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Structural characterisation and enzymic modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B39

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Abstract

Lactococcus lactis subsp. *cremoris* B39 grown on whey permeate produced an exopolysaccharide containing L-Rha, D-Gal and D-Glc in a molar ratio of 2:3:2. The polysaccharide was modified using an enzyme preparation from *Aspergillus aculeatus*, resulting in the release of Gal and a polymer with approximately the same hydrodynamic volume as the native polysaccharide. Linkage analysis and ^{1}H NMR studies of both the native and modified exopolysaccharides elucidated that terminally linked Gal was released during modification and that the chemical structure of the branches within the repeating units is: β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→. 2D NMR experiments (both ^{1}H–^{1}H and ^{1}H–^{13}C) revealed that exopolysaccharide B39 consists of a branched heptasaccharide repeating unit with the following structure:

Keywords: Exopolysaccharide; *Lactococcus lactis* subsp. *cremoris*; Structural analysis; Enzymic modification

1. Introduction

Polysaccharides are often incorporated into foods essentially to alter the balance between free and bound water and to change the rheological properties, the 'mouth feel' and the texture of the product [1]. Besides polysaccharides from higher plants and seaweeds, bacterial exopolysaccharides (EPSs) are used in foods [2].

Since lactic acid bacteria have the GRAS (generally recognised as safe) status, an increasing interest has developed in the polysaccharides produced by these bacteria [3]. To obtain insight into the structure–function relationship of EPSs, the chemical structures of several EPSs produced by lactic acid bacteria

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like lactobacilli [4–12], lactococci [13,14] and streptococci [15–18] have been reported.

The effect of structural changes on the physical properties of EPSs can be determined by examination of related polysaccharide structures [19]. One way to prepare microbial polysaccharides with altered structures is through the use of enzymes [19]. Moreover, enzymes are not only useful for studying the structure–function relationship of EPSs, but they have also proven to be very helpful in characterising the chemical structures of these polysaccharides [5,6,20–23].

Here we report the structure determination of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B39 in whey permeate. An enzyme preparation from *Aspergillus aculeatus* was used for the unravelling of the chemical structure and will be used in future research to study the physical properties of related EPS structures.

2. Experimental

Production, isolation and purification of EPS.—The production and isolation of EPS from *L. lactis* subsp. *cremoris* B39 was performed as described for EPS B40 [24] and the crude EPS was kindly supplied by NIZO food research (Ede, The Netherlands). The crude material was purified with CCl_3CO_2H and EtOH [25], followed by separation on a size-exclusion column (100 × 10 cm i.d.) of Sephacryl S500 (Amersham Pharmacia Biotech, Uppsala, Sweden) irrigated with 50 mM NaOAc, pH 5.0. The refractive index and A_{280} were measured on-line (Biopilot System; Amersham Pharmacia Biotech, Uppsala, Sweden). Fractions were assayed for their total neutral sugar content [26], pooled accordingly, dialysed and freeze dried.

Enzyme preparation.—The commercial enzyme preparation Pectinex Ultra SP-L produced by *A. aculeatus* (Novo Nordisk Ferment Ltd., Dittingen, Switzerland) was used for modifying EPS B39.

Enzymic modification of the EPS.—In the search for enzymes that are able to modify EPS B39, partially purified (using only CCl_3CO_2H and EtOH) EPS B39 (0.5 mL, 2 mg/mL in 50 mM NaOAc pH 5.0 + 0.01% (w/v) NaN_3) was incubated (24 h, 30 °C) with 50 μL Ultra SP, which was previously dialysed against the same buffer. After incubation, the enzymes were inactivated (15 min, 100 °C) and the precipitate formed was removed by centrifugation. The supernatant was analysed by high-performance size-exclusion chromatography (HPSEC) and high-performance anion-exchange chromotography (HPAEC). To obtain sufficient amounts of modified EPS B39 for sugar (linkage) analyses and NMR analyses, purified EPS B39 (30 mg) was dissolved in 50 mM NaOAc buffer pH 5.0 (15 mL) containing 0.01 (w/v) NaN_3. Ultra SP (1 mL) was dialysed against the same buffer and diluted to 10 mL. The EPS solution was incubated (24 h, 30 °C) with enzyme solution (1.5 mL) and subsequently heated (30 min, 100 °C) to inactivate the enzymes. After centrifugation, part of the supernatant was analysed by HPAEC. The remaining part was dialysed, centrifuged again and freeze dried.

Sugar composition.—Samples were pretreated in 12 M H_2SO_4 (1 h at 30 °C) and hydrolysed in 1 M H_2SO_4 (3 h at 100 °C), using inositol as internal standard. The released sugars were converted into their alditol acetates [27] and analysed by GC–FID [25].

Table 1
Sugar linkage composition of native EPS B39 (**1**) and enzymically modified EPS B39 (**2**)

Derivative	Linkage type	Molar ratio [a]	
		1	**2**
3,4-Rha [b]	1,2-Rha	1.0	1.0
2-Rha	1,3,4-Rha	0.7	0.7
2,3,4,6-Gal	t-Gal	1.0	0.4
3,4,6-Gal	1,2-Gal	1.0 [c]	1.0 [c]
2,4,6-Gal	1,3-Gal	0.8	0.8
2,3,4,6-Glc	t-Glc		0.9
2,4,6-Glc	1,3-Glc	0.6	0.6
2,3,6-Glc	1,4-Glc	1.0 [c]	0.4[c]

[a] 3,4-Rha was taken as 1.0.

[b] 1,2,5-Tri-*O*-acetyl-3,4-di-*O*-methyl-rhamnitol, etc.

[c] (1→2)-Linked Gal and (1→4)-linked Glc were not separated on GC and a mixed mass spectrum was found on GC–MS. Their total molar ratio was 2.0 for **1** and 1.4 for **2**. Based on the sugar (linkage) composition of **1**, it was supposed that in this sample both components were present in equal amounts. For **2**, the decrease in the signal was totally ascribed to (1→4)-linked Glc because of the presence of terminally linked Glc (t-Glc).

