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Note

A pseudoaminic acid-containing O-specific polysaccharide from a marine bacterium *Cellulophaga fucicola*

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Abstract—The O-polysaccharide was isolated from the lipopolysaccharide of *Cellulophaga fucicola* and studied by chemical analyses along with ^{1}H and ^{13}C NMR spectroscopy. The following new structure of the O-polysaccharide of *C. fucicola* containing 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid residue (pseudoaminic acid, Pse*p*) was elucidated as the following:

$$\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-Pse}p\text{-}(2\rightarrow$$

Keywords: O-specific polysaccharide; *Cellulophaga fucicola*; Bacterial polysaccharide structure; Marine bacterium

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The genus *Cellulophaga* belongs to the family Flavobacteriaceae of the phylum Bacteroidetes. It was created by Johansen et al.[1] to accommodate the heterotrophic aerobic Gram-negative yellow/orange pigmented gliding and agarolytic bacteria of marine origin. Currently this genus comprises five species: *Cellulophaga algicola*, *Cellulophaga baltica*, *Cellulophaga fucicola*, *Cellulophaga lytica*, and *Cellulophaga pacifica.*[2] Data on the lipopolysaccharide structure of *Cellulophaga* were reported only for *C. baltica.*[3]

The O-specific polysaccharide (OPS) of *C. fucicola* was obtained by mild acid degradation of the lipopolysaccharide (LPS) isolated from dried bacterial cells by the phenol–water procedure. Degradation of the LPS with sodium acetate buffer (pH 4.5) resulted in a high-molecular weight polysaccharide (OPS-I) and a low-molecular-weight polymer (OPS-II). Sugar analysis by GLC of the alditol acetates derived after full acid hydrolysis of the OPS-I revealed Gal and Glc in the ratios ~1:1. GLC analysis of the acetylated (*S*)-2-octyl glycosides demonstrated the D configuration of both monosaccharides.

The ^{13}C NMR spectrum of the OPS-I showed, inter alia, signals for three anomeric carbon atoms at δ 102.6, 102.8 (quaternary carbon; data from the DEPT experiment) and 104.2, two nitrogen-bearing carbon atoms of amino sugar(s) at δ 47.2 and 54.7, two CH_2OH groups at δ 61.3 and 62.2, one C–CH_2–C group at δ 36.4, two *N*-acetyl groups at δ 23.2 and 23.3 (both CH_3), one CH_3–C group at δ 17.6, and three CO groups at δ 173.2, 174.8, and 176.0. The ^{13}C NMR spectrum of the OPS-II (Fig. 1) contained also a set of minor signals belonging to terminal residues (see below).

Four signals were observed in a low-field region of the ^{1}H NMR spectrum of the OPS-I at δ 4.34–4.64, which included only two signals for anomeric protons (δ 4.45 and 4.64, both doublets, $J_{1,2}$ 8 Hz); the other

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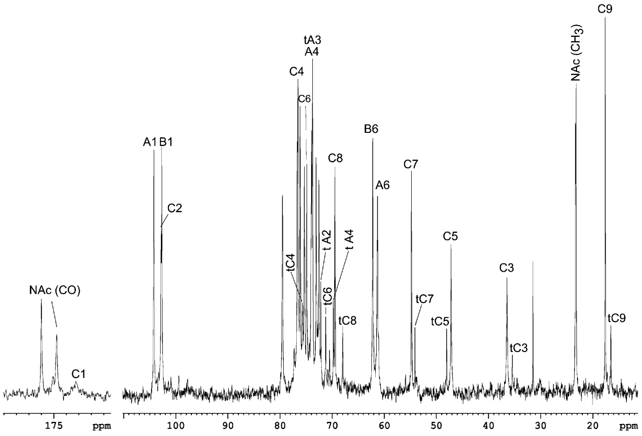

Figure 1. ^{13}C NMR spectrum of the low-molecular-weight fraction of the OPS-II of *C. fucicola*. Arabic numerals refer to the carbons in the sugar residues denoted as described in Table 1.

two signals at δ 4.34 and 4.52 were shown to belong to nonanomeric protons (see below). A high-field region of the spectrum contained two signals for *N*-acetyl groups at δ 1.94 and 2.00, one doublet (J 6 Hz) for a CH$_3$ group, and two one-proton signals at δ 2.67 (double doublets, J 5 and 13 Hz) and δ 1.77 (triplet, J 13 Hz).

