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Carbohydrate Research 340 (2005) 1097–1106
Carbohydrate RESEARCH

Revised structures for the capsular polysaccharides from *Staphylococcus aureus* Types 5 and 8, components of novel glycoconjugate vaccines

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Received 5 November 2004; received in revised form 31 January 2005; accepted 2 February 2005

Abstract—Glycoconjugate vaccines based on the capsular polysaccharides (CPSs) from *Staphylococcus aureus* serotypes 5 and 8 conjugated to genetically detoxified recombinant exoprotein A (rEPA) from *Pseudomonas aeruginosa* have been shown, in Phase 3 clinical trials, to elicit a strong bactericidal immune response in end-stage renal disease patients. Such vaccines have the potential to reduce morbidity and mortality due to methicillin-resistant *Staphylococcus aureus* (MRSA), a major cause of hospital-acquired infection. The serotype 5 and 8 polysaccharides have been fully characterized by NMR spectroscopy and full structural analyses carried out. Published structures were found incorrect and the revised structures of the repeat units of the two polysaccharides are:

Type 5 →4)-β-D-ManNAcA-(1→4)-α-L-FucNAc(3OAc)-(1→3)-β-D-FucNAc-(1→
Type 8 →3)-β-D-ManNAcA(4OAc)-(1→3)-α-L-FucNAc-(1→3)-α-D-FucNAc-(1→

Resonances indicative of the presence of peptidoglycan were observed in the spectra of both CPSs, consistent with reports that the CPS is covalently linked to peptidoglycan.

Keywords: NMR spectroscopy; *Staphylococcus aureus*; Conjugate vaccine; Polysaccharide; Capsule; Peptidoglycan

1. Introduction

Staphylococcus aureus is a Gram-positive bacterium that causes bacteremia, metastatic abscesses, septic arthritis, endocarditis, osteomyelitis, and wound infections.[1] Hospital-acquired infections are a particular problem especially as methicillin-resistant *S. aureus* (MRSA) becomes increasingly prevalent.[2] The capsular polysaccharides (CPSs) of *S. aureus* are virulence factors,[3,4] and two capsular serotypes, Types 5 and 8, predominate.[5] Antibodies against the CPSs are generally considered protective against infection.[6–8] A bivalent glycoconjugate vaccine, in which these two CPSs are covalently attached to recombinant genetically detoxified exoprotein A from *Pseudomonas aeruginosa* (rEPA) elicited strong bactericidal immune responses in volunteers,[9–11] although effectiveness in clinical trials in kidney dialysis patients was limited.[12,13] The same two serotypes are also the most common cause of mastitis in cattle[14,15] and glycoconjugate vaccines for veterinary use are also under development.[16]

The structure of the repeat unit of the Type 5 CPS was determined by Moreau et al. using ^{1}H and ^{13}C NMR and chemical degradation.[17] This revised the structure originally proposed by Vann et al.[18] The *O*-acetylated repeat unit (**1**) contains one 2-acetamido-2-deoxy-D-mannuronic acid (D-ManNAcA) and two 2-acetamido-2,6-dideoxy galactose residues with D- and L-configurations (D- and L-FucNAc). The *O*-acetyl group was reported as

Abbreviations: CPS—capsular polysaccharide; MRSA—methicillin resistant *Staphylococcus aureus*; rEPA—recombinant genetically detoxified exotoxin A from *Pseudomonas aeruginosa*.

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doi:10.1016/j.carres.2005.02.001

being located on the ManNAcA *O*-3 on the basis of ^{13}C chemical shift differences for the α-L-FucNAc C-2 resonance between the native and de-*O*-acetylated CPSs.[17]

$$\rightarrow 4)\text{-}\beta\text{-D-ManNAcA(3OAc)-}(1\rightarrow 4)\text{-}\alpha\text{-L-FucNAc-}(1\rightarrow 3)\text{-}\beta\text{-D-FucNAc-}(1\rightarrow \qquad \mathbf{1}$$

Published structural work on the Type 8 CPS is sparse. The reported structure has the same basic trisaccharide repeat but differs from the Type 5 structure in some linkages, the anomeric configuration of one of the FucNAc residues and the location of the *O*-acetyl group (**2**). Vann et al.[18] proposed the structure **2a** based largely on ^{13}C NMR evidence.

$$\rightarrow 3)\text{-}\beta\text{-ManNAcA-}(1\rightarrow 3)\text{-}\alpha\text{-FucNAc-}(1\rightarrow 3)\text{-}\beta\text{-FucNAc-}(1\rightarrow \quad \text{plus } O\text{-acetyl} \qquad \mathbf{2a}$$

