STRUCTURAL STUDIES OF THE *Klebsiella* O GROUP 9 LIPOPOLYSACCHARIDE

BENGT LINDBERG, JÖRGEN LÖNNGREN,
Institutionen för Organisk Kemi, Stockholms Universitet, S-113 27 Stockholm (Sweden)

AND WOLFGANG NIMMICH
*Institut für Medizinische Mikrobiologie und Epidemiologie der Universität Rostock,
25 Rostock (DDR)*

(Received November 12th, 1971; accepted for publication, December 6th, 1971)

ABSTRACT

The structure of the O-specific side-chains in the *Klebsiella* O group 9 lipopolysaccharide has been investigated. Methylation analysis, alone and in combination with partial hydrolysis by acid and trideuteriomethylation, were the main methods used. Partial hydrolysis of the fully methylated lipopolysaccharide, followed by reduction, trideuteriomethylation, and characterisation of a disaccharide derivative, gave further information. *O*-Acetyl groups were located by methylation analysis of chemically modified lipopolysaccharides.

As a result of these studies, it is concluded that the side chains are composed of pentasaccharide repeating units, and a structure for these units is proposed.

INTRODUCTION

The chemical composition of *Klebsiella* lipopolysaccharides (LPS) belonging to O groups 1–12 has been determined by Nimmich and Korten[1]. Structural studies of the O-10 (ref. 2) and O-5 (ref. 3) LPS indicated that their O-specific side-chains were composed of pentasaccharide repeating units, and structures for these units were proposed. The O-specific side-chains of groups O-1 and O-6 (ref. 4) were alike, however, and contained only (1→3)-linked α-D-galactopyranose residues. In this communication, studies of the O group 9 LPS, which also contains D-galactose as the only sugar in the O-specific side-chains, are reported.

RESULTS

The LPS was isolated as previously described[1] and showed $[\alpha]_D$ +52°. Analysis of a hydrolysate by g.l.c.[5]–m.s.[6] of the alditol acetates, using D-arabinose as an internal standard, showed that the LPS contained 39% of "anhydrohexose" residues. Glucose and galactose were obtained in the proportion 4:96. These results are in good agreement with those of Nimmich and Korten[1], who analysed for galactose with

D-galactose oxidase, thus establishing its D-configuration. Assuming that the main contribution to the optical rotation comes from the sugar residues, several of the D-galactose residues must be α-linked. In the previous analysis[1], small percentages of 2-acetamido-2-deoxy-D-glucose, 3-deoxyoctulosonic acid (KDO), and heptose were also found.

The LPS showed a strong absorption in the i.r. at 1735 cm^{-1}, indicating the presence of *O*-acetyl or other *O*-acyl groups, and methyl acetate was obtained on transesterification with methanolic hydrogen chloride.

The LPS was methylated by the method of Hakomori[7] and then hydrolysed, and the mixture of methylated sugars was analysed, as the alditol acetates, by g.l.c.–m.s.[8]. The results are given in Table I, column *A*.

TABLE I

METHYL ETHERS FROM THE HYDROLYSATE OF THE METHYLATED LPS

Sugars[a]	T[b]	*Mole %*	
		A[c]	*B*[d]
2,3,4,6-G	1.00	1.4	3.3
2,3,5,6-Gal	1.15	2.5	2.5
2,3,4,6-Gal	1.25	19.1	14.9
2,5,6-Gal[e]	2.25	32.9	33.5
2,4,6-Gal[e]	2.28	18.2	18.1
4,6-Gal	3.64	19.6	18.2
Others[f]		6.3	9.5

[a] 2,3,4,6-G = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc. [b] Retention times of the corresponding alditol acetates on the ECNSS-M column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [c] Methylation analysis of the original LPS. [d] Recalculated values, see the text. [e] These components were separated on an OV-225 S.C.O.T. column, on which they showed *T* 1.95 and 2.03, respectively. [f] Most of these are probably non-sugar components.

Methylation analysis gives information on the types of linkages involved but not on the mutual arrangement of the sugar residues. As the LPS contains both pyranosidic and furanosidic D-galactose residues, advantage was taken of the fact that the latter are far more susceptible to acid hydrolysis. The fully methylated polysaccharide was given a mild treatment with 90% formic acid to cleave furanosidic galactose links and was then reduced with lithium aluminium deuteride and remethylated, using trideuteriomethyl iodide. Part of the product was hydrolysed, and the mixture of methylated sugars was analysed, as the alditol acetates, by g.l.c.–m.s. (Table II) Table II shows that the major changes are an increase in the percentage of 2,3,4,6-tetra-*O*-methyl-D-galactose and a decrease in the percentage of 4,6-di-*O*-methyl-D-galactose relative to the values found in the methylation analysis of the original LPS. Analysis of the m.s. showed the positions and proportions of trideuteriomethylation in the methylated sugars. This will be discussed below.

