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STRUCTURAL STUDIES OF THE O-SPECIFIC SIDE-CHAINS OF THE *Shigella sonnei* PHASE I LIPOPOLYSACCHARIDE

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ABSTRACT

The structure of the O-specific side-chains of the *Shigella sonnei* phase I lipopolysaccharide has been investigated. The side chains are composed of disaccharide repeating-units containing two uncommon sugar components, one of which, 2-amino-2-deoxy-L-altruronic acid, has been identified previously. The other has now been identified as 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose. The uronic acid, as *N*-acetylated α-pyranosyl residues, is linked through O-4, and the diamino sugar, as β-pyranosyl residues, is linked through O-3. The pyranosyluronic acid residue assumes the $^4C_1$ conformation in the polymer, with the carboxyl group in the axial position.

INTRODUCTION

Shigella sonnei, one of the organisms that cause dysentery, occurs as two variants, phase I and phase II[1]. Phase II is an R-form, and its lipopolysaccharide (LPS) contains the *Enterobacteriaceae* R 1 core, but is devoid of O-specific side-chains. Phase I, however, is an S-form and elaborates a complete LPS. Romanowska and Reinhold showed[2] that one of the sugar components in its O-antigen is a 2-amino-2-deoxyhexuronic acid, and Kontrohr demonstrated[3] that this acid has the L-*altro* configuration. We now report further structural studies of this LPS.

RESULTS AND DISCUSSION

The polysaccharide (PS) was prepared from the LPS by mild hydrolysis with acid, and had $[\alpha]_{578}$ −31° (water). An acid hydrolysate of the PS contained small proportions of the typical core-sugars D-glucose, D-galactose, and L-*glycero*-D-*manno*-heptose.

The ^{13}C-n.m.r. spectrum (Fig. 1) of the PS contained strong signals for 16

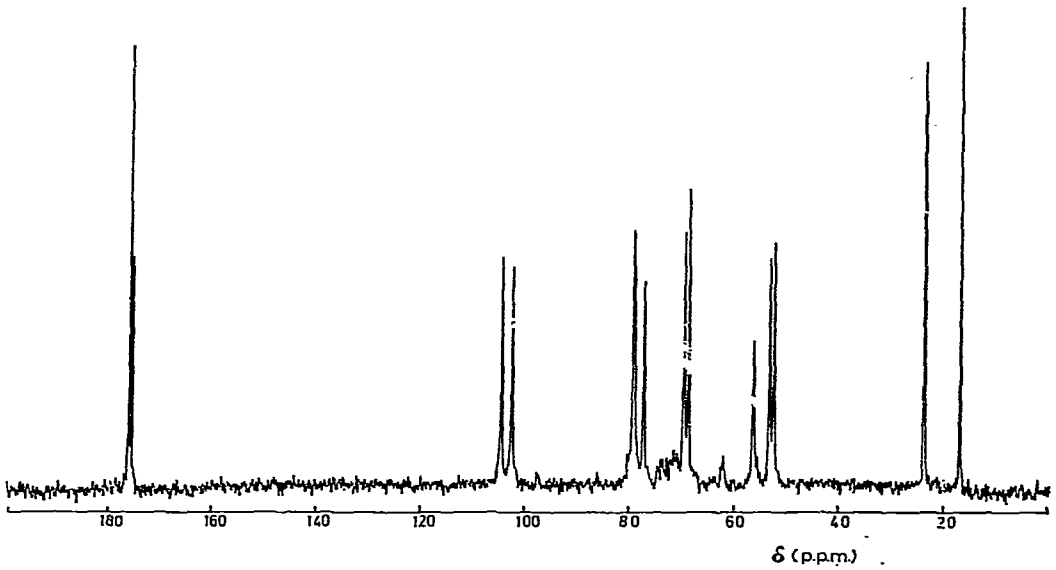


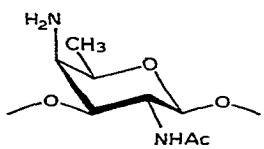
Fig. 1. ^{13}C-N.m.r. spectrum of *Shigella sonnei* PS.

