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LIPOPOLYSACCHARIDES FROM *Pseudomonas maltophilia*: STRUCTURAL STUDIES OF THE SIDE-CHAIN POLYSACCHARIDE FROM STRAIN N.C.T.C. 10257

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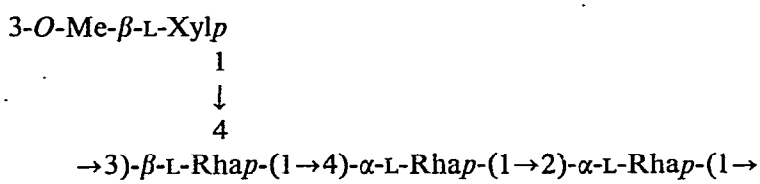
ABSTRACT

A lipopolysaccharide has been isolated from *Pseudomonas maltophilia* N.C.T.C. 10257. Monosaccharide components identified were L-rhamnose, 3-*O*-methyl-L-xylose, L-xylose, D-glucose, D-mannose, D-galacturonic acid, 2-amino-2-deoxygalactose, 2-amino-2-deoxyglucose, and a 3-deoxy-2-octulosonic acid. Heptose was absent. In this and other respects, the lipopolysaccharide resembles the corresponding products from *Xanthomonas* species. Mild hydrolysis of the lipopolysaccharide with acid, followed by chromatography of the water-soluble products on Sephadex G-50, gave a polymeric, "side-chain" fraction containing rhamnose, 3-*O*-methylxylose, and xylose residues in the molar ratios ~15:4:1. Methylation analysis, periodate oxidation, Smith degradation, and oxidation with chromium trioxide were the principal methods used in the study of this fraction. The following structure is proposed for the characteristic repeating-unit of the polymer.



INTRODUCTION

The extensive studies of bacterial lipopolysaccharides[1] have encompassed few pseudomonads other than *Pseudomonas aeruginosa*. Our interest in *Pseudomonas maltophilia* stems from the observation[2] that the organism does not exhibit the sensitivity to EDTA that characterises *P. aeruginosa* and closely related aerobic pseudomonads. Studies of the phenotypic features[3] and nucleic acid homologies[4] of the aerobic pseudomonads confirm that *P. maltophilia* is a relatively isolated species. Further stimulus to study of the species is provided by its growing importance as an opportunist pathogen[5].

Preliminary analyses[6] of the cell wall of *P. maltophilia* N.C.T.C. 10257 revealed

a high content of rhamnose and the presence of other neutral sugars, including glucose, mannose, xylose, and an unidentified pentose derivative. More recently[7], these sugars have been identified as components of a lipopolysaccharide, and the pentose derivative has been characterised as 3-*O*-methyl-L-xylose. Further data on the composition of the lipopolysaccharide and structural studies of the presumptive O-specific polysaccharide therefrom are now reported.

RESULTS AND DISCUSSION

In preliminary experiments, lipopolysaccharide was extracted from whole cells by using aqueous butan-1-ol[8] or aqueous phenol[9]. Both products contained rhamnose, xylose, 3-*O*-methylxylose, glucose, and mannose, but the phenol-extracted product was obtained only in low yield and was relatively deficient in the first three sugars. Physical heterogeneity of the butan-1-ol-extracted product (yield, ~ 4 mg . g^{-1} wet cells), attributable to the aggregation commonly found for bacterial lipopolysaccharides, was demonstrated by chromatography on Sepharose 4B. In the absence of sodium dodecyl sulphate, two fractions of similar monosaccharide composition were detected. The major fraction (86% of the total product) gave a tailing peak with V_e/V_o 1.12; the minor fraction had V_e/V_o 1.76. In the presence of sodium dodecyl sulphate, the major fraction gave a symmetrical peak with V_e/V_o 2.18. As expected, the disaggregated product was retarded more by Sepharose 2B, but was virtually excluded from Sephadex G-100. The elution profiles obtained by monitoring the chromatograms for phosphorus and for total carbohydrate were similar. However, chromatography of the lipopolysaccharide on DEAE-cellulose in the presence of Triton X-100 gave a single peak for which the later fractions had relatively high phosphorus–carbohydrate ratios. Although the lipopolysaccharide was free from nucleic acid, it was contaminated by protein (23%), despite the use of pronase in the

