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Note

Isolation and structure determination of an α-D-galactosyl-α-D-galactosyl-α-D-galactosyl-D-pinitol from the chick pea

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Low-molecular-weight carbohydrates in food legumes have been extensively investigated in recent years[1–5]. Manninotriose [*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-D-glucopyranose] has been reported by several authors[2–4] to be present in chick pea (*Cicer arietinum* L.) and lentil (*Lens esculenta*) seeds. However, t.l.c. of aqueous extracts revealed a difference between the R_F values of the presumed manninotriose and the authentic substance. The presence of a new oligosaccharide (*A*) was suspected, and this prompted us to determine its structure and that of a second unknown compound (*B*) of higher molecular weight often reported in chick peas[4–6]. When the experimental work was completed, the structure of *A*, a galactosylgalactosylpinitol, was published[6] and corresponded to our findings. The isolation of *B* is now described and its structure shown to be *O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→2)-1D-4-*O*-methyl-*chiro*-inositol.

In order to eliminate sucrose and the raffinose series of oligosaccharides, an aqueous extract of chick peas was treated with β-D-fructosidase and the resulting reducing sugars were precipitated with phenylhydrazine. Pure samples of *A* and *B* were then obtained by chromatography of the filtrate on charcoal–Celite.

Total hydrolysis of *B* by yeast α-D-galactosidase yielded only D-pinitol and D-galactose, and the products of partial enzymic hydrolysis had R_F values (t.l.c., solvent *A*) which were the same as those of D-pinitol, D-galactose, 1D-2-*O*-(α-D-galactopyranosyl)-4-*O*-methyl-*chiro*-inositol, and *A*. Only traces of *A* were detected, because the affinity of the enzyme was higher for *A* than for *B*. The retention times of their trimethylsilyl derivatives in g.l.c. were also identical to those of autenthic substances. That the liberated galactose was D was confirmed by oxidation to galactonic acid by the β-D-galactose dehydrogenase–NAD system. Furthermore, *B* was resistant to β-D-galactosidase, indicating that it contained only α linkages.

Methylation analysis of *B* gave mono-*O*-acetyl-penta-*O*-methylinositol[7], 1,5-

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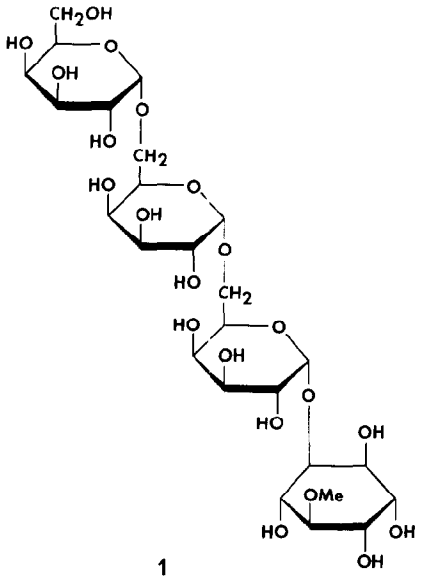
di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol (molar ratios, 1.0:1.0:2.2), identified by g.l.c.–m.s. Thus, *B* contained three (1→6)-linked D-galactosyl residues.

The point of attachment of the D-galactosyl residue to the D-pinitol residue was established by periodate oxidation of *B* followed by borohydride reduction, hydrolysis, and demethylation. Only glycerol and xylitol were formed (t.l.c., solvent *B*). The presence of xylitol indicated unambiguously that the galactosidic linkage was located at O-2 of D-pinitol. Therefore, *B* has the structure **1**.



1

The ^{13}C-n.m.r. spectrum of *B* contained signals at 95.1 (C-1 of the galactosyl residue linked to D-pinitol; *cf.* 95.5 p.p.m. for *A*), 98.1 and 98.4 (C-1 of the other galactosyl residues; *cf.* 98.6 for *A*), 60.3 (OMe of D-pinitol; *cf.* 60.2 for *A*), and 83.4 p.p.m. (C-4 of D-pinitol; *cf.* 83.1 for *A*) respectively. These resonance values were very similar to those previously reported[7] for 1D-2-*O*-(α-D-galactopyranosyl)-4-*O*-methyl-*chiro*-inositol.

EXPERIMENTAL

Materials. — Dry seeds of chick pea (*Cicer arietinum* L.) were obtained from a local supplier. D-Pinitol and 1D-2-*O*-(α-D-galactopyranosyl)-4-*O*-methyl-*chiro*-inositol, isolated from soya beans, were gifts from Dr. T. F. Schweizer from our department. The latter compound contained 10% of galactopinitol B, the isomer present in soya[2,7]. Manninotriose was produced in equimolar quantity with fructose by treatment of stachyose with β-D-fructosidase followed by ultrafiltration.

