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# THE STRUCTURE OF THE CAPSULAR POLYSACCHARIDE OBTAINED FROM A NEW SEROGROUP (L) OF *Neisseria meningitidis**

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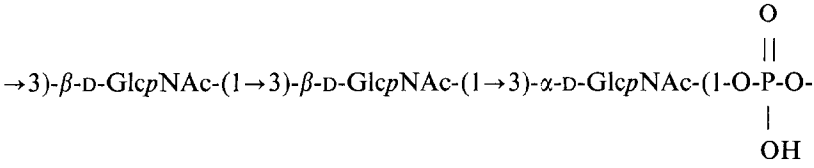
ABSTRACT

A newly isolated serogroup of *Neisseria meningitidis* (serogroup L), obtained from contacts of a patient with meningococcal meningitis, elaborates a structurally unique, capsular polysaccharide. The polysaccharide contains only 2-acetamido-2-deoxy-D-glucosyl and phosphate constituents in the molar ratio of 3 : 1, and is composed of the following repeating unit.




## INTRODUCTION

*Neisseria meningitidis* is a Gram-negative organism that has been classified serologically[1,2] into groups A, B, C, D, 29E, W135, X, Y, and Z. Except for group D, each group produces a unique, capsular polysaccharide that is the antigen responsible for group specificity[1,2]. The structures of all of these capsular polysaccharides have been determined[2]. Recently[3], a new meningococcal serogroup has been isolated from contacts of an infant with meningococcal meningitis. This organism has been classified in the genus *Neisseria meningitidis*, shown to be serologically distinct from the other known groups, and designated[3] *N. meningitidis* group L. We now report the isolation of the group L capsular polysaccharide responsible for the production of group-specific antisera to this organism, and the elucidation of its structure.

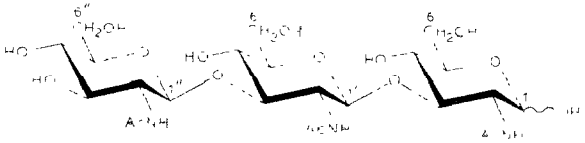
## RESULTS AND DISCUSSION

The group L capsular polysaccharide of *N. meningitidis* contains 2-acetamido-2-deoxy-D-glucosyl residues and phosphate groups in the molar ratio of 3:1. It has

*N.R.C.C. No. 20737.

$[\alpha]_D$ −10° (*c* 1.0, water), and the D configuration was assigned to the amino sugar constituent by using the method of Gerwig *et al.*[4].

Treatment of the group L polysaccharide with 48% HF yielded oligosaccharide **1**, which contained no phosphate and which, on hydrolysis, yielded only 2-acetamido-2-deoxy-D-glucose. In the methylation analysis of **1**, the individual methylated sugars detected were 3,4,6-tri-, 4,6-di-, and 1,4,5,6-tetra-*O*-methyl-(*N*-methylacetamido)-glucitol. This evidence indicated that the 2-acetamido-2-deoxyglucosyl residues of **1** are all linked by (1→3) linkages, and that its minimum size is, at least, a trisaccharide. Because of the non-quantitative response normally associated with methylated aminodeoxyalditol acetates[5], the quantification of these responses could not be used as definitive proof that **1** was a trisaccharide. However, the $^{13}$C-n.m.r. spectrum of **1** is consistent only with its being a 3-*O*-β-D-linked trisaccharide of 2-acetamido-2-deoxyglucopyranosyl residues. Some pertinent signals in the $^{13}$C-n.m.r. spectrum of **1**, and their assignments, are 102.2 (C-1′,1″), 96.0 (C-1, β-D anomer), 92.0 (C-1, α-D anomer), 82.3 (C-3, 3′ β-D anomer), 79.5 (C-3, α-D anomer), 61.7 (C-6,6′,6″), 56.8 (C-2′,2″), 55.7 (C-2, β-D anomer), 54.2 (C-2, α-D anomer), and 23.4 p.p.m. ($CH_3$ of acetamido).

