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Note

Acetyl substitution of the O-specific polysaccharide caryophyllan from the phenol phase of *Pseudomonas (Burkholderia) caryophylli*

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Abstract

The intact O-specific caryophyllan polysaccharide of the lipopolysaccharide fraction from the bacterium *Pseudomonas (Burkholderia) caryophylli* was isolated for the first time. Its structure was clarified by means of chemical and spectroscopic analysis and consisted of a homopolymer of randomly acetylated caryophyllose units. The position of acetyl groups when present is not unique: all the hydroxyl-groups on the side chain of the sugar can be substituted with a slight preference for acetylation of the C-5–C-10 tail of this unusual monosaccharide © 2001 Published by Elsevier Science Ltd.

Keywords: Caryophyllose; Lipopolysaccharide; *Pseudomonas (Burkholderia) caryophylli*; Acetyl group

A wide variety of sugars has been identified in the O-specific polysaccharides (OPSs) of bacterial lipopolysaccharides (LPSs) which may additionally bear various non-carbohydrate substituents, such as phosphates, methyl groups, amino acids or acetyl groups. The last decoration has been found frequently, often in non-stoichiometric amounts. It is added in the later steps of O-specific polysaccharide biosynthesis and is discussed as a microheterogeneic property of LPS. However, these substituents alter the spatial structure of the polysaccharide chain by exposing its surface additional groups, which can determine its immunological property, i.e., the specificity of its interactions with external agents; thus it is extremely important to determine their position and frequency on the macromolecule.

Pseudomonas (*Burkholderia) caryophylli* is a phytopathogenic bacterium responsible for carnation wilt.[1] In the water phase of the phenol–water LPS extraction two linear O-specific homopolysaccharides were found.[2] They are constituted by two novel and rather peculiar monosaccharides; the minor LPS portion contains an acetylated polysaccharide

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named caryan, built up of β-(1→7)-linked caryose (4,8-*cyclo*-3,9-dideoxy-L-*erythro*-D-*ido*-nonose) which is acetylated block-wise and only at position C-6.[3]

The OPS of the major LPS portion is a homopolysaccharide termed caryophyllan (Fig. 1), consisting of (1→7)-linked caryophyllose [3,6,10-trideoxy-4-*C*-(D-*glycero*-1-hydroxyethyl)-D-*erythro*-D-*gulco*-decose]. Both α and β configurations are present with a ratio of 8:1, respectively.[4] As with the minor OPS, the caryophyllan is partially substituted with acetyl groups.

In the present paper, we report the structural investigation on the LPS of the phenol phase which allowed us to isolate, for the first time, the intact caryophyllan OPS and to study its acetylation pattern as deduced by spectroscopic methods.

Dried cells were extracted according to the phenol method.[5] Both the water phase and the phenol phase showed the presence of lipopolysaccharidic material. Their ^{1}H NMR spectra appeared different even though compositional analysis indicated a similar monosaccharide composition. Therefore, further analyses were performed on the phenol phase. After preliminary purification of the LPS material by gel-permeation chromatography (GPC) on a Sephacryl HR 400, the lipid A-free caryophyllan was obtained from LPS by acid-hydrolysis using a 50 mM sodium acetate buffer containing 0.1% SDS (pH 4.5, 100 °C, 4 h) and purified by GPC on Sephacryl HR 100. This mild-hydrolysis condition was strong enough to cleave the Kdo glycosidic linkage, leaving caryophyllose units unaffected. Therefore, this approach led to the isolation of the intact polymer for the first time.

Methylation analysis[6] of the OPS identified exclusively, 7-substituted caryophyllose, as already found in the water phase.[2] The ^{1}H NMR spectrum of the purified product (Fig. 2(b)) showed two strong singlets at 2.17 and 2.18 ppm suggesting the presence of acetyl groups on the molecule, a feature already detected although in smaller amounts in the LPS fraction of the aqueous phase.[4]

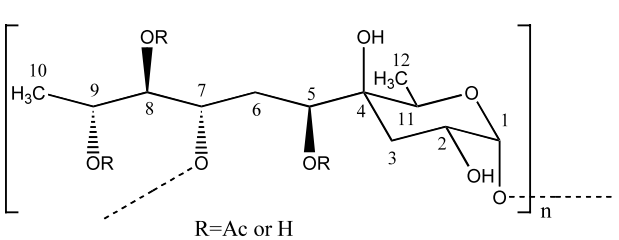

Fig. 1. The caryophyllan polysaccharide.

