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Structure of the extracellular polysaccharide produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* 291

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Abstract

The lactic acid bacterium *Lactobacillus delbrueckii* subsp. *bulgaricus* 291, when grown in skimmed milk, produced 80 mg/L exopolysaccharide with an average molecular mass of 1.4×10^3 kDa. Monosaccharide analysis, methylation analysis, MS, and 1D/2D NMR (^{1}H and ^{13}C) studies performed on the native polysaccharide, and on oligosaccharides obtained from a mild acid hydrolysate of the native polysaccharide, showed the polysaccharide to consist of branched pentasaccharide repeating units with the following structure:

β-D-Gal*p*-(1→4)-β-D-Glc*p*
1
↓
6
→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→

Keywords: *Lactobacillus delbrueckii* subsp. *bulgaricus*; Exopolysaccharide; Structural analysis

1. Introduction

Microbial exopolysaccharides (EPSs) are employed as additives in a wide variety of food products, where they serve as viscosifying, stabilizing, emulsifying or gelling agents.[1] EPSs produced by lactic acid bacteria, which carry the GRAS (generally recognized as safe) status, are used to improve body and texture of dairy products.[2] To establish connections between EPS structure and rheological behavior, structural studies are performed on EPSs produced by various species of the *Lactobacillus*, *Lactococcus*, and *Streptococcus* genera.[1,3] More recent structural investigations have been performed on EPSs produced by *Lactobacillus reuteri*,[4] *Lactococcus lactis* subsp. *cremoris*,[5,6] *Streptococcus thermophilus*,[7] and a *Lactobacillus* subsp. isolated from kefir grains.[8]

S. thermophilus strains in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* strains are used as commercial yogurt starters. The EPSs produced by *Lb. delbrueckii* subsp. *bulgaricus* characterized so far are mainly composed of Glc and Gal[9,10] or of Glc, Gal and Rha.[11–13] A detailed structural study has been performed on the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* rr having a branched heptasaccharide repeating unit composed of Gal, Glc, and Rha in a molar ratio of 5:1:1.[13]

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Table 1
Methylation analysis data of *Lb. delbrueckii* subsp. *bulgaricus* 291 EPS

Derivative	Molar amounts [a]
2,3,4,6-Gal [b]	1.2
2,3,6-Gal	0.9
2,3,6-Glc	1.9
2,3-Glc	1.0

[a] 2,3-Glc is taken as 1.0.
[b] 2,3,4,6-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol-1-*d*, etc.

Here, we report on the structural elucidation of the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* 291 in skimmed milk, utilizing native and partially acid-hydrolyzed EPS, and applying monosaccharide analysis, methylation analysis, mass spectrometry, and NMR spectroscopy.

2. Results and discussion

Isolation, purification, and composition of the polysaccharide.—The EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* 291, in reconstructed skimmed milk containing 0.35% peptone and 0.35% yeast extract, was isolated by ethanol precipitation of the protein-free culture supernatant followed by gel-filtration using Sephacryl S-500. The purity of the EPS was confirmed by ^{1}H NMR spectroscopy (vide infra). From the *Lb. delbrueckii* subsp. *bulgaricus* 291 culture, 80 mg/L of EPS with an average molecular mass of 1.4×10^3 kDa was isolated.

Quantitative monosaccharide analysis of the EPS, including the determination of absolute configurations, revealed a composition of D-Glc and D-Gal in a molar ratio of 3:2. Methylation analysis of the EPS (Table 1) demonstrated the presence of terminal Gal*p*, 4-substituted Gal*p*, 4-substituted Glc*p*, and 4,6-disubstituted Glc*p* (according to NMR experiments (vide infra) all residues are in the pyranose ring form) in a molar ratio of 1:1:2:1, indicating a branched pentasaccharide repeating unit.

The anomeric region (δ 4.4–5.0) of the 1D ^{1}H NMR spectrum (Fig. 1) of EPS **1** contained three well-resolved signals and two overlapping signals corresponding with the suggested pentasaccharide repeating unit. The overlap of the anomeric doublets was confirm-

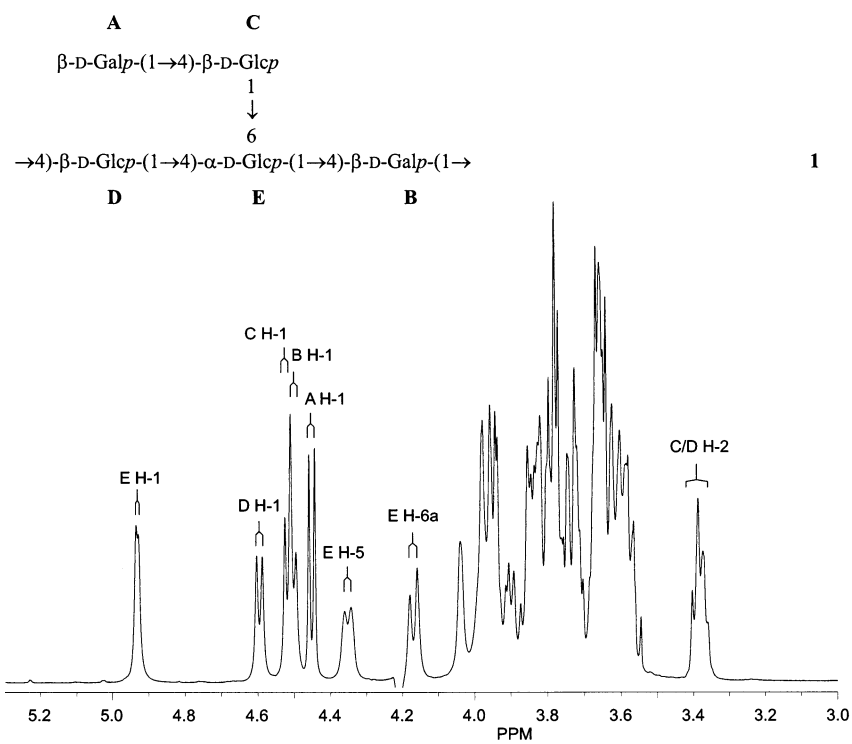


Fig. 1. 500-MHz ^{1}H NMR spectrum of EPS **1** produced by *Lb. delbrueckii* subsp. *bulgaricus* 291, recorded in D_2O at 80 °C.

