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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE FROM *Streptococcus pneumoniae* TYPE 7A

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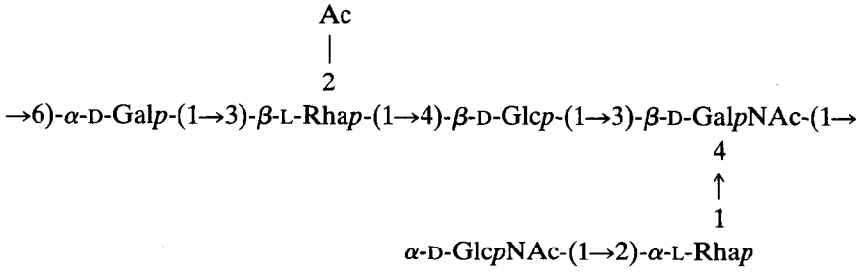
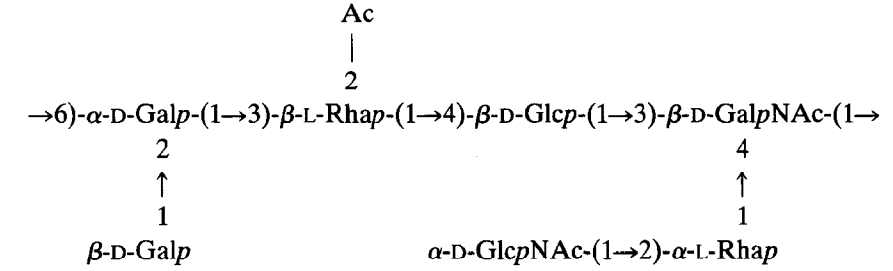
ABSTRACT

Application of methylation analysis, specific degradations, and n.m.r. spectroscopy to the capsular polysaccharide elaborated by *Streptococcus pneumoniae* type 7A indicates a hexasaccharide repeating-unit with the structure



INTRODUCTION

Streptococcus pneumoniae group 7 consists[1] of four types, 7F, 7A, 7B, and 7C. Preliminary studies of the capsular polysaccharide S7F, elaborated by type 7F, have been reported[2,3] and the heptasaccharide repeating-unit **1** was established by Moreau *et al.*[4]. We now report on the structure of the type 7A polysaccharide (S7A).



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Considering the similarity in the antigenic characters[1] of types 7F and 7A, namely 7a, 7b, and 7a, 7b, 7c, respectively, it was expected that S7F and S7A should have similar structures.

RESULTS AND DISCUSSION

S7A, which is a neutral polysaccharide, was purified from contaminating acidic C-substance by chromatography on DEAE-Trisacryl. Sugar analysis of a hydrolysate revealed L-rhamnose, D-glucose, D-galactose, 2-amino-2-deoxy-D-glucose, and 2-amino-2-deoxy-D-galactose in the molar ratios 42:15:23:10:5. The absolute configurations of the sugars were determined by the procedure of Gerwig *et al.*[5]. Methylation analysis of S7A revealed 2-linked L-rhamnose, 3-linked L-rhamnose, 4-linked D-glucose, 6-linked D-galactose, terminal 2-amino-2-deoxy-D-glucose, and 3,4-linked 2-amino-2-deoxy-D-galactose in the ratios 12:9:21:14:8:17. The stoichiometries of the sugar analysis and the methylation analysis were not good, which is not uncommon for polysaccharides that contain amino sugars.

N.m.r. studies (see below) demonstrated S7A to have a hexasaccharide repeating-unit, which sugar and methylation analyses indicated to contain two L-rhamnose residues and one residue each of D-glucose, D-galactose, 2-amino-2-deoxy-D-glucose, and 2-amino-2-deoxy-D-galactose. The n.m.r. spectra further revealed that each sugar residue is pyranosidic, that the amino sugars are *N*-acetylated, and that the polysaccharide contains one OAc group per repeating unit. The ^{1}H-n.m.r. spectrum of native S7A is shown in Fig. 1 and several of the signals of native and *O*-deacetylated S7A were assigned using COSY, relayed COSY, doubly-relayed COSY, and C–H correlation spectra (Tables I and II). The anomeric configurations were determined from the $J_{1,2}$ and $J_{H\text{-}1,C\text{-}1}$ values[6]. Thus, two sets of resonances could be assigned to the rhamnopyranosyl residues with small $J_{1,2}$ values. Comparison of the spectra of the native and the *O*-deacetylated polysaccharide showed that one of the rhamnosyl residues, evidently the 3-linked, contained an OAc group at position 2. One set of resonances could be assigned to a 6-linked α-D-galactopyranose residue, as a small value for $J_{3,4}$ was observed in the spectrum of the *O*-deacetylated S7A, as evident from the shapes of the cross-peaks, and as the signals from H-5 and H-6 were shifted substantially downfield. The amino sugars were identified from their H-2/C-2 correlated signals (δ 52.5 for β-D-Gal*p*NAc and δ 54.4 for α-D-Glc*p*NAc). That the α-linked amino sugar is terminal 2-acetamido-2-deoxy-α-D-glucopyranose was evident from the chemical shifts, which are similar to those of 2-acetamido-2-deoxy-α-D-glucopyranose.

