Carbohydrate Research, 73 (1979) 175–182

STRUCTURAL STUDIES OF THE CAPSULAR ANTIGEN FROM *Streptococcus pneumoniae* TYPE 26

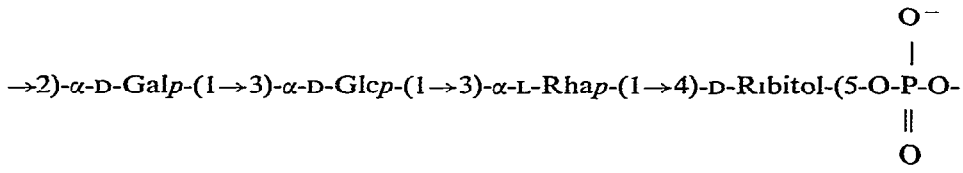
LENNART KENNE, BENGT LINDBERG, AND JOHN K. MADDEN

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

(Received October 10th, 1978; accepted for publication, November 1st, 1978)

ABSTRACT

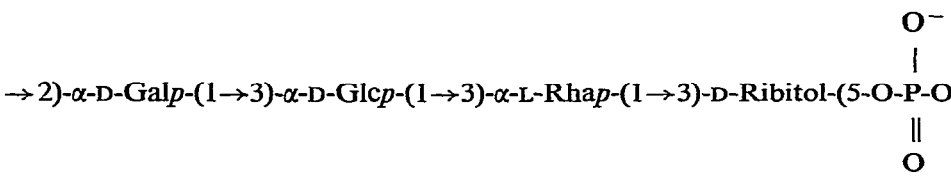
The structure of the capsular antigen from Pneumococcus type 26 has been determined by using methylation analysis, periodate-oxidation studies, and n.m.r. spectroscopy of the original and the dephosphorylated product. It is concluded that the polysaccharide is composed of repeating-units having the following structure.



The only difference between this structure and that of the type-6 antigen is that the α-L-rhamnopyranosyl residue is linked to O-4 of D-ribitol in the former, but to O-3 in the latter.

INTRODUCTION

Streptococcus pneumoniae types 6 and 26 (American nomenclature[1]) show immunological cross-reaction, as is also evident from their antigenic formulas, 6a, 6b, and 6a, 6c, respectively. According to the Danish nomenclature, these types are called 6 and 6A, but as 6A and 6B are also used, the American nomenclature will be followed here in order to avoid confusion. The structure of the type-6 capsular antigen (S-6) was investigated by Rebers and Heidelberger[2], who showed that it is composed of repeating-units having the structure **1**. The only structural feature not determined by these authors was the position to which phosphate is linked to the ribitol residue. As ribitol phosphate is most probably transferred to the repeating-unit from CDP-ribitol, in which phosphate is linked to O-5 of D-ribitol[3], it was assumed[4] that it is linked to the same position in S-6. We now report structural studies of the type-26 antigen (S-26).



1

RESULTS AND DISCUSSION

Analysis of S-26 showed that it contains the same components as S-6, namely, D-glucose, D-galactose, L-rhamnose, ribitol, and phosphate, in equimolecular proportions. The optical rotations of the two polymers were similar: $[\alpha]_D^{23}$ +91° for S-6, and $[\alpha]_D^{23}$ +86° for S-26. The ^{13}C-n.m.r. spectra (Fig. 1) were also similar, with signals for three anomeric carbon atoms at 96.7, 98.9, and 101.7 p.p.m. (S-6), and at 96.8, 98.9, and 101.1 p.p.m. (S-26), at ambient temperature. From the chemical shifts of these signals, it is concluded that all of the sugars are pyranosidic and that D-glucose and D-galactose occur as α-pyranosides in both polymers. In the ^{1}H-n.m.r. spectra, the signals for anomeric protons occurred at δ 5.58 (*J* 3 Hz), 5.09 (*J* 3 Hz), and 5.02 (*J* low) for S-6, and at δ 5.53 (*J* 3 Hz, 1 H) and 5.12 (*J* low, 2 H) for S-26, thus corroborating the foregoing assignments and further demonstrating that the L-rhamnopyranosyl residues are α-linked in both polymers.

The oligosaccharide from the S-26 polymer, obtained by treatment with 48% hydrofluoric acid, and the oligosaccharide[2] from S-6 were subjected to methylation analyses (Table I). The same methylated reducing-sugars were obtained from both products. 1,2,4,5-Tetra-*O*-methylribitol was also obtained from S-6, in agreement with the published structure, but 1,2,3,5-tetra-*O*-methylribitol was obtained from S-26.

