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Structure of the capsular antigen of *Neisseria meningitidis* serogroup K

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The capsular antigen isolated from the culture liquid of a *Neisseria meningitidis* serogroup K(1811) fermentation consists of the 2-acetamido-2-deoxymannuronic acid disaccharide repeating unit as follows:

→3)-β-D-Man*p*NAcA-(1→4)-β-D-Man*p*NAcA-(1→

The polysaccharide is *O*-acetylated at the non-glycosylated C-4. Structural evidence has been obtained from sugar analysis, methylation analysis, as well as ^{1}H and ^{13}C NMR spectroscopy.

Three new serogroups of *Neisseria meningitidis*, denominated H, I and K, have been described by Shao-Quing et al. in 1981 [1]. Recently, the isolation and structural elucidation of the capsular polysaccharide of *N. meningitidis* serogroup H has been described [2, 3]. We now report on the isolation and structure determination of the capsular antigen obtained from the serogroup K strain.

MATERIALS AND METHODS

Bacterial growth and isolation of the K polysaccharide

The *Neisseria meningitidis* strain K(1811) organism was grown in a modified Frantz' medium, at pH 7.0, with pO_2 20 mbar (10% air saturation) and at 35°C. After 16 h the polysaccharide was isolated by Cetavlon precipitation and purified [4].

General methods

GLC analyses were carried out on a Varian 3700 gas chromatograph, with flame-ionization detection, in combination with a Varian CDS-101 electronic integrator. Separations were accomplished on a fused silica WCOT column (25 m × 0.32 mm i.d.), coated with a polymethylsiloxane stationary phase (CP-Sil 5). Nitrogen was used as carrier gas at an average linear gas velocity of 15 cm/s. GLC-MS analyses were performed on a Carlo Erba GC/Kratos MS80/Kratos DS55 system, operating in the electron impact mode at a potential of 70 eV. For GLC separations the above column was used. Infrared spectroscopy was carried out with a Perkin Elmer 621 spectrophotometer; the polysaccharide (0.3%) was measured in KBr. The optical rotation of the polysaccharide was established with a Perkin Elmer 241 polarimeter, at ambient temperature, using a 10-cm micro-cell. The nucleic acid content of the preparation was measured according to [5], the protein content according to [6], the phosphorus content according to [7], the *O*-acetyl content according to [8], and the calcium content according to [9]. The presence of acetamido sugars was detected colorimetrically according to [10].

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Abbreviations. p, pyranose; ManNAc, 2-acetamido-2-deoxymannose; ManNAcA, 2-acetamido-2-deoxymannuronic acid; HexNAcA, 2-acetamido-2-deoxyhexuronic acid; GLC, gas-liquid chromatography; GLC-MS, gas-liquid chromatography/mass spectrometry.

Sugar analyses

Gas chromatographic sugar analyses were carried out as described previously [11, 12]. For the separation of the trimethylsilylated methyl glycosides on CP-Sil 5 an oven temperature program of 130 → 220°C at a rate of 2°C/min was used. Identifications were confirmed by GLC-MS, applying the same gas chromatographic conditions as for GLC. The absolute configuration of the sugars was determined by GLC of the corresponding trimethylsilylated (−)-2-butyl glycosides [13], using the GLC conditions mentioned above.

Carboxyl reduction of the polysaccharide

Carboxyl reduction of the K polysaccharide was achieved according to [14]. The polysaccharide (10 mg) was dissolved in water (10 ml) and mixed with 250 mg 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-*p*-toluene-sulfonate (Aldrich). During conjugation at room temperature (approximately 2 h) the solution was kept at pH 4.75 with 0.01 M HCl. Subsequently, 5.3 ml 2 M freshly prepared aqueous sodium borohydride was added in about 30 min, while the incubation mixture was kept at pH 7.0 with 1 M HCl. The solution was then acidified to pH 3, dialyzed and lyophilized. This procedure was repeated twice. The carboxyl-reduced polysaccharide was purified via filtration over Sephadex G-25 (120 × 1.8 cm) with water as the eluent at a flow of 30 ml/h. The 2-ml fractions were monitored for carbohydrate with 5% phenol/sulfuric acid at 492 nm. The carbohydrate material was recovered by lyophilization.

