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Structural analysis of the O-antigen of the lipopolysaccharide of *Rhizobium tropici* CIAT899

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Abstract

The structure of the O-antigen chain of the lipopolysaccharide isolated from *Rhizobium tropici* CIAT899, by the phenol–water procedure, and recovered from the phenol layer, has been investigated by hydrolysis, methylation analysis and 1D and 2D ^{1}H and ^{13}C NMR spectroscopy of the complete polysaccharide and of oligosaccharides obtained by partial hydrolysis. The O-antigen has the repeating unit

$$\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow 3)\text{-6-deoxy-}\alpha\text{-D-Tal}p\text{-}(1 \rightarrow 3)\text{-}\alpha\text{-L-Fuc}p\text{-}(1 \rightarrow$$
$$\begin{matrix} 2 \\ \uparrow \\ \text{OAc} \end{matrix}$$

Keywords: O-Antigen; Lipopolysaccharide; *Rhizobium tropici* CIAT899

1. Introduction

Rhizobia are Gram-negative bacteria which are able to form nitrogen-fixing symbiotic relationships with leguminous plants. As Gram-negative bacteria, they present surface lipopolysaccharides, which have been hypothesized to be involved in the molecular mechanism of symbiotic infections. Mutants having lipopolysaccharides that lack their

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O-antigenic polysaccharide, or have modified core components, are either defective in the formation of infection threads or have the nodules unoccupied [1–6].

The complete chemical structures of several core oligosaccharides of lipopolysaccharides from Rhizobiaceae have been described [7,8] but no complete structure of the O-antigen component of any of the fast-growing strains has been proposed except for *Rhizobium trifolii* 4S [9]. We now report on the structure of the O-antigen chain of the lipopolysaccharide isolated from *Rhizobium tropici* CIAT899.

2. Experimental

General methods.—Descending paper chromatography was performed on Whatman No. 3MM paper using 1-BuOH–EtOH–H_2O (2:1:1) as eluent. GLC–MS was performed with a Kratos MS80RFA instrument fitted with a CP-Sil5 CB WCOT column (25 m × 0.32 mm i.d.). The temperature programme for the trimethylsilylated methyl glycosides was isothermal at 140°C for 2 min followed by an 8°C/min gradient up to 250°C, while that for the trimethylsilylated (+)-2-butyl analogues was isothermal at 130°C followed by a 2°C/min gradient up to 250°C. The protocol for the partially methylated alditol acetates was isothermal at 100°C for 1 min followed by a 5°C/min gradient up to 250°C. The ionization potential was 70 eV. Positive FABMS was performed with a neutral Xe atom beam. The sample was dissolved in water, NaI was added as cationizing agent and glycerol was used as matrix. Optical rotation was measured using a Perkin–Elmer Model 241 MC polarimeter.

Isolation and purification of O-antigen.—Bacteria were grown for 3 days at 28°C in a shaken TY medium [10]. Ten litres of bacterial culture in stationary phase were centrifuged, and the pellet was washed three times with 0.5 M NaCl to remove the exopolysaccharide. The LPS was extracted from the lyophilized bacterial cells (5 g) with 100 mL of hot phenol–water mixture [11] and two phases (phenolic and aqueous) were separated. The phenolic phase was dialysed against water, concentrated, and passed through an Amberlite IRA 400 (AcO^-) anion-exchange resin (1.6 × 30 cm) using water as eluent. The eluate was lyophilized and then dissolved in 10 mM $MgSO_4$ and 50 mM Tris–HCl solution (100 mL, pH 7.0). DNase I (1 mg) and RNase A (1 mg) were added and the solution was stirred overnight at 5°C and then lyophilized [3]. The LPS was chromatographed on Sephacryl S-500 (2.6 × 65 cm) using 0.05M EDTA–TEA (pH 7.0) as eluent and the fraction containing the carbohydrates was dialysed and lyophilized. A 0.1% solution of LPS in 1% AcOH was heated at 100°C for 90 min, and the lipid A precipitated was separated by centrifugation. The supernatant was extracted with CH_2Cl_2, and the aqueous phase containing the O-antigen and core oligosaccharides was fractionated by gel permeation chromatography on BioGel P-2 (1.6 × 60 cm) using 1% AcOH as eluent. Carbohydrate fractions were detected by a refractive index detector (Model 2162, LKB, Bromma, Sweden) and by the orcinol–sulfuric acid method [12] on TLC plates. The sugar fraction in the void volume was dialysed and lyophilized.

