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Carbohydrate Research 304 (1997) 165–172

CARBOHYDRATE RESEARCH

# Structural elucidation of the capsular polysaccharide from *Streptococcus pneumoniae* type 18B

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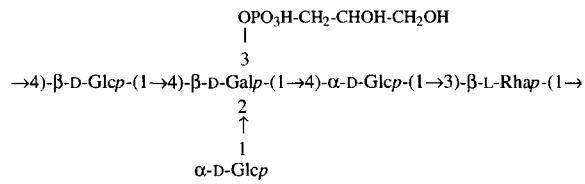
Received 20 February 1997; accepted 2 June 1997

**Abstract**

The structure of the capsular polysaccharide from *Streptococcus pneumoniae* type 18B has been determined using NMR spectroscopy and methylation analysis as the principal methods. It is concluded that the polysaccharide is composed of pentasaccharide repeating units with a glycerol phosphate substituting the 3-position of the branch point residue. The carbohydrate backbone in type 18B is identical to that in *S. pneumoniae* type 18F but without the O-acetyl groups present in that type.




In this structure, the absolute configuration of the glycerol phosphate moiety has not been determined but should be D, in analogy with that determined for the capsular polysaccharide from *S. pneumoniae* type 18A [T. Rundlöf, G. Widmalm, *Anal. Biochem.*, 243 (1996) 228–233]. 

*Keywords:* *Streptococcus pneumoniae*; NMR structure; Pneumococcus; Capsular polysaccharide

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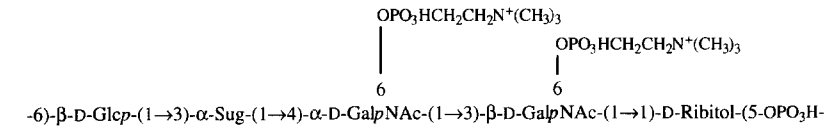
0008-6215/97/$17.00 
*PII* S0008-6215(97)00228-0

## 1. Introduction

The pneumococcus is a component of the normal microflora and most humans carry pneumococci in the upper respiratory tract. If the pneumococci enter other areas of the respiratory tract they may cause pneumonia or invasive diseases. It is known that a capsular polysaccharide is of vital importance for the virulence and that antibodies specific to the capsule exert type-specific protection.

The pneumococci produce two common antigenic polysaccharides; C-polysaccharide (or C-substance) and F-antigen. The C-substance is composed of a cell wall teichoic acid that is linked to cell wall peptidoglycan. The structure of the teichoic acid part has been established [1–3] and found to be composed of tetrasaccharides that are joined together through a ribitol phosphate diester, and it is therefore classified as a ribitol teichoic acid [4].




Sug = 2-acetamido-4-amino-2,4,6-trideoxygalactopyranoside

In addition, the C-substance contains two phosphorylcholine substituents per repeating unit. The F-antigen is a lipoteichoic acid, i.e., a teichoic acid covalently linked to a lipid. The structure of the teichoic acid part of the F-antigen is identical to that of the C-substance [2,5].

Today, 90 different capsular types of *S. pneumoniae* are known which are divided into 46 types or groups numbered 1–48 (numbers 26 and 30 are not in use) [6,7]. *S. pneumoniae* group 18 consists of four different types, 18F, 18A, 18B, and 18C, each of which produces its own, type-specific capsular polysaccharide. The corresponding antigenic formulas (18a, 18b, 18c, 18e, 18f) (own observations), (18a, 18b, 18d), (18a, 18b, 18e, 18g), and (18a, 18b, 18c, 18e), respectively [6,7], indicate that the capsular antigens elaborated by these types should have related chemical structures. The structures of the capsular polysaccharides from types 18F [8], 18A [9], and 18C [10] have been established. All three are composed of pentasaccharide repeating-units and contain a glycerol phosphate substituent. The capsular polysaccharide from types 18F and 18C are both composed of three residues of D-glucose and one residue each of D-galactose, L-rhamnose, glycerol, and phosphate. In addition, that from 18C is substituted with one *O*-acetyl group per repeating unit of undetermined location, and capsular polysaccharide 18F is substituted with two *O*-acetyl groups per repeating unit, at the terminal glucosyl group at O-6 and on the rhamnosyl residue at O-2. The anomeric configuration of the rhamnose residue in the 18C polysaccharide differs from that in types 18F and 18A but preliminary data indicate that it should be the same, i.e., β [11]. In that from type 18A one of the D-glucose residues is exchanged for a 2-acetamido-2-deoxy-D-glucose residue. We now report structural studies of the type 18B capsular polysaccharide.

