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The structure of the rough-type lipopolysaccharide from *Shewanella oneidensis* MR-1, containing 8-amino-8-deoxy-Kdo and an open-chain form of 2-acetamido-2-deoxy-D-galactose

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Abstract

The LPS from *Shewanella oneidensis* strain MR-1 was analysed by chemical methods and by NMR spectroscopy and mass spectrometry. The LPS contained no polysaccharide O-chain, and its carbohydrate backbone had the following structure: (1*S*)-GalNAc*o*-(1→4,6)-α-Gal-(1→6)-α-Gal-(1→3)-α-Gal-(1-*P*-3)-α-DDHep-(1→5)-α-8-aminoKdo4R-(2→6)-β-GlcN4*P*-(1→6)-α-GlcN1*P*, where R is *P* or EtN*PP*. There are several novel aspects to this LPS. It contains a novel linking unit between the core polysaccharide and lipid A moieties, namely 8-amino-3,8-dideoxy-D-*manno*-octulosonic acid (8-aminoKdo) and a residue of 2-acetamido-2-deoxy-D-galactose (*N*-acetylgalactosamine, GalNAc*o*) in an open-chain form, linked as cyclic acetal to O-4 and O-6 of D-galactopyranose. The structure contains a phosphodiester linkage between the α-D-galactopyranose and D-*glycero*-D-*manno*-heptose (DDHep) residues.

Keywords: LPS; Core; *Shewanella*; Kdo

1. Introduction

Genus *Shewanella* represent Gram-negative bacteria found in many environments such as sediments, oil drilling, and foods. These bacteria play a major role in the turnover of nutrients in different ecological niches.[1] In a few cases *Shewanella* were found in patients with bacteremia.[2] *Shewanella oneidensis* MR-1 is a subsurface bacterium that is capable of respiring certain iron oxides by using the Fe(II)/Fe(III) couple as a terminal electron acceptor.[3] An important aspect of this respiration is the closeness of fit between the outer membrane of the bacterium and the mineral surface. A previous publication using SDS-PAGE analysis[4] indicated that the LPS of this strain contains no polysaccharide O-chain, which may be important for adhesion to a mineral surface, since this polymeric substituent could affect the closeness of the bacterial attachment and inhibit mineral respiration. Recently, the complete genome sequence of this strain has been determined.[5]

Several structures of the *Shewanella* O-specific polysaccharides and core part of the LPS have been determined so far.[6–10] This article describes structural analysis of another rough type *Shewanella* LPS.

2. Results and discussion

LPS from the cells of *S. oneidensis* MR-1 was isolated by the procedure of Darveau and Hancock.[11] SDS-PAGE with subsequent silver staining showed the presence of one low-molecular-mass component without

Abbreviations: LPS, lipopolysaccharide; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; 8-aminoKdo, 8-amino-3,8-dideoxy-*manno*-oct-2-ulosonic acid; DDHep, D-*glycero*-D-*manno*-heptose; *P*, phosphate; *PP*, pyrophosphate; EtN, ethanolamine.

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the O-chain.[4] Alkaline deacylation of the LPS led to a mixture of the oligosaccharide **1**, isolated after desalting by gel chromatography. The oligosaccharide represented only part of the carbohydrate backbone of the LPS; other components strongly degraded in alkaline conditions and were not analysed. Its COSY, TOCSY, ROESY, ^{1}H-^{13}C HSQC, ^{1}H-^{31}P HMQC, and gHMBC NMR spectra were recorded and completely assigned following the methods described by Duus and coworkers[12] (Table 1). The NMR spectra showed the presence of two glucosamine residues, one heptose and one 8-amino-3,8-dideoxy-α-*manno*-oct-2-ulosonic acid (8-aminoKdo). DDHep and GlcN were identified by GC as their respective alditol acetates.

The presence of the amino group at C-8 of the oct-2-ulosonic acid residue was inferred from the high-field position of the C-8 signal at 44.8 ppm. The relative configuration of this monosaccharide was the same as of Kdo, which was concluded from close similarity of the proton coupling constants (Table 1) with those observed for an α-Kdo residue.[13] A strong NOE between H-1 of DDHep residue E and H-7 of 8-aminoKdo was observed. This correlation is always observed between Hep and Kdo in the α-Hep-(1→5)-Kdo fragment of the LPS, and indicates identical absolute configurations of these monosaccharides.[14] It would not be possible in the case of different absolute configurations of the monosaccharides. Thus one can conclude that 8-aminoKdo has the D configuration.