Sugar analysis.—Monosaccharides were determined as their trimethylsilylated methyl glycosides. The polysaccharide was treated with 0.625 M methanolic HCl at 80°C for 16 h and then silylated with pyridine–BSTFA (1:1) for 16 h at 80°C. Isobutanol was added to the mixture and then dried under a stream of nitrogen.

Determination of D- *and* L-*configuration.*—The O-antigen was methanolized with 0.625 M methanolic HCl, treated with 0.625 M (+)-2-butanolic–HCl under the same conditions and then trimethylsilylated. The derivatives were analysed by GC–MS [13].

Partial hydrolysis.—A 0.1% solution of the polysaccharide in 2% AcOH was heated for 6.5 h at 100°C. The acid was neutralized with 5% NH_3 solution and the hydrolysate was chromatographed on BioGel P-2 with water as eluent. Several oligosaccharides were isolated.

Methylation.—The polysaccharide was methylated twice with potassium methylsulfinylmethanide and methyl iodide [14]. The product was further purified by reversed-phase chromatography on a Sep-Pak C_{18} cartridge [15] and hydrolysed with aqueous 88% HCOOH and 2 M CF_3COOH [16]. The products were then reduced and acetylated by using the method described by Blakeney [17].

Location of O-*acetyl groups.*—The O-antigen was methylated by using the method of Prehm [18]. The methylated polysaccharide was further methylated conventionally (see above) but using trideuteriomethyl iodide, then hydrolysed, and the products were reduced, acetylated and subjected to GC–MS [19].

NMR spectroscopy.—Samples were deuterium exchanged several times by freeze-drying from D_2O and then examined as solutions in 99.98% D_2O. The spectra were recorded at 25°C with a Varian Unity 500 instrument at 500 MHz. Chemical shifts are reported in ppm, using the HDO signal (δ_H 4.76 ppm) and external dioxane (δ_C 67.4 ppm) as references.

The double-quantum filtered DQF-COSY experiment [20] was performed in the phase-sensitive mode using the Varian standard pulse sequence. A data matrix of 256 × 2K points was used to digitize a spectral width of 4500 Hz; 16 scans were used per increment with a relaxation delay of 2 s. Prior to Fourier transformation, zero-filling was used in F_1 to expand the data to 2K × 2K. The 2D-TOCSY (HOHAHA) experiment [21] was carried out in the phase-sensitive mode using MLEV-17 for isotropic mixing. The mixing time was 165 ms. The spectral width, relaxation delay and number of scans were the same as the DQF-COSY experiment. Squared cosine-bell functions were applied in both dimensions and zero-filling was used to expand the data to 2K × 2K. The one-bond proton–carbon correlation experiment [22] was collected in the ^{1}H-detection mode using the HMQC pulse sequence and a reverse probe. A data matrix of 256 × 2K points was used to resolve a spectral width of 4500 and 25 000 Hz in F_2 and F_1; 16 scans were used per increment with a relaxation delay of 2 s and a delay corresponding to a J value of 145–150 Hz. A BIRD pulse was used to minimize the proton signals bonded to ^{12}C. Experiments, both with and without ^{13}C coupling were performed. ^{13}C decoupling was achieved by the WALTZ scheme. Squared cosine-bell functions were applied in both dimensions and zero-filling was used to expand the data to a 2K × 2K matrix. The long-range proton–carbon correlations experiment (HMBC) [23] was collected in the ^{1}H-detection mode. A delay time of 80 ms between the first and second pulses and 96 scans per increment were used. The data were processed as described for HMQC. The pure absorption 2D NOESY experiments were carried out with mixing times of 500 ms. Similar acquisition and processing data as for DQF-COSY and TOCSY were used. The 2D rotating frame NOE (CAMELSPIN, ROESY) experiment [24] was recorded in the phase-sensitive mode. The spin-lock period consisted of a

train of 30° pulses (2.5 μs), separated by delays of 50 μs. The total mixing time was 250 ms. The r.f. carrier was set as δ 6.0 ppm in order to minimize spurious Hartmann–Hahn effects. A data matrix of 256 × 2K points was used to resolve a spectral width of 4800 Hz; 32 scans were used per increment. Squared sine-bell functions shifted by $\pi/3$ were applied in both dimensions and zero-filling was used to expand the data to 2K × 2K.