Other publications[9,10,19] cite structure **2b**

$$\rightarrow 3)\text{-}\beta\text{-D-ManNAcA(4OAc)-}(1\rightarrow 3)\text{-}\alpha\text{-L-FucNAc-}(1\rightarrow 3)\text{-}\beta\text{-D-FucNAc-}(1\rightarrow \qquad \mathbf{2b}$$

without experimental evidence, and conflicting with the work of Vann et al.[18] who reported that the anomeric configuration of one of the FucNAc residues differed between the Type 5 and Type 8 CPS. Whilst the Type 8 CPS is reported to be identical to the antigen from the T strain, which was shown to contain D-FucNAc,[20] there is no published evidence for the presence of L-FucNAc. Tzianabos et al. provide NMR evidence for incomplete *N*-acetylation of one of the FucNAc residues in the Type 8 CPS in a strain-dependent manner.[19] A study of the ability of native and de-*O*-acetylated CPS and derived conjugates to produce bactericidal antibody responses[21] concluded that two distinct specificities of antibodies are elicited, against the *O*-acetyl groups and against the saccharide backbone and the latter are sufficient for bactericidal activity. This seems to mirror the case in meningococcal Group C vaccines.[22] Current developmental staphylococcal conjugate vaccines are prepared using *O*-acetylated CPS.

NMR spectroscopy is used to confirm the identity and degree of *O*-acetylation of CPSs for vaccine manufacture,[23–25] and full assignment of the NMR spectra of these CPSs is required to validate this approach. These assignments were inconsistent with the published structures: this report details revised structures for the repeat units from these CPSs. The spectra also indicated the presence of peptidoglycan.

2. Experimental

Polysaccharides samples were material intended for vaccine manufacture. Samples of polysaccharide (2 mg) were deuterium exchanged by lyophilization from deuterated water (0.3 mL, Sigma–Aldrich Chemical Co., UK, 99.9% ^{2}H) and resuspended in the same deuterated water (0.35 mL) for analysis. Samples were introduced into 5 mm susceptibility-matched NMR tubes (Shigemi, Tokyo). The sample in the NMR tube was subjected to sonication in a water bath at ca. 50 °C for 24 h. NMR spectra were recorded on a Varian Inova 500 spectrometer equipped with a 5 mm PFG inverse detection probe at an indicated probe temperature of 50 °C. The spectrometer was controlled through VNMR version 6.1C. Standard Varian pulse programs were used apart from the introduction of spin echo sequences into the TOCSY and ROESY experiments, the HSQC experiment of Wider and Wüthrich[26] and variants of the HMQC-TOCSY and HMQC-NOESY experiments of Crouch et al.[27] using the HSQC sequence. Chemical shifts are referenced to internal TSP-d_4 at zero ppm (^{1}H) or −1.8 ppm (^{13}C) according to Wishart et al.[28] The HSQC spectrum was optimized for $^{1}J_{C,H}$ of 150 Hz, the mixing times in the TOCSY, ROESY, and HSQC-NOESY experiments were 80, 150, and 50 ms, respectively.

De-*O*-acetylation was performed in two ways. To quantify the degree of *O*-acetylation, the sample in the NMR tube was treated with NaOD in D_2O (40% w/v, Goss Scientific Instruments, UK) to a final concentration of 200 mM. The tube was allowed to stand at room temperature for 10 min prior to replacement in the NMR spectrometer. To prepare de-*O*-acetylated material for detailed analysis, CPS (4 mg) was treated with 200 mM NaOH for ca. 3 h at room temperature, desalted by passage through a pad of BioRad AG50Wx8 200–400 mesh H^+ form, neutralized with ammonia and lyophilized.

3. Results and discussion

3.1. One-dimensional NMR spectra of the native polysaccharides

The CPS samples used were material intended for vaccine manufacture. The 500 MHz ^{1}H NMR spectra

of the Type 5 and Type 8 CPS are shown in Figures 1a and 2a, respectively. The spectra of both samples contain an intense resonance at 3.67 ppm, due to a Tris(hydroxymethyl)-aminomethane (Tris) counterion. The spectrum contains a number of minor resonances, which are inconsistent with the published structures, and these are indicated by boxes in Figures 1 and 2. The full assignment of the spectra is described below.

