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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE FROM *Streptococcus pneumoniae* TYPE 18A

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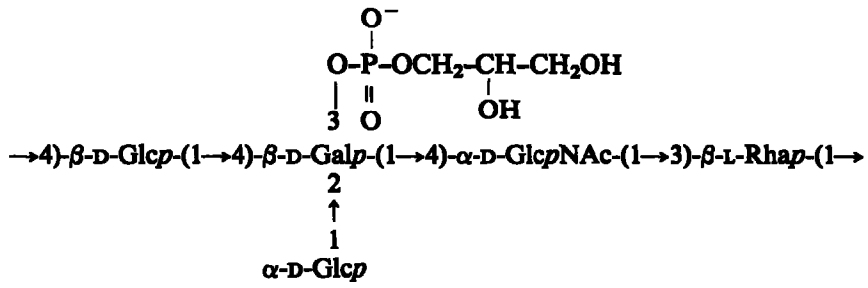
ABSTRACT

The structure of the capsular polysaccharide (S18A) elaborated by *Streptococcus pneumoniae* type 18A has been investigated by using methylation analysis and n.m.r. spectroscopy. It is concluded that the polysaccharide is composed of pentasaccharide repeating-units having the following structure.



In this structure, the absolute configuration of the glycerol 1-phosphate moiety has not been determined but is assumed to be D from biosynthesis considerations. The structure of S18A is, as expected, closely similar to those determined for S18F and S18C.

INTRODUCTION

Streptococcus pneumoniae group 18 consists of four different types[1], 18F, 18A, 18B, and 18C. The corresponding antigenic formulas (18a, 18b, 18c, 18f), (18a, 18b, 18d), (18a, 18b, 18e, 18g), and (18a, 18b, 18c, 18e), respectively, indicate that the capsular antigens elaborated by these types should have related structures.

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The structures of the antigens from types 18F[2] and 18C[3] have been determined. They are polysaccharides, composed of pentasaccharide repeating-units, and contain a glycerol 1-phosphate substituent. The antigen from type 18A (S18A) has been investigated by Heidelberger and co-workers[4], who showed that it is composed of D-galactose, D-glucose, rhamnose, 2-acetamido-2-deoxyglucose, glycerol, and phosphate. Unlike S18F and S18C, it does not contain *O*-acetyl groups. Their studies further indicated that it is a polysaccharide proper and not a polymer of the teichoic acid type. We now report further studies of S18A.

RESULTS AND DISCUSSION

A hydrolysate of S18A contained phosphate, glycerol, L-rhamnose, D-glucose, D-galactose, and 2-acetamido-2-deoxy-D-glucose, in agreement with the results of Heidelberger *et al.*[4]. The absolute configurations were determined by the method devised by Gerwig *et al.*[5]. Dephosphorylation of S18A, by treatment with aqueous 48% hydrogen fluoride, yielded a polymeric product, the hydrolysate of which contained L-rhamnose, D-glucose, D-galactose, and 2-amino-2-deoxy-D-glucose in the approximate molar proportions 11:52:25:12. N.m.r. spectra indicated that the 2-amino-2-deoxy-D-glucose is *N*-acetylated, that S18A is composed of pentasaccharide repeating-units, and that it does not contain *O*-acetyl groups.

Methylation analyses of S18A and dephosphorylated S18A gave the sugars listed in Table I, columns *A* and *B*. The results demonstrate that S18A contains a terminal D-glucopyranosyl group, an L-rhamnopyranosyl residue linked through O-3, a D-glucopyranosyl residue linked through O-4, a 2-acetamido-2-deoxy-D-glucopyranosyl residue linked through O-4, and a D-galactopyranosyl residue linked through O-2 and O-4. The results further show that phosphate is linked to O-3 of the branching D-galactopyranosyl residue. In this interpretation, it is assumed that all

TABLE I

METHYLATION ANALYSIS OF NATIVE AND MODIFIED S18A[a]

