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Structure of the core oligosaccharide in the lipopolysaccharide isolated from *Aeromonas salmonicida* ssp. *salmonicida* *

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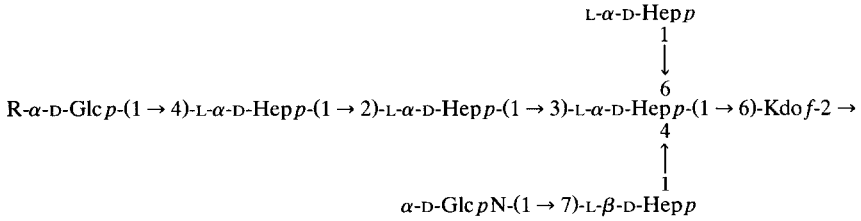
ABSTRACT

The core oligosaccharide isolated from the lipopolysaccharide of *Aeromonas salmonicida* ssp. *salmonicida* has been investigated by methylation analysis, NMR spectroscopy (^{13}C and ^{1}H), oxidation with periodate and chromium trioxide, and Smith degradation. The following structure is proposed:



with R = α-D-Gal*p*-(1 → 4)-α-D-Gal*p*NAc-(1 → 6)-

INTRODUCTION

Aeromonas salmonicida is one of the more lethal aquatic bacteria and causes the disease furunculosis in salmonid fish. Along with other members of the family Vibrionaceae, particularly *V. anguillarum*, *V. ordalii*, and *A. hydrophila*, diseases caused by these organisms can rapidly decimate the population of an aquaculture

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operation. The annual world-wide losses of farmed fish (both marine and freshwater) to disease involve millions of dollars. With the increasing concern about the use of antibiotics on species which will be used as food, considerable effort is being expended on developing vaccines for the most prevalent fish diseases.

We have described the O-antigens[1,2] and the core oligosaccharides[3,4] of the lipopolysaccharides (LPSs) of a selection of the above species as part of investigations directed toward an understanding of the role that LPS can play in the protection of fish against specific diseases, and to determine whether various LPS fractions conjugated to proteins[5] or encapsulated in liposomal carriers can be used as protective vaccines. Reports in the literature clearly indicate that the LPSs of *Edwardsiella tarda*[6] and *V. anguillarum*[7] can protect fish against the diseases caused by these organisms. Whether this effect is a long-lasting specific immunity, or a more generalised immune response remains to be determined in view of recent reports that large polysaccharides can cause short-term non-specific immunity in fish[8]. In spite of being able to elicit strong antibody production in rabbits and fish, the protective ability of the LPS from *A. salmonicida* has not yet been demonstrated.

The structure of the heptose region of the core oligosaccharide now reported is consistent with that found[9] in the core from a deep-rough mutant of *A. salmonicida*.

RESULTS AND DISCUSSION

The LPS, prepared as described[1], was electrodialysed[10] and the core oligosaccharide, isolated (12.2%) after hydrolysis, had $[\alpha]_D + 42°$ (*c* 1.56, H_2O).

Hydrolysis of the core oligosaccharide with aqueous trifluoroacetic or sulphuric acid and conversion of the monosaccharides released into alditol acetates revealed the components to be D-galactose, D-glucose, L-*glycero*-D-*manno*-heptose, and 2-amino-2-deoxy-D-galactose (later shown to be *N*-acetylated) plus an unhydrolysed disaccharide, in the molar ratios 1 : 1 : 4 : 1 : 1 (Table I). The disaccharide was shown by methylation analysis and GLC–MS to be identical to the 7-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-L-*glycero*-D-*manno*-heptose isolated[11] from the core oligosaccharide of *A. hydrophila*. The proportion of the disaccharide relative to those of the other sugars (Table I) was ascertained by analysis of the two aminodeoxy sugars (amino acid analyser) and calculation based on the ratio of the 2-amino-2-deoxyglucose and 2-amino-2-deoxygalactose determined by GLC of the acetylated alditols. Deamination of the oligosaccharide afforded 2,5-anhydro-D-mannose and, after hydrolysis, the appearance of an extra mole of L-*glycero*-D-*manno*-heptose, and no loss of 2-amino-2-deoxygalactose (Table I). This finding is consistent with the presence of 2-amino-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-galactose. The extra heptose residue is that which was a component of the disaccharide. Analysis of the oligosaccharide for 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) was essentially negative by the mild hydrolysis method (0.25 M H_2SO_4,

