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Determination of the structure of the exopolysaccharide produced by *Lactobacillus sake* 0-1

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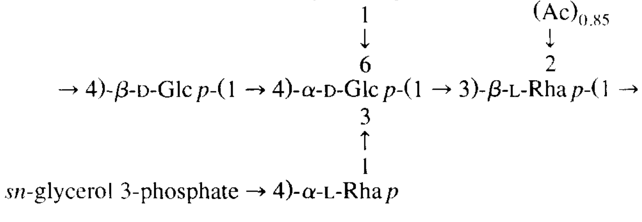
Abstract

The exopolysaccharide produced by *Lactobacillus sake* 0-1 in a semi-defined medium was found to have an average molecular mass of 6×10^6 Da and a composition of D-glucose, L-rhamnose, and *sn*-glycerol 3-phosphate (3:2:1). The polysaccharide is partially *O*-acetylated. By means of partial acid hydrolysis, *O*-deacetylation, deglycerophosphorylation, methylation analysis, and 1D/2D NMR (^{1}H, ^{13}C, and ^{31}P) studies the polysaccharide was shown to be composed of repeating units with the following structure:



$$\begin{array}{cccc}
 & \beta\text{-D-Glc}p & (\text{Ac})_{0.85} & \\
 & 1 & & \\
 & \downarrow & \downarrow & \\
 & 6 & 2 & \\
 & \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1 & \rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1 & \rightarrow 3)\text{-}\beta\text{-L-Rha}p\text{-}(1 \rightarrow \\
 & & 3 & \\
 & & \uparrow & \\
 & & 1 & \\
 & sn\text{-glycerol 3-phosphate} \rightarrow & 4)\text{-}\alpha\text{-L-Rha}p &
\end{array}$$

Keywords: Lactic acid bacteria; *Lactobacillus sake*; Polysaccharide; Phosphoglycerol; Structural analysis

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1. Introduction

Bacterial exopolysaccharides (EPSs) are widely applied in the food industry as gelling and thickening agents. Recently, a growing interest has been developed in EPSs produced by lactic acid bacteria, because these bacteria are Food Grade. Exopolysaccharides produced by GRAS (Generally Recognized As Safe) micro-organisms, like lactic acid bacteria, will possibly serve as a new generation of food thickeners. In this context, detailed structural studies have been performed on the EPSs from *Streptococcus thermophilus* [1], *Lactococcus lactis* subsp. *cremoris* SBT 0495 [2], *Lactococcus lactis* subsp. *cremoris* H414 [3], *Lactobacillus delbrückii* subsp. *bulgaricus* rr [4], and *Lactobacillus helveticus* TY1-2 [5].

Based on its outstanding rheological properties, out of 30 EPSs from lactic acid bacteria, the EPS produced by *Lactobacillus sake* 0-1 was selected for structural studies. The viscosity and shear thinning properties of the ''0-1'' EPS are comparable to those of xanthan gum [6].

To obtain insight into the relationship between the rheological properties of polysaccharides and their three-dimensional structures, knowledge of the primary structures is a prerequisite. Here we report the characterization of the exopolysaccharide produced by *Lactobacillus sake* 0-1 in a semi-defined medium.

2. Experimental

Culture conditions of the microorganism and isolation of the exopolysaccharide.—*Lactobacillus sake* 0-1 was grown in semi-defined medium [6] for 2 days at 20°C (non-shaken, non-aerated), then trichloroacetic acid was added at a final concentration of 4% (w/v) and the culture was stirred for 2 h. Cells and precipitated proteins were removed by centrifugation (27000 *g*, 30 min, 4°C). The supernatant solution was collected and the EPS was precipitated with two volumes of cold EtOH. An aqueous solution of the precipitated material was extensively dialysed against bidistilled water, and, after removal of insoluble material by centrifugation, two more volumes of EtOH were added. The precipitate formed was dissolved in water, and subsequently subjected to a fractionated precipitation at 30%, 40%, 50%, 60%, and 70% (v/v) acetone. The precipitated material collected from the 40% acetone fraction was further purified by gel filtration on a column (150 × 2.2 cm) of Sephacryl S-500 (Pharmacia), irrigated with 50 mM NH_4HCO_3, using refractive index monitoring (Bischoff 8100 RI detector).

Molecular mass determination.—The average molecular mass of the native polysaccharide was determined, using a method combining gel permeation chromatography, capillary viscometry, low-angle light scattering and differential refraction analysis, as described [7].

Protein assay.—Protein contents were determined with the Pierce Protein Assay Reagent, using bovine serum albumin as a standard.

Anion-exchange chromatography of the native polysaccharide.—To test the presence of negative charge, a solution of polysaccharide (1 mg) in water (2 mL) was transferred to a column containing 5 mL of Dowex AG 1-X2 anion-exchange resin (100–200 mesh, acetate form, Bio-Rad). The column was eluted with five column volumes of water, yielding a neutral fraction, followed by five column volumes of 1 M NH_4HCO_3, yielding an acidic fraction. Both fractions were lyophilized, and the sugar content was determined by quantitative monosaccharide analysis.

Phosphorus determination.—Phosphorus was determined as described [8].

Gas–liquid chromatography and mass spectrometry.—GLC analyses were performed on a Varian 3700 gas chromatograph, equipped with an SE-30 fused-silica capillary column (25 m × 0.32 mm, Pierce) or a CPSil43 WCOT fused-silica capillary column (25 m × 0.32 mm, Chrompack) using a temperature program of 130–220°C at 4°C/min. GLC data were collected and processed with a Shimadzu integrator.

GLC-MS analyses were carried out on a JEOL JMS-AX505W mass spectrometer (electron energy, 70 eV), coupled to a Hewlett–Packard 5980 gas chromatograph, equipped with a CPSil5 (Chrompack), a HT-5 (Chrompack), or an AT SE-54 (Pierce) capillary column (25 m × 0.32 mm).

For the determination of the absolute configuration of the 1-phosphoglycerol moiety, GLC-MS experiments were carried out on a Fisons MD800/8060 system (electron energy, 70 eV; carrier gas, He), equipped with a DB-1 fused-silica capillary column (30 m × 0.32 mm, J&W Scientific). Samples were injected using a split injector (split flow 1/10), and a temperature program of 140–150°C at 4°C/min followed by isothermal elution.

A positive-ion mode FAB mass spectrum was recorded using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer (accelerating voltage, 6 kV; Xe-beam). The methylated oligosaccharide sample was measured over a mass range of m/z 10–1500 in a matrix of *m*-nitrobenzyl alcohol.

Monosaccharide analysis.—For monosaccharide analysis, three procedures were followed. (1) Oligo/polysaccharides were subjected to methanolysis (methanolic 1 M HCl, 18 h, 85°C), and the resulting mixtures of methyl glycosides were trimethylsilylated with 1:1:5 (v/v/v) hexamethyldisilazane–chlorotrimethylsilane–pyridine, and quantitatively analyzed by GLC on SE-30 [9,10]. In addition, the absolute configurations of the monosaccharides were determined by GLC analysis of the trimethylsilylated (−)-2-butyl glycosides [11,12] on SE-30. (2) For the detection of phosphorylated components [13], the native polysaccharide (500 μg) was hydrolyzed with 2 M CF_3CO_2H (0.5 mL, 2 h, 120°C). After evaporation of CF_3CO_2H in vacuo, the residue was reduced with $NaBD_4$ in 1 M ammonia (18 h, room temperature). The solution was neutralized with AcOH, desalted with Dowex (H^+), and lyophilized, and boric acid was removed by co-evaporation with MeOH under reduced pressure. The residue was trimethylsilylated, and, after evaporation of the solvent under a stream of dry N_2, taken up into 0.5 mL of 10% MeOH in ether. Then, 0.5 mL of a saturated solution of diazomethane in ether was added and the mixture was kept at 0°C for 20 min. After evaporation of the solvent under a stream of N_2 at room temperature, the residue was taken up into 100 μL of fresh silylating reagent. The resulting mixture of trimethylsilylated (phosphate-methylated) alditols was qualitatively analyzed by GLC on SE-30 and by GLC-MS on AT SE-54. (3) Free monosaccharides isolated from a partial acid hydrolysate of the native polysaccharide were trimethylsilylated and qualitatively analyzed by GLC on SE-30.

Determination of the absolute configuration of 1-phosphoglycerol.—A sample of native EPS (1 mg) was permethylated using methyl trifluoromethanesulfonate in trimethyl phosphate according to [14], and subsequently treated with 100 μL of aq 48% HF (2 h, 0°C). After removal of HF in vacuo, the residue was treated with (1*S*,4*R*)-(−)-camphanic acid chloride and 4-dimethylaminopyridine in 0.5 mL of 1:1 CH_2Cl_2–Et_3N, as described by Beynon and Richards [15]. The resulting product was analyzed by GLC-MS on DB-1, using selected ion monitoring at *m/z* 153 and 255. Standards, prepared from D- or DL-glyceric acid according to the literature [15], were also analyzed by GLC-MS using the same experimental set-up. 1-*O*-Camphanoyl-2,3-di-*O*-methyl-*sn*-glycerol and 3-*O*-camphanoyl-1,2-di-*O*-methyl-*sn*-glycerol, prepared from DL-glyceric acid, were eluted on DB-1 at 22.68 and 23.21 min, respectively, with baseline separation.

