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Structure of stewartan, the capsular exopolysaccharide from the corn pathogen *Erwinia stewartii*

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Abstract

Stewartan, the acidic exopolysaccharide of *Erwinia stewartii*, was purified from agar grown cells. To facilitate its structural analysis, chemical and enzymatic depolymerizations were carried out using hydrofluoric acid and *E. amylovora* phage ϕ-Ea1h, respectively. Structural characterization of the resulting oligosaccharides was performed by a combination of mass spectrometric and one- and two-dimensional (1D/2D) NMR spectroscopic techniques. A branched repeating unit with seven monosaccharide residues, all in their pyranose forms, was found:

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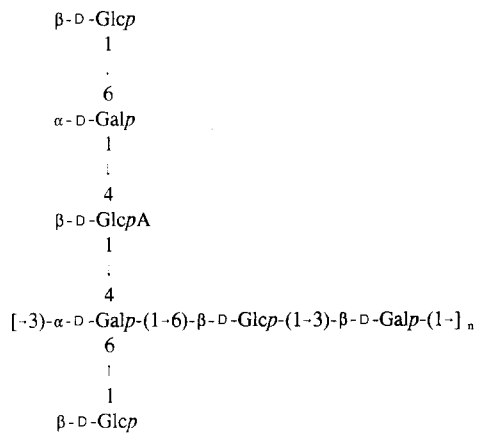


Keywords: Exopolysaccharide, structure; *Erwinia stewartii*; Stewart's wilt; Blight of corn

1. Introduction

Erwinia stewartii is the causal agent of Stewart's wilt and blight of corn. The disease is transmitted by the corn flea beetle. The capsular exopolysaccharide (EPS) produced by *E. stewartii*, called stewartan, is an important virulence factor of the pathogen. Stewartan-deficient mutants of *E. stewartii* failed to cause wilting and watersoaking, although they can still incite restricted necrotic lesions [1,2]. In this report the structural characterization of stewartan is described, and the structure of the polysaccharide is compared with that of the recently reported EPS of the closely related bacterium *E. amylovora* [3].

2. Experimental

Purification of stewartan and its enzymatic depolymerization.—E. stewartii strain DC283 has been described previously [4]. Stewartan was purified from cells grown on plates with CPG-agar. The inoculum was spread in five streaks on a cellophane disk layered on the top of the agar. After three days, the mucoid cells were suspended in water (3 mL). After vigorous shaking of the suspension followed by low speed centrifugation, the supernatant was spun in a preparative ultracentrifuge (Beckman rotor 60Ti, 4 h at 40,000 rpm) and then extensively dialyzed against water. The dialysate was lyophilized and the EPS stored at room temperature.

The EPS was cleaved with phage depolymerase, and the repeating units were purified as monomers and dimers as reported previously [3]. The reaction mixture was applied to a HPLC gel-permeation column (Toyopearl HW-40S), which was further processed as described.

Chemical cleavage and isolation of the resulting pentasaccharide.—Native polysaccharide (20–70 mg) was partially hydrolyzed using anhydrous liquid HF (10 mL) at −32 °C. The cleavage products were isolated and purified as described previously [3].

Analysis by GC/MS.—Carbohydrate compositional and methylation analyses as well as the determination of the absolute configuration of the monosaccharide residues were performed as described [5]. Stewartan (100 μg) was hydrolyzed in 4, 1, or 0.1 M trifluoroacetic acid for 2 h, and the resulting partial hydrolysis products were analyzed directly by GC/MS after reduction ($NaBD_4$) and permethylation as described previously [3].

600-MHz ^1H NMR Spectroscopy.—^{1}H NMR spectra at 600 MHz were recorded at 300 K on a Bruker AVANCE DMX 600 NMR spectrometer incorporating a gradient unit as described [3].

Electrospray ionization tandem mass spectrometry (ESI-MS/MS).—A Finnigan MAT TSQ 700 triple quadrupole mass spectrometer equipped with a Finnigan electrospray ion source (Finnigan MAT corp., San Jose, CA) was used for ESI-MS as described [3].

3. Results

Properties of stewartan released from suspension cells.—Stewartan is loosely attached to the cell surface of *E. stewartii* as a capsule and is released into the environment especially by mechanical stress. The EPS, from supernatants of suspension cultures, was concentrated by pressure dialysis on a nitrocellulose membrane. The average molecular mass was found to be approximately 1.4 MDa. Taking into account the present results (see below), this is equivalent to about 1000 repeating units per molecule of EPS [6]. In contrast to the recently described EPS from the closely related bacterium *E. amylovora* [3], no lower molecular-mass glucan-like polymer was detected in stewartan preparations.

