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The structure of stewartan, a capsular polysaccharide produced by *Erwinia stewartii* strain DC283

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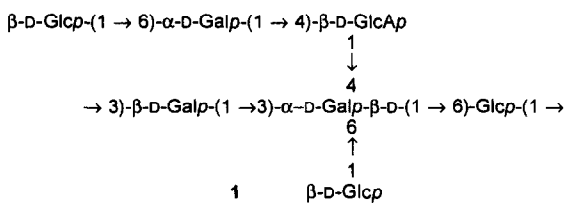
Abstract

Stewartan is a capsular polysaccharide produced by *Erwinia stewartii*, the causative agent of Stewart's wilt of maize. The structure of stewartan is shown, by a combination of methylation analysis, Li/ethylenediamine degradation, 1D and 2D NMR spectroscopy, partial acid hydrolysis and isolation of oligosaccharides, Smith degradation, MALDI-TOFMS analysis and exoglycosidase digestion, to have the following repeating unit:



Keywords: *Erwinia stewartii*; Capsular polysaccharide; Stewart's wilt; Corn

Abbreviations: GLC, gas liquid chromatography with FID detector; GLC–MS, gas liquid chromatography with mass selective detector; HPAEC–PAD, high pH anion exchange chromatography with pulsed amperometric detection; MALDI-TOFMS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; MALDI-TOFMS–PSD, matrix assisted laser desorption/ionization time-of-flight mass spectrometry with post source decay analysis

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1. Introduction

Erwinia stewartii, the causative agent of Stewart's wilt of maize, frequently causes severe losses, particularly in sweet corn [1–3]. The bacterium produces a capsular polysaccharide, stewartan, which is necessary for the pathogenicity of the organism [4–7]. Along with the study of the genetics of capsular polysaccharide production [5,6], the structure of stewartan has now been reported [8] and confirmed by the present independent study using submicro technology. It was of interest to compare its structure with the polysaccharides produced by different strains of *Erwinia chrysanthemi* [9,10] and *E. amylovora* [11].

2. Experimental

Source of stewartan.—The 10 mg sample of stewartan from *Erwinia stewartii* DC283, was a kind gift of Dr Klaus Geider, Max Planck-Institute for Medical Research, Ladenburg, Germany.

Purification of the EPS.—The crude EPS was purified by gel-filtration and anion-exchange chromatography. Briefly, crude polysaccharide (2 mL, 1 mg mL^{-1}) was chromatographed on a ToyoPearl HW65F column (1.5 × 85 cm; TosoHaas, Montgomeryville, PA) eluting with 1% (w/v) NaCl. Carbohydrate-containing fractions (determined by the phenol–sulfuric acid method) were pooled, dialyzed and re-chromatographed on a ToyoPearl DEAE 650 M column (1 × 17.5 cm), eluting with a 0–250 mM gradient of KCl. Fractions containing carbohydrate were pooled appropriately, dialyzed against deionized water and lyophilized.

The purified stewartan was electrodialyzed for the 1D ^1H NMR experiments.

Analytical methods.—Monosaccharide composition.—The composition of stewartan, after hydrolysis (2 M CF_3CO_2H, 121 °C, 1 h), was determined by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC–PAD) and by GLC and GLC–MS of the alditol acetates as previously described [10]. The absolute configuration of the monosaccharides was determined by GLC of the CH_3Si derivatives of their *R*-(−)-butan-2-ol glycosides, by the method of Gerwig et al. [12]. Carboxyl-reduction of the D-glucosyluronic residues in stewartan was accomplished by reduction of the water-soluble carbodiimide-activated carboxyl group (prepared from 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDAC), with $NaBD_4$ as described by Taylor and Conrad [13].

Glycosyl-linkage composition analysis.—Methylation analyses and GLC and GLC–MS analyses of the alditol acetates were carried out by the method of Anumula and Taylor [14] as modified by Yang et al. [10]

The methylation analysis of small amounts of oligosaccharides (< 1 μg) required careful attention to detail and was performed, as follows, using silanized vials. The HPAEC-purified oligosaccharide (700 ng) was dissolved in H_2O (100 μL), transferred to a 2 mL vial with a teflon-lined screw cap and reduced for 4 h with $NaBD_4$ (100 μL of a 10 mg mL^{-1} solution in H_2O). The excess $NaBD_4$ was decomposed by the addition of glacial AcOH and the resulting borate removed by coevaporation 3 times with 10% (v/v) AcOH in MeOH and 3 times from MeOH under a flow of N_2 at 40 °C.

A 1 mL spin-column of cation exchange resin (Bio-Rad AG50W-X12, H^+), built in a 1 mL automatic pipette tip with the end plugged with silanized glass-wool, was washed 5 times with water (300 μL) by low-speed centrifugation. The reduced oligosaccharide dissolved in H_2O (200 μL) was spun through the spin column, the effluent being collected in a 2 mL microfuge tube. The resin column was washed 3 times with deionized (300 μL) water and the original effluent and washings combined and lyophilized.

The oligosaccharide, in a 300 μL silanized vial with a conical bottom (Reacti-Vial), was methylated by treatment with NaOH in Me_2SO and MeI as described previously [10]. The clear solution was evaporated under a stream of N_2 to near dryness at 40 °C, anhydrous $CHCl_3$ (400 μL) was added and the vial mixed vigorously (vortex mixer). After a brief low-speed centrifugation to collect all the sample in the bottom of the vial, the $CHCl_3$ phase was carefully removed without disturbing the precipitate and transferred to a fresh silianized 2 mL vial. The precipitate was washed 3 times with $CHCl_3$ (200 μL), the washings being pooled with the original $CHCl_3$ phase. After reducing the volume of the pooled $CHCl_3$ phases to about 500 μL, water (500 μL) was added and the solution was mixed vigorously (vortex mixer). The phases were separated by low-speed centrifugation and the aqueous phase was carefully removed. The washing of the $CHCl_3$ phase was repeated three times. Sufficient MeOH (200 μL) was added to the aqueous $CHCl_3$ phase to form a single phase and the solvent removed under a stream of N_2 at 40 °C.

The per-*O*-methylated oligosaccharide was dissolved in CH_2Cl_2 (200 μL), care was taken to wash the sides of the vial and to collect all the solvent in the bottom of the vial by low-speed centrifugation, and transferred to a 300 μL silanized Reacti-Vial with a Pasteur pipette drawn to a fine tip and pre-wetted with solvent. The CH_2Cl_2 was carefully evaporated under a gentle stream of N_2 at 40 °C. The entire transfer procedure was repeated 5 times.

After dissolution of the per-*O*-methylated oligosaccharides in Super-Deuteride (100 μL, 1 M $LiB(Et)_3D$ in tetrahydrofuran, Aldrich), reduction of any methyl ester was allowed to proceed for 4 h at room temperature. The excess Super-Deuteride was decomposed by the addition of glacial AcOH and the solvent evaporated under a stream of N_2 at 40 °C. The borate was removed by coevaporation 3 times with MeOH (200 μL).

