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Structural characterisation of the exopolysaccharide produced by *Lactobacillus delbrückii* subspecies *bulgaricus* rr grown in skimmed milk

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ABSTRACT

The exopolysaccharide of *Lactobacillus delbrückii* subsp. *bulgaricus* rr, isolated from skimmed milk, is a heteropolymer of D-galactopyranosyl, D-glucopyranosyl, and L-rhamnopyranosyl residues in the molar ratio 5:1:1. The structure was established by linkage analysis and 1D and 2D NMR spectroscopy of the native polysaccharide, in combination with characterisation of oligosaccharide fragments, obtained by Smith degradation and partial acid hydrolysis, using methylation analysis, EIMS, and 1D and 2D ^{1}H NMR spectroscopy. The polysaccharide has a branched heptasaccharide repeating unit with the following structure: $\rightarrow$ 2)-[β-D-Gal*p*-(1 $\rightarrow$ 3)]-α-D-Gal*p*-(1 $\rightarrow$ 3)-β-D-Glc*p*-(1 $\rightarrow$ 3)-[β-D-Gal*p*-(1 $\rightarrow$ 4)]-β-D-Gal*p*-(1 $\rightarrow$ 4)-[α-L-Rha*p*-(1 $\rightarrow$ 3)]-α-D-Gal*p*-(1 $\rightarrow$.

INTRODUCTION

Only a few of the more than 50 *Lactobacillus* species are involved in milk fermentations, with *L. delbrückii* subsp. *bulgaricus* and *L. acidophilus* the most extensively used[1]. For the exopolymers, which play a role in the rheological behaviour and texture of the fermented milks produced by *L. delbrückii* subsp. *bulgaricus* strains, different sugar compositions have been reported. The slime of strain rr has[2] a carbohydrate content of 85%, with Gal and Glc in the molar ratio 2:1. The main constituent of the exopolysaccharide was reported[3] to be Gal, with Ara, Man, and Glc as minor components. The polymer from strain CRL 420

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contained[4] Glc and Fru in the molar ratio 1:2. The polysaccharide from strains CNRZ 416, CNRZ 737, and CRL 420 is composed[5] of Gal, Glc, and Rha in the molar ratio 4:1:1. Finally, it has been suggested[6] that the polymer produced by strain NCFB 2772 is a glycoprotein, although it seems that the protein is loosely associated with the carbohydrate.

We now describe the isolation and characterisation of the exopolysaccharide produced by *L. delbrückii* subsp. *bulgaricus* rr grown in skimmed milk.

EXPERIMENTAL

Growth of the organism and isolation of the exopolysaccharide.—*L. delbrückii* subsp. *bulgaricus* rr was cultured in skimmed milk for 20 h at 37°C, trichloroacetic acid was added to 4%, and the bacterial cells and precipitated proteins were removed by centrifugation (20 min, 13 000*g*, 4°C). Acetone (1 vol) was added to the supernatant solution, and the precipitated polysaccharide was collected, dialysed against running tap water (48 h) and bidistilled water (24 h, 5 × 5 L), and then lyophilised. The product was dissolved in bidistilled water and, after removal of insoluble material by centrifugation (30 min, 30 000*g*, 4°C), fractionally precipitated with acetone at 30, 40, and 50% with intermediate centrifugation. The exopolysaccharide was precipitated at 40% acetone (yield 79%) and, after lyophilisation, contaminating protein was removed by gel filtration on a column (150 × 2.2 cm) of Sephacryl S-500 (Pharmacia) by elution with 50 mM NH_4HCO_3 at 25 mL/h (5-mL fractions), using refractive index monitoring (Bischoff RI-detector 8100) of the eluate.

Analytical methods.—Samples (0.2–0.5 mg) were methanolysed (methanolic M HCl, 24 h, 85°C) and the trimethylsilylated (*N*-reacetylated) methyl glycosides were analysed on an SE-30 fused-silica capillary column (25 m × 0.32 mm, Pierce), using a Varian 3700 gas chromatograph (temperature program 130 → 220°C at 4°C/min)[7]. The absolute configuration of the monosaccharides was determined by GLC of the trimethylsilylated (*N*-reacetylated) (−)-2-butyl glycosides[8,9].

Protein content was determined with the Pierce Protein Assay Reagent with bovine serum albumin as the standard.

Total carbohydrate content was measured by the phenol–H_2SO_4 assay[10] with a mixture of D-galactose, D-glucose, and L-rhamnose in the molar ratio 5:1:1 as the standard.

Methylation analysis.—Polysaccharides and oligosaccharide-alditols were each methylated according to the method of Kvernheim[11], and the products were hydrolysed with aq 90% formic acid (1 h, 100°C), then 2 M trifluoroacetic acid (1 h, 120°C). The resulting partially methylated monosaccharides were reduced with $NaBD_4$ and the products were acetylated[12]. The partially methylated alditol acetates were analysed by GLC on a CPsil 43 WCOT fused-silica capillary column (25 m × 0.32 mm, Chrompack), using a Varian 3700 gas chromatograph (temperature program 170 → 220°C at 4°C/min, followed by 15 min at 220°C), and by

GLC–MS using a Carlo Erba GC/Kratos MS80/Kratos DS 55 system (electron energy, 70 eV; accelerating voltage, 2.7 kV; ionising current, 100 mA; CPsil 43 capillary column). The permethylated trisaccharide-alditols were analysed by GLC–MS using a HP GC/JEOL AX505W/HP 9000 system (electron energy, 70 eV; accelerating voltage, 3.0 kV; ionising current, 100 mA; SE-30 capillary column).

Smith degradation.—To a solution of the polysaccharide (50 mg) in 0.1 M NaOAc buffer (50 mL, pH 3.9) was added sodium metaperiodate to 0.05 M, and the solution was kept in the dark for 5 days at 4°C. The excess of periodate was reduced with ethylene glycol (2 mL), and the mixture was stored for 2 h at room temperature, then dialysed against running tap water (48 h) and bidistilled water (24 h, 5 × 5 L), and lyophilised. To a solution of the oxidised polysaccharide in bidistilled water (10 mL) was added $NaBH_4$ (100 mg), the mixture was stored for 18 h at ambient temperature, the excess of $NaBH_4$ was destroyed by addition of Dowex AG 50W-X8 (H^+) resin (Bio-Rad), and boric acid was removed by co-concentration with MeOH under reduced pressure. A solution of the oxidised and reduced polysaccharide in aq 90% formic acid (10 mL) was kept for 1 h at 40°C, then co-concentrated with bidistilled water, and lyophilised. The resulting polymer was subjected twice more to a periodate oxidation–reduction cycle, then hydrolysed (1 h, 40°C) in aq 90% formic acid (7.5 mL), co-concentrated with bidistilled water, and lyophilised. A solution of the residue in bidistilled water (10 mL) was treated (18 h, ambient temperature) with $NaBD_4$ (50 mg). The excess of $NaBD_4$ was removed as described above. The final product was eluted from a column (95 × 1.2 cm) of Bio-Gel P-2 (200–400 mesh, Bio-Rad) with bidistilled water at 9 mL/h (1.1-mL fractions) and refractive index monitoring of the eluate.

