



# History of *Meta*-Topolin and the Aromatic Cytokinins

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### Abstract

Following the discovery of kinetin (Miller et al., J Am Chem Soc 78 (7):1375–1380, 1956), there was a period of intensive synthesis of new cytokinins, which led to the preparation of the highly efficient and easily prepared aromatic cytokinin 6-benzylaminopurine (BAP). A huge number of biological experiments were then performed with this cytokinin derivative, which significantly contributed to our understanding of cytokinin functions in plants. Findings of naturally occurring BAP subsequently led to the search for other aromatic cytokinins. In order to rapidly detect these substances in plants, a unique procedure based on screening of HPLC fractions using specific ELISAs with antisera against individual aromatic cytokinins has been introduced. Isolation of these substances and their subsequent identification by mass spectrometry confirmed the natural occurrence of highly active endogenous ARCK *meta*-topolin (6-(3-hydroxybenzylamino)purine), but also a less active *ortho*-topolin analogue (6-(2-hydroxybenzylamino)purine) and their metabolites. The structure of topolins suggests biosynthetic and metabolic biosynthetic pathways that are likely to be similar to closely related isoprenoid cytokinins like zeatins. It also seems likely that the molecular mechanisms of action will be based on very closely related principles, although differences certainly exist, at least at the level of the signals and their recognitions carried by these phytohormones. This review

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attempts to summarise the current state of knowledge about ARCK and to point out its practical use in agriculture and biotechnology.

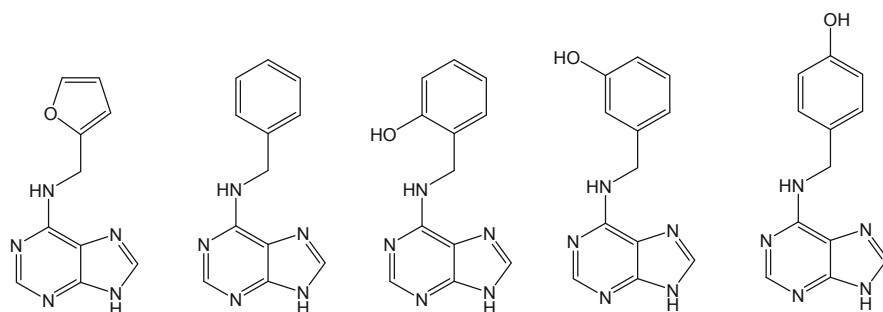
### Keywords

Aromatic cytokinins · History · Occurrence · *Meta*-topolin · Topolins

## Abbreviations

ARCK	Aromatic cytokinin
BAP	6-Benzylaminopurine
CBP	Cytokinin-binding protein
ELISA	Enzyme-linked immunosorbent assay
HPLC	High-performance liquid chromatography
iP	N <sup>6</sup> -Isopentenyladenine
ISCK	Isoprenoid cytokinin
K	Kinetin
<i>mT</i>	<i>Meta</i> -topolin
<i>mTR</i>	<i>Meta</i> -topolin riboside
<i>oT</i>	<i>Ortho</i> -topolin
<i>oTR</i>	<i>Ortho</i> -topolin riboside
<i>pT</i>	<i>Para</i> -topolin
<i>pTR</i>	<i>Para</i> -topolin riboside
<i>tZ</i>	<i>Trans</i> -zeatin

The history of cytokinins (ARCK) began in 1955, when Miller et al. isolated the first biologically active member of this group of natural phytohormones, 6-furfurylamino-purine (Fig. 1.1), from autoclaved herring sperm DNA. Due to its ability to stimulate cytokinesis in various plant tissues, this compound was named kinetin (K) (Miller et al. 1955a, b, 1956, Fig. 1.1). K thus became the first



**Fig. 1.1** Chemical structure of aromatic cytokinins. From left to right: kinetin (K, 6-furfurylamino-purine; BAP, 6-benzylaminopurine; *ortho*-topolin (*oT*, 6-(2-hydroxybenzylamino)purine); *meta*-topolin (*mT*, 6-(3-hydroxybenzylamino)purine); *para*-topolin, (*pT*, 6-(4-hydroxybenzylamino)purine)

representative of plant hormones, which were subsequently named cytokinins and which can be characterised as N<sup>6</sup>-substituted adenine derivatives (Skoog et al. 1965). Therefore, the first attempts to prepare cytokinins (CKs) started with kinetin, followed by another cytokinin substituted by an aromatic ring at the N<sup>6</sup>-position 6-benzylaminopurine (BAP, Miller et al. 1956). 6-(3-Hydroxybenzylamino)purine (*meta*-topolin) was synthesised a bit later. It was described for the first time by Okumura et al. (1959). The authors prepared BAP derivatives bearing hydroxyl, methyl, methoxy, amino, nitro and sulfonic acid groups on the benzyl ring. These functional groups were introduced into *ortho*-, *meta*- and *para*-positions of the N<sup>6</sup>-benzyl substituent (Okumura et al. 1956, 1959, Fig. 1.1).