The sequence of the monosaccharides was determined from interresidual NOE (NOE E1C5, E1C7, B1A6 were observed) and HMBC (E1C5, C2B6, B1A6) correlations and confirmed by ^{13}C NMR chemical shifts.

Compound **1** contained four phosphate residues: at O-1 of α-GlcN A (^{31}P signal at 1.02 ppm), O-4 of β-GlcN B (3.03 ppm), O-4 of 8-aminoKdo (3.12 ppm), at O-3 of Hep E (3.12 ppm). A minor (~15%) amount of the oligosaccharide with the Hep residue phosphorylated at O-2 instead of O-3 (^{31}P signal at 3.38 ppm) was also detected. The molecular mass of 1071 Da was determined by ESIMS (expected 1071.7 Da) in agreement with the proposed formula.

α-$\overset{E}{\text{D}}$DHep3*P*-(1→5)-α-8-$\overset{C}{\text{a}}$minoKdo4*P*-(2→6)-β-$\overset{B}{\text{G}}$lcN4*P*-(1→6)-α-$\overset{A}{\text{G}}$lcN1*P*

(**1**)

Mild acid hydrolysis of the LPS gave oligosaccharides **2a** (major) and **2b** as a mixture. This mixture was reduced with $NaBH_4$ to give **3a**,**b**. Monosaccharide analysis of **2a**,**b** (GC of alditol acetates) showed the presence of galactose and galactosamine. NMR data suggested the presence in the oligosaccharides **2a** and **3a** of the open-chain acetal form of the *N*-acetylgalactosamine residue Z (Table 1, only data for **3a** are shown for clarity, since the presence of the anomeric forms of the residue W in **2a**,**b** leads to splitting of all signals of other residues; data for **3b** are of no special interest). The coupling constants pattern, incompatible with galactopyranose structure, was similar to the one observed previously in *Proteus* LPSs, containing an open-chain GalNAc residue linked as a cyclic acetal to positions 4 and 6 of the GalN residue.[15,16] The key experiment for the confirmation of this type of the linkage is an HMBC spectrum, where correlations from the Z1 proton to Gal Y C-4 and C-6 were observed (Fig. 1). Another observation was zero coupling between H-5 and H-6a,6b protons in the residue Y. This situation is well known for the 4,6-*O*-benzylydene derivatives of galactopyranose. Proton H-5 in this case has 60° dihedral angles with both H-6 protons, leading to very small coupling constants. An NOE from the Z1 proton to the Y4 and Y6 protons indicates axial position of the Z1 proton and thus the (*S*)-configuration at Z1. Another indicator of this type of the linkage between monosaccharides is the high-field position of the C-5 signal of the glycosylated monosaccharide: Y C-5 was observed at 64.5 ppm.

(1*S*)-$\overset{Z}{\text{G}}$alNAc*o*-(1→4,6)-α-$\overset{Y}{\text{G}}$al-(1→6)-α-$\overset{X}{\text{G}}$al-(1→3)-$\overset{W}{\text{G}}$al

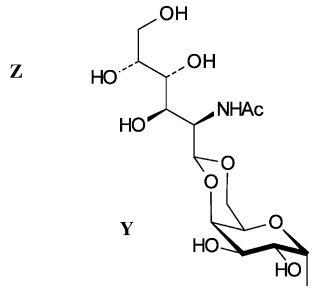
(**2a**)

α-$\overset{Y}{\text{G}}$al-(1→6)-α-$\overset{X}{\text{G}}$al-(1→3)-$\overset{W}{\text{G}}$al (**2b**)

(1*S*)-$\overset{Z}{\text{G}}$alNAc*o*-(1→4,6)-α-$\overset{Y}{\text{G}}$al-(1→6)-α-$\overset{X}{\text{G}}$al-(1→3)-$\overset{W}{\text{G}}$al-ol

(**3a**)

α-$\overset{Y}{\text{G}}$al-(1→6)-α-$\overset{X}{\text{G}}$al-(1→3)-$\overset{W}{\text{G}}$al-ol (**3b**)