The per-*O*-methylated and carboxyl-reduced oligosaccharide was dissolved in MeOH (300 μL) and quantitatively transferred to a silanized 2 mL vial. The Reacti-vial was rinsed twice with MeOH (300 μL), combined with the original solution, and evaporated under a gentle stream of N_2 at 40 °C. The dry residue was dissolved in $CHCl_3$ (750 μL) and H_2O (750 μL) was added. The solution was shaken vigorously (vortex mixter) and the phases separated by low-speed centrifugation and the upper aqueous phase (which was found to be neutral) was carefully removed without disturbing the interface. The washing procedure was repeated twice. Sufficient MeOH (200 μL) was added to create a single phase system and the solvent was carefully evaporated as described above.

The per-*O*-methylated carboxyl-reduced oligosaccharides were quantitatively transferred to a 300 μL Reacti-Vial as described above and dissolved in EtOH (25 μL) from which 0.5 μL was removed and analyzed by MALDI-TOFMS.

The residual EtOH was evaporated and the oligosaccharides were hydrolyzed with 2 M CF_3CO_2H for 1 h at 121 °C. The CF_3CO_2H was removed by evaporation 2 times in the presence of *i*-PrOH (120 μL). The residue was dissolved in 95% EtOH (75 μL) and reduced with $NaBD_4$ (75 μL of 10 mg mL^{-1} in 1 M NH_4OH) for 1 h at room temperature. After decomposing the excess $NaBD_4$ with glacial AcOH, the sample was transferred to a 2 mL silanized vial where the borate was removed as described above.

Acetylation and extraction of the alditol acetates were as previously described [9]. The *O*-methyl alditol acetates were dissolved in EtOAc (75 μL) and 1 μL was analyzed by GLC and GLC–MS.

Gas–liquid chromatographic and mass-spectrometric analysis.—The *O*-methyl alditol acetates were analyzed by splitless injection on a gas chromatograph fitted with a FID as described previously [9,10] or by cool on-column injection on a DB5 capillary column (0.25 mm × 30 m, J & W Scientific, Folsom, CA) in a Hewlett–Packard Series II gas chromatograph and a Hewlett–Packard 5971A mass selective detector. Analysis conditions for the cool on-column injections were as follows: the head pressure was maintained at 5 psi giving a flow of He through the column of 25 cm s^{-1}. Oven tracking for the injector was switched on, thus maintaining the injector temperature 3 °C above that of the oven throughout the analysis. The initial oven temperature was 50 °C for 3 min and increased to 180 °C at 30 °C min^{-1} where it was held for one min. Thereafter, it was increased to 260 °C at 5 °C min^{-1}, and maintained at this temperature until the end of the analysis.

Matrix assisted laser desorption / Ionization–time of flight mass spectrometry (MALDI-TOFMS).—MALDI-TOFMS was performed on a Voyager-RP Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA) operating in the positive ion mode with an accelerating voltage of 30.0 kV. Between 100 and 256 shots, aimed at different parts of the target with a N_2 laser operating at 337 nm (3 ns pulses), were accumulated until an acceptable signal/noise ratio (> 25:1) was attained. Spectra were acquired at a vacuum of 1×10^{-6} Torr or better with 2,5-dihydroxybenzoic acid (10 mg mL^{-1} in 10% EtOH) as a matrix. Two microliters of matrix and half a microliter of per-*O*-methylated oligosaccharide (0.1–100 pmol) were added sequentially to the target and allowed to dry at room temperature in a desiccator. Absolute EtOH (0.5 μL) was added to the sample well and the sample/matrix was allowed to recrystallize at room temperature. Per-*O*-methyl maltoheptose was used to calibrate the MALDI-TOFMS.

Post source decay analysis was performed in the positive-ion mode at 25.0 kV on the same samples previously analyzed by MALDI-TOFMS.

^1H NMR analysis.—^1H NMR spectra of the samples in D_2O, were recorded on a Bruker 600 MHz NMR spectrometer as described previously [9,10].

Degradation of polysaccharide by lithium in ethylenediamine.—The degradation of stewartan (800 μg) by Li in ethylenediamine was performed as described by Lau et al. [15], and the Li-degradation products were fractionated by chromatography on a Bio-Gel P-2 column (1.0 × 45 cm, 200–400 mesh; Bio-Rad Laboratories, Hercules, CA) eluting with water. The high molecular weight fractions, eluting in the void volume of the column, were pooled and lyophilized.

The fractions containing the side-chain (low molecular weight carbohydrate eluting in the disaccharide region) were pooled and reduced with $NaBH_4$. After decomposition of

the excess $NaBH_4$ with glacial AcOH and removal of the borate as the volatile methyl borate, the side-chain fraction was dissolved in H_2O and further purified by HPAEC-PAD on a CarboPac PA-1 column (4.0 × 250 mm) eluting isocratically with 100 mM NaOH. Fractions were collected manually, immediately placed on ice and neutralized with glacial AcOH (pH paper). The samples were passed through a small column (2 mL) of cation exchange resin (Bio-Rad AG50-X8, H^+) to remove the Na^+ and lyophilized.

Preparation of oligosaccharides by fuming HCl.—Purified stewartan (700 μg Glc equivalent) was dissolved in ice-cold fuming HCl (100 μL) with cooling in an ice bath and then warmed to 40 °C for 40 min. The HCl was removed by coevaporation twice with 95% aqueous EtOH (200 μL) under a gentle stream of N_2 at 40 °C. The oligosaccharides were dissolved in water (1 mL) and fractionated by chromatography on a Bio-Gel P-4 column (1.5 × 86 cm, 200–400 mesh), eluting with 100 mM NH_4OAc. Fractions eluting in the disaccharide region and the larger oligomers (determined by reference to chromatography of glucosyluronate–rhamnose) were pooled, lyophilized and separated by HPAEC–PAD on a CarboPac PA-1 column (4.0 × 250 mm), eluting with 40 mM NaOH and a 25 min gradient (100–300 mM) of NaOAc. Fractions were collected manually and immediately placed on ice. After neutralization with glacial AcOH (pH paper), the Na^+ was removed by chromatography on a column (2 mL) of Bio-Rad AG50-X8 (H^+) and the fractions were lyophilized.

Smith degradation.—A Smith degradation of carboxyl-reduced stewartan (300 μg) was performed as described previously [9,10] and the product was purified by column chromatography on Bio-Gel P-2 and by HPAEC as described above.

Exoglycosidase digestion.—β-Galactosidase.—The reaction mixture for the β-galactosidase digestion of the product obtained from a Smith degradation of carboxyl-reduced stewartan contained Smith product, **4**, (4 μg), 25 mM NH_4OAc buffer, pH 4.0 (18 μL), and β-galactosidase (Jack Bean, Oxford Glycosystems, Rosedale, NY; 0.2 U in 2 μL). An aliquot (4 μL) was immediately removed, boiled for 10 min and cooled. After a brief centrifugation step to collect all the sample in the bottom of the tube, H_2O (96 μL) was added and an aliquot of the sample (25 μL) was analyzed by HPAEC–PAD using the monosaccharide program (isocratic 18 mM NaOH) with a full-scale detector response of 100 nA. The samples were incubated at 37 °C for 3 h, and a 4 μL aliquot was removed, treated and analyzed in the same way. A further 1 μL aliquot was removed and analyzed by MALDI-TOFMS. The digestion mixtures remaining were frozen until completion of the HPAEC-PAD analyses.

