PALACKÝ UNIVERSITY OLOMOUC
FACULTY OF SCIENCE

LABORATORY OF GROWTH REGULATORS
&
DEPARTMENT OF BOTANY

Michal Karády

New Methods of Qualitative and Quantitative Analysis of Cytokinins

Summary of the Ph.D. thesis

P 1501 BIOLOGY – BOTANY

Supervisor – Mgr. Karel Doležal, Dr.

Olomouc
2013

Acknowledgements

The research performed and presented in this thesis was carried out at the Laboratory of Growth Regulators, Palacky University, under the supervision of Mgr. Karel Doležal, Dr.. His support, encouragement and patience are greatly appreciated. I would also like to thank prof. Miroslav Strnad, the head of the laboratory, for the material support, doc. Petr Tarkowski and Mgr. Ondrej Novak for excellent scientific and technical assistance and all my colleagues for creating a friendly and supportive environment.

Olomouc, January 2013

Content

Declaration I

Hereby I declare that the Ph.D. thesis is my original work. The literature used is listed in the Supplements section.
In Olomouc, 31.1.2013

Michal Karády

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals I-IV. The papers are appended at the end of the thesis in the Supplement section.

I. Mala J, Machova P, Cvrckova H, **Karady M**, Novak O, Mikulik J, Hauserova E, Greplova J, Strnad M, Dolezal K: Micropropagation of Wild Service Tree (*Sorbus torminalis* [L.] Crantz): The Regulative Role of Different Aromatic Cytokinins During Organogenesis. *J Plant Growth Regul* 2009, **28**(4):341-348.

II. **Karady M**, Novak O, Horna A, Strnad M, Dolezal K: High Performance Liquid Chromatography-Electrochemistry-Electrospray Ionization Mass Spectrometry (HPLC/EC/ESI-MS) for Detection and Characterization of Roscovitine Oxidation Products. *Electroanalysis* 2011, **23**(12):2898-2905.

III. Mala J, Machova P, Cvrckova H**, Karady M**, Novak O, Mikulik J, Strnad M, Dolezal K: The role of cytokinins during micropropagation of wych elm**.** *Biologia Plantarum* 2013, **57**(1):174-178.

IV. **Karady M**, Novak O, Horna A, Strnad M, Dolezal K: High Performance Liquid Chromatography-Electrochemistry-High Resolution Electrospray Ionization Mass Spectrometry (HPLC/EC/HR ESI-MS) Characterization of Selected Cytokinins Oxidation Products. (Manuscript)

Contribution report

Paper I. Co-author – performed the solid phase extractions of cytokinins from plant samples, compiled measured CK profile results

Paper II. First author – LC/EC/ESI-MS method development, optimization, interpretation of the results, writing the manuscript

Paper III. Co-author – carried out all of the solid phase extractions of cytokinins from plant samples, CK measurement results compilation, optimization of the extraction

Paper IV. First author – formulation of the research problem, experimental work, LC/EC/ESI-MS method development, optimization, interpretation of the results, writing the manuscript

Abbreviations

ACN	acetonitrile
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
APCI	atmospheric pressure chemical ionization
*At*CKX	*Arabidopsis thaliana* cytokinin dehydrogenase
*At*IPT	*Arabidopsis thaliana* isopentenyltransferase
ATP	adenosine-5'-triphosphate
BAP	6-benzylaminopurine
BAP9G	6-benzylaminopurine-9-β-d-glucopyranosyl
BAPR	6-benzylaminopurine riboside
CK	cytokinin
CKX	cytokinin dehydrogenase
CYP735A	cytochrome P450 monooxygenase
*c*Z	*cis*-zeatin
*c*ZMP	*cis*-zeatin riboside-5'-monophosphate
DAD	diode array detector
DHZ	dihydrozeatin
DMAPP	dimethylallyldiphosphate
DZOG	dihydrozeatin-O-glucoside
EC	electrochemistry
EC cell	electrochemical cell
ESI	electrospray ionization
FAB	fast atom bombardment
GC	gas chromatography
HMBDP	hydroxymethylbutenyldiphosphate
HPLC	high performance liquid chromatography
HPLC-MS	high performance liquid chromatography – mass spectrometry
HPLC-MS/MS	high performance liquid chromatography – tandem mass spectrometry
HPLC-QqTOF-MS	high performance liquid chromatography – quadrupole time-of-flight mass spectrometry
HR	high resolution
iP	N^6-(2-Isopentenyl)adenine; isopentenyladenine
iPDP	N^6-(2-Isopentenyl)adenosine 5'-diphosphate
iPMP	N^6-(2-Isopentenyl)adenosine 5'-monophosphate
iPR	N^6-(2-Isopentenyl)adenosine
iPTP	N^6-(2-Isopentenyl)adenosine 5'-triphosphate
IPT	isopentenyltransferase
K	kinetin
KR	kinetin riboside
LC	liquid chromatography
LC-MS	liquid chromatography with mass spectrometric detection
LOG	the "Lonely Guy" mutant
MALDI	matrix assisted laser desorption ionization
MeOH	Methanol
MeOBAPR	6-(3-methoxybenzylamino)purine-9-ß-D-ribofuranoside
MEP	methylerythritolphosphate
MRM	multiple reaction monitoring
MS	mass spectrometry
MS^n	multiple steps of MS analysis

mT	*meta*-topolin
MVA	Mevalonate
m/z	mass-to-charge ratio
NAA	α-Naphthylacetic acid
NP	normal phase
oT	*ortho*-topolin
oTR	*ortho*-topolin riboside
oTRDP	*ortho*-topolin riboside-5'-diphosphate
oTRMP	*ortho*-topolin riboside-5'-monophosphate
oTRTP	*ortho*-topolin riboside-5'-triphosphate
P450	P450 monooxygenase
PDA	photo diode array
Q-TOF	quadrupole time-of-flight
QqQ	triple quadrupole
RP	reversed phase
SIM	selected ion monitoring
SPE	solid phase extraction
TOF	time of flight
tRNA	transfer ribonucleic acid
t_R	retention time
TS	thermospray
*t*Z	*trans*-zeatin
*t*ZMP	*trans*-zeatin riboside-5'-monophosphate
*t*ZOG	*trans*-zeatin-O-glucoside
*t*ZR	*trans*-zeatin riboside
UPLC	ultra high performance liquid chromatography
UV	ultraviolet

1. Introduction

A wide range of analytical methods is being used for the study of endogenous metabolites in various biological systems. These techniques have to be capable of selective and accurate monitoring of many known and unknown molecules which characteristics span through a broad chemical spectrum. Investigations, typically carried out *in vitro* or *in vivo*, are mostly aimed toward understanding the current metabolic homeostasis associated with a given developmental or physiological state. Particularly for comprehensive studies dealing with complex processes - hormone responses, drug efficacy, xenobiotic toxicity etc., it's always crucial to examine carefully as much as possible of the occurring metabolites. Natural biological activity and different environmental factors stepping into this systems, for example the presence of a foreign compound, present a further challenge. By using new instrumental approaches, mimicking the reactions which occur naturally in *in vivo* and *in vitro* environment, we can significantly raise our ability to assess the risk or benefit, to predict and identify such foreign or natural entities and the products and effects of their metabolism.

2. Aims and scopes

Cytokinins play a crucial role in the regulation of proliferation and differentiation of plant cells. They also control various processes in plant growth and development, such as delay of senescence, transduction of nutritional signals or control of the balance of shoot/root growth. Virtually, all naturally occurring cytokinins identified to date, are adenine species substituted at N^6 with an isoprenoid or aromatic side chain. Additional modification of a cytokinin molecule can lead to dramatic changes of action in the control of growth and development - a specific group of synthetic cytokinins, namely C-2, N-9 substituted 6-benzylaminopurine derivatives, specifically inhibit CDK2-related kinases, frequently deregulated in cancer cells. To study the existing and other possible cytokinins and their metabolites, a new approach has been developed.

The overall aims of this doctoral thesis are:

1. Analyze and compare the influence of three different aromatic cytokinin derivatives on *in vitro* multiplication and rhizogenesis of the wild service tree (*Sorbus torminalis* (L.) Crantz) and their influence on quantitative CK profile of the plants
2. Examine and assess the effect of two aromatic cytokinin derivatives, 6-benzylaminopurine (BAP) and *meta*-topolin (mT), on *in vitro* senescence and multiplication of wych elm (*Ulmus glabra* Huds.)
3. Verify and examine the possibilities of using a coulometric electrochemical cell, in combination with high-performance liquid chromatography/mass spectrometry (HPLC/MS), for qualitative analysis of cytokinins
4. To mimic oxidative cytokinin metabolism of cytokinins and cytokinin-derived compounds (roscovitine)
5. To characterize the resulting oxidative products
6. To compare *in vivo/in vitro* and electrochemically achieved oxidation
7. To attain an upscaled electrochemical generation of various oxidation products for further examination in various assays, NMR characteristics etc.

3. Literature review

3.1 Plant hormones

Plant hormones act on many crucial levels of controlling the growth and development of plants. Although metabolism does provide the power and the building blocks, it is the hormones that regulate the tempo of growth of the individual parts and integrate them to produce the form that we recognize as a plant (Davies, 2004). Their simplest definition may be that it is a molecule that at micromolar or lower concentrations acts as a messenger within or between plant cells. The fundamental difference between plant hormones and those found in animals is their ability to act as an internal messenger within the cell that produces it (Cleland, 1999). Plant hormones, called also phytohormones, are essential for plants, therefore they are considered to be primary metabolites, together with lipids, aminoacids etc. They represent a wide group of compounds with various chemical nature and interconnected metabolic pathways. Phytohormones consist of five "classic" groups – abscisic acid, auxins, cytokinins, ethylene and gibberellins (Repcak, 2002). More recently few other compound classes have been identified as phytohormones, namely brassinosteroids (Grove et al., 1979) and strigolactones (Umehara et al., 2008). Their biological effects, biosynthesis, crosstalk and mode of action are still not fully understood. Also other compounds, such as salicylic acid, jasmonic acid, nitric oxide etc., possess some regulatory functions (Repcak, 2002).

3.1.1 Cytokinins

Cytokinins (CKs) are phytohormones affecting various developmental and growth processes in plants, such as, apical dominance, shoot/root growth and leaf senescence. They can also be characterized by an ability to induce cell division in tissue culture (in the presence of auxin) (Sakakibara, 2006). Since the discovery of first cytokinin - kinetin in autoclaved products of herring sperm by Miller and Skoog (Miller et al., 1955), identification of other CK compounds followed, such as naturally occurring *trans*-zeatin (Letham, 1963) or synthetic diphenylurea (Shantz and Steward, 1955).

Natural CKs are adenine derivatives, with an aromatic or isoprene-derived substitution at N^6. These families are therefore called isoprene CKs and aromatic CKs, respectively. They usually occur as nucleosides, nucleotides and nucleobases or as conjugates with glucose, xylose and amino acid residues (Mok and Mok, 2001). Most common endogenous CK free bases are isopentenyladenine (iP), *cis*- *trans*- zeatin (*c*Z, *t*Z) and dihydrozeatin (DHZ). Selected CKs are displayed on Fig.1 in the next section.

3.1.1.1 Cytokinin biosynthesis

The most important factor in discovering the key enzymes and genes responsible for CK biosynthesis was the deciphering of Arabidopsis genome. Before that, presumably all previous attempts to isolate and characterize the enzymes for cytokinin biosynthesis by biochemical methods have failed (Sakakibara and Takei, 2002), except for prokaryotes, where the first cytokinin biosynthetic gene *Tmr* (Tumour morphology group) was

identified and cloned from gall-forming *Agrobacterium tumefaciens*, a common plant parasitic bacterium (Lichtenstein et al., 1984). Main biosynthetic pathways are outlined in Fig. 1.

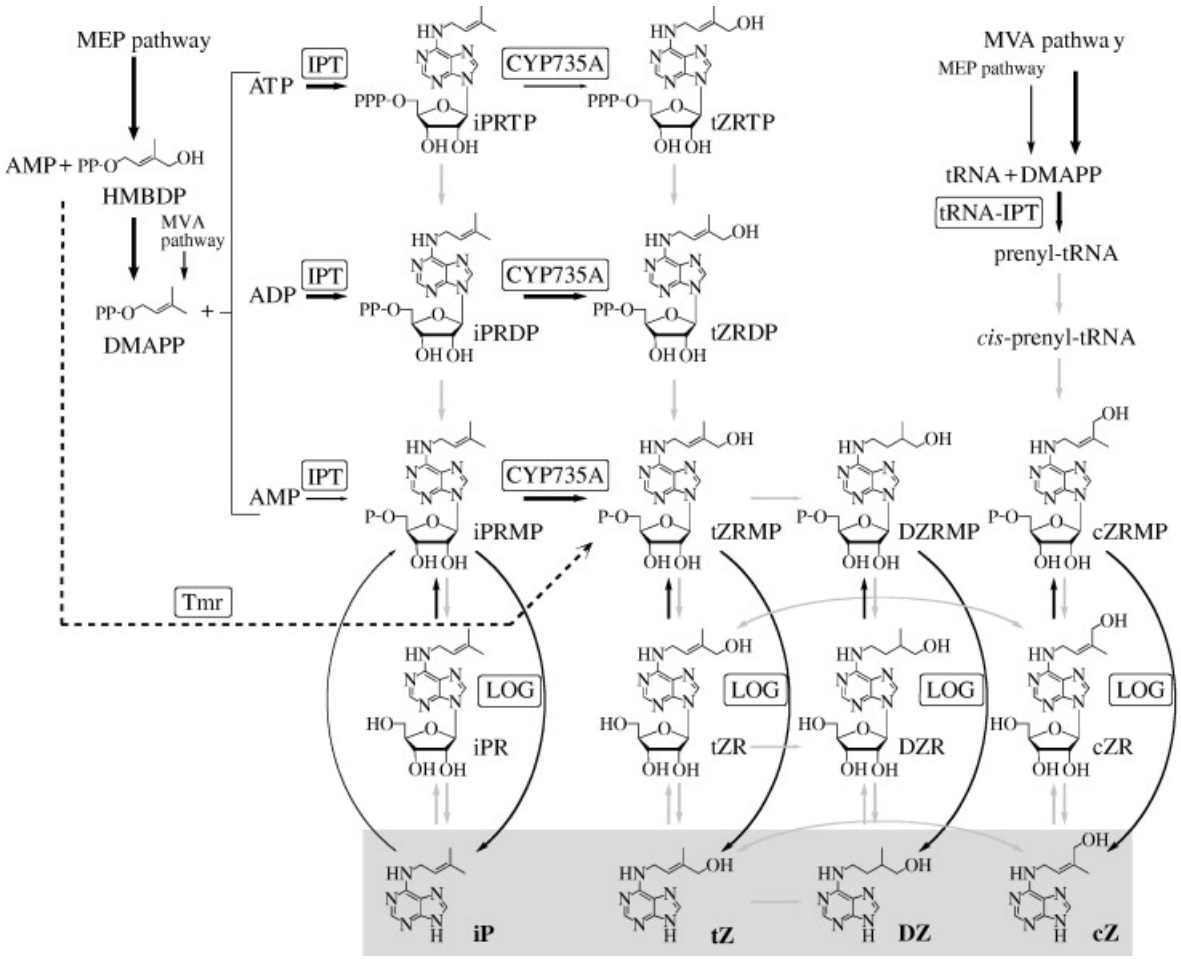


Fig. 1 – Isoprenoid CK biosyntheis model adopted from Kamada-Nobusada 2009. Gray arrows display pathways with currently unidentified enzymes.

The first and rate-limiting step in CK biosynthesis is the transfer of an isoprenoid moiety to the N^6-position of the adenine nucleotide catalysed by an IPT (adenosine phosphate-isopentenyltransferase) enzyme. Such an enzyme activity was first described and the enzyme itself partially isolated from a slime mold (*Dyctiostellum discoideum* L.) by Taya and co-workers (Taya et al., 1978). Generally, two types of structurally similar IPT enzymes are known to produce CKs in living organisms, both derived from a common ancestral gene and differing in their target of isopentelation. The tRNA-IPT enzyme is modifying some tRNAs by adding the dimethylallylpyrophosphate (DMAPP) isopentenylmoiety to the adenine residue adjacent to the anticodon. Second IPT, adenylate IPT, is a free nucleotide-forming enzyme known to be the main contributor to the CK pool (Spichal, 2012). They were discovered after deciphering the Arabidopsis genome, by *in silico* (bioinformatics) method of search for *Tmr* homologs in the genome (Kakimoto 2001, Takei 2001). Nine homologous genes named *At*IPT1- *At*IPT9 were found. *At*IPT2 and *At*IPT9 encode the enzymes for tRNA isopentelation, *At*IPT1 and *At*IPT3- *At*IPT8 are encoding enzymes involved in *de novo* biosynthesis (Kakimoto,

2001; Takei et al., 2001). Plant IPTs were also found and functionally proved in species such as mulberry (*Morus alba* L.), rice (*Oryza sativa* L.) or maize (*Zea mays* L.) (Abe et al., 2007; Sakamoto et al., 2006; Brugière et al., 2008). All IPTs prefer DMAPP as a side-chain donor, and ATP or ADP but not AMP as the accepting moiety to form N^6-(2-Isopentenyl)adenosine-5'-triphosphate (iPTP) or iPDP, as the precursors of active CKs (Kakimoto, 2001).

For formation of *trans*-zeatin type cytokinins, the most abundant and active type of CKs, two biosynthetic pathways are considered. An isopentenyladenine-dependent pathway, where an iP nucleotide is produced first and is later hydroxylated by cytochrome P450 monooxygenases, whose genes CYP735A1 and CYP735A2 have been identified in *A.thalia* genome (Takei et al., 2004). The second is an isopentenyladenine-independent pathway, suggested by Åstot (Åstot et al., 2000), where a direct transfer of hydroxylated side-chain from HMBDP to the adenine forms a *trans*-zeatin. A zeatin reductase can reduce the double bond of *trans*-zeatin to form dihydrozeatin (Martin et al., 1989) or it can undergo *trans* to *cis* isomerisation, although the responsible enzyme was only partially purified (Bassil et al., 1993).

Isoprenoid synthesis in plants occurs through two pathways. Mevalonate pathway (MVA) found in the cytosol, whereas methylerythritol phosphate pathway (MEP) is located in the plastids. The side-chain donor DMAPP can be readily synthesized by both of them. DMAPP for iP and *t*Z synthesis descents from MEP pathway, *c*Z side-chain from MVA pathway (Kasahara et al., 2004).

3.1.1.2 Metabolism of endogenous CK

CK metabolism is generally performed by several structural changes, that determine the function and compartmentalisation of the respective CK metabolite. To become biologically active, CK nucleotides produced by IPTs and CYP735As, have to be converted into free bases. Conversion of iP-, *t*Z- and *c*Z-riboside-5'-monophosphate to their active forms occurs by two pathways – the LOG and two-step pathway. During the LOG pathway, cytokinin ribozide-5'-monophosphates are directly converted to free-base cytokinins by a CK specific phosphoribohydrolase "lonely guy" LOG (Kurakawa et al., 2007). In the latter pathway, ribotides are first dephosphorylated to the ribosides and then converted to free-base cytokinins (Chen and Kristopeit, 1981), although the corresponding genes have not been found yet. Ribosides are conceived to be the transport forms of CKs. Other frequent modifications of CKs are glucosylation of *N-3*, *N-7* and *N-9* positions of the purine ring, or the formation of alanyl conjugates (Duke et al., 1978; Shaw, 1994). Generally, *N*-glucosides are not active in bioassays and *N-7* and *N-9* glucosylation irreversibly inactivates CKs (Mok and Mok, 2001). *N-3* glucosylation is on the contrary reversible (Brzobohaty et al., 1993). O-glucosylation can occur at the hydroxyl group of N^6-side chain of *trans*-zeatin and dihydrozeatin by O-glucosyl or xylosyltransferase. The biological importance has been shown in protecting the O-glycosylated CKs from N^6 side-chain cleavage by CK oxidase (Armstrong, 1994). They can be easily converted to active form by β-glucosidases (Brzobohaty et. al., 1993) and the O-glycosylated form is believed to be the stable and inactive storage form of CKs in plant cells (Mok and Mok 2001).

3.1.1.3 Cytokinin degradation

The main CK degradation pathway is executed by an irreversible cleavage of the N^6-side chain. The enzyme responsible for this action is CKX (CK dehydrogenase) and its activity was first described by Pačes et

al. (1971) in a crude tobacco tissue. CKXs remove the unsaturated isoprenyl side chain of CKs (McGaw and Horgan, 1983), recognizing its unsaturated double bond, therefore affecting less O-glycosides (Armstrong, 1994) and showing higher affinity toward iP and *t*Z (Sakakibara, 2006). There are seven CKX genes (CKX1-CKX7) in *Arabidopsis*, each encoding an isoenzyme possessing different biochemical properties and expression patterns (Kowalska et al., 2010). CKX proteins have been identified in many other species besides *Arabidopsis*, for example in maize, orchid (*Dendrobium sonia*), wheat (*Triticum aestivum* L.) (Frebort et al., 2011) and prokaryotic bacteria *Rhodococcus fascians* (Pertry et al., 2010). Current state of isoprenoid basic CK metabolism and degradation is displayed in Fig. 2.

