Demonstration of an Octasaccharide Repeating Unit in the Extracellular Polysaccharide of *Rhizobium meliloti* by Sequential Degradation

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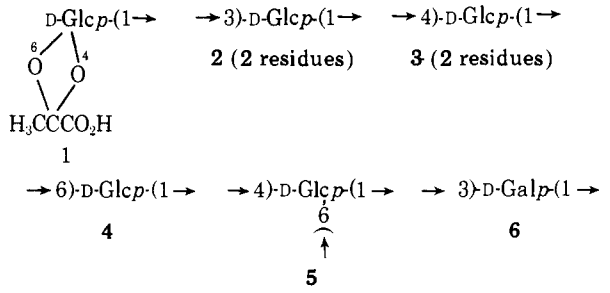
Abstract: The structure of the extracellular polysaccharide from *Rhizobium meliloti*, a microsymbiont in the nitrogen fixing symbiosis, has been investigated. The polysaccharide contains a terminal β-D-glucopyranosyl group with pyruvic acid ketalically linked to its 4 and 6 positions. After removal of this substituent from the methylated polysaccharide the four sugar residues in the side chain were removed sequentially by specific degradations; each of these steps involved oxidation, β-elimination by treatment with base, and, when necessary, acid hydrolysis under mild conditions. The result of each degradation was followed by trideuteriomethylation, hydrolysis, and analysis of the product by GLC/MS. The sequence of the sugar residues in the main chain was determined using a modified Smith degradation in which the polyalcohol, obtained after periodate oxidation–borohydride reduction, was methylated before the mild acid hydrolysis. As a result of these studies, it is concluded that the polysaccharide is composed of octasaccharide repeating units with the structure **25.**

Biosynthetic and structural studies have revealed that many bacterial polysaccharides are composed of oligosaccharide repeating units[2] and the largest of these which have been conclusively demonstrated are hexasaccharide repeating units (cf. ref 2–4). There is strong justification for this occurrence of small repeating units since larger ones would require more complex systems of enzymes for their biosynthesis. Despite this, however, preliminary studies on the extracellular polysaccharide from *Rhizobium meliloti* have indicated that it should either be composed of octasaccharide repeating units or have a less regular structure.[5,6] We now report the structural elucidation of this polysaccharide, using specific degradations.

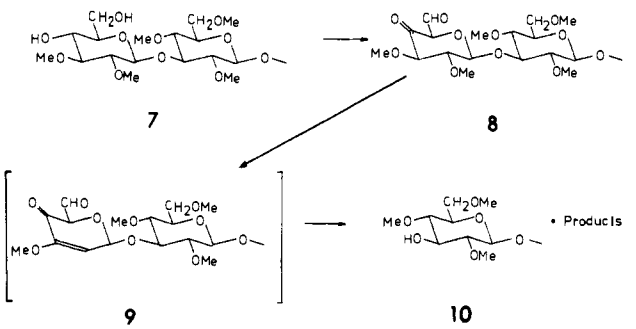
Results and Discussion

The polysaccharide (PS) contained D-glucose, D-galactose, pyruvic acid, and *O*-acetyl groups in the approximate proportions 7:1:1:1.[5] Methylation analysis,[7,8] including GLC/MS analysis of partially methylated alditol acetates (Table I, line A), showed that it contained the structural elements **1**–**6**.[5,6]



The low optical rotation of the PS, $[\alpha]_{578}$ −4°, indicated that all sugar residues were β linked. This was supported by the ^{1}H NMR spectrum of the PS, in which signals in the region for anomeric protons were observed at δ 4.5–4.8. α-Linked D-glucopyranosyl or D-galactopyranosyl residues should give signals at lower fields. *O*-Acetyl groups had been located on O-6 of the 3- and 4-substituted D-glucopyranosyl residues in earlier work.[5]

We have recently described a method for the specific degradation of polysaccharides starting from a derivative which is methylated in all but a few defined positions.[9,10] Such a polysaccharide is oxidized and subjected to β-elimination by base treatment and subsequent mild acid hydrolysis, with the result that the residues with free hydroxyls are degraded, and the sugars linked to these residues are released as nonreducing and reducing moieties. The terminal group **1** in the *Rhizobium meliloti* PS offers a suitable point of attack for this degradation. After carboxyl reduction,[11] performed in order to render the ketalic linkages of the pyruvic acid moieties more sensitive to acid hydrolysis, the PS was methylated and hydrolyzed with acid under conditions chosen to preclude cleavage of glycosidic linkages. The treatment was monitored by methylation analysis of part of the material using trideuteriomethyl iodide; 1 mol of 2,3,4,6-tetra-*O*-methyl-D-glucose with trideuteriomethyl groups at O-4 and O-6 was obtained in place of 1 mol of 2,3-di-*O*-methyl-D-glucose (Table I, line B). Thus, this treatment gave a modified PS with the terminal **7** (in this and other formulas, the structure to be demonstrated by a degradation is anticipated). On oxidation of this material with chlorine-dimethyl sulfoxide–triethylamine[12] to **8,** carbonyl groups were