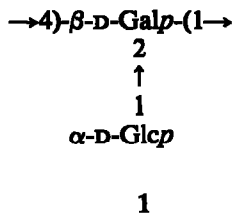
Sugar[b]	T[c]	Mole %	
		A	B
2,4-Rha	0.92	14	12
2,3,4,6-Glc	1.00	34	27
2,3,6-Glc	1.29	28	30
3,6-Gal	1.53	14	25
6-Gal	1.67	4	
3,6-GlcNAc	2.13	6	6

[a]Key: *A*, methylated polysaccharide; *B*, dephosphorylated methylated polysaccharide. [b]2,4-Rha = 2,4-di-*O*-methyl-L-rhamnose, *etc.* [c]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 SE-54 column at 170° for 3 min, 170–220°, 3°/min.

sugars are pyranosidic, as evident from the n.m.r. studies discussed below. The stoichiometry of the methylation analyses are not very good, due to partial dephosphorylation during methylation, incomplete hydrolysis of phosphate esters, and some *N*-deacetylation preceding hydrolysis of 2-acetamido-2-deoxy-D-glucopyranosidic linkages.

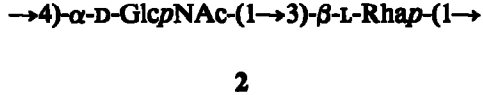
The signals given by the protons and carbon atoms of the individual sugar residues in dephosphorylated S18A (except the H-6 and C-6 signals of the D-glucose, D-galactose, and 2-acetamido-2-deoxy-D-glucose residues) were assigned by means of homo- and hetero-nuclear COSY, relayed and double-relayed homonuclear COSY (Table II). A detailed discussion of the assignments is given in the experimental section. The information on linkages for all of the residues could further be corroborated from the downfield shifts for the glycosyloxylated carbon atoms.

The mutal order of the sugar residues was determined from the 2D-n.O.e. spectrum of dephosphorylated S18A. Starting with H-1 of α-D-Glc (δ 5.31), it shows n.O.e. contacts with H-2, at δ 3.55, in the same sugar, and with H-2 of β-D-Gal, at δ 3.77, thus establishing the partial structure **1**.

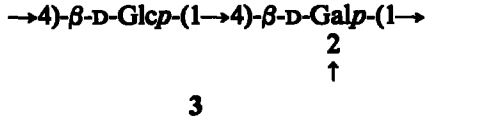


1

The signal given by H-1 of α-D-GlcNAc (δ 5.07) shows n.O.e. contacts with H-2 in the same residue, at δ 3.98, and with H-2 and H-3 of β-L-Rha, at δ 4.19 and 3.63 (weak), establishing the structural element **2**.



The signal given by H-1 of β-D-Glc, at δ 4.65, shows n.O.e. contact with H-5 in the same residue, at δ 3.50, and with H-4 of β-D-Gal, at δ 4.18, establishing the structural element **3**.



The structural elements **1**–**3** could be combined into the unique structure **4**, which represents the pentasaccharide repeating-unit of dephosphorylated S18A.

TABLE II

CHEMICAL SHIFTS (P.P.M.) OF THE SIGNALS IN THE ^{1}H- AND THE ^{13}C-N.M.R. SPECTRA[a] OF THE DEPHOSPHORYLATED *Streptococcus pneumoniae* TYPE 18A POLYSACCHARIDE

	H/C					
Sugar residue	*1*	*2*	*3*	*4*	*5*	*6*
α-D-Glc*p*	5.31	3.55[b]	3.74[b,c]	3.45[d]	4.07[b]	n.a.[f]
	98.8	72.3	73.8	70.4	72.6	
	(170)					
α-D-Glc*p*NAc	5.07	3.98[b]	~3.99[b,c]	~3.87[e]	4.11[e]	n.a.
	94.7	54.2	70.1	77.4	71.5	
	(173)					
β-L-Rha*p*	4.83	4.19[b]	3.63[b]	3.47[b]	3.41[b]	1.34[b]
	101.3	68.2	77.6	71.2	73.0	17.6
	(161)					
β-D-Gal*p*	4.67	3.77[b]	3.88[c]	4.18[d]	~3.74[e]	n.a.
	102.3	76.6	72.8	79.4	75.2	
	(163)					
β-D-Glc*p*	4.65	3.37[b]	3.66[b,c]	~3.64[d]	3.50[e]	n.a.
	104.7	74.7	76.4	78.4	75.5	
	(160)					

[a] $J_{C\text{-}1,H\text{-}1}$ coupling constants are given in brackets. [b] COSY spectrum. [c] Relayed COSY spectrum. [d] Double-relayed COSY spectrum. [e] Reasonable assignment. [f] n.a., Not assigned.

