Synthesis and Chemistry of Meta-Topolin and Related Compounds

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Lucie Plíhalová

Contents

Abstract

The chapter illustrates the gradual way in which the molecule of the active aromatic cytokinin, *meta*-topolin, was created. The historical development of the synthetic method from the first attempts to prepare aromatic cytokinins to professional organic synthesis is described here. The chapter also covers the preparation of second-generation aromatic derivatives of *meta*-topolin, e.g. C2- and/or N9-derivatives. The added value of such cytokinin derivatives over the original *meta*-topolin molecule is highlighted.

The stability and chemistry of N9-substituted *meta*-topolin derivatives were studied, and the mechanisms of action have been suggested. The benefits of these non-toxic compounds as well as the potential of specific functionalized derivatives of value to plants are discussed.

L. Plíhalová (✉)
Department of Chemical Biology, Faculty of Science, Palacký University, Olomouc, Czech Republic

Laboratory of Growth Regulators, Institute of Experimental Botany ASCR, Olomouc, Czech Republic
e-mail: lucie.plihalova@upol.cz

N. Ahmad, M. Strnad (eds.), *Meta-topolin: A Growth Regulator for Plant Biotechnology and Agriculture*, https://doi.org/10.1007/978-981-15-9046-7_2

Keywords

Aromatic cytokinin synthesis · Meta-topolin synthesis development · Modern synthetic approach

Abbreviations

ARCK	Aromatic cytokinins
BAP	6-Benzylaminopurine, N^6-benzyladenine
CK	Cytokinin
KIN	6-Furfurylaminopurine, N^6-furfuryladenine
mTR	*Meta*-topolin riboside, 6-(3-hydroxybenzylamino)purine riboside
oTR	*Ortho*-topolin riboside, 6-(2-hydroxybenzylamino)purine riboside
THF	Tetrahydrofuran-2-yl
THP	Tetrahydropyran-2-yl
WLSA	Detached wheat leaf senescence assay

2.1 From the First Artificial Aromatic Cytokinins to *Meta*-Topolin

The synthesis of the first cytokinins was initiated by the discovery of 6-furfurylaminopurine (kinetin, KIN) in autoclaved DNA samples in 1955. The compound was identified as a factor with the ability to influence cytokinesis (Miller et al. 1955, 1956). Despite the currently known fact that most purine-based cytokinins occurring naturally in plants are more likely substituted by an isoprenoid side chain at the N6 position of the adenine moiety, the first cytokinin discovered was accompanied by an aromatic furfuryl substituent. Therefore, the first attempts to prepare cytokinins (CKs) in the laboratory started with KIN, followed by another CK substituted by an aromatic benzyl moiety at the N6 position/6-benzylaminopurine (BAP, Miller et al. 1956).

Miller and his colleagues prepared KIN by the condensation of furfuryl chloride with adenine under alkaline conditions using sodium bicarbonate (Miller et al. 1956). BAP was prepared differently by a method based on the reaction of 6-methylthiopurine with benzylamine at a temperature of 140 °C for 16 h. The resulting yield was approximately 40–60% (Miller et al. 1955).

However, the process of aromatic cytokinin (ARCK) preparation was time consuming, and the resulting yields were not very satisfactory. Hence, over the years, a number of synthetic improvements were implemented. Currently, using nucleophilic substitution of SN_2 with 6-chloropurine by an appropriate amine under alkaline conditions is apparently provided by an excess of the appropriate amine or by the addition of trimethylamine. The reaction was for the first time published by Christensen and Daly for BAP synthesis (Daly and Christensen 1956). The reaction time was gradually shortened to approximately 3 h. The yields of the prepared CKs increased to 80% by further improvements of the reaction (Mik et al. 2011a). The

excess of trimethylamine to provide the alkaline medium was used against excess of the appropriate amine. Finally, the reaction time of such synthesis has been recently shortened to several minutes due to the implementation of modern technologies such as microwave reactor synthesis. The yield was also increased to approximately 98% (Huang et al. 2007; Plíhalová et al. 2016). Of course, it must be noted that the reaction itself lasts for several minutes, but an hour of reaction condition tuning is necessary, and after the reaction, there are procedures linked with the purification of the product. The development of current synthetic chemistry tools led to phenomenal progress, especially in conversion rates.

