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Identification of a Benzyladenine Disaccharide Conjugate Produced during Shoot Organogenesis in *Petunia* Leaf Explants[1]

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Prior studies of benzyladenine (BA) metabolism in *Petunia hybrida* Vilm. leaf explants during shoot organogenesis revealed the presence of an abundant unidentified BA conjugate. The BA conjugate, compound C, made up to 39% of the total pool of BA conjugates in two *Petunia* lines and was associated with an increased shoot organogenic response when compared with a third *Petunia* line that did not produce any compound C. Structural analysis of compound C utilizing fast atom bombardment mass spectrometry, two methods of carbohydrate analysis, ultraviolet absorption spectra, and Fourier transform infrared spectra identified it as a new cytokinin conjugate, 6-benzylamino-9-[*O*-glucopyranosyl-(1→3)-ribofuranosyl]-purine. Based on our prior biological studies and the similarity of this compound to related cytokinin conjugates, this disaccharide cytokinin conjugate may be part of the interconvertible pool of cytokinins active in *Petunia* shoot organogenesis.

In our previous research, leaf explants from three *Petunia hybrida* Vilm. lines were exposed to BA in tissue culture media, and patterns of BA uptake and metabolism during shoot organogenesis were compared with the developmental commitment to shoot production (Auer et al., 1992a, 1992b). BA was shown to be rapidly converted to a variety of conjugates, including BAR, benzyladenosine-5′-monophosphate, benzyladenosine-5′-diphosphate, benzyladenosine-5′-triphosphate, BA-9G, BA-7G, and three unidentified compounds. One unidentified metabolite, designated compound C, was found to be a major component of the total pool of BA metabolites. Using mild acid hydrolysis, we determined that this compound was a BA conjugate.

In two *Petunia* lines, MD1 and St40, the total pool of BA conjugates within the leaf explants during organogenesis contained up to 25 and 39% compound C, respectively. The presence of the conjugate was associated in lines MD1 and St40 with an early commitment to shoot organogenesis (between 6 and 10 d of exposure to BA) and production of shoots on 100% of the explants. In contrast, compound C was never produced in *Petunia* line TLV1 during shoot organogenesis, and this was correlated with a delayed commitment to shoot organogenesis (between 12 and 24 d) and production of shoots on only 75% of the explants. Thus, the production of compound C was associated with increased shoot organogenesis in *Petunia* leaf explants.

In this paper, we report the subsequent structural analysis of compound C. Compound C has now been shown to be a disaccharide conjugate of BA that has not been previously described. On the basis of FAB MS, carbohydrate analysis, UV spectroscopy, and FTIR spectroscopy, compound C has been identified as BA-9RG.

MATERIALS AND METHODS

Extraction of BA Conjugate

To provide samples for structural analysis, the BA conjugate C was extracted from *Petunia* leaf explants. Surface-sterilized leaf explants of *Petunia* line MD1 were placed in tissue culture on 20 mL of Murashige-Skoog media containing 22 μM BA (Auer et al., 1992b). To produce some conjugate with a radioactive label, five Petri plates were prepared containing 20 mL of Murashige-Skoog medium with 4.4 μM BA and approximately 315,000 dpm of [^{3}H]BA (labeled on the benzyl ring, specific activity 740 MBq μmol^{-1}; CEA, Gif-sur-Yvette, France). Ten leaf explants were positioned in each plate. After 10 d, leaf explants were removed from the media, rinsed gently in H_2O, and frozen in liquid N_2. Frozen explants were ground in 150 mL of methanol in a Sorvall[2] grinder. The sample was centrifuged, the supernatant was collected, and the pellet was washed with 80% methanol. Combined supernatants were reduced to aqueous phase in vacuo.

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Abbreviations: BA-3G, benzyladenine-3-β-D-glucopyranoside; BA-7G, benzyladenine-7-β-D-glucopyranoside; BA-9G, benzyladenine-9-β-D-glucopyranoside; BA-9RG, 6-benzylamino-9-[*O*-glucopyranosyl-(1→3)-ribofuranosyl]-purine; BAR, benzyladenosine; FAB, fast atom bombardment; FTIR, Fourier transform infrared spectroscopy; m/z, mass-to-charge ratio; Rt, retention time.