Methylation analyses

The carboxyl-reduced polysaccharide (10 mg), dissolved in dimethylsulfoxide (1 ml), was methylated wih methyl iodide (1 ml) in the presence of 2 M sodium methylsulfinylmethanide in dimethylsulfoxide (1 ml) [15, 16]. After dialysis and lyophilization, solvolysis of the methylated material was accomplished with 90% aqueous formic acid for 1 h at 100 °C. The reaction mixture was concentrated to dryness and the residue was treated with 4 M HCl for 2 h at 100 °C. Subsequently, the solvent was evaporated under diminished pressure and residual HCl was removed by repetitive co-distillation with methanol. The partially methylated sugars were analyzed as their [1-^{2}H]alditol acetates [16] by GLC-MS, using an oven temperature program of 130 → 220 °C, at a rate of 2 °C/min.

Solvolysis with anhydrous hydrogen fluoride

The native and carboxyl-reduced polysaccharide (2 mg) were each dissolved in 2 ml anhydrous hydrogen fluoride (dried over solid CoF_3) under magnetic stirring at −30 °C [17]. For the incubation an all-Kel F, closed distillation system was used (Protein Research Foundation, Minoh Osaka, Japan). After 2 h at room temperature the hydrogen fluoride was evaporated under diminished pressure. The residues were dissolved in 5 ml 5% aqueous acetic acid and kept at room temperature for 30 min. Afterwards the solutions were freeze-dried.

O-*Deacetylation*

The native polysaccharide (100 mg) was treated with 48% aqueous hydrogen fluoride (2 ml) for 48 h at 4 °C. The mixture was then frozen and evaporated in a vacuum desiccator over solid sodium hydroxide. The residue was dissolved in water (2 ml) and the remaining hydrogen fluoride was neutralized by addition of saturated, aqueous lithium hydroxide. After filtration the solution was extensively dialyzed and the polysaccharide material recovered by lyophilization. For NMR spectroscopy the material was converted into its calcium salt as follows. The polysaccharide was treated three times with an excess of 1 M ammonia, with intermediate evaporation of the solvent. The obtained ammonium salt was treated with 1 M calcium chloride (200 ml), followed by dialysis against water and subsequent lyophilization. Finally, the preparation was purified by filtration over Sephadex G-25 (120 × 1.8 cm) with water as the eluent at a flow rate of 30 ml/h and recovered by freeze-drying.

Nuclear magnetic resonance spectroscopy

500-MHz ^{1}H-NMR spectra were recorded on a Bruker WM-500 spectrometer (SON hf-NMR Facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands). Prior to analysis the polysaccharide materials were repeatedly treated with ^2H_2O at room temperature, with intermediate lyophilization. Finally, the polysaccharides were dissolved in ^2H_2O (99.96 mol% ^{2}H). The spectra were recorded at a probe temperature of 70 °C. Resolution enhancement was achieved by Lorentzian-to-Gaussian transformation [18]. Chemical shifts (δ) were expressed in ppm downfield from internal sodium, 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ = 2.215 ppm at 70 °C).

Proton-coupled and decoupled ^{13}C-NMR spectra were recorded on a Bruker WP-200 (50.76 MHz) spectrometer, in ^2H_2O at a probe temperature of 27 °C. Chemical shifts (δ) are given in ppm downfield from external tetramethylsilane, but were actually measured by reference to internal acetone (δ = 31.4 ppm).

RESULTS AND DISCUSSION

General characteristics of the polysaccharide

The capsular polysaccharide was obtained from the culture liquid of a *Neisseria meningitidis* serogroup K(1811) fermentation in a yield of 29 mg/l. It turned out to be substantially free from protein contamination, but a small amount of nucleic acid (0.2%) was still present; no phosphate groups could be detected. The polysaccharide had a calcium content of 1.67 mmol/g and an *O*-acetyl content of 1.6 mmol/g was found. The colorimetric assay on acetamido sugars was positive. Infrared spectroscopy indicated the occurrence of carboxylate groups (absorption band at 1620 cm^{-1}), *N*-acetyl functions (absorption bands at 1550 cm^{-1} and 1650 cm^{-1}) and *O*-acetyl functions (absorption band at 1720 cm^{-1}). The polysaccharide had $[\alpha]_D^{20}$ of −46° (*c* 1.2, water).