The ^{1}H NMR spectrum of the OPS-I was assigned using 2D ^{1}H,^{1}H COSY, TOCSY, and ROESY experiments (Table 1). The analysis of the correlations in the 2D spectra showed the presence in the repeating unit of spin systems for β-Gal*p* (**A**), β-Glc*p* (**B**) and a 5,7-diacylamido-3,5,7,9-tetradeoxynon-2-ulosonic acid (**C**). The last sugar residue was identified as 5,7-diacetylamido-3,5,7,9-tetradeoxy-β-L-*glycero*-L-*manno*-non-2-ulosonic acid (β-pseudaminic acid, β-Pse) on the basis of the following data:[4]

(i) The ^{13}C NMR spectrum contained signals characteristic for C-1 (δ 173.2), C-2 (δ 102.8), C-3 (δ 36.4), C-5, C-7 (δ 47.2, 54.7), and C-9 (δ 17.6) of the 5,7-diamino-3,5,7,9-tetradeoxynon-2-ulosonic acids.

(ii) The positions of H-5 and H-7 (δ 4.34 and 4.02, correspondingly) in the ^{1}H NMR spectrum were typical of protons at N-acylated carbons. The presence of only two *N*-acetyl groups in the repeating unit showed that the carbons C-5 and C-7 were N-acetylated.

(iii) A large coupling constant ($J_{\text{H-3ax,H-4}}$ 13 Hz) indicated an axial position for H-4, small coupling constants $J_{\text{H-4,H-5}}$ and $J_{\text{H-5,H-6}}$ (4 and 2 Hz, correspondingly) displayed an equatorial position for H-5, and coupling constants $J_{\text{H-6,H-7}}$ (11 Hz) and $J_{\text{H-7,H-8}}$ (3 Hz) were characteristic for L-*glycero*-L-*manno* isomer of the 5,7-diamino-3,5,7,9-tetradeoxynon-2-ulosonic acids.

(iv) A large difference in the chemical shifts of H-3e and H-3a (0.9 ppm) was typical of the ulosonic acids with the axial position of the COOH group, that is the β-anomeric configuration of the pseudaminic acid residue.

A 2D ^{1}H,^{13}C HSQC experiment was applied for the assignment of the ^{13}C NMR spectrum of the OPS-I (Table 1). Significant downfield displacement of the signals for C-4 of all sugar residues (**A**, **B**, and **C**), as

Table 1. ^{1}H and ^{13}C NMR data of the OPS-I and OPS-II of the OPS of *C. fucicola* (δ, ppm)[a]

Sugar residue		1	2	3	4	5	6 (6a,6b)	7	8	9
OPS-I										
→4)-β-D-Gal*p*-(1→	^{1}H	4.45	3.57	3.66	4.52	3.71	3.80; 3.98			
A	^{13}C	104.2	72.1	73.1	73.7	76.7	61.3			
→4)-β-D-Glc*p*-(1→	^{1}H	4.64	3.23	3.65	3.62	3.61	3.66; 3.70			
B	^{13}C	102.6	73.9	75.3	79.5	76.1	62.2			
→4)-β-Pse*p*-(2→	^{1}H			1.77 (ax) 2.67 (eq)	4.06	4.34	3.73	4.02	4.15	1.16
C	^{13}C	173.2	102.8	36.4	76.5	47.2	74.8	54.7	69.4	17.6
β-D-Gal*p*-(1→	^{1}H	n.d	3.53	3.66	3.93	n.d	n.d			
tA (from OPS-II)	^{13}C	n.d	72.2	73.7	69.8	n.d	n.d			
→4)-α-Pse*p*	^{1}H			1.91 (ax) 2.10 (eq)	4.34	4.40	4.03	4.16	4.12	1.10
tC (from OPS-II)	^{13}C	n.d	n.d	35.9	75.7	48.0	71.2	54.1	68.0	16.5