3. Results and discussion

Monosaccharide analysis.—Lipopolysaccharide was extracted from bacterial cells by the phenol–water method and, after hydrolysis, the O-antigen and the core oligosaccharides were fractionated by gel permeation chromatography. Monosaccharide analysis of the void-volume fraction gave fucose, glucose and an unknown sugar in molar ratio 1:1:1. The unknown sugar was isolated by hydrolysis with 4 M CF_3COOH at 100°C for 4 h, and preparative paper chromatography, and examined by ^1H and ^{13}C NMR spectroscopy. The analysis of the ^1H NMR and HMQC spectra showed the presence of an equilibrium among the α- and β-pyranose and furanose forms of a deoxysugar [25]. Analysis of the proton and carbon chemical shifts concluded that the unknown sugar was 6-deoxytalose [26]. Analysis of the trimethylsilylated (+)-2-butyl glycosides derivatives by GLC showed that the glucose had the D-configuration whereas fucose had the L-configuration. The $[\alpha]^{20}$ +17.5° (0.2, H_2O) value of the 6-deoxytalose indicated D-configuration. Conventional methylation analysis of the O-antigen revealed the presence of 4-linked D-glucose, 3-linked L-fucose and 3-linked 6-deoxy-D-talose (Table 1) in a ca. 1:1:1 molar ratio. However, conditions which retain *O*-acyl groups [18,19] gave a different result for the deoxytalose residue. The mass spectrum (*m/z* 59, 72, 89, 99, 104, 120, 131, 176, 201 and 236) corresponds to 1,3,5-tri-*O*-acetyl-6-deoxy-4-*O*-methyl-2-*O*-trideuteriomethyl-hexitol, and the absence of the peaks at *m/z* 117 and 233 identified the presence of an acyl substituent at O-2 of all the 3-linked-6-deoxy-D-talose residues of the polysaccharide.

Table 1
Methylation analysis data for the O-antigen (O-A) and the trisaccharide **O-1**

Methylated sugars (as alditol acetates)	T [a]	Molar ratio [b]		
		O-A	**O-1**	**O-1 Prer.**
2,3,4,6-Me_4-Glc [c]	1.00	–	1.0	1.0
2,3,6-Me_3-Glc	1.17	1.3	–	–
2,4-Me_2-6-deoxy-Tal	0.89	1.0	1.0	1.0
2,4-Me_2-Fuc	0.96	1.3	0.5	–
2,5-Me_2-Fuc	0.94	–	0.8	–

[a] Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on a WCOT column of CP-SIL5 CB.
[b] Key: **O-A**, O-antigen; **O-1**, trisaccharide O-1; **O-1 Prer.**, pre-reduced trisaccharide O-1.
[c] 2,3,4,6-Me_4-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.

Table 2
^{1}H NMR chemical shifts (δ, ppm) and coupling constants (J, Hz) for trisaccharide O-2

Unit	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
A	5.21	3.87	3.89	3.87	4.21	1.19	–
B	5.13	3.35	4.40	3.95	4.21	1.26	–
C	5.12	5.35	4.40	3.95	4.21	1.26	–
D	4.60	3.31	3.49	3.41	3.45	3.73	3.90
E	4.59	3.54	3.69	3.80	3.81	1.23	–
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$	$J_{5,6b}$	Other
A	3.7	10.0	n.d.	n.d.	6.5		
B	ca. 1	3.8	3.7	ca. 1	6.7		
C	ca. 1	3.8	3.7	ca. 1	6.7		
D	8.1	9.4	8.9	9.7	5.4	2.4	$J_{6a,b} = -12.6$
E	7.7	9.9	3.5	ca. 1	6.4		

Oligosaccharide identification.—Several oligosaccharides were detected by FABMS and three of them were isolated from the partial hydrolysis: O-1, with molecular mass 472 corresponding to one hexose and two deoxyhexose moieties, O-2, with a molecular mass 514 corresponding to one hexose, two deoxyhexoses and one acetate group, and O-3 with molecular mass 926 corresponding to two hexoses and four deoxyhexose residues.

Both the native and pre-reduced oligosaccharide O-1 were subjected to methylation analysis. The results (Table 1) demonstrated that the non-reducing sugar was glucose and that the reducing sugar was fucose.

