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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE FROM *Streptococcus pneumoniae* TYPE 12A

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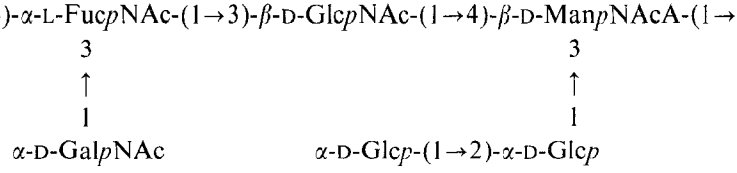
ABSTRACT

The structure of the capsular polysaccharide from *Streptococcus pneumoniae* type 12A has been investigated. Using n.m.r. spectroscopy, methylation analysis, and specific degradations as the principal methods of structural investigation, it is concluded that the polysaccharide is composed of hexasaccharide repeating-units having the following structure.



INTRODUCTION

There are 82 different types of *Streptococcus pneumoniae*, some of which show antigenic relationships and have been brought together in groups. Thus, group 12 consists of the types 12F and 12A (12 and 83 according to the American nomenclature) with the antigenic formulas 12a, 12b, 12d and 12a, 12c, 12d, respectively[1]. We recently determined the structure of the capsular polysaccharide from type 12F (S 12F)[2], which is included in a "core" vaccine covering a high percentage of the infections caused by *S. pneumoniae*. We now report similar studies of S 12A which, for serological reasons, should have significant structural features in common with S 12F.

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RESULTS AND DISCUSSION

The polysaccharide S-12A was not very soluble in water or dimethyl sulfoxide and was therefore degraded by treatment with 48% aqueous hydrogen fluoride at 6° for 48 h. The resulting material, which gave clear solutions of low viscosity, had $\overline{M}_w$ 10,900 on gel filtration and was used for n.m.r. studies. The optical rotations and the sugar and methylation analyses for this material and the original polysaccharide were essentially the same. Thus, S-12A had $[\alpha]_{5^{\circ}8}$ + 5° (water) and contained 2-acetamido-2-deoxy-L-fucose, D-glucose, 2-acetamido-2-deoxy-D-glucose, and 2-acetamido-2-deoxy-D-galactose in the relative proportions 0.7:2.0:1.0:1.0. In addition, 1,5-dideoxy-1,5-imino-D-mannitol was obtained in the relative proportion 0.3. This product was an artefact, also obtained[2] in the sugar analysis of S-12F, and was formed from 2-acetamido-2-deoxy-D-mannuronic acid *via* *N*-deacetylation, lactonisation, and reduction with sodium borohydride[3]. The sugars were shown to have the absolute configurations indicated, using the method of Leontein *et al.*[4,5]. The 2-acetamido-2-deoxy-D-mannuronic acid residues in the product obtained on Smith degradation of S-12A were reduced to 2-acetamido-2-deoxy-D-mannose residues before this determination.

The ^{13}C-n.m.r. spectrum of S-12A (Table II, column A) showed, *inter alia*, six signals for anomeric carbons, four signals for carbon atoms linked to nitrogen, and one signal for a *C*-methyl group. This result, in conjunction with the results of

TABLE I

METHYLATION ANALYSES OF ORIGINAL AND MODIFIED CAPSULAR POLYSACCHARIDES OF *Streptococcus pneumoniae* TYPE 12A

Methylated sugar[a]	T[b]	*Mole %*[c]					
		A	*B*	*C*	*D*	*E*	*F*
2,3,4,6-Glc	1	23			30		
1,2,4,5-FucNAc	1.2					44	
3,4,6-Glc	1.4	19			13		
1,2,3,5,6-ManNAc	2.1						24
2,3-FucNAc	2.7		38	36	26		
2,3,4,6-GlcNAc	3.2						76
2-FucNAc	3.4	18	14	7	7		
2,3,4,6-GalNAc	3.6	20				56	
2,3,6-ManNAc	4.7			16			
2,4,6-GlcNAc	4.9	19	48	41	24		

[a]2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,2,4,5-FucNAc = 3-*O*-acetyl-2,6-dideoxy-1,4,5-tri-*O*-methyl-2-N-methylacetamido-L-galactitol, *etc.* [b]Retention times of the corresponding alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an SE-30 column at 190°. [c]Polysaccharide: A, original; B, Smith-degraded polysaccharide, C, Smith-degraded and carboxyl-reduced polysaccharide; D, Partially Smith-degraded polysaccharide, E, alditol of oligosaccharide **1**; F, alditol of oligosaccharide **3**

