhydrous methanol with the presence of magnesium or sodium methoxides as drying agents to yield the Shiff′s base followed by the imin reduction with sodium borohydride to obtain e.g., K-2′-dR (2′-deoxy-N^6-furfuryladenosine) in good yield without further purification (**Scheme 14**). The alkyl aldehyde reaction in this case needed to be catalyzed with acetic acid with the excess of aldehyde and the reduction of Shiff′s base was performed using borane dimethylsulfide complex with the final extraction step.



Scheme 14: The synthesis of kinetin-2′-deoxyriboside using aromatic aldehyde (Adamska, Barciszewski and Markiewicz, 2012).

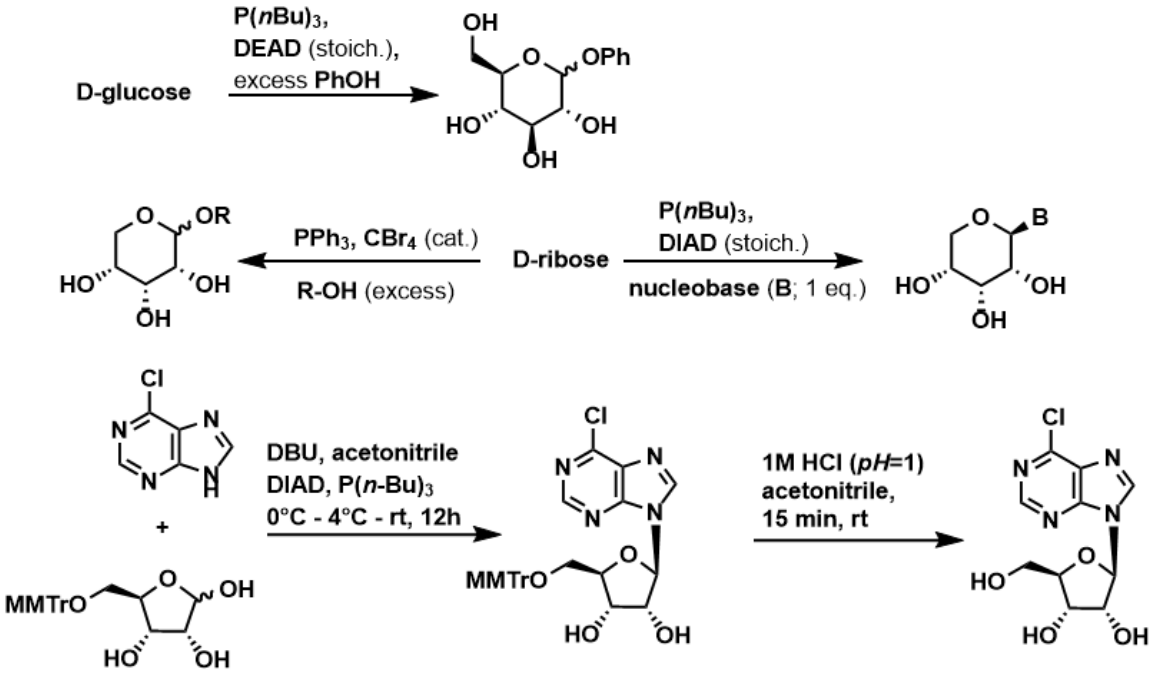
Furthermore, the method using Dimroth rearrangement was described, starting with the alkylation of nucleoside with methyliodide to form 1-*N*-methyl-nucleoside which is further rearranged to N^6-methylnucleoside. The utilization of alkylhalides and 2′,3′,5′-tri-*O*-acetyladenosine in the presence of KI and $Ba(CO_3)_2$ also led to the product (Jones and Robins, 1963; Robins and Trip, 1973; Oslovsky, Drenichev and Mikhailov, 2015).



Scheme 15: The example of the Dimroth rearrangement after 1-N-alkylation of purine nucleosides leading to e.g., iPR or iPdR (Oslovsky, Drenichev and Mikhailov, 2015).

For the preparation of dideoxynucleosides, the multistep synthesis of protected 2,3-dideoxyribose starting from L-glutamic acid was described in 1998 (Okabe *et al.*, 1988) and with modification of the glycosylation reaction (Mitsunobu conditions) was also used in this thesis - the synthetic procedure is described in the experimental section.

The highly employed method for nucleoside synthesis utilizes Mitsunobu conditions. In 2017, the Mitsunobu reaction for the synthesis of 2′-deoxynucleosides was employed (Seio *et al.*, 2017) using protected adenine 2-deoxy-3,5-*O*-bis(*tert*-butylsilyl)-D-ribose and tri(*n*-butyl)phosphine, however, the conditions were already examined in 1979 for the glycosylation reaction of unprotected D-glucose in an excess of phenol to yield the phenyl-glycoside (Grynkiewicz, 1979) and the reaction was further improved (Schmalisch and Mahrwald, 2013; Seio *et al.*, 2017). The Mitsunobu conditions are, in general, advantageous for the synthesis of hydroxyderivatives with no need to protect them and the method utilizes the reaction of N^6-acetyl-2′,3′,5′-tri-*O*-acetyladenosine with appropriate alkyl halide in the presence of DBU (1,8,-diazabicyclo(5.4.0)undec-7-ene or K_2CO_3 as a catalyst (Tararov *et al.*, 2011). In 2015, Downey (Downey *et al.*, 2015) published the one-pot method of glycosylation under Mitsunobu conditions using unprotected ribose in good yield.



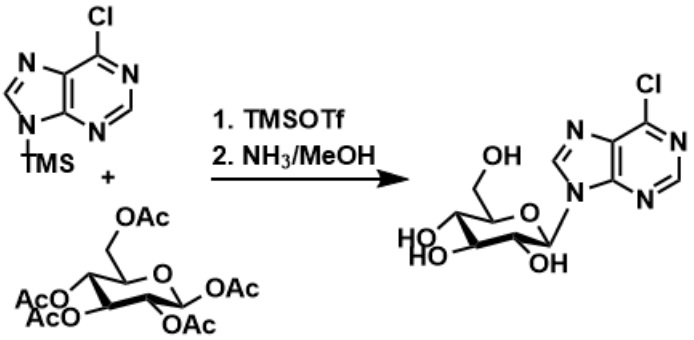
Scheme 16: The method comparison of glycosylation of D-glucose with an excess of phenol (Grynkiewicz, 1979) with the glycosylation of D-ribose with an excess of alcohol leading to anomeric mixtures (in favour of *β*-anomer) (Schmalisch and Mahrwald, 2013) *vs.* the one-pot glycosylation of unprotected (or two-step reaction with monoprotected with 4-methoxytrityl protecting group) D-ribose with nucleobase (e.g., 6-chloropurine) leadig to *β*-anomer only (Downey *et al.*, 2015) using Mitsunobu conditions.

As it was summarized in the review from 2016 (Drenichev 2016), the mostly used methods for the synthesis of N^6-substituted adenosines are the reaction of 6-chloropurine nucleosides with alkyl/aryl amines and the regioselective Mitsunobu reaction of N^6-acetyl-2′,3′,5′-tri-O-acetyladenosine with alcohols, followed by the condensation of inosine derivative with alkyl amines, the reduction of N^6-acyladenosine, the reduction of adenosine aldimines, the Dimroth rearrangement of 1-*N*-alkylated adenosines, and the glycosylation of N^6-substituted purines. However, the search of novel methods to facilitate the preparation of nucleoside analogues is of great interest.

The number of fast and solvent-friendly methods using microwaves have been published (Elgemeie and Mohamed, 2019; Grimaldi *et al.*, 2020), however, there is a need to optimize the reaction conditions for the labile nucleoside bond (Kochetkov and Budovskii, 1972; Véliz and Beal, 2001).

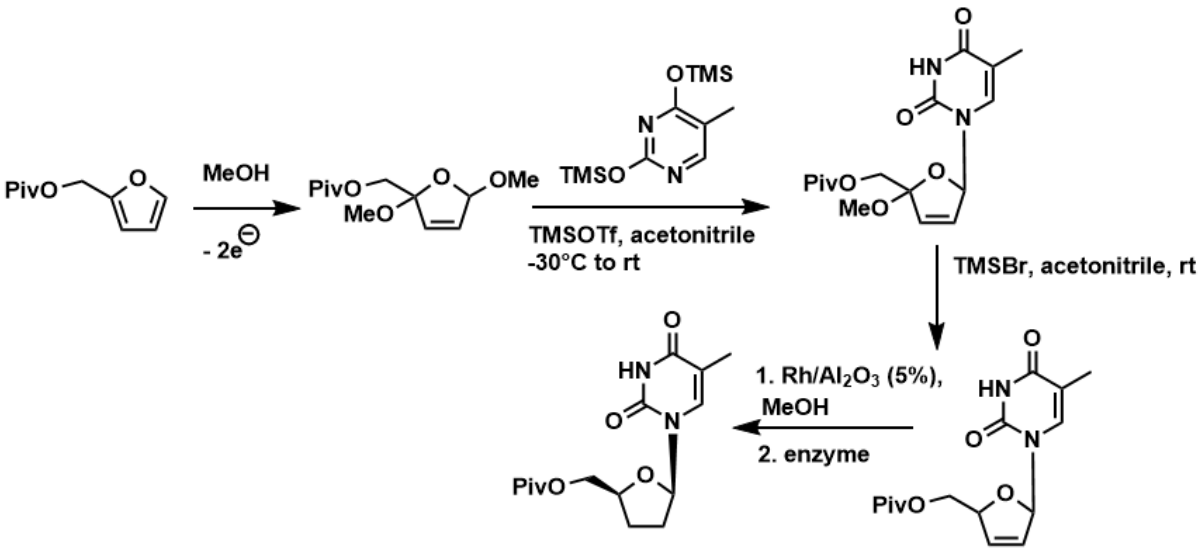
Additionally, the facile prepration of 1′,2′-*cis*-(*β*)-pyranosyladenine nucleosides from the corresponding protected 1′,2′-*trans*-(*β*)-glycosyl-6-chloropurine derivatives was published in 2007 (Ando *et al.*, 2007). They epimerized the hydroxyl group of glucose or galactose by S_N2 substitution of the O-trifluoromethanesulfonyl (TfO) group (with either tetrabutylammonium

acetate (TBAA) or CsOAc under mild conditions followed by deprotection and amidation at the position 6. Nevertheless, the sugar-base condensation reaction was performed with the peracetylated *β*-D-glucose or *β*-D-galactose (using acetanhydride), transformed into reactive cations with trimethylsilyl trifluoromethanesulfonate (TMSOTf), and with silylated 6-chloropurine under reflux (**Scheme 17**). The resulting peracetylated *β*-anomeric glucosyl or galactosyl-6-chloropurine derivatives were then deprotected by ammonolysis with methanolic ammonia (Ando *et al.*, 2007).



Scheme 17: The condensation reaction of peracetylated *β*-D-glucose with silylated 6-chloropurine resulting in the formation of *β*-anomeric purin nucleoside (Ando *et al.*, 2007).

The synthetic routes aiming to the synthesis of 2′-deoxynucleosides are more abundant than the routes leading to 2′,3′-dideoxynucleosides. The *de novo* approach to nucleosides starting from furan was described in 1996 where the Pd-catalyzed desymmetrization of *cis*-2,5-diacyloxy-2,5-dihydrofuran (prepared from furan with lead tetraacyloxylates) enabled to introduce the nucleobase to form both enantiomeric D- and L-nucleosides. However, the new method for the synthesis of 2′,3′-dideoxynucleoside analogues has been developed (Albert *et al.*, 2002) where the 2-substituted furans (2,5-dimethoxy-2,5-dihydrofurans) are electrochemically activated and coupled with the TMS-purine/pyrimidine base giving the planar furyl nucleosides, further hydrogenated *cis*-selectively (with Rh/Al_2O_3 in methanol) to yield (*β*)-2′,3′-dideoxynucleosides in racemic mixture. The enzymatic kinetic resolution then led to (*β*)-D- and (*β*)-L-configured products in high purity (e.g., (*β*)-D-2′,3′-dideoxynucleoside was separated from the mixture by the specific enzymatic hydrolysis of the (*β*)-L-product with pig liver esterase (Albert *et al.*, 2002).



Scheme 18: The synthetic approach for the synthesis of 2′,3′-dideoxynucleosides employing enzymatical resolution of anomers from the mixture; the thymine base was drawn as an exemplar base (Albert *et al.*, 2002).

11. Material and methods

The employed methods and relevant procedures as well as characterizations of appropriate prepared compounds are described in detail in the attached publications and the relative supporing informations.

General procedures

The starting materials and solvents were obtained from commercial suppliers (Sigma Aldrich®, TCI Chemicals®, VWR®, Jena Bioscience® or OlChemIm®). The organic solvents were evaporated on a rotary evaporator Heidolph® below 45 °C.

The reaction processes were controlled by thin layer chromatography (TLC) using silicagel $60WF_{245}$ plates (Merck®). Generally, $CHCl_3$/MeOH (9:1 or 3:1, v/v), $CHCl_3$/MeOH/NH_3 (9:1:0,05,v/v), toluene/acetone; (1:1, v/v), hexane/ethyl acetate (1:1, v/v) or EtOAc/MeOH/NH_3 (34:4:2, v/v) were used as the mobile phases. Davisil R LC60A 40–63 μm silica gel was used for purification of compounds by column chromatography.

The prepared compounds were characterized using an Alliance 2695 separations module (Waters®) linked to UV-VIS PDA 2996 detector (Waters®) and Q-Tof micro (Waters®) benchtop quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer. First, samples were dissolved in methanol and diluted with the mobile phase to 10 $\mu g.mL^{-1}$. Afterwards, 10 μL of the solution were injected onto a RP-column (150 × 2.1 mm; 3.5 μm; Symmetry C18, Waters®) which was kept in a thermostat at 25 °C. Solvent (A) consisted of 15 mM formic acid adjusted to pH 4.0 by ammonium hydroxide. Methanol was used as the organic modifier (solvent B). The effluent was introduced into the DAD (diode array detector; scanning range 210–400 nm with 1.2 nm resolution) equipped with an electrospray source (source temperature: 120 °C for positive mode, capillary voltage +3.0 kV, cone voltage +20 V, desolvation temperature 300°C). Nitrogen was used as both the desolvation gas (500 $L \cdot h^{-1}$) and cone gas (50 $L \cdot h^{-1}$). The mass spectrometer was operated in positive (ESI+) ionization mode. Data were acquired over the 50–1000 m/z range (fullscan mode).

For preparative purification of several compounds, HPLC-MS chromatography (Agilent 1290 Infinity II) coupled to UV-VIS and a mass detector (Agilent InfinityLab LC/MSD) with an Agilent column (5Prep-C18 5x21.2 mm) was used. Exact mass was determined by QTOF-MS (Synapt G2-Si, Waters®, UK) operating in positive ion mode.

Melting points were measured on the Büchi B-540 analyzer and elemental analysis was performed using an analyzer EA1112 CHN (Thermo Finnigan).

The Jeol 500 SSspectrometer at a temperature of 300 K and frequencies of 500.13 MHz (^{1}H NMR) and 125.03 MHz (^{13}C NMR) was employed for NMR spectra measurement. Tetramethylsilane (TMS) was used as an internal standard and DMSO-d^6 and $CDCl_3$ were used as solvents for the analysis (NMR spectra were calibrated against the residual solvent signal).

General synthetic procedures

The method using Castro's reagent was employed for the synthesis of all compounds of interest. Another methods were also performed, however, they were not as convenient as this method.

One-step method using Castro's reagent (BOP**)** (Wan *et al.*, 2005, 2007)

2′-Deoxyinosine (2′-deoxy-9-(*β*)-D-inosine; *M*r 252.23; 1 eq) or 2′,3′-dideoxyinosine (2′,3′-dideoxy-9-(*β*)-D-inosine; *M*r 236.23; 1 eq) and BOP ((benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; *Mr* 442.28; 1.2 eq) were dissolved in dry DMF (2 mL) under inert atmosphere (N_2) with DIPEA (1.5 – 5 eq) and after 5 min appropriate amine (1.2 – 4 eq) was added. The reaction was stirred at rt for 24 h under N_2 and monitored by TLC. The mixture was then evaporated (and co-evaporated with *n*-heptane) and with/without extraction (water/ethylacetate) pre-purified by column chromatography and then purified with preparative HPLC-MS chromatography or precipitated.

The specific reaction, TLC, precipitation or purification conditions are described in appropriate publications (Matušková *et al.*, 2020, 2023).

The method using the preparation of 6-chloro-2′-deoxy-9-(*β*)-D-ribofuranosylpurine for nucleophilic substitutions at the position 6

3′,5′-Di-O-acetyl-2′-deoxyadenosine (Seela, Herdering and Kehne, 1987)

2′-Deoxyadenosine monohydrate (2.2 g; 8 mmol) was dissolved in pyridine (26 mL) and acetanhydride (8 mL) was added dropwise at room temperature. Next, the reaction mixture was cooled in a crushed ice bath and DCM (100 mL) was added, followed by extraction with 10% aqueous $NaHCO_3$ (24 mL) and then water (20 mL). The organic solvent was evaporated and three times coevaporated with toluene to give a dry residue, which was then dissolved in absolute ethanol and crystallized in a refrigerator overnight.

6-Chloro-2′-deoxy-9-(β)-D-ribofuranosylpurine (Francom and Robins, 2003)

3′,5′-Di-*O*-acetyl-2′-deoxyadenosine (2 g; 5.8 mmol) in anhydrous DCM (150 mL) was placed into a dried three-neck flask under a nitrogen atmosphere (balloon). While stirring, the suspension was cooled in an ice bath to 0 °C and TMS-Cl (7.4 mL; 58 mmol) was added dropwise through the septum. Afterwards, a solution of *tert*-butyl nitrite (3.5 mL; 29 mmol) in dry DCM (30 mL) was slowly added and the reaction mixture was stirred for 2 h at 0 °C and then at room temperature overnight. Then, DCM (300 mL) was added and the organic phase was washed with 5% aqueous $NaHCO_3$ (2 × 300 mL), water (3 × 100 mL) and brine (3 × 100 mL), dried over powdered Na_2SO_4 and evaporated as a yellow oil (1.6 g). The residue was without further purification deprotected in 5% solution of NH_3 in methanol (30 mL) at 0 °C to give 6-chloro-2′-deoxy-9-(*β*)-D-ribofuranosylpurine (HPLC-MS of the residue: 77.7%). Column chromatography purification ($CHCl_3$/MeOH (9:1), v/v) was proceeded to obtain white-yellow solid product (0.7 g). The resulting 6-chloro-2′-deoxy-9-(*β*)-D-ribofuranosylpurine was subsequently used for nucleophilic substitutions with variably substituted benzylamines in *n*-propanol with base (TEA).

Following reactions were used, according to the literature, to the synthesis of *6-chloro-2′*,3´-*dideoxy-9-(β)-D-ribofuranosylpurine* ready for further nucleophlic substitution with amines, however, the laborious pathway led to the minimal yield of *β*-anomer. Nevertheless, *α-t*ZddR was prepared.

Multistep synthesis of *trans*-zeatin-9-(*α*)-D-2´,3´-dideoxyriboside (*α-t*ZddR)

(S)-γ-Butyrolactone-γ-carboxylic acid (**A**)

According to the literature (Okabe *et al.*, 1988), L-glutamic acid (1.0 mol; 147 g) was dissolved in H_2O (500 mL), cooled in ice-bath to 15 °C and solution of 5.6 M HCl (1.4 mol; 250 mL) was added dropwise to adjust pH 1. Then, the solution of $NaNO_2$ (1.5 mol; 104 g) in H_2O (144 mL) was added during 4h at 15-20 °C and the reaction mixture was stirred at rt overnight. Next day, the reaction mixture was controlled by TLC (toluene/acetone; 1:1; ninhydrin staining) which demonstrated full conversion of L-glutamic acid. The reaction mixture was co-evaporated with toluene and the white oil-solid residue (243 g) was diluted with EtOAc (1 L) and dried over powdered Na_2SO_4 (100 g). The precipitates were removed by filtration and washed by EtOAc. The combined organic extracts were stirred with 200-400 mesh H+ form (25 g) for 30 min to remove residual amino acid. After filtration and evaporation, the yellow oily residue was dried by evaporation with toluene (4 x 100 mL). The waxy residue (116 g) was dissolved in Et_2O

(200 ml), stirred at rt for 1h and cooled in the refrigerator overnight. The precipitate was filtered to give a white solid: *M*r 130.10; 91 g; yield 70 %. The structure was confirmed by elemental analysis, NMR (500 Hz; $CDCl_3$), GC-MS and melting point (62.6-63.2 °C).

(S)-γ-(Hydroxymethyl)-γ-butyrolactone (Okabe *et al.*, 1988) (**B**)

Borane-dimethylsulfide complex (Corey, Bakshi and Shibata, 1987) (BMS; 0.15 mol; 12.23 mL) was added in to (*S*)-γ-butyrolactone-γ-carboxylic acid (**A**, *M*r 130.10; 0.115 mol; 15 g) in dry THF (70 mL) during 40 min under 40°C (ice bath). The reaction was stirred for 2h at rt and quenched by MeOH (25 mL) while cooling. MeOH was evaporated 3 times (to remove formed trimethylborate) to give a light yellow oil: *M*r 116.12; 13.7 g; yield 102 %. The reaction was monitored by TLC (toluene/acetone; 1:1, v/v) and the structure was confirmed by NMR analysis.

(S)-γ-[[(Tert-butyldimethylsilyl)oxy]methyl]-γ-butyrolactone (Okabe *et al.*, 1988) (**C**)

(*S*)-γ-(hydroxymethyl)-γ-butyrolactone (**B**, *M*r 116.12; 0.11 mol; 13 g) and imidazole (0.14 mol; 9.7 g) in dry DCM (55 mL) were cooled to 0 °C under inert N_2 atmosphere. TBDMS-Cl (0.13 mol; 20 g) was added and the mixture was stirred 15 min at 0 °C and then 2h at rt. The solution was poured into cold H_2O (1 L) and 300 mL of DCM was added, and the water layer was washed with DCM (2 x 100 mL). Combined organic layers were washed with H_2O (2 x 400 mL) and brine (200 mL), dried with Na_2SO_4 overnight and evaporated to give light yellow oil: *M*r 230.38; 26 g; yield 100 %. For TLC mobile phase was hexane/EtOAc 3:1 with staining by 5% solution of H_2SO_4 in EtOH. The structure was confirmed by NMR analysis.

(S)-γ-[[(Tert-butyldimethylsilyl)oxy]methyl]-γ-butyrolactol (Okabe *et al.*, 1988) (**D**)

DIBAL in THF was slowly added during 1 h to (*S*)-γ-[[(tert-butyldimethylsilyl)oxy]methyl]-γ-butyrolactone (**C**, *M*r 230.38; 5 g) in THF under inert N_2 atmosphere, cooled by dry ice and acetone to -74 °C. The temperature was kept at -68 °C. After stirring for 5 min and TLC control (hexane/EtOAc 3:1) the reaction mixture was quenched by MeOH (5 mL) and the temperature was left to raise to rt. EtOAc (85 mL) and saturated $NaHCO_3$ (4.5 mL) were added and the mixture was stirred 3.5 h. Then Na_2SO_4 (25 g) was added and the mixture was stirred overnight. The solution was filtered, washed with EtOAc and the filtrate concentrated to give a colorless oil residue: *M*r 232.40; 5 g; yield 99 %. The structure was confirmed by NMR analysis.

6-Chloro-9-((5S)-5-(((isopropyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-9H-purine; *6-Cl-ddPR-OTBDMS* (Mitsunobu reaction) (Mitsunobu, 1981; Seio *et al.*, 2017) (**E**).

The solution of (*S*)-*γ*-[[(tert-butyldimethylsilyl)oxy]methyl]-*γ*-butyrolactol (**D**), *M*r 232.40; 11.7 mmol; 2.71 g) with 6-chloropurine (9.7 mmol; 1.5 g) in THF or DMF (0.07 M solution of 6-chloropurine obtained with 140 mL of THF and 10 mL of DMF) under inert N_2 atmosphere was cooled to 15 °C (ice bath). Then triphenylphosphine (14.5 mmol; 3.8 g) and DIAD (14.7 mmol; 2.9 ml) were added and the reaction was stirred for 1h (change of colour) and monitored by TLC (hexane/EtOAc, 1:1, v/v). The reaction mixture was then evaporated and co-evaporated with toluene and diluted with toluene (30 ml) to give a brown oil which was cooled overnight to precipitate $POPh_3$ (white solid). After filtration and washing with toluene, the crystallization process was repeated and the crude product (anomeric mixture) was roughly purified by column chromatography (hexane/EtOAc 1:1) to give 2 g of thick to solid yellow oil (a mixture of *α* and *β* anomer in favour of one anomer, according to the TLC). The HPLC analysis with acidic mobile phase (pH 4) and the cone voltage 20 eV led to molecule breakage and 6-chloropurine was observed in high percentage even if TLC analysis showed no visible amount of 6-chloropurine. Therefore, the conditions for HPLC analysis were adjusted to pH 5.3 of the mobile phase and the cone voltage set to 10 eV to lower the molecule breakage throughout the analysis. There were several attempts to purify each anomer by column chromatography, but due to the close Rf of both products the separation was unsuccessful. Elemental analysis of anomeric mixture was proceeded: calculated %: N(15.19), C(52.09), H(6.83); the first measurement N(14.88), C(51.77), H(7.51) and the second measurement N(14.90), C(51.46), H(7.57) and melting temperature range 77.4 – 77.9 °C. Preparative HPLC-MS was proved to be the best tool for the separation of individual anomers, but we decided to firstly nucleophilically substitute the position 6-Cl.

(E)-4-((9-((5S)-5-(((Isopropyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-9H-purin-6-yl)amino)-2-methylbut-2-en-1-ol; *tZ-ddPR-OTBDMS* (**F**)

6-Chloro-9-((5*S*)-5-(((isopropyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-9*H*-purine (**E**, *M*r 368.94; 0.9 mmol; 334 mg) and (*2E*)-2-methyl-4-aminobut-2-en-1-ol hydrochloride (Mr 137.61 g/mol; 1.4 mmol; 187 mg) were stirred for 5 min in *n*-propanol (6 mL) under inert N_2 atmosphere. DIPEA (2.7 mmol; 465 μL) was then added and the mixture was refluxed in a sealed tube and stirred for 5 h. The reaction was monitored by TLC (EtOAc/hexane; 6:1, v/v) and upon completion, the mixture was evaporated to give a yellow oil: *M*r 433.63; 393 mg. Due

to the close Rf of each anomers, the crude residue was used in the next deprotection without further purification.

Trans-zeatin-9-(α)-D-2′,3′-dideoxyriboside (α-*t*ZddR)

The anomeric mixture of the protected product (**F**), *M*r 433.63; 0.9 mmol; 393 mg) was dissolved in dry THF (20 mL) and while cooling, TBAF trihydrate (0.9 mmol; 286 mg) in THF (10 mL) was slowly added, the mixture was stirred at rt for 2.5 h and controlled by TLC (hexane/EtOAc 1:1 or chloroform/MeOH 9:1). The deprotected anomeric mixture was evaporated and purified by column chromatography (approximately 3:1; 93 % HPLC-UV) and the anomer with the greater yield was successfully separated by preparative HPLC-MS (Mr 319.37, to give a pale-yellow oil, 165 mg; yield 57 % of the last step; HPLC-UV purity >99 %). The structure was characterized by HRMS, and NMR. The 2D-NMR experiment NOESY found that the structure probably corresponds to the α anomer due to the presence of $-CH_2$-OH group of the ribose moiety as well as the purine scaffold in the *cis* position towards the planar sugar ring.

Biological activity testing

Cytotoxicity evaluation

The resazurin reduction assay was used to evaluate the effects of 72 h treatment with the compounds on the viability of human skin fibroblasts BJ, keratinocytes HaCaT and retinal pigment epithelium cells ARPE-19. The cell lines were obtained from the American Type Culture Collection, Manassas, VA, USA (BJ, ARPE-19) and the German Cancer Research Center (*DKFZ*), Heidelberg, Germany (HaCaT). The cells were maintained in DMEM (BJ, HaCaT) or DMEM/F12 (ARPE-19) culture medium (Sigma) supplemented with fetal bovine serum (10%), glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) under standard cell culture conditions (37 °C, 5% CO_2, humid environment) and sub-cultured two or three times a week. For testing, about 5000 cells were seeded into each well of a 96-well plate about 24 h before treatment with the test compounds at six concentrations up to 100 μM or DMSO. The final concentration of DMSO did not exceed 0.1%. An 11x concentrated solution of resazurin in DMEM medium was added to the cells to a final concentration of 100 μM after 72 h. Fluorescence intensity (λ ex/em = 570/610 nm) was measured after 3 h incubation.

The *in vitro* assay was performed at the Department of Experimental Biology, Faculty of Science, Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, Czech Republic under the supervision of Mgr. Jiří Voller, Ph.D.

Cytokinin bioassays

Amaranthus bioassay

Amaranthus caudatus (L.), 1753 var. atropurpurea seeds were sterilized on their surface, placed on a Petri dish containing paper tissue soaked with deionized H_2O 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 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 Petri 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.

The *Amaranthus* bioassay was performed by Jana Balonová (compound series from **Publication I**) and Mgr. Hana Vylíčilová, Ph.D. (compound series from **Publication II**) at the Department of Chemical Biology, Faculty of Science, Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, Czech Republic.

Wheat leaf senescence bioassay

Wheat seeds (*Triticum aestivum* (L.), 1753 cv. Aranka) were washed with 96% EtOH and H_2O and 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 to give a total weight of 0.1 g (±1 mg) per well, immersed by the basal part in a well containing solution of the test compound (DMSO solution diluted in deionized H_2O, 150 µl/well), and cultivated in a closed box at 24 °C for 96 h in the dark. Residual chlorophyll was extracted by heating the leaf material in 5 ml of 80% EtOH 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 and extracts of leaves cultivated in deionized H_2O with DMSO (0.002%, v/v).

The wheat leaf senescence bioassay was performed by Jana Balonová (compound series from **Publication I**) and Mgr. Hana Vylíčilová, Ph.D. (compound series from **Publication II**)

at the Department of Chemical Biology, Faculty of Science, Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, Czech Republic.

Tobacco callus bioassay

Cytokinin-dependent tobacco callus cells (*Nicotiana tabacum* (L.), 1753 cv. Wisconsin 38) were cultivated on solid MS medium (3 ml/well) containing different concentrations of the test compound (from 10^{-9} to 10^{-4} M solution in DMSO) in 6-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) or the respective cytokinin base.

The tobacco callus bioassay was performed by Jarmila Balonová (compound series from **Publication I**) and Mgr. Hana Vylíčilová, Ph.D. (compound series from **Publication II**) at the Department of Chemical Biology, Faculty of Science, Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, Czech Republic.

Bacterial receptor cytokinin assay (Cytokinin signaling assay)

Transgenic *E. coli* strains KMI001 harboring a PINIII/AHK4 or pSTV28/AHK3 plasmid and expressing the *β*-galactosidase gene (*ΔrcsC, cps::lacZ*) under the control of cytokinin receptors were used for this assay. Expression of the *β*-galactosidase gene was induced by cultivation of 200 μL of the precultures diluted by M9 medium with antibiotic (1:600) and test compound (50, 10, 1 or 0.1 μM) with shaking (450 rpm) at 25 °C for 17 h. At the end of the incubation period, 50 μL of the bacterial cultures was transferred to a new 96-well plate and the activity of *β*-galactosidase was determined by measuring the fluorescence intensity (λex/em − 365/460 nm) after incubation with 2 μL of 10 mM (25 mM for AHK3) chromogenic substrate (MUG) at 37 °C for 10 min (AHK4) or 30 min (AHK3) and addition of 100 μL of Stop buffer (132 mM glycine, 83 mM Na_2CO_3).

The *in vitro* assay was performed by Mgr. Zuzana Pěkná under the supervision of Mgr. Lukáš Spíchal, Ph.D. (compound series from **Publication I**) and Mgr. Hana Vylíčilová, Ph.D. (compound series from **Publication II**) at the Department of Chemical Biology, Faculty of Science, Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, Czech Republic.

Competitive ligand binding assay

The *in vitro* assay using the *Escherichia coli* BL21(DE3) strain expressing cytokinin receptor AHK2, AHK3 and CRE1/AHK4 (kindly provided by Thomas Schmülling) to cytokinin competitive ligand binding of compounds from **Publication II.** with [^{3}H] *trans*-zeatin, as was reported previously (Yamada *et al.*, 2001), was performed by Mgr. Hana Vylíčilová, Ph.D. at the Department of Chemical Biology, Faculty of Science, Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, Czech Republic.

Antioxidant capacity testing

The *in vitro* assay examining the total phenolic content (TPC) expressed as gallic acid equivalents of compounds from **Publication I**. was performed by Mgr. Jana Slobodianová at the Laboratory of Growth Regulators, The Czech Academy of Sciences, Institute of Experimental Botany and Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, Czech Republic under the supervision of Mgr. Jiří Grúz Ph.D.

Antiviral activity studies

The experimental procedures to examine the antiviral activity of compounds from **Publication I.** on MHV-68 were performed by RNDr. Katarína Briestenská, Ph.D. at the Institute of Virology, Biomedical Center SAS, Dúbravská cesta 9, 845 05 Bratislava, Slovak Republic under the supervision of and Prof. RNDr. Jela Mistríková, DrSc.and are described in detail in the **Publication I.**

12. Survey of published results

Publication I.

Matušková, V., Zatloukal, M., Voller, J., Grúz, J., Pěkná, Z., Briestenská, K., Mistríková, J., Spíchal, L., Doležal, K., Strnad, M.: **New aromatic 6-substituted 2′-deoxy-9-(*β*)-D-ribofuranosylpurine derivatives as potential plant growth regulators**. *Bioorganic & Medicinal Chemistry*. 2019; 28. 115230.

A series of 33, when compared with respective ribosides non-cytotoxic, N^6-substituted aromatic 9-(*β*)-D-2′-deoxyribofuranosylpurines was prepared using the reaction of unprotected 2′-deoxyinosine with Castro′s reagent (BOP) (**Scheme 7**) and appropriate amine under mild conditions. Although one different synthetic approach was also exmined – the nonaqueous diazotiation/dediazoniation of protected 2′-deoxyadenosine resulting to the protected 6-chloroderivative. The second method was not as convenient as the BOP method, however, prepared in larger scale it would be a great starting material after deprotection for further nucleophilic substitutions of various amines in one step.

Despite the lower yields, the library of compounds was tested in classical cytokinin bioassays (*Amaranthus,* tobacco callus and wheat leaf senescence (WLSA)) and compared with BAP as well as with BAP-2′-deoxyriboside. Surprisingly, the presence of 2′-deoxyribosyl moiety generally contributed to the anti-senescence activity of the compounds. The 3-hydroxy-benzylaminopurine-9-(*β*)-D-2′-deoxyriboside (3-OH-BAPdR), 3-methoxy-benzylaminopurine-9-(*β*)-D-2′-deoxyriboside (3-OMe-BAPdR) and 4-fluoro-benzylaminopurine-9-(*β*)-D-2′-deoxyriboside (4-F-BAPdR) delayed the senescence approximately 3-times more than BAP. 3-OH-BAPdR and 3-OMe-BAPdR had also great results in *Amaranthus* and tobacco callus assays comparable to that of BAP, however, with different behaviour at highest concentrations (> 10^{-5} M) than BAP which did not result in dramatic drop of activity due to the toxic effects.

When the AHK2, AHK3 and CRE1/AHK4 receptor activation was examined none of the tested 2′-deoxyribosides approached the activity of the standard *trans*-zeatin. Therefore, cytokinin receptor activation by aromatic purine-2′-deoxyribosides was not observed but the activity in cytokinin assays was obvious.

In the study of the antioxidant properties of compounds, the presence of phenolic hydroxygroup positively contributed, especially 2-OH, 4-OH, 2-OH-3-OMe, 4-OH-3-OMe or 3-OH-4-OMe.

Furthermore, the opportunity to test the series in Vero cells infected by murine herpesvirus (MHV-68) led, unfortunately, to no interesting result, nevertheless, the potential antivirotic activity towards another viruses would be advantageous to examine and the non-cytotoxicity of these substances is beneficial.

Publication II.

Matušková V., Zatloukal, M., Pospíšil, T., Voller, J., Vylíčilová, H., Doležal, K., Strnad, M.: **From synthesis to the biological effect of isoprenoid 2'-deoxyriboside and 2',3'-dideoxyriboside cytokinin analogues**. *Phytochemistry*. 2023; 205:113481.

The commercially available isoprenoid cytokinin bases and (*β*)-D-ribosides (iP, *t*Z, *c*Z, iPR, *t*ZR, *c*ZR) were examined together with prepared analogous series of isoprenoid (*β*)-D-2′-deoxyribosides and (*β*)-D-2′,3′-dideoxyribosides (iP-2′-dR, *t*Z-2′-dR, *c*Z-2′-dR, iP-2′,3′-ddR, *t*Z-2′,3′-ddR, *c*Z-2′,3′-ddR) in various bioassays. The synthetic approach using the Castro′s reagent was employed (**Scheme 7**) starting from 2′-deoxyinosine to form 2′-deoxyribosides and from 2′,3′-dideoxyinosine to form 2′,3′-dideoxyribosides. Another laborious synthetic pathways were employed, however, the one-step method proved its usefulness. Nevertheless, the *α*-anomer of *t*Z-2′,3′-ddR was prepared and also tested in all assays.

In the bacterial receptor assay, as well as in the *ARR5::GUS* assay or ligand binding assay, the receptor activation trend played in favor of bases as follows: base > riboside > 2′-deoxriboside > 2′,3′-dideoxyriboside proving the generally decreasing cytokinin signaling activity connected with the removal of 2′ and 3′ hydroxygroup from the sugar moiety.

From the prepared series only iP-2′,3′-dideoxyriboside considerably caused the moderate root growth inhibition in comparison with *c*Z-2′,3′-dideoxyriboside and *t*Z-2′,3′-dideoxyriboside, 2′-deoxyribosides did not exhibit any activity in this assay; only iP, *t*Z and their ribosides inhibited primary root growth as a result of cytokinin action.

Emphasizely, the betacyanin production in *Amaranthus* bioassay as well as the callus growth were highly supported by isoprenoid 2′-deoxyribosides which can be partly influenced by the N9-substituent hydrolysis. Nevertheless, the WLSA assay did not show any significant effect of isoprenoid 2′-deoxyribosides or 2′,3′-dideoxyribosides.

Publication III.

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(6):832.

The review summarizes the role of artificial and endogenous N9-substituted cytokinin conjugates in plants. The advances in knowledge of 9-substituted cytokinin conjugates from the discovery to current state-of-art are described. The emphasis is placed on the efforts toward enhancement of the efficiency of novel derivatives in agriculture and crop production or in anticancer or antiviral therapies.

Various known cytokinin conjugates and their historical evolution as well as practical outcomes, activation or inactivation, are described: N7- and N9-glucosides; isoprenoid and aromatic N9-ribosides; N9-2′-deoxyribosides; N9-arabinosides; cytokinin disaccharide conjugates or N9-non-sugar substituted analogues including N9-tetrahydropyranyl derivatives are described in details.

The development of novel cytokinin compounds based on the current knowledge and obtaining the new information can lead to syntheses of substances with predetermined properties in certain type of plant and thus the success in cultivation.

Publication IV. (Utility model)

Matušková, V., Zatloukal, M., Koprna, R., Doležal, K.: **Směs pro ošetření zemědělských plodin pro zvýšení výnosu a odolnosti proti houbovým chorobám a přípravek pro foliární aplikaci, obsahující tuto směs**. Užitný vzor (Utility model) CZ 32628 U1, 2018.

The utility model presents the use of substances 3-hydroxy-benzylaminopurine-9-(*β*)-D-2′-deoxyriboside (3-OH-BAPdR) and 3-methoxy-benzylaminopurine-9-(*β*)-D-2′-deoxyriboside (3-OMe-BAPdR) in a mixture with macronutrients (N, P, K) and micronutrients in foliar application to agricultural crops (spring barley *Francin* and winter wheat *Turandot*) and shows an increase in yield and resistance to fungal diseases based on field trials.

After the dissolution of substance in 1 mL of DMSO the solution was diluted to the final concentration of 5 μM and foliary applied on the field area of 10 m^2 (in 5 repetitions).

13. Conclusion and perspectives

The library of the 33 N^6-variously substituted aromatic cytokinin 9-(β)-D-2′-deoxyribosides was synthesized using predominantly one-step approach utilizing the Castro′ reagent. The biological activity was evaluated and compared with the respective bases and ribosides. Despite the inability to activate cytokinin receptors many of these substances succeeded in wheat leaf senescence, tobacco callus and *Amaranthus* bioassays and showed higher activity than the standard benzylaminopurine. The antiviral activity towards murine herpesvirus has not been proved, however further study should yield new knowledge.

The series of isoprenoid cytokinin 9-(β)-D-2′-deoxyribosides and 9-(β)-D-2′,3′-dideoxyribosides derived from iP, *t*Z and *c*Z was also synthesized and the biological effects were compared with the respective bases and ribosides. Similarly to the aromatic 2′-deoxyribosides the activation of cytokinin receptor was not significant for isoprenoid 2′-deoxyriboides and 2′,3′-dideoxyribosides. Nevertheless, the outcome in bioassays was significant, especially in *Amaranthus* and tobacco callus bioassays.

The aromatic 2′-deoxyribosides, as well as isoprenoid 2′-deoxy- and 2′,3′-dideoxy-derivatives were prepared by convenient one-step method but different approaches were also employed.
In both cases, the possible partial hydrolysis of the sugar moiety during bioassays has to be mentioned.

All the prepared 2′deoxyribosides and 2′,3′-dideoxyribosides are non-cytotoxic and thus suitable for further (e.g. antiviral) examination.

14. List of publications

I. **Matušková, V**., Zatloukal, M., Voller, J., Grúz, J., Pěkná, Z., Briestenská, K., Mistríková, J., Spíchal, L., Doležal, K., Strnad, M.: New aromatic 6-substituted 2´-deoxy-9-(*β*)-D-ribofuranosylpurine derivatives as potential plant growth regulators. *Bioorganic & Medicinal Chemistry*. 2019; 28. 115230. 10.1016/j.bmc.2019.115230

II. **Matušková V**., Zatloukal, M., Pospíšil, T., Voller, J., Vylíčilová, H., Doležal, K., Strnad, M.: From synthesis to the biological effect of isoprenoid 2'-deoxyriboside and 2',3'-dideoxyriboside cytokinin analogues. *Phytochemistry*. 2023; 205:113481. 10.1016/j.phytochem.2022.113481. PMID: 36283448.

III. 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(6):832. 10.3390/biom10060832

IV. **Matušková, V**., Zatloukal, M., Koprna, R., Doležal, K.: Směs pro ošetření zemědělských plodin pro zvýšení výnosu a odolnosti proti houbovým chorobám a přípravek pro foliární aplikaci, obsahující tuto směs. Užitný vzor (Utility model) CZ 32628 U1, 2018.

15. Contribution report

I. First author – synthesis, characterization of compounds, interpretation of results, writing the manuscript and supporting information

II. First author – synthesis, characterization of compounds, interpretation of results, writing the manuscript and supporting information

III. Co-author – writing the chapter 2.3

IV. Co-author – synthesis and characterization of compounds, writing the synthetic procedures

16. References

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Supplementary material I.

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New aromatic 6-substituted 2′-deoxy-9-(β)-D-ribofuranosylpurine derivatives as potential plant growth regulators

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ABSTRACT

Cytokinins are naturally occurring substances that act as plant growth regulators promoting plant growth and development, including shoot initiation and branching, and also affecting apical dominance and leaf senescence. Aromatic cytokinin 6-benzylaminopurine (BAP) has been widely used in micropropagation systems and biotechnology. However, its 9-glucoside (BAP9G) accumulates in explants, causing root inhibition and growth heterogenity. To overcome BAP disadvantages, a series of ring-substituted 2′-deoxy-9-(β)-D-ribofuranosylpurine derivatives was prepared and examined in different classical cytokinin bioassays. *Amaranthus*, senescence and tobacco callus bioassays were employed to provide details of cytokinin activity of 2′-deoxy-9-(β)-D-ribosides compared to their respective free bases and ribosides. The prepared derivatives were also tested for their recognition by cytokinin receptors of *Arabidopsis thaliana* AHK3 and CRE1/AHK4. The ability of aromatic N^6-substituted adenine-2′-deoxy-9-(β)-D-ribosides to promote plant growth and delay senescence was increased considerably and, in contrast to BAP, no loss of cytokinin activity at higher concentrations was observed. The presence of a 2′-deoxyribosyl moiety at the N9-position led to an increase in cytokinin activities in comparison to the free bases and ribosides. The antioxidant capacity, cytotoxicity and effect on the MHV-68 gammaherpesvirus strain were also examined.

1. Introduction

Plant hormone cytokinins, N^6 substituted derivatives of the purine base adenine, are responsible for many events in plant growth and development, such as cell division, initiation of shoots, apical dominance and leaf senescence.[1,2] According to the substitution in position N^6 of the adenine scaffold, cytokinins can be divided into two main groups: isoprenoid, such as N^6-isopentenyladenine (iP), dihydrozeatin, *cis*-zeatin (cZ), *trans*-zeatin (tZ) and aromatic, such as kinetin (K), 6-benzylaminopurine (BAP) and hydroxylated derivatives topolins (*o*T, *m*T, *p*T).[3] BAP is one of the most effective and affordable cytokinins used in micropropagation systems and biotechnology.[4] However, BAP has disadvantages in some crops as the main metabolite BAP-9-glucoside (identified for example in *Spatiphyllum floribundum* or *Harpagophytum procumbens*[5,6]), accumulates in explants and its slow release can cause growth heterogenity and rooting inhibition.[7] One way to overcome the negative effects is to use cytokinins conjugated at position 9 of the purine ring, e.g., with a ribose moiety (nucleoside) or tetrahydropyranyl group, or to use substituted BAP analogues (e.g., hydroxylated) on the phenyl ring.[4] These compounds show more divergent metabolism and yield lower levels of the 9-glucoside (9G).[6,8] Another possibility is to use a hydroxylated cytokinin base, such as 6-(3-hydroxybenzylamino)purine, known as *meta*-topolin. Horgan et al.[9,10] were the first to isolate *meta*-topolin and *ortho*-topolin from poplar leaves. Strnad et al.[11–14] later isolated these compounds together with their ribosides and 9-glucosides. Subsequently, *meta*-topolin was tested for its potential use in the micropropagation industry.[4,8,15,16] The significant difference in cytokinin activity observed for the hydroxylated derivative of BAP prompted further investigation of structural changes and their impact on cytokinin activity. Series where the benzyl ring was

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substituted with aromatic purine cytokinin bases[17,18] and ribosides[19] have been studied. In addition to cytokinin activity, cytotoxicity has also been examined.[19] However, the presence of the ribose moiety was found to be crucial for the cytotoxic effect.[19]

In this project, one-step synthesis of a series of 6-substituted 2′-deoxy-9-(β)-D-ribofuranosyl purines using variously substituted benzylamines was performed. A phosphonium salt mediated C—N bond formation reaction with unprotected nucleosides and arylamines under mild and non-metal-catalyzed conditions was used as described by Wan et al.[20] Castro's reagent[21] (BOP) and PyBOP[22] are (benzotriazolyloxy) phosphonium reagents frequently used in peptide synthesis,[23] particularly cyclic peptides.[24] BOP is also often used for difficult couplings of amino acids to secondary amines[25] or weakly nucleophilic heteroaromatic amines.[26] Its capability to convert aldoximes to nitriles was described in 2009.[27] The most promising and significant use of BOP has so far been to activate heterocyclic lactams and ureas, leading to formation of substituted aromatic heterocycles.[28,29] This approach has been utilized for preparation of a wide variety of nucleosides and nucleoside derivatives[20,30–33] and for the synthesis of kinetin and the kinase inhibitor olomoucin.[34] In 2017, BOP was employed for functionalization at the C4 position of pyrimidine nucleosides by amide group activation, allowing facile introduction of various nucleophiles[35] and cementing the present significance of the reagent.

In this study, we compared the above mentioned method using BOP reagent with the halogen-derivative approach to synthesize a novel series of substituted 6-benzylamino-2′-deoxy-9-(β)-D-ribofuranosylpurine derivatives and then analyzed their cytokinin activity. Their anti-senescence (retardation of wheat leave chlorophyll degradation), antioxidant, and non-cytotoxic activities were examined. The antiviral activity of BAPR derivatives and BAPdR has previously been studied.[36–39] Evaluation of the antiviral effect of the presented derivatives was started by testing murine alfa gammaherpesvirus (MHV-68). However, a more advanced antiviral study is planned in the future.

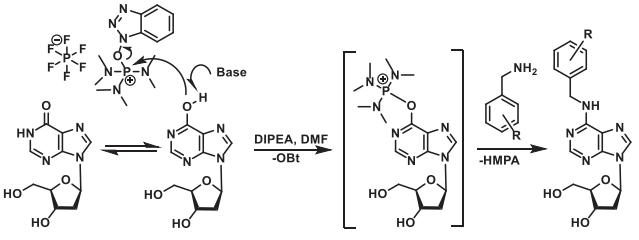
2. Results and discussion

2.1. Chemistry

The synthetic chemistry of 2′-deoxy-9-(β)-D-ribofuranosylpurines stretches far back into history, e.g., in 1914, Fischer and Helferich[40] obtained nucleosides by the chloromercuri method and Davoll and Lowy prepared 9-(β)-D-ribopyranosyladenine[41] from chloromercuri-6-acetamidopurine[42] in xylene with triacetyl D-ribopyranosyl chloride[43]. For syntheses of purine nucleoside derivatives, it is advantageous to have a suitable halogen derivative. In the early 1960s, 6-chloro-9-(2′-deoxy-(β)-D-*erythro*-pentofuranosyl)purine was prepared by base-sugar coupling using the chloromercuri procedure[44] or fusion method[45] but with only about 6% yield. In 1984, Kazimierczuk[46] described the synthesis of 6-chloro-2′-deoxyadenosine using 6-chloropurine and sodium hydride in anhydrous acetonitrile, followed by alkylation with a suitable protected sugar and ammonolysis. For synthesis of 6-halogen-2′-deoxynucleoside derivatives, attempts at direct chlorination of acylated 2′-deoxyinosine under conditions used for the halogenation reaction of tri-*O*-acylated inosine failed due to the acid and heat instability of purine 2′-deoxynucleosides.[47–49] Specific conversion of ribonucleosides to 2′-deoxynucleosides, as described by Robins and Wilson[50], can be achieved by selective protection of 3′,5′-hydroxyl groups with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane in pyridine to give a nucleoside in which only the 2′-hydroxyl group is available for further modification. In 2000, Lin and Robins[51] reported development of mild and efficient procedures for transformation of protected inosine and 2′-deoxyinosine into C6-substituted purine derivatives using I_2/Ph_3P/EtN(*i*-Pr)$_2$ in the presence of secondary amines (morpholine, piperidine or imidazole) in CH_2Cl_2 or toluene. The prepared 9-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-6-(imidazol-1-yl)purine was stirred in benzylamine at 80 °C for 2 days to form 6-*N*-(benzyl)-2′-deoxyadenosine. They also presented displacement of imidazole with benzylthiolate and oxidation of the resulting benzylsulfide to give 6-(benzylsulfonyl)purine derivatives, which can undergo substitution with an arylamine. However, the imidazoyl group was not displaced as readily as a halogen. This method is more suitable for functionalization of 6-oxopurine 2′-deoxynucleoside derivatives than halo-deoxygenations using Vilsmeier-Haack reagents and phosphoryl-halides[52–54] because of the lability of 2′-deoxynucleosides to acidic environments.[55] On the other hand, imidazolyl group can be substituted by nucleophiles but not as readily as a halogen. Nonaqueous diazotiation/halo-dediazotiations (the term ¨dediazoniation¨ was introduced by Bunnett[56] in 1954) provides efficient transformation of aminopurine nucleosides, as presented by Francom and Robins.[57] Weiss and Wagner[58] postulated that nitrosylhalides can be generated in situ in chlorinated hydrocarbons by reaction of trimethylsilyl halides with alkyl nitrites and there is no need to prepare these gases separately for reaction of nucleoside derivatives. By this procedure, 6-chloro-2′-deoxy-9-(β)-D-ribofuranosylpurine can be obtained from 2′-deoxyadenosine. The first step involves acetylation of a free hydroxy group of the sugar moiety and this intermediate compound then undergoes a chloro-dediazoniation reaction in dried dichloromethane with TMS-Cl and benzyltriethylammonium nitrite (or *tert*-butyl nitrite) under an inert atmosphere. Adamska, Barciszewski and Markiewicz[59] published an approach for syntheses of N^6- and N^4-substituted adenines and cytosines, and 6-arylamino-substituted 2′-deoxypurineribosides, particularly 2′-deoxy-N^6-furfuryladenosine (kinetin 2′-deoxyriboside), using unprotected 2′-deoxyadenosine as a starting material and an appropriate aromatic aldehyde in methanol. The reaction was catalyzed by magnesium methanolate, followed by reduction of a Schiff base by sodium borohydride. After workup, the reaction yield ranged from 40% to 64% of 2′-deoxy-N^6-furfuryladenosine as a white solid. In 2017, Seio[60] *et* al. published a synthesis for 2′-deoxynucleosides by the Mitsunobu[61] reaction starting from unprotected adenine 2-deoxy-3,5-*O*-bis(*tert*-butylsilyl)-D-ribose and tri(*n*-butyl)phosphine. Th synthesis gave a 65% yield of an anomer mixture (70:30, β:α) after column chromatography.

In the present work, a series of novel 2′-deoxy-9-(β)-D-ribofuranosylpurine derivatives (**2–34**) was synthesized via a one-step reaction[20,28] of unprotected 2′-deoxyinosine with 1*H*-benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP), resulting in conversion to O^6-(benzotriazol-1-yl) derivatives able to easily replace 1-hydroxybenzotriazolide (BtO^-) with a nucleophile (amine) (Scheme 1). The main procedure, under mild conditions, was similar for the whole series. The starting material, i.e., 9-(2′-deoxy-(β)-D-ribofuranosyl)hypoxanthine (2′-deoxyinosine), was dissolved in dry DMF with Castro's reagent under inert conditions (atmosphere N_2), followed by addition of DIPEA (base) and then, after few minutes, a benzylamine as the last reactant. This order was important and if it was not followed, the reaction hardly occured. The mixture was stirred at 55 °C overnight.

Only when using unsubstituted benzylamine did the reaction occur at room temperature under stirring and the product was precipitated from ethanol without the need for column chromatography purification. Despite the fact that Wan et al.[20] prepared 6-benzylamino-derivative from 2′-deoxyinosine by the same method in 99% yield, we were



Scheme 1. Preparation of substituted 6-benzylamino-2′-deoxy-9-(β)-D-ribofuranosylpurine derivatives by a method using Castro's reagent.

only able to obtain the isolated product in 25% yield but with excellent purity due to the crystallization procedure.

In the case of substituted benzylamines, different reaction conditions were needed. The reaction temperature was optimized at 55 °C; room temperature or higher temperature (70–80 °C) did not result in sufficient yield. Use of a microwave reactor (80 °C and 50 W, 30 min) to shorten the reaction time and increase the yield resulted in a mixture of unknown fragments. Therefore, this treatment was deemed too harsh for this type of derivative. The reaction time was optimized at 24 h; prolonging the reaction to 48 h did not increase the starting material consumption or ammount of reacted reagent (BOP) or base (DIPEA).

The best ratio of the reaction components was established as follows: 9-(2′-deoxy-(β)-D-ribofuranosyl) hypoxanthine (1 eq.); BOP (1.2 eq.); DIPEA (3 eq. were better than 2 eq.) and the corresponding benzylamine (1.2 eq.).

After partial purification by extraction, the evaporated residue was purified by column chromatography in mobile phase system $CHCl_3$/MeOH/NH_3 (9:1:0,05, v/v) or EtOAc/MeOH/NH_3 (34:4:2, v/v). Crystallization or precipitation of the product without previous purification by column chromatography or preparative TLC (mostly both) was impossible due to the large amount of residual starting material and many impurities occurring close to the Rf (retention factor) of the product. Based on the literature[20,28], the normal measured byproduct is the 2′-deoxyribosyl-cleaved 6-HOBt derivative of purine ($M + H^+ = 254.58$; $M = 253.22$). However, among the many small impurities, there was a significant unidentified impurity with $M + H^+ = 280.77$ close to the Rf of the product. Also, HMPA (*hexamethylphosphorotriamide*) with mass $M = 179.20$ in the crude residue was detected but was successfully removed by a purification process.

Finally, after successful purification to a HPLC-MS purity of approximately 85–90%, a residue was precipitated from various combinations of solvents, predominantly absolute ethanol, diethylether, acetone and dichloromethane. For example, compounds **5** and **19** were precipitated in diethylether; **7**, **17**, **21**, **30** and **31** in a combination of diethylether and dichloromethane; and derivatives **6**, **8** and **15** in absolute ethanol. After purification and isolation, the newly prepared 2′-deoxyadenosines were characterized by HPLC-PDA-MS and ^1H and ^{13}C NMR. Expected proton positions on the main scaffold were confirmed by COSY-NMR. In Table 2, the structure, purity and yields of the prepared derivatives are summarized. A procedure using a halogen derivative (Scheme 2) obtained by a halo-dediazotiation reaction[57,62] was also employed to compare two different approaches. Use of a OH-protected chloroderivative (6-chloro-3′,5′-di-O-acetyl-2′-deoxy-9-(β)-D-ribofuranosylpurine) as a starting material for nucleophilic substitution with a relevant benzylamine proved to be counterproductive and unnecessary because a mixture of byproducts (partially unprotected derivatives) was formed.

Although we compared two different methods, all final compounds presented here were prepared by activation of the starting material by Castro's reagent, followed by nucleophilic substitution (Scheme1) according to Wan et al.[20,28] The main aim of the work was to obtain biological activity test results.

Overall, the procedure using Castro's reagent (BOP) was found to be the most convenient and straightforward method for preparing the series of derivatives. On the other hand, purification was more complicated due to the large amount of impurities occurring close to the Rf of the product. Another difficulty was insufficient conversion of the starting material, resulting in reduced yields. Use of the halogen derivative method was considered advantageous if a large amount of the 6-chloro-2′-deoxy-9-(β)-D-ribofuranosylpurine is prepared in high purity. Following nucleophilic substitution, this method yielded a less complex mixture that was easier to purify by conventional chromatographical methods, but it was not possible to avoid significant cleavage of the nucleoside bond. Eventually, both methods gave an approximately similar percentage of isolated yields (data not shown). The stability of the prepared derivatives in solution was related to the pH of the solvent due to the chemical lability[63] of the glycosylic hemiacetal-type nucleoside bond. The purity of exemplary compounds dissolved only in DMSO and stored in a refrigerator remained unchanged for at least a month. The purity of exemplary compounds stored in the solid state at room temperature also remained unchanged for at least two years (Table 1).

Table 1
Comparison of HPLC-UV stability of exemplar samples stored in DMSO in a refrigerator and measured after different storage times.

Compound in DMSO	HPLC-UV purity in time (%)				
	0 h	**24 h**	**7 days**	**2 weeks**	**1 month**
1	98	98	98	98	96
3	98	98	98	> 97	> 97
6	99	99	98	98	98

2.2. Biology

In 2006, Doležal et al.[17] prepared and studied structure-activity relationships (SARs) of BAP derivatives in plants. Three cytokinin bioassays were employed, based on stimulation of different physiological processes in plants: *Amaranthus*, tobacco callus and wheat senescence bioassays. The prepared panel of derivatives was also tested if they were recognized by cytokinin AHK3 and CRE1/AHK4 receptors. This study revealed that *para*-substitution in monosubstituted derivatives reduced cytokinin activity, whereas *ortho*- and *meta*-substitution caused a significant increase. In particular, 2- and 3-methoxy-BAP[18] and 3-fluoro-BAP derivatives[17] exceeded the activity of BAP by two fold in the senescence bioassay. The converse applied to the 2-hydroxyderivative,[64] for which a H-bond formed between the 2-OH group and nitrogen N1 of the purine ring, essential for cytokinin activity.[65] Similar findings were described previously[66] and help to explain differences between the cytokinin activity of the *cis*- and *trans*-isomer of zeatin. In another large study[19] of substances derived from BAP, i.e., benzyl ring substituted 6-benzylamino-9-(β)-D-ribofuranosylpurines, the cytokinin activity was shown to be influenced by the nature and position of the substituent. As in the previously mentioned study, in our work, *para*-substitution generally caused lower activity, whereas *ortho*- or *meta*-substitution had the opposite effect. However, the *meta*-position showed better efficacy in plant bioassays.

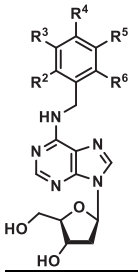
It is important to emphasize that the presence of a ribose moiety in the N^9 position of the purine scaffold is crucial for the cytotoxicity of these cytokinin-derived analogues. The presence of an *ortho*-hydroxy group on the phenyl ring is also connected with stronger cytotoxicity.[19,67]

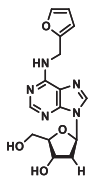
In the present study, biological activity tests were conducted on another large series of purine derivatives, i.e., aromatic 6-substituted purine-2′-deoxy-9-(β)-D-ribosides.

2.2.1. Cytotoxic activity testing on human non-cancer cells

Whereas cytokinin bases are not toxic to human cells, cytokinin ribosides, e.g., iPR, KR, BAPR and its *ortho*-hydroxylated derivatives *o*TR and 2-hydroxy-3-methoxy-BAPR, are cytotoxic and hence have been studied as candidate anti-cancer drugs.[67–70] As their toxicity depends on intracellular conversion to the corresponding ribotides, the presence of the ribose moiety is considered critical for the toxic effect. The mechanism of cell death includes ATP-depletion, which has not been observed only in the case of 2-hydroxy-3-methoxy-BAPR.[70] Examples of inactive derivatives include not only cytokinin bases but also cytokinin 9-glucosides[69] and derivatives with a tetrahydropyran-1-yl group[71] or acyclic polyols[72] at position 9 of the purine ring. Here, we show that 9-(2′-(β)-D-deoxyribosides) exhibited no or limited toxicity to human cell lines at 30 μM. The 2′-deoxyribose analogues of KR, BAPR, oTR and 2-hydroxy-3-methoxy-BAPR are among the non-toxic compounds.

Table 2
Structure, yield, purity, melting temperature, molar mass and mass of positively charged molecular ions of prepared derivatives analyzed by HPLC-MS.



Compound	R^2	R^3	R^4	R^5	R^6	Yield	HPLC-UV	Melting range/point	Mr	ES-MS [M+H$^+$]	Antioxidant capacity GAE
1	H	H	H	H	H	25%	> 98%	172	341.2	342.5	
2	OH	H	H	H	H	46%	> 96%	74–76	357.4	358.7	0.714 ± 0.004
3	H	OH	H	H	H	69%	96%	98–100	357.4	358.2	0.046 ± 0.001
4	H	H	OH	H	H	49%	99%	142	357.4	358.5	0.644 ± 0.003
5	OMe	H	H	H	H	39%	99%	128–129	371.4	373.1	0.042 ± 0.000
6	H	OMe	H	H	H	38%	99%	155–157	371.4	372.8	0.038 ± 0.003
7	H	H	OMe	H	H	52%	97%	150–152	371.4	372.9	0.007 ± 0.000
8	OH	OMe	H	H	H	21%	95%	186	387.4	388.8	0.826 ± 0.017
9	H	OMe	OH	H	H	39%	> 99%	189–191	387.4	388.5	0.582 ± 0.022
10	OMe	H	H	OMe	H	51%	> 96%	140–142	401.4	402.6	0.024 ± 0.001
11	OMe	OMe	H	H	H	28%	> 96%	218–220	401.4	402.7	0.011 ± 0.000
12	H	OH	OMe	H	H	30%	> 96%	190–193	387.4	389.0	1.531 ± 0.029
13	OMe	H	OMe	H	OMe	34%	> 95%	98–100	431.4	432.1	0.019 ± 0.010
14	H	OMe	OMe	OMe	H	45%	95%	175–177	431.4	432.8	0.015 ± 0.000
15	F	H	H	H	H	48%	> 97%	180–181	359.4	360.7	0.005 ± 0.000
16	H	F	H	H	H	44%	> 96%	190–192	359.4	360.6	0.009 ± 0.000
17	H	H	F	H	H	56%	96%	158–161	359.4	360.4	0.008 ± 0.000
18	Cl	H	H	H	H	53%	97%	158	375.8	376.7, 378.7	0.007 ± 0.001
19	H	Cl	H	H	H	38%	96%	145–147	375.8	376.8, 378.8	0.003 ± 0.000
20	H	H	Cl	H	H	47%	> 95%	149–151	375.8	376.3, 378.3	0.018 ± 0.000
21	Br	H	H	H	H	22%	98%	170	420.3	420.8, 422.7	0.005 ± 0.000
22	H	Br	H	H	H	21%	> 98%	143–145	420.3	420.6, 422.6	0.005 ± 0.000
23	H	H	Br	H	H	28%	95%	230–232	420.3	420.7, 422.7	0.006 ± 0.001
24	H	I	H	H	H	33%	> 97%	146–148	467.3	468.6	0.049 ± 0.002
25	H	H	I	H	H	36%	95%	78–80	467.3	467.9	0.064 ± 0.003
26	Me	H	H	H	H	19%	98%	135–137	355.4	356.8	0.006 ± 0.000
27	H	Me	H	H	H	29%	> 98%	125–127	355.4	356.4	0.004 ± 0.000
28	H	H	Me	H	H	37%	> 97%	218–220	355.4	356.8	0.004 ± 0.000
29	H	OCF_3	H	H	H	20%	> 99%	52	425.4	426.9	0.001 ± 0.000
30	H	H	OCF_3	H	H	48%	> 97%	153–155	425.4	426.9	0.002 ± 0.000
31	CF_3	H	H	H	H	17%	> 99%	110–111	409.4	410.9	0.008 ± 0.004
32	H	CF_3	H	H	H	66%	94%	136–137	409.4	410.4	0.008 ± 0.000
33	H	H	CF_3	H	H	72%	> 96%	157–159	409.4	410.4	0.018 ± 0.000
34						20%	> 93%	58–59	331.3	332.4	0.031 ± 0.001

At present, it is unclear whether human cell lines do not accumulate the compounds, are unable to phosphorylate the deoxyribose moiety or the deoxyribotides are not toxic. In the future, we plan to test the compounds on various infection agents, including viruses and parasitic protozoa as it is known that they are sensitive to various (deoxy)ribosides non-toxic for human cells[36–39,73] due to differences in their enzymatic equipment.

