Carbohydrate Research, 151 (1986) 349–358
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURAL STUDIES OF THE O-SPECIFIC SIDE-CHAINS OF THE *Escherichia coli* O 10 LIPOPOLYSACCHARIDE*

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

CZESLAW LUGOWSKI,
Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław (Poland)

AND STEFAN B. SVENSON
Department of Bacteriology, National Bacteriological Laboratory, S-105 21 Stockholm (Sweden)
(Received November 4th, 1985; accepted for publication, December 5th, 1985)

ABSTRACT

The structure of the O-specific side-chains of the *Escherichia coli* O 10 lipopolysaccharide has been investigated. Methylation analysis, n.m.r. spectroscopy, and various specific degradations were the principal methods used. It is concluded that the polysaccharide is composed of pentasaccharide repeating-units having the following structure, in which D-Fuc4NAcyl is 4-amino-4,6-dideoxy-D-galactose, acylated with acetic acid (60%) or D-3-hydroxybutyric acid (40%).

$$\begin{array}{l}\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 2 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \alpha\text{-D-Fuc}p\text{4NAcyl}\end{array}$$

INTRODUCTION

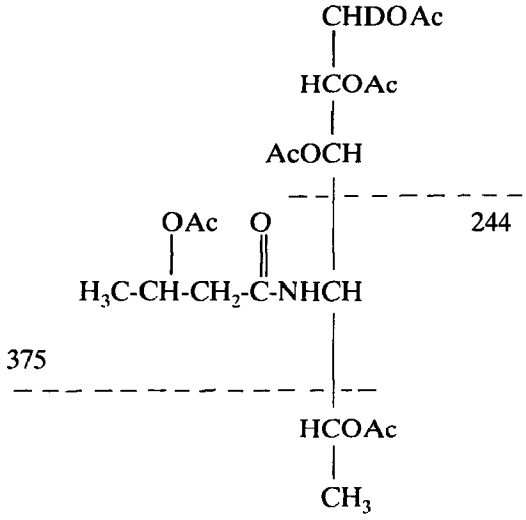
In their studies of sugar components of different bacterial lipopolysaccharides, Ørskov *et al.*[1] found several unusual sugars, almost certainly deriving from the O-specific side-chains of the lipopolysaccharides. One of these sugars, 4-amino-4,6-dideoxy-D-galactose, was a component of the *E. coli* O 10 lipopolysaccharide. Recently, this sugar has also been found as a component of the enterobacterial common antigen[2,3]. We now report structural studies of the O-antigen from *E. coli* O 10.

*Dedicated to Roger W. Jeanloz.

0008-6215/86/$ 03.50

RESULTS AND DISCUSSION

The lipid-free polysaccharide (PS) was prepared from the lipopolysaccharide by hydrolysis with dilute acetic acid[4] and showed $[\alpha]_{578}^{20}$ +84° in water. For sugar analysis, the PS was first treated with anhydrous hydrogen fluoride[5] and then with acid under mild conditions. *N*-Acyl groups are not cleaved during these conditions, and a good yield of the 4-amino-4,6-dideoxy-D-galactose, which was assumed to be *N*-acylated, should be obtained. The sugars were then analysed, as the alditol acetates, by g.l.c.–m.s. In addition to L-rhamnose, 4-acetamido-4,6-dideoxy-D-galactose, D-galactose, and 2-acetamido-2-deoxy-D-glucose, in the approximate proportions 2.1:0.6:0.8:1.0, a fifth component, having higher retention-time than the others, was obtained in the relative proportion 0.4. From the mass spectrum of its alditol (*e.g.* **1**), it was identified as deriving from a 4-amino-4,6-dideoxyhexose, *N*-acylated with a hydroxybutyric acid. The origins of the two major, primary fragments are indicated in the formula.



1

That this acid is not *O*-acetylated in the PS was evident from the ^1H- and ^{13}C-n.m.r. spectra. On hydrolysis of **1** and acetylation, an alditol acetate indistinguishable from that of 4-amino-4,6-dideoxy-D-galactose was obtained, indicating that a part of this sugar, in the PS, is acylated with acetic acid and another part with the hydroxybutyric acid.