Two negative controls, one consisting of the addition of boiled enzyme to **4** and the other the addition of enzyme to melibiose (to determine α-galactosidase activity), were also run.

α-Galactosidase.—The reaction mixture for the α-galactosidase (Recombinant *Escherichia coli*, Calbiochem, La Jolla, CA) digestion of **4** was identical to that described above except that the buffer was 25 mM NH_4OAc, pH 6.1. Treatment of the 0 h and the 3 h samples were as described above. Similar positive and negative controls to those described above were incorporated into the experiment.

Sequential β-galactosidase and α-galactosidase digestion.—The 3 h β-galactosidase digests, including the boiled enzyme blank, were boiled for 10 min, cooled and briefly

centrifuged to collect all the sample in the bottom of the tube. The NH_4OAc buffers were removed under vacuum on a SpeedVac concentrator (Savant Instruments, Farmingdale, NY), evaporation from water was repeated twice. The samples were dissolved in water (4 μL) and 2 μL of each (equivalent to 1.2 μg of original substrate) was subjected to digestion with α-galactosidase. The volumes of buffers and α-galactosidase indicated above were halved. At 0 h, a 3 μL aliquot was removed from each vial, and worked up as described above. The sample was dissolved in 50 μL of water for analysis by HPAEC–PAD. Digestion was continued for 24 h at 22–24 °C, under an atmosphere of toluene and a 3 μL sample was removed, boiled, worked up and analyzed as above. A 1 μL aliquot was removed from the 0 h and 24 h digests and analyzed by MALDI-TOFMS.

An enzyme blank containing boiled α-galactosidase was run in parallel. Similar positive controls to those described above, i.e. digestion of lactose and melibiose by β- and α-galactosidase respectively, were run in parallel.

β-D-Glucosiduronase digestion.—Aldobiouronic acid (4 μg) was digested with β-D-glucosiduronase (Sigma type VII-A, 100 Fishman units) in 20 μL of 50 mM potassium phosphate buffer, pH 6.8 at 37 °C. A sample (2.5 μL) was immediately removed, diluted to 100 μL and analyzed by HPAEC–PAD on a Carbopac PA-1 column, eluting isocratically with 40 mM NaOH–150 mM NaOAc. After removal of a second sample (2.5 μL) from the reaction mixture at 20 h, a further 200 Fishman units in 10 μL of buffer was added and incubation continued for a further 24 h after which the reaction mixture was analyzed as above. A blank in which the enzyme was substituted with boiled enzyme was run in parallel.

As a positive control, β-D-GlcA-(1 → 2)-L-Rha, isolated from the EPS of *Erwinia chrysanthemi* strain CU643 (Gray, Yang, and Montgomery, unpublished work) was digested as above; as a negative control, α-D-GlcA-(1 → 4)-D-Man, purified from a partial acid digest of *E. chrysanthemi* strain SR260 [9], was digested.

3. Results and discussion

Purification of stewartan.—The crude stewartan contained 52% carbohydrate by weight, as determined by the phenol–sulfuric acid method [16] using Glc as a standard.

Stewartan eluted as a single peak from the ToyoPearl HW65F column. Similarly, stewartan eluted as a single peak at about 130 mM KCl from the ToyoPearl DEAE 650 M anion-exchange column. The constant monosaccharide composition of fractions across the peaks from both the gel filtration and from the anion exchange columns constitute evidence of the presence of a single polysaccharide species. After purification, > 90% of the mass of the stewartan was carbohydrate.

Monosaccharide composition and linkage analysis of stewartan.—Carboxyl-reduced stewartan was found to contain D-Gal and D-Glc in the ratio 3:4, the absolute configurations of the monosaccharides was determined as the CH_3Si derivatives of the *R*-(−)-butan-2-ol glycosides. The incorporation of ^2H into the 6 position of one of the Glc residues, as determined by GLC–MS of the alditol acetates, provides evidence that one

Table 1
Methylation analysis of carboxyl-reduced stewartan and the main chain produced by degradation of stewartan with Li in ethylenediamine

Me sugar [a]	Stewartan	Stewartan main chain produced by Li/ethylenediamine degradation
	relative molar ratio	
2,3,4,6-Me_4Glc	2.0	1.0
2,3,6-Me_3-6,6′-dideuterio Glc	0.9	0.0
2,4,6-Me_3Gal	1.1	1.2
2,3,4-Me_3Glc	1.2	1.0
2,3,4-Me_3Gal	1.1	0.0
2,4-Me_2Gal	0.0	1.1
2-MeGal	1.2	0.0

[a] 2,3,4,6-Me_4Glc = 1,5-di-*O*-acetyl-1-deuterio-2,3,4,6-tetra-*O*-methylglucitol etc.

of the Glc residues is derived from D-GlcA. This is corroborated by the detection of the CH_3Si derivative of the *R*-(−)-butan-2-ol glycoside of D-GlcA after butan-2-olysis of stewartan.

Methylation analysis of the carboxyl-reduced EPS (Table 1, column 1) revealed the presence of terminal Glc (two residues), 1,3-linked Gal (one residue), 1,6-linked Glc and Gal (one residue of each), 1,4-linked Glc (one residue, arising from GlcA and detected as 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-1,6,6′-trideuterio-Glc-ol) and 1,3,4,6-linked Gal (one residue).

Seven anomeric signals are observed in the 600 MHz 1D ^{1}H NMR spectrum of stewartan (Fig. 1, lower panel), two of which are characteristic of *α-gluco* or *galacto* residues (δ 5.472, 1 H, $J_{1,2}$ 3.0 Hz; δ 5.061, 1 H, $J_{1,2}$ 3.6 Hz) and five which are characteristic of *β-gluco* or *galacto* residues (δ 4.960, 1 H, $J_{1,2}$ 7.5 Hz; δ 4.726, 1 H, $J_{1,2}$ 7.8 Hz; δ 4.669, 1 H, $J_{1,2}$ 7.3 Hz; δ 4.514, 1 H, $J_{1,2}$ 7.9 Hz; δ 4.457, 1 H, $J_{1,2}$ 7.9 Hz).

These data are all consistent with stewartan being composed of a heptameric repeat unit containing D-Gal, D-Glc, and D-GlcA in the ratio 3:3:1.

No signals due to the presence of pyruvate or acetate were detected in the ^{1}H NMR spectrum of stewartan acquired at 338 K. Two signals, a doublet at δ 1.412, $J_{1,2}$ 7.0 Hz (0.7 H) and a quintiplet centred at δ 1.280, $J_{1,2}$ 6.6 Hz (1.2 H) appear in the high field region of the spectrum; the origin of these signals is unknown.