Partial acid hydrolysis.—A solution of the polysaccharide (50 mg) in M trifluoroacetic acid (12.5 mL) was kept for 2 h at 85°C, then lyophilised, and the residue was eluted from a column (90 × 1.5 cm) of Bio-Gel P-2 with bidistilled water at 13 mL/h (1.6-mL fractions) and refractive index monitoring of the eluate.

TLC.—Silica Gel 60 F_{254} (Merck) and 3:2:2 1-butanol–EtOH–water were used, with detection by orcinol–H_2SO_4.

High-performance anion-exchange chromatography with pulsed amperometric detection.—Oligosaccharide fractions were subjected to HPAEC–PAD on a Dionex LC system, consisting of a Dionex Bio-LC quaternary gradient module, a model PAD-2 detector, and a CarboPac PA-1 pellicular anion-exchange column (250 × 9 mm), and elution programs with the following ratios of eluents *A* (0.1 M NaOH) and *B* (0.1 M NaOH containing M NaOAc) at ambient temperature and 5 mL/min (*1–4*) or 4 mL/min (*5*): *1*, 93:7 for 0.3 min then to 60:40 in 60 min; *2*, 94:6 for 0.3 min then to 65:35 in 60 min; *3*, 97:6 for 0.3 min then to 70:30 in 60 min; *4*, 98:2 for 0.3 min then to 90:10 in 60 min; *5*, 99:1 for 0.3 min then to 70:30 in 60 min. The PAD involved a gold working electrode and triple-pulse amperometry, comprising the following pulse potentials and durations: E_1 0.05 V and t_1 300 ms, E_2 0.65 V and t_2 60 ms, E_3 −0.95 V and t_3 180 ms; response time, 1 s. Data were collected and plotted by a Shimadzu C-R3A integrator. Fractions

were neutralised immediately with M HCl, lyophilised, and desalted on a column (95 × 1.2 cm) of Bio-Gel P-2 by elution with bidistilled water and refractive index monitoring. Residual NaOAc and/or NaCl was removed on a mixed-bed column (6 × 0.7 cm) of Dowex AG 50W-X8 (H^+) resin (200–400 mesh) and Dowex AG 1-X2 (HO^-) resin (100–200 mesh) by elution with bidistilled water.

FABMS.—Positive-ion FAB-mass spectra were recorded with a JEOL AX505W mass spectrometer (Xe-beam at 6 keV; acceleration voltage, 3 kV) equipped with a HP 9000 data system, and with thioglycerol as the matrix (Department of Mass Spectrometry).

NMR spectroscopy.—The proton-decoupled ^{13}C NMR spectrum (external Me_4Si, internal MeOH δ 49.00) was recorded with a Bruker AC-300 spectrometer (Department of Organic Chemistry) for a solution in D_2O at 70°C.

For the ^1H NMR spectra, carbohydrate samples were repeatedly exchanged in D_2O (99.9 atom% D) with intermediate lyophilisation, then dissolved in 0.4 mL of D_2O (99.96 atom% D). Resolution-enhanced ^1H NMR spectra (internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, internal acetone δ 2.225) were recorded with a Bruker AC-300, AM-500 (Department of NMR Spectroscopy), or AM-600 (SON-hf-NMR facility, Department of Biophysical Chemistry, Nijmegen University) spectrometer at various probe temperatures.

2D Homonuclear Hartmann–Hahn (HOHAHA) spin-lock experiments were recorded using the pulse sequence 90°–t_1–SL–acq[13–16], where SL stands for a multiple of the MLEV-17 sequence. The spin-lock field strength corresponded to a 90° pulse width of 27 μs. The total spin-lock mixing time ranged from 25 to 45 ms. The spectral width ranged from 1000 to 4000 Hz in each dimension.

2D Rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY) was carried out using the pulse sequence 90ϕ°–t_1–SL–acq[17], wherein SL stands for a continuous spin-lock pulse of 250 ms at a field strength corresponding to a 90° pulse width between 100–110 μs. The carrier frequency was placed at the left side of the spectrum at 5.7 ppm in order to minimise HOHAHA-type magnetisation transfer. The HOD signal was suppressed by presaturation during 1.0 s. The spectral width ranged from 2000 to 4000 Hz in each dimension.

2D Nuclear Overhauser enhancement spectroscopy (NOESY) of the native polysaccharide at 600 MHz was carried out at 67°C with a mixing time of 200 ms. The spectral width was 4000 Hz in each dimension.

For the HOHAHA spectra, 256 or 560 experiments of 2K data points were recorded, for the ROESY spectra, 512, 600, or 640 experiments of 2K data points, and for the NOESY spectrum, 512 experiments of 2K data points. The time-proportional phase-increment method (TPPI)[18] was used to create t_1 amplitude modulation. Each data matrix was zero-filled to 2K × 4K for the HOHAHA and ROESY spectra, and to 1K × 2K for the NOESY spectrum, and multiplied in each time domain with a phase-shifted sine function, shifted $\pi/3$ for the HOHAHA and $\pi/2$ for the ROESY and NOESY, prior to phase-sensitive FT.

RESULTS AND DISCUSSION

Isolation and composition of the polysaccharide.—The exopolysaccharide preparation was isolated as an acetone precipitate from the culture supernatant solution of strain rr, grown in skimmed milk. The purified exopolysaccharide was obtained by fractional precipitation of the total acetone precipitate from aqueous solution at 40% acetone in a yield of 79%. After gel filtration on Sephacryl S-500, no protein was detected. The polysaccharide had a carbohydrate content of 100% (w/w) and contained D-galactose, L-rhamnose, and D-glucose in the molar ratio 5:1:1, as shown by GLC of the trimethylsilylated methyl glycosides and (−)-2-butyl glycosides.

NMR spectroscopy.—The 500-MHz ^{1}H NMR spectrum at 70°C of the native polysaccharide (Fig. 1) contained seven H-1 signals at δ 5.628 (bs, residue **D**), 5.277 (d, $J_{1,2}$ 3.3 Hz, residue **A**), 5.085 (s, residue **G**), 4.779 (d, $J_{1,2}$ 7.8 Hz, residue **E**), 4.760 (d, $J_{1,2}$ 7.5 Hz, residue **B**), 4.748 (d, $J_{1,2}$ 7.6 Hz, residue **C**), and 4.664 (d, $J_{1,2}$ 7.7 Hz, residue **F**) in nearly equimolar ratios, indicative of a heptasaccharide repeating unit with a rhamnosyl residue and 2 α- and 4 β-D-hexosyl residues.

The ^{13}C NMR spectrum recorded at 70°C (not shown) contained signals for C-1 at δ 103.85 (2 C), 103.18, 102.82, 101.70, 95.87, and 95.08, respectively. The CH_3 signal of Rha was observed at δ 16.96 and the C-6 signals of six hexosyl residues at δ 61.05 (2 C), 60.88 (2 C), 60.78, and 60.65, respectively. The positions of the C-6 signals indicate the absence of 6-linked hexosyl residues and the occurrence of pyranose rings only (Me-α-D-Gal*p* δ 61.6, Me-β-D-Gal*p* δ 61.4, Me-α-D-Gal*f* δ 63.5, Me-β-D-Gal*f* δ 63.0, Me-α-D-Glc*p* δ 61.0, Me-β-D-Glc*p* δ 61.2, Me-α-D-Glc*f* δ 63.6, Me-β-D-Glc*f* δ 64.1[19]; values corrected for methanol at δ 49.0).

