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Analysis of the polysaccharide components of the lipopolysaccharide fraction of *Pseudomonas caryophylli* [1]

Matteo Adinolfi [a], M. Michela Corsaro [a], Cristina De Castro [a], Antonio Evidente [b], Rosa Lanzetta [a], Paola Lavermicocca [c], Michelangelo Parrilli [a,*]

[a] *Dipartimento di Chimica Organica e Biologica, Università di Napoli Federico II, via Mezzocannone 16, 80134 Naples, Italy*

[b] *Dipartimento di Scienze Chimico-Agrarie, Università di Napoli Federico II, via Università 100, 80055 Portici, Italy*

[c] *Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, viale L. Einaudi 51, 70125 Bari, Italy*

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Abstract

The composition of the polysaccharide moieties of the lipopolysaccharide (LPS) fraction of *Pseudomonas caryophylli* is described. Two homopolysaccharide chains, the major one built up of (1 → 7)-linked α-caryophyllose [3,6,10-trideoxy-4-*C*-(D-*glycero*-1-hydroxyethyl)-D-*erythro*-D-*gulo*-decose] residues and the minor one made up of (1 → 7)-linked β-caryose (4,8-cyclo-3,9-dideoxy-L-*erythro*-D-*ido*-nonose) residues, were identified in the LPS fraction. In addition, experimental evidence of a third polysaccharide fraction mainly composed of heptose and glucose is also reported. The structural analysis was performed by chemical and spectroscopic methods.

Keywords: Pseudomonas caryophylli; Homopolysaccharide; Caryophyllose; Caryose

1. Introduction

Recently [1,2] and in the accompanying paper [3], we have described the complete structure of two novel monosaccharides, caryophyllose, which is 3,6,10-trideoxy-4-*C*-

* Corresponding author.

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(D-*glycero*-1-hydroxyethyl)-D-*erythro*-D-*gulo*-decose [2] (**1**), and caryose (4,8-cyclo-3,9-dideoxy-L-*erythro*-D-*ido*-nonose, **2**). They are contained in the LPS fraction of *Pseudomonas caryophylli*, a phytopathogenic bacterium responsible for the wilting of carnations [4,5]. We now report the structures of two homopolysaccharide chains present in the LPS fraction and some structural information on a third polysaccharide fraction.

2. Experimental

General.—The ^{1}H and ^{13}C NMR spectra were obtained at 400 and 100 MHz, respectively, with a Bruker AM 400 spectrometer equipped with a dual probe. The ^{13}C and ^{1}H chemical shifts were measured in D_2O, if not otherwise specified, using 1,4-dioxane (67.4 ppm) and TSP (sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$), respectively, as external standards. DEPT experiments were performed using a polarisation transfer pulse of 135° and a delay adjusted to an average C,H coupling of 160 Hz. NOEs were measured in difference mode, utilising a Bruker microprogram. Optical rotations were determined on a Perkin–Elmer 141 polarimeter. Mass spectra were recorded with a VG ZAB HF instrument equipped with an FAB source. TLC was carried out on Silica Gel F_{254} (Merck). Total carbohydrates were determined by the phenol–H_2SO_4 test [6]. All compounds were revealed by spraying plates with a saturated solution of Cr_2O_3 in concentrated H_2SO_4 followed by heating at 120 °C for 15 min.

UV absorbance was determined on a Perkin–Elmer Lambda 7 instrument. GLC was performed with a Carlo Erba EL 490 instrument equipped with a flame-ionisation detector. Me_3Si ethers of methyl glycosides and methyl esters of lipids were analysed on an SPB-1 capillary column (Supelco, 30 m × 0.25 mm i.d.; flow rate, 1 mL/min; N_2 as carrier gas), with the temperature programme: 160° for 3 min, 160 → 200 °C at 2 °C/min, 200 → 260 °C at 10 °C/min, 260 °C for 15 min. Partially methylated alditol acetates were analysed by GLC–MS on a Hewlett–Packard 5890 instrument, using an SP-2330 capillary column (Supelco, 30 m × 0.25 mm i.d.; flow rate, 0.8 mL/min; He as carrier gas), with the temperature programme: 80 °C for 2 min, 80 → 170 °C at 30 °C/min, 170 → 240 °C at 4 °C/min, 240 °C for 15 min.

Preparation of cellular lipopolysaccharides.—*Pseudomonas caryophylli* strain 2151, obtained from the National Collection of Plant Bacteria (NCPPB), Harpenden, UK, was grown in 200-mL flasks containing 100 mL of Woolley's medium [7] supplemented with 1.5% (w/v) protease peptone at 27 °C with shaking (120 rpm) for 6 days [5]. The culture (2.6 L) was centrifuged (10,000 *g* × 10 min) and the harvested cells were washed three times with 85% (w/v) NaCl and lyophilised. Dry cells (5.5 g) were suspended in 100 mL of ultrapure Milli-Q water and extracted with phenol according to the conventional procedure [8]. The resulting aqueous phase was dialysed (cut-off 12,000–14,000) for 2 days. Contents of tubes were lyophilised; the residue was dissolved in ultrapure Milli-Q water (50 mL) and brought to 1.5% (w/v) NaCl and 1.5% (w/v)

[2] The name of the branch, indicated in parentheses, has been changed with respect to ref. [2] in accordance with the IUPAC-IUB nomenclature rules for branched-chain monosaccharides (see ref. [13]).

Cetavlon (hexadecyltrimethylammonium bromide) while stirring at room temperature for 10 min. This mixture was centrifuged (7000 rpm at 5 °C for 45 min) and the supernatant solution was mixed with 10 volumes of absolute EtOH and left overnight at −20 °C. The resulting precipitate was collected by centrifugation (7000 rpm at 5 °C for 1 h), dissolved in ultrapure Milli-Q water (40 mL), and dialysed as described above. The content was lyophilised to yield "crude" LPS (306 mg).

