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DIHYDROZEATIN METABOLISM IN RADISH SEEDLINGS

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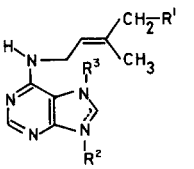
Abstract—(±)-[8-^{14}C]Dihydrozeatin was fed to derooted radish seedlings After three days the plants were harvested and the cytokinin metabolites purified by reversed and ordinary phase HPLC prior to identification by degradation, UV and MS The most abundant metabolite was shown to be dihydrozeatin-7-D-glucopyranoside, but, unexpectedly, significant quantities of dihydrozeatin-3-D-glucopyranoside and dihydrozeatin-9-D-glucopyranoside were also produced These results are discussed in relation to mechanisms which may control cytokinin levels and the enzymology of *N*-glucosylation

INTRODUCTION

When zeatin (Z) (**2**) or N^6-(Δ^2-isopentenyl) adenine (2iP) (**1**) are applied to plant tissues they are rapidly metabolised either to products of cytokinin oxidase catabolism (i e where the N^6 side chain has been removed) or to various conjugates [1–6] Though limited quantities of the biologically active 9-ribosides are produced by some tissues, the most abundant conjugation products of Z (**2**) or 2iP (**1**) are the 7-glucosides (**3**) (as a minor metabolite in corn seedlings [7], and as a major metabolite in radish tissues [8–10] and tobacco cell suspensions [3]), 9-glucosides (**4**) (in corn roots [7]) and 9-alanyl derivatives (**6**) (in lupin shoots and leaves [11]) All of these conjugates exhibit little activity in bioassay [12] They are also extremely stable (both when found as metabolites and when applied externally [8, 10, 11]) and are probably incapable of further metabolism For this reason they are regarded as detoxification or inactivation products [6, 13, 14] rather than storage forms, a role proposed for the *O*-glucosides (**5**) [4, 6, 10, 11, 14] Functionally therefore, oxidative side chain cleavage and the formation of *N*-glucoside and *N*-alanyl conjugates may be equivalent, in the sense that they appear to result in the irreversible destruction of cytokinin activity

In most tissues that have been examined there is a balance between conjugative and oxidative metabolism of externally applied cytokinins [1, 6] In radish tissues, however, there is negligible cytokinin oxidase-type metabolism and *N*-glucosides are the predominant metabolites For this reason radish has been used extensively in studies of cytokinin glucosylation

When Z (**2**) [8, 9] and 2iP (**1**) [6] were fed to derooted radish seedlings zeatin-7-D-glucopyranoside (**3**) (Z7G) and N^6-(Δ^2-isopentenyl) adenine-7-D-glucopyranoside (2iP7G) were the major metabolites On the other hand benzyladenine-7-D-glucopyranoside (BA7G), benzyladenine-9-D-glucopyranoside (BA9G) and benzyladenine-3-D-glucopyranoside (BA3G) were formed when benzyladenine (BA) was fed to radish seedlings [15] and cotyledons [10] These 7- and 3-substituted nucleosides are of great interest because they are extremely rare in nature, the only previously known compounds of this type are ribofuranosides 3-ribosyl uric acid from bovine blood [16] and compounds related to vitamin B_{12} [17]



	cytokinin	R^1	R^2	R^3
1	2iP	H	H	—
2	*trans*-Z	OH	H	—
3	Z7G	OH	—	β-D-glucosyl
4	Z9G	OH	β-D-glucosyl	—
5	ZOG	*O*-β-D-glucosyl	H	—
6	9A1Z	OH	alanyl	—

Fig 1 Various cytokinin structures

The enzymology of *N*-glucosylation has been studied in detail using a partially purified glucosyltransferase from radish cotyledons [18, 19] Only UDPG and TDPG could serve as glucose donors, but a wide variety of naturally occurring and synthetic cytokinins were able to act as acceptor molecules Of particular relevance to this paper BA, *trans*-Z (**2**), N^6-isopentyl adenine (DH2iP) and dihydrozeatin (**7**) (DHZ) were readily glucosylated, but whilst BA and *trans*-Z gave 7-/9-glucoside ratios of 1 65 and 10 5 respectively, DH2iP and DHZ (**7**) gave only traces of 9-glucoside (< 3% of the total glucoside formed) No 3-glucosides were produced by any of the substrates investigated To a certain extent these results parallel the previously discussed metabolic work with BA and Z (**2**) (where only the former gave 9-glucosides)

