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Note

Further studies of the capsular polysaccharide of Pneumococcus Type II

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In a recent publication[1], we reported structural studies of the capsular polysaccharide (SII) from *Diplococcus pneumoniae* Type II The methylation analysis was an essential part of these studies, and the results, to some extent, conflicted with previous structural proposals It was concluded that SII is composed of hexasaccharide repeating units, containing one D-glucuronic acid, two D-glucose, and three L-rhamnose residues The D-glucuronic acid was terminal and α-linked to the 6-position of a D-glucose residue The other D-glucose residue was 4-*O*-substituted, two L-rhamnose residues were 3-*O*-substituted, and one was 2,3-di-*O*-substituted. Additional structural evidence was provided by methylation analysis of SII material, which had been hydrolysed under mild conditions, during which essentially only the L-rhamnosidic linkages should be cleaved

We have now subjected SII to a new type of degradation It is based upon the observation that fully acetylated glycopyranosides in which the aglycon group occupies an equatorial position in the most stable chair-form (generally the β-anomer) are oxidised to esters of 5-hexulosonic acids by chromium trioxide in acetic acid[2,3] The corresponding α-glycosides are reasonably stable under these conditions By combining this oxidation with the methylation analysis, we may deduce which of the sugar residues in a polysaccharide are β-linked When a sugar is β-linked to an α-pyranose residue, this procedure also reveals the position of substitution[4], as the ester linkage is cleaved during the Hakomori methylation and replaced by a methoxyl group

Treatment of SII with methyl vinyl ether and an acidic catalyst yielded an acetylated product that was soluble in organic solvents This product was treated with lithium aluminium deuteride, and subsequent removal of the acetal groups yielded a carboxyl-reduced SII in which the newly formed, primary alcohol groups were dideuterated (C-6 of some glucose residues) The results of methylation analyses of this product before and after the acetylation–chromium trioxide treatment are given in Table I. Only one of the sugar residues in the repeating unit, a 3-*O*-substituted L-rhamnose residue, was destroyed by oxidation, demonstrating that this residue is β-linked and that the other five residues are α-linked The decrease in

2,3,6-tri-*O*-methyl-D-glucose and the corresponding increase in 2,3,4,6-tetra-*O*-methyl-D-glucose further demonstrate that the β-L-rhamnose residue is linked to position 4 of an α-D-glucose residue

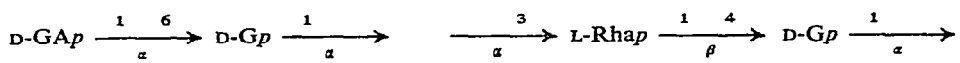
TABLE I

PARTIALLY METHYLATED SUGARS FROM THE HYDROLYSATE OF METHYLATED CARBOXYL-REDUCED SII (*A*) AND ACETYLATED, OXIDISED, AND METHYLATED SII (*B*)

Sugars	T[a]		*Molar proportions*	
	ECNSS-M	*OV 225*	A	B
2,3,4,6-Tetra-OMe-D-G[b]	1 00	1 00		0 8
2,3,4,6-Tetra-OMe-D-G-$6d_2$[b]	1 00	1 00	1 0	1 0
2,3,4-Tri-OMe-D-G	2 48	2 22	1 0	1 0
2,3,6-Tri-OMe-D-G	2 50	2 32	1 0	0 2
2,4-Di-OMe-L-Rha	0 99	0 92	2 0	1 1
4-Mono-OMe-L-Rha	1 72	1 57	1 0	1 0

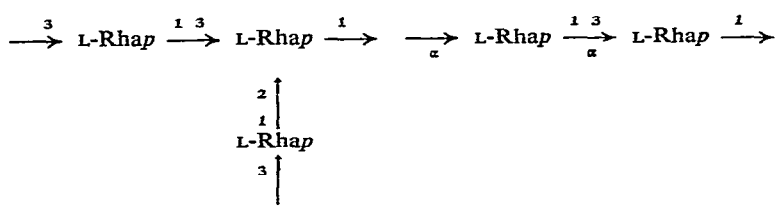
[a]Retention times of the corresponding alditol acetate on the ECNSS-M and OV 225 columns, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol [b]The relative proportions of these sugars were determined by comparing the relative intensities of appropriate *m/e*-values in the m s of the mixture

