Glycobiology Advance Access published January 21, 2010

doi: 10.1093/glycob/cwp205

Against the Rules: a Marine Bacterium, *Loktanella rosea,* Possesses a Unique Lipopolysaccharide

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Running title: Lipopolysaccharide structure from *Loktanella rosea*

Keywords: (Lipopolysaccharide, lipid A, Loktanella rosea, NMR spectroscopy)

Abstract

Bacteria are an inimitable source of new glyco-structures potentially useful in medicinal and environmental chemistry. Lipopolysaccharides (endotoxins) are the major components of the outer membrane of Gram-negative bacteria; being exposed toward the external environment they can undergo structural changes and thus, they often possess peculiar chemical features that allow them to thrive in harsh chemical and physical environments. Marine bacteria have evolved and adapted over millions of years in order to succeed in different environments, finding a niche for their survival characterized by severe physical or chemical parameters. The present work focuses on the structural investigation of the lipopolysaccharide from *Loktanella rosea*, a marine Gram-negative bacterium. Through chemical analysis, 2D NMR and MALDI Mass spectrometry investigations, a unique LPS carbohydrate backbone has been defined. The lipid A skeleton consists of a trisaccharide backbone lacking the typical phosphate groups and is characterized by two β-GlcN and an α-Galacturonic acid. The core region is built up of three ulosonic acids, with two 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid residues one of which carrying a neuraminic acid. This carbohydrate structure is an exceptional variation from the typical architectural skeleton of endotoxins which consequently implies a very different biosynthesis.

Introduction

Gram-negative bacteria possess an asymmetric outer membrane which surrounds a thin layer of peptidoglycan. In these micro-organisms, the external membrane is the first and immediate line of defense against harsh environment and antimicrobial molecules and makes the cytoplasmatic membrane more efficiently protected. Lipopolysaccharides (LPS), the major components of the outer membrane, are pivotal molecules for bacterial life and possess, within their chemical structure some invariable moieties that are strictly saved by any biosynthesis such as the lipid A carbohydrate backbone which is built up of two aminosugars, one β– and another α- anomeric configured. Actually, in the most cases, it possesses a β-GlcN-(1→6)-α-GlcN disaccharide backbone phosphorylated at positions 1 and 4' and acylated with primary 3-hydroxy fatty acids at positions 2 and 3 of both GlcN residues; the hydroxyl groups of the primary fatty acids can be further acylated by secondary acyl moieties. (Holst and Molinaro, 2009). LPS lacking the O-chain portion are termed rough LPSs (R-LPSs) or lipooligosaccharides (LOSs). In the core oligosaccharide region, an inner and outer core oligosaccharide region are present: the inner core, proximal to the lipid A, consists of archetypical residues among which the 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid (Kdo), a chemical hallmark for Gram negative bacteria; moreover, the inner core is often rich in negatively charged residues as phosphate groups. The outer core region is more variable and is usually composed by hexoses.

The present work focuses on the structural investigation of the lipopolysaccharide (LPS) from *Loktanella rosea*, a mesophilic and chemo-organotroph marine Gram-negative bacterium (Ivanova et al., 2005; Van Trappen et al., 2004) isolated from sediments of Chazma Bay, Sea of Japan. The genus *Loktanella* was created in 2004 in order to classify some new heterotrophic *Alphaproteobacteria* collected from Antartic lakes.

The LPS from *Loktanella rosea* has been defined through sugar analysis, 2D NMR and MALDI mass spectrometry investigation. A unique highly negatively charged carbohydrate backbone has been identified. The lipid A skeleton lacks the typical phosphate groups and is characterized by two β-GlcN

and an α-Galacturonic acid (GalA). This is the first example of a lipid A saccharide backbone in which the-α-GlcN-phosphate residue is replaced by a β-GlcN-(1→1)-α-GalA in a mixed trehalose like linkage. The core region is built up of three ulosonic acids, with two 3-deoxy-D-*manno*-oct-2-ulosonic acid residues, one of which is carrying a neuraminic acid. The overall carbohydrate structure is an exceptional variation from the typical architectural skeleton of endotoxins and also implies a very different biosynthesis.

Results

Extraction of LOS and compositional analysis.

Dried cells were extracted and SDS-PAGE of *L. rosea* LPS fraction revealed that it was a rough type LPS, i.e., a LOS. Structural investigations on extracted LOS revealed the following monosaccharide composition: 2-amino-2-deoxy-D-glucose (D-GlcN), D-galacturonic acid (D-GalA), 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (Neu or neuraminic acid). Methylation analysis showed the presence of 6-substituted GlcN, terminal GalA, 4,8-di-substituted Kdo, and terminal Neu, all in pyranose form. Fatty acids analysis revealed the presence of (*R*)-3-hydroxydecanoic (C10:0 (3-OH)) and dodecanoic acid (C12:0), confirming chemotaxonomic data (Ivanova et al., 2005). Phosphate assays gave negative results.

Isolation and structural elucidation of fully deacylated oligosaccharide (OS) from L. rosea.

