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STRUCTURAL INVESTIGATIONS ON THE CORE OLIGOSACCHARIDE OF *Aeromonas hydrophila* (CHEMOTYPE III) LIPOPOLYSACCHARIDE

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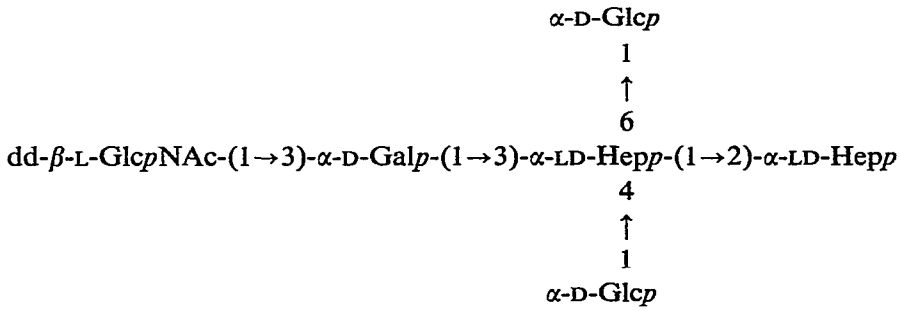
ABSTRACT

The core oligosaccharide of *Aeromonas hydrophila* (Chemotype III) lipopolysaccharide has been investigated. The studies involved the use of methylation analysis, oxidation with chromium trioxide, partial hydrolysis with acid, periodate oxidation, Smith degradation, and tagging of the reducing end-group. The core is unusual in having 3-acetamido-3,6-dideoxy-L-glucose as a constituent. As a result of these studies the following structure* is proposed.



INTRODUCTION

The Gram-negative bacterium *Aeromonas hydrophila* is an important cause of mortalities in salmonid fishes, particularly in hatchery or aquaculture ventures. The taxonomy of the species is confusing, but recent investigations have indicated three major sub-groups by traditional biochemical reactions[1]; a similar grouping has been determined by chemotyping using the sugars in the core oligosaccharide of the surface lipopolysaccharide[2]. This study was conducted on Chemotype III, which corresponds to *Aeromonas sobria* in the biochemical scheme. It has recently been determined that, of the three groupings, *A. sobria* (or Chemotype III) is unlikely to be pathogenic

*Abbreviations: dd-L-GlcNAc, 3-acetamido-3,6-dideoxy-L-glucose; LD-Hep, L-*glycero*-D-*manno*-heptose.

to fish[3], but is rather, a normal inhabitant of the intestinal flora of salmonid fishes, and of the aquatic milieu that they inhabit. Studies conducted on the serological identity of members of the *A. hydrophila* group indicated an extraordinary amount of heterogeneity[4].

The detailed core-structures of the three different chemotypes of *A. hydrophila* have not been examined to date. In the present investigation, we report the structure of the core oligosaccharide of Chemotype III.

RESULTS AND DISCUSSION

The lipopolysaccharide (lps) was isolated by the aqueous phenol method of Westphal and Jann[5]. The core oligosaccharide was obtained by mild hydrolysis of the lipopolysaccharide with acid, followed by purification by chromatography on a column of Sephadex G-50. In contrast to other species of *Aeromonas* that have been examined (*e.g., A. salmonicida*), the core oligosaccharide of *A. sobria* is the main constituent of the polysaccharide portion of the lps.

Analysis of the core oligosaccharide indicated that it was composed of residues of D-galactose, D-glucose, L-*glycero*-D-*manno*-heptose, and 3-acetamido-3,6-dideoxy-L-glucose in the molar ratios of ~1:2:2:1 (see Table I). The optical rotations of the sugars isolated from the hydrolyzate established that the galactose and the glucose had the D configuration. The amino sugar, isolated by high-voltage electrophoresis of the hydrolyzate, was ascertained to have the L configuration by circular dichroism[6]. The heptose was identified as L-*glycero*-D-*manno*-heptose by the retention time of its alditol acetate in g.l.c. Analytical studies on the core oligosaccharide indicated that it did not contain phosphorus[7], fatty acid[8], 2-aminoethanol[9], protein[10], or 3-deoxy-D-*manno*-2-octulosonic acid[11] (KDO).