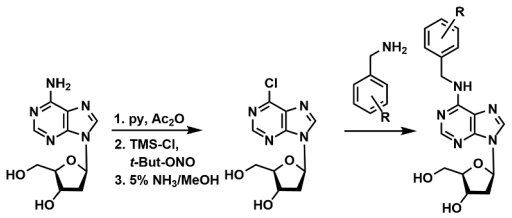
An assay based on the metabolic conversion of resazurin into a fluorescent resofurin[74] was used to evaluate toxic effects of the compounds toward proliferating human cells - skin fibroblasts BJ, keratinocytes HaCaT and retinal pigment epithelium cells ARPE-19.

At a concentration of 30 μM, the majority of the compounds had no or limited toxic effect. A decrease of the resazurin signal by more than 10% but less than 15% at the highest concentration tested (30 μM) was observed for compounds **10**, **22**, **23**, **24**, **29** and **32** in BJ cells and for **20** in HaCaT cells. Compound **28** decreased the resazurin signal in HaCaT by almost 30%.

At a concentration of 10 μM, no cytotoxic effect was observed (data not shown), with the exception of **20** (decrease of ~ 15% in HaCaT) and **24** (decrease of ~ 7% in BJ).

2.2.2. Cytokinin signaling bioassay

Escherichia coli strains expressing Arabidopsis cytokinin receptors AHK3 and CRE1/AHK4 and the down-stream-activated reporter gene cps::lacZ[75] were used to examine the ability of the receptors to be recognized and activated by these compounds. In the AHK3 receptor activation assay, *t*Z was used as a positive control and the activity at 1 μM was set as 100%. The CRE1/AHK4 receptor is generally more



Scheme 2. Preparation of substituted N^6-benzylamino-2́-deoxy-9-(β)-D-ribofuranosylpurine derivatives by a method using a halogen derivative.

selective and less sensitive[75] than the receptor AHK3. In the CRE1/AHK4 receptor activation assay, *t*Z was used as a standard and its activity at 10 μM was set as 100%. AHK3 receptor recognition was tested for compounds **2**, **3**, **5**, **6**, **8** and **11**, but none of them showed significant activity. Only compounds **3** and **6** slightly activated the AHK3 receptor.

The CRE/AHK4 receptor activation test was examined for compounds **1** (BAPdR), **2**, **3**, **5**, **6**, **7**, **8**, **10**, **11**, **13**, **15**, **16**, **17**, **18**, **19**, **20**, **21**, **22**, **23**, **24**, **26**, **27**, **28**, **30** and **31**. Among them, the greatest activity was recorded for compound **3** (13% of activity of *trans*-zeatin at a concentration of 10 μM vs. ~ 80% at 50 μM). Mild activation of the CRE1/AHK4 receptor was recognized for compounds **1** (39%), **26** (19%) and **19** (14%) at the concentration of 50 μM.

2.2.3. Cytokinin activity

The newly prepared series consisting of 33 cytokinin derivatives was tested in three classical cytokinin bioassays based on cytokinin-specific functions in plant tissues[76]: *Amaranthus*, detached wheat leaf senescence, and tobacco callus bioassays (Table 3). Results for a series of 2′-deoxy-9-(β)-D-ribofuranosylpurine derivatives (**2–34**) were calculated relative to those of BAP (100%), which is widely used in plant *in vitro* micropropagation, and compared with their parent substituted 6-benzylaminopurines[17] and ribosides[19] obtained previously to examine the importance and effects of the 2′-deoxyribosyl part in the structure of the aromatic cytokinin derivatives. All data obtained are described below and summarized in Table 3.

2.2.3.1. Amaranthus assay. The *Amaranthus* assay is based on the dark induction of betacyanin synthesis in *Amaranthus caudatus* var. *atropurpurea* cotyledons in the presence of cytokinin.[77] In this bioassay, compounds **3**, **15**, **17**, **18**, **19**, **21**, **22**, **26** and **31** exceeded the activity of tZ: the highest effects were for compounds **3** (by 33%), **19** (23%), **21** (44%), **22** (26%) and **31** (27%). Compounds **6** and **27** reached the same cytokinin activity in this assay as tZ. Positive effects on the activity in this assay were mainly for derivatives with the following functional groups: 3-OH, 4-F, 3-Cl, 2-Br, 3-Br, and 2-CF_3. Cytokinin activities of 2′-deoxyriboside derivatives were compared to those of their ribosides and free bases (Table 3**)**. For instance, chloro- and bromo- substitution in position *ortho*- or *para*- of the benzyl ring led to approximately the same activity (around 100% of BAP) for all three forms (base, riboside and 2′-deoxyriboside). Despite this, there was a clear concentration-dependent increasing trend in activity (Fig. 1**)**, in comparison with the sharp decrease of BAP activity at the highest concentration.

Cytokinin BAP, if added at high dosage to plants, induces programmed cell death by accelerating senescence processes[78] and can also inhibit plant cyclin-dependent kinases (CDKs).[17] Furthermore, the main metabolite of BAP, BAP9G, is more stable but has a negative impact on rooting and acclimatization[79] (e.g., in cultures of *Spatiphyllum floribundum*).

This trend was noticed over the whole series for compounds with cytokinin activity of BAP around 50% and more. On the other hand, 4-Cl and 4-Br substitution showed almost no activity among the compared derivative forms. Fluoro-derivatives showed about the same activity as BAP at all three positions and for all three compared forms, with the exception of 4-F-BAP and 4-F-BAPR. 4-F-BAPdR even exceeded the activity of BAP by 22%. Methyl-substituted derivatives showed a similar trend to those of chloro- and bromo-derivatives.

The 2′-deoxyriboside analog (**8**) of the cytotoxic 2-OH-3-OMe-BAPR derivative showed no activity at all, as well as compounds **5**, **7**, **14**, **30** and **33**. In the *Amaranthus* bioassay, BAPdR showed a slightly lower activity than BAP.

2.2.3.2. Wheat leaf senescence assay. This assay depends on the ability of derivatives to retard chlorophyll degradation in excised wheat leaves (*Triticum aestivum* cv. Hereward) in the dark. The results of this bioassay differed the most among the free bases, ribosides and 2′-deoxyribosides and position of substitution on the benzyl ring. All three hydroxy-substituted derivatives were active: the 2-OH derivative (**2**) demonstrated similar activity to that of BAP, the 4-OH derivative (**4**) showed double the BAP activity, the 3-OH-BAPdR (**3**) showed triple the BAP activity. The altered substituent position of the *ortho*- and *meta*-methoxy-BAPdR derivatives (**5** and **6**, respectively) resulted in different activity in the senescence bioassay. Compound **5** showed no activity, whereas compound **6** (see Fig. 2) showed almost triple the BAP cytokinin activity. The *para*-methoxy- position was not as advantageous. For the methoxy- disubstituted derivative **10,** two times higher activity than BAP was recorded. However, the methoxy-trisubstituted compounds **13** and **14** did not even reach the BAP activity. All fluoro-, chloro-, and bromobenzylamino-substituted purine 2′-deoxyribosides significantly delayed the chlorophyll degradation: the highest activities were for compounds **16** (291% of BAP), **17** (329% of BAP), **18** (232% of BAP) and **22** (211% of BAP). The methyl-substituted derivatives were active in all three forms but only for *ortho*- and *meta*-methyl substitution; *para*-methyl substitution did not positively influence the senescence delay. The same trend was noted for trifluoromethyl- analogs. BAPdR showed about 80% higher activity in this bioassay than BAP, suggesting that the results were influenced by the presence of the 2′-deoxyribose moiety. Kinetin-2′-deoxyriboside (KdR) exceeded the BAP activity by 156%.

2.2.3.3. Tobacco callus assay. The tobacco callus bioassay is based on the ability of cytokinins to promote cell division of cytokinin-dependent tobacco calluses (*Nicotiana tabacum* L. cv. Wisconsin 38) in the presence of auxin. Generally, in this bioassay, the newly tested compounds showed lower relative activity than BAP, but compounds **3**, **6**, **17**, **18**, **19**, **21**, **26**, **27** and **34** achieved approximately 80% or higher of the BAP activity.

The activity of BAP was slightly exceeded by compounds **17** and **34** (Fig. 3), but only by 1% and 2%, respectively. BAPdR exceeded the activity of BAP by 12%. Interestingly, compounds **2**, **3**, **4**, **5**, **6**, **10**, **12**, **13**, **14**, **17**, **18**, **21**, **26**, **27**, **31**, **32**, **33** and **34** showed an increasing trend in activity dependent on the concentration (contrary to the significant drop of activity of BAP). However, most of the compounds never reached the activity of BAP.

The data obtained from the three cytokinin bioassays suggested that the presence of the 2′-deoxyribose moiety most positively influenced the capability of compounds to promote plant growth and delay senescence. The remarkable activity increase was seen especially in the senescence bioassay among most of the tested derivatives. Interesting differences in cytokinin activity were recorded for methoxy-, trifluoromethyl-, and methyl- substitutions: among the methoxy-substituted derivatives, the *ortho*- and *para*- substituted compounds showed greatly suppressed activity, whereas among the methyl- substituted derivatives, the lowest activity was exhibited by the *para*-substituted derivative. The highest activities in the senescence bioassay were exhibited by all the fluoro-, chloro-, and bromo-derivatives, with derivatives **19**, **20**, **21**, **22** and **23** showing the greatest change of cytokinin activity of bases/ribosides versus 2′-deoxyribosides.

2.2.4. Antiviral activity studies

In 2008, Pohjala et al.[36] tested 29 nucleosides, among them N^6-benzyladenosine (BAPR) and non-cytotoxic N^6-benzyl-2′-

Table 3
Relative cytokinin activities of prepared 6-substituted purine-9-(β)-D-2́-deoxyribosides in three cytokinin bioassays at optimal concentration. Data are compared to those of parent ribosides and bases (taken from cited sources). All results presented are compared with the activity of benzylaminopurine (BAP), where 100% is equivalent to 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 bioassay		Senescence bioassay		Tobacco callus bioassay	
Compound		Optimal concentration (mol/L)	Relative activity (%)	Optimal concentration (mol/L)	Relative activity (%)	Optimal concentration (mol/L)	Relative activity (%)
1	**BAPdR**	10^{-4}	88 (± 21)	10^{-4}	179 (± 6)	10^{-5}	112 (± 5)
	2-OH-BAP		nt		nt		nt
	2-OH-BAPR		nt		nt		nt
2	**2-OH-BAPdR**	**10^{-4}**	**56 (± 10)**	**10^{-4}**	**119 (± 11)**	**10^{-5}**	**75 (± 5)**
	3-OH-BAP		nt		nt		nt
	3-OH-BAPR		nt		nt		nt
3	**3-OH-BAPdR**	**10^{-4}**	**133 (± 23)**	**10^{-4}**	**306 (± 5)**	**10^{-4}**	**84 (± 2)**
	4-OH-BAP		nt		nt		nt
	4-OH-BAPR		nt		nt		nt
4	**4-OH-BAPdR**	**10^{-4}**	**21 (± 4)**	**10^{-4}**	**195 (± 22)**	**10^{-4}**	**60 (± 3)**
	2-OMe-BAP[18]	10^{-5}	77 (± 5)	10^{-6}	203 (± 46)	10^{-4}	79 (± 5)
	2-OMe-BAPR[19]	10^{-5}	86 (± 4)	10^{-4}	198 (± 12)	10^{-5}	108 (± 1)
5	**2-OMe-BAPdR**	**10^{-6}**	**3 (± 1)**	**10^{-7}**	**6 (± 1)**	**10^{-4}**	**34 (± 3)**
	3-OMe-BAP[18]	10^{-5}	90 (± 16)	10^{-6}	178 (± 16)	10^{-4}	76 (± 20)
	3-OMe-BAPR[19]	10^{-4}	98 (± 10)	10^{-4}	209 (± 8)	10^{-6}	92 (± 1)
6	**3-OMe-BAPdR**	**10^{-4}**	**100 (± 13)**	**10^{-4}**	**295 (± 19)**	**10^{-4}**	**85 (± 4)**
	4-OMe-BAP[17]	10^{-4}	23 (± 2)	10^{-4}	79 (± 6)	10^{-6}	39 (± 17)
	4-OMe-BAPR[19]	10^{-4}	17 (± 8)	10^{-4}	65.5 (± 15)	10^{-6}	2 (± 1)
7	**4-OMe-BAPdR**	**10^{-6}**	**2 (± 1)**	**10^{-4}**	**14 (± 1)**	**10^{-8}**	**45 (± 3)**
	2-OH-3-OMe-BAP[17]	10^{-4}	19 (± 3)	10^{-5}	34 (± 5)		nt
	2-OH-3-OMe-BAPR[19]	10^{-4}	18 (± 5)	10^{-4}	10 (± 1)		nt
8	**2-OH-3-OMe-BAPdR**		**0**		**0**	**10^{-9}**	**55 (± 3)**
	4-OH-3-OMe-BAP[17]		nt		nt		nt
	4-OH-3-OMe-BAPR[19]		nt		nt		nt
9	**4-OH-3-OMe-BAPdR**	**10^{-4}**	**14 (± 2)**		**0**	**10^{-6}**	**28 (± 2)**
	2,5-di-OMe-BAP[17]		nt		nt		nt
	2,5-di-OMe-BAPR[19]		nt		nt		nt
10	**2,5-di-OMe-BAPdR**	**10^{-4}**	**13 (± 4)**	**10^{-4}**	**183 (± 7)**	**10^{-4}**	**30 (± 2)**
	2,3-di-OMe-BAP[17]	10^{-4}	22 (± 3)	10^{-4}	109 (± 5)	10^{-6}	7 (± 3)
	2,3-di-OMe-BAPR[19]	10^{-4}	21 (± 7)	10^{-4}	106 (± 17)	10^{-6}	5 (± 2)
11	**2,3-di-OMe-BAPdR**	**10^{-4}**	**18 (± 5)**	**10^{-4}**	**75 (± 3)**	**10^{-6}**	**8 (± 1)**
	3-OH-4-OMe-BAP[17]		nt		nt		nt
	3-OH-4-OMe-BAPR[19]		nt		nt		nt
12	**3-OH-4-OMe-BAPdR**	**10^{-4}**	**10 (± 2)**		**0**	**10^{-4}**	**69 (± 4)**
	2,4,6-tri-OMe-BAP[17]	10^{-4}	3 (± 3)		nt		nt
	2,4,6-tri-OMe-BAPR[19]		nt		nt		nt
13	**2,4,6-tri-OMe-BAPdR**	**10^{-4}**	**11 (± 2)**	**10^{-4}**	**71 (± 4)**	**10^{-6}**	**36 (± 3)**
	3,4,5-tri-OMe-BAP[17]	10^{-4}	2 (± 2)	10^{-4}	25 (± 2)		nt
	3,4,5-tri-OMe-BAPR[19]		nt		nt		nt
14	**3,4,5-tri-OMe-BAPdR**	**10^{-4}**	**7 (± 2)**	**10^{-8}**	**26 (± 2)**	**10^{-4}**	**26 (± 2)**
	2-F-BAP[17]	10^{-4}	116 (± 3)	10^{-4}	169 (± 20)	10^{-6}	111 (± 21)
	2-F-BAPR[19]	10^{-5}	96 (± 2)	10^{-4}	118 (± 39)	10^{-6}	100 (± 9)
15	**2-F-BAPdR**	**10^{-4}**	**104 (± 11)**	**10^{-4}**	**190 (± 8)**	**10^{-5}**	**78 (± 4)**
	3-F-BAP[17]	10^{-4}	140 (± 5)	10^{-4}	200 (± 25)	10^{-5}	135 (± 8)
	3-F-BAPR[19]	10^{-5}	92 (± 6)	10^{-4}	220 (± 16)	10^{-5}	91 (± 6)
16	**3-F-BAPdR**	**10^{-4}**	**94 (± 7)**	**10^{-4}**	**291 (± 16)**	**10^{-5}**	**79 (± 8)**
	4-F-BAP[17]	10^{-5}	44 (± 4)	10^{-4}	95.5 (± 3.5)	10^{-6}	122 (± 12)
	4-F-BAPR[19]	10^{-5}	71 (± 3)	10^{-4}	148 (± 2)	10^{-6}	100 (± 6)
17	**4-F-BAPdR**	**10^{-4}**	**122 (± 7)**	**10^{-4}**	**329 (± 11)**	**10^{-4}**	**101 (± 5)**
	2-Cl-BAP[17]	10^{-5}	109 (± 8)	10^{-4}	116.5 (± 6.5)	10^{-6}	93 (± 4)

(*continued on next page*)

Table 3 (*continued*)

	Compound	*Amaranthus* bioassay		Senescence bioassay		Tobacco callus bioassay	
		Optimal concentration (mol/L)	Relative activity (%)	Optimal concentration (mol/L)	Relative activity (%)	Optimal concentration (mol/L)	Relative activity (%)
	2-Cl-BAPR[19]	10^{-5}	113 (± 4)	10^{-4}	119 (± 9)	10^{-6}	93 (± 4)
18	**2-Cl-BAPdR**	**10^{-4}**	**107 (± 21)**	**10^{-4}**	**232 (± 14)**	**10^{-4}**	**89 (± 4)**
	3-Cl-BAP[17]	10^{-5}	96 (± 5)	10^{-4}	82 (± 2)	10^{-5}	94 (± 6)
	3-Cl-BAPR[19]	10^{-5}	139 (± 3)	10^{-4}	72 (± 8)	10^{-5}	96 (± 5)
19	**3-Cl-BAPdR**	**10^{-4}**	**123 (± 11)**	**10^{-4}**	**189 (± 12)**	**10^{-5}**	**79 (± 4)**
	4-Cl-BAP[17]	10^{-5}	35 (± 7)	10^{-4}	64.5 (± 13.5)	10^{-6}	64 (± 8)
	4-Cl-BAPR[19]	10^{-5}	35 (± 4)	10^{-4}	104 (± 6)	10^{-6}	46 (± 14)
20	**4-Cl-BAPdR**	**10^{-4}**	**26 (± 2)**	**10^{-4}**	**194 (± 8)**	**10^{-5}**	**40 (± 4)**
	2-Br-BAP[17]	10^{-4}	94 (± 6)	10^{-4}	52 (± 14)	10^{-5}	102 (± 5)
	2-Br-BAPR[19]	10^{-4}	147 (± 9)	10^{-5}	86 (± 29)	10^{-5}	100 (± 5)
21	**2-Br-BAPdR**	**10^{-4}**	**144 (± 21)**	**10^{-4}**	**154 (± 11)**	**10^{-4}**	**95 (± 3)**
	3-Br-BAP[17]	10^{-4}	71 (± 5)	10^{-4}	48 (± 6)	10^{-6}	85 (± 11)
	3-Br-BAPR[19]	10^{-4}	151 (± 7)	10^{-4}	89 (± 10)	10^{-5}	82 (± 10)
22	**3-Br-BAPdR**	**10^{-4}**	**126 (± 13)**	**10^{-4}**	**211 (± 11)**	**10^{-5}**	**74 (± 3)**
	4-Br-BAP[17]	10^{-5}	17 (± 7)	10^{-5}	30 (± 15)	10^{-6}	15 (± 9)
	4-Br-BAPR[19]	10^{-4}	30 (± 5)	10^{-4}	76 (± 11)	10^{-6}	16 (± 11)
23	**4-Br-BAPdR**	**10^{-4}**	**12 (± 1)**	**10^{-4}**	**176 (± 14)**	**10^{-5}**	**62 (± 2)**
	3-I-BAP[17]	10^{-5}	79 (± 3)	10^{-4}	83.5 (± 23)	10^{-5}	76 (± 8)
	3-I-BAPR[19]	10^{-4}	102 (± 18)	10^{-4}	58 (± 19)	10^{-6}	45 (± 12)
24	**3-I-BAPdR**	**10^{-4}**	**62 (± 15)**	**10^{-4}**	**64 (± 5)**	**10^{-7}**	**42 (± 8)**
	4-I-BAP[17]		nt		nt		nt
	4-I-BAPR[19]		nt		nt		nt
25	**4-I-BAPdR**	**10^{-4}**	**19 (± 4)**	**10^{-4}**	**28 (± 2)**	**10^{-9}**	**33 (± 3)**
	2-Me-BAP[17]	10^{-5}	98 (± 22)	10^{-4}	158 (± 29)	10^{-6}	118 (± 3)
	2-Me-BAPR[19]	10^{-4}	99 (± 27)	10^{-4}	141 (± 5)	10^{-6}	98 (± 4)
26	**2-Me-BAPdR**	**10^{-4}**	**105 (± 7)**	**10^{-4}**	**229 (± 11)**	**10^{-4}**	**82 (± 4)**
	3-Me-BAP[17]	10^{-5}	84 (± 14)	10^{-4}	111 (± 16)	10^{-6}	79 (± 5)
	3-Me-BAPR[19]	10^{-5}	96 (± 7)	10^{-4}	143 (± 9)	10^{-6}	90 (± 2)
27	**3-Me-BAPdR**	**10^{-4}**	**100 (± 10)**	**10^{-4}**	**257 (± 17)**	**10^{-4}**	**87 (± 5)**
	4-Me-BAP[17]	10^{-5}	26 (± 10)	10^{-4}	35 (± 23)	10^{-6}	52 (± 8)
	4-Me-BAPR[19]	10^{-4}	49 (± 13)	10^{-4}	54.5 (± 3)	10^{-6}	35 (± 6)
28	**4-Me-BAPdR**	**10^{-4}**	**56 (± 11)**	**10^{-4}**	**14 (± 1)**	**10^{-6}**	**70 (± 5)**
	3-OCF_3-BAP[17]		nt		nt		nt
	3-OCF_3-BAPR[19]	10^{-4}	104 (± 5)	10^{-4}	90 (± 19)	10^{-6}	86 (± 12)
29	**3-OCF_3-BAPdR**	**10^{-4}**	**28 (± 9)**	**10^{-4}**	**34 (± 2)**	**10^{-7}**	**36 (± 5)**
	4-OCF_3-BAP[17]		nt		nt		nt
	4-OCF_3-BAPR[19]	10^{-4}	21 (± 1)	10^{-4}	3 (± 2)		nt
30	**4-OCF_3-BAPdR**	**10^{-4}**	**6 (± 2)**	**10^{-7}**	**90 (± 4)**	**10^{-5}**	**41 (± 4)**
	2-CF_3-BAP[17]		nt		nt		nt
	2-CF_3-BAPR[19]	10^{-4}	121 (± 2)	10^{-4}	40 (± 6)		nt
31	**2-CF_3-BAPdR**	**10^{-4}**	**127 (± 15)**	**10^{-4}**	**232 (± 15)**	**10^{-4}**	**71 (± 2)**
	3-CF_3-BAP[17]		nt		nt		nt
	3-CF_3-BAPR[19]	10^{-4}	93 (± 7)	10^{-4}	95 (± 7)	10^{-6}	85 (± 8)
32	**3-CF_3-BAPdR**	**10^{-4}**	**74 (± 11)**	**10^{-4}**	**185 (± 6)**	**10^{-7}**	**50 (± 4)**
	4-CF_3-BAP[17]		nt		nt		nt
	4-CF_3-BAPR[19]	10^{-4}	14 (± 1)	10^{-4}	52 (± 10)		nt
33	**4-CF_3-BAPdR**	**10^{-4}**	**5 (± 1)**	**10^{-4}**	**44 (± 4)**	**10^{-4}**	**63 (± 4)**
	Kinetin		nt		nt		nt
	Kinetin-R[19]		nt		nt		nt
34	**Kinetin-dR**	**10^{-4}**	**71 (± 8)**	**10^{-4}**	**256 (± 22)**	**10^{-4}**	**102 (± 3)**

deoxyadenosine (BAPdR), against two alphaviruses, SFV (Semliki Forest virus) and Sindbis virus, with positive results. Later, antiviral activity of BAPR against the replication of human enterovirus 71 (*Picornaviridae)* was described[41]. Additionally, naturally occurring plant cytokinin nucleosides N^6-isopentenyladenosine and N^6-furfuryladenosine (kinetin riboside) were also shown to exhibit potent

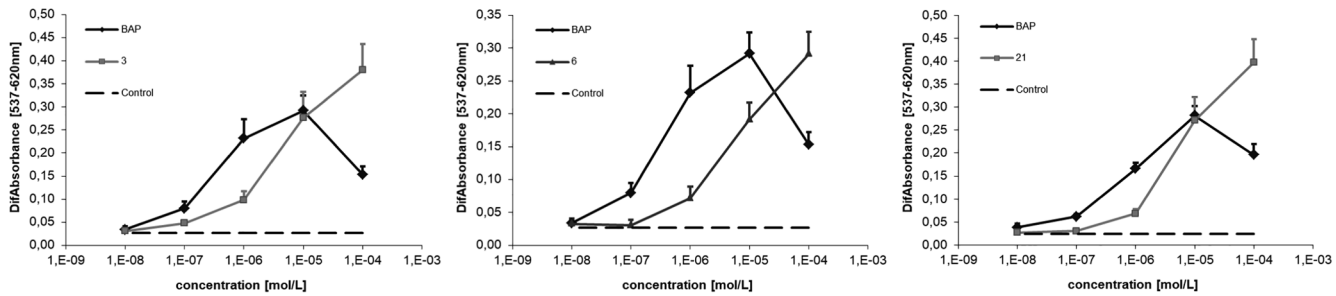


Fig. 1. *Amaranthus* bioassay. Dose-response curves for cytokinin-induced betacyanin synthesis in *Amaranthus caudatus* var. *atropurpurea* cotyledons. Dashed lines indicate values for the control treatment without any cytokinin. Exemplar derivatives (**3**, **6** and **21**) are compared to BAP.

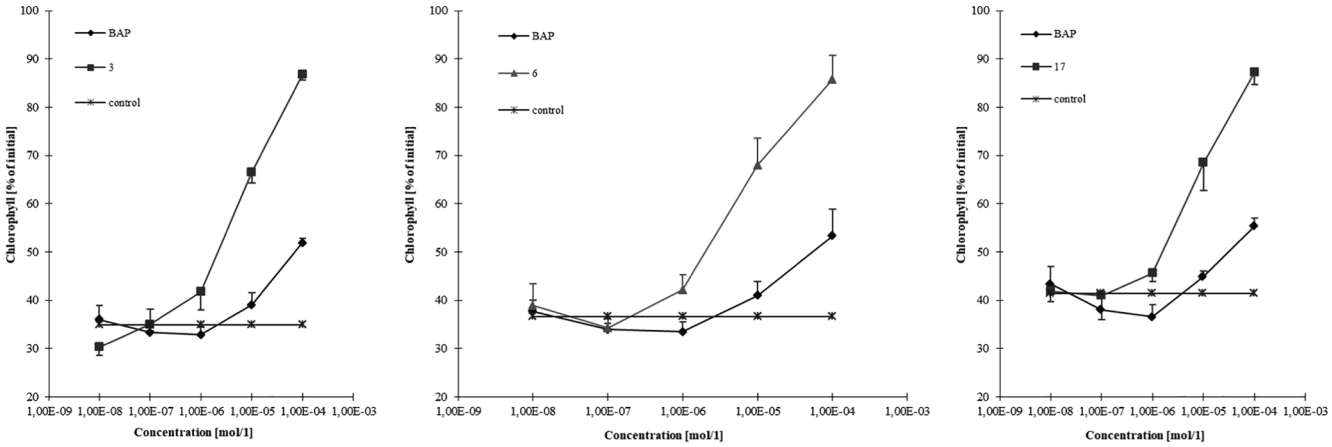


Fig. 2. Senescence bioassay. Dose-response curves for cytokinin-induced delay of chlorophyll degradation in excised wheat leaves. Horizontal lines indicate values for the control treatment without any cytokinin. Exemplar derivatives (**3**, **6**, and **17**) are compared to BAP.

antiviral activity on EV71.[37,38] In 2017, antiviral effects on the replication of EV71 were shown to be slightly increased in fluorinated BAPR derivatives (on the benzyl ring) and significantly increased in derivatives with two fluorine atoms (N^6-(2,6-difluorobenzyl)adenosine) or with trifluoromethyl group on the benzyl ring of BAPR.[39]

Some purine nucleoside derivatives are known to act as NRTI's (nucleoside reverse transcriptase inhibitors) and are already used for the treatment of HIV virus infection[80–84]. Hence, the similar structural features of the novel 6-substituted purine 2′-deoxy-9-(β)-D-ribosides with these antiviral agents inspired us to screen their potential antiviral activity, specifically on gammaherpesvirus (depending on the availability of antiviral testing).

The antiviral activity of all the prepared derivatives was examined on Vero cells infected by murine gammaherpesvirus (MHV-68)[85] with DMSO and ganciclovir as controls. The non-cytotoxicity of substances was examined on Vero cells prior to antiviral activity testing. The results showed no antiviral effect on this type of virus. Further antiviral studies and investigations of the effects of purine-2′-deoxy-9-(β)-D-ribosides on different types of viruses are being conducted.

2.2.5. Antioxidant capacity[86,87]

Senescence-delaying activity of BAP has been reported on harvested cauliflower curds treated with an aqueous solution of BAP and stored at room temperature.[88] Increased lipid peroxidation, loss of membrane integrity and processing of reactive oxygen species were successfully delayed, prolonging the period between a fresh and unsalable product. The antioxidant activity[89] of selected cytokinins (kinetin, BAP, iP, *p*-topolin) has been measured *in vitro* using various assays (hydroxyl radical scavenging ability by 2-deoxyribose degradation assay[90,91] (2-DRA), Trolox equivalence antioxidant capacity TEAC[92] assay, and oxygen radical absorbance capacity ORAC[93] assay). The results showed that the activity depends upon the chemical structure of the group present at the N^6 position of the compound and its concentration. For example, in the ORAC assay, the antioxidant activity of kinetin was the highest up to a concentration of 1 μM, whereas *p*-topolin was the most efficient antioxidant at higher concentrations. On the other hand, in the 2-DRA assay, iP succeeded in protecting 2-deoxyribose from degradation by hydroxyl radicals the most.

The total phenolic content method was used for examination of the antioxidant activities of the prepared derivatives in comparison with a known antioxidant, i.e., gallic acid. The results are summarized in Table 2. A significant response was observed for derivative **12** and also for compounds **2**, **4**, **8** and **9**. Compound **12** showed even higher antioxidant capacity than gallic acid on an equimolar basis.

3. Material and methods

The majority of reagents, substances and solvents were purchased from commercial suppliers (Sigma Aldrich®, TCI Chemicals®, VWR®, Jena Bioscience® or OlChemIm®). Organic solvents were evaporated in

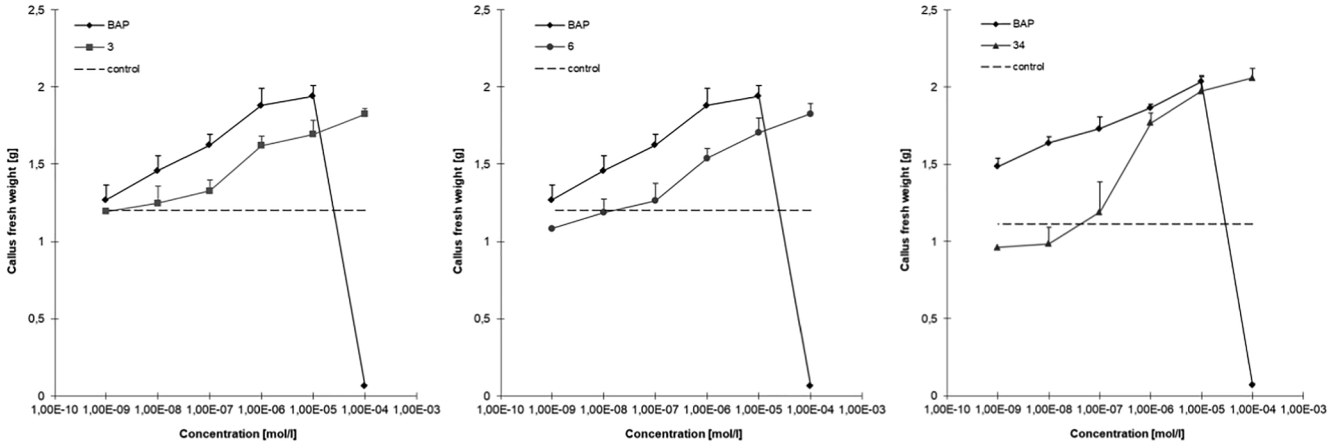


Fig. 3. Tobacco callus bioassay. Dose-response curves for cytokinin-induced promotion of cell division of cytokinin-dependent tobacco callus. Dashed lines indicate values for the control treatment without any cytokinin. Exemplar derivatives (**3**, **6** and **34**) are compared to BAP.

a rotary evaporator (Heidolph®) below 45 °C. The chromatographic purity of the compounds was determined using a HPLC-PDA-MS assembly. An Alliance 2695 separations module (Waters®) linked simultaneously to UV–VIS detector PDA 2996 (Waters®,) and a Q-Tof micro (Waters®) benchtop quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer were used. Samples were first dissolved in methanol and diluted to a concentration of 10 μg ·mL^{-1} in the mobile phase (initial conditions). Next, 10 μL of the solution were injected onto a RP-column (150 × 2.1 mm; 3.5 μm; Symmetry C18, Waters®). The column was kept in a thermostat at 25 °C. Solvent (A) consisted of 15 mM formic acid adjusted to pH 4.0 by ammonium hydroxide. Methanol was used as the organic modifier (solvent B). A flow rate of 0.2 mL ·min^{-1} and the following binary gradient were used: 0 min, 90% A; 0 – 25 min, linear gradient to 10% A, followed by 10 min isocratic elution of 10% A. At the end of the gradient, the column was reequilibrated to the initial conditions for 10 min. The effluent was introduced into the DAD (diode array detector; scanning range 210–400 nm, with 1.2 nm resolution) equipped with an electrospray source (source temperature: 120 °C for positive mode, capillary voltage +3.0 kV, cone voltage +20 V, desolvation temperature 300 °C. Nitrogen was used as both the desolvation gas (500 L·h^{-1}) and cone gas (50 L h^{-1}). The mass spectrometer was operated in positive (ESI +) ionization mode. Data were acquired over the 50–1000 *m/z* range (FULLSCAN mode). NMR spectra were measured on a Jeol 500 SS spectrometer at a temperature of 300 K and frequencies of 500.13 MHz (^{1}H NMR) and 125.03 MHz (^{13}C NMR). Tetramethylsilane (TMS) was used as an internal standard and DMSO-d_6 and $CDCl_3$ were used as solvents for the analysis (NMR spectra were calibrated against a residual solvent signal). In the presented data, chemical shifts are given in ppm (δ) and the multiplicities are marked as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; bs, broad singlet. The reaction processes were controlled by thin layer chromatography (TLC) using silica gel 60 WF_{245} plates (Merck®). $CHCl_3$/MeOH (9:1 or 3:1, v/v), $CHCl_3$/MeOH/NH_3 (9:1:0,05, v/v) or EtOAc/MeOH/NH_3 (34:4:2, v/v) were used as the mobile phase. For purification by column chromatography, Davisil R LC60A 40–63 μm silica gel was employed. For the unsuccessful experimental attempts at using microwaves to improve reaction course of BOP reaction, a Discover CEM microwave reactor was used (conditions: 80 °C, 50 W, 30 min).

3.1. Synthesis

3.1.1. General procedure

A. One-step method using Castro's reagent (BOP)[20,28]

Optically pure 9-(2′-deoxy-(*β*)-D-ribofuranosyl)hypoxanthine; (2′-deoxy-9-(*β*)-D-inosine) (100 mg; 0.4 mmol; Jena Bioscience®) and BOP ((*benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate*) (210 mg; 0.5 mmol) were dissolved in dry DMF (3 mL) under nitrogen atmosphere at 55 °C and DIPEA (101.5 μL; 0.6 mmol) was added, followed by substituted benzylamine (1.2 eq.) as the last component. The reaction mixture was then stirred at 55 °C under a nitrogen atmosphere overnight and completion of the reaction was controlled by TLC ($CHCl_3$/MeOH/NH_3 (9:1:0,05, v/v)). After further evaporation of residual solvent and coevaporation with *n*-heptane and absolute ethanol, the resulting dark yellow oily residue was dissolved in water/ethyacetate and the aqueous layer was washed by ethylacetate three times. The combined organic layers were dried by powdered Na_2SO_4 and evaporated. The resulting residue was carefully purified by column chromatography to give the desired product, which in some cases could be precipitated from various solvents.

All described and tested compounds were prepared by method A. Procedure B was only examined as a possible advantageous method in the need for synthesizing libraries of compounds since a larger amount of halogen derivative in high purity was obtained for nucleophilic substitutions, leading to final products without many impurities and byproducts.

B. Procedure using halogen derivative

3′,5′-Di-O-acetyl-2′-deoxyadenosine[62]

2′-Deoxyadenosine monohydrate (2.2 g; 8 mmol) was dissolved in pyridine (26 mL) and acetanhydride (8 mL) was added dropwise at room temperature. Next, the reaction mixture was cooled in a crushed ice bath and DCM (100 mL) was added, followed by extraction with 10% aqueous $NaHCO_3$ (24 mL) and then water (20 mL). The organic layer was vacuum-evaporated and three times coevaporated with toluene to give a dry residue, which was then dissolved in absolute ethanol and crystallized in a refrigerator overnight.

6-Chloro-2′-deoxy-9-(β)-D-ribofuranosylpurine[57]

3′,5′-Di-*O*-acetyl-2′-deoxyadenosine (2 g; 5.8 mmol) in anhydrous DCM (150 mL) was placed into a dried three-neck flask under a nitrogen atmosphere (balloon). While stirring, the suspension was cooled in an ice bath to 0 °C and TMS-Cl (7.4 mL; 58 mmol) was added dropwise through the septum. Afterwards, a solution of *tert*-butyl nitrite (3.5 mL; 29 mmol) in dry DCM (30 mL) was slowly added and the reaction mixture was stirred for 2 h at 0 °C and then at room temperature overnight. The next day, DCM (300 mL) was added and the organic phase was washed with 5% aqueous $NaHCO_3$ (2 × 300 mL), water (3 × 100 mL) and brine (3 × 100 mL), dried over powdered Na_2SO_4 and evaporated as a yellow oil (1.6 g). The residue was without further purification deprotected in 5% solution of NH_3 in methanol (30 mL) at 0 °C to give 6-chloro-2′-deoxy-9-(*β*)-D-ribofuranosylpurine (HPLC-MS of the residue: 77.7%). Column chromatography purification ($CHCl_3$/MeOH (9:1), v/v) was needed, resulting in a white-yellow solid product (0.7 g). The resulting 6-chloro-2′-deoxy-9-(*β*)-D-ribofuranosylpurine was subsequently used for nucleophilic substitutions with variously substituted benzylamines in *n*-propanol with base (TEA).

3.1.2. Experimental

3′,5′-Di-O-acetyl-2′-deoxyadenosine[62] (M = 335.32 g/mol), HPLC-MS purity: 99.6%, yield 82%, measured as: ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm: 2.08 (s, 3H); 2.12 (s, 3H); 2.60–2.64 (m, 1H); 2.91–2.97 (m, 1H); 4.32–4.36 (m, 2H); 4.39–4.42 (m, 1H); 5.41–5.43 (m, 1H); 5.78 (bs, 2H); 6.41–6.44 (m, 1H); 7.98 (s, 1H); 8.35 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm: 170.43, 155.43, 152.99, 149.72, 138.76, 120.26, 84.66, 82.63, 74.61, 63.87, 37.64, 21.04, 20.90.

6-Chloro-2′-deoxy-9-(β)-D-ribofuranosylpurine[57] measured as: ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.31–2.36 (m, 1H); 2.69–2.76 (m, 1H); 3.47–3.52 (m, 1H); 3.55–3.61 (m, 1H); 3.83–3.87 (m, 1H); 4.38–4.43 (m, 1H); 4.95–4.97 (t, J = 5.5, 1H); 5.36–5.37 (d, J = 4, 1H); 6.41–6.44 (t, J = 7, 1H); 8.76 (s, 1H); 8.86 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 61.84, 70.89, 84.74, 88.61, 131.91, 146.32, 149.71, 151.75, 152.18, 172.10.

6-*benzylamino-2′-deoxy-9-(β)-D-ribofuranosylpurine*[51] (**1**), white solid precipitated from absolute ethanol without the need for column chromatography purification. The NMR spectrum was as previously

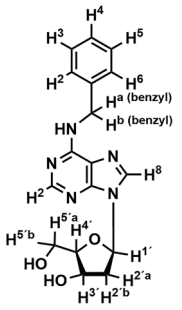


Fig. 4. Proton assignment determination by 2D COSY-NMR experiment.

described and measured as ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.23–2.28 (m, 1H, C$^{2'}$H^a); 2.69–2.76 (m, 1H, C^{2}H^b); 3.50–3.53 (m, 1H, C^{5}H^a); 3.60–3.64 (m, 1H, C^{5}H^b); 3.87–3.90 (m, 1H, C^{4}H); 4.39–4.43 (m, 1H, C^{3}H); 4.70 (bs, 2H, CH_2-*benzyl*); 5.21 (bs, 1H, C^{5}OH); 5.31 (bs, 1H, C^{3}OH); 6.34–6.37 (t, *J* = 7.5 Hz, 1H, C^{1}H); 7.18–7.21 (t, *J* = 7.0 Hz, 1H, Ar-H^4); 7.26–7.29 (t, *J* = 7.0 Hz, 2H, Ar-H^3 and Ar-H^5); 7.31–7.33 (d, *J* = 7.0 Hz, 2H, Ar-H^2 and Ar-H^6); 8.20 (s, 1H, *purine* C^8-H); 8.36 (s, 1H, *purine* C^2-H); 8.44 (bs, 1H, N−H). Proton signal assignment was performed based on 2D-NMR COSY experiment (Fig. 4).

6-*(2-hydroxybenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**2**), slightly brown waxy solid after column chromatography (9/1/0,05; $CHCl_3$/MeOH/NH_3), ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.25–2.29 (m, 1H); 2.68–2.77 (m, 1H); 3.04–3.09 (m, 1H); 3.50–3.53 (m, 1H); 3.60–3.63 (m, 1H); 3.87–3.88 (m, 1H); 4.40–4.42 (m, 1H); 4.61 (bs, 2H); 6.34–6.37 (t, *J* = 6.5 Hz, 1H); 6.69–6.72 (t, *J* = 7.5 Hz, 1H); 6.80–6.81 (d, *J* = 8 Hz, 1H); 7.03–7.07 (t, *J* = 7.5 Hz, 1H); 7.08–7.09 (m, 1H); 8.22 (s, 1H); 8.37 (bs, 1H, N−H); 8.40 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 45.41, 61.70, 70.81, 83.92, 84.14, 88.04, 115.28, 118.92, 119.48, 125.39, 126.49, 127.83, 128.08, 139.83, 148.06, 151.87, 154.04, 154.92.

6-*(3-hydroxybenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**3**), beige solid foam after column chromatography (9/1/0,05; $CHCl_3$/MeOH/NH_3), ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.27 (m, 1H); 2.71–2.76 (m, 1H); 3.50–3.54 (m, 1H); 3.60–3.63 (m, 1H); 3.88–3.89 (m, 1H); 4.39–4.43 (m, 1H); 4.62 (bs, 2H); 5.22–5.24 (t, *J* = 5.0 Hz, 1H); 5.31–5.32 (m, 1H); 6.34–6.37 (t, *J* = 6.5, 1H); 6.57–6.58 (d, *J* = 8.0 Hz, 1H); 6.71 (s, 1H); 6.72–6.74 (d, *J* = 7.5 Hz, 1H); 7.05–7.08 (dt, *J* = 8.0 Hz, *J′* = 1.0 Hz, 1H); 8.19 (bs, 1H); 8.36 (s, 1H); 8.38 (bs, 1H); 9.25 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.74, 61.92, 71.00, 84.02, 88.05, 93.75, 113.41, 113.78, 117.66, 119.66, 129.17, 139.61, 141.49, 152.39, 154.50, 155.15, 157.31.

6-*(4-hydroxybenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**4**), dark brownish waxy residue after column chromatography (9/1/0,05; $CHCl_3$/MeOH/NH_3), ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.23–2.28 (m, 1H); 2.70–2.77 (m, 1H); 3.50–3.52 (m, 1H); 3.60–3.63 (m, 1H); 3.85–3.91 (m, 1H); 4.41 (bs, 1H); 4.57 (bs, 2H); 5.24 (bs, 1H); 5.33 (bs, 1H); 6.33–6.36 (dt, *J* = 8.0, *J′* = 2.0 Hz, 1H); 6.66–6.68 (dd, *J* = 8.0 Hz, *J′* = 2.0 Hz, 2H); 7.13–7.15 (dd, *J* = 7.5, *J′* = 1.5 Hz, 2H); 8.20 (bs, 1H); 8.31 (bs, 1H); 8.34 (s, 1H); 9.26 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.21, 45.47, 62.04, 70.99, 84.02, 88.14, 114.85, 119.54, 128.55, 130.16, 139.51, 148.23, 152.34, 154.37, 156.16.

6-*(2-methoxybenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**5**), yellow solid after column chromatography and diethylether precipitation, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.38–2.42 (m, 1H); 2.76–2.81 (m, 1H); 3.53–3.56 (m, 1H); 3.63–3.65 (m, 3H); 3.90–3.93 (m, 1H); 4.45–4.47 (m, 2H); 5.02 (bs, 1H); 5.43 (bs, 2H); 6.50–6.52 (t, *J* = 7.0 Hz, 1H); 7.53–7.57 (m, 1H); 7.63–7.67 (m, 1H); 7.78–7.80 (d, *J* = 8.5 Hz, 1H); 8.20–8.21 (d, *J* = 8.5 Hz, 1H); 8.51 (s, 1H); 8.91 (s, 1H). *Signal at 3.40 ppm contains residual peak of H_2O and probably CH_3hydrogens.* ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 45.72, 61.45, 70.51, 79.27, 84.00, 84.41, 88.20, 109.50, 119.17, 120.02, 125.47, 128.61, 129.46, 142.87, 145.45, 151.12, 153.87, 158.24.

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm: 2.25–2.29 (m, 1H); 3.00–3.13 (m, 1H); 3.75–3.78 (m, 1H); 3.87 (s, 3H); 3.94–3.96 (m, 1H); 4.19 (s, 1H); 4.76–4.77 (d, 1H); 4.82–4.83 (bs, 2H); 6.25–6.28 (m, 1H); 6.50 (s, 1H); 6.87–6.90 (m, 1H); 7.24–7.28 (m, 1H, *contains residual solvent $CDCl_3$ peak*); 7.34–7.36 (d, *J* = 7.0 Hz, 1H); 7.73 (s, 1H); 8.34 (s, 1H). *Measured in $CDCl_3$ due to the hidden methyl peak in DMSO-d_6 signal if measured in DMSO-d_6.* ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm: 40.29, 40.93, 55.47, 63.61, 73.62, 87.95, 89.86, 110.40, 120.61, 121.58, 126.08, 129.11, 129.70, 139.51, 147.47, 152.60, 155.14, 157.76.

6-*(3-methoxybenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**6**), white solid after column chromatography purification (9/1/0,05; $CHCl_3$/MeOH/NH_3) and absolute athanol precipitation, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.28 (m, 1H); 2.71–2.76 (m, 1H); 3.49–3.54 (m, 1H); 3.60–3.64 (m, 1H); 3.70 (s, 3H); 3.87–3.89 (m, 1H); 4.39–4.42 (m, 1H); 4.67 (bs, 2H); 5.21–5.23 (t, *J* = 5.5 Hz, 1H); 5.31–5.32 (d, *J* = 3.5 Hz, 1H); 6.34–6.37 (t, *J* = 6.5, 1H); 6.76–6.78 (d, *J* = 7.5 Hz, 1H); 6.89–6.90 (m, 2H); 7.18–7.21 (t, *J* = 7.5 Hz, 1H); 8.20 (s, 1H); 8.37 (s, 1H); 8.42 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.78, 45.26, 54.93, 61.90, 70.98, 83.99, 88.03, 111.78, 112.94, 119.29, 119.71, 129.29, 139.64, 141.60, 148.32, 152.35, 154.44, 159.23.

6-*(4-methoxybenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**7**), white solid after column chromatography followed by diethylether-dichloromethane precipitation, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.23–2.28 (m, 1H); 2.70–2.75 (m, 1H); 3.50–3.54 (m, 1H); 3.59–3.63 (m, 1H); 3.70 (s, 3H); 3.87–3.89 (m, 1H); 4.39–4.42 (m, 1H); 4.62 (bs, 2H); 5.21–5.24 (t, *J* = 4.5 Hz, 1H); 5.31–5.32 (d, *J* = 4.0 Hz, 1H); 6.33–6.36 (t, *J* = 7.25 Hz, 1H); 6.83–6.86 (m, 2H); 7.25–7.27 (d, *J* = 8,5, 2H); 8.20 (s, 1H); 8.35 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.80, 45.69, 55.09, 61.92, 70.99, 83.99, 88.03, 113.36, 113.85, 128.16, 128.51, 128.87, 131.97, 148.26, 152.35, 154.36, 158.10.

6-*(2-hydroxy-3-methoxybenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**8**), white solid after column chromatography and absolute ethanol precipitation, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.28 (m, 1H); 2.71–2.78 (m, 1H); 3.49–3.53 (m, 1H); 3.60–3.63 (m, 1H); 3.76 (s, 3H); 3.87–3.89 (m, 1H); 4.39–4.42 (m, 1H); 4.61 (bs, 2H); 5.17–5.19 (m, 1H); 5.30–5.31 (m, 1H); 6.34–6.36 (m, 1H); 6.65–6.68 (m, 2H); 6.82–6.83 (d, *J* = 8.0 Hz, 1H); 8.20 (s, 1H); 8.27 (bs, 1H); 8,37 (s, 1H); 9.34 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 38.71, 41.39, 55.79, 61.92, 71.04, 83.96, 88.02, 110.68, 118.65, 120.18, 125.40, 126.26, 139.72, 143.93, 147.72, 148.17, 152.13, 154.31.

6-*(4-hydroxy-3-methoxybenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**9**), slightly brown solid after column chromatography, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.23–2.27 (m, 1H); 2.69–2.75 (m, 1H); 3.49–3.53 (m, 1H); 3.60–3.63 (m, 1H); 3.71 (s, 3H); 3.87–3.89 (m, 1H); 4.40–4.41 (m, 1H); 4.58 (bs, 2H); 5.32 (bs, 1H); 6.33–6.36 (t, *J* = 6.5 Hz, 1H); 6.66–6.67 (d, *J* = 8 Hz, 1H); 6.72–6.73 (d, *J* = 7.5 Hz, 1H); 6.97 (s, 1H); 8.21 (bs, 1H); 8.30 (bs, 1H); 8.35 (s, 1H); 8.81 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.64, 45.57, 55.54, 61.91, 70.99, 83.99, 88.04, 111.91, 115.14, 119.77, 122.36, 130.74, 139.47, 145.36, 147.35, 148.23, 152.30, 154.44.

6-*(2,5-dimethoxybenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**10**), white solid after column chromatography, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.25–2.29 (m, 1H); 2.72–2.77 (m, 1H); 3.51–3.54 (m, 1H); 3.59 (s, 3H); 3.61–3.64 (m, 1H); 3.78 (s, 3H); 3.88–3.89 (m, 1H); 4.40–4.44 (m, 1H); 4.64 (bs, 2H); 5.21–5.24 (m, 1H); 5.33–5.35 (m, 1H); 6.35–6.38 (t, *J* = 6.5, 1H); 6.68 (bs, 1H); 6.73–6.76 (dd, *J* = 8.5 Hz, *J′* = 3.0 Hz, 1H); 6.85–6.93 (m, 1H); 8.18 (s, 1H); 8.21 (bs, 1H); 8.38 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 38.25, 45.72, 55.28, 55.77, 61.95, 71.03, 84.06, 88.08, 110.82, 111.15, 113.96, 119.80, 128.61, 139.74, 148.33, 150.71, 152.44, 153.06, 154.66.

6-*(2,3-dimethoxybenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**11**), dark beige waxy solid after column chromatography followed by preparative TLC purification, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.20–2.24 (m, 1H); 2.68–2.71 (m, 1H); 3.30 (s, 3H); 3.46–3.50 (m, 1H); 3.56–3.59 (m, 1H); 3.75 (s, 3H); 3.84 (bs, 1H); 4.35–4.37 (m, 1H); 4.68 (bs, 2H); 5.21 (bs, 1H); 5.33 (bs, 1H); 6.29–6.33 (t, *J* = 6.5, 1H); 6.74–6.76 (d, *J* = 7.5, 1H); 6.86–6.92 (m, 2H); 8.14 (s, 1H); 8.21 (bs, 1H); 8.33 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 37.86, 45.77, 55.71, 59.99, 61.95, 71.04, 84.06, 88.08, 111.44, 119.40, 119.80, 123.76, 133.22, 139.66, 146.08, 148.31, 152.27, 152.43, 154.60.

6-*(3-hydroxy-4-methoxybenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**12**), yellow waxy solid after column chromatography, preparative TLC and diethylether-acetone precipitation, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.23–2.28 (m, 1H); 2.70–2.75 (m, 1H); 3.50–3.53 (m, 1H); 3.60–3.63 (m, 1H); 3.70 (s, 3H); 3.87–3.89 (m, 1H); 4.40–4.42 (m, 1H); 4.55 (bs, 2H); 6.33–6.36 (m, 1H); 6.68–6.70 (d, *J* = 7.5, 1H); 6.75–6.76 (d, *J* = 2.0, 1H); 6.79–6.81 (d, *J* = 8.5, 1H);

8.19 (s, 1H); 8.30 (bs, 1H); 8.35 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.45, 45.61, 55.68, 61.89, 70.96, 83.99, 88.04, 112.12, 114.56, 117.63, 123.45, 128.75, 140.15, 144.50, 146.52, 148.24, 152.37, 154.40.

6-*(2,4,6-trimethoxybenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**13**), white solid purified by column chromatography and then preparative TLC, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.27 (m, 1H); 2.69–2.75 (m, 1H); 3.34 (s, 6H + residual H_2O); 3.50–3.55 (m, 1H); 3.60–3.63 (m, 1H); 3.76 (bs, 1H); 3.78 (s, 3H); 3.84 (s, 1H); 4.41 (s, 1H); 4.62 (bs, 2H); 5.26 (s, 1H); 5.33 (s, 1H); 6.25 (s, 2H); 6.32–6.35 (m, 1H); 6.80 (bs, 1H); 8.26 (s, 1H); 8.31 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 40.85, 55.29, 55.83, 61.90, 70.89, 83.99, 87.97, 90.82, 105.79, 119.51, 139.46, 145.40, 146.63, 152.36 159.30, 160.53

6-*(3,4,5-trimethoxybenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**14**), slightly yellow waxy solid after column chromatography, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.25–2.28 (m, 1H); 2.68–2.75 (m, 1H); 3.51–3.53 (m, 1H); 3.60 (s, 3H); 3.63–3.65 (m, 1H); 3.71 (s, 6H); 3.86–3.88 (m, 1H); 4.40–4.41 (m, 1H); 4.63 (bs, 2H); 5.26 (bs, 1H); 5.36 (bs, 1H); 6.33–6.36 (t, J = 7.0 Hz, 1H); 6.70 (s, 2H); 8.22 (s, 1H); 8.37 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 43.16, 55.80, 59.98, 61.91, 70.87, 83.98, 87.92, 104.70, 128.51, 135.68, 136.26, 139.60, 148.28, 152.34, 152.85, 154.40.

6-*(2-fluorobenzylamino)-2̂-deoxy-9-(β)-D-ribofuranosylpurine* (**15**), white solid after column chromatography and absolute ethanol precipitation, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.29 (m, 1H); 2.71–2.76 (m, 1H); 3.50–3.53 (m, 1H); 3.60–3.63 (m, 1H); 3.87–3.88 (m, 1H); 4.41 (m, 1H); 4.75 (bs, 2H); 5.21 (bs, 1H); 5.32 (bs, 1H); 6.34–6.37 (t, J = 6.0 Hz, 1H); 7.09–7.12 (t, J = 7.5 Hz, 1H); 7.15–7.19 (t, J = 8.5, 1H); 7.25–7.32 (m, 2H); 8.20 (s, 1H); 8.39 (s, 1H); 8.43 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 36.75, 56.05, 61.89, 70.98, 83.99, 88.04, 114.92, 115.10, 119.79, 124.28, 126.50, 128.54, 128.84, 139.78, 148.38, 152.33, 154.38, 159.04, 160.97.

6-*(3-fluorobenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**16**), yellowish solid after column chromatography, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.28 (m, 1H); 2.71–2.76 (m, 1H); 3.50–3.53 (m, 1H); 3.61–3.63 (m, 1H); 3.87–3.88 (m, 1H); 4.41 (s, 1H); 4.70 (bs, 2H); 5.21 (s, 1H); 5.33 (s, 1H); 6.34–6.37 (t, J = 6.5 Hz, 1H); 7.01–7.05 (t, J = 8.5 Hz, 1H); 7.11–7.13 (d, J = 10.0 Hz, 1H); 7.16–7.17 (d, J = 7.5 Hz, 1H); 7.31–7.35 (m, 1H); 8.20 (s, 1H); 8.38 (s, 1H); 8.50 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.46, 61.89, 70.98, 84.00, 88.03, 113.32, 113.48, 113.64, 113.81, 119.72, 123.10, 130.16, 130.23, 139.76, 143.14, 143.17, 148.38, 152.35, 154.36, 161.23, 163, 17.

6-*(4-fluorobenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**17**), white solid after column chromatography-preparative TLC and diethylether-dichloromethane precipitation, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.23–2.28 (m, 1H); 2.70–2.76 (m, 1H); 3.50–3.54 (m, 1H); 3.60–3.63 (m, 1H); 3.87–3.89 (m, 1H); 4.40–4.42 (m, 1H); 4.67 (bs, 2H); 5.20–5.22 (m, 1H); 5.31–5.32 (d, J = 4.0 Hz, 1H); 6.34–6.36 (m, 1H); 7.09–7.13 (m, 2H); 7.35–7.38 (m, 2H); 8.20 (s, 1H); 8.37 (s, 1H); 8.46 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.19, 45.71, 61.90, 70.99, 83.99, 88.04, 114.85, 115.03, 119.73, 129.09, 129.15, 136.21, 139.68, 148.31, 152.36, 154.35, 160.15, 162.06.

6-*(2-chlorobenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**18**), slightly beige solid after column chromatography and diethylether precipitation, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.26–2.29 (m, 1H); 2.74–2.76 (m, 1H); 3.50–3.54 (m, 1H); 3.60–3.64 (m, 1H); 3.89 (m, 1H); 4.42 (m, 1H); 4.75 (bs, 2H); 5.20–5.21 (m, 1H); 5.32–5.33 (d, J = 4.0 Hz, 1H); 6.35–6.38 (t, J = 6.0 Hz, 1H); 7.25–7.26 (m, 3H); 7.44–7.46 (m, 1H); 8.19 (s, 1H); 8.41 (s, 1H); 8.46 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 40.97, 56.05, 61.90, 70.99, 84.01, 88.16, 119.89, 127.13, 127.80, 128.32, 129.09, 131.71, 136.71, 139.87, 148.41, 152.38, 154.44.

6-*(3-chlorobenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**19**), white solid after column chromatography and diethylether precipitation, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.28 (m, 1H); 2.71–2.76 (m, 1H); 3.49–3.54 (m, 1H); 3.60–3.63 (m, 1H); 3.87–3.88 (m, 1H); 4.41 (m, 1H); 4.69 (bs, 2H); 5.19–5.21 (t, J = 5.0 Hz, 1H); 5.31–5.32 (m, 1H); 6.34–6.37 (t, J = 7.5 Hz, 1H); 7.26–7.34 (m, 3H); 7.37 (s, 1H); 8.20 (s, 1H); 8.38 (s, 1H); 8.50 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.41, 61.80, 71.04, 84.00, 88.03, 125.74, 126.66, 126.89, 130.18, 132.79, 139.79, 142.74, 148.40, 152.36, 153.83, 154.30.

6-*(4-chlorobenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**20**), beige solid after column chromatography and preparative TLC purification, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.28 (m, 1H); 2.72–2.74 (m, 1H); 3.50–3.53 (m, 1H); 3.61–3.62 (m, 1H); 3.87–3.89 (m, 1H); 4.41 (m, 1H); 4.67 (bs, 2H); 5.20–5.22 (m, 1H); 5.32–5.33 (t, J = 4.0 Hz, 1H); 6.34–6.37 (m, 1H); 7.34–7.35 (m, 1H); 8.20 (s, 1H); 8.38 (s, 1H); 8.48 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.39, 45.60, 61.90, 70.98, 84.00, 88.04, 119.75, 128.19, 129.00, 131.14, 139.07, 139.73, 148.33, 152.35, 154.35.

6-*(2-bromobenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**21**), white solid after column chromatography and diethylether-dichloromethane precipitation, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.25–2.29 (m, 1H); 2.74–2.77 (m, 1H); 3.50–3.54 (m, 1H); 3.60–3.64 (m, 1H); 3.88–3.89 (m, 1H); 4.42 (m, 1H); 4.70 (bs, 2H); 5.19–5.21 (t, J = 5.5 Hz, 1H); 5.32–5.33 (d, J = 4.0 Hz, 1H); 6.35–6.38 (t, J = 7.0 Hz, 1H); 7.17–7.22 (m, 2H); 7.28–7.31 (t, J = 8, 1H); 7.61–7.62 (dd, J = 8.0 Hz, J' = 1.0 Hz, 1H); 8.19 (s, 1H); 8.42 (s, 1H); 8.47 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 43.54, 61.90, 70.99, 84.01, 88.05, 119.79, 121.97, 127.69, 127.89, 128.65, 132.32, 138.11, 139.90, 148.46, 152.39, 154.38.

6-*(3-bromobenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**22**), white solid after column chromatography and diethylether-acetone precipitation, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.28 (m, 1H); 2.70–2.75 (m, 1H); 3.50–3.54 (m, 1H); 3.60–3.64 (m, 1H); 3.87–3.89 (m, 1H); 4.39–4.41 (m, 1H); 4.69 (bs, 2H); 5.18–5.20 (t, J = 5.0 Hz, 1H); 5.30–5.32 (d, J = 4.0 Hz, 1H); 6.34–6.37 (m, 1H); 7.24–7.27 (t, J = 8.0 Hz, 1H); 7.33–7.34 (d, J = 7.0 Hz, 1H); 7.39–7.41 (d, J = 7.5 Hz, 1H); 7.52 (s, 1H); 8.20 (s, 1H); 8.38 (s, 1H); 8.50 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.35, 61.86, 70.94, 83.96, 88.01, 121.54, 126.21, 129.50, 129.78, 130.47, 131.34, 131.71, 139.77, 142.97, 148.37, 152.33.

