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Note

The structure of the core region of the lipopolysaccharide from *Shewanella algae* BrY, containing 8-amino-3,8-dideoxy-D-*manno*-oct-2-ulosonic acid

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Abstract—The structure of the carbohydrate backbone of the lipid A–core region of the LPS from *Shewanella algae* strain BrY was analysed. The LPS was *N*,*O*-deacylated to give three products, which were isolated and studied by chemical methods, NMR and mass spectrometry:

α-Hep-(1→2)┐
α-Glc-(1→2)-α-Hep-(1→6)-α-DDHep-(1→5)-α-8-amino-Kdo4*P*-(2→6)-β-GlcN4*P*-(1→6)-α-GlcN1*P*
α-L-Rha-(1→3)-β-GlcNR-(1→7)┘

where R = Ac or H

α-Glc-(1 → 4)-β-Gal-(1 → 4)-α-DDHep-(1 → 5)-α-8-amino-Kdo4*P*-(2 → 6)-β-GlcN4*P*-(1 → 6)-α-GlcN1*P*

All monosaccharides except L-rhamnose had the D-configuration.

This LPS presents a second example (after *S. oneidensis*) of the structure with a novel linking unit between the core and lipid A moieties, 8-amino-3,8-dideoxy-D-*manno*-oct-2-ulosonic acid (8-amino-Kdo).

Keywords: LPS; Core; *Shewanella*; Kdo; 8-Amino-Kdo

1. Introduction

Shewanella spp. are Gram-negative bacteria found in many environments such as sediments, oil drilling fluids and foods. It plays a major role in the turnover of nutrients in many different ecological niches[1] and has been found in patients with bacteremia.[2] Recently we analysed the structure of the core part of the LPS from *S. oneidensis* MR-1, which contained a novel derivative of Kdo linking the core and lipid A moieties—8-amino-3,8-dideoxy-D-*manno*-oct-2-ulosonic acid (8-amino-Kdo).[3] Here we present the results of the structural analysis of *Shewanella algae* BrY LPS core, which contains the same monosaccharide. The structure of the polysaccharide part of the *S. algae* BrY LPS has been published.[4]

2. Results and discussion

LPS from the cells of *S. algae* BrY was isolated by the procedure of Darveau and Hancock.[5] Alkaline treatment of the LPS resulted in the formation of three products **1a**,**b** and **2**:

Abbreviations: LPS, lipopolysaccharide; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; 8-Amino-Kdo, 8-amino-3,8-dideoxy-*manno*-oct-2-ulosonic acid; Hep, L-*glycero*-D-*manno*-heptose; DDHep, D-*glycero*-D-*manno*-heptose; P, phosphate; HPAEC, high-performance anion-exchange chromatography; ESI-MS, electrospray ionization mass spectrometry.

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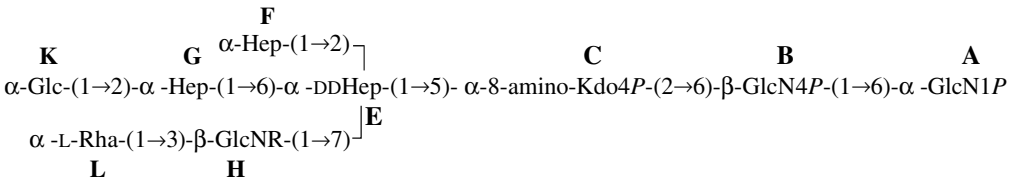
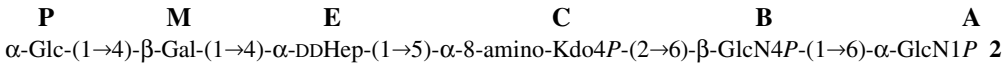


