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Carbohydrate Research 339 (2004) 2241–2248
Carbohydrate RESEARCH

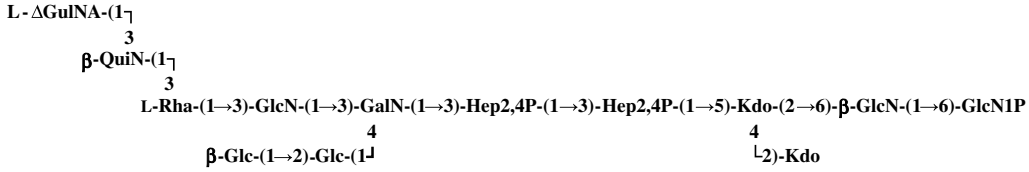
The structure of the phosphorylated carbohydrate backbone of the lipopolysaccharide of the phytopathogen bacterium *Pseudomonas tolaasii*

Alba Silipo, Serena Leone, Antonio Molinaro,* Rosa Lanzetta and Michelangelo Parrilli

Dipartimento di Chimica Organica e Biochimica, Università di Napoli Federico II, via Cintia 4, 80126 Napoli, Italy

Received 15 April 2004; received in revised form 14 June 2004; accepted 27 June 2004
Available online 22 July 2004

Abstract—A novel core-lipid A backbone oligosaccharide was isolated and identified from the lipopolysaccharide fraction of the mushrooms pathogen bacterium *Pseudomonas tolaasii*. The oligosaccharide was obtained by alkaline treatment of the lipopolysaccharide fraction. Since the repeating unit of the O-antigen contained one residue of →4)-α-L-Gul*p*NAcAN, the hydrolysis was accompanied by β-elimination on this residue and following depolymerization, producing a mixture of oligosaccharides. The complete structural elucidation showed the presence of a single core glycoform and was achieved by chemical analysis and by ^{1}H, ^{31}P, and ^{13}C NMR spectroscopy applying various 1D and 2D experiments.



All sugars are α-D-pyranoses, if not stated otherwise. Hep is L-*glycero*-D-*manno*-heptose, Kdo is 3-deoxy-D-*manno*-oct-2-ulosonic acid, P is phosphate. QuiN and ΔGulNA are present in nonstoichiometric amount.

Keywords: *Pseudomonas tolaasii*; Lipooligosaccharide; NMR spectroscopy; Phytopathogen bacteria

1. Introduction

Pseudomonas tolaasii is a mushroom pathogenic Gram-negative bacterium, responsible of the brown blotches disease on cultivated mushrooms as *Agaricus bisporus*, *Pleurotus ostreatus*, *Flammulina velutipes*, and *Lentinula edodes*. The diseases caused by this bacterium are recognized as the main cause of the crop losses in mushrooms growing houses, for an estimated amount of 8–10% world wide.[1,2]

Like the majority of Gram-negative bacteria, *P. tolaasii* possesses lipopolysaccharides (LPSs) in the outer leaflet of the external cellular membrane and these are deemed to play a key role in the interaction of bacterial cells with host organism, by activating defensive system and inducing the production of antimicrobial peptides.[3] LPSs are structurally composed of three regions, chemically and biogenetically different, named the lipid A, core and O-polysaccharide chain (or O-antigen). The occurrence of all three components allows to indicate a LPS as 'S-LPS'.

While the O-chain region of *P. tolaasii* strain NCPPB 2196 was already investigated,[4] nothing is known about the structure of the core region of this lipopolysaccharide. The structure of lipid A-core region as well as the

* Corresponding author. Tel.: +39-081-674124; fax: +39-081-674393; e-mail: molinaro@unina.it

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doi:10.1016/j.carres.2004.06.015

linkage between core and the O-chain of *P. tolaasii* lipopolysaccharide was elucidated within this frame. The complete structural characterization was achieved by chemical analysis and 2D NMR spectroscopy of the product obtained by hot alkaline degradation of the LPS fraction. Actually, exploiting the alkaline reactivity of the 4-substituted guluronamide residues present in the O-polysaccharide, an oligosaccharide was obtained.[5]

2. Results and discussion

LPS from *P. tolaasii* NCPPB 2196 was completely deacylated by mild hydrazinolysis and strong alkaline hydrolysis, following the same procedure of Knirel and co-workers.[5] Since the O-antigen contained in the repeating unit one residue of →4)-α-L-Gul*p*NAcAN, the hydrolysis was accompanied by β-elimination on this residue and depolymerization, producing a mixture of oligosaccharides deprived of O-polysaccharide.

