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STRUCTURAL STUDIES OF THE O-ANTIGEN POLYSACCHARIDE OF *Salmonella thompson*, SEROGROUP C_1 (6,7)*

BENGT LINDBERG,

Department of Organic Chemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm (Sweden)

KARIN LEONTEIN, ULF LINDQUIST, STEFAN B. SVENSON, GÖRAN WRANGSELL,

Department of Vaccine Development and Production, National Bacteriological Laboratory, S-105 21 Stockholm (Sweden)

ANNE DELL, AND MARK ROGERS

Department of Biochemistry, Imperial College of Science and Technology, Imperial Institute Road, London SW7 2A2 (Great Britain)

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ABSTRACT

The structure of the O-antigen polysaccharide of *Salmonella thompson*, serogroup C_1 (6,7) has been investigated mainly by methylation analysis, n.m.r. spectroscopy, specific degradations by a phage-associated enzyme, *N*-deacetylation–deamination, and f.a.b.-m.s. It is concluded that the structure involves the following repeating unit.

→2)-β-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-β-D-Man*p*-(1→3)-β-D-Glc*p*NAc-(1→
3
↑
R R = H or α-D-Glc*p*

There are two populations of chains, with and without α-D-glucopyranosyl groups, 3-linked to an α-D-Man*p* residue, and only the latter type is hydrolysed by the phage enzyme. The α linkage of the third Man*p* residue is cleaved by the Ø14 phage enzyme. The structure, with or without the α-D-glucopyranosyl group, represents the biological repeating-unit.

INTRODUCTION

The *Salmonella* species most harmful to man belong to serogroups A, B, C_1, and D. The structures of the O-antigen polysaccharides from groups A, B, and D are closely related, the main difference being that they contain different 3,6-di-

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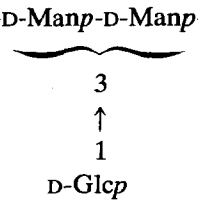
deoxy-α-D-hexopyranosyl end-groups[1]. The structure of the C_1 O-antigen polysaccharide, however, remained to be determined.

Fuller and Staub[2] reported the partial structure **1** for the repeating unit of the O-antigen polysaccharide from *S. cholerae suis*, serogroup C_1 (6,7).



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Both α- and β-D-Man*p* residues were observed. They also studied a phage-converted strain, Ø14 C_1, and found that it had a releated structure, the difference being that the terminal α-D-Glc*p* group was 3-linked to the fourth D-Man*p* residue in **1**.

At about the same time, we started structural studies of the O-antigen polysaccharide from *S. thompson*, serogroup C_1 (6,7), which should have the same structure as that from *S. cholerae suis*. The presence of a hexasaccharide repeating-unit with the same components as in **1** was confirmed with all of the Man residues 2-linked and the D-GlcNAc residue β. However, attempts to determine the anomeric configurations of the individual D-Man*p* residues and the location of the terminal α-D-Glc*p* group gave inconsistent results. Since more powerful structural methods are now available, this investigation has been resumed, the results of which are now reported.

RESULTS AND DISCUSSION

The lipopolysaccharide (LPS) from *S. thompson* IS 40 was delipidated to give the polysaccharide (PS), sugar analysis of which gave D-mannose, D-glucose, and 2-amino-2-deoxy-D-glucose, in the ratios 70:14:16, as the main components. Methylation analysis (Table I, column *A*) revealed terminal D-Glc*p*, 2- and 2,3-linked D-Man*p*, and 3-linked D-Glc*p*NAc. The ^{1}H- and ^{13}C-n.m.r. spectra of this product were complex and not in agreement with a regular structure, but they showed that the PS contained β-D-Glc*p*NAc, β-D-Man*p*, α-D-Man*p*, and α-D-Glc*p* in the ratios ~1:2:2:0.3. The polysaccharide prepared from another batch of LPS, not used in the structural studies, contained these residues in the ratios ~1:2:2:0.7.

