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Note

Identification of a digalactosyl ononitol from seeds of adzuki bean (*Vigna angularis*)

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Received 6 June 2003; received in revised form 5 July 2003; accepted 13 July 2003

Abstract

A digalactosyl ononitol was isolated from seeds of adzuki bean (*Vigna angularis* [Willd.] Ohwi et Ohasi). Analysis of hydrolysis products and NMR spectroscopy established its structure as *O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→3)-4-*O*-methyl-D-*myo*-inositol.

Keywords: *Vigna angularis*; Adzuki bean; Seed; Digalactosyl ononitol; Galactosyl cyclitol; D-Ononitol

Galactosyl derivatives of cyclitols are widely distributed in plant seeds.[1] The most prominent galactosyl cyclitol, galactinol (*O*-α-D-galactopyranosyl-(1→1)-L-*myo*-inositol), acts as the galactosyl donor in the biosynthesis of raffinose family of oligosaccharides from sucrose.[2] In addition, homologue series of α-D-galactosides of D-*chiro*-inositol (fagopyritols) and D-pinitol (galactopinitols) with a degree of polymeriztion of up to four have been identified.[3,4] In seeds accumulating raffinose oligosaccharides, these galactosyl cyclitols may have a metabolic function as galactosyl donors, but in some species they replace the latter as the major soluble carbohydrates.[1] There is some evidence that galactosyl cyclitols protect membranes and other cellular structures during seed desiccation or storage in the dry state.[5]

A monogalactoside of D-ononitol (1D-4-*O*-methyl-*myo*-inositol), isolated from adzuki bean seeds, was found to be present in seeds of 60 species from 21 plant families.[6,7] Its structure was originally proposed to be *O*-α-D-galactopyranosyl-(1→5)-4-*O*-methyl-D-*myo*-inositol.[6] We have recently revised its structure to *O*-α-D-galactopyranosyl-(1→3)-4-*O*-methyl-D-*myo*-inositol.[8]

In continuation of this work, we here report on the identification of a digalactoside of D-ononitol.

Adzuki bean seeds have been reported to contain sugars (sucrose, raffinose and stachyose), cyclitols (D-ononitol and *myo*-inositol) and their respective monogalactosyl derivatives (galactosyl ononitol and galactinol).[9] These seed constituents were identified by GC-MS by comparison of retention times and mass fragmentation patterns of trimethylsilyl derivatives with those of authentic standards. Digalactosyl *myo*-inositol and a digalactosyl glycerol were tentatively identified by their reported GC retention times.[9,10] In addition, analysis of adzuki bean seed extracts revealed the presence of a hitherto unknown compound, which was isolated after elimination of sugars by charcoal-Celite chromatography. A final purification step by HPLC was necessary to remove residual contaminants. Acid hydrolysis afforded D-ononitol and galactose (molar ratio, 1:2) as confirmed by GC-MS of trimethylsilyl derivatives. The liberated galactose was oxidized by β-D-galactose dehydrogenase, indicating that the galactose had the D configuration. The compound was resistant to β-galactosidase, but was hydrolyzed by α-galactosidase, demonstrating α anomeric linkages for both galactose residues. Partial enzymic digest with α-galactosidase gave galactosyl ononitol, galactose and D-ononitol.

The ^{1}H NMR spectrum of the isolated compound in D_2O showed 2 anomeric proton signals (at δ 5.04 and

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doi:10.1016/S0008-6215(03)00339-2