Purification of LPS.—The crude LPS sample (306 mg) was purified by chromatography on Bio-Gel A-15m (Bio-Rad). The column (2.5 × 50 cm) was equilibrated and eluted with 300 mM TEA (triethylamine) neutralised to pH 7 with HCl. The sample was dissolved in a solution of 300 mM TEA and 10 mM EDTA, adjusted to pH 7 with HCl prior to application to the column. On the basis of the chromatographic profile the eluted fractions were pooled in two fractions which showed identical ^{1}H and ^{13}C NMR spectra. Furthermore, GLC of Me_3Si ethers of methanolysis crude products revealed an identical monosaccharide composition for both fractions.

Attempts at further purification of the LPS sample with hydroxylapatite, with both SDS (sodium dodecyl sulfate) [9] and DOC (deoxycholic acid sodium salt), and Q-Sepharose fast flow, with and without SDS, failed. Therefore the further investigation was carried out on the whole sample, from now onwards referred to as the LPS fraction.

Acid hydrolysis of the LPS fraction with 1 M AcOH.—The LPS fraction (23 mg) was treated with 1 M AcOH in a sealed tube for 3 h at 110 °C. After cooling, the sample was centrifuged at 4000 rpm for 20 min. The solid consisted of lipid fraction (4 mg). The supernatant liquid was adjusted to pH 5 with pyridine and applied to a Sephadex G-50 (Pharmacia) column. The sample was eluted with pyridine acetate buffer at pH 4.26. The polysaccharide fraction eluted in the void volume was dialysed and lyophilised (1 mg). Its ^{1}H NMR spectrum showed anomeric signals of comparable intensities at δ 5.34, 5.24, 5.17, 5.01, and 4.98.

Methanolysis of the LPS fraction.—A sample of the LPS fraction (100 mg) was treated with 1 M HCl–MeOH as described in the accompanying paper [3]. An aliquot of the crude reaction product was submitted to GLC analysis of Me_3Si ethers. By Bio-Gel P-2 chromatography followed by preparative TLC the methyl glycosides of caryophyllose [2] and of caryose [3] were separated. In addition, from the more polar fractions the methyl glycosides of glucose (4 mg) and of L-*glycero*-D-*manno*-heptose (15 mg), tentatively identified by comparison of its ^{1}H NMR data with those of the allyl α-glycoside [10], were isolated.

Methylation, acid hydrolysis and acetylation of the LPS fraction.—To a solution of the LPS fraction (5 mg) in Me_2SO (2 mL) were added 6.6 M KCH_2SOMe (500 μL) and MeI (1.5 mL), according to Hakomori [11]. After evaporation of MeI by a stream of N_2, the crude mixture was extracted with water–$CHCl_3$. The organic phase, after solvent evaporation, was hydrolysed with 2 M CF_3CO_2H at 120 °C for 1 h. After the usual workup of the crude residue, preparative TLC (SiO_2, 95:5 $CHCl_3$–MeOH) gave a main product (2 mg), which by acetylation [1:1 Ac_2O–pyridine (300 μL), 120 °C, 20 min] yielded **3a** (2 mg, colourless syrup); NMR data: see text.

Another sample (1 mg) was methylated under the same conditions, hydrolysed with acid, reduced with $NaBD_4$, and analysed as alditol acetates by GLC–MS. The only product related to caryophyllose was the partially acetylated alditol corresponding to **3a**.

All the other relevant peaks were identified and assigned to: terminal Glc, terminal Gal, terminal Hep, 2-substituted Rha, 6-substituted Glc, 2,6-substituted Glc, one peak related to caryose, 3,4-substituted Hep, 7-substituted Hep, and 3,7-substituted Hep.

Periodate degradation of the LPS fraction.—Described in ref. [2].

Acid hydrolysis of the LPS fraction with 1% AcOH.—In a preliminary test, a sample (20 mg) of the LPS fraction was treated with 1% AcOH (1 mL) firstly at 50 °C for 4 h, then at 70 °C for 1.5 h, and finally at 80 °C for 3.5 h. Only under the last conditions did a light opalescence, due to the hydrolysed lipid moiety, appear. The solution was centrifuged and a portion of the supernatant was methanolysed. The crude methanolysis mixture was extracted with hexane, and this extract still contained lipids (GLC analysis). Therefore, the temperature was raised to 100 °C and the solution was kept until no lipids were detected in the polysaccharide moiety (6 h).

On the basis of these results, a solution of the LPS fraction (50 mg) in 1% AcOH (1 mL) was kept for 7 h at 100 °C, with stirring. A solid lipid (6 mg) was collected by centrifugation. The supernatant liquor was applied to a Bio-Gel P-10 (Bio-Rad, 1.0 × 119 cm) column. Three fractions were collected by elution with water. The first fraction eluted (14 mg) showed ^{1}H NMR and ^{13}C spectra very similar to those of the LPS fraction, the only difference being a less intense anomeric signal at δ 4.96. The most retained fraction (7 mg) appeared, by ^{1}H and ^{13}C NMR analysis, to consist mainly of caryophyllose. The intermediate fraction (20 mg) was chromatographed on Bio-Gel P-2 (Bio-Rad, 1.5 × 91 cm), yielding, besides an excluded fraction, two oligosaccharide fractions. The more retained of these two was the disaccharide **5** (5 mg, amorphous solid); ^{1}H NMR spectrum: see text. That less retained was the trisaccharide **6** (4 mg, amorphous solid); ^{1}H NMR spectrum: see text.

Reduction of **5** *and* **6** *with* $NaBD_4$.—Samples of **5** (1 mg) and **6** (1 mg) were separately treated with $NaBD_4$ (3 mg) in water for 1 h at room temperature. After quenching with AcOH, the mixtures were treated with MeOH, evaporated by a stream of N_2, desalted on Dowex 50W-X8 (H^+ form), and lyophilised. The residues (1 mg) were submitted to ^{1}H NMR and FABMS analyses: see text.