TABLE I

Sugar analysis of the core oligosaccharide and derived products (molar proportions)

Sugar	Original core	Partially hydrolysed core	CrO_3 oxidised core	IO_4^- oxidised and reduced core	Deaminated core
D-Galactose	0.96	–	1.16	–	1.06
D-Glucose	1.00	0.87	1.00	–	0.94
2-Acetamido-2-deoxy-D-galactose	0.91	0.13	0.80	0.93	0.78
2-Amino-2-deoxy-D-glucose	–	–	0.32	–	–
L-*glycero*-D-*manno*-Heptose	4.02	3.00	4.09	1.00	5.00
Disaccharide [a]	1.06	1.04	–	–	–
Glycerol	–	–	–	3.91	–
Threitol	–	–	–	0.96	–
Erythritol	–	–	–	0.81	–

[a] 7-*O*-(2-Amino-2-deoxy-α-D-glucopyranosyl)-L-*glycero*-D-*manno*-heptose

8 min, 100°), irrespective of whether the thiobarbituric acid or diphenylamine assay was used, but did give ~ 0.1 mol equiv of Kdo after vigorous hydrolysis (4 M HCl, 30 min, 100°) followed by assay using thiobarbituric acid. The oligosaccharide was free of phosphorus.

The ^{13}C-NMR spectrum of the core oligosaccharide contained, inter alia, signals attributable to two C–N groups at δ 53.34 and 54.96, and one at δ 23.09 for CH_3CO. This result is consistent with the presence of 2-amino-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-galactose in the molecule; the 2-amino-2-deoxy-D-glucose is known to have a free amino group[11]. The signals for anomeric carbons at δ 96.29, 96.44, 99.84, 100.23, 101.12, 102.25, 102.85, 102.99, and 103.66 confirmed the presence of nine sugar residues. The $J_{C\text{-}1,H\text{-}1}$ values were in the range 169–174 Hz for eight of these signals; this value was 162 Hz for the other signal (δ 103.66), suggesting that only one residue was β. ^{1}H-NMR spectroscopy confirmed the presence of an NAc group (δ 2.090) and nine anomeric protons with signals in the range δ 4.52–5.66.

Partial hydrolysis of the core oligosaccharide with 0.005 M H_2SO_4 (24 h, 100°) did not yield any new disaccharide, but heptose, galactose, and 2-acetamido-2-deoxygalactose were released (PC) after 30, 30, and 120 min, respectively. After partial hydrolysis for 24 h, chromatography of the products on Sephadex gave the three sugars, in the low molecular weight fraction, in the respective proportions 46.2, 32.0, and 21.8, and the ease of release indicated that they were in the peripheral region of the molecule. A minor proportion of glucose was also released. The degraded oligosaccharide eluted from the Sephadex, when hydrolysed with 2 M trifluoroacetic acid (12 h, 100°), afforded glucose, L-*glycero*-D-*manno*-heptose, and the original disaccharide in the ratios ~ 1:3:1 (Table I).

