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Structural elucidation of an extracellular polysaccharide produced by *Lactobacillus helveticus*

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Abstract

An extracellular polysaccharide produced by a strain of *Lactobacillus helveticus* isolated from cheese milk has been investigated. Sugar and methylation analysis together with ^{1}H and ^{13}C NMR spectroscopy revealed that the polysaccharide is composed of hexasaccharide repeating units. The sequence of sugar residues was determined by use of two-dimensional nuclear Overhauser effect spectroscopy and heteronuclear multiple-bond correlation experiments. The structure of the repeating unit of the exopolysaccharide from *L. helveticus* is as follows:

$$\rightarrow 6)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow$$
$$\begin{array}{c} 6 \\ \uparrow \\ 1 \\ \beta\ \text{D Gal}p \end{array}$$

Keywords: *Lactobacillus helveticus*; Exopolysaccharide; NMR

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1. Introduction

The use of lactic acid bacteria in dairy production is of considerable potential interest since these bacteria can give desirable products during the fermentation process. These lactic acid bacteria produce, besides lactic acid, exopolysaccharides which may have suitable rheological properties. Studies of the composition of extracellular polysaccharides produced by lactic acid bacteria [1] have been followed by structural studies of exopolysaccharides produced by the genus *Lactobacillus* [2]. A capsular polysaccharide produced by *L. kefiranofaciens* K_1 was proposed to contain branched hexa- or heptasaccharide repeating units with D-glucose and D-galactose [3]. A gel-forming exopolysaccharide produced by *L. hilgardii* had a dextran-like structure [4]. *L. delbrueckii* ssp. *bulgaricus* rr produced a polysaccharide with branched heptasaccharide repeating units containing D-glucose, D-galactose, and L-rhamnose [5]. The isolation and physicochemical properties of an antitumour polysaccharide produced by *L. helveticus* var *jugurti* was reported by Oda et al. [6]. The components of this polysaccharide were D-glucose and D-galactose in the ratio 2:1. Recently Yamamoto et al. [7] showed that the exopolysaccharide produced by *L. helveticus* TY1-2 had branched heptasaccharide repeating units with D-glucopyranosyl, D-galactopyranosyl, and 2-acetamido-2-deoxy-D-glucopyranosyl residues in the ratios 3.0:2.8:0.9.

This report describes the isolation and structural elucidation of the exopolysaccharide produced by a strain of *L. helveticus* isolated from cheese milk.

2. Results and discussion

A crude polysaccharide preparation was obtained from a 40% ethanol precipitate of the MRS [8] culture supernatant of *L. helveticus*. The polysaccharide was purified by anion-exchange chromatography from which the neutral fraction was collected and freeze-dried. The purity of the isolated polysaccharide was confirmed by reversed-phase high-performance liquid chromatography (RP-HPLC) which showed a single peak (data not shown).

A hydrolysate of the exopolysaccharide from *L. helveticus* contained galactose and glucose in the ratio 2:1. The absolute configuration analysis showed that galactose and glucose had the D configuration. Methylation analysis of the polysaccharide revealed 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-D-galactose and 2,4-di-*O*-methyl-D-galactose in the ratios 12:18:18:16:16:15. From the ^{1}H NMR spectrum (Fig. 1) it was possible to identify six protons in the anomeric region at δ 5.16, 4.62, 4.54, 4.54, 4.51, and 4.48. The ^{13}C NMR spectrum (Fig. 2) contained, inter alia, six signals in the anomeric region. A DEPT experiment showed that three ^{13}C signals from hydroxymethyl groups reside at $\sim$ 61 ppm and the other three at $\sim$ 70 ppm, confirming the three 6-substituted sugar residues. The above sugar and methylation analysis together with ^{1}H and ^{13}C NMR spectra show that the polysaccharide is composed of a hexasaccharide repeating unit. From the methylation analysis and NMR spectra (vide infra), it is evident that the sugars are pyranosides.

