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Structure of the capsular polysaccharide from *Alteromonas nigrifaciens* IAM 13010T containing 2-acetamido-2,6-dideoxy-L-talose and 3-deoxy-D-*manno*-octulosonic acid

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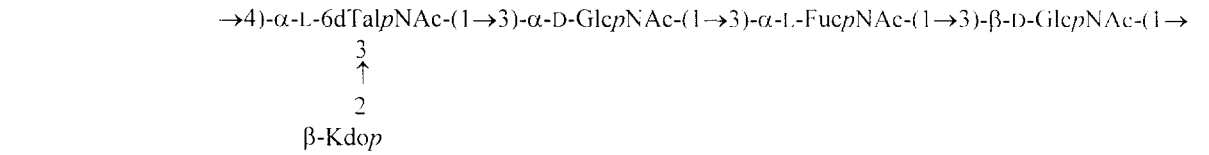
Abstract

A capsular polysaccharide was obtained from *Alteromonas nigrifaciens* IAM 13010T by saline extraction. On the basis of ^{1}H and ^{13}C NMR spectroscopy, including one-dimensional (1D) NOE spectroscopy, 2D rotating-frame NOE spectroscopy (ROESY), and ^{1}H-detected heteronuclear $^{1}H,^{13}C$ multiple-quantum coherence (HMQC), it was concluded that the polysaccharide contained inter alia an acidic sugar, 3-deoxy-D-*manno*-octulosonic acid (Kdo), and a rare amino sugar, 2-acetamido-2,6-dideoxy-L-talose (L-6dTalNAc, *N*-acetylpneumosamine), and has a pentasaccharide repeating unit of the following structure:



Keywords: *Alteromonas nigrifaciens*; Capsular polysaccharide; Structure; NMR spectroscopy; 2-Acetamido-2,6-dideoxy-L-talose; 3-Deoxy-D-*manno*-octulosonic acid

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1. Introduction

Structures of the capsular polysaccharides of four strains of marine bacteria of the genus *Alteromonas* have been established, and a number of unusual acidic and *N*-acylamino sugars have been identified as their components [1–4]. In this paper, we report the structure of the capsular polysaccharide (CPS) of *Alteromonas nigrifaciens* IAM 13010T.

2. Results and discussion

CPS was extracted from the microorganism with 0.9% aqueous saline using ultrasonication and purified by anion-exchange chromatography. Sugar analysis of CPS using GLC–MS of derived alditol acetates revealed the presence of 2-amino-2-deoxyglucose, 2-amino-2,6-dideoxyfucose, and another 2-amino-2,6-dideoxyhexose which differed by retention time from both *gluco* and *galacto* isomers and was identified as 2-amino-2,6-dideoxytalose (see below). CPS gave a positive reaction with the thiobarbituric acid reagent and, hence, contained additionally a 3-deoxyaldulosonic acid.

Mild hydrolysis of CPS with 1% aqueous acetic acid at 100 °C followed by GPC resulted in a degraded polysaccharide (DCPS). Besides that, a low-molecular-mass fraction was isolated, which contained the 3-deoxyaldulosonic acid cleaved from CPS.

The ^{13}C NMR spectrum of DCPS (Fig. 1, Table 1) indicated the presence of a tetrasaccharide repeating unit (there were signals for four anomeric carbons at δ 99.1, 99.8, 102.5, and 102.8). The spectrum also contained signals for two CH_3-C groups of 6-deoxy sugars at δ 16.5 and 16.9, two $HOCH_2$-C groups (C-6 of hexoses) at δ 62.2 (a signal with the double integral intensity), four *N*-acetyl groups (those for CH_3 at δ 23.7 with quaternary integral intensity and for CO at δ 174.9–175.4), four carbons bearing nitrogen in the region δ 49.8–57.2, and 12 other sugar ring carbons in the region δ 65.3–80.8.

Therefore, the repeating unit of DCPS includes four monosaccharides carrying four *N*-acetylamino groups, and two of the monosaccharides are 6-deoxy sugars. The absence from the ^{13}C NMR spectrum of any sugar signals for non-anomeric carbons in lower field than δ 82 indicated the pyranoid form of all sugar residues [5].

