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Structural Studies on the M-Antigen from Two Mucoid Mutants of *Salmonella typhimurium*

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The structures of the extracellular polysaccharides (M-antigen) from two mucoid mutants of *Salmonella typhimurium* 395MRO, M1 and M2, have been investigated by methylation analyses of the original polysaccharides and of polysaccharides obtained by mild acid hydrolysis and by Smith degradation. It is concluded that the polysaccharides are composed of hexasaccharide repeating units. On the basis of the present results and those of previous studies, a complete structure of this hexasaccharide unit may be proposed. The terminal β-D-galactopyranoside residue in the repeating unit carries a 3,4-*O*-ethylidene group in one, and a 3,4-*O*-carboxyethylidene group in the other polysaccharide.

When grown under the appropriate conditions, many strains from the genera *Salmonella*, *Escherichia*, and *Aerobacter* of the Enterobacteriaceae family produce an extracellular polysaccharide designated as the M-antigen. The early work on this antigen has been summarized by Lüderitz *et al.*[1] Strains producing large amounts of the polysaccharide are recognized by their ability to form mucoid colonies on solid media. The M-antigenic polysaccharide appears to be common to the mucoid forms of these bacteria. The sugar composition has been shown to be identical for a number of preparations of M-antigen from different species and strains of Enterobacteriaceae.[2] On acid hydrolysis it yields L-fucose, D-galactose, D-glucose, and D-glucuronic acid.[1] A polysaccharide with the above characteristics, isolated from a strain of *E. coli* was called colanic acid by Goebel.[3] A study of its occurrence in the Enterobacteriaceae has recently been published.[2]

Many strains of the *Salmonella-Escherichia* groups which are not commonly regarded as mucoid could be induced to form the extracellular polysaccharide when grown at room temperature [4] or in the presence of *p*-fluorophenylalanine.[5] The composition of the nutrient medium was also important for the production.[6] The genetics of the formation of the polysaccharide has been studied by Markovitz *et al.*[7]

In a previous communication,[8] a preliminary account of some structural studies on the M-antigen of *Salmonella typhimurium* 395MRO-M, now designated M1, was given. The purpose of the present communication is to provide a more detailed description of this work and also the results of further investigations on a similar antigen, obtained from another mutant of the same organism, designated M2.

Each polysaccharide contained L-fucose, D-galactose, D-glucose, and D-glucuronic acid in an approximate molar ratio of 2:2:1:1. In addition, mild acid hydrolysis and conversion into the appropriate 2,4-dinitrophenylhydrazones showed that one polysaccharide released acetaldehyde and the other pyruvic acid. A small amount of acetone was also obtained from the former polysaccharide, but its structural significance is uncertain. The latter polysaccharide also contained *O*-acetyl groups, the presence of which was shown by the characteristic carbonyl absorption in the IR as well as by the demonstration (GLC) of methyl acetate in the product after methanolysis. The other polysaccharide did not show the corresponding absorption band in the IR and therefore probably contains no acetyl. In the following discussion, the ethylidene acetal-containing material is designated polysaccharide 1, and the other, containing carboxyethylidene ketal and *O*-acetyl groups, polysaccharide 2.

Polysaccharide 1 on analytical ultracentrifugation moved as a single, homogeneous band, indicating a narrow molecular weight distribution. Partial acid hydrolysis of the material, separation of neutral and acidic fragments on an anion exchange resin followed by elution of the acidic fragments with aqueous formic acid gave a material containing essentially *O*-D-glucopyranosyluronic acid-D-galactose, $[\alpha]_{578}$ +1° (water). The material on more drastic acid hydrolysis yielded D-glucuronic acid and D-galactose. This, in conjunction with the optical rotation and with the methylation studies described below, shows that the aldobiouronic acid is identical with *O*-β-D-glucopyranosyluronic acid (1 → 3)-D-galactose, previously isolated from other M-antigens.[9]