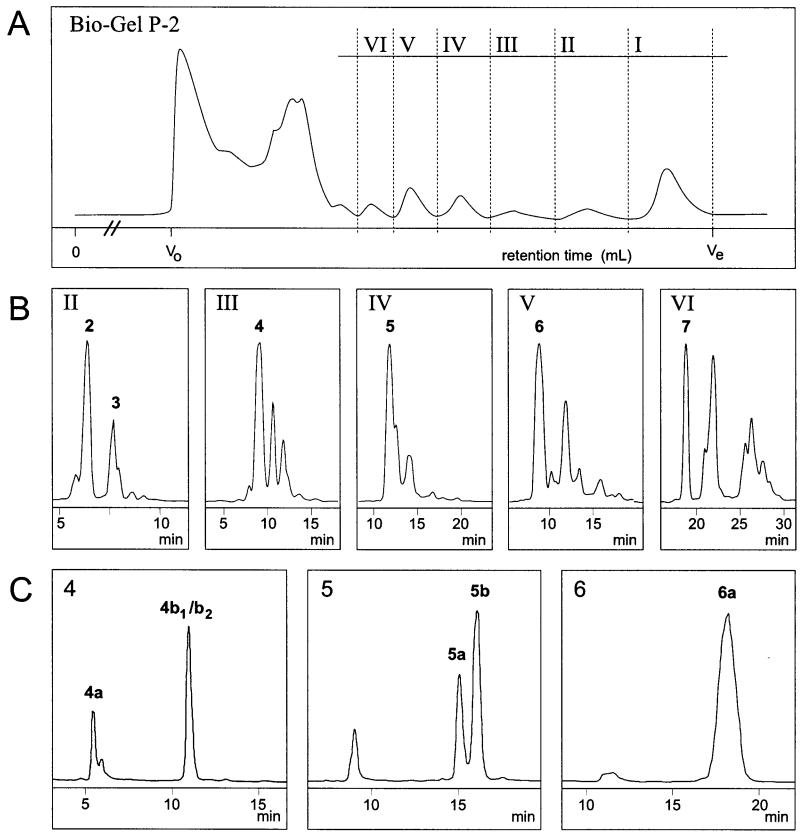


Fig. 2. (A) Bio-Gel P-2 elution profile of partially acid-hydrolyzed EPS; (B) HPAEC-PAD fractionation patterns of Bio-Gel P-2 fractions II–VI on CarboPac PA-1; (C) HPAEC-PAD fractionation patterns of fractions 4–6, after reduction with $NaBD_4$, on CarboPac PA-1.

ed by a $^{13}C-^{1}H$ HMQC experiment (vide infra). The five monosaccharide units were labeled **A**–**E** according to increasing chemical shift values of their anomeric protons. Based on observed $^{3}J_{1,2}$ values and chemical shifts, residues **A** ($^{3}J_{1,2}$ 7.9 Hz), **B** ($^{3}J_{1,2} \sim 8$ Hz), **C** ($^{3}J_{1,2} \sim 8$ Hz), and **D** ($^{3}J_{1,2}$ 7.3 Hz) were allocated the pyranose ring form and β anomeric configuration, and residue **E** ($^{3}J_{1,2}$ 3.4 Hz) was assigned the pyranose ring form and α anomeric configuration.

Partial acid hydrolysis.—The complex mixture of oligosaccharides, obtained after partial acid hydrolysis of the EPS, was fractionated on Bio-Gel P-2, yielding fractions I–VI (Fig. 2(A)). Fraction I contained the monosaccharides Glc and Gal, as demonstrated by GLC and ^{1}H NMR spectroscopy (data not shown). Fractions II–VI were subfractionated on CarboPac PA-1 (Fig. 2(B)), and the resulting HPAEC-fractions **2**–**7** were reduced with $NaBD_4$ and desalted prior to analysis. A further fractionation of fractions **4**–**6** after reduction was performed on CarboPac PA-1, yielding subfractions **4a**–**6a** (Fig. 2(C)). Quantitative monosaccharide analysis of fraction **2**–**7**, including MALDI-TOF measurements, revealed the composition of the isolated alditols (Table 2).

Table 2
Monosaccharide and MALDI-TOF analysis data of oligosaccharide alditols **2**–**7** obtained from partially acid-hydrolyzed EPS

Fraction	Component	$[M+Na]^{+}$ [a]
2	Gal_1Glc-ol-1-*d*	368
3	Glc_1Glc-ol-1-*d*	368
4a	Gal_1Glc_1Glc-ol-1-*d*	530
4b	Glc_2Gal-ol-1-*d*	530
5a	Gal_1Glc_2Gal-ol-1-*d*	692
5b	Glc_3Gal-ol-1-*d*	692
6a	Gal_1Glc_3Gal-ol-1-*d*	854
7	Gal_2Glc_3Gal-ol-1-*d*	1016

[a] Pseudo-molecular ions in *m*/*z*.

