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THE STRUCTURE OF THE ACIDIC POLYSACCHARIDE SECRETED BY *Rhizobium phaseoli* STRAIN 127 K44[†,*]

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ABSTRACT

The acidic polysaccharide secreted by *Rhizobium phaseoli* strain 127 K44 was found to differ in structure from the polysaccharides of two other strains of *Rhizobium phaseoli*, namely, strains 127 K36 and 127 K38. The 127 K44 polysaccharide has a repeating unit (**1**) of nine glycosyl residues and one pyruvic acetal group, as shown on page 158. All of the glycosyl residues were found to be in the D configuration and in the pyranoid ring-form.

INTRODUCTION

The acidic polysaccharides secreted by two strains of *Rhizobium phaseoli*, 127 K38 and 127 K36, have been shown to have different structures[1,2]. We now describe an investigation of the structure of the acidic polysaccharide secreted by a third strain of the same *Rhizobium* species, strain 127 K44.

EXPERIMENTAL

Rhizobium phaseoli strain 127 K44 was obtained from Dr. J. Burton, Nitragin Company, Milwaukee, WI. Experiments were conducted to confirm that strain 127 K44 is capable of nodulating bean plants. The acidic polysaccharide secreted by strain 127 K44 was purified, and structurally characterized, by procedures previously described[1–

[†]Host–Symbiont Interactions. Part XII. For Part XI, see ref. 1.

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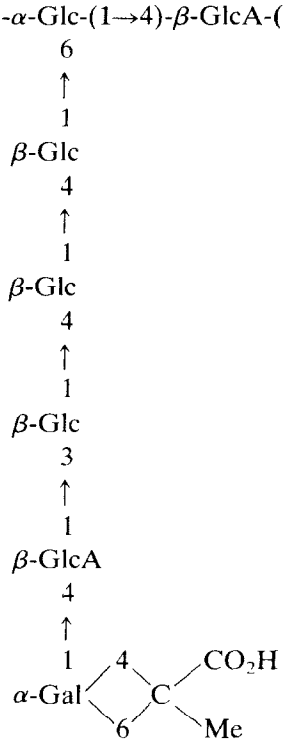

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RESULTS AND DISCUSSION

Composition of the polysaccharide. — The glycosyl composition of the purified polysaccharide was determined by methanolysis, dideuterio-reduction of the carboxyl groups, hydrolysis, reduction, and acetylation, followed by gas–liquid chromatography (g.l.c.) and g.l.c.–mass spectrometry (m.s.) analysis of the alditol

TABLE I

GLYCOSYL COMPOSITION OF THE ACIDIC POLYSACCHARIDE SECRETED BY *Rhizobium phaseoli* STRAIN 127 K44

Glycosyl residue	*Glycosyl composition (mol %)*	*Relative amount*
Galactosyl	11	1
Glucosyl[a]	57	5.2
Glucosyluronic acid[a]	32	2.9

[a]The relative amounts of glucosyluronic acid and glucosyl residues were determined by g.l.c.–m.s. analysis after reduction of the glucosyluronic residues as described[1].

acetates, as previously described[1]. The results of this analysis, which are presented in Table I, suggest a repeating unit of nine sugars, consisting of one galactosyl, three glucosyluronic acid, and five glucosyl residues. All of the glucosyl and glucosyluronic acid residues were, by using methods previously described[1,5], found to be in the D configuration.

Colorimetric determination of the pyruvic[6] and acetic acid[7] substituents gave values of 5.0 and 6.2%, respectively. These values are equivalent to 0.97 mol of pyruvic acid and 2.5 mol of acetic acid per repeating unit of nine glycosyl residues.

