Carbohydrate Research, 148 (1986) 87–99
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE STRUCTURE OF THE EXOPOLYSACCHARIDE FROM *Rhizobium* SP. STRAIN ANU280 (NGR234)

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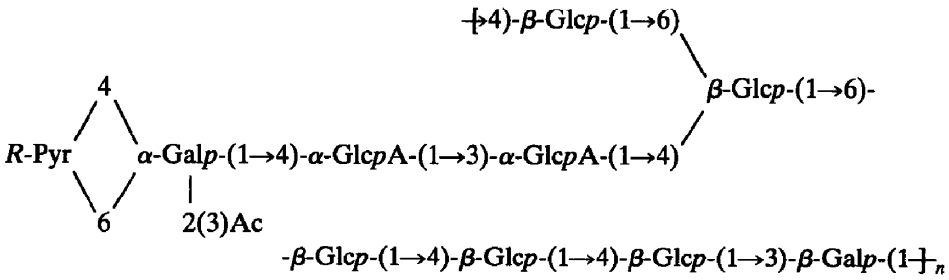
(Received August 13th, 1985; accepted for publication, September 26th, 1985)

ABSTRACT

The structure of an exopolysaccharide from *Rhizobium* sp. strain ANU280 (derivative of the broad host-range strain NGR234) has been determined. Fragments generated by partial acid hydrolysis were fractionated by sequential ion-exchange and gel chromatography, and their structures were assigned by ^{13}C-n.m.r. spectroscopy. Extensive overlap of structure between the fragments, together with the results of periodate oxidation and colorimetric analyses, permitted assignment of the nonasaccharide repeating-unit shown below. The terminal galactosyl group carries a 4,6-*O*-(1-carboxyethylidene) group and, probably, an acetyl group.



INTRODUCTION

A characteristic of many wild-type strains of *Rhizobium* is their ability to produce copious amounts of exopolysaccharides and form mucoid colonies. It has been proposed that the polysaccharide has important functions in the plant-*Rhizobium* symbiosis, such as determination of host specificity[1].

We have reported[2] the use of transposon mutagenesis to isolate more than ninety exopolysaccharide-defective strains of the broad host-range, fast-growing, cowpea strain NGR234. As part of a programme to determine the role of these

0008-6215/86/$ 03.50

polysaccharides, the structure of the exopolysaccharide from *Rhizobium* sp. strain ANU280, an antibiotic-resistant isolate of NGR234, has been determined.

EXPERIMENTAL

General. — *Rhizobium* sp. strain ANU280 is[3] a spontaneous rifampicin-resistant isolate of ANU240, which is a streptomycin-resistant derivative of NGR234.

Descending p.c. was performed on Whatman No. 1 paper with *1*, 1-butanol–pyridine–water (6:4:3); *2*, ethyl acetate–acetic acid–formic acid–water (18:3:1:4); *3*, 1-butanol–acetic acid–water (5:1:2); and *4*, 1-butanol–pyridine–water (40:11:6, solvent) with 1-butanol–acetic acid–pyridine–water (25:5:25:15, atmosphere); and detection with alkaline silver nitrate[4] or *p*-anisidine trichloroacetate[5]. Preparative p.c. was carried out with Whatman No. 3MM paper, using solvents *1* and *3*.

Hexose and uronic acid were determined by the phenol–sulphuric acid[6] and carbazole[7] methods, respectively, pyruvate as the 2,4-dinitrophenylhydrazone[8], and acetate as the ferric hydroxamate[9].

β-D-Glucosidase (from almonds, cat. G4511) was purchased from Sigma. C.d. spectra were measured with a Jasco J-20 automatic recording spectropolarimeter.