TABLE II

METHYL ETHERS FROM THE HYDROLYSATE OF THE METHYLATED, PARTIALLY HYDROLYSED, REDUCED, AND TRIDEUTERIOMETHYLATED LPS

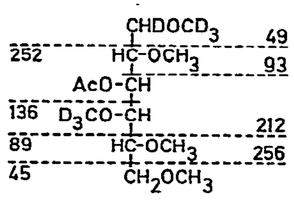
Sugars	T	*Mole %*	*Location of* $-OCD_3$	*Analogue with* $-OCD_3$ (%)
1,2,4,5,6-Gal[a]	0.42	7.8	C-1 and C-4	100
2,3,4,6-G	1.00	3.3		
2,3,5,6-Gal	1.15	3.3	C-3	60
2,3,4,6-Gal	1.25	22.2	C-3	34
2,5,6-Gal	2.25	25.7		
2,4,6-Gal	2.28	16.6	C-2	38
4,6-Gal	3.64	11.6		
Others		9.5		

[a] This component is obviously derived from a D-galactitol residue, the others from sugar residues. For other comments, see Table I.

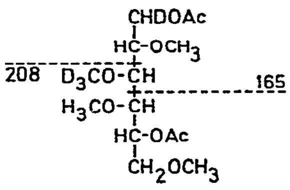
From its m.s., the 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methyl-D-galactitol (**1**) contains a deuterium atom at C-1 and trideuteriomethoxyl groups at C-1 and C-4. This alditol is thus derived from hydrolysed and reduced furanosidic residues, linked in the 3-position. The origins of some of the pertinent ions are indicated below.

Part of the 2,3,4,6-tetra-*O*-methyl-D-galactose (34%) contained a trideuteriomethoxyl group at C-3, as demonstrated by the relative intensities of relevant ions in the m.s., *e.g.*, *m/e* 162–165 and 205–208. The origins of these ions from the alditol acetate (**2**) of the deuterated analogue is indicated below. About one third of this alditol acetate is therefore derived from a 3-linked galactopyranose residue.

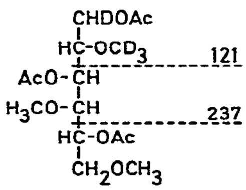
Part of the 2,4,6-tri-*O*-methyl-D-galactose (38%) contained a trideuteriomethoxyl group at C-2, as demonstrated by the relative intensities of pertinent ions in the m.s., *e.g.*, *m/e* 118–121 and 234–237. The origins of these from the alditol acetate (**3**) of the deuterated analogue are indicated below. About one third of this alditol acetate is obviously derived from the cleavage of a galactofuranose linkage to the 2-position of the branching galactopyranose residue.



1



2

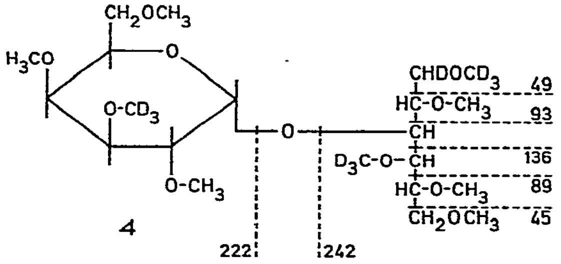


3

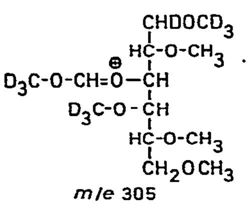
By similar arguments, it was found that 60% of 2,3,5,6-tetra-*O*-methyl-D-galactose contained a trideuteriomethoxyl group at C-3. The other trideuteriomethylated sugars, which are found in much higher yields, probably resulted from cleavage of furanosidic residues, but the labelling in this ether almost certainly results from cleavage of pyranosidic linkages.

Table II contains all the information for reconstruction of the original methylation analysis. Although some volatile 1,2,4,5,6-penta-*O*-methyl-D-galactitol and its acetate were probably lost during concentrations, and the estimates of the percentages of trideuteriomethoxyl groups in the sugars from m.s. are probably not very accurate, the agreement between the original and the reconstructed methylation analyses is reasonably good (Table I, column *B*). In this calculation, it is assumed that the percentage of pyranosidic linkages hydrolysed, as indicated by the deuterium labelling in 2,3,5,6-tetra-*O*-methyl-D-galactose, is equally distributed between the terminal, the 3-linked, and the 2,3-linked D-galactopyranose residues.