The LPS was partially delipidated by treatment with base under conditions during which essentially ester linkages, but not amide linkages, should be hydrolysed. This product was then hydrolysed, under dialysing conditions[3], by treatment with the Ø14 phage enzyme and the product was fractionated by gel-permeation chromatography. Several oligosaccharides (Table II) were obtained, and were

TABLE I

METHYLATION ANALYSES OF NATIVE AND MODIFIED C_1 POLYSACCHARIDE AND THE DEGRADATION PRODUCTS

Sugar[a]	T[b]	*Mole %*					
		A[c]	B	C	D	E	F
1,3,4,5,6-Mannitol	0.50				14	13	9
1,4,6-2,5-AnMannitol	0.53			12			
2,3,4,6-Man	1.00			} 33	26	18	15
2,3,4,6-Glc	1.00	12	17				
3,4,6-Man	1.52	65	54	47	39	55	60
4,6-Man	2.06	10	15	8			
2,4,6-GlcNAc	4.45	13	14		21	14	16

[a]2,3,4,6-Man = 2,3,4,6-tetra-*O*-methyl-D-mannose, *etc.* [b]Relative retention time, conditions given in Experimental part. [c]Key: *A*, Native polysaccharide; *B*, polysaccharide after phage-catalysed hydrolysis; *C*, product obtained after deamination; *D*, pentasaccharide; *E*, octasaccharide; *F*, decasaccharide.

characterised by sugar analysis, methylation analysis, n.m.r. spectroscopy, and f.a.b.-m.s.

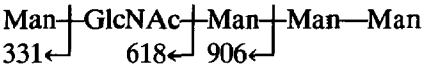
Sugar analyses showed that the oligosaccharides contained only D-Man and D-GlcNAc residues, and methylation analyses (Table I, columns *D–F*) of the alditols showed that each oligosaccharide was linear and had a D-Man residue as the reducing and non-reducing terminal. The molecular ions, $(M + H)^+$, obtained by f.a.b.-m.s., demonstrated that they were a penta- ($Man_4GlcNAc$), octa- ($Man_7GlcNAc$), deca- [$Man_8(GlcNAc)_2$], trideca- [$Man_{11}(GlcNAc)_2$], and pentadeca-saccharide [$Man_{12}(GlcNAc)_3$]. The ions $(M + NH_4)^+$ and $(M + Na)^+$ were also observed, and the spectra further showed that there had been some overlap between the fractions.

The sequences of the sugar residues in the penta-, octa-, and deca-saccharides were determined by f.a.b.-m.s. of their acetates. Such derivatives fragment[4] to give mainly the A_1-type ions, as indicated below for the acetylated penta- (**2**), octa- (**3**), and deca-saccharide (**4**).

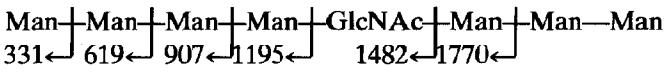
TABLE II

OLIGOSACCHARIDES OBTAINED FROM PHAGE-CATALYSED HYDROLYSIS OF PARTIALLY DELIPIDATED *Salmonella* C_1 LPS

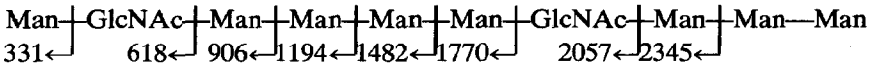
$[M + 1]^+$ (m/z)	*Composition*	*Yield (mg)*
870	$Man_4GlcNAc$	22
1356	$Man_7GlcNAc$	11
1721	$Man_8(GlcNAc)_2$	193
2207	$Man_{11}(GlcNAc)_2$	25
2572	$Man_{12}(GlcNAc)_3$	60



2 (M + H)$^+$ *m/z* 1542



3 (M + H)$^+$ *m/z* 2406



4 (M + H)$^+$ *m/z* 2981

The combined yield of the penta-, deca-, and pentadeca-saccharides was considerably higher than the combined yield of the octa- and trideca-saccharides. A reasonable explanation for this observation is that the phage enzyme catalyses the hydrolysis at a unique position in the pentasaccharide repeating-unit, giving mainly oligosaccharides containing $5n$ sugar residues, and that the oligosaccharides with $(5n + 3)$ sugar residues are derived from the terminal of the O-antigen polysaccharide. The biological repeating-unit should consequently have the structure **5**, and the phage enzyme catalyses the hydrolysis of the linkage between Man(III) and Man(IV).

