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Structural studies of the *Vibrio salmonicida* lipopolysaccharide

Per Edebrink [a], Per-Erik Jansson [b,*], Jarl Bøgwald [c], James Hoffman [c]

[a] *Department of Organic Chemistry, Stockholm University, S-106 91 Stockholm, Sweden*
[b] *Clinical Research Centre, Analytical unit, Karolinska Institute, Huddinge Hospital, NOVUM, S-141 86 Huddinge, Sweden*
[c] *Norwegian Institute of Fisheries and Aquaculture Ltd., Tromsø, Norway*

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Abstract

The oligosaccharide part of the *Vibrio salmonicida* (strain NCMB 2262) lipopolysaccharide was isolated by mild acid hydrolysis followed by gel-permeation chromatography. The structure was established mainly by methylation analysis, mass spectrometry, and NMR spectroscopy. It is concluded that the oligosaccharide has the following structure, in which L-α-D-Hep*p* is L-*glycero*-α-D-*manno*-heptopyranose, D-α-D-Hepp is D-*glycero*-α-D-*manno*-heptopyranose, α-D-Fuc*p*4N is 4-amino-4,6-dideoxy-α-D-galactopyranose, α-NonA is 5-acetamidino-7-acetamido-3,5,7,9-tetradeoxy-L-*glycero*-α-D-*galacto*-nonulosonic acid, BA is (*R*)-3-hydroxybutanoyl, and PEA is phosphoethanolamine. The substitution pattern of the branching heptosyl residue was deduced from ^{1}H NMR chemical shifts and conformations of the branching region, obtained by molecular modelling. The absolute configuration for NonA was determined by NMR spectroscopy from NOE correlations to the neighbouring sugar and ^{13}C NMR chemical shift data. It could also be shown that assignments of nonulosonic acids with the D-*glycero*-L-*galacto* configuration, reported by previous investigators, are erroneous and should be changed to L-*glycero*-D-*galacto*. The oligosaccharide is assumed to be linked to the 5-position of a Kdo residue, phosphorylated in the 4-position as observed for other lipopolysaccharides from Vibrionaceae.

* Corresponding author.

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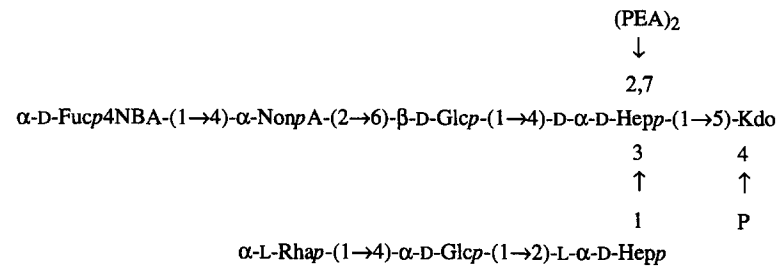


Keywords: Lipopolysaccharide; Structure; Salmon; NMR; Nonulosonic acid

1. Introduction

Vibrio salmonicida, a Gram-negative bacterium belonging to the Vibrionaceae family, is highly pathogenic for Atlantic salmon, and causes cold water vibriosis which has given significant losses in the aquaculture industry of Norway. Vaccines to cold water vibriosis are available and successful, but the reasons for the success are largely obscure. The immunogenicity resides mainly in two surface molecules, a 24-kDa protein and a lipopolysaccharide (LPS) [1,2]. The general structure of the latter is similar to those for LPSs in rough-type Gram-negative bacteria, that is, it comprises a lipid A and an oligosaccharide portion. Relatively little attention has been given to bacterial pathogens of fish, but the structures of the oligosaccharide parts of the LPSs from two types of *V. anguillarum* strain ST 40 [3] and strain V123 (serogroup JO-2) [4] have been determined; both contain unusual sugars. Some LPSs for the related genus *Aeromonas* have also been investigated (e.g., refs. [5,6]). We now report studies of the oligosaccharide part of the *V. salmonicida* LPS (strain NCMB 2262).

2. Results and discussion

Preparation of LPS and its oligosaccharide part (OS).—Freeze-dried bacteria were extracted with phenol–$CHCl_2$–petroleum ether [7] to yield a short-chain LPS. SDS(sodium dodecyl sulfate)-PAGE of the LPS showed a single component of high mobility [1], typical of such LPSs. Treatment of the LPS in a 0.1 M acetate buffer of pH 4.4, containing 0.1% sodium dodecyl sulfate, for 2 h at 100 °C, released the OS, which was purified by gel-permeation chromatography.

Constituents of the OS.—Hydrolysis of the OS with 2 M trifluoroacetic acid for 1 h at 120 °C yielded L-rhamnose, D-glucose, and L-*glycero*-D-*manno*-heptose (LD-Hep) in the approximate proportions 1:2:1, as indicated by GLC of the alditol acetates. On treatment of the OS with anhydrous hydrogen fluoride, two further sugars, D-*glycero*-D-*manno*-heptose (DD-Hep) and a 4,6-dideoxy-3-hydroxybutanamidohexose (Fig. 1), later shown to have the *galacto* configuration, were obtained (Table 1). It should be

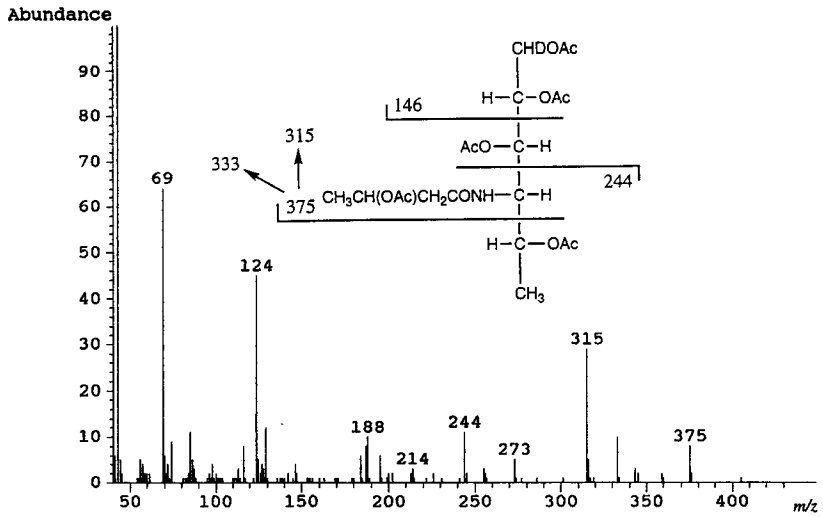


Fig. 1. The EIMS spectrum of the alditol acetate of 4,6-dideoxy-4-[(*R*)-3-hydroxybutanamido]-D-galactose. Diagnostic fragment ions are indicated.

emphasised that these analyses are only qualitative as it is not possible to obtain quantitative cleavage of glycosidic linkages without degradation. From GLC analysis on a chiral column of its methyl ester trifluoroacetate it was concluded that the acid is (*R*)-3-hydroxybutanoic acid. The absolute configurations of rhamnose, glucose, the heptoses, and 4-amino-4,6-dideoxygalactose were determined by GLC–MS analysis of their acetylated (*S*)-2-butyl glycosides essentially as determined by Gerwig et al. [8], and were shown to be L, D, L-*glycero*-D-*manno*, D-*glycero*-D-*manno*, and D, respectively. The 4-amino-4-deoxy sugar could not be detected in the trifluoroacetic acid hydrolysate since 4-amino sugars are converted into pyrrole derivatives upon treatment with strong aqueous acids [9].

The ^{1}H NMR spectrum of the OS (Fig. 2) showed that at least one further sugar residue must be present. Thus, signals for four methyl groups at δ 1.12 (d), 1.19 (d),

Table 1
Sugar analyses of the oligosaccharide from the *V. salmonicida* OS

Sugar	Detector response (%) [a]	
	A	**B**
Rha	24	11
Glc	52	36
LD-Hep	24	11
DD-Hep		18
Fuc4NBA		24

[a] Key: **A**, hydrolysis of oligosaccharide using CF_3CO_2H; **B**, solvolysis of oligosaccharide using anhydrous HF.