6-*(4-bromobenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**23**), beige solid after column chromatography and preparative TLC purification, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.28 (m, 1H); 2.72–2.75 (m, 1H); 3.50–3.53 (m, 1H); 3.60–3.63 (m, 1H); 3.87–3.88 (m, 1H); 4.41 (m, 1H); 4.66 (bs, 2H); 5.20 (bs, 1H); 5.32 (bs, 1H); 6.33–6.36 (m, 1H); 7.27–7.29 (d, J = 7.5, 2H); 7.47–7.48 (d, J = 7.5, 2H); 8.19 (s, 1H); 8.37 (s, 1H); 8.48 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.32, 61.88, 70.96, 83.99, 88.02, 119.59, 119.69, 119.76, 129.36, 131.08, 139.51, 139.72, 148.34, 152.32, 154.35.

6-*(3-iodobenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**24**), white solid after column chromatography and diethylether-dichloromethane precipitation, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.28 (m, 1H); 2.70–2.75 (m, 1H); 3.50–3.53 (m, 1H); 3.61–3.63 (m, 1H); 3.87–3.88 (m, 1H); 4.40–4.42 (m, 1H); 4.65 (bs, 2H); 5.22 (bs, 1H); 5.35 (bs, 1H); 6.34–6.36 (m, 1H); 7.08–7.11 (t, J = 8.0, 1H); 7.34–7.35 (d, J = 7.5, 1H); 7.56–7.58 (d, J = 7.5, 1H); 7.71 (s, 1H); 8.20 (s, 1H); 8.38 (s, 1H); 8.48 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.22, 61.86, 68.15, 70.83, 83.95, 88.02, 94.65, 126.55, 130.50, 135.35, 135.61, 139.63, 142.01, 142.82, 152.34, 153.16, 154.28.

6-*(4-iodobenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**25**), white solid after column chromatography and preparative TLC purification, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.27 (m, 1H); 2.71–2.74 (m, 1H); 3.48–3.54 (m, 1H); 3.60–3.63 (m, 1H); 3.88 (m, 1H); 4.39–4.41 (m, 1H); 4.63 (bs, 2H); 5.19–5.22 (m, 1H); 5.30–5.32 (m, 1H); 6.33–6.36 (m, 1H); 7.12–7.15 (d, J = 8.0 Hz, 2H); 7.63–7.66 (d, J = 8.0 Hz, 2H); 8.19 (s, 1H); 8.36 (s, 1H); 8.47 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.44, 45.70, 61.89, 70.98, 83.99, 88.03,

92.33, 119.71, 129.53, 136.94, 139.71, 139.94, 148.38, 152.34, 154.31.

6-*(2-methylbenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**26**), white solid after column chromatography and preparative TLC, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.25–2.30 (m, 1H); 2.33 (s, 3H); 2.7–2.75 (m, 1H); 3.50–3.53 (m, 1H); 3.61–3.63 (m, 1H); 3.88 (m, 1H); 4.41 (m, 1H); 4.67 (bs, 2H); 5.25 (bs, 1H); 5.36 (bs, 1H); 6.34–6.37 (t, J = 7.0 Hz, 1H); 7.06–7.13 (m, 2H); 7.14–7.17 (d, J = 7.0 Hz, 1H); 7.18–7.21 (d, J = 6.0 Hz, 1H); 8.18 (s, 1H); 8.34 (bs, 1H); 8.37 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 18.91, 57.02, 58.90, 61.89, 70.96, 84.00, 88.15, 125.67, 126.47, 126.54, 127.08, 129.80, 135.19, 139.56, 139.67, 148.18, 148.31, 152.42.

6-*(3-methylbenzylamino)-2́-deoxy-9-(β)-D-ribofuranosylpurine* (**27**), beige-brownish waxy solid after column chromatography, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.23–2.28 (m, 1H); 2.25 (s, 3H); 2.71–2.76 (m, 1H); 3.50–3.54 (m, 1H); 3.60–3.63 (m, 1H); 3.87–3.89 (m, 1H); 4.40–4.41 (m, 1H); 4.66 (bs, 2H); 5.21–5.24 (t, J = 6.0 Hz, 1H); 5.31–5.32 (d, J = 4.0 Hz, 1H); 6.34–6.36 (m, 1H); 7.01–7.02 (d, J = 7.5 Hz, 1H); 7.10–7.18 (m, 3H); 8.19 (s, 1H); 8.36 (s, 1H); 8.41 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 21.10, 42.78, 62.01, 70.99, 84.02, 87.95, 119.69, 124.22, 127.27, 127.65, 128.15, 137.24, 139.61, 139.98, 148.33, 152.38.

6-*(4-methylbenzylamino)-2́-deoxy-9-(β)-D-ribofuranosylpurine* (**28**), brownish solid after column chromatography, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24 (s, 3H); 2.23–2.29 (m, 1H); 2.72–2.75 (m, 1H); 3.50–3.53 (m, 1H); 3.60–3.63 (m, 1H); 3.88 (m, 1H); 4.41 (m, 1H); 4.64 (bs, 2H); 5.21–5.23 (m, 1H); 5.30–5.32 (m, 1H); 6.33–6.36 (t, J = 6.5 Hz, 1H); 7.08–7.09 (d, J = 7.5 Hz, 2H); 7.20–7.22 (d, J = 7.5 Hz, 2H); 8.19 (s, 1H); 8.35 (s, 1H); 8.38 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 20.68, 42.59, 61.91, 70.99, 84.01, 88.03, 119.71, 127.12, 128.76, 135.61, 136.99, 139.58, 148.28, 152.35, 154.44.

6-*(3-trifluoromethoxybenzylamino)-2́-deoxy-9-(β)-D-ribofuranosylpurine* (**29**), slightly yellow waxy solid after column chromatography, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.28 (m, 1H); 2.70–2.76 (m, 1H); 3.50–3.53 (m, 1H); 3.60–3.63 (m, 1H); 3.86–3.89 (m, 1H); 4.41 (m, 1H); 4.73 (bs, 2H); 5.20 (bs, 1H); 5.31–5.32 (d, J = 3.5 Hz, 1H); 6.34–6.37 (m, 1H); 7.20–7.22 (d, J = 8.0 Hz, 1H); 7.32 (s, 1H); 7.35–7.37 (d, J = 7.5 Hz, 1H); 7.41–7.45 (t, J = 8.0 Hz, 1H); 8.20 (s, 1H); 8.39 (s, 1H); 8.53 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.93, 54.08, 62.38, 71.47, 84.48, 88.45, 119.75, 119.93, 126.71, 130.84, 140.31, 143.50, 143.52, 148.90, 152.83, 154.72.

6-*(4-trifluoromethoxybenzylamino)-2́-deoxy-9-(β)-D-ribofuranosylpurine* (**30**), white solid foam after column chromatography and diethylether-dichloromethane precipitation, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.28 (m, 1H); 2.70–2.76 (m, 1H); 3.50–3.54 (m, 1H); 3.60–3.64 (m, 1H); 3.87–3.89 (m, 1H); 4.40–4.42 (m, 1H); 4.71 (bs, 2H); 5.18–5.21 (t, J = 6.0 Hz, 1H); 5.31–5.32 (d, J = 4.5 Hz, 1H); 6.34–6.37 (m, 1H); 7.28–7.30 (d, J = 8.5 Hz, 1H); 7.44–7.45 (d, J = 8.5 Hz, 1H); 8.20 (s, 1H); 8.38 (s, 1H); 8.50 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 58.89, 73.31, 84.48, 88.53, 112.49, 121.04, 121.29, 121.42, 121.63, 121.81, 129.25, 129.58, 140.09, 140.21, 140.40, 147.56, 151.86, 154.84.

6-*(2-trifluoromethylbenzylamino)-2́-deoxy-9-(β)-D-ribofuranosylpurine* (**31**), white solid foam after column chromatography and diethylether-dichloromethane precipitation, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.26–2.29 (m, 1H); 2.74–2.77 (m, 1H); 3.50–3.54 (m, 1H); 3.60–3.63 (m, 1H); 3.88 (m, 1H); 4.42 (bs, 1H); 4.90 (bs, 2H); 5.18–5.20 (t, J = 5.5 Hz, 1H); 5.32–5.33 (d, J = 4.0 Hz, 1H); 6.36–6.38 (t, J = 7.0 Hz, 1H); 7.42–7.45 (t, J = 8.0 Hz, 1H); 7.56–7.59 (t, J = 7.5 Hz, 1H); 7.72–7.74 (d, J = 7.5 Hz, 1H); 8.19 (s, 1H); 8.43 (s, 1H); 8.50 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 56.69, 61.96, 70.98, 84.08, 88.18, 123.55, 125.79, 154.43, 125.68, 127.08, 127.47, 132.72, 138.13, 149.94, 147.63, 148.35, 152.42.

6-*(3-trifluoromethylbenzylamino)-2́-deoxy-9-(β)-D-ribofuranosylpurine* (**32**), white solid after column chromatography, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.28 (m, 1H); 2.71–2.76 (m, 1H); 3.50–3.54 (m, 1H); 3.60–3.63 (m, 1H); 3.87–3.89 (m, 1H); 4.39–4.41 (m, 1H); 4.77 (bs, 2H); 5.19–5.21 (t, J = 6.0 Hz, 1H); 5.31–5.32 (d, J = 4.0 Hz, 1H); 6.34–6.37 (t, J = 8.0 Hz, 1H); 7.52–7.55 (t, J = 7.5 Hz, 1H); 7.58–7.59 (d, J = 7.5 Hz, 1H); 7.63–7.65 (d, J = 7.0 Hz, 1H); 7.70 (s, 1H); 8.21 (s, 1H); 8.39 (s, 1H); 8.56 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.53, 61.88, 70.97, 83.93, 88.04, 123.47, 123.70, 125.36, 128.82, 129.07, 129.25, 131.26, 139.83, 141.49, 152.24, 154.26, 154.33.

6-*(4-trifluoromethylbenzylamino)-2́-deoxy-9-(β)-D-ribofuranosylpurine* (**33**), white solid after column chromatography, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.28 (m, 1H); 2.73–2.74 (m, 1H); 3.50–3.53 (m, 1H); 3.60–3.63 (m, 1H); 3.88 (m, 1H); 4.41 (m, 1H); 4.77 (bs, 2H); 5.20–5.22 (m, 1H); 5.31–5.33 (m, 1H); 6.33–6.37 (m, 1H); 7.53–7.54 (m, 2H); 7.65–7.67 (m, 2H); 8.18 (s, 1H); 8.39 (s, 1H); 8.56 (bs, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 42.64, 45.59, 61.90, 70.99, 84.01, 87.96, 119.75, 123.33, 125.18, 127.72, 139.83, 144.93, 148.42, 152.36, 154.36.

6-*(furfurylamino)-2́-deoxy-9-(β)-D-ribofuranosylpurine* (**34**), brownish waxy solid after column chromatography, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.24–2.28 (m, 1H); 2.70–2.75 (m, 1H); 3.50–3.54 (m, 1H); 3.60–3.63 (m, 1H); 3.87–3.89 (m, 1H); 4.40–4.42 (m, 1H); 4.68 (bs, 2H); 5.20–5.22 (t, J = 6.0 Hz, 1H); 5.31–5.32 (d, J = 4.0 Hz, 1H); 6.22–6.23 (d, J = 2.5 Hz, 1H); 6.34–6.37 (m, 2H); 7.53–7.54 (dd, J = 1.5 Hz, J́= 0.2 Hz, 1H); 8.24 (s, 1H); 8.31 (bs, 1H); 8.37 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 36.48, 45.75, 61.79, 70.82, 83.96, 87.98, 106.66, 110.53, 119.76, 138.23, 139.55, 141.75, 148.46, 152.21, 152.73.

3.2. Biological testing

3.2.1. Cytotoxic activity testing

The effects of 72 h treatment with the compounds on the viability of human skin fibroblasts BJ, keratinocytes HaCaT and retinal pigment epithelium cells ARPE-19 were evaluated using the resazurin reduction assay. The cell lines were obtained from the American Type Culture Collection, Manassas, VA, USA (BJ, ARPE-19) and the German Cancer Research Center (*DKFZ*), Heidelberg, Germany (HaCaT). The cells were maintained in DMEM (BJ, HaCaT) or DMEM/F12 (ARPE-19) culture medium (Sigma) supplemented with fetal bovine serum (10%), glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL) under standard cell culture conditions (37 °C, 5% CO_2, humid environment) and sub-cultured two or three times a week.

For testing, about 5,000 cells were seeded into each well of a 96-well plate about 24 h before treatment with the test compounds (10 or 30 μM) or DMSO vehiculum. The final concentration of DMSO did not exceed 0.1%. An 11× concentrated solution of resazurin in DMEM medium was added to the cells to a final concentration of 100 μM after 72 h. Fluorescence intensity (ex = 570 nm, em = 610 nm) was measured after 3 h incubation.

3.2.2. Cytokinin bioassays

Cytokinin bioassays were performed according to the literature[76], with BAP used as a positive control for all three bioassays. Results were recorded as the highest activities of the derivatives for the concentration range tested.

3.2.2.1. 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.

3.2.2.2. Wheat leaf senescence bioassay. Wheat seeds (*Triticum aestivum* L. cv. Hereward) 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/8h; 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.

3.2.2.3. 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^{-9} 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).

3.2.3. Cytokinin signalling bioassay

3.2.3.1. Bacterial receptor assay[75]. Transgenic *E. coli* strains KMI001 harboring a PINIII/AHK4 or pSTV28/AHK3 plasmid and expressing the *β*-galactosidase gene (*ΔrcsC, cps::lacZ*) under the control of cytokinin receptors were used for this assay. Bacterial precultures were grown in liquid M9 medium supplemented with 0.1% casamino acids and an antibiotic (ampicillin, 100 $\mu g{\cdot}mL^{-1}$ for CRE1/AHK4, and chloramphenicol, 20 $\mu g{\cdot}mL^{-1}$ for AHK3), with shaking (300 rpm) at 25 °C for 24 h. Expression of the β-galactosidase gene was induced by cultivation of 200 µL of the precultures diluted by M9 medium with antibiotic (1:600) and test compound (50, 10, 1 or 0.1 µM) with shaking (450 rpm) at 25 °C for 17 h. At the end of the incubation period, 50 µL of the bacterial cultures was transferred to a new 96-well plate and the activity of β-galactosidase was determined by measuring the fluorescence intensity (λex/em − 365/460 nm) after incubation with 2 µL of 10 mM (25 mM for AHK3) chromogenic substrate (MUG) at 37 °C for 10 min (AHK4) or 30 min (AHK3) and addition of 100 µL of Stop buffer (132 mM glycine, 83 mM Na_2CO_3).

3.2.4. Antioxidant capacity testing[86,87]

Antioxidant capacity was determined by the common method of total phenolic content (TPC). Briefly, 67 µL of the solution of test compound (concentration 5.10^{-2} mol/L; 1.10^{-2} mol/L for **6**, **8**, **11**, **22**, **26** and **31**), 3 µL of Folin-Ciocalteu reagent and 130 µL of 2% Na_2CO_3 were added into wells of microplates. The microplates were kept in the dark at laboratory temperature for 30 min. Subsequently, the absorbance was read at 750 nm in an Infinite M200 reader (TECAN). The linear range used for analysis was 0.2 – 2 µg gallic acid per well. All results (obtained in triplicate) were expressed as gallic acid equivalents (GAE) on an equimolar basis.

3.2.5. Antiviral activity studies

3.2.5.1. Cell culture and virus. African green monkey kidney (Vero) cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) containing 9% fetal bovine serum (FBS) and supplemented with L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 µg/ml). Cell cultures were grown in a 5% CO_2 humidified atmosphere at 37 °C. To test the antiviral activity of a particular compound, a prototype strain of murine gammaherpesvirus (MHV-68)[85] was used. Virus stock was prepared by infection of Vero cells at low multiplicity of infection, cells were harvested after 96 h, centrifuged, the supernatant was collected and the virus titer determined by plaque assay.

Stock solutions of the tested compounds (2 mg/ml) were prepared by dissolving in dimethyl sulfoxide (DMSO), filtering through a 0.2 µm filter and diluting to appropriate concentrations in DMEM. Ganciclovir (GCV; Sigma-Aldrich) was dissolved in distilled water and used at final concentration of 10 µg/ml for *in vitro* experiments.

3.2.5.2. Cytotoxicity assay. Vero cells were seeded in 96-well plates at a density of 8×10^3 cells/well in 100 µL of DMEM (9% FBS). After 24 h incubation, the culture medium was removed and replenished with 100 µL of DMEM (2% FBS) containing different concentrations (5 µg/ml or 10 µg/ml) of the compounds. At final dilutions, the concentration of DMSO never exceeded 0.5%. Each compound concentration was tested in duplicate. DMSO vehicle controls were run simultaneously. Cells not treated with compounds or DMSO were used as control cells. Cells were then incubated for 4 days at 37 °C in a 5% CO_2 humidified atmosphere. Cell morphology was observed daily by microscopically detecting morphological alterations, such as loss of confluency, cell rounding and shrinking.

3.2.5.3. Cytopathic effect (CPE) reduction assay. To determine the potential antiviral activity of compounds against MHV-68, the CPE reduction assay was performed. Vero cells were seeded in 96-well plates at a density of 8×10^3 cells/well in 100 µL of DMEM (9% FBS). The next day, the culture medium was removed and the cells were infected with MHV-68 at a multiplicity of infection of 0.02. After adsorption for 90 min at 37 °C, residual virus was removed and 100 µL of DMEM (2% FBS) containing non-cytotoxic concentrations of the compounds was added to each well. Each compound concentration was tested in duplicate. Infected cells treated with medium alone or DMSO were included as negative controls, whereas infected cells with GCV were used as a positive control. Cells were then incubated at 37 °C in a 5% CO_2 humidified atmosphere. Viral CPE was recorded microscopically based on detectable alterations of the cell morphology as soon as it reached completion in the negative control wells (at 4 days post infection).

4. Conclusion

A novel series of non-cytotoxic 6-substituted aromatic 2′-deoxy-9-(*β*)-D-ribofuranosylpurines was prepared and cytokinin activity was examined. Generally, the presence of the 2′-deoxyribosyl moiety significantly contributed to the anti-senescence activity of 2′-deoxy-9-(*β*)-D-riboside derivatives in comparison with their ribosides or bases. For example, compounds **3** and **6** showed increasing activity (with increasing concentration) in all three CK bioassays as compared with BAP. Derivatives possessing a phenolic OH group also showed considerable antioxidant activity, especially compounds **12** and **8**. The antiviral (and also antiprotozoal) activity will be examined further in future work.

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Appendix A. Supplementary material

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Supporting information

New aromatic 6-substituted 2´-deoxy-9-(β)-D-ribofuranosylpurine derivatives as potential plant growth regulators

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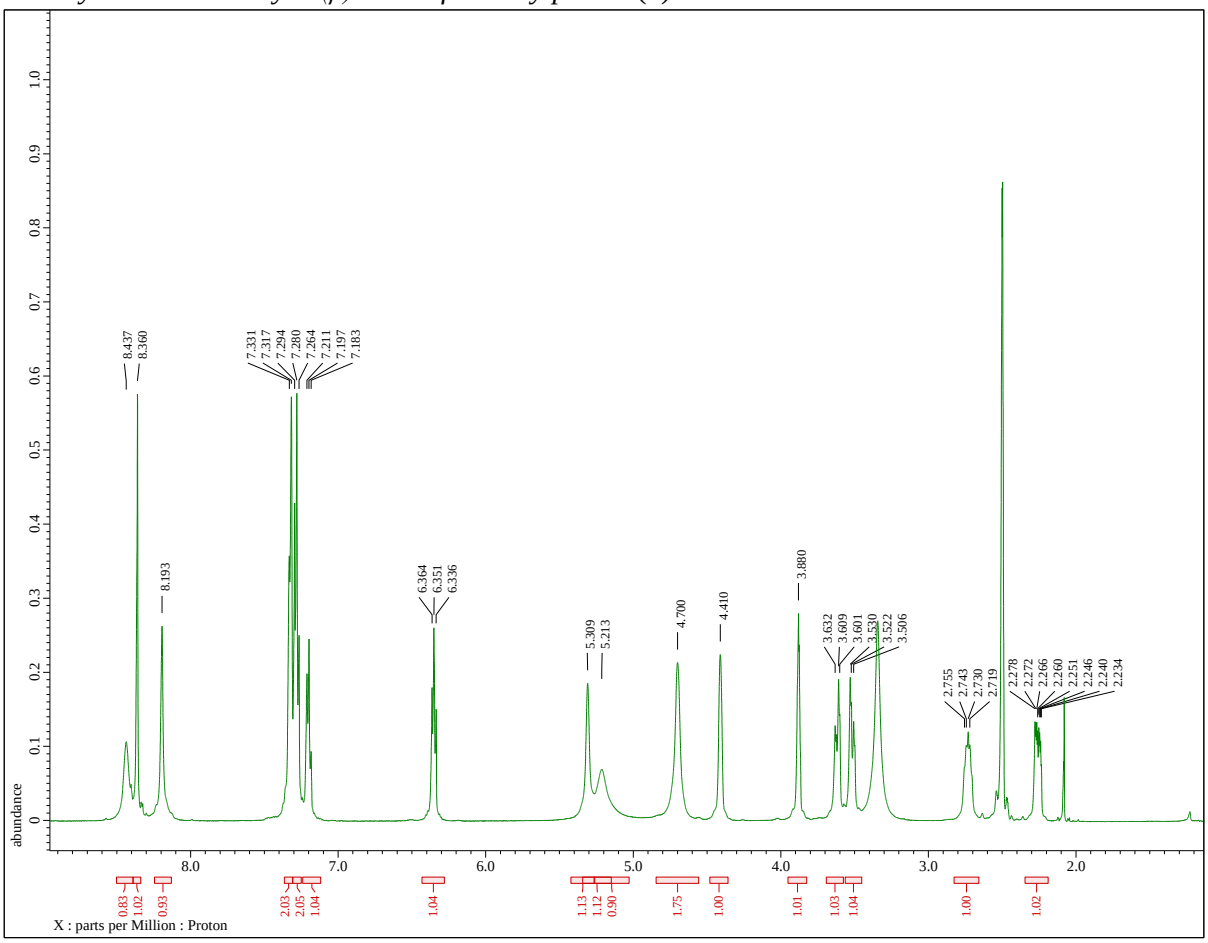
Content: copies of ^{1}H NMR spectra of prepared substituted benzylamino-2´-deoxy-9-(β)-D-ribofuranosylpurines

6-*benzylamino-2´-deoxy-9-(β)-D-ribofuranosylpurine* (**1**)



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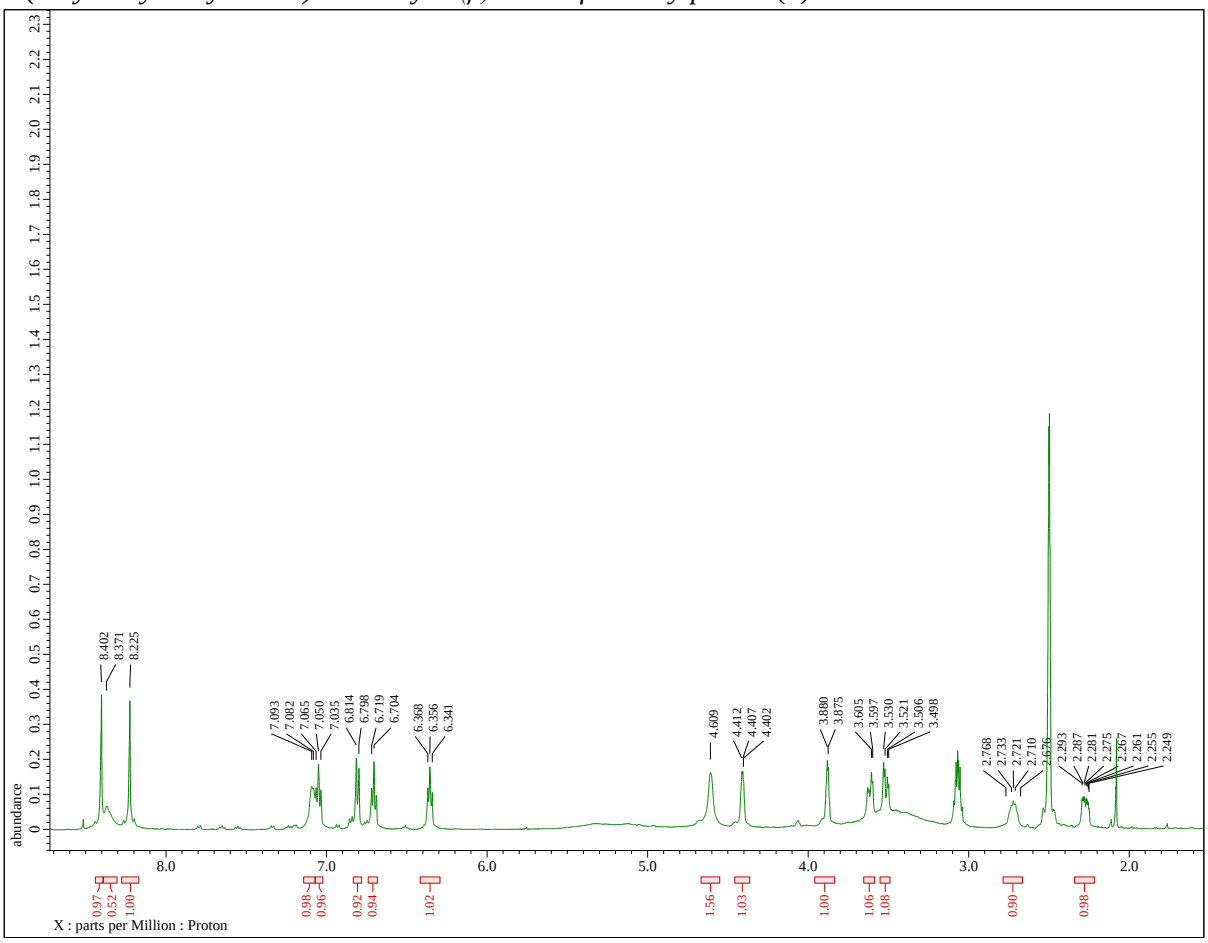

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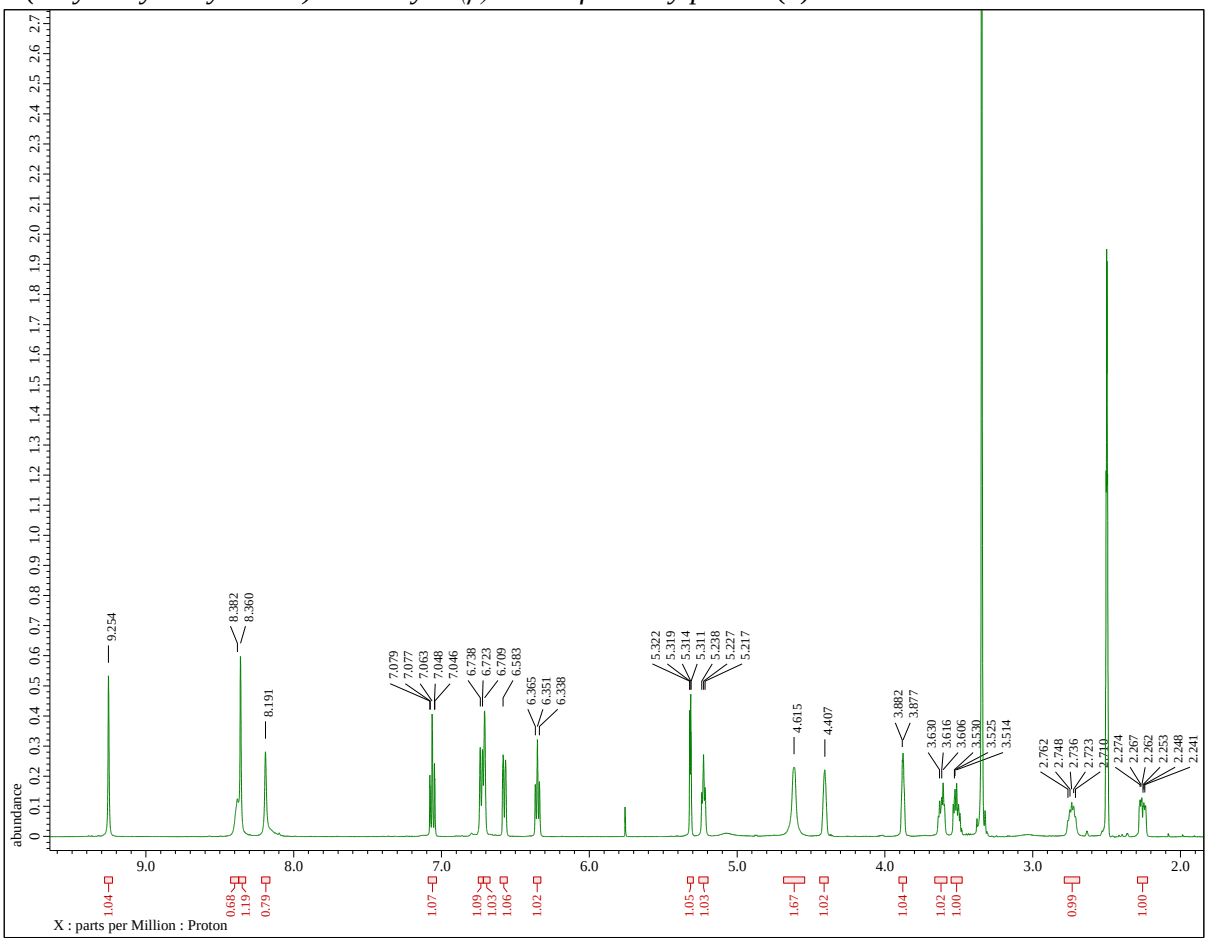

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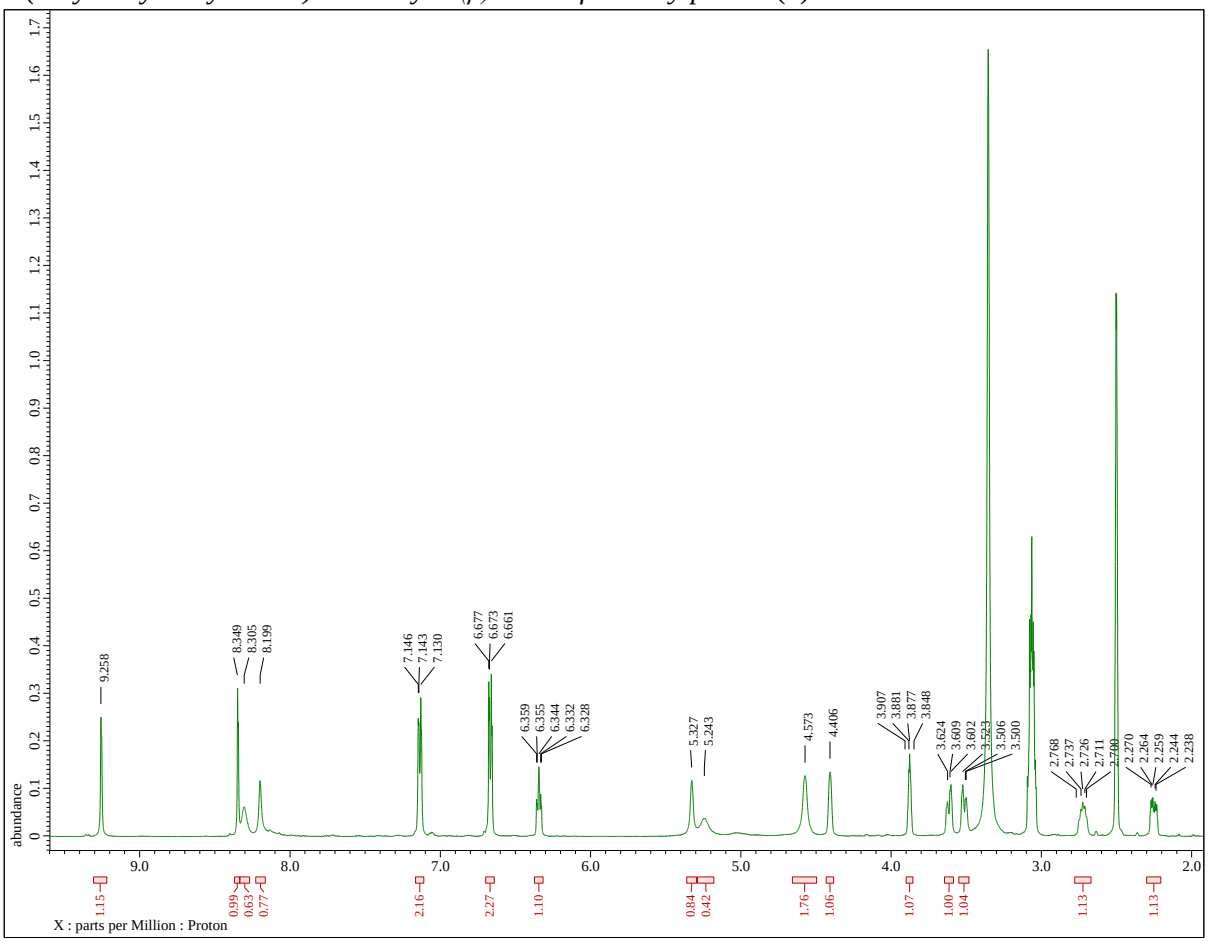

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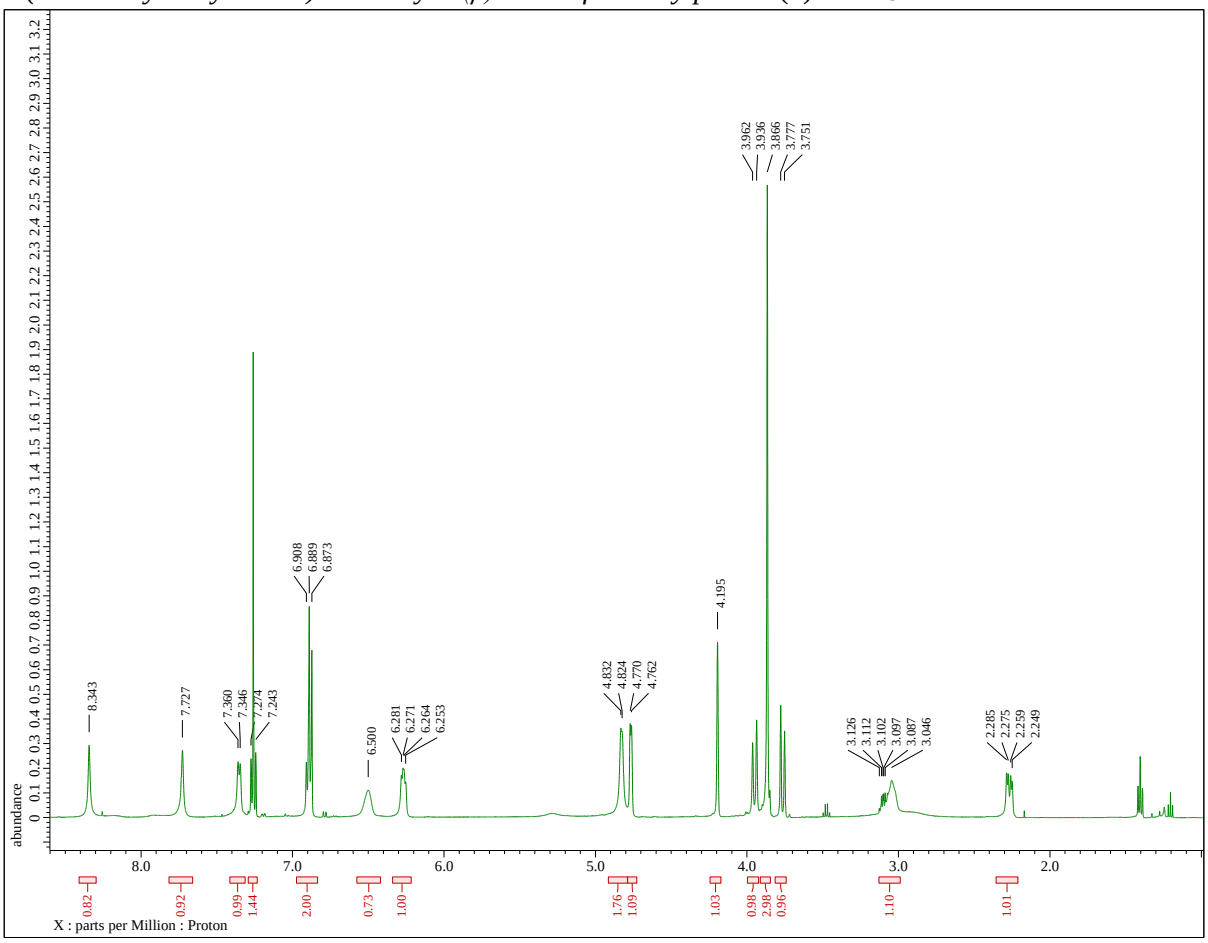

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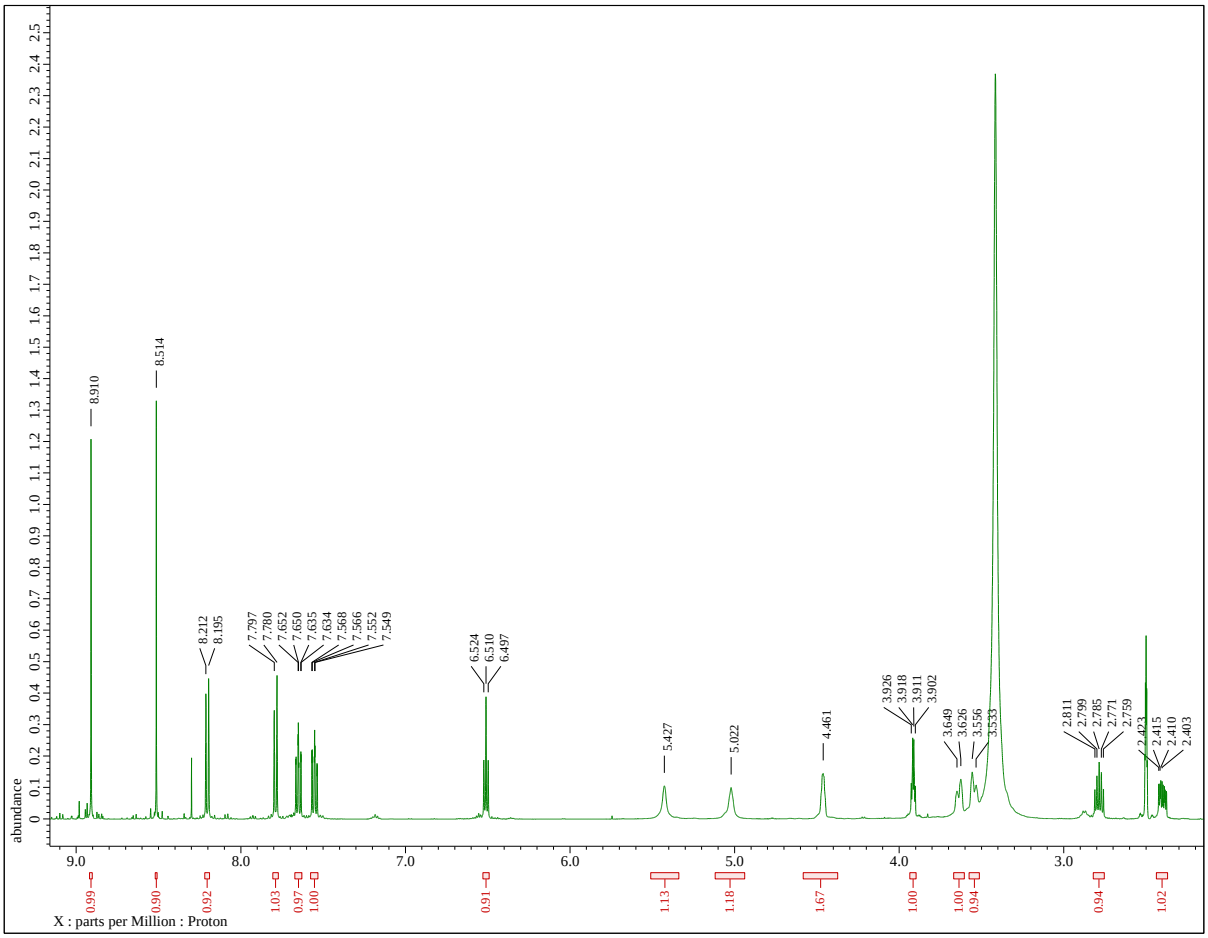

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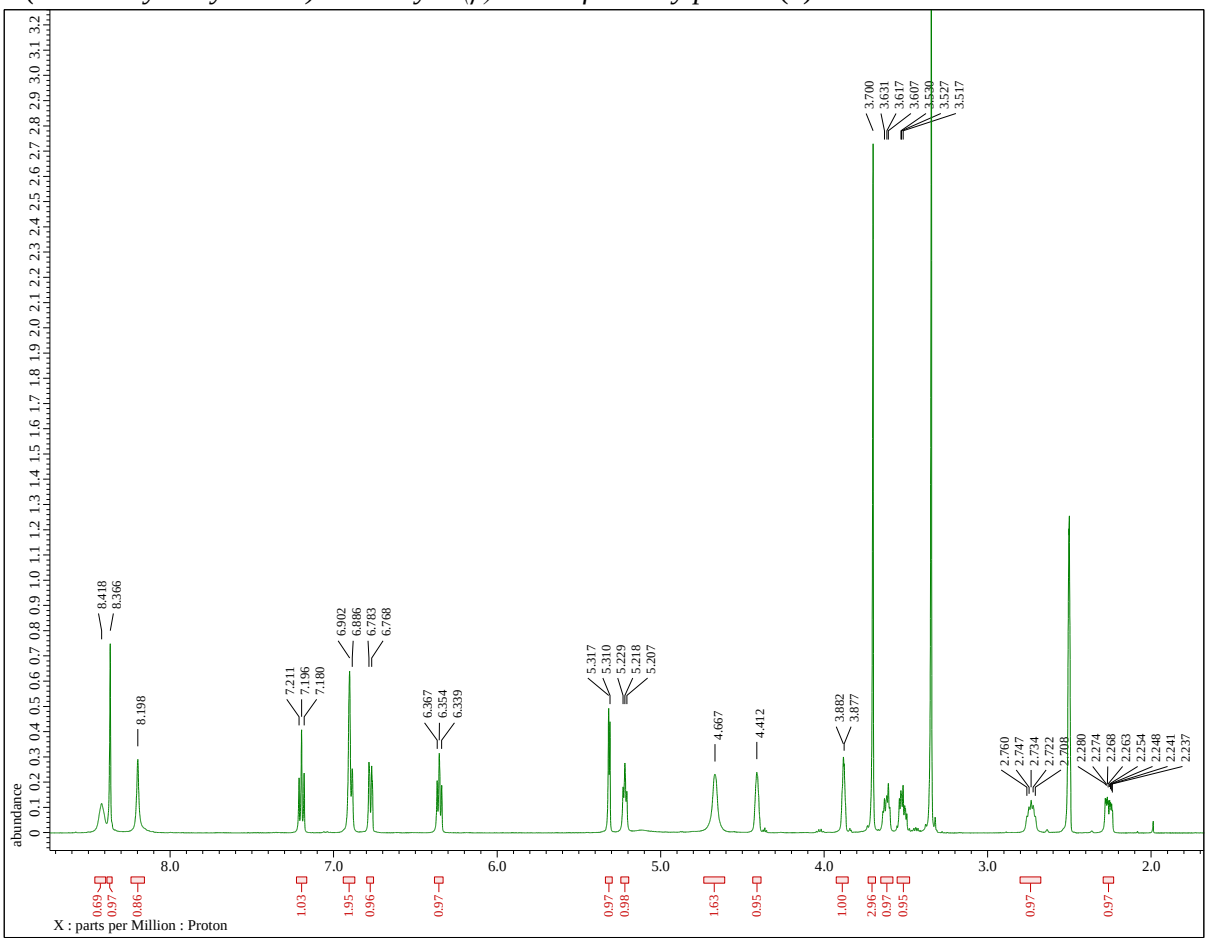

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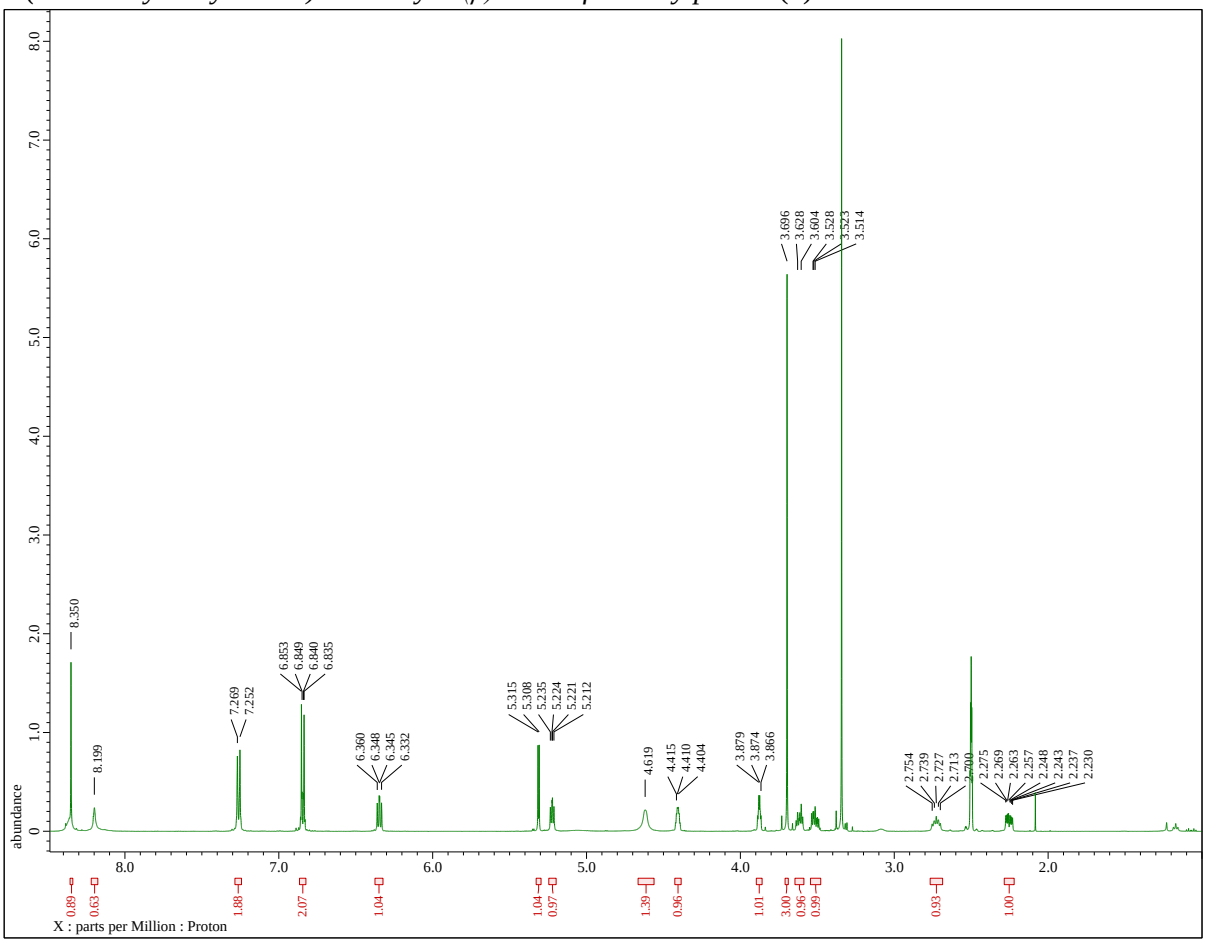

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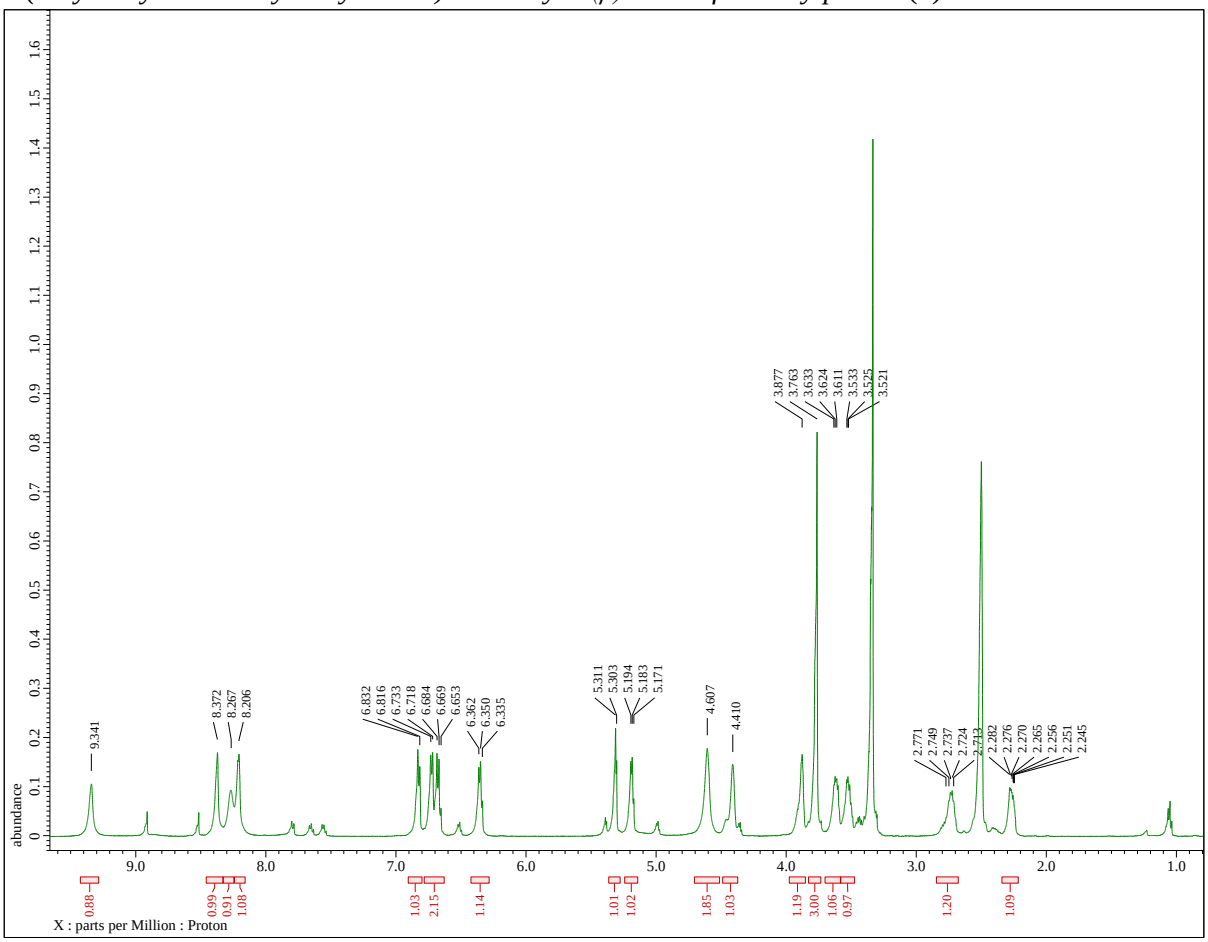

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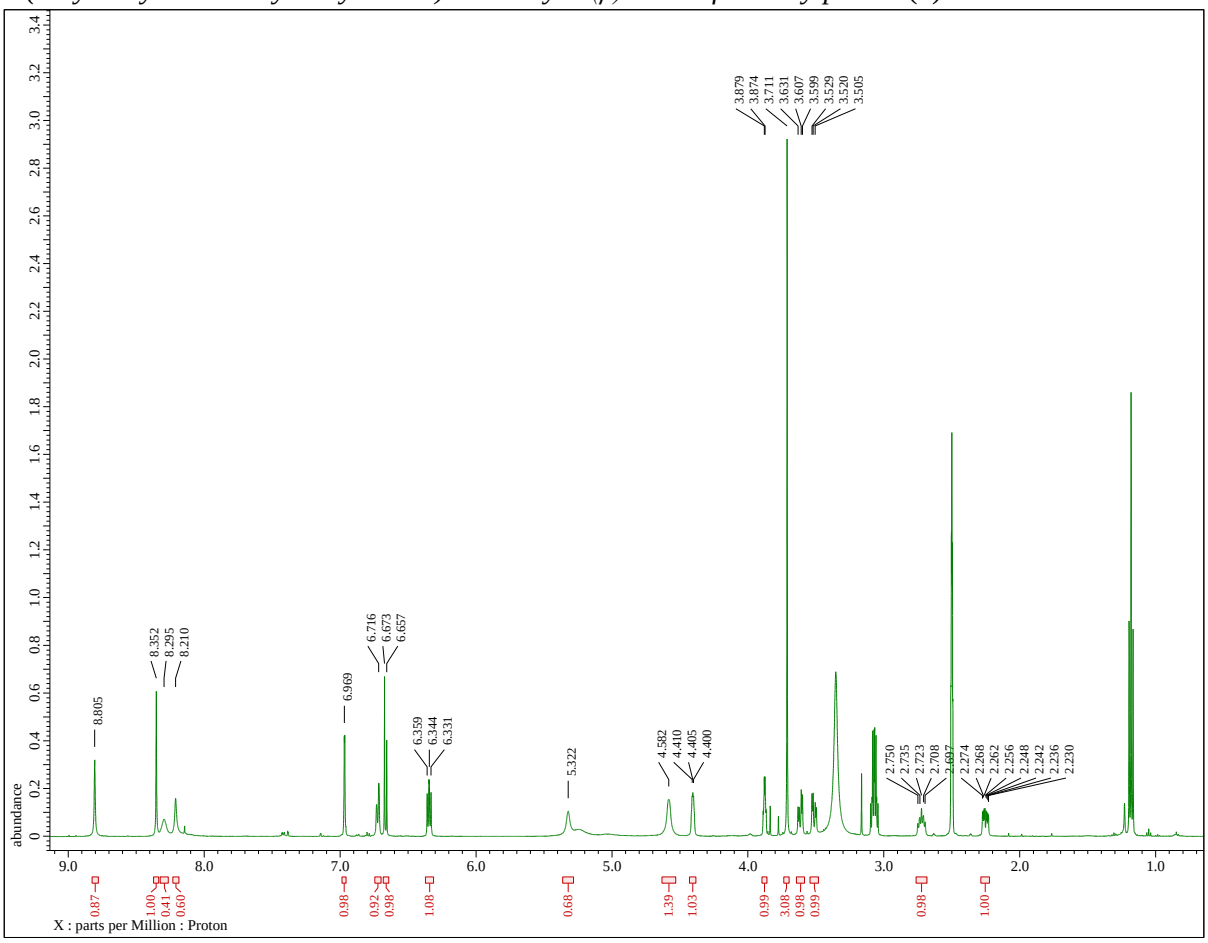

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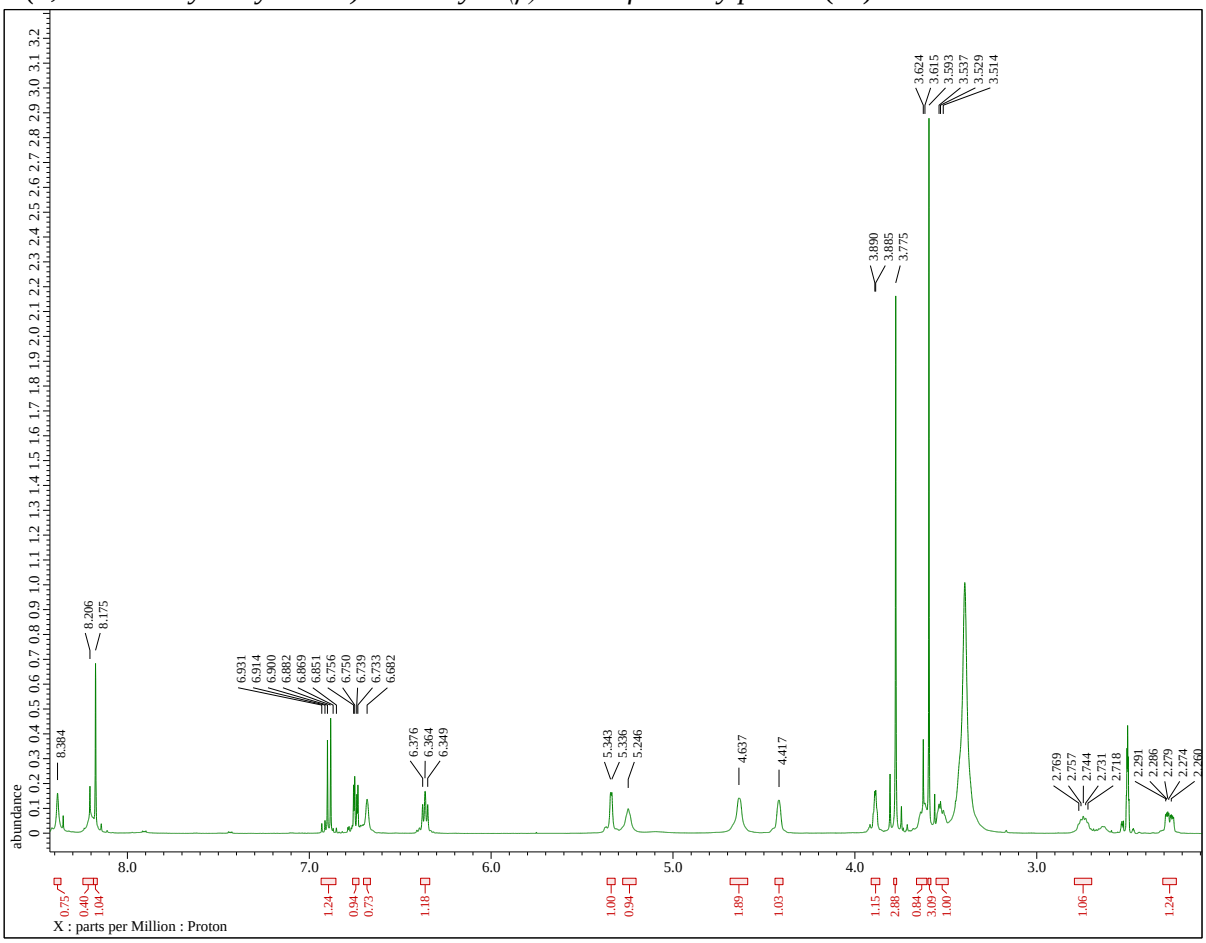

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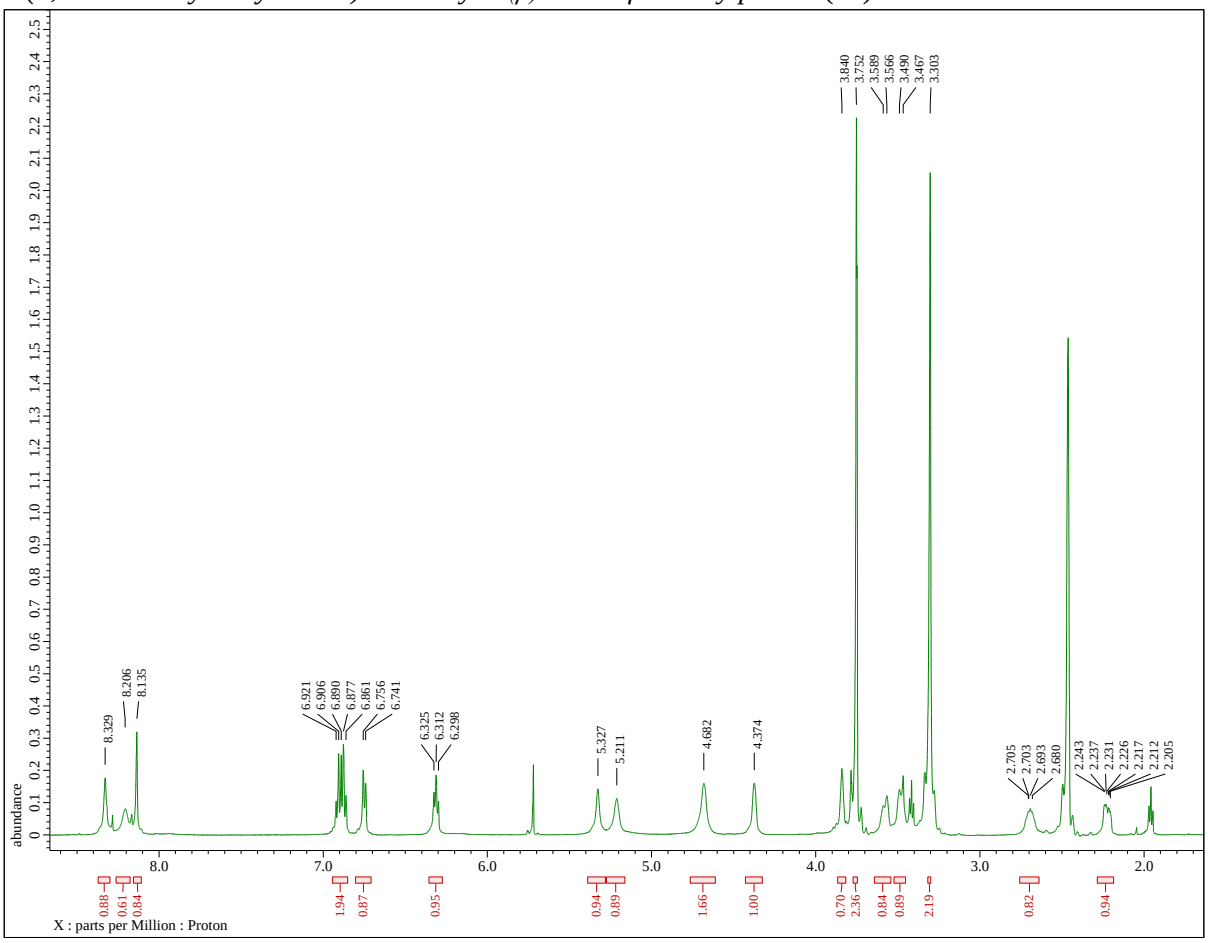