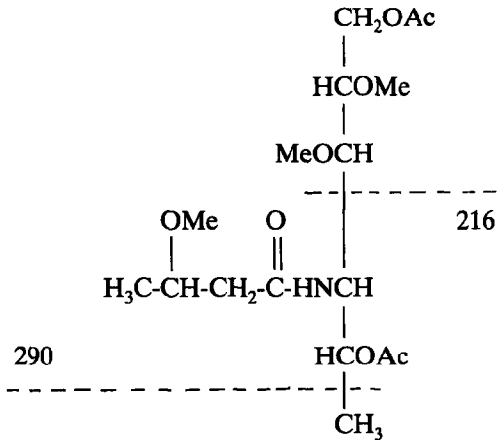
Treatment of the PS with methanolic hydrogen chloride and analysis of the chloroform-soluble part of the product by g.l.c.–m.s. showed the presence of a major component that gave the same mass spectrum as authentic methyl 3-hydroxybutyrate, prepared from the racemic acid. The acid was identified as D-3-hydroxy-

butyric acid (or *R*-3-hydroxybutanoic acid), as it was oxidized by D-3-hydroxybutyrate dehydrogenase[6] (EC 1.1.1.30) in the presence of β-nicotinamide adenine dinucleotide. Amidically linked 3-hydroxybutyric acid, of unknown absolute configuration, has recently been demonstrated in lipopolysaccharides from *Pseudomonas aeruginosa* and *Shigella boydii* strains[7].

From the sugar analysis it therefore seems as if the O-specific side-chains are composed of L-rhamnose, D-galactose, 2-acetamido-2-deoxy-D-glucose, and 4-amino-4,6-dideoxy-D-galactose in the relative proportion 2:1:1:1 and that some 60% of the 4-amino sugar is present as the *N*-acetyl derivative and the remaining 40% as the *N*-D-3-hydroxybutyrate.

Methylation analysis of the PS and analysis of the methylated sugars by g.l.c.–m.s. of their alditol acetates[8] gave the sugars listed in Table I, column A. These results are in agreement with a pentasaccharide repeating-unit in which all sugars are pyranosidic and the acylated 4-amino-4,6-dideoxy-D-galactosyl residue is terminal, one of the L-rhamnosyl residues is linked through the 2- and 3-positions, and the remaining sugars are linked through their 3-positions.

N-Acylamino sugars are generally *N*-methylated during Hakomori methylation, although not completely. The 4-*N*-acetyl- and 4-*N*-(D-3-hydroxybutyryl)-4,6-dideoxy-D-galactopyranosyl groups in the PS were, however, not *N*-methylated, as is evident from the mass spectra of the corresponding alditol acetates, *e.g.*, **2**.



2

The acylated 4-amino-4,6-dideoxy-D-galactosyl group is the only sugar in this repeating unit that should be oxidised by periodate. Methylation analysis of the polymeric product obtained after Smith degradation (Table I, column B) demonstrated that it was linear and all sugars were linked through their 3-positions. The terminal 4-amino-4,6-dideoxy sugar is consequently linked to the 2-position of the branching L-rhamnopyranosyl residue.

TABLE I

METHYLATION ANALYSIS OF ORIGINAL (A), SMITH-DEGRADED (B), AND *N*-DEACETYLATED (C) POLYSACCHARIDE

Methylated sugar[a]	T[b]	*Detector response, %*		
		A	*B*	*C*
2,4-Rha	0.85	26	55	42
4-Rha	1.20	15		
2,4,6-Gal	1.53	21	28	58
2,3-Fuc4NAc	2.49	8		
2,3-Fuc4NAcyl[c]	4.24	8		
2,4,6-GlcNAc	5.09	22	17	

[a]2,4-Rha = 2,4-di-*O*-methyl-L-rhamnose, etc. [b]Retention time of the corresponding alditol acetate, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an SE-54 fused-silica capillary column at 200°. [c]2,3-Fuc4NAcyl = 4,6-dideoxy-2,3-di-*O*-methyl-4-*N*-(D-3-methoxybutyryl)-D-galactose.

Partial solvolysis of the PS with anhydrous hydrogen fluoride and fractionation of the product by gel filtration yielded a disaccharide and a mixture of monosaccharides. The disaccharide, which was composed of 2-acetamido-2-deoxy-D-glucose and L-rhamnose, was reduced to the alditol. The ^{1}H-n.m.r. spectrum of the alditol showed, *inter alia*, a signal for an anomeric proton at δ 4.64, J 7.8 Hz, demonstrating that it was a 2-acetamido-2-deoxy-β-D-glucopyranoside. The ^{1}H- and ^{13}C-n.m.r. spectra of the disaccharide and its alditol further demonstrated that the disaccharide has structure **3**.