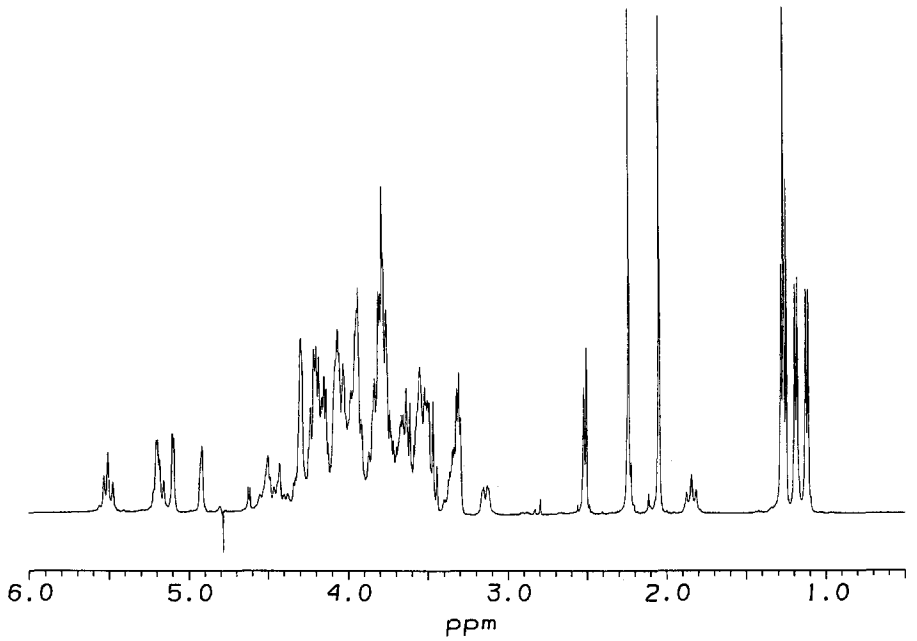


Fig. 2. The 400-MHz ^{1}H NMR spectrum of the *V. salmonicida* OS.

1.26 (d), and 1.28 (d), one *N*-acetyl group at δ 2.05, and a methyl group at δ 2.25, later shown to be derived from an acetamidino group, were present. Furthermore, signals for methylene protons were observed at δ 2.52 (d, 2 H), 1.84 (dd, 1 H), 3.15 (dd, 1 H), and 3.32 (m, ~4 H). The signal at δ 2.52 was assigned to the methylene group in the 3-hydroxybutanamido group and the signal at δ 3.32 to the protons of the nitrogen-bearing carbons of two ethanolamine groups. From data presented below it is evident that the ethanolamine groups are present as phosphoethanolamine (PEA) groups. One of the methyl groups, the acetyl group, and the acetamidino group are not accounted for by the sugars listed above, but were shown by NMR to belong to a diaminotetradeoxynonulosonic acid, referred to as nonulosonic acid below. From signals in the one-dimensional (1D) spectrum as well as from COSY and CH-correlated spectra it was evident that signals for anomeric protons were present at δ 4.51 ($J_{H\text{-}1,H\text{-}2}$ ~ 8 Hz), 4.92 ($J_{H\text{-}1,H\text{-}2}$ not resolved), 5.10 ($J_{H\text{-}1,H\text{-}2}$ ~ 4 Hz), 5.16–5.20 ($J_{H\text{-}1,H\text{-}2}$ not resolved), 5.20 ($J_{H\text{-}1,H\text{-}2}$ ~ 4 Hz), and 5.48–5.53 ($J_{H\text{-}1,H\text{-}2}$ not resolved).

The ^{13}C NMR spectrum (Fig. 3) showed signals for six methyl carbons (δ 16.4, 17.4, 19.1, 19.7, 22.7, and 22.9), signals for three carbons linked to nitrogen (δ 54.3, 54.5, and 55.0), and signals for several methylene carbons at δ 37.6, 41.1, 41.2, and 45.5, two of which (41.1 and 41.2) were doublets ($^3J_{C,P}$ ~ 7 Hz) and identified as signals for the nitrogen-bearing carbons of two PEA groups. Signals for several other methylene groups were observed in the region δ 60.9–66.2. Two doublets at δ 62.6 and 62.7, with $^2J_{C,P}$ values ~ 5.5 Hz each, corresponded to the oxymethyl carbons of the two PEA groups. Four major signals corresponding to anomeric carbons were observed at δ 95.6 ($^1J_{C,H}$

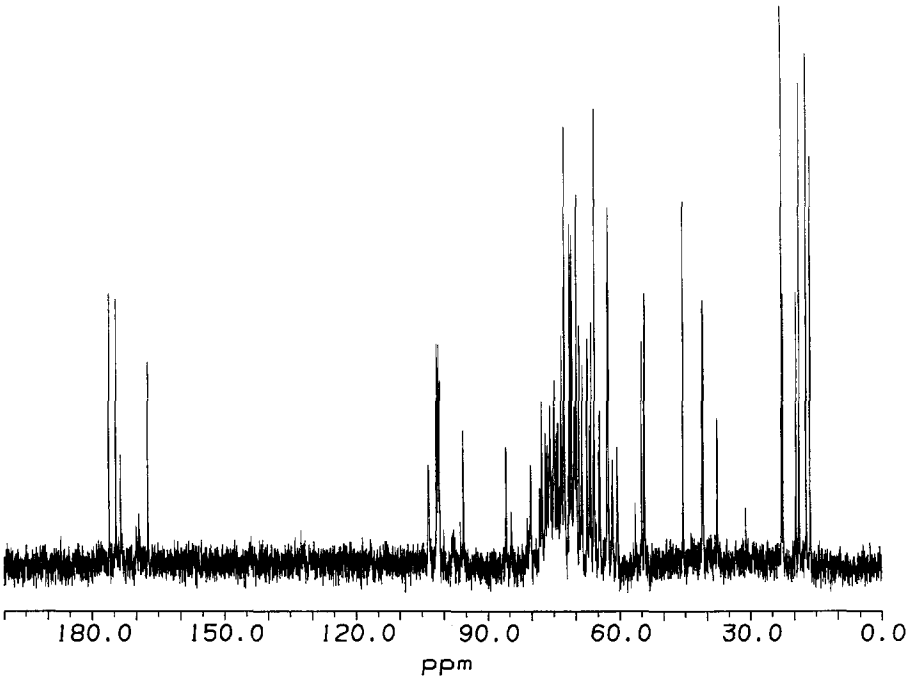


Fig. 3. The 100-MHz ^{13}C NMR spectrum of the *V. salmonicida* OS.

173 Hz), 101.3 ($^{1}J_{C,H}$ 174 Hz), 101.6 ($^{1}J_{C,H}$ 172 Hz), and 103.5 ($^{1}J_{C,H}$ 162 Hz), as well as several small signals at $\delta \sim 98$ and ~ 101. Signals for four carbonyl carbons were observed at δ 167.6, 173.3, 174.5, and 176.1.

A ^{1}H-decoupled ^{31}P NMR spectrum of the OS showed the presence of two resonances at δ −0.61 and 0.26. A P,H COSY spectrum demonstrated that the ^{31}P resonance at δ −0.61 correlated to a ^{1}H NMR resonance at δ 4.46; the other ^{31}P resonance, at δ 0.26, correlated to the ^{1}H NMR resonances at δ 4.16 and 3.32. A P,P-correlated COSY spectrum showed no cross-peaks between the two ^{31}P signals. From the combined data it was therefore concluded that no pyrophosphate was present in the OS.

