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STRUCTURAL STUDIES OF THE *Rhizobium trifolii* EXTRACELLULAR POLYSACCHARIDE

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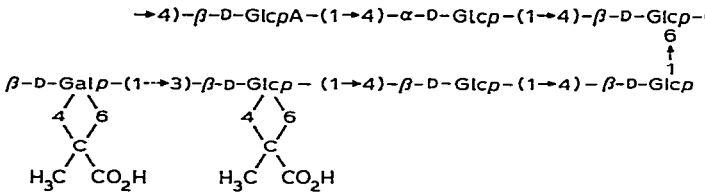
ABSTRACT

The structure of the extracellular polysaccharide of *Rhizobium trifolii* has been investigated. Methylation analysis, sequential degradations by oxidation and elimination of oxidized residues, uronic acid degradation, and degradation by oxidation of the acetylated polysaccharide with chromium trioxide in acetic acid were the main methods used. It is proposed that the polysaccharide is composed of heptasaccharide repeating-units having the following structure:



An unusual feature is that some of the repeating units are incomplete and lack the terminal β-D-galactopyranosyl group. The polysaccharide contains *O*-acetyl groups (somewhat more than 1 mol. per unit), linked to O-2 and O-3 of 4-*O*-substituted D-glucopyranosyl chain-residues. A previous finding that the polysaccharide contains 2-deoxy-D-*arabino*-hexose (2-deoxy-D-glucose) residues is erroneous.

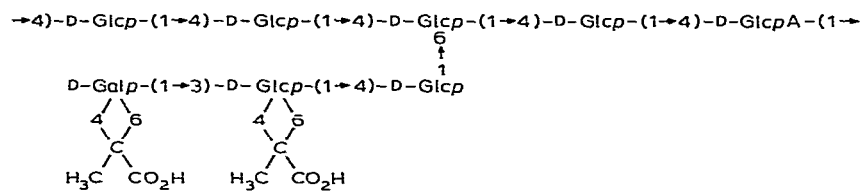
INTRODUCTION

The bacterium *Rhizobium trifolii* invades the roots of clover, and forms nodules from which the nitrogen fixed by the bacterium is transferred to the plant. The capsular polysaccharide elaborated by this organism has been investigated by several groups, the early work being summarized by Stacey and Barker[1].

Zevenhuizen[2] reported that the polysaccharide was composed of D-glucose, D-galactose, D-glucuronic acid, pyruvic acid, and *O*-acetyl groups in the proportions 5:1:2:2:3. The linkages present were determined by methylation analysis in conjunction with carboxyl-reduction. He also demonstrated that the extracellular poly-

saccharides from *Rh. leguminosarum* and *Rh. phaseoli* have closely similar, or possibly identical, structures.

Bishop and his co-workers[3] found D-glucose, D-galactose, D-glucuronic acid, pyruvic acid, and *O*-acetyl groups in the proportions 6:1:1.4:2:2. They subjected the polysaccharide to methylation analysis, also in combination with carboxyl-reduction of the methylated product and remethylation, and obtained results which were in good agreement with those of Zevenhuizen. They further investigated the products obtained on acid hydrolysis under mild conditions, when only ketal-linked pyruvic acid was removed, and under more severe conditions, when the terminal D-galacto-pyranosyl group and the penultimate D-glucopyranosyl residue, both pyruvylated, were selectively removed. They assumed that the polysaccharide was composed of octasaccharide repeating-units, and gave a structure (**1**) that summarized their results. Because of the low optical rotation of the polysaccharide, most sugars residues should be β-linked. This structure is tentative only, as some features, such as the structure at the branching point, the number of sugar residues in the side chain and in the main chain, respectively, and the mutual order of some sugar residues, were not determined.



Sømme[4] found D-glucose, D-galactose, D-glucuronic acid, pyruvic acid, and *O*-acetyl groups in the molar proportions 5:1:1:2:4 in the polysaccharide from one strain of *Rh. trifolii*. Polysaccharides from other strains of this species and from *Rh. leguminosarum* and *Rh. phaseoli* had similar compositions. She also reported the formation of oligosaccharides having two contiguous D-glucuronic acid residues on graded, acid hydrolysis of the polysaccharide[5]. If the polysaccharide is composed of oligosaccharide repeating-units, these should, from the composition reported by her, contain at least 14 sugar residues, which is a remarkably high number.

Dazzo and Hubbell[6] reported that the polysaccharide from *Rh. trifolii* contained D-glucose, D-galactose, and 2-deoxy-D-*arabino*-hexose (2-deoxy-D-glucose) residues. The latter sugar, which has not previously been found in polysaccharides, was thought to be responsible for the reaction between the polysaccharide and a lectin present in clover. However, their evidence for the presence of 2-deoxy-D-*arabino*-hexose in a hydrolysate of the polysaccharide is not only ambiguous, as it is based upon g.l.c. only, but also incorrect, as any 2-deoxy-D-*arabino*-hexose released during the hydrolysis (performed with 0.5M sulfuric acid at 100° for 8 h) should have been completely degraded.