The ^{1}H-n.m.r. spectrum of the core oligosaccharide[12] showed, *inter alia*, a signal at 2.18 p.p.m., assigned to the *N*-acetyl protons; a signal at 1.25 p.p.m., assigned to the methyl protons of the 6-deoxy function; and several signals in the anomeric region, one of which indicated the β configuration. The ^{13}C-n.m.r. spectrum showed six anomeric carbon atoms, resonating at 96.00, 100.27, 101.81, 102.41, 103.60, and 105.26 p.p.m. Due to the lack of reported chemical shifts of L-*glycero*-D-*manno*-heptose, no attempt has been made to assign these anomeric signals, but the shifts suggest that the majority of the glycosidic linkages may have the α configuration. The chemical shifts at 60.96, 61.60, and 61.95 p.p.m. correspond to the nonlinked C-6 of primary hydroxylmethyl groups of hexoses.

The chemical shifts of 176.19, 55.26, 27.75, and 18.10 p.p.m. were respectively assigned to the acetamido carbonyl carbon atom of the 3-acetamido-3,6-dideoxy-glucose, the acetyl ($CO\text{-}CH_3$) carbon atom, and the methyl carbon atom of the 6-deoxy function of the amino sugar. The optical rotation, $[\alpha]_D^{23}$ +94° (*c* 0.5, H_2O), may also indicate that the majority of the glycosidic linkages have the α configuration.

Methylation of the core oligosaccharide, with subsequent hydrolysis, reduction, and derivatization as the alditol acetates, indicated that the core is composed of six

TABLE I

SUGAR ANALYSIS OF THE CORE OLIGOSACCHARIDE AND DERIVED PRODUCTS

Sugar	*Lps*	*Core*	*Chromium trioxide-oxidized core*	*Oligo-saccharide* **2**	*Di-saccharide* **3**	*Di-saccharide* **4**	*Smith-degraded oligo-saccharide* **5**	*Periodate-oxidized, Smith-degraded product* (**6**)	*Tagged core*
D-Galactose	22.1 (1)	16.58 (1)	20.2 (1)	—	—	48.04 (1)	33.5 (1)	30.41 (1)	20.45 (1)
D-Glucose	42.3 (2)	33.45 (2)	42.0 (2)	61.34 (2)	—	—	—	—	38.41 (2)
D-Mannose	—	—	—	—	—	—	—	32.05 (1)	—
L-*glycero*-D-*manno*-Heptose	38.9 (2)	34.69 (2)	36.1 (2)	28.05 (1)	100	—	33.4 (1)	—	15.5 (1)
3-Acetamido-3,6-dideoxy-L-glucose	16.7 (1)	14.16 (1)	—	—	—	52.0 (1)	33.6 (1)	33.1 (1)	19.7 (1)