Cytokinin research is concentrated mainly on members of the isoprenoid class (ISCKs) represented by *cis*- and *trans*-zeatin, N<sup>6</sup>-isopentenyladenine, dihydrozeatin and their metabolites. On the other hand, ARCKs are represented by BAP; *ortho*-, *meta*- and *para*-topolins (hydroxylated BAPs); and also kinetin. Aromatic cytokinins have long been considered purely synthetic cytokinins. For example, kinetin was considered an artificial product of DNA rearrangement (Hall and De Ropp 1955), although some scientists thought that it could be formed *in vivo* through DNA degradation (Skoog 1994). The first evidence of its natural occurrence came in 1996, when K was identified in extracts from nodes of the root of *Casuarina equisetifolia* infected with bacteria *Frankia* (Raman and Elumalai 1996). Barciszewski et al. (1996) discovered K in plant cell samples, in DNA isolated from *in vitro* cultured human fibroblasts and in commercial samples of calf thymus DNA (Barciszewski et al. 1996, 1997a, b). Subsequently, K was also found in the urine of cancer patients (10–100-fold less in healthy patients; Barciszewski et al. 2000), in coconut milk (Ge et al. 2005) and in the brain and liver tissue of transgenic mouse with familial dysautonomia (Shetty et al. 2011). In many cases, K has not however been detected in different tissues and organisms. Our efforts to prove the occurrence of K have so far been quite unsuccessful. For example, we have not demonstrated its occurrence in extracts from *Caenorhabditis elegans* and *Escherichia coli* (Kadlecová et al. 2018). Orr and colleagues were also unable to find K in native mouse and rat brain tissue (Orr et al. 2017). We have also failed to demonstrate the occurrence of K in many plant samples of different evolutionary origin (unpublished data), suggesting that the question of the natural occurrence of kinetin is probably more complicated than previously thought.

Cytokinins with a benzyl side chain have also been detected and identified in different plant tissues, but occur also much more rarely than their isoprenoid counterparts which are commonly present in all plant species. For example, Horgan et al. (1973, 1975) identified the first ARCK from mature poplar leaves and identified it as 6-(2-hydroxybenzylamino)-9-β-D-ribofuranosylpurine (*ortho*-topolin riboside, *o*TR). This ARCK metabolite was also discovered in *Zantedeschia aethiopica* fruits together with its 2-methylthio-9-glucopyranosyl derivative (Chaves das Neves and Pais 1980a, b). The glucosyl moiety of the last compound was assigned a furanosyl ring, but a 9-glucopyranoside structure is more probable by analogy with other cytokinin 9-glucosides (see MacLeod et al. 1976). The discoveries were followed by isolation and tentative identification of BAP and its

metabolites in different plant tissues (Ernst et al. 1983; Nandi et al. 1989a, b). From the published data, we concluded that hydroxybenzylaminopurines could occur naturally as analogues of the commonly used cytokinin BAP (Strnad et al. 1992a, b). We were also disappointed with the results from studies on cytokinin-binding proteins (CBP) that ARCKs must be more widespread phytohormones. The first ARCK-binding protein CBF-1, studied in detail by Fox and his co-workers (Fox and Erion 1975, 1977; Erion and Fox 1981; Brinegar and Fox 1985a, b, 1987; Brinegar et al. 1985), is probably the best characterised of all plant hormone-binding proteins. It has amino acid similarity to vicilin-type seed storage proteins (Brinegar et al. 1988). It is unclear why this CBP has relatively high affinity ( $K_d = 10^{-7}$  M) for BAP, but much lower affinity for natural ISCKs, nonactive cytokinins and adenines (Keim et al. 1981). In one case, the biological activity of various ARCKs in callus bioassay even agreed closely with their relative order of affinity for the binding protein (Sussman and Kende 1978). These data further supported our idea that cytokinins bearing a benzyl ring at the N<sup>6</sup>-position must be more widespread in plant tissues than appeared.

