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Note

Rhizobium extracellular polysaccharides: isolation of 6-deoxy-*O*-methyl-hexoses

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Only a small number of strains of *Rhizobium japonicum* and *Rhizobium* sp. ("Cowpea" rhizobia) can be induced to fix nitrogen in pure culture, and we are investigating characteristics of these organisms in an attempt to explain why this ability should have such a limited distribution. A number of strains belonging to the slow-growing group of *Rhizobium* has been included in this survey, and some results pertaining to nitrogenase activity, antigenic affinity (seventy strains each), and extracellular polysaccharide composition (ten strains) have been reported[1].

The slow-growing rhizobia are known to synthesise extracellular polysaccharides that are markedly heterogeneous[2] and often contain *O*-methyl sugars[3–6]; in the present investigation, six strains were found to contain *O*-methyl sugars not previously reported in *Rhizobium* polysaccharides. Their identification as 3-*O*-methyl-

TABLE I

THE NEUTRAL SUGAR CONTENT[a] OF *Rhizobium* EXTRACELLULAR POLYSACCHARIDES

Sugar	Rhizobium *strain*					
	CB 627	*CB 2364*	*NZP 2073*	*NZP 2186*	*NZP 5052*	*QA 549*
Galactose	–	+	+	–	–	+
Glucose	+	+	+	+	+	+
Mannose	+	+	+	+	+	+
Ribose	–	–	tr	–	–	tr
6-*O*-Methyl-D-galactose	–	–	–	–	–	tr
3-*O*-Methyl-D-glucose	–	–	–	–	–	tr
3-*O*-Methyl-D-ribose	–	–	–	–	–	+
L-Fucose	–	+	+	–	–	–
L-Rhamnose	+	–	tr	+	+	+
3-*O*-Methyl-L-fucose	–	+	+	–	–	–
2-*O*-Methyl-L-rhamnose	–	–	–	–	–	–
3-*O*-Methyl-L-rhamnose	–	–	–	+	+	+

[a] +, Present; tr, trace component; –, not detected.

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L-fucose, 3-*O*-methyl-L-rhamnose, and 2-*O*-methyl-L-rhamnose is described in this paper.

Table I shows the identity of the neutral sugars found in acid hydrolysates of extracellular polysaccharides. It is to be noted that these preparations are crude, total polysaccharides; emphasis has been on the identification of all the neutral sugars present, rather than on the purification and analysis of a specific polysaccharide. Of the strains used, NZP2073, NZP2186, and NZP5052 are associated with *Lotus* species[2], whereas strains CB627, CB2364, and QA549 belong to the Cowpea group.

Identification of 3-O-methyl-L-fucose. — During paper chromatography (p.c.) of acid hydrolysates of polysaccharides in solvent *1*, this sugar was detected as a compound having mobility intermediate between rhamnose and 6-deoxytalose (R_{Rha} 1.3, *vs.* 1.0 and 1.55, respectively). It gave the colour reaction of a 6-deoxyhexose with aniline hydrogenphosphate[7]. The sugar was purified by preparative p.c. in solvent *1*. Following reduction with sodium borohydride and acetylation with acetic anhydride–pyridine[8], g.l.c. gave a single peak having T_{Man} 0.20 (T_{Rha} 1.0), and g.l.c.–m.s. gave a spectrum showing major fragments at *m/e* 43, 87, 101, 117, 129, 143, 189, and 203. This spectrum is that expected[9] for a 3-*O*-methyl-6-deoxyhexose. Kaufmann *et al.*[10] have shown that all of the possible 6-deoxyhexose and 3-*O*-methyl-6-deoxyhexose isomers may be distinguished by a combination of p.c. in solvent *2*, paper electrophoresis (p.e.) in borate buffer, and t.l.c. The first two techniques are sufficient to identify most of these sugars, and t.l.c. is only necessary to separate the *allo*, *gluco*, and *gulo* 3-*O*-methyl-6-deoxyhexose isomers. The 3-*O*-methyl-6-deoxyhexose described here had R_{Rha} 1.5 in solvent *2*; on p.e. it had M_{Glc} ~0.52 and migrated as a markedly elongated spot. These characteristics indicate that the sugar is 3-*O*-methylfucose (digitalose). This identification was confirmed by demethylation[11] of the sugar with boron tribromide. The product was identified as fucose by p.e., and by p.c. in solvents *1* and *2*. The 3-*O*-methylfucose from hydrolysates of strain NZP2073 polysaccharide was found to have a negative optical rotation, indicating the L configuration[10]; there was insufficient material to allow accurate calculation of a specific optical rotation.