An FAB mass spectrum, obtained in the positive mode, of the OS in the ammonium form showed a pseudomolecular ion, $[M + H]^+$, at m/z 1867.8. The molecular mass of an oligosaccharide comprising two hexoses, one deoxyhexose, two heptoses, one Kdo, one 4,6-dideoxy-4-hydroxybutanamidohexose, and one nonulosonic acid residue, with one acetamido, one acetamidino, and two PEA groups, is 1884.6. The observed mass is thus consistent with an anhydro derivative of this OS. When a glycoside of Kdo is phosphorylated at O-4, different anhydro-Kdo residues are formed under delipidation conditions [10]. It is therefore assumed that such a 4-phosphate group is present and that the reducing end of the oligosaccharide is occupied by anhydro-Kdo present in multiple forms. The FAB mass spectrum of the dephosphorylated OS showed an $[M + H]^+$ ion at m/z 1447.5, in agreement with the loss of two PEA groups.

Table 2
Methylation analyses of the oligosaccharide from the *V. salmonicida* OS

Sugar [a]	t_R [b]	Detector response (%) [c]		
		A	**B**	**C**
2,3,4-Rha	0.74	29	14	17
2,3,4,6-Glc	1.00			16
2,3,6-Glc	1.26	36	18	24
2,3,4-Glc	1.32	22	20	14
3,4,6,7-LD-Hep	1.76	13	14	19
2*,6,7*-DD-Hep	1.90			11
2,3,3'-Fuc4NBA	2.18		10	
2,3,4,3'-Fuc4NBA	2.51		16	
6-DD-Hep	2.60		8	

[a] 2,3,4-Rha = 2,3,4-tri-*O*-methylrhamnose, etc.; *, trideuteriomethyl group.
[b] Retention times relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on an HP-5 column using the temperature program: 180 °C for 3 min to 220 °C at a rate of 3 °C/min.
[c] Key: **A**, OS, hydrolysis with CF_3CO_2H; **B**, OS, solvolysis with anhydrous HF; **C**, methylated, dephosphorylated, and remethylated (CD_3I) OS.

Methylation analysis.—Methylation analysis of the OS (Table 2) demonstrated one residue each of terminal rhamnose, 4- and 6-substituted glucose, and 2-substituted heptose. The 2-substituted heptose is the only heptose appearing in the methylation analysis when using trifluoroacetic acid as hydrolysing agent, and it is therefore concluded, in agreement with sugar analysis data, that it is LD-Hep. When the methylation analysis was performed using solvolysis with anhydrous HF, derivatives of DD-Hep and 4,6-dideoxy-4-[(*R*)-3-hydroxybutanamido]galactose were also observed, the latter partially *N*-methylated. The MS of the amino sugar showed the presence of methyl groups at O-2 and O-3 as evident from the fragments at *m/z* 305 and 291, demonstrating that the 4-amino-4,6-dideoxygalactopyranosyl residue is terminal (Fig. 4). A 6-*O*-methyl-DD-Hep was obtained in a methylation analysis with HF solvolysis. In an additional methylation analysis, methylated OS was treated with aq 40% HF at 4 °C for 72 h and remethylated with trideuteriomethyl iodide. The hydrolysate contained a new methyl ether, 2,6,7-tri-*O*-methyl-DD-Hep with trideuteriomethyl groups at O-2 and O-7. This indicated that the DD-Hep was a branch-point residue, substituted at O-3 and O-4, and that the PEA groups occupy O-2 and O-7. Thus, methylation analysis indicates the presence of a branched structure in the OS where a rhamnose and a 4,6-dideoxy-4-[(*R*)-3-hydroxybutanamido]galactose residue terminate the two branches.

Relative and anomeric configuration of the sugar residues in the OS.—The ^1H and ^{13}C NMR resonances of residues **A**–**G** were assigned by homo- and hetero-nuclear 2D correlation experiments (Table 3). Two or three different spin systems were obtained for some residues, due to heterogeneous environments induced by multiple anhydro-Kdo forms. From the absence of low-field signals (> 104 ppm) in the anomeric region, and the presence of signals only attributable to the Kdo residues, at δ 83–86, it is concluded that all sugar residues are pyranosidic. Methylation analysis data are in accord with this conclusion.

Table 3
NMR chemical shifts at 25 °C for the *V. salmonicida* OS

Atom	Chemical shifts (δ) [a]													
	α-D-Fuc*p*4NBA-(1 → **D**		→ 4)-α-NonA-(2 → **G**		→ 6)-β-D-Glc*p*-(1 → **F**		→ α-L-Rha*p*-(1 → **E**		→ 4)-α-D-Glc*p*-(1 → **B**		→ 2)-L-α-D-Hep*p*-(1 → **A**		(PEA)$_2$-2,7-D-α-D-Hep*p*-(1- **C**	↓4 3↑
1	5.10	95.6		173.3	4.51	103.5	4.92	101.6	5.20	101.3	5.51	101.3	5.19	97.8
	(4)	(173)			(8)	(162)	n.r. [b]	(172)	(4)	(174)	n.r.	(> 175)	n.r.	(172)
2	3.78	68.6		~ 101	3.45	74.3	4.04	71.4	3.55	73.1	4.30	75.7	4.46	~ 79
3ax	3.82	72.6	1.84	37.6		76.9	3.78	71.1	3.82	72.5	3.95		4.01	
3eq			3.15											
4	4.30	54.3	3.78	74.4			3.54	72.6	3.64	77.7				
N*H*-4	8.05													
5	3.85	66.5	3.62	54.5			4.07	69.8	3.98	71.0				
N*H*-5			9.53											
6	1.12	16.4	4.08	71.1			1.28	17.4						
7			3.94	55.0										
N*H*-7			8.49											
8			4.06	67.4										
9			1.19	19.1										
	BA [c]		**Am** [d]			**Ac** [e]							**PEA** [f]	
1		176.1	8.97 [g], 8.71 [g]		167.6		174.5						4.16	62.6, 62.7
2	2.52	45.5	2.25		19.7	2.05	22.7						3.32	41.1, 41.2
3	4.22	65.8												
4	1.26	22.9												

[a] Values for $^{3}J_{H,H}$ and $^{1}J_{C,H}$ (Hz) are given in parentheses.
[b] n.r. = Not resolved.
[c] (*R*)-3-Hydroxybutanamido group.
[d] Acetamidino group.
[e] Acetamido group.
[f] Phosphoethanolamine groups.
[g] Amidinium protons.

