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Note

Presence of β-glycosyl linkages in caryophyllan: the main polysaccharide from the *Pseudomonas caryophylli* LPS fraction

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Abstract

A new significant structural feature of the more abundant polysaccharide (caryophyllan) of the lipopolysaccharide fraction (LPS) from *Pseudomonas caryophylli* is reported. In particular, the occurrence in the caryophyllan structure of β-linkages, about 11%, besides the more abundant α-linkages, has been evidenced by spectroscopic investigation of the polymer-degraded compounds.

Keywords: Caryophyllan; *Pseudomonas caryophylli*; O-chain; Phythopatogen

Recently [1], we described the structures of two homopolysaccharides derived from the LPS of *Pseudomonas caryophylli*, the bacterium responsible for the wilting of carnation. The structure of the more abundant caryophyllan consisted of (1→7)-linked α-caryophyllose [3,6,10-trideoxy-4-*C*-(D-*glycero*-1-hydroxyethyl)-D-*erythro*-D-*gulo*-decose] units and the minor polysaccharide (caryan) was made up of (1→7)-linked β-caryose (4,8-cyclo-3,9-dideoxy-L-*erythro*-D-*ido*-nonose) units.

In the previous paper [1] we described the FABMS spectrum and the ^{1}H NMR anomeric signals of the caryophyllose disaccharide **1** obtained by partial acid hydrolysis of the *Pseudomonas caryophylli* LPS fraction. During that study a ^{13}C NMR spectrum could not be recorded owing to the very small amount of material available in our hands. We have now isolated the same disaccharide fraction in a larger amount and its ^{13}C spectrum (Fig. 1a) showed, besides the expected [1] anomeric signals at δ 101.0 of the α-glycosyl unit and those of the reducing residue (C-1α, δ 92.0 and C-1β, δ 98.9; see Table 1), another signal at δ 104.4, which was

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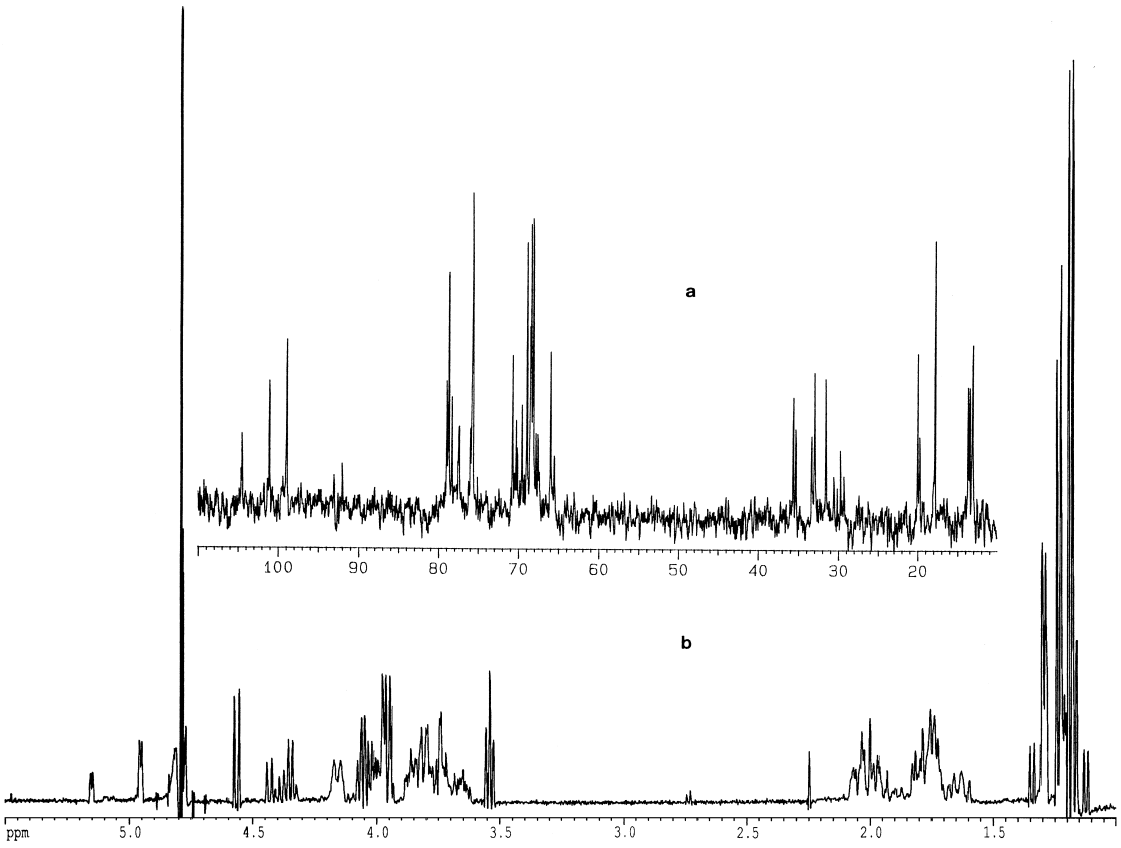


