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Effect of Stereospecific Hydroxylation of N^6-(Δ^2-Isopentenyl)adenosine on Cytokinin Activity

M. Kamínek[1], V. Pačes[2], J. Corse[3], and J.S. Challice[4]

[1] Institute of Experimental Botany, Czechoslovak Academy of Sciences,
[2] Institute of Molecular Genetics, Czechoslovak Academy of Sciences, 166 10 Prague 6, Czechoslovakia,
[3] Western Regional Research Center, U.S. Department of Agriculture, Agricultural Research Service, Berkeley, CA 97410, USA, and
[4] Long Ashton Research Station, University of Bristol, Bristol BS 18 9AF, U.K.

Abstract. The cytokinin activities of cis and trans ribosylzeatin isomers and that of N^6-(Δ^2-isopentenyl)adenosine were compared in four bioassays. The trans isomer was found to be more active than the cis isomer in stimulation of cucumber cotyledon expansion (100×), retention of chlorophyll in detached leaf pieces (7×), induction and stimulation of chlorophyll synthesis in cucumber cotyledons (20×) and of betacyanin synthesis in *Amaranthus caudatus* seedlings grown in the dark (60×). The N^6-(Δ^2-isopentenyl) adenosine was less active than the trans ribosylzeatin in all four bioassays and more active than the cis ribosylzeatin in induction and stimulation of betacyanin and chlorophyll synthesis. These results show that the hydroxylation of the trans methyl group in the N^6 side chain of N^6-(Δ^2-isopentenyl)adenosine increases the biological activity and that this activity is either decreased or not significantly changed when the cis methyl group is hydroxylated.

Key words: *Amaranthus* – *Avena* – *Cucumis* – Cytokinin bioassays.

Introduction

The two geometric isomers of zeatin and its riboside seem to have different regulatory functions in plants. While the cis isomer of ribosylzeatin (c-io^{6}Ado) was identified as a predominant cytokinin-active component of plant tRNA, the trans isomer (t-io^{6}Ado) is a free form of zeatin that occurs in plant tissues. The former isomer is thought to be essential for ensuring specific codon-anticodon interaction and the latter is responsible for most of the cytokinin activity in plant tissues (see Kamínek, 1975, for a review). It has been suggested that this functional differentiation is the result of an evolutionary process that eliminated the interference in hormonal regulation of cytokinins of tRNA origin. This process should also include adaptation of specific sites on hormone receptors in such a way that they preferentially interact with one isomer (trans) and discriminate against that of tRNA origin (Kamínek, 1974).

According to this assumption, the hydroxylation of N^6-(Δ^2-isopentenyl)adenosine (i^{6}Ado) in the trans position should increase the cytokinin activity of the compound while the hydroxylation of the cis methyl group should have the opposite effect. This was shown in the tobacco callus bioassay in which the trans: cis activity ratio of ca. 40 and 5, respectively, were found for zeatin and ribosylzeatin isomers (Vreman et al., 1974). It is reported here that t-io^{6}Ado is more active than the c-io^{6}Ado in four bioassays, which, in contrast to the tobacco callus bioassay, are not based on induction and stimulation of cell division.

Materials and Methods

Adenosine and adenine were purchased from Sigma, Los Angeles. The t-io^6-Ado (for comparison in HPLC) and i^{6}Ado are products of Calbiochem.

Synthesis and Purity of t-io^{6}Ado and c-io^{6}Ado

The two isomers of ribosylzeatin were synthesized as described elsewhere (Kuhnle et al., 1977). Nuclear magnetic resonance spectroscopy confirmed the trans and cis structures, respectively, and purity > 95%. The purity of preparations was also checked by

Abbreviations: i^{6}Ado: N^6-(Δ^2-isopentenyl)adenosine or 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine; t-io^{6}Ado: trans-ribosylzeatin or 6-(4-hydroxy-3-methyl-trans-2-butenylamino)-9-β-D-ribofuranosylpurine; c-io^{6}Ado: cis-ribosylzeatin or 6-(4-hydroxy-3-methyl-cis-2-butenylamino)-9-β-D-ribofuranosylpurine; HPLC: high pressure liquid chromatography