Culture of bacteria. — *Rhizobium* sp. strain ANU280 was grown on a glutamic acid–D-mannitol–salts medium containing K_2HPO_4, 1.0 g; KH_2PO_4, 1.0 g; $FeCl_3 \cdot 6\ H_2O$, 0.01 g; $MgSO_4 \cdot 7\ H_2O$, 0.25 g; $CaCl_2 \cdot 6\ H_2O$, 0.10 g; sodium glutamate, 1.1 g; mannitol, 10 g; thiamine, 1.0 mg; biotin, 0.5 mg; nicotinic acid, 1.0 mg; pyridoxin HCl, 1.0 mg; $MnSO_4 \cdot H_2O$, 10 mg; H_3BO_3, 3.0 mg; $ZnSO_4 \cdot 7\ H_2O$, 3.0 mg; and $Na_2MoO_4 \cdot 2\ H_2O$, 2.5 mg in 1 L of distilled water (pH 7.1–7.2). Erlenmeyer flasks (2 L) containing 400 mL of medium were inoculated with 10 mL of preculture and shaken vigorously at 28° for 7 days.

Isolation and purification of the exopolysaccharide. — Cells and associated polysaccharide were isolated by using an Amicon DC1OL hollow-fibre filtration system fitted with a 0.1-μm filter, and subjected to exhaustive diafiltration with distilled water to remove salts and other nutrients. Both cells and exopolysaccharide were retained by the filter. The cells were removed by centrifugation, and washed three times with distilled water, and the combined supernatants were concentrated under reduced pressure at 45° and then freeze-dried. The resulting crude exopolysaccharide was purified by precipitation as the cetyltrimethylammonium (CTAB) salt[10].

Sugar analysis. — The exopolysaccharide (50 mg) was hydrolysed with 2M trifluoracetic acid (5 mL) overnight at 95°. The acid was removed by several evaporations with water under reduced pressure. P.c. (solvents *1* and *2*) of the residue revealed glucose, galactose, glucuronic acid, and uronic acid-containing oligomers. For quantitative studies, the exopolysaccharide was dried *in vacuo* over sulphuric acid (typically, 14% loss in weight). The neutral sugars in the hydrolysate

were then quantified by g.l.c. of the alditol acetates with *myo*-inositol as internal standard, a 2-m glass column packed with 5% of SP2340 on Gas Chrom Q (100–120 mesh), and an oven temperature of 220°. The values obtained (Table I) were extrapolated to zero time to give the estimates in Table II.

Graded acid hydrolysis of the exopolysaccharide. — To a solution of the polysaccharide (1.0 g) in distilled water (100 mL) was added 13M trifluoracetic (8.4 mL). The mixture was kept in a sealed vessel at 95° for 2 h and then concentrated to dryness under reduced pressure at 45°, and an aqueous solution of the residue was freeze-dried.

The product was eluted from a column (1.5 × 40 cm) of DEAE-Sephadex A25 equilibrated with 25mM Tris-HCl (pH 7.5). Neutral components were eluted at 8 mL/h with 90 mL of the same buffer, and acidic components with a convex gradient formed from 90 mL of 25mM Tris-HCl (pH 7.5) and 100mM sodium chloride in 25mM Tris-HCl (limit buffer, 300 mL). Aliquots of the 2.0-mL fractions were analysed for hexose and uronic acid. Appropriate fractions were combined and freeze-dried, and each residue was eluted from a column (1.5 × 95 cm) of Biogel P-2 with 200mM trimethylammonium formate (pH 3.3) to effect further purification and removal of salt[11]. Aliquots of the 1.0-mL fractions were monitored as before. The total overall recovery was always >90%.

In pilot experiments, the products of hydrolysis were fractionated on a column (5 × 1 cm) of Dowex 1 resin. Neutral components were eluted with water (10 mL), and the acidic products with 2M formic acid (20 mL). The eluates were concentrated under reduced pressure and the residues were subjected to p.c.