Methylation analysis.—Samples (polysaccharide or oligosaccharide alditols) were permethylated using methyl iodide and solid NaOH in dimethyl sulfoxide as described previously [16]. After hydrolysis with 2 M CF_3CO_2H (2 h, 120°C), the partially methylated monosaccharides were reduced with $NaBD_4$. Conventional work-up, comprising neutralization and removal of boric acid by co-evaporation with MeOH, followed by acetylation with acetic anhydride (3 h, 120°C) yielded mixtures of partially methylated alditol acetates, which were analyzed by GLC on CPSil43 and by GLC-MS on CPSil5 [9,17].

O-*Deacetylation of the native polysaccharide.*—The native EPS (20 mg) was *O*-deacetylated by treatment with aq 5% NH_4OH (20 mL) for 8 h at room temperature. The *O*-deacetylated polysaccharide was recovered by lyophilization.

Dephosphorylation and O-*deacetylation of the native polysaccharide.*—To a solution of EPS (10 mg) in bidistilled water (5 mL) was added 4 M NaOH (5 mL). The solution was heated for 4 h at 80°C and, after cooling to room temperature, was neutralized with 2 M AcOH. After desalting on a small column of Dowex (H^+), followed by lyophilization, the residue was fractionated on a Superose-6 gel filtration column (30 × 1 cm, Pharmacia), irrigated with 50 mM NH_4HCO_3, using a Pharmacia FPLC system and refractive index monitoring. The *O*-deacetylated, deglycerophosphorylated polysaccharide was collected in the void volume peak.

Partial acid hydrolysis.—Polysaccharide (30 mg) was hydrolyzed in 0.3 M CF_3CO_2H (30 mL) for 120 min at 100°C. Then, CF_3CO_2H was removed by repeated lyophilization, and the residue was fractionated on a column (90 × 1.5 cm) of Bio-Gel P-2 (200–400 mesh), irrigated with bidistilled water, using refractive index monitoring. Subfractionation of Bio-Gel P-2 fractions was performed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a CarboPac PA-1 pellicular anion-exchange column (25 cm × 9 mm), using a Dionex LC system. The column was eluted with a gradient of NaOAc in 0.1 M NaOH (20–250 mM NaOAc at 5 mM/min) at a flow rate of 4 mL/min. The PAD-detection was made with a gold working electrode and triple-pulse amperometry (pulse potentials and durations: E_1 0.05 V, 480 ms; E_2 0.60 V, 120 ms; E_3 −0.60 V, 60 ms) was used. Data were collected and processed by a Shimadzu C-R3A integrator. The fractions were neutralized with 2 M AcOH, immediately after collection, followed by desalting on a cation-exchange resin (Dowex AG 50W-X12, 100–200 mesh, H^+-form, Bio-Rad), and lyophilization.

NMR spectroscopy.—Proton-decoupled 75.469-MHz ^{13}C NMR spectra were recorded for solutions in D_2O on a Bruker AC-300 spectrometer (Department of Organic Chemistry), equipped with a 5-mm broad-band probe, at a probe temperature of 67°C. Chemical shifts are expressed in ppm downfield from the signal for external tetramethylsilane, but were actually measured by reference to external MeOH (δ 49.00). The data were collected in 16K data sets and zero-filled to 64K. After Fourier transformation using an exponential multiplication, the spectra were baseline corrected with a fourth-order polynomial function.

121.496-MHz ^{31}P NMR spectra were recorded for solutions in D_2O on a Bruker AC-300 spectrometer, equipped with a 5-mm broad-band probe, at a probe temperature of 27°C. Chemical shifts are referenced to external 85% phosphoric acid (δ 0.00). The data were collected in 32K data sets and a Lorentzian-to-Gaussian transformation was applied.

Resolution-enhanced 1D ^1H NMR spectra were recorded on a Bruker AMX-500 or a Bruker AMX-600 spectrometer (Bijvoet Center, Department of NMR Spectroscopy) at a probe temperature of 27°C for oligosaccharides and 80°C for polysaccharides. Prior to analysis, samples were exchanged twice in D_2O (99.9 atom% D, Isotec) with intermediate lyophilization, and then dissolved in 99.96 atom% D_2O (Isotec). Chemical shifts are expressed in ppm by reference to internal acetone (δ 2.225). The 1D spectra were recorded with a spectral width of 5000 Hz at 500 MHz or 6000 Hz at 600 MHz in 16K data sets. Suppression of the HOD signal was achieved by applying the WEFT pulse sequence as described [18]. Resolution enhancement of the spectra was performed by a Lorentzian-to-Gaussian transformation and the final spectra were baseline corrected with a fourth-order polynomial fit when necessary.

2D Homonuclear Hartmann–Hahn (HOHAHA) spectra were recorded using MLEV mixing sequences of 25–125 ms. The spin-lock field strength corresponded to a 90° pulse width of ca. 27 μs. The spectral width was 3300 Hz in each dimension. The HOD signal was presaturated for 1 s during the relaxation delay. 464–512 Spectra of 2K data points with 8–64 scans per t_1 increment were recorded.

2D Nuclear Overhauser enhancement spectroscopy (NOESY) was performed with a mixing time of 250 ms. The spectral width was 3300 Hz in each dimension. The HOD signal was presaturated for 1 s during the relaxation delay. 464–512 Spectra of 2K data points with 8–16 scans per t_1 increment were recorded.

2D Rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY) was carried out with a mixing time of 225 ms. The spectral width was 3800 Hz in each dimension. The spin-lock field strength corresponded to a 90° pulse width of ca. 110 μs. The HOD signal was presaturated for 1 s during the relaxation delay. 350 Spectra of 2K data points with 16 scans per t_1 increment were collected.

Double-quantum filtered correlation experiments (COSY) were recorded in 464–512 data sets of 2K data points; 16–64 scans per t_1 increment were recorded. The HOD signal was suppressed by presaturation for 1 s during the relaxation delay. The spectral widths were 3300–3700 Hz in each dimension.

A phase-sensitive ^{13}C–^1H 2D heteronuclear multiple quantum coherence (HMQC) experiment with inverse detection [19] was carried out with a spectral width of 4000 Hz for t_2 and 25 000 Hz for t_1, using a 5-mm broad-band probe. The HOD signal was presaturated for 1 s and ^{12}C-bound protons were suppressed using a TANGO pulse sequence. Carbon-13 decoupling was not applied during the acquisition of the ^{1}H FID. 601 Spectra of 4K data points with 56 scans per t_1 increment were recorded.

All 2D NMR data were processed on Silicon Graphics IRIS workstations (Indigo, Indigo 2 or 4D/35), using TRITON software (Bijvoet Center, Department of NMR Spectroscopy). The time domain data sets were multiplied with a phase-shifted sine bell and, after Fourier transformation and zero-filling, data sets of 1024×2048 points (2048×2048 points for the HMQC experiment) were obtained, which were baseline corrected with a fourth-order polynomial function when necessary.

3. Results and discussion

Isolation, purification, and composition of the polysaccharide.—The exopolysaccharide produced by *Lactobacillus sake* 0-1 in a semi-defined medium [6,20] was isolated via ethanol precipitation of the cell-free medium, followed by a fractionated acetone precipitation. The polysaccharide, collected in the 40% (v/v) acetone precipitate, was further purified by gel filtration chromatography on Sephacryl S-500. The average molecular mass of the polysaccharide was determined to be 6×10^6 Da [6]. The protein content of the purified polysaccharide preparation was ca. 1%. The strongly negative character of the polysaccharide is evident from its behaviour on Dowex AG1-X2. Elution with water did not release any carbohydrate material and elution with 1 M NH_4HCO_3 released only 2% of the bound polysaccharide.

Quantitative monosaccharide analysis of the native EPS (n-EPS, **1**), including the determination of absolute configurations (procedure 1), revealed the presence of D-Glc and L-Rha in a molar ratio of 1.6:1.0. A qualitative GLC-MS analysis (procedure 2), focused on the detection of phosphorylated components, afforded glucitol-*1-d*, rhamnitol-*1-d*, and phosphoglycerol. The phosphorus content was colorimetrically determined to be 2.5%, which corresponds to 0.8 mol/mol repeating unit, taking into account a pentasaccharide repeating unit (vide infra). The ^{1}H and ^{13}C NMR data indicated that n-EPS is partially *O*-acetylated (vide infra).

In terms of absolute configuration, the phosphoglycerol was shown to be *sn*-glycerol 3-phosphate via GLC-MS analysis of the mono-*O*-camphanoyl-di-*O*-methylglycerol derivative, prepared via permethylation (methyl trifluoromethanesulfonate) of n-EPS [14], followed by hydrolysis with HF and subsequent camphanoylation [15]. The glycerol derivative coeluted with 3-*O*-camphanoyl-1,2-di-*O*-methyl-*sn*-glycerol, prepared from D-glyceric acid as described [15]. It should be noted that, for the chirality determination, methylation of the polysaccharide has to be carried out under mild conditions in order to prevent migration of the phosphate group. Methylation with methyl iodide–solid sodium hydroxide [16] yielded, after HF-treatment and camphanoylation, a 1:1 mixture of (2*R*)- and (2*S*)-1-*O*-camphanoyl-2,3-di-*O*-methylglycerol.