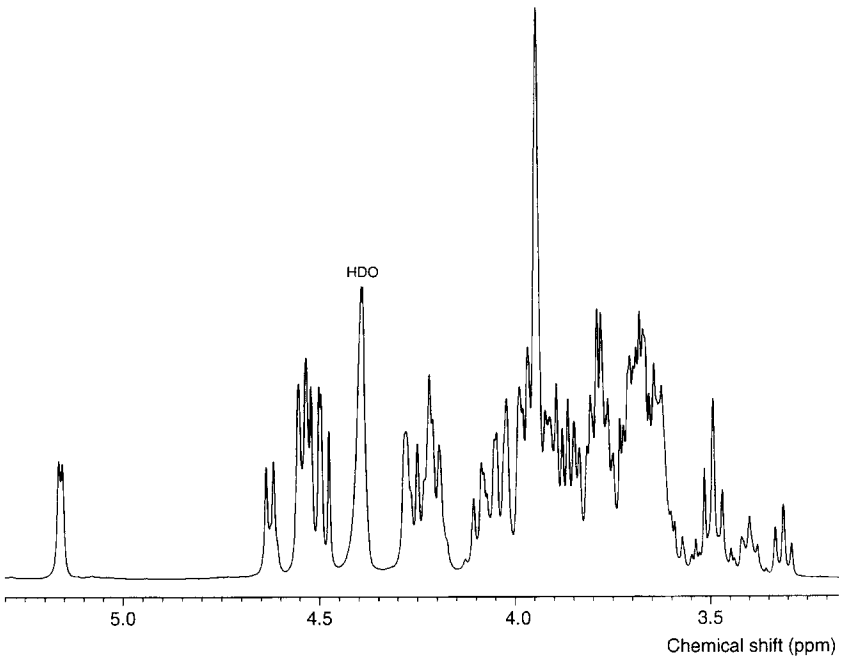


Fig. 1. The ^{1}H NMR spectrum at 400 MHz of the *L. helveticus* exopolysaccharide.

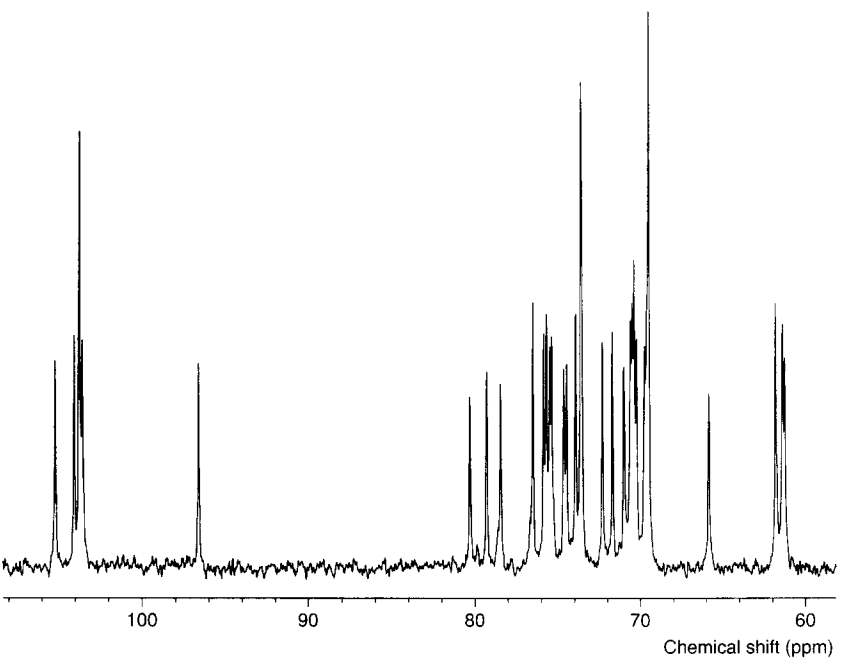


Fig. 2. The ^{13}C NMR spectrum at 100 MHz of the *L. helveticus* exopolysaccharide.