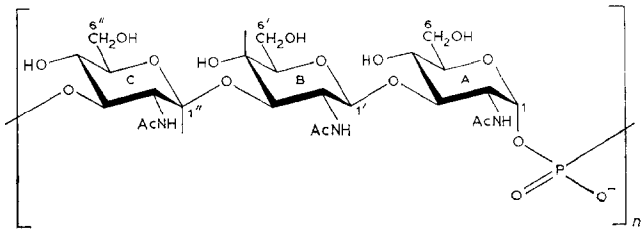


**1**

The same signals following the reduction of **1** with sodium borodeuteride were 102.4 (C-1′,1″), 82.5 (C-3,3′), 61.8, 62.0, 63.9 (C-6,6′,6″), and 54.7, 55.9, and 56.9 p.p.m. (C-2,2′,2″). The chemical shift of the linkage carbon atoms (C-3,3′) (82.3 p.p.m.) is consistent with a 3-linkage, as the alternative 4-linkage would have produced a chemical shift of 80.8 p.p.m., as in the $^{13}$C-n.m.r. spectrum of chitotriose. That both of the (1→3) linkages of **1** are in the β-D configuration may be deduced from the characteristic, chemical shift[6] (102.4 p.p.m.) assigned to both C-1′ and C-1″ in the $^{13}$C-n.m.r. spectrum of reduced **1**. Also, following the reduction of **1**, its C-2 and C-6 signals were each resolved into three signals of equal intensity, indicating that **1** is a trisaccharide.

The group L polysaccharide contains phosphate and 2-acetamido-2-deoxy-glucose in the molar ratio of 1:3, which is consistent with its structure's being composed of repeating units of **1** linked together by phosphoric diester bonds. Therefore, in order to complete the structural analysis of the group L polysaccharide, all that is required is to determine the configuration and linkage position of the phosphoric diester bonds. The following evidence is consistent only with the group L polysaccharide's having a structure composed of repeating units of **2**

Confirmation of the trisaccharide phosphate as the repeating unit, and of the



2

configuration of the phosphoric diester bond were obtained from the $^1$H-n.m.r. spectrum of the group L polysaccharide. The spectrum exhibited a doublet of doublets at $\delta$ 5.47 ($J_{1,2}$ 3.7, $^3J_{H1,P}$ 8.3 Hz), and a doublet at $\delta$ 4.69 ($J_{1,2}$ 9.0 Hz), in the intensity ratio of 1 : 2. Three methyl (acetamido) singlets were also detected, at $\delta$ 2.14, 2.08, and 2.05, in the intensity ratios of 1 : 1 : 1. The ratio of the former anomeric doublets to the acetamido singlets was 1 : 3, which is consistent with the group L polysaccharide's being composed of tri-(2-acetamido-2-deoxy-glucosyl) phosphate repeating-units. The higher-field, anomeric-proton signal, at $\delta$ 4.69, could be assigned to the overlapping signals of the 1′ and 1″ protons, and the characteristic, large value of the coupling constant (9.0 Hz) is consistent with the previously assigned $\beta$-D configuration of the residues equivalent to B and C in **1**. Therefore, the low-field signal at $\delta$ 5.47 could be assigned to C-1. The detection of a 3-bond, $^1$H–$^{31}$P coupling in this signal is consistent with residue A's having an anomeric phosphate linkage[7], and the value of $J_{1,2}$ (3.6 Hz) in this signal is consistent with the $\alpha$-D configuration of this linkage, as shown in **2**.

The position of linkage of the phosphoric diester bond to residue C of **2** was established by methylation analysis of the group L polysaccharide according to the method of Fiege *et al.*[8]. Following methylation of **2**, the phosphate groups were removed with hydrogen fluoride, to generate a partially methylated oligosaccharide,

TABLE I

CARBON-13 CHEMICAL SHIFTS OF THE GROUP L MENINGOCOCCAL POLYSACCHARIDE[a]

| *Constituent* | *$^{13}$C chemical shift (p.p.m.) of the constituent carbon atom* 1 | 2 | 3 | 4 | 5 | 6 | $CH_3$ (*NHCOCH$_3$*) | $C=O$ (*NHCOCH$_3$*) |
|---|---|---|---|---|---|---|---|---|
| C | 102.1[b] | 56.2[b] | 79.7(—)[c] | 70.6[b](—)[d] | 76.6[b] | 61.8 | 24.0[b] | 175.9[b] |
| B | 101.9[b] | 55.9[b] | 82.5 | 69.6[b] | 76.4[b] | 61.8 | 23.6[b] | 175.3[b] |
| A | 95.3 (5.7Hz)[e] | 54.1 (8.5Hz)[f] | 79.5 | 69.3[b] | 73.7 | 61.8 | 23.6[b] | 174.9[b] |

