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Full ^{1}H NMR assignment and detailed *O*-acetylation patterns of capsular polysaccharides from *Neisseria meningitidis* used in vaccine production

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Abstract

We report essentially complete ^{1}H NMR assignments for the capsular polysaccharides from *Neisseria meningitidis* serotypes A, C, W-135, and Y. These polysaccharides are components of current polysaccharide vaccines against meningococcal infection and of the polysaccharide-protein conjugate vaccines under development. From these NMR data the pattern of *O*-acetylation was determined. *O*-Acetylation of the W-135 polysaccharide is reported for the first time. We also show that, for the Types C and W-135 polysaccharides, a migration of *O*-acetyl groups occurs during storage in solution, and demonstrate that high field ^{1}H NMR represents a simple and sensitive method to define the *O*-acetylation pattern of individual batches of these polysaccharides. © 1996 Elsevier Science Ltd.

Keywords: Meningococcal polysaccharides; *O*-Acetylation; NMR spectroscopy

1. Introduction

Purified capsular polysaccharides from *Neisseria meningitidis* serotypes A, C, W-135, and Y (the meningococcal polysaccharides Types A, C, W-135, and Y) used in various combinations have proven effective as vaccines against meningococcal infection [1]. Polysaccharide vaccines have a number of limitations, however, including lack of

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immunogenicity in infants, limited duration of protection and poor tolerance of revaccination. The polysaccharide from the Type B serotype, the clinically most important strain in developed countries [2], has very poor immunogenicity [1] and is ineffective in polysaccharide vaccines. A new generation of meningococcal polysaccharide-protein conjugate vaccines are under development [3–5] following the successful introduction of *Haemophilus influenzae* Type B (Hib) polysaccharide-protein conjugate vaccines [6]. These Hib conjugate vaccines have been shown to be very effective at raising a protective T-cell dependent immune response in infants [7–9] and are now used in mass vaccination campaigns in many countries.

The basic structures of the repeating units of the meningococcal polysaccharides (Table 1) were determined by a combination of chemical methods [10,11] and an early demonstration of the power of NMR spectroscopy applied to polysaccharides [12–14]. They have simple anionic mono- or di-saccharide repeat units with *O*-acetylation in many cases, and their anionic character depends on the presence of either a phosphodiester link (in the Type A polysaccharide) or neuraminic acid (in the Types B, C, W-135, and Y). The detailed pattern of *O*-acetylation in some of these polysaccharides remains undefined, and there is little data on the relationship between the degree and location of the substituents and the extent and specificity of the antibody response [15], or the influence of *O*-acetylation on immunogenicity [16]. In the capsular polysaccharides from *E. coli*, *O*-acetylated epitopes have been reported to be immunodominant [17]. Proton NMR spectroscopy has proved an excellent method to determine the acetylation pattern of polysaccharides [18], and, once the methodology is established, provides a simple, non-destructive and routine method to quantify the substitution pattern. In this paper the full assignment of the proton NMR spectra of the A, C, W-135, and Y polysaccharides is reported, and the patterns of *O*-acetylation defined. Spectroscopic conditions, particularly the sample temperature, were chosen to be consistent with ongoing and future work to characterise the intact conjugate vaccines.

2. Experimental methods

The meningococcal polysaccharides studied were samples of material intended for vaccine production from a number of manufacturers. De-*O*-acetylation of the Type A polysaccharide was achieved in the NMR tube by dissolving 2.2 mg of material in 0.7 mL of a 0.01 M solution of NaOD in D_2O. The progress of the reaction was monitored by recording ^1H spectra at five minute intervals. De-*O*-acetylation of the Type C and Type W-135 polysaccharides was achieved by dissolving 15 mg of material in sodium hydroxide solution (10 mL, 1 M) and incubating for four hours at 37 °C [12]. Samples were neutralised with 0.1 M hydrochloric acid, dialysed against 5 mM phosphate buffer (pH 6.9) for two days at room temperature, and lyophilised. Samples (typically 2–3 mg) were dissolved in 0.7 mL of deuterated water (99.9% deuterium, Apollo Scientific Ltd, Stockport, UK) for analysis. NMR spectra were obtained on a Varian Unity 500 NMR spectrometer equipped either with a 5 mm proton-detect triple resonance, a 5 mm inverse (ie. proton detect) or a 5 mm broadband probe. Standard pulse sequences were used throughout, and phase-sensitive spectra were collected using the method of States

et al. [19]. All spectra were collected at an indicated probe temperature of 30 °C unless otherwise indicated. VNMR software versions 3.2 and 4.3 were used. Proton chemical shifts are referenced to internal TSP-d_4 at 0 ppm, ^{31}P chemical shifts to external 85% H_3PO_4 at 0 ppm, in a sealed capillary, and ^{13}C chemical shifts to internal TSP-d_4 at −1.8 ppm.

