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SYNTHESIS AND CYTOKININ ACTIVITY OF (R)-(+)- AND (S)-(−)-DIHYDROZEATINS AND THEIR RIBOSIDES

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Key Word Index—Dihydrozeatin; optical isomer; dihydrozeatin riboside; cytokinin activity; absolute configuration/activity relationships; tobacco callus; lettuce seed; radish cotyledon.

Abstract—(R)-(+)- and (S)-(−)-dihydrozeatins [(R)-(+)- and (S)-(−)-6-(4-hydroxy-3-methylbutylamino)purines, **1a** and **1b**] and their ribosides {(−)-6-[(R)-4-hydroxy-3-methylbutylamino]- and (−)-6-[(S)-4-hydroxy-3-methylbutylamino]-9-β-D-ribofuranosylpurines, **3a** and **3b**} were synthesized and tested for their cytokinin activity by four bioassay systems, the growth of tobacco callus, the seed germination of lettuce, the fr. wt increase of excised radish cotyledons and the retardation of chlorophyll degradation in radish cotyledons. In tobacco callus bioassay, **1a** was more active than **1b**. The ribosides **3a** and **3b** were not less active than their corresponding aglycones **1a** and **1b**. In other bioassays used the activity followed the order: **1a** > **3a** > **1b** > **3b**. In tobacco callus bioassay and lettuce seed germination, *trans*-zeatin [6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine] showed stronger cytokinin activity than **1***a*.

INTRODUCTION

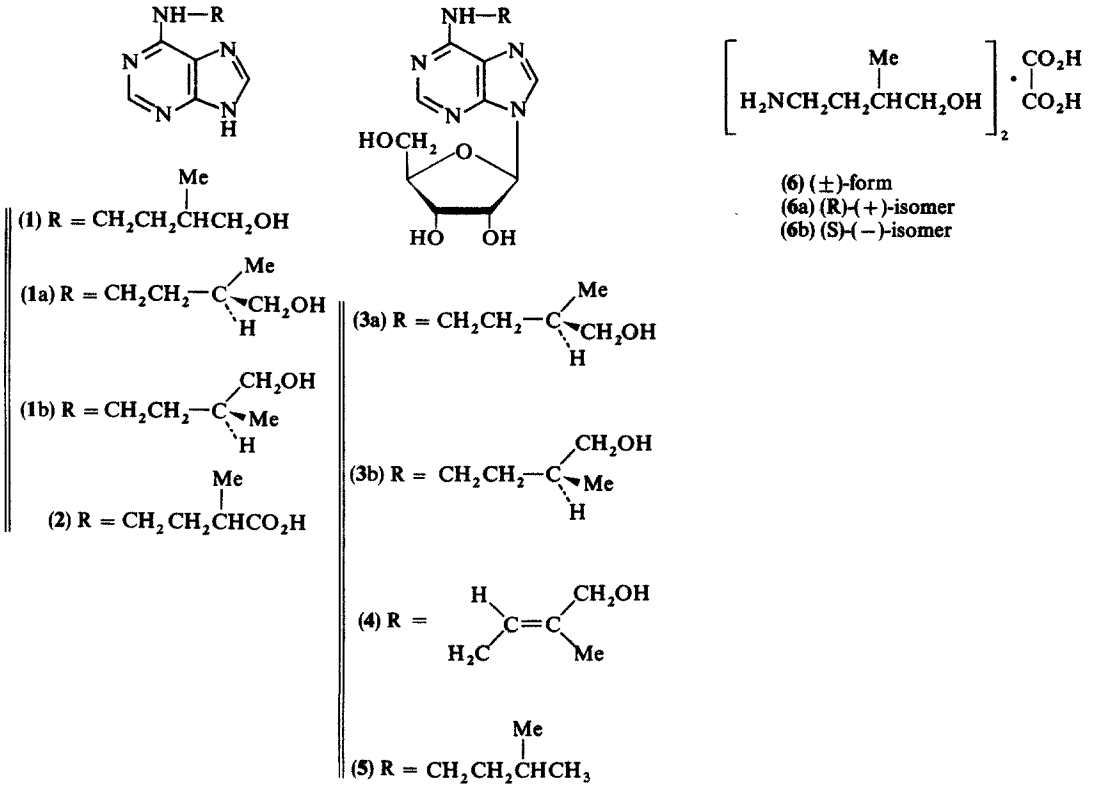
In a previous paper [1] we examined cytokinin activity of racemic dihydrozeatin [(±)-**1**], the (−)-antipode of which is a natural cytokinin isolated from immature seeds of *Lupinus luteus* [2], and revealed in several bioassays that (±)-**1** was less active than two natural cytokinins, *trans*-zeatin and 6-(3-methyl-2-butenylamino)purine [1]. Similar results were obtained later in tobacco callus bioassay [3, 4] and in radish cotyledon bioassay [5]. In all these studies, however, the absolute configuration of **1** which may affect the cytokinin activity was ignored. Different cytokinin activity between enantiomers of (R)- and (S)-configurations in several N^6-(optically active alkyl or aralkyl)-substituted adenines was reported [6–8]. Recently Fujii and Ogawa [9] determined the absolute configuration of natural (−)-dihydrozeatin [(−)-**1**] to be (S) (**1b**). These reports prompted us to examine the activity of **1** in relation to absolute configuration. In this paper, syntheses of (R)-(+)-**1** (**1a**), (S)-(−)-**1** (**1b**), and the corresponding ribosides (**3a,b**) as well as their cytokinin activities determined by four bioassay systems are presented.

