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The structure of the *O*-polysaccharide of the *Pseudoalteromonas rubra* ATCC 29570^T lipopolysaccharide containing a keto sugar

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Abstract—The structure of the phenol-soluble polysaccharide from *Pseudoalteromonas rubra* type strain ATCC 29570^T has been elucidated using ^{1}H and ^{13}C NMR spectroscopy, including 2D COSY, TOCSY, gNOESY, ROESY, ^{1}H,^{13}C gHMQC and gHMBC experiments. It is concluded that the trisaccharide repeating unit of the polysaccharide has the following structure:

→4)-α-L-Gal*p*NAm3AcA-(1→3)-α-Sug*p*-(1→4)-β-D-Glc*p*NAc3NAcylA-(1→

where Sug is 2-acetamido-2,6-dideoxy-D-*xylo*-hexos-4-ulose, Am is acetimidoyl and Acyl is a malic acid residue, which is *O*-acetylated in ~70% of the units.

Keywords: *Pseudoalteromonas rubra*; Polysaccharide; NMR spectroscopy; 2-Acetamido-2,6-dideoxy-D-*xylo*-hexos-4-ulose; Malic acid

1. Introduction

Genus *Pseudoalteromonas* belongs to the family *Alteromonadaceae* of the γ-subclass of Proteobacteria and shares similar phenotypic, genotypic and phylogenetic characteristics.[1] These heterotrophic bacteria include both free-living and symbiotic forms, which are essential components of the marine environment and have diverse habitats including coastal and open water areas, deep sea and hydrothermal vents, bottom sediments as well as marine plants and animals.[2,3] The *Pseudoalteromonas* group is relatively heterogeneous, with inter-strain similarity values ranging from 90% to 99.9% of their 16S rRNA gene sequences, and encompasses more than 30 pigmented and nonpigmented species.[2,3] Pigmented species that synthesize a variety of pigments (prodigiosin-like, carotinoids and some others) are divided into six clusters: (i) *Pseudoalteromonas citrea* and *Pseudoalteromonas aurantica*, (ii) *Pseudoalteromonas ruthenica*, (iii) *Pseudoalteromonas rubra*, *Pseudoalteromonas luteoviolacea*, *Pseudoalteromonas peptidolytica*, *Pseudoalteromonas piscicida*, *Pseudoalteromonas flavipulchra* and *Pseudoalteromonas maricaloris*, (iv) *Pseudoalteromonas tunicata* and *Pseudoalteromonas ulva*, (v) *Pseudoalteromonas denitrificans* and (vi) *Pseudoalteromonas bacteriolytica*.[4] Among the red-pigmented bacteria, *P. rubra* with the type strain NCMB 1890^T (= ATCC 29570^T) was originally isolated from the Mediterranean sea off Nice in 1976 by Gauthier.[5] Most of the pigmented species, including *P. rubra*, have been known as producers of inhibitory (including antifungal) compounds.[6] The type strain of *P. rubra* has been found to produce

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extracellularly a polyanionic antibiotic that modifies bacterial respiration[4,7] and cell-bound fatty acids and phospholipids with surface activity.[8] These observations provide evidence of ecophysiological diversification of the *Pseudoalteromonas* and in particular the remarkable metabolic capacity of *P. rubra*, which may play an important role in coexistence and survival of numerous bacterial taxa in marine environments.

This study is an extension of our previous investigations into the structural diversity of the polysaccharides of *Pseudoalteromonas* species. A recent review[9] shows that most of the strains studied revealed the combination of unusual acidic and *N*-acylamino sugars along with noncarbohydrate substituents. Now we report on the structure of an acidic polysaccharide from *P. rubra* type strain ATCC 29570^T.

