Eur. J. Biochem. *197*, 93 – 103 (1991)

0014295691002136

The structure of the *O*-specific polysaccharide chain of *Proteus penneri* strain 16 lipopolysaccharide

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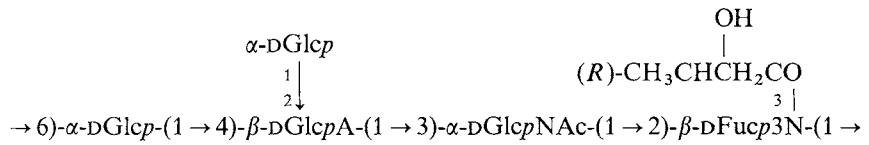
(Received July 13/November 11, 1990) – EJB 900843

O-Specific polysaccharide was obtained by mild acid degradation of *Proteus penneri* strain 16 lipopolysaccharide and found to contain D-glucose, D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose, and 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]-D-galactose in the ratio of 2:1:1:1 as well as a small proportion of *O*-acetyl groups. On the basis of one-dimensional ^{1}H-NMR ^{13}C-NMR and NOE spectroscopy, two-dimensional homonuclear-shift-correlated spectroscopy with one-step and two-step relayed coherence transfer and heteronuclear ^{1}H/^{13}C NMR shift-correlated spectroscopy, it was concluded that the *O*-specific polysaccharide of *P. penneri* strain 16 has the following structure:



This structure was confirmed by methylation analysis and structural analysis of a linear tetrasaccharide fragment prepared by cleavage of the polysaccharide with anhydrous hydrogen fluoride followed by conversion of the α-tetrosyl fluoride obtained in to the corresponding free oligosaccharide and alditol. *O*-Acetyl groups were tentatively located at position 3 of the glucuronic acid residue and at position 4 of the 6-substituted glucose residue, the degree of acetylation being less than 20% of the total.

Cross-reactions of *P. penneri* strain 16 anti-(*O*-specific polysaccharide) antiserum with lipopolysaccharides from several other *Proteus* strains and the role of 3,6-dideoxy-3-(*R*)-3-hydroxybutyramido-D-galactose in the serological specificity of *P. penneri* strain 16 are discussed.

Recently the name *Proteus penneri* has been proposed for strains formerly called *Proteus vulgaris* biogroup 1 [1, 2]. The genetic and metabolic differences of the novel *Proteus* species in comparison with other species of this genus could be reflected in the composition and structures of their lipopolysaccharides (LPS). The LPS from twenty strains of *P. penneri* were chemically analysed and their chemotypes discussed [3]. An unusual amino sugar has been found in *P. penneri* strain 16 LPS and identified as 3,6-dideoxy-3-(*R*)-3-hydroxybutyramido-D-galactose 1 [4]. In this paper we describe the structural elucidation of the *O*-specific polysaccharide chain of this LPS together with some serological data.

MATERIALS AND METHODS

Miscellaneous methods

GLC of sugar alditol acetates and partially methylated alditol acetates was performed on a Hewlett-Packard 5890 instrument equipped with a flame-ionisation detector linked to a glass capillary column (0.2 mm × 25 m) coated with OV-1 stationary phase using a linear-temperature gradient 160 – 290 °C at a rate of 10 °C/min. Combined GLC/MS was performed on a Varian MAT 311 instrument, using an ionisation potential of 70 eV and the same chromatographic conditions as in GLC.

For sugar analysis, samples were hydrolysed with 2 M trifluoroacetic acid in sealed tubes at 120° C for 2 h. Neutral sugars were analysed on a column (0.6 cm × 20 cm) of Durrum DAx4 anion-exchange resin in 0.5 M sodium borate buffer, pH 9.0, at 55 °C. Elution was monitored by the orcinol/sulfuric acid reaction using a Technicon Autoanalyzer II. Uronic acid was identified on a Biotronik LC-2000 analyzer using a column (0.37 cm × 15 cm) of Dionex DAx8-11 resin in 1 M potassium borate buffer, pH 9.6, at 65 °C. Amino sugars were analysed on a Microtechna T339 amino acid analyzer.

HPLC was performed on a column (25 cm × 0.46 cm) of Lichrosorb C_{18} and, unless otherwise stated, eluted with 0.05% trifluoroacetic acid in 4% methanol, monitored by a Knauer variable-wavelength monitor at 220 nm. Gel chromatography was carried out on a column (2.5 cm × 40 cm) of

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Abbreviations. LPS, lipopolysaccharide; d, doublet.

Sephadex G-50 in pyridine acetate pH 4.5 or on a column (1.6 cm × 80 cm) of Fractogel TSK HW 40 in 1% acetic acid and monitored by the phenol/sulfuric acid reaction or with a Knauer differential refractometer.

Ascending paper chromatography was on Whatman 3 paper in pyridine/ethyl acetate/acetic acid/water system (5:5:1:3, by vol.). Substances were detected with alkaline silver nitrate reagent.

Optical rotations were measured with a Jasco Dip 360 polarimeter in water at 25°C. Solutions were freeze-dried or evaporated *in vacuo* at 40°C.

NMR spectroscopy

^{1}H-NMR spectra were recorded on a Bruker WM-250 instrument in D_2O at 70°C for oligosaccharides and 80°C for polysaccharides. ^{13}C-NMR spectra were recorded with a Bruker AM-300 instrument in D_2O at 30°C for oligosaccharides and 70°C for polysaccharides. Acetone was used as an internal standard (δ_H, 2.23 ppm; δ_C, 31.45 ppm).

The standard Bruker software was used to obtain homonuclear-shift-correlated spectra with one-step and two-step relayed coherence transfer and heteronuclear ^{13}C/^{1}H-shift-correlated spectra with ^{1}H decoupling.

Experimental conditions for ^{1}H/^{1}H-shift-correlated experiments were as follows: 90° pulse 5.8 μs, time domain in f_2 of 1 K; spectral window 1300 Hz in both dimensions and a relaxation delay of 1 s. The relaxation delays D2 and D3 were 0.03 s (optimal for $J_{max} \approx 10$ Hz) and 512 spectra were collected with 80 scans. Prior to Fourier transformation the time-domain matrix was extended by zero filling in the f_1 dimension and the signals processed with an unshifted sine-bell function in both dimensions.

^{13}C/^{1}H shift-correlated experiments were performed under the following conditions: 90° pulse ^{1}H for 25 μs (or 14 μs for ^{13}C), time domain in f_2 of 2 K; 128 spectra collected with 1000 scans; spectral window 4200 Hz in f_2 domain and 740 Hz in f_1 domain (the region of ring carbons and protons); the relaxation delay D1 was 0.8 s; D3 and D4 3.2 μs and 1.6 μs, respectively (optimal for $^1J_{C,H} \approx 150$ Hz). Fourier transformation was performed with an unshifted sine-bell function in both dimensions.

One-dimensional NOE spectra were obtained using the Bruker Noemult program for the difference mode where the on-resonance irradiated spectrum was subtracted from that in which the irradiation frequency was off-resonance.

Antisera and serological technique

Antisera were raised against washed, heat-killed (100°C, 2.5 h) cells of *P. penneri* strains 10, 16 and 18 in New Zealand white rabbits (approximately 2.0–2.8 kg). The animals were immunised with bacterial suspensions (10^{10} cells/ml) in doses of 0.25, 0.5, and 1 ml over a three week period via the marginal ear vein. Blood was sampled by cardiac puncture six days after the last injection. Sera were separated and stored at 4°C after the addition of thiomersalate (0.01%).

Quantitative microprecipitation and its inhibition were performed as described earlier [5].

SDS/PAGE and Western blotting

SDS/PAGE was carried out in the buffer system of Laemmli [6] with a 12.5% acrylamide running gel and a 5% stacking gel. Staining was with silver nitrate according to Tsai and Frasch [7].