Treatment of n-EPS with 5% ammonia at room temperature resulted in complete *O*-deacetylation, but no dephosphorylation of the polysaccharide (dAc-EPS, **2**), as determined by NMR spectroscopy (vide infra). Treatment of n-EPS with 2 M NaOH for 4 h at 80°C resulted in complete *O*-deacetylation and almost complete deglycerophosphorylation of the polysaccharide as judged by ^{1}H NMR spectroscopy (vide infra). Since the polysaccharide still eluted in the void volume peak of Superose-6, the phosphoglycerol element has to be located in a side-chain of the polysaccharide. The *O*-deacetylated, deglycerophosphorylated EPS (dAc-dGP-EPS, **3**) had the same monosaccharide composition as n-EPS (Glc : Rha = 1.6:1.0). Treatment of n-EPS with cold 48% HF resulted in only partial deglycerophosphorylation, accompanied by breakdown of the polysaccharide backbone (as shown by ^{1}H NMR spectroscopy and gel-filtration chromatography on Superose-6). The cleavage of rhamnosyl glycosidic bonds upon treatment with HF has been reported previously for polysaccharides containing phosphoglycerol side-chain substituents and rhamnose in their backbones [21,22].

1D ^{31}P, ^{1}H, and ^{13}C NMR spectroscopy.—The ^{31}P NMR spectra of n-EPS and dAc-EPS (spectra not shown) each show one signal at δ 1.28, indicative of a phosphodiester [23].

The ^{1}H NMR spectra (Fig. 1) of n-EPS, dAc-EPS and dAc-dGP-EPS all show five signals of equal intensity in the anomeric region (δ 5.3 –4.4), suggesting a pentasaccharide repeating unit. In each case two high-field doublets (δ ~ 1.3, $^3J_{5,6}$ ~ 6 Hz) are present, arising from the methyl groups of two rhamnose residues. In the spectrum of dAc-dGP-EPS, the coupling constants of the anomeric signals at δ 4.479 ($^3J_{1,2}$ 7.9 Hz) and δ 4.580 ($^3J_{1,2}$ 7.5 Hz) indicate the occurrence of two β-glucopyranose residues. The spectrum of n-EPS also shows a low-field signal at δ 5.621 for the proton at the *O*-acetylated carbon, and a signal at δ 2.186, assigned to the protons of an acetyl group. Comparison of the spectra of n-EPS and dAc-EPS shows that the native polysaccharide is not fully *O*-acetylated. Based on integration of signals in the spectrum of n-EPS, the extent of *O*-acetylation was determined to be 85%. However, it has to be kept in mind that during the isolation and purification procedure of n-EPS partial *O*-deacetylation may have occurred.

The ^{13}C NMR spectra (Fig. 2, Table 1) of the three polysaccharide samples confirm the proposed pentasaccharide repeating unit. The spectrum of dAc-dGP-EPS contains five anomeric signals at δ 102.37 (sharp), 101.53, 100.91, 100.56, and 95.83. Furthermore, the signals from the methyl carbons of the two rhamnosyl residues are present at 16.70 and 16.94 ppm. Comparison of the spectrum of dAc-dGP-EPS with that of n-EPS reveals the signals from the carbon atoms of a 1-phosphoglycerol moiety (*sn*-glycerol 3-phosphate, vide supra) in n-EPS (δ 66.75, d, C-1, $^2J_{P,C}$ 5.4 Hz; δ 71.09, d, C-2, $^3J_{P,C}$ 7.0 Hz; δ 62.59, s, C-3). The signals reflecting the presence of 1-phosphoglycerol are also present in the spectrum of dAc-EPS (δ 66.71, d, C-1, $^2J_{P,C}$ 5.6 Hz; δ 71.08, d, C-2, $^3J_{P,C}$ 7.4 Hz; δ 62.58, s, C-3). The assignment of the signals of the 1-phosphoglycerol moiety in both n-EPS and dAc-EPS is supported by the ^{13}C NMR parameters of reference 1-phosphoglycerol (δ 65.60, d, C-1, $^2J_{P,C}$ 4.9 Hz; δ 72.04, d, C-2, $^3J_{P,C}$ 7.5 Hz; δ 63.14, s, C-3) [23]. Finally, the ^{13}C NMR spectrum of n-EPS shows two signals at δ 173.85 and δ 20.53, typical for the carbon atoms of an *O*-acetyl group.

Methylation analysis.—Methylation analyses were performed on n-EPS, dAc-EPS, and dAc-dGP-EPS. Furthermore, a methylation analysis was carried out on n-EPS after mild acid hydrolysis (30 min, 0.3 M CF_3CO_2H, 100°C), hyd-EPS. The data, collected in Table 2, indicate that n-EPS contains terminal and 3-substituted rhamnosyl residues and terminal, 4-substituted, and 3,4,6-trisubstituted glucosyl residues. Comparison of the methylation analysis data of hyd-EPS and n-EPS shows that mild acid hydrolysis causes

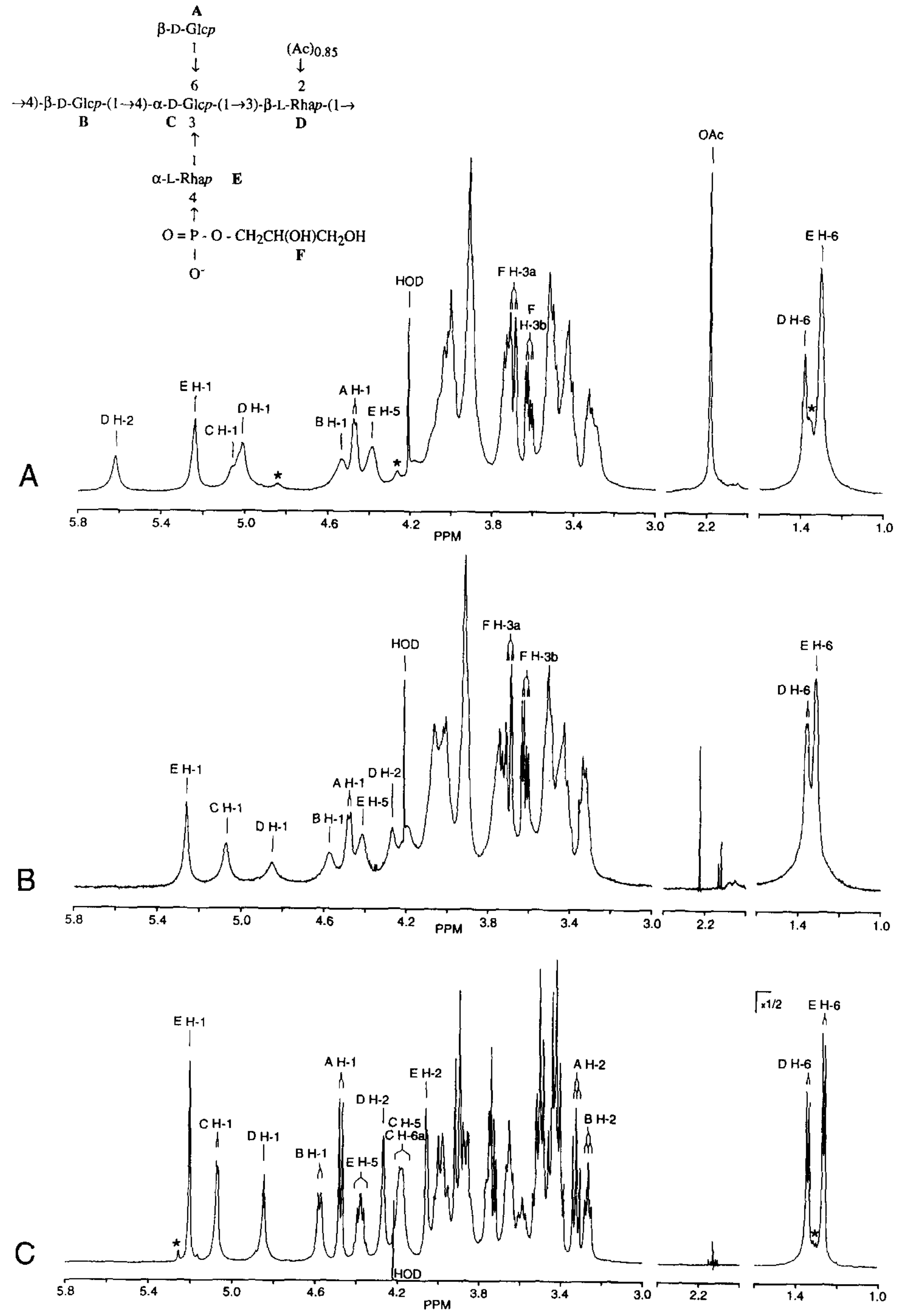


Fig. 1. 500-MHz ^{1}H NMR spectra of (A) n-EPS, (B) dAc-EPS, and (C) dAc-dGP-EPS, recorded in D_2O at 80°C. Signals marked with an asterisk (∗) in (A) stem from protons of *O*-deacetylated rhamnose **D**. Signals marked with an asterisk in (C) originate from protons of phosphorylated rhamnose **E**.