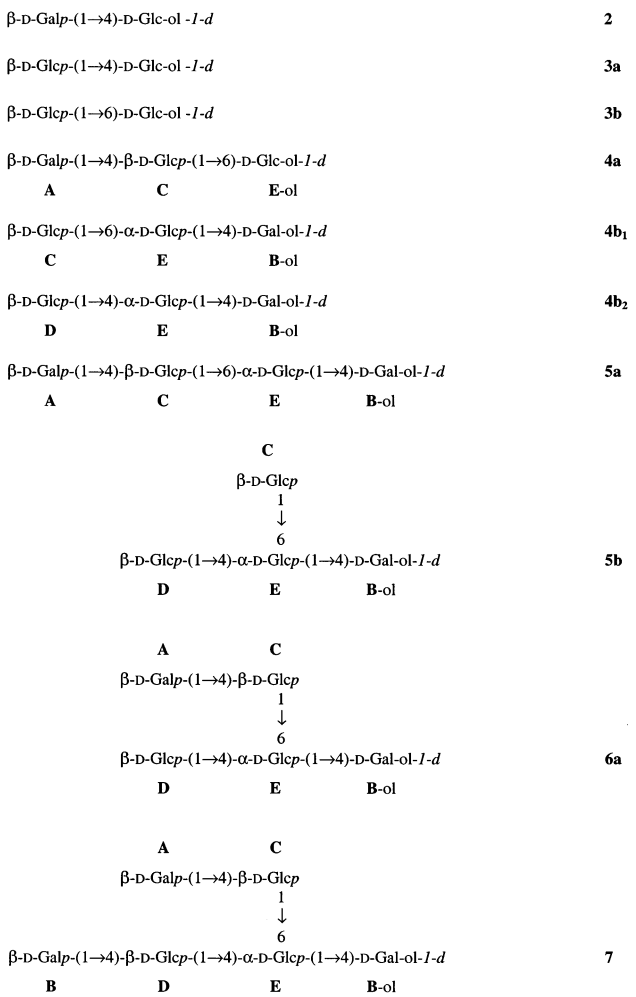


Fig. 3. Structures of oligosaccharide alditols **2**–**7** obtained from partially acid-hydrolyzed EPS.

Fractions **2** *and* **3**.—According to methylation analysis, and 1D ^{1}H NMR spectroscopy, the disaccharide alditol-1-*d* in fraction **2** is lactitol and the alditols in fraction **3** are cellobiitol **3a** and gentiobiitol **3b**, respectively (Fig. 3).

Fractions **4**–**7**.—1D ^{1}H NMR analysis of Fractions **4a**, **5a**, **5b**, **6a**, and **7** (Fig. 4; spectrum of fraction **4a** not shown) indicated single alditols for all fractions. Assignments of the ^{1}H chemical shifts and coupling constants of the alditols (Table 3), including the determination of the monosaccharide sequence were performed by means of 2D TOCSY experiments (mixing times 25–250 ms) and 2D ROESY experiments, essentially as described for the ^{1}H assignment of EPS **1** (vide infra). Based on the joint analytical data, the structures of **4a**, **5a**, **5b**, **6a**, and **7** can be formulated as illustrated in Fig. 3. Methylation analysis performed on the isolated fractions (data not shown) fit the proposed structures. Furthermore, the methylation analysis on fraction **4b** pointed to a mixture of two trisaccharide alditols differing in the substitution pattern of the internal residue as illustrated in Fig. 3.

2D NMR spectroscopy of the native polysaccharide.—The ^{1}H chemical shifts of the EPS (Table 3) were assigned by means of 2D TOCSY (mixing times 10–200 ms) and 2D NOESY experiments (mixing time 200 ms). The TOCSY spectrum, with a mixing time of 120 ms, is depicted in Fig. 5. Starting points for the interpretation of the spectra were the anomeric signals of residues **A**–**E**. Comparison of TOCSY spectra with increasing mixing times allowed the assignment of the sequential order of the chemical shifts belonging to a single spin system.

The TOCSY **A** H-1 track (δ 4.456) showed cross-peaks with **A** H-2,3,4. Via the **A** H-4 signal, the **A** H-5 TOCSY track (δ 3.724) was found, and on this track cross-peaks with **A** H-4,6a,6b were observed. The **B** H-1 TOCSY track (δ 4.510) showed a cross-peak with **B** H-4. The **B** H-2,3 chemical shifts, which could not be assigned accurately on the **B** H-1 track due to overlap with cross-peaks on the **C** H-1 track (δ 4.525), were assigned on the **B** H-4 TOCSY track (δ 4.041). The resonance for **B** H-5 was found via an intraresidual cross-peak between **B** H-1 and **B** H-5 in the NOESY spectrum (vide infra). The chemical shifts of **B** H-6a,6b were derived from NOEs on the **E** H-1 track in the NOESY spectrum, since residue **E** is linked to residue **B** (vide infra). On the TOCSY **C** H-1 track (δ 4.525) the complete series of cross-peaks with **C** H-2,3,4,5,6a,6b were observed. The H-2,3,4,5,6a,6b resonances of residue **D** were identified on the basis of cross-peaks on the **D** H-1 TOCSY track (δ 4.605) and the complete series of the well-resolved **E** H-2,3,4,5,6a,6b connectivities were found on the **E** H-1 TOCSY track (δ 4.935).

Residues **A** and **B** were assigned to β-Gal*p* based on their TOCSY spin systems. Furthermore, residues **C** and **D** could be assigned as

β-Glc*p* by the characteristic upfield shift of their H-2 resonances,[14] leaving residue **E** to be α-Glc*p*.

All ^{13}C resonances of the EPS (Table 4) could be assigned using a 2D $^{13}C-^{1}H$ HMQC spectrum (Fig. 6). The $^{1}J_{C\text{-}1,H\text{-}1}$ values of the anomeric ^{13}C atoms of residues **A** ($^{1}J_{C\text{-}1,H\text{-}1}$ 162 Hz), **B** ($^{1}J_{C\text{-}1,H\text{-}1}$ 161 Hz), **C** ($^{1}J_{C\text{-}1,H\text{-}1}$ 161 Hz), and **D** ($^{1}J_{C\text{-}1,H\text{-}1}$ 163 Hz) are in agreement with β anomeric configurations, and that of residue **E** ($^{1}J_{C\text{-}1,H\text{-}1}$ 173 Hz) with α anomeric configuration.[15]

Combining the ^{13}C NMR data and the methylation analysis data of the EPS, taking into account published ^{13}C chemical shift data of methyl aldosides,[16] demonstrated residue **A** to be terminal β-D-Gal*p* and residue **B** to be 4-substituted β-D-Gal*p* (**B** C-4 δ 78.4; β-D-Gal*p*1Me, $\delta_{C\text{-}4}$ 69.7). The downfield chemical shifts of **C** C-4 (δ 79.7) and **D** C-4 (δ 79.8) indicated that residues **C** and **D** accord with 4-substituted β-D-Glc*p* units (β-D-Glc*p*1Me, $\delta_{C\text{-}4}$ 70.6). The remaining residue **E** could be assigned as 4,6-disubstituted α-D-Glc*p*, since **E** C-4 (δ 79.3) and **E** C-6 (δ 68.3) are shifted downfield (β-D-Glc*p*1Me, $\delta_{C\text{-}4}$ 70.6, $\delta_{C\text{-}6}$ 61.6).

