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Note

Characterisation of a tetrasaccharide released on mild acid hydrolysis of LPS from two rough strains of *Shewanella* species representing different DNA homology groups

Anne L. Moule,[a] Lesley Galbraith,[a] Andrew D. Cox[b] and Stephen G. Wilkinson[a,*]

[a]*Department of Chemistry, University of Hull, Hull HU6 7RX, UK*
[b]*Institute for Biological Sciences, National Research Council, 100 Sussex Drive, Ottawa, ON, Canada K1A 0R6*

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Abstract—A reducing tetrasaccharide of the following structure was released by mild acid hydrolysis of R-type LPS from *Shewanella putrefaciens* strains NCIMB 10472 and 10473. The same tetrasaccharide containing acetal-linked open-chain GalNAc is present in the core region of LPS from *S. oneidensis* strain MR-1 [*Carbohydr. Res.* **2003**, *338*, 1991–1997] and may be characteristic of genomic groups II and III of *S. putrefaciens* and related strains.

(1*S*)-D-Gal*a*NAc-(1 → 4,6)-α-D-Gal*p*-(1 → 6)-α-D-Gal*p*-(1 → 3)-D-Gal.

Keywords: Lipopolysaccharide; *Shewanella putrefaciens*; Core; Acetal-linked *N*-acetylgalactosamine

The diverse Gram-negative bacteria assigned to the genus *Shewanella* are variously of environmental, clinical and biochemical interest and include strains responsible for the spoilage of cold-stored, protein-rich foods. The type species *Shewanella putrefaciens* has undergone several changes of nomenclature and classification, and its intraspecific heterogeneity is well documented.[1–4] Two of the four genomic groups identified by Owen et al.[1] have recently been excised as *S. baltica* (group II)[5] and *S. algae* (group IV)[3], and a relationship between *S. oneidensis* and a group III strain (not the type strain) has been evaluated.[4,5] In a previous comparative study,[6] lipopolysaccharides (LPS) from strains representing all four genomic groups were characterised by type (R, lacking an O-specific side-chain) and composition. LPSs from strain NCIMB 10472 (group II) and NCIMB 10473 (group III) were very similar in composition and in behaviour on mild acid hydrolysis (1% acetic acid, 100 °C, 1–2 h). In both cases, the pool of water-soluble, low-M_r products included the same reducing oligosaccharide containing D-Gal and D-GalN (molar ratio 3:1). Attempts to determine the structure of this oligosaccharide were frustrated by unexpected results from methylation analysis and puzzling features of the NMR spectra (vide infra). The recent discovery[7] of acetal-linked open-chain GalN in other LPSs provided the missing clue leading to establishment of the structure. The oligosaccharides containing Gal and GalN described in this paper were released by mild acidic hydrolysis of LPS from strains NCIMB 10472 and 10473, and were eluted from Sephadex G-15 in about the same position as maltopentaose and ahead of free D-GalA, material containing heptose, and a variety of phosphates (including orthophosphate and pyrophosphate, each free and ester-linked to ethanolamine).

Abbreviations: LPS, lipopolysaccharide; DD-Hep, D-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; 8-aminoKdo, 8-amino-3,8-dideoxy-D-*manno*-oct-2-ulosonic acid; Gal*a*NAc, open-chain GalNAc.; *P*, orthophosphate; *PP*, pyrophosphate; EtN, ethanolamine

* Corresponding author at present address: 185 Beverley Rd., Kirkella, Hull HU10 7AG, UK. Tel.: +44-1482-651152; fax: +44-1482-466410

