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MONOMETHYL SUGARS IN EXTRACELLULAR POLYSACCHARIDES FROM SLOW-GROWING *Rhizobia**

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ABSTRACT

Two monosaccharides present as components of extracellular gums produced by several strains of slow-growing *Rhizobia* have been shown to be 4-*O*-methyl-D-galactose and 4-*O*-methyl-D-glucose. The occurrence of mannose in several of these polysaccharides has been confirmed.

INTRODUCTION

Because *Rhizobia* are organisms of major economic importance to New Zealand, we are investigating their polysaccharides. *Rhizobia* belong to two distinct classes, somewhat independent of species, cross-inoculation groups, and host plants nodulated. They are the fast-growing, acid producers and slow-growing, non-acid producers. Extracellular polysaccharides from the first class have often been studied and do not appear to vary much in composition. Polysaccharides from many strains of the slow-growing varieties showed[1], in contrast, marked variations in monosaccharide composition, including the presence[1] of three unidentified sugars. Two of these sugars behaved as simple aldohexoses and, as they could not be demethylated by boron trichloride[2], they were not considered to be methylhexoses. We have now identified these sugars as 4-*O*-methyl-D-galactose and 4-*O*-methyl-D-glucose, partly by the use of boron tribromide[2].

While this work was in progress, Dudman[3] identified 4-*O*-methyl-D-galactose as a component of some other rhizobial polysaccharides.

RESULTS AND DISCUSSION

The bacterial strains used in the present study all belong to the slow-growing group of *Rhizobia*, and all are associated with *Lotus* species. All strains were from the Divisional collection maintained by R. M. Greenwood; the monosaccharide composition of their extracellular polysaccharides has been described[1]. Polysaccharides

*Dedicated to the memory of Professor Edward J. Bourne.

which yielded 4-*O*-methyl-D-galactose were from strains NZP2087 and NZP5088, and the 4-*O*-methyl-D-glucose was isolated from the polysaccharide produced by NZP2154. Polysaccharides from strains NZP2073, NZP2186, NZP5026, and NZP5052 were re-investigated for mannose content.

Identification of 4-O-methyl-D-galactose. This sugar, designated[1] X_1, had R_{Glc} 1.70 and 1.25 in solvents *1* and *2*, respectively, thus moving behind altrose, talose, and idose, but ahead of all other aldohexoses. Colour reactions with a variety of reagents were characteristic of a simple aldohexose. On g.l.c., the derived alditol acetate[4] was separated from all the acetylated hexitols and had a mobility between those of acetylated talitol and galactitol, with a T_{Man} of 1.09.

Mass spectrometry of the alditol acetate gave major fragments at *m/e* 43, 87, 129, 189, and 261. After reduction of the sugar with NaB^2H_4, the major fragments of the deuterated alditol acetate were at *m/e* 43, 87, 129, 189, and 262. These data are characteristic of a 4-*O*-methylhexose[5,6].

Attempted demethylation of X_1 with boron trichloride[2], as previously described[1], left the sugar unchanged; 3-*O*-methyl-D-glucose was also unaffected under similar reaction conditions. Demethylation[2] was effected with boron tribromide, and produced galactose (p.c.). The product was destroyed by treatment with D-galactose oxidase, thus establishing the D configuration.

The unknown sugar X_1 was identical with authentic 4-*O*-methyl-D-galactose kindly given by Dr. W. Dudman[3].

Identification of 4-O-methyl-D-glucose. This sugar, designated[1] X_2, also gave colour reactions (p.c.) characteristic of an aldohexose. It had R_{Glc} 2.6 in solvent *1*, and 1.3 in solvent *2*, moving slower than idose and faster than all other aldohexoses. On g.l.c., the derived alditol acetate had the same mobility as acetylated galactitol (T_{Man} 1.15) and moved more rapidly than acetylated glucitol and iditol.

The mass spectra of the alditol acetate and the 1-deuterio derivative were very similar to those of the corresponding derivatives of 4-*O*-methyl-D-galactose.