TABLE II

METHYLATION ANALYSIS OF THE CORE OLIGOSACCHARIDE AND DERIVED PRODUCTS

Methylated sugar	*Core*	*CrO_3 oxidized core*	*Oligo-saccharide* **2**	*Di-saccharide* **3**	*Di-saccharide* **4**	*Smith-degraded product* (5)	*Periodate-oxidized, Smith-degraded product* (6)	*Tagged core*	*Linkages*
2,3,4,6-Me_4-D-Glc	28.61 (2)	24.2 (2)	67.58 (2)	—	—	—	—	√	Glc-(1→
2,3,4,6-Me_4-D-Gal	—	13.3 (1)	—	—	—	—	—	—	Gal-(1→
2,4,6-Me_3-D-Gal	14.9 (1)	—	—	—	41.2 (1)	28.31 (1)	26.9 (1)	√	→3)-Gal-(1→
2,4,6-Me_3-D-Man	—	—	—	—	—	—	29.5 (1)	—	→3)-Man-(1→
2,3,4,6,7-Me_5-LD-Hep	—	—	—	47.34 (1)	—	—	—	—	Hep-(1→
3,4,6,7-Me_4-LD-Hep	15.03 (1)	14.01 (1)	—	48.84 (1)	—	—	—	—	→2)-Hep-(1→
2,4,6,7-Me_4-LD-Hep	—	—	—	—	—	30.28 (1)	—	—	→3)-Hep-(1→
2,3,7-Me_3-LD-Hep	—	—	35.32 (1)	—	—	—	—	—	→4)-Hep-(1→ 6 ↑
3,6-Dideoxy-2,4-Me_2-3-(*N*-methylacetamido)-L-Glc	15.69 (1)	—	—	—	44.8 (1)	26.45 (1)	24.31 (1)	√	ddGlcNAc-(1→ ↓ 6
2,7-Me_2-LD-Hep	14.04 (1)	10.1 (1)	—	—	—	—	—	√	→4)-Hep-(2→ 3 ↑

sugar units. Comparative studies on the hydrolysis products of the methylated core-oligosaccharide showed that reasonable stoichiometry was found only when 2M trifluoroacetic acid was used to cleave the permethylated core, instead of other methods of hydrolysis, such as acetolysis[13] and 90% formic acid followed by 0.5M sulfuric acid[14]. Analysis of the hydrolysis products by g.l.c.–m.s. gave the results shown in Table II.

The presence of a 2,7-di-*O*-methylheptose is attributable to a double branch point, and that of 2,3,4,6-tetra-*O*-methylglucose to two terminal residues. The derivatives 2,4,6-tri-*O*-methylgalactose and 3,4,6,7-tetra-*O*-methylheptose are assigned to two residues linked through O-3 and O-2, respectively. The amino sugar fragment 3,6-dideoxy-2,4-di-*O*-methyl-3-(*N*-methylacetamido)glucose indicates that it is present as one terminal residue.

The anomeric configurations of the various glycosyl groups were also ascertained. The core oligosaccharide was acetylated, and the product subjected to oxidation with chromium trioxide. During the oxidation, only the 3-acetamido-3,6-dideoxy-L-glucose was oxidized, indicating that it might be β-linked, and the other sugars, α-linked (see Table I).

Methylation analysis of the oxidized core-oligosaccharide (see Table II) indicated the disappearance of the amino sugar fragment, namely, 3,6-dideoxy-2,4-di-*O*-methyl-3-(*N*-methylacetamido)glucose. This indicates that the 2,4,6-tri-*O*-methylgalactose found in the analysis of the original material had been replaced by 2,3,4,6-tetra-*O*-methylgalactose, and that the amino sugar is linked to O-3 of the D-galactopyranosyl residue, as in the partial structure **1**.

dd-β-L-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→

1

Partial hydrolysis of the core oligosaccharide with 0.5M trifluoroacetic acid for 2 h at 100°, followed by purification on Sephadex G-15, gave saccharides **2**, **3**, and **4** (see Fig. 1, peaks 1, 2, and 3, respectively).

Analysis of fragment **2** showed that it was composed of two mol of D-glucose and one mol of L-*glycero*-D-*manno*-heptose (see Table I). Methylation analysis, hydrolysis, reduction, and g.l.c.–m.s. of the alditol acetates gave 2,3,4,6-tetra-*O*-methylglucose (2) and 2,3,7-tri-*O*-methylheptose (1) (see Table II), indicating that two D-glucosyl groups were linked to the LD-heptosyl residue through O-4 and O-6; the structure of the trisaccharide **2** was thus established as being the following.

