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Structure of the unusual trisaccharide lipopolysaccharide component produced by a symbiotically defective mutant of *Rhizobium leguminosarum* biovar *viciae*

Rawle I. Hollingsworth [a,*], Yuanda Zhang [a], Ursula. B. Priefer [b]

[a] *Departments of Chemistry and Biochemistry, Michigan State University, East Lansing, MI 48824, USA*
[b] *Ökologie des Bodens, RWTH Aachen, Aachen, Germany*

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Abstract

The structure of an unusual trisaccharide component isolated from the lipopolysaccharide (LPS) of a Tn5 mutant of *Rhizobium leguminosarum* biovar *viciae* VF39 which is defective in infection of its host plant has been elucidated. This mutant also appears to be defective in the synthesis of a tetrasaccharide component normally synthesized by the wild-type organism. The three glycosyl components are galactose, mannose, and 3-deoxy-D-*manno*-2-octulosonic acid (Kdo). Mannose is linked to the 5-position and galactose to the 7-position of the 3-deoxy-2-octulosonic acid residue (Kdo). Both hexosyl components are in the α-pyranosyl form. In the isolated molecule the octulosonic acid appears to be present as its γ-lactone. However, in the lipopolysaccharide molecule, it is most likely present in the pyranosyl form. The structure was determined by ^{1}H NMR spectroscopy and methylation analysis as well as by fast-atom-bombardment mass spectrometry of the peracetylated and per(trideuterio) acetylated oligosaccharides. Small amounts of the methylation analysis product of another tetrasaccharide different to normal tetrasaccharide component made by the wild-type organisms were detected. This indicates that in this mutant, there is a block in the synthesis of the normal tetrasaccharide component in addition to a switch in the synthesis of the LPS type.

Keywords: Rhizobium; Lipids; Lipopolysaccharide; Mutants; Kdo-lactone; R-Core

* Corresponding author.

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1. Introduction

In an earlier study [1] we described structural changes in the lipopolysaccharides of two symbiotically-defective Tn5 mutants of *Rhizobium leguminosarum* biovar *viciae* VF39. These two mutants, strains VF39-32 and VF39-86, are both defective in their ability to be released from their infection thread into the nodules of host plants [2]. This defect is very consistent with an alteration of surface chemistry. The lipopolysaccharide (LPS) of one of the mutants (VF39-86) was found to be lacking a tetrasaccharide component [3,4] found in the wild-type organism and to contain a disaccharide derived from it instead [1]. The other mutant LPS was also found to lack the tetrasaccharide component but to contain an unusual, unidentified trisaccharide component instead [1]. This work extends that study and describes the complete structural characterization of the unusual trisaccharide component from the LPS of the latter mutant (VF39-32).

2. Experimental

General methods.—Growth and maintenance of the microorganisms and LPS isolation and purification, as well as the isolation and purification of the trisaccharide component are described in detail in the earlier publication [1]. NMR analyses were performed on a Varian VXR500 spectrometer operating at 500 MHz for protons. Spectra were recorded in D_2O at 25°C. Gas chromatography–mass spectrometry analyses were performed on a Jeol 505 instrument using a DB225 column. The starting temperature for unmethylated alditols was 180°C and for partially methylated alditols 150°C. The temperature was increased at a rate of 2°C min to 230°C in both cases. Fast-atom-bombardment mass spectrometry was performed on a Jeol HX-110-HF instrument. Spectra were obtained in both positive- and negative-ion mode. In the former case a 1:1 mixture of glycerol and thioglycerol was used as matrix. In the latter case triethanolamine was used as matrix.

Preparation of alditol acetate derivatives.—A sample of the trisaccharide (~ 50 μg) was hydrolyzed with 2 M CF_3CO_2H for 2 h at 120°C. The solution was concentrated to dryness under a stream of N_2 and the free sugars reduced by treatment with ~ 100μg of $NaBH_4$ in 100 μL of water for 1 h. The solution was treated with AcOH (50 μL) to decompose excess reducing agent and then concentrated to dryness. Methanol (0.5 mL) was added and the solution was evaporated to dryness. Four more 0.5-mL volumes of MeOH were added and the solution again concentrated to dryness after each addition to remove boric acid. The residue was then treated with dry pyridine (200 μL) and Ac_2O (100 μL) for 2 h at 80°C to convert of alditols into alditol acetates. The mixture was then concentrated to dryness and partitioned between water (200 μL) and $CHCl_3$ (400 μL). The $CHCl_3$ layer was dried (Na_2SO_4) and analyzed by GC and GC–MS.