→2)-D-Man*p*-(1→2)-D-Man*p*-(1→2)-D-Man*p*-(1→2)-D-Man*p*-(1→3)-D-Glc*p*NAc-(1→
I II III IV

5

A similar observation was made during studies of the O-antigen polysaccharide of *Escherichia coli* O8 (ref. 5).

Assignment of the resonances for anomeric protons and carbons of the penta-, octa-, and deca-saccharide are given in Tables III and IV, respectively.

Man-GlcNAc-Man-Man-Man
IV I II III

6

Man-Man-Man-Man-GlcNAc-Man-Man-Man
I II III IV I II III

7

Man-GlcNAc-Man-Man-Man-Man-GlcNAc-Man-Man-Man
IV I II III IV I II III

8

TABLE III

SIGNALS GIVEN BY ANOMERIC PROTONS IN THE ^{1}H-N.M.R. SPECTRUM OF THE C_1 POLYSACCHARIDE, THIS POLYSACCHARIDE ENRICHED IN α-D-GLUCOPYRANOSYL GROUPS, AND OLIGOSACCHARIDES OBTAINED ON PHAGE-CATALYSED HYDROLYSIS[a]

Assignments	*Penta*	*Octa*	*Deca*	*PS*[b]	*PS*[c]
β-ManIV			4.70 (n.r.)		
β-GlcNAc			5.01 (8.4)		
β-ManI		4.76 (1.0)	4.77 (n.r.)	4.78 (n.r.)[d]	4.75 (n.r.)
α-ManII		5.15 (1.8)	5.17 (1.3)	5.17 (n.r.)	5.24 (n.r.)
α-ManIII		5.34 (1.5)	5.32 (1.5)	5.33 (n.r.)[d]	5.31 (n.r.)
β-ManIV	4.71 (<1)	4.74 (n.r.)	4.74 (n.r.)	4.75 (n.r.)[d]	4.75 (n.r.)
β-GlcNAc	5.02 (8.4)	5.00 (8.1)	4.99 (8.2)	5.00 (7.9)	4.99 (8.1)
β-ManI	4.79 (<1)	4.78 (n.r.)	4.78 (n.r.)		
α-ManII	5.18 (1.7)	5.17 (1.5)	5.17 (1.3)		
α-ManIII[e]	5.33 (1.5)	5.32 (1.7)	5.32 (1.7)		
α-Glc				5.26 (3.5)[f]	5.26 (3.5)

[a]Chemical shifts in p.p.m. Coupling constants, $J_{1,2}$ in Hz, in parenthesis. Some assignments may be interchanged. [b]Native polysaccharide, O.3 α-D-glucopyranosyl group per repeating-unit. [c]Polysaccharide, enriched in α-D-glucopyranosyl groups. [d]In addition to this signal, a weaker signal (ratio 7:3) appeared at the same field as the corresponding signal in the spectrum of the glucose-enriched polysaccharide. [e]Reducing terminal. [f]Weak signal, corresponding to ~0.3 glucosyl group per repeating-unit.

TABLE IV

SIGNALS OF ANOMERIC CARBONS IN THE ^{13}C-N.M.R. SPECTRUM OF THE C_1 POLYSACCHARIDE, THIS POLYSACCHARIDE ENRICHED IN α-D-GLUCOPYRANOSYL GROUPS, AND OLIGOSACCHARIDES OBTAINED ON PHAGE-CATALYSED HYDROLYSIS[a]

Assignments	*Penta*	*Octa*	*Deca*	*PS*[b]	*PS*[c]
β-ManIV			101.27		
β-GlcNAc			100.77		
β-ManI		99.42	99.26[d]	99.58	100.00
α-ManII		100.87	100.05	100.08	99.49
α-ManIII		100.39	100.38	100.42	100.43
β-ManIV	101.26	100.78	100.77	100.72	100.73
β-GlcNAc	100.75	100.87	100.77	100.87	100.81
β-ManI	99.23	99.42	99.44[d]		
α-ManII	99.91	100.04	100.05		
α-ManIII[e]	93.38	93.41	93.37		
α-Glc				101.39[f]	101.41

[a]Chemical shifts in p.p.m. [b]See Table III. [c]See Table III. [d]These assignments may be interchanged. [e]Reducing terminal. [f]Weak signal.