Identification of 3-O-methyl-L-rhamnose. — This compound was observed as a fast-moving component, having R_{Rha} 1.8 in solvent *1* and the colour reaction of a 6-deoxyhexose. After preparative p.c. in solvent *1*, followed by reduction and acetylation, the compound gave a single peak on g.l.c. having T_{Man} 0.195–0.20, and g.l.c.–m.s. gave major fragments at *m/e* 43, 87, 101, 117, 129, 143, 189, and 203. This spectrum was essentially the same as that obtained from 3-*O*-methyl-L-fucose, and shows that the sugar in question is a 3-*O*-methyl-6-deoxyhexose[9]. The polysaccharide from strain QA549 was also found to contain a 3-*O*-methylribose, which was incompletely resolved from the 3-*O*-methyl-6-deoxyhexose in solvent *1* [R_{Rha} values of 1.7 (ref. 5) *vs.* 1.8, respectively]. For this strain, the two sugars were isolated as a mixture by preparative p.c. in solvent *1* and were reduced and acetylated as before. When NaB^2H_4 was used, g.l.c.–m.s. gave major fragments at *m/e* 43, 87, 88, 101, 117, 129, 130, 143, 189, 190, and 203; corresponding to a mixture of 3-*O*-methyl-6-deoxyhexose

and 3-*O*-methylpentose. The 3-*O*-methyl-6-deoxyhexose had R_{Rha} 2.1 on p.c. in solvent *2* and M_{Glc} 0.43 on p.e., consistent only with identification as 3-*O*-methylrhamnose (acofriose)[10]. In confirmation of this proposal, demethylation[11] with boron tribromide gave rhamnose, identified by p.c. in solvents *1* and *2* and by p.e. The optical rotation of the 3-*O*-methylrhamnose from strain NZP5052, and of the derived rhamnose from strain QA549 (purified by preparative p.c. in solvent *1* and shown to be free of ribose) were both positive, indicating the L configuration[10]. In neither case was there sufficient material available to allow calculation of an accurate, specific optical rotation.

Identification of 2-O-methyl-L-rhamnose. — This sugar was detected in polysaccharide hydrolysates as a very fast-moving compound in solvent *1* (R_{Rha} 1.9), showing the colour reaction of a 6-deoxyhexose. After preparative p.c. (solvent *1*), followed by reduction and acetylation, g.l.c. gave a single peak having T_{Man} 0.155, and g.l.c.–m.s. gave major fragments at *m/e* 43, 58, 87, 99, 113, 117, 129, 159, 173, 201, and 275 This spectrum establishes the sugar as a 2-*O*-methyl-6-deoxyhexose[9]. Demethylation[11] gave rhamnose, identified by p.c. in solvents *1* and *2*, and by p.e. The 2-*O*-methylrhamnose had R_{Rha} 2.6 in solvent *2*, and M_{Glc} 0.09 on p.e. The optical rotation was positive, indicating the L configuration[10]; there was insufficient material to allow calculation of an accurate, specific optical rotation.

Identification of other sugars. — 3-*O*-Methyl-D-ribose[5], 3-*O*-methyl-D-glucose[6] and 6-*O*-methyl-D-galactose[6] have recently been reported as constituents of *Rhizobium* polysaccharides. In the present work, these sugars were identified as previously described[5,6]: by g.l.c.–m.s., demethylation and p.c. of the product in solvent *1*, as well as by chromatographic identity with the previously isolated samples, and (for the last two) with authentic material. Shortage of material precluded determination of configurations. The remaining sugars were identified by g.l.c.–m.s. and p.c. in solvent *1*; plus p.c. in solvent *2* and p.e. for rhamnose and fucose. The utility of p.c. in solvent *1*, combined with g.l.c.–m.s., has previously been noted[6]. It has not yet proved possible to separate the 6-deoxyhexoses and their 3-methyl ethers by g.l.c. sufficiently well to allow quantitative analyses.