An outline of the chemical degradations of stewartan and the structures of the fragments generated are presented in Schemes 1 and 2, respectively.

Degradation of stewartan by lithium in ethylenediamine.—Two fragments were generated by degradation of stewartan with lithium in ethylenediamine and were separated by chromatography on Bio-Gel P-2.

The high molecular weight backbone fragment (**3**) eluted in the void volume of the column and contained equimolar amounts of Glc and Gal. Methylation analysis of **3** revealed a repeat unit of four residues: terminal Glc (1.0 mol), 1,3-linked Gal (1.2 mol), 1,6-linked Glc (1.0 mol) and 1,3,6-linked Gal (1.1 mol) (Table 1, column 2).

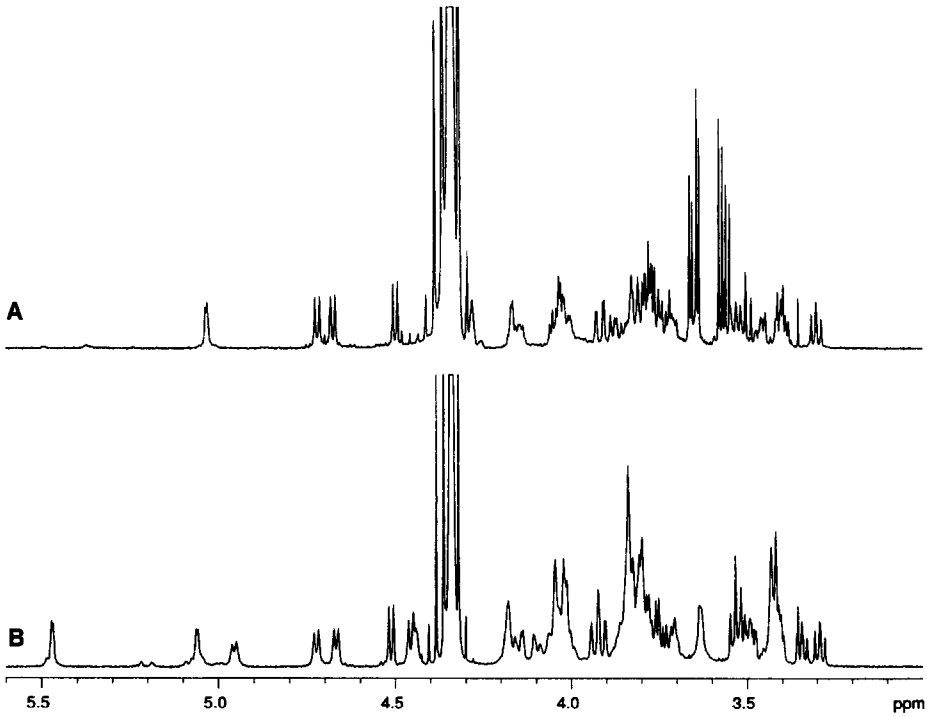


Fig. 1. 600 MHz ^{1}H NMR spectra of (A) the stewartan main chain produced by Li/ethylenediamine degradation of the polysaccharide and (B) stewartan.

Four anomeric signals are observed in the 600 MHz ^{1}H NMR spectrum (Fig. 1, upper panel) of fragment **3**, confirming the presence of a tetrameric repeat in the stewartan backbone. One of the signals is typical of an α-linked *gluco-* or *galacto*-residue (δ 5.034, $J_{1,2}$ 3.1, 1 H) and three are characteristic of β-linkages (δ 4.722, $J_{1,2}$ 7.9 Hz, 1 H; δ 4.677, $J_{1,2}$ 7.4 Hz, 1 H; δ 4.501, $J_{1,2}$ 7.9 Hz, 1 H) (Fig. 1, upper panel). Accordingly, the resonances observed at δ 5.472, 4.960, and 4.457 arise from the side chain of stewartan.

The low molecular weight fragment (**2**) eluted in the disaccharide region of the chromatogram and contained only Glc and Gal-ol together with a small amount of Gal. Two peaks were observed upon HPAEC–PAD analysis of **2**, a major peak eluting in a position typical of a disaccharide alditol and a minor peak eluting in the disaccharide region. Only a single peak was observed by HAPEC–PAD after reduction of **2** with $NaBH_4$. This is as expected since degradation of uronic acid-containing polysaccharides by Li/ethylenediamine produces a mixture of fragments, some terminated in an aldose and the others terminated with an alditol [15].

A single anomeric resonance was observed in the 600 mHz ^{1}H NMR spectrum of reduced **2** due to the β-linked Glc residue (δ 4.457, $J_{1,2}$ 7.9 Hz, 1 H).

Methylation analysis of this fragment revealed non-reducing terminal Glc and

6-*O*-acetyl-1,2,3,4,5-penta-*O*-methyl-1-deuterio-Gal-ol (data not shown); hence the sequence of the side chain is

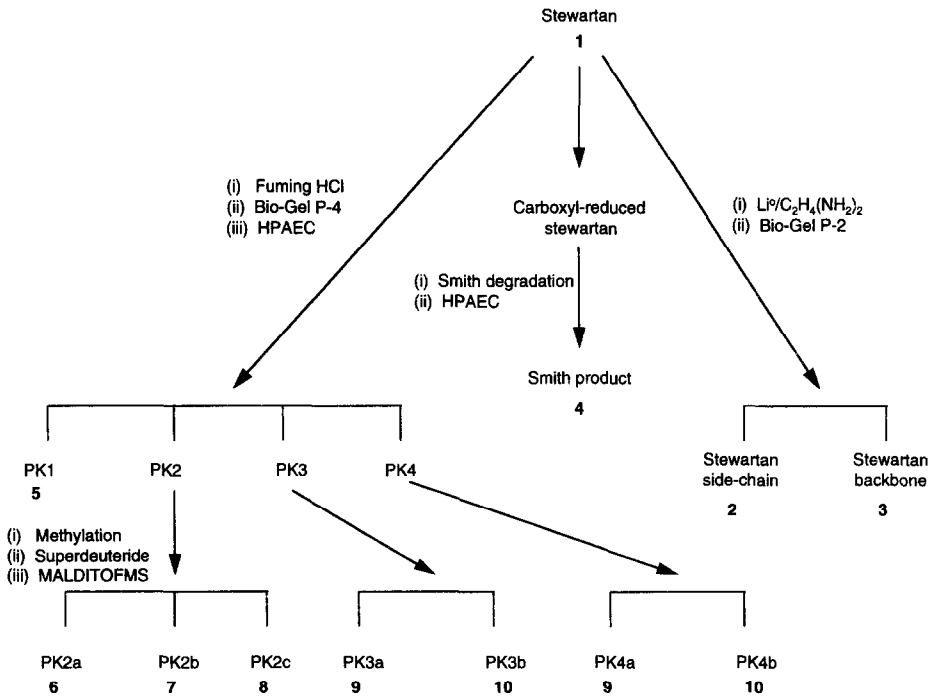
β-D-Glc*p*-(1 → 6)-D-Gal*p*-(1 → 4)-D-GlcA*p*-(1 →