## 2. Results and discussion

Pneumococcal type 18B cells were cultured and harvested by centrifugation, whereafter they were lysed with a deoxycholate-containing Tris-buffer solution. Cell debris was precipitated and centrifuged off. Proteins and lipids were removed by treatment with chloroform and butanol followed by centrifugation. Precipitation with $CaCl_2$ and centrifugation removed most nucleic acids. Treatment with DNAse, RNAse, and trypsin removed the remaining nucleic acids and proteins. Finally, the capsular polysaccharide was purified by gel filtration.

A hydrolysate of the polysaccharide obtained using aqueous trifluoroacetic acid contained glycerol, rhamnose, glucose, and galactose in the proportions 2:13:66:19. GLC analysis of the di-(1-phenylethyl)-dithioacetals [12,13] demonstrated that the glucose and galactose residues have the D configuration and the rhamnose residue has the L configuration.

Methylation analysis of the peracetylated polysaccharide revealed the presence of 3-substituted rhamnose, terminal and 4-substituted glucose, 2,4-disubstituted and 2,3,4-trisubstituted galactose in the

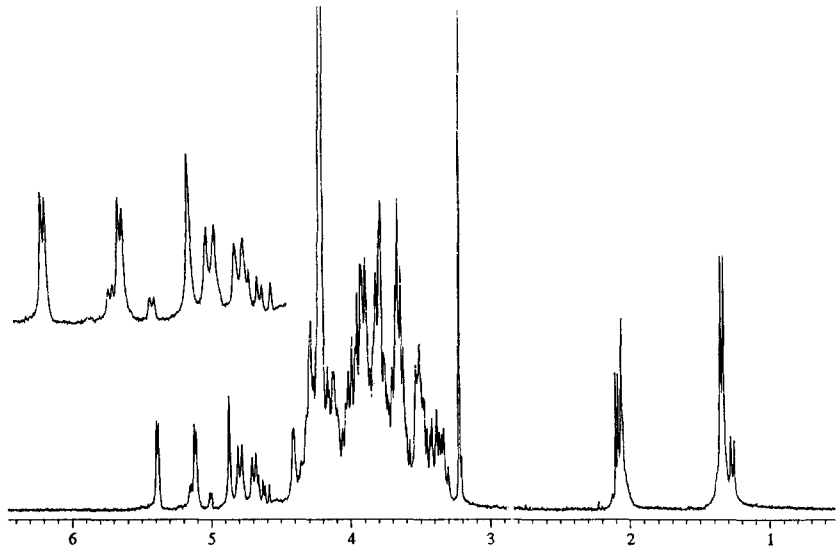



Fig. 1.