TABLE I

COMPOSITION OF THE LIPOPOLYSACCHARIDE FROM *Pseudomonas maltophilia*

Component	*Composition (%)*	*Component*	*Composition (%)*
Total carbohydrate	61[a]	KDO[i]	0.3
Total pentose	13[b]	Aldoheptose	0
L-Rhamnose	42[c], 41[d,e]	2-Amino-2-deoxygalactose	1.1[d]
3-*O*-Methyl-L-xylose	10.2[c]	2-Amino-2-deoxyglucose	3.3[d]
L-Xylose	1.8[c]	Total amino acids	1.9[d]
D-Glucose	2.8[f,g], 3.1[c,d]	Phosphorus	1.8
D-Mannose	2.0[g], 1.8[c]	Lipid A	22
D-Galacturonic acid	+[h]		

[a]Phenol–sulphuric acid method. [b]Orcinol–hydrochloric acid method. [c]G.l.c. of alditol acetate. [d]Autoanalysis. [e]Cysteine–sulphuric acid method. [f]D-Glucose oxidase method. [g]Hexokinase method. [h]Present, but not determined. [i]3-Deoxy-2-octulosonic acid.

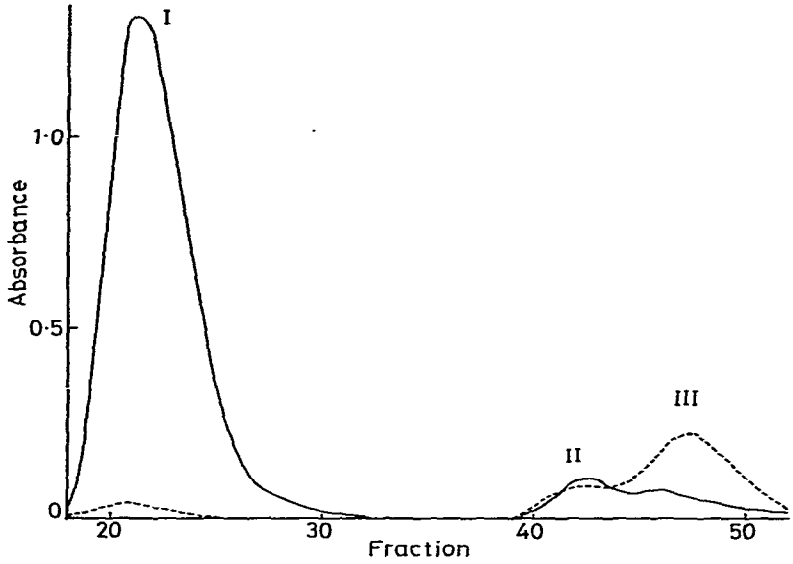


Fig. 1. Chromatography of the partly degraded polysaccharide from *P. maltophilia* N.C.T.C. 10257 on Sephadex G-50. The sample (110 mg) was applied to a column (38 × 2.5 cm) and eluted with aqueous pyridine–acetic acid buffer (pH 5.4) at a flow rate of 20 ml.h^{-1}. Fractions (4 ml) were analysed for total carbohydrate by the phenol–sulphuric acid method (absorbance at 490 nm, ——) and for phosphorus (absorbance at 830 nm, – – – –), and then combined as indicated (I, II, and III).

purification procedure. Further study of the butan-1-ol-extracted material was therefore discouraged, and attention was given to the use of isolated cell-walls, from which essentially pure lipopolysaccharide could be obtained by the aqueous phenol method.

