



# Cytokinin Properties of *Meta*-Topolin and Related Compounds

# 3

Jaroslav Nisler

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## Abstract

6-Benzylaminopurine, *meta*-topolin and their derivatives have been and are being used in and tested for the micropropagation of plants using tissue culture techniques. *Meta*-topolin and *meta*-methoxy-topolin were synthesized and tested for cytokinin activity in the late 1960s, but interest in these substances has accelerated since the 1990s. The family of topolin and methoxy-topolin cytokinins was first discovered in plants. Synthetic derivatives were then prepared, and this enabled the cytokinin activity of a wide variety of aromatic cytokinins to be evaluated in various bioassays. The latter revealed that aromatic cytokinins and most notably their *N9*-ribosides were very effective in retarding chlorophyll degradation, i.e. possessing strong senescence delaying effect. This led to the development of topolin derivatives with other *N9*-substituents. Such substances, in addition to their anti-senescence activity, show a milder negative effect on root growth and development than classical cytokinins. A combination of these two properties was crucial for choosing topolins for use and testing in plant tissue cultures. The uniqueness of the aromatic cytokinins is being gradually revealed using the molecular techniques of today. This chapter chronologically overviews what has been discovered so far about the cytokinin activity of topolins and compounds related to them.

J. Nisler (✉)

Laboratory of Growth Regulators, Institute of Experimental Botany, AS CR & Palacký University, Olomouc, Czech Republic

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*Meta*-topolin · Topolin · Cytokinin · Anti-senescence · Plant tissue culture

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### 3.1 The History of *Meta*-Topolin Activity Discovery

The history of the discovery of *meta*-topolin is closely related to that of kinetin and its cell division stimulatory activity (Miller et al. 1955). When this was first described, Japanese researchers synthesized a wide range of kinetin analogues which included among others the *ortho*-, *meta*- and *para*-substituted 6-benzylaminopurine (BAP) derivatives (Kuraishi 1959; Okumura et al. 1959). Their series contained *meta*-hydroxy-BAP which was later called *meta*-topolin (*mT*, Strnad et al. 1997). The compounds were tested as possible cytokinetic agents in the radish (*Raphanus sativus* L.) leaf disc expansion assay (Kuraishi and Okumura 1956). *Ortho*-methyl-BAP and *ortho*-chloro-BAP were slightly more active than unsubstituted BAP which was a little more active than *ortho*- and *meta*-hydroxy-BAP (this refers to *ortho*-topolin (*oT*) and *mT*, respectively, Okumura et al. 1959). The effect of different substituents introduced into the benzyl ring of BAP on the promotion of the growth of tobacco callus was analysed by Iwamura et al. (1980), but no significant effect of the substitution was found. These results appeared to be consistent with the results reported by Keim et al. (1981) who showed that BAP, *oT* and *mT* all bind to CBF-1 protein with similar high affinity, although the affinity of *oT* was a little lower. CBF-1 is a soluble protein isolated from wheat germ which binds cytokinins with relatively high affinity and specificity (Fox and Erion 1975).

It was not until 28 years later from the first *mT* synthesis that Kaminek et al. (1987) reported significant biological activity of *meta*-hydroxy-benzyladenosine (*mTR*) over the standard *N*6-benzyladenosine (BAPR) in the tobacco callus and wheat leaf senescence assay (WLSA). In the discussion section of their paper, the authors noted that *mT* was slightly more active than *mTR* in the *Amaranthus* assay, while the two compounds exhibited about the same activity in WLSA. *Ortho*- and *para*-hydroxy-BAPRs showed significantly lower activity than BAPR in all bioassays (Kaminek et al. 1987). The latter was the first report showing that *meta*-hydroxylation of BAPR leads to a significant increase in cytokinin activity, while *ortho*- and *para*-hydroxylation of BAPR have the opposite effect. These results contrasted sharply with the results of the radish leaf disc expansion assay (Kuraishi and Okumura 1956). Interestingly, the cytokinin activity of *mTR* was as high as that of *trans*-zeatin in all tested bioassays, including the *Amaranthus* assay and pea bud formation assay (Kaminek et al. 1987) apart from the two aforementioned. In this report, *mTR* was first introduced and tested for cytokinin activity, which was the beginning of *mTR* exploration. In the same year, Kaminek and Vaněk (1987) patented *mT* and *mTR* for use in agriculture and horticulture as shooting- and branching-stimulating plant growth regulators. As is so far apparent from the overview, since the discovery of *mT* and its cytokinin properties, very little attention had been paid to this molecule for almost three decades. This was about to change in the 1990s.

