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THE STRUCTURE OF THE TYPE-SPECIFIC POLYSACCHARIDE OF *Pneumococcus* TYPE XIX*

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ABSTRACT

The structure of the capsular polysaccharide of Type XIX *Streptococcus pneumoniae* (S-XIX) has been elucidated by ^{1}H- and ^{13}C-n.m.r. spectroscopy. Mild hydrolysis of S-XIX with acid yielded a major oligosaccharide, the repeating unit of S-XIX, which was shown to be *O*-2-acetamido-2-deoxy-β-D-mannopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→2)-L-rhamnose 4″-phosphate. Phosphoric acid forms a diester linkage in the S-XIX molecule, which explains the instability of S-XIX towards acid or alkali. The phosphodiester linkages in S-XIX join HO-1 of α-L-rhamnose and HO-4 of the 2-acetamido-2-deoxy-D-mannopyranosyl residue in the next repeating-unit. Treatment of S-XIX with alkali or alkaline-$NaBH_4$ produced the repeating units in a lower yield. The proposed structure of S-XIX is

$$[\text{-P(=O)(-O)-O-4-}\beta\text{-ManNAc-(1}\rightarrow\text{4)-}\alpha\text{-Glc}p\text{-(1}\rightarrow\text{2)-}\alpha\text{-Rha-O-}]_n$$

INTRODUCTION

An antigenic, pneumococcal type XIX capsular polysaccharide (S-XIX) is composed of repeating units that each contains 2-acetamido-2-deoxy-D-mannose, L-rhamnose, D-glucose, and phosphoric acid. The phosphoric acid residue is involved in a diester linkage[1], so that S-XIX is unstable towards mild acid or alkali. A partial structure of S-XIX, elucidated by chemical and destructive techniques, has been reported[1–4]. It was proposed that S-XIX was composed of a tetrasaccharide repeating-unit, which was prepared by treatments with mild alkali, acid, and then phosphomonoesterase[4]. In addition, S-XIX contains 4-*O*-(2-acetamido-2-deoxy-β-D-mannopyranosyl)-D-glucose 4′-phosphate, which is an acid-resistant moiety[5].

We now report on the application of ^{1}H- and ^{13}C-n.m.r. spectroscopy to S-XIX.

*Polysaccharides of Type XIX *Pneumococcus*, Part V. For Part IV, see ref. 5.

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EXPERIMENTAL

Materials and methods. — The pneumococcal type XIX capsular polysaccharide and anti-pneumococcal type XIX horse-serum (anti-Pn XIX) were provided by (the late) Professor J. K. N. Jones, and were purified by the method described previously[3].

Ascending p.c. and t.l.c. were performed on Toyo Roshi No. 50 filter papers and Merck 5577 cellulose sheets at room temperature, respectively, with *A*, ethyl acetate–pyridine–acetic acid–water (10:10:1:6); or *B*, ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Sugars were detected with alkaline silver nitrate[6]. Radioactive fractions were counted in 7 ml of tT76 emulsion[7] with a Packard 3330 liquid scintillation counter.

^{13}C-N.m.r. spectra were recorded at room temperature on a JEOL-FX 100 spectrometer at 25.0 MHz, in the pulsed Fourier-transform mode with complete proton-decoupling. Chemical shifts are expressed as p.p.m. downfield from the signal (49.8 p.p.m.) for MeOH. ^{1}H-N.m.r. spectra were recorded at room temperature for solutions in D_2O (internal TSP) with JEOL-PS 100 (continuous wave mode) and JEOL-FX 100 spectrometers (pulsed Fourier-transform mode). Optical rotations were measured for solutions in 0.1-dm semimicro-tubes with a JASCO DIP-4 Digital polarimeter. Phosphomonoesterase treatment[8], *N*-acetylation[9], and determinations of reducing power[10] and neutral sugars[11] were performed according to the methods described in the literature.