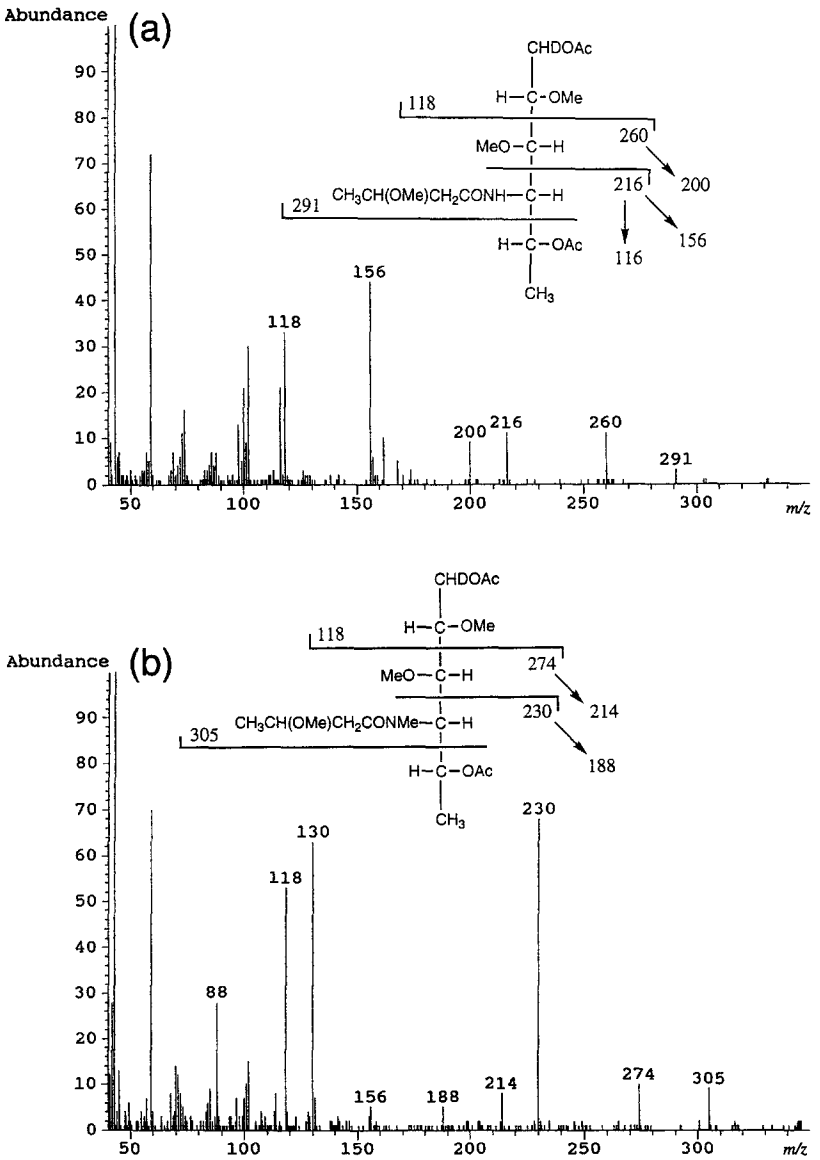


Fig. 4. The EIMS spectra of the alditol acetate of 4,6-dideoxy-4-[(*R*)-3-methoxybutanamido]-2,3-di-*O*-methyl-D-galactose (a) and 4,6-dideoxy-4-[(*R*)-3-methoxy-*N*-methylbutanamido]-2,3-di-*O*-methyl-D-galactose (b). Diagnostic fragment ions are indicated.

Residue **A** had the α-anomeric configuration as determined from the $J_{C\text{-}1,H\text{-}1}$ values > 175 Hz; this was further indicated by the chemical shift values for the H-1 signal (δ 5.48–5.53). The small $J_{H\text{-}1,H\text{-}2}$ value (< 2.5 Hz) indicated **A** to have the *manno* configuration. The high chemical shift values for H-2 (δ 4.30) and C-2 (δ 75.7) signals

show that residue **A** is the 2-substituted LD-Hep*p*. The small $J_{H-1,H-2}$ value, in combination with the heterogeneity due to the proximity to the Kdo residue, made full assignment of the ^{1}H and ^{13}C signals impossible.

A $J_{H-1,H-2}$ value ~ 4 Hz for residue **B** (δ 5.20), a $J_{C-1,H-1}$ value 174 Hz, and $^{3}J_{H,H}$ values (> 8 Hz) of the signals for all sugar ring protons are consistent with an α-D-glucopyranosyl residue. The signal for C-4 at δ 77.2 is 8.2 ppm downfield of the corresponding chemical shift for α-D-glucose, and the H-4 (δ 3.64) signal is also shifted downfield, 0.22 ppm, indicating that **B** is the 4-substituted α-D-glucopyranosyl residue.

Residue **C**, like residue **A**, gives several signals for its anomeric proton because of the heterogeneous environment, and the small $J_{H-1,H-2}$ values (< 2.5 Hz) made full assignment of the ^{1}H and ^{13}C signals impossible. The $J_{C-1,H-1}$ values are 172 Hz and the $J_{H-1,H-2}$ values are < 2.5 Hz, therefore H-1 and H-2 should have a *trans*-diequatorial disposition. The H-2 signal at δ 4.46 is clearly coupled to phosphorus (δ −0.61) and it is therefore concluded that residue **C** is a 2,7-bisphosphorylated 3,4-disubstituted D-*glycero*-α-D-*manno*-heptopyranosyl residue.

All six proton resonances of residue **D** were assigned. The large $J_{H-2,H-3}$ value (> 8 Hz) and the small values for $J_{H-3,H-4}$ and $J_{H-4,H-5}$ (< 4 Hz) indicate that the sugar has the *galacto* configuration. The $J_{H-1,H-2}$ value (~ 4 Hz) and the $J_{C-1,H-1}$ value (173 Hz) demonstrate that it has the α configuration. The chemical shift of the C-4 signal is 54.3 ppm, that is, in the region for nitrogen-bearing carbons. These results indicate that **D** is the terminal 4-amino-4,6-dideoxy-α-galactopyranosyl group. The chemical shift of the C-6 signal, δ 16.4, is typical of a 6-deoxy sugar with an axial substituent at C-4. The connectivity pattern in the COSY spectrum shows that the *N*-acyl group is a 3-hydroxybutanoyl group.

All ^{1}H NMR signals of the spin system of residue **E** were assigned. The small $J_{H-1,H-2}$ value (< 2.5 Hz) and the fact that C-6 is a methyl carbon indicate that residue **E** is the rhamnosyl group. The $J_{C-1,H-1}$ value (172 Hz) and the $^{3}J_{H,H}$ values indicated an α-rhamnopyranosyl group. The observed ^{1}H and ^{13}C NMR chemical shifts are in good agreement with such a group, with the exception that the H-5 signal (δ 4.07) is shifted 0.21 ppm downfield. The reason for this is discussed below.

Residue **F** was assigned to a β-glucopyranosyl residue based on the chemical shift of the H-1 signal (δ 4.51) and the $J_{C-1,H-1}$ and $J_{H-1,H-2}$ values 162 and ~ 7 Hz, respectively. Thus, **F** must be the 6-substituted glucose residue. The TOCSY slice, through the H-1 signal of **F** at δ 4.51, contained overlapping signals in the region where signals for H-3 to H-5 for a β-glucopyranosyl residue are expected to be, that is, at δ 3.49–3.57.

Starting either from the signal at δ 1.84, corresponding to H-3ax, or from the signal at δ 1.19, corresponding to the H-9 protons, the full spin system of residue **G** was unravelled from different H,H-correlation spectra. The signals corresponding to C-5 (δ 54.4) and C-7 (δ 55.0) appeared in the region for nitrogen-bearing carbons. Atoms C-4, C-6, and C-8 have signals in the region for secondary carbons substituted with hydroxy or glycosyloxy groups, δ_{C-4} 74.4, δ_{C-6} 71.1, and δ_{C-8} 67.4. The chemical shifts for the signals of C-3 (δ 37.6) and C-9 (δ 19.1) corresponded to a methylene and a methyl group, respectively. Finally, the signals for a carbonyl, C-1 (δ 173.3), and a quaternary carbon atom, C-2 (δ ~ 101), were observed. From the NMR data it was

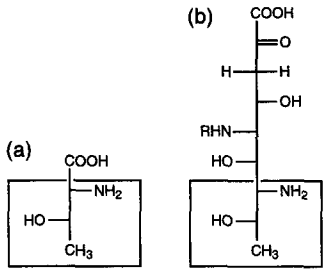


Fig. 5. Structure of D-threonine (a) and 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-nonulosonic acid (b) with boxes indicating the similar parts.

concluded that residue **G** is a 5,7-diamino-3,5,7,9-tetradeoxynonulosonic acid. Such aminodeoxy sugars with the L-*glycero*-L-*manno* configuration (pseudaminic acid) (e.g., refs. [11,12]) and the D-*glycero*-L-*galacto* configuration (legionaminic acid) [13–17] have previously been reported in the literature. A detailed examination of the original assignment of the latter nonulosonic acid shows that in fact it should be assigned the L-*glycero*-D-*galacto* configuration. The C-7–C-9 fragment of the nonulosonic acid is analogous to threonine, and it was earlier concluded that it was homomorphic with D-threonine. However, D-threonine is not homomorphic with the C-7–C-9 fragment of D-*glycero*-L-*galacto*-nonulosonic acid, but with L-*glycero*-D-*galacto*-nonulosonic acid, as indicated in Fig. 5.