Smith degradation. — To a solution of polysaccharide (1.0 g) in 50mM sodium

TABLE I

RELEASE OF HEXOSES FROM *Rhizobium* STRAIN ANU280 EXOPOLYSACCHARIDE BY ACID HYDROLYSIS

Time (h)	1	2	4	8	16	40
Gal (%)	3.8	13.3	18.0	19.7	18.0	13.2
Glc (%)	0.3	20.1	41.0	48.7	48.1	39.6

TABLE II

ANALYTICAL DATA FOR *Rhizobium* STRAIN ANU280 EXOPOLYSACCHARIDE

Component	*Amount (nmol/mg)*	*Molar ratio*	*Amount (anhydro residue, % w/w)*
Glucose	2930	5.00	47.5
Galactose	1110	1.89	18.0
Glucuronic acid	1120	1.91	19.7
Pyruvate	520	0.89	3.6
Acetate	560	0.96	2.3

acetate buffer (pH 5.2, 250 mL) was added sodium metaperiodate (5.0 g), and the mixture was stored in the dark at room temperature for 7 days. Ethylene glycol (5.0 mL) was added, and the mixture was stored for 8 h and then dialysed overnight against three changes of distilled water. The pH was adjusted to 10 with sodium hydroxide, sodium borohydride (2 g) was added, and the solution was stored at room temperature for 1 h. Acetic acid was added to decompose excess of reductant, and the solution was made 0.5M in trifluoracetic acid and kept overnight at 37°. The mixture was then concentrated to dryness under reduced pressure, and a solution of the residue in water was freeze-dried. Recovery of unreacted carbohydrate was estimated by colorimetric analysis to be 30–35%. P.c. of the hydrolysate revealed galactose and glucuronic acid, but no glucose. Fractionation on Biogel P-2 as described above gave **P1a** (fractions 78–85), **P1b** (88–98), and **P1c** (104–112), which were characterised by p.c. and n.m.r. spectroscopy (Table V).

Glycosidase treatments. — A solution of each oligosaccharide (0.5–2 mg) in 50mM phosphate buffer (pH 5.2, 0.5 mL) was incubated with β-D-glucosidase (1 U) for 30 min at 37°. A control experiment, using maltose as substrate, was carried out to demonstrate lack of significant α-D-glucosidase activity.

N.m.r. spectroscopy. — Spectra (200 MHz for ^1H, 50.1 MHz for ^{13}C) were recorded with a Varian XL-200 spectrometer for solutions in D_2O at 21° (90° for polysaccharide samples). Chemical shifts were measured relative to that of HDO at 4.815 p.p.m. (4.161 p.p.m. at 90°) for ^1H, and methanol at 50.04 p.p.m. for ^{13}C (external Me_4Si taken as 0 p.p.m.)[12].

The number of protons attached to each carbon was determined using the DEPT pulse sequence[13]. On occasion, ^1H,^{13}C chemical-shift correlation spectroscopy[14], combined with homonuclear proton decoupling, was used to assign the carbon resonances.

The presence of an *R*-(1-carboxyethylidene) acetal and an acetyl group in the exopolysaccharide was shown[15] by ^1H resonances at 1.46 and 2.16 p.p.m., respectively, and by ^{13}C resonances at 27.0 and 22.0 p.p.m. The high viscosity precluded accurate quantification of the groups by this method.

RESULTS AND DISCUSSION

Analysis of purified exopolysaccharide from *Rhizobium* sp. strain ANU280 revealed glucose, galactose, glucuronic acid, pyruvic acid, and acetate in the ratios ~5:2:2:1:1 (Table II) and accounted for 91% of the polysaccharide. The ^1H-n.m.r. spectrum indicated a preponderance of β linkages and the presence of an *R*-(1-carboxyethylidene) acetal and an acetyl group. The polymer was therefore broadly comparable in content to those from other fast-growing *Rhizobium* strains[16].

Characterisation of uronic acids in polysaccharides is hampered by the resistance of uronosyl linkages towards acid and the instability of the sugars released under the conditions of hydrolysis[17]. These problems can be avoided by carboxyl-reduction of the polymer, often with incorporation of a deuterium label, before hydrolysis or methylation analysis[18–19].