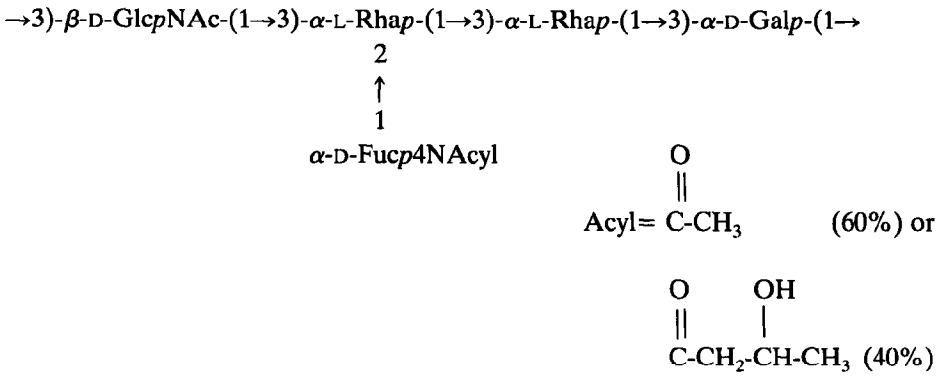
β-D-Glc*p*NAc-(1→3)-L-Rha

3

The ^{13}C- (Table II) and ^{1}H-n.m.r. spectra (Table III) of the PS confirmed that it is composed of pentasaccharide repeating-units. The $J_{C,H}$ values for the five anomeric carbon atoms demonstrate that four are α-linked and one, known to be that of the 2-acetamido-2-deoxy-D-glucopyranosyl group, is β-linked. Some splitting of signals was observed, especially in the ^{1}H-n.m.r. spectrum, and is explained by remote substituent-effects from the two different *N*-acyl substituents on the 4-amino sugar.

The signals, in the ^{13}C-n.m.r. spectrum, given by anomeric and glycosyloxylated carbon atoms, in oligo- and poly-saccharides, depend not only on the parent sugar and its anomeric configuration but also upon the chemical nature of its neighbours, the aglycon and the glycosyloxylating sugar, respectively, and several principles have been established[9,10]. It was therefore possible, as will be discussed next, to deduce the sequence of the sugars in the pentasaccharide repeating-unit (**4**) of the PS from results already discussed and from the ^{13}C-n.m.r.

spectrum of the native PS and the modified PS obtained on Smith degradation (Table II).



4

The α-D-galactopyranosyl residue could either be linked to the 3-position of the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue or to the 3-position of an α-L-rhamnopyranosyl residue. For the former alternative, the chemical shift of its anomeric carbon atom is estimated to be ~100 p.p.m., for the latter to be ~96 p.p.m. No signal is observed in the region of 96 p.p.m. and the former alternative is therefore correct, and the sequence of the sugar residues in the chain is thereby established. The sequence is also supported by the chemical shifts for the α-L-rhamnopyranosyl residues. When linked as in **4**, the chemical shifts for both anomeric carbon atoms are estimated to be ~103 p.p.m., in agreement with the observed values. If, however, one of the α-L-rhamnopyranosyl residues had been

TABLE II

^{13}C-N.M.R. DATA[a] OF NATIVE (A) AND SMITH DEGRADED (B) *E. coli* O 10 POLYSACCHARIDE, AND CALCULATED VALUES FOR THE ANOMERIC CARBON ATOMS (C) OF THE SMITH-DEGRADED POLYSACCHARIDE

Sugar residue	*A*		*B*		*C*
	C-1	$J_{C,H}$	*C-1*	*C-3*	*C-1*
→3)-α-D-Galp-(1→	100.01	172	100.07	77.89	100.0
→3)-β-D-GlcpNAc-(1→	103.34	162	103.06	80.73	103.8
→3)-α-L-Rhap-(1→	102.78	171	102.77	79.14	102.8
→3)-α-L-Rhap-(1→	100.35	170	102.68	80.93	103.1
2 ↑ α-D-Fucp4NAcyl(1→	98.18	168			

[a]Chemical shifts are given relative to internal 1,4-dioxane (δ 67.4).