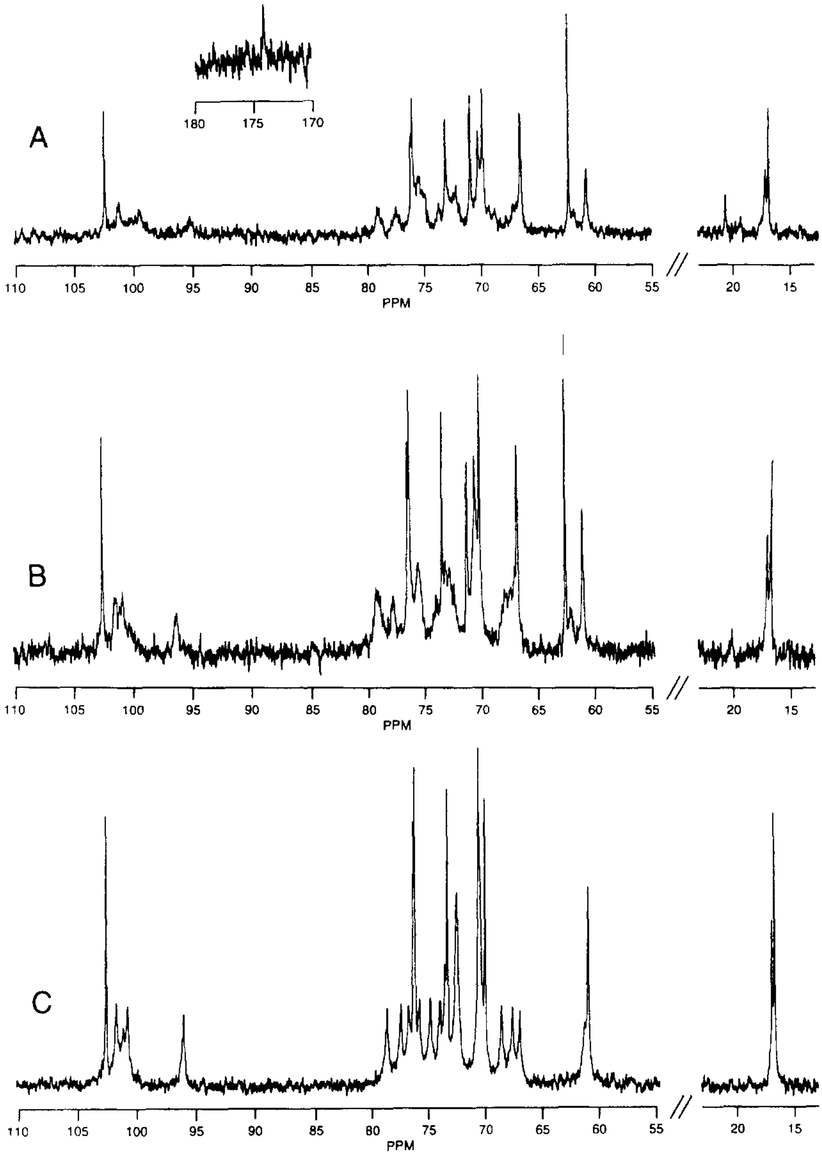


Fig. 2. 75-MHz ^{13}C NMR spectra of (A) n-EPS, (B) dAc-EPS, and (C) dAc-dGP-EPS, recorded in D_2O at 67°C.

a conversion of 3,4,6-trisubstituted glucose into 4,6-disubstituted glucose. This finding demonstrates that in n-EPS the 3,4,6-trisubstituted glucose residue is substituted at O-3 by a relatively acid-labile group, probably one of the rhamnose residues. According to NMR experiments (vide infra), all monosaccharides are in the pyranose ring form.

The low relative intensity of terminal rhamnose in the analyses of n-EPS and

Table 1
^{13}C NMR chemical shifts [a] of n-EPS (**1**), dAc-EPS (**2**), and dAc-dGP-EPS (**3**), recorded at 67°C. The assignments of residues **A–E** are based on the 2D HMQC experiment carried out on **3** at 80°C (see Table 4) [b]

Carbon atom	**1**	**2**	**3**
A C-1	102.35	102.35	102.37
A C-2	73.19	73.20	73.21
A C-3	76.10 [c]	76.14 [d]	76.14
A C-4	69.96	69.96	69.96
A C-5	76.04 [c]	76.02 [d]	76.02
A C-6	61.10	61.07	61.08
B C-1	101.13	101.36	101.53
B C-6	n.d. [e]	n.d.	61.38
C C-1	95.08	96.02	95.83
D C-1	99.48	100.67	100.56
D C-6	17.11	17.13	16.94
-*C*OCH$_3$	173.85	—	—
-CO*C*H$_3$	20.53	—	—
E C-1	100.12	~ 100.8	100.91
E C-6	16.83	16.81	16.70
F C-1	66.75 (−5.4) [f]	66.71 (−5.6) [f]	—
F C-2	71.09 (7.0) [g]	71.08 (7.4) [g]	—
F C-3	62.59	62.58	—

[a] In ppm relative to the signal of external methanol (δ 49.00). [b] For the codes **A–E** used to indicate the different monosaccharide residues, see Fig. 1. [c–d] Marked assignments may have to be interchanged within one column. [e] n.d., Not determined. [f] $^{2}J_{P,C}$ (Hz). [g] $^{3}J_{P,C}$ (Hz).

dAc-EPS (compared to a much higher intensity in the analysis of dAc-dGP-EPS) suggests that the 1-phosphoglycerol substituent may be linked to this rhamnose residue in n-EPS, probably via O-4 as suggested by the presence of trace amounts of 4-substituted rhamnose. A poor stoichiometry in the methylation analysis of phosphorylated

Table 2
Methylation analysis data of n-EPS (**1**), dAc-EPS (**2**), dAc-dGP-EPS (**3**), and n-EPS after mild hydrolysis (hyd-EPS)

Derivative	Molar amounts (%)			
	1	**2**	**3**	hyd-EPS
2,3,4-Rha [a]	6	6	15	6
2,4-Rha	22	17	20	19
2,3-Rha	trace	trace	—	—
2,3,4,6-Glc	28	29	20	35
2,3,6-Glc	23	26	25	21
2,3-Glc	—	—	—	15
2-Glc	20	22	20	4

[a] 2,3,4-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-rhamnitol-*1-d*, etc.

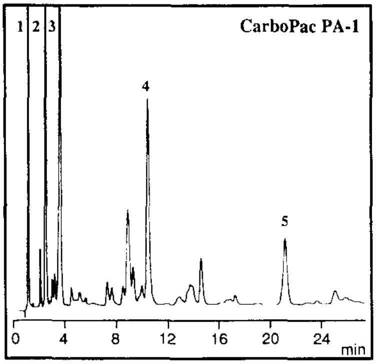
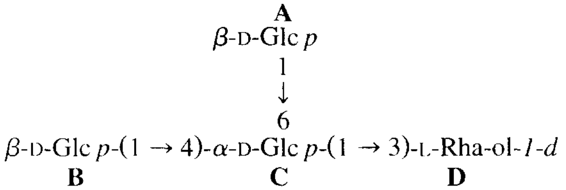


Fig. 3. HPAEC-PAD elution profile on CarboPac PA-1 of a partial acid hydrolysate of n-EPS.

polysaccharides, due to dephosphorylation during methylation and incomplete hydrolysis of phosphate esters, has been reported previously [24].

Partial acid hydrolysis.—A partial acid hydrolysate of n-EPS was fractionated on CarboPac PA-1 (Fig. 3), yielding five major fractions 1–5. Fractions 1, 2, and 3 were identified as glycerol, rhamnose, and glucose, respectively, by ^{1}H NMR spectroscopy and GLC (procedure 3).

Fraction 4 was reduced with NaBD$_4$ and then analyzed by different methods as described below, showing the presence of exclusively the following oligosaccharide alditol-*1-d* **4**:



$$\begin{array}{ccc} & \textbf{A} & \\ & \beta\text{-D-Glc}p & \\ & 1 & \\ & \downarrow & \\ & 6 & \\ \beta\text{-D-Glc}p\text{-}(1\rightarrow & 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow & 3)\text{-L-Rha-ol-}1\text{-}d \\ \textbf{B} & \textbf{C} & \textbf{D} \end{array}$$

4

Quantitative monosaccharide analysis (procedure 1) of **4** showed a composition of glucose and rhamnitol in a molar ratio of 3:1, and methylation analysis demonstrated the presence of 3-substituted rhamnitol-*1-d*, terminal glucopyranose, and 4,6-disubstituted glucopyranose in molar ratios of 1:2:1. The positive-ion mode FAB mass spectrum of permethylated **4** contained an intense $[\mathrm{M}+\mathrm{Na}]^+$ pseudomolecular ion at m/z 872, corresponding to a composition of (Hex$_3$Deoxyhex$_1$)-ol-*1-d*.