RESULTS AND DISCUSSION

Synthesis and chemistry

The enantiomers (**1a, b**) of dihydrozeatin were prepared by heating a *n*-BuOH solution of 6-chloropurine [10] with (R)-(+)- (**6a**) [11] or (S)-(−)-2-methyl-4-amino-1-butanol oxalate (**6b**) [11] in the presence of Et_3N. Similar reactions of 6-chloro-9-β-D-ribofuranosylpurine [12] with the optically active amine salts (**6a,b**) produced the corresponding ribosides (**3a,b**). Alternatively, both ribosides (**3a,b**) were obtained by the use of the racemic amine oxalate [(±)-**6**][11] instead of the optically active one in this condensation followed by fractional recrystallization of the resulting diastereoisomeric mixture. The high stereochemical purity of the sample of **3a** thus obtained was confirmed by its acid hydrolysis to the corresponding aglycone (**1a**). Chromatographic separation (PC, TLC, HPLC *etc.*) of **3a** or **3b** from the diastereoisomeric mixture was extremely difficult.

Yet another method conceivable for preparation of **3a,b** would be an asymmetric reduction of *trans*-zeatin riboside (**4**) which can be easily synthesized [13–15]. When catalytic hydrogenation of **4** in MeOH was carried out over Adams catalyst, a mixture of **3a** and **3b** and N^6-isoamyladenosine (**5**) [16] were obtained in 70% and 4% yields. Formation of **5** is probably due to the hydrogenolytic C—O bond cleavage of **4** in the allylic alcohol moiety followed by saturation of the double bond. Hydrolysis of the mixture of **3a** and **3b** with 0.5 N HCl gave (±)-**1**, suggesting that the diastereoisomeric ratio of the mixture was 1:1. Replacement of Adams catalyst by Raney Ni in the hydrogenation described above suppressed the hydrogenolysis reaction, affording a 1:1 mixture of **3a** and **3b** in 90% yield. Thus, in either case the asymmetric reduction seemed not to have occurred to any appreciable extent. The lack of stereoselectivity observed may be explained by intramolecular



Scheme 1. (R)-(+)- and (S)-(−)-Dihydrozeatins and their ribosides.

remoteness of the chiral ribosyl group from the double bond to be reduced. Leonard *et al.* [3] also reduced 4 using H_2 and Pd/C as a catalyst and isolated a sample of dihydrozeatin riboside of unspecified stereochemical purity.

For comparison, racemic dihydrozeatin [(±)-1] was synthesized from 6-chloropurine and (±)-6 in a manner similar to that described for 1a, b. In alternative synthesis of (±)-1, 6-chloropurine was condensed with (±)-2-methyl-4-aminobutyric acid [11] in boiling aqueous Na_2CO_3 to give 2-methyl-4-(purin-6-ylamino)butyric acid [(±)-2] in a good yield. The $LiAlH_4$ reduction of (±)-2 in boiling THF provided (±)-1, but in a poor yield.

