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STRUCTURAL ELUCIDATION, USING H.P.L.C.–M.S. AND G.L.C.–M.S., OF THE ACIDIC POLYSACCHARIDE SECRETED BY *Rhizobium meliloti* STRAIN 1021*†

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ABSTRACT

An improved, glycosyl-sequencing method has been used for elucidating the structure of the acidic polysaccharide secreted by *Rhizobium meliloti* strain 1021. The polysaccharide was methylated, the ether partially hydrolyzed, the products were reduced, and the alditols ethylated; the resulting mixture of peralkylated oligosaccharide-alditols was analyzed by h.p.l.c.–m.s. Chemical ionization (c.i.) mass spectra were obtained at 2-s intervals, as the various, peralkylated oligosaccharide-alditols were eluted from the h.p.l.c. column. Peralkylated mono-, di-, tri-, and tetra-saccharide-alditols could be readily detected, and at least partially identified, by their M + 1 ions, in conjunction with other characteristic ions present in their c.i.-mass spectra. The peralkylated di- and tri-saccharide-alditols were further analyzed by g.l.c.–m.s. in the electron-impact mode. The structure of the acidic polysaccharide secreted by *R. meliloti* strain 1021 is the same as that of the previously characterized polysaccharide secreted by *R. meliloti* strain U-27.

INTRODUCTION

A general method[1,2], recently described, for sequencing the glycosyl residues of complex carbohydrates is based on fragmentation of the permethylated, complex carbohydrate, and subsequent purification by reverse-phase h.p.l.c. of the partially methylated, partially ethylated oligosaccharide-alditols derived from the partially methylated oligosaccharide fragments[2]. The glycosyl sequence of the peralkylated oligosaccharide-alditols is generally determined unambiguously by formation, and

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g.l.c.–m.s. analysis, of their constituent, partially methylated, partially ethylated alditol acetates. This sequencing method has now been improved by better chromatographic separation and more-sensitive detection, and structural analysis of the intact, peralkylated oligosaccharide-alditol fragments. Improvement of the method in these areas has been provided by combined h.p.l.c.–m.s. and by g.l.c.–m.s. analysis of the peralkylated oligosaccharide-alditols.

This improved method of sequencing is illustrated by the structural characterization of the acidic polysaccharide secreted by *Rhizobium meliloti* strain 1021, a polysaccharide selected in order to test the new procedures, because we are particularly interested in *R. meliloti* 1021 as it is the parent of non-nodulating nitrogen-fixation mutants[2a] that we are examining with regard to their ability to produce their normal complement of cell-surface and extracellular polysaccharides. Another reason for selecting the *R. meliloti* 1021 polysaccharide was that the acidic polysaccharide secreted by *R. meliloti* strain U-27 had previously been characterized by other methods[3]. We reasoned that the U-27 and 1021 polysaccharides might have similar structures and, therefore, knowledge of the structure of the U-27 polysaccharide might assist our studies of the 1021 polysaccharide. However, we realized that, like the acidic polysaccharides secreted by different *R. phaseoli* strains[3a], the two *R. meliloti* polysaccharides might not have the same structure.

EXPERIMENTAL

Isolation of the acidic polysaccharide secreted by R. meliloti *strain 1021.* — *Rhizobium meliloti* 1021 was obtained from Drs. Sharon Long and Frederic M. Ausubel of Harvard University, and was shown to nodulate alfalfa. The *Rhizobium* was grown in a defined liquid medium[4]. At late exponential phase, normally 3–4 days after inoculation, the bacteria were separated from the medium by centrifugation at 7000g for 10 min. The extracellular, acidic polysaccharide was isolated from the supernatant solution by precipitation with a quaternary ammonium salt, dissolved in 10% sodium chloride, and reprecipitated with 2 volumes of acetone[5,6]. The yield of lyophilized polysaccharide was ~150 mg/L of bacterial culture.

Glycosyl-composition analysis. — After hydrolysis with 2M trifluoroacetic acid (TFA) for 2 h at 120°, the glycosyl composition of the polysaccharide was determined by the alditol acetate method[7]. The absence of glycosyluronic residues from the polysaccharide was demonstrated by using the colorimetric method of Blumenkrantz and Asboe-Hansen[8].

Determination of the absolute configuration of the glycosyl residues. — The polysaccharide (1 mg) was hydrolyzed with 2M TFA for 2 h at 120°, and the solution was cooled, and evaporated. Half of the hydrolyzed sample was incubated with D-glucose oxidase, and the other half with D-galactose oxidase (Sigma Chemical Company). The oxidized samples were then per(trimethylsilyl)ated, and the compositions analyzed by g.l.c.–m.s., and compared to glucose and galactose standards.

Methylation analysis. — The polysaccharide (25 mg) was dried overnight in a vacuum oven at 40°. Dimethyl sulfoxide (5 mL) was added, and the slurry was stirred for 4 h at room temperature, and then sonicated for 36 h. Potassium dimethylsulfinyl anion[5] (1.0 mL of 5.0M) was added, and the mixture was stirred overnight. The solution was chilled to 0°, methyl iodide (1.0 mL) was added, and the mixture was stirred for 6 h at room temperature. The excess of methyl iodide was evaporated, and the methylated polysaccharide was dialyzed for 3 days against tap water, lyophilized, the residue taken up in chloroform, the suspension filtered through glass wool, and the filtrate evaporated to dryness.

Glycosyl-linkage composition-analysis. — Methylated polysaccharide (0.25 mg) was hydrolyzed with 2M TFA for 2 h at 120°, the sugars reduced with $NaBD_4$, the alditols acetylated, and the glycosyl-linkage composition determined as described[1]. The identity of the partially methylated alditol acetates was established by their g.l.c. retention-times and by their mass spectra[9].

Preparation of partially methylated, partially ethylated oligosaccharide-alditols. — The conditions of formolysis for optimum formation of di- and tri-saccharide fragments were determined by a g.l.c.–m.s. procedure[2]. Methylated polysaccharide (25 mg) was partially hydrolyzed with 90% formic acid (15 mL) for 45 min at 80°, the sugars reduced with $NaBD_4$, and the alditols ethylated as described[2].

Separation of the peralkylated oligosaccharide-alditols by h.p.l.c., using a mass spectrometer as the detector. — The mixture of partially methylated, partially ethylated oligosaccharide-alditols (2 mg) was separated by h.p.l.c. (Hewlett–Packard 1084 B liquid chromatograph) on a column of Dupont Zorbax ODS, by using a linear, 45-min gradient of 50–65% acetonitrile (Burdick and Jackson, distilled in glass) in water (Baker, h.p.l.c. grade) as the solvent. The eluate from the h.p.l.c. column was analyzed directly by chemical ionization (c.i.) m.s., using a Hewlett–Packard liquid-chromatograph probe interfaced directly to a Hewlett–Packard 5985 mass spectrometer equipped with a cryogenic pump for l.c.–m.s. analysis. A c.i. mass spectrum, from m/z 200–1000, of the h.p.l.c. eluate was obtained every two seconds. The h.p.l.c. solvent (acetonitrile in water) served as the c.i. reactant gas. The source temperature was 150°, and the source pressure was ~1.5 torr. Using this technique, ~3% of the h.p.l.c. effluent was diverted into the mass spectrometer for analysis. The remaining 97% was collected in 0.5-mL fractions.

Analysis of the h.p.l.c. fractions by g.l.c.–m.s. — H.p.l.c. fractions that contained partially methylated, partially ethylated di- or tri-saccharide-alditols were located by their c.i. mass spectra, and were further fractionated and analyzed by g.l.c.–m.s., using splitless injection on a capillary column (25 m) of SE-30. The g.l.c. was programmed to remain at 160° for 2 min, and then to rise from 160 to 220° at 30° per min, and finally from 220 to 330° at 8° per min. The g.l.c. effluent was analyzed directly by electron impact (e.i.) m.s. (Hewlett–Packard model 5985 GC/MS; 70 eV; source temperature, 150°).

