Carbohydrate Research, 201 (1990) 285–297
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Structure of the type 5 capsular polysaccharide of *Staphylococcus aureus*

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(Received June 7th, 1989; accepted for publication, October 1st, 1989)

ABSTRACT

The *Staphylococcus aureus* type 5 capsular polysaccharide is composed of 2-acetamido-2-deoxy-L-fucose (1 part), 2-acetamido-2-deoxy-D-fucose (1 part), and 2-acetamido-2-deoxy-D-mannuronic acid (1 part). On the basis of methylation analysis, optical rotation, high-field one- and two-dimensional ^{1}H- and ^{13}C-n.m.r. experiments, and selective cleavage with 70% aqueous hydrogen fluoride, the polysaccharide was found to be a partially *O*-acetylated (50%) polymer of the repeating trisaccharide unit, [→4)-3-*O*-Ac-β-D-Man*p*NAcA-(1→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Fuc*p*NAc-(1→]$_n$.

INTRODUCTION

Despite the extensive use of antibiotics, *Staphylococcus aureus* remains one of the major causes of hospital-related infections. Control of disease has proved difficult since bacteria have become resistant to a wide range of antibiotics[1,2], and an immunological approach has to be considered for prevention of infections caused by this pathogen.

Capsular polysaccharides are virulence factors of both Gram-positive and Gram-negative bacteria[3]. Karakawa and associates have shown that most isolates from patients with bacteremia were encapsulated[4] and established a method of typing the organisms[5]. The occurrence of a capsule has been confirmed by electron microscopy[6,7]. Among the 11 different types of *S. aureus* identified to date, two types, 5 and 8, account for 70% of the *S. aureus* disease isolates[8]. The structures of several of the capsular polysaccharides have been studied: Strain M (refs. 9, 10), strain Smith[11], and strain T (ref. 12). These polysaccharides cross react with staphylococcal polysaccharide types 1, 2 and 8, respectively. In this paper, we report on the structure of the staphylococcal type 5 capsular polysaccharide.

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EXPERIMENTAL

Nuclear magnetic resonance studies. — Spectra were recorded, at 37, 57, or 70°, for solutions (0.4 mL) in deuterium oxide (Merck, Sharp, and Dohme; 99.8 atom %^{2}H) of polysaccharide samples (30 mg/mL) that had been twice lyophilized from deuterium oxide, with Bruker AM-500 or AM-200 spectrometers, equipped with Aspect 3000 computers operating in the pulsed f.t. mode with quadrature detection using 5-mm probes. ^{1}H-N.m.r. spectra at 500 MHz were recorded by use of a spectral width of 2.5 kHz, and a 90° pulse. Before Fourier transformation, resolution enhancement was achieved by the Gaussian line-shape transformation[13] with typical values of −2.0 to −4.0 Hz for the line-broadening factor and a Gaussian broadening-factor of +0.1 or +0.2. Chemical shifts are expressed relative to the signal of internal 0.1% acetone (δ 2.225). N.O.e difference spectra[14] were obtained by use of a selective low-power presaturation pulse applied to the ^{1}H resonance for 200 ms, followed by a 90° observation pulse. Broad-band ^{1}H-decoupled ^{13}C spectra, recorded at 125 MHz, were obtained for a 25 kHz spectral width using a 90° pulse and composite-pulse decoupling[15] (WALTZ). Heteronuclear $^1J_{C,H}$ coupling constants for the anomeric carbon atoms were measured by use of gated decoupling[16]. Chemical shifts are referenced to the signal of external 1,4-dioxane (δ 67.67).

Two-dimensional, homonuclear-shift correlation (COSY) and relayed-coherence transfer (Relay COSY) were measured by use of the conventional pulse sequences[17–19]. Using matrices ($t_1 \times t_2$) of 256 × 2048 data points that were zero filled to 1024 × 2048 points. After resolution enhancement in both dimensions by means of a nonshifted, sine-bell window function[20], the doubly transformed data were processed to give magnitude spectra[21] with symmetrization[22]. A spectral width of 2370 Hz was employed to cover the whole spectrum, recycle delay of 1.8 s for both experiments, and a fixed delay of 32 ms for Relay COSY experiments. Thirty-two transients were collected for each value of the incrementable delay. Heteronuclear C–H chemical shift correlations were obtained by use of the CHORTLE pulse sequence as previously described[23].

Analytical methods. — The quantitative methods used were the method of Reissig *et al.*[24] for 2-amino-2-deoxyhexoses, the Park and Johnson method[25] for reducing sugars, the method of Chen *et al.*[26] for teichoic acid phosphate, and the Folin method[27] for proteins.

Monosaccharides were determined by g.l.c. (Program A) of their alditol acetate derivatives. Samples of capsular polysaccharide (2 mg) were dried overnight at 70° under vacuum in Teflon vials and solvolyzed with anhydrous HF (0.2 mL). After the mixture had been kept at room temperature overnight[28], HF was removed by evaporation *in vacuo* or under a stream of N_2. The residues were treated with 0.02M NaOH for 5 min at 100° and the released monosaccharides were applied to a column (1 × 10 cm) of AG 1 (OH^-) ion-exchange resin. Neutral monosaccharides were recovered by elution with water, and acidic monosaccharides by elution with 0.1M formic acid. The sugars were converted into alditol acetates by reduction with $NaBH_4$ and acetylation in presence of 1-methylimidazole[29] and the peracetylated derivatives were then recovered by extraction with dichloromethane.