The sample was loaded onto a DEAE-Sephadex (acetate form) column in a 10-mL plastic syringe body on top of two Baker SepPak C_{18} columns so that the three columns were in series. After the C_{18} columns were rinsed with H_2O, they were removed, and the sample was eluted from the C_{18} with 15 mL of methanol. The sample was evaporated in vacuo, resuspended in 10% methanol, and injected onto a 15-cm LiChrospher 60 RP-Select B reverse-phase column (E. Merck). A linear solvent gradient that went from solvent A (0.01 M triethylamine in 10% methanol, pH 5.4) to solvent B (100% methanol) during 27 min was used with a flow rate of 0.8 mL min^{-1}. Authentic standards of BA, BA-7G, BA-9G, and BAR (Apex Organics, Leicester, UK) were used to establish Rts. Radioactive fractions corresponding to the Rt of compound C were collected, evaporated, and resuspended in methanol. The sample was spotted on a Silica Gel 60, Kieselguhr F254 TLC plate (E. Merck) along with BA-7G and BA-9G standards. The TLC solvent was *n*-butanol:NH_2OH:H_2O, 6:1:2. After 6 h, a fluorescent band containing compound C was removed from the TLC plate, and the silica gel was washed with methanol to elute the compound. The sample was evaporated, resuspended in 10% methanol, and injected on a 15-cm Novapak C_{18} 5-μm HPLC column (Waters). The solvents were the same as described above for the reverse-phase LiChrospher HPLC column, using a linear gradient that went from 100% solvent A to 100% solvent B during 37 min at a flow rate of 0.8 mL min^{-1}. Radioactive fractions corresponding to the Rt of compound C were collected and evaporated in vacuo. The samples were resuspended in 10% methanol and again chromatographed on the reverse-phase LiChrospher HPLC column using solvent without triethylamine and the gradient program described above. Fractions corresponding to the Rt of compound C were collected for structural analysis.

Monosaccharide Composition Analysis

The monosaccharides were identified using hydrolysis followed by production of alditol acetate derivatives (York et al., 1985). Compound C and 10 μg of inositol were dried under N_2, 0.5 mL of 2 M TFA was added, and hydrolysis was continued at 121°C for 2 h. The acid was removed under N_2, and 0.25 mL 1 M ammonium hydroxide containing 10 mg mL^{-1} of sodium borodeuteride was added. After 1 h at room temperature, several drops of glacial acetic acid were added, followed by 0.5 mL of 9:1 methanol:acetic acid mixture. This was evaporated under N_2, resuspended in methanol:acetic acid, and dried three times. The sample was taken up in 0.1 mL of acetic anhydride and 0.1 mL of pyridine and kept at 121°C for 20 min. The sample was reduced to dryness under N_2 dissolved in 0.5 mL of chloroform, and 0.5 mL of H_2O was added. After centrifugation, the chloroform phase was removed, placed in a clean vial, and evaporated with N_2. The sample was taken up in 50 μL of acetone for GLC. The GLC regimen was initially 80°C, increased 30°C min^{-1} to 235°C, and held at this temperature for 35 min. Monosaccharide peaks were identified by Rt; authentic standards of rhamnose, Fuc, Rib, Ara, Xyl, Man, Gal, Glc,and inositol were run under identical conditions.

Glucosyl Linkage Analysis

The linkage of the monosaccharides to each other was determined using partial methylation of alditol acetates and a modified Hakomori procedure (York et al., 1985). A sample of compound C was dried under N_2 and dissolved in 0.5 mL of DMSO under N_2. One-half milliliter of 2 M DMSO anion was added, and the solution was stirred overnight. The mixture was cooled in an ice bath, and 1 mL of iodomethane was slowly added while N_2 was blown over the reaction mixture. The sample was allowed to stir at room temperature for 2 h, after which 0.5 mL of H_2O was added. N_2 was bubbled through the mixture until one layer was obtained, i.e. until the excess methyl iodide was removed. The methylated sample was loaded onto a SepPak C_{18} cartridge and eluted with 3 mL of acetonitrile, which was dried with N_2. Hydrolysis and acetylation were accomplished as described above for monosaccharide composition analysis. The final sample was dissolved in 25 μL of acetone, and 1 μL was introduced into the GLC-MS for analysis. The initial oven temperature was 80°C; the temperature was increased by 30°C min^{-1} until it reached 170°C, increased by 4°C min^{-1} until it reached 240°C, and then held at this temperature for 10 min. The alditol acetates were identified by comparing their Rts with those of authentic standards and by their fragmentation pattern on MS analysis.

FAB MS

The compound C sample was inserted into a VG Instruments ZAB2F mass spectrometer equipped with a cesium gun using a glycerol matrix and analyzed by FAB MS. Fomblin was used for mass calibration.