Though DHZ (**7**) is very active in cytokinin bioassays [12] it is not a substrate for cytokinin oxidase [4, 20] In the leaves of decapitated and disbudded *Phaseolus vul-*

garis plants DHZ (7) was mainly converted to dihydrozeatin-*O*-glucoside (DHZOG) [21], whilst in stem segments dihydrozeatin-9-ribotide (DHZMP) was the most abundant metabolite [22] These are the only systems in which DHZ (7) metabolism has been examined and it appears that conversion of DHZ (7) to the cytokinin oxidase labile Z (2) derivatives does not occur thereby limiting the control of its biological activity to conjugative processes

In this paper we report on the metabolism of DHZ (7) in derooted radish seedlings

RESULTS AND DISCUSSION

Since DHZ (7) gave only traces of dihydrozeatin-9-glucoside (9) (DHZ9G) in the cell free system [19] it was expected that DHZ (7) would be metabolised much like Z (2) when fed to derooted radish seedlings (i e give only the 7-glucoside product)

The metabolites were initially purified by reversed phase HPLC (Fig 2) following the feeds with (±)-[8-^{14}C]DHZ The bulk of the radioactivity (60% of the extractable activity) eluted in fractions 9, 10 and 11 These fractions were re-chromatographed using normal phase HPLC In this way, fraction 9 was shown to contain two compounds The minor component (peak 1), which contained 8% of the extractable radioactivity, eluted first in the normal phase system, gave a UV spectrum (λ_{max} 292 nm) [23] characteristic of a 3-substituted purine and was shown to contain a glucose moiety The mass spectrum of the per TMS derivative of peak 1 (Table 1) is consistent with the dihydrozeatin-3-D-glucopyranoside (10) (DHZ3G) structure proposed for this compound

The second compound in fraction 9 (peak 2) and the single compound in fraction 10 (peak 3), which, respectively, contained 31% and 26% of the extractable radioactivity, both gave typical 7-substituted UV spectra (λ_{max} 275 5 nm) [23] and were shown to be glucosides Their mass spectra (both direct probe and GC/MS) were identical (Table 1) and we conclude that they are the (+) and (−) enantiomers of dihydrozeatin-7-D-glucopyranoside (8) (DHZ7G). The separation of these enantiomers on reversed phase HPLC has been observed before [19]

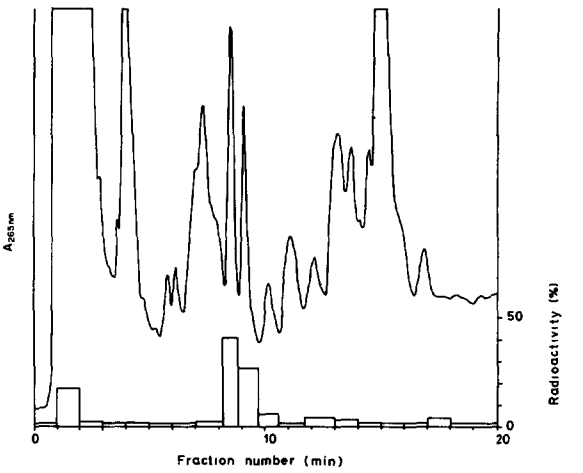


Fig 2 Reversed phase HPLC UV trace and bar diagram showing the proportion of total extracted radioactivity in each fraction Approximately 80% of the applied radioactivity was recovered at this stage

Fraction 11 (Fig 2) contained 5% of the extractable radioactivity and gave a single compound (peak 4) that was also a glucoside The UV spectrum (λ_{max} 266 1) [23] and the mass spectrum (Table 1) are consistent with this compound being dihydrozeatin-9-D-glucopyranoside (9) (DHZ9G)

Previous work [24] on the GC/MS of TMS derivatives of the *N*-glucosides of BA failed to differentiate between the 3-β-and 9-β-D-glucopyranosides An intermolecular migration of the sugar moiety from N-3 to N-9 was proposed In contrast to this we were able to obtain readily distinguishable mass spectra (both on direct probe

