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Full assignment of the proton and carbon NMR spectra and revised structure for the capsular polysaccharide from *Streptococcus pneumoniae* Type 17F

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Abstract

Full proton, ^{13}C and ^{31}P NMR assignments for the capsular polysaccharide from *Streptococcus pneumoniae* Type 17F are reported, and a revised structure differing in the anomeric configuration of the sidechain β-Gal*p* residue proposed. This polysaccharide is a component of the current 23-valent polysaccharide vaccine. The implications of this revised structure for published work are discussed.

Keywords: *Streptococcus pneumoniae*; Capsular polysaccharide; NMR spectroscopy

1. Introduction

Pneumococcal disease remains a significant cause of morbidity and mortality worldwide [1], and with increasing numbers of antibiotic-resistant pneumococci has come a resurgence of interest in improved prophylaxis. Ninety pneumococcal serotypes have been defined [2] on the basis of serological and chemical differences between their capsular polysaccharides (CPSs), but only a limited number are normally associated with disease. CPS is essential for the virulence of *Streptococcus pneumoniae*, and the overwhelming evidence that protection against pneumococcal diseases is mediated by anti-CPS antibodies led to the use of purified CPSs as vaccines [3]. Because carbohydrates tend to elicit T-independent immune responses, pneumococcal CPS–protein conjugate vaccines are being developed to provide better anamnestic responses and offer better protection to infants [4]. The CPS from *S. pneumoniae* Type 17F is a component of the 23-valent polysaccharide vaccine and, potentially, of future polysaccharide–protein conjugate vaccines. Traditionally, control of the identity and purity of polysaccharide-based vaccines has relied on a combination of wet chemical tests, to establish composition, and immunological identity tests, but NMR spectroscopy is an alternative approach that not only confirms the structure of the CPS but also shows the presence of contaminants such as C-polysaccharide, the cell wall-associated polysaccharide found in all pneumococcal serotypes that is commonly associated with pneumococcal CPSs.

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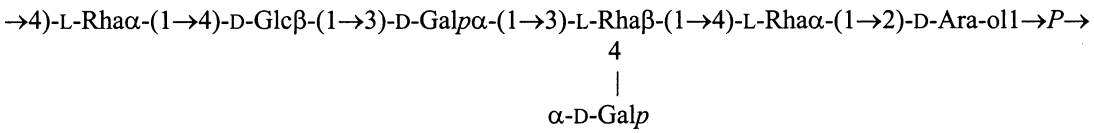
Two structures have been proposed, in review articles without experimental data, for the repeat unit of the Type 17F CPS. Both are teichoic acid like, with a hexasaccharide alditol phosphate repeat unit. Structure **1** was reported in 1983 [5].



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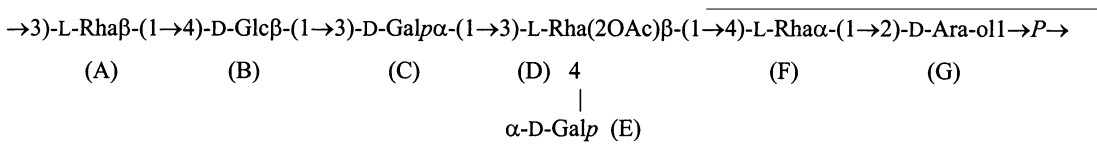
whilst a revised structure **2** was first reported in 1990 [6,7].



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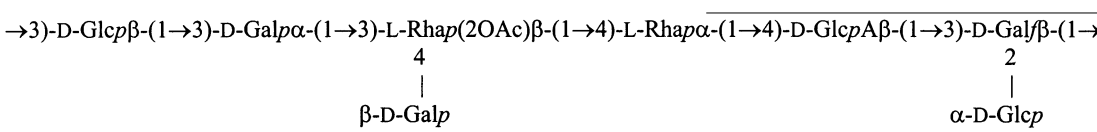
The two structures differ in (a) the linkage of the phosphodiester to the Rha residue, (b) the anomeric configuration of the phosphorylated Rha, and (c) the O-acetylation of the branch point Rha residue.

Extensive immunological cross-reactivity exists between the Type 17F CPS and another pneumococcal group 17 CPS, the Type 17A. The structure of the repeat unit of the Type 17A CPS was determined by Jansson et al. [8] and has an O-acetylated octasaccharide repeat lacking an alditol phosphate, **3**.



