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Note

Elucidation of the O-chain structure from the lipopolysaccharide of *Agrobacterium tumefaciens* strain C58

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Abstract

A linear homopolysaccharide built of 3-α-L-6dTal*p* residues, randomly acetylated at position C-4, is described for the O-specific polysaccharide of *Agrobacterium tumefaciens* strain C58. This structure, determined by spectroscopical and chemical methods, is strictly correlated to that of *Rhizobium loti* strain NZP2213, which differs for the degree and the position of O-acetylation.

Keywords: *Agrobacterium tumefaciens*; O-Chain; LPS; 6-Deoxytalose; 6-Deoxytalan

Agrobacterium tumefaciens is a soil pathogen, which causes crown gall, a tumourigenic disease in many dicotyledonous plants a species.[1] The tumour formation is due to the ability of pathogen to transfer the T-DNA, a part of the tumour-inducing plasmid Ti, into the nuclear genome of host cells. This peculiar working way makes *A. tumefaciens* a suitable tool for the genetic engineering of plants. Although the mechanism of pathogenesis is still not completely clear, the involvement of the lipopolysaccharides (LPSs) of the bacterial external membranes is well accepted. In particular, the O-specific polysaccharide moiety (O-chain) of LPSs seems to be implied in the host recognition, in the modulation of the bacterial virulence and in the adhesion to the host.[2] Despite the wealth of investigations on the biological role of the *Agrobacterium* LPS, only recently the first complete structural determination of the O-chain LPS from an *Agrobacterium* species has been published.[3] In the framework of a systematic investigation on the LPS primary structure of *Agrobacterium* bacteria, we now describe the O-specific polysaccharide structure of *A. tumefaciens* strain C58.

The LPS of *A. tumefaciens* strain C58 was isolated from the phenol phase of the phenol/water treatment and showed a ladder-LIKE profile on SDS-PAGE electrophoresis in agreement with a S-type LPS (Fig. 1); more in detail: the gel position of this profile suggested a relative low molecular weight especially if compared with the reference LPS from *Escherichia coli* O111:B4. The GC–MS chromatogram of O-acetylated methyl glycosides of the purified LPS showed, besides significative amounts of Kdo, GlcN, Gal, Man Rha (probably components of the core region), an intense signal whose MS fragmentation fitted with a derivative of a 6-deoxyaldohexose. The methanolysis of the LPS allowed us to isolate the methyl α-L-6-deoxytalopyranoside, which was identified on the basis of its ^{1}H and ^{13}C NMR spectra (Table 1) and by molecular rotation (*c*, 2.0 in H_2O, $[\alpha]_D^{20} = -104$, literature value: *c*, 2.1 in H_2O, $[\alpha]_D^{20} = -104$). Since this uncommon monosaccharide has been found both as LPS[4] and exopolysaccharide (EPS)[5] residue, the possibility of having in our LPS also an EPS component was surmised. In order to isolate the putative EPS component, the LPS was treated with a 14:6:2 mixture of $CHCl_3$–methanol–water as reported by Zahringer[6], to separate and LPS from the 6d-talan EPS. The two phases were checked both by SDS-PAGE and chemical composition, and surprisingly, LPS was detected in both layers, as revealed by SDS-PAGE

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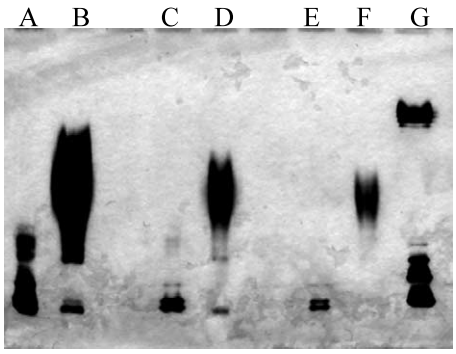
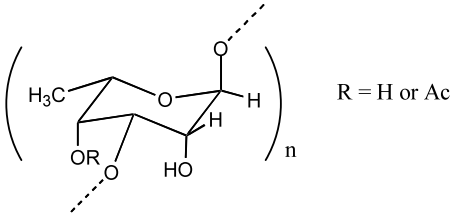


Fig. 1. SDS-PAGE of *Agrobacterium tumefaciens* membrane constituents: lanes A, C and E: (4, 1 and 0.4 μg) water extract; lanes B, D and F (4, 1 and 0.4 μg): phenol extract, lane G: reference LPS from *Escherichia coli* O111:B4, 1 μg.

electrophoresis, in addition the GC–MS methyl glycoside chromatogram of the LPS of each phase showed the identical value for the 6dTal/Kdo ratio. On the base of these results, the possibility that in one of the phases a 6d-talan EPS was also present has been excluded.

