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## Note

# Extracellular polysaccharides of *Rhizobium*: identification of monosaccharides from strain CB756*

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This report describes the identification of 6-deoxy-L-talose, 3-*O*-methyl-D-glucose, 6-*O*-methyl-D-galactose, and seven other aldoses in hydrolysates of the extracellular polysaccharide of *Rhizobium* strain CB756. 6-*O*-Methyl-D-galactose had been incorrectly identified as fucose in an earlier investigation of this polysaccharide.

Previous publications from this Division have presented[1] an extensive survey of the composition of extracellular polysaccharides from *Rhizobium*, noted[1] the existence of several unidentified sugars in these polymers, and reported[2,3] the identification of three of them. The genus *Rhizobium* is commonly divided into two groups on the basis of their cultural behaviour; slow-growing, non-acid-producing organisms and fast-growing, acid-producing organisms. The former group synthesises extracellular polysaccharides in pure culture that are markedly heterogeneous in composition, in contrast to the latter group[1], which is not known to synthesise any unusual sugars. Mono-*O*-methyl sugars have been found in all three taxonomic divisions of the slow-growing group (*Rhizobium japonicum*, *Rhizobium lupini*, and the Cowpea group of *Rhizobium*); 4-*O*-methyl-D-galactose[2,4], 4-*O*-methyl-D-glucose[2], and 3-*O*-methyl-D-ribose[3] have been reported.

The Cowpea strain, CB756, was previously reported[3] to contain 3-*O*-methyl-D-ribose and at least one unidentified component. The present publication describes the identification of all the neutral sugars detected in hydrolysates of the extracellular polysaccharide of this strain; 6-*O*-methyl-D-galactose, 3-*O*-methyl-D-glucose, 6-deoxy-L-talose, and three more-common sugars have been found, in addition to components previously reported[1,3].

### RESULTS

*Identification of 6-deoxy-L-talose.* — The original work[1] reported an unknown sugar (designated "$X_3$"), provisionally identified as a 6-deoxyhexose. More recently[3],

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

the isolation and chromatographic properties [g.l.c., paper chromatography (p.c.) solvents *1* and *2*] of "$X_3$" have been described, and it was shown to be a mixture containing 3-*O*-methyl-D-ribose. Analytical p.c. in solvent *3* resolved the mixture into two components giving the colour reactions (aniline hydrogenphosphate[5]) of a pentose ($R_{Glc}$ 2.10) and a 6-deoxyhexose ($R_{Glc}$ 1.88), and preparative p.c. allowed isolation of small quantities of the individual compounds, together with a larger quantity of unresolved mixture. After reduction of the 6-deoxyhexose with sodium borohydride, and acetylation of the product with acetic anhydride–pyridine[6], g.l.c.–m.s. gave a spectrum having major fragments at *m/e* 43, 115, 170, 187, 231, 289, and 303. When $NaB^2H_4$ was used, the major fragments occurred at *m/e* 43, 116, 171, 188, 231, 290, and 303. These spectra are those expected[7] for a 6-deoxyhexose, and were essentially identical to those from corresponding derivatives of authentic L-rhamnose and L-fucose. The spectra previously obtained[3] from reduced and acetylated "$X_3$" were those expected for a mixture of 3-*O*-methylpentose and 6-deoxyhexose derivatives, with no extra peaks.

It has been shown[8] that all eight possible 6-deoxyhexoses (disregarding enantiomorphs) may be distinguished by a combination of p.c., t.l.c., and electrophoresis. The unknown 6-deoxyhexose chromatographed as a single compound in all three systems described[8], and in each had a mobility (relative to rhamnose) close to that reported for 6-deoxytalose. The relative mobilities of authentic L-fucose were also close to those reported[8]. The optical rotation was found to be negative, indicative of the L configuration[8]; with the instrumentation available there was insufficient material to allow accurate calculation of the specific optical rotation.

*Identification of* 3-O-*methyl*-D-*glucose.* — This sugar was detected moving slightly behind ribose ($R_{Glc}$ 2.8 *vs.* 3.0) in solvent *1*, and gave the colour reaction of a hexose. Preparative p.c. removed all but traces of ribose, and after reduction and acetylation, g.l.c.–m.s. gave major fragments at *m/e* 43, 87, 129, 189, and 261 (*m/e* 43, 88, 130, 190, and 261 for the 1-deuterio derivative). These spectra are characteristic[7] of a 3-*O*-methylhexose and were identical to those from derivatives of authentic 3-*O*-methyl-D-glucose. Demethylation[9] with boron tribromide gave glucose (p.c.), which was decomposed by D-glucose oxidase, thus establishing the D configuration. The chromatographic properties (p.c. solvents *1* and *2*, g.l.c.) of the compound were identical with those of authentic 3-*O*-methyl-D-glucose.

*Identification of 6-O-methyl-*D-*galactose.* — Although this compound closely resembled fucose in solvent *1*, its $R_{Glc}$ value was found to be slightly lower (2.2 *vs.* 2.3). After preparative p.c. followed by reduction and acetylation, g.l.c. gave a single peak having $T_{Man}$ 0.53 ($T_{Man}$ 0.24 for fucitol acetate), and g.l.c.–m.s. gave major fragments at *m/e* 43, 45, 87, 115, 129, 139, 157, 184, and 259 (*m/e* 43, 45, 87, 116, 129, 140, 158, 185, and 260 for the 1-deuterio derivative). These spectra were identical to those from corresponding derivatives from authentic 6-*O*-methyl-D-galactose, and the p.c. and g.l.c. properties of the unknown were also identical to those of authentic material. Demethylation[9] with boron tribromide gave galactose (p.c.); this was decomposed by D-galactose oxidase, thus demonstrating the D configuration.

