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Note

# O-Specific chain structure from the lipopolysaccharide fraction of *Pseudomonas reactans:* a pathogen of the cultivated mushrooms

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**Abstract**

An O-specific polysaccharide containing 2-acetamidino-2-deoxy-β-D-glucopyranose (Glc*p*2Am), 2,4-diacetamido-2,4,6-trideoxy-β-D-glucopyranose (Qui*p*NAc4NAc, bacillosamine) and 2,4-di-(*N*-acetyl-L-alanylamino)-2,4,6-trideoxy-β-D-glucopyranose (Qui*p*-NAlaAc4NAlaAc) was isolated from the phenol-soluble lipopolysaccharide fraction of the mushroom-associated bacterium *Pseudomonas reactans*. The structure, determined by means of chemical analysis and 1D and 2D NMR spectroscopy, showed a linear trisaccharide-repeating unit, as shown below:

→3)-β-D-Qui*p*NAlaAc4NAlaAc-(1→3)-α-D-Glc*p*2Am-(1→3)-α-D-Qui*p*NAc4NAc(1→

To our knowledge, this is the first complete O-chain structure reported for the lipopolysaccharide of a mushroom-associated bacterium. 

*Keywords:* O-Specific polysaccharide; Mushrooms; *Pseudomonas reactans*; Lipopolysaccharide, bacillosamine

A great number of Gram-negative bacteria belong to the genera *Pseudomonas* and *Xanthomonas* and several O-specific polysaccharides (OPSs) have been hitherto elucidated.[1] About 80% of these structures are built up of a branched rhamnopyranose backbone bearing arms mainly consisting of one residue (variously: Xyl; Fuc; Rha; Fuc3Nac; GlcNAc).

*Pseudomonas reactans* is still an unclassified bacterial entity which is considered as a saprophytic form associated with cultivated mushrooms.[2] The bacterium is useful in the 'white line' assay for the identification of *Pseudomonas tolaasii*, the causal agent of brown and yellow blotch diseases of *Agaricus bisporus* and *Pleurotus ostreatus*.[3] In this assay the growth, side by side on agar of the above bacteria, determine the formation of a white precipitate which is apparently due to the interaction of tolaasiin and WLIP (white line inducing principle), lipodepsipeptides produced in cultures by virulent strains of *P. tolaasii* and *P. reactans*, respectively.[4] However, recent investigations have shown that the above mushroom diseases are characterised by a complex aetiology. In fact, both *P. tolaasii* and *P. reactans* have been constantly isolated from lesions on *A. bisporus* and *P. ostreatus* sporocarps and furthermore, pure cultures of these bacteria reproduced the disease symptoms, although differences in the pathogenetical capability between the two bacteria have been observed. *P. reactans* has also been shown to be the causal agent of the yellowing of *Pleurotus eryngii*, a mushroom species whose cultivation has been recently established in Southern Italy.[5]

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In this paper, the isolation and the structural determination of the OPS present in the phenol-soluble LPS from *P. reactans* are reported. SDS PAGE electrophoresis of water and phenol extracts arising from phenol–water treatment of *P. reactans* cells showed that only the phenol phase had the typical banding pattern of smooth LPS. Accordingly, the compositional analysis of the aqueous-phase fraction showed large amounts of glucose but neither Kdo nor 3-hydroxy fatty acids, which are typical constituents of LPSs, were present. Methylation analysis of the latter fraction revealed the presence of terminal-Glc and 4-substituted-Glc units and furthermore, its $^1$H NMR spectrum (data not shown) showed an anomeric signal at 5.41 ppm, as a broad singlet, in addition to carbinolic signals in the range 3.48–4.21 ppm. All these data suggested an amylose-like structure characterised by an average-molecular weight of 300 KDa as obtained by gel-permeation chromatography.