Part of the methylated, partially hydrolysed, reduced, and trideuteriomethylated LPS was investigated by g.l.c.–m.s., using an XE-60 column. A single peak was obtained in the disaccharide region, showing T_{MEL} 0.60 (retention time relative to fully methylated melibiitol). G.l.c.–m.s. of permethylated disaccharide alditols has been studied by Kärkkäinen[9], and m.s. of methylated methyl glycosides, containing one or several trideuteriomethoxyl groups in different positions, has been studied by Kochetkov and Chizhov[10,11]. Using their results, the m.s. of the methylated disaccharide alditol **4** was readily interpreted. Ions at *m/e* 45, 49, 89, 93, 136, and 242 show that the alditol part is derived from a hexofuranose residue, substituted at C-3; *cf.* the m.s. of **1**. The ion *m/e* 222 is derived from the D-galactopyranose residue and demonstrates that it contains a trideuteriomethoxyl group. The virtual absence of *m/e* 219 indicates that the amount of the corresponding analogue, non-deuterated in the D-galactopyranose residue, is negligible. The relative intensities of the pairs *m/e* 88–91 (base peak), 101–104, and 187–190, which are derived from the hexose part, are approximately the same as observed for the fully methylated methyl α-D-glucopyranoside or methyl β-D-galactopyranoside, containing a OCD_3 group at C-3[10,11]. The ion at *m/e* 305, finally, is a rearranged fragment and contains the alditol part, C-1, and the methoxyl from C-3 (**5**). It is analogous to the fragment *m/e* 78, obtained from the fully methylated methyl β-D-galactopyranoside, containing a trideuteriomethoxyl group at C-3[11], and also to the fragment *m/e* 295 seen in permethylated disaccharide alditols[9].



4



5

In order to locate the *O*-acetyl groups, the free hydroxyl groups in the LPS were protected as acetals[12] by treatment with methyl vinyl ether in methyl sulphoxide, using toluene-*p*-sulphonic acid as catalyst, and the modified LPS was methylated by the Hakomori procedure[7], which replaces *O*-acyl groups with *O*-methyl groups. Acid hydrolysis of the methylated product yielded a mixture of sugars and methylated sugars, the methyl groups in the latter corresponding to the *O*-acetyl groups in the original LPS. Of the total D-galactose content, 13% was recovered as 2-*O*-methyl-D-galactose and 11% as 6-*O*-methyl-D-galactose.

Further information on the location of the *O*-acetyl groups was obtained by treating the LPS with methyl vinyl ether followed by methylation, using trideuteriomethyl iodide. The modified LPS was then subjected to a mild hydrolysis with acid, which essentially removed only the acetal protecting groups. The product was then methylated with methyl iodide and hydrolysed, and the mixture of methylated sugars was analysed as the alditol acetates, by g.l.c.–m.s. The positions of the trideuteriomethoxyl groups and the relative percentages of trideuteriomethylated analogues were determined from the m.s., as described above. The results are given in Table III. The galactofuranose residues contained deuteriomethoxyl groups at both the 2- and the 6-positions, the galactopyranose residues only at the 6-positions.

TABLE III

METHYL ETHERS OF D-GALACTOSE OBTAINED AFTER ACETALATION, TRIDEUTERIOMETHYLATION, MILD HYDROLYSIS WITH ACID, REMETHYLATION, AND HYDROLYSIS OF THE LPS

Sugars	T	*Mole %*	*Location of* $-OCD_3$	*Analogue with* $-OCD_3$ (%)	
				A[a]	*B*[b]
2,3,5,6-Gal	1.15	1.8	C-2	20	0.4
			C-6	10	0.2
2,3,4,6-Gal	1.25	20.7	C-6	23	5
2,5,6-Gal	2.25	31.0	C-2	30	9
			C-6	10	3
2,4,6-Gal	2.28	17.4	C-6	8	1
4,6-Gal	3.64	19.0	C-6	20	4

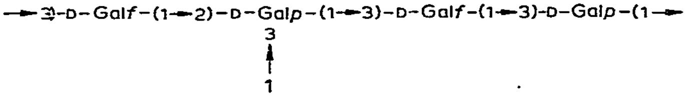
[a] Percentage of the D-galactose derivative in question. [b] Percentage of the total D-galactose content. For other comments, see Table I.