Prehm methylation analysis was tried to determine the acetyl position,[7] but it gave unsatisfactory results mainly because of the poor solubility of the polymer in the required solvent. To determine the position of acetyl groups, NMR spectroscopy was used, first on the de-acetylated polymer obtained by mild-alkaline hydrolysis and then on the native one (Fig. 2(a)).

Spectroscopical characterisation was performed employing different 2D homo- and heteronuclear NMR experiments (COSY, TOCSY, NOESY, HSQC and HMBC) at 400 MHz (Table 1). The caryophyllose monosaccharide gives rise to three unconnected spin systems, i.e., H-1–H-3 and H-11–H-12 and the side chain H-5–H-10. Each of these spin systems could be assigned using 2D COSY and TOCSY spectra and separate spin systems connected through NOE contacts and long range ^{1}H,^{13}C coupling constants. The glycosidic linkage in the polymer was confirmed by the long-range connectivities (HMBC) between H-1–C-7 and H-7–C-1. The results for the deacetylated polysaccharide are reported in Table 1. The ^{1}H NMR spectrum also showed two other weak signals (relative to anomeric protons); the first at 5.00 ppm attributed to caryan, which is present in low percentage (ca. 17%) as estimated by integration, and the second at 4.38 ppm due to β-caryophyllose; because of the small amounts of both these species, the present analysis focuses on the caryophyllose residues with α configuration. All these data taken together indicated the presence of the same OPS in both water and phenol phase LPSs, except for the acetyl substitution.

The same spectroscopic approach was applied to the native polymer and focused first on the anomeric region. It showed three clusters of signals at 4.92, 5.00 and 5.16 ppm, whose nature was revealed by analysis of the HSQC and HMBC. The HSQC spectrum showed that the first two signals contained