The presence of Kdo, dodecanoic acid (12:0), 3-hydroxydecanoic acid, [10:0 (3-OH)], 3-hydroxydodecanoic acid, [12:0 (3-OH)], as components of the phenol phase confirmed the LPS nature of this fraction. The mild-acid hydrolysis of the fraction removed, as precipitate, the lipid A moiety leaving in the supernatant, the saccharide part which, purified by gel-permeation chromatography, gave rise to two fractions and the one in the void volume represented the polysaccharidic part.

The GC–MS analysis of the high-molecular-weight polymer (see Experimental) indicated: the presence of Glc*p*NAc, Qui*p*NAc4NAc, and alanine (Ala); the D configuration of both sugar residues; the L configuration of Ala. The compositional analysis was completed by the methylation data which indicated the presence of 3-substituted glucosamine arising from Glc*p*2Am and 3-substituted bacillosamine.

The $^1$H and $^{13}$C NMR spectra (Figs. 1 and 2) showed three anomeric signals of the same integral intensity occurring at $\delta$ 5.199, 4.971 and 4.325 and at $\delta$ 95.8, 98.0 and 100.7, 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, α and β for **A**, **B** and **C** residues, respectively, by their chemical shift values and positively confirmed on the basis of their $^3J_{H-H}$ and $^1J_{C-H}$ values, 179, 177 and 164 Hz measured from a coupled HSQC experiment. For residue **C**, intraresidue NOE cross-peaks (H-1–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 and heteronuclear experiments (DQF-COSY, TOCSY, ROESY, HSQC, HMBC). Despite the crowded ring-proton region of the $^1$H NMR spectrum, the assignment of each resonance was straightforward since all three residues had gluco-configuration with large vicinal values of coupling constants (about 10 Hz). Thus starting from the anomeric signals of **A**, **B** and **C** in the COSY spectrum, the H-2 protons were identified at $\delta$ 4.039, 4.103 and 3.745, respectively. Moreover for the two deoxy-residues (**A** and **C**), it was possible also to start from their C-6/H-6 shielded signals which were correlated in the COSY at $\delta$ 3.893 and 3.461. The remaining signals were identified by means of their TOCSY and ROESY spectra. The $^{13}$C NMR spectrum was also informative in showing: several signals of carbons bearing nitrogen in the range 50.4–56.8 ppm; a hydroxymethyl signal; five methyl signals in the range 19.7–16.4 ppm; and at low field (166.6 ppm), a signal correlated by long-range scalar coupling to the methyl group at $\delta$ 2.209. These last two signals were assigned to a C=N and a $CH_3$ group, respectively, of an acetamidino group (Am), the presence of which was established by treatment of the OPS with triethylamine which converts the acetamidino into an acetyl group as shown by the disappearance of the methyl group at $\delta$ 2.209 and the appearance of an extra acetyl signal at $\delta$ 2.015.[6]

The localisation of the acetamidino group was inferred by the ROESY spectrum (Fig. 3). The presence of two NOE contacts of the methyl group at $\delta$ 2.209 with the signals at $\delta$ 4.971 and 4.103 previously assigned to H-1 and H-2 of the glucosamine residue,

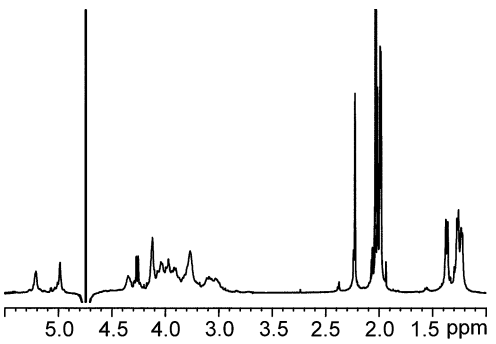



Fig. 1. The $^1$H NMR spectrum of the O-chain polysaccharide.