The yield of lipopolysaccharide from a single treatment of defatted cell-walls with hot, aqueous phenol was 18–19%. Table I gives analytical data for a representative batch. Significant batch variations for the proportions of hexoses to rhamnose and pentoses pointed to chemical heterogeneity consistent with mixtures of S- and R-form lipopolysaccharides. This interpretation was supported by the results of sodium dodecyl sulphate–polyacrylamide gel electrophoresis. All batches of lipopolysaccharide gave a major, slow-moving band and a minor, fast-moving band, the proportion of which increased with the hexose content of the lipopolysaccharide. By analogy with other lipopolysaccharides[10], the bands should correspond to S- and R-form lipopolysaccharides, respectively. Further evidence for these assignments was obtained by Sephadex chromatography of the water-soluble products from mild hydrolysis of the lipopolysaccharides with acid. Most of the material applied (61% in the fractionation shown in Fig. 1, which relates to products from the lipopolysaccharide for which data are given in Table I) was recovered in the phosphorus-depleted fraction I, V_e/V_o 1.16 on Sephadex G-50, 1.33 on Sephadex G-75. Fraction I contained all of the rhamnose, xylose, and 3-*O*-methylxylose, and only traces of other sugars. Fraction II (which was cleanly separated from fraction III by further

chromatography on Sephadex G-15) contained most of the glucose, mannose, and 2-amino-2-deoxygalactose, and part of the galacturonic acid. Different batches of fractions I and II gave consistent analyses, but the proportions of the two fractions varied according to the hexose content of the parent lipopolysaccharides. Thus, although serological confirmation has not been sought, fraction I is probably the O-specific side-chain with attached core-unit derived from S-form lipopolysaccharide, while fraction II is the phosphorylated core from R-form lipopolysaccharide lacking the O-specific substituent. The presence in fraction I of "core-specific" components, and the different chromatographic properties of disaggregated lipopolysaccharide and fraction I indicate that the latter is not simply a polysaccharide contaminant of the former. Structural studies of fractions II and III, and of the lipid-A fraction, will be reported elsewhere, but the absence from the lipopolysaccharide of an aldoheptose (Table I) and its low content of KDO (both typical components of the core units of enterobacterial lipopolysaccharides and those of the more representative pseudomonads[1]) are noteworthy.

Fraction I, $[\alpha]_D$ −12°, was essentially a rhamnose–pentose polymer: total carbohydrate 91% (determined as rhamnose by the phenol–sulphuric acid method); rhamnose 61%, 3-*O*-methylxylose 14%, xylose 3% (determined as alditol acetates). It contained little phosphorus (0.15%) and only traces of other monosaccharides, presumably present in a terminal core-unit. The absence of *O*-acyl groups was confirmed by i.r. and ^{1}H-n.m.r. spectroscopy. Each of the sugars specific to fraction I was the L isomer[7], and their proportions, determined both for lipopolysaccharide and for fraction I, were indicative of a tetrasaccharide repeating-unit (rhamnose–pentose, 3:1), in which xylose replaced its 3-*O*-methyl derivative in every fourth or fifth unit. The results of methylation analysis (Table II) support this inference. The pentoses occurred exclusively as unsubstituted pyranosyl groups, whereas the rhamnose occurred mainly as 2-substituted, 4-substituted, and 3,4-disubstituted residues in similar amounts. Possible explanations for the small proportion of 3-substituted rhamnose are discussed below; the rather low recovery of xylose may be attributed to the relative ease of destruction during hydrolysis of the methylated

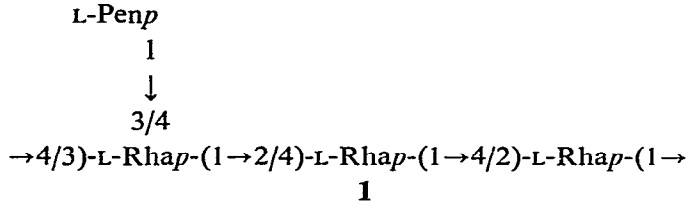
TABLE II

METHYLATION ANALYSIS OF FRACTION I

Methylated sugar[a]	T[b]	*Detector response*	*Primary m.s. fragments* (m/e)
2,3,4-Xyl	0.56	0.69	117, 161
3,4-Rha	0.74	1.07	131, 189
2,3-Rha	0.79	1.06	117, 203
2,4-Rha	0.87	0.13	117, 131, 233, 247
2-Rha	1.16	1.00	117, 275

