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Note

Structural studies of the capsular antigen from *Haemophilus influenzae* type f

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Haemophilus influenzae is divided into six types (a–f), each of which produces its type-specific capsular antigen. Four of these antigens (from types a, b, c, and f) are of the teichoic acid type; the two others seem to be conventional polysaccharides. The structures of the antigens from types a[1], b[2,3], and c[4] have been determined. A cursory study of the type-f antigen[5] indicated that it is composed of 2-amino-2-deoxygalactose residues and phosphate groups in the molar ratio 2 : 1. It was proposed that the polymer was composed of 2-acetamido-2-deoxy-β-D-galactopyranosyl 2-acetamido-2-deoxy-β-D-galactopyranoside residues, joined through phosphoric diester groups at the 3- and 4′-positions. We now report structural studies of this antigen.

The teichoic acid from *H. influenzae* type f was purified by precipitation with cetyltrimethylammonium bromide, followed by chromatography on DEAE-Sepharose, and had $[\alpha]_D$ +63°. The antigen contained 5.2% of phosphorus, and 2-amino-2-deoxy-D-galactose was the only sugar detected after dephosphorylation and acid hydrolysis.

In the ^{1}H-n.m.r. spectrum, signals were observed at δ 5.50 (H), 5.17 (H), 4.53 (H), 2.16 (3 H), and 2.07 (6 H), indicating that the teichoic acid contains one *O*-acetyl group per two 2-acetamido-2-deoxy-D-galactosyl residues.

The signal at δ 5.50 is a doublet of a doublet, with coupling constants of 6.7 and 3.3 Hz. It collapsed into a doublet (*J* 6.7 Hz) on irradiation of the ring protons (δ 3.5–4.5). These results indicate that *J* 3.3 Hz is due to H–H coupling and *J* 6.7 Hz to P–H coupling and, together with evidence given below, that the signal derives from H-1 of a 2-acetamido-2-deoxy-α-D-galactopyranosyl phosphate residue. This is in good agreement with earlier ^{1}H-n.m.r. studies of α-D-galactopyranosyl phosphate, for which the signal given by the anomeric proton appeared[6] at δ 5.68 ($J_{H,H}$ ~1.0 and $J_{H,P}$ 7.0 Hz). It is also in agreement with the assumed biosynthetic route[7], according to which the 2-acetamido-2-deoxy-α-D-galactopyranosyl phosphate residue

TABLE I

^{13}C-N.M.R. SHIFTS OF *H. influenzae* TYPE f ANTIGEN AND RELEVANT REFERENCE SUBSTANCES

Compound	*Chemical shifts (p.p.m.)*																	
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C'-1*	*C'-2*	*C'-3*	*C'-4*	*C'-5*	*C'-6*	*-NHAc*				*-OAc*	
													CH_3		$C=O$		CH_3	$C=O$
β-D-GalNAc	96.5	54.9	72.3	69.0	76.3	62.2							23.5		175.9			
α-D-GalNAc							92.2	51.4	68.6	69.7	71.6	62.4		23.3		175.7		
H.i. type f	104.2	53.6[a] (5.8)	77.0[a] (5.5)	69.2[a] (<1[e])	76.5[b]	62.5[c]	96.4[a] (5.5)	50.2[a] (7.3)	73.4[d]	75.6[b]	72.5[d]	63.0[c]	24.5	24.0	176.5	176.1	22.2	175.4

[a]Coupled signals; coupling constants in parenthesis. [b,c,d]The assignments could be reversed. [e]Low coupling-constant; only a broadening of the signal was observed.

derives from UDP-2-acetamido-2-deoxy-D-galactose. The signal at δ 5.50 disappeared after autohydrolysis of the antigen and was replaced by a signal at δ 5.20 (J small); a more complex pattern was obtained between δ 4.3–4.7. The susceptibility of the antigen to acid hydrolysis also accords with the presence of a sugar-1-phosphate linkage.