Demethylation[2] with boron tribromide gave D-glucose, identified by p.c. and reaction with D-glucose oxidase. Therefore, X_2 is 4-*O*-methyl-D-glucose.

The presence of these two sugars is further evidence of the diversity of the extracellular polysaccharides produced by slow-growing *Rhizobia*, particularly in comparison with those of the fast-growing group. *Rhizobium japonicum*, a species defined by its ability to nodulate soya beans, also belongs to the slow-growing group. Five strains of this species produce polysaccharides containing 4-*O*-methyl-D-galactose[3]. A variety of *O*-methyl sugars have been found in Gram-negative bacteria, but this appears to be the first report of the isolation of 4-*O*-methyl-D-glucose from a bacterial polysaccharide. It is evident that care is needed in interpreting results from demethylations using boron trichloride.

Mannose in rhizobial polysaccharides. In the original study of the lotus rhizobial polysaccharides[1], mannose was reported to be a common constituent. Fast-growing, acid-producing, rhizobial polysaccharides only contained small proportions of mannose, and recent work[7] suggests that this arose from contamination by traces

of yeast mannan present in the medium. This explanation seems unlikely for the slow-growing rhizobial polysaccharides, as the levels of mannose are much higher. Four strains were therefore grown on a purely synthetic medium free from mannose polymer. Hydrolysates of the isolated polysaccharides showed a component which was identical (p.c., solvents *1* and *2*) to mannose. Quantitative g.l.c. data showed that the levels of mannose relative to the other monosaccharides were in agreement with the analyses previously reported[1].

EXPERIMENTAL

General methods. — Solutions were concentrated in a rotary evaporator with bath temperatures less than 40°. G.l.c. was performed on a Varian 1440 instrument with a flame-ionization detector. A stainless-steel column (10 ft × 0.125 in.) containing 3% of ECNSS-M on Gas-Chrom Q, 100–120 mesh (Applied Science Laboratories), at 185° was used with on-column injection. G.l.c.–m.s. was effected with a Pye 104 gas chromatograph coupled to an AEI MS-30 mass spectrometer *via* either a silicone elastomer membrane or an all-glass jet separator (Scientific Glass Engineering, Melbourne, Australia). Mass spectra were recorded at an ionization potential of 70 eV and a source temperature of 200°. The following solvents were used for descending paper chromatography (p.c.) on Whatman No. 1 or (for preparative purposes) No. 3MM papers: (*1*) butyl acetate–pyridine–ethanol–water[1] (8:2:2:1), (*2*) ethyl acetate–pyridine–water[1] (12:5:4).

Sugars were demethylated[8] with boron tribromide in dichloromethane (1:1, v/v) for 3 days at room temperature. Residual reagent and solvent were removed under vacuum, and the residue, after repeated evaporations of methanol therefrom, was examined by p.c.

Isolation of the polysaccharide and purification of the sugars. — Organisms were grown and the polysaccharide was isolated as previously described[1]. For some experiments, cells were grown in a defined medium containing: K_2HPO_4 0.2 g/l, KH_2PO_4 0.3 g/l, $MgSO_4 \cdot 7H_2O$ 0.1 g/l, $CaCl_2$ 40 mg/l, $FeCl_3$ 10 mg/l, Na glutamate 0.5 g/l, mannitol 10 g/l, thiamin, biotin, and Ca pantothenate, 1 mg/l of each. The medium was adjusted to pH 6.8 with KOH; vitamins were sterilized by filtration, and added after the remainder of the medium had been autoclaved. Dialysed and freeze-dried polysaccharide was hydrolysed with 0.5M sulphuric acid (0.1 ml/mg, 4 h at 100°). Hydrolysates were neutralized with barium carbonate, filtered, and concentrated. 4-*O*-Methyl-D-glucose and 4-*O*-methyl-D-galactose were isolated by preparative p.c. or after fermentation of other sugars in the hydrolysates by yeast[1].

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