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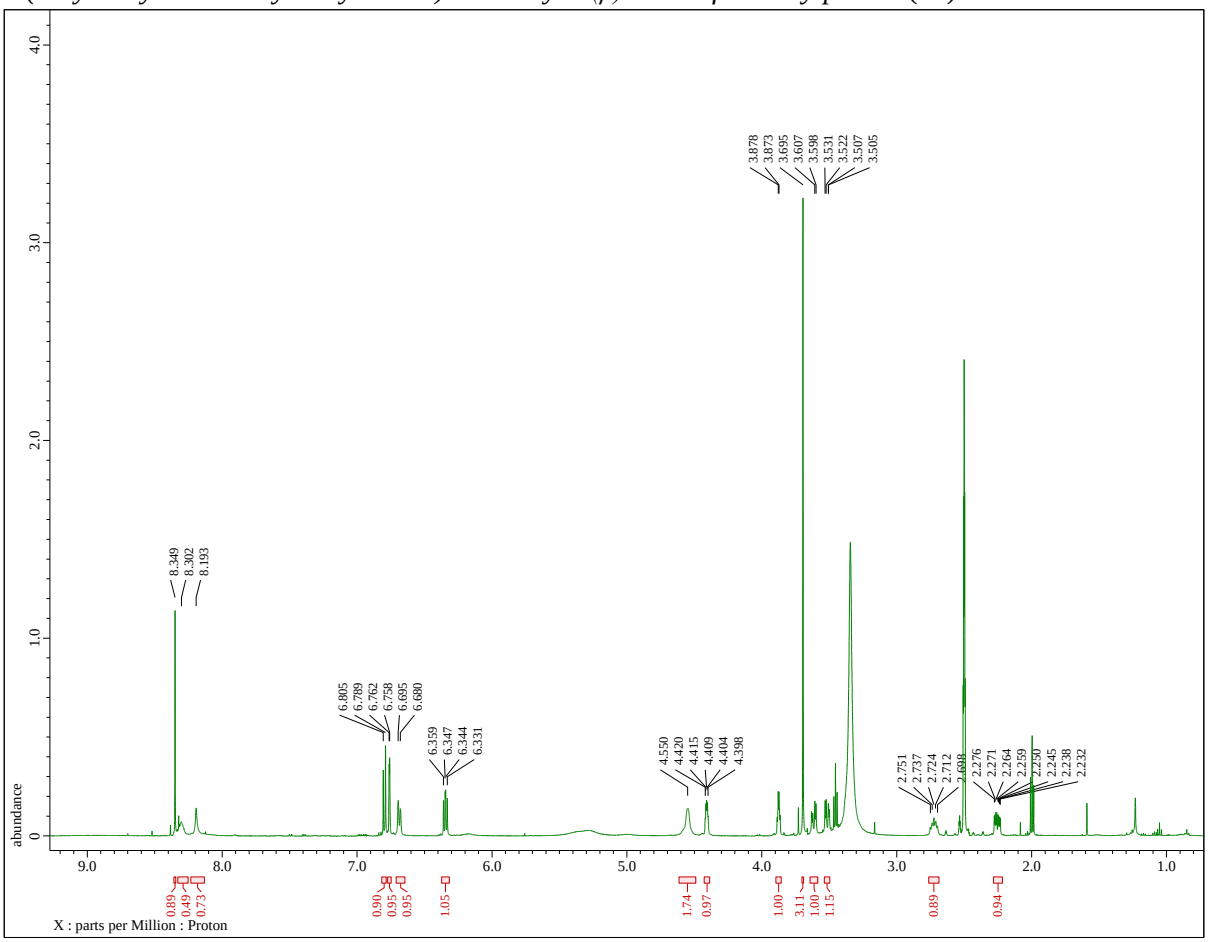
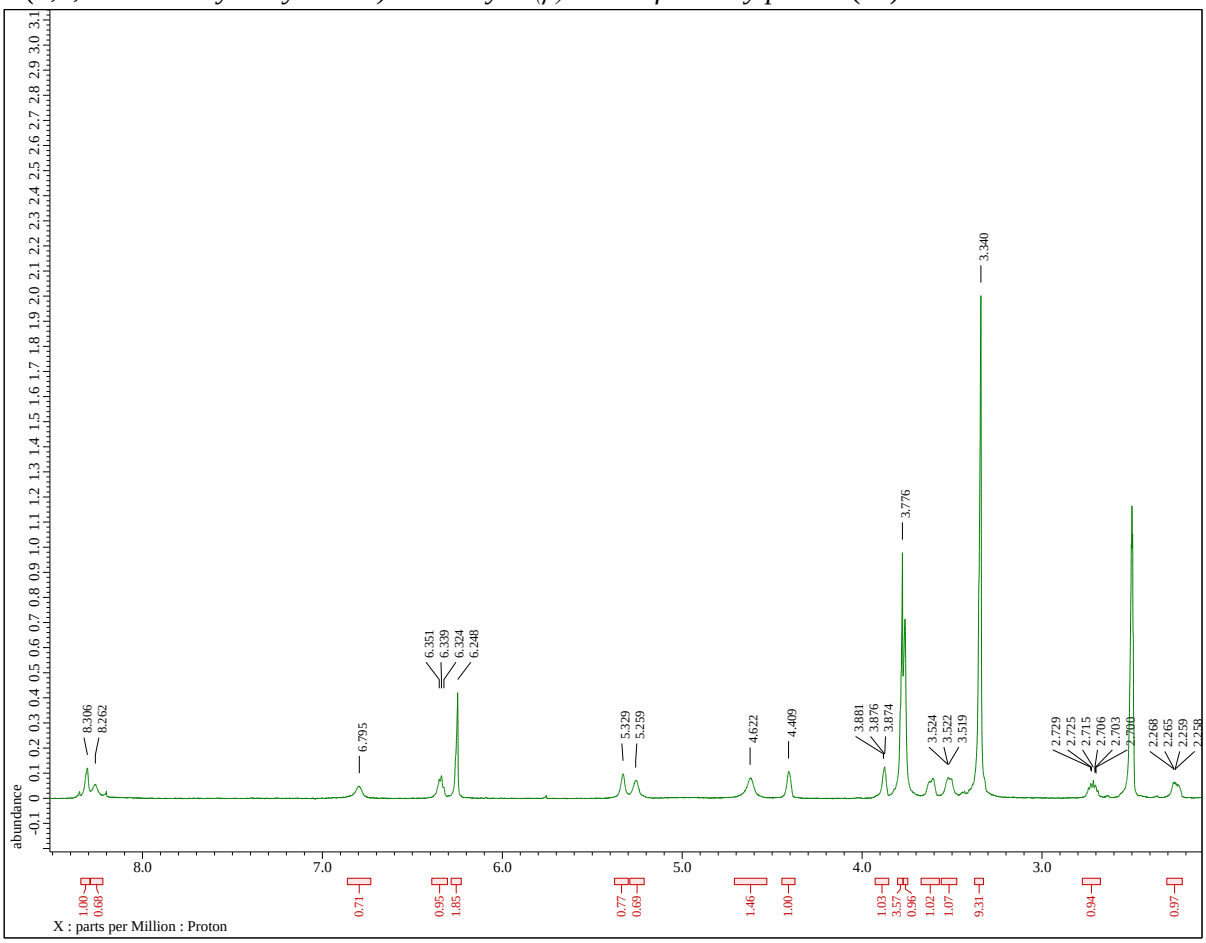


6-*(2,4,6-trimethoxybenzylamino)-2′-deoxy-9-(β)-D-ribofuranosylpurine* (**13**)



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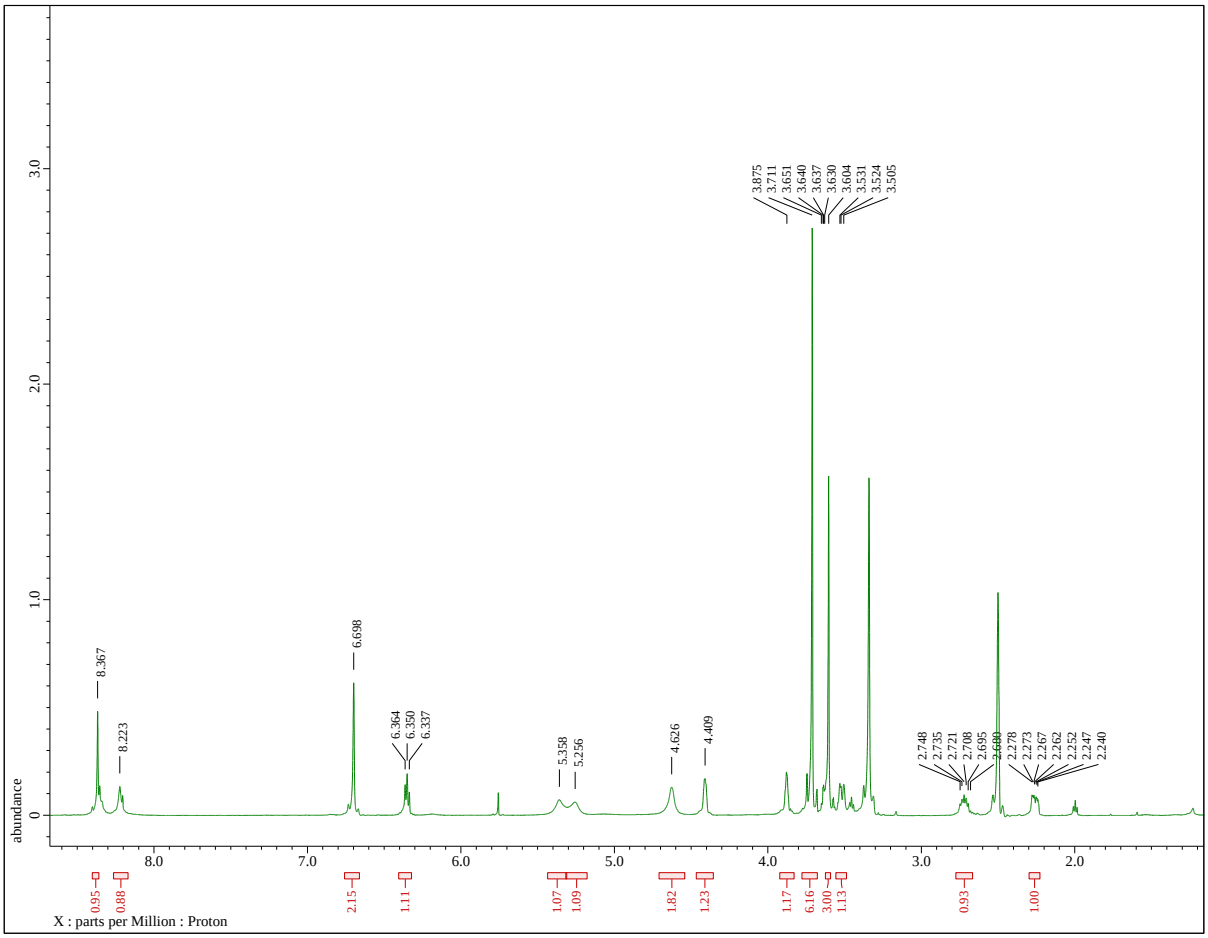

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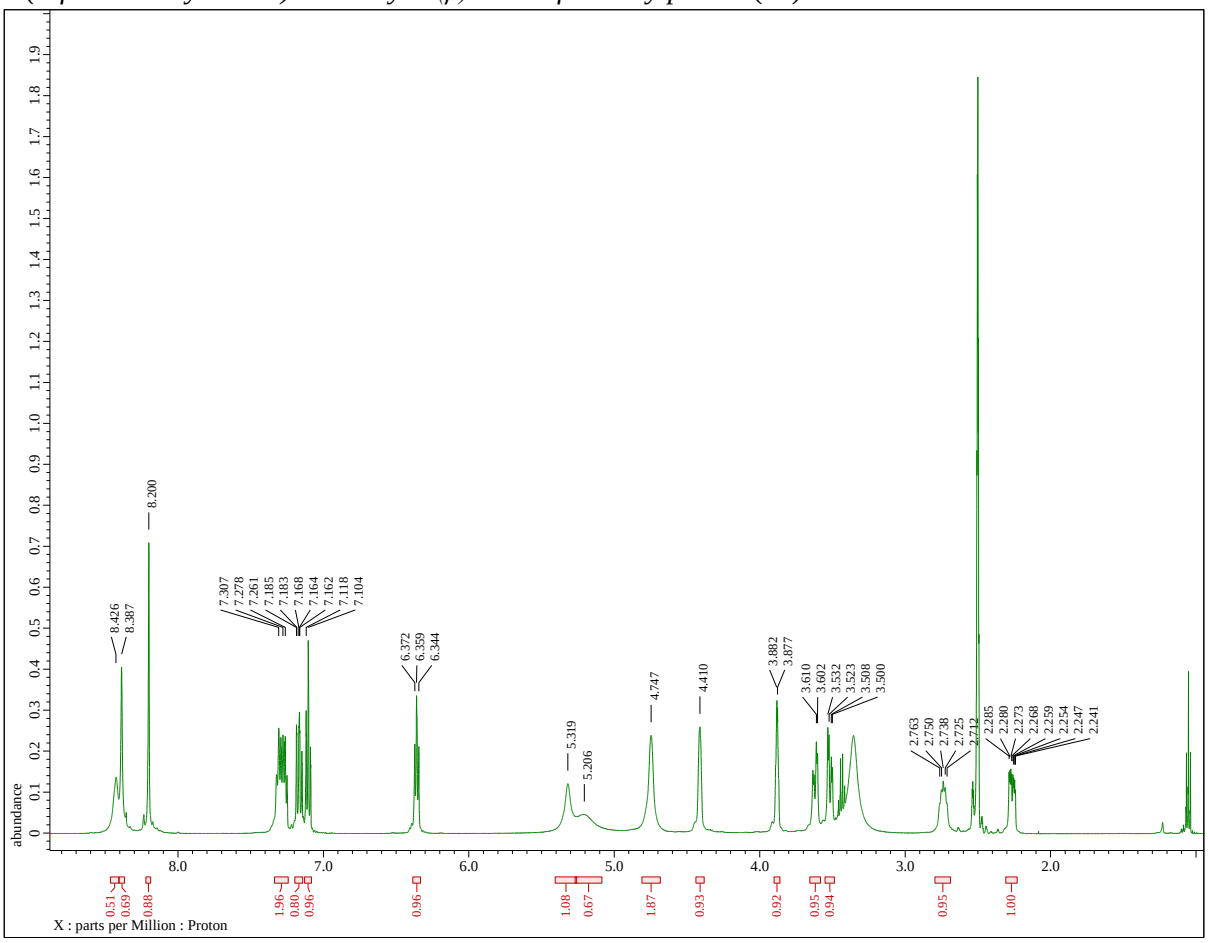

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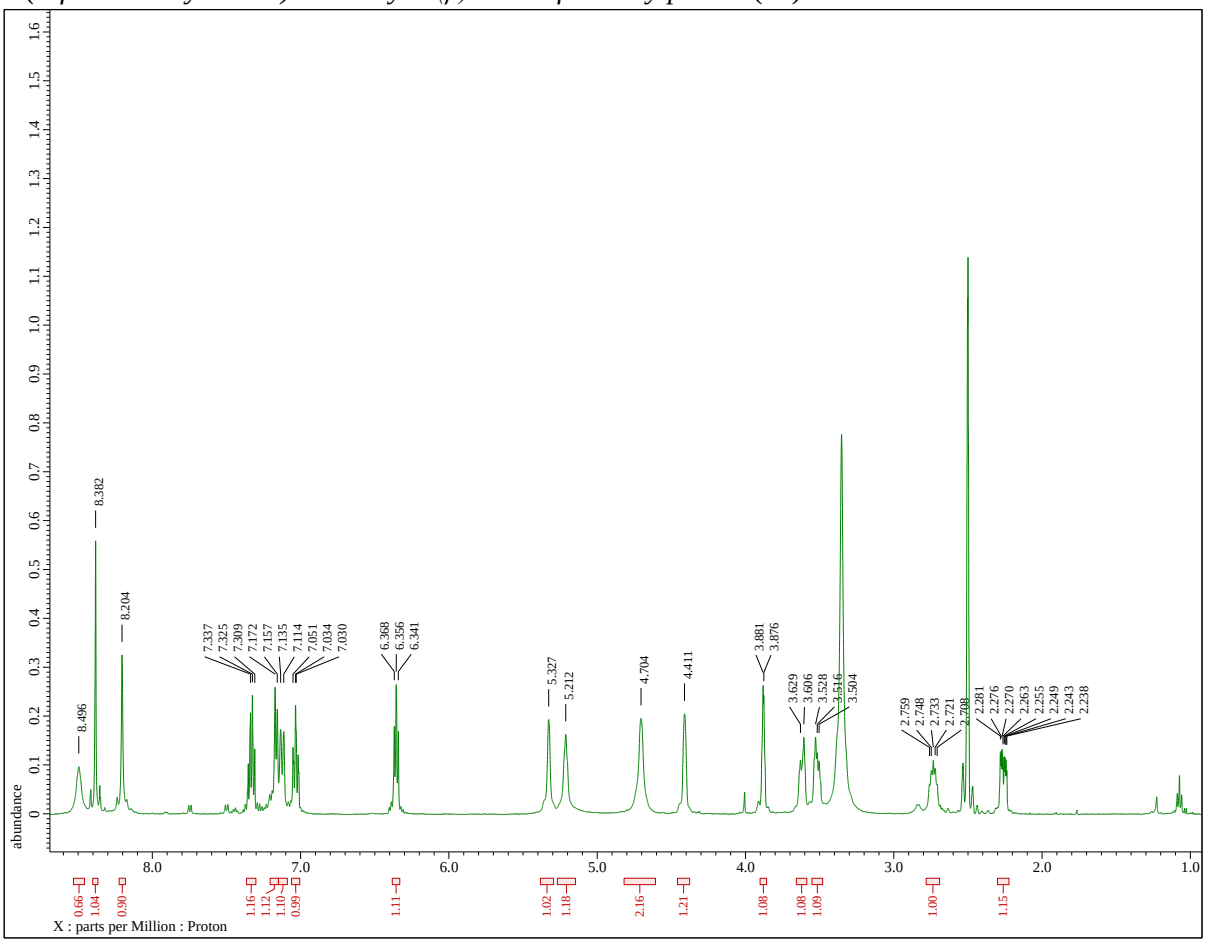

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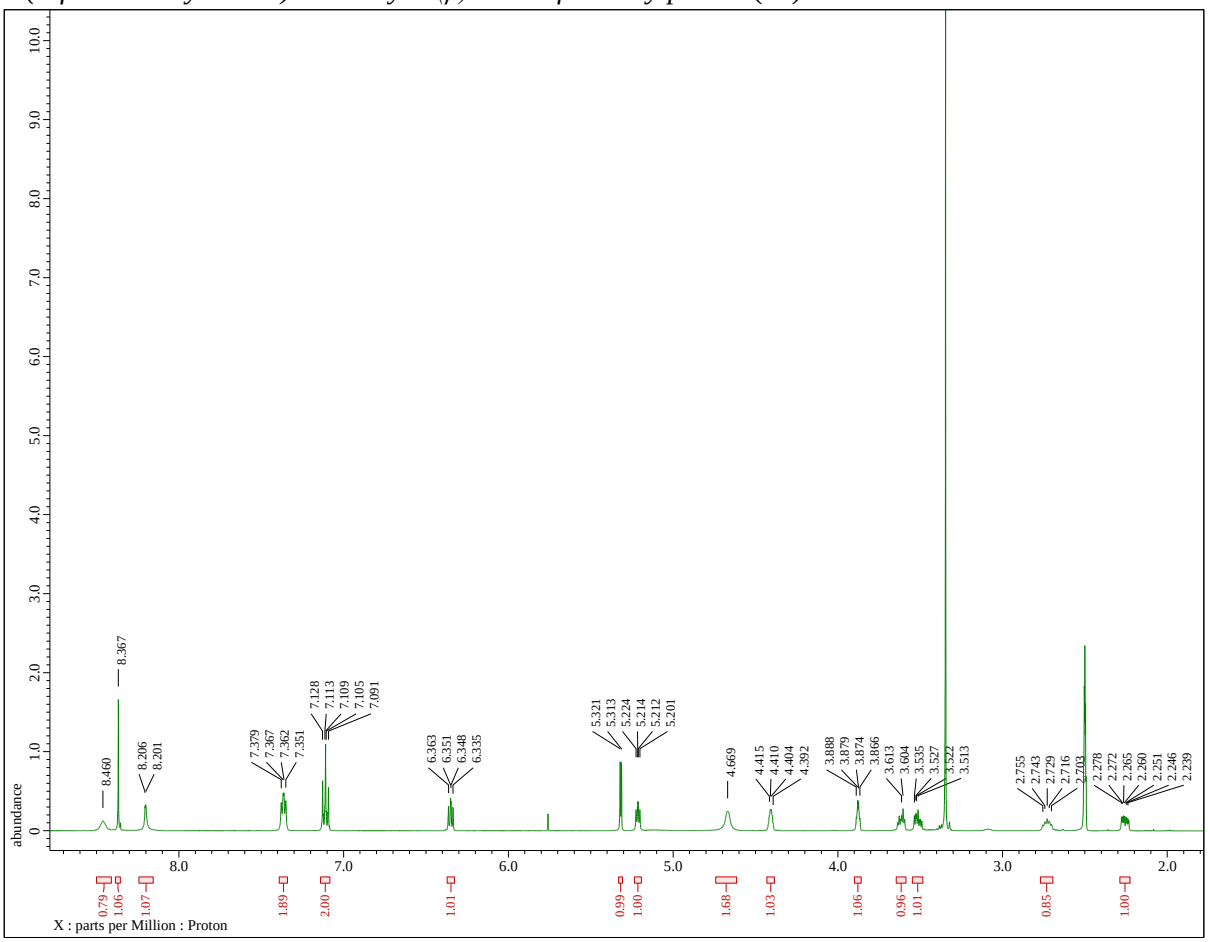

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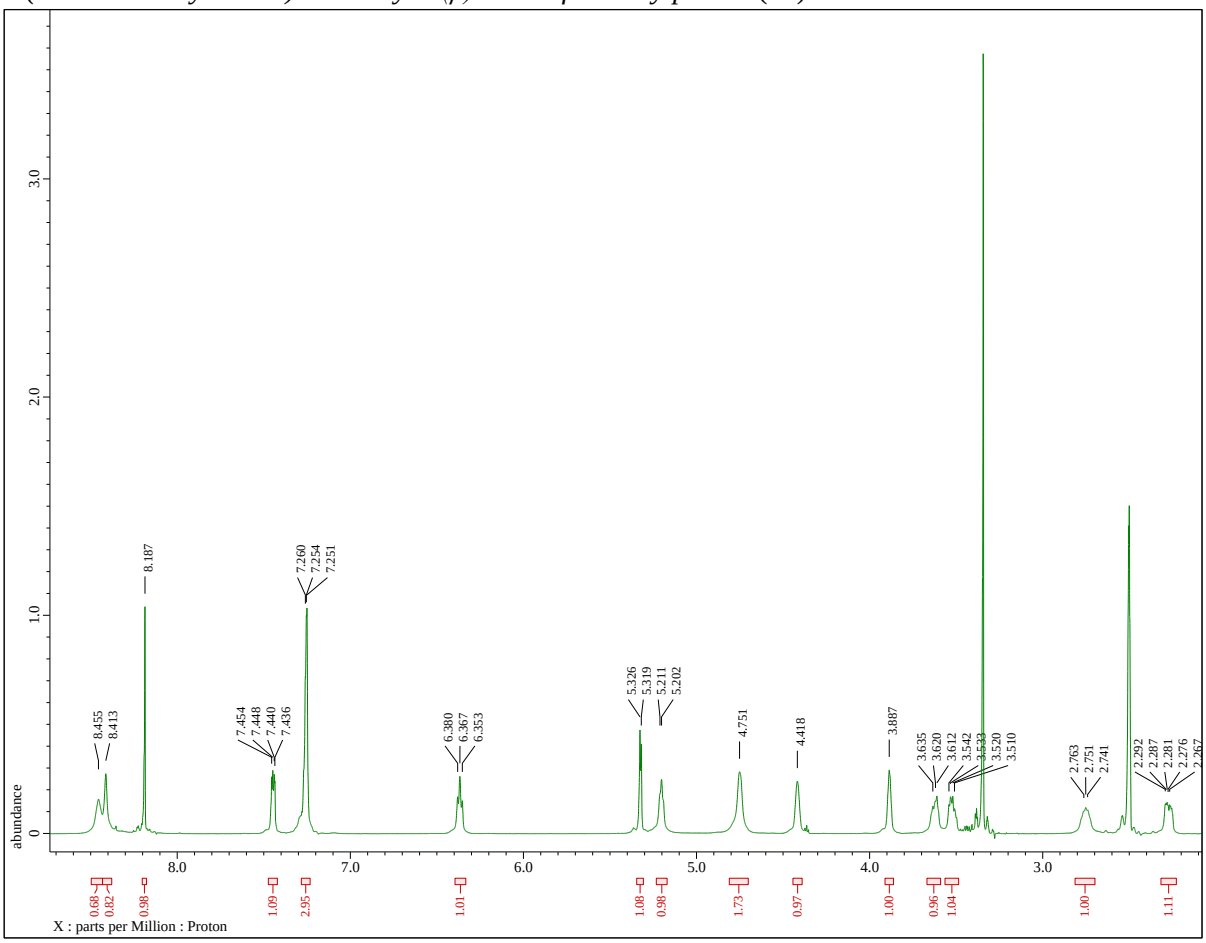

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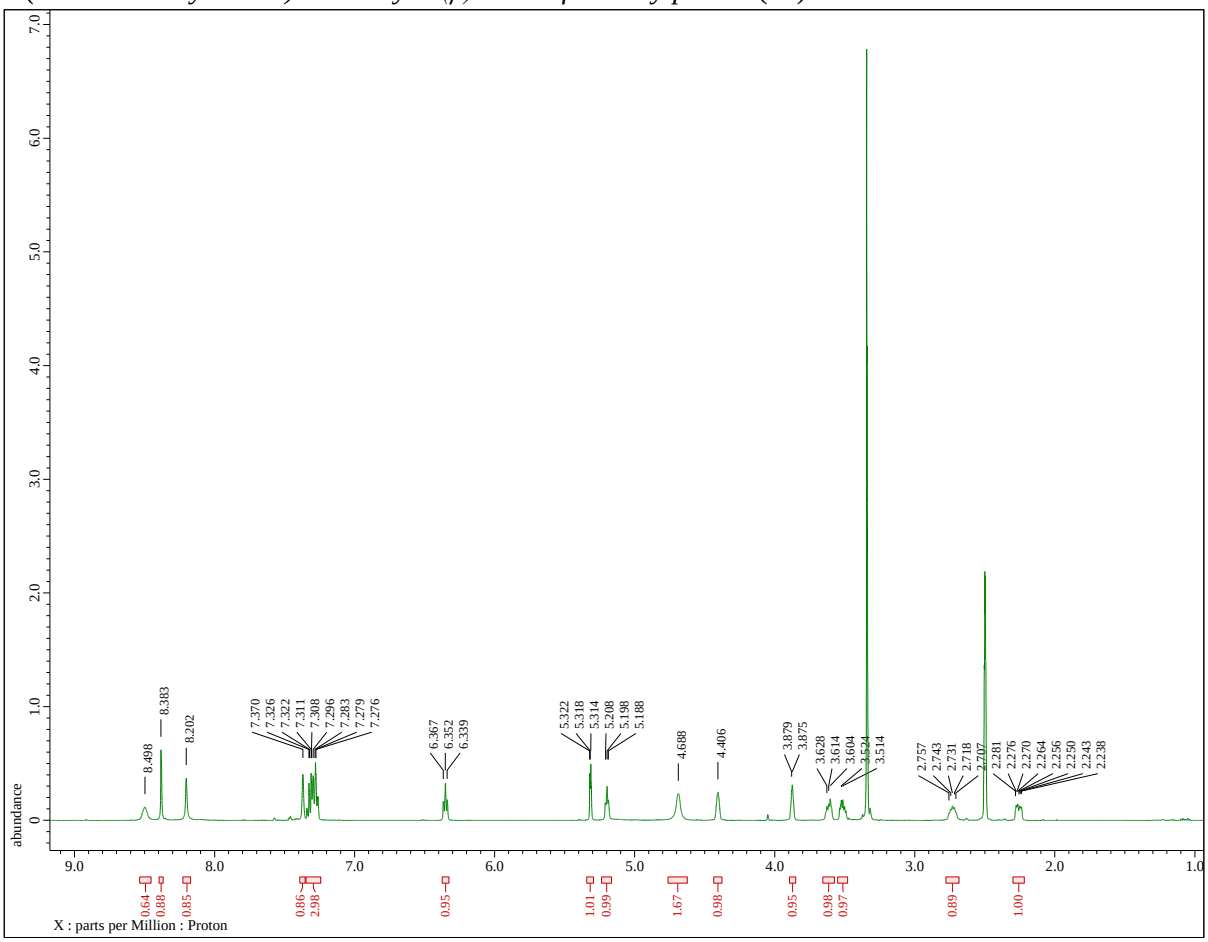

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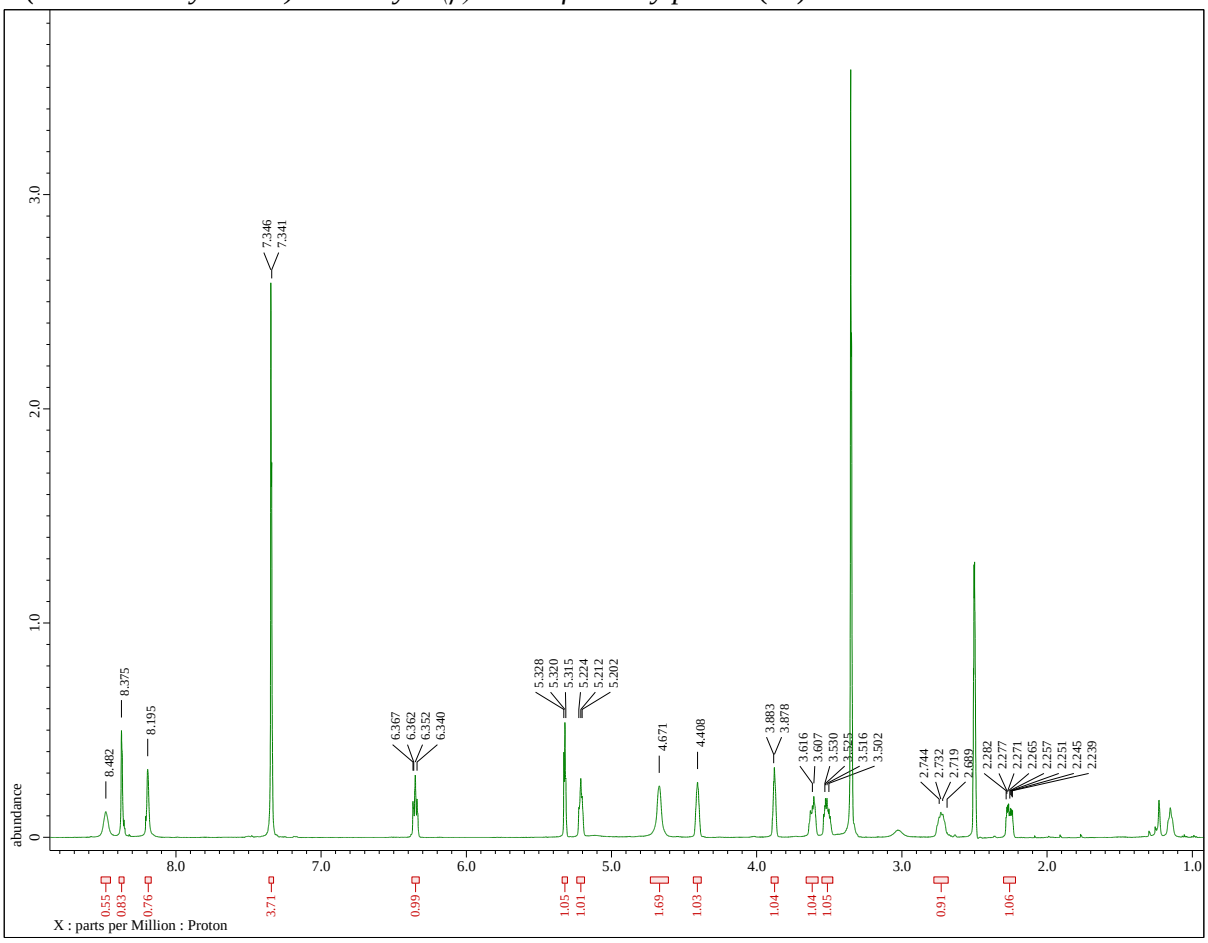

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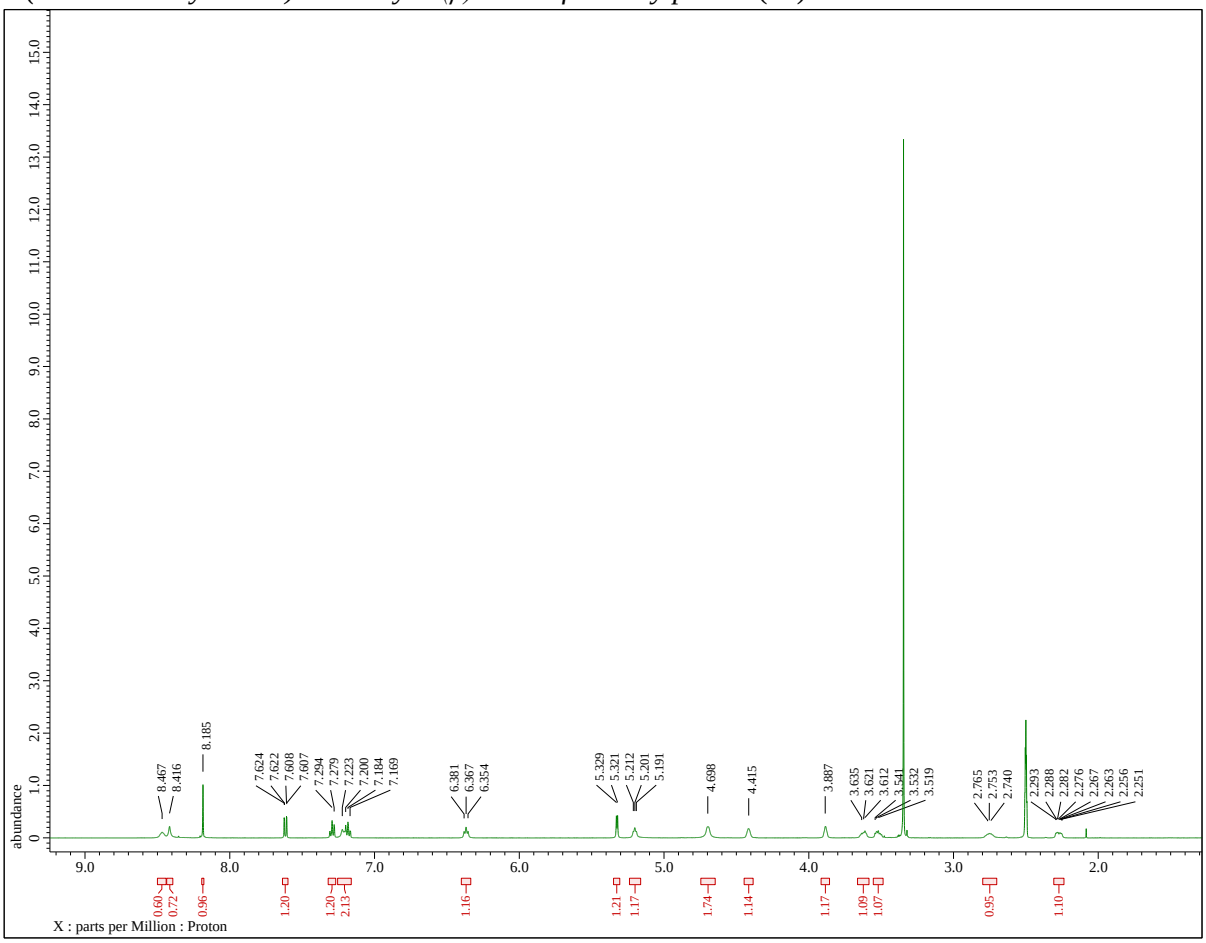

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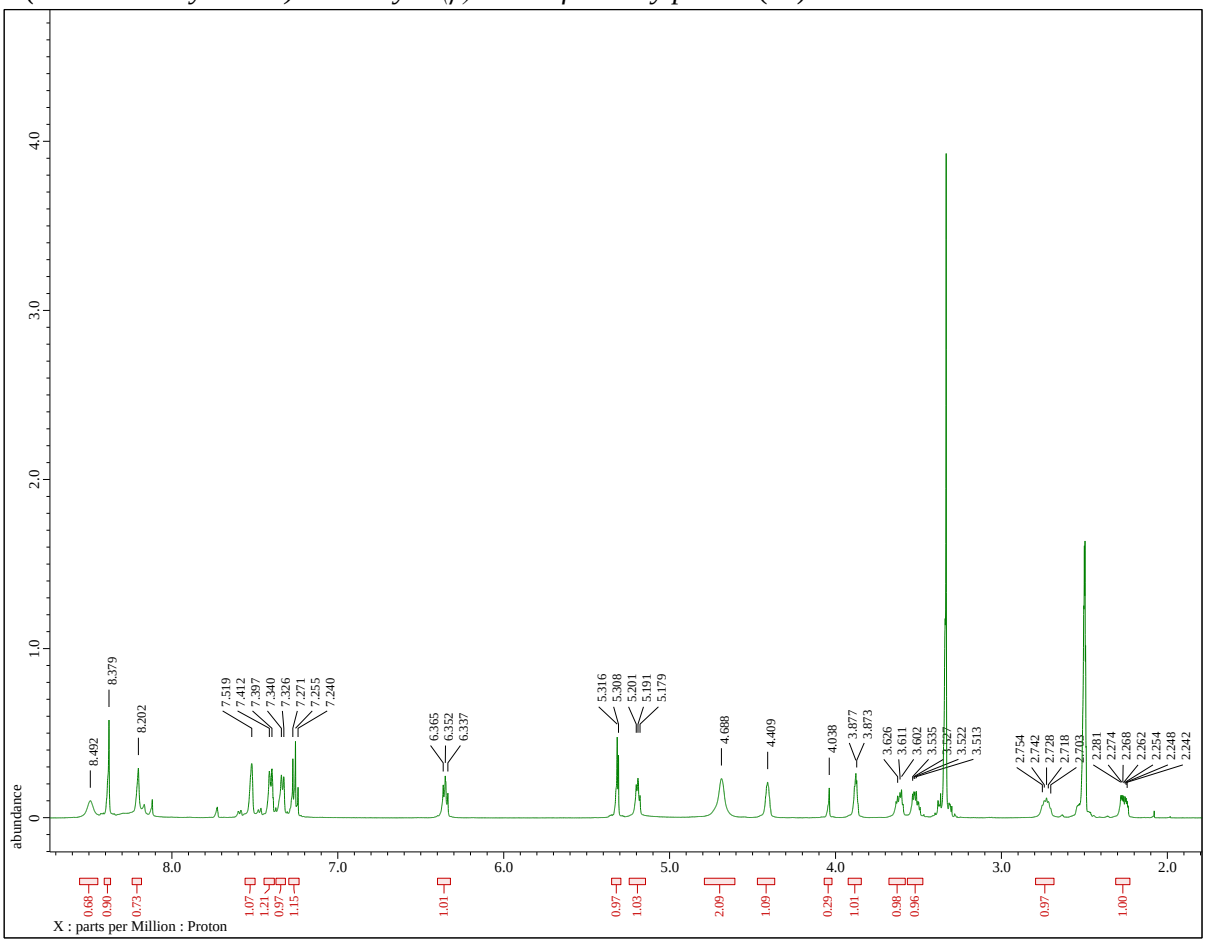

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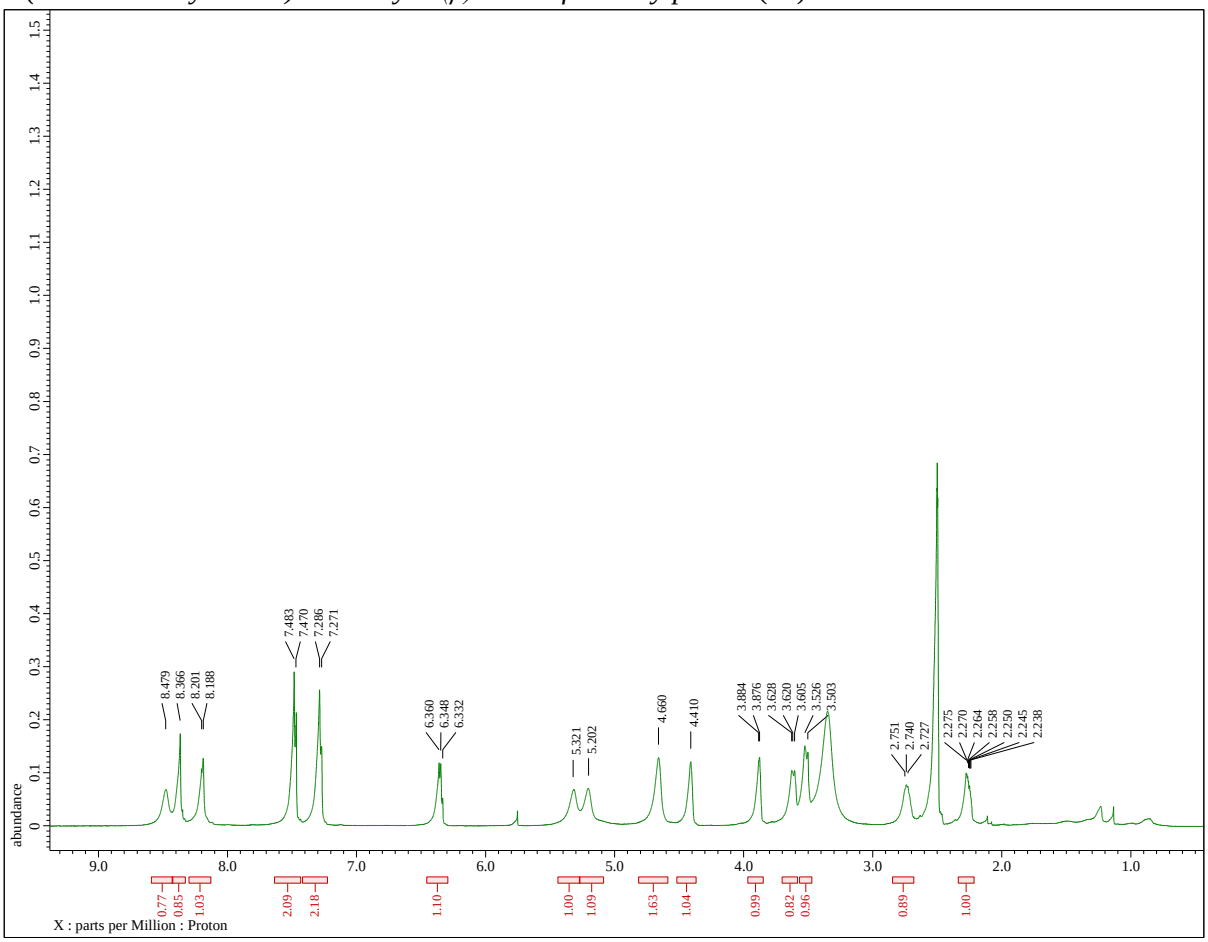

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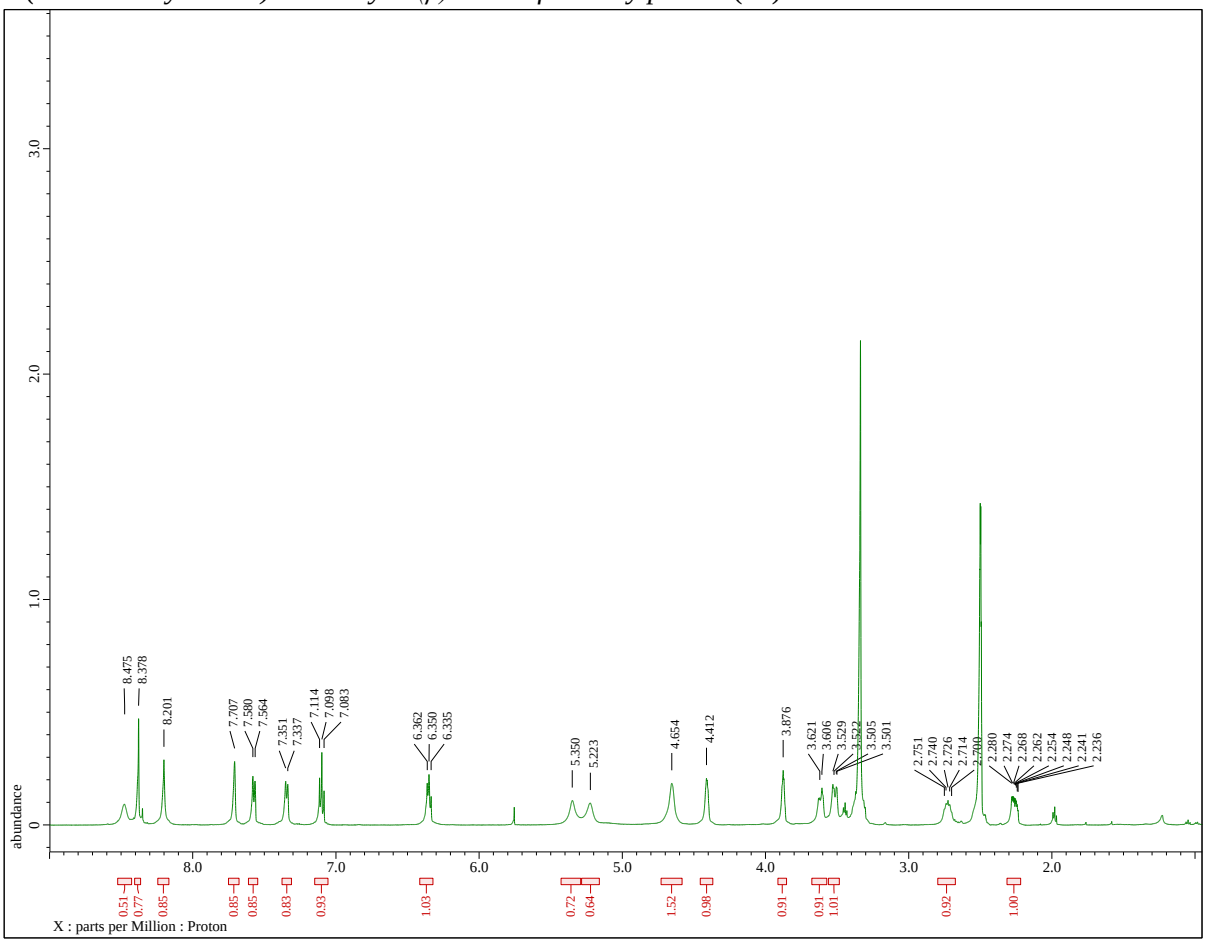

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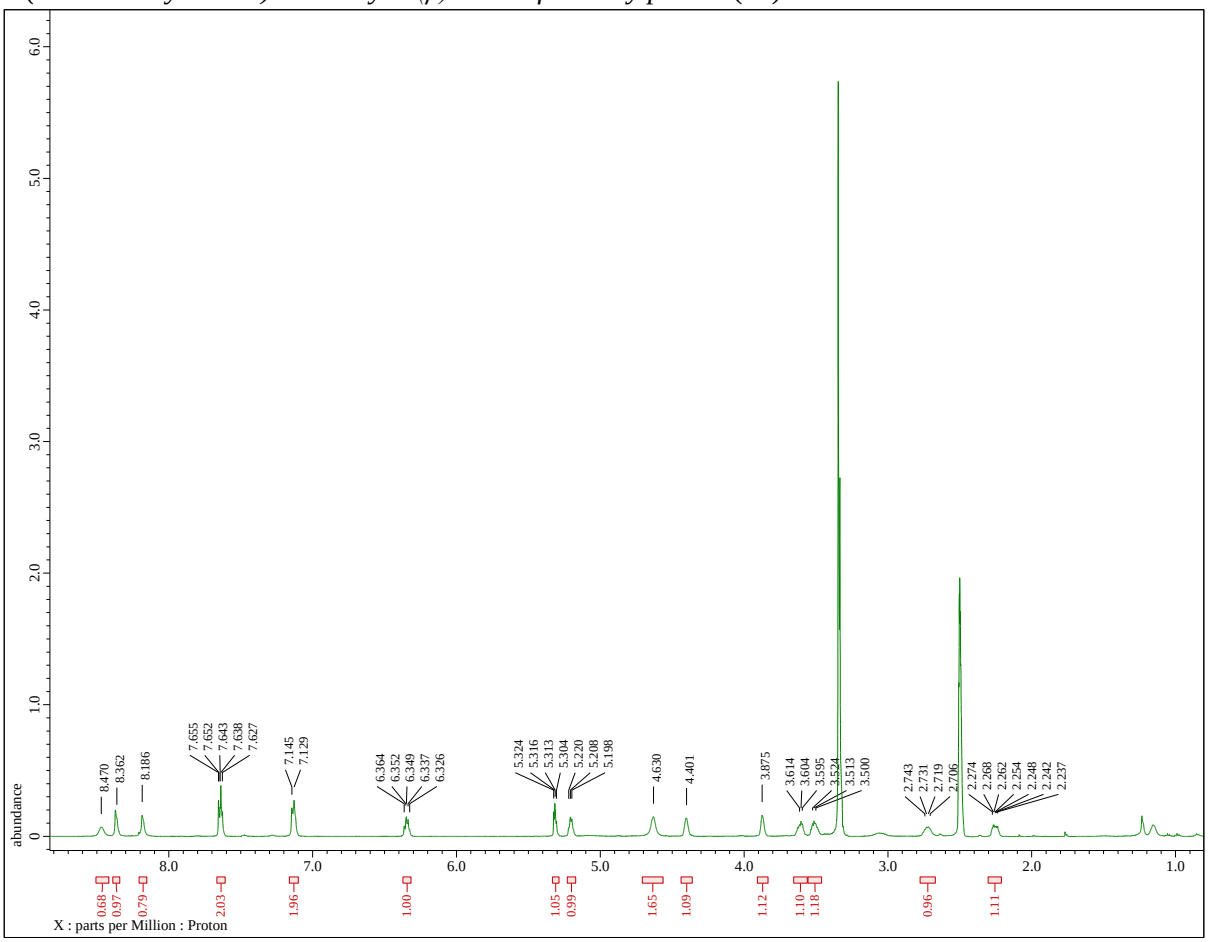

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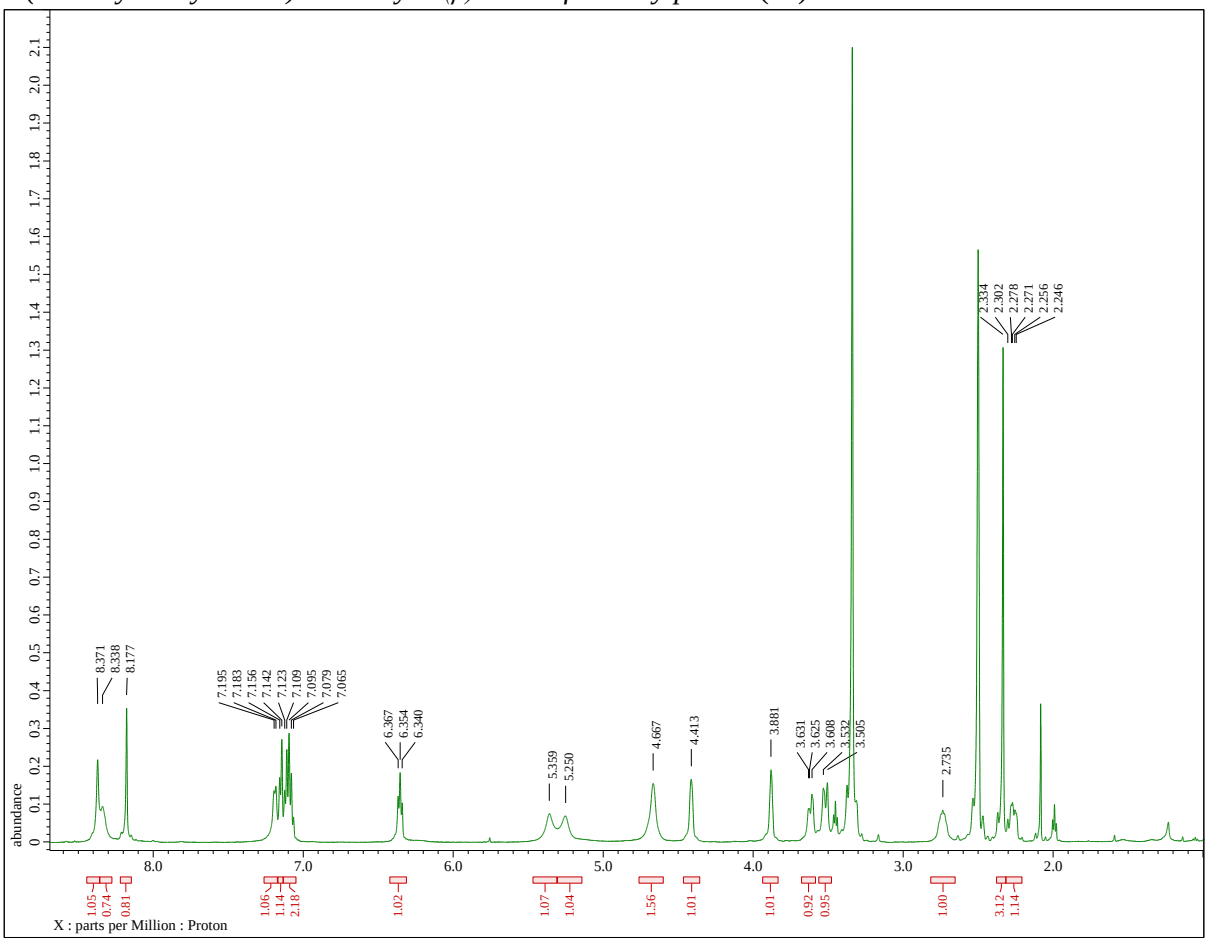

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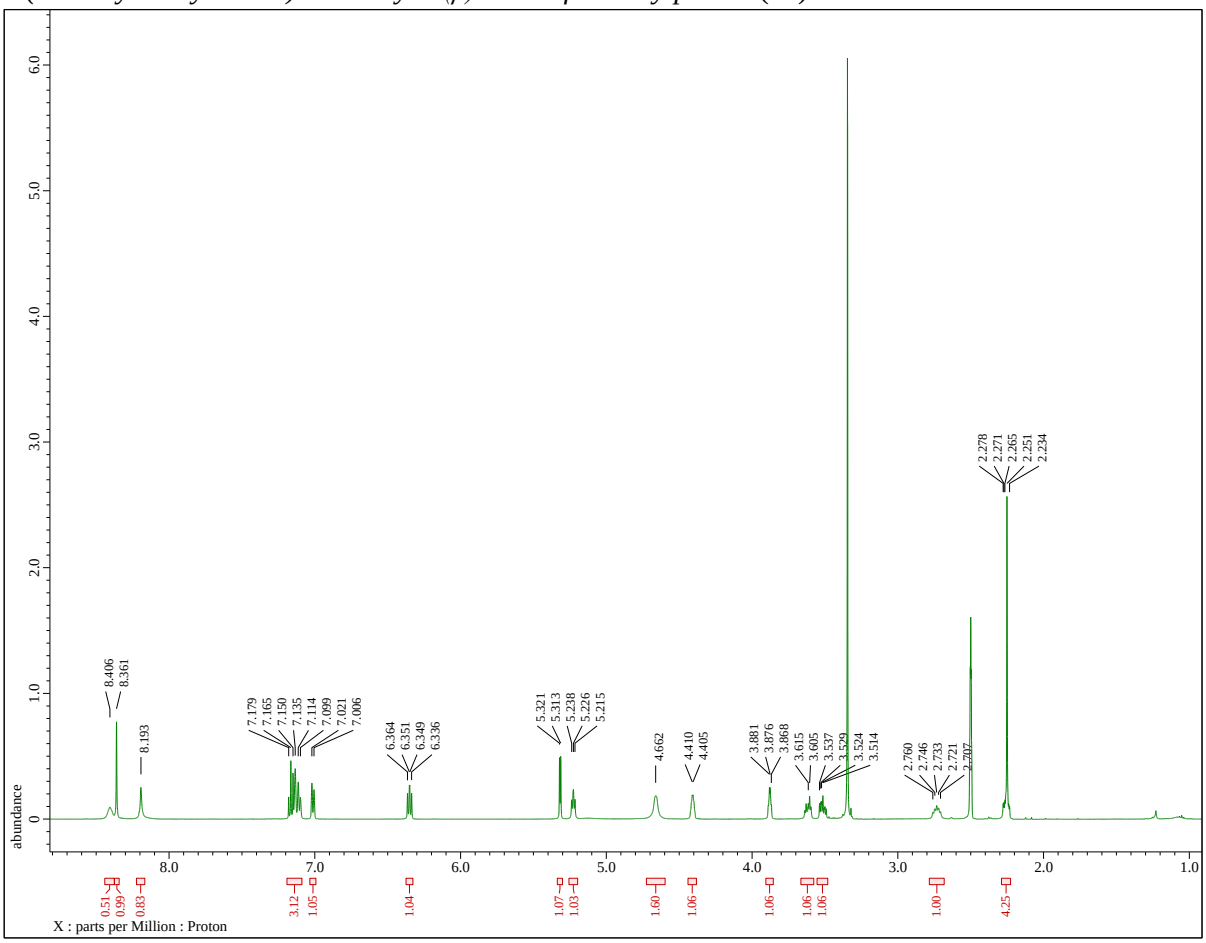

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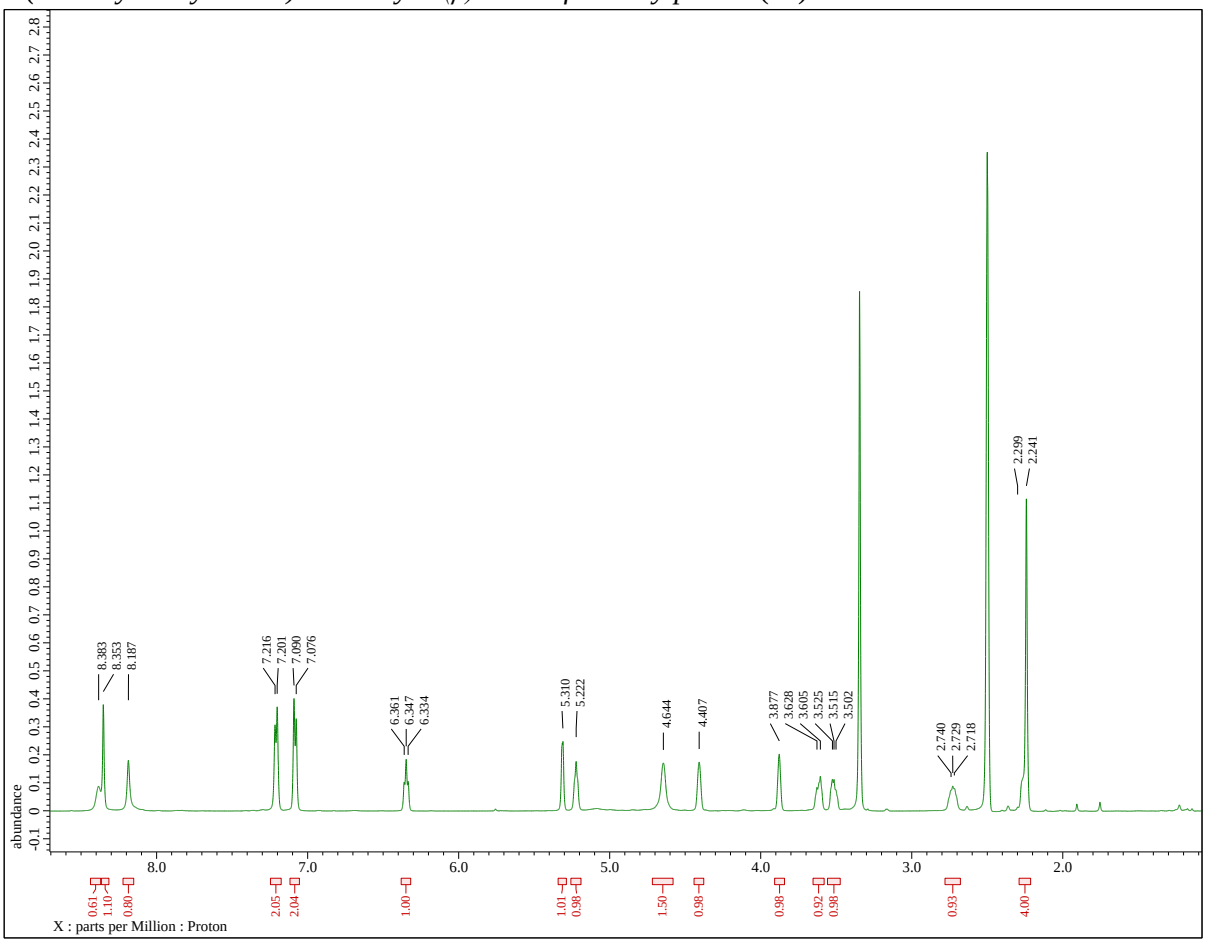

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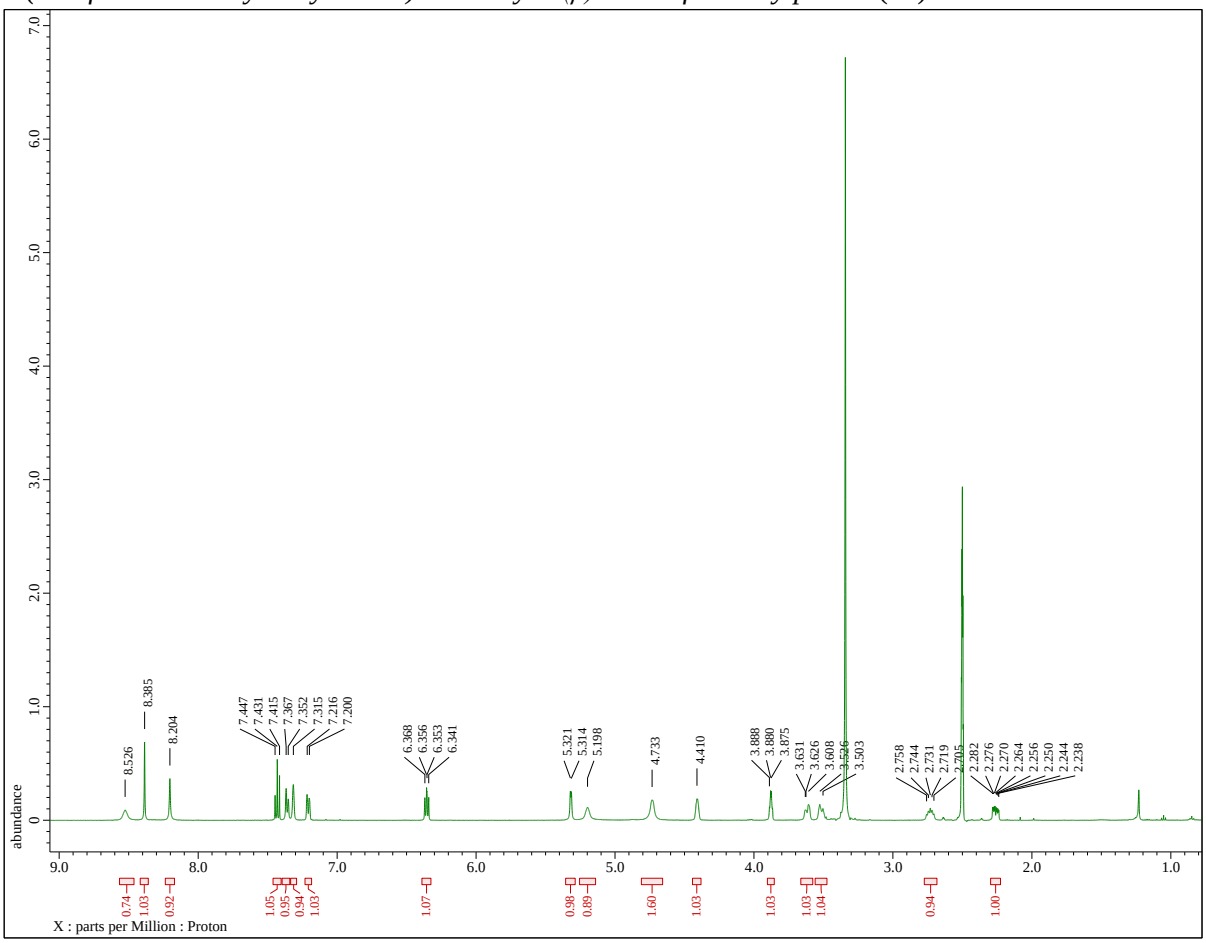

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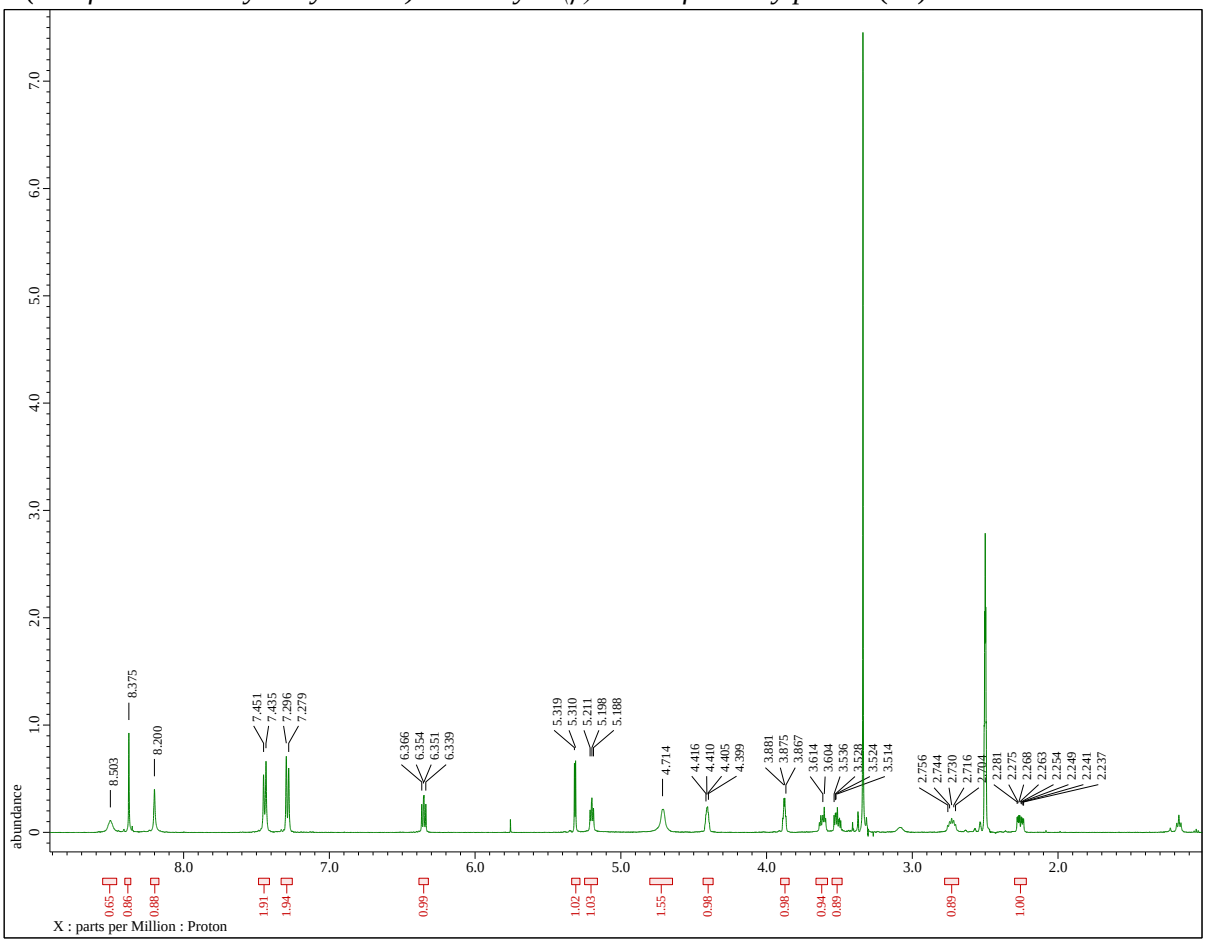

