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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE FROM *Streptococcus pneumoniae* TYPES 15B AND 15C

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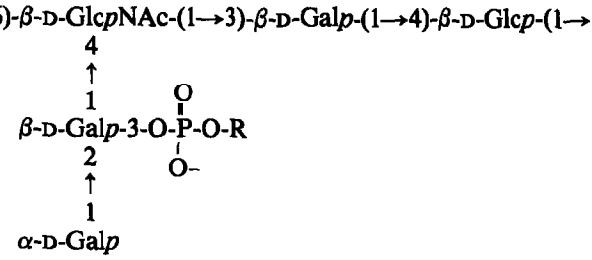
ABSTRACT

The structures of the capsular polysaccharides (S-15B and S-15C) from *Streptococcus pneumoniae* types 15B and 15C have been investigated by using n.m.r. spectroscopy, methylation analysis, and various specific degradations. It is concluded that the polysaccharides are composed of pentasaccharide repeating-units having the following structure:



In this structure, R is H (80%) or $CH_2CH_2N^+Me_3$ (20%). S-15B further contains *O*-acetyl groups, approximately 0.7 per repeating unit, which have not been located. The capsular polysaccharides S-15F and S-15A, which have been studied previously, are also composed of pentasaccharide repeating-units, containing the same sequence of sugars, but in a linear arrangement.

INTRODUCTION

Streptococcus pneumoniae group 15 has been divided into four types[1], 15F, 15A, 15B, and 15C. The structures of the capsular polysaccharides (S-15F and S-15A) elaborated by the two former types have been determined[2,3]. They both contain the same pentasaccharide repeating-unit, with phosphate (S-15F) or glycerol phosphate (S-15A) linked to the 3-position of a β-D-galactopyranosyl residue. S-15F further contains *O*-acetyl groups (~2 per repeating unit). We now report structural studies of S-15B and S-15C, elaborated by the corresponding types 15B and 15C.

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RESULTS AND DISCUSSION

The polysaccharides S-15B and S-15C were purified by ion-exchange chromatography on DEAE-Sepharose. The former contained *O*-acetyl groups, approximately 0.7 per *N*-acetyl group, according to the ^{13}C- and ^{1}H-n.m.r. spectra. S-15C and the product obtained on *O*-deacetylation of S-15B, by treatment with base under mild conditions, proved to be identical as their ^{1}H- and ^{13}C-n.m.r. spectra were superposable. The subsequent structural studies will therefore only be reported for S-15C.

Sugar analysis of S-15C gave D-glucose, D-galactose, and 2-amino-2-deoxy-D-glucose in the relative proportions 1:3:1. The absolute configurations of the sugars were determined as described by Leontein *et al.*[4]. The polysaccharide further contained phosphate (3.2% as P) corresponding to ~1 phosphate group per 5 sugar residues. On dephosphorylation with aqueous 48% hydrogen fluoride, the still-polymeric material contained less than 0.1% of P. On dephosphorylation of S-15C with alkaline phosphatase, only ~75% of the phosphate was removed, indicating that this part is present as monoesters and the remaining 25% as diesters. In agreement with this, signals in the ^{31}P-n.m.r. spectrum of a solution of S-15C at pH7 were observed at approximately −1 p.p.m. (80%) and +1 p.p.m. (20%), relative to aqueous 85% phosphoric acid.

The ^{1}H-n.m.r. spectrum of the dephosphorylated S-15C contained, *inter alia*, signals of equal intensities at δ 5.31, 4.75, 4.65, 4.51, and 4.43. The first had $J_{1,2}$ ~4 Hz, the other four had $J_{1,2}$ ~7 Hz, indicating that the polysaccharide was composed of pentasaccharide repeating-units, containing one α-linked and four β-linked pyranosidic sugar residues. A signal at δ 2.01 (3 H) further indicated the 2-amino-2 deoxy-D-glucose to be *N*-acetylated. In agreement with these observations, the ^{13}C-n.m.r. spectrum contained, *inter alia*, signals at δ 175.3, 104.7, 104.2 (2 C), 103.1, 99.7, 57.3, and 24.0.