From this experiment, the percentages of *O*-acetyl groups at C-2 and C-6 in the D-galactose residues could be estimated as approximately 9 and 13%, respectively, in good agreement with the direct determination.

DISCUSSION

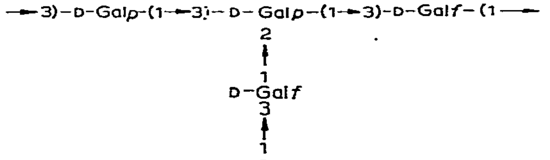
The O-specific side-chains in LPS are generally composed of oligosaccharide repeating units. From the result of the methylation analysis (Table I), the smallest possible repeating unit for the present LPS contains five D-galactose residues. Two of these are furanosidic and linked in the 3-position. The three others are pyranosidic and terminal, pyranosidic and linked in the 3-position, and pyranosidic and linked in the 2- and 3-positions. The optical rotation of the LPS indicates that several of the residues are α-D linked. A small amount of 2,3,4,6-tetra-*O*-methyl-D-glucose detected in the methylation analysis is probably derived from a basal core of the LPS and has also been found in studies of other *Klebsiella* LPS[2].

The results of the modified methylation analysis, including methylation, mild hydrolysis with acid, reduction, and trideuteriomethylation (Table II), demonstrate that one of the furanosidic residues is linked to the 3-position of the chain D-galactopyranose residue and the other to the 2-position of the branching D-galactopyranose residue. From these results, three different pentasaccharide repating units (6–8) may be constructed. Each of these, on methylation, mild hydrolysis with acid, reduction, and trideuteriomethylation, would give a fully methylated 3-*O*-D-galactopyranosyl-D-galactitol. That obtained from **6** should be trideuteriomethylated at C-3 in the D-galactose residue, that from **7** should contain an ordinary methoxyl in the same position, whereas both these derivatives would be formed from **8**. Only the isomer expected for structure **6** was obtained, so that **6** is the actual repeating unit. Attempts to split off the terminal D-galactopyranose residue, using α-D-galactosidase from three different sources, were unsuccessful. These results lend further support to structure **6**, in which this residue is linked directly to the main chain.



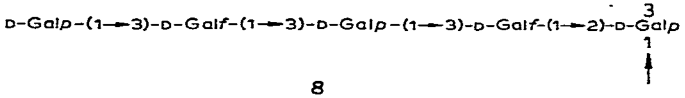
$$\rightarrow 3)\text{-D-Gal}f\text{-}(1\rightarrow 2)\text{-}\underset{\substack{3\\ \uparrow \\ 1 \\ \text{D-Gal}p}}{\text{D-Gal}p}\text{-}(1\rightarrow 3)\text{-D-Gal}f\text{-}(1\rightarrow 3)\text{-D-Gal}p\text{-}(1\rightarrow$$

6



$$\rightarrow 3)\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\underset{\substack{2\\ \uparrow \\ 1 \\ \text{D-Gal}f \\ 3 \\ \uparrow \\ 1 \\ \text{D-Gal}p}}{\text{D-Gal}p}\text{-}(1\rightarrow 3)\text{-D-Gal}f\text{-}(1\rightarrow$$

7

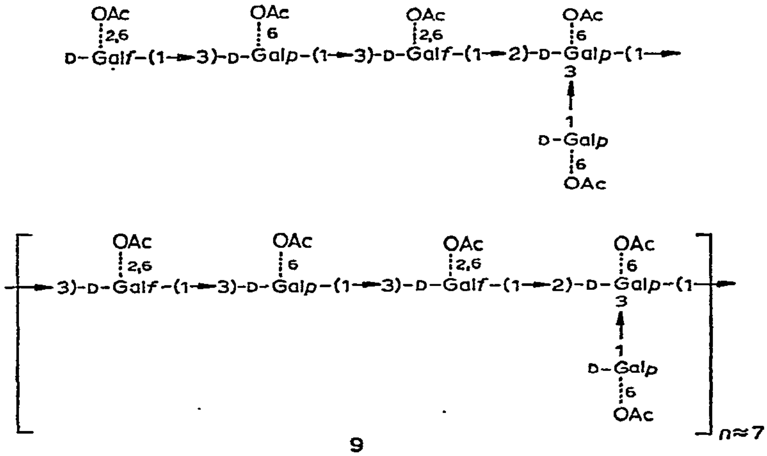


$$\text{D-Gal}p\text{-}(1\rightarrow 3)\text{-D-Gal}f\text{-}(1\rightarrow 3)\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-D-Gal}f\text{-}(1\rightarrow 2)\text{-}\overset{\substack{\uparrow \\ 3}}{\underset{\substack{1 \\ \uparrow}}{\text{D-Gal}p}}$$