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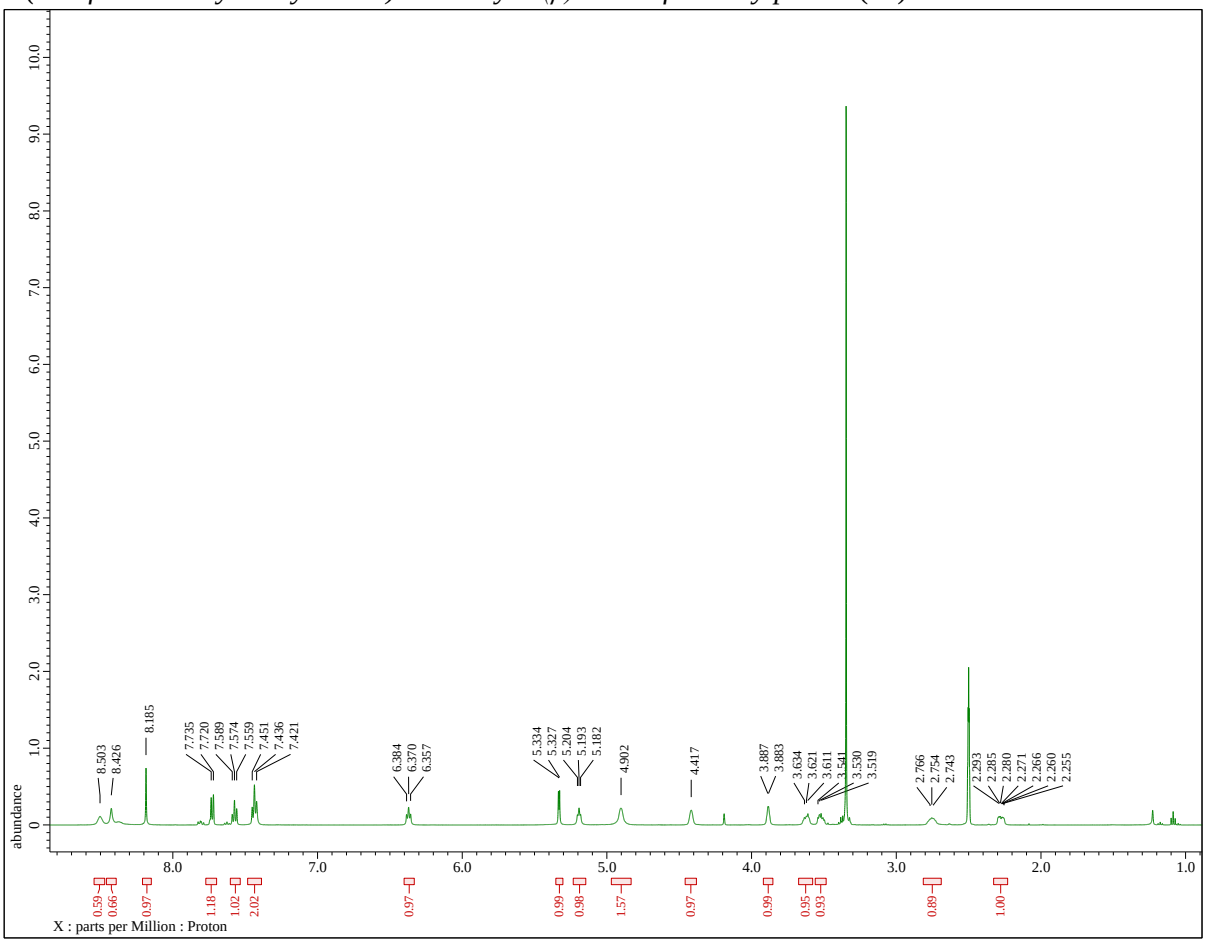

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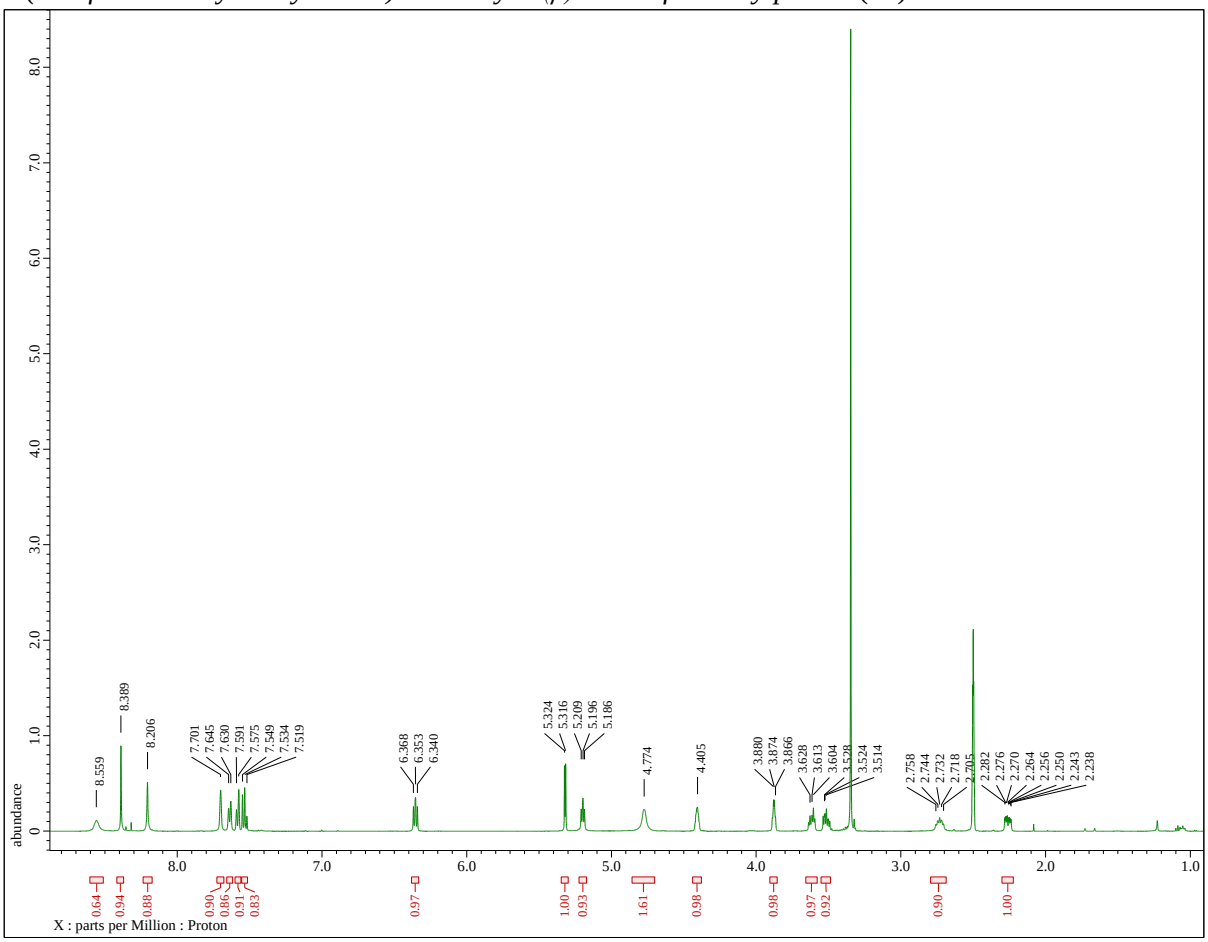

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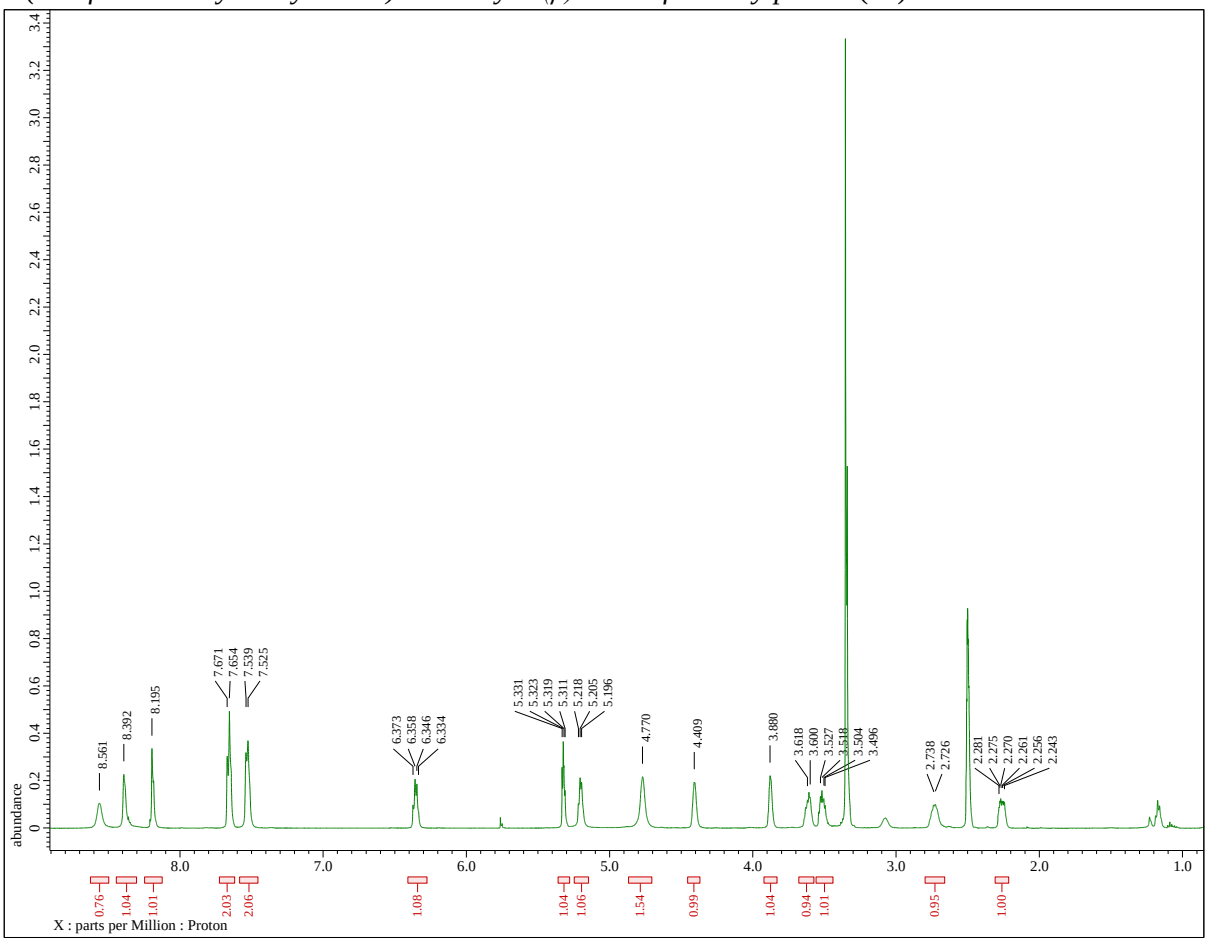

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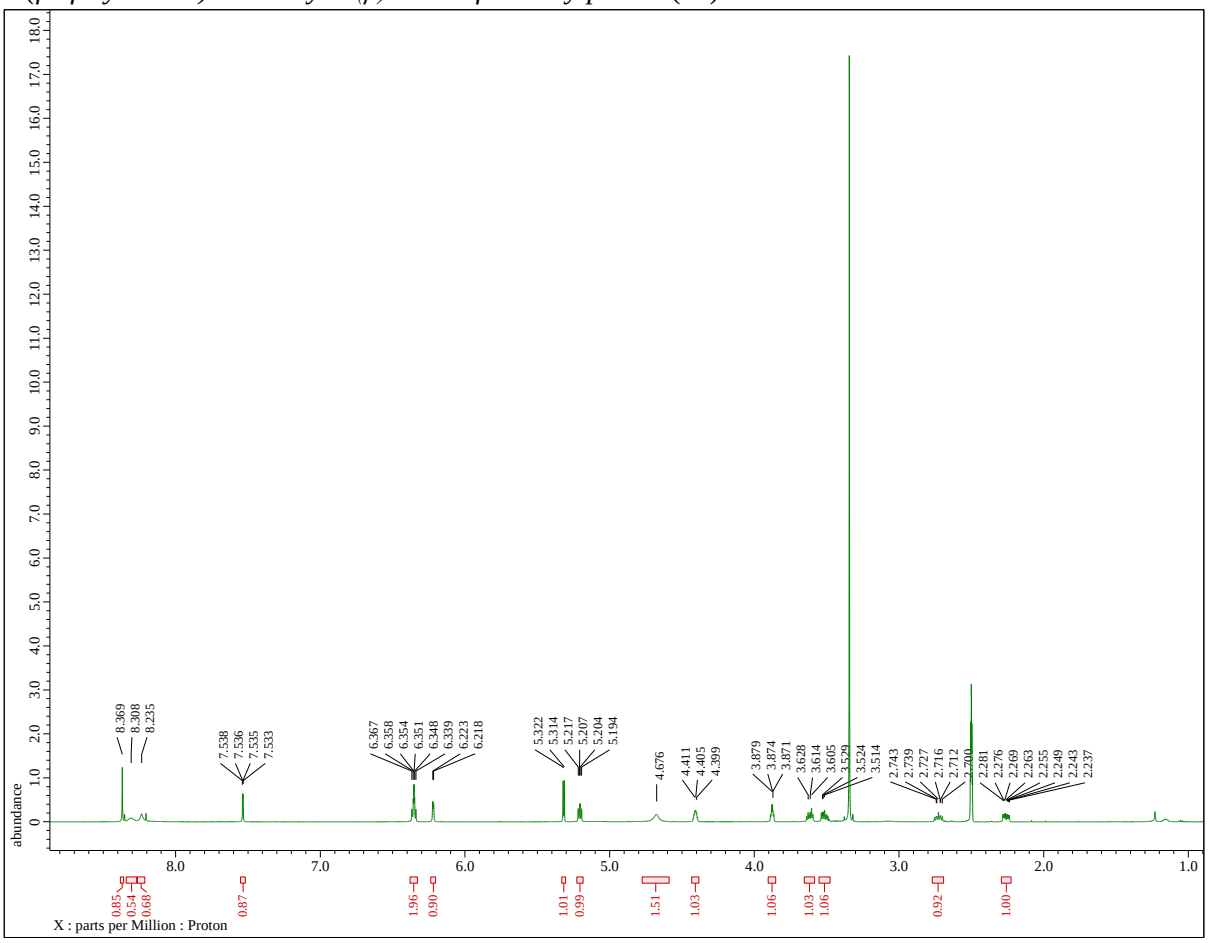

Supplementary material II.

Matušková V., Zatloukal, M., Pospíšil, T., Voller, J., Vylíčilová, H., Doležal, K., Strnad, M.: From synthesis to the biological effect of isoprenoid 2'-deoxyriboside and 2',3'-dideoxyriboside cytokinin analogues. *Phytochemistry*. 2023; 205:113481.

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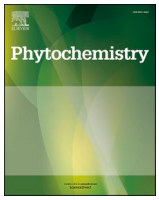

From synthesis to the biological effect of isoprenoid 2′-deoxyriboside and 2′,3′-dideoxyriboside cytokinin analogues

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ABSTRACT

Isoprenoid cytokinins are a class of naturally occurring plant signaling molecules. A series of prepared compounds derived from isoprenoid cytokinins (isopentenyladenine, *trans*-zeatin and *cis*-zeatin) with attached 2′-deoxy-D-ribose or 2′,3′-dideoxy-D-ribose at the N^9 position of the purine were prepared and their biological activities were examined. Different synthetic approaches were employed. The final compounds were characterized with variety of physicochemical methods (TLC, HPLC-MS, and NMR) and their cytokinin activity was determined in classical bioassays such as *Amaranthus*, tobacco callus, detached wheat leaf senescence and *Arabidopsis thaliana* root elongation inhibition assay. In addition, compounds were screened for activation of the cytokinin signaling pathway (bacterial receptor, competitive ligand binding and *ARR5::GUS* assay) to provide a detailed assessment of CK structure-activity relationship. The prepared compounds were found to be non-toxic to human cells and the majority of assays exhibited the highest activity of free bases while 2′,3′-dideoxyribosides had very weak or no activity. In contrast to the free bases, all 2′-deoxyriboside derivatives were not toxic to tobacco callus even at the highest tested concentration (10^{-4} moL/l) and compound **1** (iPdR) induced betacyanin synthesis at higher concentration even stronger than iP free base in the *Amaranthus* bioassay. The general cytokinin activity pattern base > riboside >2′-deoxyriboside > 2′,3′-dideoxyriboside was distinguished.

1. Introduction

Cytokinins represent an important group of plant hormones that fundamentally regulate plant growth and development. These mobile signal molecules are effective at very low concentration in all plant tissues and affect a wide range of functions including cell division and differentiation, leaf senescence, seed germination, control shoot and root meristem, nutrient mobility, defence responses against plant pathogens etc. (Choi et al., 2010; Großkinsky et al., 2013; Mok and Mok, 1994). Naturally, cytokinins are adenine derivatives substituted at the N^6 position with the isoprenoid side chain or aromatic ring. The terminal position of the isoprenoid chain can be hydroxylated to form *cis*- or more common *trans*-zeatin, which was named after the first identification in maize (*Zea mays* (L.), 1753) in 1963 (Frébort et al., 2011; Letham, 1963). Unlike aromatic, isoprenoid cytokinins are more abundant in plants, this group includes isopentenyladenine (iP), *trans*-zeatin (*tZ*), *cis*-zeatin (*cZ*) and dihydrozeatin (DHZ) having a saturated side chain (Kieber, 2002). *Trans*-zeatin and iP are the most active cytokinins and *cZ*, oppositely, shows weaker biological response and low content in some plants (Hluska et al., 2021). The distribution of individual isoprenoid cytokinins in plants varies depending on the plant species and developmental stage (Frébort et al., 2011; Hirose et al., 2008; Quesnelle and Emery, 2007). Cytokinin action is organ-specific, therefore they positively affects the shoot apical meristem and negatively regulate the root apical meristem and inhibit primary root growth (Dello Ioio et al., 2007; Kurakawa et al., 2007; Werner et al., 2003). Cytokinins are not limited to plants, but also occur in other prokaryotic and eukaryotic organisms such as bacteria, algae, fungi or insect (Evidente et al., 1989, 1991; Scarbrough et al., 1973; Strzelczyk et al., 1989; Surico et al., 1985). The bacterium *Pseudomonas savastanoi* pv. *savastanoi* (Psv) (Gardan et al., 1992; Rodríguez-Moreno et al., 2009) affects olive trees (*Olea europaea* L.) as well as other *Oleaceae* members (ash; *Fraxinus*

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excelsior L., jasmine) and oleander (*Nerium oleander* L.) causing hyperplastic symptoms (knots and galls) due to the bacterial secretion of phytohormones (indole-3-acetic acid and cytokinins) (Iacobellis et al., 1994; Sisto et al., 2004) disturbing the hormone balance in infected tissues. The study of cytokinins accumulated in the culture medium filtrate extracts of pathogenic isolates of *Pseudomonas syringae* pv. *savastanoi* (Evidente et al., 1991; Surico et al., 1985) revealed the presence of *t*Z and *t*ZR as well as other naturally occurring isoprenoid cytokinins further characterized as 1′-methylzeatin (1′Met*Z*) with its 9-(*β*)-D-riboside (1′′Met*Z*R) and zeatin 2′-deoxyriboside (*t*ZdR) and lead to further study of these derivatives (De Napoli et al., 1990; Fujii et al., 1989b, 1990, 1993, 1994; Itaya et al., 1986).

Cytokinin homeostasis is strictly controlled and enzymatically regulated by their biosynthesis, interconversion and degradation in plant cells (Zhang et al., 2013). In particular, cytokinin free bases are thought to be the most active species in plant tissues (Lomin et al., 2015; Oshchepkov et al., 2020; Spíchal et al., 2004). However, cytokinins are also presented as ribosides, ribotides or glucosides which represent transport or storage forms (ribosylphosphates (Sakakibara, 2006), ribosides (Schmitz et al., 1972), *O*-glucosides (Brzobohaty et al., 1993; Sakakibara, 2006)). Ribosides and nucleotides are often more abundant in plants, the free bases represent only a small part of the total cytokinin pool (Simonović et al., 2020). The first steps of cytokinin biosynthesis in plants lead to the N^6-(Δ^2-isopentenyl)adenine riboside 5′-mono, -di or tri-phosphate by IPT (isopentenyltransferase) that can be hydroxylated by cytochrome P450 monooxygenase (CYP735A) to *t*ZR mono-, di- or tri-phosphates (Sakakibara, 2006). To obtain the free base it is necessary to convert cytokinin nucleotides by nucleotidase and nucleosidase (Chen and Kristopeit, 1981a, 1981b). Additionally, cytokinin ribosides are converted after phosphorylation to their active form by phosphoribohydrolase (LONELY GUY) (Osugi and Sakakibara, 2015). While cytokinin sugar conjugates *O*-glucosides can be reversibly hydrolysed by *β*-glucosidase to active forms, *N*-glucosides (N^7, N^9) are generalized to be metabolically stable and thus serve as a storage forms (Brzobohaty et al., 1993; Hošek et al., 2020; Lomin et al., 2015; Pokorná et al., 2020). Moreover, irreversible degradation of endogenous cytokinin is regulated by removal of the side chain by cytokinin dehydrogenases (CKX) (Brzobohaty et al., 1993; Hošek et al., 2020; Lomin et al., 2015; Pokorná et al., 2020) with different substrate preferences. Recent research (Hošek et al., 2020; Hoyerová and Hošek, 2020) has shown a possible role of isoprenoid cytokinins, particularly N^9-glucosides (*t*Z, iP and DHZ), in the chlorophyll retention in senescence assay. It has been reported (Hallmark and Rashotte, 2020) that iP and its N^9-glucoside retained comparable amount of chlorophyll levels in *Arabidopsis thaliana* (L.) Heyhn., 1842 cv. Col-0 cotyledons, but an analogical trend was not observed for iP N^7-glucoside. Delaying senescence by iP and its N^9-glucoside treatment was performed with the *cyp735A1, 2* double mutant, which block conversion of iP to *t*Z or DHZ (Kiba et al., 2013), and suggest that iP and its N^9-glucoside appear to be sufficient in anti-senescent capability (Hallmark and Rashotte, 2020). Despite the current progress, knowledge about cytokinin *N*-glycosides is very limited.

In the literature, the purine 2′-deoxyribonucleoside and 2′,3′-dideoxyribonucleoside derivatives showed promising antiviral and anticancer activity (Fayzullina et al., 2022; Seley-Radtke and Yates, 2018). For example, 5′-*O*-masked-6-chloropurine-9-(*β*)-D-2′-deoxyriboside exhibited higher potency against hepatitis C virus infection than the conventional antiviral drug ribavirin bearing the ribosyl residue (Ikejiri et al., 2007). Substituted 2′,3′-dideoxypurine nucleosides have been reported as promising anti-HIV agents (Saran and Ojha, 1996). Furthermore, the nucleoside prodrug 6-methylpurine-2′-deoxyriboside was used as a non-toxic substrate for drug-activating enzyme in suicide gene therapy inserted in tumour cells (Bharara et al., 2005). On the other hand, a study of various iP derivatives with different modifications of the hydroxyl group on the sugar moiety revealed that the ribosyl residue is necessary to maintain antiproliferative activity unlike 2′-deoxynucleoside or 2′,3′-dideoxynucleoside (Ottria et al., 2010).

At present, the interest in the chemical synthesis of new cytokinin nucleoside analogues for agriculture and biotechnology is enormous (Bryksová et al., 2020a, 2020b). The first attempt to synthesize iP nucleoside by rearrangement of N^1-alkylated derivatives to N^6 isomers was described by Leonard et al. (Fleysher et al., 1969; Leonard et al., 1966; Leonard and Fujii, 1964). Later, riboside, 2′-deoxyriboside and 2′,3′-dideoxyriboside derivatives of iP were also prepared (Ottria et al., 2010) by N^1-alkylation followed by the Dimroth rearrangement (Oslovsky et al., 2015). The naturally occurring *tZ*-2′-deoxyriboside was first isolated from the plant pathogenic organism (*Pseudomonas amygdali*) and its structure was confirmed by chemical synthesis (Evidente et al., 1989). Coupling of 6-chloropurine-2′-deoxy-9-(*β*)-D-riboside with (*Z*)-4-amino-2-methyl-2-buten-1-ol ethandioate affording *cis*-zeatin-9-(*β*)-D-2′-deoxyriboside was reported (Evidente et al., 1989, 1992) but enzymatic approaches for nucleosides synthesis can also be applied (Inoue et al., 1991).

Our previous work (Matušková et al., 2020) reported the synthesis and biological activity of aromatic 6-substituted purine 2′-deoxy-9-(-*β*)-D-ribosides with the different substitutions on the benzyl ring. The presence of 2′-deoxyribose moiety significantly improved the anti-senescence effect and showed a non-cytotoxic effect of the prepared compounds. In this work, we follow up with the synthesis of isoprenoid 2′-deoxy-9-(*β*)-D-riboside and 2′,3′-dideoxy-9-(*β*)-D-riboside cytokinin analogues with the focus on their biological activity in various cytokinin bioassays.

2. Results and discussion

2.1. Synthesis

Although aromatic cytokinins are highly active in cytokinin bioassays, we have attempted to modify the sugar residue of the widespread natural isoprenoid cytokinin (iP, *t*Z, *c*Z) and study their biological properties. We modified the structure of the sugar moiety attached to the N^9 position of the purine with 2′-deoxyribose or 2′,3′-dideoxyribose. It has been reported several times, that the removal of the hydroxyl group from the 2′ and 3′ position of the sugar ring can significantly change biological properties of cytokinin glycosides (Evidente et al., 1992; Matušková et al., 2020; Oslovsky et al., 2015; Ottria et al., 2010). We synthesized the chemical library of isoprenoid 6-substituted purine 2′-deoxy-9-(*β*)-D-ribosides (dR) and purine 2′,3′-dideoxy-9-(*α*/*β*)-D-ribosides (ddR) (Fig. 1) and the structure-activity relationship in comparison with appropriate cytokinins (free bases) and their ribosides was studied. The synthesis of isoprenoid 6-substituted purine 2′-deoxy-9-(*β*)-D-ribosides (Leonard et al., 1969) and 2′,3′-dideoxy-9-(*α*/*β*)-D-ribosides (Koszalka et al., 1989) was proceeded using three different synthetic strategies. The HPLC-UV purities and yields of final compounds are summarized in Table 1.

Firstly, the most of the compounds (**1**, **2**, **4b**, **5**, and **6** (Greco et al., 1992)) were prepared by the common one-step reaction (Wan et al., 2005, 2007) outlined in Scheme S1 using Castro's reagent. The unprotected precursor 2′-deoxyinosine or 2′,3′-dideoxyinosine was treated with different amines, in the presence of BOP and DIPEA in dry DMF under N_2 atmosphere and stirred overnight, as described in the previously published protocol (Matušková et al., 2020). The optimal reaction temperature was determined to be 50 °C, in the case of compound **1** the reaction was performed at ambient temperature. The water-soluble crude was pre-purified by the column chromatography. The final products were separated by the preparative HPLC-MS chromatography to give the gel-like products in the yield ranging 20–44%.

Secondly, the amino group at the N^6 terminus of adenine-2′-deoxy-9-(*β*)-D-riboside was chlorinated to produce 6-chloropurine-2′-deoxy-9-(*β*)-D-riboside according to previously published protocol (Francom and Robins, 2003; Seela et al., 1987) in Scheme S2. The final reaction step was performed by nucleophilic substitution at the *C6* position of the purine with (2*E*)-2-methyl-4-aminobut-2-en-1-ol hydrochloride in the

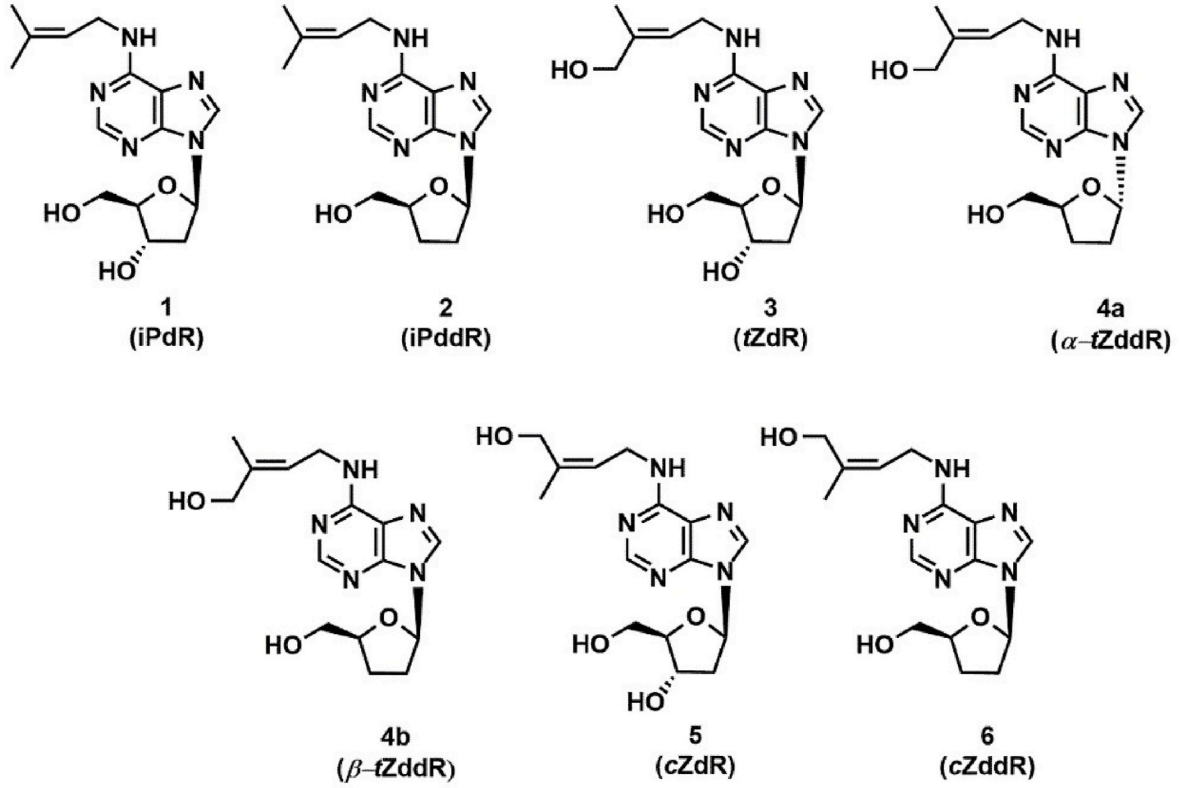

Fig. 1. Structures of prepared derivatives: iPdR (**1**), iPddR (**2**), *t*ZdR (**3**), *α*-*t*ZddR (**4a**), *β*-*t*ZddR (**4b**), *c*ZdR (**5**) and *c*ZddR (**6**) with abbreviation or numbering used in this study.

Table 1
Molecular weight, mass of positively charged molecular ions, high resolution mass of positively charged molecular ions with HRMS mass accuracy, HPLC-UV purity and yield of prepared derivatives analysed by HPLC-MS.

Compound	MW [g.mol^{-1}]	MS (M + H$^+$)	HRMS (M + H$^+$)	HRMS mass accuracy	HPLC-UV purity [%]	Yield [%]
1 (iPdR)	319.37	320.20	320.1723	+0.6 mDa, +1.9 ppm	>99	30
2 (iPddR)	303.37	304.25	304.1773	−0.1 mDa, −0.3 ppm	>98	44
3 (*t*ZdR)	335.36	336.29	336.1672	−0.4 mDa, −1.2 ppm	>99	57
4a (*α*-*t*ZddR)	319.37	320.27	320.1723	+0.7 mDa, +2.2 ppm	>99	57
4b (*β*-*t*ZddR)	319.37	320.27	320.1717	+0.6 mDa, +1.9 ppm	>97	20
5 (*c*ZdR)	335.36	336.29	336.1672	−0.2 mDa, −0.6 ppm	>99	26
6 (*c*ZddR)	319.37	320.27	320.1723	+0.7 mDa, +2.2 ppm	>99	23

presence of DIPEA to give the *trans*-zeatin-2′-deoxy-9-D-(*β*)-riboside (*t*ZdR, **3**).

Other synthetic strategy (Corey et al., 1987; Mitsunobu, 1981; Okabe et al., 1988; Seio et al., 2017) for the preparation of *trans*-zeatin-2′,3′-dideoxy-9-D-(*α*/*β*)-ribosides (**4a**/**b**) was chosen according to Scheme S3. The protected 2′,3′-dideoxy-9-D-ribosyl moiety was synthesized through the several reaction steps and then joined to the N^9 position of the purine scaffold. Firstly, *L*-glutamic acid was treated with $NaNO_2$ in HCl to produce (*S*)-γ-butyrolactone-γ-carboxylic acid which was then reduced and protected with TBDMS to give (*S*)-γ-[[(tert-butyldimethylsilyl)oxy]methyl]-γ-butyrolactone followed by reduction of the lactone to the lactol using DIBAL in THF. This intermediate was then attached to the N^9 position of the 6-chloropurine core with DIAD and PPh_3 under Mitsunobu conditions. Nucleophilic substitution of the appropriate amine: ((2*E*)-2-methyl-4-aminobut-2-en-1-ol) to the *C6* position of 6-chloropurine and deprotection of the 5′-OH group lead to the anomeric mixture of *trans*-zeatin-2′,3′-dideoxy-9-(*α*/*β*)-D-ribosides (**4a**/**b**). The yield of the *α*-anomer predominated in the resulting mixture of this stereospecific glycosylation, as confirmed by 2D-NMR experiments. We assumed that the steric hindrance of silyl groups could contribute to the predominant formation of the *α*-anomer. To prevent the formation of the *α*-anomer, the *β*-anomer can be preferably formed with the BOP coupling agent (Wan et al., 2005, 2007) with unprotected nucleoside analogues, unfortunately the reaction is accompanied by relatively small yields.

Detailed chemical procedures for the preparation of presented compounds and NMR data are listed in the Supplementary Material.

2.2. Biological activity

2′-Deoxyriboside and 2′,3′-dideoxyriboside derivatives of naturally occurring isoprenoid cytokinins (iP, *c*Z, *t*Z) were prepared and tested in classical cytokinin bioassays (*Amaranthus*, tobacco callus and wheat leaves senescence), bacterial receptor assay and competitive ligand binding assay, *in vivo ARR::GUS* assay, and root elongation inhibition assay to examine the differences in cytokinin activity caused by altered N^9-substitution.

The study of structure-activity relationship of isoprenoid cytokinins began with their isolation and identification (e.g. Hecht et al., 1970b; Sakakibara, 2006). For instance, it was revealed that senescence depends mainly on the N^6 part of the purine molecule and iP itself shows weak antisenescence activity compared to *t*Z (Hallmark, 2020; Mik

et al., 2011; Pokorná et al., 2020). Furthermore, the *trans* stereochemistry of the double bond of the N^6-side chain is important for cytokinin activity (Evidente et al., 1991, 1992). The presence of the double bond in the iP and *t*Z side chains, in contrast to DHZ, contributed to higher cytokinin activity in the tobacco callus bioassay (Hecht et al., 1970a, 1970b; Matsubara et al., 1968; Skoog et al., 1967). It was also reported that *c*ZR was more active than corresponding 2′-deoxyriboside in the tobacco callus assay (Fujii et al., 1994). It was also revealed that mild N^6-side chain alteration is beneficial in some assays and also the conformation is important as the natural 1′*R* form of 1′Me*t*Z showed similar activity in the tobacco callus and the lettuce seed germination bioassays as the unsubstituted *t*Z while the unnatural enantiomer (*1′S*) showed lower activity (Fujii et al., 1989a, 1989b). Additionally, the 1′Me*t*Z derivatives showed higher chlorophyll synthesis stimulating activity in etiolated cucumber cotyledons than 2′-deoxyzeatin riboside (Evidente et al., 1991). The 1′-methylated *cis*-zeatin isomers ((*1′R*)-1′MecZ and (*1′R*)-1′MecZR) were less active in tobacco callus and lettuce seed germination assays than 1′-unsubstituted *cis*-zeatin and (*1′R*)-1′-methylated-*trans*-zeatin derivatives ((*1′R*)-1′Me*t*Z and (*1′R*)-1′Me*t*ZR) (Fujii et al., 1990) which correlated with the lower cytokinin activity in tobacco callus assay of the *cis*-zeatin and its riboside compared to the *trans*-zeatin and its riboside (Matsubara, 1980). However, the highest cytokinin activity belonged to *trans*-zeatin (Fujii et al., 1994).

The cytokinin free bases are the most active cytokinin species in plant tissues (Lomin et al., 2015; Mok et al., 2000; Oshchepkov et al., 2020; Romanov and Schmülling, 2022; Spíchal et al., 2004; Yamada et al., 2001). However, significant cytokinin activity in different cytokinin receptor assays has been also detected for naturally occurring cytokinin ribosides (Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004). In many bioassays the cytokinin activity of free bases and ribosides *per se* is comparable, nevertheless, this does not directly prove that ribosides are active (Lomin et al., 2015; Oshchepkov et al., 2020; Spíchal et al., 2004). However, it was also reported that ribosides have a relatively higher activity with AHK3 than with CRE1/AHK4 and the ability of *t*ZR to activate CRE1/AHK4 did not increase with prolonged incubation periods as a result of nucleosidase activity (Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004) suggesting a genuine AHK3 biological activity of ribosides (*t*ZR). The activation of three different cytokinin receptors of maize by ribosides was also reported (Yonekura-Sakakibara et al., 2004). The predominant preference for the free base binding was also confirmed for maize receptors (ZmHKs) and different ZmHK-dependent cytokinin-induced *lacZ* expression in *E. coli* was reported as well. Hence, ZmHK1 shown its preference for iP and ZmHK2 was more responsive for *t*Z. Additionally, a weak response of ZmHK1 was detected to iPR but shortening of the treatment period lead to decreased response suggesting the iPR degradation by internal nucleosidase (Yonekura-Sakakibara et al., 2004). However, ZmHK2 receptor showed a similar response for free bases and nucleosides, even after shortening of the treatment period, suggesting the genuine ZmHK2 sensitivity to nucleosides, but not excluding the influence of ribose moiety cleavage (Yonekura-Sakakibara et al., 2004).

The conflict differences in cytokinin activity of free bases and their ribosides in bacterial *E. coli* systems and other heterologous assays (Lomin et al., 2012; Spíchal et al., 2004; Steklov et al., 2013; Suzuki et al., 2001; Yamada et al., 2001) led to the development of novel quantitative assay analysing the cytokinin receptor-ligand interaction in microsomes isolated from tobacco leaves expressing individual cytokinin receptor and thus allowing to obtain natural receptor membrane environment (Lomin et al., 2015). In this plant membrane test system ribosides and other N^9-substituted cytokinin derivatives showed highly decreased activities, underachieving the activity of the free base (Lomin et al., 2015; Savelieva et al., 2018). The 3D-structure of the CRE1/AHK4 binding site (Hothorn et al., 2011) showed that N^9-substituted cytokinin base, not only by ribose, cannot fit well enough as the cytokinin base and, moreover, the ribose residue prevents the receptor cavity closure necessary for the receptor activation (Lomin et al., 2015; Romanov and Schmülling, 2022). Moreover, no single example of high-affinity binding of cytokinin riboside to the receptor in a model system that excludes metabolic changes in the ligand has been reported. Therefore, the cytokinin activity of N^9-substituted cytokinins in bacterial and plant assays could depend on the enzymatic interconversions between cytokinin free bases and ribosides or on a hydrolytical removal of the ribose moiety (Kieber and Schaller, 2018; Lomin et al., 2015; Oshchepkov et al., 2020; Romanov and Schmülling, 2022).

Our results correspond with these findings. In the bacterial assays (bacterial receptor assay, competitive ligand binding assay) as well as in test systems using cultivation of transgenic plant seeds (*ARR::GUS assay*) or in classical plant cytokinin bioassays the highest activities were observed for the free bases. Some activity was, however, reported for riboside and 2′-dideoxyriboside derivatives in the bacterial receptor assays (Figs. 2 and 3). The 2′,3′-dideoxyribosides were inactive in the bacterial receptor assays whereas ribosides and 2′-deoxyribosides interacted with CRE1/AHK4 and, mainly, AHK3 receptor, consistently with the literature (Spíchal et al., 2004). In the competitive ligand binding assay ribosides and 2′-deoxyribosides were able to compete with radiolabelled *t*Z for the AHK2, AHK3 or CRE1/AHK4 binding site. Obviously, we cannot exclude the possibility of the partial N^9-substitution hydrolysis. Nevertheless, in the *Amaranthus* bioassay mainly 2′-deoxyribosides (iPdR, **1**; *t*ZdR, **3**) showed not only comparable but even higher activity than the corresponding free base. This trend was also observed in the tobacco callus bioassay where especially 2′-deoxyribosides (**2**, **3**, **5**) and several 2′,3′-dideoxyribosides (**4a**, **4b**) did not cause toxic effect at the highest concentration while the same concentration of cytokinin ribosides as well as the free bases did not support the tobacco callus growth. These results suggests that cytokinin 2′-deoxyribosides could be at least partially active *per se* in bacterial receptor assays and are capable of causing significantly different results in bioassays (*Amaranthus*, tobacco callus) in comparison with cytokinin ribosides or free bases.

However, we should not forget that in a model system *in vivo* the results of exogenous hormone exposure depend on many parameters, such as the efficiency of hormone penetration into the cell, its metabolism inside and outside the cell, sequestration inside certain cellular compartments, the cell response to the hormone excess by the fast feedback reaction, etc. For example, when the transport system of ribosides will be particularly active in the tissue, the concentration of free bases released from them can be equal or even higher than a similar concentration of hormones added directly as bases. Our methodological possibilities to answer these questions, such as tissue- and/or compartment-specific phytohormone analyses, are still very limited, however, current development of novel approaches with high spatial resolution is promising (Pařízková et al., 2022; Skalický et al., 2018).

2.2.1. Cytotoxicity testing on human non-cancer cells

The cytotoxicity of the prepared derivatives **1**–**6** was evaluated in cell lines BJ (skin fibroblasts), HaCaT (spontaneously immortalized keratinocytes) and ARPE-19 (retinal pigment epithelium cells) using resazurin reduction assay. The compounds were tested at six concentrations up to 100 μM (data not shown) and showed no or limited (at 100 μM) cytotoxicity (resazurin signal 95–105% of control) after 3 days.

The favourable toxicity profiles on human cells show that the compounds do not interfere with nucleic acid metabolism. Cytotoxic activity reported for cytokinin ribosides studied as anticancer drugs including iPR requires conversion to the respective 5′-monophosphates as demonstrated by the protective effect of the cotreatment with an inhibitor of adenosine kinase (Ishii et al., 2003; Mlejnek and Doležel, 2005; Voller et al., 2010). Cytokinins have been studied as anti-aging, neuroprotective, anti-inflammatory, anti-aggregatory and antiviral agents (Voller et al., 2017, 2019). Preventing the conversion into toxic metabolites by designing analogues without 5′-hydroxyl can therefore provide new compounds for a range of pharmacological studies.

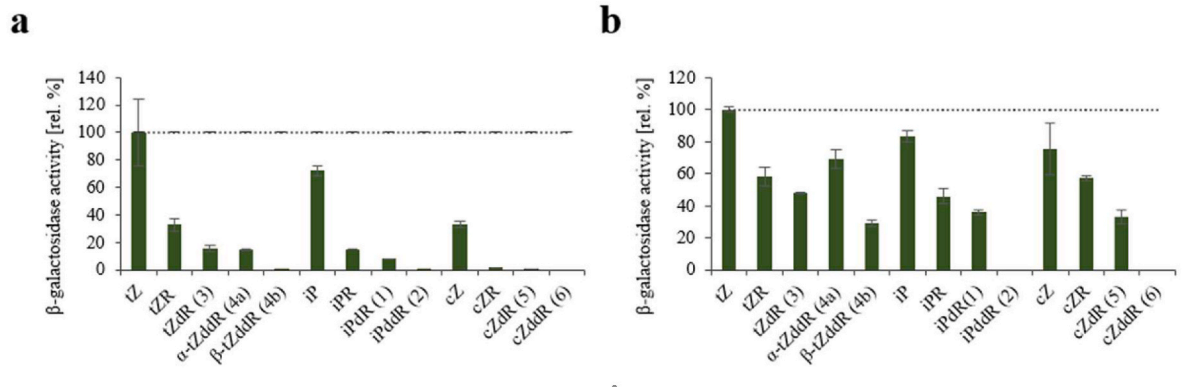

Fig. 2. Bacterial receptor assays. Comparison of relative *β*-galactosidase activities of test compounds (*t*Z, iP, *c*Z series) at the final concertation of 10 μM in *E. coli* expressing **a** CRE1/AHK4 and **b** AHK3 receptor. The standard *t*Z was used as a positive control and set to 100% (dashed line). Error bars represent standard deviation (n = 6).

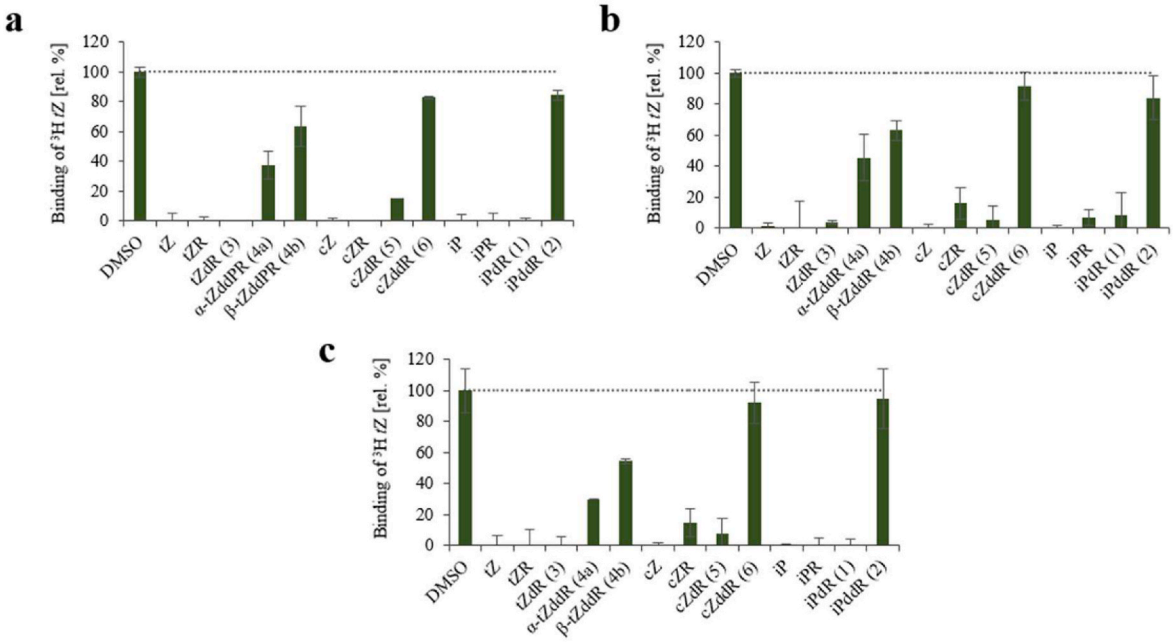

Fig. 3. Competitive ligand binding assays. The ability of test compounds at the final concentration of 10 μM to block the binding of radiolabelled *t*Z to **a** AHK2, **b** AHK3, and **c** CRE1/AHK4 receptor. The value of obtained with ^{3}H-*t*Z (3 nM) was set as 100% activation (dashed line). An excess of unlabelled *t*Z (1000-fold) was used to distinguish between specific and non-specific binding, and this value was subtracted from the data. Error bars represent standard deviation (n = 3).

Our intention was to determine whether or not the prepared 2′-deoxyriboside and 2′,3′-dideoxyriboside cytokinin derivatives are cytotoxic and therefore, the cancerous cell lines were not employed in this study.

2.2.2. *Bacterial cytokinin receptor assays*

Generally, cytokinin is recognized by three *Arabidopsis thaliana* histidine kinases (AHK2, AHK3 and CRE1/AHK4) known as cytokinin membrane receptors. Upon cytokinin binding, the signaling cascade is phosphorylated and the signal is transmitted to the nuclear *Arabidopsis thaliana* response regulators (ARRs) encoding cytokinin-responsive genes (Inoue et al., 2001; Suzuki et al., 2001). The ability of prepared compounds to activate cytokinin signaling was studied *in vitro* in the bacterial cytokinin receptor assay using cytokinin-dependent expression of *cps::lacZ* in heterologous *Escherichia coli* system (Inoue et al., 2001; Suzuki et al., 2001).

The receptor recognition/activation effect of all prepared substances on these two cytokinin receptors, CRE1/AHK4 (Fig. 2a) and AHK3 (Fig. 2b), was examined using the downstream activated reporter gene *cps::lacZ* (Spíchal et al., 2004). In both cases, *t*Z was used as a positive control, set as 100% at 10 μM, and the respective base, 2′-deoxyriboside and 2′,3′-dideoxyriboside were tested to compare the influence of the altered N^{9}-substituent.

The declining trend of receptor activation depending on the isoprenoid *C6* substituent was confirmed in the bacterial CRE1/AHK4 assay as follows: *t*Z > iP > *c*Z. Similarly, in each cytokinin group the base was more active than the riboside, and correspondingly, the riboside was more active than the 2′-deoxyriboside (**1**, **3**, **5**). The 2′,3′-*β*-dideoxyribosides (**2**, **4b**, **6**) were inactive in this assay. Generally, *t*Z derivatives were the most active in comparison with iP and *c*Z analogues, however, *t*ZR reached only one third of *t*Z activity. Interestingly, the *α*-*t*ZddR (**4a**) activated the AHK4 receptor comparably as *t*ZdR (**3**) at 10 μM.

In the bacterial AHK3 assay the receptor activation results were more significant at 10 μM, nevertheless, the declining activity was as follows: base > riboside >2′-deoxyriboside (**1**, **3**, **5**) > 2′,3′-dideoxyriboside (**2**, **4a/b**, **6**) noticeable mainly for *t*Z, iP and *c*Z series in *β*-stereochemistry.

However, the α-anomer *t*ZddR (**4a**) showed 2-fold enhancement in AHK3 receptor activation compared to the β-anomer (**4b**) and exceeded the activity of *t*ZdR (**3**), the most active 2′-deoxyriboside tested. Furthermore, iPddR (**2**) and *c*ZddR (**6**) retained almost unaffected through the AHK3 receptor.

Overall, the bases of isoprenoid cytokinins (especially iP and *t*Z) were well percieved through the CRE1/AHK4 and AHK3 receptors while the N^9-substituent (ribose, 2′-deoxyribose or 2′,3′-dideoxyribose) caused a remarkable decrease of cytokinin signaling activity, which was more significant for prepared isoprenoid 2′,3′-dideoxyribosides (**2**, **4a/b**, **6**) than 2′-deoxyribosides (**1**, **3**, **5**) or appropriate ribosides. This is relevant with the reported reduced affinity of N^9-ribosides toward cytokinin receptors in the newly developed assay system (Lomin et al., 2015) emphasizing the weak activity of cytokinin ribosides and highlighting the role of free bases as the most active cytokinin forms (Lomin et al., 2015) which is also in good agreement with our results. Generally, the reduction of the sugar hydroxyl group of the β-anomers led to suppression of the receptor activation at the tested receptors (AHK3, CRE1/AHK4) in the bacterial receptor assay. Moreover, the less common configuration of the α-diastereomer (**4a**) also exhibited observable activity in bacterial receptor assay. Although some anomeric mixture can be chromatographically separated, the individual α/β anomers due to mutarotation in aqueous solution can be dynamically interconverted back to the original diastereomeric mixture. Nevertheless, the different bioactivity of the individual anomers would help to control the dynamic balance towards the bioactive anomer or better receptor binding due to a more efficient spatial arrangement (Goff and Thorson, 2014).

2.2.3. Competitive ligand binding assay

The ability of the prepared compounds to bind directly to the cytokinin receptors (AHK2, AHK3, CRE1/AHK4) can be verified in competitive ligand binding assay. We measured the specific binding activity of the tested compounds (10 μM) in competition with radiolabelled *t*Z (^{3}H-*t*Z; set as 100% affinity) to the AHK2 (Fig. 3a), AHK3 (Fig. 3b), and CRE1/AHK4 receptor (Fig. 3c). In general, the relative activity of the tested substances was comparable for all receptors. The 2′-deoxyribosides (**1**, **3**) with a small exception of *c*ZdR (**5**) showed a higher affinity for the tested receptor comparable to the parent free base and riboside and the lowest ligant binding activity was observed for 2′,3′-dideoxyribosides (**2**, **4a/b**, **6**). The highest binding activity was detected mainly for the bases, followed by ribosides and 2′-deoxyribosides. Thus, the obtained results suggest that the prepared 2′-deoxyribosides bind to the receptor comparably to natural isoprenoid cytokinins. On the other hand, the ligand-binding activity of 2′,3′-dideoxyriboside derivatives was strongly suppressed. However, when comparing the α- and β-anomers of *t*ZddR (**4a/b**), the α-anomer shown also observable activity, which confirms the results of bacterial receptor assays.

2.2.4. In vivo ARR5::GUS assay

The β-glucuronidase (GUS) activity of the prepared substances was quantitatively determined for the activation of the cytokinin-dependent promoter *Arabidopsis thaliana* response regulator 5 (*ARR5*) which is associated with the cytokinin signaling pathway. It was performed in the *ARR5::GUS* assay (Fig. 4), where transgenic *Arabidopsis thaliana* plants were cultured with the presence of test compounds at 5 μM. In this assay, no significant differences of β-glucuronidase activity were observed between N^9-unsubstituted and N^9-substituted analogues in a number of tested derivatives of iP and *t*Z series. This could be due to the integration of responses from several putative cytokinin signaling pathways, as this assay may not distinguish between different cytokinin receptors (Spíchal et al., 2004). At the same time, probably due to mutarotation equilibrium in water between the α- and β-anomer of *t*ZddR (**4a**, **4b**), they showed relatively similar results in favour of the compound **4b**. On the contrary, among *c*Z series a notable difference of activity was observed. The *c*Z base and riboside showed comparable activity as overall iP and *t*Z series, analogically to the activity of BAP at 1 μM. However, *c*ZdR (**5**) and *c*ZddR (**6**) showed significantly reduced relative β-glucuronidase activity. Hence, in this bioassay, no significant difference between isoprenoid cytokinin 2′-deoxyriboside (**1**, **3**, **5**) and 2′,3′-dideoxyriboside (**2**, **4a/b**), **6**) was observed.

2.2.5. Root elongation inhibition assay

The biological activity of the prepared compounds could be also related to the growth and developmental system in plants. Therefore, *in vivo* root assay of *Arabidopsis thaliana* seedlings was performed to study the effects of exogenous cytokinins with various N^9-substituted glycosides compared to the parent compound on the primary root length (Fig. 5a–c) and number of lateral roots (Fig. 6a–c). Compared to BAP, generally used as a positive control inhibiting root development, substances of *c*Z series (*c*Z, *c*ZR, **5**, **6**) did not inhibit primary root growth and lateral root formation at the concentration of 0.1 μM at all. On the other hand, at this concentration, N^9-unsubstituted iP and *t*Z strongly inhibited the root growth as well as lateral root formation, analogically to BAP, while iPR as well as *t*ZR exhibited lower inhibitory effect on root

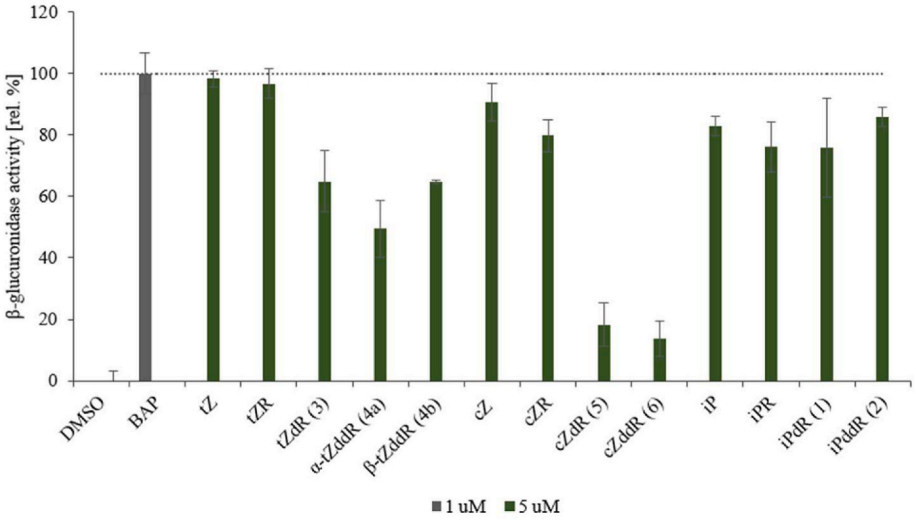

Fig. 4. Quantitative evaluation of GUS activity in *ARR5::GUS* transgenic *A. thaliana* seedlings (dashed line). The activity of test compounds (*t*Z, *c*Z, iP series) at the final concertation of 5 μM and 0.1% DMSO (solvent control) was compared to the activity of 1 μM BAP (used as s standard) which was set as 100% (dashed line). Error bars represent standard deviation (n = 3).

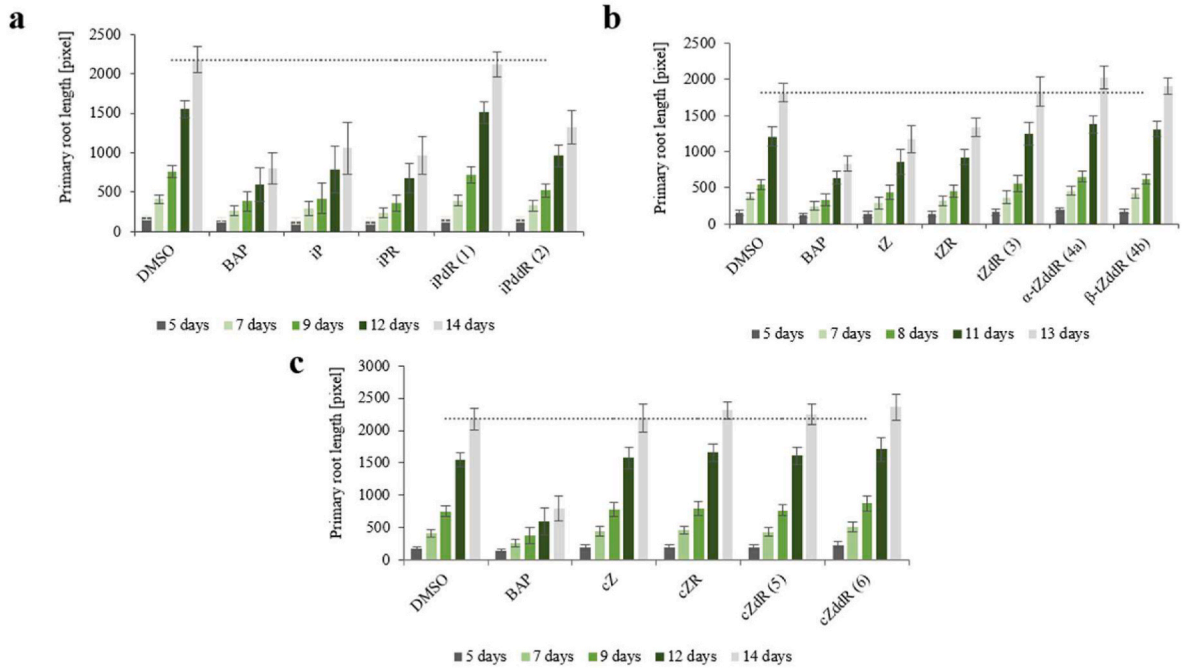

Fig. 5. Inhibition of primary root elongation in A. thaliana (Col-0) treated with BAP (positive control), 0.001% DMSO which was used as solvent control (dashed line) and **a**: iP, iPR, iPdR (**1**), iPddR (**2**), (iP series); **b**: *t*Z, *t*ZR, *t*ZdR (**3**), *t*ZddR (**4a**, **4b**), (*t*Z series); **c**: *c*Z, *c*ZR, *c*ZdR (**5**), *c*ZddR (**6**), (*c*Z series) The compounds were applied at the final concertation of 0.1 μM. Error bars represent standard deviation (n > 30).

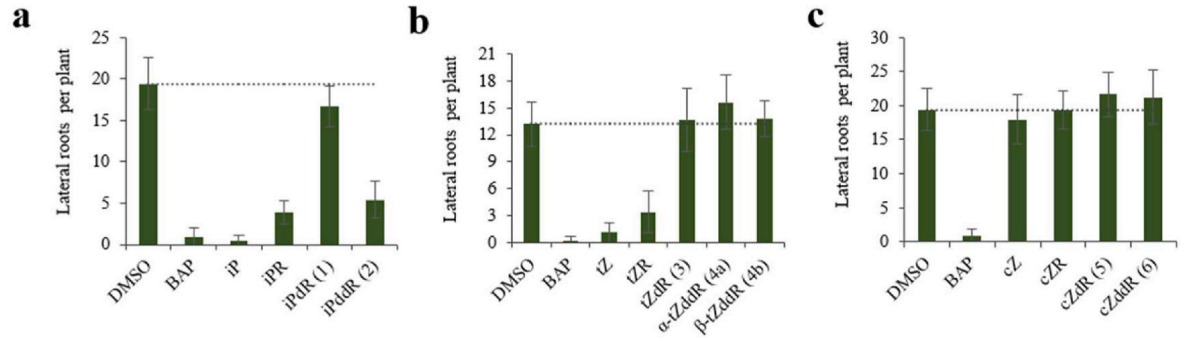

Fig. 6. Number of lateral roots per plant (Col-0) treated with BAP 0.001% DMSO which was used as solvent control (dashed line) and **a**: iP, iPR, iPdR (**1**), iPddR (**2**), (iP series); **b**: *t*Z, *t*ZR, *t*ZdR (**3**), *t*ZddR (**4a**, **4b**), (*t*Z series); **c**: *c*Z, *c*ZR, *c*ZdR (**5**), *c*ZddR (**6**), (*c*Z series). The lateral roots of the seedlings were measured 13/14 days after germination. The compounds were applied at the final concertation of 0.1 μM. Error bars represent standard deviation (n > 30).