The chemical shifts for the ^{1}H and ^{13}C resonances of the trisaccharide O-2 (Tables 2 and 3) were assigned from COSY, TOCSY and HMQC experiments. The five anomeric protons observed in the 1D ^{1}H NMR spectrum (Fig. 1a) were labelled A–E from low to high field. From δ and ^3J values, residue A was identified as α-fucopyranose; residues B and C were identified as 6-deoxytalopyranose having an α-configuration; two signals appear for this anomeric proton due to the anomerization equilibrium at the reducing end. Residue D was identified as β-glucopyranose and residue E was β-fucopyranose. The signals at 4.59 and 4.60 ppm with coupling constants > 7 Hz were assigned to the β-anomers of glucose and fucose. The two signals at ca. 5.13 ppm, with coupling constant 1 Hz were assigned to the α-anomer of 6-deoxytalose, affected, as mentioned above, by the anomerization equilibrium at the reducing end. The doublet at 5.21 ppm

Table 3
^{13}C NMR chemical shifts (δ, ppm) for trisaccharide O-2

Unit	C-1	C-2	C-3	C-4	C-5	C-6
A	93.2	68.0	78.6	72.6	67.0	
B	103.9	68.0	72.3	70.8	68.1	
C	101.1	68.0	72.3	70.8	68.1	
D	101.3	73.7	76.2	70.4	76.7	61.4
E	97.0	71.6	81.9	72.0	71.4	