Methylation analysis.—A sample of the trisaccharide (~ 50 μg) was treated with $NaBH_4$ (~ 50 μg) in water (100 μL) for 5 h. The solution was treated with 20 μL

of AcOH, and then reduced to dryness and treated with 1% methanolic HCl in for 2 h at 55°C. The solution was again evaporated to dryness and treated with $NaBH_4$ (50μg) in 1:1 MeOH–water (200 μL) for 12 h. The excess borohydride was decomposed by treatment with 50 μL of AcOH. The mixture was evaporated under a stream of N_2 and then treated with 200 μL of a 1.5 M solution of sodium methylsulfinyl anion in dry Me_2SO. The mixture was stirred for 24 h at room temperature and then treated with 100 μL of MeI. The resulting suspension was stirred for 4 h and then evaporated. The residue was then diluted to 1 mL with water and passed through a C-18 reverse-phase cartridge. The cartridge was eluted sequentially with 4 mL each of water, 2:1 MeOH–water and finally pure MeOH. Each eluate was collected separately and subjected to acid hydrolysis with aqueous 2 M CF_3Cl for 1 h at 120°C. The hydrolyzates were then reduced with $NaBH_4$ and acetylated as described earlier. The partially methylated, peracetylated alditols were analyzed by gas GC–MS.

Peracetylation of oligosaccharide.—A sample of the oligosaccharide was treated with pyridine (50 μL) and Ac_2O (50 μL) for 24 h at room temperature. The mixture was then concentrated to dryness under a stream of N_2 and the residue was analyzed by fast-atom-bombardment mass spectrometry. Per(trideuterio) acetates were prepared in the same manner using perdeuterated Ac_2O as the acylating reagent.

3. Results and discussion

Gas chromatography–mass spectrometry indicated that mannose and galactose were present in the oligosaccharide in equal amounts. Two other minor peaks were present in similar proportions to each other. The ammonia chemical-ionization mass spectrum of the two minor components indicated that they differed by 14 amu and that one was, therefore, probably a methylated derivative of the other. The chemical ionization mass spectrum of the earlier eluting peak contained a quasimolecular ion ($M + NH_4^+$) at m/z 352. The corresponding peak in the spectrum of the later eluting peak appeared at m/z 366. The later eluting component is probably formed by methylation of a carboxylic acid methyl ester residue present in the first eluting peak with methanol and acid during the derivitization process. The two minor components eluted several minutes before the alditol acetates of galactose and mannose. This indicated that if they were derived from Kdo, they were due to dehydration products.

The ^1H NMR spectrum of the oligosaccharide (Fig. 1) contained peaks which could be assigned to the anomeric protons of mannose and galactose residue in the α-pyranose form. The anomeric proton for the mannose residue appeared at 4·98 ppm and the one for the galactose residue appeared at 4·89 ppm. Two upfield sets of signals were assignable to a methylene group on a carbon atom to which no oxygen atoms were attached. The chemical shift and coupling constants were inconsistent with them being due to a methylene group in a pyranose ring-system. Both signals were doublets of doublets. The most upfield of the pair ($J = 14.5 + 1.5$