Carbohydrate compositional and methylation analysis.—Stewartan was found to contain glucose, galactose, and glucuronic acid in molar ratios of approximately 3:3:1. The absolute configuration of the monosaccharide constituents was found to be D in all cases. No acyl groups were detected by IR spectroscopy. Methylation analysis (Table 1) showed the presence of terminal and 6-substituted glucose in a molar ratio of approximately 2:1, and 3-, 6-, and 3,4,6-trisubstituted galactose residues. A 4-substituted glucuronic acid residue was detected as 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylglucitol-6-d_2 after additional $NaBD_4$-reduction of the permethylated polysaccharide. The presence of monosaccharide residues in their furanose form could be excluded from the NMR and MS analyses of polysaccharide fragments (see below). Taken together, compositional and methylation analysis data suggest a branched heptasaccharide repeating unit of stewartan.

Analysis of derivatized hydrolytic fragments by GC/MS.—In order to obtain sequence information of the monosaccharide constituents described above, the native polysaccharide was partially hydrolyzed with trifluoroacetic acid at three different concentrations. In each case, the resulting oligosaccharide mixture was reduced with

Table 1
Methylation analyses of the polysaccharide and oligosaccharide fragments produced by chemical or enzymatic cleavage. Uncorrected molar ratios of the derivatives are given

Peracetylated derivative of	Substituted in position	Stewartan	Penta-saccharide **8**	Hepta-saccharide **9**	Tetradeca-saccharide **10**
Galactitol					
1,2,4,5,6-Penta-*O*-methyl-	3	–	–	0.6	0.8
1,2,5,6-Tetra-*O*-methyl-	3,4	–	0.1	–	–
2,4,6-Tri-*O*-methyl-	3	1.0	1.0	–	0.7
1,2,5-Tri-*O*-methyl-	3,4,6	–	0.5	–	–
2,3,4-Tri-*O*-methyl-	6	1.0	–	1.0	2.0
2,3-Di-*O*-methyl-	4,6	–	–	0.5	0.6
2-Mono-*O*-methyl-	3,4,6	0.5	–	–	0.5
Glucitol					
2,3,4,6-Tetra-*O*-methyl-	terminal	2.1	2.0	2.1	4.2
2,3,4-Tri-*O*-methyl-(6-d_2)- [a]	terminal	–	0.4	–	–
2,3,4-Tri-*O*-methyl-	6	0.9	–	1.0	1.9
2,3-Di-*O*-methyl-(6-d_2)- [a]	4	0.5	–	0.5	1.1

[a] These derivatives were obtained by an additional $NaBD_4$-reduction of the permethylated sample.

$NaBD_4$, then permethylated, and directly analyzed by GC/MS. Seven alditol compounds were detected and will be briefly described in the order of their elution and their EI mass spectrometric fragments are listed in Table 2.

In the monosaccharide region, only glucitol and galactitol were detected. In the disaccharide-alditol region, four compounds could be separated. Compound **1** was shown to be Hex-(1 → 3)-Hex-ol-*1-d*, confirming the detection of 3-substituted galactose by methylation analysis. This compound corresponds to the fragment **E–F** in Fig. 1. The second disaccharide-alditol (**2**) turned out to be HexA-(1 → 4)-Hex-ol-*1-d*, compatible with residues **C–D** in Fig. 1. Both compounds **3** and **4**, clearly separated on the GC-column, were identified as Hex-(1 → 6)-Hex-ol-*1-d*. They can be generated from the fragments **D–E** and **A–B** in Fig. 1 and are compatible with the detection of both 6-substituted galactose and glucose by methylation analysis.

Three compounds eluted in the trisaccharide-alditol region. Compound **5** was shown to have a branched structure, since characteristic fragments of both terminal Hex and terminal HexA were observed, but no fragment indicating a monosubstituted hexitol residue. Complementary fragments generated by the cleavage of the bond between C-3 and C-4 of the alditol moiety indicated substitution at O-3 by a hexose residue (the respective fragment was labelled by deuterium) and at O-4 by a hexuronic acid (the respective fragment was not labelled by deuterium). In a similar way, **6** could be identified as HexA-(1 → 4)[Hex-(1 → 6)]-Hex-ol-*1-d*. These two compounds define the branched core of stewartan, arising from the fragments **C–(F)D** and **C–(G)D** in Fig. 1. A third linear trisaccharide-alditol-*1-d* (**7**) with a 6-substituted hexitol residue and a terminal hexuronic acid was detected, comprising the sequence **C–D–E** in Fig. 1. Compounds **2**, **5**, and **6** are identical to the respective hydrolysis products from the EPS of *E. amylovora*, recently characterized by us [3], demonstrating a close structural relationship between the EPSs of these bacteria.

Table 2
EI mass spectral data of the reduced ($NaBD_4$) and permethylated oligosaccharides obtained by partial hydrolysis from stewartan (diagnostic fragments have been underlined, relative abundance in parentheses). The exact structure of all compounds is given. The identity of the individual monosaccharide units and the anomeric configurations of the linkages was inferred from methylation analyses and NMR data of the isolated stewartan fragments

Compound **1** (Glc *p*-β(1 → 3)-Gal-ol-*1-d*): *m / z* 88 (100), 89 (31), 90 (19), 101 (90), 111 (40), 172 (10), 187 (50), 219 (8), 236 (20), 296 (2), 350 (1), 382 (1), 471 (M^+) n.d.

