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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE FROM *Streptococcus pneumoniae* TYPE 5

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ABSTRACT

The structure of the capsular polysaccharide (S5) elaborated by *Streptococcus pneumoniae* type 5 has been investigated by using n.m.r. spectroscopy, methylation analysis, and various specific degradations. It is concluded that the polysaccharide is composed of pentasaccharide repeating-units having the following structure:

$$\begin{array}{l}\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Fuc}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-Sug}p\text{-}(1\rightarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad 3 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad 1 \\ \alpha\text{-L-Pne}p\text{NAc-}(1\rightarrow 2)\text{-}\beta\text{-D-Glc}p\text{A}\end{array}$$

In this structure, L-PneNAc stands for 2-acetamido-2,6-dideoxy-L-talose (pneumosamine) and D-Sug for 2-acetamido-2,6-dideoxy-D-*xylo*-hexos-4-ulose. The latter sugar accounts for the lability of S5 towards alkali. N.m.r. spectra indicate heterogeneity in S5, most probably associated with the hexosyl-4-ulose residue.

INTRODUCTION

The capsular polysaccharide (S5) elaborated by *Streptococcus pneumoniae* type 5 was studied by Stacey and his co-workers in the early 1960's. Most of this work is summarised in ref. 1. The polysaccharide, which had $[\alpha]_D$ −83°, yielded D-glucose, D-glucuronic acid, 2-amino-2,6-dideoxy-L-galactose (L-fucosamine), and 2-amino-2,6-dideoxy-L-talose (pneumosamine) on hydrolysis with acid. The two amino sugars, which were *N*-acetylated in the polysaccharide, had not been found in Nature before. An aldobiouronic acid (**1**) and an aldotriouronic acid (**2**) were obtained on treatment of S5 with acid under milder conditions.

Methylation analysis[2] of S5 yielded 2,3,6-tri-*O*-methyl-D-glucose and 3,4-di-*O*-methyl-D-glucuronic acid as major components. Another significant result was that all sugar residues in S5, except the *N*-acetylfucosamine residues, were oxidised

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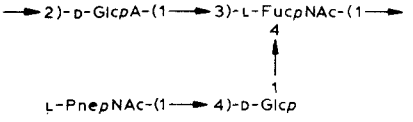
by periodate, indicating that the pneumosamine residues are present as terminal groups. On the basis of these results, it was suggested, in a survey of bacterial polysaccharides[3], that S5 was composed of tetrasaccharide repeating-units having the partial structure **3**.



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S5 is labile towards alkali. Thus, Heidelberger *et al.*[4] demonstrated that the precipitating power of S5 towards homologous rabbit antiserum fell to 18 and 7% of the original value on treatment, at room temperature, with 0.1M sodium hydroxide for 3 and 6 days, respectively. Structure **3**, however, does not account for this alkali-lability, and it is evident that S5 must contain some further structural element. We now report the results of a re-investigation of S5, using techniques more advanced than those available to the earlier investigators.

RESULTS AND DISCUSSION

The sample of S5, labelled Squibb, was a gift from Professor Michael Heidelberger and was most probably from the same batch as that investigated by Stacey and his co-workers. It was a brown powder and was purified by dialysis followed by chromatography on a DEAE-Sepharose column. The purified polysaccharide had $[\alpha]_{578}$ −82°. In all sugar and methylation analyses, the sample was first solvolysed with anhydrous hydrogen fluoride[5], followed by acid hydrolysis. By this treatment, glycosidic linkages were completely cleaved. A sugar analysis revealed *N*-acetylpneumosamine, *N*-acetylfucosamine, and glucose in the proportions 26:33:41. Glucuronic acid was not accounted for in this analysis.

Sugar analysis of a sample which had been subjected to carboxyl-reduction[6] gave the same sugars (with the expected increase in the proportion of glucose), but also a fourth sugar, in the proportions 20:24:41:9. The latter sugar was identified as 2-acetamido-2,6-dideoxyglucose (*N*-acetylquinovosamine) by comparison (g.l.c.–m.s., and g.l.c. on two different columns) of its alditol acetate with that of an authentic sample. That it had the D configuration was demonstrated using the method of Gerwig *et al.*[7].