2′

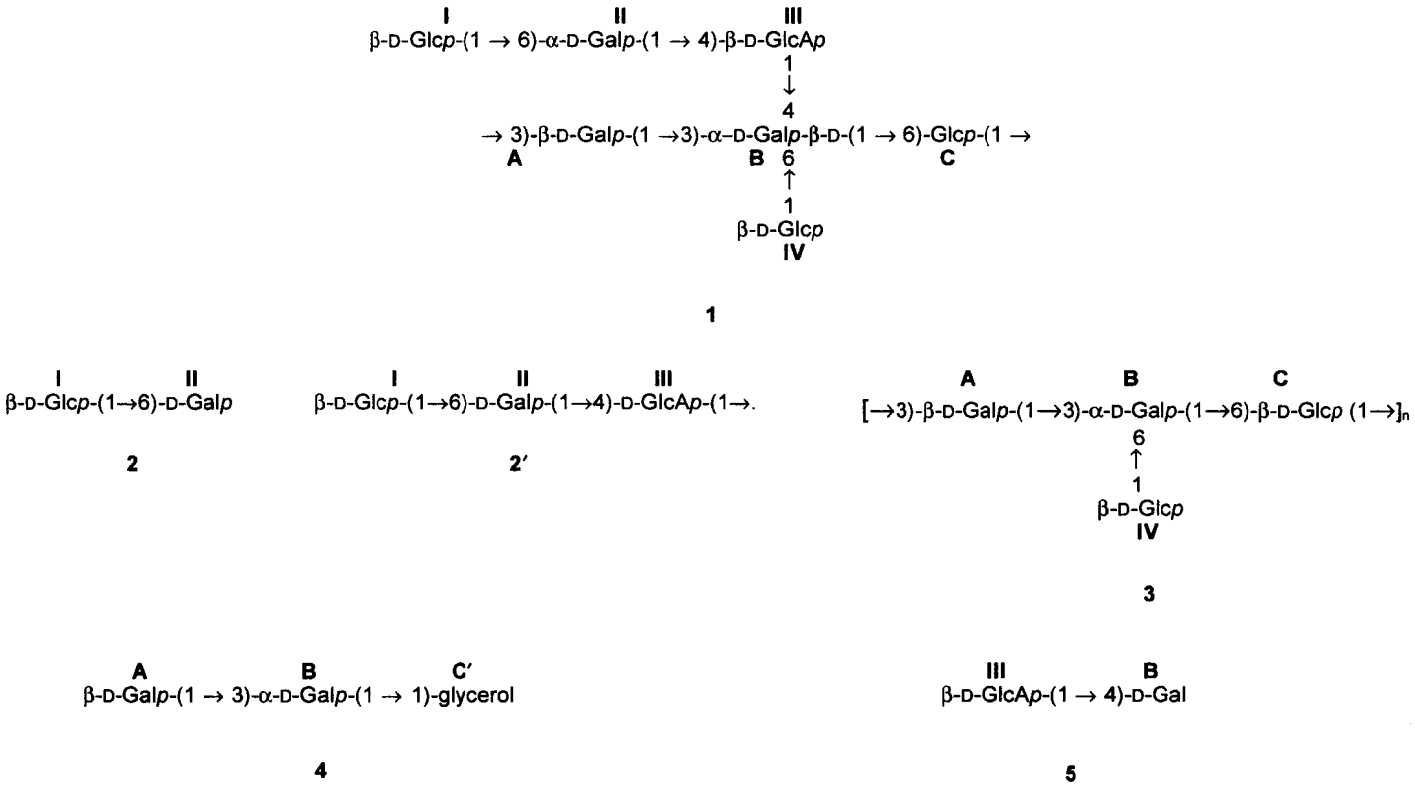
Smith degradation.—Only Gal and glycerol (from the oxidative cleavage of a 1,6-linked hexose), in a 2:1 ratio, are detected in the product (**4**) derived from a Smith degradation of carboxyl-reduced stewartan. Methylation analysis revealed the presence of a terminal Gal residue and a 1,3-linked Gal residue. As this element can only be derived from the backbone (since all the other residues in stewartan are susceptible to periodate oxidation), the sequence of the main chain is

→ ?)-Galα/β-(1 → 3)-Galα/β-(1 → 6)-Glcβ-(1 →

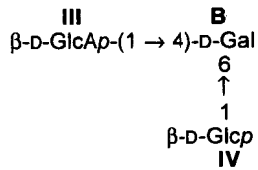
^{1}H NMR analysis of (**4**) shows that one of the Gal residues is α-linked (δ 4.980, $J_{1,2}$ 3.5 Hz, 1 H) and the other is β-linked (δ 4.600, $J_{1,2}$ 7.7 Hz, 1 H). Since ^{1}H NMR



Scheme 1. Chemical degradations of stewartan (**1**).



Scheme 2. Structures of the oligosaccharides produced from stewartan (**1**).



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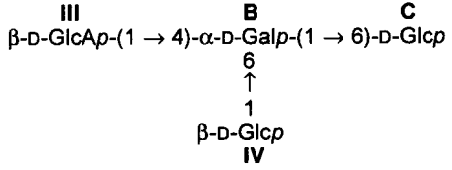

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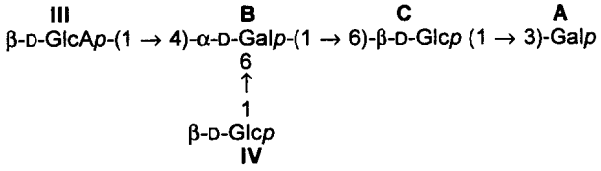

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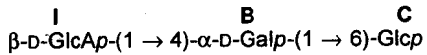

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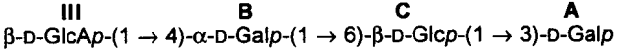

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Scheme 2 (continued).

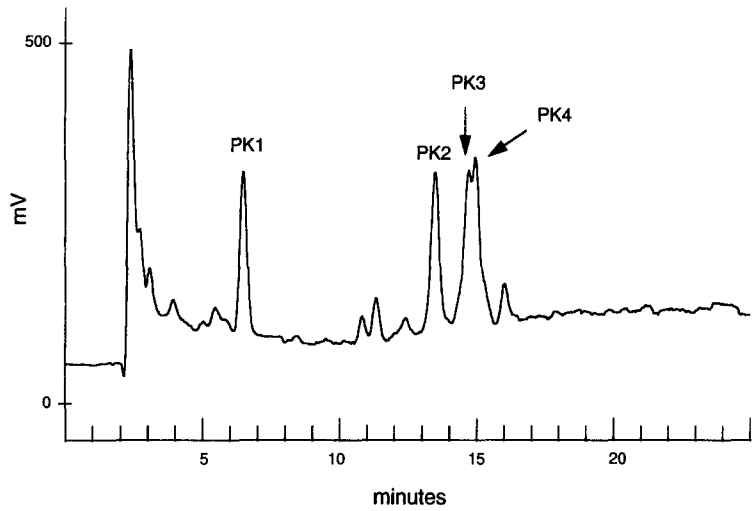


Fig. 2. HPAEC–PAD analysis of the oligosaccharides produced by the action of fuming HCL on stewartan. The PAD sensitivity was 100 nA full-scale.

analysis reveals that there is only one α-linked residue in the backbone of stewartan and this is now accounted for by one of the Gal residues, both Glc residues in the main chain must be β-linked.