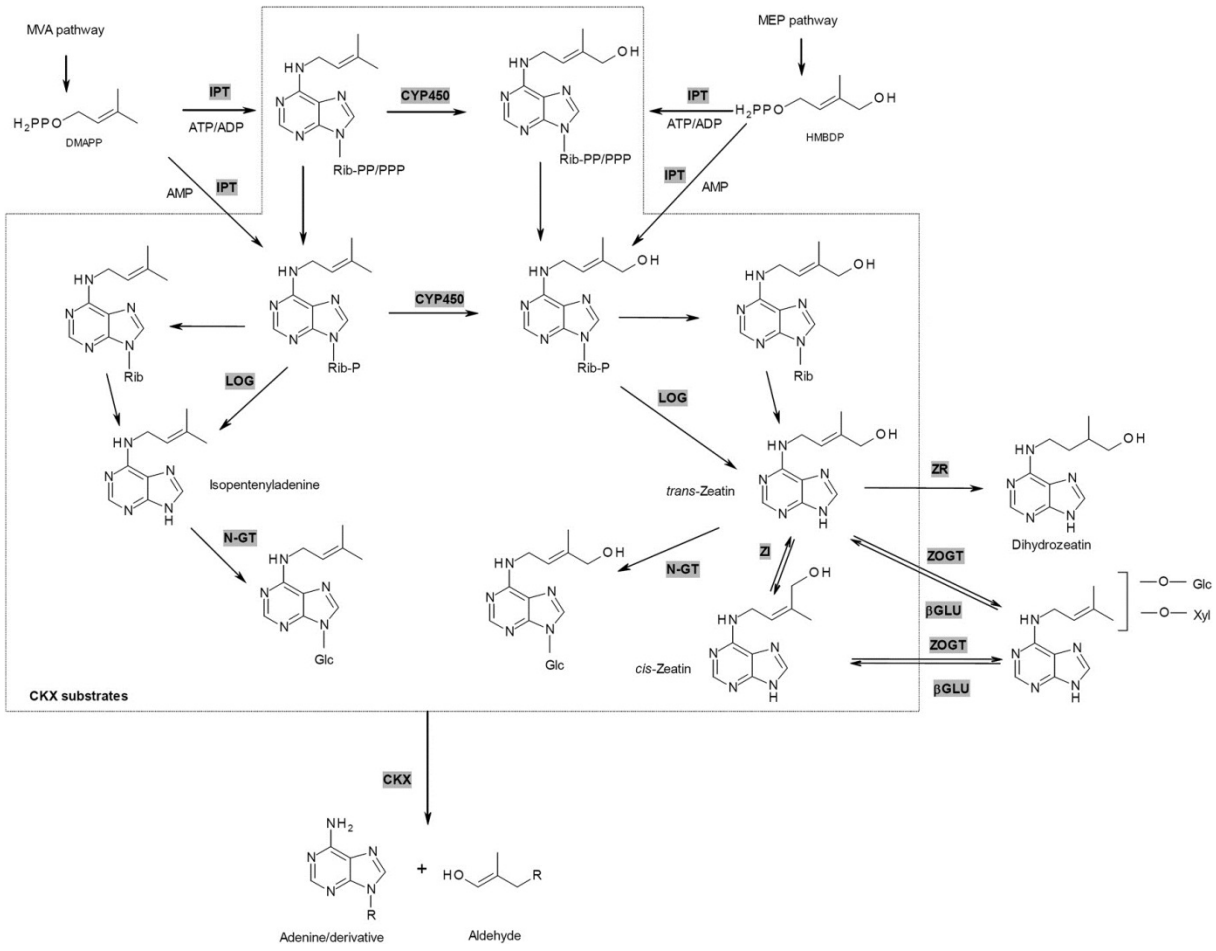


Fig. 2 Basic current state of isoprenoid CK metabolism and degradation, modified scheme adopted from Frebort et al. (2011)

3.1.1.4 Cytokinin derived CDK inhibitors

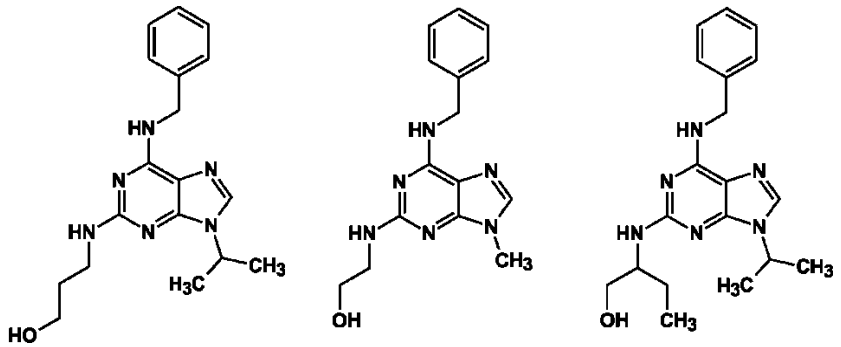
The mammalian cell cycle is a complex process divided into several phases. Its accurate regulation is essential for the normal cell growth and development. A strict control of the cell cycle progress in higher eukaryotic cells is carried out by an organized combination of proteins, which integrate extra- and intracellular

signals to ensure a full coordination. Cyclin-dependent kinases (CDKs) drive the events of the eukaryotic cell cycle by functioning like a clock, timing these events (Morgan, 1997).

Protein kinases represent a large family of enzymes catalyzing protein phosphorylation, which results in a change in functions of proteins such as their enzymatic activity or location. Phosphorylation of certain proteins plays a central role in the regulation of cell proliferation, differentiation and apoptosis. Deregulation of kinase activity can therefore lead to massive changes of these complicated processes (Cicenas and Valius, 2011). Deregulated kinases very often exhibit oncogenetic functions and can be central for the survival of cancer cells (Hunter 1985).

CDKs are Ser/Thr protein kinases with molecular weight of 30-40 kDa, playing a crucial role in the regulation of cell cycle, transcription, posttranscriptional modifications, cell proliferation and apoptosis. To date, the family of human CDKs consists of 13 members. (Knockaert et al., 2002; Malumbres and Barbacid, 2005). They associate with cyclin-subunits, whose concentrations oscillate during the cell cycle, maintaining the stage-specific timing of the CDKs (Nigg, 1996).

Many different classes of CDK inhibitors have been characterized during the past decades, including purine-based compounds (DeAzevedo et al., 1997), alkaloids, flavonoids, indirubins and paullones. The most efficient ones have entered clinical trials as candidate drugs against various diseases, such as cancer (Krystof and Uldrian, 2010a) or neurodegenerative and cardiac disorders (Knockaert et al., 2002; Krystof et al., 2010b). The purine heterocycle scaffold was one of the first systematically investigated potential CDK inhibitor, leading to the discoveries of olomoucine, bohemine and roscovitine (Vesely et al., 1994; Havlicek et al., 1997) (Fig 3). Roscovitine is a plant cytokinin-derived purine analog, first synthesized and described by Havlicek 1997. Since its identification as a CDK inhibitor (Meijer et al., 1997), roscovitine has also been shown to inhibit RNA synthesis in human neonatal fibroblasts and colon carcinoma cells (Ljungman and Paulsen, 2001), affect apoptosis (Hahntow et al., 2004), or to have effect on *in vitro* reproduction of mammalian oocytes (Mermillod et al., 2000). CYC202 (Seliciclib), a pure and chirally defined form (Wang et al., 2001) of roscovitine, was chosen for the further development as anti-cancer drug from a large set of substituted purine analogues (Raynaud et al., 2004) and is currently undergoing phase II. clinical trial studies in Nasopharyngeal Cancer (NPC) and Non-Small Cell Lung Cancer (NSCLC) (Le Tourneau et al., 2010).



Bohemine Olomoucine Roscovitine

Fig. 3 Structures of selected purine-derived CDK inhibitors

3.2 Analysis of phytohormones

Plant hormones represent a structurally unrelated collection of small molecules that play a pivotal role in regulating several aspects of plant growth, development and response to a wide palette of biotic and abiotic stress. Diverse analytical methods have been developed in the past decades for the accurate identification and quantitative determination of plant hormones and related compounds. Each class of phytohormones is present and possesses its characteristic biological effects at trace concentrations, which makes their identification and quantification difficult (Pan and Wang, 2009). Various phytohormones are being involved in many different processes and they do not act in isolation, but are prone to crosstalk in a way of modulating each other's biosynthesis or responses (Pieterse et al., 2009). Therefore a simultaneous profiling of multiple classes of plant hormones is naturally desired for identifying of this complex and interconnected hormone crosstalks (Pan et al., 2010). In many cases though, a profiling of single phytohormone class, for example CKs (Novak et al., 2008) or an analysis of the most active or most analysis-technique suitable compound of the selected class is used in such studies (Kojima et al., 2009). The determination of plant hormones in complex biological samples requires extensive sample preparation techniques prior to instrumental analysis. Sample preparation has become a major bottleneck, with a wide array of techniques being used, including sampling, extraction, purification derivatization and concentration methods (Du et al., 2012). Detection techniques generally involve bioassays, immunoassays, electroanalysis, and most importantly chromatographic methods such as capillary electrophoresis (CE) or high-performance liquid chromatography (HPLC) connected to various detectors. Especially HPLC coupled with a mass spectrometry detector has shown good separation, robustness and qualitative abilities, therefore representing a powerful and up-to-date tool for the detection of various phytohormones in complex plant samples (Du et al., 2012).

3.2.1 Liquid chromatography

Chromatography is a physical separation method in which the components to be separated are selectively distributed between two immiscible phases, a mobile phase flowing through a stationary phase. In liquid chromatography, the mobile phase is a liquid and carries the injected samples through the stationary phase which usually represents a filling of a separation column. A complex interaction between the samples and the two phases results in different retention time as the samples emerge from the column into a detector. The retention time is characteristic for each compound, under defined conditions, (Ardrey, 2003). Since the discovery of liquid chromatography technique, upgrade from older procedures (thin-layer chromatography, paper chromatography etc.) in equipment, material, techniques and application of theory results in modern HPLC (high-performance liquid chromatography). This technique arose by combining high pressure pumps (35-400 bar) and strengthened packed columns with ~5µm particles (Horvath, 1986). HPLC is suitable mostly for compounds which bear one or more of following specifications – high polarity, high molecular weight, thermal instability and tendency to ionize in solution (Niessen and Greef, 1982). In 2004 ongoing advances made possible the development of columns with smaller particles (1.7 µm) and specialized instrumentation withstanding 1000 bar pressure allowed to achieve a new level of performance known as UPLC (ultra-performance liquid chromatography) (Nguyen et al., 2006).

3.2.2 Mass spectrometry

Mass spectrometry (MS) represents the most sensitive method for molecular analysis among the wide range of spectrometric techniques, allowing detection and measurement of picomole to femtomole levels of many compounds (Sumner et al., 2003). A mass spectrometer is an instrument that is able to effectively separate moving ions on the basis of their mass to charge ratios (*m/z*) and consequently records the intensities of the ions (Hoffman and Stroobant, 2002). It consists of five basic parts: sample introduction or inlet, ion source, mass analyzer, detector and data handling system. After introducing the sample via the inlet system, the sample is converted into gaseous ions in the ion source. The gaseous ions are then accelerated and dispersed in the mass analyzer according to their *m/z*. Afterwards a detector converts the beam of ions into an electric signal directly proportional to their abundance (Hoffman and Stroobant, 2007).

Modern MS provides a highly specific chemical information directly related to the chemical structure such as accurate mass, isotope distribution patterns or characteristic fragment ions. It offers a vast array of technologies differing in operational principles and performance. These variations include ionization technique, resolving power, technology of the mass analyzer and mass accuracy (Lei et al., 2011). Most common ion source techniques nowadays are electrospray ionization (ESI) (Bedair and Sumner, 2008) and atmospheric pressure chemical ionization (APCI). Mass analyzers with different resolving power include, for example, Fourier transformation ion cyclotron resonance MS (FT-ICR), orbitrap MS, quadrupole time-of-flight Q-TOF (high resolution analyzers) and quadrupoles or ion-trap systems (low resolution). Each platform has its specific advantages and limitations and its selection depends on the goal of the project, throughput and instrumental cost (Lei et al., 2011).

3.2.3 Liquid chromatography/mass spectrometry in CK analysis

Coupling liquid chromatography with mass spectrometry offers an excellent solution for the analysis of individual compounds and complex mixtures. In general, chromatographic separation prior to MS analyses provides several advantages, like reducing matrix effect, separation of isomers, additional data (retention time) and allows more accurate quantification of individual compounds (Lei et al., 2011). This approach is vastly applied for determination of individual or multiple classes of plant hormones and their metabolites, like simultaneous analysis of seven classes of phytohormones (Pan and Wang, 2009).

HPLC itself shows a high suitability for CKs analysis as CKs exhibit good gradiations in polarity and can be detected by a widely employed UV absorbance detector (Chen, 1987). HPLC also enables rapid purification and identification of CKs from plant extracts prior to MS analysis. However their quantification is skewed because absorbance at single UV wavelength is inadequate for this purpose, therefore the most widely used procedure of choice for CKs quantification is isotope-dilution MS, especially with LC-electrospray ionization-MS (Tarkowski et al., 2009).

The first LC-MS analysis and quantification of CKs was performed by Yang et al. in 1993, using an APCI ionization interface. Fast atom bombardment (FAB) interface was used by Åstot et al. in 1998. Applying of ESI interface was established by Prinsen et al. in 1998, using a triple quadrupole mass detector. Novak et al. (2003) achieved baseline separation of 20 nonderivatized naturally occurring CKs employing a single quadrupole. Quantitative analysis is commonly performed by specialized quadrupole modes - selected ion

monitoring mode (SIM) (Novak et al., 2003) or more recently by multiple reaction monitoring mode MRM (Novak et al., 2008). UPLC-MS methods have been established and proved in last years to be a suitable system for the study of cytokinins. Schwartzenberg et al. in 2007 successfully applied UPLC-MS to establish the profile of 40 cytokinins found in *Physcomitrella patens.* Dolezal et al. in 2007 successfully isolated new cytotoxic members of aromatic cytokinins and Novak et al. in 2008 developed and UPLC-MS method for complex CK profiling in plant tissues, which is four times faster than the previously used HPLC methods.

3.2.4 Electrochemistry on-line with LC/MS

Electrochemistry (EC) represents a distinct chemistry field where electron transfer reactions occurring between electrodes and solutions are studied. One of the specific applications of EC is inducing oxidations in order to simulate oxidative drug metabolism. This is usually performed by employing different kinds of electrodes, which behave as an oxidant that can be tuned by an applied potential to perform charge-transfer reactions (Nouri-Nigjeh et al., 2011). Such application is particularly useful in the examination and simulation of *in vivo* oxidative metabolism reactions for newly developed drugs, as it represents a crucial point in pharmaceutical industry (Lohmann et al., 2010). Generally, metabolism of xenobiotics can be divided into three phases. Phase I consists of primary functionalization like oxidation, dehydrogenation, hydrolysis, isomerization etc. Phase II metabolism represents conjugation reactions like sulfation, methylation, condensation, glucuronidation etc. . Phase III metabolism is related to transport processes such as renal excretion. Various electrochemical approaches, aimed to investigate the potential of EC towards mimicking these natural phases, have shown their highest potential for mimicking phase I (Jurva et al., 2003), which pathways are *in vivo* initiated through oxidation reactions catalyzed by enzymes of the cytochrome P450 family (CYP) (Josephy et al., 2005). The main classic instrumentation for examination of phase I drug metabolism are *in vitro* simulations based on CYP enzymes originating from the liver including liver slices, hepatocytes and liver-cell microsomes or *in vivo* animal models. These methods are often time consuming and possess only limited reproducibility (Lohmann et al., 2010). EC combined with mass spectrometry and/or liquid chromatography (EC-(LC)-MS) has become the preferred technique for these simulation experiments as it may provide exhaustive information about the chemical properties of the electrochemically generated oxidation products. Employing this method reduces the complexity of simulation system to the basic factors decreasing the influence of biological matrix, exposes sites in the molecule labile toward oxidations, enables generation and identification of products at one site thus saving time, cost and effort, etc. (Lohmann and Karst, 2009).

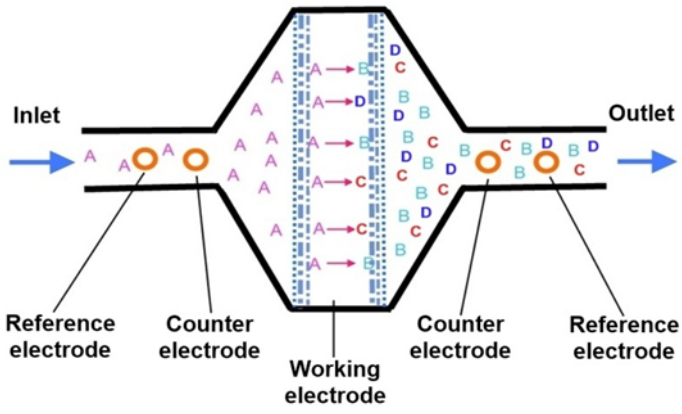


Fig.4 Schematic drawing of a coulometric flow-through cell with a total conversion rate of the passing compound A

The majority of the studies towards EC-LC-MS simulation of oxidative metabolism have been performed by commercially available coulometric electrochemical cells. Other employed types include wall-jet,

amperometric or thin-layer cells. All these types share a common design with a three electrode set-up consisting of a working electrode, reference and a counter electrode (Baumann and Karst, 2010). The oxidation potential is set between the counter and working electrode by a potentiostat. Unstable working potentials are compensated by the reference electrode most commonly made from Pd/H_2 . A schematic drawing of a coulometric EC cell is displayed in Fig. 4. . Its main feature is a working electrode consisting of porous glassy carbon providing high conversion rate for introduced compound. Other profound materials used instead of porous glassy carbon are boron-doped diamond or platinum. The cell position in the LC-MS system can be varied according to a specific application, placing it after the column to examine the redox potential of the compounds or before the column to separate the oxidation products, (Fig. 5), or dropping the column altogether for obtaining first-hand results. Off-line EC batch reactors are also used (Lohmann et al., 2010).

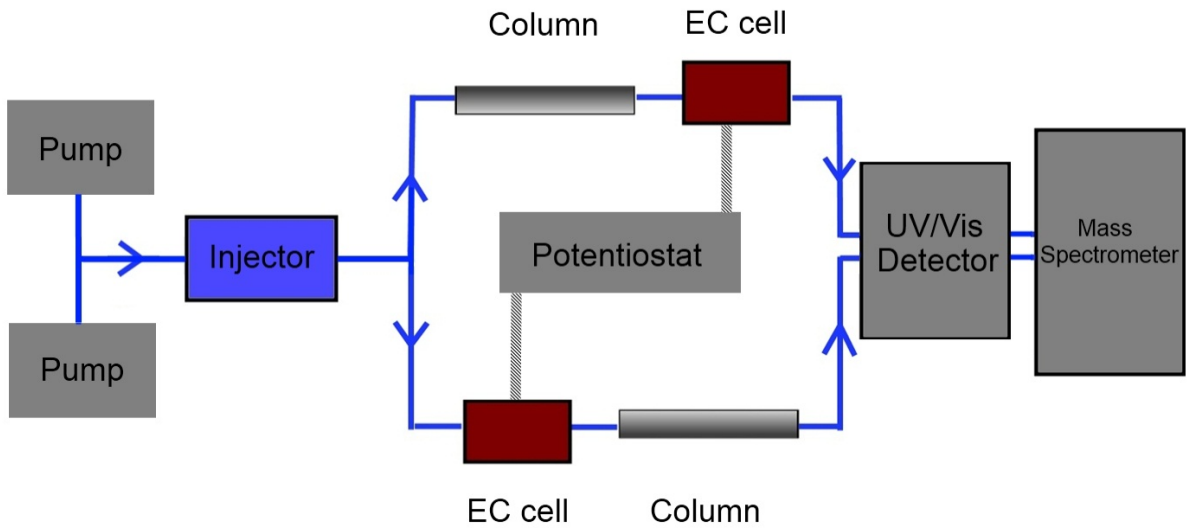


Fig. 5 Schematic LC/EC/MS setup with an optional UV/Vis detector prior to mass spectrometer

The first record of EC coupled together with mass spectrometry (MS) was a study performed by Gadde and Bruckenstein (1971) based on the detection of volatile intermediates obtained during reactions on a porous platinum electrode. In 1981 Shono et al. utilized direct electrochemical oxidation for the imitation of biotransformation of lisuride, diazepam, methylsergide, and imipramine. In a study conducted by Jurva et al. the N-dealkylation of lidocaine was readily performed electrochemically, whereas other reactions, such as O-dealkylation, failed. A conclusion was drawn since then, that electrochemical oxidation is able to provide relevant oxidative drug metabolites only if it's initiated by one or more one-electron oxidations, moreover only if these reactions do not require higher oxidation potentials than the oxidation potential of the used solvent (usually water) (Jurva et al., 2003). Besides N-dealkylation, biotransformations that could be imitated by direct electrochemical oxidation include alcohol oxidation, P-oxidation, S-oxidation and dehydrogenation whereas oxidative metabolites resulting from hydrogen atom abstraction, such as hydroxylation of the nonactivated aromatic rings or O-dealkylation, cannot be imitated by direct electrochemistry (Jurva et al., 2003). A vast array of molecules and their classes have been analyzed during recent period by EC-(LC)-MS including guanosine, clozapine, paracetamol, diclofenac, resveratrol, selected nucleotides etc., with an excellent recent review covering the field (Jahn and Karst, 2012). There is also a possibility of using a miniaturized microfluidic chip (Odijk 2012), the ability to detect reactive, unstable, or volatile intermediates (Jahn and Karst, 2012), and a

promising field of application - preparative synthesis of drug metabolites for identification, bioassays etc. (Simon et al., 2012). Considering usage of LC-EC-MS in cytokinin analysis, besides the aforementioned nucleotide analyses, only a study of roscovitine has been performed so far (Karady et al., 2011), although classical EC approaches, including application of voltammetry and/or mercury electrodes, were carried out for specific CKs - *t*Z (Hernandez et al., 1997), DHZ (Blanco et al., 2000), kinetin (Ballesteros et al., 2002) or BAP (Tarkowska et al., 2003).

4. Material and methods

4.1 Chemicals

- All chromatographic solvents and chemicals were of analytical grade or higher purity from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), Merck (Darmstadt, Germany) and Roche Diagnostics (Mannheim, Germany) (I.-IV.)

- All CK standards were obtained from Olchemim Ltd (Olomouc, Czech Republic) (I.-IV.)

- A Milli-Q deionization unit (Millipore, France) was used for preparation of the purified water for mobile phases and solutions. (I.-IV.)

4.2 Biological material

- Multiapex explant cultures of the wild service tree (*Sorbus torminalis* (L.) Crantz), derived from three clones, found in the Explant Bank of the Forestry and Game Management Research Institute, Czech Republic, grown for 12 months on the multiplication medium were selected, with general description in Supplement I.

- Multiapex cultures derived from 15 clones of wych elm (*Ulmus glabra* Huds.) growing in the Explant Bank of the Forestry and Game Management Research Institute, Czech Republic, were used. Common description is included in Supplement III.

- No biological material was employed in articles contained in Supplements II. and IV. .

4.3 Equipment

- The cytokinins were analyzed using an Acquity UPLC™ (Waters, Milford, MA), equipped with a BEH C-18 (1.7 μm, 2.1 × 150 mm) column linked to a Quattro micro™ API detector (Waters MS Technologies, Manchester, UK) triple quadrupole mass spectrometer equipped with an electrospray interface. (I., III.)

