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Note

Minor structures in β-D-(1→2)-linked D-glucopyranans from *Agrobacterium tumefaciens* and *Agrobacterium radiobacter**†

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The exocellular D-glucan[1,2] formed by the crown-gall organism, *Agrobacterium tumefaciens*, was found to consist principally of β-D-(1→2)-linked pyranoside residues by virtue of its specific rotation[1] (−9°), and by the shift of specific rotation occurring on going from aqueous to cuprammonium solution, which resembled that of methyl 2-*O*-methyl-β-D-glucopyranoside[3]. This finding was confirmed by methylation data[4] and because the glucan consumed 1.0 mol of sodium metaperiodate per mol of glucose residues with little production of acid[5]. Similar structures were shown to be present in D-glucans of *Agrobacterium radiobacter*, *Agrobacterium rhizogenes*, and *Agrobacterium rubi*[5]. A later investigation[6] of two strains of *A. tumefaciens* revealed the presence of D-glucose, D-galactose, pyruvic acid, and *O*-acetyl groups in the approximate proportion of 12:2:2:3 (succinic acid esters are also present in exocellular polysaccharides of *A. radiobacter*[7] and *A. tumefaciens*[8]). Methylation and examination of resulting *O*-methylalditol acetates by gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.) indicated (1→3)-linked D-glucopyranosyl (24%), (1→3)-linked D-galactopyranosyl (15%), (1→4)-linked D-glucopyranosyl (20%), and (1→6)-linked D-glucopyranosyl (18%) residues. 4,6-Di-*O*-substituted D-glucopyranosyl branch-residues (12%) and those substituted at positions 4 and 6 by pyruvic acid acetals (11%) were also present. All residues had the β-D configuration. As these data[6] show structures differing from those originally reported, a reinvestigation was conducted on exocellular polysaccharides of *A. tumefaciens* and *A. radiobacter*[5] by g.l.c.–m.s. and ^{13}C-nuclear magnetic resonance (n.m.r.) techniques.

β-D-Glucan from A. tumefaciens *IIBV7.* — The ^{13}C-n.m.r. spectrum of a solu-

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*Dedicated to Professor Stephen J. Angyal on the occasion of his retirement.

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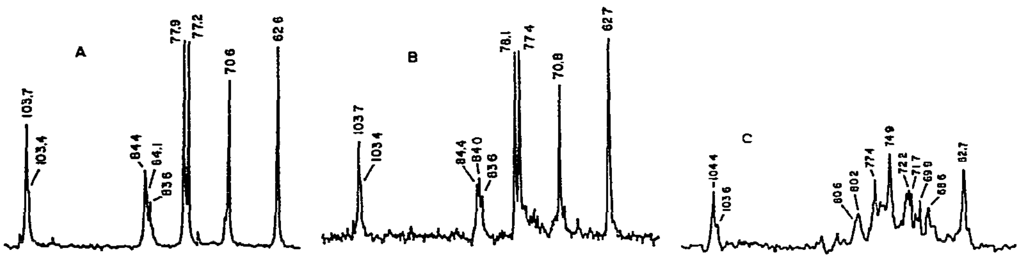

Fig. 1. ^{13}C-N.m.r. spectra of β-D-glucans from *A. tumefaciens* IIBV7 (A), *A. radiobacter* 1000 (B), and *A. radiobacter* ATCC 19358 (C), in the region δ_C 62–104.

tion of the *A. tumefaciens* glucan in D_2O at 70° showed major signals at δ_C 103.7, 84.4, 77.9, 77.2, 70.6, and 62.7 (Fig. 1A). These data appear to correspond to a predominantly (1→2)-linked β-D-glucopyranose structure, as the C-1 signal has a shift characteristic of the β-D configuration[9], and because shifts of the other signals are different from signals of β-D-(1→3)- (ref. 10), β-D-(1→4)- (ref. 11), and β-D-(1→6)-linked[12] structures. However, in addition to the foregoing signals, there were minor ones at δ_C 103.4 (C-1) and 83.6 and 84.1 (*O*-glycosylated carbon atoms). Signals at δ_C ~17 or ~27, corresponding to the CH_3 group of a pyruvic acid acetal (having the *R* or *S* configuration, respectively[13]) were absent, as were *O*-acetyl signals.

