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Pyruvated galactose and oligosaccharides from *Erwinia chrysanthemi* Ech6 extracellular polysaccharide

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Abstract

The acidic extracellular polysaccharide of Ech6 was depolymerized by fuming HCl. The pyruvated sugars were isolated and characterized by methods that included a combination of low-pressure gel-filtration and high-pH anion-exchange chromatographies, methylation linkage analyses, mass (GC–MS and MALDI-TOF MS) and ^{1}H NMR (1D and 2D) spectroscopies. The following pyruvated sugars were obtained: 4,6-*O*-(1-carboxyethylidene)-D-Gal*p*; 4,6-*O*-(1-carboxyethylidene)-α-D-Gal*p*-(1→4)-β-D-GlcA*p*-(1→3)-D-Gal*p*; 4,6-*O*-(1-carboxyethylidene)-α-D-Gal*p*-(1→4)-β-D-GlcA*p*-(1→3)-α-D-Gal*p*-(1→3)-L-Fuc*p*; 4,6-*O*-(1-carboxyethylidene)-α-D-Gal*p*-(1→4)-β-D-GlcA*p*-(1→3)-α-D-Gal*p*-(1→3)-L-[β-D-Glc*p*-(1→4)]-Fuc*p*. These oligosaccharides present potential haptenes for the development of specific antibodies and confirm the partial structure proposed previously for the extracellular polysaccharide from *Erwinia chrysanthemi* Ech6 [Yang, B. Y.; Gray, J. S. S.; Montgomery, R. *Int. J. Biol. Macromol.*, **1994**, *16*, 306–312].

Keywords: Erwinia chrysanthemi; Pyruvated sugars; Extracellular polysaccharide; Structure

1. Introduction

Erwinia chrysanthemi Ech6 is gram-negative bacterial phytopathogen to potatoes. The bacterium produces an extracellular polysaccharide (EPS6), when grown on solid or liquid media. The resulting EPS was previously determined to be a branched hexsaccharide repeating unit,[1] in which the pyruvic acid was linked as an acetal to the 4,6-positions of the terminal galactose residue of the side chain.

Along with the study of the role of EPS6 in the pathogenic process, the preparation of oligosaccharides as potential haptens in the preparation of specific antigens was studied. The presence of the pyruvated galactose residues in EPS6 directed the study with particular focus to pyruvated oligosaccharides, so EPS6 was subjected to partial acid hydrolysis under several conditions. Somewhat surprisingly, the optimum condition from this research was hydrolysis with fuming HCl. The resulting pyruvated galactose and pyruvated oligosaccharides were isolated and characterized.

2. Experimental

Production and purification of polysaccharide.—The aqueous medium for the preparation of EPS6 in Fernbach flasks contained: glucose (20.0 g/L); proteose peptone (2.0 g/L); yeast extract (0.5 g/L); $MgSO_4 \cdot 7H_2O$ (0.5

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g/L); KH_2PO_4 (0.5 g/L). Calcium carbonate (2.5 g/L) was added after sterilization by autoclaving. A 100-mL amount of this medium was inoculated with a culture of *E. chrysanthemi* Ech6 grown on nutrient agar. After 3 days, 25 mL of the inoculum was added to 1 L of medium in a Fernbach flask, which was shaken at 30 °C and 150 rpm for the first day, increasing the shaking to 200 rpm for 2 more days. The viscous solution was centrifuged at 14,000 rpm for 2 h or until the supernatant was clear. To the supernatant was added NaCl (30 g/L) and the EPS6 was precipitated with 2.0–2.5 volumes of EtOH. The crude EPS6 was dissolved in 5% NaCl, centrifuged and the EPS6 reprecipitated with EtOH. The yield from *E. chrysanthemi* Ech6 was 3.0–4.0 g/L.

Analytical and general methods.—Methods used for methylation analysis, gas liquid chromatography with FID detector (GLC) and mass selective detector (GC–MS), monosaccharide analysis by high-pH anion-exchange chromatography pulsed amperometric detection (HPAEC-PAD), matrix assisted laser desorption–ionization time of flight mass spectrometric analysis (MALDI-TOF MS) of per-*O*-methylated derivatives, and ^1H NMR spectroscopy have been described previously.[2,3]

Partial hydrolysis of EPS6 by HCl.—The EPS6 (1.1 g dried in vacuo at 80 °C for 16 h), was dissolved by shaking gently in a round-bottom flask (250 mL) containing 50 mL (600 mmol) of HCl (37.4%, Fisher Scientific, Pittsburgh, PA) that had been cooled in dry ice. The solution was then kept at rt (25 °C), when the reaction temperature increased gradually. After 30 min, the resulting cloudy solution was poured with stirring into 300 mL of ice-cold aq AcONa solution (51.7 g, 630 mmol) in a 2-L round-bottom flask. The resulting solution (pH 3) was concentrated to dryness in vacuo by co-evaporation with toluene in a rotary-evaporator at 43 °C.

