Structural Determination of the O-Specific Chain of the Lipopolysaccharide from *Pseudomonas cichorii*

Jesus Jimenez-Barbero,[a] Cristina de Castro,*[b] Antonio Evidente,[c][‡] Antonio Molinaro,[b] Michelangelo Parrilli,[b] and Giuseppe Surico[d][‡‡]

Dedicated to the memory of Professor Guido Sodano

Keywords: Molecular mechanics / Natural products / NMR spectroscopy / O-chain / Structure elucidation

The complete structure of the O-specific polysaccharide isolated from the phytopathogenic bacterium *Pseudomonas cichorii* strain 5707 has been determined by spectroscopic, computational, and chemical techniques. It consists of a linear tetrasaccharide repeating unit as represented in the formula 3)-α-L-Fuc*p*2NAc-(1→3)-α-D-Qui*p*2NAc-(1→3)-α-L-Fuc*p*2NAc-(1→2)-β-D-Qui*p*3NAc-(1→

Introduction

Pseudomonas cichorii is a Gram-negative bacterium that causes a disease known as "varnish spot" on lettuce. *P. cichorii* was originally isolated from chicory and subsequently reported from many other hosts such as cabbage, tomato, celery, tobacco, coffee and several ornamental plants, grown in open-field or in greenhouse. On lettuce the disease is characterised by large necrotic brown-blackish areas that occur on the blades and petioles of leaves. With frequent rain or high relative humidity, the leaf spots turn into large soft rots.

Lipopolysaccharides (LPSs) are thought to be involved in various processes during interactions between plants and bacteria, as demonstrated by different experiments. Treatment of pepper (*Capsicum annuum*) leaves[1] with LPS either from plant pathogenic bacteria or from enteric bacteria, for instance, alters several aspects of the plant response to subsequent inoculation with phytopathogenic xanthomonads. As a matter of fact, LPS pretreatment prevents hypersensitive reaction and induces early synthesis of potentially antimicrobial feruloyl− and coumaroyl−tyramine phenolic conjugates.

The above evidence, together with other experiments, indicates a key role for LPSs in plant−bacterium interactions. Knowledge of their structures is thus fundamental for obtaining more information about the pathogenesis mechanism.

These molecules are amphiphilic essential outer membrane constituents; chemically, they comprise three regions: the O-specific polysaccharide, the core region and the Lipid A, which together comprise a smooth-form (S-form) LPS, named according to the smooth aspect of the bacterium under electronic microscopy. A rough-form (R-form) LPS (LOS) lacks the O-specific polysaccharide either partially or totally. This can occur in both wild and laboratory strains possessing mutations in the genes encoding O-specific polysaccharide biosynthesis. Preliminary experiments revealed that *P. cichorii* produces both S- and R-type LPSs. The characterization of the O-chain of the S-form is discussed in this paper.

Results and Discussion

Analysis of both water and phenol extracts of the cells by SDS-PAGE (Sodium Dodecyl Sulfate Poliacrylamide Gel Electrophoresis) revealed the presence of both LPS and LOS species (Figure 1). In fact, the silver-stained LPS fraction in the phenol phase showed the typical ladder appear-

[‡] LPS extraction and cooperation with plant pathologist
[‡‡] Bacterial growth and biological aspects
[a] Instituto de Química Orgánica, CSIC,
Juan de la Cierva 3, 28006 Madrid, Spain
[b] Dipartimento di Chimica Organica e Biochimica, Università di Napoli "Federico II", Complesso Universitario Monte Sant' Angelo,
Via Cintia 4, 80126 Napoli, Italy
Fax: (internat.) + 39-081/674393
E-mail: decastro@unina.it
[c] Dipartimento di Scienze Chimico-Agrarie, Università di Napoli "Federico II",
80055 Portici, Napoli, Italy
[d] Dipartimento di Biotecnologie Agrarie, Università di Firenze, 50144 Firenze, Italy

ance of S-form bacteria in the upper part of the gel. In the water extract, low molecular weight species were present instead.