The signals in the ^{1}H NMR spectrum of DCPS were assigned using sequential, selective spin-decoupling performed in the difference mode [6] and 2D shift-correlated spectroscopy (COSY) (Fig. 2). Analysis of the chemical shifts and coupling constant values (Table 2) confirmed the pyranoid form of the sugar residues [7,8] and showed that two of them are GlcNAc residues, one being α-linked and the other β-linked ($J_{1,2}$ 4 and 8 Hz, respectively). Of two remaining sugars, one is an α-linked residue of 2-

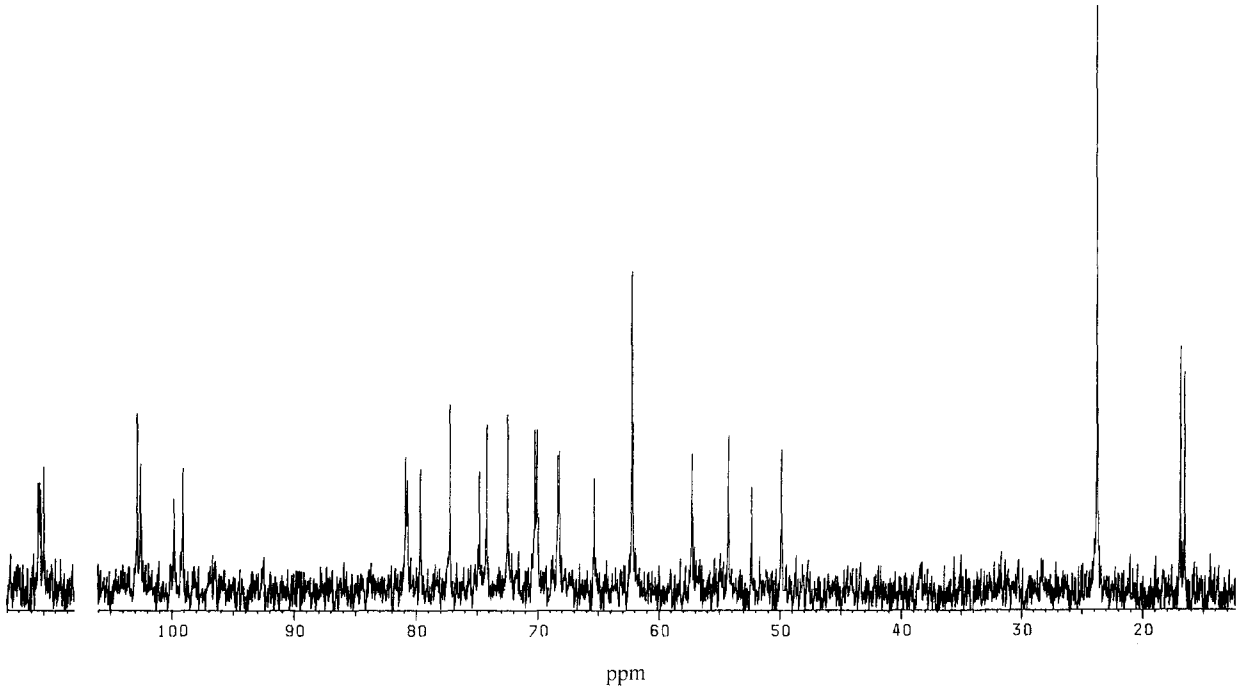


Fig. 1. ^{13}C NMR spectrum of DCPS.

Table 1
^{13}C NMR data (δ, ppm) [a]

Sugar residue	Carbon							
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
DCPS								
A: → 4)-α-L-6dTal*p*NAc-(1 →	102.5	52.3	65.3	80.8	68.3	16.5 [b]		
B: → 3)-α-D-Glc*p*NAc-(1 →	99.8	54.2	80.7	70.2	74.2	62.2		
C: → 3)-α-L-Fuc*p*NAc-(1 →	99.1	49.8	74.8	72.4	68.2	16.9 [b]		
D: → 3)-β-D-Glc*p*NAc-(1 →	102.8	57.2	79.6	70.0	77.2	62.2		
CPS								
A: → 4)-α-L-6dTal*p*NAc-(1 → 3 ↑	102.3	53.8	67.5	79.8	68.8	16.6		
B: → 3)-α-D-Glc*p*NAc-(1 →	99.8	54.8	79.9	70.5	74.4	62.4		
C: → 3)-α-L-Fuc*p*NAc-(1 →	99.1	50.0	74.4	72.5	68.3	16.8		
D: → 3)-β-D-Glc*p*NAc-(1 →	102.9	57.4	79.5	70.3	77.6	63.0		
E: β-Kdo*p*-(2 →	174.0	102.0	36.5	69.2	67.1	74.4	70.5	65.9
Sodium (methyl 3-deoxy-D-*manno*-octulosid)onate [c]								
α-Kdo*p*-(2 →	176.5	102.5	35.2	67.4	67.1	72.5	70.5	64.2
β-Kdo*p*-(2 →	174.8	102.4	35.5	68.6	66.5	74.6	70.3	65.2