Preparation of B-Unit[1]. — A solution of S-XIX in 0.2M NaOH was diluted with water (1 vol.), kept at 37° for 48 h, and then cooled and neutralised (dilute HCl). The solution was dialysed against distilled water, and the external solution was concentrated and eluted from a column of Dowex-1 ($HCOO^-$) resin with a linear gradient of 0→0.6M NaCl. The main oligosaccharide (B-unit) was eluted at 0.25M NaCl, and the appropriate fractions were concentrated and desalted by using a column (1.5 × 90 cm) of Sephadex G-10. The B-unit was similar to non-reduced G-25-2 described later.

Treatment of S-XIX with alkaline-[^{3}H]-NaBH$_4$. — A solution of S-XIX (7 mg) in water (100 μl) was treated with 0.2M [^{3}H]-$NaBH_4$ (67.1 mCi/mmol, 200 μl) and 0.2M NaOH (100 μl) at 37° for 48 h. The reaction was terminated by adding acetone, the mixture was neutralised with NH_4Cl and concentrated to dryness, and the residual borate and ^3H_2O were removed by repeated distillation of MeOH and water from the residue, which was then dissolved in a small quantity of water and desalted by using a column of Bio Gel P-2. The radioactive fraction was re-treated with alkaline [^{3}H]-$NaBH_4$ at 48° for 8 h, then treated as described above, and passed through a column of Sephadex G-25.

α-L-Rhamnopyranosyl phosphate. — This compound (α-L-Rha-1P), synthesised from α-L-rhamnose tetra-acetate by the method of Chatterjee and MacDonald[12] and purified by the method of Ishihara *et al.*[13] as the cyclohexylammonium salt, had $[\alpha]_D$ −17°. ^{1}H-N.m.r. data: δ 5.33 (q, $J_{1,2}$ 1.5, $J_{1,P}$ 8 Hz, H-1).

Rates of hydrolysis of S-XIX, B-Unit, and α-L-Rha-1P. — S-XIX, B-Unit, and

α-L-Rha-1P were treated severally with equal volumes of 20mM HCl at 85°. After the appropriate time, each hydrolysate was cooled, and neutralised with NaOH. The rate of hydrolysis was measured by the Park–Johnson method[10].

Mild hydrolysis of B-Unit with acid was effected as described above. The 45-min and 6-h hydrolysates were examined by t.l.c.

Mild, acid hydrolysate of S-XIX. — S-XIX (30 mg) was hydrolysed in 10mM HCl for 15 min at 85°. The hydrolysate was lyophilised, and the residue was eluted from a column (2 × 13 cm) of Dowex-1 ($HCOO^-$) resin with water followed by a linear gradient (600 ml) of 0→0.6M NaCl, and then with 2M NaCl (100 ml) and 2M HCl (100 ml). Sugar-containing fractions were collected, and desalted by passing through a column (1.5 × 140 cm) of Sephadex G-25.

RESULTS

Hydrolysis of S-XIX. — (*a*) The hydrolysis of S-XIX, B-unit, and α-L-Rha-1P in 10mM HCl at 85° is shown in Fig. 1. Whereas the hydrolysis of α-L-Rha-1P was complete within 30 min, that of the B-unit was relatively slow, but, after 6 h, much of the Rha had been released together with *N*-deacetylation of ManNAc. The latter phenomena were detectable after 45 min. The hydrolysis of S-XIX proceeded rapidly during the first 15 min and at a lower rate thereafter. As estimated from the results of α-L-Rha-1P and B-unit, the phosphodiester linkages in S-XIX would be hydrolysed within 15 min and the Rha residue in S-XIX would be hydrolysed at a relatively low rate. Therefore, 10mM HCl at 85° for 15 min was used for the subsequent hydrolysis of S-XIX.

(*b*) *Mild hydrolysis.* Fig. 2 shows that the mild, acid hydrolysis of S-XIX (30 mg) gave four peaks, of which DF-3 (13.3 mg) was the major oligosaccharide, $[\alpha]_D$ +66° (*c* 0.5, water). Gel filtration of DF-1 gave four peaks; DF-1-1 (V_O), DF-1-2 ($V_{Raffinose}$), DF-1-3 ($V_{Maltose}$), and DF-1-4 (V_{Rha}). DF-4 was eluted in the void volume from Sephadex G-25.