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high pressure liquid chromatography (HPLC) (Challice, 1975). A glass column 1 m long (diameter 4 mm) was used. Injected samples of 200–400 µg per 1–10 µl were eluted with 0.05 M KH_2PO_4, pH 4.5 at 40° C (flow rate 1.5 ml/min, pressure 350 psi). Ultraviolet detection was carried out at 254 nm. Two kinds of column packings were used: Zipax SCX strong cation resin (System 1) and Pellidon Polyamide (System 3) obtained from Du Pont, Hertfordshire, and Reeve-Angel, Maidstone, respectively.

Amaranthus Bioassay

The sensitized bioassay of Conrad (1974) using *Amaranthus caudatus* seedlings was employed. The final concentration of phenol in the medium was decreased to one-half that used in the original procedure. The concentration of the betacyanin pigment was calculated as the difference between absorbance at 542 nm and 620 nm (ΔA).

Chlorophyll Synthesis Bioassay

A modified bioassay of Fletcher and McCullagh (1971) was used. Cucumber seeds (*Cucumis sativus* L. cv. Mělnická nakládačka) were sown in vermiculite soaked with distilled water and germinated at 26° C in the dark. After 7 days the cotyledons were excised under a dim green light and transferred into 5-cm Petri dishes containing 5 ml of cytokinin solution under test. Closed dishes were kept in darkness at 26° C for 16 h and then exposed to fluorescent light (3,000 lx) for another 6 h. Twelve cotyledons from each treatment were dried on a filter paper, weighed, and boiled in 6 ml 80% ethanol for 10 min. The chlorophyll concentration was estimated by measuring absorbance at 665 nm.

Cotyledon Expansion Bioassay

The increase of fresh weight of detached cucumber cotyledons in response to cytokinins described by Narain and Laloray (1974) also occurred in the previous bioassay. This allowed us to estimate the fresh weight of cotyledons just before chlorophyll extraction and to calculate the fresh weight increase induced by cytokinins.

Chlorophyll Retention Bioassay

The oat leaf bioassay of Thimann and Sachs (1966) was used. The reported insensitivity of this bioassay to natural cytokinins (Varga and Bruinsma, 1973) was overcome by selecting conditions under which the differences in response to natural and synthetic cytokinins are minimized (Kamínek and Luštinec, 1978). The chlorophyll content was estimated by measuring absorbance of 80% acetone extracts at 665 nm.

For all bioassays cytokinins were dissolved in twice distilled water. All bioassays were repeated at least three times with four (chlorophyll retention bioassay) and three (all other bioassays) replicates. For easy comparison the results are expressed in percentage of controls with no cytokinin.

Statistics

The statistical evaluation of results was based on the assumption that values expressing the physiologic response follow a curve that is continually rising and does not exceed lower and upper asymptotes. This property has a logistic curve which in this case has the following form:

$$y = \frac{1}{p_1 \exp(-p_2 x) + p_3}$$

where p_1, p_2, and p_3 are unknown regression coeficients, x is the independent variable (concentration) and y represents the physiologic response. The parameters were calculated using the program for nonlinear regression of the Health Science Computing Facility, University of California, Los Angeles. The significance of differences in the effects of individual substances was estimated on the basis of variance analysis calculated for standard concentrations.

Results

Purity of t-io^{6}Ado and c-io^{6}Ado and Their Separation by HPLC

The two isomers of zeatin riboside were clearly separated on Zipax SCX resin (Table 1). Interestingly the cis isomer has longer retention time than the trans isomer on cation exchange resin, which indicates that the separation was due more to differences in ionic properties ($K_{cis} > K_{trans}$ by analogy with the cinnamic acids, Challice and Williams, 1965) than to a differential absorption effect (Challice, 1975; Challice and Williams, 1966) on the polystyrene matrix, as originally suspected. Both isomers used in this study were found to be pure and uncontaminated by each other.