Methylation of **3a**,**b** with the analysis of the products as the alditol-1*d* acetates led to the identification of terminal (minor), 6-, and 4,6-substituted galactopyranose products, and of 3-substituted galactitol. However, no products derived from GalNAc was identified. This is the result of the instability of the permethylated GalNAc, which has no possibility of hemiacetal ring

Table 1
Assigned NMR spectral data (ppm) for the isolated oligosaccharides [a]

Unit, compound	Nucleus	**1** $J_{1,2}$ or $J_{3a,3e}$	**2** or **3a** $J_{2,3}$ or $J_{3a,4}$	**3** or**3e** $J_{3,4}$ or $J_{3e,4}$	**4** $J_{4,5}$	**5** $J_{5,6a}$ or $J_{5,6}$	**6** $J_{6a,6b}$ or $J_{6,7}$	**7** or **6b** $J_{5,6b}$ or $J_{7,8a}$	**8a** or **7b** $J_{8a,8b}$	**8b** $J_{7,8b}$
A, **1**	^{1}H	5.72	3.43	3.90	3.51	4.11	4.28	3.89		
	^{13}C	93.4	55.7	71.7	71.3	74.2	70.9			
B, **1**	^{1}H	4.87	3.15	3.89	3.94	3.78	3.79	3.50		
	^{13}C	100.8	57.2	73.3	75.8	75.4	64.1			
C, **1**	^{1}H		2.15	2.25	4.60	4.34	3.86	4.10	3.04	3.50
	J, Hz	12.5	12.5	4.4	~1	~1	9	9.1	13	4
	^{13}C		101.2	35.6	71.6	75.2	75.5	67.3	44.8	
C4*P*, **4a**	^{1}H		2.03	2.26	4.46	4.25	3.77	4.04	3.23	3.47
	^{13}C			35.9	69.4	74.7	75.2	67.2	44.3	
C4*PP*, **4b**	^{1}H		2.10	2.33	4.68	4.29	3.77	4.04	3.23	3.47
	^{13}C			35.5	72.0	75.0	75.2	67.2	44.3	
E, **1**	^{1}H	5.10	4.28	4.35	3.91	4.10	4.08	3.79	3.71	
	^{13}C	102.5	70.9	77.4	67.6	74.5	74.5	64.0		
E, **4a**	^{1}H	5.09	4.28	4.43	3.81	4.34	4.14	3.68	3.78	
	^{13}C	102.3	70.8	76.1	67.0	74.3	73.4	63.7		
E, **4b**	^{1}H	5.0.7	4.28	4.42	3.86	4.05	4.12			
	^{13}C	102.3	70.8	76.1	66.3	74.3	74.6	64.3		
Z, **2a**,**3a**	^{1}H	4.85	4.32	4.11	3.34	3.90	3.64	3.61		
	J, Hz	4.7	~0	10.1	2.5					
	^{13}C	101.4	52.9	68.8	70.3	71.1	64.4			
Z, **4**	^{1}H	4.89	4.33	4.10	3.38	3.93	3.64	3.68		
	^{13}C	101.6	53.1	69.4	70.9	71.4	64.5			
Y, **3a**	^{1}H	4.99	3.92	3.99	4.21	3.87	4.12	3.99		
	J, Hz	3.5	10.7	3.9	~0	~0	13	~0		
	^{13}C	100.3	69.2	69.2	77.0	64.5	69.8			
Y, **4**	^{1}H	5.01	3.91	4.01	4.21	3.90	4.00	4.13		
	^{13}C	99.7	69.4	69.2	77.3	64.6	69.9			
X, **3a**	^{1}H	5.10	3.82	3.86	4.02	4.18	3.87	3.68		
	^{13}C	101.2	69.5	70.2	70.5	71.1	68.6			
X, **4**	^{1}H	5.18	3.85	4.09	4.01	4.18	3.69	3.91		
	^{13}C	94.6	69.6	70.5	70.8	70.6	68.4			
W, **3a**	^{1}H	3.75/3.72	4.05	3.79	3.87	4.04	3.65	3.65		
	^{13}C	64.2	73.0	79.8	71.0	71.3	64.2			
W, **4**	^{1}H	5.65	3.99	3.86	4.28	3.98	3.75	3.80		
	^{13}C	98.0	68.2	74.0	65.7	73.0	62.1			
α-W, **2a**	^{1}H	5.30	3.94	3.94	4.22	4.03	3.76	3.73		
	^{13}C	93.4	67.8	74.7	66.5	71.6	62.3			
β-W, **2a**	^{1}H	4.64	3.61	3.72	4.16	3.66	3.78	3.75		
	^{13}C	97.5	71.5	78.8	66.1	76.2	62.1			
EtN in **4b**	^{1}H	4.22	3.30							
	^{13}C	63.8	41.3							