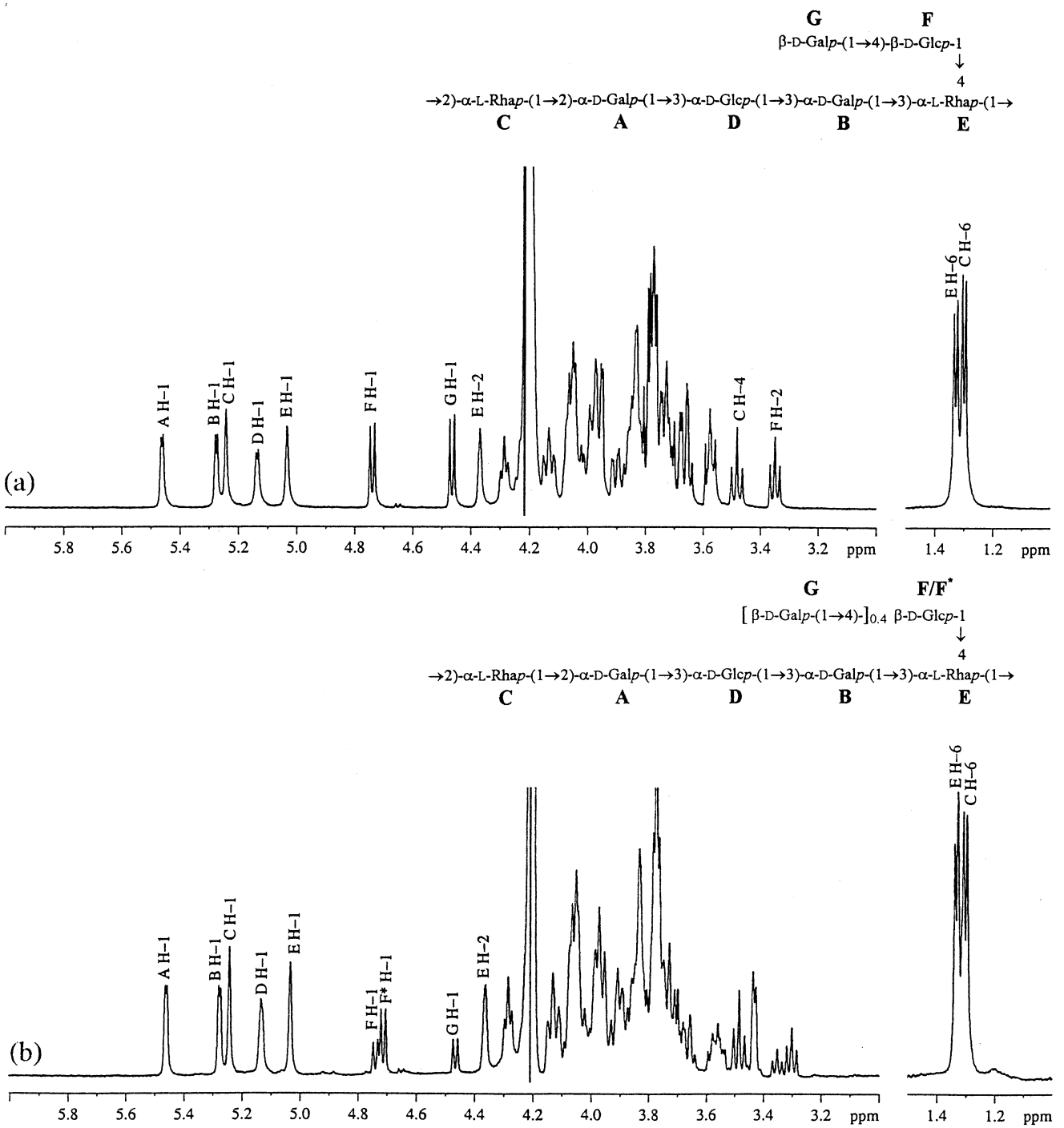

Fig. 1. 500-MHz ^{1}H NMR spectra of EPS B39 (**1**) (a) and modified EPS B39 (**2**) (b), recorded in D_2O at 80 °C. In modified EPS B39, the code **F** refers to (1→4)-linked β-D-Glc*p*, whereas **F*** refers to terminally linked β-D-Glc*p*. The approximate relative amount of remaining terminally linked β-D-Gal*p* residues in **2** is indicated by $[\ ]_{0.4}$ (100% = 1.0).

Sugar linkage composition.—Samples were methylated according to Hakomori [28] and subsequently dialysed against water and freeze dried. The methylated samples were hydrolysed in 2 M CF_3CO_2H (1 h at 121 °C). After evaporation (stream of air, < 20 °C), the partially methylated sugars were converted into alditol acetates [27] and analysed by GC–FID as described by Vincken et al. [29]. Partially methylated alditol acetates were quantified according to their effective carbon response [30]. Identification of the compounds was confirmed by GC–MS [25].

Absolute configurations of monosaccharides.—The absolute configurations of the monosaccharides were determined as described by Gerwig et al. [31]. The trimethylsilated (−)-2-butyl glycosides were analysed using GC–FID [20].

HPAEC analysis of the digest.—The release of monomers and/or oligomers caused by enzymic hydrolysis of EPS B39 was verified by HPAEC, which was performed as described earlier [20].

Hydrodynamic volume.—HPSEC was performed as described earlier [20,25].

NMR spectroscopy.—Prior to NMR analysis, the samples were exchanged thrice in D_2O (99.9 at% D, Cambridge Isotope Laboratories, USA) with intermediate freeze drying. Finally, samples were dissolved in 99.96% D_2O (Cambridge Isotope Laboratories, USA). NMR spectra were recorded at probe temperatures of 80 °C on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. Incidentally, 1D ^{1}H, 2D COSY and TOCSY spectra of EPS B39 were recorded at 70 °C in order to shift the HOD signal. Chemical shifts are expressed in ppm relative to internal acetone; δ 2.225 for ^{1}H and δ 31.55 for ^{13}C.

In the 1D ^{1}H NMR spectra suppression of the HOD signal was achieved by using presaturation during relaxation delay for 1 s. Proton-decoupled ^{13}C spectra were recorded at 125.77 MHz. For 1D ^{1}H and ^{13}C NMR spectra data sets of 16,384 and 32,768 data points were recorded.