The LOS was completely deacylated with a strong alkaline treatment as reported (Holst and Molinaro, 2009) and then purified by gel-permeation chromatography that yielded the oligosaccharide fraction (**OS**). The monosaccharide analysis of **OS** gave the same results of intact LOS that means that no sugar was lost or altered following chemical treatment. The oligosaccharide product was characterized through a full 2D NMR analysis and supported by MS data. In the anomeric region of **OS** ^{1}H-NMR spectrum (Figure 1) three anomeric signals were identified (**A-C)** (Table 1). Furthermore, the signals at 1.62/2.67, 1.65/2.05 and 1.73/2.01 ppm were identified as three pairs of H-3 methylene protons belonging to the

non substituted Neu (**D**) and Kdo (**E**) residues and to the 4,8-di-substituted-D-Kdo residue (**F**). A complete assignment of the spin systems was carried out assigning the proton resonances in DQF-COSY and TOCSY spectra, and the carbon resonances in the HSQC spectrum. The anomeric configuration of each aldose monosaccharide unit was assigned on the basis of the $^{3}J_{H1,H2}$ coupling constants obtained by the DQF-COSY, by the $^{1}J_{C1,H1}$ [1] derived by *F2-coupled* HSQC (Figure 3, main text and Table 1) and confirmed by the *intra*-residual NOE contacts observed in the ROESY and NOESY spectra (Figure 2, main text and Table 1), whereas the values of vicinal $^{3}J_{H,H}$ ring coupling constants allowed the identification of the relative configuration of hydroxyl groups within each sugar residue.

Spin system **A** (H-1 at 5.23 ppm) was identified as the α-galacturonic acid. Its $^{3}J_{H3,H4}$ and $^{3}J_{H4,H5}$ values (3 Hz and 1 Hz, respectively) were diagnostic of a *galacto*-configuration; the chemical shifts of H-1 and C-1 (5.23 and 100.6 ppm), the $^{1}J_{C1,H1}$ and $^{3}J_{H1,H2}$ values (172.5 Hz and 3.6 Hz respectively) and the *intra*-residual NOE contact of H-1 with H-2 were all in agreement with an α-anomeric configuration. Furthermore, in the $^{1}H,^{13}C$ HMBC spectrum (Figure 3), it was visible a correlation of H-5 (4.33 ppm) with a carboxyl group (175.6 ppm), thus defining residue **A** as a uronic acid residue.

Spin systems **B** and **C** (H-1 at 4.86 and 4.48 ppm, respectively) were both identified as GlcN residues as indicated by their large $^{3}J_{H,H}$ coupling constants of ring protons (around 10 Hz). Furthermore, the $^{1}H,^{13}C$ HSQC spectrum showed the correlation of both H-2 **B** (3.09 ppm) and H-2 **C** (2.92 ppm) with nitrogen bearing carbon signals at 55.4 ppm and at 55.7 ppm, respectively. The chemical shifts of H-1 and C-1 of **B** (4.86 and 99.6 ppm) and **C** (4.48 and 100.8 ppm), the $^{3}J_{H1,H2}$ (8.4 Hz), the $^{1}J_{C1\text{-}H1}$ (162.0 Hz for **B** and 163.1 Hz for **C,** Figure 1) and the *intra*-residual NOE contacts (Figure 2, main text) of H-1 with H-3 and H-5 univocally and surprisingly demonstrated β-anomeric configuration for both residues.

Spin systems **E** and **F** were identified as Kdo residues and were assigned starting from the diastereotopic H-3 methylene protons, resonating in a shielded region at 1.65/2.05 and 1.73/2.01 ppm ($H\text{-}3_{ax}$ and $H\text{-}3_{eq}$ respectively for **E** and **F**). The α-anomeric configuration at C-2 for both residues was attributed by the chemical shift values of H-3 and by $^{3}J_{H7,\ H8a}$ and $^{3}J_{H7,\ H8b}$ coupling constants (Birnbaum et al., 1987; Holst et al., 1994). Residue **D** was identified as the neuraminic acid whose 5-*N*-Acyl group was removed

under alkaline treatment. The spin system was assigned starting from its diastereotopic H-3 methylene protons resonating at 1.62/2.67 ppm (H-3_{ax} and H-3_{eq} respectively). The $^{1}H,^{13}C$ HSQC spectrum showed the correlation of H-5 **D,** at 3.44 ppm, with a nitrogen bearing carbon signal at 51.8 ppm, indicating the presence of the amino group at position C-5. The α-anomeric configuration was inferred by the presence of the three bond correlation between C-1 and H-3_{ax} observed in $^{1}H,^{13}C$ HMBC spectrum (Figure 3), indicative of the large value of $^{3}J_{C\text{-}1,H\text{-}3ax}$ that is in agreement with an axial orientation of carboxyl group, as reported (Ali et al., 2006).

The analysis of the HSQC spectrum allowed the identification of glycosylation sites (Figure 3); the saccharide sequence was assigned by the $^{n}J_{C,H}$ long range correlations in the HMBC spectrum and the *inter*-residual NOE contacts in NOESY and T-ROESY spectra. Residue **A** of α-GalA was sitting at O-1 of β-GlcN **B** through its anomeric position as inferred by scalar correlations visible in the HMBC spectrum between C-1 **A** and H-1 **B** and between C-1 **B** and H-1 **A** (Figure 3**)**; the β-(1→1)-α linkage was also confirmed by the NOE contact between H-1 **A** and H-1 **B** (Figure 2**)**. Residue **B** was glycosylated at its O-6 by β-GlcN **C** as confirmed by HMBC correlation between C-1 **C** and C-6 **B** and by NOE contact of H-1 **C** with H-6 **B**. Residue **C** was glycosylated at its O-6 by α-Kdo **F** as attested by the weak C-6 **C** down-field shift, typical of ketose glycosylation, and by the long range correlation in the HMBC spectrum of C-2 **F** with H-6 **C**. In addition, unit **F** was glycosylated at its O-4 by the terminal α-Kdo **E** as shown by the *inter*-residue NOE contacts between H_{3eq} of 4,8-Kdo **F** and H-6 of t-Kdo (**E**) (Figure 3**),** that were diagnostic for the α-D-Kdo-(2→4)-α-D-Kdo linkage (Bock et al., 1992). Kdo **F** was further glycosylated by residue **D** of α-Neu at its O-8, as attested by the scalar correlation present in HMBC spectrum between C-2 **D** and H-8 **F (**Figure 3**)** and by the NOE contacts of both axial and equatorial H-3 of residue **D** with H-8 of **F** (Figure 2**).**