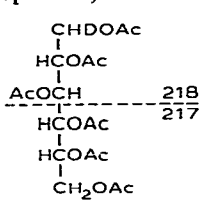
Most bacterial polysaccharides are composed of oligosaccharide repeating-units, generally containing 2–6 sugar residues. The capsular polysaccharide from *Rh. meliloti* is composed of octasaccharide repeating-units[7], the largest repeating-unit

that has been conclusively demonstrated. It seems possible that the *Rh. trifolii* capsular polysaccharide should also be composed of fairly large repeating-units, as suggested by Bishop and his co-workers[3]. It also seems possible that observed differences in composition between polysaccharides elaborated by various strains of *Rh. trifolii* and the closely related species *Rh. leguminosarum* and *Rh. phaseoli*[2-4] were due to imperfect analytical methods rather than to significant differences in structure. We now report further structural studies of the capsular polysaccharide from *Rh. trifolii*.

RESULTS AND DISCUSSION

The polysaccharide (PS), which was purified by precipitation with cetyltrimethylammonium bromide, had $[\alpha]_{578}^{22}$ −17°. An acid hydrolysate of the PS contained D-glucose and D-galactose in the ratio 7:1. The ^{1}H-n.m.r. spectrum showed, *inter alia*, signals at δ 1.48 (3.8 H), 2.14 (2.5 H), 4.53 (5.3 H), and 5.38 (1 H), assigned to methyl protons of pyruvic acid residues, methyl protons of *O*-acetyl groups, and anomeric protons of β-pyranosidic and α-pyranosidic sugar residues, respectively. The integrals were not very accurate, as the solution was viscous, leading to considerable broadening of the signals. Signals for protons linked to C-2 of presumed 2-deoxy-D-*arabino*-hexose residues were not observed and could hardly have been overlooked. The absence of such residues was also evident from the ^{13}C-n.m.r. spectrum.

In order to determine the proportion of uronic acid residues, the PS was hydrolysed with 2M trifluoroacetic acid at 100° for 2 h, the mixture of mono- and oligo-saccharides was reduced[8] with sodium borodeuteride and hydrolysed, the resulting monomers were reduced with sodium borodeuteride and acetylated, and the alditol acetates were analysed by g.l.c.–m.s. The D-glucitol hexa-acetate derived from D-glucose residues (**2**) gives the primary fragments *m/e* 217 and 218, whereas that derived from D-glucuronic acid residues (**3**) gives the corresponding fragments *m/e* 218 and 219. The proportions of *m/e* 217, 218, and 219, averaged from several spectra, were 5:6:1, thus giving a ratio for D-glucose and D-glucuronic acid of 5:1.



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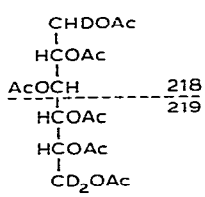

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Methylation analysis of the PS (Table I, column *A*) showed that it contains D-glucopyranosyl residues linked through O-4, through O-4 and O-6, and through O-3, O-4, and O-6, and D-galactopyranosyl residues linked through O-4 and O-6. Analysis of PS that had been methylated and carboxyl-reduced, using lithium borohydride, showed (Table I, column *B*) an increase of 2,3-di-*O*-methyl-D-glucose, demonstrating that the D-glucopyranosyluronic acid residues are linked through O-4. This finding was confirmed by analysing the remethylated material (Table I, column *C*),

TABLE I

METHYLATION ANALYSIS OF ORIGINAL AND CHEMICALLY MODIFIED *Rh. trifolii* EXTRACELLULAR POLYSACCHARIDES

Methylated sugar[a]	T[b]	T[c]	*Mole %*[d]							
			A	A_{calc}	B	B_{calc}	C	C_{calc}	D	E
2,3,4,6-Glc	1	1	—	—	—	—	2	—	10[e]	5
2,3,4,6-Gal	1.19	1.14	—	—	—	—	—	—	7[e]	10
2,4,6-Glc	1.82	1.72	—	—	—	—	—	—	7[e]	12
2,3,6-Glc	2.32	1.94	49	53	43	45	66	60	63	56
2,3-Glc	4.5	3.50	24	23	38	35	15	20	13	17
2,3-Gal	4.7	3.66	12	12	9	10	7	10	—	
2-Glc	6.6	5.06	15	12	9	10	9	10	—	

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, *etc.* [b]Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an OV-225 column at 180°. [c]As in *b*, but on an SP-1000 glass-capillary column at 220°. [d]Polysaccharide (for details, see text) *A*, original; *B*, methylated and reduced; *C*, methylated, reduced, and remethylated; *D*, methylated, reduced, remethylated, depyruvylated, and trideuteriomethylated; *E*, carboxyl-reduced, depyruvylated. [e]Partially trideuteriomethylated, see text.

when an increase in 2,3,6-tri-*O*-methyl-D-glucose was observed. The remethylation was also performed with trideuteriomethyl iodide. From the proportions of pertinent peaks in the mass spectrum of the alditol acetate derived from 2,3,6-tri-*O*-methyl-D-glucose, *e.g.*, *m/e* 233 and 236, it was evident that 25% of that sugar was derived from D-glucuronic acid residues. This confirms the 5:1 ratio for D-glucose and D-glucuronic acid residues in the PS.