*Meta*-topolin riboside (*mTR*) has been tested by Czech scientists as a crop yield-increasing substance in barley (Hradecká and Petr 1992a) and wheat (Trčková et al. 1992; Hradecká and Petr 1992b; Borkovec and Prochazka 1995). Relative plant growth rate, 1000-grain weight, number of grains per plant and other yield-forming parameters were improved after application of *mTR*. In 1991, Holub presented his teams' findings at the 14th IPGSA conference in Amsterdam that *mT* is slightly more active than *mTR* in the tobacco callus and WLSA, making it the most active compound of the hydroxylated aromatic cytokinins studied so far in these assays (Holub et al. 1991). Such observation led to the idea whether *mT* could be an alternative to BAP in tissue culture (Werbrouck et al. 1996). Strnad and his co-workers further developed an analytical HPLC-ELISA screening system specific for BAP, *oT* and *mT* and their N9-substituted derivatives (Strnad et al. 1992a; Strnad 1996). This enabled the screening of plant tissue extracts for these types of cytokinins. Shortly afterwards, a brand new family of endogenous aromatic cytokinins, including *mT*, was discovered in plants, characterized and labelled as topolins (Strnad et al. 1992b, 1994, 1997; Jones et al. 1996).

The structure-activity relationships of natural cytokinins, namely, *trans*-zeatin, BAP, *oT*, *mT* and their N9-ribosides and N9-glucosides, were then examined and compared in three cytokinin bioassays (Holub et al. 1998). The bioassays were based on the stimulation of tobacco callus growth (tobacco callus assay), retention of chlorophyll in excised wheat leaves (wheat leaf senescence assay, WLSA) and dark induction of betacyanin synthesis in *Amaranthus* cotyledons (*Amaranthus* assay). The results showed what could have been read between the lines, but was never shown in one publication. Hydroxylation of the benzyl ring in the *meta*-position significantly increased the activity of BAP and BAPR in WLSA. In this assay, the activity of *mT* as well as *mTR* was doubled compared to BAP and BAPR, respectively. In the tobacco callus assay, the activity was increased only in the case of the *mT* free base but not in the case of *mTR*. Interestingly, in the *Amaranthus* assay, *meta*-hydroxylation led to a decrease in BAP and BAPR activity. *Ortho*-hydroxylation reduced the activity of all compounds in all bioassays, and the N9-glucosylation inactivated all the compounds completely (Holub et al. 1998). The authors further reported that the N9-ribosylation had no significant effect on the cytokinin activity of the compounds; however, N9-ribosylation slightly diminished their activity in tobacco callus and *Amaranthus* assays but markedly improved their activity in WLSA. Even from the graphs in Holub's work, it is clear that N9-ribosides retained more of the chlorophyll in the detached wheat leaves than cytokinin free bases. This effect was later observed by other authors and further investigated. It can be definitively affirmed that the WLSA played an irreplaceable role in the future development of *meta*-topolin derivatives, their analogues and all aromatic cytokinins in general.

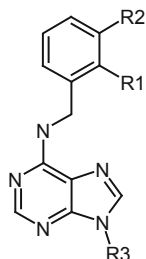
### 3.2 *Meta*-Topolin and Related Compounds in a New Millennium

For the family of topolin compounds, a new millennium began with the discovery of their very close relatives—methoxy-topolins, 6-(2-methoxybenzylamino)purine (*ortho*-methoxy-topolin, MeoT) and 6-(3-methoxybenzylamino)purine (*meta*-methoxy-topolin, MemT). Their N9-ribosides (MeoTR and MemTR, respectively) were found again in *Populus x canadensis* leaves and in *Arabidopsis thaliana* plants (Tarkowská et al. 2003). The latter evaluated the cytokinin activity of the four new hormones in the three abovementioned cytokinin bioassays. It is not very surprising that MemT and MemTR were highly active in the WLSA. Their *ortho*-analogues were highly active as well. All derivatives exhibited plus/minus double activity relative to BAP (Table 3.1). In the other two bioassays, the compounds had an equal or a slightly lower activity than BAP (Tarkowská et al. 2003). These results demonstrated that the cytokinin activity of the benzyl ring-substituted BAPs and BAPRs is not limited to their *meta*-derivatives. The need for a comprehensive review of the cytokinin activity of individual BAP and BAPR derivatives was accomplished by Doležal et al. (2006, 2007). Their systematic approach made it possible to identify key substituents for achieving high cytokinin and particularly high anti-senescence activity of BAP and BAPR derivatives. Of the substituted BAPs, two fluorinated compounds, 3F-BAP and 2F-BAP, showed the highest cytokinin activities (Doležal et al. 2006 and Table 3.1). Apropos BAPR derivatives, many of them showed high activity in WLSA (Doležal et al. 2007). From the available results (Holub et al. 1998; Doležal et al. 2007; Hönig et al. 2018), it is clear that the presence of ribose at the N9-position alone significantly increased the compound's anti-senescence activity. This is very well demonstrated in Table 3.1. Based on the disproportion in activity of aromatic cytokinins in WLSA and in the other cytokinin bioassays, Doležal et al. suggested that the effect of aromatic cytokinins including topolins is predominantly associated with protection of the photosynthetic apparatus. This is consistent with the work of other authors. It was shown that in *Rosa hybrida* cuttings, MemTR exhibited the best anti-senescent effects of all tested cytokinins (Bogaert et al. 2006). On the other hand, the compounds 3F-BAP and 3F-BAPR showed the best shoot multiplication rate. MemTR was also superior to BAP in the multiplication of *Petunia hybrida* (Bogaert et al. 2006). Further, *mT* was evaluated as the most effective cytokinin in stimulating chlorophyll and protein biosynthesis at low concentrations in cucumber cotyledons (*Cucumis sativus* L., Çağ et al. 2007). *Meta*-topolin was also very efficient in retarding the leaf senescence of *Pelargonium* cuttings with only marginal negative effect on the root development. This was in contrast with the use of thidiazuron which severely affected root growth (Mutui et al. 2012).