Cytokinin activity

Effects of 1a, 1b, their ribosides 3a,b and *trans*-zeatin on the growth of tobacco callus are shown in Figure 1. Compound 1a was apparently more active than 1b. The maximal yield of the callus was obtained at 10^{-1} μM in 1a and at 4×10^{-1} μM in 1b. The ribosides 3a and 3b were nearly as active as the aglycones 1a and 1b respectively. *trans*-Zeatin tested at the same time was more active than 1a. Thus, the cytokinin activity followed the order: *trans*-zeatin > 1a = 3a > 1b = 3b.

The cytokinins used in the present study promoted the lettuce seed germination (Fig. 2) and the fr. wt increase of radish cotyledons (Fig. 3), and inhibited chlorophyll degradation in radish cotyledons (Fig. 4). In these bioassays 1a was more active than 1b. The

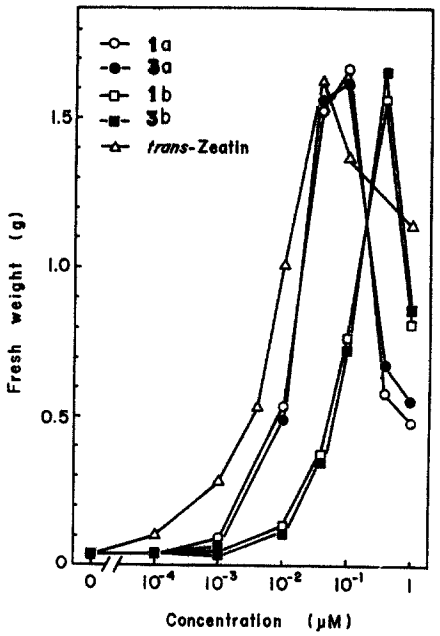


Fig. 1. Effect of 1a, 1b, their ribosides 3a,b and *trans*-zeatin on fresh weight yield of tobacco callus cultured on Linsmaier and Skoog medium.

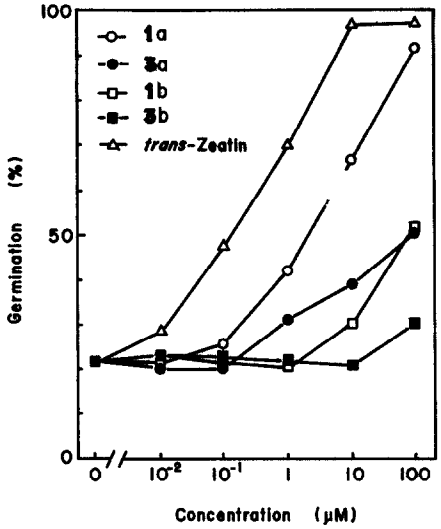


Fig. 2. Effect of 1a, 1b, their ribosides 3a,b and *trans*-zeatin on lettuce seed germination.

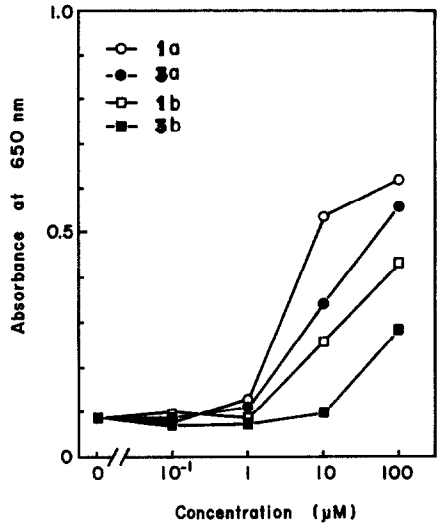


Fig. 4. Effect of 1a, 1b and their ribosides on retardation of chlorophyll degradation in senescing radish cotyledons.

ribosides 3a and 3b were less active than the corresponding purine bases respectively. Hence, the order of activity, 1a > 3a > 1b > 3b, was obtained. In the lettuce seed germination *trans*-zeatin tested together was more active than 1a.

