Pergamon

PII: S0031-9422(97)00373-7

Phytochemistry, Vol. 46, No. 5, pp. 801–804, 1997

Printed in Great Britain
0031-9422/97 $17.00+0.00

SYNTHESIS OF CYTOKININ GLUCURONIDES FOR THE SELECTION OF TRANSGENIC PLANT CELLS

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(*Received in revised form* 24 *March* 1997)

Key Word Index—benzyladenine; cytokinin glucuronides; isopentenyladenine; selection; synthesis; genetic transformation.

Abstract—Glucuronide derivatives of cytokinins have been synthesized for use as agents for the selection of plant cells transformed with a β-glucuronidase (GUS) gene. In this selection system, the GUS gene functions as both a selectable, as well as a screenable gene. GUS liberates active cytokinin from inactive cytokinin glucuronides which then stimulates growth and regeneration of the transformed cells. The frequently used cytokinin N^6-benzyladenine was conjugated to glucuronic acid at N-3 or at N-9 but only the former was a substrate for GUS. The glucuronide of isopentenyladenine was also made, by coupling at the N-3. This compound was readily hydrolysed by GUS. © 1997 Elsevier Science Ltd

INTRODUCTION

Production of transgenic plants requires a selection method which enables the transformed cells to grow and develop on selective media while the fraction of cells which have remained untransformed are arrested in development. The most frequently employed selection methods are based on resistance to antibiotics like kanamycin [1] or, to some extent, herbicides [2].

Antibiotic resistance genes are only useful during selection and after transgenic shoots have been identified such genes are redundant and often undesirable. Another feature of these selection methods is that the non-transformed cells are killed by the selective agent leaving the transgenic cells encapsulated in necrotic tissue which may release toxic substances and also prevent uptake of nutrients from the medium [2].

To overcome these drawbacks which may reduce the number of emerging transformed shoots significantly, a novel selection principle was recently presented in which the non-transformed cells are starved but not killed while the growth and regeneration of transgenic cells are stimulated [3]. This selection method exploits the fact that most plant tissues cultured *in vitro* require exogenous cytokinin for optimal growth. By adding cytokinin as an inactive glucuronide, active cytokinin is released only in cells which have been transformed with a β-glucuronidase (GUS) gene which is very frequently used as a screenable gene in transformation studies [4]. In this paper we describe the chemical synthesis of cytokinin glucuronides useful for the selection of transgenic plant cells in the absence of antibiotic or herbicide resistance genes.

RESULTS AND DISCUSSION

In this selection system, the GUS gene serves the dual purpose of being both selectable and screenable gene. Therefore, no other auxiliary genes like antibiotic resistance genes are required, facilitating construction of transgene cassettes and presumably also approvals by environmental authorities.

However, cytokinin glucuronides which must be hydrolysable by the *Escherichia coli* GUS are required to accomplish selection according to this principle. Moreover, the liberated cytokinin must be able to sustain proliferative growth of the particular plant species in trial. Therefore, we have synthesized glucuronide derivatives of two of the most widely used cytokinins in plant tissue culture, namely benzyladenine and isopentenyladenine.

Benzyladenine N3-glucuronide (BA3GN, Fig. 1) was synthesized *via* the condensation of N^6-benzyladenine with methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide) uronate (MBTG). Hydrolysis of the resulting peracetyl BA3GN methyl ester afforded the sodium salt of BA3GN. The ^{1}H NMR spectrum contained signals corresponding to the adenine (δ 8.62 and 7.88), the benzyl group (δ 7.25–

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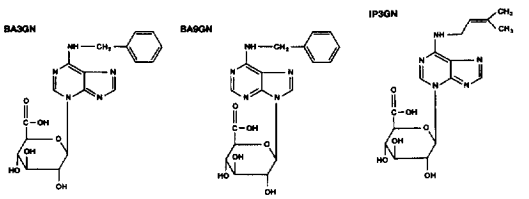

Fig. 1. The structures of the cytokinin glucuronides BA3GN, BA9GN and IP3GN for selection of plant cells transformed with a GUS gene.

