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THE STRUCTURE OF CAPSULAR POLYSACCHARIDE OF THE PNEUMOCOCCUS TYPE II*

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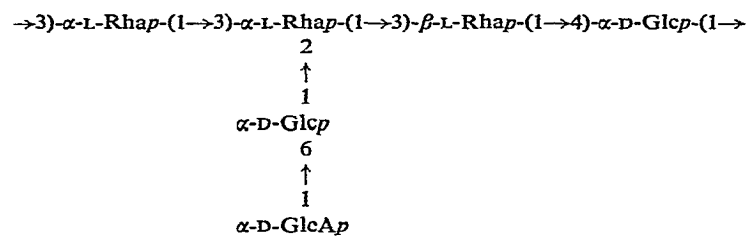
ABSTRACT

The pneumococcus type II capsular polysaccharide (SII) is composed of singly-branched hexasaccharide repeating units, for which three alternative structures have been proposed[1,2]. The correct structure (**1**) has now been determined by consecutive eliminations of the sugar residues in the side chain. The terminal D-glucuronic acid group was eliminated by treating the fully methylated and esterified SII polysaccharide first with base, and then with weak acid. The hydroxyl group at C-6 in the penultimate D-glucose residue released by this elimination was transformed into the 6-deoxy-6-tosyl derivative, and the residue thereafter eliminated by treatment with base. As the side-chains were eliminated by these reactions, it is considered that they contain only two sugar residues, which thus excludes two of the three alternative structures. Structure **1** was further confirmed by subjecting SII to a Smith degradation, which yielded the tetrasaccharide L-Rha*p*-(1→3)-L-Rha*p*-(1→3)-L-Rha*p*-(1→2)-erythritol, characterised by methylation analysis.

INTRODUCTION

In our reinvestigation[1,2] of the structure of the capsular polysaccharide (SII) from *Diplococcus pneumoniae* type II, we postulated three alternative structures (**1–3**). Each of these contains a hexasaccharide repeating unit, composed of one D-glucuronic acid, two D-glucose, and three L-rhamnose residues. The structural studies involved methylation analysis, partial hydrolysis studies, and oxidation of the carboxyl-reduced, fully acetylated polysaccharide by chromium trioxide in acetic acid, followed by methylation analysis. Disaccharide derivatives were obtained by partial hydrolysis of the fully methylated polysaccharide, followed by reduction with sodium borodeuteride and trideuteriomethylation. From the m.s. of one of these disaccharide derivatives, a methylated rhamnitol glucoside, it was concluded that structure **1** was less probable than structures **2** or **3**. We now report further studies on SII, the results of which confirm previous results and also establish the correct structure.

*Dedicated to Professor Michael Heidelberger in honor of his 87th birthday.



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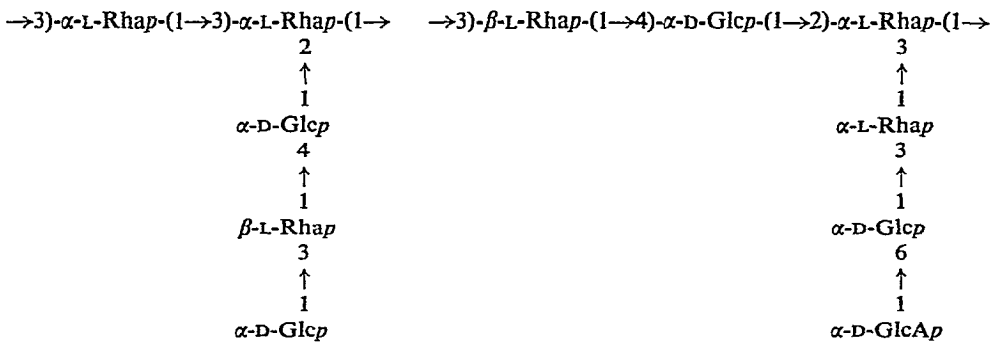
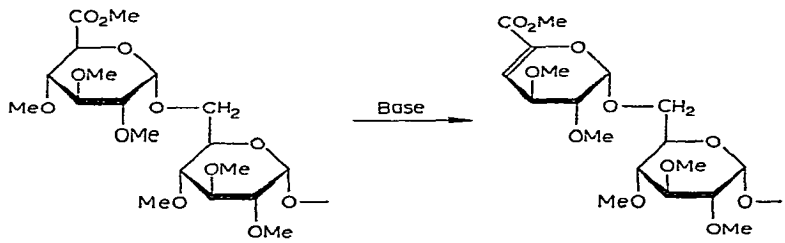