Table 1
$^{1}$H- and $^{13}$C-NMR data for *S. pneumoniae* type 18B

| Sugar residue | Chemical shifts[a] ($\delta$) | | | | | |
|---|---|---|---|---|---|---|
| | H-1/C-1 | H-2/C-2 | H-3/C-3 | H-4/C-4 | H-5/C-5 | H-6/C-6 |
| ↓ | | | | | | |
| 4 | 4.69 [7.4] | 4.02 | 4.31 | 4.42 | 3.77 | n.a.[b] |
| →3)-β-D-Gal*p*-(1→ | (0.16) | (0.57) | (0.72) | (0.53) | (0.12) | |
| 2 | 103.1 | 72.0 | 76.0 | 75.3 | 75.3 | |
| **A** ↑ | (5.7) | (-1.0) | (2.2) | (5.6) | (-0.6) | |
| →4)-β-D-Glc*p*-(1→ | 4.78 [7.8] | 3.33 | 3.67 | 3.63 | 3.51 | n.a. |
| **B** | (0.14) | (0.08) | (0.17) | (0.21) | (0.05) | |
| | 103.8 | 74.5 | 76.0 | 77.7 | 75.3 | |
| | (7.0) | (-0.7) | (-0.8) | (7.0) | (-1.5) | |
| →3)-β-L-Rha*p*-(1→ | 4.87 [n.r.][c] | 4.29 | 3.68 | 3.50 | 3.44 | 1.35 |
| **C** | (0.02) | (0.36) | (0.09) | (0.12) | (0.05) | (0.05) |
| | 101.6 | 68.1 | 78.5 | 71.3 | 72.9 | 17.5 |
| | (7.2) | (-4.1) | (4.7) | (-1.5) | (0.1) | (-0.1) |
| →4)-α-D-Glc*p*-(1→ | 5.11 [3.8] | 3.64 | 3.95 | 3.78 | 4.10 | n.a. |
| **D** | (-0.12) | (0.10) | (0.23) | (0.36) | (0.26) | |
| | 96.0 | 72.0 | 72.1 | 76.9 | 71.2 | |
| | (3.0) | (-0.5) | (-1.7) | (6.2) | (-1.2) | |
| α-D-Glc*p*-(1→ | 5.39 [3.5] | 3.51 | 3.88 | 3.38 | 4.12 | n.a. |
| **E** | (0.16) | (-0.03) | (0.16) | (-0.04) | (0.28) | |
| | 98.2 | 72.8 | 73.7 | 70.5 | 71.9 | |
| | (5.2) | (0.3) | (-0.1) | (-0.2) | (-0.5) | |
| —O | 3.93 | n.a. | 3.62 | | | |
| \| | 4.01 | | 3.69 | | | |
| O=P-O-Glycerol | 67.5 | n.a. | 63.4 | | | |
| \| | | | | | | |
| OH **F** | | | | | | |

[a] Chemical shift differences compared to monomers are given in parentheses and $J_{H\text{-}1,H\text{-}2}$ values [Hz] are given in square brackets.
[b] n.a. = not assigned.
[c] n.r. = not resolved.

proportions 13:19:45:17:6. The presence of both 2,4-di- and 2,3,4-trisubstituted galactose indicates that the D-galactosyl residue is linked through positions 2 and 4, and further, that the glycerol phosphate residue is linked to position 3 of the same residue but has been partially split off during the methylation.