α-D-Galactosidase was obtained by growing yeast (*Saccharomyces carlsbergensis*) in a medium containing 0.5% of yeast extract and 1% of melibiose. After harvesting, the cells were inactivated by incubation at 50° in 0.5M sodium acetate buffer (pH 5.0) for 180 min, washed with distilled water, and freeze-dried. This crude preparation contained cell-bound α-D-galactosidase and β-D-fructosidase, but was free from β-D-galactosidase.

General. — All evaporations were conducted under diminished pressure at ≯40°.

T.l.c. was performed on silica gel 60 (Merck, 5631) with *A*, chloroform–acetic acid–water (6:7:1); *B*, 2-propanol–ethyl acetate–water (7:2:1). With solvent *A*, a three-fold development was used[8]. Xylitol, L-arabinitol, L-threitol, and glycerol were completely resolved in solvent *B*. Detection was effected with the diphenylamine–aniline–phosphoric acid reagent[9] at 120° or the lead tetra-acetate–sodium fluorescein reagent[10].

Capillary g.l.c. of trimethylsilylated derivatives[11] was carried out on a Carlo Erba Model 4160 gas chromatograph, using an ID WCOT glass-capillary column (10 m × 0.3 mm) coated with SE-30, and hydrogen at 0.55 bar as carrier gas. The temperature programme was 15°/min 80° → 140°, 4°/min → 180°, and 10°/min → 330°.

G.l.c.–m.s. was performed with a Hewlett–Packard 5880 A gas chromatograph coupled to a Kratos MS 30 spectrometer and a DS-55 data-processing system. Column DB-5 (J & W Scientific, Inc.) was used for g.l.c. with the following programme: 2-min hold at 60°, 30°/min → 150°, 1-min hold, 4°/min → 300°. Mass spectra were recorded at 70 eV with an ion-source temperature of 200°.

^{13}C-N.m.r. spectra (20 MHz) were recorded with a Varian FT-80A spectrometer for solutions in D_2O. Chemical shifts were assessed relative to internal 1,4-dioxane (δ 67.4).

Isolation of pinitol galactosides from chick peas. — Chick peas (30 g) in water (300 mL) were autoclaved for 20 min at 120° and then ground in a Waring Blendor. This treatment was repeated once, the pH of the suspension was adjusted to 4.0 with HCl, and the mixture was centrifuged. The supernatant solution was incubated with β-D-fructosidase (30 mg, Boehringer Mannheim GmbH) for 22 h at 25°. After the addition of ethanol (4 vol.) and centrifugation, the resulting supernatant solution was concentrated and freeze-dried (residue, 2.0 g). Reducing sugars were precipitated as their phenylosazones[2] and the filtrate was eluted from a column (29 × 300 mm) of Darco G60–Celite 535 (1:1) with water and then with a water–ethanol gradient. Fractions containing pure compounds were combined and concentrated. D-Pinitol, *myo*-inositol, and their respective galactosides were isolated. Precipitation from ethanol yielded *B* (8 mg), $[\alpha]_D^{23}$ +179° (*c* 0.5, water).

Enzymic hydrolysis. — Pinitol galactosides (5 μL; ~2 mg/mL) were incubated at 45° in the presence of yeast α-D-galactosidase (10 μL; 10 mg/mL in 0.5M sodium acetate buffer, pH 5.0). At intervals, the supernatant solution (3 μL) was subjected to t.l.c. (solvent *A*).

Methylation analysis. — Methylation was performed according to the method of Hakomori[12], as described by Conrad[13]. The methylated product was hydrolysed at 100° for 1 h in aqueous 90% formic acid and for 4 h after the addition of 3 vol. of 0.2M HCl. The products were reduced and acetylated[14], and then analysed by g.l.c.–m.s.

Periodate oxidation. — A solution of *B* (133 μg) in 0.03M sodium metaperiodate (100 μL) was stored at 4° for 90 h in the dark. The solution was then passed through a column of Amberlite IRA-400 (AcO^-) resin and concentrated to 50 μL. A freshly prepared solution of sodium borohydride (50 μL; 10 mg/mL) was added, the mixture was kept overnight at room temperature and then treated with Amberlite IR-120 (H^+) resin, and boric acid was removed by three successive concentrations to dryness in the presence of methanol (250 μL). The residue was dissolved in water (100 μL), an aliquot (10 μL) was concentrated to dryness, and the residue was hydrolysed with 0.1M hydrochloric acid (10 μL) for 4 h at 100°. After treatment with Amberlite IRA-400 (AcO^-) resin and concentration to dryness, the product was demethylated with aqueous 56% hydrogen iodide (10 μL) at 100° for 10 min. The mixture was concentrated to dryness, and a solution of the residue in water (10 μL) was treated with Amberlite IRA-400 (AcO^-) resin and analysed by t.l.c. (solvent *B*).

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