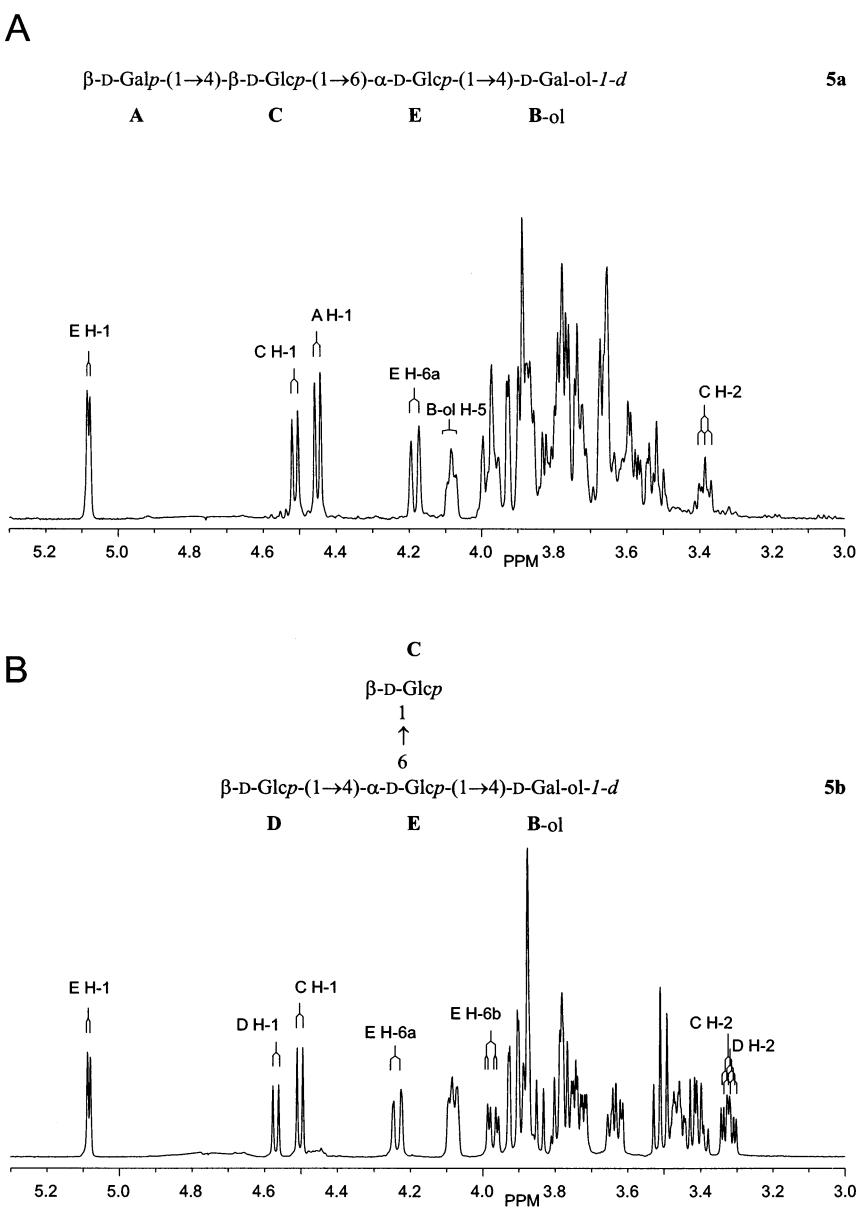
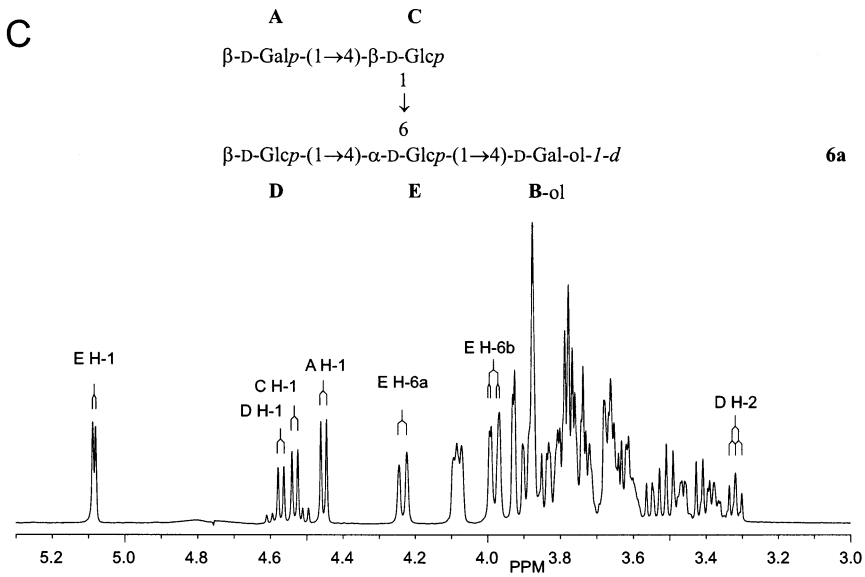


Fig. 4. 500-MHz ^{1}H NMR spectra of (A) tetrasaccharide alditol **5a**; (B) tetrasaccharide alditol **5b**; (C) pentasaccharide alditol **6a**; and (D) hexasaccharide alditol **7**, recorded in D_2O at 27 °C.