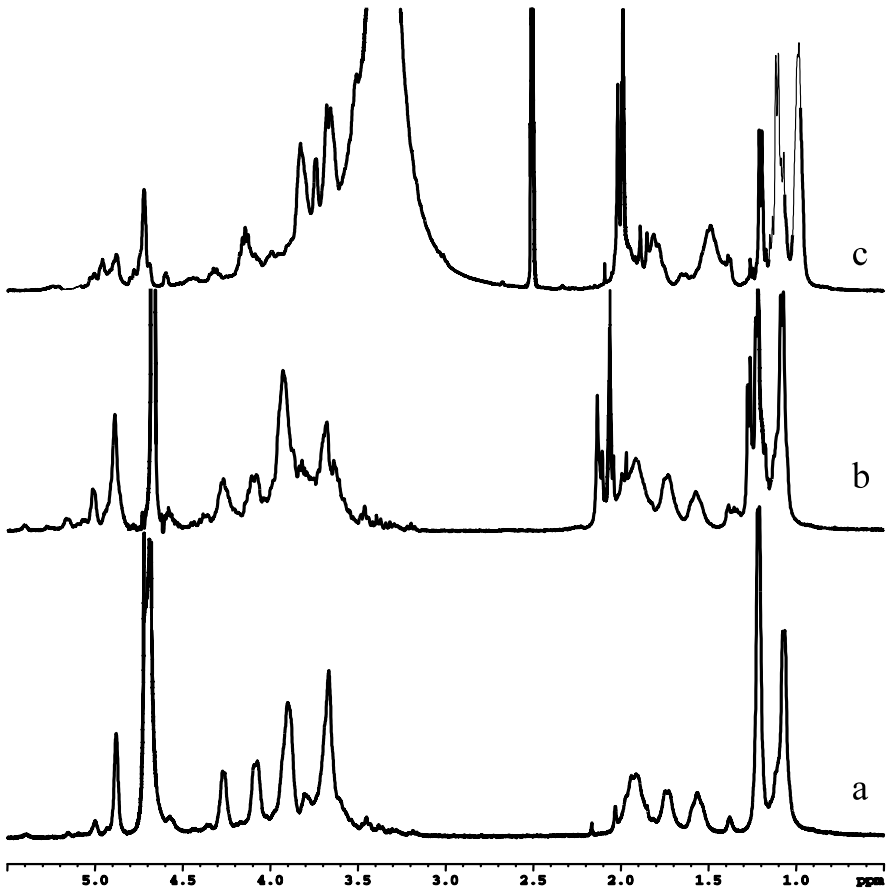

Fig. 2. ^{1}H NMR spectra of: (a) deacetylated and (b) native OPS in D_2O at 30 °C; (c) native OPS in Me_2SO-d_6 at 60 °C.

Table 1
^{1}H and ^{13}C NMR chemical shifts of the deacetylated polysaccharide

Residue	1	2	3 (ax–eq)	4	5	6	7	8	9	10	11	12
^{1}H	4.88	3.93	1.93 1.73		3.90	1.89 1.54	4.08	3.67	3.67	1.21	4.26	1.07
^{13}C	99.9	77.1	30.1	74.6	68.6	28.1	77.0	66.78	77.62	18.5	66.8	11.2

two different species of protons, in both cases an anomeric and a carbinolic proton, while the third signal at 5.16 ppm was relative to a carbinolic proton since it correlated only to a carbon at 72.3. The low field ^{1}H NMR chemical shift of these three signals was explained as a consequence of the acetylation shift. Accordingly, in the HMBC spectrum there was a long-range correlation with a signal at ca. 174 ppm attributed to a carbonyl of the acetyl group.

Further information was obtained by analysing the high-field area of the HMBC spectrum (Fig. 3). It contained three main methyl signals. The signal at 1.08 ppm was recognised as that positioned on the caryophyllose ring and showed two clear long-range correlations with the carbons at 74.6 and 67.0 ppm, identified as C-4 and C-11, respectively. Only one signal was present for the C-4 and had the same chemical shift as the de-acetylated product; this suggested that the corresponding hydroxyl function is never substituted.

Nearly all resonances were assigned by analysing both the COSY and TOCSY spectra. In this way it was possible to assign the signal at δ 4.92, partially overlapping the caryophyllose anomeric proton. It was recognised as the proton H-9, since it is correlated

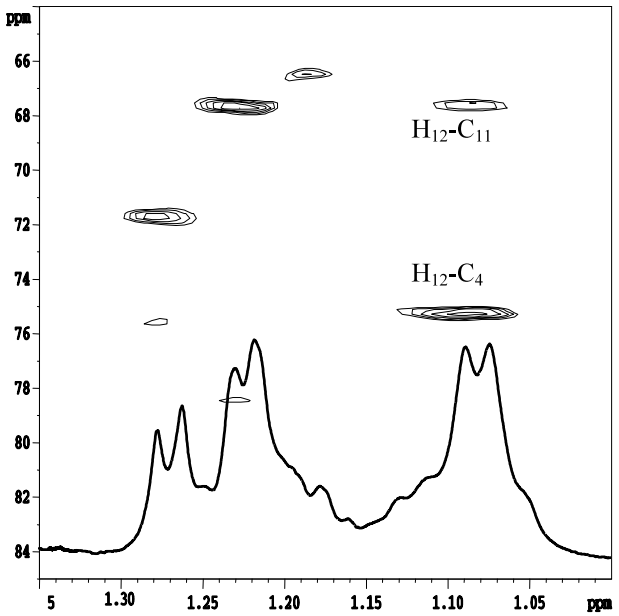

Fig. 3. High field section of HMBC spectrum.

both to a methyl group and to the proton H-8; similarly the signal at δ 5.00 contained two different protons: the anomeric signal of caryan polysaccharide and a carbinolic one, identified as the proton H-8 because of its correlation to two carbinolic protons. Finally, it was also possible to assign the less intense proton at 5.16 ppm; located on position C-5 of a caryophyllose unit since it was coupled only with the methylene protons H-6.