Native type 18B polysaccharide initially gave NMR spectra with broad lines, but heating the sample for 12 h at 70 °C at pH 7.8 followed by 8 h in an ultrasonic bath gave a sample more suitable for NMR experiments. The $^1$H-NMR spectrum of the resulting material (Fig. 1) showed signals for five anomeric protons at $\delta$ 4.69, 4.78, 4.87, 5.11, and 5.39 corresponding to approximately one proton each. In addition, four smaller signals at $\delta$ 4.59, 4.64, 4.99, and 5.15 were present and assigned to the C-substance. The assignment of these signals was based on comparison to NMR data of authentic C-substance. The C-substance is present in most preparations of type-specific polysaccharides. It has been shown that at least some of it is covalently linked to peptidoglycan to which also C-substance is linked [14]. Treatment with enzymes degrades most of the peptidoglycan but the reactions are seldom quantitative, thereby leaving some peptidoglycan and C-substance in the preparations. From $^1$H-NMR spectra of different preparations of S18B, it was evident that C-substance mostly was abundant. The sample used in this study is however relatively free of C-substance. According to the intensity difference between the anomeric signals from type 18B polysaccharide and the anomeric signals from C-substance, the sample contains the two in a 5:1 ratio. The amount of peptidoglycan is difficult, however, to estimate. The signals at $\delta$ 5.11 and 5.39 have $J_{H1,H2}$ values of about 4 Hz, each typical for $\alpha$-*gluco*- and *galacto*-sugars; the signals at $\delta$ 4.69 and 4.78 have $J_{H1,H2}$ values of about 8 Hz, each typical for $\beta$-*gluco*- and *galacto*-sugars; and the remaining signal at $\delta$ 4.87 has a $J_{H1,H2}$ value below 1 Hz, probably a $\beta$-L-rhamnose residue. Evidence presented below showed this to be the case. In the high-field region the signal for the $CH_3$ group in L-rhamnose appeared at $\delta$ 1.35. In addition, the signal for the $CH_3$ group in 2-acetamido-4-amino-2,4,6-trideoxygalactose of C-substance appeared at $\delta$ 1.25 and signals for four *N*-acetyl groups, anticipated to belong to C-substance and peptidoglycan, appeared at $\delta$ 2.05, 2.06, 2.09, and 2.11 (see below). Also, a signal at $\delta$ 3.23 corresponding to the three $CH_3$ groups of the phosphocholine residues in C-substance was present. In accordance with the $^1$H-NMR data and a repeating unit of five sugars, the $^{13}$C-NMR spectrum had signals for five anomeric carbons at $\delta$ 95.7, 97.8, 101.2, 102.7, and 103.4, respectively. A signal at $\delta$ 17.5 corresponds to the methyl group in L-rhamnose. Also, in accord with the $^1$H-NMR data, signals at $\delta$ 94.5, 99.1, 102.5, and 105.0 were attributed to the four anomeric carbons of the sugar residues in C-substance and signals at $\delta$ 16.1, 22.8 (2C), 22.9, 23.2, 175.9, 176.0, 176.1, and 176.2 indicated the presence of the methyl group in the 2-acetamido-4-amino-2,4,6-trideoxygalactose residue and *N*-acetyl groups.

In order to assign the $^1$H-NMR chemical shifts $^1$H,$^1$H-correlated COSY, relayed COSY and HOHAHA experiments were performed. The sugar residues are named **A**–**E** according to increasing chemical shift of the signals from the anomeric pro-

Table 2
Observed interresidue NOE correlations for *S. pneumoniae* type 18B

| Residue | Anomeric proton | Interresidue NOE contact to proton | | |
|---|---|---|---|---|
| | $\delta$ | $\delta$ | Intensity[a] | Residue, atom |
| $\rightarrow$ 3)-$\beta$-D-Gal$p$-(1 $\rightarrow$ (4↓, 2↑) | | | | |
| **A** | 4.69 | 3.78 | s | **D**, H-4 |
| | | 3.88 | s | **D**, H-3 |
| $\rightarrow$ 4)-$\beta$-D-Glc$p$-(1 $\rightarrow$ | 4.78 | 4.42 | m | **A**, H-4 |
| **B** | | | | |
| $\rightarrow$ 3)-$\beta$-L-Rha$p$-(1 $\rightarrow$ | 4.87 | 3.63 | s | **B**, H-4 |
| **C** | | | | |
| $\rightarrow$ 4)-$\alpha$-D-Glc$p$-(1 $\rightarrow$ | 5.11 | 3.68 | s | **C**, H-3 |
| **D** | | 4.29 | w | **C**, H-2 |
| $\alpha$-D-Glc$p$-(1 $\rightarrow$ | 5.39 | 4.02 | s | **A**, H-2 |
| **E** | | | | |

[a] w = weak, m = medium and s = strong intensities.