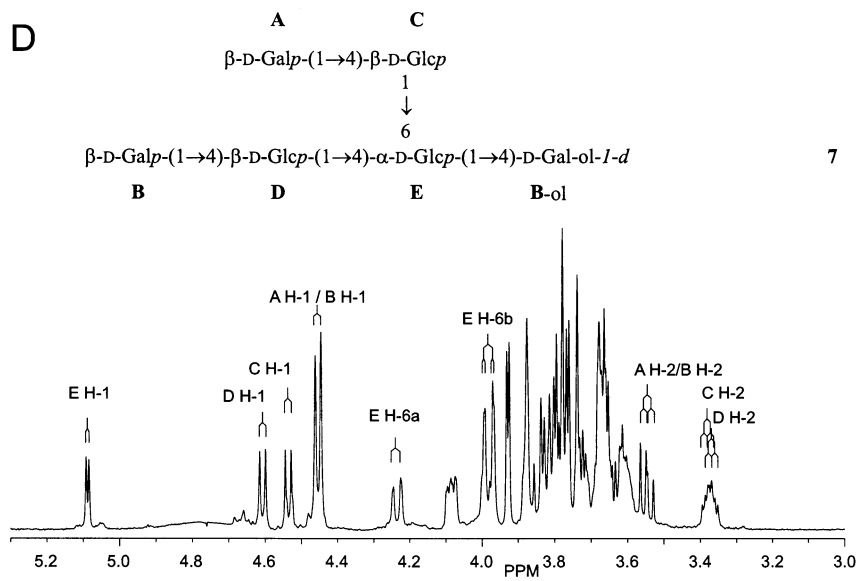


Fig. 4. (*Continued*)

The assignment of interresidual cross-peaks in the 2D NOESY spectrum (Fig. 7) and of long-range couplings in the HMBC spectrum (Fig. 8) allowed the determination of the sequence and the linkage positions of the residues within the repeating unit of the EPS. On the NOESY **C** H-1 track strong NOEs with **E** H-6a,6b were observed, suggesting a **C**(1→6)**E** linkage. This linkage was confirmed by a long-range $^{1}H-^{13}C$ coupling between **C** H-1 and **E** C-6, observed in the HMBC spectrum. The interresidual connectivities **D** H-1,**E** H-3,4,5,6a,6b indicated a linkage between residue **D** and **E**. Taking into account the (1→6) linkage between residue **C** and **E** and the substitution pattern of residue **E** (vide supra), the NOE between **D** H-1 and **E** H-4 must reflect the **D**(1→4)**E** linkage. The additional NOEs between residues **D** and **E** were brought about by spin diffusion. Besides the **C**(1→6)**E** linkage, also the **D**(1→4)**E** linkage was confirmed by a long-range $^{1}H-^{13}C$ coupling in the HMBC spectrum. Interresidual connectivities between **E** H-1 and **B** H-4,6a,6b in the NOESY spectrum together with a long-range $^{1}H-^{13}C$ coupling between **E** H-1 and **B** C-4 in the HMBC spectrum indicated a **E**(1→4)**B** linkage. On the **A** H-1 NOESY track,

Table 3
^{1}H NMR chemical shifts [a] of EPS (**1**), recorded in D_2O at 80 °C and oligosaccharide alditols **4**–**7**, recorded in D_2O at 27 °C [b]

Residue	Proton	**1**	**4a**	**5a**	**5b**	**6a**	**7**
A	H-1	4.456 (7.9)	4.449 (7.8)	4.451 (7.8)		4.453 (7.9)	4.452 (7.8)
	H-2	3.56	3.546	3.55		3.55	3.544 [d]
	H-3	3.66	3.66	3.66		3.67	3.66
	H-4	3.94	3.93	3.93		3.93	3.93
	H-5	3.72	3.72	3.72		3.72	3.73
	H-6a	3.79	nd [c]	3.77		3.77	3.77
	H-6b	3.79	nd	3.77		3.77	3.77
B	H-1	4.510 (~8)					4.452 (7.8)
	H-2	3.59					3.548 [d]
	H-3	3.74					3.66
	H-4	4.041					3.93
	H-5	3.79					3.73
	H-6a	3.91					3.77
	H-6b	3.78					3.77
C	H-1	4.525 (~8)	4.529 (7.8)	4.514 (7.8)	4.502 (7.9)	4.532 (7.9)	4.534 (7.8)
	H-2	3.39	3.377	3.385	3.327	3.38	3.374
	H-3	3.67	3.66	3.66	3.50	3.67	3.67
	H-4	3.65	3.66	3.66	3.40	3.67	3.66
	H-5	3.59	3.61	3.60	3.45	3.60	3.60
	H-6a	3.97	3.986 (12.7)	3.99	3.92	3.98	3.97
	H-6b	3.81	3.81	3.81	3.73	3.82	3.82
D	H-1	4.605 (7.3)			4.568 (7.8)	4.571 (8.3)	4.605 (7.8)
	H-2	3.38			3.320	3.318	3.368
	H-3	3.67			3.50	3.51	3.67
	H-4	3.66			3.42	3.42	3.67
	H-5	3.62			3.47	3.47	3.61
	H-6a	3.98			3.92	3.91	3.97
	H-6b	3.83			3.73	3.74	3.82
E	H-1	4.935 (3.4)		5.082 (3.9)	5.084 (3.9)	5.084 (3.9)	5.086 (4.0)
	H-2	3.62		3.58	3.62	3.63	3.63
	H-3	3.86		3.73	3.84	3.85	3.85
	H-4	3.76		3.52	3.79	3.80	3.79
	H-5	4.37		3.97	4.08	4.08	4.08
	H-6a	4.17		4.184 (10.8)	4.235 (10.3)	4.234 (10.3)	4.234 (9.8)
	H-6b	3.98		3.86	3.971	3.979	3.980
B-ol	H-4			3.88	3.88	3.88	3.88
	H-5			4.084	4.08	4.08	4.08
	H-6a			3.78	3.77	3.77	3.78
	H-6b			3.78	3.77	3.77	3.78
E-ol	H-1a		3.64				
	H-1b		3.72				
	H-2		3.83				
	H-3		3.84				
	H-4		3.73				
	H-5		3.92				
	H-6a		4.145 (10.8)				
	H-6b		3.82				

[a] In ppm relative to the signal of internal acetone at δ 2.225.
[b] Coupling constants are included in parentheses.
[c] nd, not determined.
[d] Signals may have to be interchanged.