Table 1
Chemical shift (δ, ppm) of the signals in the ^{1}H and ^{13}C NMR spectra of the exopolysaccharide from *L. helveticus*

Sugar residue	^{1}H / ^{13}C						
	1	2	3	4	5	6	
→ 4)-α-D-Gal *p*-(1 →	5.16 (3.7)	3.96	4.05	4.28	4.25	3.76	3.87
A	96.7	69.6	70.7	79.3	71.0	61.4	
→ 6)-β-D-Gal *p*-(1 →	4.62 (7.3)	3.62	3.69	3.95	3.92	3.97	4.03
B	105.3	72.3	73.7	69.6	74.7	70.5	
→ 4)-β-D-Glc *p*-(1 →	4.54 (~ 8)	3.40	3.68	3.67	3.62	3.82	4.00
C	103.6	73.7	75.4	80.4	75.5	61.3	
→ 3,6)-β-D-Gal *p*-(1 →	4.54 (~ 8)	3.71	3.79	4.22	3.94	~ 3.95	4.08
D	103.8	70.2	78.5	65.9	74.5	69.7	
→ 6)-β-D-Glc *p*-(1 →	4.51 (8.2)	3.31	~ 3.49	~ 3.49	3.63	3.89	4.21
E	103.8	73.9	76.5	70.4	75.7	69.8	
β-D-Gal *p*-(1 →	4.48 (7.9)	3.57	3.67	3.95	3.71	3.80	3.80
F	104.1	71.7	73.7	69.6	75.9	61.9	

[a] $J_{H\text{-}1,H\text{-}2}$ values are given in Hz in parentheses.

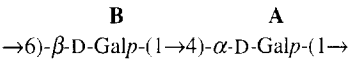
The chemical shifts for the protons and carbons together with coupling constants for anomeric protons are given in Table 1. The assignments of the spin system for each sugar residue were performed using homo- and hetero-nuclear two-dimensional techniques. Homonuclear COSY gave the chemical shifts of the H-2 signals for residues **A**–**F**. Relayed COSY and double-relayed COSY gave the $\delta_{H\text{-}3}$ and $\delta_{H\text{-}4}$, respectively, for residues **A**–**F**. Intra-residue NOE correlations between H-1 and H-5 in a NOESY experiment gave the $\delta_{H\text{-}5}$ for residues **B**, **C**, **D**, and **E**. Other intra-residue NOE correlations used in the assignment of spin systems were, inter alia, H-3 (δ 4.05) in **A** to its H-5 (δ 4.25), H-5 (δ 3.92) in **B** to its H-6 (δ 4.03), H-5 (δ 3.62) in **C** to its H-6 (δ 3.82, 4.00), H-4 (δ 4.22) in **D** to its H-5 (δ 3.94) and its H-6 (δ 4.08), H-5 (δ 3.94) in **D** to its H-6 (δ 4.08), H-5 (δ 3.63) in **E** to its H-6 (δ 3.89, 4.21), and H-5 (δ 3.71) in **F** to its H-6 (δ 3.80). The chemical shifts of the H-6 protons of the glucose residues **C** and **E** could also be interpreted from a TOCSY spectrum. From the heteronuclear COSY spectrum the assignments of the carbon chemical shifts for residue **A**, C-1, C-3–C-4; **B**, C-1, C-2, C-5; **C**, C-2–C-4; **D**, C-2–C-5; **E**, C-2–C-4; and **F**, C-1, C-2, C-5 could be performed. Further assignments were deduced from intra-residue correlations via heteronuclear couplings over two and three bonds employing ^{1}H detected HMBC experiments. The intra-residue correlations were, inter alia, H-3 (δ 4.05) in **A** to its C-2 (δ 69.6), H-4 (δ 4.28) in **A** to its C-2 (δ 69.6), H-5 (δ 4.25) in **A** to its C-4 (δ 79.3) and its C-6 (δ 61.4), H-6 (δ 3.76) in **A** to its C-5 (δ 71.0), H-2 (δ 3.62) in **B** to its C-3 (δ 73.7), H-5 (δ 3.92) in **B** to its C-6 (δ 70.5), H-6 (δ 4.03) in **B** to its C-5 (δ 74.7), H-2 (δ 3.40) in **C** to its C-1 (δ 103.6), H-6 (δ 3.82) in **C** to its C-5 (δ 75.5), H-2 (δ 3.71)

in **D** to its C-1 (δ 103.8), H-2 (δ 3.31) in **E** to its C-1 (δ 103.8), H-4 (δ 3.49) in **E** to its C-5 (δ 75.7), H-6 (δ 4.21) in **E** to its C-4 (δ 70.4), H-2 (δ 3.57) in **F** to its C-3 (δ 73.7), H-5 (δ 3.71) in **F** to its C-3 (δ 73.7), C-4 (δ 69.6), and C-6 (δ 61.9), and H-6 (δ 3.80) in **F** to its C-5 (δ 75.9).

