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# Structure of a new ribitol teichoic acid-like O-polysaccharide of a serologically separate *Proteus vulgaris* strain, TG 276-1, classified into a new *Proteus* serogroup O53

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**Abstract**—An unusual ribitol teichoic acid-like O-polysaccharide was isolated by mild acid degradation of the lipopolysaccharide from a previously non-classified *Proteus vulgaris* strain TG 276-1. Structural studies using chemical analyses and 2D $^{1}$H and $^{13}$C NMR spectroscopy showed that the polysaccharide is a zwitterionic polymer with a repeating unit containing 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose (D-FucNAc4N) and two D-ribitol phosphate (D-Rib-ol-5-*P*) residues and having the following structure:

→3)-β-D-Fuc*p*NAc4N-(1→1)-D-Rib-ol-5-*P*-(O→1)-D-Rib-ol-5-*P*-(O→,

where the non-glycosylated ribitol residue is randomly mono-O-acetylated. Based on the unique O-polysaccharide structure and the finding that the strain studied is serologically separate among *Proteus* bacteria, we propose to classify *P. vulgaris* strain TG 276-1 into a new *Proteus* serogroup, O53.


*Keywords:* *Proteus vulgaris*; Ribitol phosphate; Serological classification; Deamination; NMR spectroscopy

## 1. Introduction

Gram-negative bacteria of the genus *Proteus* from the family *Enterobacteriaceae* are opportunistic pathogens that cause urinary tract infections, the most frequently diagnosed kidney and urological disorders. These bacteria are seen predominantly in hospitalized patients, who may have received antibiotics or instrumentation, including catheterization, of the urinary tract.[1] In the genus *Proteus,* there are four clinically important named species: *P. mirabilis*, *P. vulgaris*, *P. penneri* and *P. hauseri* as well as three unnamed genomospecies 4, 5 and 6.[2,3] Virulence factors and properties of *Proteus* sp. that mediate the infectious process are swarming phenomenon, adherence due to the fimbriae or glycocalyx, flagella, hemolysins, invasiveness, enzymes, including proteases and ureases, capsular polysaccharide, and outer membrane lipopolysaccharide (LPS, endotoxin).[4]

As in other Gram-negative bacteria, the O-specificity of *Proteus* is defined by the structure of the carbohydrate moiety (O-polysaccharide, O-antigen) of the LPS. Based on the O-antigens, strains of two species, *P. mirabilis* and *P. vulgaris*, have been classified into up to 60 O-serogroups.[5–7] Recently, we have established

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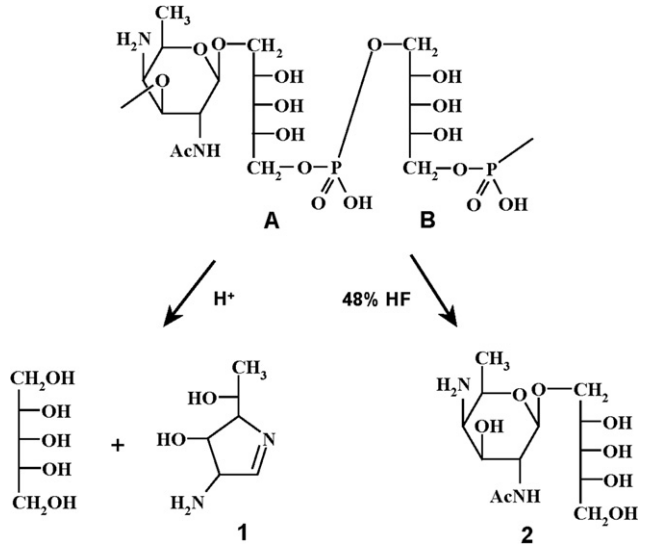
the O-polysaccharide structures of most classified and a number of non-classified strains of these and other *Proteus* species and, based on structural and serological data, proposed some 15 more O-serogroups.[8–13] Now we report on a new O-polysaccharide structure of a previously non-classified serologically separate *P. vulgaris* strain, which is a candidate for a further new *Proteus* O-serogroup.