Analysis by direct inlet probe e.i.-m.s. of isolated, peralkylated tetrasaccharide-alditols. — Isolated, peralkylated tetrasaccharide-alditols (~5 μg) were placed on the direct inlet probe, and inserted into the mass spectrometer source (Hewlett–Packard, Model 5895). These peralkylated tetrasaccharide-alditols could not be directly analyzed by g.l.c.–m.s., as they are not eluted from the g.l.c. column. Spectra were obtained at 70 eV, with a source temperature of 150°.

Analysis of the partially methylated, partially ethylated oligosaccharide-alditols by formation of their constitutive alditol acetates. — H.p.l.c. fractions containing a purified, peralkylated oligosaccharide-alditol were combined, hydrolyzed with 2M TFA for 2 h at 120°, the sugars reduced with $NaBD_4$, the alditols acetylated, and the acetates analyzed by g.l.c.–m.s.[1].

^{1}H-N.m.r. spectroscopy. — Polysaccharide (5 mg) was dissolved in 2 mL of deuterium oxide (99.7 atom% D) and freeze-dried. This procedure was repeated a second time, and then the dried polysaccharide was taken up in 1 mL of deuterium oxide (100 atom% D), and the suspension centrifuged. The supernatant solution was analyzed in a Nicolet 360-MHz, Fourier-transform, n.m.r. spectrometer at 90°. The n.m.r. parameters included 45° pulse, 16-k memory, and 2 s between pulses. Chemical shifts were assigned relative to an internal standard of sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (Stohler Isotope Chemicals), defined as having $\delta = 0.00$.

RESULTS AND DISCUSSION

Determination of the glycosyl composition, the absolute configuration of the glycosyl residues, and the glycosyl-linkage composition of the polysaccharide. — Glycosyl-composition analysis showed that the acidic extracellular polysaccharide secreted by *R. meliloti* 1021 is composed of glucosyl and galactosyl residues in the molar ratio of 7:1. No glycosyluronic residues could be detected by the colorimetric assay. The polysaccharide is not readily soluble in water. However, the dissolved material

TABLE I

GLYCOSYL-LINKAGE COMPOSITION OF THE EXTRACELLULAR, ACIDIC POLYSACCHARIDE SECRETED BY *Rhizobium meliloti* STRAIN 1021. THE POLYSACCHARIDE WAS METHYLATED, THE ETHER HYDROLYZED, THE SUGARS REDUCED, THE ALDITOLS ACETYLATED, AND THE ACETATES ANALYZED BY G.L.C. AND G.L.C.–M.S.

Glycosyl residue	*Determined position of O-methyl groups*	*Deduced points of attachment of glycosyl residues and pyruvyl groups*	*Retention time*[a]	*Mole percent*	*Number of residues per repeating unit*
Glucosyl	2,4,6	3	0.59	22.1	2
Glucosyl	2,3,6	4	0.61	28.1	2
Glucosyl	2,3,4	6	0.64	11.6	1
Glucosyl	2,3	4,6	0.80	25.5	2
Galactosyl	2,4,6	3	0.62	12.7	1

[a] Retention times relative to hexa-*O*-acetyl-*myo*-inositol as unity.

and the undissolved material, isolated by centrifugation, were shown to have the same glycosyl composition.

The absolute configuration of the glycosyl residues of the polysaccharide was determined by first converting the glucosyl and galactosyl residues of the polysaccharide into the free sugars by acid hydrolysis. The free glucose was completely oxidized by incubation with D-glucose oxidase, and the free galactose by incubation with D-galactose oxidase, showing that all of the glucosyl and galactosyl residues of the polysaccharide are in the D configuration.

Glycosyl-linkage composition-analysis (see Table I) indicated that the polysaccharide consists of two 3-linked glucosyl residues, two 4-linked glucosyl residues, one 6-linked glucosyl residue, one 3-linked galactosyl residue, and two 4,6-linked glucosyl residues, suggesting that the polysaccharide is composed of an octasaccharide repeating-unit. One of the 4,6-linked residues originates from a terminal glucosyl group bearing a 1-carboxyethylidene group (see later).

Formolysis of the permethylated polysaccharide. — Fragmentation of the methylated polysaccharide by heating in 90% formic acid for 45 min at 80° cleaved ~12% of the glycosyl linkages of the 4,6-linked glucosyl residues, ~30% of the glycosyl linkages of the 3-linked, 4-linked, and 6-linked glucosyl residues, and ~50% of the glycosyl linkages of the 3-linked galactosyl residues. These conditions give good yields of partially methylated di- and tri-saccharide fragments[2].

Advantages of c.i. mass-spectrometric detection of the liquid chromatograph effluent. — There are several advantages to using m.s., rather than refractive index, to detect peralkylated oligosaccharide-alditols in the effluent of a h.p.l.c. column. The most apparent advantage is the ability to use a concentration gradient of the eluting solvent that provides better resolution, and more-concentrated peaks that require less material for analysis. Concentration gradients cannot be used with a refractive index monitor. The use of a solvent gradient also permits the fractionation, in one sample, of peralkylated oligosaccharide-alditols of very different molecular weights. Another important advantage of m.s. detection is that c.i. mass spectra of the peralkylated oligosaccharide-alditols are obtained as they are eluted from the column.

C.i. mass spectra of peralkylated oligosaccharide-alditols give several important pieces of information. One is the molecular weight of each peralkylated oligosaccharide-alditol, up to and including peralkylated tetrasaccharide-alditols. The molecular weight determines whether the peralkylated oligosaccharide-alditol is a derivative of a di-, tri-, or tetra-saccharide fragment, as well as the number of *O*-ethyl substituents. The molecular weights and diagnostic ions from the c.i. mass spectra, in conjunction with the glycosyl-linkage composition of the intact polysaccharide, provide considerable information about the sequence of the glycosyl residues in the polysaccharide. It is, perhaps, more important that the c.i. mass spectra allow assessment of the purity of the collected, h.p.l.c. effluent-fractions, and indicate which peralkylated oligosaccharide-alditols and which h.p.l.c. fractions should be further analyzed in order

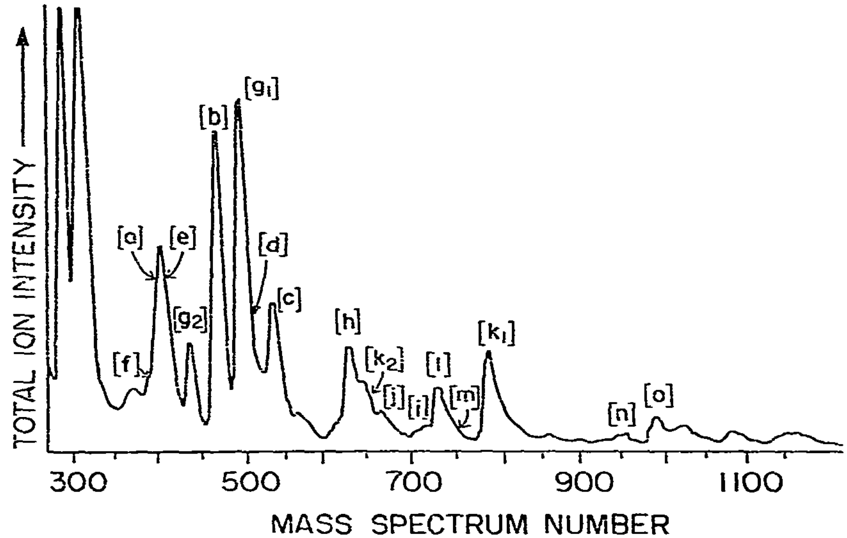


Fig. 1. Reverse-phase, h.p.l.c. elution-profile of the partially methylated, partially ethylated oligosaccharide-alditols derived from the oligosaccharide fragments produced by partial fragmentation of the acidic polysaccharide secreted by *R. meliloti* strain 1021. The profile is the chemical ionization total-ion response of 3% of the effluent from the h.p.l.c. column introduced directly into the source of the mass spectrometer. The mass spectrometer scanned from m/z 200 to 1000, once every 2 s. A linear gradient from 1:1 (v/v) acetonitrile–water to 13:7 acetonitrile–water, over 45 min, was used to elute the peralkylated oligosaccharide-alditols. Each peralkylated oligosaccharide-alditol that has been structurally characterized has been assigned a letter which shows where it elutes from the h.p.l.c. column. The structures of the identified fragments are shown in Fig. 10 and in Tables II and III.