- The electrochemical system from ESA (Chelmsford, MA, USA) comprised an electrochemical detector and potentiostat Coulochem III equipped with a model 5021 conditioning cell (EC cell). The EC cell contained a porous glassy carbon coulometric working electrode with a large surface area , a Pd/H_2 reference electrode and a Pd counter electrode.(II., IV.)

- The electrochemical system was on-line connected to an Alliance 2690 Separation Module (Waters, Milford, MA, USA) with a Symmetry C18 RP-column (150mm x 2.1mm x 5μm, Waters (II., IV.).

- For the detection of electrochemically generated products a ZMD 2000 single quadrupole mass spectrometer equipped with an ESI electrospray interface (Micromass, Manchester, UK) was used (II.)

- For high resolution mass analysis and detection of EC oxidation products a hybrid mass analyzer Q-TOF *micro* (Micromass, Manchester, UK), equipped with an ESI electrospray interface (Micromass, Manchester, UK) was employed (II., IV.)

4.4 Methods

- After 12 weeks of cultivation with supplemented media, the shoot cultures (100-200 mg per sample) of three different clones, grown on media containing three different cytokinins (mT, MeoBAPR, BAP) for wild service tree (*Sorbus torminalis* (L.) Crantz) (I.), or two CKs (BAP, mT) for wych elm (*Ulmus glabra* Huds.) (III.), were taken, deep frozen and prepared for the analyses. Fifty endogenous cytokinin derivatives were purified and their concentration measured by ultra-performance liquid chromatography combined with electrospray mass spectrometry

[UPLC-ESI(+)-MS/MS] according to Novak et al., 2008. The analytes were quantified by multiple-reaction monitoring of [M + H]+ and the appropriate product ion. The identity of all measured cytokinin metabolites was verified by comparing the mass spectra and chromatographic retention times with those of authentic standards, all measurements were done in triplicate (I., III.).

- Roscovitine (10^{-4}g) was dissolved in 1ml of mobile phase (initial conditions) and 10 μL were injected. The setup consisting of on-line LC/EC/ESI-MS was optimized by varying its conditions, without separating the resulting oxidation products. For all LC/EC/ESI-MS measurements the working electrode was kept at a constant potential and the system was equilibrated for 10 minutes after the EC cell was switched on. The electrochemical conversions were carried out at potential 0 – 1800 mV in 100 mV steps. Different pHs of the aqueous part of the mobile phase (Solvent A) were tested, namely unbuffered formic acid (pH 2.8), formic acid (pH 4) and ammonium formate (pH 7.4) buffered to their respective pH by ammonium hydroxide; all tested buffer solutions were 15mM. Methanol and acetonitrile were used and compared for all tested pHs as the organic phase. Also water/methanol (90:10 v/v) and water/acetonitrile (90:10 v/v) mixtures were used as solvent A. At a flow rate of 250 μl/min the following binary gradient was used - solvent B was raised from initial 10% to 75% in 20 minutes and further raised to 100% in 5 minutes, where it was held constant for 3 minutes. The gradient then returned in 2 minutes to its initial conditions and the column was equilibrated for 5 minutes. There were also different negative modes tested, with a negative potential applied to either EC cell and ESI electrospray interface, with no usable results (data not shown). The effluent was introduced into the ZMD 2000 electrospray source with optimized conditions (source block temperature 100^{o}C, capillary voltage +3.0 kV, cone voltage +20 V, desolvation temperature 250^{o}C). Nitrogen was used both as a desolvation gas (400 L/h) and as a cone gas (50 L/h). All measurements were done in positive ion mode with scan range *m/z* 50-820 (II.).

- For identification of electrochemically generated R-roscovitine oxidation products, as well as for MS/MS fragmentation experiments, the LC conditions used were the same as above; the EC Cell potential was kept constant for 1800 mV. On-line setup was modified and the EC cell was placed before the column. Mobile phase consisted of 15 mM ammonium formate/ammonium hydroxide (Solvent A) buffered to pH 7.4 and acetonitrile (Solvent B). The QqTOF accurate mass MS analyses were done in positive ion mode with the following optimized parameters: source block/desolvation temperature 100^{o}C/350^{o}C, capillary/cone voltage 2500/25 V, spray/cone gas

flow (N_2) 500/50 L/h, scan range *m/z* = 50-820. Lock spray was used for internal calibration with a mixture of 0.1 M NaOH/10% formic acid (v/v) and acetonitrile (1:1:8, v/v/v) as a reference. Accurate masses were calculated and used for the determination of the elementary composition of the analytes, with fidelity of 5.5 ppm or better (II.).

- iP, *t*Z and *c*Z (10^{-4}g) were dissolved in 1ml of mobile phase, identical with initial conditions of analysis (10% A/ 90% B), and 10 µl were injected into the system. Mobile phase consisted of 15mM ammonium formate/ammonium hydroxide solution buffered to pH 7.4 (solvent B) and organic phase (solvent A) represented by acetonitrile. The conditions, pH and type of mobile phase were selected as optimal for adenine with different N^6-side chain type compounds, based on our previous article (Karady et al., 2011). Flow rate was constantly set to 100 µl . The LC gradient started at 10% of solvent A held for one minute and then continually raised to 90% in 24 minutes. Then it was kept constant for 10 minutes and returned to 10% in one minute with subsequent ten minute long equilibration. The EC, LC and MS components were coupled together on-line with EC cell placed between the HPLC and RP column for separation of the oxidation products. The EC cell potential was kept constant for 1200 mV. QqTOF settings were the same as for (II.), with slightly lower max QqTOF fidelity – 6.0 ppm (IV.).

5. Survey of results

- The highest multiplication rate (number of newly grown shoots per culture) of wild service tree (*Sorbus torminalis* (L.) Crantz) was achieved using multiplication medium supplemented with BAP (24 ± 4 shoots). Only 55-60% of the multiplication rate observed with BAP was achieved in the media containing MeOBAPR and mT. There were no significant differences in the number of new shoots among clones cultivated on the same media. On the other hand, the highest percentages (15 ± 2.5%) of rooted plantlets were achieved after transferring the microcuttings from medium with MeOBAPR into one-third MS medium without NAA (I.).

- The levels of 27 cytokinin species were found to be present above the detection limit. The highest concentration (2.4 ± 0.8 pmol g−1 FW) of BAP9G was determined in the samples grown on BAP, whereas levels of this metabolite in the explants grown on MeoBAPR and/or mT were found to be significantly lower (0.41 ± 0.35 and 0.08 ± 0.06 pmol g−1 FW, respectively). Detectable levels of BAP as well as mT cytokinins were measured in the *S. torminalis* explants grown on media containing MeoBAPR. Among the isoprenoid cytokinins found, the highest concentrations detected were for the nucleotides, namely, iPMP, *t*ZMP, and *c*ZMP. On the other hand, levels of *t*Z, *t*ZR, and iPR, which are considered the most active endogenous isoprenoid cytokinins, were suppressed in explants grown on BAP in comparison with the other two treatments. Dihydrozeatin and *cis*-zeatin derivatives were almost unaffected by these cytokinin treatments (I.).

- Six roscovitine oxidation products were observed under various pH and mobile phase conditions. The highest number of new compounds formed was observed at pH 7.4 with acetonitrile. Under all investigated pHs, except for the pure water, the oxidation began at 800 mV, with best results obtained at 1800 mV, thus these conditions (pH 7.4, acetonitrile, 1800 mV) were used for QqTOF accurate mass measurements. From the six major products, none was structurally identical to those identified previously *in vitro.* R-roscovitine in the EC cell underwent N-dealkylation of the isopropyl moiety, hydroxylation of the aromatic side-chain, dihydroxylation, methoxylation and dimer formation. The hydroxylation product was identified as Olomoucine II (a potent CDK inhibitor), the occurrence of which, as a roscovitine metabolite, has not yet been observed *in vitro* (II.).

- The explants of wych elm (*Ulmus glabra* Huds.), cultivated on multiplication medium supplemented with mT produced six times more shoots than counterparts cultivated with BAP (31.9 ± 28.1 versus 5.88 ± 4.03). There was no significant difference between the average length of shoots produced by explants grown on mT (3.4 ± 1.4 cm) and those on BAP (3.6 ± 1.2 cm). The levels of 33 cytokinin species were found to be present at levels exceeding the detection limit of the UPLC-MS/MS quantification method used (Novak et al., 2008). We found much higher in-tissue concentrations of BAP in the explants grown in the BAP-containing medium than of mT. Hydroxylated aromatic cytokinins were also substantially more abundant in the tissue micropropagated on BAP. We did not find any significant differences in content of most isoprenoid cytokinin free bases – *t*Z, *c*Z and DHZ (III.)

- The electrochemical oxidation of isopentenyladenine resulted in five products, including *trans*-zeatin and dehydrogenated products, which correlates very well with its *in vitro* metabolism. Electrochemical conversion of *trans*-zeatin revealed six products with two dehydrogenation products corresponding to its *in vitro* occuring metabolites. *Cis*-zeatin oxidation in the electrochemical cell gave rise to eight products, which structures are resembling similarity to *trans*-zeatin oxidation products. All three compounds have underwent a complete turnover mainly through two oxidation reactions occurring in the electrochemical cell – dehydrogenation and a less typical aliphatic hydroxylation (IV.).

6. Conclusion and perspectives

This thesis describes an examination of different CKs effect in plant culture micropropagation and investigates the possibility of EC hyphenated methods for CKs and CK-derived compounds analysis and metabolism prediction. It concludes that:

- the optimal endogenous concentration of cytokinins, their metabolites and other plant hormones, auxin and ethylene, is crucial for successful organ development including root emergence and formation from *in vitro* multiplicated explants of *Sorbus terminalis* and *Ulmus glabra*

- CKs, and derived compounds, are under certain circumstations, readily oxidizable by an EC cell. Investigating and employing the right conditions, pH, oxidation potential etc., are crucial.

- EC cell failed to mimick *in vivo/in vitro* metabolism of roscovitine, although the products resembled similarity due to appearing through the same reaction type

- Most of the resulting products of iP, *t*Z and *c*Z EC cell oxidation, especially *trans*-zeatin from isopentenyladenine and -2H dehydrogenation products, are in excellent correlation with their known *in vitro/in vivo* metabolism

In summary, besides underlining the importance of using a well-balanced phytohormone mixture for the micropropagation of plants, a new LC-EC-MS method for CK analysis was developed. Although the EC cell failed to mimick *in vivo/in vitro* metabolism for roscovitine and was partially successful for observed CKs, it holds a great promise for the future. Moreover there is a possibility to further enhance EC cell application, by employing an EC synthetic cell connected to a preparative LC, to readily obtain the oxidation products in yields high enough for their additional utilization, like bioassays, NMR identification etc. .

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8. Supplements

Supplement I.

J Plant Growth Regul (2009) 28:341–348
DOI 10.1007/s00344-009-9099-2

Micropropagation of Wild Service Tree (*Sorbus torminalis* [L.] Crantz): The Regulative Role of Different Aromatic Cytokinins During Organogenesis

Jana Malá · Pavlína Máchová · Helena Cvrčková · Michal Karady · Ondřej Novák · Jaromír Mikulík · Eva Hauserová · Jarmila Greplová · Miroslav Strnad · Karel Doležal

Received: 30 January 2009 / Accepted: 10 March 2009 / Published online: 24 April 2009

Abstract The influences of three different aromatic cytokinin derivatives [6-benzylaminopurine, *meta*-topolin, and 6-(3-methoxybenzylamino)purine-9-ß-D-ribofuranoside (MeOBAPR)] on in vitro multiplication and rhizogenesis of the wild service tree (*Sorbus torminalis* [L.] Crantz) were compared. The highest micropropagation rate (24 new shoots per explant after 3 months of cultivation) was achieved on media containing BAP. On the other hand, the best rooting microcuttings were those multiplied on a medium containing MeoBAPR. To compare these results with the levels of endogenous cytokinins in multiplied explants, a newly developed UPLC-ESI(+)-MS/MS method was used to determine levels of 50 cytokinin metabolites in explants cultivated 12 weeks on media supplemented by BAP and of the two other aromatic cytokinin analogs used. Several significant differences among the levels of endogenous cytokinins, extracted from the explants, were found. The concentration of BAP9G, an important metabolite suspected to be responsible for inhibition of rooting and acclimatization problems of newly formed plantlets, was found to be the highest in microcuttings grown on media supplemented with BAP. This agrees well with the results of our rooting experiments; the lowest percentages of rooted plantlets 6 weeks after transferring shoots on rooting medium were present on explants multiplied on BAP. In contrast, BAP was still the most effective for the induction of bud formation on primary explants. Levels of the most active endogenous isoprenoid cytokinins, tZ, tZR, and iPR, as well as O-glucosides were also suppressed in explants grown on BAP compared with those of explants treated with other cytokinin derivatives. This may be the result of a very high BAP uptake into the explants grown on this cytokinin. On the other hand, endogenous concentrations of *cis*-zeatin derivatives as well as dihydrozeatin derivatives were not affected. Differences in the production of another plant hormone, ethylene, that plays an important role in controlling organogenesis in tissue culture, were also observed among *S. torminalis* plantlets grown in vitro on media containing different cytokinins tested. The highest ethylene levels were detected in the vessels containing media supplemented with mT. They were two to four times higher compared with the production by the *S. torminalis* explants cultivated on other media used. Finally, the levels of free IAA were also determined in the explants. *S. torminalis* plantlets grown on media containing BAP contained the lowest level of auxin, which is again in good agreement with their loss of rooting capacity. The results found in this study about optimal plant hormone concentrations may be used to improve in vitro rooting efficiency of the wild service tree and possibly also of other plant species.

J. Malá · P. Máchová · H. Cvrčková
Institute of Forest and Game Management,
Strnady 136, 252 02 Jíloviště, Czech Republic

M. Karady · O. Novák · J. Mikulík · E. Hauserová · J. Greplová · M. Strnad · K. Doležal (✉)
Laboratory of Growth Regulators, Palacký University,
and Institute of Experimental Botany, AS CR,
783 71 Olomouc-Holice, Czech Republic
e-mail: Karel.Dolezal@upol.cz

Keywords Wild service tree · Micropropagation · Rooting · Cytokinins · Ethylene · IAA

Abbreviations

BAP	6-Benzylaminopurine
MeOBAP	6-(3-Methoxybenzylamino) purine

*m*T	6-(3-Hydroxybenzylamino) purine
*o*T	6-(2-Hydroxybenzylamino) purine
iP	N^6-isopentenyladenine
cZ	*cis*-Zeatin
tZ	*trans*-Zeatin
DHZ	Dihydrozeatin
*R	9-β-D-ribofuranosyl derivative
*9G	9-β-D-glucopyranosyl derivative
*OG	O-β-D-glucopyranosyl derivative
*5′MP	5′-Monophosphate derivative
LC-MS	Liquid chromatography combined with mass spectrometry
UPLC-ESI(+)-MS/MS	Ultra-performance liquid chromatography combined with positive electrospray mass spectrometry
MS	Murashige-Skoog medium
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
NAA	α-Naphthylacetic acid

Introduction

The wild service tree (*Sorbus torminalis* [L.] Crantz) is one of a scarce species among forest trees and occurs in scattered populations at a low density in central Europe. It is a slow-growing tree that reaches a maximum height of 20–25 m at around 80–100 years. According to the latest data, the species is disappearing from forests of the Czech Republic and it is considered to be endangered. Nowadays, the wild service tree is rated as one of the most valuable hardwoods with a great potential for wider use in forestry and forest ecology; it is also important in the timber industry. Since the 1990s it has been among the highest-priced timber species in Europe (Demesure and others 2000).

The genetic diversity of this scattered species may be at risk, particularly in dense forests, which contain a high proportion of long-lived tree species. Because it grows in small fragmented populations, it is sensitive to high levels of interspecific competition. Such conditions could be a cause of a low gene exchange among populations (Hoebee and others 2006) and consequently of a strong decrease in genetic diversity in the species through reduction of population size. The regeneration of the wild service tree is gradually weakened, which could eventually lead to its local extinction. As for all forest trees, the wild service tree is particularly threatened by habitat fragmentation due to its low population density (Demesure and others 2000; Oddou-Muratorio and others 2001; Prat and Daniel 1993).

For long-term sustainability of wild service tree genetic resources, micropropagation technologies could prove to be useful (Chalupa 1992; Dujíčková and others 1992; Malá and others 2005). Clonal propagation could preserve the genetic diversity of the species over a long period of time, even if environmental conditions might worsen during the course of succession. At present there are 95 clones derived from mature elite wild service trees from various forest regions in the Czech Republic deposited in the Bank of Explants in the Forestry and Game Management Research Institute of the Czech Republic. Despite this, for standardized micropropagation procedures for the wild service tree and for its effective exploitation in forestry, some difficulties during the rooting stage of organogenesis remain to be solved.

Cytokinins, N^6-substituted purine derivatives, are an important class of plant hormones that regulate a large number of physiologic and developmental processes in plants (Letham and Palni 1983). They are classified as isoprenoid or aromatic, depending on the structure of the N^6-substituent (Strnad 1997). Endogenously, cytokinins can occur in different metabolic forms, including free bases, ribosides (R), N-glucosides (G), O-glucosides (OG), and nucleotides (5′MP) (Letham and Palni 1983).

In micropropagation technology, 6-benzylaminopurine (BAP) is widely used as one of the most effective and affordable cytokinins. Nevertheless, it often induces disproportional growth or inhibition of rooting in a number of plant species (Werbrouck and others 1995, 1996), including the wild service tree. Thus, research to find alternative phytohormones for micropropagation purposes remains important.

The high morphogenetic activity of *meta*-topolin [6-(3-hydroxybenzylamino)purine; mT] and its derivatives was previously described (Werbrouck and others 1996). Our LC-MS-based identification of naturally occurring aromatic cytokinins in plants and plant-pathogenic bacteria recently led to the discovery of several new phytohormones, including methoxytopolins (Tarkowská and others 2003). A large group of methoxytopolin analogs has been synthesized and characterized, and selected derivatives have been tested for use in micropropagation (Doležal and others 2006, 2007). 6-(3-Methoxybenzylamino)purine-9-ß-D-ribofuranoside (MeOBAPR), an endogenously occurring cytokinin and one of the most active derivatives from this large group discovered by us so far, was compared in this study with BAP and mT—standard and commercially available cytokinin free bases.

Ethylene is another plant hormone that plays an important role in controlling organogenesis in tissue culture, although its role in rooting has not been fully

elucidated (Kepczynski and others 2006). Increased ethylene levels caused reduction of root formation from pea cuttings (Nordstrom and Eliasson 1993). Moreover, exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) inhibited root formation in apple shoot cultures by delaying root emergence and increasing callus formation at the bases of shoots (Ma and others 1998). The same authors also reported promotion of root formation by using various ethylene inhibitors (Ma and others 1998). On the other hand, ethylene was found to stimulate root formation from hazelnut cotyledons (Gonzalez and others 1991) as well as kiwi (*Actinidia deliciosa*) explants (Arigita and others 2003). Root formation in *Populus tremula* L. explants was either inhibited or stimulated by ethylene depending on its concentration within the culture vessels (Gonzalez and others 1997). This implies that the ethylene effect on rooting emergence and promotion is concentration dependent and knowledge about its optimal concentration may be used to improve in vitro rooting efficiency (Ma and others 1998).

Root formation has many practical implications in horticulture, agronomy, and forestry. There is a lot of commercial interest in root formation because of the many plant species that are difficult to root (Davies and others 1994). The auxins were the first plant hormone group to be used to stimulate rooting of cuttings. Their important role in root initialization and formation has been studied intensively for many years (although it is still not completely understood on a molecular level) (Ludwig-Müller and others 2005).

In this study we compared the influence of three different aromatic cytokinins (BAP, mT, and MeOBAPR) on the multiplication of explants and the rooting of microcuttings of the wild service tree. Subsequently, the endogenous cytokinin concentrations in the explants, with the focus on the level of potentially root-inhibiting cytokinin metabolites and their ethylene production and endogenous auxin (IAA) levels, were determined.

Material and Methods

Plant Material

Multiapex explant cultures derived from three clones of wild service tree from the Explant Bank of the Forestry and Game Management Research Institute and which had grown for 12 months on the multiplication medium:agar solidified MS (Murashige and Skoog 1962) medium with 0.2 mg dm^{-3} of BAP, 0.1 mg dm^{-3} of IBA, 100 mg dm^{-3} of glutamine, and 30 g dm^{-3} of sucrose (pH 5.8) were selected. The explants were cultivated under white fluorescent light (36 W/33 Philips tubes, Eindhoven, the Netherlands; irradiance of 30 μmol m^{-2} s^{-1} with 12-h photoperiods) and at 24°C.

Experimental Setup

Shoots derived from three clones were divided into three groups: control shoots cultivated on multiplication medium (see above) (30 cultures), shoots cultivated on a modified medium (multiplication medium with 0.2 mg dm^{-3} of mT instead of BAP) (30 cultures), and shoots cultivated on another modified medium (multiplication medium with 0.3 mg dm^{-3} of MeOBAPR instead of BAP) (30 cultures). All cultures were cultivated for 12 weeks (with transfer every 4 weeks into fresh multiplication medium of the same composition) under the same cultivation conditions. Ten shoot cultures were taken from each group for chemical analyses. For induction of rooting, 20 shoots (microcuttings derived from one clone) from each group were placed onto the agar rooting medium [one-third-strength MS medium enriched with NAA (14 mg dm^{-3}), that is, without cytokinins]. Transferred shoots were cultured in black boxes in an air-conditioned room at 25°C. After 1 week the shoots were transferred into fresh rooting medium (one-third-strength MS medium without NAA) and cultured under white fluorescent light (36 W/33 Philips tubes) with a 12-h photoperiod (irradiance = 30 μmol m^{-2} s^{-1}). The experiment was repeated three times.

Evaluation of Changes in Endogenous Cytokinin Levels

After 12 weeks of cultivation, the shoot cultures (100 mg per sample) of three different clones, grown on media containing different cytokinins, were taken for analyses. The endogenous concentrations of 50 cytokinin derivatives were determined by means of ultra-performance liquid chromatography combined with electrospray mass spectrometry [UPLC-ESI(+)-MS/MS] (Novák and others 2008). The cytokinins were analyzed using an Acquity UPLC™ (Waters, Milford, MA), equipped with a BEH C-18 (1.7 μm, 2.1 × 150 mm) column linked to a Quattro *micro*™ API detector (Waters MS Technologies, Manchester, UK) triple quadrupole mass spectrometer equipped with an electrospray interface.

