Carbohydrate Research, 163 (1987) 81-90
Elsevier Science Publishers B.V., Amsterdam - Printed in The Netherlands

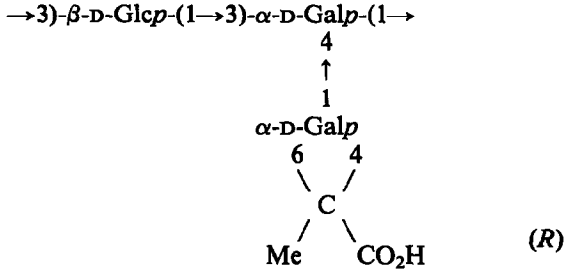
STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE FROM *Escherichia coli* O9:K37 (A 84a)

A. NIXON ANDERSON, HARALAMBOS PAROLIS, AND LESLEY A. S. PAROLIS
School of Pharmaceutical Sciences, Rhodes University, Grahamstown, 6140 (Republic of South Africa)

(Received September 2nd, 1986; accepted for publication, December 5th, 1986)

ABSTRACT

The structure of the capsular polysaccharide from *E. coli* O9:K37 (A 84a) has been studied, using methylation analysis, Smith degradation, and graded acid hydrolysis. The configurations at the anomeric centres were assigned by ^{1}H-n.m.r. spectroscopy of the polysaccharide and its derivatives and oligosaccharide fragments. The polysaccharide has the following trisaccharide repeating-unit which is unique in the *E. coli* series of capsular polysaccharides in possessing a 1-carboxyethylidene group as the sole acidic function.



INTRODUCTION

E. coli capsular polysaccharides have been classified[1] into seventy-four serotypes. The structures of about twenty of these polysaccharides have been elucidated, one of which, K29, has been reported[2] to contain a 1-carboxyethylidene group. In continuation of a programme aimed at establishing the structural basis for the serology and immunochemistry of the *E. coli* capsular antigens, we now report on the structure of the capsular polysaccharide from *E. coli* O9:K37.

RESULTS AND DISCUSSION

Composition and n.m.r. spectra. — The bacteria were grown on an agar medium, and the acidic polysaccharide was isolated and purified by precipitation

0008-6215/87/$03.50

with cetyltrimethylammonium bromide (CTAB). The polysaccharide had $[\alpha]_D$ + 105° (water), which indicates[3] a preponderance of α linkages.

Gel-permeation chromatography (Fig. 1A) showed the native capsular extract to be polydisperse (three peaks having M_r = 6.3 × 10^6, 4.6 × 10^6, and 2.0 × 10^6); however, after treatment with a cation-exchange resin, a product giving a single broad peak (M_r = 12,000) was obtained (Fig. 1B). Analysis for phosphate, using the Molybdenum Blue method[4] after hydrolysis of the polysaccharide with 5M sulfuric acid, revealed 1 mol of phosphate per 9 mol of trisaccharide repeating-unit (see below), suggesting that short polysaccharide chains are substituted by phospholipid. This inference accords with reports[5] that the polysaccharides of *E. coli* K2, K5, K12, K62, and K82 are linked *via* their reducing ends to 1,2-dipalmitoylglycerol by labile phosphodiester bridges. The high molecular weights of the native K37 polysaccharide, as determined by gel filtration, can be ascribed to large micellar aggregates which are broken down to low-molecular-weight polysaccharide chains on treatment with the acidic resin. The native K37 polysaccharide was also treated with dilute alkali[1], but this failed to produce the same kind of deaggregation as reported for other *E. coli* polysaccharides[6-9] (Fig. 1C).