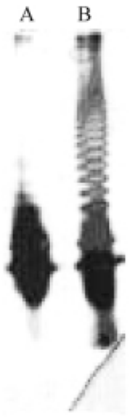

Figure 1. Silver-stained SDS-PAGE electrophoresis of water (lane A: 4 μg) and phenol (lane B: 4 μg) extracts of *P. cichorii* cells

The material extracted in the phenol phase was directly purified by SEC (Size Exclusion Chromatography); the chromatographic support used was Sephacryl HR 400, and under the conditions used the LPS molecules were eluted in the void volume, due to their high apparent molecular weight; the other contaminants, such as low molecular weight carbohydrates (mainly glucans) and proteins, were strongly retained from these conditions.

The purified fraction was subjected to methanolysis in order to determine both the lipid and the monosaccharide compositions. Analysis of the fatty acid methyl esters revealed the presence of the following components: lauryl-, 2-hydroxylauryl-, 3-hydroxylauryl- and 3-hydroxycaproic methyl esters, characteristic of the *Pseudomonas* sp., while unsaturated fatty acid methyl esters, probably due to contamination by glycolipids, were present in small amounts.

More interesting was the monosaccharide composition (Figure 2); besides the acetylated methyl glycosides of rhamnose, glucose and 2-acetamido-2-deoxyglucose (glucosamine), the presence of Kdo was detected, supporting the LPS nature of the fraction isolated, while a cluster of peaks with a similar fragmentation pattern, consistent with the structure of acetamidodideoxy sugars, was also present.

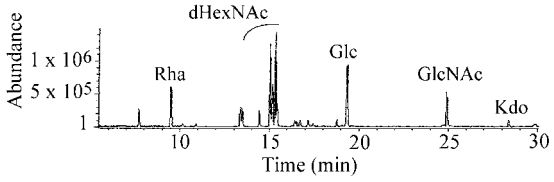

Figure 2. GC-MS profile of monosaccharide components of lipopolysaccharide material extracted from the phenol phase

The O-specific polysaccharide part associated with this LPS was isolated after mild acid hydrolysis and gel chromatography on a Sephacryl HR 300 column, and was extensively studied by NMR spectroscopy. Its ^{1}H and HSQC NMR spectra (Figure 3) suggested a regular structure built up of a tetrasaccharide repeating unit. In particular, this polymer was composed of four acetamido-6-deoxy sugars, in agreement with the four signals (two were coincident) for methyl doublets at high field, four *N*-acetyl signals at $\delta \approx 1.9$ ppm, and four signals of nitrogen-bearing carbon atoms, in the range of $\delta = 49-58$ ppm.

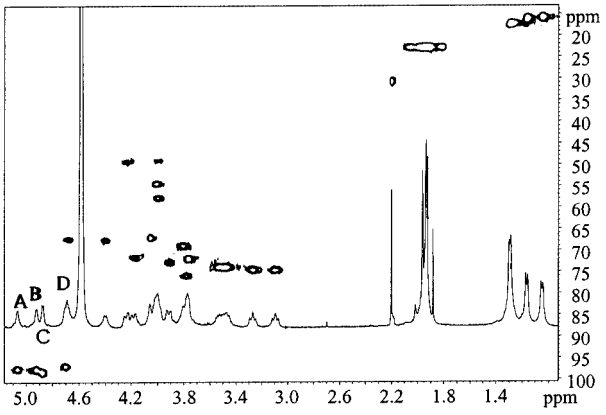

Figure 3. Superimposition of proton and HSQC spectra of the O-chain fraction, measured at 318 K on 400 MHz spectrometer

The proton anomeric region showed five signals: four anomeric signals with the same intensity at $\delta = 5.08$, 4.93, 4.88 and 4.70 ppm, named **A**−**D** respectively, and one at $\delta = 4.69$ ppm, correlated with the low-field methyl group signal at $\delta = 1.05$ ppm by DQF-COSY and with its carbon signal at $\delta = 67.5$ ppm by the HSQC spectrum; combination of the information provided by the HSQC spectrum with that from DQF-COSY, TOCSY and NOESY experiments allowed the complete assignment of both carbon and proton signals of all four residues (Table 1).