The analytes were quantified by multiple-reaction monitoring of $[M + H]^+$ and the appropriate product ion (Novák and others 2008). The identity of all measured cytokinin metabolites was verified by comparing the mass spectra and chromatographic retention times with those of authentic standards. The measurements were done in triplicate.

Ethylene Measurement

Analysis was performed using the gas chromatography-flame ionization detector (GC-FID) method (Fišerová and others 2001). Cultivation vessels were ventilated 1 h before sampling. Subsequently, 1 ml of air was taken from each vessel and analyzed using a Finnigan Trace GC Ultra equipped with a FID detector and 50-m capillary column (HP-AL/S stationary phase, 15 μm, i.d. = 0.535). Injection temperature was set to 200°C, oven temperature to 40°C, and detector temperature to 220°C. The measurements were done in triplicate from three different vessels of each clone and cultivation medium used.

Determination of Endogenous IAA

Material was ground and homogenized with liquid nitrogen and weighed out into Eppendorf tubes. A cold solution of 0.05 M phosphate buffer (pH 7.0) containing 0.02% DEDTCA was used for extraction of material. To check the recovery during purification and to validate the quantification, a labeled internal standard of $13C_6$-IAA was added to each sample. Samples were stirred for 15 min at 4°C. After centrifugation (5 min at 12,000 rpm, 4°C), the supernatant was collected and acidified to pH 2.7 with 1 M HCl. Finally, samples were purified by solid-phase extraction on Varian BondElut C_8 (500 mg/3 ml) columns, evaporated to dryness, and store at -20°C before LC/MS analysis.

An Acquity UPLC System (Waters), including a binary solvent manager and a sample manager, and a Micromass Quattro *micro* API detector (Waters MS Technologies) were used for cytokinin analysis. All data were processed by MassLynx™ software with QuanLynx™ and QuantOptimize™ programs (ver. 4.0, Waters).

Samples were dissolved in 20 μl of ACN/water (10/90) and filtered (Micro-spin® filter tube; 0.2 μm; 3 min at 8000 rpm; Grace, USA); then 15 μl of sample was injected onto a reversed-phase column (BetaMax Neutral; 150 mm × 1 mm; particle size = 5 μm; Thermo Fisher Scientific, Waltham, MA) with UNIGUARD™ column protection (Hypurity advance; 10 mm × 1 mm; 5 μm; Thermo Fisher Scientific). The samples were eluted with a 20-min gradient as follows: 0–5 min 90/10 A/B, 5–10 min 80/20 A/B, 10–18 min 70/30 A/B, 18–19 min 50/50 A/B, 19–20 min 10/90 A/B (v/v), where A was 1% ACN with 0.1% formic acid and B was 95% ACN with 0.1% formic acid. At the end of the gradient the column was equilibrated to initial conditions for 5 min. The flow rate was 0.05 ml min^{-1} and the column was eluted at ambient temperature. Under these conditions, retention time for the monitored IAA was 15.36 min. The effluent was passed to the tandem mass spectrometer without post-column splitting. Indole-3-acetic acid was quantified by multiple-reaction monitoring of $[M + H]^+$ and the appropriate product ion.

For the selective multiple reaction monitoring mode (MRM) experiment, optimized conditions were as follows: capillary voltage, 3.0 kV; source/desolvation gas temperature, 100/250°C; cone/desolvation gas flow rates, 2.0/450 L h^{-1}; LM/HM resolution, 10.0; ion energy 1, 1.0 V; ion energy 2, 1.4 V; multiplier voltages, 700 eV. Argon was used as collision gas with an optimized pressure of 5×10^{-3} mbar. The dwell times (0.80 s), cone voltages (20 V), and collision energy (15 eV) for diagnostic transitions were optimized to maximize sensitivity.

Growth and Morphogenetic Evaluations

The newly developed shoots were counted in all shoot cultures after 3 months of cultivation. At the same time a qualitative evaluation of growth was carried out (numbers of new shoots per explant and average length of shoot).

Statistical Evaluation

The data were analyzed by analysis of variance (ANOVA) and Duncan's multiple-range test (Duncan 1955).

Evaluation of Rooting

The percentages of rooted plantlets were determined 6 weeks after transferring shoots onto rooting medium (see subsection "Experimental setup").

Results

Growth and Morphologic Evaluations

The highest multiplication rate (number of newly grown shoots per culture) was achieved using multiplication medium supplemented with BAP (24 ± 4 shoots). Only 55-60% of the multiplication rate observed with BAP was achieved in the media containing MeOBAPR and mT (Table 1). There were no significant differences in the number of new shoots among clones cultivated on the same media. The average numbers of new shoots in three clones is presented in Table 1. None of these cytokinins influenced either the morphologic formation or the length of shoots (3.2 ± 0.6 cm on average in all clones).

Rooting

Percentages of successfully rooted plantlets were determined after 6 weeks of cultivation in the rooting medium (see above). The highest percentages (15 ± 2.5%) of

Table 1 Comparison of the average numbers of new shoots influenced by different cytokinins (values combined from 3 experiments)

Cytokinin	No. of new shoots ± SD	mT	MeoBAPR	BAP
mT	14.2 ± 4.96	X	NS	*
MeoBAPR	13.3 ± 4.76	NS	X	*
BAP	24.3 ± 4.41	*	*	X

NS not significant

* Significant according to Duncan's multiple-range test at the 0.05 level (Duncan 1955)

Table 2 Comparison of the average percentages of rooted plantlets (values combined from 3 experiments)

Cytokinin	Percentage of rooted plantlets ± SD	MeoBAPR	mT	BAP
MeoBAPR	15.3 ± 2.5	X	*	*
mT	8.7 ± 3.5	*	X	NS
BAP	7.0 ± 3.0	*	NS	X

NS not significant

* Significant according to Duncan's multiple-range test at the 0.05 level (Duncan 1955)

rooted plantlets were achieved after transferring the microcuttings from medium with MeOBAPR into one-third MS medium without NAA (see subsection "Experimental setup"). This rate was significantly higher compared with the other two cytokinins tested (8.7 ± 3.5 for mT and 7.0 ± 3.0 for BAP) (Table 2).

Cytokinin Levels in the Explants

Fifty endogenous cytokinin metabolites were determined in the samples of *S. torminalis* plantlets grown in vitro on media containing three different cytokinin species. The levels of 27 cytokinin species were found to be present above the detection limit of the UPLC-MS/MS quantification method used (Novák and others 2008) and therefore could be quantified and compared in relation to cytokinins used in the cultivation media. The highest concentration (2.4 ± 0.8 pmol g^{-1} FW) of BAP9G was determined in the samples grown on BAP, whereas levels of this metabolite in the explants grown on MeoBAPR and/or mT were found to be significantly lower (0.41 ± 0.35 and 0.08 ± 0.06 pmol g^{-1} FW, respectively) (Table 3). Moreover, detectable levels of corresponding mT and MeoBAPR metabolites, mT9G and MeoBAP9G, were not found even in plantlets grown on mT and MeoBAPR, respectively. It is interesting to note that formation of BAP cytokinins was observed after application of mT to the culture medium (Table 3). Moreover, detectable levels of BAP as well as mT cytokinins were measured in the *S. torminalis* explants grown on media containing MeoBAPR (Table 3).

Among the isoprenoid cytokinins found, the highest concentrations detected were for the nucleotides, namely, iPR-5′MP, tZR-5′MP, and cZR-5′MP. On the other hand, levels of tZ, tZR, and iPR, which are considered the most active endogenous isoprenoid cytokinins, were suppressed in explants grown on BAP in comparison with the other two treatments (Table 3). Dihydrozeatin and *cis*-zeatin derivatives were almost unaffected by these cytokinin treatments.

Ethylene Induction

Levels of ethylene were measured in cultivation vessels during the incubation of *S. torminalis* plantlets in vitro on media containing three different cytokinins (Table 4). The highest concentration (272–392 nl L^{-1} depending on the clone used) of ethylene was determined in the vessels containing mT, whereas levels of this gaseous plant hormone produced by the explants grown on BAP and/or MeoBAPR were found to be significantly lower (Table 4).

IAA Levels in the Explants

Using UPLC-ESI(+)-MS/MS technology, endogenous IAA concentrations were measured in samples of *S. torminalis* plantlets (two different clones) grown in vitro on media containing three different cytokinins. A calibration curve ($y = 0.6167x - 0.0047$; $R^2 = 0.9972$) was created by plotting the known concentration of unlabeled/labeled analyte ratio against the calculated response area of the analyte/internal standard ratio. The indole-3-acetic acid limit of detection (LOD = 9.3 fmol) and quantification (LOQ = 30.8 fmol) were calculated from the signal-to-noise ratio of 3:1 and 10:1, respectively. The lowest concentration (29.4 ± 1.2 ng g^{-1} FW for clone 1 and 27.3 ± 0.7 ng g^{-1} FW for clone 3, respectively) of IAA was present in the plantlets cultivated on BAP. Levels of this compound in the explants grown on mT were found to be significantly higher in both clones (31.0 ± 0.2 ng g^{-1} FW in clone 1 and 51.2 ± 3.8 ng g^{-1} FW in clone 3, respectively) (Table 5). Whereas IAA concentration in the plantlets of clone 3, grown on another topolin derivative, MeoBAPR, were also significantly higher (33.8 ± 1.8 ng g^{-1} FW) compared with those grown on BAP, the IAA increase in clone 1 was not significant (Table 5).

Discussion

Plant micropropagation represents a complex process consisting of successive developmental stages controlled

Table 3 Cytokinin levels (pmol g^{-1} FW) in *S. torminalis* shoots determined by UPLC-ESI(+)-MS/MS

	tZ	tZR	tZR-5′MP	cZ	cZR	cZR-5′MP	DHZ	DHZR	iP
BAP	0.83 ± 0.34	0.69 ± 0.23	4.7 ± 1.3	0.30 ± 0.13	0.51 ± 0.14	4.8 ± 1.7	0.03 ± 0.02	0.12 ± 0.05	2.1 ± 1.3
MeOBAPR	3.4 ± 2.1	2.2 ± 0.9	3.7 ± 1.2	0.37 ± 0.21	0.64 ± 0.26	5.1 ± 2.3	0.11 ± 0.06	0.22 ± 0.12	1.2 ± 0.2
mT	5.7 ± 2.9	1.5 ± 0.3	2.7 ± 0.4	0.28 ± 0.07	0.52 ± 0.17	4.2 ± 1.0	0.24 ± 0.19	0.17 ± 0.05	0.8 ± 0.2
	iPR	iPR-5′MP	BAP	BAPR	BAP9G	BAPR-5′MP	mT	mTR	oT
BAP	0.95 ± 0.2	10.8 ± 2.8	2214 ± 611	261 ± 64	2.4 ± 0.8	753 ± 134	6.3 ± 4.3	0.60 ± 0.26	3.9 ± 1.5
MeOBAPR	2.1 ± 0.8	15.9 ± 3.6	69.3 ± 23	17 ± 12	0.41 ± 0.35	16.9 ± 6.4	37 ± 17.5	25.9 ± 12	1.6 ± 1.4
mT	1.4 ± 0.3	12.3 ± 2.3	24.1 ± 8.5	3.3 ± 1.8	0.08 ± 0.06	12.5 ± 7.6	167 ± 125	52 ± 41	3.9 ± 1.2
	oTR	MeOBAP	MeOBAPR	tZOG	tZROG	cZOG	cZROG	mTOG	mTROG
BAP	0.43 ± 0.22	<LOD	<LOD	3.15 ± 1.6	1.17 ± 0.54	0.53 ± 0.11	0.43 ± 0.11	5.7 ± 3.5	13.3 ± 3.6
MeOBAPR	0.46 ± 0.09	2177 ± 724	2255 ± 869	5.0 ± 2.5	1.96 ± 0.82	0.62 ± 0.17	0.58 ± 0.11	166 ± 41	160 ± 38
mT	0.44 ± 0.12	1.45 ± 0.3	<LOD	5.5 ± 0.5	1.90 ± 0.23	0.39 ± 0.14	0.43 ± 0.13	798 ± 428	523 ± 148

Amount ± SD represents mean values of three measurements of real samples. Only compounds above the detection limit are included. Twelve-week-old *S. torminalis* shoots (250 mg) were extracted. The extracts were purified by SPE followed by immunoaffinity chromatography, and then measured by UPLC-ESI(+)-MS/MS technique

Table 4 Ethylene production (nl L^{-1}) by *S. torminalis* explants determined by GC-FID 1 h after ventilation

Cytokinin	Clone number		
	1	2	3
MeoBAPR	33 ± 12	291 ± 28	175 ± 77
mT	392 ± 92	357 ± 68	272 ± 55
BAP	81 ± 16	119 ± 15	177 ± 18

The measurements were done in triplicate

Table 5 IAA levels (ng g^{-1} FW) in *S. torminalis* explants determined by UPLC-ESI(+)-MS/MS

Cytokinin	Clone number	
	1	3
MeoBAPR	30.9 ± 1.1	33.8 ± 1.8
mT	31.0 ± 0.2	51.2 ± 3.8
BAP	29.4 ± 1.2	27.3 ± 0.7

The measurements were done in triplicate

by various endogenous and exogenous stimuli which also regulate growth processes in vitro (Centeno and others 1996). However, cross-talk between auxins, cytokinins, and other active substances such as polyamines and phenolic acid derivatives that participate in morphogenetic processes during organ differentiation is far from being fully understood (Altamura and others 1993; Cvikrová and Hrubcová 1999; Scholten 1998). It has been shown that root formation proceeds through several growth stages that are characterized by a high level of endogenous auxins, particularly indole-3-acetic acid (Nag and others 2001). The presence of cytokinins is also essential for the induction of cell division at the beginning of root formation (De Klerk and others 2001), but in contrast to auxins, higher levels of cytokinins inhibit adventitious rooting (Bollmark and others 1988).

Problems with the rooting of microcuttings are why the wide utilization of micropropagation techniques are not used for many important tree species (Malá and others 2005) This is especially true for *S. torminalis*, plantlets of which root poorly, even when a protocol previously used successfully to micropropagate other poorly rooting broad-leaf tree species, for example, oak, is used (Malá and others 2000). Decreased ability to root adventitiously could be related to an increased level of BAP9G, mainly in the basal parts of explants. Accumulation of this metabolite at the plant base might be a reason for various acclimatization problems such as heterogeneity in growth and inhibition of rooting (Werbrouck and others 1995). The aim of the current work was to test other aromatic cytokinin derivatives, use of which could have strong organogenesis activity, comparable to that of BAP, but a minimal effect on subsequent rooting. Simultaneously, the levels of corresponding 9-glucosides, responsible for the decreased rooting capacity of newly formed explants, should be lowered. We confirmed the formation of this metabolite in *S. torminalis* explants, with the highest concentrations detected in the microcuttings grown on media containing BAP (Table 3). Accordingly, the lowest percentage of rooted plantlets 6 weeks after transferring shoots on rooting medium was among the explants multiplied on BAP (Table 2). On the other hand, this compound was still the most effective in the induction of adventitious buds as well as axillary bud

formation on primary explants. In this case the excised apical meristems were from dormant buds (Table 1).

To analyze this problem in more detail, we used our newly developed UPLC-ESI(+)-MS/MS method to determine levels of BAP, BAP9G, and 48 other endogenous cytokinin metabolites in the explants studied. Several significant differences were found between levels of the compounds extracted from the explants cultivated on media supplemented with BAP and those supplemented with cytokinin analogs. Levels of the most active endogenous isoprenoid cytokinins, tZ, tZR, their O-glucosides, and iPR, were suppressed in explants grown on BAP compared with other treatments. This suppression could probably be caused by very high concentrations of BAP cytokinins present in the explants grown on this cytokinin (Table 3). In contrast, mT exhibited massive formation of the corresponding O-glucosides (mTOG and mTROG) (Table 3), which are considered as storage forms and located in the vacuole (Fusseder and Ziegler 1988). Futhermore, MeoBAPR offers an even broader spectrum of possible metabolites (after alternative demethylation) (Table 3). On the other hand, endogenous concentrations of *cis*-zeatin and dihydrozeatin derivatives were comparable (Table 3). We describe here, also for the first time, limited formation of BAP derivatives after the application of *meta*-topolin as well as formation of mT and BAP cytokinins in MeOBAPR-treated explants (Table 3). Such metabolic conversions have not yet been described for isoprenoid cytokinins. However, the mechanism for such an in vivo transformation remains to be elucidated.

To analyze in more detail the different rooting abilities of explants multiplied on media supplemented with different cytokinins, the ethylene concentrations in cultivation vessels were also measured. In our experiment, the highest ethylene levels (272–392 nl L^{-1} depending on the clone) were detected in the vessels containing media supplemented with mT. They were several times higher compared with that produced by the *S. torminalis* explants cultivated on other media used (Table 4). This finding, that explants grown on media supplemented with mT have slightly elevated ethylene production, correlates well with the rooting ability of these explants (Table 2) as well as with earlier experiments performed on *Populus tremula* L. (Gonzalez and others 1991).

Although our work was focused on the role and practical use of another important plant hormone group, aromatic cytokinins, on the multiplication of explants, and on the rooting of microcuttings of the wild service tree, to get a complete picture of optimal endogenous plant hormone concentrations and their dependence on different exogenous cytokinins used in the cultivation media in relation to in vitro rooting efficiency of the wild service tree, we also analyzed their endogenous auxin (IAA) levels. As expected, the lowest level of IAA in the explant *S. torminalis* plantlets grown on media containing BAP was confirmed (Table 5). Because auxin is well known as the major factor in *de novo* root formation (Casson and Lindsey 2003), this finding is again in good agreement with the limited rooting capacity of the explants grown on BAP.

On the other hand, experiments on the CRE1/AHK4 cytokinin receptor mutant already showed that cytokinin is also required for correct vascular morphogenesis in the root (Casson and Lindsey 2003) and root formation (Higuchi and others 2004). However, the precise role of cytokinin in other aspects of root development is still to be determined. Moreover, we have shown that different cytokinin derivatives can exhibit different root-inducing capacities. It is possible that cytokinin, along with auxin (Sabatini and others 1999), provides positional cues required for meristem organization.

It is becoming clear that the optimal concentration of cytokinins and their metabolites and of the other plant hormones, including auxin and ethylene, is crucial for successful organ development including root emergence and formation from multiplicated explants. Subsequently, the results about optimal endogenous plant hormone concentrations and their dependence on different exogenous cytokinins used in the cultivation media may help to improve in vitro rooting efficiency of the wild service tree and possibly other plant species.

Acknowledgments This work was supported by the Grant Agency of the Czech Republic (GA 206/07/0570), by the Ministry of Agriculture of the Czech Republic (MZE 0002070203), and the Czech Ministry of Education (MSM 6198959216, 1M06030). We thank Petra Amakorová for her skilled technical assistance.

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Supplement II.

High Performance Liquid Chromatography-Electrochemistry-Electrospray Ionization Mass Spectrometry (HPLC/EC/ESI-MS) for Detection and Characterization of Roscovitine Oxidation Products

Michal Karady,[a] *Ondřej Novák*,[a] *Aleš Horna*,[c, d] *Miroslav Strnad*,[a, b] *Karel Doležal**[a, b]

[a] Laboratory of Growth Regulators, Palacky University and Institute of Experimental Botany ASCR, Šlechtitelů 11, 783 71, Olomouc, Czech Republic
[b] Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Šlechtitelů 11, CZ 783 71, Olomouc, Czech Republic
[c] RADANAL Ltd., Okružní 613, Pardubice, 530 03, Czech Republic
[d] Tomas Bata University in Zlín, University Institute, Food Research Centre, Mostní 539, CZ-760 01 Zlín, Czech Republic
*e-mail: karel.dolezal@upol.cz

Received: July 19, 2011
Accepted: September 28, 2011

Abstract
On-line LC-EC/ESI-MS has been established as a fast and simple method to mimic some types of oxidation reaction of various drugs and to study the formation and structure of the resulting products. This technique has been applied to a 2,6,9-trisubstituted purine, R-roscovitine, which is known to be an inhibitor of some cyclin-dependent kinases (CDKs) and a potential anticancer drug. Oxidation of R-roscovitine in an electrochemical cell (EC), operated under various conditions, resulted in appearance of 6 major products. These were further analyzed by high-resolution mass spectrometry, their structures were elucidated by accurate mass measurement and compared to previously identified R-roscovitine in vitro/in vivo metabolites. Although none of the observed products was structurally identical to those identified previously in vitro/in vivo, all of them, except for the methoxylated products, resembled similarity due to appearing through the same reaction type. R-roscovitine in the EC cell underwent *N*-dealkylation of the isopropyl moiety, hydroxylation of the aromatic side-chain, dihydroxylation, methoxylation and dimer formation. The hydroxylation product was identified as Olomoucine II, a R-roscovitine derivative, which displays 10-times higher CDK-inhibiting activity than R-roscovitine and the occurrence of which, as R-roscovitine product, has not yet been observed in vitro/in vivo.

Keywords: Electrochemistry, Mass spectrometry, R-roscovitine, Oxidation

DOI: 10.1002/elan.201100383

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/elan.201100383.

1 Introduction

One of the major bottlenecks in small molecule drug development is the synthesis of substances derived from investigated compound. These related substances include degradants and metabolites that are commonly a result of oxidative processes [1]. Metabolism of various xenobiotics can be generally divided into two different phases. Phase I reactions are mostly oxidation reactions, catalyzed in the liver by enzymes from the cytochrome P450 family [2]. The resulting metabolite is excreted directly by the kidneys or in the bile, or it can undergo a Phase II metabolism step, in which small and hydrophilic biomolecules, such as glutathione, are coupled to the Phase I metabolite [3]. A common approach is to use in vitro systems using hepatocytes or microsomes [4] or in vivo methods, in which various radioactively labeled drug compounds are administered [5]. There is an increasing interest in new methods that could provide reliable information on possible metabolites. Applying these methods at an early stage of metabolic studies may lead to a more efficient and economic procedure in drug development. Oxidation performed by an electrochemical cell has several advantages in comparison with chemical oxidation, as there is virtually no need to remove reagents and an electrochemical flow cell can be readily coupled on-line to a mass spectrometer (MS), allowing fast analysis of oxidation reactions. Incorporation of a HPLC separation step facilitates the analysis of complex mixtures. Coupled EC/LC/MS techniques have only been developed in the last decade. However, they have many possible applications, such as study and mimicking of in vivo oxidation and metabolism of natural compounds and drugs, or enhancing the efficiency of ionization of analytes for various mass spectrometry techniques [6]. Several studies, utilizing electrochemical (EC) flow cells online with MS, have

demonstrated that electrochemically derived products can often accurately predict biological metabolites and chemical degradants [7]. Some of the typical metabolism reactions simulated by an EC system include *N*-dealkylation [7–8], aromatic hydroxylation [7,9–11], benzylic hydroxylation [7], alcohol oxidation [7,12] or dehydrogenation [9,13–15].