## 2. Results and discussion

### 2.1. Elucidation of the O-polysaccharide structure

The O-polysaccharide was obtained by mild acid hydrolysis of the LPS, isolated from dried bacterial cells of *P. vulgaris* TG 276-1 by the phenol–water procedure,[14] followed by GPC on Sephadex G-50. Full acid hydrolysis of the polysaccharide resulted in ribitol, which was identified by GLC after acetylation. Further studies showed that the polysaccharide also contains 2-acetamido-4-amino-2,4,6-trideoxygalactose (FucNAc4N), but no FucN4N could be detected in the hydrolysate owing to full destruction of this acid-labile monosaccharide.[15] Instead, an amino compound with a high retention time on a cation-exchange resin was detected in amino acid analysis, which was isolated by cation-exchange chromatography. Its MALDI-TOF mass spectrum showed a pseudomolecular $[M+H]^+$ ion peak at $m/z$ 145.15, which may belong to a FucN4N degradation product **1** (calculated molecular mass 144.09 Da), shown in Scheme 1. In addition, there were mass peaks in the region $m/z$ 317–393 for putative products of polymerization of **1** or/and other primary degradation product(s).




**Scheme 1.** Structure and degradations of the O-polysaccharide of *P. vulgaris* TG 276-1 (O53) and its degradation products (**1**, **2**).

The $^{13}C$ NMR spectrum of the O-polysaccharide (Fig. 1A) indicated an irregularity, most likely, owing to non-stoichiometric O-acetylation as there were signals for *O*-acetyl groups at $\delta$ 21.7–21.8 ($CH_3$) and 174.5 (CO). Indeed, the spectrum of the O-deacetylated polysaccharide (Fig. 1B) showed a pattern typical of a regular polymer. It contains signals for one mono-N-acetylated 6-deoxydiaminohexose (FucN4N), including those for an anomeric carbon (C-1) at $\delta$ 102.4, $CH_3$–C group (C-6) at $\delta$ 16.8, two nitrogen-bearing carbons at $\delta$ 52.4 and 55.2 and an *N*-acetyl group at $\delta$ 23.6 ($CH_3$) and 176.2 (CO). In addition, there were 10 more signals, from which four signals at $\delta$ 67.7–68.0 and 71.9 belonged to O$CH_2$–C groups (data of a DEPT-135 experiment). Taking into account the GLC analysis data (see above), these signals could be assigned to C-1 and C-5 of two ribitol residues. The $^1H$ NMR spectrum showed, inter alia, signals for an anomeric proton (H-1) at $\delta$ 4.64 and $CH_3$–C group (H-6) at $\delta$ 1.36 of FucN4N as well as one *N*-acetyl group at $\delta$ 2.08. The $^{31}P$ NMR spectrum contained two signals for monophosphate groups at $\delta$ −0.29 and 1.59.

The $^1H$ and $^{13}C$ NMR spectra of the O-deacetylated polysaccharide were assigned (Table 1) using 2D $^1H$, $^1H$ COSY, TOCSY and $^1H$, $^{13}C$ HSQC spectroscopy. The monosaccharide was identified as 2,4-diamino-2,4,6-trideoxy-β-galactose (β-FucN4N) based on $^1H$, $^1H$ coupling constants characteristic of the β-*galacto* configuration ($J_{1,2}$ 8.5, $J_{2,3}$ 10.5, $J_{3,4}$ 3.2, $J_{4,5}$ <2, $J_{5,6}$ 6.6 Hz) and correlations in the HSQC spectrum of the protons at the nitrogen-bearing carbons (H-2 and H-4) to the corresponding carbons (C-2 and C-4) at $\delta$ 52.4 and 55.2. The NMR data also confirmed the presence of two pentitol (ribitol) residues. Asymmetric chemical shift patterns of both residues excluded the occurrence of 1,5-poly(ribitol phosphate) as a separate chain or a block(s), which would have a symmetric pattern (compare published data[16] shown in Table 1).

The 2D ROESY spectrum of the O-deacetylated polysaccharide showed a correlation between H-1 of FucN4N and H-1a,1b of one of the ribitol residues (unit **A**) at $\delta$ 4.64/3.85 and 4.64/3.96. This finding indicated glycosylation of unit **A** at position 1, which was confirmed by a downfield displacement, due to an α-effect of glycosylation, of the C-1 signal from $\delta$ 62.8 in non-substituted ribitol[16] to $\delta$ 71.9 in unit **A**.

