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Structural determination of the *O*-deacetylated O-chain of lipopolysaccharide from *Burkholderia* (*Pseudomonas*) *cepacia* strain PVFi-5A

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Abstract

On the basis of chemical degradation methods and one- and two-dimensional ^{1}H and ^{13}C NMR experiments the novel following structure was established for the *O*-deacetylated repeating unit of the O-chain of the main *Burkholderia* (*Pseudomonas*) *cepacia* (strain PVFi-5A) lipopolysaccharide:

→4)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→6)-α-D-Glc*p*NAc-(1→

Keywords: *Burkholderia* (*Pseudomonas*) *cepacia*; O-chain; Lipopolysaccharide; Bacterial polysaccharide

1. Introduction

Burkholderia (Pseudomonas) cepacia, a non-fermentative, Gram-negative, motile ubiquitous bacterium, was first reported as a phytopathogenic bacterium by Burkholder [1], and subsequently as a biological control agent of plant disease [2–5] and as an opportunistic human pathogen [6] which could be isolated from cystic fibrosis patients, contaminated medical devices and chemical solution employed in hospital practices [7,8]. This last ability of clinical *B. cepacia* strains raises the risk of an environmental release of this bacterium. In order to facilitate the selection of those *B. cepacia* strains that may be safely used as biocontrol agents,

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strains of the bacterium recovered from soil, water and clinical environments have previously been distinguished according to their cultural, biochemical, physiological and molecular differences [9–13]. Other approaches to distinguish them were by characterisation of lipopolysaccharide (LPS) components of the bacterial cell [14]. We now report on the structural determination of the O-chain of the LPS fraction of *B. cepacia* strain PVFi-5A. This strain was isolated from the roots of healthy tomato plants and is currently being used experimentally as a biocontrol agent of bacterial and fungal disease of tomato plants.

2. Results and discussion

The LPS fraction was isolated from phenol phase of phenol-water treatment [15] of dried bacterial cells. The finding of the LPS fraction almost exclusively in the phenol phase, instead of in the aqueous phase, suggested the presence of many hydrophobic functional groups. Accordingly, the ^{1}H NMR of LPS fraction showed several acetyl signals and many signals of different intensities in the anomeric region suggesting an abundant (23%) and non-stoichiometric *O*-acetylation of the polymer. This was confirmed by mild alkaline treatment of the LPS fraction which, hydrolysing *O*-acyl linkages, afforded a fraction (LPS–OH), the ^{1}H NMR spectrum of which showed only two acetyl signals, attributable to *N*-acylamino sugars, and a more clear anomeric region.

The *O*-deacetylated polysaccharide part (O-chain) of the LPS fraction was obtained, as a peak eluted shortly after the void volume, by gel-filtration chromatography on Bio-Gel P-100 of the acetic acid hydrolysate of LPS–OH fraction. Methanolysis gave a monosaccharide composition, as TMS-ether derivatives, consisting of Gal, GalNAc, GlcNAc and Rha in the ratios 1.0:0.9:0.8:1.3 and traces of Glc. The main signals in the anomeric region of the ^{1}H NMR spectrum of O-chain (Fig. 1) were three doublets of the same integral intensities. Two of these, occurring at δ 4.98 (3.8 Hz) and 4.96 (3.9 Hz), showed chemical shifts and $J_{1,2}$ values in accordance with an α anomeric configuration. The other at δ 4.75 (8.4 Hz) had values indicating a β anomeric configuration. At higher field two acetyl signals occurred at δ 2.08 and 2.04 in a 1:1 ratio and minor signals, attributable to 6-Me of Rha units, appeared at δ 1.28.

