ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 330 (2001) 231–239

CARBOHYDRATE RESEARCH

Structure of an acidic polysaccharide from a marine bacterium *Pseudoalteromonas distincta* KMM 638 containing 5-acetamido-3,5,7,9-tetradeoxy-7-formamido-L-*glycero*-L-*manno*-nonulosonic acid☆

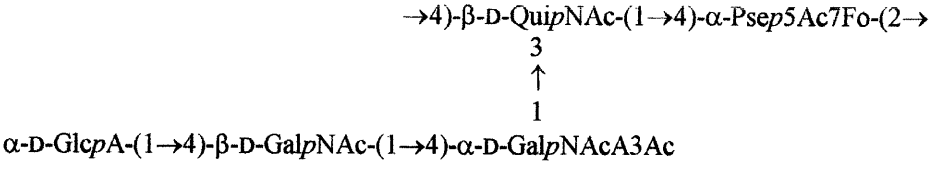
Jimmy Muldoon,[a] Alexander S. Shashkov,[b] Sof'ya N. Senchenkova,[b] Svetlana V. Tomshich,[c] Nadezhda A. Komandrova,[c] Lyudmila A. Romanenko,[c] Yuriy A. Knirel,[b] Angela V. Savage [a,*]

[a] *Department of Chemistry, National University of Ireland, Galway, Ireland*
[b] *N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow 117913, Russia*
[c] *Pacific Institute of Bioorganic Chemistry, Far East Branch of the Russian Academy of Sciences, Vladivostok 690022, Russia*

Received 2 December 1999; received in revised form 16 October 2000; accepted 23 October 2000

Abstract

An acidic polysaccharide was obtained from the lipopolysaccharide of *Pseudoalteromonas distincta* strain KMM 638, isolated from a marine sponge, and found to contain D-GlcA, D-GalNAc, 2-acetamido-2,6-dideoxy-D-glucose (D-QuiNAc) and two unusual acidic amino sugars: 2-acetamido-2-deoxy-D-galacturonic acid (D-GalNAcA) and 5-acetamido-3,5,7,9-tetradeoxy-7-formamido-L-*glycero*-L-*manno*-nonulosonic acid (Pse5Ac7Fo, a derivative of pseudaminic acid). Oligosaccharides were derived from the polysaccharide by partial acid hydrolysis and mild alkaline degradation and characterised by electrospray ionisation (ESI) MS and ^{1}H and ^{13}C NMR spectroscopy. Based on these data and NMR spectroscopic studies of the initial and O-deacetylated polysaccharides, including quaternary carbon detection, 2D COSY, TOCSY, ROESY, H-detected $^{1}H,^{13}C$ HMQC and HMBC experiments, the following structure of the branched pentasaccharide repeating unit was established:



where the degree of O-acetylation of D-Gal*p*NAcA at position 3 is ~60%.

Keywords: Structure; *Pseudoalteromonas distincta*; O-specific polysaccharide; 2-Acetamido-2-deoxy-D-galacturonic acid; 5-Acetamido-3,5,7,9-tetradeoxy-7-formamido-L-*glycero*-L-*manno*-nonulosonic acid

☆ Preliminary data were presented at the 10th European Carbohydrate Symposium, July 1999, Galway, Ireland.

* Corresponding author. Tel.: +353-91-750447; fax: +353-91-525700.
E-mail address: angela.savage@nuigalway.ie (A.V. Savage).

0008-6215/00/$ - see front matter
PII: S0008-6215(00)00280-9

1. Introduction

The bacterial genera *Alteromonas* and *Pseudoalteromonas* originate from the division of the former genus *Alteromonas* which now includes only one species, *Alteromonas macleodi*, while all other species fall in the genus *Pseudoalteromonas*.[1] These bacteria are ubiquitous aerobic marine gram-negative heterotrophic prokaryotes.[2,3] They produce a wide range of biologically active compounds, such as antibiotics, enzymes, antitoxins, antitumour and antiviral agents.[4,5] Studies on the lipopolysaccharides of *Alteromonas* and *Pseudoalteromonas* have recently commenced with data on the structure of the polysaccharides being obtained.[6–15] Here we report the structure of a new polysaccharide isolated from the lipopolysaccharide of a melanin-producing species *Pseudoalteromonas distincta*, strain KMM 638. Based on phenotypic and genotypic properties, as well as DNA–DNA hybridisation data, strain KMM 638 was classified originally to a new species *Alteromonas distincta*,[16] and later reclassified to *P. distincta*.[17]

2. Results and discussion

Mild acid degradation of the lipopolysaccharide of *P. distincta* KMM 638 with dilute acetic acid resulted in polysaccharide and oligosaccharide fractions which were separated by GPC on Sephadex G-50. The polysaccharide chain of the lipopolysaccharide was partially depolymerised during degradation, as indicated by a marked decrease in the molecular mass and formation of more oligosaccharides following prolonged treatment.

Sugar analysis of the polysaccharide hydrolysate by GLC of alditol acetates or using an amino acid analyser failed to reveal any monosaccharide in substantial amounts. GLC, after methanolysis of the polysaccharide followed by acetylation, indicated the presence of GlcA. In addition, based on the characteristic ions for glycosyl cations, a 2-amino-2-deoxyhexose, a 2-amino-2,6-dideoxyhexose and a 2-amino-2-deoxyhexuronic acid were identified by GLC–MS analysis of the acetylated methyl glycosides. NMR studies of the polysaccharide (see below) demonstrated that these amino sugars are GalN, 2-amino-2,6-dideoxyglucose (QuiN) and 2-amino-2-deoxygalacturonic acid (GalNA). Determination of the absolute configurations by GLC of acetylated glycosides with (+)-2-octanol[18] showed that GlcA, GalN and QuiN have the D configuration. The D configuration of GalNA was determined from ^{13}C NMR data (see below).