When the methylated, carboxyl-reduced, and remethylated material was treated with 50% acetic acid at 100° for 90 min, the modified ketal groups were hydrolysed. The product was remethylated, using trideuteriomethyl iodide, and hydrolysed, and the sugars were analysed by g.l.c.–m.s. as their alditol acetates (Table I, column *D*). The appearance of 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,4,6-tri-*O*-methyl-D-glucose, both with trideuteriomethyl groups at O-4 and O-6, demonstrates that pyruvic acid is linked to these positions of terminal D-galactopyranosyl and 3-linked D-glucopyranosyl residues. The high percentage of 2,3,4,6-tetra-*O*-methyl-D-glucose was unexpected. Analysis of the mass spectrum of the derived alditol acetate demonstrates that it comes from three different sources. One part, with trideuteriomethyl groups at O-3, O-4, and O-6, must be due to hydrolysis of D-galactopyranosidic linkages during the mild hydrolysis with acid. Another part, with a trideuteriomethyl group at O-4, is probably due to β-elimination from non-reduced uronic acid residues. These terminals had already been observed in the methylated, reduced, and remethylated product (Table I, column *C*). However, the major part of the 2,3,4,6-tetra-*O*-methyl-D-glucose had trideuteriomethyl groups at O-4 and O-6, and must be derived from terminal D-glucopyranosyl groups, with pyruvic acid linked to O-4 and O-6, present in the original PS.

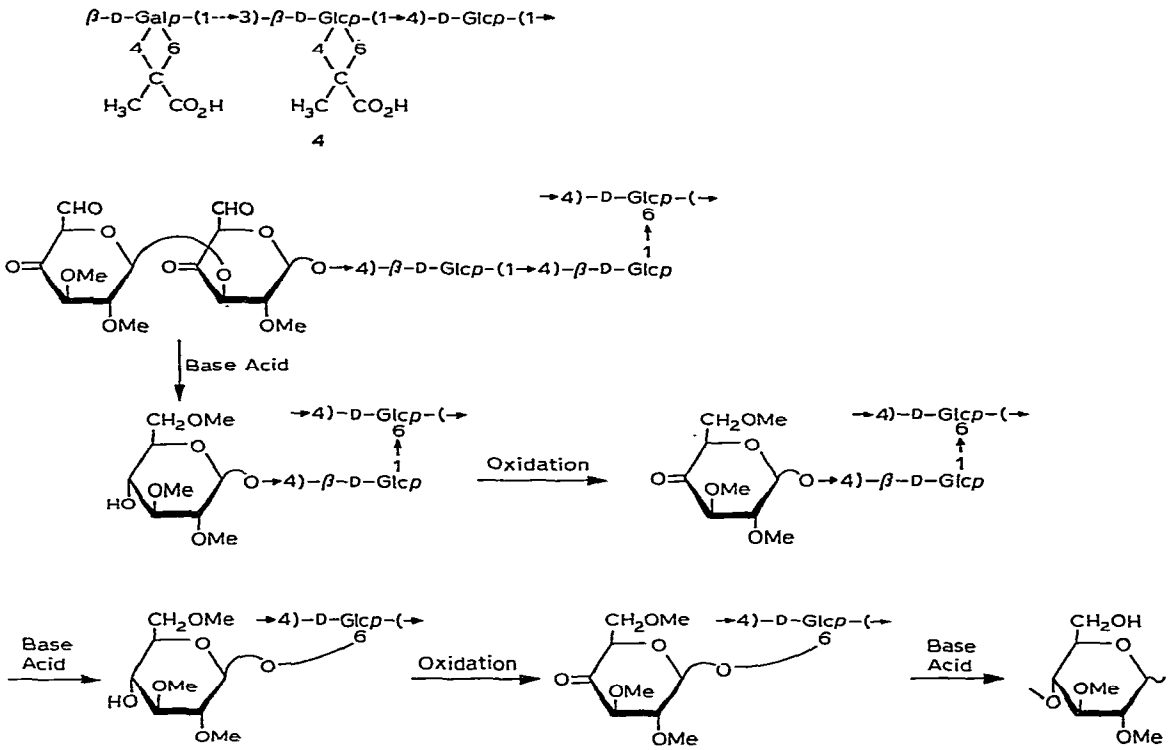
That some of the D-glucopyranosyl residues are terminal is evident from another experiment, in which the PS was carboxyl-reduced by the method of Taylor and Conrad[8], treated with 50% acetic acid at 100° for 2 h, and then subjected to methylation analysis (Table I, column *E*). It proved difficult to reduce the carboxyl groups of the uronic acid residues, and comparison with the analysis in Table I, columns *C* and *D*, indicates that only part of them had been reduced. The analysis further demonstrates that approximately two-thirds of the side chains in the PS are terminated by a D-galactopyranosyl group and one-third by a D-glucopyranosyl group.

