Carbohydrate Research 344 (2009) 479–483

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Structure of a polysaccharide from the lipopolysaccharide of *Vibrio vulnificus* CECT4602 containing 2-acetamido-2,3,6-trideoxy-3-[(*S*)- and (*R*)-3-hydroxybutanoylamino]-L-mannose

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ARTICLE INFO

Article history:
Received 2 December 2008
Received in revised form 10 December 2008
Accepted 12 December 2008
Available online 25 December 2008

Keywords:
Lipopolysaccharide
Bacterial polysaccharide structure
2,3-Diamino-2,3,6-trideoxy-L-mannose
3-Hydroxybutanoyl group
Vibrio vulnificus

ABSTRACT

A polysaccharide was isolated by GPC after mild acid treatment of the lipopolysaccharide of *Vibrio vulnificus* CECT4602 and found to contain L-Rha, D-Glc*p*NAc and 2-acetamido-2,3,6-trideoxy-3-(3-hydroxybutanoylamino)-L-mannose (L-RhaNAc3NHb). GLC analysis of the trifluoroacetylated (*S*)-2-octyl esters derived by full acid hydrolysis of the polysaccharide showed that ~80% of the 3-hydroxybutanoic acid has the *S* configuration and ~20% the *R* configuration. The following structure of the polysaccharide was established by ^{1}H and ^{13}C NMR spectroscopies, including 2D ROESY and $^{1}H/^{13}C$ HMBC experiments:

β-L-Rha*p*NAc3NHb-(1→3)┐
→4)-α-D-Glc*p*NAc-(1→3)-α-L-Rha*p*-(1→3)-β-D-Glc*p*NAc-(1→

1. Introduction

A Gram-negative bacterium *Vibrio vulnificus* is able to infect fish and humans causing a disease called vibriosis. Human vibriosis occurs after infection of pre-existing wounds with seawater, after injury while fishing or handling fish or after ingestion of raw seafood.[1] The disease can lead to septicaemia and death depending on the host innate defences status.[1] Fish vibriosis is produced by gill or intestine colonisation, after immersion in contaminated water or after contact with diseased fish or carriers.[2,3]

Based on phenotypic characteristics and host range criteria, *V. vulnificus* isolates were primarily grouped into two biotypes:[4] indole^{+} and ornithine decarboxylase^{+} (ODC^{+}) biotype 1 strains associated with pathogenicity in humans and indole^{-} and ODC^{-} biotype 2 strains pathogenic for eels. Later, this subdivision has been questioned in several studies which showed that indole^{-} and ODC^{-} strains were pathogenic also for humans as well as the existence of indole^{+} and/or ODC^{+} strains which were pathogenic for eels. On the other hand, a third *V. vulnificus* biotype has been described in Israel[5] and comprises cellobiose-negative strains isolated from patients who handled Saint Peter's fish (*Tilapia* sp.).

All biotypes of *V. vulnificus* are potentially pathogenic for humans, but biotype 1 strains are most frequently isolated from clinical specimens.[1] Eel-pathogenic *V. vulnificus* (formerly biotype 2) strains have been subdivided into four O-antigenic groups, including serovars A, E (O4), O3 and O3/O4.[6] Serovar E strains (VSE) are distributed worldwide and primarily considered as a pathogen of eels (vibriosis) and secondary of humans.[7] VSE strains are homogeneous in respect to the lipopolysaccharide (LPS) O-antigen.

In this report we characterise, for the first time, a polysaccharide from the LPS of a VSE representative strain *V. vulnificus* CECT4602.

2. Results and discussion

The lipopolysaccharide was isolated by phenol/water extraction of dried cells of *V. vulnificus* CECT4602. A high-molecular-mass polysaccharide was released by mild acid degradation of the lipopolysaccharide with dilute acetic acid and isolated by GPC on Sephadex G-50.

Sugar analysis of the polysaccharide by GLC of the alditol acetates derived after full acid hydrolysis of the polysaccharide revealed Rha, GlcN and a 2,3-diamino-2,3,6-trideoxyhexose. Determination of the absolute configurations by GLC of the acetylated (*S*)-2-octyl glycosides indicated that GlcN is D and Rha is L.