The ^{13}C NMR spectrum (Fig. 2) showed the following intense signals: three for anomeric

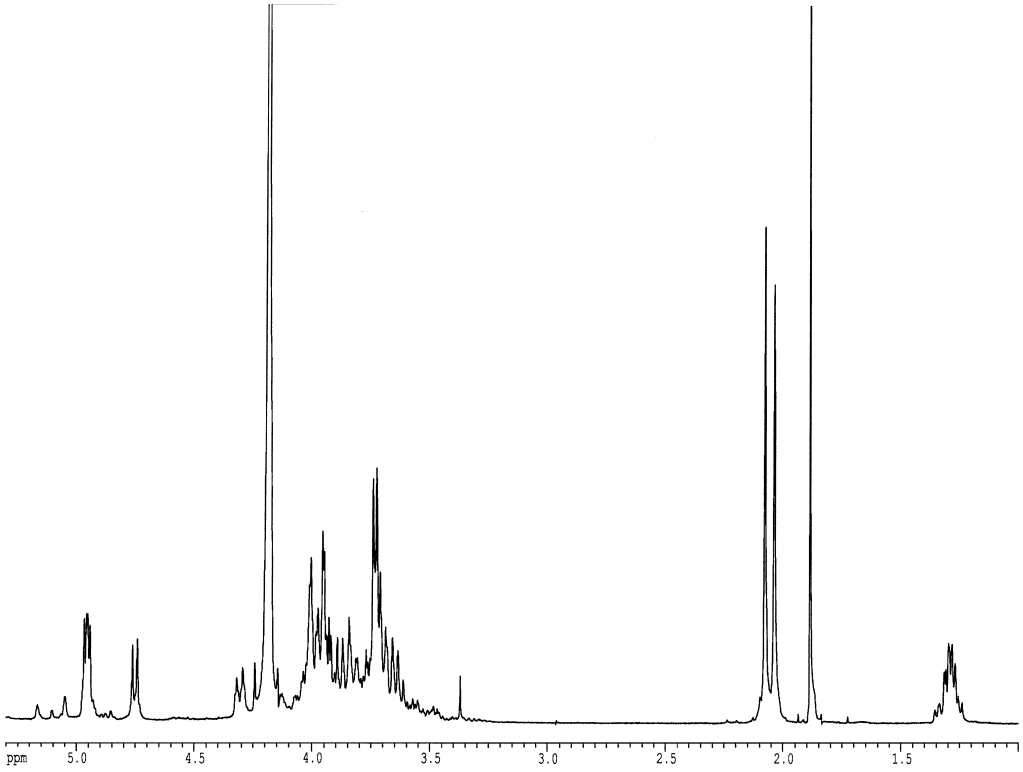


Fig. 1. ^{1}H NMR of the de-*O*-acetylated O-chain of *B. cepacia* at 70 °C.

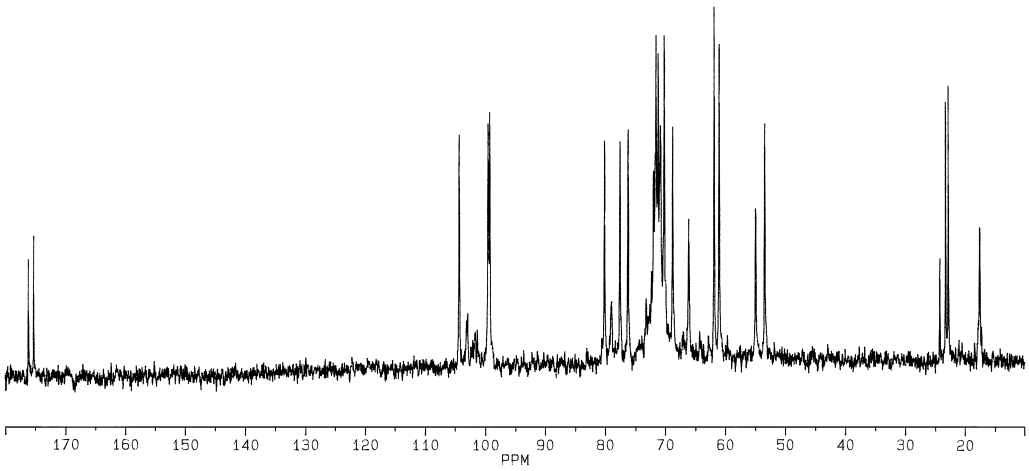


Fig. 2. ^{13}C NMR of the de-*O*-acetylated O-chain of *B. cepacia* at 30 °C.

carbons at δ 104.8, 100.0 and 99.7, two for carbons bearing nitrogen at δ 55.4 and 53.9, three for hydroxymethyl carbons of which two are unsubstituted, since occurred at δ 62.3 and 61.5, and the other is substituted in agreement with its downfield shift [16] at δ 66.6 (from DEPT experiment). In addition, signals for two acetyl carbons occurred at δ 176.6, 175.7, 23.7 and 23.3 due to the carbonyls and the methyls, respectively. The signals for the other sugar ring carbons were present in the region δ 80.5–69.2. Minor signals attributable to rhamnose units were also found.