[a] The chemical shifts for the *N*-acetyl groups are δ_{H} 1.94 and 2.00; δ_{C} 23.2, 23.3 (both Me), and 174.8, 176.0 (2 CO).

compared with their position in the corresponding nonsubstituted monosaccharides,[5] indicated the linkage position in each sugar residue.

The 2D ROESY experiment revealed strong inter-residue cross-peaks at δ 4.45/3.62,3.66,3.70; 4.64/4.06,4.34; 2.67/4.64, and 1.77/4.52, which were assigned to **A** H-1, **B** H-4, 6a, 6b; **B** H-1, **C** H-4, 5; **C** H-3e, **B** H-1, and **C** H-3a, **A** H-4 correlations, respectively. Therefore, the repeating unit of the OPS-I has the following structure:

$$\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-Pse}p\text{-}(2\rightarrow$$

A **B** **C**

This conclusion was confirmed by a 2D ^{1}H,^{13}C HMBC experiment (Fig. 2), which showed the following interresidue peaks: **A** H-1, **B** C-4; **B** H-1, **C** C-4, and **A** H-4, **C** C-2.

Methylation analysis of the polysaccharide resulted in identification of 2,3,6-tri-*O*-methylglucose and 2,3,6-tri-*O*-methylgalactose.

The analysis of 2D spectra of the OPS-II showed that the minor signals in the ^{1}H (Fig. 3) and ^{13}C NMR spectra (Fig. 1) belonged to a 4-substituted α-Pse[4] residue and nonsubstituted β-D-Gal*p*. The integral intensity of minor signals from α-Pse demonstrated that the OPS-II contained from 5 to 6 trisaccharide repeating units (**ABC)** with α-Pse at the reducing end, and, accordingly, nonsubstituted β-Gal at the nonreducing end:

$$\beta\text{-D-Gal}\,p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}\,p\text{-}(1\rightarrow 4)\text{-}\beta\text{-Pse}p\text{-}(2\rightarrow\ [\mathbf{ABC}]_{5\text{-}6}\rightarrow 4)\text{-}\beta\text{-D-Gal}\,p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}\,p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-Pse}p$$

tA **B** **C** **A** **B** **tC**

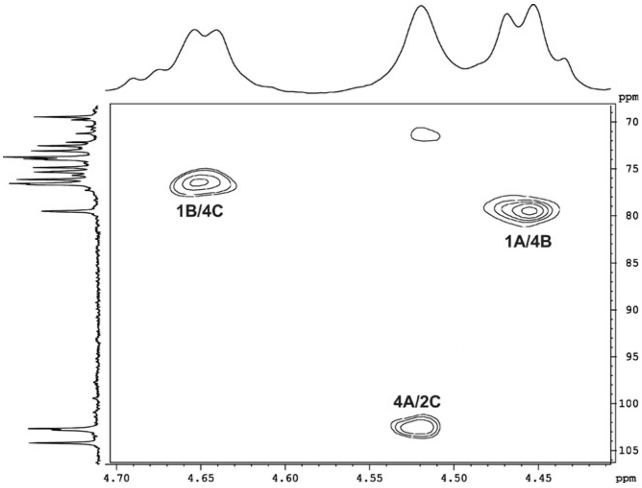

Figure 2. Partial ^{1}H,^{13}C HMBC spectrum of the OPS-II of *C. fucicola*. The corresponding parts of the ^{1}H and ^{13}C NMR spectra are shown along the horizontal and vertical axes, respectively. Arabic numerals before a slash refer to protons and those after the slash refer to carbon in sugar residues denoted by capital letters as shown in Table 1.