The present work exploited the acid resistance of the uronosyl linkages during graded acid hydrolysis. Under optimised conditions, a mixture of neutral and acidic oligosaccharides was obtained, which was fractionated by application of ion-exchange and gel chromatography in sequence. Efficient gel chromatography of acidic oligosaccharides can be carried out with an acidic eluant[11].

When the exopolysaccharide was treated with trifluoracetic acid, and the acidic (**A**) and neutral (**N**) products were fractionated on Dowex 1 (formate) resin and analysed by p.c. (solvents *1* and *2*), it was found that extended treatment (2M acid, 100°, 8 h) caused almost complete cleavage of the hexosyl linkages, but that small proportions of acidic oligomers remained. Milder treatment (M acid, 100°, 2 h) gave much larger proportions of acidic oligomers, up to d.p. 6, together with neutral oligosaccharides. The latter conditions were used for preparation of the fragments.

Ion-exchange chromatography of the mixture of fragments on DEAE-Sephadex separated the neutral and acidic components and effected some resolution of the acidic oligosaccharides (Fig. 1). Subsequent gel chromatography of the unretained fraction gave neutral (**N2–N5**) oligosacharides up to d.p. 5. The di- and tri-saccharide fractions were mixtures and were resolved by preparative p.c. (solvent *1*). Gel chromatography of the acidic fraction gave oligomers (**A2–A6**) of d.p. 2–6. The oligosaccharide sizes were determined from their behaviour in gel chromatography[11].

β-Gal*p*-(1→4)-Glc **N2a**

β-Glc*p*-(1→6)-Glc **N2b**

β-Glc*p*-(1→4)-Glc **N2c**

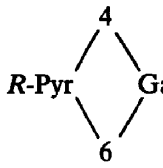
β-Glc*p*-(1→3)-Gal **N2d**

β-Glc*p*-(1→6)-β-Glc*p*-(1→4)-Glc **N3a**

β-Glc*p*-(1→4)-β-Glc*p*-(1→4)-Glc **N3b**

β-Glc*p*-(1→4)-β-Glc*p*-(1→4)-β-Glc*p*-(1→3)-Gal **N4**

β-Glc*p*-(1→6)-β-Glc*p*-(1→4)-β-Glc*p*-(1→4)-β-Glc*p*-(1→3)-Gal **N5**



A1

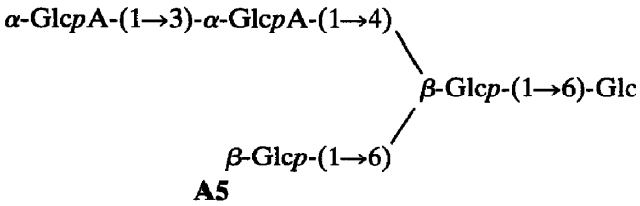
α-Glc*p*A-(1→3)-GlcA **A2**

α-Glc*p*A-(1→3)-α-Glc*p*A-(1→4)-Glc

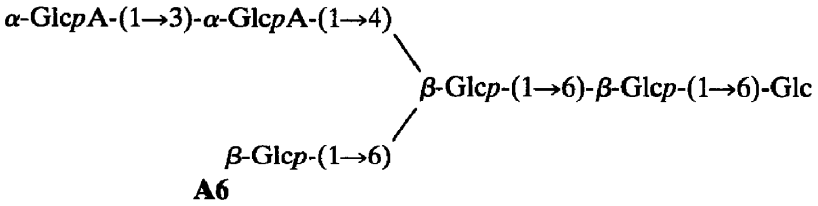
A3

α-Glc*p*A-(1→3)-α-Glc*p*A-(1→4)-β-Glc*p*-(1→6)-Glc

A4



A5



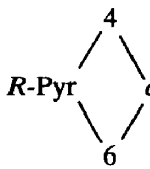
A6

All oligomers were homogeneous by p.c. (solvents *1* and *3* for neutral sugars, solvents *2* and *4* for acidic sugars). After extensive hydrolysis (2M trifluoracetic acid, 16 h, 95°), the resulting sugars were determined by p.c. (solvents *1* and *2*) and by g.l.c. of their alditol acetates. Only two oligosaccharides, **N2a** and **N5**, contained galactose.