The absolute configuration of the monosaccharides was determined on the carboxyl-reduced polysaccharide[30,31] and the HF solvolysis products[28]. Monosaccharides were released by hydrolysis with 4M HCl for 3 h at 100°, followed by *N*-reacetylation[35]. The (−)-2-butyl glycosides were prepared according to the procedure of Gerwig *et al.*[31], and analyzed by g.l.c.–m.s. of the corresponding acetylated (2-acetamido-2-deoxymannose) derivatives by use of program C.

Trisaccharide A (4 mg) was reduced with $NaBH_4$ and the product treated with 2M methanolic HCl for 16 h at 80°. The liberated methyl glycosides were hydrolyzed and converted into the peracetylated (−)-2-butyl 2-acetamido-2-deoxyglycosides[31].

Chromatographic methods. — *(a) Paper electrophoresis.* Acidic monosaccharides were separated by high-voltage electrophoresis on a Shandon Model L24 apparatus using Whatman 1 MM paper in 5:2:293 pyridine–acetic–water buffer at pH 5.3 for 45 min (2000 V/60 cm) or in 1% $K_3B_2O_7$ buffer at pH 9.2 for 60 min (2000 V/60 cm), and detected by the $AgNO_3$ reagent[32]. Reference components were 2-acetamido-2-deoxy-D-mannuronic acid obtained by HF solvolysis of *Escherichia coli* K7 polysaccharide[33] (donated by Dr. W. Egan, FDA), 2-acetamido-2-deoxy-D-galacturonic acid obtained from the Vi antigen[34] (donated by Dr. J. B. Robbins, NIH), and D-glucuronic acid.

(b) Gas–liquid chromatography. G.l.c. was performed with a Perkin–Elmer Sigma 3B gas chromatograph under the following conditions: (*A*) A glass column (2 mm × 2 m) containing 3% OV 225 and the temperature program 180–210° at 2°.min;$^{-1}$ and (*B*), a fused-silica capillary column (0.3 mm × 25 m) containing 3% OV 17 and the temperature program 180–240° at 2°.min^{-1}. (*C*) Other analyses were performed with a Hewlett–Packard model 5710A gas chromatograph equiped with a fused-silica capillary column (0.3 mm × 25 m) containing 3% OV 17 and the temperature program 180 (delay 2 min)–240° at 2°.min^{-1}. Retention times are reported relative to that of peracetylated inositol (T_{IA}) or 1,4,5-tri-*O*-acetyl-2,6-dideoxy-3-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol (T_{FM}). G.l.c.–m.s. was done with a Hewlett-Packard 5985 system employing conditions (*B*) and (*C*) described above.

(c) Gel filtration. Gel filtration of polysaccharide was carried out on columns of Sepharose 4B CL (2 × 85 cm) and Sephacryl S300 (2 × 85 cm), eluted with 0.02M ammonium acetate at pH 6. Oligosaccharides were separated on a column (2 × 100 cm) of Bio-Gel P-2 (200–400 mesh) eluted with 0.02M ammonium acetate. Column effluents were monitored with a Waters R403 differential refractometer and fractions were assayed for reducing sugar[25]. The distribution coefficients K_{AV} of the eluted materials were determined relative to that of 2-acetamido-2-deoxy-D-glucose.

Preparation of S. aureus *type 5 polysaccharide.* — The polysaccharide was prepared according to the method described by Fournier *et al.*[36]. *S. aureus* type 5 (strain Reynolds) was cultivated overnight on Columbia medium agar (Difco), the cells were suspended in phosphate-buffered saline (PBS), and autoclaved for 60 min at 120°. The suspension was centrifuged and the supernatant was incubated at 37° with deoxyribonuclease and ribonuclease (Sigma) for 6 h, and then with protease overnight. The crude polysaccharide was recovered after dialysis against de-ionized water and lyophilized.

Crude polysaccharide was separated from contaminating teichoic acid[36] on an

ion-exchange DEAE-Sephacel (Pharmacia) column, equilibrated with 0.05M sodium acetate (pH 6) and eluted with 0.15M NaCl in 0.05M sodium acetate. The teichoic acid was eluted with 0.2M NaCl in the same buffer. The polysaccharide-containing fractions, detected by capillary precipitation with anti-type 5 serum, were further purified by repeated gel filtration on Sepharose 4B CL.

Carboxyl group reduction of type 5 capsular polysaccharide. — The polysaccharide was reduced according to the method described by Taylor and Conrad[30]. To a solution of the polysaccharide (20 mg) in water (10 mL) was added 1-ethyl-3-(3-methylaminopropyl)carbodiimide·HCl (260 mg) and the pH of the solution was maintained at pH 4.75 by addition of 0.1M HCl. When no more acid was consumed ($\sim$ 1 h), the pH was adjusted to pH 7, and a solution (20 mL) of $NaBH_4$ (10 mg·mL^{-1}) was added dropwise while the pH was maintained at pH 6.5–7.5 by the addition of 4M HCl. The reduced product was dialyzed and the reduction procedure was repeated a second time. The extent of reduction was monitored by colorimeric analysis for 2-amino-2-deoxyhexose.

O-*Deacetylation.* — The type 5 polysaccharide was treated with 0.1M NaOH for 15 h at 37°, neutralized, desalted on Dowex 50 (H^+) cation-exchange resin, and lyophilized.

Solvolysis of type 5 polysaccharide — The *O*-deacetylated type 5 polysaccharide (10 mg) was treated with 70% aqueous HF (0.5 mL), which had been prepared by distillation of anhydrous HF onto a known volume of ice. After 3 h at 4°, HF was promptly removed from the solution by evaporation under a stream of N_2. Oligosaccharides were separated by filtration on a Bio-Gel P-2 column and purified by paper electrophoresis to afford Trisaccharide **A**.