D-Quinovosamine was also obtained on sugar analysis of a sample that had been treated with sodium borohydride or sodium borodeuteride. With the latter reducing agent, one deuterium atom was incorporated, as demonstrated by g.l.c.–m.s. of the alditol acetate. From the evidence given below, it was evident that this deuterium atom was linked to C-4 and that the third amino sugar in S5 consequently is 2-acetamido-2,6-dideoxy-D-*xylo*-hexos-4-ulose. Reduction of this sugar might also yield *N*-acetyl-D-fucosamine. No significant increase in *N*-acetylfucosamine was observed in the sugar analysis, and no incorporation of deuterium in this sugar could be detected. In addition, no D isomer could be found on determination of the absolute configurations of *N*-acetylfucosamine. Thus, the formation of this sugar on reduction was insignificant.

The amount of *N*-acetyl-D-quinovosamine formed was less than 50% of the calculated amount, assuming that S5 is composed of pentasaccharide repeating-units. The most probable explanation of this discrepancy seems to be that a considerable part of the 2-acetamido-2,6-dideoxy-D-*xylo*-hexosyl-4-ulose residues in S5 had been modified during storage of the sample and was not accounted for in the sugar analysis of the reduced sample.

In agreement with this assumption, the ^{1}H- and ^{13}C-n.m.r. (Fig. 1) spectra of S5 were more complex than expected for a polysaccharide composed of pentasaccharide repeating-units and indicated heterogeneity. No signal for a carbonyl group was detected, indicating that this group is hydrated. In the ^{13}C-n.m.r. spectrum, the signal at δ 12.2, an exceptionally high field for C-6 of a 6-deoxyhexose residue, is almost certainly given by C-6 of the 2-acetamido-2,6-dideoxy-D-*xylo*-hexosyl-4-ulose residue. It is weaker than the other signals in this region and was shifted to the more normal value of δ 17.7 on borohydride reduction. The spectrum further exhibited peaks at δ 16.5 and 16.9 for methyl groups of 6-deoxyhexoses,

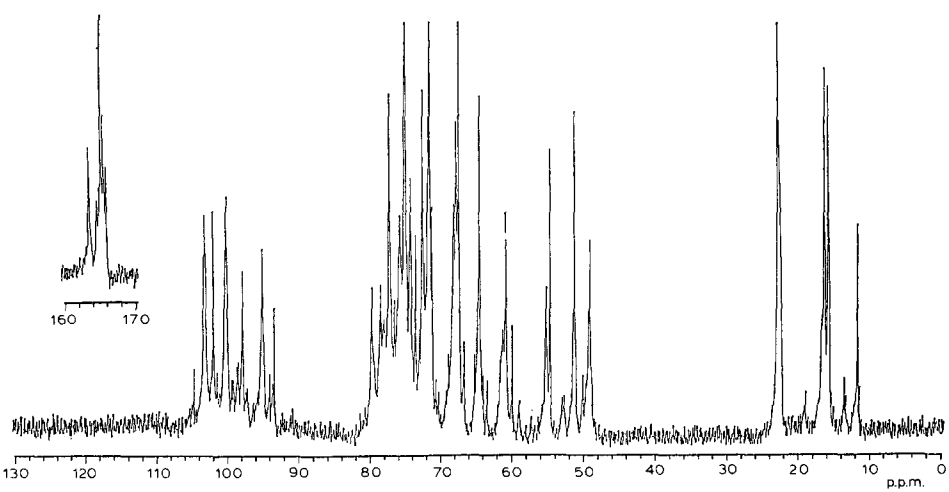

Fig. 1. ^{13}C-N.m.r. spectrum of S5.

indicative of sugars having an axial substituent at C-4. In the region for carbon linked to nitrogen, peaks were found at δ 49.5, 51.6, 55.0, and 55.5, indicating the presence of α- and β-linked amino sugars. A signal at δ 64.9 appeared where the signal for C-6 of O-6-substituted hexopyranosides and hydroxymethyl groups in acyclic alditols are normally found; however, from the evidence given below, this signal can be assigned to C-3 of the pneumosamine. In the anomeric region, three major signals were found at δ 95.0, 100.5, and 103.5. The peak at δ 100.5 is larger than the others and corresponds to two carbon atoms. From the evidence given below, it is concluded that both the glucose and the glucuronic acid are β-linked. One of these sugar residues should consequently give a signal at δ 100.5, an unusually high field. Most probably this signal can be assigned to the 2-linked glucuronic acid, because of the induced β-shift. Three minor signals were also found at δ 93.6, 98.0, and 102.2. This indicates heterogeneity that is most likely due to the hexosylulose residue.