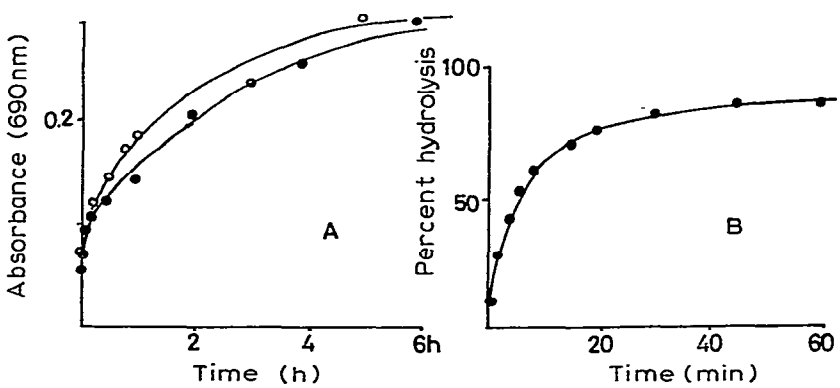


Fig. 1. Hydrolysis of S-XIX (A, —●—), B-unit (A, —○—), and α-L-Rha-1P (B) in 10mM HCl at 85°.

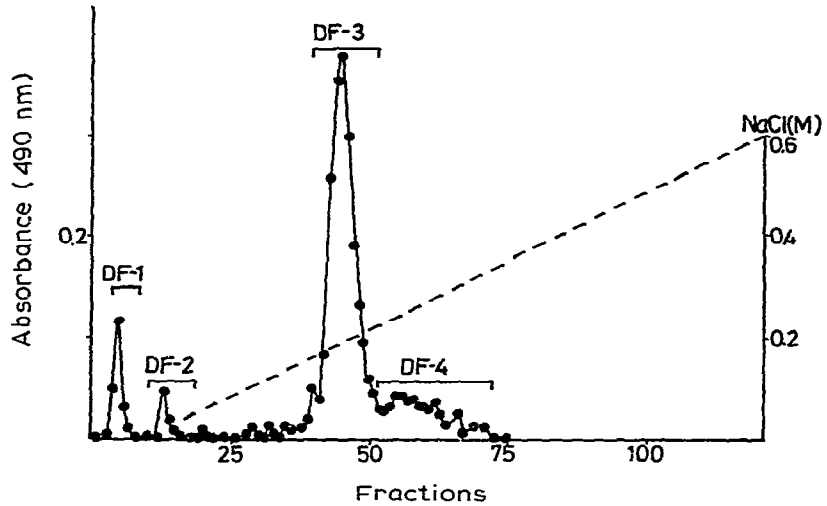


Fig. 2. Fractionation of the mild, acid hydrolysate of S-XIX on Dowex-1 ($HCOO^-$) resin.

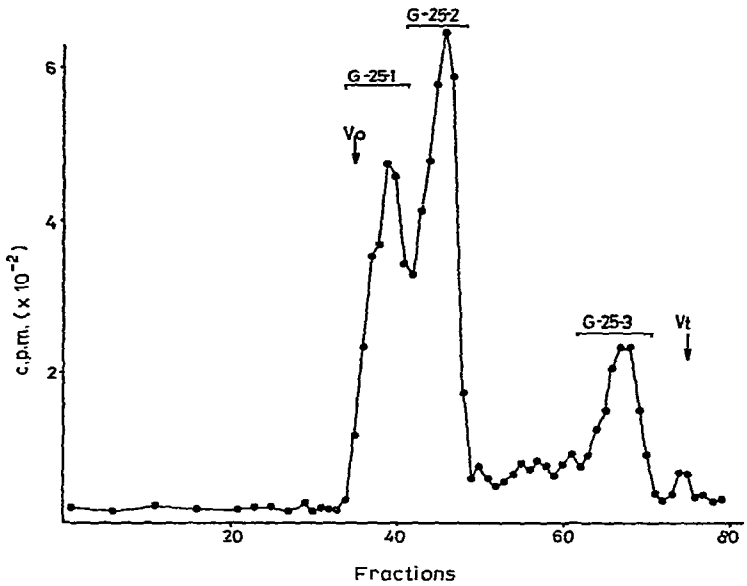


Fig. 3. Fractionation of the alkaline-[^{3}H]-$NaBH_4$ degradation product of S-XIX on Sephadex G-25.