The 2D COSY spectra (70 and 80 °C) were acquired in the magnitude mode. In addition, a 2D DQF-COSY spectrum at 80 °C was recorded according to Derome and Williamson [32]. 2D TOCSY and ROESY spectra were basically acquired as described by Fransen et al. [33] using the time-proportional phase increment (TPPI) method [34]. The 2D NOESY experiment was performed with a mixing time of 250 ms. For ^{1}H–^{13}C 2D-heteronuclear proton-detected multiple bond correlation spectroscopy (HMBC) spectra [35] a standard gradient-enhanced ^{1}H–^{13}C 2D-heteronuclear proton detected multiple-quantum coherence (HMQC) pulse-sequence delivered by Bruker was changed into a HMBC sequence by setting the delay between the first proton and first carbon pulse to 40, 60, 80 and 140 ms, respectively. For 2D HMQC spectra, this delay was 3.0 ms. For all homonuclear 2D experiments, 512 experiments of 2048 data points were acquired with 80–120 scans per increment; all heteronuclear 2D spectra were acquired in 1024 experiments of 2048 data points, except for the decoupled HMQC, which was acquired in 1024 experiments of 1024 data points.

Time-domain data were multiplied by phase-shifted (squared-)sine-bell functions or with Lorentzian-to-Gaussian multiplication. After zero filling and Fourier transformation, data sets of 2048 × 1024 (homonuclear experiments) or 2048 × 2048 points (heteronuclear experiments) were obtained, which were baseline corrected when necessary.

3. Results

Isolation, purification and chemical characterisation of EPS B39.—Crude EPS produced by *L. lactis* subsp. *cremoris* B39 was purified by CCl_3CO_2H extraction and EtOH precipitation, followed by size-exclusion chromatogra-

Table 2
^{1}H NMR chemical shifts [a] of EPS B39 (**1**), recorded in D_2O at 80 °C

Residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	CH_3
A (1,2-α-D-Gal*p*)	5.462	3.96	4.07	4.05	4.286	3.76	3.76	
B (1,3-α-D-Gal*p*)	5.276	4.05	4.12	4.19	4.23	3.77	3.77	
C (1,2-α-L-Rha*p*)	5.243	4.07	3.90	3.482	3.83			1.302
D (1,3-α-D-Glc*p*)	5.136	3.73	3.99	3.73	4.03	3.83	3.83	
E (1,3,4-α-L-Rha*p*)	5.033	4.369	4.13	3.96	3.86			1.331
F (1,4-β-D-Glc*p*)	4.739	3.351	3.69	3.65	3.56	3.98	3.84	
G (t-β-D-Gal*p*)	4.464	3.57	3.66	3.95	3.73	3.79	3.79	

[a] In ppm relative to the signal of acetone at δ 2.225.