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Whilst undertaking the full NMR assignments for the Type 17F CPS, we noted that these were not in accord with either structure **1** or **2**: in particular, the sidechain Gal residue was found to have the β-anomeric configuration, as observed in the Type 17A CPS. We report here the full NMR assignments and the revised structure for the Type 17F CPS.

2. Results

The CPS used was a sample of material intended for vaccine production, and its identity was confirmed as Type 17F by the use of type-specific antiserum. The anti-Type 17F IgG titre of Factor Serum 17b was 3.8 when assayed against this sample, whilst it failed to bind to a sample of the Type 17A CPS.

One-dimensional NMR spectra.—The one-dimensional proton spectrum (Fig. 1(a)) showed the presence of two highfield anomeric resonances at 4.611 and 4.672 ppm, with $^3J_{H1,H2}$ of ca. 8 Hz and only a single major lowfield resonance ($\delta_H = 5.139$ ppm) with $^3J_{H1,H2}$ of ca. 3.5 Hz, indicating that of the three Glc and Gal*p* residues, two are in the β-anomeric form, at variance with both reported structures. The anomeric region of the proton spectrum also contained characteristic resonances for *manno* H-1s, with small coupling to the H-2s. Also apparent in the spectra were resonances for a single *O*-acetyl group present on approximately 80% of the repeat units, and a lowfield resonance (5.727 ppm) subsequently assigned as the H-2 of the 2-O-acetylated Rha residue. Three C-methyl groups from 6-deoxysugars were apparent. The sample contained low levels (ca. 3.5 mol% of the repeat unit) of C-polysaccharide, as assessed from the intensity of the phosphocholine *N*-methyl peak at 3.21 ppm [9,10] and peptidoglycan [11]. The resonances from these contaminants did not interfere with the spectral analysis of the CPS. The one-dimensional ^{13}C NMR spectrum (Fig. 1(b)) of the CPS was consistent with a repeat unit containing three

hexoses, three 6-deoxyhexoses, no amino sugars, and an absence of furanose ring forms. There is additional heterogeneity due to incomplete O-acetylation. Two resonances, a major and a minor, appeared to show coupling with phosphorus. The one-dimensional ^{31}P spectrum contained a single intense resonance at a chemical shift ($\delta_P = -0.47$ ppm) consistent with its assignment as a phosphodiester, and three weak resonances (one a shoulder on the major peak) of comparable intensity arising from the C-polysaccharide (Fig. 1(c))

Spectral assignment.—The proton spectrum was fully assigned by a combination of homo- and heteronuclear methods, including TOCSY and double quantum-filtered COSY methods,