The compositional monosaccharide analysis of the oligosaccharide fraction led to the identification, of L,D-Hep, D-HexN (successively identified as GalN and GlcN), D-QuiN, L-Rha D-Glc, Kdo. Methylation analysis showed the presence of terminal Kdo, terminal glucose, terminal QuiN, 6-substituted-GlcN, 3-substituted-Hep, 2-substituted-Glc, 3-substituted-GlcN, 3-substituted-Rha, 3-substituted-QuiN, 3-substituted-GlcN, 4,5-disubstituted-Kdo, and 3,4-disubstituted-GalN. The ^{1}H NMR spectrum (Fig. 1) showed a highly crowded anomeric region due to the presence of several modifications of the oligosaccharide. The unique olefin signal of H-4 of ΔHexNA at δ 5.82 ppm was evident and, in addition, signals were apparent for the H-3_{ax}/H-3_{eq} of two Kdo residues (**K** and **L**) at δ 1.66/1.95 and δ 1.90/2.21 ppm, respectively. Methyl signals of 6-deoxy-residues were also recognizable in the region between 1.10 and 1.22 ppm. Because of the low amount of the starting product, it was not worth purifying the oligosaccharide fraction by HPAEC, and so, the 2D NMR characterization was carried out on the mixture.

A full 2D NMR analysis (DQF-COSY, TOCSY, NOESY, HSQC, and HMBC) was very significant to clarify the primary structure of this heterogeneous product. Anomeric and relative configurations were assigned on the basis of chemical shifts and ring proton $^{3}J_{H,H}$ values obtained from the DQF-COSY spectrum. All sugar residues were present as pyranose rings, according to either ^{13}C chemical shift values and the occurrence of long range correlations between C-1/H-1 and H-5/C-5 in the ^{1}H, ^{13}C HMBC spectrum (for Kdo residues between C-2 and H-6).

A main series of spin systems was identified whose anomeric protons occurred at 5.32 (**A**), 5.26 (**B**), 5.15 (**C**), 5.22 (**D**, **D′**), 5.12 (**E**), 5.09 (**E′**), 4.46 (**F**), 4.27 (**H**) ppm, and, furthermore, the AB diasterotopic methylene signals of two Kdo (**K** and **L**) residues (Table 1). The assignment of the inner part of the core-lipid A

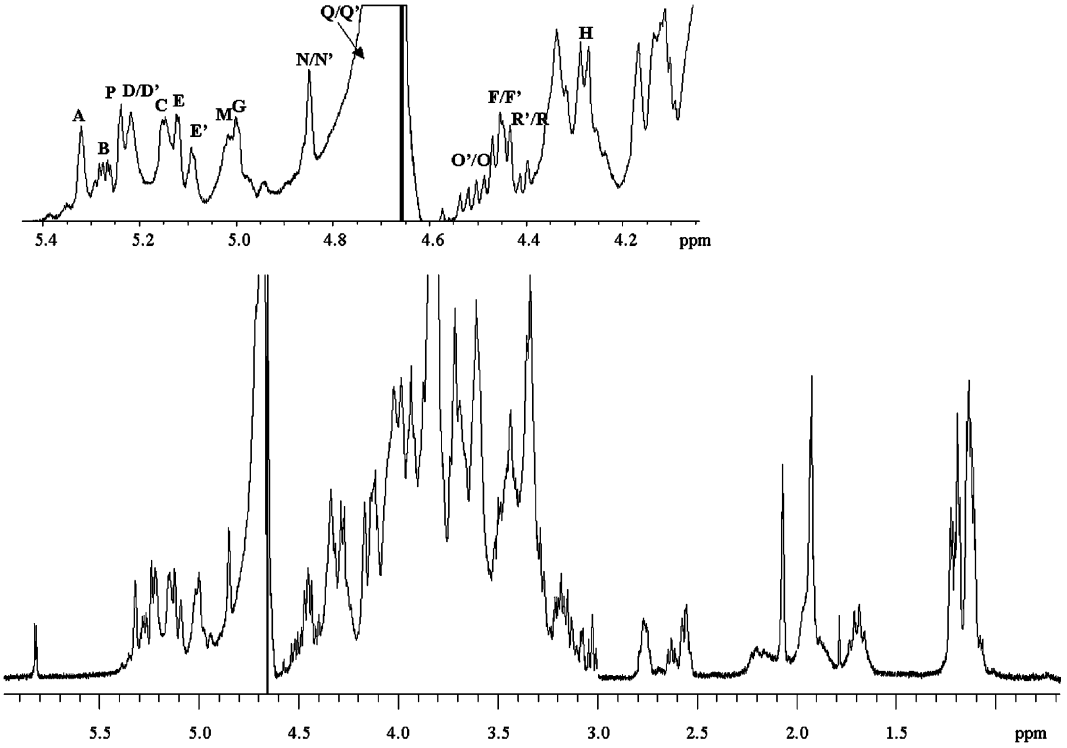
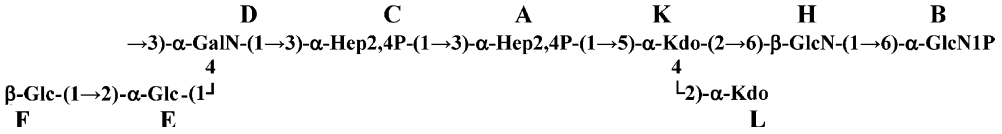