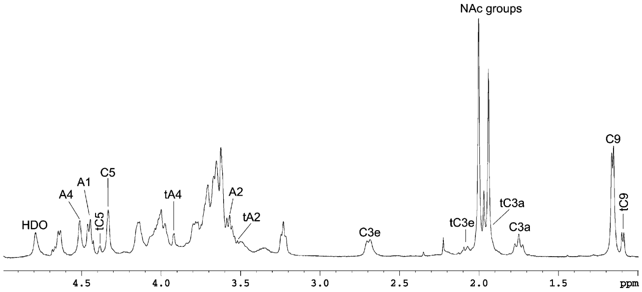

Figure 3. ^{1}H NMR spectrum of the low-molecular-weight fraction of the OPS-II of *C. fucicola*. Arabic numerals refer to the protons in the sugar residues denoted as described in Table 1.

1. Experimental

1.1. Bacterial strains and isolation of lipopolysaccharide

Cellulophaga fucicola strain NN015860^T, isolated from brown alga *Fucus serratus* L, which inhabits the North Sea, Atlantic Ocean, was received from Dr. Preben Nielsen (Enzyme Research, Novo Nordic, Denmark). The bacterium was cultivated for 48 h at ambient temperature on a medium consisting of (L^{-1}) 5 g Bacto Peptone (Difco), 2 g Bacto Yeast Extract (Difco), 1 g glucose, 0.02 g KH_2PO_4 and 0.05 g $MgSO_4{\cdot}7H_2O$ in 50% (v/v) natural seawater and 50% (v/v) distilled water. Lipopolysaccharide was isolated in a yield of 5.2% from dried bacterial cells by hot phenol–water extraction[6] and purified by treatment with cold aq 50% CCl_3CO_2H, followed by dialysis of the supernatant.

1.2. Degradation of lipopolysaccharide

Delipidation of the lipopolysaccharide of *C. fucicola* (300 mg) was performed with aq sodium acetate buffer (pH 4.5, 100 °C, 4 h). The precipitate was removed by centrifugation (15,000*g*, 30 min). The supernatant was sequentially fractionated by GPC on columns of TSK-50 and TSK-40 to give the high-molecular-weight polysaccharide (38 mg, OPS-I) and low-molecular-weight polymer (90 mg, OPS-II).

1.3. Chemical analyses

The OPS-I of *C. fucicola* was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h). Monosaccharides were identified by GLC of the alditol acetates on a Hewlett–Packard 5890 chromatograph equipped with an Ultra-1 column using a temperature gradient of 160–290 °C at 10 °C min^{-1}. The absolute configuration of the monosaccharides was determined by GLC of the acetylated (*S*)-2-octyl glycosides.[7,8] Methylation of the polysaccharide was performed with CH_3I in dimethyl sulfoxide in the presence of sodium methylsulfinylmethanide.[9] Partially methylated monosaccharides were derived by hydrolysis under the same conditions as in sugar analysis, converted into alditol acetates, and analyzed by GLC–MS using the same chromatographic conditions as in GLC.

1.4. NMR spectroscopy

The sample was deuterium exchanged by freeze drying two times from D_2O and then examined in a solution of 99.96% D_2O, using internal acetone as reference (δ_H 2.225, δ_C 31.45). NMR spectra were recorded at 30 °C on a Bruker DRX-500 spectrometer equipped with a SGI Indy/Irix 5.3 workstation and XWINNMR software. Mixing times of 100 and 200 ms were used in ROESY and TOCSY experiments, respectively. The HMBC experiment was optimized for coupling constant $J_{H\text{–}C}$ 8 Hz.

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