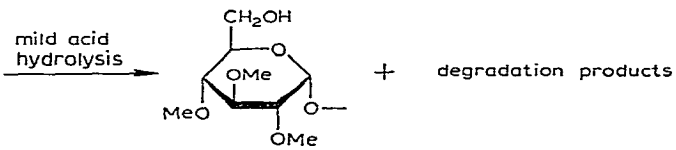
2 **3**

RESULTS AND DISCUSSION

In the approach adopted in these structural studies, the sugar residues in the side chain of the repeating unit were eliminated sequentially, and the eliminations were followed by methylation analysis. The terminal D-glucuronic acid group was eliminated by treating the fully methylated and esterified SII with base, followed by mild acid hydrolysis (Scheme 1) as previously described for other polysaccharides[3]. Part of the degraded product was remethylated with trideuteriomethyl iodide. Analyses of hydrolysates of the carboxyl-reduced, methylated SII and of the just described product (Table I, columns A and B), as the alditol acetates[4], showed that most of the uronic acid residue had been eliminated. Thus, virtually all the 2,3,4-tri-*O*-methyl-D-glucose observed in the analysis of nondegraded SII had been replaced by 2,3,4,6-tetra-*O*-methyl-D-glucose, with a trideuteriomethoxyl group at C-6.

The first degradation confirms previous results and produced a modified SII, in which all hydroxyl groups in the pentasaccharide repeating unit, except that at C-6 of the terminal D-glucose group, were methylated. By successive tosylation, treatment with sodium iodide, and thereafter with sodium toluene-*p*-sulphinate, these groups were transformed into 6-deoxy-6-tosyl groups. On alkaline treatment, these terminal,





Scheme 1

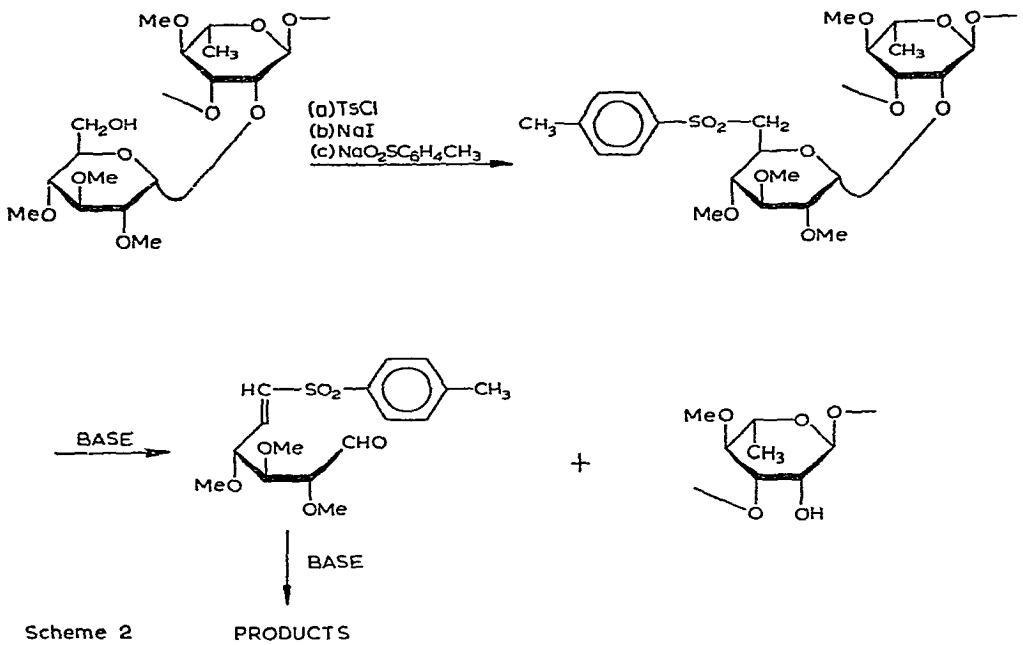
TABLE I

METHYL ETHERS OBTAINED FROM THE HYDROLYSATE OF THE METHYLATED, REDUCED (A), GLUCURONIC ACID DEGRADED, REMETHYLATED (CD_3I) (B), AND SULPHONE DEGRADED, REMETHYLATED (CD_3I) (C) POLYSACCHARIDE

Sugars and location of methoxyl groups[a]	R_T[b]	*Mole* (%)		
		A	*B*	*C*
2,4-Rha	0.94	28	42	42
2*,4-Rha[c]	0.94			8
2,3,4,6*-Glc	1.00		13	7
2-Rha	1.21	18	21	16
2,3,6-Glc	1.75	17	19	23
2,3,4-Glc	1.88	37	5	4

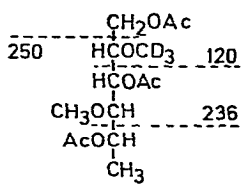
[a]The asterisk indicates the location of the trideuteriomethyl group. [b]Retention time of the corresponding alditol acetate, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an OS 138 S.C.O.T. column at 200°. [c]Calc. from fragments *m/e* 117/120 and *m/e* 233/236 of the m.s. of 2,4-Rha.