Both ARCKs, KIN and BAP became very useful in biotechnologies, in particular in plant tissue culture (PTC), and the optimization of BAP preparation caused massive use of such CKs in PTC of many economically important plants such as banana (Arinaitwe et al. 2000; Aremu et al. 2012a), roses (Pati et al. 2006), strawberries (Borkowska 2001), apples (Dobránszki and Teixeira da Silva 2010), melons (Milazzo et al. 1999) and medicinal plants (Fajinmi et al. 2014; Bairu et al. 2007, 2009; Werbrouck et al. 1995). Several described BAP side effects such as inhibition of root growth and rooting of cultured explants provoked interest in research and development of new, improved BAP derivatives.

6-(3-Hydroxybenzylamino)purine (*meta*-topolin) was prepared for the first time by Okumura and his co-workers in 1959 (Okumura et al. 1959). They prepared BAP derivatives containing hydroxyl, methyl, methoxy, amino, nitro and sulfonic acid groups and introduced these functional groups into *ortho*-, *meta*- and *para*-positions of the benzyl ring (Okumura et al. 1956, 1959). The synthesis was performed by the condensation of 6-(methylmercapto)purine with the appropriate amines in a sealed and/or open tube at temperatures higher than 125 °C (Okumura et al. 1959). In order to gain more knowledge of the biological properties of CK derivatives containing the N^6 aromatic side ring, BAP derivatives substituted at the *ortho*-, *meta*- and *para*-positions on the benzyl ring, including *o*-, *m*- and *p*-topolin, were systematically prepared (Doležal et al. 2006). Their biological activity was tested using classical cytokinin bioassays, such as detached wheat leaf senescence assay (WLSA), Amaranthus bioassay and tobacco callus bioassay. The synthesis of *meta*-topolin and related analogues was based on the previously described procedures for BAP, in which the selected amine (3-hydroxybenzylamine in the case of *meta*-topolin) was mixed with trimethylamine and heated to reflux in a suitable alcohol, most often butanol, for 3 to 4 h (Fig. 2.1).

A total of 38 BAP derivatives were prepared and tested, including hydroxy (topolins) and methoxy groups (methoxy-topolins). Some of the methoxy-topolins had very strong antisenescence activity, especially *ortho*- and *meta*-methoxy-topolins (Doležal et al. 2004, 2006). It was found that the position of functional groups on the benzyl ring can significantly influence the biological properties of the CK derivatives. The biological activities were strongly dependent on the bioassay used (Doležal et al. 2004, 2006, 2007; Szüčová et al. 2009). The rapid development in the synthesis of the ARCKs enabled the construction of a large library of such substances. These derivatives were tested not only in a number of plant bioassays but

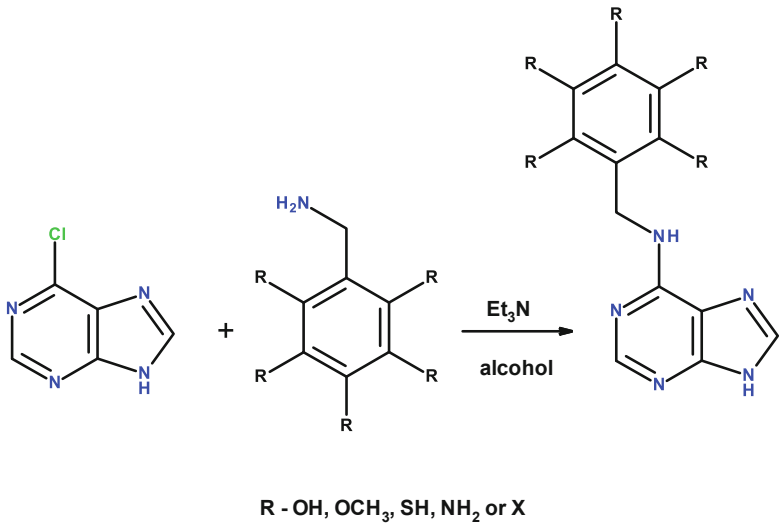

Fig. 2.1 Recent procedure, SN2 used for preparation of aromatic cytokinins

also in several biotechnological and agricultural applications (Plíhalová et al. 2016; Koprna et al. 2016—UV).