The ^{13}C NMR spectrum showed that the polysaccharide lacks strict regularity, most likely owing to non-stoichiometric O-acetylation (there was a signal for an *O*-acetyl group at δ 21.7). An attempt to O-deacetylate the polysaccharide by treatment with aqueous ammonia (12%) resulted in its depolymerisation to an oligosaccharide, which was identified as tetrasaccharide **1** (see below). Therefore, an O-deacetylated polysaccharide was obtained by a milder (8%) alkaline degradation of the intact lipopolysaccharide followed by mild acid hydrolysis.

The ^{13}C NMR spectrum of the O-deacetylated polysaccharide (Fig. 1, Table 1) contained signals for five anomeric carbons at δ 97.6–103.7. The signal at δ 97.6 was assigned to a quaternary carbon using the QUAT pulse sequence experiment, and hence a keto sugar is present. A signal at δ 35.3 suggested a C–CH_2–C group, and two signals at δ 16.9 and 17.8 belonged to CH_3–C groups of deoxy sugars. Two signals of double intensity at δ 23.3 and 23.7 (Me) indicated the presence of four *N*-acetyl groups. Signals for one *N*-formyl group (Fo) were observed at δ 165.3 (*Z*-Fo) and 171.3 (*E*-Fo). The absence from the ^{13}C NMR spectrum of any signals for non-anomeric sugar carbons at a lower field than δ 81 demonstrated the pyranoid form of all sugar residues.[19]

The $^1J_{C\text{-}1,H\text{-}1}$ coupling constant values determined from the gated-decoupling spectrum of the polysaccharide confirmed that the four aldo sugar residues are in the pyranoid form[20] and showed that two of them are β-linked ($^1J_{C\text{-}1,H\text{-}1}$ 161–162 Hz) and two others α-linked ($^1J_{C\text{-}1,H\text{-}1}$ 171–172 Hz).[21]

A low-field region of the ^1H NMR spectrum of the O-deacetylated polysaccharide (Fig. 2,

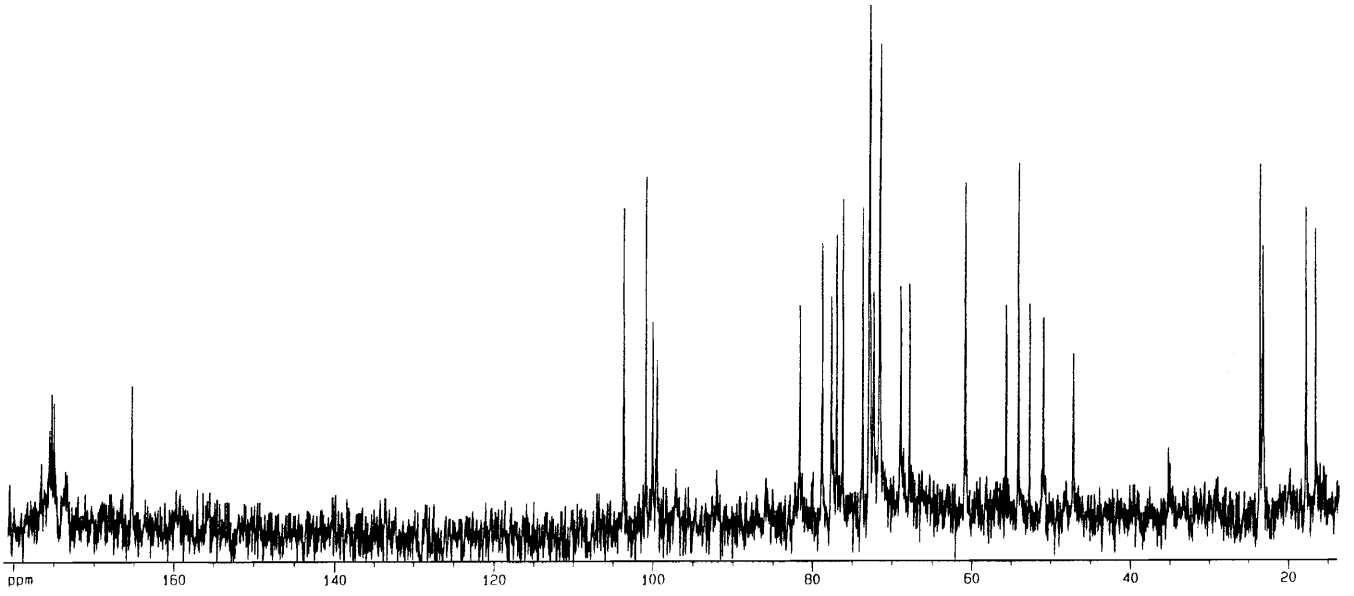


Fig. 1. 125-MHz ^{13}C NMR spectrum of the O-deacetylated polysaccharide (50 °C, pD 2.5).

Table 1
^{13}C NMR data (δ in ppm) [a]

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
Tetrasaccharide ***1*** *(pD 2.5, 45 °C)*									
α-D-Glc*p*A-(1→ (**A**)	100.1	73.2	73.8	73.2	72.8	174.4			
→4)-β-D-Gal*p*NAc-(1→ (**B**)	104.0	54.2	71.6	77.2	76.7	61.5			
→4)-α-D-Gal*p*NAcA-(1→ (**C**)	100.0 [b]	51.2 [b]	69.4	79.6 [b]	72.2				
	99.5 [c]	51.0 [c]		79.4 [c]					
→3)-D-Qui*p*NAc (**D**)									
α	92.2	54.3	79.4	78.0	68.8	18.0			
β	96.1	57.0	81.0	77.8	73.2	18.0			
O-Deacetylated polysaccharide ***2*** *(pD 2.5, 50 °C)*									
α-D-Glc*p*A-(1→ (**A**)	101.0	73.0	73.8	73.0	72.8	175.5			
→4)-β-D-Gal*p*NAc-(1→ (**B**)	103.7	54.3	71.7	77.3	76.4	61.4			
→4)-α-D-Gal*p*NAcA-(1→ (**C**)	99.4	51.0	69.3	79.1	72.2	174.3			
→3,4)-β-D-Qui*p*NAc-(1→ (**D**)	100.0	55.7	81.3	77.9	73.0	17.8			
→4)-α-Pse*p*5Ac7Fo-(2→ (**E**)		97.6	35.3	72.9	47.1 [d]	71.7 [d]	52.8 [d]	68.2 [d]	16.9
					47.0 [e]	70.8 [e]	54.2 [e]	66.7 [e]	