2. Result and discussion

The ^{13}C NMR spectrum of the intact polysaccharide (PS-1, Fig. 1 (bottom), Table 2) contained *inter alia* signals for four anomeric carbons (94.5–103.6 ppm) and four nitrogen-bearing carbons (51.3–55.0 ppm), two signals of differing intensity at δ 41.5 and 38.9 characteristic for C–*C*H$_2$–C groups and one signal for C-6 of a 6-deoxy sugar with an unusually low chemical shift of 12.1 ppm. The signals at δ 20.1 and 167.5 were assigned to an *N*-acetimidoyl group, an intense signal at δ 21.5 to an *O*-acetyl group(s), and three signals at δ 23.3 and 23.6 (2C) to *N*-acetyl groups. The signals for carbonyl groups at δ 174.3–176.1 had different intensities, most likely owing to nonstoichiometric *O*-acetylation.

Treatment of the polysaccharide with aqueous ammonia resulted in an *O*-deacetylated polysaccharide (PS-2). Its ^{13}C NMR spectrum (Fig. 1, top) was typical of a regular polymer and contained signals for four anomeric carbons at 94.5–102.3, with that at δ 94.5 ppm belonging to a quaternary carbon while each of the other three carbons carried a proton (from the data of attached proton test[10]). Eight signals were observed for oxygen-bearing carbons at δ 67.9–76.6 ppm and four signals for nitrogen-bearing carbons at δ 50.9–55.15. The signals for C–*C*H$_2$–C (δ 41.5), OCO*C*H$_3$ (δ 23.3–23.6, 3C), and *C*H$_3$–C (δ 12.3) groups were seen upfield and seven signals for carbonyl groups (δ 173.2–177.8) downfield in the spectrum. The signals for the *N*-acetimidoyl group were absent from the spectrum of PS-2, thus demonstrating conversion of the *N*-acetamidoyl group to an *N*-acetyl group upon treatment with aqueous ammonia.[11]

The ^{1}H NMR spectrum of PS-2 showed *inter alia* four signals in the low-field region at δ 4.7–5.1 ppm, three intense signals for CH$_3$CO groups (δ 1.99, 2.01 and 2.02, all s), as well as signals for the C–CH$_2$–C– group (δ 2.52 and 2.66, both dd) and the CH$_3$–C group (δ 1.17, d). The 2D ^{1}H,^{1}H COSY and TOCSY spectra revealed five isolated proton spin systems (Table 1). Two of these, consisting of five protons each, belonged to pyranosidic hexuronic acids with the β-*gluco* and α-*galacto* configuration (δ_H 4.71, $J_{1,2}$ 8 Hz and δ 5.08, $J_{1,2}$ 3 Hz, respectively). The third spin system consisted of three protons, including a proton resonating in the anomeric region at δ 5.11 (d, $J_{1,2}$ 3 Hz) and two others at δ 4.21 (dd, $J_{2,3}$ 10 Hz) and δ 3.78 (d). Protons of a CH$_3$–CHO group at δ 1.17 (d, J 6 Hz) and 3.76 (q) created the fourth spin system. The fifth spin system consisted

