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# COVALENT STRUCTURE OF THE EXTRACELLULAR POLYSACCHARIDE FROM *Xanthomonas campestris*: EVIDENCE FROM PARTIAL HYDROLYSIS STUDIES*

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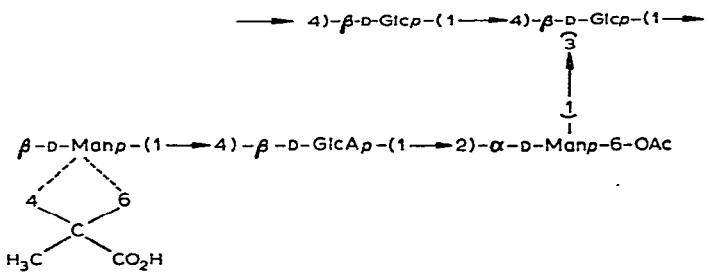
ABSTRACT

Partial, acid hydrolysis of the extracellular polysaccharide from *Xanthomonas campestris* gave products that were identified as cellobiose, 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose, *O*-(β-D-glucopyranosyluronic acid)-(1→2)-*O*-α-D-mannopyranosyl-(1→3)-D-glucose, *O*-(β-D-glucopyranosyluronic acid)-(1→2)-*O*-α-D-mannopyranosyl-(1→3)-[*O*-β-D-glucopyranosyl-(1→4)]-D-glucose, and *O*-(β-D-glucopyranosyluronic acid)-(1→2)-*O*-α-D-mannopyranosyl-(1→3)-[*O*-β-D-glucopyranosyl-(1→4)]-*O*-β-D-glucopyranosyl-(1→4)-D-glucose. This and other evidence supports the following polysaccharide structure (**1**) which has been proposed independently by Jansson, Kenne, and Lindberg:




**1**

## INTRODUCTION

The extracellular polysaccharide produced by the bacterium *Xanthomonas campestris* NRRL B-1459 is industrially useful because of the unusual physical properties of its solutions, including pseudoplasticity[1], the anomalous variation of viscosity with temperature[2], and its ability to form mixed gels with certain other polysaccharides such as galactomannans[3]. In preliminary studies[4], we have shown that

*Editorial Footnote:* Because the paper cited as Ref. 6 formed part of a memorial issue, a proposal by Professors Lindberg and Rees for the simultaneous publication of their related papers proved to be impracticable.

these properties can be traced to the existence of an ordered conformation in aqueous solutions at room temperature which "melts" reversibly at higher temperatures, and which can bind co-operatively to polysaccharides having unbranched sequences of β-(1→4)-linked D-mannose, D-glucose, or D-xylose residues. The ordered conformation can also be detected in the condensed phase, by X-ray diffraction studies on oriented fibres[5]. Further progress in these conformational studies was hampered, however, by the lack of definitive evidence on the covalent structure, and for this reason we have reinvestigated this problem. When our work was in progress, we learned that Lindberg and his co-workers had almost completed the definition of structure by elegant, new stepwise-degradation procedures[6]. Our own conclusions are reached by different methods but are entirely consistent, and we describe them here to support the structure which they have proposed.

It was already known[7,8] that the polysaccharide contains residues of D-glucose, D-mannose, and D-glucuronic acid, with acetate and pyruvate groups, in the proportions shown in Table I. Periodate oxidation[9] followed by reduction with sodium borohydride and acid hydrolysis yielded, among other products, 2-*O*-β-D-glucopyranosyl-D-erythritol. From the acid hydrolysate of the polysaccharide[7], an aldobiouronic acid and two higher acidic oligosaccharides were isolated, and evidence was presented suggesting the aldobiouronic acid was 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose. Preliminary investigations of the two higher oligosaccharides suggested that they were composed of additional D-glucose residues linked to the reducing end of the aldobiouronic acid.

TABLE I

COMPOSITION OF POLYSACCHARIDE FROM *X. campestris*[7,8]

| *Component* | *Molar proportion* |
|---|---|
| D-Glucuronic acid | 2.0 |
| D-Glucose | 3.8 |
| D-Mannose | 3.7 |
| Acetate | 1.7 |
| Pyruvate | 0.51–0.63 |