Partial acid hydrolysis of stewartan with fuming HCl and isolation of oligosaccharides.—Two items of information are still required in order to complete the structure of stewartan; which of the two Gal residues in the backbone is the branch-point and what is the anomeric linkage of this residue? The first question was deemed to be best answered by the isolation of acidic oligosaccharides incorporating the branch-point Gal and one or more of the other monosaccharides present around this residue, namely, the side-chain Glc residue together with other residues of the stewartan backbone. The oligosaccharides generated by treatment with fuming HCl were purified by HPAEC.

After fractionation of the mixture on a Bio-Gel P-4 column, oligosaccharides larger than the reference compound, glucosyluronate–rhamnose, were pooled, recovered by freeze-drying and fractionated further by HPAEC on a CarboPac PA-1 column (Fig. 2). The four major peaks were collected manually and labelled PK1 to PK4 (Fig. 2).

The only monosaccharides detected in PK1 by HPAEC–PAD are Gal and GlcA, whereas PK2, PK3 and PK4 contained Glc in addition to Gal and GlcA.

MALDI-TOFMS, MALDI-TOFMS–PSD, and methylation analyses of PK1 to PK4.—Each peak was reduced with $NaBD_4$ to mark the reducing end and then methylated. The glucuronosyl methyl esters in each fraction were reduced with Super-Deuteride and a small portion (~ 2%) of each was removed for MALDI-TOFMS and MALDI-TOFMS–PSD analysis. The remainder of each fraction was converted to the methylated alditol acetates and subjected to GLC–MS analysis.

Analysis of PK1.—Analysis of PK1 by MALDI-TOFMS revealed a single peak, containing **5**, which after methylation, gave a mass of 478 Da, consistent with the

Na-adduct of a methylated disaccharide. Methylation analysis confirms that the aldobiouronic acid has the sequence

GlcA-(1 → 4)-Gal.

5

From the results of the Li/ethylenediamine degradation, this fragment is derived from the branch-point of stewartan and incorporates both the side-chain GlcA and the branch-point Gal residues.

Analysis of PK2.—MALDI-TOFMS analysis of PK2 revealed the presence of three methylated oligosaccharide alditols, a major peak at *m/z* 685 (70% of the signal), and two minor peaks at *m/z* 888 (24% of the signal), and *m/z* 1092 (6% of the signal) (Fig. 3). These masses are consistent with the Na-adducts of a per-*O*-methylated trisaccharide alditol (calculated *m/z* 686), a tetrasaccharide (calculated *m/z* 890) and a pentasaccharide, respectively (calculated *m/z* 1094).

The methylation analysis of PK2 confirmed the MALDI-TOFMS data, revealing the presence of three 1-*O*-methyl alditols, **6**, **7**, and **8** (Table 2, column 1), albeit in slightly

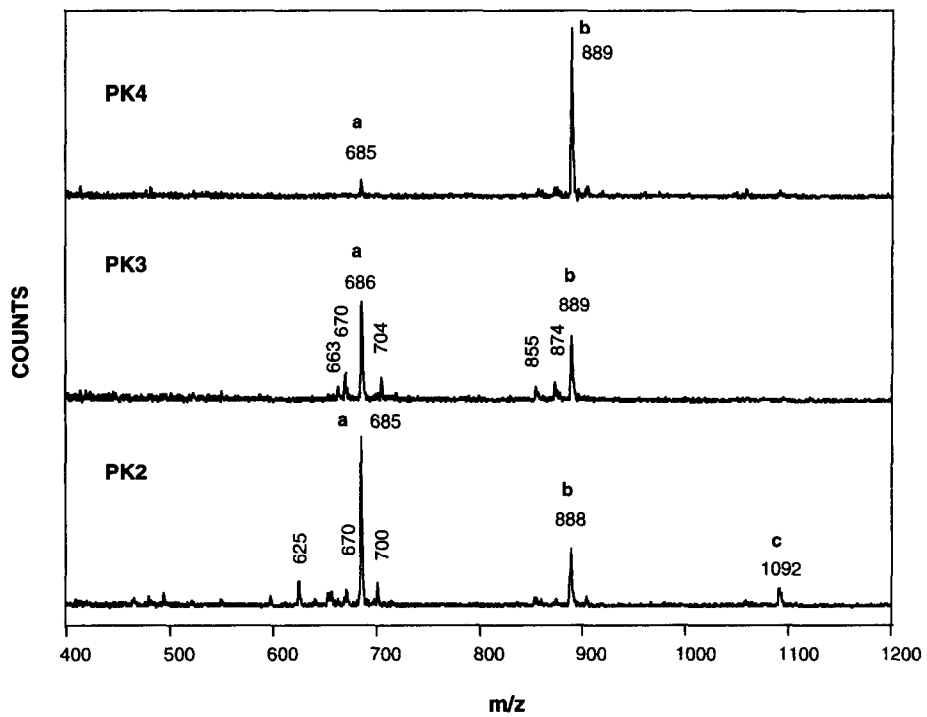


Fig. 3. MALDI-TOFMS analysis of the methylated and carboxyl-reduced oligosaccharidiotols PK2, PK3 and PK4.

different proportions for the trisaccharide (73%), tetrasaccharide (18%), and the pentasaccharide (9%).

Only two of the methylated alditol acetates present in the methylation analysis of PK2 can arise from non-reducing terminal residues, namely, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-6,6′-dideuterio-Glc-ol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-Glc-ol (Table 2, column 1). Since these are present in equimolar proportions, they must represent the non-reducing termini of all three oligosaccharides (**6**, **7**, and **8**).

The appearance of the MALDI-TOFMS–PSD spectra from PK2a, PK2b and PK3c are very similar (Fig. 4), suggesting that all three oligosaccharides share a common core.

Two strong signals, one at *m/z* 481 (M + Na-2,3,4-tri-*O*-methyl-6,6′-dideuterio-Glc) and one at *m/z* 468 (M + Na-2,3,4,6-tetra-*O*-methyl Glc) are observed in the MALDI-TOFMS–PSD analysis of **6** (PK2a, Scheme 1, Fig. 4A). The loss of two non-reducing terminal residues from the trisaccharide alditol requires that the major 1-*O*-methyl-1-deuterioalditol be present as a branch point, as is indeed the case (Table 2, column 1).

The structure of the trisaccharide represented by PK2a is therefore

GlcA-(1 → 4)-[Glc-(1 → 6)]-Gal.

