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APPLICATION OF HIGH-RESOLUTION N.M.R. SPECTROSCOPY TO THE ELUCIDATION OF THE STRUCTURE OF THE SPECIFIC CAPSULAR POLYSACCHARIDE OF *Streptococcus pneumoniae* TYPE 7F*

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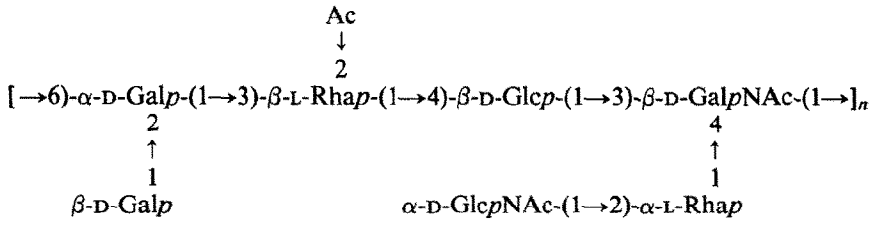
ABSTRACT

The specific capsular polysaccharide of *Streptococcus pneunomiae* type 7F (American type 51) is a high-molecular-weight neutral polymer composed of 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-glucose, D-glucose, D-galactose, L-rhamnose, and 2-*O*-acetyl-L-rhamnose residues. N.m.r. spectroscopy (^1H and ^{13}C), in conjunction with composition and methylation analyses, and periodate oxidation data, showed the polysaccharide to be a branched polymer with a repeating heptasaccharide unit having the following structure.



INTRODUCTION

The chemical and immunological properties of the cell-surface capsular polysaccharides of *Streptococcus pneumoniae* serotypes have received considerable attention because of their use as vaccines for the prevention of pneumococcal infections[1,2]. Eighty-four serologically-defined types of *S. pneumoniae* are known, of which the highly cross-reacting types have been classified into groups[3]. Group 7 is

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composed of four serotypes, 7F, 7A, 7B, and 7C, with the type 7F accounting for 96% of diseases caused by group 7 pneumococci and it is included as a component of a 23-valent pneumococcal vaccine[4].

The composition of the type 7F pneumococcal polysaccharide has been reported on by two groups[5-7] who found D-Gal, D-Glc, L-Rha, D-GlcNAc, and D-GalNAc as the component glycoses, but in different molar proportions. Although immunological and chemical studies revealed several features of structural significance, structural analysis of the type 7F polysaccharide, employing many of the conventional methods, provided only partial information. We now report the application of ^{1}H-(500 MHz) and ^{13}C-n.m.r. spectroscopy (125 MHz) in the structural elucidation of the type 7F polysaccharide.

EXPERIMENTAL

Type 7F pneumococcal polysaccharide. — Type 7F polysaccharide was obtained by precipitation with ethanol from the growth medium of *S. pneumoniae* type 7F, after digestion with trypsin, ribonuclease, and deoxyribonuclease, and fractional precipitation with 2-propanol with serological probing[8].

Chromatographic methods. — (*a*) *Ion-exchange chromatography.* Crude type 7F polysaccharide (50 mg) was deionized with Rexyn 101 (H^+) resin (5 mL). The concentrated eluate was applied to a column (3 x 40 cm) of DEAE-Sephacel (Pharmacia), equilibrated with 0.05M Tris-HCl buffer (pH 7.2), and eluted with the buffer (50 mL) followed by a 0→2M NaCl gradient in the same buffer (100 mL). Fractions (5 mL) were collected and monitored.

(*b*) *Gel filtration.* Samples of polysaccharide were eluted from columns (2.2 x 30 cm) of Sephadex G-50 with 0.05M pyridinium acetate (pH 4.5) or from columns (1.6 x 70 cm) of Sepharose 6B with 0.01M phosphate-buffered saline (pH 6.8). Fractionations of samples of oligosaccharide were made on a column (1.5 x 95 cm) of Bio-Gel P2 (−400) eluted with distilled water.

Column eluates were monitored, using a Waters R403 differential refractometer, and fractions were assayed colorimetrically for neutral glycose[9], aminodeoxyglycose[10], and phosphate[11]. The gel-filtration properties of the eluted materials are expressed as $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_o is the void volume of the system, V_e is the elution volume of the specific material, and V_t is the total volume of the system.

(*c*) *T.l.c.* Analytical and preparative t.l.c. was performed on Silica Gel 60 (Merck), using *A*, 1-propanol–0.88 ammonia–water (6:3:1); *B*, ethyl acetate–acetic acid–methanol–water (12:3:3:5). The mobility of components (R_{SUC}) are recorded relative to that of sucrose.

(*d*) *G.l.c.* A Hewlett-Packard model 5710A chromatograph was used with a hydrogen flame-ionization detector, a model 3380A electronic integrator, and a fused-silica OV17-coated capillary column (0.3 mm x 25 m), with the following temperature programs: *A*, 180° (for 2 min) → 240° at 4°/min; *B*, 180° (for 2 min)

→ 320° at 10°/min; *C*, 200° (for 2 min) → 240° at 2°/min. The carrier gas was dry nitrogen at 30 mL/min and retention times are given relative to those of D-glucitol hexa-acetate (T_{GA}), 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (T_{GM}), or methylated cellobiitol (T_{CM}).

G.l.c.-m.s. was performed with a Hewlett-Packard 5958B system, employing conditions *A*–*C*, by e.i. (70 eV) or c.i. (isobutane).

Analytical methods. — The phenol–sulfuric acid method was used for neutral glycoses[9], the modified Elson–Morgan method for aminodeoxyglycoses[10], and the method of Chen *et al.*[11] for phosphate.

Glycoses were determined by g.l.c. (program *A*) of their alditol acetate derivatives[12], using inositol as internal standard. Samples (2 mg) were hydrolyzed in sealed glass tubes with 2M trifluoroacetic acid (1 mL) for 6 or 15 h at 100°, or with 10M hydrochloric acid (1 mL) for 20 min at 90°, followed by concentration to dryness *in vacuo*. In other analyses, samples (2 mg) of polysaccharide were hydrolyzed with M hydrochloric acid (0.5 mL) for 12 h at 100°, the hydrolysates were concentrated to dryness, and the aminodeoxyglycose-containing residues were treated[13] with nitrous acid.

The configurations of the glycoses were determined[14] by g.l.c. (program *A*) of their trimethylsilylated (−)-2-butyl glycosides[14]. Prior to derivatization, samples of polysaccharides were hydrolyzed and the glycoses were separated by t.l.c. (solvent *B*).

The identity of each glycose was established by comparison (g.l.c.-m.s.) with authentic compounds.

Methylation analysis. — Samples (2 mg) of polysaccharide were methylated[15], using sodium methylsulfinylmethanide and methyl iodide in methyl sulfoxide, and the products were purified by filtration through C_{18} Sep-pak cartridges (Waters)[16]. Oligosaccharides were methylated by the above procedure, but the products were recovered by extraction into dichloromethane and analyzed by g.l.c.-m.s. (program *B*).

Methylated products were hydrolyzed with 2M trifluoroacetic acid for 15 h at 100°, then reduced with sodium borodeuteride, acetylated[17], and analyzed by g.l.c.-m.s. (program *C*).