After mild acetic acid hydrolysis the lipid A moiety was precipitated whereas the O-chain part (PS) was recovered from the supernatant liquor. The ^{1}H NMR spectrum of PS showed (Fig. 2) a rather crowded anomeric region, the two more intense signals appeared as broad singlets at δ 5.14 and 5.11. The first one was strongly correlated to a carbon signal at δ 70.7 and in a weakly way to two anomeric carbon signals at δ 97.3 and 99.8, suggesting that at δ 5.14 occurred not only proton anomeric signals but protons geminal to acetoxy groups as well. The second signal at δ 5.11 was correlated only to the carbon anomeric signals at δ 97.1 and 99.7. Other intense unresolved signals occurred at about δ 4.05 and 3.95, which were both correlated to carbon signals around δ 68.3–68.7 and 69.9–71.1. More diagnostic intense signals were the singlet at δ 2.15 (Me of acetyl groups) and the doublet at δ 1.25, assignable to the methyl signals of 6-deoxytalopyranose residues, correlated to carbon signals at δ 21.8 and 15.6, respectively. This spectral situation strongly suggested a heterogeneous structure probably due to a non regular distribution of *O*-acetyl groups. Actually a mild alkaline hydrolysis gave O-deacetylated product (dPS) with a regular structure as inferred by its ^{1}H and ^{13}C NMR spectra (Fig. 3). The minor anomeric signals clearly visible in the proton spectra are probably due to sugars of the 'core' region of LPS, their significative intensities supported a rather short O-chain, as already suggested by the gel electrophoresis profile (Fig. 1). Two-dimensional NMR experiment (gCOSY, TOCSY, gHSQC) allowed us to assign easily all the intense signals (Table 1). The chemical shift values of dPS were almost identical to those of the O-deacetyled O-specific polysaccharide obtained from the LPS of *Rhizobium loti* strain NZP2213[5] and identified as a linear homopolymer built of 3-α-6-deoxy-L-talopyranose residues. The methylation analysis of dPS and the C-3 lower and C-4 higher field NMR shifts with respect to the ^{13}C chemical shift values of the corresponding carbons in the reference methyl glycoside[6] (Table 1) confirmed the presence of 3-linked-6-deoxytalose unit.

The location of the O-acetylation sites in the PS was deduced from the COSY and TOCSY spectra which showed no correlation of the acetoxy geminal proton signals with the anomeric signals suggesting that each residue of the polysaccharide is O-acetylated exclusively at the position 4, because the position 3 involved in the glycosidic linkage. Therefore, the heterogeneity of PS is due only to the random O-acetylation at position C-4. This conclusion is supported by the fact that H-5 signals occur in a large chemical shift range, from δ 3.8 to 4.4, in agreement with a random β-O-acetylation shift. On the other hand, the narrow anomeric chemical shift range from δ 5.06 to 5.15, support the lack of *O*-acetyl at position C-2. Therefore, the structure of the polysaccharide can be as follows:



This structure differs from that of the O-specific chain of *R. loti* strain NZP2213 by the O-acetylation site, which in the latter is stoichiometric and exclusively at position C-2.[4]

Table 1
^{1}H and ^{13}C NMR chemical shifts of α-L-6d-talose methyl glycopyranoside (400 and 100 MHz, 303 K, D_2O) and O-deacetyled O-chain (dPS; 500 and 125 MHz, 323 K, D_2O)

	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6
α-L-6dTal*p*OMe	4.79	3.81	3.83	3.73	4.00	1.27
	102.9	70.8	66.6	73.2	68.2	16.6
dPS	5.16	4.03	4.08 [a]	3.96 [a]	4.18	1.28
	100.2	70.6	72.5	70.4	68.9	16.7

[a] Assignments reversed with respect to.[4]