3.2. De-*O*-acetylation of the polysaccharides

The polysaccharides were de-*O*-acetylated in situ by treatment with 200 mM NaOD in the NMR tube[23,25] and spectra obtained on these samples, under basic conditions. These spectra are shown in Figures 1b and 2b, respectively. The chemical shift of the Tris resonance moved upfield to 3.52 ppm, consistent with deprotonation. These spectra in 200 mM NaOD allow the degree of *O*-acetylation of the original polysaccharide to be estimated, by comparison of the integral of the acetate anion resonance with a resonance from an appropriate resonance arising from the polysaccharide chain, either an *N*-acetyl resonance or the FucNAc H-6s. For both serotypes, these values are approximately 90%. The failure to observe resonances from non-*O*-acetylated repeat units in the HSQC spectra suggests the degree of *O*-acetylation is close to 100%. For spectral assignments and structure confirmation, de-*O*-acetylated CPS samples were neutralized with cation exchange resin and ammonium salts formed. The spectra of the de-*O*-acetylated Types 5 and 8 polysaccharides recovered from these treatments are shown in Figures 1c and 2c, respectively.

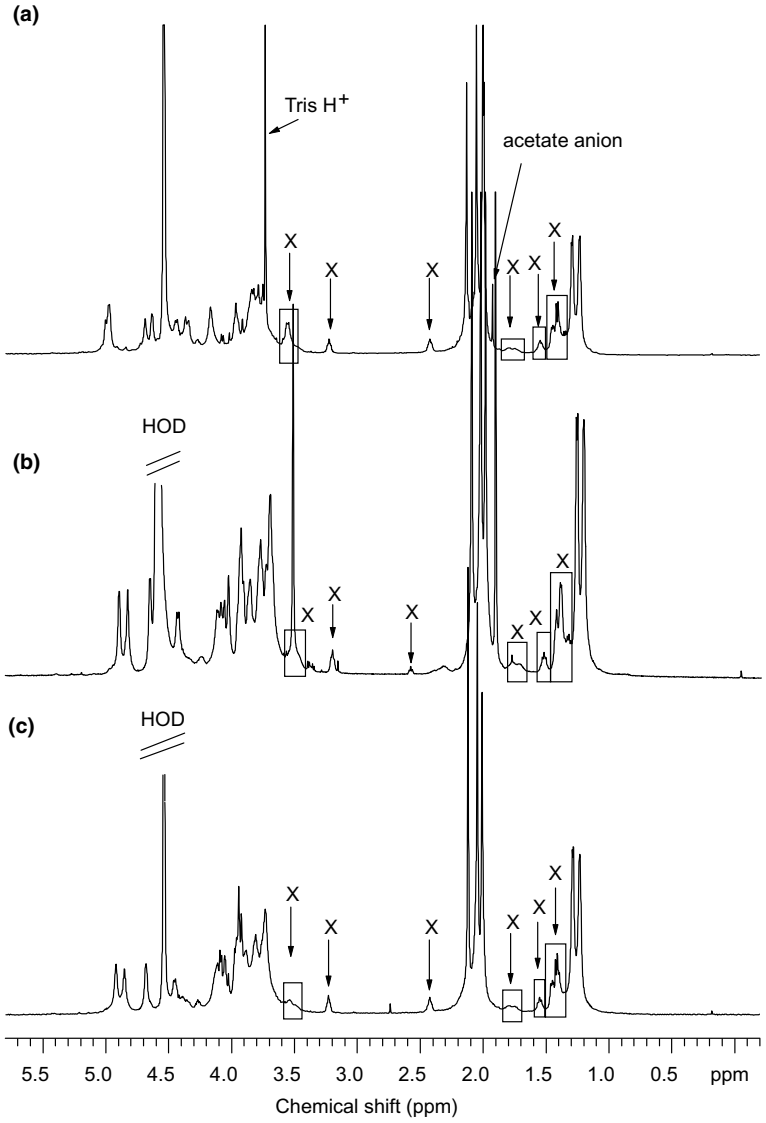

Figure 1. Partial 500 MHz ^{1}H NMR spectra of the native *S. aureus* Type 5 CPS as supplied by the manufacturer, (b) the same sample de-*O*-acetylated in situ by the addition of NaOD to a final concentration of 200 mM, and (c) the same samples after desalting, neutralization and lyophilization. Spectra were obtained at an indicated probe temperature of 50 °C. Identified and unidentified impurities are indicated by an 'X'.