The C-5 signal of unit **A** as well as C-1 and C-5 signals of the other ribitol residue (unit **B**) were also shifted downfield but to a lesser degree (to $\delta$ 67.7–68.0) due to phosphorylation (Table 1). A similar shift from $\delta$ 68.9 (Table 1) to 73.3 was observed also for the C-3 signal of FucN4N, thus suggesting the presence of a phosphate group at position 3 of the sugar moiety. Accordingly, the H-3 signal of FucN4N at $\delta$ 4.50 stood apart from other signals in the low-field region of the $^1H$ NMR spectrum and was additionally split by coupling to phos-

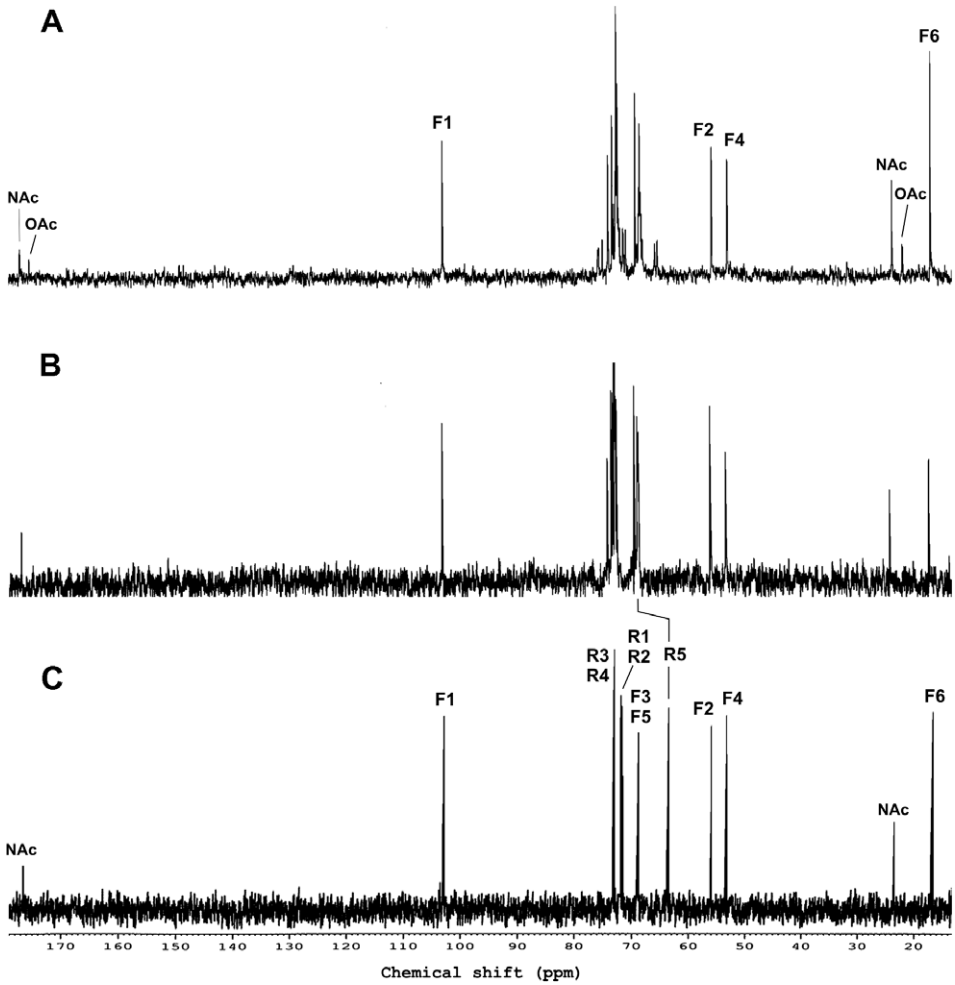



**Figure 1.** $^{13}C$ NMR spectra of the O-polysaccharide of *P. vulgaris* TG 276-1 (A), O-deacetylated polysaccharide (B) and glycoside **2** (C). Arabic numerals refer to carbons in FucNAc4N (F) and ribitol unit **A** (R).

phorus. The phosphorylation pattern in the repeating unit was confirmed by the $^{1}H$, $^{31}P$ HMQC spectrum, which showed correlations of the phosphate group at $\delta$ 1.59 with H-5a,5b of unit **A** and H-1a,1b of unit **B** and that at $\delta$ −0.29 with H-5a,5b of unit **B** and H-3 of FucN4N. Therefore, one phosphate group 1,5-interlinks two ribitol residues and the other links ribitol residue **B** to O-3 of FucN4N.