N.m.r. spectroscopy. — Measurements were made at 37° on solutions (0.4 mL; 0.03–0.05M for oligosaccharides, 25 mg/mL for polysaccharides in D_2O) (99.8 atom %) of samples which had been lyophilized twice from D_2O. Spectra were obtained using Bruker AM500 or AM200 spectrometers equipped with Aspect 3000 computers, operating in the pulsed F.t. mode, with quadrature detection using 5-mm probeheads, and standard Bruker software.

^{1}H-N.m.r. spectra (500 MHz) were recorded using a spectral width of 2.5 kHz, a 16 K data block for a digital resolution of 0.3 Hz/point, an acquisition time of 3.2 s, and a 90° pulse. Before Fourier transformation, resolution enhancement was achieved by the Gaussian line-shape transformation[18] with typical values of −2.0 to −4.0 Hz for the line-broadening factor and a Gaussian broadening factor of +0.1

or +0.2. Chemical shifts are expressed relative to internal acetone (0.1%; 2.225 p.p.m.). T_1 relaxation times were determined by the non-selective inversion recovery method[19]. N.O.e. difference spectra[20] were obtained by applying a selective low-power presaturation pulse (44dB below 0.2 W) for 200 ms followed by a 90° observation pulse. The sum of the acquisition time and the relaxation delay did not exceed four T_1 values. Each line of a multiple resonance was irradiated sequentially for 25 ms during the presaturation delay[21], eight transients were acquired for irradiation of each multiplet and for an off-resonance reference spectrum, and the entire sequence was cycled to achieve a satisfactory signal-to-noise ratio.

Broad-band proton-decoupled ^{13}C-n.m.r. spectra were obtained at 125 MHz for polysaccharides or 50 MHz for oligosaccharides. For the polysaccharides, a 25-kHz spectral width was empolyed, using a 90° pulse (8 μs) and a 32 K data set for a digital resolution of 1.5 Hz/point. Heating effects were minimized by employing composite pulse decoupling (WALTZ) (~ 1 W)[22]. The $^1J_{C,H}$ values for the anomeric carbons were measured using gated decoupling[23]. DEPT spectra[24] were obtained with broad-band proton decoupling, a 135° proton pulse, and a 3.3-ms delay [$0.5/(^1J_{C,H})$] between pulses to distinguish between methine and methylene carbon resonances. Chemical shifts are referenced to external 1,4-dioxane (67.40 p.p.m.).

2D Homonuclear shift-correlated (COSY) experiments involved the conventional pulse sequence (90°-t_1-90°-t_2-T)[25-27]. Spectra were obtained using matrices (t_1 x t_2) of 256 x 1024 or 512 x 2048 data points that were zero-filled to 512 x 1024 or 1024 x 2048 points, respectively, and, following resolution enhancement in both dimensions by means of a non-shifted sine bell window function[28], the doubly transformed data were processed to give magnitude spectra[29] with symmetrization[30]. Spectral widths of 2500 or 1200 Hz were employed. A minimum recycle delay (T) of 2.0 s was used and 32 transients were collected for each value of the incrementable delay (t_1). 2D J-Resolved spectra[31,32] were obtained using a data matrix of 64 x 2048 points and spectral widths of 2702 Hz and ±21.1 Hz in the chemical shift (t_2) and the coupling constant (t_1) dimensions, respectively. The data matrix was zero-filled to 128 x 2048 points and a non-shifted sine bell window function[28] was applied in both time domains. The transformed data matrix was tilted 45°[32].

^{1}H–^{13}C chemical shift correlations were achieved using the CHORTLE technique[33]. Four "cosine"/"sine" pairs of ^{13}C-n.m.r. spectra were acquired for the τ values, 0.16, 1.00, 2.40, and 3.20 ms, using 8 K data sets. The 90° pulses were 20 μs for the ^{1}H decoupler and 12.5 μs for the ^{13}C transmitter, a 1.5 recycle delay was employed, and 3584 transients were acquired for each of the four spectra. The ^{1}H resonance offsets were calculated from the ^{13}C intensities by a non-linear least-squares calculation, using a program developed by Dr. J.-R. Brisson (N.R.C., Ottawa).

O-Deacetylation of the type 7F polysaccharide. — A solution of the type 7F polysaccharide (30 mg) in aqueous 5% ammonia was kept for 5 h at 37° and then lyophilized.

Periodate oxidation. — Polysaccharide (30 mg) was treated in the dark with

0.05M sodium metaperiodate (30 mL) for 4 days at 4° and, following reduction of the excess of periodate with ethylene glycol (0.4 mL), the oxidized polymer was reduced with sodium borohydride (300 mg) for 15 h at 4°. The solution was neutralized with dilute acetic acid and then dialyzed against water until salt-free. Smith-type hydrolysis[34] of the product was effected with aqueous 1% acetic acid (7 mL) for 4 h at 100° followed by concentration to dryness. The products were treated with sodium borodeuteride prior to fractionation by gel filtration on Bio-Gel P2.

Deacetylation and deamination of the type 7F polysaccharide. — A solution of the type 7F polysaccharide (30 mg) in 2M sodium hydroxide (3 mL) containing sodium borohydride (1 mg) was heated for 4 h at 100°, then cooled, neutralized with 2M HCl, and decationized with Rexyn 101 (H^+) resin (2 mL). The partially *N*-deacetylated polysaccharide was recovered by lyophilization and a solution in aqueous 16% acetic acid (1 mL) was treated with freshly prepared aqueous 5% sodium nitrite (0.5 mL). After storage for 30 min at 25°, the mixture was deionized with Rexyn 101 (H^+) resin (2 mL) and lyophilized, and the residue was fractionated on Sephadex G-50.

Immunodiffusion. — Double diffusion studies[35] were made at 4° in 1% agarose gels, using polysaccharide concentrations of 1 mg/mL, and precipitin lines were allowed to develop during 3 days. Type 7F pneumococcal antiserum (H 937 Bled, 11-4-37) was a gift from Dr. M. Heidelberger (New York University Medical Center). Mouse serum from BALB/c mice bearing plasmatoma MOPC-603, used as a source of myeloma protein specific for phosphocholine[36], was obtained from Dr. N. M. Young (N.R.C.). Pneumococcal C-substance, isolated from the cell walls of *S. pneumoniae* type 1 organisms[37], was obtained from Dr. H. J. Jennings (N.R.C.) and was used at a concentration of 4 mg/mL.

General. — Commercial reagents and solvents were analytical grade. Concentrations were made under reduced pressure at $<40°$. Optical rotations were determined at 22° in 10-cm microtubes, using a Perkin-Elmer 243 polarimeter.

RESULTS AND DISCUSSION

The crude specific capsular polysaccharide, obtained from the growth medium of *S. pneumoniae* type 7F, was a water-soluble white powder. On fractionation of this material on DEAE-Sephacel, the specific polysaccharide was eluted as the void-volume fraction (65%) and a second polysaccharide-containing fraction (35%) was eluted as a broad peak at the beginning of the salt gradient. The two fractions showed similar contents of hexose and hexosamine, but the latter fraction also contained PO_4 (3.9%). Each fraction gave a positive precipitin reaction in immunodiffusion against type 7F pneumococcal antisera. The major fraction gave a single sharp precipitin line, whereas the minor fraction gave an additional more diffuse band and also gave a positive precipitin reaction against a myeloma protein[36] known to be specific for phosphocholine substituents. The presence of a phosphocholine substituent was also indicated by the ^{1}H-n.m.r. spectrum of this fraction, which

contained a sharp singlet (3.28 p.p.m.) characteristic of choline methyl protons which was absent from the spectrum of the major component. The ^{13}C-n.m.r. spectra of the two fractions were similar, but the minor fraction contained additional minor resonances at 105.2, 102.4, 99.3, 94.6 (anomeric carbons), 55.9, 51.8, 50.5, 49.4 (amino-substituted carbons), 55.1 (CH_3 of choline), and 16.6 p.p.m. (CH_3 of 6-deoxyhexose), which are attributed to pneumococcal C-substance[37,38], a common phosphocholine-containing cell-wall polysaccharide observed in several strains of *S. pneumoniae*. Authentic pneumococcal C-substance, on immunodiffusion, gave a positive precipitin reaction against type 7F pneumococcal antisera which was similar to, but not continuous with, the diffuse band produced by the minor fraction.