[a] NAc methyl signal in **2** and **3** are at 2.05/23.1 ppm (^{1}H/^{13}C).

formation in the conditions of acid hydrolysis. *Proteus* LPSs contain 4- or 5-substituted open-chain GalNAc, which partly survives hydrolysis after methylation.

Compounds **2b** and **3b** without the residue Z were obtained from the fraction of the LPS naturally lacking this residue, which was confirmed during the analysis of the O-deacylated LPS.

The absolute configurations of D-galactose and D-galactosamine in **2a** were determined by GC of the acetates of their respective (*S*)-2-butyl glycosides.

In order to determine the connection between the fragments obtained after acid and base treatments within the whole LPS, NMR analysis of the hydrazine O-deacylated product **4a**,**b** was performed. Products

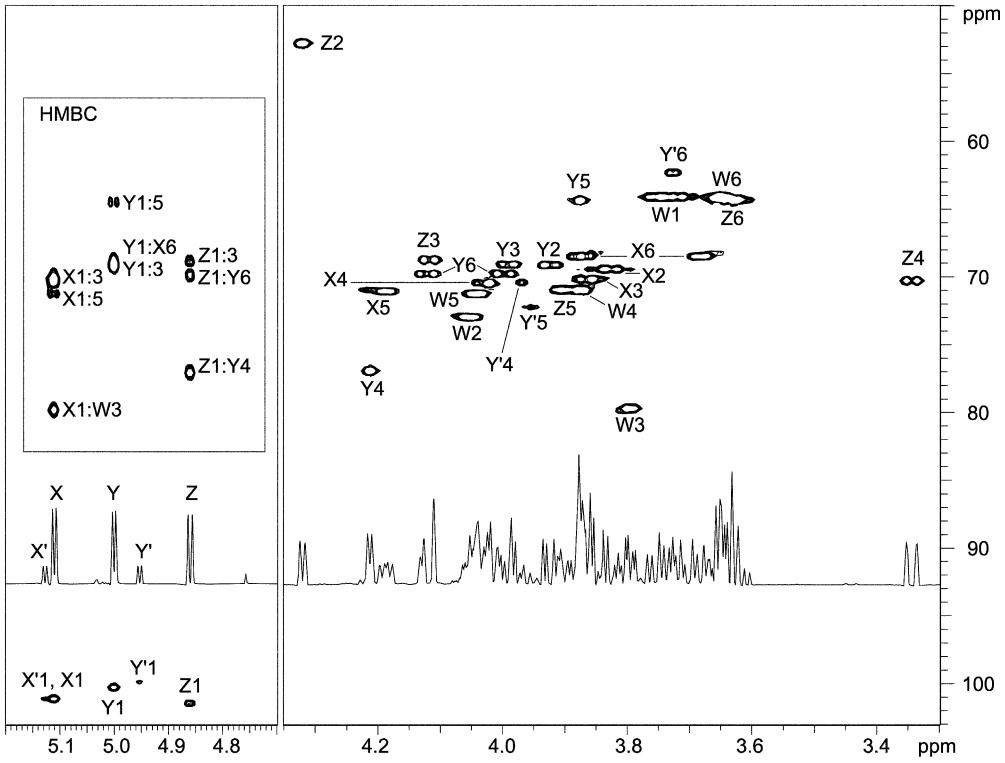


Fig. 1. ^{1}H-^{13}C NMR HSQC and HMBC correlation spectra of the compound **3a**. Left panel contains fragments of HSQC and HMBC (insert) spectra. Right panel contains the HSQC spectrum. Minor signals labeled with a '−sign belong to the compound with the missing residue Z.

