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THE REPEATING SEQUENCE OF THE CAPSULAR POLYSACCHARIDE OF *Staphylococcus aureus* M*

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ABSTRACT

The anomeric configuration of the sugar residues of the capsular polysaccharide antigen of *Staphylococcus aureus* M were established by ^{13}C-n.m.r. spectroscopy, and the linkage positions by g.l.c.–m.s. after methylation, indicating a →4)-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyluronic acid)-(1→4)-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyluronic acid)-(1→3)-*O*-(2-acetamido-2-deoxy-α-D-fucopyranosyl)-(1→ repeating unit. A taurine residue is linked by an amide bond, on the average, to every fourth 2-acetamido-2-deoxy-D-galactopyranosyluronic acid residue.

INTRODUCTION

The isolation and identification of the components of the capsular-polysaccharide antigen of *Staphylococcus aureus* M[1,2] have been previously reported. This polysaccharide is composed of taurine, 2-acetamido-2-deoxy-D-fucose, and 2-acetamido-2-deoxy-D-galacturonic acid in the molar ratios of 1:2:4. The present report describes the anomeric configurations and linkage positions of the sugar residues of the repeating unit of the polysaccharide.

EXPERIMENTAL

General. — Unless otherwise indicated, the materials and methods used in the isolation and identification of the components of the capsular polysaccharide of

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S. aureus M are the same as previously described[1-3]. Other materials used in this study were obtained from the following sources: sodium hydride and sodium borohydride from Metal Hydrides Inc. (Beverly, MA 01915); sodium borodeuteride and deuterium oxide from Aldrich Chemical Co., Inc. (Milwaukee, WI 53233); dimethyl sulfoxide and 3-(dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride from Pierce Chemical Co. (Rockford, IL 61105); and methyl iodide from Fisher Scientific Co. (Pittsburgh, PA 15219).

Purification of the capsular polysaccharide. — The isolation of *S. aureus* M polysaccharide was modified from that previously described[1] as follows. Cultures were grown in brain–heart infusion broth (1000 mL) (Difco Laboratories, Detroit, MI 48232) in 2-L flasks. Incubation was at 37° on a rotary shaker for 20 h. Cells were harvested by centrifugation at 10 000*g* for 30 min and used without washing. The packed cells were resuspended in 10% trichloroacetic acid (3–5% of the initial culture volume) and heated at 85–90° for 30 min with magnetic stirring. Cellular debris was sedimented by centrifugation at 25 000*g* for 40 min. The pellet was discarded because additional extractions failed to improve the yield of capsular polysaccharide. The yellowish-brown supernatant was dialyzed for 48 h at 4° with several changes of distilled water.

The dialyzed material was evaporated to dryness *in vacuo*, the residue was redissolved in a minimum volume of distilled water, and absolute ethanol (4–5 vol.) was slowly added at room temperature. The resulting precipitate was collected by centrifugation at 25 000*g*. The supernatant solution was discarded and the pellet was carefully dissolved in a minimum volume of 0.5M sodium chloride. Slow addition of absolute ethanol (4 vol.) again precipitated the capsular polysaccharide, which was collected by centrifugation as described earlier. It was dissolved in a minimum volume of distilled water and the solution lyophilized. This crude material was slightly brown and served as starting material for the isolation of the pure polysaccharide. The polysaccharide had the tendency to form clear, thin films in test tubes, and it was important to dissolve precipitates by vigorous mixing with a Vortex mixer in order to prevent losses.

Amino acid analyses of the crude material revealed the presence of taurine, 2-amino-2-deoxygalacturonic acid, 2-amino-2-deoxyfucose, large proportions of alanine and 2-amino-2-deoxyglucose, and traces of amino acids derived from proteins. The presence of alanine and 2-amino-2-deoxyglucose indicated a contamination by teichoic acid. The yield of crude, lyophilized material was approximately 50 mg/L. In addition to methods previously used to remove teichoic acid[1], including depolymerization of the teichoic acid with hot 10% trichloroacetic acid, various chromatographic procedures were tried. The method that consistently gave the best results for the quantitative removal of teichoic acid from the capsular polysaccharide was chromatography on QAE-Sephadex A-50 (Pharmacia Inc., Piscataway, NJ 08854). A typical purification is as follows. Dry QAE-Sephadex A-50 was soaked in 0.01M hydrochloric acid overnight and a column (1.4 × 26 cm, packed dimensions) was prepared. The lyophilized, crude polysaccharide (100 mg) was