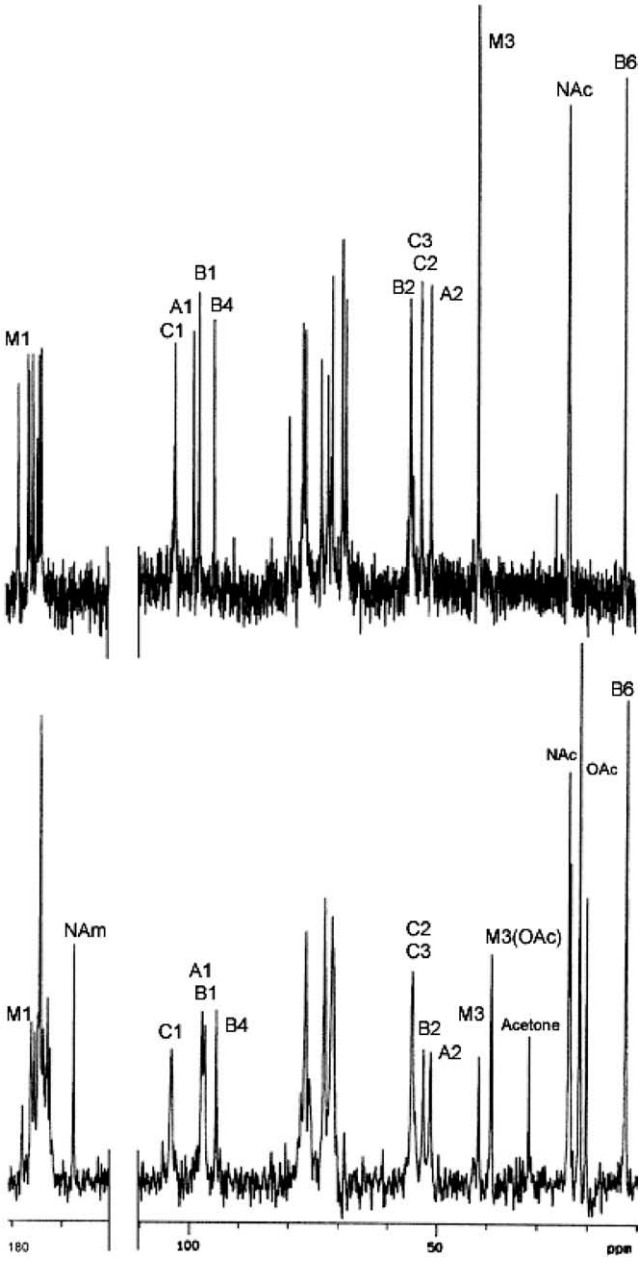


Figure 1. The ^{13}C NMR spectra of the *O*-polysaccharide (PS-1) of *Pseudoalteromonas rubra* ATCC 29570^T (bottom) and *O*-deacetylated polysaccharide (PS-2) (top).

Table 1. ^{1}H NMR chemical shifts of the polysaccharides (δ, ppm)

Residue	H-1	H-2	H-3	H-4	H-5	H-6	NH-2[a]	NH-3[a]
PS-3								
→4)-α-L-Gal*p*NAcA-(1→ (**A**)	5.08	4.12	3.95	4.52	4.97			
→3)-α-D-Qui*p*NAc-(1→ (**B′**)	5.11	4.02	3.71	3.22	3.66	1.23		
→3)-α-D-Fuc*p*NAc-(1→ (**B″**)	5.15	4.28	3.88	3.73	3.93	1.16		
→4)-β-D-Glc*p*NAc3NA-(1→ (**C**)	4.78	3.79	4.32	3.96	4.06			
$HO_2CCH(OH)CH_2CO$ (**M**)		4.42	2.55					
			2.67					
PS-2								
→4)-α-L-Gal*p*NAcA-(1→ (**A**)	5.08	4.15	4.01	4.52	5.02		7.79	
→3)-α-Sug*p*-(1→ (**B**)	5.11	4.21	3.78		3.76	1.17	8.29	
→4)-β-D-Glc*p*NAc3NA-(1→ (**C**)	4.71	3.79	4.32	3.96	4.03		7.80	8.22
$HO_2CCH(OH)CH_2CO$ (**M**)		4.44	2.52					
			2.66					
PS-1								
→4)-α-L-Gal*p*NAm3AcA-(1→ (**A**)	5.03	4.26	5.18	4.71	4.97			
→3)-α-Sug*p*-(1→ (**B**)	5.28	4.27	3.88		3.76	1.18		
→4)-β-D-Glc*p*NAc3NA-(1→ (**C**)	4.69	3.80	4.30	4.03	4.03			
$HO_2CCH(OH)CH_2CO$ (**M**)		5.05	2.73					
			2.68					

Signals for NAc, OAc and NAm are at δ 1.99–2.07, 2.08–2.16 and 2.26, respectively.
[a] For solution in a 9:1 H_2O–D_2O mixture.

of the ABX protons of the fragment –C–$CH(OH)CH_2$–C– (δ 4.44, dd; δ 2.52, dd, ^{2}J 13 Hz and ^{3}J 5 Hz; and δ 2.66, dd, ^{3}J 2 Hz).