Full dephosphorylation of the O-polysaccharide with aq 48% HF resulted in glycoside **2** (Scheme 1) isolated by GPC on Sephadex G-25. The expected structure of **2** was confirmed by the MALDI-TOF mass spectrum, which showed an $[M+H]^+$ ion peak at *m*/*z* 339.0 (calculated molecular mass 338.2 Da), and by NMR spectroscopy as described above for the O-deacetylated polysaccharide (for the $^{13}C$ NMR spectrum see Fig. 1C; assignments of $^{1}H$ and $^{13}C$ NMR chemical shifts are given in Table 1). Deamination of glycoside **2** with $HNO_2$ followed by acid hydrolysis afforded, among other products, 2-amino-2,6-dideoxy-D-glucose (D-quinovosamine),[15] which was identified using amino acid analyzer and GLC of the acetylated (*R*)-2-octyl glycosides. Hence, FucN4N is mono-N-acetylated at position 2 and has the D configuration. Interestingly, FucN4N occurs rarely in bacterial polysaccharides but, when present,[17] is mono-N-acetylated at position 2 and is D. A report[18] that FucNAc4NMe in the LPS of *Bordetella pertussis* is L requires an additional confirmation.

The absolute configuration of ribitol in glycoside **2** was established by oxidation using 2,2,6,6-tetramethyl-1-piperidinyloxy radical (TEMPO) followed by acid hydrolysis and identification of the resultant L-ribonic acid by GLC of the acetylated (*R*)-2-octyl ester.[19] As ribitol has the D configuration by definition,[20] this finding indicates that D-ribitol in **2** (unit **A**) is glycosylated at position 1. The absolute configuration of the other ribitol residue **B** in the polysaccharide was not confirmed.

When dephosphorylation of the polysaccharide was performed for a shorter time, glycoside **2** was accompanied by a higher-molecular-mass product. MALDI-TOF