The primary structure of the oligosaccharide fraction **OS** derived by NMR investigations is reported in Figure 4 and sketched below:

D **E** **F** **C** **B** **A**

α-Neu-(2→8)[α-D-Kdo-(2→4)]-α-D-Kdo-(2→6)-β-D-GlcN-(1→6)-β-D-GlcN-(1→1)-α-D-GalA

The **OS** oligosaccharide structure was also confirmed through MALDI mass spectrometry. The mass spectrum acquired in negative polarity gave a peak at *m/z* 1186.3, matching with the ion [**OS** - H_2O]$^-$ (Figure 5), composed by a GalA, a Neu, two Kdo and two GlcN residues.

Discussion

The LOS turned out to be characterized by a novel and unique hexasaccharide skeleton comprising: i) a very small core region exclusively composed of ulosonic sugars and containing a neuraminic acid attached to a Kdo unit: α-Neu-(2→8)[α-D-Kdo-(2→4)]-α-D-Kdo-(2→, and ii) an exceptional lipid A backbone: β-D-GlcN-(1→6)-β-D-GlcN-(1→1)-α-D-GalA in which both GlcN residues were present with a β anomeric configuration. Moreover, it lacked the classical phosphate residues at O-4'and O-1, this latter was replaced by an α-GalA linked in a mixed trehalose-like linkage. At the best of our knowledge this kind of glycosydic linkage was never found in biomolecules; its presence obviously implies profound biosynthetic differences from the canonical LPS lipid A pathway (Raetz et al., 2007). In fact, current studies showed that, in Gram-negative bacteria, the initial steps of lipid A biosynthesis are common and catalyzed by a set of intracellular enzymes constitutively expressed and generally not subjected to regulation (Raetz et al., 2007). These earlier stages lead to $(Kdo)_2LipidIV_A$ intermediate constituted by a β-(1→6)-glucosamine disaccharide backbone phosphorylated at positions 1 of the reducing α-configured unit (GlcN I) and at 4' of the non reducing β-configured unit (GlcN II), acylated at positions 2 and 3 by 3-hydroxy fatty acids and glycosylated at O-6 of GlcN II by a α-(2→4)-Kdo disaccharide. Instead, lipid A modifications, that characterize the maturation process, are catalyzed by extracytoplasmic enzymes and are different from bacteria to bacteria since they are generally induced and strongly influenced by growth conditions. For instance, it has been proven the existence of two inner membrane phosphatases, LpxE and LpxF, as described for the highly infectious human pathogen

Francisella tularensis lipid A, that act on the periplasmic surface of the inner membrane and promote the cleavage of the phosphate groups on the lipid A moiety (Raetz et al., 2007). Likewise, in *L. rosea* the lipid A precursor could be processed by two homologues of 1- and 4'-phosphatase, thus explaining the lack of both phosphate groups. For what concerns the presence of the two GlcNs exclusively in the β anomeric orientation, it can be speculated that the galacturonic acid tranferase only recognizes the equatorial GlcN I free anomer obtained by phosphatase cleavage or, conversely, it catalyses the transfer of GalA on a different substrate, the canonical phosphorylated α-GlcN, thus achieving an inversion of configuration at the anomeric position.

The replacement of phosphates with GalA residues has been already detected in lipid A deriving from other bacterial lipid A (De Castro et al., 2008), for example a lipid A with a 2,3 diamino-2,3-dideoxyglucose (DAG) disaccharide backbone substituted at both 1 and 4' positions by α-D-GalA moieties has been identified for the first time in the extremely thermophilic bacterium *Aquifex pyrophilus* (Plötz et al., 2000); further, the lipid A from the symbiontic bacterium *Mesorhizobium huakuii* (Choma et al., 2004) consists of a DAG backbone bearing an α-galacturonic acid only at O-1 of distal DAG residue. In other lipid A belonging to *Rhizobiaceae,* as for *Rhizobium etli* and *R.leguminosarum* (De Castro et al., 2008; Que et al., 2000; Jeyaretnam et al., 2002), LPS lacking phosphate groups are constituted by a mixture of species bearing α-galacturonic acid moiety at 4' and carrying the GlcN I oxidized to aminogluconate; for what concerns GalA units, in a later step of the biosynthesis $(Kdo)_2$lipid IV_A precursor is processed by 1-phosphatase and then by a galacturonic acid tranferase (Raetz et al., 2007). The uronic acids are known to be more resistant to the hydrolysis than the phosphate groups thus reinforcing the lipid A and, consequently, membrane stability. The absence of the phosphate group at position 4' has been reported so far in few lipid A species as the case of *Azospirillum lipoferum* (Choma et al., 2008), a plant-growth-promoting Rhizobacterium, and of the genus *Francisella* as mentioned above (Raetz et al., 2007; Vinogradov et al., 2002; Wang et al., 2006), whose absence

strongly contribute to the LPS low endotoxicity (Loppnow et al., 1989). The reason of the absence in *L. rosea* of the 4' phosphate group is unknown.

The "wrong" anomeric configuration on the GlcN I might be crucial in the supramolecular assembly of the LOS and so in determining the rheological and biological properties of outer membrane. In fact, the intrinsic conformation of the lipid A strongly influences its supramolecular properties and the inclination angle of the glycoside linkage allows the reducing GlcN to be exposed toward the external environment (Brandenburg et al., 2004).