G.l.c. analysis of the acetylated aldononitriles derived from the products in an acid hydrolysate of the polysaccharide, with and without prior reduction of the acidic function, indicated that it is composed of glucose and galactose in the molar ratio 1.0:2.3 (Table I, column I). ^{1}H-N.m.r. spectroscopy (Table II) of a partially autohydrolysed sample of the polysaccharide indicated, *inter alia*, the presence of three anomeric protons corresponding to two α linkages (δ 5.09 and 5.31) and one

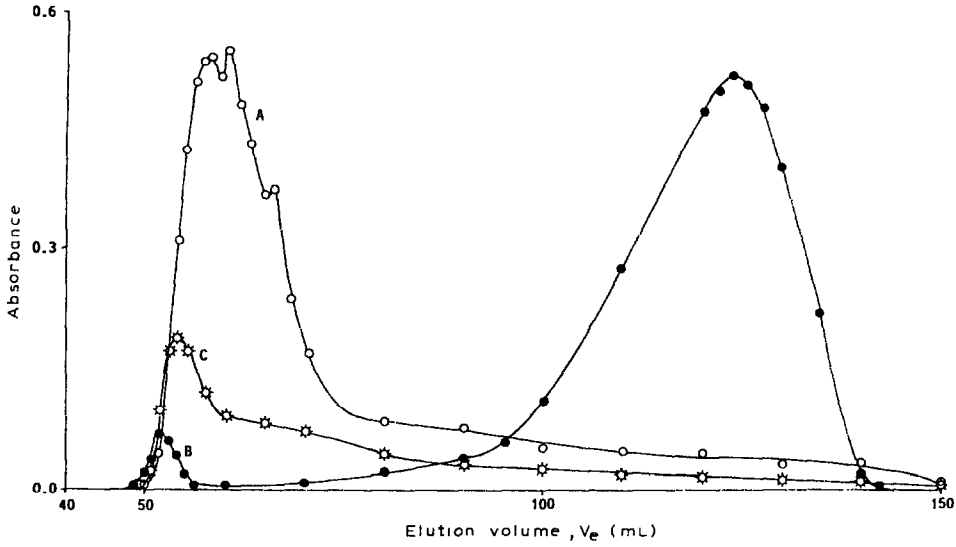


Fig. 1. Gel-permeation chromatography on Sepharose 4B: A, K37 polysaccharide (O); B, K37 polysaccharide after treatment with Amberlite IR-120 (H^+) resin (●); C, alkali-treated (8 h) K37 polysaccharide (☼).

β linkage (δ 4.72, $J_{1,2}$ 7 Hz). A single 1-carboxyethylidene (pyruvate) group was present per trisaccharide repeating-unit. The ^{13}C-n.m.r. data for the polysaccharide substantiated these results (Table II)[10]. The ^{13}C signals at 25.83 and 174.10 p.p.m. were attributed to the equatorial methyl and axial carboxyl groups, respectively, of the 1-carboxyethylidene unit[11]. The assignment of the signals of the anomeric protons in the ^{1}H-n.m.r. spectrum of the native polymer was accomplished on the basis of the spectra of derived poly- and oligo-saccharides (see below). The above results suggest that the K37 polysaccharide consists of a trisaccharide repeating-unit with a 1-carboxyethylidene group as the sole acidic function.

TABLE I

SUGAR ANALYSIS OF *E. coli* K37 POLYSACCHARIDE AND DERIVED PRODUCTS

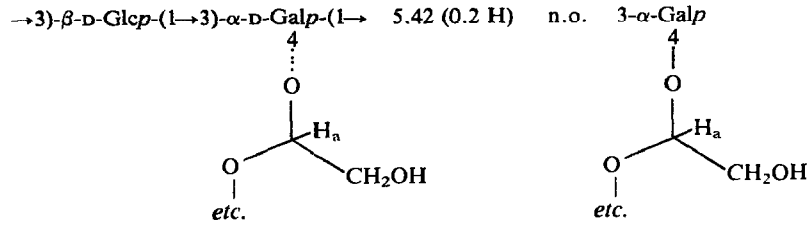
Sugar (as peracetylated aldononitrile)	*Molar ratio*[a,b]				
	I	*II*	*III*	*IV*	*V*
Glucose	1.00	1.00	1.00	—	1.00
Galactose	2.31	1.60	1.17	1.00	—
Galactitol[c]	—	—	1.37	—	1.32

[a]Determined on a capillary DB-225 column at 225°. [b]I, Native K37 polysaccharide; II, Smith-degraded polysaccharide (SD); III, *A*-alditol; IV, monosaccharide *B*; V, *C*-alditol. [c]Galactitol hexa-acetate.