For Western blot analysis, substances were electrophoretically transferred from gels to nitrocellulose sheets [8] and probed with rabbit polyclonal antiserum raised against *P. penneri* strains, followed by incubation with goat anti-(rabbit IgG) serum labelled with horseradish peroxidase (Jackson Immunoresearch Laboratories Inc., USA). Horseradish peroxidase colour-development reagent containing 4-chloro-1-naphthol (Bio-Rad) and H_2O_2 was used as a substrate.

Bacterial strains, isolation and degradation of LPS

Strains of *P. penneri* were kindly provided by Professor D. J. Brenner (Center for Diseases Control, Atlanta, USA). Dry bacterial cells were obtained from aerated liquid cultures as described [9].

LPS (420 mg) was isolated from dry bacterial cells (10 g) of *P. penneri* strain 16 by phenol/water extraction [10] and purified by ultracentrifugation followed by digestion with nucleases [11]. Alkali-treated LPS was prepared by saponification of LPS with 0.25 M sodium hydroxide (56°C, 2 h) followed by precipitation with ethanol.

LPS (200 mg) was cleaved with 1% acetic acid [9] in order to split off the lipid A moiety and the water-soluble polysaccharide material was separated in to three fractions by gel chromatography on Sephadex G-50. The first high-molecular-mass fraction (74 mg) represented *O*-specific polysaccharide, the second fraction (39 mg) represented core polysaccharide and the third fraction (26 mg) contained mainly 3-deoxyoctulosonic acid.

De-O-acetylation of O-specific polysaccharide

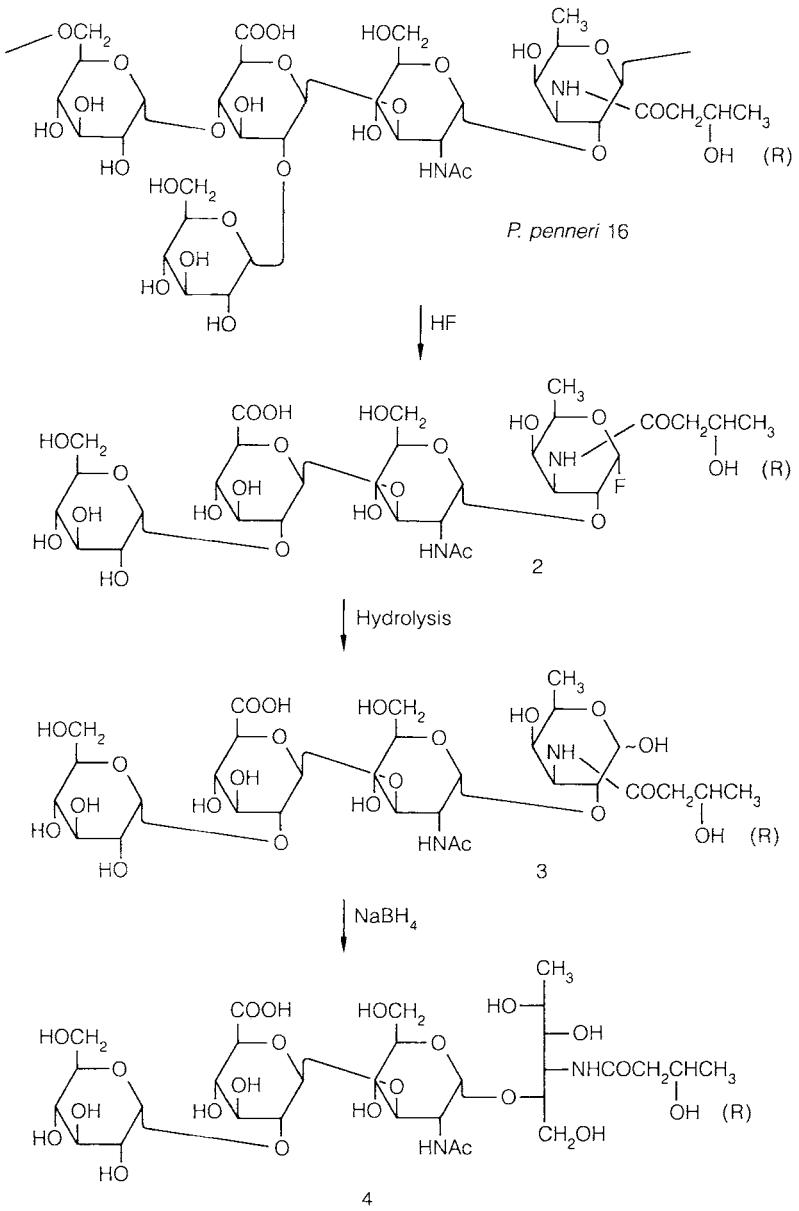
Polysaccharide (70 mg) was dissolved in 12% aqueous ammonia solution (5 ml), maintained for 4 h at 25°C and evaporated to dryness. The de-*O*-acetylated polysaccharide (60 mg) was isolated by gel filtration on Fractogel TSK HW 40.

Determination of absolute configurations of monosaccharides

Polysaccharide (40 mg) was hydrolysed with 2 M trifluoroacetic acid (120°C, 6 h). After evaporation, the residue was dissolved in water, the amino sugar components adsorbed on a KU-2 (H^+) cation-exchange column (5 cm × 1 cm) which was washed with water, then eluted with 1 M hydrochloric acid. The neutral eluate was evaporated and the residue separated by preparative paper chromatography to yield D-glucose, $[\alpha]_D$ +45°C, cf. +52.2°C (water) [12], and D-glucuronic acid, $[\alpha]_D$ +32°C ($c = 0.5$), cf. +36.5°C (water) [12]. D-Glucosamine was isolated from the acid eluate by preparative paper chromatography and evaporated with hydrochloric acid to give D-glucosamine hydrochloride, $[\alpha]_D$ +37°C ($c = 0.5$), cf. +72°C (water) [12].

Methylation analysis

Methylation was performed according to the Hakomori procedure [13]. The methylated substances were recovered using a Sep-Pak C_{18} cartridge [14]. A portion of the methylated polysaccharide was hydrolysed with 2 M trifluoroacetic acid (120°C, 1 h) and the monosaccharides conventionally converted into partially methylated alditol acetates and ana-



Scheme 1. *Selective cleavage of de-O-acetylated polysaccharide and subsequent modification of oligosaccharide 2*

lysed by GLC/MS. The second portion was reduced with an excess of lithium borohydride in 70% aqueous 2-propanol (75 ml, 20 °C, 2 h), diluted with water. The product was isolated using a Sep-Pak C_{18} cartridge, hydrolysed and analysed as described above.

Solvolysis with anhydrous hydrogen fluoride

For the isolation of monosaccharide 1 (Scheme 1), the polysaccharide (30 mg) was treated with anhydrous hydrogen fluoride (approximately 10 ml, 25 °C, 2 h) and the solvolysate evaporated *in vacuo* to give a mixture of sugars from which monosaccharide 1 was isolated by reverse-phase HPLC in water.

For the isolation of α-oligosyl fluoride 2, the polysaccharide (70 mg) was dissolved in anhydrous hydrogen fluoride (approximately 10 ml), stirred for 1 h with acetonitrile/solid carbon dioxide (−40 °C) and poured into cold ether (200 ml). The precipitate was separated by filtration through a stainless-steel filter, washed several times with cold ether, dissolved in water and α-oligosyl fluoride 2 isolated by gel chromatography on TSK HW 40 followed by purification with the help of reversed-phase HPLC.

Oligosaccharide 2 was heated in water (2 ml, 90 °C, 3 h) to give oligosaccharide 3, which was reduced with an excess of sodium borohydride. The solution was desalted by gel filtration on TSK HW 40 and oligosyl alditol 4 finally purified by reverse-phase HPLC.