The results of the methylation analysis[8] are given in Table II. The major components are consistent with the results of periodate oxidation[9], and indicate that the only terminal non-reducing residues are mannoses, the other mannose residues present are (1→2)-linked, the glucuronic acid residues are (1→4)-linked, and there are two types of D-glucose residue. One type of D-glucose residue is linked (1→4), and the other is a branching point with linkages through positions 3 and 4. The minor components may be due to incomplete methylation and/or the presence of pyruvate groups linked to positions 4 and 6 of glucopyranosyl or mannopyranosyl residues. (The pyruvate group would be removed by the methanolysis step and hence 2,3-di-*O*-methylglucosides or 2,3-di-*O*-methylmannosides would result). Sloneker and co-workers[9,10] have repeatedly suggested that the pyruvate is linked to positions 4 and 6

of a glucopyranosyl residue. The evidence for this is based on the isolation of 1,3-*O*-(1-carboxyethylidene)-L-erythritol from periodate-oxidised polysaccharide which had been subsequently reduced and hydrolysed. However, 1,3-*O*-(1-carboxyethylidene)-L-erythritol could have arisen from a pyruvate-substituted mannopyranose instead. The position of attachment of the acetate group was not definitely established, although it was suggested that periodate-oxidation studies[9] pointed to its location on position 6 of the internal mannose residue.

TABLE II

METHYLATION ANALYSIS OF *X. campestris* POLYSACCHARIDE BY G.L.C.[8]

| *Component* | *Molar ratio* |
|---|---|
| 2,3,4,6-Tetra-*O*-methyl-D-mannose | 3 |
| 2,3,6-Tri-*O*-methyl-D-glucose | 2 |
| 3,4,6-Tri-*O*-methyl-D-mannose | 2 |
| 2,6-Di-*O*-methyl-D-glucose | 3 |
| 2,3-Di-*O*-methyl-D-glucuronic acid | 3 |
| 2,3-Di-*O*-methyl-D-mannose | 0.3 |
| 3,4-Di-*O*-methyl-D-mannose | 0.25 |
| 2,3-Di-*O*-methyl-D-glucose | 0.25 |

Our approach to the structure of the *X. campestris* polysaccharide was to isolate and characterize the oligosaccharides produced by partial, acid hydrolysis. If sufficient oligosaccharides were characterized so that they "overlapped", then the whole repeating sequence would be obtained.

## EXPERIMENTAL

*Chromatography.* — P.c. was performed on Whatman No. 1 paper, using *A*, 10:4:3 ethyl acetate–pyridine–water; *B*, 18:3:1:4 ethyl acetate–acetic acid–formic acid–water; *C*, 18:8:3:9 ethyl acetate–acetic acid–formic acid–water; and detection with aniline oxalate at 120–130°. $R_F$ values are given relative to that ($R_{GLCA}$) of glucuronic acid.

Preparative p.c. was carried out on washed Whatman No. 3 paper, using solvent *B*. The time for the separation was 4 days unless stated otherwise.

Liquid chromatography (l.c.) was performed on a Pye Liquid Chromatograph, using a column (50 × 0.4 cm) of Aminex A-6 ($Li^+$) resin (Bio-Rad Labs.) equilibrated with 85% ethanol–water at 75°. The column effluent was continuously monitored by using a modified moving-wire detector[11]. The determinations were kindly carried out by Mr. J. S. Hobbs.

G.l.c. was performed on a Pye 104 chromatograph, using (*1*) a column (100 × 0.3 cm) of 5% of polyethyleneglycol 20M on 100–120 mesh acid-washed, silanised Celite at 170°, and (*2*) a column (150 × 0.3 cm) of 5% of neopentylglycol adipate on 100–120 mesh acid-washed, silanised Celite at 165°, unless otherwise stated. Retention times (*T*) were measured relative to that of methyl 2,3,4,6-tetra-*O*-

methyl-β-D-glucoside, which had an absolute retention time of 1.50 min on column *1* and 1.22 min on column *2*.

G.l.c.–m.s. was performed on a Pye 104 chromatograph linked to an A.E.I. MS-902 mass spectrometer, using a column (0.3 × 150 cm) of 5% of polyethyleneglycol 20M on 100–120 mesh acid-washed, silanised Celite at 180°. The collaboration of Mr. D. Welti on this part of the work is acknowledged.

*General.* — P.m.r. spectra were recorded on a Bruker–Spectrospin instrument at 90 MHz. Chemical shifts were referenced to standard, external sodium 4,4-dimethyl-4-silapentanesulphonate (0 p.p.m.). Optical rotations were measured with a Perkin–Elmer 141 polarimeter at room temperature in a 1-dm cell, using the appropriate solvent. Compounds were dried over phosphorus pentaoxide under vacuum at 60° to give powders of constant weight. DEAE Sephadex (A25) was allowed to swell overnight in water and then washed three times alternately with 0.5M sodium hydroxide and 0.5M hydrochloric acid before being generated in the formate form by stirring with 15% formic acid. The resin was washed with water to remove excess formic acid and packed in a column (3 × 35 cm). Dialysis was performed in a closed system against distilled water, which was changed daily. Solutions were concentrated on a Buchi rotary evaporator at bath temperatures not exceeding 45°.