TABLE II

N.M.R. DATA (500 MHZ) FOR *E. coli* K37 POLYSACCHARIDE AND DERIVED PRODUCTS

Compound[a]	^1H			^{13}C	
	δ[b] (*p.p.m.*)	$J_{1,2}$ (*Hz*)	*Assignment*[c]	*δ* (*p.p.m.*)	*Assignment*
→3)-β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→ 4 ↑ 1 α-D-Gal*p* 6 4 \ / pyr (*R*)	5.31 (1 H)	4	3,4-α-Gal*p*	174.10	CO_2H of pyr[d]
K37 Polysaccharide	5.09 (1 H)	n.o.[e]	α-Gal*p* 6 4 \ /	105.20	3-β-Glc*p*
			pyr	100.67	α-Gal*p* 6 4 \ / pyr
	4.72 (1 H)	7	3-β-Glc*p*		
	1.50 (3 H)		Me of pyr	99.77	3,4-α-Gal*p*
				25.83	Me of pyr



(SD)

	5.38 (0.8 H)	n.o.	3-α-Gal*p*		
	4.80 (t, 0.2 H)		H_a		
	4.74 (1 H)	7	3-β-Glc*p*		
β-Glc*p*-(1→3)-Gal*p* 4 ↑ 1 α-Gal*p* 6 4 \ / pyr (*R*)	5.33 (~0.4 H)	n.o.	3,4-α-Gal*p*	176.90	CO_2H of pyr
				105.21	β-Glc*p*
				100.74	α-Gal*p* 6 4 \ / pyr
(*A*)	5.06 (~0.4 H)	4	3-α-Gal*p* 4 ↑ 1 α-Gal*p* 6 4 \ / pyr	97.11	3,4-β-Gal*p*
	5.05 (~0.6 H)	4	3-β-Gal*p*- 4 ↑ 1 α-Gal*p* 6 4 \ / pyr	93.07	3,4-α-Gal*p*
				26.02	Me of pyr
	4.68 (~0.6 H)	8	β-Glc-(1→3)-β-Gal*p*		
	4.66 (~0.4 H)	8	β-Glc-(1→3)-α-Gal*p*		
	4.65 (~0.6 H)	8	3,4-β-Gal*p*		
	1.48 (3 H)		Me of pyr		
A-alditol	5.22 (1 H)	~3	α-Gal*p* 6 4 \ / pyr	175.10	CO_2H of pyr
				104.68	β-Glc*p*
	4.54 (1 H)	8	β-Glc*p*		
	1.56 (3 H)		Me of pyr	101.33	α-Gal*p* 6 4 \ / pyr
				25.83	Me of pyr

Gal*p* 6 4 \ / pyr	5.32 (0.4 H)	4	α-Gal*p* 6 4 \ / pyr
(*B*)	4.62 (0.6 H)	8	α-Gal*p* 6 4 \ / pyr
	1.48 (1.8 H)		Me of pyr (β)
	1.46 (1.2 H)		Me of pyr (α)
β-Glc*p*-(1→3)-Gal (*C*)	5.30 (0.3 H)	n.o.	3-α-Gal*p*
	4.70 (0.3 H)	8	β-Glc*p*-(1→3)-α-Gal*p*
	4.69 (0.7 H)	8	β-Glc*p*-(1→3)-β-Gal*p*
	4.63 (0.7 H)	8	3-β-Gal*p*

[a]For origin of compounds, see text. [b]Chemical shift relative to that of sodium 4,4-dimethyl-4-silapentane-1-sulphonate (DSS). [c]3-β-Glc*p* connotes the anomeric proton of a 3-linked β-glucopyranosyl residue, *etc.* The absence of a numerical prefix indicates a (terminal) non-reducing group. [d]1-Carboxyethylidene acetal (pyruvate). [e]Not observed.

Methylation analysis. — The native polysaccharide was methylated by the Hakomori method[12], as modified by Sandford and Conrad[13], and the derived alditol acetates were analysed by g.l.c.–m.s. (Table III, column I). The results confirm the concept of a trisaccharide repeating-unit, establish the presence of a branch point, and locate the 1-carboxyethylidene group on one of the galactose residues.