Qualitative analysis of scalar couplings from a DQF-COSY spectrum allowed us to establish the stereochemistry of residues **A** and **B** as *galacto* and of **C** and **D** as *gluco*. The sequence of residues was deduced from the dipolar couplings (Table 2) measured from the NOESY spectrum for the anomeric protons of each residue; the linkage points were supported by analysis of the partially methylated alditol acetates arising from the permethylated derivative of the O-chain. Finally, the $^3J_{H1,H2}$ values indicated α anomeric configurations for the first three sugars (**A**−**C**, 2.0−3.6 Hz), but β for the fourth residue (**D**, 6.5 Hz).

The following sequence was inferred from the data in Table 2:

→3)-α-Fuc*p*2NAc-(1→3)-α-Qui*p*2NAc-(1→3)-α-Fuc*p*2NAc-(1→2)-β-Qui*p*3NAc-(1→

A **C** **B** **D**

In order to determine the absolute configurations of the three residues, we used both a computational approach and the Exciton Coupling Method;[2] the combination of these

Table 1. ^{1}H and ^{13}C chemical shifts [ppm] and coupling constants in parentheses [Hz] of the O-specific polysaccharide

	1-H C-1	2-H C-2	3-H C-3	4-H C-4	5-H C-5	6-H C-6
A	5.08 d (2.0)	4.00	4.19	4.06	4.40	1.16 d (6.2)
3)-α-L-Fuc*p*2NAc-(1	97.4	49.1	71.5	67.0	67.6	16.0
B	4.93 d (3.6)	4.29	3.92	3.78	4.69	1.05 d (6.1)
3)-α-L-Fuc*p*2NAc-(1	97.5	49.1	72.8	71.8	67.5	15.9
C	4.88 d (3.5)	4.01	3.72	3.27 t (9.5)	3.79	1.29d (5.4)
3)-α-D-Qui*p*2NAc-(1	98.4	54.4	75.9	74.5	68.9	17.3
D	4.70 d (6.5)	3.47	4.00	3.09 t (9.4)	3.53	1.29 d (5.4)
2)-β-D-Qui*p*3NAc-(1	98.0	73.9	57.5	74.5	73.8	17.3

Table 2. Interresidue dipolar couplings and their intensities, measured for anomeric protons of each unit of O-specific polysaccharide

Residue	NOE observed	
A: →3)-α-L-Fuc*p*2NAc-(1→	C_3 strong	C_4 weak
B: →3)-α-L-Fuc*p*2NAc-(1→	D_2 strong	D_4, D_3 weak
C: →3)-α-D-Qui*p*2NAc-(1→	B_3 strong	B_4 weak
D: →2)-β-D-Qui*p*3NAc-(1→	A_4 strong	A_3 medium

methods overcame the problems associated with the low quantity of available O-chain material and the lack of monosaccharide references.

Methyl 2-acetamido-3,4-di-*O*-(*p*-bromobenzoyl)-2-deoxy-α-fucopyranoside was obtained by methanolysis of the O-chain and *p*-bromobenzoylation of the methyl 2-acetamido-2-deoxy-α-fucopyranoside isolated by TLC chromatography, and its ΔO.D. (Circular Dichroic Absorption) spectrum was measured. The L configuration was established for the Fuc*p*2NAc residues from the negative (Figure 4) Cotton effect exhibited by the compound, which indicated a counterclockwise arrangement of the two *p*-bromobenzoate chromophores.