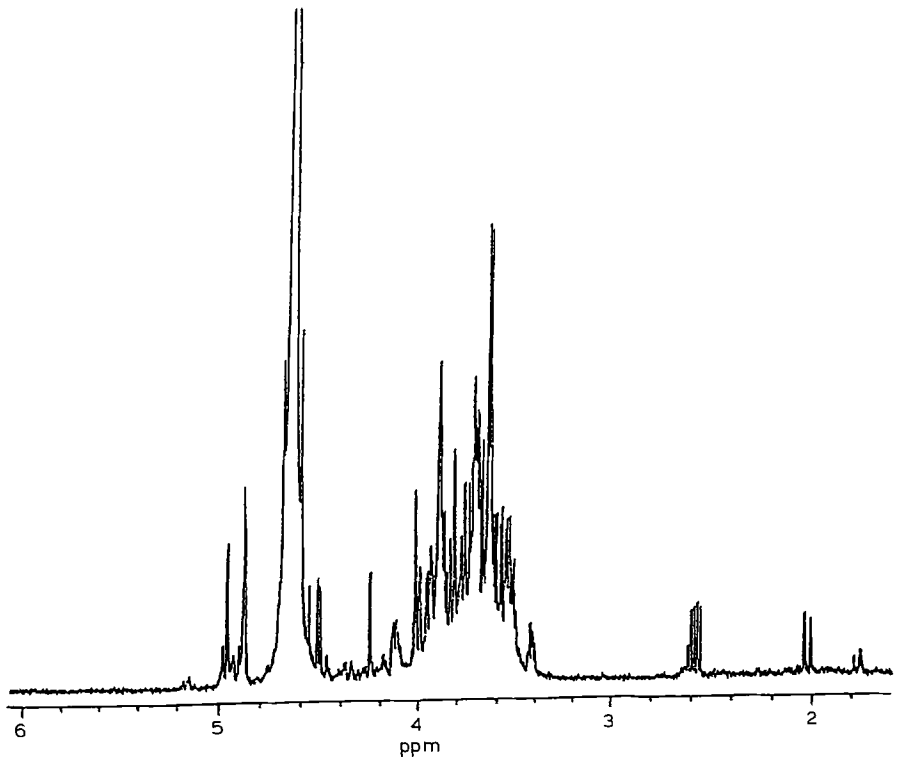


Fig. 1. ^{1}H NMR spectrum of the oligosaccharide. Note the two anomeric protons at 4·98 and 4·89 ppm and the methylene signals from the deoxy group between 2·0 and 2·7 ppm.

Hz) appeared at 2.02 ppm. The second doublet of doublets ($J = 14.5 + 7.6$ Hz) appeared at 2.60 ppm. The coupling constants were consistent with the protons being part of a furanose ring. However, the unusually large chemical shifts indicated that they were adjacent to an electron-withdrawing center such as a carbonyl group. Irradiation of the more upfield doublet of doublets at 2.02 ppm (Fig. 2) led to the loss of the larger splitting of the second doublet of doublets. In addition, there was the loss of the smaller splitting of another downfield doublet of doublets at 4.53 ppm ($J = 7.6 + 1.5$ Hz). This latter signal was first thought to be due to an anomeric proton [1] but was later assigned to a proton on a carbon bearing an acyloxy function. Irradiation of the doublet of doublets at 2.60 ppm (Fig. 3) led to the collapse of the one at 2.02 ppm to a doublet (J 1.5 Hz) as well as loss of the large coupling from the one at 4.53 ppm. This result confirmed the assignment of the methylene function, the 14.5 Hz splitting being due to germinal coupling. The signal at 4.53 ppm was the most downfield resonance in the NMR spectrum except for those which were due to the two anomeric protons. This was the main fact in favor of its earlier tentative assignment to an anomeric proton resonance [1]. However, evidence of residual coupling to another proton beside the methylene group was inconsistent with this. The large chemical shift indicated that