Compound **2** (Glc *p*A-β(1 → 4)-Gal-ol-*1-d*): *m / z* 45 (73), 46 (53), 59 (47), 75 (72), 88 (73), 89 (67), 90 (38), 101 (89), 134 (38), 141 (43), 172 (37), 201 (100), 233 (55), 236 (37), 245 (3), 296 (3), 363 (3), 395 (2), 408 (1), 420 (1), 435 (1), 495 (M^+) n.d.

Compound **3** (Gal *p*-α(1 → 6)-Glc-ol-*1-d*): *m / z* 88 (85), 89 (25), 90 (30), 101 (100), 111 (26), 134 (20), 146 (35), 172 (15), 178 (12), 187 (42), 190 (6), 219 (11), 222 (2), 236 (52), 250 (3), 337 (3), 471 (M^+) n.d.

Compound **4** (Glc *p*-β(1 → 6)-Gal-ol-*1-d*): *m / z* 88 (90), 89 (30), 90 (35), 101 (100), 111 (25), 133 (15), 134 (9), 146 (27), 172 (13), 178 (9), 187 (32), 190 (4), 219 (6), 222 (1), 236 (32), 250 (2), 337 (2), 471 (M^+) n.d.

Compound **5** (Glc *p*A-β(1 → 4)[Gal *p*-β(1 → 3)-]-Gal-ol-*1-d*): *m / z* 88 (77), 89 (24), 90 (9), 101 (77), 111 (41), 172 (28), 187 (44), 201 (100), 219 (13), 233 (8), 250 (4), 306 (< 1), 319 (< 1), 440 (2), 454 (8), 500 (< 1), 514 (< 1), 643 (< 1), 644 (< 1), 689 (M^+) n.d.

Compound **6** (Glc *p*A-β(1 → 4)[Glc *p*-β(1 → 6)-]-Gal-ol-*1-d*): *m / z* 88 (87), 89 (23), 90 (12), 101 (92), 111 (35), 134 (8), 172 (28), 187 (24), 201 (100), 219 (6), 233 (8), 250 (2), 317 (2), 349 (2), 440 (1), 454 (10), 500 (< 1), 689 (M^+) n.d.

Compound **7** (Glc *p*A-β(1 → 4)-Gal *p*-α(1 → 6)-Glc-ol-*1-d*): *m / z* 88 (98), 90 (23), 101 (83), 116 (17), 134 (12), 146 (18), 172 (19), 178 (8), 190 (4), 201 (100), 222 (1), 233 (11), 236 (25), 250 (1), 291 (1), 296 (4), 319 (1), 376 (2), 405 (< 1), 436 (< 1), 500 (< 1), 523 (< 1), 555 (< 1), 598 (< 1), 689 (M^+) n.d.

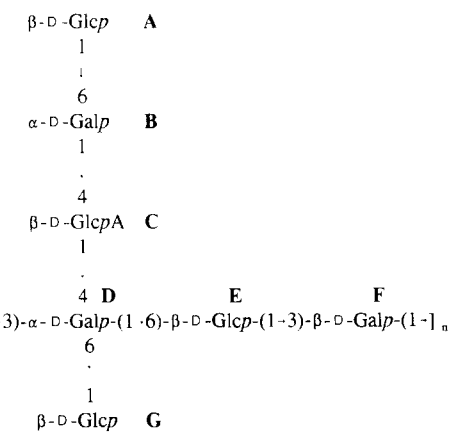
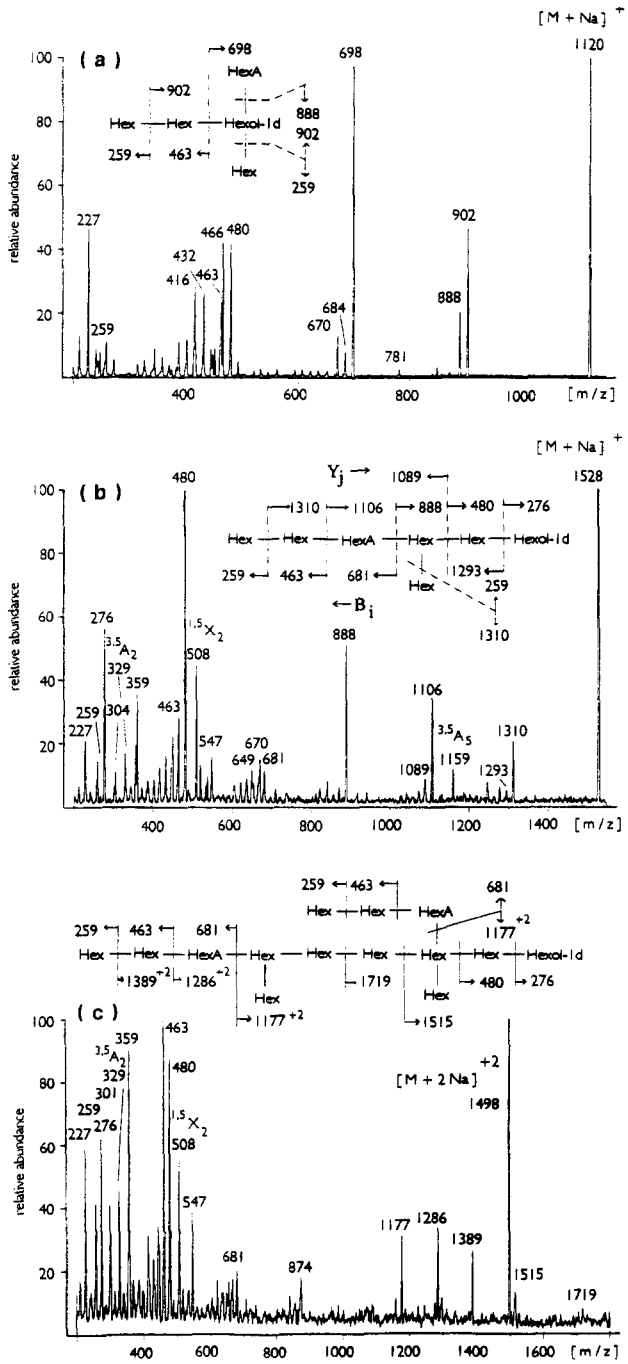