Sugar composition of the polysaccharide

Methanolysis of the native polysaccharide (1 M methanolic HCl, 24 h, 85 °C), followed by *N*-reacetylation and trimethylsilylation of the solvolyzed material gave rise to a GLC-MS pattern of methyl glycosides of HexNAcA forms. This pattern is identical to that obtained by subjection to methanolysis of 2-acetamido-2-deoxy-mannofuranurono-6,3-lactone [19]. The sugar analysis of the carboxyl-reduced polysaccharide showed the presence of only 2-acetamido-2-deoxy-mannose. Since polysaccharides which contain uronic acid or 2-acetamido-2-deoxyhexose residues are fairly resistent towards solvolysis under moderate acidic conditions, the methanolysis procedure was also carried out on the native and carboxyl-reduced polysaccharides being first solvolyzed by treatment with anhydrous hydrogen fluoride. The results obtained in this way were identical to those in the direct methanolysis experiments. The absolute configuration of the ManNAcA units was established on the basis of the carboxyl-reduced polysaccharide, solvolyzed by treatment with anhydrous hydrogen fluoride. Subjection of this material to butanolysis, followed by GLC-MS of the trimethylsilylated, *N*-acetylated (−)-2-butyl glycosides [13] exclusively revealed D-ManNAc forms. Hence it was concluded that the K polysaccharide consisted exclusively of D-ManNAcA residues.

Structure of the repeating unit

GLC-MS analysis of the partially methylated [1-^{2}H]-alditol acetates obtained from the permethylated carboxyl-reduced polysaccharide shows 1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-2-deoxy-2-*N*-methylacetamido[1-^{2}H]mannitol (compound 1) and 1,3,5-tri-*O*-acetyl-4,6-di-*O*-methyl-2-deoxy-2-*N*-methylacetamido-[1-^{2}H]mannitol (compound 2), in the molar ratio of 1.3:1. The GLC retention data of compounds 1 and 2 on CP-Sil 5 are 1.09 and 1.12, respectively (per-*O*-acetylated glucitol = 1.00). The 70 eV electron impact mass spectra of compounds 1 and 2 are shown in Fig. 1. The spectrum of compound 1 is consistent with that published for the unlabeled compound [20]. The spectra of compounds 1 and 2

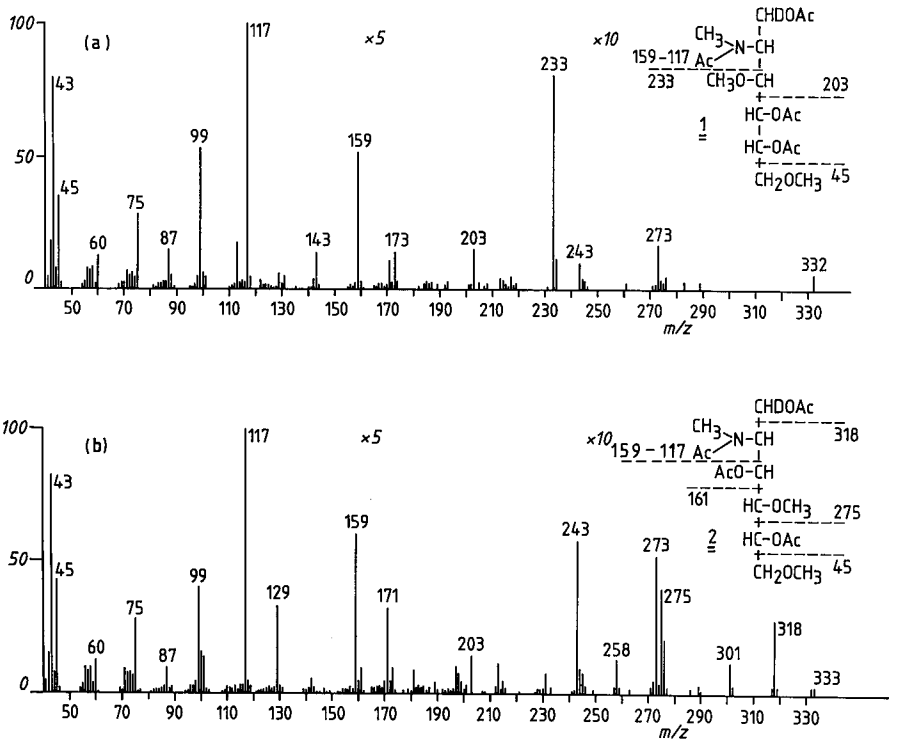

Fig. 1. *Mass spectra (70 eV) and fragmentation schemes of (a) 1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-2-deoxy-2-*N*-methylacetamido-[1-^{2}H]mannitol (compound 1) and (b) of 1,3,5-tri-*O*-acetyl-4,6-di-*O*-methyl-2-deoxy-2-*N*-methylacetamido-[1-^{2}H]mannitol (compound 2)*

differ only slightly from those of the corresponding *N*-acetylglucosaminitol derivatives [21, 22]. The detection of compounds 1 and 2 point to the occurrence of two differently glycosylated ManNAcA residues: →4)-D-ManNAcA(1→ and →3)-D-ManNAcA-(1→, respectively. This may suggest that the polysaccharide is built up from disaccharide repeating units resulting in a linear polymer of alternating 3-glycosylated and 4-glycosylated D-ManNAcA units.