As for the core region of *L. rosea* LPS, even its carbohydrate sequence is very peculiar. Actually, the lipid A moiety is glycosylated by a Kdo unit which is further substituted at O-4 and O-8 positions by two 2-ulosonic residues: a further Kdo, sitting at O-4 and a neuraminic acid at position O-8. Neu5Ac residues have been found in the outer core region, as the case of *Campylobacter jejuni*, *Helicobacter pylori* (Holst et al., 2002) that can express either Lewis antigens, or resemble structural similarities with glycosphingolipids of the ganglioside group, all attempts to evade host immune response although has never been found in an environmental or marine bacterial LPS. *Chlamydia trachomatis* produces a deep-rough LPS characterized by a linear sequence of three Kdo residues: α-Kdo-(2→8)-α-Kdo-(2→4)-α-Kdo-(2→6)-lipid A, whereas in *Chlamydophila psittaci* a non-stoichiometric fourth Kdo is located at O-4 of the second one (Holst, 2002). However, in few cases the first Kdo has been found carrying two ulosonic acid residues, as the case of the LPS from *Acinetobacter lwoffii* F78[16] (Hanuszkiewicz et al., 2008) but never a neuraminic acid residue has been found directly linked to the Kdo residue.

The deep-rough LPS from *Loktanella rosea* has a short oligosaccharide moiety that at physiological pH is negatively charged: overall, of six residues, four bear negatively charged carboxyl groups. This peculiar sugar backbone confers to the whole LOS the ability to strongly and extensively interact with divalent cations, especially Ca^{2+} and Mg^{2+}, on the surface of the outer membrane. The establishment of such wide network of ionic bridges can contribute to the network of interactions that shield the outer membrane and give rigidity and resistance to bacterial cells. This could give a key explanation on why this class of microorganisms is allowed to survive in conditions of unusual pressure and salinity.

MATERIAL AND METHODS

Bacterial growth and LOS extraction

The type strain of *Loktanella rosea* KMM 6003^T was cultivated on a liquid medium containing (g/L): pepton - 5, yeast extract - 1, citric acid - 0.1, $FeSO_4$ - 0.07 and sea water (1 L), pH 7.6. Cells were collected by centrifugation, washed with water and next dried with acetone (three times) to yield around 10 g of dried cells from 20 L of the cultural fluid.

Dried cells (6 g) were treated with Westhpal method as reported (Westhpal et at., 1965). In details, they were suspended in hot 90% phenol and extracted three times with hot water. Then the two phases obtained were dialyzed and purified through enzymatic treatment, the LOS was present only in the water phase (3% of bacterial dried mass). After this, a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE 13.5 %) was performed in order to detect LPS and LOS after staining with silver nitrate.

Isolation of oligosaccharide OS

An aliquot of LOS (10 mg) was dissolved in 1 mL of anhydrous hydrazine, stirred at 37°C for 90 min, cooled poured into ice-cold acetone (10 mL) and allow to precipitate. The precipitate was collected by centrifuging (4°C, 7000 rpm, 30 min), washed twice with ice-cold acetone and dried. The sample was further N-deacylated adding 4M KOH (120°C, 16 h). After desalting on a Sephadex G-10 column, the resulting oligosaccharide was purified on a Biogel P-2 column.

General and analytical methods

Determination of sugars residues and of their absolute configuration, GLC and GLC-MS were all carried out as described (Leontein et al., 1978) Monosaccharides were identified as acetylated O-methyl glycosides derivatives. After methanolysis (2M HCl/MeOH, 85°, 2 h) and acetylation with acetic anhydride in pyridine (85°, 30 min) the sample was analyzed by GLC-MS. Linkage analysis was carried out by methylation of the complete core region as described (Hakomori, 1964) The sample was

hydrolyzed with 2 M trifluoroacetic acid (100°C, 2 h), carbonyl-reduced, carboxy-methylated, carboxyl-reduced with $NaBD_4$ (4°C, 20 h) acetylated and analyzed by GLC-MS.

Total fatty acid content was obtained by acid hydrolysis. LOS was first treated with 4M HCl (4h, 100°C) and then neutralized with 5M NaOH (30 min, 100°C). Fatty acids were then extracted in $CHCl_3$, methylated with diazomethane and analyzed by GLC-MS. The absolute configuration of fatty acids was determined as described (Rietschel, 1976)

NMR Spectroscopy

For structural assignments, 1D and 2D ^{1}H-NMR spectra were recorded on a solution of 0.5 mg in 0.5 mL of D_2O, at 300 K, at pD 7 (uncorrected value); on Bruker 600 DRX equipped with a cryo probe. Spectra were calibrated with internal acetone [δ_H 2.225, δ_C 31.45]. 2D-DQF COSY spectra were acquired with 4096×512 data points in both F_2 and F_1 dimensions. Quadrature indirect dimensions were achieved through States-TPPI method; spectra were processed applying a Qsine function to both dimensions and data matrix was zero-filled by factor of 2 before Fourier transformation. Coupling constants were determined on a first order basis from 2D phase sensitive DQF-COSY (Piantini et al., 1982; Rance et al., 1983). NOESY and (ROESY) and spectra were measured using data sets ($t_1 \times t_2$) of 4096×256 points, mixing times of 200-400 ms were used. Total correlation spectroscopy experiments (TOCSY) were performed with a spinlock time of 100 ms, using data sets ($t_1 \times t_2$) of 4096×256 points. In these homonuclear experiments the data matrix was zero-filled in the *F1* dimension to give a matrix of 4096 x 2048 points and was resolution enhanced in both dimensions by a 90° shifted Qsine function before Fourier transformation. Heteronuclear single quantum coherence (HSQC), HSQC-TOCSY and heteronuclear multiple bond correlation (HMBC) experiments were measured in the ^{1}H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain, using data sets of 2048×256 points. Experiments were carried out in the phase-sensitive mode according to the method of States *et al*. (States et al., 1982) ^{1}H,^{13}C HMBC spectra were optimized for 4, 6 an 10 Hz coupling constants, and ^{1}H,^{31}P HSQC for 8 Hz coupling constant. In all heteronuclear experiments the data matrix was extended to 2048×1024 points using forward linear prediction extrapolation. *F2-coupled* HSQC was recorded