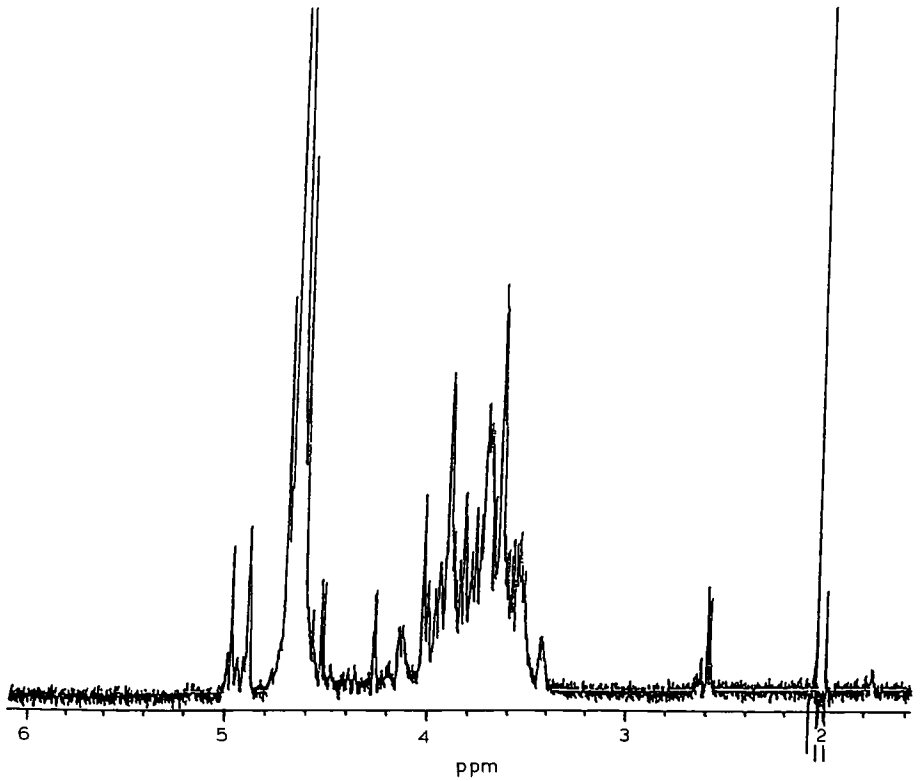


Fig. 2. ^{1}H NMR spectrum of the oligosaccharide after irradiating the signals at 2·02 ppm.

the carbon bearing the proton at 4.53 was substituted by an electron withdrawing function such as an acyl group. This suggested the possibility of a γ-lactone. The possibility of 3-deoxy-2-octulosonic acid in a γ-lactone form was therefore entertained. The methylene group was, therefore, assigned to the 3-position of Kdo.

Permethylation of the pre-reduced oligosaccharide and elution of the product from a C-18 cartridge sequentially with 2:1 methanol–water and pure methanol gave a major product in the 2:1 methanol–water fraction. Conversion of this into alditol acetates followed by GC–MS analysis gave a profile containing three major peaks (Fig. 4). The first two were due to terminally-linked mannose and galactose. The later eluting peak had a mass spectrum (Fig. 5) consistent with a peracetylated 3-deoxy-2-deuterio-6,8-di-*O*-methyloctitol, indicating that not only was the lactone function resistant to reduction, but that the 2-borate ester formed by reduction of the 2-keto function was stable. The fragments due to primary cleavage α- to the 6-methoxy group appeared at *m/z* 161 and 348 (Fig. 5). The latter fragment showed characteristic sequential losses of acetic acid, and acetic anhydride to give ions at *m/z* 288 and 186, respectively. A loss of acetic acid from the fragment at *m/z* 288 to yield an ion at *m/z* 228 was also observed. A sequence beginning at *m/z* 245 was also observed. This ion originates from the one at *m/z* 348 by the

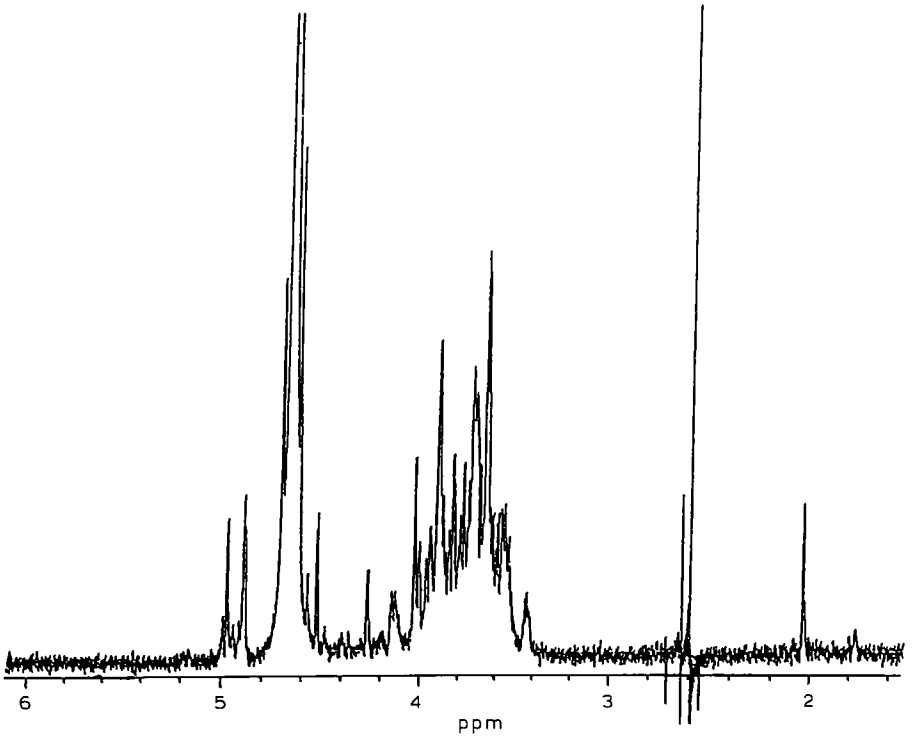


Fig. 3. ^{1}H NMR spectrum of the oligosaccharide after irradiating the signals at 2·60 ppm.

