Carbohydrate Research, 182 (1988) 111–117
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE FROM *Streptococcus pneumoniae* TYPE 2, A REINVESTIGATION

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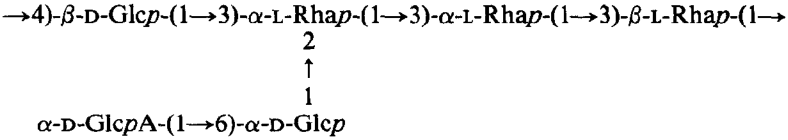
(Received January 21st, 1988; accepted for publication, March 2nd, 1988)

ABSTRACT

The structure of the capsular polysaccharide from *Streptococcus pneumoniae* type 2 has been reinvestigated, specific degradations and n.m.r. spectroscopy being the main methods used. It is concluded that the polysaccharide is composed of hexasaccharide repeating-units having the following structure, which differs from that previously proposed.



$$\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-}\underset{\substack{2\\ \uparrow\\ 1\\ \alpha\text{-D-Glc}p\text{A-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p}}{\text{Rha}p}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow$$

INTRODUCTION

The anomeric configurations of sugar residues in polysaccharides are generally determined by using ^{1}H- and ^{13}C-n.m.r. spectroscopy. Before the introduction of Fourier-transform n.m.r. spectroscopy, other methods had to be used, one of these being oxidation of the fully acetylated polysaccharide with chromium trioxide in acetic acid. Acetylated hexopyranosides with an equatorially attached aglycon are oxidised to 5-hexulosonates, but those with an axially attached aglycon are oxidised only slowly[1]; several acetylated oligo- and poly-saccharides behaved analogously[2]. The method has been useful in several studies but failed in others, and there is reason to reinvestigate structures based upon the results of such oxidation. Thus, the ^{1}H-n.m.r. spectrum of the *Streptococcus pneumoniae* type 2 capsular polysaccharide (S-2) contained, *inter alia*, a signal for an anomeric proton at δ 4.68 ($J_{1,2}$ 8.1 Hz). This is in disagreement with the proposed structure[3] (**1**), in which all three sugar

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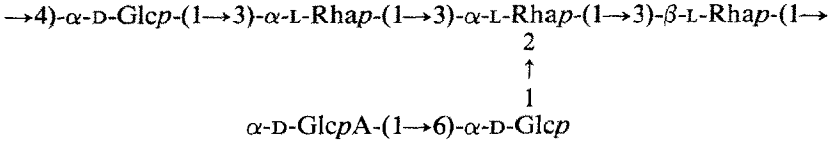
residues with the *gluco* configuration are α-linked. We have therefore reinvestigated the structure of S-2.



$$\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-}\underset{\substack{2\\ \uparrow\\ 1\\ \alpha\text{-D-Glc}p\text{A-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p}}{\text{Rha}p}\text{-}(1\rightarrow 3)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow$$

1

RESULTS AND DISCUSSION

The methylation analysis of S-2, without and with carboxyl-reduction of the methylated polysaccharide (Table I, columns A and B), showed good agreement with previous analyses. The ^{1}H-n.m.r. spectrum (Table II) showed, *inter alia*, signals for six anomeric protons. Of the three sugar residues with the *gluco* configuration, two are α-linked and one is β-linked. In agreement with this, the ^{13}C-n.m.r. spectrum contained, *inter alia*, signals for six anomeric carbons at δ 104.9 ($J_{C,H}$ 163 Hz), 102.8 ($J_{C,H}$ 168 Hz), 101.2 ($J_{C,H}$ 158 Hz), 100.5 ($J_{C,H}$ 171 Hz), 99.2 ($J_{C,H}$ 170 Hz), and 98.5 ($J_{C,H}$ 170 Hz). From the coupling constants[4], it is concluded that two sugar residues in S-2 are β-linked and the other four are α-linked. One of the L-rhamnopyranosyl residues is consequently β-linked. The results discussed above confirm that S-2 is composed of hexasaccharide repeating-units, and that the sugar residues are linked through the same positions as in **1**.