The *O*-methylalditol acetates derived from the methylated glucan were examined by g.l.c.–m.s.[14]. A glass capillary column containing OV-17 gave 3,4,6-tri-*O*-methylglucitol triacetate (61%) as the principal peak, as shown by its retention time, which differs from those of the 2,3,4-, 2,3,6-, and 2,4,6-tri-*O*-methyl derivatives, and also from its characteristic electron-impact mass spectrum. This g.l.c. system does not separate the 2,3,4 and 2,3,6 isomers. Also detected were minor peaks for the 2,3,4,6-tetra- (7%), and 4,6- (26%), and 3,4-di-*O*-methylglucitol acetates (4%). Of these, the peak of the 4,6- isomer was larger than that of the 2,3,4,6 isomer and could arise from undermethylation. The glucan was, therefore, oxidized for 3 days by sodium metaperiodate and the oxidized product successively reduced with sodium borohydride and hydrolyzed. The presence of glucose, in addition to glycerol, showed that the 4,6-di-*O*-methyl fragment was, in part, a product of full *O*-methylation. Thus the D-glucan consists of (1→2)-linked β-D-glucopyranosyl residues and minor 2,3- and 2,6-di-*O*-substituted β-D-glucopyranosyl residues. The extent of branching is ~8%, as estimated from periodate oxidation, the nonreducing end-groups being degraded to 0.08 mol of formic acid per mol of glucose residues[5]. Partial hydrolysis of the glucan was conducted in order to obtain the possible central core of the polysaccharide. An ethanol-soluble fraction (25%) was obtained, but its fine structure from the evidence of methylation analysis differed little from that of the native glucan. The following *O*-methylglucitol acetates were obtained: 2,3,4,6-tetra- (13%), 3,4,6-tri- (63%), 2,3,6-tri- (4%), 4,6-di- (15%), and 3,4-di- (5%).

β-D-Glucan from A. radiobacter *1000.* — The ^{13}C-n.m.r. spectrum of the D-glucan from *A. radiobacter*, obtained from a solution in D_2O at 32°, showed six principal signals corresponding to a β-D-(1→2)-linked D-glucopyranan. One broad signal, apparently arising from C-2, fell at δ_C 83.1 and exhibited a small shoulder on the upfield side[15]. Re-examination at 70° revealed three signals in this region, and two signals for C-1, resulting in a spectrum (Fig. 1B) showing signals similar in shift to those of the glucan from *A. tumefaciens*.

The glucan of *A. radiobacter*, which contains ~7% of galactose residues, was methylated, and the permethylated product degraded to *O*-methylhexitol acetates. These were shown, by their characteristic g.l.c. retention-times and mass spectra, to be 3,4,6- (52%) and 2,3,6-tri- (24%)*, and 4,6- (8%) and 2,3-di-*O*-methylglucitol (18%) derivatives, the percentage figures representing the relative peak-areas. Thus, in addition to the structures present in the glucan from *A. tumefaciens*, there are 4-*O*-substituted and 4,6-di-*O*-substituted glucopyranosyl residues. The failure to detect 2,3,4,6-tetra-*O*-methylglucitol acetate arises from the presence of pyruvic acetals on the 4 and 6 positions of nonreducing end-groups. The ^{13}C-n.m.r. spectrum (Fig. 1B) of the glucan (galactose content 9%) showed a small signal for CH_3 at δ_C 26.1 corresponding to an acetal of pyruvic acid. Degradation of the glucan with acid provided an ethanol-insoluble fraction that did not give rise to this signal. Methylation analyses showed the degraded polysaccharide to contain nonreducing end-groups and to have fewer (1→2) linkages, the peak percentages being 2,3,4,6-tetra- (11%), 3,4,6- (16%), 2,4,6- (17%), and 2,3,6-tri- (33%), and 4,6- (5%) and 2,3-di-*O*-methylglucitol acetates (9%). 2,3,4-Tri-*O*-methylgalactitol acetate (7%) was also present. In agreement with the results of Zevenhuizen[6], the presence of the 2,3,4,6-tetra-*O*-methyl isomers, following removal of the pyruvic acid acetal, indicates that they are 4,6-*O*-substituents of nonreducing end-groups. Also, the 2,3-di-*O*-methylglucitol acetate produced is consistent with presence of 4,6-di-*O*-substituted D-glucopyranosyl branch-residues. The formation of only 16% of 3,4,6-tri-*O*-methyl derivative indicates preferential hydrolysis of (1→2) linkages. This result is paralleled by the increase of the 2,4,6 isomer to 17%, corresponding to removal of (1→2) linkages from 2,3-di-*O*-substituted branch-residues.