loss of CH_3COOD followed by ketene (a total of 103 mass units). The ion at 203 is derived from the one at m/z 245 by the loss of ketene. The formation of this methylated product in the methylation analysis is to be expected if the γ-lactone is reduced at the 2-position by borodeuteride during the pre-reduction and the resulting borate ester at this position survives methylation. The spectrum is rationalized in Fig. 6. It was possible, after prolonged treatment, to effect (some) reduction of the lactone group. This reduction and the ketone function pre-reduction were carried out using sodium borodeuteride. The mass spectrum of the partially methylated, peracetylated alditol (not shown) contained primary fragments at m/z 268, 161, 152, and 92 and secondary fragments at 236 (268 – CH_3OH), 203 (236 – CH_3OD), and 119 (152 – CH_3OD). This is consistent with the expected peracetylated 1,2,4,6,8-penta-*O*-methyl 3-deoxyoctitol.

The relative positions of substitution of the γ-lactone by the mannosyl and galactosyl protons was determined by NOE measurements. On irradiating the doublet of doublets at 2.60 ppm, an increase in intensity of the mannosyl anomeric proton was observed. This indicated that the mannosyl residue was closest to the methylene group. The site of linkage of this residue was, therefore, assigned to the 5-position. The galactosyl residue was assigned to the 7-position.

In order to confirm the proposed structure, the oligosaccharide was peracety-

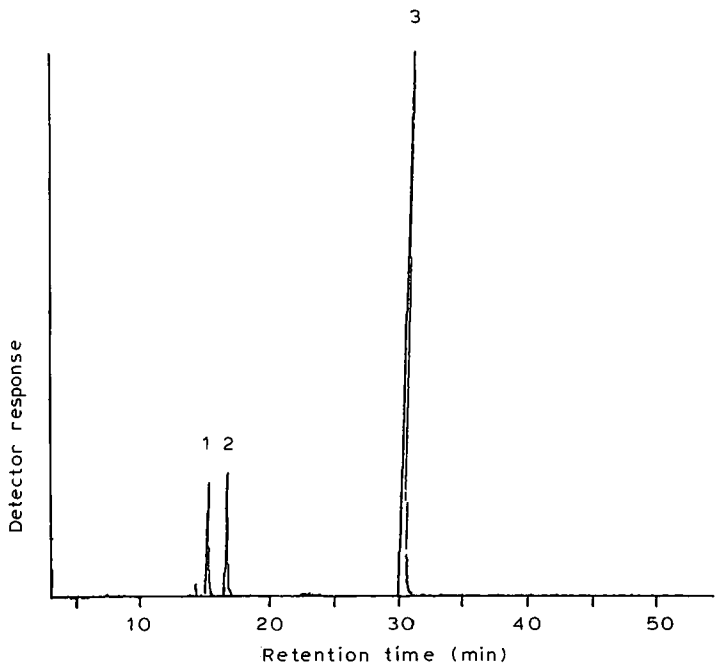


Fig. 4. GC profile of the partially methylated alditol acetate derivatives formed during methylation analysis of the oligosaccharide.

lated and the fast-atom-bombardment mass spectrum was obtained (Fig. 7). The quasimolecular ion at m/z 965 was one mass unit higher than the molecular weight of the proposed structure after the introduction of ten acetate groups. This increase of one mass unit could be attributed to the loss of an OH species from the hydrate of the keto group or by hydride transfer from the matrix to the analyte. Losses of 44 and 28 amu, attributable to losses of carbon dioxide and carbon monoxide, respectively, from the α-ketolactone function of the parent ion were observed. The mass spectrum of the per(trideuterio)acetyl derivative (Fig. 8) confirmed the presence of ten acetate groups since there was a shift in the molecular ion of 30 amu. The same losses of 44 and 28 amu were again observed.