tons, **F** being the glycerol phosphate residue. A proton decoupled proton–carbon correlated HSQC experiment gave the $^{13}$C-NMR chemical shifts. The $^{1}$H- and $^{13}$C-NMR data are shown in Table 1. Residue **C** was assigned to the 3-substituted L-rhamnose residue from the chemical shift of the C-6 signal at $\delta$ 17.5. Residues **B** and **D** were assigned to 4-substituted glucose residues, from the large glycosylation shifts for the C-4 signals. Similarly from the glycosylation shifts of the signals from C-2, C-3, and C-4 of residue **A** it is evident that it is the 2,3,4-trisubstituted D-galactose residue. Finally, residue **E** have relatively small $^{13}$C-NMR glycosylation shifts and was therefore assigned to the terminal D-glucose residue. The NMR data for C-substance will not be further discussed as they are in agreement with those published [1–3]. The ratio of choline methyl and acetyl methyl groups was 1:2, which indicates a significant portion of peptidoglycan. The $J_{C1,H1}$ value for the L-rhamnose residue was taken from a coupled proton–carbon correlated HMQC spectrum and was found to be 160 Hz ($\delta$ 101.6) corresponding to the β-configuration. Also the chemical shift value for the signal from H5 ($\delta$ 3.44) is in accord with β-configuration (cf H5 in α-Rha, $\delta$ 3.86).

A NOESY experiment (mixing time 100 ms) was run in order to obtain information about the sequence (Table 2). An NOE contact was observed between H-1 in the β-D-Gal*p* residue (**A**, $\delta$ 4.69) and H-4 in one of the α-D-Glc*p* residues (**D**, $\delta$ 3.78), thus establishing the structural element **A–D**. Furthermore, the anomeric proton of residue **C** ($\delta$ 4.87) gave a cross-peak to a signal at $\delta$ 3.67, probably arising from both H-3 in **C** ($\delta$ 3.68) and H-4 in **B** ($\delta$ 3.63). From the methylation analysis, it was evident that residue **B** is linked through position 4 and together with data given below it is evident that residue **C** is linked to H-4 in residue **B**. The anomeric proton of residue **D** ($\delta$ 5.11) had NOE contacts to H-2 and H-3 in residue **C** ($\delta$ 4.29 and 3.68, respectively). Both contacts are expected from the stereochemical arrangement around the glycosidic linkage with a γ-gauche interaction between H-1 in **D** and H-2 in **C**. Also, the small glycosidation shifts for the signals from C-1 in **D** and C-3 in **C** and the large upfield shift for the signal for C-2 in **C** are all in agreement with such a γ-gauche interaction. Thus, the structural element **D–C** is established. Also, an NOE contact was observed between the anomeric proton of the terminal α-D-Glc*p* residue (**E**, $\delta$ 5.39) and H-2 in the branch point residue (**A**, $\delta$ 4.02), thus demonstrating the element **E–A**. In addition, at a longer mixing time (300 ms) an NOE contact from H-1 in residue **B** ($\delta$ 4.78) to H-4 in residue **A** ($\delta$ 4.42) was observed, thus establishing the structural element **B–A**.

In order to confirm the result from the NOESY experiments a long-range proton–carbon correlated HMBC experiment was run (Table 3). A correlation between $\delta$ 4.69 (H-1 in residue **A**) and a resonance at $\delta$ 76.9 (C-4 in residue **D**) confirmed the element **A–D**. Another, between $\delta$ 103.1 (C-1 in residue **A**) and a resonance at $\delta$ 3.78 (H4 in residue **D**), corroborated the element. The anomeric protons of residues **D** ($\delta$ 5.11) and **C** ($\delta$ 4.87) showed connectivities to C-3 ($\delta$ 78.5) of residue **C** and to C-4 ($\delta$ 77.7) of residue **B**, respectively, confirming the element **D–**