The acid-degraded polysaccharide gave a ^{13}C-n.m.r. spectrum showing a broad signal at δ_C 80.0 (Fig. 2E) that could arise from C-4 atoms of consecutive, (1→4) linkages, as with the internal residues of β-D-(1→4)-linked D-glucopyranosyl oligosaccharides[11]. However, this signal could not be detected in the spectrum of the parent polysaccharide, and the proportion of such consecutive linkages must be very small.

The foregoing data show the presence of a branched, pyruvylated structure containing 4-, and 4,6-di-*O*-substituted, and possibly other linkage-types in a β-D-glucan. This structure is in addition to another one having (1→2) linkages with a possible (1→3)-linked core, as suggested by the detection of 2,4,6-tri-*O*-methylglucitol acetate from the acid-degraded polysaccharide. In such an acid degradation,

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*This was preferred to the unresolved 2,3,4-tri-*O*-methyl isomer as the mass spectrum displayed a large peak having[14] *m/z* 113.

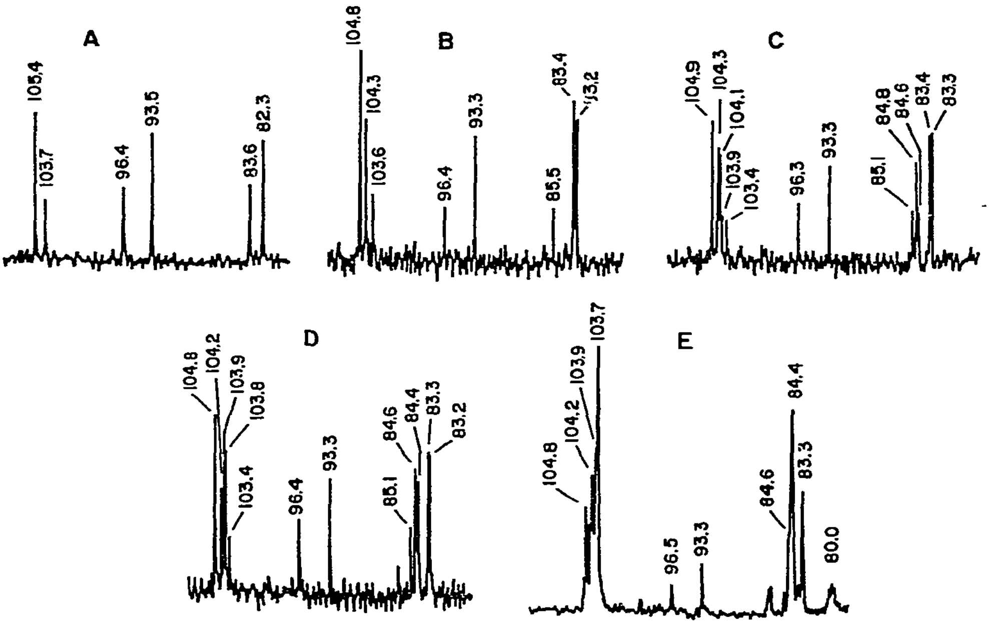

Fig. 2. The δ_C 80–106 regions of the ^{13}C-n.m.r. spectra of β-D-(1→2)-linked D-glucopyranose disaccharide (A), trisaccharide (B), tetrasaccharide (C), and pentasaccharide (D), and of acid-degraded polysaccharide (E).