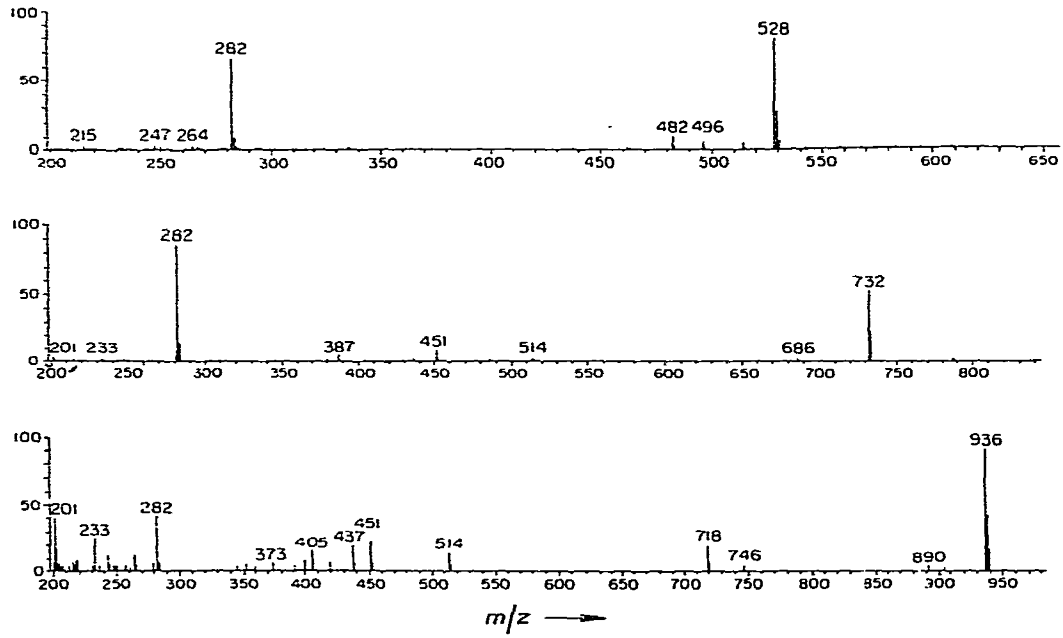


Fig. 2. Chemical ionization, mass spectra of partially methylated, partially ethylated di-, tri-, and tetra-saccharide-alditol fragments [g1], [k1], and [o] (see Figs. 1 and 4, and Tables II and III; see also, Fig. 10). The c.i. mass spectra were obtained while the fragments were being eluted from the h.p.l.c. column (see Fig. 1).

to give information that would be the most helpful in completing the elucidation of the structure of the polysaccharide.

Peralkylated oligosaccharide-alditols consisting of five or more glycosyl residues have molecular weights >1000, and, as this is the maximum ion detectable by the quadrupole, mass spectrometer used, their M + 1 ion cannot be detected by h.p.l.c.–m.s. However, their fragment ions are detected, and, therefore, location of the larger, peralkylated oligosaccharide-alditols in the eluting stream is possible. Still another advantage of the m.s. detector is that it is more sensitive than refractive index monitoring.

H.p.l.c. and c.i.–m.s. of peralkylated oligosaccharide-alditols. — The partially methylated oligosaccharide fragments of the *Rhizobium* 1021 polysaccharide were reduced, and the alditols ethylated. The resulting mixture of partially methylated, partially ethylated oligosaccharide-alditols was separated by h.p.l.c., using a linear solvent-gradient of acetonitrile and water, and detection by c.i.–m.s. The total-ion chromatogram of the h.p.l.c. effluent is presented in Fig. 1. Many c.i.-mass spectra were obtained of each peralkylated oligosaccharide-alditol eluted from the h.p.l.c. column.

Representative c.i. mass spectra of peralkylated oligosaccharide-alditols [g_1], [k_1], and [o], obtained during the elution of these fragments from the h.p.l.c.

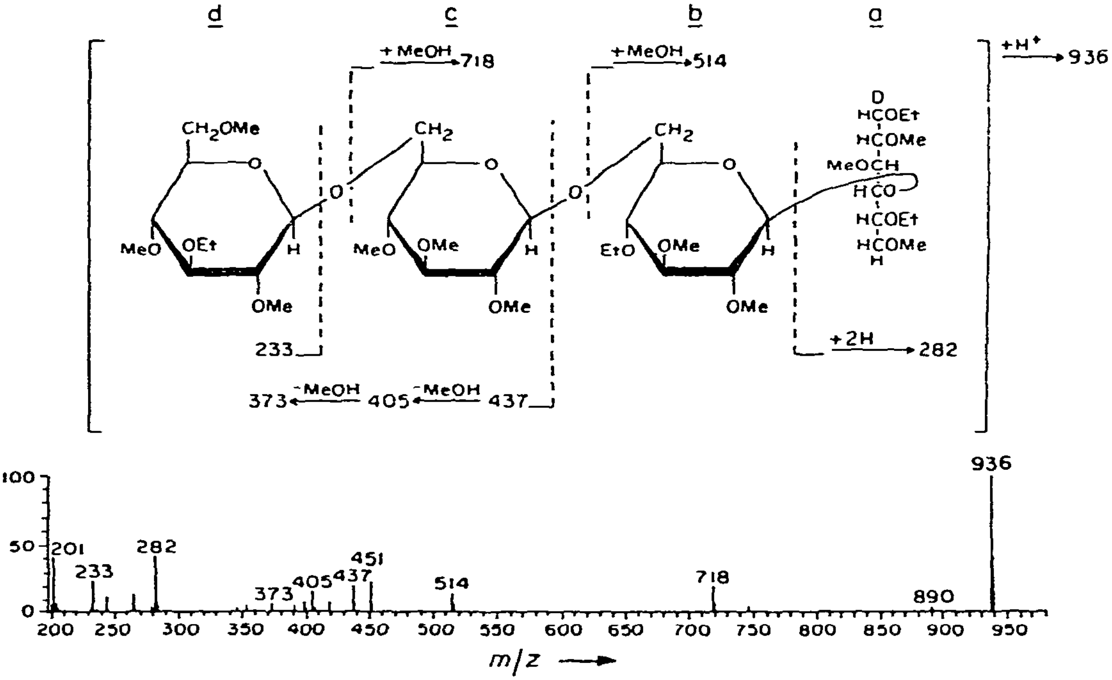


Fig. 3. C.i. mass spectrum, and the origin of the fragment ions produced during c.i.–m.s., of partially methylated, partially ethylated tetrasaccharide-alditol [o] (see Figs. 1, 4, and 10).

column, are illustrated in Fig. 2. As c.i. mass spectra of permethylated di- and trisaccharide alditols have previously been discussed[10], the spectra reported here will be described by using the nomenclature of that report[10]. Intense ions at *m/z* 528, 732, and 936 represent the M + 1 ions of the di-, tri-, and tetra-saccharide derivatives, respectively. Less-abundant ions, representing the losses of methanol and ethanol from the M + 1 ion, are also discernible.

The origin of the AOH$_2^+$ ion and of some other diagnostic ions of peralkylated tetrasaccharide-alditols [o] are illustrated in Fig. 3. The intense AOH$_2^+$ ion at *m/z* 282 in the c.i. mass spectrum of each of the peralkylated oligosaccharide-alditols originates by cleavage of the alditol residue. The ions at *m/z* 514 and 718 are probably formed by an alkoxyl or alkyl transfer[10]. The results obtained during this investigation suggest that the transfer occurs from C-4 of the neighboring glycosyl residue, that is, from C-4 of residue "d" for the ion at *m/z* 714, and from residue "c" for the ion at *m/z* 514. The ions at *m/z* 233 and 437 are from the G series[10], with the ions at *m/z* 201 being derived from the *m/z* 233 ion, and the ions at *m/z* 405 and 373 from the ion at *m/z* 437. The A$^-$ ion[10] is at *m/z* 264.

