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Note

O-Allyl decoration on α-glucan isolated from the haloalkaliphilic *Halomonas pantelleriensis* bacterium

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Abstract—An α-glucan containing the unprecedented peculiar *O*-allyl substituent was isolated from the haloalkaliphilic Gram-negative *Halomonas pantelleriensis* bacterium. Its dextran-like structure was deduced from chemical degradative and spectroscopic methods.
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Surface microbial polysaccharides play a crucial role for the surviving and growth of microorganisms in their specific ecological niches, even when they grow under extreme life conditions. In particular, the surface polysaccharidic repertoire for Gram-negative bacteria almost always consists of lipopolysaccharides, the main component of outer membranes, and, occasionally of capsular polysaccharides, that in some cases are closely connected to lipopolysaccharides.[1] During our recent investigation[2] on the chemical structure of the O-chain repeating unit of the LPS from *Halomonas pantelleriensis*, a Gram-negative haloalkaliphilic bacterium, we isolated a new polysaccharide whose structural determination is now described.

After cultivation and harvesting, the bacterial cell mass was washed with an isotonic solution, lyophilised and then extracted utilizing the phenol/water method, as already reported.[2] The aqueous phase, after dialysis, was ultracentrifuged and the supernatant was treated with RNAse and DNAse in order to remove nucleic acids. After dialysis, the inside liquor was lyophilised and the crude LPS was hydrolysed with 1% acetic acid to cleave the lipid A moiety, that was removed from the solution by centrifugation. The supernatant was submitted to gel-chromatography yielding, in addition to the O-chain retained peak, an excluded polysaccharide peak, whose sugar analysis by GLC–MS of acetylated methyl glycosides indicated the presence of Glc besides another unidentified sugar. The ^{1}H NMR spectrum of the unknown polysaccharide (Fig. 1a) showed two anomeric signals at δ 5.08 (broad singlet) and δ 4.90 (broad singlet), in addition to two doublets at δ 5.32 ($^3J_{\mathrm{H,H}}$ 17.1 Hz) and δ 5.28 ($^3J_{\mathrm{H,H}}$ 10.5 Hz), a multiplet at δ 5.92 and a doublet signal at δ 4.16, with a splitting of 6.2 Hz. The latter showed an intensity that was double compared with that of each signal at 5.28, 5.32 and 5.92, respectively. A ^{1}H, ^{13}C DEPT-HSQC (Fig. 1b) experiment showed two anomeric signals at δ 98.9 and δ 97.0, and two signals, in the sp^2 region, at δ 120.0 and δ 135.1 in addition to the carbinolic carbon signals. The proton signal at δ 5.92 was correlated to the carbon signal at δ 135.1 and those at δ 5.32 and δ 5.28 were both correlated in anti-phase mode to the carbon signal at δ 120.0, indicating the presence of an unsaturated methylene carbon atom. Moreover the proton signal at δ 4.16

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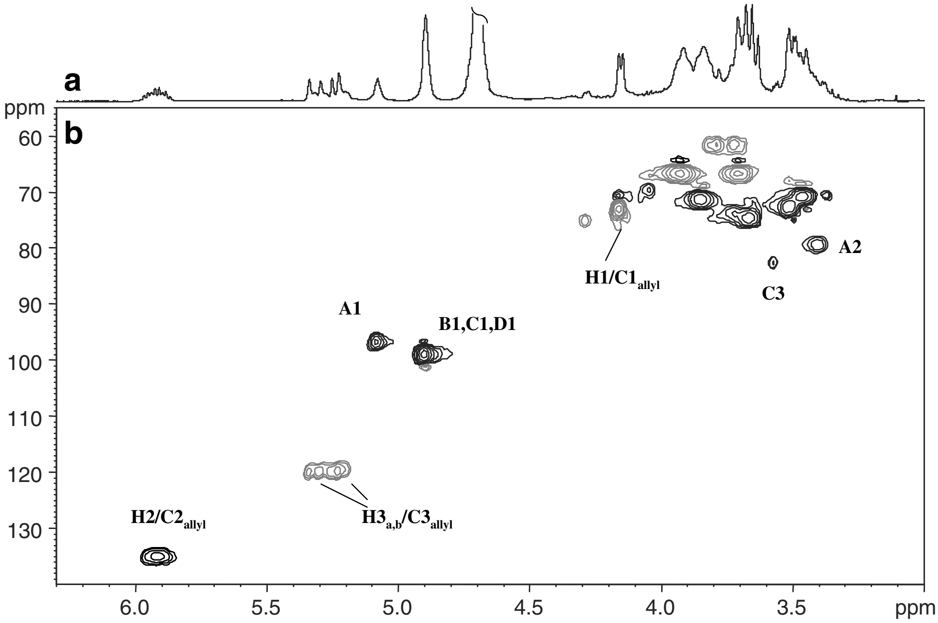