For the Types C, W-135, and Y, and for the de-*O*-acetylated Types C and W-135, the following spectra were recorded: a series of TOCSY [20] spectra with mixing times of 20, 40, and 60 or 80 ms, a double quantum filtered COSY (DQFCOSY) [21] spectrum, NOESY [22] spectra (mixing time of 150 ms or 200 ms), $^{13}C-^{1}H$ correlations [23] (HMQC spectra, and an HMBC spectrum for the de-*O*-acetylated W-135). For the Type A polysaccharide, a TOCSY spectrum with a mixing time of 60 ms, a $^{13}C-^{1}H$ correlation (HMQC) and a $^{31}P-^{1}H$ correlation (HSQC) were recorded. The spectral widths were 5000 Hz in both f1 and f2 dimensions in homonuclear spectra, and for heteronuclear experiments, the spectral widths were 5000 Hz in f2 and 20,000 Hz in f1 (^{13}C). Typically, 2048 or 4096 data points were collected in the t2 domain and 660 in the t1 domain. The latter was extended to 1024 points by zero filling. Proton-detected heteronuclear spectra were collected without heteronucleus decoupling. ^{31}P spectra employed broadband proton noise decoupling and ^{13}C spectra used inverse-gated WALTZ-16 decoupling.

3. Results and discussion

The reported structures of the repeating units of the four polysaccharides included in this study and for comparison the structure of the repeat unit of the meningococcal Type B polysaccharide are shown in Table 1. Full assignments of the proton and ^{13}C NMR spectra of the Type B have been reported [24,25]. The primary aim of this work was to assign the ^{1}H NMR spectra of the acetylated polysaccharides and to define the detailed *O*-acetylation pattern. Substitution of a sugar hydroxyl with *O*-acetyl causes a small downfield shift of the C_α resonance, typically less than 2 ppm [26], which may fail to be diagnostic. The effects of *O*-acetylation on the proton NMR spectrum are more dramatic, with a downfield shift on a H_α of a secondary hydroxyl of 1–1.5 ppm, and downfield shifts on the two H_αs at a primary hydroxyl group of ca. 0.5 ppm [18]. Effects on the chemical shifts of the H_βs are usually much less and small long-range, conformation-dependent shifts may be observed. Thus ^{1}H NMR provides an excellent

Table 1
Reported structures of the repeating units of the four meningococcal polysaccharides investigated in this study, and that of the closely related Type B polysaccharide

Type A:	→6)-Man*p*NAc-(α1→OPO_3→	ca. 70% *O*-acetylation at O-3	Ref. [14]
Type C:	→9)-Neu*p*NAc-(α2→	*O*-acetylation at O-7 or O-8	Ref. [12]
Type W-135:	→6)-Gal*p*-(α1→4)-Neu*p*NAc-(α2→	*O*-acetylation not reported	Ref. [13]
Type Y:	→6)-Glc*p*-(α1→4)-Neu*p*NAc-(α2→	*O*-acetylation at O-7, O′-3 or O′-4	Ref. [13]
Type B:	→8)-Neu*p*NAc-(α2→	not *O*-acetylated	Ref. [12]

method to locate *O*-acetyl groups, but application of this technique requires extensive assignment of the proton NMR spectrum, including minor *O*-acetylation variants. In this work the availability of spectra on materials with different *O*-acetylation patterns proved valuable, particularly in those cases where the chemical shift assignment for one residue depended on the substitution pattern of an adjacent residue.