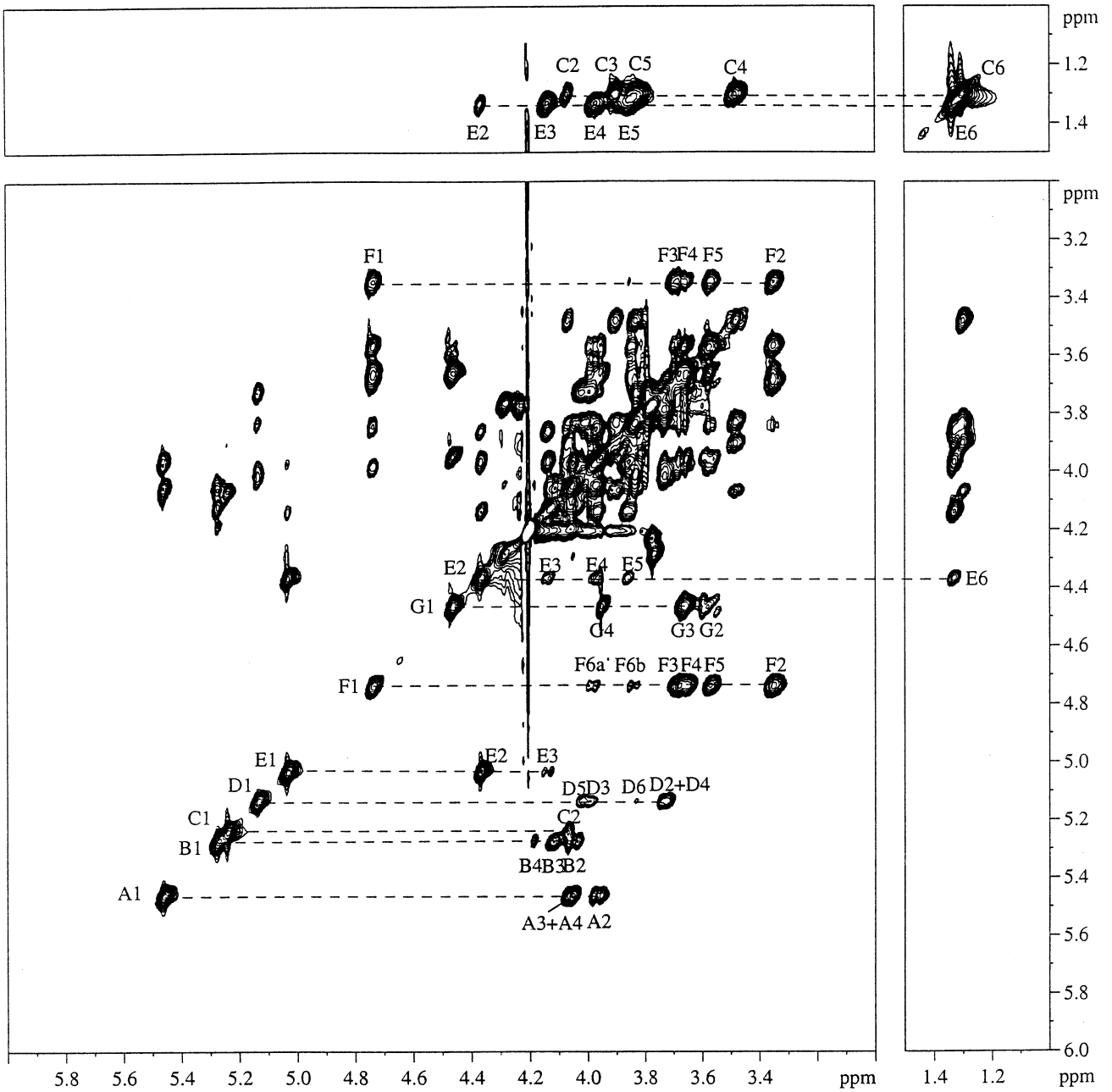

Fig. 2. 500-MHz 2D TOCSY spectrum (mixing time = 140 ms) of EPS B39 (**1**) recorded in D_2O at 80 °C. Diagonal peaks of the anomeric protons of H-2, of residues **E** and **F**, and of H-6 of residues **C** and **E** are indicated. Cross-peaks belonging to the same scalar-coupling network are indicated near a dotted line starting from the corresponding diagonal peak.

phy on Sephacryl S-500. The purification step using CCl_3CO_2H and ethanol removed most of the proteins present in the crude EPS, while size-exclusion chromatography successfully removed a mannan population (probably originating from the medium) and remnants of protein. No A_{280} absorbing material co-eluted with EPS B39, indicating that the EPS was (almost) free of protein.

Sugar composition analysis of native EPS B39 (**1**) and determination of absolute configurations revealed the presence of L-Rha, D-Gal and D-Glc in a molar ratio of 2.0:3.0:2.0. Sugar linkage analysis of **1** showed the presence of 2-substituted rhamnose, 3,4-disubstituted rhamnose, terminally linked galactose, 2-substituted galactose, 3-substituted galactose, 3-substituted glucose and 4-substituted glucose (Table 1). According to

these results, the results of enzymic modification of EPS B39 (vide infra) and NMR experiments (vide infra), all hexose residues are in the pyranose ring form.

Modification of EPS B39.—After incubation of EPS B39 with Ultra SP, analysis by HPAEC showed that monomeric galactose had been released, while no oligomers were found. The hydrodynamic volume of the modified EPS had only been decreased very slightly compared with the native EPS (HPSEC). Sugar composition analysis of modified EPS B39 (**2**) resulted in a molar ratio of Rha, Gal and Glc of 2.0:2.4:2.1. The lower amount of galactose in **2** compared with **1** is in accordance with the release of galactose as analysed by HPAEC. Sugar linkage analysis (Table 1) showed a decrease in terminally linked galactose and (1→4)-linked glucose, while a new type of sugar linkage appeared,

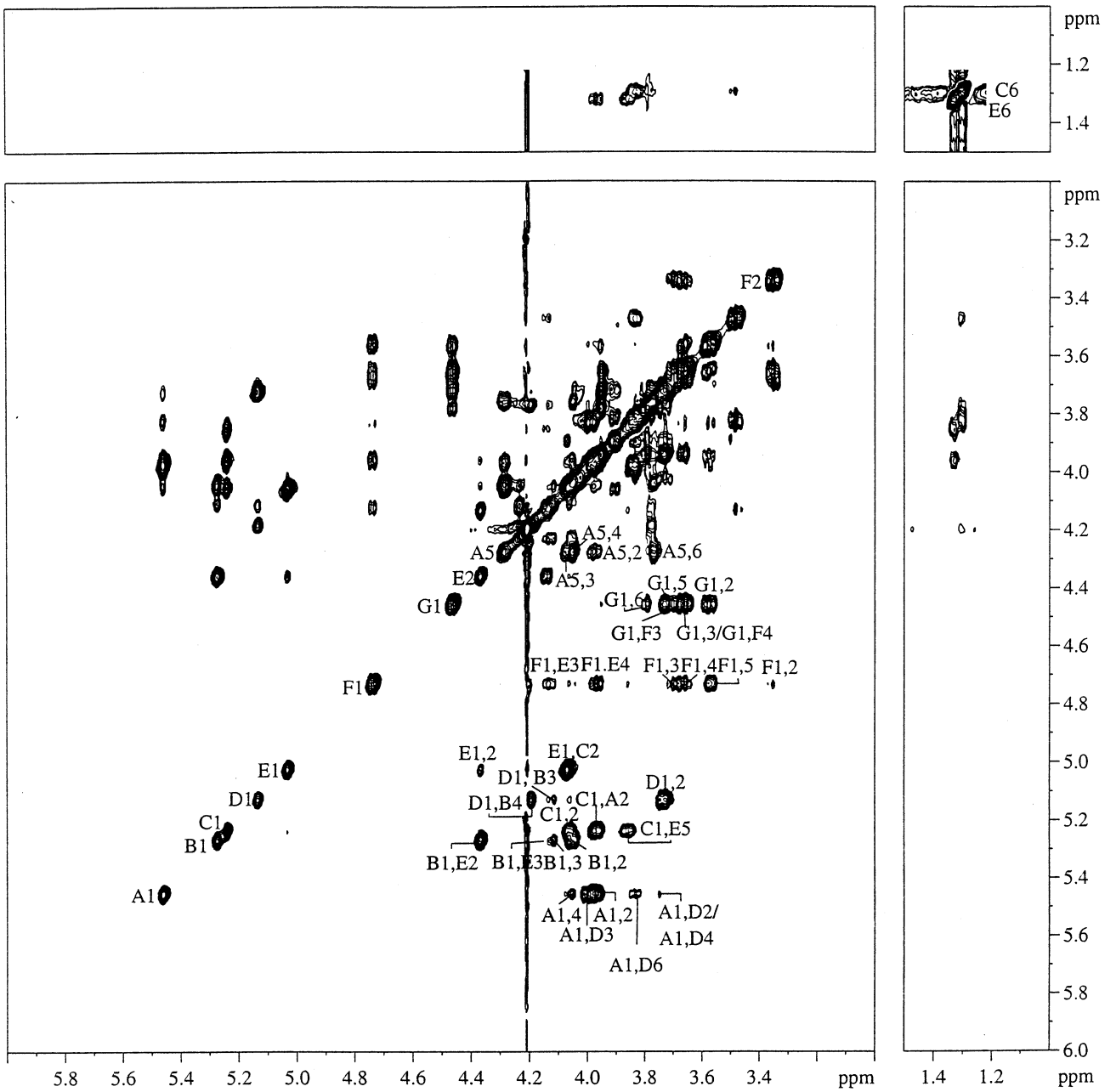

Fig. 3. 500-MHz 2D ROESY spectrum (mixing time = 250 ms) of EPS B39 (**1**) recorded in D_2O at 80 °C. The code A1 corresponds to the diagonal peak belonging to **A** H-1; A1,2 refers to an intra-residual cross-peak between **A** H-1 and **A** H-2; A1,D3 refers to an inter-residual connectivity between **A** H-1 and **D** H-3, etc.