Fig. 1. Structure and letter code of the monosaccharide residues of the repeating unit of stewartan.


[M + Na]+
(a)
1120
698
902
888
480
466
432
463
416
684
670
781
259
227
relative abundance
[m/z]
(b)
1528
1089
1310
1106
888
480
276
1293
681
463
259
276
508
359
329
304
227
547
670
649
1159
(c)
[M + 2 Na]+2
1498
1389
1286
1177
1515
1719
874

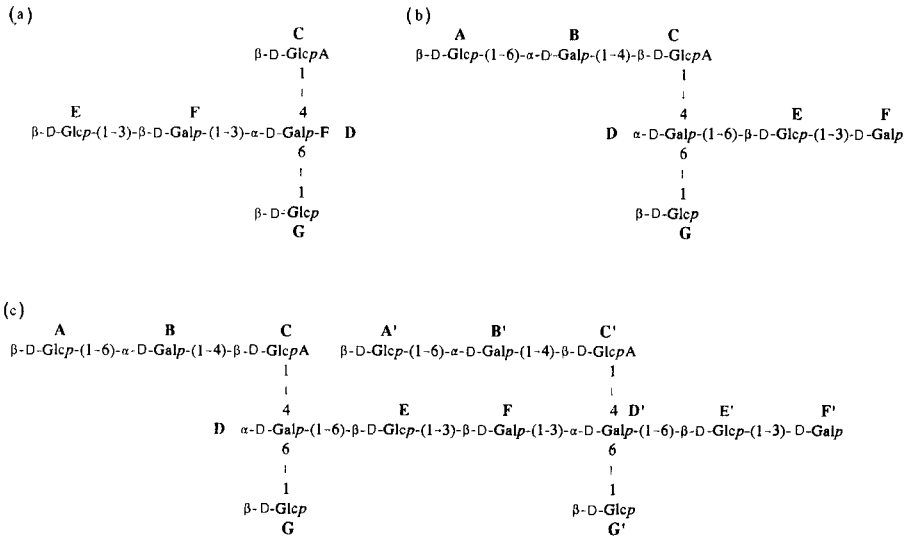
Structural elucidation of a pentasaccharide produced by HF-treatment of stewartan. —Treatment of stewartan with liquid HF at −32 °C, conditions which are expected to cleave α-linkages of hexoses but neither α- nor β-linkages of uronic acids [7], led to the production of predominantly di- (not analyzed further) and penta-saccharides. Negative-ion mode ESI-MS of the isolated compound **8** afforded a deprotonated molecular ion at *m/z* 843 corresponding to $[Hex_3HexAHex\,F - H]^-$ demonstrating the presence of a pentasaccharide fluoride. The additional presence of an ion at *m/z* 841 indicates that approximately 30% of the material is hydrolyzed. Compound **8** was contaminated with about 25% of a tetrasaccharide fluoride (*m/z* 681, $[Hex_2HexAHex\,F - H]^-$) and its hydrolyzed derivative. Positive-ion mode ESI-MS of the $NaBD_4$-reduced and permethylated compound yielded the expected sodiated molecular ion at *m/z* 1120 ($[Hex_3HexAHex\text{-}ol\text{-}1\text{-}d + Na]^+$) of the main component, and the daughter-ion spectrum depicted in Fig. 2a, when subjected to collision-induced dissociation (CID). The branching pattern, in particular the presence of a trisubstituted hexitol moiety, was deduced from the fragmentation pattern, as is illustrated in Fig. 2a. The weak fragment at *m/z* 480, however, can only be explained by small amounts of a contaminating isomeric structure lacking one hexose residue at the hexitol moiety. Methylation analysis of reduced **8** (Table 1), especially the detection of 3,4,6-tri-*O*-acetyl-1,2,5-tri-*O*-methyl-galactitol, indicative of a trisubstituted reducing monosaccharide residue, strongly suggests a structure comprising the fragment **E–F–(C)[G]D** in Fig. 1 as dominant component. MS/MS of the parent ion at *m/z* 916 ($[Hex_2HexAHex\text{-}ol\text{-}1\text{-}d + Na]^+$) of the contaminating permethylated tetrasaccharide-alditol-*1-d* clearly showed the absence of the terminal hexose residue linked to the hexitol residue. This was confirmed by the detection of small amounts of 3,4-di-*O*-acetyl-1,2,5,6-tetra-*O*-methylgalactitol in the methylation analysis.