Each of polysaccharides 1 and 2 was methylated according to the Hakomori procedure.[10] Part of the methylated polysaccharide 1 was reduced with lithium aluminium hydride.[11] The three methylated materials were hydrolyzed and then converted into the corresponding alditol acetates.[12] The resulting mixtures were examined by combined gas-liquid chromatography[13] – mass spectrometry[14] (GLC–MS). In this procedure, uronic acids are not accounted for, unless the carboxyl groups in the methylated polysaccharide have been reduced. A typical chromatogram is shown in Fig. 1, and the results of the methylation analyses in Table 1.

The high proportion of 2-*O*-methylfucose indicates a considerable degree of branching. However, the polysaccharide appears devoid of end groups. This seeming anomaly was resolved when it was found that mild hydrolytic treatment of the polysaccharide yielded acetaldehyde (from 1) and pyruvic acid (from 2). The identities of the carbonyl compounds were shown by subjecting the polysaccharide to mild acid treatment in the presence of 2,4-dinitrophenylhydrazine and demonstrating the presence of the appropriate 2,4-dinitrophenylhydrazones in the hydrolysates. Accordingly, polysaccharide 1 was subjected to mild acid treatment, and the product then methylated,

Table 1. Methyl ethers from the hydrolysates of methylated polysaccharides 1 and 2.

Polysaccharide		1	1	2
Sugars	T[a]	%[b]	%[c]	%[b]
2,3-Di-*O*-methyl-L-fucose	1.18	19	15	19
2-*O*-Methyl-L-fucose	1.67	23	19	22
2,4,6-Tri-*O*-methyl-D-glucose	1.95	22	19	22
2,4,6-Tri-*O*-methyl-D-galactose	2.28	18	16	17
2,6-Di-*O*-methyl-D-galactose	3.65	18	18	20
2,3-Di-*O*-methyl-D-glucose	5.39	–	13	–

[a] Retention times of the corresponding alditol acetates on the ECNSS-M column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
[b] Not carboxyl-reduced.
[c] Carboxyl-reduced.

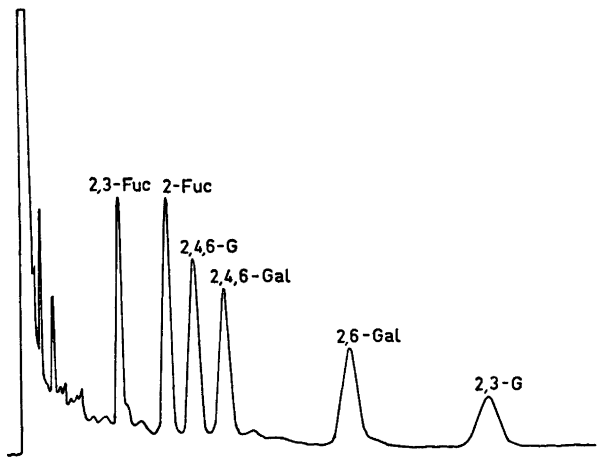


Fig. 1. GLC-separation of methylated sugars, as their alditol acetates, obtained from the hydrolysate of the fully methylated, carboxyl-reduced polysaccharide 1. 2,3-Fuc means the alditol derived from 2,3-di-*O*-methyl-L-fucose, *etc.* Peaks not accounted for are of non-carbohydrate nature (demonstrated by mass spectrometry).

hydrolyzed, and converted into the corresponding alditol acetates; these were then examined by GLC – MS.[13,14] The results, shown in Table 2, clearly indicate the attachment of the ethylidene groups to O-3 and O-4 of the non-reducing, terminal galactose end-units in the original polysaccharide. The 2,6-di-*O*-methylgalactose from methylated polysaccharide 1 shown in Table 1 thus originates from terminal 3,4-*O*-ethylidene-D-galactopyranose units. Since the same di-*O*-methyl ether is obtained in nearly the same proportions from the methylated polysaccharide 2, and since this polysaccharide contains pyruvic acid, it would seem reasonable to assume that the ketal in this polysaccharide is also attached to O-3 and O-4 of terminal non-reducing galactose. In