Treatment of S-XIX with alkaline-[^3H]-$NaBH_4$. — After treatment of S-XIX with alkaline-[^{3}H]-$NaBH_4$, the product was passed through a column of Sephadex G-25 (Fig. 3), and three peaks, G-25-1,2,3, were obtained. G-25-1 contained Rha, rhamnitol (RhaOH), Glc, ManNAc, and phosphoric acid, and gave a precipitin band against anti-Pn XIX. G-25-3 contained no sugars and might reflect an impurity in the [^{3}H]-$NaBH_4$. G-25-2 contained RhaOH, Glc, ManNAc, and phosphoric acid,

but gave no precipitin band against anti-Pn XIX. G-25-2 ($R_{Glucitol}$ 0.3, solvent *A*) gave RhaOH on acid hydrolysis, and the product formed after treatment with phosphomonoesterase had $R_{Glucitol}$ 0.82 (solvent *A*). This value was the same as that of the reduced and phosphomonoesterase-treated B-unit.

Characterisation of the oligosaccharides. — Oligosaccharide DF-3, described above, was composed of Rha, Glc, ManNAc, and phosphoric acid, and its ^{1}H-n.m.r. spectrum contained, *inter alia*, signals at δ 2.10 (s, 3 H, NAc of ManNAc) and 1.13 (d, 3 H, *J* 6 Hz, Me of Rha).

Acid hydrolysis DF-3 yielded the disaccharide unit[5] PO_4-4-β-ManN-(1→4)-Glc and Rha. Reduction of DF-3, followed by hydrolysis, liberated RhaOH. From these results, it was concluded that DF-3 was PO_4-4-β-ManNAc-(1→4)-Glc(1→?)-Rha.

The ^{13}C-n.m.r. data for DF-3 are summarised in Table I. The configuration of Glc was assumed to be α, since the signal (98.6 p.p.m.) was in the appropriate region and up-field of that for C-1β. Signals for C-1 and C-2 of α- and β-Rha (94.7, 92.4, 81.8, 78.2 p.p.m.) were in good agreement with (1→2) substitution. Thus, it is reasonable to assume that Glc is attached to position 2 of Rha.

The ^{1}H-n.m.r. spectra of the minor oligosaccharides, DF-1-2 and DF-4, obtained by mild, acid hydrolysis of S-XIX, showed a pattern similar to that of DF-3. In the spectrum of the other minor product, DF-2, there was no signal for *N*-acetyl. These results suggest that DF-1-2, DF-2, and DF-4 were de-phosphorylated DF-3, *N*-deacetylated DF-3, and an incomplete-degradation product of S-XIX, respectively.

Hydrolysis (M HCl, 100°, 3 h) of the B-unit gave PO_4-4-β-ManN-(1→4)-Glc and Rha, and all of the phosphoric acid in the B-unit was liberated by phosphomonoesterase. The ^{1}H- and ^{13}C-n.m.r. spectra of the B-unit (Table I) were essentially the same as those of DF-3, except that the B-unit gave several minor signals due to the effect of alkali on the reducing, terminal Rha. These results indicated that S-XIX liberated the same oligosaccharide on treatment with mild acid or alkali.

Characterisation of S-XIX. — The thermal instability of S-XIX limited ^{1}H-n.m.r. spectroscopy to room temperature. The spectrum showed signals at δ 2.10 (s, 3 H) and 1.32 (d, 3 H) in the ratio 1:1 and attributable to NAc of ManNAc, and Me of Rha, respectively. Three distinct signals were observed in the anomeric region, at δ 5.50, 5.02, and 4.83. The signal at δ 4.83 (*J* 5 Hz) was assigned to H-1 of the β-linked ManNAc residue, since the chemical shift was similar to that of H-1 in the β-linked ManNAc residue in the disaccharide unit and DF-3. The coupling constant (8 Hz) of the signal at δ 5.50 is similar to that for $J_{1,P}$ of α-L-Rha-1P; this signal was absent from the spectrum of DF-3. The signal at δ 5.50 is assigned to H-1 of phosphorylated Rha.