Fig. 1. ^{13}C (a) and ^{1}H (b) NMR spectra of disaccharide **1** in D_2O.

correlated by a HETCOR experiment to a doublet at δ 4.43 (see ^{1}H NMR spectrum, Fig. 1b) with a coupling constant of 8.2 Hz. This finding indicated the presence in the disaccharide fraction of an axial anomeric proton, strongly suggesting the presence of the disaccharide with the β-glycosyl residue as well. Integration of the NMR signal at δ 4.43 and that of the α-anomer at δ 4.96 (3.4 Hz) indicated a ratio of about 3:2 between the α and β-disaccharides. This ratio might not be representative of the caryophyllan α/β ratio owing to the different acid hydrolysis rate of α and β linkages. Therefore, we exploited periodate oxidation followed by $NaBH_4$ reduction of the LPS fraction with the aim of evaluating the actual α/β ratio of the glycosidic linkages in the caryophyllan. From previous work

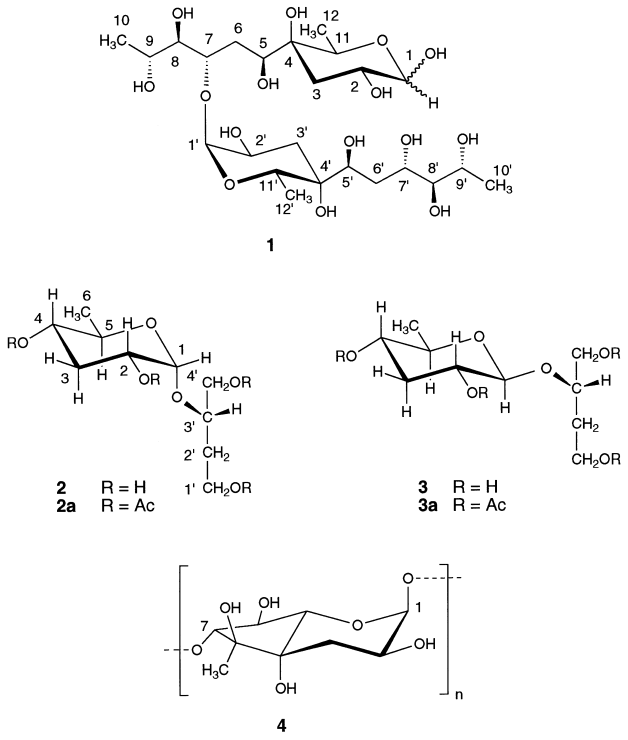
Table 1
$^{1}H/^{13}C$ data of α- and β-anomers of **1** obtained by COSY, 1D HOHAHA and HSQC experiments. Proton coupling constants are given in parentheses