Characterisation of the oligosaccharides by n.m.r. spectroscopy was greatly simplified by the isolation of several fragments of different size. Where reference samples or published spectra were not available, rigorous characterisation of spectra of key parent oligomers (*e.g.*, **N2b** and **A2**) was carried out (see below).

All neutral oligomers, except **N2a**, were completely degraded by β-D-glucosidase, which proved that the constituent sugars were β-D. Galactose and glucuronic acid, isolated by preparative p.c., were shown to be D by comparison of their c.d. spectra with those of authentic samples.

Exhaustive periodate oxidation of the exopolysaccharide at pH 5.2 and borohydride reduction of the products gave material that became almost completely dialysable, as judged by colorimetric analysis. Therefore, the final hydrolysis was carried out without preliminary dialysis. Fractionation of the products using Biogel P-2 gave **P1a, P1b**, and galactose[11,20].



α-Gal*p*-(1→3)-L-erythronic acid

P1a

α-Glc*p*A-(1→2)-erythritol

P1b

Gal

P1c

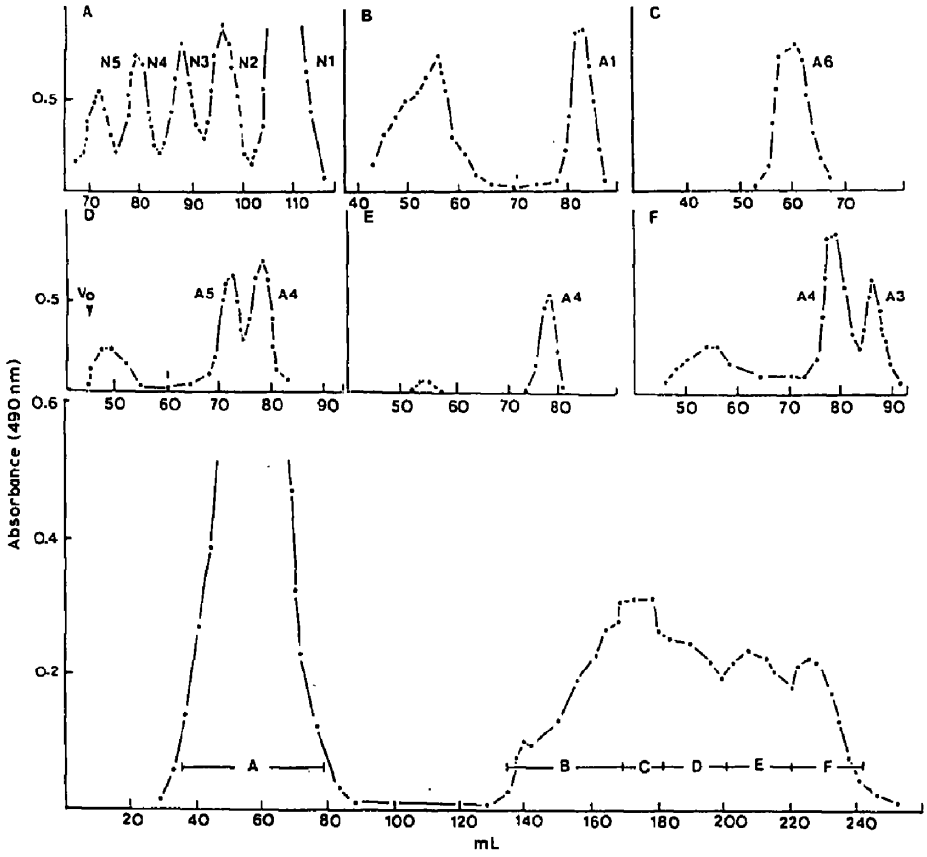


Fig. 1. Chromatography of the hydrolysis fragments from *Rhizobium* sp. strain ANU280 exopolysaccharide on DEAE-Sephadex A25 and rechromatography of fractions A–F on Biogel P-2 (panels A–F) (see Experimental for details).