Figure 1. ^{1}H NMR spectrum of oligosaccharide mixture obtained by alkaline degradation of the whole LPS and expansion of the anomeric region. Anomeric signals are designated by capital letters.

region was straightforward owing to the low heterogeneity of NMR signals of the residues and the close NMR data similarity to those already published on the inner core region of *Pseudomonas* lipopolysaccharides.[5] Briefly, spin systems **A** and **C** were identified as α-heptose residues since they possessed low $^{3}J_{H-1,H-2}$ and $^{3}J_{H-2,H-3}$ values, diagnostic of H-2 equatorial orientation, and, in the TOCSY spectrum from H-2 it was possible to assign all the other cross peaks. Spin systems **B**, **E**, **F**, and **H** all possessed large ring proton $^{3}J_{H,H}$ values typical of *gluco*-configuration, spin system **D** possessed low $^{3}J_{H-3,H-4}$ and $^{3}J_{H-4,H-5}$ values (3 and 1 Hz, respectively) and thus H-5 resonance was confirmed by NOE correlation with H-4 in the NOESY spectrum. The proton resonances of the two Kdo residues (**K** and **L**) were assigned by COSY, TOCSY, and NOESY, in particular, NOESY experiment was useful in the identification of H-6 resonance of both residues, given the very low $^{3}J_{H-5,H-6}$ value (less than 1 Hz). The ^{13}C NMR chemical shifts could be assigned by a HSQC experiment, using the assigned proton resonances and the full data provided by 2D NMR spectra allowed the identification of the inner part of the core-lipid A region as follows: 6-α-GlcN (**B**), 6-β-GlcN (**H**), 3-α-Hep (**A** and **C**), 3,4-α-GalN (**D**), 2-α-Glc (**E**), and t-β-Glc (**F**), 4,5-Kdo (**K**), and terminal Kdo (**L**).

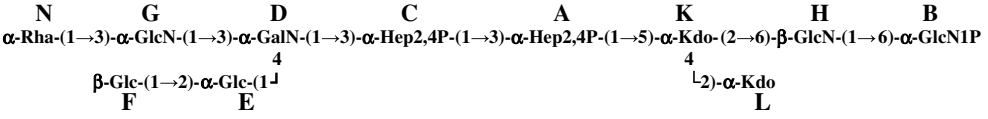
The ^{1}H NMR resonance values for residues heptoses **A** and **C** suggested phosphorylation at O-2 and O-4 of both residues. This hypothesis was confirmed by a ^{1}H, ^{31}P HSQC spectrum, in which H-2 and H-4 of both **A** and **C** residues correlated to ^{31}P signals (Table 1). In addition, the expected correlation between H-1**B** and a ^{31}P signal (3.61 ppm) was also found, confirming phosphorylation at the anomeric position of α-GlcN of lipid A. On the other hand, no correlation was apparent for the expected phosphate group at O-4 of β-GlcN. Therefore, one can presume that in the LPS, at this position, a phosphodiester/pyrophosphate group could be attached, that in harsh alkaline conditions is totally cleaved.

The sequence of monosaccharides of the inner core region was established according to the NOE signals found in the NOESY spectrum (Fig. 2). NOE cross peaks were found between H-1**H**/H-6**B**, H-3$_{eq}$**K**/H-6**L**, and H-1**A**/H-5**K**, these data allowed to confirm the typical inner core backbone composed of α-L,D-Hep-(1→5)-[α-Kdo-(2→4)]-α-Kdo.[5–7] Moreover, interresidual dipolar couplings occurred between H-1**C**/H-3**A**, H-1**D**/H-3**C**, H-1**E**/H-4**D**, H-1**F**/H-2**E**. NOE data together with ^{13}C glycosylation shifts, methylation data, and HMBC spectrum analysis allowed the complete sequence assignment of the inner part of the core region. All data merged into the following partial substructure:



In the 2D NMR spectra other spin systems were visible. One of these (**G**) possessed the typical chemical shifts and $J_{H,H}$ values of α-*gluco*-configured monosaccharide and, in the ^{1}H–^{13}C HSQC spectrum, its H-2 signal correlated to a nitrogen bearing carbon signal at 54.1 ppm, therefore, it was identified as α-GlcN (**G**). In the NOESY spectrum, H-1**G** gave NOE with H-3**D**, thus, indicating attachment of **G** residue at O-3 of GalN **D** (Fig. 2).