These results, in conjunction with previous results, establish the presence of structural elements **1** and **2** in SII

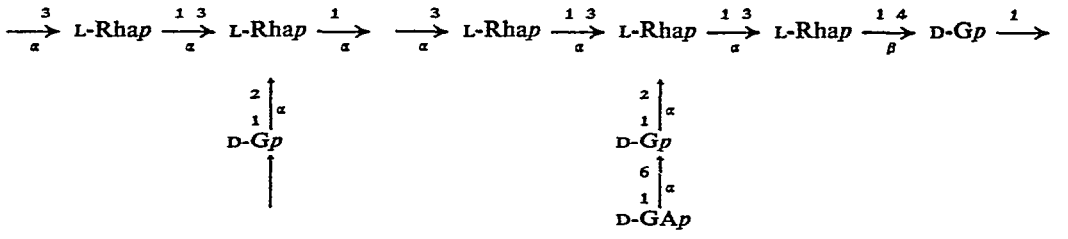


1 **2**

In the previous investigation, the fragment **3** was proposed, but may now be excluded as one of the three L-rhamnose residues is already engaged in structure **2** The remaining, conceivable alternatives to **3** are fragments **4** and **5** (where possible, the anomeric nature of the sugar residues is given)

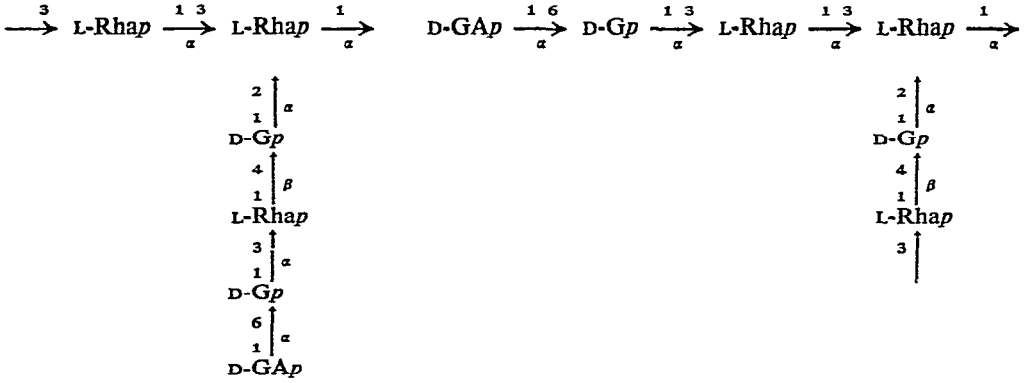


3 **4**



5 6

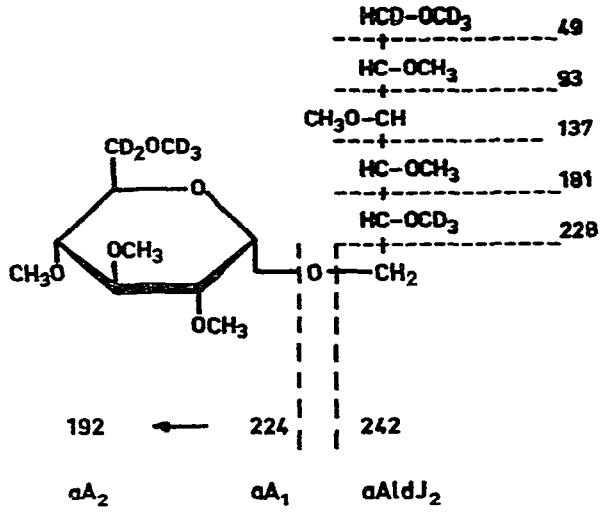
The D-glucose residue in **5** could either form part of the terminal element **1**, giving structure **6** for the hexasaccharide repeating unit, or form part of the chain element **2**, giving two further alternatives, **7** and **8**, for the repeating unit.