Partial hydrolysis of S-2 with acid, followed by borodeuteride reduction and gel-permeation chromatography, yielded a trisaccharide-alditol. Methylation

TABLE I

METHYLATION ANALYSIS OF NATIVE AND MODIFIED S-2 AND SOME DEGRADATION PRODUCTS

Sugar[a]	T[b]	*Mole%*			
		A[c]	B	C	D
1,3,4,5-Rhamnitol	0.24			28[e]	
2,3,4-Rha	0.66				30
2,4-Rha	0.88	39	29		70
4-Rha	1.22	20	16		
2,3,6-Glc	1.57	25	20		
2,3,4-Glc	1.66	16	35[d]	72[d]	

[a] 2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, *etc.* [b] Retention time of the derived alditol acetate, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an Ultra 2 column (see Experimental). [c] Key: *A*, Native S-2; *B*, methylated and carboxyl-reduced S-2 (using sodium borodeuteride); *C*, oligosaccharide from partial acid hydrolysis; *D*, Smith-degradation product. [d] Half of this component dideuterated at C-6. [e] Monodeuterated at C-1.

TABLE II

^{1}H-N.M.R. CHEMICAL SHIFTS FOR S-2

Sugar residue	*Chemical shifts (p.p.m.)*^{a}		
	H-1	*H-2*	*H-3*
→3)-α-L-Rha*p*-(1→ 2 ↑	5.15[n.r.] (0.03)	4.30 (0.38)	4.10 (0.29)
→3)-α-L-Rha*p*-(1→	5.04[n.r.] (−0.08)	4.16 (0.24)	3.96 (0.15)
→6)-α-D-Glc*p*-(1→	5.03[4.0] (−0.20)	3.56 (0.02)	3.77 (0.05)
α-D-Glc*p*A-(1→	4.96[3.7] (−0.27)	3.60 (0.06)	3.76 (0.04)
→3)-β-L-Rha*p*-(1→	4.87[n.r.] (0.02)	4.17 (0.24)	3.66 (0.07)
→4)-β-D-Glc*p*-(1→	4.68[8.1] (0.04)	3.28 (0.03)	3.63 (0.13)

^{a}Chemical shift differences relative to the monomers are given in parenthesis and $J_{1,2}$ values (Hz) in brackets.

analysis, with carboxyl-reduction ($NaBD_4$) of the methylated product, gave the sugars listed in Table I, column C. The ^{1}H-n.m.r. spectrum contained, *inter alia*, signals for anomeric protons at δ 5.18 ($J_{1,2}$ 3.4 Hz) and 4.95 ($J_{1,2}$ ~3 Hz). From these results, it is concluded that the trisaccharide-alditol has structure **2**.

α-D-Glc*p*A-(1→6)-α-D-Glc*p*-(1→2)-L-Rhamnitol

2

Trisaccharide **2** accounts for the two sugar residues in the repeating unit of S-2 with the α-*gluco* configuration, and the 4-linked D-glucopyranosyl residue consequently has the β-configuration.

Smith degradation[5] of S-2 and fractionation of the product by gel filtration yielded a product eluted in the trisaccharide region. N.m.r. spectra indicated that this was a mixture, and it was fractionated into two components by h.p.l.c. Methylation analysis of the major component (Table I, column D) showed that it contained one terminal and two 3-linked rhamnopyranosyl residues. N.m.r. spectroscopy, discussed in detail below, demonstrated that it has structure **3**.

α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→2)-D-Erythritol

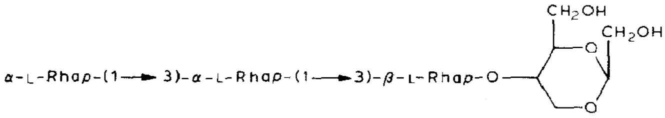
3

The ^{1}H- and ^{13}C-n.m.r. spectra of **3** could be fully assigned (Table III) by using 2D-techniques. The residue giving signals for its anomeric carbon and proton at δ 103.0 and 5.07, respectively, was identified as α-linked because of the $J_{H-1,H-2}$ value and the low-field position of the H-5 signal, and as terminal because of the insignificant shifts of the C-3, H-3, and H-4 signals, relative to the corresponding signals of α-L-rhamnopyranose. The other groups of signals were assigned to the 3-linked α- and β-L-rhamnopyranosyl residues because of substantial shifts for the signals given by C-3, H-3, and H-4. The mutual order of these residues in **3** could be determined from the ^{13}C-n.m.r. signals. From previous studies[6], the shifts of the C-2 and C-4 signals of the third L-rhamnopyranosyl residue in the structures **3** and **4** could be estimated as −0.5 and −0.4 for **3** and −1.7 and −3.1 for **4**. The actual values are −0.4 and −0.9 p.p.m. for the α-linked and −0.7 and −0.6 p.p.m. for the β-linked residue, thus strongly supporting the mutual order of these residues given in structure **3**.