6

Three fragments are observed in the PSD spectrum of PK2b (*m/z* 889, M + Na) at *m/z* 685 (M + Na-2,3,4-tri-*O*-methyl-6,6′-dideuterio-Glc), *m/z* 672 (M + Na-2,3,4,6-tetra-*O*-methyl-Glc) and *m/z* 469 (M + Na-2,3,4-tri-*O*-methyl-6,6′-dideuterio-Glc-2,3,4,6-tetra-*O*-methyl-Glc) (Fig. 4B). No fragmentation beyond the disaccharide alditol (*m/z* 469) is observed. The reducing end of the oligosaccharide represented by PK2b must be Glc because of the similar proportions of 6-*O*-acetyl-1,2,3,4,5-penta-*O*-methyl-

Table 2
Methylation analysis of the oligosaccharide alditols produced by treatment of stewartan with fuming HCl

Me sugar [a]	Oligosaccharide alditol, relative molar ratio									
	PK2 total [b]	PK2a [c]	PK2b [c]	PK2c [c]	PK3 total [b]	PK3a [c]	PK3b [c]	PK4 total [b]	PK4a [c]	PK4b [c]
1,2,4,5,6-Me_5Gal	0.1			0.1	0.1		0.1	0.6		0.6
1,2,3,4,5-Me_5Glc	0.2		0.2	0.0	0.8	0.8		0.2	0.2	
2,3,4,6-Me_4Glc	1.1	0.8	0.2	0.1	0.0			0.0		
1,2,3,5-Me_4Gal	0.8	0.8			0.0			0.0		
2,3,6-Me_3Gal	0.0				1.3	1.1	0.2	1.2	0.2	1.0
2,3,4-Me_3Glc	0.1			0.1	0.2		0.2	0.7		0.7
2,3,4-Me_3-6,6′-dideuterio Glc	1.0	0.8	0.2	0.1	1.0	0.8	0.2	1.0	0.2	0.8
2,3-Me_2Gal	0.2		0.2	0.1	0.0			0.0		

[a] 1,2,4,5,6-Me_5Gal = 3-*O*-acetyl-1-deuterio-1,2,4,5,6-penta-*O*-methylgalactitol etc.
[b] Methylation analysis of PK2, PK3 or PK4.
[c] Theoretical methylation analysis obtained by apportioning the methylated alditol acetates according to the molar ratios of the oligosaccharide alditols.

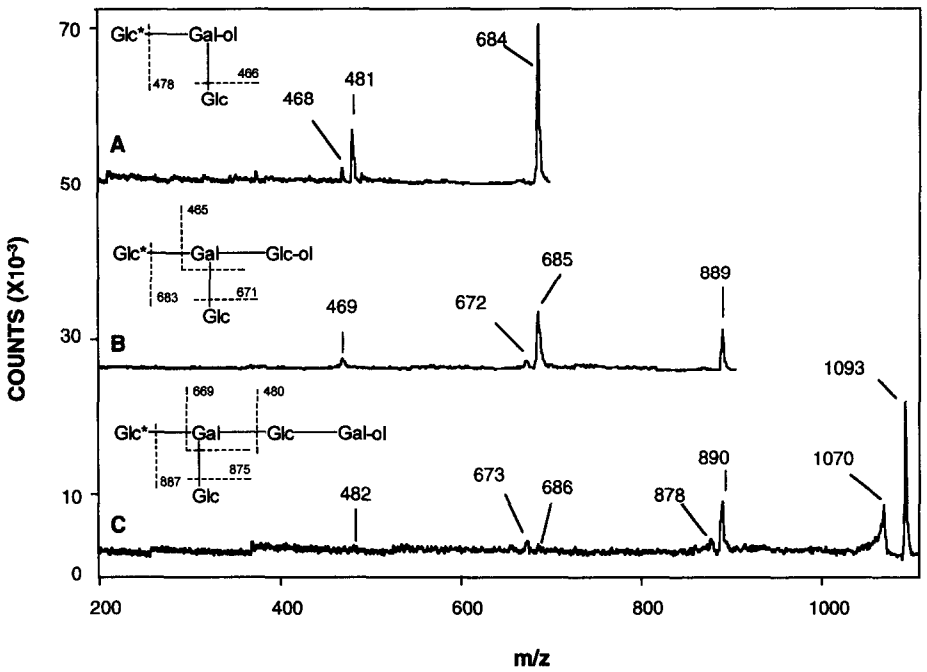


Fig. 4. Sequencing of methylated PK2a, PK2b, and PK2c by MALDITOFMS–PSD analysis. The Glc labelled with an asterisk is derived from GlcA and is present as a 2,3,4-tri-*O*-methyl-6,6′-dideuterio Glc residue.

1-deuterio-Glc-ol in the methylation analysis (18%, Table 2, column 1) and the intensity of the signal arising from PK2b in the MALDI-TOFMS analysis of PK2 (24%, Fig. 3).

Therefore the structure of the tetrasaccharide represented by PK2b has the sequence

GlcA-(1 → 4)-[(Glc-(1 → 6)]-Gal (1 → 6)-Glc.

7

Using similar arguments, the structure of the pentasaccharide represented by PK2c is deduced to be

GlcA-(1 → 4)-[(Glc-(1 → 6)]-Gal-(1 → 6)-Glc-(1 → 3)-Gal.

8

The complex methylation analysis of PK2 can now be rationalized by apportioning the methylated alditol acetates according to the molar ratios of the methylated oligosaccharide alditols (Table 2, columns PK2 (a, b and c)).

Analysis of PK3 and PK4.—PK3 and PK4 are incompletely resolved by HPAEC (Fig. 2) and this is reflected in the MALDI-TOFMS analyses of the permethylated derivatives of these two fractions (Fig. 3). A number of peaks are seen in PK3 but only two of these can be assigned to the Na-adducts of a methylated trisaccharide alditol (observed *m/z* 686, calculated *m/z* 686) and a tetrasaccharide alditol (observed *m/z* 889, calculated *m/z* 890); these are present in the ratio 6:4. Some of the signals arise from the K-adducts of the per-*O*-methylated oligosaccharidi tols (i.e. those that are 16 amu higher). PK4 contains mainly a methylated tetrasaccharide alditol (observed *m/z* 889, calculated *m/z* 890) and the trace of a methylated trisaccharide alditol (observed *m/z* 685, calculated *m/z* 686) (Fig. 3).

The presence of only two methylated oligosaccharide alditols in PK3 is supported by the presence of two 1-*O*-methyl-1-deuteriomethylated alditol acetates in the methylation analysis of this peak (Table 2, column 5). The methylation data indicates that the trisaccharide alditol has Glc-ol on its reducing end and the tetrasaccharide alditol has Gal-ol on its reducing end.

These two 1-*O*-methyl-1-deuteriomethylated alditol acetates are also present in the methylation analysis of PK4 suggesting that the same reducing end termini are present in the tri- and tetra-saccharide alditol in this fraction (Table 2, column 8).