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The isolated oligosaccharides had identical ^{1}H NMR spectra and were confirmed as tetrasaccharides by soft-ionisation MS. The positive-ion spectrum obtained by FAB-MS contained diagnostic peaks at m/z 708.2 $[M+H]^+$ and 730.2 $[M+Na]^+$, and the negative-ion spectrum contained peaks at m/z 706.2 $[M-H]^-$ and 742.2. $[M+Cl]^-$. Both spectra also indicated that the major component with M_r 707.2 was contaminated by a minor product with M_r 848.1 (not detected by LD-FT-ICR-MS). Accurate mass measurement gave the value of 707.2469 for M_r of the major product, corresponding to $C_{26}H_{45}O_{21}N$ and the composition $Gal_3{\cdot}GalNAc_1$ for the tetrasaccharide. Evidence supporting and extending these conclusions was obtained by LD-FT-ICR-MS (giving pseudomolecular ions with m/z 730 $[M+Na]^+$ and 746 $[M+K]^+$) and by MS–MS. Collision-induced fragmentation of the $[M+H]^+$ ion from FAB-MS produced successive losses of 18, 162, 162 and 162 amu to leave the fragment of m/z 204 indicative of a GalNAc residue at the nonreducing terminus.[8] The corresponding fragmentation[8] for the $[M-H]^-$ ion also involved consecutive elimination of three hexose residues.

Reduction ($NaBH_4$) of the tetrasaccharide simplified the ^{1}H NMR spectrum, which showed the presence of three one-proton signals in the anomeric region with δ 5.17 ($J_{1,2}$ 3.2 Hz), 5.06 ($J_{1,2}$ 3.5 Hz) and 4.92 ($J_{1,2}$ 4.5 Hz), and a methyl singlet with δ 2.11. Corresponding signals for anomeric carbons with δ 100.4, 100.0 and 99.3 and an *N*-acetyl group with δ 174.7 and 22.1 were present in the ^{13}C NMR spectrum.

On treatment of the parent tetrasaccharide with $NaIO_4$ all monosaccharide residues were oxidised. Methylation analysis of the tetrasaccharide, monitored by GLC–MS of the *d*-labelled methylated alditol acetates, identified the major products as the derivatives of 3-substituted Gal*p* (**A**), 6-substituted Gal*p* (**B**) and 4,6-disubstituted Gal*p* (**C**). A minor product from 3-substituted Gal*f* (**D**) pointed to the presence of 3-substituted Gal at the reducing terminus of the tetrasaccharide, consistent with the result from periodate oxidation. This was confirmed by the replacement of the products from **A** and **D** by the derivative from 3-substituted galactitol (**E**) on methylation analysis of the oligosaccharide alditol. Although residue **C** suggested a branched structure for the oligosaccharide, no unsubstituted Gal was present and no derivative from GalNAc was detected in methylation analyses.

Closer examination of the NMR spectra for the oligosaccharide alditol revealed anomalous data for the GalNAc residue. In particular, the chemical shift for H-2 (Table 1) and the value of 1.1 Hz for $J_{2,3}$ are inconsistent with an α-Gal*p*NAc residue, but are in general accord with data reported[7,9–11] for an open-chain terminal residue of GalNAc (**F**, Gal*a*NAc) acetal-linked to positions 4 and 6 of the adjacent sugar. Thus, the putative structural sequence **F** → **C** → **B** → **A/D** could be postulated for the tetrasaccharide from strains NCIMB 10472 and 10473, for which the α-configuration could be assigned to the internal Gal residues **B** and **C** from the $J_{1,2}$ values.

Since the completion of this study, isolation of the identical tetrasaccharide from the R-type LPS of *S. oneidensis* strain MR-1 has been reported.[12] The NMR data reported for the derived alditol are in good overall agreement with those found here (Table 1), permitting the 1*S*-configuration to be assigned to the Gal*a*NAc residue as shown in structure **1**. In the case of *S. oneidensis*, the parent tetrasaccharide is linked to D-*glycero*-D-*manno*-heptose in a novel inner core-lipid A region via an acid-labile glycosidic phosphodiester as shown in the structure **2** for the O-deacylated LPS. The inner core region of LPS was not included in the present study, but the distinctive structure **2**, possibly with minor variations (e.g., the incorporation of GalA and

Table 1. NMR shift data for the oligosaccharide alditol[a]

Atom		Residue			
		→6)-α-Gal*p*-(1→ **B**	→4,6)-α-Gal*p*-(1→ **C**	Gal*a*NAc-(1→ **F**	→3)-Gal-ol **E**
1	H	5.17	5.06	4.92	~3.8
	C	100.0	99.3	100.4	63.1
2	H	3.88	3.98	4.37	4.12
	C	68.4	68.2	52.2	72.0
3	H	3.93	4.05	4.16	3.85
	C	69.2	68.2	67.9	78.8
4	H	4.10	4.26	3.42	3.99
	C	69.4	75.9	69.5	70.0
5	H	4.23	3.92	3.96	4.10
	C	70.0	63.5	70.1	70.3
6	H	3.74, 3.94	4.07, 4.18	~3.7	~3.7
	C	67.4	68.7	63.5	63.2

