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STRUCTURE OF THE CAPSULAR POLYSACCHARIDE FROM *Streptococcus pneumoniae* TYPE 23

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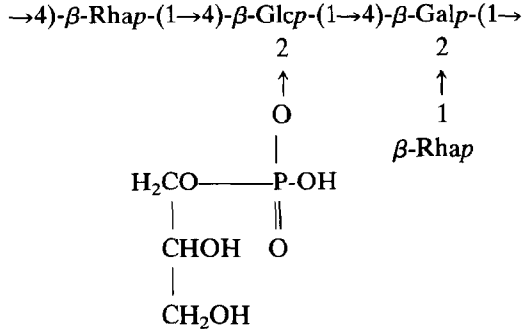
ABSTRACT

The capsular polysaccharide from *Streptococcus pneumoniae* Type 23 (S-23) was found to contain D-galactose, D-glucose, L-rhamnose, glycerol, and phosphorus in the ratios of 1:1:2:0.6:1. Methylation analysis provided information about the linkages of the different sugar units. The sequence of the different sugar residues was confirmed by Smith degradation. Oxidation of S-23 with chromium trioxide indicated that all of the sugar units have the β configuration. The results suggest the following structure for the repeating unit.



INTRODUCTION

The immunological properties of pneumococcal polysaccharides have been extensively studied by Heidelberger and co-workers[1]. These polysaccharides and their antisera have also been used in the immunological characterization of other polysaccharides. Structural studies of these polysaccharides are, therefore, very important for providing a clear idea about the relationship between structure and immunological specificity. Heidelberger and co-workers[2] found that S-23 is composed of D-galactose, D-glucose, and L-rhamnose, together with some phosphorus. Their immunochemical studies indicated that the polymer contains terminal L-rhamnosyl groups. The present work deals with the structure of S-23.

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RESULTS AND DISCUSSION

The polysaccharide S-23 (Lot 2b), obtained through the courtesy of K. Amiraian, was found to give a single peak when examined in a Sephadex G-100 column. The homogeneity of the polysaccharide was revealed by ultracentrifugal analysis using Schlieren optics, when a single, sharp peak was obtained. The polysaccharide also gave a single, strong precipitin line in the Ouchterlony gel-diffusion test with the homologous, antipneumococcal type 23 serum. The polysaccharide had $[\alpha]_{589\ 6}^{23}$ −3.7° (*c* 0.27, water).

Hydrolysis of the polysaccharide with 0.5M sulfuric acid for 20 h at 100°, followed by paper chromatography in solvent systems *1* and *2*, gave spots corresponding to galactose, glucose, and rhamnose. Analysis of the alditol acetates (from the sugars in the hydrolyzate) by g.l.c. (column *A*) gave peaks corresponding to galactose, glucose, rhamnose, and glycerol, in the ratios of 1:1:2:0.6. The results are given in Table I The presence of glycerol was also noted on studying the acetates of the sugars in the hydrolyzate of native S-23 (without treatment with sodium

TABLE I

ACID HYDROLYSIS OF NATIVE, DEPHOSPHORYLATED, AND PERIODATE-OXIDIZED S-23

Sugars as alditol acetates	*Mole percent of sugars in*		
	S-23	*Dephosphorylated S-23*	*Periodate-oxidized S-23*
Glycerol	12.0		
Rhamnose	43.5	49.7	
Galactose	21.4	26.0	51.3
Glucose	23.1	24.3	48.7

TABLE II

VISCOSITIES OF S-23 SOLUTION AT 29°, BEFORE AND AFTER TREATMENT WITH ALKALI

Solutions examined[a]	*Flow time (s)*	*η (mPa.s)*
A	239	1.010
B	238	1.005
C	232	0.981
D	194	0.818

[a]Key: A, 0.043% solution of native S-23 in M sodium hydroxide; B, 0.043% solution of native S-23 in M sodium hydroxide, after heating for 3 h at 100°; C, M sodium hydroxide solution; D, distilled water at 29° (the value of η already known).