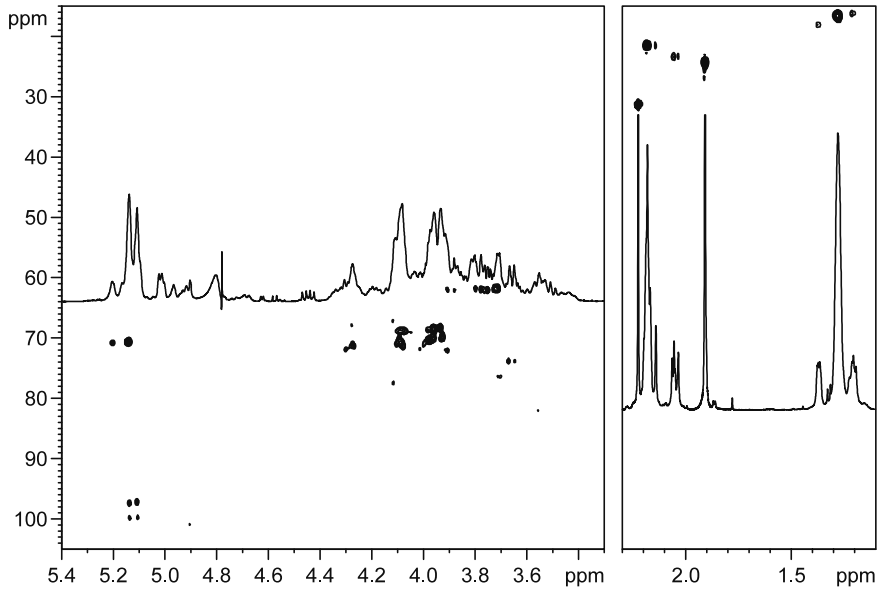


Fig. 2. 500 MHz, 323 K, gHSQC and proton spectra of native O-Chain fraction.

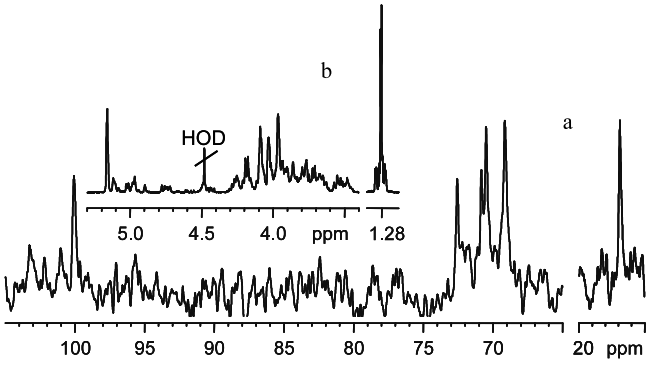


Fig. 3. 323 K in D_2O: (a) 125 MHz carbon; and (b) 500 MHz proton spectra of O-deacetyled O-chain fraction.

It is noteworthy the structural identity of the O-chain polysaccharide backbones between these two strains belonging to two different species, *Agrobacterium* and *Rhizobium*, strictly correlated from biological point of view. Both are rhizosphere inhabitants and both are able to induce growth of tumours with a similar mechanism, even though the first is pathogenic for a broad range of hosts, while the second is selective symbiotic producing nitrogen-fixing nodules. Since the only chemical difference between the two O-chains is due to the O-acetylation, this feature might be important in the biological relationship between these bacteria. To this regard it is interesting to remind the case of another 6d-talan obtained from *Actinobacillus actinomycetemcomitans* whose antigen of serotype a is the α-D-2AcO-6dTal*p* residue and that of serotype *c* is the α-L-4AcO-6dTal*p* unit, if both the antigens are O-deacetyled no precipitation line with the corresponding antiserum is obtained in the immunodiffusion tests.[5] It would be interesting to compare the serological activities of *A. tumefaciens* strain C58 and of *R. loti* strain NZP2213 but, unfortunately, they are not yet available at present.

1. Experimental

Agrobacterium tumefaciens strain DSM 5178, referred as C58 as well, was grown at 27 °C in liquid shake culture (200 rpm) in Nutrient Broth (DIFCO). Cells were pelleted at 2000*g*, washed with distilled water and freeze-dried.