Another spin system at δ 4.85/101.5 ppm (residue **N**) was characterized by low $^{3}J_{H-1,H-2}$ and $^{3}J_{H-2,H-3}$ values, indicative of α-*manno*-configuration, and in addition, starting from H-2**N** proton signal and from a methyl signal at δ 1.13 ppm, all other cross peaks within the spin system were assigned by TOCSY spectrum, leading to identification of residue **N** as rhamnose. In the NOESY spectrum, H-1**N** gave NOE effect with H-3**G**, thus, indicating linkage of **N** residue at O-3 of GlcN **G**. ^{13}C carbon resonances were in agreement with the presence of **N** as terminal residue. So, the first complete oligosaccharide (**1a**) is the following:



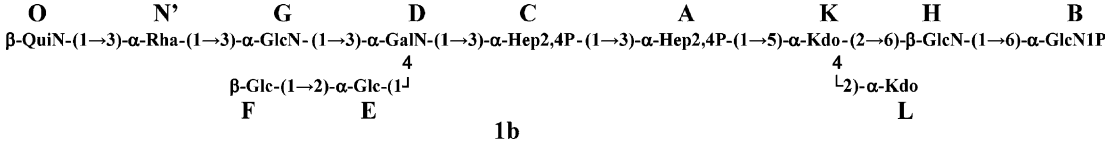
1a

A variant of this oligosaccharide was characterized by an additional spin system (**O** residue, H-1/C-1 at 4.51/105.8 ppm) together with some alterations of resonances for **N** (**N**′, δ 4.84/101.8 and H-3/C-3 at 3.80/77.0 ppm). Spin system **O** showed typical chemical shifts and $J_{H,H}$ values of a β-*gluco*-configured residue, moreover, in the TOCSY spectrum correlations were visible from H-1 proton signal to a methyl signal at δ 1.19 ppm whereas in HSQC spectrum H-2**O** proton signal correlated to a nitrogen bearing carbon 57.6 ppm. The other ring ^{13}C carbon resonances were in agreement with the presence of **O** as terminal residue. Spin system **O** was consequently identified as terminal β-QuiN, the first monosaccharide of the O-polysaccharide chain and a NOE correlation between H-1**O** and H-3**N**′ indicated

Table 1. ^{1}H, ^{13}C (bold), and ^{31}P (italic) NMR chemical shifts (ppm) of deacylated lipopolysaccharide from *P. tolaasii*

	1(3)	2(4)	3(5)	4(6)	5(7)	6(8)	7
A	5.32	4.29	3.99	4.33	4.24	4.06	3.92/3.72
α-Hep	**97.6**	**73.8**	**77.2**	**73.0**	**72.9**	**69.4**	**63.4**
		4.28		*5.23*			
B	5.26	2.57	3.79	3.43	4.14	3.88/4.19	
α-GlcN	**92.4**	**55.9**	**70.3**	**71.2**	**69.2**	**70.3**	
	3.61						
C	5.15	4.23	4.03	4.48	4.01	4.01	3.75/4.09
α-Hep	**102.2**	**71.9**	**78.1**	**72.8**	**74.1**	**69.9**	**63.7**
		4.00		*5.23*			
D	5.22	3.11	3.87	4.16	4.11	3.92	
α-GalN	**101.5**	**51.4**	**76.7**	**77.5**	**65.7**	**61.8**	
D′	5.22	3.16	3.89	4.16	4.10	—	
α-GalN	**101.5**	**50.8**	**76.7**	**77.5**	—	—	
E	5.12	3.46	3.82	3.36	3.69	3.80	
α-Glc	**100.9**	**82.2**	**70.7**	**75.9**	**72.8**	**61.1**	
E′	5.09	3.43	3.86	3.30	3.79	3.81	
α-Glc	**100.5**	**82.2**	**71.3**	**75.9**	**73.5**	**61.2**	
F	4.46	3.32	3.60	3.80	3.58	3.24/3.26	
α-Glc	**103.0**	**76.6**	**72.4**	**73.0**	**70.8**	**61.7**	
G	5.00	2.74	3.60	3.79	3.60	3.35/3.27	
β-GlcN	**97.4**	**54.1**	**82.9**	**73.1**	**73.5**	**61.8**	
H	4.27	2.92	3.78	3.38	3.56	3.54/3.65	
α-GlcN	**104.7**	**56.6**	**74.1**	**71.2**	**75.5**	**63.5**	
K	1.90/2.22	4.00	4.11	3.61	3.92	3.82	
α-Kdo	**34.6**	**73.2**	**70.2**	**70.8**	**69.2**	**64.2**	
L	1.66/1.95	4.12	4.25	3.73	3.94	3.44	
α-Kdo	**34.6**	**66.0**	**67.5**	**72.8**	**69.1**	**64.2**	
M	5.02	4.05	3.71	3.41	3.73	3.80	
α-GlcN	**97.4**	**52.9**	**79.9**	**71.2**	**71.5**	**61.2**	
N′	4.85	3.99	3.67	3.34	3.89	1.13	
α-Rha	**101.5**	**70.2**	**70.3**	**69.7**	**69.3**	**16.8**	
N′	4.84	4.14	3.80	3.49	3.93	1.14	
α-Rha	**101.8**	**69.3**	**77.0**	**68.6**	**69.3**	**16.8**	
O	4.51	2.55	3.22	3.02	3.38	1.18	
β-QuiN	**105.8**	**57.6**	**76.0**	**75.6**	**72.2**	**17.2**	
O′	4.53	2.77	3.43	3.16	3.41	1.19	
β-QuiN	**104.4**	**57.8**	**85.2**	**73.5**	**69.2**	**17.3**	
P	5.24	3.07	4.10	5.82			
α-GulNAΔ	**100.2**	**50.6**	**67.8**	**107.1**	**144.4**	**169.5**	
Q	4.82	4.00	3.89	3.34	3.90	1.11	
α-Rha	**101.1**	**72.9**	**68.8**	**69.7**	—	**16.7**	
Q′	4.81	3.81	4.02	—	—	—	
α-Rha	**101.5**	**70.8**	**80.2**	—	—	—	

