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Structure of a new *N*-acetylisomuramic acid-containing O-specific polysaccharide of *Proteus penneri* strains 19 and 35

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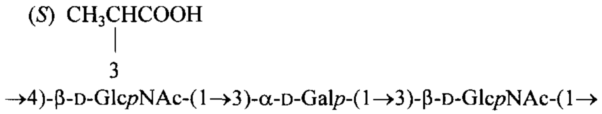
Abstract

O-Specific polysaccharides, together with oligosaccharide products of their degradation, were isolated by GPC after mild acid delipidation of lipopolysaccharides of *Proteus penneri* strains 19 and 35. The polysaccharides had the same trisaccharide repeating unit containing one residue each of D-galactose, 2-acetamido-2-deoxy-D-glucose, and 2-acetamido-3-*O*-[(*S*)-1-carboxyethyl]-2-deoxy-D-glucose (*N*-acetylisomuramic acid). On the basis of 1D and 2D ^{1}H and ^{13}C NMR spectroscopy, including 2D correlation spectroscopy (COSY), rotating-frame NOE spectroscopy (ROESY), and H-detected heteronuclear ^{1}H,^{13}C multiple-quantum coherence (HMQC), the following structure of the repeating unit was established:



$$\begin{array}{l} (S)\ \mathrm{CH_3CHCOOH} \\ \qquad\quad | \\ \qquad\quad 3 \\ \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow \end{array}$$

The oligosaccharide products formed by cleavage of the glycosidic linkage of GlcNAc represent a chemical trisaccharide repeating unit of the polysaccharide and its oligomer homologs. The ease of hydrolysis of the polysaccharide is associated with the closeness of the glycosyl group and the

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lactic acid residue in *N*-acetylisomuramic acid. The polysaccharides studied are structurally related to the O-specific polysaccharides of *P. penneri* strains 62 and 71 studied by us earlier.

Keywords: Proteus; Bacterial polysaccharide, structure; O-Antigen; Lipopolysaccharide, acid degradation; *N*-Acetylisomuramic acid

1. Introduction

Structural studies of O-specific polysaccharide chains of lipopolysaccharides of a number of *Proteus penneri* strains showed that they are acidic due to the presence of hexuronic acids [1–3], their amides with amino acids [4–6], or an ether-linked lactic acid residue [7,8]. In particular, the O-specific polysaccharide of *P. penneri* strain 35 has been reported to include 2-acetamido-4-*O*-[(*S*)-1-carboxyethyl]-2-deoxy-D-glucose [8], an isomer of *N*-acetylmuramic acid. However, later studies revealed that this strain was actually strain 65, taken erroneously as strain 35, and the structure of the strain 65 O-specific polysaccharide containing the sugar mentioned above has been established [1].

In the present paper, we report the structure of the O-specific polysaccharides of the 'true' strain 35 and *P. penneri* strain 19 which have the same trisaccharide repeating unit and, like the O-specific polysaccharide of *P. penneri* strain 62 [7], contain another isomer of *N*-acetylmuramic acid, namely 2-acetamido-3-*O*-[(*S*)-1-carboxyethyl]-2-deoxy-D-glucose (*N*-acetylisomuramic acid).

2. Results and discussion

Lipopolysaccharides were isolated from bacterial cells of *P. penneri* strains 19 and 35 by the phenol–water procedure [9]. Hydrolysis of the lipopolysaccharides with aqueous 1% acetic acid resulted in cleavage of the lipid moiety. GPC on Sephadex G-50 indicated the high heterogeneity of the carbohydrate moiety by molecular weight. Thus, together with the expected polymeric material (O-specific polysaccharide), there were oligosaccharides of higher and lower molecular weight, down to a trisaccharide (see below). Further hydrolysis of higher molecular weight fractions under the same conditions led to lower molecular weight fractions, and, hence, the presence of the latter was, at least partially, due to degradation of the polysaccharide chain during mild acid delipidation of the lipopolysaccharides. In an attempt to avoid the degradation, aqueous 1% acetic acid was replaced by 0.1 M sodium acetate buffer (pH 4.5), but oligosaccharide compounds (more from strain 19 and less from strain 35) were still present in the hydrolysate of the lipopolysaccharides.