The dried residue was mixed with 150 mL of dimethyl sulfoxide, and sonicated to produce a slurry. The supernatant was recovered by centrifugation. This step was repeated twice (2 × 100 mL). The supernatants were combined and the oligosaccharides precipitated by adding $CHCl_3$ (750 mL) slowly with stirring to minimize heating. The resulting cloudy solution, after standing overnight, was decanted and the solids recovered by centrifugation. The residue was washed repeatedly with $CHCl_3$ (4 × 60 mL), and finally dried in vacuo. The product was redissolved in water (42 mL) and separated into anionic and neutral species by eluting sequentially on a strong cation-exchange column (80 mL, AG50W-X8, H-form, 200–400 mesh, Bio-Rad) and a weak anion-exchange column (80 mL, AG3-X4, OH-form, 100–200 mesh, Bio-Rad). The neutral fraction was isolated from the eluate. The resulting bound anionic species were eluted with 200 mL of 1.5 M pyridinum acetate (pH 5.5) and the eluent evaporated to dryness in vacuo.

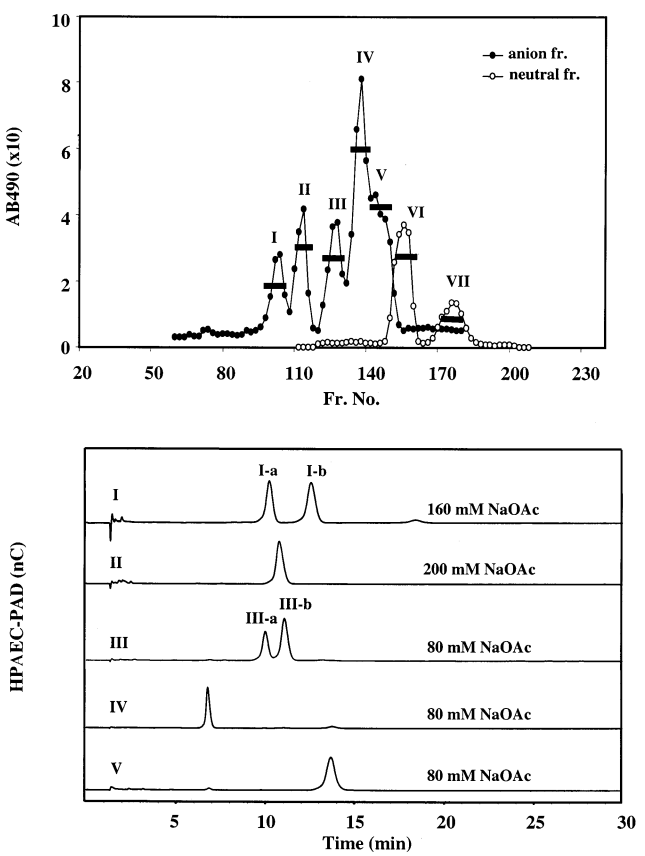

Fig. 1. Separation of acidic oligosaccharides by low-pressure gel-filtration chromatography (upper panel) on a BioGel P-2 column (2.5 × 83 cm) and subsequent analyses by HPAEC-PAD (lower panel) on a CarboPac PA100 column (4.5 × 250 mm). BioGel P-2 column eluted with 0.1 M pyridinum acetate (pH 5.5) and CarboPac PA100 column eluted with various concentration of AcONa as just described in the presence of 40 mM NaOH. The fractions under the bars were pooled for purification on a semi-preparative CarboPac PA100 column (9 × 250 mm). I-a, pyruvated pentasaccharide; I-b, pyruvated tetrasaccharide; II, pyruvated trisaccharide; III-a, de-pyruvated I-b; III-b, depyruvated trisaccharide; IV, pyruvated galactose; V, aldobiuronic and glucuronic acid.