Figure 1. (a) ^{1}H NMR spectrum and (b) ^{1}H, ^{13}C HSQC-DEPT experiment of glucan from *Halomonas pantelleriensis* in D_2O at 500 MHz (30 °C). Some diagnostic signals are indicated in the spectrum. Grey cross-peaks refer to CH_2 carbon.

was assigned to an oxymethylene group, as this signal was correlated to the carbon signal at δ 72.9 in anti-phase mode. The chemical shifts and the proton connectivities of the above signals, obtained by a COSY experiment, allow to easily identify an *O*-allylic substituent: $–OCH_2–CH{=}CH_2$. In order to support this hypothesis, an EI mass spectrum of the unidentified acetylated methyl glycoside was carefully investigated. The spectrum (data not shown) showed an ion peak at *m*/*z* 329, that corresponds to an A_1 ion[3] of an *O*-allyl substituted hexose. To confirm the presence of an *O*-allyl decoration, the polysaccharide was treated with $PdCl_2$, the usual reagent to remove the *O*-allyl substituent,[4] and the following glycosyl analysis of the treated polysaccharide revealed the presence of only glucose. The location at O-2 of the allyl substituent and the linkage analysis were inferred on the basis of the EI mass spectra of partially methylated alditol acetates. In particular, in Figure 2 the fragmentation pattern of 1,5-di-*O*-acetyl-2-*O*-allyl-3,4,6-tri-*O*-methyl-D-glucitol-1-*d* is reported. The spectrum allowed to identify the presence of 2-*O*-allylglucose as terminal units in the polysaccharide. Moreover the methylation analysis indicated the presence of 6-linked (2-*O*-allyl)-Glc, in addition to terminal Glc, 3,6-linked Glc and 6-linked Glc units. The anomeric configuration of glucopyranose residues was assigned to be α on the

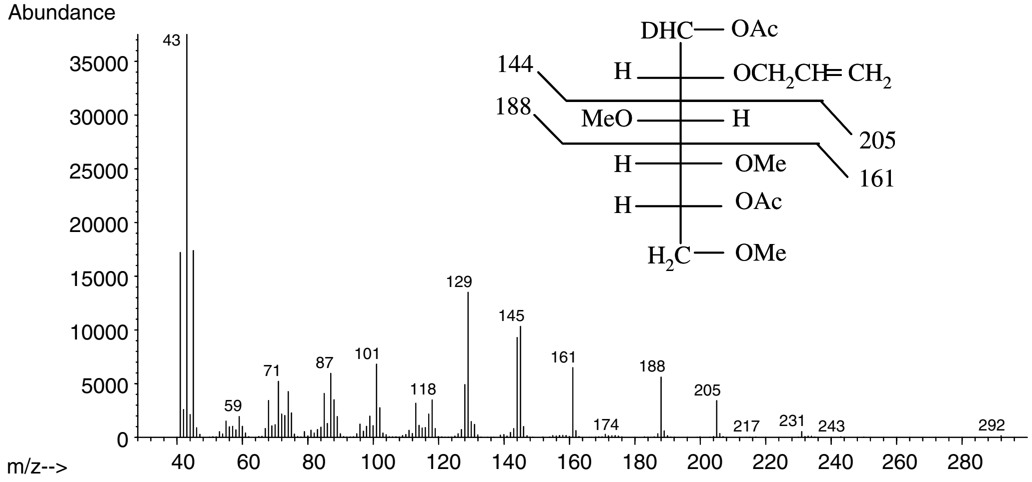

Figure 2. EIMS spectrum of 1,5-di-*O*-acetyl-2-*O*-allyl-3,4,6-tri-*O*-methyl-D-glucitol-1-*d*.