The formation of **P1b** was consistent with the structures of the fragments obtained by acid hydrolysis (see above), but the formation of galactose is surprising. Evidently, it was hydrolysis of the Gal linkage, under the essentially neutral conditions of dialysis, that caused depolymerisation and loss after reduction of the polyaldehyde.

The isolation of **P1a** showed that the galactose was linked to one of the uronic acid residues and its resistance to oxidation indicated that it was substituted at O-2 or O-3. It is likely that the substituent was the single acetyl group that is present in each repeating unit. The terminal position of this galactose residue was consistent with its rapid removal under conditions of mild hydrolysis. This is evident from the data in Table I, but the isolation of large amounts of pyruvate-substituted galactose (**A1**) after acid hydrolysis indicates that even greater amounts of the residues are released in the early stages of hydrolysis than are observed by g.l.c. of alditol acetates.

TABLE III

^{13}C-N.M.R. DATA FOR THE NEUTRAL OLIGOSACCHARIDES (N) OBTAINED FROM *Rhizobium* STRAIN ANU280 EXOPOLYSACCHARIDE

Carbon assignment[a]		N2a	N2b	N2c	N2d	N3a	N3b	N4	N5
Unit a	1	93.0, 97.0[b]							
	2	72.4, 75.0							
	3	72.2, 75.6							
	4	79.7, 79.5							
	5	71.6, 75.9							
	6	61.2, 61.3							
Unit b	1		103.9			103.9			103.8[e]
	2		74.3			74.3			74.3[f]
	3		76.9			77.1			77.1
	4		70.8			70.8			70.6
	5		76.9			76.6			76.6
	6		61.9			61.9			61.9
Unit c	1		93.4, 97.2	103.8		103.9	103.7[c]	103.8[d]	103.9[e]
	2		72.6, 75.2	74.3		74.3	74.3	74.1	74.1[f]
	3		74.5, 77.1	76.7		76.8	77.2	77.2	76.8
	4		70.7, 70.7	70.7		70.8	70.6	70.7	70.8
	5		71.6, 72.1	77.2		76.0	76.0	76.6	75.9
	6		69.9, 70.0	61.8		69.9	61.7	61.8	69.8
Unit d	1			93.0, 97.0		93.0, 96.9	103.5[c]	103.5[d]	103.5[e]
	2			72.5[i], 74.5		72.2, 75.6	74.1	74.3	74.3[f]
	3			72.4[i], 75.5		72.7, 75.9	76.7	76.1	76.0
	4			80.0, 79.9		80.6, 80.4	79.5	79.6[j]	80.1
	5			71.3, 75.1		71.1, 75.0	75.2	75.2	75.2[g]
	6			61.1, 61.3		61.1, 61.3	61.1	61.0[k]	61.0[h]
Unit e	1				105.0		93.0, 96.9	104.8	104.8
	2				74.3		72.0, 75.4	74.3	74.2[f]
	3				76.0		72.3, 75.8	76.1	76.0
	4				70.6		79.5, 79.5	79.4[j]	79.4
	5				76.7		71.3, 75.1	75.2	75.4[g]
	6				61.7		61.1, 61.1	61.1[k]	61.1[h]

Unit f	1	104.1	93.4, 97.4	93.4, 97.4	93.4, 97.4
	2	72.1	68.6, 72.1	68.5, 72.1	68.5, 72.0
	3	73.8	80.7, 83.8	80.0, 83.9	80.9, 83.9
	4	71.4	70.4, 69.6	70.2, 69.6	70.2, 69.6
	5	76.6	71.3, 77.0	71.3, 75.8	71.3, 75.8
	6	62.3	62.3, 62.2	62.3, 62.1	62.3, 62.1

[a]Unit designations as in structure **A9**. [b]The first value is for the α and the second for the β anomer. [c–k]Assignments may be interchanged.