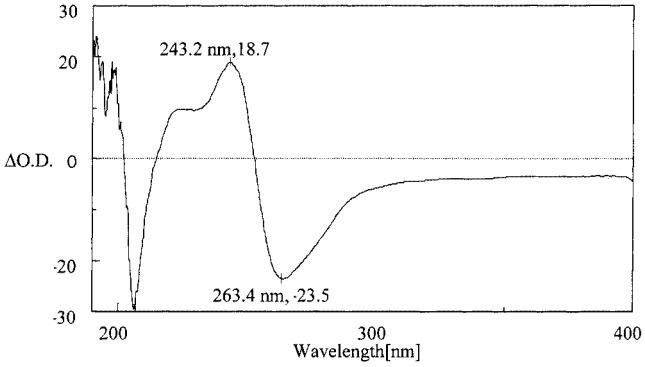

Figure 4. ΔO.D. (dichroic absorption) spectrum of the *p*-bromobenzoate derivative of the Fuc*p*2NAc residue

In order to gain insight into the absolute configuration of the quinovosamine units, a technique based on comparison of experimentally obtained NMR results (NOESY) with those predicted for different models was used. In particular, eight distinct disaccharide entities − namely **A**−**C**, **C**−**B**, **B**−**D**, and **D**−**A** (residues **A** and **B** drawn with L configurations, whereas for residues **C** and **D**, both D and L configurations were considered) − were constructed with the MACROMODEL package and subjected to extensive calculations with the MM3* force field. Ensemble average interproton distances for each disaccharide entity considered were extracted from these calculations, which were translated into expected NOEs by a full-matrix relaxation approach. The NOEs predicted for a given disaccharide (D/L or L/L) were compared with those obtained experimentally. This approach is in fact an extension of that proposed by Lipkind et al.,[3] who exploited the relative intensities of NOEs measured between the anomeric protons of each residue and the protons at the α and β positions of corresponding glycosylated units. Indeed, for every disaccharide fragment, the NOE values depend on the absolute configurations of the constituent residues and on the configuration of the anomeric centre of the glycosylating moiety. Comparison of the simulated NOEs with those obtained for the authentic O-chain sample (Table 2) allowed us to evaluate the absolute stereochemistries of residues **C** and **D**, which in both cases proved to be D.

In fact, the strong NOE measured for 1-H[β-Qui*p*3NAc (**D**)]/4-H[α-L-Fuc*p*2NAc (**A**)] (Table 2) suggested a D configuration for the glycosylating β-Qui*p*3NAc (**D**) moiety. For the corresponding L residue, no such NOE was predicted at all. In addition, the weak NOE of the analogous 1-H[α-Qui*p*2NAc (**C**)]/4-H[α-L-Fuc*p*2NAc (**B**)] NOE probably suggests a D configuration for entity **C**, since this 1-H/4-H is marginal only for α-linked D-Qui*p*2NAc residues linked to α-L-Fuc*p*2NAc units.

On the basis of these considerations, it was possible to propose the following repeating unit for the O-specific polysaccharide of the lipopolysaccharide from *Pseudomonas cichorii*:

3)-α-L-Fuc*p*2NAc-(1→3)-α-D-Qui*p*2NAc-(1→3)-α-L-Fuc*p*2NAc-(1→2)-β-D-Qui*p*3NAc-(1→
A **C** **B** **D**

A view of a possible three-dimensional structure of the polysaccharide, constructed by combination of the different global minima for each disaccharide entity and in agreement with the experimental NOEs, is shown in Figure 5.

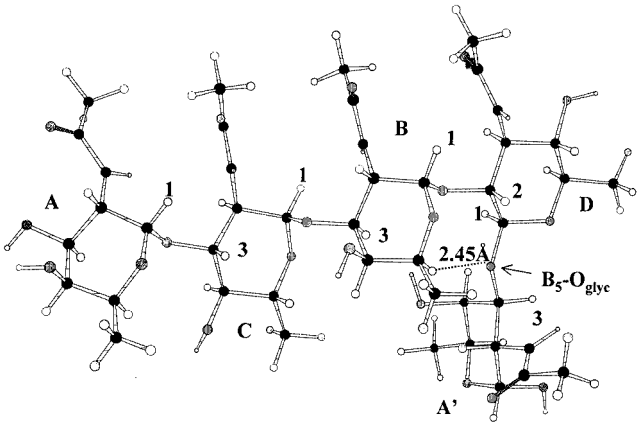

Figure 5. View of a possible three-dimensional structure of the polysaccharide repeating unit: the different units are indicated with their letter and the more relevant protons for NOEs are marked with a number; the distance between proton B-5 and the glycosidic oxygen atom of residue **D** is indicated