1a, R = Ac; **1b**, R = H



Although **1a** and **1b** separate well on HPAEC under analytical conditions (Fig. 1), they were not separated in a preparative run, and were analysed as a mixture. Compound **2** was isolated pure. COSY, TOCSY, ROESY, ^{1}H–^{13}C HSQC, ^{1}H–^{31}P HMQC and gHMBC 2D NMR spectra were recorded and completely assigned for **1a**,**b** and **2** following the methods described in[6] (Table 1, Fig. 2; NMR data for **1b** are not presented for brevity). The NMR spectra of **1a**,**b** showed the presence of three glucosamine residues, two LD- and one DD-heptose, α-glucose, α-rhamnose and one 8-amino-3,8-dideoxy-α-*manno*-oct-2-ulosonic acid (8-amino-Kdo). The configuration of the heptose residues F and G was determined as LD on the basis of the high-field position of C-6 signals (~70 ppm[7,8]). Since DD-Hep was also found during monosaccharide analysis, it must be the residue E (NMR data for this residue are not indicative due to the substitution). The DD-Hep in this position has also been found in other *Shewanella* strains.[3,4] Identification of 8-amino-Kdo was based on NMR data, as described in a previous publication.[3] All monosaccharides except 8-amino-Kdo were identified by GC as alditol acetates. The absolute configuration of all monosaccharides except 8-amino-Kdo was achieved by the GC identification of the acetates of 2-butyl glycosides with optically pure (*S*)-2-butanol with the respective standards prepared from known monosaccharides and (*S*)-and (*R*)-2-butanol.[9]

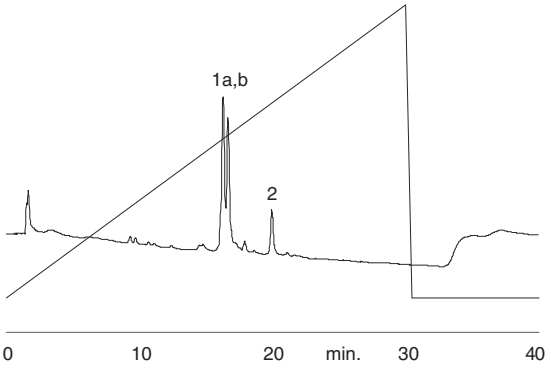


Figure 1. HPAEC separation of the alkaline deacylation products of *S. algae* LPS.

Oligosaccharides **1a** and **1b** differed in the acylation of N-2 of the GlcN residue H. The position of the H-2 signals of residue H at 3.85 in **1a** and 3.21 ppm in **1b** indicated that N-2 of this residue is acetylated in **1a** and free in **1b**. HMBC correlation between H-2 of this residue and CO of the acetyl group was observed for **1a.** The formation of **1a** is a result of the incomplete *N*-deacylation. However, the identification of this product is useful, because it shows that the amino group of the GlcN H in the LPS is acetylated (maybe partially).

The sequence of the monosaccharides was determined from the interresidual NOE correlations: B1A6, E1C5, E1C7, E1F5, F1E2, G1E5, G1E6, G1K5, K1G1, K1G2, H1E7 (weak) and L1H3, leading to the structures presented. The respective HMBC correlations were also observed: E1C5, F1E2, G1E6, K1G2, H1E7 and L1H3.

Both **1a** and **1b** contained three phosphate residues at O-1 of α-GlcN A (^{31}P signal at 1.63 ppm), O-4 of β-GlcN B (3.50 ppm), and O-4 of 8-amino-Kdo (3.33 ppm). A molecular mass of 1886.4 and 1844.4 Da was determined by ESI-MS for **1a** and **1b**, respectively, in agreement with the proposed structures.

A similar analysis was carried out for a shorter oligosaccharide **2**, leading to the structure presented.

Mild acid hydrolysis of the LPS yielded no core-related products, and thus the presence of possible alkali-labile components in the core part was not investigated. Hydrazine-*O*-deacylated LPS was analysed by ESI-MS, but no prominent peaks, which could be attributed to the *R*-form of LPS were observed.

The core region of the *S. algae* LPS contains a novel Kdo derivative, 8-amino-Kdo, previously detected in *S. oneidensis*.[3] The LPS of many bacteria contain Kdo, linking the core oligosaccharide and the lipid A, and having charged substituents at O-8 (usually 4-amino-4-deoxy-β-L-arabinopyranose or phosphoethanolamine[10,11]). The presence of a charged substituent at this position seems to be functionally important, and direct

Table 1. NMR spectral data (δ, ppm) for the oligosaccharides **1a** and **2**. Spectra were recorded in D_2O at 25 °C. The NAc methyl signal in **1a** is at 2.05/23.1 ppm ($^1H/^{13}C$)