[a] Chemical shifts for NAc are δ 23.3, 23.7 (Me), 176.0–176.5 (CO), for NFo δ 165.3 (*Z*) and 171.3 (*E*).
[b] Linked to α-D-Qui*p*NAc.
[c] Linked to β-D-Qui*p*NAc.
[d] *Z*-Fo at N-7 of Pse5Ac7Fo.
[e] *E*-Fo at N-7 of Pse5Ac7Fo.

Table 2) contained signals for four anomeric protons at δ 4.70, 4.72, 5.40 and 4.98 and a signal from H-5 of α-GlcA at δ 4.66 (see below). Signals at δ 8.07 and 7.77 were due to an *N*-formyl group existing as two stereoisomers (*Z*-Fo and *E*-Fo, respectively).[22] A high-field region of the ^1H NMR spectrum contained typical signals for H-3_{ax} and H-3_{eq} (δ 2.04 and 1.93) and two CH_3–C groups (δ 1.11 and 1.31) of deoxy sugars, as well as signals for four *N*-acetyl groups at δ 1.96–2.04.

The ^1H NMR spectrum of the O-deacetylated polysaccharide was assigned using 2D

COSY and ^{1}H,^{1}H relayed COSY experiments (Table 2). Spin-systems for two sugars having the *gluco* configuration (GlcA and QuiN) and two sugars with the *galacto* configuration (GalN and GalNA), all in the pyranoid form, were identified on the basis of the characteristic $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ coupling constant values. GalN was distinguished from GalNA by the characteristic pH dependence of the H-5 chemical shift (see below). The $J_{1,2}$ coupling constant values showed that GlcA and GalNA are α-linked ($J_{1,2} < 3$ Hz), whereas GalN and QuiN are β-linked ($J_{1,2} >$ 8 Hz).

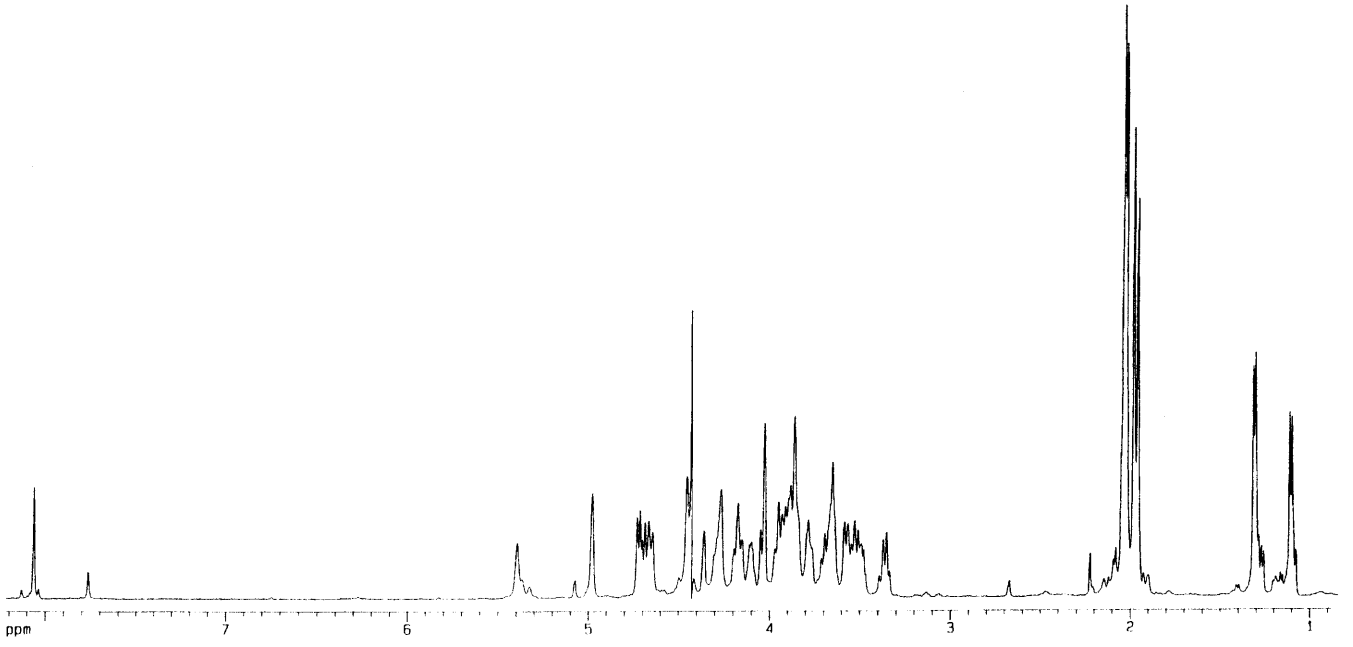


Fig. 2. 500-MHz ^{1}H NMR spectrum of the O-deacetylated polysaccharide (50 °C, pD 2.5).