5.17) and the singlet of a methoxy group (at δ 3.62). The ^{13}C NMR spectrum revealed 19 signals, including two anomeric carbons (at δ 97.29 and 100.46). A one-bond $^{1}H-^{13}C$ correlation spectrum (HSQC) was used to link the carbon signals to the corresponding proton resonances. Analysis of the DQF-COSY spectrum allowed to trace all connectivities of the ring system lacking an anomeric proton and most of the connectivities of the hexose residues. The remaining signals (H-5″ and H-6″) were established using complementary information from the DQF-COSY and a long-range $^{1}H-^{13}C$ connectivity (HMBC) spectrum. Vicinal coupling constants of the protons of the cyclitol residue confirmed that its configuration corresponded to a *myo*-inositol residue (Table 1). The chemical shift values of the hexoses compare well with those of the residues in other digalactosyl cyclitols.[3] Due to overlap of signals in the distal galactopyranosyl ring, however, it was not possible to determine accurate vicinal coupling constants for all protons. The linkages between the galactose units and the cyclitol residue were established based on long-range couplings in the HMBC spectrum. A cross-peak was observed between the anomeric carbon at δ 97.29 (C-1′) and the cyclitol proton at δ 3.73 (H-3). This proton was flanked by the only equatorial proton of the cyclitol ring (H-2 at δ 4.30) and a proton at δ 3.52 (H-4), which in its turn showed a long-range coupling to the methyl carbon. The stereochemistry of this partial structure corresponds to that in galactosyl ononitol.[8] Cross-peaks were also observed between the carbon at δ 68.91 (C-6′) and the anomeric proton H-1″ and between C-1″ and both H-6′ protons. The low-field shift of C-6′ supported substitution at this carbon. The interglycosidic linkages were consistent with cross-peaks detected by a NOESY experiment (tm = 800 ms). Correlations were observed between H-1″ and the H-6′ protons as well as between H-1′ and the protons H-2 and H-3 of the cyclitol ring. As a result of these analyses, the compound was assigned as *O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→3)-4-*O*-methyl-D-*myo*-inositol (Fig. 1).

Table 1
^{1}H (500.13 MHz) and ^{13}C (125.8 MHz) NMR data for digalactosyl ononitol in D_2O at 25 °C

Position (no.)	δ_H (ppm)	δ_C (ppm)	Coupling constants (J, Hz)	
1	3.440	73.54	$J_{1,2}$	2.8
2	4.296	70.20	$J_{2,3}$	3.8
3	3.733	77.05	$J_{3,4}$	9.4
4	3.523	83.78	$J_{4,5}$	9.4
5	3.368	76.56	$J_{5,6}$	9.4
6	3.700	74.93	$J_{6,1}$	10.0
CH_3	3.619	63.31		
1′	5.171	97.29	$J_{1',2'}$	3.7
2′	3.892	70.72	$J_{2',3'}$	10.2
3′	3.951	72.19	$J_{3',4'}$	3.4
4′	4.106	71.87	$J_{4',5'}$	~1.5
5′	4.300	71.38	$J_{5',6'a}$	7.6
6′a	3.882	68.91	$J_{5',6'b}$	4.7
6′b	3.752		$J_{6'a,6'b}$	11.2
1″	5.039	100.46	$J_{1'',2''}$	3.0
2″	3.833	70.91	$J_{2'',3''}$	10.6
3″	3.827	72.30	$J_{3'',4''}$	4.0
4″	3.995	71.80	$J_{4'',5''}$	~1.0
5″	3.990	73.58	$J_{5'',6''a}$	6.3
6″	3.757	63.63	$J_{5'',6''b}$	6.3

1. Experimental

1.1. General methods

Evaporations were performed under reduced pressure at 40 °C. Optical rotations were measured with a Perkin–Elmer 241 polarimeter. GC of trimethylsilyl derivatives[11] was carried out on a Hewlett Packard 6890 instrument equipped with a cool on-column injector and a HP-5 column (25 m × 0.20 mm i.d., 0.33 μm film thickness). The carrier gas was Helium and the temperature program was 1 min at 85 °C, 85–220 °C at 10 °C/min, 220–325 °C at 12 °C/min, 15 min at 325 °C. GC-MS was carried out with a Varian 3400CX coupled to a Varian Saturn 3 ion trap mass spectrometer. A DB-5ms column (30 m × 0.25 mm i.d., 0.1 μm film thickness) was used with Helium as the carrier gas and a temperature gradient of 110–320 °C at 8 °C/min. NMR spectra (^{1}H, ^{13}C, DQF-COSY, phase-sensitive NOESY,[12] gradient-enhanced HSQC[13] and HMBC[14]) were recorded in D_2O using a Bruker DRX 500 spectrometer with d_6-TSP (sodium trimethylsilyl propionate) as internal standard (500.1 MHz for ^{1}H NMR or 125.75 MHz for ^{13}C).