[a]2,3,4-Xyl = 2,3,4-tri-*O*-methyl-L-xylose, *etc.* [b]Retention time for the corresponding alditol acetate, relative to that for 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on OS-138 at 180°.

polysaccharide and the greater volatility of the methylated alditol acetate. The recovery after hydrolysis by the method of Percival[11] was significantly worse than after hydrolysis by the method[12] of Lindberg's group, which was used to obtain the data in Table II. The identities of the methylation products were confirmed by g.l.c. comparison with reference samples of the alditol acetates on ECNSS-M, by g.l.c.–m.s. (primary fragments are listed in Table II), and by methylation analysis of fraction I after periodate oxidation. Only the residues of 3-*O*-methylxylose, 3-substituted rhamnose, and 3,4-disubstituted rhamnose survived oxidation. The consumption of periodate (4.0 μmol . mg^{-1}) approached the theoretical value (4.1 to 4.3 μmol . mg^{-1}, depending on the ratio of xylose to 3-*O*-methylxylose) for a polymer with a repeating unit of partial structure **1**.



1

Pen = Xylose or 3-*O*-methylxylose

In order to locate the pentose residue and to determine the sequence of rhamnose residues in the chain, the polysaccharide was subjected to Smith degradation. Paper chromatography of a total acid hydrolysate of the major product, apparently a trisaccharide alditol, revealed the presence of rhamnose, 3-*O*-methylxylose, and a butane-1,2,3-triol (R_{Rha} 1.5 in solvent system *B*; not resolved from 3-*O*-methylxylose in solvent system *A*), but no glycerol. Acetylation followed by g.l.c. on column *4* (which separates the diastereoisomeric triol acetates) identified the triol as 1-deoxyerythritol. Methylation analysis of the Smith-degradation products, monitored by g.l.c. (column *2*) and g.l.c.–m.s. of the alditol acetates, confirmed that the 3-*O*-methylxylose residue was unsubstituted and showed that the rhamnose was mainly 4-substituted (a small proportion was unsubstituted). The partial structure **2** can therefore be inferred for the major Smith-degradation product; minor products containing unsubstituted rhamnose residues could arise from repeating units containing xylose or no pentose residue (a possible explanation for the presence of some 3-substituted rhamnose in fraction I, Table II), or as a result of partial loss of 3-*O*-methylxylose residues during the hydrolytic step of the Smith degradation.

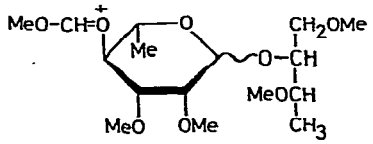
3-*O*-Me-L-Xylp-(1→4)-L-Rhap-(1→3)-D-dEryl

2

dEryl = 1-Deoxyerythritol

Structure **2** for the trisaccharide alditol was confirmed by g.l.c.–m.s. of its permethylated derivative[13,14]. On column *3*, the derivative had a retention time of 3.36 relative to that of permethylated maltitol. Fragment ions of the A series[15] at *m/e* 175 (aA_1), 143 (aA_2), and 111 (aA_3) establish that the pentose residue is at the

non-reducing terminus, while the deoxytetritol residue is indicated by the ions at *m/e* 177 (bcJ_1) and 117 (cA_1). Rhamnosyl substitution of the deoxytetritol residue is shown by the ions at *m/e* 351 ($abcJ_1$, **3**), 291 (bcA_1, **4**), and 259 (bcA_2). Fragmentation by the bcA pathway and the occurrence of the base peak at *m/e* 88, as observed, also support a (1→4) linkage between the pentose and 6-deoxyhexose residues[13].



