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The structure of the capsular polysaccharide from *Streptococcus pneumoniae* type 7B

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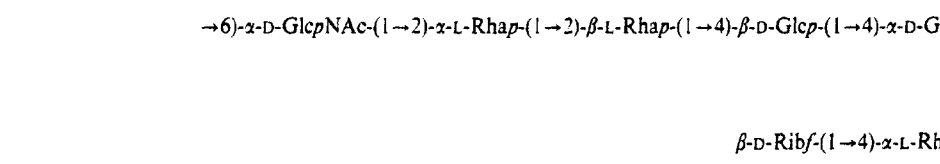
ABSTRACT

The capsular polysaccharide elaborated by *Streptococcus pneumoniae* type 7B is composed of the following heptasaccharide repeating-units.



The identities and modes of linkage of the constituents were established using sugar, methylation, and phosphorus analysis, together with 1D- and 2D-n.m.r. spectroscopy. The sequence was established from inter-residue n.O.e. data. The structure was corroborated by n.m.r. spectroscopy, f.a.b.-m.s., and methylation analysis of the oligosaccharides isolated after partial acid hydrolysis of the polysaccharide with aqueous 48% hydrogen fluoride.

It is suggested that the structural basis for the common antigenic formula in the group 7 serotypes of *S. pneumoniae* is the disaccharide element α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→.

INTRODUCTION

Streptococcus pneumoniae consists of 83 capsular types, 56 of which are divided into 19 groups in the Danish nomenclature system. Each of the 83 types elaborates its own, type-specific capsular polysaccharide. Group 7[1] consists of the serotypes 7F, 7A, 7B, and 7C. The capsular polysaccharides from types 7F (antigenic formulas 7a and 7b)[2] and 7A (7a, 7b, and 7c)[3] differ by only one sugar residue present as a second branch in the former polysaccharide. Serotype 7B (7a, 7d, 7e, and 7h) has only one antigenic component in common with 7F and 7A. In order to increase the knowledge about primary structure, conformation, and their relation to cross-reactivity, *Streptococcus*

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pneumoniae type 7B was chosen, and we now report on the structure of its capsular polysaccharide (S7B).

RESULTS AND DISCUSSION

Streptococcus pneumoniae type 7B, strain 1348/39, was grown overnight and crude polysaccharide material was obtained after treatment of the lysed cells with chloroform–1-butanol and fractional precipitation with ethanol. After extraction of the crude polysaccharide with phenol[4] and anion-exchange chromatography, a protein-free polysaccharide (S7B) was isolated.

Solvolysis of S7B with anhydrous hydrogen fluoride followed by hydrolysis with trifluoroacetic acid yielded glucose, 2-amino-2-deoxyglucose, rhamnose, and ribose in the ratios 2.0:0.4:1.8:0.4. The absolute configurations, determined by g.l.c. of the trimethylsilylated (+)-2-butyl glycosides[5], were L for the rhamnose and D for the other sugars. Methylation analysis of S7B revealed terminal D-ribose, 2- and 4-linked L-rhamnose, 4- and 3,4-linked D-glucose, and 6-linked 2-amino-2-deoxy-D-glucose (Table I). The low value for the proportion of the terminal D-ribose is probably due to the lower effective carbon response, *i.e.*, that it has five carbons and the other sugars six. A comparison of the molar ratios obtained when either trifluoroacetic acid or anhydrous hydrogen fluoride followed by trifluoroacetic acid was used to degrade S7B showed (Table I, columns A and B) that, with the latter, the proportion of 2-linked rhamnose had approximately doubled and that the proportion of 2-amino-2-deoxyglucose had increased, indicating the D-GlcN residue to be linked to position 2 of L-Rha in a repeating unit of seven sugar residues.