Periodate oxidation and Smith degradation. — The native K37 polysaccharide consumed 0.97 mol of periodate per trisaccharide repeating-unit, thus supporting the results of the methylation analysis. Smith degradation[14] of the polysaccharide yielded a polymeric product (SD), analysis of which revealed that a side-chain galac-

TABLE III

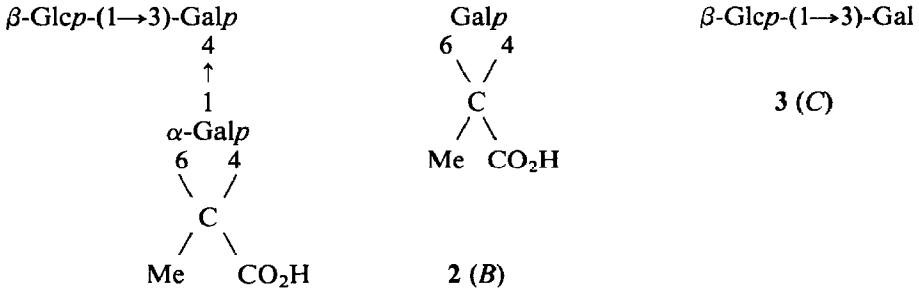
METHYLATION ANALYSES OF *E. coli* K37 POLYSACCHARIDE AND DERIVED PRODUCTS

Methylated sugar[a] *(as alditol acetate)*	*Molar ratio*[b,c]				
	I	*II*	*III*	*IV*	*V*
1,2,4,5,6-Gal	-	-	-	-	0.66
1,2,3,5-Gal	-	-	-	1.00	-
2,3,4,6-Glc	-	-	1.00	-	1.00
1,2,5,6-Gal	-	-	0.87	-	-
2,4,6-Glc	1.00	1.00	-	-	-
2,4,6-Gal	-	0.45	-	-	-
2,3-Gal	1.17	-	0.74	-	-
2,6-Gal	1.08	0.57	-	-	-

[a]1,2,4,5,6-Gal = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol, *etc.*; all substitution patterns were confirmed by g.l.c.–m.s. [b]Determined on a DB-225 capillary column at 205°. [c]I, Native K37 polysaccharide; II, Smith-degraded polysaccharide (SD); III, *A*-alditol; IV, *B*-alditol; V, *C*-alditol.

tose residue had been cleaved from the parent polysaccharide. This conclusion was confirmed by the ^{1}H-n.m.r. spectrum of SD, which showed two major signals for anomeric protons (Table II) and thus allows the α-signal at δ 5.09 in the ^{1}H-n.m.r. spectrum of the autohydrolysed polysaccharide to be assigned to the side-chain galactose. The appearance of 2,4,6-tri-*O*-methylgalactose in the hydrolysate of methylated SD (Table III, column II) indicates that the pyruvalated side-chain galactose unit was linked to position 4 of an in-chain galactosyl residue. The signal at δ 5.42 (0.2 H) in the ^{1}H-n.m.r. spectrum of SD can be assigned to the α-galactose to which is attached an uncleaved acetal arising from incomplete Smith hydrolysis of the pyruvalated side-chain fragment. This result can be ascribed to stabilisation of the acyclic acetal bond by the carboxylic acid group of the pyruvate. The proton of this acetal linkage gave a triplet at δ 4.80 which integrated for 0.2 H. These results are consistent with the results of the methylation analysis.

Partial acid hydrolysis. — Partial acid hydrolysis of the native polysaccharide gave (p.c.) four fractions (*A*–*D*). Fractions *A*–*C* were characterised by methylation analysis, acid hydrolysis, and n.m.r. spectroscopy. *A* and *C* were reduced to the alditols, and g.l.c analysis of *A*-alditol, *B*, and *C*-alditol, after acid hydrolysis, gave the constituent sugars listed in Table I, columns III–V. The results indicated galactose to be the reducing sugar in *A* and *C*, *A* to be a trisaccharide, *C* to be a disaccharide, and *B* to contain only galactose. ^{1}H-N.m.r. spectroscopy of *A*–*C* showed *A* to be a trisaccharide corresponding to the repeating unit of the polysaccharide, *B* to be a pyruvalated galactose, and *C* to be a disaccharide. *A* and *C* were reduced with $NaBD_4$ and *B* was reduced with $NaBH_4$. Each alditol was then methylated and hydrolysed. The results of the g.l.c. analyses (as alditol acetates) are shown in Table III, columns III–V, and are consistent with structures **1**–**3** for *A*–*C*.