Unit, compound	Nucleus	1	2 (3a)	3 (3e)	4	5	6	7 (6b)	8a	8b
α-GlcN A, **1a**, **2**	^1H	5.73	3.44	3.93	3.53	4.15	3.91	4.31		
	^{13}C	92.3	54.8	70.3	70.4	73.2	70.1			
β-GlcN B, **1a**, **2**	^1H	4.90	3.17	3.92	3.96	3.80	3.48	3.74		
	^{13}C	100.0	56.3	72.3	74.9	74.5	62.9			
8-amino-Kdo C, **1a**	^1H		2.05	2.25	4.62	4.31	3.83	4.03	3.12	3.53
	^{13}C	174.6	100.6	35.0	70.7	75.7	74.4	66.6	43.9	
8-amino-Kdo C, **2**	^1H		2.05	2.26	4.62	4.38	3.88	4.05	3.14	3.53
	^{13}C			35.0	70.7	73.7	74.4	66.6	44.0	
α-DDHep E, **1a**	^1H	5.15	4.06	4.11	3.75	3.98	4.21	3.87	4.08	
	^{13}C	101.3	79.1	70.8	67.8	73.9	76.1	69.7		
α-DDHep E, **2**	^1H	5.10	4.12	4.06	3.94	4.31	4.22	3.75	3.88	
	^{13}C	100.9	70.5	70.6	79.2	73.8	71.7	62.7		
α-Hep F, **1a**	^1H	5.10	4.06	3.84	3.87	3.64	4.06	3.77	3.77	
	^{13}C	102.9	70.9	71.4	66.9	73.3	69.9	64.1		
α-Hep G, **1a**	^1H	5.27	3.93	3.92	3.92	3.70	4.03	3.72	3.72	
	^{13}C	97.3	81.0	71.3	67.2	72.9	70.1	64.5		
β-GlcN H, **1a**	^1H	4.63	3.85	3.59	3.52	3.52	3.76	3.96		
	^{13}C	101.1	55.8	82.9	69.4	76.8	61.7			
α-Glc K, **1a**	^1H	5.02	3.59	3.75	3.47	3.84	3.75	3.84		
	^{13}C	101.6	72.5	73.6	70.3	72.8	61.5			
α-Rha L, **1a**	^1H	4.84	3.78	3.74	3.43	3.99	1.24			
	^{13}C	102.3	71.5	70.9	72.6	69.7	17.2			
β-Gal M, **2**	^1H	4.54	3.62	3.76	4.03	3.82	3.85	3.95		
	^{13}C	104.3	71.7	73.0	78.0	76.5	61.2			
α-Glc P, **2**	^1H	4.92	3.53	3.77	3.45	4.15	3.78	3.82		
	^{13}C	101.0	72.9	73.7	70.3	72.8	61.0			

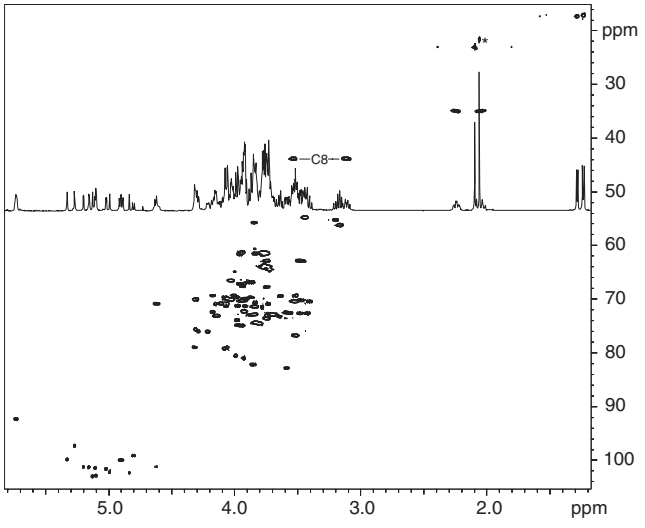


Figure 2. ^1H–^{13}C NMR HSQC correlation spectrum of the mixture of oligosaccharides **1a** and **1b**. The signal of the H/C-8 of 8-amino-Kdo is labelled C8. * signal of the residual acetic acid.

introduction of the amino group at C-8 of Kdo residue is another way of achieving this.

An interesting feature of the analysed LPS is the presence of two different structures in the core region, represented by the oligosaccharides **1** and **2**. Normally the core is heterogenous, but all variants are usually mere truncations of one maximal structure.