The ^{13}C NMR spectrum of PS-2 was assigned using 2D ^{1}H,^{13}C HSQC and HMBC experiments (Table 2). The former revealed that carbons C-2 (δ 55.1) and C-3 (δ 55.15) of the β-*gluco* sugar and C-2 (δ 50.9) of the α-*galacto* sugar bear nitrogen. Hence, these monosaccharides are derivatives of 2,3-diamino-2,3-dideoxy-β-glucopyranuronic acid (β-Glc*p*N3NA) and 2-amino-2-deoxy-α-galactopyranuronic acid (α-Gal*p*NA). H-1, C-1, H-2,C-2 and H-3,C-3 correlations at δ_H/δ_C 5.11/97.4, 4.21/52.9 and 3.78/76.2 suggested that the third proton system may belong to the C-1–C-3 fragment of another sugar, whose C-2 also bears nitrogen. The HMBC spectrum revealed the other carbons of this monosaccharide and connected the third and the fourth proton spin systems together by H-1,C-3 and H-1,C-5 correlations at δ_H/δ_C 5.11/76.2 and 5.11/70.7 as well as H-6,C-5 and H-6,C-4 correlations at δ_H/δ_C 1.17/70.7 and 1.17/94.5, respectively. The chemical shift δ 94.5 for C-4 indicated a hydrated keto group $–CH(OH)_2$.[12] Therefore, the third constituent monosaccharide of PS-2 is a derivative of a 2-amino-2,6-dideoxyhexos-4-ulose (Sug). All protons of the fifth, C–$CH(OH)CH_2$– spin system showed correlations with

Table 2. ^{13}C NMR chemical shifts of the polysaccharides (δ, ppm)

Residue	C-1	C-2	C-3	C-4	C-5	C-6
PS-3						
→4)-α-L-Gal*p*NAcA-(1→ (**A**)	98.2	51.0	68.0	79.5	71.3	173.6
→3)-α-D-Qui*p*NAc-(1→ (**B′**)	97.4	54.5	75.6	74.8	69.9	17.9
→3)-α-D-Fuc*p*NAc-(1→ (**B″**)	97.3	49.2	74.2	72.1	68.6	16.6
→4)-β-D-Glc*p*NAc3NA-(1→ (**C**)	102.3	55.1	55.2	73.0	76.8	173.3
$HO_2CCH(OH)CH_2CO$ (**M**)	177.8	68.7	41.5	173.2		
PS-2						
→4)-α-L-Gal*p*NAcA-(1→ (**A**)	98.5	50.9	67.9	79.4	71.5	173.7
→3)-α-Sug*p*-(1→ (**B**)	97.4	52.9	76.2	94.5	70.7	12.3
→4)-β-D-Glc*p*NAc3NA-(1→ (**C**)	102.3	55.1	55.15	72.9	76.6	173.5
$HO_2CCH(OH)CH_2CO$ (**M**)	177.8	68.6	41.5	173.2		
PS-1						
→4)-α-L-Gal*p*NAm3AcA-(1→ (**A**)	97.4	51.3	71.2	76.4	71.2	173.7
→3)-α-Sug*p*-(1→ (**B**)	96.7	52.6	75.8	94.5	71.2	12.1
→4)-β-D-Glc*p*NAc3NA-(1→ (**C**)	103.6	55.0	55.0	72.7	75.8	172.8
$HO_2CCH(OH)CH_2CO$ (**M**)	177.9	72.6	38.9	172.3		

Signals for NAc and OAc are at δ 23.3–23.6 and 21.5 (all CH_3), respectively, and 174.3–176.1 (C=O), signals for NAm are at 20.1 (CH_3) and 167.5 (C=N).

two carbonyl groups at δ_H/δ_C 2.52, 2.66, 4.44/177.8, 173.2 in the HMBC spectrum, thus indicating the presence of malic acid. Attempts to confirm malic acid and to determine its absolute configuration after acid hydrolysis of the polysaccharide were unsuccessful.