NMR spectroscopy provides the ultimate proof for the structure of the repeating unit, as well as the configuration of the anomeric centers and the ring form of the sugars. Since the native polysaccharide contains *O*-acetyl groups, which could complicate the NMR spectra, an *O*-deacetylation was carried out by subjecting the native polysaccharide to 48% aqueous hydrogen fluoride. Simultaneously, a number of glycosidic linkages were cleaved, reducing the chain length and thus the viscosity of the aqueous polysaccharide solution. Attempts to remove quantitatively the *O*-acetyl functions under alkaline reaction conditions, using ammonia [2], were unsuccessful. The acidic conditions however yielded an essentially *O*-deacetylated polymer, as was concluded from a comparison of the ^{1}H and ^{13}C NMR spectra of the native polysaccharide and of that treated with 48% hydrogen fluoride. In the 500-MHz ^{1}H-NMR spectrum a complete collapse of the resonance of the *O*-acetyl protons at δ = 2.11 ppm was observed upon subjection of the polysaccharide to aqueous hydrogen fluoride. Also in the ^{13}C-NMR spectrum the *O*-acetyl ^{13}C resonances (δ = 21.7 ppm for $-CH_3$ and δ = 173.9 ppm for $-C=O$) virtually disappeared after treatment of the polysaccharide; see also Figs 2 and 3.

The ^{13}C-NMR spectrum of the *O*-deacetylated polysaccharide (Fig. 3) could be assigned completely on the basis of data of related polysaccharides (Table 1) [23, 24]. In the anomeric region the equally intense resonances at δ = 97.0 and 100.0 ppm indicate that the polysaccharide is composed of dimeric repeating units. This is confirmed by the two resonance peaks for the C-2 atoms (δ = 50.9 and δ = 53.7 ppm) and by the fact that six resonances are found for the C-3, C-4 and C-5 atoms. The typical acetamido methyl ^{13}C resonance at δ = 23.1 ppm and the carbonyl ^{13}C resonances at δ = 176.3 and δ = 176.9 ppm indicate that the sugar residues are *N*-acetylated. This justifies the *N*-reacetylation step following the methanolysis process during sugar analysis. The resonance positions of the C-2 atoms (δ = 50.9 and δ = 53.7 ppm) locate these acetamido groups at C-2 and C-2′. The chemical shift values for the anomeric carbons at δ = 97.0 and δ = 100.0 ppm show that the ManNAcA units possess the pyranose ring forms, since the signals for furanoid structures are generally found at higher chemical shift values [25]. The anomeric configuration of the ManNAcA residues is primarily deduced from the values of the C-1 $^1J(^{13}C\text{-}^1H)$ coupling constants (163.0 Hz and 164.9 Hz, respectively) and from the chemical shift values of the C-5 and C-5′ resonances (δ = 77.4 and δ = 76.5 ppm, respectively). Literature data for mannose-related monosaccharides reveal that values for $^1J(^{13}C\text{-}^1H)$ less than 165 Hz are associated with β-configuration [23, 24, 26–29], with concomitant chemical shift values for the C-5 resonance being $\delta \approx 77$ ppm [23, 24, 28, 30]. The corresponding values for the α-configuration are typically 170 Hz and $\delta \approx 74$ ppm. Therefore the data found for the ManNAcA units in the *O*-deacetylated polysaccharide indicate β-anomeric configuration for both types of sugar residue.

In view of this result the chemical shift values for the H-1 resonances at δ = 4.75 ppm and δ = 4.92 ppm in the ^{1}H-NMR spectrum have to correspond to β-anomeric configuration (see also below). The ^{13}C-NMR spectrum of the *O*-deacetylated polysaccharide further confirms that the D-ManNAcA units are glycosylated at C-3 and C-4′.