with 4096× 128 data points, the FID was apodised in both dimensions with a 90° shifted cosine function and zero-filled to give, after Fourier transformation, a 2D spectrum of 8192 × 512.

MALDI-TOF analysis

MS analysis of the *core* oligosaccharide was performed in reflector mode and in negative polarity on a Perseptive (Framingham, MA, USA) Voyager STR instrument equipped with delayed extraction technology. Such acidic OS was first converted in the ammonium form by a home-made miniaturized column of cation-exchange resin (Dowex 50WX8-200, Sigma Aldrich) previously equilibrated with a 5% NH_4OH solution. The obtained sample was analyzed in a matrix solution of dihydroxybenzoic acid (DHB) 50 mg/ml in TFA 0.1%-ACN 80/20, utilizing the classic dried drop method: 1 µl of a sample/matrix solution mixture (1:1, v/v) was deposited onto the MALDI sample plate and left to dry at room temperature.

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Figure caption

Figure 1. a) The ^{1}H-NMR spectrum of oligosaccharide (OS) isolated by the LOS from *Loktanella rosea*. Spin system are as described in Table 1; **b)** Zoom of the anomeric region of the proton and the *F2-coupled* HSQC spectra in which are shown the diagnostic heteronuclear anomeric constants.

Figure 2. Zoom of the TOCSY (black) and T-ROESY (red) spectra in which the key interresidual NOE contacts are indicated.

Figure 3. Zoom of the HSQC (red) and HMBC (black) spectra in which the key interresidual scalar long range correlations are indicated.

Figure 4. The structure of the oligosaccharide isolated by the LOS from *L. rosea.* Monosaccharides are labelled as in Table 1.

Figure 5. Negative-ion MALDI mass spectrum of the OS obtained after O/N de-acylation of *Loktanella rosea* R-LPS. The mass spectrum acquired in negative polarity shows a peak at *m/z* 1186.3, matching with the ion [**OS -** H_2O]$^-$. The ion [**OS -** H_2O+Na]$^-$is also visible.

Table 1. ^{1}H, ^{13}C NMR chemical shifts (ppm) and anomeric coupling constants of sugar residues of the core region of the LOS extracted from *L. rosea*

Chemical shift δ ($^{1}H/^{13}C$)									
Unit	1	2	3	4	5	6	7	8	9
A	5.23	3.76	3.81	4.17	4.33	---			
1–α-GalA	100.6	68.0	69.2	70.6	72.7	175.9			
	$^{1}J_{C,H}$=172.5 Hz		$^{3}J_{H1,H2}$=3.6 Hz						
B	4.86	3.09	3.55	3.33	3.53	3.69/4.11			
6–β-GlcN	99.6	55.4	71.9	69.6	75.5	71.1			
	$^{1}J_{C,H}$=162.0 Hz		$^{3}J_{H1,H2}$=8.4 Hz						
C	4.48	2.92	3.66	3.42	3.45	3.79/3.60			
6–β-GlcN	100.8	55.7	71.6	69.3	74.8	62.2			
	$^{1}J_{C,H}$=163.1 Hz		$^{3}J_{H1,H2}$=8.4 Hz						
D	---	---	1.62/2.67	3.70	3.44	4.01	3.80	3.88	3.60/3.8
t-α-Neu	172.7	100.5	36.3	68.2	51.8	71.6	69.0	71.2	62.7
E	---	---	1.65/2.05	3.99	3.88	3.63	3.88	3.67/3.81	
t-α–Kdo	---	99.5	34.4	65.9	66.3	72.1	70.1	62.3	
F	---	---	1.73/2.01	4.07	3.99	3.38	3.85	3.78/3.65	
4,8–α-Kdo	176.7	98.2	33.8	68.3	63.9	72.1	68.9	62.2	