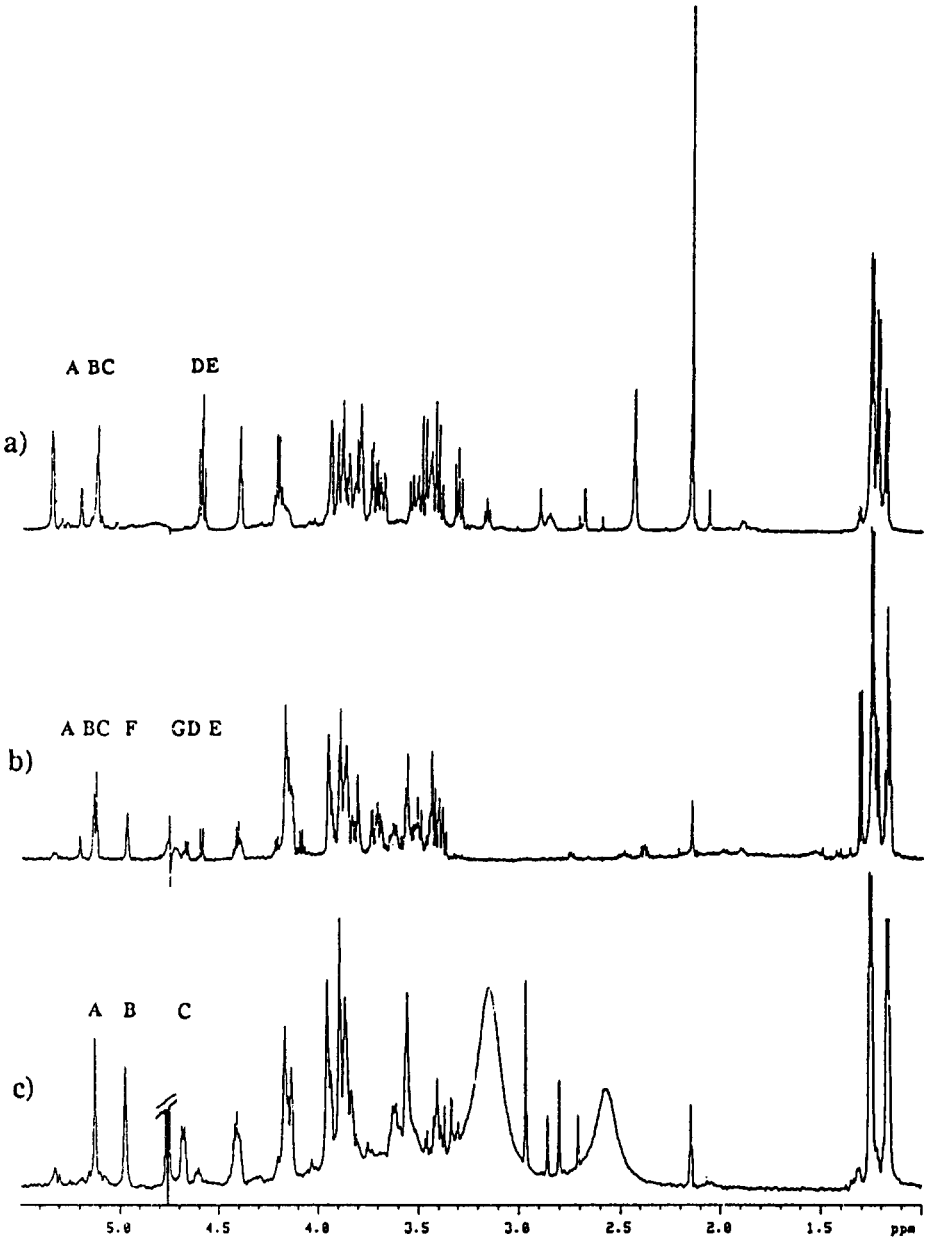


Fig. 1. 1D ^{1}H NMR spectra of (a) trisaccharide O-2, (b) hexasaccharide O-3, and (c) O-antigen polysaccharide. Anomeric protons are labelled A–E, A–G and A–C, respectively.

($J = 3.7$ Hz) was assigned to the α-anomer of fucose. Three doublets at ca. 1.25 ppm ($J = 6.5$ Hz) were assigned to the methyl groups of the 6-deoxy sugars, while the singlet at 2.15 ppm was identified as an acetate signal. The signal at 5.35 ppm was assigned to

the H-2 proton of 6-deoxytalose shifted downfield by the acetyl group at position 2. This assignment was confirmed by an HMQC spectrum (corresponding carbon atom at 68 ppm). Comparison of the observed ^{13}C chemical shift values with those previously