Type A polysaccharide: Bundle et al. [14] reported the presence of *O*-acetylation at O-3 on approximately 70% of the ManNAc residues. The samples examined here were more highly substituted (approximately 95% of residues), as determined from the integral of the ManNAc H-2 resonances (see below). Assignments are reported in Table 2 and the one-dimensional proton NMR spectrum is shown in Fig. 1(a). In two dimensional proton spectra, four distinct spin systems were observed in an approximate intensities ratio of 80:7:8:5 although this varied between samples. One of these systems was assigned as non-acetylated residue, from the lack of a low field non-anomeric resonances. Two were assigned as 3-*O*-acetylated from the chemical shift of the H-3 resonance and one as 4-*O*-acetylated, from the presence of a lowfield triplet. Two ^{31}P resonances were observed in the ^{31}P–^{1}H HSQC spectrum, the minor highfield one correlated with the H-6/H-6′ of the 4-*O*-acetylated residue and with the H-1 of a spin system assigned as one of the ManNAc(3-*O*Ac)'s. This suggested the two ManNAc(3-*O*Ac) spin systems differed due to the *O*-acetylation status of the distal residue. This was confirmed by monitoring the ^{1}H spectrum of the polysaccharide during the mild base de-*O*-acetylation in the NMR tube. Four ManNAc H-2 resonances are observed and are diagnostic of the *O*-acetylation status of that residue and its distal neighbour. The relative intensity of the H-2 resonance at 4.45 ppm, arising from non-*O*-acetylated ManNAc, increased during de-*O*-acetylation. Initially, the intensities of the peaks at 4.55 and 4.50 ppm decrease faster than that at 4.59 ppm. The proportion of residues lacking *O*-acetylation is given by the ratio of the integral of H-2 peak at 4.45 ppm to that of all the H-2 peaks (from 4.59 to 4.45 ppm).

Type C polysaccharide: This polysaccharide is an ($\alpha 2 \rightarrow 9$)-linked NeuNAc homopolymer with *O*-acetylation on O-7 or O-8. ^{13}C NMR assignments for the native and de-*O*-acetylated material [12] and proton assignments for chemically de-*O*-acetylated material [27] have been reported. Fig. 1(b) shows the proton NMR spectrum of a sample of the *O*-acetylated polysaccharide containing all the important species.

Comparing the spectra of native and de-*O*-acetylated material, the *N*-acetyl and *O*-acetyl methyl peaks could be assigned. On average, 85% of the NeuNAc residues were *O*-acetylated. The assignment process for the ring and sidechain protons was complicated by the dependence of the chemical shifts from one residue both on its own *O*-acetylation state and that of its neighbours. The chemical shifts of H-3 to H-6 of a particular residue depend both on the *O*-acetylation of its own sidechain and that of the aglycone, whilst for H-7, H-8, and the two H-9s the chemical shifts reflect both local (on the same residue) and distal *O*-acetylation. Moreover, the spectrum of freshly dissolved material differed from that obtained when the sample had been allowed to stand at room temperature for a period of time.

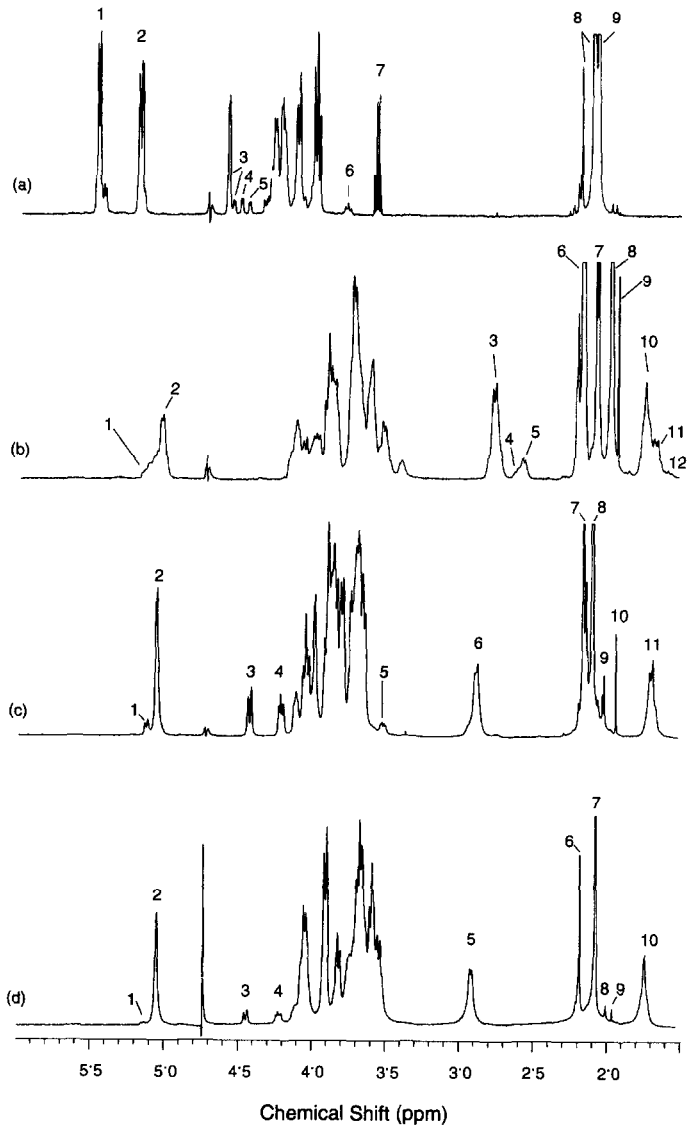
Combining data from a series of TOCSY spectra, obtained with mixing times of 20, 40 and 60 ms (Fig. 2), with information on the ratios of the integrals of the various H-3_{ax} (from 1.5 to 1.75 ppm), NAc methyl (1.96 and 2.06 ppm) and H-3_{eq} (from 2.5 to