Table 2
^{1}H NMR data (δ in ppm) [a]

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-7 (H-6b)	H-8	H-9
Tetrasaccharide ***1*** *(pD 1, 25 °C)*									
α-D-Glc*p*A-(1→ (**A**)	5.03	3.61	3.88	3.56	4.78				
→4)-β-D-Gal*p*NAc(1→ (**B**)	4.74	3.96	3.85	4.04	3.68	3.80	3.86		
→4)-α-D-Gal*p*NAcA(1→ (**C**)	5.32 [b]	4.07 [b]	4.03	4.48	4.46				
	5.34 [c]	4.17 [c]							
→3)-D-Qui*p*NAc (**D**)									
α	5.08	3.99	3.80	3.39	3.95	1.27			
β	4.68	3.72	3.62	3.39	3.49	1.30			
O-Deacetylated polysaccharide ***2*** *(pD 2.5, 50 °C)*									
α-D-Glc*p*A (1→ (**A**)	4.98	3.58	3.87	3.53	4.66				
→4)-β-D-Gal*p*NAc (1→ (**B**)	4.72	3.93	3.85	4.03	3.66	3.77	3.89		
→4)-α-D-Gal*p*NAcA-(1→ (**C**)	5.40	4.17	3.95	4.46	4.27				
→3,4)-β-D-Qui*p*NAc-(1→ (**D**)	4.70	3.70	3.66	3.34	3.50	1.31			
→4)-α-Pse*p*5Ac7Fo-(1→ (**E**)			1.93 (ax)	4.28	4.36 [d]	4.07 [d]	4.19 [d]	4.10 [c]	1.11
			2.04 (eq)		4.42 [e]	3.97 [e]	3.88 [e]	4.09 [e]	

[a] Chemical shifts for NAc are δ 1.96–2.04, for NFo δ 8.07 (*Z*) and 7.77 (*E*).
[b] Linked to α-D-Qui*p*NAc.
[c] Linked to β-D-Qui*p*NAc.
[d] *Z*-Fo at N-7 of Pse5Ac7Fo.
[e] *E*-Fo at N-7 of Pse5Ac7Fo.

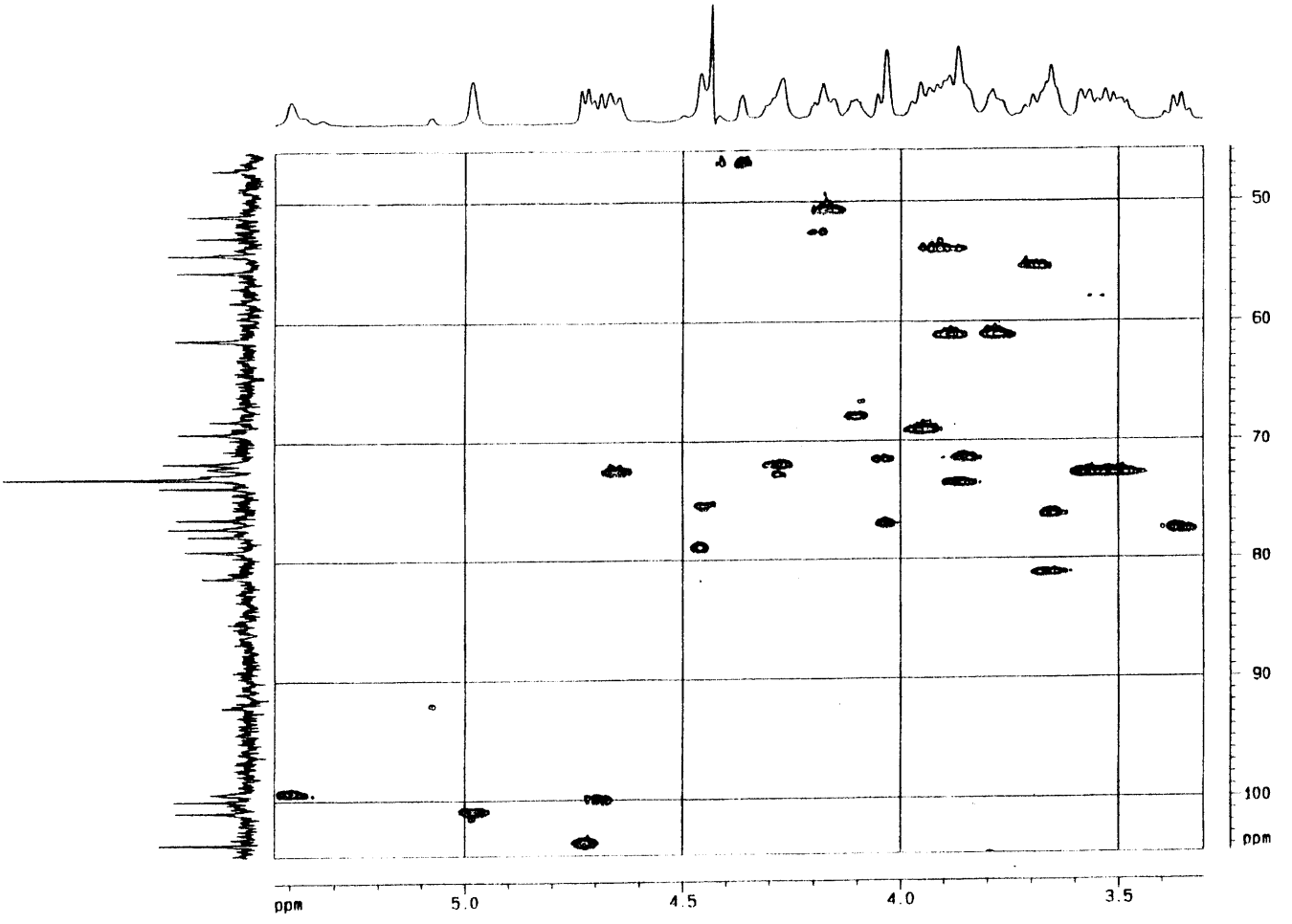


Fig. 3. Part of an H-detected $^{1}H,^{13}C$ NMR spectrum of the O-deacetylated polysaccharide (50 °C, pD 2.5). The corresponding parts of the ^{13}C and ^{1}H NMR spectra are displayed along the vertical and horizontal axes, respectively.

