Phytochemistry, Vol. 20, No. 4, pp. 683–686, 1981.
Printed in Great Britain.

0031-9422/81/04683-04 $02.00/0

CYTOKININS IN IMMATURE SEEDS OF *DOLICHOS LABLAB*

TAKAO YOKOTA, JUNICHI UEDA* and NOBUTAKA TAKAHASHI

Department of Agricultural Chemistry, The University of Tokyo, Bynkyo-ku, Tokyo, Japan

(*Revised received* 30 *July* 1980)

Key Word Index—*Dolichos lablab*; Leguminosae; cytokinins; *trans*-zeatin; ribosyl-*trans*-zeatin; ribosyl-*cis*-zeatin; glucosyl-*trans*-zeatin; glucosyl ribosyl-*trans*-zeatin.

Abstract—Five cytokinins, *trans*-zeatin, 9-β-D-ribofuranosyl-*trans*-zeatin, 9-β-D-ribofuranosyl-*cis*-zeatin, 6-(*trans*-4-*O*-β-D-glucopyranosyl-3-methyl-2-butenylamino)purine and 6-(*trans*-4-*O*-β-D-glucopyranosyl-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine were identified from immature seeds of *Dolichos lablab*.

INTRODUCTION

Seeds of higher plants are known as one of the sites of cytokinin biosynthesis [1] and are a rich source of cytokinins [2–4]. Characterization of endogenous seed cytokinins has been reported by a large number of workers but in most cases identifications were based on ambiguous techniques, e.g. chromatographic behavior. Recent advances in HPLC and GC–MS, however, have made it easier to isolate and identify very small amounts of endogenous cytokinins. Using such techniques we have now surveyed the endogenous cytokinins contained in the seeds of a cultivated leguminous plant, *Dolichos lablab*.

RESULTS

The crude extract of immature seeds was extracted with *n*-butyl alcohol at pH 9 after ethyl acetate-soluble acidic materials were removed. Both the butyl alcohol fraction and the aqueous phase showed strong cytokinin activity in the *Amaranthus* betacyanin test [5]. To the aqueous fraction was added methanol and the resulting precipitate was removed by filtration. The filtrate and butyl alcohol fractions were combined and purified with a cation exchange resin. The ammoniacal eluate was chromatographed on a polyvinyl-pyrrolidone (PVP) column [6], and then on a Sephadex LH-20 column [7], yielding 4 active fractions, A, B, C and D.

The most active fraction B was purified by PC and TLC using various solvent systems. Final purification was carried out by HPLC using Lichrosorb DIOL and Nucleosil C18, affording two pure compounds B-1 and B-2. Each compound was hydrolysed with β-glucosidase. The hydrolysate was purified by HPLC and converted to the corresponding TMSi derivative, which was then analysed by GC–MS. The product from B-1 was identified as $(TMSi)_2$ *trans*-zeatin from the R_t and mass spectrum: *m/e* 363 (M^+, 19), 348 (21), 273 (93), 264 (5), 260 (100), 258 (21), 246 (5), 232 (35), 220 (9), 192 (19), 156 (41). The product from B-2 was likewise identified as $(TMSi)_4$ ribosyl-*trans*-zeatin from the R_t and mass spectrum: *m/e* 639 (M^+, 21), 624 (16), 549 (27), 536 (41), 320 (41), 276 (13), 259 (28), 245 (27), 243 (15), 230 (49), 217 (43), 201 (100), 188 (54), 169 (12), 156 (87). B-1 and B-2 were treated with methanolic hydrogen chloride and the products analysed by GLC after silylation. B-2 was found to produce *ca* equimolecular amounts of methyl riboside and methyl glucosides, whilst B-1 generated only methyl glucosides. Upon permethylation using methylsulfinylcarbanion and methyl iodide [8], B-1 was converted to the hexamethyl derivative (MS: *m/e* 465 (M^+, 2), 450 (1), 434 (4), 230 (100), 199 (5), 188 (17), 164 (9), 162 (8), 101 (10), 88 (10)), whilst B-2, was converted to the octamethyl derivative (MS: *m/e* 625 (M^+, 2), 610 (1), 594 (4), 390 (100), 348 (9), 216 (89), 174 (13), 159 (13), 135 (14), 129 (13), 115 (15), 111 (21), 101 (43), 88 (26)). The above data indicate that B-1 and B-2 are the glucosides of *trans*-zeatin and ribosyl-*trans*-zeatin respectively.