The ^{1}H-n.m.r. spectrum of S7B (Fig. 1) contained signals for seven anomeric

TABLE I

Methylation analysis of S7B and its hydrolysis products[a]

Sugar[b]	*Mole%*			
	A	B	C	D
2,3,4-Rha			12	
1,2,3,5,6-Glucitol				24[c]
1,2,5,6-Glucitol			36[c]	
2,3,5-Rib	16	3		
3,4-Rha	23	35	28	36
2,3-Rha	20	16		
2,3,6-Glc	22	18	15	26
2,3,4,6-GlcNAc			9	15
2,6-Glc	19	20		
2,3,4-GlcNAc		7		

[a] Key: *A*, hydrolysis with CF_3COOH; *B*, solvolysis with HF followed by hydrolysis with CF_3COOH; *C*, oligosaccharide HF-1; *D*, oligosaccharide HF-2. [b] 2,4-Rha = 2,4-di-*O*-methyl-L-rhamnose, *etc.* [c] Deuterium-labelled at C-1.

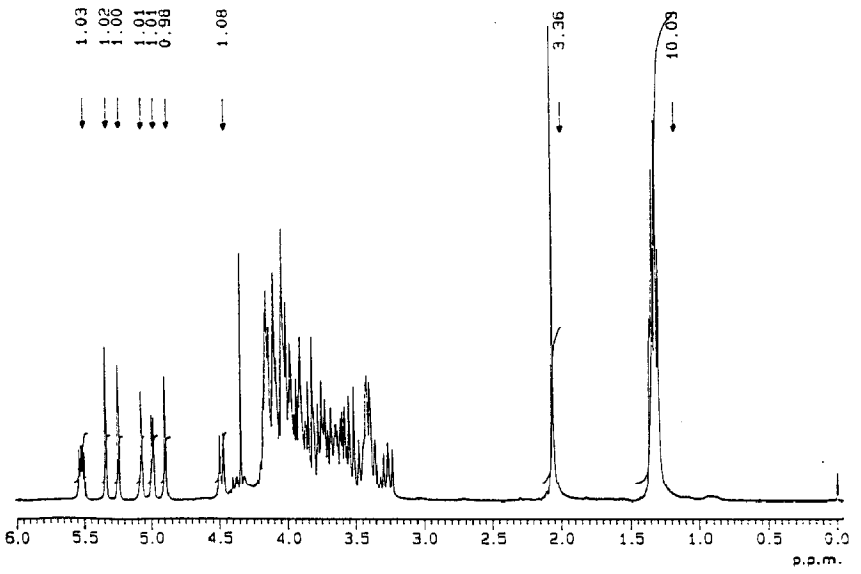


Fig. 1. ^{1}H-N.m.r. spectrum of S7B. The figures above the arrows refer to the integrals of the signals from the anomeric and deoxy protons.

protons with similar intensities. That the doubly coupled signal at δ 5.52 corresponded to an anomeric proton was evident from the corresponding ^{13}C signal at δ 96.0 ($^2J_{C,P}$ 6.0 Hz), and the fact that the signal had only one associated cross-peak in the H,H-COSY spectrum. The chemical shift of the ^{1}H resonance and the presence of the extra coupling indicated an anomerically linked phosphate[6] (see below). Thus, S7B was of the teichoic acid type. The ^{31}P signal at δ −0.64 verified the presence of a phosphate, and analysis[9] revealed phosphorus that corresponded to 7.3% of phosphate or ~1 phosphate per repeating unit. The ^{1}H-n.m.r. spectrum of S7B also contained signals for three CH*Me* groups (δ 1.25–1.40, 9 H). A signal at δ 2.06 (s, 3 H) and the stability of the acetate substituent to treatment with 0.1M NaOH for 15 h at room temperature indicated that the D-GlcN residue was *N*-acetylated.