Table 1
MALDI-TOF MS analyses and composition analyses of the oligosaccharides derived from partial acid hydrolysis of EPS6 by fuming HCl

Oligosaccharides	Composition analysis [a]	m/z (M+Na) [b]	
		Observed	Calculated
I-a	$PyGal_2GlcAFuc$ Glc	1129.1	1129.5
I-b	$PyGal_2GlcAFuc$	925.4	925.4
II	$PyGal_2GlcA$	751.5	751.3
III-a	$Gal_2GlcAFuc$	869.5	869.4
III-b	GlcAGalFuc	665.7	665.3
IV	PyGal	329.9	329.2
V	GlcAGal	491.8	491.2

[a] HPAEC-PAD analyses of monosaccharides released by 2 M TFA, 120 °C, 1 h and HPLC analyses of pyruvic acid released by 5 mM H_2SO_4, 120 °C, 1 h.

[b] MALDI-TOF analyses of the per-*O*-methylated oligosaccharide derivatives and detected as sodium adducts.

Gel-filtration and high-pH ion-exchange chromatographic purification of oligosaccharides.—Each species separated as just described by ion-exchange chromatography was further fractionated on a gel-filtration column (BioGel P-2, 2.5 × 82 cm), eluting with 0.1 M pyridinum acetate (pH 5.5). The elution was followed by phenol–H_2SO_4 analysis[4] and appropriately pooled fractions (see Fig. 1) were analyzed by MALDI-TOF MS as their per-*O*-methylated derivatives. The composition of the fractions was determined by HPAEC-PAD (Dionex Corp., Sunnyvale, CA, USA) after hydrolysis with 2 M trifluoroacetic acid (TFA) at 120 °C for 1 h. Pyruvated oligosaccharides were further purified on a semi-preparative column (CarboPac PA100, 9 × 250 mm) by HPAEC-PAD (Fig. 1).

HPLC analyses for pyruvate.—Pyruvate was hydrolyzed from the sugars or EPS6 by mild acid hydrolysis (5 mM H_2SO_4, 120 °C, 1 h) and was analyzed by high-performance cation-exchange chromatography (HPLC) on a Dionex BioLC system (Dionex Corp., Sunnyvale, CA, USA), consisting of a guard and cation-exchange analytical column (Bio-Rad Aminex HPX-87H, 300 × 7.8 mm) and a UV detector at 210 nm, as described elsewhere.[5]

3. Results and discussion

Partial acid hydrolysis of EPS6 with fuming HCl.—Recognizing the relative sensitivities of

Table 2
Methylation analyses of oligosaccharides derived from partial acid hydrolysis of the EPS6 by fuming HCl

Me sugar [a]	Relative molar ratio of oligosaccharide						
	EPS6 [e]	I-a	I-b	II	III-a	III-b	V
2,3-Me_2Fuc	1.0						
2,4-Me_2Fuc			1.1		1.0	0.9	
2-MeFuc	1.1	1.1					
2,3,4,6-Me_4Glc		0.8					
2,3,6-Me_3Glc [b]	0.8						
2,4,6-Me_3Glc	1.1						
2,3,4,6-Me_4Gal					0.8		
2,4,6-Me_3Gal	1.1	1.0	1.0	1.0	1.0	1.0	1.0
2,3-Me_2Gal	0.9	1.1	1.0	1.0			
2,3,4-Me_3GlcA [c]					1.0	1.0	1.1
2,3-Me_2GlcA [d]		1.1	1.0	1.0			

[a] 2,4-Me_2Fuc = 1,3,5-tri-*O*-acetyl-1-deuterio-2,4-di-*O*-methylfucitol.

[b] Derived from methylation of carboxyl-reduced EPS6.

[c] Observed as 1,5,6-tri-*O*-acetyl-1,6,6′-trideuterio-2,3,4-tri-*O*-methylglucitol derived from the reduction of the methyl ester of 2,3,4-Me_3GlcA with super-deuteride.

[d] Observed as 1,4,5,6-tetra-*O*-acetyl-1,6,6′-trideuterio-2,3-di-*O*-methylglucitol derived from the reduction of the methyl ester of 2,3-Me_2GlcA with super-deuteride.

[e] From Ref. 1.

glycosidic linkages and substituent groups in polysaccharides to acid hydrolysis,[6–9] many conditions are described to achieve partial depolymerization[10] and isolation of oligosaccharides.[11] As in the determination of the composition of the polysaccharide, each monomer unit may have its optimum condition for release, and also for partial hydrolysis of the polysaccharide. The desired oligomers will determine the optimum conditions to use. Examination of several conditions, such as acid concentration and reaction temperature, for the preparation of pyruvated oligosaccharides from EPS6, showed that fuming HCl was the best. The effective removal of excess HCl when the reaction is scaled up presented problems because of the generation of large amounts of salts. In this work, both effective desalting and the use of minimal volumes of solvents were achieved by extraction of the oligosaccharides with Me_2SO. A 90–95% recovery of sugar was realized, as determined by the phenol–sulfuric acid method. The oligosaccharides were finally recovered as precipitates by back-extraction of the Me_2SO with chloroform. This procedure removed excess of salts ($>95\%$) and the resulting residue could be subjected to ion-exchange chromatography for the fractionation of the sugar mixtures.