The ^{13}C-n.m.r. data for S-XIX are shown in Table I. Signals for three anomeric carbons were observed. Those at 100.3 and 98.5 p.p.m. were assigned to the β-ManNAc and α-Glc residues, respectively, since these signals were also observed in the spectrum of DF-3. The signal at 94.7 p.p.m. ($J_{C\text{-}1,P}$ 7.0 Hz) was assigned to C-1 of the L-Rha-1P moiety. If the configuration of L-Rha-1P in S-XIX is β, then the signals for C-3,4,5 would be expected, from studies with oligosaccharides derived

TABLE I

^{13}C-CHEMICAL SHIFTS AND CARBON–PHOSPHORUS COUPLINGS (Hz, IN PARENTHESES) OF S-XIX AND RELATED SUBSTANCES[a]

Atom		A[b]	B	C	D	E	F	G
ManNAc	C-1α	93.9						
	β	94.0	100.3		100.3	100.4	100.2	100.1
	C-2α	54.1						
	β	55.0	53.9		53.7	53.5	54.3	53.9
	C-3α	69.8						
	β	73.0	72.9(1.4)[c]		72.9[d](2.4)	72.9[d]	72.9	72.6
	C-4α	67.7						
	β	67.5	71.7(5.4)[c]		71.4(6.1)	71.3(4.9)	67.5	71.1(5.8)
	C-5α	73.0						
	β	77.3	76.6(4.9)		76.8(6.1)	77.0(7.4)	77.4	76.3(5.0)
	C-6α	61.3						
	β	61.3	61.1		60.6	60.7	60.7	60.7
	-N-C=Oα	175.6						
	β	176.5	176.4		176.3	176.1	176.3	176.1
	C-CH$_3$α	23.0						
	β	22.9	23.0		23.0	23.0	23.0	22.9
Glc	C-1α	92.9	92.7		98.6	98.5	98.7	98.5
	β	96.7	96.7					
	C-2α	72.3	72.3		72.1[d]	72.1[d]	72.2[d]	72.1
	β	75.0	74.9					
	C-3α	73.6	72.2		72.1[d]	72.1[d]	72.2[d]	72.1
	β	76.7	75.1					
	C-4α	70.5	79.6		79.7	79.6	79.6	79.6
	β	70.5	79.9					
	C-5α	72.3	70.8		71.2	71.2	71.2	71.2
	β	76.8	75.4					
	C-6α	61.6	61.3		61.4	61.4	61.3	61.3
	β	61.7	61.3					
Rha	C-1α	94.9		96.5(4.9)	92.4	92.4	92.7	94.6(7.0)
	β	94.4			94.7	94.7	94.8	
	C-2α	71.7		71.6(8.8)	78.2	78.2	78.4	77.5(8.5)
	β	72.2			81.8	81.8	81.9	
	C-3α	70.9		70.6	70.2	70.2	70.3	70.0
	β	73.7			73.2[d]	72.8[d]	72.9	
	C-4α	73.1		73.0	72.7[d]	72.9[d]	72.9	72.6
	β	72.7			72.4[d]	72.3[d]	72.4[d]	
	C-5α	69.2		70.2	69.5	69.5	69.5	71.0
	β	72.9			72.7[d]	72.9[d]	72.9	
	C-6α	17.8		17.7	17.6	17.7	17.7	17.5
	β	17.8			17.6	17.7	17.7	

[a]Key: *A*, authentic monosaccharide; *B*, *N*-acetylated disaccharide unit[5]; *C*, α-L-Rha-1P; *D*, DF-3; *E*, B-unit; *F*, phosphomonoesterase-treated B-unit; *G*, S-XIX. [b]Data from refs. 20 and 21. [c]Calculated value from the disaccharide unit[5]. [d]Assignments not unequivocal.

from S-XIX, to have chemical shifts of ~72.9 p.p.m. The signal at 70.0 p.p.m. was assigned to C-3 of the α-L-Rha-1P residue. The configuration of L-Rha-1P in S-XIX was concluded to be α.