Permethylation analysis. — Polysaccharide and oligosaccharide samples (2–5 mg) were methylated in dimethyl sulfoxide solution (0.5 mL) by use of potassium methylsulfinylmethanide (0.5 mL) and methyl iodide (0.3 mL) according to the Hakomori procedure[37], and the permethylated products were recovered by extraction with dichloromethane. The methylated polysaccharide was solvolyzed with anhydrous HF for 7 h at room temperature. The residues were reduced and acetylated as described above and analyzed by g.l.c.–m.s. (programs *B* and *C*).

Nitrous acid deamination. — The carboxyl-reduced polysaccharide was *N*-deacetylated by hydrolysis with 4M HCl for 2 h at 100° followed by evaporation to dryness. The residue was treated with 5% $NaNO_2$ (0.5 mL) and 33% acetic acid (0.5 mL). After 45 min at room temperature, the solution was desalted on Dowex AG 50 (H^+) at cation-exchange resin[38]. The released components were reduced and peracetylated for g.l.c. analysis (program *A*). Reference components were prepared similarly by deamination of 2-amino-2-deoxy-D-mannose, 2-amino-2-deoxy-D-galactose, and aminodeoxyhexoses obtained from the capsular polysaccharide[39] of *Pneumococcus* Type 4.

RESULTS AND DISCUSSION

Composition. — The acidic staphylococcal type 5 polysaccharide was purified free

of teichoic acid by phosphate and antibody detection on DEAE-Sephacel and by repeated gel filtration on Sephacryl S300 or Sepharose 4B CL. Neither 2-acetamido-2-deoxyglucose nor ribitol[40] could be detected by g.l.c. analysis. The polysaccharide was found to be a high-molecular-weight polymer as indicated from its elution point on Sepharose 4B CL (K_{av} 0.6) and Sephacryl S300 (K_{av} 0.3), and showed $[\alpha]_D$ −101° (*c* 1, water).

S. aureus type 5 was found to be particularly resistant to hydrolysis under a variety of acidic conditions, but solvolysis with anhydrous hydrogen fluoride[28] (15 h, 20°) yielded 90% of reducing sugar and 90% of 2-acetamido-2-deoxyglycose by colorimetric assays, suggesting near complete cleavage of the polymer. These results indicated that the polysaccharide is composed only of acetamidodeoxy sugars. G.l.c.–m.s. analysis of the monosaccharides released by hydrogen fluoride treatment of the native polysaccharide showed a single component, which was identified as per-*O*-acetyl-2-amino-2,6-dideoxygalactitol from its retention time (T_{IA} 0.91) and its m.s. fragmentation pattern. A single acidic component was detected by paper electrophoresis of sodium hydroxide treated hydrogen fluoride solvolyzate. The component mobilities at pH 5.5 (R_{GA} 0.88) and pH 9.2 (R_{GA} 0.71) were identical with those of an authentic sample of 2-acetamido-2-deoxy-D-mannuronic acid. In agreement with this finding, 2-amino-2-deoxymannose was identified in solvolyzates of the carboxyl-reduced polysaccharide as its alditol acetate derivative by g.l.c.–m.s. (T_{IA} 1.76, program *A*) in addition to 2-amino-2,6-deoxygalactose. Treatment of these hydrolyzates with nitrous acid gave glucose.

The absolute configurations of the monosaccharide units in the carboxyl-reduced polysaccharide were determined according to the method of Gerwig *et al.*[34]. 2-Acetamido-2,6-dideoxy D- and L-galactose were identified in approximately equal molar amounts, and the 2-acetamido-2-deoxymannose was found to have the D-configuration. These results indicated a repeating trisaccharide unit composed of 2-acetamido-2,6-dideoxy-D-galactose, 2-acetamido-2,6-dideoxy-L-galactose, and 2-acetamido-2-deoxy-D-mannuronic acid.

N.m.r. assignments. — The ^{1}H- and ^{13}C-n.m.r. spectra of the native type 5 capsular polysaccharide were complex owing to partial *O*-acetylation as evidenced by the alkali-sensitive signals observed at δ 2.0 and 21.2. The ^{13}C-n.m.r. spectrum of the *O*-deacetylated polysaccharide (Fig. 1B) showed three signals at δ 100.3, 100.8, and 102.4 corresponding to the three anomeric carbons of the repeating trisaccharide unit. The sets of signals in the region characteristic for C-2 of a 2-acetamido-2-deoxyhexose unit (δ 51.1, 52.2, 52.8), the methyl carbon signals (δ 23.7, 24.0, 24.2) for the *N*-acetyl groups, and the signals at δ 16.2 and 16.3 for C-6 of the 6-deoxyhexoses, suggested that the repeat unit contains three acetamidodeoxy sugar residues, of which two are 6-deoxyhexoses. A more detailed description of the repeat structure was obtained from the 2D-n.m.r. experiments described below.