Determination of the absolute configuration by GLC of acetylated octyl glycosides [17] showed that GalNAc, Gal and GlcNAc were the D isomers.

From the data above it can be inferred that the major polymer of the O-chain was a regular polysaccharide based on a trisaccharide repeating unit constituted of GlcNAc, GalNAc and Gal. Taking into account the large amount of rhamnose found by methanolysis of the O-chain, the minor polysaccharide component was probably built up of a rhamnan (rhamnose is a minor component of several lipopolysaccharides from *P. cepacia* [18,19]).

The results of the methylation analysis, monitored by GLC and MS of the methylated alditol acetates, indicated, for the major polymer, a linear structure built up of 4-substituted Gal*p*NAc, 3-substituted Gal*p* and 6-substituted Glc*p*NAc residues (apparent molar ratio 0.2:1:0.3). The finding of low ratios for amino sugars in the methylation analysis was already reported [20]. The complete ^{1}H and ^{13}C NMR assignment of O-chain spectra (Table 1), which was achieved by combining the data of 1D (DEPT and HOHAHA) and 2D (COSY, HSQC and HMBC) experiments, allowed confirmation of the above structural features and the gain of further structural information. The resonances at δ 4.96 and 4.75 were assigned to the anomeric protons of Glc*p*NAc and Gal*p*NAc, respectively, by HSQC correlations of their associated H-2 signals (δ 3.99 and 4.02) with the resonances at δ 55.4 and 53.9, respectively. These carbon chemical shifts suggested also the α anomeric configuration for Glc*p*NAc and β for Gal*p*NAc residues in agreement with the anomeric proton values for chemical shifts and $J_{1,2}$. The chemical shifts of anomeric carbons at δ 104.8,

Table 1
^{1}H and ^{13}C NMR data (δ in ppm), coupling constant in parentheses (Hz) for O-chain[a]

Sugar residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
→6)-α-D-Glc*p*NAc-(1→	4.96 (3.9)	3.99	3.86	3.68	4.36	4.08–3.61
	100.0	55.4	72.2	71.6	72.0	66.6
→4)-β-D-Gal*p*NAc-(1→	4.75 (8.4)	4.02	3.86	4.00	3.76	≈3.75
	104.8	53.9	71.3	78.0	76.6	61.5[b]
→3)-α-D-Gal*p*-(1→	4.98 (3.8)	3.90	3.99	4.22	3.58	≈3.74
	99.7	69.2	80.5	70.6	72.4	62.3[b]

[a] Chemical shifts for *N*-acetyl groups are δ 2.08 and 2.04 for methyl protons, and δ 23.7, 23.3 and 176.6, 175.7 for methyl and carbonyl carbons, respectively.
[b] Interchangeable values.

100.0 and 99.7 gave further support to the anomeric configuration assignments β, α, α for Gal*p*NAc, Glc*p*NAc and Gal*p*, respectively, and indicated also the pyranose ring size for all of the residues. Downfield displacement of the signals for C-3 of Gal*p* to δ 80.5, for C-4 of Gal*p*NAc to δ 78.0 and for C-6 of Glc*p*NAc to δ 66.6 as compared with their positions in the spectra of the corresponding unsubstituted monosaccharides [16] confirmed the substitution pattern of O-chain defined by methylation analysis.