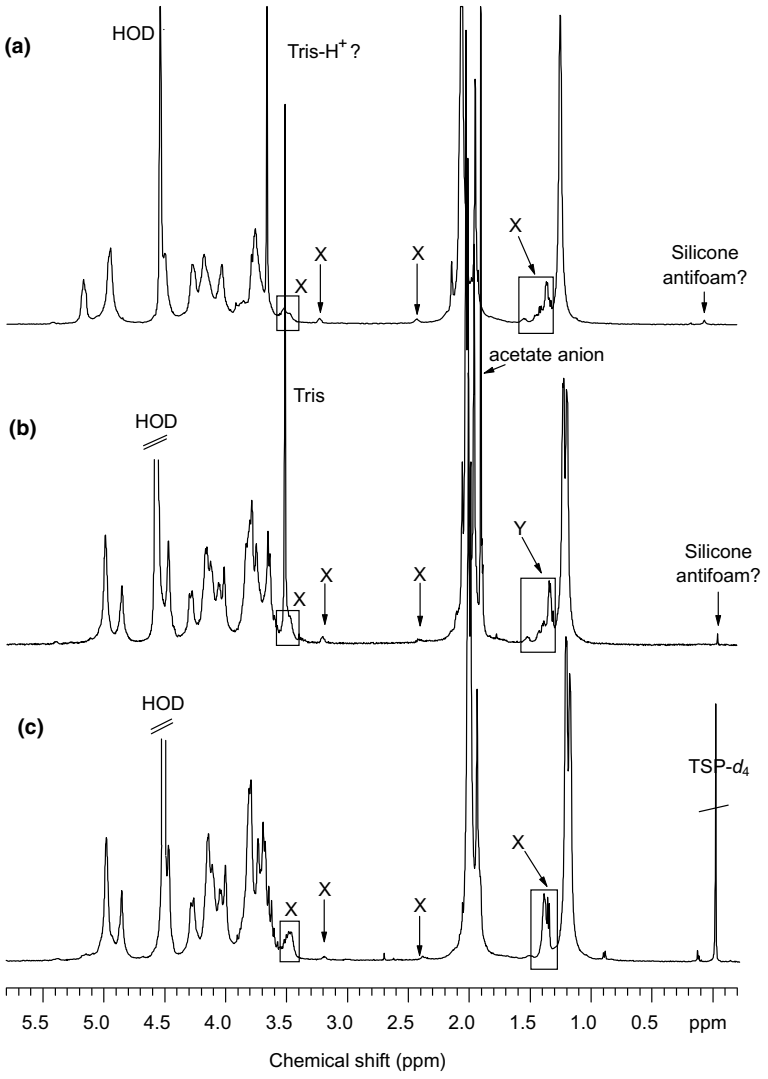

Figure 2. Partial 500 MHz ^{1}H NMR spectra of the native *S. aureus* Type 8 CPS as supplied by the manufacturer, (b) the same sample de-*O*-acetylated in situ by the addition of NaOD to a final concentration of 200 mM, and (c) the same samples after desalting, neutralization and lyophilization. Spectra were obtained at an indicated probe temperature of 50 °C. Identified and unidentified impurities are indicated by an 'X'.

3.3. Full assignment of the ^{1}H and ^{13}C NMR spectra of the de-*O*-acetylated Type 5 CPS

The NMR spectra of the de-*O*-acetylated polysaccharides were assigned using conventional two-dimensional homo- and hetero-nuclear NMR techniques at 50 °C. The assignments are listed in Table 1. The key starting resonances for the assignment were the anomeric protons, the ManNAcA H-2 and the FucNAc methyl resonances. The CPS was clearly contaminated with another material, which was identified from the NMR spectra. There are clear resonances from a minor ManNAcA spin system (see below).

3.4. Anomeric configurations of the sugar residues in the Type 5 CPS

The anomeric configurations of the sugar residues were determined from the values of $^{1}J_{\mathrm{H\text{-}1,C\text{-}1}}$ determined in an HSQC experiment without proton decoupling and from the ^{1}H and ^{13}C chemical shift data. The $^{1}J_{\mathrm{H\text{-}1,C\text{-}1}}$ values obtained were 162 Hz for the β-ManNAcA H-1, 164 Hz for the β-FucNAc H-1 and 173 Hz for the α-FucNAc H-1.[29] No useful information could be obtained from the unresolved $^{3}J_{\mathrm{H\text{-}1,H\text{-}2}}$ of the ManNAcA, but a value of 8 Hz was determined for the β-FucNAc H-1. The expected 3 Hz splitting on the α-FucNAc H-1 was unresolved.

3.5. Absolute configurations of the sugar residues in the Type 5 CPS

Previously published work indicates that the Type 5 CPS contains both enantiomers of FucNAc, and the evidence of their sequence within the repeat unit provided by Moreau et al.[17] is convincing. Whilst NMR spectroscopy cannot provide information on the absolute confi-

Table 1. NMR assignments for the native *O*-acetylated and chemically de-*O*-acetylated *S. aureus* Type 5 capsular polysaccharide