Sugar analysis of the isolated polysaccharide fractions, by acid hydrolysis followed by ion-exchange chromatography, revealed the presence of galactose, GlcN, and an acidic amino sugar. GLC of acetylated (*S*)-2-butyl glycosides [10] showed that the galactose and GlcN are D.

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[1] Authors' unpublished data.

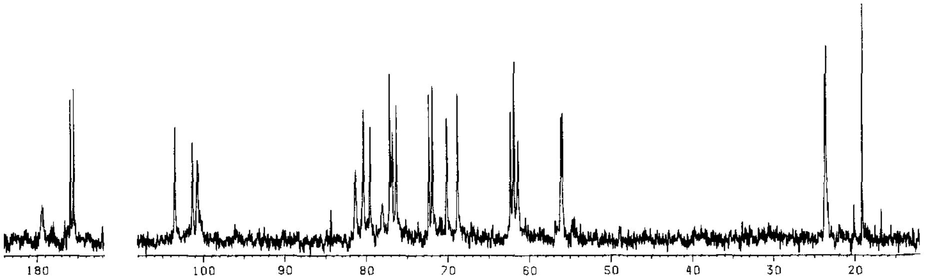


Fig. 1. ^{13}C NMR spectrum of *P. penneri* strain 35 O-specific polysaccharide.

In cation-exchange chromatography, the acidic amino sugar was different from muramic acid but had the same retention time as 2-amino-3-*O*-[(*S*)-1-carboxyethyl]-2-deoxy-D-glucose (isomuramic acid), which had been identified previously as a component of the O-specific polysaccharide of *P. penneri* strain 62 [7]. The structure and configuration of the monosaccharide from the newly studied strains were confirmed by NMR spectroscopic data (see below).

The ^{13}C NMR spectra of the polysaccharides from both strains studied (Fig. 1, Table 1) were practically identical, and further studies were performed with the polysaccharide from strain 19. The spectrum contained signals for three anomeric carbons at δ 103.4, 101.3, and 100.7, three $HOCH_2$–C groups (C-6) at δ 62.3, 61.8, and 61.4, two carbons bearing nitrogen at δ 56.1 and 55.9, two *N*-acetyl groups (Me at δ 23.7 and 23.5, CO at

Table 1
^{13}C NMR chemical shifts (δ in ppm) [a]

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	C-1′	C-2′	C-3′
O-Specific polysaccharide **1**									
→ 4)-β-Sug*p*-(1 →	103.4	56.1 [b]	79.5	78.0	76.3	61.4	179.3	77.1	19.1
→ 3)-α-D-Gal*p*-(1 →	100.7	68.8	80.3	70.1	71.9	61.8			
→ 3)-β-D-Glc*p*NAc-(1 →	101.3	55.9 [b]	81.4	72.3	76.8	62.3			
Trisaccharide **2**									
β-Sug*p*-(1 →	103.5	56.8	84.3	70.7	77.0	61.9 [c]		80.2	20.1
→ 3)-α-D-Gal*p*-(1 →	100.6 [d]	68.8	80.4	70.1	71.9	62.0 [c]			
→ 3)-α-D-Glc*p*NAc	92.4	53.8	78.7	72.7	72.2	61.9 [c]			
→ 3)-β-D-Glc*p*NAc	96.1	56.6	80.8	72.2	77.0	61.9 [c]			
Hexasaccharide **3**									
β-Sug*p*-(1 →	103.5	56.8	84.3	70.7	77.0	61.9 [c]		80.0	20.1
→ 3)-α-D-Gal*p*-(1 →	100.5	68.8	80.4	70.1	71.8	62.0 [c]			
→ 3)-β-D-Glc*p*NAc-(1 →	101.3	55.9 [b]	81.1	72.2	76.9	62.2			
→ 4)-β-Sug*p*-(1 →	103.5	56.0 [b]	79.4	78.0	76.3	61.3		77.1	19.1
→ 3)-α-D-Gal*p*-(1 →	100.5 [d]	68.8	80.4	70.1	71.8	61.9 [c]			
→ 3)-α-D-Glc*p*NAc	92.4	53.7	78.7	72.7	72.2	61.9 [c]			
→ 3)-β-D-Glc*p*NAc	96.1	56.6	80.8	72.2	77.0	61.9 [c]			

[a] Chemical shifts for NAc are δ 23.4–23.7 (Me) and 175.2–175.9 (CO).
[b,c] Assignment could be interchanged.
[d] In the predominant oligosaccharide with α-GlcNAc; 100.3 ppm in the oligosaccharide with β-GlcNAc.