Analysis of the interglycosidic linkages of **5** *and* **6**.—Samples of **5** (1 mg) and **6** (1 mg) were separately methylated and hydrolysed as above. The mixtures were reduced with $NaBD_4$ (3 mg), acetylated [1:1 Ac_2O–pyridine (300 μL), 120 °C, 20 min], and submitted to GLC–MS analysis.

Acid hydrolysis of the LPS fraction with 0.125 M CF_3COOH.—A sample of the LPS fraction (50 mg) was treated with 0.125 M CF_3COOH as previously described [1]. After the usual workup the residue (45 mg) was chromatographed on Bio-Gel P-2 (1.5 × 91 cm; flow rate, 14 mL/h). By water elution, two main fractions were collected. The more retained (22 mg) consisted of caryophyllose and a small amount of galactose. The excluded fraction (12 mg) was further chromatographed on Bio-Gel P-10 (1.0 × 119 cm; flow rate, 15 mL/h). Elution by water gave two fractions (**A** and **B**). Fraction **B** (eluted second; 4 mg), $[\alpha]_D$ −52.5° (*c* 1.2, H_2O), was submitted to ^{1}H and ^{13}C NMR analysis: see text. An M_r of 2000 was evaluated on a Bio-Gel P-10 column calibrated with dextran standards under the condition described above. An aliquot of this fraction (1 mg) was methanolysed and analysed by GLC as Me_3Si ethers. Fraction **A** (eluted first; 8 mg) showed relevant anomeric signals at δ 5.39, 5.14, 5.00, 4.90, and 4.87 in the ^{1}H NMR

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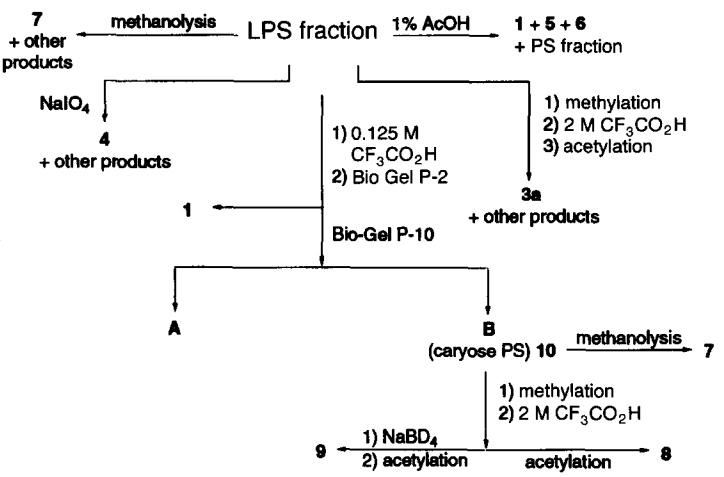


Scheme 1.

spectrum and at δ 100.9, 98.6, 97.9, 97.2, and 96.9 in the ^{13}C NMR spectrum. It was in part (1 mg) submitted to methanolysis and the crude mixture was analysed by GLC as Me_3Si ethers.

Methylation, acid hydrolysis and acetylation of fraction **B**.—A sample of fraction **B** (3 mg) was treated as described for the LPS fraction, giving **8** (2 mg, colourless syrup); ^1H NMR: see text.

Preparation of **9**.—An aliquot (1 mg) of methylated and acid-hydrolysed **B** was reduced with $NaBD_4$ (3 mg) in EtOH (250 μL) at room temperature for 1 h. After the usual workup, the crude mixture was acetylated as above. Partition with CH_2Cl_2–H_2O afforded **9** (1 mg, colourless syrup). FABMS: see text.

A summary of the LPS fraction degradation pathways is reported in the chart of Scheme 1.

3. Results and discussion

Both ^1H and ^{13}C NMR spectra (Fig. 1) of the LPS fraction, isolated from *Pseudomonas caryophylli* strain 2151 as described in the Experimental section, displayed, in the anomeric regions, very intense peaks at δ 4.96 and 100.6, respectively, besides minor signals, suggesting a heterogeneous lipopolysaccharide fraction containing a homopolysaccharide as the major component. The mixture could not be purified further.

Treatment of this LPS fraction with 1 M acetic acid for 3 h at 110 °C yielded a small amount of lipid-free polysaccharide, whose ^1H NMR spectrum did not show any of the intense anomeric signal, suggesting an alteration with acid of the more abundant component of the lipopolysaccharide fraction. Therefore, we decided to study the major component directly on the whole LPS fraction.