Residue	H-1 C-1 $^1J_{H-1,C-1}$	H-2 C-2 $^3J_{H-1,H-2}$	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6	NAc	OAc
De-O-acetylated Type 5 CPS, neutral, 50 °C								
→4)-β-D-ManNAcA-(1→	4.860 100.46 164 Hz	4.680 53.18 NR	3.900 71.43	3.819 79.18	3.738 78.14		2.018[a] 23.59[a]	
→4)-α-L-FucNAc-(1→	4.935 100.30 173 Hz	4.105 51.25 NR	3.940 68.01	4.060 80.53	4.142 68.33	1.242 16.37	2.057[a] 23.31[a]	
→3)-β-D-FucNAc-(1→	4.461 102.08 162 Hz	3.970 52.22 8 Hz	3.760 78.18	3.715 71.62	3.820 71.89	1.298 16.27	2.126[a] 23.08[a]	
Native O-acetylated Type 5 CPS neutral, 50 °C								
→4)-β-D-ManNAcA-(1→	4.698 101.21	4.645 53.22	3.855 71.39	3.804 80.18	3.572 78.78		2.011[a] 23.62[a]	
→4)-α-L-FucNAc(3OAc)-(1→	4.981 100.31	4.368 48.19	5.005 71.03	4.382 79.40	4.175 67.95	1.242 16.37	2.023[a] 23.23[a]	2.070 21.39
→3)-β-D-FucNAc-(1→	4.461 102.39	3.992 52.25	3.782 78.39	3.738 71.63	3.837 71.94	1.300 16.45	2.149[a] 23.17[a]	

Data collected at a nominal probe temperature of 50 °C and referenced to internal TSP-d_4 at zero ppm (^{1}H) and −1.8 ppm (^{13}C). NR = not resolved.
[a] Assignments may be reversed.

gurations of the sugar residues, the glycosylation shift and inter-proton NOEs are sensitive to the 'relative absolute configuration' of adjacent residues, so that if the absolute configuration of one residue can be established by independent means (or assumed), then the absolute configuration of the other residues can be deduced. The effects of these factors on ^{13}C glycosylation shifts and inter-residue NOEs have been codified by the group of Kochetkov and co-workers.[30–32] Minor, low intensity spin systems were observed for the ManNAcA and α-FucNAc residues. The minor ManNAcA spin system is characterized by a relatively high-field H-4 resonance at 3.63 ppm, suggesting that this residue is terminal, which would indicate that the biological repeat unit, the unit that is polymerized to form the CPS, is the same as that shown as the chemical repeat unit **3**. The H-1/C-1 and H-2/C-2 crosspeaks of the minor ManNAcA spin system are resolved in the HSQC spectrum, and integration of their volumes showed that they have approximately 8% of the intensity of the in-chain residues. This corresponds to an average of 12–13 repeat units per glycan chain, an average molecular weight of ca. 7500–8000 Da in this material, which has been subjected to extensive sonication. There is no indication of heterogeneity which might arise from reducing termini, consistent with a lack of extensive random depolymerization during sonication, and that the glycan chains are covalently attached to the peptidoglycan chain.

3.6. Spectra of the intact Type 5 CPS and confirmation of the position of *O*-acetylation

The HSQC spectrum of the native, *O*-acetylated Type 5 CPS showed the presence of a single low-field proton resonance correlated to a relatively high-field ^{13}C resonance, indicative of a single *O*-acetylation site (Fig. 3). The ^{1}H and ^{13}C spectra were assigned using conventional techniques and using the information derived from the de-*O*-acetylated sample (Fig. 4). This showed that the *O*-acetylation site was the α-FucNAc *O*-3, on the basis of the low-field chemical shift of the α-FucNAc H-3 and effects of de-*O*-acetylation on the chemical shifts of adjacent atoms. Moreau et al. locate the *O*-acetyl group on the basis of a change in the chemical shift of one of the C-2 resonances from 48 to 51 ppm, assigned as the α-FucNAc C-2.[17] Together, these data show that the structure of the Type 5 CPS is **3** below.

→4)-β-D-ManNAcA-(1→4)-α-L-FucNAc(3OAc)-(1→3)-β-D-FucNAc-(1→ **3**

3.7. Assignment of the ^{1}H and ^{13}C NMR spectra of the Type 8 CPS

The NMR spectra of the native and de-*O*-acetylated Type 8 CPSs were assigned using two-dimensional