A conventional structural formula of the polysaccharide is shown together with the three-dimensional view, in order to display the stereochemistry of the monosaccharide residues involved more clearly (Figure 6).

Analysis of the secondary structure revealed that proton B-5 was located 2.45 Å distant from the glycosidic oxygen atom of residue **D**; its location and the deshielding nature of the oxygen atom may account for the peculiar chemical shift found for this proton signal, which appeared at δ = 4.69 ppm, the anomeric area of the ^{1}H NMR spectrum.

It is interesting to underline that this LPS structure is practically unique to phytopathogenic bacteria. It is totally constituted of acetamido sugars, all 6-deoxy; this last feature is very frequent in the case of LPSs from phytopathogenic bacteria[4] and is probably responsible for the adhesion of the bacteria on the plant cell wall. More work to establish the LPS's role in the phytopathogenesis mechanism is necessary.

Experimental Section

***Pseudomonas cichorii* and Bacterial Cultivation:** *P. cichorii* strain 5707 (from *Cichorium endiva* L.) was grown in IMM.[5] Bacterial suspension (8 mL, A_{600} = 0.35) was added to 1-L Erlenmeyer flasks containing 400 mL of medium and incubated at 25 °C. The bacterial suspension was centrifuged (9000 g × 20 min) and harvested cells were washed three times with 0.85% NaCl and lyophilised.

Isolation and Purification of the LPS Fraction: Dried cells (1.9 g) were extracted by the phenol/water method.[6] Both phases were separately dialysed against distilled water and freeze-dried to yield 77 mg of LPS fraction in the phenol phase (4.1% yield) and 7.6 mg in the water phase (0.4% yield). Both fractions were analysed by discontinuous SDS-PAGE electrophoresis[7] on a 12% gel with a Bio-Rad miniprotean gel system; the samples (4 μg) were run at constant voltage (150 V) and stained according to the procedure of Kittelberger[8] (Figure 1). LPS fraction from the phenol phase was further purified from proteic material on Sephacryl HR 400 (Pharmacia, 1.5 × 90 cm, eluent NH_4HCO_3 50 mM, flow 0.4 mL/min), eluate was monitored with an R.I. refractometer (R410 Waters), and the collected peaks were again screened for LPS fraction on SDS-PAGE, to provide 40 mg of purified LPS fraction (2.1% yield with respect to dry cells).

Sugar and Lipid Analysis of the LPS Fraction: LPS (1 mg) was dried in a desiccator over P_2O_5 for 1 h under vacuum and then treated with 1 M methanolic HCl at 80 °C for 18 h. After cooling, the solution was directly extracted twice with equal volumes of *n*-hexane; the top phase (*n*-hexane) was dried and the fatty acid methyl esters were analysed by GC-MS. The methanolic phase was dried and the methyl glycosides were acetylated as follows: first with Ac_2O (200 μL) at 80° C for 15 min, followed by drying in a stream of air, and then by treatment with dry pyridine (200 μL) and Ac_2O (100 μL) at the same temperature for 30 min. The reactants were removed by evaporation and the mixture of peracetylated methyl glycosides was analysed by GC-MS. GS-MS analysis conditions for both fatty acids and methyl glycoside derivatives were the same, and were run with a Hewlett−Packard 5890 instrument, on an SPB-5 capillary column (Supelco, 30 m × 0.25 i.d. flow rate, 0.8 mL/min; He as carrier gas), with the temperature program: 150 °C for 5 min, 150 → 300 °C at 5.0 °C/min, 300 °C for 15 min. The