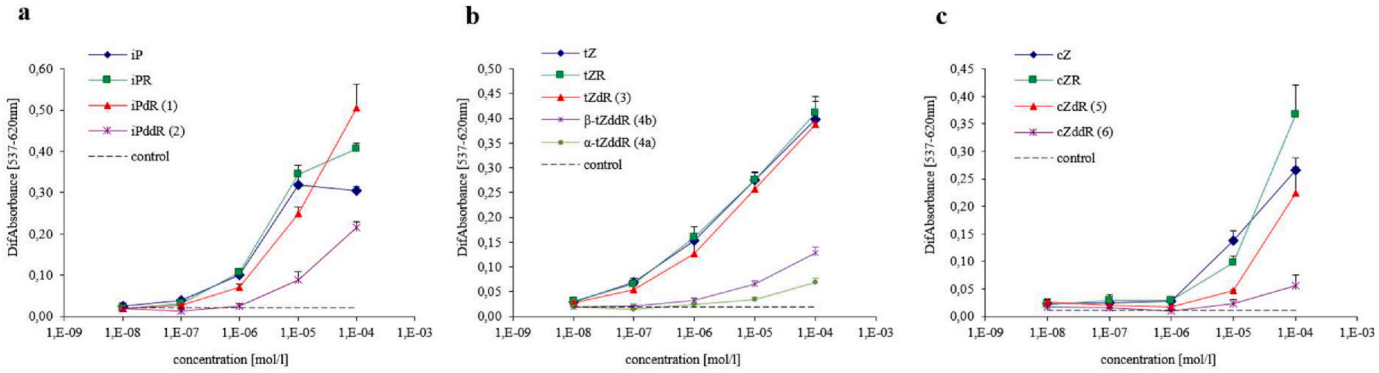

Fig. 7. Dose response curves for cytokinin-induced betacyanin synthesis in *Amaranthus caudatus* var. *Artropurpurea* cotyledons treated with **a**: iP, iPR, iPdR (**1**), iPddR (**2**), (iP series); **b**: *t*Z, *t*ZR, *t*ZdR (**3**), *t*ZddR (**4a**, **4b**), (*t*Z series) and **c**: *c*Z, *c*ZR, *c*ZdR (**5**), *c*ZddR (**6**), (*c*Z series). Dashed lines indicate values for the control treatment without any cytokinin. Error bars represent standard deviation (n = 5).

elongation. Moreover, iPdR (**1**), *t*ZdR (**3**) and both anomers of *t*ZddR (**4a/b**) showed no negative effect on root development. However, compound iPddR (**2**) inhibited primary root length comparably to iPR and iP accompanied by a lower number of lateral roots. Taken together, with increasing replacement of OH groups by hydrogen on the sugar ring, inhibition of primary root development is supressed, except for iPddR (**2**).

2.2.6. Cytokinin bioassays

Three classical cytokinin bioassays (Holub et al., 1998) (*Amaranthus*, detached wheat leaf senescence, and tobacco callus bioassay) were used to determine the cytokinin activity of the prepared compounds with different N^9 substituents compared to the corresponding cytokinin bases (iP, *t*Z and *c*Z). The results of cytokinin activity are reported as the average of at least 2–3 assay replicates (Figs. 7–9 and Tables S1–S3).

2.2.6.1. Amaranthus assay. The *Amaranthus* bioassay is based on the dark induction of betacyanin synthesis in *Amaranthus caudatus* (L.), 1753 var. atropurpurea cotyledons in the presence of cytokinin (Bamberger and Mayer, 1960). In this study, the parent compound of iP, *t*Z, or *c*Z (10^{-4} mol/L) were used as the positive controls and set to 100% (Fig. 7). Among prepared iP-derivatives, iPdR (**1**) showed higher activity followed with iPR compared to iP. On the other hand, iPddR (**2**) caused lower betacyanin synthesis. However, constantly increasing effect (Fig. 7a) with higher concentrations was reported in the results of the compounds **1** and **2**. Although *t*ZdR (**3**) and *t*ZR showed the same activity as the parent base (*t*Z), *t*ZddR (**4a**) was almost inactive (Fig. 7b). Among the *c*Z-derivatives, *c*ZR exceeded the activity of *c*Z followed by less active *c*ZdR (**5**). Moreover, *c*ZddR (**6**) exhibited very low activity even at the highest concentration (Fig. 7c).

In the cytokinin bioassays, the *in situ* activity of iPdR (**1**), *t*ZdR (**3**) and *t*ZR exceeded the activity of corresponding base compared to the receptor bioassays.

2.2.6.2. Senescence bioassay. For the senescence bioassay the respective base (iP, *t*Z and *c*Z) at the concentration of 10^{-4} mol/L was used as a reference and set as 100% (Fig. 8). The wheat leaf senescence assay (Holub et al., 1998) depends on the ability of derivatives to retard chlorophyll degradation in darkness after 5 days. We measured the residual chlorophyll in detached wheat leaves (*Triticum aestivum* (L.), 1753 cv. Aranka) treated with test compounds compared to fresh leaves in the final concentration of 10 μM and 100 μM (Fig. 8). Both iP and *c*Z-types of compounds (**1**, **2** and **5**, **6**) and their appropriate free bases iP (Fig. 8a) and *c*Z (Fig. 8c) were not able to retard senescence process. No apparent correlation was observed between the structure of free bases (iP, *c*Z), their prepared glycosides (2′-deoxyriboside, 2′,3′-didexyriboside) and anti-senescence properties. On the other hand, *t*Z-derivatives (Fig. 8b) exhibited strong anti-senescence activity compared to iP and *c*Z-derivatives. It was shown that most *t*Z derivatives (*t*Z, *t*ZR) exceeded double activity of the chlorophyll degradation than untreated control. Regarding *t*Z group, the anti-senescence activity decreased in order: *t*ZR > *t*Z > *t*ZdR (**3**) > α-*t*ZddR (**4a**) > β-*t*ZddR (**4b**). These results confirmed that the anti-senescence effects are related to the nature of the N^6 substituent and the *t*Z-type, which maintains chlorophyll levels more effectively compared to the iP and *c*Z-counterparts. In addition, it has been observed that the various glycosyl N^9 substituents of the *c*Z and iP-derivatives do not affect senescence process, with the exception of the *t*Z-derivatives. On the other hand, in contrast to the ability of chlorophyll retardation, the ability of *t*ZdR (**3**) to stimulate the synthesis of chlorophyll (in etiolated cucumber cotyledons) was reported as insignificant (Evidente et al., 1991).

2.2.6.3. Tobacco callus bioassay. The tobacco callus bioassay (Holub et al., 1998) is based on the ability of cytokinins to promote cell division of cytokinin-dependent tobacco callus (*Nicotiana tabacum* (L.), 1753 cv. Wisconsin 38) in the presence of auxin. The iP derivatives (iPR, **1**, **2**) possessed activities comparable to iP (Fig. 9). Simultaneously, *t*ZdR (**3**) and *t*ZddR (**4a**, **4b**) does not have a cytotoxic effect at 10^{-4} mol/L showing inhibition of callus growth. The callus tissue supplied with *c*ZdR (**5**) did not cause a rapid decrease of callus weight at the highest concentration, typical for compounds *c*Z, *c*ZR and *c*ZddR (**6**) in this assay. Nevertheless, callus fresh weight was the lowest among *c*Z derivatives, after *c*ZdR treatment at all concentrations except the highest, corresponding to the previously published results (Fujii et al., 1994). This trend was observed for all the three 2′-deoxyriboside derivatives: iPdR (Fig. 9a), *t*ZdR (Fig. 9b) and *c*ZdR (Fig. 9c) suggesting the importance of the presence of 2′-deoxyribosyl moiety in attempt to reach better callus growth supported by cytokinin. Nevertheless, the positive effect of N^9-substituted cytokinin over nucleobase at ultra-high concentrations does not prove the inherent cytokinin activity.

3. Conclusion

In summary, a series of isoprenoid cytokinin analogues were prepared by using three different synthetic strategies and their structures were verified by various physicochemical methods. The biological activities of the prepared compounds were studied in various cytokinin *in vitro/in vivo* bioassays.

In the bacterial cytokinin receptor (AHK2, AHK3, CRE1/AHK4) assays, the recognition of tested derivatives was demonstrated according

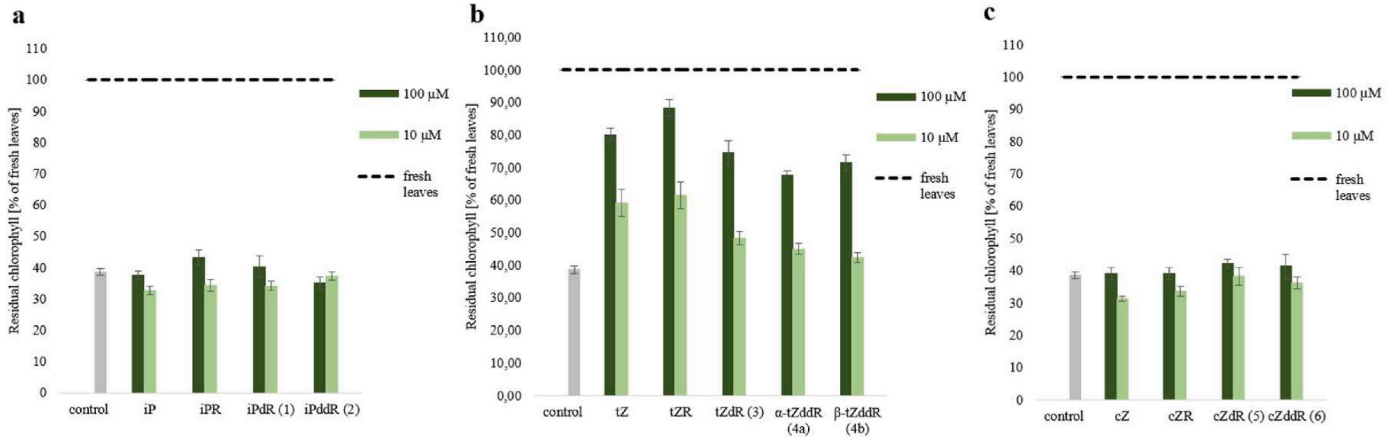

Fig. 8. Relative amount of residual chlorophyll in excised wheat leaves treated with **a**: iP, iPR, iPdR (**1**), iPddR (**2**), (iP series); **b**: *t*Z, *t*ZR, *t*ZdR (**3**), *t*ZddR (**4a**, **4b**), (*t*Z series) and **c**: *c*Z, *c*ZR, *c*ZdR (**5**), *c*ZddR (**6**) (*c*Z series) at the final concentration of 10 μM and 100 μM and 0,002% DSMO (solvent control). The residual chlorophyll in the fresh wheat leaves was set to as 100% (dashed line). Error bars represent standard deviation (n = 5).

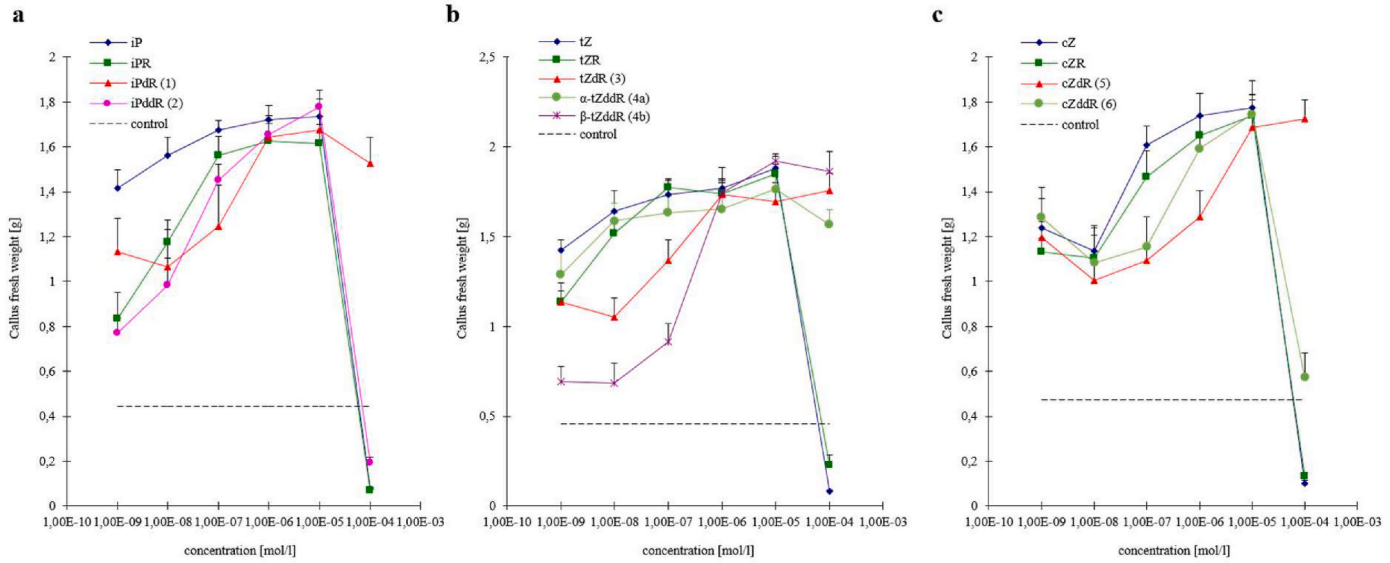

Fig. 9. Dose-response curves for cytokinin-dependent tobacco callus growth treated with **a**: iP, iPR, iPdR (**1**), iPddR (**2**), (iP series); **b**: *t*Z, *t*ZR, *t*ZdR (**3**), *t*ZddR (**4a**, **4b**), (*t*Z series) and **c**: *c*Z, *c*ZR, *c*ZdR (**5**), *c*ZddR (**6**), (*c*Z series). Dashed lines indicate values for the control treatment without any cytokinin. Error bars represent standard deviation (n = 6).

to the trend: base > riboside >2′-deoxyriboside > 2′,3′-dideoxyriboside. Interestingly, *α*-*t*ZddR (**4a**) differed from this trend especially in the AHK3 and showed to be a better ligand than *β*-*t*ZddR (**4b**). The comparison in the ligand binding assay showed that the isoprenoid bases, ribosides and 2′-deoxyribosides interact better with the cytokinin receptors rather than 2′,3′-dideoxyribosides (**2**, **6**). Additionally, no significant differences in *β*-glucuronidase activity were observed in iP series in the *ARR5::GUS* assay. These results shown that the removal of OH group from the 2′ and 3′ position of the sugar moiety of isoprenoid cytokinin causes a generally decreasing trend on the cytokinin signaling function in the *t*Z series (**3**, **4a**/**b**) and mainly in the *c*Z series (**5**, **6**).

In the *c*Z series (*c*Z, *c*ZR, *c*ZdR, *c*ZddR), no cytokinin inhibitory effect was found on primary root development and lateral root initiation, while iP and *t*Z and their ribosides (iPR, *t*ZR) inhibited primary root growth. Inability to inhibit root growth was also observed for iP and *t*Z-derived 2′-deoxyribosides (**1**, **3**). In the case of 2′,3′-dideoxyribosides, iPddR (**2**) considerably inhibited root development compared to *c*ZddR (**6**) and *α*/*β*-*t*ZddR (**4a**/**b**). In the *Amaranthus* bioassay (Fig. 7), the results showed a great efficacy of isoprenoid 2′deoxyribosides (**1**, **3**, **5**) which generally had an equal or even higher cytokinin activity than their parent bases or corresponding ribosides. On the other hand, presence of 2′,3′-dideoxyribosyl moiety did not specifically support the betacyanin production. The whole tested series of iP and *c*Z derivatives (base, riboside, 2′-deoxyriboside and 2′,3′-dideoxyriboside) did not retard chlorophyll degradation in senescent leaves. As expected, *t*Z 2′-deoxyriboside/2′,3′-dideoxyriboside (**3**, **4a**/**b**) showed weak antisenescence properties, but less than *t*Z and *t*ZR. Thus, the elimination of the OH group on the ribose moiety does not significantly improve the antisenescence properties. On the other hand, it was shown that the presence of 2′-deoxyribose moiety (**1**, **3**, **5**) positively contributes to cytokinin-dependent callus growth (Fig. 9) as these derivatives have no cytotoxic effect at the highest concentration tested. The prepared 2′-deoxyribosides and 2′,3′-dideoxyribosides were not cytotoxic to non-cancer human cell lines at the concentrations up to 100 μM. Antiviral activity of cytokinin 2′-deoxyribosides and 2′,3′-dideoxyribosides would be of great interest for further study.

Taken together, the cytokinin receptor interaction ability, mainly of the free bases, especially in bacterial bioassays, was confirmed. Moreover, our results are well consistent with claims that the presence and also alterations of N^9 ribose substitution significantly reduce AHK2, AHK3, and CRE1/AHK4 receptor perception (Lomin et al., 2015; Šmehilová et al., 2016). However, the presence of ribose or 2′-deoxyribose did not prevent the bacterial receptor assays to reveal at least minor genuine activity. Additionally, 2′-deoxyriboside derivatives (especially **1** and **3**) supported the growth of tobacco callus or the induction of betacyanin synthesis in *Amaranthus* cotyledons in the similar manner or even better in comparison with the corresponding free bases which can be partly influenced by the N^9-susbtituent hydrolysis during bioassay.

4. Experimental

4.1. Biological procedures

4.1.1. Cytotoxicity bioassay

The effects of 72 h treatment with the compounds on the viability of human skin fibroblasts BJ, keratinocytes HaCaT and retinal pigment epithelium cells ARPE-19 were evaluated using the resazurin reduction assay. The cell lines were obtained from the American Type Culture Collection, Manassas, VA, USA (BJ, ARPE-19) and the German Cancer Research Center (*DKFZ*), Heidelberg, Germany (HaCaT). The cells were maintained in DMEM (BJ, HaCaT) or DMEM/F12 (ARPE-19) culture medium (Sigma) supplemented with fetal bovine serum (10%), glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) under standard cell culture conditions (37 °C, 5% CO_2, humid environment) and sub-cultured two or three times a week.

For testing, about 5000 cells were seeded into each well of a 96-well plate about 24 h before treatment with the test compounds at six concentrations up to 100 μM (data not shown) or DMSO vehiculum. The final concentration of DMSO did not exceed 0.1%. An 11x concentrated solution of resazurin in DMEM medium was added to the cells to a final concentration of 100 μM after 72 h. Fluorescence intensity (λ ex/em = 570/610 nm) was measured after 3 h incubation.

4.1.2. Bacterial receptor assay (Cytokinin signaling bioassay) (Spíchal et al., 2004)

Transgenic *Escherichia coli* strains KMI001 harbouring PINIII/AHK4 or pSTV28/AHK3 plasmid expressing the *β*-galactosidase gene (*ΔrcsC, cps::lacZ*) under the control of cytokinin receptors were used for this assay. Bacterial precultures were grown in M9 liquid medium supplemented with 0.1% casamino acids and an antibiotic (ampicillin, 100 μg

ml^{-1} for CRE1/AHK4 or chloramphenicol, 25 μg ml^{-1} for AHK3) at 25 °C for 24 h. Expression of the β-galactosidase gene was induced by cultivation of 200 μl of the precultures diluted by M9 medium with antibiotic (OD_{600}0.5) and test compound at the final concertation of 10 μM and shaking (450 rpm) at 25 °C for 17 h. After measuring of OD_{600}, 50 μl of the sample was incubated with 2 μl 25 mM MUG (4-methyl-umbelliferyl galactoside) at 37 °C for 10 min (AHK4) or 60 min (AHK3). Fluorescence was measured (λ ex/em − 365/460 nm) using a Synergy LX Multi-Mode Microplate Reader (Biotech, USA) after the addition of stop buffer (100 μl).

4.1.3. Amaranthus bioassay (Holub et al., 1998)

Amaranthus caudatus (L.), 1753 var. atropurpurea seeds were sterilized on their surface (10 min with 10% sodium hypochloride, washed with H_2O (0.5 L), 10 min with 70% EtOH, washed with H_2O (0.5 L), placed on a Petri dish containing paper tissue soaked with deionized H_2O 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 Petri 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.

4.1.4. Wheat leaf senescence bioassay (Holub et al., 1998)

Wheat seeds (*Triticum aestivum* (L.), 1753 cv. Aranka) were washed with 96% EtOH and H_2O, sown in vermiculite 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 solution of the test compound (DMSO solution diluted in deionized H_2O, 150 μl/well), and cultivated in a closed box at 24 °C for 96 h in the dark. Residual chlorophyll was extracted by heating the leaf material in 5 ml of 80% EtOH 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 and extracts of leaves cultivated in deionized H_2O with DMSO (0.002%, v/v).

4.1.5. Tobacco callus bioassay (Holub et al., 1998)

Cytokinin-dependent tobacco callus cells (*Nicotiana tabacum* (L.), 1753 cv. Wisconsin 38) were cultivated on solid MS medium (3 ml/well) containing different concentrations of the test compound (from 10^{-9} to 10^{-4} M solution in DMSO) in 6-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) or the respective cytokinin base.

4.1.6. Competitive ligand binding assay (Romanov et al., 2005, 2006; Yamada et al., 2001)

The *Escherichia coli* BL21(DE3) strain expressing cytokinin receptor AHK2, AHK3 and CRE1/AHK4 (kindly provided by Thomas Schmülling) was used in the cytokinin competition ligand binding assay with [^{3}H] *trans*-zeatin as was reported previously (Yamada et al., 2001). Each sample contained 1 ml of bacterial culture (OD_{600} 0.7–1), 2 μL of 1.5 nM ^{3}H-*t*Z and tested competitors or unlabelled *t*Z at the final concertation of 10 μM. DMSO (0.1%, v/v) was used a negative control. The samples were mixed, incubated in a refrigerator (4 °C) for 30 min and then centrifuged (4 °C, 6 min, 8000 rpm). The supernatant was carefully removed, and the bacterial pellet was resuspended in H_2O (20 μl) and then in a 1 ml of scintillation cocktail (Ultima-Flo M, PerkinElmer, USA). Radioactivity was measured on a Beckman LS 6500 Scintillation Counter (Beckman, USA). An excess of unlabelled *t*Z (1000-fold) was used for competition to distinguish between specific and non-specific binding.

4.1.7. ARR5::GUS assay

This assay was carried out according to the protocol (Romanov et al., 2002) with little modification. After the stratification for 3 days at 4 °C in the darkness, transgenic *Arabidopsis thaliana ARR5::GUS* seeds were grown in six-well plates on an orbital shaker in a growth chamber (21 °C) under long-day conditions (16 h light/8 h dark) for 3 days. The seedlings were added to the liquid growth solution contained tested compound (5 μM), a standard (BAP, 1 μM) or pure DMSO (solvent, final conc. 0.1%) and cultured for additional 16 h. The specific activity of β-glucuronidase in the plant extract was spectrophotometrically measured (λ ex/em − 365/450 nm) using a Fluoroscan Ascent microplate reader (Labsystems, Helsinki, Finland).

4.1.8. Root elongation inhibition assay

The root test was performed on *Arabidopsis thaliana* (L.), Heyhn. 1842 cv. Columbia-0 seedlings. The solution of test compounds (0.1 μM in DMSO) was added to ½ MS medium supplemented with 1% (w/v) sucrose, 0,8% (w/v) Phytagel, pH 5.8 (Thermofisher, Germany) and poured into 12 × 12 cm square Petri dishes. The corresponding free bases and BAP were used as positive controls and DMSO as the negative control. *Arabidopsis thaliana* sterile seeds were applied to the culture plates in a row of approx. ten pieces and four dishes were prepared for each substance. After the stratification at 4 °C for 2 days, seeds were grown on vertically oriented plates in a growth chamber under a photoperiod of 16 h light/8 h dark at 18 °C and at light intensity of 8.5–10 μmol $m^{-2}s^{-1}$. The seedlings were scanned (800 dpm) using a horizontal scanner and the root length was measured with ImageJ software. The experiments were repeated twice.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2022.113481.

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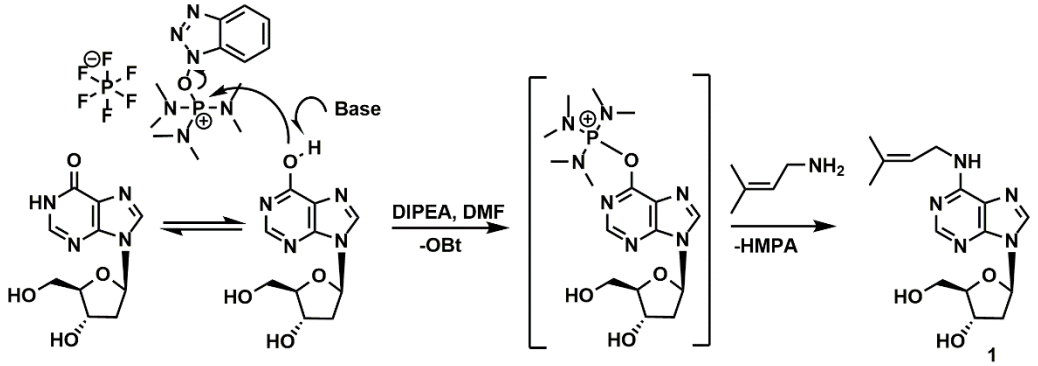
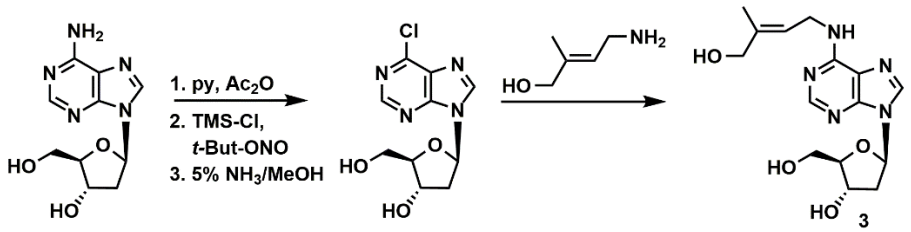
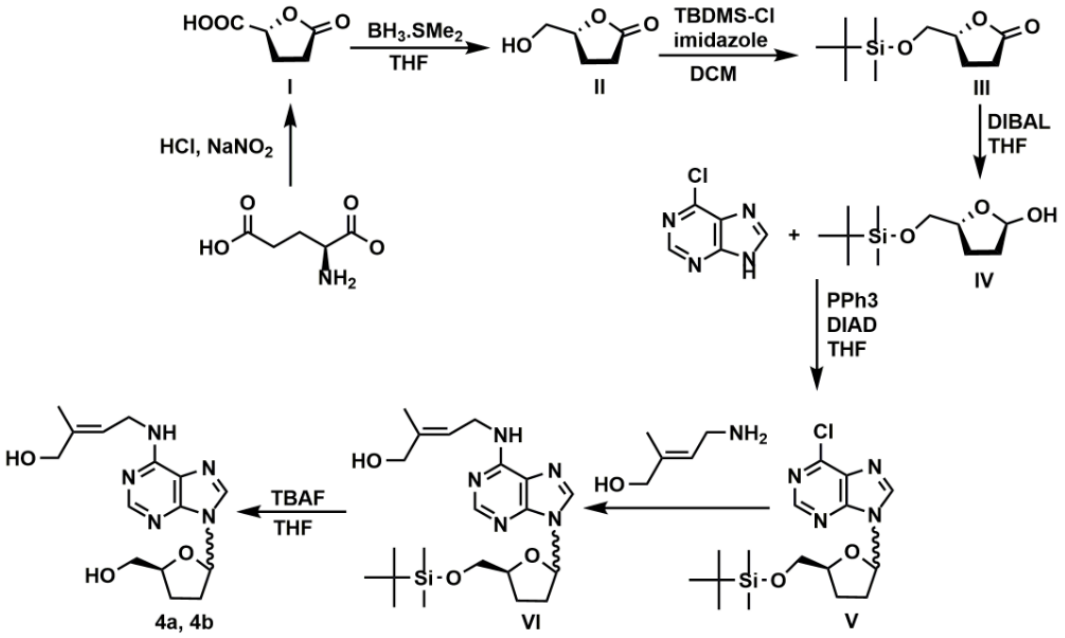
Supplementary Material



Scheme S1: General synthesis (Wan et al., 2007, 2005) by the method using Castro´s reagent given in the example for iPdR (**1**), also used for the preparation of *c*ZdR (**5**) iPddR (**2**), *β*-*t*ZddR (**4b**) and *c*ZddR (**6**).



Scheme S2: Reaction scheme (Francom and Robins, 2003; Seela et al., 1987) for the synthesis of *t*ZdR (**3**).



Scheme S3: Multi-step reaction scheme (Mitsunobu, 1981; Okabe et al., 1988; Seio et al., 2017) for *α*-*t*ZddR (**4a**) and *β*-*t*ZddR (**4b)**

Chemical procedures

Material and methods

The reagents, substances and solvents were obtained from commercial suppliers (Sigma Aldrich®, TCI Chemicals®, VWR®, Jena Bioscience® or OlChemIm®). The organic solvents were evaporated on a rotary evaporator (Heidolph®) below 45 °C.

The reaction processes were controlled by thin layer chromatography (TLC) using silica gel 60 WF_{245} plates (Merck®). Toluene/acetone; (1:1, v/v), hexane/ethyl acetate (1:1, v/v), $CHCl_3$/MeOH 9:1 or $CHCl_3$/MeOH/NH_3 (9:1:0.05, v/v) were used as the mobile phase. Silica gel (Davisil R LC60A 40-63 μm) was used for column chromatography purification. The chromatographic purity of the derivatives was determined using a HPLC-PDA-MS assembly. An Alliance 2695 Separations Module (Waters®) linked simultaneously to the UV-VIS detector PDA 2996 (Waters®,) and QDa Mass Spectrometer (Waters®) with ESI system was used. Samples (10 μl), dissolved in MeOH and diluted to a concentration of 10 μg $\cdot ml^{-1}$ in the mobile phase (initial conditions), were injected onto a RP-column (150 × 2.1 mm; 3.5 μm; Symmetry C18, Waters®). The column was kept in a thermostat at 25 °C. Solvent (A) consisted of 15 mM formic acid adjusted to pH 4.0 by ammonium hydroxide. MeOH was used as the organic modifier (solvent B). A flow rate of 0.2 ml × min^{-1} and the following binary gradient were used: 0 min, 90% A; 0 – 25 min, linear gradient to 10% A, followed by 10 min isocratic elution of 10% A. At the end of the gradient, the column was reequilibrated to the initial conditions for 10 min. The effluent was introduced into the DAD (diode array detector; scanning range 210-400 nm, with 1.2 nm resolution) equipped with an electrospray source (source temperature: 120 °C for positive mode, capillary voltage +3.0 kV, cone voltage +15 V, desolvation temperature 300 °C. Nitrogen was used as both the desolvation gas (500 l $\cdot h^{-1}$) and cone gas (50 l $\cdot h^{-1}$). The mass spectrometer was operated in positive (ESI+) ionization mode. Data were acquired in the range of 50-1000 *m/z* range (FULLSCAN mode).

Preparative HPLC-MS chromatography (Agilent 1290 Infinity II) coupled to UV-VIS and a mass detector (Agilent InfinityLab LC/MSD) with an Agilent column (5Prep-C18 5x21.2 mm) was used to separate the final products. The samples *c*ZddR (**6**), iPddR (**2**) were dissolved in 50% MeOH. Mobile phase (MeOH A/H_2O B) with flow rate 20 ml/min and following linear gradients was used: 0 min (90% A, 10% B); 10 min (10% A, 90% B); *c*ZdR (**5**): 0 min (90% A, 10% B); 10 min (40% A, 60% B); *t*ZdR (**3**): 0 min (80% A, 20% B); 6,5 min (40% A, 60% B); iPdR (**1**): 0 min (80% A, 20% B); 5,5 min (10% A, 90% B). Exact mass was determined by QTOF-MS (Synapt G2-Si, Waters®, UK) operating in positive ion mode.

The compound (**I**) was confirmed using Shimadzu® GC-MS QP2010 Plus chromatograph equipped with capillary column DB5-MS (Agilent® Technologies; 20 m, 0.18 mm, 25 μm), splitless injection (250°C) and He as a carrier gas.

NMR spectra were measured with a Jeol 500 SS spectrometer operating at 300 K and 500 MHz (^{1}H NMR) or 126 MHz (^{13}C NMR). DMSO-d_6 or $CDCl_3$ were used as the solvent. (NMR spectra were calibrated to a residual solvent signal). Chemical shifts (δ, ppm) were referenced from tetramethylsilane (TMS) and the following abbreviations were used: s, singlet; bs, broad singlet; d, doublet; dd, doublet of doublet; ddd, doublet of doublet of doublets; t, triplet; tt, triplet of triplets; dt, doublet of triplets; td, triplet of doublets; dtd, doublet of triplet of doublets; m, multiplet; bs, broad singlet.

N^6-(2-isopentenyl)adenine-9-(β)-D-2′-deoxyriboside (**1**, *iPdR*) (Leonard et al., 1969; Wan et al., 2007, 2005)

2′-Deoxyinosine (2′-deoxy-9-(*β*)-D-inosine; *M*r 252.23; 0.4 mmol; 100 mg) and BOP ((benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; 0.5 mmol; 210 mg) were dissolved in dry DMF (2 ml) under inert atmosphere (N_2) with DIPEA (1.98 mmol; 345 μl) and after 5 min (3-methylbut-2-en-1-yl)amine hydrochloride (Mr 121.61; 97 mg) was added. The reaction was stirred at rt for 24 h under N_2 and monitored by TLC

($CHCl_3$/MeOH/NH_3 9:1:0.05; v/v). The mixture was then evaporated (and co-evaporated with *n*-heptane) and without extraction (due to partial solubility in water) pre-purified by column chromatography ($CHCl_3$/MeOH/NH_3 9:1:0.05; v/v) to give the product with approx. 70% HPLC-UV purity followed with preparative HPLC-MS chromatography to give a white solid wax: *M*r 319.37; 38 mg; yield 30 %; HPLC-UV purity >99 %.

^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 1.66 (s, 3H), 1.69 (s, 3H), 2.25 (ddd, *J* = 13.1, 6.1, 2.8 Hz, 1H), 2.72 (ddd, *J* = 13.4, 7.8, 5.8 Hz, 1H), 3.51 (ddd, *J* = 12.0, 6.7, 4.2 Hz, 1H), 3.62 (dt, *J* = 11.8, 4.6 Hz, 1H), 3.88 (td, *J* = 4.2, 2.7 Hz, 2H), 4.05 (s, 2H), 4.41 (dtd, *J* = 6.0, 3.0, 2.8 Hz, 1H), 5.26 (dd, *J* = 6.7, 4.9 Hz, 1H), 5.25 – 5.34 (m, 1H), 5.32 (d, *J* = 4.0 Hz, 1H), 6.34 (dd, *J* = 7.8, 6.2 Hz, 1H), 7.92 (s, 1H), 8.20 (s, 1H), 8.33 (s, 1H).

^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm): 17.9, 25.4, 37.7, 39.4, 61.9, 71.0, 84.0, 88.0, 119.7, 122.1, 133.2, 139.4, 148.1, 152.4, 154.3.

N^6-(2-isopentenyl)adenine-9-(β)-D-2′,3′-dideoxyriboside (**2**, *iPddR*) (Koszalka et al., 1989; Wan et al., 2007, 2005)

2′,3′-Dideoxyinosine (2′,3′-dideoxy-9-(*β*)-D-inosine; *M*r 236.23; 0.4 mmol; 100 mg) and BOP ((benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; 0.76 mmol; 337 mg) were dissolved in dry DMF (2 ml) under inert atmosphere (N_2) with DIPEA (3.8 mmol; 652 µl) and after 5 min (3-methylbut-2-en-1-yl)amine hydrochloride (1.6 mmol; 193 mg) was added. The reaction was stirred at 50 °C for 24 h under N_2 and controlled by TLC ($CHCl_3$/MeOH/NH_3 9:1:0.05; v/v). The mixture was then evaporated (and co-evaporated with *n*-heptane) and without extraction pre-purified by column chromatography ($CHCl_3$/MeOH/NH_3 9:1:0.05; v/v) to give approx. 70% HPLC-UV purity of the product which was then purified by preparative HPLC-MS chromatography to give a pale yellow wax: *M*r 303.37; 56 mg; yield 44 %; HPLC-UV purity >98 %.

^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 1.66 (s, 3H), 1.69 (s, 3H), 1.98 – 2.13 (m, 2H), 2.37 – 2.45 (m, 2H), 3.50 (dt, *J* = 12.0, 4.7 Hz, 1H), 3.62 (dt, *J* = 11.5, 4.1 Hz, 1H), 4.05 (s, 2H), 4.11 (tt, *J* = 7.0, 4.2 Hz, 1H), 5.07 (t, *J* = 5.2 Hz, 1H), 5.28 – 5.32 (m, 1H), 6.22 (dd, *J* = 6.0, 4.6 Hz, 1H), 7.85 (s, 1H), 8.20 (s, 1H), 8.34 (s, 1H).

^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm): 17.9, 25.4, 25.7, 31.8, 37.7, 62.9, 81.7, 84.4, 119.5, 122.2, 133.1, 138.8, 148.0, 152.4, 154.2.

Trans-zeatin-9-(β)-D-2′-deoxyriboside (**3**, *tZdR*) (Evidente et al., 1989; Fujii et al., 1989; Itaya et al., 1986; Napoli et al., 1990)

The starting 6-chloropurine-2′-deoxy-9-(*β*)-D-riboside was prepared according to the method described in our previous article or literature (Francom and Robins, 2003; Seela et al., 1987). 6-Chloropurine-2′-deoxy-9-(*β*)-D-riboside (Mr 270.67; 0.4 mmol; 100 mg) was dissolved with *n*-propanol (4 ml) in a reaction ampoule and (*2E*)-2-methyl-4-aminobut-2-en-1-ol hydrochloride (0.4 mmol; 61 mg) were added followed by DIPEA (1.1 mmol; 190 µl). The reaction was stirred 5 h at 90 °C and controlled by TLC ($CHCl_3$/MeOH/NH_3 9:1:0.05; v/v). After completion of the nucleophilic substitution, the reaction mixture was evaporated and purified by preparative HPLC-MS chromatography to give a pale yellow oil: *M*r 335.36; 71 mg; yield 57 %; HPLC-UV purity >99 %.

^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 1.65 (s, 3H), 2.25 (ddd, *J* = 13.1, 6.1, 3.0 Hz, 1H), 2.72 (ddd, *J* = 13.1, 7.6, 5.5 Hz, 1H), 3.51 (ddd, *J* = 11.6, 6.7, 4.2 Hz, 1H), 3.62 (dt, *J* = 11.6, 4.6 Hz, 1H), 3.77 (d, *J* = 5.4 Hz, 3H), 3.88 (td, *J* = 4.2, 2.6 Hz, 1H), 4.10 (s, 1H), 4.41 (dtd, *J* = 5.8, 3.0, 2.8 Hz, 1H), 4.75 (t, *J* = 5.7 Hz, 1H), 5.26 (dd, *J* = 6.6, 4.9 Hz, 1H), 5.32 (d, *J* = 4.0 Hz, 1H), 5.51 (t, *J* = 5.9 Hz, 1H), 6.34 (dd, *J* = 7.8, 6.2 Hz, 1H), 7.97 (s, 1H), 8.21 (s, 1H), 8.33 (s, 1H).

^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm): 13.7, 37.3, 61.9, 65.9, 71.0, 84.0, 88.1, 119.8, 120.7, 137.3, 139.4, 148.1, 152.4, 154.3.

Multistep synthesis of *trans*-zeatin-9-(*α*)-D-2′,3′-dideoxyriboside (**4a**, *α-t*ZddR):

(S)-γ-Butyrolactone-γ-carboxylic acid (**I**)

According to the literature (Okabe et al., 1988), L-glutamic acid (1.0 mol; 147 g) was dissolved in H_2O (500 ml), cooled in ice-bath to 15 °C and solution of 5.6 M HCl (1.4 mol; 250 ml) was added dropwise to adjust pH 1. Then, the solution of $NaNO_2$ (1.5 mol; 104 g) in H_2O (144 ml) was added during 4h at 15-20 °C and the reaction mixture was stirred at rt overnight. Next day, the reaction mixture was controlled by TLC (toluene/acetone; 1:1; ninhydrin staining) which demonstrated full conversion of L-glutamic acid. The reaction mixture was co-evaporated with toluene and the white oil-solid residue (243 g) was diluted with EtOAc (1 L) and dried over powdered Na_2SO_4 (100 g). The precipitates were removed by filtration and washed by EtOAc. The combined organic extracts were stirred with 200-400 mesh H^+ form (25 g) for 30 min to remove residual amino acid. After filtration and evaporation, the yellow oily residue was dried by evaporation with toluene (4 x 100 ml). The waxy residue (116 g) was dissolved in Et_2O (200 ml) (DCM used in the literature did not lead to a crystallization process), stirred at rt for 1h and cooled in the refrigerator overnight. The precipitate was filtered to give a white solid: *M*r 130.10; 91 g; yield 70 %. The structure was confirmed by elemental analysis, NMR (500 Hz; $CDCl_3$), GC-MS and melting point (62.6-63.2 °C).

(S)-γ-(Hydroxymethyl)-γ-butyrolactone (Okabe et al., 1988) (**II**)

Borane-dimethylsulfide complex (Corey et al., 1987) (BMS; 0.15 mol; 12.23 ml) was added in to (*S*)-*γ*-butyrolactone-*γ*-carboxylic acid (**I**, *M*r 130.10; 0.115 mol; 15 g) in dry THF (70 ml) during 40 min under 40°C (ice bath). The reaction was stirred for 2h at rt and quenched by MeOH (25 ml) while cooling. MeOH was evaporated 3 times (to remove formed trimethylborate) to give a light yellow oil: *M*r 116.12; 13.7 g; yield 102 %. The reaction was monitored by TLC (toluene/acetone; 1:1, v/v). The structure was confirmed by NMR analysis.

(S)-γ-[[(Tert-butyldimethylsilyl)oxy]methyl]-γ-butyrolactone (Okabe et al., 1988) (**III**)

(*S*)-*γ*-(hydroxymethyl)-*γ*-butyrolactone (**II**, *M*r 116.12; 0.11 mol; 13 g) and imidazole (0.14 mol; 9.7 g) in dry DCM (55 ml) were cooled to 0 °C under inert N_2 atmosphere. TBDMS-Cl (0.13 mol; 20 g) was added and the mixture was stirred 15 min at 0 °C and then 2h at rt. The solution was poured into cold H_2O (1L) and 300 ml of DCM was added, and the water layer was washed with DCM (2 x 100 ml). Combined organic layers were washed with H_2O (2 x 400 ml) and brine (200 ml), dried with Na_2SO_4 overnight and evaporated to give light yellow oil: *M*r 230.38; 26 g; yield 100 %. TLC hexane/EtOAc 3:1, stained by 5% solution of H_2SO_4 in EtOH. The structure was confirmed by NMR analysis.

(S)-γ-[[(Tert-butyldimethylsilyl)oxy]methyl]-γ-butyrolactol (Okabe et al., 1988) (**IV**)

DIBAL in THF (we used THF instead of toluene as a solvent used in the literature(Okabe et al., 1988)) was slowly added during 1 h to (*S*)-*γ*-[[(tert-butyldimethylsilyl)oxy]methyl]-*γ*-butyrolactone (**III**, *M*r 230.38; 5 g) in THF under inert N_2 atmosphere, cooled by dry ice and acetone to -74 °C. The temperature was kept at -68 °C. After stirring for 5 min and TLC control (hexane/EtOAc 3:1) the reaction mixture was quenched by MeOH (5 ml) and the temperature was left to raise to rt. EtOAc (85 ml) and saturated $NaHCO_3$ (4.5 ml) were added and the mixture was stirred 3.5 h. Then Na_2SO_4 (25 g) was added and the mixture was stirred overnight. The solution was filtered, washed with EtOAc and the filtrate concentrated to give a colorless oil residue: *M*r 232.40; 5 g; yield 99 %. The structure was confirmed by NMR analysis.

6-Chloro-9-((5S)-5-(((isopropyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-9H-purine; *6-Cl-ddPR-OTBDMS* (Mitsunobu reaction) (Mitsunobu, 1981; Seio et al., 2017) (**V**)

The solution of (*S*)-γ-[[(tert-butyldimethylsilyl)oxy]methyl]-γ-butyrolactol (**IV**, *M*r 232.40; 11.7 mmol; 2.71 g) with 6-chloropurine (9.7 mmol; 1.5 g) in THF or DMF (0.07 M solution of 6-chloropurine obtained with 140 ml of THF and 10 ml of DMF due to the worse solubility of 6-chloropurine in THF) under inert N_2 atmosphere was cooled to 15 °C (ice bath). Then triphenylphosphine (14.5 mmol; 3.8 g) and DIAD (14.7 mmol; 2.9 ml) were added and the reaction was stirred for 1h (change of colour from orange, yellow and green to brown-green at the end was observed) and monitored by TLC (hexane/EtOAc, 1:1, v/v). The reaction mixture was evaporated and co-evaporated with toluene and diluted with toluene (30 ml) to give a brown oil which was cooled overnight to precipitate $POPh_3$ (white solid). After filtration and washing with toluene, the crystallization process was repeated and the crude product (anomeric mixture) was roughly purified by column chromatography (hexane/EtOAc 1:1) to give 2 g of thick to solid yellow oil (a mixture of *α* and *β* anomer in favour of one anomer, according to TLC). The HPLC analysis with acidic mobile phase (pH 4) and the cone voltage 20 eV led to molecule breakage and 6-chloropurine was observed in high percentage even if TLC analysis showed no visible amount of 6-chloropurine. Therefore, the conditions for HPLC analysis were adjusted to pH 5.3 of the mobile phase and the cone voltage set to 10 eV to lower the molecule breakage throughout the analysis. There were several attempts to purify each anomer by column chromatography, but due to the close Rf the separation was unsuccessful. Elemental analysis of anomeric mixture was proceeded: calculated % N(15.19), C(52.09), H(6.83); the first measurement N(14.88), C(51.77), H(7.51) and the second measurement N(14.90), C(51.46), H(7.57) and melting temperature range 77.4 – 77.9 °C. Preparative HPLC-MS was proved to be the best tool for the separation of individual anomers, but we decided to firstly nucleophilically substitute the position 6-Cl.

(E)-4-((9-((5S)-5-(((Isopropyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-9H-purin-6-yl)amino)-2-methylbut-2-en-1-ol; *tZ-ddPR-OTBDMS* (**VI**)

6-Chloro-9-((5*S*)-5-(((isopropyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-9*H*-purine (**V**, *M*r 368.94; 0.9 mmol; 334 mg) and (*2E*)-2-methyl-4-aminobut-2-en-1-ol hydrochloride (Mr 137.61 g/mol; 1.4 mmol; 187 mg) were stirred for 5 min in *n*-propanol (6 ml) under inert N_2 atmosphere. DIPEA (2.7 mmol; 465 µl) was then added and the mixture was refluxed in a sealed tube and stirred for 5 h. The reaction was monitored by TLC (EtOAc/hexane; 6:1, v/v) and upon completion, the mixture was evaporated to give a yellow oil: *M*r 433.63; 393 mg. Due to the close Rf of each anomers, the crude residue was used in the next deprotection without further purification.

Trans-zeatin-9-(α)-D-2′,3′-dideoxyriboside (**4a**, *α-t*ZddR)

The anomeric mixture of the protected product (**VI**, *M*r 433.63; 0.9 mmol; 393 mg) was dissolved in dry THF (20 ml) and while cooling, TBAF trihydrate (0.9 mmol; 286 mg) in THF (10 ml) was slowly added, the mixture was stirred at rt for 2.5 h and controlled by TLC (hexane/EtOAc 1:1 or chloroform/MeOH 9:1). The deprotected anomeric mixture was evaporated and purified by column chromatography (approximately 3:1; 93 % HPLC-UV) and the anomer with the greater yield was successfully separated by preparative HPLC-MS (Mr 319.37, to give a pale yellow oil, 165 mg; yield 57 % of the last step; HPLC-UV purity >99 %). The structure was characterized by HRMS, and NMR. The 2D-NMR experiment NOESY found that the structure probably corresponds to the *α* anomer due to the presence of –CH2-OH group of the ribose moiety as well as the purine scaffold in the *cis* position towards the planar sugar ring.

^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 1.66 (s, 3H), 1.80 – 1.91 (m, 1H), 2.20 – 2.31 (m, 1H), 2.41 – 2.49 (m, 2H), 3.41 (dt, *J* = 11.6, 4.9 Hz, 1H), 3.46 (dt, *J* = 11.6, 4.5 Hz, 1H), 3.78 (d, *J* = 4.5 Hz, 2H), 4.11 (s, 2H), 4.38 (tt, *J* = 6.8, 4.7 Hz, 1H), 4.74 (t, *J* = 5.6 Hz, 1H), 4.80 (t, *J* = 5.5 Hz, 1H), 5.51 (t, *J* = 6.1 Hz, 1H), 6.29 (dd, *J* = 6.4, 4.7 Hz, 1H), 7.88 (s, 1H), 8.22 (s, 1H), 8.27 (s, 1H).

^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm): 13.7, 26.4, 31.0, 37.2, 63.3, 65.9, 81.1, 84.5, 119.7, 120.8, 137.2, 139.0, 148.2, 152.5, 154.3.

Trans-zeatin-9-(β)-D-2′,3′-dideoxyriboside (**4b**, *β-t*ZddR)

2′,3′-Dideoxyinosine (2′,3′-dideoxy-9-(*β*)-D-inosine; didanosine) (Mr 236.23; 0.4 mmol; 100 mg) and BOP ((benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (0.5 mmol; 225 mg) were dissolved in dry DMF (4 ml) under inert atmosphere (N_2) and DIPEA (2.4 mmol; 0.4 ml) was added, followed with (*2E*)-2-methyl-4-aminobut-2-en-1-ol.HCl (0.8 mmol; 110 mg) after 5 min and the reaction mixture was stirred overnight under N_2 at 50 °C. The reaction was controlled by TLC using $CHCl_3$/MeOH/NH_3 (9:1:0.05; v/v). The mixture was then evaporated (and co-evaporated with *n*-heptane) and pre-purified without extraction by column chromatography ($CHCl_3$/MeOH/NH_3 9:1:0.05; v/v) to give the product with approximately 60% HPLC-UV purity. The final product was then purified by preparative HPLC-MS chromatography to give yellow oil: *M*r 319.37; 27 mg; yield 20 %; HPLC-UV purity >97 %.

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.66 (s, 3H), 1.94 – 2.15 (m, 2H, 20), 2.34 – 2.43 (m, 2H, 21), 3.50 (dt, *J* = 11.6, 4.8 Hz, 1H, 22'), 3.62 (dt, *J* = 11.6, 4.8 Hz, 1H, 22''), 3.77 (d, *J* = 5.3 Hz, 2H, 15), 4.11 (m, 3H, 12, 19), 4.74 (t, *J* = 5.6 Hz, 1H, 17), 5.06 (t, *J* = 5.5 Hz, 1H, 23), 5.51 (t, *J* = 6.3 Hz, 1H, 13), 6.22 (t, *J* = 5.3 Hz, 1H, 11), 7.90 (s, 1H, 10), 8.20 (s, 1H, 2), 8.35 (s, 1H, 8).

^{13}C NMR (101 MHz, DMSO-D_6) δ (ppm): 13.7 (16), 25.7 (20), 31.8 (21), 37.3 (12), 62.9 (22), 65.9 (15), 81.7 (19), 84.4 (11), 119.6, 120.8 (13), 137.2, 138.8 (8), 148.0 (4), 152.4 (2), 154.2 (6).

Cis-zeatin-9-(β)-D-2′-deoxyriboside (**5**, *c*ZdR) (Greco et al., 1992; Wan et al., 2007, 2005)

2′-Deoxyinosine (2′-deoxy-9-(*β*)-D-inosine; *M*r 252.23; 0.4 mmol; 100 mg) and BOP ((benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate) (0.47 mmol; 210 mg) were dissolved in dry DMF (4 ml) under inert atmosphere (N_2). DIPEA (3.1 mmol; 0.54 m l) was added, followed with (*2Z*)-2-methyl-4-aminobut-2-en-1-ol hemioxalate (Mr 262.26; 0.4 mmol; 105 mg) after 5 min and the reaction mixture was stirred for 24 h under N_2 at 50 °C. The reaction was controlled by TLC using $CHCl_3$/MeOH/NH_3 (9:1:0.05; v/v). The mixture was then evaporated (and co-evaporated with *n*-heptane) and pre-purified without extraction by column chromatography ($CHCl_3$/MeOH/NH_3 9:1:0.05; v/v) to give the product with approximately 70% HPLC-UV purity. The final product was purified by preparative HPLC-MS chromatography to give a yellow wax: *M*r 335.36; 36 mg; yield 26 %; HPLC-UV purity >99 %.

^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 1.69 (s, 3H, 14), 2.25 (ddd, *J* = 13.1, 6.1, 2.8 Hz, 1H), 2.72 (ddd, *J* = 13.3, 7.8, 5.8 Hz, 1H), 3.51 (dt, *J* = 11.3, 4.9 Hz, 1H), 3.62 (dt, *J* = 11.5, 3.7 Hz, 1H), 3.88 (td, *J* = 4.2, 2.7 Hz, 1H), 4.03 (d, *J* = 3.7 Hz, 2H), 4.11 (s, 2H), 4.41 (bs, 1H), 4.72 (bsf, 1H), 5.24 (t, *J* = 5.0 Hz, 1H), 5.33 (m, 2H, 12), 6.34 (dd, *J* = 7.7, 6.2 Hz, 1H), 7.88 (s, 1H), 8.20 (s, 1H), 8.33 (s, 1H).

^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm): 21.2, 37.0, 39.4 (overlapped with DMSO-d_5), 59.8, 61.9, 71.0, 84.0, 88.0, 119.8, 123.1, 129.1, 137.8, 139.4, 148.1, 152.3, 154.2.

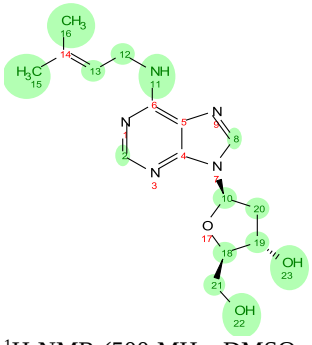
Cis-zeatin-9-(β)-D-2′, 3′-dideoxyriboside (**6**, *c*ZddR) (Wan et al., 2007, 2005)

2′,3′-Dideoxyinosine (2′,3′-dideoxy-9-(*β*)-D-inosine; *M*r 236.23; 0.6 mmol; 150 mg) and BOP ((benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; 0.8 mmol; 337 mg) were dissolved in dry DMF (5 ml) under inert N_2 atmosphere. DIPEA (5 mmol; 870 µl) was added, followed with (*2Z*)-2-methyl-4-aminobut-2-en-1-ol hemioxalate (0.8 mmol; 200 mg) after 5 min and the reaction mixture was stirred for 24 h under N_2 at 50 °C. The reaction was controlled by TLC using $CHCl_3$/MeOH/NH_3 9:1:0.05; v/v. The mixture was then evaporated (and co-evaporated with *n*-heptane) and pre-purified without extraction by column chromatography ($CHCl_3$/MeOH/NH_3 9:1:0.05; v/v) to give the product with approximately 70% HPLC-UV purity. The final product was then purified by preparative HPLC-MS chromatography to give a light yellow wax: *M*r 319.37; 47 mg; yield 23 %; HPLC-UV purity >99 %.

^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 1.69 (d, J = 1.5 Hz, 3H), 1.99 – 2.12 (m, 2H), 2.38 – 2.44 (m, 2H), 3.50 (ddd, J = 11.7, 5.8, 4.6 Hz, 1H), 3.62 (ddd, J = 11.7, 5.3, 4.0 Hz, 1H), 4.03 (d, J = 5.6 Hz, 2H), 4.11 (tt, J = 8.0, 4.1 Hz, 3H), 4.72 (t, J = 5.5 Hz, 1H), 5.05 (t, J = 5.6 Hz, 1H), 5.33 (t, J = 6.5 Hz, 1H), 6.22 (dd, J = 6.1, 4.4 Hz, 1H), 7.82 (s, 1H), 8.20 (s, 1H), 8.35 (s, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm): 21.2, 25.7, 31.8, 36.9, 59.8, 62.9, 81.7, 84.4, 119.5, 123.2, 137.7, 138.9, 148.0, 152.3, 154.1.

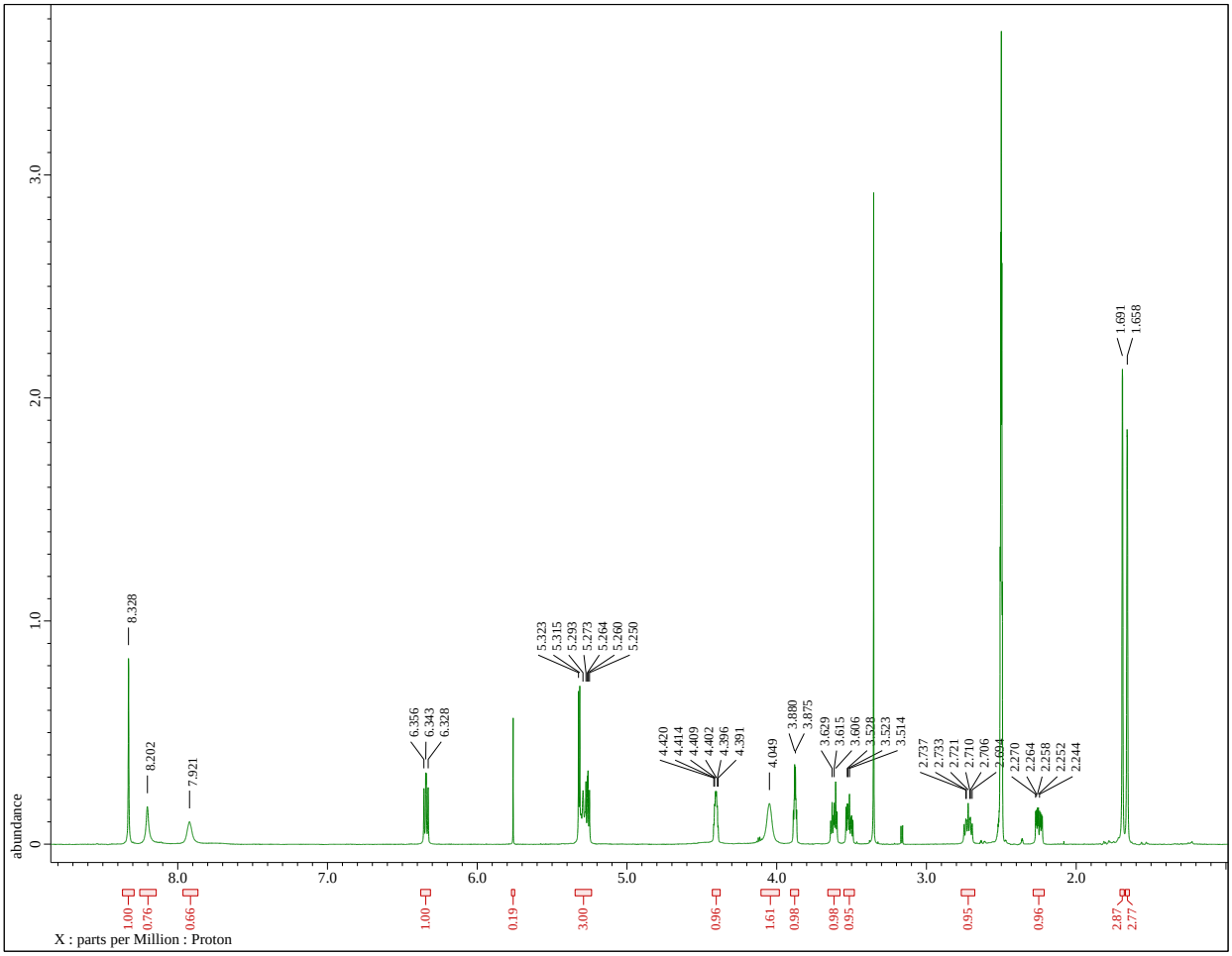
NMR data:

***N*^{6}-(2-isopentenyl)adenine-9-(*β*)-D-2′-deoxyriboside (1, iPdR)**

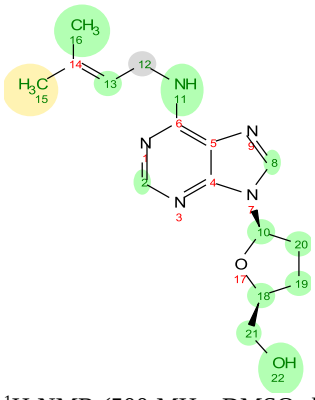


^{1}H NMR (500 MHz, DMSO-d_6) δ 1.66 (s, 3H, 15 or 16), 1.69 (s, 3H, 15 or 16), 2.25 (ddd, *J* = 13.1, 6.1, 2.8 Hz, 1H, 20''), 2.72 (ddd, *J* = 13.4, 7.8, 5.8 Hz, 1H, 20'), 3.51 (ddd, *J* = 12.0, 6.7, 4.2 Hz, 1H, 21''), 3.62 (dt, *J* = 11.8, 4.6 Hz, 1H, 21'), 3.88 (td, *J* = 4.2, 2.7 Hz, 2H, 18), 4.05 (s, 2H, 12''), 4.41 (dtd, *J* = 6.0, 3.0, 2.8 Hz, 1H, 19), 5.26 (dd, *J* = 6.7, 4.9 Hz, 1H, 22), 5.25 – 5.34 (m, 1H, 13), 5.32 (d, *J* = 4.0 Hz, 1H, 23), 6.34 (dd, *J* = 7.8, 6.2 Hz, 1H, 10), 7.92 (s, 1H, 11), 8.20 (s, 1H, 2), 8.33 (s, 1H, 8).

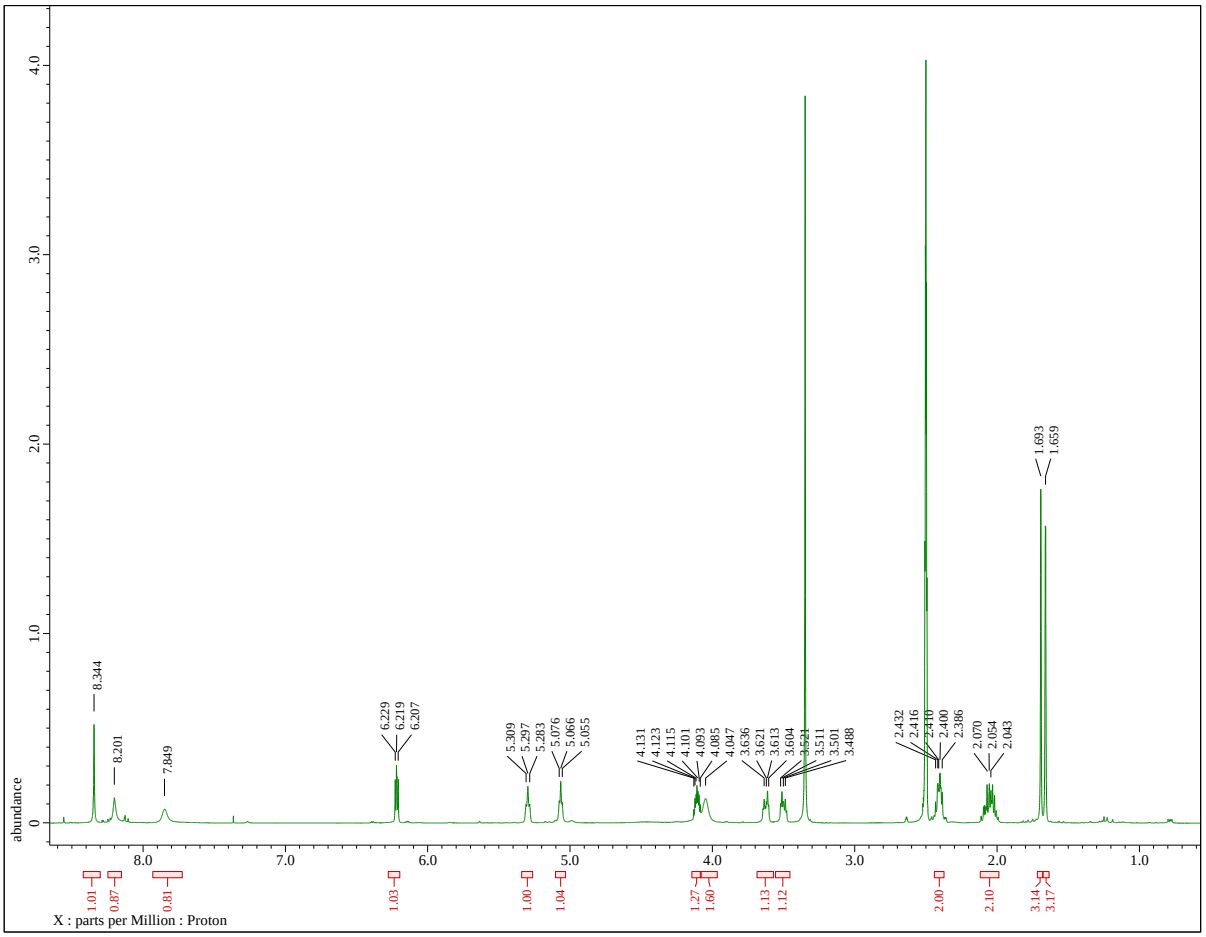
^{13}C NMR (126 MHz, DMSO-d_6) δ 17.9 (15 or 16), 25.4 (15 or 16), 37.7 (12), 39.4 (20), 61.9 (21), 71.0 (19), 84.0 (10), 88.0 (18), 119.7 (5), 122.1 (13), 133.2 (14), 139.4 (8), 148.1 (4), 152.4 (2), 154.3 (6).