The lactone form of 3-deoxy-2-octulosonic acid is quite rare. It should be pointed out that this Kdo residue must have been present in the intact LPS molecule in the regular pyranose form. However, once released, the substitution pattern makes the pyranose hemiacetal form less stable than the γ-lactone. The reason is probably the close steric contact between the pendant glycosyl groups at the 5 and 7 positions in the pyranose form. The carbonyl group in this lactone was found to be resistant to borohydride reduction. Reduction occurred only after extended reaction times.

Recently, an oligosaccharide with a different composition and a very similar ^{1}H NMR spectrum was reported to be present in the LPS of a *Bradyrhizobium iaponictum* strain [5]. The 3-deoxy-2-octulosonic acid residue in that molecule was

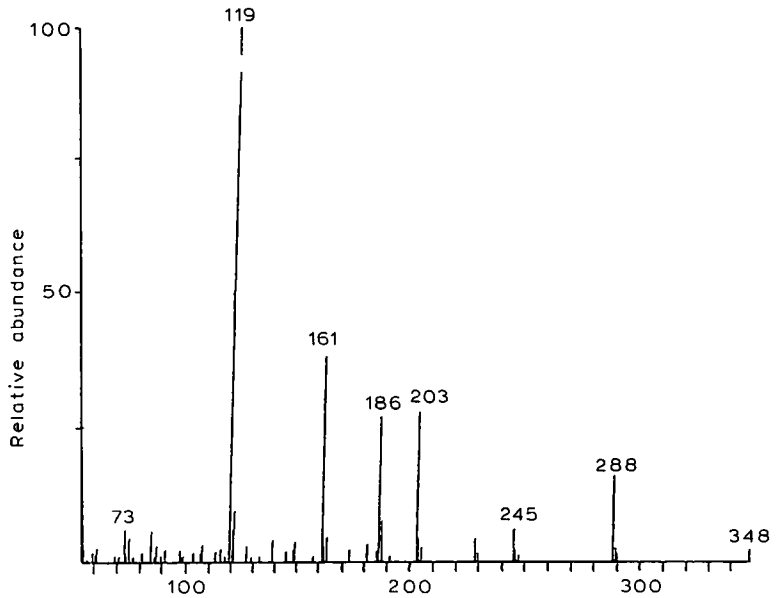


Fig. 5. Mass spectrum of the non-hexose derived component of the methylation analysis mixture (latest eluting peak in Fig. 4). The ion at m/z 348 can be assigned to the large cleavage fragment α to the 6-methoxy group.

proposed to be present in the 2,7-anhydro form. The methylation analysis product from the octulosonic acid residue was never recovered in that study and the conclusions were based on ^{1}H NMR results. However, the results we report here

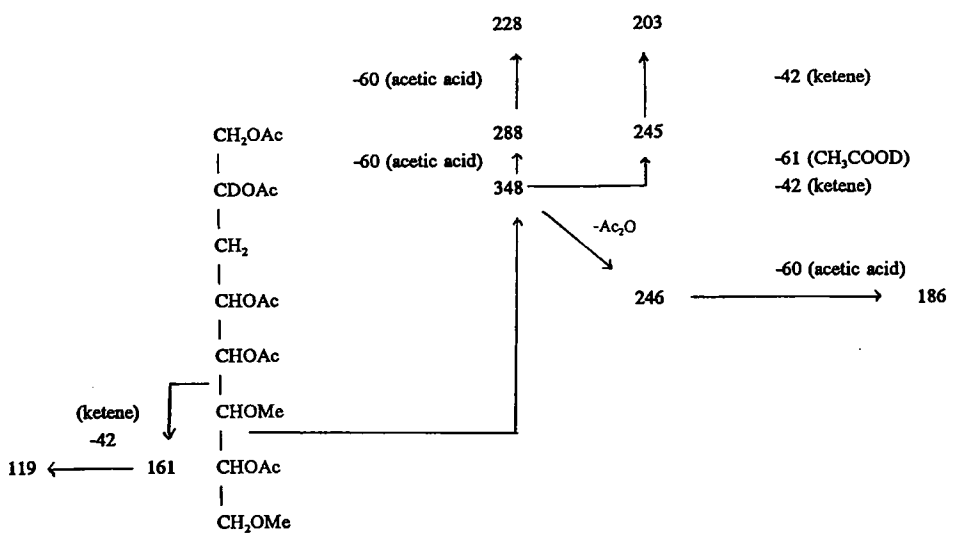


Fig. 6. Detailed mechanism for the fragmentation pattern observed in Fig. 5.