introduced. The oxidation was monitored by hydrolyzing an aliquot; a decrease in the amount of 2,3-di-*O*-methyl-D-glucose was observed (Table I, line C). It has been demonstrated, using model substances,[13] that the glycosidic linkage of a dicarbonyl sugar residue (as in **8**) is broken when it is treated first with alkali to give the hypothetical intermediate **9,** and subsequently with acid under mild conditions. Part of the polymeric residue of the PS (**10**) obtained after this treatment was remethylated, using trideuteriomethyl iodide, and hydrolyzed. Analysis of the hydrolysate (Table I, line D) showed, inter alia, that it contained 2,3,4,6-tetra-*O*-methyl-D-glucose with a trideuteriomethyl group at O-3. The terminal groups in the PS are consequently linked to 3 positions in D-glucopyranose residues.

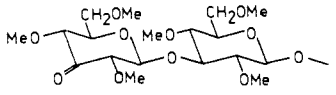
The modified polysaccharide **10,** which was methylated in all positions except at O-3 of the new terminal, was subjected

Table I. Analyses of Original and Degraded *R. meliloti* Polysaccharides

Polysaccharide material	Methylated sugar,[a] mol % (relative proportions)				
	2,3,4,6-Glc T^b = 1.00	2,4,6-Glc T^b = 1.72	2,3,6-Glc and 2,4,6-Gal T^b = 1.94	2,3,4-Glc T^b = 2.00	2,3-Glc T^b = 3.50
A		28 (2.5)	34 (3)	15 (1.3)	23 (2.0)
B	14[c] (1.3)	28 (2.5)	33 (3)	14 (1.3)	12 (1.1)
C		30 (2.3)	40 (3)	16 (1.2)	14 (1.1)
D	14[d] (1.1)	17 (1.3)	38 (3)	17 (1.3)	13 (1.0)
E		18 (1.2)	45 (3)	17 (1.1)	20 (1.3)
F	13[d] (0.9)	7 (0.5)	44 (3)	18 (1.2)	19 (1.3)
G		10 (0.6)	51 (3)	18 (1.1)	22 (1.3)
H	21[e] (1.2)	8 (0.5)	51 (3)	7 (0.4)	13 (0.8)
I		8 (0.4)	58 (3)	12 (0.6)	22 (1.1)
J	17[f] (0.9)	9 (0.5)	38[g] (2.1) 18 (1)	9 (0.5)	8 (0.4)

[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 SP-1000 W.C.O.T. column at 220 °C. [c] Trideuteriomethyl groups at O-4 and O-6. [d] Trideuteriomethyl group at O-3. [e] Present as the 6-*O*-trideuteriomethyl and 3-*O*-trideuteriomethyl derivatives in the ratio 3:1. [f] Present as either the 6-*O*-trideuteriomethyl or 3-*O*-trideuteriomethyl derivative. [g] 2,3,6-Tri-*O*-methyl-D-glucose partially labeled with a trideuteriomethyl group at O-6 and 2,4,6-tri-*O*-methyl-D-galactose were separated on an OS-138 S.C.O.T. column.

to a second degradation involving oxidation and treatment with base. This oxidation was monitored as before; a decrease in the amount of 2,4,6-tri-*O*-methyl-D-glucose was observed (Table I, line E). The glycosidic linkage of the oxidized group **11** was cleaved on treatment with base as had been demonstrated in model experiments.[14,15] Using the same methods as above it was shown that a new terminal D-glucopyranosyl group (**12**), with a free hydroxyl at C-3, was formed (Table I, line F).