Bacterial polysaccharides are generally composed of oligosaccharide repeating-units containing 2–6 sugar residues, but there are examples of repeating-units having 7 and 8 sugar residues. From the experimental evidence discussed above, it is not possible to construct such a repeating-unit for the *Rh. trifolii* PS. It seems possible, however, that the polysaccharide is composed of units containing five D-glucosyl residues and one D-glucosyluronic acid residue linked and substituted as indicated above, and that two-thirds of these units further contain a terminal, pyruvylated D-galactopyranosyl residue. The calculated values for methylation analysis of such a polysaccharide, with and without carboxyl-reduction and remethylation, are given in Table I (columns A_{calc}, B_{calc}, and C_{calc}). The formation of a methylated sugar, 2,3-di-*O*-methyl-D-glucose, from three different sources in the analysis of the methylated and carboxyl-reduced material, namely from branching residues, terminal pyruvylated groups, and carboxyl-reduced uronic acid residues, is unusual. The agreement between observed and calculated values is good and strongly supports the hypothesis that one-third of the "repeating-units" lack the terminal, pyruvylated D-galactopyranosyl group.

In the biosynthesis[9] of bacterial polysaccharides having a regular structure, the repeating-unit, linked to the pyrophosphate of an isoprenoid C_{55} alcohol, is first constructed by stepwise transfer of glycosyl groups from sugar nucleotides. The repeating-units are then transferred to the polymer. Although, to the best of our knowledge, polysaccharides in which some of the "repeating-units" are defective and lack a sugar residue have not been observed before, it seems possible that the transfer of a relatively large unit to the polymer may occur before the last glycosyl group in a side chain has been added. A somewhat similar situation exists in the *Xanthomonas campestris* capsular polysaccharide, which is composed of pentasaccharide units, but the terminal D-mannopyranosyl group in these is only pyruvylated in part of the units[10].

In order to study the sequence of sugar residues, the PS was methylated, carboxyl-reduced, remethylated, and treated with acid under mild conditions, during which essentially only the modified ketal groups should be hydrolysed. The depyruvylated residues were then degraded and eliminated by oxidation of the alcohol groups to carbonyl groups with chlorine–dimethyl sulfoxide–triethylamine[11], followed by treatment with base and then with acid under mild conditions (Scheme 1), a degradation previously used in structural studies of the capsular polysaccharide from *Rhizobium meliloti*[7]. Part of the oxidized product was hydrolysed, and the methylated

sugars were analysed, as their alditol acetates, by g.l.c.–m.s. (Table II, column *A*). The analysis demonstrated that the originally pyruvylated residues had been completely degraded. Part of the degraded product was remethylated, using trideuteriomethyl iodide, hydrolysed, and analysed as described above (Table II, column *B*). The 2,3,4,6-tetra-*O*-methyl-D-glucose observed in this analysis, with a trideuteriomethyl group at O-4, shows that a terminal D-glucopyranosyl group having a free hydroxyl at C-4 has been released during the degradation. ^{1}H-N.m.r. spectroscopy



Scheme 1

TABLE II

ANALYSIS OF DIFFERENT, DEGRADED *Rh. trifolii* POLYSACCHARIDES (FROM THE SEQUENTIAL DEGRADATION)

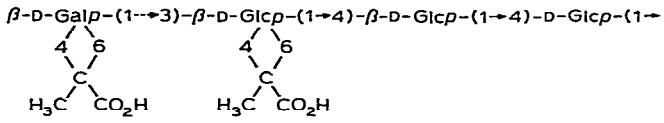
Methylated sugar[a]	*Mole %*[b]					
	A	B	C	D	E	F
2,3,4,6-Glc	2	22[c]	4	32[c]	4	34[c]
2,3,6-Glc	80	64	74	54	71	61[d]
2,3-Glc	18	14	22	14	25	4

[a]For explanation and retention times, see Table I. [b]Polysaccharide (for details, see text) *A*, first degradation, oxidized; *B*, first degradation, degraded and trideuteriomethylated; *C*, second degradation, oxidized; *D*, second degradation, degraded and trideuteriomethylated; *E*, third degradation, oxidized; *F*, third degradation, degraded and trideuteriomethylated. [c]Labelled at O-4 essentially. [d]25% labelled at O-6.

of the degraded, polymeric material demonstrated that it still contains the α-glycopyranosyl residue present in the original PS. The partial structure **4**, already established by Bishop and his co-workers[3], is thereby confirmed.

In the analysis of the degraded material, the percentage of 2,3,4,6-tetra-*O*-methyl-D-glucose, from terminals, is considerably higher than that of 2,3-di-*O*-methyl-D-glucose, from branching residues. The most probable explanation for this result is that the carboxyl-reduction of uronic acid residues was incomplete, resulting in β-elimination and formation of new end-groups during the subsequent treatment with base.