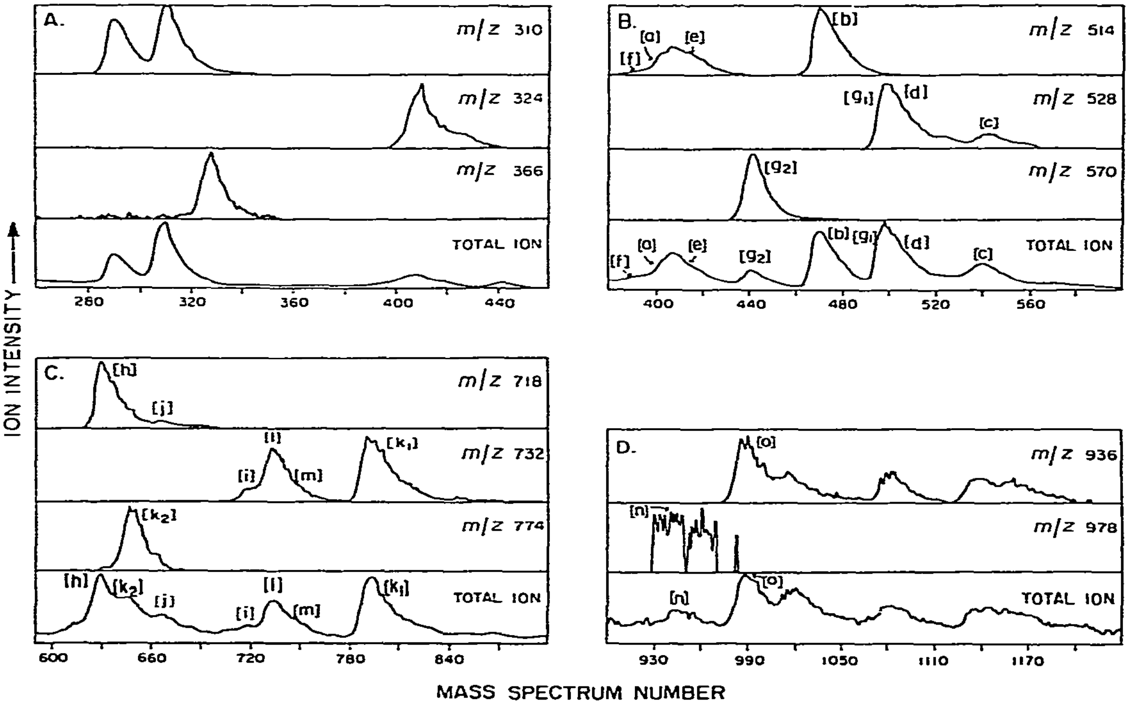


Fig. 4. Selected portions of the reverse-phase, h.p.l.c.–m.s., total-ion elution profile illustrated in Fig. 1, compared with the reconstructed, ion chromatograms of the protonated, molecular ion (M + 1 ion) of each peralkylated mono-, di-, tri-, and tetra-saccharide-alditol that could theoretically be produced from a polysaccharide having the glycosyl-linkage composition (see Table I) of the *R. meliloti* 1021 polysaccharide. The identities of the derivatized fragments in the peaks labelled with letters are given in Tables II and III, and Fig. 10.

Attachment of glycosyl residue "b" to different carbon atoms of glucitol residue "a" results in c.i. mass spectra having different relative-abundances of the A^+ and the AOHE ions[10]. The ratios of the abundance of the A^+ to the AOH_2^+ ions for glucosyl linkage to O-3, O-4, and O-6 of the alditol residue are ~0.03, 0.09, and 0.3, respectively.

The *m/z* values of the M + 1 ions for all of the theoretically possible, peralkylated mono-, di-, tri-, and tetra-saccharide-alditols that can be derived from the glycosyl residues composing the polysaccharide, as determined from the glycosyl linkage composition (see Table I), were calculated. Ion chromatograms of the theoretical M + 1 ions, compiled by the computer from the ~1,400 c.i. mass spectra obtained during elution of the peralkylated oligosaccharide-alditols from the h.p.l.c. column, are presented in Fig. 4. The M + 1 ions of peralkylated mono-, di-, and tri-saccharide-alditols containing three *O*-ethyl substituents have *m/z* values of 310, 514, and 718, respectively. The ion chromatograms computed from the calculated M + 1 ions (see Fig. 4) clearly show where such peralkylated oligosaccharide-alditols are eluted from the h.p.l.c. column. It is notable that no ions at *m/z* 922, equivalent to a theoretical, linear, peralkylated tetrasaccharide-alditol having three *O*-ethyl substituents, could be detected.

The M + 1 ions of the peralkylated mono-, di-, tri-, and tetra-saccharide-alditols which contain four *O*-ethyl substituents have *m/z* values of 324, 528, 732, and 936, respectively (see Fig. 4). The presence of four *O*-ethyl groups in these peralkylated oligosaccharide-alditols means that these fragments contain what had been a branched glycosyl residue, or a residue having a 1-carboxyethylidene substituent, in the underivatized polysaccharide.

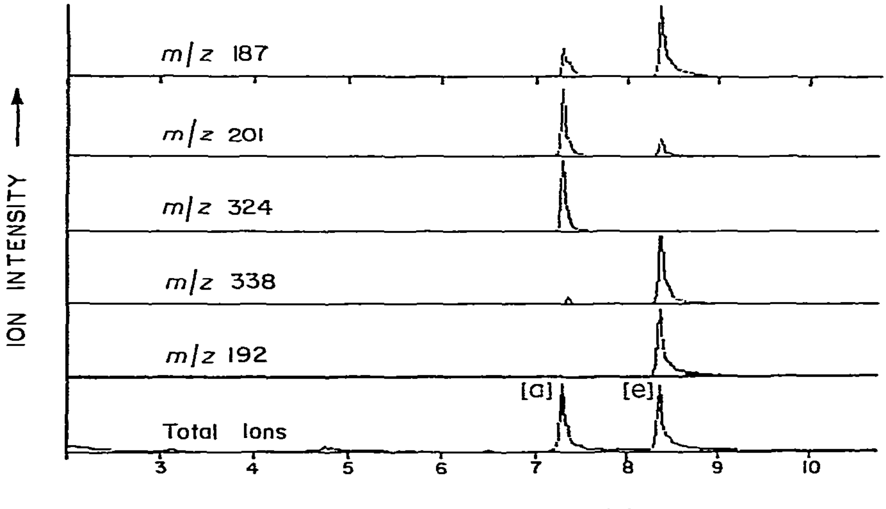


Fig. 5. Total-ion chromatogram and selected, reconstructed, ion chromatograms resulting from g.l.c.–m.s. (e.i.) analysis of partially methylated, partially ethylated disaccharide-alditols [a] and [e] (see Table II and Fig. 10) which were eluted from the h.p.l.c. column (see Figs. 1 and 4) when mass spectra 405 through 415 were being obtained. The collected h.p.l.c.-fraction containing these alditols was dried, and dissolved in chloroform, and an aliquot of the solution was injected into the g.l.c.–m.s. column, and analyzed.

The M + 1 ions of peralkylated mono-, di-, tri-, and tetra-saccharide-alditols, which, after the formolysis, have retained a ketosidically linked pyruvyl residue that was subsequently ethyl-esterified, have *m/z* values of 366, 570, 774, and 978, respectively. All of the components in the total ion chromatogram (see Fig. 1) can be accounted for by at least one component in the reconstructed, M + 1 ion chromatograms (see Fig. 4).

Determination by g.l.c.–m.s. (e.i.) of the purity of peralkylated oligosaccharide-alditols in the h.p.l.c. fractions. — Some of the h.p.l.c. fractions contained more than one peralkylated oligosaccharide-alditol. Fractions containing mixtures of either peralkylated di- or tri-saccharide-alditols were further fractionated by capillary-column g.l.c., and the column effluent was analyzed directly by e.i.-m.s. As an example, peralkylated disaccharide-alditols [a] and [e], which were not separated by h.p.l.c. (see Fig. 1), were clearly separated by g.l.c. (see Fig. 5). The purity of the components separated by g.l.c. is assessed by reconstructed, ion chromatograms. The ion profiles coincide for a pure component, as they do in Fig. 5.