Atom	α	β	Atom	α	β
1	5.15 d (3.4)/92.0	4.56 d (8.3)/98.9	1′	4.96 d (3.4)/101.0	4.43 d (8.2)/104.4
2	4.00/65.9	3.66 ddd (11.5, 8.3, 5.2)/68.5	2′	3.98/65.9	3.70/68.5
3	1.97–1.78/33.3	2.05–1.74/35.3	3′	2.00–1.80/32.9	2.05–1.78/35.6
4	—/75.9	—/75.9	4′	—/75.9	—/75.9
5	3.95/n.i.	3.95/n.i.	5′	3.79/n.i.	3.79/n.i.
6	1.85–1.58/29.7 [a]	1.85–1.58/31.5 [a]	6′	1.80–1.68/32.9	1.80–1.68/33.3
7	4.15/78.3	4.15/78.3	7′	3.85/68.9	3.85/68.9
8	3.70/77.3	3.70/77.3	8′	3.53/78.7	3.53/78.7
9	3.81/68.1	3.81/68.1	9′	3.95/68.4	3.95/68.4
10	1.28 d (6.5)/17.8	1.23 d (6.5)/17.8	10′	1.18 d (6.5)/19.6	1.18 d (6.5)/19.9
11	4.38 q (6.5)/68.1	4.07/75.6	11′	4.35 q (6.5)/68.1	4.07/75.6
12	1.13 d (6.5)/13.6	1.20 d (6.5)/13.0	12′	1.15 d (6.5)/13.4	1.20 d (6.5)/13.0

[a] Interchangeable.
n.i., not identified.

[1,2] it could be deduced that these reactions would degrade caryophyllan while leaving unchanged the glycosidic linkages, thus allowing us to isolate, besides the expected α-glycoside **2**, the suspected glycoside **3**. On the other hand caryan, which is the minor polysaccharide component of the LPS fraction, would be resistant to the above reactions owing to its peculiar structure **4** [1].

The ^{1}H NMR spectrum of the *Pseudomonas caryophylli* LPS fraction showed weak acetyl signals indicating a partial *O*-acetylation. Therefore, in order to obtain a quantitative evaluation of the α/β glycosyl ratio the fraction was de-*O*-acylated before performing the periodate oxidation. To this purpose the LPS fraction was treated with hydrazine [3] yielding fraction LPS–OH, which was devoid of both *O*-acetyl and *O*-acyl groups of the polysaccharide and lipid moiety, respectively. Both ^{1}H and ^{13}C NMR spectra of LPS-OH showed the anomeric signals attributable to caryophyllan (δ 4.88, 100.7) and caryan (δ 5.16, 97.0) [1], in addition to a minor signal at δ 104.0, which was correlated to a signal occurring at δ 4.36, in agreement with the presence of an anomeric axial proton.



Scheme 1.