Methylation analysis of the oligosaccharide afforded seven acetylated partially methylated monosaccharide-alditols, in approximately equimolar quantities, and the methylated disaccharide demonstrated[11] to be present in *A. hydrophila* core

TABLE II

Methylation analysis of the core oligosaccharide and derived products (molar proportions)

Methyl sugar	Core oligosaccharide	Degraded core from mild hydrolysis	Expected IO_4^- oxidation product	Indicated linkage
2,3,4,6-Me_4-D-Gal	0.94	–	Glycerol	Gal-(1 →
2,3,4,6-Me_4-D-Glc	–	0.85	n.a. [a]	Glc-(1 →
2,3,4-Me_3-D-Glc	1.24	–	Glycerol	→ 6)-Glc-(1 →
2,3,4,6,7-Me_5-LD-Hep	1.19	0.12	Glycerol	Hep-(1 →
2,3,6,7-Me_4-LD-Hep	1.00	0.81	Erythritol	→ 4)-Hep-(1 →
3,4,6,7-Me_4-LD-Hep	1.03	1.05	Glycerol	→ 2)-Hep-(1 →
3,6-Me_2-D-GalN	0.74	0.05	2-Amino-2-deoxy-galactose	→ 4)-GalNAc-(1 →
Methylated disaccharide [b]	0.78	0.91	Threitol + glycerol	GlcN-(1 → 7)-Hep-(1 →
2,6,7-Me_3-LD-Hep	–	0.96	n.a.	→ 3)-Hep-(1 → 4 ↑ ↓ 6
2,7-Me_2-LD-Hep	0.78	–	Heptose	→ 3)-Hep-(1 → 4 ↑

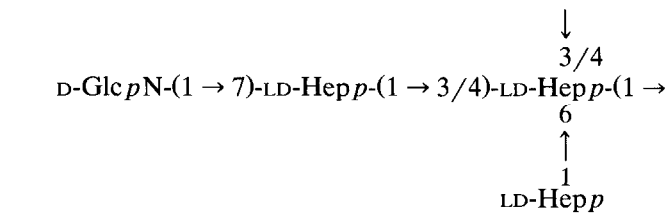
[a] Not applicable. [b] 1,5-Di-*O*-acetyl-7-*O*-(2-deoxy-2-dimethylamino-3,4,6-tri-*O*-methyl-α-D-glucopyranosyl)-2,3,4,6-tetra-*O*-methyl-L-*glycero*-D-*manno*-heptitol.

oligosaccharide (Table II). The formation of 1 mol equiv each of 2,3,4,6-tetra-*O*-methylgalactose and of 2,3,4,6,7-penta-*O*-methylheptose (i.e., end groups) is consistent with the ease with which galactose and 1 mol equiv of L-*glycero*-D-*manno*-heptose were released by partial acid hydrolysis. Methylation analysis of the degraded core oligosaccharide, isolated after mild acid hydrolysis for 48 h (Table II), afforded derivatives of 2,3,4,6-tetra-*O*-methylglucose, 2,3,4,6,7-penta-*O*-methylheptose, 2,3,6,7-tetra-*O*-methylheptose, 3,4,6,7-tetra-*O*-methylheptose, 2,6,7-tri-*O*-methylheptose, and the methylated disaccharide in the molar ratios 0.85 : 0.12 : 0.81 : 1.05 : 0.96 : 0.91. The formation of 2,3,4,6-tetra-*O*-methylglucose and 2,6,7-tri-*O*-methylheptose from the methylated degraded oligosaccharide, in place of the original 2,3,4-tri-*O*-methylglucose and 2,7-di-*O*-methylheptose, respectively, and in similar ratios, proved that the partial hydrolysis removed residues attached to position 6 of glucose and to the doubly branched heptose residue. Coupled with evidence gained from periodate oxidation, this finding demonstrates that the galactose → 2-acetamido-2-deoxygalactose fragment was linked to the glucose, whereas the end-group heptose was linked to the doubly branched heptose residue of the inner core. Because of a minor loss of glucose (0.13 mol equiv) during partial hydrolysis, a small proportion of 2,3,4,6,7-penta-*O*-methylheptose (0.12 mol equiv) was formed in place of a similar proportion of 2,3,6,7-tetra-*O*-methylheptose (0.19 mol equiv) in the original methylated oligosaccharide.