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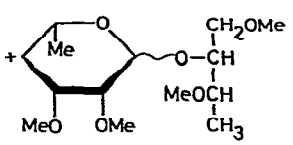

4

The assignment of structure **2** to the major product of Smith degradation defines the repeating unit of fraction I as structure **5**. In order to determine the anomeric configurations, a sample of fraction I was acetylated and the product treated with chromium trioxide in acetic acid (a procedure known to oxidise β-pyranosyl residues selectively[16]). About 59% of the rhamnose (assumed two-thirds), all of the xylose, but none of the 3-*O*-methylxylose residues survived the treatment. Methylation analysis of the degraded polysaccharide revealed the presence of 2-substituted and unsubstituted rhamnose residues in similar amounts, but no 3-substituted, 4-substituted, or 3,4-disubstituted rhamnose residues. The unsubstituted rhamnose must arise from an α-rhamnopyranosyl residue substituted in the 4-position by a β-rhamno-

L-Pen*p*
1
↓
4
→3)-L-Rha*p*-(1→4)-L-Rha*p*-(1→2)-L-Rha*p*-(1→

5

Pen = Xylose or 3-*O*-methylxylose

pyranosyl residue (the residue at the branch point). The structure of the preponderant repeating unit in fraction I can therefore be finalised as **6**. The ^{1}H-n.m.r. spectrum of fraction I contained clear signals for the anomeric protons of the rhamnopyranosyl

3-*O*-Me-β-L-Xyl*p*
1
↓
4
→3)-β-L-Rha*p*-(1→4)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→

6

residues at δ 5.19, 4.82, and 4.70 with the expected low coupling-constants. The signal

for the anomeric proton of the 3-*O*-methylxylopyranosyl group at δ ~4.4 (J ~9 Hz) was partly obscured by the side band of the HOD signal.

The fact that no terminal rhamnose residue was detected by methylation analysis of fraction I suggests that structure **6** may also represent the biological repeating-unit of the polymer. The presence in the polymer of small proportions of terminal α-linked xylopyranose and 3-substituted β-rhamnopyranose residues also raises the possibility that these are components of a modified repeating-unit which terminates biosynthesis of the polymer. However, no direct evidence for these hypotheses has been obtained.

Whereas L-rhamnose is a common component of bacterial polysaccharides, including lipopolysaccharides, 3-*O*-methyl-L-xylose has only been found in lipopolysaccharides from *Rhodopseudomonas viridis*[17,18] and *P. maltophilia*. In its overall composition, the lipopolysaccharide from *P. maltophilia* strikingly resembles those of *Xanthomonas* species[19–22]. In addition to a high content of rhamnose, distinctive features of these products include the presence of D-galacturonic acid, the absence of a heptose, a low content of KDO, and an unusual complement of fatty acids[23,24]. Studies of nucleic acid homologies[4] also demonstrate the close relationship between *P. maltophilia* and *Xanthomonas* species.

EXPERIMENTAL

General methods. — Concentrations were performed under reduced pressure at bath temperatures not exceeding 40°. G.l.c. was performed with a Pye Series 104 instrument. Glass columns of the sizes indicated were packed with the following stationary phases: *1*, 3% of ECNSS-M on Gas Chrom Q (1.6 m × 2 mm); *2*, 10% of OS-138 and 1% of Adpet 80 on Chromosorb W (1.5 m × 3 mm); *3*, 3% of OV-225 on Gas Chrom Q (0.8 m × 2 mm); and *4*, 30% of SE-52 on Gas Chrom Q (2.5 m × 3 mm). For quantitative g.l.c., a Supergrator-2 integrator (Columbia Scientific Instruments) was used. G.l.c.–m.s. was performed with an LKB spectrometer (model 2091) coupled to a Pye Series 104 chromatograph. Paper chromatography was performed on Whatman No. 1 paper with *A*, 5:2:5 ethyl acetate–pyridine–water (upper phase); and *B*, 4:1:1 butan-1-ol–ethanol–water. Reagents used for the detection of sugars and polyols were alkaline silver nitrate, aniline hydrogen oxalate, and the periodate–Schiff reagents. Gel-permeation chromatography on Sephadex (G-75, G-50, G-15, or G-10) was performed with 10:4:986 pyridine–acetic acid–water (pH 5.4) as the eluent. Sodium dodecyl sulphate–polyacrylamide electrophoresis[10] was performed with slab gels and 0.01M phosphate buffer (pH 7.0); bands were detected with the periodate–Schiff reagents. Optical rotations were determined with a Bendix polarimeter (model 143A). ^{1}H-N.m.r. spectra for polysaccharide fraction I in D_2O at ~85° were recorded with a JEOL 4H-100 spectrometer and sodium 4,4-dimethyl-4-silapentane-1-sulphonate as the internal standard.