Table 1 GC/MS data for the TMS derivatives of peaks 1–5

Peak	Compound(s)	*m/z* (rel intensity)
1	DHZ3G (10)	743 (3 9), 728 (3 4), 654 (0 4), 640 (0 5), 612 (0 5), 599 (0 5), 450 (19 6), 361 (10 4), 360 (13 8), 271 (11 3), 218 (15 5), 217 (52 1), 205 (3 6), 204 (10 8), 162 (7 2), 148 (10 0), 147 (32 5), 129 (14 6), 103 (17 4), 77 (10 0), 75 (35 5), 74 (12 7), 73 (100)
2 and 3	(−)-DHZ7G and (+)-DHZ7G (8)	743 (13 9), 728 (11 9), 654 (6 5), 640 (4 2), 612 (3 7), 599 (12 4), 450 (7 8), 436 (9 6), 361 (10 2), 360 (1 9), 294 (10 5), 292 (10 0), 218 (17 3), 217 (56 1), 205 (8 8), 204 (22 8), 162 (12 8), 148 (17 5), 147 (61 9), 133 (14 1), 129 (30 4), 117 (14 4), 103 (36 1), 101 (10 9), 77 (15 5), 75 (71 3), 74 (30 7), 73 (100)
4	DHZ9G (9)	743 (7 3), 728 (8 5), 654 (1 0), 640 (1 6), 612 (1 5), 599 (0 9), 451 (17 8), 450 (43 8), 361 (24 9), 360 (40 6), 322 (12 7), 292 (10 0), 271 (18 0), 218 (20 9), 217 (69 1), 205 (6 8), 204 (19 1), 162 (13 3), 148 (14 8), 147 (44 5), 129 (20 0), 117 (13 4), 103 (28 0), 75 (37 0), 74 (20 8), 73 (100)
5	DHZ (7)	365 (17 9), 350 (17 6), 262 (10 2), 236 (22 6), 235 (26 7), 234 (76 5), 222 (16 7), 221 (24 0), 220 (48 2), 209 (12 7), 207 (46 4), 75 (29 9), 74 (11 5), 73 (100)

Only major ions and those discussed in the text are included Direct probe spectra for peaks 1–4 were almost identical

and GC/MS) for the TMS derivatives of all three DHZ glucosides (**8–10**) (Table 1), all of which gave clear molecular ions DHZ9G (**9**) and DHZ3G (**10**) gave the most similar spectra, but in both probe and GC/MS (where they separated readily) the $[M]^+/[M-15]^+$ ion abundance ratios were quite different DHZ9G (**9**) gave a more intense $[M-15]^+$ ion whilst the $[M]^+$ was the most intense in DHZ3G (**10**) and DHZ7G (**8**) Interestingly these ratios parallel those of the TMS derivatives of the 7- and 9-glucopyranosides of BA and Z [24] As with the TMS derivatives of BA9G, Z9G (**4**) and adenine-9-D-glucopyranoside [24], DHZ9G (**9**) gave intense ions at *m/z* 450 [TMS glucose – H], 361 [TMS glucose – TMS-OH] and 360 [TMS glucose – H-TMS-OH] underlining the ease with which the cleavage of the glycosidic bond (with retention of charge on the sugar) can occur in 9-glucopyranosides 9-Glucofuranosides do not appear to undergo this cleavage to any appreciable extent [24] DHZ3G (**10**) also gives moderately intense ions at these *m/z* values, but in the DHZ7G (**8**) spectrum (as with BA7- and Z7-glucopyranosides) they are much less significant In all the spectra of the DHZ glucoside metabolites, however, the ions at *m/z* 204 were more intense than those at *m/z* 205 This is characteristic of glucopyranosides (in glucofuranosides the order is reversed [24])

The ions at *m/z* 612 and 162 are present in all the DHZ glucoside spectra (although less intense than the corresponding ions at *m/z* 610 and 160 for the TMS derivatives of the Z glucosides [24]) and represent the so-called cyclisation ion derived from intramolecular alkylation of the N^1 by the side chain Similarly the ions derived from loss of TMS-O (*m/z* 654) and the TMS-OCH_2· radical (*m/z* 640) from the side chain of the DHZ glucosides are small in comparison to those of the Z glucosides The only exception to this is the intense ion at *m/z* 599 in the DHZ7G (**8**) spectra which is probably derived from cleavage of the entire N^6 side chain This ion was insignificant in the spectra of the TMS derivatives of DHZ3G (**10**) and DHZ9G (**9**)

Fractions 13 and 14 from the reversed phase HPLC (Fig 2) were shown to contain a single compound and represented 6% of the total extractable radioactivity The compound (peak 5) was identified by co-chromatography with authentic material, UV and MS (Table 1) as unmetabolised DHZ (**7**)