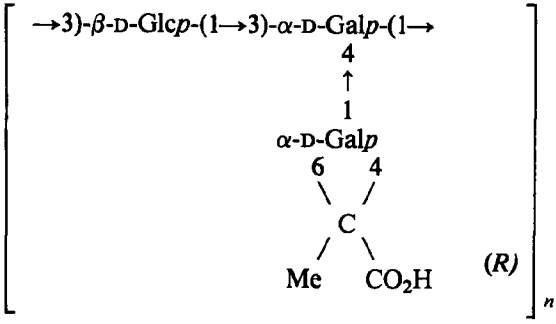
1 (*A*) **2** (*B*) **3** (*C*)

The 1-carboxyethylidene group has the *R*-configuration[11]. This follows from the values for the ^{13}C resonances of the methyl group in *A* and the polysaccharide. The structural unit *B* is a constituent of many biological polysacharides and has been isolated from acid hydrolysates of agar (from *Gelidium cartilagineum*)[15], carrageenan (from *Petrocelis middendorfii*)[16], and *Corynebacterium insidiosum*

polysaccharide[17].

Fraction *D* of the partial hydrolysate of K37 is a mixture of monosaccharides. These were converted into the acetylated (−)-2-octyl glycosides[18] and were shown by g.l.c. to have the D configuration.

The results described above allow the following structure to be written for the *E. coli* K37 capsular antigen.



This polysaccharide is the first in the *E. coli* series of capsular antigens shown to owe its acidity solely to a 1-carboxyethylidene acetal and the second reported to contain this group as a structural unit, the other being *E. coli* K29 (ref. 2).

EXPERIMENTAL

General methods. — Acid hydrolysates were co-concentrated with water under reduced pressure at $\gtrsim 40°$ (bath). Optical rotations were measured at 21–23° with a Perkin–Elmer model 141 polarimeter for aqueous solutions in a 1-cm cell. I.r. spectra were recorded with a Beckman IR8 spectrophotometer for solutions in dry chloroform. Acetylation of alditols was performed with acetic anhydride–pyridine (1:1) at 100° for 1 h.

Descending p.c. was performed on Whatman No. 1 paper with *A*, 18:3:1:4 ethyl acetate–acetic acid–formic acid–water; *B*, 40:11:9 1-butanol–ethanol–water; *C*, 8:2:1 ethyl acetate–pyridine–water; and *D*, 5:1:5:3 ethyl acetate–acetic acid–pyridine–water; and detection with alkaline silver nitrate[19].

^{1}H- and ^{13}C-n.m.r. spectra (internal acetone; δ 2.23 for ^{1}H and 31.07 p.p.m. for ^{13}C, measured against external sodium 4,4-dimethyl-4-silapentane-1-sulfonate) were recorded with a Bruker WM 500 F.t. spectrometer at 30° and at 95°. Samples were deuterium-exchanged by freeze-drying solutions in D_2O. Gel-filtration chromatography was performed on columns (1.6 × 60 cm) of Sepharose 4B, calibrated with dextrans, and eluted with M NaCl at 15 mL/h. Fractions (1 mL) were analysed by the phenol–sulfuric acid method[20]. Analytical g.l.c. was performed at 205° and 225°, using a Hewlett-Packard 5890A gas chromatograph fitted with flame-ionisation detectors, a 3392A recording integrator, a DB-225 bonded-phase capillary column (30 m × 0.25 mm) having a film thickness of 0.25 μm, and helium

as the carrier gas. G.l.c.–m.s. was conducted with a VG Micromass 16F spectrometer with an ionisation energy of 40 eV and an ion-source temperature of 170°.