DISCUSSION

Our previous suggestion that S-XIX is built up *via* a phosphodiester linkage is confirmed by ^{1}H- and ^{13}C-n.m.r. spectroscopy of S-XIX and its mild, acid hydrolysate.

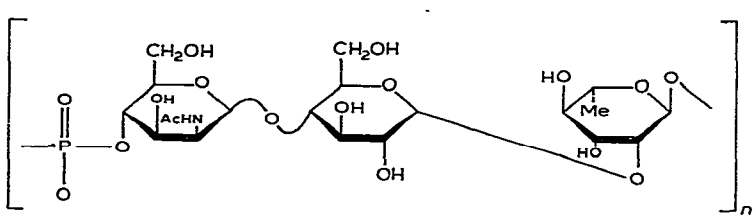
Mild, acid hydrolysis of S-XIX cleaved the phosphodiester without significant hydrolysis of glycosidic linkages and gave a major oligosaccharide, DF-3, which could be the repeating unit. In the ^{13}C-n.m.r. spectrum of DF-3, the signals for C-1 and C-2 of α- and β-Rha (94.7, 92.4, 81.8, 78.2 p.p.m.) were characteristic of (1→2) substitution. A similar observation was made for (1→2)-β-linked mannobiose[14], which gave two signals for C-1 involved in the inter-sugar linkage, due to the presence of α- and β-forms of the reducing unit. Only one signal was observed for C-1 of Glc of DF-3, but it is possible that one of the signals for C-1 of Glc overlapped with that for C-1 of ManNAc. In the region of the ^{1}H-n.m.r. spectrum for anomeric protons, the signals were complex and no assignments could be made.

Usui *et al.*[15] noted that the ^{1}H-n.m.r. signals for the anomeric protons associated with the inter-sugar linkages of some D-gluco-oligosaccharides overlapped, except for the (1→2)-linked glucobiose. The complexity of the ^{1}H-n.m.r. spectrum of DF-3 could be due to a similar effect and reflect the presence of an α-Glc-(1→2)-Rha unit in DF-3.

Treatment of S-XIX with alkali or alkaline-$NaBH_4$ also produced the repeating units (B-unit), but in a lower yield. Thus, the phosphodiester linkages in S-XIX are unstable to both acid and alkali, but more so in acid. The ^{13}C-n.m.r. spectrum of the B-unit showed several minor signals, possibly due to changes at the Rha terminal by the alkali treatment. The repeating unit of S-XIX is better prepared by acid treatment.

The phosphodiester linkage in S-XIX, assigned to α-L-Rha-1P on the basis of the ^{13}C-n.m.r. data, was also suggested by the $[\alpha]_D$ values: +66° for DF-3, +37° for S-XIX, and −17° for α-L-Rha-1P.

From the data described above, the structure **1** is proposed for S-XIX.



1

Previous work[1–4] suggested that S-XIX was composed of L-Rha, D-Glc, D-ManNAc, and phosphoric acid in the proportions ~2:1:1:1. It is now concluded

that the ratios are 1:1:1:1. In the previous papers[1-4], the component sugars were quantified by using colorimetric methods and paper chromatography. However, S-XIX contains one residue (Rha) which is acid-labile and cleaved by 10mM HCl at 85°. The residual disaccharide unit[5] is not completely hydrolysed, even by 4M HCl at 100° for 3 h. It is impossible, therefore, to set up hydrolysis conditions suitable for determination of the molar composition of the units in S-XIX.

The mechanism of biosynthesis of phosphodiester-containing polysaccharides, such as teichoic acid, has been widely investigated[16,17]. Partridge *et al*[18]. suggested that the anomeric configuration of the glycosyl 1-phosphate is the same as that of the corresponding sugar nucleotide[18]. Larm and Lindberg[19] assumed that the L-rhamnosyl phosphate residue in S-XIX had the β-L configuration, because the L-rhamnosyl phosphate was most probably derived from TDP-β-L-rhamnose.

The foregoing data show that the L-rhamnosyl residue in S-XIX has the α configuration. It is possible that another biosynthetic pathway is operative.

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