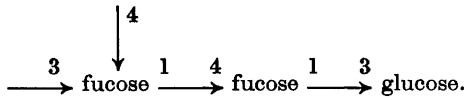
another communication [15] it will be shown that the M-antigens from various Enterobacteriaceae all apparently contain the same polysaccharide framework, the non-reducing, terminal galactose units of which carry various alkylidene residues. From the results in Table 2, some tentative conclusions

Table 2. Methyl ethers from the hydrolysate of methylated, partially degraded polysaccharide 1.

	T[a]	%[b]
2,3,4-Tri-*O*-methyl-L-fucose	0.65	2
2,3,4,6-Tetra-*O*-methyl-D-glucose	1.00	4
2,3-Di-*O*-methyl-L-fucose	1.18	17
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.25	10
2-*O*-Methyl-L-fucose	1.67	24
2,4,6-Tri-*O*-methyl-D-glucose	1.95	18
2,4,6-Tri-*O*-methyl-D-galactose	2.28	16
2,6-Di-*O*-methyl-D-galactose	3.65	9

[a, b] See Table 1.

regarding the actual sequence of the various units in the polysaccharide(s) may be drawn. In addition to the terminal galactose, discussed above, the hydrolysate also contains 2,3,4,6-tetra-*O*-methylglucose and 2,3,4-tri-*O*-methylfucose, demonstrating the presence of small amounts of terminal, non-reducing glucose and fucose in the degraded polysaccharide 1. Assuming that the fucosyl bonds are the most readily cleaved glycosidic bonds in the molecule, the aforementioned methylated sugars must arise from the sequences fucose → glucose and fucose → fucose, which combined afford the sequence



The methylation analyses (Tables 1 and 2) show that all sugars, except fucose, are pyranosidic. Had, however, the latter sugar occurred in the furanose form, the fucosyl bonds would have been cleaved far more rapidly than was

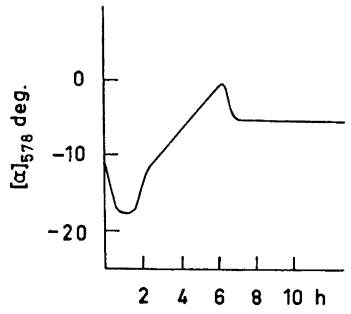
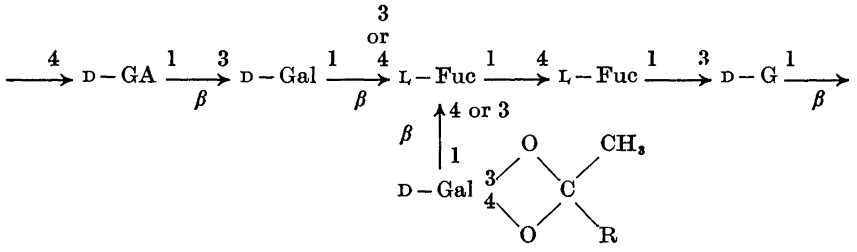


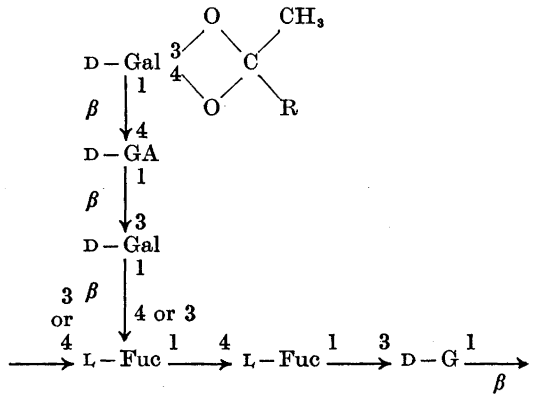
Fig. 2. Optical rotation *versus* time on acid hydrolysis of polysaccharide 2.