Table 1. ^{1}H and ^{13}C NMR data of glucan fraction from *Halomonas pantelleriensis* in D_2O at 30 °C

Residue	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6a/C6	H6b
→6)-α-D-(2-*O*-allyl)-Glc	5.08	3.40	3.71	3.47	3.83	3.92	3.71
A	97.0	79.6	73.8	70.7	71.3	66.7	
→6)-α-D-Glc	4.90	3.51	3.66	3.43	3.85	3.93	3.71
B	98.9	72.6	74.6	70.7	71.3	66.7	
Terminal α-D-Glc	4.90	3.49	3.66	3.36	3.85	3.79	3.72
C	98.9	72.6	74.6	70.7	71.3	61.7	
→3,6)-α-D-Glc	4.90	3.44	3.57	3.54	3.85	3.93	3.71
D	98.9	72.6	82.6	70.7	71.3	66.7	
Allyl	4.16	5.92	5.32, 5.28				
	72.9	135.1	120.0				

basis of the proton and carbon chemical shifts and the values of 3.3 Hz for $J_{H1,H2}$ coupling constants, measured on a high resolved ^{1}H NMR spectrum. The complete assignment of ^{1}H and ^{13}C NMR chemical shifts (Table 1) was achieved by 1D and 2D experiments (COSY, TOCSY, NOESY, DEPT-HSQC and HMBC).

The proton anomeric signal at δ 5.08 was assigned to the 2-*O*-allyl substituted glucoses on the basis of the upfield shift of their carbon anomeric signal due to the α-substitution. The downfield shift of C-2 at 79.6 ppm, compared with the value of 72.5 ppm for an unsubstituted glucose[5] was in agreement with the linkage at position 2 of the *O*-allyl substituent. In particular, these assignments were attributed to the 6-linked residue, which was indicated by **A** in Table 1, as its C-6 resonance occurred at the low-field chemical shift value of 66.7 ppm. It was not possible to identify the proton and carbon signals for the terminal 2-*O*-allylglucose units, due to their very low amount (Table 2). Residues **B** and **D** were assigned to 6-linked and 3,6-linked glucopyranoses, respectively, on the basis of the down-field chemical shift of their substituted carbon signals. The residue **C** was assigned to the terminal α-glucose, since all its carbon chemical shifts were in good agreement with that of an unsubstituted glucose.

These data suggested for the polysaccharide a glucan with a backbone constituted by α-(1→6)-linked glucose units, branched at C-3. The mole percentage of methylated derivatives (Table 2) suggested a low branching degree, on the basis of terminal glucose units. In fact the mole percentage value of (3→6)-linked glucose could not be considered due to the overlapping of its signal in the GLC–MS chromatogram with that of the 6-linked 2-*O*-allylglucose. These methylation data should suggest a 2-*O*-allylglucose/glucose ratio lower than that indicated by the integration of their proton anomeric signals, which is 1:3. This discrepancy could be attributed to the different response factors of methylated derivatives and/or the non-stoichiometric ratio between nodal and terminal residues, as reported by Seymour et al.[6]

Table 2. Mole percentage of methylated monosaccharides from the hydrolysis of methylated glucan

2,3,4,6-Tetra-*O*-methylglucose	15	2,4-Di-*O*-methylglucose 2-*O*-Allyl-3,4-di-*O*-methylglucose	21
2,3,4-Tri-*O*-methylglucose	62	2-*O*-Allyl-3,4,6-tri-*O*-methylglucose	2

On the basis of the above data this glucan seems to belong to the dextran family, even though to our knowledge, this is the first time that an *O*-allyl decoration is reported.

The absolute configuration determination was carried out on the O-deallylated polysaccharide and the GLC–MS analysis of the acetylated octyl glycosides[7] showed that Glc has the D configuration. From this result a D configuration for the 2-*O*-allylglucose was also deduced.