7 8

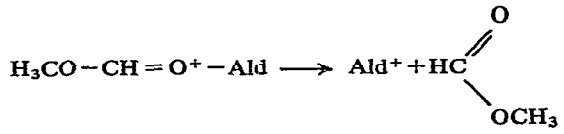
Graded, acid hydrolysis of a methylated polysaccharide, followed by reduction with sodium borodeuteride, remethylation with trideuteriomethyl iodide, and analysis (by g l c –m s) of the oligosaccharide alditols formed, has earlier been used to elucidate the sequence of sugar residues in polysaccharides[5]

When SII was subjected to this treatment, g l c showed two peaks in the disaccharide region The slower of these, from its m s , corresponded to the isomaltitol derivative **9** The aA_1 fragment (using the nomenclature proposed by Kochetkov and Chizhov[6]) of *m/e* 224 contains five deuterium atoms and consequently is derived from the D-glucuronic acid residue The aA_2 ion is formed from the aA_1 fragment by elimination of methanol The fact that only methanol and no trideuteriomethanol was eliminated proves that the trideuteriomethoxyl group in aA_1 is located[6] at position 6. The alditol moiety of **9** gives all the expected fragments, demonstrating that the uronic acid residue is linked to C-6 of a chain D-glucose residue A fragment $aAldJ_1$, formed by a rearrangement, consists of the aglycon group (Ald), C-1, and the methoxyl group from C-3 in the D-glucopyranose moiety[6]. The formation of a



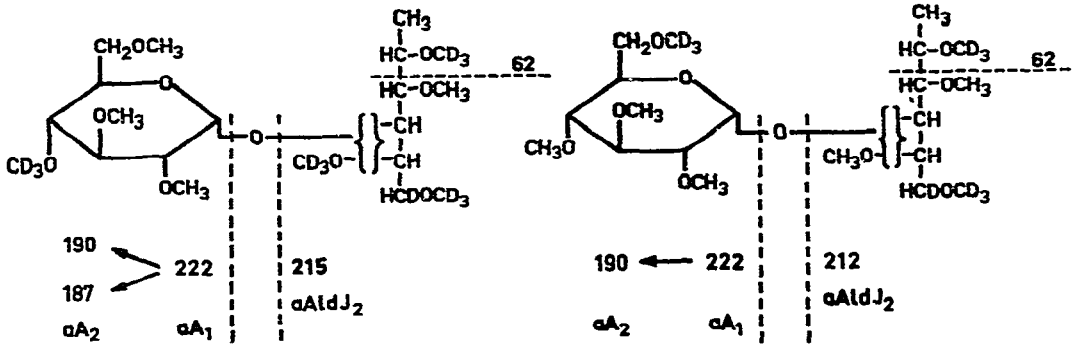
9

fragment $aAldJ_2$, containing the algycon part, is not completely understood[7], but probably involves elimination of methyl formate from $aAldJ_1$ as indicated below



$aAldJ_1$ $aAldJ_2$

The m s of the material in the other peak was more complex and indicated that it was a mixture of the L-rhamnitol glycoside derivatives **10** and **11**.



10 11

The D-glucopyranose residue should have a trideuteriomethoxyl group at either C-4 or C-6, accounting for the aA_1 ion at *m/e* 222 This ion loses either methanol or trideuteriomethanol to form aA_2 From the ratio for *m/e* 190 and 187, it is concluded that most of the trideuteriomethoxyl should be at position 4 It is not possible to establish whether the L-rhamnitol part is substituted at position 2 or 3 The fragment $aAldJ_1$ was observed at *m/e* 275 and 272, and the corresponding, $aAldJ_2$, at *m/e* 215 and 212 The preponderance of the ion with the highest mass number in both cases strongly suggests that most of the L-rhamnitol is derived from the branched L-rhamnose residue in SII These results thus demonstrate that the 4-substituted D-glucose residue in SII is linked to the branched L-rhamnose residue Structure **6** for the repeating unit may thereby be excluded, and either **7** or **8** should represent the actual structure.