The ^{1}H-n.m.r. spectrum of the native S-15C was similar to that of the dephosphorylated product and, in addition, contained signals in the region for anomeric protons at δ 4.57 (0.2 H) and 4.52 (0.7 H) and also a signal at δ 3.19 (1.5 H). The former signals may be due to the proton on the carbon atom carrying the phosphate ester group. The latter corresponds to the signal given by a choline phosphate residue. A signal in the ^{13}C-n.m.r. spectrum at δ 56.0, which disappeared after dephosphorylation, supports this assumption. The percentage (~20%) of choline phosphate obtained from these analyses corresponds reasonably well with the percentage of phosphodiesters, as discussed above.

Methylation analysis of the fully dephosphorylated S-15C gave the sugars listed in Table I, column A. The results of the analysis supports the assumption that S-15C is composed of pentasaccharide repeating-units and indicates that these contain a terminal D-galactopyranosyl group, a D-glucopyranosyl residue linked through O-4, a D-galactopyranosyl residue linked through O-2, a D-galactopyranosyl residue linked through O-3, and a 2-acetamido-2-deoxy-D-glucopyranosyl residue

TABLE I

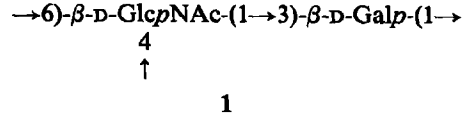
METHYLATION ANALYSIS OF NATIVE AND MODIFIED S-15C

Sugar[a]	T[b]	*Mole %*		
		A[c]	B	C
2,3,4,6-Gal	1.19	24	26	31
2,3,6-Glc	2.27	32	38	34
3,4,6-Gal	2.08	22	—	13
2,4,6-Gal	1.92	20	24	15
4,6-Gal	2.69	1	11	7
2,3-GlcNAc[d]	-	+	+	+

[a]2,3,4,6-Gal = 2,3,4,6-tetra-*O*-methyl-D-galactose, *etc.* [b]Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on an SP-2340 column. [c]*A*, Dephosphorylated S-15C; *B*, native PS; *C*, partially dephosphorylated PS. [d]Determined using an SE-54 column and a temperature programme.

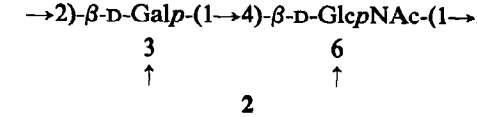
linked through O-4 and O-6. Methylation analysis of original S-15C (Table I, column B) demonstrates that phosphate is linked to O-3 of the 2-*O*-substituted D-galactopyranosyl residue. Methylation of the sample of S-15C which had been treated with alkaline phosphatase (Table I, column C) finally confirmed that only part of the phosphate ester group was removed by this treatment.

Smith degradation of dephosphorylated S-15C gave an oligosaccharide which, on methylation analysis, gave 2,4,6-tri-*O*-methyl-D-galactose and 2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-D-glucose. The ^{1}H-n.m.r. spectrum of this product showed, *inter alia*, signals for two anomeric protons at δ 4.70 (H, $J_{1,2}$ 8.5 Hz) and 4.45 (H, $J_{1,2}$ 7.5 Hz). These results demonstrate the presence of partial structure **1**.