RESULTS

Characterisation and composition of P. penneri *strain 16 O-specific polysaccharide*

P. penneri strain 16 *O*-specific polysaccharide had the specific optical rotation $[\alpha]_D$ +30 °C ($c = 1$).

The ^{13}C-NMR spectrum of the polysaccharide (Fig. 1), contained signals with smaller intensities in addition to the

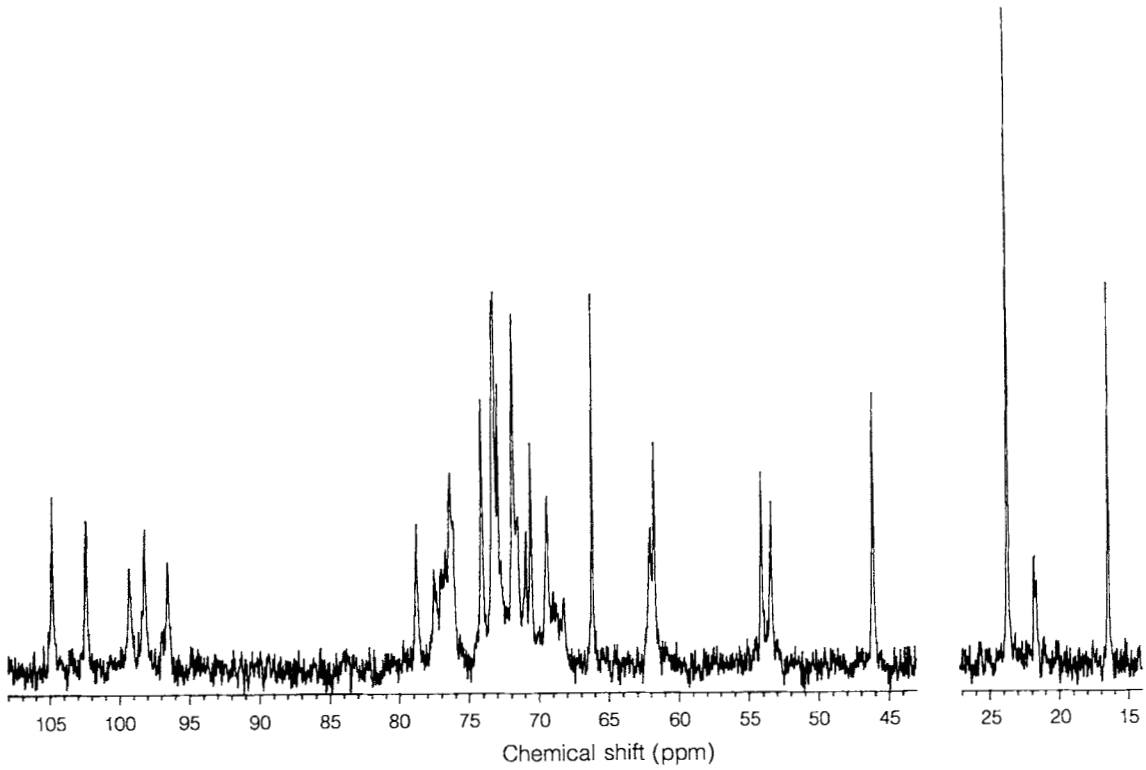


Fig. 1. ^{13}*C-NMR spectrum of* P. penneri *strain 16* O*-specific polysaccharide (except the signals for CO groups)*

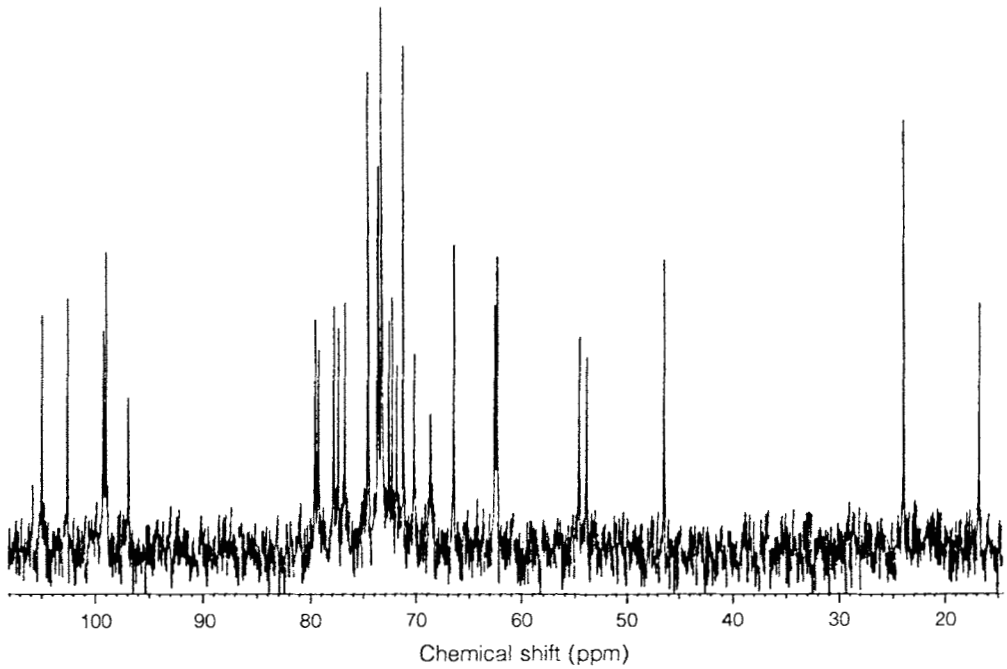


Fig. 2. ^{13}*C-NMR spectrum of de-*O*-acetylated polysaccharide (except the signals for CO groups)*

major signals, indicating that this polymer is not strictly regular, most probably due to the presence of *O*-acetyl groups in non-stoichiometric amounts (there were two signals corresponding to CH_3 in the region of 21.7 ppm which had integral intensities much less than those of the major signals). These signals disappeared after de-*O*-acetylation of the polysaccharide by treatment with aqueous ammonia, and the spectrum of the de-*O*-acetylated polysaccharide was typical for a regular polymer (Fig. 2).

The ^{13}C-NMR spectrum showed that the de-*O*-acetylated polysaccharide is built up of pentasaccharide repeating units (there were signals for five anomeric carbons at 96.9, 98.9, 99.2, 102.5 and 104.9 ppm). The repeating unit contains two amino sugars (signals of carbons bearing nitrogen atoms at

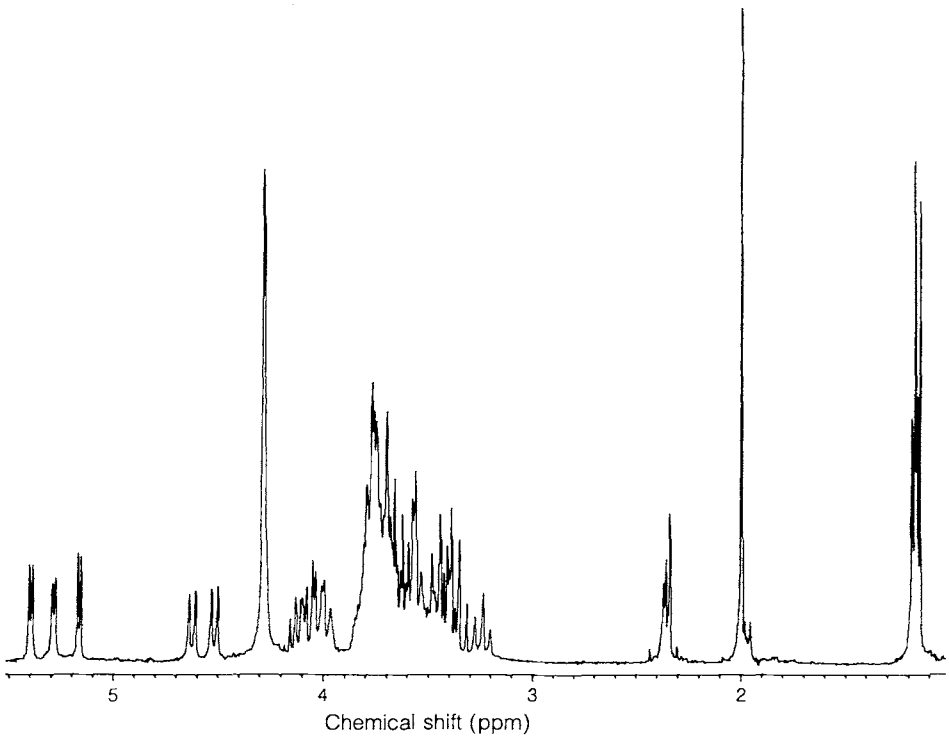
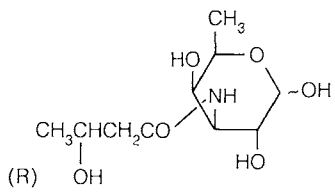