dissolved in 0.01M hydrochloric acid (3–4 mL) and any insoluble material removed by centrifugation. After loading the sample onto the column, it was eluted stepwise with 3–4 column-volumes each of 0.01M hydrochloric acid, and 0.25M and 0.5M sodium chloride. The QAE-Sephadex A-50 shrank considerably during the sodium chloride elution-steps, precluding the use of a concentration gradient. Amino acid analysis of the fractions indicated that small proportions of contaminating proteins were eluted with 0.01M hydrochloric acid, teichoic acid was quantitatively recovered in the 0.25M sodium chloride eluate, and the capsular polysaccharide was recovered in the 0.5M sodium chloride eluate. Salt was removed by dialysis against distilled water at 4°, and the sample was lyophilized. The final product was a white, fluffy product, with a yield of about 20% of the starting, crude material. Complete hydrolysis of the polysaccharide was obtained with a mixture of sulfuric acid and acetic acid[4]; amino acid analysis showed the presence of taurine, 2-amino-2-deoxygalacturonic acid, and 2-amino-2-deoxyfucose. In the hydrolyzate with hydrochloric acid, some oligosaccharide intermediates were observed, owing to incomplete hydrolysis. All other amino acids were absent, and this pure material was used in all subsequent studies.

Chromatographic procedures. — Amino acid analyses were performed with a Beckman 121 C automated amino acid analyzer as previously described[1], except that it was equipped with a Spectra Physics 4000 integrator. T.l.c. was performed on Eastman thin-layer chromatograms (13254 cellulose). In addition to the previous solvent systems[1], 5:1:3 (v/v) pyridine–butyric acid–water was used. This system was especially good for separating the components of the hydrolyzed polysaccharide. G.l.c. of alditol acetates was performed with a Perkin–Elmer Sigma I gas–liquid chromatograph equipped with a 10-m OV-225 capillary column; the initial column temperature of 180° was held for 5 min after sample injection, and then increased 1°/min to 210° where it was held constant.

Chemical procedures. — Carbodiimide reduction of 2-amino-2-deoxygalacturonic acid into 2-amino-2-deoxygalactose residues was performed with the carbodiimide procedure of Taylor and Conrad[5] using sodium borodeuteride. The reaction was monitored by both t.l.c. and amino acid analysis of hydrolyzed samples. In both systems, 2-amino-2-deoxygalactose was resolved from other components.

Removal of taurine residues from the polysaccharide, prior to methylation analysis, was carried out as follows. Carbodiimide-reduced (2 cycles) polysaccharide (30 mg) was treated with M sodium hydroxide–M sodium borodeuteride[6]. After completion of the reaction, the sample was acidified at 4° with acetic acid to neutralize sodium hydroxide and to decompose the excess of sodium borodeuteride. After evaporation of the sample to dryness *in vacuo*, several additions and evaporations of methanol removed boric acid. The residue was dissolved in water by sonication and the solution passed through a Sephadex G-25 column (1 × 50 cm) with water as eluent. Fractions (2 mL) were monitored with a spectropolarimeter (Autopol III, Rudolph Research, Fairfield, NJ 07006). Optically ac-

tive fractions emerged before the salt fractions were pooled and lyophilized. The elution pattern of the detaurinated polysaccharide from the Sephadex G-25 column indicated that considerable depolymerization had occurred during the removal of taurine. Amino acid analysis showed the quantitative removal of taurine. The carbodiimide-reduced, detaurinated polysaccharide was selectively *N*-acetylated with acetic anhydride and sodium hydrogencarbonate[7] at room temperature. After removal of sodium ions by passage through a Dowex 50(H^+) column (0.3 × 10 cm), the sample was lyophilized, and then subjected to an additional cycle of reduction by the carbodiimide procedure, this time with sodium borohydride, to reduce the carboxyl groups liberated by the removal of taurine. Salts and carbodiimide reaction products were removed by passage through the Dowex-50(H^+) column. The lyophilized product is slightly brownish, and the amino acid analysis showed the presence of 2-amino-2-deoxygalactose, 2-amino-2-deoxyfucose, and a small proportion (5%) of unreduced 2-amino-2-deoxygalacturonic acid.