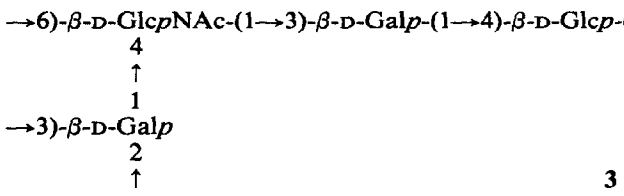
1

Smith degradation of the original S-15C and fractionation of the product on Bio-Gel P-2 gave a product that was eluted in the trisaccharide region and did not contain phosphate. This indicates that some cleavage of phosphate ester bonds occurred during the Smith hydrolysis. This product, on methylation analysis, yielded comparable amounts of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose, and 2-deoxy-3,6-di-*O*-methyl-2-*N*-methylacetamido-D-glucose. The ^{1}H-n.m.r. spectrum contained, *inter alia*, three signals for anomeric protons at δ 4.77 (H, $J_{1,2}$ ~7 Hz, somewhat contaminated), 4.47 (H, $J_{1,2}$ 7.6 Hz), and 4.46 (H, $J_{1,2}$ 7.6 Hz). In agreement with this, the ^{13}C-n.m.r. spectrum showed signals at δ 103.9, 103.8, and 103.2. These results demonstrate the presence of partial structure **2**.



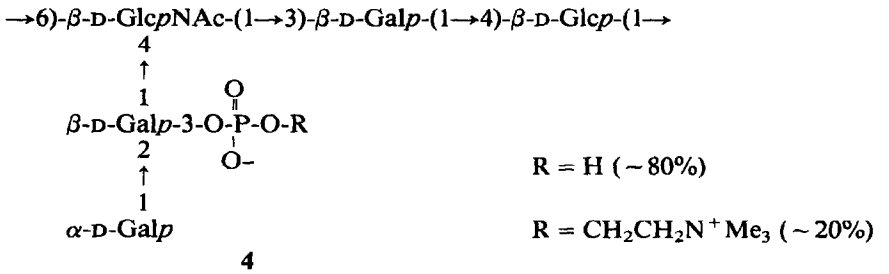
2

In the original S-15C, the terminal D-galactopyranosyl group, which contains a pair of *cis*-hydroxyl groups, should be more readily oxidised by periodate than the 4-substituted β-D-glucopyranosyl residue. Therefore, S-15C was subjected to a modified Smith-degradation, using only 1.1 mol of periodate per pentasaccharide repeating-unit. A phosphate-free, polymeric product was obtained after fractionation on Bio-Gel P-2. Methylation analysis of this product gave comparable amounts of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-galactose, and 2-deoxy-3-*O*-methyl-2-*N*-methylacetamido-D-glucose. The ^{1}H-n.m.r. spectrum contained signals in the region for anomeric protons at δ 4.8–4.4, but not at lower fields, demonstrating that all sugar residues in the product were β-linked. The terminal D-galactopyranosyl group removed on oxidation is consequently α-linked. The result thus demonstrates the presence of the structural element **3**.



3

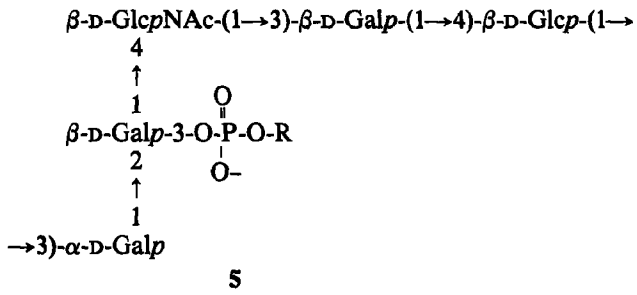
From the combined results, structure **4** is suggested for the oligosaccharide repeating-unit of S-15C.



4

S-15B has the same basic structure, but in addition contains *O*-acetyl groups (~0.7 group per repeating unit), the locations of which have not been determined.

S-15F and S-15A are also composed of pentasaccharide repeating-units. These contain the same carbohydrate backbone but differ in two respects. S-15F contains phosphate residues and *O*-acetyl groups, whereas S-15A contains glycerol phosphate residues but no *O*-acetyl groups. These polysaccharides are linear, but their pentasaccharide repeating-unit (**5**, written in a non-conventional way) contains the same pentasaccharide element as S-15B and S-15C, and it is phosphorylated in the same position.