8

The LPS contains *O*-acetyl groups linked in two different ways. Those linked to 6-positions are found in all the D-galactose residues, those linked to 2-positions are found only in the galactofuranose residues. It has not been determined whether the latter groups are linked exclusively to one of the two D-galactofuranose residues or are divided between them. As no 2,6-di-*O*-methyl-D-galactose was detected in the direct determination, the proportion of galactofuranose residues containing *O*-acetyl groups in both the 2- and the 6-positions is insignificant.

From these results, the structure **9** is proposed for the O-specific side-chains of *Klebsiella* O group 9 LPS. The low percentage of 2,3,5,6-tetra-*O*-methyl-D-galactose found in the methylation analysis is most probably derived from the terminal sugar of the O-specific side-chains. The "biological" repeating unit should consequently also be terminated by a D-galactofuranose residue. One alternative is structure **6**, the other is that given in structure **9**, which also includes the *O*-acetyl groups. The dotted lines to these indicate that only some of the positions indicated carry such a group. From the percentage of 2,3,5,6-tetra-*O*-methyl-D-galactose, the average number of repeating units in the O-specific side chains is approximately 8.



9

The technique involving methylation of a polysaccharide, mild hydrolysis with acid, reduction, and trideuteriomethylation, followed either by acid hydrolysis and analysis of the sugars by g.l.c.–m.s. or by direct analysis of the methylated oligosaccharide alditols by g.l.c.–m.s., has not previously been used. It is an alternative to ordinary fragmentation analysis, that is graded hydrolysis of the polysaccharide, followed by isolation and identification of oligosaccharides, and is especially valuable when only small amounts of polysaccharide are available. It gives no information on the anomeric nature of sugar residues, but gives, in some respects, more-detailed information than ordinary fragmentation analysis.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at bath temperatures not exceeding 40°. G.l.c. was performed on Perkin–Elmer 900 or 990 instruments fitted with flame-ionisation detectors. Separations were performed on (*a*) glass columns (180×0.15 cm) containing 3% ECNSS-M on Gas Chrom Q (100/120 mesh) at 170° (partially methylated alditol acetates) or 190° (alditol acetates), (*b*) OV-225 S.C.O.T. columns (50 ft×0.020 in.) at 190° (partially methylated alditol acetates), (*c*) glass columns (180×0.15 cm) containing 5% XE-60 on Chromosorb W

(80/100 mesh) at 190° (permethylated disaccharide alditol derivative). For mass spectrometry, a Perkin–Elmer 270 g.l.c.–m.s. instrument fitted with OV-225 S.C.O.T. or XE-60 columns was used. Mass spectra were recorded at an ionisation potential of 70 eV, an ionisation current of 80 μamps, and an ion-source temperature of 80°. Optical rotations were recorded by using a 10-cm micro-cell in a Perkin–Elmer 141 instrument, and i.r. spectra were recorded on a Perkin–Elmer 257 instrument.

The LPS was isolated from strain *Klebsiella* O9:K72 (1205), as previously described[1]. The LPS showed $[\alpha]_D^{20}$ +52° (*c* 0.5, water).

Sugar and methylation analyses. — For sugar analysis, the LPS (3 mg) and D-arabinose (0.5 mg) were treated with 0.25M sulphuric acid at 100° for 14 h. The solution was neutralised with barium carbonate, and the sugars were converted into alditol acetates as previously described[13]. The mixture was then analysed by g.l.c.–m.s. For methylation analysis, a 5-mg sample of LPS was methylated by the method of Hakomori, as previously described[7,14]. Sodium borodeuteride was used for the reduction of sugars to alditols.