Table 2
^{1}H and ^{31}P NMR assignments for the Type A polysaccharide

O-acetylation at residue [a]		Chemical shifts of the *n*-th residue										Integral of H-2 in the *n*-th residue
n-th	$(n-1)$-th	^{1}H									^{31}P	
		NAc methyl	OAc methyl	H-1	H-2	H-3	H-4	H-5	H-6	H-6′	OPO_3	
3	3/−	2.08	2.12	5.49	4.59	5.21	3.99	4.12	4.29	4.23	−2.75	80
3	4	2.08	2.12 [b]	5.44	4.55	5.19	3.99	4.08	4.29	4.23	−2.75	7
−	+/−	2.11	−	5.44	4.45	4.14	3.78	4.02	4.13	4.03	−2.75	5
4	+/−	2.08	2.20 [b]	5.47	4.50	4.21	5.20	4.32	4.13	4.03	−3.5	8

[a] The $(n-1)$-th residue is the aglycone of the *n*th residue. '3' denotes a 3-*O*-acetylated residue: →6)-ManNAc(3OAc)-(α1-OPO_3→. '4' denotes a 4-*O*-acetylated residue: →6)-ManNAc(4OAc)-(α1-OPO_3→. '−' denotes a non-acetylated residue: →6)-ManNAc-(α1-OPO_3→. +/− Chemical shifts of the residue *n* are independant of the *O*-acetylation state of this residue.
[b] These assignments of OAc methyl can be interchanged.

2.8 ppm) resonances, assignments for the majority of the peaks were obtained. A series of assigned, characteristic resonances were used to quantify non-acetylated and 7-*O*- or 8-*O*-acetylated residues. A NOESY spectrum, obtained at 15 °C with a mixing time of 200 ms, allowed the assignment to be completed (Table 3); particularly the NAc methyl resonances and to link the ring and sidechain spin systems, where the small value of $^{3}J_{H\text{-}6,H\text{-}7}$ leads to weak correlations. The spectral changes observed when the polysaccharide was incubated at room temperature were thereby ascribed to the migration of an *O*-acetyl substituent from the NeuNAc O-8 to O-7. A similar phenomenon has been reported in di-*O*-acetyl NeuNAc ketosides [28]. A freshly dissolved sample of Type C



polysaccharide shows an 8-*O*Ac to 7-*O*Ac ratio of 5:1, after a few days at 22 °C, this ratio reaches an equilibrium value of 1:3. A detailed study of *O*-acetyl migration between NeuNAc O-7 and O-9 [29] demonstrated the strong dependence of the migration rate on pH, and a similar sensitivity must be expected for the polysaccharide. Fig. 3 shows spectra of a Type C polysaccharide sample run after different periods of incubation at room temperature. These spectra proved valuable in confirming our previous assignments. The proportion of non-*O*-acetylated NeuNAc residues did not increase from 15% over the incubation period. No evidence was found for acetylation of NeuNAc O-4 or for the presence of di-*O*-acetylated residues.