When the minor fraction was rechromatographed on DEAE-Sephacel, pure specific phosphate-free polysaccharide was obtained together with fractions enriched in pneumococcal C-substance as evidenced by their ^{1}H- and ^{13}C-n.m.r. spectra. Similar elution profiles on DEAE-Sephacel have been observed with specific polysaccharide preparations of *S. pneumoniae* type 33F[39], and other type 7F polysaccharide preparations were heavily contaminated with pneumococcal C-substance which proved difficult to separate from the specific polysaccharide[5,6]. Therefore, it was concluded that the material not retained on the DEAE-Sephacel was pure type-specific polysaccharide and all subsequent investigations were made on this material.

The purified type 7F pneumococcal polysaccharide had $[\alpha]_D + 68°$ (*c* 1, water), a high molecular weight as evidenced by its elution point (K_{av} = 0.1) on Sepharose 6B, and, in agreement with previous reports[5–7], contained both neutral

TABLE I

COMPOSITION OF THE *S. pneumoniae* TYPE 7F POLYSACCHARIDE[a]

Glycose	T_{GA} *(alditol acetate)*	*Composition (% "anhydroglycose")*
L-Rha	0.45	20[b], 24[c], 18[d]
D-Glc	1.00	18[b,c]
D-Gal	1.03	34[b,c]
D-GlcN	1.57	13[b,c], 13[d,e]
D-GalN	1.66	12[b,c], 15[d,f]

[a]Determined by g.l.c (program *A*) of the derived alditol acetates, employing the acid hydrolysis conditions in *b–d*. [b]Hydrolysis with 2M trifluoroacetic acid (6 h, 100°). [c]Hydrolysis with 10M hydrochloric acid (20 min, 90°). [d]Hydrolysis with 0.5M hydrochloric acid (15 h, 100°), followed by deamination with nitrous acid. [e]Determined as 2,5-anhydromannitol (T_{GA} 0.53). [f]Determined as 2,5-anhydrotalitol (T_{GA} 0.58).

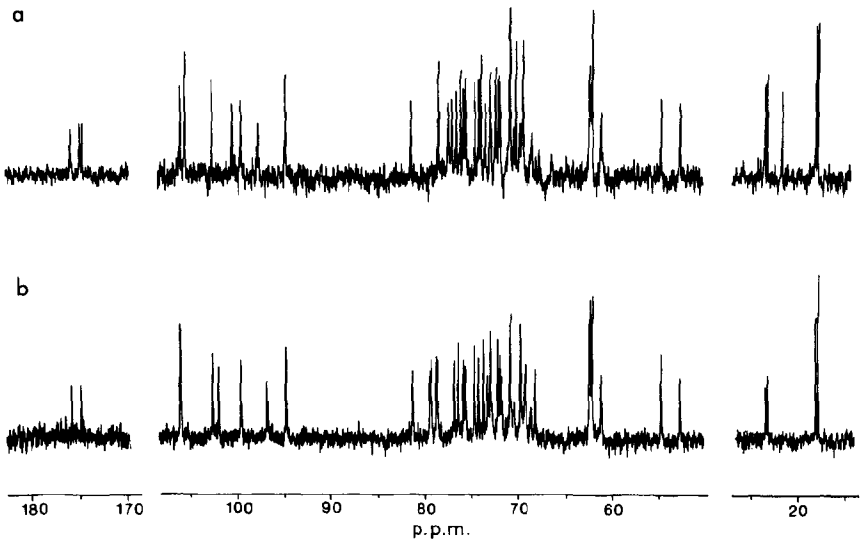


Fig. 1. Proton-decoupled ^{13}C-n.m.r. spectra of the type 7F *S. pneumoniae* specific capsular polysaccharide recorded at 37°: (a) native polysaccharide, (b) *O*-deacetylated polysaccharide.

(71%) and aminodeoxyglycose (27%) components. The presence of L-Rha, D-Glc, D-Gal, D-GlcN, and D-GalN was established and their absolute configurations were determined by g.l.c. of the trimethylsilyl derivatives of their R-(−)-2-butyl glycosides[14] and of the derived acetylated alditols[12]. After employing various conditions

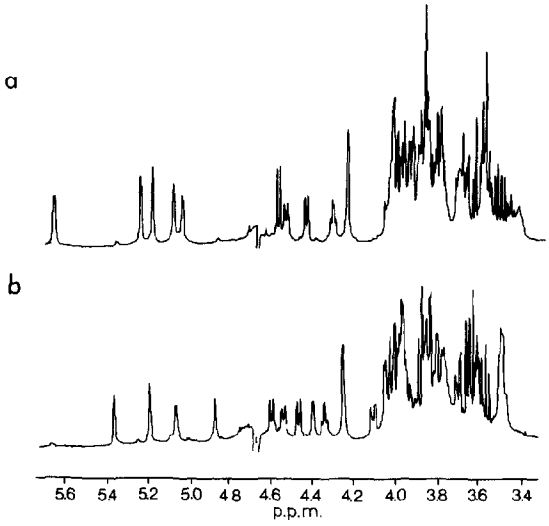


Fig. 2. ^{1}H-N.m.r. spectra for the ring-proton region of the type 7F *S. pneumoniae* specific capsular polysaccharide recorded at 37°: (a) native polysaccharide, (b) *O*-deacetylated polysaccharide.

TABLE II

METHYLATION DATA FOR THE NATIVE TYPE 7F POLYSACCHARIDE AND ITS PARTIALLY *N*-DEACETYLATED AND DEAMINATED PRODUCT

Derivative	T_{GM}	*Mole ratio*[a]	
		Native polysaccharide	*Partially* N-*deacetylated deaminated polysaccharide*
1,5-Di-O-acetyl-2,3,4-tri-*O*-methyl-L-rhamnitol-*1-d*	0.62[b]		0.59
1,2,5-Tri-*O*-acetyl-3,4-di-*O*-methyl-L-rhamnitol-*1-d*	0.84	0.43	trace
1,3,5-Tri-*O*-acetyl-2,4-di-*O*-methyl-L-rhamnitol-*1-d*	0.92	0.94	1.18
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol-*1-d*	1.08	1.00	1.00
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol-*1-d*	1.45	1.03	0.97
1,2,5,6-Tetra-*O*-acetyl-3,4-di-*O*-methyl-D-galactitol-*1-d*	2.25	0.95	0.92
1,5-Di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol-*1-d*	2.72	0.35	trace
1,3,4,5-tetra-*O*-acetyl-2-deoxy-6-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol-*1-d*	4.12	0.66	0.36

[a]Relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol. [b]G.l.c. (program *C*).

of acid hydrolysis, including initial deamination with nitrous acid (Table I), it appeared that L-Rha, D-Glc, D-Gal, D-GlcN, and D-GalN were present in the molar ratios ~1:1:2:1:1. This conclusion, however, was not supported by the ^{1}H- and ^{13}C-n.m.r. data. Moreover, methylation analysis indicated the content of L-Rha to be twice that noted above.