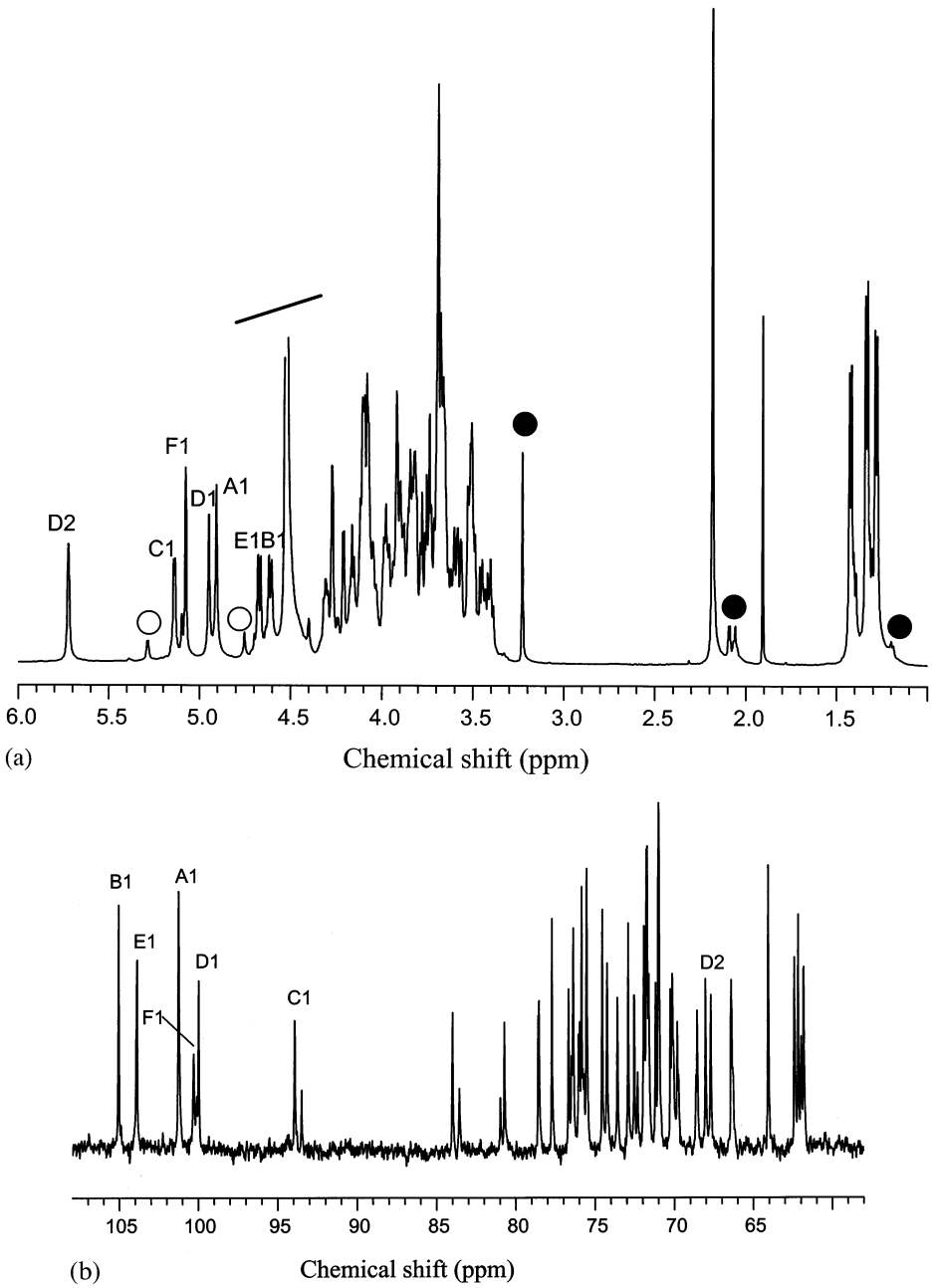

Fig. 1. (a) One-dimensional 500 MHz proton NMR spectrum of the pneumococcal Type 17F CPS at 50 °C, (b) one-dimensional 125 MHz ^{13}C NMR and (c) one-dimensional 202 MHz ^{31}P NMR spectra of the same sample. The residues are defined as in Table 1, where A = →3)-Rhaβ-(1→, B = →4)-Glcβ-(1→, C = →3)-Gal*p*α-(1→, D = →3,4)-Rha(2OAc)β-(1→, E = terminal Gal*p*β-(1→, and F = →4)-Rhaα-(1→. Resonances marked with an open circle arise from the non-O-acetylated repeat unit, and those marked with a closed circle arise from C-polysaccharide.

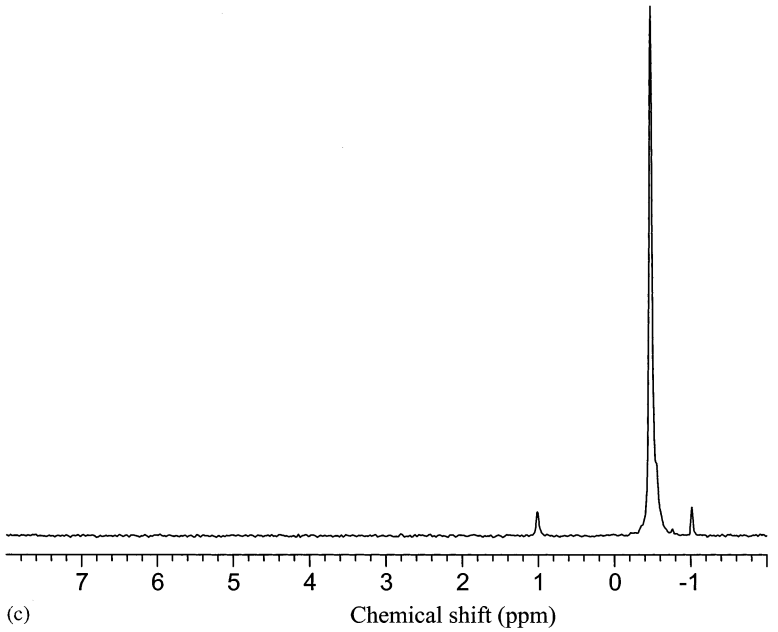

Fig. 1. (*Continued*)