This correction thus changes the configurational assignment of the nonulosonic acid residue in the O-specific polysaccharides from *Pseudomonas aeruginosa* O13 [13], *Yersinia ruckeri* O1 [14], *Legionella pneumophila* serogroup 1 [15], *Salmonella arizonae* O61 [16], and *Vibrio alginolyticus* [17].

To conform with the methylation analysis data, residue **G** must be substituted by a glycosyl residue either in the 4- or the 8-position. Based on observed NOE and heteronuclear three-bond correlations, see below, it was demonstrated that residue **D** substituted the 4-position of the nonulosonic acid. A correlation in a COSY spectrum of the OS in 9:1 H_2O–D_2O was observed between the signal for H-5 (δ 3.62) and a signal at δ 9.53 ($^3J_{H,H}$ 10.5 Hz). A correlation in a NOESY spectrum of the OS, in the same mixture, between the signal at δ 9.53 and the signal for the acetamidino methyl group (δ 2.25) was also observed (Table 4, Fig. 6). These two correlations demonstrated that C-5 of residue **G** is substituted with an acetamidino group. A COSY correlation between the signal for H-7 in **G** (δ 3.94) and a signal at δ 8.49 ($^3J_{H,H}$ 9.2 Hz), and an NOE correlation between the latter signal and the signal for the acetamido methyl group (δ 2.05) demonstrated that C-7 of residue **G** is substituted with an acetamido group. These observations were corroborated by correlations observed in an HMBC (heteronuclear multiple-bond correlation) spectrum (data not shown).

From a combination of ^{1}H and ^{13}C NMR data the configuration of the nonulosonic acid could be established. Carbon atoms C-4–C-6 all had axial protons as seen from the large $^3J_{H,H}$ values between the ring proton resonances, $J_{H\text{-}3ax,H\text{-}4}$ 11.9, $J_{H\text{-}4,H\text{-}5}$ 10.2, and $J_{H\text{-}5,H\text{-}6}$ 10.0 Hz. Thus, the C-4–C-6 fragment of residue **G** has the *arabino* configuration, which is the configuration proposed previously [13–17]. From observed inter-

Table 4
NOE correlations between NH and other protons in the *V. salmonicida* OS

			NOE to		
Residue, Atom	δ	$^3J_{H,H}$ (Hz)	Residue, Atom	δ	Intensity [a]
G, NH-5	9.53	10.5	**G**, H-6	4.08	s
			G, H-7	3.94	m
			G, H-4	3.78	m
			G, Am (CH_3)	2.25	s
G, AmHa	8.97	n.r. [b]	**D**, H-3	3.82	w
			G, H-5	3.62	w
			G, Am (CH_3)	2.25	s
G, AmHb	8.71	n.r.	**D**, H-3	3.82	w
			G, H-5	3.62	s
			G, Am (CH_3)	2.25	w
G, NH-7	8.49	11.0	**G**, H-8	4.06	m
			G, H-7	3.94	w
			G, H-5	3.62	w
			G, Ac (CH_3)	2.05	s
D, NH-4	8.05	11.0	**D**, H-4	4.30	w
			D, H-2	3.78	s
			D, BA (CH_2)	2.52	s

[a] Intensities are given as strong (s), medium (m), or weak (w).
[b] n.r. = Not resolved.

residue NOE correlations and from ^{13}C NMR chemical shift data, see below, it was concluded that the absolute configuration of the fragment C-4–C-6 is L-*arabino*.

The C-7–C-9 fragment of the nonulosonic acid is analogous to threonine or allothreonine. The chemical shift of the signal for the methyl carbon is ~ 19 ppm for threonine and ~ 17 ppm for allothreonine. The present chemical shift of the signal for C-9 is 19.1 ppm. Thus, the *threo* configuration is indicated for the C-7–C-8 fragment of residue **G**.

From the fragments, two sugars can be constructed, one having the L-*glycero*-D-*galacto* configuration and the other the D-*glycero*-L-*altro* configuration. Molecular mechanics calculations of these structures yielded two low-energy conformations for the former and one for the latter (Table 5). The dihedral angle set by C-5–C-6–C-7–C-8 was found to be *trans* in all three low-energy conformations. This results in a *gauche* relation between H-6 and H-7 in the nonulosonic acid having the L-*glycero*-D-*galacto* configuration and a *trans* relation between H-6 and H-7 in that having the D-*glycero*-L-*altro* configuration. A small $J_{H\text{-}6,H\text{-}7}$ value, ~ 2 Hz, was observed which is in accord with a *gauche* relation between the protons, thus indicating the L-*glycero*-D-*galacto* configuration. Small $J_{H\text{-}6,H\text{-}7}$ values, < 2.5 Hz, have also been observed for the nonulosonic acids from the O-polysaccharides studied earlier (see above). However, the $J_{H\text{-}6,H\text{-}7}$ value for pseudaminic acid (L-*glycero*-L-*manno*) was large, 10 Hz [11,12]; this is a value which would be expected for the nonulosonic acid having the D-*glycero*-L-*altro* configuration, since the C-6–C-7 fragments of both compounds have the *erythro* configuration. Another result of the molecular mechanics calculations is that proton–proton distances are estimated. For the nonulosonic acid having the L-*glycero*-D-*galacto*

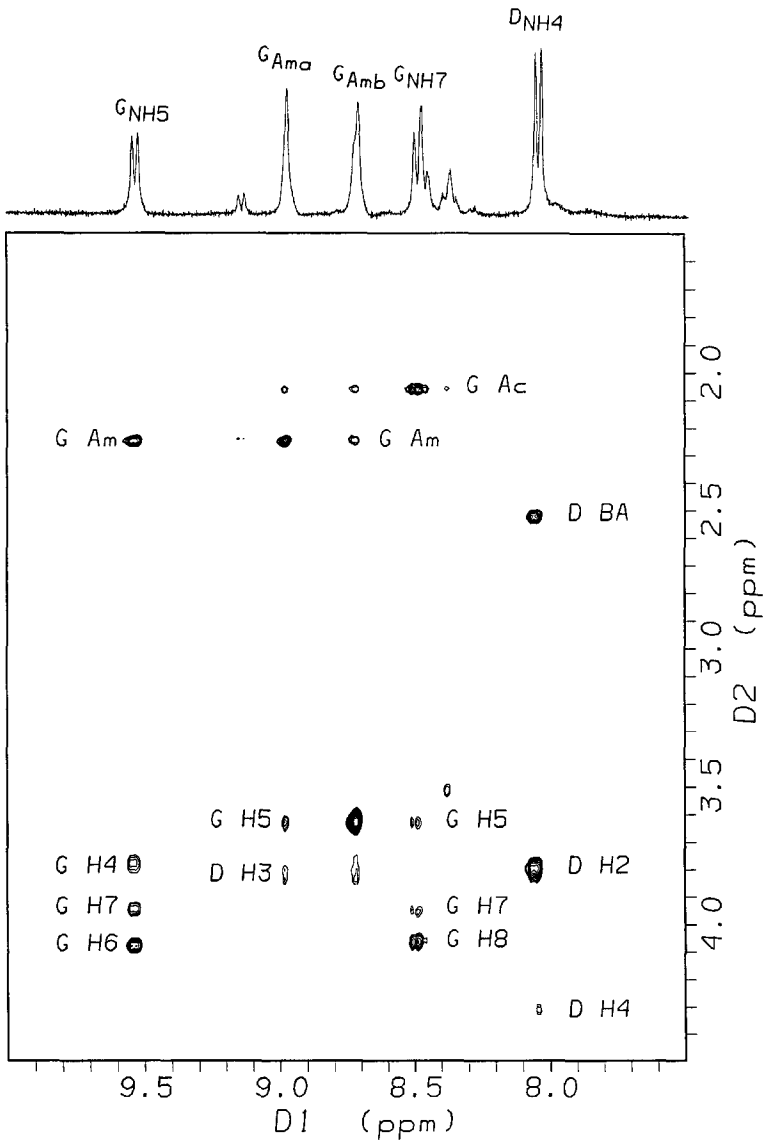
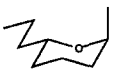