TABLE II

PERTINENT ^{13}C-N.M.R. CHEMICAL SHIFTS[a] FOR THE *Streptococcus pneumoniae* TYPE 12A CAPSULAR POLYSACCHARIDE (A), THE POLYSACCHARIDE FROM THE SMITH DEGRADATION (B), THE POLYSACCHARIDE FROM PARTIAL SMITH-DEGRADATION (C), THE ALDITOL OF OLIGOSACCHARIDE **1** (D), AND THE ALDITOL OF OLIGOSACCHARIDE **3** (E)

Sugar unit	*Carbon atom*	*A*	*B*	*C*	*D*	*E*
α-D-Glc*p*-(1→	C-1	96.2		100.9		
→2)-α-D-Glc*p*-(1→	C-1	97.3				
→4)-α-L-Fuc*p*NAc-(1→	C-1	99.6[b]				
3	C-2	50.0[c]				
↑	C-6	17.1				
→4)-α-L-Fuc*p*NAc-(1→	C-1		99.4 ($^1J_{C,H}$ 171 Hz)	99.4		
	C-2		51.5	51.5		
	C-6		16.7	16.7		
→3)-L-FucNAc-ol	C-2				52.3[d]	
	C-6				19.9	
→4)-β-D-Man*p*NAcA-(1→	C-1	101.3		101.7		
3	C-2	53.8		53.7		
↑						
→4)-β-D-Man*p*NAcA-(1→	C-1		101.1 ($^1J_{C,H}$ 163 Hz)			
	C-2		53.7			
→4)-D-ManNAc-ol	C-2					54.4
α-D-Gal*p*NAc-(1→	C-1	99.3[b]			100.0	
	C-2	50.9[c]			51.2[d]	
→3)-β-D-Glc*p*NAc-(1→	C-1	102.9	102.4 ($^1J_{C,H}$ 163 Hz)	103.0		
	C-2	56.9	56.8	57.0		
β-D-Glc*p*NAc-(1→	C-1					102.4
	C-2					57.1

[a]In D_2O relative to external tetramethylsilane. [b–d]May be interchanged.

sugar analysis, indicates that S 12A is composed of hexasaccharide repeating-units containing two D-glucose residues and one residue each of 2-acetamido-2-deoxy-L-fucose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, and 2-acetamido-2-deoxy-D-mannuronic acid. That all amino sugars are *N*-acetylated was evident from the ^{1}H- and ^{13}C-n.m.r. spectra.

Methylation analysis of S 12A, in which the methylated sugars were analysed by g.l.c.–m.s. of their alditol acetates[6], gave the sugars listed in Table I, column A. As usual, essentially all of the *N*-acetylamino groups were *N*-methylated during the Hakomori methylation. The analysis shows that S 12A contains a D-glucopyranosyl group, a 2-acetamido-2-deoxy-D-galactopyranosyl group, a D-glucopyranosyl residue linked through O-2, and a 2-acetamido-2-deoxy-D-glucopyranosyl residue linked through O-3. Evidence that the 2-acetamido-2-deoxy-L-fucosyl residue is also pyrano-

sidic is given below, and it is consequently linked through O-3 and O-4. The 2-acetamido-2-deoxy-D-mannuronic acid residue is not accounted for in this analysis, as carboxyl-reduction, using the method of Taylor *et al.*[7], was unsuccessful. The finding of two terminal groups, but only one branching residue, indicates, however, that this is also a branching residue and, if pyranosidic, linked through O-3 and O-4. The SE-30 glass-capillary column proved to be most convenient in sugar and methylation analysis of polysaccharides which, like S 12A, contain several different amino sugars.

