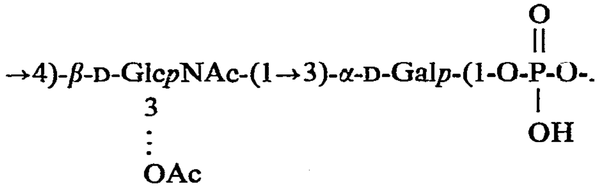
Carbohydrate Research, 76 (1979) 197–202

STRUCTURAL STUDIES OF THE CAPSULAR ANTIGEN OF *Haemophilus influenzae* TYPE c

PAULA BRANEFORS-HELANDER,
Institute of Medical Microbiology, University of Gothenburg, S-413 46 Gothenburg (Sweden)
BJÖRN CLASSON, LENNART KENNE, AND BENGT LINDBERG
Department of Organic Chemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm (Sweden)
(Received January 9th, 1979; accepted for publication, January 29th, 1979)

ABSTRACT

The structure of the capsular antigen from *Haemophilus influenzae* type c has been investigated, n.m.r. spectroscopy being the principal method used. It is concluded that the antigen is composed of repeating-units having the following structure:



$$\rightarrow 4)\text{-}\beta\text{-D-}\underset{\substack{3\\ \vdots \\ \text{OAc}}}{\text{Glc}p\text{NAc}}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\text{-O-}\overset{\substack{\text{O}\\ \|}}{\underset{\substack{| \\ \text{OH}}}{\text{P}}}\text{-O-}.$$

O-Acetyl groups are present in ~90% of the repeating-units.

INTRODUCTION

Haemophilus influenzae is divided into six types (a–f), which produce different, type-specific capsular antigens. The structures of the type a[1] and type b[2,3] antigens are similar and involve 4-*O*-*β*-D-glucopyranosyl-D-ribitol and 1-*O*-*β*-D-ribofuranosyl-D-ribitol residues, respectively, joined through phosphoric diester linkages.

Several capsular polymers, elaborated by bacteria, consist of mono- or oligosaccharides joined through phosphoric diester linkages. The term polysaccharide is often used for these substances, but is not adequate. Following a suggestion by Baddiley[4], we therefore call them capsular teichoic acids.

A cursory study of the type-c teichoic acid[5] indicated that it is composed of hexose and phosphate residues. We now report structural studies of this teichoic acid.

RESULTS AND DISCUSSION

The teichoic acid from *H. influenzae* type c was purified by chromatography on DEAE-Sepharose and had $[\alpha]_{578}$ +46°. Analyses revealed that it is composed of D-galactose, 2-acetamido-2-deoxy-D-glucose, phosphate, and *O*-acetyl groups in the proportions 1:1:1:0.9. In the ^{1}H-n.m.r. spectrum, the proportions of the signals for the anomeric protons of the two sugars and the methyl protons of *N*- and *O*-acetyl groups were 1:1:3:2.7. Glycerol and ribitol, which are components of several teichoic acids, were not detected.

Autohydrolysis of the acidic form of the teichoic acid, followed by dephosphorylation and simultaneous *O*-deacetylation by treatment with alkaline phosphatase, yielded a disaccharide. This was reduced with sodium borodeuteride to the alditol, $[\alpha]_{578}$ −17°. Methylation analysis of the alditol yielded 2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-D-glucose and 1,2,4,5,6-penta-*O*-methyl-D-galactitol-*1-d*, analysed by g.l.c.–m.s. of their alditol acetates. The disaccharide is therefore 3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-galactose (**1**). In the ^{1}H-n.m.r. spectrum of the disaccharide alditol, a signal at δ 4.59, $J_{1,2}$ 8 Hz, confirms the β linkage.

β-D-Glc*p*NAc-(1→3)-D-Gal
1

In the ^{13}C-n.m.r. spectrum of the teichoic acid (Table I), some of the 16 signals expected for the disaccharide moiety appear as doublets, with relative intensities 9:1. The spectrum of the *O*-deacetylated product contained 14 signals, and the doublets in the former spectrum are obviously due to the fact that only 90% of the repeating-units are *O*-acetylated. In the ^{1}H-n.m.r. spectrum of the teichoic acid, the methyl protons from the *N*-acetyl group also give two signals (δ 1.98 and 2.08), with the relative intensities 9:1.

