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Structural characterization of the exopolysaccharide produced by *Lactobacillus acidophilus* LMG9433

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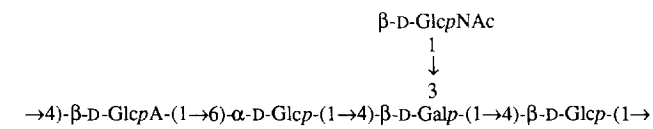
Abstract

The exopolysaccharide produced by *Lactobacillus acidophilus* LMG9433 in a semi-defined medium was found to be a charged heteropolymer, with a composition of D-glucose, D-galactose, D-glucuronic acid, and 2-acetamido-2-deoxy-D-glucose in molar ratios of 2:1:1:1. By means of methylation analysis, uronic acid degradation, de-*N*-acetylation/deamination, partial acid hydrolysis, and 1D/2D NMR studies the polysaccharide was demonstrated to consist of repeating units with the following structure:



Keywords: Lactic acid bacteria; *Lb. acidophilus*; Exopolysaccharide structure

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1. Introduction

In the food industry, microbial exopolysaccharides (EPSs) are widely applied as thickening, gelling, and stabilizing agents [1]. In the search for a new generation of 'green' food thickeners, much attention is currently being given to EPSs produced by lactic acid bacteria. Because these microorganisms are food grade, the application of the excreted EPSs into food products is expected to be very promising.

In order to obtain insight into the relation between the physico-chemical properties and the three-dimensional structures of these polysaccharides, knowledge of the primary structures is a prerequisite. In this framework, since 1990 several structural studies have been reported on EPSs produced by different strains of lactic acid bacteria [2–10]. Here we report the structural analysis of the exopolysaccharide produced by *Lb. acidophilus* LMG9433 in a semi-defined medium.

2. Experimental

Production, isolation and purification of the exopolysaccharide.—The production and isolation of the EPS from *Lb. acidophilus* LMG9433 was carried out as previously described for the EPS from *Lb. paracasei* 34-1 [10]. The crude material was dissolved in water and subsequently 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). The protein content of the purified sample was determined with the Pierce protein assay reagent, using bovine serum albumin as a standard.

Monosaccharide and methylation analysis.—For monosaccharide analysis, poly- or oligosaccharides were methanolyzed, followed by trimethylsilylation and GLC analysis as described [8,11,12]. The absolute configurations of the monosaccharides were determined according to refs. [13,14]. For methylation analysis, samples were permethylated according to a modified Hakomori procedure, using potassium methylsulfinylmethanide and methyl iodide [15]. Then, the permethylated material was carboxyl reduced with 1 M $Li(Et_3)BD$ in THF (200 μL) [16,17] and subsequently hydrolyzed with CF_3CO_2H. After reduction with $NaBD_4$, and acetylation with Ac_2O [8], the partially methylated alditol acetates were analyzed by GLC–EIMS on DB-1 [12,18].

Uronic acid degradation.—To a solution of carefully dried methylated polysaccharide (0.5 mg) in DMSO (200 μL), containing 2,2-dimethoxypropane (25 μL) and a trace of *p*-toluenesulfonic acid, was added potassium methylsulfinylmethanide (250 μL, 1 M), and the mixture was stirred for 1 h. Then, the sample was cooled on ice, and CD_3I (125 μL) was added. After removal of the ice-bath and stirring for 10 min at room temperature, the mixture was diluted with water (1 mL), and the product was extracted with CH_2Cl_2 [19]. The material obtained was analyzed by FAB–MS and GLC–EIMS on DB-5, before and after conversion into partially methylated alditol acetates.

De-N-acetylation.—A solution of polysaccharide (10 mg) in anhydrous hydrazine (1 mL), containing a trace of hydrazine sulfate, was kept for 4 h at 95 °C [20]. Then, the sample was concentrated in vacuo, and residual hydrazine was removed by repeated

coevaporation with toluene. The residue was dissolved in water (0.75 mL) and the solution was desalted by gel filtration on a High Trap Desalting column (Sephadex G-25 Superfine, bed volume 2 × 5 mL, Pharmacia), eluted with 5 mM NH_4HCO_3, at a flow rate of 2 mL/min. De-*N*-acetylated polysaccharide (7 mg) was recovered by lyophilization.

Deamination of the de-N-*acetylated EPS.*—De-*N*-acetylated polysaccharide (7 mg) was treated with 3.9 M $NaNO_2$/0.28 M HOAc (1.6 mL; pH 4.1) for 2 h at room temperature [21,22]. After cooling on ice, the solution was adjusted to pH 8.0 with 2 M NH_4OH, and subsequently treated with $NaBH_4$ (20 mg) for 3 h at room temperature. The residue obtained after conventional work-up was desalted on a High Trap Desalting column as described above.

Partial acid hydrolysis.—Native polysaccharide (5 mg) was hydrolyzed in 1.0 M CF_3CO_2H (5 mL) for 2 h at 100 °C. Then, the solution was lyophilized, and the residue was fractionated by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex LC system, equipped with a CarboPac PA-1 pellicular anion-exchange column (25 cm × 9 mm). The column was eluted with a gradient of NaOAc in 0.1 M NaOH (20–165 mM NaOAc in 17 min, followed by 165–190 mM NaOAc in 5 min) at a flow rate of 4 mL/min. PAD-detection was carried out according to [8]. Immediately after collection, the fractions were neutralized with 2 M HOAc, lyophilized, re-*N*-acetylated (fractions I and II) with Ac_2O (25 μL) in saturated aq $NaHCO_3$ (1 mL), and finally desalted on a cation-exchange resin (Dowex AG 50W-X12, 100–200 mesh, H^+-form, Bio-Rad).

