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Note

A novel capsular polysaccharide from *Rhizobium rubi* strain DSM 30149

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Abstract—*Rhizobium rubi*, strain DSM 30149, is a Gram negative phytopathogenic bacterium which produces a linear polysaccharide with the following repeating unit:

[4)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-β-L-3OMe-Rha*p*-(1→]$_n$

This new structure was determined by spectroscopical and chemical methods. It presents similar lipophilic features reported for another strain of *R. rubi*. These contrast with features already known for capsular polysaccharide species from symbiontic members of the *Rhizobiaceae* family, namely highly anionic polymers.

Keywords: Capsular polysaccharide; Acofriose; Structure; NMR

Rhizobium rubi, a member of the *Rhizobiaceae* family,[1] is a Gram-negative soil pathogen bacterium which causes the crown gall disease in many dicotyledonous plant species. It preferentially attacks plant species belonging to *Rosideae* family, in particular bushes such as raspberries and blackberries. The disease mechanism, similar to that of the pathogenic *Rhizobium radiobacter*, is due to the ability of the bacterium to transfer T-DNA,[2] part of the tumor-inducing plasmid Ti, into the nuclear genome of host cells.

The external layer of the outer membrane of *R. rubi* is composed of lipopolysaccharides (LPS) and the current study evidenced the occurrence of a putative capsular polysaccharide as well. The importance of this component is demonstrated for the symbiontic *Rhizobiaceae* species, where the outcome of the infection thread and the final settlement of the symbiosome depends on its presence.[3] So far, data regarding the biological activity of the capsular polysaccharides from the pathogenic *Rhizobia* are poor as well as information on their chemical composition. Only the structure of the capsular polysaccharide of the type strain of *R. rubi* is known and it presents the neutral residue 6-deoxy-talose and an acetylation pattern modulated during the growth stage of the organism.[4]

The bacterium currently investigated produces a polymer chemically different from that of the type strain; this polysaccharide is never decorated with acetyl groups and it is constituted from three units of rhamnose with one methylated at O-3.

This polysaccharide was recovered in the phenol layer of the phenol/water extraction[5] of the dry cells, and purified from LPS traces by size exclusion chromatography on Sephacryl HR-500. Three fractions were pooled on the basis of the refractive index profile of the eluate and assayed for the occurrence of LOS material by SDS–PAGE and ^{1}H NMR spectroscopy. The second fraction contained the pure polysaccharide and it was the object of further analyses.

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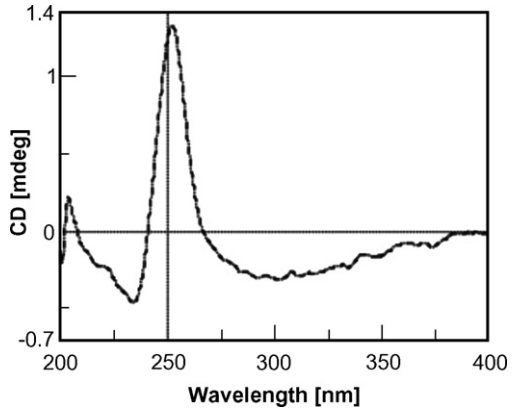

Figure 1. CD spectrum of the 4-bromobenzoate derivative of acofriose methyl glycoside.

Chemical analysis defined the presence of rhamnose and its 3-*O*-methyl derivative: acofriose, whereas methylation analysis identified the occurrence of 2-, 3- and 4-linked derivatives.

The L-configuration was assigned to rhamnose by analyzing the 2-(+)-octyl derivative,[6] whereas configuration of acofriose was established through CD measurement of its methyl 4-bromobenzoyl glycoside. The dichroic curve (Fig. 1) of this derivative showed a strong positive Cotton effect at 253 nm and a negative one at 234 nm, due to the clockwise arrangement of the two chromophores, a situation consistent with the L isomer of the monosaccharide.[7]

The ^{1}H NMR spectrum of the polymer (Fig. 2) showed three anomeric signals (4.75–5.11 ppm), labeled in the order of decreasing chemical shift with capital letters **A**, **B**, **C**. Other three intense signals occurred at about 1.3 ppm assignable to the methyl groups of 6-deoxypyranose residues, and an intense singlet at 3.35 ppm due to a methoxyl group.