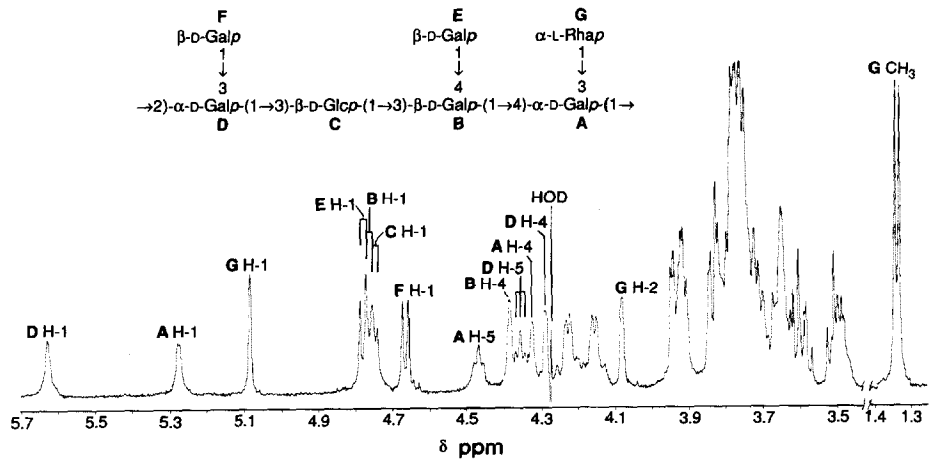


Fig. 1. Assignments in the 500-MHz ^{1}H NMR spectrum of the native polysaccharide (**PS**) recorded at 70°C.

TABLE I

Methylation analysis data of the native polysaccharide (**PS**), Smith-degraded PS (**PS-sd**), **1**, borodeuteride-reduced IIIA, and **5**

Derivative	Molar ratio				
	PS	**PS-sd**	**1**	IIIA[a]	**5**[a]
2,3,4-Rha[b,c]	0.7				
2,3,4,6-Gal[c]	0.9			0.3	
2,3,6-Gal		0.6		1.0	
2,4,6-Gal		0.8	1.0		1.0
3,4,6-Gal		0.6			
2,6-Gal	1.6				
4,6-Gal	1.1				
2,3,4,6-Glc[c]			0.6		0.6
2,4,6-Glc	1.0	1.0			

[a] Due to its high volatility, the 'reducing end' was not observed. [b] 2,3,4-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol-*1-d*, etc. [c] Because of the relatively high volatility of these residues, the values are lower than expected.

Methylation analysis.—GLC of the partially methylated alditol acetates, obtained from the methylated exopolysaccharide, revealed terminal Gal and Rha residues, a 3-linked Glc residue, and 3,4- and 2,3-linked Gal residues (Table I).

Smith degradation.—Periodate oxidation of the polysaccharide, followed by reduction and mild acid hydrolysis, gave a product that contained Gal and Glc in the molar ratio 3.0:1.0, indicating the degradation of one Rha and two Gal residues in the heptasaccharide repeating unit. Methylation analysis of the degraded polysaccharide (Table I) revealed an unbranched structure with a 3-linked Glc residue and 2-, 3-, and 4-linked Gal residues. The conversion of 3,4-linked Gal into 3- and 4-linked Gal, and of 2,3-linked Gal into 2-linked Gal, supported the occurrence of three branch points with one Rha and two Gal residues as side chains in the native material. The 500-MHz ^{1}H NMR spectrum of the Smith-degraded polysaccharide recorded at 70°C contained signals for H-1α at δ 5.43 and 5.11, and for H-1β at δ 4.62 and 4.55. Taking into account the ^{1}H NMR data for the native polysaccharide, it was concluded that both side-chain Gal residues were β.

After a second Smith-degradation (periodate oxidation and reduction were performed twice because of incomplete oxidation), the product was fractionated on Bio-Gel P-2. The major fraction was fractionated further on CarboPac PA-1 with program *5*. The main constituent (**1**) contained Glc, Gal, and threitol in the molar ratio 1.0:1.0:0.6, and FABMS indicated a molecular mass of 488. The 300-MHz ^{1}H NMR spectrum of **1** recorded at 27°C contained signals for H-1β at δ 4.678 (d, $J_{1,2}$ 7.7 Hz) and 4.624 (d, $J_{1,2}$ 7.7 Hz), together with a signal (t) at δ 5.050. A similar triplet was found in the ^{1}H NMR spectrum of the Smith-degradation product of the galactan of *Lactococcus lactis* subsp. *cremoris* H414, which was shown to be an oligosaccharide-(1 → 2)-threitol, in which the threitol unit was

O-2′-hydroxyethylidenated[20]. These derivatives can be formed during mild acid hydrolysis[21] of polyalcohols that contain periodate-oxidised and borohydride-reduced 4-linked residues. Methylation analysis of **1** (Table I) revealed a terminal Glc residue and a 3-linked Gal residue. The above data indicate the structure depicted for **1**.

β-D-Glc*p*-(1 → 3)-β-D-Gal*p*-(1 → 2)-[2′-hydroxyethylidene]threitol

C **B**

1

In view of the results for the native polysaccharide, the linear polymer obtained after one Smith-degradation cycle, and **1**, the structure for the backbone of the native polysaccharide is **2**.

↓ ↓ ↓
3 4 3
→ 2)-α-D-Gal*p*-(1 → 3)-β-D-Glc*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-α-D-Gal*p*-(1 →

D **C** **B** **A**

2

Partial acid hydrolysis.—In order to determine the positions of the terminal Rha and Gal residues, oligosaccharides were prepared by partial acid hydrolysis of the polysaccharide. Gel filtration of the hydrolysate on Bio-Gel P-2 gave the major fractions I–V (Fig. 2). Fractions IA and IB contained Gal and Rha, respectively (TLC). Fractions II–V were subfractionated on CarboPac PA-1 (Fig. 3) to give IIA, IIIA, IIIB, IVA, VA, VB, and VC.

Fraction IIA.—The ^{1}H NMR spectrum of fraction IIA at 27°C was identical to that of the disaccharide β-D-Gal*p*-(1 → 3)-D-Gal (**3**) isolated from the partial acid hydrolysate of the galactan[20] of *Lactococcus lactis* subsp. *cremoris* H414.

Fraction IIIA.—Monosaccharide analysis of $NaBD_4$-reduced IIIA revealed Gal and Gal-ol in the molar ratio 2.0 : 0.7. The 600-MHz ^{1}H NMR spectrum at 27°C suggested the occurrence of two trisaccharide-alditols in the molar ratio 5 : 1. For

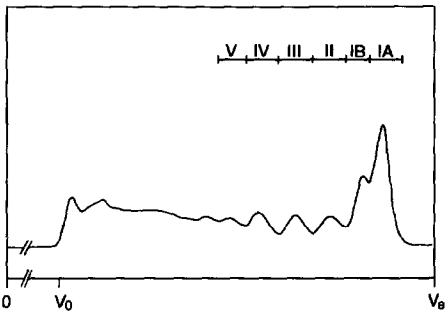


Fig. 2. Elution profile on Bio-Gel P-2 of the oligosaccharides obtained by partial acid hydrolysis of the native polysaccharide (**PS**).