To determine the sequence of residues, a HMBC experiment was carried out. The three-bond inter-residue connectivities of the anomeric protons (Table 2) indicated that Gal*p*NAc unit was linked to the Gal*p* and that to the Glc*p*NAc unit. The intra-residue correlations gave further support to the assignment of the anomeric signal at δ 4.75 to the Gal*p*NAc unit and gave the C-3 assignments of Gal*p*NAc and Glc*p*NAc, which could not be assigned by HSQC correlations since the associate H-3 signals occurred at the same chemical shifts. In the same way the C-5 signal of Gal*p* was assigned. Further support for the sequence determined by HMBC was obtained from ROESY spectra of the O-chain (Table 3). Inter-residue NOE contacts were measured between the anomeric proton of each glycosyl residue (column 1) and the protons of the attachment points of glycosylated sugars (column 2). Intra-residue NOE contacts (column 3) confirmed the anomeric configuration assignments for all of the residues. The monosaccharide sequence of repeating unit was chemically confirmed by Smith degradation [21] of the O-chain. Bio-Gel P-2 chromatography of the crude reaction product gave three main peaks A–C in order of the increasing elution time.

Fraction C was the expected diglycosyl-alditol **1**. Its structure was supported by NMR data, chemical analysis and FABMS. The ^{1}H NMR spectrum (Fig. 3) showed two anomeric doublets at δ 4.94 (3.9 Hz) and 4.65 (8.4 Hz) due to α-D-Gal*p* and β-D-Gal*p*NAc units, respectively. In addition only one *N*-acetyl methyl signal at δ 2.06 occurred. The ^{13}C NMR spectrum showed 17 signals of which two for anomeric carbons at δ 103.7 (Gal*p*NAc) and 99.1 (Gal*p*), eight for oxygen-bearing methine carbons (δ 79.7 (C-3 Gal*p*), 76.4, 71.3, 71.0, 70.9, 69.6, 68.2 and 67.9), four for hydroxymethyl carbons (from DEPT) (δ 69.1, 63.0, 61.6 and 61.5), one for nitrogen-bearing methine carbon at δ 53.1 (C-2 Gal*p*NAc) and two for the carbons of the *N*-acetyl group at δ 175.6 and 22.8.

β-D-Gal*p*NAc(1→3)-α-D-Gal*p*(1→1)glycerol

1

Methanolysis of **1** gave, as TMS ether derivatives, a hexose composition of Gal and GalNAc in the 1:0.9 ratio and the methylation analysis gave a 1:0.5 ratio of 3-linked Gal*p* and terminal Gal*p*NAc.

Table 2
Heteronuclear inter-residue and intra-residue correlations for anomeric protons in 2D HMBC spectrum of O-chain

		Inter-residue correlations			Intra-residue correlations	
Residue	δ_{H-1}	δ_C	Atom	Residue	δ_C	Atom
→6)-α-D-Glc*p*NAc-(1→	4.96	78.0	C-4	Gal*p*NAc	≈72.1	C-3 and/or C-5
→4)-β-D-Gal*p*NAc-(1→	4.75	80.5	C-3	Gal*p*	53.9	C-2
					71.3	C-3
→3)-α-D-Gal*p*-(1→	4.98	66.6	C-6	Glc*p*NAc	80.5	C-3
					72.4	C-5

Table 3
ROESY data for O-chain

		Inter-residue NOE contacts			Intra-residue NOE contacts	
Residue	δ_{H-1}	δ_H	Atom	Residue	δ_H	Atom
→6)-α-D-Glc*p*NAc-(1→	4.96	4.00	H-4	Gal*p*NAc	3.99	H-2
		3.76	H-5	Gal*p*NAc		
→4)-β-D-Gal*p*NAc-(1→	4.75	3.99	H-3	Gal*p*	3.76	H-5
					3.86	H-3
→3)-α-D-Gal*p*-(1→	4.98	3.62, 4.07	H-6,6′	Glc*p*NAc	3.90	H-2