It was evident from the results of the methylation analysis that two of the sugar residues in the repeating unit of S7A, namely the 3-substituted L-rhamnopyranosyl residue and the 3,4-disubstituted 2-acetamido-2-deoxy-D-galactopyranosyl residue, should not be oxidized by periodate. The *O*-deacetylated S7A was therefore subjected to a Smith degradation[7]. Fractionation of the products on Bio-Gel P-2 gave two oligosaccharides, and the ^{1}H-n.m.r. data of their acetates

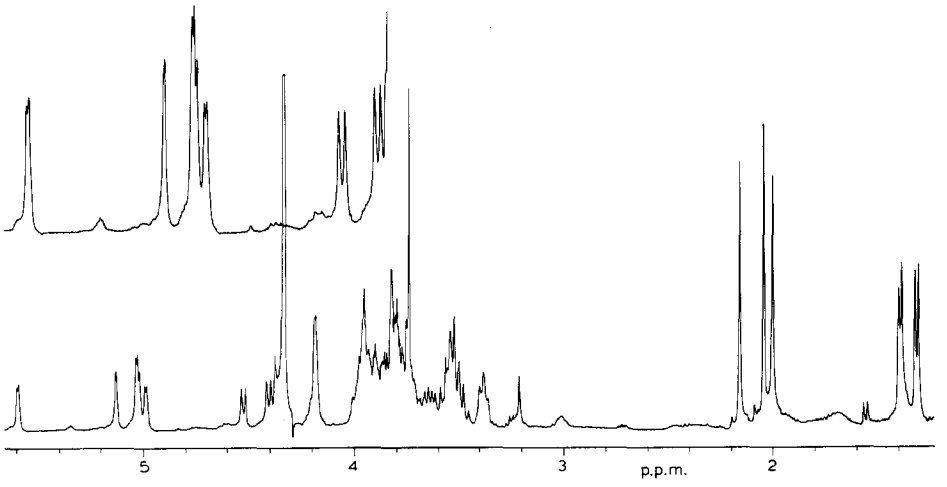


Fig. 1. ^{1}H-N.m.r. spectrum of native S7A.

(Table III) indicated them to be erythritol-2-yl β-L-rhamnopyranoside and glycerol-1-yl 2-acetamido-2-deoxy-β-D-galactopyranoside. These results, in conjunction with those of the methylation analysis, demonstrate the presence of the structural elements **2** and **3** in S7A.

→3)-β-L-Rha*p*-(1→4)-D-Glc*p*-(1→ →3)-β-D-Gal*p*NAc-(1→6)-D-Gal*p*-(1→
4
↑

2 **3**

Hydrolysis of *O*-deacetylated S7A by treatment with 0.25M trifluoroacetic acid at 77° for 4 h gave a mixture of mono- and oligo-saccharides from which a pure tetrasaccharide was isolated after chromatography on Bio-Gel P-2. A hydrolysate of the tetrasaccharide contained L-rhamnose, D-glucose, 2-amino-2-deoxy-D-galactose, and 2-amino-2-deoxy-D-glucose. Most of the signals in the ^{1}H-n.m.r. spectrum of this tetrasaccharide were assigned using COSY, relayed COSY, and doubly-relayed COSY experiments (Table IV). The ^{1}H-n.m.r. data indicated that the 2-acetamido-2-deoxy-D-galactose residue is the reducing unit and 3,4-substituted, and that the D-glucose residue is terminal and β-pyranosidic, and this was confirmed by the results of methylation analysis. The two remaining residues were identified from the $J_{1,2}$ values. The 2-acetamido-2-deoxy-D-galactopyranose residue showed signals for the α and the β forms in the ratio ~0.55:0.45. Also, H-1 of the terminal β-D-glucopyranosyl group gave two signals in the same ratio because of long-range effects, indicating that this group is linked close to C-1 of the 2-acetamido-2-deoxy-D-galactopyranose residue. Thus, the tetrasaccharide has structure **4** and this was confirmed by the presence of all expected interglycosidic