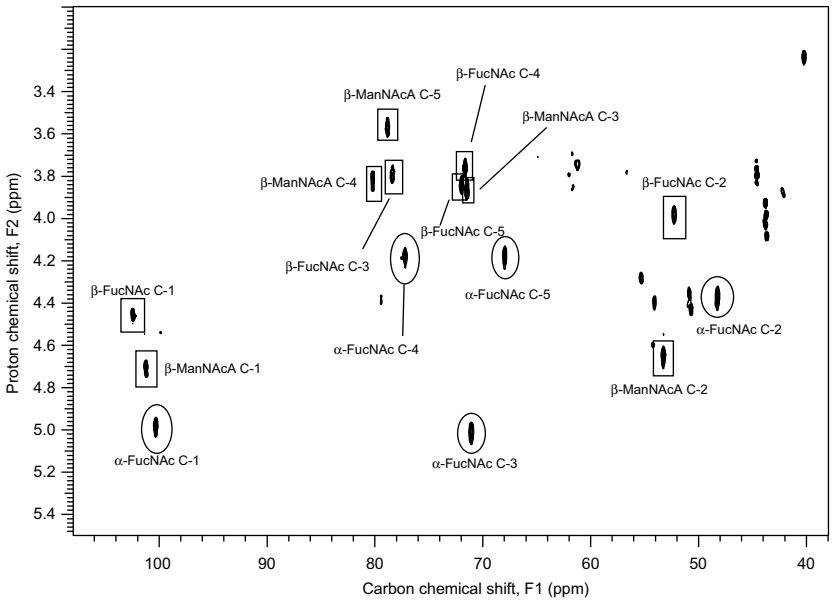

Figure 3. Anomeric and ring carbon region of the 500 MHz 150 Hz HSQC spectrum of the native CPS from *S. aureus* Type 5 obtained at 50 °C. Crosspeaks in ovals arise from the α-FucNAc residue and whose chemical shifts change significantly between the native and de-*O*-acetylated CPS.

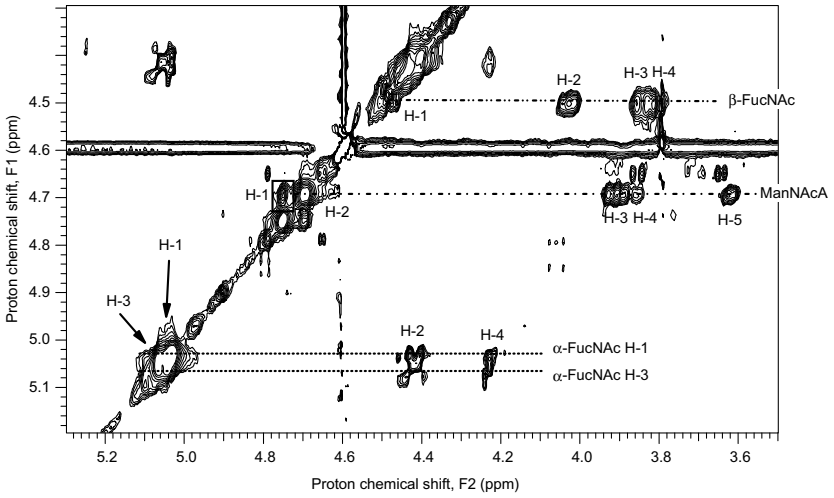

Figure 4. Partial 500 MHz TOCSY spectrum of the native Type 5 CPS, showing correlations, which demonstrate the 3-*O*-acetylation of the α-FucNAc residue.

heteronuclear methods, and the assignments are shown in Table 2. The chemical shifts for the two FucNAc residues suggest that both are in the α-anomeric configuration, with characteristic low-field chemical shifts for H-5 and high-field chemical shifts for C-5: this was confirmed by measurement of $^{1}J_{\mathrm{H\text{-}1,C\text{-}1}}$. The values obtained were 172 and 174 Hz for the two FucNAc residues and 164 Hz for the ManNAcA residue. The high value of $^{1}J_{\mathrm{H\text{-}1,C\text{-}1}}$ of approximately 170 Hz is characteristic of an anomeric proton in an axial orientation (the usual case for an α-anomer).[29]

The HSQC spectrum of the native Type 8 CPS contained a single low-field proton resonance attached to a nonanomeric carbon, at $\delta_{\mathrm{H}} = 5.17$ and $\delta_{\mathrm{C}} = 71.85$ ppm. This resonance, a 10 Hz triplet, correlated in the TOCSY spectrum to the ManNAcA H-2, H-3, and H-5, indicating that it arose from the ManNAcA H-4.

3.8. Absolute configurations of the sugar residues in the Type 8 CPS

There is little published information on the absolute configurations of the FucNAc residues in the Type 8 CPS. As discussed above, glycosylation shifts and inter-residue NOEs provide information about the relative absolute configurations of adjacent residues.[30–32] The NOE data is particularly useful when one of the protons on the carbons adjacent to the glycosylation site is equatorial, which is true for all three residues in the repeat unit. The absolute configuration of the