Gas–liquid chromatography and mass spectrometry.—GLC and GLC–EIMS analyses of trimethylsilylated (methyl ester) methyl glycosides and partially methylated alditol acetates were performed with a Varian 3700 gas chromatograph and a Fisons MD800/8060 system, respectively, using conditions as described previously [9]. GLC–EIMS and positive-ion mode FAB–MS analyses of the products obtained from uronic acid degradation of the permethylated polysaccharide (UD-EPS) were carried out on a JEOL JMS-AX505W instrument and a JEOL JMS-SX/SX102A tandem mass spectrometer, respectively, essentially according to [8]. GLC–MS of UD-EPS was performed with a DB-5 fused-silica capillary column, using the following oven conditions: on-column injection at 60 °C; 60–250 °C at 30 °C/min; 250–320 °C at 10 °C/min; thereafter isothermal elution at 320 °C.

NMR spectroscopy.—Proton-decoupled 75.469 MHz ^{13}C NMR spectra were recorded in D_2O on a Bruker AC-300 spectrometer at probe temperatures of 67 °C or 80 °C. ^{13}C Chemical shifts are referenced to external MeOH (δ 49.00). 1D ^1H and 2D NMR spectra were recorded on a Bruker AMX-500 or a Bruker AMX-600 spectrometer at probe temperatures of 10 °C, 27 °C, 57 °C, or 80 °C. 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). ^1H Chemical shifts are expressed in ppm by reference to internal acetone (δ 2.225). Suppression of the HOD signal was achieved either by applying a WEFT pulse sequence [23] in 1D ^1H NMR experiments, or by presaturation for 1s in 2D NMR experiments.

2D HOHAHA spectra were recorded using MLEV-17 mixing sequences with an effective spin-lock time of 25–125 ms. 2D NOESY experiments were performed with a

mixing time of 200 ms. A homospoil pulse of 10 ms, followed by a recovery of 30 ms was applied during the mixing time. DQF-COSY spectra were collected according to [24]. 2D ROESY spectra were performed with mixing times of 200–250 ms. The spin-lock field strength corresponded to a 90° pulse width of about 110 μs. Phase-sensitive $^{13}C-^{1}H$ 2D HMQC experiments with inverse detection [25] were carried out at a ^{1}H frequency of 500.139 MHz (125.769 MHz for ^{13}C), using a TANGO pulse sequence to suppress signals stemming from ^{12}C)-bound protons. ^{13}C Decoupling was not applied during the acquisition of the ^{1}H FID. A proton-detected $^{13}C-^{1}H$ 2D HMBC experiment was performed at a ^{1}H frequency of 600.140 MHz (150.916 MHz for ^{13}C) using the pulse sequence described in [26]. The delay time for the detection of long-range $^{13}C-^{1}H$ couplings was set to 60 ms. All 2D NMR data were processed on Silicon Graphics IRIS workstations (Indigo and Indigo 2), using TRITON software (Bijvoet Center, Department of NMR spectroscopy).

3. Results and discussion

Purification and composition of the polysaccharide.—The crude exopolysaccharide produced by *Lb. acidophilus* LMG9433 in a semi-defined medium was purified by gel filtration chromatography on Sephacryl S-500, which afforded the EPS in a broad void volume peak. The protein content of the purified material was determined to be less than 1%.

Monosaccharide analysis of native EPS (**1**), including the determination of absolute configurations, revealed the presence of D-glucose, D-galactose, D-glucuronic acid, and 2-acetamido-2-deoxy-D-glucose in molar ratios of 2:1:1:1. Methylation analysis of **1** (Table 1), including carboxyl reduction of the permethylated EPS prior to acid hydrolysis, demonstrated the presence of 4-substituted glucose, 6-substituted glucose, 3,4-disubstituted galactose, 4-substituted glucuronic acid, and terminal 2-acetamido-2-de-

Table 1
Methylation analysis data (including carboxyl reduction prior to acid hydrolysis) of native EPS (**1**), de-*N*-acetylated, deaminated EPS (**3**), disaccharide alditol-*1-d* (**4**), and uronic acid degraded EPS (UD-EPS)

	Detector response			
Derivative [a]	**1**	**3**	**4**	UD-EPS
1,2,3,4,5-Glc	—	—	0.8	—
2,3,4,6-Glc [b]	—	—	—	0.9
2,3,6-Gal	—	0.9	—	—
2,3,6-Glc	1.0	1.0	—	0.4
2,3,4-Glc	0.9	1.0	—	—
2,3,4-Glc [c]	—	—	1.0	—
2,6-Gal	1.0	0.1	—	1.2
2,3-Glc [c]	0.8	1.0	—	—
2,3,4,6-GlcNAc	0.8	—	—	0.9

[a] 1,2,3,4,5-Glc = 6-*O*-acetyl-1,2,3,4,5-penta-*O*-methyl-D-glucitol-*1-d*, etc.
[b] Labeled with CD_3 at O-6.
[c] Labeled with D_2 at C-6.

oxyglucose in approximately equimolar amounts. According to NMR experiments (vide infra) all residues are in the pyranose ring form.

The 1D ^{1}H NMR spectrum of **1** (Fig. 1A) showed five signals in the anomeric region (δ 5.0–4.4) suggesting a pentasaccharide repeating unit. The five monosaccharide residues in the native EPS were arbitrarily designated **A**–**E**, as indicated in the spectrum. The singlet originating from the protons of the *N*-acetyl group of the GlcNAc residue could be observed at δ 2.028. Each of the residues **B**, **C**, **D**, and **E** was allocated the pyranose ring form and b configuration based on the observed $^3J_{1,2}$ values of about 8 Hz for the corresponding anomeric signals. Residue **A** ($^3J_{1,2}$ 2.7 Hz) was assigned the a configuration and pyranose ring form. The 1D ^{13}C NMR spectrum of **1** (Fig. 2A) contained five signals in the anomeric region (δ 105–95), confirming the proposed pentasaccharide repeating unit. Typical signals for the GlcNAc residue appeared at δ 22.42 (NDCO*C*H$_3$) and δ 56.01 (C-2). Furthermore, three signals at δ 60.21, 60.51, and 61.66, clearly reflected the presence of three hydroxymethyl carbons.

