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N9-substituted derivatives of kinetin: Effective anti-senescence agents

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ABSTRACT

The first isolated cytokinin, 6-furfurylaminopurine (kinetin or Kin), was identified almost 55 years ago. Its biological effects on plant cells and tissues include influences on such processes as gene expression, cell cycle, chloroplast development, chlorophyll biosynthesis, stimulation of vascular development, delay of senescence, and mobilization of nutrients. In the present study we prepared a series of eight N9-substituted Kin derivatives, and characterized them with available physicochemical methods such as CI+ mass spectrometry and ^{1}H NMR spectroscopy. All compounds were tested in three classical cytokinin bioassays: a tobacco callus assay, an *Amaranthus* assay, and a senescence assay with excised wheat leaves. The ability of the compounds to interact with *Arabidopsis* cytokinin receptors CRE1/AHK4 and AHK3 was tested in a bacterial receptor assay. Prepared derivatives with certain substitutions of the N9-atom of the purine moiety enhanced the cytokinin activity of the parent compound in the bioassays to a remarkable degree but negatively affected its perception by CRE1/AHK4 and AHK3. The ability of compounds to delay the senescence of excised wheat leaves in both dark and light conditions, was highly correlated with their ability to influence membrane lipid peroxidation, which is a typical symptom of senescence. Our results were corroborated by gene expression profiling of those genes involved in cytokinin metabolism and perception, plant senescence, and the stress response, and suggest that prepared kinetin derivatives might be used as potent anti-senescence agents.

1. Introduction

6-Furfurylaminopurine (commonly known as kinetin or Kin) was first isolated from naturally occurring material and characterized by Miller et al. (1955a,b, 1956). Kinetin belongs to a group of plant growth regulators known as cytokinins. Many of the cytokinins are structurally based on adenine, substituted at the N^{6}-atom, either by an isoprenoid side-chain or an aromatic ring. Cytokinins participate in a number of developmental processes such as cell division and differentiation, chloroplast development, chlorophyll biosynthesis, cellular senescence delay, differentiation of vascular tissue, as well as having roles in the development of flowers and fruits (Mok and Mok, 2001; Davies, 2004; Schmülling, 2004).

Leaf senescence forms the final stage of leaf development. It involves the loss of chlorophyll integrity and is influenced by hormonal modulation, as well as the catabolism of proteins, lipids and nucleic acids, and is associated with nutrient relocation (Zavaleta-Mancera et al., 2007; Dhindsa et al., 1982; Kar and Mishra, 1976; Srivalli and Khanna-Chopra, 2009). The cells in senescing leaves undergo highly coordinated changes in their structure, metabolism and gene expression (Noodén et al., 1997). The idea that exogenous cytokinins can control senescence was suggested following the measurement of endogenous cytokinin levels during plant senescence, and results from experiments with transgenic tobacco plants (Gan and Amasino, 1996). These transgenic plants were characterized by the expression of the cytokinin biosynthetic gene, isopentenyltransferase (*ipt*), which is under the control of the promotor from a senescence-associated gene (SAG12). The transgenic plants showed a significant delay of leaf senescence (Gan and Amasino, 1995, 1997) and enhanced flower longevity (Chang et al., 2003). Richmond and Lang (1957) observed the delay of leaf senescence after the external application of Kin to detached

Abbreviations: Kin, kinetin; BAP, 6-benzylaminopurine; CRE1/AHK4, cytokinin response 1/*Arabidopsis* histidine kinase 4; AHK3, *Arabidopsis* histidine kinase 3; *ipt*, isopentenyltransferase; *cps::lacZ*, fusion gene of capsular polysaccharide synthesis operon and reporter gene coding for β-galactosidase; DMF, dimethylformamide; DMSO, dimethylsulphoxide; ROS, reactive oxygen species; MDA, malondialdehyde; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; SOD, superoxide dismutase; GR, glutathione reductase; CAT, catalase; *APX*, ascorbate peroxidase; *SAG12*, cystein protease senescence associated gene; *VP14*, 9-cis-epoxycarotenoid dioxygenase gene; ARR, A-type response regulators; UGT, glucosyl transferases.

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Xanthium leaves. Kin, as well as a large number of cytokinin derivatives such as 2-methylthio-6-(3-methylbut-2-en-1-ylamino)purine, *trans*-zeatin, 6-benzylaminopurine or 6-benzylamino-9-(tetrahydropyran-2-yl)purine, were tested for their ability to delay senescence in the detached leaves of various plant species such as wheat and soybean (Kuhnle et al., 1977; Zhang and Letham, 1989). The exogenous application of Kin onto leaf segments delayed leaf senescence of oats (Varga and Bruinsma, 1973), barley (Kuroda et al., 1990), wheat (Kuhnle et al., 1977) and rice (Kar and Mishra, 1976) and prolonged the life span of cut carnation flowers (Eisinger, 1977). The anti-senescence and anti-aging activity of Kin has also been observed in *in vitro* cell cultures of animal and human cells (Barciszewski et al., 1999; Rattan and Clark, 1994; Sharma et al., 1997). An *in vitro* Fenton reaction proved that Kin is able to protect DNA from the hydrogen peroxide-induced formation of mutagenic 8-oxodeoxyguanine (Olsen et al., 1999). We can therefore state that Kin can protect proteins against oxidative and glyoxidative damage (Verbeke et al., 2000). Kin has also been shown to delay aging and dramatically prolong the lifespan of the fruit fly *Zaprionus indianus* when it is added to the fly's diet (Sharma et al., 1997). Furthermore, Kin has been shown to delay the onset of several age-related characteristics in human skin fibroblast cultures (Rattan and Clark, 1994).