According to the methylation analysis, three of the six sugar residues in S 12A should be oxidised by periodate, namely the D-glucopyranosyl group, the 2-linked D-glucopyranosyl residue, and the 2-acetamido-2-deoxy-D-galactopyranosyl group. The polysaccharide was therefore subjected to a Smith degradation[8] (periodate oxidation, borohydride reduction to a "polyalcohol", and hydrolysis with acid under mild conditions). Sugar analysis of the "polyalcohol" confirmed that the above-mentioned sugars had been oxidised. However, selective cleavage of the acetalic linkages of the modified residues proved to be difficult but was finally achieved by treatment with 48% aqueous hydrogen fluoride at 6° for 48 h. Sugar analysis of the degraded material, which was polymeric and had $[\alpha]_{5-8}$ + 107° (water), gave 2-acetamido-2-deoxy-L-fucose, 1,5-dideoxy-1,5-imino-D-mannitol, and 2-acetamido-2-deoxy-D-glucose in the relative proportions 1.0 : 0.4 : 1.0. Methylation analysis (Table I, column B) demonstrated that the degraded material was a linear polysaccharide in which the 2-acetamido-2-deoxy-D-glucopyranosyl residue was linked through O-3 and the 2-acetamido-2-deoxy-L-fucosyl residue, assumed to be pyranosidic, was linked through O-4. The small proportion of 2-deoxy-2-methylacetamido-L-fucose found indicates that the Smith hydrolysis was incomplete. Again, the 2-acetamido-2-deoxy-D-mannosyluronic acid residue was not accounted for in the analysis. After four, successive carboxyl-reductions of the degraded polysaccharide, only ~50% of the uronic acid residues had been reduced (Table I, column C). The 2-acetamido-2-deoxymannose obtained on hydrolysis of this material had the D configuration.

The low value for the optical rotation of the degraded polysaccharide suggests that the 2-acetamido-2-deoxy-L-fucose is α-linked and that the two D-sugars are β-linked. That the former is α-linked and pyranosidic is evident from the ^{13}C-n.m.r. spectrum, in which the signal for C-1 appears at δ 99.4. The signal given by C-2, at δ 51.5, is also typical[9-11] for 2-amino-2-deoxy-α-D-galactopyranosides. Of the two other signals in the region for anomeric carbons, that at δ 102.4 was assigned to the 2-acetamido-2-deoxy-D-glucopyranosyl residue, which should be β-linked. From the chemical shift of the third signal, δ 101.1, it could be inferred that the 2-acetamido-2-deoxy-D-mannosyluronic acid is pyranosidic, but not whether it is α- or β-linked. This problem was solved by determining the $^1J_{C,H}$ values, which were 171 Hz for the δ 99.4 signal and 163 Hz for the signals at δ 101.1 and 102.4. These values[12] demonstrate that the 2-acetamido-2-deoxy-L-fucopyranosyl residue is α-linked and that the two other sugar residues are β-linked.

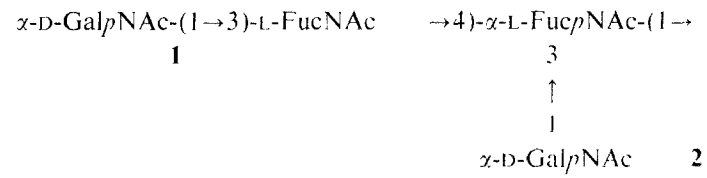
Three signals for anomeric carbons in the ^{13}C-n.m.r. spectrum of S 12A, at δ 96.2, 97.3, and 99.3, disappeared on Smith degradation. From the values of these

chemical shifts, it was concluded that the sugars eliminated by the degradation, two residues of D-glucose and one of 2-acetamido-2-deoxy-D-galactose, were all α-linked. This is also in agreement with the considerable shift in specific rotation, from $[\alpha]_{578}$ −5° to −107°, on Smith degradation.

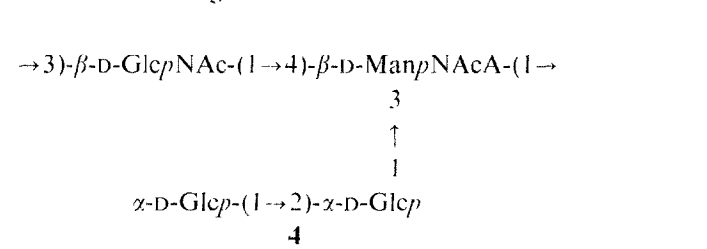
In the structural studies of S 12F, it was possible to selectively oxidise an α-D-galactopyranosyl group with periodate and eliminate it by Smith degradation. Of the sugar residues in S 12A which are oxidised by periodate, only the 2-acetamido-2-deoxy-α-D-galactopyranosyl group contains a pair of vicinal *cis*-hydroxyl groups, and a similar, partial Smith-degradation was therefore attempted. However, this could not be achieved, as this group and the α-D-glucopyranosyl group were oxidised at similar rates. Sugar analysis of the "polyalcohol", in which all of the original 2-acetamido-2-deoxy-D-galactose had been modified, gave 2-acetamido-2-deoxy-L-fucose, D-glucose, 1,5-dideoxy-1,5-imino-D-mannitol, and 2-acetamido-2-deoxy-D-glucose in the relative proportions 1.0:0.8:0.4:0.8. Methylation analysis of the degraded product (Table I, column D), which had $[\alpha]_{578}$ −55° (water), demonstrated that O-3 in the 2-acetamido-2-deoxy-α-L-fucopyranosyl residue had been exposed and that a considerable percentage of 2-linked D-glucose had been transformed into terminal D-glucose. Although it seemed likely that the 2-acetamido-2-deoxy-α-D-galactopyranosyl group is linked to O-3 of the 2-acetamido-2-deoxy-α-L-fucopyranosyl residue and that a 2-*O*-α-D-glucopyranosyl-α-D-glucopyranosyl group is linked to the 2-acetamido-2-deoxy-β-D-mannopyranosyluronic acid residue, by analogy with the situation in S 12F, the evidence for this assumption is not conclusive. The ^{13}C-n.m.r. spectrum of the product is given in Table II, column C.