The fact that appearance of the latter compound was accompanied by loss of 2,3,4,6,-tetra-*O*-methylglucose (0.15 mol equiv) indicates that the glucose residue is 4-linked to the first heptose residue of the inner core. The preceding results are consistent with the presence of the partial structures **1** and **2** in the oligosaccharide.

D-Gal*p*-(1 → 4)-D-Gal*p*NAc-(1 → 6)-D-Glc*p*-(1 → 4)-LD-Hep*p*-(1 →

1



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Chromium trioxide oxidation was used in an attempt to confirm which one of the nine residues in the oligosaccharide was β. Table I indicates that the disaccharide was absent after oxidation and hydrolysis. In its place, 2-amino-2-deoxyglucose was formed and one mol equiv of heptose was lost, which confirmed that it was the heptose residue forming part of the disaccharide attached at position 3 or 4 of the doubly branched heptose residue that was β. These results are consistent with the ^{13}C-NMR data. Although chromium trioxide oxidation is not absolutely reliable for determining anomeric configurations, particularly for *manno* compounds, it was reported[4] that the above disaccharide was α-linked to the backbone in the core of *A. hydrophila* LPS and that there was no degradation by this technique, thus enhancing the present interpretation. Methylation analysis of the oxidised degradation product was not unequivocal and the molar relationships indicated that the degradation had not proceeded as clearly as might be predicted. However, the results indicated that the methylated disaccharide and the 2,7-di-*O*-methylheptose were no longer present and that the only new methylated residue formed was 2,4,7-tri-*O*-methylheptose. This result is consistent only with the disaccharide side chain being situated on C-4 of the original double-branched heptose residue. Thus, the rest of the backbone must be attached to C-3 of the same residue.

The core oligosaccharide was oxidised with periodate, then reduced, and subjected to chromatography on Sephadex G-25. Acid hydrolysis of the product (Table I) indicated that 1 mol equiv each of 2-acetamido-2-deoxygalactose and L-*glycero*-D-*manno*-heptose survived the oxidation, and that the other residues variously afforded erythritol, threitol, and glycerol in the molar ratios 0.81 : 0.96 : 3.91. Although the amount of glycerol appears to be ~ 1 mol equiv less than that predicted from the structure (cf. Table II), loss of this percentage of glycerol during the work-up procedure is not unusual. Apart from this minor

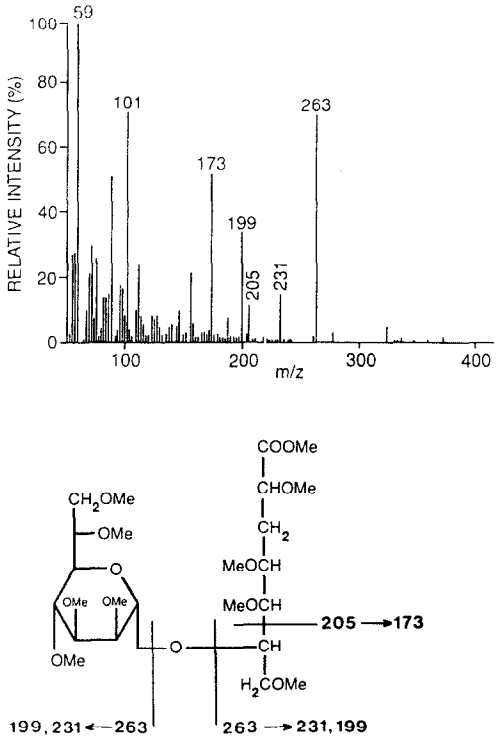


Fig. 1. EI-mass spectrum and fragmentation of the methylated disaccharide from periodate oxidation of the core oligosaccharide.

discrepancy, the results of periodate oxidation accorded with those of the methylation analysis.