Since the depolymerization of EPS6 was for only 30 min, a quick and effective quenching of excess HCl was necessary to avoid further hydrolysis. A slight excess (5 mol%) of sodium acetate was used to avoid any free HCl when the solution was concentrated to dryness in vacuo. Upon reconstitution of the residue in water, the solution gave pH 7.

Isolation of oligosaccharides by chromatographic procedures.—Oligosaccharides were separated into anionic and neutral species before gel-filtration chromatography. Each species was further fractionated on a low-pressure gel-filtration column (Fig. 1). Neutral species was separated into disaccharide (VI) and monosaccharide (VII) fractions, of which glucosylfucose was the principal disaccharide. The anionic species were separated into five components (I–V). Fraction V, which contained aldobiuronic acid, glucuronic acid, and pyruvic acid, was rechromatographed to separate the aldobiuronic and glucuronic acids, but pyruvic acid coeluted with the aldobiuronic acid (data not shown). Further purification of each oligosaccharide was conducted on a semi-preparative column as described in Fig. 1.

Monosaccharide composition and linkage analyses of oligosaccharides.—The analyses of the fractions summarized in Table 1 show Peak I-a, pyruvated pentasaccharide; Peak I-b, pyruvated tetrasaccharide; Peak II, pyruvated trisaccharide; Peak III-a, de-pyruvated I-b; III-b, trisaccharide; Peak IV, pyruvated galactose; Peak V, aldobiuronic acid.

Methylation analyses of oligosaccharides (Table 2), in which the methyl ester of glucuronic acid from per-*O*-methylation was reduced with super-deuteride [$LiB(Et)_3D$], was followed by conventional procedures of hydrolysis, reduction and acetylation. All of the sugar residues were pyranosides. The reducing end of each oligosaccharide was determined by GC–MS analysis of the alditol acetate derivatives derived by acetylating the acid hydrolyzates of the pre-reduced oligosaccharidiotol. The overall linkage analyses and ^1H NMR study (see below) permit the oligosaccharides to be defined as follows:

α-D-Py(4,6)Gal*p*-(1 → 4)-β-D-GlcA*p*-(1 → 3)-α-D-Gal*p*-(1 → 3)-L-[β-D-Glc*p*-(1 → 4)]-Fuc