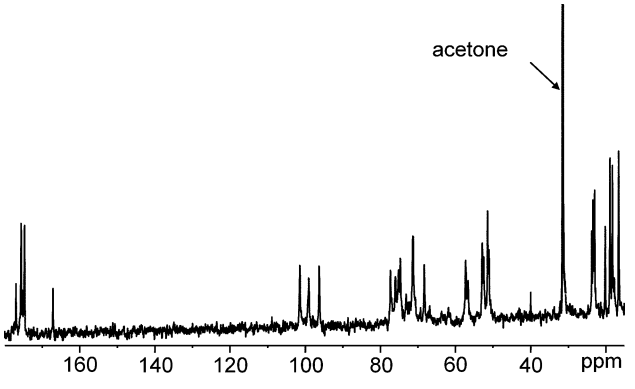
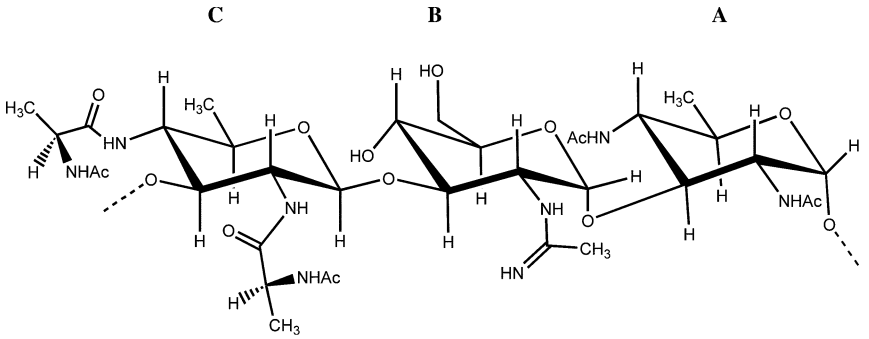



Fig. 2. The $^{13}$C NMR spectrum of the O-chain polysaccharide.

Table 1
The $^{1}H$ and $^{13}C$ NMR resonances for each residue and substituent are shown




| Residue | H-1/C-1 | H-2/C-2 | H-3/C-3 | H-4/C-4 | H-5/C-5 | H-6/C-6 |
|---|---|---|---|---|---|---|
| Qui*p*NAc4NAc | 5.199 | 4.039 | 3.933 | 3.751 | 3.893 | 1.239 |
| (**A**) | 95.8 | 52.1 | 74.4 | 56.4 | 67.4 | 16.4 |
| Acetyl ($\times 2$) | | 1.987 | | | | |
| | 174.0 | 23.2 | | | | |
| GlcAm | 4.971 | 4.103 | 3.777 | 3.969 | 3.986 | 3.866, 3.854 |
| (**B**) | 98.0 | 50.4 | 76.6 | 74.6 | 72.5 | 61.1 |
| Amidino group | | 2.209 | | | | |
| | 166.6 | 19.7 | | | | |
| Qui*p*NAlaAc4NAlaAc | 4.325 | 3.745 | 3.766 | 3.579 | 3.461 | 1.191 |
| (**C**) | 100.7 | 56.8 | 76.5 | 56.1 | 71.0 | 17.6 |
| Ala1 residue | | 4.239 | 1.324 | | | |
| | 176.6 | 51.0 | 18.0 | | | |
| Ala2 residue | | 4.099 | 1.274 | | | |
| | 177.4 | 51.9 | 17.0 | | | |
| Ac Ala1 | | 1.983 | | | | |
| | 174.2 | 22.7 | | | | |
| Ac Ala2 | | 1.973 | | | | |
| | 176.3 | 22.8 | | | | |

respectively, allowed the localisation of this substituent at the C-2 position of the residue **B**. The HMBC spectrum confirmed this hypothesis, showing a cross peak correlating the signal at $\delta$ 166.6 and the H-2 proton of the α-glucosamine residue at $\delta$ 4.103.