Table 2
^{1}H NMR chemical shifts for the O-specific polysaccharide (δ in ppm) [a]

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	H-2′	H-3′
→ 4)-β-Sug*p*-(1 →	4.75	3.80	3.66	3.90	3.47	3.70	3.58	4.46	1.31
→ 3)-α-D-Gal*p*-(1 →	5.35	3.89	3.82	4.16	3.86	3.72			
→ 3)-β-D-Glc*p*NAc-(1 →	4.57	3.80	3.81	3.62	3.40	3.89	3.71		

[a] Chemical shifts for NAc are δ 2.04 and 2.02.

δ 175.9 and 175.5), C-1 and C-3 of a 1-carboxyethyl group at δ 179.3 and 19.1, respectively (cf. published data [7,8]), and for 10 other sugar ring carbons and C-2 of the 1-carboxyethyl group in the region δ 68.8–81.4.

The ^{1}H NMR spectrum of the polysaccharide contained, inter alia, signals for three anomeric protons at δ 5.35, 4.75, and 4.57, two *N*-acetyl groups at δ 2.04 and 2.02, a 1-carboxyethyl group (H-2 at δ 4.46, H-3 at δ 1.31, cf. published data [7,8]), and for other sugar protons in the region δ 3.4–4.2.

These data suggested that the polysaccharide has a trisaccharide repeating unit containing one residue each of D-galactose, D-GlcNAc, and *N*-acetylisomuramic acid (Sug).

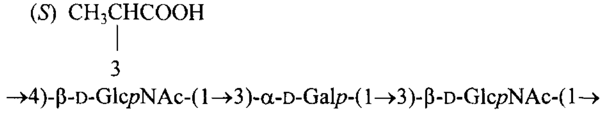
The ^{1}H NMR spectrum of the polysaccharide was assigned using 2D correlation spectroscopy (COSY, Table 2). Coupling constant values $J_{2,3} \approx J_{3,4} \approx J_{4,5}$ 9–10 Hz confirmed the *gluco* configuration of both amino sugar residues [11], including that containing the lactic acid residue. The value $J_{1,2}$ 8.5 Hz showed that GlcNAc and Sug are β-linked, while the value $J_{1,2}$ 3.5 Hz indicated that Gal is α-linked.

A 2D rotating-frame NOE (ROESY) experiment revealed the following correlations between the transglycosidic protons: Sug H-1,Gal H-3 at δ 4.75/3.82, Gal H-1,GlcNAc H-3 at δ 5.35/3.81, and GlcNAc H-1,Sug H-4 at δ 4.57/3.90. These data suggested the positions of substitution and the sequence of the monosaccharide residues in the O-specific polysaccharide as shown below (structure **1**). In addition to the interresidue correlations listed above, intraresidue correlations H-1,H-3 and H-1,H-5 of GlcNAc at δ 4.57/3.81 and 4.57/3.40 and of Sug at δ 4.75/3.66 and 4.75/3.47, respectively, and H-1,H-2 of Gal at δ 5.35/3.89 were observed in accordance with the configurations of the glycosidic linkages of the monosaccharide residues.

With the ^{1}H NMR spectrum assigned, the ^{13}C NMR spectrum of the polysaccharide was interpreted using a 2D H-detected heteronuclear ^{1}H,^{13}C multiple-quantum coherence (HMQC) experiment. The position of the signals for C-2 of both sugars with the *gluco* configuration in the region of carbons bearing nitrogen (δ 56.1 and 55.9) confirmed that both of them are derivatives of GlcN.

The relatively low-field positions of the C-3 signals of GlcNAc and Gal and the C-4 signal of Sug at δ 81.4, 80.3, and 78.0, respectively, as compared with their positions in the ^{13}C NMR spectra of the corresponding nonsubstituted monosaccharides [7,12], confirmed the substitution pattern of the polysaccharide. A low-field position of the C-3 signal of Sug at δ 79.5, as compared with the position of the C-3 signal of β-GlcNAc at δ 74.8 [12], was consistent with the attachment of the lactic residue at position 3 of the sugar moiety. The position of the C-1 signal of Sug at δ 103.4 proved the same absolute configuration of Sug and Gal since C-1 of Sug would resonate near δ 98 [13] in the case of their different configuration.