Glycosyl-linkage composition of the polysaccharide. — The methylated polysaccharide contained (1→3)-linked, (1→4)-linked, and 4,6-di-*O*-substituted glucosyl residues and 4,6-di-*O*-substituted galactosyl residues (see Table II, column A). Analysis of the methylated, dideuterio-reduced carboxyl polysaccharide showed the presence of (1→4)-linked glucosyluronic residues (see Table II, column B). The quantitative data in Table II show that the repeating unit of the polysaccharide contains one (1→3)-linked glucosyl, one 4,6-di-*O*-substituted glucosyl, one 4,6-di-*O*-substituted galactosyl, three (1→4)-linked glucosyluronic acid, and three, or four, (1→4)-linked glucosyl residues. The sequence analysis next described established that only three (1→4)-linked glucosyl residues were actually present in the repeating unit.

Partial hydrolysis of the O-methylated dideuterio-reduced carboxyl polysaccharide, followed by reduction and O-ethylation, and then separation and analysis of the per-O-alkylated oligosaccharide-alditol. — The polysaccharide was per-*O*-methylated and the resulting methyl-esterified residues were reduced with lithium aluminum deuteride, as previously described[1]. Partial hydrolysis of the carboxyl-

TABLE II

GLYCOSYL-LINKAGE COMPOSITION OF THE EXTRACELLULAR POLYSACCHARIDE OF *Rhizobium phaseoli* STRAIN 127 K44

Glycosyl residues	*Positions of O-methyl groups*	*R.t.*[b]	*Sample*[a]	
			A (mol %)	*B*
Glucosyl	2,4,6	0.67	15	11
Glucosyl	2,3,6	0.69	54	39
Glucosyl	2,3	0.84	19	10
Galactosyl	2,3	0.85	12	9
Glucosyluronic acid[c]	2,3	0.84	—	31

[a]Sample A was *O*-methylated and hydrolyzed, and the resulting, partially *O*-methylated aldoses were reduced ($NaBD_4$), and the alditols acetylated. Sample B was *O*-methylated, carboxyl-reduced ($LiAlD_4$), and hydrolyzed, and the resulting, partially *O*-methylated aldoses were reduced ($NaBD_4$), and the alditols acetylated. [b]Retention time relative to *myo*-inositol hexaacetate on a capillary column (10 m) of SP-2100, programmed from 150 to 240° at 4°/min. [c]This residue results from the reduction of glucosyluronic acid residues to 6,6-dideuterio-D-glucosyl residues. [The relative amounts of the 2,3-di-*O*-methyl-D-glucosyl and -glucosyluronic acid residues were determined by g.l.c.–m.s.]

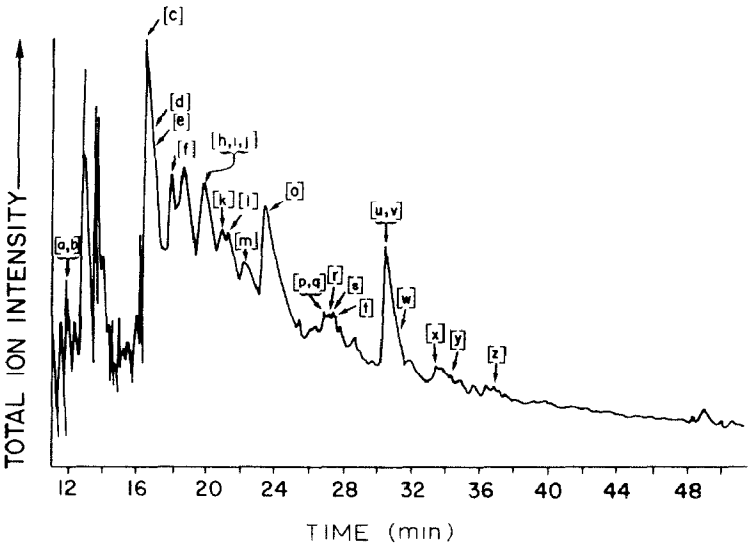

Fig. 1. Reverse-phase, l.c. elution-profile of the partially *O*-methylated, partially *O*-ethylated oligosaccharide-alditols derived from the oligosaccharide fragments produced by partial hydrolysis of the *O*-methylated, carboxyl-reduced polysaccharide. [The profile is the chemical-ionization, total-ion response of ~3% of the effluent from the l.c. column, which was introduced directly into the source of the mass spectrometer. The mass spectrometer scanned from *m/z* 200 to 1000, once every 3 s. Each per-*O*-alkylated oligosaccharide-alditol that has been structurally characterized has been assigned a letter indicating where it is eluted from the l.c. column (see Tables III, IV, and V, and Fig. 2).