Cytokinins play a crucial role in the regulation of proliferation and differentiation of plant cells. They also control various processes in plant growth and development, such as delay of senescence, transduction of nutritional signals, control of the balance of shoot/root growth and increased crop productivity [16]. Virtually, all naturally occurring cytokinins identified to date, are adenine species substituted at N6 with an isoprenoid or aromatic side chain. Additional modification of a cytokinin molecule can lead to dramatic changes of action in the control of growth and development. Olomoucine and its derivatives R-roscovitine and bohemine, are examples of C2, N9-substituted 6-benzylaminopurine derivatives, which specifically inhibit CDK2-related kinases [17], frequently deregulated in cancer cells. CDK inhibition causes cell cycle arrest, forcing the cancer cell into programmed cell death. Since the first report [18] of the selective inhibition of CDK2 by Olomoucine, there has been a substantial progress in the development of more potent analogues. Among these, CYC202 (seliciclib), a pure and chirally defined form [19] of R-roscovitine, was chosen for the further development as anticancer drug from a large set of substituted purine analogues [20] and is currently undergoing phase II clinical trials studies in Nasopharyngeal Cancer (NPC) and Non-Small Cell Lung Cancer (NSCLC) [21]. Its metabolism has been studied on in vivo/in vitro based models [22–24], showing that R-roscovitine undergoes mainly oxidation and N-dealkylation.

2 Experimental

2.1 Chemicals

R-roscovitine and Olomoucine II standards were obtained from Olchemim Ltd (Olomouc, Czech Republic). Preparation of 2,4 and 2,5-dihydroxy derivatives of Olomoucine II was described previously [25–27]. Water used for preparation of the mobile phases and solutions was purified with a Milli-Q deionization unit (Millipore, France). All other chromatographic solvents and chemicals were of analytical grade or higher purity from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

2.2 Instrumentation

The electrochemical system from ESA (Chelmsford, MA, USA) comprised an electrochemical detector Coulochem III equipped with a model 5021 conditioning cell (EC cell). The EC cell contained a porous glassy carbon coulometric working electrode with a large surface area, a Pd/H_2 reference electrode and a Pd counter electrode. All potentials described in this article were given versus the Pd/H_2 reference electrode. An in-line polyetherketone (PEEK) filter was placed upstream of the EC cell inlet to protect the working electrode.

The LC system consisted of an Alliance 2690 Separation Module (Waters, Milford, MA, USA) with a Symmetry C18 RP-column (150 mm × 2.1 mm × 5 μm, Waters), with the column thermostat set to 30 °C.

Coupling of LC/MS on-line with electrochemistry was done in two ways – the EC cell was placed either between the HPLC column and the ESI interface of the LC/MS system used (Figure 1A), or the cell was placed before the reversed phase column (Figure 1B). Figure 1A was used for optimization of the LC/EC/ESI-MS system, with no metabolites separation. Figure 1B was used for metabolites separation, identification and MS/MS experiments. The connection between the EC cell and the ESI source, or between the cell and the column, was kept as short as possible to minimize band dispersion and to prevent short-lived products from being degraded.

For setup displayed in Figure 1A, with the LC and EC system remaining unchanged, the ZMD 2000 single quadrupole mass spectrometer equipped with an ESI electrospray interface (Micromass, Manchester, UK) was used. Data were processed by MassLynx software (Data Handling System for Windows, version 4.0, Micromass, Altrincham, UK).

Accurate mass measurements and MS/MS fragmentation experiments (Figure 1B) were carried out using above described LC and EC components connected to a hybrid mass analyzer Q-TOF *micro* (Micromass, Manchester, UK), equipped with an ESI electrospray interface (Micromass, Manchester, UK) and MassLynx software (Data Handling System for Windows, version 4.0, Micromass, Altrincham, UK).

2.3 HPLC Conditions

Roscovitine (10^{-4} g) was dissolved in 1 mL of mobile phase (initial conditions) and 10 μL were injected. The mobile phase used was Solvent A consisting of different 15 mM buffer/water solutions as described below and Solvent B consisted of acetonitrile or methanol as organic phase. At a flow rate of 250 μL/min the following binary gradient was used–solvent B was raised from initial 10 % to 75 % in 20 minutes and further raised to 100 % in 5 minutes, where it was held constant for 3 minutes. The gradient then returned in 2 minutes to its initial conditions and the column was equilibrated for 5 minutes.

2.4 Optimization of the LC/EC/ESI-MS System

The setup shown in Figure 1A was optimized by varying its conditions, without separating the resulting oxidation products. For all LC/EC/ESI-MS measurements the working electrode was kept at a constant potential and the system was equilibrated for 10 minutes after the EC cell was switched on. The electrochemical conversions were

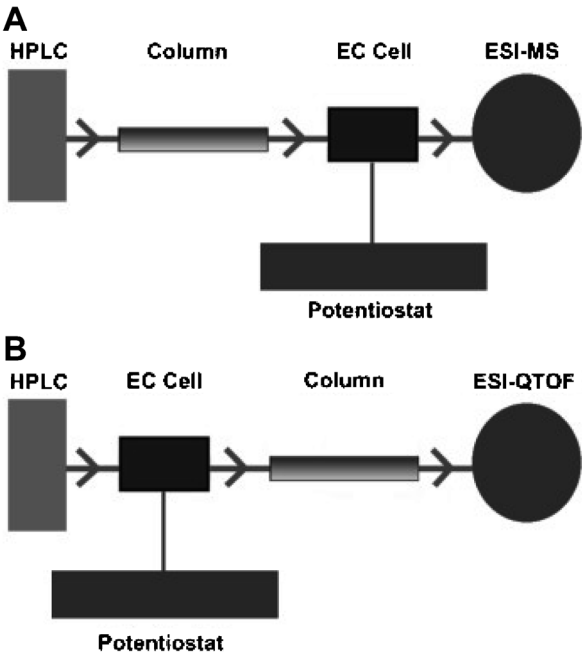

Fig. 1. Different setups for the simulation of oxidation processes of R-roscovitine. A) Setup for LC/EC/ESI-MS configuration used for investigation of the effect of different mobile phases and EC Cell voltages (voltages ramped from 0 to 1800 mV) with no metabolites separation. B) Configuration for LC/EC/ESI-QqTOF with metabolites separation, identification and fragmentation experiment.

carried out at potential 0–1800 mV in 100 mV steps. Different pHs of the aqueous part of the mobile phase (Solvent A) were tested, namely unbuffered formic acid (pH 2.8), formic acid (pH 4) and ammonium formate (pH 7.4) buffered to their respective pH by ammonium hydroxide, all tested buffer solutions were 15 mM. Methanol and acetonitrile were used and compared for all tested pHs as the organic phase. Also water/methanol 90 : 10 v/v and water/acetonitrile 90 : 10 v/v mixtures were used as solvent A. There were also different negative modes tested, with a negative potential applied to either EC cell and ESI electrospray interface, with no usable results (data not shown). The effluent was introduced into the ZMD 2000 electrospray source with optimized conditions (source block temperature 100 °C, capillary voltage +3.0 kV, cone voltage +20 V, desolvation temperature 250 °C). Nitrogen was used both as a desolvation gas (400 L/h) and as a cone gas (50 L/h). All measurements were done in positive ion mode with scan range *m/z* 50–820

2.5 LC/EC/ESI-QqTOF

The setup displayed in Figure 1B was used for separation and subsequent identification of electrochemically generated R-roscovitine oxidation products, as well as for MS/MS fragmentation experiments. The EC Cell potential was kept constant for 1800 mV, the LC conditions used were the same as above – mobile phase consisted of 15 mM ammonium formate/ammonium hydroxide (Solvent A) buffered to pH 7.4 and acetonitrile (Solvent B). The QqTOF accurate mass MS analyses were done in positive mode with following optimized parameters: source block/desolvation temperature 100 °C/350 °C, capillary/cone voltage 2500/25 V, spray/cone gas flow (N_2) 500/50 L/h, scan range $m/z = 50$–820. Lock spray was used for internal calibration with a mixture of 0.1 M NaOH/10% formic acid (v/v) and acetonitrile (1 : 1 : 8, v/v/v) as a reference. Accurate masses were calculated and used for the determination of the elementary composition of the analytes, with fidelity of 5.5 ppm or better.

3 Results and Discussion

3.1 LC/EC/ESI-MS

The used electrochemical flow-through cell utilizes a porous glassy carbon working electrode. Its large surface area is able to provide a good conversion rate at high flow conditions, depending on the pH, analyte attributes and other conditions [28]. An EC Cell connected online to an LC/MS can provide an easy to set-up oxidation-mimicking system, with generated oxidation products and resulting potential metabolites subsequently detected by ESI-MS. In a drug molecule, it can also indicate drug sites labile towards potential oxidation [28]. Various types of purine based substances have already been examined by LC/EC/ESI-MS [29–31]. R-roscovitine is a promising potential anticancer drug. Chosen for further development from a large set of substituted purine analogues, it is currently undergoing phase II clinical trials [32]

First part of results for the electrochemical behavior of R-roscovitine were obtained using the LC/EC/ESI-MS setup shown in Figure 1A, with a single quadrupole mass spectrometer, without separation of the produced ions. These results with corresponding masses, acquired by using different mobile phases, are displayed in Table 1.

Six products were observed under various pH and mobile phase conditions and these are examined further in the next section.

Depending on a normal silica-based column operating pH range, Table 1 clearly shows the significance of choosing the right mobile phase for investigation. At pH 2.8, the number of products is low, consisting of hydroxylated products and it shows the dimer formation as well. With the addition of an ammonium hydroxide to buffer and an increase in pH to 4, the number of products increases slightly and the formation of dimers occurs again. Mixture of pure solvents (Table 1, H_2O) showed almost no additional peaks, probably due to the low conductivity of the solvent. The highest number of new compounds formed was observed at a weak alkaline pH of 7.4, which also corresponds with physiological pH of human arterial blood. Thus, the selected pH and mobile phase chosen for further studies (Figure 1B) was 7.4 combined with acetonitrile, since it showed the best suitability in terms of peak dispersion and amount of discovered oxidation

Table 1. The occurence of **1a–f** masses using the LC/EC/ESI-MS setup, depending on different pHs of chromatographic solvents (phase A), H_2O being pure water without any chemical modifiers.

	Found mass (m/z)	H_2O	pH 2.8	pH 4	pH 7.4
1a	329.2	–	–	–	X
1b	371.4	–	X	X	X
1c	385.2	–	–	X	X
1d	387.2	X	X	X	X
1e	401.2	–	–	X	X
1f	706.4	–	X	X	X

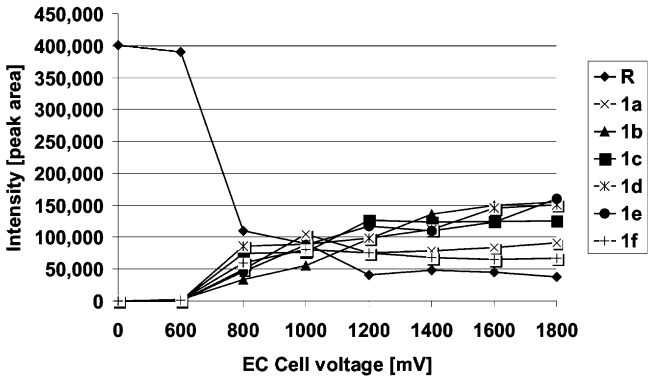

Fig. 2. Intensity (peak areas) of corresponding masses of EC (**1a–f**) products depending on used voltage in the LC/EC/ESI-MS setup (pH 7.4).

products. At all investigated pHs, except for the pure water, the oxidation began at 800 mV, with best results obtained at 1800 mV (Figure 2).

3.2 LC/EC/ESI-QqTOF

An LC/EC/ESI-QqTOF system was employed to obtain further details of the structure of the obtained oxidation products. Using the setup displayed in Figure 1B, the electrochemically generated R-roscovitine metabolites from the EC cell were separated under the optimized LC conditions described above – (ammonium formate/ammonium hydroxide)/acetonitrile with ph 7.4 as mobile phase and EC Cell operated at 1800 mV. This resulted in the total ion chromatograms (TIC) presented in Figure 3. The extracted ion chromatograms (XIC) and TOF mass spectra for products **1a** (retention time $t_R = 12.34$ min), **1b** ($t_R = 15.93$ min), **1c** ($t_R = 19.37$ min), **1d** ($t_R = 13.78$ min), **1e** (t_R 14.06 min) and **1f** ($t_R = 25.70$ min) from Table 1 are also displayed in Figure 3. All of these peaks exhibit the distinct isotopic pattern of R-roscovitine. The R-roscovitine itself has, under the same conditions, $t_R = 16.58$ min.

The resulting accurate mass spectra for these products, and the molecular formula calculated from them, are provided in Table 2. The differences between the theoretical and calculated monoisotopic molecular masses for all measured compounds were below 5.5 ppm. Based on these results, we propose the corresponding molecular structures shown in Figure 4, which are discussed further.

Product **1a** represents a *N*-dealkylation change by loss of the isopropyl group at position 9, with hydroxylation of the benzene ring . Such *N*-dealkylation of a tertiary amine has been previously successfully performed using lidocaine and an EC/LC/MS setup [33]. The product itself shows the shortest retention time, and thus has the highest polarity of all the observed products. This is understandable due to loss of the nonpolar isopropyl chain and the further increased polarity resulting from the introduction of an –OH group onto the benzene ring. The basis of choosing the benzene ring as the site of hydroxylation, is based on product **1b**, the molecular mass of which corresponds to hydroxylated R-roscovitine; its molecular formula is identical to the commercially available R-roscovitine derivate, Olomoucine II. This was verified by comparison of the retention time for Olomoucine II synthetic standard and the product ion spectrum. Product **1b** shows also higher polarity than R-roscovitine based on its shorter retention time, which is in accordance with its proposed structure. Due to presence of one hydroxyl group in product **1a**, we suggest that its position should be identical as in product **1b** and that the hydroxylation of the benzene ring occurs primarily to the *N*-dealkylation, with product **1b** as a substrate. Hydroxylation of a benzene ring has already been performed by different EC/ESI-MS setups [7, 9–11].

Product **1d** corresponds to a dihydroxylated R-roscovitine, the proposed structure of which is highly hypothetical. However, due to occurrence of Olomoucine II, we believe that the product **1b** might be the first step substrate for the second hydroxylation of the R-roscovitine structure. Its retention time, which is lower than **1b**, correlates well to the expected increase in polarity caused by the presence of a second hydroxyl group. Hydroxylation of a benzene ring bearing another hydroxyl group by an EC Cell has been previously reported on some compounds [34].

However comparing t_R of the product **1d** with the retention times of synthesized 2,4- and 2,5-dihydroxy roscovitine derivatives standards excluded the possibility of their formation in the EC Cell, therefore the second hydroxylation is predicted to be located on the oxidation site at the 8-position, as proposed before for guanine [35–37]. This proposition is supported by a MS/MS experiment (see Supporting Information Figure1).

The molecular formula of product **1c** shows a CH_3O addition, which should represent a methoxylation. This

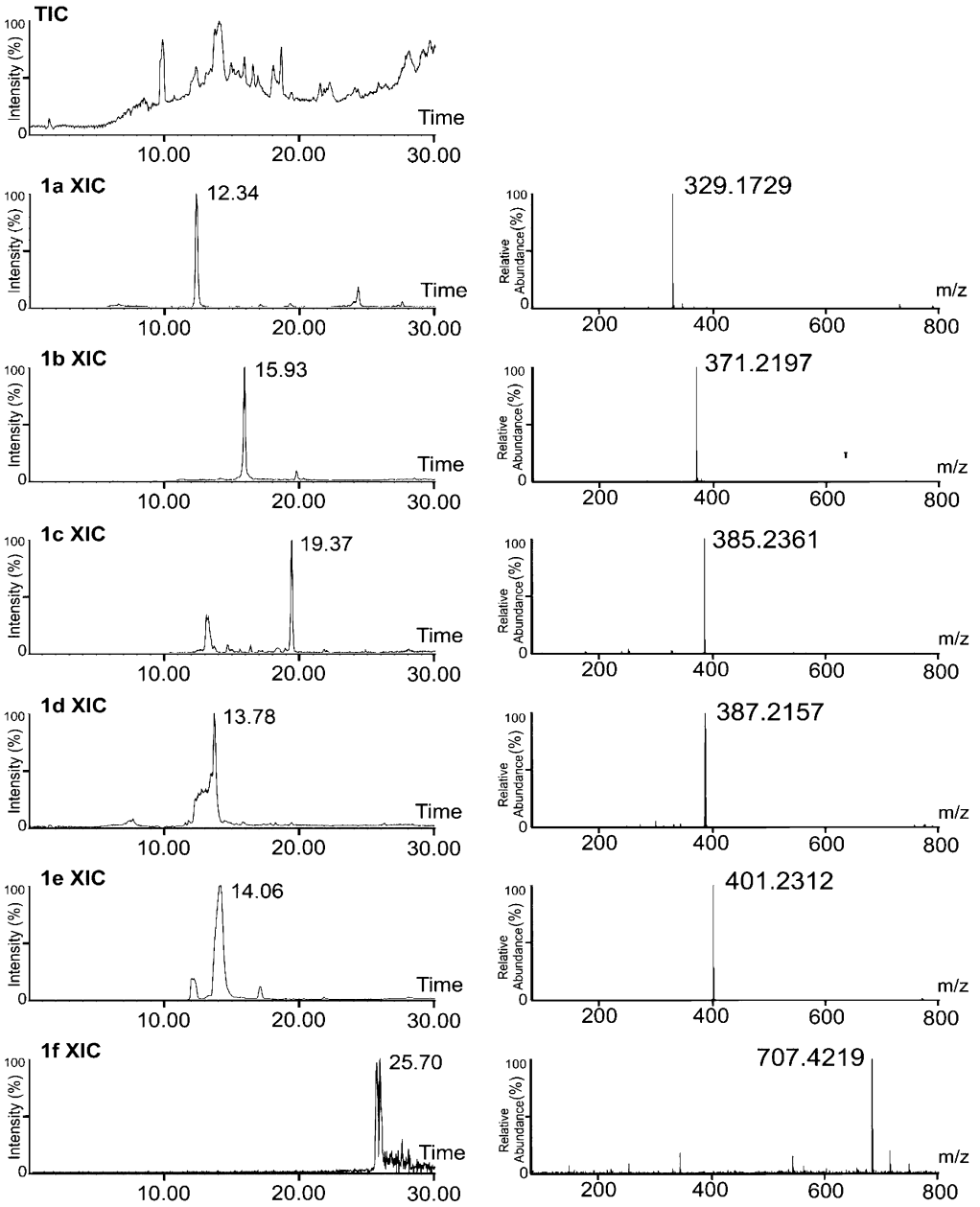

Fig. 3. Total ion (TIC), extracted ion chromatograms (XIC) and MS spectra of products **1a–f** determined by the LC/EC/ESI-QqTOF system.

type of reaction, although mediated by significantly different EC conditions–an anodic oxidation in methanol, was also previously observed [38]. We suggest this option instead of possible separated methylation and hydroxylation on different molecule sites, as methylation was not observed in an LC/EC/ESI-MS study before. Likewise compound **1e** should correspond to an identically methoxylated product, with an additional hydroxylation on the same site as product **1d**.

Product **1f** is predicted to be a R-roscovitine dimer. Such dimerisation of purine based compounds was described previously in EC and EC/MS studies for adenine [29], guanine and xanthine [35], guanosine [36,39], dideoxy adenine [40], cyclic 1.N^2-propanoguanosine [41] and adenosine monophosphate [42]. Guanosine derivatives are also known to self-assembly into different aggregates [43]. However, some substances, such as boscolide (2-chloro-*N*-(4′-chloro(1,1′-biphenyl)-2-yl)-3-pyridinecarboxamide), show no dimer formation in in vitro assays in contrast to their formation in the EC cell [10]. The proposed structure and the site of dimerisation was based on the report by Pitterl et al.[36]. Dimerisation seems to be a common purine reaction in an EC cell, although it was not reported in case of some purine monophosphates [37].