^{1}H and ^{13}C NMR chemical shifts (Table 1) were assigned using COSY, TOCSY, and ^{1}H/^{13}C HSQC spectra, whereas sequencing of the residues employed NOESY and gHMBC experiments.

Anomeric signals **A**, **B**, and **C** presented small $^{3}J_{H1,H2}$ and $^{3}J_{H2,H3}$ coupling constants values in agreement with the *manno* configuration expected, and analysis of the ^{1}H–^{13}C HSQC spectrum added more details on the nature of the three monosaccharides.

Residue **A** was glycosylated at O-4 on the basis of the low field displacement of its C-4 (84.8 ppm) with respect to the characteristic standard value reported for the non substituted glycoside (73.1 ppm).[8] The anomeric α configuration was inferred on the basis of the C-5 carbon chemical shift, at 68.7 ppm, which occurred at a slightly higher field (68.7 ppm) with respect to the standard value due to the β-glycosylation displacement induced from the glycosylation at C-4. The C-5 carbon chemical shift, although influenced by the vicinal substitution, was consistent with the α configuration at the anomeric centre.[8]

Following the same approach, residue **B** was identified as an α-rhamnose unit glycosylated at O-3, whereas the β-configuration was assigned to residue **C** on the basis of its C-5 chemical shift (73.8 ppm) with substitution at both C-2 and C-3. This pattern, which apparently contrasts with the methylation data, was due to

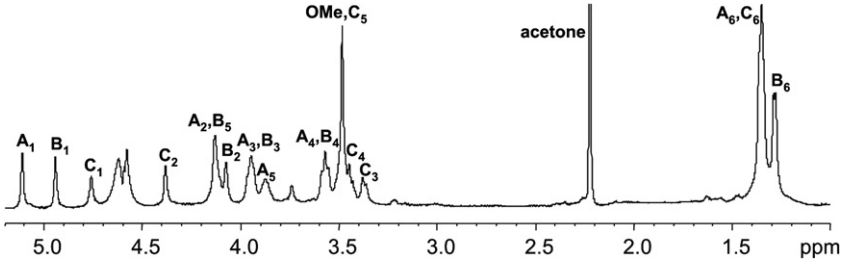

Figure 2. ^{1}H NMR spectrum (500 MHz, 45 °C, D_2O) of the capsular polysaccharide produced from *Rhizobium rubi* strain DSM 30149. Signals of the sugar residues are labeled **A** to **C** according to the sequence in the repeating unit.

Table 1. (500 MHz, 45 °C, D_2O) ^{1}H (plain text) and ^{13}C (italic) chemical shift of the capsular polysaccharide from *Rhizobium rubi*, strain DSM 30149

Residue	1	2	3	4	5	6
A	5.11	4.13	4.15	3.94	4.57	1.36
4)-α-L-Rha-(1→	*102.9*	*70.8*	*70.5*	*84.8*	*68.7*	*18.0*
B	4.94	4.07	3.95	3.57	4.12	1.28
3)-α-L-Rha-(1→	*101.6*	*71.5*	*78.1*	*72.7*	*70.1*	*18.0*
C	4.75	4.37	3.37	3.45	3.51	1.34
2)-β-L-3-*O*MeRha-(1→	*101.6*	*72.9*	*84.3*	*72.5*	*73.8*	*18.0*

Methyl group at O-3 of residue **C** was at 3.35 (^{1}H) and 58.7 ppm (^{13}C).

the occurrence of a methyl group at O-3 of residue **C**, as evidenced from gHMBC analysis and is in agreement with the presence of the acofriose residue.