reduced, *O*-methylated polysaccharide with 88% formic acid for 100 min at 80° yielded a mixture of partially *O*-methylated oligosaccharide fragments. The mixture of fragments thus obtained was reduced with $NaBD_4$, and the product *O*-ethylated, to yield a mixture of partially *O*-methylated, partially *O*-ethylated oligosaccharide-alditols. These were separated by 3.5-MPa l.c., as described[1] (see Fig. 1). The effluent was monitored by mass spectrometry, as described[4].

The per-*O*-alkylated oligosaccharide-alditols were located, in the fractions obtained from the l.c. column, by reconstructed, selected-ion chromatograms using the (M + 1) ions (calculated from the data of Tables I and II) of all possible per-*O*-alkylated disaccharide-, trisaccharide-, and tetrasaccharide-alditols. Fractions containing per-*O*-alkylated oligosaccharide-alditols were further examined by g.l.c.–m.s., using electron impact (e.i.) fragmentation to establish the identities of the per-*O*-alkylated oligosaccharide-alditols. Nine per-*O*-alkylated disaccharide-alditols (see Table III), nine per-*O*-alkylated trisaccharide-alditols (see Table IV), and seven per-*O*-alkylated tetrasaccharide-alditols (see Table V) were detected, and their glycosyl sequences determined. It should be noted that, as a result of deuterio-reduction before *O*-ethylation, each glucosyluronic residue was converted into a 6,6-dideuterioglucosyl residue having an ethyl group on O-6. The structures of fragments [f], [i], [o], [p], and [u] were confirmed by hydrolysis, and analysis of

TABLE III

I C AND G L C RETENTION-TIMES AND DIAGNOSTIC IONS FROM C I - AND E I -M S OF PARTIALLY *O*-METHYLATED, PARTIALLY *O*-ETHYLATED DISACCHARIDE-ALDITOL FRAGMENTS OBTAINED FROM THE POLYSACCHARIDE OF *Rhizobium phaseoli* STRAIN 127 K44

Oligosaccharide	*Fragment*[a]	*R t*[b] (I c)	*(M + 1) ion*	*R t*[c] (g l c)	*Electron-impact mass-spectral fragment-ions*[d]			
					aJ_2	aJ_1	bA_1	bA_2
Et→3Glc→4Glc→	[a]	12 20	514	10 65	264 (48 3)	338 (4 9)	233 (19 6)	187 (100)
Et→4Glc→4Glc→	[b]	12 20	514	10.62	264 (63 8)	324 (11 2)	233 (23 6)	201 (100)
Et→4GlcA→3Glc→	[c]	16 60	530	10 75	264 (25 0)	324 (11 2)	249 (5 5)	217 (100)
Et→4Glc→6Glc→ (4 ↑ Et; Et ↓ 6)	[e]	16 90	528	11 87	278[e] (85 2)	338 (46 5)	233 (16 0)	201 (100)
Et→4Gal→4GlcA→	[f][f]	18 05	544	11 47	280 (87 8)	340 (26 5)	247 (32 0)	215 (28 1)
Et→4GlcA→4Glc→	[g]	18 24	530	11 08	264 (100)	324 (32 3)	249 (17 5)	217 (83 1)
Et→4Glc→4Glc→ (6 ↑ Et; Et ↓ 6)	[h]	19 82	528	11 15	278[g] (57 9)	338 (7 5)	233 (7 1)	201 (100)
Et→4Glc→4GlcA→	[j][f]	20 25	544	10 65	280 (56 0)	340 (11 1)	247 (18 0)	215 (100)
Et→4GlcA→4GlcA→	[m]	22 32	546	11 03	280 (94 7)	340 (19 6)	249 (20 7)	217 (100)