[a] The spectra of DCPS and CPS were assigned using H-detected ^{1}H,^{13}C HMQC and HMQC-COSY, respectively. Chemical shifts of additional signals for NAc are δ 23.7–23.8 (Me) and 174.9–175.4 (CO).
[b] Assignment could be interchanged.
[c] Data from ref. [15].

acetamido-2,6-dideoxygalactose (FucNAc, $J_{1,2}$ 4 Hz), and the other has the α-*talo* configuration ($J_{1,2} < 2$, $J_{2,3} \approx J_{3,4} \sim 3$, $J_{4,5} < 2$ Hz, cf. published data [9]) and, thus, is an α-linked residue of 2-acetamido-2,6-dideoxytalose (6dTalNAc, *N*-acetylpneumosamine).

Linkage and sequence analysis of DCPS was carried out using 1D NOE and 2D ROESY experiments. In the 1D NOE experiment with pre-irradiation of H-1 of 6dTalNAc (unit A) at δ 4.74, a strong NOE was observed on H-3 of α-GlcNAc (unit B) at δ 3.72, together with much smaller NOEs on some neighbouring protons (most likely, these were accounted for by spin-diffusion typical of polysaccharides). On pre-irradiation of H-1 of unit B at δ 4.94, two interresidue NOEs were observed on H-3 (strong) and H-4 (weak) of FucNAc (unit C) at δ 3.97 and 3.83, respectively. Pre-irradiation of H-1 of unit C at δ 4.98 caused a NOE on H-3 of β-GlcNAc (unit D) at δ 3.64. These data suggested the sequence A-(1 → 3)-B-(1 → 3)-C-(1 → 3)-D. A 2D ROESY experiment with DCPS (Fig. 3) revealed the expected interresidue cross-peaks between the transglycosidic protons A H-1,B H-3 at δ 4.74/3.72, B H-1,C H-3 at δ 4.94/3.97, and C H-1,D H-3 at δ 4.98/3.64 and, thus, confirmed the monosaccharide sequence.

Due to partial coincidence of the signals for H-1 of unit D (δ 4.41) and H-5 of unit C (δ 4.37), these protons were jointly pre-irradiated to afford a strong NOE at $\delta \sim 3.85$. This could be interpreted as an interresidue NOE on H-4 of unit A (δ 3.86), thus indicating a linear polysaccharide, but, alternatively, as a NOE on H-4 of unit C (δ 3.83), which might be both intraresidue (from H-5 of unit C) and interresidue (from H-1 of unit D) in the case of a branched polysaccharide. In the same experiment other intraresidue NOEs were observed on H-3,6 of unit C and H-3,4,5 of unit D. Similar results were obtained using a 2D ROESY experiment (Fig. 3), where a cross-peak on the coordinate of H-1 chemical shift of unit D (δ 4.41) might be assigned either to D H-1,A H-4 (δ 4.41/3.86) or to D H-1,C H-4 (δ 4.41/3.83).

The uncertainty in the interpretation of the NOE data was eliminated by using a ^{1}H,^{13}C HMQC experiment [10], which allowed assignment of the ^{13}C NMR spectrum of DCPS (Table 1). It was found that of two signals, for C-4 of unit A and C-4 of unit C, only the former shifted downfield (to δ 80.8), evidently due to glycosylation at position 4, while the latter was at δ 72.4, which is typical of an unsubstituted α-FucNAc residue [7]. Therefore, unit D is attached to unit A at position 4 and DCPS has a linear structure.