The compounds at fractions 2 and 18 (which contained respectively 18% and 4% of the extractable radioactivity) were not identified It was thought that the early running material may be nucleotide or products of purine catabolism (i e adenine, adenosine, AMP, adenine-7-glucoside etc), but it was not possible to obtain a conclusive identification

The production of 3-, 7- and 9-glucosides of DHZ (**8–10**) was unexpected, both because of its close structural similarity to Z (**2**) which only gives Z7G (**3**) [8, 9] and because of the results obtained with the partially purified radish glucosyltransferase preparation [19] In view of the ability of the glucosylating enzyme's ability to discriminate between the presence and absence of a double bond in the side chain it was also surprising that approximately equal quantities of (+)- and (−)-DHZ7G were formed *S*-(−)-DHZ (**7**) is the naturally occurring enantiomer in *Lupinus luteus* [25–27] DHZ7G (**8**), which has now been identified as a minor cytokinin in tobacco crown gall tissue [Dr I M Scott, personal communication], co-chromatographs with the first running

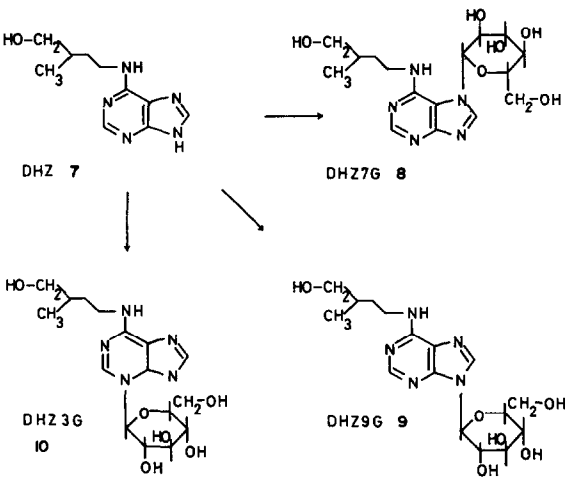


Fig 3 Metabolic products of dihydrozeatin (**7**) in derooted radish seedlings

DHZ7G fraction (peak 2) indicating that this is the *laevo* antipode

The synthesis of the biologically inactive 7- and 9-glucosides in response to external cytokinin application may be a mechanism designed to control the levels of cytokinin activity On the other hand the 3-glucosides are very active in bioassay [12, 15] The biologically active *O*-glucosides (**5**) are also produced by many plant tissues in response to external application of cytokinins [1, 6] The latter are readily converted back to Z (**2**) by the action of almond β-glucosidase (emulsin) and it is thought that this may account for their biological activity [4, 6, 14] Similarly the 3-glucosides are hydrolysed by almond β-glucosidase [15] and may not be active cytokinins *per se*

The 3-glucosides have not been isolated as endogenous cytokinins from any source The only previous report of a 3-glucoside metabolite is BA3G [15] DHZ3G (**10**) is therefore the first example of a naturally occurring cytokinin conjugated in this manner

EXPERIMENTAL

Plant tissue Raphanus sativus L cv Yates Long Scarlet, Market strain, were obtained from Arthur Yates Co Ltd, Auckland, New Zealand Ten days after sowing the seedlings were uprooted and the roots surgically removed

Chemicals (±)-[8-^{14}C]DHZ (11 7 mCi/mmol) was synthesized by condensing 6-chloro[8-^{14}C]purine (Radiochemical Centre, Amersham, U K) with 4-hydroxy-3-methylbutylamine [28] The DHZ was purified by HPLC [29] prior to use

Derivatisation TMS derivatives were prepared by heating the dry (P_2O_5) sample in a sealed capillary tube with *N,O*-bis(trimethylsilyl)-acetamide (5 μl) and dry C_5H_5N (5 μl) at 70° for 1 hr

GC/MS 10 m × 0 3 mm i d BP1 capillary column (film thickness = 0 5 μ), He 8psi, temp programme 35–280° (ballistic) and then to 300° at 8°/min, ionizing voltage 70 eV, source, separator and inlet temperatures 190°, 250° and 240°, respectively, scan speed 1 sec/decade

Probe spectra. direct insertion, 70 eV, source temp 190°, scan speed 3 sec/decade The probe temp was taken from 35° to 350° at 30°/min