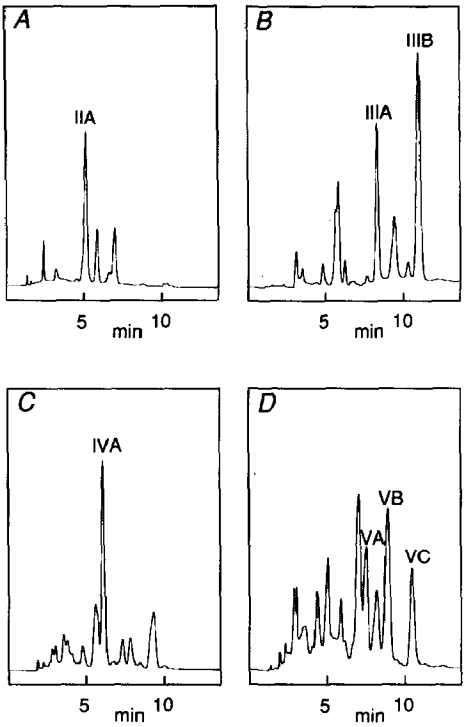


Fig. 3. HPAEC–PAD fractionation patterns on CarboPac PA-1 of Bio-Gel P-2 fractions II (*A*), III (*B*), IV (*C*), and V (*D*) by programs *1–4*, respectively.

the major component **4**, H-1β signals of equal intensity were observed at δ 4.597 (d, $J_{1,2}$ 7.8 Hz) and 4.526 (d, $J_{1,2}$ 7.9 Hz). The minor component is represented by H-1α and H-1β signals of equal intensity at δ 5.251 (d, $J_{1,2}$ 3.9 Hz) and 4.589 (d, $J_{1,2}$ 7.8 Hz), respectively. After methylation of the sample, GLC–EIMS of the major carbohydrate-containing peak indicated that methylated **4** had a molecular mass of 675 [*m/z* 585 (M^+ – CHDOMeCHOMe), 236 (cA_1)], and the sequence Hex–Hex–Hex-ol-*l-d* followed[22] from the fragment ions with *m/z* 219 [$aA_1 \rightarrow 187$ (aA_2) $\rightarrow 155$ (aA_3)], 423 [$baA_1 \rightarrow 391$ (baA_2)], 440 [$bcA_1 \rightarrow 408$ (bcA_2) $\rightarrow 376$ (bcA_3)], 236 [$cA_1 \rightarrow 204$ (cA_2) $\rightarrow 172$ (cA_3)], 296 (bcJ_1), and 500 ($abcJ_1$). The linkage between the internal Hex and Hex-ol-*l-d* was established to be (1 → 4), because of the presence of the fragment ions *m/z* 134 (CHDOMeCHOMeCHO-Me)$^+$ and 585, and the absence of the ion *m/z* 177/178 (containing four carbon atoms of the alditol chain with methoxyl groups)[22]. Linkage analysis of the methylated reduced sample revealed an internal 4-linked Hex residue (Table I), indicated by an intense peak with *m/z* 296 (bcJ_1) together with the predominance of bcA ions over baA ions in the EI-mass spectrum of methylated **4**, diagnostic for (1 → 4) and (1 → 6) linkages[22]. Hence, the structure **4** is assigned.

β-D-Gal*p*-(1 → 4)-β-D-Gal*p*-(1 → 4)-D-Gal-ol-*l-d* **4**

E **B** **A-ol**

Fraction IIIB.—Monosaccharide analysis of $NaBD_4$-reduced IIIB revealed Gal, Glc, and Gal-ol in the molar ratio 1.0:1.1:0.8. The 600-MHz ^1H NMR spectrum at 27°C contained H-1β signals of equal intensity at δ 4.677 (d, $J_{1,2}$ 7.9 Hz) and 4.557 (d, $J_{1,2}$ 7.9 Hz) indicative of a trisaccharide-alditol **5**. GLC–EIMS demonstrated that methylated **5** had a molecular mass of 675 [m/z 675 (M^+), 643 ($M^+ - MeOH$), 630 ($M^+ - CH_2OMe$), and 585 ($M^+ - CHDOMeCHOMe$)], and the sequence Hex–Hex–Hex-ol-*1-d* followed from the fragment ions with m/z 219 [$aA_1 \rightarrow 187$ (aA_2) $\rightarrow 155$ (aA_3)], 423 [$baA_1 \rightarrow 391$ (baA_2) $\rightarrow 359$ (baA_3)], 440 [$bcA_1 \rightarrow 408$ (bcA_2)], and 236 [$cA_1 \rightarrow 204$ (cA_2) $\rightarrow 172$ (cA_3)]. The linkage between the internal Hex and Hex-ol-*1-d* was established to be (1 → 4), because of the presence of the fragment ions m/z 134 $(CHDOMeCHOMeCHOMe)^+$ and 585, together with the absence[22] of the ion m/z 177/178. The linkage between Hex and Hex was established as (1 → 3), indicated by an intense peak at m/z 159 (composed probably of fragments of the internal Hex residue[22]), the predominance of the baA_1 ion over the bcA_1 ion[22], and further supported by the linkage analysis of methylated **5** (Table I) that revealed terminal Glc and 3-linked Gal and confirmed the structure **5**.

β-D-Glc*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Gal-ol-*1-d* **5**

C **B** **A**-ol

Fraction IVA.—Monosaccharide analysis of IVA revealed Gal and Glc in the molar ratio 3.0:1.1. After reduction with $NaBD_4$, Gal, Glc, and Gal-ol were present in the molar ratio 2.0:1.1:0.8. The 500-MHz ^1H NMR spectrum of $NaBD_4$-reduced IVA at 12°C contained H-1 signals of equal intensity at δ 4.797 (d, $J_{1,2}$ 7.9 Hz, residue **E**), 4.682 (d, $J_{1,2}$ 7.9 Hz, residue **C**), and 4.574 (d, $J_{1,2}$ 7.9 Hz, residue **B**), indicating a tetrasaccharide-alditol **6** with three β-Hex residues, in accordance with the data on monosaccharide analysis. The assignments of the non-anomeric protons of these residues (Table II) are based on cross-peaks observed in the HOHAHA and ROESY spectra. The signals at δ 4.104 (d) and 4.011 (t), assigned to H-2 and H-5 of the Gal-ol-*1-d* residue (**A**-ol), respectively, were used as starting points for the identification of the other resonances of the **A**-ol residue via cross-peaks observed in the HOHAHA and ROESY spectra (Table II). The sequence **C** → [**E** →]**B** → **A**-ol and the linkage positions followed from inter-residue NOEs observed in the ROESY spectrum. On the basis of the connectivities **C** H-1,**B** H-3 (strong) and H-2 (weak), and **E** H-1,**B** H-4, the linkages **C**-(1 → 3)-**B** [**C**-(1 → 2)-**B** can be excluded on the basis of the structure **1**] and **E**-(1 → 4)-**B** could be established. Taking into account the structure of **1**, the observed inter-residue NOEs **B** H-1,**A**-ol H-2,3,4 have to be correlated with a

TABLE II

^{1}H NMR chemical shifts[a] of oligosaccharide-alditols obtained by partial acid hydrolysis–reduction ($NaBD_4$), and of the native polysaccharide (PS)