α-D-Glc*p*
1
↑
6
α-D-Glc*p*-(1→4)-α-LD-Hep*p*

2

Fragment **3** was found to be composed of LD-heptose only (see Table I). The

methylation analysis (see Table II) yielded 2,3,4,6,7-penta-*O*-methylheptose (1) and 3,4,6,7-tetra-*O*-methylheptose (1), indicating that one LD-heptosyl group is linked to an LD-heptose residue through O-2; the structure of disaccharide **3** was thus established as being that shown.

α-LD-Hep*p*-(1→2)-α-LD-Hep*p*

3

Disaccharide **4** was isolated from the Sephadex fraction (that also contained monosaccharides) by high-voltage electrophoresis; it had M_{GlcN} 0.58, and analysis indicated that it was composed of D-galactose and 3-acetamido-3,6-dideoxy-L-glucose. Methylation analysis yielded 2,4,6-tri-*O*-methylgalactose and 3,6-dideoxy-2,4-di-*O*-methyl-3-(*N*-methylacetamido)glucose, reconfirming that the amino sugar is linked to the D-galactopyranosyl residue through O-3 (see Table II). The structure of disaccharide **4** is therefore established as shown.

dd-β-L-Glc*p*NAc-(1→3)-α-D-Gal*p*

4

A sample of the core was oxidized with periodate, and the product was reduced. Sugar analysis of the resulting polyol indicated the presence of D-galactose, LD-heptose, and 3-acetamido-3,6-dideoxy-L-glucose in the ratios of 1 : 1 : 1. Glycerol was identified by paper chromatography. This result showed that 2 mol of D-glucose and 1 mol of LD-heptose had been oxidized, thus confirming the results of the methylation analysis.

The core oligosaccharide was subjected to a Smith degradation, that is periodate oxidation, borohydride reduction, and mild hydrolysis with acid. Methylation analysis of the product yielded 3,6-dideoxy-2,4-di-*O*-methyl-3-(*N*-methylacetamido)-glucose, 2,4,6-tri-*O*-methylgalactose, and 2,4,6,7-tetra-*O*-methylheptose (see Table II). Hence, the structure of the Smith-degraded oligosaccharide **5** is as shown.

dd-β-L-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→3)-α-LD-Hep*p*-(1→2)-glycerol

5

The Smith degraded product (**5**) was subjected to another periodate oxidation, the product reduced, and the product hydrolyzed. Methylation analysis of this new product (**6**) yielded 3,6-dideoxy-2,4-di-*O*-methyl-3-(*N*-methylacetamido)-glucose (1), 2,4,6-tri-*O*-methylgalactose (1), and 2,4,6-tri-*O*-methylmannose (1). The structure of this product is shown in formula **6**. The latter results indicate that the D-galactose

dd-β-L-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→3)-α-D-Man*p*-(1→2)-glycerol

6

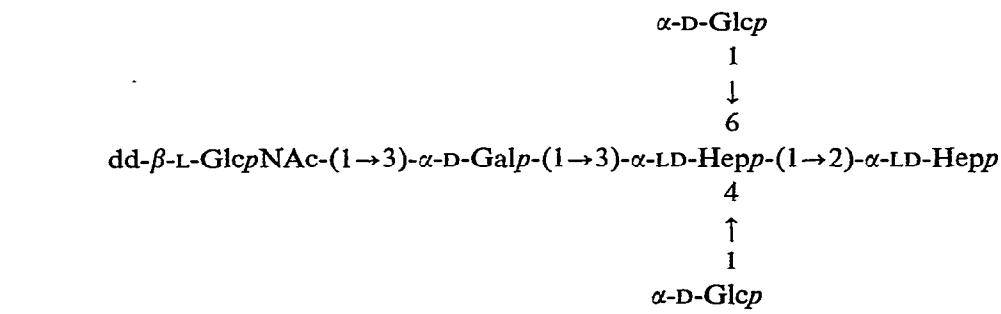
is linked to the LD-heptosyl residue through O-3 in the Smith-degraded product **5**; similarly, the D-galactose is linked to the D-mannosyl residue through O-3 in the periodate-oxidized, Smith-degraded product **6**.