Preparation and properties of K37 polysaccharide. — A culture of *E. coli* O9:K37 (A84a), obtained from Dr. I. Ørskov (Copenhagen), was grown on Mueller–Hinton agar (containing 2% of NaCl) for 4 days at 37°. The acidic capsular polysaccharide was isolated and purified by CTAB as described previously[21]. The polysaccharide had $[\alpha]_D$ + 105° (*c* 2.1, water). An aqueous solution of the acid form of the polysaccharide was heated (0.5 h) at 90°, dialysed, and freeze-dried, and the product was examined by ^{1}H- and ^{13}C-n.m.r. spectroscopy (Table II). Gel filtration (Fig. 1A) of the native polysaccharide (3.0 mg) indicated a heterogeneous product (three peaks). However, after passage of the polysaccharide through Amberlite IR-120 (H^+) resin, the polymer showed only one large peak (Fig. 1B). Treatment of the native polysaccharide with 0.25M NaOH at 60° for 1, 2, 4, 8, and 12 h, respectively, caused decreases in molecular mass (Fig. 1C).

Sugar composition. — The polysaccharide (11 mg) was hydrolysed with 2M trifluoroacetic acid overnight at 100° and the acid was then evaporated. P.c. of the hydrolysate (solvents *A–C*) revealed two major products corresponding to galactose and glucose. The products of hydrolysis were converted into the acetylated aldononitriles[22] and analysed by g.l.c. (Table I, column I). Dried polysaccharide (9 mg) was treated with refluxing methanolic 3% hydrogen chloride at 80° for 19 h, the mixture was then neutralised, and the products were reduced with $NaBH_4$ in anhydrous methanol and then hydrolysed with 2M trifluoroacetic acid (100°, 16 h). The products were converted into the acetylated aldononitriles and examined by g.l.c.

Methylation analysis. — K37 polysaccharide (23.8 mg), in the acid form, was methylated once by the Hakomori method[12] and then by the Kuhn method[23]. The product showed no i.r. absorption for hydroxyl but a strong absorption at 1100 cm^{-1} (methoxyl). The methylated polymer was hydrolysed with 2M trifluoroacetic acid at 100° overnight. After evaporation of the acid, the product was reduced with $NaBH_4$ and then acetylated. The partially methylated alditol acetates were analysed by g.l.c. and g.l.c.–m.s. (Table III, column I).

Periodate oxidation and Smith degradation[14]. — A solution of the K37 polysaccharide (26.4 mg) in water (5 mL) was treated with 0.03M sodium metaperiodate (5 mL) at room temperature in the dark. The uptake of periodate was monitored spectrophotometrically[14] (223 nm). After 48 h, 0.97 mol of periodate had been consumed per trisaccharide repeating-unit. The excess of periodate was then reduced with ethylene glycol, and the solution was dialysed against running water (2 days) and then freeze-dried. This product was hydrolysed (0.5M trifluoroacetic acid, 60°, 0.5 h), and the hydrolysate was dialysed and then freeze-dried to give a polymeric product (SD). G.l.c. of the acetylated aldononitriles derived from SD gave the results in Table I, column II. A sample (7.2 mg) of SD was methylated by the Hakomori method followed by a Kuhn methylation. G.l.c.–m.s. of the alditol acetates derived from the products in an acid hydrolysate of methylated SD gave the results in Table III, column II.

Partial hydrolysis. — The polysaccharide (251 mg) was treated with refluxing 0.5M trifluoroacetic acid (50 mL) at 100° for 1.5 h. The acid was evaporated and p.c. (solvent *D*) of the residue yielded *A* (24.1 mg), $[\alpha]_D$ +94° (*c* 8, water); *B* (33.8 mg), $[\alpha]_D$ +13° (*c* 5.5); *C* (40.3 mg), $[\alpha]_D$ +21° (*c* 7); and *D* (28.4 mg). The ^{1}H-n.m.r. data for *A*–*C* are given in Table II.