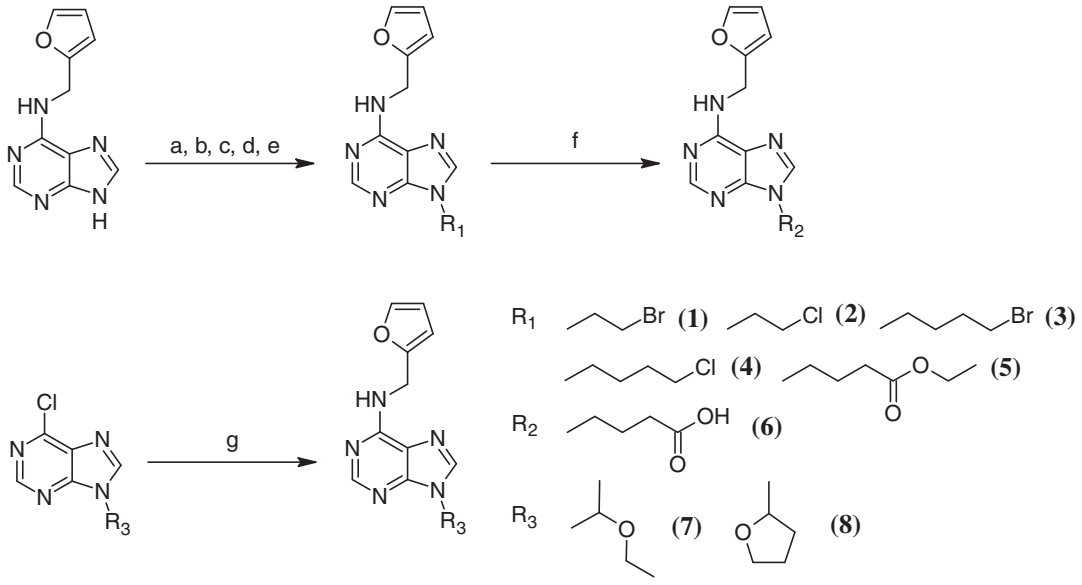
Anti-senescent activity has also been described for some N9-substituted aromatic cytokinin derivatives such as ribosides (Doležal et al., 2007) and tetrahydropyran-2-yl, as well as tetrahydrofuran-2-yl derivatives (Szüčová et al., 2009). The introduction of a tetrahydropyran-2-yl and tetrahydrofuran-2-yl moiety into the N9-position of 6-benzylaminopurine (BAP) doubled the retardation of soybean leaf senescence compared to the free base. 6-Benzyl-9-(4-chlorobutyl)purine has been shown to retard soybean leaf senescence slightly more than BAP (Zhang and Letham, 1989). Moreover, 6-[(3-methylbut-2-en-1-yl)amino]-9-(tetrahydropyran-2-yl)purine was found to be markedly more effective in the retention of the chlorophyll content in tobacco leaf discs than the parent cytokinin, 6-[(3-methylbut-2-en-1-yl)amino]purine (Letham et al., 1982). The introduction of tetrahydropyran-2-yl to the N9-position of Kin also enhanced its anti-senescence as well as its anti-aging activity, producing a substance that was significantly more active than Kin in a detached wheat leaf senescence bioassay (Szüčová et al., 2008). The activity of 6-furfurylamino-9-(tetrahydropyran-2-yl)purine on human diploid fibroblasts *in vitro*, as well as many *in vivo* tests on human skin, have been published so far. 6-Furfurylamino-9-(tetrahydropyran-2-yl)purine has recently been marketed under the registered trade name Pyratine-6® in several anti-aging skin preparations (www.senetek.com, Szüčová et al., 2008; McCullough et al., 2008).

In the present study, we prepared several novel derivatives of Kin substituted at the N9-position of the purine moiety by various alkyl side-chains. The prepared compounds were subjected to a range of different cytokinin assays, namely the tobacco callus assay, the *Amaranthus* assay, and the wheat leaf senescence bioassay in both dark and light conditions. The effects of the new compounds on lipid peroxidation in senescing detached leaves, and on root growth, were studied in plant assays and compared to the effects generated by the parent Kin compound. Finally, cytokinin-responsive gene expression profiling was used to describe the action of the novel compounds in more detail.

2. Results and discussion

2.1. Synthesis

Compounds **1**–**6** were prepared from kinetin by alkylation in an alkaline environment using the appropriate alkylation agent (a–e) as given in Fig. 1; compounds **7** and **8** were prepared in a two-step synthesis from their 6-chloropurine intermediates. Although the alkylation is more or less regioselective to the N9-position, a small amount of N7 isomer was also formed, necessitating the purification of the products by VersaFlash chromatography. The purity of prepared substances was verified by high performance liquid chromatography (HPLC). C, H and N elemental analysis data, melting points, and CI^+ MS data are given in Table 1. The details relating to the syntheses of the compounds, as well as ^1H NMR data, are given in Section 4.



Reaction scheme: a) **1** - 1,2-dibromoethane; b) **2** - 1-bromo-2-chloroethane; c) **3** - 1,4-dibromobutane; d) **4** - 4-bromo-1-chlorobutane; e) **5** - ethyl 4-bromobutyrate; f) **6** - prepared from compound **5**; g) **7, 8** - furfurylamine

Fig. 1. Schematic representation of the synthetic pathways of the new derivatives described in this study.