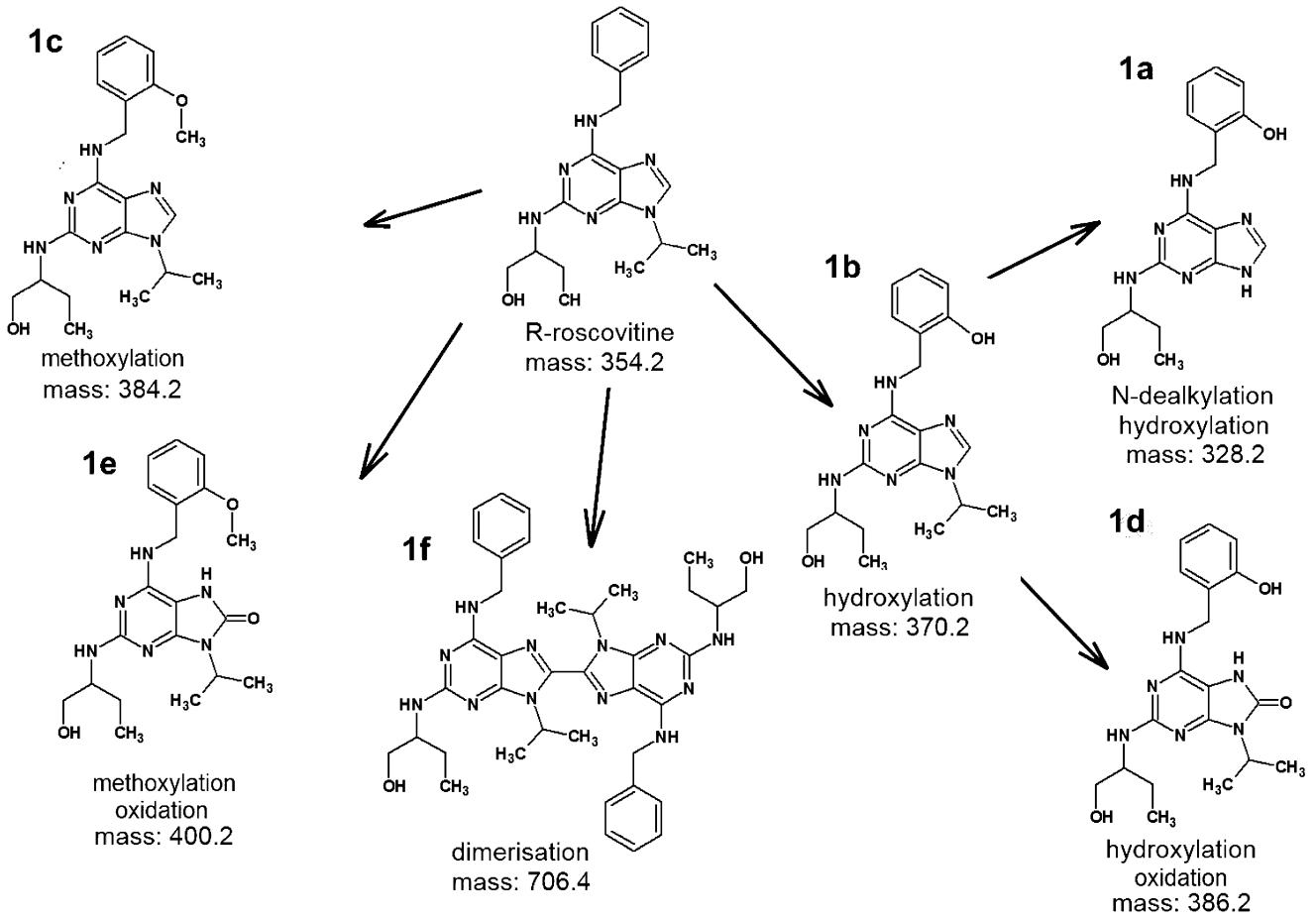

Fig. 4. Proposed oxidation products of R-roscovitine in the EC Cell determined by the LC/EC/ESI-QqTOF setup accurate mass measurement.

Table 2. Accurate mass measurements and molecular formula calculations of R-roscovitine (R) and electrochemically derived products **1a–f** determined by the LC/EC/ESI-QqTOF system.

	Measured mass (m/z)	Theoretical mass $[M+H]^+$	Molecular formula	Transformation	Deviation [ppm]
R	355.2244	355.2246	$C_{19}H_{26}N_6O$		−0.5
1a	329.1729	329.1726	$C_{16}H_{20}N_6O_2$	$-[C_3H_7]+H+OH-H$	0.9
1b	371.2197	371.2195	$C_{19}H_{26}N_6O_2$	$+OH-H$	0.5
1c	385.2361	385.2352	$C_{20}H_{28}N_6O_2$	$+[CH_3O]-H$	2.3
1d	387.2157	387.2145	$C_{19}H_{26}N_6O_3$	$+2OH-2H$	3.1
1e	401.2312	401.2301	$C_{20}H_{28}N_6O_3$	$+[CH_3O_2]-H$	2.7
1f	707.4219	707.4258	$C_{38}H_{50}N_{12}O_2$	$+[C_{19}H_{25}N_6O]-2H$	−5.5

4 Conclusions

4.1 Comparison with Previous in vitro Experiments

To verify, that the electrochemically generated products are comparable with those obtained by pharmacologically relevant cytochromes P450, we compared our results with previously published metabolic studies. Metabolism of the similar trisubstituted purine, bohemine, has been studied to some degree with the determination of certain primary metabolism routes [44,45]. in vivo metabolism of R-roscovitine has been examined after administration to rats [22,24], mice [23] and its in vitro metabolism was examined by an interaction with selected human and animal microsomes [22]. There are five main R-roscovitine metabolites mentioned (Table 3), with the carboxylic roscovitine PMF30−128 (m/z 369.2) being quantitatively the major R-roscovitine metabolite observed in pharmacological studies [22,46,47].

In Table 3, shown data compare the products from the EC reactions that were observed for R-roscovitine, as well as the metabolites obtained by the conventional in vitro technique using different animal and human microsomes [22].

Table 3. Comparison of occurrence of EC (**1a–f**) products according to previously published in vitro (**Iv**) R-roscovitine (**R**) metabolites [22].

	Found mass (*m*/*z*)	Molecular formula $[M–H]^+$	Transformation	EC	In vitro
R	355.2	$C_{19}H_{26}N_6O$	–	X	X
1a	329.2	$C_{16}H_{20}N_6O_2$	$-[C_3H_7]+H+OH–H$		X
1b	371.2	$C_{19}H_{26}N_6O_2$	$+OH–H$	X	X
1c	385.2	$C_{20}H_{28}N_6O_2$	$+[CH_3O]–H$	X	
1d	387.2	$C_{19}H_{26}N_6O_3$	$+2OH–2H$	X	
1e	401.2	$C_{20}H_{28}N_6O_3$	$+[CH_3O_2]–H$	X	
1f	707.4	$C_{38}H_{50}N_{12}O_2$	$+[C_{19}H_{25}N_6O]–2H$	X	
Iv	283.2	$C_{15}H_{18}N_6$	$–C_4H_8O$		X
Iv	313.2	$C_{16}H_{20}N_6O$	$–C_3H_6$		X
Iv	353.2	$C_{19}H_{24}N_6O$	$–2H$		X
Iv	369.2	$C_{19}H_{24}N_6O_2$	$–2H+O$		X
Iv	371.2	$C_{19}H_{26}N_6O_2$	$+OH–H$	X	X

There are no dimers reported as in vitro/in vivo metabolites, but this probably results from failure to detect them with methods used, none of which measured masses above 600 Da [22] or 650 Da [23] or used a selective MRM approach with highest precursor ion *m*/*z* 371 [24], which made it impossible to trace unknown metabolites as dimers. However, the newest FDA and ICH issued guidelines, dealing with the safety testing of metabolites, strongly recommends the issue of metabolite profiling in humans as an important early stage in plans for clinical drug development. Moreover, all metabolites with relative content higher than 10% of the total drug derived material or of the parent drug in human plasma, require an evaluation of their toxicokinetic coverage in preclinical species [48]. A large number of purine derived substances undergo dimerisation under similar electrochemical conditions to those described above. The biological activity of the dimers formed should also be taken into consideration, as one of the guanosine dimers has been found to be toxic, causing nephritis with edema in albino mice [49].

The main roscovitine metabolite found in in vitro/in vivo studies, carboxylate metabolite PMF30−128 (*m*/*z* 369.2), was not found in our EC studies. Its absence can be explained by the nature of the oxidations happening in the EC Cell. They occur almost exclusively via a one-electron oxidation pathway, including *N*-dealkylation of amines, dehydrogenation reactions, oxidation of alcohols, or hydroxylation of activated aromatics [28]. On the other hand, cytochrome P450 -mediated oxidations can be initiated both by an electron extraction, but also by a deprotonisation step [12]. All cytochrome P450 enzymes are monooxygenases, i.e. they cleave dioxygen and incorporate one oxygen atom into the substrate, while the remaining oxygen atom is reduced by two-electron reaction to give water. This brings us to the conclusion, that the oxidation of alcohols or aldehydes to carboxylic acids (CYP-catalyzed reactions occurring in vivo), will not be observable under electrochemical conditions [12]. Therefore, the preferred EC Cell reaction is the one-electron step oxidation of the aromatic ring (products **1a**, **1b**, **1d**).

We present here a purely instrumental method for investigation of the R-roscovitine oxidation pathway, based on EC cell located oxidation in combination with HPLC separation and identification provided by exact mass measurements of the resulting products. We have successfully shown an electrochemical preparation of Olomoucine II (product **1b**). It is a 2,6,9-trisubstituted purine derivative which displays 10 times higher CDK inhibitory activity than R-roscovitine [50]. Its identity has been confirmed by a comparison of its retention time with that of a synthetic Olomoucine II standard and by the QqTOF accurate mass MS analysis. A similar hydroxylation of the benzene ring was observed in in vitro study of bohemine [45].

Another hydroxylated product, **1d**, a dihydroxylated derivative of R-roscovitine, can be also described as a hydroxy derivative of Olomoucine II. A recent study of Olomoucine II in vitro P450 metabolism reveals a potential hydroxylated metabolite with *m*/*z* 387 (the same *m*/*z* as product **1d**), which has been identified as 2,5-dihydroxyroscovitine [51]. However, as shown above, comparison with a synthetic standard failed to reveal the product occurence in the EC cell. Moreover, the performed MS/MS experiment (Supporting Information Figure 1) suggested the position of second hydroxyl group at the purine skeleton.

Acknowledgements

This work was supported by the *Czech Ministry of Education* (MSM 6198959216), and Grant No. ED0007/01/01 from the *Centre of the Region Haná for Biotechnological and Agricultural Research*.

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Supplement III.

biologia plantarum

Micropropagation of wych elm (Ulmus glabra, Huds.): the regulative role of two different aromatic cytokinins during organogenesis.

Journal:	*Biologia Plantarum*
Manuscript ID:	2011-07-1434
Manuscript Type:	Brief Communications
Date Submitted by the Author:	29-Jul-2011
Complete List of Authors:	Malá, Jana; Institute of Forest and Game Management Machova, Pavlina; Institute of Forest and Game Management Cvrckova, Helena; Institute of Forest and Game Management Karady, Michal; Palacky University and Institute of Experimental Botany AS CR, Laboratory of Growth Regulators Novák, Ondrej; Palacky University and Institute of Experimental Botany AS CR, Laboratory of Growth Regulators Mikulík, Jaromír; Palacky University and Institute of Experimental Botany AS CR, Laboratory of Growth Regulators Dostál, Jaroslav; Institute of Forest and Game Management Strnad, Miroslav; Palacky University, Centre of the Region Haná for Biotechnological and Agricultural Research; Palacky University and Institute of Experimental Botany AS CR, Laboratory of Growth Regulators Dolezal, Karel; Palacky University, Centre of the Region Haná for Biotechnological and Agricultural Research; Palacky University and Institute of Experimental Botany AS CR, Laboratory of Growth Regulators
Keywords:	wych elm, micropropagation, rooting, cytokinins, meta-topolin, ethylene, senescence

Micropropagation of wych elm (*Ulmus glabra*, Huds.): the regulative role of two different aromatic cytokinins during organogenesis.

Jana Malá[1], Pavlína Máchová[1], Helena Cvrčková[1], Michal Karady[2], Ondřej Novák[2], Jaromír Mikulík[2], Jaroslav Dostál[1], Miroslav Strnad[2,3], Karel Doležal[2,3] *

[1]Institute of Forest and Game Management, Strnady 136, 252 02 Jíloviště, The Czech Republic, tel. +420 257 892 222

[2]Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany AS CR, 783 71 Olomouc-Holice, The Czech Republic, tel. +420585634940

[3]Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Šlechtitelů 11, CZ 783 71 Olomouc, Czech Republic

*Corresponding author, e-mail: Karel.Dolezal@upol.cz

Summary
We have compared the influence of two aromatic cytokinin derivatives, 6-benzylaminopurine (BAP) and meta-topolin (mT), on the *in vitro* multiplication and senescence of wych elm (*Ulmus glabra*, Huds.). After 3 months of cultivation, the micropropagation rate was higher (approx. six times more shoots developed) on Murashige Skoog (MS) medium supplemented with mT than on MS supplemented with BAP. A newly developed UPLC-ESI(+)-MS/MS method was used to determine concentrations of 50 endogenous cytokinin metabolites in explants cultivated 12 weeks on media supplemented by BAP or mT. Analysis of concentrations of 50 cytokinin metabolites, using a recently developed UPLC-ESI(+)-MS/MS method, showed there were significant differences in the endogenous cytokinin pools of explants cultivated for 12 weeks on media supplemented with BAP or mT, and their levels of metabolites of the added cytokinins. Correlative differences in their photosynthetic rates and ethylene production levels were also detected.'

Key words
wych elm, micropropagation, rooting, cytokinins, ethylene

Abbreviations
BAP – 6-benzylaminopurine; cZ – cis-zeatin; mT – 6-(3-hydroxybenzylamino)purine; DHZ – dihydrozeatin; *9G – 9-β-D-glucopyranosyl derivative; iP – N6-isopentenyladenine; *5´MP – 5´-monophosphate derivative; *OG – O-β-D-glucopyranosyl derivative; oT – 6-(2-hydroxybenzylamino)purine; *R – 9-β-D-ribofuranosyl derivative; tZ – trans-zeatin; IBA – indole-3-butyric acid, MS – Murashige-Skoog medium, LC-MS - liquid chromatography combined with mass spectrometry; UPLC-ESI(+)-MS/MS - ultra performance liquid chromatography combined with positive electrospray mass spectrometry

Elms (*Ulmus* sp.) are trees that are highly valued for their great strength, tightly twisted grain, durability and tolerance of both cold and salt (Heybroek et al., 1982). Wych elm (*Ulmus glabra*, Huds.) is a native European species that is especially common in mountainous regions of the Czech Republic. However, during the 1970's, the second Dutch elm disease epidemic destroyed most elm populations in Europe (Brasier 1990, Fenning et al.

1993). Hence, a program for conserving genetic resources of elms has been initiated, within the EUFORGEN framework, which includes establishment of collections of trees (clonal archives), presuming trees that survived the epidemic to have a high degree of resistance against repeated infection. Elite elm trees can be vegetatively propagated by grafting and cuttings, but micropropagation is the most promising option (Gartland et al. 2001, Gartland et al. 2004). However, although various micropropagation systems have been reported for numerous species and hybrids (Biondi et al., 1984, Fink et al., 1986, Chalupa 1983, Chalupa 1994, McCown and McCown 1987, Fenning et al. 1993, Corchete et al. 1993, Cheng and Shi 1995), some obstacles still remain during organogenesis, notably early senescence and low multiplication rates, both of which decrease the micropropagation efficiency.

Optimizing micropropagation is not straightforward since it is a complex process, involving a sequence of developmental stages that are influenced by numerous endogenous and exogenous stimuli. These stimuli include phytohormones, especially auxins and cytokinins. The focus here is on cytokinins, which have been shown to play important regulative roles during organogenesis in numerous studies (e.g. D´Angeli et al. 2001; Caboni et al. 2002). However, it should be noted that auxin/cytokinin ratios during growth in culture are also important (Skoog and Miller 1957), and the levels (absolute and relative) of these hormones are influenced by rates of both their biosynthesis and degradation/inactivation (Hausman et al. 1994, Mok and Mok 2001), as well as by exogenous plant growth regulators added to the culture media. The addition of exogenous cytokinins is essential, *inter alia*, for the induction of root formation (De Klerk et al. 2001), but (unlike auxins), high levels of cytokinins inhibit adventitious rooting (Bollmark et al. 1988). Thus, deeper understanding of the roles of endogenous cytokinins, and effects of exogenous cytokinins, could substantially facilitate the development of more effective micropropagation techniques. BAP is an important aromatic cytokinin derivative that is routinely utilized to induce organogenesis in micropropagation, but in some cases it may negatively influence growth, rooting and acclimatization (Werbrouck et al. 1995, Werbrouck et al. 1996). Hence, another aromatic cytokinin derivative (mT) appears to be more advantageous during the micropropagation and acclimatization of numerous plant species, because differences in its metabolism ameliorate some of these adverse effects (Werbrouck et al. 1996, Bairu et al. 2009, Valero-Aracama et al. 2010, Wojtania 2010).

In the study presented here we compared the effects of these two cytokinin derivatives on the *in vitro* multiplication and senescence of wych elm explants. In addition, we determined concentrations of 50 cytokinin metabolites in explants cultivated for 12 weeks on media supplemented with BAP or mT, to compare their metabolism, their effects on endogenous cytokinin pools, the explants' photosynthetic rates and their production levels of the senescence-related hormone ethylene.

Plant material, used for the explant cultures, was collected from donor trees, 40 - 80 years old, in 2009 and is conserved in the Explant Bank of the Forestry and Game Management Research Institute, The Czech Republic.

To establish experimental explant cultures, multiapex cultures derived from 15 clones of wych elm growing in the Explant Bank were used. The multiapex cultures were cultivated for 18 months at 24 °C on multiplication medium consisting of agar-solidified MS (Murashige and Skoog 1962) medium supplemented with 0.5 $mg.l^{-3}$ BAP, 0.1 $mg.l^{-3}$ IBA, 100 $mg.l^{-3}$ glutamine and 30 $g.l^{-3}$ sucrose, pH 5.8, under white fluorescent lamps (36W/33 Philips tubes, Eindhoven, the Netherlands), providing irradiance of 30 $\mu mol.m^{-2}.s^{-1}$ in 12 h photoperiods. A pair of explant cultures derived from the multiapex cultures of each of the 15 clones was grown on MS multiplication medium with 0.5 $mg.l^{-3}$ BAP, while another pair was grown on the same medium with 0.5 $mg.l^{-3}$ mT. All cultures were cultivated for 12 weeks (with transfer every 4 weeks to fresh MS multiplication medium) under the same cultivation

conditions as in the Explant Bank. The new shoots that developed from each multiapex culture were then counted, and their lengths were measured. In addition, four shoot cultures were randomly selected from the sets (of 30) growing in the presence of each supplementary cytokinin derivative to analyze their endogenous cytokinin pools and levels of metabolites of the added cytokinins.

Triplicate portions (app. 200 mg) of the samples were extracted for 3 h in ice-cold 70% ethanol (v/v), and a mixture of deuterium-labeled standards was added to each sample to check recovery during purification and to validate determinations (Novák et al. 2008).After extraction, the combined supernatants were concentrated to approximately 1.0 ml under vacuum at 35 ºC, diluted to 20 ml with ammonium acetate buffer (40 mM, pH 6.5) and purified using a DEAE-Sephadex (1.0 x 5.0 cm) column coupled to an octadecylsilica (0.5 x 1.5 cm) column followed by immunoaffinity chromatography (IAC) with a generic cytokinin monoclonal antibody (Faiss et al. 1997). This resulted in three fractions containing: (1) the free cytokinin bases, ribosides and N-glucosides, (2) a ribotide fraction, and (3) an O-glucoside fraction. Fractions 2 and 3 were further purified by IAC, following treatment with alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) and β-glucosidase (G-0395, Sigma, St. Louis, USA), respectively. The eluates from the IAC columns were evaporated to dryness and dissolved in 20 μL of the initial mobile phase used for quantitative analysis. The concentrations of 50 endogenous cytokinins and metabolites of the added cytokinins were then determined by ultra performance liquid chromatography combined with tandem mass spectrometry (UPLC-MS/MS) (Novák et al., 2008), using an ACQUITY UPLC ultra-performance liquid chromatograph (Waters), equipped with a BEH C 18 (1.7 μm; 2.1 x 150 mm) column, linked to a Quattro micro API (Waters MS technologies) triple quadrupole mass spectrometer equipped with an electrospray interface. The compounds were quantified by multiple reaction monitoring of $[M+H]^+$ ions and an appropriate product ion (Novák et al. 2008). The identities of all measured cytokinin metabolites were verified by comparing their mass spectra and chromatographic retention times with those of authentic standards. The measurements were done in triplicate.

Three clones, exhibiting signs of senescence (leaf yellowing and shedding), growing in the long-term BAP medium, were transferred to two multiplication media containing either 0.2 $mg.l^{-3}$ mT or 0.2 $mg.l^{-3}$ BAP. After 6 weeks of cultivation their ethylene productions levels were measured using the GC-FID method described by Fišerová et al. (2001). Briefly, an hour before sampling, cultivation vessels were ventilated. Then, 1 ml of air was taken from each vessel and analyzed using a Finnigan Trace GC Ultra, equipped with an FID detector and 50 m capillary column (HP-AL/S stationary phase, 15 μm, ID 0.535 mm., injection, oven and detector temperatures set to 200, 40 and 220 ºC, respectively). The measurements were done in triplicate, using samples from each of three vessels containing cultures grown on each medium. Finally, the photosynthetic activity of the sets of 30 explants grown on both cultivation media was determined using an Imaging-PAM Chlorophyll Fluorometer (Heinz Walz GmbH), leaf samples with surface areas of ca. 0.25 cm^2 and 3 $\mu mol.m^2.s^{-1}$ pulse-amplitude-modulated light (Waltz 2003

Between-treatment differences in mean numbers of shoots produced, the length of the shoots, levels of measured endogenous cytokinins, ethylene production and photosynthetic activity were explored by two-way analysis of variance (ANOVA), and their significance was evaluated by the Tukey-Kramer test using QC Expert and NCSS software .

Number of growing shoots increased differently after 12 weeks in relation to the kind of cytokinin in nutrient media. Explants cultivated on multiplication medium supplemented with mT produced six times more shoots than counterparts cultivated with BAP (31.9 ± 28.1 versus 5.88 ± 4.03; a significant difference at the 0.01% probability level, according to the ANOVA and Tukey-Kramer test). Further, despite great clonal variability in numbers of new

shoots, the mT medium induced higher numbers of adventitive shoots in every case. These findings are consistent with results obtained in experiments with other plant species, e. g. *Aloe ferox* (Bairu et al. 2009), *Pelargonium* (Wojtania et al. 2010), *Pinus sylvestris* (De Diego et al. 2010) and *Prunus microcarpa* (Nas et al. 2010). Several authors have also reported that medium supplemented with mT induces stronger growth of various species (Werbrouck et al. 1996, Bairu et al. 2007, Valero-Aracama et al. 2010, Wojtania et al. 2010). However, there was no significant difference between the average length of shoots produced by explants grown on mT (3.4±1.45) and those grown on BAP (3.6 ±1.22).

As described above, shoots in long-term BAP medium cultures, in which signs of senescence (leaf yellowing and shedding) appeared, were replanted into multiplication media with 0.2 $mg.l^{-3}$ mT or 0.2 $mg.l^{-3}$ BAP. After 6 weeks of cultivation, the ageing signs persisted only in cultures with BAP. Accordingly, substantially higher ethylene concentrations were detected in the vessels containing these cultures (99±13 $nl.l^{-1}$, versus 41±17 $nl.l^{-1}$ in vessels containing the cultures grown with mT). Similar results have also been obtained in experiments with cultured *Rosa hybrida* tissue (Doležal et al. 2003) and various senescence bioassays (Holub et al. 1998; Cag et al. 2003).