Analysis of methylated, partially hydrolysed, and trideuteriomethylated LPS. — The LPS (7 mg) was methylated by Hakomori's procedure[7,14] and recovered by dialysis against tap water overnight, followed by concentration to dryness. The product was then treated with 90% formic acid (3 ml) at 70° for 45 min, concentrated to dryness, suspended in water, and freeze-dried. The product and lithium aluminium deuteride (30 mg) were dissolved in a mixture of dichloromethane (2 ml) and ethyl ether (8 ml) and the solution was refluxed for 4 h. Excess lithium aluminium deuteride was destroyed by adding ethyl acetate and then water, and the solution was neutralised with M phosphoric acid. The precipitate was filtered off, the solution concentrated to dryness, and the residue dried over phosphorous pentoxide. The product was remethylated, using trideuteriomethyl iodide, and the methylated material was recovered by partition between chloroform and water. Part of this product was hydrolysed, and the methylated sugars were converted into alditol acetates and analysed by g.l.c.–m.s. (Table II). The other part was analysed by g.l.c.–m.s. without further treatment. The m.s. of the component having T_{MEL} 0.60 showed, *inter alia*, the following peaks (relative intensities in brackets): 45(72), 49(20), 88(23), 89(34), 91(100), 93(26), 101(80), 104(71), 136(17), 187(37), 190(25), 222(16), 242(32), and 305(8).

Location of O*-acetyl groups in the LPS.* — A strong absorption at 1735 cm^{-1} (KBr) was observed in the i.r. spectrum of the LPS, indicating the presence of *O*-acyl groups. The LPS (2 mg) in 4% methanolic hydrogen chloride (0.2 ml) was heated, in a sealed tube, at 100° for 1 h. G.l.c., on a Carbowax 20 M column at 25°, demonstrated the presence of methyl acetate in the methanolysate.

The LPS (5 mg) was dissolved in methyl sulphoxide (2 ml), methyl vinyl ether (condensed at −35°; 1 ml) and toluene-*p*-sulphonic acid (8 mg) were added, and the mixture was kept at 15° for 3.5 h. The solution was added to the top of a Sephadex LH-20 column (25 x 2 cm) which was irrigated with anhydrous acetone. The eluate was monitored polarimetrically, and the acetalated product was collected and concentrated, yielding a light-yellow syrup (12 mg). This material was methylated as above and hydrolysed, and the methylated sugars were analysed, as their alditol acetates, by g.l.c.–m.s. Another sample of the LPS (5 mg) was acetalated as above and trideuteriomethylated. The recovered material was treated with 50% acetic acid (3 ml) at 100° for 2 h. The solution was then concentrated to dryness, dissolved in water, and freeze-dried. This partially trideuteriomethylated material was then subjected to methylation analysis, using ordinary methyl iodide, and after hydrolyses and conversion into alditol acetates was analysed by g.l.c.–m.s. (Table III).

ACKNOWLEDGMENTS

The skilled technical assistance of Mrs Jana Cederstrand is acknowledged. This work was supported by grants from the Swedish Natural Science Research Council, from the Swedish Medical Research Council (B72-40X-2522-04), from Harald Jeanssons Stiftelse and from Stiftelsen Sigurd och Elsa Goljes Minne.

REFERENCES

1 W. NIMMICH AND G. KORTEN, *Pathol. Microbiol.*, 36 (1970) 179.
2 H. BJÖRNDAL, B. LINDBERG, AND W. NIMMICH, *Acta Chem. Scand.*, 24 (1970) 3414.
3 B. LINDBERG, J. LÖNNGREN, AND W. NIMMICH, *Acta Chem. Scand.*, in the press.
4 H. BJÖRNDAL, B. LINDBERG, AND W. NIMMICH, *Acta Chem. Scand.*, 25 (1971) 750.
5 J. S. SAWARDEKER, J. H. SLONEKER, AND A. R. JEANES, *Anal. Chem.*, 37 (1965) 1602.
6 O. S. CHIZHOV, L. S. GOLOVKINA, AND N. S. WULFSON, *Izv. Akad. Nauk SSSR, Ser. Khim.*, (1966) 1915.
7 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205.
8 H. BJÖRNDAL, C. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem.*, 82 (1970) 643.
9 J. KÄRKKÄINEN, *Carbohyd. Res.*, 14 (1970) 27.
10 N. K. KOCHETKOV AND O. S. CHIZHOV, *Advan. Carbohyd. Chem.*, 21 (1966) 39.
11 N. K. KOCHETKOV AND O. S. CHIZHOV, *Tetrahedron*, 21 (1965) 2029.
12 A. N. DE BELDER AND B. NORRMAN, *Carbohyd. Res.*, 8 (1968) 1.
13 H. BJÖRNDAL, B. LINDBERG, AND S. SVENSSON, *Acta Chem. Scand.*, 21 (1967) 1801.
14 C. G. HELLERQVIST, B. LINDBERG, S. SVENSSON, T. HOLME, AND A. A. LINDBERG, *Carbohyd. Res.*, 8 (1968) 43.