GLC-MS of the permethylated **4** (code a(a′)bc-ol; for coding system see [25]) shows fragment ions in the A-series [26] at m/z 219 (aA$_1$/a′A$_1$), m/z 187 (base peak, aA$_2$/a′A$_2$), m/z 155 (aA$_3$/a′A$_3$), m/z 627 [ba(a′)A$_1$], m/z 595 [ba(a′)A$_2$], and m/z 614 (abc-olA$_1$/a′bc-olA$_1$). The absence of A-series ions at m/z 423, 391, 359, and a J$_1$ ion at m/z 470, characteristic for a linear tetrasaccharide-alditol-*1-d*, suggests branching at residue b. In the J-series two intense peaks are observed at m/z 266 (bc-olJ$_1$) and m/z 674 [α(a′)bc-olJ$_1$] [27,28]. The presence of the J$_1$ ion at m/z 266 and the absence of the corresponding J$_0$ ion at m/z 252 [29] suggest that the internal glucose residue is not substituted at O-3, consistent with the methylation analysis. Ions

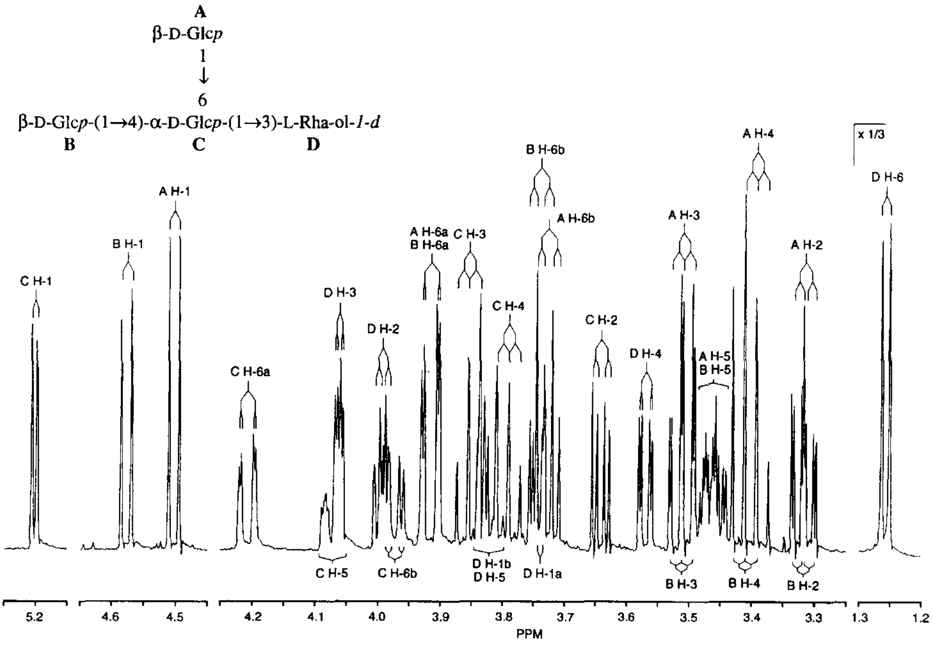


Fig. 4. 500-MHz ^{1}H NMR spectrum of tetrasaccharide-alditol-*1*-*d* **4**, recorded in D_2O at 27°C.

at m/z 59 (CH_3CHOMe), m/z 103 ($CH_3CHOMeCHOMe$), m/z 790 (M − CH_3CHOMe), and m/z 759 (M − CHOMeCHDOMe) confirm the Glc-(1 → 3)-Rha-ol-*1*-*d* linkage. An intense ion at m/z 424 ($C_{19}H_{34}O_{10}D_1$; observed m/z 424.2343, calculated m/z 424.2293) was assigned to an intramolecular rearrangement in residue a of the abc-olA$_1$ ion (abc-olA$_1$ − [CHOMeCHOMe + CH_2OMeCHCHOMe]).

In the 1D ^{1}H NMR spectrum of **4** (Fig. 4) three anomeric signals are observed at δ 5.201 ($^3J_{1,2}$ 3.9 Hz), δ 4.577 ($^3J_{1,2}$ 7.9 Hz), and δ 4.502 ($^3J_{1,2}$ 7.9 Hz), indicating the presence of one α- and two β-glucopyranosyl residues. 2D NMR spectroscopy (COSY, HOHAHA, ROESY) allowed the assignment of all proton resonances, as presented in Table 3. The HOHAHA-tracks for H-1 of the residues **A** and **B** both show cross-peaks with H-2,3,4,5,6a,6b. The H-1 track for residue **C** shows cross-peaks with H-2,3,4,5. Starting from **C** H-5 in the COSY and HOHAHA spectra, **C** H-6a,6b were found. The ^{1}H resonances for rhamnitol-*1*-*d* residue **D** were traced starting from the H-6 protons (δ 1.256, $^3J_{5,6}$ 6.2 Hz) in the HOHAHA spectrum, showing cross-peaks with **D** H-5,4,3. On the HOHAHA track of **D** H-3, cross-peaks with **D** H-2 and with **D** H-1 in both isotopic diastereomers (**D** H-la and **D** H-lb), which were obtained after reduction of the oligosaccharide with $NaBD_4$, are observed. Finally, in the 2D ROESY spectrum of **4** strong interresidual ROE connectivities are observed for **A** H-1,**C** H-6b, for **B** H-1,**C** H-4, and for **C** H-1,**D** H-3, supporting **A**(1 → 6)**C**, **B**(1 → 4)**C**, and **C**(1 → 3)**D** linkages.

Fraction 5 was analyzed by NMR spectroscopy and shown to be 1-phosphoglycerol. The ^{1}H and ^{13}C NMR spectra (sodium salt; ^{1}H: δ 3.765–3.872, m, H-1a,1b,2; δ 3.691, dd, H-3a, $^2J_{3a,3b}$ −11.8 Hz, $^3J_{3a,2}$ 4.4 Hz; δ 3.617, dd, H-3b, $^3J_{3b,2}$ 5.7 Hz; ^{13}C: δ

Table 3

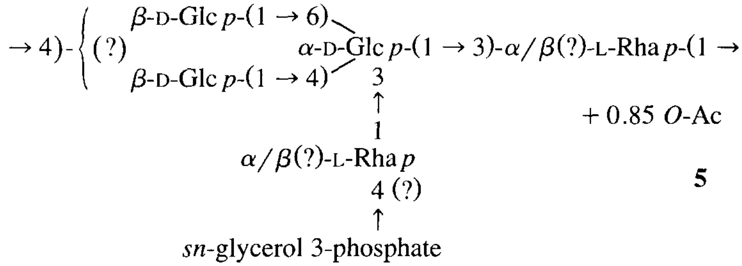
^{1}H NMR chemical shifts[a] of n-EPS (**1**), dAc-EPS (**2**), and dAc-dGP-EPS (**3**), recorded at 80°C, and tetrasaccharide-alditol-*1-d* (**4**), recorded at 27°C. Coupling constants (Hz) are given in brackets

Residue	Proton	**1**	**2**	**3**	**4**
A	H-1	4.486 (7.4)	4.467 (7.4)	4.479 (7.9)	4.502 (7.9)
	H-2	3.32	3.32	3.311 (9.1)	3.315 (9.5)
	H-3	3.49	3.50	3.51	3.509 (9.2)
	H-4	3.40	3.42	3.43	3.393 (9.4)
	H-5	3.43	3.44	3.45	3.457
	H-6a	3.89	3.89	3.92	3.916 (2.2, −12.4)
	H-6b	3.73	3.73	3.74	3.720 (5.8)
B	H-1	4.538	4.579	4.580 (7.5)	4.577 (7.9)
	H-2	3.28	3.31	3.270 (9.1)	3.318 (9.6)
	H-3	3.52	3.59	3.65	3.513 (9.2)
	H-4	3.50	3.51	3.60	3.411 (9.4)
	H-5	3.52	3.51	3.49	3.473
	H-6a	n.d. [b]	n.d.	3.79	3.916 (2.2, −12.4)
	H-6b	n.d.	n.d.	3.84	3.745 (5.6)
C	H-1	5.05	5.073	5.075 (3.2)	5.201 (3.9)
	H-2	3.72	3.76	3.76	3.642 (9.8)
	H-3	4.00	4.05	4.00	3.855 (9.4)
	H-4	3.92	3.92	3.90	3.790 (9.9)
	H-5	4.22	4.19	4.20	4.073
	H-6a	n.d.	4.16	4.19	4.207 (2.0, −11.6)
	H-6b	n.d.	3.96	4.00	3.975
D	H-1a	5.02	4.852	4.852	3.739 [c]
(**D**-ol)	H-1b	—	—	—	3.835 [c]
	H-2	5.621	4.274	4.271	3.997 (4.2)
	H-3	3.88	3.66	3.66	4.062 (2.0)
	H-4	3.52	3.49	3.52	3.569 (8.5)
	H-5	3.52	3.44	3.42	3.833
	H-6	1.384	1.352	1.344 (6.0)	1.256 (6.2)
E	H-1	5.240	5.264	5.207	—
	H-2	4.04	4.06	4.062	—
	H-3	4.06	4.09	3.87	—
	H-4	4.01	4.02	3.45 (9.1)	—
	H-5	4.39	4.41	4.383	—
	H-6	1.308	1.316	1.267 (6.0)	—
F	H-1a	3.92 [d]	3.92 [e]	—	—
	H-1b	4.00 [d]	3.99 [e]	—	—
	H-2	3.90	3.90	—	—
	H-3a	3.700 (3.7/ −11.8)	3.702 (4.0/ −11.8)	—	—
	H-3b	3.621 (5.5/ −11.8)	3.622 (5.6/ −11.8)	—	—
O-Acetyl		2.186	—	—	—

[a] In ppm relative to the signal of internal acetone at δ 2.225. [b] n.d., Not determined. [c-e] Marked assignments may have to be interchanged within one residue.

65.22, C-1; δ 71.25, C-2; δ 62.43, C-3) match those of reference 1-phosphoglycerol [23]. The ^{31}P NMR spectrum shows one signal at δ 4.12, in agreement with the presence of a phosphomonoester [23].