2.2 9-Substituted Topolin Derivatives

Substitutions at the N9 purine atom in CKs are common in plants. They are represented by a variety of natural conjugates, such as nucleosides, nucleotides and alanine derivatives (Davies 2007). CK-9-glucosides are highly abundant metabolites in plants. They are considered to be inactive forms of CK for their very low or zero CK activity. The binding of glucose to the N9 purine atom is considered irreversible in plants (Davies 2007; Kotek et al. 2010; Zahajská et al. 2019; Sakakibara 2006). In addition, naturally occurring 9-ribosides of ARCKs were discovered (Horgan et al. 1975; Strnad et al. 1997). Hydroxylated BAP derivatives substituted by ribose (BAPRs) were the first ARCK metabolites discovered in poplar leaves (*Populus x canadensis* Moench., cv. Robusta) (Horgan et al. 1975; Strnad 1997). Naturally occurring BAPRs were uniquely identified as *ortho*- and *meta*-topolin ribosides (oTR and mTR; 6-(2- and 3-hydroxybenzylamino)purine riboside) which were also recognized as active CK in standard bioassays such has tobacco callus, *Amaranthus* or WLSA. Especially in WLSA, ARCK ribosides exhibited significant biological activity. Although oTR was less active than BAPR, mTR was the most active of the three tested cytokinins (Holub et al. 1998; Zhang and Letham 1989). Bases of these nucleosides were named "topolins" according to the poplar or "topol" in the Czech language. A number of ARCK ribosides were later prepared by Doležal et al and their synthesis as well as biological activity, especially

antisenescence and anticancer activity, was protected by several patents (Doležal et al. 2007, 2012). ARCK ribosides are active CK with significant biological activity despite the fact that most 9-substituted CK derivatives are unable to activate CK receptors (Doležal et al. 2007; Zhang and Letham 1989; Kende and Tavares 1968). On the other hand, it is interesting to note that these substances often trigger a CK response regardless of the level of CK receptor recognition and often have considerable biological activity in plant bioassays and in plants (Podlešáková et al. 2012; Spíchal et al. 2004; Vylíčilová et al. 2016).

Mok and Mok (2001) found that if the N9 substitution of CK is made by special protection groups, such as tetrahydropyran-2-yl (THP), tetrahydrofuran-2-yl (THF), 4-chlorbutyl or methyl, the arising 9-substituted CK derivatives show even higher biological activity than the original free bases. Especially in case of THP and THF, they mimic the structure of sugars, and the comparison with ribosides comes into consideration. On the other hand, it was shown that neither THP nor THF derivatives are toxic as comparable molecules accompanied by ribose (Doležal et al. 2007; Szüčová et al. 2007, 2009). These findings are supported by many other reports (Mok and Mok 2001; Fleysher et al. 1969; Szüčová et al. 2009; Young and Letham 1969; Weaver et al. 1965; Kende and Tavares 1968). It appears that 9-substitution is related to block of the purine moiety position for naturally occurring 9-glucosylation that inactivates CKs. Bearing in mind that the majority of 9-substituted CK still possess CK activity, blocking the N9 atom by suitable protecting group can serve as a barrier to the in situ formation of 9-glucosides and prolongs CK persistence in living plant systems (Bairu et al. 2011; Podlešáková et al. 2012).

As mentioned above, 9-substitution by THP or THF group can significantly increase the antisenescence activity of topolins (Young and Letham 1969; Szüčová et al. 2009). Combination of the appropriate 9-substitution with the presence of hydroxyl or methoxy group in the benzyl ring enables these second-generation ARCK molecules to avoid 9-glucosylation and promote reversible but less often O-glucosylation of CKs. 9-THP and 9-THF derivatives of *meta*-topolin and *meta*-methoxy-topolin in particular are revealed to be interesting compounds for use in plant biotechnologies and agriculture.