Methylation analyses. — Reduced, detaurinated material (1–5 mg) was subjected to methylation with sodium methylsulfinylmethylide and methyl iodide[8,9]. The methylated material was purified by partition in chloroform–water, followed by gel filtration on Sephadex LH-20 (Pharmacia) in acetone. On the basis of optical rotation, individual fractions were pooled and evaporated to dryness. Each sample was hydrolyzed[4] with glacial acetic acid (3.60 mL) and 2M sulfuric acid[4] (0.40 mL) for 10 h at 100°. Acetic acid was removed by repeated codistillation with water, not allowing the sample volume to fall below 0.4 mL. After neutralization with solid barium carbonate, the monomers were reduced with sodium borodeuteride and acetylated with acetic anhydride–pyridine[4] to form the methylated alditol acetates.

Carbon-13 n.m.r. analysis of capsular polysaccharide. — The proton-decoupled ^{13}C-n.m.r. spectrum of the capsular polysaccharide was recorded at 22.5 MHz with a JEOL FX-90Q Fourier-transform NMR spectrometer, equipped with a 5-mm insert. The spectrum was obtained for a solution of the polysaccharide in deuterium oxide (60 mg/mL) at 90°, by use of a 57° pulse, 5000-Hz spectral-width, 1.0-s repetition rate, 8192 data points, 181 000 scans, and heteronuclear noise-decoupling. Similar conditions were employed for the proton-coupled spectrum, except that the repetition rate was 0.6 s (0.4-s acquisition and 0.2-s delay), 128 000 scans were taken, and the decoupler was gated-off during data acquisition. The spectra were standardized against the signal of the methyl group (δ 1.6) of acetonitrile in deuterium oxide, in a separate run.

Combined g.l.c.–m.s. — Analysis of alditol acetates was carried out with an LKB model, 9000 gas chromatograph–mass spectrometer, equipped with a 3% OV-225 column (2 mm × 180 cm), and with helium as carrier gas. The initial temperature of 180° was held for 5 min after injection, then increased by 2°/min to 220°, and thereafter held constant. The temperature of both the molecule separator and ion source was 250°. The energy of the bombarding electrons was 70 eV and the ionizing current was 60 μA. Mass spectra were recorded with a scan speed of 4–6 s (m/z 40–450). All data were acquired and reduced with a PDP-12 computer.

RESULTS AND DISCUSSION

Identification of anomeric configuration of the sugars residues of the polysaccharide by ^{13}C-n.m.r. spectroscopy. — Previous studies[1-3] established that the *S. aureus* M capsular polysaccharide contains 2-acetamido-2-deoxy-D-galacturonic acid, 2-acetamido-2-deoxy-D-fucose, and taurine in the molar ratios of 4:2:1. A disaccharide was isolated and characterized by g.l.c.–m.s. as *O*-(2-acetamido-2-deoxy-D-galactopyranosyluronic acid)-(1→3)-2-acetamido-2-deoxy-D-fucose. The anomeric configuration of the linkage could not be established with the g.l.c.–m.s. technique.

The present investigation has established the anomeric configuration of the sugar residues in the polymer, and the position of the linkages. When a sufficient amount of material is available, optical rotation is a suitable method for deducing the anomeric configuration of sugars. Optical rotation was used for the characterization of 2-amino-2-deoxy-D-galacturonic acid and 2-amino-2-deoxy-L-fucose obtained from the polysaccharide[1].