actually observed; in all probability therefore, these units also are pyranosidic. The change in optical rotation of polysaccharide 2 in 0.025 M aqueous sulphuric acid is shown in Fig. 2. For polysaccharide 1, the curve was similar, except for the higher initial rotation of the polysaccharide ($[\alpha]_{578}$ +24°). The initial rapid decrease in the rotation, probably due to the hydrolysis of the alkylidene groups, is followed by a complex change in optical rotation. Since the fucosyl bonds are expected to be more acid labile than other glycosidic bonds, this change could be explained by the cleavage of α-L-fucosidic linkages hydrolyzing at a somewhat faster rate than β-L-fucosidic ones. The low optical rotation of the polysaccharide indicates that except for this one fucosidic bond, the other glycosidic bonds are β.

The above findings allow two structural alternatives for the repeating unit, I and II:



I.



II.

In polysaccharide 1: R=H.
In polysaccharide 2: R=COOH.

The polysaccharides contain either 3,4-*O*-ethylidene-β-D-galactopyranosyl terminal, non-reducing end-units (polysaccharide 1) or the corresponding 3,4-*O*-carboxyethylidene unit (polysaccharide 2). Ethylidene acetals have not previously been found in polysaccharides from natural sources. Pyruvic acid ketals are well documented,[16-18] however, the galactose ketals previously found have involved O-4 and O-6, and not O-3 and O-4 as in polysaccharide 2.

In order to distinguish between the two structural alternatives, polysaccharide 2 was subjected to Smith degradation.[19] After periodate oxidation, borohydride reduction and mild acid hydrolysis, neutral and acidic fragments (from uronate residues) were separated by ion exchange. The neutral portion was then subjected to Hakomori methylation;[10] the methylated material was hydrolyzed, and the product converted into the corresponding alditol acetates. The results, which are presented in Fig. 3 and in Table 3, in conjunction with the above findings, clearly indicate the presence in the polysaccharide of the fragment III.*

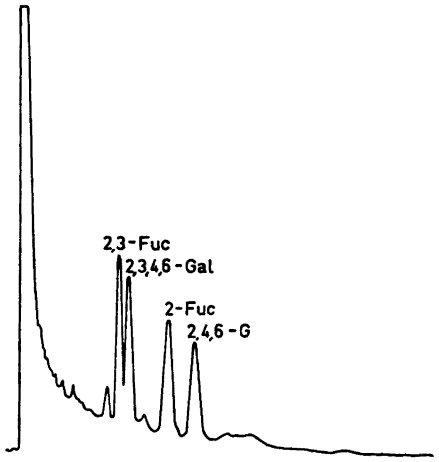
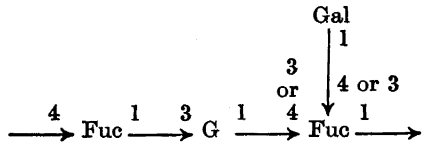


Fig. 3. GLC-separation of methylated sugars, as their alditol acetates, obtained from the hydrolysate of the Smith-degraded, fully methylated polysaccharide 2. 2,3-Fuc means the alditol derived from 2,3-di-*O*-methyl-L-fucose, *etc.* Peaks not accounted for are of non-carbohydrate nature (demonstrated by mass spectrometry).

Table 3. Methylation analysis of the neutral fragment (from polysaccharide 2) arising from periodate oxidation, borohydride reduction, partial acid hydrolysis, followed by removal of acidic fragments.

Sugars	T[a]	%
2,3-Di-*O*-methyl-L-fucose	1.18	26
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.25	25
2-*O*-Methyl-L-fucose	1.67	25
2,4,6-Tri-*O*-methyl-D-glucose	1.95	24

[a] See Table 1.