Because the data reflects the *O*-acetylation state of neighbouring residues, a simple model was developed to test for non-randomness in the acetylation pattern. Within the limits of this model, the distribution of 7-*O*- or 8-*O*-acetylated residues and unacetylated residues was random. The *O*-acetylation pattern may be deduced by integration of the *N*- and *O*-acetyl methyl resonances: the global extent of substitution by comparing the ratio of the areas of the *O*-acetyl methyl at 2.17 ppm with those of the *N*-acetyl methyls at 2.07 and 1.97 ppm. The proportion of 7-*O*-acetyl NeuNAc is deduced from the ratio of the areas of the peak at 1.97 ppm and that at 2.07 ppm arising from non- and 8-*O*-acetyl NeuNAc.

Meningococcal Type C polysaccharide-protein conjugate vaccines effective in infants are now available [3–5] and are likely to be widely used in mass paediatric vaccination campaigns. Depolymerisation, activation and coupling of the polysaccharide to the protein provide opportunities for loss or migration of *O*-acetyl groups, and this may prove an important factor in determining the efficacy of such vaccines. A method to determine the extent and pattern of *O*-acetylation *in the conjugate* is obviously necessary for clinical evaluation of this factor. The one dimensional NMR spectrum of a CRM_{197}-meningococcal Type C conjugate showed intense resonances from the carbohydrate component with linewidths comparable to those of the unconjugated polysaccharide and from which the *O*-acetylation pattern could readily be determined (X. Lemercinier and C. Jones, unpublished results).

Fig. 1. Partial 500 MHz NMR spectra of (a) the meningococcal polysaccharides Type A, pH 6.4 (uncorrected), (b) Type C, pH 7.4 (uncorrected), (c) Type W-135, pH 6.2 (uncorrected), and (d) Type Y, pH 6.2 (uncorrected). The peaks are labelled as follows: in (a) 1: ManNAc H-1; 2: ManNAc H-3 in 3-*O*Ac and H-4 in 4-*O*Ac residues; 3 and 4: ManNAc H-2 in 3-*O*Ac and 4-*O*Ac residues; 5: ManNAc H-2 in non-acetylated residues; 6: ManNAc H-4 in non-acetylated residues; 7: Ethanol; 8: ManNAc OAc methyl; 9: ManNAc NAc methyl. In (b) 1: NeuNAc H-8 in 8-*O*Ac residues; 2: NeuNAc H-7 in 7-*O*Ac residues; 3: NeuNAc $H\text{-}3_{eq}$ in 7-*O*Ac residues 4: NeuNAc $H\text{-}3_{eq}$ in 8-*O*Ac residues flanked at their reducing side by an 8-*O*Ac residue; 5: NeuNAc $H\text{-}3_{eq}$ in 8-*O*Ac residues flanked by a 7-*O*Ac residue; 6: OAc methyl of acetylated residues; 7: NAc methyl of 8-*O*Ac and non-acetylated NeuNAc residues; 8: NAc methyl of 7-*O*Ac residues; 9: Acetate anion; 10: $H\text{-}3_{ax}$ in 7-*O*Ac NeuNAc residues flanked by a 7-*O*Ac residue; 11: $H\text{-}3_{ax}$ in 7-*O*Ac NeuNAc residues flanked by a 8-*O*Ac residue and in 8-*O*Ac NeuNAc residues flanked by a 7-*O*Ac residue; 12: $H\text{-}3_{ax}$ in 8-*O*Ac NeuNAc residues flanked at their reducing side by a 8-*O*Ac residue. In (c) 1: H-7 in 7-*O*Ac NeuNAc; 2: α-Gal H-1; 3 and 4: H-9 and H-9′ in 9-*O*Ac NeuNAc; 5: H-9′ of 7-*O*Ac NeuNAc; 6: NeuNAc $H\text{-}3_{eq}$ 7: OAc methyl of 7- and 9-*O*Ac NeuNAc; 8: NAc methyl of 9-*O*Ac and non-acetylated NeuNAc; 9: NAc methyl of 7-*O*Ac NeuNAc; 10: acetate anion; 11: NeuNAc $H\text{-}3_{ax}$. In (d) 1: H-7 in 7-*O*Ac NeuNAc; 2: α-Glc H-1; 3 and 4: H-9 and H-9′ in 9-*O*Ac NeuNAc; 5: NeuNAc $H\text{-}3_{eq}$; 6: OAc methyl of 7- and 9-*O*Ac NeuNAc; 7: NAc methyl of 9-*O*Ac and non-acetylated NeuNAc; 8: NAc methyl of 7-*O*Ac NeuNAc; 9: acetate anion; 10: NeuNAc $H\text{-}3_{ax}$.