These observations correspond very well with the ability of each compound to activate cytokinin receptors in plants. 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). It has been reported that the receptor CRE1/AHK4 is responsible for the root inhibitory activity of cytokinins in *Arabidopsis* (Riefler et al.

**Table 3.1** Comparison of the cytokinin activities of BAP and BAPR derivatives in wheat leaf senescence assay and tobacco callus assay. The values for BAPR, *o*T, *m*T, *o*TR and *m*TR were calculated from estimated values extracted from the graphs published by Holub et al. (1998). Further, it is important to note that the activities of all compounds were evaluated by the authors only according to the activity of the substance in the highest concentration tested, and it was compared to BAP activity. The highest activity of BAP could differ slightly among these assays, but this value in % would strongly affect the activity of other substances (in % of BAP activity)

**R** =  $\beta$ -D-ribose



Compound abbreviation	R1	R2	R3	Senescence assay (% of BAP activity)	Tobacco callus a. (% of BAP activity)	Reference
BAP				100	100	
<i>o</i> T	OH	H	H	20	55	Holub et al. (1998)
<i>m</i> T	H	OH	H	210	130	Holub et al. (1998)
MeoT	OMe	H	H	203 $\pm$ 46	79 $\pm$ 5	Tarkowska et al. (2003)
MemT	H	OMe	H	178 $\pm$ 16	76 $\pm$ 20	Tarkowska et al. (2003)
2F-BAP	F	H	H	169	111 $\pm$ 21	Doleal et al. (2006)
3F-BAP	H	F	H	200	135 $\pm$ 8	Doleal et al. (2006)
BAPR			<b>R</b>	205	108	Holub et al. (1998)
<i>o</i> TR	OH	H	<b>R</b>	120	40	Holub et al. (1998)
<i>m</i> TR	H	OH	<b>R</b>	320	110	Holub et al. (1998)
MeoTR	OMe	H	<b>R</b>	198 $\pm$ 12	108 $\pm$ 1	Tarkowska et al. (2003)
MemTR	H	OMe	<b>R</b>	209 $\pm$ 8	92 $\pm$ 1	Tarkowska et al. (2003)
2F-BAPR	F	H	<b>R</b>	118 $\pm$ 39	100 $\pm$ 9	Doleal et al. (2007)
3F-BAPR	H	F	<b>R</b>	220 $\pm$ 16	91 $\pm$ 6	Doleal et al. (2007)

2006), while the AHK3 receptor is the key element in cytokinin-mediated leaf longevity (Riefler et al. 2006; Kim et al. 2006). Spíchal et al. (2004) showed that TDZ is a strong activator of both mentioned cytokinin receptors, while *mT* activates preferably the receptor AHK3. The relative activity of *mT* at AHK3 and CRE1/AHK4 receptors was 80% and 30% of *tZ* activity, respectively. *Meta*-topolin riboside 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). Recently, it has also been shown that cytokinin receptors from *Brassica napus* bind *mT* and with higher affinity than BAP (Kuderová et al. 2014).

The continuous search for compounds with cytokinin activity but with limited root growth inhibitory activity led to the development of topolin derivatives with N9-substituents, such as tetrahydropyran-2-yl, 9-tetrahydrofuran-2-yl and 4-chlorobutyl (Szüčová et al. 2009; Plíhal et al. 2013; Podlešáková et al. 2012). Such compounds are reported to maintain high anti-senescence activity (Szüčová et al. 2009) while exhibiting negligible negative effects on root growth and development. It is assumed that the reasons for this are (1) the compounds do not activate the CRE1/AHK4 cytokinin receptor; and (2) they are preferably transported from roots to shoots, (3) where the free base is slowly released and takes action (Plíhal et al. 2013). It has been also speculated that such compounds may act as CRE1/AHK4 receptor antagonists, which would further favour their indifference to the root system (Plíhal et al. 2013). This is feasible given that two other derivatives of BAP were shown to be cytokinin receptor antagonists. A compound known as PI-55, which is (6-(2-hydroxy-3-methylbenzylamino)purine), is an antagonist of the CRE1/AHK4 receptor (Spíchal et al. 2009). The compound LGR-991 (2,5-dihydroxy-BAP) showed antagonistic effects on AHK3 and CRE1/AHK4 receptors (Nisler et al. 2010).

For the reasons described above, it is clear that the N9-substituted derivatives of aromatic cytokinins have great potential for improving plant in vitro regeneration techniques.

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