For accurate and fast detection of the aromatic cytokinins (ARCKs) in plant tissues, we developed a very new approach based on combined high-performance liquid chromatography-enzyme-linked immunosorbent assay (HPLC-ELISA) which showed high selectivity for BAP; *ortho*-, *meta*- and *para*-hydroxybenzylaminopurine; and their 9-substituted derivatives (Strnad et al. 1992a; Strnad 1996). A typical protocol most commonly used for the isolation of new substances of natural origin is generally based on bioassay-guided fractionation, which consists of the gradual fractionation of bioactive substances based on differences in their physicochemical properties and evaluation of certain types of biological activity; the separation and testing steps are repeated several times (Weller 2012). The new cytokinin analytical strategy applied here for screening different plant tissues was much faster, precise and more robust being based on selective antibody recognition properties. The extracts were fractionated by HPLC similar to classical natural product discovery techniques, but the fractions were analysed by group-specific ELISA assays (ELISA selective for BAP; *ortho*-, *meta*- and *para*-topolin; and their appropriate 9-riboside and 9-glucosides). Immunoactivity ELISA analysis of HPLC fractions detected major immunoactive peaks co-chromatographing with the ARCK standards. Using this approach, we have been able to discover a brand new family of endogenous aromatic cytokinins centred around the highly active compound, 6-(3-hydroxybenzylamino)purine (*meta*-topolin, *mT*; Strnad et al. 1997). Because this compound was first detected in poplar leaves, we adopted the trivial name '*meta*-topolin' (*mT*), derived from 'topol', the Czech word for a poplar (Strnad et al. 1997). This new family of cytokinin-related growth regulators include, apart from *mT*, the highly active BAP and the much less active 6-(2-hydroxybenzylamino)purine (*ortho*-topolin, *oT*), 6-(4-hydroxybenzylamino)purine (*para*-topolin, *pT*) and their metabolites. Other hydroxyl and methoxy derivatives as well as their sugar conjugates have also been identified in other plant species than poplar leaves (Strnad et al. 1992b, 1994;

Goicoechea et al. 1995, Jones et al. 1996; Doležal et al. 2002; Tarkowská et al. 2003).

A recent study reports that topolins are also produced by *Mycobacterium tuberculosis*, a human-exclusive pathogen. They are formed by the activity of Rv1205, a homologue of the plant enzyme LONELY GUY, from respective riboside 5'-monophosphates (Samanovic et al. 2015). Furthermore, the intracellular accumulation of *para*-hydroxybenzaldehyde (*p*HBA) in the LOG-deficient mycobacterial strain, which is a breakdown product of *para*-topolin, is likely to be responsible for the sensitisation of proteasomal degradation mutants to NO. The addition of *p*HBA to mycobacteria was lethal selectively in the NO presence but had no effect on similarly treated cultures of *E. coli*, suggesting that this is probably the mechanism by which LOG and cytokinins in the absence of the proteasome lead to NO sensitivity (Zhu and Javid 2015). I would like to mention here that Log-like homologues were identified in several other bacterial species, including the important human and animal pathogens *Bordetella* spp. and *Staphylococcus aureus*. It can be assumed that elucidation of the role of cytokinins in the bacterial pathophysiology of humans, animals and plants will undoubtedly provide more insights into their future important functions.

Targeted perturbation of this pathway by, for example, cytokinin analogues, may provide a novel therapeutic approach to tuberculosis treatment. As LONELY GUY homologues are present in the genomes of other bacterial pathogens, including *Staphylococcus aureus*, such an approach could have a significant impact on the treatment of bacterial infections. *Para*-topolin riboside (*p*TR) has been studied mainly in connection with neuroprotection. It was identified as one of the cytoprotective substances in plant *Gastrodia elata* that is used in Chinese traditional medicine for the treatment of headaches, dizziness and epilepsy (Huang et al. 2007). Overall, the most recent data show that *p*TR has promising activity in the treatment of neurological diseases (Huang et al. 2011; Visentin et al. 2013; Chou et al. 2015; Hong et al. 2019).

It is also interesting to note that a recent 12-week clinical study on 39 subjects (Garcia et al. 2018) demonstrated beneficial effects of topical *para*-topolin application (*p*T, designated 4HBAP in this study). At the concentration of 0.1%, *p*T improved the appearance of fine and coarse wrinkles and reduced skin roughness and hyperpigmentation. It also increased skin hydration and had a positive effect on both facial erythema and non-inflammatory acne lesions.