Complete assignment of the proton resonances of the ^{1}H-n.m.r. spectrum of the *O*-deacetylated polysaccharide (Fig. 2) was achieved by 2-D-homonuclear chemical-

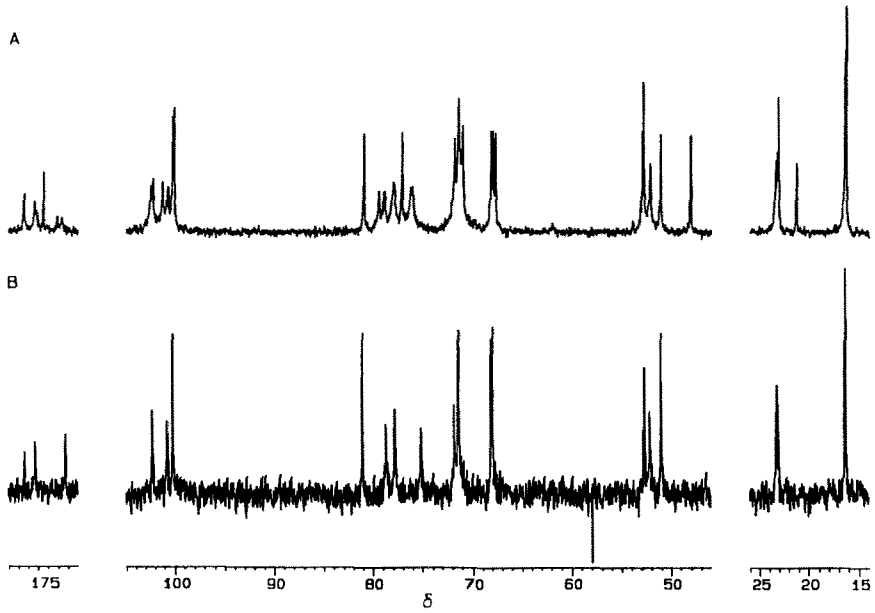

Fig. 1. ^{13}C-N.m.r. spectra of the type 5 *Staphyloccocus aureus* capsular polysaccharide recorded at 57°: (A) Native polysaccharide; (B) *O*-deacetylated polysaccharide.

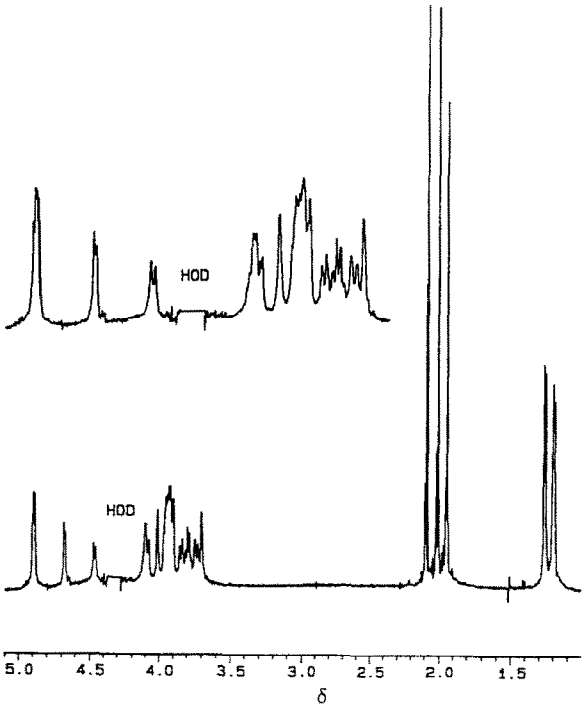

Fig. 2. ^{1}H-N.m.r. spectrum for the ring-proton region of the *O*-deacetylated type 5 *Staphyloccocus aureus* capsular polysaccharide recorded at 70°. Inset: expansion of the ring-proton region (δ 5.0–3.6).

TABLE I

^{1}H-Chemical shifts (δ) for the type 5 *O*-deacetylated staphylococcal polysaccharide[a]

Residue	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*
→4)-α-L-Fuc*p*NAc(1→	4.93(3.8)[b]	4.10(9)[c]	3.94	4.05	4.12	1.22
→4)-β-D-Man*p*NAcA(1→	4.92(<1)[b]	4.71(3)[c]	3.94	3.85	3.94	–
→3)-β-D-Fuc*p*NAc(1→	4.48(8.3)[b]	3.97(9)[c]	3.76	3.77	3.82	1.30

[a] Recorded at 57° with acetone as internal standard. [b] Vicinal couplings, $J_{1,2}$ (Hz), in parentheses. [c] Vicinal couplings, $J_{2,3}$ (Hz), in parentheses.

shift-correlation experiments (COSY and Relay COSY), (see Table I). Four ^{1}H resonances were observed in the anomeric region (δ 5.0–4.4) of the spectrum: two unresolved signals centered at δ 4.93, and two doublets at δ 4.71 (J 3.0 Hz) and 4.48 (J 8.3 Hz) (Fig. 2). From the values of the coupling constants, $J_{1,2}$, determined from the appropriate cross-peaks in the 2D-n.m.r. spectra (Table I), it was evident that the H-1 signal corresponding to a 2-acetamido-2,6-dideoxy-α-L-galactosyl residue at δ 4.93 ($J_{1,2}$ 3.8 Hz) was overlapping the anomeric singlet of the 2-acetamido-2-deoxy-D-mannosyluronic acid residue (δ 4.92, $J_{1,2} < 1$ Hz). The coupling constant ($J_{1,2}$ 8.3 Hz) of the H-1 signal at δ 4.48 was indicative of the other 2-acetamido-2,6-dideoxy-β-D-galactosyl residue. Identification of a connectivity between the singlet at δ 2.92 and the doublet (J 3.0 Hz) at δ 4.71 in the COSY spectrum indicated unambiguously that the latter signal corresponds to H-2 of the D-mannosyluronic residue, the small values of the coupling constants $J_{1,2}$ and $J_{2,3}$, being indicative of the *manno*-pyranosyl configuration[41]. The magnitude of the $J_{1,2}$ constant did not permit one to establish the anomeric configuration of the 2-acetamido-2-deoxymannosyluronic acid residue. This configuration was determined from the $^1J_{C,H}$ value[42] which was measured from the proton-coupled ^{13}C-n.m.r. spectrum. A CHORTLE experiment was carried out to identify the connectivities between each proton and the resonance of its directly attached carbon atom. The ^{13}C-resonance assignments are given in Table II. The ^{13}C signal at δ 100.8 was correlated to the H-1 resonance of the 2-acetamido-2-deoxy-D-mannosyluronic acid residue and the $^1J_{C,H}$ value of 164 Hz indicated the β-D configuration[43]. Thus, the trisaccharide