*Hydrolyses.* — Each oligosaccharide (5 mg) was hydrolysed overnight in M sulphuric acid (0.5 ml) in a sealed tube at 100°. The solution was neutralised with barium carbonate, filtered, treated with Amberlite IR-120($H^+$) resin, and concentrated to a syrup.

*Reduction.* — To a solution of each oligosaccharide (5 mg) in water (0.5 ml), sodium borohydride (10 mg) was added and the solution was stored overnight. The sodium ions were removed from the solution with Amberlite IR-120($H^+$) resin. The solution was then concentrated five times with methanol to give the oligosaccharide alditol.

*Methanolysis.* — Each oligosaccharide (5 mg) was treated with 4% methanolic hydrogen chloride (2 ml) in a sealed tube at 100° for 6 h. The solution, containing methyl glycosides and the methyl ester of methyl glucopyranosiduronic acid, was neutralised with silver carbonate, filtered, and concentrated to a syrup.

*Kuhn methylation.* — A solution of each oligosaccharide in *N,N*-dimethylformamide (0.1 ml) (the amounts given in this description are per mg of oligosaccharide) was shaken with iodomethane (0.2 ml) and silver oxide (0.1 g) in a sealed tube in the dark for 48 h. The mixture was filtered and the residue was washed with chloroform. The combined filtrate and washings were concentrated, and *N,N*-dimethylformamide was removed by co-distillation with toluene under diminished pressure.

*Haworth methylation.* — To a solution of each carbohydrate in water (0.4 ml) (the amounts are per mg of carbohydrate), methyl sulphate (0.05 ml) and 30% aqueous sodium hydroxide (0.1 ml) were added dropwise during 1 h whilst the mixture was stirred vigorously under nitrogen and the temperature was kept at 0°. The mixture was then stirred overnight at room temperature. Further additions of

methyl sulphate (0.2 ml) and 30% aqueous sodium hydroxide (0.4 ml) were made on each of 5 subsequent days, each addition taking 4 h; 24 h after the final addition, the reaction mixture was heated at 100° for 1 h. The solution was then adjusted to pH 4 with dilute sulphuric acid and poured into 8 vol. of ethanol. The precipitated sodium sulphate was collected, and washed with ethanol. The filtrate was adjusted to pH 8 and the solution was concentrated to dryness.

*Purification of polysaccharide.* — *Xanthomonas campestris* polysaccharide, purchased from A.B.M. Chemicals Ltd., Cheshire, was dissolved in water and dialysed. After dialysis for 6 days, the polysaccharide solution was freeze-dried.

*Isolation of oligosaccharides.* — To a solution of the freeze-dried polysaccharide (15.1 g) in water (750 ml), 4M $H_2SO_4$ (750 ml) was added and the solution was heated for 2 h at 100°. The solution was treated with $Ba(OH)_2$ followed by $BaCO_3$ to pH 5.1. After filtration and evaporation of most of the water, the solution was treated with Amberlite IR-120($H^+$) resin and concentrated to dryness. A solution of the residue (13.8 g) in water (100 ml) was applied to a column of DEAE Sephadex and eluted successively with water and 0.05M formic acid (4.7 l). The 15-ml fractions were analysed by p.c. and combined as appropriate. Formic acid was removed by extraction with 5% dioctylmethylamine in chloroform, and then with chloroform. The combined fractions were concentrated to syrups and dried. According to p.c. (solvents *A* and *B*), the material (7.8 g) eluted by water (fraction *1*) contained glucose and mannose, with cellobiose as a minor component.

The disaccharide (32 mg) was isolated by preparative p.c. of a portion (1 g) of fraction *1* (16 h). The material (30 mg) was conventionally[12] acetylated with acetic anhydride (2.5 ml) and sodium acetate to give β-cellobiose octa-acetate, m.p. 189–191°, mixture m.p. 190–192°. Authentic β-cellobiose octa-acetate, prepared in the same manner, had m.p. 194–196°.

The material (4.3 g) eluted by formic acid (fraction *2*) was separated into five components by preparative p.c.: *2A* (650 mg), *2B* (25 mg), *2C* (70 mg), *2D* (75 mg), and *2E* (25 mg).

