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STRUCTURAL STUDIES ON THE EXTRACELLULAR ACIDIC POLYSACCHARIDE FROM *Rhizobium trifolii* 4S

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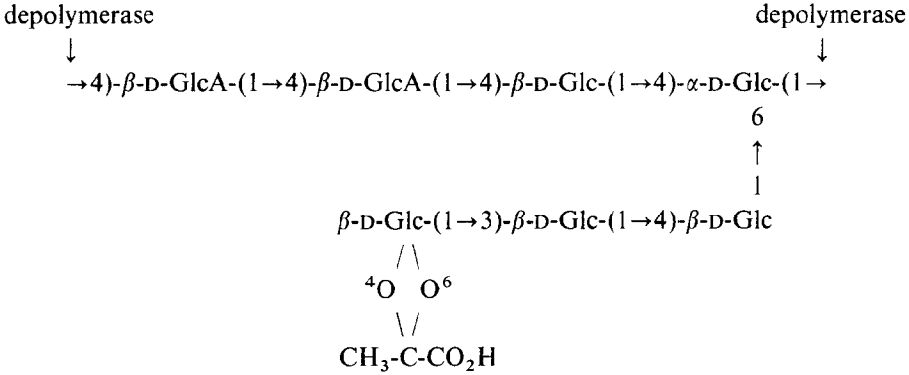
ABSTRACT

The structure of an extracellular acidic polysaccharide produced by *Rhizobium trifolii* 4S was studied by fragmentation with phage-induced depolymerase, methylation analysis, digestion with exo-β-D-glycanases, and n.m.r. spectroscopy. The polysaccharide is composed of D-glucose, D-glucuronic acid, pyruvic acid, and acetic acid in the molar ratio of 5:2:1:2. The repeating unit is a heptasaccharide containing (*a*) terminal 4,6-*O*-(1-carboxyethylidene)-D-glucose (1 residue), (*b*) (1→3)-linked D-glucose (1 residue), (*c*) (1→4)-linked D-glucose (2 residues), (*d*) (1→4)-linked D-glucuronic acid (2 residues), and (*e*) (1→4), (1→6)-linked D-glucose (1 residue). A possible structure for the polysaccharide is proposed. The phage-induced depolymerase hydrolyzes linkages of 4-*O*-α-D-glucosyl-β-D-glucuronic acid adjacent to branching points.



INTRODUCTION

Most extracellular, acidic polysaccharides of *Rhizobium trifolii* reported thus far[1–6] contain D-glucose, D-galactose, and D-glucuronic acid as sugar constituents.

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However, Ghai *et al.*[7] reported that *R. trifolii* J60 produces a polysaccharide typical of *R. meliloti* that does not contain D-glucuronic acid. Higashi and Abe[8] studied the sugar composition of polysaccharides of *Rhizobium* strains and showed that the polysaccharide from one (strain 4S) of six strains of *R. trifolii* did not contain D-galactose. We are interested in this aberrant polysaccharide because a role of polysaccharides of *Rhizobium* in the symbiotic relation has been proposed by several workers[9-13]. We now report structural studies on this polysaccharide.

EXPERIMENTAL

General methods. – Descending paper chromatography (p.c.) was conducted on Toyo filter paper No. 50 with 6:4:3 (v/v) 1-butanol–pyridine–water. Sugars were detected with silver nitrate. Gas–liquid chromatography (g.l.c.) was performed with a Shimadzu GC 7A gas chromatograph fitted with a flame-ionization detector. Carboxyl reduction of the polysaccharide was performed by the procedure of Taylor and Conrad[14].

^{1}H-N.m.r. spectra were recorded with a JEOL JNM-FX100 instrument (99.7 MHz) for solutions in deuterium oxide at 85° with sodium 4,4-dimethyl-4-silapentane-1-sulfonate as the internal standard.