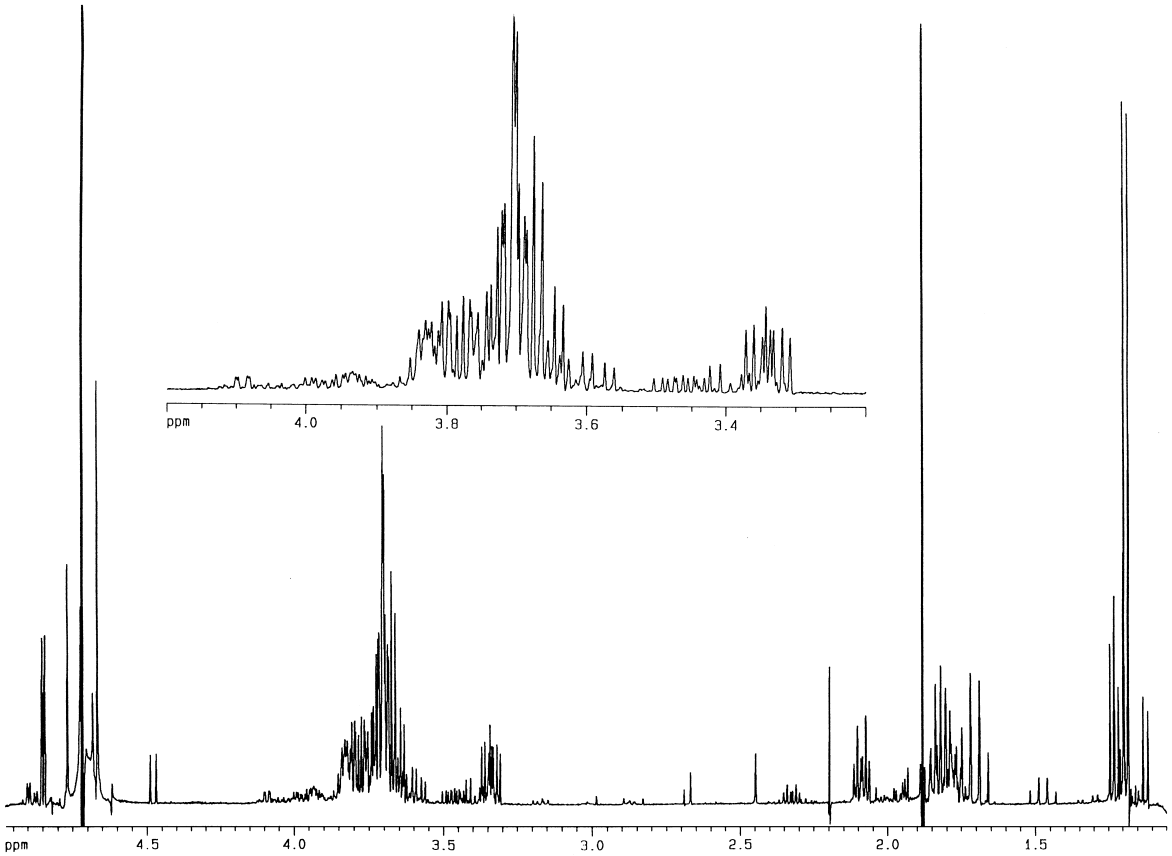


Fig. 2. ^{1}H NMR spectrum of the mixture of α and β glycosides **2** and **3** in D_2O and, in the inset, the expanded 3.2–4.2 ppm region.

Table 2
^{1}H NMR data of **2**, **3** and 4-epimer of **2**

H	**2** [a]	**3**	4-epi of **2**
1	4.85 d, $J_{1,2}$ 3.4 Hz	4.48 d, $J_{1,2}$ 8.1 Hz	4.90 d, $J_{1,2}$ 3.4 Hz
2	3.78 ddd, $J_{2,3a}$ 11.2, $J_{2,3b}$ 4.4 Hz	3.45 ddd, $J_{2,3a}$ 12, $J_{2,3b}$ 5.2 Hz	3.97 ddd, $J_{2,3a}$ 11.8, $J_{2,3b}$ 6.0 Hz
3eq	2.09 dt, $J_{3eq,3ax}$ 11.2, $J_{3eq,4}$ 4.4 Hz	2.32 dt, $J_{3eq,3ax}$ 12, $J_{3eq,4}$ 3.9 Hz	1.94 [b]
3ax	1.71 q, $J_{3ax,4}$ 11.2 Hz	1.47 q, $J_{3ax,4}$ 12 Hz	1.81 [b]
4	3.34 ddd, $J_{4,5}$ 10.7 Hz	3.34	3.85 [b]
5	3.72 [b]	3.43 dq, $J_{4,5}$ 9.4 Hz	4.11 dq, $J_{4,5}$ 1.6 Hz
6	1.19 d, $J_{6,5}$ 6.3 Hz	1.24 d, $J_{6,5}$ 6.3 Hz	1.13 d, $J_{6,5}$ 6.7 Hz
1′	3.70 [b]	n.i.	n.i.
2′	1.81 m	n.i.	n.i.
3′	3.83 m	n.i.	n.i.
4′	3.65 [b]	n.i.	n.i.

[a] From ref. [2].
[b] Signals buried.
n.i., not identified.