Thus, in all the bioassays employed here, the unnatural (R)-(+)-antipode (1a) always showed higher cytokinin activity than naturally-occurring (S)-(−)-dihydrozeatin (1b). A similar relationship was also found between their ribosides (3a,b). Difference in configuration of several optically active cytokinins was reflected in their physiological activities on retardation of chlorophyll degradation [6], on bud formation of tobacco callus [7] and on tobacco callus growth [8]. All these results suggested that the three-dimensional structure around the asymmetric carbon atom in the side-chain of cytokinins has an important role in determining the cytokinin activity. At present, however, the biological implication of the fact that the compounds with the side-chain of natural absolute configuration are less active than those of unnatural absolute configuration is not clear.

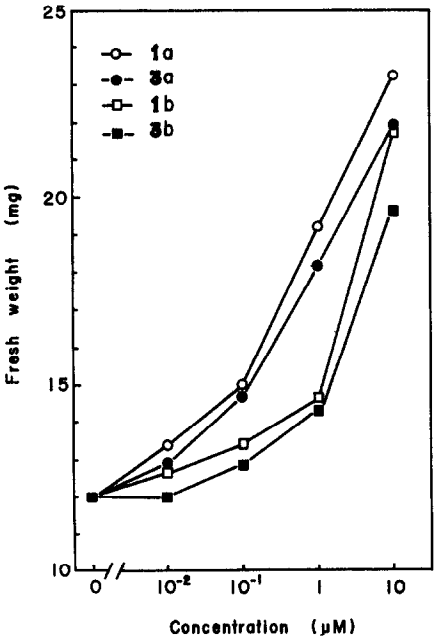


Fig. 3. Effect of 1a, 1b and their ribosides on fresh weight increase of excised radish cotyledons.

Schmitz *et al.* [4] reported that ribosidation at the 9-position of the purine ring reduced the cytokinin activity. In the present study, 1a and 1b were more active than 3a and 3b respectively in three bioassay systems except the tobacco callus bioassay. This result was consistent with the conclusion obtained by Schmitz *et al.* [4]. In the tobacco callus bioassay, however, 3a and 3b were not less active than corresponding purine bases respectively. This bioassay was repeated four times, but with similar results. Such inconsistency in the results of different bioassays was sometimes reported [1, 17].

Hecht *et al.* [18] pointed out that side-chain planarity is important in imparting the highest order of cytokinin activity, and that if side-chain planarity is disturbed by adding substituents to the double bond, cytokinin activity is lowered. Schmitz *et al.* [4] demonstrated that saturation of the double bond in side-chain of cytokinins reduced the cytokinin activity. The present study revealed that 1a and 1b which had no double bond in the side-chain were apparently less active than the unsaturated analog, *trans*-zeatin. This result was consistent with the earlier findings.

EXPERIMENTAL

All mp's are corrected. Elemental analyses were performed by Mr. Y. Itatani and his associates at Kanazawa University.

(±)-*Dihydrozeatin* [(±)-1]. (a) *From* (±)-6. A soln of 6-chloropurine [10] (155 mg) and (±)-6 [11] (159 mg) in *n*-BuOH (5 ml) containing Et_3N (1 ml) was refluxed for 3 hr. The mixture was evaporated *in vacuo* and the residue was dissolved in H_2O

(20 ml). The aq. soln was passed through a column packed with Amberlite IRA-402 (HCO_3^-) (6 ml) and the column was eluted with H_2O (300 ml). Evaporation of the eluate under vac. left a solid, which was recrystallized from EtOH–MeCN (1:2) to give (±)-**1** (160 mg, 72%) as colourless platelets, mp 165–167° (lit. [2] mp 167–168°); (Found: C, 54.38; H, 6.77; N, 31.40. Calc. for $C_{10}H_{15}ON_5$: C, 54.28; H, 6.83; N, 31.66%); MS, UV, IR, and NMR spectra, see lit. [9]. *Picrate.* Yellow needles (from EtOH), mp 199–201° (sintered at 194°) [lit. [2] mp 188–190° (sinters above 176°)]; (Found: C, 42.87; H, 4.14; N, 25.00. Calc. for $C_{16}H_{18}O_8N_8$: C, 42,67; H, 4.03; N, 24.88%); IR and NMR spectra, see lit. [9].