4a,**b** gave well-resolved spectra in D_2O solution containing deuterated SDS and NH_3 at elevated temperature (Fig. 2), which were completely assigned (Table 1). Spin systems of all components of the oligosaccharides **1** and **2a**,**b** were identified in the spectra. The H-1 signal of the Gal residue W in the α-anomeric form showed correlation with the ^{31}P signal at −1.2 ppm, which also correlated with H-3 of DDHep E, thus indicating linkage α-Gal-(1-*P*-3)-α-DDHep (Fig. 3). The spectra contained two sets of signals for the residues Y, X, W due to the partial absence (~ 20 %) of the component Z. Two sets of signals for DDHep and 8-aminoKdo of nearly equal intensity were present. This was a result of the alternative presence of the phosphate or ethanolamine pyrophosphate substituents at O-4 of 8-aminoKdo. ^{1}H-^{31}P HMQC spectra showed the correlation between H-4 of one 8-aminoKdo with the pyrophosphate ^{31}P signal at −10.3 ppm, and of H-4 of another 8-aminoKdo with the phosphate signal at 4.5 ppm. The signal of H-1 of the EtN component correlated with pyrophosphate at −10.3 ppm. Thus product **4** contained components **4a** with *P* at C4, and **4b** with *PP*EtN at C4; both **4a** and **4b** were present in variants with and without (minor) the Z residue.

Other phosphate groups were at A1 (δ_P 3.1 ppm) and B4 (δ_P 4.6 ppm). DDHep E phosphorylated at O-2 was not detected; thus its presence in the products of the alkaline deacylation was due to phosphate migration in alkaline conditions.

Mass spectra of the O-deacylated LPS contained peaks corresponding to the molecular mass of 2214 Da, which is expected for compound **4a**, as well as peaks of another compound of the molecular mass of 2335.8 Da, which corresponds to **4b** with an additional *P*EtN substituent.

$$\begin{aligned}&\overset{Z}{}\\&(1S)\text{-GalNAc}o\text{-}(1\rightarrow 4,6)\text{-}\overset{Y}{}\alpha\text{-Gal-}(1\rightarrow 6)\text{-}\overset{X}{}\alpha\text{-Gal-}(1\\&\rightarrow 3)\text{-}\overset{W}{}\alpha\text{-Gal-}(1\text{-}P\text{-}3)\text{-}\alpha\text{-}\overset{E}{}\text{DDHep-}(1\\&\rightarrow 5)\text{-}\alpha\text{-}\overset{C}{}\text{8-aminoKdo4R-}(2\rightarrow\\&6)\text{-}\underset{B}{\beta\text{-GlcNac4}P}\text{-}(1\rightarrow 6)\text{-}\underset{A}{\alpha\text{-GlcNac1}P}\end{aligned}$$

Both ac are C14:0(3-OH)

4a, R = *P*

4b, R = *PP*EtN

The analysed LPS shows another example of the novel type of glycosidic linkage—an open-chain acetal linkage, previosly found in the core part of many *Proteus* LPSs.[15,16] It also contains a Kdo derivative, 8-aminoKdo, and a phosphodiester linkage between the monosaccharides. The presence of the novel component linking core and lipid A fragments of the LPS is highly