Fig. 3. *^{1}H-NMR spectrum of de-O-acetylated polysaccharide*

53.8 ppm and 54.5 ppm). One 6-deoxy sugar (a signal for C-6 at 16.7 ppm), one uronic acid (a signal for C-6 in the region 175.2 – 175.5 ppm), three hexoses or hexose derivatives, one of which is substituted at position 6 (a signal for C-6 at 68.6 ppm) and two others not substituted (signals for C-6 at 62.3 ppm and 62.5 ppm), one acetamido and one 3-hydroxybutyramido group [signals for CH_3 at 24.0 ppm (of double intensity), CO in the region 175.2 – 175.5 ppm, C-2 and C-3 of the 3-hydroxybutyramido group at 46.4 ppm and 66.4 ppm, respectively]; the signals for the other sugar-ring carbons were in the region 68 – 80 ppm. The absence of signals in the region 80 – 90 ppm indicated that all the sugar residues are in pyranose form.

The ^{1}H-NMR spectrum of the de-*O*-acetylated polysaccharide (Fig. 3) contained the signals for five anomeric protons at δ 4.51 ppm (d, $J_{1,2}$ 7.5 Hz), δ 4.60 ppm (d, $J_{1,2}$ 7.6 Hz), δ 5.16 ppm (d, $J_{1,2}$ 3.5 Hz), δ 5.26 ppm (d, $J_{1,2}$ 3.5 Hz), and δ 5.39 ppm (d, $J_{1,2}$ 3.5 Hz). The values of the coupling constants indicated that the first two signals belong to β-pyranoses and the last three signals to α-pyranoses. In the spectrum there were also signals for methyl groups of one 6-deoxy sugar at δ 1.16 ppm (d, $J_{5,6}$ 7 Hz), one *N*-acetyl group at 1.99 ppm, one 3-hydroxybutyryl group at δ 1.14 ppm (d, $J_{3,4}$ 6.5 Hz); for the methylene group (C-2) of 3-hydroxybutyryl at δ 2.33 ppm and for other sugar protons in the region of δ 3.2 – 4.2 ppm.

Acid hydrolysis of the polysaccharide revealed the presence of glucose and glucosamine in the ratio of approximately 2:1 as well as glucuronic acid and 3-amino-3,6-dideoxygalactose. Glucuronic acid and 3-amino-3,6-dideoxygalactose were not quantitated because of partial decomposition of the former and incomplete removal of *N*-acyl substituents in the latter under the hydrolysis conditions. The *N*-acyl amino sugar present in the hydrolysate was also obtained by solvolysis of the polysaccharide with anhydrous hydrogen fluoride at room temperature. It was further converted in to the corresponding



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Structure 1

alditol acetate and GLC/MS analysis showed it to be 3,6-dideoxy-3-(3-hydroxybutyramido)galactose (Structure 1). The structure and the D configuration of this sugar, isolated from the *P. penneri* strain 16 LPS as well as the *R* configuration of the 3-hydroxybutyryl group have been established previously [4]. All other constituent sugars were isolated from the hydrolysate of the polysaccharide and proved to have the D configuration on the basis of their specific optical rotation values.

As judged from these data, the repeating unit of *P. penneri* *O*-specific polysaccharide contains two residues of D-glucose, one of each residue of D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose, and monosaccharide 1 as well as a small proportion of *O*-acetyl groups.

The ^{13}C-NMR spectra of the *O*-specific polysaccharides of *P. penneri* strain 10 and 18 were identical to that of *P. penneri* strain 16 and hence they have the same structure of repeating unit.

NMR study of de-O-acetylated polysaccharide

The assignment of the signals in the ^{1}H-NMR spectrum of *P. penneri* strain 16 de-*O*-acetylated polysaccharide was performed with the help of two-dimensional homonuclear-shift-correlated spectroscopy with one-step and two-step re-

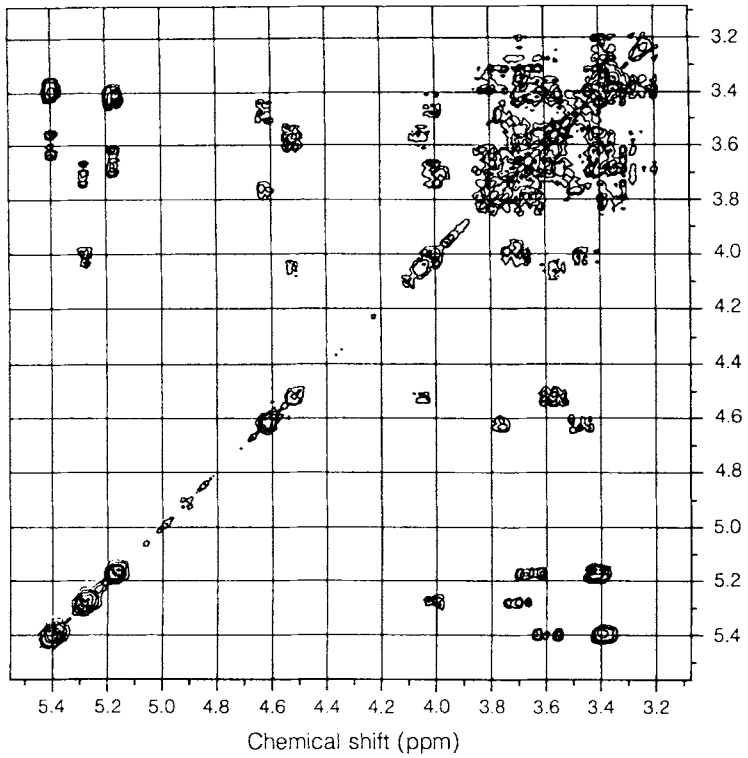

Fig. 4. *Two-dimensional homonuclear shift-correlated spectrum with one-step relayed coherence transfer of de-O-acetylated polysaccharide shown in the region 3.1—5.5 ppm.* Cross peaks of H-1 and H-2 as well as H-1 and H-3 are observed