The first attempts for the preparation of 6,9-disubstituted derivatives resulted in the mixtures of 6,7- and 6,9-derivatives, which generally contained preferentially 9-isomers. For this reason, the synthetic procedure had to be subjected to further modification, and later, due to the implementation of modern BOP and DIPEA catalysts, it was possible to prepare regional-specific 6,9-isomers (Wan et al. 2005). N9 atom substitution can be performed in two steps using 6-chloropurine as the starting material. In the first step, the N9 atom of purine is substituted by the chosen protecting group, such as THP or ribose. The second step is realized via nucleophilic substitution of N6 atom by the appropriate amine, similar to the preparation of free bases (Szüčová et al. 2009, Fig. 2.2).

In addition to CK activities, some of the 6,9-disubstituted derivatives were cytotoxic to human tumour cells, e.g. ribosides, and hence, there was considerable interest in their development (Legraverend et al. 2000).

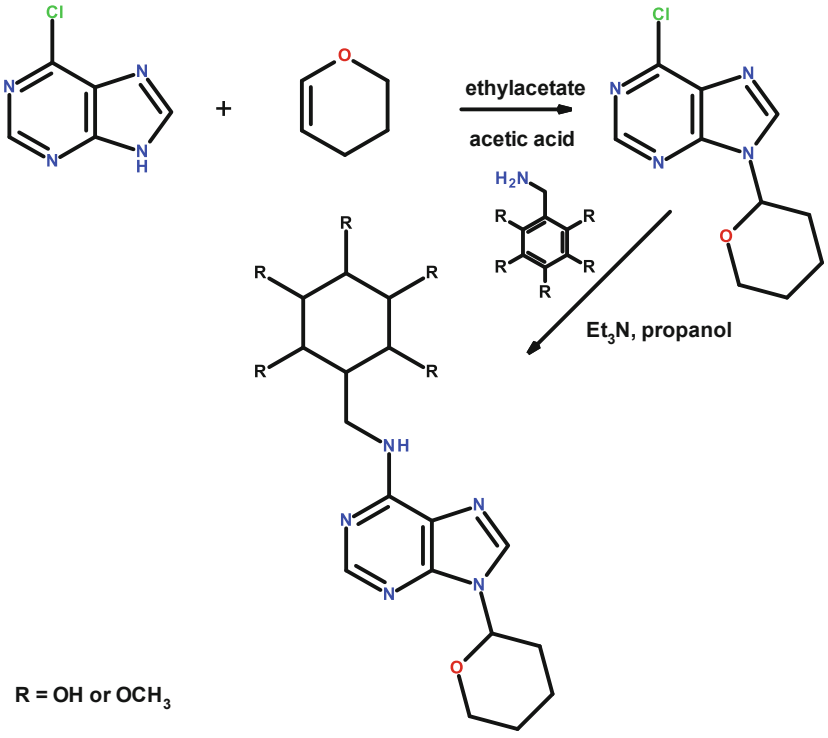

Fig. 2.2 Preparation of N9-(tetrahydropyran-2-yl)purine derivatives of substituted BAPs (Szüčová et al. 2009)

This aside, these compounds have a number of benefits for plant tissue culture, such as stimulation of root formation and its architecture in explants (Bairu et al. 2009), improvement of acropetal transport of CK and regulation of leaf senescence (Plíhal et al. 2013; Szüčová et al. 2011). 9-Substituted topolins also resist cytokinin oxidase/dehydrogenase (CKX) enzymatic degradation for a longer period of time (Podlešáková et al. 2012). In addition, 9-substituted KIN derivatives have been shown to actively protect lipid membranes against reactive oxygen species (ROS, Mik et al. 2011b).

2.3 2,6-Disubstituted and 2,6,9-Trisubstituted Aromatic Cytokinin Derivatives

2-Methyl-BAP was recognized as a compound with the ability to protect green plant material from harm, and its synthesis was firstly mentioned in the patent of Shell Oil Company nearly 60 years ago (Shell Patent 1966; Van Overbeek 1961).