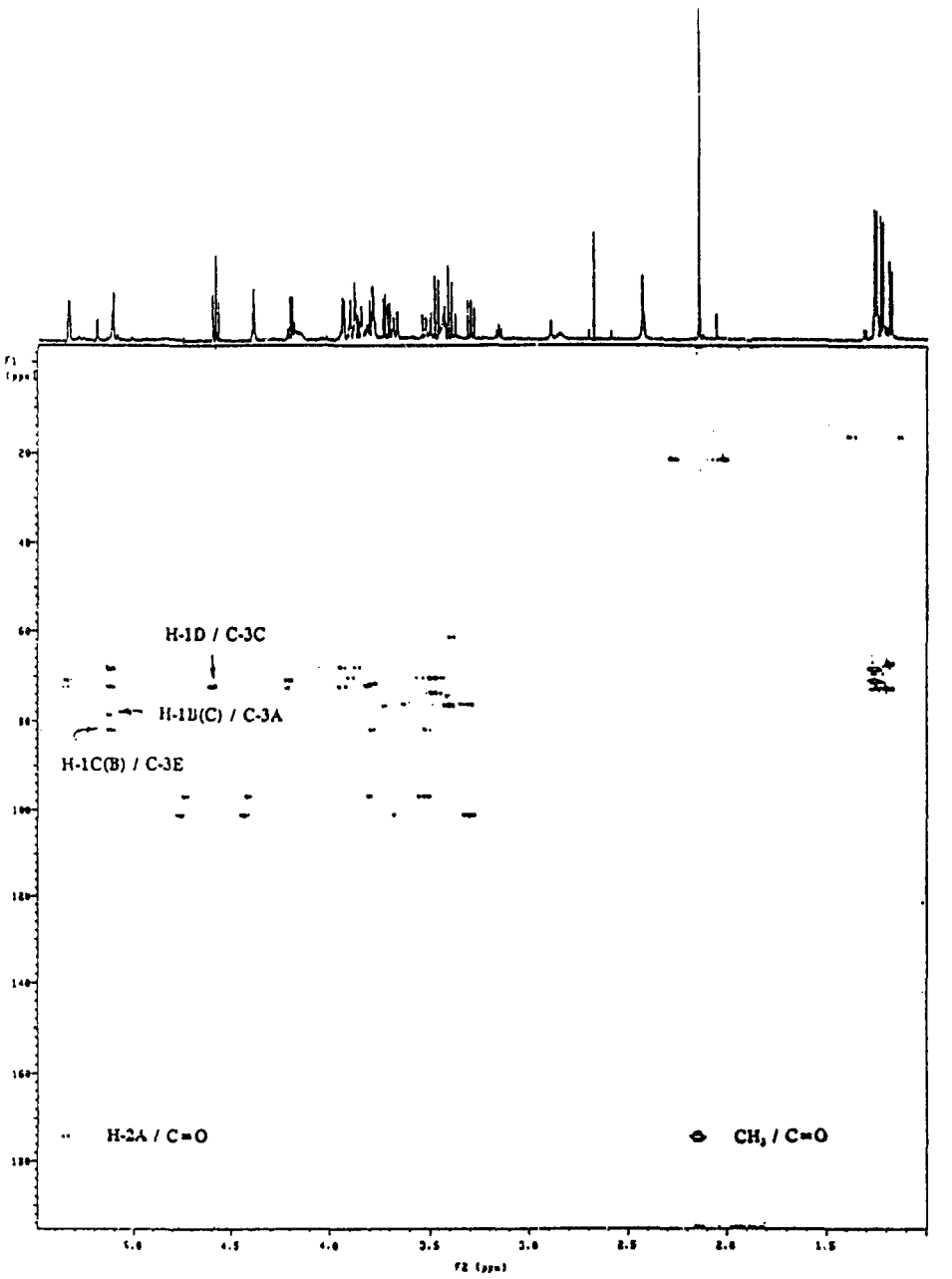


Fig. 2. Long-range correlated spectrum HMBC of the trisaccharide O-2. Significant cross-peaks are labelled.

reported for the corresponding residues [26,27] showed that C-3 of the fucose and 6-deoxytalose rings were shifted downfield. Therefore, at this point, it is possible to conclude that the glucopyranose moiety is the non-reducing end, while fucose is the reducing end. Nevertheless, the sequence was confirmed by the use of ROESY and HMBC experiments. The ROESY spectrum showed cross-peaks between H-1 of D and H-2 of B(C), H-1 of D and H-3 of B(C), H-1 of B and H-3 of A, and H-1 of C and H-3 of E. These correlations suggest that D is linked to position 3 of B(C), B is linked to position 3 of A and C is linked to position 3 of E. In addition, the HMBC map (Fig. 2) showed cross-peaks between H-1 of B(C) and C-3 of A, H-1 of B(C) and C-3 of E, H-1 of D and C-3 of C and the C=O and H-2 of B(C).

Therefore, the conclusions of the NMR experiments are in agreement with the analytical results and indicate the following structure for the acetylated trisaccharide O-2:

$$\begin{array}{llll} \beta\text{-D-Glc}p\text{-}(1 \rightarrow 3)\text{-6-deoxy-}\alpha\text{-D-Tal}p\text{-}(1 \rightarrow 3)\text{-L-Fuc}p \\ \mathbf{D} & \mathbf{B/C} & \begin{matrix}2\\ \uparrow\\ \text{OAc}\end{matrix} & \mathbf{A/E} \end{array}$$

Therefore, trisaccharide O-1 corresponds to deacylated O-2. The ^{1}H NMR spectrum of the hexasaccharide O-3 (Fig. 1b) showed seven anomeric signals, four of them being very similar to those of the previously analysed trisaccharide. The anomeric signals were labelled A–G. The chemical shifts values for the anomeric signals of the residues A, B, C and G were the same as those of the trisaccharide. The signals at 5.35 and 2.15 ppm were not observed, indicating that the hexasaccharide was not acetylated at position 2 of the 6-deoxytalose. A new anomeric resonance, F, at 4.97 ppm ($J = 2.5$ Hz) was assigned to a α-fucopyranose residue, while the two signals at 4.67 and 4.69 ppm (D and G, respectively) having $J > 7$ Hz, were assigned to β-glucopyranose moieties.