The positions of substitution and the sequence of the sugar residues were established using NOESY and HMBC experiments. GalNA, Sug and GlcN3NA were designated as units **A**–**C**, respectively, according to their sequence in the repeating unit (see below). The NOESY spectrum showed the following correlations between anomeric and linkage protons: **A** H-1,**B** H-3; **B** H-1,**C** H-4 and **C** H-1,**A** H-4. Accordingly, the HBMC spectrum displayed the following inter-residue cross-peaks: δ_H/δ_C **C** H-1,**A** C-4; **A** H-4,**C** C-1; **B** H-3,**A** C-1 and **C** H-4,**B** C-1. These data defined the sequence of the monosaccharides and demonstrated that units **A** and **C** are 4-substituted, while unit **B** is 3-substituted.

The location of the malic acid residue (**M**) was determined by NMR spectroscopic analysis of the *O*-deacetylated PS-2 sample in a 1:9 D_2O–H_2O mixture. The NH signals of the amino sugars were assigned using a COSY experiment (Table 1). The assignment and the sequence of the monosaccharides were confirmed by intense **B** NH-2,**A** H-1 and **C** NH-3,**B** H-1 cross-peaks in the NOESY spectrum (Fig. 2). The NOESY experiment showed correlations of NH-2 of units **A**, **B** and **C** with the CH_3CO groups and NH-3 of unit **C** with both protons of **M** CH_2. Therefore, malic acid is linked by COOH-4 to N-3 of GlcN3NA.

Borohydride reduction of PS-2 converted the keto group of Sug into a hydroxyl group to give PS-3. Sugar analysis of PS-3, including determination of the absolute configurations, indicated the presence of 2-amino-2,6-dideoxy-D-glucose (D-QuiN) and 2-amino-2,6-dideoxy-D-galactose (D-FucN), the C-4 epimers derived from 2-amino-2,6-dideoxy-D-hexos-4-ulose, in the ratio ~2.5:1, respectively. Similar analysis of the carboxyl-reduced[13] PS-3 resulted in the identification of L-galactosamine. The ^{13}C NMR spectrum of PS-3 contained signals of different intensities for four anomeric carbons, five nitrogen-bearing carbons and two CH_3–C groups (Table 2). The ^1H NMR spectrum showed five signals for anomeric protons and H-5 of GalNAcA at δ 4.78–5.15, three of which, at 5.08, 5.11 and 5.15, were of differing intensities. The spectrum contained also signals for the C–CH_2–C group of the malic acid residue and two signals for CH_3–C groups of QuiN and FucN at δ 1.23 and 1.16 in the ratio ~2:1, respectively.

The ^1H and ^{13}C NMR spectra of PS-3 were assigned by COSY, TOCSY, ROESY and ^1H,^{13}C HSQC experiments (Tables 1 and 2), and, in particular, spin systems for α-QuiN (**B′**) and α-FucN (**B″**) were identified. No significant changes were observed for the ^1H and ^{13}C chemical shifts of two other units **A** and **C**. The ROESY spectrum of PS-3 showed **A** H-1,**B′**/**B″** H-3; **B′**/**B″** H-1,**C** H-4 and **C** H-1,**A** H-4 correlation peaks and, thus, confirmed the sequence →4)-**A**-(1→3)-**B**-(14)-**C**-(1→.

The absolute configurations of GalNA (**A**) and GlcN3NA (**C**) were established by analysis of glycosylation effects on the ^{13}C NMR chemical shifts as described.[14] A small negative glycosylation effect (−0.3 ppm) for C-4 of unit **B″** was observed for the **A**-(1→3)-**B″** disaccharide fragment and was expected with different absolute configurations of the sugar residues. In the case of the same absolute configuration, the glycosylation effect would have been much higher (ca. −4 ppm). Similarly, a small negative glycosylation effect (−0.7 ppm) for C-4 of unit **C** was observed and is characteristic for the same absolute configuration of units **B′**/**B″** and **C** (a higher effect of −1.5 to −2 ppm would have been observed in the case of differing absolute configurations).