I-a

α-D-Py(4,6)Gal*p*-(1 → 4)-β-D-GlcA*p*-(1 → 3)-α-D-Gal*p*-(1 → 3)-L-Fuc*p*

I-b

α-D-Py(4,6)Gal*p*-(1 → 4)-β-D-GlcA*p*-(1 → 3)-D-Gal*p*

II

α-D-Gal*p*-(1 → 4)-β-D-GlcA*p*-(1 → 3)-α-D-Gal*p*-(1 → 3)-L-Fuc*p*

III-a

β-D-GlcA*p*-(1 → 3)-α-D-Gal*p*-(1 → 3)-L-Fuc*p*

III-b

D-Py(4,6)Gal*p*

IV

β-D-GlcA*p*-(1 → 3)-D-Gal*p*

V

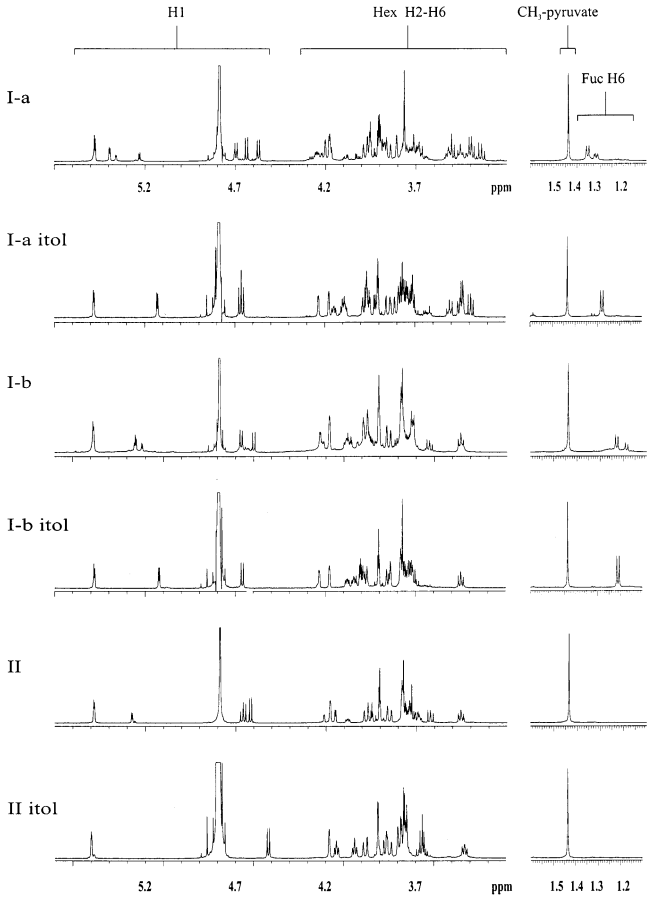

Fig. 2. Comparison of the 600-MHz 1D ^{1}H NMR spectra of oligosaccharides before and after reduction with $NaBH_4$. The spectra were obtained of the oligosaccharides, I-a (10 mM), I-b (20 mM), II (40 mM), and of the corresponding oligosaccharidiotols (2 mM), I-a itol, I-b itol, II itol in D_2O at 25 °C and pD 6.5.

The structures of these oligosaccharides corroborated the sequence of the EPS6 hexasaccharide repeating unit published previously[1] except that the original structure presented the α anomeric form instead of the now corrected β-D-GlcA*p*. This error was due to the native EPS6 giving a poor ^{1}H NMR spectrum that persisted under all conditions of temperature and ionic strength.

^{1}H NMR spectroscopy of oligosaccharides and oligosaccharidiotols.—As confirmation of the structures assigned to the oligosaccharides, the 600 MHz ^{1}H NMR spectra were obtained of oligosaccharides before and after reduction with $NaBH_4$ (Fig. 2) and analyzed, in particular detail for I-a.

The spectrum of each oligosaccharide (10–40 mM in D_2O) was complex due to the presence of α and β anomers of the reducing terminal residue, noted in particular with the pyruvated tetrasaccharide (I-b). However the spectra of oligosaccharidiotols (2 mM in D_2O) were much simpler. The signals of anomeric protons from the spectra of oligosaccharidiotols were observed in the predicted stoichiometric ratio and the results are summarized in Table 3. Based on the chemical shifts and coupling constants, Py(4,6)galactose and 1,3-linked galactose residues are present in the α configuration, and the 1,4-linked glucuronic acid and non-reducing terminal glucose residues are β anomers.

The non-reducing terminal galactose residue in the pyruvated compounds was quantitatively capped with pyruvic acid, as determined by the area ratio (1.0:3.0) of anomeric protons to methyl protons (Table 3). The chemical shift of the methyl group of the pyruvic acid indicates an equatorial conformation of the methyl group, accordingly the *R* configuration to the pyruvic acid substituent.[12]

The 1D ^{1}H NMR spectrum of the pyruvated pentasaccharide, I-a (Figs. 2 and 3) shows eight anomeric resonances. Based on the chemical shifts and coupling constants, four of the resonances, δ 5.476 ($J_{1,2}$ 3.6 Hz), δ 5.395 ($J_{1,2}$ 3.0 Hz), δ 5.360 ($J_{1,2}$ 3.0 Hz), δ

Table 3
^{1}H NMR chemical shifts and coupling constants for anomeric protons of oligosaccharidiotols (reduced with $NaBH_4$) in D_2O (pD 6.5) at 25 °C

Residues	δ [a] (ppm) of H-1 and $^3J_{1,2}$ (Hz)			
	I-a itol	I-b itol	II itol	V itol
α-D-Py (4,6)Gal*p*-(1→	5.482	5.481	5.497	
	(1.0) [b]	(1.0)	(1.0)	
$J_{1,2}$	3.0	3.0	2.4	
→3)-α-D-Gal*p*-(1→	5.130	5.123		
	(0.9)	(0.9)		
$J_{1,2}$	3.0	3.6		
→4)-β-D-GlcA*p*-(1→	4.672	4.662	4.518	4.495
	(0.9)	(1.0)	(1.0)	
$J_{1,2}$	8.4	7.8	7.8	7.8
β-D-Glc*p*-(1→	4.658			
	(0.9)			
$J_{1,2}$	8.4			
CH_3-pyruvate	1.435	1.437	1.434	
	(3.0)	(3.0)	(3.0)	

[a] Relative to the internal signal of acetone (δ 2.225 ppm).
[b] Relative peak intensity in parenthesis.