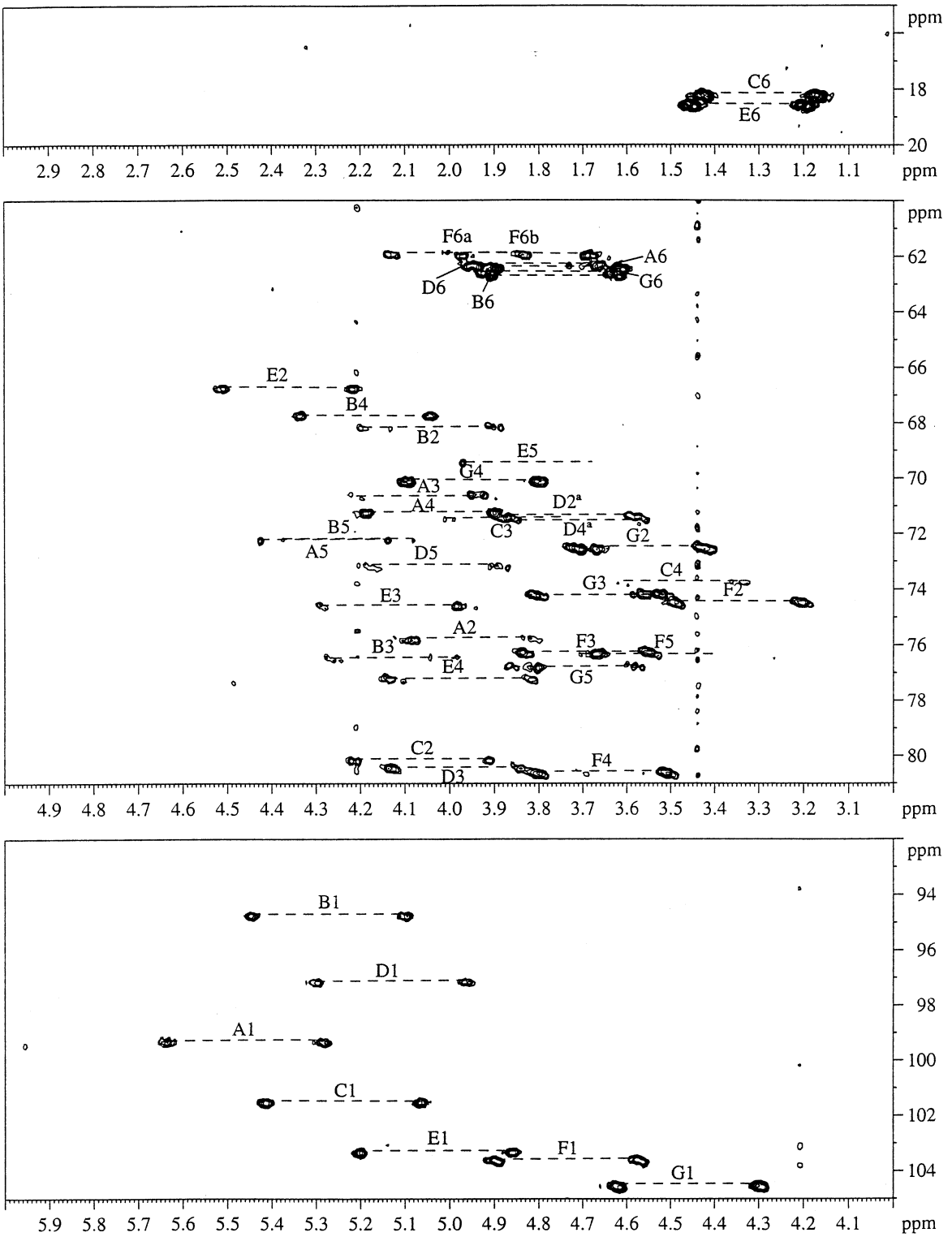

Fig. 4. 500-MHz 2D $^{1}H-^{13}C$ undecoupled HMQC spectrum of EPS B39 (**1**) recorded in D_2O at 80 °C. A1 refers to the set of cross-peaks between C-1 and H-1 of residue **A**, etc. [a] Assignments may have to be interchanged.

terminally linked glucose. Consequently, terminally linked galactose is attached to the (1→4)-linked glucose residue and the branches attached to the backbone of native EPS B39 contain at least two sugar residues.

During incubation of EPS B39 with Ultra SP not all terminally linked galactosyl residues

were removed (Table 1). Nevertheless, the enzymic modification provided important structural information about EPS B39.

1D NMR spectroscopy.—The ^{1}H NMR spectrum of **1** (Fig. 1(a)) showed seven signals with similar intensity in the anomeric region (δ 5.5–4.4), suggesting a heptasaccharide repeating unit. The seven monosaccharide residues were designated **A**–**G**, as indicated in the spectrum. The chemical shifts and coupling constants of the signals at δ 5.462 (**A** H-1, $^3J_{1,2}$ 3.51 Hz), δ 5.276 (**B** H-1, $^3J_{1,2}$ 3.81 Hz) and δ 5.136 (**D** H-1, $^3J_{1,2}$ 3.66 Hz) indicated the presence of three α-hexopyranosyl residues, whereas the chemical shifts and coupling constants of the signals at δ 4.739 (**F** H-1, $^3J_{1,2}$ 7.93 Hz) and δ 4.464 (**G** H-1, $^3J_{1,2}$ 7.78 Hz) suggested two β-hexopyranosyl residues. The coupling constants $^3J_{1,2}$ of the signals at δ 5.243 (**C** H-1) and δ 5.033 (**E** H-1) were too small to be determined. Together with two high-field doublets (δ 1.331, $^3J_{5,6}$ 5.95 Hz and δ 1.302, $^3J_{5,6}$ 6.10 Hz), arising from methyl groups, these signals are indicative of two rhamnosyl residues.

