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Note

The structure of the O-specific polysaccharide chain of *Proteus penneri* strain 42 lipopolysaccharide

Yuriy A. Knirel [a,*], Aleksander S. Shashkov [a], Eugeny V. Vinogradov [a,1], Anna Swierzko [b], Zygmunt Sidorczyk [c]

[a] *N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Pr. 47, 117913 Moscow, Russian Federation*

[b] *Center of Microbiology and Virology, Polish Academy of Sciences, Dabrowskiego str. 251, 93-231 Lodz, Poland*

[c] *Institute of Microbiology and Immunology, University of Lodz, Banacha str. 12/16, 90-237 Lodz, Poland*

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Recently [1,2], the name *Proteus penneri* has been proposed for strains formerly called *Proteus vulgaris* biogroup 1. The composition and immunological properties of lipopolysaccharides of this novel species have been studied [3–9] and structures of the O-specific polysaccharide chains of lipopolysaccharides of a number of *P. penneri* strains have been established [4–6,8]. We now report the structure of a new O-specific polysaccharide of *P. penneri* strain 42.

The polysaccharide was obtained by mild acid degradation of the lipopolysaccharide isolated from dry bacterial cells by the phenol–water procedure [10]. Acid hydrolysis of the polysaccharide revealed the presence of glucose, galacturonic acid, and 2-amino-2-deoxyglucose identified using a sugar and an amino acid analyser and by GLC of derived alditol acetates. To prove the D configuration, glucose was oxidised with D-glucose oxidase. The D configuration of the other constituent sugars was determined by analysis of the glycosylation effects in the ^{13}C NMR spectrum of the polysaccharide (see below).

* Corresponding author.

[1] Present address: M.M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya str. 10/16, 117871 Moscow, Russian Federation.

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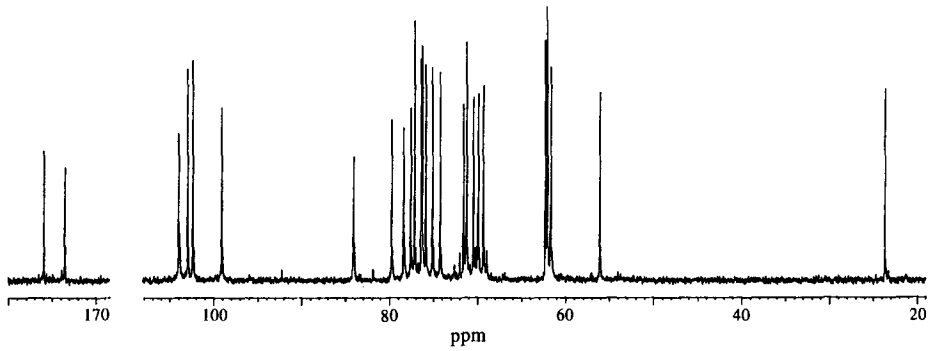

Fig. 1. 75-MHz ^{13}C NMR spectrum of *P. penneri* 42 O-specific polysaccharide.

Table 1
75-MHz ^{13}C NMR data (δ in ppm)

C-1	C-2	C-3	C-4	C-5	C-6	CH_3CON	CH_3CON
→ 2)-β-D-Glc*p*-(1 →	(unit **A**)						
102.3	78.3	77.0	71.1	75.8	62.2		
→ 4)-β-D-Glc*p*-(1 →	(unit **B**)						
103.9	74.1	75.0	77.5	76.2	62.0		
→ 3)-β-D-Glc*p*NAc-(1 →	(unit **C**)						
102.9	56.0	84.0	69.8	76.3	61.5	23.6	175.8
→ 4)-α-D-Gal*p*A-(1 →	(unit **D**)						
99.0	69.2	70.4	79.6	71.5	173.4		