De-N-*acetylation / deamination.*—Treatment of **1** with anhydrous hydrazine resulted in almost complete de-*N*-acetylation (> 95%), as judged by ^{1}H (Fig. 1B) and ^{13}C (Fig. 2B) NMR spectroscopy. The 1D ^{1}H NMR spectrum of the de-*N*-acetylated EPS (**2**; Fig. 1B) contained a typical signal at δ 2.937 which could be assigned to H-2 of the Glc*p*NH$_2$ residue. Deamination, followed by borohydride reduction of **2** resulted in a linear polysaccharide as demonstrated by methylation analysis and NMR spectroscopy. Methylation analysis, including carboxyl reduction, of the de-*N*-acetylated, deaminated EPS (**3**) showed a composition of 4-substituted glucopyranose, 6-substituted glucopyranose, 4-substituted galactopyranose, and 4-substituted glucopyranuronic acid (Table 1), which proved that in the native EPS (**1**) the terminal GlcNAc residue was linked to O-3 of the 3,4-disubstituted galactopyranosyl residue. The 1D ^{1}H and ^{13}C NMR spectra of **3** (Fig. 1C and Fig. 2C, respectively) each contained four anomeric signals, in agreement with a tetrasaccharide repeating structure.

Partial acid hydrolysis.—A partial acid hydrolysate of **1** was fractionated on CarboPac PA-1 (Fig. 3), yielding three major fractions I–III. Re-*N*-acetylated fraction I consisted of only GlcNAc as determined by GLC(–MS) analysis, and fraction II was demonstrated to be a mixture of Gal and Glc by GLC(–MS) and ^{1}H NMR spectroscopy. Monosaccharide analysis of fraction III revealed a composition of Glc and GlcA in a molar ratio of 1:1. After reduction with NaBD$_4$, fraction III was analyzed by methylation analysis including carboxyl reduction (Table 1), which demonstrated 6-substituted Glc-ol-*1-d* and terminal Glc*p*A, suggesting a disaccharide alditol. The 1D ^{1}H NMR spectrum of fraction III contained one anomeric signal at δ 4.496 ($^3J_{1,2}$ 8.0 Hz), which established fraction III to contain a single disaccharide alditol with the following structure:

β-D-Glc*p*A-(1→6)-D-Glc-ol-*1-d* **4**

D **A**-ol

A complete assignment of all ^{1}H resonances of **4** as obtained from 2D COSY and HOHAHA experiments is presented in Table 2. Interresidual cross-peaks between **D** H-1 and **A**-ol H-6a/6b in the 2D ROESY spectrum of **4** confirmed the 1 → 6 linkage.

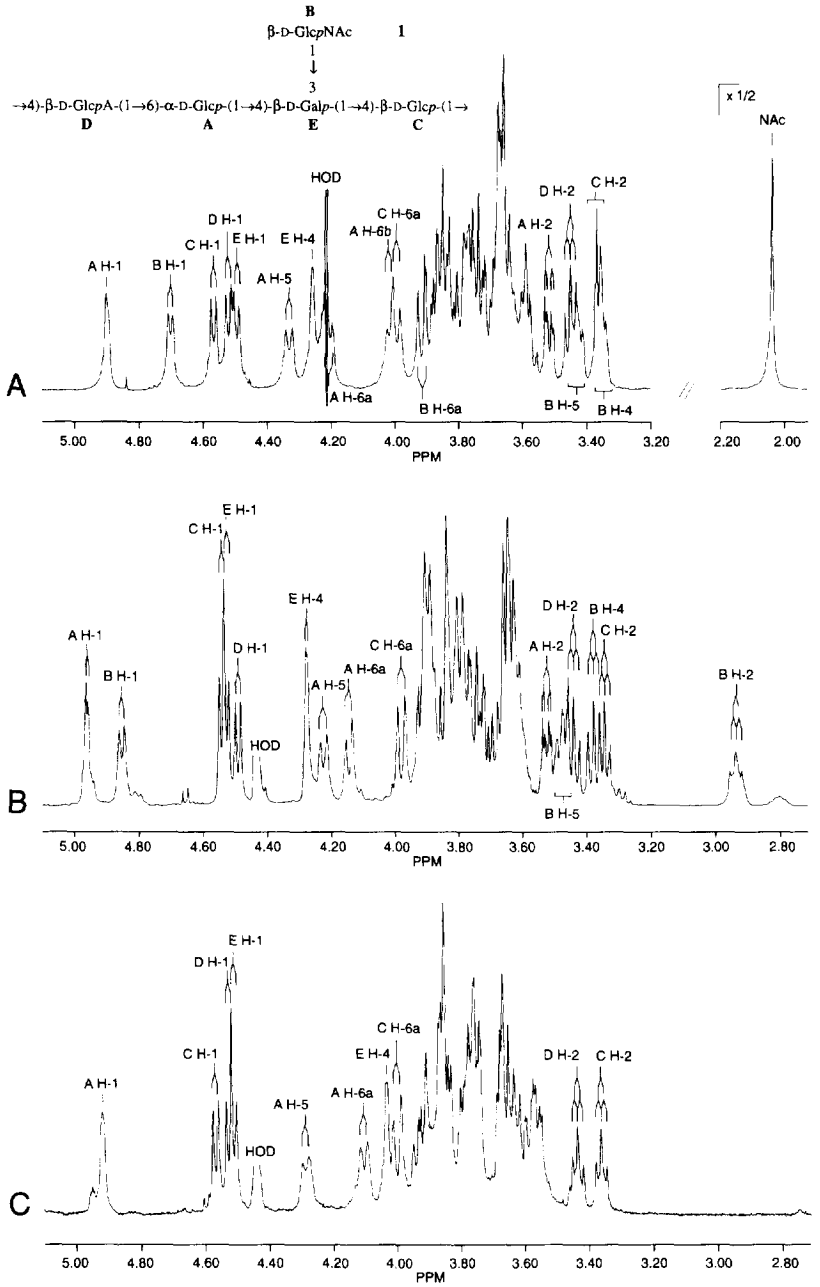
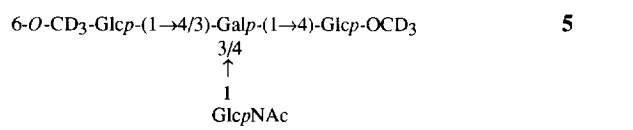


Fig. 1. 500 MHz ^{1}H NMR spectra of (A) native EPS (**1**), (B) de-*N*-acetylated EPS (**2**), and (C) de-*N*-acetylated, deaminated EPS (**3**), recorded in D_2O at 80 °C (A) or 57 °C (B and C).

