STRUCTURAL STUDIES ON PNEUMOCOCCUS TYPE II CAPSULAR POLYSACCHARIDE*

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ABSTRACT

The structure of the capsular polysaccharide of *Diplococcus pneumoniae* Type II has been investigated. Methylation analyses of the original polysaccharide and of a product obtained on mild, acid hydrolysis of the polysaccharide, together with the characterization of an aldobiouronic acid derived therefrom, have provided the essential information in this study. Possible structures for the capsular polysaccharide are discussed.

INTRODUCTION

The capsular polysaccharides from different types of *Diplococcus pneumoniae* have been extensively studied by chemical and immunochemical methods[1,2]. For several of these polysaccharides, a complete structure has been proposed, and the correlation between their chemical structure and immunological properties is well understood. This is, however, not true of the Type II capsular polysaccharide (SII); there is conflicting evidence concerning its structure, and the immunological properties observed seem inconsistent with structural features proposed. Goodman and Kabat[3] studied inhibition of the cross reaction of Type II antipneumococcal horse serum with dextran, a cross reaction involving about 18 percent of the antibody, and showed isomaltopentaose to be the best inhibitor. Ninety-five percent of the binding energy was contributed by the terminal, nonreducing α-isomaltosyl group. D-Glucuronic acid was found to be a much better inhibitor of this cross reaction than D-glucose, but was less active than isomaltose, and was the only inhibitor of the homologous SII–anti-SII reaction. Recently, Heidelberger *et al.*[4] have shown that isomaltobiouronic acid is more efficient than other *O*-D-glucopyranosyluronic-D-glucoses (including cellobiouronic acid) in inhibiting the type II–anti-type II system, a result that strongly indicates the presence of terminal isomaltobiosyluronic groups in SII. On the other hand, Barker *et al.*[5], who made structural studies by use of essentially

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enzymic degradations and periodate oxidation, proposed a structure containing a terminal cellobiosyluronic group in a dodecasaccharide repeating-unit. Other features in their structure are not in agreement with results from previous studies[6,7] that used methylation analysis. A re-investigation of the structure of SII has therefore been undertaken, the results of which are now given.

RESULTS

The $SIIB_1$ preparation used was reported[2] to have $[\alpha]_D$ +53°, and to contain 50% of 6-deoxyhexose and 0.62% of N. A hydrolyzate of carboxyl-reduced SII contained equimolecular parts of L-rhamnose and D-glucose (analyzed, as their alditol peracetates, by g.l.c.[8]–m.s.[9]). Reduction of the carboxyl groups was performed with lithium aluminum deuteride, and, from the intensities of pertinent peaks in the mass spectrum (m.s.) of the D-glucitol peracetate, it was concluded that one third of the D-glucitol had been derived from D-glucuronic acid. The relative proportions of L-rhamnose, D-glucose, and D-glucuronic acid are therefore 3:2:1, a result that was confirmed by the methylation analysis (see later). A quantitative sugar analysis, with L-arabinose as the internal standard, revealed that these sugars account for 98% of the SII preparation.

Polysaccharide SII was methylated by treatment with methylsulfinylsodium and methyl iodide in methyl sulfoxide[10]. Part of the methylated polysaccharide was hydrolyzed, and the resulting mixture of methylated sugars was analyzed, as their alditol peracetates, by g.l.c.–m.s.[11] (see Table I, column A). The rest of the methylated SII was treated with methanolic hydrogen chloride, the product reduced with lithium aluminum deuteride, the product hydrolyzed, and the hydrolyzate analyzed as before (see Table I, column B). In the former analysis, the D-glucuronic acid residues are not accounted for. A considerable proportion of the glycosiduronic

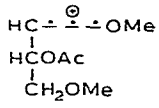
TABLE I

METHYLATED ETHERS OBTAINED FROM THE HYDROLYZATE OF METHYLATED SII (A), METHYLATED-REDUCED SII (B), AND PARTIALLY HYDROLYZED, METHYLATED SII (C)