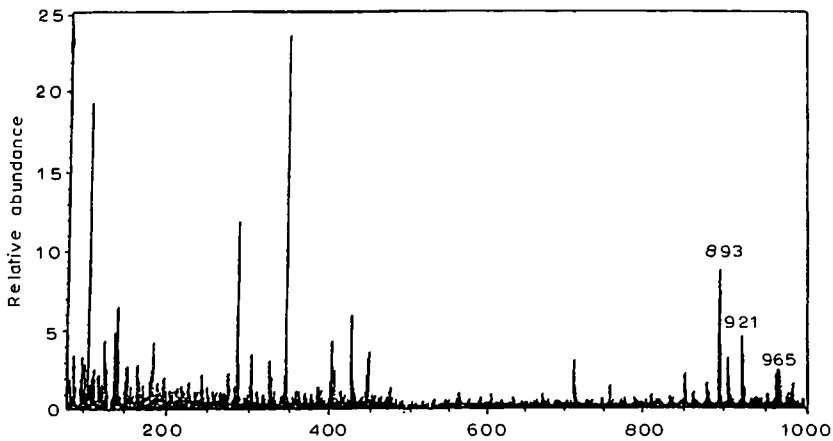


Fig. 7. Negative-ion fast-atom-bombardment mass spectrum of the peracetylated oligosaccharide.

are inconsistent with that structure. In the 2,7-anhydro form, only one hydroxyl group is free on the 3-deoxy-2-octulosonic acid residue. This would lead to only nine positions for acetylation and not ten as we have demonstrated. In addition, the methylation analysis results would have been quite different. Only two methoxy groups can be introduced in the anhydro form since the C-2 position would not be reduced in the pre-reduction. The C-2 and C-5 positions would, therefore, not be methylated. In fact, if the carboxyl groups were not reduced, only one other position would be available for methylation. The proposed structure of the trisaccharide is shown in Structure **1**.

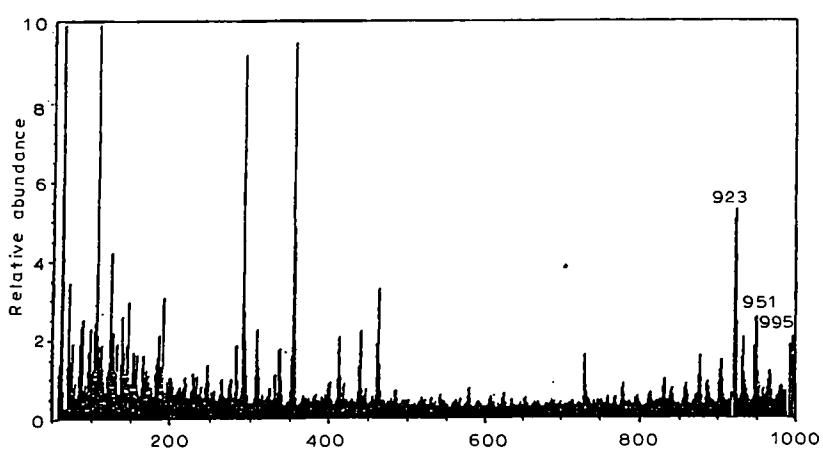
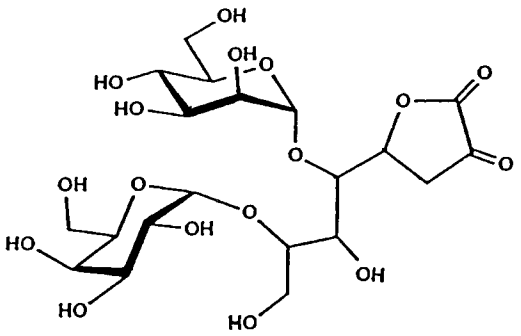


Fig. 8. Negative-ion fast-atom-bombardment mass spectrum of the per(trideuterio) acetylated oligosaccharide.



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The methylation analysis yielded a trace of material in the methanol fraction from the C-18 cartridge with composition and linkage pattern (by GC–MS of alditol acetates) different to the tetrasaccharide component normally made by the wild-type organism. This is an interesting finding since it indicates that the bacterium is capable of carrying out a number of modifications in LPS structure in response to a single genetic perturbation. The small quantity of the material precluded further characterization.

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