Finally, the sequence was deduced from the *inter*-residual NOE contacts displayed in the NOESY spectrum (Fig. 3). These correlations were in agreement with those in the gHMBC spectrum, with inter-related H-1 of **A** with H-3 of **B**, H-1 of **B** with H-2 of **C**, and H-1 of **C** with H-4 of **A**, as summarized in the following repeating unit:

[4)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-β-L-3OMeRha*p*-(1→]$_n$

A **B** **C**

According to the NMR data and the methylation analysis, the structure of the repeating unit of the polysaccharide produced by *R. rubi* DSM 30149 was deduced. It appears new in presenting two units of α-L-rhamnose, and one unit of β-L-acofriose. These residues confer rather hydrophobic properties to the resulting polysaccharide, directing its partition in the phenol phase of the water/phenol extraction. This behavior is similar to that reported for the related strain, *R. rubi*^{T} DSM 6772,[4] which produces a capsular polysaccharide containing the C-4 epimer of rhamnose, namely a 6d-L-talopyranoside unit, with a growth modulated acetylation pattern.

The occurrence of lipophilic capsular polysaccharides is apparently characteristic of *R. rubi* species, whereas the taxonomically close symbiontic *Rhizobia* produce exo- and capsular polysaccharides without deoxy-residues which are highly acidic because these are rich in uronic acids and/or substituents such as succinic or pyruvic acid.

The lipophilic features of the capsular polysaccharide investigated in this work, may be of importance for the comprehension of the plant pathogenic activity of *R. rubi*, and constitute the molecular basis for future studies.

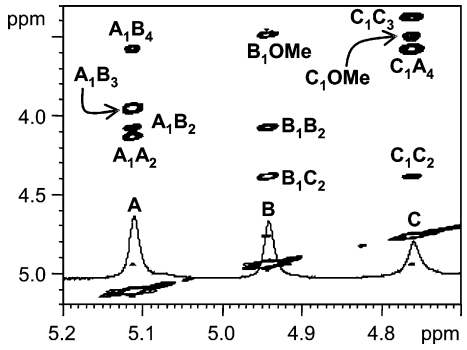

Figure 3. Expansion of the NOESY spectrum (500 MHz, 45 °C, D_2O) of the capsular polysaccharide produced by *Rhizobium rubi* DSM 30149. Letters refer to carbohydrate residues as defined in Table 1, and arabic numerals to the protons in the respective residues. Inter- and intra-residue contacts are labeled.

1. Experimental

1.1. Bacterial cultivation, extraction and electrophoresis conditions

R. rubi strain DSM 30149 (DSMZ collection) was grown at 28 °C in Nutrient Broth, cells were collected by centrifugation (rt, 7000 rpm), washed with distilled water, and freeze-dried.

Dry cells (yield 0.130 g/L) were extracted[5] and both water and phenol phases were separately dialyzed against distilled water and freeze-dried (yield: 39 mg/g_{cell} and 50 mg/g_{cell}, respectively).

SDS–PAGE[9] was performed on a Bio-Rad miniprotean gel system selecting a 12% separating gel; samples were run at a constant voltage (150 V) and stained as reported.[10]

1.2. Monosaccharides chemical analysis

The sample (1 mg) was dried over P_2O_5 for 1 h under diminished pressure and treated with methanolic HCl at 80 °C for 18 h. The soln was extracted twice with equal vols of *n*-hexane to remove contaminant fatty acid methyl esters, the methanolic phase was dried, and the methyl glycosides were acetylated with dry pyridine (200 μL) and Ac_2O (100 μL) at 80 °C for 30 min. The reactants were removed by evaporation and the mixture of peracetylated methyl glycosides was analyzed by GC–MS.

Methylation analysis was performed according to the procedure described by Sandford and Conrad,[11] and rhamnose absolute configuration was determined through the analysis of 2-(+)-octyl derivatives, as reported.[6] All the samples were analyzed with the same temperature program, using a Hewelett–Packard 5890 instrument, equipped with a SPB-5 capillary column (Supelco, 30 m × i.d. flow rate, 0.8 mL/min, He as a carrier gas), with the temperature program: 150 °C for 5 min, 150→300 °C at 10 °C/min, 300 °C for 12 min. EI mass spectra were recorded with an ionization energy of 70 eV and an ionizing current of 0.2 mA.