α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-D-Erythritol

4

The second, minor component from the Smith degradation gave, on methylation analysis, the same methylated sugars as **3**, but gave two additional signals in the ^{13}C-n.m.r. spectrum, one in the "anomeric region" and one in the "bulk region". The product is therefore most probably a derivative of **3** in which glycolaldehyde is linked acetalically to the erythritol moiety, as in **5**. The high-field position of the new acetalic carbon signal at δ 100.8 or 98.7 indicates the presence of a 1,3-dioxane ring.



5

Similar by-products in the Smith degradation have been observed[5]. Comparison of the ^{13}C-n.m.r. spectra of **3** and **5** shows that the signal for C-1 of the α-L-rhamnopyranosyl residues are virtually the same, whereas that for C-1 of the β-L-rhamnopyranosyl residue at δ 100.0 has shifted to either δ 100.8 or 98.7 in **5**. This lends further support to the sequence of rhamnosyl residues given in **3** and **5**.

The results discussed above show that S-2 is composed of hexasaccharide repeating-units having the structure **6** or an alternative structure in which the side chain is linked to another L-rhamnopyranosyl residue in the main chain. In order to distinguish between these possibilities, the signals for H-1, H-2, and H-3 of the six

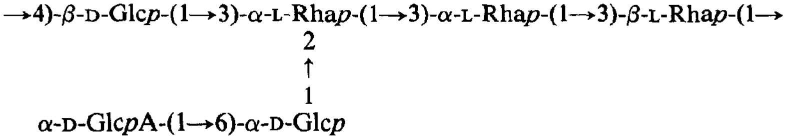
TABLE III

^{1}H- AND ^{13}C-N.M.R. CHEMICAL SHIFTS FOR THE SMITH-DEGRADATION PRODUCT

Sugar residue	*Chemical shifts (p.p.m.)*[a] *1*	*2*	*3*	*4*	*5*	*6*
α-L-Rha*p*-(1→	(C) 103.0 (H) 5.07 [1.5]	71.1 4.08	71.2 3.85	73.0 3.48	69.9 3.86	17.5 1.34
→3)-α-L-Rha*p*-(1→	102.9 5.03 [1.8]	70.9 4.15	79.2 3.93	72.2 3.57	70.1 3.91	17.5 1.34
→3)-β-L-Rha*p*(1→	100.0 4.79 [n.r.]	71.6 4.11	81.3 3.65	72.2 3.50	72.9 3.43	17.5 1.35
→2)-Erythritol	61.5 3.79	72.1 ~3.89	80.7 ~3.85	63.6 3.74		
α-L-Rhamnose	94.8 5.12	71.8 3.92	71.0 3.81	73.2 3.45	69.1 3.86	17.6 1.28

[a] $J_{1,2}$ values (Hz) in brackets.

sugar residues in the repeating unit were assigned, using 2D-COSY experiments (Table II). The H-1 signals given by the rhamnopyranosyl residues were recognised from their low $J_{1,2}$ values. The signal at δ 5.15 was assigned to the branching α-L-rhamnopyranosyl residue because of the large downfield shifts of the H-2 and H-3 signals, caused by the substitution in the 2-position. The signals at δ 5.03 and 4.96, assigned to the α-D-glucopyranosyl residue and the α-D-glucopyranosyluronic acid group, were distinguishable because of the presence of n.O.e. signals for the anomeric proton to H-1 and H-2 in the residue having its anomeric proton at δ 5.15 for the former and the absence of n.O.e. interactions from the anomeric proton of the latter. The assignments of the other signals were straightforward and require no comments.



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6

The mutual order of the sugar residues was determined using n.O.e. experiments. Interactions between H-1 of the 2,3-linked α-L-rhamnopyranosyl residue and H-3 of the 3-linked α-L-rhamnopyranosyl residue, between H-1 of the latter residue and H-3 of the 3-linked β-L-rhamnopyranosyl residue, and between H-1 of the β-D-glucopyranosyl residue and H-3 of the branching α-L-rhamnopyranosyl residue are all in agreement with structure **6** and exclude the other, alternative structures.