The product from the first degradation, with free hydroxyl groups at C-4 in the terminal D-glucopyranosyl group, was subjected to a second degradation, involving oxidation and treatment with base followed by acid under mild conditions, during which these groups should be eliminated[12,13]. Parts of the oxidized and the degraded materials were analysed as above (Table II, columns *C* and *D*). Again, a terminal D-glucopyranosyl group having a free hydroxyl at C-4 was released, and ^{1}H-n.m.r. spectroscopy demonstrated that the α-linked residue had not been affected. The partial structure **5** is thereby established.



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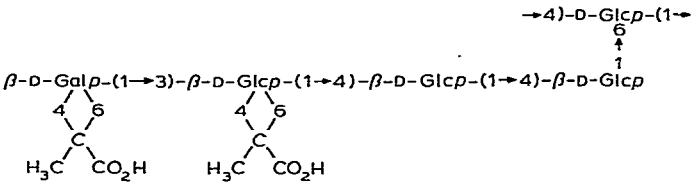
The product from the second degradation was subjected to a third degradation by the same procedure (Scheme 1). Analyses of the oxidized and the degraded, trideuteriomethylated materials are given in Table II (columns *E* and *F*). Only a small proportion of 2,3-di-*O*-methyl-D-glucose was found in the analysis of the latter product, indicating that the whole side-chain had been eliminated. As 2,3,6-tri-*O*-methyl-D-glucose was the only trimethyl ether found, the side chains should be linked to O-6 of the branching D-glucopyranosyl residue. This inference was confirmed by analysis of the mass spectrum of the derived alditol acetate, which showed that ~25% of the product had a trideuteriomethyl group at O-6. No such labelling was observed in 2,3,6-tri-*O*-methyl-D-glucose after the first or second degradation. Despite the side reactions, giving a high percentage of 2,3,4,6-tetra-*O*-methyl-D-glucose, it is therefore established that the PS has tetrasaccharide side-chains linked to O-6 of D-glucopyranosyl residues in the main chain. The degraded material still contained the α-linked residue, as demonstrated by ^{1}H-n.m.r. spectroscopy, and all sugar residues in the side chain are therefore β-linked, as in partial structure **6**.



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With four sugars in the side chain, each "repeating-unit" can contribute only three sugar residues to the main chain of the PS, namely, one D-glucopyranosyl residue linked through O-4 and O-6, one D-glucopyranosyl residue linked through O-4, and one D-glucopyranosyluronic acid residue linked through O-4. According to ^{1}H-n.m.r. evidence, one of these residues should be α-linked and the two others β-linked. The sequence of these residues was determined by uronic acid degradation[12] of a material that had been depyruvylated by acid hydrolysis under mild conditions. That some glycosidic linkages had simultaneously been hydrolysed was evident from the high percentage of 2,3,4,6-tetra-*O*-methyl-D-glucose obtained on methylation analysis of this material (Table III, column *A*). The depyruvylated product was methylated and treated with base, when the sugar linked to O-4 of the uronic acid residue was released by β-elimination and further degraded (Scheme 2). The hydrolysate of this product contained only a small proportion of 2,3-di-*O*-methyl-D-glucose (Table III, column *B*), demonstrating that it is the branching D-glucopyranosyl residue that is linked to this position. If not already cleaved during the treatment with base[14], the glycosidic linkage of the modified uronic acid should be released during the subsequent mild hydrolysis with acid. It was uncertain whether the sugar linked to O-4 of the modified branching-residue should be released during this treatment. In order to account for this, the product was reduced with sodium borohydride before trideuteriomethylation and hydrolysis. Analysis of the product (Table III, column *C*) showed that hydrolysis of this linkage was insignificant, as only traces of penta-*O*-methylglucitol were observed. About 50% of the 2,3,4,6-tetra-*O*-methyl-D-glucose found in this analysis was trideuteriomethylated on O-4, showing that the D-glucopyranosyluronic acid is glycosidically linked to this position of the chain D-glucopyranosyl residue, as in partial structure **7**.

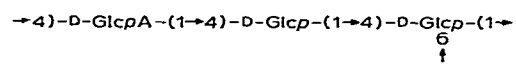

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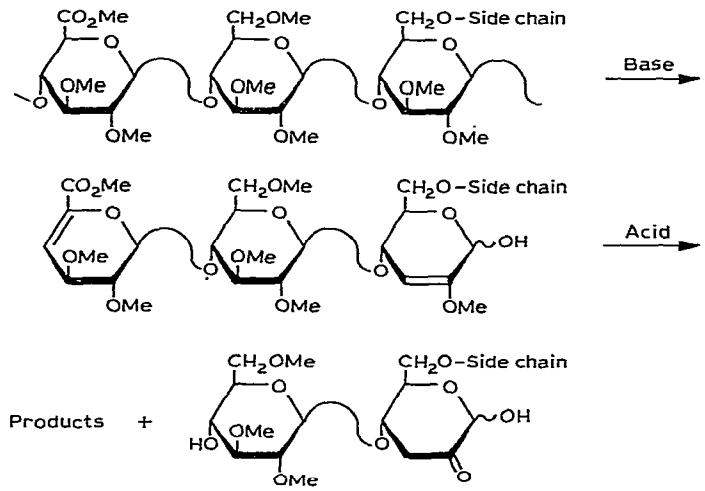
TABLE III