Samples of *B* (1.2 mg) and *C* (1.2 mg) were hydrolysed (2M trifluoroacetic acid, 100°, 4 h), and the products in the hydrolysates were converted into the acetylated aldononitriles and analysed by g.l.c. (Table I, columns IV and V). A second sample of each, as well as one of *A* (1.9 mg), was reduced with $NaBH_4$ and then hydrolysed and analysed as above. *B*-Alditol ($NaBH_4$ reduced) and *A*- and *C*-alditols ($NaBD_4$ reduced) were methylated (Hakomori) and then analysed, after hydrolysis, by g.l.c.–m.s. (alditol acetates, Table III, columns III–V).

Determination of absolute configuration[18]. — To a solution of fraction *D* (5.7 mg) in (−)-2-octanol (0.5 mL) was added 1 drop of trifluoroacetic acid. The solution was sealed in an ampoule, heated overnight (130°), and then concentrated to dryness under vacuum at 55°. The residue was acetylated and the acetylated octyl glycosides were analysed by g.l.c. (DB-225 column at 230°).

ACKNOWLEDGMENTS

We thank Dr. I. Ørskov (International Escherichia Centre, Copenhagen) for the culture of *E. coli* O9:K37, Professor A. M. Stephen (University of Cape Town) for arranging the mass spectrometry, the C.S.I.R. (Pretoria) for financial support (of H.P.) and for post-doctoral and doctoral bursaries (granted to L.A.S.P. and A.N.A., respectively), and Mr. I. Antonowitz of the National Chemical Research Laboratory for recording the n.m.r. spectra.

REFERENCES

1 I. Ørskov, F. Ørskov, B. Jann, and K. Jann, *Bacteriol. Rev.*, 41 (1977) 677–710.
2 Y.-M. Choy, F. Fehmel, N. Frank, and S. Stirm, *J. Virol.*, 16 (1975) 581–590.
3 C. S. Hudson, *J. Am. Chem. Soc.*, 31 (1909) 66–86.
4 A. I. Vogel, *Textbook of Quantitative Inorganic Analysis*, 4th edn., Longmans, London, 1979, p. 756.
5 M. A. Schmidt and K. Jann, *FEMS Microbiol. Lett.*, 14 (1982) 69–74.
6 D. Hungerer, K. Jann, B. Jann, F. Ørskov, and I. Ørskov, *Eur. J. Biochem.*, 2 (1967) 115–126.
7 K. Jann, B. Jann, F. Ørskov, I. Ørskov, and O. Westphal, *Biochem. Z.*, 342 (1965) 1–22.
8 K. Jann, B. Jann, K. F. Schneider, F. Ørskov, and I. Ørskov, *Eur. J. Biochem.*, 5 (1968) 456–465.
9 L.-B. Nahn, B. Jann, and K. Jann, *Eur. J. Biochem.*, 21 (1971) 226–234.
10 K. Bock and C. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 27–103.
11 P. J. Garegg, P.-E. Jansson, B. Lindberg, F. Lindh, J. Lönngren, I. Kvarnström, and W. Nimmich, *Carbohydr. Res.*, 78 (1980) 127–132.
12 S.-I. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
13 P. A. Sandford and H. E. Conrad, *Biochemistry*, 5 (1966) 1508–1517.
14 I. J. Goldstein, G. W. Hay, B. A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, 5 (1965) 361–370.

15 P. A. J. GORIN AND T. ISHIKAWA, *Can. J. Chem.*, 45 (1967) 521–532.
16 V. L. DINNINO, R. A. BELL, AND E. L. MCCANDLESS, *Biomed. Mass Spectrom.*, 5 (1978) 671–673.
17 P. A. J. GORIN AND J. F. T. SPENCER, *Can. J. Chem.*, 42 (1964) 1230–1232.
18 K. LEONTEIN, B. LINDBERG, AND J. LÖNNGREN, *Carbohydr. Res.*, 62 (1978) 359–362.
19 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London)*, 166 (1950) 444–445.
20 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
21 K. OKUTANI AND G. G. S. DUTTON, *Carbohydr. Res.*, 86 (1980) 259–271.
22 G. D. MCGINNIS, *Carbohydr. Res.*, 108 (1982) 284–292.
23 R. KUHN, H. TRISCHMANN, AND I. LOW, *Angew. Chem.*, 67 (1955) 32.