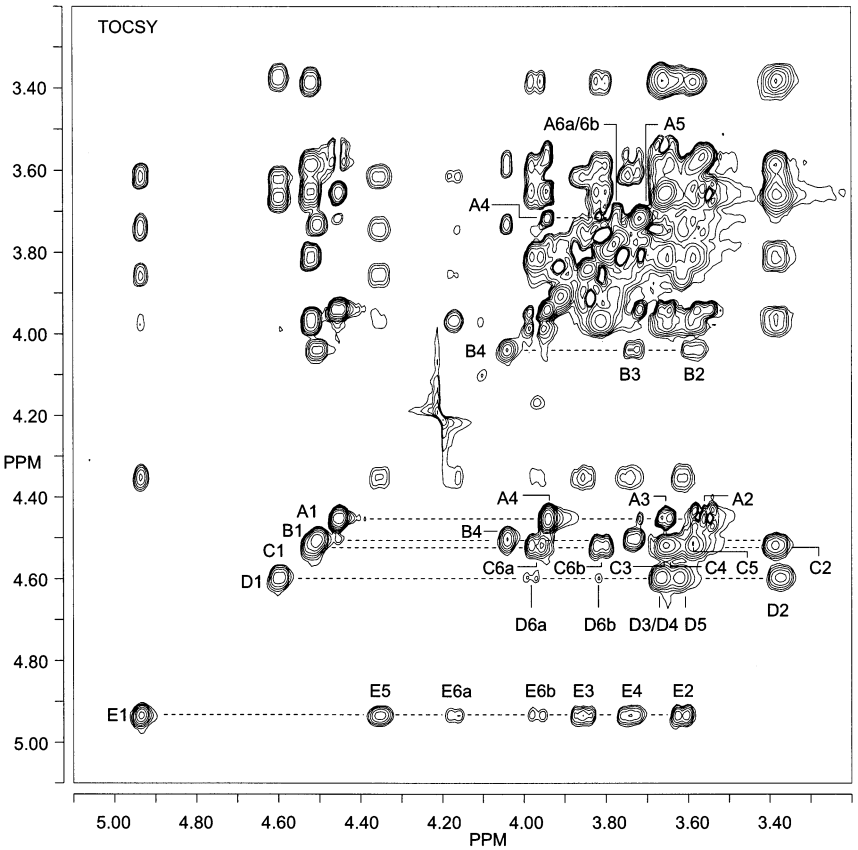


Fig. 5. 500-MHz 2D TOCSY spectrum (mixing time 200 ms) of EPS **1**, recorded in D_2O at 80 °C. Diagonal peaks of the anomeric protons, of H-4 of residue **B**, and of H-5 of residue **A** are indicated. Cross-peaks belonging to the same scalar-coupling network are indicated near a dotted line starting from the corresponding diagonal peak. A1 means **A** H-1, etc.

cross-peaks with **C** H-6a,6b were observed. Since the chemical shifts of both **A** H-3 (δ 3.66) and **C** H-4 (δ 3.65) were nearly identical, the NOE between **A** H-1 and **C** H-4 could not be distinguished from the intraresidual connectivity **A** H-1,**A** H-3. As residue **C** is 4-substituted β-Glc*p* (vide supra), the **A**(1→4)**C** linkage is supported. Finally, the interresidual NOESY connectivity between **B** H-1 and a signal at $\delta \sim 3.65$ established the **B**(1→4)**D** linkage. In the HMBC spectrum the long-range $^1H-^{13}C$ couplings **A** H-1,**C** C-4 and **B** H-1,**D** C-4 confirmed the **A**(1→4)**C** and **B**(1→4)**D** linkages, respectively.

Concluding remarks.—Based on monosaccharide analysis, methylation analysis, and MS and 1D/2D NMR studies on EPS **1** and on oligosaccharides obtained from a partial acid hydrolysate of the EPS, the primary structure of the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* 291 was shown to be built up from the following pentasaccharide repeating units:

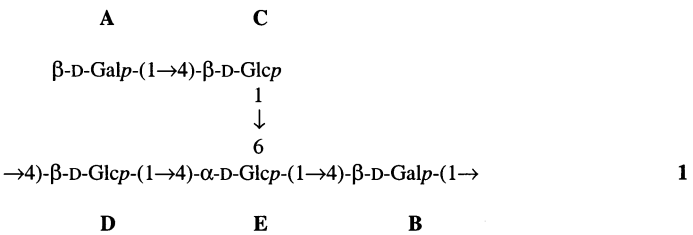


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Table 4
^{13}C NMR chemical shifts [a] of EPS (**1**), as determined from a 2D $^{13}C-^1H$ HMQC spectrum, recorded at 80 °C [b]

Residue	C-1	C-2	C-3	C-4	C-5	C-6
A	103.6 (162)	71.9	73.6	69.5	76.1	61.9
B	104.0 (161)	71.9	73.1	78.4	76.2	61.2
C	103.1 (161)	73.9	75.2	79.7	75.6	61.2
D	102.9 (163)	73.9	75.2	79.8	75.6	61.1
E	100.5 (173)	72.4	72.2	79.3	70.6	68.3

[a] In ppm relative to the α anomeric signal of external [1-^{13}C]glucose at δ 92.9.

[b] $^1J_{C\text{-}1,H\text{-}1}$ values are included in parentheses.