Downfield displacements to δ 80.7, 74.8, and 79.6 of the signals for C-3 of units B, C, and D, respec-

tively, as compared with their positions in the spectra of the corresponding unsubstituted monosaccharides [7], confirmed substitution of all these units at position 3. In addition, the ^{13}C NMR data confirmed that all four constituent monosaccharides of DCPS are 2-acetamido-2-deoxy sugars, as followed from the

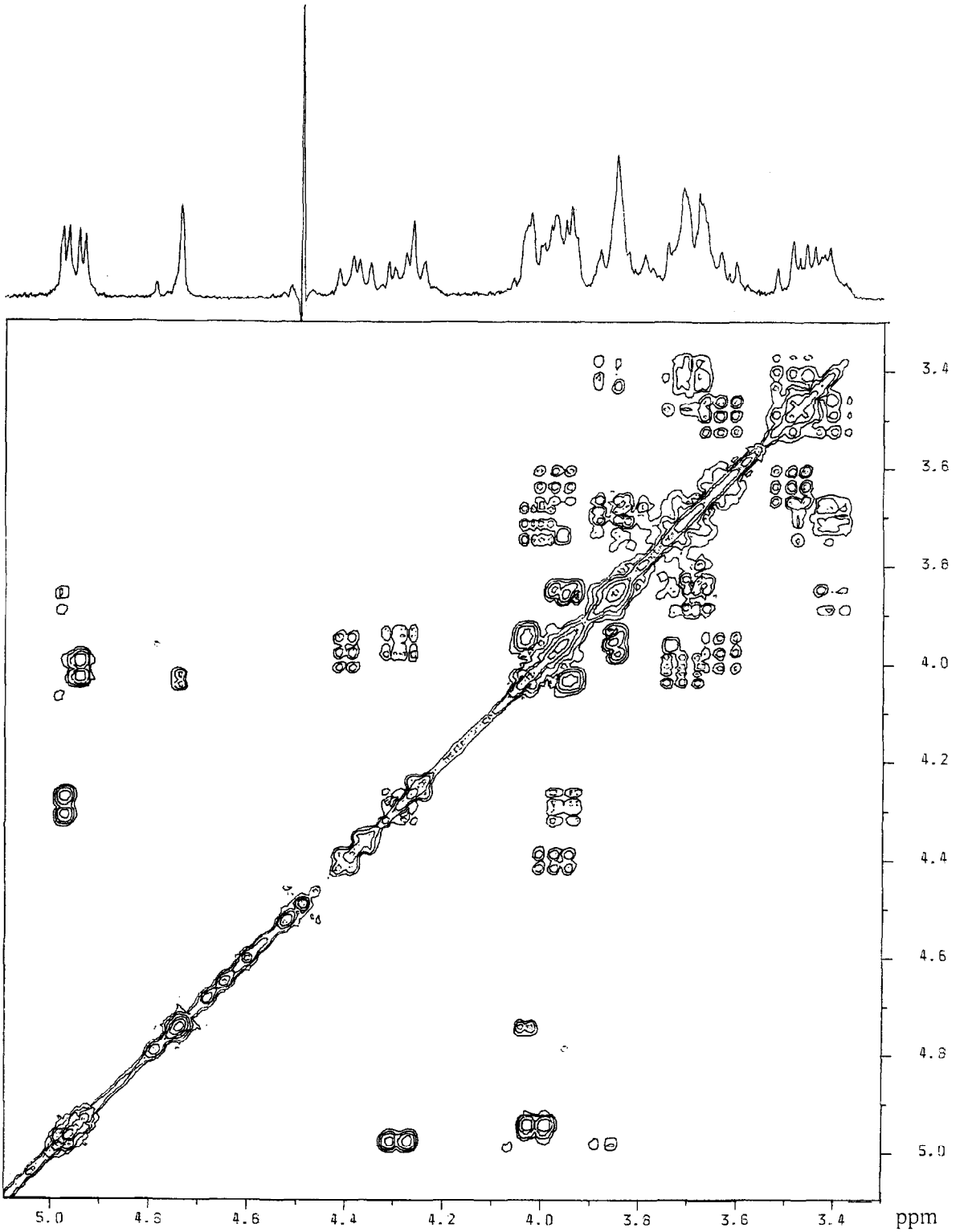


Fig. 2. Part of a COSY spectrum of DCPS. The corresponding part of the ^{1}H NMR spectrum is shown along the horizontal axis.