The ^{1}H-n.m.r. spectrum of native S5 was complex and difficult to interpret. However, two signals at δ ~5.10 and 4.95 could be assigned to α linkages, and several overlapping peaks at δ ~4.55 to β linkages. The number of signals for *N*-acetyl groups and methyl groups of 6-deoxyhexoses also indicated heterogeneity. S5 that had been treated with base for different times gave ^{13}C-n.m.r. spectra which were similar to that of native S5. The most significant difference was that the former spectra contained signals with narrower line-widths; this should indicate depolymerisation, giving products of lower molecular weight.

Methylation analysis of S5 (without or with carboxyl-reduction ($NaBD_4$) of the methylated product), borodeuteride-reduced S5, and carboxyl-reduced S5 gave the sugars listed in Table I (columns *A–D*). The results show that the *N*-acetylpneumosamine residue is terminal, that the glucose and glucuronic acid residues are linked through O-4 and O-2, respectively, and that the *N*-acetylfucosamine residue is linked through O-3 and O-4, in perfect agreement with the previous results. It further demonstrates that the 2-acetamido-2,6-dideoxy-D-*xylo*-hexosyl-4-ulose residue is linked through O-3. A small proportion of 2,3,4,6-tetra-*O*-methyl-D-glucose was also found, indicating some degradation of the hexosyl-4-ulose residue. The alditol acetate (**4**) of the quinovosamine derivative obtained from S5, which had been reduced with borodeuteride, contained deuterium at C-4, as evident from its mass spectrum. The origins of some pertinent fragments are indicated in formula **4**.

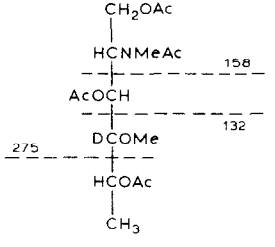

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TABLE I

METHYLATION ANALYSIS OF NATIVE AND MODIFIED S5

Sugar[a]	T[b]	*Mole %*						
		A[c]	B	C	D	E	F	G
2,3,4,6-Glc	1.00	6				13	7	32
3,4,6-Glc	1.46				26			
2,3,6-Glc	1.58	37	26	38	25	56	34	35
2,3,4-Glc	1.66						35[d]	
3,4-PneNAc	1.82	34	26	32	17			
3,4-Glc	2.37		27[d]					
2,4-QuiNAc	2.95			9	10			7
2-FucNAc	3.23	23	21	21	22	31	24	26

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, *etc.* [b]Retention time of the derived alditol acetate on an SE-54 column (temperature programme: 150°, 2 min; 150→220°, 2°/min). [c]Key: *A*, Native material. *B*, Native material with carboxyl-reduction of the methylated product (using sodium borodeuteride). *C*, Sodium borohydride-reduced. *D*, Carboxyl-reduced. *E*, Polymeric product after treatment with aqueous 48% HF. *F*, Product as in *E* with carboxyl-reduction of the methylated product (using sodium borodeuteride). *G*, polymeric product after treatment of carboxyl-reduced material with aqueous 48% HF. [d]Dideuterated at C-6.

In the foregoing discussion, it is assumed that the glucose and *N*-acetylfucosamine residues are pyranosidic. Evidence for this will be given below.

Treatment of S5 with aqueous 48% hydrogen fluoride at 4° for 24 h selectively hydrolysed the *N*-acetylpneumosamine residues. Methylation analysis of the polymeric product, without or with carboxyl-reduction of the methylated product with sodium borodeuteride (Table I, columns *E* and *F*), demonstrates that *N*-acetylpneumosamine is linked to the 2-position of glucuronic acid.

Comparison of the ^{1}H-n.m.r. spectra of original S5 and the partially hydrolysed sample showed that a signal at δ 5.10 was present in the former, but not in the latter, spectrum. This signal could be assigned to the anomeric proton of the *N*-acetylpneumosamine residue and indicates that it is α-linked. Similar comparison of the ^{13}C-n.m.r. spectra demonstrated that signals at δ 95.0, 64.9, and 51.6 are given by the *N*-acetylpneumosamine residues. The first and the last of these signals were assigned to C-1 and C-2, respectively, and that at δ 64.9 could be assigned to C-3 by comparison with the spectrum of methyl 2-acetamido-2,6-dideoxy-α-L-talopyranoside (see Experimental).