UV and FTIR Spectroscopy

To provide evidence for the position of linkage between BA and the ribosyl group, both UV absorption and FTIR spectroscopy were conducted. For UV absorbance spectroscopy, compound C and authentic standards of BA-7G, BA-3G, BA-9G, and BAR were analyzed in a Hewlett-Packard 8452A diode array spectrophotometer in aqueous solutions at pH 2, 7, and 11 (Leonard et al., 1965).

FTIR spectra of the same compounds were collected using a Nicolet 60SX spectrometer with a MCT-A detector recording 100 scans per sample. Compound C was applied in methanol to potassium bromide in a 3-mm pellet and used for FTIR spectral analysis.

RESULTS

In previous research we showed that an abundant unidentified cytokinin conjugate extracted from *Petunia* leaf explants was a conjugate of BA (Auer et al., 1992b). Using FAB MS, carbohydrate analysis, UV absorption, and FTIR spectroscopy, we identified the BA conjugate as BA-9RG (Fig. 1).

A sample of the purified BA conjugate was analyzed by FAB MS, which provided a clear $(M + H)^+$ peak of m/z 520 (Fig. 2) and a mol wt for the compound of 519. This mol wt is equivalent to that of BA (mol wt 225) plus one pentosyl (mol wt 116) and one hexosyl group (mol wt 179). After this

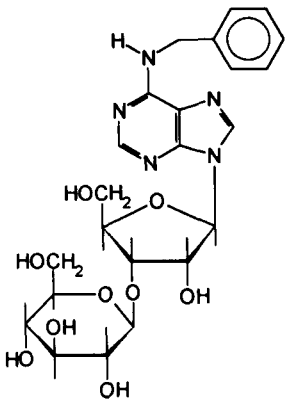

Figure 1. Proposed structure of BA disaccharide conjugate BA-9RG extracted from *Petunia* leaf explants during shoot organogenesis.

Table I. *Major ions of partially methylated alditol acetate sugar residues obtained by GLC-MS of BA conjugate compound C*

Diagnostic ion fragments are shown, with the percentage of abundance for each fragment in parentheses. Diagnostic ions were compared with standards to determine carbohydrate linkages.

Compound	Diagnostic Ion Fragments
3-linked pentose	233 (10), 202 (5), 173 (4), 160 (7), 129 (12), 118 (100), 113 (15)
1-linked hexose	205 (29), 173 (2), 162 (41), 161 (36), 145 (66), 129 (71), 118 (61), 102 (100)

finding, two methods of carbohydrate analysis were carried out to identify the monosaccharides and to determine the linkage between monosaccharide conjugates.

Monosaccharide composition analysis was performed and the GLC trace showed two peaks with Rts of 5.77 and 8.99 min. Authentic standards of rhamnose, Fuc, Rib, Ara, Xyl, Man, Gal, Glc, and inositol were run on GLC; an authentic standard of Rib had an Rt of 5.76 min, and Glc had an Rt of 9.01. Therefore, it was determined that two monosaccharides, Rib and Glc, were present in the BA conjugate.

To determine the intersugar glycosidic linkage of the two monosaccharides, a partial methylation of alditol acetate residues was performed. In GLC-MS analysis, two large peaks were identified with Rts of 16.35 and 16.63 min, consistent with the Rt for a 3-linked Rib sugar and a terminal (1-linked) Glc sugar, respectively (York et al., 1985). Ion fragments from GLC-MS were also consistent for a 3-linked pentose and a terminal 1-linked hexose (Table I). Therefore, using monosaccharide analysis and linkage analysis, we believe that a pentose sugar, Rib, is linked at the 3 position to a hexose sugar, Glc, at the terminal, or 1, position.

Because of previous structural studies of cytokinin conjugates, it is probable that the Rib has a β-linkage to BA (Letham and Palni, 1983). However, our results cannot establish the anomeric configuration (α or β) in the conjugate. Evidence for the D or L forms of the sugars also awaits further characterization.

The question remained as to where the Rib was attached to BA. Two spectroscopic methods, UV absorption and FTIR, were used to provide evidence regarding which N within the adenine structure was linked to the Rib. The presence of BAR (with an N^9-linked ribosyl group) in *Petunia* leaf explant tissue suggested a linkage at the N^9 position. UV absorption has been recognized as a valuable tool in determining substitution positions on adenine and cytokinin conjugates (Leonard et al., 1965; Horgan and Scott, 1987). In Table II, the UV absorption maxima are shown for compound C and relevant standards (BAR, BA-9G, BA-7G, and BA-3G) at pH 2, 7, and 11. The absorption maximum of 266 nm exhibited by C is consistent with the maxima of N^9-substituted BA