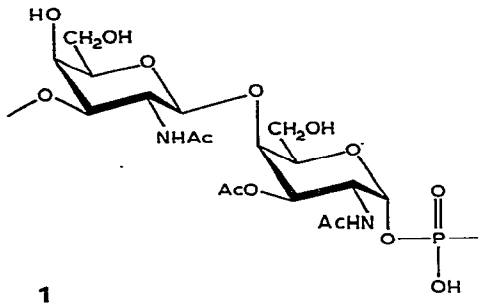
The signal at δ 5.17 is a doublet of a doublet, with coupling constants of 11 and 2.5 Hz, and collapsed to a singlet when irradiated as described above. The observed resonance must be due to the proton on the acetoxylated carbon atom. The coupling constants indicate that this proton is axial, as is one of the vicinal protons, whilst the other is equatorial. The *O*-acetyl group is consequently linked to O-3 of one of the 2-acetamido-2-deoxy-D-galactopyranosyl residues. In the ^{1}H-n.m.r. spectrum of methyl 3-*O*-acetyl-α-D-galactopyranoside[8], the signal for H-3 appeared at δ 4.98 ($J_{2,3}$ 11 and $J_{3,4}$ 3 Hz).

The signal at δ 4.53 ($J_{1,2}$ 7.7 Hz) is assigned to the anomeric proton of a 2-acetamido-2-deoxy-β-D-galactopyranosyl residue. The finding that one of the sugar residues is α-linked and the other β-linked accords with the observed optical rotation of the teichoic acid.

In addition to signals given by *N*-acetyl and *O*-acetyl groups, 12 signals were observed in the ^{13}C-n.m.r. spectrum of the teichoic acid (see Table I), consistent with a regular structure composed of disaccharide repeating-units. By comparing this spectrum with those of 2-acetamido-2-deoxy-α- and -β-D-galactopyranose[9], it was possible to assign several of the signals to defined carbon atoms. The spectra further confirm that both sugar residues in the teichoic acid are pyranosidic.

Both the readily recognizable signals given by C-2 carbon atoms show P–C coupling. One of these couplings is due to interaction with phosphate on C-1 of the 2-acetamido-2-deoxy-α-D-galactopyranosyl residue. The fact that the other C-2 signal is coupled demonstrates that phosphate is linked to C-3 of the 2-acetamido-2-deoxy-β-D-galactopyranosyl residue. This inference is further supported by the downfield shift of the signal for C-3 and the observed P–C couplings for the C-3 and C-4 signals.

Phosphorylation, acetylation, or glycosylation of a position in a sugar residue causes a downfield shift of the corresponding signal in the ^{13}C-n.m.r. spectrum. One such shift, involving the signal for C-3 in the 2-acetamido-2-deoxy-β-D-galactopyranosyl residue, has been accounted for. The signal given by C-4 in this residue, which shows P–C coupling, is not significantly shifted and C-4 is consequently unsubstituted. The signals given by the two C-6 atoms also appear at their normal values, demonstrating that the 6-positions are unsubstituted. The only remaining positions for acetylation or glycosylation are O-3 and O-4 in the 2-acetamido-2-deoxy-α-D-galactopyranosyl residue, and downfield shifts of these signals were observed. As discussed above, ^{1}H-n.m.r. spectroscopy demonstrates that the *O*-acetyl group is linked to O-3, and the 2-acetamido-2-deoxy-β-D-galactopyranosyl residue is consequently linked to O-4. From the combined results, structure **1** is proposed for the repeating-unit of the capsular teichoic acid of *H. influenzae* type f.



1

EXPERIMENTAL

Material. — The teichoic acid, from *H. influenzae* type f, strain NCTC 8473, was isolated and purified by precipitation with cetyltrimethylammonium bromide, followed by chromatography on DEAE-Sepharose, as previously described[4].

Phosphorus was determined as described by Chen *et al.*[10].

2-Amino-2-deoxy-D-galactose, $[\alpha]_{578}$ +70° (*c* 0.3, water), was isolated after dephosphorylation (48% aqueous hydrogen fluoride, 12 h, 25°) followed by hydrolysis of the polymer with 0.5M trifluoroacetic acid at 100° for 18 h.

Autohydrolysis was performed as earlier described[4].

N.m.r. spectroscopy. — N.m.r. spectra were recorded, for solutions in deuterium oxide at 85°, with a JEOL FX-100 spectrometer and with external tetramethylsilane (^{13}C n.m.r.) or internal 4,4-dimethyl-4-silapentane-1-sulfonate (^{1}H n.m.r.) as references. The ^{13}C-n.m.r. spectrum was run with complete proton-decoupling, using 46,000 accumulations of free induction decay; pulse width, 11 μs (70° flip angle); acquisition time, 0.95 s; and pulse repetition time, 1.0 s. In order to assign the low-field signals in the ^{1}H-n.m.r. spectrum, irradiation was performed at δ 4.0, affecting all signals between δ 3.5 and 4.5.

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