(b) *Reduction of* (±)-**2**. A stirred mixture of (±)-**2** (470 mg), $LiAlH_4$ (380 mg), and THF (110 ml) was refluxed for 7 hr. After addition of H_2O the THF was evaporated *in vacuo*. The residue was treated with 0.1 N NaOH (20 ml) and an insoluble material was removed by filtration. The filtrate was neutralized with 10% aq. HCl and evaporated to dryness *in vacuo*. Extraction of residue with EtOH and evaporation of the EtOH from the extract left an oil (*ca* 350 mg), which was chromatographed on a 35-g Al_2O_3 column using $CHCl_3$–EtOH (4:1) as eluent to give crude (±)-**1** (*ca* 50 mg). Purification of this sample by PC [R_f 0.70, *n*-BuOH–H_2O–conc NH_4OH (4:1:1)] followed by recrystallization from EtOAc–EtOH (10:1) produced (±)-**1** (23 mg, 5.2%), mp 164–166°, identical with an authentic sample.

(*R*)-(+)-*Dihydrozeatin* (**1a**). (a) *From* **6a**. Prepared from 6-chloropurine [10] and **6a** [11] (mp 179–180°; $[\alpha]_D^{23}$ +15.4° (50% aq. EtOH)) in 63% yield by a procedure similar to that described under method-(a) in the preparation of (±)-**1**. Colorless prisms [from EtOH–MeCN (1:2)], mp 153–154°; $[\alpha]_D^{21}$ +11.7° (MeOH; *c* 0.974); (Found: C, 54.61; H, 6.88; N, 31.50. $C_{10}H_{15}ON_5$ requires: C, 54.28; H, 6.83; N, 31.66%); ORD, UV, IR, and NMR spectra, see lit. [9]. *Picrate.* Yellow needles (from EtOH), mp 187–189°. The IR (KBr disc) spectrum of the picrate was superimposable on that of the picrate of **1b**.

(b) *Acid hydrolysis of* **3a**. The sample (282 mg) of **3a**, obtained by fractional recrystallization of the product from the reaction of 6-chloro-9-β-D-ribofuranosylpurine and (±)-**6** (see below), was heated in 0.5 N HCl (9.6 ml) at reflux for 1.5 hr. HCl was evaporated *in vacuo* and H_2O (10 ml) was added to the residue. The aq. soln was passed through a column of Amberlite IRA-402 (HCO_3^-) (11.4 ml) and the column was eluted with H_2O. The eluate was evaporated to dryness *in vacuo* and the residue was chromatographed [Al_2O_3 (30 g), $CHCl_3$–MeOH (15:1)] to give **1a** (100 mg, 57%), mp 154–155° (from EtOH–MeCN (1:2)); $[\alpha]_D^{20}$ +13.1° ± 0.5° (MeOH; *c* 1.02). This sample was identical with the one prepared by method-(a).

(*S*)-(−)-*Dihydrozeatin* (**1b**). Prepared from 6-chloropurine [10] and **6b** [11] (mp 180–181°; $[\alpha]_D^{22}$ − 15.4° (50% aq. EtOH)) in 75% yield in a manner similar to that described above for **1a**. Colorless prisms (from EtOH–MeCN (1:2)), mp 154–155° (lit. [2] mp 165–166°); $[\alpha]_D^{21}$ − 12.1° (MeOH; *c* 0.972); (Found: C, 54.12; H, 6.98; N, 31.59. Calc. for $C_{10}H_{15}ON_5$: C, 54.28; H, 6.83; N, 31.66%); ORD, UV, IR, and NMR spectra, see lit. [9]. *Picrate.* Yellow needles (from EtOH), mp 187–189° (lit. [2] mp 188–191° (sinters above 176°)); (Found: C, 42.96; H, 4.14; N, 25.25. Calc. for $C_{16}H_{18}O_8N_8$: C, 42.67; H, 4.03; N, 24.88%)