The identification of a spin system with a specific sugar residue and substitution pattern as determined from the methylation analysis was done as follows. For residue **A** the value of $J_{H\text{-}1,H\text{-}2}$ was 3.7 Hz, showing the α configuration, and for residues **B**–**F** the $J_{H\text{-}1,H\text{-}2}$ values were around 8 Hz showing β configurations. The low chemical shifts of the H-2 signals for residues **C** and **E** are typical of glucose and the H-3 signals in the relayed COSY experiment show two large couplings; these residues were thus assigned to Glc*p* residues. The remaining residues **A**, **B**, **D**, and **F** could then be assigned to Gal*p* residues in accordance with sugar and methylation analysis. The downfield chemical shifts for the C-4 signals, δ 79.3 and δ 80.4, of residues **A** and **C**, respectively, compared with α-D-Gal*p* and β-D-Glc*p* [9] demonstrated that these were 4-substituted. Methylation analysis then indicated that residue **E** had to be 6-substituted. Residue **D** was assigned to the 3,6-substituted galactose supported by the downfield chemical shift of the C-3 signal, δ 78.5, compared with β-D-Gal*p*. The chemical shift of the H-5 signal, δ 3.92, of residue **B** compared with the expected values for terminal β-D-Gal*p* (H-5, δ 3.65) and 6-substituted β-D-Gal*p* (H-5, δ 3.87) [10] indicated that residue **B** was 6-substituted. Residue **F** could then be assigned as terminal in agreement with the lack of chemical shift displacement of its H-5.

In order to determine the sequence of the sugar residues, ^{1}H-detected HMBC and NOESY experiments were performed. In the HMBC experiment there are, in addition to correlations within a sugar residue, inter-residue correlations from the anomeric protons as well as the anomeric carbons, over the glycosidic linkage to carbons and protons on the adjacent residue (Table 2). Inter-residual NOE correlations are compiled in Table 3.

The galactose residue **B** shows a three-bond correlation from the anomeric proton over the glycosidic linkage to C-4 of the 4-substituted galactose residue **A** and from C-1 to H-4 of the same residues. An inter-residue NOE between H-1 in residue **B** and H-4 in residue **A** further confirms partial structure **1**.



1

The galactose residue **A** has three-bond correlations between H-1 in **A** and C-3 of the 3,6-disubstituted residue **D** and also between C-1 in **A** and H-3 of residue **D**. Two NOE correlations between H-1 in residue **A** and H-3 and H-4 in residue **D** further corroborate partial structure **2**.

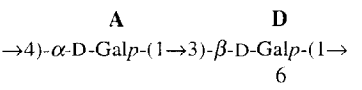


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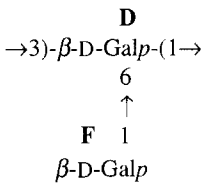
Table 2
Observed inter-glycosidic three-bond connectivities in HMBC experiments for the anomeric atoms of the exopolysaccharide from *L. helveticus*

Residue	Anomeric atom		Connectivity to		Residue, atom
	δ_H	δ_C	δ_C	δ_H	
→ 4)-α-D-Gal *p*-(1 →	5.16		78.5		**D**, C-3
A		96.7		3.79	**D**, H-3
→ 6)-β-D-Gal *p*-(1 →	4.62		79.3		**A**, C-4
B		105.3		4.28	**A**, H-4
→ 4)-β-D-Glc *p*-(1 →	4.54		69.8		**E**, C-6
C		103.6		3.89	**E**, H-6a
				4.21	**E**, H-6b
→ 3,6)-β-D-Gal *p*-(1 →	4.54		80.4		**C**, C-4
D					
→ 6)-β-D-Glc *p*-(1 →	4.51		70.5		**B**, C-6
E		103.8		3.97	**B**, H-6a
				4.03	**B**, H-6b
β-D-Gal *p*-(1 →	4.48		69.7		**D**, C-6
F		104.1		4.08	**D**, H-6b