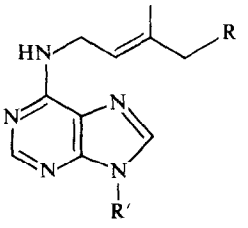
B-1 exhibited UV spectra characteristic of N^6-monosubstituted adenines [9, 10], indicating that the sugar is linked to the side-chain oxygen. This is also supported by the base peak at *m/e* 230 in the permethylated B-1, which arises by expulsion of the side-chain glucosyloxy group from the M^+. This allylic fission seems to be the predominant reaction in *O*-alkylated zeatin derivatives [8, 11]. Thus, the structure of B-1 was established as 6-(*trans*-4-*O*-β-D-glucopyranosyl-3-methyl-2-butenylamino)purine (**1**).

B-2 had a UV spectrum characteristic of N^6, 9-disubstituted adenines [9]. Therefore, the glucosyl moiety must be attached to either the side chain or ribosyl moiety. The base peak at *m/e* at 390 observed in permethylated B-2, due to the loss of the glucosyloxy group from the M^+, indicates that the side chain hydroxyl is glucosylated (*vide supra*). Thus, B-2 is assigned the structure 6-(*trans*-4-*O*-β-D-glucopyranosyl-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**).

The structures B-1 and B-2 were confirmed by their ^1H NMR spectra (see Experimental) and also by the fact that the mass spectra of permethylated B-1 and B-2 are in close resemblance to published data on permethylated derivatives of glucosylzeatin and glucosyl ribosylzeatin isolated from crown gall callus, respectively [12].

Fraction C was purified by HPLC. The fast-moving major compound and a slow-moving minor one were

* Present address: College of Integrated Arts and Sciences, University of Osaka Prefecture, Sakai, Osaka, Japan.



1 R = *O*-β-D-glucosyl, R′ = H
2 R = *O*-β-D-glucosyl, R′ = β-D-ribosyl
3 R = H, R′ = β-D-ribosyl
4 R = R′ = H

observed, the R_ts being the same as those of ribosyl-*trans*-zeatin and its *cis* isomer respectively. These were collected and analysed by GC–MS after silylation. The former was identified as ribosyl-*trans*-zeatin (**3**) since the R_t and mass spectrum were identical with those of $(TMSi)_4$ ribosyl-*trans*-zeatin. This compound crystallized from ethanol–acetonitrile and the mp of the crystals (186°) also supported the identity. The latter was likewise identified by GC–MS as ribosyl-*cis*-zeatin, its mass spectrum (as the $(TMSi)_4$ derivative) being identical to that of the *trans* isomer.

Fraction D was also purified by HPLC. The major compound eluted faster than the minor one: their R_ts coincided with those of *trans*-zeatin and *cis*-zeatin, respectively. The former was identified as *trans*-zeatin (**4**) by GC–MS after silylation. Two peaks in the total ion current trace were observed, which showed mass spectra assignable to $(TMSi)_3$-*trans*-zeatin (fast-moving major derivative) and $(TMSi)_2$-*trans*-zeatin (slow-moving minor one) respectively. It was not clear why zeatin was derivatized mainly to the $(TMSi)_3$ derivative which usually only partially formed from zeatin under the conditions employed here. The *cis*-zeatin-like compound eluted from the HPLC column could not be characterized by GC–MS.

Fraction A which appeared to contain the most polar cytokinin from its chromatographic behavior was purified successively by charcoal chromatography, PC and cellulose TLC. The active fraction was finally purified by HPLC to afford an active compound, which had a R_t slightly less than that of adenine. The amount isolated was estimated to be either 11 μg *trans*-zeatin equivalents from its UV absorption or 7 μg *trans*-zeatin equivalents from its activity in the *Amaranthus* test, indicating this compound has nearly the same amount of activity as that of zeatin. This polar compound, which showed a UV maximum at 269 nm (10% MeOH) characteristic of cytokinins, does not seem to be a nucleotide cytokinin, since it remained unchanged after alkaline phosphatase treatment. Further structural analysis is in progress.

DISCUSSION

Ribosylzeatins were a 34:1 mixture of ribosyl-*trans*-zeatin and its *cis* isomer.Recently, the *cis* isomer has been reported to occur in several species of plants [13–15]. Our findings may strengthen the possibility that ribosyl-*cis*-zeatin could be an ubiquitous constituent in plants. On the other hand it is questionable whether *cis*-zeatin is present or not, since a peak with the same R_t as that of *cis*-zeatin was detected by HPLC, but GC–MS analysis of this peak did not give a positive result. It is interesting to note that glucoside cytokinins exist exclusively as *trans* isomers.