Stimulation of Betacyanin Synthesis

The t-io^{6}Ado was about 15 times more active and c-io^{6}Ado about four times less active than i^{6}Ado (Fig. 1). The trans:cis activity ratio was about 60 (calculated at Δ A=400% of control). Using this bioassay the t-io^{6}Ado, i^{6}Ado, and c-io^{6}Ado can be detected at concentrations of 7×10^{-9} M, 2×10^{-7} M, and 9×10^{-7} M, respectively, i.e., at concentrations that give about 200% increase in betacyanin formation as compared with the control.

Stimulation of Chlorophyll Synthesis

The i^{6}Ado was three times less active than t-io^{6}Ado and seven times more active than c-io^{6}Ado in stimulation of chlorophyll synthesis in cucumber cotyledons. The trans:cis activity ratio was about 20 (calculated at A_{665}=180% of the control). Significant greening of cotyledons was recorded at concentrations starting from 1.7×10^{-8} M of t-io^{6}Ado, 10^{-7} of i^{6}Ado, and 7×10^{-7} M of c-io^{6}Ado (A_{665}=125% of control, Fig. 2).

Table 1. HPLC separation of cytokinins

Compound	Retention time (min)	
	System 1	System 3
MeOH solvent peak	3.4	3.4
Adenosine (Sigma)	3.5	3.4–3.8
trans-Ribosylzeatin (Calbiochem)	4.6	3.8
trans-Ribosylzeatin (synthesized)	4.6	3.8
cis-Ribosylzeatin (synthesized)	5.1	3.9
Adenine (Sigma)	6.2	3.4–3.8
Kinetinriboside	4.4	4.3
N^6-(Δ^2-isopentenyl)adenosine	20.8	5.0
N^6-(Δ^2-isopentenyl)adenine	>60.0	6.35
N^6-benzyladenosine	7.4	5.6
N^6-benzyladenine	>60.0	7.9

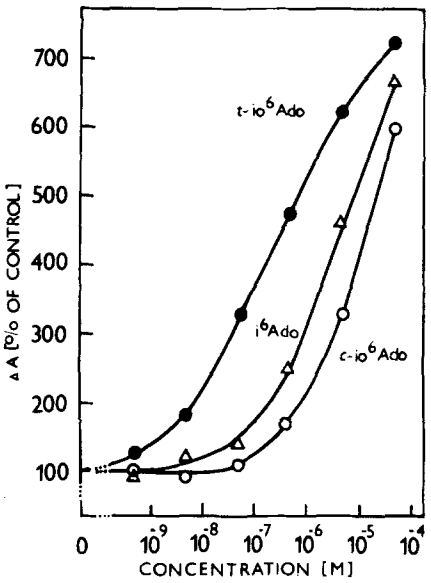

Fig. 1. Activities of trans-ribosylzeatin (t-io^{6}Ado), cis$^-$ ribosylzeatin (c-io^{6}Ado), and N^6-(Δ^2-isopentenyl)adenosine (i^{6}Ado) in the *Amaranthus* bioassay. The t-io^{6}Ado is significantly more active than the c-io^{6}Ado and i^{6}Ado at or above 5×10^{-9} M (1% and 5% confidence limit, respectively). The i^{6}Ado is more active than c-io^{6}Ado at concentrations 5×10^{-8} M and higher (5% confidence limit)