A more extended analysis of these spectra led us to assign all the spin systems connected with these acetylated protons, thus identifying four types of residues; the unacetylated residue A, a C-9 acetylated residue A′, a C-8 acetylated residue B and a C-5 acetylated residue C (Table 2). It was thus clear that most of the caryophyllose units were substituted by no more than one acetyl residue, always located on its side chain. A further characteristic is that the signals of the ring protons are not influenced by the substitution on the chain.

The partial overlapping of the signals in the anomeric area made it impossible to integrate the shifted signals in order to determine the substitution degree of the polysaccharide. This problem was circumvented by measuring and analysing the spectra of the same sample in a different solvent, i.e., Me_2SO-d_6.

Fig. 2(c) shows the proton spectrum recorded in Me_2SO-d_6 at 60 °C; the very small amount of solved polysaccharide prevented us from obtaining 2D heteronuclear-correlated spectra, so the analysis was performed employing only 2D homonuclear spectroscopy techniques. The chemical shifts are reported in Table 3. The anomeric region shows a group of signals at δ 5.02, 4.96, 4.88, 4.72 and 4.69 labelled as D_5, C_8, B_9, A_1 and A_1', respectively; they all belong to different caryophyllose residues. Analysis of the 2D NMR spectra allowed us to confirm the features found in the previous case and to identify five different residues A, A′, B, C and D, neither residue A nor A′ is acetylated, B, C and D residues are acetylated at position C-9, C-8 and C-5, respectively. The area between 5.02 and 4.88 ppm was populated by carbinolic protons

Table 2
^1H and ^{13}C NMR chemical shifts of the deacetylated polysaccharide obtained in D_2O

Residue		1	2	3 (ax–eq)	4	5	6	7	8	9	10	11	12
A	^1H	4.88	3.93	1.91 1.73		3.87	1.54, 1.91	4.09	3.68	3.68	1.22	4.27	1.08
	^{13}C	99.9	77.1	30.1	74.6	69.0	28.1	77.5	78.5	67.5	18.8	67.0	12.0
A′	^1H	4.88	3.93	1.91 1.73		3.87	1.54, 1.91	3.93	3.93	4.92	1.26	4.27	1.08
	^{13}C	99.9	77.1	30.1	74.6	69.0	28.1	77.5	75.1	71.3	15.0	67.0	12.0
	CO									174.0			
B	^1H	4.88	3.93	1.91 1.73		3.95	1.97	3.78	5.00	4.00	1.18	4.27	1.08
	^{13}C	99.9	77.1	30.1	74.6	69.0	n.d.	n.d.	79.5	66.0	18.6	67.0	12.0
	CO								174.2				
C	^1H	4.88	3.93	1.91 1.73		5.16	1.73	3.93	3.68	3.68	1.22	4.27	1.08
	^{13}C	99.9	77.1	30.1	74.6	72.3	n.d.	77.5	78.5	67.5	18.8	67.0	12.0
	CO					n.d.							

Table 3
^{1}H and ^{13}C NMR chemical shifts of the deacetylated polysaccharide obtained in Me_2SO-d_6 at 60 °C

Residue	1	2	3 (ax–eq)		5	6		7	8	9	10	11	12
A	4.72	3.82	1.80	1.48	n.d.	1.80	1.48	3.82	3.51	3.66	1.10	4.14	0.99
A′	4.69	3.82	1.80	1.48	n.d.	1.80	1.48	3.82	3.51	3.66	1.10	4.14	0.99
B	4.72	3.82	1.80	1.48	3.83	1.41	1.95	3.74	3.74	4.88	1.20	4.14	0.99
C	4.72	3.82	1.80	1.48	3.85	1.55	1.93	4.02	4.96	3.83	1.07	4.14	0.99
D	4.72	3.82	1.80	1.48	5.02	1.64	2.07	3.71	3.51	3.66	1.10	4.14	0.99