7.41) and the glucuronide portion of the molecule. The chemical shift of the 1′-hydrogen (δ 5.76) is confirmation of the conjugation of the sugar to the adenine. The widely spaced pair of singlets corresponding to the adenine hydrogens confirms that conjugation has taken place at position-3 of the adenine ring. ^{13}C NMR data were consistent with the structure, the spectrum containing signals corresponding to benzyladenine and the glucuronide portion of the molecule. The ^{13}C NMR spectrum also indicated a high level of purity of the sample. The UV spectra of Na-BA3GN displayed absorption maxima at 297 nm in ethanol, at 291 nm in ethanol–acetic acid and at 297 nm in ethanol–ammonia. These values are identical to those reported by Leonard *et al.* [5] for BA-3-β-D-glucopyranoside and N^3, N^6-disubstituted adenine. TLC and HPLC analysis showed purities of more than 98%.

Enzymatic analysis of BA3GN was carried out using *E. coli* β-glucuronidase. Analysis of the reaction mixture by HPLC showed virtually complete disappearance (more than 99%) of the BA3GN peak with the production of a peak which co-chromatographed with authentic BA. This further confirmed the identity of the product as a conjugate of BA and β-D-glucuronic acid.

The amide corresponding to BA3GN was synthesized by treating BA3GN with anhydrous ammonia in anhydrous methanol (not shown). Although this compound, BA3GNamide, was not substrate for GUS, it nevertheless acted as free benzyladenine when added to GUS-transformed tobacco leaf discs (not shown) suggesting that inside the cells the amide was converted to BA3GN and then hydrolysed by GUS. As BA3GNamide is devoid of the charged carboxyl group which may restrict uptake of cytokinin glucuronides, BA3GNamide might be useful for certain plant species where glucuronide uptake is limited.

BA9GN (Fig. 1) was prepared by catalytic oxidation of N^6-benzyladenine-9-β-D-glucopyranoside, as this compound is commercially available, as well as by condensation of 6-chloropurine and MBTG with subsequent base hydrolysis. Both methods yielded the same product which was identified as the sodium salt of BA9GN. ^1H NMR analysis indicated that the sugar portion of the molecule was attached to position-9 of the adenine ring since the signals corresponding to the adenine hydrogens (δ 8.24 and 8.32) have moved closer together relative to their positions in BA3GN. The UV-spectra of Na-BA9GN dissolved in 95% ethanol, 95% ethanol–0.1 M HCl or 95% ethanol–0.1 M sodium hydroxide all displayed absorption maxima at 270 nm with extinction coefficients of 17 400, 16 200 and 17 400, respectively. These data are consistent with the structure of an N^6, N^9-disubstituted adenine. The extinction coefficients are virtually the same as pure BA9G indicating freedom from non-UV absorbing contaminants. HPLC analysis was performed on a 10×0.46 cm octadecyl silica column, the UV-monitor recording at 270 nm. Isocratic (50% methanol–0.2 M acetic acid, 2 ml min^{-1}) and gradient HPLC (0–60% methanol–0.2 M acetic acid, 2 ml min^{-1}) showed a sharp, symmetrical peak for BA9GN, which eluted just before the corresponding glucoside. HPLC purity of BA9GN was more than 98% with no detectable (<2%) α-anomer or other impurities, including BA.

Mineral acid converts cytokinin glucosides to the corresponding free cytokinin base. Treatment of Na-BA9GN (1 mg ml^{-1}) with 1 M HCl at 100° overnight produced a single spot on TLC which co-chromatographed with authentic BA. This test confirmed that the synthesized BA9GN was an acid-labile conjugate of BA.

Tsou and Seligman [6] prepared 2-naphthyl-β-D-glucopyraronoside from 2-naphthyl-β-D-glucopyranoside, also by platinum-black catalysed oxidation and the glucuronide product was readily hydrolysed by GUS. Enzymatic analysis of our BA9GN with GUS was performed as for BA3GN. However, HPLC and TLC analysis of the reaction mixture after incubation for 18 hr showed no detectable production of BA. Further incubation at room temperature for 3 days showed no hydrolysis. Thus, BA9GN is not susceptible to hydrolysis by GUS and can not be used as selective agent. There are a few examples of glucuronides like BA9GN which are not hydrolysable by GUS (β-glucuronido-N-acetylglucosamine and β-glucuronido-6-galactose [7]) although the aglycone part of glucuronides generally is considered to be of minor importance for determining whether a glucuronide is substrate for GUS [8].