[a]In parts per million from external tetramethylsilane. [b]Due to the closeness of their chemical shifts, these assignments could be interchanged vertically. [c]$^2J$ $^{31}$P–$^{13}$C, not measurable; overlapping signals. [d]$^3J$ $^{31}$P–$^{13}$C, not resolved. [e]$^2J$ ($^{31}$P–$^{13}$C). [f]$^3J$ ($^{31}$P–$^{13}$C).

which was methylated with trideuteriomethyl iodide, in order to label the hydroxyl group originally involved in the phosphate linkage. In g.l.c.–m.s. studies on the hydrolyzed, methylated oligosaccharide obtained from **2**, only two methylated aminodeoxyalditol acetates were detected, namely, 3,4,6-tri- and 4,6-di-*O*-methyl-(*N*-methylacetamido)glucitol, the first of which was labeled with deuterium at O-3. This corresponded to the original linkage position (O-3) of the phosphoric diester in **2**. Although this method of locating phosphate groups has not yet been applied to sufficient numbers of differently linked phosphorylated polysaccharides to establish its general applicability, the method performed well in this particular case, as was demonstrated by the independent location of the phosphoric diester groups of **2** by $^{13}$C-n.m.r. spectroscopy.

The chemical shifts of the signals of the individual carbon atoms of **2** are listed in Table 1, together with the assignments for these signals. The $^{13}$C-n.m.r. spectrum of **2** revealed three anomeric carbon atoms, at 95.3, 101.9, and 102.1 p.p.m. On the basis of $^{13}$C-n.m.r. studies on **1**, the second two signals were assigned to C-1′ and C-1″, leaving the lower-field signal, at 95.3 p.p.m., assignable to C-1. The lower-field position of this signal in relation to those of C-1′ and C-1″ is supportive of the previous assignment of the α-D configuration to residue A by use of $^{1}$H-n.m.r.-spectral data, and the detection of $^{13}$C–$^{31}$P coupling in both the C-1 and C-2 signals is also consistent with the residue's having a phosphoric ester as the aglycon.

Linkage to O-3 of all three residues (A, B, and C) of **2** was verified by the characteristic pattern of chemical-shift displacements[9] on C-2, 3, and 4 of residues A, B, and C of **2**, relative to the previously assigned chemical shifts of the same carbon atoms of the appropriate 2-acetamido-2-deoxy-α- or -β-D-glucopyranose monomer[10]. The readily assignable, characteristic chemical-shifts of C-2, 2′, and 2″ of **2** were respectively displaced upfield by −1.2, −2.1, and −1.8 p.p.m., indicative of a linkage on vicinal C-3, 3′, and 3″. Compatible with these data, the signals of the latter linkage-carbon atoms were respectively displaced downfield by +7.5, +7.3, and +4.5 p.p.m. and those of the other carbon atoms (C-4, 4′, and 4″), vicinal to the linkages, were displaced upfield by −1.9, −1.8, and −0.8 p.p.m., respectively.

It was not possible to measure the $^{13}$C–$^{31}$P coupling-constants for the signals (C-2″, 3″, and 4″) associated with the phosphoric diester linkage to residue C. The signal of C-3″ overlapped that of C-3 and those of C-2″ and 4″ did not manifest identifiable doublets, or even any significant diminution in intensity, despite the location of C-2″ and 4″ adjacent to the phosphoric diester linkage on C-3″. Failure to detect three-bond, $^{13}$C–$^{31}$P coupling where it had been anticipated has been reported[11] for C-4 of the 3-phosphate-linked 2-acetamido-2-deoxy-α-D-galactopyranosyl residue of the meningococcal group Z polysaccharide, and it was attributed to the extremely small value of the coupling constant. Three-bond, $^{13}$C–$^{31}$P coupling is dihedral-angle-dependent[12], and the coupling constant becomes extremely small[11] as this angle approaches 90°.

## EXPERIMENTAL

*Growth of the organism.* — The *Neisseria meningitidis* strain L.C.D.C. No. 78189, originally isolated from a contact of an infant with meningococcal meningitis[3], is now in the culture collection of the National Reference Center for *Neisseria*. The strain, confirmed[3] as *N. meningitidis*, was grown in a chemically defined medium (NCDM), and the capsular polysaccharide was isolated and purified as previously described[13], using an initial Cetavlon precipitation to obtain the crude polysaccharide.