Of the Man residues in the pentasaccharide **6**, one is a reducing terminal, one is α, and two are β (1,1,2). The corresponding numbers for the octa- (**7**) and decasaccharide (**8**) are 1,3,3 and 1,3,4, respectively. From these figures, it is evident that Man(III) is α and Man(IV) is β. The latter assignment is also in agreement with the chemical shift data for the resonance of C-3 in the β-D-Glc*p*NAc residue. The expected chemical shifts on substitution with an α- and a β-D-Man*p* residue are δ 80–81 and >83, respectively, and the observed signal was at δ 83.1.

In the ^{1}H-n.m.r. spectrum of the pentasaccharide-alditol, prepared by reduction with sodium borodeuteride, only one signal in the region for anomeric protons was shifted significantly, namely, from δ 5.18 to 5.14. This may indicate that Man(II), adjacent to the reducing terminal, is α. In the spectra of the PS and the oligosaccharides, the signal for H-1 of the β-D-Glc*p*NAc residue appears at an exceptionally low-field position (δ 5.0, $J_{1,2}$ 8 Hz), probably because the proton is deshielded by its proximity to O-1 of Man(I), which should be β. As one α [Man(III)] and one β residue [Man(IV)] are also identified, the assignment of the β configuration to Man(I) is in agreement with the tentative assignment of the α configuration to Man(II). From the results discussed above, the structures of the pentasaccharide (**9**) and the biological repeating-unit (**10**) of the phage-enzyme-hydrolysable part of the PS are determined.

β-D-Man*p*-(1→3)-β-D-Glc*p*NAc-(1→2)-β-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-D-Man*p*
IV I II III

9

→2)-β-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-β-D-Man*p*-(1→3)-β-D-Glc*p*NAc-(1→
I II III IV

10

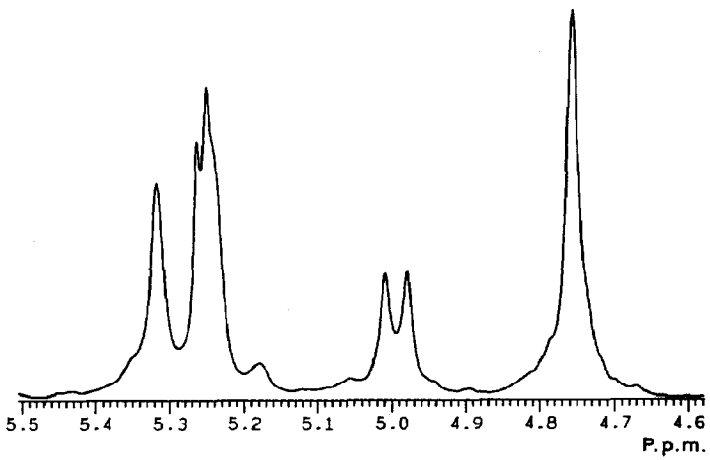


Fig. 1. ^{1}H-N.m.r. spectrum (anomeric region) of PS after treatment with phage endo-glucosidase.

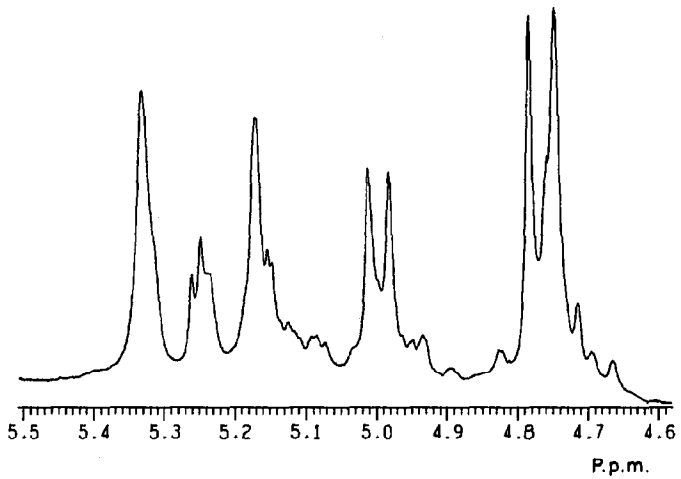
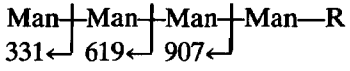


Fig. 2. ^{1}H-N.m.r. spectrum (anomeric region) of the original polysaccharide.