Polysaccharide S 12A was also subjected to graded hydrolysis by treatments[2,13] first with liquid hydrogen fluoride at 20° for 3 h and then with aqueous acid under mild conditions. Two main oligomers, a neutral and an acidic disaccharide, were obtained, and were isolated by chromatography on DEAE-Sepharose.

The neutral disaccharide was reduced with sodium borodeuteride, and the resulting disaccharide-alditol was purified by gel filtration on Sephadex G-15, followed by h.p.l.c. on a Dextropak column. This product, which had $[\alpha]_{578}$ +159° (water), was hydrolysed, reduced with sodium borohydride, and acetylated, giving equal parts of fully acetylated 2-amino-2-deoxy-D-galactitol and 2-amino-2-deoxy-L-fucitol-*1-d*. Methylation analysis of the disaccharide-alditol (Table I, column E) demonstrated that the 2-acetamido-2-deoxy-L-fucitol was linked through O-3. The optical rotation and the signal for the anomeric proton in the ^{1}H-n.m.r. spectrum at δ 5.10 ($J_{1,2}$ 4 Hz) confirmed the assignment of α configuration to the 2-amino-2-deoxy-D-galactopyranosyl group. The neutral disaccharide consequently has structure **1** and this demonstrates the presence of the structural element **2** in the hexasaccharide repeating-unit. The other branch in the repeating-unit should therefore be a 2-*O*-α-D-glucopyranosyl-α-D-glucopyranosyl group.

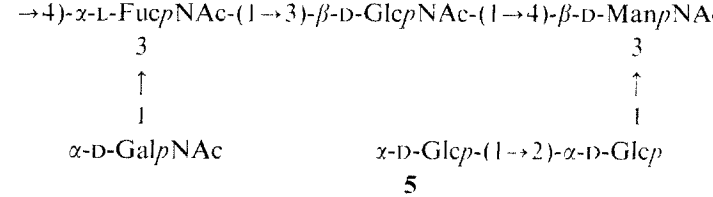


The acidic disaccharide was treated with hydrochloric acid and freeze-dried in order to transform it into the lactone, and the product was reduced with sodium borodeuteride and purified by chromatography on Sephadex G-15. This material, on acid hydrolysis, borohydride reduction, and acetylation, gave equal parts of 2-acetamido-2-deoxy-D-glucitol and 2-acetamido-2-deoxy-D-mannitol-*1-d*-6-d_2. Methylation analysis (Table I, column F) further revealed that the 2-acetamido-2-deoxy-D-mannitol is linked through O-4. The $[\alpha]_{578}$ value of −12° (water) and the signal for the anomeric proton in the ^{1}H-n.m.r. spectrum, at δ 4.65 ($J_{1,2}$ 7 Hz), confirm the β configuration of the 2-acetamido-2-deoxy-D-glucopyranosyl residue. The acidic disaccharide is consequently a pseudoaldobiouronic acid having the structure **3**. The identification of this component defines the structural element **4** in S 12A; as the other side-chain, the 2-acetamido-2-deoxy-α-D-galactopyranosyl group had already been located in structural element **2**.



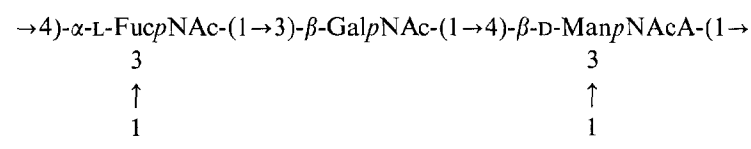
A pseudoaldobiouronic acid derivative, which was carboxyl-reduced on treatment with sodium borohydride, was also formed on similar treatment of S 12F and the capsular polysaccharide from *Haemophilus influenzae* type e[2,14]. The product from the latter is identical with **3**.