The photosynthetic activity of the explants was determined as the Fv/Fm ratio (variable fluorescence/maximal fluorescence), i.e. potential quantum yield of fluorescence of chlorophyll in the dark-adapted state (Papageorgiou and Govindjee 2004). The results showed that the samples of the explants growing in the mT medium had higher photosynthetic activity (Tab. 1) than those growing in the BAP medium, in accordance with their lack of visible signs of senescence (yellowing).

Fifty endogenous cytokinin metabolites were determined in the samples of wych elm plantlets grown *in vitro* on media containing the two cytokinins. The levels of 33 cytokinin species were found to be present at levels exceeding the detection limit of the UPLC-MS/MS quantification method used (Novák et al. 2008), and thus could be quantified and compared in relation to the cytokinins added to the cultivation media. We found much higher in-tissue concentrations of BAP in the explants grown in the BAP-containing medium than of mT in explants grown in the presence of mT (Tab. 2), in accordance with results of previous experiments with *Sorbus torminalis* (Malá et al. 2009). In contrast, plants grown on both cytokinins generally contained very similar levels of their major metabolic forms (ribosides, nucleotides and 9-glucosides). However, much higher concentrations of O-glucosides were detected in the explants grown on mT, as previously reported in *Spathiphyllum* (Werbrouck et al. 1996), while 9-glucosides were the major metabolites in explants grown with BAP. These findings may be related to the amelioration of acclimatization problems when mT is used. Levels of hydroxylated aromatic cytokinins were also substantially more abundant in the tissue micropropagated on BAP (Tab. 2).'

In addition, we analyzed levels of endogenously formed isoprenoid cytokinins in relation to the exogenously applied cytokinin in the media. In contrast to previously published results of experiments with *S. torminalis* (Malá et al. 2009) we did not find any significant differences in levels of most isoprenoid cytokinin free bases – tZ, cZ, and DHZ. However, levels of their other metabolic forms – ribosides, nucleotides and 9-glucosides – were slightly higher in plants grown on media with mT, while levels of iP-type cytokinins were slightly higher in BAP micropropagated plants (Tab. 2).

Clearly, optimal concentration of cytokinins and their metabolites, as well as other plant hormones, are crucial for successful and efficient plant *in vitro* micropropagation. Hence, determinations of optimal endogenous plant hormone concentrations and their dependence on exogenous cytokinins used in cultivation media, may improve both the *in-vitro* micropropagation efficiency and the quality of *ex vitro* acclimatized plants of wych elm, and possibly other plant species.

Acknowledgements
This work was supported by grants from the Ministry of Agriculture of the Czech Republic (NAZV QI 92A247), Czech Ministry of Education (MSM 6198959216), ED0007/01/01 Centre of the Region Haná for Biotechnological and Agricultural Research.

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Tab. 1: Comparison of photosynthetic activities (mean Fv/Fm ratios) of wych elm explants cultivated on medium supplemented with BAP or mT by two-way analysis of variance (ANOVA) and the Tukey-Kramer test. The difference between the values in both cases is significant at the 0.05 probability level.

Cytokinin used in the medium	Number of samples	Mean Fv/Fm
BAP	30	0.667
mT	30	0.804

Tab. 2.
Cytokinin levels (pmol.g^{-1} FW) in wych elm (*Ulmus glabra,* Huds.) shoots determined by UPLC-ESI(+)-MS/MS. 12-week-old *U. glabra* shoots (250 mg) were extracted, the extracts were purified by SPE followed by immunoaffinity chromatography and their cytokinin contents were then measured by UPLC-ESI(+)-MS/MS.

	tZ	tZR	tZR-5´MP	cZ	cZR	cZR-5´MP	DHZROG	DHZR	iP
BAP	1.24±0.60	1.88±1.63	3.41±3.02	0.24±0.11	0.13±0.05	<LOD	0.07±0.03	0.09±0.07	1.98±0.5
mT	1.30±0.43	4.32±1.52	6.27±2.47	0.28±0.16	0.50±0.25	1.59±0.87	0.11±0.06	0.13±0.08	1.07±0.36
	iPR	iPR-5´MP	BAP	BAPR	BAP9G	BAPR-5´MP	mT	mTR	oT
BAP	2.56±0.59	9.86±3.19	17798±11595	92.5±21.5	4.71±0.57	460±148	7.10±1.80	1.66±0.31	0.55±0.25
mT	1.48±0.57	4.31±2.30	2.61±1.49	0.34±0.29	<LOD	0.75±0.61	1141±651	173±116	<LOD
	oTR	tZ9G	mTR-5´MP	tZOG	tZROG	cZOG	mT9G	mTOG	mTROG
BAP	8.16 ± 1.55	0.06±0.01	3.16±1.08	2.00 ± 1.19	0.43 ± 0.32	0.13±0.07	<LOD	9.86 ± 3.09	11.4±2.74
mT	<LOD	0.44±0.13	224±84	2.07±0.56	0.67±0.22	0.13±0.06	5.42±3.37	1885 ± 434	1197±349

[a] **Amounts** ±SD represent mean values of 3 independent technical replicates of 4 different clones. Only compounds above the detection limit are included.

Supplement IV.

High Performance Liquid Chromatography-Electrochemistry-High Resolution Electrospray Ionization/Mass Spectrometry (HPLC-EC-HR ESI/MS) Characterization of Selected Cytokinins Oxidation Products

Michal Karady[a], Ondřej Novák[a,b], Aleš Horna[c,d], Miroslav Strnad[a,b] and Karel Doležal[a,b]*

[a] Laboratory of Growth Regulators, Palacky University and Institute of Experimental Botany ASCR, Šlechtitelů 11, 783 71, Olomouc, Czech Republic

[b] Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Šlechtitelů 11, CZ 783 71, Olomouc, Czech Republic

[c] RADANAL Ltd., Okružní 613, Pardubice, 530 03

[d] Tomas Bata University in Zlín, University Institute, Food Research Centre, Mostní 539, CZ-760 01 Zlín

* e-mail: karel.dolezal@upol.cz

Abstract

Electrochemistry combined with mass spectrometry represents an emerging analytical technique used to mimick phase I metabolism of various drugs and *in vivo* occurring compounds, showing a capability to generate many known metabolites or new oxidation products. An on-line LC-EC-ESI/MS method had been used to investigate the oxidation of selected cytokinin compounds. This setup allowed rapid identification and structure elucidation of the obtained products. An electrochemical oxidation of isopentenyladenine resulted in five products, including *trans*-zeatin and dehydrogenated products, which correlates very well with its *in vivo* metabolism. Electrochemical conversion of *trans*-zeatin revealed six products, with two dehydrogenation products corresponding to its *in vivo* occuring metabolites. *cis*-Zeatin oxidation in the electrochemical cell gave rise to eight products, resembling similarity to *trans*-zeatin oxidation. All three compounds have undergone a complete turnover mainly through two oxidation reactions occurring in the electrochemical cell – dehydrogenation and a less typical aliphatic hydroxylation. The resulting products, especially *trans*-zeatin from isopentenyladenine and -2H dehydrogenation products are in excellent correlation with their known *in vivo* metabolism.

Keywords: Electrochemistry, Cytokinin, Oxidative metabolism, Mass Spectrometry

1. Introduction

Cytokinins (CKs) are phytohormones directly involved in various developmental, growth and stress processes in plants, such as apical dominance, shoot/root growth and counteraction of senescence. Naturally occurring CKs are adenines, which bear an isoprenoid or aromatic side chain at the N^6 position[1]. In living systems, their metabolism can be characterized by modification of their purine moiety, by N^6-side chain modifications or cleavage. These structural alterations can lead to reversible or irreversible removal of CK activity, and therefore they determine the function and subsequent compartmentalisation of the resulting CK metabolite. Frequent endogenous modifications of

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CKs are glycosylation of *N-3*, *N-7* and *N-9* positions of the purine ring, or the formation of alanyl conjugates[2]. Formation of *trans*-zeatin type cytokinins, the most abundant and active type of CKs, involves hydroxylation of the isopentenyladenine side-chain catalysed by two cytochrome P450 mono-oxygenases[3]. A zeatin reductase can reduce the double bond of the resulting *trans*-zeatin to form dihydrozeatin[4] or it can undergo *trans* to *cis* isomerisation, although the responsible enzyme was only partially purified[5]. A key metabolic regulation is represented by an irreversible cleavage of the of the N^6-side chain by CK oxidase (CKX)[6]. During the development process of a new chemical compound, metabolism studies are often crucial[7]. Electrochemistry represents one of the most versatile and susceptible methods available for these studies[8]. The on-line EC-LC-MS technique of coupling together an electrochemical cell (EC) with mass spectrometry (MS) or liquid chromatography (LC), provides a steady analysis of possible metabolites, without a need for product isolation, purification or derivatization and enhancing the ionization efficiency of analytes for various mass spectrometry techniques. Many classes of compounds have been readily analysed with a special emphasis on phase I and phase II metabolites and oxidation products of various drugs and xenobiotics as EC has been shown to be able to readily mimick one-electron oxidations initiated in a human body by P450 cytochromes. Applying these methods at an early stage of metabolic studies may lead to a more efficient and economic procedure in drug development[9]. EC was shown capable of mimicking various type of oxidative reactions, including N-oxidation, benzylic hydroxylation, N-dealkylation, dehydrogenation, S-oxide formation etc.[9]. Various classes of purine-based compounds have been recently investigated by means of EC by several groups[10,11,12] showing a promising potential of EC to oxidize and mimick metabolism of such molecules. In this study, the coupling of EC and HR ESI/MS (high resolution electrospray ionization-mass spectrometry) was used to assess the possibilities of examining the behaviour of selected CKs at an electrochemical cell, with an emphasis to imitate their *in vivo/in vitro* metabolism.

2. Experimental

Chemicals

N^6-(2-Isopentenyl)adenine (iP), 6-(4-Hydroxy-3-methylbut-2-enylamino)purine (*trans*-Zeatin, *t*Z) and 6-(4-Hydroxy-3-methyl-cis-2-butenylamino) purine (*cis*-Zeatin, *c*Z) standards were obtained from Olchemim Ltd (Olomouc, Czech Republic). A Milli-Q deionization unit (Millipore, France) was used for preparation of the purified water for mobile phases and solutions. All other chromatographic solvents and chemicals were of analytical grade or higher purity from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Instrumentation

For the electrochemical conversion of cytokinin standards a commercially available system from ESA (Chelmsford, MA, USA) was used. It comprised a model 5021 conditioning cell (EC cell) connected to a Coulochem III electrochemical detector and potentiostat. A porous glassy carbon coulometric working electrode was employed in the EC cell, together

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[9] Jahn S, Karst U: **Electrochemistry coupled to (liquid chromatography/) mass spectrometry-Current state and future perspectives**. *J Chromatogr A* 2012, **1259**:16-49.
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[11] Pitterl F, Chervet JP, Oberacher H: **Electrochemical simulation of oxidation processes involving nucleic acids monitored with electrospray ionization-mass spectrometry**. *Anal Bioanal Chem* 2010, **397**(3):1203-1215.
[12] Karady M, Novak O, Horna A, Strnad M, Dolezal K: **High Performance Liquid Chromatography-Electrochemistry-Electrospray Ionization Mass Spectrometry (HPLC/EC/ESI-MS) for Detection and Characterization of Roscovitine Oxidation Products**. *Electroanal* 2011, **23**(12):2898-2905.

with a Pd/H_2 reference electrode and a Pd counter electrode. The potentials described in this article are given versus the Pd/H_2 reference electrode. By default an in-line PEEK filter was placed at the inlet line of the EC cell for protection of the working electrode. The LC system consisted of an Alliance 2695 HPLC separation module (Waters, Milford, MA, USA) with all separations done on a Symmetry C18 RP column (150mm x 2.1mm x 5µm, Waters) with the column thermostat set to 30°C. MS analysis, accurate mass measurements and in-source fragmentation experiments were carried out on a hybrid mass analyzer Q-Tof *micro* (Micromass, Manchester, UK) employing an ESI electrospray interface (Micromass, Manchester, UK). All data were processed using the MassLynx software (Data handling system for Windows, v4.1, Micromass, Altrincham, UK).

The EC, LC and MS components were coupled together on-line with EC cell placed between the HPLC and RP column (Fig.1). The PEEK tubing between the instruments was kept as short as possible to minimize dispersion. Under all conditions, there was no disturbing electrical current from ESI interface observed at the EC cell.

HPLC conditions

iP, *t*Z and *c*Z (10^{-4}g) were dissolved in 1ml of mobile phase, identical with initial conditions of analysis, and 10 µl were injected into the system. Mobile phase consisted of 15mM ammonium formate/water solutions (solvent B) and organic phase (solvent A) represented by acetonitrile. The conditions, pH and type of mobile phase were selected as optimal for adenine with different N^6-side-chain type compounds, based on our previous article[12]. Flow rate was constantly set to 100µl. The gradient profile used for all analyses and separations is shown in Table 1.

Time (min.)	0.00	1.00	24.00	34.00	35.00	45.00
% A	10	10	90	90	10	10

Table 1 Gradient profile used for the analysis and separation of oxidation products

QqTOF conditions

The QqTOF accurate mass high-resolution MS analyses were done in positive mode with following optimized parameters: source block/desolvation temperature 100°C/350 °C, capillary/cone voltage 2500/25 V, spray/cone gas flow (N_2) 500/50 L/h, scan range *m/z* = 50-820. Lock spray was used for internal calibration with a mixture of 0.1 M NaOH/10% formic acid (v/v) and acetonitrile (1:1:8, v/v/v) as a reference. Accurate masses were calculated and used for the determination of the elementary composition of the analytes, with fidelity of 6.0 ppm or better.

The on-line HPLC/EC/HR ESI-MS setup used in this study is displayed in Fig. 1.

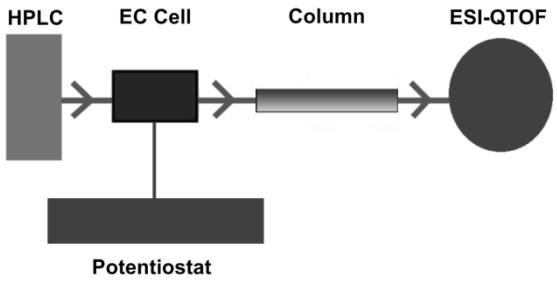

Fig.1 LC/EC/HR ESI-MS setup for the simulation of oxidation processes of the respective cytokinins.Voltage of the EC cell was set constantly to 1200mV with oxidation products separation and subsequent identification by HR mass spectroscopy

3. Results and discussion

3.1 LC/EC/ESI-QqTOF of CK standards

The process of new *in vitro* metabolites or products identification can be divided into two steps. First one is the generation of product. This step involves incubations of known or new chemical entitities in the presence of different cellular systems like liver microsomes, intact cells etc. The second one is their separation and identification. This is usually achieved by different analytical methods, mostly LC/MS and LC/MS/MS used for detection, separation and characterization of products[13]. Mimicking this

[13] Baranczewski P, Stanczak A, Kautiainen A, Sandin P, Edlund PO: **Introduction to early in vitro identification of metabolites of new chemical entities in drug discovery and development**. *Pharmacol Rep* 2006, **58**(3):341-352.

steps by using an electrochemical cell involves using the EC as a substitute for cellular systems, with the second step remaining unchanged. As described before, a LC/EC/ESI-QqTOF system was employed for the study of the electrochemically generated products, under defined conditions, from CK standards. Metabolism of CKs was very well described by a recent article[2]. CK metabolism and modification is generally performed by several structural changes, that crucially determine the function and compartmentalisation of the respective CK metabolite, involving different enzymes and chemical reactions like hydroxylation, glycosylation, hydrogenation etc[2], some of which can be readily mimicked by an EC cell[9]. EC reactions can be also useful by pointing to sites labile or prone to oxidation[14] .

3.1.1. N6-(2-Isopentenyl)adenine (iP)

The total ion chromatogram (TIC) of the electrochemically generated oxidation products of iP at 1200mV is shown in Fig. 1, together with corresponding accurate mass spectra, molecular formulas and presumed structures for them. Almost all of the mass spectra bear additional fragment ions, which are observable throughout all compounds investigated in this manuscript. As the analyses were made only in full-scan mode, origin of these ions could be assigned to fragmentation at the extractor part of the used QqTof or at its collision cell, since a specific collision energy is applied regardless of the scan mode performed[15]. A similar phenomena - thermally induced fragmentation in the APCI source, was observed for electrochemically modified lidocaine[16]. Full-scan fragmentation ions with various abundance, using soft ionizationg techniques like ESI, APCI or APPI, are known, especially for APCI source[17]. Employing accurate mass measurement is highly useful, as it enables to determine the elemental formula of observed target compound thus vastly limiting the number of possible solutions and giving valuable insight onto the oxidation transformations that the compound has undergone. Nevertheless this kind of data does not provide conclusive information about the site of modification[18]. Depending on the molecule structure, some cases can be resolved by further fragmentation reactions or by employment of another ionization technique – unfortunately, these experiments are not yet shown in this study, although the additional fragmentation ions (explained above) provide valuable information.

Five main peaks iA-iE were observed and investigated, with their principal data summarized in Table 2 and Fig. 2. No iP was observed after the electrochemical conversion, meaning that it has undergone a quantitative turnover in the used porous glassy carbon working electrode. Products iA and iE represent dehydrogenated iP molecules as accurate mass measurement reveals for both a loss of 2H atoms. -2H iP has been investigated and fragmentation data are provided by Popelková et al. 2006[19] - iA and iE structures were made solely on this base, as the presence of fragmentation ions leaves no other option than the dehydrogenation of the side-chain and therefore matching the structures with[19]. Dehydrogenation of aliphatic chain has been directly performed by EC[20] . iA and iE are tautomers, assigning the two possible structures to their two largely differing elution times was made by presuming that the exposed double bond in iA is responsible for increased polarity and thus shorter retention time. Product iC has m/z 220 and a distinct fragmentation pattern identical to zeatin type molecule[21]. Although there are few options where hydroxylation on the side-chain can occur (double bond, N-oxidation) the retention time of iC perfectly matches with retention time of tZ standard under the same conditions, therefore the structure should be identical.

[14] Baumann A, Karst U: **Online electrochemistry/mass spectrometry in drug metabolism studies: principles and applications**. *Expert Opin Drug Met* 2010, **6**(6):715-731.

[15] Micromass Q-Tof *micro* Mass Spectrometer Operator's Guide (71500058502 Revision A), 2002, Waters Inc.

[16] Nouri-Nigjeh, E., et al., *Lidocaine Oxidation by Electrogenerated Reactive Oxygen Species in the Light of Oxidative Drug Metabolism.* Analytical Chemistry, 2010. **82**(18): p. 7625-7633.

[17] Holčapek M, Volná K, Ansorgová D (2007) Dyes Pigm 75:156–165 .

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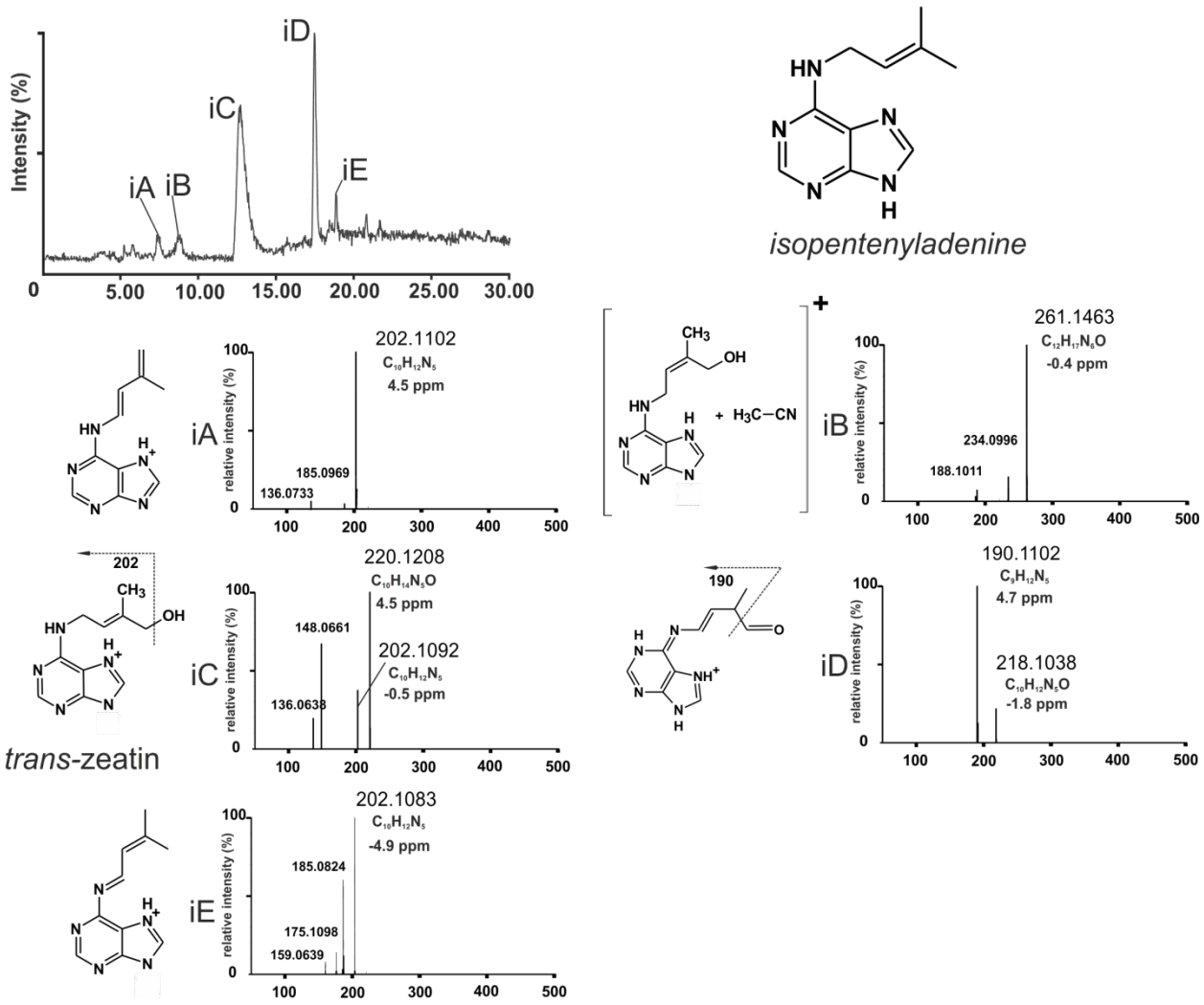

Fig. 2 Total ion TIC chromatogram and accurate mass spectra of **iA-iE** products determined by the LC/EC/QqTOF system

Despite the fact, that such aliphatic hydroxylation is rather a rare electrochemical reaction formerly presumed to be not possible[22], tetrazepam was found to resemble a similar reaction[23]. iB, based on its molecular formula, should represent an acetonitrile adduct [M+H+ACN]+ of product iC. The last observed product, iD should represent a dehydrogenated *t*Z. Although its retention time is lower than iP, which is in good correlation with the presence of oxygen atom, it doesn't match with any retention times of the *m/z* 218 compounds stemming from *t*Z (Table 2). This is also underlined by the presence of *m/z* 190 fragment ion, also unseen in *t*Z *m/z* 218 compounds, and also not present in dehydrogenated *t*Z mass spectra presented by[19]. The structure of iD is therefore highly putative and further investigation is needed.