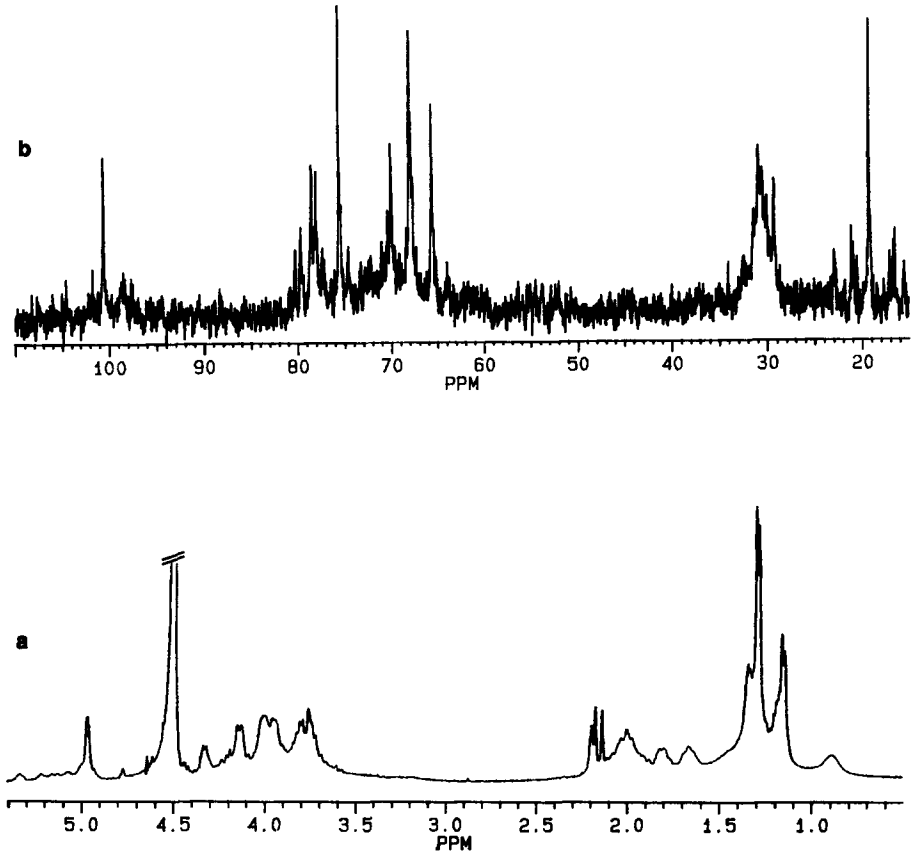


Fig. 1. ^{1}H (a) and ^{13}C (b) NMR spectra of the LPS fraction.

Methanolysis of the LPS fraction gave, after lipid extraction with hexane, a carbohydrate fraction whose GLC Me_3Si ether analysis (Fig. 2a) revealed the presence of peaks corresponding to the methyl glycosides of caryophyllose [2] and peaks due to the methyl glycosides of caryose [3]. In addition, peaks attributable to the methyl glycosides of glucose, L-*glycero*-D-*manno*-heptose (probably), rhamnose, and galactose, together with traces of those of Kdo, glucosamine, and another heptose, were also identified in the GLC chromatogram. By taking into account the molar ratios of the above products (Table 1), it is suggested that the more abundant LPS component contains a polysaccharide moiety derived only from caryophyllose. From a preparative point of view, besides the methyl glycosides of caryophyllose and caryose, the methyl glycosides of L-*glycero*-D-*manno*-heptose (tentatively assigned) and glucose were also isolated.

Methylation of the LPS fraction followed by acid hydrolysis and acetylation led us to isolate, by TLC, compound **3a** as the main product, whose structure was defined on the basis of the comparison of its ^{1}H NMR data with those of **3b** [1], the more abundant pyranose form in the isomeric mixture of caryophyllose (Table 2). The presence of

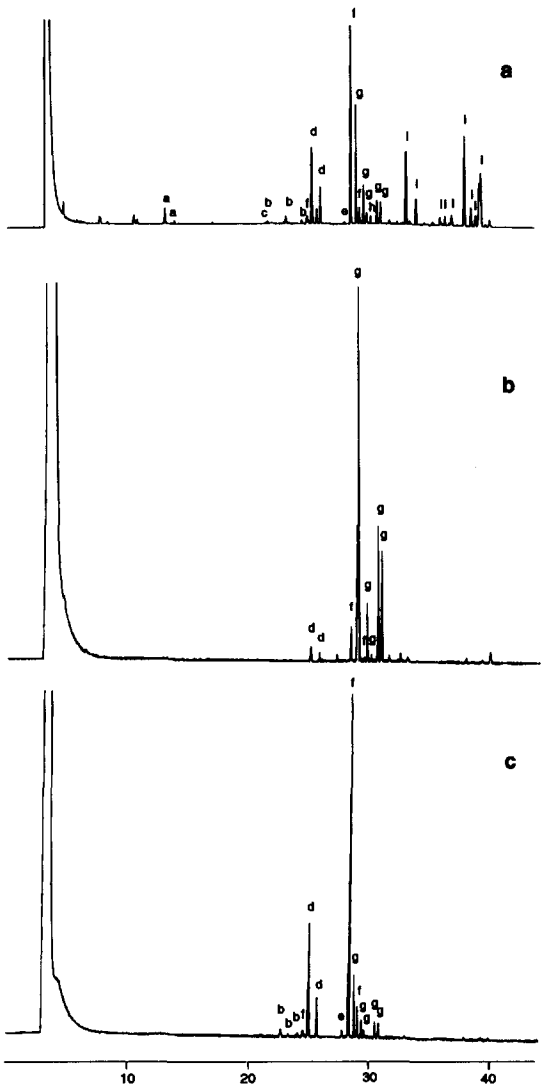


Fig. 2. Me_3Si ether GLC chromatograms of methyl glycosides from (**a**) LPS carbohydrate moiety; (**b**) fraction **B**; (**c**) fraction **A**. Rha (a), Gal (b), GlcN (c), Glc (d), Kdo (e), L,D-Hep (f), caryose (g), heptose (h), caryophyllose (i).

O-acetyl groups at positions 1 and 7 indicated the latter as the position involved in the interglycosidic linkages. The GLC–MS analysis of the crude partially methylated alditol acetates allowed us to identify all the relevant peaks. The interglycosidic linkage at position 7 was also confirmed by the fragmentation of the alditol acetate from **3a** (GLC–MS spectrum: see Fig. 3).