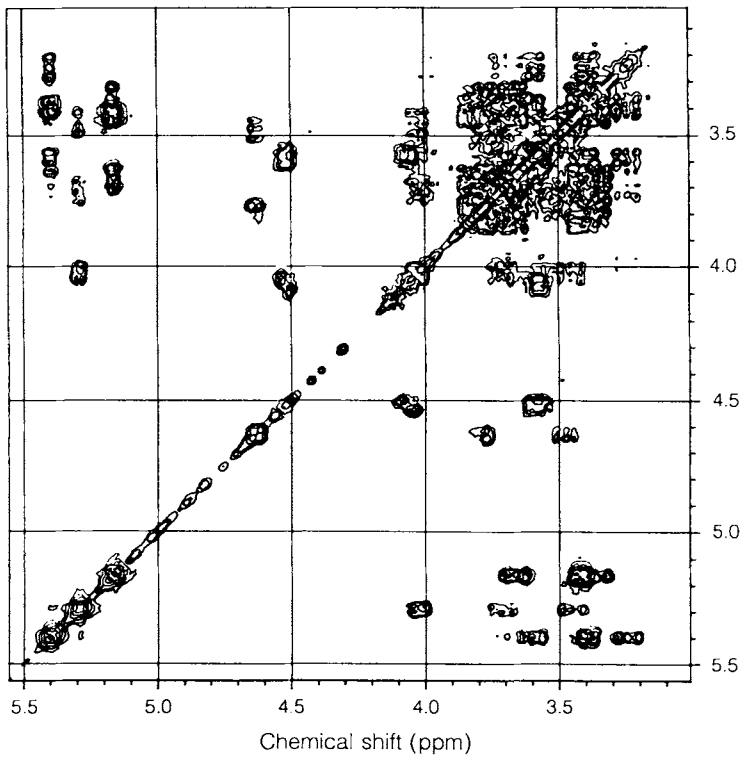

Fig. 5. *Two-dimensional homonuclear shift-correlated spectrum with two-step relayed coherence transfer of de-O-acetylated polysaccharide in the region 3.1—5.5 ppm.* Cross peaks of H-1 and H-2, H-1 and H-3, H-1 and H-4 are observed

layed coherence transfer. The cross peaks of H-1 and H-2, H-1 and H-3, H-1 and H-4 of all the sugar residues were clearly recognised in these spectra (Figs 4 and 5). Sequential, selective homonuclear spin-decoupling experiments allowed us to determine the positions of H-5 of all but one sugar residue and H-6 for two of the sugar residues (Table 1).

The sugar residue (unit D) characterised by the coupling constant $J_{3,4}$ 3 Hz must have the *galacto* configuration and

Table 1. *Chemical shifts in the* ^{1}H-NMR spectra
PS, *O*-specific polysaccharide. The chemical shift for the acetamido group was 1.99 ppm and 2.03 ppm for the de-*O*-acetylated polysaccharide and oligosaccharide 3, respectively. d, doublet; t, triplet; dd, doublet of doublet; ddd, doublet of doublet of doublet

Compound	Residue	Proton	Chemical shift	Visible multiplicity	Coupling constants
			ppm		Hz
De-*O*-acetylated PS	α-glucose (unit A)	H-1	5.39	d	$J_{1,2}$ 3.5
		H-2	3.40	d	$J_{2,3}$ 9
		H-3	3.58	t	$J_{3,4}$ 9
		H-4	3.22	t	$J_{4,5}$ 9
		H-5	3.71	m	
		H-6	3.57	dd	
		H-6′	3.98	dd	
	β-glucuronic acid (unit B)	H-1	4.60	dd	$J_{1,2}$ 7.6
		H-2	3.47	dd	$J_{2,3}$ 9
		H-3	3.80	m	
		H-4	3.80	m	
		H-5	3.76	m	
	α-glucosamine (unit C)	H-1	5.26	d	$J_{1,2}$ 3.5
		H-2	4.03	dd	$J_{2,3}$ 9
		H-3	3.72	t	$J_{3,4}$ 9
		H-4	3.46	t	$J_{4,5}$ 9
	3-amino-3,6-dideoxy-β-galactose (unit D)	H-1	4.51	d	$J_{1,2}$ 7.5
		H-2	3.56	dd	$J_{2,3}$ 9
		H-3	4.06	dd	$J_{3,4}$ 3
		H-4	3.57	dd	$J_{4,5}$ <2
		H-5	3.79	q	$J_{5,6}$ 7
		H-6	1.16	d	
	α-glucose (unit E)	H-1	5.16	d	$J_{1,2}$ 3.5
		H-2	3.42	dd	$J_{2,3}$ 9
		H-3	3.67	t	$J_{3,4}$ 9
		H-4	3.35	t	$J_{4,5}$ 9
		H-5	3.83	ddd	
	3-hydroxybutyramido	H-2	2.33	m	
		H-3	4.10	m	$J_{3,4}$ 6.5
		H-4	1.14	d	
Oligosaccharide 3	β-glucuronic acid (unit B)	H-1	4.77	d	$J_{1,2}$ 7.5
		H-2	3.55	m	$J_{2,3}$ 9
		H-3	3.60	m	
		H-4	3.62	m	
		H-5	3.80	m	
	α-glucosamine (unit C)	H-1	5.10	d	$J_{1,2}$ 3.5
		H-2	4.10	dd	$J_{2,3}$ 11
		H-3	4.02	dd	$J_{3,4}$ 8
		H-4	3.60	dd	$J_{4,5}$ 10
	3-amino-3,6-dideoxy-β-galactitol (unit D)	H-6	1.16	d	$J_{5,6}$ 6.5
	α-glucose (unit E)	H-1	5.27	dd	$J_{1,2}$ 3.5
		H-2	3.49	d	$J_{2,3}$ 9
		H-3	3.73	t	$J_{3,4}$ 9
		H-4	3.42	t	$J_{4,5}$ 9
		H-5	3.95	dd	
	3-hydroxybutyramido	H-2	2.48	m	$J_{3,4}$ 6.5
		H-3	4.20	m	
		H-4	1.22	d	

Table 2. *NOE observed in the de-O-acetylated polysaccharide*
+, NOE in the range 10–30%

NOE response	unit	Irradiation of H-1 unit				
		A	B	C	D	E
H-2	A	+				
H-4	A			+		
H-6,6	A				+	
H-2	B		+			+
H-3	B		+			
H-4	B	+				
H-5	B		+			
H-2	C			+		
H-3	C		+			
H-2	D			+	+	
H-3	D				+	
H-5	D				+	
H-2	E					+

is thus the β-linked residue of monosaccharide 1 ($J_{1,2}$ 7.5 Hz). This conclusion was confirmed by the presence of a 6-deoxy group in this residue (δ 1.16 ppm, $J_{5,6}$ 7 Hz) and a relatively low-field position of the signals for H-3 at δ 4.06 ppm which is due to the attachment of the acylamino group to C-3. Analogously, the low-field position of the H-2 signal for another sugar residue (unit C) at δ 4.03 ppm is due to the attachment of the acylamino group to C-2. Hence, unit C of 2-acetamido-2-deoxyglucose is α-linked ($J_{1,2}$ 3.5 Hz). All signals for one of the glucose residues (unit A), which is α-linked ($J_{1,2}$ 3.5 Hz), were identified in the spectrum, whereas the series of signals for the two remaining residues, namely glucuronic acid and the second glucose residues (units B and E), were not distinguished from each other at this stage.

In the NOE experiments with sequential irradiation of H-1 protons of all the sugar residues effects on some protons of the irradiated residue and inter-residue effects were observed (Table 2) which allowed us to determine the corresponding adjacent sugar residues. From these data the following sequence of the units in the polysaccharide was deduced:

$$\begin{array}{c} A-B-C-D \\ \quad\; | \\ \quad\; E \end{array}$$

Furthermore, unit B was proven to be 2,4-disubstituted, and hence, taking into account the methylation analysis data discussed below, this unit is the residue of glucuronic acid which is β-linked ($J_{1,2}$ 7.6 Hz). Therefore, unit E, which is attached to the glucuronic acid residue at position 2, represents the second glucose residue which is α-linked ($J_{1,2}$ 3.5 Hz). The NOE data also verify the substitution pattern of the other sugar residues (units A, C and D). The appearance of a significant NOE signal for H-4 of the glucose residue substituted at C-6 (unit A) after irradiation of the H-1 of unit C is not a contradiction since the preferred conformation of the trisaccharide fragment C–D–A has H-1 of unit C in spatial proximity to H-4 of unit A, as judged from the corresponding molecular model.