The neutral fragments were mostly oligomers of D-glucose and their structures were assigned by using the generalisation that the ^{13}C resonance at the site of glycosyl substitution undergoes a downfield shift of ~9 p.p.m., whereas those of the carbon atoms on either side move upfield[21] by ~0.5 p.p.m. relative to the unsubstituted sugar. For glucose and galactose, the chemical shifts of the signals for substituted C-2, C-3, and C-4 are usually sufficiently distinctive to permit assignment but, if the residue is at the reducing terminus, the difference between the signals of the α and β forms is also characteristic[21,22], being ~2.5 p.p.m. for C-2 and C-3, and negligible for C-4. For this reason, the availability of a series of fragments of increasing size was particularly useful, as the addition of a new residue at the reducing terminus immediately gives the anomeric configuration of the penultimate sugar and the site of its attachment (Table III).

The ^1H-n.m.r. spectrum of the periodate-oxidation fragment **P1b** was assigned on the basis of decoupling experiments; $^{13}C,^1H$ correlation spectroscopy then permitted complete assignment of the ^{13}C-n.m.r. spectrum (Table V). This, together with published assignments for D-glucuronic acid[23], enabled assignment of the ^{13}C-n.m.r. spectrum of **A2**. The spectrum of trisaccharide **A3** was used to verify the (1→3) linkage between the glucuronic acid residues. Using proton decoupling of the characteristic H-1 and H-5 resonances of the uronic acids, it was possible to locate the resonances of H-2′ and H-2″, and H-4′ and H-4″, but not to distinguish within the pairs. The information was used to assign the C-2 and C-4 resonances in the heteronuclear correlated spectrum. One C-2 peak was shifted to higher field by ~1 p.p.m. The two C-4 peaks were close to the normal position. The central glucuronic acid residue was therefore 3-substituted and the C-3′ resonance was as expected. The isolation of periodate-oxidation fragment **P1b** confirms this conclusion.

The higher members of the acidic oligosaccharide series were assigned by methods similar to those employed for the neutral fragments (Table IV).

The structure of the periodate-oxidation product **P1a** was also determined by heteronuclear correlation spectroscopy. The chirality of the pyruvate acetal is *R*, on the basis of the chemical shifts of the signal of its methyl group[15], and the downfield shifts induced by the substituent (Table V) are smaller than those observed for glycosyl substitution[21]. The acetalated galactose was also recovered as **A1**, the smallest fragment of acid hydrolysis (Fig. 1, Table V).

The extensive overlap between oligosaccharides permits the identification of an octasaccharide repeating unit. The remaining residue is acetalated terminal galactose, which is represented by **A1** and **P1a**. The resulting repeating unit **A9** generally resembles those of other fast-growing *Rhizobium* strains[16], but is unusual in that it has a neutral main-backbone chain with a high concentration of acidic groups in the branches.