Fraction *2A* was chromatographically pure, $R_{GLCA}$ 0.41 (solvent *B*), 0.75 (solvent *C*), and hydrolysis gave glucuronic acid and mannose (p.c., solvents *A* and *B*). Hydrolysis of the oligosaccharide glycitol gave glucuronic acid as the sole reducing sugar (p.c., solvents *A* and *B*). After conversion into the methyl ester methyl glycosides followed by methanolysis, then reduction with sodium borohydride and hydrolysis, glucose and mannose were detected (p.c., solvents *A* and *B*) in the ratio ~1:1,

A solution of a portion (5 mg) of fraction *2A* in oxygen-free lime-water (2 ml) was stored at room temperature. After 10 days, the calcium ions were removed with Amberlite IR-120($H^+$) resin and the solution was concentrated. P.c. (solvents *A* and *B*) of the syrupy residue showed one major spot with the same $R_F$ value as the original oligosaccharide and a 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannopyranose standard.

Samples of the oligosaccharide and the oligosaccharide glycitol were methylated (Kuhn) and methanolysed. The products were examined by g.l.c. (see Table III).

TABLE III

ASSIGNMENT OF PEAKS OBTAINED ON G.L.C. OF METHYLATED–METHANOLYSED *X. campestris* FRACTIONS *2A*, *2B*, *2C*, *2D*, AND *2E*[a]

| *Retention times*[b] (*Column* 1) | | | | | | *Methyl glycoside* | *Retention times*[b] (*Column* 2) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Standard* | 2A | 2B | 2C | 2D[c] | 2E | | *Standard* | 2A | 2B | 2C | 2D | 2E |
| 0.71m | | | | 0.72w | 0.72w | 2,3,4,6-Tetra-*O*-methylglucoside | 0.72m | | | | 0.73w | 0.72w |
| 1.00s | | | | 1.00s | 1.00s | | 1.00s | | | | 1.00s | 1.00 s |
| 2.16 | 2.22 | | (2.17) | (2.20) | (2.17) | 3,4,6-Tri-*O*-methylmannoside | 1.69 | 1.70 | | 1.70 | 1.69 | 1.73 |
| 2.19 | | | (2.17) | | | 2,4,6-Tri-*O*-methylglucoside | 1.83m | | | (1.85) | | |
| 3.22 | | | 3.22 | | | | 2.62s | | | 2.62 | | |
| 1.71m | 1.72 | 1.71 | 1.71 | 1.71 | 1.69 | Methyl 2,3,4-tri-*O*-methylglucopyranosiduronate | 1.48m | 1.48m | 1.48m | 1.49 | 1.49 | 1.48 |
| 2.21s | (2.22) | 2.21 | (2.17) | (2.20) | (2.17) | | 1.87s | 1.88s | 1.90s | (1.85) | (1.92) | — |
| 8.45m | | | | 8.65m | 8.54m | 2,6-Di-*O*-methylglucoside | — | | | | | |
| 11.14s | | | | 11.37s | 11.19s | | — | | | | | |
| | | 2.15 | | | | 3,4,6-Tri-*O*-methylglucoside | | | 1.71 | | | |
| 2.41m | | | | 2.43m | 2.43m | 2,3,6-Tri-*O*-methylglucoside | 1.95m | | | | (1.92) | 1.96m |
| 3.21s | | | | 3.22s | 3.19s | | 2.61s | | | | 2.61 | 2.62s |

[a]Key: s, strong; m, medium; w, weak. Retention times in brackets indicate overlapping peaks. [b]Where reference samples were not available, peaks were identified by comparison with retention times cited in the literature[18]. [c]No methyl 2,3,4,6-tetra-*O*-methylmannoside was obtained by temperature-programming under conditions known to separate it and the corresponding glucoside (3% of ECNSS/M, 50–150° at 1°/min.).

TABLE IV

G.L.C. OF METHYLATED–METHANOLYSED FRACTIONS AFTER REDUCTION WITH SODIUM BOROHYDRIDE[a]

| *Retention times* (*Column* 1) | | | | *Sugar* | *Retention times* (*Column* 2) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Standard* | 2A | 2B | 2D | | *Standard* | 2A | 2B | 2D | 2E |
| 0.71m | | | 0.69w | Methyl 2,3,4,6-tetra-*O*-methylglucoside | 0.72m | | | 0.72w | 0.72w |
| 1.00s | | | 0.98s | | 1.00s | | | 1.00s | 1.00s |
| 2.16 | | | (2.13) | Methyl 3,4,6-tri-*O*-methylmannoside | 1.69 | | | 1.72 | (1.67) |
| 2.19 | | | 2.36 | Methyl 2,4,6-tri-*O*-methylglucoside | 1.83m | | | (1.92) | (1.90) |
| 3.22 | | | (3.07) | | 2.62s | | | 2.63 | 2.64 |
| 1.71m | 1.72m | 1.71m | 1.64 | Methyl (methyl 2,3,4-tri-*O*-methylglucosiduronate) | 1.48m | 1.49m | (1.52) | 1.50 | 1.46 |
| 2.21s | 2.24s | 2.23s | (2.13) | | 1.87s | 1.89s | 1.96 | (1.92) | (1.90) |
| 1.64 | | | | 1,2,3,5,6-Penta-*O*-methylglucitol | 1.69 | | | | (1.67) |
| | | 1.52 | | 1,3,4,5,6-Penta-*O*-methylglucitol | | | (1.63) | | |
| | 1.87 | | | 1,3,4,5,6-Penta-*O*-methylmannitol | | 1.77 | | | |
| | | | (3.07) | 1,2,5,6-Tetra-*O*-methylglucitol | | | | 2.74 | |

[a]For conditions, see Table III.