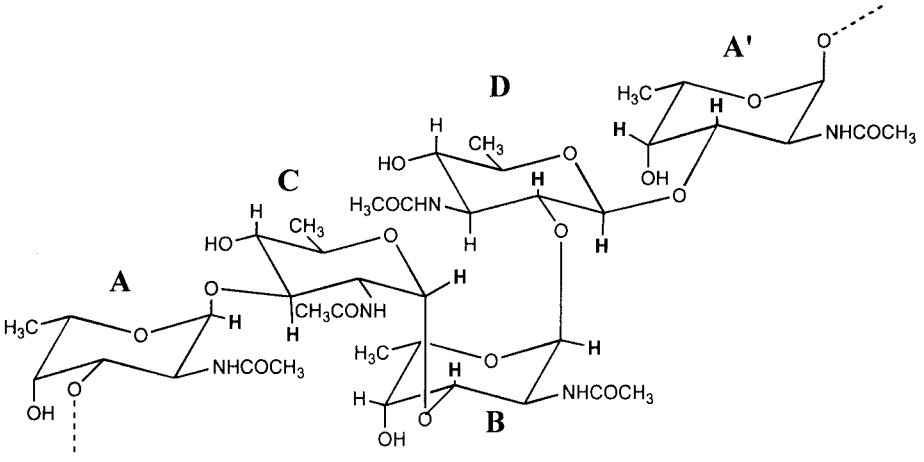

Figure 6. Conventional structural formula of the O-chain moiety: for clarity the hydrogen atoms displayed are those showing NOEs, the protons in bold are involved in strong or medium NOEs, whereas those in plain have only weak NOEs, as in Table 2

mass spectra were recorded with an ionization energy of 70 eV and an ionizing current of 0.2 mA.

Glycosyl-linkage Analysis of LPS: LPS (1 mg) was dried overnight in a desiccator over P_2O_5 and methylated as described by Sandford.[9] The permethylated lipopolysaccharide was recovered in the organic layer of the water/chloroform extraction, dried and hydrolysed with TFA (2 M, 200 μL) at 120 °C for 2 h. The acid was removed by repeated evaporation with 2-propanol, and the partially methylated monosaccharides were dissolved in EtOH (200 μL) and reduced with $NaBD_4$ (3 mg) at room temp. for 1 h. Borates were removed by evaporation with methanol and a few drops of glacial acetic acid, and acetylation was performed with Ac_2O and pyridine (150 μL each) at 120 °C for 20 min. The partially methylated and acetylated alditols were analysed by GC-MS, with the temperature program: 80 °C 2 min, 80 → 240 °C at 4 °C min, 240 °C for 15 min.

Isolation of the O-Specific Polysaccharide Fraction: LPS fraction (30 mg) was dissolved in 2 mL of 1% acetic acid solution and kept at 100 °C for 2 h. After cooling, the solution was centrifuged at 6000 rpm for 20 min and the clear supernatant was freeze-dried. Further purification of this sample was performed by SEC on Sephacryl HR 300 (Pharmacia, 1.5 × 70 cm, NH_4HCO_3 50 mM, flow 0.4 mL/min) and the eluate was monitored by refractive index as mentioned above. The O-chain (16 mg) was isolated in approx. 40% yield from purified LPS.