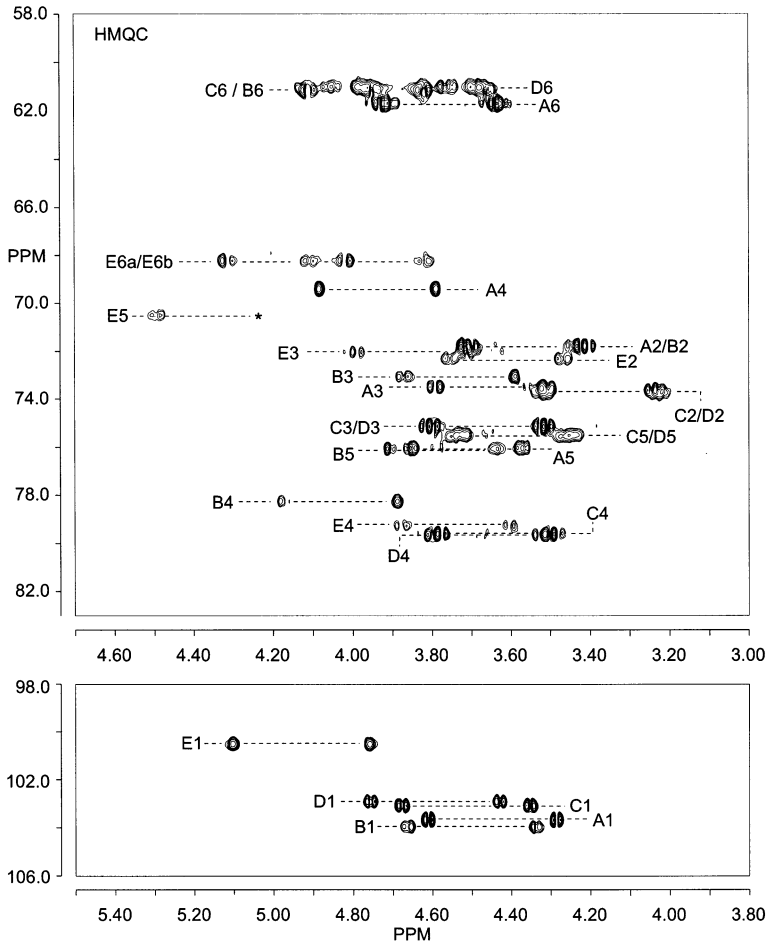

Fig. 6. 500-MHz 2D $^{13}C-^{1}H$ undecoupled HMQC spectrum of EPS **1**, recorded in D_2O at 80 °C. A1 stands for the set of cross-peaks between H-1 and C-1 of residue **A**, etc. The asterisk (*) indicates the right-hand side of the E5 doublet, which is lost due to HOD suppression.

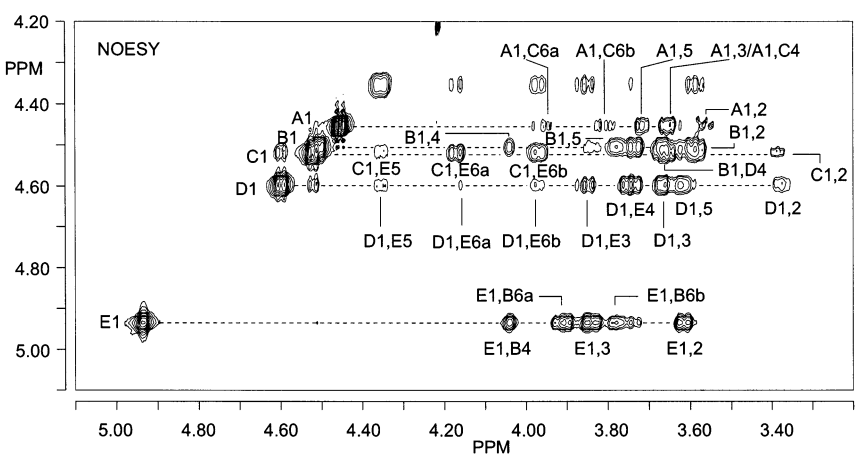

Fig. 7. Partial 500-MHz 2D NOESY spectrum (mixing time 200 ms) of EPS **1**, recorded in D_2O at 80 °C. E1 corresponds to the diagonal peak belonging to residue **E** H-1; E1,2 refers to an intraresidual cross-peak between **E** H-1 and **E** H-2, and E1,B4 means an interresidual connectivity between **E** H-1 and **B** H-4, etc.

The structural elucidations of the oligosaccharides isolated from the partial acid hydrolysate of EPS **1** do not give complete evidence for the repeating unit of the EPS. Even though, the size and structure of oligosaccharide **7** suggests a full elucidation of

the repeating unit, it must be kept in mind that residues **A** and **B** in this oligosaccharide are chemically equivalent, leaving the accurate determination of the **A**(1→4)**C** and **B**(1→4)**D** fragments within the EPS **1** to be essential for the correct determination of the primary structure of the EPS.

The repeating unit of the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* 291 consists of a trimeric main chain, containing one Gal and two Glc residues, and carrying a lactosyl side chain. This type of side chain has been reported before for the EPSs produced by several strains of *Lb. helveticus*[17–19] and *L. lactis* subsp. *cremoris*[5,6] and may confer these EPSs specific biological properties.[19] Surprisingly, the repeating unit of the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* 291 was found to be identical to the O-deacetylated repeating unit of the EPS produced by *L. lactis* subsp. *cremoris* B891.[6] Repeating units, only differing in the presence or absence of *O*-acetyl groups have been reported before for capsular polysaccharides produced by *S. pneumoniae* strains belonging to the same serotype.[20] However, the absence of O-acetylation in the repeating unit of the *Lb. delbrueckii* subsp. *bulgaricus* 291 EPS as compared to the *L. lactis* subsp. *cremoris* B891 EPS can also be the result of differences in culturing conditions, as known for xanthan, produced by *Xanthomonas campestris*.[21] Furthermore, it must be kept in mind that differences in the procedure used for the isolation of the *Lb. delbrueckii* subsp. *bulgaricus* 291 EPS might have resulted in O-deacetylation of the EPS.

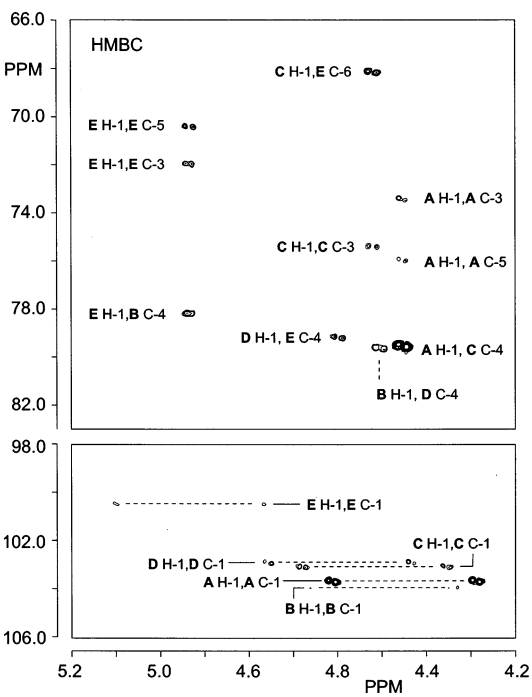

Fig. 8. Partial 500-MHz 2D $^{13}C-^{1}H$ undecoupled HMBC spectrum of EPS **1**, recorded in D_2O at 80 °C. **E** H-1,**B** C-4 corresponds to a cross-peak between **E** H-1 and **B** C-4, etc.