TABLE I

METHYLATION ANALYSIS OF ORIGINAL AND MODIFIED OLIGOSACCHARIDES FROM S-6 AND S-26

Methylated sugar[a]	T[b]	*Mole %*[c]		
		A	*B*	*C*
1,2,4,5-Ribitol[d]	0.13	18		
1,2,3,5-Ribitol[d]	0.15		13	
2,4-Rha	0.94	28	29	48
2,3,4,6-Glc	1.00			52
2,3,4,6-Gal	1.19	28	28	
2,4,6-Glc	1.82	26	29	

[a]2,4-Rha = 2,4-di-*O*-methyl-L-rhamnose, *etc.* [b]Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an OV-225 column at 190°. [c]A, S-6 oligosaccharide; B, S-26 oligosaccharide; and C, S-26 oligosaccharide after Smith degradation. [d]These derivatives are volatile and were partially lost during concentrations.

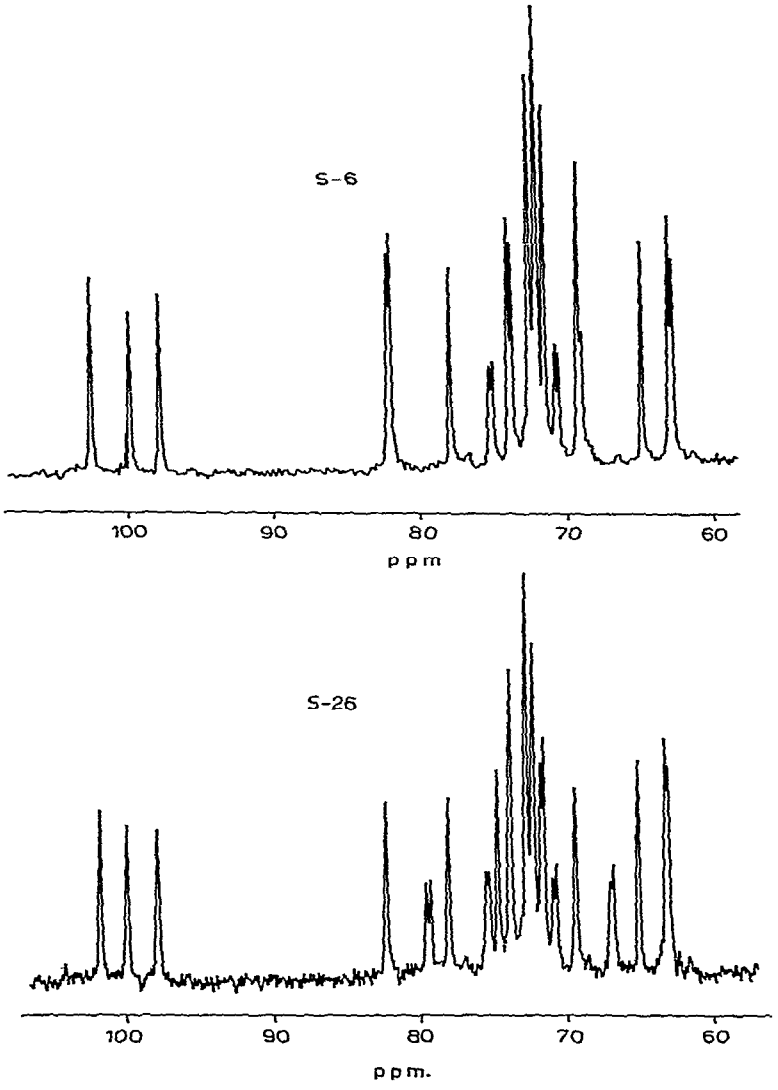
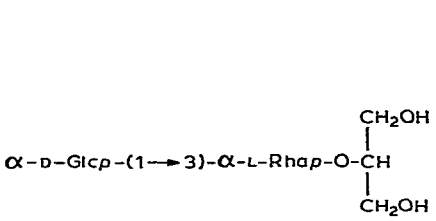


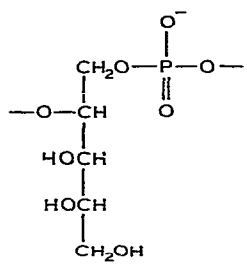
Fig. 1. ^{13}C-N.m.r. spectra of S-6 and S-26. The spectra were determined at 85° and are better resolved than those determined at ambient temperature, also given in Table II. The high-field region, with the signal for C-6 of the L-rhamnopyranosyl residue, is omitted.