In order to determine the anomeric configuration of the sugar residues and their sequence, the ^{1}H and ^{13}C resonances were assigned *via* H,H- and H,C-2D-n.m.r. techniques. The ^{13}C-n.m.r. spectrum of S7B is given in Fig. 2, and the ^{1}H- and ^{13}C-n.m.r. data in Tables II and III, respectively. The sugar residues are designated A–G according to the decreasing chemical shift of the H-1 signals. Residue B, with a C-1 signal at δ 109.2, was β-furanosidic. Ribose was furanosidic according to the methylation analysis (Table I) and, therefore, was identified as residue B. Residues C, D, and F were each recognised as rhamnose since each signal for H-1 had a small coupling constant. The chemical shift (δ 5.24) of the signal for H-1 indicated residue C to be α. The $J_{H\text{-}1,C\text{-}1}$ values for residues C, D, and F were 179, 170, and 159 Hz, respectively, which indicated that they were α, α, and β, respectively. The signal at δ 54.6 for residue E was assigned to C-2 in the 6-linked 2-acetamido-2-deoxy-D-glucose residue, which was α according to the $J_{1,2}$ value of 3.3 Hz for H-1. The remaining residues (A and G) must each be D-glucose, and

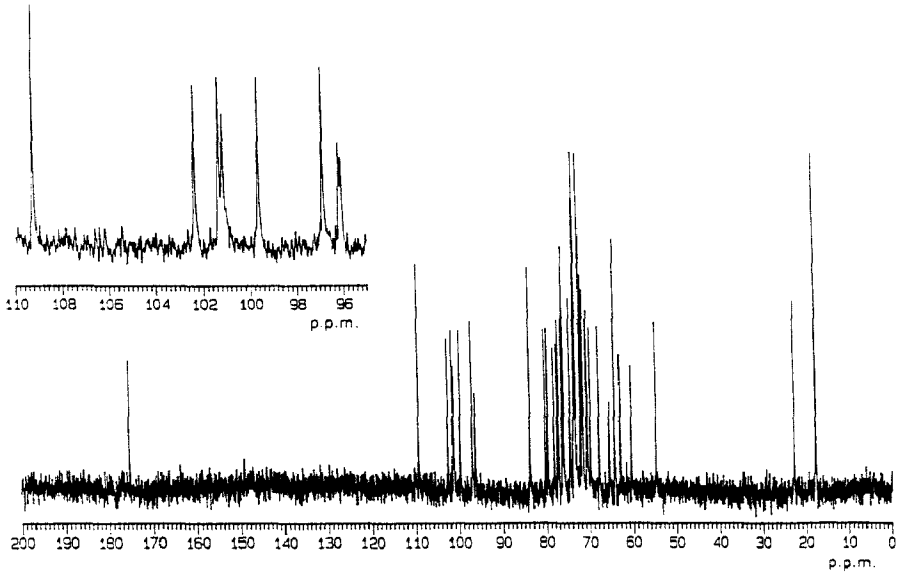


Fig. 2. ^{13}C-N.m.r. spectrum of S7B.

as the latter had a signal for H-1 at δ 4.48 (7.8 Hz) it was β. In a phase-sensitive H,H-COSY spectrum, the signal at δ 5.52 (dd) had only one associated cross-peak. The cross-peak contained a signal with coupling constants of 3.5 and 6.6 Hz from active and passive couplings, respectively. Thus, residue A was α. The chemical shift of the H-1 resonance and the additional 6.6 Hz coupling indicated the presence of an anomeric phosphate[6], which was verified by the 6.0 Hz coupling to the corresponding C-1. For all residues, glycosylation shifts of >2 p.p.m. were observed for at least one ^{13}C signal other than that of C-1, thus demonstrating the position of the linkage. Residue A had low glycosidation shifts (2.1 and 2.7 p.p.m., respectively) for the signals from C-3 and C-4, which can be attributed to the 3,4-substitution[7].

Two NOESY experiments with mixing times of 400 and 850 ms gave information on most of the inter-residue n.O.e.'s (Table IV). From these data and the linkage analysis, the presence of the disaccharide elements **1**–**6** was established.