By comparison with spectra of substances of low molecular weight, given in Tables I and II, it is possible to identify most of the signals in the original and the *O*-deacetylated teichoic acid. The signal at 103.4 p.p.m. is assigned to C-1 of the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue and that at 97.3 p.p.m. (*J* 7.1 Hz) to C-1 of an α-D-galactopyranosyl phosphate residue. This conclusion is in agreement with the assumed biosynthetic route, which indicates that phosphate should be linked to this position in disaccharide **1** and that the D-galactopyranosyl residue should have the α configuration, as in the precursor UDP-galactose[4]. That the D-galactosyl residue is pyranosidic is evident from the ^{13}C-n.m.r. spectrum, as some low-field signals, typical for galactofuranosides, were not observed[6].

The phosphorylated disaccharide alditol (Table II), obtained by autohydrolysis followed by borohydride reduction, was subjected to ^{13}C-n.m.r. spectroscopy. Most signals could be identified by comparison with spectra of 3-*O*-methyl-D-galactitol[7], methyl 2-acetamido-2-deoxy-β-D-glucopyranoside, and 2-acetamido-2-deoxy-β-D-glucopyranose 4-phosphate[8]. Inspection of Table II shows that phosphate is linked to C-4 of the 2-acetamido-2-deoxy-D-glucosyl moiety. As expected, the spectra of the monophosphates are pH-dependent, and the mutual order of the C-3 and C-4 signals

TABLE I

SIGNALS IN THE ^{13}C-N.M.R. SPECTRA OF THE CAPSULAR TEICHOIC ACID OF *H. influenzae* TYPE C AND RELEVANT REFERENCE SUBSTANCES

Substance	*2-Acetamido-2-deoxy-*D*-glucose*						D*-Galactose*						N-*Acetyl*		O-*Acetyl*	
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C'-1*	*C'-2*	*C'-3*	*C'-4*	*C'-5*	*C'-6*	*>C=O*	*-CH$_3$*	*>C=O*	*-CH$_3$*
Antigen, 85°	103.9	56.3	76.4	73.7^{b}	77.2^{b}	62.5^{a}	97.8^{b}	69.2^{b}	81.2	70.9	73.4	62.9^{a}	176.6	24.0	175.3	22.5
Antigen	103.4	55.4	75.5	72.9^{b}	76.1^{b}	61.5^{a}	97.3^{b}	68.4^{b}	80.5	70.2	72.7	62.2^{a}	176.2	23.3	174.8	21.9
Antigen, *O*-DeAc	104.3	57.0	74.1^{b}	75.1^{b}	76.2^{b}	61.5^{a}	97.6^{b}	68.7^{b}	80.1	70.3	72.9	62.2^{a}	176.6	23.6		
β-D-GlcNAc[15]	96.2	58.0	75.2	71.2	77.2	62.0							175.9	23.5		
3-OAc-β-D-GlcNAc[15]	95.6	56.3	77.0	69.1	77.0	61.8							175.7	23.0	175.1	21.5

^{a}The assignments of these signals may be reversed. ^{b}Coupled signals. Coupling constants are given in Table III.

TABLE II

SIGNALS IN THE ^{13}C-N.M.R. SPECTRA OF DISACCHARIDE DERIVATIVES OBTAINED FROM THE CAPSULAR TEICHOIC ACID OF *H. influenzae* TYPE C, AND RELEVANT REFERENCE SUBSTANCES

Substance	*2-Acetamido-2-deoxy-*D*-glucose*						D*-Galactitol*						N-*Acetyl*		
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C'-1*	*C'-2*	*C'-3*	*C'-4*	*C'-5*	*C'-6*	*>C=O*	*-CH$_3$*	*MeO*
3-*O*-(GlcNAc-4-phosphate)-D-galactitol, pH 11	103.1	56.8	75.0^{a}	73.7^{a}	76.3^{a}	62.0	63.8	71.1^{b}	79.3	72.3^{b}	70.7^{b}	64.2	175.9	23.5	
Same, pH 4	103.1	56.8	74.4^{a}	75.1^{a}	76.0^{a}	62.0	63.8	71.1^{b}	79.4	72.4^{b}	70.8^{b}	64.3	176.0	23.6	
3-*O*-(GlcNAc)-D-galactitol	103.2	57.1	74.9	70.7	76.8	62.2	63.7	71.1^{b}	79.3	72.3^{b}	70.9^{b}	64.3	176.1	23.4	
3-*O*-Me-galactitol[7]							64.2	71.5	80.7	72.1	70.1	64.7			61.5
β-D-GlcNAc-4-phosphate, NH_4^{+}[8]	96.0	57.5	74.4^{a}	74.8^{a}	76.2^{a}	61.7							175.7	23.5	
Methyl β-D-GlcNAc	103.2	56.7^{c}	75.2	71.2	77.1	62.0							176.1	23.3	58.3^{c}