Table 2. NMR assignments for the *S. aureus* Type 8 capsular polysaccharide

	H-1 C-1 $^1J_{H-1,C-1}$	H-2 C-2 $^3J_{H-1,H-2}$	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6	NAc NAc	OAc
De-O-acetylated polysaccharide								
→3)-β-D-ManNAcA-(1→	4.858 96.37 164 Hz	4.470 53.97 NR	3.789 80.42	3.791 69.97	3.646 78.09		2.018 23.33	
→3)-α-L-FucNAc-(1→	4.994 99.97 172 Hz	4.166 48.73 NR	4.172 73.92	4.017 68.85	4.059 67.88	1.237 16.38	1.959 23.18	
→3)-α-D-FucNAc-(1→ Major	4.983 174 Hz	4.291 101.18 NR	3.825 49.85	3.750 75.03	4.123 72.31	1.198 68.25	2.036 16.53	22.97
Native O-acetylated polysaccharide								
→3)-β-D-ManNAcA(4OAc)-(1→	4.956 96.03 162 Hz	4.504 54.05 NR	4.146 75.59	5.171 71.85	3.785 75.74		2.068 23.76	2.148 21.58
→3)-α-L-FucNAc-(1→	4.980 100.15 173 Hz	4.190 49.00 NR	4.188 74.26	4.035 69.06	4.054 68.28	1.260 16.73[a]	1.957 23.53	
→3)-α-D-FucNAc-(1→	4.953 100.23 174 Hz	4.272 49.85	3.765 72.55	3.746 74.96	4.282 68.20	1.260 16.89[a]	2.068 23.33	

Data collected at a nominal probe temperature of 50 °C and referenced to internal TSP-d_4 at zero ppm (^{1}H) and −1.8 ppm (^{13}C). NR = not resolved.
[a] Assignments may be reversed.

ManNAcA residue is assumed to be D, as in the Type 5 CPS. No ^{1}H or ^{13}C chemical shift data has been reported for either α- or β-FucNAc or for β-ManNAcA, the usual starting points from which to calculate glycosylation shifts. Predicted chemical shifts for these species were estimated by applying the differences in chemical shifts between Gal and GalNAc to Fuc data, and the differences between β-Glc and β-GlcA to β-ManNAc data.[33]

3.8.1. The ManNAcA-FucNAc linkage. The work of Lipkind et al.[32] indicates that a strong transglycosidic NOE would be expected between the β-ManNAcA H-1 and the FucNAc(1) H-4 only if the two residues have opposite absolute configuration. Such an NOE is observed in both the HSQC-NOESY and ROESY spectra of the de-*O*-acetylated Type 8 CPS. The pattern of ^{13}C glycosylation shifts is also consistent with the L-configuration of the FucNAc residue, with an observed shift of −3.2 ppm on the FucNAc C-4. This is predicted[31] to be −3.3 ± 0.6 ppm if L-FucNAc is present or −0.4 ± 0.4 ppm in the D-FucNAc case.

3.8.2. The FucNAc-FucNAc linkage. The glycosylation shifts of the FucNAc(2) C-2, C-3, and C-4 are almost identical to those observed in the de-*O*-acetylated Type 5 CPS, with a known α-L-FucNAc-(1→3)-D-FucNAc substructure. This suggests that the two FucNAc residues in the Type 8 CPS have opposite absolute configuration. The observation of a correlation between FucNAc(1) H-5 and FucNAc(2) H-4 and the failure to observe a transglycosidic NOE between the FucNAc(1) H-1 and the FucNAc(2) H-4 in either the HSQC-NOESY or ROESY spectra is consistent with them having opposite absolute configuration.[32]

3.8.3. The FucNAc-ManNAcA linkage. An unusual inter-residue NOE between the α-FucNAc(2) H-5 and the ManNAcA H-2 observed in the HSQC-NOESY and ROESY spectra is consistent with both residues having the same absolute configuration, and suggests that the unit D-FucNAc(2)-α-(1→3)-D-ManNAcA is present, by analogy with the specific inter-residue NOE between Gal H-5 and ManNAc H-2 observed in the sequence D-Gal*p*-α-(1→3)-D-ManNAc.[34] The observation of a very weak transglycosidic NOEs between the FucNAc(2) H-1 and the ManNAcA H-2 in the HSQC-NOESY or ROESY spectra is consistent with this.

All of these data are consistent with a structure for the repeat unit of the Type 8 CPS of

→3)-β-D-ManNAcA(4OAc)-(1→3)-α-L-FucNAc-(1→3)-α-D-FucNAc-(1→ **4**

3.9. Peptidoglycan and other possible impurities

Analysis of the two dimensional NMR spectra showed the presence of spin systems, which were assigned as arising from Ala, Gln, or Glu, and Lys (Fig. 5, Table 3). The HSQC spectra of the de-*O*-acetylated Type 5 and 8 CPSs showed some evidence of two unsubstituted hydroxymethyl groups, which would be consistent with the presence of the β-GlcNAc and β-MurNAc residues present in the peptidoglycan.[35] Two minor crosspeaks observed in the anomeric region of the HSQC spectrum of the de-*O*-acetylated Type 8 CPS are consistent with the GlcNAc and MurNAc repeat unit in peptidoglycan, structure **5**.