β-D-Rib*f*-(1→4)-α-L-Rha*p*-(1→

1

→2)-α-L-Rha*p*-(1→2)-β-L-Rha*p*-(1→

3

→2)-β-Rha*p*-(1→4)-β-D-Glc*p*-(1→

5

→4)-α-L-Rha*p*-(1→3)-α-D-Glc*p*-(1→PO_3^-

4

↑

2

→6)-α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→

4

→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→PO_3

3

↑

1

6

TABLE II

^{1}H-N.m.r. data for *S. pneumoniae* type 7B polysaccharide

Sugar residue	*Chemical shift*[a] *(δ)*						
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*
→4)-α-D-Glc*p*-(1→	5.52[b] [3.5]	3.76	4.00	3.87	4.00	3.91	3.91
A 3	(0.28)	(0.22)	(0.28)	(0.45)	(0.16)	(0.19)	(0.01)
↑							
β-D-Rib*f*-(1→	5.33 [n.r.]	4.08	4.15	4.03	3.83[c], 3.71[d]		
B	(−0.04)	(0.08)	(−0.05)	(0.03)	(0.01, 0.05)		
→4)-α-L-Rha*p*-(1→	5.24 [n.r.]	~4.02	~4.01	3.55	4.36	1.31	
C	(0.12)	(0.10)	(0.20)	(0.10)	(0.50)	(0.03)	
→2)-α-L-Rha*p*-(1→	5.07 [n.r.]	4.09	3.96	3.51	4.06	1.30	
D	(−0.05)	(0.17)	(0.15)	(0.06)	(0.20)	(0.02)	
→6)-α-D-Glc*p*NAc-(1→[e]	4.99 [3.7]	3.95	3.82	3.60	4.14	~4.18	~4.18
E	(−0.22)	(0.07)	(0.07)	(0.11)	(0.28)	(0.40)	(0.33)
→2)-β-L-Rha*p*-(1→	4.89 [n.r.]	4.14	3.66	~3.40	~3.40	1.29	
F	(0.04)	(0.21)	(0.07)	(0.02)	(0.01)	(−0.01)	
→4)-β-D-Glc*p*-(1→	4.48 [7.7]	3.26	3.61	3.35	3.44	3.71[c]	4.06[d]
G	(−0.16)	(0.01)	(0.11)	(−0.07)	(−0.02)	(−0.01)	(0.16)

[a] *J* values (Hz) in square brackets and chemical shift differences compared to monomers in parentheses; n.r., not resolved. [b] $^3J_{H,P}$ 6.6 Hz. [c] pro-*R*. [d] pro-*S*. [e] *δ* 2.06 (NAc).

TABLE III

^{13}C-N.m.r. spectrum data for the *S. pneumoniae* type 7B polysaccharide

Sugar residue	*Chemical shift*[a] *(δ)*					
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
→4)-α-D-Glc*p*	96.0[b] [175]	73.6[c]	75.8	73.4	73.2	60.4
A 3	(3.0)	(1.1)	(2.0)	(2.7)	(0.7)	(−1.4)
↑						
β-D-Rib*f*-(1→	109.2[177]	76.0	72.0[d]	83.6	64.1	
B	(7.3)	(−0.2)	(0.6)	(0.1)	(0.7)	
→4)-α-L-Rha*p*-(1→	101.1[175]	71.4[e]	71.7[e]	80.2	67.7	17.8
C	(6.3)	(−0.4)	(0.7)	(7.0)	(−1.4)	(0.1)
→2)-α-L-Rha*p*-(1→	99.6[170]	77.4	70.5	73.1	69.7	17.8
D	(4.8)	(5.6)	(−0.5)	(−0.1)	(0.6)	(0.1)
→6)-α-D-Glc*p*NAc-(1→[f]	96.8[171]	54.6	71.6	70.7	72.1[d,g]	65.4[h]
E	(5.0)	(−0.4)	(−0.1)	(−0.6)	(−0.4)	(3.6)
→2)-β-L-Rha*p*-(1→	101.2[159]	78.0	74.3	73.1	73.4	17.8
F	(6.8)	(5.8)	(0.5)	(0.3)	(0.6)	(0.2)
→4)-β-D-Glc*p*-(1→	102.3[161]	74.4	77.0	79.4	76.2	62.9
G	(5.5)	(−0.8)	(0.2)	(8.7)	(−0.6)	(1.1)

[a] Chemical shift differences relative to monomers are given in parentheses and $J_{C\text{-}1,H\text{-}1}$ values are given in square brackets. [b] $^2J_{C,P}$ 6.0 Hz. [c] $^3J_{C,P}$ 7.8 Hz. [d] Interchangeable. [e] Interchangeable. [f] *δ* 24.0 ($NCOCH_3$), *δ* 175.9 ($NCOCH_3$). [g] $^3J_{C,P}$ 5–9 Hz. [h] $^2J_{C,P}$ 5.0 Hz.