its linkage to O-3 of Rha**N′**. Thus, the second core variant (**1b**) possesses one more monosaccharide, which allowed the identification of the mode and site of linkage of O-polysaccharide to core.



1b

Furthermore, it was possible to recognize a third variant (**1c**) of this oligosaccharide possessing another spin system (**P**) and differences of chemical shifts for **O** residue (**O**)′, for example, C-3 signal of **O′** spin system was found with a large downfield displacement at 85.2 ppm, indicative of glycosylation at that position. A NOE correlation between H-3**O′** and H-1**P** was visible in the NOESY spectrum, thus indicating that **P** residue is attached at O-3**O′**. Residue **P** was identified as the remainder of 4-α-L-Gul*p*NAcAN left after β-elimination, since in the TOCSY spectrum its anomeric signal correlated to H-4**P** olefin signal at 5.82 ppm. These other data allowed the recognition of the third and last oligosaccharide variant (**1c**) below described:

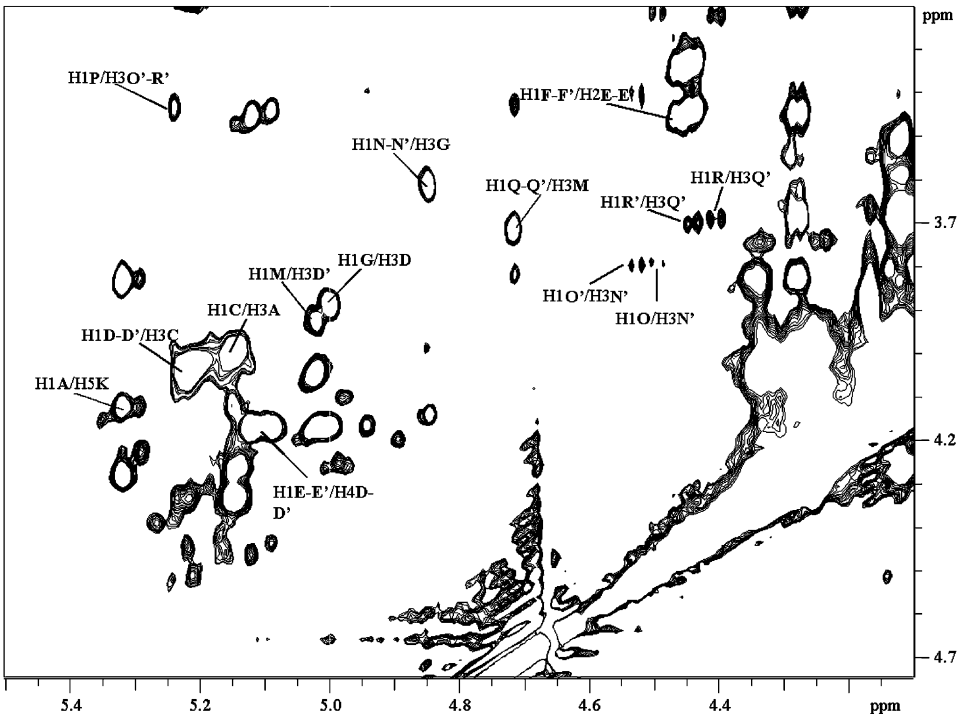
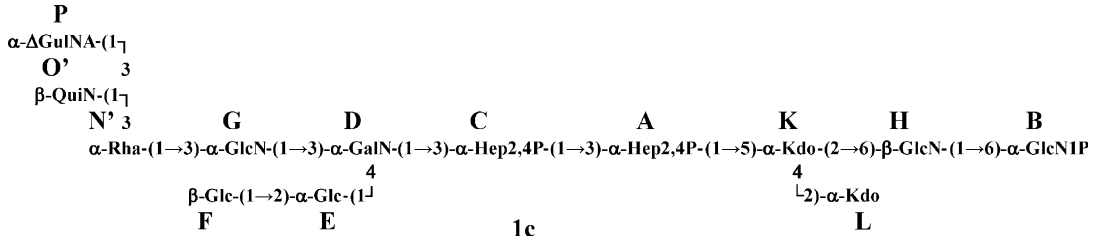


Figure 2. Section of the NOESY spectrum of oligosaccharide mixture. Annotations refer to interresidual cross peaks. The capital letters refer to residues as denoted in Table 1.