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0008-6215/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2008.12.012

The diamino sugar was identified as 2,3-diamino-2,3,6-trideoxy-L-rhamnose (L-RhaN3N) by GLC–MS of the trifluoroacetylated (*S*)-2-octyl glycosides and comparison with the authentic samples of L-RhaN3N derived from the O-polysaccharide of *Proteus penneri* 2.[8] In addition to the monosaccharide, 3-hydroxybutanoic acid was found in the polysaccharide hydrolysate and shown to be a 4:1 mixture of the *S* and *R* isomers by GLC of the trifluoroacetylated (*S*)-2-octyl esters.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) showed signals for four anomeric carbons at δ 95.3, 100.5, 101.5 and 102.4, two CH_3–C groups (C-6) of Rha and RhaN3N at δ 18.0 and 18.5, two $HOCH_2$–C groups (C-6) of GlcN at δ 60.9 and 62.4, four nitrogen-bearing carbons at δ 52.8, 53.3, 55.0 and 57.5, other sugar ring carbons at δ 68.8–83.0, one *N*-(3-hydroxybutanoyl) group at δ 23.3 (CH_3), 46.2 (CHOH) and 66.4 (CH_2), three *N*-acetyl groups at δ 23.5–23.6 (CH_3) and the corresponding CO groups at δ 175.4–176.2. The ^{1}H NMR spectrum of the polysaccharide contained five low-field signals, including four signals for anomeric protons at δ 4.52, 4.87, 4.98 and 5.06 and one non-anomeric proton at δ 4.58 (H-2 of RhaN3N, see below), two CH_3–C groups (H-6) of Rha and RhaN3N at δ 1.25 and 1.35, other sugar protons at 3.29–4.02, one *N*-(3-hydroxybutanoyl) group at δ 1.14 (CH_3), 2.36 (CHOH) and 4.17 (CH_2) and three *N*-acetyl groups at δ 2.02–2.04.

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned using 2D ^{1}H/^{1}H COSY, TOCSY and ^{1}H/^{13}C NMR spectroscopy (Table 1). Spin systems for α-Rha, α-GlcN, β-GlcN and β-RhaN3N were revealed by tracing connectivity from H-1 for GlcN and from H-1, H-2 and H-6 for Rha and RhaN3N. The ^{1}H NMR signals within each spin system were assigned using COSY. The amino sugars were confirmed by correlation of protons at the nitrogen-bearing carbons to the corresponding carbons resonating in the region δ 52–58. $^3J_{H,H}$ coupling constants confirmed the configurations and the pyranose form of the monosaccharides as well as the configurations of the GlcN linkages. The α configuration of Rha[9] and β configuration of RhaN3N[8] were inferred by comparison of the ^{1}H and ^{13}C NMR chemical shifts with published data for the α- and β-anomers of the corresponding free monosaccharides.

A downfield position of the signals for C-3 of α-Rha, C-3 and C-4 of α-GlcN to δ 76.5–77.5 and C-3 of β-GlcN to δ 83.0, as compared with those of the corresponding non-substituted monosaccharides at δ 71.3–72.0 and 75.1, respectively, demonstrated the glycosylation pattern of the constituent sugars with the α-GlcN residue at the branching point. The ^{13}C NMR chemical shifts for C-2–C-6 of β-RhaN3N in the polysaccharide were similar to those of free β-RhaNAc3NAc,[9] thus confirming the terminal position of this monosaccharide in the side chain.

Sequence analysis was carried out by a ^{1}H/^{13}C HMBC experiment (Fig. 2), which revealed the following correlations between the anomeric protons and protons at the linkage carbons: RhaN3N H-1,α-GlcN H-3; α-GlcN H-1,α-Rha H-3; α-Rha H-1,β-GlcN H-3 and β-GlcN H-1,α-GlcN H-4. These data define the arrangement of the monosaccharides in the branched tetrasaccharide repeating unit of the polysaccharide, which was confirmed by a 2D ROESY experiment (data not shown).