Periodate degradation performed on the LPS fraction yielded, as the main product,

Table 1
Molar ratios among the monosaccharides constituting the polysaccharides of the LPS fraction evaluated from methanolysis

Monosaccharide	Molar ratio
Caryophyllose	4.40 [a]
L-*glycero*-D-*manno*-Heptose	1.41 [b]
Caryose	1.32 [c]
Glucose	1.00
Rhamnose	0.21
Galactose	0.17

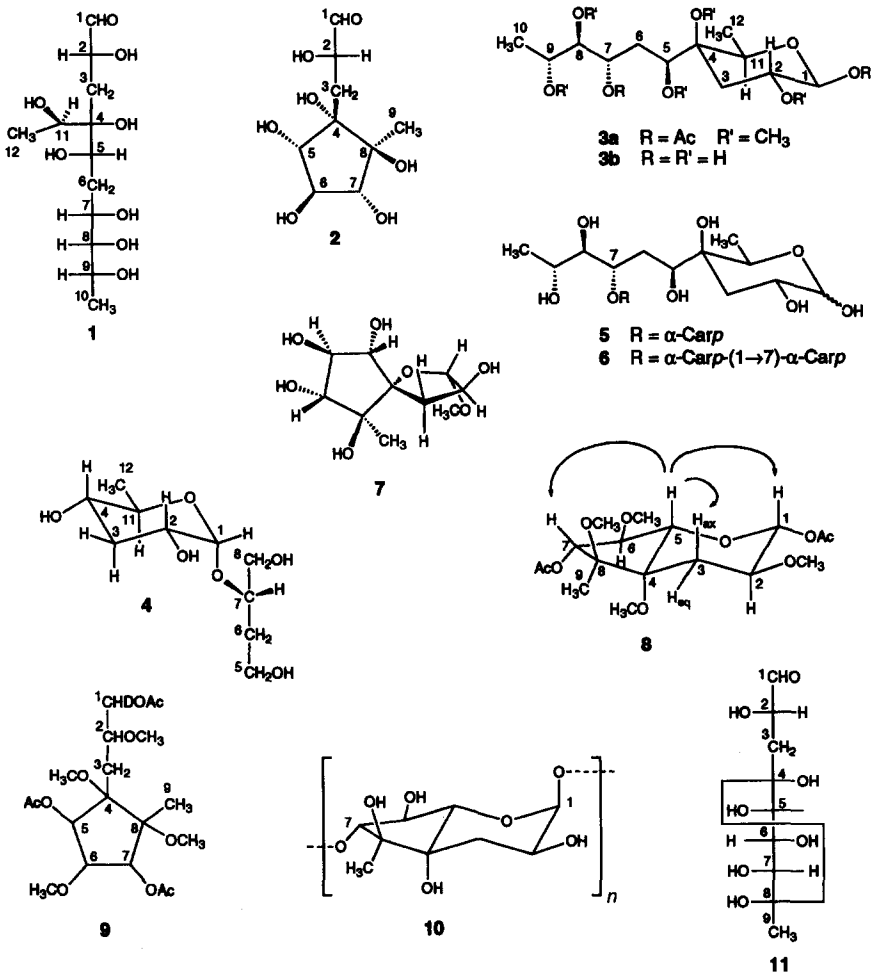
[a] The response factor has been estimated in this work.
[b] The response factor has been estimated on a sample of D-*glycero*-D-*galacto*-heptose purchased from Fluka.
[c] A unitary response factor for this monosaccharide has been assumed.

glycoside **4**, whose structure, already described [2], was assigned on the basis of ^{1}H NMR data (Table 2). In particular, the $^3J_{1,2}$ value of 3.4 Hz indicated an α configuration of the anomeric carbon in the polysaccharide, in agreement with the high-field chemical shift of the anomeric carbon (δ 100.6) of the more abundant component of the LPS fraction.

Table 2
^{1}H NMR chemical shifts (δ) and apparent coupling constants in parentheses (Hz) of compounds **3a**, **3b**, and **4**

Proton [a]	**3a** [b]	**3b** [c]	**4** [d]
1	5.92 d (7.8)	4.56 d (8.2)	4.85 d (3.4)
2	3.67 ddd (11.2; 7.8; 5.9)	3.66 ddd (11.5; 8.2; 5.2)	3.78 ddd (11.2; 4.4; 3.4)
3eq	2.41 dd (14.7; 5.9)	2.04 dd (13.2; 5.2)	2.09 dt (11.2; 11.2; 4.4)
3ax	1.98 dd (14.7; 11.2)	1.74 [f]	1.71 q (11.2)
4			3.34 ddd (11.2; 10.7; 4.4)
11	3.97 q (6.3 Hz)	4.06 q (6.5)	3.72 [g]
12-H_3	1.36 d (6.4)	1.20 d (6.5)	1.19 d (6.3)
5	3.15 dd (9.8; 2.4)	3.79 m	3.70 [g]
6-H_2	2.30 ddd (12.7; 10.7; 2.4)	1.74 [f]	1.81 m
	1.87 ddd (12.7; 9.8; 2.4)		
7	5.69 dt (10.7; 2.4)	3.85 m	3.83 m
8	3.29 [e]	3.54 m	3.65 [g]
9	3.29 [e]	3.96 dq (6.5; 6.5)	
10-H_3	1.22 d (6.4)	1.23 d (6.5)	
OCH_3	3.36 s; 3.30 s; 3.25 s; 3.20 s; 3.10 s		
$OCOCH_3$	1.71 s; 1.69 s		

[a] The numbering used for the cyclic form of caryophyllose is different from that utilised in refs. [1,2], but in accordance with that of the acyclic form **1**.
[b] Measured at 30 °C in C_6D_6 (δ from solvent signal at δ 7.15).
[c] From ref. [1]. Measured in D_2O at 30 °C.
[d] From ref. [2]. Measured in D_2O at 30 °C.
[e] Overlapped signals.
[f] Overlapped signals.
[g] Overlapped signals.



Since only **3a** and **4**, as compounds derived from caryophyllose, were obtained from the methylation and the periodate degradation, respectively, the structure of the polysaccharide moiety of the more abundant component of the LPS fraction comprised a linear chain of 3,6-dideoxy 4-*C*-(D-*altro*-1,3,4,5-tetrahydroxyhexyl)-D-*xylo*-hexopyranose (Car*p*) residues linked through α linkages to positions 7, i.e., at C-3 of the side chains.