carbon atoms, some of which overlapped, and a number of weak signals, as expected for an O-antigen having a simple, regular structure, linked to a core. In the region for anomeric carbons, two signals (104.6 and 102.6 p.p.m.) were observed, indicating that the O-antigen is composed of disaccharide repeating-units. Three signals in the region for carbonyl carbons, at 176.0 (2 C) and 175.6 p.p.m., and two signals at 24.2 and 24.1 p.p.m. indicate that the repeating unit contains two *N*-acetyl groups and one carbonyl group. A signal at 17.3 p.p.m. may be due to the methyl group of a 6-deoxyhexose. Three signals, at 53.0, 53.7, and 56.8 p.p.m., may be assigned to carbon atoms substituted with amino or acetamido groups. The ^{1}H-n.m.r. spectra of the original and *N*-acetylated PS demonstrated that the original PS contains two *N*-acetyl groups and one amino group per methyl group of the suspected 6-deoxyhexose. From the evidence given below, it is concluded that the amino group is present in a new sugar. If one sugar component of the PS is 2-acetamido-2-deoxy-L-altruronic acid, the other should be a mono-*N*-acetylated diaminotrideoxyhexose having a deoxy group at C-6.

The signal at 56.8 p.p.m. in the ^{13}C-n.m.r. spectrum was shifted to 54.0 p.p.m. on *N*-acetylation. A similar upfield-shift has been observed on acetylation of the axial amino group in 2-amino-2-deoxy-D-mannose, whereas a corresponding shift on *N*-acetylation of 2-amino-2-deoxy-D-glucose is not observed[4]. In *N*-deacylated *Vibrio cholerae* O-antigen[5], the signal for C-4 of the 4-amino-4,6-dideoxy-α-D-mannopyranosyl residue is not shifted on *N*-acetylation. On *N*-acetylation of the *S. sonnei* PS, the signal for the C-6 methyl group in the ^{1}H-n.m.r. spectrum was shifted upfield by 0.18 p.p.m., suggesting that the amino and methyl groups are adjacent.

When the ^{1}H-n.m.r. spectrum of the PS was determined at pD 10, a signal appeared at δ 3.15 (J low); this is assigned to the hydrogen on the carbon atom carrying the amino group and demonstrates the absence of *trans*-diaxial relationships between this and the vicinal protons. The n.m.r. evidence therefore indicates that the free amino group is axial and linked to C-4 of a 6-deoxyhexose.

Attempts to isolate the diaminotrideoxyhexose by acid hydrolysis of the original or *N*-acetylated PS were unsuccessful. Treatment of the *N*-acetylated PS with anhydrous hydrogen fluoride, during which glycosidic linkages should be cleaved but amide linkages should be resistant[6], was also unsuccessful. In order to identify this sugar component, the PS was therefore deaminated with nitrous acid; considerable depolymerisation occurred during the deamination, as shown by gel filtration of the product. The product was subjected to sequential hydrolysis, reduction with sodium borohydride, acetylation, and investigation by g.l.c.–m.s. The main component was indistinguishable from the alditol acetate derived from 2-amino-2,6-dideoxy-D-glucose (quinovosamine). The derivatives of other 2-amino-2-deoxyhexoses were well-separated from the *gluco* derivative on the columns used[7]. The ^{1}H-n.m.r. spectrum of the amino sugar was indistinguishable from that given by authentic 2-acetamido-2,6-dideoxy-D-glucose and the $[\alpha]_D$ value indicated the D configuration.



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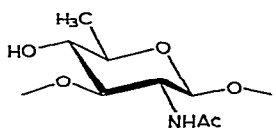

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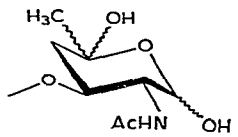

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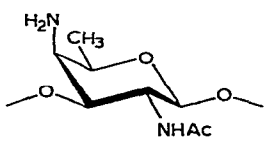

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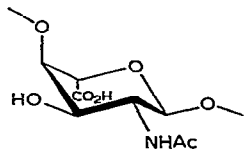

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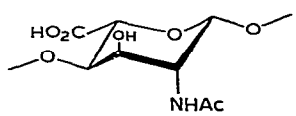