TABLE II

^{13}C-Chemical shifts (δ) and anomeric $^1J_{C,H}$ values (Hz)[b] for the type 5 *O*-deacetylated staphylococcal polysaccharide[b]

Residue	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*
→4)-α-L-Fuc*p*NAc-(1→	102.4(171)	51.1	71.5	81.2	68.4
→4)-β-D-Man*p*NAcA-(1→	100.8(164)	52.8	68.3	78.9	75.4
→3)-β-D-Fuc*p*NAc-(1→	100.3(164)	52.2	78.07	71.6	72.0

[a] In parentheses, [b] Recorded at 57° with external 1,4-dioxane as standard.

TABLE III

G.l.c.–m.s. methylation analysis of the carboxyl-reduced type 5 polysaccharide

Alditol acetate derivative	T_{PM}[a]	*Fragmentation*[b] *(m/z)*	*Molar proportion (%)*
1,4,5-Tri-*O*-acetyl-2,6-deoxy-3-*O*-methyl-2-(*N*-methylacetamido)galactitol	0.95	87,116,143,158,203	34
1,3,5-Tri-*O*-acetyl-2,6-deoxy-4-*O*-methyl-2-(*N*-methylacetamido)galactitol	1.00	116,131,158,242,274,288	46
1,4,5-Tri-*O*-acetyl-2-deoxy-3-*O*-methyl-2-(*N*-methylacetamido)mannitol	1.05	116,142,158,202,233	19

[a] G.l.c. program *B*. [b] Characteristic m.s. fragment ions.

repeating unit of the type 5 polysaccharide was composed of one 2-acetamido-2,6-dideoxy-α-L- and one -β-D-galactopyranosyl residue, and one acetamido-2-deoxy-β-D-mannopyranosyluronic acid residue.

Substitution pattern. — In order to determine the linkage positions of the monosaccharides in the repeating unit, the carboxyl-reduced type 5 polysaccharide was subjected to methylation analysis. The permethylated polysaccharide was cleaved with anhydrous HF and the components were analyzed as their alditol acetate derivatives by g.l.c.–m.s.. Three aminodeoxyalditols were detected by selective-ion monitoring at *m/z* 158 (Table III). The mass spectra were indicative of derivatives derived from 3-*O*- and 4-*O*-substituted 2-acetamido-2,6-dideoxygalactosyl, and 4-*O*-substituted 2-acetamido-2-deoxymannosyl residues. The sequence of these residues in the polysaccharide was determined by n.O.e. measurements on the *O*-deacetylated polymer and from specific chemical degradation studies.

Owing to the overlapping ^{1}H-1 signals of the 2-acetamido-2,6-dideoxy-α-L-galactosyl and the 2-acetamido-2-deoxy-β-D-mannosyluronic acid residues, the use of n.O.e. difference experiments for completely sequencing the polysaccharide was difficult. Nevertheless, saturation of the ^{1}H-1 signal of the 2-acetamido-2,6-dideoxy-α-L-galactosyl residue resulted in an *inter*-residue n.O.e. at H-4 (δ 3.85) of the 2-acetamido-2-deoxy-β-D-mannosyluronic acid residue, establishing the presence of the partial sequence, →)-β-Fuc*p*NAc-(1→4)-β-D-Man*p*NAcA (1→, in the repeating unit. Confirmation of this result was obtained by cleavage of the *O*-deacetylated polysaccharide with 70% aqueous HF to afford Oligosaccharide **A** which was isolated by gel filtration on Bio-Gel P-2 (Fig. 3); the major component had a K_{AV} 0.71 and a mobility on paper electrophoresis at pH 5.5 of R_{GA} 0.48.

The ^{13}C-n.m.r. spectrum of reduced Oligosaccharide **A** suggested a trisaccharide. Three signals were observed in the low-field region of the ^{1}H-n.m.r. spectrum of reduced Trisaccharide **A** (Fig. 4) at δ 4.90, (d,$J_{1,2}$ 3.6 Hz), 4.85(s), and 4.64 (d,J 3 Hz), which could readily be assigned to H-1 of the 2-acetamido-2,6-dideoxy-α-L-galactosyl residue,