Based on the results obtained thus far, a partial structure **5** can be proposed for n-EPS, as follows:



5

Analysis of the 2D COSY, HOHAHA, NOESY, and HMQC spectra of n-EPS (**1**), dAc-EPS (**2**), and dAc-dGP-EPS (**3**) allowed the determination of (i) the anomeric configurations of the two rhamnose residues; (ii) the location of the *O*-acetyl group; (iii) the attachment site of the 1-phosphoglycerol substituent; (iv) the positions of the two (1 → 4)-linked β-glucose residues.

2D NMR spectroscopy.—The ^{1}H NMR chemical shifts and coupling constants of **3**, **2**, and **1**, as determined from a 2D NMR analysis, are presented in Table 3.

All resonances in the ^{1}H NMR spectrum of dAc-dGP-EPS (**3**, Fig. 1C) were assigned by means of 2D COSY and HOHAHA (mixing times 25, 50, and 100 ms) measurements. As starting points for the interpretation of the 2D spectra, the H-1 signals of the monosaccharide residues **A**–**E** and the methyl signals of the rhamnose residues **D** and **E** were used. As a typical example, the HOHAHA spectrum of **3** with a mixing time of 100 ms is depicted in Fig. 5. The H-1 signals at δ 4.479 ($^3J_{1,2}$ 7.9 Hz) and δ 4.580 ($^3J_{1,2}$ 7.5 Hz) were assigned to the two β-glucopyranose residues **A** and **B**, respectively. The HOHAHA-track of **A** H-1 (δ 4.479) shows the complete series of cross-peaks with **A** H-2,3,4,5,6a,6b. On the HOHAHA track of **B** H-1 (δ 4.580), cross-peaks are observed with **B** H-2,3,4,5. The resonances for **B** H-6a,6b were found via connectivities with **B** H-5 in both the COSY and the HOHAHA spectra. For the identification of the ^{1}H resonances of the rhamnose residues **D** and **E**, the methyl resonances of both residues (δ 1.344, **D** H-6; δ 1.267, **E** H-6) were chosen as starting points. On the HOHAHA tracks of both **D** H-6 and **E** H-6, cross-peaks are found with H-5,4,3,2. The correlation of the rhamnose H-1 signals with these respective spin-coupling systems was made possible by the observation of cross-peaks on the **D** H-1 track (δ 4.852) with **D** H-2 and on the **E** H-1 track (δ 5.207) with **E** H-2,3. The chemical shift of **D** H-5 at δ 3.42 strongly suggests the β configuration for residue **D**. Likewise, the chemical shift of **E** H-5 at δ 4.383 supports the α configuration for residue **E** [30,31]. The remaining anomeric signal at δ 5.075 ($^3J_{1,2}$ 3.2 Hz) was assigned to H-1 of the trisubstituted α-glucopyranose residue **C**. On the HOHAHA **C** H-1 track, the cross-peaks with **C** H-2,3,4,5 are found, whereas in the COSY spectrum the complete spin-coupling system of residue **C**, including H-6a,6b, is observed.

By making use of the ^{1}H NMR data, the 2D ^{13}C–^{1}H HMQC spectrum of dAc-dGP-

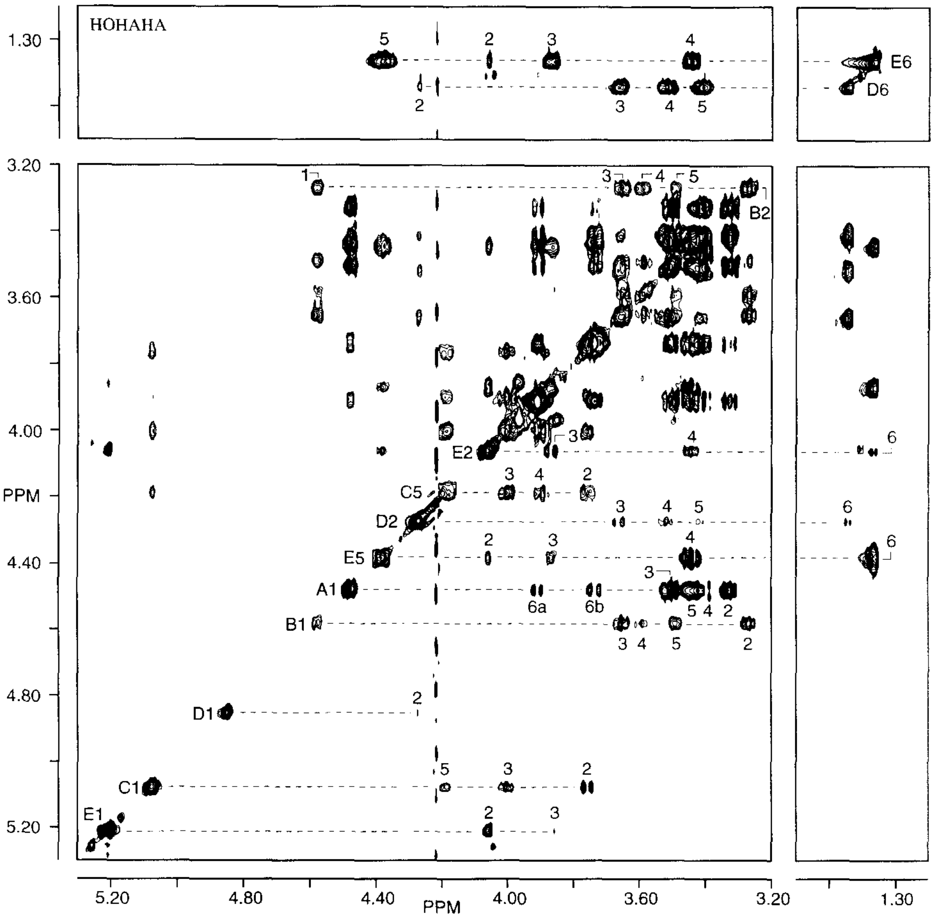


Fig. 5. 500-MHz 2D HOHAHA spectrum (mixing time 100 ms) of dAc-dGP-EPS **3**, recorded in D_2O at 80°C. Diagonal peaks of the anomeric protons, of H-2 of residues **B**, **D**, and **E** of H-5 of residues **C** and **E**, and of H-6 of residues **D**, and **E** are indicated. The numbers near cross-peaks refer to the protons of the scalar-coupling network belonging to a diagonal peak.

EPS (Fig. 6) allowed the complete assignment of the corresponding ^{13}C NMR spectrum (Fig. 2C; Table 4). The observed $^{1}J_{C\text{-}1,H\text{-}1}$ coupling constants of 160–161 Hz for residues **A**, **B**, **D** and 170–171 Hz for **C**, **E** confirm the assignments of the different anomeric configurations made above [32]. Inspection of the ^{13}C data of β-glucopyranose residue **B** demonstrates that this residue is substituted at C-4, since the chemical shift of **B** C-4 (δ 77.11) is ~ 6.5 ppm downfield compared to Me-β-D-Glc*p* ($\delta_{C\text{-}4}$ 70.6 [33]). Furthermore, the low-field chemical shifts for **C** C-3 (δ 76.38), **C** C-4 (δ 73.34), and **C** C-6 (δ 66.89) confirm that residue **C** is substituted at C-3, C-4, and C-6 (Me-α-D-Glc*p*, $\delta_{C\text{-}3}$ 74.1, $\delta_{C\text{-}4}$ 70.6, $\delta_{C\text{-}6}$ 61.6 [33]). Likewise, the downfield chemical shift for **D** C-3 (δ 78.29) shows that rhamnose **D** is substituted at C-3 (Me-β-L-Rha*p*, $\delta_{C\text{-}3}$ 73.0 [33]).

By means of the 2D NOESY spectrum (Fig. 7) of dAc-dGP-EPS (**3**), sequencing of the different monosaccharide residues in the EPS was performed. Evaluation of the