and $^{1}H-^{13}C$ correlation methods optimised for $^{1}J_{C-H}$ and for $^{n}J_{C-H}$ of 20 Hz. The assignments were completed by essentially conventional methods, and only unusual features will be commented on. Assignment of the α-Gal residue could not be completed from homonuclear correlation methods, due to the typically small $^{3}J_{H4,H5}$, but the C-5 resonance was located by the observation of long-range correlation to the H-1 in a WHSQC spectrum optimised for $^{n}J_{C,H}$ of 20 Hz (called the 20 Hz WHSQC). The relatively lowfield hydroxymethyl resonance (66.42 ppm) correlating to strongly coupled lowfield proton resonances (4.102 and 4.126 ppm) was assigned as the C-1 of the pentitol residue, shown to be arabinitol by GC analysis, an assignment confirmed by the presence of a long-range correlation to the arabinitol C-2, which correlated in turn with the Rha H-1 in the 20 Hz WHSQC. The arabinitol H-3 and H-4 were the most difficult resonances to assign, due to the near correspondence of the H-3, H-4 and H-5a resonances. When the assignments were nearly complete, two major crosspeaks in the 150 Hz WHSQC spectrum remained unassigned: one 70.97 ppm/3.693 ppm showed a weak long-range correlation to a proton resonance at 4.161 ppm (arabinitol H-2); this correlation was also present in the 20 Hz WHSQC and 20 ms WHSQCTOCSY. This crosspeak is tentatively assigned as the arabinitol C-3/H-3, and the other peak (at 71.76 ppm/3.693 ppm) as the arabinitol C-4/H-4. In the 150 ms ROESY spectrum, the resonance at 4.161 ppm showed a strong correlation to a signal (or signals) at 3.693 ppm. These assignments are reported in Table 1.

The proton and ^{13}C chemical shifts of the β-Gal*p* residue were consistent with it being present as a non-reducing terminus, whilst those of the α-Gal*p* residue indicated that it was monosubstituted on O-3 by a β-D-*gluco* or -*galacto* residue. The methods used to assign anomeric configuration are discussed below.

Heterogeneity arises because approximately 20% of the repeat units are not O-acetylated. The minor resonances from non-O-acetylated repeat units were assigned and are reported in Table 2. The degree of O-acetylation can be estimated from the relative peak heights of minor resolved Rha H-1 at 5.097 ppm (compared with its position at 5.077 ppm in the acetylated repeat unit) or the relative intensities of minor and major →3)-Galα-(1→H-1 resonances at 5.287 and 5.139 ppm. None of the data obtained suggested heterogeneity in the position of O-acetylation.

All of the $^{3}J_{H,H}$ values estimated from the DQFCOSY experiment were consistent with the expected $^{4}C_{1}$ or $^{1}C_{4}$ conformations for D-

and L-sugars, respectively. The absolute configuration of the hexoses and 6-deoxyhexoses were determined by GC analysis of the (−)-butyl glycosides obtained after acid butanolysis of the intact polysaccharide [12]. The absolute configuration of the arabinitol residue has not been confirmed, and is assumed to be D- as reported for structures **1** and **2**. The proton and ^{13}C chemical shifts found for a synthetic tetrasaccharide [13] are similar to those we have assigned for the intact CPS, supporting this assignment of the D-configuration.

Anomeric configurations of the residues.—The anomeric configurations of the residues were determined from the chemical shifts of the anomeric protons and carbons (Table 1), $^3J_{H1,H2}$ data (for the Glc and Gal residues), and $^1J_{H1,C1}$ values [14] determined in a WHSQC experiment where ^{13}C coupling was not suppressed (Table 1). Additionally, for the three Rha residues, the pattern of chemical shifts for Rha H-5 and H-6s [15] was also considered. By all of these criteria, the sidechain Gal*p* residue was shown to have the β-anomeric configuration ($\delta_{H1} = 4.672$ ppm, $\delta_{C1} = 103.98$ ppm, $^3J_{H1,H2} = 8$ Hz, and $^1J_{H1,C1} = 164.5$ Hz), in contrast to both previously reported structures. In the ROESY spectrum, crosspeaks were observed between the resonance of the axial H-1 and the H-3 and H-5 resonances, again indicating β-anomeric configuration. The data for other residues were consistent with the structure reported by Jennings [6,7], with the phosphorylated Rha having the β-anomeric configuration.