Methylations were performed as described by Hakomori[15] with sodium methylsulfinyl methanide and methyl iodide in dimethyl sulfoxide. The methylated samples were hydrolyzed and analyzed as their alditol acetates by g.l.c. on a column (3 mm × 3 m) of 0.3% OV275–0.4% GEXF1150 on Shimalite W, as described previously[16]. Methylated sugars were identified by comparison with methylated sugars derived from the polysaccharide[16] of *Alcaligenes faecalis* var. *myxogenes* 10C3 and by mass spectrometry.

Quantitative analysis of neutral sugars was performed by g.l.c. on a column (3 mm × 2 m) of 4% ECNSS-M on Gas-Chrom Q at 180° as described previously[7].

Glucuronic acid was determined by the modified carbazole reaction of Galambos[17] and identified by g.l.c. after reduction of the carboxyl group to give glucose.

Pyruvic acid and *O*-acetyl groups were assayed by the methods of Koepsell and Sharpe[18] and McComb and McCready[19], respectively. These acids were identified by ^{1}H-n.m.r. spectroscopy.

Organisms. – *Rhizobium trifolii* 4S (ref. 8) was isolated from a nodule of white clover (*Trifolium repens*). *R. trifolii* A1 (ref. 8) was isolated as a non-infectious strain from strain 4S by spontaneous mutation. *R. leguminosarum* KP (ref. 8) was isolated from a nodule of pea (*Pisum sativum*).

Culture medium. – The composition of the medium used to isolate extracellular acidic polysaccharide was as follows (per 100 mL of distilled water): D-glucose (4 g); $(NH_4)_2HPO_4$ (0.15 g); KH_2PO_4 (0.1 g); $MgSO_4 \cdot 7H_2O$ (0.05 g); NaCl (1 mg); $MnCl_2 \cdot 4H_2O$ (1 mg); $CaCl_2$ (1 mg), thiamine (20 μg); biotin (2 μg); and $CaCO_3$ (0.5 g); pH 7.0.

Preparation of extracellular acidic polysaccharide. – Extracellular acidic poly-

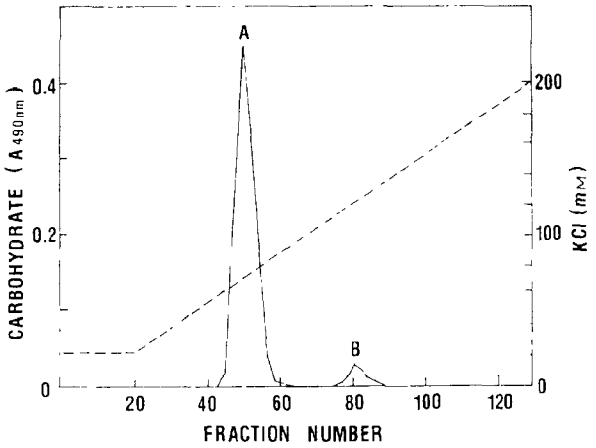


Fig. 1. DEAE-cellulose chromatography of depolymerase-digested polysaccharide of *R. trifolii* 4S. Polysaccharide was digested with the enzyme described in the text. The digest was separated on a column (2.5 × 15 cm) of DEAE-cellulose equilibrated with 20mM potassium chloride, and material was eluted with 1 L of a linear gradient of 20–200mM potassium chloride (-----). Fractions of 9.1 mL were collected and analyzed for carbohydrate by the phenol–sulfuric acid method (——). The first peak (A) and second peak (B) of the digest are designated as oligosaccharide 1 and oligosaccharide 2, respectively.

saccharide was prepared from the supernatant liquor of cultures grown for 5 days at 30° in the synthetic medium. The method used, involving precipitation with acetone and cetylpyridinium chloride, has been described previously[7]. The polysaccharide obtained was dissolved in water, treated with Amberlite IR-120 (H^+), resin and lyophilized. The H^+-form polysaccharide was used for analyses.

Preparation of phage-induced depolymerase. — Phage-induced depolymerase was prepared as described previously[8]. One unit of enzyme activity is defined as the amount liberating 1 μmol of aldehyde groups as glucose per h.