In recent years, ^1H- and ^{13}C-n.m.r. spectra have been used extensively to determine the anomeric configuration of carbohydrates. ^1H-N.m.r. spectra of carbohydrates[10-12] are often complex due to the many protons in the structures. Assignment of configurations is complicated by the fact that whereas the anomeric resonances are well separated from the signals of the other protons, they fall within a narrow range with some overlap between the values observed for α and β configurations. In cases where both H-1 and -2 are axial, *e.g.*, for the β-D-glucosyl residue, secure assignments can be made from vicinal coupling constants. In the present case, no useful information could be obtained from the ^1H-n.m.r. spectrum (90 MHz) of the intact capsular polysaccharide. Instrumental advances, especially Fourier-transform techniques, have improved sensitivity to such a degree that natural abundance, ^{13}C-n.m.r. spectra can be obtained. ^{13}C-N.m.r. spectra of carbohydrates have been used successfully by several investigators[13-17]. The resonances for the various carbon atoms are well separated, and the signals for the anomeric carbon atoms appear in an area not occupied by those of other carbon atoms. Anomeric carbon atoms of pyranoses[16] with an α configuration exhibit resonances in the δ 95–103 region, and those with the β configuration at δ 103–108. A major advantage of ^{13}C-n.m.r. spectroscopy is that the intact polysaccharide can be used without the necessity of isolating intermediate oligosaccharides where the composition of the polysaccharide is not too complex. In a test trial with soluble starch, six sharp resonances were observed, one for each carbon atom of the repeating D-glucose unit. A single resonance was present at δ 95–103, namely, at δ 96.04, which was assigned to the known α-D anomeric carbon atom. If both anomers are present in a polysaccharide, it may still be necessary to isolate intermediate oligosaccharides to obtain positive identification.

Solutions of highly purified samples of *S. aureus* M capsular polysaccharide (60 mg/mL) in deuterium oxide were analyzed by ^{13}C-n.m.r. spectroscopy at 90°.

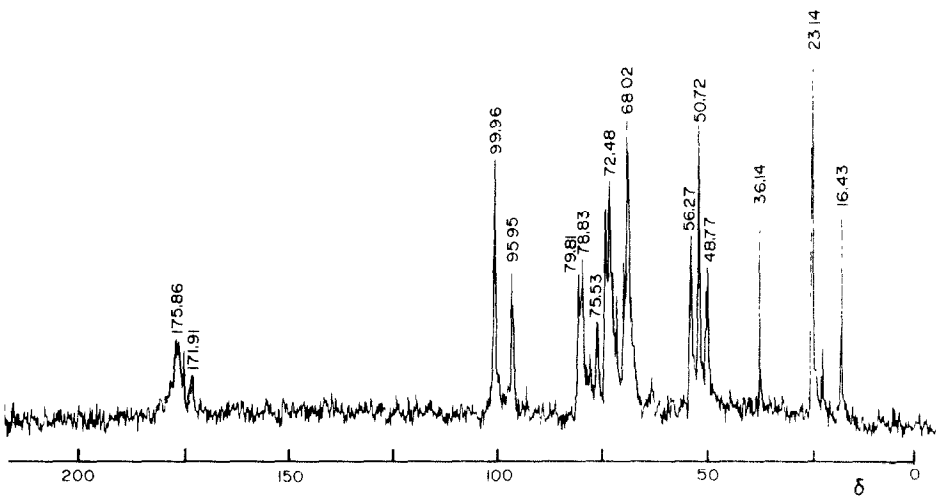


Fig. 1. ^{13}C-N.m.r. spectrum of *Staphylococcus aureus* M capsular polysaccharide

A proton-decoupled spectrum is shown in Fig. 1. The spectrum is complex and specific assignments cannot be made for all the individual carbon atoms. The most significant signals are the two at δ 95–103, namely, 95.95 and 99.96, which can be assigned to anomeric carbon atoms of pyranoses having the α configuration. No resonances were present in the region for β anomers at δ 103–108. Confirmation of the α anomer assignment was obtained from a proton-coupled spectrum, which revealed that the one-bond coupling constants ($^1J_{C,H}$) for the two signals were 171 and 173 Hz, respectively, values that are consistent with structures having equatorial anomeric protons; that is, α configuration. The coupling constants for structures in which these protons are in axial position are ~10 Hz less [18]. The ratio of the areas of the peaks at δ 99.96 and 95.95 is ~2:1, in agreement with the analytical data for a ratio of 2-acetamido-2-deoxy-D-galacturonic acid to 2-acetamido-2-deoxy-D-fucose of 2:1. Therefore, the resonance at δ 99.96 was assigned to C-1 of the 2-acetamido-2-deoxy-D-galacturonic acid residues and the resonance at δ 95.95 to C-1 of 2-acetamido-2-deoxy-D-fucose residues. The ^{13}C-n.m.r. data thus clearly indicate that all glycosidic linkages in Strain M capsular polysaccharide are in the α-D configuration.