Table 3
NOE data for the anomeric protons of the exopolysaccharide from *L. helveticus*

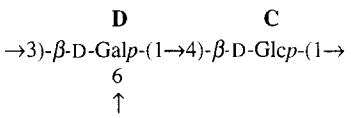
Residue	Anomeric proton	NOE to proton	Residue, atom
	δ	δ	
→ 4)-α-D-Gal *p*-(1 →	5.16	3.79	**D**, H-3
A		3.96	**A**, H-2
		4.22	**D**, H-4
→ 6)-β-D-Gal *p*-(1 →	4.62	3.69	**B**, H-3
B		3.92	**B**, H-5
		4.28	**A**, H-4
→ 4)-β-D-Glc *p*-(1 →	4.54	3.62	**C**, H-5
C		3.89	**E**, H-6a
		4.21	**E**, H-6b
→ 3,6)-β-D-Gal *p*-(1 →	4.54	3.67	**C**, H-4
D		3.79	**D**, H-3
		3.94	**D**, H-5
→ 6)-β-D-Glc *p*-(1 →	4.51	3.51	**E**, H-3
E		3.63	**E**, H-5
		4.03	**B**, H-6b

The terminal galactosyl group **F** has three-bond correlations between H-1 in **F** and C-6 of the 3,6-substituted galactose residue **D**. There is also a correlation between C-1 of **F** and H-6 (δ 4.08) of residue **D**. These data give structural element **3**.



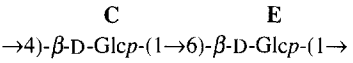
3

From the HMBC spectrum one can observe an inter-residue correlation between H-1 in residue **D** and C-4 of the 4-substituted residue **C**. Together with an observed NOE between H-1 in residue **D** and H-4 of residue **C**, structure element **4** is defined as follows:



4

The 4-substituted glucose residue **C** shows a three-bond correlation between H-1 in **C** and C-6 of the 6-substituted residue **E**. There is also correlation between C-1 in **C** and H-6 (δ 3.89, 4.21) of the 6-substituted residue **E**. H-1 in residue **C** has also an NOE to the H-6 protons of residue **E**. These data give partial structure **5**.



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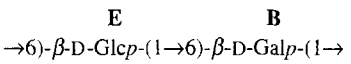
Finally, the 6-substituted residue **E** has three-bond correlations between H-1 in **E** and C-6 of the 6-substituted residue **B** and between C-1 in **E** and H-6 (δ 3.97, 4.03) of residue **B**, and also an NOE correlation from H-1 in **E** to H-6 (δ 4.03) of residue **B**, which give structural element **6**.



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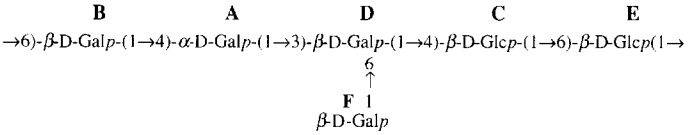
From the combined results, it is concluded that the neutral exopolysaccharide from *L. helveticus* is composed of a hexasaccharide repeating unit with structure **7**.



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3. Experimental

Growth of the organism.—*L. helveticus* strain (Valio Culture Collection Number 2091) was obtained from Valio Ltd., Research and Development Centre, Helsinki, Finland. The bacteria were grown in MRS broth (Lab M, Bury, UK) at 37 °C for 45 h. The culture broth (1 liter) was then filtered through a Pellicon Cassette System (Millipore Co., Milford, MA, USA) at 10 °C for 45 min to remove the bacterial cells.

Isolation of the polysaccharide.—The cell-free supernatant was concentrated by lyophilisation on a DURA-DRY freeze-dryer (FTS Systems Inc., Stone Ridgeny, NY, USA) to 10% of the original volume, and was then fractionally precipitated with ethanol at 40, 50, 60, 70, 80, 90, and 95% with intermediate centrifugation (30 min, 10500 rpm, 4 °C). The crude polysaccharide that precipitated at 40% (500 mg) was used for the structural studies.