modified D-glucose residues should be eliminated (Scheme 2). Similar degradations have been performed with a fungal galactan[5] and with a dextran[6]. Methylation analysis (CD_3I) of the product (Table I, column C), showed that the degradation was, however, incomplete, probably because of incomplete *O*-tosylation. Some of the terminal D-glucose groups had, however, been eliminated. The proportions of 2,3,4,6-tetra-*O*-methyl-D-glucose (OCD_3 at C-6) and 4-*O*-methyl-L-rhamnose were considerably reduced and a corresponding increase in 2,4-di-*O*-methyl-L-rhamnose was observed. The additional 2,4-di-*O*-methyl-L-rhamnose, compared to that given by the



Scheme 2

starting material (Table I, column B) contains a trideuteriomethyl group at C-2, as demonstrated by the m.s. of the acetylated alditol (**4**). It is evident from the results of these two degradations that the side-chain contains only two sugar residues and that **1** is the correct structure of the hexasaccharide repeating unit.



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This result was confirmed by subjecting SII to a Smith degradation. Only the L-rhamnose residues should be resistant to periodate oxidation. An oligomeric product was isolated that, on hydrolysis, yielded L-rhamnose and erythritol in the molar proportion 3:1. The fully methylated product on hydrolysis yielded 2,3,4-tri-*O*-methyl-L-rhamnose and 2,4-di-*O*-methyl-L-rhamnose in the molar proportion ~1:2. The results of this experiment demonstrate the presence of three consecutive L-rhamnose residues in the hexasaccharide repeating unit. Although the alternative structure **2** is thereby excluded, this experiment does not discriminate between structures **1** and **3**.

The anomeric configurations of the sugar residues were assigned mainly on the basis of the results from the oxidation of the carboxyl-reduced, fully acetylated SII

with chromium trioxide in acetic acid. N.m.r. of the fully methylated polysaccharide lends some support to this assignment as a broad 5-proton signal at about δ 5.1 and a 1-proton signal at δ 4.6 were observed. The material obtained after the elimination of the uronic acid residue gave a similar spectrum, with the integral of the former, broad signal corresponding to 4 protons. The L-rhamnosyl-L-rhamnosyl-L-rhamnosyl-erythritol, obtained by Smith degradation of SII, showed $[\alpha]_D$ $-11°$. The calculated value, based on the published values for methyl α- and β-L-rhamnopyranoside, Hudson's rules for isorotation, and two α- and one β-linked L-rhamnopyranose residues being assumed, is $-9°$, in good agreement with the observed value.

Previous results based on the m.s. of the methylated rhamnitol glucoside[2], referred to in the earlier discussion, were consequently misleading. In this m.s., a fragment (Aldol J_2) representing the alditol part of a fully methylated disaccharide alditol was identified which, if derived from the branching L-rhamnose residue, should contain three trideuteriomethyl groups and give an ion *m/e* 215 and, if derived from a chain residue, should only contain two such groups and give an ion *m/e* 212. Although both these ions were observed, the former was stronger, and was assumed to be structurally diagnostic. It would now appear that the *m/e* 215 ion could have several different origins.

The proposed structure (**1**) is based upon the assumption that SII is composed of hexasaccharide repeating units. Although this has not been rigorously substantiated, it is strongly supported by the good stoichiometry observed in the sugar analysis, and in the methylation analysis of the original polysaccharide and of different chemically modified polysaccharides. Oligosaccharide repeating units also seem to be a common feature of pneumococcal and other bacterial polysaccharides[7].

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure, at bath temperatures not exceeding 40°. G.l.c. was conducted with a Perkin–Elmer model 990 instrument, equipped with the following columns: (a) OS-138 S.C.O.T. column at 200°, for partially methylated alditol acetates; and (b) ECNSS-M, 3% on Gas Chrom Q at 160°, for alditol acetates. For g.l.c.–m.s., a Perkin–Elmer 270 gas chromatograph–mass spectrometer was used. Gel filtration was performed with Sephadex LH-20, irrigated with acetone–chloroform (1:2), and the eluate was monitored polarimetrically.

Methylation of SII. — SII polysaccharide (120 mg) was dissolved in dimethyl sulphoxide (20 ml), in a sealed bottle in an ultrasonic bath. 2M Methylsulphinyl anion in dimethyl sulphoxide (10 ml) was added and the mixture was agitated in an ultrasonic bath for 30 min, and kept for 18 h at room temperature. Methyl iodide (10 ml) was added under external cooling; the reaction mixture was agitated in an ultrasonic bath for 30 min, poured into water, dialysed, and freeze-dried to yield 120 mg of fully methylated product. Part of this material (5 mg) was heated at reflux with 3% hydrogen chloride in methanol (5 ml) for 18 h. The reaction mixture was neutralised

with silver carbonate, concentrated to dryness, and reduced with lithium aluminium deuteride (30 mg) in dichloromethane–ethyl ether (1:1, 10 ml). The resulting mixture of methyl glycosides was converted into partially methylated alditol acetates and analysed by g.l.c.–m.s.