The ^{13}C NMR spectrum of the polysaccharide was typical of a regular polymer (Fig. 1, Table 1). It pointed to a tetrasaccharide repeating unit (there were signals for four anomeric carbons at 103.9, 102.9, 102.3, and 99.0 ppm) containing one residue of an *N*-acetylated amino sugar (GlcNAc, signals for a carbon bearing nitrogen at 56.0 ppm and for Me and CO of an *N*-acetyl group at 23.6 and 175.8 ppm, respectively), and one residue of uronic acid (GalA, a signal for COOH at 173.4 ppm). The three residues of hexose and aminohexose (2 Glc and GlcNAc) were unsubstituted at position 6 (the signals for C-6 lie at 62.2, 62.0, and 61.5 ppm).

The ^1H NMR spectrum of the polysaccharide (Fig. 2, Table 2) contained signals for four anomeric protons at 4.54, 4.68, 4.76 (all d, $J_{1,2}$ 8 Hz), and 5.40 ppm (d, $J_{1,2}$ 3.5 Hz). Therefore, the first three signals belong to β-pyranoses and the fourth signal belongs to an α-pyranose. Two additional signals in the low-field region of the spectrum at 4.48 (d, $J_{3,4}$ 3 Hz) and 4.91 ppm (s, $J_{4,5} < 1$ Hz) were assigned to H-4 and H-5 of GalA (see below).

The polysaccharide was methylated and the partially methylated monosaccharides derived by acid hydrolysis were analysed by GLC/MS as alditol acetates. As a result, 3,4,6-tri-*O*-methylglucose, 2,3,6-tri-*O*-methylglucose, and 2-deoxy-4,6-di-*O*-methyl-2-methylaminoglucose were identified. When the methylated polysaccharide was carboxyl-reduced prior to the hydrolysis, 2,3-di-*O*-methylgalactose derived from GalA was

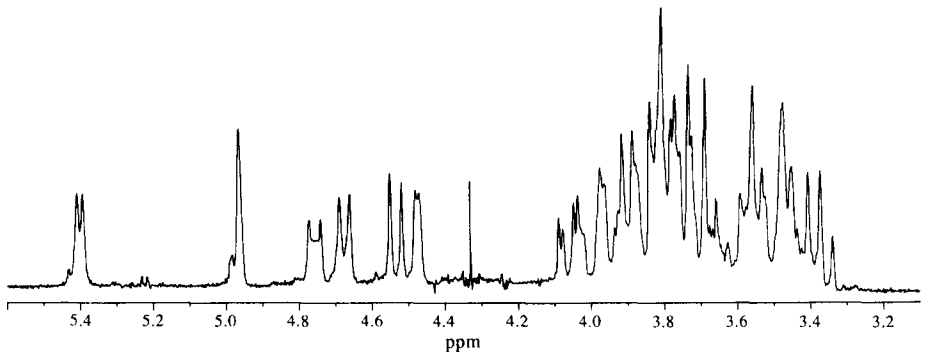

Fig. 2. 250-MHz ^{1}H NMR spectrum of *P. penneri* 42 O-specific polysaccharide. The signal for Me of the *N*-acetyl group at 2.08 ppm is not shown.

Table 2
250-MHz ^{1}H NMR data (δ in ppm, J in Hz)

H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	CH_3CON
→ 2)-β-D-Glc*p*-(1 →	(unit **A**)						
4.68	3.54	3.47	3.47	3.57	3.82		
$J_{1,2}$ 8	$J_{2,3}$ 9	[a]	[a]				
→ 4)-β-D-Glc*p*-(1 →	(unit **B**)						
4.54	3.38	3.68	3.73	3.67	3.85	4.00	
$J_{1,2}$ 8	$J_{2,3}$ 9	$J_{3,4}$ 9	$J_{4,5}$ 9				
→ 3)-β-D-Glc*p*NAc-(1 →	(unit **C**)						
4.76	3.82	3.82	3.55	3.42	3.75	3.90	2.08
$J_{1,2}$ 8	[a]	[a]	$J_{4,5}$ 10				
→ 4)-α-D-Gal*p*A-(1 →	(unit **D**)						
5.40	3.79	4.06	4.48	4.96			
$J_{1,2}$ 3.5	$J_{2,3}$ 10	$J_{3,4}$ 3	$J_{4,5}$ < 1				