→4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*NAc-(1→3)-β-L-Rha*p*-(1→

2
↑
1
α-D-Glc*p*

4

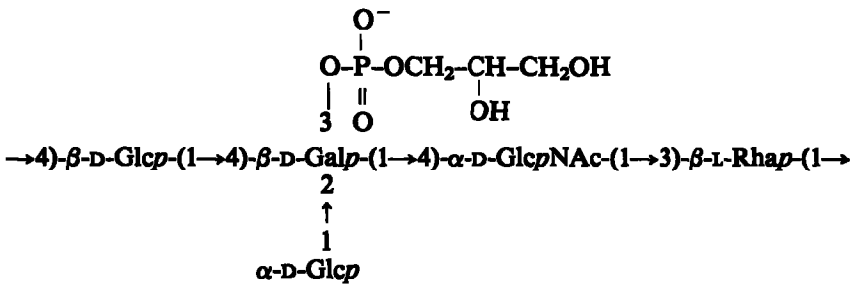
N.O.e. contacts were also observed between H-1 of β-L-Rha, at δ 4.83, and H-3 in the same molecule as well as H-4 in β-D-Glc, both at δ ~3.64, and between H-1 of β-D-Gal, at δ 4.67, and H-5 of the residue at δ ~3.74, H-3 of the same residue and H-4 of α-D-GlcNAc, the last two at δ ~3.87. No conclusive structural assignments could be made from these results which, however, are not in disagreement with structure 4.

As discussed above, methylation analyses of S18A and dephosphorylated S18A demonstrate that phosphate is linked to O-3 of the β-D-galactopyranosyl residue. In agreement with this, a well separated signal in the ^{13}C-n.m.r. spectrum of dephosphorylated S18A appears at δ 79.4 and is assigned to C-4 of β-D-Gal. The signal of this carbon atom appears at higher field (not assigned) in the ^{13}C-n.m.r. spectrum of native S18A, and an upfield shift is expected on phosphorylation of the 3-position.

Two signals, of the same intensity as the other strong signals, at δ 63.2 and

67.4, $J_{C,P}$ 4.9 Hz, in the ^{13}C-n.m.r. spectrum of native S18A could be assigned to C-3 and C-1 of a glycerol 1-phosphate residue, and were absent from the spectrum of the dephosphorylated S18A. The absolute configuration of this residue has not been determined but, from biosynthesis considerations[6], it is assumed to be D-glycerol 1-phosphate (*sn*-glycerol 3-phosphate).

From the combined evidence, structure **5** is proposed for the pentasaccharide repeating-unit of S18A. It differs from the pentasaccharide repeating-unit of S18F, in that the latter contains an α-D-glucopyranosyl residue replacing the 2-acetamido-2-deoxy-α-D-glucopyranosyl residue in S18A. S18F further contains *O*-acetyl groups.



5

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at <40° (bath) or at room temperature by flushing with air. For g.l.c., a Hewlett-Packard 5830A instrument fitted with a flame-ionisation detector was used. Separation of partially methylated alditol acetates and of alditol acetates was performed on an HP high-performance SE-54 capillary column. G.l.c.-m.s. was performed on a Hewlett-Packard 5970 instrument, using the same phase. Absolute configurations of the sugars were determined according to the procedure of Gerwig *et al.*[5]. Hydrolyses were performed with 0.5M trifluoroacetic acid for 1 h at 120° followed by 16 h at 100°. Methylation analyses and work-up were performed essentially as previously described[7,8].