Figure 1

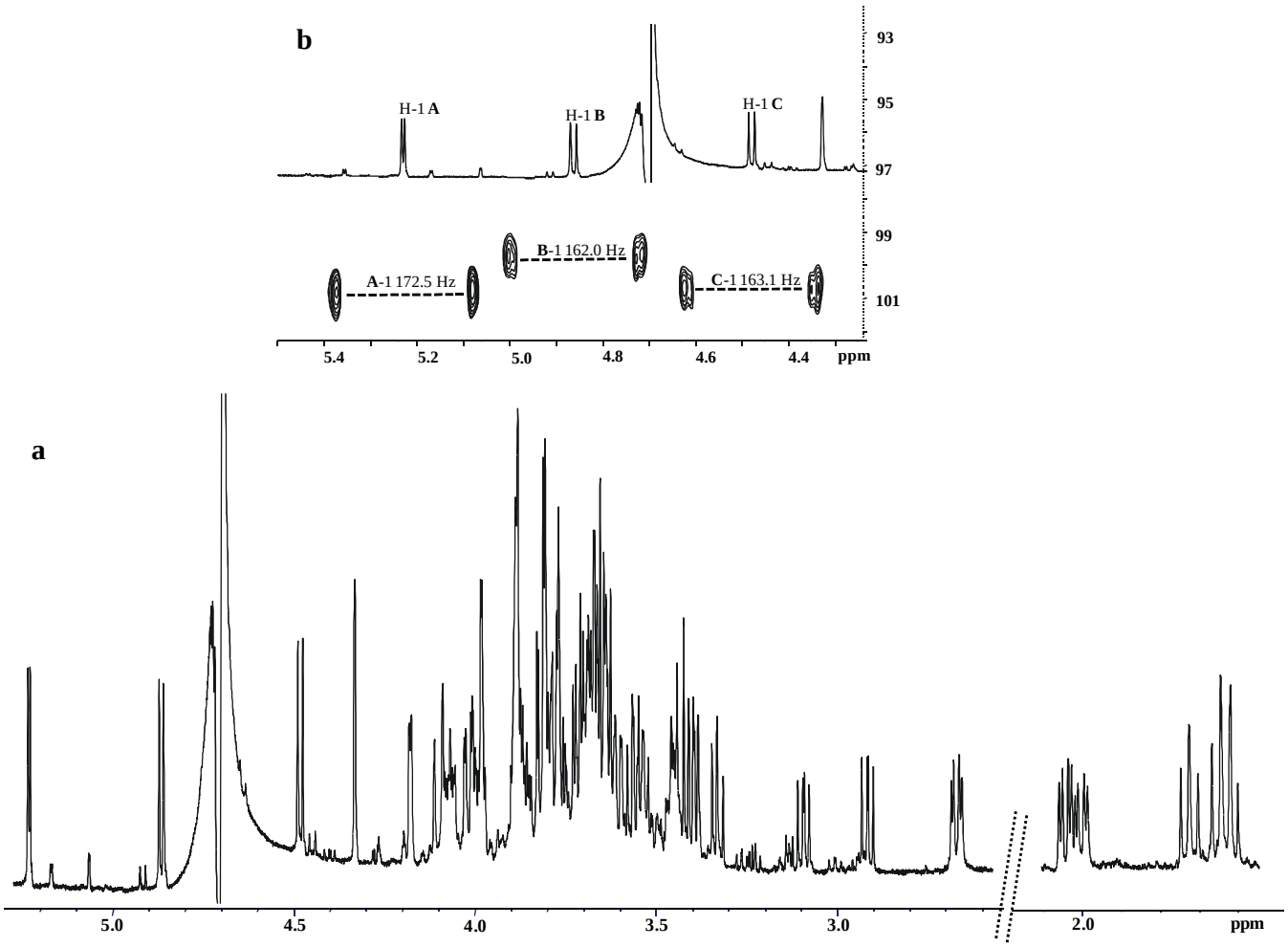


Figure 2

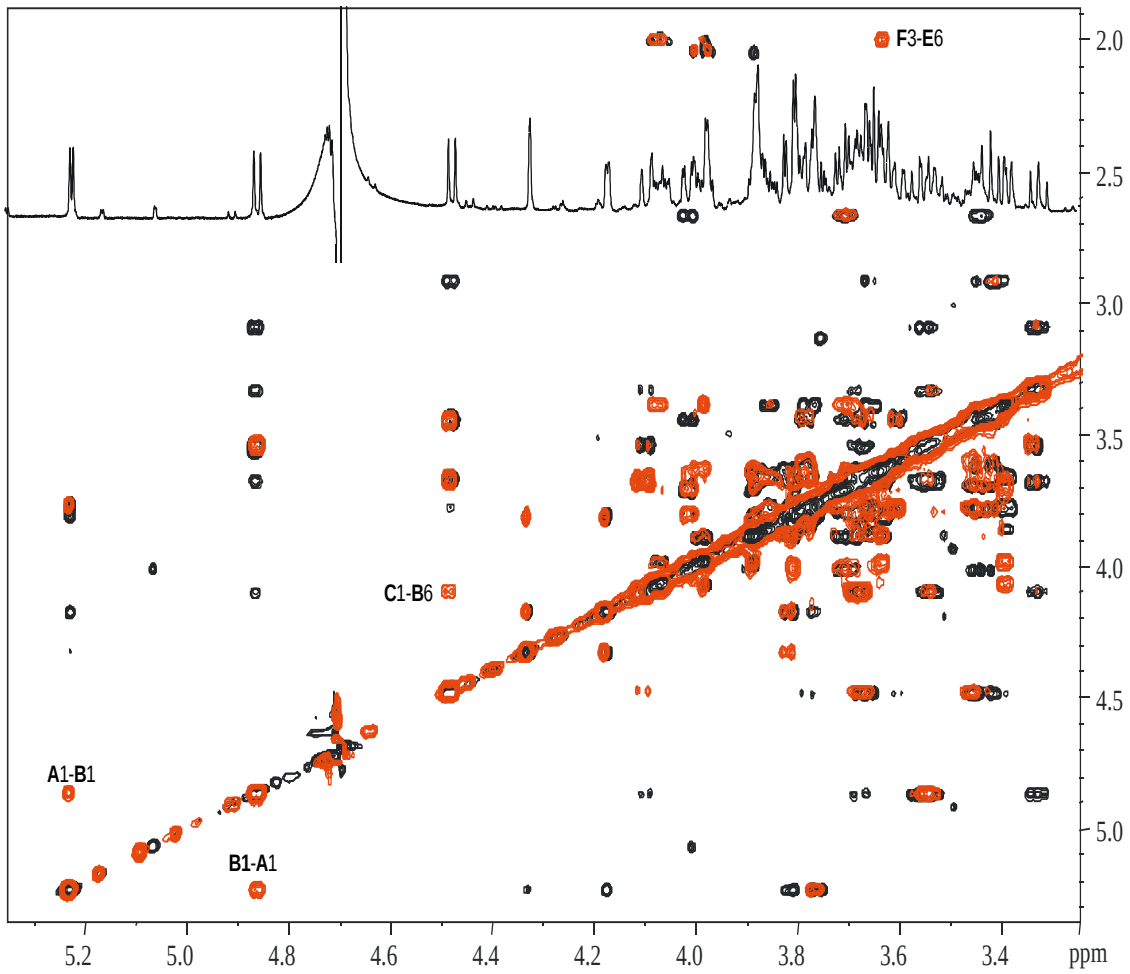