The proton and carbon signals of the two alanine residues (named **ala1** and **ala2**) were clearly identified starting from nitrogen-bearing carbon at $\delta$ 51.0 and 51.9 which were correlated in the HMBC with the methyl doublet at $\delta$ 1.324 (**ala1**) and $\delta$ 1.274 (**ala2**), respectively. Moreover, the correlations between the methine proton of each alanine residue, occurring at $\delta$ 4.239 and 4.099 respectively, with two carbonyl carbons (174.2 and 176.3 ppm), in turn correlated to methyl acetyl signals, indicated that both the alanine residues were acetylated. The location of both alanine units on the residue **C** was positively established by the observation of the connectivities from the H-2 ($\delta$ 3.745) and H-4 ($\delta$ 3.579) protons of the residue **C** with the carbonyl signals at $\delta$ 177.4 and 176.6 belonging to **ala2** and **ala1**, respectively. Therefore **ala1** is located at C-4 and **ala2** at C-2 of residue **C**. These data were positively confirmed by NOE contacts found in the ROESY spectrum between the methine protons of each alanine residue and the respective ring proton H-2 and H-4. The other two acetyl signals present in the $^{1}H$ and $^{13}C$ spectra were located at C-2 and C-4 of the residue **A** on the basis of the long-range correlation between the protons at $\delta$ 4.039 and 4.090 with carbonyl carbons at $\delta$ 174.0.

The sequence of the residues was deduced by ROESY spectrum (Fig. 3), whereby each anomeric proton showed an interresidual contact with the corresponding proton at the glycosylated positions. In particular, the anomeric proton of residue **A** showed a strong NOE contact with H-3 of **C**, the anomeric

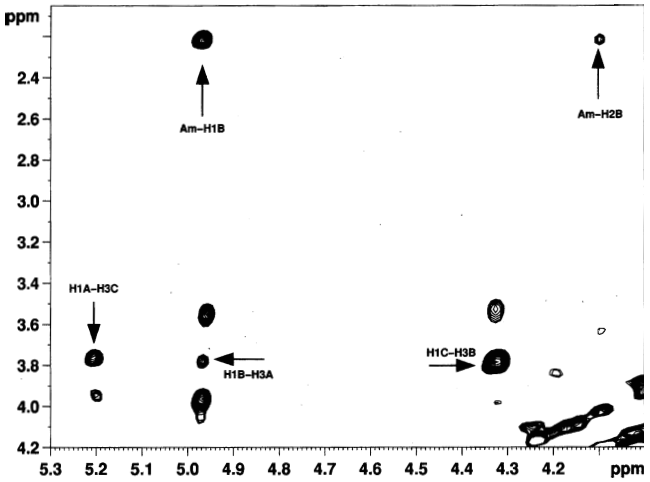



Fig. 3. Section of ROESY spectrum of the O-chain polysaccharide.

proton of residue **B** was correlated in the same spectrum to H-3, and in a more intense way, to H-4 of **A**. This is in accordance with the D absolute configurations of both **B** and **A** units of their $1\rightarrow3$ interglycosidic linkage and of the $\alpha$ anomeric configuration of **B** residue.[7] Moreover, an NOE contact was observed between the anomeric proton of residue **C** and H-3 of residue **B** in agreement with a linkage at C-3 for the GlcAm unit. These data, together with the downfield shift of the respective carbons and with the expected correlations found in the HMBC spectrum, validated the proposed structure.

Since we were unable to find in the literature previous reports of O-chain structures of lipopolysaccharides from bacteria that are mushroom pathogens, we presume that this is the first such report.

The peculiarity of these monosaccharides, together with other characters, will allow further insight into *P. reactans* classification and possibly, to sanction its differentiation from the correlated *P. tolaasii*.[4] In this regard the structural investigation on the O-chain of the latter mushroom pathogen are in progress.