oligosaccharides containing up to six residues were also isolated. They were shown to have β-D-(1→2)-linked D-glucopyranose structures[5], a finding that is currently confirmed by g.l.c.–m.s. after methylation. ^{13}C-N.m.r. examination of each oligosaccharide of up to five residues revealed two C-1 signals of reducing end-groups, their δ_C values of 93.3 and 96.4 being characteristic of 2-*O*-substituted residues having the α and β configuration, respectively (Fig. 2A,B,C,D). Signals in the rest of the C-1 region, and the portion of the spectrum at δ_C 83–85, could not be reliably interpreted in terms of chemical structure as they were, unlike many other homologous series of oligosaccharides, sensitive to their position in the chain relative to the non-reducing and reducing ends, and to chain length. Such a phenomenon probably arises from variation of the torsion angles, ϕ and ψ, of the glycosidic bonds.

The ^{13}C-n.m.r. spectrum of the glucan strain of *A. radiobacter*, ATCC 19358 (Fig. 1C), differs from that of the presently investigated strain, thus confirming the strain-dependence of chemical structure found by Zevenhuizen[6].

EXPERIMENTAL

Culture conditions and isolation of the polysaccharides. — The polysaccharides were produced by using *A. radiobacter* strain 1000 and *A. tumefaciens* (virulent strain IIBV7) as previously described[5].

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra of the D-glucans of *A.*

tumefaciens were obtained by using 200 mg of sample in D_2O (2 mL) at 70°. Only 40 mg of *A. radiobacter* polysaccharide was used, as the solution was viscous, although the viscosity decreased at high temperatures. Oligosaccharides obtained on partial hydrolysis were examined by using the total amounts isolated by column chromatography (see later). Chemical shifts were recorded relative to external tetramethylsilane. Other conditions for obtaining spectra were as previously described[5].

Partial hydrolysis of A. radiobacter *and isolation of oligosaccharides and glucan.* — The conditions for hydrolysis were as described earlier[5]. An aqueous solution of hydrolyzate obtained from glucan (2.95 g) was treated with an excess of ethanol, yielding insoluble, acid-degraded polysaccharide (1.10 g). The mother liquor was fractionated by chromatography on a cellulose column, with 2-L volumes of acetone–water mixtures, the ratios being 7:1, 4:1, 3:1, 7:3, 13:7, and 3:2. Thus isolated were di- (160 mg), tri- (110 mg), tetra- (90 mg), penta- (80 mg), and hexa-saccharide (20 mg). Similar conditions of partial hydrolysis were used to obtain acid-degraded glucan of *A. tumefaciens*, and the glucan was isolated in 25% yield by precipitation from water with an excess of ethanol.

Methylation analyses. — Glucans were methylated by successive treatments by the Haworth[16] and Kuhn[17] procedures. The products were hydrolyzed, the free *O*-methylglucoses reduced with sodium borohydride, the alditols acetylated, and the acetates characterized by their retention times on a 30-m glass capillary column containing SP-2250 (OV-17) programmed from 95 to 230° (at 2°/min), and by their electron-impact pattern in g.l.c.–m.s.[14]. Under such conditions, the tri-*O*-methylglucitol acetates had the following retention times (min): 2,3,4- and 2,3,6-, 53.10; 3,4,6-, 51.69; and 2,4,6-, 52.42. 2,3,6-Tri-*O*-methylgalactitol acetate had a retention time of 52.83 min and the 2,3,4 isomer, 52.86 min. The retention time of 2,3,4,6-tetra-*O*-methylglucitol acetate was 45.86 min, and values for 2,3- and 4,6-di-*O*-methylglucitol acetates were 59.09 and 57.69 min, respectively.

Oligosaccharides were methylated directly by the Kuhn procedure and the per-*O*-methylated derivatives processed similarly.

Smith degradation of A. tumefaciens *glucan.* — The D-glucan (10 mg) in water (2 mL) containing sodium metaperiodate (100 mg) was oxidized for 3 days. The solution was deionized with mixed-bed ion-exchange resin and the product successively reduced with sodium borohydride and hydrolyzed with 0.5M sulfuric acid for 3 h at 100°. Paper chromatography revealed the presence of glycerol and glucose.

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