Fraction *2A* was 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannopyranose, and had $[\alpha]_D$ −45° (*c* 1.2, water); lit.[13,14] −32° (*c* 0.83), −31° (*c* 1).

Fraction *2B* was chromatographically pure, $R_{GLCA}$ 0.66 (solvent *C*). Hydrolysis gave glucuronic acid, glucose, and traces of mannose (p.c., solvents *A* and *B*). Hydrolysis of the oligosaccharide glycitol gave glucuronic acid and traces of glucose and mannose, whereas hydrolysis of the reduced methyl ester methyl glycosides gave glucose and traces of mannose (p.c., solvents *A* and *B*).

Treatment (10 days) of fraction *2B* with lime-water (as described for *2A*) gave a mixture of two substances having the $R_F$ values of the starting material and 2-*O*-(β-D-glucosyluronic acid)mannose. Samples of the oligosaccharide and the oligosaccharide glycitol were methylated (Kuhn), methanolysed, and examined by g.l.c. (see Table III).

Fraction *2B* was 2-*O*-(β-D-glucopyranosyluronic acid)-D-glucopyranose and had $[\alpha]_D$ −8° (*c* 0.75, water); lit.[15] −1.6° (*c* 2).

TABLE V

MASS SPECTRA OF PARTIALLY METHYLATED METHYL GLYCOSIDES BY G.L.C.–M.S.

| *Methyl 2,3,4-tri-O-methylglucosiduronate* | | *3,4,6-Tri-O-methyl-mannoside* | | *2,4,6-Tri-O-methyl-glucoside* | | *2,3,6-Tri-O-methylglucoside* | |
|---|---|---|---|---|---|---|---|
| *1st peak* | | *1st peak* | | *1st peak* | | *1st peak* | |
| m/e | % | m/e | % | m/e | % | m/e | % |
| 101 | 100 | 71 | 100 | 71 | 100 | 88 | 100 |
| 88 | 62 | 75 | 94 | 74 | 63 | 75 | 62 |
| 75 | 25 | 88 | 71 | 101 | 60 | 45 | 34 |
| 45 | 16 | 45 | 67 | 45 | 35 | 73 | 21 |
| 73 | 16 | 74 | 62 | 102 | 26 | 71 | 17 |
| 85 | 11 | 101 | 32 | 75 | 17 | 85 | 13 |
| 89 | 8 | 89 | 31 | 59 | 12 | 101 | 12 |
| 102 | 6 | 87 | 26 | 87 | 12 | 87 | 12 |
| 59 | 4 | 161 | 20 | 88 | 10 | 161 | 11 |
| 74 | 4 | 59 | 20 | 89 | 9 | 74 | 9 |
| *2nd peak* | | *2nd peak* | | *2nd peak* | | *2nd peak* | |
| m/e | % | m/e | % | m/e | % | m/e | % |
| 101 | 100 | 75 | 100 | 71 | 100 | 88 | 100 |
| 88 | 64 | 71 | 100 | 101 | 68 | 75 | 57 |
| 75 | 24 | 45 | 75 | 74 | 66 | 45 | 35 |
| 45 | 15 | 74 | 57 | 45 | 43 | 73 | 19 |
| 73 | 14 | 88 | 44 | 102 | 31 | 71 | 15 |
| 85 | 12 | 89 | 33 | 88 | 23 | 101 | 14 |
| 89 | 8 | 101 | 32 | 75 | 19 | 85 | 12 |
| 102 | 5 | 87 | 30 | 87 | 17 | 87 | 12 |
| 59 | 4 | 59 | 20 | 59 | 13 | 74 | 10 |
| 74 | 4 | 161 | 16 | 89 | 9 | 161 | 9 |