IP3GN (Fig. 1) was synthesized by the condensation of N^6-(2-isopentyl)-adenine and MTBG. Hydrolysis of the peracetyl IP3GN methyl ester afforded the sodium salt of IP3GN. ^1H NMR analysis confirmed the structure of the product. Signals corresponding to the adenine portion of the molecule (δ 8.56 and 7.88) were well spaced indicating addition to position-3 of the adenine ring. The two methyl groups of the isopentenyl portion of the molecule appeared as two sharp singlets at (δ 1.76 and 1.74) and the olefinic hydrogen resonated as a triplet (J = 7 Hz) at δ 5.34. TLC analysis of Na-IP3GN on silica using 1-butanol–acetic acid–water (12:3:5) gave a single, sharp spot (R_f 0.32) at 100 μg loading with no detectable N^6-(2-isopentenyl)adenine (R_f 0.66) or other contaminants. Purity was more than 99.5%.

Enzymatic analysis of Na-IP3GN using GUS was

performed as for BA3GN. After incubation for 12 hr at 37° TLC analysis showed removal of the UV spot corresponding to IP3GN with the production of a new UV spot which co-chromatographed with authentic IP.

EXPERIMENTAL

^1H and ^{13}C NMR spectra recorded at 400 and 100 MHz, respectively, using a JEOL GX-400 instrument. Chemical shifts are given in δ values (ppm) with tetramethylsilane (TMS) as int. standard and coupling constants (J) are given in Hz.

3-β-D-glucopyranuronosyl-6-benzylaminopurine, sodium salt (Na-BA3GN). N^6-benzyladenine (BA, 2.8 g) and methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide) uronate (MBTG, 5.5 g) were suspended in 50 ml anhydrous DMF and condensed by heating at 100° for *ca* 10 hr [9]. Most of the DMF was removed under vacuum and the crude product was dissolved in $CHCl_3$ (300 ml) and partitioned with H_2O (3 × 300 ml). After drying over anhydrous Mg_2SO_4 the $CHCl_3$ extract was evaporated under vacuum and a dark syrup was recrystallised from EtOH. The crude product was purified over 100 g silica packed in $CHCl_3$ eluted with a gradient of 0–4% EtOH in $CHCl_3$. Crude peracetyl BA3GN methyl ester was recrystallised from EtOH. Yield: 280 mg of a colourless, amorphous solid.

Peracetyl BA3GN methyl ester was hydrolysed by treatment with 5% NaOH in 50% EtOH at room temp. 5 min after the solid had dissolved the reaction mixt. was carefully neutralised with HCl while cooling on ice. After drying under vacuum, crude BA3GN sodium salt was purified by reverse-phase chromatography (over 100 g octadecylsilica) eluting with H_2O (1 l) followed by 20% MeOH followed by recrystallisation from EtOH. Pure BA3GN sodium salt (150 mg) was obtained as a colourless, microcrystalline solid which was dried to constant wt over $CaCl_2$ under vacuum. UV λ_{max}^{EtOH} nm: 297; $\lambda_{max}^{EtOH\ HOAc}$ nm: 291; $\lambda_{max}^{EtOH-NH_3}$ nm: 297. ^1H NMR (CD_3OD–D_2O, 400 MHz): δ 3.69 (*t*, J = 9.5 Hz, H-2′), 3.76 (*t*, J = 9.5 Hz, H-3′), 3.95 (*d*, J = 9.5 Hz, H-5′), 4.19 (*t*, J = 9.5 Hz, H-4′), 4.91 (*s*, —NH—C<u>H</u>$_2$—Ar), 5.76 (*d*, J = 9.5 Hz, H-1′), 7.25–7.41 (5 H *m*, 5 × ArH), 7.88 (*s*, H-8), 8.62 (*s*, H-2). ^{13}C NMR (CD_3OD–D_2O, 100 MHz): δ 45.57 (C-10), 72.97 (C-4′), 73.13 (C-2′), 78.17 (C-3′), 80.02 (C-5′), 89.56 (C-1′), 121.22, 128.69, 129.81 (all Ar—C), 139.56 (C-6), 144.69 (C-8), 149.58 (C-5), 153.01 (C-2), 155.05 (C-4), 175.93 (C-6′).