G.l.c. and e.i.-mass spectrometric analysis of the h.p.l.c. fractions. — E.i. mass spectra of partially methylated, partially ethylated oligosaccharide-alditols have not previously been reported. Electron-impact mass spectra of permethylated oligosaccharides are, however, well known$^{11-14}$. The nomenclature of Kochetkov and Chizhov^{14} will be used in describing e.i. mass spectra. Particularly important are the ions from the A and J series (see Fig. 6), for these give information about the sequence of the glycosyl residues in the oligosaccharide. Further glycosyl-residue sequence information can be obtained by direct cleavage of the alditol chain, leading to frag-

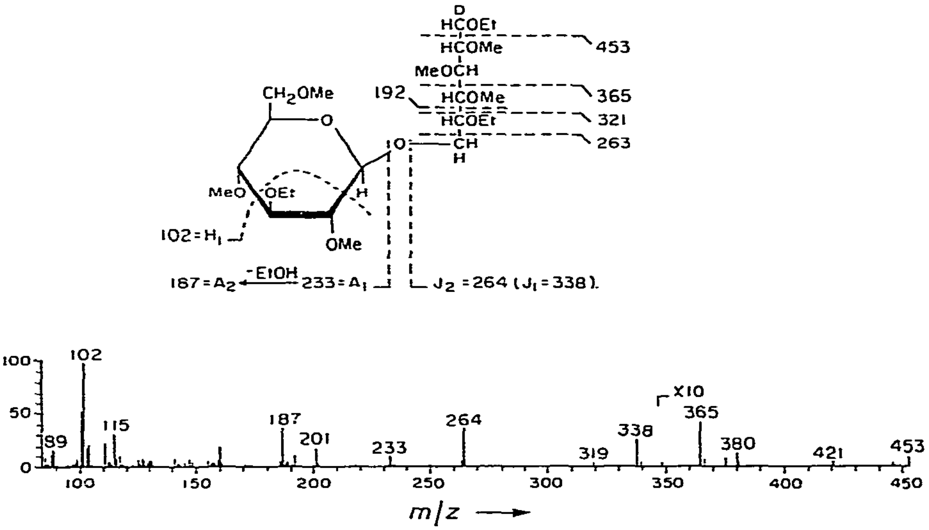


Fig. 6. E.i. mass spectrum of partially methylated, partially ethylated disaccharide-alditol [e] (see Figs. 1, 4, 5, and 10, and Table II).

ment ions that often identify the point of attachment of the glycosyl residue to the alditol.

A typical e.i. mass spectrum of a peralkylated disaccharide-alditol {fragment [e] in Figs. 1, 4, and 5} is shown in Fig. 6. The fragment ion at *m/z* 233 agrees with the mass expected of an A_1 ion, and indicates that the nonreducing glycosyl group is a hexosyl group bearing one *O*-ethyl group. A_1 ions from partially methylated, partially ethylated oligosaccharide-alditols can lose methanol or ethanol, to yield A_2 ions. Alkyl groups are more readily lost from O-3 than from O-4, and from O-4 more readily than from O-6. The loss of ethanol from the A_1 ion at *m/z* 233 of the peralkylated oligosaccharide-alditol, shown in Fig. 6, yields the predominant A_2 ion at *m/z* 187, indicating that O-3 of the glycosyl residue is substituted with an ethyl group. The presence of an ethyl substituent on O-3 is confirmed by the abundant H_1 ion at *m/z* 102.

The fact that the alditol of the peralkylated disaccharide-alditol is a hexitol bearing two *O*-ethyl groups is ascertained from the J_2 ion at *m/z* 264. The presence of an ethyl group on O-3 of the (nonreducing) glycosyl group is confirmed by the mass of the J_1 ion at *m/z* 338. The J_1 ion is formed by a complicated fragmentation-process[14] involving the peralkylated glycosyl residue attached to the point of J_2 fragmentation. The covalent bonds between C-1 and C-2, and between C-1 and the ring-oxygen atom, of the attached, peralkylated glycosyl residue are cleaved, with migration of the ethyl group from O-3 to O-1 of that glycosyl residue[14]. The addition of the 3-*O*-ethyl group, and of C-1, H-1, and O-1 to the alditol, results in the J_1 ion at *m/z* 338.

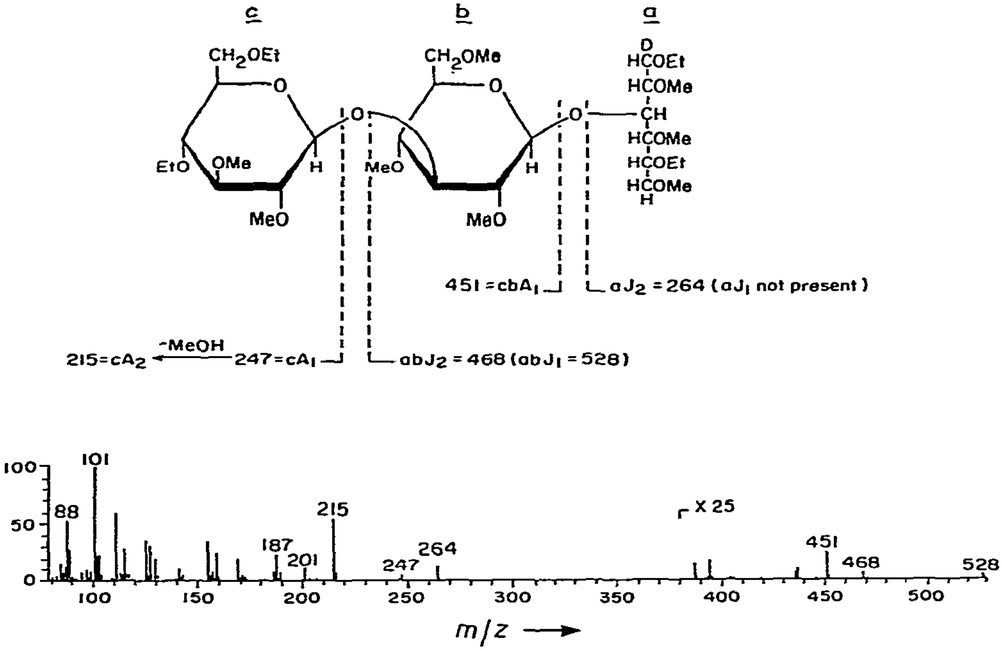


Fig. 7. E.i. mass spectrum of partially methylated, partially ethylated trisaccharide-alditol [k1] (see Figs. 1, 4, 5, and 10, and Table III).

Ions at *m/z* 192, 365, and 453 and a weaker ion at *m/z* 263 are consistent with cleavages of the alditol chain. These ions establish that the peralkylated disaccharide-alditol is 6-linked.

The e.i. mass spectrum of a peralkylated trisaccharide-alditol {fragment [k$_1$] in Figs. 1, 4, and 5} is shown in Fig. 7. This peralkylated trisaccharide-alditol was eluted from the h.p.l.c. column (Figs. 1 and 4) while the c.i. mass spectra 785 through 795 were being obtained. The c.i. mass spectrum of [k$_1$] is illustrated in Fig. 2.

The mass of peralkylated glycosyl residue "c" of [k$_1$] (see Fig. 7) is obtained from the cA$_1$ ion at *m/z* 247. The mass of this ion shows that "c" is a hexosyl residue bearing two *O*-ethyl groups. The ratio of the cA$_2$ ions at *m/z* 215 and at *m/z* 201 establishes that O-3 of residue "c" is substituted with a methyl group. As two *O*-ethyl groups are present on residue "c", the ethyl groups must be on O-4 and O-6, because the glycosyl-linkage analysis (see Table I) shows that all of the glycosyl residues have a methyl group on O-2 (there are no 2-linked glycosyl residues).

The cbA$_1$ ion at *m/z* 451 (see Fig. 7) provides, by subtraction of the mass of residue "c", the mass of glycosyl residue "b". This ion shows that residue "b" is a hexosyl derivative containing three *O*-methyl groups and no *O*-ethyl groups. The loss of one, or two, methanol residue(s), respectively, from the cbA$_1$ ion leads to cbA$_2$ at *m/z* 419, and cbA$_3$ at *m/z* 387.