Therefore, the data obtained allowed assignment of structure **1** for the O-specific polysaccharide of *P. penneri* strains 19 and 35.



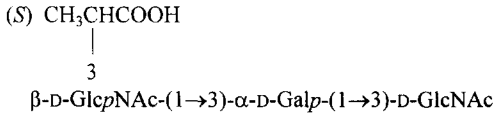
$$\begin{array}{l} \quad\quad\quad (S)\ \mathrm{CH_3CHCOOH} \\ \quad\quad\quad\quad\quad\quad | \\ \quad\quad\quad\quad\quad\quad 3 \\ \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow \end{array}$$

1 (*P. penneri* strains 19 and 35)

The oligosaccharide products of degradation of the lipopolysaccharide of strain 19 with aqueous 1% acetic acid were fractionated on Sephadex G-50 to give four fractions, which were studied by ^{13}C NMR spectroscopy (Table 1).

The fraction eluted last was a trisaccharide representing a chemical repeating unit of the polysaccharide. Its ^{13}C NMR spectrum (Table 1) contained the signals for reducing α- and β-GlcNAc and terminal nonreducing β-Sug. The C-3 and C-4 signals of Sug shifted significantly from δ 79.5 and 78.0 in the spectrum of the polysaccharide to δ 84.3 and 70.7, respectively, in the spectrum of the trisaccharide, which reflected the removal of the glycosyl substituent at position 4 in the latter. It is worth noting that this also influenced the position of the C-2 and C-3 resonances of the lactic acid residue, which appeared at δ 77.1 and 19.1 in the spectrum of the polysaccharide but at δ 80.2 and 20.1, respectively, in the spectrum of the trisaccharide. The signals of Gal had almost the same chemical shifts in both spectra.

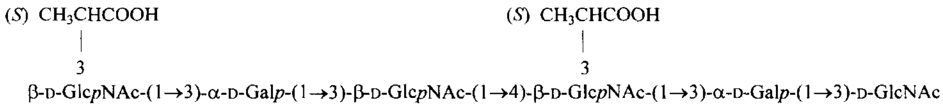
These data suggested that the trisaccharide has the structure **2**.



$$\begin{array}{l} (S)\ \mathrm{CH_3CHCOOH} \\ \quad\quad\quad | \\ \quad\quad\quad 3 \\ \beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-D-GlcNAc} \end{array}$$

2

Comparison of the ^{13}C NMR spectrum of the fraction eluted last but one (Table 1) with the spectra of polysaccharide **1** and the trisaccharide **2** showed that the product is a hexasaccharide, which, like **2**, has GlcNAc at the reducing end and contains one terminal nonreducing and one 4-substituted Sug residue. Hence, this product is a dimer of **2** and has the structure **3**.



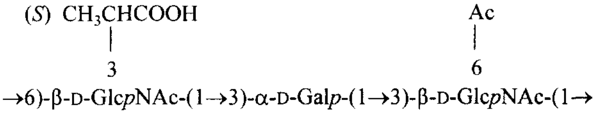
$$\begin{array}{l} (S)\ \mathrm{CH_3CHCOOH} \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad (S)\ \mathrm{CH_3CHCOOH} \\ \quad\quad\quad | \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad | \\ \quad\quad\quad 3 \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad 3 \\ \beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-D-GlcNAc} \end{array}$$

3

Finally, each of two fractions eluted between the polysaccharide and the hexasaccharide **3** appeared to be a mixture of higher oligomer-homologs of the trisaccharide **2**. As judged by the ratio of the signals at δ 20.1 and 19.1, which belong to C-3 of the lactic acid residue attached to the terminal and 4-substituted monosaccharide residues, respectively, a trimer of the trisaccharide **2** was predominant in the lower molecular weight fraction. This fraction also contained the cyclic enterobacterial common antigen, and these data will be published elsewhere.

Therefore, the lower molecular weight products of the degradation of the lipopolysaccharide are oligomer-homologs of the O-antigen trisaccharide repeating unit derived by selective cleavage of the β-(1 → 4) linkage between GlcNAc and *N*-acetylisomuramic acid.