Further support for this structure and evidence for the α configuration for the interglycosidic linkage were achieved by partial acid degradation of the LPS fraction. With the aim of finding the appropriate acid conditions for obtaining the lipid-free homopolysaccharide, a sample of the LPS fraction was hydrolysed with 1% acetic acid at progressively higher temperatures, monitoring the progress of the hydrolysis of the lipid moiety by methanolysis and GLC analysis of the polysaccharide residue. The reaction with 1% acetic acid at 100 °C for 7 h allowed us to obtain a lipid-free carbohydrate fraction, containing a polysaccharide fraction (PS fraction, Scheme 1)

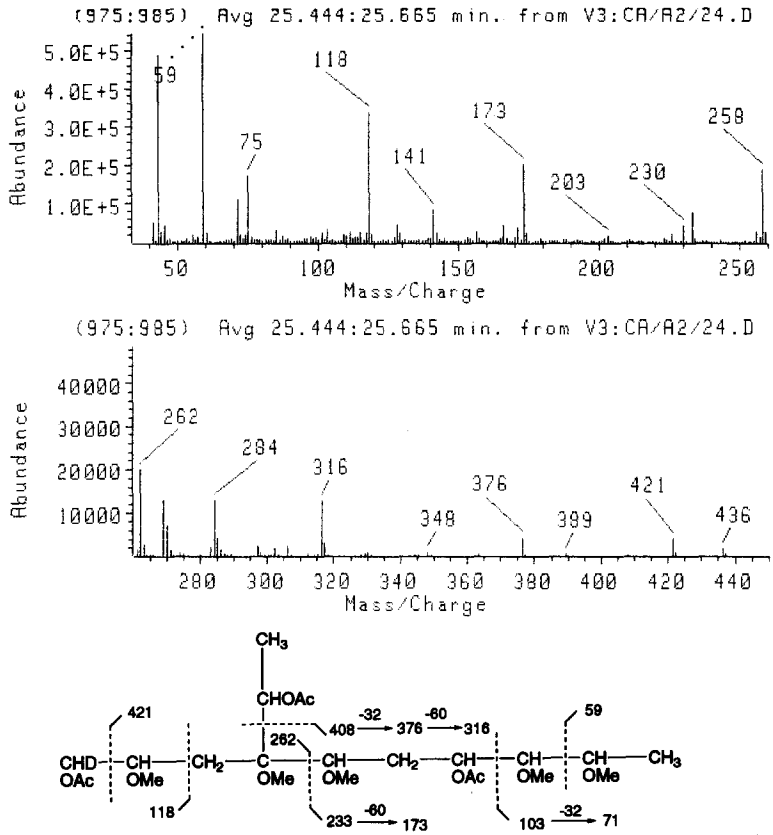


Fig. 3. EIMS spectrum of the alditol-*1-d* acetate **3a**.

composed, besides other polysaccharides, of the partially depolymerised caryophyllose polysaccharide.

Actually, the PS fraction, obtained by gel chromatography of the hydrolysate, showed in the ^{1}H and ^{13}C NMR spectra a pattern of anomeric signals similar to that of the LPS fraction. Furthermore caryophyllose and an oligosaccharide fraction were also isolated. The latter was further chromatographed to give the caryophyllose disaccharide **5** and the trisaccharide **6**. The ^{1}H NMR spectrum of **5** showed, besides the anomeric signals of the reducing unit at δ 5.15 (3.4 Hz, α anomer) and 4.57 (8.3 Hz, β anomer), that of the non-reducing unit at δ 4.96 d (3.4 Hz), which survived at δ 5.02 d (3.9 Hz) when **5** was reduced with $NaBD_4$. The size of the disaccharide was confirmed by the FABMS spectrum of its deuterated alditol derivative, which showed a pseudomolecular ion peak $[M + H]^+$ at m/z 578. The ^{1}H NMR spectrum of **6** showed two anomeric signals centred at δ 4.96 assignable to two non-reducing units of caryophyllose, as confirmed by the proton spectrum of its alditol derivative which displayed two anomeric signals at δ 5.01 d (3.9 Hz) and 4.97 d (3.9 Hz). The FABMS spectrum of the deuterated alditol derivative displayed a pseudomolecular peak at m/z 856, in agreement with a reduced

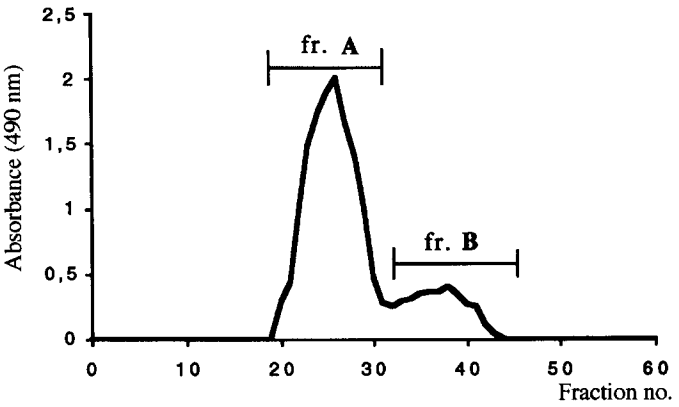


Fig. 4. Bio-Gel P-10 gel-filtration chromatography of the 0.125 M CF_3CO_2H hydrolysed LPS fraction.

trisaccharide. The α configuration for the glycosidic linkages for both **5** and **6** was indicated by the $^3J_{1,2}$ values and the attachment points by methylation and analysis of the alditol acetates. Therefore the structures α-Car*p*-(1 → 7)-Car and α-Car*p*-(1 → 7)-α-Car*p*-(1 → 7)-Car could be assigned to **5** and **6**, respectively.