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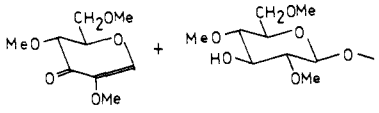

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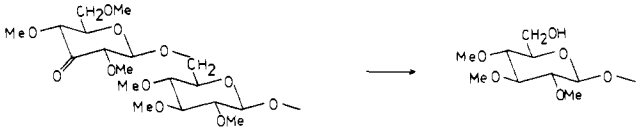
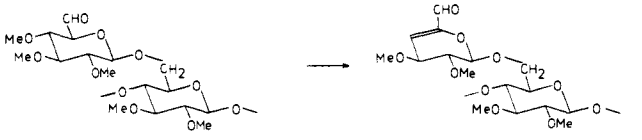
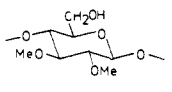
The third degradation involved oxidation of **12** to **13** followed by treatment with base; it was analogous to the second degradation, except that a polymer (**14**) with a terminal D-glucopyranosyl group, having a free hydroxyl at C-6, was formed. The oxidation step was again monitored by hydrolyzing and analyzing part of the material (Table I, line G) while the degradation was monitored by methylation analysis



13 **14**



15 **16**



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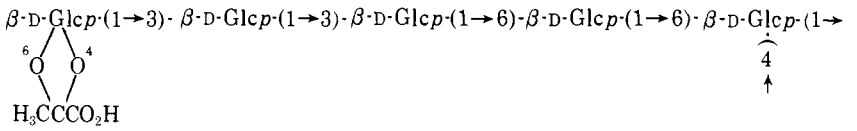
of part of the material using trideuteriomethyl iodide (Table I, line H).

On oxidation of **14** a polymer (**15**) in which the terminal sugar residue had an aldehyde group at C-6 was produced. (The oxidation was monitored as before (Table I, line I).) The degradation of such residues by treatment with base followed by mild acid hydrolysis is analogous to the degradation of fully methylated, esterified uronic acid residues[16] and has been previously investigated.[17] The unsaturated residue (e.g., that in **16**) most probably gives a furan derivative and the substituents at O-1, O-2, O-3, and O-4 are released. Analysis of the polymeric product (**17**) (Table I, line J) showed that the hydroxyl at C-6 of the originally branching residue in the PS had been exposed as a result of the fourth degradation.

It should, in principle, be possible to subject **17** to a fifth degradation, but this would prove difficult in practice. Firstly, such a degradation would be more complicated than the previous degradations during which only nonreducing terminal sugar groups were eliminated; in this reaction the substituent sugar at C-4 would be eliminated as a reducing sugar residue which would be further degraded under the alkaline conditions. Secondly, although proceeding to a high extent, the previous degradations had not been complete and material from incomplete reaction sequences had accumulated (cf. Table I, lines H and J) and might therefore have obscured the results of the fifth degradation.

The four consecutive degradations, each of which gave an unambiguous result, demonstrated the structural element **18,** containing the side chain and the branching point of the PS. Residues **1, 2** (both residues), **4,** and **5** were thus accounted for and the finding of an ordered structure for them strongly suggested that the whole PS had a regular structure and that it was composed of octasaccharide repeating units.

The remaining residues, **3** (both residues) and **6,** should be present in the main chain, and there were three possible alternatives for their mutual arrangement. In order to decide between these, the native PS was subjected to a Smith degradation.[10,18] The only sugar residue in this part of the polymer (**19**) which should not be oxidizable by periodate is the D-galactopyranose residue (**6**) and hence reduction of the periodate oxidized PS gave the polyalcohol **20a.** It should be possible, by mild acid treatment of **20a,** to hydrolyze selectively the noncyclic acetals, yielding 2-*O*-β-D-galactopyranosyl-D-erythritol (**21**), but, since the erythritol moiety in **21** could derive from either a 4-substituted or a 4,6-disubstituted D-glucopyranose residue, the isolation of this substance would



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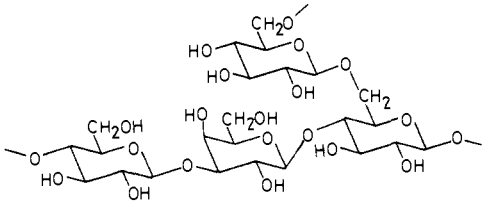

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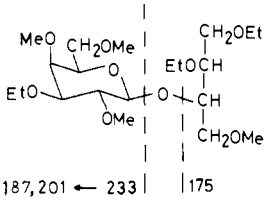

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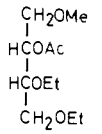

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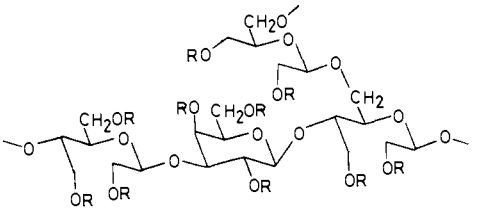
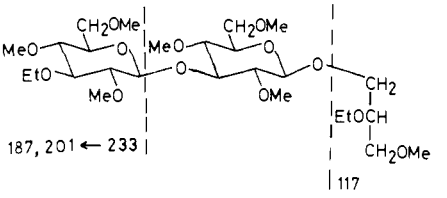