Today, 2,6-dichloropurine or 2-fluoro-6-chloropurine (Zatloukal et al. 2008) is used as a starting material for preparing 2-substituted CK derivatives. 2-Substituted

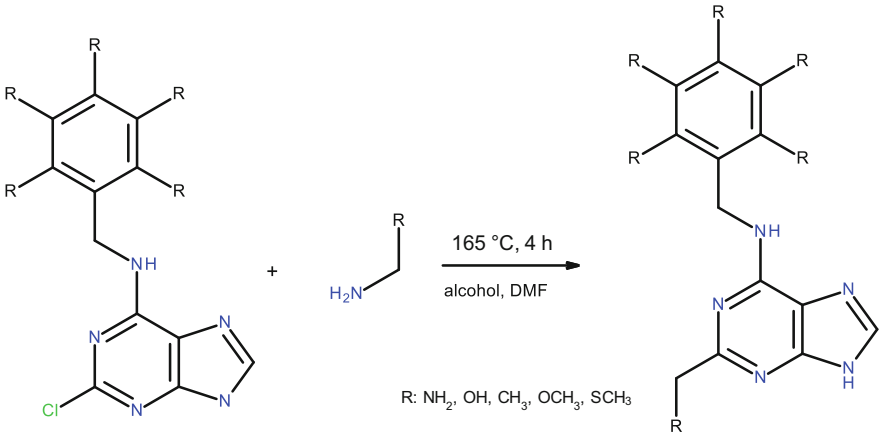

Fig. 2.3 Reaction scheme for the preparation of ARCKs substituted on purine C2

derivatives of *meta*-topolin were prepared from 2,6-dichloropurine or 2-fluoro-6-chloropurine (Zatloukal et al. 2008). The first step in the synthesis is the substitution of the C6-attached chlorine by suitable nucleophile, e.g. amine. In a subsequent step, C2-halogen can be suitably substituted with another functional group, e.g. amine, hydroxyl, methyl or methylthio group. However, not all functional groups can be added with equal efficiency, and the conversion rate and product purity may vary (Langli et al. 1996; Zatloukal et al. 2008) (Fig. 2.3).

2,6-Disubstituted derivatives have been shown to be biologically active in plant bioassays. For example, very interesting and highly biologically active *meta*-methoxy-topolin derivatives have been prepared by shortening the bond between the benzyl ring and the N^6-adenine by one carbon atom (Zatloukal et al. 2008) and by the addition of halogen atom to the C2 atom of the purine moiety. Specifically, 2-chloro-6-(3-methoxyphenylamino)purine and 2-fluoro-6-(3-methoxyphenylamino)purine were prepared and described as highly active inhibitors of the enzyme cytokinin oxidase/dehydrogenase (AtCKX2) from *A. thaliana* (Zatloukal et al. 2008; Spíchal et al. 2004, 2012). The inhibition of such enzyme can postpone the degradation of endogenously present CK in plants (Spíchal et al. 2004). From the example of these two derivatives, it is self-evident how the discovery of the molecular mechanisms of CK action can effectively be used to prepare fertilizers capable of significantly influencing plant growth and yield and enable plants to overcome abiotic stress. The compound, inhibitor of cytokinin degradation (INCYDE), described above, is capable of significantly affecting the response of plants to saline or mineral (cadmium) stress at a concentration starting from 10 nM and improving the yield of a range of crops and vegetables such as tomatoes, *Bulbine natalensis* Baker (Asphodelaceae) and *Rumex crispus* L. (Polygonaceae) (Gemrotová et al. 2013). In addition, it is also used in the micropropagation of some plants, for example, *Eucomis autumnalis* (Aremu et al. 2015) and banana trees (Aremu et al. 2012b).