In most of the lipopolysaccharide structures studied so far, three molecules of KDO (3-deoxy-D-*manno*-2-octulosonic acid) were found to join the core oligo-

saccharide to the lipid A; in our present study, KDO appears to be absent. It seems probable that the L-*glycero*-D-*manno*-heptose serves as a link between the core oligosaccharide and lipid A. Attempted determination of the reducing end and the reducing power of the core oligosaccharide by known methods[15,16] failed. It is suggested that the reducing end of the LD-heptose residue may either be blocked by cyclization to the 1,6-anhydro ring that L-*glycero*-D-*manno*-heptose seems prone to form[17], or that it could be glycosidically linked to decomposition fragments resulting from hydrolysis of the lps with 1% acetic acid. However, the reducing end of the core oligosaccharide was linked to 2-aminopyridine by reductive amination with cyanoborohydride[18], giving a fluorescent-tagged core that was hydrolyzed with 0.2M HCl for 15 min at 100°. The tagged LD-heptose obtained from this hydrolyzate was, in high-voltage electrophoresis, electrophoretically identical with synthetic, tagged heptose.

The rest of the unhydrolyzed core was recovered, and it was subjected to an analysis (see Table II) that indicated that one molecule of LD-heptose per molecule had been lost in the tagging process. The methylation analysis indicated the absence of 3,4,6,7-tetra-*O*-methylheptose. This result proved that the LD-heptose constitutes the reducing end of the core oligosaccharide (see Table II).

From the combined evidence, the core oligosaccharide from *Aeromonas hydrophila* chemotype III has the following structure.



EXPERIMENTAL

General methods. — Analytical paper-chromatography was performed on Whatman No. 1 paper, and Whatman No. 3 MM paper was used for preparative purposes, with 8:2:1 (v/v) ethyl acetate–pyridine–water as the solvent. Chromatograms were developed with alkaline silver nitrate. High-voltage paper-electrophoresis was conducted for 90 min in a Shandon flat-bed, high-voltage, electrophoresis apparatus, using a buffer of 5:2:43 (v/v) pyridine–acetic acid–water at pH 5.4, 2 kV, and 170 mA. The amino sugar was detected by spraying with ninhydrin, or by using the alkaline silver nitrate reagent. The amino sugar and the disaccharide (containing the amino sugar) were eluted from the paper electropheretogram with 0.05% HCl. Thin-layer chromatography was conducted on precoated cellulose

(100 μm) plates (E. Merck, Darmstadt) with a solvent system of 5:5:1:3 ethyl acetate–pyridine–acetic acid–water in an atmosphere saturated[19] with 40:11:6 ethyl acetate–pyridine–water.

Gas–liquid chromatography of the acetylated alditols and partially methylated alditol acetates was performed on packed columns (1.83 m × 2 mm i.d.) of 1.5% of Silar 7CP on Gas Chrom Q (100–120 mesh) in a Perkin–Elmer Model 3920 gas chromatograph operated isothermally at temperatures varied from 190 to 220°, with a helium flow of 40 mL/min. We have found that Silar 7CP makes an excellent substitute for ECNSS-M, and has almost identical McReynolds constants. In gas–liquid chromatography, Silar 7CP maintains its concentration on the solid phase indefinitely, leading to much greater long-term stability in absolute and relative retention-times. G.l.c. was also performed in[20] a 25-m column of W.C.O.T. CP-Sil 5 (0.25 μm) (Chrompack, The Netherlands) at 180°.

Combined g.l.c.–mass spectrometry was performed in a Hewlett–Packard Model 5981A g.l.c.–m.s. instrument controlled by a 5939A data system, with a membrane separator, a source temperature of 160°, and an ionizing voltage of 70 eV. Carbon-13 and ^{1}H-n.m.r. spectra were recorded at 20 and 79.7 MHz, respectively, in the pulsed, Fourier-transform mode, with a Varian CFT-20 spectrometer. The ^{1}H-n.m.r. spectra were recorded at 85°, and the chemical shifts expressed relative to sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate. Carbon-13 shifts are expressed relative to external tetramethylsilane.