Carboxyl-reduced S5 was subjected to the same treatment with aqueous 48% hydrogen fluoride. Methylation analysis of the product (Table I, column *G*) confirmed that *N*-acetylpneumosamine is linked to O-2 of glucuronic acid and, in conjunction with previous results, also demonstrated that the 2-acetamido-2,6-dideoxy-D-*xylo*-hexosyl-4-ulose residues are located in the main chain.

A sample of S5 was subjected to hydrolysis with acid under mild conditions, and the aldobio- and aldotrio-uronic acids formed were isolated by gel filtration. Methylation analysis of the oligosaccharide-alditols, prepared by borodeuteride re-

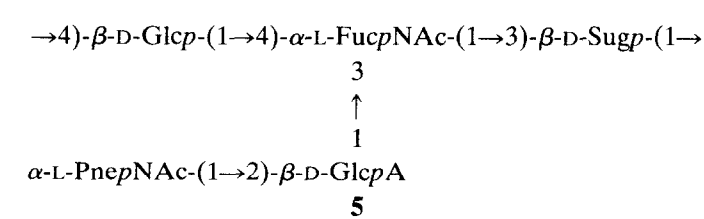
TABLE II

METHYLATION ANALYSES OF OLIGOSACCHARIDES OBTAINED ON DEGRADATION OF S5

Sugar[a]	T[b]	*Mole %*			
		A[c]	B	C	D
2,3,4,6-Glc	1.00		37		
1,2,4,5-FucNAc	1.25	35[d]			
1,2,4,5-QuiNAc	1.28				40[d]
2,3,4-Glc	1.66	65[e]	44[e]		
1,2,5-FucNAc	1.70		19[d]		
2,3,4-FucNAc	2.35			58	60
2,4-QuiNAc	2.95			42	

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, *etc.*, or corresponding alditol. [b]Retention time of the derived alditol acetate on an SE-54 column (temperature programme: 150°, 2 min; 150→220°, 2°/min). [c]Key: Reducing oligosaccharides were transformed into alditols and, when relevant, carboxyl-reduced after methylation. *A*, Acidic disaccharide (**1**) from partial acid hydrolysis. *B*, Acidic trisaccharide (**2**) from partial acid hydrolysis. *C* and *D*, Oligosaccharides **6** and **7**, respectively, obtained on Smith degradation. [d]Monodeuterated at C-1. [e]Dideuterated at C-6.

duction, and carboxyl-reduction of the methylated products, also with deuterated reagent, gave the sugars listed in Table II, columns *A* and *B*. The analyses demonstrate that the oligosaccharides giving these alditols are those (**1** and **2**) already isolated and characterised by Stacey and his co-workers. The ^{1}H- and ^{13}C-n.m.r. spectra of the disaccharide-alditol showed, *inter alia*, signals for an anomeric proton and an anomeric carbon at δ 4.59 ($J_{1,2}$ 8 Hz) and 102.9. The corresponding signals for the trisaccharide-alditol were δ 4.67 ($J_{1,2}$ 8 Hz) and 4.55 ($J_{1,2}$ 8 Hz), and δ 104.6 and 102.6, respectively. These figures demonstrate that the glucose and glucuronic acid residues are pyranosidic and β-linked.



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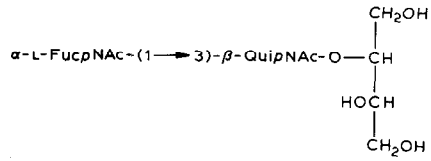
From the combined results, it is concluded that the pentasaccharide repeating-unit of S5 has structure **5**. The only structural features not discussed (but given in the formula) are the anomeric natures of the *N*-acetylfucosamine and hexosyl-4-ulose residues. In order to determine these, carboxyl-reduced S5 was subjected to a Smith degradation[8] (periodate oxidation, borohydride reduction, and hydrolysis with acid under mild conditions). The resulting oligosaccharide (**6**) on hydrolysis yielded *N*-acetylfucosamine, *N*-acetylquinovosamine, and erythritol. The ^{1}H- and ^{13}C-n.m.r. spectra showed, *inter alia*, signals for anomeric protons and carbons at

δ 4.97 ($J_{1,2}$ 4 Hz) and 4.52 ($J_{1,2}$ 8 Hz), and at δ 103.3 and 99.6, respectively, demonstrating that one of the sugars is α-linked and the other β-linked. This conclusion was confirmed by the shifts of the *N*-substituted carbon atoms. The signal at δ 51.1 could be assigned to an α-linked *N*-acetylfucosamine residue and that at δ 57.5 to a β-linked *N*-acetylquinovosamine residue.