Further information about the complex nature of the LPS fraction was obtained by its acid hydrolysis with 0.125 M CF_3CO_2H at 100 °C for 30 min [1]. Under these conditions, rhamnose and all of the caryophyllose were liberated with destruction of the homopolysaccharide component of the LPS fraction. By gel chromatography a fraction containing the minor polysaccharide components was isolated. This fraction showed several anomeric signals with comparable intensities both in the ^1H and ^{13}C NMR spectra. GLC of the crude methanolysate of this fraction did not show peaks assignable to caryophyllose or rhamnose, the main peaks being those from heptose, galactose, glucose, and caryose. This fraction was further resolved, by gel chromatography, into the two fractions **A** and **B** (Fig. 4). The M_r of this latter was established to be 2000.

The more retained fraction **B** displayed ^1H and ^{13}C NMR spectra (Fig. 5) which suggested a homopolysaccharide structure since both showed essentially only one anomeric signal at δ 5.17 and 97.2, respectively. Accordingly, GLC of the crude methanolysate of this fraction showed almost exclusively the peaks of the methyl glycosides of caryose (Fig. 2b). The ^{13}C NMR spectrum displayed nine signals: the anomeric one at δ 97.2, four signals due to carbinolic methine carbons (δ 88.4, 78.5, 75.2, 65.7), two of tertiary carbinolic carbons (δ 79.7, 79.3), one of a methylene carbon (δ 31.5), and one of a methyl carbon (δ 16.8). The ^1H NMR spectrum, in accordance with the caryose structure, displayed the signals of methylene protons, in the range 1.8–2.1 ppm, and of a methyl singlet at δ 1.25, indicating a methyl group linked to an unprotonated carbon.

By methanolysis of this polysaccharide, compound **7**, described in the accompanying paper [3], was isolated, while methylation of the polysaccharide, acid hydrolysis, and acetylation gave compound **8**. The ^{13}C NMR spectrum of **8** showed an anomeric signal at δ 97.5, suggesting a pyranose structure. The ^1H NMR assignments (Table 3) were

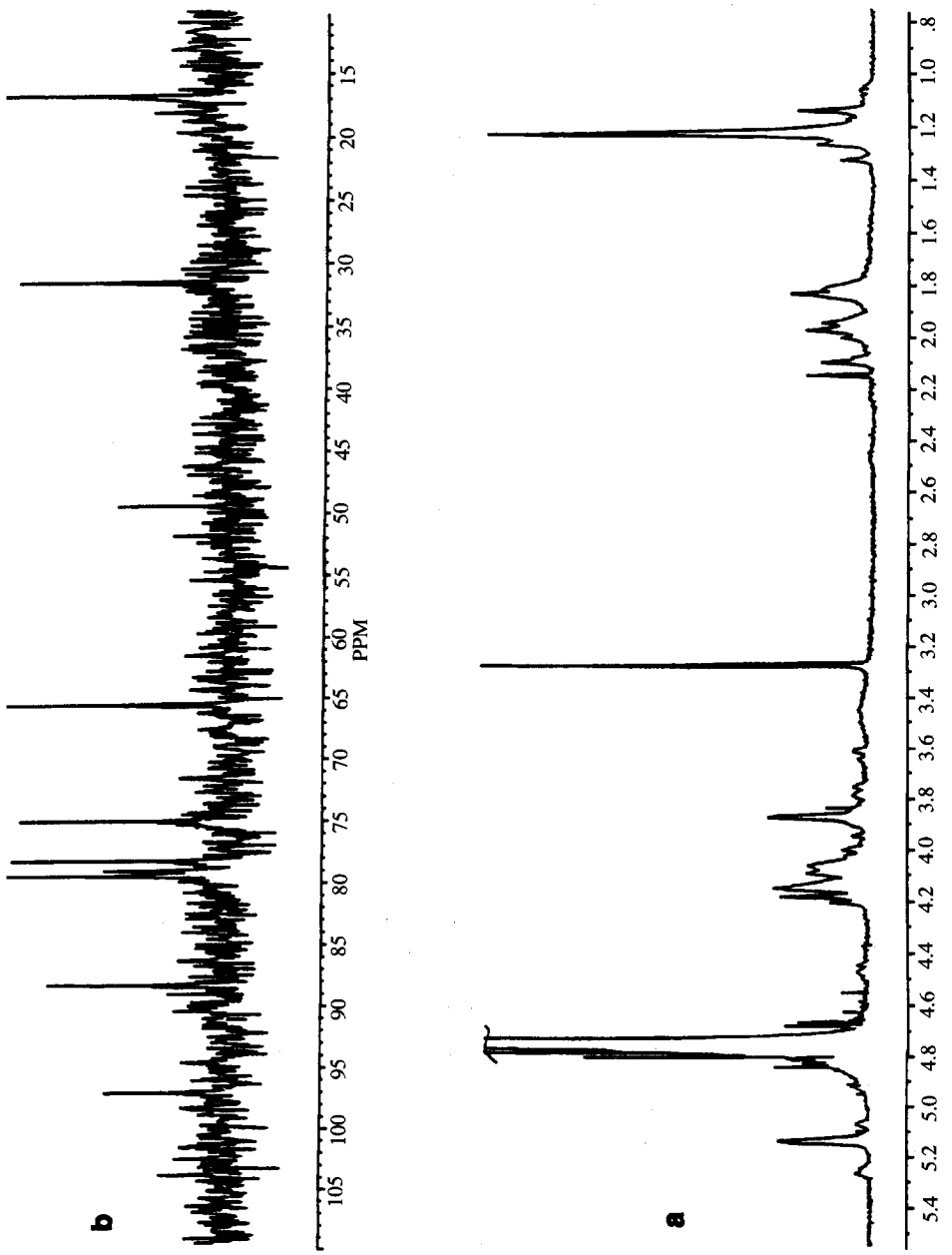


Fig. 5. ^{1}H (a) and ^{13}C (b) NMR of fraction **B**.