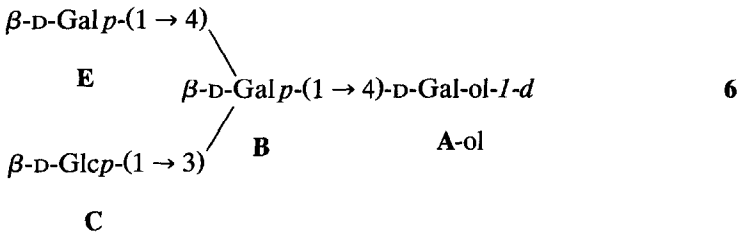
Residue	Proton	Oligosaccharide-alditol[b]				PS[c]
		6	7	8	9	
A	H-1		5.265	5.241		5.277*
	H-2		3.934	3.950		4.17
	H-3		3.98	3.986		4.14
	H-4		4.257	4.269		4.323*
	H-5		4.11	4.128		4.468*
	H-6a[d]		3.76	3.77		3.74
	H-6b[d]		3.82	3.83		3.78
B	H-1	4.574	4.697	4.655	4.558	4.760*
	H-2	3.814	3.852	3.73	3.684	3.82
	H-3	3.935	3.94	3.85	3.840	3.92
	H-4	4.418	4.414	4.188	4.188	4.383*
	H-5	3.74	3.73	3.70	3.71	
C	H-1	4.682	4.679	4.678	4.710	4.748*
	H-2	3.386	3.393	3.376	3.475	3.499
	H-3	3.505	3.504	3.506	3.68	3.73
	H-4	3.394	3.393	3.423	3.66	3.634
	H-5	3.451	3.450	3.44	3.45	3.48
	H-6a	3.920	3.92	3.89	3.87	3.91
	H-6b	3.73	3.73	3.74	3.74	3.73
D	H-1				5.428	5.628*
	H-2				3.995	4.21
	H-3				4.065	4.24
	H-4				4.305	4.288*
	H-5				4.349	4.354*
	H-6a				3.71	3.75
	H-6b				3.71	3.75
E	H-1	4.797	4.800			4.779*
	H-2	3.564	3.557			3.58
	H-3	3.66	3.66			3.65
	H-4	3.89	3.885			3.92
F	H-1			4.534	4.605	4.664*
	H-2			3.566	3.608	3.59
	H-3			3.679	3.665	3.65
	H-4			3.891	3.91	3.947
	H-5			3.73	3.69	
G	H-1					5.085*
	H-2					4.083*
	H-3					3.83
	H-4					3.509
	H-5					3.77
	CH_3					1.338*

TABLE II (continued)

Residue	Proton	Oligosaccharide-alditol[b]				PS[c]
		6	7	8	9	
A-ol	H-1	3.674			3.67	
	H-2	4.104			4.086	
	H-3	3.87			3.87	
	H-4	3.91			3.93	
	H-5	4.011			4.00	
	H-6a[d]	3.71			3.68	
	H-6b[d]	3.80			3.83	
D-ol	H-1a[e]		3.87	3.97		
	H-1b[e]		3.80	3.91		
	H-2		4.107	4.188		
	H-3		3.82	4.097		
	H-4		3.72	3.83		
	H-5		3.986	4.27		
	H-6a		3.68	3.70		
	H-6b		3.68	3.70		

[a] In ppm relative to the signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (using internal acetone at δ 2.225) in D_2O. [b] Chemical shifts at 12°C (**6**) and 7°C (**7–9**). [c] Chemical shifts at 70°C (marked with an asterisk) or 67°C. [d] Chemical shifts for H-6a and H-6b may be interchanged. [e] Chemical shifts for H-1a and H-1b may be interchanged.

(1 → 4) linkage between **B** and **A**-ol (see also **4** and **5**), and the structure **6** is assigned.



Fraction VA.—Monosaccharide analysis of VA revealed Gal and Glc in the molar ratio 4.0:1.1. After reduction with $NaBD_4$, Gal, Glc, and Gal-ol were present in the molar ratio 3.0:1.1:0.6. The 500-MHz ^1H NMR spectrum of $NaBD_4$-reduced VA at 7°C contained H-1 signals of equal intensity at δ 5.265 (d, $J_{1,2}$ 3.6 Hz, residue **A**), 4.800 (d, $J_{1,2}$ 7.9 Hz, residue **E**), 4.697 (d, $J_{1,2}$ 8.4 Hz, residue **B**), and 4.679 (d, $J_{1,2}$ 8.8 Hz, residue **C**), indicating a pentasaccharide-alditol **7** with one α- and three β-Hex residues, in accordance with the data on monosaccharide analysis. Assignments of the non-anomeric protons of these residues (Table II) were made on the basis of cross-peaks observed in the HOHAHA and ROESY spectra. The signals at δ 4.107 (d) and 3.986 (t) assigned to H-2 and H-5 of the Gal-ol-*1-d* residue (**D**-ol), respectively, were used as starting points for the determination of the other protons of **D**-ol via cross-peaks observed on the H-2 and H-5 tracks in the HOHAHA and ROESY spectra (Table II). On

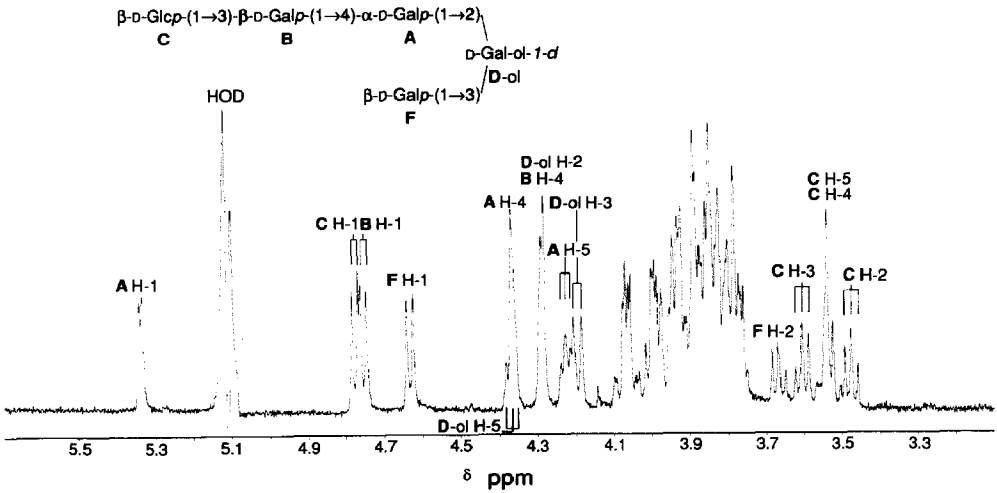
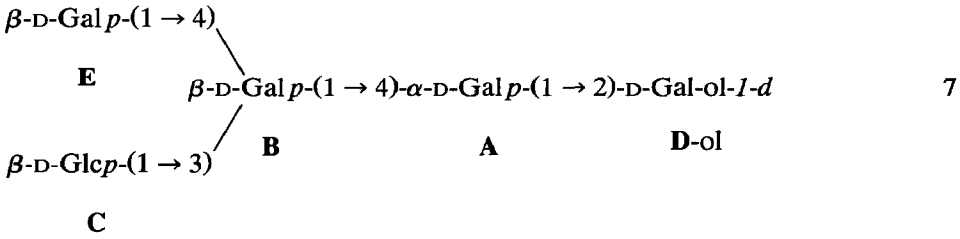


Fig. 4. Assignments in the 500-MHz ^{1}H NMR spectrum of oligosaccharide-alditol **8** recorded at 7°C.

the H-2 track in the HOHAHA spectrum, two cross-peaks were observed at δ 3.87 and 3.80, due to H-1. The occurrence of H-1a and H-1b can be ascribed to the different orientations of H and D in the two Gal-ol-*1-d* residues obtained after $NaBD_4$ reduction. In general, both H-1a and H-1b of Gal-ol-*1-d* resonate at the same position, but, due to the 2-substitution of the **D**-ol residue (as shown below), their resonances have different chemical shifts. The ROESY spectrum showed inter-residue NOEs for **C** H-1,**B** H-3, **E** H-1,**B** H-4, **B** H-1,**A** H-4, and **A** H-1,**D**-ol H-2 and H-1a which indicated the sequence **C** → [**E** →]**B** → **A** → **D**-ol and the linkage positions. Hence, the structure **7** was assigned.