The COSY and TOCSY spectra of PS-1 demonstrated the location of the *O*-acetyl groups at position 3 of unit **A** (~100%) and at position 2 of malic acid **M** (~70%). The *O*-acetylation caused a rather high downfield displacement of **A** H-3 (by 1.17 ppm) and **M** H-2 (by 0.61 ppm) in the ^1H NMR spectrum of PS-1 as compared with their positions in the spectrum of PS-2 (Table 1). Typical effects were also observed in the ^{13}C NMR spectrum of PS-1, which was assigned using a ^1H,^{13}C HSQC experiment. Thus, the downfield displacements for **A** C-3 and **M** C-2 were +3.3 and +4.0 ppm (α-effects of *O*-acetylation) and the upfield displacements for **A** C-4 and **M** C-3 were −3 and −2.6 ppm (β-effects of *O*-acetylation), respectively (Table 2). In contrast, the difference between the **A** C-2 chemical shift in the spectra of PS-1 and PS-2 was +0.4 ppm, which could be accounted for by simultaneous *O*-acetylation at position 3, which should cause an upfield shift, and replacement of the

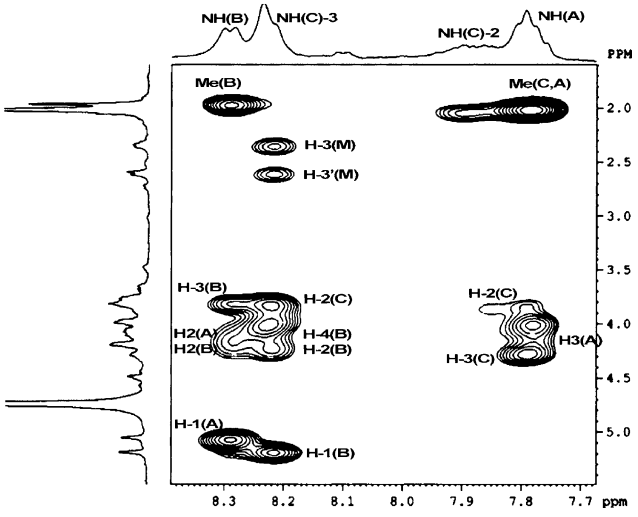
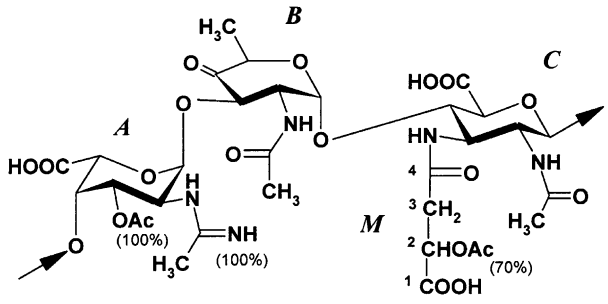


Figure 2. Part of a NOESY spectrum of PS-2 in a 9:1 H_2O–D_2O mixture showing correlations for NH protons. The corresponding portions of the proton spectrum are shown along the axes.

N-acetyl group in PS-2 with an *N*-acetimidoyl group in PS-1 at position 2 of GalNA (the latter effect is known to be +3 ppm or more[15,16]). This finding demonstrated the presence of 2-acetimidoylamino-2-deoxygalacturonic acid (GalNAmA).