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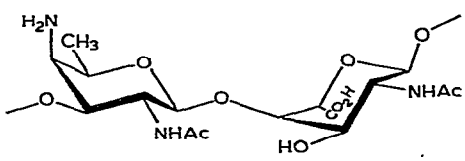

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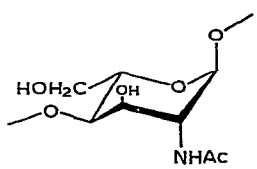

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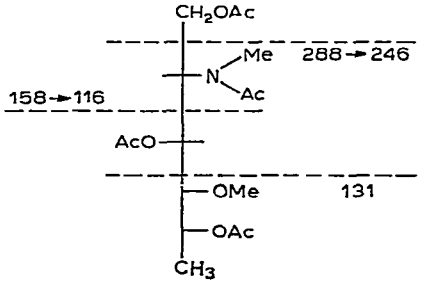
When the deaminated PS was reduced with borohydride before the acid hydrolysis, a second amino sugar was formed which, according to the mass spectrum of its alditol acetate, was a 2-amino-2,4,6-trideoxyhexose (most probably a mixture of

isomers was obtained, but was not well-separated in g.l.c.). These are the results expected on deamination of a 2-acetamido-4-amino-2,4,6-trideoxy-D-galactopyranosyl residue (**1**). The main course of this deamination should involve[8] an S_N2 reaction with water, giving a 2-acetamido-2,6-dideoxy-D-glucopyranosyl residue, and a shift of either of the axial hydrogens H-3 and H-5 to C-4. The latter reactions ultimately lead to fission of either the O–C-3 linkage or the glycosidic linkage, giving a 4-deoxyhexose residue having a carbonyl group in the 5- or 3-position. The deoxyhexosuloses formed during the acid hydrolysis should be decomposed, but the corresponding 2-acetamido-2,4,6-trideoxyhexoses formed when borohydride reduction precedes the acid hydrolysis should survive. Analogous results were obtained on deamination of the capsular polysaccharide from *Streptococcus pneumoniae* type 1, and the identification of the amino sugar as 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose was supported by model experiments[9].

The assignment of the L configuration to the 2-amino-2-deoxy-L-altruronic acid rested upon the low $[\alpha]_D$ value of the derived 2-amino-2-deoxyaltrose[3]. This assignment has now been supported by demonstrating that the ribose obtained on ninhydrin oxidation of the 2-amino-2-deoxyaltrose has the L configuration.

Methylation analysis of the *N*-acetylated and carboxyl-reduced PS gave a component which, from the mass spectrum of its alditol acetate, was a 2-deoxy-3,6-di-*O*-methyl-2-*N*-methylacetamidohexose. This component should derive from the 2-acetamido-2-deoxy-L-altruronic acid residue, which is consequently linked through O-4 in the PS.

The PS was subjected, in sequence, to deamination with nitrous acid, reduction with sodium borodeuteride, methylation, acid hydrolysis, reduction with sodium borohydride, and acetylation. G.l.c.–m.s. of the resulting product showed the presence of two components, which were identified as **4** and **5** from their mass spectra (some typical fragments are indicated in the formulas). Component **4** obviously derives from the 2-acetamido-2,6-dideoxy-D-glucopyranosyl residue (**2**) formed in the deamination reaction. The formation of **5** demonstrates that the shift of the axial H-5 to C-4 is the main rearrangement reaction on deamination of **1**, ultimately giving the hexosulose residue **3**. No product resulting from a shift of the axial H-3 was observed.



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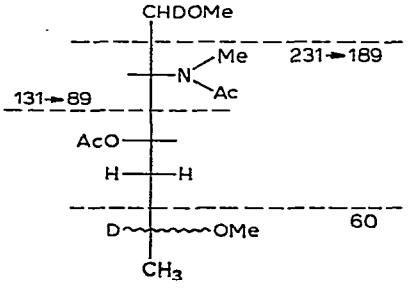