→4)-β-D-GlcNAc-(1→4)-β-D-MurNAc-(1→ **5**

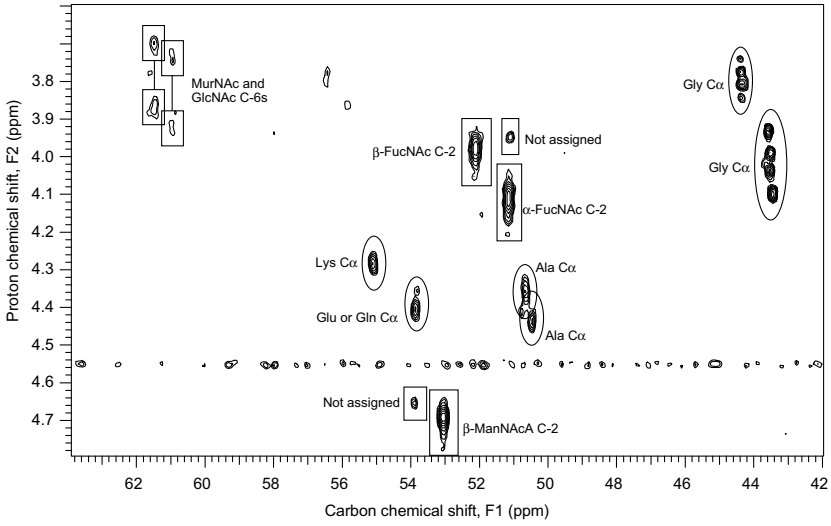

Figure 5. High-field region of the 500 MHz 150 Hz HSQC spectrum of the de-*O*-acetylated CPS from *S. aureus* Type 5 obtained at 50 °C showing the resonances from hydroxymethyl groups and CH–N systems. The resonances from the MurNAc and GlcNAc hydroxymethyl groups are shown in linked rectangles, the C-2s of the FucNAc and ManNAcA residues in single rectangles, and the lysine, alanine, glycine, and *iso*-glutamine spin systems in ellipses.

Table 3. NMR resonances from spin systems associated with peptidoglycan

Residue	Hα/Cα	Hβ/Cβ	Hγ/Cγ	Hδ/Cδ	Hε/Cε
Lysine	4.246	1.768	1.365	1.536	3.229
	55.25	31.69	23.45	28.97	40.13[a]
iso-Gln	4.380	2.169/1.998	2.41		
	54.00	28.27	32.70		
Ala(1)	4.42	1.42			
	50.62	17.91			
Ala(2)	4.39	1.41			
		19.34			
Ala(3)	4.33	1.45			
	50.78	17.91			
Ala(4)	4.30	1.45			
Ala(5) minor	4.24	1.39			
Gly(1)	3.77, 3.77		Gly(4)	3.99, 3.99	
	44.46			43.60	
Gly(2)	3.80, 3.80		Gly(5)	4.03, 4.03	
	44.42			43.61	
Gly(3)	3.93, 3.93		Gly(6)	4.09, 4.09	
	43.66			43.53	
	H-1/C-1	H-2/C-2	H-6/H-6′/C-6		
β-GlcNAc	4.59		3.74, 3.87		
	102.53		61.05		
β-MurNAc	5.22		3.74, 3.96		
	101.13		61.58		

[a] The low-field chemical shift of this resonance, compared to peptide model systems, reflects the *N*-acetylation with glycine and the formation of the inter-chain bridges in the peptidoglycan network.

Together, these indicate the presence of peptidoglycan, which may arise from the covalent attachment of the CPS, as has been found in *Streptococcus pneumoniae*.[36] No evidence was obtained for the presence of teichoic acid[37] in these preparations, which in *S. aureus* is linked to peptidoglycan through a phosphodiester.[38] A number of cell-wall associated proteins are also covalently attached to peptidoglycan[39] through disubstituted amino acids,[40] although our NMR methodology would be insensitive to their presence. Immune responses against these proteins may boost the efficacy of the vaccine.[39] No evidence was obtained for the presence in this sample of lipoteichoic acid,[41,42] or the immunogenic staphylococcal poly-β-*N*-acetylglucosamine.[43,44] No evidence for incomplete *N*-acetylation of any of the aminosugars in either serotype was observed.[19]

Acknowledgements

I would like to thank NABI Biotherapeutics (Rockville, MD) for the gift of the polysaccharides, for helpful discussions and permission to publish this work.

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