The structure of **8** was confirmed by NMR spectroscopy. Four pyranose (three *gluco-* and one *galacto-*) monosaccharide spin systems, all with β-anomeric configurations, were completely or partially assigned using 2D COSY and TOCSY NMR spectra [Table 3(a)]. For the anomeric proton of the fifth residue **D** (a galactopyranose with α-anomeric configuration) a characteristic signal with a large geminal coupling constant to fluorine (54.3 Hz) was observed. Interresidual cross-peaks in the 2D ROESY spectrum were used to determine the linkages between the monosaccharide residues, as included in Table 3(a): **C** H-1,**D** H-4; **F** H-1,**D** H-3; **E** H-1,**F** H-3. Although **G** H-1 showed cross-peaks to two signals that must belong to a H-6a,b system, the latter could not be unambiguously assigned, but they did not belong to **G** or **E**.

Structural elucidation of the heptasaccharide produced by enzymatic depolymerization of stewartan.—Extensive cleavage of stewartan with phage ϕ-Ea1h-depolymerase and size fractionation of the degradation products gave two major oligosaccharides.

Fig. 2. Daughter-ion mass spectra of reduced ($NaBD_4$) and permethylated oligosaccharides obtained by chemical or enzymatic depolymerization of stewartan. (a) Pentasaccharide **8** obtained by HF-treatment; (b) repeating unit **9** obtained by enzymatic depolymerization; and (c) dimer of the repeating unit (**10**) obtained by enzymatic depolymerization. Fragments generated by cleavage of the glycosidic bonds are explained in the affixed fragmentation schemes. Rare inner-ring fragments are marked according to the nomenclature of Domon et al. [8].

Table 3
^{1}H chemical shifts of stewartan fragments: (a) pentasaccharide **8**; (b) heptasaccharide **9**; and (c) tetradecasaccharide **10** [a]



(a) Compound **8**	(b) Compound **9**		(c) Compound **10**	
Residue **D**	Residue **F** (α)	Residue **C**	Residue **F′** (α)	Residue **C′**
H-1: 5.695	H-1: 5.276	H-1: 4.621	H-1: 5.280	H-1: 4.865
H-2: 4.08	H-2: 3.97	H-2: 3.41	H-2: 4.00	H-2: 3.38
H-3: 4.08–413 [b]	H-3: 4.00	H-3: 3.80	H-3: 4.19	H-3: 3.76–3.82 [b]
(R: **F** H-1)	(R: **E** H-1)	H-4: 3.76–3.81 [b]	(N: **E′** H-1)	H-4: 3.76–3.82 [b]
H-4: 4.51	H-4: 4.20	(R: **B** H-1)	H-4: 4.23	H-5: 3.76–3.82 [b]
(R: **C** H-1)		H-5: 3.76–3.81 [b]		
H-6a: 4.03	Residue **F** (β)		Residue **F′** (β)	Residue **C**
(R: **G** H-1) [c]	H-1: 4.615	Residue **B**	H-1: 4.627	H-1: 4.643
H-6b: 3.89	H-2: 3.66	H-1: 5.513	H-2: 3.68	H-2: 3.42
(R: **G** H-1) [c]	H-3: 3.80	H-2: 3.80	H-3: 3.82	H-3: 3.75–3.83 [b]
	(R: **E** H-1)	H-3: 3.86	H-4: 4.18	H-4: 3.75–3.83 [b]
Residue **F**	H-4: 4.16	H-4: 4.023		H-5: 3.75–3.83 [b]
H-1: 4.507	H-5: 4.09	H-5: 3.79	Residue **F**	
H-2: 3.72		H-6a,b: 3.72–3.78 [b]	H-1: 4.643	Residue **B′** + **B**
H-3: 3.78	Residue **E**	(R: **A** H-1) [c]	H-2: 3.79	H-1: 5.515
(R: **E** H-1)	H-1: 4.698 (β*F*)		H-3: 3.84	H-2: 3.80
H-4: 4.13	H-1: 4.72 (α*F*)	Residue **A**	H-4: 4.20	H-3: 3.86
	H-2: 3.39	H-1: 4.543		H-4: 4.03
Residue **E**	H-3: 3.50	H-2: 3.315	Residues **E′** + **E**	
H-1: 4.585	H-4: 3.51	H-3: 3.54	H-1: 4.725	Residue **A′** + **A**
H-2: 3.33	H-5: 3.98	H-4: 3.40	H-2: 3.41	H-1: 4.547
H-3: 3.41	H-6a,b: 3.78	H-5: 3.50	H-3: 3.51	H-2: 3.31
H-4: 3.36	(R: **D** H-1) [c]	H-6a: 3.92	H-4: 3.55	H-3: 3.52
H-5: 3.30–3.41 [b]		H-6b: 3.72	H-5: 3.65	H-4: 3.39
H-6a: 3.84				H-5: 3.5
H-6b: 3.65				H-6a: 3.72
				H-6b: 3.92