Preparation of hydrolysis products of polysaccharide with phage-induced depolymerase. — A sample (100 mg) of the polysaccharide of *R. trifolii* 4S was hydrolyzed with phage-induced depolymerase (15 units) for 24 h at 30° in 50mM Tris-HCl buffer, pH 7.2, (20 mL) containing 0.1 μmol of calcium chloride with a few drops of toluene to prevent microbial growth. After hydrolysis, the mixture was dialyzed by vacuum filtration through a collodion membrane. The dialyzable fraction was concentrated to 1 mL, and salts were removed by chromatography on a column of Sephadex G-10. Fractions in the void volume containing the oligosaccharide were concentrated to low volume and lyophilized, to yield 81 mg of material.

A sample (60 mg) of the oligosaccharide was dissolved in 20mM potassium chloride and applied to a column of DEAE-cellulose (2.5 × 15 cm) equilibrated with 20mM potassium chloride. Material was eluted with 1 L of a linear gradient of 20–200mM potassium chloride with a flow rate of 50 mL per h. As shown in Fig. 1, the oligosaccharide preparation gave two peaks of oligosaccharide (oligosaccharides **1** and **2**). The fractions in each peak were pooled and concentrated to low volume,

and salts were removed by chromatography on a column of Sephadex G-10. Oligosaccharide fractions in the void volume were concentrated and lyophilized; yields: oligosaccharide **1**, 49 mg; oligosaccharide **2**, 5.3 mg.

Preparation of deacylated oligosaccharide. — A solution of 0.1 % oligosaccharide **1** was incubated in 10mM potassium hydroxide for 5 h at 20° under nitrogen. The solution was made neutral with M hydrochloric acid, concentrated to low volume, and passed through a column of Sephadex G-10. The effluent containing sugar was concentrated and lyophilized.

Preparation of depyruvylated polysaccharide and oligosaccharide. — A solution of 0.1 % of polysaccharide or oligosaccharide **1** was heated for 2 h at 100° after adjusting the solution to pH 2 with hydrochloric acid. The polysaccharide solution was then dialyzed and lyophilized. The oligosaccharide solution was passed through a column of Sephadex G-10 and the effluent containing sugar was concentrated and lyophilized.

Preparation of carboxyl-reduced oligosaccharide. — A solution of 0.3 % of oligosaccharide **1** was carboxyl reduced by the method of Taylor and Conrad[14]. Salts in the mixture were removed by chromatography with a column of Sephadex G-10. The fully reduced oligosaccharide was obtained by passage through a column of Amberlite IR-45 (OH^-) resin.

Digestion of oligosaccharide with β-D-glucosidase. — Depyruvylated, deacylated oligosaccharide **1** (3 mg) was digested from the non-reducing terminal sugar of the side chain with almond β-D-glucosidase (Sigma Chem. Co.) at 30° in 100 μL of 50mM sodium acetate buffer (pH 5.0). Partially and extensively digested oligosaccharide was obtained by treatment with 0.5 mg of enzyme for 2 h and 3 mg of enzyme for 16 h, respectively. One drop of toluene was added to prevent microbial growth. The mixture was then boiled for 1 min, the resulting precipitate was removed, and the supernatant solution was treated with Amberlite IR-120 (H^+) resin. The solution was concentrated and subjected to p.c. The digested oligosaccharide was separated from liberated glucose by extracting the appropriate section of paper. The extract was concentrated and lyophilized.

Depyruvylated, deacylated, carboxyl-reduced oligosaccharide **1** was also digested extensively with the β-D-glucosidase, and the digested oligosaccharide was obtained by the method just described.

Digestion of oligosaccharide with β-D-glucosiduronase. — Deacylated oligosaccharide **1** (5 mg) was digested with 2 mg of bovine liver β-D-glucosiduronase (P-L Biochem. Inc.) for 16 h at 30° in 100 μL of 50mM sodium acetate buffer (pH 5.0) containing a drop of toluene. The digested oligosaccharide was obtained by p.c. as already described.