We do not know if the *O*-allyl substituent is native but, at present, it is hard to imagine more complex substituents that could result in it by degradation during either the phenol/water cells extraction or acetic acid hydrolysis. As for the nature of this polysaccharide, it cannot be considered as an exopolysaccharide since no glucan was found in the culture broth, whereas mannan was isolated. Furthermore the possibility that it represents an additional O-chain different from that already described, with a structure such as:

→4)-α-D-Gal*p*NAcA-(1→3)-β-L-Qui*p*NAc-(1→2)-β-4-*O*-[(*S*)-1-carboxyethyl]-D-Glc*p*A-(1→4)-β-D-Glc*p*A-(1→

might be, in our opinion, excluded. This was deduced on the basis of the electrophoresis of the crude LPS, that did not suggest the presence of more LPSs of different sizes,[2] in agreement with the different elution volume on gel-chromatography of glucan and O-chain polysaccharides. By exclusion the hypothesis of a putative capsular polysaccharide remains, but its very low amount prevented a microscopy analysis to support this suggestion.

The role of EPS for extremophilic bacteria has not yet been clarified but these biopolymers offer a number of novel material properties and commercial opportunities.[8,9] In our opinion, the presence of the *O*-allyl decoration in this glucan isolated from *Halomonas pantelleriensis* might be considered for potential industrial application, since it could be a convenient functional group for cross-linkage.

1. Experimental

1.1. Bacterial strain and growth conditions

Halomonas pantelleriensis (DSM 9661) was isolated from hard sand of the lake Venere in the Pantelleria island (Italy) as previously reported.[10] It was grown at 35 °C, pH 9.0 in 2-L flasks with 500 mL of medium in a rotary shaker incubator (New Brunswik) with stirring at 100 rpm. The medium composition (g/L): yeast extract 10; NaCl 160; Na_3–citrate 3; KCl 2.0; $MgSO_4 \cdot 7H_2O$ 1.0; Na_2CO_3 3.0; mg/L $MgCl_2 \cdot 4H_2O$ 0.36 and $FeSO_4$ 50. Na_2CO_3 and NaCl were autoclaved separately. The inoculum was 1% of total volume.

1.2. Polysaccharide extraction and purification

Dry cells (2 g) were suspended in ultrapure Milli-Q water (50 mL) and extracted with phenol according to the phenol/water method.[11] The resulting aqueous phase was dialysed (cut-off 3500) for 4 days. Contents of the tube were lyophilised (300 mg); the residue was dissolved in ultrapure Milli-Q water and treated with DNAse (Sigma, 15 mg), RNase (Sigma, 15 mg) for 3 h at 37 °C. After this time the sample was dialysed as above and lyophilised to yield ‘crude’ LPS (60 mg, yield 3.0% of dried cells). A sample (52 mg) of the crude LPS was hydrolysed with 1% AcOH containing 0.1% of SDS at 100 °C for 3 h. After cooling, the sample was centrifuged (10,000*g*) for 20 min. The supernatant (25 mg) was applied to a Sephacryl S-300 HR column (Pharmacia, 109 × 1.5 cm, flow rate 17 mL/h, volume fraction 2.0 mL) and eluted with 50 mM NH_4HCO_3 buffer, resulting in a polysaccharide fraction in the void volume (7 mg), the O-chain fraction (4 mg) and a core fraction (9 mg).

1.3. General and analytical methods

The determination of neutral sugars and the GLC–MS analyses were all carried out as described elsewhere.[2] The absolute configuration determination was carried out on the O-deallylated polysaccharide as reported by Leontein et al.[7] In order to detect the presence of the allyl substituent on glucose units, an aliquot of the polysaccharide (1 mg) was dissolved in a mixture of CH_3OH/CH_2Cl_2 and treated with $PdCl_2$ (8 mg) for 18 h at 20 °C. The sample was purified by extraction with CH_2Cl_2 and 5 M NaCl and then submitted to glycosyl analysis. The methylation analysis was carried out by Ciucanu and Kerek method.[12] The methylated polysaccharide was then hydrolysed with 2 M CF_3COOH at 120 °C for 1 h. The partially methylated products in the hydrolysates were reduced with $NaBD_4$, acetylated, and analysed by GLC–MS.

1.4. NMR spectroscopy

For structural assignments of glucan polysaccharide, 1D and 2D ^1H and ^{13}C NMR spectra were recorded at 30 °C using a Varian Inova 500 spectrometer. Chemical shifts were measured in D_2O using acetone, at δ 2.225 and 31.45 for proton and carbon, respectively, as internal standard. All 2D NMR spectra were registered according to Varian software. The high resolved ^1H NMR spectrum was obtained using a Gaussian function.

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