The *S. algae* BrY LPS contains an O-side chain with the following structure of the repeating unit:[4]

-3)-α-D-BacNAc4NBu-(1 → 3)-α-L-Rha-(1 → 2)-
α-L-Rha-(1 → 2)-L-malyl-(4 → 2)-α-L-FucN-(1-

where D-BacNAc4NBu is 2-acetamido-4-(D-3-hydroxybutyramido)-2,4,6-trideoxy-D-glucose, and L-malyl-(4 → 2)-α-L-FucN is 2-amino-2,6-dideoxy-α-L-galactose, *N*-acylated by the 4-carboxyl group of L-malic acid. This polymeric chain contains one amide linkage, which on alkaline treatment must degrade leaving at least the FucN residue linked to the core. However, isolated products of alkaline deacylation contained no FucN. This fact remains unexplained, and two options seem possible: the polymer is linked to the core through an alkali-labile bond (possibly a phosphodiester linkage), or the polymer is not linked to the isolated structures at all.

3. Experimental

3.1. Bacterial strain and growth conditions

S. algae BrY was grown at room temperature in trypticase soy broth under aerobic conditions to an optical density (OD_{600}) of 1.0 and the bacteria isolated by centrifugation at 6000 × g for 20 min. Cells were washed in HEPES buffer (pH 6.8) before LPS extraction was initiated.

3.2. LPS isolation

The method described by Hancock[11] was used. Bacterial cells were broken in a French press, the cell lysate was treated with DNase, RNase, and protease, and the LPS was eventually precipitated in ice-cold 95% ethanol containing 0.375 M $MgCl_2$.

3.3. NMR spectroscopy and general methods

NMR spectra were recorded at 25 °C in D_2O on a Varian Unity Inova 400 (for ^{31}P spectra only) and 600 instruments using acetone as reference (^1H, 2.225 ppm, ^{13}C, 31.45 ppm). Varian standard programs COSY, ROESY (mixing time of 300 ms), TOCSY (spinlock time 120 ms), HSQC and gHMBC (evolution delay of 100 ms) were used with digital resolution in F2 dimension <2 Hz/pt.

Hydrolysis was performed with 4 M CF_3CO_2H (110 °C, 3 h), and monosaccharides were conventionally converted into the alditol acetates and analysed by GLC on a Agilent 6850 chromatograph equipped with a DB-17 (30 m × 0.25 mm) fused-silica column using a temperature gradient of 180 °C (2 min) → 240 °C at 2 °C/min. GC–MS was performed on Varian Saturn 2000 system with an ion-trap mass spectral detector, using the same column. ESI-MS was carried out as described previously.[9]

Gel chromatography was carried out on Sephadex G-50 (2.5 × 95 cm) and Sephadex G-15 columns (1.6 × 80 cm) in pyridinium acetate buffer, pH 4.5 (4 mL pyridine and 10 mL AcOH in 1 L water) and the eluate was monitored by a refractive index detector.

For determination of the absolute configuration of the monosaccahrides, products **1a**,**b** and **2** (1 mg each) were treated with (*S*)-2-butanol–AcCl (0.25 mL, 10:1 v/v, 2 h, 85 °C),[9] dried under a stream of air, acetylated and analysed by GC in comparison with authentic standards prepared with (*S*)- and (*R*)-2-butanol.

Methylation analysis was performed using the Ciucanu–Kerek procedure,[12] methylated products were hydrolysed, monosaccharides converted to alditol-1-D acetates by conventional methods, and analysed by GC–MS.

3.4. *N*,*O*-Deacylation of LPS and preparation of backbone oligosaccharides[13]

LPS (120 mg) was dissolved in 4 M KOH (4 mL), and the solution was heated at 120 °C for 16 h, cooled, and neutralized with 3 M HCl. The precipitate was removed by centrifugation and the supernatant desalted by gel chromatography on Sephadex G-50. Oligosaccharides were separated by HPAEC on a CarboPac PA1 column (9 × 250 mm) with pulsed amperiometric detection, equilibrated in 0.1 M NaOH, using a linear gradient of 1 M sodium acetate in 0.1 M NaOH from 5% to 80% of acetate in 60 min at 3 mL/min. Fractions (3 mL) were collected and analysed by a Dionex system with an analytical CarboPac PA1 column (4.6 × 250 mm) at 1 mL/min. The separated oligosaccharides were desalted on a Sephadex G-15 column.

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