Elimination of the uronic acid group from fully methylated SII. — A solution of carefully dried, methylated polysaccharide (115 mg) and toluene-*p*-sulphonic acid (1 mg) in dimethyl sulphoxide–2,2-dimethoxypropane (20 ml; 19:1, v/v) was prepared in a serum vial, which was then sealed with a rubber cap. The vial was flushed with nitrogen and agitated ultrasonically for 30 min at room temperature, and then 2M methylsulphinyl anion in dimethyl sulphoxide (10 ml) was added with a syringe. The solution was agitated in the ultrasonic bath for a further 30 min and kept at room temperature for 16 h. Excess aqueous acetic acid was added with external cooling, and the mixture was dialysed against water. After concentration, the product was suspended in 10% aqueous acetic acid (20 ml), and kept for 1 h at 100°. The cooled solution was concentrated and the product purified by gel filtration. The material eluted with the void volume was collected. The yield of the degraded polysaccharide (SIIa) was 80 mg. Part of this product (5 mg) was trideuteriomethylated, hydrolysed, and analysed.

*Toluene-*p*-sulphonylation of SIIa.* — Toluene-*p*-sulphonyl chloride (2.0 g) was added, in portions, to a solution of SIIa (75 mg) in chloroform–pyridine (10 ml; 1:1, v/v). The resulting clear solution was kept for 72 h at room temperature, and the excess of toluene-*p*-sulphonyl chloride was destroyed by adding ethanol under external cooling. The reaction product was purified by gel filtration, and the modified polysaccharide (SIIb) (75 mg) was eluted with the void volume.

Reaction of SIIb with sodium iodide. — Sodium iodide (1.2 g) was added to a solution of SIIb (75 mg) in chloroform–*N*,*N*-dimethylformamide (7.5 ml; 1:2, v/v). The solution was kept for 18 h at 100° and the product (SIIc) (70 mg) recovered by gel filtration.

*Reaction of SIIc with sodium toluene-*p*-sulphinate.* — Sodium toluene-*p*-sulphinate (1.0 g) was added to a solution of SIIc (70 mg) in chloroform–*N*,*N*-dimethylformamide (30 ml; 1:3, v/v). The mixture was kept for 18 h at 100°, and then partitioned between water and chloroform. The chloroform phase was concentrated and the product (SIId) (65 mg) was purified by gel filtration.

Alkaline degradation of SIId. — SIId (65 mg) was dissolved in dimethyl sulphoxide (2 ml) in a serum bottle sealed with a rubber cap, and a solution (2M) of methylsulphinyl anion in dimethyl sulphoxide (4 ml) was added dropwise. The resulting mixture was agitated in an ultrasonic bath for 30 min, and kept for 18 h at room temperature. The solution was neutralised with 50% aqueous acetic acid, and then partitioned between water and chloroform. The organic phase was concentrated and, on subsequent gel filtration, the degraded polysaccharide (45 mg) was eluted with the void volume. Part of this material (5 mg) was trideuteriomethylated, hydrolysed, and analysed as just described.

Smith degradation of the SII polysaccharide. — A mixture of polysaccharide

(50 mg) in 0.15M sodium metaperiodate (50 ml) was kept in the dark for 150 h at room temperature. The excess of periodate was destroyed with ethane-1,2-diol (1 ml), the solution was dialysed overnight and concentrated to 50 ml, sodium borohydride (300 mg) was added, and the solution was kept for 9 h at room temperature. Excess borohydride was decomposed with 50% acetic acid and the solution dialysed overnight. Part of the material (1/20) was used for sugar analysis with L-arabinose as internal standard. This experiment demonstrated that 80% of the D-glucose residues had been oxidised. The rest was treated with 0.5M sulphuric acid for 24 h at room temperature, neutralised with 0.1M sodium hydroxide, concentrated, and fractionated on a column (2.6 × 100 cm) of Sephadex G-25 with water as irrigant. The fractionation was followed by determination of the optical rotation and an oligomeric product (2.1 mg) having $[\alpha]_D^{24}$ −11° (*c* 0.2, water) was eluted in the tri–tetrasaccharide region. Part of this material (0.2 mg) was used for sugar analysis with D-arabinose as internal standard. Part of the oligosaccharide (1 mg) was methylated, hydrolysed, and the resulting sugars analysed as just described.

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