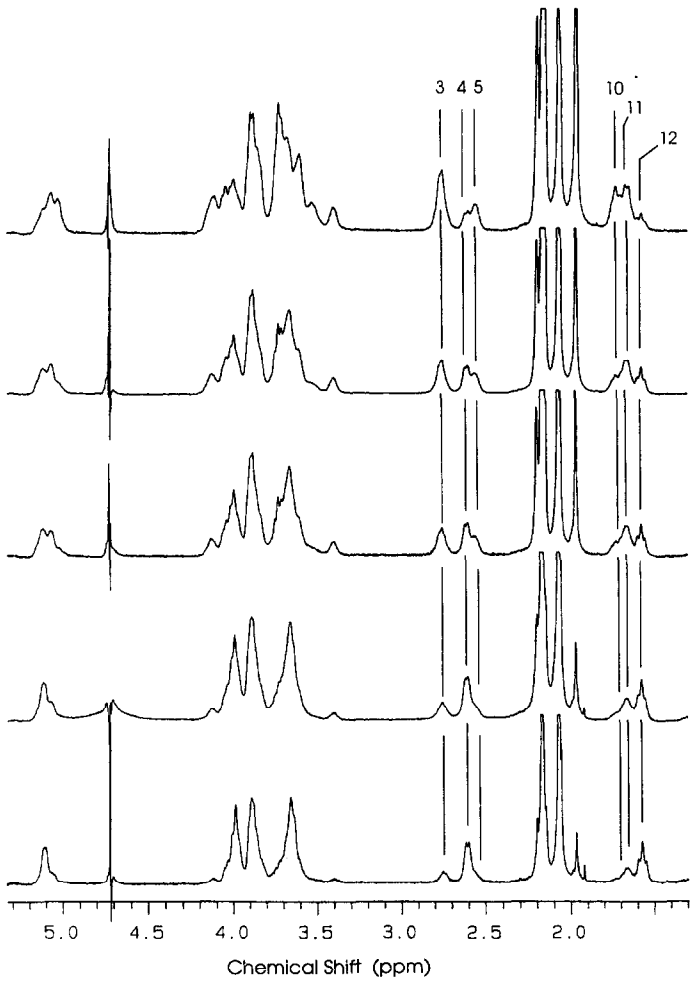


Fig. 2. 500 MHz one-dimensional ^{1}H NMR spectra of a sample of the meningococcal Type C polysaccharide obtained at different times after dissolution. From bottom to top, after: 5 min, 24 hours, 7 days, 10 days, and 34 days at 22 °C. Spectra were run at 30 °C, pH 7.4 (uncorrected). Peaks are labelled as in Fig. 1(b).

Type W-135 polysaccharide: The structure reported for the Type W-135 polysaccharide, determined primarily from ^{13}C NMR data did not contain *O*-acetyl groups [13]. However, both samples examined here showed acetylation of more than 60% of repeat units. Full proton and carbon assignments for the de-*O*-acetylated material were obtained from HMQC, HMBC and TOCSY spectra: starting from this model and published assignments for free *O*-acetylated sialic acids [30], a full assignment of the spectrum of the native polysaccharide was made (Table 4). An intense pair of coupled resonances ($^{2}J_{HH}$ = 12 Hz) at 4.43 and 4.21 ppm indicated the presence of 9-*O*-acetylated NeuNAc residues, as observed for the Type Y polysaccharide (see below) and a resonance at 5.13 ppm was assigned as H-7 of 7-*O*-acetyl NeuNAc.

Table 3
Proton NMR assignments for the type C polysaccharide [a]

O-acetylation patterns	NAc methyl	OAc methyl	H-3_{ax}	H-3_{eq}	H-4	H-5	H-6	H-7	H-8	H-9	H-9′
7-OAc 7-OAc	1.96	–	1.72	2.76	3.60	3.71	3.89	–	–	–	–
7-OAc 8-OAc	1.96	–	1.65	2.76	3.59	3.73	3.89	–	–	–	–
7-OAc **7-OAc**	–	2.17	–	–	–	–	–	5.01	4.11	3.72	3.51
8-OAc **7-OAc**	–	2.20	–	–	–	–	–	5.06	4.13	3.67	3.41
8-OAc 7-OAc	2.07	–	1.66	2.56	3.68	3.89	4.05	–	–	–	–
8-OAc 8-OAc	2.07	–	1.57	2.61	3.66	3.87	4.00	–	–	–	–
7-OAc **8-OAc**	–	2.17	–	–	–	–	–	3.87	5.06	3.96	3.67
8-OAc **8-OAc**	–	2.17	–	–	–	–	–	3.85	5.12	3.95	3.65
Non acetylated 7/8-OAc	2.07	–	1.71	2.74	3.71	3.83	3.75	n.d.	n.d.	n.d.	n.d.
Non acetylated [b]	2.04	–	1.71	2.73	3.66	3.83	3.71	3.57	3.97	3.83	3.67