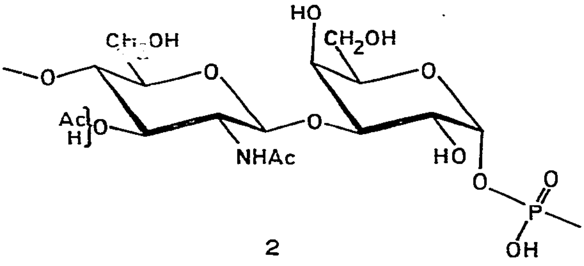
^{a}Coupled signals. Coupling constants are given in Table III. ^{b}The assignments of these signals may be reversed. ^{c}The assignments of these signals, based upon off-resonance decoupling, differ from those given in ref. 16.

in the 2-acetamido-2-deoxy-D-glucopyranosyl moiety is reversed when changing the pH from 11 to 4.

That phosphate was linked to this position was also evident from the ^{13}C-n.m.r. spectrum of the polymer and the phosphorylated disaccharide alditol. Carbon–phosphorus coupling was observed for C-3, C-4, and C-5 in the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue. The significant downfield-shift of the C-4 signal, and the smaller upfield-shifts of the C-3 and C-5 signals in the *O*-deacetylated polymer, compared to the corresponding shifts for the non-phosphorylated 2-acetamido-2-deoxy-β-D-glucopyranosyl derivatives, also support this assignment.

In the low-field part of the ^{1}H-n.m.r. spectrum of the teichoic acid, signals for anomeric protons at δ 4.90 ($J_{1,2}$ 8 Hz) and 5.50 ($J_{1,2}$ 2, $J_{\mathrm{H,P}}$ 6 Hz) are in agreement with the foregoing results. On irradiation at δ 3.8, the signal at δ 5.50 collapsed into a doublet, $J_{\mathrm{H,P}}$ 6 Hz, in agreement with the assignment of the coupling constants. A signal at δ 5.17 (J' 9, J'' 11 Hz), which does not appear in the spectrum of the *O*-deacetylated polymer, can be assigned to the proton linked to the *O*-acetylated carbon. Because of the high values of the coupling constants, this proton and those on the adjacent carbon atoms should all be axial. This excludes all protons except H-3 and H-4 of the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue. As phosphate is linked to C-4 of this residue, *O*-acetyl is consequently linked to O-3. The shift (—0.10 p.p.m.) of the protons of the *N*-acetyl group caused by the *O*-acetyl group also suggests that these groups are close. In agreement with this, an upfield shift of the C-3 signal and downfield shifts of those for C-2 and C-4 in the ^{13}C-n.m.r. spectrum are observed on *O*-deacetylation.

From the combined results, structure **2** is proposed for the repeating-unit of the capsular teichoic acid of *H. influenzae* type c. *O*-Acetyl groups are present in some 90% of these units.



2

Some information on the conformation of the polymer may also be obtained from the ^{13}C-n.m.r. spectra[9]. The three-bond ^{31}P–^{13}C coupling-constant is dependent upon the angle between the POC and OCC′ planes, allowing an estimation of the populations of the possible rotamers. Coupling constants of 8–10 Hz and 2–3 Hz are observed for *trans* (dihedral angle, 180°) and *gauche* (dihedral angle, 60°) relationships, respectively.

The values for the polymer (Table III) show that the phosphorus atom preferentially adopts a position *trans* to C-2 and *gauche* to the ring oxygen of the D-

TABLE III

^{13}C–^{31}P COUPLING CONSTANTS (Hz)[a]

Substance	D-*GlcNAc*			D-*Gal*	
	C-3	*C-4*	*C-5*	*C'-1*	*C'-2*
Teichoic acid	—	6.7	4.4	7.1	8.5
3-*O*-(GlcNAc-4-phosphate)-D-galactitol, pH 11	small	4.9	7.4		
Same, pH 4	1.6	5.5	6.1		
β-D-GlcNAc-4-phosphate, NH_4^+	1.9	6.2	6.1		

[a] Values are given only for well-resolved signals.

galactopyranosyl moiety. The same orientation has been observed for related polymers[8].

EXPERIMENTAL

The general methods were the same as in a previous publication[10].

Material. — The capsular teichoic acid was isolated from the culture filtrate by precipitation with 0.5% cetyltrimethylammonium bromide. It was further purified by chromatography on a DEAE-Sepharose column with a sodium chloride gradient (0–2M).

Analyses. — After hydrolysis with 0.5M trifluoroacetic acid at 100° for 16 h and subsequent deamination, the sugars were analysed by g.l.c. of the alditol acetates. Identifications were confirmed by g.l.c.–m.s. The absolute configuration of the D-galactose was determined as described by Leontein *et al.*[11].