TABLE IV

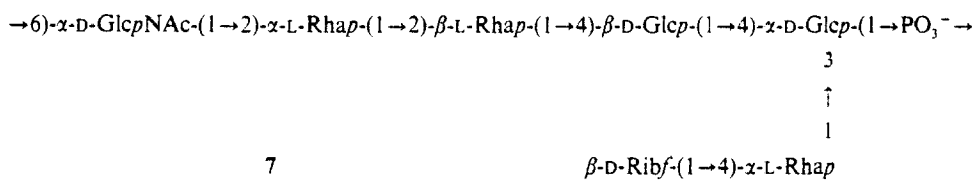
Observed n.O.e. contacts for the anomeric protons of S7B

Anomeric protons	*N.O.e. contacts to*
5.33 (Rib-B)	3.54 (Rha-C, H-4), 4.09 (Rib-B, H-2), 4.14 (Rib-B, H-3)
5.24 (→4-Rha-C)	4.00 (GlcA, H-3), 4.01 (Rha-C, H-2)
5.07 (→2-Rha-D)	4.10 (Rha-D, H-2), 4.14 (Rha-F, H-2), 4.99 (GlcNAc-E, H-1)
4.99 (→6-GlcNAc-E)	3.95 (GlcNAc-E, H-2), 5.07 (Rha-D, H-1)
4.89 (→2-Rha-F)	3.35 (Glc-G, H-4), 3.40 (Rha-F, H-5), 3.66 (Rha-F, H-3), 4.14 (Rha-F, H-2)
4.48 (→4-Glc-G)	3.26 (Glc-G, H-2), 3.44 (Glc-G, H-5), 3.61 (Glc-G, H-3), 3.87 (Glc-A, H-4), 4.00 (Glc-A, H-3)

For residue G, H-1 gave a signal at δ 4.48 and had a strong n.O.e. contact to H-4 and a weak contact to H-3 in the 3,4-linked D-glucose residue. The relative intensities of the cross-peaks that corresponded to n.O.e. contacts were approximately the same in the two NOESY experiments, which indicates that spin diffusion did not contribute to the observed n.O.e.'s and that the linkage involved O-4 in the glucose residue.

In order to test whether the β-D-Glc*p* and α-L-Rha*p* residues were either 3,4- or 4,3-linked to the branch-point residue, each of these trisaccharide elements was modelled using the GESA program[8]. For the trisaccharide with α-L-Rha*p* 3-linked and β-D-Glc*p* 4-linked, the distances between α-L-Rha*p* H-1 and H-3, and H-4 in the branch point are 2.35 and 4.49 Å, respectively, and 4.59 and 2.59 Å, respectively, for β-D-Glc*p*. In the reverse configuration, the corresponding distances are 4.53 and 2.37 Å, respectively, for α-L-Rha*p* H-1, and 2.58 and 4.54 Å, respectively, for β-D-Glc*p* H-1. These data further indicated that the n.O.e. contacts reflected the linkage positions, and that the first structure was correct.

The only residue for which no inter-residue n.O.e. contact from H-1 was observed was the branch-point residue, since the anomerically linked phosphate shielded H-1 on the neighbouring residue. Combination of the structural elements **1**–**6** yielded a heptasaccharide, which left the phosphate group as the only possibility for joining it *via* position 6 of the 2-acetamido-2-deoxy-D-glucose residue. Thus, it is concluded that the repeating unit has structure **7**.