Absolute Configuration of Methyl 2-Acetamido-2-deoxyfucopyranoside: The O-chain fraction (5 mg) was treated with 1 M methanolic HCl at 80 °C for 18 h. The excess of acid was removed by repeated evaporation with methanol, and the methyl glycosides were *N*-acetylated by treatment with dry methanol (500 μL), dry pyridine (100 μL) and acetic anhydride (50 μL), at room temperature for 30 min. Solvents were removed by evaporation, and the monosaccharide derivatives were purified by preparative TLC, eluted with $CHCl_3/CH_3OH$ (9:1). Methyl 2-acetamido-2-deoxyfucopyranoside isolated under these conditions (1 mg, 20% yield from O-chain), was *p*-bromobenzoylated with an 1.5-fold excess of the corresponding acyl chloride at room temp. for 3 h with dry pyridine as solvent. ^{1}H NMR ($CDCl_3$, 303 K): δ = 4.99 (1-H, $J_{1,2}$ = 3.30 Hz), 5.62 (2-H, $J_{2,3}$ = 11.74 Hz), 6.06 (3-H, $J_{3,4}$ = 2.93 Hz), 5.77 (4-H, coupled only with proton 3-H), 4.30 (5-H, $J_{5,6}$ = 6.30 Hz), 1.21 ppm (6-H); aromatic signals were present at δ ≈ 7.5 ppm. Circular Dichroic Absorption (ΔO.D.) was measured in HPLC-grade methanol with a Jasco J-715 instrument; the spectrum was elaborated according to the instrument software.

Molecular Mechanics and Dynamics Calculations: Molecular mechanics and dynamics calculations were performed with the MM3* force field as implemented in MACROMODEL 5.5.[10] The MM3* force field[11] implemented in MACROMODEL differs from the regular MM3 force field in the treatment of the electrostatic term, since it uses charge−charge rather than dipole−dipole interactions. For every glycosidic linkage, Φ is defined as $H^1-C^1-O-C_{aglycon}$ and Ψ as $C^1-O-C_{aglycon}-H_{aglycon}$. Both D and L configurations were considered for residues **C** and **D**, while the L configuration, as deduced from the exciton coupling analysis, was set for residues **A** and **B**. Eight disaccharide entities were therefore considered. The calculations were performed for a dielectric constant ε = 80, as an approximation for bulk water. Firstly, potential energy maps were calculated for the constituent isolated disaccharides; relaxed (Φ,Ψ) potential energy maps were calculated as described. The previous step involved the generation of the corresponding rigid residue maps by use of a grid step of 18°. Then, every (Φ,Ψ) point of this map was optimised by 200 steepest descent steps, followed by 1000 conjugate gradient iterations. From these relaxed maps, adiabatic surfaces were constructed, and the probability distributions were calculated for each φ,ψ point according to a Boltzmann function at 313 K. Ensemble average distances between intra- and interresidue proton pairs were calculated from the calculations and employed for the simulation of the experimental NOESY spectra by the previously described procedure, by a full-matrix relaxation approach.[12a−12e] In particular, the spectra were simulated from the average distances $<r^{-6}>_{kl}$ calculated from the relaxed energy maps at 313 K. By this procedure, it is possible to deduce an actual population distribution by comparison with the experimental data. Isotropic motion and external relaxation of 0.25 s^{-1} were assumed. A τ_c = 7000 ps was used to obtain the best match between experimental and calculated NOEs for the intraresidue proton pairs. The simulated NOEs were then compared to the experimental ones, and in this way it was possible to deduce both the unknown absolute configurations of the residues **C** and **D** and the major average conformation in solution for the glycosidic linkages of the polysaccharide. All the NOE calculations were automatically performed by an in-house program, available from the authors upon request.[13]

NMR Spectroscopic Analysis: NMR experiments were carried out with Bruker DRX 400 and 500 MHz machines equipped with reverse multinuclear probe at 45 °C or at 30 °C. Chemical shifts of spectra recorded in D_2O are expressed in δ relative to internal acetone (δ = 2.225 and 31.4 ppm), whereas spectra in $CDCl_3$ are referred to the chemical shifts of the solvent. Two-dimensional spectra (DQF-COSY, phase-sensitive TOCSY and ROESY, and phase-sensitive gradient HSQC) were measured with 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 10 ppm and the carrier was placed at the residual HOD peak. A mixing time of 250 ms was used in the NOESY 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. Processing and plotting was performed with the standard Bruker Xwin NMR 1.3 program.

Acknowledgments

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