Smith degradation of the polyol, Sephadex chromatography, and methylation analysis of the fragments revealed two methylated components larger than monosaccharides. The first, and larger, component had ions at *m/z* 323, 263, 231, 205, 199, and 173 in its mass spectrum (Fig. 1) and was shown to arise from L-*glycero*-D-*manno*-heptose 6-linked to oxidised (between C-7 and C-8) and reduced Kdo [i.e., LD-Hep*p*-(1 → 6)-heptonic acid]. We have demonstrated[9] a similar periodate-oxidation product [Man*p*-(1 → 6)-heptonic acid] from the core oligosaccharide of a deep-rough mutant of the same bacterium, and comparison of the mass spectra indicated that the only differences were the absence of the ions at *m/z* 219 and 187, due to primary and secondary fragmentations of the fully methylated mannose end group, and doubling of the intensities of the ions at *m/z* 263, 231, and 199 relative to those with *m/z* 205 and 173. This increase in intensities is due to the contribution made by fragments from the fully methylated end-group heptose being added to similar fragments from the heptonic acid residue. The ion at *m/z* 323 represents the abJ_1 fragment[12], an ion also found in

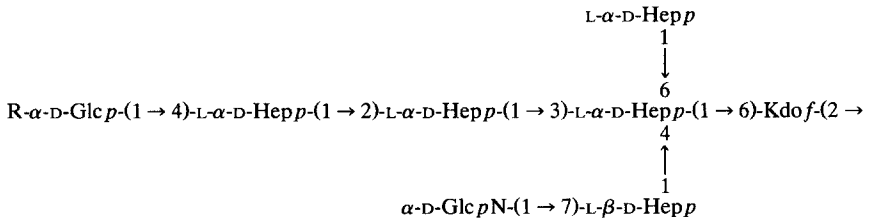
the Man*p*-(1 → 6)-heptonic acid. This result also confirms that the Kdo residue is furanose, as reported for the mutant, and this view is supported[13] by the presence of a strong signal for the C-5 resonance at δ 81.13.

The mass spectrum of the smaller component contained significant ions at *m/z* 334, 260, 228, 186, 103, and 58, which would be expected from the methylated degradation product [i.e., GalNAc-(1 → 3)-glycerol] originating from a 4-linked 2-acetamido-2-deoxygalactose residue 6-linked to glucose. Smith degradation also afforded a small proportion of 2-acetamido-2-deoxygalactose in the monosaccharide fraction eluted from Sephadex G-15. It is possible that this unexpected product arose by cleavage of at least some of the GalNAc → glycerol bonds during the Smith degradation, due to their lability even under mildly acid conditions.

Positive-ion FABMS of the methylated oligosaccharide gave major ions at *m/z* 464, 480, 728, 945, and 2170, corresponding to the following methylated fragments: Gal*p*-(1 → 4)-Gal*p*NMeAc (464), Glc*p*NMe_2-(1 → 7)-LD-Hep*p* (480), disaccharide + the doubly branched heptose residue (728), disaccharide + the doubly branched heptose + the end-group heptose (945), oligosaccharide (including Kdo) − end-group heptose (2170). Unfortunately, there was no molecular ion, presumably because of the ease with which the end-group heptose residue was removed from the molecule.

The structure and linkage of the three heptoses in the chain of the inner core and the occurrence of the Kdo in the furanose form are identical to those demonstrated[9] for the oligosaccharide isolated from a deep-rough mutant of this same bacterium.

Thus, on the basis of the above results, the structure of the core oligosaccharide may be represented as follows.



with R = α-D-Gal*p*-(1 → 4)-α-D-Gal*p*NAc-(1 → 6)

EXPERIMENTAL

Bacterial culture.—The virulent strain of *A. salmonicida* ssp. *salmonicida* was obtained from Dr. T.P.T. Evelyn (Department of Fisheries and Oceans, Nanaimo, Canada). The strain was isolated originally from Sockeye salmon and numbered B1-2-399 (Strain SJ-15 of the collection of the Northwest Atlantic Fisheries Centre). The culture was grown in Trypticase Soy Broth without added glucose

(Baltimore Biological Laboratories Inc.) for 20 h at 25° in a New Brunswick MF-128S fermentor.