**Table 1.** $^{1}H$ and $^{13}C$ NMR data ($\delta$, ppm)

| Sugar residue | Nucleus | 1 (1a,1b) | 2 | 3 | 4 | 5 (5a,5b) | 6 | $CH_3CON$ | $CH_3CON$ |
|---|---|---|---|---|---|---|---|---|---|
| *Glycoside* **2** | | | | | | | | | |
| β-D-Fuc*p*NAc4N-(1→ | $^{1}H$ | 4.57 | 3.79 | 4.09 | 3.61 | 4.06 | 1.36 | 2.06 | |
| | $^{13}C$ | 102.8 | 53.4 | 68.9 | 56.0 | 68.8 | 16.8 | 23.5 | 176.4 |
| →1)-D-Rib-ol (**A**) | $^{1}H$ | 3.83, 3.97 | 3.94 | 3.69 | 3.80 | 3.65, 3.79 | | | |
| | $^{13}C$ | 72.0 | 71.8 | 73.2 | 73.2 | 63.7 | | | |
| *O-Deacetylated polysaccharide* | | | | | | | | | |
| →*P*-(O→3)-β-D-Fuc*p*NAc4N-(1→ | $^{1}H$ | 4.64 | 3.94 | 4.50 | 3.83 | 4.09 | 1.36 | 2.08 | |
| | $^{13}C$ | 102.4 | 52.4[a] | 73.3[a] | 55.2 | 68.5 | 16.8 | 23.6 | 176.2 |
| →1)-D-Rib-ol-5→*P*-(O→ (**A**) | $^{1}H$ | 3.85, 3.96 | 3.97 | 3.75 | 3.97 | 4.01, 4.11 | | | |
| | $^{13}C$ | 71.9[a] | 71.6 | 72.7 | 72.0[a] | 67.7[a] | | | |
| →*P*-(O→1)-D-Rib-ol-5-*P*-(O→ (**B**) | $^{1}H$ | 3.96, 4.07 | 3.90 | 3.79 | 3.98 | 3.99, 4.09 | | | |
| | $^{13}C$ | 68.0[a] | 71.9[a] | 72.4 | 72.1[a] | 67.8[a] | | | |
| *1,5-Poly(ribitol phosphate)*[16] | | | | | | | | | |
| →*P*-(O→1)-D-Rib-ol-5-*P*-(O→ | $^{13}C$ | 68.1 | 72.1 | 72.8 | 72.1 | 68.1 | | | |
| *O-Polysaccharide*[b] | | | | | | | | | |
| →*P*-(O→1)-D-Rib-ol2Ac-5-*P*-(O→ | $^{1}H$ | 4.14, 4.17 | 5.29 | 3.94 | | | | | |
| | $^{13}C$ | 64.7 | 75.0[c] | 70.6[d] | | | | | |
| →*P*-(O→1)-D-Rib-ol3Ac-5-*P*-(O→ | $^{1}H$ | | 4.16 | 5.07 | 4.16 | | | | |
| | $^{13}C$ | | 70.3 | 74.4 | 70.3 | | | | |
| →*P*-(O→1)-D-Rib-ol4Ac-5-*P*-(O→ | $^{1}H$ | | | 3.94 | 5.29 | 4.09, 4.16 | | | |
| | $^{13}C$ | | | 70.8[d] | 75.2[c] | 65.1 | | | |

[a] The carbon signal is split owing to a coupling with phosphorus.
[b] Data of the atoms at the α- and β-positions to the O-acetylation sites in ribitol unit **B**.
[c–d] Assignment could be interchanged.

mass spectrum of the latter contains $[M+H]^+$ ion peaks at *m*/*z* 553.0 (major) and 595.1 (minor) for compounds containing, as compared to **2**, an additional ribitol phosphate or mono-O-acetylated ribitol phosphate (calculated molecular masses 552.2 and 594.2 Da, respectively). This finding further confirmed that both glycosylated (unit **A**) and non-glycosylated (unit **B**) ribitol residues enter into the same polysaccharide chain.

The data obtained showed that the O-deacetylated polysaccharide has the structure shown in Scheme 1.

In the $^{1}H$, $^{13}C$ HSQC spectrum of the initial O-polysaccharide, three cross-peaks for H-2,C-2; H-3,C-3 and H-4,C-4 of the ribitol unit **B** were partially shifted downfield by 1.28–1.39 and 2.0–3.1 ppm in the $^{1}H$ and $^{13}C$ dimensions, respectively, as compared with their positions in the spectrum of the O-deacetylated polysaccharide (Table 1). These displacements were due to a deshielding effect of O-acetylation and indicated the presence of *O*-acetyl groups at positions 2, 3 and 4 of unit **B** in non-stoichiometric quantities. This finding was confirmed by an observation that the signals for the carbons that are neighbouring to the O-acetylation sites in unit **B** were shifted upfield by 1.6–3.3 ppm (Table 1), which is typical of β-effects of O-acetylation.[21] Tracing connectivities in the $^{1}H$, $^{1}H$ COSY and TOCSY spectra of the initial polysaccharide starting from protons at the O-acetylation sites (H-2, H-3 and H-4) showed the occurrence of only mono-O-acetylated forms of unit **B**. Comparison of the HSQC spectra of the initial and O-deacetylated polysaccharides showed no significant displacements of the NMR signals of the ribitol unit **A** and FucNAc4N moiety, which therefore are not O-acetylated. As judged by the integral intensities of the signals of the O-acetylated and non-acetylated forms of unit **B** in the $^{13}C$ NMR spectrum, the degree of O-acetylation at each position is 20–25%.