## 1. Experimental

*Growth of bacteria, isolation of LPS and OPS.*—Strain NCPPB1311 of *P. reactans* was kept at 4 °C and routinely subcultured on KB agar slants at 25 °C. Bacterial cells for LPS extraction were obtained by growing the strain in 500 mL Erlenmeyer flasks filled with 200 mL of liquid KB on a rotary shaker at 150 rpm at 25 °C for 48 h. Cultures were centrifuged (12,000 rpm, 15 min), the pellet washed twice with saline solution (0.8% NaCl) and the cells were freeze-dried. The dried cells (3.5 g) from 1 L of culture filtrates of *P. reactans* were suspended in 100 mL of ultrapure Milli-Q water and extracted with phenol according to the conventional procedure.[8] The LPS content of both phases was checked by SDS/PAGE electrophoresis,[9] Kdo[10] and 3-hydroxy fatty acid content and it was found in the phenol phase (yield: 250 mg, 7% of bacterial dry mass). In order to obtain the O-polysaccharide chain, the LPS was hydrolysed with aq 1% AcOH for 2 h at 100 °C and centrifuged (11,000 rpm, 4 °C, 1 h). The supernatant thus obtained (OPS fraction, 202 mg, 81% of LPS) was purified by gel-permeation chromatography on a Sephacryll S300-HR column ($90\times1.5$ cm) using 0.05 M ammonium bicarbonate as eluent and monitored with a Waters differential refractometer.

*NMR spectroscopy.*—The $^1$H and $^{13}$C NMR spectra were obtained in $D_2O$ at 600 and 150 MHz, respectively, with a Bruker DRX 600 spectrometer equipped with a reverse probe, in the FT mode at pD 7, with a solution of 10 mg in 0.5 mL of $D_2O$, at 30 °C. $^{13}$C and $^1$H chemical shifts are expressed in $\delta$ relative to internal acetone (31.1 ppm) and TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-$d_4$), respectively. Two-dimensional spectra (DQF-COSY, TOCSY, ROESY, HSQC and HMBC) were measured using standard Bruker software. The homonuclear spectra were all acquired with 4096 data points in the $F^2$ dimension. The data matrix was zero-filled in the $F^1$ dimension to give a matrix of $4096\times2048$ points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. A mixing time of 200 ms was used in the ROESY experiment and 80 ms for the TOCSY experiment.

*Gas chromatography.*—GC was performed on a Hewlett–Packard 5890 instrument, SPB-5 capillary column (0.25 mm × 30 m, Supelco). For compositional and methylation analyses, the temperature program was: 150 °C for 5 min, then 5 °C/min to 300 °C. For absolute configuration analysis, the temperature program was: 150 °C for 8 min, then 2 °C/min to 200 °C for 0 min, then 6 °C/min to 260 °C for 5 min.

The monosaccharides were identified as acetylated *O*-methyl glycosides derivatives: briefly, samples were methanolysed with 2 M HCl–MeOH at 85 °C 20 h, dried under reduced pressure and then acetylated with $Ac_2O$ in pyridine at 80 °C 30 m. After work-up, the sample was analysed by GLC–MS. The absolute configuration of glucosamine was determined by GLC of acetylated glycosides of (+)-2-octanol according to the published method.[11] The absolute configuration of bacillosamine was determined by the same method comparing it with an authentic sample. The absolute configuration of alanine was determined by GLC of the octyl ester obtained with (+)-2-octanol in the above conditions according to reference.[6]

Methylation analysis was carried out with methyl iodide in dimethyl sulfoxide in the presence of sodium hydroxide according to the published method.[12] The

hydrolysis of the methylated O-polysaccharide was performed with 4 M TFA (120 °C, 4 h) and the partially methylated monosaccharides, reduced with $NaBD_4$, were converted to alditol acetates with acetic anhydride in pyridine at 80 °C 30 min and analysed by GLC–MS (bacillosamine methylated derivative, $m/z = 117$, 159, 130, 172, 329, 287, 316, 274).

## Acknowledgements

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

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