Isolation, and purification, of the core oligosaccharide. — The lipopolysaccharide was isolated by the aqueous phenol method of Westphal and Jann[5], and the core oligosaccharide was isolated as previously described[2].

Hydrolysis of the core oligosaccharide, and sugar analysis. — The core oligosaccharide (10 mg) was hydrolyzed with 0.5M sulfuric acid for 7 h at 100°. The monosaccharides liberated were converted into alditol acetates by reduction and acetylation, and they were identified by g.l.c.–m.s. The sugars were isolated by preparative paper-chromatography and thin-layer chromatography, and their optical rotations were recorded: D-glucose, $[\alpha]_D^{23}$ +45° (*c* 0.01, water); D-galactose, $[\alpha]_D^{23}$ +39° (*c* 0.5, water). The 3-acetamido-3,6-dideoxy-L-glucose was isolated by high-voltage electrophoresis, and its optical rotation was $[\alpha]_D^{23}$ −26° (*c* 0.001, water).

Methylation analysis. — The core oligosaccharide (10 mg) was dissolved in dimethyl sulfoxide, and was methylated by the Hakomori method[21]. The methylated oligosaccharide was hydrolyzed with 2M trifluoroacetic acid for 18 h at 100°. The resulting, partially methylated sugars were reduced, the products acetylated, and the alditol acetates analyzed by g.l.c.–m.s. (see Table II).

Oxidation with chromium trioxide. — The core oligosaccharide (10 mg) was dissolved in *N*,*N*-dimethylformamide (1.5 mL), and pyridine (1.5 mL) and acetic anhydride (1.5 mL) were added[23]. The solution was stirred for 16 h at room temperature, and evaporated to dryness, and toluene was added to, and evaporated from, the residue; this was dissolved in chloroform, and *myo*-inositol hexaacetate (2 mg) was added as the internal standard. The solution was divided into two portions, and

these were evaporated. One part was dissolved in glacial acetic acid (2 mL), and powdered chromium trioxide (40 mg) was added; the mixture was then agitated ultrasonically for 3 h at 50°, poured into water, extracted with chloroform (10 mL), and the extract washed twice with water, and evaporated to dryness.

Part of the oxidized core, and the remaining, original unoxidized material, were separately hydrolyzed with 0.25M sulfuric acid for 12 h at 100°, followed by reduction, acetylation, and analysis by g.l.c.–m.s. as alditol acetates (see Table I). Oxidized core-oligosaccharide was also methylated by the Hakomori method, and processed as usual, to afford partially methylated alditol acetates (see Table II).

Partial hydrolysis of the core oligosaccharide with acid. — The core oligosaccharide (60 mg) was partially hydrolyzed with 0.5M trifluoroacetic acid for 30 min at 100°, and the solution was evaporated with a stream of nitrogen, and the residue twice dissolved in water, and evaporated. The product was purified on a column of Sephadex G-15, using 47mM pyridinium acetate buffer, pH 4.26. Elution of the components was monitored with a differential, refractive-index monitor, and afforded three fractions (see Fig. 1).

A portion of each oligosaccharide was hydrolyzed with 0.5M sulfuric acid for 4 h at 100°, and then the hydrolyzates were made neutral, reduced, and acetylated (see Table I). The oligosaccharides were methylated by the Hakomori method, and the methyl ethers hydrolyzed with 2M trifluoroacetic acid. The resulting, partially methylated sugars were reduced and acetylated (see Table II).