Smith degradation[5] (periodate oxidation, borohydride reduction, and mild hydrolysis with acid) of the oligosaccharide from S-26 yielded a disaccharide derivative composed of D-glucose, L-rhamnose, and glycerol residues. Methylation analysis (Table I, column C), in conjunction with results discussed above, demonstrate that it has structure **2** and consequently that the sequence of sugar derivatives in S-26 is the same as that in S-6. For reasons discussed above, it may safely be assumed that one of the phosphate diester linkages is to O-5 of D-ribitol. In order to determine the location of the other phosphate diester linkage, and to decide whether the α-L-rhamnopyrano-

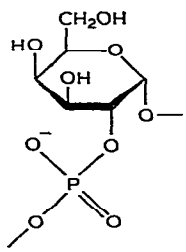
syl residue is linked to O-2 or O-4 of D-ribitol, S-26 was oxidized with periodate; three mol of oxidant were consumed and one mol of formaldehyde was formed. The formation of formaldehyde demonstrates that D-ribitol is substituted at O-4, as in **3**, and not at O-2. Two mol of periodate are consumed by the ribitol residue, leaving one for the D-galactopyranosyl residue, which is consequently substituted at O-2 or O-4. On acid hydrolysis of the oxidized and borohydride-reduced product, D-glucose, L-rhamnose, and glycerol, but no threitol, were formed, demonstrating that the D-galactopyranosyl residue is substituted at O-2, as in **4**.



2

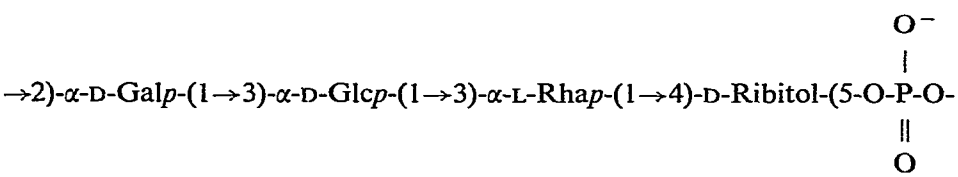


3



4

The combined results therefore demonstrate that S-26 is composed of repeating-units having the structure **5**. The only structural feature that is different in S-6 and S-26 is the position to which the α-L-rhamnopyranosyl residue is linked to D-ribitol, O-3 in the former and O-4 in the latter.



5

In the ^{13}C-n.m.r. spectra of S-6 and S-26, several signals overlap. However, structural information may be obtained from these spectra, especially when they are compared with those of the corresponding oligosaccharides (Table II). Thus, ^{31}P–^{13}C coupling is observed for phosphate-substituted carbons and their neighbours, namely, C-5 and C-4 of D-ribitol, and C-2 and C-3 of D-galactose, in both polymers. The insignificant coupling of C-1 in D-galactose is discussed below. Significant downfield-shifts are also observed when a carbon atom is glycosyloxylated or substituted with phosphate, and the signals from the adjacent carbon atoms are shifted upfield, but to a lesser extent[6]. Thus, C-1 of the galactopyranosyl residue in both S-6 and S-26 is easily recognized, as it shifts from 98.9 to 100.6 p.p.m. on cleavage of the phosphate linkage to O-2.

The only structural difference between S-6 and S-26, namely, that O-3 in D-ribitol is glycosidically substituted in the former, but that O-4 is substituted in the

TABLE II

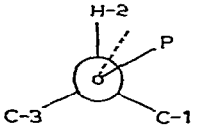
PERTINENT SIGNALS IN THE ^{13}C-N.M.R. SPECTRUM OF S-6, S-26, AND THE CORRESPONDING OLIGOSACCHARIDES

Compound	*Temperature*	*Anomeric carbons*			*Glycosyloxylated carbons*			*Other secondary carbons, coupled in the polymer*		*Primary carbons*			
Oligosaccharide from S-6	Ambient	101.4	100.6	96.7	81.1	80.8	76.5			63.9	63.6	62.1	61.3
S-6 PS	Ambient	101.7	98.9	96.7	80.6	80.6	76.3	74.0 (7 Hz)	69.5 (6 Hz)	68.1[a]	63.9	61.8	61.5
S-6 PS	85°C	102.4	99.8	97.7	82.0	81.9	77.8	75.0 (6 Hz)	70.5 (6 Hz)	68.9[b]	64.8	62.9	62.7
Oligosaccharide from S-26	Ambient	101.1	100.6	96.8	80.8	80.0	76.6			63.8	62.1	61.3	60.7
S-26 PS	Ambient	101.1	98.9	96.8	80.6	78.3 (7 Hz)	76.4	74.3 (5 Hz)	69.5 (6 Hz)	66.0[b]	63.9	61.9	61.5
S-26 PS	85°C	101.6	99.8	97.7	82.0	79.1 (8 Hz)	77.8	75.1 (5 Hz)	70.5 (6 Hz)	66.6 (5 Hz)	64.8	62.9	62.7