Glucosylzeatin and its derivatives have been partially characterized from a variety of plants [16–20]. *Vinca rosea* crown gall callus has been reported to produce cytokinin-*O*-glucosides [12]. Recently several glucosyl-zeatin derivatives have been found in the metabolites of labeled zeatin [23]. Cytokinin glucosides isolated from *D. lablab* seeds were unambiguously shown to be composed of *O*-glucosyl *trans*-zeatin and *O*-glucosyl ribosyl-*trans*-zeatin. However, most of the glucosylzeatin isolated is apparently formed by the degradation of ribosyl glucosylzeatin during the long purification procedure, since, when the seed extract was purified by an improved technique, consisting of a sequence of Dowex-50W treatment, PVP chromatography and PC and the purified glucoside fraction was analysed by HPLC, the peak height ratio of glucosyl ribosylzeatin to glucosylzeatin was 58:1. The above facts, together with the observation that during the purification step only a small amount of ribosylzeatin and/or zeatin seems to be liberated, indicate that the ribosyl linkage is more unstable than glucosyl linkage.

The presence of an unknown cytokinin which is more polar than the glucoside cytokinins was also indicated. This minor cytokinin showed a strong activity and did not seem to be a ribotide cytokinin.

In this study HPLC was recognized as an efficient method for isolating cytokinins. Cytokinin fractions which were obtained after several steps of prepurification prior to HPLC still contained impurities which interfered with the subsequent determination by UV and GC–MS. This problem could be easily overcome after using HPLC. In this sense prepurifications steps used in this work should have been simplified by applying HPLC at the earlier stage of prepurification. It should be stated that when the simplified prepurification procedure combined with HPLC were applied to purify glucoside cytokinins, their breakdown could be suppressed.

EXPERIMENTAL

GC–MS was carried out using 2% OV-1 (3 mm × 1 m) as described previously [22]. GLC data were obtained with the same column used for GC–MS. All samples tested for GC–MS and GLC were silylated by heating with a 2:1 mixture of MeCN–*N,O-bis*(trimethylsilyl)acetamide (BSA) unless otherwise stated. HPLC was carried out using a Nucleosil 7 C18 column (4 mm × 25 cm) and aq. MeOH (*ca* 2 ml/min) by monitoring at UV_{269nm} unless otherwise stated, the detailed procedure being reported previously [21]. Cytokinin activity was measured on the basis of betacyanin production by *Amaranthus caudatus* [5].

Extraction and partition. Immature seeds (5.6 kg, mean wt/seed = 0.25 g) harvested at the Tanashi farm of our University were extracted × 3 with MeOH by homogenizing. MeOH was evaporated to give an aq. soln which was extracted × 3 with EtOAc at pH 2 to remove inactive materials. The aq. phase was readjusted to pH 9 and extracted × 4 with *n*-BuOH, giving an active BuOH fraction. The aq. residue, which still retained a strong cytokinin activity, was neutralized, MeOH added and filtered to remove the inactive ppt. The filtrate and BuOH fraction were combined and condensed *in vacuo* to give the cytokinin fraction.

Cation exchange resin column. The cytokinin fraction dissolved in 300 ml H_2O (pH 4) was passed through a 700 ml column of Dowex 50W (100–200 mesh), which was washed with H_2O (1.4 l.)

and 95% EtOH (3 l.). The resin was then removed from the column, cooled to 5° and mixed with cold 3 N NH_4OH in 50% EtOH to prevent local heating. This mixture was repacked into the column, further elution being conducted at 5° with the same solvent (3 l.) and then 6N NH_4OH in 50% EtOH (2 l.). Most of the activity was recovered in the eluate with 3 N NH_4OH and the minor activity in the eluate with 6 N NH_4OH.

Insoluble PVP column. The above active fractions were purified with a column (300 g of PVP, bed vol. = 1.2 6 l.). The eluate with M/75 Pi buffer (pH 6.35) was collected in 15 ml fractions. Based on the bioassay result, fractions 71–90 (a), 91–140 (b), 141–190 (c) and 191–290 (d) were combined, respectively.

Sephadex LH-20 column. Fractions a–d were purified separately with columns (bed vol. 370 ml) using 35% EtOH as the eluting solvent (one fraction = 15 ml), affording active fractions 7–14 (A), 16–19 (B), 28–31 (C) and 30–37 (D), respectively.