Comparison of the ^{1}H NMR spectrum of **2** (Fig. 1(b)) with the ^{1}H NMR spectrum of **1** (Fig. 1(a)) showed that the intensity of the signals at δ 4.739 (residue **F**) and δ 4.464 (residue **G**) decreased, while a new anomeric signal appeared at δ 4.713. According to the sugar linkage composition analyses, the new anomeric resonance could be assigned to H-1 of a terminally linked glucopyranosyl residue (residue **F***). Furthermore, it was concluded that both sugar residues in the branches of EPS B39 have a β configuration. The combined results, from sugar linkage analysis and NMR studies, suggested that (at least) a disaccharide fragment is involved in the branches of EPS B39: β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→, corresponding to residues **G** and **F**. The specific residue assignments follow from 2D NMR experiments (vide infra).

The ^{13}C NMR spectrum of **1** (not shown) confirmed the proposed heptasaccharide repeating unit since it showed seven anomeric signals: δ 104.5, 103.6, 103.3, 101.5, 99.3, 97.1, and 94.7. Furthermore, the signals from the methyl carbons of the two rhamnosyl residues were present at 18.5 and 18.1 ppm.

2D NMR spectroscopy.—Assignments of ^{1}H chemical shifts of **1** (Table 2) were performed by means of 2D (DQF-)COSY, TOCSY, ROESY and HMQC measurements. As starting points, the anomeric protons of residues **A**–**G** and the methyl protons of the rhamnosyl residues **C** and **E** were used. From the (DQF-)COSY spectra (not shown), all protons of the rhamnosyl residues **C** and **E** could be assigned. TOCSY spectra confirmed these assignments and the spectrum with a mixing time of 140 ms is shown in Fig. 2 as a typical example. The chemical shifts of **C** H-5 at δ 3.83 and **E** H-5 at δ 3.86 (Table 2) strongly suggest the α configuration for both rhamnosyl residues [22]. The TOCSY H-1 track of residue **A** showed cross-peaks with H-2,3,4, indicating a galactosyl residue. The partial overlap of the signals of **A** H-3 and **A** H-4 was inferred from **A** H-5, **A** H-3 and **A** H-5, **A** H-4 cross-peaks in the ROESY spectrum (Fig. 3) and a weak connectivity of **A** H-5 with **A** H-4 in the DQF-COSY spectrum.

Table 3
^{13}C NMR chemical shifts [a] of EPS B39 (**1**), recorded in D_2O at 80 °C

Residue	C-1	C-2	C-3	C-4	C-5	C-6
A (1,2-α-D-Gal*p*)	99.3	75.8	70.6	71.2	72.2	62.5
B (1,3-α-D-Gal*p*)	94.7	68.2	76.5	67.8	72.2	62.8
C (1,2-α-L-Rha*p*)	101.5	80.2	71.5	73.8	nd [b]	18.1
D (1,3-α-D-Glc*p*)	97.1	71.4 [c]	80.5	71.6 [c]	73.2	62.4
E (1,3,4-α-L-Rha*p*)	103.3	66.8	74.6	77.3	69.4	18.5
F (1,4-β-D-Glc*p*)	103.6	74.5	76.3	80.6	76.3	62.0
G (t-β-D-Gal*p*)	104.5	72.5	74.2	70.1	76.8	62.5

[a] In ppm relative to the signal of acetone at δ 31.55.
[b] Not determined.
[c] Assignments may have to be interchanged.