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The results discussed above indicate that the PS is composed of a 2-acetamido-2-deoxy-L-altropyranosyluronic acid residue, linked through O-4, and a 2-acetamido-4-amino-2,4,6-trideoxy-D-galactopyranosyl residue, linked through O-3. In the ^{13}C-n.m.r. spectrum, the two signals for anomeric carbons appeared at 104.6 ($^1J_{C,H}$ 162 Hz) and 102.6 p.p.m. ($^1J_{C,H}$ 163 Hz), indicating that the anomeric protons adopt axial positions in both sugar residues[10]. The 2-acetamido-4-amino-2,4,6-trideoxy-D-galactopyranosyl residue is consequently β-linked (**6**), as this residue almost certainly adopts the $^4C_1$ conformation. There is also n.m.r. evidence, discussed above, indicating that the amino group at C-4 is axial. The foregoing results indicate that the 2-acetamido-2-deoxy-L-altropyranosyluronic acid residue may either be α-linked, assuming the $^4C_1$ conformation (**7**), or β-linked, assuming the $^1C_4$ conformation (**8**). In the ^1H-n.m.r. spectrum, four low-field signals were observed at δ 4.90 (J 8 Hz), 4.73 (J 8 Hz), and 4.49 (J low, 2 H). Two of these signals should be given by anomeric protons and one by H-5 of the uronic acid residue, but the assignment of the fourth signal is less obvious. It was demonstrated that the two former signals are actually given by the anomeric protons by running a ^{13}C-n.m.r. spectrum in which the protons giving these signals were selectively decoupled; both of the signals given by the anomeric carbons then appeared as singlets. The high values (8 Hz) of the coupling constants therefore indicate a *trans*-diaxial relation between H-1 and H-2 in both residues. Consequently, the uronic acid is α-linked and assumes the $^4C_1$ conformation, as in **7**. From the combined evidence, it is therefore proposed that the O-specific side-chains of the *Shigella sonnei* phase I LPS are composed of disaccharide repeating-units having the structure **9**.

In the ^1H-n.m.r. spectrum of the *N*-acetylated and carboxyl-reduced PS, only two signals appeared in the region for anomeric protons, at δ 4.65 ($J_{1,2}$ 8 Hz) and 4.83 ($J_{1,2}$ low). In the ^{13}C-n.m.r. spectrum of the same material, the signals for anomeric carbons appeared at 102.0 ($^1J_{C,H}$ 158 Hz) and 102.9 p.p.m. ($^1J_{C,H}$ 170 Hz). These results therefore demonstrate that the 2-acetamido-2-deoxy-α-L-altropyranosyl residue in the carboxyl-reduced PS assumes the $^1C_4$ conformation (**10**).

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at bath temperatures not exceeding 40°. G.l.c. was performed on glass columns (190 × 0.15 cm) containing (*a*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 200°, (*b*) 3% of OV-225 on Gas Chrom Q (100–120 mesh) at 200°, and (*c*) 3% of OV-17 on Gas Chrom Q (100–120 mesh) at 200°, and (*d*) on an SP-1000 W.C.O.T. glass-capillary column (25 × 0.25 mm) at 230°. G.l.c.–m.s. was recorded with a Varian MAT 311-SS 100 instrument. The n.m.r. spectra were recorded for solutions in D_2O at 85° with a JEOL FX-100 spectrometer; external tetramethylsilane (^{13}C-n.m.r.) and/or internal sodium 1,1,2,2,3,3-hexadeuterio-4,4-dimethyl-4-silapentane-1-sulfonate (^1H-n.m.r.) references were used. Undecoupled spectra were obtained by a gated decoupling technique with a sampling time of 0.4 sec and a pulse repetition

time of 1 sec. In the selective-decoupling experiment, irradiation was performed at δ 4.82 in the ^{1}H-n.m.r. spectrum and was strong enough to effect the protons resonating at δ 4.73 and 4.90.

Preparation of polysaccharide. — The polysaccharide, prepared from *S. sonnei* phase I lipopolysaccharide as previously described[2], had $[\alpha]_{578}^{23}$ −31° (*c* 1.0, water). An acid hydrolysate contained small proportions of D-galactose, D-glucose, and L-*glycero*-D-*manno*-heptose, analysed as the alditol acetates by g.l.c.[11] on column (*b*). In the ^{13}C-n.m.r. spectrum, signals were observed at 176.0 (2 C), 175.6, 104.6, 102.6, 79.6 (2 C), 72.5, 69.8, 69.0, 56.8, 53.7, 53.0, 24.2, 24.1, and 17.3 p.p.m.