Fraction VB.—Monosaccharide analysis of $NaBD_4$-reduced VB revealed Gal, Glc, and Gal-ol in the molar ratio 3.0 : 1.2 : 0.9. The 500-MHz ^{1}H NMR spectrum of reduced VB at 7°C (Fig. 4) contained H-1 signals of equal intensity at δ 5.241 (d, $J_{1,2}$ 3.5 Hz, residue **A**), 4.678 (d, $J_{1,2}$ 7.9 Hz, residue **C**), 4.655 (d, $J_{1,2}$ 7.9 Hz, residue **B**), and 4.534 (d, $J_{1,2}$ 7.9 Hz, residue **F**), indicating a pentasaccharide-alditol **8** with one α- and three β-Hex residues. On the various H-1 tracks of the Gal residues in the HOHAHA spectrum (Fig. 5), cross-peaks were detected with H-2,3,4, and on the H-1 track of the Glc residue **C**, cross-peaks with H-2,3,4,5 were observed, whereas H-6a,6b of residue **C** were found via the H-5 track. Assignments for H-5 of residues **B** and **F** were made on the basis of cross-peaks observed on the

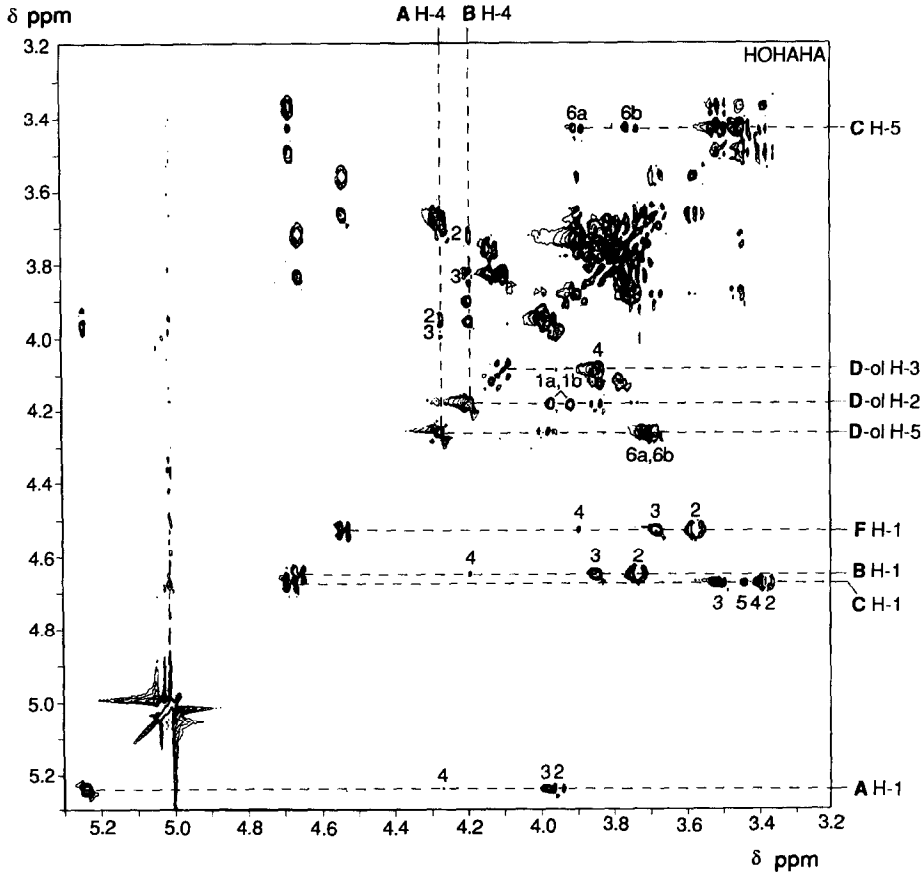


Fig. 5. Assignments in the 500-MHz 2D HOHAHA spectrum of oligosaccharide-alditol **8** recorded at 7°C with a mixing time of 35 ms.

respective H-1 tracks in the ROESY spectrum (Fig. 6), and H-5 and H-6a,6b of residue **A** were identified via cross-peaks observed on the H-4 and H-5 track, respectively. The signal at δ 4.097 (d, $J_{3,4}$ 9.9 Hz) was identified as due to H-3 of the Gal-ol-*1-d* residue (**D**-ol), and this proton showed cross-peaks with H-4 in the HOHAHA spectrum and with H-2 in the ROESY spectrum. On the **D**-ol H-4 track in the ROESY spectrum, a cross-peak with H-5 was found, which, in turn, showed cross-peaks with H-6a,6b in each spectrum. For **D**-ol H-2, cross-peaks with H-1a and H-1b were observed in the HOHAHA spectrum (cf. fraction VA). The chemical shifts are listed in Table II. The inter-residue connectivities **C** H-1,**B** H-3 (strong) and H-4 (weak), **B** H-1,**A** H-4, **A** H-1,**D**-ol H-2, and **F** H-1,**D**-ol H-3 (strong) and H-1a,1b (both weak), observed on the H-1 tracks in the ROESY spectrum, established the sequence as **C** → **B** → **A** → [**F** →]**D**-ol and the linkages between the residues **C** and **B** and between **F** and **D**-ol as (1 → 3) [**C**-(1 → 4)-**B** can