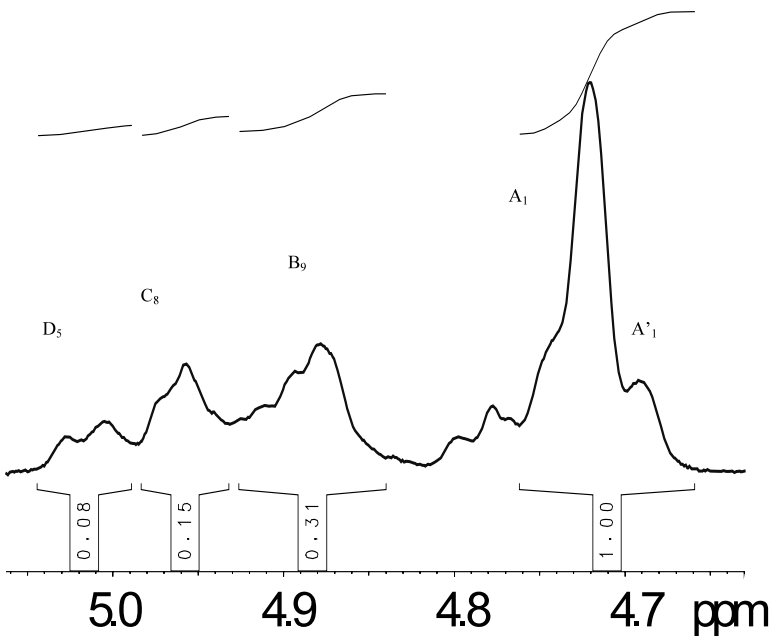

Fig. 4. Integration of the anomeric area of the ^{1}H NMR spectrum in Me_2SO-d_6.

shifted by acetylation and belongings to the sugar chain (Fig. 4). Two distinct anomeric protons, identified as A_1 and A'_1, occurred at 4.72 and 4.69 ppm. The origin of this difference was comprehensible by analysing the ROESY spectrum, actually these protons both have correlation with the protons H-7 (strong) and H-8 (medium) of the successive residue. A_1 was correlated with a unit neither non-acetylated or acetylated in position 5 (unit D); A'_1, the different chemical shift of the A′ unit, was induced by the presence of an acetyl group in position C-9 or C-8 of the subsequent residue (units B or C) as proven by the ROE correlations A'_1–C_7 and A'_1–$B_{7,8}$.

The degree of acetylation was roughly indicated by integration of the signals at low field of the ^{1}H NMR spectrum (Fig. 4) to obtain the following ratios: D_5:C_8:B_9:$A_1 + A'_1$ = 0.08:0.15:0.31:1.0. This information allows the estimation of the acetylation degree calculated as the ratio between the shifted protons versus the anomeric ones, i.e., 0.54:1.0, which indicates that this polysaccharide presents an acetylation degree of 54%. Unfortunately, since the signals of the ring of the sugar of the acetylated and non-acetylated residues were almost indistinguishable, it was not possible to identify the sequence among the different units recognised.

In conclusion, the present paper describes, for the first time, the major OPS isolated from the LPS produced by *P.* (*Burkholderia*) *caryophylli*. All previous attempts led to depolymerisation owing to the extreme acid lability of the glycosidic linkage of this sugar, and only the present conditions allowed us to isolate the polysaccharide in a good yield. The spectroscopic analysis led to the characterisa-

tion of its acetylation pattern that showed some similarities to that of caryan,[3] the minor OPS produced from this bacterium. Caryophyllan presents the same acetylation degree and a single substitution for each residue, but unlike caryan, the acetyl group is not sitting in a particular position. All the hydroxyls on the side chain of the sugar can be substituted with a slight preference for the terminal section of the tail of this unusual monosaccharide. The biosynthesis of this acetylation pattern and its impact on the biological properties of the caryophyllan are not yet understood, but the acetylation should increase the hydrophobicity of the polysaccharide which may be important for the interaction between the bacteria and plant-cell surface molecules.