Other assignments that can be made include C-6 of the 2-acetamido-2-deoxy-D-fucose residue at δ 16.43 and $COCH_3$ at δ 23.14. The signals of the two carbon atoms of taurine are at δ 36.14 and in the δ 50 region, those of the acetamido-substituted C-2 atoms are also in the δ 50 region, and those of the carbon atoms of the carboxamide and carboxylic acid groups are in the δ 175 region. The exceptionally complex region between δ 60 and 80 is of little diagnostic value, except that the most downfield signals represent carbon atoms bearing glycosyloxy substituents.

Identification of position of linkages by combined g.l.c.–m.s. analysis. — Methylation analysis of the reduced and detaurinated polysaccharide gave rise to

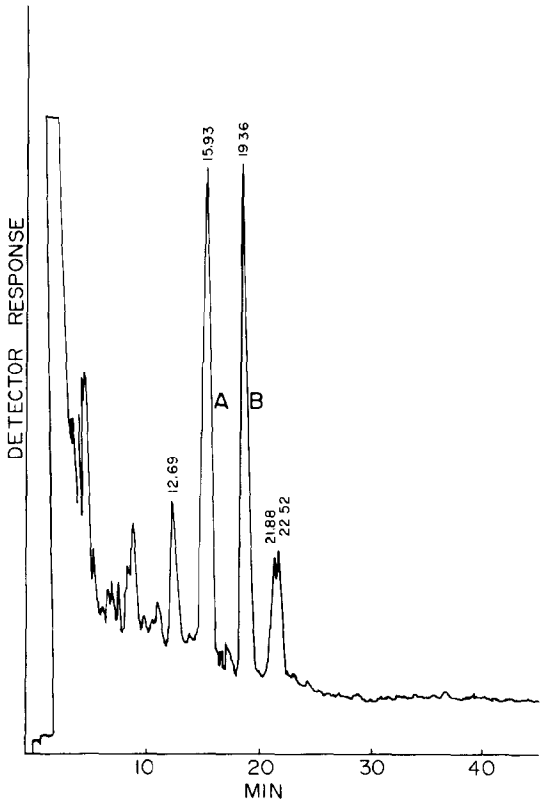
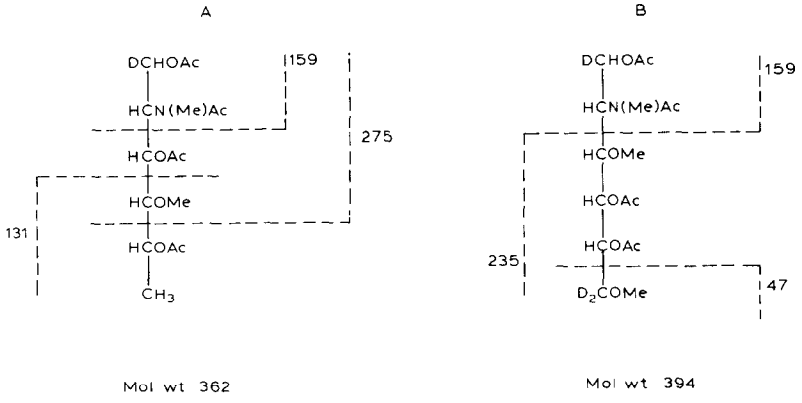


Fig. 2. G l.c. of methylated alditol acetates from *Staphylococcus aureus* M capsular polysaccharide.