[a]See Figs 1 and 2 [b]Retention time (in min) on a Brownlee column [c]Retention time (in min) on a J & W fused-silica, capillary column (30 m × 0 25 mm) of SE-30 [d]Figures in parentheses show peak intensity relative to the base peak (= 100) [e]*m/z* 206 (9 2) and 174 (18 7) are present, showing that the alditol is linked through O-6 [f]The identity (Glc or Gal) of the nonreducing termini of [f] and [j] was determined by formation of the corresponding, partially *O*-alkylated alditol acetates, followed by determination of the g.l c retention-times[g] [g]*m/z* 206 and 174 are absent, showing that the alditol is linked through O-4

TABLE IV

G.C. AND G.L.C. RETENTION TIMES, AND DIAGNOSTIC IONS FROM C.I.- AND E.I.-M.S. OF PARTIALLY O-METHYLATED, PARTIALLY O-ETHYLATED TRISACCHARIDE ALDITOL FRAGMENTS OBTAINED FROM THE POLYSACCHARIDE OF *Rhizobium phaseoli* STRAIN 127 K44

Oligosaccharides	*Fragment*[a]	*R.T.*[b] (*l.c.*)	(*M + 1*) *ion*	*R.T.*[c] (*g.l.c.*)	*Electron impact mass spectral fragment-ions*[d] aJ	aJ_1	abJ_2	abJ_1	cbA	cbA	cA_1	cA_2
Et→3Glc→4Glc→4Glc→	[d]	16.72	718	17.1	264 (32.5)	324 (4.6)	468 (0.2)	542 (0.2)	437 (0.4)	405 (0.4)	233 (4.9)	187 (100)
Et→4Gal→4GlcA→3Glc→ (Et→6 on Gal)	[h][e]	19.82	748	17.4	264 (100)	324 (34.2)	484 (1.5)	544 (0.9)	467 (0.5)	435 (0.4)	247 (35.4)	215 (33.7)
Et→4Glc→6Glc→ (4 ← Et→4Glc)	[k]	21.15	732	17.2			482 (14.6)	542 (2.7)			233 (14.7)	201 (100)
Et→4Glc→4Glc→6Glc→ (4 ← Et)	[l]	21.15	732	18.2	278 (61.4)	338 (15.2)	482 (0.6)	542 (1.2)	437 (0.8)	405 (0.4)	233 (10.4)	201 (100)
Et→4GlcA→3Glc→4Glc→	[o]	23.53	734	17.5	264 (75.7)		468 (1.4)	528 (2.7)	453 (13.4)	421 (1.3)	249 (15.6)	217 (100)
Et→4GlcA→4GlcA→4Glc→	[p]	27.18	750	17.4	264 (100)	324 (12.9)	484 (0.6)	544 (0.9)	469 (0.7)	437 (0.6)	249 (5.6)	217 (54.2)
Et→4Glc→4Glc→4GlcA→ (Et→6)	[r]	27.48	748	17.2	280 (63.5)	340 (6.4)	498 (1.8)	558 (1.1)	451 (1.1)	419 (0.6)	233 (10.4)	201 (100)
Et→4GlcA→4Glc→4Glc→ (Et→6)	[t][f]	28.77	746	17.0	278 (100)	338 (9.8)	482 (0.4)	542 (0.5)	453 (0.7)	421 (0.7)	249 (5.5)	217 (98.4)
Et→4Glc→4GlcA→4GlcA→ (Et→6)	[w]	31.13	764	17.2	280 (98.5)	340 (11.4)			467 (0.9)	435 (1.2)	247 (6.2)	215 (100)

See Footnotes c,d to Table III. The identity (Glc or Gal) of the nonreducing termini of [h] and [w] were determined by formation of the corresponding partially O-alkylated alditol acetates followed by determination of the g.l.c. retentions. [e]The presence of m/z 206 (13) and m/z 174 (28.9) shows that the alditol is linked through O-6. The data are also consistent with the structure having the internal glycosyl residue linked through O-6 and an ethyl group on O-4. Both fragments are present in the proposed structure. [f]m/z 206 and m/z 174 are absent, demonstrating that the alditol was linked through O-4.