TABLE III

METHYLATION ANALYSIS OF NATIVE, DEPHOSPHORYLATED, AND PERIODATE-OXIDIZED S-23

Methylated sugars[a] *(as alditol acetates)*	*Retention time (min)*		*Mole%*		
	Column A	*Column B*	*A*[b]	*B*[b]	*C*[b]
2,3,4-Rha	0.46	0.35	18.8	17.5	
2,3-Rha	0.98	0.92	26.0	23.8	
2,3,6-Glc	2.50	2.32	30.4	28.5	
3,6-Gal	4.35		24.8	30.2	
2,3,4,6-Glc	**1.00**	**1.00**			18.7
3,4,6-Glc	1.98	1.83			31.0
2,3,6-Gal	2.42	2.22			50.3

[a]2,3,4-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-rhamnitol, etc. [b]*A*, methylated, native S-23 polysaccharide; *B*, methylated, dephosphorylated S-23; *C*, methylated, periodate-oxidized S-23.

borohydride). The polysaccharide gave a positive test for phosphorus. Colorimetric estimation[3] showed 3.5% of phosphorus, confirming the earlier findings. This value of phosphorus corresponded to one phosphate group per four sugar units. In a separate experiment, S-23 was dephosphorylated with alkaline phosphatase, giving a phosphorus-free polymer that contained galactose, glucose, and rhamnose in the ratios of 1:1:2, but no glycerol could be found (see Table I). The viscosity of native S-23 before and after treatment with alkali remained the same. The results are shown in Table II. These two experiments suggest that, during treatment with alkali, phosphate groups are removed, together with glycerol, but that the chain remains intact.

Native S-23 was methylated, first by the Hakomori procedure[4] and then by the Kuhn method[5,6]; the absence of hydroxyl absorption in the i.r. spectrum of the product indicated complete methylation. Analysis of the alditol acetates from the product by g.l.c. (columns *A* and *B*) showed peaks for 2,3,4-tri-*O*-methylrhamnose, 2,3-di-*O*-methylrhamnose, 2,3,6-tri-*O*-methylglucose, and 3,6-di-*O*-methylgalactose in almost equimolar amounts. Similar results were obtained on methylation analysis of dephosphorylated S-23. Methylation results, given in Table III, provided information about the linkages of the different sugar units. The results also showed that the polysaccharide has nonreducing L-rhamnose units, and that the phosphate groups do not form a part of the main chain. They are probably attached, together with glycerol, to one of the sugar residues, and are removed during Hakomori methylation of the native S-23. The same conclusion was also apparent from the viscosity behavior, because removal of the phosphate groups did not change the viscosity. The presence of 3,6-dimethylgalactose showed that there is a glycosidically linked branching at O-2 of galactose.

On oxidation of the polysaccharide with sodium periodate[7], followed by treatment of the product with sodium borohydride, a polyol was obtained. Total hydrolysis of a portion of the polyol gave galactose and glucose in the ratio of 1:1

(see Table I). There was no trace of rhamnose or glycerol. The presence of galactose was evident, because its O-2 and O-4 atoms were blocked, but the complete survival of the glucose suggested that O-2 or O-3 of the glucose units might be occupied by phosphate groups that remained unaffected by periodate, and thus imparted stability to the glucose. Moreover, the absence of glycerol in the periodate-oxidized product suggested that the phosphate group is linked to O-1 of the glycerol. The rest of the polyol was hydrolyzed at room temperature, and the product was methylated[5]. Analysis, by g.l.c., of the alditol acetates prepared from the methylated material showed the presence of 2,3,4,6-tetra-*O*-methylglucose, 2,3,6-tri-*O*-methylgalactose, and another trimethylated sugar that corresponded to 2,4,6- or 3,4,6-tri-*O*-methylglucose, which have the same retention times in columns *A* and *B*. Moreover, the peak area of 2,3,6-tri-*O*-methylgalactose is exactly equal to the areas of the other two peaks (see Table III). It was, therefore, quite clear that periodate oxidation resulted in the formation of a disaccharide, namely, 4-*O*-glucopyranosyl-galactose, in which the phosphate group is linked to O-2 or O-3 of the glucose. During Kuhn methylation, the phosphate group is partially removed, and this resulted in the formation of both tetra- and tri-*O*-methylglucose, the amounts of the two compounds together being exactly equal to the amount of 2,3,6-tri-*O*-methylgalactose. In order to ascertain whether the phosphate group is linked to O-2 or O-3 of glucose, the phosphorus-containing disaccharide was again oxidized with periodate. The oxidation product, isolated as already described, showed no glucose (or any other sugar) on analysis by g.l.c. It was, therefore, most probable that the phosphate group is linked to O-2 of the glucose in the disaccharide, because a 3-substituted glucose would have survived periodate oxidation.