Uronic acid degradation.—The product obtained after treatment of permethylated **1** with potassium methylsulfinylmethanide, followed by remethylation with CD_3I (UD-EPS) was analyzed as described below, demonstrating the presence of mainly the following permethylated tetrasaccharide:



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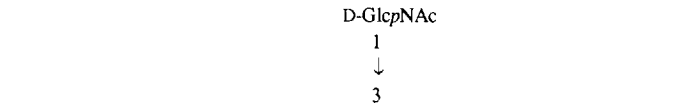
Hydrolysis of UD-EPS, followed by reduction, acetylation, and GLC–MS analysis of the resulting alditol acetates (Table 1) revealed the presence of 4-substituted Glc *p*, 3,4-disubstituted Gal *p*, terminal Glc *p*NAc, and terminal Glc *p*. The alditol acetate derivative corresponding with the terminal nonreducing Glc *p* residue was trideuteriomethylated at O-6, demonstrating that in the native EPS GlcA was linked to this position. The positive-ion mode FAB mass spectrum of UD-EPS contained two intense pseudomolecular ions at *m/z* 910 ($[M + H]^+$) and *m/z* 932 ($[M + Na]^+$), corresponding to a composition of $HexNAc(CD_3Hex)_2Hex$. GLC–MS of UD-EPS revealed two main components **5a** and **5b** (α and β anomers of **5**) in an approximate molar ratio of 2:1, eluting at 30.5 and 31.1 min, respectively, which had essentially identical mass spectra. The mass spectrum of **5a** (code: a(b)cd, in which b is the GlcNAc residue; for coding system see [27]) showed ions in the A-series [28] at *m/z* 222 (aA_1/dA_1), *m/z* 190 (base peak, aA_2/dA_2), *m/z* 155/158 (aA_3/dA_3), *m/z* 260 (bA_1), *m/z* 228 (bA_2), *m/z* 671 ($c(b)aA_1/c(b)dA_1$), and at *m/z* 639 ($c(b)aA_2/c(b)dA_2$). Furthermore, in the J-series two intense ions were detected at *m/z* 731 ($a(b)cdJ_1$) and *m/z* 693 ($b(a)cdJ_1$). The absence of A-series ions at *m/z* 426 (Hex–Hex-) and *m/z* 467 (HexNAc–Hex-) confirmed branching in **5** as indicated above. Finally, two intense high-mass ions were found at *m/z* 836 ($M-CH_3CONHCH_3$) and *m/z* 805 ($M-CH_3CONHCH_3-CH_3O$), which agreed with the proposed tetrasaccharide structure.

The combination of the results obtained thus far allowed the following partial structure to be proposed for the repeating unit of the EPS:



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In order to establish the anomeric configurations of the different monosaccharide residues in the EPS, and to obtain independent support for the sequence of the different residues as deduced from the chemical experiments described above, a 2D NMR analysis was performed on native EPS **1**, de-*N*-acetylated EPS **2**, and de-*N*-acetylated, deaminated EPS **3**.

2D NMR spectroscopy.—By means of 2D COSY, HOHAHA, NOESY, $^{13}C-^{1}H$ HMQC, and $^{13}C-^{1}H$ HMBC experiments, all ^{1}H and ^{13}C resonances in the 1D ^{1}H (Fig. 1) and ^{13}C (Fig. 2) NMR spectra of **1**, **2**, and **3** could be assigned (Tables 2 and 3).