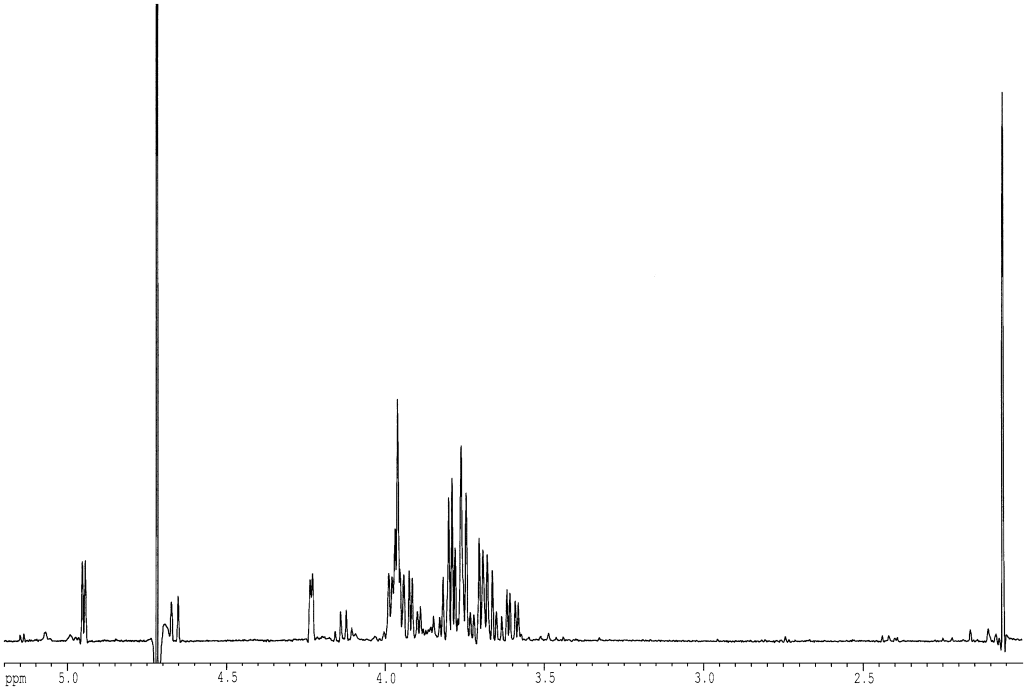
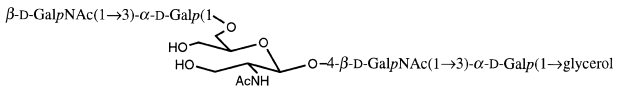


Fig. 3. ^{1}H NMR of fraction C (compound **1**) at 30 °C.

In addition, glycerol was found from methanolysis but its relative amount was not evaluated. The FAB mass spectrum (run in the positive mode) of the permethylated **1** derivative showed a pseudomolecular ion peak at *m/z* 584 $[M+H]^+$ and, *inter alia*, fragments ions at *m/z* 464 $[HexNAc\text{-}Hex]^+$ and 260 $[HexNAc]^+$ in agreement with structure **1**. The less retained peak B gave a ^{1}H NMR spectrum (Fig. 4) containing five anomeric doublets at δ 4.93 (3.5 Hz), 4.92 (3.5 Hz), 4.68 (8.4 Hz), 4.63 (8.4 Hz) and at δ 5.06 (2.3 Hz) and *N*-acetyl methyl signals at δ 2.09 and 2.06 in a 1:2 ratio. The first two were HSQC correlated to the anomeric carbon signal at δ 103.6 (two residues of Gal*p*NAc), the second two were correlated to the signal at δ 99.2 (two residues of Gal*p*) and the last one was connected to the resonance at δ 102.5. This clearly belonged to the bridge fragment between two β-D-Gal*p*NAc-(1→3)-α-D-Gal*p* disaccharide units. The bridge fragment must arise from the Glc*p*NAc residue. Actually in the ^{13}C NMR spectrum occurred three nitrogen-bearing carbons two at δ 53.4 and 53.1, due to the two Gal*p*NAc units, the other at δ 54.0, attributable to the Glc*p*NAc fragment. Methanolysis of this fraction gave, as TMS ether derivatives, a hexose composition of Gal and GalNAc in the 1:0.8 ratio and methylation analysis gave a 1:0.2:0.3 ratios of 3-linked Gal*p*, terminal Gal*p*-NAc and 4-linked Gal*p*NAc. In the methanolysis glycerol was found but its relative amount was not evaluated. All the data above suggested a structure **2** for major component of B fraction. Conclusive evidence for structure **2** was obtained from FABMS spectrum of its permethylated derivative. A pseudomolecular ion peak at *m/z* 1280 $[M+H]^+$ and the fragments ions depicted in Fig. 5 were measured. The ^{1}H NMR spectrum of excluded peak A appeared to be close similar to that of peak B. Therefore a higher polymeric structure of **2** could be suggested for peak A. The incomplete acid hydrolysis of Glc*p*NAc residues can be explained by the known difficulty in hydrolysing glycosidic linkages of 2-deoxy-2-acetamido sugars and by the mild acid condition required for the Smith degradation. On the basis of the data above it was concluded that the O-chain has structure **3**:



2

→4)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→6)-α-D-Glc*p*NAc-(1→

3

This structure, in our knowledge, is a novel one both for *Burkholderia* [14,18,22–28] species and for O-antigens in general [19].

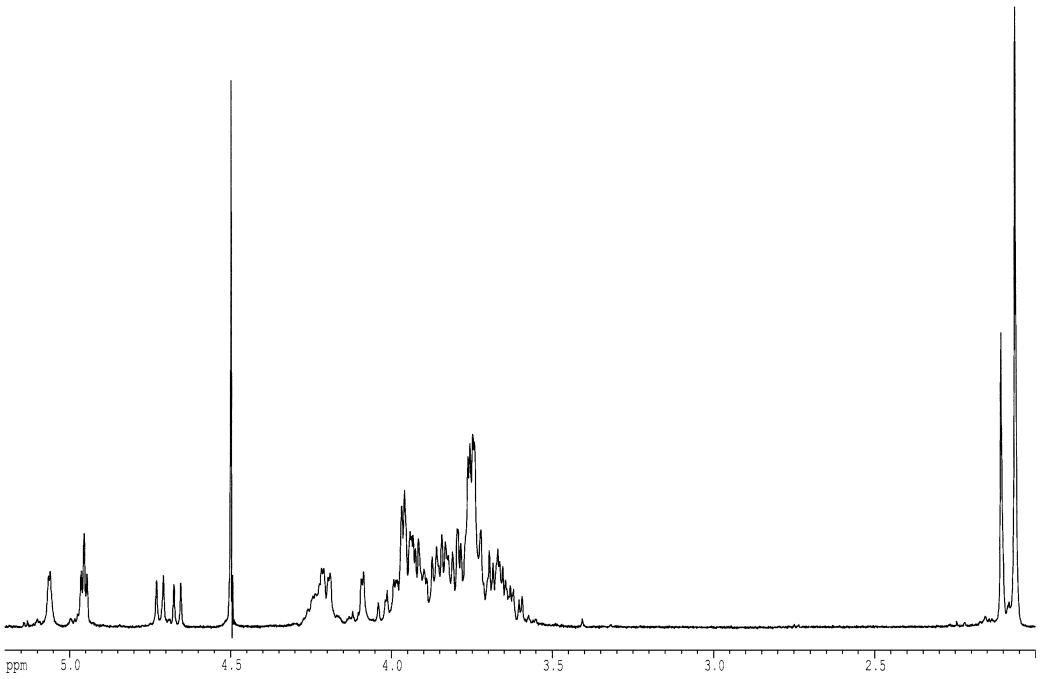


Fig. 4. ^{1}H NMR of fraction B (compound **2**) at 50 °C.

3. Experimental

General.—Mass spectra were recorded, in positive mode, with a VG ZAB HF instrument equipped with a FAB source. TLC was carried out on Silica Gel F_{254} (Merck). Total carbohydrates were determined by the phenol-H_2SO_4 test [29]. All compounds were revealed by spraying plates with a saturated solution of chromic oxide 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-ionization detector. TMS ethers of methyl glycosides were analysed by GLC on a 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: 150° for 8 min, 150°→200° at 2°/min, 200°→260° at 6°/min, 260° for 15 min.