PSD analysis of the permethylated trisaccharide alditol in PK3 gave rise to a fragment ion at *m/z* 481 (M + Na-2,3,4-tri-*O*-methyl-6,6′-dideuterio-Glc). Ions at *m/z* 686 (M + Na-2,3,4-tri-*O*-methyl-6,6′-dideuterio-Glc) and at *m/z* 481 (M + Na-2,3,4-tri-*O*-methyl-6,6′-dideuterio-Glc-tri-*O*-methyl-Hex) were observed in the PSD analysis of the permethylated tetrasaccharide alditol. No signals due to the loss of a second non-reducing terminal residue is observed in the PSD analysis of either the permethylated tri- or the tetra-saccharide alditol in PK3, as observed for PK2, indicating that these are both linear oligosaccharides. This conclusion is confirmed by the methylation analysis of PK3 (Table 2, column 8) where the presence of a single non-reducing end is indicated. The PSD analysis of the methylated tetrasaccharide alditol in PK4 gave identical data to that for the tetrasaccharide alditol in PK3, suggesting that these are the same oligosaccharide alditols and arise from the contamination of PK3 with PK4 and vice versa. A PSD analysis of the trisaccharide alditol in PK4 was unsuccessful due to its low concentration.

These data allow the sequence of the parent trisaccharide to be written as

GlcA-(1 → 4)-Gal-(1 → 6)-Glc

9

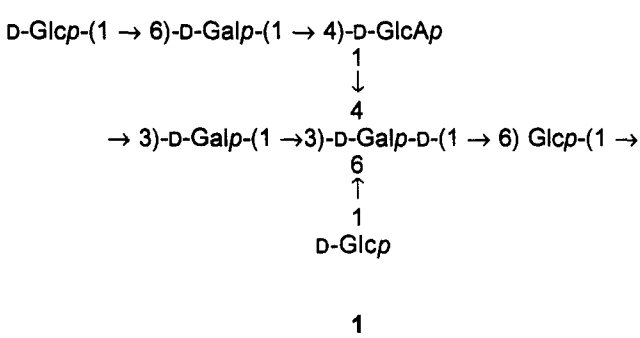
and the tetrasaccharide as

GlcA-(1 → 4)-Gal-(1 → 6)-Glc-(1 → 3)-Gal.

10

In a manner similar to that for PK2, the complex methylation analysis of PK3 and PK4 can be rationalized.

Sequence of stewartan.—All these data allow the following sequence to be proposed for stewartan:



1

Assignment of the anomeric linkages in the stewartan.—As described above seven anomeric linkages are observed in the ^{1}H NMR spectrum of stewartan, two α-linkages and five β-linkages (Fig. 1, lower panel). A comparison of the 1D NMR spectrum obtained from stewartan (Fig. 1, lower panel) and the backbone derived by Li/ethylenediamine degradation of stewartan (Fig. 1, upper panel) shows that one of the α-linkages (δ 5.061) and three of the β-linkages (δ 4.722, 4.677, 4.501) are in the stewartan main chain and that the remaining α-linkage (δ 5.472) and two β-linkages (δ 4.960, 4.457) are in the GlcA-containing side-chain. The 1D NMR spectrum of the side-chain derived from stewartan by Li/ethylenediamine degradation reveals that the terminal Glc residue is β-linked.

An unambiguous assignment of the anomeric linkage of the GlcA could not be obtained from the NMR studies because of the complexity of the spectra and the limiting amounts of material available. An alternative means of determining the anomeric linkage of the GlcA was sought. The aldobiuronic acid, **5**, purified by sequential Bio-Gel P-4 chromatography and HPAEC, was digested with β-glucosiduronase. After 19 h, 10% of the aldobiuronic acid from stewartan was digested; at 48 h, after the addition of fresh enzyme, 40% of the aldobiuronic was cleaved. The β-D-GlcA-(1 → 2)-L-Rha was digested to a similar extent in 48 h. No digestion was observed in the reaction mixtures containing boiled β-glucosiduronase nor was the aldobiouronic acid purified from a partial acid hydrolyzate of the EPS from *E. chrysanthemi* SR260 (α-D-GlcA-(1 → 4)-D-Man) digested. These data were confirmed by MALDI-TOFMS analyses of the enzyme digests.

Therefore, the GlcA is β-linked and consequently, the Gal residue in the side chain is α-linked, thus finally accounting for the one α-linkage and two β-linkages present in the side chain.

A COSY-45 2D analysis of the main-chain of stewartan was able to determine that the two Glc residues were both β-linked; consequently, there is one α- and one β-linked

Gal residue in the main-chain of stewartan. This is confirmed by the 1D NMR spectrum of **4** where one α- and one β-linkage, both assigned to the Gal residues, are seen.

The 2D NOESY experiments on stewartan (**1**) or the stewartan backbone (**2**) were unsuccessful for assigning unambiguous sequence data to these polysaccharides. Therefore, it was not possible to assign unambiguously the anomeric linkages of the two Gal residues in the main-chain by NMR other than one was α- and the other was β-linked.

Exoglycosidase digestion of **4** with α- and β-galactosidase showed that it was rapidly (within 3 h, data not shown) and completely degraded by β-galactosidase with the release of Gal, but it was undegraded by either boiled β-galactosidase or by α-galactosidase, despite the latter enzyme hydrolyzing melibiose. The Gal-glycerol produced by β-galactosidase digestion of **4** was further degraded by α-galactosidase confirming that the structure of this fragment is

$$\beta\text{-D-Gal-}(1 \rightarrow 3)\text{-}\alpha\text{-D-Gal-}(1 \rightarrow 1)\text{-glycerol}$$

4

MALDI-TOFMS analyses of the enzyme digests confirmed these data.

The structures of **4**, **5**, **6**, **7**, and **8** define the sequence of the main chain of stewartan and the initial residues of the two side-chains about the Gal branch-point. This sequence is confirmed by the structures determined for **9** and **10**. The sequence of the uronic acid containing side chain is defined by **2'**.

These data allow structure **1** to be proposed for the stewartan produced by *Erwinia stewartii* DC283 and confirm the structure of stewartan published recently by Nimtz et al. [8]. Because of the limited availability of the sample, the approach in this research was of necessity different from that of Nimtz et al. [8] with greater emphasis on the application of submicro techniques including exoglycosidase digestions and newer mass spectrometric techniques, particularly MALDI-TOFMS–PSD.

This structure is quite different from those produced by *Erwinia chrysanthemi* strains SR260 [9], Ech6 [10], Ech1 and Ech9 [17] and CU643 [18], all of which contain a 6-deoxyhexose; but it is similar to the amylovoran produced by *E. amylovora* (11).

With the modern technologies of molecular biology it may become necessary to derive structural information on carbohydrate-containing materials that are available in submicro amounts, such as may be isolated from the band of an electrophoretic gel. The present study uses techniques which are approaching this level of sophistication. It required procedures that reduced transfer losses and the application of modern technologies. The use of PSD analysis for sequencing limited amounts of sample, particularly in mixtures, is a very promising technique. In conjunction with methylation analysis, it allowed the structures of oligosaccharides, up to a pentasaccharide in this study, to be sequenced with much smaller amounts of sample than has previously been attempted. Far lower amounts of sample are required for MALDI-TOFMS–PSD analysis than for FABSMS analysis and in this respect, these two techniques complement each other well.

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