Dried cells (4.3 g) were extracted according to the phenol–water method.[7] Both phases were separately dialyzed against distilled water, freeze-dried and screened by discontinuous SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis),[8] 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 and Hilbink,[9] the R-LPS (0.7% $g_{LPS}/g_{dry\text{-}cells}$) was present in the water layer whereas the S-LPS was recovered by the phenol phase and further purified by SEC chromatography on Sephacryl HR 400 (Pharmacia, 1.5 × 70 cm, NH_4HCO_3 50 mM, flow 0.4 mL/min) to give pure LPS in a yield 3.8% of dry cells.

1.1. Chemical analysis of LPS

Monosaccharides were analysed as acetylated methyl glycosides derivatives as described.[10]

Methylation analysis was performed according the protocol described by Sandford and Conrad.[11]

1.2. Isolation of α-L-*O*-methyl-6d-talopyranoside

LPS sample (15 mg) were dried over P_2O_5 in a dessicator was treated with 1 M methanolic HCl at 80 °C for 2 h; lipids were removed by repeated extractions with *n*-hexane and the methanolic phase was dried repeatedly in order to remove the acid. The 6d-talose derivative was purified (14% mg/mg_{LPS}) by preparative TLC in the solvent system of $CHCl_3$–MeOH, 8:2 v/v and studied by NMR spectroscopy. L Configuration of 6d-talose was deduced by the comparison of its optical rotation with that reported in literature.

1.3. Isolation of the O-specific polysaccharide

The LPS in 1% AcOH (10 mg/mL) was hydrolysed at 100 °C for 2 h. After cooling, the solution was centrifuged at 6000 rpm for 20 min and the clear supernatant was freeze-dried and purified by SEC on Sephacryl HR 100 (Pharmacia, 1.5 × 70 cm, NH_4HCO_3 50 mM, flow 0.4 mL/min). The eluted material was monitored using refractometer; O-chain was eluted as major peak close to the void volume (31% yield from LPS).

1.3.1. De-O-acetylation of O-specific polysaccharide. Native O-chain has been treated with 12% ammonia at 60 °C for 2 h, and dried at reduced pressure on rotatory evaporator at 35 °C until all ammonia was removed.

1.4. NMR spectroscopic analysis

NMR experiments on the α-L-*O*-methyl-6d-talopyranoside were carried out on a Bruker DRX 400 MHz equipped with reverse multinuclear probe at 303 K; whereas spectra on the native and O-deacetylated PS were measured on Varian INOVA 500 of Consortium INCA (L488/92, Cluster 11), equipped with a reverse probe at 323 K.

In general, chemical shift are expressed in δ relative to internal acetone (2.225 and 31.4 ppm). Two-dimensional spectra (gCOSY, phase-sensitive TOCSY and NOESY, and gradient-HSQC) were measured as follows: 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 always performed with Bruker XWin-NMR 1.3 program.

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References

1. Sigee, D. C. *Bacterial Plant Pathology: Cell and Molecular Aspects*; Cambridge University Press: UK, 1993.
2. Matthysse, A. G. *CRC Crit. Rev. Microbiol.* **1986**, *13*, 281–307.
3. De Castro, C.; De Castro, O.; Molinaro, A.; Parrilli, M. *Eur. J. Biochem.* **2002**, *269*, 2885–2888.
4. Russa, R.; Urbanik-Sypniewska, T.; Shashkov, A. S.; Kochanowski, H.; Mayer, H. *Carbohydr. Polym.* **1995**, 299–303.
5. Shibuya, N.; Amano, K.; Azuma, J.; Nishihara, T.; Kitamura, Y.; Noguchi, T.; Koga, T. *J. Biol. Chem.* **1991**, *266*, 16318–16323.
6. Zahringer, U.; Rettenmaier, H.; Moll, H.; Senchenkova, S. N.; Knirel, Y. A. *Carbohydr. Res.* **1997**, *300*, 143–151.
7. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.
8. Laemmli, U. K. *Nature* **1970**, *97*, 620–628.
9. Kittelberger, R.; Hilbink, F. *J. Biochem. Biophys. Methods* **1993**, *26*, 81–86.
10. De Castro, C.; Molinaro, A.; Nunziata, R.; Grant, W.; Wallace, A.; Parrilli, M. *Carbohydr. Res.* **2003**, *338*, 567–570.
11. Sandford, P. A.; Conrad, H. E. *Biochemistry* **1966**, *5*, 1508–1517.