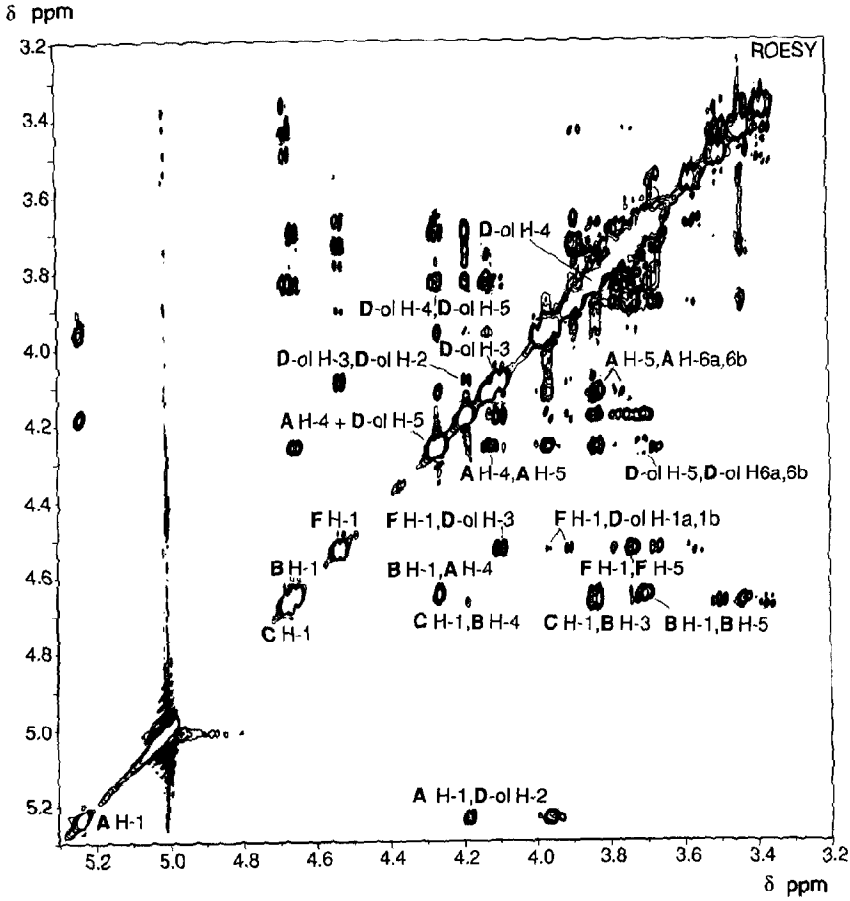
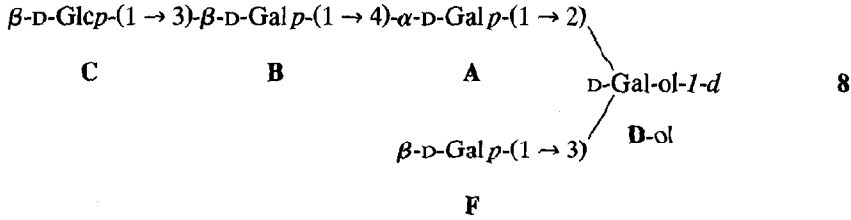


Fig. 6. 500-MHz 2D ROESY spectrum of oligosaccharide-alditol **8** recorded at 7°C with a mixing time of 250 ms: **A** H-1,**D**-ol H-2 means the cross-peak between H-1 of residue **A** and H-2 of residue **D**-ol, etc.

be excluded on the basis of structure **1**], between **B** and **A** as (1 → 4), and between **A** and **D**-ol as (1 → 2). The structure **8** is assigned.



Fraction VC.—Monosaccharide analysis of VC revealed Gal and Glc in the molar ratio 4.0 : 1.2. After reduction with $NaBD_4$, Gal, Glc, and Gal-ol were found in the molar ratio 3.0 : 1.2 : 0.9. The 500-MHz ^1H NMR spectrum of reduced VC at

7°C contained H-1 signals of equal intensity at δ 5.428 (d, $J_{1,2}$ 3.9 Hz, residue **D**), 4.710 (d, $J_{1,2}$ 8.2 Hz, residue **C**), 4.605 (d, $J_{1,2}$ 7.7 Hz, residue **F**), and 4.558 (d, $J_{1,2}$ 7.9 Hz, residue **B**), pointing to a pentasaccharide-alditol **9** with one α- and three β-Hex residues. The H-2,3,4,5 signals of residues **B** and **F**, and the H-2,3,4,5,6a,6b signals of residues **C** and **D**, were assigned from cross-peaks observed in the HOHAHA and ROESY spectra (Table II). The signals at δ 4.086 (d) and 4.00 (t), assigned to H-2 and H-5 of the Gal-ol-*1-d* residue (**A**-ol), respectively, were used as starting points for the determination of the other protons of the **A**-ol residue via cross-peaks observed on the H-2 and H-5 track in the HOHAHA and ROESY spectra (Table II). The sequence **F** → **D** → **C** → **B** → **A**-ol and the linkage positions followed from the inter-residue NOEs observed in the ROESY spectrum. On the basis of the connectivities **F** H-1,**D** H-3, **D** H-1,**C** H-3, and **C** H-1,**B** H-3 (strong) and H-4 (very weak), (1 → 3) linkages were established [**C**-(1 → 4)-**B** can be excluded on the basis of structure **1**]. The **B** H-1,**A**-ol H-4 (strong) and H-2,3 (both weak) connectivities indicated a **B**-(1 → 4)-**A**-ol linkage (see structures **4** and **5**). The structure **9** was assigned.

β-D-Gal*p*-(1 → 3)-α-D-Gal*p*-(1 → 3)-β-D-Glc*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Gal-ol-*1-d*

F **D** **C** **B** **A**-ol

9

Combination of the structures of oligosaccharide-alditols **6–9** with the backbone structure **2** yielded the positions of the two terminal Gal residues **E** and **F** in the native polysaccharide. Residue **E** is β-(1 → 4)-linked to residue **B**, whereas residue **F** is β-(1 → 3)-linked to residue **D**. Consequently, the terminal Rha residue **G** must be (1 → 3)-linked to residue **A**, but, because Rha-containing oligosaccharides were not found in the partial hydrolysate, no direct proof is available.

^{1}H NMR spectroscopy of the native polysaccharide.—2D HOHAHA and 2D NOESY experiments at 67°C were carried out on the native polysaccharide. The spin system of the Rha residue **G** was traced in the HOHAHA spectrum (Fig. 7), starting at the H-1 track at δ 5.085, giving H-2, then going via the H-2 track at δ 4.083, affording H-3,4, to the CH_3 track at δ 1.338, yielding H-3,4,5 (Table II). The α configuration of the Rha residue was deduced from the position of the H-5 signal at δ 3.77 (cf. H-5 of α-L-Rha*p*-OMe and β-L-Rha*p*-OMe at δ 3.67 and 3.39, respectively[19]), supported by the observation of only one intra-residue NOE, namely, between **G** H-1 and **G** H-2 (Fig. 8).

The assignments of the non-anomeric protons of the Hex residues **A–F** (Table II) were made on the basis of cross-peaks observed in the HOHAHA and NOESY spectra. On the H-1 tracks of the Gal residues **A**, **B**, **D**, and **E** in the HOHAHA spectrum (Fig. 7), cross-peaks were detected with H-2,3, and on the H-1 track of residue **F** with H-2,3,4. Assignments for H-4 of residues **B** and **E** were made on the basis of cross-peaks observed on the H-3 and H-2 tracks, respectively. The signals at δ 4.323 and 4.288 (Fig. 1) were assigned to H-4 of residue **A** and **D**, respectively, based on the cross-peaks with H-2,3 in the HOHAHA spectrum. Assignments for