Table 2
^{1}H NMR data (δ, ppm; J, Hz) [a]

Sugar residue	Proton							
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	
DCPS								
A: → 4)-α-L-6dTal*p*NAc-(1 →	4.74	4.03	3.93	3.86	4.26	1.12		
	$J_{1,2}$ < 2	$J_{2,3}$ 3	$J_{3,4}$ 3	$J_{4,5}$ < 2	$J_{5,6}$ 6			
B: → 3)-α-D-Glc*p*NAc-(1 →	4.94	4.02	3.72	3.45	3.65	3.84	3.70	
	$J_{1,2}$ 4	$J_{2,3}$ 10	$J_{3,4}$ 10	$J_{4,5}$ 8.5				
C: → 3)-α-L-Fuc*p*NAc-(1 →	4.98	4.30	3.97	3.83	4.37	1.13		
	$J_{1,2}$ 4	$J_{2,3}$ 10	$J_{3,4}$ 3	$J_{4,5}$ < 2	$J_{5,6}$ 6			
D: → 3)-β-D-Glc*p*NAc-(1 →	4.41	3.98	3.64	3.50	3.43	3.94	3.70	
	$J_{1,2}$ 8	$J_{2,3}$ 10	$J_{3,4}$ 10	$J_{4,5}$ 10				
CPS								
A: → 4)-α-L-6dTal*p*NAc-(1 → 3 ↑	4.79	4.04	4.68	3.75	4.31	1.12		
B: → 3)-α-D-Glc*p*NAc-(1 →	5.00	4.05	3.99	3.53	3.75	3.88	3.77	
C: → 3)-α-L-Fuc*p*NAc-(1 →	5.01	4.36	4.01	3.91	4.42	1.20		
D: → 3)-β-D-Glc*p*NAc-(1 →	4.48	4.02	3.70	3.43	3.44	3.92	3.80	
	H-3a	H-3b	H-4	H-5	H-6	H-7	H-8a	H-8b
E: β-Kdo*p*-(2 →	1.70	2.46	3.73	3.19	3.46	3.93	3.97	3.73
	$J_{3a,3e}$ 13	$J_{3e,4}$ 5	$J_{3a,4}$ 13	$J_{4,5}$ 3	$J_{5,6}$ < 2	$J_{6,7}$ 8	$J_{7,8a}$ 3	$J_{7,8b}$ 5

[a] Chemical shifts of additional signals for NAc are δ 1.95–2.10.

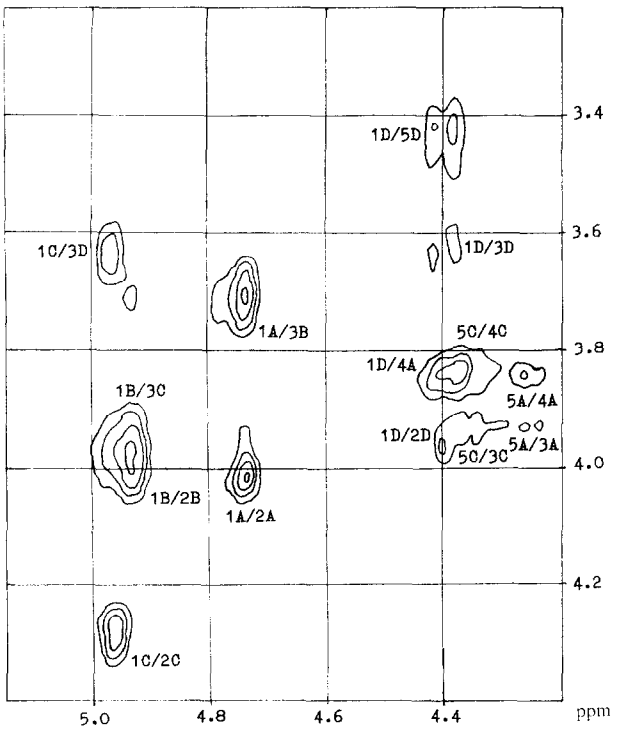


Fig. 3. Part of a ROESY spectrum of DCPS. Arabic numerals refer to the protons in the sugar residues denoted by letters (see Table 2).

positions of the signals for C-2 in the region of carbons bearing nitrogen (δ 49–58).