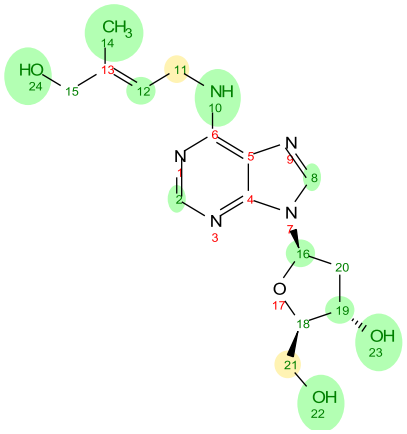
***N*^{6}-(2-isopentenyl)adenine-9-(*β*)-D-2′,3′-dideoxyriboside** (**2**, **iPddR**)



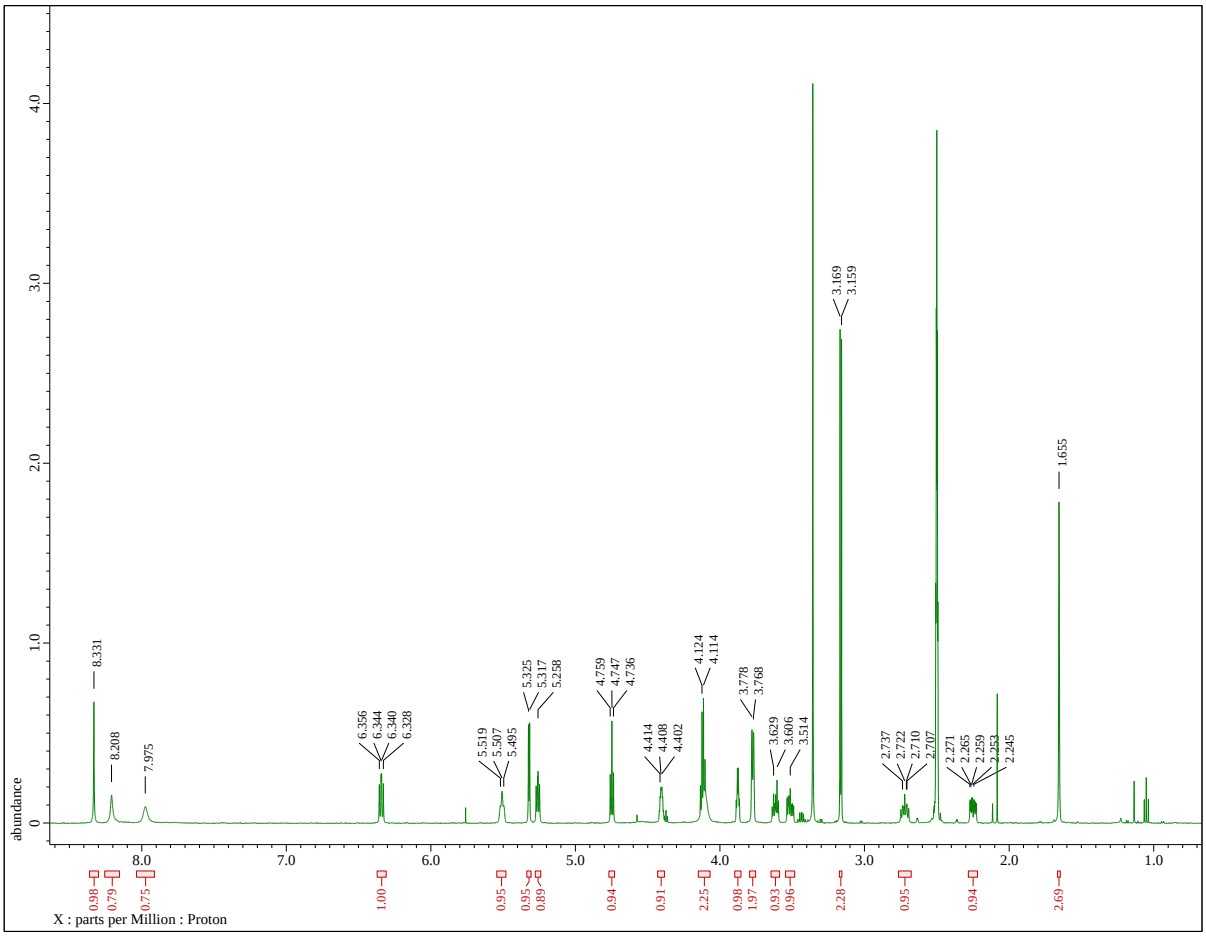
^{1}H NMR (500 MHz, DMSO-d_6) δ 1.66 (s, 3H, 15 or 16), 1.69 (s, 3H, 15 or 16), 1.98 – 2.13 (m, 2H, 19''), 2.37 – 2.45 (m, 2H, 20''), 3.50 (dt, *J* = 12.0, 4.7 Hz, 1H, 21'), 3.62 (dt, *J* = 11.5, 4.1 Hz, 1H, 21''), 4.05 (s, 2H, 12'), 4.11 (tt, *J* = 7.0, 4.2 Hz, 1H, 18), 5.07 (t, *J* = 5.2 Hz, 1H, 22), 5.28 – 5.32 (m, 1H, 13), 6.22 (dd, *J* = 6.0, 4.6 Hz, 1H, 10), 7.85 (s, 1H, 11), 8.20 (s, 1H, 2), 8.34 (s, 1H, 8).
^{13}C NMR (126 MHz, DMSO-d_6) δ 17.9 (15 or 16), 25.4 (15 or 16), 25.7 (19), 31.8 (20), 37.7 (12), 62.9 (21), 81.7 (18), 84.4 (10), 119.5 (5), 122.2 (13), 133.1 (14), 138.8 (8), 148.0 (4), 152.4 (2), 154.2 (6).



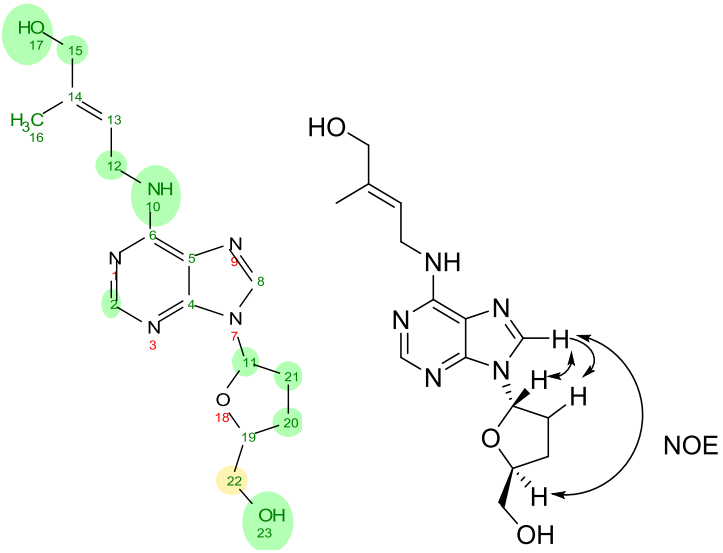
***Trans*-zeatin-9-(*β*)-D-2´-deoxyriboside (3, *t*ZdR)**



^{1}H NMR (500 MHz, DMSO-d_6) δ 1.65 (s, 3H, 14), 2.25 (ddd, *J* = 13.1, 6.1, 3.0 Hz, 1H, 20''), 2.72 (ddd, *J* = 13.1, 7.6, 5.5 Hz, 1H, 20'), 3.51 (ddd, *J* = 11.6, 6.7, 4.2 Hz, 1H, 21'), 3.62 (dt, *J* = 11.6, 4.6 Hz, 1H, 21''), 3.77 (d, *J* = 5.4 Hz, 3H, 15'), 3.88 (td, *J* = 4.2, 2.6 Hz, 1H, 18), 4.10 (s, 1H, 11''), 4.41 (dtd, *J* = 5.8, 3.0, 2.8 Hz, 1H, 19), 4.75 (t, *J* = 5.7 Hz, 1H, 24), 5.26 (dd, *J* = 6.6, 4.9 Hz, 1H, 22), 5.32 (d, *J* = 4.0 Hz, 1H, 23), 5.51 (t, *J* = 5.9 Hz, 1H, 12), 6.34 (dd, *J* = 7.8, 6.2 Hz, 1H, 16), 7.97 (s, 1H, 10), 8.21 (s, 1H, 2), 8.33 (s, 1H, 8).
^{13}C NMR (126 MHz, DMSO-d_6) δ 13.7 (14), 37.3 (11), 61.9 (21), 65.9 (15), 71.0 (19), 84.0 (16), 88.1 (18), 119.8 (5), 120.7 (12), 137.3 (13), 139.4 (8), 148.1 (4), 152.4 (2), 154.3 (6).

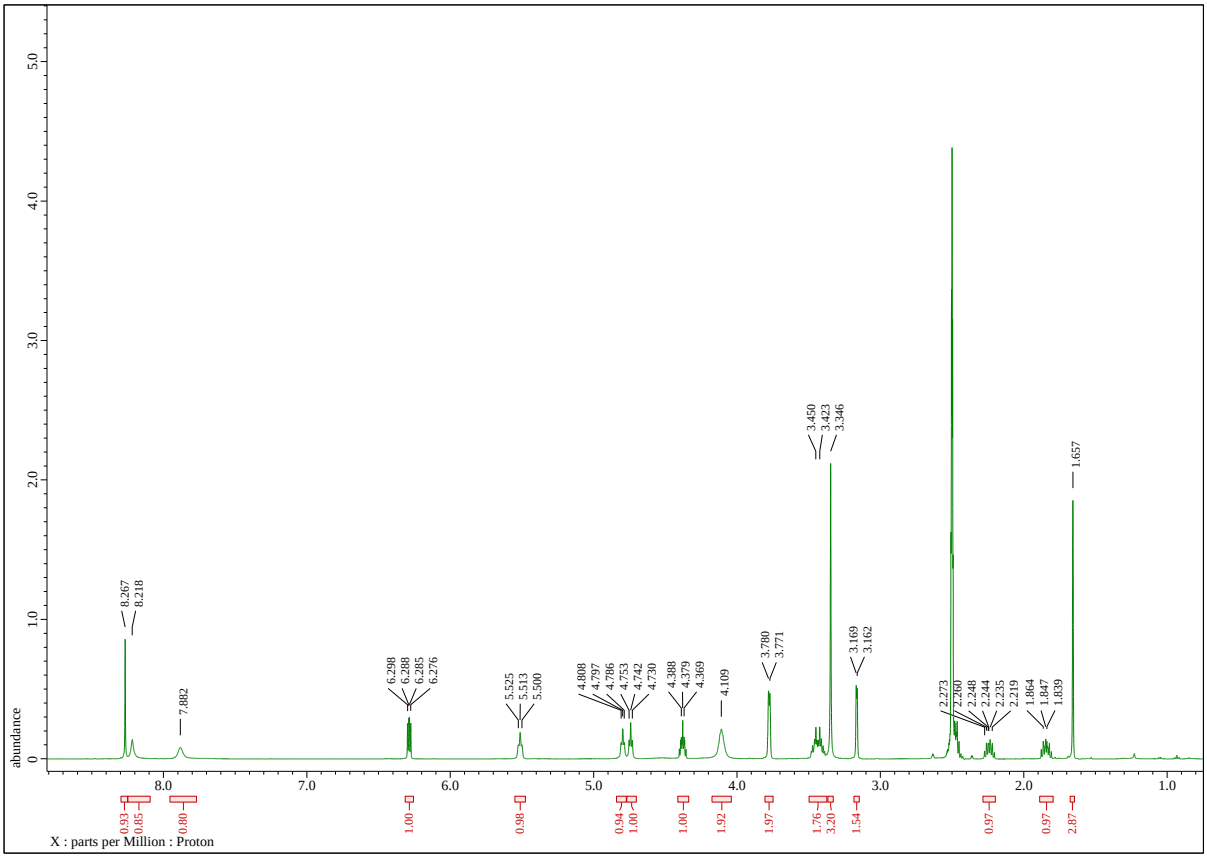


***Trans*-zeatin-9-(*α*)-D-2′,3′-dideoxyriboside (4a, *α*-*t*ZddR)**



^{1}H NMR (500 MHz, DMSO-d_6) δ 1.66 (s, 3H, 16), 1.80 – 1.91 (m, 1H, 20''), 2.20 – 2.31 (m, 1H, 20'), 2.41 – 2.49 (m, 2H, 21), 3.41 (dt, *J* = 11.6, 4.9 Hz, 1H, 22''), 3.46 (dt, *J* = 11.6, 4.5 Hz, 1H, 22'), 3.78 (d, *J* = 4.5 Hz, 2H, 15), 4.11 (s, 2H, 12), 4.38 (tt, *J* = 6.8, 4.7 Hz, 1H, 19), 4.74 (t, *J* = 5.6 Hz, 1H, 17), 4.80 (t, *J* = 5.5 Hz, 1H, 23), 5.51 (t, *J* = 6.1 Hz, 1H, 13), 6.29 (dd, *J* = 6.4, 4.7 Hz, 1H, 11), 7.88 (s, 1H, 10), 8.22 (s, 1H, 2), 8.27 (s, 1H, 8).

^{13}C NMR (126 MHz, DMSO-d_6) δ 13.7 (16), 26.4 (20), 31.0 (21), 37.2 (12), 63.3 (22), 65.9 (15), 81.1 (19), 84.5 (11), 119.7 (5), 120.8 (13), 137.2 (14), 139.0 (8), 148.2 (4), 152.5 (2), 154.3 (6).



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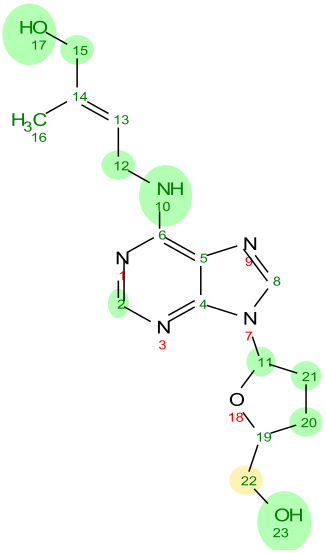
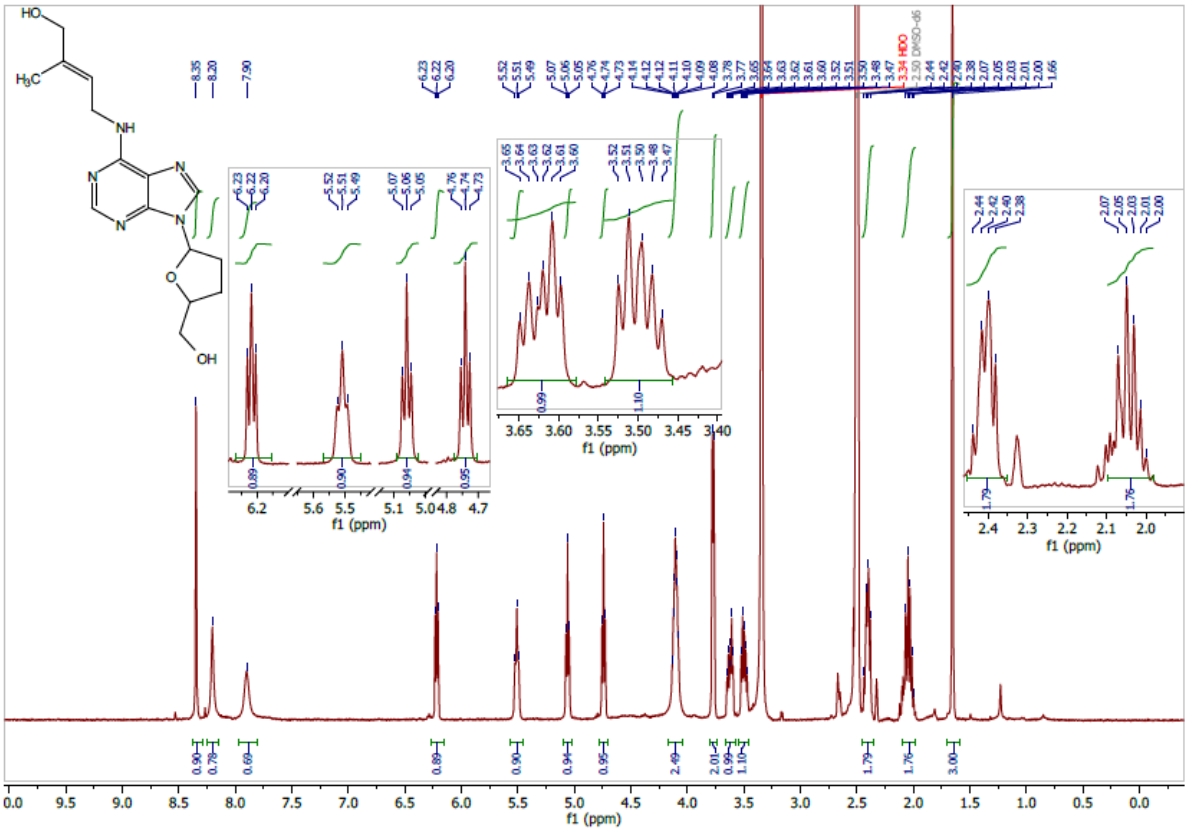
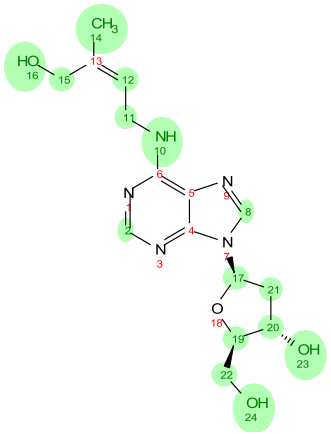


^{1}H NMR (400 MHz, DMSO-d_6) δ 1.66 (s, 3H), 1.94 – 2.15 (m, 2H, 20), 2.34 – 2.43 (m, 2H, 21), 3.50 (dt, *J* = 11.6, 4.8 Hz, 1H, 22'), 3.62 (dt, *J* = 11.6, 4.8 Hz, 1H, 22''), 3.77 (d, *J* = 5.3 Hz, 2H, 15), 4.11 (m, 3H, 12, 19), 4.74 (t, *J* = 5.6 Hz, 1H, 17), 5.06 (t, *J* = 5.5 Hz, 1H, 23), 5.51 (t, *J* = 6.3 Hz, 1H, 13), 6.22 (t, *J* = 5.3 Hz, 1H, 11), 7.90 (s, 1H, 10), 8.20 (s, 1H, 2), 8.35 (s, 1H, 8).

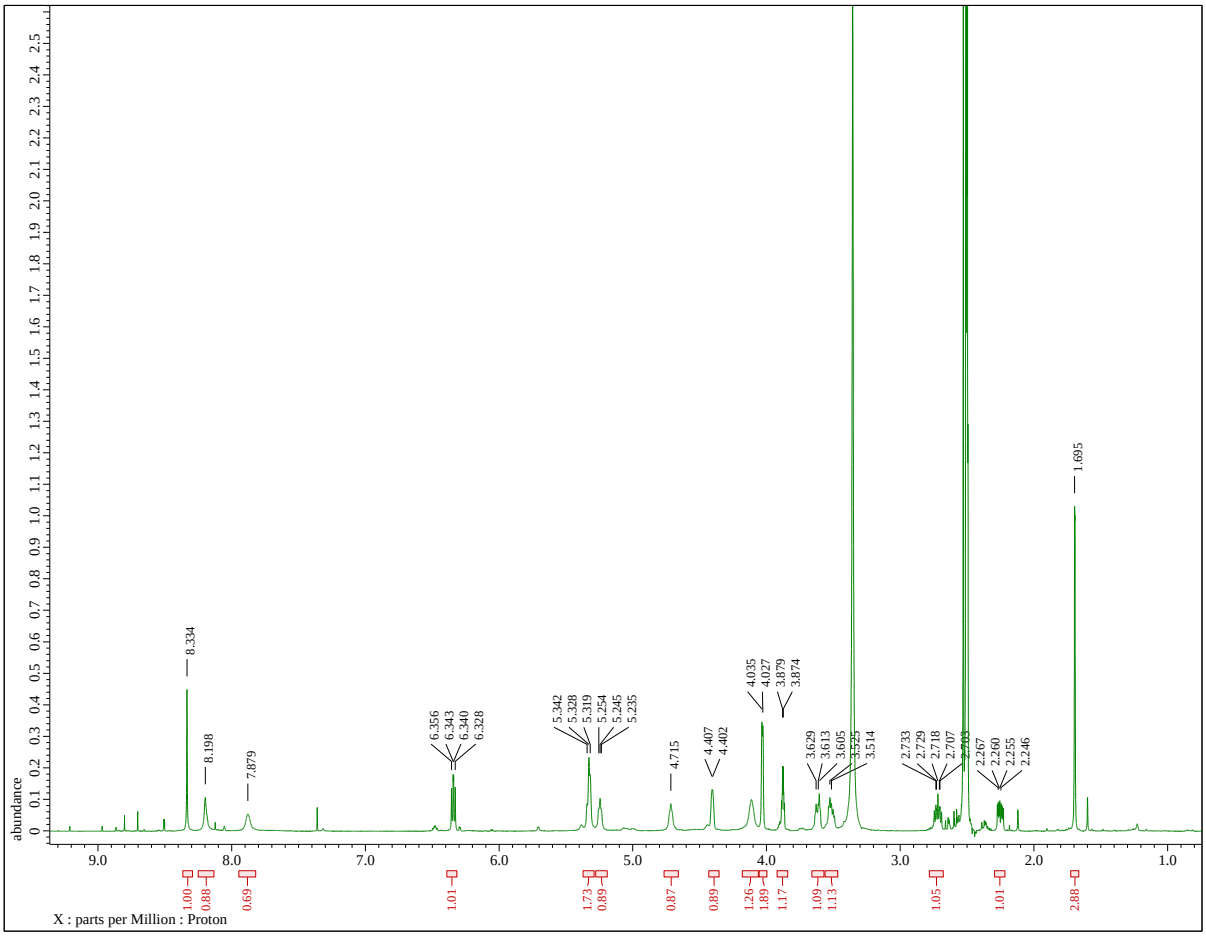
^{13}C NMR (101 MHz, DMSO-D_6) δ 13.7 (16), 25.7 (20), 31.8 (21), 37.3 (12), 62.9 (22), 65.9 (15), 81.7 (19), 84.4 (11), 119.6, 120.8 (13), 137.2, 138.8 (8), 148.0 (4), 152.4 (2), 154.2 (6).



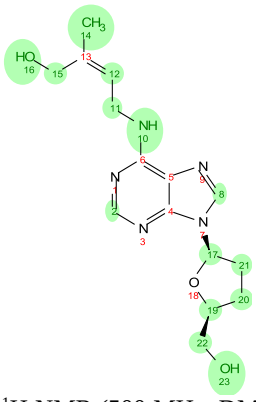
***Cis*-zeatin-9-(*β*)-D-2′-deoxyriboside (5, *c*ZdR)**



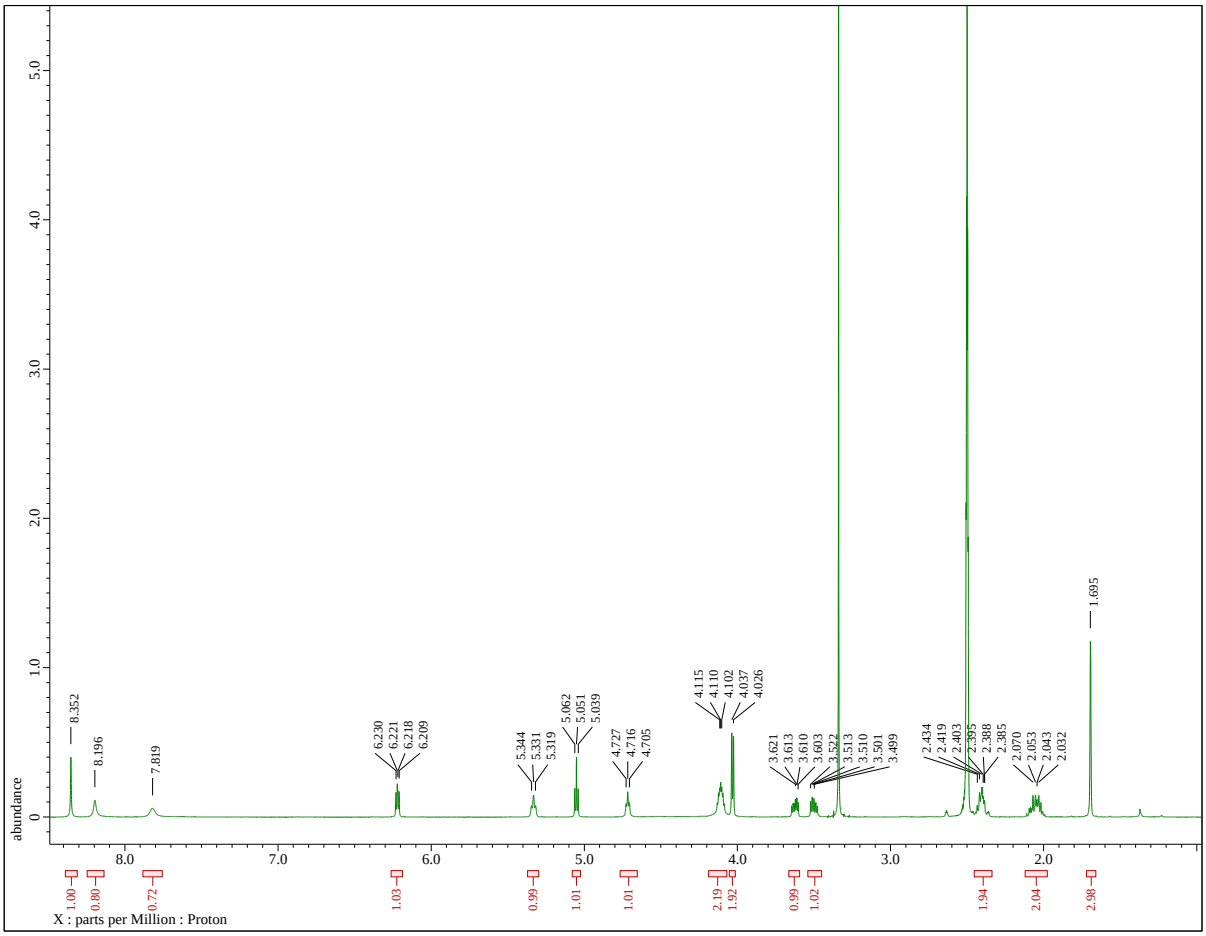
^{1}H NMR (500 MHz, DMSO-d_6) δ 1.69 (s, 3H, 14), 2.25 (ddd, J = 13.1, 6.1, 2.8 Hz, 1H, 21'), 2.72 (ddd, J = 13.3, 7.8, 5.8 Hz, 1H, 21"), 3.51 (dt, J = 11.3, 4.9 Hz, 1H, 22"), 3.62 (dt, J = 11.5, 3.7 Hz, 1H, 22'), 3.88 (td, J = 4.2, 2.7 Hz, 1H, 19), 4.03 (d, J = 3.7 Hz, 2H, 15), 4.11 (s, 2H, 11), 4.41 (bs, 1H, 20), 4.72 (bsf, 1H, 16), 5.24 (t, J = 5.0 Hz, 1H, 24), 5.33 (m, 2H, 12, 23), 6.34 (dd, J = 7.7, 6.2 Hz, 1H, 17), 7.88 (s, 1H, 10), 8.20 (s, 1H, 2), 8.33 (s, 1H, 8).
^{13}C NMR (126 MHz, DMSO-d_6) δ 21.2 (14), 37.0 (11), 39.4 (21, overlapped with DMSO-d_5) 59.8 (15), 61.9 (22), 71.0 (20), 84.0 (17), 88.0 (19), 119.8 (5), 123.1 (12), 129.1, 137.8 (13), 139.4 (8), 148.1 (4), 152.3 (2), 154.2 (6).



***Cis*-zeatin-9-(*β*)-D-2′, 3′-dideoxyriboside (6, *c*ZddR)**



^{1}H NMR (500 MHz, DMSO-d_6) δ 1.69 (d, J = 1.5 Hz, 3H, 14), 1.99 – 2.12 (m, 2H, 20), 2.38 – 2.44 (m, 2H, 21), 3.50 (ddd, J = 11.7, 5.8, 4.6 Hz, 1H, 22'), 3.62 (ddd, J = 11.7, 5.3, 4.0 Hz, 1H, 22"), 4.03 (d, J = 5.6 Hz, 2H, 15), 4.11 (tt, J = 8.0, 4.1 Hz, 3H, 19 overlaped with bs 11), 4.72 (t, J = 5.5 Hz, 1H, 16), 5.05 (t, J = 5.6 Hz, 1H, 23), 5.33 (t, J = 6.5 Hz, 1H, 12), 6.22 (dd, J = 6.1, 4.4 Hz, 1H, 17), 7.82 (s, 1H, 10), 8.20 (s, 1H, 2), 8.35 (s, 1H, 8).
^{13}C NMR (126 MHz, DMSO-d_6) δ 21.2 (14), 25.7 (20), 31.8 (21), 36.9 (11), 59.8 (15), 62.9 (22), 81.7 (19), 84.4 (17), 119.5 (5), 123.2 (12), 137.7 (13), 138.9 (8), 148.0 (4), 152.3 (2), 154.1 (6).



Cytokinin bioassays results:

Table S1: *Amaranthus*, senescence and tobacco callus bioassay results for isopentenyladenine derivatives related to iP at the concentration 10^{-4} mol/L for *Amaranthus* and senescence bioassays and 10^{-5} mol/L for tobacco callus bioassay (set as 100 % of activity).

		Amaranthus bioassay		Senescence bioassay			Tobacco callus bioassay	
Compound		Concentration [mol/L]	Relative activity [%]	Concentration [mol/L]	Chlorophyll of fresh leaves [%]	Relative activity [%]	Concentration [mol/L]	Relative activity [%]
	iP	10^{-4}	100	10^{-4}	38 ±3	100	10^{-4}	No activity
		10^{-5}	105 ± 10	10^{-5}	33 ± 4	87	10^{-5}	100
		10^{-6}	28 ± 1				10^{-6}	99 ± 4
	iPR	10^{-4}	136 ± 6	10^{-4}	43 ± 7	115	10^{-4}	No activity
		10^{-5}	114 ± 8	10^{-5}	34 ±5	92	10^{-5}	91 ± 5
		10^{-6}	30 ± 4				10^{-6}	92 ± 6
1	iPdR	10^{-4}	170 ± 23	10^{-4}	40 ± 9	107	10^{-4}	84 ± 7
		10^{-5}	80 ± 6	10^{-5}	34 ± 4	91	10^{-5}	95 ± 4
		10^{-6}	18 ± 2				10^{-6}	93 ± 6
2	iPddR	10^{-4}	68 ± 5	10^{-4}	35 ± 6	93	10^{-4}	No activity
		10^{-5}	24 ± 5	10^{-5}	37 ± 4	99	10^{-5}	103 ± 5
		10^{-6}	1 ± 1				10^{-6}	94 ± 3

Table S2: *Amaranthus*, senescence and tobacco callus bioassay results for *trans*-zeatin derivatives related to *t*Z at the concentration 10^{-4} mol/L for *Amaranthus* and senescence bioassays and 10^{-5} mol/L for tobacco callus bioassay (set as 100 % of activity). 10^{-5} mol/L (set as 100 % of activity).

		Amaranthus bioassay		Senescence bioassay			Tobacco callus bioassay	
Compound		Concentration [mol/L]	Relative activity [%]	Concentration [mol/L]	Chlorophyll of fresh leaves [%]	Relative activity [%]	Concentration [mol/L]	Relative activity [%]
	*t*Z	10^{-4}	100	10^{-4}	80 ± 2	100	10^{-4}	No activity
		10^{-5}	67 ± 4	10^{-5}	59 ± 4	74	10^{-5}	100
		10^{-6}	35 ± 4				10^{-6}	92 ± 3
	*t*ZR	10^{-4}	103 ± 7	10^{-4}	88 ± 2	110	10^{-4}	No activity
		10^{-5}	68 ± 5	10^{-5}	61 ± 4	77	10^{-5}	98 ± 3
		10^{-6}	37 ± 6				10^{-6}	90 ± 5
3	*t*ZdR	10^{-4}	97 ± 2	10^{-4}	75 ± 4	93	10^{-4}	91 ± 6
		10^{-5}	63 ± 6	10^{-5}	48 ± 2	60	10^{-5}	87 ± 6
		10^{-6}	28 ± 6				10^{-6}	90 ± 4
4a	*α*-*t*ZddR	10^{-4}	13 ± 2	10^{-4}	68 ± 1	85	10^{-4}	78 ± 5
		10^{-5}	4 ± 1	10^{-5}	45 ± 2	56	10^{-5}	92 ± 4
		10^{-6}	1 ± 1				10^{-6}	84 ± 5
4b	*β*-*t*ZddR	10^{-4}	29 ± 2	10^{-4}	71 ± 2	89	10^{-4}	99 ± 7
		10^{-5}	12 ± 2	10^{-5}	42 ± 2	53	10^{-5}	103 ± 2
		10^{-6}	4 ± 1				10^{-6}	90 ± 8

Table S3: *Amaranthus*, senescence and tobacco callus bioassay results for *cis*-zeatin derivatives related to *c*Z at the concentration 10^{-4} mol/L for *Amaranthus* and senescence bioassays and 10^{-5} mol/L for tobacco callus bioassay (set as 100 % of activity).

		Amaranthus bioassay		Senescence bioassay			Tobacco callus bioassay	
Compound		Concentration [mol/L]	Relative activity [%]	Concentration [mol/L]	Chlorophyll of fresh leaves [%]	Relative activity [%]	Concentration [mol/L]	Relative activity [%]
	*c*Z	10^{-4}	100	10^{-4}	39 ± 2	100	10^{-4}	No activity
		10^{-5}	50 ± 8	10^{-5}	31 ± 1	80	10^{-5}	100
		10^{-6}	7 ± 2				10^{-6}	97 ± 6
	*c*ZR	10^{-4}	139 ± 24	10^{-4}	39 ± 2	100	10^{-4}	92 ± 16
		10^{-5}	34 ± 5	10^{-5}	34 ± 2	86	10^{-5}	97 ± 6
		10^{-6}	7 ± 1				10^{-6}	93 ± 5
5	*c*ZdR	10^{-4}	83 ± 20	10^{-4}	42 ± 1	108	10^{-4}	No activity
		10^{-5}	14 ± 1	10^{-5}	38 ± 3	98	10^{-5}	93 ± 8
		10^{-6}	3 ± 1				10^{-6}	64 ± 7
6	*c*ZddR	10^{-4}	18 ± 6	10^{-4}	41 ± 4	106	10^{-4}	No activity
		10^{-5}	5 ± 1	10^{-5}	36 ± 2	93	10^{-5}	98 ± 6
		10^{-6}	No activity				10^{-6}	88 ± 4

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Supplementary material III.

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(6):832.

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Review

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

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

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

1. Introduction

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

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

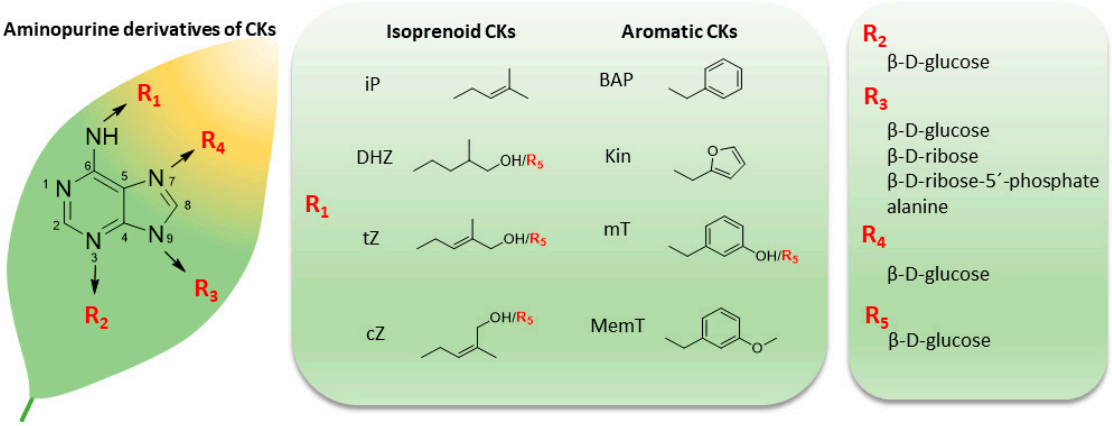

Figure 1. Structure of naturally occurring cytokinin (CK) aminopurine derivatives. The R_1 determines the type of side chain, R_2-R_5 specify the type of CK conjugate.

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

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

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

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

2. N7- and N9-Sugar Cytokinin Conjugates

2.1. Cytokinin 7- and 9-Glucosides

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

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

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

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

2.2. Cytokinin 9-Ribosides

2.2.1. IsCK 9-Ribosides

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

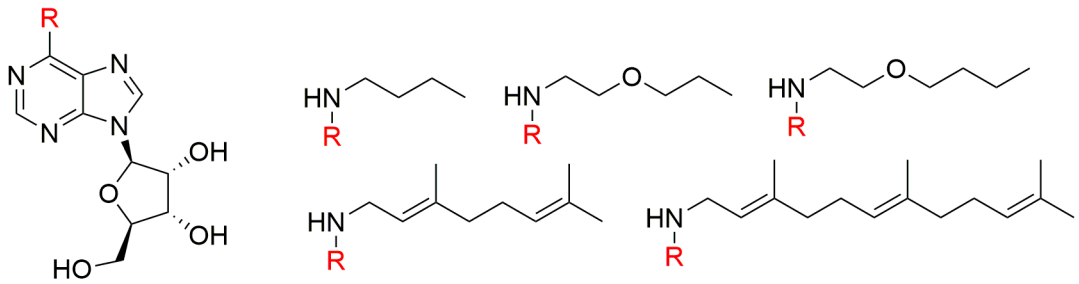

Figure 2. General structure of N6-substituted adenosines and their N6-substituents [68].

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

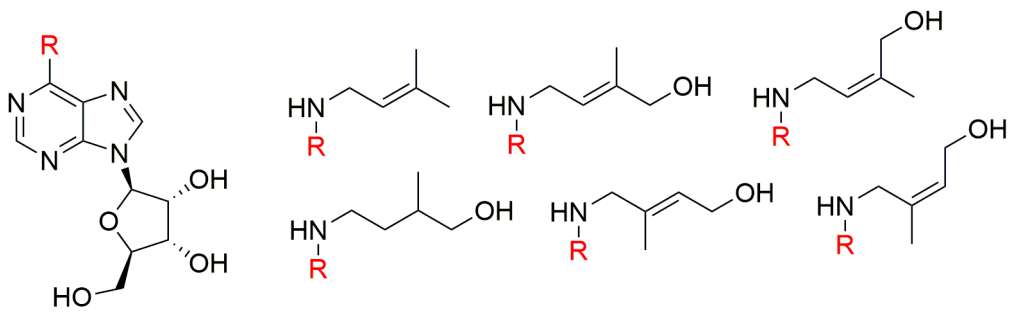

Figure 3. Compounds prepared and tested by Schmitz et al. in 1972 [69].

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

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

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

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

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

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

2.2.2. ArCK 9-Ribosides

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

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

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

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

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

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

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

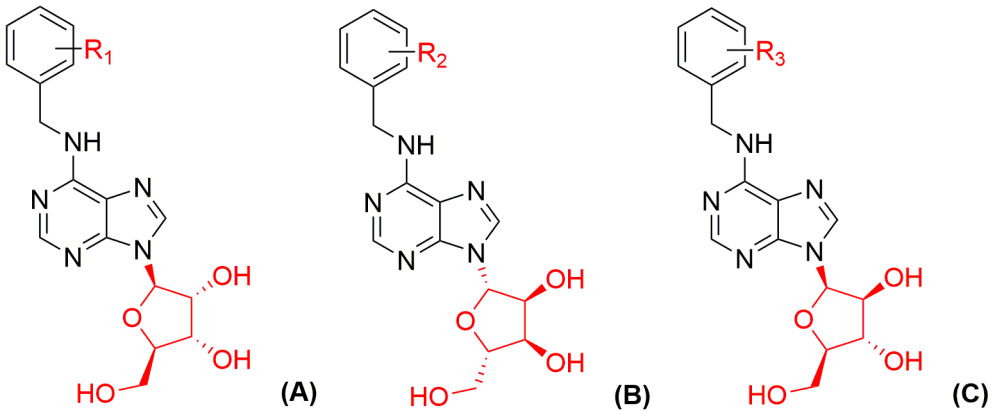

Figure 4. General structures of aromatic (**A**) 6-benzylaminopurine-9-β-D-riboside derivatives (R_1 = X, CH_3, OCH_3, OH, $OCHF_2$, OCF_3, CF_3 or a combination of these groups), (**B**) 6-benzylaminopurine-9-β-L-riboside derivatives (R_2 = H, F, Cl, OCH_3 or OH) and (**C**) 6-benzylaminopurine-9-β-D-arabinoside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

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

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

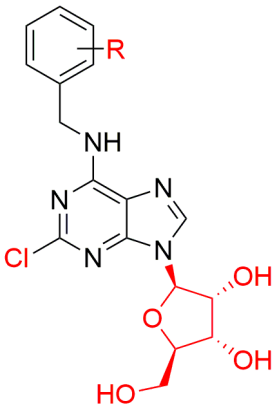

Figure 5. General structure of 2-chloro-6-disubstituted benzylaminopurine riboside derivatives, where R = halogens [46].

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

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

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

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

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

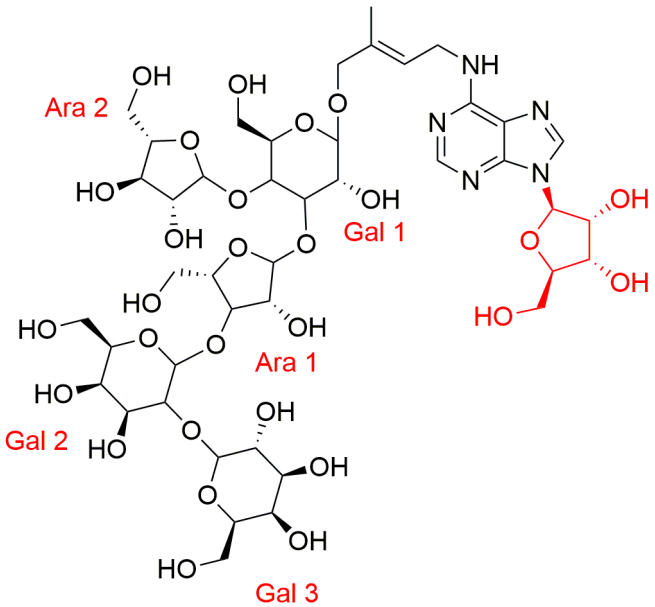

Figure 6. Structure of 14-*O*-{3-*O*-[β-D-galactopyranosyl-(1→2)-α-D-galactopyranosyl-(1→3)-α-L-arabinofuranosyl]-4-*O*-(α-L-arabinofuranosyl)-β-D-galactopyranosyl}-trans-zeatin riboside (G_3A_2-ZR) [113].

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

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

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

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

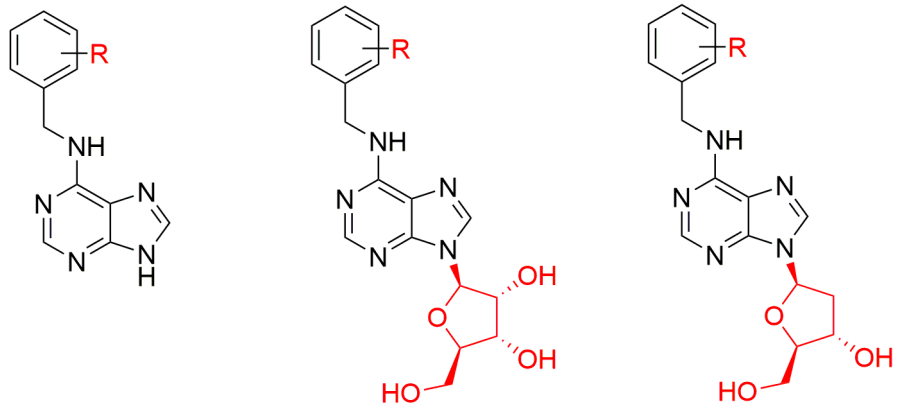

Figure 7. Comparison and general structures of free base, riboside and 2′-deoxyriboside derivatives, where R = X, OH, OCH_3, CH_3, OCF_3, CF_3 or a combination of these groups.

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

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

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

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

2.4. Purine N9-Arabinosides and Their Precursors

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

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

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

2.5. Cytokinin Disaccharide Conjugates

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

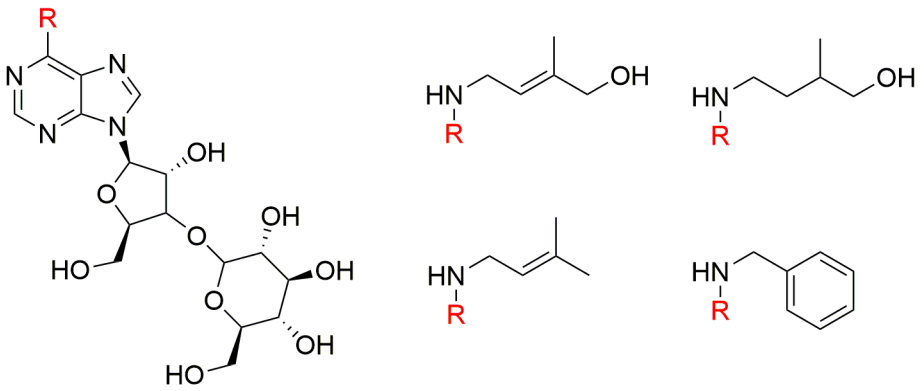

Figure 8. CK disaccharides (Z9RG, DHZ9RG, iP9RG) reported by Zhang et al. (2001) and BAP9RG reported by Auer and Cohen (1993) [138,140].

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

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

3. Non-Sugar N9-Substituted Cytokinins

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

3.1. 9-Alanyl Derivatives

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

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

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

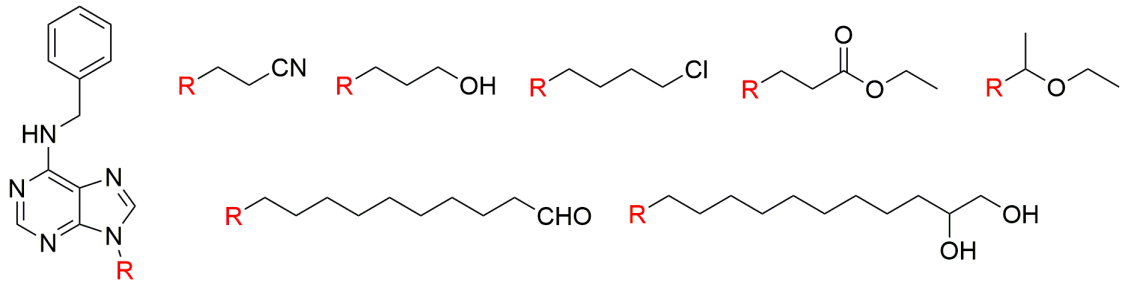

Figure 9. Structures of aliphatic chains substituted at the N9 atom of BAP [145].

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

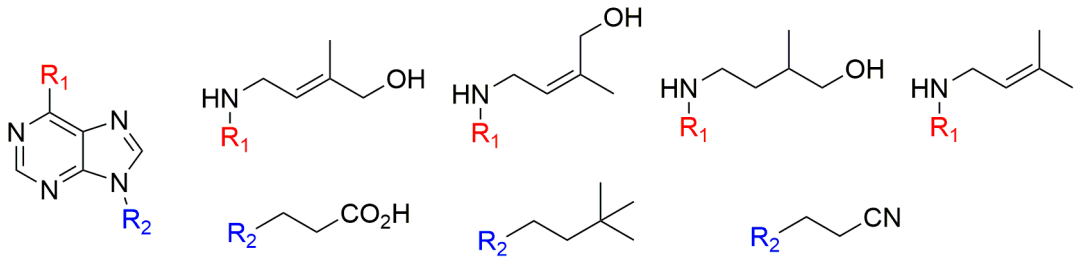

Figure 10. Structures of compounds prepared by Corse et al. (1989). On N6 (R1): tZ, cZ, DHZ, and iP side chains. On N9 (R2): 2-carboxyethyl, 2-carbo-*t*-butoxyethyl and 2-nitriloethyl) [146].

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

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

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

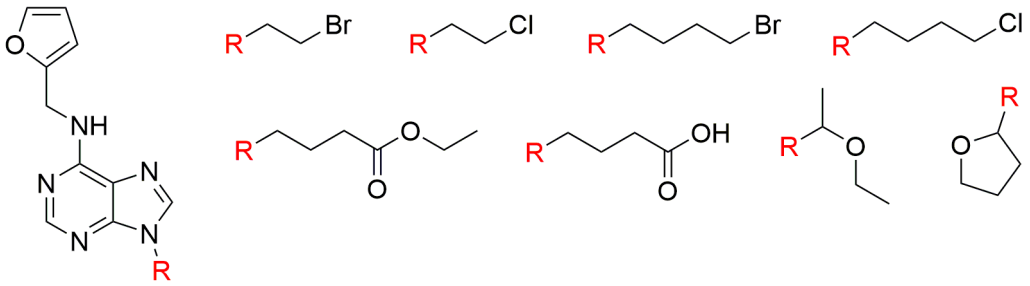

Figure 11. Structures of Kin derivatives prepared by Mik et al. (2011) [157].

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

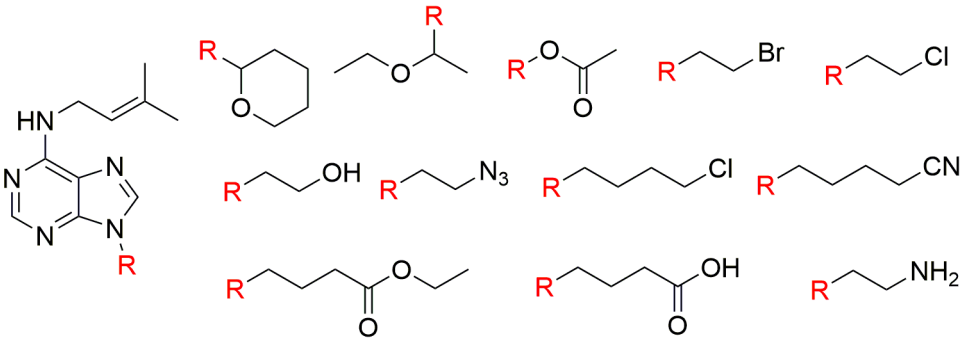

Figure 12. Structures of iP derivatives prepared by Mik et al. 2011 [158].

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

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

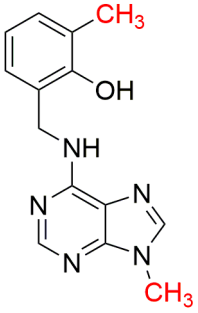

Figure 13. Structure of N9-methylated PI-55 [160].

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

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

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

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

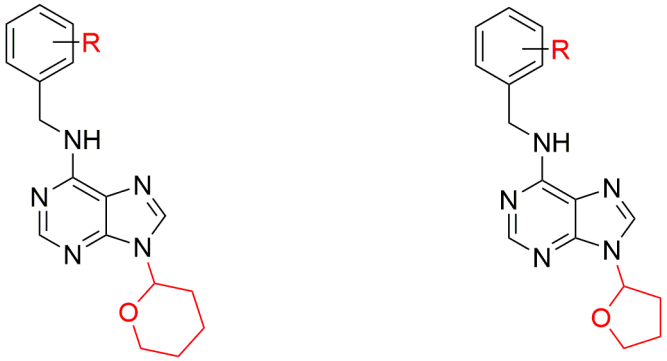

Figure 14. General structures of 9THP and 9THF ArCK derivatives (R = OH, OCH_3 or their combination) [147].

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

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

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

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

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

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

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

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

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

Table 1. *Cont.*

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

Table 1. *Cont.*

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

4. Conclusions

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

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

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Supplementary material IV.

Matušková, V., Zatloukal, M., Koprna, R., Doležal, K.: Směs pro ošetření zemědělských plodin pro zvýšení výnosu a odolnosti proti houbovým chorobám a přípravek pro foliární aplikaci, obsahující tuto směs.

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Směs pro ošetření zemědělských plodin pro zvýšení výnosu a odolnosti proti houbovým chorobám a přípravek pro foliární aplikaci, obsahující tuto směs

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Směs pro ošetření zemědělských plodin pro zvýšení výnosu a odolnosti proti houbovým chorobám a přípravek pro foliární aplikaci, obsahující tuto směs

Oblast techniky

Předkládané technické řešení se týká směsi pro ošetření zemědělských plodin formou listové aplikace postřikovou jíchou. Účinná látka je syntetický cytokinin na bázi substituovaného derivátu adeninu s rozpouštědlem. Tato směs je vhodná k listové aplikaci na zemědělské plodiny – obilniny (pšenice, ječmen, žito, oves, kukuřice), olejniny (řepka, hořčice), mák setý, bobovité (hrách, vikev, bob, peluška, sója) a dalších tržních plodin. Předkládané řešení se dále týká přípravku, který tuto směs obsahuje.

Dosavadní stav techniky

Cytokininy jsou rostlinné hormony, jejichž syntéza probíhá převážně v kořenech, odtud jsou transportovány xylémem do celé rostliny. Cytokininy mají tyto účinky: podpora buněčného dělení, podpora větvení lodyh, zábrana stárnutí listů, zvýšení odolnosti vůči vysokým teplotám fyziologické, omezení apikální dominance (opak auxinového účinku) a podpora větvení, oddálení senescence, stimulace růstu kořenů a klíčení, stimulace fotosyntézy.

Vlastní výsledky aplikace látek ovlivňujících hladinu cytokininů na jarním ječmeni prokázaly intenzivnější vzcházení a následující růst při simulaci vodního stresu. Nárůst hmotnosti kořenů byl u testovaných variant oproti kontrole o 23 až 84 %, v případě hmotnosti nadzemní části to bylo o 22 až 75 %. Výsledky z polního experimentu potvrdily v roce 2012 (rok s extrémně nízkým úhrnem srážek v dubnu až červnu) pozitivní účinek moření těmito látkami (jednalo se zejména o CK antagonisty), kdy byl výnos ošetřených variant 97 až 118 % na kontrolu.

Přirozeně se vyskytující cytokininy jsou malé organické molekuly strukturně odvozené od adeninu, které ovlivňují celou řadu fyziologických procesů v rostlinách, například růst a vývoj jednotlivých částí rostliny a jejích orgánů, diferenciaci buněk a pletiv, reakci rostliny na biotický a abiotický stres a senescenci. Cytokininy se v rostlinách vyskytují ve velmi nízkých koncentracích, interagují s dalšími rostlinnými hormony. Díky tomu je možné využít některých derivátů těchto látek, například cytokininů s aromatickou substitucí na atomu N6-adeninu v exogenní aplikaci v biotechnologiích. Cíleným designem a specifickou substitucí těchto malých organických molekul je možné získat deriváty zachovávající si biologickou aktivitu původních molekul, ba dokonce s aktivitou vyšší, a přitom docílit látek lépe rozpustných ve vodě a tím i dostupnějších pro příjem rostlinami, zejména například při exogenní aplikaci na list. Jiným způsobem zvýšení dostupnosti pro rostliny je zásadnější změna v substituci purinového/adeninového skeletu, nebo malé úpravy stávajících funkčních molekul zavedením vhodných funkčních skupin nebo vhodnou substitucí konkrétních atomů adeninu, například C2 nebo N9.

Substituované adeninové deriváty jsou známy jako fytohormony. Tyto látky mají vysokou antisenescenční aktivitu a vliv na dělení rostlinných buněk. Tyto látky mají také účinek v podobě zvýšení výnosu zemědělských plodin po jejich aplikaci. Antisenescenšní aktivita těchto látek byla sledována na pšenici seté – odrůda Hereward. V koncentraci 10 až 4 Mol byla zjištěna aktivita až 198 % ve srovnání na kontrolní variantu s BAP (benzylaminopurin).

Účinek těchto látek byl testován i na omezení výskytu nekrotických pletiv u *Dais cotinifolia,* kde bylo při koncentraci 5 až 30 µMol látky v MS médiu zjištěn snížený výskyt nekróz na úrovni 31,3 až 52,2 % na kontrolní variantu bez aplikace syntetických fytohormonů. Aplikace této látky na rostlinu *Harpagophytum procumbens* v *in vitro* podmínkách způsobil při koncentraci 5 až 30 µMol látky v MS médiu výrazné zvýšení počtu výhonů, jejich délky a hmotnosti biomasy.

Stejné biotesty na rostlině muchovníku olšolistém *(Amelanchier alnifolia).* Stejně jako v předešlém případu, došlo k výraznému nárůstu počtu výhonů, jejich délky a hmotnosti biomasy. In vitro testy na rostlině *Gymnosporia buxifolia* prokázali ve stejných koncentracích 5 až 30 µMol látky v MS médiu omezení nekróz na listech ve srovnání s kontrolou (BAP) až na úroveň 13,3 až 53,3 %. Stejně jako v předešlém případě se při zvyšující koncentraci látky v médiu snižovala plocha nekrotických pletiv na testovaných rostlinách.

Aplikace benzylaminopurinu a meta-topolinu na locika setou – syn. salátovou (*Lactuca sativa* L.), způsobila supresi plísně salátové *(Bremia lactucae).* Po 24 hodinách od aplikace těchto cytokininů, došlo k omezení sporulace tohoto patogena a jeho vývoje, bez negativního vlivu na fotosyntetický aparát rostliny (Prokopová J. a kol., 2010). Výsledky jiných autorů potvrdily vliv cytokininů na růst a patogenezi houbových patogenů. Exogenní aplikace cytokininů může vést k potlačení vývoje patogenů, nebo zvýšení rezistence rostlin (Mishina G.N. a kol., 2002; Sahran A.R.T. a kol., 1991).

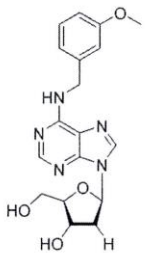
S intenzifikací zemědělství je spojeno mnoho negativních faktorů, mimo jiné snížením diverzity pěstovaných plodin. Toto opatření zkracuje období pěstování stejných plodin (příp. druhově stejných) na témže pozemku, čímž dochází ke zvýšenému plaku zejména houbových patogenů. Různé spory houbových chorob přežívají v půdě několik let, příp. se přenáší větrem z okolních pozemků a rostliny jsou pak vystavenu vysokému infekčnímu tlaku.

Většina hospodářsky významných patogenů kulturních plodin je houbového původu a odolnost rostlin (příp. tolerance) je dána mnoha geny. Tím, že tato dědičnost není dána monogenně, je obtížné zkombinovat hospodářské vlastnosti plodin s vysokou rezistencí proti patogenům do jedné odrůdy. Příkladem širokého spektra patogenů je pšenice setá *(Triticum aestivum),* která je napadána těmito houbovými patogeny: plíseň sněžná, pravý stéblolam *(Ramulispora herpotrichoides),* kořenomorka obilná *(Rhizoctonia cerealis),* fuzarióza stébel *(Fusarium spp.),* černání kořenů *(Gaeumannomyces graminis),* padlí travní *(Blueria graminis),* braničnatka plevová (*Stagonospora nodorum*), rez plevová (*Puccinia striiformis*), rez travní (*Puccinia graminis),* běloklasost a fuzariózy klasů a další.

Podstata technického řešení

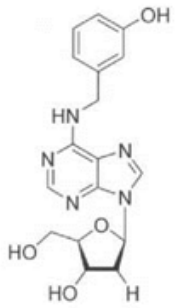
Podstatou technického řešení je směs (s výhodou roztok, suspenze, emulze, mikroemulze, mikrodisperze), vhodná pro aplikaci do zemědělských plodin (obilniny, olejniny, luskoviny, přadné a aromatické plodiny, trávy, pícniny). Směs se aplikuje formou postřiku (foliární aplikace). Směs podle předkládaného technického řešení obsahuje alespoň jednu účinnou látku, vybranou z N^6-(3-hydroxybenzylamino)-9-(β)-D-2´-deoxyribofuranosylpurinu a N^6-(3-methoxybenzylamino)-9-(β)-D-2´-deoxyribofuranosylpurinu a rozpouštědlo, s výhodou dimetyl sulfoxid (DMSO). Směs může dále obsahovat také makroprvky (N, P, K) a mikroprvky (Zn, Cu, Mo, Mo, Fe, B, Ca, Mg) ve formě solí a různém poměru.

Účinná látka N^6-(3-methoxybenzylamino)-9-(β)-D-2´-deoxyribofuranosylpurin je syntetický cytokinin podle strukturního vzorce I.



vzorec (I)

Účinná látka N^6-(3-hydroxybenzylamino)-9-(β)-D-2´-deoxyribofuranosylpurin je syntetický cytokinin podle strukturního vzorce II.



vzorec (II)

Dimethyl sulfoxid (DMSO) je bezbarvá sloučenina (kapalina), která se používá v chemii jako rozpouštědlo mísitelné s vodou. Další použitelná rozpouštědla jsou polární organická rozpouštědla, jako například polyetylenglykol (PEG BG až PEG 200), 2-(2-ethoxyethoxy)ethanol, 1-methyl-2-pyrrolidon, glycerol, dodecylsulfonát sodný a 1-methyl-2-pyrrolidon.

Předmětem předkládaného technického řešení je směs pro ošetření zemědělských plodin pro zvýšení výnosu a odolnosti proti houbovým chorobám *Ramulispora herpotrichoides, Blueria graminis, Puccinia striiformis a Puccinia graminis*, která obsahuje alespoň jednu účinnou látku, vybranou z N^6-(3-methoxybenzylamino)-9-(β)-D-2´-deoxyribofuranosylpurinu a N^6-(3-hydroxybenzylamino)-9-(β)-D-2´-deoxyribofuranosylpurinu, a polární organické rozpouštědlo, s výhodou vybrané ze skupiny zahrnující DMSO, polyetylenglykol (o molekulové hmotnosti v rozmezí od 80 do 200 Da), 2-(2-ethoxyethoxy)ethanol, 1-methyl-2-pyrrolidon, glycerol, manitol, kyselinu laurovou, myristovou, palmitovou, stearovou, arachidonovou, erukovou, parafin, parafínový olej, propylen-glykol, sorbitol, polyalkohol, glykol, přičemž koncentrace účinné látky ve směsi je v rozmezí od 0,1 mM do 0,1 M. Směs pro ošetření zemědělských plodin je ve formě postřiku pro zvýšení a stabilizaci výnosu, zejména ve stresových podmínkách a pro zlepšení zdravotního stavu rostlin, zejména pro zvýšení odolnosti proti houbovým chorobám.

Předmětem předkládaného technického řešení je dále přípravek pro foliární aplikaci do zemědělských plodin, který obsahuje směs podle předkládaného technického řešení, (tedy alespoň jednu účinnou látku, vybranou z N^6-(3-methoxybenzylamino)-9-(β)-D-2´-deoxyribofuranosylpurinu a N^6-(3-hydroxybenzylamino)-9-(β)-D-2´-deoxyribofuranosylpurinu, a polární organické rozpouštědlo, s výhodou vybrané ze skupiny zahrnující DMSO, polyetylenglykol (o molekulové hmotnosti v rozmezí od 80 do 200 Da), 2-(2-ethoxyethoxy)ethanol, 1-methyl-2-pyrrolidon, glycerol, manitol, kyselinu laurylovou, myristovou, palmitovou, stearovou, arachidonovou, erukovou, parafin, parafínový olej, propylen-glykol, sorbitol, polyalkohol, glykol; a vodu, přičemž výsledná koncentrace účinné látky v přípravku je v rozmezí od 0,1 μM do 100 μM. Přípravek pro listovou aplikaci do zemědělských plodin tedy obsahuje směs podle předkládaného technického řešení, rozpuštěnou ve vodě na výslednou koncentraci 0,1 μMol až 100 μMol N^6-(3-methoxybenzylamino)-9-(β)-D-2´-deoxyribofuranosylpurinu a/nebo N^6-(3-hydroxybenzylamino)-9-(β)-D-2´-deoxyribofuranosylpurinu, rozpouštědla (např. DMSO), což tvoří zásobní roztok účinné látky.