N.m.r. spectroscopy. — N.m.r. spectra of solutions in deuterium oxide were recorded at 70° with a JEOL GX-400 instrument, using sodium 3-trimethylsilylpropanoate-d_4, δ 0.00 (^{1}H), or 1,4-dioxane, δ 67.4 (^{13}C), as internal references, except for the ^{13}C-n.m.r. spectrum of native S18A for which acetone, δ 31.0, was used. Standard pulse sequences were used for the two-dimensional n.m.r. spectroscopy except for the double-relayed COSY which was performed according to Bax and Drobny[9]. For the NOESY spectrum, a mixing time of 400 ms was used.

N.m.r. assignments. — Chemical shifts for the signals of H-1–H-5 and C-1–C-5 for all sugar residues and of H-6 and C-6 for the L-rhamnose residue, together with

the $J_{C\text{-}1,H\text{-}1}$ values of the sugar residues of dephosphorylated S18A polysaccharide, are given in Table II. In the following discussion, the carbon–proton correlated n.m.r. spectrum was always used for all assignments together with some of the COSY spectra. All H-1,2 signals were readily assigned from the COSY spectrum. Of the five signals for anomeric protons, that at δ 5.07 was assigned to a 2-acetamido-2-deoxy-α-D-glucopyranosyl residue because of the high field for the corresponding C-2 signal and a $J_{C\text{-}1,H\text{-}1}$ value of 173 Hz. The signal at δ 4.83 could be assigned to a β-L-rhamnopyranosyl residue because of the $J_{C\text{-}1,H\text{-}1}$ value (161 Hz) and the low $J_{H\text{-}1,H\text{-}2}$ value (~1 Hz). The signal at δ 4.65 showed a high $J_{H\text{-}1,H\text{-}2}$ value (~8 Hz) and, from the chemical shift of the H-2 signal, δ 3.37, it could be assigned to a β-D-glucopyranosyl residue. Of the remaining two anomeric signals that at δ 5.31 is assigned to a terminal α-D-glucopyranosyl group because of the chemical shift of the H-2 and C-2 signals, for which chemical shift differences are small compared to α-D-glucopyranose. The final anomeric signal at δ 4.67 could thus be assigned to the β-D-galactopyranosyl residue. The $J_{C\text{-}1,H\text{-}1}$ value is 163 Hz and both the H-2 and C-2 signals are shifted downfield compared to those of β-D-galactopyranose. In agreement with this, the methylation analysis shows that the D-galactose residue is linked through O-2 (and O-4). As there are two proton signals at δ 3.74, the C-3 signal of the α-D-glucopyranosyl group could be either at δ 73.8 or 75.2. Comparison with the chemical shift for C-3 in α-D-glucopyranose indicates that the first alternative is correct.

The remaining unassigned signals, for H-4, H-5, C-4, and C-5 in the 2-acetamido-2-deoxy-α-D-glucopyranosyl residue and for H-5 and C-5 in the two β-linked sugar residues, are then finally assigned by using the following arguments. The carbon signal at the lowest field could be assigned to C-4 of the 2-acetamido-2-deoxy-α-D-glucopyranosyl residue since the linkage carbon should have a large downfield shift.

The shifts for H-5 signals in 4-linked sugar residues are positive or near zero. Thus, a signal at δ 4.11 can be assigned to the 2-acetamido-2-deoxy-α-D-glucopyranosyl residue. Using the same argument for the signals at δ 3.74 and δ 3.50, they could be assigned to the β-D-galacto- and the β-D-gluco-pyranosyl residue, respectively.

Purification of S18A. — The crude polysaccharide was purified from contaminating protein by partition between phenol and water[10]. It was further purified by chromatography on a DEAE-Trisacryl M column (300 × 15 mm). The column was first irrigated with 0.05M sodium dihydrogen phosphate, followed by a linear gradient of sodium chloride (0→0.5M, 600 mL) in the same phosphate buffer. The fractionation was monitored by the anthrone reaction. S18A was eluted at a sodium chloride concentration of 0.17–0.24M. Pure S18A was recovered by dialysis and freeze-drying.

Dephosphorylation of S18A. — A solution of the native polysaccharide (30 mg) in aqueous 48% hydrogen fluoride (2.0 mL) was kept for 96 h at 4°. 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 fractionated on a column of Bio-Gel P-2, irrigated with water. The dephosphorylated polymer (20 mg) was eluted with the void volume.

ACKNOWLEDGMENTS

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