ANALYSIS FROM URONIC ACID DEGRADATION OF *Rh. trifolii*

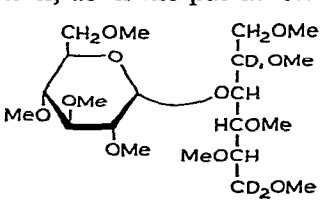
Methylated sugar[a]	*Mole %*[b]		
	A	B	C
2,3,4,6-Glc	15	17	31[c]
2,3,4,6-Gal	10	11	14
2,4,6-Glc	9	11	12
2,3,6-Glc	50	54	38
2,3-Glc	17	5	5

[a]For explanation and retention times, see Table I. [b]Polysaccharide (for details, see text) *A*, depyruvylated, methylated; *B*, uronic acid-degraded; *C*, degraded, trideuteriomethylated. [c]50% labelled at O-4.



Scheme 2

In order to locate the α-linked sugar residue, which, according to evidence discussed above, should be in the main chain, the PS was acetylated and oxidized with chromium trioxide in acetic acid. On this treatment, β-linked hexopyranosyl residues are oxidized to 5-hexulosonate residues, but the corresponding α-linked residues should be intact[12]. Sugar analysis of the product revealed that ~25% of the D-glucose, but only traces of the D-galactose, had survived. The product was reduced with sodium borohydride, during which treatment aldonic ester groups and carbonyl groups should be reduced and *O*-acetyl groups hydrolysed, and the disaccharide fraction enriched by gel filtration. A similar procedure has been used in structural studies of the *Klebsiella* type 37 capsular polysaccharide[15]. ^{1}H-N.m.r. spectroscopy of this fraction showed, *inter alia*, a strong signal at δ 5.1, assigned to the anomeric proton of an α-glucoside, and a weak signal at δ 4.5. In g.l.c.–m.s., the methylated product gave a main peak having the expected retention time and mass spectrum of permethylated maltitol. On reduction of a D-*xylo*-5-hexulosonate derivative, two products, a D-glucitol and an L-iditol derivative, should be obtained. These products were not separated in g.l.c., but a hydrolysate contained iditol and glucitol. That the alditol moiety of the disaccharide was derived from a 5-hexulosonate residue was demonstrated in an analogous experiment, in which the oxidized material was treated with sodium borodeuteride. In g.l.c.–m.s., the disaccharide component (**8**) showed the expected deuterium labelling. This experiment therefore demonstrates that the D-glucopyranosyl residue linked through O-4 in the main chain has the α-configuration, as in the partial structure **9**.



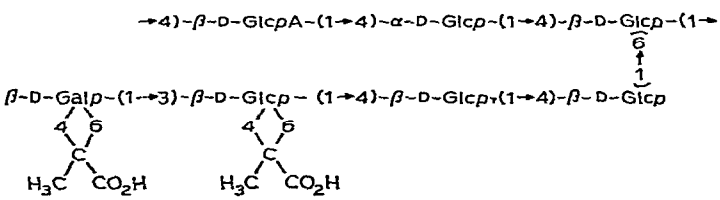
8

→4)-α-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→
6
↑

9

O-Acetyl groups in the PS were located[16] by reaction with methyl vinyl ether and an acidic catalyst, followed by methylation analysis of the product. *O*-Methyl groups in the resulting sugars are in the position of the original *O*-acetyl groups. This analysis yielded D-glucose (68%), D-galactose (10%), 2-*O*-methyl-D-glucose (5%), 3-*O*-methyl-D-glucose (9%), and 3,4-di-*O*-methyl-D-glucose (8%). Uronic acid degradation occurs during this analysis[17], and the methyl group at O-4 in 3,4-di-*O*-methyl-D-glucose is introduced after such a degradation. In another experiment, the product obtained after treatment with methyl vinyl ether was methylated, treated with acid under mild conditions that essentially removed only the acetal protecting-groups, and remethylated with trideuteriomethyl iodide. A hydrolysate of this material contained 2,3,4,6-tetra-*O*-methyl-D-glucose (15%), 2,3,6-tri-*O*-methyl-D-glucose (45%), 2,3-di-*O*-methyl-D-glucose (16%), 2,3-di-*O*-methyl-D-galactose (11%), and 2-*O*-methyl-D-glucose (13%). Nondeuterated methyl groups were detected in the tetra-*O*-methyl-D-glucose (70%) and in the 2,3,6-tri-*O*-methyl-D-glucose (25%), but not in the other sugars. The results demonstrate that the PS contains somewhat more than 1 mol. of *O*-acetyl per unit and that these groups are linked to positions 2 and 3 of the 4-linked D-glucopyranosyl chain-residues. It seems possible that, in the biosynthesis, only one of these positions is acetylated and that some acetyl migration has occurred. A major part seems to be linked to the α-D-glucopyranosyl residue in the main chain.