Treatment of S7B with aqueous 48% hydrogen fluoride for 12 h at 4° followed by gel-filtration on Bio-Gel P-2 gave a mixture of oligosaccharides. When the oligosaccharide-alditols, obtained by reduction with borodeuteride, were chromatographed on

Bio-Gel P-2, the alditols HF1 and HF2 were isolated. The ^{1}H-n.m.r. spectrum of HF1 contained signals that corresponded to five anomeric protons at δ 5.08, 5.00 (3.7 Hz), 4.96, 4.92, and 4.54 (8.0 Hz) with approximately equal intensities. In addition, signals at δ 2.07 (s, 3 H) and several signals (10 H) in the range δ 1.25–1.40 were observed, which corresponded to NAc and CH*Me* groups of 2-acetamido-2-deoxyglucose and rhamnose residues, respectively. From these data and the ions in the f.a.b.-mass spectrum at *m/z* 987.6 for $(M + H)^+$ and 1009.6 for $(M + Na)^+$, it was concluded that HF1 was a hexasaccharide that lacked the phosphate and the terminal D-Rib*f* group. The results of methylation analysis (Table I) showed that HF1 was formed by cleavage of the phosphodiester and the β-furanosidic linkages. Furthermore, it was evident that the terminal ribose was 4-linked to Rha. From these inferences and the information on disaccharide elements **1**–**6**, it was concluded that HF1 had the structure **8**.

α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→2)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Glc-ol
3
↑
1
8 α-L-Rha*p*

The fact that the 6-linked α-GlcNAc residue in S7B had been replaced by a terminal α-GlcNAc group in HF1 verified element **9**.

→3)-α-D-Glc*p*-(1→PO_3^-→6)-α-D-Glc*p*NAc-(1→
4
↑
9

The f.a.b.-mass spectrum of HF2 contained ions at *m/z* 841.8 for $(M + H)^+$ and 863.7 for $(M + Na)^+$, which indicated a pentasaccharide-alditol that constituted the repeating unit without the terminal D-Rib group and one of the L-Rha residues. This conclusion was supported by the ^{1}H-n.m.r. spectrum which contained, *inter alia*, signals for anomeric protons at δ 5.09, 5.00 (3.3 Hz), 4.92, and 4.57 (7.7 Hz), and for methyl groups at δ 2.07 (s, 3 H), and 1.25–1.40 (7 H). The 1,2,3,5,6-penta-*O*-methyl-glucitol-*1-d* formed in the methylation analysis showed that HF2 had the linear structure **10**, and confirmed that the side chain in S7B was attached to position 3 of the branch point.

α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→2)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Glc-ol
10

From the antigenic formulas[1] of type 7F (7a and 7b), 7A (7a, 7b, and 7c), and 7B (7a, 7d, 7e, and 7h), it was expected that 7B should have at least one structural element in common with 7F and 7A (**11** and **12**, respectively).

→6)-α-D-Gal*p*-(1→3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→
2 2 4
↑ ↑ ↑
1 Ac 1
R α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*

11 R = β-D-Gal*p*
12 R = H

The disaccharide **4**, which in 7F and 7A constitutes the side chain, is also present in 7B but as a part of the main chain. Estimation of the minimum energy conformations of the repeating units of S7F, S7A, and S7B, using the GESA program, indicated that **4**, which is well exposed in S7F and S7A, is also exposed to a considerable extent in S7B. This finding indicated that this disaccharide could be the structural basis for the common antigenic formula. Another disaccharide element, **13**, is also present in each of these polysaccharides but only as an internal fragment, and is thus less likely to be the common factor.

β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→
13

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at <40° or in a stream of air. For g.l.c., a Hewlett–Packard 5890 instrument fitted with a flame-ionisation detector was used. G.l.c.–m.s. (e.i.) was performed on a Hewlett–Packard 5970 MSD instrument. F.a.b.-mass spectra were recorded on a JEOL SX 102 instrument with Xe atoms (6 keV) and a matrix of glycerol.