TABLE III

^{1}H-N.M.R. DATA[a] OF NATIVE (A), SMITH-DEGRADED (B), AND *N*-DEACYLATED *E. coli* O 10 POLYSACCHARIDE

Sugar residue	*A*						*B*			*C*		
	H-1	*H-5*	*H-6*	*NAc*	CH_2	CH_3	*H-1*	*H-6*	*NAc*	*H-1*	*H-5*	*H-6*
→3)-α-D-Gal*p*-(1→	5.440						5.430			5.352		
	(3.4)						(4.2)			(3.8)		
→3)-β-D-Glc*p*NAc-(1→	4.828			2.067			4.811		2.056	4.686		
	(8.1)						(8.1)			(8.2)		
→3)-α-L-Rha*p*-(1→[b]	5.035		1.313				5.039	1.299		5.065		1.331
↓	(1.0)		(6.0)				(1.1)	(6.1)		(1.2)		(6.2)
2												
→3)-α-L-Rha*p*-(1→[b]	5.111		1.298				5.057	1.298		5.143		1.305
	(1.3)		(6.0)				(1.3)	(6.1)		(1.3)		(6.2)
α-D-Fuc*p*4NAc-(1→[c]	4.974	4.610	1.166	2.103						5.013	4.634	1.302
	(3.5)	(1.1; 6.0)	(6.0)							(3.5)	(1.7; 6.9)	(6.7)
α-D-Fuc*p*4N(D-3-OH-Butyryl)-(1→	4.974	4.624	1.181		2.527	1.263						
	(3.5)	(1.1; 6.0)				(6.2)						

[a]Chemical shifts are given relative to internal acetone (δ 2.225). Coupling constants are in parentheses. [b]The assignments of the signals given by the two rhamnopyranosyl residues could be reversed. [c]α-D-Fuc*p*4N(1→ in column C.

linked to the 3-position of the 2-acetamido-2-deoxy-β-D-glucopyranosyl residues, a shift of ~102 p.p.m. would be expected.

In the branching L-rhamnopyranosyl residue, the signals for C-1 and C-3 are shifted upfield because of the β-effect caused by the 2-substituent. By comparison of the spectra for the native PS and the Smith-degraded PS, these signals are therefore readily assigned. They appear at δ 100.35 and 78.44 in the native PS and at 102.68 and 80.93 in the modified PS. The estimated shifts given by C-3 of an α-L-rhamnopyranosyl residue linked through O-3 by a 2-acetamido-2-deoxy-β-D-glucopyranosyl residue or by another α-L-rhamnopyranosyl residue are ~81 and 79 p.p.m., respectively. The 2-acetamido-2-deoxy-β-D-glucopyranosyl residue is consequently linked to O-3 of the branching α-L-rhamnopyranosyl residue, as in **4**.

Also in other details, the ^{1}H- and ^{13}C-n.m.r. spectra were in agreement with the postulated structure. The assignments of the different signals were made by comparisons with oligosaccharide spectra and by use of the same type of principles as already discussed. Integration of pertinent peaks in the ^{1}H-n.m.r. spectrum confirmed that ~40% of the 4-amino sugar groups are *N*-acylated with D-3-hydroxybutyric acid. Structural variation in the repeating unit of a bacterial polysaccharide is not very common. It has not been decided, however, if *N*-acetyl and *N*-D-hydroxybutyryl groups occur together, in the same molecule, or if some polysaccharide chains contain only the former and other chains only the latter groups.

The sequence of the sugars in the repeating unit was also determined by chemical methods. The Smith-degraded PS was *N*-deacylated, deaminated by treatment with nitrous acid, and the product reduced with sodium borohydride. Methylation analysis of the tetrasaccharide alditol yielded 2,5-anhydro-1,4,6-tri-*O*-methyl-D-mannitol, 2,3,4-tri-*O*-methyl-L-rhamnose, 2,4-di-*O*-methyl-L-rhamnose, and 2,4,6-tri-*O*-methyl-D-galactose. The only component in this tetrasaccharide alditol that is oxidised by periodate is the terminal L-rhamnopyranosyl group, and Smith degradation yielded the trisaccharide alditol **5**, as evident on methylation analysis.

α-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→3)-2,5-anhydro-D-mannitol

5

These results therefore confirm the sequence of the four chain-sugars in **4**.