TABLE V

LC AND GLC RETENTION-TIMES AND DIAGNOSTIC IONS FROM CI- AND EI-MS OF PARTIALLY *O*-METHYLATED PARTIALLY *O*-ETHYLATED TETRASACCHARIDE-ALDITOL FRAGMENTS OBTAINED FROM THE POLYSACCHARIDE OF *Rhizobium phaseoli* STRAIN 127 K44

Oligosaccharide	*Fragment*[a]	*RT*[b] (*l.c.*)	(*M* + 1) *ion*	*RT*[c] (*g.l.c.*)	*Electron-impact mass-spectral fragment-ions*[d]							
					aJ_2	aJ_1	abJ_2	abJ_1	dcA_1	dcA_2	dA_1	dA_2
Et ↓ 4 Et→3Glc→4Glc→4Glc→6Glc→	[s]	27.62	936	24.5	278[e] (50.7)	338 (6.6)	482 (0.3)	542 (1.0)	437 (1.4)	405 (0.8)	233 (15.1)	187 (100)
Et→4GlcA→3Glc→4Glc→4Glc→	[u]	30.22	938	23.0	264 (75.3)	324 (4.4)	468 (0.6)		453 (1.2)	421 (0.7)	249 (5.2)	217 (32.2)
Et ↓ 6 Et→4Gal→4GlcA→3Glc→4Glc→	[v]	30.52	952	22.4	264 (86.0)			528 (0.7)	467 (0.5)		247 (40.0)	215 (23.3)
Et→4Glc→4Glc→6Glc→4GlcA→ 4 ↑ Et	[y][f]	34.15	952	22.0	280 (41.3)	340 (2.4)	498 (2.4)			405 (0.3)	233 (11.2)	201 (100)
Et ↓ 6 Et→4Glc→4GlcA→4GlcA→4Glc→	[z]	37.0	968	22.3	264 (97.4)	324 (8.3)	484 (0.8)	544 (0.4)	467 (0.8)	435 (0.2)	247 (6.1)	215 (100)
					$ab'J_2$	$ab'J_1$	cbA_1	cbA_2	cA_1	cA_2	$b'A_1$	$b'A_2$
c b a Et→4Glc→4Glc→6Glc→ 4 ↑ Et→4Glc b'	[q][f]	27.18	936	23.2	482 (5.8)	542 (0.3)	437 (0.7)	405 (0.4)	233 (11.6)	201 (100)	233 (11.6)	201 (100)
c b a Et→4GlcA→4Glc→4Glc→ 6 ↑ Et→4Glc b'	[x][f]	34.15	952	22.9	482 (13.5)	542 (1.0)	453 (0.6)		249 (6.8)	217 (77.9)	233 (7.1)	201 (66.3)

[a]–[d] See Footnotes *a*–*d* to Table III. [e] The presence of *m/z* 174 showed that the alditol is linked through O-6. [f] Determination of the structure of the oligosaccharides required knowledge of the structure of the per-*O*-alkylated oligosaccharide alditols [t] and [l].