3.1.2 6-(4-Hydroxy-3-methylbut-2-enylamino)purine (*trans*-Zeatin, tZ)

By applying a steady potential of 1200 mV, *t*Z gave rise to seven different products tA-tF (Fig. 3) as indicated on the total ion chromatogram by their differing retention time (t_R). For product

[22] Jurva U, Wikstrom HV, Weidolf L, Bruins AP: **Comparison between electrochemistry/mass spectrometry and cytochrome P450 catalyzed oxidation reactions**. *Rapid Commun Mass Sp* 2003, **17**(8):800-810.
[23] Baumann A, Lohmann W, Schubert B, Oberacher H, Karst U: **Metabolic studies of tetrazepam based on electrochemical simulation in comparison to in vivo and in vitro methods**. *J Chromatogr A* 2009, **1216**(15):3192-3198.

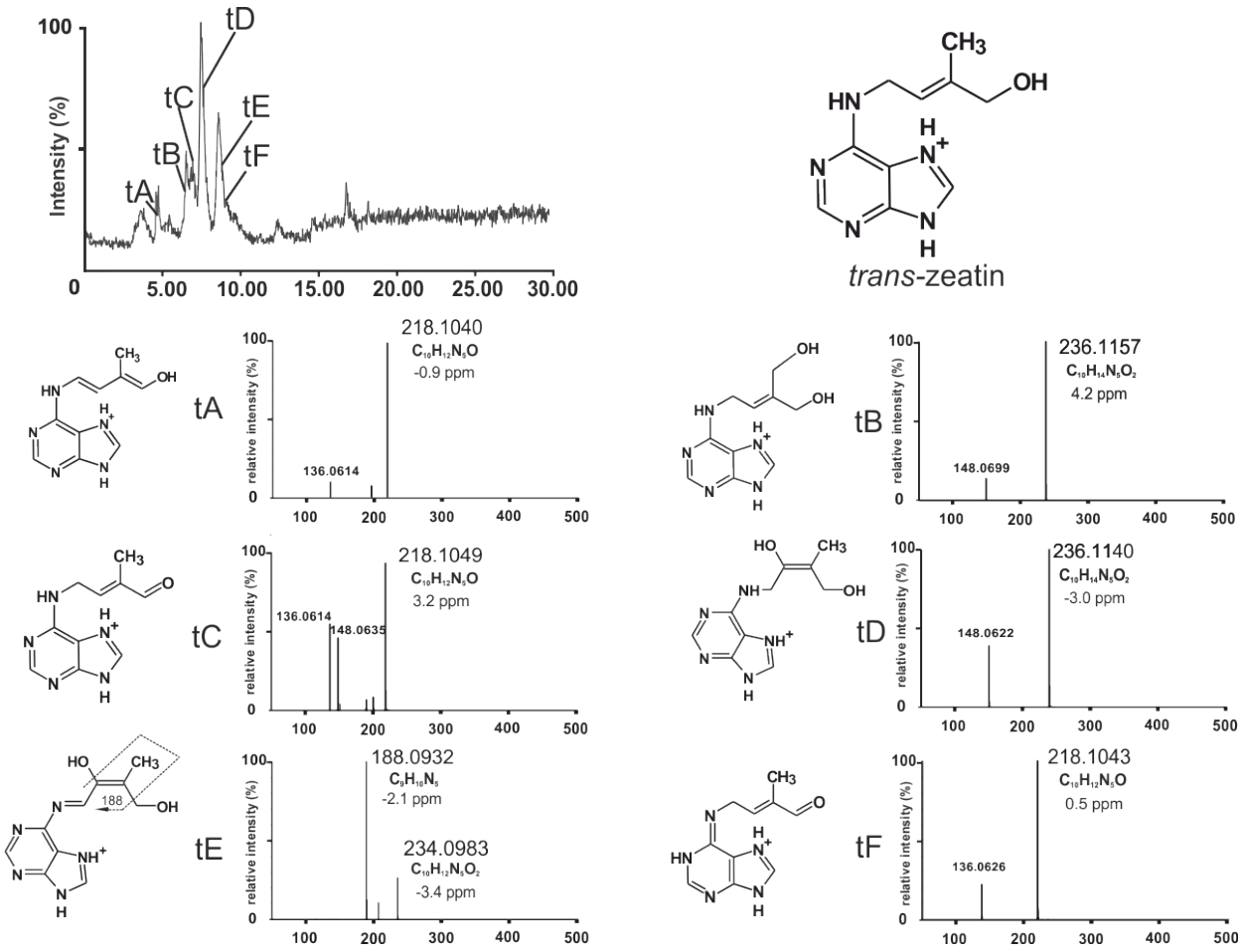

Fig. 3 Total ion TIC chromatogram and accurate mass spectra of **tA-tF** products determined by the LC/EC/QqTOF system

A, the t_R is 5.10 min, product B has a t_R 6.86 min, product C t_R is 7.20, D t_R 7.81, E t_R 8.93 min and F t_R is 9.38 min. Under the same conditions, *t*Z t_R is 12.82, which means that, considering the usage of RP column, every obtained product is more polar than *trans*-zeatin. Fig. 3 provides the resulting high resolution mass spectra for every product and the molecular formula calculated from them. Differences between the calculated and theoretical monoisotopic molecular masses were for all observed compounds below 5.0 ppm. The anticipated structures based on these results are also to be found in Fig. 3 and are discussed further. Product A is considered to be a product of a dehydrogenation at the isoprenyl side-chain, as there is a 136 *m/z* ion present in the spectrum, a characteristically occurring CK fragment representing an adenine[21]. Three such products with identical chemical formula were observed for *t*Z in total (A, C, F). By comparing their possible structures and the predicted polarity based on them, compound A with the hydroxyl group remaining intact is the most polar from them, thus having the shortest retention time. Similar aliphatic dehydrogenation, under EC conditions, involving amine dehydrogenation has been already observed by Nozaki for zotepine[24]. Compound B is supposed to bear an additional hydroxyl group. There are total three dihydroxylated tZ products observed in this study. Their mass spectra were also compared to previously examined dihydroxy zeatin spectra[25] to determine the site of hydroxylation. A 148 *m/z* fragment ion observed in the mass spectra of compound B is a known fragmentation ion resulting from *t*Z and CKs in

[24] Nozaki K, Osaka I, Kawasaki H, Arakawa R: **Application of On-line Electrochemistry/Electrospray/Tandem Mass Spectrometry to a Quantification Method for the Antipsychotic Drug Zotepine in Human Serum**. *Anal Sci* 2009, **25**(10):1197-1201.

[25] Chen CM, Smith OC, Mcchesney JD: **Biosynthesis and Cytokinin Activity of 8-Hydroxy and 2,8-Dihydroxy Derivatives of Zeatin and N6-(Delta2-Isopentenyl)Adenine**. *Biochemistry-Us* 1975, **14**(14):3088-3093.

common[21]. It represents an intact adenine with a methyl group, therefore its occurrence leaves the aliphatic side-chain as the only site for hydroxylation. Although aliphatic hydroxylation is not a common reaction observed under EC conditions, tetrazepam was found to resemble a similar reaction[23]. Product C having an identical molecular formula with products A and F, together with the 136 and 148 *m/z* fragment ions present, should represent a $-H_2$ loss, possibly by alcohol oxidation (at the hydroxyl group of *t*Z), which is a known EC reaction[22]. Product D, being alike product B, has several options of adding the second hydroxyl group, but the presence of 148 *m/z* ion leaves only the option of aliphatic hydroxylation, presumably at the chosen site. Product E is a dehydrogenated product originating from B or D. The presence of the *m/z* 188 fragment ion points to dehydrogenation at the same site as for product A. Similar dehydrogenation has been observed under different EC conditions for amphetamine[22]. Product F, the last from –2H loss products has the lowest polarity and is supposed to undergo an alcohol oxidation similar to product C and to bear the ketimine form of the molecule, which lends it the lowest polarity.

3.1.3 6-(4-Hydroxy-3-methyl-cis-2-butenylamino)purine (*cis*-Zeatin, cZ)

After the chromatographic analysis of cZ with a 1200mV potential applied at the electrochemical cell, eight putative oxidation products were detected (cA-cH). The resulting chromatogram and mass spectra are shown in Fig.3 and additional data in Table 2. For four products, however, <5ppm couldn't be achieved. cA and cC have approximately identical fragmentation and retention times, plus matching *m/z* and molecular formulas with tB and tD, therefore they should represent the same compounds. Products cB, cG and cH have the same molecular mass *m/z* 216 and molecular formula. The mass *m/z* 216 was also previously found for permethylated zeatin[26] or trimethylzeatin[27], but the molecular formula of compound cB, cG and cH contains an oxygen, hence this options are dismissed. Notably, they have the same *m/z* as another cytokinin - kinetin, which fragmentation mass spectra is available[19] and which has a t_R 19.32 min, close to cG and cH. Its fragmentation ion with *m/z* 188 from[19] is also present in cG spectra (Fig 3.). Although in this manuscript the option of such cyclization reaction at the side-chain has been dropped, since it is not a preferred electrochemical reaction[9], this possibility is worth further consideration. Putative structures with double dehydrogenation of side-chain are therefore provided. All three presented compounds with *m/z* 216 have a double bonded oxygen atom, but it's necessary to note, that they should be viewed as highly elusive, since they cannot explain the relatively vast differences in retention time. Products cD and cE have the same *m/z* as products tA, tC and tF. Since their retention times are relatively close to tC and tF, their differences in t_R can be simply explained by assigning a cis conformation to tC and tF. Product cF is an acetonitrile adduct, with -2H lower *m/z* as the similar product iB. Placing the double bond at the oxygen atom could tentatively explain their differing retention time.

3.1.4 Comparison with previous *in vitro/ in vivo* experiments

To verify that the electrochemically generated products are comparable with those obtained *in vivo* or *in vitro* established by other conditions, we compared our results with previously published metabolic studies. Complete overview of the obtained products with all the data is in Table 2. Metabolism of cytokinin type compounds has been closely examined through past decades with excellent recent reviews covering this field[28]. CKs generally are metabolised in living systems by modification of their purine moiety, or by N6-side chain modifications or cleavage[2]. Since no modification of purine moiety were observed in this manuscript, we focus on the side-chain modifications of examined CKs.

[26] Macdonald EMS, Powell GK, Regier DA, Glass NL, Roberto F, Kosuge T, Morris RO: **Secretion of Zeatin, Ribosylzeatin, and Ribosyl-1''-Methylzeatin by Pseudomonas-Savastanoi - Plasmid-Coded Cytokinin Biosynthesis**. *Plant Physiol* 1986, **82**(3):742-747.

[27] Morris RO: **Mass Spectroscopic Identification of Cytokinins - Glucosyl Zeatin and Glucosyl Ribosylzeatin from Vinca-Rosea Crown Gall**. *Plant Physiol* 1977, **59**(6):1029-1033.

[28] Kamada-Nobusada T, Sakakibara H: **Molecular basis for cytokinin biosynthesis**. *Phytochemistry* 2009, **70**(4):444-449.

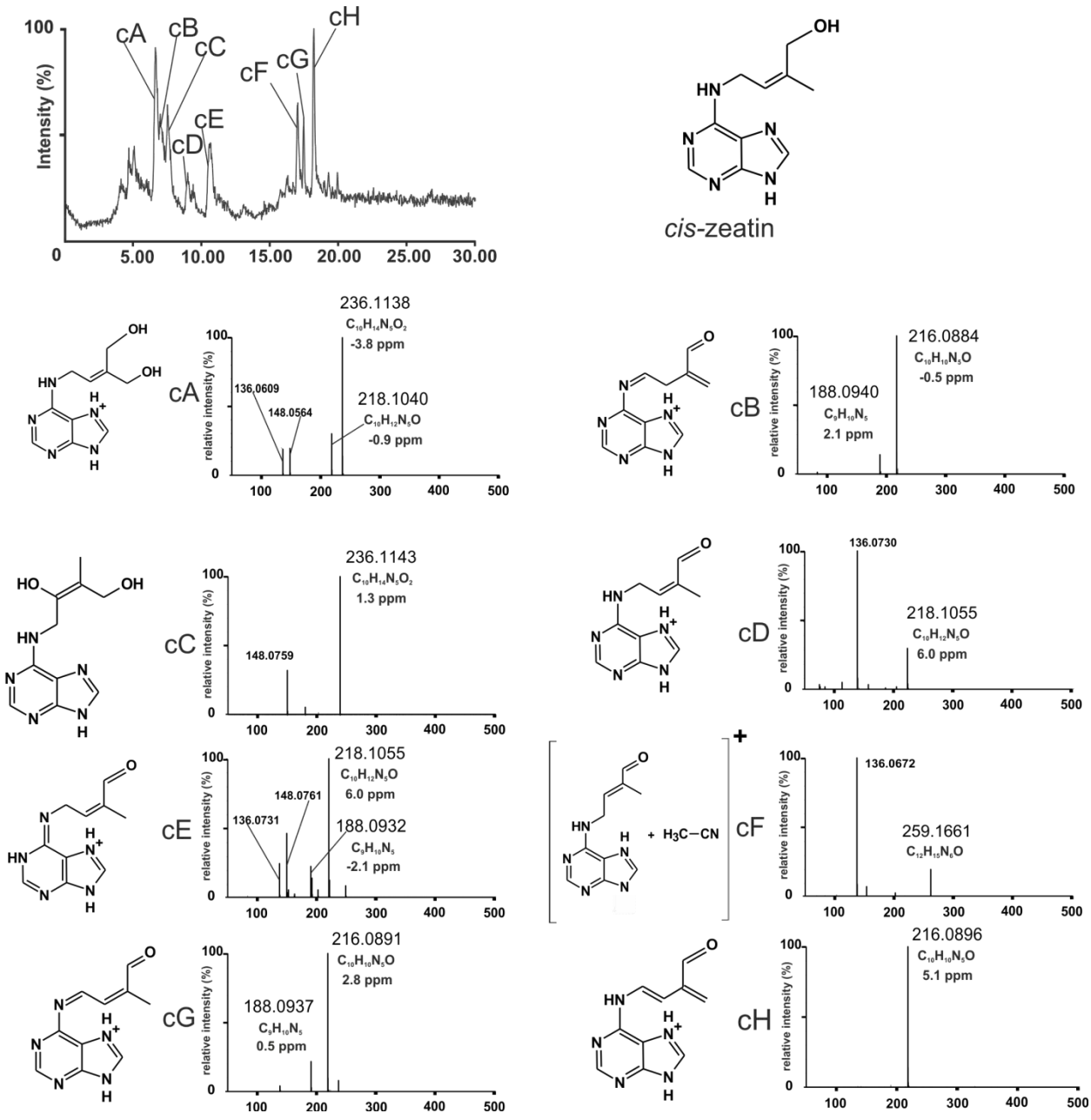

Fig. 4 Total ion TIC chromatogram and accurate mass spectra of **cA-cH** products determined by the LC/EC/QqTOF system

The absence of purine ring oxidation was quite expectable, since adenine has the highest peak oxidation potential value of purine bases[29]. No oxidation of purine-ring, meaning higher potential for oxidation of the side-chain is in conclusion with the theory of side-chain being responsible for the CK hormone activity[1], although other naturally occuring forms (ribosides etc) are not being considered since they have not been examined in this study but are known to posess physiological functions[2].

After incubation of iP with ZmCKX1 (cytokinin dehydrogenase/oxygenase from *Zea mays*), iP-2H intermediates were found[19], which is in perfect correlation with our observed reaction.

[29] G. Dryhurst, Electrochemistry of Biological Molecules, Academic Press, New York, 1977.

	Measured mass (*m/z*)	Theoretical mass $[M+H]^+$	Molecular Formula	Transformation	Deviation [ppm]	Retention time t_R (min)
iP	204.1241	204.1249	$C_{10}H_{14}N_5$	-	4.1	21.602
iA	202.1102	202.1093	$C_{10}H_{12}N_5$	-2H	4.5	6.768
iB	261.1463	261.1448	$C_{12}H_{17}N_6O$	+2C +5H +N+O-2H	-0.4	8.701
iC	220.1208	220.1198	$C_{10}H_{12}N_5O$	+O	4.5	12.717
iD	218.1038	218.1042	$C_{10}H_{12}N_5O$	+O-2H	-4.9	17.462
iE	202.1083	202.1093	$C_{10}H_{12}N_5$	-2H	-4.9	18.832

	Measured mass (*m/z*)	Theoretical mass $[M+H]^+$	Molecular Formula	Transformation	Deviation [ppm]	Retention time t_R (min)
***t*Z**	220.1190	220.1198	$C_{10}H_{14}N_5O$	-	-3.6	12.819
tA	218.1040	218.1042	$C_{10}H_{12}N_5O$	-2H	-0.9	5.081
tB	236.1157	236.1147	$C_{10}H_{14}N_5O_2$	+O	4.2	6.850
tC	218.1049	218.1042	$C_{10}H_{12}N_5O$	-2H	3.2	7.200
tD	236.1140	236.1147	$C_{10}H_{14}N_5O_2$	+O	-3.0	7.801
tE	234.0983	234.0991	$C_{10}H_{12}N_5O_2$	+O -2H	-3.4	8.926
tF	218.1043	218.1042	$C_{19}H_{26}N_6O_3$	-2H	0.5	9.381

	Measured mass (*m/z*)	Theoretical mass $[M+H]^+$	Molecular Formula	Transformation	Deviation [ppm]	Retention time t_R (min)
***c*Z**	220.1208	220.1198	$C_{10}H_{14}N_5O$	-	4.5	15.658
cA	236.1138	236.1147	$C_{10}H_{14}N_5O_2$	+O	-3.8	6.768
cB	216.0884	216.0885	$C_{10}H_{10}N_5O$	-4H	-0.5	7.012
cC	236.1143	236.1147	$C_{10}H_{14}N_5O_2$	+O	-1.7	7.527
cD	218.1055	218.1042	$C_{10}H_{12}N_5O$	-2H	6.0	9.041
cE	218.1057	218.1044	$C_{10}H_{12}N_5O$	-2H	6.0	10.707
cF	259.1661	259.1307	$C_{12}H_{15}N_6O$	+2C +3H +N-2H	-5.5	17.069
cG	216.0891	216.0885	$C_{10}H_{10}N_5O$	-4H	2.8	17.506
cH	216.0896	216.0885	$C_{10}H_{10}N_5O$	-4H	5.1	18.249

Table 2 – Summary of all measured values for iP, *t*Z and *c*Z oxidation products

We were even being able to observe both proposed tautomeric structures with same fragmentation ions as in the *in vitro* study. Furthermore, hydroxylation of iP to *t*Z, the main *t*Z biosynthesis pathway[2], was also observed, although *in vivo* conversion is limited to the hydroxylation of ribosyl-phosphate CK forms. The remaining oxidation product, iD, was not observed in any metabolic study. In the case of iP, the EC cell showed a very high ability to succesfully mimick main biological modifications of the presented molecule.

*t*Z is presumed to be the substrate for the side-chain hydrogenation of the double bond to form dihydrozeatin, a naturally occurring CK hormone[2], although this has not been confirmed directly, since only a partial purification of responsible enzyme was performed[4]. No molecule with DHZ mass-to-charge ratio (*m/z* 222) was observed in our study. Similarly, an enzyme mediating *t*Z to *c*Z conversion was also only partially purificated[5]. No other works so far have been published that exploit the physiological significance of zeatin isomerisation[2]. and no isomerization to cZ was observed after using the EC cell either. A -2H *t*Z product was observed in the before mentioned kinetic study[19]. Instead of two tautomeric products, three were observed after *t*Z oxidation in the EC cell, with similar fragmentation spectra, thus *in vitro* products were being successfully mimicked by the EC cell oxidation. Side-chain dihydroxylated products (tB and tD) were not observed in any article so far. Other *in vivo* occuring *t*Z side-chain reactions (O-oxidation with xylulose, modification to lupinic acid etc.) couldn't be observed in the EC cell for obvious reasons.

EC oxidation of *c*Z yielded the most products. Similarly to *t*Z, dihydroxylation product is not known to occur *in vivo*. The occurrence of cD and cE (*m/z* 218) should have the same origin as tC and tF products, although they were not observed in any study yet. Products cB, cG and cH represent the biggest challenge to identification. Former study, observing modifications of side-chain hydroxylated iP molecule under acidic conditions *in vitro*, confirmed the possibility of forming a cyclized intermediate, which underwent further reactions to finally form a stable cyclized compound, but without a confirmation of whether *cis* or *trans* zeatin was used as a precursor substance[30]. Cyclization of *c*Z was since then hypothesized[31] and its possible occurrence made responsible for some observed physiological reactions[32]. Besides the isomeration from *t*Z, another resource origin of *c*Z has been confirmed to tRNA (which also contains *t*Z albeit in ~40 times lower concentration)[33]. Since cB, cG and cH have the m/z and molecular formula exactly the same as a naturally occurring CK kinetin, which is also known to originate from nucleic acid by DNA oxidation[34], further investigation of these three *c*Z oxidation products is necessary, since two of them (cG and cH) have also a close retention time to kinetin (kinetin t_R under the same conditons is 19.31 min., data not shown). Furthermore, the possibility of such cyclization in EC cell has been confirmed for dopamine[35].

We present here a purely instrumental method for investigation of the iP, *t*Z and *c*Z oxidation pathway, based on EC cell located oxidation in combination with HPLC separation and identification provided by exact mass measurements of the resulting products. The EC mediated oxidation has successfully mimicked their dehydrogenation and hydroxylation *in vivo/in vitro* reactions and possible new, yet unobserved oxidation products.

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