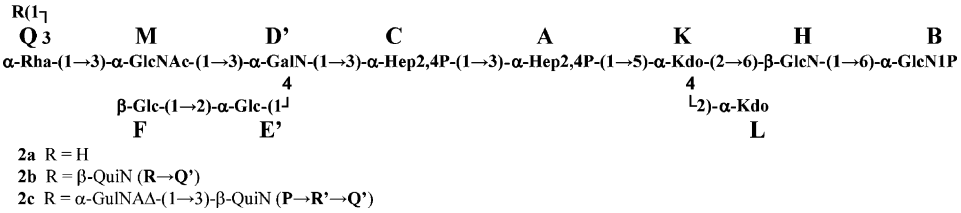
1c

In conclusion, the main oligosaccharide mixture (compounds **1a**–**c**) consists of three components that vary in the length of the fragment attached at O-3 of **G** residue. In compound **1a**, terminal Rha (**N**) is present, in **1b** disaccharide **N**′–**O** is present and this latter, in **1c**, is substituted by the O-chain remnant (**N**′–**O**′–**P**).

In the 2D NMR spectra, minor signals were present for a slightly altered oligosaccharide mixture (**2a**–**c**). An alternative spin system only differed for nitrogen substitution at C-2 of GlcN **G**. In the DQF-COSY spectrum, the anomeric proton signal of this different residue (**M**) correlated to H-2 signal at δ 4.05, indicating a downfield displacement caused by acylation at C-2 nitrogen. In agreement, the H-2**M** signal, together with a methyl signal at 2.08 ppm, correlated, in the HMBC spectrum, to a carbonyl signal at 175.6 ppm leading to the identification of α-GlcNAc (**M**). Spin systems **M** and **G** only differed in the nitrogen substitution at C-2 and thus, both accounted for a partial de-*N*-acylation of α-GlcNAc. The presence of **M** residue also caused slight alterations in **D** and **E** chemical shifts (**D**′ and **E**′). The partial de-*N*-acylation in hot alkaline environment of two contiguous 2-acetamido-2-deoxy-residues was already described and it is attributable to a structural hindrance, which prevents the full action of the alkaline reagent.[8–10] Thus, a part of the heterogeneity of the core region of the lipopolysaccharide from *P. tolaasii* is attributable to an incomplete deacylation, which yields two different residues.

The structural assignments proposed for compounds **1a**–**c** matched also for the second oligosaccharide form (**2a**–**c**), which contained, as only diversity, residue **M** (α-GlcNAc) and, of course, different spin systems of the neighboring residues identified as **Q** and **R**. Three different forms (**2a**, **2b**, **2c**) with the same structural features of oligosaccharide **1a**–**c** were detectable, thus the structural determination is not discussed even if it was worth to gain data on compounds **2** since, in this way, the presence of acetyl group present on GlcN **M** was acknowledged. NOE correlations were in full accordance with the previous results in confirming relative configurations and monosaccharides sequence of the

backbone. These results can be summarized in the following mono-acetylated oligosaccharide:



The MALDI-MS spectrum (Fig. 3) measured on the oligosaccharide mixture was in agreement with the previously proposed structure. The spectrum contained all ion peaks relative to species **1a**–**c** and **2a**–**c** and fragments arising from the very labile glycosydic bond cleavage between Kdo and the monophosphoryl lipid A moiety. This fragmentation arise from a β-elimination[11] and yields, an oligosaccharide ion (B-type ions, Domon and Costello nomenclature.[12]) The elimination provided confirmative information on the degree of phosphorylation of the core region. Since the oligosaccharide mixture is stoichiometrically penta-phosphorylated (see molecular ion peaks in Fig. 3) and the eliminated fragment only contained one phosphate group (monophosphoryl lipid A, *m*/*z* 420), the remaining core oligosaccharide must be tetra-phosphorylated, as above stated on the basis of NMR data.