TABLE I

^{1}H-N.M.R. CHEMICAL SHIFTS (δ D_2O) FOR NATIVE S7A

Sugar residue	*C-1*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*NAc*	*OAc*
→2)-α-L-Rha*p*-(1→	99.2	5.13	4.18				1.35[c]		
	(167)[a]	(~1)[b]							
→6)-α-D-Gal*p*-(1→	96.4	5.02	3.78	3.84					
	(167)	(3.7)							
α-D-Glc*p*NAc-(1→	94.9	4.99	3.92	3.82				2.04[d]	
	(169)	(4.0)							
→3)-β-L-Rha*p*-(1→	99.7	5.03	5.59	3.81	3.48		1.36[c]		2.16
	(161)	(~1)							
→4)-β-D-Gal*p*(NAc-(1→	102.2	4.52	3.96	3.88				2.00[d]	
3	(161)	(8.2)							
↑									
→4)-β-D-Glc*p*-(1→	104.5	4.40	3.38	3.54					
	(161)	(7.9)							

[a] $J_{C\text{-}1,H\text{-}1}$ (Hz). [b] $J_{H\text{-}1,H\text{-}2}$ (Hz). [c,d] These values could be interchanged.

TABLE II

^{1}H-N.M.R. CHEMICAL SHIFTS (δ, D_2O) FOR *O*-DEACETYLATED S7A

Sugar residue	*C-1*	*H-1*	*C-2*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6A*	*H-6B*	*NAc*
→2)-α-L-Rha*p*-(1→	99.3 (167)[a]	5.15 (~1)[b]		4.22	3.99	3.58	3.82	1.35[c]		
→6)-α-D-Gal*p*-(1→	97.0 (167)	5.10 (3.7)		3.86	3.93	4.00	4.01	4.29	3.71	
α-D-Glc*p*NAc-(1→	94.8 (169)	5.02 (3.7)	54.4	3.94	3.83	3.54	3.94	3.82	3.94	2.05[d]
→3)-β-L-Rha*p*-(1→	101.4 (161)	4.85 (~1)		4.26	3.64	3.47		1.36[c]		
→4)-β-D-Gal*p*NAc(1→	102.2 (161)	4.54 (7.9)	52.5	3.97	3.90	4.21				2.02[d]
3 ↑										
→4)-β-D-Glc*p*-(1→	105.6 (161)	4.43 (7.9)		3.42	3.59					

[a] $J_{C\text{-}1,H\text{-}1}$ (Hz). [b] $J_{H\text{-}1,H\text{-}2}$ (Hz). [c,d] These values could be interchanged.

TABLE III

^{1}H-N.M.R. CHEMICAL SHIFTS ($CDCl_3$) FOR THE ACETYLATED OLIGOSACCHARIDES OBTAINED AFTER SMITH DEGRADATION

Residue	*H-1*	*H-1'*	*H-2*	*H-3*	*H-3'*	*H-4*	*H-4'*	*H-5*	*H-6*	*H-6'*
β-D-Gal*p*NAc-(1→	4.52		3.90	5.21		5.30		3.85	~4.09[a]	~4.09[a]
→1)-Glycerol	3.81	3.62	5.12	4.01	4.31					
β-L-Rha*p*-(1→	4.70		5.35	~4.96[a]		~4.96[a]		3.42	1.32	
→2)-Erythritol	4.25	3.85	5.20	5.05		4.34	4.10			

[a]Not resolved.

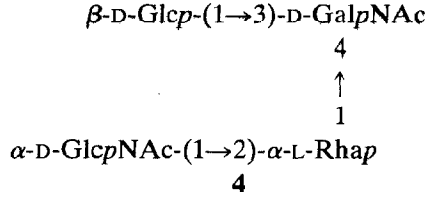
TABLE IV

^{1}H-N.M.R. CHEMICAL SHIFTS (δ D_2O) FOR THE TETRASACCHARIDE OBTAINED ON ACID HYDROLYSIS OF S7A

Sugar residue	*H-1*	J *(Hz)*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6A*	*H-6B*	*NAc*
→4)-α-D-Gal*p*NAc 3 ↑	5.23	(nr)	4.30	4.02	4.28	4.16	3.72	3.72	2.03[a]
→2)-α-L-Rha*p*-(1→	5.20	(nr)	4.20	3.96	3.58	3.78	1.33		
α-D-Glc*p*NAc-(1→	4.98	(3.7)	3.93	3.82	3.53	3.93	3.81	3.93	2.05[a]
→4)-β-D-Gal*p*NAc 3 ↑	4.73	(7.4)	3.92	3.88	4.20				2.05[a]
β-D-Glc*p*-(1→	4.50	(7.3)	3.37	3.42	3.30	3.40	3.61	3.89	
β-D-Glc*p*-(1→	4.43	(7.3)	3.38	3.42	3.30	3.40	3.61	3.89	