For confirmation of the N-acylation pattern, 1D ^{1}H NMR and 2D ^{1}H/^{1}H NMR spectra of the polysaccharide were run in a 17:3 H_2O–D_2O mixture to enable detection of resonances of nitrogen-linked protons in the region δ 7.1–8.6 (Fig. 3). The assignment of the NH protons was performed using COSY and TOCSY experiments and their NOE correlations were analysed using a ROESY experiment (Fig. 3). From them, all NH-2 protons resonating at δ 7.18, 7.83 and 8.54 gave strong cross-peaks with CH_3 of *N*-acetyl groups at δ 2.02–2.04. The NH-3 proton of RhaN3N at δ 8.10 showed only a weak peak with an *N*-acetyl group but a strong cross-peak with CH_2 of the *N*-(3-hydroxybutanoyl) group at δ 2.36. Therefore, the 3-hydroxybutanoyl group is located at N-3 of RhaN3N whereas the amino groups at position 2 of RhaN3N and both GlcN residues are N-acetylated.

The data obtained suggest that the polysaccharide from *V. vulnificus* CECT4602 has the structure shown in Chart 1. A peculiar feature of the polysaccharide is the presence of a new monosaccharide derivative L-RhaNAc3NHb. Earlier, the di-*N*-acetyl derivative of 2,3-diamino-2,3,6-trideoxy-L-mannose has been found in the O-specific polysaccharide of *Proteus penneri* 2 (O66)[8] and capsular polysaccharide of *Escherichia coli* K48.[10] 2-Acetamido-2,3,6-trideoxy-3-formamido-D-mannose has been reported as a component of the O-specific polysaccharide of *Escherichia coli* O119.[11] Remarkably, in *V. vulnificus* CECT4602, the major part (~80%) of RhaN3N is 3-N-acylated with the (*S*)-3-hydroxybutanoyl group and the rest with the corresponding *R* isomer. While, in most cases of the occurrence of the *N*-(3-hydroxybutanoyl) group in bacterial polysaccharides, it has been identified as a single isomer, both *R* and *S* isomers in a 3:1 ratio have been reported in a polysaccharide from *Sinorhizobium fredii* HH103. [12]

V. vulnificus CECT4602 is the first VSE representative strain with a defined structure of a polysaccharide from the LPS. The data obtained will be helpful for isolation of LPS O-polysaccharide mutants for studies of interaction between the O-antigens and the innate

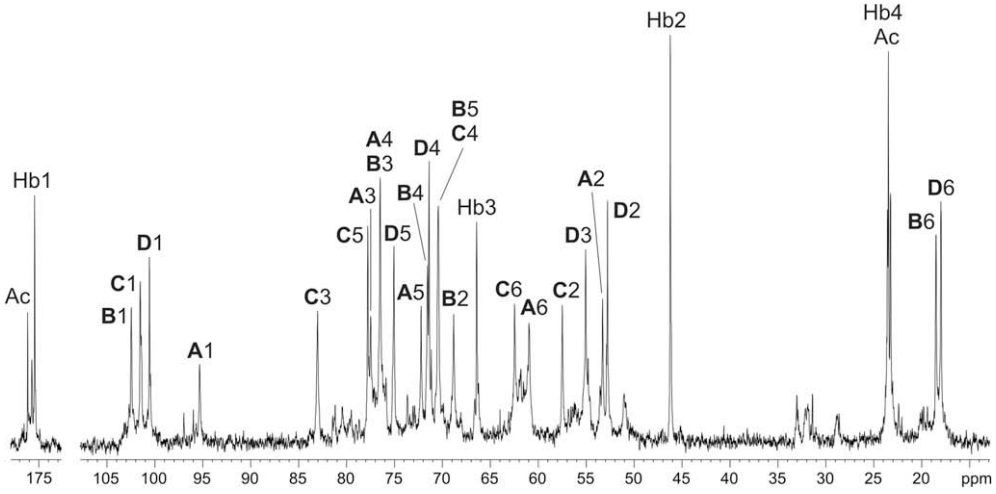

Figure 1. 125-MHz ^{13}C NMR spectrum of the polysaccharide from *Vibrio vulnificus* CECT4602. Region of the CO resonance is not shown. Arabic numerals refer to carbons in sugar residues denoted by letters as shown in Table 1.