Several core-lipid A backbone oligosaccharides from *Pseudomonas* strains have already been isolated and characterized,[13,14] principally from *Pseudomonas aeruginosa* strains.[15,16] The carbohydrate backbone of the so-called inner core region of *Pseudomonas* LPS determined so far has always been found to be identical in different strains,[13,16] which indicates a strict biosynthetic control. It contains two residues of 3-deoxy-D-*manno*-octulosonic acid (Kdo), two residues of L-*glycero*-D-*manno*-heptose (Hep) and a 2-amino-2-deoxy galactose residue (GalN), which is the branching point of the oligosaccharide. The inner core region is always characterized by the presence of a large number of negative charges, usually carried by phosphate groups, which are linked to heptose residues, in addition to the key phosphate residues attached to the lipid A backbone. The outer core is more variable than the inner part, usually resulting in two outer core glycoforms. However, the architecture of the outer core is common, with a GalN residue linked by two glucose moieties, one of which could carry a rhamnose residue, the key residue substituent for the O-polysaccharide transfer.[13–16]

The structure of the inner core region of the LOS from *P. tolaasii*, as determined in the present paper, was found to adhere to the general structure since it possesses the characteristic monosaccharide residues and four phosphate groups on heptose residues. Neverthe-

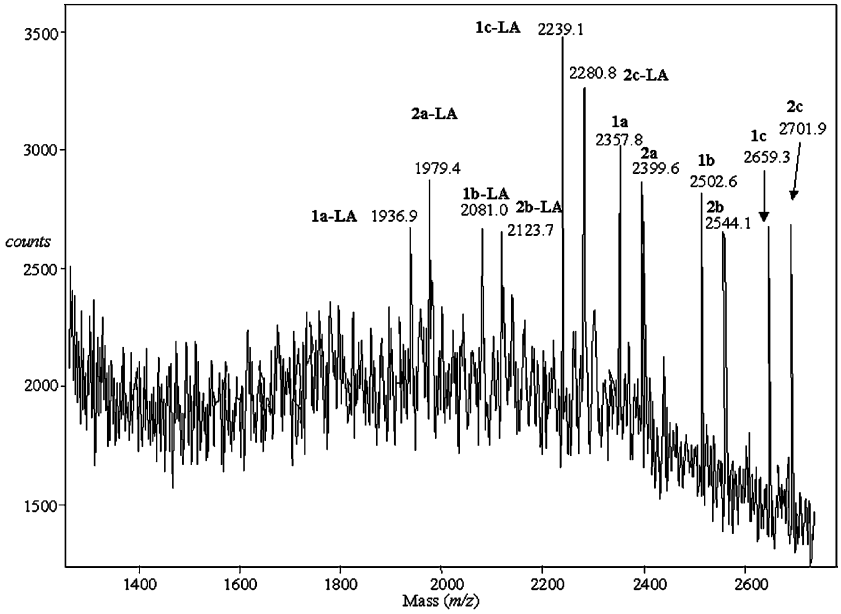


Figure 3. The MALDI-MS spectrum of the oligosaccharide mixture. In the spectrum all the ion peaks relative to species **1a**–**c**and **2a**–**c** are visible and their fragments arising from the cleavage of the very labile glycosydic bond cleavage between Kdo and the lipid A moiety (LA, monophosphoryl lipid A and H_2O, *m*/*z* 420).

less, it presents some interesting features if compared with published data on core oligosaccharides from *Pseudomonas*.[3,16] In all *Pseudomonas* core oligosaccharides, the GalN residue is substituted at O-3 and O-4 by β-glucose and α-glucose, respectively, whereas in *P. tolaasii* GalN is substituted by two α-*gluco*-configured residues, at O-3 by glucose and at O-4 by GlcNAc. To our knowledge, a GlcNAc residue directly linked to GalN is a fairly rare substitution in the core region of *Pseudomonas*, since it was only found in *P. cychorii*[17] and *P. stutzeri* LPSs.[18]

Furthermore, β-QuiN was identified as the attachment point of O-polysaccharide chain to core, and, interestingly, this residue does not possess the reverse anomeric orientation, as found in other core regions,[16] namely it was present with same anomeric linkage in the O-polysaccharide, →4)-α-L-Gul*p*NAc3AcAN-(1→3)-β-D-QuiNAc*p*-(1→.

According to the Wzy biosynthetic pathway, O-polysaccharide is polymerized by successive couplings of the oligosaccharide biological repeating unit that is previously assembled. The finding of a single β-QuiN appended as nonreducing end would not be possible if a disaccharide unit is pre-assembled by the Wzy biosynthesis. This could be explained either with a complete destruction of the uronic residue under alkaline hydrolysis,[9] or with a diverse biosynthetic pathway working for O-polysaccharide biosynthesis of the LPS from *P. tolaasii*.

Despite the numerous core structural variants isolated from LPS of *P. tolasii*, all structures agree with one single core glycoform to which O-chain is attached. The rhamnose residue can be considered the last monosaccharide of the outer core region to which is attached in one case a single-QuiN unit, otherwise the whole O-polysaccharide chain is attached.