1. Experimental

General methods.—NMR experiments were carried out at 400 MHz on a Bruker DRX equipped with reverse multinuclear probe at 30 or at 60 °C when in Me_2SO-d_6 solution. Chemical shifts of spectra recorded in D_2O are expressed in δ relative to internal acetone (2.225 and 31.4 ppm), whereas spectra in Me_2SO-d_6 are referred to the chemical shifts of the solvent. Two-dimensional spectra (gradient selected COSY, phase-sensitive TOCSY and ROESY, phase-sensitive gradient-HSQC and magnitude gradient HMBC) 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 4000 Hz and the carrier placed at the residual HOD peak. A mixing time of 200 ms was used in the ROESY 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; the HMBC spectrum was measured with 100 scans per FID and 80 ms delay for the evolution of the long range couplings. Processing and plotting was performed with standard Bruker XWINNMR 1.2 program.

GC-MS analyses was carried out on a Hewlett–Packard 5890 instrument with a SPB-5 capillary column (0.25 mm × 30 m, Supelco) and with a temperature program: 150 °C for 5 min, then 5 °C min^{-1} up to 300 °C.

Bacterial growth and culture conditions.—*P. (Burkholderia) caryophylli* strain 2151 was obtained from the National Collection of Plant Bacteria (NCPPB, Harpenden, UK). It was grown in 200 mL flasks containing 100 mL of Woolley's medium supplemented with 1.5% (w/v) protease peptone. The growth proceeded at 27 °C with shaking for 6 days. The culture (2.6 L) was centrifuged (10,000*g*, 10 min) and the harvested cells were washed three times with 85% (w/v) aq NaCl and then lyophilised. Dry cells (5.5 g) were extracted with hot phenol–water as described[5] (yield: 5% of LPS equally present in both phases).

Isolation of native caryophyllan.—The lipopolysaccharidic material was recovered from the phenol phase by exhaustive dialysis and further purified on Sephacryl HR 400 (1.5 × 120 cm, NH_4HCO_3 50 mM). LPS (100 mg) was hydrolysed with acetate buffer (20 mL) at pH 4.5, containing 0.1% SDS at 100 °C for 4 h. Then, the solution was centrifuged at 26,000 rpm at 4 °C. The supernatant was freeze dried and the solid was washed several times with cold EtOH and chromatographed on Sephacryl HR 100 (1.5 × 60 cm) and the run was monitored with a differential refractometer (Knauer). A single peak was obtained (yield 30%).

Monosaccharide analysis.—The caryophyllose was identified by GC and GC-MS as acetylated methyl glycoside. Briefly, the OPS sample (1 mg) was treated for 16 h with 1 M HCl in MeOH at 80 °C. After drying under a stream of N_2, it was treated firstly with 150 μL of Ac_2O at 80 °C for 15 min, dried and acetylated with 100 μL of Ac_2O in 200 μL of pyridine at the same temperature for 30 min. After work-up, the sample was analysed by GC-MS as above described. Methylation of

the caryophyllan was performed as described.[5] Partially methylated alditol acetates were obtained with hydrolysis (trifluoroacetic acid 2M, 200 μL, 120 °C, 1 h), reduction with $NaBD_4$, and acetylation and they were analysed by GC-MS in the same condition as above.

Deacetylation of caryophyllan.—OPS (10 mg) was solved in 2 mL of NaOH 0.1 M and incubated at 37 °C for 2 h, afterward the sample was neutralised with Dowex 50W X8, filtered and lyophilised.

References

1. Jones, L. K. *Phytopathology* **1941**, *31*, 199.
2. Adinolfi, M.; Corsaro, M. M.; De Castro, C.; Evidente, A.; Lanzetta, R.; Lavermicocca, P.; Parrilli, M. *Carbohydr. Res.* **1996**, *284*, 119.
3. Molinaro, A.; De Castro, C.; Petersen, B.; Duus, J.; Parrilli, M.; Holst, O. *Angew. Chem., Int. Ed.* **2000**, *39*, 156.
4. De Castro, C.; Evidente, A.; Lanzetta, R.; Lavermicocca, P.; Manzo, E.; Molinaro, A.; Parrilli, M. *Carbohydr. Res.* **1998**, *307*, 167.
5. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83.
6. Sandford, P. A.; Conrad, H. E. *Biochemistry* **1966**, *5*, 1508.
7. Prehm, P. *Carbohydr. Res.* **1980**, *78*, 372.