Table 1
^{1}H and ^{13}C NMR data of the polysaccharide from *Vibrio vulnificus* CECT4602

Sugar residue	Atom	δ_C	δ_H	Inter-residue correlations	
				NOE (ROESY)[a]	$^{3}J_{H,C}$ (HMBC)[b]
→3,4)-α-D-Glc*p*N-(1→	1	95.3	4.98	**A**1/**B**2	**A**1/**B**3
A	2	53.3	3.93	**A**1/**B**3	**A**3/**D**1
	3	77.5	4.02		
	4	76.5	3.91		
	5	72.2	3.97		
	6	60.9	3.68, 3.81		
NAc	1	175.4			
	2	23.6	2.04		
→3)-α-L-Rha*p*-(1→	1	102.4	4.87	**B**1/**C**3	**B**1/**C**3
B	2	68.8	3.88		
	3	76.5	3.79		
	4	71.5	3.49		
	5	70.4	4.02		
	6	18	1.25		
→3)-β-D-Glc*p*N-(1→	1	101.5	4.52	**C**1/**A**4	**C**1/**A**4
C	2	57.5	3.84		**C**3/**B**1
	3	83	3.69		
	4	70.4	3.56		
	5	77.8	3.4		
	6	62.4	3.84. 3.89		
NAc	1	176.2[c]			
	2	23.5	2.02		
β-L-Rha*p*N3N-(1→	1	100.5	5.06	**D**1/**A**3	
D	2	52.8	4.58		
	3	55.0	4.00		
	4	71.4	3.29		
	5	75	3.52		
	6	18.5	1.35		
NAc	1	175.7[c]			
	2	23.5	2.02		
NHb	1	175.4			
	2	46.2	2.36		
	3	66.4	4.17		
	4	23.3	1.14		

Arabic numerals refer [a]to protons or [b]before slash to protons and after slash to carbons in sugar residues denoted by letters.
[c] Assignment could be interchanged.