Methylation analysis of the *N*-deacylated PS gave the sugars listed in Table I, column C. As the glycosidic linkage of the methylated 2-amino-2-deoxy-D-glucopyranosyl residue should be resistant to acid hydrolysis, the sugar to which it is linked should not appear as a monomer in this analysis. The disappearance of 4-*O*-methyl-L-rhamnose therefore demonstrates that the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue is actually linked to the branching α-L-rhamnopyranosyl residue, as in **4**.

EXPERIMENTAL

General methods. — Concentration of solutions was performed under diminished pressure at bath temperatures below 40°. The n.m.r. spectra were recorded as solutions in D_2O at 85° (^1H) or 70° (^{13}C) with a JEOL GX-400 instrument, using internal sodium 4,4-dimethyl-4-silapentanoate-*2,2',3,3'-d*$_4$ (^1H) and 1,4-dioxane (^{13}C; δ 67.4) as references. For g.l.c., a Hewlett–Packard 5830A instrument fitted with a flame-ionisation detector was used. Separation of alditol acetates and partially methylated alditol acetates was performed on SP-1000 or SE-54 fused-silica capillary columns, either isothermally or by using a temperature gradient of 150°→220° at 2°/min. G.l.c.–m.s. was performed on a Hewlett–Packard 5970 instrument, using the same conditions as just specified. Identifications of mass spectra were unambiguous and are only discussed for the 4-amino-4,6-dideoxy-D-galactose derivative.

Isolation and purification of the polysaccharide. — The lipopolysaccharide was isolated from *E. coli* O 10:K5:H4 by extraction with phenol–water as described[4]. Isolation of the O-polysaccharide from the lipopolysaccharide was achieved by mild hydrolysis with acid (HOAc 1%, 100°, 1 h) and subsequent work-up[4]. The polysaccharide showed $[\alpha]_{578}^{20}$ +84° (*c* 0.5, water).

Sugar analysis. — The sample (~3 mg) was treated with anhydrous hydrogen fluoride (~2.5 mL) for 3 h at room temperature. The hydrogen fluoride was pumped off at room temperature, the residue dissolved in 0.5M trifluoroacetic acid (2 mL), and the solution kept overnight at room temperature and then evaporated. The product was successively dissolved in water, freeze-dried, reduced with sodium borodeuteride, and acetylated, using standard procedures.

Methylation analysis. — The PS was methylated and the methylated product successively recovered, solvolysed, reduced, acetylated, and analysed as previously described[2].

Identification of D-*3-hydroxybutyric acid.* — The PS (10 mg), suspended in M methanolic hydrogen chloride (2.5 mL), was kept in a sealed tube at 80° with magnetic stirring for 16 h. The solution was made neutral with silver carbonate and the cooled mixture was centrifuged. The supernatant solution was concentrated by flushing with nitrogen at 10° and analysed by g.l.c.–m.s. The main component had the same retention time on SP-1000 and SE-30 capillary columns (80°) as authentic methyl 3-hydroxybutyrate and the mass spectra were superposable.

The PS (1 mg), lyophilized but not further dried, was hydrolysed with 4M hydrochloric acid for 2.5 h at 100° and the hydrolysate analysed for D-3-hydroxybutyric acid by using D-3-hydroxybutyrate dehydrogenase[6] (EC 1.1.1.30, type IV, Sigma). The amount of D-3-hydroxybutyric acid found was 0.32 mg, compared to the value of 0.44 mg, calculated under the assumption that the PS consisted of pure, dry O-antigen and that 40% of the pentasaccharide repeating-units contained an *N*-(D-3-hydroxybutyryl) group.

Smith degradation. — A solution of the PS (25 mg) in a mixture of 0.1M

sodium metaperiodate (5 mL) and 0.2M sodium acetate buffer of pH 3.9 (5 mL) was kept in the dark for 48 h at 4°. The excess of periodate was reduced with ethylene glycol, the solution concentrated to 4 mL, and fractionated on a column (50 × 2.6 cm) of Sephadex G-25. The fractions containing polymeric material were collected and freeze-dried, dissolved in water (2 mL), and treated with sodium borohydride (50 mg) overnight at room temperature. The excess of borohydride was decomposed by addition of acetic acid and the product recovered as before. This material (20 mg) was dissolved in 0.5M trifluoroacetic acid (5 mL) and kept for 40 h at room temperature. The polymeric material was recovered by gel filtration on a Bio-Gel P2 column, followed by freeze-drying. The product showed $[\alpha]_{578}^{20}$ +33° (*c* 0.5, water).