RESULTS AND DISCUSSION

R. trifolii 4S produced 240 mg of extracellular, acidic polysaccharide per 100 mL of culture medium. The polysaccharide had $[\alpha]_D^{20}$ +12° (*c* 0.2 water) and

TABLE I

COMPONENTS OF EXTRACELLULAR ACIDIC POLYSACCHARIDE OF *R. trifolii* 4S AND OLIGOSACCHARIDES PRODUCED BY HYDROLYSIS WITH PHAGE-INDUCED DEPOLYMERASE

	D-*Glucose* *Wt %* *(mol ratio)*	D-*Glucuronic acid* *Wt %* *(mol ratio)*	*Pyruvic acid* *Wt %* *(mol ratio)*	*Acetic acid* *Wt %* *(mol ratio)*
Native polysaccharide	61.2 (5)	27.6 (2.0)	5.1 (1.0)	5.5 (1.7)
Oligosaccharide **1**	60.5 (5)	25.8 (1.9)	5.1 (1.0)	5.7 (1.8)
Oligosaccharide **2**	59.9 (5)	26.4 (2.0)	4.8 (0.9)	5.4 (1.7)

TABLE II

METHYLATION ANALYSIS OF NATIVE AND MODIFIED POLYSACCHARIDES OF *R. trifolii* 4S

	Methylated sugar (relative proportions)			
	2,3,4,6-Glc *(T[a] 1.00)*	*2,4,6-Glc* *(T[a] 1.72)*	*2,3,6-Glc* *(T[a] 2.32)*	*2,3-Glc* *(T[a] 3.63)*
Native polysaccharide	0.0	1	2.1	1.8
Depyruvylated polysaccharide	1.0	1	2.3	1.1
Carboxyl-reduced polysaccharide	0.0	1	3.6	1.8

[a]Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

was composed of D-glucose, D-glucuronic acid, pyruvic acid, and acetic acid in the molar ratio of 5:2:1:2 (Table I).

The sugars from the methylated polysaccharide identified by g.l.c. were 2,4,6-tri-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, and 2,3-di-*O*-methyl-D-glucose in the molar ratio of 1:2:2 (Table II). Depyruvylation of the polysaccharide resulted in an increase of 1 mol of 2,3,4,6-di-*O*-methyl-D-glucose and decrease of 1 mol of 2,3-di-*O*-methyl-D-glucose per mol. These results show that this polysaccharide has (1→3)- and (1→4)-linked D-glucose residues, D-glucose residues branching through a (1→4)- or (1→6)-linkage, and non-reducing terminal D-glucose residues having pyruvic acid linked to O-4 and O-6; these give 2,3-di-*O*-methyl-D-glucose.

When the polysaccharide was carboxyl-reduced, the methylated sugars showed an increase of ~1.5 mol of 2,3,6-tri-*O*-methyl-D-glucose per mol. This increase is attributable to reduction of the carboxyl groups in (1→4)-linked D-glucuronic acid residues. Although an increase of 2 mol of this methylated sugar is expected from the component analysis (Table I), it was difficult to reduce carboxyl groups of uronic acid residues completely, even after repeated reduction.

The products obtained from the native polysaccharide by digestion with phage-induced depolymerase were separated into two of oligosaccharide fractions by chromatography on a column of DEAE-cellulose (Fig. 1). Each oligosaccharide was composed of D-glucose, D-glucuronic acid, pyruvic acid, and acetic acid in the molar ratio of 5 : 2 : 1 : 2 (Table I). This ratio is the same as that of the native polysaccharide. Previously[8] Higashi and Abe reported that the ratio of these components of the oligosaccharide obtained by digestion with phage-induced depolymerase was 1 : 1 : 2 : 2, but this was wrong.