As a result of these studies, structure **10** is proposed for the "repeating-unit" of the *Rh. trifolii* capsular polysaccharide. *O*-Acetyl groups are linked to 4-substituted D-glucopyranosyl residues as discussed above. Unit **10** is not a repeating-unit in the true sense, as approximately one-third of the units lack the terminal β-D-galactopyranosyl residue. This is a most unusual structural feature. Disregarding this feature, the structural studies indicated a regular structure, and there does not seem to be any alternative interpretation of the results. The structure has many features in common with that suggested by Bishop and his group[3]. As they expected that the polysaccharide was composed of true repeating-units, they had to assume a larger repeating-unit. However, from the present results, it is not possible to draw a structure having two adjacent D-glucopyranosyluronic acid residues, as proposed by Sømme[5].



10

The structural studies were complicated by several factors. A major problem was the difficulty in achieving complete carboxyl-reduction of the uronic acid residues. The ease with which the terminal β-D-galactopyranosyl groups were hydrolysed caused some further complications. Before it was suspected that part of the "repeating-units" might be incomplete, it was also difficult to interpret some results. For these

reasons, all the degradations discussed above were repeated several times, with consistent results. We therefore believe that the proposed structure is fairly well established.

EXPERIMENTAL

General methods. — For g.l.c., Perkin–Elmer 990 or Hewlett Packard 5380A instruments, fitted with flame-ionisation detectors, were used. Separations were performed on SP-1000 WCOT glass-capillary columns (25 m × 0.25 mm) at 220° and on 3% of OV-225 on Gas Chrom Q in glass columns (180 × 0.15 cm) at 190° (for partially methylated alditol acetates), and on 3% of OV-1 on Gas Chrom Q in glass columns (180 × 0.15 cm) at 210° (for methylated oligosaccharides). G.l.c.–m.s. was performed on a Varian MAT 311 instrument, using the two OV-phases. Gel filtrations were performed on Biogel P-2 with water as irrigant, and effluents were monitored with a differential refractometer. Some separations were performed on Sephadex LH-20 with chloroform–acetone (2 : 1) as irrigant. The effluent was monitored by spot tests on t.l.c. plates (charring with sulfuric acid). For n.m.r. spectra, a JEOL FX-100 instrument was used. Samples were dissolved either in deuterium oxide (internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate reference), or in deuteriochloroform (internal tetramethylsilane). Samples in deuterium oxide were studied at 85°. Carboxyl-reductions of underivatised materials were performed according to the procedure of Taylor and Conrad[8]. Carboxyl-reduction of methylated products was performed with lithium borohydride in tetrahydrofuran. Methylations were performed according to Hakomori[18] with sodium methylsulfinylmethanide–methyl iodide in dimethyl sulfoxide, and the aliquots taken were ~1 mg. Methylated products were recovered by dialysis against water, followed by freeze-drying (Method A). For materials of low molecular weight, or when partial degradation was suspected, dimethyl sulfoxide was removed by freeze-drying, and the product was isolated by partition between chloroform and water. When necessary, it was further purified by chromatography on Sephadex LH-20 (Method B). Hydrolysis and analysis of the products was performed as previously described[19].

Isolation and purification of the polysaccharide. — Growth of *Rh. trifolii*, strain U 226, isolation, and purification were as described for *Rh. meliloti*[20].

Sequential degradations. — (*a*) *First degradation.* The polysaccharide (120 mg) was methylated, and the product (135 mg) dissolved in tetrahydrofuran (20 ml). Lithium borohydride (100 mg) was added and the mixture was boiled under reflux for 6 h. Excess of borohydride was destroyed with water, and the solution was concentrated and partitioned between chloroform and water. The resulting polysaccharide was remethylated and recovered by Method B (yield, 80 mg). Treatment with 50% aqueous acetic acid (5 ml) at 100° for 90 min, followed by concentration, yielded the depyruvylated polysaccharide (75 mg). Part of the material was withdrawn, remethylated with trideuteriomethyl iodide, and analysed (Table I, column *D*). The rest was oxidized[11] with M chlorine in dichloromethane (25 ml), dimethyl sulfoxide (9 ml), and triethylamine (7 ml) for 6 h at −45°, and the product was purified by

Method B (yield 90 mg). An aliquot was hydrolysed and analysed (Table II, column *A*). The remaining material in dichloromethane (5 ml) was treated with M sodium ethoxide in ethanol (1 ml) for 1.5 h at room temperature, neutralized with dilute hydrochloric acid, and concentrated. Aqueous acetic acid (50%, 5 ml) was added, and the mixture was kept at 100° for 3 h, concentrated, partitioned between chloroform and water, and purified by gel filtration (yield, 33 mg). Part of the material was withdrawn and remethylated with trideuteriomethyl iodide, hydrolysed, and analysed (Table II, column *B*). ^{1}H-N.m.r. spectroscopy of the degraded material showed signals for the α- and β-anomeric protons in the ratio 1:5.4.