GLC analysis of acetylated (*S*)-2-butyl glycosides derived from DCPS by a modified method [11] showed that both GlcNAc residues have the D configuration. Analysis of glycosylation effects in the ^{13}C NMR spectrum of DCPS [12,13] allowed determination of the absolute configurations of FucNAc and 6dTalNAc. Thus, the positions of C-1 of unit B at δ 99.8 and C-4 of unit C at δ 72.4 indicated that the residues of α-GlcNAc and FucNAc have different absolute configurations since in the case of their same absolute configuration the signals in question would lie near δ 94.5 and 69, respectively. A relatively high absolute value of the negative β-effect (−1.2 ppm) on C-4 of unit B, caused by glycosylation of this unit at position 3 by unit A, showed that 6dTalNAc and α-GlcNAc also have different absolute configurations (a positive β-effect or one close to zero would be observed in the case of identical absolute configurations). Since GlcNAc is D, both FucNAc and 6dTalNAc are L.

These data suggested that DCPS has a linear tetrasaccharide repeating unit of the first structure below.

As compared to the spectrum of DCPS, the ^{13}C NMR spectrum of CPS contained eight additional

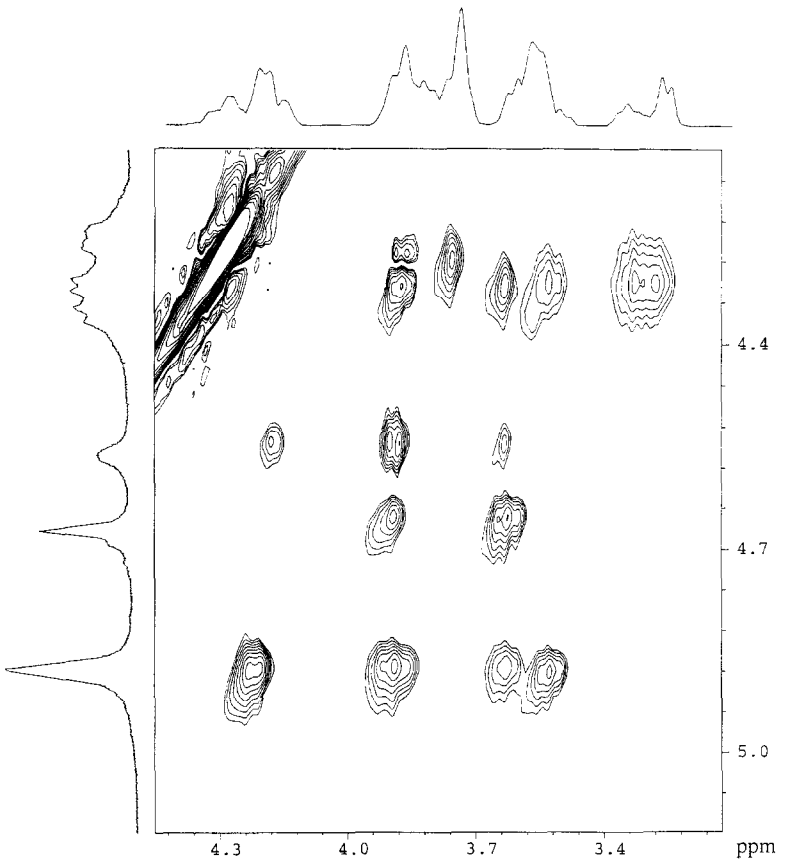


Fig. 4. Part of a ROESY spectrum of CPS. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes.