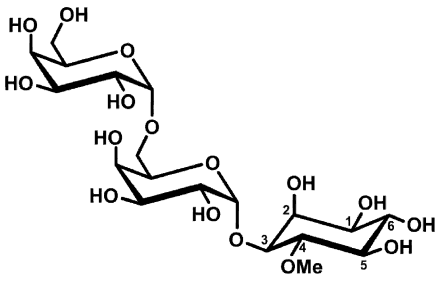

Fig. 1. Structure of digalactosyl ononitol.

1.2. Isolation of digalactosyl ononitol

A ground seed sample (100 g) was extracted with boiling H_2O (1.8 L). The extract was frozen, thawed, and filtered through a fine nylon mesh. The solution was reduced in volume and mixed with one volume of ice-cold acetone. Insolubles were removed by centrifugation, and the clarified supernatant was evaporated to dryness. The residue was dissolved in water, deionized by passage through an ion exchange column (50 mL Dowex 1×8, $HCOO^-$, overlaid by 50 mL Dowex $50W \times 8$, H^+) and reduced in volume. To eliminate sucrose and raffinose family oligosaccharides, the sample was applied to a column (23×2.5 cm i.d.) of Dowex 1×8, 100–200 mesh (OH^-), and eluted with H_2O. Fractions were collected and their content was monitored by GC. Appropriate fractions were pooled, reduced in volume and loaded onto a column (14×2.5 cm i.d.) of Darco G60-Celite 535.[15] After the column had been washed with 0.5 L H_2O, a linear gradient to 10% EtOH (1 L) was applied, followed by 0.5 L 10% EtOH. Fractions containing the compound were pooled, taken to dryness, and re-chromatographed on a small charcoal-Celite column (6×1 cm i.d.). A pure sample was obtained by semi-preparative HPLC using an Aminex HPX-87C column (300×7.8 i.d. mm). The column was eluted with H_2O at 80 °C (0.6 mL/min). The glycoside (8 mg) was obtained as a colourless solid: α_D^{20} +162.5 (*c* 0.2, H_2O); FABMS (pos): *m/z* 541 $[M+Na]^+$, 519 $[M+H]^+$; FABMS (neg): *m/z* 517 $[M-H]^-$. ^1H and ^{13}C NMR chemical shifts are shown in Table 1.

1.3. Acid and enzymic hydrolysis

For acid hydrolysis, the sample (1 mg) was incubated in 1 M HCl at 100 °C for 1 h. The acid was removed by repeated evaporation. An aliquot was analyzed by GC and GC-MS. Another sample of the hydrolyzate was incubated in a reaction mixture (2 mL) containing 20 mM Tris–HCl buffer, pH 8.65, 4 mM NAD and 0.1 U β-D-galactose dehydrogenase (from *Pseudomonas fluorescens*). Oxidation of D-galactose was monitored at 340 nm. For enzymic hydrolysis, samples were incubated at 30 °C with 1 U α-galactosidase (from green coffee beans) in 0.2 mL of McIlvaine buffer (pH 6.1), or with 0.17 U β-galactosidase (from bovine testes) in 0.2 mL of McIlvaine buffer (pH 4.1), respectively. At intervals, samples (20 μL) were removed and examined by GC.

Acknowledgements

This study was funded through grant P10917-BIO of the Austrian Science Fund (to A.R.).

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