Alditol acetates were analysed on an HP-5 capillary column (25 m × 0.20 mm × 0.33 μm) using the temperature program 200° (3 min) →250° at 2°/min or at 220° on a DB-225 column. Partially methylated alditol acetates were analysed on the HP-5 column, using the temperature program 150° (2 min) →220°. Gel filtrations were performed on Bio-Gel P-2, using water that contained 1% of 1-butanol, and monitored using a differential refractometer. H.p.l.c. was performed with a Shimadzu LC6A system, including a u.v. detector (195 nm). All fractionations were isocratic, using a reversed-phase C-18 (μBondapak) column and water containing 0.1% of acetonitrile. Phosphate was determined according to Chen *et al.*[9].

Preparation of the crude polysaccharide. — *S. pneumoniae* type 7B, strain 1348/39 (from the collection of the WHO Collaborating Centre for Reference and Research on Pneumococci, Statens Seruminstitut, Copenhagen), was grown overnight in serum broth (Statens Seruminstitut). The cells were then lysed with sodium deoxycholate, most of the proteins were removed by treatment with chloroform–1-butanol, nucleic acids were precipitated with aqueous 25% ethanol that contained 1% of $CaCl_2$, and the

polysaccharide was precipitated from the supernatant solution with aqueous 80% ethanol.

Purification of the polysaccharide. — The crude polysaccharide (300 mg) was deproteinised using phenol–water extraction[4], and the water phase was dialysed and freeze-dried (96 mg). The residue was fractionated on a column (1.6 × 20 cm) of DEAE-Trisacryl, which was irrigated first with 0.01M NaH_2PO_4 (100 mL) and then with a linear gradient of sodium chloride (0→0.5M) in 0.01M NaH_2PO_4. The polysaccharide was eluted between 0.16 and 0.20M NaCl, as detected by the anthrone test, and the solution was desalted on a column (2.5 × 90 cm) of Bio-Gel P-2 to give the polysaccharide (53 mg, 17%).

Sugar and methylation analysis. — The native and methylated polysaccharides were each kept in anhydrous hydrogen fluoride for 3 h at room temperature. After evaporation of the solvent, the residues were treated with 2M trifluoroacetic acid at 120° for 3 h. The sugars in the hydrolysates were then converted into the alditol acetates and partially methylated alditol acetates. The absolute configurations of the sugars were determined according to Gerwig *et al.*[5].

N.m.r. spectroscopy. — N.m.r. spectra of solutions in D_2O were recorded at 70° (^{13}C), 85° (^1H), and 30° (^{31}P), using either a JEOL GX-400 or GSX-270 instrument. Chemical shifts are reported in p.p.m., using sodium 3-trimethylsilylpropanoate-d_4 (TSP, δ 0.00) for ^1H and acetone (δ 31.07) for ^{13}C, as internal references, and aqueous 85% H_3PO_4 (δ 0.00) as external reference for ^{31}P. H,H-COSY, H,H-relayed COSY, NOESY, and H,C-COSY experiments were performed using JEOL standard-pulse sequences. H,H-COSY was performed in the phase-sensitive double-quantum-filter mode and the H,H-double-relayed COSY was performed according to Bax and Drobny[10]. Relayed COSY spectra were obtained using a delay time of 30 or 60 ms. The double-relayed COSY was performed with delay times of 60 and 30 ms. The $J_{H\text{-}1,C\text{-}1}$ value for each C-1 was determined by an INEPT experiment, using a delay time of 1.8 ms, and the multiplicities for ^{13}C resonances were established *via* a ^1H-decoupled DEPT experiment, using P_θ 135° and a delay time of 3.58 ms.

Partial hydrolysis. — The polysaccharide (36 mg) was treated with aqueous 48% HF (2 mL) at 4° for 12 h. The solution was concentrated to dryness under vacuum, neutralised with M NH_4OH, and applied on a column (95 × 2.5 cm) of Bio-Gel P-2. The oligosaccharide fraction (9 mg), eluted at 1.5 void volumes, was reduced with $NaBD_4$ and the product was rechromatographed on a column of Bio-Gel P-2. Two oligosaccharide-alditols were isolated, HF1 (3.5 mg) and HF2 (4.5 mg), and tested for purity with h.p.l.c. using a reversed-phase column.

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