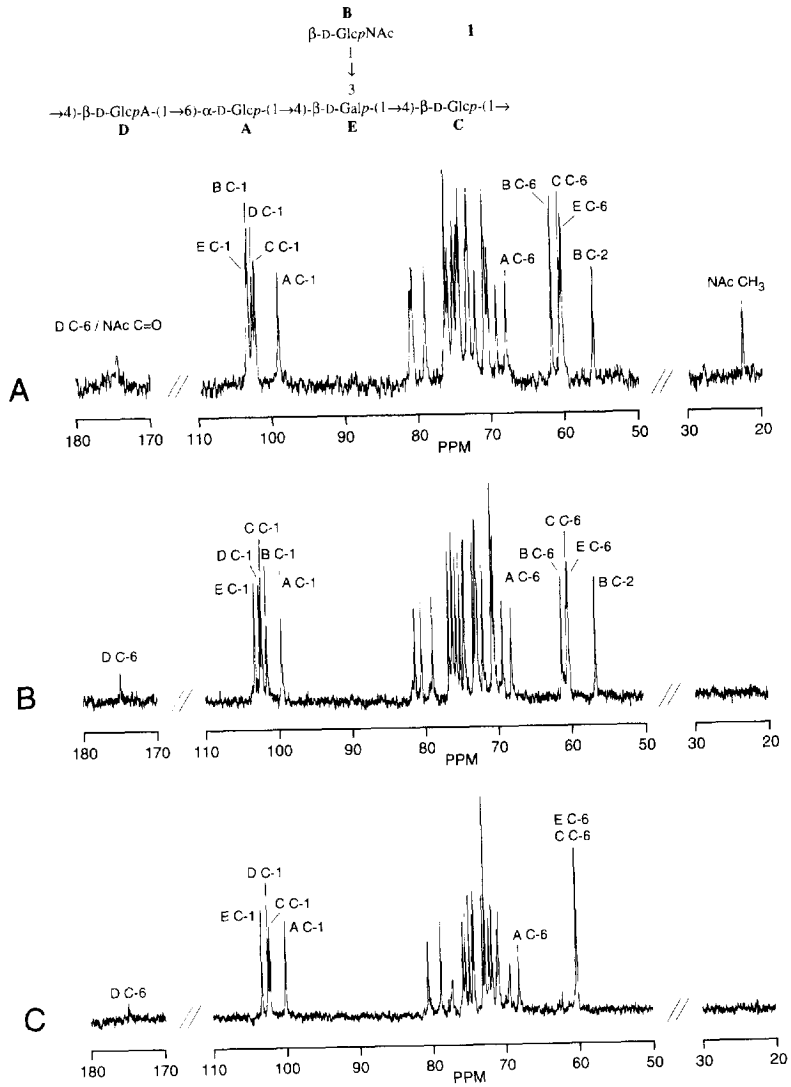


Fig. 2. 75 MHz ^{13}C NMR spectra of (A) native EPS (**1**), (B) de-*N*-acetylated EPS (**2**), and (C) de-*N*-acetylated, deaminated EPS (**3**), recorded in D_2O at 80 °C (A) or 67 °C (B and C).

In the 1D ^{1}H NMR spectrum of native EPS **1**, most of the ^{1}H resonances (H-2,3,4,5 of residues **A**, **C**, and **D**; H-2,3,4,5,6a,6b of residue **B**; H-2,3,4 of residue **E**) were assigned via connectivities with the correlative H-1 signals in the HOHAHA (Fig. 4) and COSY (not shown) spectra; the corresponding ^{13}C signals could be detected via correlation to the ^{1}H signals in the HMQC spectrum (not shown). The resonances of **A** H-6a,6b were determined via intraresidual connectivities with **A** H-5 in the NOESY spectrum of **1** (Fig. 5A), and the signals of **C** H-6a,6b were assigned from connectivities **C** H-5,6a,6b as observed on the HOHAHA **C** H-6a track. Again, the ^{13}C resonances **A**

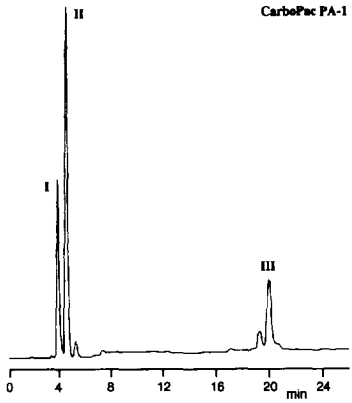


Fig. 3. HPAEC-PAD elution profile on CarboPac PA-1 of a partial acid hydrolysate of native EPS **1**.

C-6 and **C** C-6 were determined via correlation to the corresponding ^{1}H signals in the HMQC spectrum. The chemical shift of **E** H-5 was established via intraresidual NOE connectivities with both **E** H-1 and **E** H-4, and the **E** H-5,C-5 connectivity in the HMQC spectrum allowed the assignment of **E** C-5. Finally, the resonances of **E** H-6a,6b were determined via correlation with **E** C-6 (δ 60.21) in the HMQC spectrum, and from the **E** H-4,**E** H-6b connectivity in the NOESY spectrum.

In conjunction with methylation analysis, the downfield chemical shift of **A** C-6 (δ 67.89) demonstrated residue **A** to be the 6-substituted Glc*p* residue. Based on the characteristic chemical shift of **B** C-2 (δ 56.01), residue **B** was identified as the Glc*p*NAc residue. The typical set of HOHAHA cross-peaks **E** H-2,3,4 on the **E** H-1 track identified residue **E** as a Gal*p* residue. The absence of H-6a,6b signals for residue **D** allowed this residue to be attributed to the Glc*p*A residue. The assignment of the remaining residue **C** to 4-substituted Glc*p* was corroborated by comparison of the complete set of ^{1}H and ^{13}C chemical shifts with literature data [8,29,30]. The $^{1}J_{C\text{-}1,H\text{-}1}$ values of 170 Hz for residue **A**, 165 Hz for residue **B**, and 160–162 Hz for residues **C**, **D**, and **E**, confirmed the proposed anomeric configurations and ring forms (vide supra) of the different residues. From a conformational point of view, it is worth noticing that the small values of both $^{3}J_{5,6a}$ and $^{3}J_{5,6b}$ for residue **A** (< 2 Hz) imply a strong dominance of the *gg* conformation for the 1,6-linkage in the backbone of the EPS [31].

Following a similar strategy as described for **1**, all ^{1}H and ^{13}C resonances of the EPS derivatives **2** and **3** have been identified. In the case of de-*N*-acetylated EPS **2**, a ^{13}C–^{1}H HMBC experiment (Fig. 6), which focuses on long-range ^{13}C–^{1}H couplings, greatly facilitated the unambiguous assignments of the ^{1}H and ^{13}C chemical shifts of all residues. For example, the assignment of residue **D** to Glc*p*A was in the case of **1** made based on the absence of **D** H-6a/6b signals. However, for de-*N*-acetylated EPS **2** the assignment of residue **D** was firmly corroborated by two intraresidual long-range couplings **D** C-6,**D** H-5 and **D** C-6,**D** H-4 in the HMBC spectrum.