Fraction *2C* was chromatographically homogeneous, $R_{GLCA}$ 0.31 (solvent *B*) and 0.54 (solvent *C*), and had $[\alpha]_D$ +18° (*c* 0.7, water). Hydrolysis gave (p.c., solvents *A* and *B*) substances corresponding to 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose, glucuronic acid, mannose, and glucose; the ratio of glucose to mannose by l.c. was 1:1. The hydrolysate of the oligosaccharide glycitol contained the same sugars except glucose. Reduction of the methyl ester methyl glycosides followed by hydrolysis gave (p.c., solvents *A* and *B*) glucose and mannose in the ratio of ~2:1. The oligosaccharide was methylated (Kuhn) and methanolysed. G.l.c. of the products under the usual conditions (Table III) was not conclusive and therefore the conditions were modified (Table IV). The identification of methyl ethers was confirmed by g.l.c.–m.s. (Table V). The combined evidence shows that fraction *2C* is *O*-(β-D-glucopyranosyluronic acid)-(1→2)-*O*-D-mannopyranosyl-(1→3)-D-glucose.

*Fraction 2D* was chromatographically homogeneous, $R_{GLCA}$ 0.12 (solvent *B*) and 0.35 (solvent *C*), and had $[\alpha]_D$ +13° (*c* 0.6, water). Hydrolysis gave (p.c., solvents *A* and *B*) 2-*O*-(β-D-glucosyluronic acid)-D-mannose, glucuronic acid, mannose, and glucose. The ratio of glucose to mannose (l.c.) was 1.85:1. Hydrolysis of the oligosaccharide glycitol gave the same sugars but, by visual examination of the chromatogram, a reduced amount of glucose. Methanolysis, reduction, and hydrolysis gave glucose and mannose in the ratio of ~3:1.

Partial hydrolysis (0.5M $H_2SO_4$, 1.5 h, at ~100°), followed by neutralisation, gave (p.c.) mainly glucose and 2-*O*-(β-D-glucopyranosyluronic acid)mannose, with moderate amounts of mannose, cellobiose, and trisaccharide *2C*. Hydrolysis of the oligosaccharide glycitol under similar conditions gave (p.c.) a similar distribution of sugars but without cellobiose. The methanolysis products were identified by g.l.c. (Table III) after permethylation of the oligosaccharide by the Haworth procedure and the oligosaccharide glycitol by the Kuhn method.

Fraction *2E* was chromatographically homogeneous, $R_{GLCA}$ 0.03 (solvent *B*) and 0.22 (solvent *C*). Hydrolysis of the oligosaccharide glycitol and of the reduction product of the methyl glycoside methyl ester gave results (p.c.) similar to those obtained with fraction *2D*. The oligosaccharide and its glycitol were methylated by the Haworth and Kuhn procedures, respectively, and the products were identified by g.l.c. (Table III). It was not possible to obtain an accurate $[\alpha]_D$ value for fraction *2E*, owing to contamination by chromatographic support material and the small quantities available.

## RESULTS AND DISCUSSION

The results of previous work[7,8] (Tables I and II) can be re-interpreted to give a much simpler repeating-unit consisting of D-glucuronic acid, D-glucose, and D-mannose in the ratios of 1:2:2, with one acetate group per repeating unit and one pyruvate group per three repeating units. Our results, as well as those of Jansson, Kenne, and Lindberg[6], support this proposal.

The presence of acetate and pyruvate groups in the polysaccharide was con-

firmed by p.m.r. spectroscopy at 80–100°. Two peaks were observed at $\tau$ 7.8 and 8.5, corresponding to those previously[16] assigned to acetate and pyruvate groups in acidic polysaccharides. The areas of these peaks, relative to an internal standard, changed with temperature in a way that indicated an order–disorder transition[17]. This variation could be correlated with changes[2,4] in viscosity, optical rotation, and circular dichroism, and the results will be published elsewhere.

After partial hydrolysis of the polysaccharide with acid and fractionation of the resulting oligosaccharides on a column of DEAE Sephadex, a neutral fraction (*1*) and an acidic fraction (*2*) were obtained. Fraction *1* contained cellobiose, shown by conversion into the crystalline $\beta$-octa-acetate, thereby confirming the earlier identification on the basis of its chromatographic mobility and i.r. spectrum[9]. No higher cellulosic oligosaccharides were detected by p.c. Fraction *2* contained five acidic oligosaccharides (*2A–E*). The major acidic oligosaccharide was 2-*O*-($\beta$-D-glucopyranosyluronic acid)-D-mannose. This disaccharide has previously been detected in hydrolysates of the extracellular polysaccharides of *X. campestris* and the related micro-organism, *Xanthomonas oryzae*[19].

Fraction *2B* was 2-*O*-($\beta$-D-glucopyranosyluronic acid)-D-glucose; it was a very minor component and could be an artefact formed from *2A* by epimerisation during isolation.