Oxidation of the fully acetylated S-23 with chromium trioxide[8] revealed that all of the sugar units are almost completely decomposed in ~2 h (see Table IV). It was, therefore, probable that all of the sugars are in the β configuration. The two β-rhamnopyranosyl units will contribute to the rotation in the positive direction, and the β-glucosyl and β-galactosyl units will contribute to it in the negative direction. The glycerol unit attached to the phosphate also has one asymmetric center, and this will contribute to the specific rotation of the polysaccharide. Thus, the specific rotation of $-3.7°$ for the polysaccharide is not unusual. All of these results conform to structure **1**, suggested for the repeating unit of the S-23 polysaccharide.

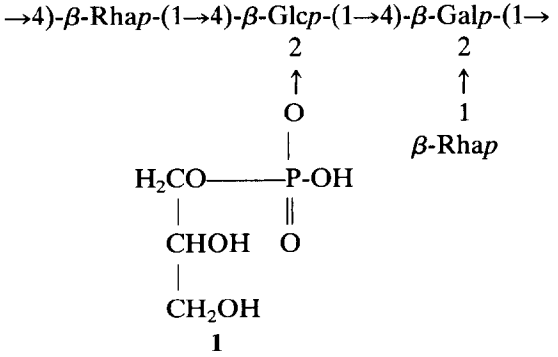

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TABLE IV

OXIDATION OF PERACETYLATED S-23 WITH CHROMIUM TRIOXIDE

Time of oxidation (h)	*Rhamnose*	*Galactose*	*Glucose*	*Inositol*
0	1.60	0.72	0.96	10
1	0.82	0.00	0.34	10
2	0.00	0.00	0.20	10

EXPERIMENTAL

General methods. — Paper partition-chromatography was performed on Whatman No. 1 papers. Solvent systems (v/v) used were (*1*) 4:1:5 1-butanol–acetic acid–water (upper layer) and (*2*) 8:2:1 ethyl acetate–pyridine–water; the spray reagent was alkaline silver nitrate. All solvents were distilled before use, and all evaporations were conducted at 50°, unless stated otherwise. Optical rotations were measured with a Perkin–Elmer Model 241 MC spectropolarimeter. Colorimetric estimations were conducted with a Hitachi Model 100-60 spectrophotometer.

Gas–liquid chromatography (g.l.c.) was performed with a Hewlett–Packard Model 5730A gas chromatograph having a flame-ionization detector, and glass columns (1.83 m × 6 mm) with (*A*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) and (*B*) 3% of OV-225 on Gas Chrom Q (100–120 mesh). All g.l.c. analyses were conducted (at 185° for unmethylated sugars, and at 170° for methylated sugars) by converting the sugars into their alditol acetates[9]. Retention times of partially methylated alditol acetates were measured with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as unity.

Purification of the polysaccharide. — The polysaccharide S-23 (35 mg) was purified by passage through a column of Sephadex G-100. The column was eluted with 0.5M ammonium hydrogencarbonate solution (pH 8.0), and 55 fractions (5 mL each) were collected. The fractions were automatically monitored with a Waters Associates' Differential Refractometer Model 403 fitted with a recorder. Fractions 24–35 contained the polysaccharide, which emerged as a single peak. The fractions containing the polysaccharide were combined, and lyophilized; yield 24 mg $[\alpha]_{589.6}^{23}$ −3.7° (*c* 0.3, water). The homogeneity of the polysaccharide was established by means of a Beckman L5-65 Ultracentrifuge having Schlieren optics, utilizing a 1% solution of the polysaccharide in 0.1M phosphate buffer for 45 min, when a single peak was obtained. The homogeneity was also confirmed by Ouchterlony gel-diffusion of a 1% solution of S-23 in normal saline against the homologous anti-pneumococcal serum in an agar plate, when a single, sharp precipitin line was obtained.

Acid hydrolysis of the polysaccharide. — The polysaccharide (1.6 mg) was dissolved in 0.5M sulfuric acid (1 mL), and the solution was heated in a sealed ampoule for 20 h at 100°. The acid was neutralized with barium carbonate, and the so-

lution was treated with Amberlite IR-120 cation-exchange resin, and the suspension filtered. The filtrate was concentrated to 2 mL and the concentrate was examined by paper chromatography in solvent systems *1* and *2*; spots for galactose, glucose, and rhamnose were found. To the rest of the hydrolyzate (~2 mL) was added sodium borohydride (10 mg), and the alditol acetates were prepared in the usual way[9] and analyzed by g.l.c. (column *A*). The results are shown in Table I. A portion of the hydrolyzate from the first experiment was acetylated, and the acetates tested by g.l.c. A clear peak for glycerol triacetate was observed.