Phosphate analyses were performed as described by Chen *et al.*[12].

Autohydrolysis. — An aqueous solution of the teichoic acid (100 mg) was passed through a small column of Dowex-50(H$^+$) resin, concentrated to 40 mL, heated at 60° for 90 min, neutralized with aqueous ammonia, and freeze-dried to yield a phosphorylated disaccharide containing ~60% of *O*-acetyl groups as indicated by n.m.r. spectroscopy.

Part of this product was reduced with sodium borohydride, and the product was treated with aqueous ammonia (pH 12) for 2 min at 100° to remove remaining *O*-acetyl groups.

Dephosphorylation. — The rest of the autohydrolysate (50 mg) was treated with alkaline phosphatase (pH 10.4) for 3 days at 37°, passed through a mixed bed of anion- and cation-exchange resin, and freeze-dried to yield the disaccharide (34 mg) free from *O*-acetyl groups.

Methylation analysis. — The dephosphorylated disaccharide (3 mg) was reduced with sodium borodeuteride (10 mg), methylated according to Hakomori[13], and hydrolysed with 0.5M trifluoroacetic acid for 16 h at 100°. The methylated sugars were analysed by g.l.c.–m.s. as their alditol acetates[14].

O-Deacetylation. — The teichoic acid was treated with aqueous ammonia (pH 11) for 4 h at room temperature. The solution was then concentrated to dryness. The product was redissolved in water and recovered by freeze-drying.

^{13}C-N.m.r. spectroscopy. — Proton-decoupled ^{13}C-n.m.r. spectra were recorded with a JEOL FX-100 spectrometer. Samples were dissolved in deuterium oxide, and chemical shifts are given in p.p.m. downfield from external tetramethylsilane. Studies of monophosphorylated glucosides (unpublished results) have shown that a carbon substituted with a phosphate group is shifted downfield ~1.5 p.p.m., whereas the vicinal carbons are shifted upfield ~0.5 p.p.m., on changing the pH from 11 to 4. This behaviour can therefore be used for assignments of signals. The values for the signals for galactitol obtained in the present work were consistently 1.3 p.p.m. higher than those given in Ref. 6. The latter values for 3-*O*-methylgalactitol were consequently increased by 1.3 p.p.m.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (B79-03X-02522-11C and B78-16X-04978-02), from Knut och Alice Wallenbergs Stiftelse, and from Stiftelsen Sigurd och Elsa Goljes Minne.

REFERENCES

1 P. Branefors-Helander, C. Erbing, L. Kenne, and B. Lindberg, *Carbohydr. Res.*, 56 (1977) 117–122.
2 R. M. Crisel, R. S. Baker, and D. E. Dorman, *J. Biol. Chem.*, 250 (1975) 4926–4930.
3 P. Branefors-Helander, C. Erbing, L. Kenne, and B. Lindberg, *Acta Chem. Scand., Ser. B*, 30 (1976) 276–277.
4 J. Baddiley, *Acc. Chem. Res.*, 3 (1970) 98–105.
5 S. Zamenhof and G. Leidy, *Fed. Proc.*, 13 (1954) 327.
6 P. A. J. Gorin and M. Mazurek, *Can. J. Chem.*, 53 (1975) 1212–1223.
7 E. Breitmaier, W. Voelter, E. B. Rathbone, and A. M. Stephen, *Tetrahedron*, 29 (1973) 3845–3848.
8 D. R. Bundle, I. C. P. Smith, and H. J. Jennings, *J. Biol. Chem.*, 249 (1974) 2275–2281.
9 H. J. Jennings and I. C. P. Smith, *Methods Enzymol.*, 50C (1978) 39–50.
10 L. Kenne, B. Lindberg, and J. K. Madden, *Carbohydr. Res.*, 73 (1979) 175–182.
11 K. Leontein, B. Lindberg, and J. Lönngren, *Carbohydr. Res.*, 62 (1978) 359–362.
12 P. S. Chen, Jr., T. Y. Toribara, and H. Warner, *Anal. Chem.*, 28 (1956) 1756–1758.
13 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
14 P. E. Jansson, L. Kenne, H. Liedgren, B. Lindberg, and J. Lönngren, *Chem. Commun., Univ. Stockholm*, 8 (1976) 1–75.
15 D. R. Bundle, H. J. Jennings, and I. C. P. Smith, *Can. J. Chem.*, 51 (1973) 3812–3819.
16 S. J. Perkins, L. N. Johnson, D. C. Phillips, and R. A. Dwek, *Carbohydr. Res.*, 59 (1977) 19–34.