3. Experimental

3.1. Growth of *P. tolaasii* and isolation of LPS

P. tolaasii strain NCPPB2192 was grown as described.[4] Bacterial cells were extracted according to the hot phenol–water method[19] and LPS recovered in water phase.[4]

3.2. Isolation of oligosaccharides

An aliquot of LPS (40 mg) was dissolved in anhydrous hydrazine (2 mL), stirred at 37 °C for 90 min, cooled, poured into ice-cold acetone (40 mL), and allowed to precipitate. The precipitate was centrifuged (3000*g*, 30 min), washed twice with ice-cold acetone, dried, and then dissolved in water and lyophilized (32 mg, 80% of LOS). This material was subsequently de-*N*-acylated with 4 M KOH as described.[17] After desalting using a column (50×1.5 cm) of Sephadex G-10 (Pharmacia), the resulting oligosaccharide mixture represented the carbohydrate backbone of the lipid A-core region (1 mg, 2.5% of the LPS).

3.3. General and analytical methods

Determination of Kdo, neutral sugars, organic bound phosphate, absolute configuration of the hexoses and heptoses, GLC, and GLC–MS were all carried out as described elsewhere.[20–24] The methylation analysis was carried out on a de-phosphorylated sample obtained with 48% HF (4 °C, 48 h). For methylation analysis of Kdo region, LOS carboxy-methylated with methanolic HCl (0.1 M, 5 min) and consecutively with diazomethane in order to improve its solubility in DMSO. Methylation was carried out as described.[22] LOS was hydrolyzed with 2 M TFA (100 °C, 1 h), carbonyl-reduced with $NaBD_4$, carboxy-methylated as before, carboxyl-reduced with $NaBD_4$ (4 °C, 18 h), acetylated and analyzed by GLC–MS. Methylation of the complete core region was carried out as described,[25] and the sample was hydrolyzed with 4 M TFA (100 °C, 4 h), carbonyl-reduced with $NaBD_4$, carboxy-methylated, carboxyl-reduced, acetylated, and analyzed by GLC–MS.

3.4. NMR spectroscopy

For structural assignments of oligosaccharide mixture, 1D and 2D ^{1}H NMR spectra were recorded in 0.6 mL D_2O at pD 14 (uncorrected value). Two-dimensional NMR experiments were carried out at 30 °C using a Varian Inova 500 spectrometer, and ^{31}P NMR spectra on a Bruker DRX-400 spectrometer. Spectra were calibrated with internal acetone [δ_H 2.225, δ_C 31.45]. Aq 85% phosphoric acid was used as external reference (0.00 ppm) for ^{31}P NMR spectroscopy.

Nuclear Overhauser enhancement spectroscopy (NOESY) was measured using data sets ($t_1 \times t_2$) of 4096×1024 points, and 32 scans were acquired. A mixing time of 200 ms was employed. Double quantum-filtered phase-sensitive COSY experiment was performed with 0.258 s acquisition time using data sets of 4096×2048 points and 64 scans were acquired. The total correlation spectroscopy experiment (TOCSY) was performed with a spinlock time of 80 ms, using data sets ($t_1 \times t_2$) of 4096×1024 points, and 32 scans were acquired. In all homonuclear experiments the data matrix was zero-filled in the *F*1 dimension to give a matrix of 4096×2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. Coupling constants were determined on a first order basis from 2D phase-sensitive double quantum-filtered correlation spectroscopy (DQF-COSY).[26,27] The heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond

correlation (HMBC) experiments spectrum were measured in the ^{1}H-detected mode with proton decoupling in the ^{13}C (or ^{31}P) domain, using data sets of 2048×512 points, and 128 scans were acquired for each t_1 value. The experiments were carried out in the phase-sensitive mode according to the method of States et al.[28] ^{1}H, ^{13}C HMBC were optimized for 6 Hz coupling constant and ^{1}H, ^{31}P HSQC for 8 Hz coupling constant. In all the heteronuclear experiments the data matrix was extended to 2048×1024 points using forward linear prediction extrapolation.[29,30]

3.5. MALDI-MS spectrometry

MALDI-TOF analysis was conducted using a Perseptive (Framingham, MA, USA) Voyager STR instrument equipped with delayed extraction technology. Ions formed by a pulsed UV laser beam (nitrogen laser, $\lambda=337$ nm) were accelerated through 24 kV. Mass spectra reported are the result of 256 laser shots. The dried samples was dissolved in $CHCl_3/CH_3OH$ (50/50 v/v) at a concentration of 25 pmol μL^{-1}. The matrix solution was prepared by dissolving trihydroxyacetophenone (THAP) in CH_3OH/0.1% TFA/CH_3CN (7/2/1 by vol) at a concentration of 75 mg mL^{-1}. A sample/matrix solution mixture (1:1 v/v) was deposited (1 μL) onto a stainless steel gold-plated 100-sample MALDI probe tip, and left drying at room temperature.

Acknowledgements

This work was supported by grants (to M.P.) from the Ministry of University and Research (PRIN/2002). NMR experiments were carried out on a 500 MHz spectrometer of Consortium INCA (L488/92, Cluster 11) at Centro Interdipartimentale Metodologie Chimico Fisiche Università di Napoli. Authors thank Prof. N. S. Iacobellis for supplying cells of *P. tolaasii* and Prof. A. Evidente for LPS extraction.

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