When the ^{1}H and ^{13}C NMR data of **2** and **1** are compared, significant differences for **B** H-2 (Δδ −0.66), **B** C-1 (Δδ −1.71), and **B** C-3 (Δδ −1.56) are observed, reflecting de-*N*-acetylation of residue **B** in **2**. Comparison of the ^{13}C chemical shifts of **3**

Table 2
^{1}H NMR chemical shifts [a] of native EPS (**1**), de-*N*-acetylated EPS (**2**), de-*N*-acetylated, deaminated EPS (**3**), recorded at 57 °C, and of disaccharide alditol-*1-d* (**4**), obtained from a partial acid hydrolysate of **1**, recorded at 10 °C. Coupling constants (Hz) are given between brackets

Residue		**1**	**2**	**3**	**4**
A	H-1(a)	4.874 (2.7)	4.961 (3.5)	4.919 (3.5)	3.71 [b]
(**A**-ol)	H-1(b)	—	—	—	3.58 [b]
	H-2	3.503 (9.8)	3.525 (10.0)	3.561 (9.9)	3.83
	H-3	3.73	3.74	3.75	3.83
	H-4	3.66	3.63	3.56	3.718
	H-5	4.350	4.225	4.288	3.914
	H-6a [c]	4.221 (< 2, −10.5)	4.145 (< 2, −10.4)	4.105 (< 2, −10.0)	4.135 (2.4, −11.0)
	H-6b [c]	4.01 (< 2)	3.90 (< 2)	3.89 (< 2)	3.809 (6.0)
B	H-1	4.648 (7.9)	4.854 (8.1)	—	—
	H-2	3.60	2.937	—	—
	H-3	3.55	3.64	—	—
	H-4	3.31	3.379	—	—
	H-5	3.41	3.478	—	—
	H-6a [c]	3.904 (< 2, −12.3)	3.913	—	—
	H-6b [c]	3.69	3.71	—	—
N-acetyl	CH_3	2.028	—	—	—
C	H-1	4.550 (7.9)	4.544 (7.6)	4.569 (8.0)	—
	H-2	3.34	3.346	3.362 (8.5)	—
	H-3	3.64	3.63	3.67	—
	H-4	3.65	3.66	3.68	—
	H-5	3.63	3.60	3.62	—
	H-6a [c]	3.99	3.982 (< 2, −10.9)	4.000 (< 2, −11.7)	—
	H-6b [c]	3.83	3.816	3.84	—
D	H-1	4.525 (7.9)	4.492 (8.1)	4.528 (8.0)	4.496 (8.0)
	H-2	3.43	3.442	3.435 (8.5)	3.359
	H-3	3.65	3.63	3.65	3.507
	H-4	3.78	3.80	3.76	3.497
	H-5	3.86	3.84	3.86	3.721
E	H-1	4.479 (8.1)	4.528 (7.1)	4.512 (7.9)	—
	H-2	3.65	3.77	3.59	—
	H-3	3.74	3.90	3.75	—
	H-4	4.259	4.278	4.030	—
	H-5	3.75	3.79	3.79	—
	H-6a [c]	3.87	3.89	3.93	—
	H-6b [c]	3.81	3.82	3.85	—

[a] In ppm relative to the signal of internal acetone at δ 2.225.
[b] The assignments of H-1 of the two isotopic diastereomers [H-1(a) and H-1(b)] may have to be interchanged.
[c] The assignments of H-6a and H-6b may have to be interchanged within one residue.

and **1** only revealed significant differences for **E** C-3 (Δδ −8.66) and **E** C-4 (Δδ +2.17), which supported that in native EPS **1** the GlcNAc side-chains are attached via O-3 to the 3,4-disubstituted Gal*p* residues.

The complete monosaccharide sequence of the EPS was unambiguously determined

Table 3
^{13}C NMR chemical shifts [a] of native EPS (**1**), at 80 °C, and of de-*N*-acetylated EPS (**2**) and de-*N*-acetylated, deaminated EPS (**3**), at 67 °C. $^{1}J_{C-1,H-1}$ values (Hz) are given between brackets

Residue		1	2	3
A	C-1	99.03 (170)	99.46 (171)	100.09 (171)
	C-2	72.07	71.88	71.88
	C-3	73.03	72.72	72.83
	C-4	69.17	68.97	69.47
	C-5	70.31	70.58	71.11
	C-6	67.89	68.00	68.25
B	C-1	103.12 (165)	101.41 (164)	—
	C-2	56.01	56.52	—
	C-3	74.18	72.62	—
	C-4	70.84	70.32	—
	C-5	76.09	76.52	—
	C-6	61.66	61.14	—
N-acetyl	CH_3	22.42	—	—
	$C=O$	~ 174.5	—	—
C	C-1	102.18 (162)	102.11 (163)	102.50 (163)
	C-2	73.25	73.15	73.15
	C-3	74.42	74.35	74.35
	C-4	78.94	78.78	78.85
	C-5	75.02	74.99	74.99
	C-6	60.51	60.35	60.32
D	C-1	102.54 (160)	102.36 (161)	102.63 (163)
	C-2	72.95	72.80	72.83
	C-3	74.60	74.50	74.53
	C-4	80.68	80.31	80.64
	C-5	76.23	75.99	75.84
	C-6	~ 174.5	174.98	174.90
E	C-1	103.32 (162)	103.05 (163)	103.36 (162)
	C-2	70.71	70.58	70.98
	C-3	80.94	81.57	72.28
	C-4	75.14	75.99	77.31
	C-5	75.80	75.54	75.52
	C-6	60.21	60.20	60.32

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

via NOESY analysis of **1**, **2**, and **3**, and from the HMBC spectrum of **2**. In the NOESY spectrum of **1** (Fig. 5A), strong interresidual connectivities **A** H-1,**E** H-4, **A** H-1,**E** H-6a, and **A** H-1,**E** H-6b demonstrated the **A** → **E** sequence, whereas the **C**(1 → 4)**D** linkage was indicated by a strong cross-peak between **C** H-1 and **D** H-4. Interresidual connectivities **D** H-1,**A** H-6a (weak) and **D** H-1,**A** H-6b (strong) revealed the structural element **D**(1 → 6)**A**, and the **E** → **C** linkage was suggested by a strong connectivity **E** H-1,**C** H-4, although this cross-peak could only be assigned tentatively, due to overlap in the spectra. The **B** → **E** linkage was not established by a connectivity in the NOESY spectrum of **1**, however, in the NOESY spectrum of **2** (Fig. 5B) such a connectivity (**B**