(±)-*2-Methyl-4-(purin-6-ylamino)butyric acid* [(±)-**2**]. (±)-2-Methyl-4-aminobutyric acid [11] (520 mg) was dissolved in H_2O (12 ml) and the soln was adjusted to pH 9 with 10% aq. NaOH. To the resulting soln were added Na_2CO_3 (742 mg) and 6-chloropurine [10] (927 mg) and the mixture was refluxed for 3.5 hr. After cooling, the mixture was brought to pH 3 with aq. HCO_2H. The ppt. that resulted was collected by filtration, washed with H_2O, and dried to give crude (±)-**2** (1.14 g), which was recrystallized from H_2O to furnish hygroscopic, colorless needles, mp 203–207° (dec); MS *m/e*: 235 M^+; UV $\lambda_{max}^{H_2O}$ (pH 1) 274 nm (log ε 4.19); $\lambda_{max}^{H_2O}$ (pH 7) 270 (4.21); $\lambda_{max}^{H_2O}$ (pH 13) 275 (4.22); PMR (60 M Hz, DMSO-d_6, TMS): δ 1.12 (3H, *d*, *J* = 7 Hz, —CH-C<u>H</u>$_3$), 1.46–2.35 (3H, *m*, —C<u>H</u>$_2$—C<u>H</u>—), 3.35–4.05 (2H, *m*, —NH—C<u>H</u>$_2$—CH$_2$—), 7.64 (1H, *t*, *J* = 6 Hz, —N<u>H</u>—CH$_2$—), 8.13 and 8.23 (1H each, *s*, purine protons), *ca* 14 (2H, broad, CO_2H and N-9).

(−)-6-[(*R*)-4-*Hydroxy*-3-*methylbutylamino*]-9-β-D-*ribofuranosylpurine* (**3a**). A soln of 6-chloro-9-β-D-ribofuranosylpurine [12] (123 mg) and **6a** [11] (83 mg) in *n*-BuOH (7.5 ml) containing Et_3N (0.6 ml) was refluxed for 3 hr. The mixture was evaporated to dryness *in vacuo* and the residue was triturated with H_2O (5 ml). The resulting insoluble solid was filtered off, washed with a little H_2O, and dried to give **3a** (126 mg, 83%). Recrystallization from EtOH yielded colourless needles, mp 224–225°; $[\alpha]_D^{22}$ − 56.8° ± 1.0° (MeOH; *c* 0.103); (Found: C, 50.68; H, 6.57; N, 19.44. $C_{15}H_{23}O_5N_5$ requires: C, 50.98; H, 6.56; N, 19.82%); MS *m/e*; 353 M^+; PMR (100 M Hz, DMSO-d_6–D_2O, TMS) δ 0.91 (3H, *d*, *J* = 6 Hz, —CH—C<u>H</u>$_3$), 1.70 (3H, *m*, —C<u>H</u>$_2$—C<u>H</u>—), 3.35 (2H, *d*, *J* = 6 Hz, —CH—C<u>H</u>$_2$O), 3.72 (4H, *m*; —NH—C<u>H</u>$_2$— and C-5'), 4.10 (1H, *m*, C-4'), 4.26 (1H, *m*, C-3'), 4.69 (1H, *m*, C-2'), 6.00 (1H, *d*, *J* = 6 Hz, C-1'), 8.35 and 8.48 (1H each, *s*, purine protons).

(−)-6-[(*S*)-4-*Hydroxy*-3-*methylbutylamino*]-9-β-D-*ribofuranosylpurine* (**3b**). Obtained from 6-chloro-9-β-D-ribofuranosylpurine [12] and **6b** in 70% yield by following a procedure similar to that described above for **3a**. Colourless needles (from EtOH), mp 199–200°; $[\alpha]_D^{21}$ − 75.1° ± 1.0° (MeOH; *c* 0.097); (Found: C, 50.61; H, 6.47; N, 19.64. $C_{15}H_{23}O_5N_5$ requires: C, 50.98; H, 6.56; N, 19.82%); MS *m/e*: 353 M^+; PMR (100 MHz, DMSO-d_6-D_2O, TMS), almost undistinguishable from that of **3a**.

Preparation of **3a** *and* **3b** *from* (±)-**6**. 6-Chloro-9-β-D-ribofuranosylpurine [12] and (±)-**6** were allowed to react in the same way as described above for **3a**. The reaction product was then separated by fractional recrystallization from EtOH into the sparingly soluble isomer (**3a**) [9% yield; mp 224–225°; $[\alpha]_D^{20}$ − 56.7° ± 1.0° (MeOH)] and the more soluble isomer (**3b**) (13% yield; mp 192–195°; $[\alpha]_D^{20}$ − 72.1° ± 1.0° (MeOH)), which were virtually identical (by IR and mmp) with authentic samples.