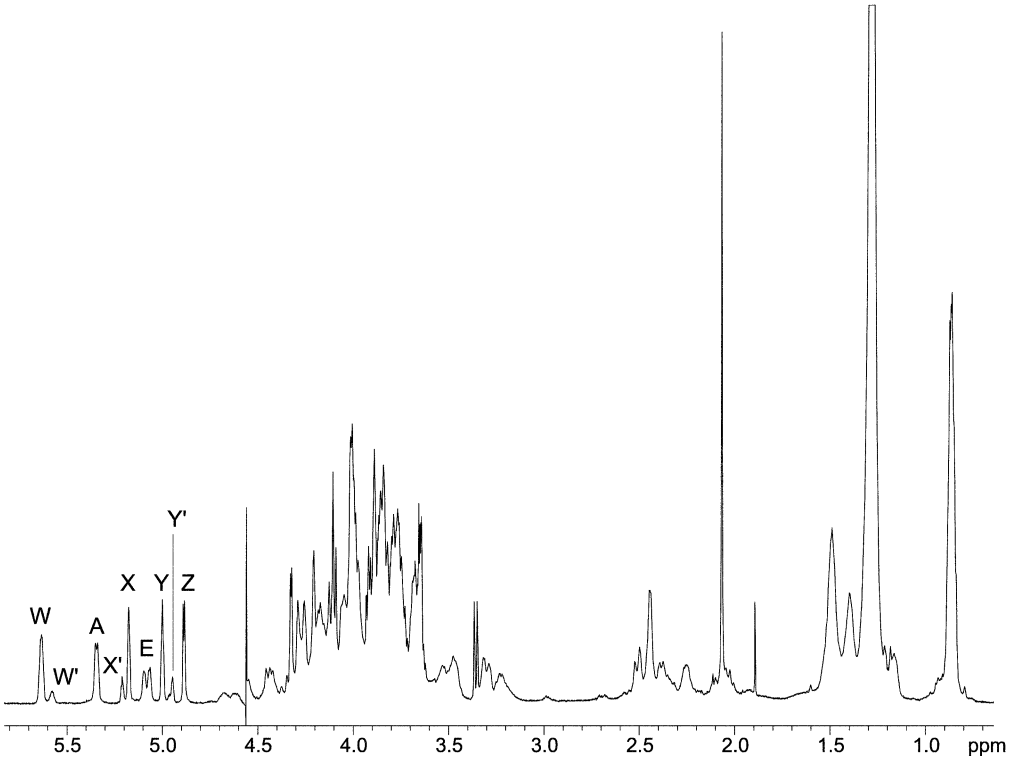


Fig. 2. ^{1}H NMR spectrum of the O-deacylated LPS (compound **4**). Labels show H-1 signals of the respective monosaccharide residues, minor signals labeled with a '−sign belong to the compound with the missing residue Z. The spectrum was recorded at 45 °C in 1% deuterated SDS in 0.5% NH_3.

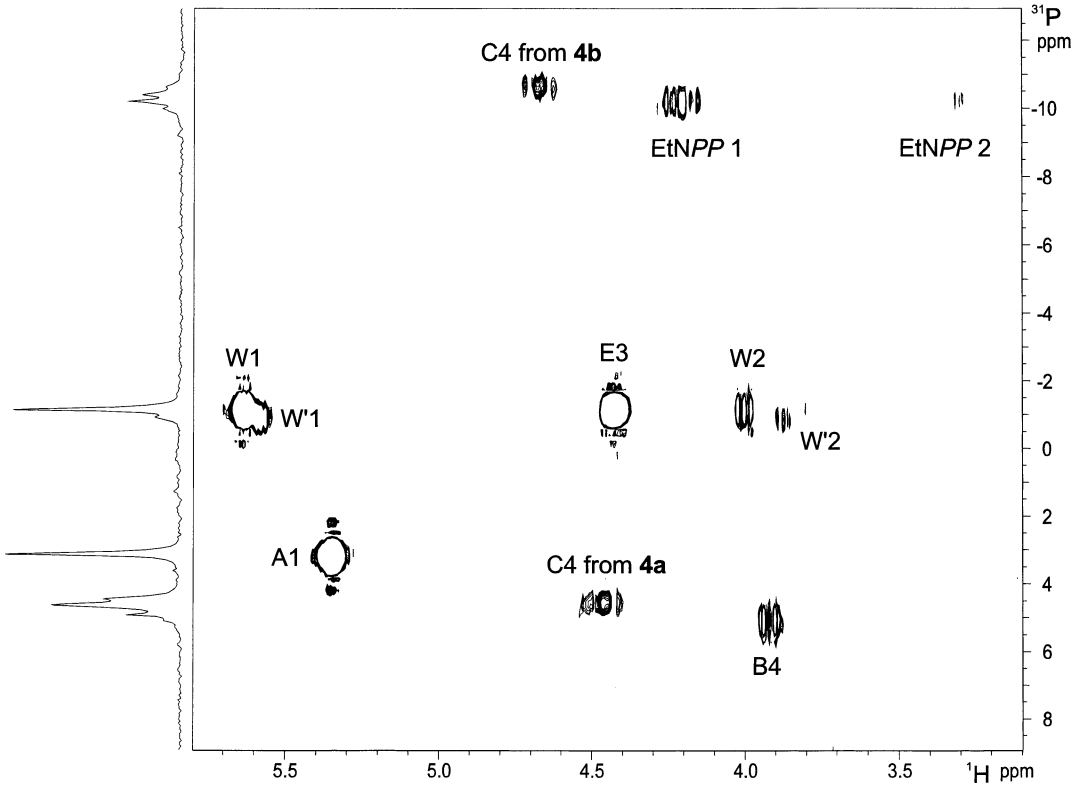


Fig. 3. ^{1}H-^{31}P NMR HMQC correlation spectrum of the product **4**. Minor signals labeled with a '−sign belong to the compound with the missing residue Z. The spectrum was recorded at 45 °C in 1% deuterated SDS in 0.5% NH_3.