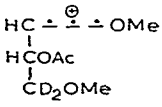
Sugars	*T*[a]		*Molar proportions* (%)		
	ECNSS-M	*OV-225*	*A*	*B*	*C*
2,3,4-Tri-*O*-methyl-L-Rha	0.46	0.35			5
2,4-Di-*O*-methyl-L-Rha	0.99	0.92	47	27	35
3,4-Di-*O*-methyl-L-Rha	0.92	0.87			7
2,3,4,6-Tetra-*O*-methyl-D-G	1.00	1.00			9
4-Mono-*O*-methyl-L-Rha	1.72	1.57	23	18	23
2,3,4-Tri-*O*-methyl-D-G	2.48	2.22	7	38(19)[b]	9
2,3,6-Tri-*O*-methyl-D-G	2.50	2.32	23	17	12

[a]Retention times of the corresponding alditol acetate on the ECNSS-M and OV-225 columns, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [b]The value in parentheses represents 2,3,4-tri-*O*-methyl-D-glucose derived from D-glucosyluronic groups.

linkages was probably not hydrolyzed, and, consequently, their glycosiduronically linked sugar residues are also not accounted for. In the latter analysis, D-glucuronic acid residues are transformed into D-glucose residues that are dideuterated at C-6. No sugar residues should, therefore, be lost because of incomplete hydrolysis, but part of the 2,4-di-*O*-methyl-L-rhamnose was probably lost as its volatile methyl glycosides during concentration of the methanolyzate. It therefore seems reasonable to assume that the ratio between 2,4-di-*O*-methyl-L-rhamnose and 4-*O*-methyl-L-rhamnose is actually 2:1, as was found in the former analysis. Considerably more 2,3,4-tri-*O*-methyl-D-glucose was obtained from the carboxyl-reduced, than from the unreduced, material. Comparison of pertinent fragments in the mass spectrum, *e.g.*, *m/e* 189 and 191, obtained by fission between C-3 and C-4 of the nondeuterated and deuterated alditol, respectively, revealed that half of the 2,3,4-tri-*O*-methyl-D-glucose was derived from terminal D-glucuronic acid residues, and half from D-glucose residues substituted at C-6.



m/e 189



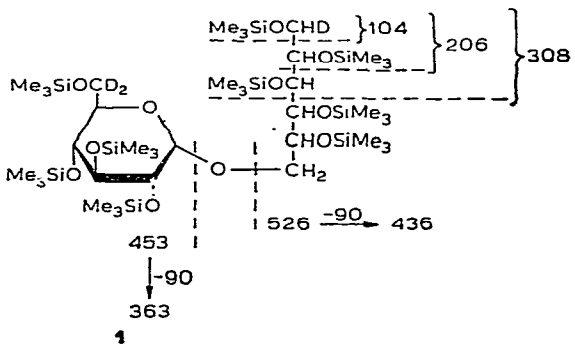
m/e 191

The low percentage of this sugar obtained from unreduced material indicates that D-glucuronic acid is linked to O-6 of D-glucose. On correcting for the part of the 2,4-di-*O*-methyl-L-rhamnose that was lost in the former analysis, as already discussed, the ratios of L-rhamnose to D-glucose to D-glucuronic acid are 3:2:1, in good agreement with the sugar analysis.

A sample of SII was hydrolyzed with acid under mild conditions such that a part of the L-rhamnosidic linkages, but an insignificant percentage of the other glycosidic linkages, should have been cleaved. The oligo- and poly-meric material was then subjected to methylation analysis, without reduction of the carboxyl groups (see Table I, column C).