(*b*) *Second degradation.* The remaining material was oxidized and worked-up as described above (yield 33 mg). An aliquot was hydrolysed and analysed (Table II, column *C*). The material was then dissolved in dichloromethane (1 ml), treated with 0.6M sodium ethoxide in ethanol (1 ml), worked-up as before, and treated with 50% aqueous acetic acid (2 ml) at 100° for 14 h. After purification of the degraded material (15 mg), an aliquot was remethylated with trideuteriomethyl iodide, hydrolysed, and analysed (Table II, column *D*). ^{1}H-N.m.r. spectroscopy of the degraded material showed signals for the α and β anomers in the ratio 1:4.4.

(*c*) *Third degradation.* This was performed as described in (*b*), and the results are recorded in Table II (columns *E* and *F*). ^{1}H-N.m.r. spectroscopy of the degraded material showed signals for the α and β anomers in the ratio 1:2.8.

Uronic acid degradation. — The polysaccharide was depyruvylated by treatment with hydrochloric acid (pH 2) for 4 h at 100°, dialysed, recovered by freeze-drying, and methylated. Analysis of this material is given in Table III (column *A*). A solution of the methylated polysaccharide (8 mg) and a catalytic amount of *p*-toluenesulfonic acid in a mixture of methanol (5 ml) and 2,2-dimethoxypropane (0.5 ml) was boiled under reflux for 30 min. Freshly cut sodium (60 mg) was added and the solution boiled under reflux for 2 h.

The analysis of the degraded material is given in Table III (column *B*). After treatment with Dowex-50(H^+) resin, the solution was concentrated, the residue dissolved in 50% aqueous acetic acid (5 ml), and the solution kept at 100° for 2 h. Partition between chloroform and water and concentration of the organic phases yielded degraded polysaccharide (4.5 mg). The product was reduced with sodium borohydride in ethanol–water (3:1, 3 ml) for 5 h at room temperature, trideuteriomethylated, and analysed (Table III, column *C*).

Chromium trioxide oxidation. — The polysaccharide (20 mg) was dissolved in a mixture of formamide (10 ml), pyridine (5 ml), and acetic anhydride (5 ml). The mixture was stirred at room temperature for 24 h, dialysed, and freeze-dried. To ensure complete acetylation, this material was kept in pyridine–acetic anhydride (1:1, 5 ml) for 1 h at 100°.

The acetylated polysaccharide was dissolved in acetic acid (5 ml), and *myo*-inositol hexa-acetate (2 mg) was added as the internal standard. Part of the solution was withdrawn for sugar analysis. Chromium trioxide (400 mg) was added to the main part, and the mixture was kept in an ultrasonic bath at 50° for 1 h. The reaction

mixture was diluted with water (5 ml) and extracted with chloroform (3 × 7 ml). The combined organic phases were washed with water (3 × 7 ml) and concentrated. An aliquot was taken for sugar analysis. D-Glucose and D-galactose were obtained in 25% and 10% of the original values. The residue was dissolved in ethanol–1,4-dioxane (1 : 1, 6 ml), sodium borohydride (100 mg) was added, and the mixture was stirred overnight. The mixture was diluted with water and treated with Dowex-50(H^+) resin, and boric acid was removed by codistillation with methanol. The resulting material was fractionated on Biogel P-2, and material in the disaccharide region was investigated by ^{1}H-n.m.r. spectroscopy and by sugar and methylation analysis. ^{1}H-N.m.r. spectroscopy showed, *inter alia*, signals at δ 5.1 and 4.5 (weak). Hydrolysis and reduction yielded glucitol and iditol in the proportion 2.1 : 1. The permethylated disaccharide alditol was analysed by g.l.c.–m.s., using an OV-1 column. The main peak was obtained at T_{Mel} (retention time relative to permethylated melibiitol) 0.71. The mass spectrum was indistinguishable from that given by permethylated maltitol.

O-*Acetyl determination.* — The polysaccharide (10 mg) and *p*-toluenesulfonic acid (1 mg) were dissolved in dimethyl sulfoxide (5 ml) in a sealed serum-flask. Methyl vinyl ether (3 ml) was added and the solution kept at 15° for 3.5 h. Excess of methyl vinyl ether was evaporated, and the resulting solution was used directly for methylation. The product was purified by gel filtration on Sephadex LH-20, by elution with acetone. An aliquot was taken for hydrolysis and analysis. D-Glucose (68%), D-galactose (10%), 2-*O*-methyl-D-glucose (5%), 3-*O*-methyl-D-glucose (9%), and 3,4-di-*O*-methyl-D-glucose (8%) were obtained. The main part of the product was treated with 25% aqueous acetic acid and acetone (9 : 1, 3 ml) for 1 h at 100°. The mixture was then remethylated, hydrolysed, and analysed. 2,3,4,6-Tetra-*O*-methyl-D-glucose (15%), 2,3,6-tri-*O*-methyl-D-glucose (45%), 2,3-di-*O*-methyl-D-glucose (16%), 2,3-di-*O*-methyl-D-galactose (11%), and 2-*O*-methyl-D-glucose (13%) were obtained.

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