Methods of quantitative analysis. — Phosphorus was determined by the method of Bartlett[25], and amino compounds by autoanalysis (Locarte bench analyser), after hydrolysis of samples with 6.1M hydrochloric acid at 105° for 4 h. Unhydrolysed

samples were used for the determination of total carbohydrate by the phenol–sulphuric acid method[26], total pentose by the orcinol–hydrochloric acid method[27], and heptose and KDO by the methods of Osborn[28]. For the determination of other monosaccharides, samples were hydrolysed with M hydrochloric acid at 105° for 4 h. The hydrolysates were neutralised with Dowex 1 (HCO_3^-) resin and deionised. Galactose was used as the internal standard both for autoanalysis of the hydrolysates with a modified Technicon analyser[29] and for g.l.c. analysis (column *I*) after conversion of the sugars into alditol acetates[30]. Rhamnose was also determined by the cysteine–sulphuric acid method[31], D-glucose by the use of D-glucose oxidase (EC 1.1.3.4) or of hexokinase (EC 2.7.1.1) with D-glucose 6-phosphate dehydrogenase (EC 1.1.1.49), and D-mannose by the use of the latter combination with D-glucose phosphate isomerase (EC 5.3.1.9) and D-mannose phosphate isomerase (EC 5.3.1.8). All enzymes were obtained from Boehringer und Soehne G.m.b.H. Standard mixtures of sugars subjected to treatment with acid under the conditions used for hydrolysis of samples were used for the calibration of all analyses.

Growth of bacteria and isolation of lipopolysaccharide. — Cells of *P. maltophilia* N.C.T.C. 10257 were grown for 16 h at 37° in Nutrient Broth No. 2 (Oxoid) with aeration at 10 litres.min^{-1}. Lipopolysaccharide was extracted from whole cells by using aqueous butan-1-ol[8] or aqueous phenol[9] and was purified by the standard methods[8,9]. For structural studies, lipopolysaccharide was extracted by the aqueous phenol method from defatted cell-walls[32].

Chromatography of disaggregated lipopolysaccharide. — The behaviour of lipopolysaccharide extracted by the aqueous butan-1-ol method and disaggregated by treatment with a surfactant (sodium dodecyl sulphate or Triton X-100) was studied by gel-permeation and ion-exchange chromatography. Elution profiles were determined both for total carbohydrate and for phosphorus. For gel-permeation studies, samples (~5 mg) of lipopolysaccharide were applied to columns (52 × 1.4 cm) of Sepharose 2B, Sepharose 4B, and Sephadex G-100, and eluted with 0.12M Tris hydrochloride buffer (pH 8.1) containing sodium dodecyl sulphate (0.5%). For ion-exchange chromatography[33], lipopolysaccharide (20 mg) in Triton X-100 (5 ml) was applied to a column (13 × 1 cm) of DEAE-cellulose (Whatman DE-32, chloride form). Elution was carried out with a linear gradient formed from 1% Triton X-100 (100 ml) and M sodium chloride in 1% Triton X-100 (100 ml). Carbohydrate- and phosphorus-containing materials were co-eluted, but the maxima in the respective elution profiles were at 25 and 35 ml.

Isolation of polysaccharide fractions. — Lipopolysaccharide (10 mg.ml^{-1}) was hydrolysed with 1% aqueous acetic acid at 100° for 1.5 h. After extraction of precipitated lipid A into chloroform, the water-soluble products were freeze-dried and then fractionated by Sephadex chromatography. The side-chain polysaccharide of high molecular weight (fraction I) was isolated after chromatography on a column (38 × 2.5 cm) of Sephadex G-50. The core polysaccharide of low molecular weight (fraction II) and hydrolytic fragments (fraction III) were resolved by further chromatography on a column (84 × 1.8 cm) of Sephadex G-15.