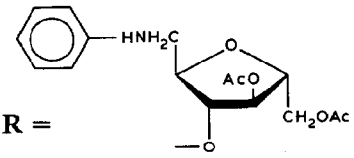
The partially delipidated LPS which remained after the phage-enzyme-catalysed hydrolysis was delipidated to the PS, which, on hydrolysis with acid, yielded D-Man, D-Glc, and D-GlcNAc in the ratios ~67:20:13. This material was consequently enriched in D-Glc, which was also evident from the data for methylation analysis (Table I, column *B*). These analyses indicate that the partially degraded PS is composed of hexasaccharide repeating-units in which an α-D-Glc*p* group is 3-linked to one of the D-Man*p* residues. The ^{1}H-n.m.r. spectrum of this material (Fig. 1) was also simpler than that (Fig. 2) of the original PS with a low content of α-D-Glc*p* groups.

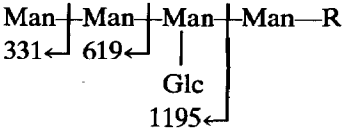
The original PS was *N*-deacetylated by treatment with aqueous sodium hydroxide in methyl sulfoxide, which required severe conditions (10M sodium hydroxide–methyl sulfoxide, 1:1, 12 h, 120°). The ^{1}H- and ^{13}C-n.m.r. spectra revealed that the *N*-deacetylation was complete. The ^{13}C-n.m.r. spectrum showed, *inter alia*, a signal for C-3 of the β-D-Glc*p*N residue at δ 88.5. The product was then deaminated by treatment with sodium nitrite in aqueous acetic acid, followed by fractionation on Bio-Gel P-2. Sugar analysis of the main fraction, after reduction with sodium borohydride, gave D-Man, D-Glc, and 2,5-anhydro-D-mannitol in ratios ~4:0.8:1. The material was a mixture of two components, most probably a glucose-free pentasaccharide and a glucose-containing hexasaccharide. The mixture was treated with aniline in the presence of sodium cyanoborohydride, and the resulting phenylamine derivatives were fractionated on a column of Spherisorb NH_2. The two components obtained (**11** and **12**) were acetylated and the products were analysed by f.a.b.-m.s. The molecular ions and the A_1-type fragments obtained demonstrated **11** to be linear, **12** to be branched, and the α-D-Glc*p* residue in **12** to be linked to Man(III).



11 $(M + H)^+$ *m/z* 1518

R =





$(M + H)^+$ *m/z* 1806

12

From the results discussed above, it is concluded that the O-antigen polysaccharide of *S. thompson*, serotype C_1, is composed of repeating units with the structure **13**, which is also the biological repeating-unit.

→2)-β-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-β-D-Man*p*-(1→3)-β-D-Glc*p*NAc-(1→
3
↑
R R = H or α-D-Glc*p*

There are two types of polysaccharide chains, with and without a terminal α-D-Glc*p* group 3-linked to Man(III), and only the latter are hydrolysed by the action of phage Ø14.

In order to decide if these two types are present in the same molecule or if there are two different populations, the PS from the original and the phage-degraded PS were subjected to gel filtration. The amount of high-molecular-weight material decreased on hydrolysis with the phage enzyme, but no significant increase in material of medium–high molecular weight was observed. This is the expected result when there are two populations of polysaccharide chains, with and without terminal Glc*p* groups. A similar result has been observed for the *S. zürich* O-antigen polysaccharide, which also consisted of two types, with and without terminal α-D-Glc*p* groups[6]. As mentioned above, the glucose content varies in polysaccharides from different batches, indicating that the ratio between the two populations is controlled by some variable factor, *e.g.*, the number of lysogenic bacteria in a given culture.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at ≯40° (bath). For g.l.c., a Hewlett–Packard 5830A instrument, fitted with a flame-ionisation detector, was used. Separations were performed on an Ultra 2 (cross-linked 5% phenyl methyl silicone) fused silica, capillary column, using a

temperature programme (150° for 2 min, 150→220° at 2°/min, 220° for 20 min). G.l.c.–m.s. was performed on a Hewlett–Packard 5790–5970 system, using the same column. A differential refractometer was used for monitoring the column effluents in gel chromatography. Methylation analyses were performed as previously described[7]. Methylated products were purified[8] on Sep-Pak C_{18}-cartridges.