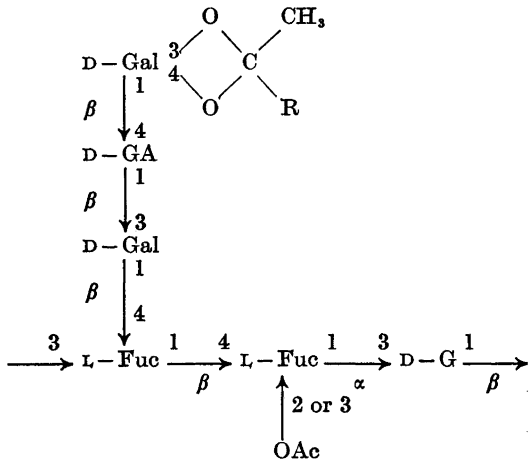


III.

* Difficulties in achieving hydrolysis of the glycol-cleaved and reduced uronic acid residues under the mild conditions normally used in the Smith degradation have been reported (Aspinall, G. O., Bhavanadan, V. P and Christensen, T. B. *J. Chem. Soc.* **1965** 2677; Bouveng, H. *Acta Chem. Scand.* **19** (1965) 953). Slightly stronger hydrolysis conditions were, however, used in the present work, which may account for the observed results.

The non-branching fucose unit should, according to the result presented in Table 1, be (1,4)-linked. It nonetheless seems to have resisted periodate oxidation. The polysaccharide 2 does, however, contain *O*-acetyl, and it therefore is possible that this fucose unit is acetylated in the polysaccharide.

The results therefore establish structure II for the repeating unit of the M-antigen from *Salmonella typhimurium* 395MRO-M2. This is in good agreement with the detailed structure proposed by Sutherland *et al.*[20a,b] for the *E. coli* K12(S53) M-antigen, except for the position and/or nature of the *O*-alkylidene group linked to the terminal D-galactose residue. It seems reasonable to assume that the polysaccharides contain the same hexasaccharide repeating unit. Sutherland, on fragmentation analyses, isolated a β-D-glucopyranosyl-L-fucose, distinguishable from 4-*O*-β-D-glucopyranosyl-L-fucose. This, in conjunction with the results of the methylation analyses, indicates that the linkage between D-glucose and L-fucose is (1 → 3). He also isolated, in small amounts, an L-fucosyl-D-glucosyl-L-fucose which, on treatment with α-L-fucosidase, yielded L-fucose and the above disaccharide. Thereby the anomeric nature of one of the L-fucose residues was determined. The final structure for the repeating unit of the M-antigen from *Salmonella typhimurium* 395MRO-M, is depicted by IV, and it is assumed that these structural features also are valid for the present polysaccharides:



IV.

In polysaccharide 1: R = H.
In polysaccharide 2: R = COOH.
– OAC is present in polysaccharide 2 only.

The configuration at the acetalic and ketalic carbon atoms of the alkylidene groups have not been determined. In proposing structure IV, the presence of a repeating unit is assumed.