Part of SII was hydrolyzed under more vigorous conditions whereby essentially only the glycosiduronic linkages should have remained intact. The hydrolyzate was reduced with sodium borodeuteride, the product pertrimethylsilylated, the product reduced with lithium aluminum deuteride, the product pertrimethylsilylated, and the ether analyzed by g.l.c.–m.s. In the disaccharide region, a single component was obtained that had the same retention time as the per(trimethylsilyl) (TMS) derivative of isomaltitol ($T_M = 0.85$, compared to $T_M = 1.00$ for the TMS derivative of melibiitol[10]). The TMS derivative of gentiobiitol showed $T_M = 0.90$ on 5% XE-60 at 170°. Allowing for the three deuterium atoms, the mass spectrum was indistinguishable from that of the TMS derivative of authentic isomaltitol. The origin of some of the main fragments is indicated in (1). These results therefore demonstrate that isomaltobiouronic acid residues are incorporated in the structure of SII. The absence of other

disaccharide derivatives indicates that all of the D-glucuronic acid residues occur in such units.



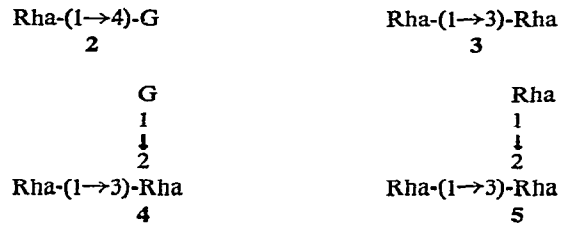
DISCUSSION

Several pneumococcal polysaccharides, as well as other bacterial polysaccharides, are composed of oligosaccharide repeating-units. As SII contains L-rhamnose, D-glucose, and D-glucuronic acid residues in the relative proportions of 3:2:1, the smallest possible repeating-unit should be a hexasaccharide. The assumption of a hexasaccharide repeating-unit is further supported by the result of the methylation analysis, according to which, SII should contain 3-*O*-substituted L-rhamnose, 2,3-di-*O*-substituted L-rhamnose, 6-*O*-substituted D-glucose, and 4-*O*-substituted D-glucose residues, and terminal D-glucosyluronic groups, in the relative proportions of 2:1:1:1:1. The analysis further demonstrated, except for the 4-*O*-substituted D-glucose, that the sugar residues are pyranosidic. The latter residue is, presumably, also pyranosidic, as a furanosidic linkage should have been hydrolyzed much faster than was actually observed. These results are different from those obtained by Kent[6] and by Butler and Stacey[7], who had available less effective methods for methylation of polysaccharides, and who obviously did not achieve complete methylation. Also, the methods for fractionation of mixtures of methylated sugars have been considerably improved since their studies were performed.

The identification of isomaltitol after acid hydrolysis followed by reductions demonstrates the presence of isomaltobiosyluronic groups in S II. As all of the D-glucosyluronic groups are terminal, the corresponding disaccharide residues should also be terminal. This conclusion confirms the results of Heidelberger *et al.*[4], and is in accord with the cross-reaction studies of Goodman and Kabat[2], but contrasts with those of Barker *et al.*[5].

Some further structural information was obtained from the methylation analysis of the sample that had been subjected to mild hydrolysis with acid. Each methyl ether that is found in column C of Table I but that is not present in column A should be the result of hydrolytic cleavage. By assuming that L-rhamnosidic linkages had mainly been broken, and by comparing the methyl ethers after methylation analysis

with those found in the original methylation analysis, the presence of the structural element **2** may be inferred, but it is not possible to distinguish between **3** and **4**, or **5**.



Furthermore, the anomeric configuration of all linkages except the D-glucosiduronic are unknown. Consequently, an unambiguous structure for a repeating unit can not be proposed until complementary information has been obtained.

The structure, however, presumably contains three L-rhamnose residues, two D-glucose residues, and one D-glucuronic acid residue, linked as demonstrated by the methylation analysis. Some information as to the mutual order of the residues is available: the structure contains a terminal 6-*O*-(α-D-glucosyluronic)-D-glucosyl group and the partial structure **2**. These structural features cannot be accommodated in the dodecasaccharide repeating-unit suggested by Barker *et al.*[5]; this contains terminal L-rhamnose, branched D-glucose, and chain D-glucuronic acid which, if present, could not have been overlooked in the present investigation. It is of interest that all studies other than that of Barker *et al.*[5] were conducted with the same preparation (Squibb), whereas the Barker sample was obtained from a different source that might not have produced exactly the same polysaccharide; this possibility should be studied further.