*Analytical methods.* — Amino sugars were detected and quantified with a Technicon auto analyzer. Acid hydrolyses of the polysaccharide were conducted with 4M HCl at 100°. Amino sugar determinations were made at intervals between 4 and 24 h, with prior incubation of the hydrolyzates with alkaline phosphate (Worthington Biochemical Corp., Freehold, N.J.). The total content of 2-amino-2-deoxyglucose was determined by extrapolation to zero time. Phosphorus was determined by the method of Chen *et al.*[14]. Chitotriose was a gift from Dr. N. M. Young of this laboratory. G.l.c. was performed with a Hewlett–Packard 5830A instrument equipped with a flame-ionization detector and a model 18850A electronic integrator. A glass column (180 × 0.15 cm) containing 3% of OV-17 on Gas Chrom Q was used at 200° to separate the partially methylated aminodeoxyalditol acetates. Combined g.l.c.–m.s. was conducted in a Hewlett–Packard 5985 instrument, using the same column and an ionization potential of 70 eV.

*Nuclear magnetic resonance spectroscopy.* — $^{13}$C-N.m.r. spectra were recorded at 37° with a Varian CFT20 spectrometer operating at 20 MHz in the pulsed, Fourier-transform mode with complete proton-decoupling. Chemical shifts are reported in parts per million (p.p.m.) downfield from external tetramethylsilane, and the $^{2}$H resonance of deuterium oxide was used as the field-frequency lock-signal. Polysaccharides and oligosaccharides were examined as solutions in deuterium oxide (25–50 mg/mL). $^{1}$H-N.m.r. spectra were recorded at 80° with a Varian 400-MHz spectrometer (Regional High Resolution N.m.r. Laboratory, University of Montreal, Quebec) in the pulsed, Fourier-transform mode. The polysaccharide (3 mg) was twice lyophilized from 99.7% $D_2O$, and examined in the same solvent. The apparent, first-order coupling-constants (Hz) were measured directly, and the chemical shifts ($\delta$) are expressed relative to external sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate.

*Treatment of the group L polysaccharide with hydrogen fluoride.* — The phosphorylated polysaccharide (75 mg) was treated at 4° with 48% hydrogen fluoride (2 mL), a reagent that has been shown to promote the facile cleavage of phosphoric esters[15]. After 2 d, the excess of hydrofluoric acid was removed under diminished pressure over solid sodium hydroxide in a desiccator, and the residue was dissolved in water and fractionated on a column (2.6 × 90 cm) of Sephadex G25, using a 0.02M pyridine acetate buffer (pH 5.4) as the eluant and a Waters Associates differential refractometer (model R403) to monitor the column eluate. One major oligosaccharide

fraction was identified that, on lyophilization, yielded oligosaccharide **1** (54 mg), which contained no phosphate.

*Methylation analysis.* Oligosaccharide **1** (prereduced with sodium borodeuteride), and the phosphorylated polysaccharide (de-ionized form), were methylated with methyl iodide in the presence of methylsulfinyl anion according to the method of Hakomori[16]. For isolation of the methylated oligosaccharide, the methyl iodide was first removed by evaporation, and then the dimethyl sulfoxide. The residue was partitioned between chloroform and water, and the chloroform phase was evaporated to dryness. The methylated oligosaccharide was purified by passage through a small column of LH-20 with 2:1 chloroform–acetone as the eluant. The methylated polysaccharide was recovered by dialysis against water, followed by lyophilization.

The location of phosphate groups in the polysaccharide was achieved essentially as described by Feige and Radziejewska-Lebrecht[8]. The methylated, phosphorylated polysaccharide was treated with 48% hydrogen fluoride to remove the phosphate groups[15,17], and the resultant, partially methylated oligosaccharide was reduced with sodium borodeuteride in 1:1 ethanol–1,4-dioxane. The borate was removed by adjusting the pH of the solution to 3 with 50% aqueous acetic acid, evaporating the solution to a residue, and adding and evaporating methanol 6 times from the residue. The reduced, methylated oligosaccharide was then remethylated, and the product isolated exactly as described for the reduced oligosaccharide, except for the use of trideuteriomethyl iodide.

The methylated oligosaccharides and polysaccharide were hydrolyzed, and the partially methylated *N*-methylhexosamines were reduced and acetylated as described by Stellner *et al.*[5]. The resultant, methylated *N*-methylhexaminitol acetates were then analyzed by g.l.c.–m.s.[18].

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