Acid hydrolysis of *2C* and its derivatives showed it to be a trisaccharide of the type *O*-($\beta$-D-glucopyranosyluronic acid)-(1→2)-*O*-D-mannopyranosyl-D-glucose. It has previously been suggested[9] that the linkage between the internal mannose and the adjacent glucose residue is (1→4). If this were so, the products of methylation and methanolysis should include 2,3,6-tri-*O*-methylglucosides. These derivatives were not formed, but 2,4,6-tri-*O*-methylglucosides were obtained, indicating a (1→3)-linkage. Although the two sets of glycosides are difficult to separate by g.l.c. and there are problems of overlap with other components, their identity was established conclusively in two ways. First, by using column *2* (neopentylglycol adipate) at a lower temperature (140°) and with a lower flow-rate of carrier gas (65 ml/min) than usual and supplementing the sample with increasing amounts of 2,3,6-tri-*O*-methylglucosides. Second, by g.l.c.–m.s. The most-abundant peaks from the mass spectra of standard, partially methylated monosaccharides are shown in Table V. The mass spectra of the fast and slow peaks of methyl 2,3,6-tri-*O*-methylglucoside are in agreement with the published[20] spectra of the $\beta$- and $\alpha$-glucosides, respectively. The two peaks of methyl 2,4,6-tri-*O*-methylglucosides gave mass spectra in agreement with those published[20] for the $\beta$-glucoside, and the two peaks of methyl (methyl 2,3,4-tri-*O*-methylglucosiduronate) gave spectra comparable to those published[21] for the $\alpha$ anomer of this compound. Apparently, mass spectra for methyl 3,4,6-tri-*O*-methylmannoside have not previously been recorded.

The first peak (*T* 1.71) from methylated and methanolysed fraction *2C* gave fragment ions at *m/e* 101, 88, and 75, in approximately the correct ratios for methyl (methyl 2,3,4-tri-*O*-methylglucopyranosiduronate). The retention time is also as expected. The leading edge of the second peak (*T* 2.19) gave signals in decreasing order

of intensity, *m/e* 71, 75, 45, 88, 89, 101, 87, 161, and 59 which, with the retention time, correspond to methyl 3,4,6-tri-*O*-methyl-α-D-mannoside. From the centre of the peak to the trailing edge, the order of decreasing intensity of fragment ions changed from *m/e* 101, 88, 75, 71, 45, 74, 89, 87, and 161 to *m/e* 101, 71, 88, 45, 75, 74, 102, and then to *m/e* 71, 101, 45, 74, 85, 102. These data suggest that the second methyl (methyl 2,3,4-tri-*O*-methylglucosiduronate) anomer follows the mannoside. Finally, the last spectrum is consistent with that of the faster methyl 2,4,6-tri-*O*-methylglucoside and not the 2,3,6-tri-*O*-methyl derivative. The third g.l.c. peak (*T* 3.22) gave fragment ions in order of decreasing intensity *m/e* 71, 101, 74, 45, 102, 88, and 75, which is consistent with methyl 2,4,6-tri-*O*-methylglucoside; the mannoside peak in g.l.c. is very weak and hence the intensities of the m.s. signals are low. Thus, 2,4,6-tri-*O*-methylglucosides are present as well as methyl 2,3,4-tri-*O*-methylglucosiduronates and 3,4,6-tri-*O*-methylmannosides; if the 2,3,6-tri-*O*-methylglucosides are present, they could at most represent 10% of the amount of the 2,4,6-tri-*O*-methyl isomers.

Based on the foregoing data, we conclude that the *2C* is an *O*-(β-D-glucopyranosyluronic acid)-(1→2)-*O*-D-mannopyranosyl-(1→3)-D-glucose. This resembles the tetrasaccharide *O*-(β-D-glucopyranosyluronic acid)-(1→2)-*O*-D-mannopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→4)-D-glucose, which has been isolated from a polysaccharide produced by a closely related micro-organism, *Xanthomonas hyacinthi*[22].

Fractions *2D* and *2E* were examined by techniques similar to those for *2A* and *2B* (see Tables III and IV for g.l.c. results), with an additional g.l.c. column (ECNSS/M), to show that 2,3,4,6-tetra-*O*-methylglucoside was present rather than the corresponding mannoside. The results indicate that *2D* is a branched tetrasaccharide *O*-(β-D-glucopyranosyluronic acid)-(1→2)-*O*-D-mannopyranosyl-(1→3)-[*O*-β-D-glucopyranosyl-(1→4)]-D-glucose, and that *2E* appears to be a homologous, branched pentasaccharide *O*-(β-D-glucopyranosyluronic acid)-(1→2)-*O*-D-manno-pyranosyl-(1→3)-[*O*-β-D-glucopyranosyl-(1→4)]-*O*-D-glucopyranosyl-(1→4)-D-glucose.