After the ^{1}H-NMR spectrum had been assigned, the ^{13}C-NMR spectrum of the de-*O*-acetylated polysaccharide was completely interpreted using heteronuclear ^{13}C/^{1}H-NMR shift-correlated spectroscopy (Fig. 6, Table 3). The high-field positions of the signals for C-2 of unit C (δ 53.8 ppm) and C-3

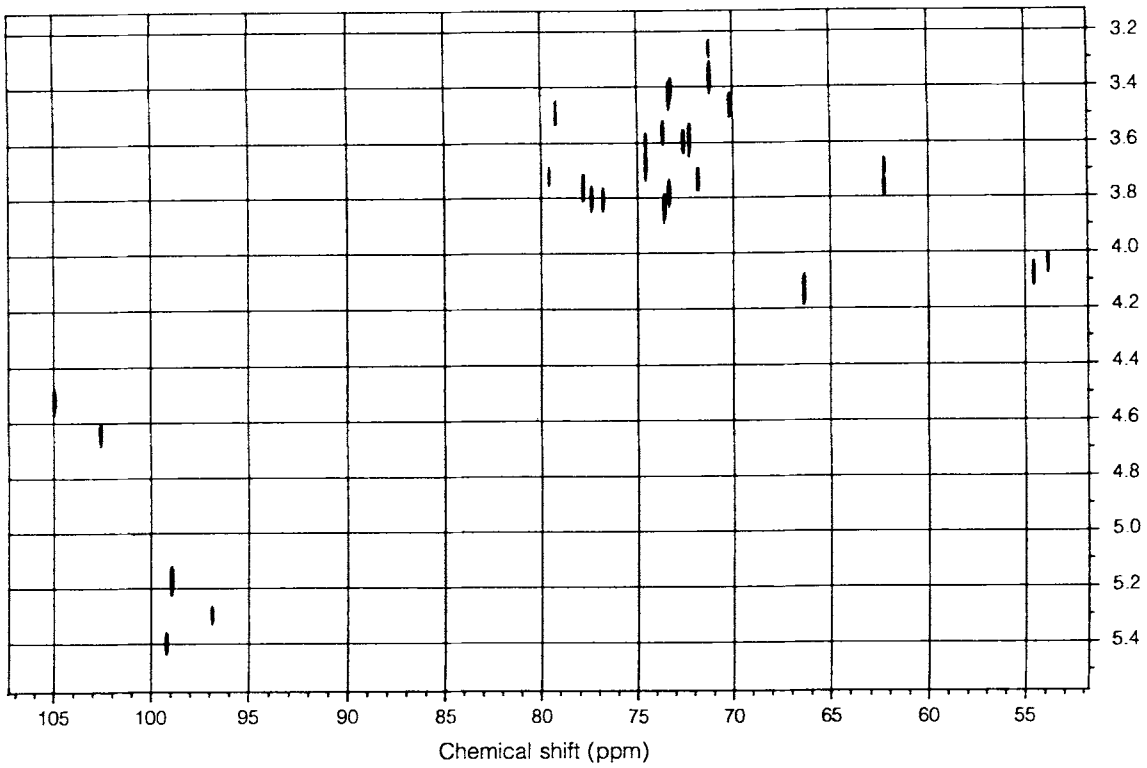

Fig. 6. *Two-dimensional heteronuclear $^{13}C/^{1}H$-NMR-shift-correlated spectrum of de-O-acetylated polysaccharide in the region 52 – 107 ppm*

Table 3. *Chemical shifts in the ^{13}C-NMR spectra*
Chemical shifts for the acetamido and 3-hydroxybutyramido group 23.3 – 24.0 ppm and 175.2 – 176.2 ppm, respectively. PS, *O*-specific polysaccharide

Compound	Residue	Chemical shifts					
		C-1	C-2	C-3	C-4	C-5	C-6
		ppm					
PS	α-glucose (unit A)	99.2	73.3	74.6	71.2	71.8	68.6
PS	β-glucuronic acid (unit B)	102.5	79.2	76.7	77.4	77.8	175.2
Oligosaccharide 3		102.0	77.5	75.8	72.7	76.2	
Oligosaccharide 1		102.6	77.1	75.7	73.0	76.4	
PS	α-glucosamine (unit C)	96.9	53.8	79.6	70.1	72.6	62.3
Oligosaccharide 3		97.7	54.0	78.7	69.8	72.9	61.9
Oligosaccharide 1		95.1	53.7	79.4	69.4	73.0	61.9
PS	3-amino-3,6-dideoxy-β-galactose (unit D)	104.9	73.7	54.5	72.2	73.3	16.7
Oligosaccharide 3		62.5	78.5	52.1	73.8	67.2	19.6
Oligosaccharide 1		105.1[a]	73.0	50.1	71.5	71.0	16.6
PS	α-glucose (unit E)	98.9	73.6	74.5	71.2	73.6	62.5
Oligosaccharide 3		98.7	73.4	74.3	70.9	73.1	61.9
Oligosaccharide 1		98.1	73.6	74.4	70.8	73.2	61.9
PS	3-hydroxybutyramido group		46.4	66.4	24.0		
Oligosaccharide 3			45.6	66.0	23.4		
Oligosaccharide 1			46.1	66.3	23.8		

[a] Doublet, $J_{C,F}$ 226 Hz.

of unit D (δ 54.5 ppm) confirmed these units to be glucosamine and 3-amino-3,6-dideoxygalactose, respectively. The two α-linked glucose residues were distinguished from each other by the position of the signal for C-5 of unit A at a higher field (δ 71.8 ppm) than that of unit E (δ 73.6 ppm) due to the β-effect of glycosylation of unit A at position 6, thus proving once again that unit E is the lateral glucose residue. The results of the ^{13}C-NMR spectrum were in full agreement with the substitution positions of the sugar residues formerly established.

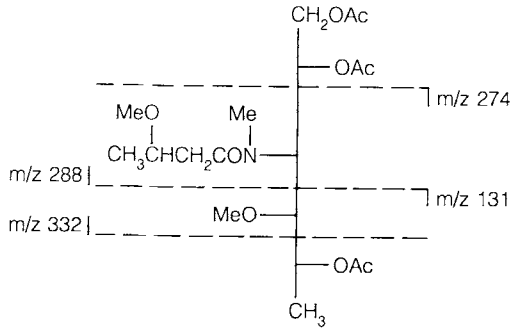

Fig. 7. *Mass spectrometric fragmentation of the partially methylated derivative of 3-(3-hydroxybutyramido)-3,6-dideoxygalactitol*

On the basis of the data obtained, it was concluded that the de-*O*-acetylated polysaccharide has the structure shown in Scheme 1.

Methylation analysis

The *P. penneri* strain 16 de-*O*-acetylated polysaccharide was subjected to methylation and partially methylated monosaccharides obtained by hydrolysis of the methylated polysaccharide analysed by GLC/MS as alditol acetates. The corresponding sugars identified were: 2,3,4,6-tetra-*O*-methylglucitol, 2,3,4-tri-*O*-methylglucitol, 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methyl)acetamidoglucitol, and 3,6-dideoxy-4-*O*-methyl-3-[(*N*-methyl)-3-methoxybutyramido]galactitol. The acetate of the last-named alditol displayed the fragmentation pattern shown in Fig. 7. In another experiment, the methylated polysaccharide was carboxyl-reduced prior to hydrolysis and, as a result, the derivative of 3-*O*-methylglucose which originated from glucuronic acid was identified in addition to the above-mentioned sugars.

The data obtained confirmed that the polysaccharide has a branched structure with a lateral glucose and 2,4-disubstituted glucuronic acid at the branch point; the second glucose residue is substituted at position 6, glucosamine at position 3, and 3-amino-3,6-dideoxygalactose at position 2.