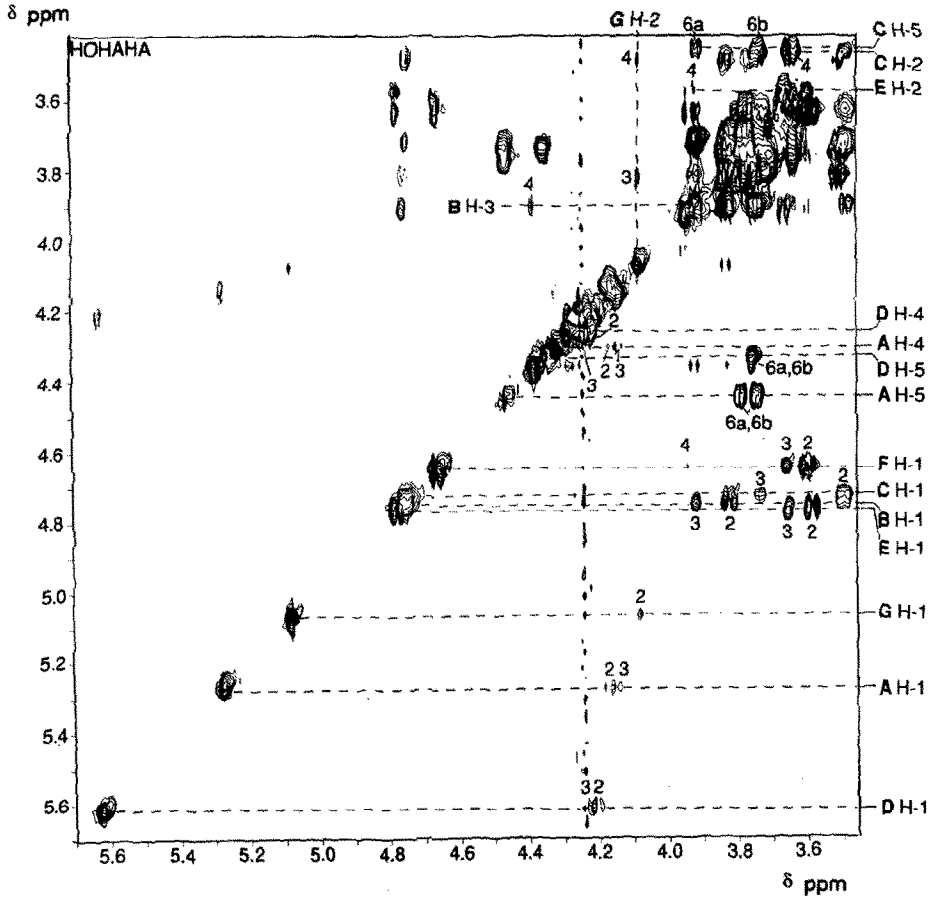


Fig. 7. The assignments in the region δ 5.7–3.45 of the 600-MHz 2D HOHAHA spectrum of the native polysaccharide (PS) recorded at 67°C with a mixing time of 25 ms.

H-5 of residues **A** and **D** were based on cross-peaks detected on the H-4 tracks in the NOESY spectrum, whereas H-6a,6b were identified from the cross-peaks on the H-5 tracks in the HOHAHA spectrum. On the H-1 track of the Glc residue **C**, cross-peaks with H-2,3 in the HOHAHA spectrum and with H-5 in the NOESY spectrum were observed. Assignments for H-4 and H-6a,6b were made on the basis of cross-peaks found on the H-2 and H-5 track in the HOHAHA spectrum, respectively.

The inter-residue connectivities **A** H-1,**D** H-1, **F** H-1,**D** H-3, **D** H-1,**C** H-3, **C** H-1,**B** H-3, **E** H-1,**B** H-4, **B** H-1,**A** H-4, and **G** H-1,**A** H-3 together with the data on methylation analysis (Table I) established the sequence and the type of linkages for the native polysaccharide, in agreement with the data from the Smith-degradation

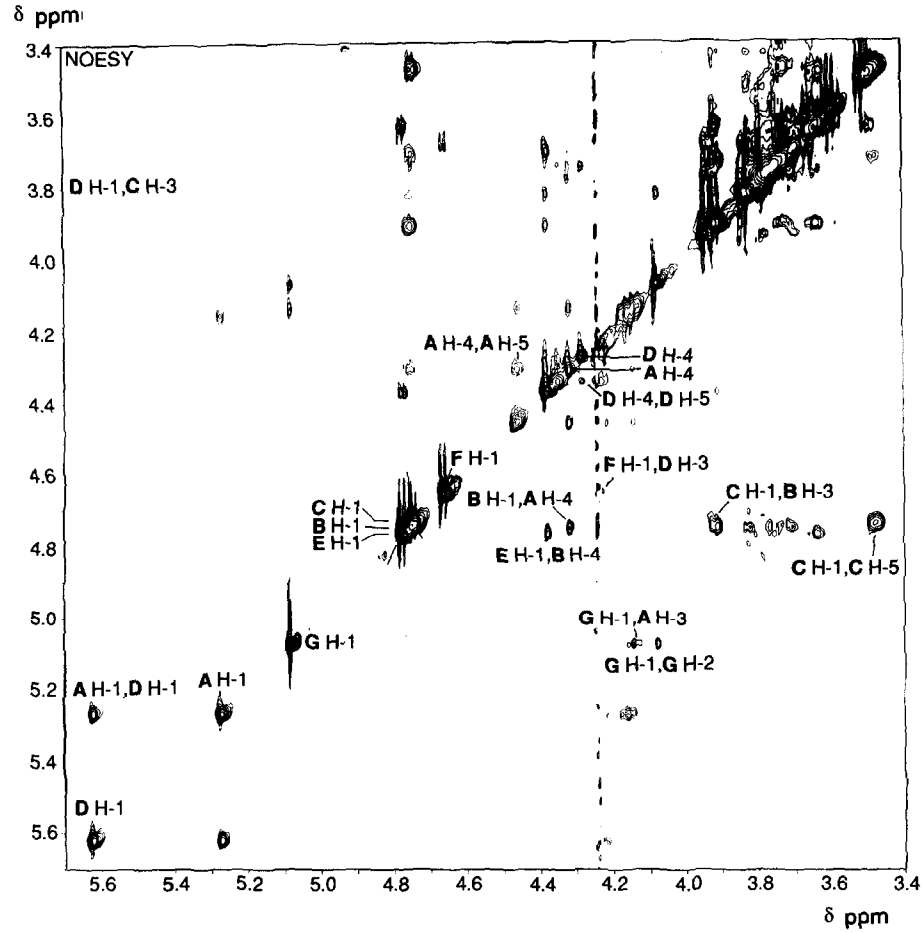
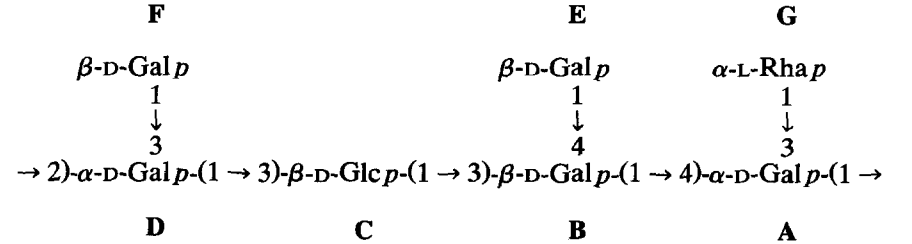


Fig. 8. The region δ 5.7–3.4 of the 600-MHz 2D NOESY spectrum of the native polysaccharide recorded at 67°C with a mixing time of 200 ms: A H-1,D H-1 means the cross-peak between H-1 of residue A and H-1 of residue D, etc.

study (backbone **2**) and the partial hydrolysis study (oligosaccharides **3–9**), to be **10**.



10

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