Partial solvolysis. — The PS (30 mg) was dissolved in anhydrous hydrogen fluoride and kept for 5 min at −20°. The solution was then poured into dichloromethane containing calcium carbonate and Dry Ice. The solvent was evaporated, the sugars extracted with water, and the solution freeze-dried. Fractionation on a column (100 × 1.6 cm) of Bio-Gel P2, equilibrated and irrigated with 0.01M pyridine–acetic acid buffer of pH 5.2, yielded the disaccharide **3** and a mixture of monosaccharides. Part of the disaccharide was reduced with sodium borohydride to the alditol.

N-*Deacylation*[11]. — The Smith-degraded PS (30 mg) was dissolved in anhydrous hydrazine (2 mL) containing hydrazine sulphate (100 mg) and kept at 105° in a sealed tube for 7 days. Hydrazine was removed by evaporation and the product (21 mg) recovered by gel filtration on a Sephadex G-50 column and freeze-drying.

N-Deacylation of the original PS was performed analogously.

Deamination. — The Smith-degraded, *N*-deacetylated PS (21 mg) was dissolved in water (1.5 mL) and solutions of 5% aqueous sodium nitrite (2.5 mL) and 33% aqueous acetic acid (2.5 mL) were added. The solution was kept for 45 min at room temperature, then treated with Dowex 50 (H^+, 4 mL) and freeze-dried. The product, and sodium borohydride (50 mg), were dissolved in water (2 mL). After 2 h at room temperature, the product was treated with Dowex 50 (H^+) and the solution concentrated. Boric acid was removed by distillation of methanol (3 × 3 mL) from the residue, which was then dissolved in water (2 mL) and fractionated on a column (400 mesh, 100 cm × 1.6) of Bio-Gel P2. The main component (7 mg) was a tetrasaccharide alditol, according to methylation analysis and n.m.r. spectra.

Smith degradation of the tetrasaccharide alditol. — The tetrasaccharide alditol (5 mg) was dissolved in 0.05M sodium metaperiodate and the solution kept for 60 h at 4°. Conventional work-up, including reduction with sodium borohydride, partial acid hydrolysis, and fractionation on a column of Bio-Gel P2, yielded the trisaccharide alditol **5**, which was characterised by methylation analysis.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (03X-02522), the National Swedish Board for Technical Development, and Stiftelsen Sigurd and Elsa Goljes Minne.

REFERENCES

1 F. ØRSKOV, I. ØRSKOV, B. JANN, K. JANN, E. MÜLLER-SEITZ, AND O. WESTPHAL, *Arch. Pathol. Microbiol. Scand.*, 71 (1967) 339–358.
2 C. LUGOWSKI, E. ROMANOWSKA, L. KENNE, AND B. LINDBERG, *Carbohydr. Res.*, 118 (1983) 171–181.
3 A. DELL, J. OATES, C. LUGOWSKI, E. ROMANOWSKA, L. KENNE, AND B. LINDBERG, *Carbohydr. Res.*, 133 (1984) 95–104.
4 S. B. SVENSON AND A. A. LINDBERG, *J. Immunol.*, 120 (1978) 1750–1757.
5 A. J. MORT AND D. T. A. LAMPORT, *Anal. Biochem.*, 82 (1977) 289–309.
6 D. H. WILLIAMSON AND J. MELLANBY, in H. U. BERGENMEYER (Ed.), *Methoden der Enzymatischen Analyse*, Verlag Chemie, Weinheim, 1974, pp. 1883–1886.
7 Y. A. KNIREL, E. V. VINOGRADOV, V. L. L'VOV, N. A. KOCHAROVA, A. S. SHASKOV, B. A. DIMITRIEV, AND N. K. KOCHETKOV, *Carbohydr. Res.*, 133 (1984) c5–c8.
8 P.-E. JANSSON, L. KENNE, B. LINDBERG, H. LIEDGREN, AND J. LÖNNGREN, *Chem. Commun., Univ. Stockholm*, (1976) 1–75.
9 N. K. KOCHETKOV, O. S. CHIZOV, AND A. S. SHASKOV, *Carbohydr. Res.*, 133 (1984) 173–185.
10 H. BAUMANN, P.-E. JANSSON, AND L. KENNE, in preparation.
11 M. A. SCHMIDT, B. JANN, AND K. JANN, *Eur. J. Biochem.*, 137 (1983) 163–171.