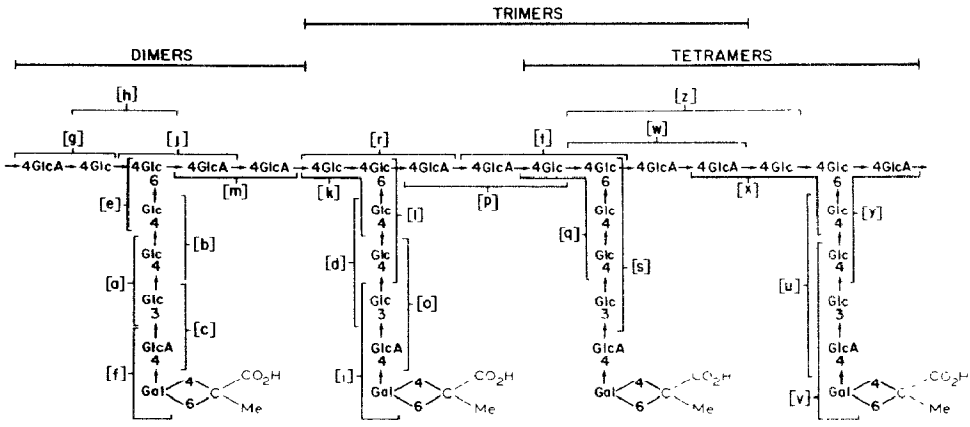

Fig. 2. Summary of the structurally characterized oligosaccharides derived from the acidic polysaccharide secreted by *R. phaseoli* strain 127 K44. [These fragments define the glycosyl sequence illustrated in the Figure. Each fragment is labeled by a letter, to identify the corresponding per-*O*-alkylated oligosaccharide-alditols in the Tables.]

the partially *O*-alkylated alditol acetates derived from them.

Pyranoid ring-forms of the glycosyl residues. — The ring forms of all of the glycosyl residues in the repeating unit of the polysaccharide were determined by identifying and characterizing per-*O*-alkylated oligosaccharide-alditols containing each glycosyl residue at either the (formerly reducing) alditol end or the nonreducing end. The ring forms of such residues are delineated by the location of the *O*-methyl and *O*-ethyl substituents[8].

Base-catalyzed degradation of the methylated polysaccharide. — The *O*-methylated polysaccharide was subjected to base-catalyzed degradation, as described[1,9]. Two major peaks were observed after reverse-phase, l.c. separation of the products. The fractions containing the major components were analyzed by e.i.-m.s. using a direct-inlet probe, by glycosyl-linkage composition-analysis, and by ^{1}H-n.m.r. spectroscopy. These data and the structures of fragments [o], [s], and [t] identified the two products of the base degradation as the methyl α- and β-glycosides of the per-*O*-alkylated pentasaccharide fragment shown in Table VI. The molecular weight of the methyl β-glycoside was confirmed by fast-atom-bombardment–mass spectrometry (f.a.b.–m.s.), which yielded an intense (M + 1) ion at *m/z* 1095.

The repeating unit of the polysaccharide. — The fragments that were identified among the reduced and *O*-ethylated products from the partial hydrolysis of the carboxyl-reduced, *O*-methylated polysaccharide are summarized in Fig. 2. The nonasaccharide repeating-unit (**1**) depicted in Fig. 2 is determined by many different sets of fragments, as, for example, fragments [i], [o], [s], [t], and [z]. The structure elucidated agrees with the data obtained by compositional analysis (see Table I), glycosyl-linkage analysis (see Table II), and base-catalyzed degradation (see

TABLE VI

DIAGNOSTIC IONS FROM THE E.I. MASS SPECTRUM OF THE PARTIALLY *O*-METHYLATED, PARTIALLY *O*-ETHYLATED PENTASACCHARIDE METHYL GLYCOSIDE OBTAINED BY BASE-CATALYZED DEGRADATION OF THE GLYCOSYLURONIC ACID RESIDUES OF THE METHYLATED POLYSACCHARIDE SECRETED BY *Rhizobium phaseoli* STRAIN 127 K44

	Diagnostic fragment-ions[a]
b′ a Et→4Glc→4Glc→OMe 6 ↑ Et→3Glc→4Glc→4Glc d c b	187 (100, dA_2) 201 (90, $b'A_2$), 233 (53, dA_1 + $b'A_1$), 405 (2.1, dcA_2), 437 (4.7, $b'aJ_2$ + dcA_1), 497 (0.6, $b'aJ_1$), 609 (0.6, $dcbA_2$), 641 (0.3, $b'abJ_2$ + $dcbA_1$), 701 (0.2, $b'abJ_1$), 905 (0.2, $dcbaJ_1$)

[a]% of base peak and fragment-ion designations are in parentheses.

Table VI) of the polysaccharide. No oligosaccharide fragments were found that contradicted the structure proposed.