Identification of glucosides The presence of the glucose moiety in peaks 1, 2, 3 and 4 was confirmed by hydrolysis of these compounds by using a cation exchange resin The resulting glucose was detected by glucose oxidase following TLC of the hydrolysate [15]

Feeding experiments DHZ (120 nM) was fed to each of two derooted radish seedlings which had been placed in glass vials containing H_2O (1 ml) When this had been taken up (2–3 days) the seedlings were harvested and extracted immediately

Extraction and purification of the metabolites The seedlings were macerated in MeOH (100 ml) and the liquor filtered (paper) before being evapd at red pres The residue was re-dissolved in H_2O (10 ml) and placed on a SEP-PAK C_{18} cartridge (Waters Associates, Northwich, U K) which was washed with H_2O (20 ml) then MeOH (20 ml) The combined washings were evapd to dryness, re-dissolved in starting solvent and applied to an analytical HPLC column (150 mm × 4 5 mm i d) of Apex ODS reversed phase material The sample was eluted at 2 ml/min with a linear gradient system of 5–10% MeCN with H_2O at pH 7 0 (triethylammonium bicarbonate) over a 20 min period [29] Fractions were collected as indicated in Fig 2 and 0 05 ml of these were counted in a liquid scintillation spectrometer The active regions were analysed separately

Fraction 2 (18% of extractable radioactivity) was rechromatographed on a 0–10% (20 min) linear gradient and most of the activity eluted in fraction 12 min UV λ_{max}, MeOH was 270 1 nm and shifted to 274 0 nm when a drop of HOAc was added When treated with calf intestinal alkaline phosphatase no alteration in HPLC retention time was observed GC/MS was not helpful in assigning a structure(s) to this compound(s) because of large amounts of non-UV absorbing impurities that co-chromatographed on reversed phase HPLC The obviously very polar compound(s) was not elutable from normal phase HPLC material

Fraction 9 (39% of the extractable radioactivity) was rechromatographed on a Partisil PAC normal phase HPLC column (250 mm × 4 5 mm i d) using MeCN–H_2O (9 1), pH 7 0, at a flow rate of 2 ml/min The first eluting compound (8% of the extractable radioactivity) in fractions 15–16 min (peak 1) gave a UV λ_{max}, MeOH of 292 0 nm and was a glucosyl conjugate Peak 1 was converted to its TMS derivative and subjected to GC/MS and direct probe MS (Table 1) which were consistent with the proposed DHZ-3-D-glucopyranoside structure

The second eluting peak from fraction 9 (peak 2) eluted in fractions 17–18 min on the normal phase system and gave a UV λ_{max}, MeOH of 275 5 nm and was also a glucosyl conjugate GC/MS and direct probe MS (Table 1) were performed and peak 2 was identified as DHZ-7-D-glucopyranoside

Fraction 10 (26% of the extractable radioactivity) from the reversed phase HPLC was also re-run on the normal phase system and gave a single peak eluting in fractions 17–18 This compound (peak 3) was identical in all aspects to peak 2 except in its retention time on Apex ODS Since naturally occurring DHZ7G co-chromatographs with peak 2 [Dr I M Scott, personal communication] and since naturally occurring DHZ is the *S*-(−)-enantiomer [25–27] peak 2 was assigned (−)-DHZ-7-D-glucopyranoside and peak 3 was assigned (+)-DHZ-7-D-glucopyranoside

Fraction 11 (5% of the extractable radioactivity) when rechromatographed on normal phase HPLC gave a single compound (peak 4) which eluted in fractions 12 and 13 This compound was a glucoside and gave a UV λ_{max}, MeOH of 266 1 nm The GC/MS and probe MS (Table 1) were consistent with peak 4 being DHZ-9-D-glucopyranoside

When fractions 13 and 14 (6% of the extractable radioactivity) from the reversed phase HPLC were re-run on normal phase (using MeCN–H_2O (22 3), pH 7 0), the only radioactive peak (peak 5) eluted in fractions 5–6 min and had a UV λ_{max}, MeOH of 267 1 nm Peak 5 co-chromatographed (in both systems) with authentic DHZ and the TMS derivative gave a GC/MS identical with authentic DHZ standard (Table 1)

The minor peak at fraction 18 (4% of the extractable radioactivity) was not identified, but did not co-chromatograph with DHZ-*O*-glucoside or DHZ-9-riboside

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