[a] Coupling constant was not determined because of the coincidence of the signals.

found in addition. These data indicated that the polysaccharide is linear with one 2-substituted and one 4-substituted glucose residue, 3-substituted GlcNAc, and 4-substituted GalA.

In the ^{1}H NMR spectrum of the polysaccharide the signals for H-1–H-4 of all sugar residues were assigned using sequential, selective spin-decoupling, 2D shift-correlated spectroscopy (COSY) and COSY with relayed coherence transfer (COSYRCT). The coupling constant value of $J_{1,2}$ 8 Hz, determined for all three sugars having the *gluco* configuration (units **A–C**), showed that they are β-linked, while GalA (unit **D**) is α-linked ($J_{1,2}$ 3.5 Hz). The positions of the signals for H-5 of the β-linked sugar residues were determined by NOE experiments making use of appearance of a strong NOE on H-5 on preirraditaion of H-1 of the same sugar residue. The position of the signal for H-5 of α-GalA was found by preirraditaion of H-4.

With the ^{1}H NMR spectrum assigned, the ^{13}C NMR spectrum of the polysaccharide was interpreted using sequential, selective ^{13}C,^{1}H heteronuclear double resonance (Table

1). The relatively high-field position of the signal for C-2 of unit **C** at 56.0 ppm showed that this unit is GlcNAc; therefore, units **A** and **B** are glucose residues. Relatively low-field positions of the signals for C-2 **A**, C-3 **C**, C-4 **B**, and C-4 **D** (at 78.3, 84.0, 77.5, and 79.6 ppm, respectively), as compared with their positions in the spectra of the corresponding free monosaccharides [11], are due to the α-effects of glycosylation and confirm the substitution pattern of the sugar residues determined by methylation (see above).

In NOE experiments with sequential, selective preirradiation of H-1 of all sugar residues, together with intraresidue NOEs, the following interresidue NOEs on the transglycosidic protons were observed: H-1 **A**–H-4 **B**, H-1 **B**–H-2 or H-3 **C**, H-1 **C**–H-4 **D**, and H-1 **D**–H-2 **A**. These data are in agreement with the positions of substitution of the sugar residues determined earlier and allowed determination of their sequence in the repeating unit.

Analysis of the β-effects of glycosylation in the ^{13}C NMR spectrum allowed determination of the absolute configurations of GlcNAc and GalA. The effect on C-3 **A** in the α-(1 → 2)-linked disaccharide fragment **D** → **A** is 1.3 ppm, which is expected if units **A** and **D** have the same absolute configuration [12]. The effects on C-3 **B** in the β-(1 → 4)-linked disaccharide fragment **A** → **B** and on C-4 **C** in the β-(1 → 3)-linked disaccharide fragment **B** → **C** are negative and larger in absolute value than 1 ppm (−2.5 and −1.6 ppm, respectively), thus indicating the same absolute configuration of units **A**, **B**, and **C** [12]. Since glucose (units **A** and **B**) has the D configuration (see above), GlcNAc (unit **C**) and GalA (unit **D**) are also D.