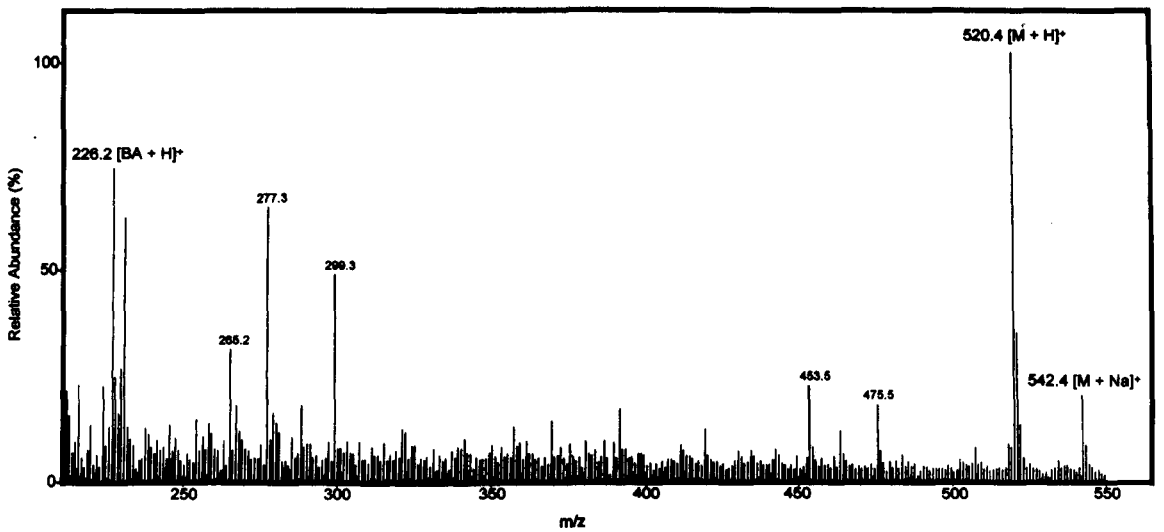

Figure 2. The mol wt of the BA conjugate was determined by FAB MS. The peak at m/z 520 is $(M+H)^+$ and indicates that the mol wt of the conjugate is 519 atomic mass units. The peak at m/z 226 is equivalent to that for a compound with the mol wt of $(BA + H)^+$.

Table II. *UV absorption maxima (λmax) of N-substituted BA conjugates at pH 2, 7, and 11*

	λmax		
	pH 2	pH 7	pH 11
		nm	
Compound C	266	266	266
BAR	264	268	266
BA-9G	266	270	270
BA-3G	290	294	296
BA-7G	284	278	278

standards such as BAR and BA-9G and is considerably different from the maximum exhibited by BA-7G or BA-3G. Comparison with UV spectra available for dibenzyl-substituted adenines and *N*-methyl zeatin compounds further strengthens the argument that the disaccharide is attached at the N^9 position (Leonard et al., 1965; Horgan and Scott, 1987).

FTIR spectroscopy of compound C and BA conjugate standards provided further evidence for an N^9 linkage. In an FTIR spectrum, compound C produced peaks of absorbance within the region of primary interest at 1630, 1410, 1262, and 1120 cm^{-1}. This spectrum showed closer similarity to spectra from BAR and BA-9G than to those of BA-7G or BA-3G. The FTIR spectrum of BAR produced peaks at 1628, 1260, and 1124 cm^{-1}, that of BA-9G produced peaks at 1630, 1408, and 1124 cm^{-1}, that of BA-3G peaks at 1630 and 1414 cm^{-1}, and that of BA-7G a peak at 1119 cm^{-1}. Until BA-9RG is chemically synthesized in the future, FTIR spectroscopy cannot by itself be considered to provide unambiguous proof of an N^9 linkage. Taken together, however, the evidence obtained suggests that the structure of compound C is BA-9RG (Fig. 1).

DISCUSSION

The conjugation and metabolism of the synthetic cytokinin BA has been investigated since the 1970s and the compounds previously identified include purine ring substitutions (N^9-riboside, N^9-riboside 5′-phosphates, N-glucosides, N^9-Ala) and products of isoprenoid side chain cleavage (adenine, adenosine, adenosine 5′-phosphates) (Laloue et al., 1981; Laloue and Pethe, 1982; Letham et al., 1982; Letham and Palni, 1983; McGaw et al., 1984). Subsequently, BA, BAR, BA-9G, and BA ribotides have been discovered as naturally occurring cytokinins (Nandi et al., 1989). In addition, N^6-(*o*-hydroxybenzyl)adenine and N^6-(*m*-hydroxybenzyl)adenine and their N^9-ribosides have been described (Strnad et al., 1992).