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After treatment of the polyalcohol with aqueous 48% hydrogen fluoride at 4°, the reducing disaccharide **7** was obtained, in agreement with the results of Jennings and Lugowski[9], who showed that the rates of hydrolysis of 2-acetamido-2-deoxy-β-D-gluco and -β-D-galacto-pyranoside with this reagent are higher than those of the corresponding α-glycosides and also higher when the aglycon is an alditol than when it is a sugar. Methylation analysis of the alditol of **7** confirmed the proposed structure. The ^{1}H- and ^{13}C-n.m.r. spectra of the alditol showed, *inter alia*, one signal in the region for anomeric protons, at δ 4.99 ($J_{1,2}$ 4 Hz), and one in the region for anomeric carbons, at δ 100.3, respectively, in agreement with the proposed α configuration of the 2-acetamido-2,6-dideoxy-L-galactopyranosyl residue.

α-L-Fuc*p*NAc-(1→3)-D-QuiNAc

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Although sugar nucleotides of hexos-4-uloses are common intermediates in transformations of sugars during biosynthesis, such sugars have not been found hitherto as components of polysaccharides. A sugar nucleotide of 2-acetamido-2,6-dideoxy-D-*xylo*-hexopyranosyl-4-ulose could actually be the precursor of 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose, a component of the *S. pneumoniae* type 1 capsular polysaccharide[10] and also of the C-substance[11], the species-specific antigen of *S. pneumoniae*. The 2-acetamido-2,6-dideoxy-D-*xylo*-hexopyranosyl-4-ulose residues in S5 account for the alkali-lability of the polysaccharide, as observed by previous investigators, and also for the degradation and browning on storage. The finding of 2,3,4,6-tetra-*O*-methyl-D-glucose on methylation analysis of S5 (Table I) also indicates partial degradation of the hexosylulose residue. The presence of another hexosulose, namely, D-*lyxo*-5-hexosulopyranuronic acid, as a component of a polysaccharide from *Sphagnum* moss, has recently been demonstrated[12].

In branched-polysaccharide antigens, side-chains are often immunodominant. It therefore seems possible that borohydride-reduced S5, which should be much

more stable than the native polysaccharide, should display similar immunological activity. It might therefore be advantageous to reduce S5 if it should be used as a vaccine.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at bath temperatures not exceeding 40°. For g.l.c., a Hewlett–Packard 5830A instrument, fitted with a flame-ionisation detector, was used. Separations were performed on an SP-1000 fused-silica capillary column at 230° or on an SE-54 cross-linked fused-silica capillary column using a temperature programme (150°, 2 min; 150→220°, 2°/min). G.l.c.–m.s. was performed on a Hewlett–Packard 5790–5970 g.l.c.–m.s. system. All separations were performed on an Ultra 2 capillary column (essentially the same as SE-54). Optical rotations were measured at room temperature (~22°) with a Perkin–Elmer 241 polarimeter. A differential refractometer was used for monitoring the Sephadex-column effluents. Carboxyl-reductions were performed as described by Taylor *et al.*[6]. Carboxyl-reductions of methylated material were performed as described by Dutton *et al.*[13]. Absolute configurations were determined by the procedure of Gerwig *et al.*[7]. Methylation analyses were performed essentially as previously described[14,15]. Methylated products were purified on Sep-Pak C_{18}-cartridges as described by Waeghe *et al.*[16].

All hydrolyses were performed[17] by solvolysis with anhydrous hydrogen fluoride for 3 h at room temperature followed by hydrolysis with 2M trifluoroacetic acid for 2 h at 100°. For n.m.r. spectroscopy, a JEOL FX-100 or a GX-400 spectrometer was used. ^{13}C-N.m.r. spectra of solutions in D_2O were recorded at 70°. Chemical shifts are given in p.p.m. relative to internal 1,4-dioxane (δ 67.4). ^{1}H-N.m.r. spectra of solutions in D_2O were recorded at 85°. Chemical shifts are given in p.p.m. downfield from 4,4-dimethyl-4-silapentane-1-sulfonate, using residual water as δ 4.17.