Periodate oxidation of the core oligosaccharide. — The core oligosaccharide (150 mg) was dissolved in distilled water (10 mL) and 0.1M sodium metaperiodate (20 mL) was added. The solution was kept in the dark at 5°. After 72 h, ethylene glycol (2 mL) was added, the product was reduced with sodium borohydride, and

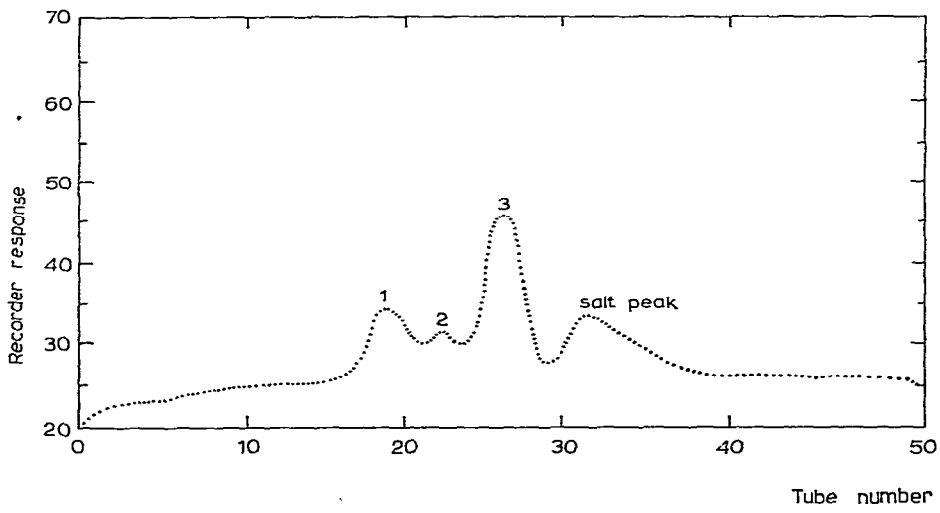


Fig. 1. Separation, on a column of Sephadex G15, of fragments obtained by partial hydrolysis of the core oligosaccharide.

the base neutralized. The polyol was purified by chromatography on a column of Sephadex G-15 eluted with pyridinium acetate buffer, pH 4.26.

Smith degradation of the core oligosaccharide. — The polyol (50 mg) obtained from the periodate oxidation of the core oligosaccharide was hydrolyzed with 0.1M hydrochloric acid for 3 h at 100°. After evaporation of the acid, the residue was purified by chromatography on a column of Sephadex G-15 eluted with a pyridinium acetate buffer. The pure Smith-degraded oligosaccharide obtained was hydrolyzed with 0.5M sulfuric acid for 4 h at 100°, made neutral, reduced, and acetylated (see Table I), and subsequently methylated by the Hakomori method (see Table II).

Periodate oxidation of the Smith-degraded product. — Smith-degraded product (10 mg) was subjected to a further periodate oxidation under the conditions described earlier, and the product was purified by chromatography on a column of Sephadex G-15.

Tagging of the reducing end of the core oligosaccharide with 2-aminopyridine. — The core oligosaccharide (10 mg) was dissolved in water (170 μL), and the solution was added to the tagging reagent (640 μL) [obtained by mixing 2-aminopyridine (332 mg), sodium cyanoborohydride (128 mg), glacial acetic acid (148 μL), and methanol (1.32 mL)]. The solution was heated in a capped tube for 6 h at 90°, cooled to room temperature, and kept overnight. Dowex-50 X-4 (H^+) resin (5.6 mL) was added to the mixture, and the resin was washed with water, and eluted with 0.6M ammonia. The tagged core-oligosaccharide was subjected to hydrolysis with 0.2M hydrochloric acid for 15 min at 100°, the solution was evaporated, and the residue was purified by high-voltage electrophoresis. The tagged heptose, M_{GlcN} 0.8, and the rest of the unhydrolyzed core, were recovered by elution from the paper electropheretogram with 0.05% hydrochloric acid. The unhydrolyzed core was subjected to sugar analysis, and permethylation.

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