The aJ$_2$ ion at *m/z* 264 (see Fig. 7) gives the mass of alditol residue "a", and shows that it has only two *O*-ethyl groups. The abJ$_2$ ion at *m/z* 468 confirms the absence of *O*-ethyl groups from residue "b". The absence of an aJ$_1$ ion at *m/z* 324 indicates that residue "b" is substituted at O-3 by glycosyl residue "c". The abJ$_1$

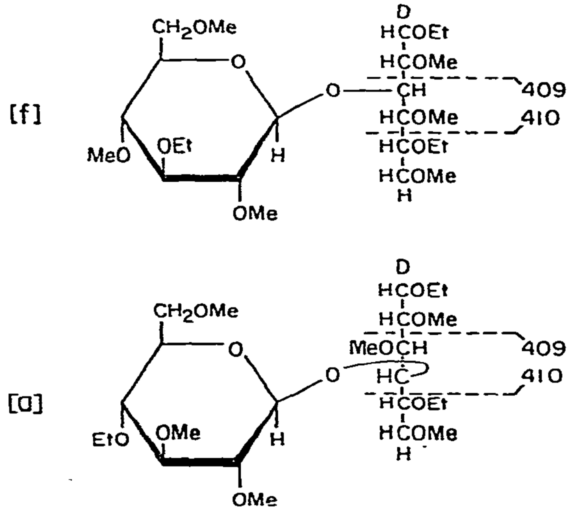
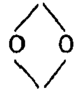


Fig. 8. Cleavage patterns for the alditol chain of partially methylated, partially ethylated, 3-linked disaccharide-alditol [f] and 4-linked disaccharide-alditol [a]. The ratio of the abundance of the *m/z* 409 to 410 fragment-ions can be used to distinguish between 3-linked (<0.2, see Table II), and 4-linked (<2, see Table II), peralkylated oligosaccharide-alditols (see text).

TABLE II

SOME CHARACTERISTICS OF THE STRUCTURALLY CHARACTERIZED, PERALKYLATED DISACCHARIDE-ALDITOLS OBTAINED FROM THE METHYLATED, PARTIALLY HYDROLYZED, REDUCED (NaBD$_4$), AND ETHYLATED *R. meliloti* POLYSACCHARIDE

Oligosaccharide structure[a]	*Oligo-saccharide fragments*	*R.t. (h.p.l.c.) (min)*	*C.i. mass spectra*			*E.i. mass spectra*										m/z *ratio*[d] *409/410; 423/424; or 465/466*	m/z *ratio*[e] *201/187*
			M + 1 m/z	*A*[b] m/z	*R.t. (g.l.c.) (min)*	*J*$_1$ m/z	*J*$_2$ m/z	*A*$_1$ m/z	*A*$_2$[c] m/z	*Alditol-chain cleavage* m/z	m/z	m/z	m/z	m/z	m/z		
Et→4Glc→4Glc→	[a]	15.1	514	282	7.3	324	264	233	201	—	—	409	410	453	468	2.2	4.5
Et→4Glc→3Gal→	[b]	17.6	514	282	7.5	324	264	233	201	—	366	409	410	453	468	0.16	4.6
Et→4Glc→4Glc→ 6 ↑ Et	[c]	20.2	528	282	7.6	324	264	247	215	—	—	423	424	467	482	2.1	—
Et→6Glc→6Glc→ 4 ↑ Et	[d]	19.6	528	296	8.3	338	278	233	201	206	—	321	379	—	467	—	14
Et→3Glc→6Glc→	[e]	15.3	514	282	8.4	338	264	233	187	192	263	321	365	—	453	—	0.25
Et→3Glc→3Glc→	[f]	14.5	514	282	7.4	338	264	233	187	—	366	409	410	453	468	0.14	0.73
Et→4Glc→3Glc→ 6 ↑ Et	[g$_1$]	18.7	528	282	7.7	324	264	247	215	—	—	423	424	467	482	0.15	—
Glc→3Glc→  MeCCO$_2$H	[g$_2$]	16.5	570	282	9.5	324	264	289	257	—	—	465	466	509	524	0.14	—

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[a] Et→4Glc→4Glc→ is equivalent to 4-*O*-ethyl-2,3,6-tri-*O*-methyl-D-glucopyranosyl-(1→4)-1,5-di-*O*-ethyl-2,3,4,6-tetra-*O*-methyl-D-glucitol-*1-d*, and so on. [b] A = Alditol fragment +18. [c] Only the most abundant A$_2$ fragment from each compound is listed. [d] See text. The ratio of the abundance of the *m/z* 423 and 424 ions and the ratio of the 465 and 466 ions are equivalent to the ratio of the 409 and 410 ions with the addition of one *O*-ethyl group and one ethyl-esterified 1-carboxylethylidene group, respectively. [e] See text.

TABLE III

SOME DIAGNOSTIC, TRISACCHARIDE FRAGMENTS FROM THE METHYLATED, PARTIALLY ACID-HYDROLYZED, REDUCED ($NaBD_4$), AND ETHYLATED *R. meliloti* POLYSACCHARIDE

Trisaccharide structure[a]	*Tri-saccharide fragment (min)*	*R.t.*[b] *(h.p.l.c.)* m/z	*Diagnostic ions of c.i. mass spectra* *M + 1* m/z	*A*[a] m/z	*R.t. (g.l.c.) (min)*	*Diagnostic fragment ions of e.i. mass spectra*[a] *aJ₁* m/z	*aJ₂* m/z	*cA₁* m/z	*cA₂* m/z	*abJ₁* m/z	*abJ₂* m/z	*cbA₁* m/z	*cbA₂* m/z
Et→4Glc→4Glc→3Gal→	(h)	23.5	718	282	13.3	324	264	233	201	528	468	437	405
Et→3Glc→6Glc→6Glc→ 4 ↑ Et	(i)	26.8	732	296	13.9	338	278	233	187	556	482	437	405
Et→3Glc→3Glc→6Glc→	(j)	24.8	718	282	15.4	324[c]	264	233	187	542	468	437	—
Et→4Glc→3Glc→3Glc→ 6 ↑ Et	(k₁)	29.5	732	282	13.3	324[c]	264	247	215	528	468	451	419
Glc→3Glc→3Glc→ / \\ O O \\ / MeCCO₂H	(k₂)	24.2	772	282	n.d.[d]	—	—	—	—	—	—	—	—
Et→6Glc→6Glc→4Glc→ 4 ↑ Et	(l)	27.5	732	282	12.8	324	264	233	201	542	482	451	419
Et→4Glc→4Glc→4Glc→ 6 ↑ Et	(m)	28.0	732	282	13.4	324	264	247	215	528	468	451	419

[a] See Table II. [b] R.t. = retention time. [c] Very weak, 3-linked residue. [d] n.d. = not determined.

ion at *m/z* 528 confirms the presence of a methyl group on O-3 of residue "c". The linkage to the alditol residue could not be determined from the e.i. mass spectra, as ions of the alditol chain containing residues "b" and "c" were not detected, and the abundance of the lower-weight ions was not sufficient to be of diagnostic value.

The foregoing examples (Figs. 6 and 7) show that the e.i. mass spectra of partially methylated, partially ethylated di- and tri-saccharide-alditols provide important information about the sequence and the linkages of the glycosyl residues, including the mass of each of the three residues, and the positions of *O*-ethyl groups on the (terminal) nonreducing group. The *O*-ethyl groups on all residues serve as markers, and are of great value in interpreting the spectra. We have also found that g.l.c.–m.s. of h.p.l.c. fractions containing peralkylated oligosaccharide-alditols is very useful for determining which fractions to pool for further analyses.

It is difficult to distinguish between peralkylated disaccharide-alditols in which the alditol is 3- or 4-linked, as the alditol chains of such compounds fragment similarly. However, we have found that, in the e.i. mass spectra, the ratio of the abundance of the *m/z* 409 to that of the *m/z* 410 ions (see Fig. 8), and, for appropriate compounds, the *m/z* 423 to 424 and *m/z* 465 to 466 ions, is ~2 for the compound containing 4-linked hexitol and <0.2 for the analogous compound containing 3-linked hexitol (see Table II).