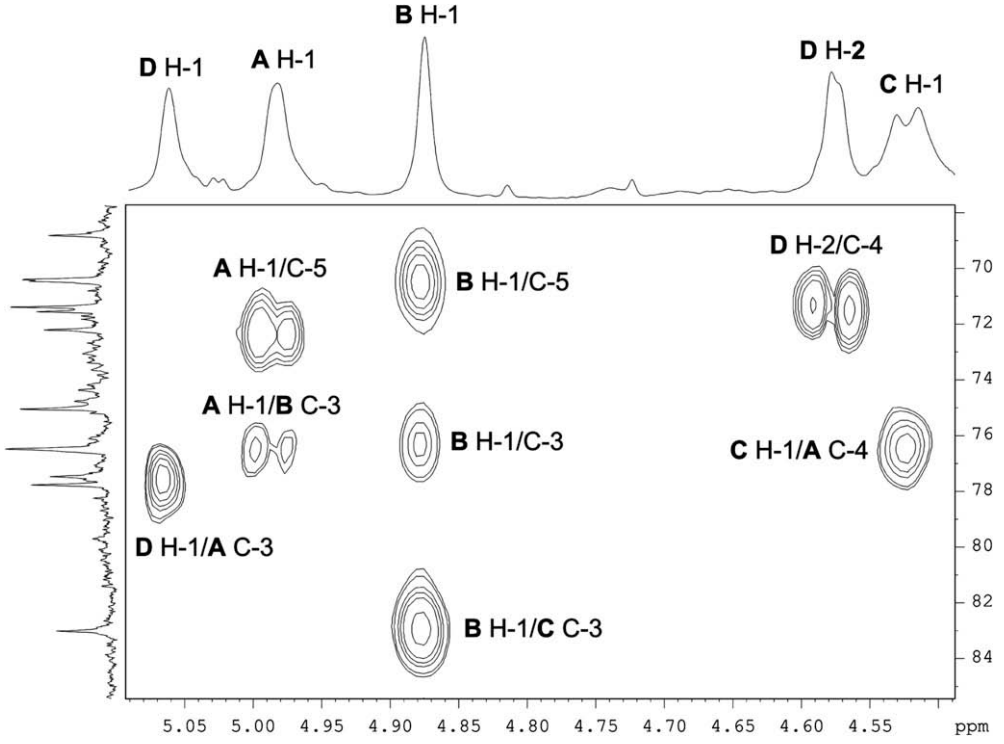

Figure 2. Part of a ^{1}H, ^{13}C HMBC spectrum of the polysaccharide from *Vibrio vulnificus* CECT4602 showing correlations for anomeric protons. The corresponding parts of the ^{1}H and ^{13}C NMR spectra are displayed along the horizontal and vertical axes, respectively. Letters denote sugar residues as shown in Table 1.

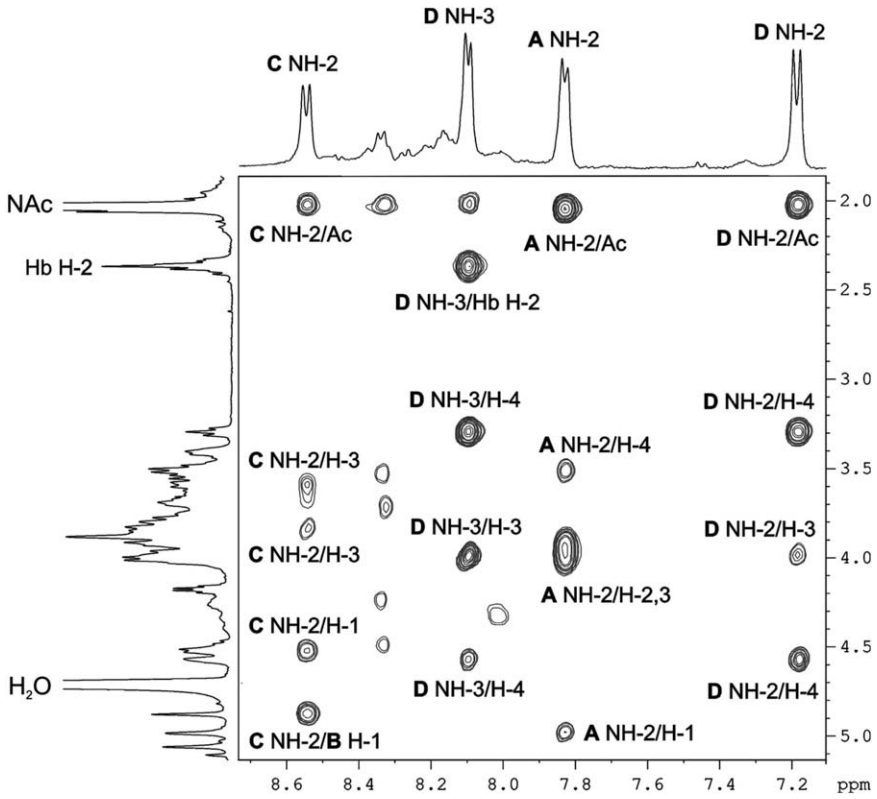

Figure 3. Part of a 2D ROESY spectrum of the polysaccharide from *Vibrio vulnificus* CECT4602 showing correlations for nitrogen-linked protons. The corresponding parts of the ^{1}H NMR spectrum are displayed along the axes. The spectra were measured in a 17:3 H_2O–D_2O mixture. Letters denote sugar residues as shown in Table 1.