Přípravek pro listovou aplikaci (postřiková jícha) může obsahovat další makroprvky (N, P, K) a mikroprvky (S, Zn, Cu, Mo, Mo, Fe, B, Ca, Mg) ve formě solí v jakémkoliv vzájemném mísitelném poměru.

V jednom provedení přípravek dále obsahuje dusíkaté hnojivo a/nebo fosfor obsahující hnojivo a/nebo draselné hnojivo.

Dusíkaté hnojivo je s výhodou vybrané ze skupiny zahrnující NH_4H_2PO4, $(NH_4)_2HPO_4$, $CO(NH_2)_2$, $Ca(NO_3)_2$, $(NH_4)_2SO_4$ a NH_4NO_3.

Fosfor obsahující hnojivo je s výhodou vybrané ze skupiny zahrnující $NH_4H_2PO_4$, $(NH_4)_2HPO_4$, KH_2PO_4, K_2HPO_4, $Ca(H_2PO_4)_2$, K_3PO_4, K_3PO_4, Na H_2PO_4, Na_3PO_4 a Na_2HPO_4.

Draselné hnojivo je s výhodou vybrané ze skupiny zahrnující KH_2PO_4, K_2HPO_4, KCl, K_2SO_4, K_3PO_4, K_3PO_4.

V jednom provedení přípravek dále obsahuje kation C^{m+}, vybraný z Ca^{2+}, Mg^{2+}, Li^{+}, Fe^{2+}, Fe^{3+}, Al^{3+}, Ag^{+}, Cu^{2}+, Zn^{2}+, Hg^{2}+, Pb^{2}+, Ba^{2}+ a NH4+,

přičemž kation C^{m+} je ve formě soli s anionem A^{n-}, vybraným z F^{-}, CI^{-}, Br^{-}, I^{-}, SO_4^{2-}, NO_3^{-} nebo $CH_3CO_2^{-}$,

nebo ve formě chelátu s DMSO.

V jednom provedení se přípravek o objemu 1 litr tedy připraví rozpuštěním 0,04 až 37,14 mg N^6-(3-methoxybenzylamino)-9-(*β*)-D-2´-deoxyribofuranosylpurinu, nebo N^6-(3-hydroxybenzylamino)-9-(*β*)-D-2´-deoxyribofuranosylpurinu v 1 ml DMSO a v 1.000 ml vody.

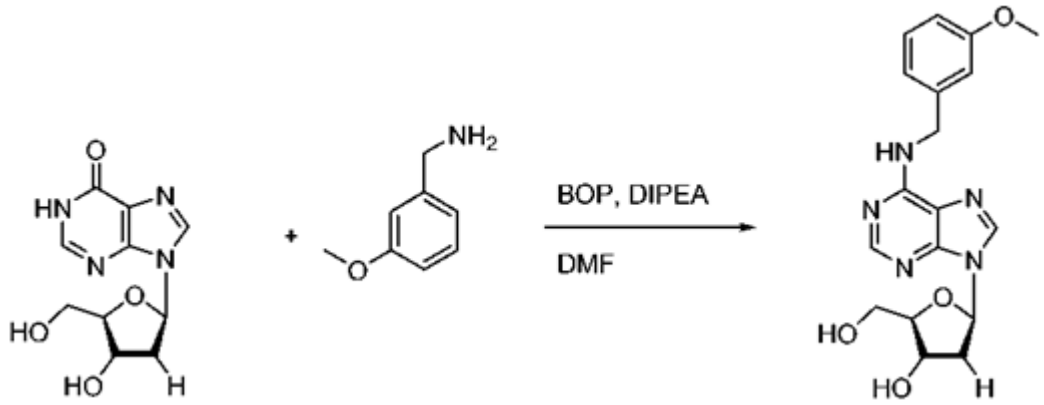
Uvedené složení odpovídá optimální koncentraci účinné látky N^6-(3-methoxybenzylamino)-9-(*β*)-D-2´-deoxyribofuranosylpurinu nebo N^6-(3-hydroxybenzylamino)-9-(*β*)-D-2´-deoxyribofuranosylpurinu a rozpouštědla DMSO ve vodě pro zvýšení a stabilizaci výnosu zemědělských plodin a snížení napadení houbovými patogeny u těchto plodin. Přípravek lze použít samostatně, nebo ve směsi s dalšími hnojivy, stimulátory a přípravky na ochranu rostlin. Doporučené dávkování vychází z koncentrace DMSO ve vodném roztoku, které je 0,1 % obj. ředění roztoku účinné látky N^6-(3-methoxybenzylamino)-9-(*β*)-D-2´-deoxyribofuranosylpurinu nebo N^6-(3-hydroxybenzylamino)-9-(*β*)-D-2´-deoxyribofuranosylpurinu rozpuštěné v DMSO ve vodě je 1:1000.

Doporučený termín aplikace přípravku podle předkládaného technického řešení je od období tvorby listů rostlin – dle dekadické stupnice BBCH je to od BBCH 14 až 16 do konce kvetení (BBCH 69). Doporučená aplikace je formou postřiku na nadzemní části rostlin, polním postřikovačem, nebo jinou formou postřiku (zádový a ruční postřikovač). Před aplikací je doporučené postřikovou jíchu se směsí s výhodou zamíchat a homogenizovat. Doporučené množství přípravku podle předkládaného technického řešení (postřikové jíchy) je 200 až 600 l v závislosti na pěstované plodině.

Termín aplikace je doporučen během vegetace, optimálně v době, kdy rostliny trpí abiotickým stresem, nebo je předpoklad přenosu patogenů ve formě spor na rostlinná pletiva a dochází k rozvoji houbových chorob. Postřik lze opakovat až 3x za vegetaci.

Příklady uskutečnění technického řešení

Příklad 1: Syntéza N^6-(3-methoxybenzylamino)-9-(β)-D-2´-deoxyribofuranosylpurinu a příprava směsi pro ošetření zemědělských plodin



Násada:

2´-deoxyinosin	300 mg (1,2 mmol)
BOP reagent	630 mg (1,4 mmol)
DIPEA	303 µL (1,8 mmol)
3-methoxybenzylamin báze	182,4 µL (1,4 mmol)
suchý DMF	8 mL

Postup:

Do suché 50 ml trojhrdlé baňky s kulatým dnem se předloží 2´-deoxyinosin (1,2 mmol; 300 mg), BOP reagent *(Castro´s reagent; benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate*; 1,4 mmol; 630 mg) a bezvodý DMF (8 mL). Směs se inertizuje proudem N_2, opatřena septy a ponechá se míchat 10 minut. Poté se k reakční směsi přidá DIPEA (1,8 mmol; 303 µl) a následně 3-methoxybenzylamin (1,4 mmol; 182,4 µL), jako poslední komponenta. Reakční směs se vloží do olejové lázně vyhřáté na 55 °C a ponechá se míchat za inertní atmosféry do dalšího dne. Průběh reakce je kontrolován pomocí TLC ($CHCl_3$:MeOH:TEA 9:1:0,05) až vizuálně dojde ke změně barvy reakční směsi do hnědo-oranžova. Po ukončení reakce se přidá směs ethylacetát/H_2O a vodná fáze se třikrát promyje ethylacetátem. Spojené organické fáze se vysuší bezvodým Na_2SO_4 a odpaří na RVO. Vzniklý olejovitý odparek se rozpustí v abolutním ethanolu a ponechá v lednici přes noc. Vzniklá pevná bílá látka (Mr = 371,39; Rf (TLC) 0,35) se zfiltruje a promyje absolutním ethanolem a následně vysuší v exsikátoru do konstantní hmotnosti.

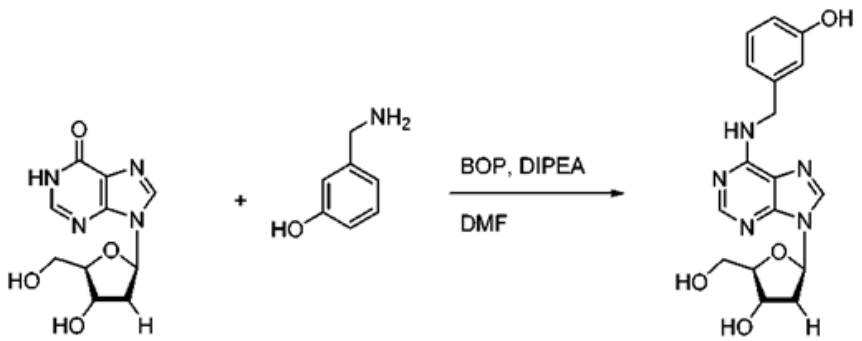
Výtěžek: 168 mg (38 %)

Čistota podle HPLC: 99 % +

Směs pro ošetření zemědělských plodin podle předkládaného technického řešení o různých koncentracích byla připravena rozpuštěním 0,04 mg až 37,14 mg připraveného N^6-(3-methoxybenzylamino)-9-(*β*)-D-2´-deoxyribofuranosylpurinu vždy v 1 ml rozpouštědla. Jako rozpouštědlo byl použit dimetylsulfoxid (DMSO), kromě tohoto lze použít i jiná organická polární rozpouštědla. Směs může obsahovat i organická i anorganická listová hnojiva obsahující

makroprvky (N, P, K) a mikroprvky (S, Zn, Cu, Mo, Mo, Fe, B, Ca, Mg) ve formě solí v jakémkoliv vzájemném mísitelném poměru těchto prvků.

Příklad 2: Syntéza N^6-(3-hydroxybenzylamino)-9-(β-D-2´-deoxyribofuranosylpurinu a příprava směsi pro ošetření zemědělských plodin



Násada:

2´-deoxyinosin	300 mg (1,2 mmol)
BOP reagent	630 mg (1,4 mmol)
DIPEA	303 μL (1,B mmol)
3-hydroxybenzylamin báze	175,B mg (1,4 mmol)
suchý DMF	8 mL

Postup:

Do suché 50 ml trojhrdlé baňky s kulatým dnem se předloží 2´-deoxyinosin (1,2 mmol; 300 mg), BOP reagent *(Castro´s reagent; benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate*; 1,4 mmol; 630 mg) a bezvodý DMF (8 mL). Směs se inertizuje proudem N_2, opatřena septy a ponechá se míchat 10 minut. Poté se k reakční směsi přidá DIPEA (1,8 mmol; 303 μL) a následně 3-hydroxybenzylamin (3-(aminomethyl)fenol) 1,4 mmol; 175,8 mg) jako poslední komponenta. Reakční směs se vloží do olejové lázně vyhřáté na 55 °C a ponechá míchat za inertní atmosféry do dalšího dne. Průběh reakce je kontrolován pomocí TLC ($CHCl_3$:MeOH:TEA 9:1:0,05) až vizuálně dojde ke změně barvy reakční směsi z čiré do žlutohnědé. Po ukončení reakce se ke směsi přidá směs ethylacetát/H_2O a vodná fáze se třikrát promyje ethylacetátem. Spojené organické fáze se vysuší bezvodým Na_2SO_4 a odpaří na RVO. Vzniklý olejovitý odparek se přečistí sloupcovou chromatografií ($CHCl_3$:MeOH:TEA 9:1:0,05) za vzniku lehce nažloutlé pevné látky (Mr = 357,36) s Rf TLC 0,21.

Výtěžek: 293 mg (69 %)

Čistota podle HPLC: 96 % +

Směs pro ošetření zemědělských plodin podle předkládaného technického řešení o různých koncentracích byla připravena rozpuštěním 0,04 až 37,14 mg připraveného N^6-(3-hydroxybenzylamino)-9-(*β*)-D-2´-deoxyribofuranosylpurinu vždy v 1 ml rozpouštědla. Jako rozpouštědlo byl použit dimetylsulfoxid (DMSO), kromě tohoto lze použít i jiná organická polární rozpouštědla. Směs může obsahovat i organická i anorganická listová hnojiva obsahující makroprvky (N, P, K) a mikroprvky (S, Zn, Cu, Mo, Mo, Fe, B, Ca, Mg) ve formě solí

v jakémkoliv vzájemném mísitelném poměru těchto prvků.

Příklad 3: Příprava přípravku pro foliární aplikaci v jarním ječmeni – odrůda Francin

Směs připravená v Příkladu 1, byla naředěna vodou na výslednou koncentraci 5 µMol účinné látky v postřikové jíše. Takto připravená směs byla přímo aplikována na rostliny v podmínkách maloparcelního pokusu o velikosti parcel 10 m^2 a počtu 5 randomizovaných opakování. Varianta látky ve směsi označená jako RR-Z byla aplikována formou postřiku na list v dávce 5 µMol a celkové dávce postřikové jíchy odpovídající 300 l/ha. Pokus proběhl v podmínkách maloparcelních pokusů o velikosti parcely 10 m^2 a 5 randomizovaných opakováních.

Z výsledků uvedených v Tabulkách 1 a 2, byl zjištěn pozitivní vliv směsi obsahující látku N^6-(3-methoxybenzylamino)-9-(β)-D-2´-deoxyribofuranosylpurin na výnos zrna, počet jednotlivých odnoží, ale také výrazné zlepšení odolnosti proti chorobám pat stébel (108 a 113 % na kontrolu) a padlí travnímu (113 a 119 % na neošetřenou kontrolu).

Tabulka 1

sklizňový rok	látka / úřipravek: aplikace	dávka /ha	termín aplikace (BBCH fáze)	Průměrný výnos (t/ha při 14 % vlhk.)	% na kontrolu	počet klasů na m2	% na kontrolu	silné odnože	% na K	střední odnože	% na K	slabé odnože	% na K	odnože celkem	% na K
2017	Kontrola			7,57		660,00		2,13		1,58		2,17		5,88	
2018	Kontrola			8,53		174,40		0,85		1,75		1,20		3,80	
2017	RR-Z 5	konc. 5 mikroMol	BBCH 30 - 33	7,99	105,55	637,60	97,55	2,21	103,92	1,00	63,16	2,46	113,46	5,67	96,45
2018	RR-Z 5	konc. 5 mikroMol	BBCH 30 - 33	8,48	99,40	179,20	104,43	0,95	111,76	2,80	160,00	1,55	129,17	5,30	139,47
Průměr				**8,23**	**102,47**	**408,40**	**100,99**	**1,58**	**107,84**	**1,90**	**111,58**	**2,00**	**121,31**	**5,48**	**117,96**
2017	RR-Z 5	konc. 5 mikroMol	BBCH 39-41	8,22	108,66	658,40	103,26								
2018	RR-Z 5	konc. 5 mikroMol	BBCH 39-41	8,64	101,26	172,80	96,43								
Průměr				**8,43**	**104,96**	**415,60**	**99,85**								

Tabulka 2

sklizňový rok	látka / úřipravek: aplikace	dávka /ha	termín aplikace (BBCH fáze)	Výška porostu (cm)	% na kontrolu	ramulariová skvrnitost (choroby pat stébel)	% na K	Rhynchosporiová skvrnitost	% na K	Padli travní	% na K	rez pšeničná (hnědá rzivost)	% na K
2017	Kontrola			67,80		6,63		7,63					
2018	Kontrola			69,60		5,00				6,20		7,80	
2017	RR-Z 5	konc. 5 mikroMol	BBCH 30 - 33	67,00	100,00	6,88	103,77	7,63	100,00				
2018	RR-Z 5	konc. 5 mikroMol	BBCH 30 - 33	68,20	101,19	5,60	112,00			7,00	112,90	7,80	100,00
Průměr				**67,60**	**100,59**	**6,24**	**107,89**	**7,63**	**100,00**	**7,00**	**112,90**	**7,80**	**100,00**
2017	RR-Z 5	konc. 5 mikroMol	BBCH 39-41	67,30	100,45	7,25	109,43	7,38	96,72				
2018	RR-Z 5	konc. 5 mikroMol	BBCH 39-41	68,60	100,59	5,80	116,00			7,40	119,35	7,20	92,31
Průměr				**67,95**	**100,52**	**6,53**	**112,72**	**7,38**	**96,72**	**7,40**	**119,35**	**7,20**	**92,31**

Příklad 4: Aplikace přípravku pro foliární aplikaci v ozimé pšenici – odrůda Turandot

Přípravek vyrobený dle Příkladu 1 byl naředěn vodou na výslednou koncentraci 5 µMol v postřikové jíše. Takto připravená směs byla přímo aplikována na rostliny v podmínkách maloparcelního pokusu o velikosti parcel 10 m^2 a počtu 5 randomizovaných opakování. Varianta látky ve směsi označená jako RR-Z byla aplikována formou postřiku na list v dávce 5 µMol a celkové dávce postřikové jíchy odpovídající 300 l/ha.

Z výsledků uvedených v Tabulkách 3 a 4, byl zjištěn pozitivní vliv směsi obsahující látku N^6-(3-methoxybenzylamino)-9-(β)-D-2´-deoxyribofuranosylpurin na výnos zrna, počet jednotlivých odnoží, ale také výrazné zlepšení odolnosti proti chorobám pat stébel (108 a 113 % na kontrolu) a padlí travnímu (113 a 119 % na neošetřenou kontrolu).

Tabulka 3

						Hodnocení odnoží ve sloupkování (v BBCH 33-36)									
sklizňový rok	látka / úřipravek: aplikace	dávka /ha	forma aplikace / termín aplikace	Průměrný výnos (t/ha při 14 % vlhk.)	% na kontrolu	silné odnože	% na K	střední odnože	% na K	slabé odnože	% na K	Odnože CELKEM	% na K	Výška porostu	% na kontrolu
2018	Kontrola			6,72	100,00	1,87	100,00	0,93	100,00	3,13	100,00	5,93	100,00	89,20	100,00
2018	RR-Z 5	konc. 5 mikroMol	BBCH 25 (plné odn.)	6,74	100,31	2,27	121,43	0,60	64,29	3,40	108,51	6,27	105,62	88,20	98,88
2018	RR-Z 5	konc. 5 mikroMol	BBCH 51 (metání)	6,91	102,89									89,70	100,56

Tabulka 4

sklizňový rok	látka / úřipravek: aplikace	dávka /ha	forma aplikace / termín aplikace	počet klasů na m2	% na kontrolu	padlí travní	% na kontrolu	listové skvrnitosti	% na kontrolu	rez plevová	% na kontrolu	běloklasost	% na kontrolu	choroby pat stébel	% na kontrolu
2018	Kontrola			432,00	100,00	7,60	100,00	5,00	100,00	5,60	100,00	9,00	100,00	7,40	100,00
2018	RR-Z 5	konc. 5 mikroMol	BBCH 25 (plné odn.)	438,40	98,74	8,00	105,26	5,00	100,00	6,20	110,71	9,00	100,00	7,80	105,41
2018	RR-Z 5	konc. 5 mikroMol	BBCH 51 (metání)	444,80	101,46	7,80	102,63	5,20	104,00	6,20	110,71	9,00	100,00	7,60	102,70

NÁROKY NA OCHRANU

1. Směs pro ošetření zemědělských plodin pro zvýšení výnosu a odolnosti proti houbovým chorobám *Ramulispora herpotrichoides, Blueria graminis, Puccinia striiformis a Puccinia graminis*, **vyznačená tím**, že obsahuje alespoň jednu účinnou látku, vybranou z N^6-(3-hydroxybenzylamino)-9-(*β*)-*D*-2´-deoxyribofuranosylpurinu a N^6-(3-methoxybenzylamino)-9-(*β*)-D-2´-deoxyribofuranosylpurinu,
a polární organické rozpouštědlo, s výhodou vybrané ze skupiny zahrnující dimethylsulfoxid, polyetylenglykol, 2-(2-ethoxyethoxy)ethanol, 1-methyl-2-pyrrolidon, glycerol, manitol, kyselinu laurylovou, myristovou, palmitovou, stearovou, arachidonovou, erukovou, parafin, parafínový olej, propylenglykol, sorbitol, polyalkohol, glykol,

přičemž koncentrace účinné látky ve směsi je v rozmezí od 0,1 mM do 0,1 M.

2. Přípravek pro foliární aplikaci do zemědělských plodin, **vyznačený tím**, že obsahuje alespoň jednu účinnou látku, vybranou z
N^6-(3-hydroxybenzylamino)-9-(*β*)-*D*-2deoxyribofuranosylpurinu a
N^6-(3-methoxybenzylamino)-9-(*β*)-*D*-2´-deoxyribofuranosylpurinu,
a polární organické rozpouštědlo, s výhodou vybrané ze skupiny zahrnující DMSO, polyetylenglykol, 2-(2-ethoxyethoxy)ethanol, 1-methyl-2-pyrrolidon, glycerol, manitol, kyselinu laurylovou, myristovou, palmitovou, stearovou, arachidonovou, erukovou, parafin, parafínový olej, propylenglykol, sorbitol, polyalkohol, glykol,

a vodu,

přičemž koncentrace účinné látky v přípravku je v rozmezí od 0,1 μM do 100 μM.

3. Přípravek podle nároku 2, **vyznačený tím**, že dále obsahuje dusíkaté hnojivo a/nebo fosfor obsahující hnojivo a/nebo draselné hnojivo.

4. Přípravek podle nároku 3, **vyznačený tím**, že dusíkaté hnojivo je vybrané ze skupiny zahrnující $NH_4H_2PO_4$, $(NH_4)HPO_4$, $CO(NH_2)_2$, $Ca(NO_3)_2$, $(NH_4)_2SO_4$ a NH_4NO_3.

5. Přípravek podle nároku 3 nebo 4 **vyznačený tím**, že fosfor obsahující hnojivo je vybrané ze skupiny zahrnující $NH_4H_2PO_4$, $(NH_4)_2HPO_4$, KH_2PO_4, K_2HPO_4, $Ca(H_2PO_4)_2$, K_3PO_4, K_3PO_4, NaH_2PO_4, Na_3PO_4 a Na_2HPO_4.

6. Přípravek podle nároku 3, 4 **vyznačený tím**, že draselné hnojivo je vybrané ze skupiny zahrnující KH_2PO_4, K_2HPO_4, KCl, K_2SO_4, K_3PO_4 a K_3PO_4.

7. Přípravek podle kteréhokoliv z nároků 3 až 6, **vyznačený tím**, obsahuje alespoň jeden kation C^{m+}, vybraný z Ca^{2+}, Mg^{2+}, Li^+, Fe^{2+}, Fe^{3+}, Al^{3+}, Ag^+, Cu^{2+}, Zn^{2+}, Hg^{2+}, Pb^{2+}, Ba^{2+} a NH_4^+, přičemž kation je ve formě soli s anionem An^-, vybraným z F^-, Cl^-, Br^-, I^-, SO_4^{2-}, NO_3^- nebo $CH_3CO_2^-$.

Palacký University Olomouc

Faculty of Science

Department of Chemical Biology

Vlasta Matušková

Summary of the Doctoral Thesis

Synthesis and study of biological activity of novel purine nucleosides

P1527 Biology

1501V019 Experimental biology

Supervisor

Mgr. Karel Doležal, Dr., DSc.

Olomouc
2023

The presented Ph.D. thesis was realized at the Department of Chemical Biology, Faculty of Science, Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, the Czech Republic within the Ph.D. program of Experimental Biology, Faculty of Science, Palacký University in Olomouc (2016-2023).

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The oral defense will take place on ……..... before the Commision for the Ph.D. thesis of the Study Program Experimental Biology, seminar room, building 52, Šlechtitelů 241/27, 783 71 Olomouc. The Ph.D. thesis and expert reviews will be available 14 days before the defense in the Study Department of the Faculty of Science (Mgr. Martina 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 the Faculty of Science, Palacký University, Šlechtitelů 241/27, 783 71 Olomouc.

prof. Ing. Miroslav Strnad, CSc. DSc.
Chairman of the Commision for the Ph.D. thesis
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Content

Introduction

The early beginnings of agriculture in the so-called Neolitic Revolution around 12 000 years ago triggered the changes in man´s lifestyle from hunter-gatherers to permanent settlements. The reliable food supply from crop and animal farming has increased the population from a several thousand to today´s eight billion and projections of United Nations (www.un.org) suggest the increase up to ten billion till 2050.

The current agroecosystems are already meeting urbanized landscapes and it will be necessary to provide additional areas for growing crops, especially the territories of Africa and South America (FAO, IFAD, UNICEF, 2022; Wright, 2022). Therefore, the pressure is placed, among other issues like water supply, on obtaining the largest possible harvest of crops on the smallest possible area.

Thus, the necessity to discover the plant signaling and how to affect the processes of plant growth and development in every detail is obvious. In addition to the study and development of novel ecological fertilizers and herbicides that would not harm the landscape and organisms but still be effective enough, the study of cytokinins and other plant hormones, as well as their novel synthetic derivatives, is also important factor to achieve success in future agricultural projects.

Furthermore, the structural features of cytokinins that mimic the structure of nucleobases or nucleosides suggests, and many times already proved (Voller *et al.*, 2019), the use of these substances and their derivatives in medicine for the treatment of proliferative diseases and viral infections.

Although this work is like a needle in a haystack on a global scale, hopefully, it will contribute to the current knowledge of cytokinin derivatives.

Aims

This thesis is aiming on the synthesis of the series of variously N^6-susbtituted analogues of aromatic cytokinin benzylaminopurine as well as kinetin with attached 2′-deoxyribosyl moiety at the position N9 to compare the biological activity with the ribosides and the influence of the missing 2′-hydroxygroup.

As well as the aromatic cytokinin 2′-deoxyriboside derivatives, another aim is focused on the synthesis and the biological activity study of analogous 2′-deoxyriboside and 2′,3′-dideoxyriboside series of isoprenoid cytokinins isopentenyladenine, *trans*-zeatin and *cis*-zeatin.

1. To review the field of N^6-substituted cytokinin analogues, their synthesis and biological activity

2. The synthesis of a series of aromatic N^6-substituted cytokinin 9-(β)-D-2′-deoxyribosides

3. The synthesis of a series of isoprenoid cytokinin 9-(β)-D-2′-deoxyribosides

4. The synthesis of a series of isoprenoid cytokinin 9-(β)-D-2′,3′-dideoxyribosides

5. Evaluation of the biological activity of all prepared drivatives with available assays and bioassays

6. Drawing conclusions based on structure-activity-relationship between the studied compounds and relative ribosides and bases

Material and methods

The employed methods and relevant procedures as well as characterizations of appropriate prepared compounds are described in detail in the attached publications and the relative supporing informations.

General procedures

The starting materials and solvents were obtained from commercial suppliers (Sigma Aldrich®, TCI Chemicals®, VWR®, Jena Bioscience® or OlChemIm®). The organic solvents were evaporated on a rotary evaporator Heidolph® below 45 °C.

The reaction processes were controlled by thin layer chromatography (TLC) using silicagel $60WF_{245}$ plates (Merck®). Generally, $CHCl_3$/MeOH (9:1 or 3:1, v/v), $CHCl_3$/MeOH/NH_3 (9:1:0,05,v/v), toluene/acetone; (1:1, v/v), hexane/ethyl acetate (1:1, v/v) or EtOAc/MeOH/NH_3 (34:4:2, v/v) were used as the mobile phases. Davisil R LC60A 40–63 μm silica gel was used for purification of compounds by column chromatography.

The prepared compounds were characterized using an Alliance 2695 separations module (Waters®) linked to UV-VIS PDA 2996 detector (Waters®) and Q-Tof micro (Waters®) benchtop quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer. First, samples were dissolved in methanol and diluted with the mobile phase to 10 $\mu g.mL^{-1}$. Afterwards, 10 μL of the solution were injected onto a RP-column (150 × 2.1 mm; 3.5 μm; Symmetry C18, Waters®) which was kept in a thermostat at 25 °C. Solvent (A) consisted of 15 mM formic acid adjusted to pH 4.0 by ammonium hydroxide. Methanol was used as the organic modifier (solvent B). The effluent was introduced into the DAD (diode array detector; scanning range 210–400 nm with 1.2 nm resolution) equipped with an electrospray source (source temperature: 120 °C for positive mode, capillary voltage +3.0 kV, cone voltage +20 V, desolvation temperature 300°C). Nitrogen was used as both the desolvation gas (500 $L \cdot h^{-1}$) and cone gas (50 $L \cdot h^{-1}$). The mass spectrometer was operated in positive (ESI+) ionization mode. Data were acquired over the 50–1000 m/z range (fullscan mode).

For preparative purification of several compounds, HPLC-MS chromatography (Agilent 1290 Infinity II) coupled to UV-VIS and a mass detector (Agilent InfinityLab LC/MSD) with an Agilent column (5Prep-C18 5x21.2 mm) was used. Exact mass was determined by QTOF-MS (Synapt G2-Si, Waters®, UK) operating in positive ion mode.

Melting points were measured on the Büchi B-540 analyzer and elemental analysis was performed using an analyzer EA1112 CHN (Thermo Finnigan).

The Jeol 500 SSspectrometer at a temperature of 300 K and frequencies of 500.13 MHz (^{1}H NMR) and 125.03 MHz (^{13}C NMR) was employed for NMR spectra measurement. Tetramethylsilane (TMS) was used as an internal standard and DMSO-d^6 and $CDCl_3$ were used as solvents for the analysis (NMR spectra were calibrated against the residual solvent signal).

General synthetic procedures

The method using Castro's reagent was employed for the synthesis of all compounds of interest. Another methods were also performed, however, they were not as convenient as this method.

One-step method using Castro's reagent (BOP**)** (Wan *et al.*, 2005, 2007)

2′-Deoxyinosine (2′-deoxy-9-(*β*)-D-inosine; *M*r 252.23; 1 eq) or 2′,3′-dideoxyinosine (2′,3′-dideoxy-9-(*β*)-D-inosine; *M*r 236.23; 1 eq) and BOP ((benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; *Mr* 442.28; 1.2 eq) were dissolved in dry DMF (2 mL) under inert atmosphere (N_2) with DIPEA (1.5 – 5 eq) and after 5 min appropriate amine (1.2 – 4 eq) was added. The reaction was stirred at rt for 24 h under N_2 and monitored by TLC. The mixture was then evaporated (and co-evaporated with *n*-heptane) and with/without extraction (water/ethylacetate) pre-purified by column chromatography and then purified with preparative HPLC-MS chromatography or precipitated.

The specific reaction, TLC, precipitation or purification conditions are described in appropriate publications (Matušková *et al.*, 2020, 2023).

The method using the preparation of 6-chloro-2′-deoxy-9-(*β*)-D-ribofuranosylpurine for nucleophilic substitutions at the position 6

3′,5′-Di-O-acetyl-2′-deoxyadenosine (Seela, Herdering and Kehne, 1987)

2′-Deoxyadenosine monohydrate (2.2 g; 8 mmol) was dissolved in pyridine (26 mL) and acetanhydride (8 mL) was added dropwise at room temperature. Next, the reaction mixture was cooled in a crushed ice bath and DCM (100 mL) was added, followed by extraction with 10% aqueous $NaHCO_3$ (24 mL) and then water (20 mL). The organic solvent was evaporated and three times coevaporated with toluene to give a dry residue, which was then dissolved in absolute ethanol and crystallized in a refrigerator overnight.

6-Chloro-2′-deoxy-9-(β)-D-ribofuranosylpurine (Francom and Robins, 2003)

3′,5′-Di-*O*-acetyl-2′-deoxyadenosine (2 g; 5.8 mmol) in anhydrous DCM (150 mL) was placed into a dried three-neck flask under a nitrogen atmosphere (balloon). While stirring, the suspension was cooled in an ice bath to 0 °C and TMS-Cl (7.4 mL; 58 mmol) was added dropwise through the septum. Afterwards, a solution of *tert*-butyl nitrite (3.5 mL; 29 mmol) in dry DCM (30 mL) was slowly added and the reaction mixture was stirred for 2 h at 0 °C and then at room temperature overnight. Then, DCM (300 mL) was added and the organic phase was washed with 5% aqueous $NaHCO_3$ (2 × 300 mL), water (3 × 100 mL) and brine (3 × 100 mL), dried over powdered Na_2SO_4 and evaporated as a yellow oil (1.6 g). The residue was without further purification deprotected in 5% solution of NH_3 in methanol (30 mL) at 0 °C to give 6-chloro-2′-deoxy-9-(*β*)-D-ribofuranosylpurine (HPLC-MS of the residue: 77.7%). Column chromatography purification ($CHCl_3$/MeOH (9:1), v/v) was proceeded to obtain white-yellow solid product (0.7 g). The resulting 6-chloro-2′-deoxy-9-(*β*)-D-ribofuranosylpurine was subsequently used for nucleophilic substitutions with variably substituted benzylamines in *n*-propanol with base (TEA).

Following reactions were used, according to the literature, to the synthesis of *6-chloro-2′*,3´-*dideoxy-9-(β)-D-ribofuranosylpurine* ready for further nucleophlic substitution with amines, however, the laborious pathway led to the minimal yield of *β*-anomer. Nevertheless, *α-t*ZddR was prepared.

Multistep synthesis of *trans*-zeatin-9-(*α*)-D-2´,3´-dideoxyriboside (*α-t*ZddR)

(S)-γ-Butyrolactone-γ-carboxylic acid (**A**)

According to the literature (Okabe *et al.*, 1988), L-glutamic acid (1.0 mol; 147 g) was dissolved in H_2O (500 mL), cooled in ice-bath to 15 °C and solution of 5.6 M HCl (1.4 mol; 250 mL) was added dropwise to adjust pH 1. Then, the solution of $NaNO_2$ (1.5 mol; 104 g) in H_2O (144 mL) was added during 4h at 15-20 °C and the reaction mixture was stirred at rt overnight. Next day, the reaction mixture was controlled by TLC (toluene/acetone; 1:1; ninhydrin staining) which demonstrated full conversion of L-glutamic acid. The reaction mixture was co-evaporated with toluene and the white oil-solid residue (243 g) was diluted with EtOAc (1 L) and dried over powdered Na_2SO_4 (100 g). The precipitates were removed by filtration and washed by EtOAc. The combined organic extracts were stirred with 200-400 mesh H+ form (25 g) for 30 min to remove residual amino acid. After filtration and evaporation, the yellow oily residue was dried by evaporation with toluene (4 x 100 mL). The waxy residue (116 g) was dissolved in Et_2O

(200 ml), stirred at rt for 1h and cooled in the refrigerator overnight. The precipitate was filtered to give a white solid: *M*r 130.10; 91 g; yield 70 %. The structure was confirmed by elemental analysis, NMR (500 Hz; $CDCl_3$), GC-MS and melting point (62.6-63.2 °C).

(S)-γ-(Hydroxymethyl)-γ-butyrolactone (Okabe *et al.*, 1988) (**B**)

Borane-dimethylsulfide complex (Corey, Bakshi and Shibata, 1987) (BMS; 0.15 mol; 12.23 mL) was added in to (*S*)-γ-butyrolactone-γ-carboxylic acid (**A**, *M*r 130.10; 0.115 mol; 15 g) in dry THF (70 mL) during 40 min under 40°C (ice bath). The reaction was stirred for 2h at rt and quenched by MeOH (25 mL) while cooling. MeOH was evaporated 3 times (to remove formed trimethylborate) to give a light yellow oil: *M*r 116.12; 13.7 g; yield 102 %. The reaction was monitored by TLC (toluene/acetone; 1:1, v/v) and the structure was confirmed by NMR analysis.

(S)-γ-[[(Tert-butyldimethylsilyl)oxy]methyl]-γ-butyrolactone (Okabe *et al.*, 1988) (**C**)

(*S*)-γ-(hydroxymethyl)-γ-butyrolactone (**B**, *M*r 116.12; 0.11 mol; 13 g) and imidazole (0.14 mol; 9.7 g) in dry DCM (55 mL) were cooled to 0 °C under inert N_2 atmosphere. TBDMS-Cl (0.13 mol; 20 g) was added and the mixture was stirred 15 min at 0 °C and then 2h at rt. The solution was poured into cold H_2O (1 L) and 300 mL of DCM was added, and the water layer was washed with DCM (2 x 100 mL). Combined organic layers were washed with H_2O (2 x 400 mL) and brine (200 mL), dried with Na_2SO_4 overnight and evaporated to give light yellow oil: *M*r 230.38; 26 g; yield 100 %. For TLC mobile phase was hexane/EtOAc 3:1 with staining by 5% solution of H_2SO_4 in EtOH. The structure was confirmed by NMR analysis.

(S)-γ-[[(Tert-butyldimethylsilyl)oxy]methyl]-γ-butyrolactol (Okabe *et al.*, 1988) (**D**)

DIBAL in THF was slowly added during 1 h to (*S*)-γ-[[(tert-butyldimethylsilyl)oxy]methyl]-γ-butyrolactone (**C**, *M*r 230.38; 5 g) in THF under inert N_2 atmosphere, cooled by dry ice and acetone to -74 °C. The temperature was kept at -68 °C. After stirring for 5 min and TLC control (hexane/EtOAc 3:1) the reaction mixture was quenched by MeOH (5 mL) and the temperature was left to raise to rt. EtOAc (85 mL) and saturated $NaHCO_3$ (4.5 mL) were added and the mixture was stirred 3.5 h. Then Na_2SO_4 (25 g) was added and the mixture was stirred overnight. The solution was filtered, washed with EtOAc and the filtrate concentrated to give a colorless oil residue: *M*r 232.40; 5 g; yield 99 %. The structure was confirmed by NMR analysis.

6-Chloro-9-((5S)-5-(((isopropyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-9H-purine; *6-Cl-ddPR-OTBDMS* (Mitsunobu reaction) (Mitsunobu, 1981; Seio *et al.*, 2017) (**E**).

The solution of (*S*)-γ-[[(tert-butyldimethylsilyl)oxy]methyl]-γ-butyrolactol (**D**), *M*r 232.40; 11.7 mmol; 2.71 g) with 6-chloropurine (9.7 mmol; 1.5 g) in THF or DMF (0.07 M solution of 6-chloropurine obtained with 140 mL of THF and 10 mL of DMF) under inert N_2 atmosphere was cooled to 15 °C (ice bath). Then triphenylphosphine (14.5 mmol; 3.8 g) and DIAD (14.7 mmol; 2.9 ml) were added and the reaction was stirred for 1h (change of colour) and monitored by TLC (hexane/EtOAc, 1:1, v/v). The reaction mixture was then evaporated and co-evaporated with toluene and diluted with toluene (30 ml) to give a brown oil which was cooled overnight to precipitate $POPh_3$ (white solid). After filtration and washing with toluene, the crystallization process was repeated and the crude product (anomeric mixture) was roughly purified by column chromatography (hexane/EtOAc 1:1) to give 2 g of thick to solid yellow oil (a mixture of *α* and *β* anomer in favour of one anomer, according to the TLC). The HPLC analysis with acidic mobile phase (pH 4) and the cone voltage 20 eV led to molecule breakage and 6-chloropurine was observed in high percentage even if TLC analysis showed no visible amount of 6-chloropurine. Therefore, the conditions for HPLC analysis were adjusted to pH 5.3 of the mobile phase and the cone voltage set to 10 eV to lower the molecule breakage throughout the analysis. There were several attempts to purify each anomer by column chromatography, but due to the close Rf of both products the separation was unsuccessful. Elemental analysis of anomeric mixture was proceeded: calculated %: N(15.19), C(52.09), H(6.83); the first measurement N(14.88), C(51.77), H(7.51) and the second measurement N(14.90), C(51.46), H(7.57) and melting temperature range 77.4 – 77.9 °C. Preparative HPLC-MS was proved to be the best tool for the separation of individual anomers, but we decided to firstly nucleophilically substitute the position 6-Cl.

(E)-4-((9-((5S)-5-(((Isopropyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-9H-purin-6-yl)amino)-2-methylbut-2-en-1-ol; *tZ-ddPR-OTBDMS* (**F**)

6-Chloro-9-((5*S*)-5-(((isopropyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-9*H*-purine (**E**, *M*r 368.94; 0.9 mmol; 334 mg) and (*2E*)-2-methyl-4-aminobut-2-en-1-ol hydrochloride (Mr 137.61 g/mol; 1.4 mmol; 187 mg) were stirred for 5 min in *n*-propanol (6 mL) under inert N_2 atmosphere. DIPEA (2.7 mmol; 465 μL) was then added and the mixture was refluxed in a sealed tube and stirred for 5 h. The reaction was monitored by TLC (EtOAc/hexane; 6:1, v/v) and upon completion, the mixture was evaporated to give a yellow oil: *M*r 433.63; 393 mg. Due

to the close Rf of each anomers, the crude residue was used in the next deprotection without further purification.

Trans-zeatin-9-(α)-D-2′,3′-dideoxyriboside (α-*t*ZddR)

The anomeric mixture of the protected product (**F**), *M*r 433.63; 0.9 mmol; 393 mg) was dissolved in dry THF (20 mL) and while cooling, TBAF trihydrate (0.9 mmol; 286 mg) in THF (10 mL) was slowly added, the mixture was stirred at rt for 2.5 h and controlled by TLC (hexane/EtOAc 1:1 or chloroform/MeOH 9:1). The deprotected anomeric mixture was evaporated and purified by column chromatography (approximately 3:1; 93 % HPLC-UV) and the anomer with the greater yield was successfully separated by preparative HPLC-MS (Mr 319.37, to give a pale-yellow oil, 165 mg; yield 57 % of the last step; HPLC-UV purity >99 %). The structure was characterized by HRMS, and NMR. The 2D-NMR experiment NOESY found that the structure probably corresponds to the α anomer due to the presence of $-CH_2$-OH group of the ribose moiety as well as the purine scaffold in the *cis* position towards the planar sugar ring.

Biological activity testing

Cytotoxicity evaluation

The resazurin reduction assay was used to evaluate the effects of 72 h treatment with the compounds on the viability of human skin fibroblasts BJ, keratinocytes HaCaT and retinal pigment epithelium cells ARPE-19. The cell lines were obtained from the American Type Culture Collection, Manassas, VA, USA (BJ, ARPE-19) and the German Cancer Research Center (*DKFZ*), Heidelberg, Germany (HaCaT). The cells were maintained in DMEM (BJ, HaCaT) or DMEM/F12 (ARPE-19) culture medium (Sigma) supplemented with fetal bovine serum (10%), glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) under standard cell culture conditions (37 °C, 5% CO_2, humid environment) and sub-cultured two or three times a week. For testing, about 5000 cells were seeded into each well of a 96-well plate about 24 h before treatment with the test compounds at six concentrations up to 100 µM or DMSO. The final concentration of DMSO did not exceed 0.1%. An 11x concentrated solution of resazurin in DMEM medium was added to the cells to a final concentration of 100 µM after 72 h. Fluorescence intensity (λ ex/em = 570/610 nm) was measured after 3 h incubation.

The *in vitro* assay was performed at the Department of Experimental Biology, Faculty of Science, Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, Czech Republic under the supervision of Mgr. Jiří Voller, Ph.D.

Cytokinin bioassays

Amaranthus bioassay

Amaranthus caudatus (L.), 1753 var. atropurpurea seeds were sterilized on their surface, placed on a Petri dish containing paper tissue soaked with deionized H_2O 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 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 Petri 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.

The *Amaranthus* bioassay was performed by Jana Balonová (compound series from **Publication I**) and Mgr. Hana Vylíčilová, Ph.D. (compound series from **Publication II**) at the Department of Chemical Biology, Faculty of Science, Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, Czech Republic.

Wheat leaf senescence bioassay

Wheat seeds (*Triticum aestivum* (L.), 1753 cv. Aranka) were washed with 96% EtOH and H_2O and 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 to give a total weight of 0.1 g (±1 mg) per well, immersed by the basal part in a well containing solution of the test compound (DMSO solution diluted in deionized H_2O, 150 µl/well), and cultivated in a closed box at 24 °C for 96 h in the dark. Residual chlorophyll was extracted by heating the leaf material in 5 ml of 80% EtOH 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 and extracts of leaves cultivated in deionized H_2O with DMSO (0.002%, v/v).

The wheat leaf senescence bioassay was performed by Jana Balonová (compound series from **Publication I**) and Mgr. Hana Vylíčilová, Ph.D. (compound series from **Publication II**)

at the Department of Chemical Biology, Faculty of Science, Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, Czech Republic.

Tobacco callus bioassay

Cytokinin-dependent tobacco callus cells (*Nicotiana tabacum* (L.), 1753 cv. Wisconsin 38) were cultivated on solid MS medium (3 ml/well) containing different concentrations of the test compound (from 10^{-9} to 10^{-4} M solution in DMSO) in 6-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) or the respective cytokinin base.

The tobacco callus bioassay was performed by Jarmila Balonová (compound series from **Publication I**) and Mgr. Hana Vylíčilová, Ph.D. (compound series from **Publication II**) at the Department of Chemical Biology, Faculty of Science, Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, Czech Republic.

Bacterial receptor cytokinin assay (Cytokinin signaling assay)

Transgenic *E. coli* strains KMI001 harboring a PINIII/AHK4 or pSTV28/AHK3 plasmid and expressing the *β*-galactosidase gene (*ΔrcsC, cps::lacZ*) under the control of cytokinin receptors were used for this assay. Expression of the *β*-galactosidase gene was induced by cultivation of 200 μL of the precultures diluted by M9 medium with antibiotic (1:600) and test compound (50, 10, 1 or 0.1 μM) with shaking (450 rpm) at 25 °C for 17 h. At the end of the incubation period, 50 μL of the bacterial cultures was transferred to a new 96-well plate and the activity of *β*-galactosidase was determined by measuring the fluorescence intensity (λex/em − 365/460 nm) after incubation with 2 μL of 10 mM (25 mM for AHK3) chromogenic substrate (MUG) at 37 °C for 10 min (AHK4) or 30 min (AHK3) and addition of 100 μL of Stop buffer (132 mM glycine, 83 mM Na_2CO_3).

The *in vitro* assay was performed by Mgr. Zuzana Pěkná under the supervision of Mgr. Lukáš Spíchal, Ph.D. (compound series from **Publication I**) and Mgr. Hana Vylíčilová, Ph.D. (compound series from **Publication II**) at the Department of Chemical Biology, Faculty of Science, Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, Czech Republic.

Competitive ligand binding assay

The *in vitro* assay using the *Escherichia coli* BL21(DE3) strain expressing cytokinin receptor AHK2, AHK3 and CRE1/AHK4 (kindly provided by Thomas Schmülling) to cytokinin competitive ligand binding of compounds from **Publication II.** with [^{3}H] *trans*-zeatin, as was reported previously (Yamada *et al.*, 2001), was performed by Mgr. Hana Vylíčilová, Ph.D. at the Department of Chemical Biology, Faculty of Science, Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, Czech Republic.

Antioxidant capacity testing

The *in vitro* assay examining the total phenolic content (TPC) expressed as gallic acid equivalents of compounds from **Publication I**. was performed by Mgr. Jana Slobodianová at the Laboratory of Growth Regulators, The Czech Academy of Sciences, Institute of Experimental Botany and Palacký University, Šlechtitelů 241/27, 783 71 Olomouc, Czech Republic under the supervision of Mgr. Jiří Grúz Ph.D.

Antiviral activity studies

The experimental procedures to examine the antiviral activity of compounds from **Publication I.** were performed by RNDr. Katarína Briestenská, Ph.D. at the Institute of Virology, Biomedical Center SAS, Dúbravská cesta 9, 845 05 Bratislava, Slovak Republic under the supervision of and Prof. RNDr. Jela Mistríková, DrSc.and are described in detail in the **Publication I.**

Survey of published results

Publication I.

Matušková, V., Zatloukal, M., Voller, J., Grúz, J., Pěkná, Z., Briestenská, K., Mistríková, J., Spíchal, L., Doležal, K., Strnad, M.: **New aromatic 6-substituted 2′-deoxy-9-(*β*)-D-ribofuranosylpurine derivatives as potential plant growth regulators**. *Bioorganic & Medicinal Chemistry*. 2019; 28. 115230.

A series of 33, when compared with respective ribosides non-cytotoxic, N^6-substituted aromatic 9-(*β*)-D-2′-deoxyribofuranosylpurines was prepared using the reaction of unprotected 2′-deoxyinosine with Castro′s reagent (BOP) (**Scheme 7**) and appropriate amine under mild conditions. Although one different synthetic approach was also exmined – the nonaqueous diazotiation/dediazoniation of protected 2′-deoxyadenosine resulting to the protected 6-chloroderivative. The second method was not as convenient as the BOP method, however, prepared in larger scale it would be a great starting material after deprotection for further nucleophilic substitutions of various amines in one step.

Despite the lower yields, the library of compounds was tested in classical cytokinin bioassays (*Amaranthus,* tobacco callus and wheat leaf senescence (WLSA)) and compared with BAP as well as with BAP-2′-deoxyriboside. Surprisingly, the presence of 2′-deoxyribosyl moiety generally contributed to the anti-senescence activity of the compounds. The 3-hydroxy-benzylaminopurine-9-(*β*)-D-2′-deoxyriboside (3-OH-BAPdR), 3-methoxy-benzylaminopurine-9-(*β*)-D-2′-deoxyriboside (3-OMe-BAPdR) and 4-fluoro-benzylaminopurine-9-(*β*)-D-2′-deoxyriboside (4-F-BAPdR) delayed the senescence approximately 3-times more than BAP. 3-OH-BAPdR and 3-OMe-BAPdR had also great results in *Amaranthus* and tobacco callus assays comparable to that of BAP, however, with different behaviour at highest concentrations (> 10^{-5} M) than BAP which did not result in dramatic drop of activity due to the toxic effects.

When the AHK2, AHK3 and CRE1/AHK4 receptor activation was examined none of the tested 2′-deoxyribosides approached the activity of the standard *trans*-zeatin. Therefore, cytokinin receptor activation by aromatic purine-2′-deoxyribosides was not observed but the activity in cytokinin assays was obvious.

In the study of the antioxidant properties of compounds, the presence of phenolic hydroxygroup positively contributed, especially 2-OH, 4-OH, 2-OH-3-OMe, 4-OH-3-OMe or 3-OH-4-OMe.

Furthermore, the opportunity to test the series in Vero cells infected by murine herpesvirus (MHV-68) led, unfortunately, to no interesting result, nevertheless, the potential antivirotic activity towards another viruses would be advantageous to examine and the non-cytotoxicity of these substances is beneficial.

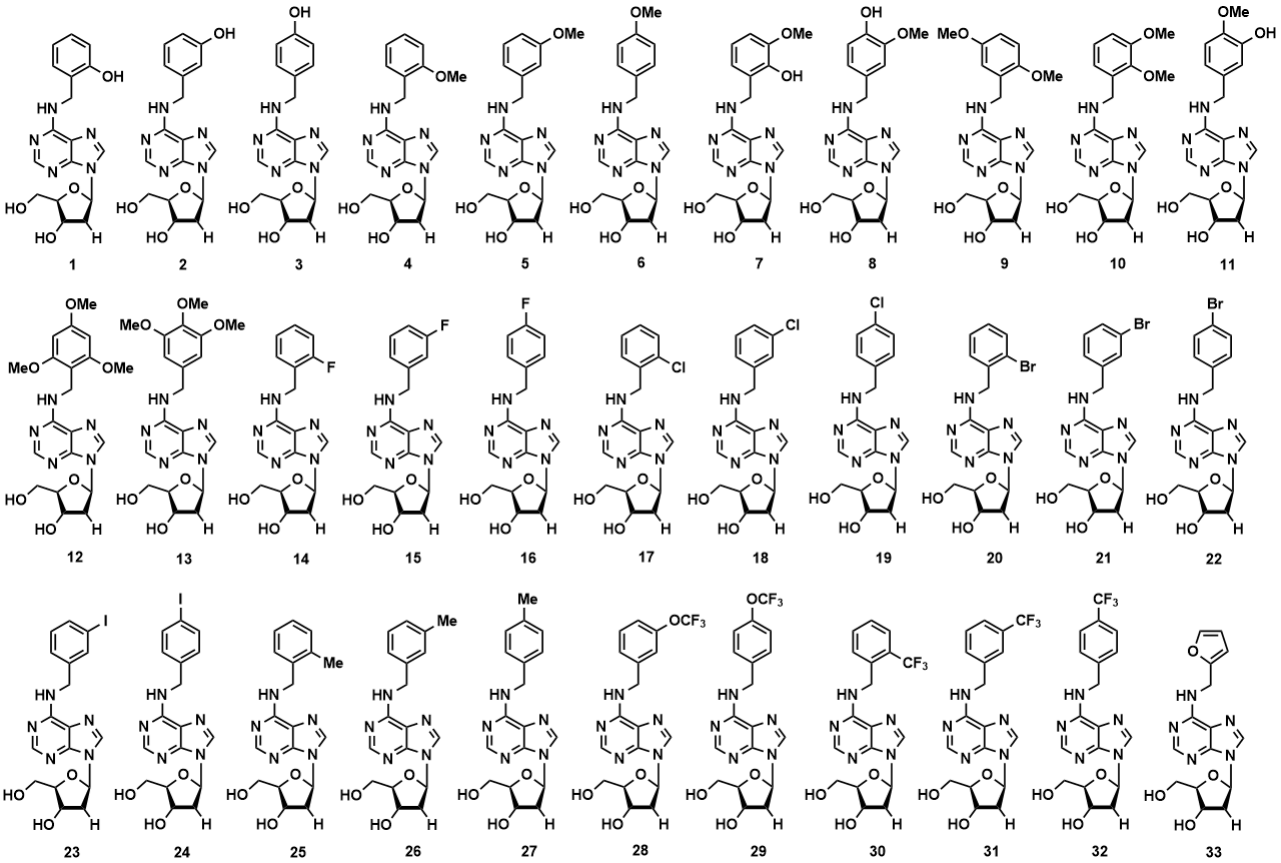

Fig. 1: Prepared and evaluated compounds in **Publication I**

Publication II.

Matušková V., Zatloukal, M., Pospíšil, T., Voller, J., Vylíčilová, H., Doležal, K., Strnad, M.: **From synthesis to the biological effect of isoprenoid 2'-deoxyriboside and 2',3'-dideoxyriboside cytokinin analogues**. *Phytochemistry*. 2023; 205:113481.

The commercially available isoprenoid cytokinin bases and (*β*)-D-ribosides (iP, *t*Z, *c*Z, iPR, *t*ZR, *c*ZR) were examined together with prepared analogous series of isoprenoid (*β*)-D-2′-deoxyribosides and (*β*)-D-2′,3′-dideoxyribosides (iP-2′-dR, *t*Z-2′-dR, *c*Z-2′-dR, iP-2′,3′-ddR, *t*Z-2′,3′-ddR, *c*Z-2′,3′-ddR) in various bioassays. The synthetic approach using the Castro´s reagent was employed starting from 2′-deoxyinosine to form 2′-deoxyribosides and from 2′,3′-dideoxyinosine to form 2′,3′-dideoxyribosides. Another laborious synthetic pathways were

employed, however, the one-step method proved its usefulness. Nevertheless, the α-anomer of *t*Z-2′,3′-ddR was prepared and also tested in all assays.

In the bacterial receptor assay, as well as in the *ARR5::GUS* assay or ligand binding assay, the receptor activation trend played in favor of bases as follows: base > riboside > 2′-deoxriboside > 2′,3′-dideoxyriboside proving the generally decreasing cytokinin signaling activity connected with the removal of 2′ and 3′ hydroxygroup from the sugar moiety.

From the prepared series only iP-2′,3′-dideoxyriboside considerably caused the moderate root growth inhibition in comparison with *c*Z-2′,3′-dideoxyriboside and *t*Z-2′,3′-dideoxyriboside, 2′-deoxyribosides did not exhibit any activity in this assay; only iP, *t*Z and their ribosides inhibited primary root growth as a result of cytokinin action.

Emphasizely, the betacyanin production in *Amaranthus* bioassay as well as the callus growth were highly supported by isoprenoid 2′-deoxyribosides which can be partly influenced by the N9-substituent hydrolysis. Nevertheless, the WLSA assay did not show any significant effect of isoprenoid 2′-deoxyribosides or 2′,3′-dideoxyribosides.

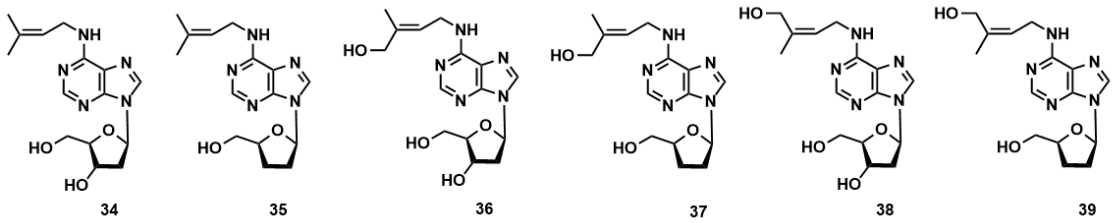

Fig. 2: Prepared and evaluated compounds in **Publication II**

Publication III.

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(6):832.

The review summarizes the role of artificial and endogenous N9-substituted cytokinin conjugates in plants. The advances in knowledge of 9-substituted cytokinin conjugates from the discovery to current state-of-art are described. The emphasis is placed on the efforts toward enhancement of the efficiency of novel derivatives in agriculture and crop production or in anticancer or antiviral therapies.

Various known cytokinin conjugates and their historical evolution as well as practical outcomes, activation or inactivation, are described: N7- and N9-glucosides; isoprenoid and aromatic N9-ribosides; N9-2′-deoxyribosides; N9-arabinosides; cytokinin disaccharide

conjugates or N9-non-sugar substituted analogues including N9-tetrahydropyranyl derivatives are described in details.

The development of novel cytokinin compounds based on the current knowledge and obtaining the new information can lead to syntheses of substances with predetermined properties in certain type of plant and thus the success in cultivation.

Publication IV. (Utility model)

Matušková, V., Zatloukal, M., Koprna, R., Doležal, K.: **Směs pro ošetření zemědělských plodin pro zvýšení výnosu a odolnosti proti houbovým chorobám a přípravek pro foliární aplikaci, obsahující tuto směs**. Užitný vzor (Utility model) CZ 32628 U1, 2018.

The utility model presents the use of substances 3-hydroxy-benzylaminopurine-9-(*β*)-D-2′-deoxyriboside (3-OH-BAPdR) and 3-methoxy-benzylaminopurine-9-(*β*)-D-2′-deoxyriboside (3-OMe-BAPdR) in a mixture with macronutrients (N, P, K) and micronutrients in foliar application to agricultural crops (spring barley *Francin* and winter wheat *Turandot*) and shows an increase in yield and resistance to fungal diseases based on field trials.

After the dissolution of substance in 1 mL of DMSO the solution was diluted to the final concentration of 5 μM and foliary applied on the field area of 10 m^2 (in 5 repetitions).

Conclusion and perspectives

The library of the 33 N^6-variously substituted aromatic cytokinin 9-(β)-D-2′-deoxyribosides was synthesized using predominantly one-step approach utilizing the Castro′ reagent. The biological activity was evaluated and compared with the respective bases and ribosides. Despite the inability to activate cytokinin receptors many of these substances succeeded in wheat leaf senescence, tobacco callus and *Amaranthus* bioassays and showed higher activity than the standard benzylaminopurine. The antiviral activity towards murine herpesvirus has not been proved, however further study should yield new knowledge.

The series of isoprenoid cytokinin 9-(β)-D-2′-deoxyribosides and 9-(β)-D-2′,3′-dideoxyribosides derived from iP, *t*Z and *c*Z was also synthesized and the biological effects were compared with the respective bases and ribosides. Similarly to the aromatic 2′-deoxyribosides, the activation of cytokinin receptor was not significant for isoprenoid 2′-deoxyriboides and 2′,3′-dideoxyribosides. Nevertheless, the outcome in bioassays was significant, especially in *Amaranthus* and tobacco callus bioassays.

The aromatic 2′-deoxyribosides, as well as isoprenoid 2′-deoxy- and 2′,3′-dideoxy-derivatives were prepared by convenient one-step method but different approaches were also employed.
In both cases, the possible partial hydrolysis of the sugar moiety during bioassays has to be mentioned.

All the prepared 2′deoxyribosides and 2′,3′-dideoxyribosides are non-cytotoxic and thus suitable for further (e.g. antiviral) examination.

List of publications

I. **Matušková, V**., Zatloukal, M., Voller, J., Grúz, J., Pěkná, Z., Briestenská, K., Mistríková, J., Spíchal, L., Doležal, K., Strnad, M.: New aromatic 6-substituted 2´-deoxy-9-(*β*)-D-ribofuranosylpurine derivatives as potential plant growth regulators. *Bioorganic & Medicinal Chemistry*. 2019; 28. 115230. 10.1016/j.bmc.2019.115230

II. **Matušková V**., Zatloukal, M., Pospíšil, T., Voller, J., Vylíčilová, H., Doležal, K., Strnad, M.: From synthesis to the biological effect of isoprenoid 2'-deoxyriboside and 2',3'-dideoxyriboside cytokinin analogues. *Phytochemistry*. 2023; 205:113481. 10.1016/j.phytochem.2022.113481. PMID: 36283448.

III. 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(6):832. 10.3390/biom10060832

IV. **Matušková, V**., Zatloukal, M., Koprna, R., Doležal, K.: Směs pro ošetření zemědělských plodin pro zvýšení výnosu a odolnosti proti houbovým chorobám a přípravek pro foliární aplikaci, obsahující tuto směs. Užitný vzor (Utility model) CZ 32628 U1, 2018.

Published abstracts

Posters:

- Synthesis and biological activity of N^6-substituted 2′-deoxy-9-(*β*)-D-ribofuranosylpurine derivatives: *International conference G4G (Green for Good) IV*, Olomouc, Czech Republic 19. – 22. 6. 2017
- Synthesis and biological activity of N^6-substituted 2′-deoxy-9-(*β*)-D-ribofuranosylpurine derivatives: *1st Alpine Winter Conference on Medicinal and Synthetic Chemistry*, Sankt Anton, Austria 28. 1. – 1. 2. 2018
- Biological activity of novel N^6-substituted purine 2′-deoxy-9-(*β*)-D-ribosides: *International conference G4G (Green for Good) V*, Olomouc, Czech Republic 10. – 13. 6. 2019
- Cytokinin and anti-senescence activity of novel N^6-substituted purine 2′-deoxy-9-(*β*)-D-ribosides: The 23rd international conference on plant growth substances, Paris, France 25. – 29. 6. 2019

Presentations:

- Synthesis and biological activity of N^6-substituted 2′-deoxy-9-(*β*)-D-ribofuranosylpurine derivatives: International conference *Chemistry and Biology of Phytohormones and Related Substances 2017*, Kouty nad Desnou, Czech Republic 21. – 23. 5. 2017
- Synthesis and biological evaluation of new purine nucleosides: International konference *Chemistry and Biology of Phytohormones and Related Substances 2018*, Luhačovice, Czech Republic 24. – 26. 5. 2018
- Biological activity of novel purine nucleosides: International konference *Chemistry and Biology of Phytohormones and Related Substances 2019*, Luhačovice, Czech Republic 19. – 21. 5. 2019

Souhrn (in Czech)

Název disertační práce: Syntéza a stadium biologické aktivity nových purinovách nukleosidů

Převážně jednokrokovou syntézou využívající Castrovo činidlo byly připraveny dvě série cytokininových derivátů: 33 N^6-substituovaných 2´-deoxyribosidů odvozených od benzylaminopurinu (BAP) (**1** - **33**) a série 2´-deoxyribosidů a 2´,3´-dideoxyribosidů odvozených od isoprenoidních cytokininů isopentenyladeninu, *trans*-zeatinu a *cis*-zeatinu (**34** - **39**).

Všechny tyto deriváty byly v biologických testech porovnány s příslušnými bázemi a ribosidy. Navzdory nezřetelné schopnosti aktivovat cytokininové receptory AHK2, AHK3 a CRE1/AHK4 vykazovaly zejména některé aromatické 2´-deoxyribosidy významnou anti-senescenční aktivitu a také odlišné chování v kalusovém a amarantovém biotestu v porovnání s BAP.

Antivirová aktivita řady benzylaminopurinových analog na herpesvirus MHV-68 nebyla potvrzena.

Isoprenoidní 2´-deoxyribosidy byly úspěšné v amarantovém a kalusovém biotestu, ale 2´,3´-dideoxyribosidy nedosahovaly významných výsledků v provedených testech.

Nebyla prokázána toxicita jak aromatických 2´-deoxyribosisů tak isoprenoidních 2´-deoxyribosidů a 2´,3´-dideoxyribosidů na nádorových i nenádorových buňkách.

Byly vyselektovány dvě látky ze série aromatických 2´-deoxyribosidů (**2** a **5**), u kterých byl prokázán pozitivní vliv na zemědelský výnos jarního ječmene a ozimé pšenice prostřednictvím polních experimentů.

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