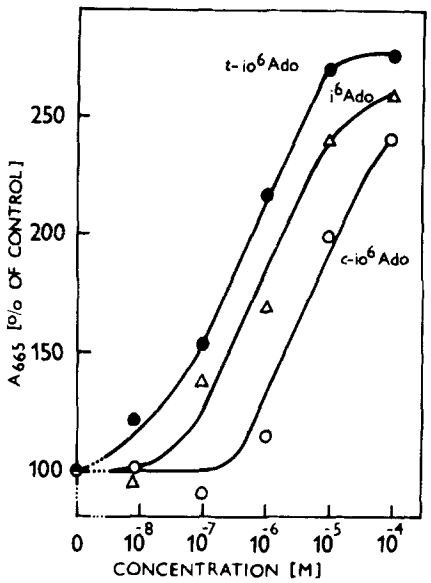

Fig. 2. Stimulation of chlorophyll synthesis in cucumber cotyledons by trans-ribosylzeatin (t-io^{6}Ado), cis-ribosylzeatin (c-io^{6}Ado), and N^6-(Δ^2-isopentenyl)adenosine (i^{6}Ado). The trans isomer is significantly more active than the cis isomer and i^{6}Ado at or above 10^{-8} M (1% confidence limit) and i^{6}Ado than the cis isomer at or above 10^{-7} M (5% confidence limit)

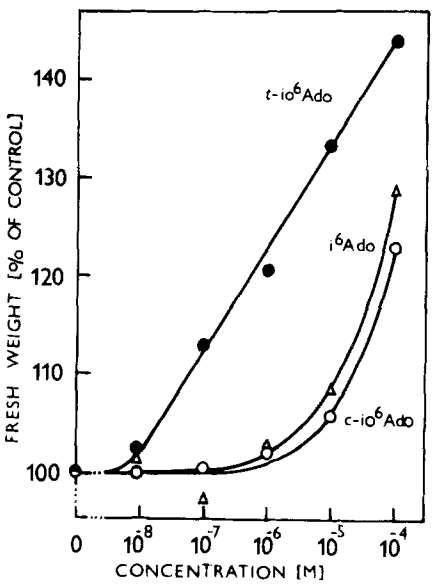

Fig. 3. The effect of trans-ribosylzeatin (t-io^{6}Ado), cis-ribosylzeatin (c-io^{6}Ado), and N^6-(Δ^2-isopentenyl)adenosine (i^{6}Ado) on expansion of cucumber cotyledons. The trans isomer is significantly more active than the cis isomer at or above 10^{-6} M (5% confidence limit)

Stimulation of Cotyledon Expansion

Comparison of cytokinin concentrations that induce 120% increase in fresh weight of cucumber cotyledons over the control showed that t-io^{6}Ado was about 100 times more active than i^{6}Ado and c-io^{6}Ado (Fig. 3). This increase in fresh weight also corresponds to concentrations at which these cytokinins can be reproducibly detected: t-io^{6}Ado 5×10^{-7} M, i^{6}Ado and c-io^{6}Ado 6×10^{-5} M.

Retention of Chlorophyll

Hydroxylation of i^{6}Ado in the trans position increased its cytokinin activity in the oat leaf senescence bioassay by a factor of 3.9 (Fig. 4). However, its activity is decreased by a factor of 1.8 when hydroxylated

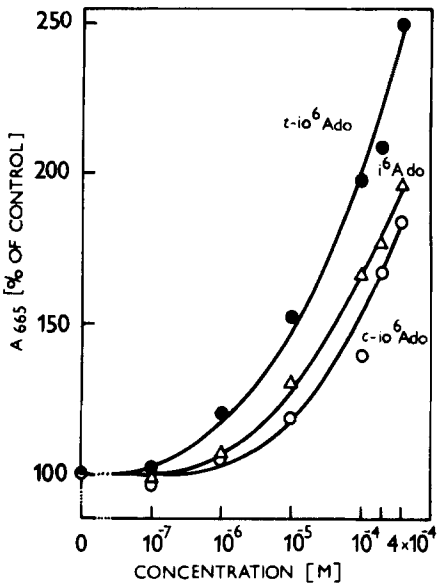

Fig. 4. The effect of trans-ribosylzeatin (t-io^{6}Ado), cis-ribosylzeatin (c-io^{6}Ado) and N^6-(Δ^2-isopentenyl)adenosine (i^{6}Ado) on chlorophyll retention in excised oat leaves. The trans isomer is significantly more active than the cis isomer and i^{6}Ado at or above 10^{-5} M (1% and 5% confidence limits, respectively) while the differences between the i^{6}Ado and c-io^{6}Ado are not statistically significant