Fig. 6. Section of the 1D ^{1}H NMR spectrum (500 MHz, top) and section of a NOESY spectrum (bottom) of the *V. salmonicida* OS in 9:1 H_2O–D_2O (pH 5.8).

configuration the distance between N*H*-5 and N*H*-7 is estimated to be ~ 4.8 Å, and the distance between N*H*-5 and H-7 ~ 2.5 Å (Table 5). The corresponding distances for the D-*glycero*-L-*altro* isomer are ~ 2.6 and 3.8 Å, respectively. The observation of an NOE correlation between the signals for N*H*-5 and H-7 and the absence of any NOE correlation between the signals for N*H*-5 and N*H*-7 is in accord with the configuration

Table 5
Selected intra-residue H–H distances for some energy-minimised conformations of 5-acetamidino-7-acetamido-3,5,7,9-tetradeoxynonulosonic acids having L-*glycero*-D-*galacto* and D-*glycero*-L-*altro* configuration

Configuration	Side-chain conformation	Potential energy (kcal/mol)	Distances (Å) from			
			N*H*-5		N*H*-7	
L-*glycero*-D-*galacto*	*trans*–*trans* [a]	4.95	H-4	2.76	H-5	3.07
			H-6	2.44	H-6	3.79
			H-7	2.53	H-8	3.69
			N*H*-7	4.82		
	cis–*trans*	5.13	H-4	2.76	H-5	2.98
			H-6	2.42	H-6	3.79
			H-7	2.55	H-8	2.55
			N*H*-7	4.76		
D-*glycero*-L-*altro*	*trans*–*trans*	6.00	H-4	2.70	H-5	4.03
			H-6	2.46	H-6	2.52
			H-7	3.76	H-8	3.76
			N*H*-7	2.57		

[a] Refers to dihedral angles at C-5–C-9.

indicated by the small $J_{H\text{-}6,H\text{-}7}$ value. Thus, it is proposed that residue **G** is 5-acetamidino-7-acetamido-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-nonulosonic acid. The anomeric configuration of residue **G** was determined to be α, since the large chemical shift difference between H-3eq (δ 3.15) and H-3ax (δ 1.84) resonances demonstrated the carboxyl group to be axial [18]. Of the two possible sites for substitution, O-4 and O-8, only the former is substituted, as shown by the high chemical shift value for the C-4 signal (δ 74.5) and the low value for the chemical shift of the C-8 signal (δ 67.4). The corresponding values for the same sugar residue in the *P. aeruginosa* O13 LPS [13], where it is substituted at O-8 but not at O-4, are δ 69.3 and 73.5, respectively.

Sequence determination of OS.—Several diagnostic fragment ions were observed in the B/E-linked scan FAB mass spectrum [19], using the pseudomolecular ion of the OS (m/z 1867.6) as precursor ion (Fig. 7). Fragment ions, given as nominal masses, formed via pathway A [20] indicated the presence of a disaccharide element consisting of residues **D** and **G** (m/z 547), and a trisaccharide element **D–G–B** or **D–G–F** (m/z 709). A pathway B fragment ion, corresponding to loss of residue **D** (m/z 1636), was observed in agreement with the presence of the structural element **D–G–B** or **D–G–F**. A fragment ion (m/z 519) formed via pathway C corresponds to a trisaccharide element containing rhamnose, glucose, and heptose. A pathway B fragment ion (m/z 1367) corresponds to loss of rhamnose, glucose, and heptose from the pseudomolecular ion.

In order to study further the sequence of the OS, H,H-NOESY experiments were employed. Inter-residue NOE contacts from anomeric protons established one trisaccharide and one disaccharide element (Table 6). Thus, H-1 in residue **E** (δ 4.92) had inter-residue contact to H-4 (δ 3.64) in residue **B**, in addition to an intra-residue contact to H-2 (δ 4.04). A correlation to a signal at δ 3.80, possibly H-6a in **B**, was also

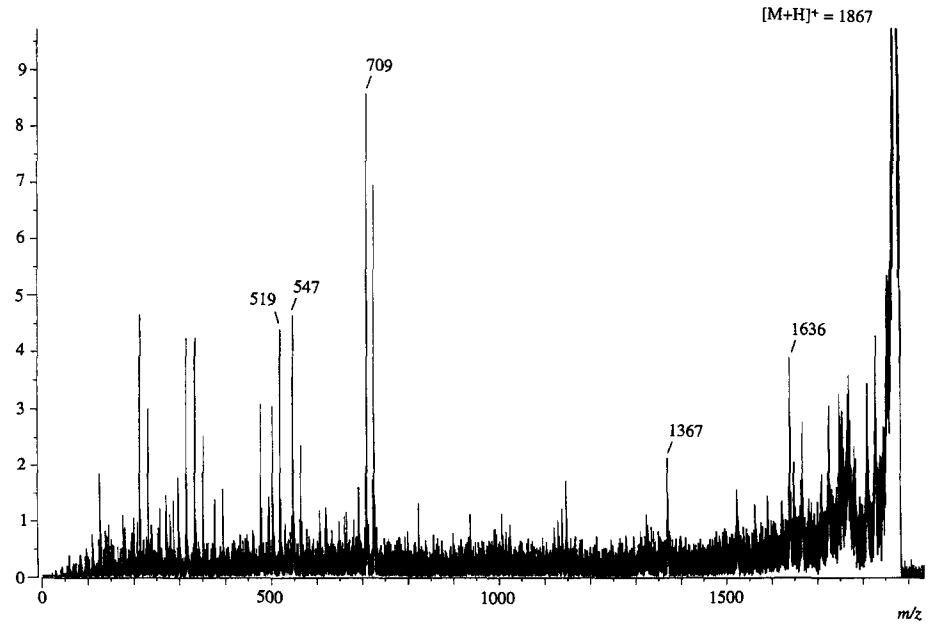


Fig. 7. The B/E-linked scan of the *V. salmonicida* OS. Diagnostic fragment ions are indicated; see text for interpretation.

Table 6
Observed NOE correlations for anomeric protons in the *V. salmonicida* OS obtained from a NOESY spectrum

NOE from anomeric proton		NOE contact to proton		
Residue	δ	Residue, atom	δ	Intensity [a]
→ 2)-L-α-D-Hep*p*-(1 →	5.51	**A**, H-2	4.30	w
A				
→ 4)-α-D-Glc*p*-(1 →	5.20	**A**, H-2	4.30	m
B		**B**, H-2	3.55	m
		G, H-3ax	1.84	w
→ 2,3,4,7)-D-α-D-Hep*p*-(1 →	5.22	**C**, H-2	4.46	w
C				
α-D-Fuc*p*4NBA-(1 →	5.11	**D**, H-2	3.78	m
D		**G**, H-4	3.78	m
		G, H-3eq	3.15	m
		G, H-3ax	1.84	w
α-L-Rha*p*-(1 →	4.93	**E**, H-2	4.04	w
E		**B**, H-4	3.64	s
→ 6)-β-D-Glc*p*-(1 →	4.51	**C**, H-7 [b]	4.30	s
F		**F**, H-3	3.54	s

[a] Intensities are given as strong (s), medium (m), or weak (w).
[b] Tentative assignment.

observed. The anomeric proton of residue **B** had an inter-residue contact to H-2 in residue **A** (δ 4.30) as well as a contact to its own H-2 (δ 3.55). Thus trisaccharide element **1** was postulated.