It is also possible (see earlier) to distinguish between 3-, 4-, and 6-linked, terminal nonreducing groups in partially methylated, partially ethylated oligosaccharide-alditols, by examining the relative abundance of the A_2 fragments. The ratio of the A_2 ions at *m/z* 201 to the A_2 ions at *m/z* 187 for the peralkylated disaccharide-alditols of the *Rhizobium* polysaccharide are listed in Table II.

A summary of the peralkylated oligosaccharide-alditols characterized, in part, by their c.i. and e.i. mass spectra. — C.i. and e.i. mass-spectral, diagnostic ions, and

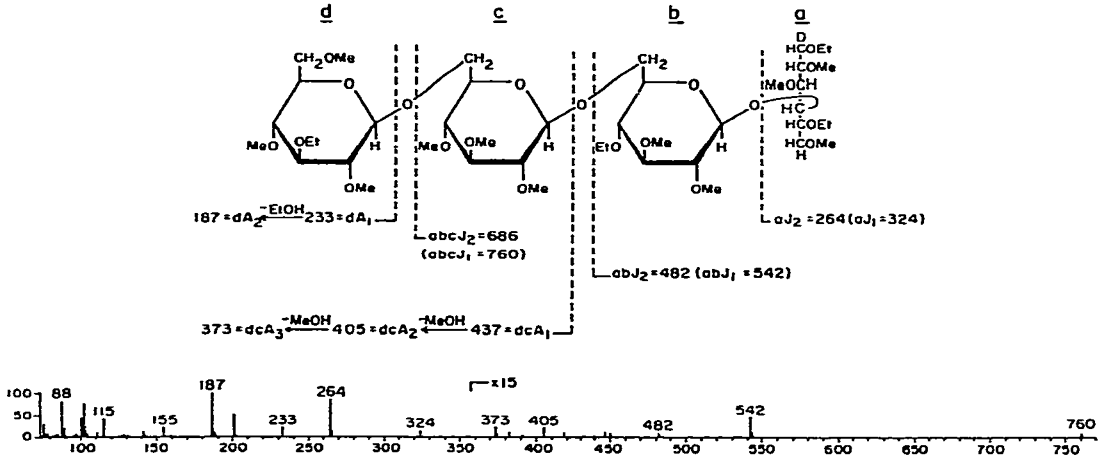

Fig. 9. E.i. mass spectrum, and the origin of the fragment ions of peralkylated tetrasaccharide-alditol [o] (see Fig. 10). This mass spectrum was obtained by direct-probe, m.s. analysis of the h.p.l.c. fraction collected while mass spectra 985 through 995 (see Figs. 1 and 4) were being obtained. The c.i. mass spectrum of this compound is illustrated in Fig. 3.

h.p.l.c. and g.l.c. retention-times of the partially methylated, partially ethylated disaccharide-alditols of the *Rhizobium* polysaccharide are summarized in Table II. The glycosyl-residue sequence of these peralkylated disaccharide-alditols, except those containing a 3-linked glucosyl or galactosyl residue, could be completely elucidated from knowledge of the glycosyl-residue composition of the intact polysaccharide (see Table I) and from their c.i. and e.i. mass spectra. The 3-linked glucosyl and galactosyl residues were distinguished by glycosyl-linkage, composition-analysis, retention times. C.i. and e.i. mass-spectral, diagnostic ions, and h.p.l.c. and g.l.c. retention-times of peralkylated trisaccharide-alditols from the polysaccharide are presented in Table III. Only partial glycosyl-sequences of the peralkylated trisaccharide-alditols may be deduced from these data.

The peralkylated tetrasaccharide-alditols [n] and [o] were located in the h.p.l.c. fractions by their c.i.–m.s. M + 1 ions at *m/z* 978 and 936, respectively (see Fig. 4). The amount of peralkylated tetrasaccharide alditol [n] was insufficient to permit e.i. mass-spectral analysis. The e.i. mass spectrum of [o], obtained by using the direct inlet probe, is illustrated in Fig. 9.

Analysis of peralkylated tetrasaccharide-alditol [o] by conversion into its constituent, partially methylated, partially ethylated alditol acetates established that a 3-*O*-ethyl-2,4,6-tri-*O*-methylglucosyl group is present at the nonreducing end of the peralkylated tetrasaccharide-alditol. This is confirmed by the dA_1 ion at *m/z* 233 and the dA_2 ion at *m/z* 187 (see Fig. 9). Analysis of the partially methylated, partially ethylated alditol acetates also established the presence of a 4-linked 1,5-di-*O*-ethyl-2,3,6-tri-*O*-methylglucitol residue at the alditol end of the peralkylated oligosaccharide-alditol. This is confirmed by the aJ_2 ion at *m/z* 264 (see Fig. 9). Finally, analysis of the partially methylated, partially ethylated alditol acetates established the presence of two internal glycosyl residues, namely, a 6-linked 2,3,4-tri-*O*-methylglucosyl residue and a 6-linked 4-*O*-ethyl-2,3-di-*O*-methylglucosyl residue. The order of these two glycosyl residues was ascertained by examination of its e.i. mass spectrum (see Fig. 9).

The order of the two internal glucosyl residues of peralkylated tetrasaccharide-alditol [o] (see Fig. 9) is defined by the masses of the abJ_2 ion at *m/z* 482 and the abJ_1 ion at *m/z* 542. These ions establish that the 6-linked 4-*O*-ethyl-2,3-di-*O*-methylglucosyl residue is directly attached to the alditol. If the 6-linked 2,3,4-tri-*O*-methylglucosyl residue had been directly attached to the alditol, the mass of the abJ_2 ion would have been 468 (instead of 482) and the mass of the abJ_1 ion would have been 528 (instead of 542). The mass of the dcA_2 ion at *m/z* 405 and the mass of the dcA_3 ion at *m/z* 373 confirm the sequence by establishing that the 6-linked 2,3,4-tri-*O*-methylglycosyl residue is adjacent to the (nonreducing) terminal glycosyl group. Thus, the glycosyl sequence of the peralkylated tetrasaccharide-alditol [o] was determined.

Formation of the constituent, partially methylated, partially ethylated alditol acetates, and determination of the ring form of the glycosyl residues of the peralkylated oligosaccharide-alditols. — The glycosyl-sequence analysis of the peralkylated oligosaccharide-alditols was completed by hydrolysis, reduction, and acetylation, to afford the constitutive, partially methylated, partially ethylated alditol acetate derivatives, and identification of these derivatives by g.l.c. retention time and by g.l.c.–m.s.[1]. This procedure completed, or confirmed, the glycosyl sequences of all of the peralkylated di-, tri-, and tetra-saccharide-alditols obtained from the polysaccharide. The procedure distinguished between the 3-linked glucosyl and 3-linked galactosyl residues, a distinction that was not made by mass-spectral analysis.

Formation of the constituent alditol acetates of the peralkylated oligosaccharide-alditols also showed that all of the glycosyl residues in the repeating unit of the polysaccharide are in the pyranoid form. This was established for the (terminal) nonreducing glucosyl group by the fact that this residue is substituted with a 1-carboxyethylidene group attached to O-4 and O-6 {compare, for example, the peralkylated oligosaccharide alditols [g_1] and [g_2] in Table II and Fig. 10}. Therefore, O-5 of this residue must participate in the pyranoid ring. The pyranoid ring-forms of the other glycosyl residues were established by characterizing the alditol acetate derivative of each residue when the residue was situated at either the reducing or the nonreducing end of a peralkylated oligosaccharide-alditol. This analysis unequivocally identifies the ring form of such residues[1]. The e.i. mass spectra obtained during g.l.c.–m.s. analysis of the peralkylated di- and tri-saccharide-alditols confirmed that each of the glycosyl residues is in the pyranoid form[14].