Inter-residue glycosidic linkages.—The sequence and linkages between the sugars were determined from a combination of long-range carbon–proton correlation data, obtained from the 20 Hz WHSQC experiment (Fig. 2), and the observation of inter-residue NOEs in a ROESY spectrum. Inter-residue carbon–proton correlations indicated the linkages Rha(A)β-(1→4)-Glc(B), Rha(E)β-(1→4)-

Table 1
Pneumococcal Type 17F CPS: proton and ^{13}C NMR assignments [a]

	Residue	H-1/H-1′ C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6	H-6′ Oac
A	→3)-Rhaβ-(1→4	4.908 101.26 [b]	4.275 70.97	*4.049* *78.56*	3.512 71.92	3.450 72.93	1.341 17.78	
B	→4)-Glcβ-(1→3	4.611 105.07 [c]	3.410 74.24	3.652 76.38	*3.662* *77.73*	3.518 75.52	3.836 61.83	3.908
C	→3)-Galα-(1→3	5.139 93.94 [d]	3.976 67.71	*4.093* *80.73*	4.212 70.26	4.312 71.64	[3.762] 62.24	3.739
D	→3,4)-Rha(2OAc)β-(1→4	4.950 99.99 [e]	5.727 68.05	*4.109* *73.60*	*3.978* *76.67*	3.665 72.54	1.425 18.29	2.184 174.12 21.31
E	terminal Galβ-(1→4	4.672 103.98 [f]	3.514 71.76	3.576 74.56	3.918 69.82	3.688 75.86	3.828 62.17	[3.768]
F	→4)-Rhaα-(1→2	5.077 [g] 100.30	4.078 71.20	3.895 70.13	*3.602* *84.01*	3.860 68.56	1.288 17.61	
G	→2)-Ara-ol1→***P***	*4.10/4.13* *66.42*	*4.161* *76.01*	3.693 [70.97]	3.693 [71.76]	3.829 64.07	3.688	

[a] Proton chemical shifts referenced to TSP-d_4 at 0 ppm and carbon chemical shifts to TSP-d_4 at −1.8 ppm, at 50 °C. Italicised figures refer to glycosylated or phosphorylated positions. [ ], assignment tentative.
[b] $^1J_{H1,C1} = 161.8$ Hz.
[c] $^1J_{H1,C1} = 159.0$ Hz.
[d] $^1J_{H1,C1} = 172.7$ Hz.
[e] $^1J_{H1,C1} = 162.4$ Hz.
[f] $^1J_{H1,C1} = 164.5$ Hz.
[g] $^1J_{H1,C1} = 170.2$ Hz.

Table 2
Pn Type 17F: minor spin systems from the O-de-acetylated repeat unit

	Residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6	H-6′
B′	→4)-Glcβ-(1→	4.631 [105.07]	3.416 [74.24]					
	differences	+0.020 [0.00]	+0.006 [0.00]					
C′	→3)-Galα-(1→4	5.287 93.51 [a]	4.072 [67.91]	4.172 81.00	4.241 70.10	4.262 71.79	3.742 62.38	3.742
	differences	+0.148 −0.31	−0.096 +0.20	+0.081 +0.27	+0.039 −0.16	−0.050 +0.15	−0.020 +0.14	+0.003
D′	→3,4)-Rhaβ-(1→	4.754 [b] 101.26	4.402 66.33	4.090 [73.56]	3.940 76.49	3.583 72.25	1.401 [18.29]	
	differences	−0.196 +1.27	−1.325 −1.72	−0.029 +0.04	−0.038 −0.18	−0.081 −0.29	−0.027 [0.00]	
E′	terminal Galβ-(1→4	4.672 [c] 103.89	3.512 71.78	[3.576] [74.56]	3.940 [69.82]			
	differences	0.00 0.00	−0.002 0.00	[0.000] [0.00]	+0.022 0.00			
F′	→4)-Rhaα-(1→2)-Ara′ol	5.097 [d] 100.14	4.103 [71.20]	3.928 70.08	3.604 83.58	[3.942] 68.64	1.311 [17.61]	
	differences	+0.020 +0.16	+0.025 [0.00]	+0.033 −0.05	+0.002 −0.43	+0.082 +0.08	+0.023 [0.00]	
G′	→2)-Ara′ol1→***P***		74.44					
	differences		−1.57					

[a] $^{1}J_{H1,C1} = 170.9$ Hz.
[b] $^{1}J_{H1,C1} = 162.2$ Hz.
[c] $^{1}J_{H1,C1} = 160.2$ Hz.
[d] $^{1}J_{H1,C1} = 169.6$ Hz.