While studying the uptake and metabolism of BA in *Petunia* leaf explants during shoot organogenesis, we recovered an abundant, previously unidentified BA conjugate (Auer et al., 1992a, 1992b). In this paper we present evidence that the structure of the unidentified BA conjugate is BA-9RG (Fig. 1). Although BA metabolism has been studied in similar in vitro plant systems, such as tobacco cell-suspension cultures and tobacco thin-cell-layer pedicel explants (Laloue et al., 1981; Laloue and Pethe, 1982; Van der Krieken et al., 1990), BA-9RG has not been previously described, nor has any other disaccharide BA conjugate been reported.

A disaccharide zeatin conjugate has been extracted from the shoots of two conifer species. Taylor et al. (1984) examined the structure of biologically active cytokinins from dormant *Pinus* shoots. By interpreting the mass spectrum of the permethylated conjugate, they suggested a glycoside of zeatin riboside in which the hexosyl moiety was attached to the ribosyl group, although the position of attachment was unknown (Taylor et al., 1984); this compound was active in a soybean hypocotyl bioassay. In buds of *Pseudotsuga*, Morris et al. (1990) identified a hexose conjugate of zeatin riboside using β-glucosidase treatment, anti-*trans*-zeatin riboside antibody cross-reactivity, and GC-MS analysis of the permethylated compound (Morris et al., 1990). In neither of these studies was the hexose identified, the linkage position between the ribosyl and hexose groups established, or the linkage position between the disaccharide and the cytokinin elucidated.

In our studies using three *Petunia* lines, BA metabolism was compared during in vitro shoot organogenesis. In two *Petunia* lines, MD1 and St40, BA-9RG represented up to 25 and 39%, respectively, of the total pool of BA conjugates within the leaf explants during organogenesis. In contrast, no BA-9RG was detected in *Petunia* line TLV1. The presence of BA-9RG was associated in lines MD1 and St40 with an early commitment to shoot organogenesis (between 6 and 10 d of exposure to BA) and production of shoots by 100% of the leaf explants. In *Petunia* line TLV1, no BA-9RG was detected during 24 d of exposure to exogenous BA, and this was correlated with a delayed commitment to shoot organogenesis (between 12 and 24 d) and ultimate production of shoots by only 75% of the leaf explants. The percentage of free BA and BA ribotides decreased in each *Petunia* line through time; no differences in free BA were observed with regard to the production of BA-9RG. The BAR concentration stayed constant in St40 and TLV1 but decreased in MD1.

The metabolic interconversions of cytokinin free bases, ribosides, and ribotides have been well established (Chen, 1981; Laloue et al., 1981; Laloue and Pethe, 1982; Letham and Palni, 1983; Koshimizu and Iwamura, 1986). These interconversions are probably the most fundamental mechanism for controlling the level of active cytokinin within the cells (Chen, 1981; Koshimizu and Iwamura, 1986). Therefore, it is important to know whether BA-9RG is part of the interconvertible pool of BA conjugates, because the production of BA-9RG may give *Petunia* leaf explants a larger pool for interconversion to free BA during shoot organogenesis. After 10 d of exposure to BA, *Petunia* leaf explants from line St40 had 32% BA-9G and 39% BA-9RG in the total pool of BA conjugates, whereas explants from line MD1 had 40% BA-9G and 25% BA-9RG. In the *Petunia* line TLV1, which did not produce BA-9RG, the conjugate that dramatically increased was BA-9G, making up 86% of the total BA pool on d 10 (Auer et al., 1992a, 1992b). At each time in our study, the concentration of BA-9G in TLV1 was approximately twice as high as that of BA-9G in St40. BA N-glucosides are believed to be inactivation products, and they are extremely stable in plant tissues, either as metabolites or

as exogenously fed compounds (Letham et al., 1982; McGaw et al., 1984). Therefore, in *Petunia* lines St40 and MD1, the production of BA-9RG may be increasing the pool of interconvertible cytokinins and decreasing the production of BA-9G, an inactivated cytokinin N-glucoside conjugate.

In conclusion, we have described a new conjugate of BA, BA-9RG, produced in *Petunia* leaf explants during shoot organogenesis. Two *Petunia* lines that manufactured BA-9RG also exhibited increased shoot organogenic activity when compared to leaf explants from a *Petunia* line that did not produce BA-9RG. Therefore, BA-9RG may be important in the metabolic regulation of cytokinins leading to shoot organogenesis in culture.

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