Purification. — Crude S5 was dialysed against tap water and then against distilled water, and the solution was freeze-dried. The recovered material was purified by chromatography on a column (3.0 × 40 cm) of DEAE-Sepharose CL-6B irrigated first with water (250 mL) and then with a linear gradient of aqueous sodium chloride (0→M, 1 L). Peaks were monitored polarimetrically, and the polysaccharide was eluted at a salt concentration of 0.4–0.5M. The material was recovered by dialysis and freeze-drying. The yield of pure S5, $[\alpha]_{578}$ −82° (*c* 1, water), was 50–60%.

Borohydride reduction of S5. — S5 (50 mg) was dissolved in water (20 mL) and sodium borohydride (100 mg) was added. After 15 h at room temperature, the excess of borohydride was decomposed by addition of aqueous acetic acid. The product, recovered by dialysis and freeze-drying, had $[\alpha]_{578}$ −79° (*c* 1, water).

Partial hydrolysis of S5. — (*a*) S5 (50 mg) was treated with aqueous 48% hydrofluoric acid (5 mL) for 24 h at 4°. The acid was removed under diminished

pressure over sodium hydroxide in a desiccator, and a solution of the residue in water was freeze-dried. The product was fractionated on a column (2.6 × 80 cm) of Sephadex G-25 by elution with water. Two main fractions were obtained; the first (35 mg), $[\alpha]_{578}$ −72° (*c* 1, water), was eluted in the void volume, and the second (10 mg) in the monosaccharide region. The latter consisted of pure *N*-acetylpneumosamine.

Partial hydrolysis of carboxyl-reduced S5 (50 mg) was performed as described above. The polymeric fraction (20 mg) had $[\alpha]_{578}$ −69° (*c* 1, water).

(*b*) S5 (50 mg) was treated with 0.01M hydrochloric acid (5 mL) for 15 h at 70° and then the solution was freeze-dried. After reduction with sodium borodeuteride, the product was fractionated on a column (2.6 × 75 cm) of Sephadex G-15 by elution with water. Two major components were obtained, namely, the alditols of **2** (9 mg) and **1** (7 mg).

Smith degradation[8]. — Carboxyl-reduced S5 (300 mg) was oxidised with 0.04M sodium metaperiodate in sodium acetate buffer (pH 3.9, 0.1M, 250 mL) for 95 h at 4°. The excess of periodate was reduced with ethylene glycol (3 mL). The solution was dialysed against water and freeze-dried. The product was dissolved in water, sodium borohydride (1.0 g) was added, and the mixture was kept for 15 h. The excess of sodium borohydride was decomposed with aqueous acetic acid. The solution was dialysed and the product (85 mg) recovered by freeze-drying. Part of the material was subjected to sugar analysis.

Part (25 mg) of this product was treated with 0.5M trifluoroacetic acid at room temperature for 48 h. The product was freeze-dried and fractionated on a column (2.6 × 75 cm) of Sephadex G-15. One major product (**6**, 8 mg) was obtained in the oligosaccharide region.

The oxidised and reduced S5 (25 mg) was treated with aqueous 48% hydrofluoric acid (3 mL) for 40 h at 4°. The acid was removed as described above and the product was fractionated on a column (2.6 × 75 cm) of Sephadex G-15. One major product was obtained in the oligosaccharide region (the alditol of **7**, 6 mg).

*Methyl 2-acetamido-2,6-dideoxy-α-*L*-talopyranoside*. — The 3,4-diacetate of the title compound was obtained by chromatography of the mother liquor from a synthesis of the corresponding L-*galacto* derivative[18], using a silica gel column with chloroform–acetone (7:3) as irrigant. The *O*-deacetylated product had $[\alpha]_{578}$ −11° (*c* 1, water). ^{13}C-N.m.r. data: δ 174.5 (C=O), 101.2 (C-1), 71.8 (C-4), 67.4 (C-5), 65.0 (C-3), 55.6 (OMe), 52.0 (C-2), 23.2 (NHAc), and 16.4 (C-6). The assignments were corroborated by selective-decoupling experiments of the ^{1}H-n.m.r. signals and by ^{13}C–^{1}H shift-correlated two-dimensional spectroscopy. On methylation of the title compound, only traces of *N*-methylation were observed. Similarly, no *N*-methylation of the *N*-acetylpneumosamine residue in S5 was observed.

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