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 900 instrument by using the following columns: (*a*) ECNSS-M, 3% on Gas-Chrom Q, at 200° for alditol peracetates, and at 165° for partially methylated alditol acetates; (*b*) OV-225, S.C.O.T. column, at 200°, for partially methylated alditol acetates; and (*c*) XE-60, 5%, on Gas-Chrom Q, at 170° for trimethylsilylated disaccharide alditols. For g.l.c.–m.s., a Perkin–Elmer 270 gas chromatograph–mass spectrometer was used. Mass spectra were recorded at a manifold temperature of 300°, an ionization potential of 60 eV, an ionization current of 80 μA, and an ion-source temperature of 80°.

Sugar analysis. — A solution of the polysaccharide (5.15 mg) and an internal standard of L-arabinose (4.25 mg) in 0.25M sulfuric acid (1 ml) was kept for 10 h at 100°. The hydrolyzate was made neutral with barium carbonate, the suspension was filtered, and the filtrate was evaporated to dryness. The resulting mixture was per(trimethylsilyl)ated[11], and the TMS derivatives were treated with lithium aluminum

deuteride (10 mg) in boiling ethyl ether (5 ml) for 8 h. After processing, the produ was hydrolyzed with 0.25M sulfuric acid for 10 h at 100°, the acid was neutralized before, and the sugars were converted into alditol acetates which were analyzed l g.l.c.[6]–m.s.[7].

Methylation analysis. — The polysaccharide (10 mg) in methyl sulfoxide (2 n was treated with 2M methylsulfinylsodium (2 ml) for 7 h. Methyl iodide (1 ml) w then added, under external cooling in ice water. After 30 min, the mixture was pour into water (10 ml), dialyzed against tap water overnight, and evaporated to dryne Half of the resulting material was treated with 90% formic acid for 2 h at 100°, t solution concentrated, and the product hydrolyzed with 0.13M sulfuric acid for 18 at 100°. The sugars in the hydrolyzate were converted into alditol acetates, and the were analyzed by g.l.c.–m.s.[11] (see Table I, column A).

The other half of the methylated polysaccharide was dissolved in 3% methano hydrogen chloride (5 ml), and the solution was boiled under reflux for 4 h. T solution was made neutral with silver carbonate, the suspension filtered, and t filtrate evaporated to dryness. The product plus lithium aluminium deuteride (5 m were dissolved in tetrahydrofuran (10 ml), and the solution was boiled under refl for 8 h. After processing, the product was hydrolyzed as before, and the methylat sugars were converted into alditol acetates which were analyzed by g.l.c.–m.s.[11] (s Table I, column B).

Methylation analysis of degraded SII. — The polysaccharide (5 mg) was hydr yzed with 0.25M sulfuric acid (2 ml) for 30 min at 100°, the acid was neutralized wi barium carbonate, the suspension was filtered, and the filtrate was concentrated. T product was subjected to methylation as already described, the product w isolated by partition between chloroform and water, and hydrolyzed, and the resulti methylated sugars were analyzed, as their alditol acetates, by g.l.c.–m.s.[11] (s Table I, column C).

Characterization of the aldobiouronic acid. — The polysaccharide (5 mg) w hydrolyzed with 0.25M sulfuric acid (2 ml) for 8 h at 100°. The hydrolyzate w processed, the product reduced with sodium borodeuteride, the product trimeth silylated, and the product reduced ($LiAlD_4$) as already described. The reduc material was per(trimethylsilyl)ated, and the product was analyzed by g.l.c.–m.s. In the disaccharide region, a single component ($T_M = 0.85$) showed the same ma spectrum as the authentic TMS derivative of isomaltitol ($T_M = 0.85$), except for pea affected by the deuterium labeling. Peaks were observed, *inter alia*, at *m/e* 526(52 453(451), 436(435), 363(361), 308(307), 218(217), 206(205), and 104(103). (Cor sponding peaks for the non-labeled isomaltitol derivative are given in parenthese The component was distinguishable from gentiobiitol ($T_M = 0.90$).

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