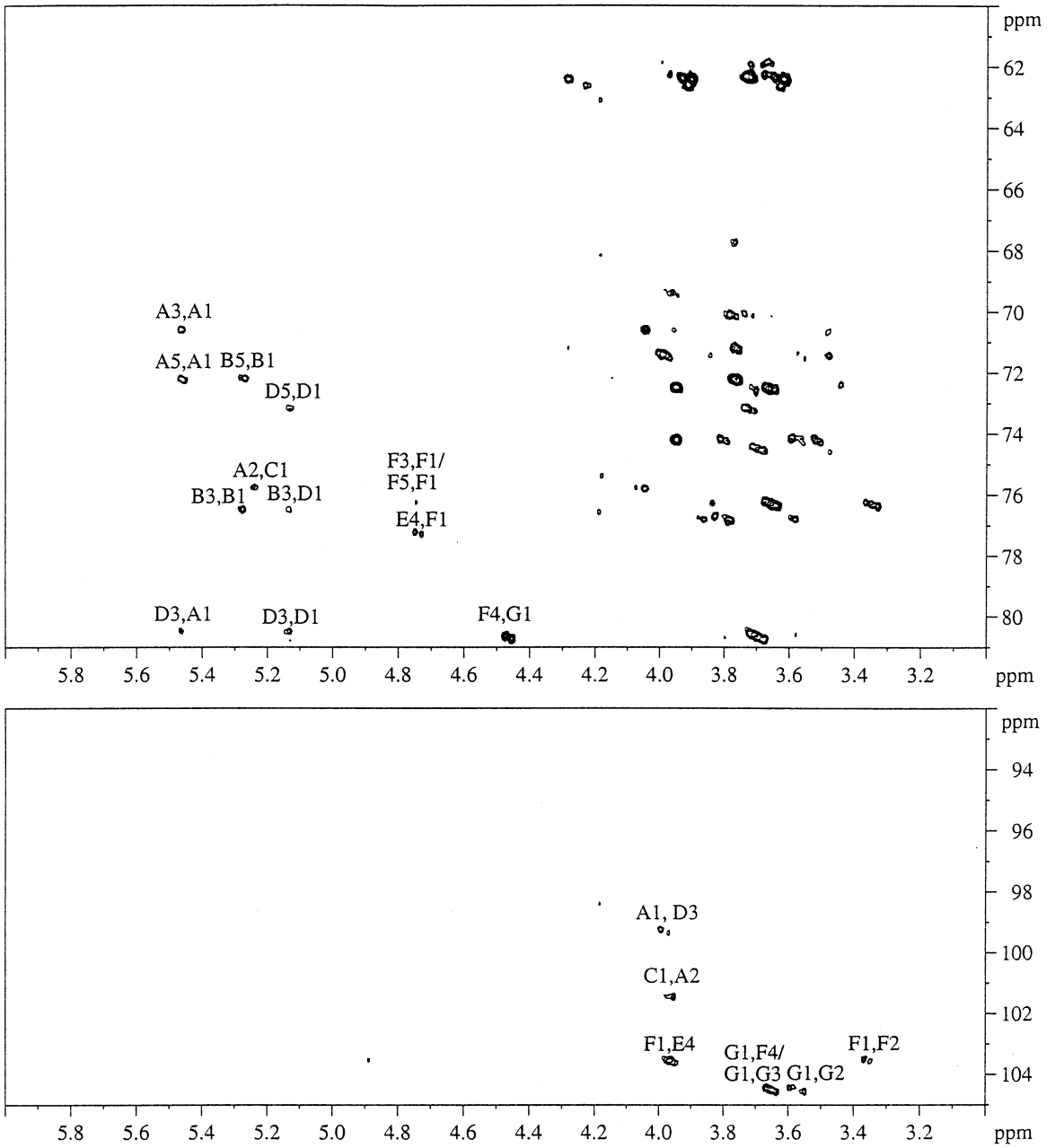

Fig. 5. Partial 500-MHz 2D $^{1}H-^{13}C$ undecoupled HMBC spectrum (delay time between the first proton pulse and the first carbon pulse = 80 ms) of EPS B39 (**1**) recorded in D_2O at 80 °C. The code G1,F4 corresponds to a long-range coupling between **G** C-1 and **F** H-4, etc.

The (un)decoupled HMQC spectra confirmed the partial overlap of **A** H-3 and **A** H-4. The resonances for **A** H-6a,6b were found via connectivities with **A** H-5 in both the (DQF-)COSY and the TOCSY spectra and since the undecoupled HMQC spectrum (Fig. 4) showed only one set of cross-peaks between **A** C-6 and **A** H-6, the resonances of **A** H-6a and **A** H-6b had to overlap. The TOCSY H-1 track of residue **B** showed cross-peaks with H-2,3,4, indicating a galactosyl residue. The resonance for **B** H-5 was found via an intra-residual connectivity with **B** H-3 in the ROESY spectrum. This assignment was confirmed in COSY and TOCSY spectra recorded at 70 °C (not shown); weak cross-peaks between **B** H-4 and **B** H-5 were found. The resonances for **B** H-6a,6b were found via connectivities with **B** H-5 in both the (DQF-)-COSY and the TOCSY spectra and the

(un)decoupled HMQC spectra indicated that the resonances of **B** H-6a and **B** H-6b overlap. The TOCSY track of **D** H-1 showed cross-peaks with **D** H-2,3,4,5,6a,6b, which is indicative of a glucosyl residue. The (DQF-)COSY and TOCSY spectra already suggested overlap between **D** H-2 and **D** H-4, and this was confirmed by the (un)decoupled HMQC spectra. The latter spectra also indicated that the resonances of **D** H-6a and **D** H-6b overlap. The TOCSY track of **F** H-1 showed the complete series of cross-peaks with **F** H-2,3,4,5,6a,6b, indicating that **F** corresponds to a glucosyl residue. Using these connectivities and the cross-peaks in the (DQF-)COSY spectrum, all protons of residue **F** could be assigned. The **G** H-1 track in the TOCSY spectrum showed cross-peaks with **G** H-2,3,4, suggesting a galactosyl residue. **G** H-5 and **G** H-6 were found via the **G** H-1 and **G** H-4 track in the ROESY spectrum. Again, the (un)decoupled HMQC spectra indicated that the resonances of **G** H-6a and **G** H-6b overlap.

The undecoupled 2D HMQC spectrum (Fig. 4) together with the decoupled 2D HMQC spectrum allowed the assignment of the ^{13}C resonances (Table 3). The one-bond coupling constants of the anomeric carbon atoms of residues **B**, **D** and **E** ($^{1}J_{C\text{-}1,H\text{-}1}$ 170–173 Hz) and residues **A** and **C** ($^{1}J_{C\text{-}1,H\text{-}1}$ 175–177 Hz) are indicative of an α configuration for these residues, whereas residues **F** and **G** ($^{1}J_{C\text{-}1,H\text{-}1}$ 162–163 Hz) are in the β configuration [36]. The relatively high coupling constants of residues **A** and **C** are most likely caused by the 2-substitution [18]. Comparison of the ^{13}C data in Table 3 with the corresponding methyl aldosides [37] shows low-field chemical shifts for **A** C-2 and **C** C-2, suggesting that residues **A** and **C** are substituted at C-2. Likewise, the downfield chemical shifts of **B** C-3 and **D** C-3 indicate that these residues are 3-substituted, whereas the downfield chemical shifts of **E** C-3 and **E** C-4 show that residue **E** is 3,4 disubstituted. Residue **F** was assigned as the (1→4)-linked residue since **F** C-4 was downfield shifted and residue **G** was identified as the terminally linked sugar residue. The assignments of the type of sugar residues and the type of linkages based on NMR experiments are in agreement with the results from the sugar linkage analysis (Table 1).