Figure 3

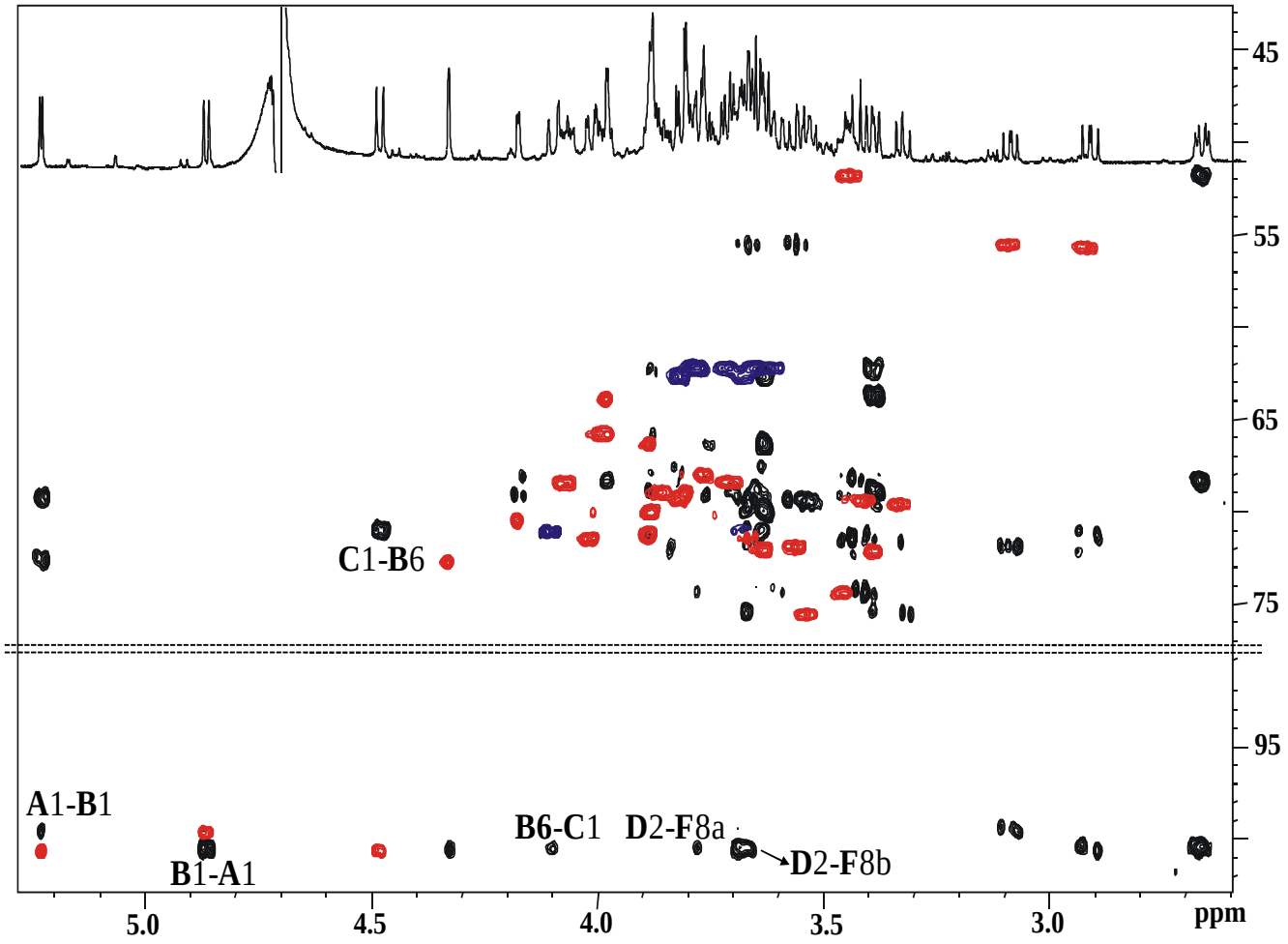
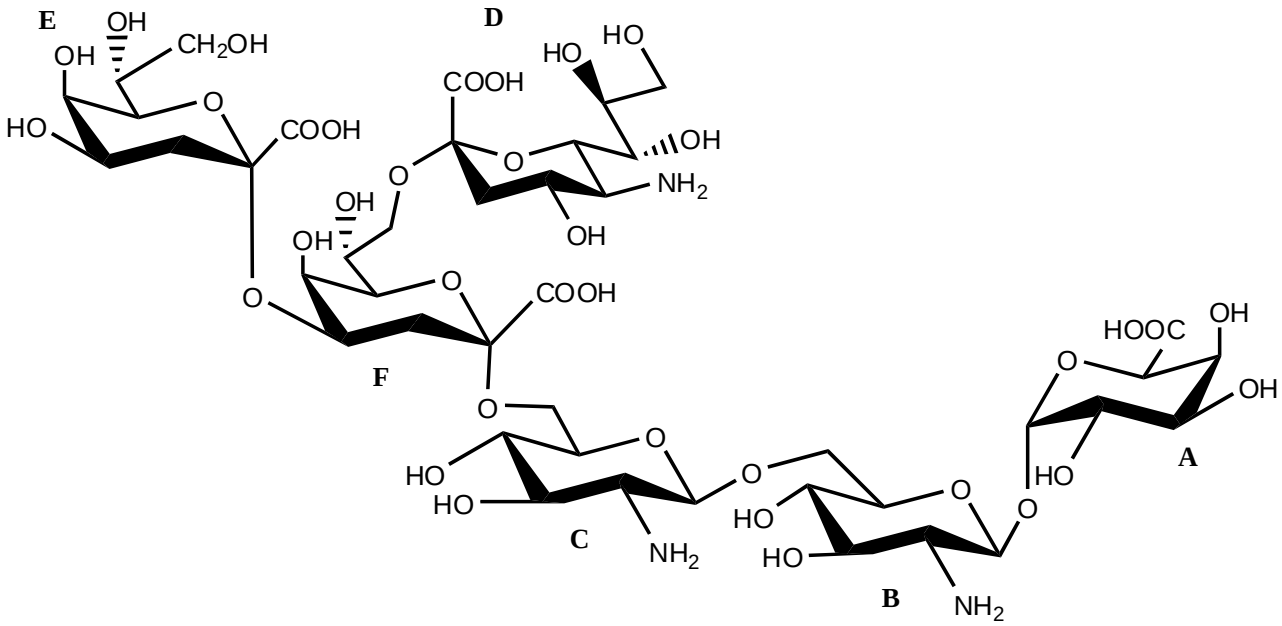