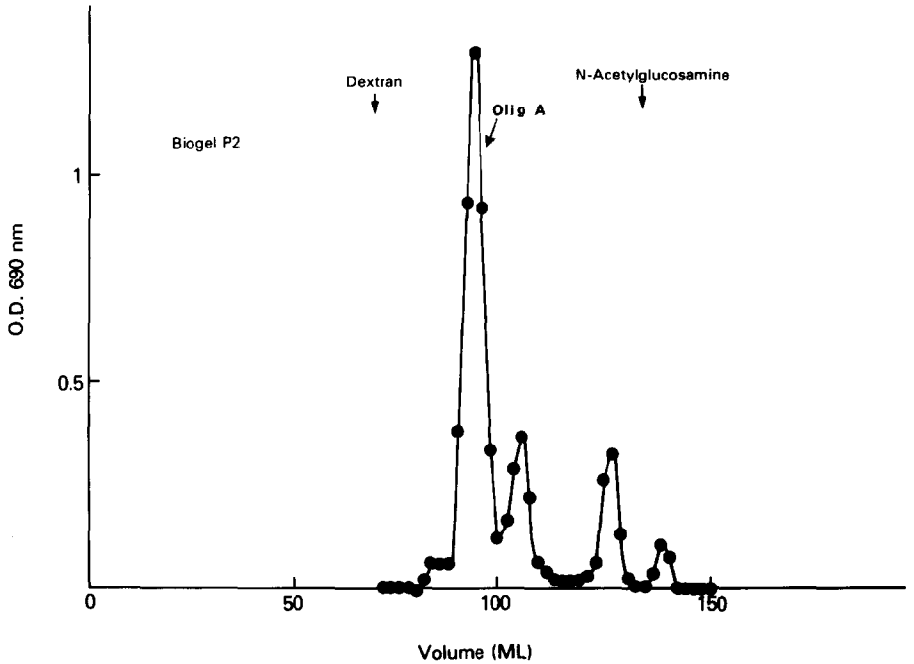

Fig. 3. Gel filtration on Bio-Gel P-2 (200 mL) of the solvolysis products in aqueous hydrofluoric acid of type 5 polysaccharide.

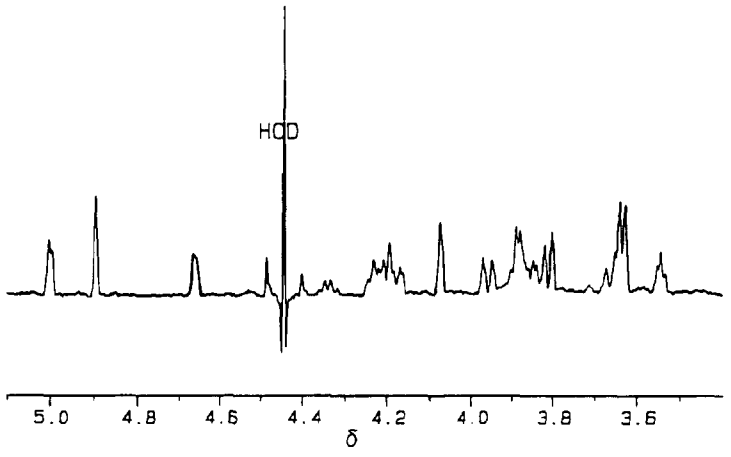

Fig. 4. ^{1}H-N.m.r. spectrum for the ring-proton region of reduced Trisaccharide A recorded at 37°.

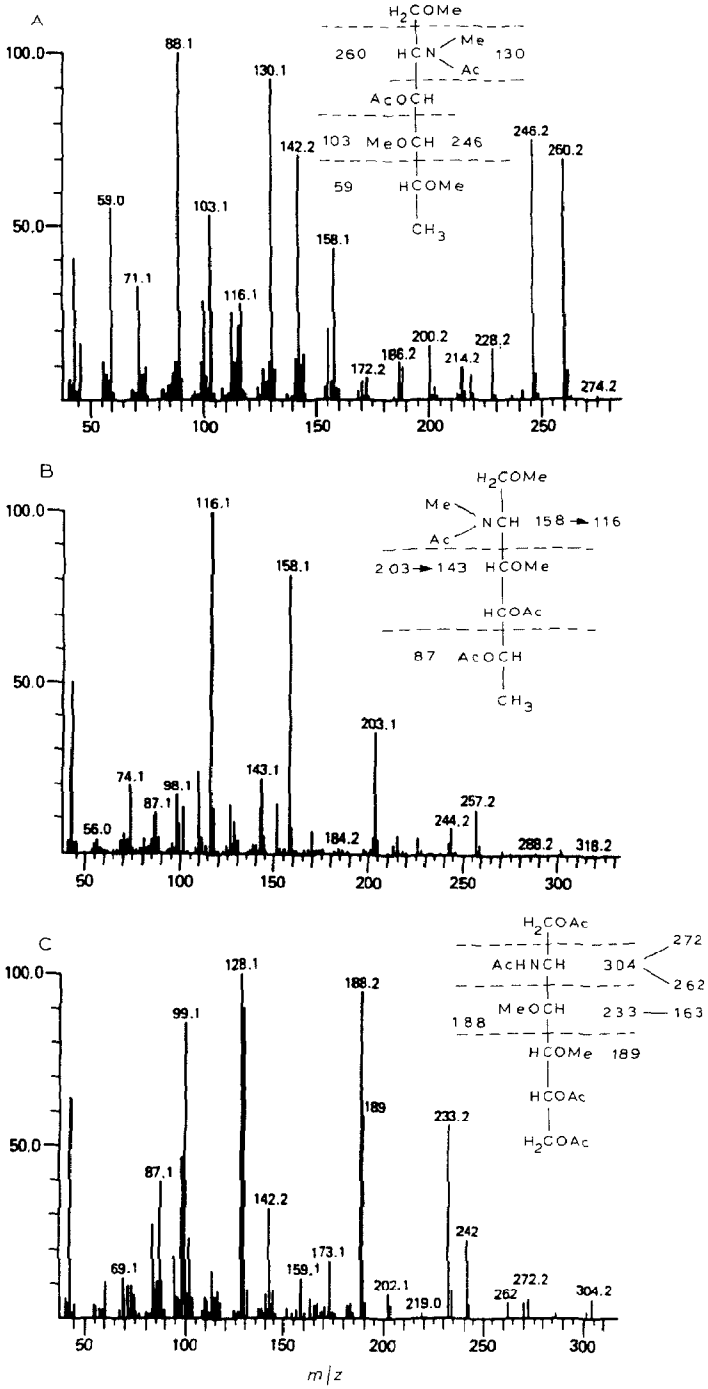

Fig. 5. Mass spectra and fragmentation patterns of the methylated products derived from reduced Trisaccharide **A** after solvolysis, reduction, and acetylation.

and to H-1 and H-2 of the 2-acetamido-2-deoxy-β-D-mannosyluronic acid residue, respectively. These assignments, together with the absence of ^{1}H resonances in the δ 4.4–4.6 region of the spectrum, indicated that the hydrolysis conditions (70% aqueous HF) led to almost exclusive cleavage of the 2-acetamido-2,6-dideoxy-β-D-galactosyl residue in the intact polysaccharide.