20 a R = H
b R = Me



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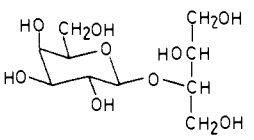

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not give any further structural information. However, by methylating the polyalcohol (as described in similar studies[3,4,10]) to **20b,** before the mild acid hydrolysis, the pattern of methyl substitution in the derived D-galactosylerythritol should depend upon whether it originated from a chain or a branching residue. The oligomeric degradation products were realkylated with ethyl iodide and investigated by GLC/MS.[19] Two components were detected, the first of which was identified from its mass spectrum as the glycosylerythritol derivative **22;** the origins of some fragments are indicated in the formula. The realkylated product was also hydrolyzed, reduced, acetylated, and investigated by GLC/MS[8] and among the hydrolysis products 2-*O*-acetyl-3,4-di-*O*-ethyl-1-*O*-methyl-D-erythritol (**23**) was identified. The results of this modified Smith degradation therefore demonstrated that the D-galactopyranose residue (**6**) was linked to O-4 of the branching D-glucopyranose residue (**5**). Identification by GLC/MS of the second component after the realkylation, a laminarobiosylglycerol derivative (**24**), further corroborated a sequence of three sugar residues already demonstrated as part of the side chain.

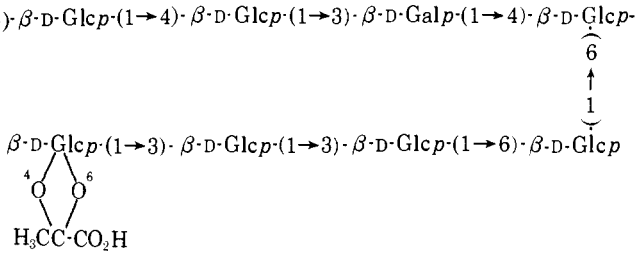
From the combined evidence structure **25** is proposed for the repeating unit of the *Rhizobium meliloti* extracellular polysaccharide. This octasaccharide is the largest repeating unit that has been demonstrated for a bacterial polysaccharide. The elucidation of its structure also illustrates the versatility of the specific degradations used.

Experimental Section

General. For GLC, Perkin-Elmer 990 or Hewlett-Packard 5380A instruments fitted with flame-ionization detectors were used. Separations were performed on (a) SP-1000 W.C.O.T. glass-capillary columns (25 m × 0.25 mm) at 220 °C (for partially methylated alditol acetates); (b) OS-138 S.C.O.T. metal-capillary columns (15 m × 0.5 mm) at 200 °C (for partially methylated alditol acetates); and (c) 3% OV-1 on Gas Chrom Q (100/120 mesh) packed in 180 × 0.15 cm glass tubing at 190 °C (for methylated oligosaccharide derivatives). GLC/MS was recorded at 70 eV using OV-225 or the last two mentioned columns in Perkin-Elmer 270 or Varian MAT 311 instruments. Gel filtrations of methylated polysaccharides were performed on columns of Sephadex LH-20 (Pharmacia Fine Chemicals) irrigated with chloroform–acetone (2:1). Separations were monitored by spot tests on TLC plates and compounds were detected by charring with sulfuric acid.

Isolation and Purification of the Polysaccharide. Growth of *Rhizobium meliloti* strain U 27 and isolation and purification of the polysaccharide were as described earlier.[5]

Methylation Analysis. Methylations were performed with sodium methylsulfinylmethanide–methyl iodide in dimethyl sulfoxide.[7,8] Other alkylations were performed analogously using appropriate alkylating agents. Methylated materials were recovered after removal of dimethyl sulfoxide by freeze drying and purified by gel filtration. Hydrolyses and transformations into alditol acetates were carried out as described earlier.[8] All analyses of methylated sugars were performed by GLC/MS of their alditol acetates[8,19] and all identifications (including those of partially trideuteriomethylated or ethylated derivatives) were unambiguous and will not be discussed.