Since all attempts to separate the components of the LPS–OH mixture were unsuccessful, the whole LPS–OH fraction was submitted to periodate–$NaBH_4$ treatment. Subsequent chromatography on Bio-Gel P-2 of the crude reaction mixture yielded a mixture of glycosides together with an excluded fraction containing caryan. The ^{1}H NMR spectrum of the glycoside mixture (Fig. 2) showed mainly two anomeric signals. The strongest signal at δ 4.85 (3.4 Hz) was that of glycoside **2**, already described in the previous paper [2], and the other, at higher field (δ 4.48), appeared as doublet with a coupling constant of 8.1 Hz, indicating a proton in axial orientation. Starting from this last signal a scalar connectivity by a COSY experiment up to a 6-Me signal was established. The multiplet patterns and the values of the coupling constants (Table 2) were in accord with the β-glycoside structure **3**. The β-configuration was supported by a NOE contact measured on H-3ax by irradiation of the proton at δ 4.48. In the ^{1}H spectrum of the glycoside mixture also a minor signal at δ 4.90 (3.4 Hz) was observed which was assigned to the α-anomeric proton of the 4-epimer of **2**, on the basis of the chemical shift and coupling constant values [4] (Table 2). This compound, which was a glycoside of abequose, was the minor product of the stereoselective $NaBH_4$ reduction at C-4 after periodate

Table 3
^{1}H and ^{13}C data of compounds **2a** and **3a** in C_6D_6

Atom	**2a**		**3a**	
1	5.15 d, $J_{1,2}$ 3.6 Hz	96.3	4.50 d, $J_{1,2}$ 7.8 Hz	102.1
2	4.83 ddd, $J_{2,3ax}$ 11.4, $J_{2,3eq}$ 5.1 Hz	69.1	5.06 ddd, $J_{2,3ax}$ 11.8, $J_{2,3eq}$ 5.0 Hz	69.3
3ax	2.13 q, $J_{3ax,4}$ 11.4 Hz	29.7	1.50 q, $J_{3ax,4}$ 11.8 Hz	34.0
3eq	2.30 dt, $J_{3eq,3ax}$ 11.4, $J_{3eq,4}$ 5.1 Hz		2.61 dt, $J_{3eq,3ax}$ 11.8, $J_{3eq,4}$ 5.1 Hz	
4	4.77 ddd, $J_{4,5}$ 9.8 Hz	71.7	4.67 ddd, $J_{4,5}$ 9.5 Hz	71.2
5	4.12	66.6	3.32 dq, $J_{5,6}$ 6.2 Hz	73.6
6	1.26 d, $J_{5,6}$ 6.3 Hz	17.4	1.22 d	17.7
1′a	4.22 m	60.5	4.42 m	61.8
1′b	4.12		4.29 m	
2′a	1.76	31.0	1.77	31.6
2′b	1.68		1.77	
3′	3.71 m	75.2	3.93 m	73.6
4′a	4.16	66.4	4.21 dd, $J_{4'a,3'}$ 6.15, $J_{4'a,4'b}$ 11.3 Hz	66.1
4′b	4.16		4.03 dd, $J_{4'a,3'}$ 4.13, $J_{4'a,4'b}$ 11.3 Hz	
Mes	1.93; 1.76; 1.75; 1.68	20.3	1.89; 1.84; 1.83; 1.71	20.5
C=Os		170.1		168.8
		169.8		168.8
		169.6		169.9
		169.0		170.7

oxidation of the LPS–OH fraction. Measurement of the integral intensities of all of the anomeric signals with a small coupling constant (spectral region δ 4.90–4.85) relative to the intensity of the signal at δ 4.48 established a 8:1 ratio for the α and β glycosides.

Conclusive evidence for the presence of β-anomeric linkages was obtained by isolation of the peracetyl derivative **3a**, separated from its anomer **2a**. In Table 3 the ^{1}H and ^{13}C NMR data of both compounds are reported, obtained by homo- and heterocorrelated 2D NMR experiments.