N-*Acetylation.* — Acetic anhydride (5 × 100 μL) was added in portions during 16 h to a solution of the PS (50 mg) in a mixture of water (2 mL) and saturated, aqueous sodium acetate (2 mL). The PS (47 mg) was recovered by dialysis and freeze-drying. In the ^{1}H-n.m.r. spectrum, the signal for the 6-deoxy protons was shifted upfield by 0.18 p.p.m. to δ 1.17, and the signals from *N*-acetyl protons were obtained at δ 2.05 (6 H) and 2.18 (3 H) after *N*-acetylation. In the ^{13}C-n.m.r. spectrum, signals were observed at 176.5, 176.2, 175.9, 175.6, 104.1, 101.3, 79.8, 77.8 (2 C), 71.8, 69.9, 54.9, 54.0 (2 C), 24.3, 24.1, 24.0, and 17.6 p.p.m.

Carboxyl-reduction of the *N*-acetylated PS (45 mg) was performed by the method of Taylor *et al.*[12], and the product (36 mg), $[\alpha]_{578}^{23}$ −17° (*c* 2.3, water), was isolated by dialysis and freeze-drying. A signal at 62.5 p.p.m. in the ^{13}C-n.m.r. spectrum demonstrated that a hydroxymethyl group had been formed.

Methylation analysis. — The *N*-acetylated and carboxyl-reduced PS (18 mg) was methylated according to Hakomori[13], and hydrolysed by treatment first with 90% formic acid at 100° for 2 h and then with 2M trifluoroacetic acid at 100° for 3 h. The acid was removed by evaporation, and the product was reduced with sodium borohydride. A column (5 × 0.5 cm) of Dowex-50(H^+) resin, was washed with water (15 mL); the product was then eluted from the washed column with M trifluoroacetic acid (5 mL). The latter fraction was concentrated to dryness, and the residue was acetylated and investigated by g.l.c.–m.s., using column (*c*). A single peak was obtained, with retention time 1.6 (relative to 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-D-glucitol) and a mass spectrum corresponding to a 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-*N*-methylacetamidohexitol[14].

Ninhydrin degradation. — *N*-Acetylated and carboxyl-reduced PS was hydrolysed with 4M trifluoroacetic acid at 100° for 4 h. The amino sugars were recovered by chromatography on Dowex-50(H^+) resin as described above, and oxidised with ninhydrin by the method of Stoffyn and Jeanloz[15]. Ribose, identified by g.l.c.–m.s. of its alditol acetate, was the main reaction product. The L configuration of the ribose was demonstrated by the method of Leontein *et al.*[16].

Deamination. — The PS (60 mg) in water (2 mL) was treated with 33% aqueous acetic acid (3 mL) and 5% aqueous sodium nitrite (3 mL) at 25° for 40 min, and the mixture was diluted with water (20 mL) and freeze-dried. On gel filtration of the product on a column (80 × 1.6 cm) of Biogel P-2 (to remove salts), part of the

material was eluted with the void, but another part was included; these materials were combined (52 mg).

Part of the product was reduced with sodium borohydride. Acid hydrolysis of this material, and analysis of the components as their alditol acetates by g.l.c.–m.s., showed the presence of a mixture of 2-amino-2,4,6-trideoxyhexoses and a 2-amino-2,6-dideoxyhexose. Retention times (column *b*), relative to glucitol hexa-acetate, were 0.74 (maximum of a broad peak) and 1.22. Mass spectra, taken at different parts of the first peak, were identical with, *inter alia*, the following ions (relative intensities in brackets): *m/e* 43(100), 44(17), 55(9), 57(10), 60(20), 69(10), 84(43), 85(23), 100(10), 102(17), 113(8), 124(9), 131(7), 137(6), 142(10), 143(9), 144(9), 154(3), 156(3), 173(4), 184(4), 202(3), and 244(2). The material in the second peak was indistinguishable from the acetylated 2-amino-2,6-dideoxyglucitol both in m.s. and in g.l.c. using the columns (*a*)–(*d*).