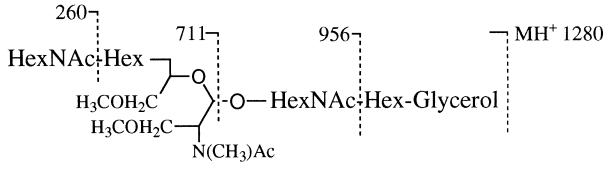


Fig. 5. FABMS fragmentations of permethylated derivative of **2**.

The quantitative evaluation was made using mannitol, as internal standard, and appropriate response factors.

Partially methylated alditol acetates were analysed by GLC–MS on a Hewlett–Packard 5890 instrument, in the following conditions: SPB-1 capillary column (Supelco, 30 m×0.25 mm i.d., flow rate 0.8 mL/min, He as carrier gas), with the temperature programme: 100° for 2 min, 100°→240° at 2°/min. The GC quantitative evaluation was made on the same column.

Bacterial strain and condition of growth.—*B. cepacia* strain PVFi-5A from the collection of the Istituto di Patologia e Zoologia Forestale e Agraria, Firenze, Italy was grown in 1 L Erlenmeyer flasks containing 400 mL of Wolley's medium [30] supplemented with 1.5% (w/v) peptone at 27 °C with shaking (100 rpm) for 5 days. The culture (5.2 L) was centrifuged (10,000 g×15 min) and harvested cells were washed three times with 85% (w/v) NaCl and lyophilized.

Preparation of cellular lipopolysaccharide fraction.—The dry cells (1.7 g) were suspended in 100 mL of ultrapure Milli-Q water and extracted with phenol according to the conventional procedure [15]. The resulting phenol phase was diluted to 400 mL with ultrapure Milli-Q water and resulting solution dialysed (cut-off 3500 Da) for 2 days. Contents of tubes were lyophilized; the residue

was dissolved in ultrapure Milli-Q water (15 mL) and brought to 1.5% (w/v) NaCl and 1.5% (w/v) Cetavlon (hexadecyltrimethylammonium bromide) while stirring at room temperature for 15 min. The mixture was centrifuged (5000 *g* for 45 min) and the supernatant solution was mixed with 10 volumes of cold (−20 °C) EtOH and left overnight at −20 °C. The resulting precipitate was collected by centrifugation (5000 *g* at 5 °C for 1 h), dissolved in ultrapure Milli-Q water (10 mL) and dialysed as described above. The content was lyophilized to yield "crude" LPS (133 mg). The aqueous phase from the initial extraction was dialysed as above described. The contents were lyophilized and the residue was treated as the crude extract of the corresponding phenol phase to give in a lower yield the same (by ^{1}H NMR) "crude" LPS (5 mg).

Purification of LPS.—The crude LPS (130 mg) was purified by chromatography on Bio-Gel A-15 m (Bio-Rad). The column (47×2.5 cm) was equilibrated and eluted with 300 mM TEA (triethylamine) neutralised to pH 7 with HCl. The sample was dissolved in 300 mM TEA and 10 mM EDTA, adjusted to pH 7 with HCl prior to application to the column. Fractions (2.5 mL) were collected and tested for sugar by phenol assay and for proteins by absorption at 280 nm. On the basis of the chromatographic profile the eluted fractions were pooled as one fraction (120 mg).

NMR spectroscopy.—All spectra were recorded on a Bruker DRX400 Avance spectrometer using a 5 mm multinuclear inverse Z-grad probe with standard Bruker pulse sequences. Spectra were recorded at 30, or 50 or 70 °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. 2D homuclear shift correlation (COSY) data were collected in phase sensitive mode using the TPPI method and using gradient pulses for selection with multiple quantum filter. Typically, data sets of 2048 (t_2)×512 (t_1) complex points were collected with 16 scans per FID, and a sweep width in both dimensions of 2 ppm. COSY spectra were processed with shifted sine-bell in both dimensions. 1D homonuclear Hartman-Hann correlation HOHAHA [31] were recorded using MLEV17 sequence for mixing selective excitation with a shaped pulse z-filter. Mixing times of 30, 50 and 100 ms were used. DEPT spectra were recorded with a 135° read pulse for polarisation transfer with decoupling during acquisition. Delays were optimised for a coupling constant of 160 Hz. A gradient heteronuclear single quantum coherence (HSQC) [32] data set was collected in phase sensitive using echo-antiecho gradient selection with decoupling during acquisition. Typically, data sets of 1024×256 complex points was acquired with 64 scans. The sweep width was 2 ppm for proton and 180 ppm for carbon. 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. The heteronuclear multiple bond correlation (HMBC) [33] spectrum was recorded with low-pass J-filter to suppress one-bond correlation, no decoupling during acquisition and using gradient pulses for selection. A delay of 60 ms was used for evolution of long-range. The spectrum was processed with a shifted sine-bell in both dimensions. 2D Rotating-frame nuclear Overhauser enhancement spectra (ROESY) [34] were recorded with cw spinlock for mixing, in phase sensitive mode using TPPI. Mixing times of 80 and 140 ms were used.