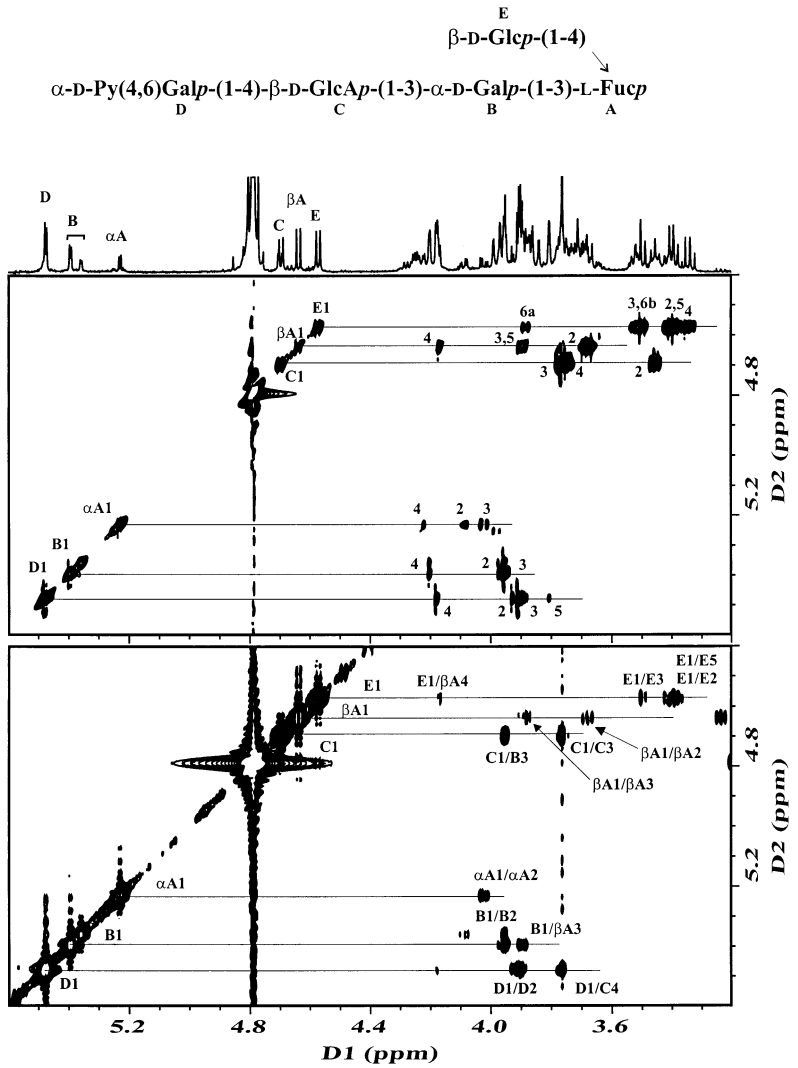

Fig. 3. Part of the 600-MHz ^{1}H 2D-TOCSY (upper panel, mixing time 116 ms) and NOESY (lower panel, mixing time 0.75 s) spectra of the pyruvated pentasaccharide (I-a). The spin system belonging to each unit and NOE contacts indicated as annotated.

5.231 ($J_{1,2}$ 4.2 Hz) were assigned to H-1 of α-linked glycosyl residues. The other four resonances, δ 4.700 ($J_{1,2}$ 7.8 Hz), δ 4.691 ($J_{1,2}$ 8.4 Hz), δ 4.639 ($J_{1,2}$ 7.8 Hz), δ 4.573 ($J_{1,2}$ 7.8 Hz) were assigned to H-1 of β-linked glycosyl residues. Two doublets, δ 5.395 (denoted Gal $_{\delta\ 5.395}$) and δ 5.360 (denoted Gal $_{\delta\ 5.360}$) were assigned to the internal α-1,3-linked galactose residue and two doublets, δ 4.700 (denoted GlcA $_{\delta\ 4.700}$) and δ 4.691 (denoted GlcA $_{\delta\ 4.691}$) were assigned to β-1,4-linked glucuronic acid.