Rha(F) and Rha(F)α1→2Ara-ol, whilst inter-residue NOEs were observed between (i) the β-Rha(A) H-1 and the β-Glc(B) H-4, (ii) between the β-Glc(B) H-1 and the H-3 and H-4 of the α-Gal(C), indicating a Glcβ-(1→3)-Gal linkage, (iii) between the α-Gal(C) H-1 and the β-Rha(D) H-2 and H-3 (confirming the Galα-(1→3)-Rha(D) linkage), (iv) between the β-Rha(D) H-1 and the H4 and H5 and/or H3 of the α-Rha(F), consistent with a Rha(D)β-(1→4)-Rha(F) linkage, and (v) between the α-Rha(F) H-1 and H-1 and H-2 of the arabinitol residue. Together, these data define the structure of the repeat unit except for the attachment of the sidechain and mainchain on the Rha(D) branchpoint, and the linkage of the phosphodiester to Rha(A). A number of inter-residue NOEs involving the non-O-acetylated Rha(D) and other residues in non-acetylated repeat units were also observed. The most important of these was between the α-Gal(C′) H-1 and the H-2 and H-3 of non-O-acetylated Rha(D′) and between the β-Gal(E′) H-1 and the H-4 of Rha(D′). These data show that the β-Gal*p* sidechain is linked to O-3 and the mainchain to O-4 of the branchpoint Rha(D). Unusually, a weak inter-residue crosspeak was observed in the TOCSY spectrum between the α-Gal H-1 resonance and the →3,4)-Rha(2OAc)β-(1→H-2 resonance (at 5.727 ppm), also consistent with the proposed pattern of substitution at the branch-point residue. These linkages are consistent with the substitution patterns of the various residues as indicated by the downfield chemical shifts of the carbon atoms compared

with model systems [12,16] and the changes in proton chemical shifts expected from various substitution patterns.

Location of the phosphate group.—The phosphodiester linkage between the arabinitol O-1 and Rha(A) O-3 was established from chemical shift and coupling constant data, and from a $^{31}P-^{1}H$ correlation experiment. The lowfield positions of the arabinitol C-1 and H-1s are consistent with phosphorylation at O-1, and as expected from the normal mode of biosynthesis of teichoic acid-like systems. Similarly, the lowfield chemical shifts of the Rha(A) H-3 and C-3 suggest phosphorylation. The value of $^{3}J_{P-O-C-H3}$ was estimated at 8.8 Hz from the resolved Rha(A) H-2/H-3

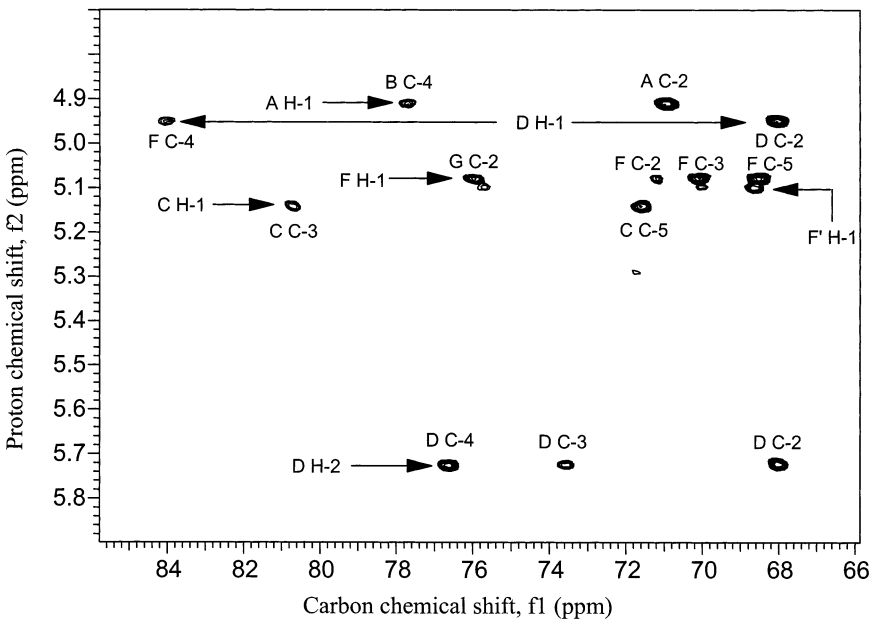

Fig. 2. Partial WHSQC spectrum of the Pn Type 17F CPS, optimised for $^{n}J_{H,C} = 20$ Hz, showing correlations involving anomeric protons. The horizontal traces are labelled by the assignment for the proton, and individual crosspeaks for the carbon correlation. The residue labelling is as in Tables 1 and 2.