Estimation of phosphorus. — Phosphorus was determined by a colorimetric method[3], using 1 mg of the polysaccharide.

Dephosphorylation[10] *of S-23.* — The polysaccharide (3.02 mg) was dissolved in M sodium hydroxide solution (7 mL). The solution was poured into an Ubbelohde viscometer placed in a constant-temperature bath (29°), and the flow time for the solution was measured. The solution was then transferred to a tube which was sealed, and heated for 3 h at 100°. The cooled solution was carefully transferred back into the viscometer, and the flow time was measured as before. The results are given in Table II. The alkali hydrolyzate was de-ionized with Dowex-50W X-8 (H^+) resin, and lyophilized. To the neutral hydrolyzate was added alkaline phosphatase in ammonium carbonate solution (1 mL), prepared by dissolving ammonium carbonate (2 mg) and alkaline phosphatase (1.5 mg) in water (2 mL). A few drops of toluene were added, and the mixture was kept for 20 h in a constant-temperature bath at 37°. The hydrolyzate was dialyzed against water, and it was found that the carbohydrate part remained inside the dialysis bag. The dephosphorylated polysaccharide was isolated by lyophilization. The alditol acetates prepared from this polysaccharide (0.5 mg) were analyzed by g.l.c. (see Table I).

Methylation analysis of native, S-23 polysaccharide. — The S-23 polysaccharide (2 mg) was dissolved in dry dimethyl sulfoxide (2 mL) by stirring overnight in a serum vial. To the clear solution, under a nitrogen atmosphere, was added methylsulfinyl carbanion[4,11] (2 mL), and the mixture was stirred for 1 h, and kept overnight. The vial was then cooled in an ice bath, methyl iodide (2 mL) was added, and the mixture was stirred for 2 h. The vial was opened, the excess of methyl iodide was removed by passing nitrogen through the solution, and the mixture was dialyzed against distilled water for 2 days. The solution from the dialysis bag was lyophilized, to afford methylated S-23. The material was remethylated by the Kuhn method[5]. The product showed no hydroxyl band in its i.r. spectrum. The permethylated, native S-23 was hydrolyzed, first with 90% formic acid for 2 h on a boiling-water bath, and then with 0.5M sulfuric acid for 20 h at 100°. The alditol acetates were prepared from the hydrolyzate, and examined by g.l.c. (columns *A* and *B*). The results are summarized in Table III. In a similar set of experiments, dephosphorylated S-23 (0.9 mg) was methylated, and analyzed as its alditol acetates. The results are given in Table III.

Oxidation of S-23 with periodate. — To a 0.05% solution of the polysaccharide (8 mL) was added 0.2M sodium periodate solution (2 mL), and the mixture

was kept in the dark for 48 h at 5°. The excess of periodate was decomposed by adding an excess of ethylene glycol (0.5 mL), and the mixture was kept for 3 h and then dialyzed against distilled water. The solution inside the dialysis bag was concentrated to a small volume, sodium borohydride (20 mg) was added, and the solution was kept for 4 h at room temperature, decationized with Dowex-50W X-8 (H^+) ion-exchange resin, and evaporated to dryness. Boric acid was removed by repeated addition and evaporation of methanol. Alditol acetates prepared from a portion of the polyol thus obtained were analyzed by g.l.c. (column *A*). The results are summarized in Table I. The rest of the polyol was hydrolyzed with 0.5M sulfuric acid for 8 h at room temperature. The acid was neutralized with barium carbonate, the suspension was filtered through a Celite bed, and the filtrate was decationized with Dowex-50W X-8 (H^+) ion-exchange resin, and lyophilized. A portion of this material was methylated, and the alditol acetates, prepared from the methylated product, were analyzed by g.l.c. (columns *A* and *B*). The results are given in Table III.

The remaining portion of the periodate-oxidation product was again subjected to periodate oxidation, in which no sugar survived, as revealed by g.l.c.

Oxidation of S-23 with chromium trioxide[8]. — This experiment was performed exactly as described earlier[6]. The results are summarized in Table IV.

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