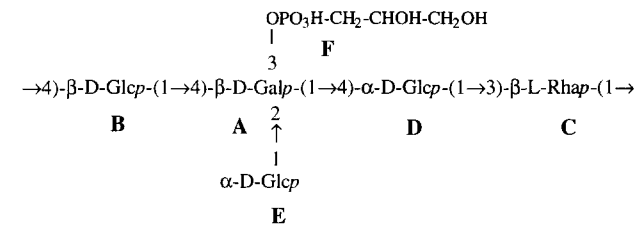
Table 3
Observed $^{2}J_{H,C}$ and $^{3}J_{H,C}$ interresidue connectivities in an HMBC experiment (60 ms delay) for the anomeric nuclei of *S. pneumoniae* type 18B

| Residue | Anomeric nucleus | | Interresidue $J_{H,C}$ connectivities to | | |
|---|---|---|---|---|---|
| | $\delta$($^{1}$H) | $\delta$($^{13}$C) | $\delta$($^{13}$C) | $\delta$($^{1}$H) | Residue, atom |
| **A** → 4)-β-D-Gal*p*-(1 → | 4.69 | 103.1 | 76.9 | 3.78 | **D**, C-4<br>**D**, H-4 |
| **B** → 4)-β-D-Glc*p*-(1 → | 4.78 | | 75.3 | | **A**, C-4 |
| **C** → 3)-β-L-Rha*p*-(1 → | 4.87 | | 77.7 | | **B**, C-4 |
| **D** → 4)-α-D-Glc*p*-(1 → | 5.11 | | 78.5 | | **C**, C-3 |
| **E** α-D-Glc*p*-(1 → | 5.39 | | 72.0 | | **A**, C-2 |

**C–B**. Also, the anomeric protons of residues **B** ($\delta$ 4.78) and **E** ($\delta$ 5.39) showed connectivities to C-4 ($\delta$ 75.3) of residue **A** and to C-2 ($\delta$ 72.0) of residue **A**, respectively. However, these connectivities can also be intramolecular; to C-5 in residue **B** and C-5 in residue **E**, respectively. Since no other connectivities were observed from residues **B** and **E**, the results are in agreement with the NOE data, confirming the elements **B–A** and **E–A**. In addition to the above interresidue correlations, a number of intra-residual correlations were obtained.

The result of the methylation analysis indicated that the glycerol phosphate residue substitutes position 3 of the 2,3,4-trisubstituted galactose residue and in order to confirm that, native 18B polysaccharide was treated with 48% HF for 96 h at 4 °C to remove the glycerol phosphate residue. This yielded a slightly degraded but mainly polymeric material which was eluted with the void volume on a Bio-Gel P-4 column. Methylation analysis of the polymeric material revealed the presence of 3-substituted rhamnose, terminal and 4-substituted glucose, and 2,4-disubstituted galactose in the proportions 18:20:41:21. NMR spectra were recorded on the modified polymer and most of the $^{1}$H- and $^{13}$C-NMR chemical shifts could be assigned (Table 4). The sequence information from NOESY spectra were in accordance with the result from the native polysaccharide with the exception that the anomeric proton in residue **A** did not show any interresidue contacts. In the HMBC spectrum interresidue correlations from anomeric atoms of residues **B**, **C**, and **E** could be observed.

From the combined results of the methylation analyses on the native and the dephosphorylated material, together with the NMR results on native material we conclude the following structure:




In this structure, the absolute configuration of the glycerol phosphate moiety has not been determined but should be D, in analogy with that determined for

Table 4
$^{1}$H- and $^{13}$C-NMR data for dephosphorylated *S. pneumoniae* type 18B