The polysaccharide studied is structurally related to another acidic O-specific polysaccharide, namely, that of *P. penneri* strain 62 (structure **4** [7]). Thus, as mentioned above, the two polysaccharides contained the same acidic amino sugar, *N*-acetylisomuramic acid, which has not been found hitherto in other natural carbohydrates. Moreover, they also have the same two other sugar components (Gal and GlcNAc) and similar structures, which differ only in the position of substitution of *N*-acetylisomuramic acid (at position 4 in strains 19 and 35 or at position 6 in strain 62) and the presence of an *O*-acetyl group in the polysaccharide of strain 62.



$$\rightarrow 6)\text{-}\beta\text{-D-}\overset{\substack{(S)\ \mathrm{CH_3CHCOOH}\\ | \\ 3}}{\mathrm{Glc}p\mathrm{NAc}}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-}\overset{\substack{\mathrm{Ac}\\ | \\ 6}}{\mathrm{Glc}p\mathrm{NAc}}\text{-}(1\rightarrow$$

4 (*P. penneri* strain 62)

No cleavage of the glycosidic linkage of *N*-acetylisomuramic acid occurred on acid hydrolysis of the lipopolysaccharide of *P. penneri* strain 62, thus indicating that the ease of hydrolytic cleavage of the polysaccharides of *P. penneri* strains 19 and 35 is associated with glycosylation of *N*-acetylisomuramic acid with a bulky glycosyl substituent (GlcNAc) at position 4 adjacent to the site of attachment of the acidic group (the lactic acid residue).

Interestingly, the acidic polysaccharide of *P. penneri* strains 19 and 35 (structure **1**) has the same carbohydrate backbone as a neutral O-specific polysaccharide of *P. penneri* strain 71 [2] and differs only in the presence of the lactic acid residue at one of the GlcNAc residues (i.e., by replacement of 2-acetamido-2-deoxyglucose with *N*-acetylisomuramic acid).

Studies of the serological relationships between these *P. penneri* strains will be reported elsewhere.

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[2] Authors' unpublished data.

3. Experimental

General methods.—The ^{1}H and ^{13}C NMR spectra were obtained with a Bruker AM-300 instrument in D_2O at 60 °C. Acetone was used as internal standard (δ_H 2.23, δ_C 31.45). 2D correlation experiments were performed using standard Bruker software. A mixing time of 200 ms was used in a ROESY experiment. A BSV-3 generator was applied to perform an HMQC experiment [14].

GLC was performed using a Hewlett–Packard Model 5890 instrument equipped with a glass capillary column (25 m × 0.2 mm) coated with OV-1 stationary phase. Neutral sugars were identified using a Biotronik LC-2000 sugar analyzer as described [15]. Cation-exchange chromatography was carried out with a Biotronik LC-2000 amino acid analyzer using standard sodium citrate buffers, 0.35 M, pH 5.25 and 0.2 M, pH 3.25 for identification of GlcN and isomuramic acid, respectively.

GPC was carried out on a column (68 cm × 2.6 cm) of Sephadex G-50 in pyridinium acetate buffer (pH 4.5) or a column (1.3 × 75 cm) of TSK HW-40 (S) in water, and monitored using a Knauer differential refractometer.

Bacterial strains, isolation and degradation of lipopolysaccharide.—*P. penneri* strains 19 (CDC-0572-78) and 35 (TGH 2200) were kindly provided by Professor J. Brenner (Center for Disease Control, Atlanta, USA). Dry bacteria were obtained from aerated liquid cultures as described [16]. Crude lipopolysaccharide preparations obtained after extraction of bacterial mass with a hot phenol–water mixture [9] were purified by treatment with cold aq 50% CCl_3CO_2H. Degradation of LPS was performed with 0.1 M NaOAc–AcOH (pH 4.5) at 100 °C for 4 h or with aq 1% AcOH at 100 °C for 2 h, and products were fractionated by GPC on Sephadex G-50. Oligosaccharides **2** and **3** were purified by GPC on TSK HW-40.

Sugar analysis.—For sugar analysis, the polysaccharide (1 mg) was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h), the hydrolysate was evaporated to dryness, sugars were conventionally reduced with $NaBH_4$ or converted into (*S*)-2-butyl glycosides [10], acetylated, and analyzed by GLC. A portion of the hydrolysate was analyzed using sugar and amino acid analyzers.

Acknowledgements

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