O-Deacylation of LPS.—The LPS fraction (80 mg) was treated overnight with 10 mM KOH (80 mL) [35] at room temperature with stirring. The crude reaction product (LPS–OH) was neutralised on a Dowex 50W X8 (H^+ form) column and then lyophilised (80 mg). In order to estimate the content of acetate an aliquot of the neutralised crude reaction was assayed in triplicates with the Boehringer UV-test.

Acid hydrolysis of LPS–OH.—The LPS–OH fraction (80 mg) was treated with 1 M AcOH [36] in sealed tube for 3 h at 110 °C. After cooling the sample was centrifuged at 4000 rpm for 20 min. The content of the supernatant liquid (70 mg) was applied to a Bio-Gel P-100 (Bio-Rad) column and eluted with 50 mM NaOAc buffer (pH 5.2). Fractions (1.5 mL) were collected, tested for sugars by the phenol assay and pooled in three fractions, of which one in the void volume (LPS–OH unreacted, 10 mg), the second eluted shortly after the void volume (O-chain, 40 mg) and the third was more retained (core sugars 12 mg).

Glycosyl analysis.—A dry sample (1 mg) was treated with 1 M HCl–MeOH for 20 h at 80 °C. After neutralisation with Ag_2CO_3, the mixture was kept overnight at room temperature with Ac_2O (50 μL) with stirring. After centrifugation, the supernatant liquid was evaporated with N_2 stream, dried on P_2O_5 and submitted to GLC analysis as Me_3Si ethers.

Determination of absolute configuration.—A sample of LPS–OH (1 mg) was hydrolysed with a mixture of 2 M H_2SO_4 (0.2 mL)/AcOH (1.8 mL) for 9 h at 100 °C [37]. After neutralisation the crude reaction was treated with (+)-2-octanol (0.5 mL) and a drop of TFA overnight at 130 °C with stirring [16]. After usual work-up, the crude reaction was acetylated with Ac_2O and pyridine. The GLC analysis was performed on a SP-2330 capillary column (30 m×0.25 mm i.d.), column conditions: 240 °C for 50 min.

Glycosyl linkage analysis.—To a solution of the sample (1 mg) in Me_2SO (1 mL) were added 6.6 M KCH_2SOMe (100 μL) and MeI (300 μL), according to the Hakomori modified procedure [38]. The methylated product was purified on Sep-pak C-18 and submitted to acid hydrolysis by H_2SO_4/AcOH at 100 °C for 9 h. The crude reaction mixture was reduced with $NaBD_4$, acetylated and analysed by GLC-MS.

Smith degradation.—The LPS–OH (20 mg) was treated with 0.1 M $NaIO_4$ (5 mL) at 6 °C for 96 h in the dark with stirring. The reaction was quenched with ethylene glycol and the crude mixture was reduced at room temperature overnight by addition of $NaBH_4$. The mixture was neutralised with AcOH, dialysed (cut-off 3500 Da) and lyophilised. The sample was then treated with 0.5 M TFA (2 mL) at room temperature for 72 h. After TFA evaporation the crude mixture was purified on a Bio-Gel P-2 (Bio-Rad, 96×1.5 cm) column, using ultrapure Milli-Q water as eluent. Fractions were pooled in three main fraction A (4 mg), B (5 mg) and C (5 mg), on the basis of TLC analysis.

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