unusual; until now only Kdo and its 3-hydroxylated derivative (Ko) have been found in known LPS structures.[17] The recent publication of the genome sequence of *S. oneidensis* MR-1[5] can be useful for the determination of biosynthetic mechanisms leading to the formation of 8-aminoKdo and the open-chain form of GalNAc.

The analysed LPS has significant structural similarity to the previously determined structure of the rough-type LPS from *S. putrefaciens* CN32,[9] containing

α-DDHep-(1 → 5)-α-Kdo4*P*-(1 → 6)-β-GlcN4*P*-(1 → 6)-α-GlcN1*P*.

3. Experimental

3.1. Bacterial strain and growth conditions

S. oneidensis MR-1 was grown at room temperature in trypticase soy broth under aerobic conditions to an optical density (OD_{600}) of 1.0, and the bacteria were 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 Darveau and 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% EtOH containing 0.375 M $MgCl_2$.

3.3. NMR spectroscopy and general methods

NMR spectra were recorded at 25 °C in D_2O (for oligosaccharides) or at 45 °C in 1% fully deuterated SDS in D_2O (0.6 mL) with 10 μL of 25% NH_3 (for compound **3**) 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, NOESY (mixing time of 300 ms), TOCSY (spinlock time 120 ms), HSQC, and gHMBC (evolution delay of 100 ms) were used with digital resolution in the F2 dimension <2 Hz/pt. Spectra were assigned using the computer program PRONTO.[18]

Hydrolysis was performed with 4 M CF_3CO_2H (110 °C, 3 h), and the monosaccharides were conventionally converted into the alditol acetates and analysed by GLC on a Agilent 6850 chromatograph equipped with DB-17 (30 m × 0.25 mm) fused-silica column using a temperature gradient of 180 (2 min) → 240 °C at 2 °C/min. GC–MS was performed on Varian Saturn 2000 system with ion-trap mass spectral detector using the same column. ESIMS was carried out as previously described.[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 Py and 10 mL AcOH in 1 L water), and the eluate was monitored by a refractive index detector.

Methylation analysis was performed using Ciucanu–Kerek procedure,[19] methylated products were hydrolysed, monosaccharides converted to alditol-1*d* acetates by conventional methods, and analysed by GC–MS.

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

3.4. O-deacylation of the LPS

LPS (100 mg) was dissolved in anhyd hydrazine (3 mL), kept 1 h at 40 °C and then poured into cold acetone. The precipitate was collected by centrifugation, washed with acetone, and liophylized. The product was separated on Hitrap Q anion-exchange column in a gradient of water–1 M NaCl over 1 h. Fractions containing O-deacylated LPS were identified by charring of the eluate spots on a TLC plate with 5% H_2SO_4. The product was desalted by gel chromatography on a Sephadex G-50 column to give product **4**.

3.5. O,N-Deacylation of LPS and preparation of backbone oligosaccharides[10]

LPS (120 mg) was dissolved in 4 M KOH (4 mL), and the solution was heated at 120 °C for 16 h, then cooled and neutralized with 3 M HCl. The precipitate was removed by centrifugation, and the supernatant was desalted by gel chromatography on Sephadex G-50. The oligosaccharide fraction was additionally purified by passing through a Seppak C18 cartridge in water to give oligosaccharide **1**.

3.6. Acetic acid hydrolysis of LPS

LPS (100 mg) was treated with 2% AcOH (5 mL, 100 °C, 3 h), the precipitate was removed by centrifugation, and the soluble products were separated on a Sephadex G50 column to provide low-molecular-mass fractions. These were separated on a Sephadex G-15 column, and the oligosaccharide product was then run on a Hitrap Q anion-exchange column in a water–1 M NaCl gradient. Product **2a**,**b** was eluted with the void volume and was

then reduced with $NaBH_4$ and desalted on a Sephadex G-15 column to give the oligosaccharides **3a**,**b**.

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