To our knowledge, there are no reports on the appearance of identical heteropolysaccharide repeating units produced by different genera of lactic acid bacteria. Therefore, the production of an identical EPS by *Lb. delbrueckii* subsp. *bulgaricus* 291 and *L. lactis* subsp. *cremoris* B891 is remarkable.

3. Experimental

Culture conditions of microorganism and isolation of polysaccharide.—*Lb. delbrueckii* subsp. *bulgaricus* 291 (WISBY, Niebüll, Germany) was inoculated into skimmed milk containing 0.35% peptone and 0.35% yeast extract. After incubation (22 h, 37 °C), trichloroacetic acid was added to a final concentration of 8% (v/v), and the culture was stirred for 45 min. Cells and precipitated coagulated proteins were removed by centrifugation (20 min, 8300*g*, 4 °C). The pH of the supernatant was adjusted to 3.7 using 10 M NaOH. EPS in the supernatant was precipitated with 1.65 vol of EtOH during 16 h at 4 °C. Precipitated material was collected through centrifugation (20 min, 8300*g*, 4 °C), re-dissolved in water and dialyzed for 24 h against running tap water. The EPS was recovered by lyophilization and further purified on a Sephacryl S-500 gel-filtration column as described.[14]

Molecular mass determination.—The average molecular mass of the polysaccharide was determined using a modified method combining gel-permeation chromatography, static light scattering, and differential refraction analysis, as described.[22]

Gas-liquid chromatography and mass spectrometry.—GLC analyses were performed on a Chrompack CP9002 gas chromatograph equipped with a CP-Sil 5CB fused silica capillary column (25 m × 0.32 mm, Chrompack). GLC–MS analyses were carried out on a

MD800/8060 system (Fisons instruments; electron energy, 70 eV), using a CP-Sil 5CB fused silica capillary column (25 m × 0.25 mm, Chrompack) or an AT-1 fused silica capillary column (30 m × 0.25 mm, Alltech). Both GLC and GLC–MS analyses were performed using conditions as described previously.[23] Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF–MS) experiments were performed using a Voyager-DE mass spectrometer equipped with a nitrogen laser. Samples were prepared by mixing directly on the target 1 μL oligosaccharide alditol solution with 2 μL aq 10% 2,5-dihydroxybenzoic acid as matrix solution.

Monosaccharide and methylation analysis.—For monosaccharide analysis, samples were methanolyzed followed by trimethylsilylation and GLC analysis as described.[24–26] The absolute configuration of the monosaccharides was determined by GLC analysis of their trimethylsilylated (−)-2-butyl glycosides.[27,28] For methylation analysis, the polysaccharide and isolated oligosaccharide alditols were subsequently permethylated, hydrolyzed, reduced, and acetylated, as described previously.[23] The obtained partially methylated alditol acetates were identified by GLC and by GLC–MS.[26,29]

Partial acid hydrolysis.—The exopolysaccharide (15 mg) was treated with 0.3 M trifluoroacetic acid (15 mL) for 1 h at 100 °C. After lyophilization, the residue was fractionated on a Bio-Gel P-2 column (200–400 mesh, 100 × 1.6 cm, Bio-Rad), eluted with 5 mM NH_4HCO_3 at a rate of 0.5 mL/min at 57 °C, monitored by differential refractive index detection (LKB Bromma). Collected fractions were subfractionated 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, Dionex). The column was eluted with a gradient of NaOAc in 0.1 M NaOH at a flow rate of 4 mL/min. Gradients were optimized for each fraction. PAD-detection was carried out with a gold working electrode and triple-pulse amperometry (pulse potentials and durations: E_1 0.05 V, 300 ms; E_2 0.65 V, 60 ms; E_3 −0.95 V, 180 ms) was used. Collected fractions were desalted on graphitized carbon according to,[30] and reduced with $NaBD_4$ in 1 M NH_4OH prior to analysis. In the cases of oligosaccharide alditol mixtures, a further fractionation was carried out on CarboPac PA-1 using the same conditions as described above, and prior to analysis, the collected fractions were desalted on graphitized carbon.

NMR spectroscopy.—Prior to NMR-spectroscopic analysis, samples were exchanged twice in 99.9 atom% D_2O (Cambridge Isotope Laboratories Inc.) with intermediate lyophilization and finally dissolved in 99.96 atom% D_2O (Isotec Inc.). NMR spectra were recorded on a Bruker AMX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy) at a probe temperature of 80 °C for the EPS and 27 °C for the oligosaccharide alditols. The HOD signal was suppressed either by applying a WEFT pulse sequence[31] in 1D ^1H NMR experiments, or by presaturation for 0.8–1 s in 2D experiments. When necessary, the remaining HOD signal was eliminated by convolution of low frequency contributions in the FID by a first order phase correction.[32] Chemical shifts were referenced to internal acetone (δ 2.225) for ^1H or to the α anomeric signal of external [1-^{13}C]glucose ($\delta_{C\text{-}1}$ 92.9) for ^{13}C. Spectra were recorded using a spectral width of 4032 and 16350 Hz for ^1H and ^{13}C, respectively. Resolution enhancement of the spectra was performed by a Lorentzian-to-Gaussian transformation or by multiplication with a squared-bell function phase shifted by $\pi/(2.3)$, and when necessary, a fifth-order polynomial baseline correction was performed. 2D TOCSY spectra were recorded using a 'clean' MLEV-17 mixing sequence with an effective spin-lock time of 10–200 ms. A total of 512 experiments of 1024 points were acquired with 16 or 32 scans per increment. 2D NOESY experiments were performed with a mixing time of 200 ms and 2D off-resonance ROESY spectra were obtained with a mixing time of 300 ms; both NOESY and ROESY spectra were acquired with 512 experiments of 1024 points with 16–32 scans per increment. Both natural abundance ^{13}C–^1H 2D HMQC and ^{13}C–^1H 2D HMBC experiments were recorded without decoupling during acquisition of the ^1H FID. A total of 512 free

induction decays of 1024 data points were acquired using 140 or 400 scans per decay. The $^{13}C-^{1}H$ 2D HMBC spectrum was recorded using a delay of 50 ms for the evolution of the long-range coupling.

All NMR data were processed using TRITON (Bijvoet Center, Department of NMR Spectroscopy) software.

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