Catalytic hydrogenation of trans-zeatin riboside (**4**). (a) *Over Pt catalyst.* A soln of **4** [13–15] (178 mg) in MeOH (200 ml) was hydrogenated over Adams catalyst (30 mg) at 22° and atm pres for 6 hr. The reduction was further continued with an additional amount (30 mg) of the catalyst for 10 hr. The catalyst was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The residue (172 mg), mp 192–204°, was chromatographed (Si gel, $CHCl_3$–MeOH (10:1)) to afford **5** (4% yield; mp 161–162° (lit. [16] mp 158°)), identical with an authentic sample, and a mixture (70% yield; mp 201–208°) of **3a** and **3b**. The mixture was hydrolyzed in a manner similar to that described under method-(b) in the preparation of **1a**, giving (±)-**1** [64% yield; mp 165–167°; $[\alpha]_D^{18}$ − 1.51° ± 0.75° (MeOH)] identical with an authentic sample. (b) *Over Raney Ni.* A soln of **4** [13–15] (178 mg) in MeOH (200 ml) was hydrogenated over Raney Ni W-2 (0.4 ml) at 22° and atm pres for 9 hr. Removal of the catalyst by filtration and evaporation of the filtrate gave a mixture [mp 193–210°; $[\alpha]_D^{21}$ −65.9° ± 1.0° (MeOH)] of **3a** and **3b** in 90% yield. The PMR spectrum and optical rotation of this sample suggested that the ratio of the diastereoisomers present was 1:1.

Bioassay procedure. For tobacco callus (*Nicotiana tabacum* L. cv Wisconsin No. 38) bioassay, the method of [19] partially modified as in [8] was used. The basal culture medium was the Linsmaier and Skoog medium [19] containing the mineral salts, 30 g/l. sucrose, 10 g/l. agar, 100 mg/l. *myo*-inositol, 2 mg/l. IAA and 0.4 mg/l. thiamine-HCl. Aq. solns of the cytokinins to be tested were filter-sterilized and added to the autoclaved basal media in 50 ml conical flasks, shortly before solidification. Each flask contained 20-ml medium and three pieces of tobacco callus (5–8 mg each fr. wt) implanted on the agar surface. Each experimental treatment contained 4 replicates of flasks. The flasks were maintained at 28° in the dark for 4 weeks, and then the fr. wt of tissues was determined. The cytokinin-assay by lettuce seed germination was undertaken as described in [1]. Lettuce (*Lactuca sativa* L. cv New York 515) seeds were sown

to be tested in a Petri dish (diam 7 cm). After 48-hr incubation in darkness at 27°, the germination percentages were determined. The bioassay using fr. wt increase of radish cotyledons was performed by the method of [20]. Radish (*Raphanus sativus* L. cv Tokinashi) seeds were germinated at 26° in darkness on well-wetted filter paper in large Petri dishes. After *ca* 48 hr, one cotyledon, a larger one of two, was excised from each seedling. The cotyledons were selected for uniformity (*ca* 5 mg fr. wt), and placed on filter paper in Petri dishes (diam 7 cm). Each dish contained a sheet of filter paper, 4 ml of cytokinin soln to be tested (prepared in 2 mM KH_2PO_4) and 10 cotyledons. After 3 days at 26° under continuous fluorescent light (*ca* 3200 lx), the cotyledons were blotted dry and weighed. The cytokinin-assay by its inhibitory action on the chlorophyll degradation in radish cotyledons was undertaken by the method described in [21] with partial modification as in [1]. Radish (*Raphanus sativus* L. cv Tokinashi) seedlings were grown for *ca* 2 weeks in a greenhouse. At the stage when the first leaves opened, 10 cotyledons with uniform size (*ca* 50 mg each fr. wt) were excised and placed on filter paper wetted with 4 ml of cytokinin soln to be tested in a Petri dish (diam 7 cm). After incubation in darkness for 4 days at 27°, chlorophyll in cotyledons was extracted with 10 ml of 80% aq. EtOH [21]. Absorbance of the extract was determined at 650 nm with a spectrophotometer.

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