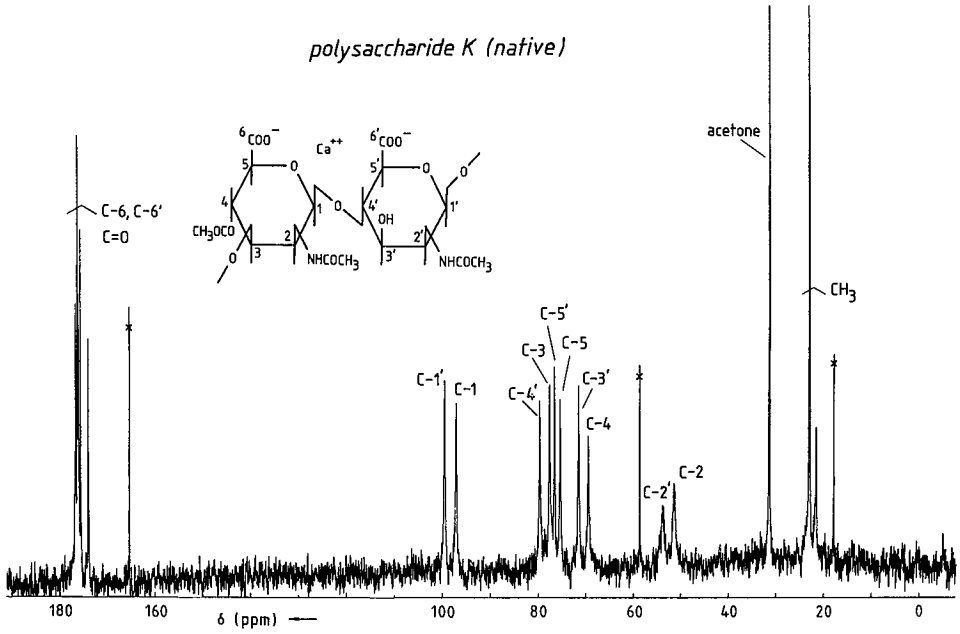

Fig. 2. ^{13}C-*NMR Spectrum of the native K polysaccharide (calcium salt form, pH 6) recorded in* ^2H_2O, *at a probe temperature of 27°C and 50.76 MHz.* Chemical shifts are given relative to external tetramethylsilane, using internal acetone (δ = 31.4 ppm)

Glycosylation generally causes $\approx 5-10$ ppm downfield shift of the signal of the contiguous carbon atom, and $\approx 0-3$ ppm upfield shift of those of adjacent carbon atoms [31]. Glycosylation at C-3 can be seen from the chemical shift alteration of $\Delta\delta$ = 5.7 ppm for the C-3 resonance and the upfield chemical shift displacements for the C-2 resonance ($\Delta\delta$ = −4.0 ppm) and the C-4 resonance ($\Delta\delta$ = −1.0 ppm), as compared with the corresponding resonances in β-D-ManNAcA [24]. In a similar way glycosylation at C-4′ effects a downfield shift of its resonance ($\Delta\delta$ = 9.9 ppm) and an upfield shift of the C-3′ resonance ($\Delta\delta$ = −1.3 ppm). The resonance position of C-5′ is less affected (δ = −0.3 ppm).

In conclusion, compatible arguments have been obtained to propose the structure of the repeating unit of the *O*-deacetylated group K polysaccharide as:

$$\rightarrow 3)\text{-}\beta\text{-D-Man}p\text{NAcA-}(1\rightarrow 4)\text{-}\beta\text{-D-Man}p\text{NAcA-}(1\rightarrow .$$
$$\quad\quad 3$$

This is the first demonstration of HexNAcA chain constituents in capsular antigens of *N. meningitidis* serological species. The C-4 glycosylated β-D-ManNAcA residue has been reported as incorporated in capsular polysaccharide from *Streptococcus pneumoniae* type 12F [32] and type 12A [26], *Haemophilus influenzae* type e [24, 30] and in enterobacterial common-antigen [20, 33, 34]. The →3)-β-D-ManNAcA(1→ structural unit has been described for the *Escherichia coli* K7 [35] and *H. influenzae* type d [23, 36] capsular polysaccharides.