The results presented above allow us to conclude that in caryophyllan about 11% of the β-glycosidic linkages occur, besides the more abundant α ones. Unfortunately, the acid lability of caryophyllan has so far precluded to obtain a large oligosaccharide which should give insight into the sequence of the α and β linkages. The finding in the disaccharide fraction of a ratio of about 3:2 for the α and β linkages, while in the caryophyllan this ratio is 8:1, indicates that α linkages hydrolysed faster than β ones, and this makes it more difficult to generate an oligosaccharide representative of the α, β distribution in caryophyllan. In fact, a reinvestigation of the earlier recorded ^{1}H NMR spectrum of the trisaccharide fraction [1] showed, besides the signals of the α-anomeric linkage, a weak signal at δ 4.43 (8.1 Hz) of the β linkage as well. However, by integration of the anomeric protons it was concluded that this fraction deals with a trisaccharide mixture, and then no information can be obtained about the α,β linkage sequence.

Finally, it is noteworthy that the acid hydrolysis behaviour of caryophyllan linkages is in accordance to that of glucosyl-glucosides and opposite to that of methylglycosides [5].

1. Experimental

General.—All spectra were recorded on a Bruker DRX-400 Avance spectrometer using a 5 mm multinuclear inverse Z-grad probe with standard Bruker pulse sequences. Spectra were recorded at 30 °C. Chemical shifts were measured in D_2O using 1,4-dioxane (δ 67.4) and sodium 3-trimethylsilylpropionate-2,2,3,3-d_4 (TSP δ 0.00), respectively, as internal standards. For the spectra in C_6D_6 the solvent peak (δ 7.15) was used as reference. 1D homonuclear Hartman–Hahn (HOHAHA) spectra [6] were recorded using a MLEV17 sequence for mixing selective excitation with a shaped pulse z-filter. Mixing times of 30, 50 and 100 ms were used. A gradient heteronuclear single quantum coherence (HSQC) [7] data set was collected in phase sensitive mode using the echo-antiecho method. Typically, data sets of 1024×256 complex points were acquired with 64 scans. The sweep width was 6 ppm for ^{1}H spectra and 180 ppm for ^{13}C spectra. Data were processed with a Lorentzian-to-Gaussian weighting function applied to t_2 and a shifted squared sinebell function and zero-filling applied to t_1. TLC was carried out on Silica Gel F_{254} (Merck). All compounds were revealed by spraying plates with a saturated solution of CrO_3 in conc. H_2SO_4, followed by heating at 120 °C for 15 min.

Preparation of cellular lipopolysaccharide.—*Pseudomonas caryophylli* strain 2151 was purchased from the National Collection of Plant Bacteria (NCPPB), Harpenden, UK and grown as already reported [1].

Purification of LPS.—The crude LPS sample was purified as reported [1].

Hydrazinolysis of the LPS fraction.—A sample of LPS (70 mg), dried at low pressure on P_2O_5 overnight, was treated with distilled NH_2NH_2 at 37 °C for 30 min. After cooling the solution in an ice bath, the polysaccharide fraction was precipitated by adding slowly acetone. The solid material was separated by centrifugation, washed several times with acetone, dissolved in H_2O, and lyophilised (56 mg, LPS–OH).

Periodate degradation of LPS–OH.—LPS–OH (15 mg) was treated with $NaIO_4$ and $NaBH_4$ as already described for the LPS fraction [2]. After usual work-up, the crude mixture was chromatographed on a column of Bio-Gel P-2 (90.6×1.5 cm, 14 mL/h, fraction volume 1.5 mL, H_2O as eluent), yielding two carbohydrate-containing peaks: one as the excluded fraction containing caryan (4 mg) and one as the glycoside fraction containing **2** and **3** (8 mg).

Acetylation of the glycoside fraction.—The sample (4 mg), dried overnight at low pressure at 40 °C, was acetylated with Ac_2O (0.5 mL) in pyridine (0.5 mL) at room temperature overnight. After usual work-up, the crude mixture was chromatographed on TLC Silica Gel (1 run; 95:5 benzene–MeOH), affording two fractions. The less polar fraction was constituted of pure α-glycoside **2a** (2 mg), the other consisted of the β-glycoside **3a** with traces of the 4-epimer of **2a**.

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