Table 3 (continued)

(a) Compound **8**	(b) Compound **9**		(c) Compound **10**	
Residue **C**	Residue **D**	Residue **G**	Residue **D'**	Residue **G'** + **G**
H-1: 4.840	H-1: 5.017	H-1: 4.491	H-1: 5.046	H-1: 4.488
H-2: 3.27	H-2: 3.92	H-2: 3.267	H-2: 4.15	H-2: 3.27
H-3: 3.45	H-3: 3.95	H-3: 3.51	H-3: 4.09	H-3: 3.51
H-4: 3.45	H-4: 4.223	H-4: 3.40	(N: **F** H-1)	H-4: 3.40
H-5: 3.63	(R: **C** H-1)	H-5: 3.45	H-4: 4.455	H-5: 3.45
	H-5: 3.71	H-6a: 3.74	(N: **C'** H-1; **F** H-1)	H-6a: 3.75
Residue **G**	H-6a: 3.91	H-6b: 3.92	H-5: 3.90	H-6b: 3.92
H-1: 4.410	(R: **G** H-1) [c]		H-6a: 3.91	
H-2: 3.20	H-6b: 4.09		(N: **G'** H-1)	
H-3: 3.41	(R: **G** H-1) [c]		H-6b: 4.09	
H-4: 3.33			(N: **G'** H-1)	
H-5: 3.30–3.41 [b]				
H-6a: 3.85			Residue **D**	
H-6b: 3.67			H-1: 5.019	
			H-2: 3.93	
			H-3: 3.97	
			H-4: 4.25	
			(N: **C** H-1)	
			H-5: 3.83	
			H-6a: 3.91	
			(N: **G** H-1)	
			H-6b: 4.09	
			(N: **G** H-1)	

[a] N: NOESY; R: ROESY.

[b] Could not be assigned more accurately due to signal overlap.

[c] Showed cross-peaks to the H-6a,b system of another monosaccharide residue, which could not be unambiguously assigned due to signal overlap.

Judged from calibration of the gel-permeation column with oligomeric dextrans, the degradation products eluted in the size range of tetradecasaccharides and heptasaccharides, respectively. Negative-ion mode ESI-MS of the heptasaccharide fraction (compound **9**) yielded a molecular ion at m/z 1165 ($[Hex_6HexA - H]^-$). In the positive-ion mode, the $NaBD_4$-reduced and permethylated sample yielded a molecular ion at m/z 1528 ($[Hex_5HexAHex\text{-}ol\text{-}1\text{-}d + Na]^+$), corresponding to the Na^+-adduct of the repeating heptasaccharide of stewartan. CID of the parent ion at m/z 1528 (Fig. 2b) clearly showed a branching at the third hexose residue from the reducing end, and the sequence Hex–Hex–HexA–(Hex)Hex–Hex–Hex-ol-*1-d* (for the fragmentation scheme, see Fig. 2b). Most of the fragment ions are generated by cleavage of the glycosidic bonds (Y- or B-type, according to the nomenclature of Domon et al. [8]). Two relatively intense fragments at m/z 359 and 1159 are generated by fragmentation of the sugar ring of the 2nd and the 5th monosaccharide residue from the non-reducing end between C-3/C-4 and C-5/O-5 ($^{3,5}A_2$ and $^{3,5}A_5$) excluding a (1 → 2)- or (1 → 3)-linkage between the respective monosaccharide units. These findings are compatible with the sequence **A–B–C–(G)D–E–F** in Fig. 1, and the (1 → 6)-bonds between the residues **A** and **B**