Therefore, it was concluded that the O-specific polysaccharide of *P. penneri* strain 42 has the following structure:

$$\rightarrow 2)\text{-}\underset{\mathbf{A}}{\beta\text{-D-Glc}p}\text{-}(1 \rightarrow 4)\text{-}\underset{\mathbf{B}}{\beta\text{-D-Glc}p}\text{-}(1 \rightarrow 3)\text{-}\beta\text{-D}$$

$$\text{-}\underset{\mathbf{C}}{\text{Glc}p\text{NAc}}\text{-}(1 \rightarrow 4)\text{-}\underset{\mathbf{D}}{\alpha\text{-D-Gal}p\text{A}}\text{-}(1 \rightarrow$$

Like most O-antigens of *P. penneri* studied previously (strains 8, 12, 14, 16, 35, 52, 62 [4–6,8,9]), the O-specific polysaccharide of *P. penneri* strain 42 is acidic. Interestingly, it has the same structure as the polysaccharide chain of the lipopolysaccharide of a mutant strain *Proteus mirabilis* R14 ("T-like" form) derived from *P. mirabilis* S1959 [13]; these data will be published in full elsewhere.

1. Experimental

General methods.—The ^{1}H and ^{13}C NMR spectra were obtained with a Bruker WM-250 and a Bruker AM-300 instrument, respectively, for solutions in D_2O at 60° C. Acetone was used as an internal standard (δ_H 2.23, δ_C 31.45). Selective spin-decoupling, 1D NOE experiments, 2D COSY, COSYRCT, and heteronuclear ^{13}C,^{1}H COSY (XHCORRD) were performed using standard Bruker software.

GPC was carried out on a column (40 × 2.5 cm) of Sephadex G-50 in pyridine acetate buffer (pH 4.5) and monitored by the phenol–H_2SO_4 reaction. GLC was

performed using a Hewlett–Packard 5890 instrument equipped with a glass capillary column (25 m × 0.2 mm) coated with OV-1 stationary phase. GLC/MS was performed using a Varian MAT 311 instrument operating at the ionisation potential 70 eV under the same chromatographic conditions as in GLC.

Neutral sugars were analysed on a column (20 × 0.6 cm) of Durrum DAx4 anion-exchange resin in 0.5 M sodium borate buffer (pH 9.0) at 55°C; the elution was monitored by the orcinol–H_2SO_4 reaction using a Technicon Autoanalyzer II. Uronic acid was identified with a Biotronic LC-2000 analyser using a column (15 × 0.37 cm) of Dionex DAx8 resin in 1 M potassium borate buffer (pH 9.6) at 65°C [14]. Amino sugar was conventionally identified using a Microtechna T3339 amino acid analyser.

Bacterial strain; isolation of lipopolysaccharide and polysaccharide.—P. penneri strain 42 was kindly provided by Professor D.J. Brenner (Centre for Diseases Control, Atlanta, USA). Dry bacterial cells were obtained from the aerated liquid culture as described [15].

The lipopolysaccharide was isolated by phenol–water extraction [10] and purified by ultracentrifugation followed by digestion with RNAse and DNAse [16]. The O-specific polysaccharide was obtained by degradation of the lipopolysaccharide with aq 1% AcOH (100°C, 2 h) followed by GPC on Sephadex G-50.

Sugar and methylation analysis.—The polysaccharide (4 mg) was hydrolysed with 2 M CF_3CO_2H (120°C, 3 h), the hydrolysate was evaporated to dryness, and the residue was investigated using a sugar and an amino acid analyser; a portion of the residue was conventionally converted into alditol acetates and analysed by GLC. Oxidation of glucose was conventionally performed with D-glucose oxidase (Glucotest, Boehringer).

Methylation of the polysaccharide (10 mg) was performed according to the Hakomori procedure [17,18]; the methylated substance was recovered using a Sep-Pak C18 cartridge. A portion of the methylated polysaccharide was hydrolysed with 2 M CF_3CO_2H (120°C, 1 h), and the resulting partially methylated monosaccharides were conventionally converted into alditol acetates and analysed by GLC/MS. Another portion was reduced with an excess of $LiBH_4$ in aq 70% 2-propanol (20°C, 2 h), then diluted with water, and the product was isolated using a Sep-Pak C18 cartridge, hydrolysed, and analysed as above.

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