The ^{1}H- and ^{13}C-n.m.r. data for the native polymer indicated that two L-Rha residues were present. Thus, the ^{1}H-n.m.r. spectrum contained two 3-proton high-field doublets at 1.33 ($J_{5,6}$ 6.2 Hz) and 1.41 p.p.m. ($J_{5,6}$ 5.5 Hz), and the corresponding ^{13}C resonances (Fig. 1a) were at 17.57 and 17.82 p.p.m. Furthermore, there were seven resonances for anomeric carbons, indicating a heptasaccharide repeating-unit. The $^1J_{C,H}$ values revealed three α and four β monosaccharide residues. Characteristic ^{13}C signals at 52.37 and 54.41 p.p.m., together with signals at 174.59, 175.58, 22.98, and 23.20 p.p.m., indicated the presence of the two

2-acetamido-2-deoxyglycose residues[40]. The ^{13}C resonances at 174.32 and 21.41 p.p.m. revealed an OAc group and *O*-deacetylation resulted in the loss of these signals (Fig. 1b). The integrated intensity of the ^{1}H resonance for OAc (1.91 p.p.m., s, 3 H), when compared to those (2.21, s, 3 H; 2.22 p.p.m., s, 3 H) of the NAc groups, suggested a single location of the OAc group. Seven resonances for anomeric protons were readily identified between 4.4 and 5.4 p.p.m. in the spectrum of the *O*-deacetylated polymer (Fig. 2b). The ^{1}H-n.m.r. spectrum of the native polysaccharide (Fig. 2a) also contained a low-field doublet at 5.63 p.p.m., which could be attributed to *H*COAc.

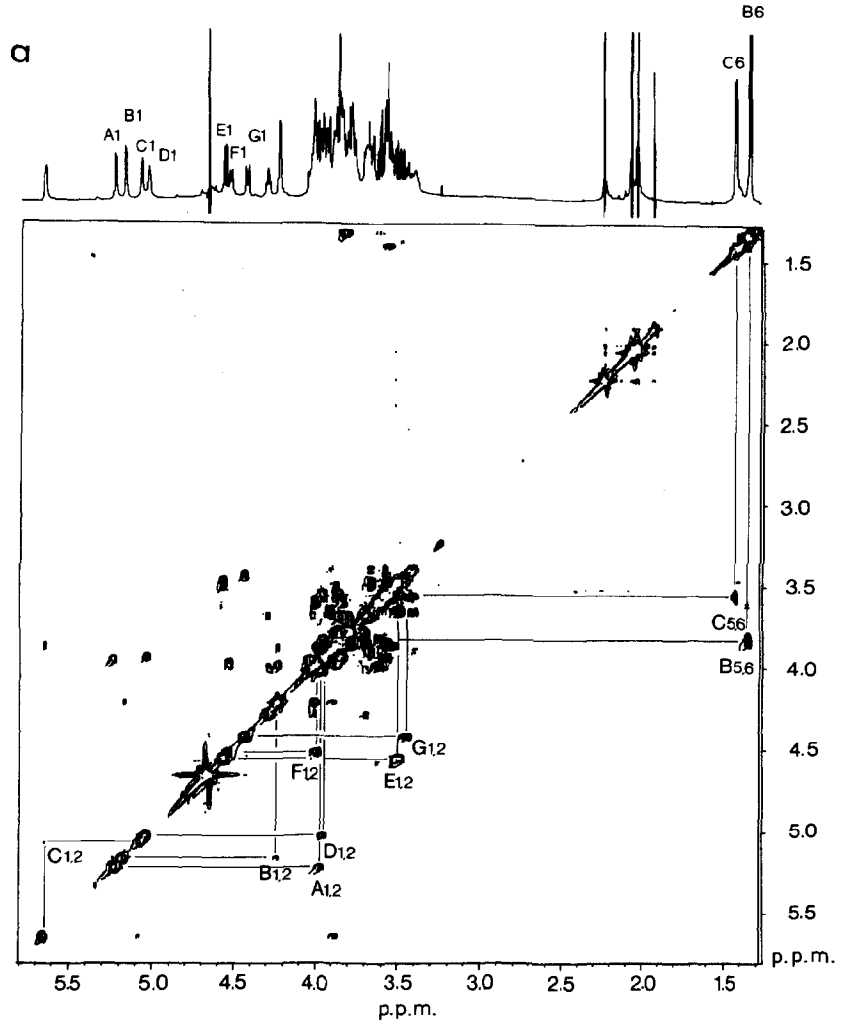


Fig. 3a. COSY contour plot of the complete spectrum (5.80-1.30 p.p.m.) for the native type 7F pneumococcal polysaccharide.

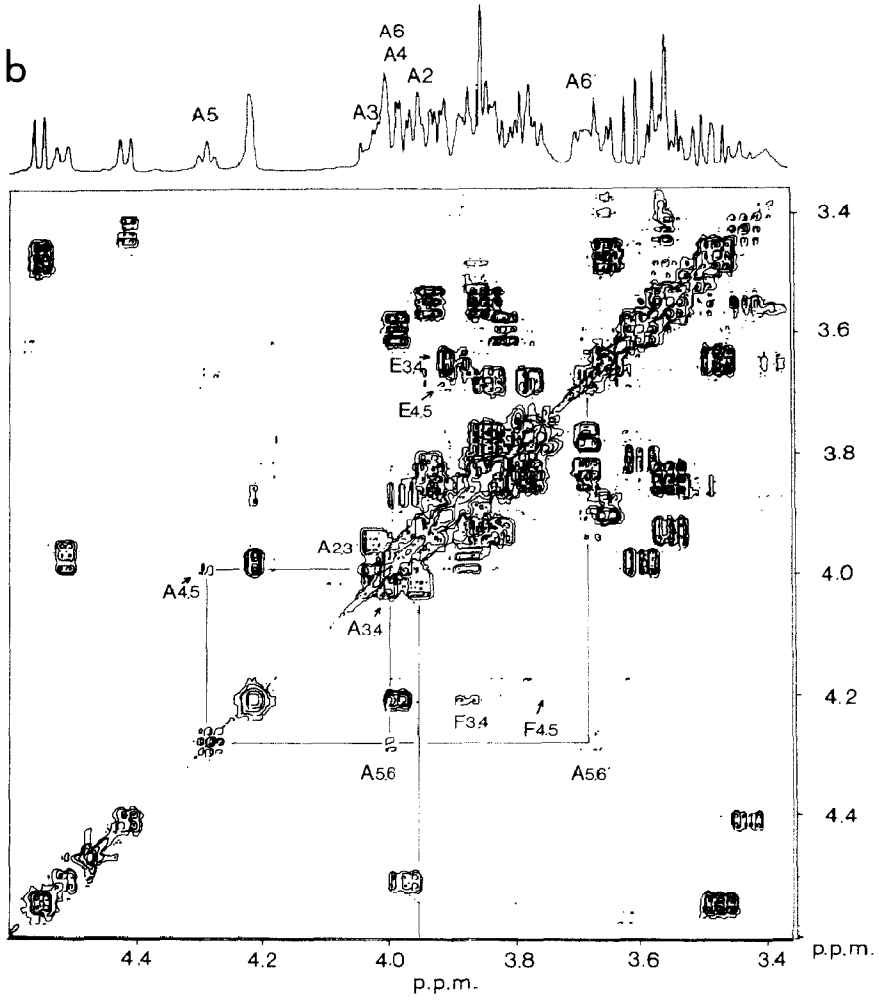


Fig. 3b. COSY contour plot for the ring proton region (4.60–3.35 p.p.m.) for the native type 7F pneumococcal polysaccharide.