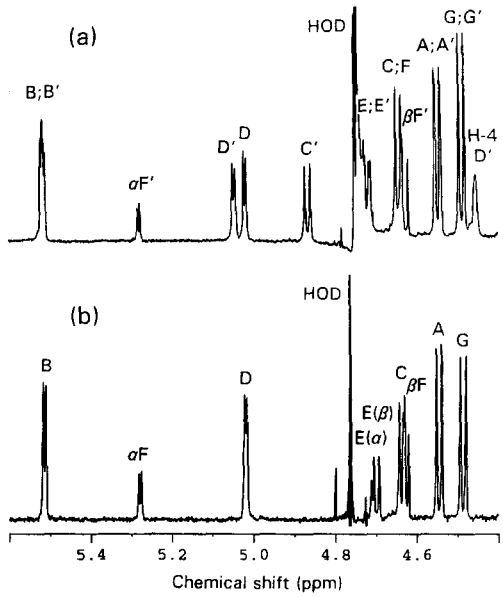


Fig. 3. 600-MHz ^{1}H 1D NMR spectra of the anomeric proton region of (a) the dimer **10** and (b) the monomer **9** of the repeating unit of stewartan obtained by enzymatic depolymerization.

and between **D** and **E**. Methylation analysis (Table 1) yielded, among others, the 4,6-disubstituted galactose derivative instead of the 3,4,6-trisubstituted derivative characteristic of the intact polymer, revealing the site of polymerization at O-3 of the branched galactose residue. This was confirmed by the complementary detection of 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol defining the reducing end of the repeating unit of stewartan.

Compound **9** was also investigated by NMR spectroscopy [Fig. 3b and Table 3(b)]. In particular, the technique allowed a clear differentiation between *galacto-* and *gluco-*spin systems. Additionally, the anomeric configurations of the constituting monosaccharides could be unequivocally determined, and most linkages could be deduced directly from 2D NMR experiments. The presence of four β-*gluco*-pyranose spin systems were shown from the chemical shifts of the respective H-2 signals, which were to high field of those of the galactose residues. Cross-peaks between **E** H-1 and **F** H-3α,β confirmed their (1 → 3)-linkage. Additionally, the (1 → 4)-linkage between residues **B** and **C** and between **C** and **D** could be verified in the same way. The three (1 → 6)-bonds, however, could not be rigorously confirmed due to signal overlap.

Structural elucidation of the tetradecasaccharide produced by enzymatic depolymerization of stewartan.—The dimer of the repeating unit **10** yielded a doubly charged deprotonated molecular ion at *m/z* 1156 corresponding to $[Hex_{12}HexA_2 - 2H]^{2-}$, when subjected to negative-ion mode ESI-MS. The $NaBD_4$-reduced and permethylated sample yielded a disodiated molecular ion at *m/z* 1498 ($[Hex_{11}HexA_2Hex\text{-}ol\text{-}1\text{-}d + 2Na]^{2+}$) in the positive-ion mode, confirming the data from the native compound. CID

of the parent ion at m/z 1498 yielded the daughter-ion spectrum depicted in Fig. 2c. In spite of the large molecular mass of the sample, a relatively complete fragment-ion series of the Y-type including the hexitol-*1-d-* residue, complemented by some B-type fragments, was obtained, confirming all expected branching points and the structure **A–B–C–(G)D–E–F–(A′–B′–C′)[G′]D′–E′–F′** (see Figs. 1 and 2c). Methylation analysis (Table 1) again confirmed the site of polymerization at O-3 of the central galactose **D**, yielding both the 4,6-di- and 3,4,6-trisubstituted derivative, characteristic of the depolymerized and polymerized branched core galactose residues, respectively.

Due to the similarity of the analogous residues of the dimer (**A** + **A′**, **B** + **B′**, **E** + **E′**, and **G** + **G′**), the ^{1}H NMR spin systems of these residues could not be clearly separated, even at 600 MHz (Table 3). The signals of the residues close to the site of polymeriza-