| Sugar residue | Chemical shifts[a] ($\delta$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | H-1/C-1 | H-2/C-2 | H-3/C-3 | H-4/C-4 | H-5/C-5 | H-6/C-6 | |
| → 4)-β-D-Gal*p*-(1 → | 4.61 [163] | 3.71 | 3.80 | 4.13 | ca. 3.72 | n.a.[b] | |
| 2 ↑ | | | | | | | |
| **A** | (0.08) | (0.26) | (0.21) | (0.24) | (0.07) | | |
| | 102.7 | 77.1 | 73.0 | 79.7 | 73.9 | | |
| | (5.3) | (4.1) | (−1.1) | (10.0) | (−2.0) | | |
| → 4)-β-D-Glc*p*-(1 → | 4.61 [163] | 3.32 | 3.58 | 3.59 | 3.45 | n.a. | |
| **B** | (−0.03) | (0.07) | (0.08) | (0.17) | (−0.01) | | |
| | 105.1 | 74.9 | 76.5 | 78.5 | 75.7 | | |
| | (8.3) | (−0.3) | (−0.3) | (7.2) | (−1.1) | | |
| → 3)-β-L-Rha*p*-(1 → | 4.81 [162] | 4.23 | 3.61 | 3.39 | 3.35 | 1.28 | |
| **C** | (−0.04) | (0.30) | (0.02) | (0.01) | (−0.03) | (−0.02) | |
| | 101.7 | 68.2 | 77.9 | 71.3 | 73.2 | 17.5 | |
| | (7.3) | (−4.0) | (4.7) | (0.4) | (−1.5) | (−0.1) | |
| → 4)-α-D-Glc*p*-(1 → | 5.06 [174] | 3.57 | 3.88 | ~ 3.76 | 4.05 | n.a. | |
| **D** | (−0.17) | (−0.03) | (0.17) | (0.34) | (0.21) | | |
| | 96.1 | 72.4 | 72.3 | 76.6 | 71.5 | | |
| | (3.1) | (−0.1) | (−1.5) | (5.9) | (−0.9) | | |
| α-D-Glc*p*-(1 → | 5.27 [172] | 3.48 | 3.65 | 3.38 | 4.00 | 3.74 | ca. 4.0 |
| **E** | (0.04) | (−0.06) | (−0.07) | (−0.04) | (0.16) | (−0.02) | |
| | 99.1 | 72.4 | 75.4 | 70.5 | 72.7 | | |
| | (6.1) | (−0.1) | (1.6) | (−0.2) | (0.6) | | |

[a] Chemical shift differences compared to monomers are given in parentheses and $J_{H\text{-}1,C\text{-}1}$ values [Hz] are given in square brackets.
[b] n.a. = not assigned.

the capsular polysaccharide from *S. pneumoniae* type 18A [15].

The close relation between the structures within pneumococcal group 18 is demonstrated by the new data on type 18B polysaccharide. The structure corresponds to the carbohydrate backbone of S18F but it is still unclear what the antigenic factor 18g corresponds to, as all monosaccharide structural features in S18B are present also in the other serotypes. If the 18g factor corresponds to a larger structural element however, one could envisage parts that are unique.

## 3. Experimental

*Bacterial strain.*—The pneumococcal type 18B reference strain from the WHO Collaborating Centre for Reference and Research on Pneumococci, Statens Serum Institut, Copenhagen was used for preparation of the capsular polysaccharide. Identity and optimal capsulation of the strain was controlled by the capsular reaction test using typing antisera from Statens Serum Institut.

*General methods.*—Concentrations were performed by flushing with air at 50 °C. For GLC, a Hewlett-Packard 5890 instrument fitted with a flame-ionisation detector was used. Separations of alditol acetates were performed on a DB-5 capillary column (15 m × 0.25 mm), using a temperature programme 160 °C (1 min) → 250 °C at 3 °C/min. GLC–MS (EI) was performed on a Delsi Di200 GC-Nermag R10-10H quadrupole MS. Partially methylated alditol acetates were separated on a DB-5 capillary column (15 m × 0.25 mm), using a temperature programme 130 °C (1 min) → 250 °C at 3 °C/min. Analysis of the acetylated di-(1-phenylethyl)-dithioacetals were performed on a DB-5 column (28 m) with the temperature program 275 °C (1 min) → 300 °C at 2 °C/min [12,13]. Gel permeation chromatography was performed on a column of Bio-Gel P-4 (Bio-Rad, USA), using aq 0.1 M pyridinium acetate buffer (pH 5.6) as eluent. Fractions were monitored by a differential refractometer.