Table 4
^{1}H NMR chemical shifts (δ, ppm) and coupling constants (J, Hz) for hexasaccharide O-3

Unit	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
A	5.21	3.90	3.90	3.88	4.22	1.19	–
B	5.14	4.15	4.18	3.96	4.16	1.26	–
C	5.13	4.15	4.18	3.96	4.16	1.26	–
D	4.67	3.42	3.50	3.44	3.45	3.73	3.89
E	4.60	3.53	3.69	3.80	3.82	1.23	–
F	4.97	3.88	3.90	3.88	4.42	1.17	–
G	4.69	3.40	3.57	3.56	3.64	3.85	3.95
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$	$J_{5,6b}$	
A	3.9	n.d.	n.d.	~1	6.6		
B	ca. 1	n.d	n.d	~1	6.6		
C	ca. 1	n.d	n.d	~1	6.6		
D	7.8				n.d	n.d	
E	7.8	n.d	n.d	~1	6.6		
F	2.5			~1	6.6		
G	7.4	n.d	n.d	n.d	n.d	n.d	

Table 5
^{13}C NMR chemical shifts (δ, ppm) for the hexasaccharide O-3

Unit	C-1	C-2	C-3	C-4	C-5	C-6
A	92.8	68.2	79.0	(73.2)	67.6	16.8
B	104.3	69.0	75.0	73.1	69.0	16.8
C	101.4	69.0	75.0	73.1	69.0	16.8
D	102.8	70.8	76.8	70.6	77.0	61.8
E	97.4	72.0	82.4	72.6	71.8	16.8
F	100.7	68.5	79.2	73.2	68.1	16.4
G	102.7	74.4	76.5	78.0	75.6	61.2

The ^{1}H and ^{13}C chemical shifts (Tables 4 and 5) were assigned from the 2D-TOCSY and HMQC experiments. The HMQC plot showed two different hydroxymethyl groups that were assigned to the two glucopyranose residues. The ^{1}H and ^{13}C resonances for one of them were very similar to those described above for the terminal moiety of the trisaccharide. The observed chemical shift values of residue G were different, although this moiety could be identified as a 4-substituted β-glucopyranose.

Therefore, the hexasaccharide sequence is:

$$\underset{D}{\beta\text{-D-Glc}p}\text{-}(1\rightarrow 3)\text{-6-d-}\alpha\text{-D-}\underset{C}{\text{Tal}p}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-}\underset{F}{\text{Fuc}p}\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-}\underset{G}{\text{Glc}p}\text{-}(1\rightarrow 3)$$

$$\text{-6-d-}\alpha\text{-D-}\underset{B/C}{\text{Tal}p}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-}\underset{A/E}{\text{Fuc}p}$$

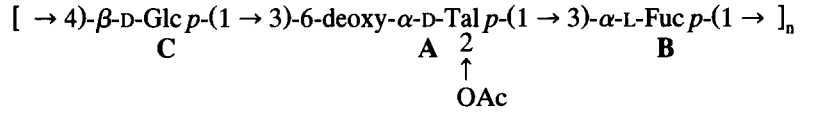
The ^{1}H NMR spectrum of the O-antigen polysaccharide (Fig. 1c) showed three anomeric signals which were labelled A, B and C from low to high field. The anomeric resonance for the residue A at 5.11 ppm and $J \approx 1$ Hz was assigned to the α-6-deoxytalose residue; the resonance at 4.96 ppm and $J \approx 2.5$ Hz (residue B) was assigned to the α-fucopyranose residue and the doublet at 4.65 ppm and $J = 7.7$ Hz (residue C) was assigned to the β-glucopyranose residue.

From the 2D experiments DQFCOSY, TOCSY and HMQC and from the comparison with the data for the tri- and hexa-saccharides, the chemical shifts for ^{1}H and ^{13}C were determined (Table 6). The 2D-NOESY spectra showed cross-peaks between H-1B and H-4C (H-3C), H-1A and H-2B (H-3B), and H-1C and H-2A (H-3A), which are expected

Table 6
^{1}H and ^{13}C NMR chemical shifts (δ, ppm) for the O-antigen polysaccharide of *Rhizobium tropici* CIAT899

Unit	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
A	5.11	4.15	4.17	3.95	4.16	1.25	
B	4.96	3.88	3.89	3.87	4.41	1.26	
C	4.67	3.41	3.56	3.56	3.63	3.84	3.94
	C-1	C-2	C-3	C-4	C-5	C-6	
A	104.8	69.4	75.4	73.5	69.3	17.0	
B	101.2	68.8	79.6	73.6	68.6	16.5	
C	103.2	75.3	77.0	78.5	76.0	61.5	

for the sequences determined above. Therefore, on the basis of the above chemical and spectroscopic data, the O-antigen of the LPS isolated in the phenolic phase from *Rhizobium tropici* CIAT899 has the following trisaccharide repeating unit:



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