Solvolysis with anhydrous hydrogen fluoride; oligosaccharides 2–4

The *P. penneri* strain 16 de-*O*-acetylated polysaccharide was cleaved selectively upon treatment with anhydrous hydrogen fluoride to give an oligosaccharide product which was identified as α-oligosyl fluoride 2 (Scheme 1). Its ^{13}C-NMR spectrum (Table 3) showed a characteristic signal for C-1 at δ 105.1 ppm which was split due to the coupling to fluorine ($J_{C,F}$ 226 Hz). The corresponding signal for H-1 at δ 5.79 ppm, split as a result of H/F coupling ($J_{H,F}$ 54 Hz), was present in the ^{1}H-NMR spectrum of oligosaccharide 2. This oligosaccharide was not completely stable in hot aqueous solution and was therefore subjected to hydrolysis to produce reducing tetrasaccharide 3 (Scheme 1). Reduction of tetrasaccharide 3 with sodium borohydride yielded oligosaccharide 4 (Scheme 1).

Sugar analysis showed that oligosaccharide 4 represents a triosyl alditol containing all components of the parent polysaccharide except one of the glucose residues. Methylation analysis showed oligosaccharide 4 to be linear and terminate in a glucose residue at the non-reducing end; two other sugar residues, namely those of glucuronic acid (unit B) and

Table 4. *Precipitation of* P. penneri *strain 16 anti-(O-specific polysaccharide) antiserum by various antigens*
Minimal amounts of antigens which were precipitated with undiluted serum are given. PS, *O*-specific polysaccharide

Bacterium	Strain	Antigen	Minimum concentration precipitated
			μg/ml
P. penneri	16	LPS	3.9
	16	alkali-treated LPS	7.8
	16	PS	2.0
	10	de-*O*-acetylated PS	2.0
	18	LPS	62.5
		LPS	125
P. vulgaris	O17	LPS	3.9
	O37	LPS	31.2
P. mirabilis	O17	LPS	15.6
	O38	LPS	31.2

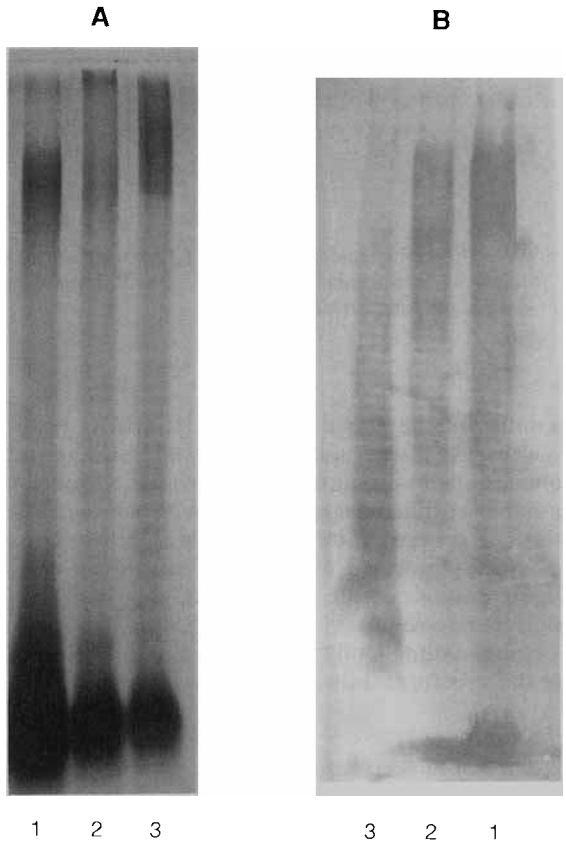

Fig. 8. *Silver-stained electrophoresis profile (A) and parallel immunoblot with* P. penneri *strain 16 O-antiserum (B) of LPS from* P. penneri *strains 10 (lane 1), 16 (lane 2) and 18 (lane 3)*

2-acetamido-2-deoxyglucose (unit C) are substituted at positions 2 and 3, respectively. The 1,2-linkage between residues of glucose and glucuronic acid proved that, of the two glucose residues present in the polysaccharide, the lateral one (unit E) entered oligosaccharide 4.

The ^{13}C-NMR spectrum of oligosaccharide 4 (Table 3), which was interpreted using the data on glycosylation effects

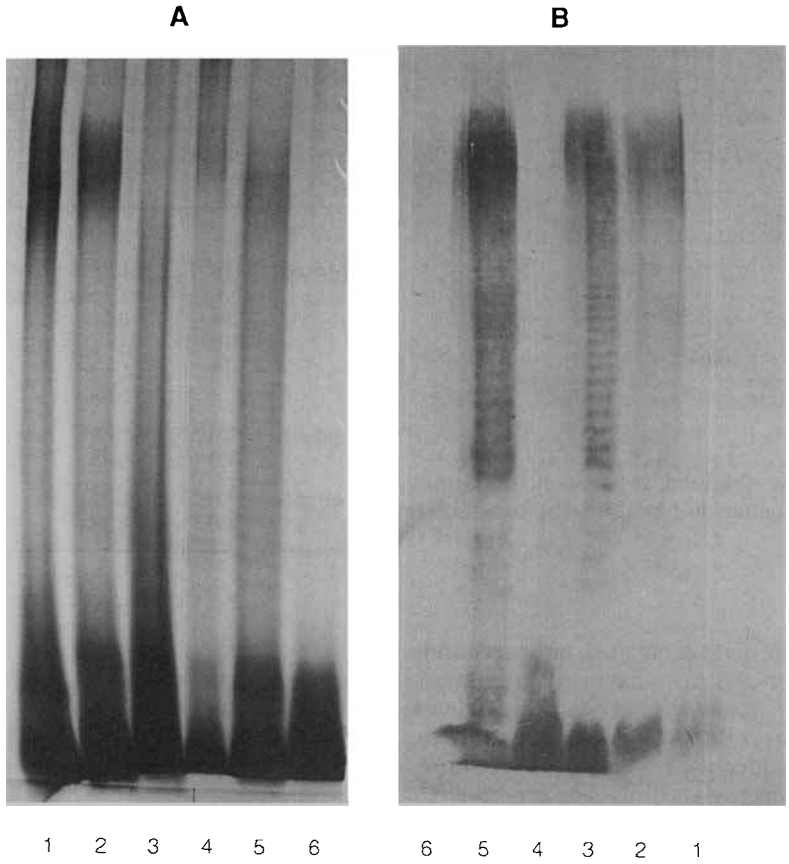

Fig. 9. *SDS/PAGE Western-blot analysis of LPS from* P. mirabilis *S1959 (lane 1)*, P. vulgaris *O17 (lane 2)*, P. vulgaris *O37 (lane 3)*, P. penneri *strain 16 (lane 4)*, P. mirabilis *O17 (lane 5), and* P. mirabilis *O38 (lane 6)*. (A) Silver stained gel; (B) Immunoblot with *P. penneri* strain 16 anti-(*O*-specific polysaccharide) antiserum

[15], confirmed the above data. The ^{1}H-NMR spectrum of oligosaccharide 4 was assigned with the help of selective homonuclear spin-decoupling and two-dimensional homonuclear shift-correlated spectroscopy with one-step and two-step related coherence transfer (Table 1) and NOE experiments were performed as described above for the de-*O*-acetylated polysaccharide. As a result, the sequence of the monosaccharide residues was established E→B→C→D, and thus oligosyl alditol 4, oligosaccharide 2 and oligosaccharide 3 have the structures shown in Scheme 1.

Localisation of O-*acetyl groups*

Comparison of the ^{13}C-NMR spectrum of the initial and de-*O*-acetylated polysaccharides of *P. penneri* strain 16 showed differences in the region near δ 77 ppm where carbons C-3, C-4 and C-5 of the glucuronic acid residue (unit B) resonate. This allowed us to propose that one of the *O*-acetyl groups substitute some of these 2,4-disubstituted residues at position 3. Another difference was observed at a higher field where the intensity of the signal at δ 68.6 ppm, belonging to the 6-substituted glucose residue (unit A) decreased and a new signal at δ 69.0 ppm appeared. This may be accounted for by the presence of a second *O*-acetyl group on some of these glucose residues at position 4. Judging by the relative intensities of the signals of the CH_3 of *O*-acetyl groups and of other methyl groups in the ^{13}C-NMR spectrum, the degree of *O*-acetylation of each of the sugar residues does not exceed 20%.