1.3. Absolute configuration of 3-*O*-methyl-rhamnose (acofriose)

The polysaccharide (10 mg) was treated with 1 M methanolic HCl at 80 °C for 2 h, the excess of acid was removed by repeated evaporation with MeOH and the methyl glycosides mixture was purified by preparative TLC, eluted with 9:1 $CHCl_3$–MeOH. Methyl 3-*O*-methyl-α-rhamnopyranoside (1.5 mg, R_f 0.7) was recognized on the basis of its ^{1}H NMR data (10 °C, D_2O): δ 4.78 (s, 1H, H-1), δ 4.17 (br s, 1H, H-2), δ 3.46 (signal overlapped with a *O*-methyl group, expected as d, 1H, $J_{3,4}$.9.8 Hz, H-3), δ 3.50 (t, 1H, $J_{4,3} = J_{4,5}$ 9.8 Hz,

H-4), δ 3.73 (dq, 1H, $J_{5,4}$ 10.1 Hz, $J_{5,6}$ 6.3 Hz, H-5), δ 1.33 (d, 3H, $J_{6.5}$ 6.25, H-6), δ 3.45 (s, 3H, 3-OCH$_3$), δ 3.43 (s, 3H, 1-OCH$_3$). Assignment of the two *O*-methyl groups can be exchanged.

The methyl glycoside was 4-bromobenzoylated with a 1.5 M excess of the corresponding acyl chloride, (50 °C, 3 h) in dry pyridine and purified by preparative TLC, then eluted with 7:3 hexane–EtOAc (R_f 0.6). The identity of the isolated compound was confirmed from ^{1}H NMR data ($CDCl_3$, 25 °C): δ 4.83 (s, 1H, H-1), δ 5.81 (br s, 1H, H-2), δ 3.85 (dd, 1H, $J_{3,2}$ 2.9, $J_{3,4}$ 10.0, H-3), δ 5.33 (t, 1H, $J_{4,3} = J_{4,5}$ 10.2 Hz, H-4), δ 3.994 (dq, 3H, $J_{5,4}$ 10.2 Hz, $J_{5,6}$ 6.3 Hz, H-5), δ 1.31 (d, 1H, $J_{6.5}$ 6.3, H-6), δ 3.46 (s, 3H, 3-OCH$_3$), δ 3.33 (s, 3H, 1-OCH$_3$). Assignment of the two *O*-methyl groups can be exchanged.

CD absorption was measured in MeOH with a Jasco J-815 instrument; the sample concentration (2.2×10^{-6} M) was calculated measuring the chromophores absorbance at 247 nm; the spectrum was elaborated according to the instrument software.

1.4. Phenol phase purification

20 mg of the material extracted in the phenol phase was purified by SEC (size exclusion chromatography) using Sephacryl HR-500 resin (Pharmacia, 1.5×65 cm, NH_4HCO_3 50 mM, flow 20 mL/h). The sample was not completely soluble in the eluent and, after centrifugation, the clear supernatant was loaded on the gel, three main peaks were obtained and screened by discontinuous SDS–PAGE (to visualize contaminating LOS) and by proton NMR spectroscopy. The capsular polysaccharide was eluted in both the first and the second chromatographic peaks (2 and 3 mg, respectively), the first fraction was discarded because it was contaminated with LOS. NMR analysis was performed on the second fraction.

1.5. NMR spectroscopy analysis

NMR experiments were carried out with a Varian Inova 500 instrument of Consortium INCA (L488/92, Cluster 11) equipped with a *z*-gradient, reverse probe. Chemical shifts are expressed in δ relative to internal acetone (^{1}H at 2.225 ppm, ^{13}C at 31.45 ppm). Two-dimensional spectra (DQ-COSY, TOCSY, NOESY, gHSQC, and gHMBC) were measured using the standard Varian software.

For the homonuclear experiment, 512 FIDs of 2048 complex data points were collected, with 40 scans per FID. The spectral width was set to 10 ppm and the frequency carrier was placed at the residual HOD peak. For the HSQC and HMBC spectra, 256 FIDS of 2048 complex points were acquired with 50 scans per FID, the GARP sequence was used for ^{13}C decoupling during acquisition. Conversion of the Varian data, processing and analysis was performed with Bruker TOPSPIN 1.3 program.

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