EXPERIMENTAL

Isolation of the polysaccharide. The mucoid mutants were isolated from mutant strains resistant to phage FO-1. The techniques used have been described earlier.[12] The parental strain was a spontaneous R mutant of *Salmonella typhimurium* 395M, designated RO.[12] For the production of the slime polysaccharide, 20 plates (diam. 19 cm), each containing 100 ml of solid medium, were used. The composition of the medium was as follows (concentrations in g/l): D-glucose 4.0, NH_4Cl 0.4, $Na_2HPO_4 \cdot 2H_2O$ 7.5, KH_2PO_4 3.0, NaCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.2, and Difco-Agar 15. The concentration of the nitrogen source, ammonium chloride, was low in order to give suboptimal growth. Each plate was inoculated with 1 ml of a bacterial suspension, containing 10^8 viable bacteria. They were incubated for 24 h at 37° followed by 48 h at room temperature. The colonies formed in this manner consisted of a dense centre of bacterial cells (diam. about 1 mm), surrounded by a confluent layer of extracellular viscous polysaccharide. 10 ml of 0.01 M phosphate buffer (pH 7) was poured onto each plate, and the colonies and slime were scraped off with the aid of a bent glass rod. The material was gently suspended in the buffer and then filtered through a double layer of gauze. The bacterial cells, constituting the dense centre of each colony, were retained by this procedure, and a homogeneous, viscous solution was obtained. The material was then dialyzed against distilled water, and freeze-dried. The average yield was 600 – 700 mg. The material was purified by phenol-water treatment,[21] and the aqueous phase processed as described by Sutherland.[22] A yield of about 200 mg polysaccharide with a purity exceeding 90 % was obtained. The purity was checked by carbohydrate analysis as previously described,[12] but with xylose as an internal standard.

General methods

Concentrations were carried out in a vacuum at bath temperatures below 40°.

GLC analyses were performed on a Perkin-Elmer Model 801 Chromatograph, fitted with glass columns, and with a flame ionization detector. The carrier gas was nitrogen and the flow rate 30 ml/min. The column packing was 3 % ECNSS-M on GasChrom Q unless otherwise stated, the column temperature about 175° for the separation of acetylated alditols, and about 150° for acetylated methyl alditols.

GLC-MS was run on a Perkin-Elmer 270 combined gas chromatograph-mass spectrometer. The mass spectra were recorded at a manifold temperature of 200°, an ionization potential of 60 eV, ionization current of 80 μA, and a temperature at the ion source chamber of 80°.

Acid hydrolyses and analyses of hydrolysates

(*a*) *Identification of acetal groups.* The polysaccharides (about 40 mg) were treated with 0.025 M aqueous sulphuric acid (1 ml) at 100° for 1 h in the presence of 2,4-dinitrophenylhydrazine (80 mg). Extraction of the mixtures with chloroform yielded the following products. *Polysaccharide 1.* 0.8 mg crystals. Examination of this product by TLC (silica gel-isopropyl ether), GLC (on a 3 % OV 1 methyl silicone on Chromosorb W column), and by MS revealed the presence of acetaldehyde (major product) and acetone (minor amounts) 2,4-dinitrophenylhydrazones. Paper chromatography (see below) showed the absence of the corresponding pyruvic acid derivative. This was confirmed by mild acid hydrolysis as described above, but omitting the 2,4-dinitrophenylhydrazine. The hydrolysate was examined by GLC (on a 10 % Carbowax polyglycol on Chromosorb W column), whereby the presence of acetaldehyde (major compound) and acetone (minor compound) was confirmed. *Polysaccharide 2.* Examination of this product by TLC (silica gel-isopropyl ether) and by GLC showed the absence of acetaldehyde and acetone 2,4-dinitrophenylhydrazones. The m.p. of the crystals was 208 – 212°. Paper chromatography (Whatman No. 1) in butanol:pyridine:water, 6:4:3, showed the presence of pyruvic acid 2,4-dinitrophenylhydrazone (m.p. 216°),[23] which was confirmed by MS.

(*b*) *Mild acid hydrolysis followed by methylation.* The polysaccharide was hydrolyzed with 0.025 M aqueous sulphuric acid at 100° for 1 h, cooled, neutralized with Dowex 3

(free base), filtered and concentrated to dryness. The last traces of water was removed by co-distillation with benzene. The dry residue was methylated according to the Hakomori procedure.[10]

(*c*) *Hydrolysis of polysaccharides.* The polysaccharides were treated with 0.25 M aqueous sulphuric acid at 100° for 16 h, cooled, neutralized, worked up, and the mixture converted into the corresponding alditol acetates as previously described.[12] The hydrolysis depicted in Fig. 2 was performed at 90° in 0.025 M aqueous sulphuric acid with an initial polysaccharide concentration of 0.1 %.