An additional spin system in the ^{1}H NMR spectrum was assigned to a 3,9-dideoxynonulosonic acid. A 2D ^{1}H-detected $^{1}H,^{13}C$ HMQC experiment (Fig. 3) revealed correlation of protons at carbons bearing nitrogen (H-5 and H-7) to the corresponding carbons (C-5 and C-7) at δ 47.1 and 52.8, and thus demonstrated the presence of amino groups at C-5 and C-7. This experiment also allowed assignment of other protonated carbon signals in the ^{13}C NMR spectrum of the polysaccharide (Table 1). The coupling constant values and the ^{13}C NMR chemical shifts (Table 1) for the higher sugar were similar to those published for 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-nonulosonic acid (pseudaminic acid, Pse)[23] and different from two other known stereoisomers.[24,25] In particular, $J_{3ax,4}$ 11 Hz, $J_{4,5} < 2$ Hz and $J_{5,6} < 2$ Hz indicated that H-4 occupies the axial position and H-5 the equatorial position, and hence the C-4,5,6 fragment has the *lyxo* configuration. A large $J_{6,7}$ value of 10 Hz showed the *erythro* configuration of the C-6,7 fragment (higher sugars with the *threo* configuration of this fragment are characterised by a small $J_{6,7}$ value of < 2 Hz).[24] The chemical shift δ 16.9 for C-9 was indicative of the *erythro* configuration of the C-7,8 fragment (chemical shifts $\delta > 19$ were observed for stereoisomers with the *threo* configuration).[23,24]

A relatively small difference (0.11 ppm) between the chemical shifts of H-3_{ax} and H-3_{eq} was typical of the equatorial orientation of the carboxyl group in 3-deoxyaldulosonic acids.[23] This was confirmed also by the ^{13}C NMR chemical shift δ 71.7 for C-6 (Pse with the axial carboxyl group is characterised by a C-6 chemical shift of $\delta > 74$).[23] Hence, Pse has the α configuration.

Marked splitting of the signals for C-6,7,8 of Pse in the ^{13}C NMR spectrum (Table 1) could be accounted for by the presence of a *N*-formyl group at N-7 which exists as two stereoisomers (*Z* and *E*). Therefore, N-5 of Pse and the amino groups of the other amino sugars are N-acetylated. The acylation pattern was confirmed by an HMBC experiment which allowed assignment of the carbonyl signals at δ 175.0, 175.5, 175.7 and 176.5 to the *N*-acetyl groups and revealed their connectivities to H-5 of Pse5Ac7Fo and H-2 of QuiNAc, GalNAc and GalNAcA at δ 4.36, 3.70, 3.93 and 4.17, respectively. The structure of Pse5Ac7Fo is shown in Fig. 4.

The data obtained showed that the polysaccharide has a pentasaccharide repeating unit containing one residue each of α-D-GlcA, β-D-GalNAc, β-D-QuiNAc, α-D-GalNAcA and α-Pse5Ac7Fo. Both rare acidic sugars present in the polysaccharide, GalNAcA and Pse5Ac7Fo, as well as QuiNAc, have been previously reported as components of the lipopolysaccharides of *Pseudomonas aeruginosa*.[26]

Oligosaccharide **1**, which was obtained by mild alkaline treatment of the polysaccharide, was purified by GPC on TSK-40 and studied by electrospray ionisation (ESI) MS and NMR spectroscopy. A negative mode ESI mass spectrum of **1** showed a peak of a pseudomolecular ion $[M-H]^-$ at m/z 800.5 which corresponded to a tetrasaccharide containing all components of the O-deacetylated polysaccharide apart from Pse5Ac7Fo. The ^1H and ^{13}C NMR data of the tetrasaccharide (Tables 1 and 2) confirmed this conclusion.

The ^1H NMR spectrum of **1** was assigned using 2D COSY and $^1H,^1H$ relayed COSY experiments (Table 2); the ^{13}C NMR spectrum of **1** was then assigned using a $^1H,^{13}C$ HMQC experiment (Table 1). The spin-systems for the four sugar residues were identified based on the coupling values and correlation of protons at carbons bearing nitrogen (H-2) to the corresponding carbons (C-2) at δ 51–57. The $J_{1,2}$ coupling constant values confirmed α-GlcA, α-GalNAcA ($J_{1,2}$ 3.9 Hz for both) and β-GalNAc ($J_{1,2}$ 8 Hz). QuiNAc was present in both α- and β-forms ($J_{1,2}$ 3.6 and 8.4 Hz, respectively), and therefore occupied the reducing end of tetrasaccharide **1**. Two corresponding sets of signals were observed not only for QuiNAc, but also for the neighbouring GalNAcA residue.

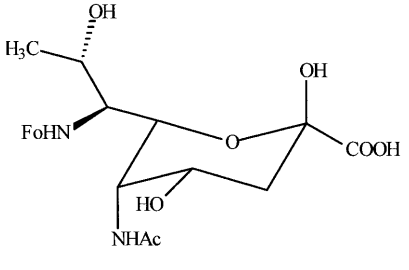


Fig. 4. Structure of 5-acetamido-3,5,7,9-tetradeoxy-7-formamido-L-*glycero*-L-*manno*-nonulosonic acid (Pse5Ac7Fo, a derivative of pseudaminic acid).