Structure **6**, which is now proposed for the repeating unit of S-2, differs from the previously proposed structure (**1**) in two respects. The anomeric configuration of the D-glucopyranosyl residue in the main chain is β instead of α, and the side chain has been moved to the other α-L-rhamnopyranosyl residue.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at bath temperatures not exceeding 40°. G.l.c. was performed on an Ultra 2 (cross-linked 5% phenyl - 95% methyl silicone) fused-silica capillary column, using a temperature programme (150° for 2 min, 150° → 220° at 2°/min, 220° for 20 min). G.l.c.-m.s. was performed on a Hewlett-Packard 5790-5970 system, using the same column. A differential refractometer was used for monitoring h.p.l.c. and gel-chromatography column effluents. H.p.l.c. was performed on a Waters Carbohydrate Analysis Column (3.9 mm x 30 cm, stainless steel column), with acetonitrile-water (70:30) at a flow rate of 1 mL/min. Gel filtration was performed on a column (2.6 x 80 cm) of Bio-Gel P-2, irrigated with water. Methylation analyses were performed essentially as previously described[7,8]. Carboxyl-reduction and purification of methylated products were performed as described by Waeghe *et al.*[8].

N.m.r. spectra of solutions in deuterium oxide were determined at 70°, using a JEOL GX-270 or GX-400 instrument. Chemical shifts are reported in p.p.m. relative to the signals for internal 1,4-dioxane (67.40 p.p.m., ^{13}C) and internal sodium 3-trimethylsilylpropanoate-d_4 (δ 0.00 p.p.m., ^{1}H). For the assignment of signals, standard homo- and hetero-nuclear COSY, relayed COSY, double-relayed COSY, and NOESY pulse sequences were used.

Purification of S-2. — Crude S-2 was purified on a column (2.6 x 30 cm) of DEAE-Sepharose CL-6B irrigated first with water (200 mL) and then with a linear gradient of aqueous chloride (0→M, 1000 mL). Peaks were monitored by polarimetry. The material was recovered by dialysis and freeze-drying.

Partial acid hydrolysis of S-2. — A solution of S-2 (12 mg) in 0.1M trifluoroacetic acid (5 mL) was kept for 2 h at 100° and then freeze-dried. A solution of the product in water containing sodium borodeuteride (10 mg) was kept overnight at room temperature, excess of borodeuteride was decomposed by adding acetic acid, and the solution was concentrated. Boric acid was removed by codistillation with 10% acetic acid in methanol (3 x 3 mL) and methanol (3 x 3 mL). The product was fractionated by gel filtration. One main product was obtained in the oligosaccharide region (2 mg).

Smith degradation of S-2. — A solution of S-2 (75 mg) was oxidised with 40mM sodium metaperiodate in sodium acetate buffer (0.1M, pH 3.9; 40 mL) for 95 h at 4°. Excess of periodate was reduced with ethylene glycol, and the product was isolated by chromatography on a column (2.6 x 80 cm) of Bio-Gel P-2. A solution of the product in water (20 mL) was treated with sodium borohydride (500 mg) for 18 h at room temperature, excess of reagent was decomposed with acetic acid, and the product was isolated by chromatography on a column (2.6 x 80 cm) of Bio-Gel P-2. Part of the material was subjected to methylation analysis which showed that the oxidation was complete.

The product (47 mg) was treated with 0.5M trifluoroacetic acid (20 mL) for 40 h at room temperature, freeze-dried, and fractionated by gel filtration. One major product (19 mg) was eluted in the oligosaccharide region. Further purification by h.p.l.c. gave I (7 mg) and II (10 mg). Hydrolysates of I and II both contained rhamnose and erythritol in the ratios 2.9:1 and 2.6:1, respectively, and were assigned to **5** and **3**, respectively.

ACKNOWLEDGMENTS

We thank Mr. Thomas Wehler for assistance with the NOESY experiment. This work was supported by grants from the Swedish Medical Research Council (03X-02533) and the Swedish Board for Technical Development.

REFERENCES

1 S. J. Angyal and K. James, *Aust. J. Chem.*, 23 (1970) 1209-1215.

2 J. Hoffmann, B. Lindberg, and S. Svensson, *Acta Chem. Scand.*, 26 (1972) 661-666.

3 L. Kenne, B. Lindberg, and S. Svensson, *Carbohydr. Res.*, 40 (1975) 69-75.

4 K. Bock and C. Pedersen, *J. Chem. Soc., Perkin Trans. 2*, (1974) 293-297.

5 I. J. Goldstein, G. W. Hay, B. A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, 5 (1965) 361-370.

6 N. K. Kochetkov, O. S. Chizhov, and A. S. Shashkov, *Carbohydr. Res.*, 133 (1984) 173-185.

7 P.-E. Jansson, L. Kenne, H. Liedgren, B. Lindberg, and J. Lönngren, *Chem. Commun., Univ. Stockholm*, 8 (1976) 1-75.

8 T. J. Waeghe, A. G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281-304.