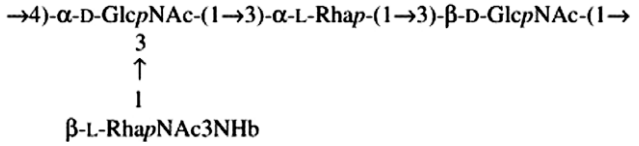

Chart 1. The polysaccharide from *Vibrio vulnificus* CECT4602. Hb stands for (*S*)- and (*R*)-3-hydroxybutanoyl (4:1, respectively).

defences, which could be crucial in the case of *V. vulnificus* as the innate defences are fundamental to avoid septicaemia.

3. Experimental

3.1. Growth conditions and isolation of the lipopolysaccharide and the polysaccharide

V. vulnificus CECT4602, a VSE representative strain from the Spanish National Collection isolated from a diseased fish, was grown on Tryptone broth (1% tryptone, 1% NaCl) at pH 7.2 and at the optimal growth temperature 28 °C for 24 h. Bacterial cells were obtained from 50 L culture by centrifugation, resuspended in 200 mL water, extensively dialysed against tap water, centrifuged and dried.

Dried cells (5 g) were extracted with hot 45% aq phenol as described,[13] after dialysis of the combined water and phenol layers, contaminations were precipitated by adding 50% aq trichloroacetic acid, the supernatant was dialysed and lyophilised to give the lipopolysaccharide in a 4.7% yield of the dried cells weight.

A portion of the lipopolysaccharide (105 mg) was degraded with 2% HOAc for 2 h at 100 °C, a precipitate was removed by centrifugation (13,000*g*, 20 min), and the supernatant was fractionated on a column (50 × 2.5 cm) of Sephadex G-50 (S) in pyridinium acetate buffer, pH 4.5, (4 mL pyridine and 10 mL AcOH in 1 L water) with monitoring using a Knauer differential refractometer. A high-molecular-mass polysaccharide was isolated in a yield 38% of the lipopolysaccharide weight.

3.2. Sugar analysis

The polysaccharide was hydrolysed with 2 M trifluoroacetic acid (120 °C, 2 h). After evaporation, the monosaccharides were converted into the alditol acetates and analysed by GLC on a Hewlett-Packard 5890 chromatograph equipped with a HP-5ms column, carrier gas N_2, using a temperature gradient of 160–290 °C at 10 °C min^{-1}. For determination of the absolute configuration of the monosaccharides,[14] the polysaccharide hydrolysate was peracetylated, subjected to methanolysis (MeOH/2 M HCl, 85 °C, 2 h), peracetylated, treated with (*S*)-2-octanol/1 M HCl (100 °C, 2 h), peracetylated and analysed by GLC–MS using an MSD 5975 instrument (Agilent) equipped with a HP-5ms column, carrier gas He, using a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C min^{-1}. For determination of the absolute configuration of 3-hydroxybutanoic acid, the polysaccharide hydrolysate was reacted with (*S*)-2-octanol/1 M HCl (100 °C, 2 h), acylated with trifluoroacetic anhydride (65 °C, 2 h) and analysed by GLC–MS as above using a temperature gradient of 100 °C (3 min) to 320 °C at 5 °C min^{-1}.

3.3. NMR spectroscopy

NMR spectra were obtained on a Bruker DRX-500 spectrometer using standard Bruker software at 40 °C in 99.95% D_2O or a 17:3 H_2O–D_2O mixture. Prior to the measurements in D_2O, samples were deuterium-exchanged by freeze-drying twice from 99.9% D_2O. A mixing time of 200 and 100 ms was used in 2D TOCSY and ROESY experiments, respectively. Chemical shifts are referenced to internal TSP (δ_H 0.00) or acetone (δ_C 31.45).

Acknowledgements

Authors thank Mr. H. Moll (Research Center Borstel, Germany) for help with GLC–MS. This work was supported in part by Plan Nacional de I + D and FIS Grants (Ministerio de Educación, Ciencia y Deporte and Ministerio de Sanidad, Spain) and by Generalitat de Catalunya (Centre de Referència en Biotecnologia).

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