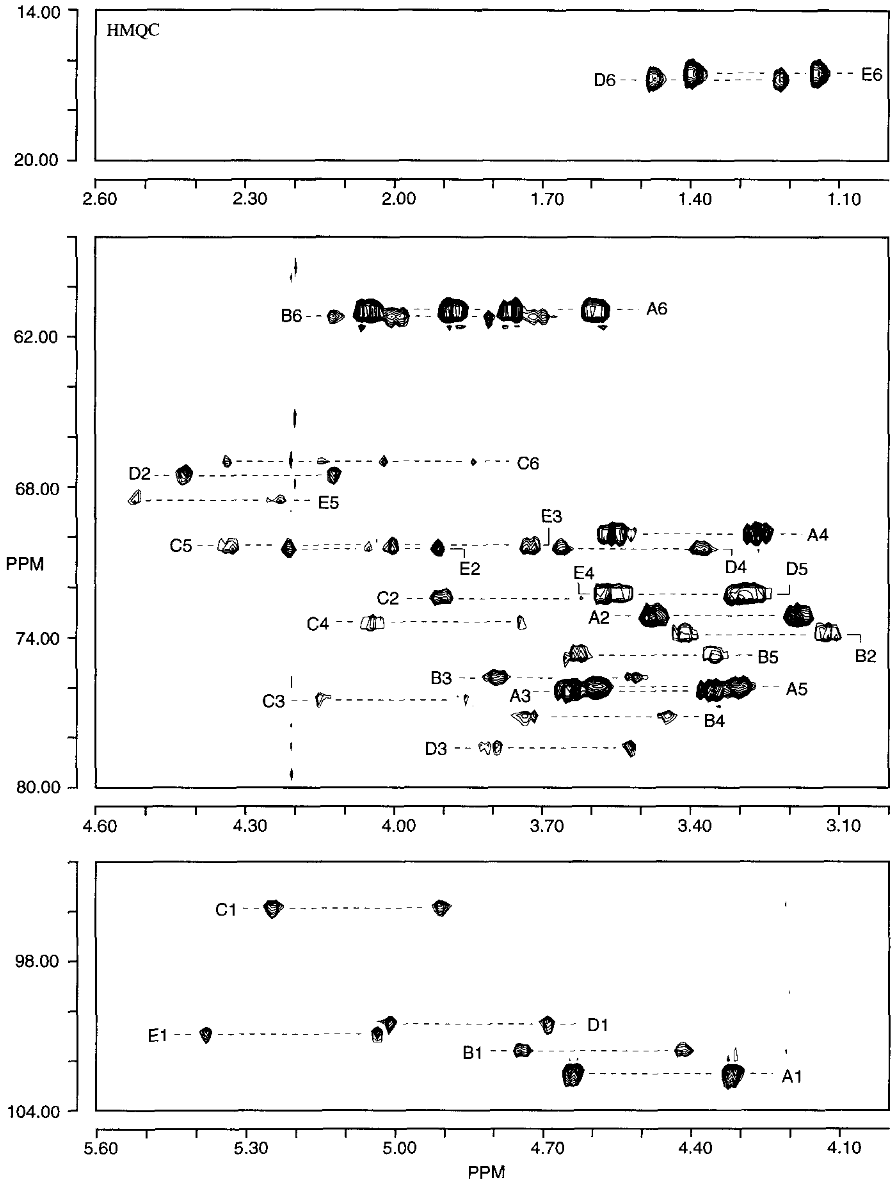


Fig. 6. 500-MHz 2D $^{13}C-^{1}H$ HMQC spectrum of dAc-dGP-EPS **3**, recorded in D_2O at 80°C: A1 denotes the set of cross-peaks between H-1 and C-1 of residue **A**, etc.

NOESY spectrum in the light of the methylation analysis data allowed the unambiguous determination of all linkages in the polysaccharide. The intraresidual connectivities confirm the anomeric configurations for all residues. A strong interresidual connectivity **E** H-1,**C** H-3 indicates the **E**(1 → 3)**C** linkage. On the **C** H-1 track, a strong connectivity is observed with **D** H-2. Since methylation analysis of **3** and the structure of tetrasaccha-

Table 4
^{13}C NMR chemical shifts [a] of dAc-dGP-EPS (**3**), as determined from the 2D HMQC experiment, recorded at 80°C. $^{1}J_{C\text{-}1,H\text{-}1}$ values (Hz) are given in brackets

Residue	C-1	C-2	C-3	C-4	C-5	C-6
A	102.37 (160)	73.06	75.96	69.81	75.87	60.89
B	101.48 (160)	73.73	75.50	77.11	74.55	61.11
C	95.77 (170)	72.27	76.38	73.34	70.26	66.89
D	100.48 (161)	67.43	78.29	70.34	72.18	16.82
E	100.86 (171)	70.38	70.29	72.18	68.34	16.60

[a] In ppm relative to the signal of external methanol at δ 49.00.

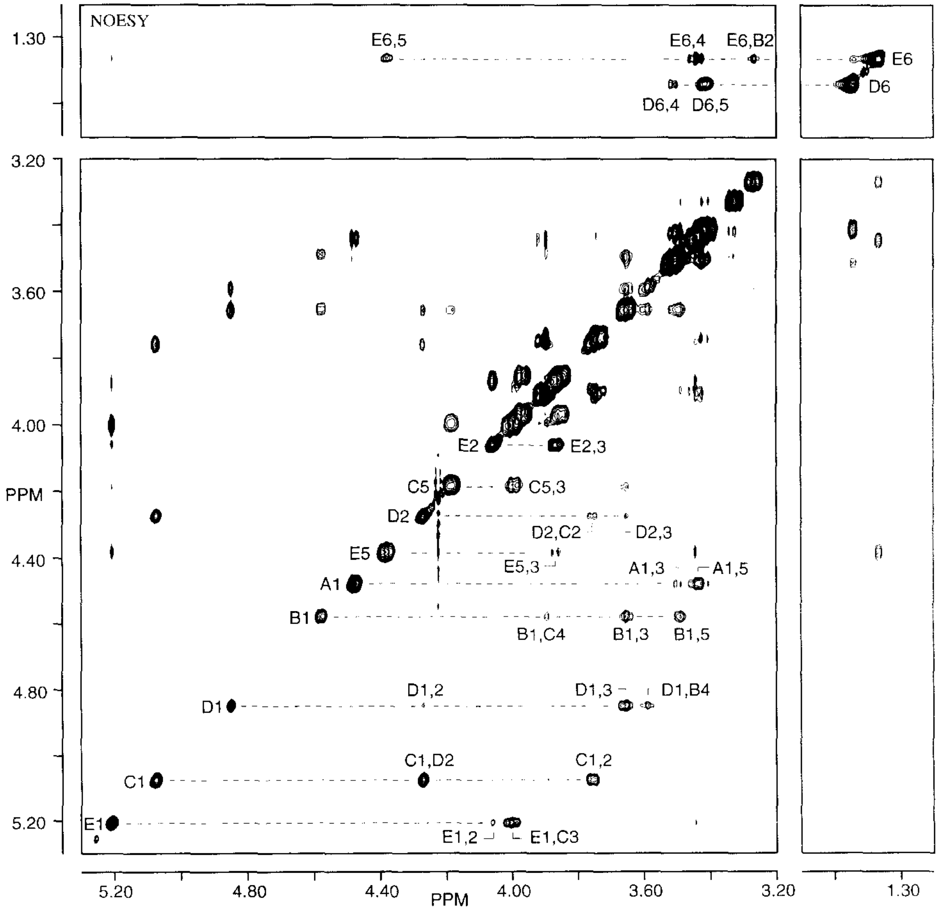


Fig. 7. 500-MHz 2D NOESY spectrum (mixing time 250 ms) of dAc-dGP-EPS **3**, recorded in D_2O at 80°C: E1 corresponds to the diagonal peak belonging to **E** H-1; E1,2 refers to an intraresidual cross-peak between **E** H-1 and **E** H-2, and E1,C3 means an interresidual connectivity between **E** H-1 and **C** H-3, etc.

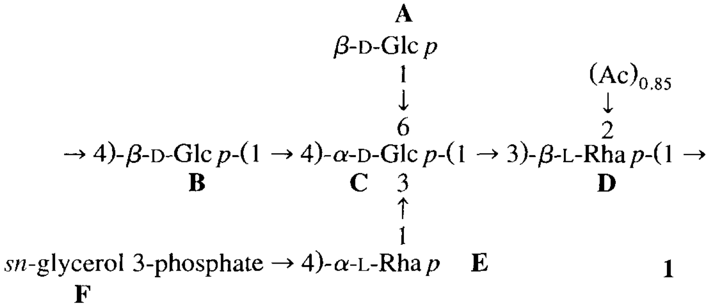
ride-alditol **4** both demonstrated a **C**(1 → 3)**D** sequence (vide supra), this NOE does not reflect the glycosidic linkage. The observation of such a non-glycosidic NOE cross-peak has been reported before for the Glc*p*-α-(1 → 3)-Rha*p* linkage [34]. Furthermore, NOE cross-peaks between **D** H-1 and **B** H-4, and between **B** H-1 and **C** H-4 are observed, demonstrating the **D**(1 → 4)**B**(1 → 4)**C** sequence. The **A** → **C** linkage could not be confirmed by NOESY analysis.

The COSY and HOHAHA spectra of dAc-EPS (**2**) contain the subspectra of the 1-phosphoglycerol moiety **F**. The two intense double doublets of small linewidth (see also Fig. 1B) at δ 3.702 ($^3J_{2,3a}$ 4.0 Hz, $^2J_{3a,3b}$ − 11.8 Hz) and δ 3.622 ($^3J_{2,3b}$ 5.6 Hz, $^2J_{3a,3b}$ − 11.8 Hz) were assigned to **F** H-3a and H-3b, respectively. The COSY spectrum shows strong connectivities between **F** H-3a/3b and **F** H-2 at δ 3.90, and on the track of **F** H-2, cross-peaks with **F** H-1a/1b are observed at δ 3.92 and δ 3.99.

Comparison of the ^{1}H NMR data of dAc-EPS, as deduced from 2D COSY and HOHAHA experiments, with those of dAc-dGP-EPS allowed determination of the position of the 1-phosphoglycerol unit in the repeating structure of the polysaccharide. In the two spectra, most of the ^{1}H resonances of the constituent monosaccharides are similar (within 0.05 ppm), but major differences are found for **E** H-3 ($\Delta\delta + 0.22$) and **E** H-4 ($\Delta\delta + 0.57$), indicating phosphorylation at O-4 of residue **E** [23,35].