Studies of the pD dependence of the ^1H NMR spectrum of **1** revealed significant upfield shifts for the H-5 signals of GlcA and GalNAcA from δ 4.46 and 4.78 to δ 4.13 and 4.49, respectively, with a change of pD from 1 to 9. These data confirmed that both uronic acids have a free carboxyl group.[27]

The ^{13}C NMR chemical shifts for α-GlcA (Table 1) were similar to those reported for the corresponding nonsubstituted monosaccharide,[28] and hence this sugar is at the nonreducing end of tetrasaccharide **1**. A low-field position of the signals for C-4 of GalNAc and GalNAcA at δ 77.2 and 79.4–79.6 indicated that both sugars are substituted at C-4 (compare published data[28,29] for the corresponding nonsubstituted monosaccharides). Comparison of the ^{13}C NMR chemical shifts for GalNAcA and D-QuiNAc with published data[29] for a pair of disaccharides, α-D-GalNAcA-(1→3)-D-QuiNAc and α-L-GalNAcA-(1→3)-D-QuiNAc, showed that GalNAcA in **1** has the D configuration.

Therefore, tetrasaccharide **1** has the following structure:

α-D-Glc*p*A-(1→4)-β-D-Gal*p*NAc-(1→4)-

α-D-Gal*p*NAcA-(1→3)-D-Qui*p*NAc

Sequence and linkage analyses of the O-deacetylated polysaccharide were performed using a HMBC pulse sequence. This revealed three of the linkages in the polysaccharide (Table 3) which defined two oligosaccharide fragments, GlcA-(1→4)-GalNAc-(1→4)-GalNAcA and QuiNAc-(1→4)-Pse5Ac7Fo. 1D NOE, 1D CAMELSPIN and 2D ROESY experiments confirmed these sequences and, in

addition, revealed a GalNAcA H-1/QuiNAc H-3 correlation at δ 5.40/3.66, demonstrating a GalNAcA-(1→3)-QuiNAc fragment. A ^{13}C-detected ^{1}H-decoupling experiment with selective irradiation of QuiNAc H-4 at δ 3.34 showed a correlation between this proton and C-2 of Pse5Ac7Fo at δ 97.6, indicating a Pse5Ac7Fo-(2→4)-QuiNAc fragment.

The terminal position of GlcA and the glycosylation modes of the other monosaccharides were confirmed by the ^{13}C NMR chemical shift data (Table 1). Low-field displacements of the signals for C-4 of GalNAc, GalNAcA and Pse5Ac7Fo by 6–10 ppm, when compared to their positions in the corresponding nonsubstituted monosaccharides,[23,28,29] were consistent with substitution at O-4. A similar large shift of the signal for C-3 of QuiNAc showed its glycosylation at O-3, whereas only a small displacement (by +1.2 ppm) was observed for the C-4 signal of QuiNAc which is typical of substitution with a keto sugar (e.g., compare published data[23]). These data were in full agreement with the structure of tetrasaccharide **1**, and, therefore, the O-deacetylated polysaccharide has structure **2**.

→4)-β-D-Qui*p*NAc-(1→4)-α-Pse*p*5Ac7Fo-(2→
3
↑
1
α-D-Glc*p*A-(1→4)-β-D-Gal*p*NAc-(1→4)-α-D-Gal*p*NAcA3R

2 (R = H), **3** (R = Ac)

Analysis of the glycosylation effects on the chemical shifts in the ^{13}C NMR spectrum of the polysaccharide confirmed the absolute configuration of Pse. As compared with the corresponding nonsubstituted monosaccharides,[23,29] the β-effect on C-5 of Pse5Ac7Fo caused by its glycosylation at O-4 is relatively large (−2.7 ppm) and the effect on C-1 of QuiNAc is relatively small (+3.9 ppm), relative to oligosaccharide **1**. Taking into account the D configuration of QuiNAc, it can be concluded that the C-4,5,6 fragment of Pse has the same configuration as the C-3,4,5 fragment of L-galactose,[23,30] and hence Pse has the L-*glycero*-L-*manno* configuration (Fig. 4).

The location of the *O*-acetyl group was determined by comparison of the ^{13}C NMR spectra of the initial and O-deacetylated polysaccharides. In the former spectrum, the signal for GalNAcA C-2 at δ 51.0 was reduced to half the intensity of the signals for C-2 of GalNAc and QuiNAc, and a new signal with a similar intensity appeared at δ 49.2. In addition, the signal for C-3 of GalNAcA at δ 69.3 decreased significantly in the spectrum of the initial polysaccharide, as compared with its intensity in the spectrum of the O-deacetylated polysaccharide. These changes suggested partial O-acetylation of GalNAcA at C-3 (compare published data on the effects of O-acetylation on the ^{13}C NMR chemical shifts[31]). The O-acetylation also caused splitting of the C-1 signals for GalNAcA (δ 99.4 and 97.6) and the neighbouring GalNAc (δ 103.7 and 103.5). An unexpectedly large difference of 1.8 ppm for the former sugar could

Table 3
Heteronuclear interresidue connectivities for transglycosidic atoms in the 2D HMBC spectrum of the O-deacetylated polysaccharide

Sugar residue	Chemical shift for correlating atom				Connecting to
	δ_{H-1}	δ_{C-1}	δ_{H-4}	δ_{C-4}	
→4)-β-D-Qui*p*NAc-(1→ 3 ↑	4.70	100.0	4.28	72.9	→4)-α-Pse5Ac7Fo-(2→
→4)-β-D-Gal*p*NAc-(1→	4.72	103.7	4.46	79.1	→4)-α-D-Gal*p*NAcA-(1→
α-D-Glc*p*A-(1→	4.98	101.0	4.03	77.3	→4)-β-D-Gal*p*NAc-(1→

be attributed not to an inductive effect of the *O*-acetyl group, but rather to a conformational change caused by different spatial interactions of the bulky glycosyl substituents at C-3 and C-4 of QuiNAc (GalNAcA or GalNAcA3Ac and Pse5Ac7Fo, respectively).