In conclusion, the *O*-polysaccharide of *P. rubra* ATCC 29570^T has the structure shown in Figure 3. It contains two rarely occurring components. One of them, malic acid, to the best of our knowledge has been hitherto identified only once in a polysaccharide from *Shewanella algae* BrY.[17] The other, 2-acetamido-2,6-dideoxy-D-*xylo*-hexos-4-ulose, has been recently found in the mutant *Rhizobium etli* strain CE166 lipopolysaccharide as the single residue that links the *O*-polysaccharide to the core and is suggested to be the biosynthetic precursor of D-QuiNAc in the non-mutant strain.[18] The same keto sugar has been reported also as a component of the repeating units of the capsular polysaccharides from *Streptococcus pneumoniae* type5,[19] *Vibrio ordalii* O:2[20] ordalii O:2[20] and the *O*-polysaccharide of *Flavobacterium columnare* ATCC 43622.[21] Remarkably, in all these polysaccharides, as in the *O*-polysaccharide of *P. rubra* studied in this work, 2-acetamido-2,6-dideoxy-D-*xylo*-hexos-4-ulose is (1→4)-linked to a β-D-*gluco* configured monosaccharide and glycosylated at position 3 by a monosaccharide having the α-L-configuration.



→4)-α-L-Gal*p*NAm3AcA-(1→3)-α-Sug*p*-(1→4)-β-D-Glc*p*NAc3NAcylA-(1→

Figure 3. The structure of the *O*-polysaccharide (PS-1) of *Pseudoalteromonas rubra* ATCC 29570^T.

In connection with this, it should be noted that the *galacto* configuration of the glycosylating sugar in the *O*-polysaccharide of *V. ordalii* O:2[20] should be revised for the *gulo* configuration (Fig. 4). Indeed, comparison of the ^{13}C NMR chemical shifts of all eight methyl 2,3-diacetamido-2,3-dideoxy-α-D-hexopyranosides[22] (Table 3) showed that the C-2 chemical shift of δ 44.6 ppm reported for 2,3-diacetamido-2,3-dideoxy-α-L-hexuronic acid in this polysaccharide[20] is characteristic only for the α-*gulo* configuration.[22] Carbon C-2 of the other model monosaccharides, including that with α-*galacto* configuration, resonates significantly downfield (compare $\delta_{C\text{-}2}$ 45.4 for α-D-Gul*p*NAc3NAc-(1→OMe, 49.1 for α-D-Gal*p*NAc3NAc-(1→OMe and δ >49.5 for the other six isomers). Comparison of the C-5 chemical shift (δ 68.4 and 72.5 reported for the *gulo* and *galacto* models, respectively,[22] and δ 68.1 for α-Hex*p*NAc3NAcA in the *V. ordalii* O:2 polysaccharide[20]) supported this

Vibrio ordalii O:2

→4)-α-L-Gul*p*NAc3NAcA-(1→3)-β-Sug*p*-(1→4)-β-D-Glc*p*NAc3N(Fo-L-Ala)AN-(1→4)-β-D-Glc*p*NAc3NAmA-(1→

Vibrio anguillarum O:2

→4)-α-L-Gul*p*NAc3NAcA-(1→3)-β-D-Qui*p*NAc4NAc-(1→4)-β-D-Glc*p*NAc3N(Fo-L-Ala)AN-(1→4)-β-D-Man*p*NAc3NAmA-(1→

Figure 4. The revised structures of two *Vibrio* polysaccharides. In the original papers,[20,23] α-L-Gul*p*NAc3NAcA has been erroneously identified as α-L-Gal*p*NAc3NAcA. Sug, 2-acetamido-2,6-dideoxy-D-*xylo*-hexos-4-ulose; Am, acetimidoyl; Fo, formyl.

Table 3. Comparison of the ^{13}C NMR chemical shifts of some 2,3-diacetamido-2,3-dideoxy-α-hexopyranose derivatives

Residue	C-1	C-2	C-3	C-4	C-5	C-6
α-D-Gal*p*NAc3NAc-(1→OMe[a,b]	99.4	49.5	51.2	68.7	72.5	62.9
α-D-Gul*p*NAc3NAc-(1→OMe[a,b]	99.8	45.8	51.9	68.6	68.4	62.7
→4)-α-L-Gul*p*NAc3NAcA-(1→[b,c]	99.7	44.9	50.7	76.8	68.0	(≈173)
→4)-α-L-Hex*p*NAc3NAcA-(1→[d,e]	98.1	44.6	50.4	78.3	68.1	175.2
→4)-α-L-Hex*p*NAc3NAcA-(1→[e,f]	98.7	44.2	50.7	76.7	67.9	175.3