N.m.r. spectra were determined on solutions in D_2O, using a JEOL GX-270 instrument. The ^{13}C-n.m.r. spectra of oligosaccharides were recorded at 30° and all other spectra at 70°. Chemical shifts are reported in p.p.m. relative to the signals for internal 1,4-dioxane (δ 67.40) for ^{13}C and internal acetone (δ 2.21) for ^{1}H. Chemical shifts for the resonances of anomeric protons and carbons in the oligosaccharides and the polysaccharides are given in Tables III and IV. Most of the resonances for anomeric protons in the oligosaccharides were readily assigned and those of the anomeric carbons were assigned from the $^{13}C,^{1}H$ correlation spectra. The $J_{C\text{-}1,H\text{-}1}$ values were in agreement with the assigned anomeric configurations.

F.a.b.-m.s. was carried out on a VG analytical ZAB HF mass spectrometer, fitted with a high-field magnet. Samples were loaded in 1–2 μL of aqueous 5% acetic acid (native oligosaccharides) or methanol (acetylated oligosaccharides) onto a drop of glycerol/thioglycerol on the stainless-steel target. The V_{acc} was at 8 kV and the M-Scan ion gun was operated at 8–10 kV, using Xenon as the bombarding gas.

Isolation and purification of the polysaccharide. — The lipopolysaccharide was isolated from *Salmonella thompson* var. berlin (O.6,7) IS 40. Bacteria were grown in TY-medium supplemented with D-glucose. The lipopolysaccharide was extracted from formaldehyde-killed bacteria with phenol–water and treated with 0.15M sodium hydroxide for 15 min at 100°. Isolation of the O-polysaccharide from the lipopolysaccharide and from the partially delipidated lipopolysaccharide which had been treated with the phage enzyme was achieved by hydrolysis with aqueous 2% acetic acid for 1 h at 100° and subsequent work-up. Each polysaccharide, on chromatography on a column (2.6 × 90 cm) of Sephacryl S-400, was eluted immediately after the void volume.

Phage endo-glycosidase-catalysed hydrolysis of alkali-treated lipopolysaccharide. — The above lipopolysaccharide (2.0 g) in ammonium carbonate buffer (pH 7.0, 5mM, 200 mL) and 1×10^{14} plaque-forming units of a clear plaque mutant of phage Ø14 were mixed in a dialysis bag which was immersed in the same buffer (2 L) and kept at 37°. After 70 h, the external buffer was renewed and the dialysis was continued for 70 h. The combined diffusates were freeze-dried to give 1080 mg of a crude oligosaccharide mixture. This material (500 mg) was fractionated on a column (2.6 × 140 cm) of Bio-Gel P-4. Five major components were obtained (Table II). The retentate was recovered by freeze-drying and was delipidated as described above, to give the glucose-enriched polysaccharide (200 mg).

N-*Deacetylation.* — A solution of the polysaccharide (100 mg) in methyl sulfoxide (15 mL) and 10M sodium hydroxide (15 mL) was stirred vigorously in a

sealed tube for 12 h at 120°, then cooled, neutralised with acetic acid, dialysed, and freeze-dried, to give the *N*-deacetylated material (40 mg).

Deamination[9]. — Aqueous 30% acetic acid (3 mL) and aqueous 5% sodium nitrite (3 mL) were added to a solution of the *N*-deacetylated polysaccharide (40 mg) in water (2 mL). The solution was kept for 1 h at room temperature and then freeze-dried. The product was fractionated on a column of Bio-Gel P-2. One major fraction was obtained in the oligosaccharide region. Part of this material was subjected to methylation analysis (Table I, column *C*).

Of the major P-2 fraction, ~75 μg was treated with a solution of sodium cyanoborohydride (2 mg) in acetic acid–methanol–aniline (0.7:6:1, 100 μL) for 1 h at 90°. The solution was cooled, injected onto a column (4.6 × 250 mm) of Spherisorb NH_2, and eluted with a linear gradient of acetonitrile–water (95:5 to 5:95) at 1.5 mL/min. Derivatised oligosaccharides were detected at 254 nm.

ACKNOWLEDGMENTS

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