*Identification of other sugars.* — Solvent *I* separates all eight aldohexoses, and the four aldopentoses; thus once a sugar has been assigned to one of these classes by m.s. it is readily identified. Allose, gulose, and mannose are not always clearly separated, but their alditol acetates are readily distinguished[10] by g.l.c. Similarly, xylose and lyxose, not always well separated on p.c., are unambiguously separated by electrophoresis[8].

Galactose, glucose, and mannose in total hydrolysates of polysaccharide co-chromatographed (p.c.) with standards; g.l.c. of reduced and acetylated total hydrolylysate also gave peaks having retention times corresponding to authentic material. Mass spectra of these three alditol acetates were very similar to each other and to mannitol hexaacetate. Configurations were not determined; they are presumed to be D by analogy with other work[4]. Arabinose and ribose were purified by preparative p.c.; they were identified by p.c. and g.l.c.–m.s. There was insufficient of either for determination of configuration. Rhamnose was isolated by preparative p.c., and identified by g.l.c.–m.s., and mobilities in the chromatographic[8] and electrophoretic[8] systems were used to identify 6-deoxytalose. Its configuration also remains undetermined.

## DISCUSSION

The extracellular polysaccharide from *Rhizobium* strain CB756 was initially reported[1] to contain galactose, glucose, mannose, fucose, and an unknown sugar, designated "$X_3$"; this was subsequently shown[3] to be a mixture containing 3-*O*-methyl-D-ribose. Reinvestigation of the polysaccharide has shown that the only other component of "$X_3$" is 6-deoxy-L-talose, and that the sugar previously identified as fucose is 6-*O*-methyl-D-galactose.

6-Deoxy-L-talose is known[11] as a constituent of cardiac glycosides, and has been isolated[12] from some *Mycobacterium* glycolipids. It is also found[13,14] in a number of lipopolysaccharides in the Enterobacteriaceae, and has been identified in the cell walls of *Actinomyces bovis*[15], *Streptococcus bovis*[16], and *Streptococcus cremoris*[17]. The D isomer has been reported[18] in the capsular polysaccharide from an unidentified, Gram-negative bacterium. The present work appears to be the first report of 6-deoxy-L-talose in *Rhizobium* or in an exocellular polysaccharide. 3-*O*-Methyl-D-glucose has been reported in Holothurin A[19], a sea-cucumber toxin, and as the non-reducing, terminal sugar of an unusual lipopolysaccharide from *Mycobacterium*[20], but not to my knowledge from any other source. 6-*O*-Methyl-D-galactose is a well-known component of some seaweed polysaccharides[21,22], but has not been reported elsewhere in Nature.

Hydrolysates of total, extracellular polysaccharide from *Rhizobium* strain CB756 thus contain 10 aldoses, and no chromatographic method has yet been found to separate all of them and allow quantitative analysis of the mixture. Interference by phthalic esters in g.l.c. analysis of sugars has been reported[23], and it has been emphasised[23] that g.l.c. retention-times do not in themselves constitute valid identification of sugar

derivatives. Semi-quantitative g.l.c. data are in general agreement with the original analysis, which reported[1] the contents of galactose, glucose, mannose, 6-*O*-methyl-D-galactose, and "$X_3$" (6-deoxy-L-talose plus 3-*O*-methyl-D-ribose) as 9, 37, 20, 1, and 13%, respectively. The "$X_3$" component is judged, on the basis of colour-reaction intensities in p.c., to have about twice as much 6-deoxy-L-talose as 3-*O*-methyl-D-ribose. The remaining aldoses identified (arabinose, ribose, 3-*O*-methyl-D-glucose, and rhamnose) each contribute less than 1% of the total weight of the polysaccharide. An eleventh monosaccharide, an as yet unidentified uronic acid, was found to make up 20% of the polysaccharide in the original analysis[1]. The complexity of this mixture makes it virtually certain that more than one polysaccharide is being produced; emphasis has been on the identification of all components present, rather than on purification of a specific polysaccharide. Ribose, in particular, is likely to be present from RNA released by lysed bacteria, although the finding of 3-*O*-methyl-D-ribose as a significant component suggests that not all the ribose is from this source.

The marked differences between the extracellular polysaccharide compositions of fast- and slow-growing *Rhizobia* have already been noted[1], and other differences are known[24]. In particular, some slow-growing strains are found to fix nitrogen in pure culture when grown in appropriate media; strain CB756 is being widely studied in this respect[25]. Further possible correlations between this nitrogen-fixing ability and polysaccharide composition are under investigation.

## EXPERIMENTAL

The general methods used have been described[2,3]. Solvents[1] used for descending p.c. on Whatman No. 1 or (for preparative p.c.) No. 3MM papers were: (*1*) 8:2:2:1, butyl acetate–pyridine–ethanol–water, (*2*) 12:5:4 ethyl acetate–pyridine–water, and (*3*) phenol, saturated with water at 20°. Electrophoresis[8] on Whatman No. 1 paper in borate buffer, pH 10.4, was conducted with a Miles Hivolt 10kV Electrophoresis Unit; 4kV for 60 min gave good separation of sugars.

*Rhizobium* strain CB756, from the Divisional collection maintained by R. M. Greenwood, was grown, and polysaccharide was isolated, as previously described[1]. The polysaccharide composition was not changed when bacteria were grown in a synthetic medium[2].

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