[a] The chemical shift values are given for the residue shown in bold face.
[b] These values were obtained from spectra of the de-*O*-acetylated material.

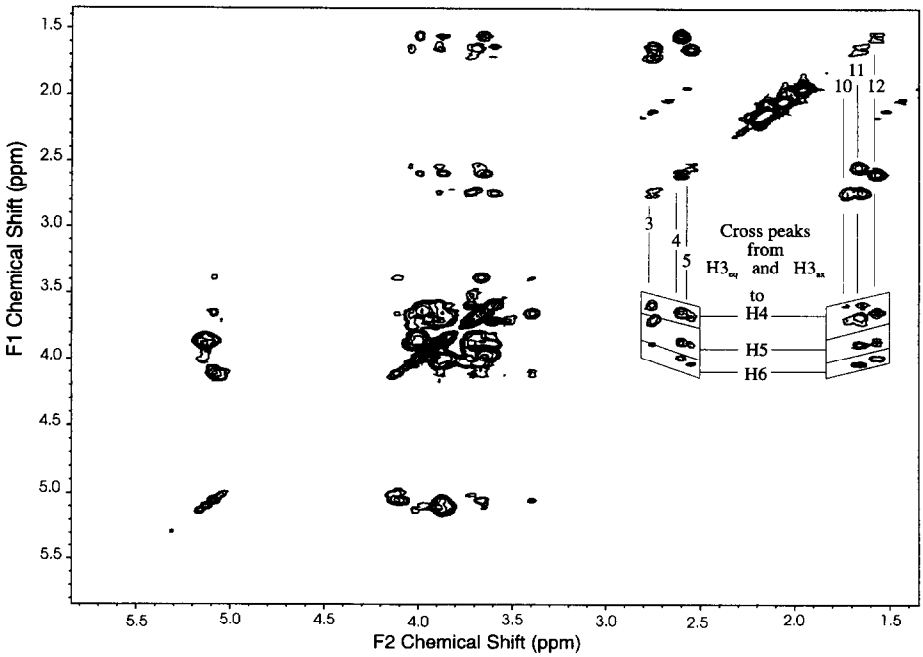


Fig. 3. 500 MHz TOCSY spectrum of the meningococcal Type C polysaccharide, obtained at 30 °C and using a 60 ms mixing time. The different spin systems are labelled as in Fig. 1(b). The vertical lines show the different H-3_{ax} and H-3_{eq} resonances of each of the spin system, showing that the chemical shift depends both on the *O*-acetylation of the residue and that of its aglycone.

Our ^{13}C assignment agrees with that reported [13] except that we change the assignments for the Gal C-2′ (to 68.9 ppm), the Gal C-3′ (to 70.2 ppm) and the NeuNAc C-8 (to 73.0 ppm). Both 7-*O* and 9-*O*-acetylation modifies the chemical shift of C-9 from that found in non-acetylated residues, and a second estimate of the *O*-acetylation pattern may also be obtained from the carbon spectrum by comparing the heights of the C-9 resonances of 7-*O*Ac-NeuNAc (62.7 ppm), 9-*O*Ac-NeuNAc (66.8 ppm) and non-acetylated (63.7 ppm) residues. As found for the Type C polysaccharide the proton spectra of freshly dissolved material differed from those obtained after the sample had stood at room temperature for a few days. From the above mentioned ^{1}H assignments, this modification could be attributed to an *O*-acetyl migration from NeuNAc O-7 to O-9. A similar migration has been reported in the case of 7-*O*-acetyl NeuNAc [29]. In the Type W-135 polysaccharide, the 7-*O*Ac to 9-*O*Ac ratio was initially 1:1 and reaches an equilibrium value of 1:2 after three days, although this rate is probably sensitive to pH. No evidence of *O*-acetylation of the Gal residue or 8-*O*-acetylation of the NeuNAc was obtained, although the 8-*O*-acetyl NeuNAc may be an intermediate in the acetyl migration. Fig. 1(c) shows the 500 MHz proton spectrum of this polysaccharide, with important resonances labelled.