[a]Overlapping signal. [b]Coupled signal, poorly resolved.

latter, is also manifested in their ^{13}C-n.m.r. spectra. Thus, in S-6, C-5 and C-3 of the D-ribitol residue appear at 68.1 and 80.6 p.p.m., respectively. In S-26, the signals of C-5 and C-4 of D-ribitol, at 66.0 and 78.3 p.p.m. respectively, have been shifted upfield because of the mutual shielding effects of the substituents (the signals for C-3 and C-4 in ribitol appear at the same field, 73.4 p.p.m.). The deshielding effected by dephosphorylation is also clearly observed.

Some information on the conformation of the polymers may also be obtained from the ^{13}C-n.m.r. spectra[6]. The three-bond ^{31}P–^{13}C coupling is dependent upon the angle between the POC and OCC′ planes, allowing an estimation of the populations of the possible rotamers. Coupling constants of 8–10 and 2–3 Hz are observed for trans (dihedral angle 180°) and gauche (dihedral angle 60°) relationships, respectively. In the ^{13}C-n.m.r. spectra of α- and β-D-glucose 2-phosphate[7] $J_{P,C-1}$ is 3 and 5 Hz, respectively, indicating a preference for gauche conformations. The virtual absence of coupling between P and C-1 of the D-galactopyranosyl residue in both S-6 and S-26, and the high coupling constant (6 Hz) for C-3 at 69.9 and 69.5 p.p.m., suggest a preference for the conformation depicted in **6**, with gauche and trans relationships, respectively. The conformation may be somewhat distorted, as indicated by the dotted line. The coupling constant (7 Hz) between ^{31}P and C-4 in the D-ribitol residue also indicates a trans relation between these atoms.



6

EXPERIMENTAL

General methods. — Concentrations were performed under reduced pressure at bath temperatures below 40°. A Perkin–Elmer 990 instrument with flame-ionisation detectors was used for g.l.c. Separations were performed on a glass column (180 × 0.15 cm) containing 3% of OV-225 on 100/120 mesh Gas Chrom Q, at 210° for alditol acetates and at 190° for partially methylated alditol acetates. Peak areas were measured with a Hewlett–Packard 3370B electronic integrator. G.l.c.–m.s. was performed with a Varian MAT 311–SS 100 instrument at an ionisation potential of 70 eV. N.m.r. spectra for solutions in D_2O were recorded in the PFT-mode with a JEOL FX-100 spectrometer, using external tetramethylsilane (^{13}C), and internal 4,4-dimethyl-4-silapentane-1-sulphonate (^{1}H) as references. Optical rotations were determined with a Perkin–Elmer 241 instrument.

Material. — The polysaccharides S-6 and S-26 were prepared at the Lederle Laboratories, Pearl River, N.Y., U.S.A. As observed at this laboratory, the polymers undergo autohydrolysis unless kept at low temperature. For further purification, each polymer (475 mg) was dissolved in water (250 ml), and 5% cetyltrimethylammonium

bromide (60 ml) was added. The resulting precipitate was collected by centrifugation, washed with water, and dissolved in 10% aqueous sodium chloride (100 ml), and the polymer was then precipitated with ethanol (500 ml). The products (~250 mg) in water (*c* 0.2) showed $[\alpha]_D^{23}$ +91° (S-6) and 86° (S-26).

Sugar analysis. — After hydrolysis with 0.25M trifluoroacetic acid for 16 h at 100°C, the sugars were analysed as described by Sloneker[8]. Identifications were confirmed by g.l.c. and m.s. Absolute configurations of the sugars were determined according to the method of Leontein *et al.*[9].

Phosphate analyses. — These were performed as described by Chen *et al.*[10].

Dephosphorylation. — S-26 (50 mg) was dissolved in 48% aqueous hydrogen fluoride[11] (3 ml). After 4 days at −16°C, the hydrogen fluoride was evaporated at reduced pressure and the residue was dissolved in water (4 ml). The pH of the solution was adjusted to 7 with ammonia, and the solution was freeze-dried. Chromatography on a column (100 × 2.5 cm) of Biogel P2 yielded the pure oligosaccharide (16 mg), $[\alpha]_D$ +95° (*c* 0.2, water). The oligosaccharide from S-6 had $[\alpha]_D$ +90° (*c* 0 2, water).