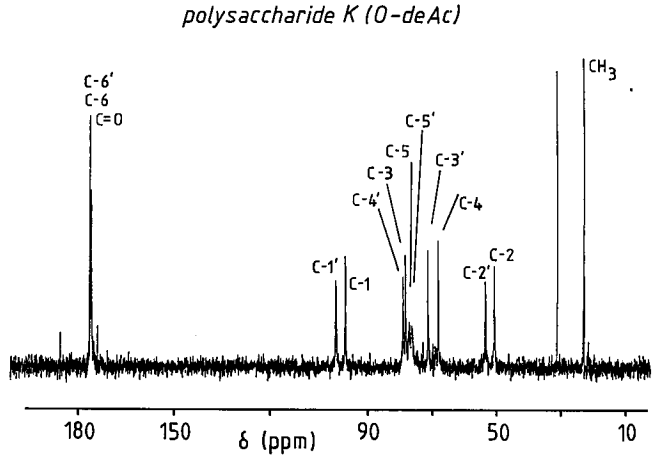

Fig. 3. ^{13}C-*NMR spectrum of the* O-*deacetylated K polysaccharide (calcium salt form, pH 6) recorded in* ^2H_2O, *at a probe temperature of 27°C and 50.76 MHz.* Chemical shifts are given relative to external tetramethylsilane, using internal acetone (δ = 31.4 ppm)

O-*Acetyl substitution of the native polysaccharide*

It is obvious from structure 3 that *O*-acetyl substituents can only be located at C-3′ and/or C-4 of the repeating unit. *O*-Acetylation of C-3′ can be excluded upon comparison of the ^{13}C-NMR spectra of the native (Fig. 2) and the *O*-deacetylated polysaccharides (Fig. 3), because no β effect of *O*-acetylation on the resonance position of C-2′ was observed. On the other hand, all effects expected for substitution at C-4 are present. An α effect of *O*-acetylation of $\Delta\delta$ = 1.1 ppm is observed for C-4, with concomitant β effects on the resonances of C-3 and C-5 ($\Delta\delta$ = −0.9 and $\Delta\delta$ = −2.1 ppm, respectively). It should be mentioned that the chemical shift displacement of $\Delta\delta$ = 1 ppm is somewhat smaller than usually found for α and β effects of *O*-acetylation, which are in the order of magnitude of $\Delta\delta$ = 2−3 ppm [37, 38]. It is established from the number of the resonance peaks and the relative intensities of the signals that the *O*-acetylation of C-4 is virtually quantitative. Also ^1H-NMR spectroscopy clarifies the *O*-acetylation pattern. The 500-MHz ^1H-NMR spectrum of the native polysaccharide shows a triplet resonance peak at δ = 4.94 ppm (J = 10.2 Hz) assigned to H-4, which disappears upon *O*-deacetylation. The intensity of the signal is

Table 1. *Pertinent ^{13}C chemical shifts and ^{13}C-^{1}H coupling constants (in parenthesis) in the ^{13}C-NMR spectra of the* Neisseria meningitidis *serogroup K polysaccharide (native, O-deacetylated and carboxyl-reduced material; analyzed in the calcium salt form, p^2H 6) and some reference compounds*
Chemical shift values are given at 27 °C, relative to external tetramethylsilane in $^{2}H_2O$, but were actually measured relative to internal acetone (δ = 31.4 ppm)

Compounds	Chemical shifts (couplings)																	
	C-1	C-2	C-3	C-4	C-5	C-6	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′	*N*-Acetyl				*O*-Acetyl	
													$-CH_3$		C=O		$-CH_3$	C=O
K polysaccharide (native)	97.1	51.5	77.6[a]	69.4	75.3	175.9[b]	99.5	53.8	71.4	79.7	76.5[a]	175.5[b]	23.0	23.1	176.1	176.5	21.7	173.9
K polysaccharide (*O*-deAc)[g]	97.0 (163.0)[f]	50.9	78.5[c]	68.3	77.4[d]	175.9[e]	100.0 (164.9)[f]	53.7	71.5	79.2[c]	76.5[d]	175.7[e]	23.1	23.1	176.3	176.9		
β-D-Man*p*NAcA[h]	94.3	54.9	72.8	69.3	76.2	–	94.3	54.9	72.8	69.3	76.2	–	23.2		176.8			
→3)-β-D-Man*p*NAcA-(1→[i]	99.3 (159)[f]	51.3	80.8	69.0	78.4	176.5							23.8		175.9			
→4)-β-D-Man*p*NAcA-(1→[h]							101.4 (164)[f]	54.4	72.2	79.5	78.8	176.0	23.8		176.5			
→3)-β-D-Man*p*NAc-(1→ 4)-β-D-Man*p*NAc-(1→[j]	97.7 (162)[f]	51.4	79.1	66.9	78.0[k]	62.1[l]	100.7 (162)[f]	54.2	72.3	77.9[k]	76.6	61.9[l]	23.6	23.6	176.2	176.5		
K polysaccharide (reduced)[m]	97.2	51.0	78.5	66.5	77.4	61.3[n]	100.8	53.7	71.9	n.r.[o]	76.1	61.2[n]	23.0	23.1	176.1	176.4	21.4	175.1