[a]These values could be interchanged.

n.O.e. contacts of the anomeric protons as determined from a NOESY spectrum (Table V).



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On treatment of **4** with base under mild conditions, the substituent linked t O-3 of the 2-acetamido-2-deoxy-D-galactopyranose residue should be released b β-elimination and, in fact, D-glucose was released, in agreement with the postulate structure.

The signals in the ^{1}H- (Fig. 1) and ^{13}C-n.m.r. spectra of native S7A (Table I were compared with those of the *O*-deacetylated S7A, the most significant diffe ence being that the signal for H-2 of the β-L-rhamnopyranosyl residue now appeare at δ 5.59 instead of at δ 4.26. Consequently, the OAc group in S7A is linked to O- of this residue, and acetyl migration is prevented because of the substituent at O-2 Comparable downfield shifts on acetylation of O-2 in a rhamnopyranosyl residu have been observed[8,9]. The corresponding downfield shift of ~0.2 p.p.m. for th signals from H-1 and H-3 are also in the expected range[10].

From the combined results, it is concluded that S7A has the hexasaccharid repeating-unit **5**. As expected, the structures of S7F (**1**) and S7A are similar. Th

TABLE V

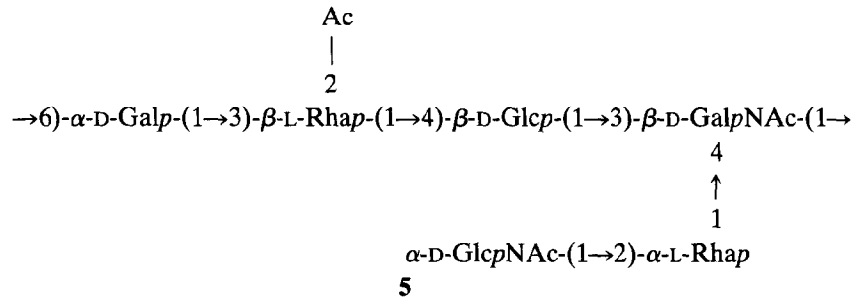
OBSERVED INTERGLYCOSIDIC N.O.E CONTACTS OF ANOMERIC PROTONS OF *O*-DEACETYLATED S7A AND C THE TETRASACCHARIDE OBTAINED ON ACID HYDROLYSIS OF S7A

Anomeric proton	*N.O.e. contact*
O-Deacetylated S7A	
5.15 (α-L-Rha)	5.02 (α-D-GlcNAc, H-1)
5.02 (α-D-GlcNAc)	5.15 (α-L-Rha, H-1), 4.22 (α-L-Rha, H-2)
5.10 (α-D-Gal)	4.26 (β-L-Rha, H-2)
4.85 (β-L-Rha)	3.48 (β-D-Glc, H-4)
4.43 (β-D-Glc)	3.90[a] (β-D-GalNAc, H-3)
Tetrasaccharide	
5.23 (α-D-GalNAc)	4.30 (α-D-GalNAc, H-2)
5.20 (α-L-Rha)	4.98 (α-D-GlcNAc, H-1), 4.28 (α-D-GalNAc, H-4), 4.20 (α-L-Rha, H-2)
4.98 (α-D-GlcNAc)	5.20, 4.20 (α-L-Rha, H-1, H-2), 3.93 (α-D-GlcNAc, H-2)
4.73 (β-D-GalNAc)	3.92 (β-D-GalNAc, H-2)
4.50 (β-D-Glc)	4.02 (α-D-GalNAc, H-3), 3.37 (β-D-Glc, H-2)
4.43 (β-D-Glc)	3.88 (β-D-GalNAc, H-3), 3.38 (β-D-Glc, H-2)

[a]Signal with low intensity.

only difference is that the terminal β-D-galactopyranosyl group linked to O-2 of the α-D-galactopyranosyl residue in the heptasaccharide repeating-unit **1** is lacking in **5**.