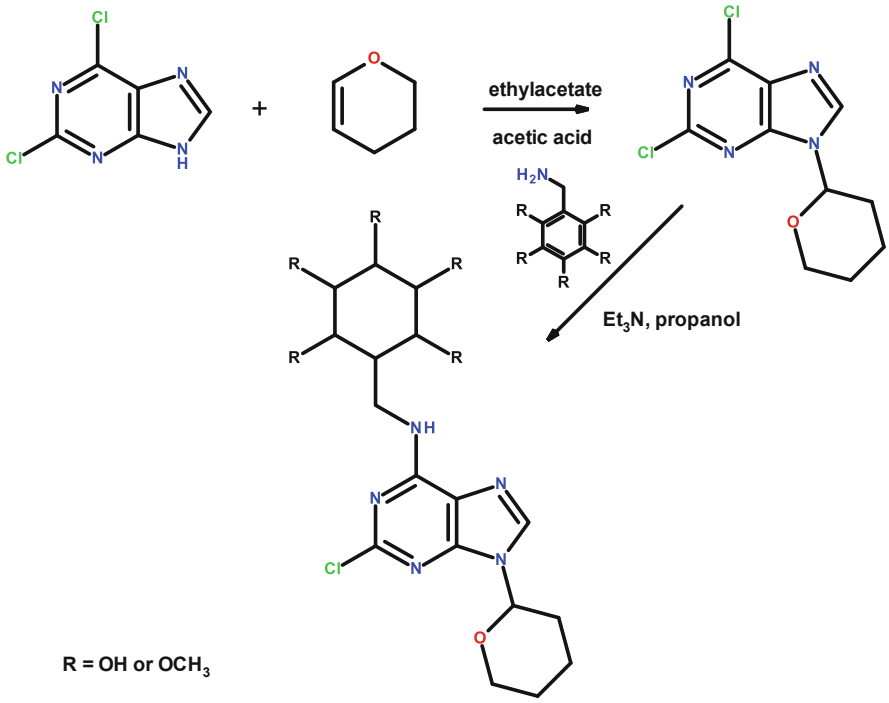

Fig. 2.4 The preparation of 2,6,9-trisubstituted ARCKs containing tetrahydropyran-2-yl (THF) at N9

It is also possible to prepare 2,6,9-trisubstituted purine derivatives using similar procedures as described above for the preparation of 6,9-disubstituted ARCKs (Szüčová et al. 2009). With this procedure, it is also possible to prepare very effective ARCKs, e.g. *meta*-topolin derivatives, which are viable as antisenescence agents (Fig. 2.4).

2.4 Stability of Meta-Topolin and N9-Substituted Meta-Topolin Derivatives

Although *meta*-topolin is a relatively stable compound, its 9-substituted derivatives, especially those covered by ribose, THP or THF groups, may undergo pH-dependent disintegration (Szüčová et al. 2009). It is for this reason that ribose or THP/THF are commonly used as protective groups in organic synthesis when further substitution of the molecule is needed and can be elegantly removed under controlled acidic conditions (Szüčová et al. 2009). The stability of THP/THF derivatives including *meta*-topolin-THP was described previously, and it was shown that the compounds are stable in pH around 5 and higher. Under this pH, gradual disintegration occurs and the free base of *meta*-topolin is released (Szüčová et al. 2009).

2.5 Summary

The preparation of ARCKs, including *meta*-topolin, has evolved over the past few decades when yields of prepared ARCKs were increased, syntheses were improved to produce higher HPLC purity products, while reaction time periods were gradually decreased. Improved syntheses over the past three decades have made ARCKs very readily available molecules that can be prepared in one- or two-step syntheses. More than 60 years after the preparation of the first ARCK, there has also been a significant development in the recognition of their effects and metabolism, and therefore more potent derivatives could be prepared that followed the natural molecular mechanisms of action. Key knowledge and understanding of the mechanism of action of O- and N9-glucosylation enabled the preparation of effective ARCKs, but also biologically active 9-substituted derivatives, which we now call second-generation ARCKs. All topolins and BAPs including their 2- or 9-substituted derivatives combined with the functionalization of the benzyl ring are protected by international patent applications (PCT) all over the world for use in agriculture and plant biotechnology, including plan tissue culture applications (Doležal et al. 2004). These derivatives effectively prevent the formation of N9-glucosides and thereby reduce ARCK inhibitory effects on root growth. Together with their high antisenescence activity and the prevention of unwanted side effects, these molecules form a new generation of ARCKs more and more utilized in plant tissue cultures of many crops and economically important plants.

Acknowledgement We would like to thank Mr. Alex Oulton for the careful reading of the manuscript. The work was supported by an ERDF project entitled "Development of Pre-Applied Research in Nanotechnology and Biotechnology" (No. CZ.02.1.01/0.0/0.0/17_048/0007323). The author of this review is grateful to Alexander Oulton, PhD., for the critical reading of the chapter.

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