Scheme 1. Primary fragmentation patterns from m.s. of methylated alditol acetates: (A)1,3,5-tri-*O*-acetyl-2,6-dideoxy-4-*O*-methyl-2-(*N*-methylacetamido)-D-[1-^{2}H]galactitol, and (B) 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D- [1,6,6-$^2H_3$]galactitol.

two major peaks in g.l.c. (Fig. 2). The mass spectrum of the first major chromatographic peak (A) showed fragments at *m/z* 43, 75, 85, 89, 99, 117, 131, 159, 275, and 302. The signals at *m/z* 131, 159, and 275 (Scheme 1) correspond to the primary fragments expected[19,20] from the derivative of a 2-acetamido-2,6-dideoxyhexose residue methylated at O-4. The signal at *m/z* 302 corroborates this conclusion because it corresponds to M − 60 (acetic acid). Because 2-amino-2-deoxy-D-fucose is the only aminodeoxy sugar in the polysaccharide, it can be deduced that the 2-amino-2-deoxy-D-fucosyl residues are linked 1→3, in agreement with previous studies in which a disaccharide containing a 1→3 linkage was isolated[2]. The m.s. of the second major chromatographic peak (B) showed primary fragments at *m/z* 43, 45, 47, 75, 87, 99, 117, 129, 159, 175, 233, 235, 332, and 334. The original 2-acetamido-2-deoxy-D-galacturonic acid residues in the polymer were reduced with sodium borodeuteride, whereas those residues that had been detaurinated were reduced with sodium borohydride. The signals at *m/z* 332 and 334 thus represent M − 60 (acetic acid) of 2-acetamido-2-deoxy-di-*O*-methylhexose derivatives not deuterated and deuterated at C-6, respectively (Scheme 1). The presence of signals at *m/z* 45 and 47 demonstrates that O-6 is methylated, and at *m/z* 233 and 235 that the second methyl group is present at O-3. From these results, it can be deduced that the 2-acetamido-2-deoxy-D-galacturonic acid residues are linked at O-4 in the original polysaccharide. The ratio of the signals at *m/z* 233 to 235 was ~1:3, indicating that one out of four of the 2-acetamido-2-deoxy-D-galacturonic acid residues was substituted with a taurine residue. This conclusion is corroborated by examining the ratio of the signals at *m/z* 173–175, which arose from those at *m/z* 233 and 235, respectively, by elimination of acetic acid[20], again ~1:3. These findings are in agreement with the analytical data for the polysaccharide[7].

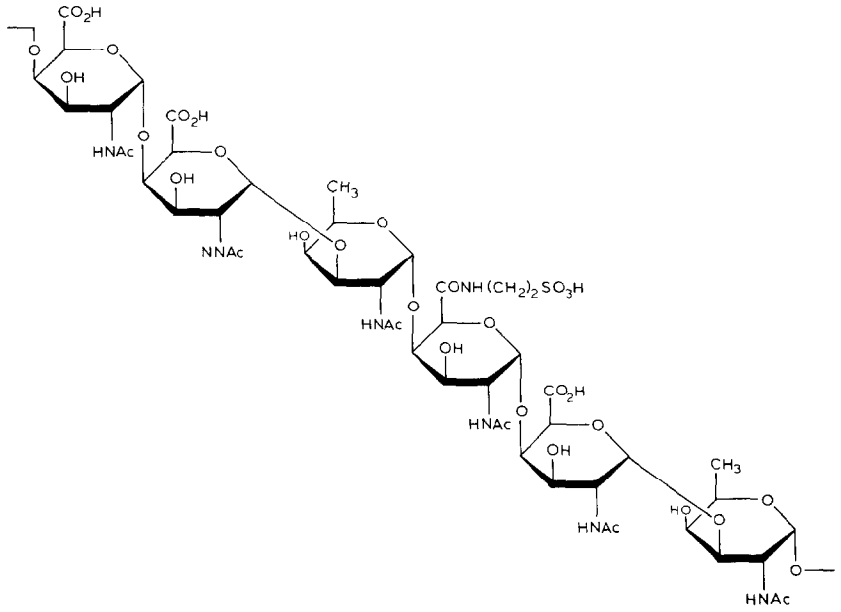
In the area of fully methylated amino sugars was a small peak having a retention time of 12.69 min (Fig. 2). Major *m/z* signals were at 43, 45, 47, 75, 89, 99, 117, 119, 147, 159, 161, 163, 203, 205, 207, 304, and 306. These fragments are consistent with a 3,4,6-tri-*O*-methyl alditol acetate of a 2-acetamido-2-deoxyhexose. These data indicate that the nonreducing end of the polysaccharide chain is a 2-acetamido-2-deoxy-D-galacturonic acid residue, which is in agreement with the isolation of a disaccharide having a 2-acetamido-2-deoxy-D-fucose residue at the reducing end and a 2-acetamido-2-deoxy-D-galacturonic acid residue at the nonreducing end [2].