Determination of the anomeric configuration of the glycosyl linkages by ^{1}H-n.m.r. spectroscopy. — The ^{1}H-n.m.r. spectrum of the methylated polysaccharide showed two α-anomeric signals and multiple β-anomeric signals (see Table VII). Five of the per-*O*-alkylated oligosaccharide-alditols ([c], [d], [i], [o], and [p]) isolated after partial hydrolysis of the *O*-methylated polysaccharide, and the pentasaccharide methyl glycoside obtained after base-catalyzed elimination, were also examined by ^{1}H-n.m.r. spectroscopy (see Table VII).

The data in Table VII allow the anomeric configuration of all of the glycosyl residues to be unambiguously assigned as illustrated in **1**. However, no fragment containing the branch-point glucosyl residue and the adjacent glucosyluronic acid residue was available in sufficient quantities, or sufficiently free from other oligomers, to allow ^{1}H-n.m.r. examination. This glycosidic linkage was deduced to be an α-linkage, because the other eight glycosidic linkages had been unambiguously characterized in isolated oligosaccharide fragments, and only one was an α-linkage. The ^{1}H-n.m.r. spectrum of the polysaccharide clearly showed the presence of two α-linkages. Further evidence that the glycosidic linkage of the branched glucosyl residues is in the α configuration came from consideration of the l.c. retention-times of the per-*O*-alkylated disaccharide-alditols [j] and [m]. These two fragments differ only by containing two and four deuterium atoms, respectively, and, because the two fragments are identical in the distribution of methyl and ethyl groups, they would be predicted to have virtually indistinguishable retention-times on the l.c. column if they had the same anomeric configuration of their glycosidic bond. The fact that their l.c. retention-times are different (see Table III) indicates that they differ in the anomeric configuration of the glycosidic linkages between their constituent residues[4]. The glycosidic linkage of fragment [m] was established to be in the β-anomeric configuration by ^{1}H-n.m.r. analysis of fragment [p] (see Table VII and Fig. 2). Therefore, fragment [j] contains an α-anomeric linkage.

TABLE VII

^{1}H-N M R CHEMICAL SHIFTS AND COUPI ING CONSTANTS OF THE ANOMERIC PROTONS OF THE *O*-METHYI ATED POLYSACCHARIDE, OF ISOLATED PER-*O*-ALKYLATED OLIGOSACCHARIDI -AI DITOLS AND OF A PER-*O*-ALKYLATED PENTASACCHARIDE ME THYL GLYCOSIDE

Material	*Fragment*	*Chemical shift* (δ)[a]	*Coupling constant* $J_{1,2}$ (*Hz*)	*Anomeric configuration assigned*
Methylated polysaccharide		5 49	3 0	α [1 proton]
		5 43	not resolved	α [1 proton]
		multiple signals centered at 4.3	not resolved	β [~9 protons]
Et→4GlcA→3Glc→	[c]	4 46	8.0	β
Et→4Gal→4GlcA→3Glc→	[i]	5 36	3.0	α
6 ↑ Et		4 50	not resolved	β
Et→3Glc→4Glc→4Glc→	[d]	4.30	not resolved	β,β
Et→4GlcA→3Glc→4Glc→	[o]	4 70	7 8	β
		4.47	8.4	β
Et→4GlcA→4GlcA→4Glc→	[p]	4 40	not resolved	β,β
Et→4Glc→4Glc→OMe		4 82	3 5	α (Me glycoside)[b]
6 ↑ Et→3Glc→4Glc→4Glc		multiple signals centered at 4.34	not resolved	β

[a]Relative to the signal for internal chloroform at δ 7 26 [b]See ref 9

The structure of the polysaccharide secreted by *Rhizobium phaseoli* strain 127 K44 has been determined to be that depicted in **1**. This structure has the same backbone as previously characterized polysaccharides secreted by *Rhizobia phaseoli* strain[1] 127 K38 and *R. phaseoli* strain[2] 127 K36, but it contains a side chain different from that of either of the previously described *R. phaseoli* polysaccharides.

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