The 1D ^{1}H NMR spectrum of n-EPS (**1**, Fig. 1A) reveals the glycerol signals **F** H-3a and **F** H-3b as sharp double doublets at δ 3.700 ($^3J_{2,3a}$ 3.7 Hz, $^2J_{3a,3b}$ − 11.8 Hz) and δ 3.621 ($^3J_{2,3b}$ 5.5 Hz), respectively, and via the COSY spectrum the signals for **F** H-2 (δ 3.90) and **F** H-1a/1b (δ 4.00 and δ 3.92) could be traced. By comparison of the ^{1}H NMR data of n-EPS, as determined from 2D COSY and HOHAHA experiments, with those of dAc-EPS, the site of partial *O*-acetylation could be determined. Most of the ^{1}H resonances in the spectrum of n-EPS have chemical shifts that are close to the corresponding signals in the spectrum of dAc-EPS, with the exception of the signals of residue **D**. The differences in chemical shift for **D** H-1 ($\Delta\delta + 0.17$), **D** H-2 ($\Delta\delta + 1.347$), and **D** H-3 ($\Delta\delta + 0.22$) demonstrate that n-EPS is acetylated at O-2 of residue **D**. It should be noted that the structural element α-D-Glc*p*/Gal*p*-(1 → 3)-[2-*O*-acetyl]-β-L-Rha*p*-(1 → 4)-β-D-Glc*p* is also present in capsular polysaccharides isolated from *Streptococcus pneumoniae* type 18F and 45 [21,36]. The chemical shifts of the β-rhamnose residues in these polysaccharides, before and after *O*-deacetylation, are within 0.05 ppm of those of the β-rhamnosyl residues **D** in n-EPS and dAc-EPS/dAc-dGP-EPS, respectively. Finally, the assignment of the low-field signal at δ 5.621 to H-2 of the *O*-acetylated rhamnosyl residue **D** was confirmed by the observation of a strong intraresidual connectivity **D** H-1,H-2 in the NOESY spectrum of n-EPS.

From the combined evidence, the EPS produced by *Lactobacillus sake* 0-1 was deduced to be composed of pentasaccharide repeating units with the following structure:



$$\begin{array}{ccccc}
 & & \textbf{A} & & \\
 & & \beta\text{-D-Glc}p & & \\
 & & 1 & & (\text{Ac})_{0.85}\\
 & & \downarrow & & \downarrow\\
 & & 6 & & 2\\
\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1 & \rightarrow & 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1 & \rightarrow & 3)\text{-}\beta\text{-L-Rha}p\text{-}(1 \rightarrow\\
\textbf{B} & & \textbf{C}\;\;3 & & \textbf{D}\\
 & & \uparrow & & \\
 & & 1 & & \\
sn\text{-glycerol 3-phosphate} & \rightarrow & 4)\text{-}\alpha\text{-L-Rha}p\;\;\textbf{E} & & \\
\textbf{F} & & & &
\end{array}$$

1

An interesting feature, observed in both the ^{1}H and ^{13}C NMR spectra of the investigated polysaccharides n-EPS, dAc-EPS, and dAc-dGP-EPS, is the significant difference in linewidth between the signals of the constituent residues within one spectrum, reflecting significant differences in mobility of the various residues. For instance, inspection of the anomeric region of the 1D ^{1}H NMR spectrum of n-EPS (Fig. 1A) demonstrates that the H-1 signals of the side-chain residues **A** and **E** are relatively sharp, whereas those of the backbone residues **B**, **C**, and **D** are much broader. In the bulk region of the spectrum, the signals for **F** H-3a/3b are very sharp, demonstrating the high mobility of the glycerol residue.

In the 2D NOESY spectra of n-EPS, dAc-EPS, and dAc-dGP-EPS, strong interresidual connectivities are observed between **E** H-6 (methyl group) and **B** H-2. These long-range NOE contacts suggest for all three polysaccharides a conformation in which residue **E** is close to residue **B**. When building a model of the repeating unit of the polysaccharide, it is clear that in such a conformation **E** H-5 is close to three oxygen atoms, namely, the ring oxygens of residues **B** and **E**, and the oxygen of the **B** → **C** glycosidic bond. This may explain why the chemical shift for **E** H-5 (δ 4.383) is more downfield than is usually observed for H-5 in an α-rhamnosyl residue [30,31]. In the 2D NOESY spectra of n-EPS and dAc-EPS, strong interresidual connectivities between **E** H-6 and **F** H-2 provide further evidence for the proximity of residue **F** to residue **E**.

The native and the *O*-deacetylated EPSs have similar viscosities and shear thinning properties. Furthermore, their ^{1}H NMR spectra show a similar resolution. However, the *O*-deacetylated, deglycerophosphorylated EPS is much less viscous and the resolution of its ^{1}H NMR spectrum is much better, which allowed the recording of well-resolved 2D NMR spectra. Therefore, it can be assumed that the 1-phosphoglycerol moiety contributes significantly to the specific rheological properties of the "0-1" exopolysaccharide.

Acknowledgements

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References

[1] T. Doco, J.-M. Wieruszeski, and B. Fournet, *Carbohydr. Res.*, 198 (1990) 313–321.

[2] H. Nakajima and T. Hirota, *Carbohydr. Res.*, 224 (1992) 245–253.

[3] M. Gruter, B.R. Leeflang, J. Kuiper, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 231 (1992) 273–291.

[4] M. Gruter, B.R. Leeflang, J. Kuiper, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 239 (1993) 209–226.

[5] Y. Yamamoto, S. Murosaki, R. Yamauchi, K. Kato, and Y. Sone, *Carbohydr. Res.*, 261 (1994) 67–78.

[6] D.J.C. van den Berg, G.W. Robijn, A.C. Janssen, M.L.F. Giuseppin, R. Vreeker, J.P. Kamerling, J.F.G. Vliegenthart, A.M. Ledeboer, and C.T. Verrips, *Appl. Environ. Microbiol.*, in press.

[7] B. Tinland, J. Mazet, and M. Rinaudo, *Makromol. Chem.*, 9 (1988) 69–73.

[8] G. Rouser, S. Fleischer, and A. Yamamoto, *Lipids*, 5 (1969) 494–496.

[9] J.P. Kamerling and J.F.G. Vliegenthart, in A.M. Lawson (Ed.), *Clinical Biochemistry— Principles, Methods, Applications. Vol. 1, Mass Spectrometry*, Walter de Gruyter, Berlin, 1989, pp 176–263.

[10] M.F. Chaplin, *Anal. Biochem.*, 123 (1982) 336–341.

[11] G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.

[12] G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 77 (1979) 1–7.

[13] M.A.J. Ferguson, in M. Fukuda and A. Kobata (Eds.), *Glycobiology: A Practical Approach, Vol. 125, GPI Membrane Anchors: Isolation and Analysis*, Oxford University Press, Oxford, 1993, pp 351–383.

[14] P. Prehm, *Carbohydr. Res.*, 78 (1980) 372–374.

[15] L.M. Beynon and J.C. Richards, *Carbohydr. Res.*, 252 (1994) 263–268.

[16] I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.

[17] P.-E. Jansson, L. Kenne, H. Liedgren, B. Lindberg, and J. Lönngren, *Chem. Commun., Univ. Stockholm*, 8 (1976).

[18] K. Hård, G. van Zadelhoff, P. Moonen, J.P. Kamerling, and J.F.G. Vliegenthart, *Eur. J. Biochem.*, 209 (1992) 895–915.

[19] A. Bax, R.H. Griffey, and B.L. Hawkings, *J. Magn. Reson.*, 55 (1983) 301–315.

[20] D.J.C. van den Berg, A. Smits, B. Pot, A.M. Ledeboer, K. Kesters, J.M.A. Verbakel, and C.T. Verrips, *Food Biotechnol.*, 7 (1993) 189–205.

[21] P.-E. Jansson, B. Lindberg, J. Lindberg, and J. Henrichsen, *Carbohydr. Res.*, 173 (1988) 217–225.

[22] C. Lugowski and H.J. Jennings, *Carbohydr. Res.*, 131 (1984) 119–129.

[23] M. Moreau, J.C. Richards, and M.B. Perry, *Biochemistry*, 27 (1988) 6820–6829.

[24] P.-E. Jansson, N.S. Kumar, B. Lindberg, and G. Widmalm, *Carbohydr. Res.*, 173 (1988) 227–233.

[25] J. Lönngren and S. Svensson, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 41–106.

[26] N.K. Kochetkov and O.S. Chizhov, *Adv. Carbohydr. Chem.*, 21 (1966) 39–94.

[27] J. Kärkkäinen, *Carbohydr. Res.*, 17 (1971) 1–10.

[28] J. Kärkkäinen, *Carbohydr. Res.*, 17 (1971) 11–18.

[29] J.K. Sharp and P. Albersheim, *Carbohydr. Res.*, 128 (1984) 193–202.

[30] A. de Bruyn, M. Anteunis, and R. de Gussem, *Carbohydr. Res.*, 47 (1976) 158–163.

[31] P.-E. Jansson, J. Lindberg, K.M.S. Wimalasiri, and M.A. Dankert, *Carbohydr. Res.*, 245 (1993) 303–310.

[32] K. Bock and C. Pedersen, *J. Chem. Soc., Perkin Trans. 2*, (1974) 293–297.

[33] K. Bock and C. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 27–66.

[34] G.M. Lipkind, A.S. Shashkov, S.S. Mamyan, and N.K. Kochetkov, *Carbohydr. Res.*, 181 (1988) 1–12.

[35] J.C. Richards and M.B. Perry, *Biochem. Cell Biol.*, 66 (1988) 758–771.

[36] M. Moreau, J.C. Richards, and M.B. Perry, *Carbohydr. Res.*, 182 (1988) 79–99.