E **B** **A**

α-L-Rha*p*-(1→4)-α-D-Glc*p*-(1→2)-L-α-D-Hep*p*-(1→

1

The signal for H-5 in residue **E** is shifted downfield 0.21 ppm, compared to α-rhamnose; this is believed to be a consequence of a short distance between H-5 of **E** and O-3 of **B**. A simple molecular model and molecular mechanics calculations indicated that such a short distance is present. The disaccharide element **D–G**, established from the B/E-linked scan FAB mass spectrum, was corroborated, as inter-residue contacts from H-1 in **D** to H-3eq (δ 3.15) and H-3ax (δ 1.84) in residue **G** were observed. The contacts from H-1 in residue **D** to H-3eq and H-3ax of **G** are conclusive, but a further correlation to a signal at δ 3.78 is not, as both H-4 in **G** and H-2 in **D** resonate with this chemical shift.

D **G**

α-D-Fuc*p*4NBA-(1→4)-α-Non*p*A-(2→

2

The chemical shift of the signal for C-3 in **G** is δ 37.6, the value for the corresponding residue in the LPS from *P. aeruginosa* O13, which is not substituted at the 4-position, is δ 42.3. Thus, the signal for C-3 in **G** in the *V. salmonicida* OS is shifted significantly upfield, ~4.5 ppm. Such a large change can only result from substitution at C-4 and a highly defined stereochemistry at the glycosidic linkage [21]. Thus, a so-called *γ-gauche* interaction must be present, in which an anomeric proton and an inter-residue neighbouring equatorial proton are close to each other. This arrangement is associated with small downfield shifts of the signals for the anomeric carbon and the linkage carbon in addition to a large upfield shift of the signal for the neighbouring carbon carrying the equatorial proton. A small downfield shift, compared to the monomer, is observed for the signal for C-1 in residue **D** (δ 95.6), and this together with the NOE contacts between H-1 in residue **D** and the methylene protons in residue **G** and NOE between both amidino protons and H-3 in residue **D** demonstrate the relative configuration of the constituent monosaccharides in disaccharide **2** (Fig. 8). As the absolute configuration for Fuc4N was determined to be D, it follows that the absolute configuration of NonA is L-*glycero*-D-*galacto*. From the discussion above it is clear that this is the only nonulosonic acid found, having the *glycero–galacto* configuration, and also that D is the only configuration found for Fuc4N. From the combined evidence

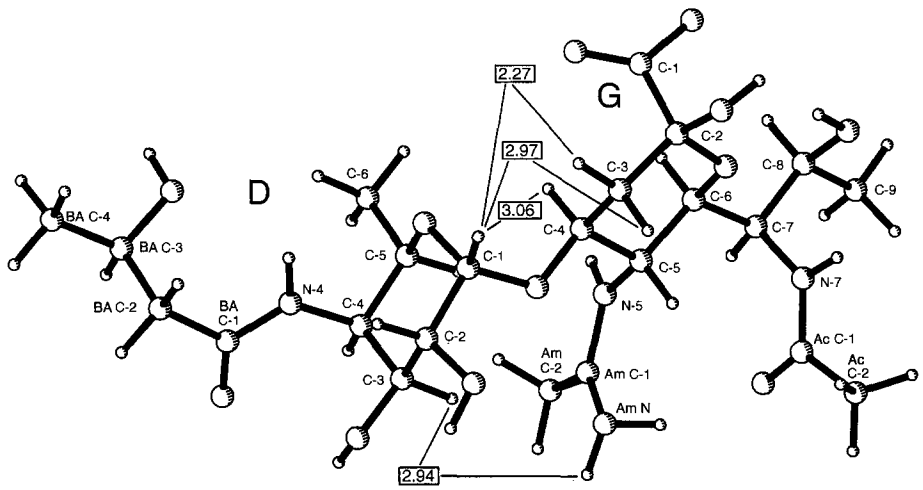


Fig. 8. Disaccharide element **2** as indicated by molecular modelling and NMR data. The short distances (Å) are calculated and observed as NOE correlations and extraordinary δ values.

based on MS and NMR data, partial structure **3** is indicated, as **B**, the other D-glucopyranosyl residue, is already engaged in structure **1**.

D **G** **F**

α-D-Fuc*p*4NBA-(1→4)-α-Non*p*A-(2→6)-β-D-Glc*p*-(1→

3

The two trisaccharide side-chains (**1** and **3**) are linked to the 3- and 4-positions of DD-Hep, which in turn is linked to Kdo. The chemical shift for the H-1 signal of residue **A** has an unusually high value (δ 5.48–5.53). This indicates a defined geometry at the branch-point residue **C**, in which the anomeric proton in **A** should have close contact with one or several oxygen atoms. This phenomenon has been observed previously, for example, with the LPS from *Neisseria gonorrhoea* F62 which also contains a 3,4-disubstituted heptosyl residue [22]. In order to investigate this, the low-energy conformations of the two possible branched trisaccharide elements **4** and **5** were estimated by molecular mechanics calculations (Fig. 9).

F **C**

β-D-Glc*p*-(1→4)-D-α-D-Hep*p*

3

↑

1

L-α-D-Hep*p*

A

4

A **C**

L-α-D-Hep*p*-(1→4)-D-α-D-Hep*p*

3

↑

1

β-D-Glc*p*

F

5

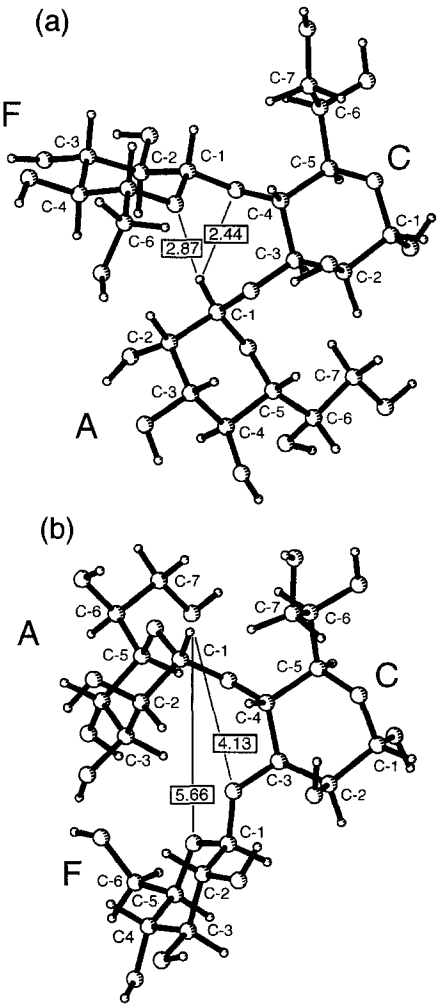
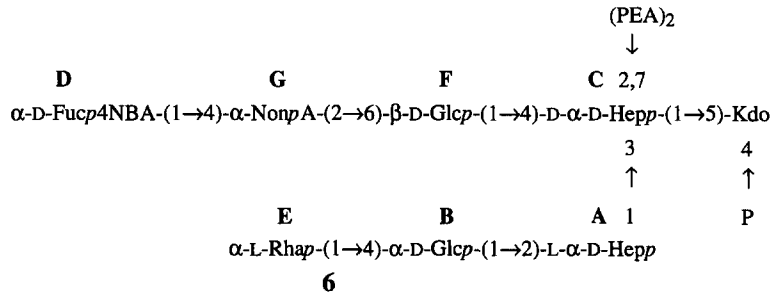


Fig. 9. Energy-minimised conformations of **4** (a) and **5** (b) with indicated short distances (Å) to oxygen atoms from the anomeric proton in residue **A**.