5

This may indicate that the same pentasaccharide unit is synthesised by all types of *Streptococcus pneumoniae* group 15, but that there are two different routes for the polymerisation of this pentasaccharide. If this view is correct, **4** and **5** should represent the biological repeating-units of S-15B and S-15C and of S-15F and S-15A, respectively.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at bath temperatures not exceeding 40°. For g.l.c., a Perkin-Elmer 990 instrument, fitted with a flame-ionisation detector, was used. Separations were performed on glass columns containing 3% of SP-2340 on Gas Chrom Q at 220° or on an SE-54 capillary column, using a temperature program 150→220° at 2°/min. G.l.c.-m.s. was performed on a Varian MAT 311 instrument, using the SP-2340 column.

Methylation analyses were performed as previously described[5], and the methylation products were purified on Sep-Pak C_{18}-cartridges[6]. Phosphorus was determined according to Chen *et al.*[7]. Hydrolyses were performed by solvolysis with anhydrous hydrogen fluoride for 3 h at room temperature, followed by treatment with 2M trifluoroacetic acid for 2 h at 100°.

For n.m.r. spectroscopy, a JEOL FX-100 or a GX-400 spectrometer was used. ^{13}C-N.m.r. spectra of solutions in D_2O were recorded at 70° and chemical shifts are given in p.p.m. relative to internal 1,4-dioxane (δ 67.40) ^{1}H-N.m.r. spectra of solutions in D_2O were recorded at 85° and chemical shifts are given in p.p.m. downfield from the signal for sodium 4,4-dimethyl-4-silapentane-1-sulfonate. ^{31}P-N.m.r. spectra of solutions in D_2O were recorded at 85° and chemical shifts are given in p.p.m. relative to aqueous 85% phosphoric acid.

Purification of S-15B and S-15C. — A solution of the polysaccharide (100 mg) in water (5 mL) was added to a column of DEAE-Sepharose CL-6B (3.0 × 32 cm), which was irrigated first with water (250 mL) and then with a linear gradient of aqueous sodium chloride (0→M, 1000 mL). Peaks were monitored polarimetrically, and the polysaccharide was eluted at a salt concentration of 0.4–0.5M. The material was recovered by dialysis and freeze-drying.

Deacetylation. — A solution of S-15B (50 mg) in 0.1M aqueous sodium

hydroxide (20 mL) was kept at room temperature for 15 h and then neutralised, and the polysaccharide was recovered by dialysis and freeze-drying.

Dephosphorylation. — (*a*) A solution of S-15C (55 mg) in aqueous 48% hydrogen fluoride (6 mL) was kept at 4° for 96 h. The acid was removed under diminished pressure over sodium hydroxide in a desiccator. A solution of the residue in water was freeze-dried, and the residue was purified by chromatography on a column of Sephadex G-15. The main fraction (40 mg) was eluted in the void volume.

(*b*) S-15C (20 mg) was dissolved in M aqueous diethanolamine (5 mL), alkaline phosphatase (45 μL, Sigma) was added, the solution was kept at 37° for 7 days, then stirred with aqueous 80% phenol (5 mL) for 30 min, and centrifuged, and the aqueous phase was dialysed and freeze-dried.

Smith degradation[8]. — (*a*) A solution of dephosphorylated S-15C (15 mg) in 50mM sodium metaperiodate (5 mL), buffered to pH 3.9 with 0.1M sodium acetate, was kept in the dark at 4° for 96 h. Excess of periodate was reduced with ethylene glycol, and the product was isolated by chromatography on a column (2.6 × 75 cm) of Bio-Gel P-2. A solution of the product in water (20 mL) was treated with sodium borohydride (200 mg) at room temperature for 15 h, excess of reagent was decomposed with acetic acid, and the polyalcohol was isolated by chromatography on the column of Bio-Gel P-2. It was then treated with 0.5M trifluoroacetic acid at room temperature for 50 h. The solution was concentrated by freeze-drying and the residue was fractionated on a column of Bio-Gel P-2.

(*b*) A solution of native S-15C (40 mg) was oxidised and the product worked-up essentially as described under (*a*).

(*c*) A solution of native S-15C (40 mg) was treated with 13mM sodium metaperiodate (15 mL; buffered to pH 3.9) in the dark at 4° for 96 h and worked-up as described under (*a*).

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