[a] Compared with literature data,[12] chemical shifts are displaced ~0.06 ppm downfield (^{1}H) and ~1 ppm upfield (^{13}C), attributable to the use of different operating parameters (referencing, temperature). Additional signals from the *N*-acetyl group were at δ_H 2.11, δ_C 174.7 and 22.1.

greater diversity in the complement of amide-bound 3-hydroxy acids[6,13]) may be a chemotaxonomic marker for genomic groups II and III, and closely related organisms. A preliminary study[6] of the putative core material from two group IV strains (NCTC 10738 and 10763) indicated the absence of acid-stable phosphates (elution profiles for carbohydrate and phosphorus in mild hydrolysates do not match) and the presence of fructose (acid-labile) and quinovosamine (2-amino-2,6-dideoxyglucose) in addition to Gal, GalN, Kdo and DD-Hep. In contrast, core fractions from two group I strains [the type strain (NCTC 10471) and NCIMB 8768] contain acid-stable phosphates, Gal (much of it released by mild acid hydrolysis), Glc, Kdo and DD-Hep.[6,14] 3-Amino-3,6-dideoxyglucose is also present for the type strain.[6] Structure **3** has been proposed[15] for the core region of LPS from *S. putrefaciens* CN32, apparently belonging to group I. Although it once appeared that *Shewanella* strains produce only R-type LPS, O-specific or capsular polysaccharides were elaborated by about half of the strains examined in a recent survey.[16]

1.2. Structural methods

Methods used to identify, quantify and assign configurations to monosaccharides and to study other products have been described.[6] Treatment with $NaBH_4$ was used to convert reducing tetrasaccharides to the alditols. Oxidation of oligosaccharides was carried out with 50 mM $NaIO_4$ at 4 °C for 1–3 days. Methylation analyses were carried out by standard methods.[17,18] GLC-MS of methylated alditol acetates employed a Finnigan 1020B instrument.

FAB-MS was carried out with a ZAB-2SE instrument with caesium ion bombardment, thioglycerol as the matrix, and recording both positive and negative ion spectra. Calibration with polyethylene glycol was used for accurate mass determination. MS–MS of pseudomolecular ions was carried out with helium as the collision gas and linked scanning in the first field-free region. LD-FT-ICR-MS was carried out with a Nicolet FTMS-2000 instrument and the sample dissolved in methanolic NaCl–KBr.

F C B A/D

(1*S*)-D-Gal*a*NAc-(1→4,6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→3)-D-Gal

1

(1*S*)-D-Gal*a*NAc-(1→4,6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→3)-α-D-Gal-(1-*P*-3)-

α–DD-Hep*p*-(1→5)-α-8-aminoKdo4R^1-(2→6)-β-D-GlcNR^{2}4*P* -(1→6)-α-D-GlcNR^{2}1*P*

2

R^1 = *P* or EtN*PP*; R^2 = 3-OH-14:0

β-D-Gal*f*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-α-DD-Hep*p*2*P*EtN-(1→5)-α-

Kdo4*P*-(2→

3

1. Experimental

1.1. Growth of bacteria, isolation and fractionation of LPS

Strains NCIMB 10472 and 10473, received as *S. putrefaciens*, were grown as described.[6] Cell walls were prepared by mechanical disintegration, and LPS was extracted from the defatted walls by the aqueous-phenol method and recovered from the aqueous phase after exhaustive dialysis and freeze drying.[6] The water-soluble products produced by mild hydrolysis (1% acetic acid, 100 °C, 1–2 h) were fractionated on Sephadex G-10 or G-15.

NMR spectra (1D, COSY, relayed COSY and HETCOR) were recorded with a Bruker WH-400 spectrometer for samples in D_2O at 45 °C, and using an external reference of sodium 3-trimethylsilylpropanoate-d_4 (^{1}H) or Me_4Si (^{13}C).

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