Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 338 (2003) 567–570
CARBOHYDRATE RESEARCH
www.elsevier.com/locate/carres

Note

The O-specific chain structure of the major component from the lipopolysaccharide fraction of *Halomonas magadii* strain 21 MI (NCIMB 13595)

Cristina de Castro,[a,*] Antonio Molinaro,[a] Rosa Nunziata,[a] William Grant,[b] Andrew Wallace,[b] Michelangelo Parrilli[a]

[a] *Dipartimento di Chimica Organica e Biochimica, Università di Napoli Federico II via Cynthia 4, 80126 Napoli, Italy*
[b] *Department of Microbiology and Immunology, University of Leicester, Leicester, UK*

Received 31 July 2002; received in revised form 11 November 2002; accepted 23 November 2002

This paper is dedicated to Professor Lorenzo Mangoni on occasion of his 70th birthday

Abstract

An O-specific polysaccharide containing D-galactose and D-glucose, was isolated from the water-soluble lipopolysaccharide fraction of the alkaliphilic bacterium *Halomonas magadii*. The structure, determined by means of chemical analysis and 1D and 2D NMR spectroscopy, showed a trisaccharide repeating unit, as shown below:

4)-β-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→
4
↑
α-D-Glc*p*-(1

Keywords: O-Specific polysaccharide; Lipopolysaccharide; Alkaliphile; Gram-negative extremophile

Halomonas magadii is a Gram-negative extremophile and alkaliphilic bacterium isolated from Lake Magadi, located in the East African Rift Valley. Recently, several members of the halomonad group of bacteria were shown to inhabit the alkaline brines, including a new member, *H. magadii* strain 21 MI (NCIMB 13595), an organism that grows at high pH and relatively high salt concentration.[1] Very recently we described[2] the O-chain structure of the minor LPS component of this Gram-negative bacterium that is constituted by the following repeating unit:

→4)-α-L-Gul*p*NAcA-(1→6)-α-D-Glc*p*-(1→4)

-α-L-Gul*p*NAcA-(1→

We now report the structural elucidation of the O-chain of the major component obtained prolonging the batch culture up to approx 2 weeks under the conditions already described,[1] in these conditions LPS-2 was present in higher proportion and a new batch of bacterial cells was produced.

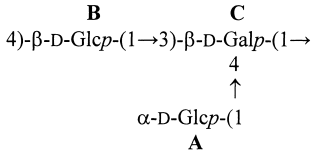
The compositional analysis of the LPS fraction contained in the aqueous phase of this second production analyzed here showed the presence of both glucose and galactose, traces of heptose and KDO and surprisingly, only traces of gulosaminuronic acid which had been a main component of LPS-1. Methylation analysis revealed the almost exclusive presence of terminal-Glc, 4-substituted-Glc units and, galactose branched at C-3 and -4; the D configuration was assigned to the glucose and galactose residues by analysing the 2-(+)-octyl derivatives.

Mild acid hydrolysis and the successive chromatographic separation led to the isolation of the O-chain

* Corresponding author. Tel.: +39-081-674124; fax: +39-081-674393
E-mail address: decastro@unina.it (C. de Castro).

0008-6215/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
PII: S0008-6215(02)00502-5

moiety that was studied by NMR spectroscopy. The ^{1}H and the HSQC spectrum spectra (Fig. 1) showed three anomeric signals of the same integral intensity occurring at δ 4.96, 4.70 and 4.55 correlated to carbon signals at 100.5, 105.6 and 104.4 ppm respectively, suggesting a trisaccharide repeating unit. Sugar moieties are named with **A**–**C** letters in order of decreasing chemical shift (Table 1). The anomeric configurations were established to be α, for **A**, and β for **B** and **C** residues, respectively on the basis of both their H-1/H-2 coupling constants and C-1 and C-5 proton and carbon chemical shifts. For residues **B** and **C**, *intra* residue NOEs cross peaks (H-1 to H-3 and H-5) were found in full agreement with the expected β configuration. The complete assignment of all proton and carbon signals was achieved by 2D homonuclear experiments (COSY and TOCSY) starting from the anomeric signals of each residue, whereas information about the sequence of the residues were deduced by NOESY spectrum (Fig. 2). Each anomeric proton showed an interresidual contact with the corresponding proton at the glycosylated position, in particular, the anomeric proton of residue **A** showed a strong NOE contact with H-4 of **C**. The anomeric proton of residue **B** was correlated in the same spectrum to H-3 of **C**, but the anomeric proton correlation of the latter residue was less clear, due to the overlapping of protons H-3 and H-4 of residue **B**: the substitution of this residue at C-4 was performed on the basis of the methylation data. The attachment points of each residue is supported also by the low-field shift of ^{13}C signals of glycosylated carbons (B-4, C-3 and C-4 Table 1) with respect to the corresponding carbons of the unsubstituted residues.[3] Combining both the spectroscopical and the chemical information, the O-chain structure of the major lipopolysaccharidic component from *H. magadii*, is the following:



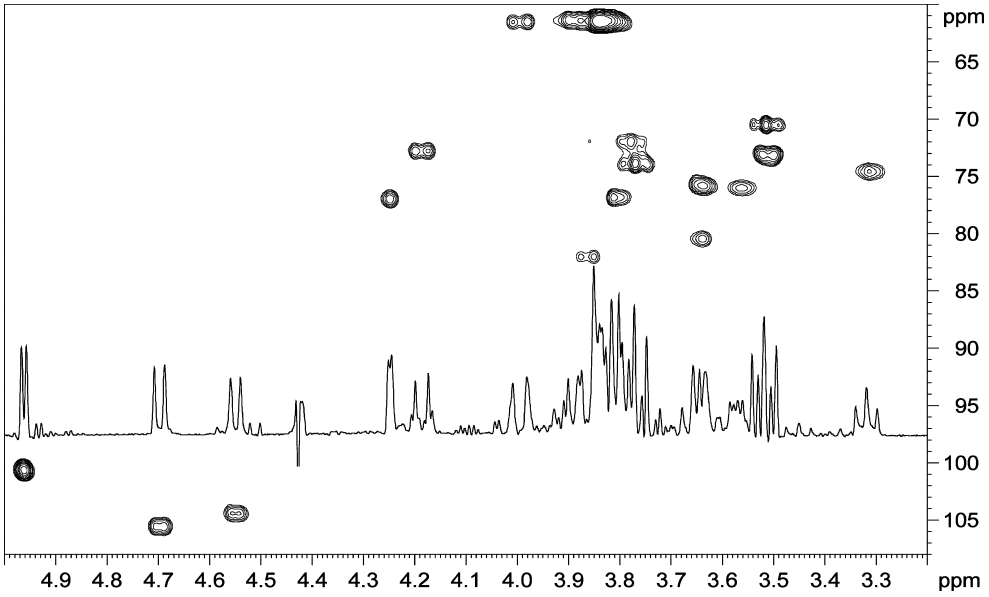


Fig. 1. 400 MHz ^{1}H and HSQC spectra measured at 328 K of the O-chain polysaccharide.

Table 1
400 MHz ^{1}H, and 125 MHz ^{13}C (italic) NMR data of O-chain polysaccharide, in D_2O at 328 K

	1	2	3	4	5	6
α-D-Glc^{A}*p*-(1→	4.96 *100.5*	3.50 *73.2*	3.77 *73.9*	3.51 *70.5*	4.19 *72.9*	3.84–3.86 *61.5*
4)-β-D-Glc^{B}*p*-(1→	4.70 *105.6*	3.31 *74.6*	3.64 *75.9*	3.64 *80.5*	3.56 *76.1*	4.00–3.89 *61.5*
3,4)-β-D-Gal^{C}*p*-(1→	4.55 *104.4*	3.78 *72.0*	3.86 *82.1*	4.25 *77.1*	3.81 *76.9*	3.89–3.86 *61.5*

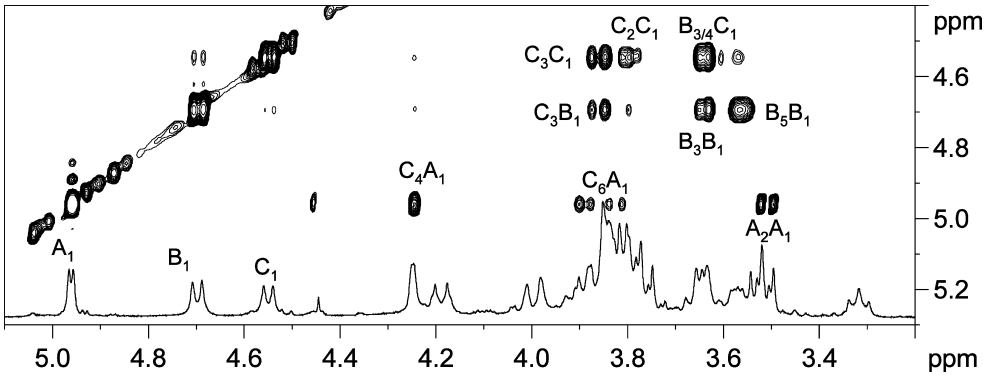


Fig. 2. 400 MHz and 328 K, NOESY spectrum of O-chain polysaccharide.

1. Experimental

H. magadii strain was isolated from Lake Magadi, Kenya and grown at 37 °C for 2 weeks, in liquid shake culture (200 rpm) in the alkaline medium already described.[1] Cells were pelleted at 2000*g*, washed with the salts component of the medium and freeze dried. Dried cells (10 g) were extracted according to the phenol–water method.[4] Both phases were separately dialyzed against distilled water, freeze-dried and screened by discontinuous SDS-PAGE,[5] with a 12% gel on a miniprotean gel system from Bio-Rad; the samples where run at constant voltage (150 V) and stained according the procedure of Kittelberger:[6] the lipopolysaccharide material was recovered in the water phase (5%).