Methylation analyses. — The oligosaccharides were subjected to methylation analysis, as previously described[12], the permethylated products being isolated by partitioning between water and chloroform. The product was hydrolysed with 0.25M trifluoroacetic acid for 16 h at 100°C and the resulting mixture of methylated sugars was analysed, as their alditol acetates, by g.l.c.–m.s.

Smith degradation of the oligosaccharide from S-26. — A solution of the oligosaccharide (16 mg) in a mixture of 0.1M sodium acetate buffer (pH 3.9, 12 ml) and 0.1M sodium metaperiodate (4 ml) was kept at +5° for 4 days. Excess of periodate was reduced with ethylene glycol (0.2 ml), sodium borohydride was added, and the solution was kept at room temperature overnight. Excess of borohydride was decomposed by addition of 50% acetic acid. Chromatography on a column of P-2 yielded a pure product (4 mg). This material was treated with 0.25M trifluoroacetic acid for 20 h at room temperature, the acid was removed by evaporation, and the material was chromatographed on a column (70 × 1.6 cm) of P-2. The product (3 mg) had $[\alpha]_D$ +78° (*c* 0.2, water). An acid hydrolysate of the product contained glycerol, rhamnose, and glucose.

Periodate-oxidation studies of S-26. — A solution of S-26 (5 mg) in 25mM sodium metaperiodate was kept at 24°. Consumption of periodate was followed spectrophotometrically[13] at 223 nm and was complete after 48 h, when 3.2 mol had been consumed. The formaldehyde produced (1.0 mol) was determined by reaction with chromotropic acid[14].

A solution of S-26 (25 mg) in 0.1M sodium acetate buffer (pH 3.9, 24 ml) and 0.1M sodium metaperiodate (8 ml) was kept at 4° for 5 days. Excess of periodate was reduced with ethylene glycol (0.3 ml), and the mixture was dialysed overnight and freeze-dried. The product was reduced with sodium borohydride (200 mg) in water (5 ml), and the solution was then acidified with 50% acetic acid, dialysed, and freeze-dried. A hydrolysate of the residue was reduced and acetylated. The presence of glucitol, rhamnitol, and glycerol acetates was demonstrated by g.l.c.–m.s , but no threitol acetate was detected.

ACKNOWLEDGMENTS

The authors thank Dr. John Robbins, Bureau of Biologics, FDA, Bethesda, for suggesting the problem, and Dr. Francis Cano, Lederle Laboratories, Pearl River, for generous samples of S-6 and S-26. A Swedish Institute scholarship to one of us (J.K.M.) is gratefully acknowledged. This work was supported by the Swedish Medical Research Council (B79-03X-02522-11C), Knut och Alice Wallenbergs Stiftelse, Harald Jeanssons Stiftelse, and Stiftelsen Sigurd och Elsa Goljes Minne.

REFERENCES

1 F. KAUFFMANN, E. LUND, AND B. E. EDDY, *Int. Bull. Bacteriol. Nomencl. Taxon*, 10 (1960) 30–41.
2 P. A. REBERS AND M. HEIDELBERGER, *J. Am. Chem. Soc.*, 83 (1961) 3056–3059.
3 J. BADDILEY, *Acc. Chem. Res.*, 3 (1970) 98–105.
4 O. LARM AND B. LINDBERG, *Adv. Carbohydr. Chem. Biochem.*, 33 (1976) 295–322.
5 I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS, AND F. SMITH, *Methods Carbohydr. Chem.*, 5 (1965) 361–370.
6 H. J. JENNINGS AND I. C. P. SMITH, *Methods Enzymol.*, 50C (1978) 39–50.
7 Unpublished results from the authors' laboratory.
8 J. H. SLONEKER, *Methods Carbohydr. Chem.*, 6 (1972) 20–24.
9 K. LEONTEIN, B. LINDBERG, AND J. LÖNNGREN, *Carbohydr. Res*, 62 (1978) 359–362.
10 P. S. CHEN, JR., T. Y. TORIBARA, AND H. WARNER, *Anal. Chem*, 28 (1956) 1756–1758.
11 P. PREHM, S. STIRM, B. JANN, AND K. JANN, *Eur. J. Biochem*, 56 (1975) 41–55.
12 P. E. JANSSON, L. KENNE, H. LIEDGREN, B. LINDBERG, AND J. LÖNNGREN, *Chem. Commun, Univ. Stockholm*, 8 (1976) 1–75.
13 R. D. GUTHRIE, *Methods Carbohydr. Chem*, 1 (1962) 435–441.
14 J. C. SPECK, JR., *Methods Carbohydr. Chem*, 1 (1962) 441–445.