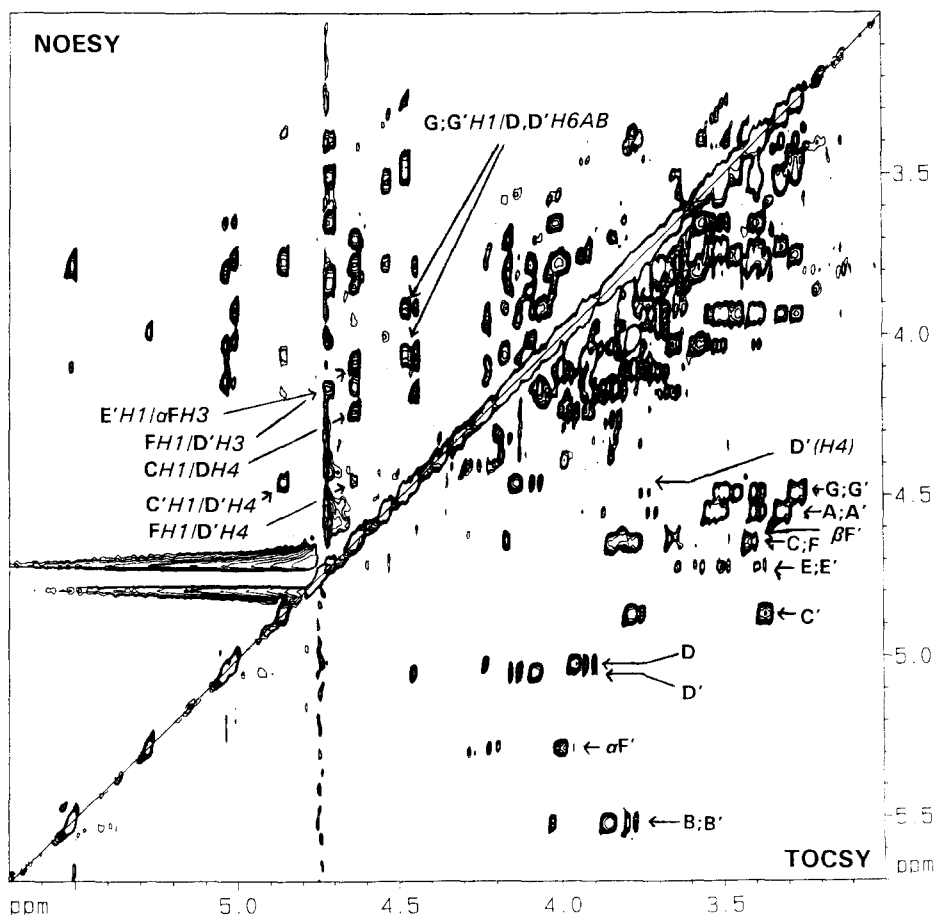


Fig. 4. 600-MHz ^{1}H 2D NOESY (upper) and TOCSY (lower) spectra of the tetradecasaccharide **10** produced by enzymatic depolymerization of stewartan. The spin systems belonging to the various units are indicated in the TOCSY spectrum and the most important linkage cross-peaks are indicated in the NOESY spectrum.

tion, however, exhibited characteristic shifts in their resonances dependent on their proximity to the polymerized or depolymerized central galactose, respectively. The anomeric protons of the residues **C** and **C′** showed the largest differences, although residues **D** and **D′**, and **F** and β**F′** also were clearly differentiated (Fig. 3). The linkages between the residues **C** and **D**, **C′** and **D′**, and, most importantly, between the residues **F** and **D′**, could be unequivocally confirmed by cross-peaks in the NOESY spectrum [Fig. 4 and Table 3(c)].

4. Discussion

The isolation of overlapping fragments of stewartan produced by enzymatic and chemical procedures, and the application of complementary MS and NMR methods for structural elucidation, allowed the complete characterization of the repeating unit of this polysaccharide. The basic structure is similar to the EPS amylovoran of the closely related bacterium *E. amylovora* [3]. In particular, the backbone of amylovoran differs only in the exchange of β-glucose (residue **E**) for β-galactose. The GlcA-containing side chain of stewartan is terminated by β-Glc*p*-(1 → 6)-α-Gal*p* (**A**–**B**) instead of a 4,6-pyruvated α-Gal residue for amylovoran, linked identically at O-4 of glucuronic acid. The β-Glc residue **G**, linked to O-6 of the central galactose of the repeating unit of stewartan, is present in only 10% of the amylovoran repeating units. Finally, in contrast to amylovoran, no *O*-acetyl groups in the trisaccharide side chain were detected. Beside these small differences, the similarities of the monosaccharide residues and their linkages point to similarities in the genetic make-up of the chromosomal regions involved in EPS synthesis. The structural genes of *E. stewartii* (*cps*) [9] and *E. amylovora* (*ams*) [10] are clustered in an area of about 20 kb. Twelve *ams*-genes are transcribed as an operon. They are succeeded by two genes involved in precursor synthesis. The high degree of gene-to-gene homology is interrupted in the central parts for genes *cps*D/*ams*C, *cps*F/*ams*E, and *cps*G/*ams*D. We assume that the corresponding gene products cause the observed differences in monosaccharide composition of stewartan and amylovoran. By complementation of defective genes in these areas, it might be possible to exchange glucose and galactose in the backbone and to synthesize side chains terminated by either glucose or pyruvate residues. The synthesis of amylovoran in *E. stewartii*- or stewartan in *E. amylovora*-EPS mutants restored virulence for *E. stewartii*, but not for *E. amylovora* [11]. The substitution of single monosaccharides could identify the requirement of a specific EPS structure for virulence better than the conversion of one EPS-type into another.

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