Purification of fraction A. This was purified by charcoal chromatography using 50 g charcoal (Wako's product for chromatography). Elution was conducted successively with H_2O (1.3 l.), 0.5 N NH_4OH in 50% MeOH (1 l.), pyridine–H_2O (1:9, 1 l.) and pyridine–EtOH–M NH_4OH (2:5:3, 1 l.). Activity was found in the latter two fractions. The 3rd fraction was chromatographed on Toyo No. 526 paper using H_2O–satd. *sec*-BuOH to reveal a major activity at R_f 0.1–0.17, with the last fraction, at R_f 0.4–0.6 (glucoside region). The former fraction was purified successively with Toyo No. 526 paper (R_f 0.13–0.38), Toyo No. 50 paper (R_f 0.09–0.16) and cellulose TLC (R_f 0.31–0.38), in which the same solvent (*n*-BuOH–HOAc–H_2O, 12:3:5) was used. Further purification by HPLC (10% MeOH) afforded an active compound at R_t = 6.8 min: cf. R_t of adenine = 8.8 min.

Purification of fraction B. Fraction B (1.1 g) was purified in the following order: (1) Toyo No. 526 paper, H_2O–satd *sec*-BuOH (R_f 0.28–0.53), (2) the same paper, *sec*-BuOH–15N NH_4OH = 4:1 (R_f 0.17–0.4), (3) Merck silanised Si gel GF_{254} plate, 40% MeOH (R_f 0.5–1.0), (4) Merck precoated Si gel GF_{254} plate, *n*-BuOH–HOAc–H_2O = 12:3:5 (R_f 0.15–0.31), (5) Toyo No. 50 paper, H_2O–satd *n*-BuOH (R_f 0.04–0.26) and (6) Eastman Kodak precoated F_{254} cellulose plate, *n*-BuOH–HOAc–H_2O, 12:3:5. In the last step, coherent factors were separable into B-1 (R_f 0.63) and B-2 (R_f 0.58). Sufficient purity could be obtained after HPLC using Lichrosorb DIOL (10% MeOH in $CHCl_3$, 3 ml/min) and then Nucleosil 7 C18 column (30% MeOH). B-1 and B-2 were coeluted in the DIOL column (R_t = 4.8 min), while they were well separated in the C18 column (R_t: B-1 = 5.2 min, B-2 = 6 min). Final yields of B-1 and B-2 were 560 and 300 μg respectively, based on the assumption that UV-extinction coefficients of B-1 and B-2 should be close to those of zeatin and zeatin riboside respectively. ^{1}H FT NMR (99.55 MHz, CD_3OD) of B-1: δ 1.84 (*s*, Me), 5.74 (*t*, *J* = 7 Hz, olefinic H), 8.05, 8.24 (*s*, *s*, 2-H and 8-H). B-2: δ 1.84 (*s*, Me), 5.74 (*t*, olefinic H), 5.94 (*d*, *J* = 7 Hz, ribose 1-H), 8.23 (*s*, 2-H and 8-H). The other signals could not be assigned because of the overlapping with the solvent and H_2O. UV spectra of B-1: λ_{max} 270, λ_{min} 235 (25% MeOH); λ_{max} 275, λ_{min} 237 (0.1 N HCl in 25% MeOH); λ_{max} 276, λ_{min} 243 (0.1 N NaOH in 25% MeOH). B-2: λ_{max} 269, λ_{min} 232 (25% MeOH); λ_{max} 266, λ_{min} 235 (0.1 N HCl in 25% MeOH); λ_{max} 270, λ_{min} 241 (0.1 N NaOH in 25% MeOH).

Purification of fraction C. Fraction C (22 mg) was purified by HPLC (27% MeOH), affording ribosyl-*trans*-zeatin at 10.5 min and its *cis*-isomer at 12.8 min, the yields of which were determined by peak hts to be 1714 and 51 μg respectively. Using GC–MS (oven 220°), the *trans*-isomer appeared at R_t 5.4 min [$(TMSi)_4$ derivative], whilst the *cis*-isomer appeared at R_t 4.7 min [$(TMSi)_4$ derivative].

Purification of fraction D. This fraction (25 mg) was purified by HPLC (30% MeOH), affording *trans*-zeatin (18.5 μg based on the peak ht) at R_t 8.6 min and a compound (1.1 μg *cis*-zeatin equivalents at 10.6 min, the same R_t as that of *cis*-zeatin). By GC–MS (oven 175°) the former appeared at R_t 3.9 min [$(TMSi_3$ derivative] and 4.7 min [$(TMSi)_2$ derivative].

Enzymatic hydrolysis of B-1 and B-2. Each compound (15 μg) was incubated at 37° for 3 hr with 0.1 ml of 0.1% β-glucosidase (Sigma, almond emulsin) soln in 0.05 M acetate buffer. The reaction mixture was directly purified by HPLC (30% MeOH) and after silylation was analysed by GC–MS. $(TMSi)_2$ *trans*-zeatin (R_t = 2.3 min; oven 180°) was detected in the hydrolysate of B-1, while $(TMSi)_4$ ribosyl-*trans*-zeatin (R_t = 4.6 min; oven 220°), was identified in that of B-2.