The repeating unit of the Rhizobium *1021 polysaccharide.* — The glycosyl sequence of the octasaccharide repeating-unit of the *R. meliloti* strain 1021 poly-

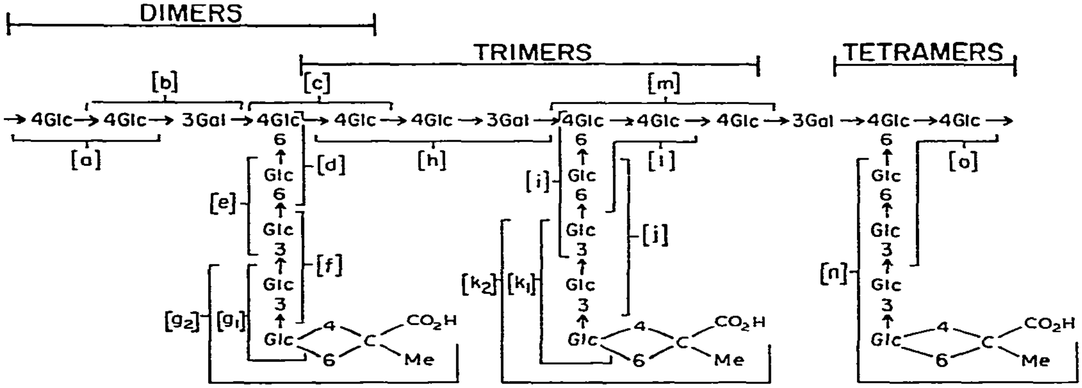

Fig. 10. Summary of the structurally characterized oligosaccharides derived from the acidic polysaccharide secreted by *R. meliloti* strain 1021. These fragments define the sequence illustrated in this Figure. Each fragment is labelled by a letter that identifies where the corresponding peralkylated oligosaccharide-alditol is eluted in the h.p.l.c.-m.s. elution-profile (see Fig. 1). The 1-carboxyethylidene groups of fragments [g_1] and [k_1] were cleaved during the partial hydrolysis of the polysaccharide, leading to formation of these fragments. Therefore, ethyl groups are present on O-4 and O-6 of the terminal glucosyl group in the peralkylated oligosaccharide-alditols derived from these fragments. On the other hand, the 1-carboxyethylidene groups of fragments [g_2], [k_2] and [n] were not cleaved during formation of the fragments. Therefore, the terminal glucosyl group in the peralkylated oligosaccharide-alditols derived from these fragments contains a 1-carboxyethylidene group whose carboxyl group is ethyl-esterified.

saccharide (see Fig. 10) can be elucidated by considering only the structure of the eight peralkylated disaccharide-alditols (see Table II and Fig. 10) in conjunction with the glycosyl-linkage composition (see Table I). Peralkylated disaccharide-alditols [g_1] and [g_2] show that the 1-carboxyethylidene group is linked to O-4 and O-6 of a terminal glucosyl group which, in turn, is glycosidically linked to O-3 of another glucosyl residue. Disaccharide fragment [f] shows that one 3-linked glucosyl residue is linked to O-3 of the only other 3-linked glucosyl residue (see Table I), and disaccharide fragment [e] shows that this 3-linked glucosyl residue is linked to O-6 of the only 6-linked glucosyl residue (see Table I).

Disaccharide fragment [d] (Table II and Fig. 10) establishes that the only 6-linked glucosyl residue is linked to O-6 of the only branched, 4,6-linked glucosyl residue (Table I), and disaccharide fragment [d] shows that the branched residue is linked to O-4 of a glucosyl residue. Disaccharide fragment [a] establishes that this 4-linked glucosyl residue is directly linked to the only other 4-linked glucosyl residue (see Table I), and disaccharide fragment [b] shows that this 4-linked residue is attached to the only 3-linked galactosyl residue (see Table I). Thus, all eight glycosyl residues in the repeating unit are accounted for. It may be concluded that the 3-linked galactosyl residue is glycosidically attached to O-4 of the branched, 4,6-linked glucosyl residue (see Fig. 10), as this is the only point of attachment not accounted for.

The trisaccharide fragments by themselves, for example, trisaccharide fragments [k_2], [i], [m], and [h], confirm the glycosyl sequence determined by the disaccharide fragments, and the tetrasaccharide fragments add additional confirmation of the structure. In fact, all of the partially methylated, partially ethylated di-, tri-, and tetra-saccharide-alditols that were isolated from the polysaccharide, and whose glycosyl sequences have been elucidated, fit in the repeating unit illustrated in Fig. 10. Interestingly, all of the possible peralkylated di- and tri-saccharide-alditols were identified in this single, h.p.l.c. fractionation, except those three which would have contained the acid-labile, galactosyl linkage. Nevertheless, the glycosyl sequence of the polysaccharide is unambiguous, even without isolation of peralkylated oligosaccharide-alditols containing the galactosyl linkage.

^{1}H-N.m.r. analysis of the underivatized polysaccharide. — ^{1}H-N.m.r. analysis of the polysaccharide provided additional information about its structure. An envelope of signals in the β-anomeric-proton region, δ 4.5–4.8, accounted for all eight of the glycosyl linkages. No signals in the α-anomeric-proton region were detected. A signal at δ 1.46 (s) was assigned to the methyl protons of the 1-carboxyethylidene groups, showing that these groups had the (*S*) configuration[15]. A signal at δ 1.15 (s) was assigned to the methyl protons of acetoxyl groups[3]. Signals at δ 2.47 (t) and 2.63 (t) were assigned to the methylene protons of a succinyl substituent. Integration of these signals indicated the presence of ~1.5 acetyl groups and ~1 succinyl group per 1-carboxyethylidene group. These ^{1}H-n.m.r. results are in good agreement with those previously reported[3,16] for the acidic polysaccharide secreted by *R. meliloti* strain U-27.

Comparison of the acidic polysaccharides secreted by R. meliloti *strains 1021 and U-20.* — The *R. meliloti* strain 1021 polysaccharide and the previously investigated *R. meliloti* strain U-27 polysaccharide[3,17] have identical structures with respect to glycosyl composition, glycosyl-linkage composition, glycosyl sequence, location of the 1-carboxyethylidene groups, and the anomeric configuration of the glycosyl linkages. It is now established that all of the glycosyl residues of the *R. meliloti* strain 1021 polysaccharide are in the pyranoid form and that their absolute configuration is D. It seems probable that the residues of the U-27 polysaccharide have the same configuration. We have shown that the anomeric configuration of all of the glycosyl linkages are β, and the absolute configuration of the 1-carboxyethylidene group is (*S*), which is in agreement with the structure of the U-27 polysaccharide[3]. The polysaccharides from both strains contain acetyl and succinyl groups[3,16]. However, it cannot yet be established that the polysaccharides from the two strains have exactly the same structure. The acetyl groups of the U-27 polysaccharide are situated on O-6 of some of the glucopyranosyl residues[3,16], and the succinyl group is attached to a 3- or 6-linked glucosyl residue of the side chain[16]. The locations of these substituents have not been determined for the 1021 polysaccharide. Comparison of these substituents of the polysaccharides of different *Rhizobium* strains is difficult, as the content of acetyl groups of the extracellular polysaccharide has been reported to change with the age of the culture[18].

GENERAL DISCUSSION

H.p.l.c.–m.s. of a mixture of peralkylated oligosaccharide-alditols and g.l.c.–m.s. of fractions of the h.p.l.c. effluent combine two entirely different separation-techniques; this is illustrated in Tables II and III on comparing the h.p.l.c. retention-times with the g.l.c. retention-times of the peralkylated oligosaccharide-alditols. The c.i. and e.i. mass spectra of the peralkylated oligosaccharide-alditols give their molecular weight, and provide much information about the glycosyl sequence and the points of attachment of other glycosyl residues in the intact polysaccharide.

It is important to have general and sensitive methods for determining the structures of complex carbohydrates, as the carbohydrates of biological importance are often very complicated structures and are usually available in only small quantities. The sensitivity of this method may be further enhanced by use of selected-ion-monitoring m.s., a technique not used in this study. The methods described herein offer improved separations of complex carbohydrate fragments, a decrease in the quantity of material needed for structural analysis of complex carbohydrates by chemical methods, and a significant shortening of the time needed for such analyses.

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