*Preparation of capsular polysaccharide.*—Capsular polysaccharide was prepared from an autolysate of the type 18B pneumococcal strain. The strain was cultured anaerobically for 8 h in a 50 L fermentor at 37 °C using 30 L trypsin broth (Statens Serum Institut) as medium. During growth, the pH was kept constant at 7.0 with 1 M NaOH. The fermentor was then cooled by tap water (15 °C) until the bacterial cells were harvested by the use of a flow centrifuge (Biofuge, Heraeus Instruments). The cells were lysed in 300 mL 0.15 M Tris–HCl buffer pH 7.5 containing 0.1% sodium deoxycholate. In order to kill viable pneumococci, the lysate was heated to 50 °C for 30 min. After cooling to room temperature the pH was adjusted to 5.0 by adding HOAc, and cell debris and deoxycholate, precipitated at the low pH, was then removed by centrifugation. Proteins were removed by shaking with 0.25 volumes of 5:1 chloroform–butanol followed by centrifugation. Capsular polysaccharide was purified after the deproteinization by fractionation with EtOH in two steps (25% and 80% v/v) in the presence of $CaCl_2$ (1% w/v) at 5 °C. The precipitate was treated with DNase, RNase and trypsin according to the procedure given by the manufacturer (Sigma). The polysaccharide was then fractionated by gelfiltration on a Sephacryl S300 column (Pharmacia). The high-molecular-weight fractions containing the capsular polysaccharide were pooled and lyophilized.

*NMR spectroscopy.*—NMR spectra of solutions in $D_2O$ were recorded at 70 °C with a JEOL EX270 instrument, except for the decoupled HSQC spectrum which was run on a Varian Unity 600 instrument. Chemical shifts are reported in ppm, using acetone ($\delta_C$ 31.00) and sodium 3-trimethylsilylpropanoate-$d_4$ ($\delta_H$ 0.00) as internal references. The native polysaccharide was converted to its ammonium salt by passage through a CM Sepharose cation-exchange column, which resulted in that the pH became 7.8. Heating at 70 °C for 12 h followed by ultrasonication in a bath for 8 h made the sample more suitable for spectroscopy. $^1$H,$^1$H-COSY and relayed COSY experiments were performed using JEOL standard pulse-sequences. $^1$H,$^1$H-HOHAHA and NOESY experiments were performed in the phase-sensitive mode. The mixing times in the NOESY experiments were 100 and 300 ms and $^1$H,$^1$H-HOHAHA experiments were obtained using mixing times of 40, 80 and 120 ms. Proton–carbon correlated spectra (HMQC) were obtained with or without decoupling.

*Sugar and methylation analysis.*—Hydrolyses of native and methylated polysaccharide were performed by treatment with 2 M $CF_3CO_2H$ at 120 °C for 2 h. For the methylation analysis peracetylated polysaccharide was used; 2 mg of native S18B was dissolved in a mixture of formamide (600 μL), pyridine (200 μL) and acetic anhydride (200 μL). The mixture was stirred at room temperature for 24 h, dialysed and freeze-dried [16]. Methylation was performed with methyl iodide in the presence of sodium methyl sulfinyl methanide and the methylated prod-

ucts were purified by absorbing them on Sep-Pak $C_{18}$-cartridges and subsequently eluting them with acetonitrile. The sugars in the hydrolysates were converted into alditol acetates and partially methylated alditol acetates according to standard methods [17,18]. Identification of the methylated sugars were made by MS and by comparison with references.

*Dephosphorylation.*—Native polysaccharide (15 mg) in aq 48% HF (2 mL) was kept for 96 h at 4 °C. The acid was removed by flushing with air. The material was redissolved in water and fractionated on a column of Bio-Gel P-4, irrigated with a 0.1 M pyridinium acetate buffer (pH 5.6). The dephosphorylated polymer was eluted mainly with the void volume.

## Acknowledgements

This work was supported by grants from the Swedish Natural Science Research Council and the Swedish Research Council for Engineering Sciences.

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