Isolation and purification of the core oligosaccharide.—The LPS was isolated by the aqueous phenol method[14] and the core oligosaccharide was prepared by hydrolysis in boiling aqueous 1% acetic acid for 90 min followed by chromatography on Sephadex G-50 as described[1].

Analytical methods.—The $[\alpha]_D$ values were determined with an Atago Polax-D polarimeter. Aminodeoxy sugar residues were determined by the method of Rondle and Morgan[15], and in an amino acid analyser (Durum D-500). Kdo was assayed by the modified thiobarbituric acid[16] and diphenylamine[17] methods. Phosphorus was determined as reported by Chen et al.[18]. Sugars in the core oligosaccharide containing free amino groups were identified on the basis of the anhydro sugars formed[19] on deamination with nitrous acid.

Hydrolyses.—The core oligosaccharide was hydrolysed with either 2 M trifluoroacetic acid (to identify the neutral sugars) or 0.5 M H_2SO_4 (to determine the 2-acetamido-2-deoxy sugar) at 100° for 12 or 4 h, respectively. The methylated oligosaccharide was either acetolysed[20] or hydrolysed with M trifluoroacetic acid for 12 h at 100°. Partial hydrolysis was carried out with 0.005 M H_2SO_4 for 24 h at 100°.

Chromium trioxide oxidation.—The core oligosaccharide (100 mg) was acetylated and then oxidised by the method of Hoffman et al.[21]. The product was hydrolysed and also subjected to methylation analysis.

Periodate oxidation and Smith degradation.—The core oligosaccharide was treated with 0.05 M sodium metaperiodate for 7 days at 5° in the dark. Excess of periodate was reduced with a slight excess of ethylene glycol, and the product was reduced with sodium borohydride for 16 h, then recovered by chromatography on Sephadex G-25. Smith degradation was achieved in 0.5 M trifluoroacetic acid for 40 h at ~ 23°, and the products were reduced with sodium borohydride, subjected to chromatography on Sephadex G-15, and analysed by GLC–MS of the acetylated alditols and by methylation analysis.

Methylation analysis.—Methylation was effected by the method of Hakomori[22] and the products were isolated by elution from a column of Sephadex LH-20 with chloroform. Identification of the individual methylated residues formed by acetolysis or hydrolysis was by GLC–MS of the derived acetylated alditols.

GLC–MS.—Sugar residues and methylated sugar residues were analysed as the acetylated alditols on either a DB-210 megabore (J&W Scientific) column (30 m × 0.53 mm id) at 215° (for acetylated alditols of neutral and amino sugars) or on a 25-m WCOT CP-Sil 5CB (0.25-μm film) fused-silica capillary column (Chrompack) held isothermally at 180° for 32 min, then programmed to 250° at 8°/min and held for 16 min at 250°. GLC–EIMS was performed with a Hewlett–Packard 5970 mass selective detector coupled to a 5890A gas chromatograph. Positive FABMS was carried out on a JEOL Model 505H instrument with an accelerating voltage of 3 kV, and with *m*-nitrobenzyl alcohol as the matrix. The absolute

configuration of the Kdo was not determined but was assumed to be the same as that reported generally. The absolute configurations of the hexose, heptose, and aminodeoxy sugar residues were determined by polarimetry.

NMR spectroscopy.—^{13}C-NMR spectra were recorded with proton decoupling on a Varian Gemini-300 spectrometer in the pulsed FT mode at 75.5 MHz and 25°. The $J_{C\text{-}1,H\text{-}1}$ values were determined by the gated ^{1}H decoupling technique. Chemical shifts are reported relative to that of external Me_4Si. ^{1}H-NMR spectra were measured on the same instrument at 300.1 MHz and 25°.

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