The ESI mass spectrum of the oligosaccharide fraction obtained by mild acid degradation of the lipopolysaccharide (see above) showed four intense peaks for pseudomolecular ions $[M-H]^-$ at m/z 800.7, 842.5, 1102.3 and 1144.3. These ions were evidently derived from tetrasaccharide **1**, mono-*O*-acetylated **1** and two corresponding pentasaccharides with an additional Pse5Ac7Fo residue which, most likely, was at the reducing end. Comparison of the ^{13}C NMR chemical shifts of the two pairs (data not shown) confirmed this suggestion, as well as the location of the *O*-acetyl group at C-3 of GalNAcA and allowed estimation of the degree of O-acetylation as $\sim 60\%$.

On the basis of the data obtained, it was concluded that the O-specific polysaccharide of *P. distincta* KMM 638 has structure **3**.

Remarkably, the polysaccharide contained three acidic components in the pentasaccharide repeating unit. The failure to obtain information from the conventional sugar analysis of this polysaccharide may be accounted for by the stability of the glycosidic linkages of uronic acids towards acid hydrolysis. However, the glycosidic linkage of the 3-deoxynonulosonic acid (Pse) is labile in the acidic conditions leading to an easy selective cleavage of the polysaccharide. Pse was also found to be cleaved in mild alkaline conditions used for O-deacetylation.

3. Experimental

Bacterial strain, growth and isolation of the lipopolysaccharide.—*P. distincta* strain KMM 638 was isolated from a marine sponge collected at a depth of 350 m near the Komandorskie Islands. The bacterium was grown on a medium containing (g/L) 1 glucose, 5 pepton, 2.5 yeast extract, 0.2 K_2HPO_4, 0.05 $MgSO_4$, sea water (750 ml), and distilled water (250 ml).

Lipopolysaccharide was isolated from dried cells of *P. distincta* KMM 638 using the phenol–water method;[32] the water layer was dialysed and freeze-dried. The yield of the lipopolysaccharide was about 6% of the dried cells weight.

Isolation and degradations of the lipopolysaccharide and the O-specific polysaccharide.—The lipopolysaccharide (55 mg) was hydrolysed with 1% aq HOAc at 100 °C for 1.5 h and a lipid precipitate was removed by centrifugation. The carbohydrate portion was fractionated by GPC on Sephadex G-50 in 0.05 M pyridinium acetate buffer (pH 4.5) and monitored using a Knauer differential refractometer to give a high-molecular mass mannan (15 mg), an O-specific polysaccharide (**3**, 13.8 mg) and an oligosaccharide fraction (16.7 mg after additional purification by GPC on TSK HW-40 in water).

The polysaccharide was treated with aq 8% NH_3 for 18 h at ambient temperature to give an oligosaccharide (**1**, 8.1 mg) which was isolated by GPC on TSK-40 in water. O-Deacetylation of the lipopolysaccharide (200 mg) was performed with aq. 12% NH_3 for 18 h at ambient temperature. The product (80 mg) was hydrolysed with aq 1% HOAc as above and fractionated by GPC on Sephadex G-50 to give polysaccharide (**2**, 9 mg) and oligosaccharide (47 mg) fractions.

Sugar analysis.—Methanolysis of the polysaccharide (2 mg) was performed with 1 M HCl–MeOH (80 °C, 16 h), and the methanolysate was acetylated with Ac_2O in C_5H_5N (100 °C, 1 h). A portion of the acetylated methyl glycosides preparation was subjected to (+)-2-octanolysis in the presence of concd CF_3COOH (120 °C, 16 h) followed by acetylation. The acetylated derivatives were analysed by GLC and GLC–MS.

GLC and GLC–MS.—GLC was performed on a Varian 3700 chromatograph (Varian) equipped with a fused-silica gel SPB-5 column using a temperature gradient 150 °C (3 min) to 320 °C at 5°/min. GLC–MS was performed on a HP 5989A instrument (Hewlett–Packard) equipped with an HP-5 column under the same chromatographic conditions as in GLC. Electron impact (EI) mass spectra were recorded at 70 eV. Ammonia was used as a reactant gas in chemical ionisation (CI) MS.

ESIMS.—ESIMS was performed in the negative mode using a VG Quattro triple quadrupole mass spectrometer (Micromass, Altrincham) with MeCN as the mobile phase at a flow rate of 10 μL/min. Samples were dissolved in aq 50% MeCN at a concentration of about 50 pmol/μL, and 10 μL was injected via a syringe pump into the electrospray source.

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying three times from D_2O and then examined as solutions in 99.97% D_2O. A solution of tetrasaccharide **1** was adjusted to pD 9 using solid Na_2CO_3 and to pD 1 using 2 M $(CF_3CO)_2O$ in D_2O. Spectra were recorded on a JEOL 400 MHz spectrometer equipped with a DEC AXP 300 computer workstation or a Bruker DRX-500 MHz spectrometer equipped with an SGI INDY computer workstation. A mixing time of 1 s was used in 1D NOE experiments. The parameters used for 2D experiments were essentially the same as described previously.[11,15]

Acknowledgements

We are grateful to Mrs G. Alvelius (Karolinska Institute, Clinical Research Centre, Huddinge University Hospital, Sweden) and Mr H. Moll (Forschungszentrum Borstel, Germany) for performing the ESIMS and EI/CIMS, respectively. We thank the Wellcome Trust (London) for a grant to A.V.S. towards the purchase of the NMR instrument, BioResearch Ireland for funding the visit of A.S.S to Galway and Enterprise Ireland for an International Collaboration grant to J.M. and A.V.S.