(*d*) *Hydrolyses of methylated polysaccharides.* The methylated polysaccharide after purification was dissolved in 90 % formic acid (3 ml) and kept at 100° for 3 h, after which it was concentrated to dryness. The residue was treated with 0.25 M aqueous sulphuric acid at 100° for 16 h and then transformed into the corresponding alditol acetates as previously described.[12]

Methanolyses. Each of the two polysaccharides (10 mg) was treated with 2 % hydrogen chloride in methanol (0.5 ml) at 100° for 1 h. The solutions were examined for the presence of methyl acetate by GLC (on a 10 % Carbowax on Chromosorb W column) at 40°.

Methylation analyses. The various materials were methylated essentially as described by Hakomori.[10] The polysaccharide (10 mg) was dispersed in methyl sulphoxide (2 ml) in an ultrasonic bath (Aerograph Ultrasonic cleaner) in a capped serum bottle under nitrogen at room temperature. The methyl sulphinyl anion was generated by adding sodium hydride (50 mg, freed from adhering oil from the container by repeated washings with hexane under nitrogen) to methyl sulphoxide (2 ml), previously purified by distillation and by drying over Union Carbide Molecular Sieve 4 Å) under nitrogen in a capped serum bottle in the ultrasonic bath. After 4 h at room temperature, the sodium methylsulphinylmethide solution (2 ml) was added to the polysaccharide dispersion. After 1 h at room temperature in the ultrasonic bath under nitrogen, followed by standing for at least 5 h at room temperature, methyl iodide (1 ml, freshly distilled from phosphorus pentoxide) was added under cooling in ice-water. The serum bottle was then placed in the ultrasonic bath and kept there for 1 h. The content was poured into water (20 ml), and the methylated polysaccharide extracted with three portions (25 ml each) of chloroform. The combined chloroform extracts were washed with three portions (25 ml each) of water, dried over magnesium sulphate, filtered, and concentrated to dryness.

Lithium aluminium hydride reductions were performed essentially as previously described.[11] The methylated polysaccharide (15 mg, previously dried in a vacuum over phosphorus pentoxide overnight) was dissolved in dry tetrahydrofuran (5 ml) and treated with lithium aluminium hydride (or deuteride, when required for MS purposes) (30 mg), and the mixture refluxed for 4 h. Excess reducing agent was destroyed by adding in turn ethyl acetate, ethanol, and water, and the mixture was then neutralized with 2 M aqueous phosphoric acid. The salts were removed by filtration, and the filtrate concentrated to dryness.

Smith degradation. Polysaccharide 2 (100 mg) was treated with sodium metaperiodate (107 mg) in water (50 ml) in the ultrasonic bath at 24°. Attempts to follow the periodate consumption quantitatively by spectrophotometry as described by Aspinall and Ferrier [24] were not successful due to the opalescence of the solutions. Although the uncertainty of the absorption of the solution at zero time precluded quantitative measurements, the method nevertheless qualitatively showed the progress of the reaction. After 70 h, when no more periodate was consumed, the excess periodate was removed by adding excess glycol and then dialyzing the material for 48 h. The product in water (3 ml) was treated with sodium borohydride (100 mg) at 25° for 48 h, neutralized with Dowex 50 (H^+ form), concentrated, and then hydrolyzed with 0.05 M aqueous sulphuric acid at 25° for 72 h; the borohydride reduction, neutralization and hydrolysis were all carried out in the ultrasonic bath. The hydrolysate was neutralized with Dowex 2 (acetate form). Filtration and concentration yielded a neutral material which was lyophilized and then dried in a vacuum over phosphorus pentoxide. The material did not move from the starting line on paper chromatography (solvent, ethyl acetate:acetic acid:water, 3:1:1).

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