Table 3
^{1}H NMR chemical shifts (δ) and apparent coupling constants in parentheses (Hz) of compound **8** [a]

Proton	**8**
1	5.95 (7.8)
2	3.46 ddd (11.7; 7.8; 4.9)
3ax	1.99 dd (13.7; 11.7)
3eq	2.19 dd (13.7; 4.9)
5	4.29 d (9.3)
6	4.42 dd (9.3; 4.4)
7	5.43 d (4.4)
9	0.92 s
OAc	1.66 s; 1.65 s
OCH_3	3.39 s; 3.18 s; 3.16 s; 3.11 s

[a] Measured at 30 °C in C_6D_6 (δ from solvent signal at δ 7.15).

based on decoupling and NOE experiments. The anomeric signal at δ 5.95 with a vicinal coupling constant value of 7.8 Hz indicated a *trans*-diaxial relationship between H-2 and H-1. By irradiation of the latter, enhancements of the signals at δ 4.29 and 1.99 were found, allowing assignment of these signals to H-5 and H-3*ax*, respectively, and indicating that the pyranose ring is *trans*-fused at position 5 and not *cis*-fused at position 8, this latter pyranose closure occurring for the monosaccharide caryose [3]. Since the signal at δ 4.29 was J_{vic}-connected to that at δ 4.42 and this, in turn, was connected to that at δ 5.43, the last two of these signals were assigned to H-6 and H-7, respectively. The latter and the anomeric proton, on the basis of their chemical shifts, must each be geminal to an acetoxyl group, whereas any other proton (H-5 excepted) linked to an oxygen-bearing carbon was geminal to a methoxyl group. Therefore, the position involved in the interglycosidic linkage was C-7. In addition, the signals of four methoxyl and two acetyl groups and of a methyl group attached to an unprotonated carbon were also found in the ^{1}H NMR spectrum.

Part of the product obtained by methylation and acid hydrolysis of fraction **B** was reduced with $NaBD_4$ and acetylated to give **9**. The FABMS spectrum of **9**, measured in positive mode, showed two peaks at m/z 422 and 439 assignable to the pseudomolecular ions $[M + H]^+$ and $[M + NH_4]^+$, respectively. The EIMS spectrum of **9** (Fig. 6) derived from GLC–MS analysis showed, as the only identified fragments, those at m/z 118 and 347.

Since the only compound related to caryose and isolated from the methylation was **8**, the polysaccharide of fraction **B** was composed of a linear chain of caryopyranose residues linked through (1 → 7) glycosidic bonds. The β configuration [3] for the anomeric centres was inferred from NMR data of fraction **B** polysaccharide, in particular, the value of 170 Hz for $^1J_{C,H}$ of the anomeric carbon [12] and its high-field chemical

[3] In the accompanying paper [3], we proposed for caryose the systematic name 4,8-cyclo-3,9-dideoxy-L-*erythro*-D-*ido*-nonose (see formula **11**). On this basis the β notation can be applied to the anomeric centre of **10**.

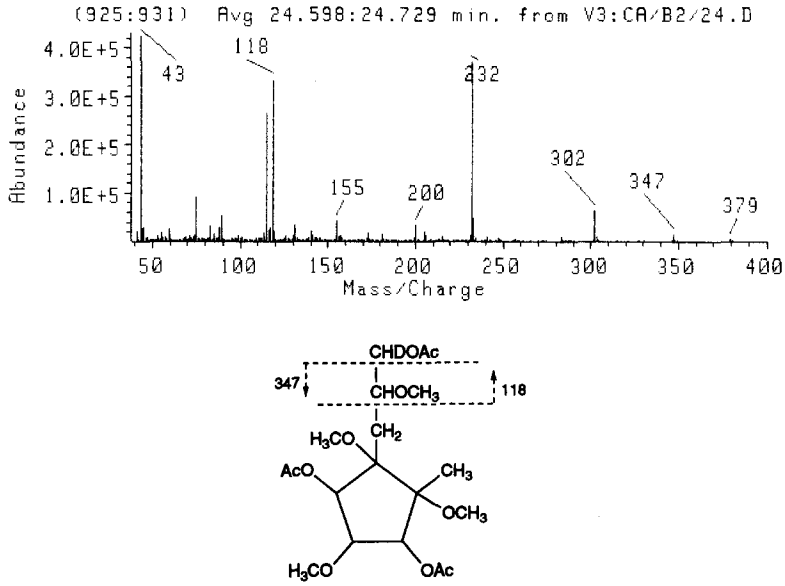


Fig. 6. EIMS spectrum of the alditol-*1-d* acetate **9**.

shift (δ 97.2), supported by the appearance as a singlet of the corresponding anomeric proton signal. The repeating unit of the caryose homopolysaccharide is depicted in **10**.

The less retained fraction **A** still showed several anomeric signals of comparable intensities in the ^{1}H and ^{13}C NMR spectra, the signal of the caryose polysaccharide included, whose presence might be due to incomplete chromatographic separation. In agreement with these data, GLC of the crude methanolysate of this fraction (Fig. 2c) showed mainly peaks attributable to heptose and glucose, besides minor ones corresponding to caryose and galactose. This suggested that the polysaccharide of fraction **A** was composed mainly of heptose and glucose residues with different types of interglycosidic linkages, in agreement with the LPS methylation data (see Experimental section). The structure of the heptose is tentatively suggested to be L-*glycero*-D-*manno*-heptose from the ^{1}H NMR spectrum of its methyl glycoside, isolated from the crude methanolysate of the LPS fraction. This heptose is a common component of the oligosaccharide core of LPS, as is D-glucose.

Work is in progress to establish the structure of the fraction **A** polysaccharide and the relationship among caryophyllose, caryose, the heptose polysaccharides, and the lipid moiety.

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