at the cis position. The trans:cis activity ratio was about 7 (calculated at $A_{665}=175\%$ of control). The minimum detectable concentrations of t-io^6-Ado, i^{6}Ado, and c-io^{6}Ado were approximately 10^{-5} M, 4×10^{-5} M, and 10^{-5} M, respectively. These concentrations increased the retention of chlorophyll to 150% as compared with controls.

Discussion

The cis-trans isomerism in the N^6-side chain of ribosylzeatin is an important factor determining its cytokinin activity. It is known that the trans isomers of zeatin, ribosylzeatin, and their 2-methylthio derivatives are more active than the corresponding cis isomers in the tobacco callus bioassay (Leonard et al., 1971; Schmitz et al., 1972; Vreman et al., 1974). Although the relative activities of various cytokinins can be very different and even in reverse orders in different bioassays (Letham, 1967; Conrad, 1971), our results show that the activity pattern of the two ribosylzeatin isomers is much the same in four bioassays not based on the stimulation of cell division.

The two isomers differ most in their ability to stimulate expansion of cucumber cotyledon. This stimulation is based primarily on increased water uptake (Farineau and Rousaux, 1975). It seems to be related to the effect of cytokinin on cell permeability (Feng, 1973) and other membrane functions (Göring and Mardanov, 1976).

A high trans:cis activity ratio (60) was also found in the *Amaranthus* bioassay. Such a large difference may be a result of high sensitivity of the *Amanthus* bioassay to cytokinin-active ribosides (Conrad, 1971).

Chlorophyll synthesis is also affected to different extents by the two isomers. Pretreatment of excised dark-grown cucumber cotyledons with a cytokinin eliminates the lag phase and stimulates chlorophyll formation (Fletcher and McCullagh, 1971). Differentiation of chloroplasts is also stimulated (Farineau and Roussaux, 1975). Twenty times higher activity of t-io^{6}Ado than that of the c-io^{6}Ado indicates that the trans isomer is a powerful regulator of plastid development.

The smallest difference in activities of cis and trans ribosylzeatin was found in oat chlorophyll retention bioassay. This may be due to a preferential enzymatic degradation of t-io^{6}Ado in oat leaves. Synthetic cytokinins are known to have high activity in this assay (Varga and Bruinsma, 1973; Kamínek and Luštinec, 1978), and these cytokinins are not substrates for cytokinin oxidase (Whitty and Hall, 1974). Cytokinin oxidase, however, has higher affinity for the trans than for the cis isomer of zeatin riboside (Pačes and Kamínek, 1967). Kuhnle et al. (1977) found that t-io^{6}Ado is much more active than i^{6}Ado in retention of chlorophyll in wheat leaves while the c-io^{6}Ado is almost inactive. The order of activities t-io^{6}A-do > i^{6}Ado > c-io^{6}Ado was, however, the same as in the oat leaves.

The i^{6}Ado is less active than the t-io^{6}Ado in all four bioassays. Its activity is higher than that of c-io^{6}Ado in bioassays based on stimulation of betacyanin and chlorophyll synthesis. It is evident that hydroxylation of the trans methyl group in the N^6-side chain of i^{6}Ado results in an increase of cytokinin activity. The hydroxylation of the cis methyl group either decreases activity or is without effect. The relation between the stereospecific hydroxylation and biological activity may be a part of mechanism(s) that regulate(s) the cytokinin activity in plant cells by stereospecific enzymatic synthesis (Muira and Hall, 1973) and degradation (Whitty and Hall, 1974; Pačes and Kamínek, 1976) of the two isomers of ribosylzeatin.

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