The reducing-terminal sugar of each oligosaccharide was determined to be D-glucose by reduction of the reducing-terminal sugar with sodium borohydride, hydrolysis of the reduced oligosaccharide, and identification of the alditol by g.l.c. on a silicone OV-17 column as the acetylated derivative. The ratio of D-glucose to D-glucitol on the gas chromatogram was 4 : 1 for the oligosaccharide of the main fraction (oligosaccharide **1**) and 9 : 1 for the oligosaccharide of the minor fraction (oligosaccharide **2**). Thus, oligosaccharide **1** is the repeating-unit heptasaccharide of the polysaccharide, consisting of five D-glucose and two D-glucuronic acid residues, and oligosaccharide **2** is the dimer of the repeating-unit heptasaccharide. Oligosaccharide **2** was hydrolyzed completely to the monomer by further treatment with phage-induced depolymerase.

The methylated sugars of oligosaccharide **1** were the same as those of the native polysaccharide (Table III). Thus, the repeating unit retained the branching D-glucose residue, and the nonreducing-terminal sugar produced by hydrolysis with

TABLE III

METHYLATION ANALYSIS OF NATIVE AND MODIFIED OLIGOSACCHARIDE **1**

	Methylated sugars (relative proportions)				
	2,3,4,6-Glc (T 1.00)	*1,2,3,5-Glc (T 1.07)*	*2,4,6-Glc (T 1.72)*	*2,3,6-Glc (T 2.32)*	*2,3-Glc (T 3.63)*
Oligosaccharide **1**	0.0	0.0	1.0	2	1.9
Deacylated oligosaccharide **1**	0.0	0.0	1.0	2	1.7[a]
Depyruvylated, deacylated (DPDA) oligosaccharide **1**	1.0	0.0	1.0	2	0.8[a]
β-Glucosiduronase-digested, deacylated oligosaccharide **1**	0.5	0.0	1	1.6	1.7[a]
β-Glucosidase-digested DPDA oligosaccharide **1** (partially digested)	1.0	0.0	0.6	2	0.8[a]
β-Glucosidase-digested DPDA oligosaccharide **1** (extensively digested)	1.1	0.0	0.0	1	0.8[a]
Reduced oligosaccharide **1**	0.0	0.8	1.0	2	0.9

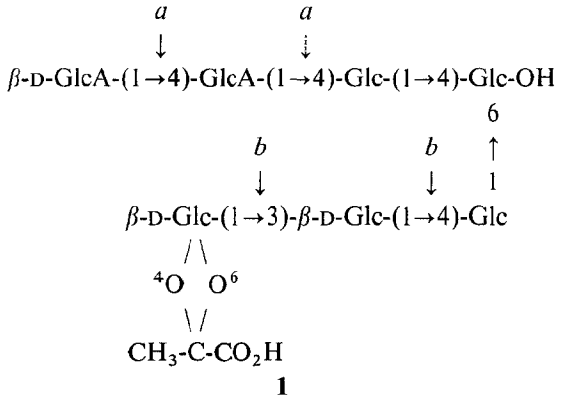
[a]Lower values may be due to partial decomposition of the reducing-terminal D-glucose during deacylation

phage-induced depolymerase was D-glucuronic acid. The reducing-terminal sugar was D-glucose, as described before.

When deacylated oligosaccharide **1** was treated with β-D-glucosiduronase, D-glucuronic acid was liberated from the nonreducing terminal. This conversion was monitored by p.c. Colorimetric analysis of the enzyme-digested oligosaccharide showed that 1.4 mol of D-glucuronic acid per mol was removed. We could not remove the 2 mol of acids in the oligosaccharide completely, even by further treatment. Methylation analysis of the oligosaccharide showed a decrease of 0.5 mol of 2,3,6-tri-*O*-methyl-D-glucose and an increase of 0.5 mol of 2,3,4,6-tetra-*O*-methyl-D-glucose per mol (Table III). These results indicate that D-glucuronic acid residues exist contiguously at the non-reducing terminal and that the D-glucose residue is linked to the D-glucuronic acid residue through a (1→4)-linkage. We also confirmed the existence of this D-glucuronosyl-(1→4)-D-glucose linkage by methylation analysis of the acidic oligosaccharide obtained by partial hydrolysis of the native polysaccharide with 2M trifluoroacetic acid for 3 h at 100°.