The methylation analysis data for the type 7F polysaccharide are recorded in Table II. Approximately equimolar amounts of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4-di-*O*-methyl-L-rhamnose, 2,3,6-tri-*O*-methyl-D-glucose, and 3,4-di-*O*-methyl-D-galactose were obtained, whereas smaller amounts of 2-deoxy-3,4,6-tri-*O*-methyl-2-methylamino-D-glucose, 2-deoxy-6-*O*-methyl-2-methylamino-D-galactose, and 3,4-di-*O*-methyl-L-rhamnose were obtained. Although low values for the proportion of partially methylated amino sugars are not uncommon, it is unclear why a low yield of one of the rhamnose residues was obtained, both in the methylation analysis (Table II) and after employing various conditions for acid hydrolysis (Table I).

Thus, the type 7F polysaccharide has a heptasaccharide repeating-unit composed of L-Rha, D-Glc, D-Gal, D-GlcNAc, D-GalNAc, and OAc in the molar ratios 2:1:2:1:1:1. One D-Gal is 2,6-disubstituted, the D-GlcNAc is 3,4-disubstituted,

one D-Gal and D-GlcNAc are non-reducing end groups, and the remaining monosaccharide residues are mono-substituted.

The sequence of sugars in repeating units was determined by 1D- and 2D-n.m.r. techniques.

The COSY spectra of the native type 7F polysaccharide are shown in Fig. 3. The anomeric proton resonances were well resolved in the low-field region (4.4–5.4 p.p.m.) of the spectrum and were readily identified on the basis of the ^3J values. These resonances were assigned arbitrarily the notation *A*1–*G*1 in order of decreasing chemical shift (5.22–4.41 p.p.m.). The cross-peaks relating H-1,2 were well resolved for each residue and lead to the location of *A*2–*G*2 as indicated in Fig. 3a. Thus, the signal at lowest field (5.63 p.p.m.; cross-peaks at 5.06 and 3.86 p.p.m.) was assigned to H-2 of residue *C*. Residues *C* and *B* were identified as the two L-Rha residues (small $J_{1,2}$ and $J_{2,3}$ values). The signals for H-5 of the two L-Rha residues were located from the high-field position of the H-6 resonances (1.33 and 1.41 p.p.m.) which gave cross-peaks in an unobscured spectral region.

Many of the cross-peaks in the region 3.4–4.0 p.p.m. were close to the diagonal and were not well resolved in the full-scale COSY spectrum (Fig. 3a). When the COSY experiment was made using a narrow sweep-width (1200 Hz) and a larger data set, much better digital resolution was achieved (Fig. 3b) which permitted assignment of almost all the proton resonances (Table III). Thus, the connectivity pathway relating H-2,3,4 of residue *A* was readily discernible, even though the relevant cross-peaks ($A_{2,3}$ and $A_{3,4}$) were close to the diagonal. The enhanced resolution of these cross-peaks also facilitated the measurement of the $J_{1,2}$ (3.4 Hz), $J_{2,3}$ (9.7 Hz), and $J_{3,4}$ (3.3 Hz) values, which indicated residue *A* to be a α-D-Gal*p* residue. The cross-peak $A_{4,5}$, relating H-4 (3.99 p.p.m.) and H-5 (4.28 p.p.m.) of residue *A*, was of low intensity and not resolved completely from that ($A_{5,6}$) relating H-5 and H-6 (4.01 p.p.m.). The low intensity of the cross-peak correlating the H-4,5 resonances reflects the small value (~1 Hz) of $J_{4,5}$ typical of Gal*p*. The cross-peaks relating H-4,5 of residues *E* and *F* were also of low intensity and n.O.e measurements confirmed the assignments of *E*5 (3.68 p.p.m.) and *F*5 (3.76 p.p.m.). Assignment of the remaining ^{1}H resonances was straightforward except for H-6,6′ of residues *D* and *F*, which, together with the H-6,6′ of residue *E*, occurred in a relatively crowded region of the spectrum (3.77–3.83 p.p.m.). Since the corresponding methylene carbon atoms could easily be identified from a ^{13}C-DEPT experiment[24], the assignment of the H-6 resonances of residues *D*–*F* were confirmed by ^{1}H/^{13}C correlation.

A heteronuclear ^{1}H/^{13}C chemical shift correlation experiment performed on the native polysaccharide, using the CHORTLE pulse sequence[33], led to the assignment of the ^{13}C resonances in uncrowded regions of the ^{13}C-n.m.r. spectrum (*cf.* Fig. 1a). Correlation of the ^{13}C resonances with the corresponding ^{1}H signals, present in the more crowded regions of both spectra, was then made by comparison with ^{13}C data from the literature[41–44] (Table IV).

The component monosaccharides were identified from the chemical shift data

TABLE III

PROTON CHEMICAL SHIFTS[a] AND COUPLING CONSTANTS (Hz)[b] FOR THE *Streptococcus pneumoniae* TYPE 7F POLYSACCHARIDE[c,d]

Residue	*Glycose unit*	*H-1* ($J_{1,2}$)	*H-2* ($J_{2,3}$)	*H-3* ($J_{3,4}$)	*H-4* ($J_{4,5}$)	*H-5*	*H-6* ($J_{5,6}$)	*H-6'* ($J_{5,6'}$, $J_{6,6'}$)
A	→6)-α-D-Gal*p*-(1→ 2 ↑	5.22 [5.33][d] (3.4)	3.95 (9.7)	4.02 [4.08] (3.3)	3.99 [4.02] (~1)	4.28 [4.31]	4.01 (~6.6)	3.68 (~6.6, -)[f]
B	→2)-α-L-Rha*p*-(1→	5.16 (s)[e]	4.21 (4.1)	3.98 (10.3)	3.60 (9.7)	3.81	1.33 (6.2)	
C	→3)-β-L-Rha*p*-(1→	5.06 [4.85] (s)[e]	5.63 [4.37] (3.1)	3.86 [3.63] (10.1)	3.50 [3.44] (~10)	3.55 [3.47]	1.41 [1.38] (5.5)	
D	α-D-Glc*p*NAc-(1→	5.01 [5.04] (3.1)	3.93 (9.8)	3.85 (9.6)	3.55 (9.7)	3.93	3.82 (ur)	3.82 (ur)
E	β-D-Gal*p*-(1→	4.54 [4.57] (8.1)	3.47 [3.62] (9.1)	3.65 [3.67] (3.4)	3.91 [3.94] (~1)	3.68 [3.73]	3.84 [3.81] (6.9)	3.77 [3.81] (4.0, 11.1)
F	→3)-β-D-Gal*p*NAc-(1→ 4 ↑	4.51 (8.8)	3.97 (9.4)	3.87 (3.3)	4.21 (~1)	3.76	~3.82 (ur)	~3.82 (ur)
G	→4)-β-D-Glc*p*-(1→	4.41 (7.5)	3.43 (9.4)	3.55 [3.57] (ur)[g]	3.57 (ur)	3.39 [3.47]	3.89 [3.96] (~1)	3.65 [3.73] (6.0, 11.2)

[a]Measured at 37° (internal acetone). [b]Observed first-order coupling constants. [c]Data are for the native type 7F polysaccharide. [d]Chemical shift values in square brackets are for proton resonances shifted in the *O*-deacetylated polysaccharide. [e]Singlet, $w_{1/2}$ <3 Hz. [f]Not determined. [g]Unresolved multiplet.