[a–e, k, l, n] Assignments may be reversed.
[f] $^{1}J(^{13}C$-$^{1}H)$ in Hz.
[g] *O*-Deacetylated by treatment with 48% aqueous hydrogen fluoride.
[h] Taken from [24]. These spectra were recorded at 85 °C and p^2H 8.
[i] Taken from [23]. These spectra were recorded at 85 °C and p^2H 8.
[j] Taken from reference [28]. This spectrum was recorded at 70 °C; as has been demonstrated for Man*p*NAc resonances are positioned ≈0.5 ppm more downfield as compared with resonances determined at ambient temperature [28, 40].
[m] Small additional signals are observed at δ = 100.3 ppm, 75.0 ppm, near 72 ppm and at δ = 66.4 ppm and 65.1 ppm.
[o] Signals not resolved.

equal to those of the β anomeric protons at $\delta = 4.81$ ppm and 4.74 ppm [24, 35, 39]. Furthermore the spectrum shows three equally intense signals in the resonance region of the *N*-acetyl and *O*-acetyl methyl protons at $\delta = 1.98$ ppm, 2.06 ppm and 2.11 ppm. The intensity of these signals is approximately three times higher than that of the H-4 triplet and the both anomeric resonances. The resonance peak at $\delta = 2.11$ ppm could be assigned to the *O*-acetyl methyl protons (see above). The resonances at $\delta = 1.98$ ppm and 2.06 ppm are accordingly assigned to acetamido methyl protons. In agreement with this, resonance values for such protons have been reported between $\delta = 1.99$ ppm and 2.08 ppm [35, 39]. These ^{1}H-NMR data confirm the complete *O*-acetylation of the non-glycosylated C-4 atoms. In addition, the resonances of the acetamido methyl protons indicate the *N*-acetylation of the native polysaccharide, thus supporting the evidence obtained from ^{13}C-NMR spectroscopy. A phenomenon not clearly understood is observed in the overlapping of the two acetamido methyl signals at $\delta = 2.06$ ppm, after *O*-deacetylation of the native polysaccharide.

The ^{13}C-NMR data of the carboxyl-reduced polysaccharide are also compiled in Table 1. According to the results of the sugar analysis, the polysaccharide has been reduced completely, but the ^{13}C-NMR spectrum shows that the material remains at least partially *O*-acetylated, as can be seen from the typical resonances at $\delta = 21.4$ ppm ($-CH_3$) and 175.1 ppm ($-C=O$). The spectrum appears to be consistent with a →3)-β-D-Man*p*NAc-(1→4)-β-D-Man*p*NAc-(1→ repeating unit [28], but it complexity means that the pattern does not indicate the location of *O*-acetyl substituents.

In summary, the NMR-spectroscopic data show that the *Neisseria meningitidis* serogroup K polysaccharide is quantitatively *O*-acetylated at C-4 and thus is composed of the following repeating unit:

→3)-β-D-Man*p*NAcA-(1→4)-β-D-Man*p*NAcA-(1→.
|
4-OAc

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REFERENCES

1. Shao-Quing, D., Ren-Bang, Y. & Huan-Chun, Z. (1981) *J. Biol. Stand. 9,* 307–315.
2. Van der Kaaden, A., Van Doorn-Van Wakeren, J. I. M., Kamerling, J. P., Vliegenthart, J. F. G. & Tiesjema, R. H. (1984) *Eur. J. Biochem. 141,* 513–519.
3. Michon, F., Roy, R., Jennings, H. J. & Ashton, F. E. (1984) *Can. J. Chem. 62,* 1519–1524.
4. Bundle, D. R., Jennings, H. J. & Kenny, C. P. (1974) *J. Biol. Chem. 249,* 4797–4801.
5. Wetmur, J. G. & Davidson, N. (1968) *J. Mol. Biol. 31,* 349–370.
6. Lowry, O. H., Rosebrough, N. J., Farr, A. J. & Rondall, R. J. (1951) *J. Biol. Chem. 193,* 265.
7. Chen, P. S., Toriba, J. U. & Warner, H. (1965) *Anal. Chem. 28,* 1756.
8. Hestrin, S. (1949) *J. Biol. Chem. 180,* 249–261.
9. Calcium Kit, Biomérieux, Charbonnières-les Bains, France.
10. Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955) *J. Biol. Chem. 217,* 959–966.
11. Kamerling, J. P., Gerwig, G. J., Vliegenthart, J. F. G. & Clamp, J. R. (1975) *Biochem. J. 151,* 491–495.
12. Kamerling, J. P. & Vliegenthart, J. F. G. (1982) *Cell Biol. Monogr. 10,* 95–125.
13. Gerwig, G. J., Kamerling, J. P. & Vliegenthart, J. F. G. (1979) *Carbohydr. Res. 77,* 1–7.
14. Taylor, R. L., Shively, J. E. & Conrad, H. E. (1976) *Methods Carbohydr. Chem. 7,* 149–151.
15. Hakomori, S. (1964) *J. Biochem. (Tokyo) 55,* 205.
16. Jansson, P. E., Kenne, L., Liedgren, H., Lindberg, B. & Lönngren, J. (1976) *Chem. Commun. Univ. Stockholm 8,* 1–75.
17. Jansson, P. E., Lindberg, B., Lönngren, J., Ortega, C. & Svenson, S. B. (1984) *Carbohydr. Res. 131,* 277–283.
18. Ernst, R. R. (1966) *Adv. Magn. Res. 2,* 1–135.
19. Darakas, E., Hultberg, H., Leontein, K. & Lönngren, J. (1982) *Carbohydr. Res. 103,* 176–180.
20. Männel, D. & Mayer, H. (1978) *Eur. J. Biochem. 86,* 361–370.
21. Schwarzmann, G., Reinhold, V. & Jeanloz, R. W. (1974) *Colloq. Int. Cent. Natl Rech. Sci. 221,* 85–94.
22. Schwarzmann, G. O. H. & Jeanloz, R. W. (1974) *Carbohydr. Res. 34,* 161–168.
23. Branefors-Helander, P., Kenne, L., Lindberg, B., Petersson, K. & Unger, P. (1981) *Carbohydr. Res. 97,* 285–291.
24. Branefors-Helander, P., Kenne, L., Lindberg, B., Petersson, K. & Unger, P. (1981) *Carbohydr. Res. 88,* 77–84.
25. Bock, K. & Thøgersen, H. (1982) *Annu. Rep. NMR Spectroscopy 13,* 1–57.
26. Leontein, K., Lindberg, B., Lönngren, J. & Carlo, D. J. (1983) *Carbohydr. Res. 114,* 257–266.
27. Bock, K. & Pedersen, C. (1974) *J. Chem. Soc. Perkin Trans. 2,* 293–297.
28. Cherniak, R., Dayalu, K. I. & Jones, R. G. (1983) *Carbohydr. Res. 119,* 171–190.
29. Walker, T. E., London, R. E., Barker, R. & Matwiyoff, N. A. (1978) *Carbohydr. Res. 60,* 9–18.
30. Tsui, F.-P., Schneerson, R. & Egan, W. (1981) *Carbohydr. Res. 88,* 85–92.
31. Jennings, H. J. & Smith, I. C. P. (1978) *Methods Enzymol. 50,* 39–50.
32. Leontein, K., Lindberg, B. & Lönngren, J. (1981) *Can. J. Chem. 59,* 2081–2085.
33. Lugowski, C., Romanowska, E., Kenne, L. & Lindberg, B. (1983) *Carbohydr. Res. 118,* 173–181.
34. Dell, A., Oates, J., Lugowski, C., Romanowska, E., Kenne, L. & Lindberg, B. (1984) *Carbohydr. Res. 133,* 95–104.
35. Tsui, F.-P., Boykins, R. A. & Egan, W. (1982) *Carbohydr. Res. 102,* 263–271.
36. Tsui, F.-P., Schneerson, R., Boykins, R. A., Karpas, A. B. & Egan, W. (1981) *Carbohydr. Res. 97,* 293–306.
37. Bundle, D. R., Smith, I. C. P. & Jennings, H. J. (1974) *J. Biol. Chem. 249,* 2275–2281.
38. Bhattacharjee, A. K., Jennings, H. J., Kenny, C. P., Martin, A. & Smith, I. C. P. (1975) *J. Biol. Chem. 250,* 1926–1932.
39. Goustin, A. S., Krick, T. P. & Anderson, J. S. (1983) *Carbohydr. Res. 119,* 258–262.
40. Bundle, D. R., Jennings, H. J. & Smith, I. C. P. (1973) *Can. J. Chem. 51,* 3812–3819.