The complete monosaccharide sequence of EPS B39 was determined via a 2D HMBC spectrum with a delay time of 80 ms (Fig. 5) and 2D ROESY (Fig. 3) and NOESY analyses. In other HMBC spectra (delay times 40, 60, 140 ms, not shown) no new inter-residual connectivities could be detected. Since the inter-residual connectivities in the NOESY and ROESY experiments were essentially identical, only the results in the ROESY spectrum are

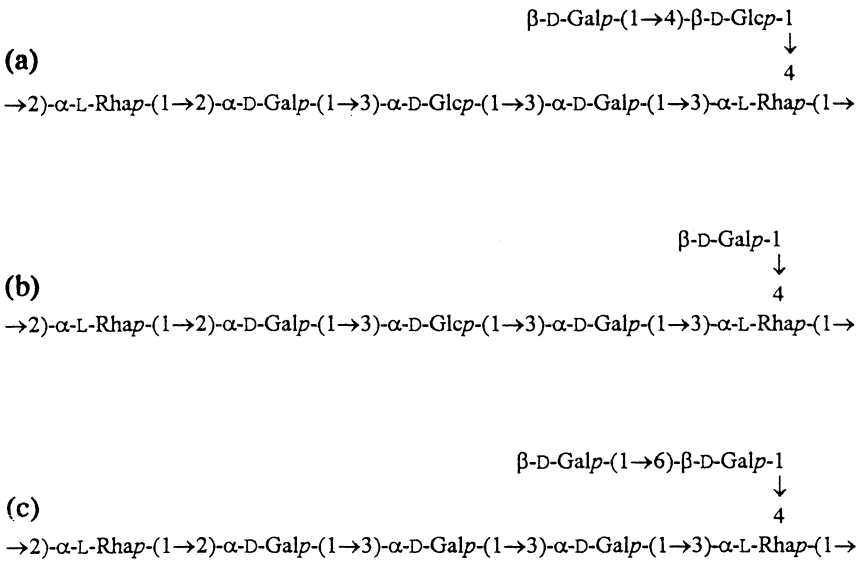

Fig. 6. Chemical structures of EPSs produced by *S. thermophilus* with close structural similarities to the EPS produced by *L. lactis* subsp. *cremoris* B39 (a): SFi12 [16] (b), OR 901 [17] and Rs and Sts [18] (c).

described. In the latter spectrum inter-residual connectivities **A** H-1, **D** H-3, **A** H-1, **D** H-2 and/or **A** H-1, **D** H-4 and **A** H-1, **D** H-6 demonstrated the **A**→**D** sequence, which was also proven by **A** C-1, **D** H-3 and **D** C-3, **A** H-1 cross-peaks in the HMBC spectrum. The connectivities in the ROESY spectrum between **D** H-1, **B** H-3 and **D** H-1, **B** H-4 indicated the **D**→**B** sequence. Moreover, a weak inter-residual connectivity in the HMBC spectrum was found between **B** C-3 and **D** H-1, confirming the **D**-(1→3)-**B** linkage. The **B**-(1→3)-**E** linkage could not be revealed by HMBC analyses, but methylation analysis in combination with carbon chemical shifts demonstrated that residue **E** is 3,4-disubstituted. Therefore, the strong connectivity of **B** H-1 and **E** H-2 and the weaker connectivity of **B** H-1 and **E** H-3 in the ROESY spectrum showed the **B**→**E** sequence, but the strongest connectivity did not reflect the glycosidic linkage. The observation of a strong inter-residual non-glycosidic NOE cross-peak was reported before for this α-(1→3) linkage [18]. The strong inter-residual cross-peak of **E** H-1,**C** H-2 in the ROESY spectrum indicated the **E**-(1→2)-**C** linkage, but this could not be confirmed by HMBC analyses. The strong cross-peak in the ROESY spectrum between **C** H-1, **A** H-2 revealed the structural element **C**-(1→2)-**A** and this sequence was proven by **C** C-1, **A** H-2 and **A** C-2, **C** H-1 connectivities in the HMBC spectrum. The **F**→**E** linkage was suggested by connectivities in the ROESY spectrum, **F** H-1,**E** H-3 and **F** H-1,**E** H-4, and inter-residual cross-peaks between **F** C-1, **E** H-4 and **E** C-4, **F** H-1 in the HMBC spectrum confirmed the **F**-(1→4)-**E** sequence. The **G**-(1→4)-**F** linkage could only be tentatively assigned by ROESY experiments since **G** H-1, **G** H-3 and **G** H-1, **F** H-4 overlap. However, the **G**-(1→4)-**F** linkage was proven by inter-residual connectivities between **G** C-1, **F** H-4 and **F** C-4, **G** H-1 in the HMBC spectrum.

In both 2D NOESY and ROESY spectra long-range NOE contacts between **C** H-1 and **E** H-5 were found, indicating a conformation for the polysaccharide in which residue **C** is close to residue **E**.

The combined results from enzymic modification, chemical studies and NMR experiments demonstrated that the EPS produced by *L. lactis* subsp. *cremoris* B39 is composed of the heptasaccharide repeating unit with the structure as shown in Fig. 1(a).

4. Discussion

In order to be able to study the effect of structural changes in the physical properties of exopolysaccharides, the chemical structure of the EPS from *L. lactis* subsp. *cremoris* B39 was elucidated. To our knowledge, this chemical structure (Fig. 6(a)) has not been reported before for EPS produced by lactic acid bacteria. However, related EPS structures (Fig. 6(b) and (c)) have been described for different strains of *Streptococcus thermophilus*. The backbone of EPS B39 is identical to the backbone reported for EPS SFi12 [16] (Fig. 6(b)) and this was reflected in the similar ^{1}H NMR chemical shifts of the backbone sugar residues (within 0.06 ppm). The only difference between these two EPSs is the (1→4)-linked glucosyl residue in the branches of EPS B39, which is absent in EPS SFi12. Another structure that is related to EPS B39 was reported for EPS from *S. thermophilus* OR 901 [17], Rs and Sts [18], three presumably different strains with an identical repeating unit [18] (Fig. 6(c)). In comparison with EPS B39, all glucosyl residues were replaced by galactosyl residues in this EPS and the terminally-linked galactosyl residue is linked to the 6-position instead of the 4-position of the next sugar residue.

It has been noted before [19] that different microbial species or strains may produce a range of polysaccharides with close structural similarities. Examination and comparison of the physical properties of all available, related EPS structures, including enzymically modified EPS, enables us to determine the effect of structural changes on the physical properties of these macromolecules. Work on the comparison of physical properties of related EPS structures is in progress.

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