For trisaccharide **4**, in which residue **A** is linked to O-3 in **C**, the distances between H-1 in **A** and the two closest oxygen atoms, O-4 in **C** and O-5 in **F**, were estimated to be 2.44 and 2.87 Å, respectively. For trisaccharide **5** the distances between H-1 in **A** and O-3 in **C** and between H-1 in **A** and O-5 in **F** were estimated to be 4.13 and 5.66 Å, respectively. As no other short inter-residue distances from H-1 in **A** to oxygen atoms were calculated for trisaccharide **5**, this strongly indicates that **4** is actually the trisaccharide element present in the OS. It may be assumed that the DD-Hep residue substitutes the 5-position of the Kdo-4-phosphate residue, as has been observed for other

LPS from Vibrionaceae [23]. Thus, from the combined evidence, structure **6** is proposed for the oligosaccharide part of the *V. salmonicida* lipopolysaccharide.



6

3. Experimental

General methods.—Concentrations were performed under diminished pressure at < 40 °C or by flushing with air or nitrogen at room temperature. For GLC, a Hewlett–Packard 5830 instrument fitted with a flame-ionisation detector was used. GLC–MS (EI) was performed on a Hewlett–Packard 5970 MSD. A JEOL SX102 instrument was used for the FABMS. The spectra were acquired from the OS containing a dideuterated anhydro-Kdo residue, arising from exchange in D_2O in connection with the NMR studies, but masses are given for the protiated compound for simplicity. Ions were produced by a beam of Xe atoms (6 keV), using a 1:1 mixture of glycerol and thioglycerol as matrix. Separations of alditol acetates, partially methylated alditol acetates, and acetylated 2-butyl glycosides were performed on an HP-5 fused-silica capillary column (25 m × 0.20 mm), using a temperature program: 180 °C for 3 min to 250 °C at 3 °C/min. GPC was performed on a column of Superdex 30 (Pharmacia AB), using aq 0.07 M pyridinium acetate buffer (pH 5.4) as irrigant, and monitored with a differential refractometer.

Preparation of the oligosaccharide (OS).—The LPS was obtained after phenol–$CHCl_3$–petroleum ether extraction [7]. The LPS was suspended in aq 0.1 M NaOAc (0.2 mL/mg) containing 0.1% sodium dodecyl sulfate (SDS). The pH was adjusted to 4.4 by addition of glacial AcOH, and the solution was kept at 100 °C for 30 min [24,25], cooled in an ice bath, and lyophilised. The product was washed in absolute EtOH, suspended in H_2O, and centrifuged. This procedure was repeated once and the volume of the combined aqueous solutions was reduced. The solution was added to a Sep-Pak C18 cartridge with water as eluant in order to remove the last traces of lipids. The lyophilised eluate was purified by GPC on a Superdex 30 column. Only one peak in the region for large oligosaccharide was obtained. The fraction was lyophilised and the OS was obtained in ~ 30% yield.

NMR spectroscopy.—The ^{1}H and ^{13}C NMR spectra were recorded with a JEOL Alpha 400 or GSX 270 spectrometer or a Varian unity 500 spectrometer, using standard pulse sequences. Spectra were processed using standard software of the Felix program v2.3 software (Biosym Technologies Inc.) The ^{1}H and ^{13}C NMR spectra of D_2O solutions, containing a 20-mM Na_3PO_4 buffer of pD 6.2, were recorded at 25 °C. For 9:1 H_2O–D_2O solutions with the same buffer (pH 5.8), spectra were recorded at 27 °C. Chemical shifts are reported in ppm, using sodium 4,4-dimethyl-4-silapentanoate-d_4 (δ_H 0.00) or acetone (δ_C 31.0) as internal reference, and 2% phosphoric acid in D_2O was used as external reference for ^{31}P NMR spectroscopy (δ_P 0.00). Positive δ values always indicate a downfield shift. ^{1}H NMR chemical shifts were obtained from 1D spectra when possible, or from proton–proton-correlated 2D spectra (COSY) or total proton–proton-correlated 2D spectra (TOCSY). $J_{H,H}$ values were obtained from 1D or proton–proton-correlated 2D spectra. The TOCSY spectra were recorded using spin-lock times of 30, 60, and 90 ms. NOESY spectra were recorded using mixing times of 100 and 200 ms; a mixing time of 200 ms was used for the experiment performed on the H_2O solution. Typically, a digital resolution of 3.4 Hz/point was used for the H,H-correlated spectra. Proton–carbon-correlated spectra (HMQC, HSQC) were obtained with or without decoupling; the long-range proton–carbon-correlated spectrum (HMBC) was performed using a delay time of 60 ms. The $J_{H,C}$ values were obtained from either a coupled HMQC experiment or an INEPT experiment. The methylene carbon signals were identified by a DEPT experiment with the Θ pulse set to 135°.

Dephosphorylation of the OS.—The OS was kept in aq 40% HF (5 mg/mL) at −15 °C for 3 weeks and the products were monitored by FABMS. The HF was removed in a stream of air. The resulting material was devoid of phosphate but a small amount of terminal Rha was also removed. When the experiment was performed at +4 °C a significant amount of Rha and some nonulosonic acid were removed.

Sugar analysis.—A solution of OS in 2 M CF_3CO_2H (0.2 mL) was kept at 120 °C for 2 h and the sugars in the hydrolysate were then converted into alditol acetates. Alternatively the PS, dried in vacuo over P_2O_5 for 16 h, was dissolved in anhydrous HF (~ 1 mL) and kept at room temperature for 3 h. The HF was removed under diminished pressure, and traces of HF were removed after co-distillation with diethyl ether. The resulting glycosyl fluorides were hydrolysed in aq 50% AcOH at 40 °C for 16 h, and the resulting monosaccharides were converted into alditol acetates.

Methylation analysis.—Methylation was carried out essentially as described earlier [26,27]. The methylated OS was hydrolysed with CF_3CO_2H or solvolysed with HF as described for the OS, and converted into partially methylated alditol acetates. For the location of PEA groups, the methylated OS was dephosphorylated by treatment with aq 40% HF (0.2 mL) for 48 h at 4 °C, remethylated with trideuteriomethyl iodide, hydrolysed with CF_3CO_2H, and finally converted into partially methylated alditol acetates as described for the OS.

Determination of absolute configurations.—The residues **A**–**F** were converted into their acetylated (*S*)-butyl glycosides and analysed by GLC [8]. The identity of the sugars was determined by MS analysis and comparison with references. The A_1 fragment served as a useful marker in this analysis, and other fragments were also in accord with reference data. Enterobacterial common antigen (ECA) [28] which contains 4-amino-

4,6-dideoxy-D-galactose was kindly provided by Professor Hubert Mayer, Max-Planck-Institut für Immunbiologie, Freiburg, Germany.

Determination of absolute configuration of the 3-hydroxybutanoic acid.—Dried OS (5.1 mg) was kept in 2 M methanolic HCl (1 mL) at 80 °C for 18 h under N_2 atmosphere in a sealed tube. The solution was concentrated to dryness under a stream of N_2. The product was partitioned between H_2O and CH_2Cl_2, and the combined CH_2Cl_2 phases were evaporated to dryness. The product was treated with trifluoroacetic anhydride (50 μL) at room temperature for 30 min, and excess of anhydride was evaporated. The sample was analysed at 60 °C by GLC on a Lipodex A (Macherey Nagel) column (50 m × 0.25 mm). The relative retention times of the 3-hydroxybutanoic acid derivative and standards prepared from the (*R*) and (*S*) enantiomers were 1.00, 1.00, and 1.02, respectively.

Molecular modelling.—Energy minimisations were made using the CHARMM forcefield with standard parameters as supplied in the QUANTA–CHARMM program version 4.0 (Molecular Simulations Inc.). The nonulosonic acids were treated as uncharged species, but depicted as charged. Gridsearches with fixed dihedrals were used to find conformations of low energy. These conformations were then relaxed with no constraints. The dihedrals in the side-chains were essentially staggered also after the relaxation.

Acknowledgements

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