A solution of the deaminated PS (26 mg) in 0.5M trifluoroacetic acid was kept at 100° for 16 h and then concentrated to dryness, the residue was *N*-acetylated, and the hydrolysis procedure was repeated. The amino sugars were isolated by chromatography on Dowex-50(H^+) resin, as described above. In order to remove impurities, this material was acetylated and partitioned between chloroform and water. The chloroform-soluble material was *O*-deacetylated by treatment with a catalytic amount of sodium methoxide in methanol, and the solution treated with Dowex-50(H^+) resin and concentrated. The ^{1}H-n.m.r. spectra of this material and of authentic 2-acetamido-2,6-dideoxy-D-glucose were superposable. The sugar had $[\alpha]^{23}_{578}$ +10° (*c* 0.1, water), demonstrating that it has the D configuration; lit.[18] $[\alpha]_D$ +15°. The yield of the sugar was low and it was quantified by ^{1}H-n.m.r. spectroscopy, using sodium acetate as the internal standard.

Methylation analysis of the deaminated material. — Deaminated material (5 mg) was reduced with sodium borodeuteride, and methylated as described by Hakomori[13]. After purification by gel filtration on a column (50 × 1 cm) of Biogel P-2 eluted with water, the product was hydrolysed with 0.5M trifluoroacetic acid at 100° for 16 h, the hydrolysate concentrated to dryness, and the residue reduced with sodium borohydride. The material was isolated by chromatography on Dowex-50(H^+) resin, as described above, acetylated, and analysed by g.l.c.–m.s. using column (*c*). Two peaks were obtained with retention times 0.27 and 0.98, relative to 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-D-glucitol. The following ions were observed, *inter alia*, for the faster component (relative intensities in brackets): *m/e* 60(62), 89(100), 131(45), 157(11), 171(6), 189(13), and 231(19); and for the slower component: 74(28), 98(40), 116(60), 131(13), 158(30), 212(10), 246(3), and 288(1).

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REFERENCES

1 S. G. WILKINSON, in I. W. SUTHERLAND (Ed.), *Surface Carbohydrates of the Prokaryotic Cell*, Academic Press, London, 1977, pp. 97–175.
2 E. ROMANOWSKA AND V. REINHOLD, *Eur. J. Biochem.*, 36 (1973) 160–166.
3 T. KONTROHR, *Carbohydr. Res.*, 58 (1977) 498–500.
4 D. R. BUNDLE, I. C. P. SMITH, AND H. J. JENNINGS, *J. Biol. Chem.*, 249 (1974) 2275–2281.
5 L. KENNE, B. LINDBERG, P. UNGER, T. HOLME, AND J. HOLMGREN, *Carbohydr. Res.*, 68 (1979) c14–c16.
6 A. J. MORT AND D. T. A. LAMPORT, *Anal. Biochem.*, 82 (1977) 289–309.
7 M. B. PERRY AND V. DAOUST, *Can. J. Biochem.*, 51 (1973) 1335–1339.
8 J. M. WILLIAMS, *Adv. Carbohydr. Chem. Biochem.*, 31 (1975) 9–79.
9 B. LINDBERG, B. LINDQVIST, J. LÖNNGREN, AND D. A. POWELL, *Carbohydr. Res.*, 78 (1980) 111–117.
10 K. BOCK AND C. PEDERSEN, *J. Chem. Soc., Perkin Trans 2*, (1974) 293–297.
11 J. H. SLONEKER, *Methods Carbohydr. Chem.*, 6 (1972) 20–24.
12 R. L. TAYLOR, J. E. SHIVELY, AND H. E. CONRAD, *Methods Carbohydr. Chem.*, 7 (1976) 149–151.
13 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
14 G. O. H. SCHWARZMANN AND R. W. JEANLOZ, *Carbohydr. Res.*, 34 (1974) 161–168.
15 P. J. STOFFYN AND R. W. JEANLOZ, *Arch. Biochem. Biophys.*, 52 (1954) 373–379.
16 K. LEONTEIN, B. LINDBERG, AND J. LÖNNGREN, *Carbohydr. Res.*, 62 (1978) 359–362.
17 R. KUHN, W. BISTER, AND W. DAFELDECKER, *Ann. Chem.*, 617 (1958) 115–128.