References

1. Gauthier, G.; Gauthier, M.; Christen, R. *Int. J. Syst. Bacteriol.* **1995**, *45*, 755–761.
2. Baumann, L.; Baumann, P.; Mandel, M.; Allen, R. D. *J. Bacteriol.* **1972**, *110*, 402–429.
3. *Bergey's Manual of Systematic Bacteriology*; Holt, J. C., Ed.; Williams & Wilkins: Baltimore, 1984; Vol. 1, pp. 343–352.
4. Austin, B. *J. Appl. Bacteriol.* **1989**, *67*, 461–470.
5. Fenical, W. *Chem. Rev.* **1993**, *93*, 1673–1683.
6. Gorshkova, R. P.; Nazarenko, E. L.; Zubkov, V. A.; Ivanova, E. P.; Ovodov, Y. S.; Shashkov, A. S.; Knirel, Y. A. *Bioorg. Khim.* **1993**, *19*, 327–336.
7. Nazarenko, E. L.; Gorshkova, R. P.; Zubkov, V. A.; Shashkov, A. S.; Ivanova, E. P.; Ovodov, Y. S. *Bioorg. Khim.* **1993**, *19*, 733–739.
8. Nazarenko, E. L.; Zubkov, V. A.; Shashkov, A. S.; Knirel, Y. A.; Gorshkova, R. P.; Ivanova, E. P.; Ovodov, Y. S. *Bioorg. Khim.* **1993**, *19*, 740–751.
9. Zubkov, V. A.; Nazarenko, E. L.; Gorshkova, R. P.; Ivanova, E. P.; Shashkov, A. S.; Knirel, Y. A.; Paramonov, N. A.; Ovodov, Y. S. *Carbohydr. Res.* **1995**, *275*, 147–154.
10. Gorshkova, R. P.; Nazarenko, E. L.; Zubkov, V. A.; Shashkov, A. S.; Knirel, Y. A.; Paramonov, N. A.; Meshkov, S. V.; Ivanova, E. P. *Carbohydr. Res.* **1997**, *299*, 69–76.
11. Hanniffy, O.; Shashkov, A. S.; Senchenkova, S. N.; Tomshich, S. V.; Komandrova, N. A.; Romanenko, L. A.; Knirel, Y. A.; Savage, A. V. *Carbohydr. Res.* **1998**, *307*, 291–298.
12. Komandrova, N. A.; Tomshich, S. V.; Isakov, V. V.; Romanenko, L. A. *Biochemistry* (*Moscow*) **1998**, *63*, 1410–1415.
13. Gorshkova, R. P.; Nazarenko, E. L.; Isakov, V. V.; Zubkov, V. A.; Gorshkova, N. M.; Romanenko, L. A.; Ivanova, E. P. *Bioorg. Khim.* **1998**, *24*, 839–841.
14. Gorshkova, R. P.; Nazarenko, E. L.; Zubkov, V. A.; Shashkov, A. S.; Ivanova, E. P.; Gorshkova, N. M. *Carbohydr. Res.* **1998**, *313*, 61–64.
15. Hanniffy, O.; Shashkov, A. S.; Senchenkova, S. N.; Tomshich, S. V.; Komandrova, N. A.; Romanenko, L. A.; Knirel, Y. A.; Savage, A. V. *Carbohydr. Res.* **1999**, *321*, 132–138.
16. Romanenko, L. A.; Mikhailov, V. V.; Lysenko, A. M.; Stepanenko, V. I. *Mikrobiologiya* **1995**, *64*, 74–77.
17. Ivanova, E. P.; Chun, J.; Romanenko, L. A.; Matte, M. H.; Mikhailov, V. V.; Frolova, G. M.; Huq, A.; Colwell, R. R. *Int. J. Syst. Microbiol.* **1999**, *321*, 132–138.
18. Leontein, K.; Lindberg, B.; Lönngren, J. *Carbohydr. Res.* **1978**, *62*, 359–362.
19. Bock, K.; Pedersen, C. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 27–65.
20. Cyr, N.; Perlin, A. S. *Can. J. Chem.* **1979**, *57*, 2504–2511.
21. Bock, K; Pedersen, C. *J. Chem. Soc., Perkin Trans. 2* **1974**, 293–297.
22. Katzenellenbogen, E.; Romanowska, E.; Kocharova, N. A.; Shashkov, A. S.; Knirel, Y. A.; Kochetkov, N. K. *Carbohydr. Res.* **1995**, *273*, 187–195.
23. Knirel, Y. A.; Kocharova, N. A.; Shashkov, A. S.; Dmitrev, B. A.; Kochetkov, N. K.; Stanislavsky, E. S.; Mashilova, G. M. *Eur. J. Biochem.* **1987**, *163*, 639–652.
24. Knirel, Y. A.; Vinogradov, E. V.; Shashkov, A. S.; Dmitrev, B. A.; Kochetkov, N. K.; Stanislavsky, E. S.; Mashilova, G. M. *Eur. J. Biochem.* **1987**, *163*, 627–637.
25. Knirel, Y. A.; Moll, H.; Helbig, J. H.; Zähringer, U. *Carbohydr. Res.* **1997**, *304*, 77–79.
26. Knirel, Y. A. *CRC Crit. Rev. Microbiol.* **1990**, *17*, 273–304.
27. Vinogradov, E. V.; Knirel, Y. A.; Shashkov, A. S.; Kochetkov, N. K. *Carbohydr. Res.* **1987**, *170*, C1–C4.
28. Jansson, P.-E.; Kenne, L.; Widmalm, G. *Carbohydr. Res.* **1989**, *188*, 169–191.
29. Knirel, Y. A.; Kocharova, N. A.; Shashkov, A. S.; Kochetkov, N. K.; Kholodkova, E. V.; Stanislavsky, E. S. *Eur. J. Biochem.* **1987**, *166*, 189–197.
30. Shashkov, A. S.; Lipkind, G. M.; Knirel, Y. A.; Kochetkov, N. K. *Magnet. Reson. Chem.* **1988**, *26*, 735–747.
31. Jansson, P.-E.; Kenne, L.; Schweda, E. *J. Chem. Soc., Perkin Trans. 1* **1987**, 377–383.
32. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.