[a] Ref. 22.
[b] Published chemical shifts[15,18] are re-calculated related to acetone δ_C 31.07.
[c] Ref. 15.
[d] Ref. 20.
[e] α-L-Gal*p*NAc3NAcA reported in the original papers[20,23] should be revised to α-L-Gul*p*NAc3NAcA.
[f] Ref. 23.

conclusion (Table 3). A similar revision in favour of α-L-Gul*p*NAc3NAcA is required for α-L-Gal*p*NAc3NAcA in the structurally related O-specific and capsular polysaccharides of *Vibrio anguillarum* O:2[23] (Table 3, Fig. 4). The original assignment of the *galacto* configuration for this monosaccharides was based on the $J_{2,3}$ coupling constant, which was erroneously measured as 8.9 Hz due to the heterogeneity in chemical shifts caused by the equal population of the α- and β-forms of a monosaccharide at the reducing end of an oligosaccharide derived from the *V. anguillarum* O:2 polysaccharide (J.-R. Brisson, personal communication).

3. Experimental

3.1. Bacterial strain, growth and isolation of the polysaccharide

P. rubra type strain ATCC 29570^T was grown on modified Youschimizu-Kimura medium.[24] Wet bacterial cells were extracted with hot aq 45% phenol,[25] the resulting mixture was centrifuged, the aqueous layer dialyzed, freed from insoluble contaminations by centrifugation, concentrated in a vacuum and freeze dried to yield a lipopolysaccharide (860 mg from 20 L of cultural fluid).

The lipopolysaccharide (500 mg) was hydrolyzed with 1% HOAc (100 °C, 2 h) to give polysaccharide and lipid portions (40% and 4% of the lipopolysaccharide weight, respectively). The polysaccharide was purified by GPC on a column (1 × 100 cm) of TSK-50 (F) to give PS-1 (180 mg).

3.2. *O*-Deacetylation and reduction of the polysaccharide

The intact polysaccharide PS-1 (50 mg) was treated with aq 12.5% ammonia (60 °C, 3 h), and the *O*-deacetylated polysaccharide (PS-2, 45 mg) was isolated by GPC on a column (100 × 1.5 cm) of TSK HW-40 (F) (Toyo Soda, Japan) in water monitored with a RIDK-101 differential refractometer.

The *O*-deacetylated polysaccharide PS-2 (99 mg) was reduced with $NaBH_4$ (55 mg) in 1 M ammonia (5 mL) for 16 h at 20 °C, neutralized with HOAc, and the product (PS-3, 85.3 mg) was isolated by GPC on TSK HW-40 in aq 1% HOAc.

3.3. Compositional analysis

The carbonyl-reduced polysaccharide (PS-3, 1 mg) was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h), the monosaccharides (FucN and QuiN) were identified by GLC as the alditol acetates[26] using a Hewlett–Packard 5880 instrument with a DB-5 capillary column and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C min^{-1}. The absolute configurations of FucN and QuiN were determined by GLC of the acetylated glycosides with (−)-2-octanol[27] under the same chromatographic condition as above.

3.4. NMR spectroscopy

The samples were deuterium-exchanged by freeze-drying twice from D_2O and then examined as solutions in 99.97% D_2O. The NMR spectra were recorded, using internal TSP (δ_H 0.0) or acetone (δ_H 2.225 and δ_C 31.45) at 30 °C as references. ^1H and ^{13}C NMR spectra were run using a JEOL Lambda 400 MHz spectrometer equipped with a DEC AXP 300 computer workstation or a Bruker DRX 500 MHz spectrometer and processed with IRIX5.3 XWINNMR 2.6 software on an SGI workstation. 2D spectra were recorded using standard pulse sequences for COSY, TOCSY (spin-lock time 300 ms), NOESY and ROESY (mixing time 200 ms), gHSQC and gHMBC (optimized for a 8 Hz coupling constant).

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