An earlier study reported[2] on the extracellular polysaccharide compositions of a number of *Rhizobia*, including strains NZP2073, NZP2186, and NZP5052. The present work adds 3-*O*-methyl-L-rhamnose to the previously known components of the last two strains. The original analysis[2] of strain NZP2073 polysaccharide reported galactose, glucose, mannose, and an unknown sugar, designated "X_2". This last sugar "X_2" was later isolated from strain NZP2154 and shown[4] to be 4-*O*-methyl-D-glucose. However, 4-*O*-methyl-D-glucose is not present in strain NZP2073 polysaccharide, and the previously unidentified sugar is fucose. The present work shows that 3-*O*-methyl-L-fucose and traces of ribose and rhamnose also occur in strain NZP2073 polysaccharide.

3-*O*-Methylfucose (digitalose) is known as a constituent of cardiac glycosides[10], as the free sugar from a brown seaweed[12], and in glycolipids from shellfish[13] and *Mycobacterium*[14]. The configuration of the sugar from the last three sources was not

determined. The present work appears to be the first report of 3-*O*-methylfucose of either configuration as a polysaccharide constituent, and the first definite identification of the L isomer outside the plant kingdom. 3-*O*-Methyl-L-rhamnose (L-acofriose) has been reported in cardiac glycosides[10], and in polysaccharides from plant resins[15] and a fresh-water red alga[16]. It has also been found in glycolipids from *Mycobacterium*[14,17,18] and lipopolysaccharides from *Klebsiella*[19], *Rhodopseudomonas*[20] and *Anabaena*[21], but has not previously been reported in *Rhizobium* or as a bacterial extracellular polysaccharide component. 2-*O*-Methyl-L-rhamnose is known as a constituent of soil polysaccharides[2] and the antibiotics Aranciamycin[23] and Scopamycin A[24], whereas 2-*O*-methylrhamnose of undetermined configuration has been found in *Mycobacterium* glycolipids[14,25]. The present report is thus the first of 2-*O*-methyl-L-rhamnose in a bacterial polysaccharide.

While these 6-deoxyhexose derivatives are not common in Nature and were previously unknown in *Rhizobium*, they have been identified in six strains out of 18 studied in the present work. On the other hand, Bailey *et al.*[2] did not report any of these compounds in a survey of the extracellular polysaccharides from 54 slow-growing *Rhizobium* strains. Although present work has shown some of these analyses to be incomplete, it is likely that few omissions remain, as it has been found in the present work that a methylated 6-deoxyhexose is always accompanied by the parent sugar, and few bacteria examined in the previous survey[2] produced a 6-deoxyhexose-containing polysaccharide. It seems, therefore, that *O*-methyl-6-deoxyhexoses are not common components in *Rhizobium* strains thus far examined, and thus presumably in rhizobia in general. Any significance they may have with regard to other properties of the bacteria remains obscure. Although the presence of *O*-methyl sugars in extracellular polysaccharides appears to be restricted to slow-growing strains of *Rhizobium*, in contrast to the fast-growing group which produces polysaccharides that are generally similar in composition and without unusual sugars[2], 2-*O*-methylfucose has recently been identified[26] in a lipopolysaccharide from *Rhizobium leguminosarum*, a member of the fast-growing group.

EXPERIMENTAL

The general methods used have been described[4,5]. Solvents[2,10] used for descending p.c. on Whatman No. 1 or (for preparative p.c.) No. 3 MM papers were: (*1*) 8:2:2:1 butyl acetate–pyridine–ethanol–water, (*2*) 1:2 toluene–1-butanol, saturated with water at 20°; the latter was used with water-loaded paper as described[10]. Electrophoresis[10] on Whatman No. 1 paper in borate buffer, pH 10.4, was performed with a Miles Hivolt 10-kV electrophoresis unit; 4 kV for 60 min gave good separation of sugars. Spray reagents used were aniline hydrogenphosphate[7] and *p*-aminohippuric acid–phthalic acid[10] for p.c. and p.e., respectively. For g.l.c. of sugars (as the alditol acetate derivatives), a stainless-steel column (2 m × 2 mm) containing 3% ECNSS-M on Gas-Chrom Q was used with on-column injection[8].

Rhizobium strains, from the Divisional collection maintained by R. M. Greenwood, were grown, and polysaccharide was isolated, as previously described[2].

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