9-β-D-glucopyranuronosyl-6-benzylaminopurine, sodium salt (Na-BA9GN). Method 1: catalytic oxidation of N^6-benzyladenine-9-β-D-glucopyranoside (BA9G). BA9G (50 mg) was suspended in 50 mM Na_2CO_3 (25 ml) with platinum black (200 mg). The mixt. was heated at 80° on a water bath while O_2 was passed through vigorously. A further 100 mg of platinum catalyst was added after 4 hr. More than 95% of BA9G was converted to the corresponding 9-β-D-glucopyranuronic acid and some BA with 20 hr of treatment. The mixt. was neutralised and the product Na-BA9GN was purified by reverse-phase chromatography (over 20 g octadecylsilica) eluting with 200 ml H_2O followed by 20% MeOH. Pure Na-BA9GN, free of glucoside and BA, was dried to a colourless solid over $CaCl_2$ under vacuum.

Method 2: Condensation of 6-chloropurine and MBTG. 6-Chloropurine (1.13 g, dried over phosphorus pentoxide), MBTG (3.87 g) and freshly-dried K_2CO_3 (1.5 g) were stirred in anhydrous propylene carbonate (30 ml) at room temp. for 24 hr. The dark mixt. was filtered and purified by CC over silica (100 g) eluting with a 0–80% gradient of EtOAc in $CHCl_3$. The main fr. (other than unreacted 6-chloropurine) was dried and recrystallised from boiling EtOH to yield methyl 6-chloropurine-9-(2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranuronate) as a pale yellow solid (450 mg) which was dried over phosphorus pentoxide under vacuum. HPLC analysis indicated a purify of more than 95%.

Methyl 6-chloropurine-9-(2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranuronate (312 mg) and benzylamine (171 μl) were heated together in 1-BuOH (13.5 ml) at 100° for 1 hr. The solid dissolved readily to give a clear yellow soln. Most of the BuOH was removed under vacuum to yield a colourless solid which was shaken with 5% NaOH in 50% EtOH (25 ml) for 1–2 hr at room temp. After neutralization the product was dried under vacuum to remove traces of BuOH and crude Na-BA9GN was purified by reverse-phase chromatography as in Method 1. The pure product was dried under vacuum over $CaCl_2$ and phosphorus pentoxide to yield a colourless solid (210 mg). UV λ_{max}^{EtOH} nm: 270; $\lambda_{max}^{EtOH-HCl}$ nm: 270; $\lambda_{max}^{EtOH-NaOH}$ nm: 270. ^1H NMR (CD_3OD–D_2O, 400 MHz): δ 3.65 (*m*, H-2′ and H-3′), 3.90 (*d*, J = 9.5 Hz, H-5′), 4.07 (*t*, J = 9.5 Hz, H-4′), 4.88 (*s*, —NH—C<u>H</u>$_2$—Ar), 5.60 (*d*, J = 9.5 Hz, H-1′), 7.20–7.5 (5 H *m*, 5 × ArH), 8.24 (*s*, H-8), 8.32 (*s*, H-2). ^{13}C NMR (CD_3OD–D_2O, 100 MHz): δ 44.46 (C-10), 73.53 (C-4′ and C-2′). 78.72 (C-3′), 79.10 (C-5′), 84.67 (C-1′), 120.51, 128.35, 128.66, 129.68, 130.02, 130.25 (all Ar—C), 134.73 (C-5), 140.44 (C-8), 141.17 (C-2), 154.11 (C-4), 156.22 (C-6), 176.21 (C-6′).