Our reasons for investigating the structure of the polysaccharide from *Xanthomonas campestris* were to use the information in elucidation of the conformation and its relation to physical and biological properties. For this purpose, it is as essential to determine the configurations of glycosidic linkages as it is to determine their positions. We found that the native polysaccharide was precipitated by Concanavalin A, which indicates that the mannose residue at either the non-reducing terminal[23] or in the internal position[24] had the α-D configuration. Since the optical rotations for the unmethylated (see below) and the methylated (+7.5°) polysaccharide are low, it is probable that other anomeric linkages are β, but this will be fully discussed.

The difference between the molecular rotation of trisaccharide *2C* and that of the disaccharide *2B* was +253° (or +202°, using a literature value for the disaccharide[13,14]). That this is a clear indication for an α-D configuration at the linkage by which the third residue is added, *i.e.*, at the (1→3)-linkage between mannose and glucose, is confirmed by comparison with similar calculations for related di- and

oligo-saccharides of known configuration. This is a more-reliable approach than straightforward application of Hudson's rules, since, in principle, it can take conformational influences[25] at the linkage into account. The following pairs of sugars differ by an α-D-linked sugar residue and have the difference in molecular rotation which is shown in brackets: D-glucose and 3-*O*-α-D-glucopyranosyl-D-glucose (+377°), D-mannose and 3-*O*-α-D-mannopyranosyl-D-mannose (+171°), D-mannose and 4-*O*-α-D-mannopyranosyl-D-mannose (+141°), D-glucose and 3-*O*-α-D-glucopyranosyl D-mannose (+203°), D-glucose and 4-*O*-α-D-glucopyranosyl-D-mannose (+298°), 3-*O*-α-D-mannopyranosyl-D-mannose and *O*-α-D-mannopyranosyl-(1→3)-*O*-α-D-mannopyranosyl-(1→3)-D-mannose[26] (+205°), 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose and *O*-(β-D-glucopyranosyluronic acid)-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose[27] (+134°), the latter trisaccharide and *O*-(β-D-glucopyranosyluronic acid)-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→3)-D-glucose[27] (+264°), *O*-(β-D-glucopyranosyluronic acid)-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-D-glucose and *O*-(β-D-glucopyranosyluronic acid)-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-D-galactose (+380°). Values for pairs which differ by a β-D-linked sugar residue are as follows: D-mannose and 4-*O*-β-D-mannopyranosyl-D-glucose (+38°), D-glucose and 3-*O*-β-D-glucopyranosyl-D-glucose (−40°). D-mannose and 4-*O*-β-D-mannopyranosyl-D-mannose (−54°), D-glucose and 3-*O*-β-D-glucopyranosyl-D-mannose (±0°), D-glucose and 4-*O*-β-D-glucopyranosyl-D-mannose (−52°), the latter disaccharide and *O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-mannopyranosyl-(1→4)-D-mannose (−83°), cellobiose and *O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-D-glucose[28] (−59°), cellotriose and *O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-D-glucose[29] (−60°), cellobiouronic acid and *O*-(β-D-glucopyranosyluronic acid)-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-D-glucose (+35°). Except where indicated otherwise, optical rotation values are taken from standard Tables[30,31]. Even without allowance for the different residue rotations of glucose and mannose and other secondary effects, it is clear that α and β linkages are easily distinguished and that the linkage in question falls in the α category. By extension of this approach, it emerges that the difference in molecular rotation between trisaccharide *2C* and tetrasaccharide *2D* is −6°, indicating that the added linkage is β-D. The configuration of the other glucosyl linkage in the repeating unit, namely that which joins the branched glucose residue to the unbranched glucose residue follows from the previous isolation of 2-*O*-β-D-glucopyranosyl-D-erythritol[9] after periodate degradation. The only remaining linkage to be characterized is that which attaches the non-reducing terminal mannose residue. Calculations for the pentasaccharide repeating-unit, using the approach described above, predict that the specific rotation of the native polysaccharide should be about +20° if this configuration were α-D and about −4° if it were β-D. The value measured is temperature dependent[4,17], but is in the range −0.5 to −2°, pointing clearly to a β-D linkage.

Our results are entirely consistent with the structure **1** that has been derived for the polysaccharide in another way[6]. Although we have confirmed most features of

this structure, our evidence alone would not have been sufficient to establish it with complete confidence because (*a*) we have obtained no indication of the site of the *O*-acetyl substituent, and (*b*) the elimination of an alternative structure having single-unit, mannose side-chains would rest rather heavily on the identification of the pentasaccharide *2E*, which we would not wish to claim was rigorous at this stage.

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