TABLE IV

^{13}C CHEMICAL SHIFTS AND $J_{C\text{-}1,H\text{-}1}$ VALUES (Hz) FOR THE NATIVE *Streptococcus pneumoniae* TYPE 7F CAPSULAR POLYSACCHARIDE[a,b]

		Carbon atom							
Residue	*Glycose residue*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*-C=O*	*-CH₃*
A	→6)-α-D-Gal*p*-(1→ 2 ↑	97.37 (171)	78.18	69.50[c]	70.15[c]	72.55[c]	68.14[d]		
B	→2)-α-L-Rha*p*-(1→	99.15 (171)	78.18	70.54[c]	73.14	69.19[c]	17.57		
C	→3)-β-L-Rha*p*-(1→ 2 ↑ Ac	100.09 (164)	70.59[c]	76.76	71.51	73.14[c]	17.82	174.32	21.41
D	α-D-Glc*p*NAc-(1→	94.45 (169)	54.41	71.56	70.59	72.63	61.77[e]	175.58[f]	23.20[g]
E	β-D-Gal*p*-(1→	105.16 (162)	72.07	73.63	69.89	75.82	62.08		
F	→3)-β-D-Gal*p*NAc-(1→ 4 ↑	102.31 (158)	52.37	81.13	74.27	75.52	60.85[e]	174.59[f]	22.98[g]
G	→4)-β-D-Glc*p*-(1→	105.68 (164)	73.83	76.31	77.12	75.28	61.91		

[a]Measured at 37° (external 1,4-dioxane). [b]Unless indicated, assignments made from the $^{13}C/^{1}H$ chemical shift correlation experiment. [c]Tentative assignment, made by comparison with reference compounds (*cf.* ref. 44). [d]Identified by DEPT experiment. [e-g]Assignments may be reversed.

and the magnitude of the $^{3}J_{H,H}$ values. The ^{3}J values were determined from the 1D ^{1}H-n.m.r. spectrum, using resolution enhancement techniques[18], from the appropriate cross-peaks in the COSY spectrum, or from a 2D homonuclear *J*-resolved spectrum[45], and are recorded in Table III. The ^{3}J values indicated[46] the glycose residues to be pyranosides having the $^{4}C_1$(D) or $^{1}C_4$(L) conformation. Residues *A*, *E*, and *F* were identified as *galacto* compounds from the small $J_{3,4}$ and $J_{4,5}$ values (3.3–3.4 and ~1 Hz, respectively), whereas the larger values (~10 Hz) for $J_{2,3}$, $J_{3,4}$,and $J_{4,5}$ exhibited by residue *D* indicated the *gluco* configuration. On the basis of the $J_{1,2}$ values and ^{1}H chemical shifts[47], residues *A*, *E*, and *F* were identified as the α-D-Gal*p*, β-D-Gal*p*, and β-D-Gal*p*NAc residues. Correlation of the resonances of H-2 (3.97 p.p.m.) and C-2 (52.37 p.p.m.) indicated[40] *F* to be a 2-acetamido-2-deoxyglycose. Similarly, residue *D* was identified as α-D-Glc*p*NAc on the basis of its $J_{1,2}$ value (3.1 Hz) and a $^{1}H/^{13}C$ connectivity between H-2 (3.93 p.p.m.) and C-2 (54.41 p.p.m.). Resonances corresponding to H-3,4 of residue *G* were almost coincident and strong coupling precluded direct measurement of the $J_{3,4}$ and $J_{4,5}$ values. The ^{1}H chemical shift data for residue *G*, particularly those of the H-2 (3.43 p.p.m.) and H-5 (3.39 p.p.m.) resonances, were diagnostic[48] of a

β-D-Glc*p* residue. Small $J_{1,2}$ and $J_{2,3}$ values indicated residues *B* and *C* to have the *manno* configuration. Due to the axial disposition of HO-2, the anomeric configurations of the two L-Rha*p* residues were not evident from the $J_{1,2}$ values (< 3 Hz)[46], but could be determined[49] form the $^1J_{C,H}$ values for the anomeric carbons (171 Hz) for *B* and (164 Hz) for *C*, which indicated them to be α and β, respectively, In agreement with these assignments, the chemical shift values of the H-5 resonance of residues *B* (3.81 p.p.m.) and *C* (3.55 p.p.m.) also pointed[50] to them being α and β, respectively.

The low-field position of the H-2 resonance of the β-L-Rha*p* residue (*C*) was indicative[51] of 2-*O*-acetylation, which was confirmed by analysis of the ^{1}H-n.m.r. spectrum of the *O*-deacetylated polysaccharide. The connectivities and assignments

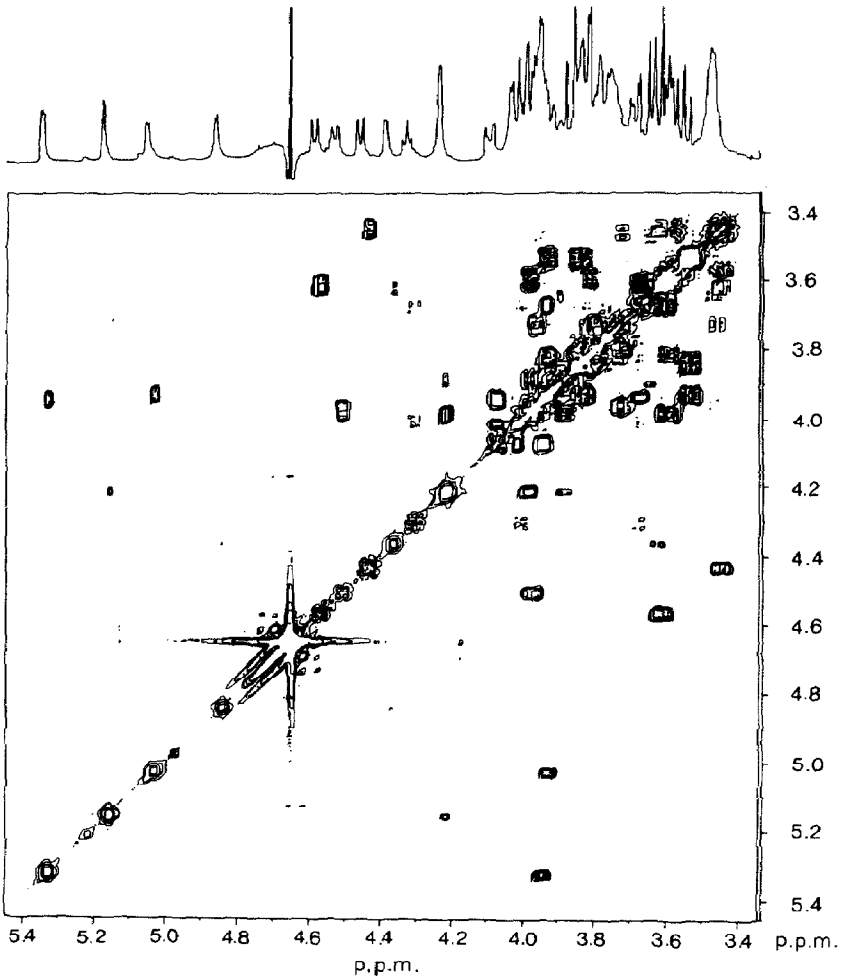


Fig. 4. Contour plot of the COSY spectrum of the *O*-deacetylated type 7F pneumococcal polysaccharide for the ring-proton region (5.45–3.35 p.p.m.).

of the ^1H resonances of the *O*-deacetylated polysaccharide were achieved by analysis of the corresponding COSY experiment (Fig. 4), and the resonances of those protons which showed a significant change in chemical shift (>0.01 p.p.m.) from the values obtained for the native polysaccharide are indicated in Table III.