These structures should, at the present stage, be regarded as tentative, and independent evidence is needed in order to confirm the structural details and effect a final choice

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, using the following columns (*a*) ECNSS-M, 3% on Gas-Chrom Q, at 200° for alditol acetates and at 165° for partially methylated alditol acetates, (*b*) OV-225 S C O T column, at 190° for partially methylated alditol acetates, XE 60 5% on Gas-Chrom Q, at 200° for permethylated 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 ionisation potential of 70 eV, an ionisation current of 80 μamp, and an ion-source temperature of 80°

Carboxyl reduction of SII — Methyl vinyl ether (3 ml) and toluene-*p*-sulphonic acid (5 mg) were added to a solution of SII (25 mg) in methyl sulphoxide (10 ml) The resulting solution was kept at 15° for 3 h The excess of methyl vinyl ether was removed by evaporation, and the resulting mixture was added to a column (60 × 5 cm) of Sephadex LH-20 which was then irrigated with acetone. The acetalated SII (35 mg) was recovered with the void volume

Acetalated SII (30 mg) was dissolved in dichloromethane–ethyl ether (2 1) and treated with lithium aluminium deuteride (50 mg) at reflux temperature for 8 h Excess of reductant was destroyed by the addition of M phosphoric acid After filtration and concentration, the yield of reduced, acetalated SII was 30 mg I r showed no carbonyl absorption band Reduced, acetalated SII (25 mg) was dissolved in 5% aqueous acetic acid and the solution was heated at 100° for 1 h The product, isolated by concentration of the solution, was purified by passage through a column (60 × 2 cm) of Sephadex G 15; yield of carboxyl-reduced SII, 20 mg The result of a methylation analysis of this material is shown in Table I (column *A*).

Chromium trioxide oxidation of carboxyl-reduced SII. — Carboxyl-reduced SII

(10 mg) was dissolved in formamide–pyridine (30 ml, 2 1), acetic anhydride (10 ml) was added, and the resulting mixture was kept at room temperature for 18 h Water (10 ml) was added dropwise to the cooled and stirred solution, and the mixture was dialysed against distilled water Concentration and purification by passage through a column of Sephadex LH-20, as described above, yielded carboxyl-reduced, acetylated SII (15 mg).

Powdered chromium trioxide (30 mg) was added to the solution of acetylated, carboxyl-reduced SII (10 mg) in glacial acetic acid (0 3 ml) The resulting suspension was agitated in an ultrasonic bath at 50° for 1 h. The dark reaction mixture was fractionated on a column of Sephadex LH-20 by irrigation with acetone The oxidised SII material (10 mg) was eluted with the void volume, free of reagents Methylation analysis of this product was performed as previously described[8], but the methylated material was isolated from the reaction mixture by partitioning between water and chloroform, and not by dialysis

Partial hydrolysis of methylated SII — Methylated SII (25 mg) was hydrolysed with 90% aqueous formic acid at 80° for 70 min After evaporation to dryness, the oligosaccharide mixture was dissolved in dichloromethane–ether (30 ml, 2 1), and lithium aluminium deuteride (50 mg) was added The mixture was refluxed and, after 8 h, the excess of reductant was destroyed by the addition of M phosphoric acid

After processing, the residue was methylated with trideuteriomethyl iodide, as described above The mixture of permethylated oligosaccharide alditols was analysed by g l c–m s Two peaks were obtained in the disaccharide region *A*, *T*-value 0 68 (relative to permethylated isomaltitol), and *B*, *T*-value 1 00 The m s of *A* had, *inter alia*, peaks at *m/e* 43(24), 45(21), 55(19), 57(26), 62(24), 69(25), 71(30), 74(15), 75(12), 78(11), 83(10), 85(12), 88(100), 91(12), 96(12), 97(13), 101(24), 104(40), 106(10), 111(13), 119(15), 148(9), 187(6), 190(24), 212(4), 215(9), 222(4), 272(3), 275(11), and *B* at *m/e* 43(25), 49(18), 50(12), 61(9), 63(8), 71(15), 73(16), 75(23), 78(12), 88(100), 89(9), 93(21), 101(53), 104(22), 105(16), 111(29), 137(10), 149(22), 181(9), 192(43), 196(2), 224(5), 228(1), 242(17), 302(4)

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