The resonances at δ 5.231 and 4.639 were due to the H-1 protons of the α and β anomers of the reducing fucosyl residues, in which the relative intensity of α-H-1/β-H-1 was determined to be 0.39/0.61. The same ratio was also observed with the methyl protons of fucosyl residue between the α anomer at δ 1.319 and the β anomer at δ 1.354. The two doublets of α-1,3-linked galactose residue (H-1-Gal $_{\delta\ 5.395}$/H-1-Gal $_{\delta\ 5.360}$ = 0.36/0.64) and the two doublets of β-1,4-linked glucuronic acid residue (H-1-GlcA$_{\delta\ 4.700}$/H-1-GlcA$_{\delta\ 4.691}$ = 0.37/0.63) were attributed to the anomers of the reducing terminal fucose. This effect was further supported by inspection of the spectrum derived from the corresponding pyruvated pentasaccharidtol, in which only four anomeric resonances were observed at δ 5.482 ($J_{1,2}$ 3.0 Hz), δ 5.130 ($J_{1,2}$ 3.0 Hz), δ 4.672 ($J_{1,2}$ 8.4 Hz) and δ 4.658 ($J_{1,2}$ 8.4 Hz), each in the ratio corresponding to one proton (see I-a itol in Fig. 2 and Table 3).

Table 4
^{1}H NMR chemical shifts and coupling constants for the pyruvated galactose (IV), pyruvated trisaccharide (II), and pyruvated pentasaccharide (I) in D_2O at 25 °C and pD 6.5

Sugar	δ [a] (ppm)/$^3J_{H/H}$ (Hz)						
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
Residue of IV							
α-D-Py [b](4,6)Gal*p*	5.294	3.924	3.900	4.220	3.885	4.042	3.848
$J_{a,b}$	3.0	10.2	3.0	1.2	1.8	9.0	
β-D-Py(4,6)Gal*p*	4.618	3.597	3.687	4.157	3.593	4.020	3.903
$J_{a,b}$	7.8	9.6	3.6	1.2	1.8	13.2	
Residue of II							
α-D-Py [b](4,6) Gal*p*-(1→	5.483	3.914	3.894	4.177	3.779	3.978	3.849
$J_{a,b}$	3.0	10.2	2.4	1.2	2	13.2	
→3)-α-D-[R]Gal*p* [c]-(1→	5.274	3.959	3.933	4.211	4.080	3.89	3.69
$J_{a,b}$	3.0	9.0	2.4	1.2	1		
→4)-β-L-GlcA*p*-(1→	4.667, 4.652	3.455	3.762	3.751	3.725		
$J_{a,b}$	8.4, 7.8	9.0	12.0	9.6			
→3)-β-D-[R]Gal*p* [c]-(1→	4.619	3.622	3.746	4.149	3.673	3.725	
$J_{a,b}$	7.8	9.6	3.6	0.6	1.2	10.2	
Residue of I							
D α-D-Py [b](4,6) Gal*p*-(1→	5.476	3.919	3.902	4.179	3.807	3.980	3.852
$J_{a,b}$	3.6	10.2	3.0	0.6	<1	13.2	
B →3)-α-D-Gal*p*-(1→	5.395, 5.360	3.960	3.934	4.203	4.247		
$J_{a,b}$	3.0, 3.0	10.8	2.4	1.2			
αA →3, 4)-α-L-[R]Fuc*p* [c]-(1→	5.231	4.022	4.090	4.222	4.261	1.319	
$J_{a,b}$	4.2	10.8	3	<1			
C →4)-β-L-GlcA*p*-(1→	4.700, 4.696	3.456	3.764	3.764	3.713		
$J_{a,b}$	7.8, 8.4	8					
βA →3,4)-β-L-[R]Fuc*p* [c]-(1→	4.639	3.680	3.890	4.171	3.876	1.354	
$J_{a,b}$	7.8	10.2	3.0	3	7		
E →3)-β-D-Glc*p*-(1→	4.573	3.394	3.504	3.339	3.402	3.878	3.528
$J_{a,b}$	7.8	9.0	10.2	9.0	1.8		

[a] Relative to the internal signal of acetone (δ 2.225 ppm).
[b] CH_3 of pyruvic acid observed as a singlet at δ 1.450 (α anomer IV), at δ 1.461 (β anomer IV), δ 1.435 (II) and at δ 1.436 (I).
[c] R, reducing terminal residue.

The non-reducing terminal residue, Py(4,6)Gal, was also affected by anomerization of the reducing terminal fucose residue, but only to an extent of the distorted peak shape of H-1.

Further analyses for the pyruvated pentasaccharide (I-a) were performed by the combination of two-dimensional (^{1}H, ^{1}H) correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and nuclear Overhauser enhancement spectroscopy (NOESY) (see Fig. 3).