Collectively, *m*T, *o*T, *p*T and BAP and their metabolites constitute natural aromatic cytokinins, distinguishable from the isoprenoid cytokinins both chemically and because of their spectrum of biological activities. The aromatic cytokinins found in *P. × canadensis* were recently remeasured by sophisticated UHPLC-MS/MS analyses of leaf extracts from the same tree, from 12 gene bank accessions as well as during the season (Jaworek et al. 2019, 2020). While *o*T derivatives were found, those of *m*T were not. Currently, we cannot exclude the possibility that topolins are not products of poplar metabolism but endophyte products (Wang et al. 2019). Observed fluctuations could account for the changes in endophyte growth. This hypothesis is at least partially supported by identification of endogenous ARCKs in

different algae strains (Stirk et al. 2003; Ordog et al. 2004) and also by identification of *o*TR in poplar tRNA (Jaworek et al. 2019). In algae, all three isomers of topolins are present (*ortho*-, *meta*- and *para*-), with *o*T occurring at higher concentrations than the other isomers. For O-glucosides, *meta*-isomers (*meta*-topolin-O-glucoside and *meta*-topolin riboside-O-glucoside) were present in higher concentrations than other isomers. It is important to note that no N-glucosides were detected in micro- and macroalgae (Stirk et al. 2003; Ordog et al. 2004). The main difference between the two groups of algae (micro- and macroalgae) was that the BAP-type cytokinins were found in higher percentages in microalgae (1%–28%) than in macroalgae (below 1%) (Stirk et al. 2003). The origin of topolins in plants remains unclear and needs to be further investigated in detail.

To conclude, the ARCKs as a group of plant growth regulators are already of immense economic importance. The micropropagation industry is based on the ability of cytokinin to relax apical dominance so that axillary buds grow outwards, proliferating the numbers of shoots for cultivation. The future of plant biotechnology rests with genetically engineered plants, for which cytokinin-induced shoot formation is an obligatory step. For both of these processes, unwanted side effects of BAP treatment and the more or less permanent suppression of root formation and growth, for instance, are serious problems (Aremu et al. 2012). Evidence for the unique biological activity of *meta*-topolin in many plant *in vitro* systems (Werbrouck et al. 1996) has been followed up by a synthetic and biochemical study of new, more efficient ARCK derivatives as clearly seen from the many reviews in this book.

The important role of ARCKs in plant cell growth and differentiation has also attracted the attention of researchers working with animal and human cells. Here, cytokinins and their derivatives were demonstrated to either promote or inhibit the division of mammalian cells depending on the cytokinin and experimental system used (Strnad 1997; Voller et al. 2017). Some also induce differentiation of various cell lines, including keratinocytes and certain leukemias (see Voller et al. 2017). Many other pharmacological activities are reported, including neuroprotective, immunomodulatory and anti-angiogenic (e.g. see Voller et al. 2019).

The fact that plants and also animals have two related groups of cytokinins with overlapping spectra of biological activity gives us a fascinating insight into the potential complexity of developmental control. What is clear at this stage is that ARCKs and ISCKs are not merely alternative forms of the same signal. Clear differences are emerging. First, although the biosynthetic pathway of ARCKs is not firmly established, the evidence points to an entirely separate pathway for ARCKs, implying separate control of biosynthesis. It is also possible that the endogenous levels of compounds in the two groups are functionally linked in some way, either directly or inversely. Second, there would seem to be a difference in the nature of the receptors for ARCKs and ISCKs. It is an extraordinary fact that ARCK-specific binding proteins that do not bind ISCKs have been rejected for years as a puzzling artefact. Three cytokinin receptors, AHK2, AHK3 and CRE1/AHK4, have been described in *Arabidopsis* (Inoue et al. 2001; Suzuki et al. 2001; Yamada et al. 2001). Spíchal et al. (2004) showed that the most active ISCK *trans*-zeatin (*tZ*) is a strong activator of the cytokinin receptors, while *mT* activates preferably the

receptor AHK3. The relative activity of *mT* at AHK3 and CRE1/AHK4 receptors was found to be 80% and 30% of *tZ* activity, respectively. *mTR* did not activate these receptors, but it was able to activate the expression of *ARR5* (*Arabidopsis* cytokinin response regulator 5) in *Arabidopsis* plants (Spíchal et al. 2004). It has also been shown that cytokinin receptors from different plant species bind *mT* and with higher affinity than BAP and *oT* (Spíchal et al. 2004; Kuderová et al. 2014; Jaworek et al. 2020). To resolve the relationships between ARCKs and ISCKs at the receptor level will necessitate further controlled experimental manipulation of the levels of hormonal signals and receptor activity.

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