In the region of the chromatogram corresponding to undermethylated compounds were two other small, fused peaks having retention times of 21.88 and 22.52 min. Mass spectra recorded through this double peak showed fragments that suggested the presence of both 3- and 6-*O*-methyl derivatives of a 2-deoxy-2-(*N*-methylacetamido)hexitol acetate. Therefore, these two peaks most likely represent small amounts of 2-acetamido-2-deoxy-D-galacturonic acid in the polysaccharide that were not fully methylated.

Data obtained from the two major peaks permit the conclusion that, in the polysaccharide, the 2-acetamido-2-deoxy-D-fucose residues are linked (1→3) and

2-acetamido-2-deoxy-D-galacturonic acid residues (1→4), and, on the average, one taurine residue is linked to every fourth 2-acetamido-2-deoxy-D-galacturonic acid residue. No other amino sugar derivative having the distinguishing *m/z* ion 159 was detected in the mass spectrogram.

The results from the ^{13}C-n.m.r. analysis of the intact capsular polysaccharide and the g.l.c.–m.s. analysis of methylated alditol acetates of the hydrolyzed polysaccharide permit the conclusion that the repeating unit is a trisaccharide having the structure →4)-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyluronic acid)-(1→4)-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyluronic acid)-(1→4), *O*-(2-acetamido-2-deoxy-α-D-fucopyranosyl)-(1→ (**1**). The smallest unit that will accommodate one taurine residue is a hexasaccharide, and its exact location is unknown.



The ^{13}C-n.m.r. spectrum of the polysaccharide was complex and further study is required to make specific assignments for all the individual carbon atoms. Fortunately, the resonances for the anomeric carbon atoms could be assigned unambiguously to the C-1 atoms of the 2-acetamido-2-deoxy-D-galactopyranosyluronic acid and 2-acetamido-2-deoxy-D-fucopyranosyl residues, both having clearly the α-D configuration. This finding eliminated the necessity of isolating intermediate oligosaccharides for determination of the anomeric configurations.

Alkaline instability of the carboxyl-reduced polysaccharide, with attendant sample losses, was observed in both the detaurination and methylation steps. Also, low yields of methylated amino sugars were experienced on g.l.c.–m.s., a finding in common with those of other investigators[21–23]. In spite of the low yields, the mass-

spectral data were unambiguous and, combined with the anomeric configuration data from ^{13}C-n.m.r. spectrometry, permit the assignment of the repeating sequence as **1**.

Several staphylococcal, capsular polysaccharides have been described. In addition to Strain M used in this study, other strains and their compositions include the Smith Diffuse containing L-alanine and 2-amino-2-deoxy-D-glucuronic acid[24], the T containing 2-amino-2-deoxy-D-fucose and 2-amino-2-deoxy-D-mannuronic acid[25], the 7007 containing 2-amino-2-deoxy-D-fucose and 2-amino-2-deoxy-D-mannuronic acid[26], and the D containing mainly 2-amino-2-deoxygalacturonic acid[27]. There are other reports of encapsulated staphylococci scattered in the literature, but capsular materials of these organisms have not been rigorously characterized. Hanessian and Haskell[24] methylated the Smith Diffuse, carboxyl-reduced, capsular polysaccharide and established that the linkages were (1→4). On the basis of the high-negative, optical rotation of the intact polysaccharide, they proposed that the anomeric configurations of the residue was β-D. These results have not been confirmed by other methods and are in contrast to the α-D linkages found for the Strain M polysaccharide. However, the results appear to indicate that staphylococci are capable of synthesizing capsular polysaccharides containing both α- and β-D-linked residues.

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