With respect to the reducing fucosyl residue, the H-2 resonance of the α-Fuc was assigned from the COSY experiment at δ 4.022 due to its cross peak to H-1 at δ 5.231 and a similar cross-peak relationship lead to the assignments of H-3 at δ 4.090, H-4 at δ 4.222 and H-5 at δ 4.261, where H-5 clearly showed a cross-peak to H-6 at δ 1.319. Because an equatorial–axial relationship of H-4 with both H-3 ($J_{3,4}$ 3 Hz) and H5 ($J_{4,5} < 1$ Hz), the H-4 and H-5 signals in the fucosyl residue were observed as a doublet at δ 4.222 and as a quartet at δ 4.261, respectively. The TOCSY spectrum across H-1 at δ 5.231 α-Fuc shows multiplets at δ 4.022, 4.090, and 4.222 (Fig. 3), which were consistent with H-2, H-3 and H-4 resonances assigned from the COSY spectrum. Similarly, the H-2 resonance for the

Table 5
NOE data for the pyruvated pentasaccharide (I-a)

Residue	Anomeric proton (H1)	Connectivity residue and chemical shifts	
	δ (ppm)		δ H (ppm)
D α-D-Py(4,6)Gal*p*-(1→	5.476	D1-D2	3.919
		D1-C4	3.764
B →3)-α-D-Gal*p*-(1→	5.395	B1-B2	3.960
		B1-βA3	3.890
αA →3,4)-α-L-^{R}Fuc*p*-(1→	5.231	αA1-αA2	4.022
C →4)-β-L-GlcA*p*-(1→	4.700	C1-B3	3.934
		C1-C3	3.764
βA →3, 4)-β-L-^{R}Fuc*p*-(1→	4.639	βA1-βA3	3.890
		βA1-βA2	3.680
E →3)-β-D-Glc*p*-(1→	4.573	E1-βA4	4.171
		E1-E3	3.504
		E1-E2	3.394
		E1-E5	3.402

β-Fuc residue at δ 3.680 was assigned from the COSY spectrum by its cross-peak to the H-1 at 4.639. Considered together with the TOCSY spectra, the spin systems of the glycosyl residues were assigned (Table 4).

The spin systems of the glucosyl residue were observed furthest upfield and well separated from the spin system of the other glycosyl residues. This permitted the complete assignments of proton resonances of the glucose residue from the COSY spectrum. The β-D-glucose has large vicinal coupling constants, which allows magnetization transfer from H-1 all the way to H-6 during the mixing period in the TOCSY experiment,[13] and consequently all signals from H-1 to H-6 were also observed in the subspectrum.

In the 1D ^{1}H NMR spectrum of I-a, a marked anomerization effect was observed with two inter-residues of the oligosaccharide, 1,3-linked galactose and 1,4-linked glucuronic acid, between two nonreducing and reducing termini, particularly with anomeric protons of the two residues. In the COSY and TOCSY experiments the two doublets of H-1 resonance of α-1,3-linked galactose residue, the Gal $_{\delta\ 5.395}$ and the Gal $_{\delta\ 5.360}$, were coupled to and superimposed on the same H-2 resonance at δ 3.960, consequently the subsequent assignments of the remaining protons were not discernable from each other. This was true with the two doublets of the H-1 resonance of the β-1,4-linked glucuronic acid residue.

In the NOESY spectrum there were clear inter-residue connective signals as well as intra-residue cross-peaks (Fig. 3 and Table 5). The inter-residue contacts between H-1 of D and H-4 of C, H-1 of C and H-3 of B, H-1 of B and H-3 of β-A, and H-1 of E and H-4 of β-A supported the sequence of monosaccharide residues in the pyruvated pentasaccharide (I-a), which was consistent with the structure deduced from the analyses of a series of oligosaccharides.

The structure of all the oligosaccharides are consistent with the partial structure of EPS6 published previously, except for the anomeric proton configuration of the β-D-glucuronosyl residue, as noted earlier.

4. Summary

Fuming HCl has been used for oligosaccharide preparation by partial acid hydrolysis of polysaccharides.[2,3,14] However, there has always been some concern that side reactions, such as transglycosylation or pyruvic acid migration, might occur during the partial acid hydrolysis, in addition to the cleavages of glycosidic linkages.

The oligosaccharides obtained in this study are consistent with the structure of the EPS6 established previously. No unexpected residues were detected in these oligosaccharides. Accordingly, all the oligosaccharides

isolated were derived by degradation of EPS6 without transglycosylation or migration of pyruvic acid residues.

Complete depyruvation from the EPS6 and other EPS (such as amylovoran, not published) could be achieved by mild acid hydrolysis of per-*O*-methylated EPS (10 mM HCl in 50% aqueous ethanol, 100 °C, 4 h), as determined by methylation analyses. Under such a condition, depyruvation occurs sometimes with concomitant cleavage of minor amounts (10–20%) of the terminal residue linked to pyruvic acid. However, fuming HCl reacts differently in this regard.

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