Serological studies

The native and alkali-treated LPS, native and de-*O*-acetylated polysaccharide of *P. penneri* strain 16 precipitated homologous and (*O*-specific polysaccharide) antiserum (Table 4). In a quantitative precipitation test, this antiserum reacted with the *O*-specific polysaccharide giving precipitation curves typical for IgG antibodies. 50 µg polysaccharide completely precipitated antibodies from 50 µl undiluted serum in the equivalence zone.

Monosaccharide 1 and tetrasaccharide 3 showed some inhibitory activity. 0.4 mg tetrasaccharide 3 and 1 mg monosaccharide 1 caused 37% and 42% inhibition, respectively.

LPS from only two out of 20 various *P. penneri* strains, namely those of strains 10 and 18, cross-reacted with *P. penneri* strain 16 anti-(*O*-specific polysaccharide) antiserum (Table 4). Cross-reactions with LPS of strains of *P. mirabilis* O17 and O38 and *P. vulgaris* O17 and O37 were also observed.

Similar results were obtained on SDS/PAGE and Western immunoblot analyses. In SDS/PAGE the above mentioned LPS gave a ladder-like migration pattern which was typical for the S form of LPS containing a varying number of repeating units in the polysaccharide side chain (Figs 8A and 9A). *P. penneri* strain 16 anti-(*O*-specific polysaccharide) antiserum recognized all the LPS and reacted with their *O*-specific parts (Figs 8B and 9B). Only some fast-migrating molecules of *P. penneri* strain 18 LPS, which probably contain a lipid-A-core moiety substituted only by a few *O*-specific repeating units, were recognized by this antiserum. The lowest molecular

mass bands of silver-stained *P. penneri* strain 18 LPS (Fig. 8A, lane 3), which did not react with *P. penneri* strain 16 anti-(*O*-specific polysaccharide) antiserum, seem to consist of lipid A and core unsubstituted by the *O*-specific chain. It is also noteworthy that both slow-migrating and fast-migrating fractions of *P. vulgaris* O17 and *P. mirabilis* O17 LPS were reactive whereas in the case of *P. vulgaris* strain O37 and *P. mirabilis* O38 only fast-migrating fractions reacted which correspond to low-molecular-mass molecules of LPS (Fig. 9).

DISCUSSION

The structure of the *O*-specific polysaccharide of strain 16 is the first described for a new species of *P. penneri*. It was established mainly by a non-destructive method which included one-dimensional and two-dimensional ^{1}H-NMR and ^{13}C-NMR spectroscopy and led to the complete structural determination of the repeating unit, and tentative location of *O*-acetyl groups present in an amount much less than that predicted from the stoichiometry. The structure was confirmed by methylation analysis and selective cleavage of the polysaccharide by solvolysis with anhydrous hydrogen fluoride. The main oligosaccharide product of the solvolysis was identified as oligosyl-(1 → 2)-3-acylamino-3,6-dideoxy-α-galactopyranosyl fluoride 2, which was stable in aqueous solution at room temperature. Formation of an oligosyl-(1 → 2)-α-glucopyranosyl fluoride stable enough to survive under the conditions of an overnight NMR recording at elevated temperature has been observed [16]. This was not the case for oligosyl fluoride 2 and it was converted in to the corresponding stable oligosyl alditol 4 by hydrolysis followed by borohydride reduction of the free oligosaccharide 3 obtained.

The present study showed that an unusual *N*-aminoacyl sugar, discovered formerly in *P. penneri* strain 16 LPS and identified as 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]-D-galactose [4] enters the *O*-specific polysaccharide chain. 3-Acetamido-3,6-dideoxygalactose and the (*R*)-3-hydroxybutyryl group have been found in various bacterial polysaccharides, but to the best of our knowledge this is the first demonstration of the existence of monosaccharide 1 in nature. It easily isolated from the polysaccharide by solvolysis with anhydrous hydrogen fluoride and also, due to the relatively high stability of the amide linkage, by acid hydrolysis (0.1 M hydrochloric acid, 100 °C, 48 h [4]).

The inhibitory activity of monosaccharide 1 in precipitation tests indicated its important role in serological specificity of the *P. penneri O*-specific polysaccharide. The higher inhibitory activity of oligosaccharide 3 may be explained by retention of the neighbouring sugar of monosaccharide 1 and/or by the presence of some other immunodeterminant fragment, for example that related to the lateral glucose residue.

Serological studies revealed the close relatedness of *P. penneri* strains 10, 16 and 18 which correlate with the structures of the *O*-specific polysaccharides established by ^{13}C-NMR spectroscopy. The chemical basis for the observed cross-reactivity of *P. penneri* strain 16 with strains of *P. mirabilis* O17 and O38, as well as *P. vulgaris* O17 and O37 remains obscure, since the structure of their *O*-specific polysaccharides have not been established yet.

This work was supported in part by grant CPBP 04.01. We thank Dr S. N. Senchenkova, Mgr K. Zych, and Mgr M. Lipinska for excellent technical assistance.

REFERENCES

1. Hickman, F. W., Steigerwalt, A. G., Farmer, J. J. III & Brenner, D. J. (1982) *J. Clin. Microbiol. 15*, 1097–1102.
2. List No. 11 (1983) *Int. J. Syst. Bacteriol. 33*, 672–674.
3. Zych, K. & Sidorczyk, Z. (1989) *Carbohydr. Res. 188*, 105–111.
4. Zähringer, U., Moll, H. & Sidorczyk, Z. (1990) Abstract 15th Int. Carbohydrate Symposium, Yokohama, P.B001.
5. Vinogradov, E. V., Kaca, W., Shashkov, A. S., Krajewska-Petrasik, D., Rożalski, A., Knirel, Y. A. & Kochetkov, N. K. (1990) *Eur. J. Biochem. 188*, 645–651.
6. Laemmli, V. K. (1970) *Nature 277*, 680–685.
7. Tsai, C. & Frasch, C. E. (1982) *Anal. Biochem. 119*, 115–119.
8. Bitter, M., Kuppler, P. & Morris, C. F. (1980) *Anal. Biochem. 102*, 458–471.
9. Kotelko, K., Gromska, M., Papierz, M., Sidorczyk, Z., Krayjewska, D. & Szer, K. (1977) *J. Hyg. Epidemiol. Microbiol. Immunol. (Prague) 21*, 277–274.
10. Westphal, O. & Jann, K. (1965) *Methods Carbohydr. Chem. 5*, 83–91.
11. Gmeiner, J. (1975) *Eur. J. Biochem. 58*, 621–626.
12a. Stanek, J., Černy, M., Kocourek, J. & Pácák, J. (1963) *The monosaccharides*, p. 99, Publishing house of the Czechoslovak academy of sciences, Prague.
12b. Stanek, J., Černy, M., Kocourek, J. & Pácák, J. (1963) *The monosaccharides*, p. 498, Publishing house of the Czechoslovak academy of sciences, Prague.
12c. Stanek, J., Černy, M., Kocourek, J. & Pácák, J. (1963) *The monosaccharides*, p. 99, 498, 701, Publishing house of the Czechoslovak academy of sciences, Prague.
13. Hakomori, S. (1964) *J. Biochem. (Tokyo) 55*, 205–208.
14. Waeghe, T. J., Darvill, A. G., McNeil, M. & Albersheim, P. (1983) *Carbohydr. Res. 123*, 281–304.
15. Lipkind, G. M., Shashkov, A. S., Knirel, Y. A., Vinogradov, E. V. & Kochetkov, N. K. (1988) *Carbohydr. Res. 175*, 59–75.
16. Zubkov, V. A. & Gorshkova, R. P. (1990) *Abstr. 15th Int. Carbohydr. Symp.* Yokohama. P.CO47.