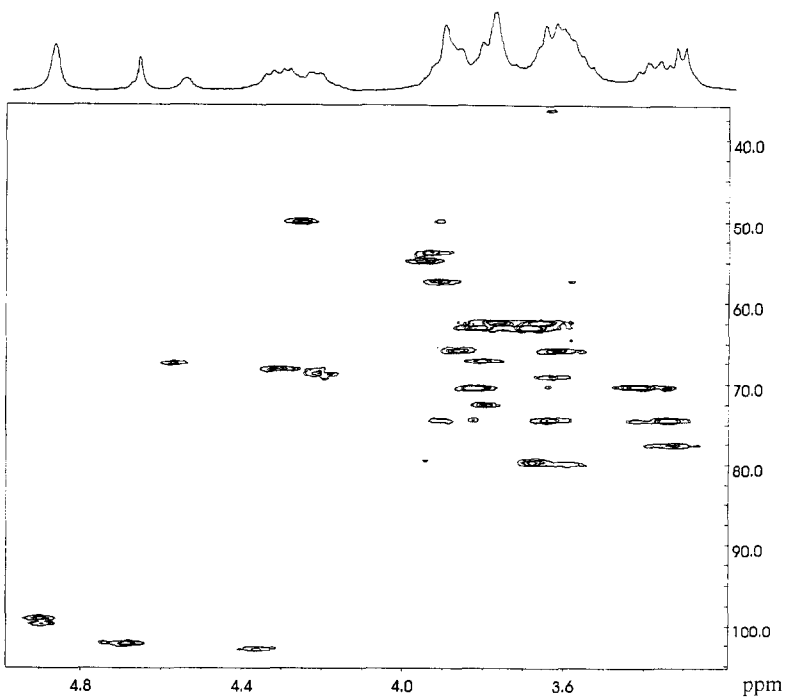


Fig. 5. Part of a HMQC-COSY spectrum of CPS. The corresponding part of the ^{1}H NMR spectrum is shown along the horizontal axis.

signals. Using the attached-proton test data [14], it was concluded that four of these signals at δ 36.5, 65.9, 102.0, and 174.9 belonged to a methylene group (a deoxy unit), a hydroxymethyl group, a non-protonated carbon (C-2 of a keto sugar), and a carboxyl group, respectively.

These data were consistent with the positive reaction of CPS with the thiobarbituric acid reagent and indicative of the presence in CPS of a 3-deoxyoctulosonic acid, most likely, of the well-known 3-deoxy-D-*manno*-octulosonic acid (Kdo). Having a highly acid-labile glycosidic linkage, Kdo was completely eliminated from CPS by mild hydrolysis with 1% acetic acid without depolymerisation of the polymeric chain, and, hence, this monosaccharide is attached as a lateral sugar residue.

The ^{1}H NMR spectrum of CPS (Table 2, Fig. 4) was completely assigned using 2D COSY and 2D ROESY, and the coupling constant values were determined by sequential, selective spin-decoupling performed in the difference mode [6]. With the ^{1}H NMR spectrum assigned, the ^{13}C NMR spectrum of CPS was interpreted using a ^{1}H,^{13}C HMQC-COSY experiment (Table 1, Fig. 5). The ^{13}C chemical shifts for the 3-deoxyoctulosonic acid residue in CPS were similar to those in sodium (methyl 3-deoxy-β-D-*manno*-octulosid)onate [15] (Table 1), thus confirming finally that the fifth sugar constituent of CPS is a β-linked Kdo residue.

A downfield displacement to δ 4.68 of the signal for H-3 of unit A, as compared with its position at δ 3.93 in the spectrum of DCPS (Table 2), suggested that Kdo is attached at position 3 of the 6dTalNAc residue. That unit A is the site of attachment of Kdo was confirmed by the presence in the 2D ROESY spectrum of a cross-peak Kdo H-3e,6dTalNAc H-4 at δ 2.46/3.75. The final evidence for glycosylation of unit A at position 3 followed from comparative analysis of the ^{13}C NMR spectra of DCPS and CPS. Thus, in the former spectrum, of the signals for carbons bearing an unsubstituted hydroxyl group, the signal for C-3 of 6dTalNAc at δ 65.3 shifted most markedly and appeared in the latter spectrum at δ 67.5 (a low positive α-glycosylation effect is typical of keto sugars lacking the anomeric proton; e.g., cf. published data [15,16]).