Monosaccharides were analyzed as acetylated methylglycoside derivatives as follows: LPS (1 mg) was dried in a desiccator over P_2O_5 for 1 h under vacuum and then treated with 1 M methanolic HCl at 80 °C for 18 h. After cooling, the solution was directly extracted twice with equal volumes of *n*-C_6H_{14} in order to remove lipids components. The methanolic phase was dried and the methyl glycosides were acetylated as follows: first with Ac_2O (200 μL) at 80 °C for 15 min, dried in a stream of air and then treated with dry Py (200 μL) and Ac_2O (100 μL) at the same temperature for 30 min. After evaporation, the mixture of peracetylated methyl glycosides was analyzed by GC–MS under the same conditions used for 2-(+)-octyl-glycosides (see below) derivatives. Absolute configurations were deduced by analysis of the chiral 2-octyl glyco-derivatives according to the procedure of Leontein and Lonngren.[7]

Derivatives were run on Hewlett-Packard 5890 instrument, using a SPB-5 capillary column (Supelco, 30 m × 0.25 i.d. flow rate, 0.8 mL/min; He as carrier gas), with the temperature program: 150 °C for 5 min, 150–300 °C at 5.0 °C/min, 300 °C for 15 min, the mass spectra were recorded using a ionization energy of 70 eV and a ionizing current of 0.2 mA.

LPS (1 mg) was dried in a desiccator over P_2O_5 overnight and methylated as described by Sandford[8] The permethylated lipopolysaccharide was recovered in the organic layer of the water–$CHCl_3$ extraction, dried and hydrolyzed with TFA (2 M, 200 μL) at 120 °C for 2 h, the acid was removed by repeated evaporations with *i*-propanol and the partially methylated monosaccharides were dissolved in EtOH (200 μL) and reduced with $NaBD_4$ (3 mg) at room temperature for 1 h, borates were removed by evaporation with MeOH and few drops of glacial AcOH, acetylation was performed with Ac_2O and Py (150 μL each) at 120 °C for 20 min. The partially methylated and acetylated alditols were analyzed by GC–MS, with the following temperature program: 80 °C 2 min, 80–240 °C at 4 °C min, 240 °C for 15 min.

Typically, LPS fraction was dissolved in 1% AcOH solution (10 mg/mL) and kept at 100 °C for 2 h. After cooling, the solution was centrifuged at 6000 rpm for 20 min and the clear supernatant freeze-dried. The dried supernatant was further purified by SEC on Sephacryl HR 100 (Pharmacia, 1.5 × 70 cm, NH_4HCO_3 50 mM, flow 0.4 mL/min) and the eluate monitored by refractive index as above mentioned; O-chain was eluted as major peak close to the void volume (75% yield from LPS).

NMR experiments were carried out on a Bruker DRX 400 MHz equipped with reverse multinuclear probe at 328 K in order to avoid the overlapping of the anomeric proton signals with the residual water peak; chemical shift are expressed in δ relative to internal C_3H_6O (2.225 and 31.4 ppm). Two-dimensional spectra (DQF-COSY, phase-sensitive TOCSY and NOESY, and gradient-HSQC) were measured using standard Bruker software. For homonuclear experiments, typically 512 FIDs of 1024 complex data points were collected, with 40 scans per FID. In all cases, the spectral width was set to 10 ppm and the carrier placed at the residual HOD peak. A mixing time of 200 ms

was used for both NOESY experiment. For the HSQC spectrum, 256 FIDS of 1024 complex points were acquired with 50 scans per FID, the GARP sequence was used for ^{13}C decoupling during acquisition. Processing and plotting was performed with standard Bruker XWIN-NMR 1.3 program.

Acknowledgements

We thank the Centro di Metodologie Chimico-Fisiche of the University Federico II of Naples for the NMR spectra, and CNR for financial support.

References

1. Duckworth, A. W.; Grant, W. D.; Jones, B. E.; Meijer, D.; Marquez, M. C.; Ventosa, A. *Extremophiles* **2000**, *4*, 53–60.
2. De Castro, C.; Molinaro, A.; Wallace, A.; Grant, W.D.; Parrilli, M. *Eur. J. Org. Chem.* **2003**, in press.
3. Lipkind, G. M.; Shashkov, A. S.; Knirel, Y. A.; Vinogradov, E. V.; Kochetcov, N. K. *Carbohydr. Res.* **1988**, *175*, 59–75.
4. Westphal, O.; Jann, K. *Meth. Carbohydr. Chem.* **1965**, *5*, 83–91.
5. Laemmli, U. K. *Nature* **1970**, *97*, 620–628.
6. Kittelberger, R.; Hilbink, F. *J. Biochem. Biophys. Meth.* **1993**, *26*, 81–86.
7. Leontein, K.; Lönngren, J. *Meth. Carbohydr. Chem.* **1978**, *62*, 359–362.
8. Sandford, P. A.; Conrad, H. E. *Biochemistry* **1966**, *5*, 1508–1517.