Methanolysis of B-1 and B-2. The 10 μg sample was dissolved in 0.3 ml of N MeOH–HCl. The soln, after being maintained at 100° for 3 hr, was evaporated to dryness *in vacuo*. The dried material was silylated with a 3:3:1 mixture of pyridine–BSA–TMCS and analysed by GLC (oven 150°; N_2 25 ml/min). In the hydrolysate of B-2, Me riboside (R_t = 2.1 min) and Me glucosides (R_t = 9.4, 10.5 min) were observed, while in the hydrolysate of B-1, only Me glucosides were detected.

Permethylation of B-1 and B-2. A M soln of dimethylsulfinylcarbanion prepared from a 55% oil dispersion of NaH and DMSO freshly dist. from CaH was used as base [8, 11]. B-1 (82 μg) was dissolved in 0.1 ml of the reagent, intermittently shaken for 30 min and 10 μl of MeI added with cooling. After 1 hr reaction, the mixture was diluted with 1 ml of H_2O and partitioned × 3 with 2 ml of $CHCl_3$. The combined $CHCl_3$ extracts were washed × 3 with 1.5 ml of H_2O. Evaporation of the $CHCl_3$ gave an aq. emulsion, which was diluted with H_2O and freeze-dried to give the product. B-2 (50 μg) was similarly methylated. GC–MS was carried out at 230° for permethylated B-1 (R_t = 4.3 min) and at 250° for permethylated B-2 (R_t = 8.7 min).

Acknowledgements—We are indebted to Dr. N. Watanabe, Mr. S. Kobayashi and Mr. C. Sakamaki for assistance.

REFERENCES

1. Hahn, H., deZacks, R. and Kende, H. (1974) *Naturwissenschaften* **61,** 170.
2. Letham, D. S. (1973) *Phytochemistry* **12,** 2445.
3. Koshimizu, K., Matsubara, S., Kusaki, T. and Mitsui, T. (1967) *Agric. Biol. Chem.* **31,** 795.
4. Watanabe, N., Yokota, T. and Takahashi, N. (1978) *Plant Cell Physiol.* **19,** 617.
5. Biddington, N. L. and Thomas, T. H. (1973) *Planta* **111,** 183.
6. Biddington, N. L. and Thomas, T. H. (1976) *J. Chromatogr.* **121,** 107.
7. Armstrong, D. J., Burrows, W. J., Evans, P. K. and Skoog, F. (1969) *Biochem. Biophys. Res. Commun.* **37,** 451.
8. Macleod, J. K., Summons, R. E. and Letham, D. S. (1976) *J. Org. Chem.* **41,** 3959.
9. Leonard, N. J., Carraway, K. L. and Helgeson, J. P. (1965) *J. Heterocycl. Chem.* 2, 291.
10. Letham, D. S., Shannon, J. S. and McDonald, I. R. C. (1967) *Tetrahedron* 23, 479.
11. Young, H. (1977) *Analyt. Chem.* 79, 226.
12. Morris, R. O. (1977) *Plant Physiol.* **59,** 1029.
13. Watanabe, N., Yokota, T. and Takahashi, N. (1978) *Agric. Biol. Chem.* **42,** 2415.
14. Mauk, L. S. and Langile, A. R. (1978) *Plant Physiol.* **62,** 438.
15. Kimura, K. Sugiyama, T. and Hashizume, T. (1978) *Nucleic Acid Research*, Special Publication No. 5, p. 339.
16. Yoshida, R. and Oritani, T. (1972) *Plant Cell Physiol.* **13,** 337.

17. Wang, T. L., Thompson, A. G. and Horgan, R. (1977) *Planta* **135,** 285.
18. Henson, I. E. and Wheeler, C. T. (1977) *J. Exp. Botany* **28,** 1076.
19. Van Staden J. and Dimalla, G. G. (1978) *J. Exp. Botany* **29,** 1077.
20. Van Staden, J. (1978) *Bot. Gaz.* **139,** 32.
21. Yokota, T. and Takahashi, N. (1980) *Phytochemistry* **19,** 2367.
22. Watanabe, N., Yokota, T. and Takahashi, N. (1978) *Plant Cell Physiol.* **19,** 1263.
23. Summons, R. E., Entsch, B. Letham, D. S., Gollnow and MacLeod (1980) *Planta* **147,** 422.