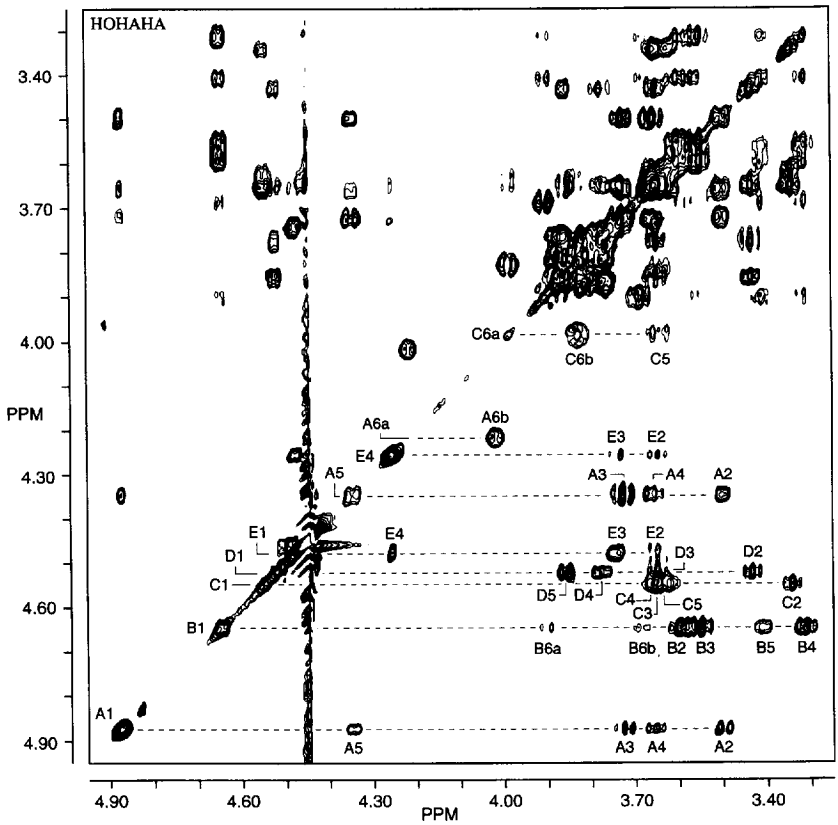


Fig. 4. 500-MHz 2D HOHAHA spectrum (mixing time 100 ms) of **1**, recorded in D_2O at 57 °C. Diagonal peaks of the anomeric protons, of H-4 of residue **E**, of H-5 of residue **A**, and of H-6a of residues **A** and **C** are indicated. Cross-peaks belonging to the same scalar-coupling network are indicated near a dotted line starting from the corresponding diagonal peak.

H-1,**E** H-3) could be observed. Furthermore, the NOESY spectra of **2** and **3** (not shown) completely agree with the monosaccharide sequence as deduced from the NOESY analysis of **1**.

In the 2D $^{13}C-^{1}H$ HMBC spectrum of **2** (Fig. 6), intraresidual two- and three-bond $^{13}C-^{1}H$ couplings can be observed, as well as interresidual three-bond connectivities over the glycosidic linkages. The **D**(1 → 6)**A** linkage was verified by a strong cross-peak between **A** C-6 and **D** H-1 in the HMBC spectrum. Furthermore, each of the three 1,4-linkages in the EPS was convincingly confirmed by two long-range couplings in the HMBC spectrum: **A** C-1,**E** H-4 and **E** C-4,**A** H-1 (**A** → **E** sequence); **E** C-1,**C** H-4 and **C** C-4,**E** H-1 (**E** → **C**); **C** C-1,**D** H-4 and **D** C-4,**C** H-1 (**C** → **D**). Finally, the strong connectivities **B** C-1,**E** H-3 and **E** C-3,**B** H-1 corroborated the 1,3-linkage of the Glc*p*NAc side-chains to the Gal*p* backbone residues.

The combined results from chemical and NMR studies have demonstrated that the

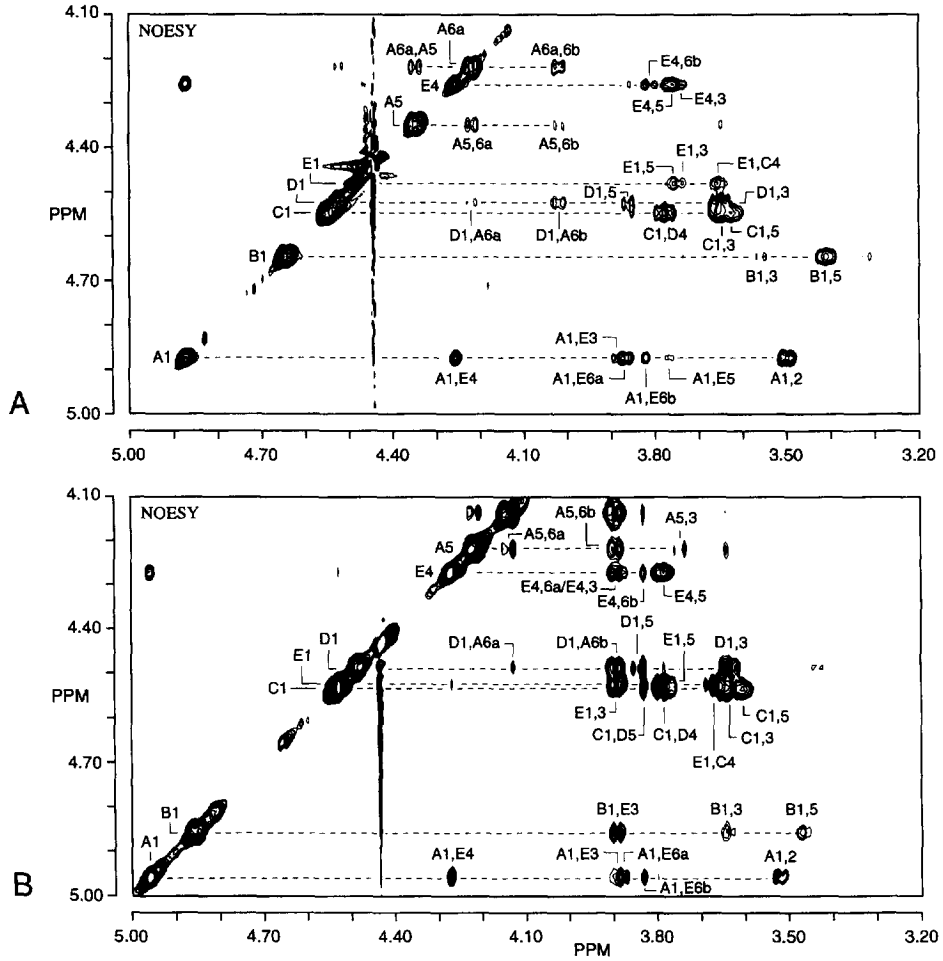
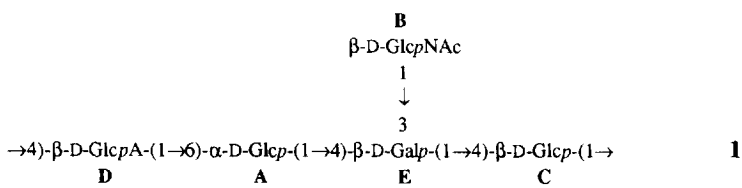