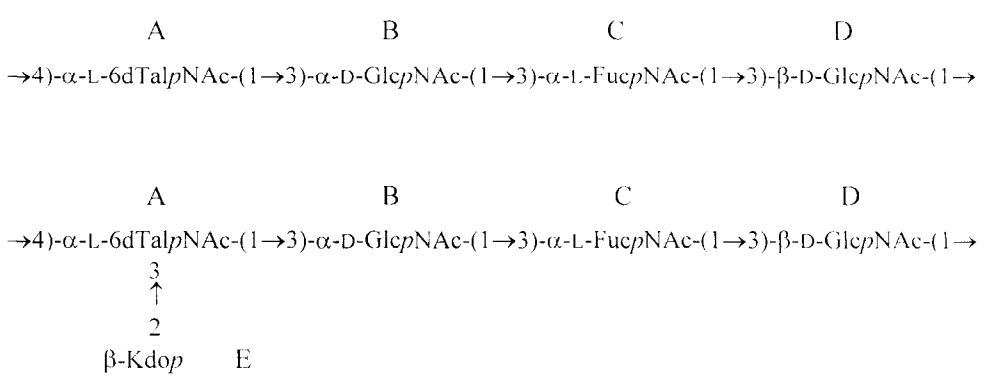
Therefore, on the basis of the data obtained, it was concluded that the capsular polysaccharide from *A. nigrifaciens* IAM 13010T has the second structure below.

This polysaccharide contains a rare sugar, 2-acetamido-2,6-dideoxy-L-talose (*N*-acetylpneumosamine). To the best of our knowledge, this amino sugar has been hitherto found only once, as a component of a *Pneumococcus* type Y antigenic capsular polysaccharide [17,18], and its structure was confirmed by synthesis of the authentic sample [19]. Another remarkable sugar constituent of the *A. nigrifaciens* CPS is Kdo, which is common for the lipopolysaccharide core of Gram-negative bacteria [20] and has also been found in some capsular polysaccharides, mainly in those of *Escherichia coli* [21].

3. Experimental

Chromatography.—GLC was carried out on a Hewlett–Packard 5890A instrument using a capillary column (0.2 mm × 25 m) of Ultra-1 stationary phase and a temperature program of 80 → 290 °C at 10 °C/min. Anion-exchange chromatography was performed on a column (2.5 × 50 cm) of DEAE-TSK 650M eluted with 50 mM Tris–HCl buffer, pH 7.0, and then with aq 0.5 M NaCl in the same buffer and monitored with a RIDK 101 refractometer. GPC was carried out on a column (2.5 × 90 cm) of TSK 50(F) in water.

NMR spectroscopy.—^{1}H and ^{13}C NMR spectra were recorded on Bruker WM-250 and Bruker AMX-



400 spectrometers for solutions in D_2O at 40 and 60 °C, respectively (internal acetone, δ_H 2.225, δ_C 31.45). A ROESY experiment with DCPS was carried out on a modified Bruker WM-250 spectrometer using the proposed pulse sequence [22] and a mixing time of 0.23 s; HDO signal was suppressed by irradiation for 1 s. A mixing time of 0.15 s was used in a ROESY experiment with CPS. Standard Bruker software was used to obtain 2D spectra.

Bacterial strain, growth, isolation of CPS and DCPS.—The *Alteromonas nigrifaciens* strain IAM 13010T was kindly provided by Dr. S. Suzuki (Kochi University, Japan). Growth of bacteria and isolation of CPS were performed as described earlier [1]. CPS was purified by anion-exchange chromatography; it was eluted with aq 0.5 M NaCl. CPS (200 mg) was hydrolysed with 1% aq CH_3CO_2H at 100 °C for 1 h, and the resulting mixture was fractionated by GPC to yield DCPS (130 mg), which was eluted shortly after the void volume of the column (K_D 0.9–0.95), and a low-molecular-mass material (40 mg).

Sugar analysis.—Hydrolysis of CPS (0.3 mg) was performed with 2 M CF_3CO_2H at 120 °C for 2 h, after conventional borohydride reduction and full acetylation, alditol acetates derived were analysed by GLC and GLC–MS.

CPS (3 mg) was treated with (*S*)-2-butanol (0.2 mL) in the presence of conc CF_3CO_2H (1 drop) at 130 °C for 4 h, the mixture was evaporated to dryness, acetylated with $(CH_3CO)_2O$ in pyridine, and analysed by GLC using the corresponding authentic samples prepared from GlcNAc and (*S*)- and (*R*)-2-butanol.

Acknowledgements

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