The structural elements **2** and **4** contain all of the components of the hexasaccharide repeating-unit, which consequently has structure **5**.



Pertinent signals in the ^{13}C-n.m.r. spectrum of S 12A and its degradation products are given in Table II. The assignments, some of which are tentative only, were made by using spectral data from review articles[10,11,15] and by comparison with spectra of polysaccharides containing similar or identical structural elements, namely S 12F[2] and the capsular polysaccharides from *Haemophilus influenzae* type e[14] and *Escherichia coli* type K7[16]. The ^1H-n.m.r. spectra of S 12A and its degradation products were also in agreement with the postulated structures. However, only pertinent signals in the spectra of the oligosaccharides **1** and **3** are reported above.

Polysaccharide S 12F is also composed of hexasaccharide repeating-units (**6**); these have a structure closely related to that of S 12A (**5**). The only difference is that an α-D-galactopyranosyl group and a 2-acetamido-2-deoxy-β-D-galactopyranosyl residue in S 12F are replaced by a 2-acetamido-2-deoxy-α-D-galactopyranosyl group and a 2-acetamido-2-deoxy-β-D-glucopyranosyl residue in S 12A. In other respects, namely, sugars, linkages, and anomeric configurations, the structures are identical.



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EXPERIMENTAL

General methods. — For g.l.c., Hewlett–Packard 5830 and Perkin–Elmer 990 instruments equipped with flame-ionisation detectors were used. Separations of monosaccharide derivatives were performed on glass-capillary columns (0.25 mm × 25 m) of SE-30 or SP-1000. Separations of permethylated oligosaccharide-alditols were performed on a column (180 × 0.15 cm) of 3% OV-17 at 230°. G.l.c.–m.s. was performed with a Varian MAT 311-SS 100 m.s.–computer system. For h.p.l.c., a Waters RCM-100 unit fitted with a Dextropak column was used. For n.m.r. spectroscopy, a JEOL FX-100 instrument operated in the pulsed F.t.-mode was used. Chemical shifts are given relative to external Me_4Si (^{13}C) and relative to the HDO peak at δ 4.16 (^1H, 85°). The $^1J_{CH}$ values of Smith-degraded S 12A were determined by using an INEPT[17] pulse sequence. For treatment with liquid hydrogen fluoride, an apparatus (Type II) from Peninsula Laboratories, Inc. (P.O. Box 1111, San Carlos, CA 94070, U.S.A.) was used. Methods for sugar and methylation analyses have been described[2,6].

Material. — *S. pneumoniae* type 12A was obtained from Dr. Robert Austrian (University of Pennsylvania). The organism was grown and the capsular polysaccharide isolated essentially as described earlier[18]. Crude material (100 mg), which had $\overline{M}_w > 10^6$, was dissolved in water (4 mL) and loaded onto a column (2.5 × 40 cm) of DEAE-Sepharose CL 6B. The column was washed with water (300 mL) followed

by a linear gradient of sodium chloride (0→M, 1 L); S 12A was eluted as a homogeneous peak. The appropriate fractions were combined, dialysed, and freeze-dried (89 mg). In order to obtain a more-soluble material, a solution of S 12A (60 mg) in 48% aqueous hydrogen fluoride (5 mL) was kept at 6° for 48 h. The solution was concentrated to dryness and a solution of the residue in water was freeze-dried. An aqueous solution of the product was neutralised with aqueous sodium hydroxide and fractionated on a column (2.5 × 80 cm) of Sephadex G-15 by elution with water. The material (36 mg) eluted in the polymeric region was isolated by freeze-drying and had $[\alpha]_{578}^{25}$ −5° (*c* 1, water). The molecular weight of the material was determined by gel filtration[19] on Sepharose CL 6B.

Carboxyl-reduction. — Attempted carboxyl-reduction of S 12A, partially degraded S 12A, and Smith-degraded S 12A (see below) was performed as described elsewhere[2,7].