3-β-D-glucopyranuronosyl-6-(3-methyl-but-2-enylamino)purine, sodium salt (Na-IP3GN). N^6-(2-Isopentenyl)adenine (IP, 9.48 g) and MBTG (22.1 g) were heated in anhydrous DMF (170 ml) at 100° for 12 hr. Most of the DMF was removed under vacuum on a boiling water-bath and the cooled syrup taken up in $CHCl_3$ (500 ml). The $CHCl_3$ soln was extracted with H_2O (3 × 500 ml) and the organic extract dried over anhydrous Na_2SO_4. Most of the $CHCl_3$ was removed under vacuum and the syrup chromatographically purified over silica developed with a gradient of 0 to 3.75% MeOH in $CHCl_3$. Crude peracetyl IP3GN methyl ester was recrystallised from MeOH with charcoal decolourisation to yield pure, colourless peracetyl IP3GN methyl ester (3.2 g) which was dried under

vacuum over $CaCl_2$. A portion of peracetyl IP3GN methyl ester (1.2 g) was hydrolysed by dissolving in *ca.* 1 l. of 75% MeOH containing 5% NaOH and stirring the mixt. for 10 min at room temp. The mixt. was cooled on ice, carefully neutralised with HCl and reduced to a syrup under vacuum. The crude product was purified by successive chromatography over XAD-2 resin and octadecyl-silica to yield IP3GN sodium salt as a colourless, microcrystalline solid which was dried over calcium chloride (810 mg). IP3GN sodium salt is soluble in H_2O at 2 mg ml^{-1}.

^1H NMR (CD_3OD–D_2O, 400 MHz): δ 1.74 and 1.76 (both *s*, 13-H_3 and 14-H_3), 3.72 (*t*, $J = 9.5$ Hz, H-2′), 3.86 (*t*, $J = 9.5$ Hz, H-3′), 4.14 (*d*, $J = 9.5$ Hz, H-5′), 4.26 (*d*, $J = 9.5$ Hz, 10-H_2), 4.34 (*t*, $J = 9.5$ Hz, H-4′), 5.34 (*t*, $J = 7$ Hz, H-11), 5.73 (*d*, $J = 9.5$ Hz, H-1′), 7.88 (*s*, H-8), 8.56 (*s*, H-2). ^{13}C NMR (CD_3OD–D_2O, 100 MHz): δ 18.19 and 25.93 (C-13 and C-14), 40.07 (C-10), 71.65 (C-4′), 77.48 (C-2′), 77.63 (C-3′), 79.54 (C-5′), 89.98 (C-1′), 120.31 (C-11), 121.16 (C-12), 138.60 (C-6), 144.89 (C-8), 148.38 (C-5), 152.76 (C-2), 154.74 (C-4), 173.44 (C-6′).

Enzymatic analysis. Enzymatic analysis was conducted by dissolving 500 μg cytokinin glucuronide in 500 μl of 50 mM Na phosphate buffer pH 7.0 added 2500 'Fishman' units [7] of *E. coli* β-glucuronidase (Sigma G7896) and incubated for 18 hr at 37°. Analysis of the reaction mixt. was carried out by HPLC using a 10 × 0.46 cm column of octadecyl silica, eluting isocratically with 60% MeOH containing 10% HOAc at 1 ml min^{-1}.

REFERENCES

1. Fraley, R. T., Rogers, S. G. and Horsch, R. B., *CRC Critical Reviews in Plant Science*, 1986, **4**, 1.
2. Bowen, B. A., in *Transgenic Plants*, Vol. 1, ed. S. D. Kung and R. Wu. *Academic Press*, New York, ISBN 0-12-428781-6, 1993, p. 89.
3. Joersbo, M. and Okkels, F. T., *Plant Cell Reports*, 1996, **16**, 219.
4. Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W., *EMBO Journal*, 1987, **6**, 3901.
5. Leonard, N. J., Carraway, K. L. and Helgeson, J. P., *Journal of Heterocyclic Chemistry*, 1965, **2**, 291.
6. Tsou, K.-C. and Seligman, A. M., *Journal of the American Chemical Society*, 1953, **75**, 1042.
7. Meyer, K., Linker, A. and Rapport, M. M., *Journal of Biological Chemistry*, 1951, **192**, 275.
8. Fishman, W. H., *Advances in Enzymology*, 1955, **16**, 361.
9. Bollenback, G. N., Long, J. W., Benjamin, D. G. and Lindquist, J. A., *Journal of the American Chemical Society*, 1955, **77**, 3310.