Methylation analyses. — Samples (2–5 mg) of polysaccharide fraction I, before and after oxidation with sodium periodate or chromium trioxide, and of an oligosaccharide alditol derived from a Smith degradation of fraction I were methylated by the use of sodium methylsulphinylmethanide and methyl iodide in methyl sulphoxide[12]. Polymeric products were purified by dialysis, and the oligomeric product by partition between chloroform and water. The purified products were treated with 90% formic acid at 100° for 2 h and then with 0.13M sulphuric acid at 100° for 12 h, followed by neutralisation of the acid with barium carbonate. The partially methylated sugars were converted into their alditol acetates and identified by g.l.c. (columns *1* and *2*) and g.l.c.–m.s.

Periodate oxidation. — For the determination of periodate consumption[34], fraction I (10 mg) was oxidised with 0.06M sodium periodate (1 ml) at 4° for 3 days. For product analysis, the excess of periodate was reduced with ethane-1,2-diol (7 μl), and the oxidised polysaccharide treated with sodium borohydride (20 mg) at room temperature for 2 h. After the dropwise addition of 2M acetic acid to decompose the excess of sodium borohydride, the product was deionised on a column (36 × 2.6 cm) of Sephadex G-50.

Smith degradation. — For preparative work, a larger sample (47 mg) of fraction I was oxidised with sodium periodate, and the product treated with sodium borohydride under conditions comparable with those described above. The final product (33 mg) was hydrolysed with 0.5M hydrochloric acid at room temperature for 8 h. After neutralisation with Dowex 1 (HCO_3^-) resin and deionisation, the hydrolysate was fractionated on a column (85 × 1.5 cm) of Sephadex G-10. The major product, apparently a trisaccharide alditol, was hydrolysed with M hydrochloric acid at 100° for 4 h, and the components were identified by paper chromatography (solvents *A* and *B*) and by g.l.c. (columns *1* and *4*) of the derived alditol acetates. Further samples of the Smith-degradation products were subjected to methylation analysis and also examined by g.l.c.–m.s. (column *3*) after permethylation.

Chromium trioxide oxidation. — Fraction I (25 mg) in formamide (5 ml) was treated with acetic anhydride (2 ml) and pyridine (2.5 ml) at room temperature for 20 h. The reaction mixture was poured into ice-cold water (100 ml), and the peracetate was purified by dialysis. After the addition of *myo*-inositol hexa-acetate as internal standard, part of the product (6 mg) was treated with chromium trioxide (50 mg) in acetic acid (1 ml) at 50° for 2 h with sonication. After addition of the reaction mixture to water, the chloroform-soluble product was extracted and its solution dried ($MgSO_4$). Both the oxidised peracetate and an unoxidised control-sample were subjected to sugar analysis (by g.l.c. of the alditol acetates) and to methylation analysis.

Synthesis of reference compounds. — 3-*O*-Methyl-D-xylose, prepared from 1,2-*O*-isopropylidene-5-*O*-tosyl-α-D-xylofuranose (Sefochem-Fine Chemicals Ltd., Israel) by the use of standard reactions, had m.p. 95°, $[\alpha]_D$ +14.6° (*c* 4, water); lit.[35] m.p. 99.5–101°, $[\alpha]_D$ +15°; lit.[36] m.p. 96°, $[\alpha]_D$ +19°.

The diastereoisomeric butane-1,2,3-triols were prepared by the application of

standard hydroxylation reactions[37] to but-2-en-1-ol, mainly the *E* isomer. Hydroxylation with alkaline potassium permanganate gave mainly 1-deoxythreitol, b.p. 120–123°/0.5 mmHg; lit.[38], for the D isomer, b.p. 98–99°/0.15 mmHg. Hydroxylation with performic acid gave mainly 1-deoxyerythritol, b.p. 113–116°/0.3 mmHg; lit.[38], for the D isomer, b.p. 94°/0.05 mmHg.

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