Smith degradation of S 12A. — S 12A (50 mg) was dissolved in water (50 mL), and sodium metaperiodate (230 mg) in water (5 mL) was added. The solution was kept in the dark at 6° for 8 days. Excess of periodate was reduced with ethylene glycol (3 mL), the mixture was dialysed and concentrated to 25 mL, and sodium borohydride (250 mg) was added. After stirring at room temperature overnight, excess of borohydride was decomposed with 50% acetic acid, and the solution was dialysed and freeze-dried (50 mg). Sugar analysis showed that virtually all of the 2-acetamido-2-deoxy-D-galactose was modified and that only traces of D-glucose remained. The polyalcohol was hydrolysed under mild conditions (48% aqueous hydrogen fluoride, 6°, 2 days) and the hydrolysate worked-up as described earlier. The product (16 mg) had $[\alpha]_{578}^{25}$ −107° (*c* 0.9, water). Part of this material was subjected to methylation analysis (Table I, column B). Another part (10 mg) was carboxyl-reduced (4 treatments) and then subjected to methylation analysis (Table I, column C) and to sugar analysis (see text).

Modified Smith-degradation. — To a solution of S 12A (15 mg) in water (12 mL) was added a solution of sodium metaperiodate (68 mg) in water (4 mL). The mixture was kept in the dark at 6° for 2 h and then processed as described above. Sugar analysis of the polyalcohol showed that all of the 2-acetamido-2-deoxy-D-galactose had been modified and that ~50% of the original D-glucose remained. The modified polysaccharide (7 mg), recovered after mild hydrolysis with acid (see above), had $[\alpha]_{578}^{25}$ −55° (*c* 0.3, water). Part of this material was subjected to methylation analysis (Table I, column D), and another part to sugar analysis (see text).

Treatment of S 12A with hydrogen fluoride. — Polysaccharide S 12A (100 mg) was treated with liquid hydrogen fluoride (10 mL). After 3 h at room temperature, the hydrogen fluoride was evaporated under diminished pressure and the residue was treated with 5% aqueous acetic acid for 1 h at room temperature. The solution was concentrated to dryness, and a solution of the residue in water was freeze-dried. The product was applied to a column (1.5 × 25 cm) of DEAE-Sepharose CL 6B which was irrigated with water (100 mL) and then with 0.5M ammonium hydrogencarbonate

(50 mL). The material eluted with water (75 mg) was recovered by freeze-drying, reduced with sodium borodeuteride, and fractionated on a column (2.5 × 80 cm) of Sephadex G-15 irrigated with water. The material (15 mg) eluted in the disaccharide region was further purified by h.p.l.c. on a Dextropak column (elution with water) to yield the alditol of **1** (5 mg), $[\alpha]_{578}^{25}$ +159° (*c* 0.3, water). Part of this material was subjected to sugar analysis, and another part was methylated and analysed by g.l.c.–m.s. The methylated product showed T_{CHI} 1.02 (retention time relative to methylated chitobi-itol). The mass spectrum of this compound showed, *inter alia*, peaks at *m/z* (relative intensities in brackets and some assignments[20] in square brackets): 89(100), 101(64), 103(24), 129(88)[H], 131(68), 228(84)[aA_2], 247(44)[bA_1], and 260(76)[aA_1]. Part of the methylated material was hydrolysed, and the product was converted into alditol acetates and analysed (Table I, column E). The material (42 mg) eluted from the DEAE-Sepharose column with ammonium hydrogencarbonate was recovered by freeze-drying. A solution of this material (consisting of crude **3**) in 0.5M hydrochloric acid (4 mL) was kept at 100° for 5 min. Hydrochloric acid was then removed by distillation *in vacuo* at 20°; water was added at the beginning of the distillation in order to avoid a high concentration of acid. A solution of the product in water was freeze-dried, and the residue was reduced with sodium borodeuteride and purified on a column (2.5 × 80 cm) of Sephadex G-15 (elution with water). The main component in the disaccharide region (*i.e.*, reduced **3**; 14 mg) had $[\alpha]_{578}^{25}$ −12° (*c* 0.4, water). Part of this material was subjected to sugar analysis, and another part was methylated and analysed by g.l.c.–m.s. The methylated product showed T_{CHI} 0.96. Its mass spectrum showed, *inter alia*, peaks at *m/z* (relative intensities in brackets and some assignments in square brackets[20]): 89(100), 91(25), 101(84), 129(52)[H], 131(46), 175(19), 228(51)[aA_2], 260(37)[aA_1], and 279(51)[bA_1]. Part of the methylated material was hydrolysed, and the product was converted into alditol acetates and analysed (Table I, column F).

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