The sequence of Trisaccharide **A** was determined by methylation analysis of the reduced oligosaccharide. Three components were detected by g.l.c.–m.s. (program *D*); they were characterized (Scheme 1) from their m.s. fragmentation patterns as 3-*O*-acetyl-2,6-dideoxy-1,4,5-*O*-methyl-2-(*N*-methylacetamido)galactitol (T_{FM} 0.5), 1,3,5-tri-*O*-acetyl-2,6-dideoxy-4-*O*-methyl-2-(*N*-methylacetamido)galactitol (T_{FM} 1.00), and 1,5,6-tri-*O*-acetyl-2-deoxy-3,4-di-*O*-methyl-2-(*N*-acetamido)mannitol (T_{FM} 1.33). These results established that the 3-*O*-substituted 2-acetamido-2,6-dideoxygalactosyl residue is the terminal reducing residue, that the 4-*O*-substituted 2-acetamido-2,6-dideoxy-α-L-galactosyl residue occupies the central position, and that the 2-acetamido-2-deoxy-β-D-mannosyluronic acid group is at the nonreducing end; thus, reduced Trisaccharide **A** has structure **1**. Combined with the aforementioned n.O.e. result, the identification of the structure of the trisaccharide established unambiguously the

β-D-Man*p*NAcA-(1→4)-α-Fuc*p*NAc-(1→3)-Fuc*p*NAcol

1

sequence of the repeating unit of the staphylococcal type 5 polysaccharide as, →4)-β-D-Man*p*NAcA-(1→4)-α-Fuc*p*NAc-(1→3)-β-Fuc*p*NAc-(1→.

The absolute configurations of the 2-acetamido-2,6-dideoxy-α- and -β-galactosyl residues were determined[31] on the reduced trisaccharide that contained a single 2-acetamido-2,6-dideoxygalactosyl residue. G.l.c.–m.s. analysis showed peracetylated derivatives corresponding to only (*R*)-2-butyl-2-acetamido-2-deoxy-L-galactoside, clearly indicating that the →4)-α-Fuc*p*NAc-(1→ residue in the trisaccharide is the L enantiomer. Since a similar analysis of the polysaccharide indicated that both the L and the D forms of these aminodeoxyhexoses are present in the repeating unit, it follows that the →3)-β-Fuc*p*NAc-(1→ residue has the D chirality. Thus, the type 5 capsular polysaccharide has the structure; →4)-β-D-Man*p*NAcA-(1→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Fuc*p*NAc(1→. The anomeric and absolute configurations of the monosaccharide components within this structure are consistent with the high negative value of the optical rotation ($[\alpha_D]$ −101°) of the polysaccharide. The presence of both the D- and L-enantiomers of 2-acetamido-2,6-dideoxygalactose in the same oligomer is relatively rare, but has been previously observed as a structural feature of the O-chain of the LPS from *Pseudomonas aeroginosa*[44] and of the *S. aureus* type 8 polysaccharide[45].

Location of the O-*acetyl group.* — Comparison of the ^{13}C-n.m.r. spectra of the native and *O*-deacetylated polysaccharides (Fig. 1) revealed that removal of the *O*-acetyl group resulted in a two-fold increase of the C-2 resonance at δ 51.1 of the 2-acetamido-2-deoxy-β-D-mannosyluronic acid residue and a loss of a signal at δ 48.1.

The observed downfield-shift (3 p.p.m.) to give an increase in the signal at δ 51.1 suggested that the *O*-acetyl substituent was located on the carbon atom (C-3) adjacent to the acetamido group of the 2-acetamido-2-deoxy-β-D-mannosyluronic acid residue in the native polysaccharide.

In conclusion, the structure of the *S. aureus* type 5 polysaccharide was determined to be **2**. The β-D-(1→4) linked 2-acetamido-2,6-dideoxygalactosyl residues were found to be more labile to 70% HF than other linkages in the polysaccharide. The type 5 and type 8 (ref. 45) polysaccharides are associated with a major portion of *S. aureus* clinical isolates; they are composed of the same aminodeoxyhexosyl residues, but differ in mode of substitution and the nature of the anomeric configuration of one of the 2-acetamido-2,6-dideoxygalactose units.

$$[\rightarrow 4)\text{-}3\text{-}O\text{-Ac-}\beta\text{-D-Man}p\text{NAc-}(1\rightarrow 4)\text{-}\alpha\text{-L-Fuc}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-Fuc}p\text{NAc-}(1\rightarrow)]_n$$

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ACKNOWLEDGMENTS

The authors thank Dr. Perry for his kindness in permitting this work to be completed and Mr. P. F. Cooper for g.l.c.–m.s. analyses.