TABLE IV

^{13}C-N.M.R. DATA FOR THE ACIDIC OLIGOSACCHARIDES (**A**) OBTAINED FROM *Rhizobium* STRAIN ANU280 EXOPOLYSACCHARIDE

Carbon assignment[a]		**A2**	**A3**	**A4**	**A5**	**A6**
Unit a	1				103.8	103.9
	2				74.1	74.2
	3				77.2	77.2
	4				70.6	70.6
	5				76.2	76.2
	6				61.8	61.8
Unit b	1		93.1, 96.8[b]	103.7	103.7	103.9
	2		72.5, 75.2	74.2	74.2	74.3
	3		74.3, 77.3	77.2	76.7	76.6
	4		78.7, 78.5	78.5	78.5	78.6
	5		71.3, 75.8	75.8	75.8	76.2
	6		61.7, 61.9	61.8	70.2	70.3
Unit c	1			93.3, 97.2	93.3, 97.1	103.5
	2			72.6, 75.2	72.5, 75.2	75.8
	3			74.3, 76.9	74.3, 76.8	76.7
	4			70.6, 70.7	70.8, 70.7	70.9
	5			71.6, 76.1	71.7, 76.0	76.0
	6			69.8, 70.0	69.9, 69.7	69.8
Unit d	1					93.0, 97.0
	2					72.4, 75.3
	3					72.5, 75.4
	4					80.3, 80.3
	5					?, 75.1
	6					61.2, 61.2
Unit h	1	100.0, 99.8	100.2	100.2	100.2	100.2
	2	73.1	72.7	72.7	72.7	72.7
	3	73.8	73.9	73.9	73.8	73.9
	4	73.2	73.2	73.2	73.1	73.2
	5	73.7	?	?	?	74.2
	6	178.2[c]	178.2	178.1	177.8	177.9
Unit i	1	93.4, 97.1	101.2, 101.1	101.2	101.2	101.2
	2	72.6, 72.6	71.4, 71.3	71.3	71.3	71.3
	3	82.5, 80.2	80.4, 80.2	80.2	80.2	80.2
	4	73.2, 73.8	73.5	73.5	73.4	73.5
	5	77.4	?	?	?	?
	6	177.0[c]	177.3	177.3	177.1	177.2

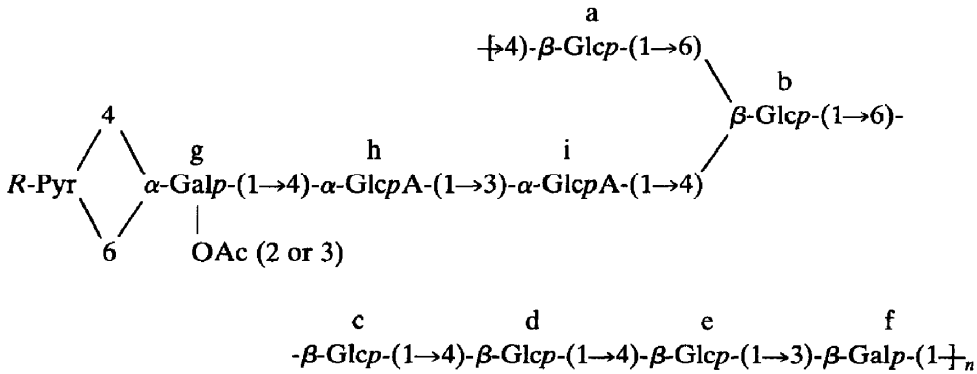
[a]Unit designations as in structure **A9**. [b]The first value is for the α and the second for the β anomer. [c]Assignments interchangeable in all oligosaccharides.

TABLE V

^{13}C-N.M.R. DATA FOR THE PYRUVATE ACETAL FRAGMENTS OBTAINED FROM *Rhizobium* STRAIN ANU280

Carbon assignment		**A1**	**P1a**	**P1b**
Sugar	1	93.9, 97.2[a]	100.5	98.9
	2	69.2[b], 72.6[c]	69.1[d]	72.5
	3	63.2, 67.2	64.0	73.9
	4	72.7, 72.2	72.7	73.2
	5	68.9[b], 72.8[c]	69.5[d]	73.8
	6	66.3, 66.2	66.2	175.2
Carboxyethylidene	1	177.1	178.2	
	2	101.8	101.7	
	3	26.3	26.4	
Oxidation fragment	1		178.2	60.8
	2		73.6	79.7
	3		81.8	72.6
	4		61.3	63.7

[a]The first value is for the α and the second for the β anomer. [b–d]Assignments may be interchanged.



Our analytical procedure lacks the sensitivity of the best protocols based on methylation analysis[18,19]. Because of the excellent chromatographic recoveries, however, total analysis can be carried out on less than 1 g of polysaccharide. The method is experimentally simple, yields direct information concerning ring types and substitution, and is the method of choice when gram amounts of an acidic polysaccharide are available.

ACKNOWLEDGMENTS

We thank Mrs. Brigid Roberts and Miss Helen Elliot for skillful assistance, and one of us (S.P.D.) acknowledges the award of an A.N.U. Scholarship.

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