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Sequential Degradations. First Degradation. PS (400 mg) was carboxyl reduced using 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate–sodium borohydride,[11] dialyzed against water, and freeze dried (yield 375 mg). This material was methylated, hydrolyzed with aqueous acetic acid (90%, 75 mL) for 100 min at 100 °C, concentrated, and purified by gel filtration (yield 332 mg). An aliquot (2 mg) was remethylated with trideuteriomethyl iodide, hydrolyzed, and analyzed (Table I, line B). The remaining material was oxidized[12] in two equal portions with chlorine (1 M in 25 mL of methylene chloride) and dimethyl sulfoxide (9 mL)–triethylamine (7 mL) at −45 °C for 7 h; after concentration dimethyl sulfoxide was removed by freeze drying and the material purified by gel filtration (yield 266 mg). An aliquot (1 mg) was hydrolyzed and analyzed (Table I, line C). The remaining material, in methylene chloride (20 mL), was treated with sodium ethoxide in ethanol (1 M, 2 mL) for 1.5 h at room temperature, neutralized with glacial acetic acid, and concentrated to dryness. Aqueous acetic acid (50%, 10 mL) was added and the mixture was kept at 100 °C for 4 h, concentrated, and purified by gel filtration (yield 233 mg). An aliquot (2 mg) was remethylated with trideuteriomethyl iodide, hydrolyzed, and analyzed (Table I, line D).

Second Degradation. The material from the first degradation was oxidized and worked up as before (yield 170 mg). An aliquot (1 mg) was hydrolyzed and analyzed (Table I, line E). The remaining material, in methylene chloride (10 mL), was treated with sodium ethoxide in ethanol (0.8 M, 10 mL) for 1.5 h at room temperature, neutralized with glacial acetic acid, partitioned between chloroform and water, and purified by gel filtration (yield 130 mg). An aliquot (2 mg) was remethylated with trideuteriomethyl iodide, hydrolyzed, and analyzed (Table I, line F).

Third Degradation. This was performed essentially as described for the second degradation. The yield of degraded material was 110 mg. The oxidation step was monitored by hydrolyzing and analyzing an aliquot (1 mg) (Table I, line G) and the degradation was monitored by remethylation with trideuteriomethyl iodide, hydrolysis, and analysis of an aliquot (2 mg) (Table I, line H).

Fourth Degradation. The material from the third degradation was oxidized essentially as described for the second degradation (yield 100 mg). An aliquot (1 mg) was hydrolyzed and analyzed (Table I, line I). The remaining material, in methylene chloride (10 mL), was treated with sodium ethoxide in ethanol (1.2 M, 5 mL) for 1.25 h at room temperature, neutralized with glacial acetic acid, and partitioned between chloroform and water. This material was treated with aqueous acetic acid (50%, 10 mL) for 14 h at 100 °C, concentrated, and purified by gel filtration (yield 55 mg). An aliquot (2 mg) was remethylated with trideuteriomethyl iodide, hydrolyzed, and analyzed (Table I, line J).

Smith Degradation. PS (65 mg) was oxidized in the dark with sodium metaperiodate (0.03 M in 0.1 M sodium acetate buffer, pH 3.9, 76 mL) for 5 days at 4 °C. Excess periodate was destroyed by addition of ethylene glycol (2 mL) and the solution was dialyzed and concentrated to 50 mL. Sodium borohydride (1 g) was added, the solution was kept at room temperature overnight, and excess of borohydride was destroyed by addition of aqueous acetic acid. The solution was dialyzed and freeze dried. In order to obtain complete oxidation, the oxidation–reduction cycle was repeated (yield 27 mg). The material was methylated and an aliquot (2 mg) was hydrolyzed and on analysis was shown to contain 2,4,6-tri-*O*-methyl-D-glucose and 2,4,6-tri-*O*-methyl-D-galactose in the ratio 2.1:1. Only trace amounts of other methyl ethers were found, indicating the oxidation to have been almost complete. The remaining methylated material was hydrolyzed with aqueous formic acid (90%, 5 mL) for 1 h at 40 °C and evaporated at dryness. This material was realkylated with ethyl iodide and analyzed by GLC/MS using an OV-1 column. Two peaks were obtained showing T_{mel} (retention time relative to methylated melibiitol) 0.41 and 4.9. The mass spectrum of the former compound (**22**) showed, inter alia, the following peaks (relative intensities in parentheses and some assignments[19] in brackets: 102 (100), 175 (9) [bA_1], 187 (4) [aA_2], 201 (3) [aA_2], 233 (1) [aA_1], 249 (7) [abJ_1]. The latter compound (**24**) showed, inter alia, the following peaks: 45 (100), 101 (53), 102 (78), 117 (22) [cA_1], 187 (25) [aA_2], 201 (12) [aA_2], 233 (4) [aA_1]. Part of the realkylated material was hydrolyzed to completion and analyzed. 3-*O*-Ethyl-2,4,6-tri-*O*-methyl-D-galactose, 3-*O*-ethyl-2,4,6-tri-*O*-methyl-D-glucose, and 2,4,6-tri-*O*-methyl-D-glucose were obtained in the proportions 1.2:1.2:1 together with 3,4-di-*O*-ethyl-1-*O*-methyl-D-erythritol.

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