Type Y polysaccharide: Two Y polysaccharide samples were investigated which showed 0.18 and 0.05 *O*-acetyl groups per repeat unit, well below the value of 1.3

Table 4
Proton NMR assignments for the Type W-135 polysaccharide [a]

	NAc methyl	OAc methyl	H-1	H-2	H-3/H-3_{ax}	H-3_{eq}	H-4	H-5	H-6	H-6′	H-7	H-8	H-9	H-9′
→6)**Gal**(α1→4)	–	–	5.06	3.79	3.74	–	3.99	3.84	3.90	3.65	–	–	–	–
NeuNAc(α2→ [b]														
→6)Gal(α1→4)	2.10	–	–	–	1.69	2.88	3.70	4.03	3.80	–	3.64	3.87	3.89	3.64
NeuNAc(α2→														
→6)Gal(α1→4)	2.02 [c]	2.13	–	–	1.69	2.91	3.72	3.85 [d]	4.00 [d]	–	5.13	4.07	3.70	3.52
NeuNAc7OAc(α2→	2.06 [c]													
→6)Gal(α1→4)	2.10	2.15	–	–	1.69	2.88	3.72	4.03	3.80	–	3.68	4.12	4.43	4.21
NeuNAc9OAc(α2→														

[a] The chemical shift values are given for the residue shown in bold face.
[b] The chemical shifts in the Gal residue are not altered by *O*-acetylation of the NeuNAc residue.
[c] This resonance was split, probably due to the effect of neighbouring residues.
[d] Tentative assignments.

Table 5
Proton NMR Assignments for the Type Y polysaccharide [a]

	NAc methyl	OAc methyl	H-1	H-2	H-3/H-3_{ax}	H-3_{eq}	H-4	H-5	H-6	H-6′	H-7	H-8	H-9	H-9′
→6)**Glc**(α1→4)NeuNAc(α2→ [b]	–	–	5.06	3.55	3.60	–	3.52	3.63	4.02	3.70	–	–	–	–
→6)Glc(α1→4)**NeuNAc**(α2→	2.03	–	–	–	1.69	2.88	3.73	4.04	3.80	–	3.65	3.88	3.88	3.65
→6)Glc(α1→4)**NeuNAc7OAc**(α2→	1.92	2.14	–	–	n.d.	n.d.	n.d.	n.d.	n.d.	–	5.15	4.01	3.68	3.51
→6)Glc(α1→4)**NeuNAc9OAc**(α2→	2.03	2.14	–	–	1.69	2.88	3.73	4.04	3.80 [c]	–	3.68	4.09	4.41	4.19

[a] The chemical shift values are given for the residue shown in bold face.
[b] The chemical shifts of resonances from the Glc residue are unaffected by *O*-acetylation of the NeuNAc.
[c] Tentative assignment.

reported previously [13]. Two lowfield resonances at 4.19 ppm and 4.41 ppm were attributed to H-9 and H-9′ of 9-*O*-acetylated NeuNAc. In the more highly acetylated sample, a minor resonance at 5.15 ppm indicated the presence of 7-*O*-acetylated NeuNAc. The full proton assignment is given in Table 5. Our ^{13}C assignment agrees with the published data except that the assignments are reversed for the NeuNAc C-8, at 72.9 ppm, and Glc C-2′ at 72.0 ppm. No evidence was found to suggest *O*-acetylation of the glucose residues in the samples we examined, or migration of *O*-acetyl groups from the O-9 position. Fig. 1(d) shows the 500 MHz proton NMR spectrum of this polysaccharide, with important resonances labelled.

The proton NMR spectra of the four meningococcal polysaccharides currently used in vaccine production have been assigned and the heterogeneous pattern of *O*-acetylation for each polysaccharide determined. Integration of resolved resonances allows the *O*-acetylation pattern of individual batches of material to be established rapidly and non-destructively, and this methodology opens the way to detailed biological studies of the influence of heterogeneous or variant *O*-acetylation on the immunogenicity and antigenic specificity of these systems.

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