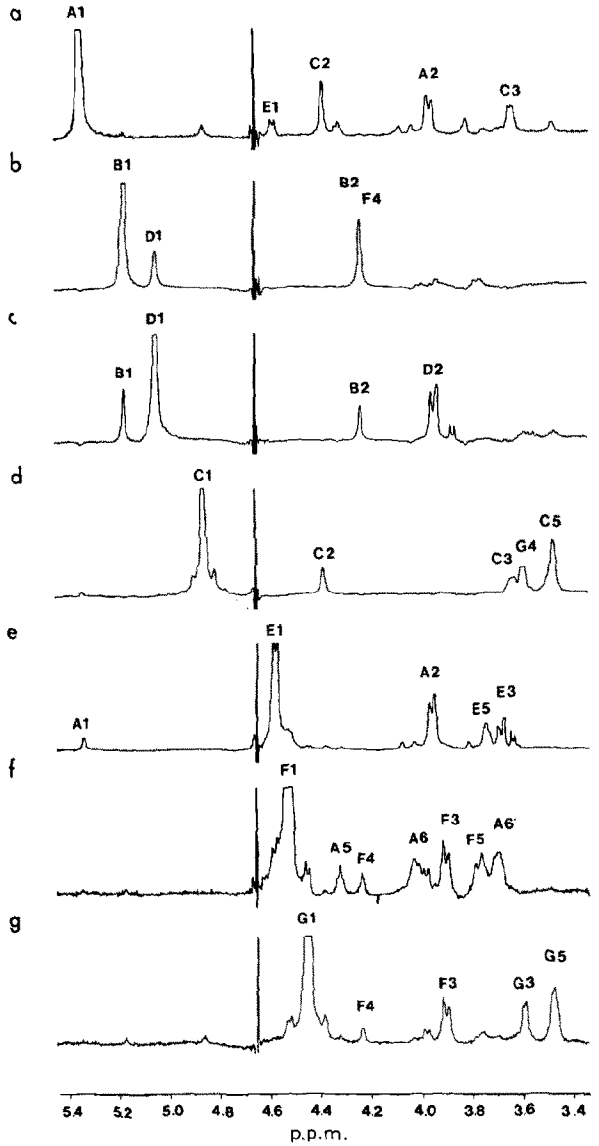


Fig. 5. N.O.e. difference spectra of the *O*-deacetylated type 7F polysaccharide for saturation of the anomeric proton resonances of (a) residue *A* (*A*1), (b) residue *B* (*B*1), (c) residue *D* (*D*1), (d) residue *C* (*C*1), (e) residue *E* (*E*1), (f) residue *F* (*F*1), and (g) residue *G* (*G*1).

The sequence of residues in the repeating unit was determined from *inter*-residue ^1H–^1H n.O.e. measurements[52,53] on the native and the *O*-deacetylated polysaccharides, and the difference spectra obtained by saturation of the respective H-1 resonances of the *O*-deacetylated polymer are shown in Fig. 5. The results of the n.O.e. experiments on the native polysaccharide were similar, but spectral overlap of several resonances rendered their interpretation difficult.

Irradiation of the anomeric proton of the α-D-Gal*p* residue (*A*) (Fig. 5a) showed, in addition to the *intra*-residue n.O.e. at H-2 (*A*2), which is a characteristic feature of α-linked glycopyranosyl residues, *inter*-residue effects at H-2 and H-3 of the β-L-Rha*p* residue (*C*) establishing the presence of a (1→3) linkage between these residues. The occurrence of significant *inter*-residue n.O.e.'s at both H-2 and H-3 of residue *C* is a consequence of the conformation about the *A*–*C* glycosidic bond[54] and also accompanied saturation of the H-1 resonance of residue *A* in the native polysaccharide in which the β-L-Rha*p* residue carries a 2-*O*-acetyl group. An *inter*-residue n.O.e. involving H-1 of the β-L-Rha*p* residue (*C*) (Fig. 5d) and either H-3 or H-4 of the β-D-Glc*p* residue (*G*) indicated the sequence *C*–*G* in the repeating unit. That the β-D-Glc*p* residue was 4-linked was established by a comparison of the ^{13}C chemical shift data for residue *G* (Table IV) with data in the literature[55] and from the methylation analysis data (Table II). Saturation of the H-1 resonance of residue *C* (Fig. 5d) resulted in *intra*-residue n.O.e.'s at H-2 and also at the *trans*-diaxial protons, H-3 and H-5, confirming that this L-Rha*p* residue was β.

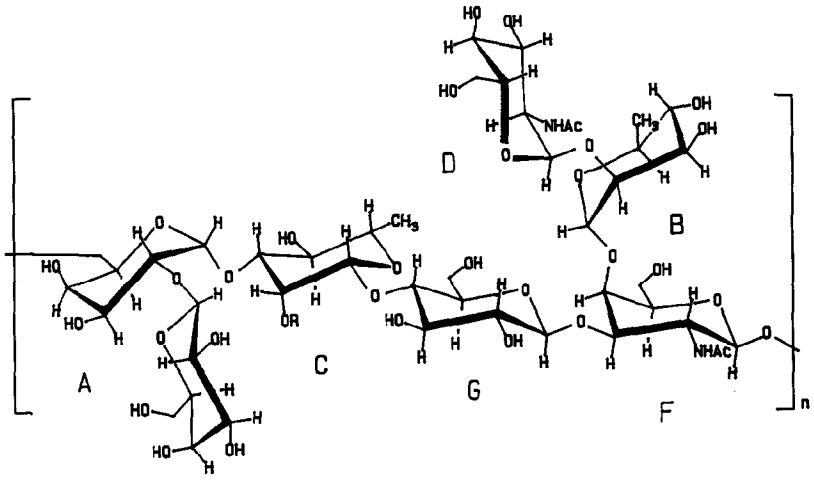
The β-D-Glc*p* residue (*G*) was 3-linked to the β-D-Gal*p*NAc residue (*F*) since a substantial *inter*-residue n.O.e. at the resonance of H-3 of residue *F*, together with a smaller effect at H-4 (*F*4), accompanied saturation of the anomeric proton of residue *G* (Fig. 5g). Dipolar interactions across the glycosidic bond at both the H-3 and H-4 resonances of 3-linked D-Gal*p* residues have been noted[56].