N.O.e. contacts between some anomeric protons in *O*-deacetylated S7A were determined (Table IV), namely, between H-1 in α-D-Glc*p*NAc and H-1 and H-2 in α-L-Rha*p*, H-1 in α-D-Gal*p* and H-2 in α-L-Rha*p*, H-1 in β-L-Rha*p* and H-4 in β-D-Glc*p*, and H-1 in β-D-Glc*p* and H-3 in β-D-Gal*p*NAc, confirming four of the six disaccharide elements present in **5**.



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EXPERIMENTAL

General methods. — Concentrations were carried out under diminished pressure at 40° (bath) or in a stream of air. For g.l.c., a Hewlett–Packard 5830 instrument fitted with a flame-ionisation detector was used. Separations of alditol acetates were performed on an HP-54 column, using a temperature programme 200° (3 min) →250° at 2°.min^{-1}. Partially methylated alditol acetates were fractionated on an SE-54 column, using a temperature programme 150° (2 min) →220°, at 2°.min^{-1}. G.l.c.–m.s. was performed on a Hewlett–Packard 5970 MSD instrument. Absolute configurations of the sugars were determined according to the procedure of Gerwig *et al.*[5].

Methylation analyses were performed as previously described[11,12].

N.m.r. spectroscopy. — N.m.r. spectra (^{1}H at 400 or 270 MHz, and ^{13}C at 100 or 67 MHz) were recorded for solutions in D_2O at 70° with a JEOL GX-400 or GSX-270 spectrometer. Chemical shifts are given in p.p.m., using internal sodium 3-trimethylsilyltetradeuteriopropanoate (δ_H 0.00) and 1,4-dioxane (δ_C 67.40) as references. COSY, relayed COSY, NOESY, and C–H correlation spectroscopy experiments were performed according to JEOL standard pulse sequences, and the double-relayed COSY experiment was performed according to Bax and Drobny[13]. For the correlation spectroscopy, a 90° mixing pulse was used. The NOESY experiments were performed with a mixing time of 300 ms for the polysaccharide and 400 ms for the tetrasaccharide.

Purification of the polysaccharide. — A solution of the polysaccharide was applied to a column (20 × 1.6 cm) of DEAE-Trisacryl, which was irrigated first with water and then with a gradient (0→M) of sodium chloride. The main part of

the material was eluted by water, the C-substance and some polysaccharide being retained. The water fraction was freeze-dried to give the pure polysaccharide.

O-Deacetylation. — A solution of the polysaccharide in 0.1M sodium hydroxide was kept for 14 h at 20°, then neutralised, dialysed, and freeze-dried.

Smith degradation. — A solution of the polysaccharide (42 mg) and sodium metaperiodate (320 mg) in 0.1M sodium acetate buffer (pH 6, 33 mL) was kept for 2 days in the dark at 8°. Excess of periodate was reduced with ethylene glycol, and the solution was dialysed and freeze-dried. To a solution of the residue in water (8 mL) was added sodium borohydride (28 mg), and the solution was kept overnight at room temperature, neutralised with aqueous 50% acetic acid, and freeze-dried. Sugar analysis and ^{1}H-n.m.r. spectroscopy showed that the oxidation was complete. A solution of the product (30 mg) in 0.5M trifluoroacetic acid (8 mL) was kept at 25° for 48 h, then diluted with water (5 mL), and concentrated, and a solution of the residue in water (2 mL) was freeze-dried. To a solution of the residue (25 mg) in water (5 mL) was added sodium borodeuteride (29 mg), and the solution was kept overnight at room temperature, then neutralised with acetic acid, dialysed, and freeze-dried. Elution of the products from a column (90 × 3 cm) of Bio-Gel P-2 with water and monitoring of the fractions with a differential refractometer gave, after freeze-drying, two main fractions, **2** (2 mg) and **3** (3 mg).

Partial acid hydrolysis. — The polysaccharide (13 mg) was treated with 0.25M trifluoroacetic acid (1 mL) at 77° for 4 h. The solution was freeze-dried and the product was eluted from a column (80 × 3 cm) of Bio-Gel P-2 with water. A fraction (2 mg) eluted in the tri-/tetra-saccharide region contained pure tetrasaccharide **4**. Part of **4** was treated with 5mM sodium hydroxide at 40° for 30 min followed by reduction with sodium borohydride. After conventional work-up and acetylation of the product, g.l.c. demonstrated that glucose was the only monosaccharide released on treatment with base.

ACKNOWLEDGMENTS

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