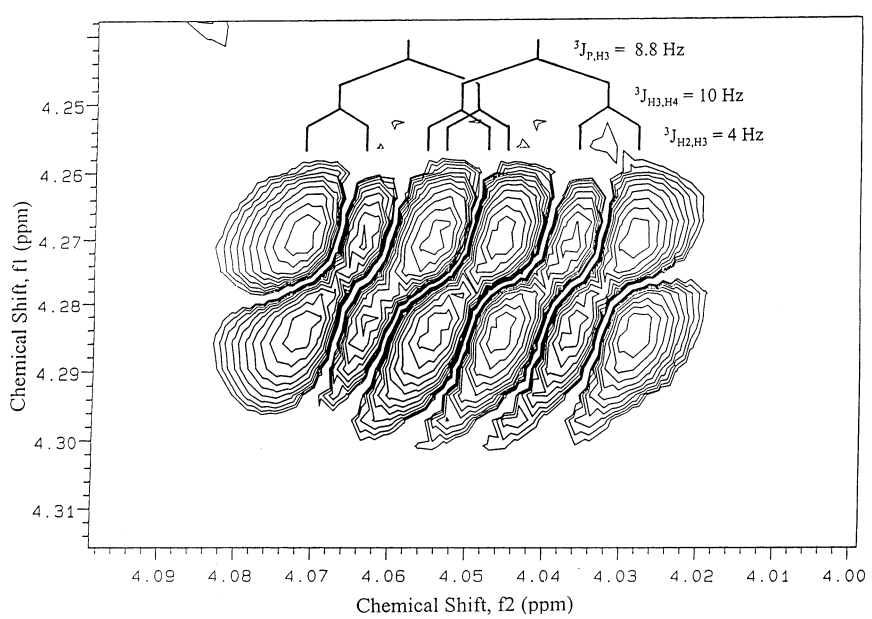

Fig. 3. Expansion of the Rha(A) H-2/H-3 crosspeak from the 500 MHz DQFCOSY spectrum of the Pn Type 17F CPS, showing the passive $^{3}J_{POCH}$ coupling of 8.8 Hz.

Rhaα-(1→2)-Arabinitol-(1→***P***→linker

Rhaα-(1→2)-Arabinitol-(1→***P***→4)-Rhaα-(1→linker

Rhaα-(1→2)-Arabinitol-(1→***P***→4)-Rhaα-(1→4)-Glcβ-(1→linker

Fig. 4. Structures of the oligosaccharide prepared by Veeneman et al. [13], related to structure **1**.

crosspeak in the double-quantum-filtered COSY spectrum (Fig. 3). Two resonances (one major, one minor) in the one-dimensional ^{13}C spectrum showed coupling attributed to $^{n}J_{C,P}$, assigned as arising from the arabinitol C-2. Other peaks that might be expected to show such coupling (Rha(A) C-3, C-4 or C-5; arabinitol C-1) were either unresolved or failed to show a coupling. An ^{1}H-detected $^{1}H-^{31}P$ correlation spectrum optimised for $^{n}J_{H,P}$ of 8 Hz showed correlations between the major ^{31}P resonance and signals at 4.10 and 4.13 ppm and an apparent doublet at 4.04 ppm, corresponding to the two arabinitol H-1s and the Rha(A) H-3 resonances, respectively.

3. Discussion

We have fully assigned the NMR spectrum of the pneumococcal Type 17F CPS, and used this approach to determine the structure of the repeat unit. This study (i) shows that the sidechain Gal*p* residue has the β-anomeric configuration, not α- as in both the previously reported structures, (ii) confirms that the phosphorylation occurs on the Rha O-3 rather than the O-4, as in the structure reported by Jennings [6,7], (iii) confirms the β-anomeric configuration of the phosphorylated Rha residue, as reported by Jennings [6,7] but in contradiction to the report of Perry et al. [5], and (iv) confirms the other linkages and anomeric configurations as previously reported. These data thus show that the structure of the repeat unit of the pneumococcal Type 17F CPS is:

→3)-L-Rhaβ-(1→4)-D-Glcβ-(1→3)-D-Gal*p*α-(1→3)-L-Rha(2OAc)β-(1→4)-L-Rhaα-(1→2)-D-Ara-ol1→***P***→
(A) (B) (C) (D) 4 (F) (G)
|
β-D-Gal*p* (E)

4

The structural element:

D-Glcβ-(1→3)-D-Gal*p*α-(1→3)-[D-Gal*p*β-(1→4)]-L-Rha(2OAc)β-(1→4)-L-Rhaα-(1→

is common between the two Group 17 CPSs and presumably accounts for the immunological cross-reactivity between these two serotypes.