The difference spectrum obtained for irradiation of the H-1 resonance of the β-D-Gal*p*NAc residue (*F*) (Fig. 5f) was complex and showed direct *intra*-residue n.O.e.'s at H-3 and H-5 as well as indirect *intra*-residue effects at H-4 and H-2, presumably arising from an effective spin-diffusion process[57]. However, *inter*-residue n.O.e.'s at H-6, H-6′, and H-5 of the α-D-Gal*p* residue (*A*) indicated that residue *F* was 6-linked to residue *A*. Thus, the sequence *A*→*C*→*G*→*F*→*A* is established, indicating the main chain of the polymer to be comprised of the repeating tetrasaccharide-unit

$$[\rightarrow 6)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow]_n,$$

in which the α-D-Gal*p* and β-D-Gal*p*NAc residues are the two branch points as deduced from methylation analysis data (Table II).

The n.O.e. data, in agreement with results from methylation analysis (Table II), points to residues *E* (β-D-Gal*p*) and *D* (α-D-Glc*p*NAc) as being the terminal non-reducing groups. That these residues are unsubstituted is also reflected in their ^{13}C chemical shift data (Table IV) which do not differ significantly from those for



R = H
R = Ac **1**

the corresponding methyl glycosides[42].

The strong n.O.e. produced at the H-2 resonance of residue *A* upon irradiation of H-1 of residue *E* (Fig. 5e) established that the terminal β-D-Gal*p* unit (*E*) is 2-linked to the →6)-α-D-Gal*p*-(1→ residue (*A*). Saturation of the H-1 resonance of the α-D-Glc*p*NAc unit (*D*) (Fig. 5c) results in *inter*-residue n.O.e.'s at H-2 and H-1 of the α-L-Rha*p* residue (*B*), indicating that the terminal α-D-Glc*p*NAc residue is 2-linked to the α-L-Rha*p* unit (*B*), thereby establishing the disaccharide *D*→*B* as a side chain. Thus, the second branched glycose, the 3,4-disubstituted β-D-Gal*p*NAc residue (*F*) must be 4-substituted by the side chain, α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→, which is consistent with the observation of a significant n.O.e. between H-1 of the α-L-Rha*p* residue (*B*) and the resonance (4.21 p.p.m.) corresponding to both H-2 of residue *B* (*intra*-residue effect) and H-4 of residue *F* (*inter*-residue effect) (Fig. 5b). The resonances of these protons (*B*2 and *F*4) also showed identical chemical shifts in the ^{1}H-n.m.r. spectrum of the native polymer (Table III) and, as with the *O*-deacetylated polysaccharide (Fig. 5b), n.O.e.'s were only observed at the signal corresponding to these protons and the H-1 resonance of residue *D* when the anomeric proton resonance of residue *B* of the native polysaccharide was saturated.

The accumulated evidence permits the repeating heptasaccharide unit of the pneumococcal type 7F polysaccharide to be assigned the structure **1**.

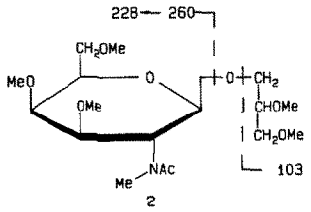
Supporting evidence for the structure **1** was obtained from the oligosaccharide fragments produced by periodate oxidation, and by deamination with nitrous acid of the type 7F polysaccharide. Periodate oxidation followed by borohydride reduc-

TABLE V

^{13}C-N.M.R. CHMICAL SHIFTS OF THE OLIGOSACCHARIDES DERIVED FROM THE PNEUMOCOCCAL TYPE 7F POLYSACCHARIDE FOLLOWING SMITH DEGRADATION

Oligosaccharide	*Chemical shift*[a]											
	Glycose								*Aglycon*			
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*CH$_3$*	*C=O*	*C-1'*	*C-2'*	*C-3'*	*C-4'*
β-D-Gal*p*NAc-(1→1)-D-glycerol (**A**)	102.87	53.21	71.70[b]	71.22[b]	75.89	61.76	22.95	178.73	68.55	71.46[b]	63.15	
β-L-Rha*p*-(1→2)-D-erythritol (**B**)	100.14	71.68[c]	73.52[d]	73.01[d]	72.87	17.49			61.26	80.29	71.83[e]	73.50

[a]Measured at 37° on a solution in D_2O. [b-d]Assignments may be reversed.



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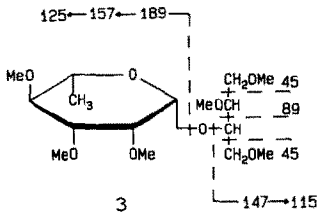
tion and Smith-type hydrolysis[34] afforded aminodeoxyglycose-containing (**A**) and neutral (**B**) disaccharides which were isolated by gel filtration on Bio-gel P2.

Oligosaccharide **A** was composed of D-Gal*p*NAc and glycerol (1:1). G.l.c.–m.s. (c.i.) of methylated **A** gave a single peak showing a molecular ion *m/z* 380 (M + 1), and the e.i. fragmentation pattern (**2**) was consistent with the structure 1-*O*-(2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-β-D-galactopyranosyl)-2,3-di-*O*-methylglycerol. The ^{1}H-n.m.r. spectrum of **A** showed a single resonance (4.48 p.p.m.) for anomeric protons and a $J_{1,2}$ value (8.2 Hz) characteristic of a β-D-glycosyl derivative. Eleven signals were observed in the ^{13}C-n.m.r. spectrum in which the ^{13}C resonances corresponding to the terminal aminodeoxyglycose were assigned (Table V) by comparison with those reported[40] for β-D-Gal*p*NAc, and the chemical shifts of the remaining signals were consitent with 1-substitution of the glycerol moiety. Thus, **A** was identified as 1-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-glycerol and, since the glycerol moiety could arise only from a 6-linked glycopyranosyl unit, the sequence →3)-β-D-Gal*p*NAc-(1→6)-α-D-Gal*p*-(1→ is identified as part of the repeating unit.

Disaccharide **B** was composed of L-Rha and erythritol (1:1), and gave characteristic ^{1}H-n.m.r. signals at 4.79 (d, 1 H, $J_{1,2}$ 1.2 Hz, H-1) and 1.32 p.p.m. (d, 3 H, $J_{5,6}$ 6.0 Hz, H-6). The 10 signals in the ^{13}C-n.m.r. spectrum (Table V) were assigned to a terminal β-L-Rha*p* unit and a 2-substituted erythritol moiety by comparison with reference compounds[41,58] and on g.l.c.–m.s. analysis of the methylated derivative, the e.i. mass spectral fragmentation pattern (**3**) was consistent with the structure 1,3,4-tri-*O*-methyl-2-*O*-(2,3,4-tri-*O*-methyl-β-L-rhamnopyranosyl)erythritol.

Identification of **B** as 2-*O*-β-L-rhamnopyranosyl-D-erythritol confirms the presence of →3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→ in the repeating unit.

The presence of the α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1- side chain in the re-



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peating unit was confirmed as follows. Treatment of the type 7F polysaccharide with 2M NaOH (4 h, 100°) led to almost exclusive hydrolysis of the NAc group of the β-D-Glc*p*NAc residue, and, following deamination[59], a high-molecular-weight product was obtained by filtration through Sephadex G-50. The products of hydrolysis obtained from the methylated modified polysaccharide (Table II) were similar to those obtained from methylated native material, except that only trace amounts of 2-deoxy-3,4,6-tri-*O*-methyl-2-methylamino-D-glucose and 3,4-di-*O*-methyl-L-rhamnose were obtained together with the major new component, 2,3,4-tri-*O*-methyl-L-rhamnose, which established that the terminal β-D-Glc*p*NAc group was 2-linked to the α-L-Rha*p* residue in the polysaccharide.

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