When depyruvylated, deacylated oligosaccharide **1** was treated with β-D-glucosidase, D-glucose was shown by p.c. to be liberated from the nonreducing terminal of the side chain. Methylation analysis of depyruvylated, deacylated oligosaccharide **1** digested partially or extensively with the enzyme showed that one mol of the terminal D-glucose and subsequently one mol of (1→3)-linked D-glucose per mol were removed by the digestion (Table III). As one mol of 2,3-di-*O*-methyl-D-glucose derived from branching D-glucose remained, at least one undigested D-glucose residue is attached to the branching D-glucose residue. This glucose residue could not be hydrolyzed, even by further addition of enzyme or longer incubation.

From these data, the possible structure of oligosaccharide **1** may be as shown in the formula. The linkages hydrolyzed



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readily with β-D-glycosidases are β (arrows *a* and *b* show the linkages hydrolyzed with β-D-glucosiduronase and β-D-glucosidase, respectively). The reducing-terminal sugar is branching D-glucose. This was confirmed as follows: the reducing-terminal D-glucose of oligosaccharide **1** was reduced to D-glucitol with sodium borohydride

and the reduced oligosaccharide **1** was methylated. The methylated sugars of the product (Table III) showed a decrease of one mol of 2,3-di-*O*-methyl-D-glucose with an increase of one mol of 1,2,3,5-tetra-*O*-methyl-D-glucose per mol, as compared with unreduced oligosaccharide **1**. The latter methylated sugar was identified by its retention time and by mass spectrometry. If the reducing-terminal D-glucose were not branching, penta-*O*-methyl-D-glucose would be produced from the reduced oligosaccharide by reduction.

The anomeric linkages were studied by ^{1}H-n.m.r. analysis. The spectra of the native and depyruvylated polysaccharides indicated the presence of α- and β-linked residues, but the resolution was not good. The spectrum of oligosaccharide **1** produced

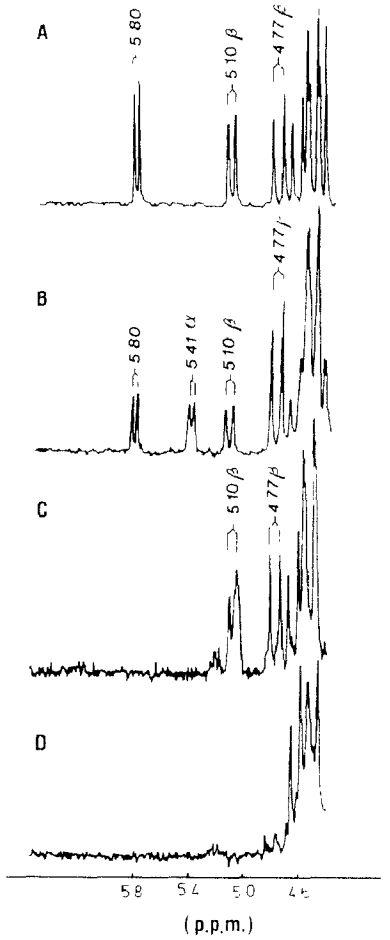
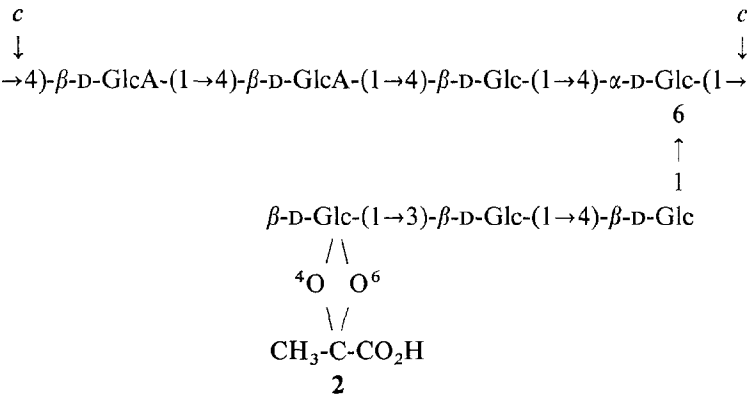