Fig. 5. Partial 500 MHz 2D NOESY spectra (mixing times 200 ms) of (A) **1** and (B) **2**, recorded in D_2O at 57 °C. The code A1 corresponds to the diagonal peak belonging to **A** H-1; A1,2 refers to an intraresidual cross-peak between **A** H-1 and **A** H-2, and A1,E4 means an interresidual connectivity between **A** H-1 and **E** H-4, etc.

EPS produced by *Lb. acidophilus* LMG9433 is composed of pentasaccharide repeating units with the following structure:



1

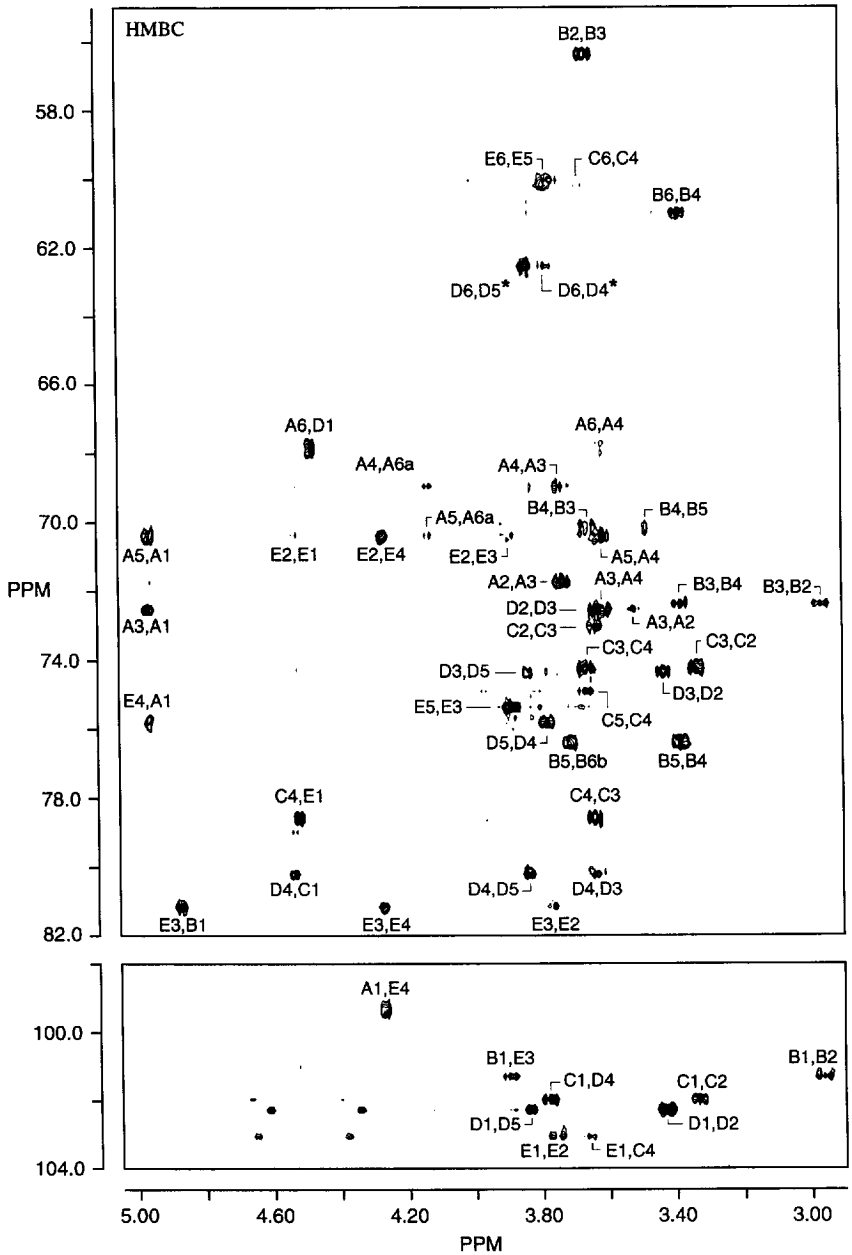


Fig. 6. 600 MHz 2D $^{13}C-^{1}H$ HMBC spectrum of **2**, recorded in D_2O at 57 °C. The code A1,E4 corresponds to a long-range coupling between **A** C-1 and **E** H-4, etc. The cross-peaks marked with an asterix (*), projected at an apparent ^{13}C chemical shift of δ 62.5 due to backfolding of the spectrum, correspond to an actual ^{13}C chemical shift of δ 174.9.

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