After washing, the polysaccharide precipitate was dissolved in water and the solution was filtered through an Acrodisc PF syringe filter (0.8 μm/0.2 μm, Gelman Sciences, MI, USA), and then freeze-dried. A part of the crude polysaccharide ($\sim$ 20 mg) was redissolved in water, and applied to an anion-exchange chromatographic column on Fractogel TSK DEAE-650(S) gel (Merck, Darmstadt, Germany). A Bio-Rad Econo system was used: Econo-Column chromatographic column (1.5 $\times$ 25 cm), Econo pump (Model EP1), system controller (Model ES1), and a fraction collector (Model 2110). The column effluent was eluted at a flow rate of 1.10 mL/min; firstly with water for 80 min, and subsequently with 0.06 M NH_4OAc (Riedel–deHaën AG, Seelze, Germany) adjusted to pH 5.5 with AcOH (Sequencer Grade, Rathburn Chemicals Ltd., Scotland) for 120 min. A fraction was collected every 8 min, and the presence of sugar was tested qualitatively with a Molisch test [11]. Fractions reacting positively were collected and freeze-dried. The purity of the neutral polysaccharide was checked by RP-HPLC using a refractive index detector.

General methods.—Alditol acetates and partially methylated alditol acetates were separated on an HP-5 fused-silica column (0.20 mm $\times$ 25 m) using a temperature program of 180 °C for 1 min followed by 3 °C/min to 250 °C. Hydrogen was used as carrier gas. The column was fitted to a Hewlett–Packard model 5890 series II gas chromatograph (Hewlett–Packard, Palo Alto, CA, USA) equipped with a flame-ionisa-

tion detector. GC-MS analysis was performed on a Hewlett–Packard model 5970 mass spectrometer equipped with an HP-5MS fused-silica column (0.20 mm × 25 m). A temperature program of 170 °C for 3 min followed by 3 °C/min to 250 °C was used with He as carrier gas.

NMR spectroscopy.—NMR spectra of solutions in D_2O were recorded at 65 °C and pD 5.5, using a Jeol GSX-270, Jeol Alpha-400, or Varian Unity+ 600 instrument. Chemical shifts are reported in ppm relative to sodium 4,4-dimethyl-4-sila(2,2,3,3-$^2H_4$)pentanoate (TSP, δ_H 0.00) or acetone (δ_C 31.00) as internal reference. Data processing was performed using standard Jeol software, VNMR software, or Felix 2.3 (Biosym/MSI, San Diego, CA, USA). $^1H,^1H$-correlated spectroscopy (COSY), relayed COSY, double-relayed COSY, total correlation spectroscopy (TOCSY), $^{13}C,^1H$-COSY, gradient-enhanced heteronuclear single quantum coherence (gHSQC) [12] and heteronuclear multiple-bond correlation (HMBC) [13] experiments were used to assign signals and performed according to standard pulse sequences. For inter-residue correlations, two-dimensional nuclear Overhauser effect spectroscopy (NOESY) experiments with mixing times of 300 and 400 ms, and HMBC experiments with 60- and 90-ms delays for the evolution of long-range couplings were used.

Sugar analysis.—The samples were hydrolysed with 2 M trifluoroacetic acid at 120 °C for 2 h. After reduction with $NaBH_4$ and acetylation, the samples were analysed by GC. The absolute configuration of the sugars present in the exopolysaccharide from *L. helveticus* was determined essentially as devised by Leontein et al. [14], but with (+)-2-butanol [15].

Methylation analysis.—The analysis was performed according to Hakomori [16] using sodium methylsulfinylmethanide and MeI in dimethyl sulfoxide. The methylated compounds were recovered by use of Sep-Pak C_{18} cartridges (Millipore) [17]. The purified methylated sample was then hydrolysed (2 M trifluoroacetic acid, 120 °C, 2 h), reduced, and acetylated. The partially methylated alditol acetates were analysed by GC-MS.

Acknowledgements

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