Fig. 2 ^{1}H-N.m.r. spectra of solutions in deuterium oxide at 85 : only the anomeric-proton region is shown. A, reduced oligosaccharide **1**; B, reduced oligosaccharide **2**; C, carboxyl-reduced oligosaccharide **1**; D, β-D-glucosidase-digested, deacylated, depyruvylated, carboxyl-reduced oligosaccharide **1**.

by digestion with phage-induced depolymerase showed a signal assigned to an α linkage at δ 5.19 ($J_{1,2}$ 3.4 Hz). When the reducing-end D-glucose was reduced to D-glucitol with sodium borohydride, the two signals at δ 5.19 ($J_{1,2}$ 3.4 Hz) and δ 4.97 ($J_{1,2}$ 8.8 Hz), assigned to α- and β-linkages, respectively, disappeared. Thus, these two signals were derived from free reducing-end anomeric protons. This reduced oligosaccharide **1** has no other α linkage (Fig. 2a), whereas reduced oligosaccharide **2** has another signal at δ 5.41 ($J_{1,2}$ 3.4 Hz) indicating an α linkage (Fig. 2b). Therefore, phage-induced depolymerase hydrolyzes this α linkage between branching D-glucose and D-glucuronic acid residues. The signal at δ 5.80 seems to be derived from H-5 of glucuronic acid residues, because this signal disappeared by carboxyl reduction of the oligosaccharide (Fig. 2c). We assigned the signal at δ 5.10 ($J_{1,2}$ 5.6 Hz) as the chemical shift of a β-D anomeric proton because this signal disappeared by digestion of the depyruvylated, deacylated, carboxyl-reduced oligosaccharide **1** with β-D-glucosidase (Fig. 2d).

From these results we concluded that the structure of the acidic polysaccharide of *R. trifolii* is as follows:



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Arrows *c* represent linkages hydrolyzed with phage-induced depolymerase. Two moles of *O*-acetyl groups exist per repeating-unit heptasaccharide.

The glycosyl-residue sequence of the backbone is the same as that of the backbone of polysaccharides from *R. leguminosarum* strains 128c53 and 128c63 and *R. trifolii* strains NA30 and 0430 elucidated by Robertsen *et al.*[6]. Differences are in the structure of the side chain. Our polysaccharide lacks the terminal, pyruvylated D-galactose residue, and the next pyruvylated D-glucose residue is attached through a (1→3) linkage, not a (1→4) linkage. The structure of the polysaccharide from *R. trifolii* U226 proposed by Jansson *et al.*[5] shows that one-third of the side chains do not contain terminal pyruvylated D-galactose. However, our polysaccharide has no galactose. Preparations obtained from cultures of different ages (logarithmic, early stationary, or late stationary) also had no galactose. The *R. trifolii* 4S used for this study is infectious and nodulates white clover (*Trifolium repens*) efficiently. Thus,

the difference in the structure of the side chain of extracellular, acidic polysaccharides of *R. trifolii* does not seem to be important for the infection processes.

The acidic polysaccharides of a non-infectious strain (A1) obtained from *T. trifolii* 4S, and one (strain KP) of six strains of *R. leguminosarum* examined (all of which are infectious to pea) were also composed of D-glucose, D-glucuronic acid, and pyruvic acid in the molar ratio of 5:2:1 with some acetic acid. These polysaccharides were also hydrolyzed by phage-induced depolymerase to their repeating-unit heptasaccharides. The methylated sugars from the native polysaccharides and the repeating units were the same as those of *R. trifolii* 4S. Therefore, the structures of these three polysaccharides seem to be identical.

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