WHO Requirements [17] and the European Pharmacopoeia Monograph [18] for this CPS require a methylpentose content greater than 20% by dry weight, and a phosphorus content between 0 and 3.5% of dry weight, which indirectly limits the content of the phosphate-rich C-polysaccharide. From the structures of Types 17F and 17A CPS, the expected values are 42.1 and 26.5% (methyl pentose) and 2.6 and 0%, so that, curiously, WHO and EP Requirements are more consistent with the Type 17A than the Type 17F CPS.

Veeneman et al. [13] synthesised a series of oligosaccharides (Fig. 4) related to structure **1** (i.e., with 4-phosphorylated α-Rha) incorporating a linker for conjugation to a carrier protein. They report full NMR assignments for their oligosaccharides, but these and the reported long-range $^{31}P-^{1}H$ and $^{31}P-^{13}C$ coupling constants are inconsistent with those determined here for the intact polysaccharide **4** and provide additional evidence that structure **1** is incorrect. The conclusions of a study of the immunological responses to the peptide-conjugated synthetic oligosaccharides prepared by Veeneman and co-workers [19], assuming structure **1**, must also be considered suspect.

4. Experimental

General methods.—The polysaccharides used were bulk material destined for production of the 23-valent vaccine. Approximately 6 mg of polysaccharide was lyophilised once from 500 μL of deuterated water (> 99.8%, M&G Chemicals, Stockport, UK), redissolved

in 350 μL of deuterated water and introduced into a 5 mm susceptibility-matched Shigemi tube. NMR spectra were collected on a Varian Unity 500 NMR spectrometer using a 5 mm PFG triple resonance probe and at an indicated probe temperature of 50 °C. The spectrometer was running VNMR version 5.3B and standard Varian pulse sequences were used apart from the introduction of an echo sequence into the TOCSY and ROESY spectra, the use of pulsed field gradients to act as homospoil pulses in the $^{1}H-^{13}C$ HSQC spectrum (called WHSQC), according to Wider and Wüthrich [20], and the implementation of a WHSQCTOCSY experiment related to the HMQCTOCSY experiment described by Crouch et al. [21]. Proton chemical shifts are referenced to internal TSP-d_4 at zero ppm, carbon chemical shifts to internal TSP-d_4 at -1.8 ppm [22], and ^{31}P chemical shifts to internal (sealed capillary) 85% phosphoric acid. The ^{1}H-detected $^{1}H-^{31}P$ correlation experiment was an HSQC without the use of field gradients, and with the tau delay set to 5 ms.

Methylation analysis and absolute configurations of the sugars.—Methylation analysis was carried out as previously described [23], and the absolute configurations of the monosaccharides established using the method of Gerwig et al. [12].

Enzyme-linked immunosorbant assay for detection of anti-PS17F IgG antibody.—IgG antibody against pneumococcal Type 17F CPS was measured by ELISA after pre-absorption with cell-wall polysaccharide (Statens Serum Institut, Copenhagen). Microtitre plates (Maxisorp F96 Immuno plates, Life Technologies Inc.) were coated with the Type 17F CPS antigen overnight and blocked with 1% bovine serum albumin (BSA). Plates were then incubated consecutively with pre-absorbed, serially diluted Factor Serum 17b (diagnostic antiserum for the identification of the Type 17F CPS, Statens Serum Institut), biotinylated sheep anti-rabbit IgG (Pharmingen) and peroxidase-conjugated streptavidin, (Serotec). Enzyme substrate (*o*-phenylenediamine and urea–hydrogen peroxide) was added and the reaction terminated after 30 min by the addition of 3 M HCl. Optical density (OD) was measured at 492 nm using an automated microplate reader. Background OD values were obtained from wells with phosphate buffered saline containing 1% BSA instead of serum and were subtracted from all readings prior to calculation of anti-Type 17F CPS IgG titre using the following formula: Anti-Type 17F IgG titre $= -\log_{10}$ [highest serum dilution producing $> 3 \times$ background OD].

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