Figure 4



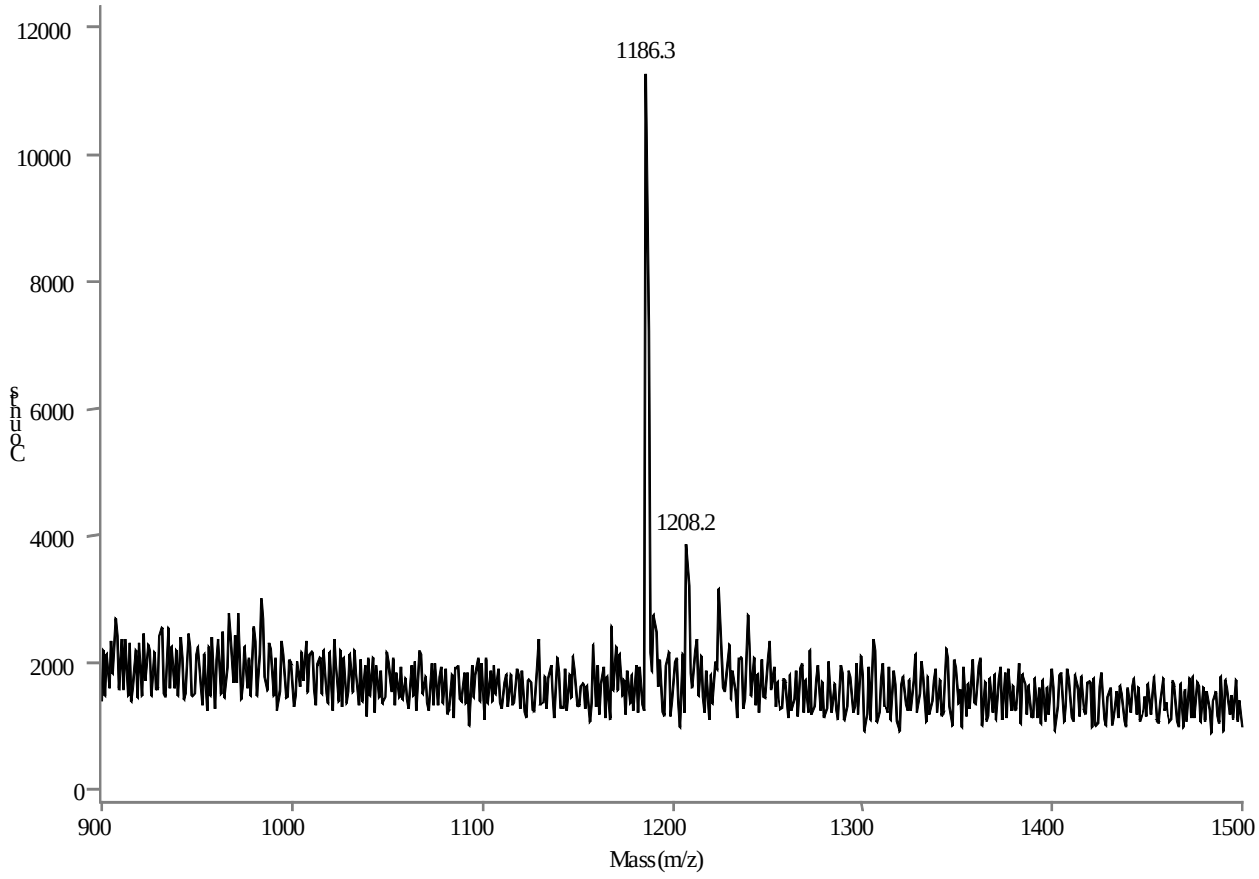


Figure 5