REFERENCES

1 K. Rosendal, O. Jessen, V. Faber, and M. W. Bentzon, *Scand. J. Infect. Dis., Suppl.*, 41 (1983) 19–29.
2 J. N. Sheagren, *N. Eng. J. Med.*, 310 (1984) 1368–1373.
3 H. Smith, *Bacteriol. Rev.*, 41 (1977) 475–500.
4 W. W. Karakawa and W. F. Vann, *Semin. Infect. Dis.*, 4 (1982) 285–293.
5 W. W. Karakawa, J. M. Fournier, W. F. Vann, R. Arbeit, R. Schneerson, and J. B. Robbins, *J. Clin. Microbiol.*, 22 (1985) 445–447.
6 D. Sompolinski, Z. Samra, W. W. Karakawa, W. F. Vann, R. Schneerson, and Z. Malik, *J. Clin. Microbiol.*, 22 (1985) 828–834.
7 H. K. Hochkeppel, D. G. Braun, W. Vischer, A. Imm, S. Sutter, U. Staeubli, R. Guggenheim, E. L. Kaplan, A. Boutonnier, and J.-M. Fournier, *J. Clin. Microbiol.*, 25 (1987) 526–530.
8 R. D. Arbeit, W. W. Karakawa, W. F. Vann, and J. B. Robbins, *Diagn. Microbiol. Infect. Dis.*, 2 (1984) 85–91.
9 S. V. Murthy, M. A. Melly, T. M. Harris, C. G. Hellerqvist, and J. H. Hash, *Carbohydr. Res.*, 117 (1983) 113–123.
10 D. F. Liau and J. H. Hash, *J. Bacteriol.*, 131 (1977) 194–200.
11 S. Hanessian and T. H. Haskell, *J. Biol. Chem.*, 239 (1964) 2758–2764.
12 T. C. M. Wu and J. T. Park, *J. Bacteriol.*, 108 (1971) 874–884.
13 A. G. Ferrige and J. C. Lindon, *J. Magn. Reson.*, 31 (1978) 337–340.
14 R. Richarz and K. Wuthrich, *J. Magn. Reson.*, 30 (1978) 147–150.
15 A. J. Shaka, J. Keeler, T. Frenkiel, and R. Freeman, *J. Magn. Reson.*, 52 (1983) 335–338.
16 D. A. Gansow and W. Schittlenhelm, *J. Am. Chem. Soc.*, 93 (1971) 4294–4295.
17 W. P. Aue, E. Bartholdi, and R. R. Ernst, *J. Chem. Phys.*, 64 (1976) 2229–2246.
18 A. Bax, R. Freeman, and G. Morris, *J. Magn. Reson.*, 42 (1981) 164–168.
19 A. Bax and R. Freeman, *J. Magn. Reson.*, 44 (1981) 542–561.
20 A. de Marco and K. Wuthrich, *J. Magn. Reson.*, 24 (1976) 201–204.
21 L. C. Lindon and A. G. Ferrige, *J. Magn. Reson.*, 36 (1979) 277–280.

22 R. Baumann, G. Wilder, R. R. Ernst, and K. Wuthrich, *J. Magn. Reson.*, 44 (1981) 402–406.
23 M. Moreau, J. C. Richards, M. B. Perry, and P. J. Kniskern, *Carbohydr. Res.*, 182 (1988) 79–99.
24 J. L. Reissig, J. L. Strominger, and L. F. Leloir, *J. Biol. Chem.*, 217 (1956) 959–964.
25 J. T. Park and M. J. Johnson, *J. Biol. Chem.*, 181 (1949) 149–151.
26 P. S. Chen, T. Y. Toribara, and M. Warner, *Anal. Chem.*, 28 (1956) 1756–1758.
27 O. H. Lowry, J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
28 A. J. Mort and D. T. A. Lamport, *Anal. Biochem.*, 82 (1977) 289–309.
29 R. J. Henry, A. B. Blakeney, P. J. Harris, and B. A. Stone, *J. Chromatogr.*, 256 (1983) 419–427.
30 R. L. Taylor and H. E. Conrad, *Biochemistry*, 11 (1972) 1383–1388.
31 G. J. Gerwig, J. P. Kamerling, and J. F. G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
32 W. E. Trevelyan, D. P. Procter, and J. S. Harrison, *Nature (London)*, 166 (1950) 444–445.
33 F. P. Tsui, R. A. Boykins, and W. Egan, *Carbohydr. Res.*, 102 (1982) 263–271.
34 J. D. Robbins and J. B. Robbins, *J. Infect. Dis.*, 150 (1984) 436–449.
35 G. A. Levvy and A. McAllen, *Biochemistry*, 73 (1959) 127–132.
36 J. M. Fournier, K. Hannon, M. Moreau, W. W. Karakawa, and W. F. Vann, *Ann. Inst. Pasteur Microbiol.*, 138 (1987) 561–567.
37 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
38 B. A. Dmitriev, L. V. Bakinowsky, V. L. Lvov, and N. K. Kotchetkov, *Eur. J. Biochem.*, 50 (1975) 539–547.
39 P. E. Jansson, B. Lindberg, and U. Lindquist, *Carbohydr. Res.*, 95 (1981) 73–80.
40 J. Coyette and J. M. Ghuysen, *Biochemistry*, 7 (1968) 2385–2389.
41 C. Altona and C. P. G. Haasnoot, *Org. Magn. Reson.*, 13 (1980) 417–428.
42 K. Bock and H. Thogersen *Annu. Rep. NMR Spectrosc.*, 13 (1982) 1–57.
43 K. Leontein, B. Lindberg, and J. Lönngren, *Can. J. Chem.*, 59 (1981) 2081–2085.
44 B. A. Dmitriev, Y. A. Knirel, N. A. Kochrova, N. K. Kochetkov, E. S. Stanislavsky, and G. M. Mashilova, *Eur. J. Biochem.*, 106 (1980) 643–651.
45 M. Moreau and W. F. Vann, unpublished results.