### 2.2. Serological studies of the lipopolysaccharide

Polyclonal rabbit O-antiserum against *P. vulgaris* TG 276-1 was tested with LPS from 94 strains representing all *Proteus* O-serogroups. From them, only the homologous LPS reacted in passive immunohemolysis and enzyme immunosorbent assay with reciprocal titres 51,200 and 512,000, respectively, whereas no cross-reaction was observed. Both LPS and alkali-treated LPS inhibited the reaction in both assays (minimal inhibitory dose 0.5–1.0 ng). In Western blot with anti-*P. vulgaris* TG 276-1 O-antiserum, the homologous LPS showed a typical banding pattern (not shown). These data demonstrated a high specificity of the reaction in the homologous system.

The serological behaviour of the LPS is consistent with the O-antigen structure of *P. vulgaris* TG 276-1, which is unique among the known structures of bacterial polysaccharides. Based on these data, we propose to classify *P. vulgaris* strain TG 276-1 into a new, separate *Proteus* serogroup, O53, in which this strain is at present the single representative.

## 3. Experimental

### 3.1. Bacterial strains and growth

*P. vulgaris* TG 276-1 was kindly provided by J. Penner (Department of Medical Genetics, University of Ontario, Canada). Thirty-nine strains of *P. mirabilis* and 27 strains of *P. vulgaris* were from the Czech National Collection of Type Cultures (CNCTC, National Institute of Public Health, Prague). *P. myxofaciens* CCUG 18769 was kindly provided by E. Falsen from the Cultures Collection University of Goeteborg (CCUG, Goeteborg, Sweden). *Proteus* genomospecies 4 (ATCC 51469) and 24 strains of *P. penneri* were kindly provided by C. M. O'Hara and D. J. Brenner (Centre for Disease Control and Prevention, Atlanta, GA).

*P. vulgaris* TG 276-1 was grown under aerobic conditions on nutrient broth (BTL, Łódź, Poland). Dry bacterial mass was obtained from an aerated, liquid culture as described.[22]

### 3.2. Isolation and degradation of the lipopolysaccharide

LPS was obtained by extraction of bacterial mass with phenol–water mixture[14] and purified by treatment with cold aq 50% $CCl_3CO_2H$ followed by dialysis of the supernatant.[22]

Acid degradation of the LPS was performed with 1% aq HOAc at 100 °C for 2 h until lipid A precipitation. The precipitate was removed by centrifugation (13,000*g*, 20 min), and the supernatant was fractionated on a column (56 × 2.6 cm) of Sephadex G-50 (Amersham Biosciences, Sweden) in 0.05 M pyridinium acetate buffer, pH 4.5, monitored using a differential refractometer (Knauer, Germany). A high-molecular-mass polysaccharide was obtained in a yield of 12% of the LPS weight.

### 3.3. O-Deacetylation and dephosphorylation of the polysaccharide

A polysaccharide sample (15 mg) was treated with 12% aq ammonia for 3 h at 50 °C, after evaporation of the solution an O-deacetylated polysaccharide was isolated by GPC on a column (80 × 1.6 cm) of TSK HW-50 (Merck, Germany) in 0.1% aq HOAc monitored as above.

Another portion of the polysaccharide (15 mg) was dephosphorylated with aq 48% HF for 16 h at 5 °C, HF was flushed out by a stream of air and glycoside **2** was isolated by GPC on a column (130 × 2.6 cm) of Sephadex G-25 in 0.1% aq HOAc. Partial dephosphorylation with aq 48% HF for 1 h at 18 °C resulted in a mixture of **2** and a compound containing FucNAc4N and two ribitol residues, which was separated by GPC on the same column (elution volumes 205 and 170 mL, respectively).

### 3.4. Chemical analyses

Polysaccharide samples (0.5 mg each) were hydrolyzed with 2 M $CF_3CO_2H$ (120 °C, 2 h) or 4 M $CF_3CO_2H$ (100 °C, 2 h), either acetylated with an $Ac_2O$–pyridine mixture (1:1) and analyzed by GLC on an HP-5ms column (25 m × 0.25 mm) using a Hewlett-Packard 5890 instrument (USA) and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C $min^{-1}$ or studied using a Biotronik LC-2000 amino acid analyzer (Germany) equipped with a column (10 × 0.5 cm) with an Ostion LG AN B cation-exchange resin in 0.2 M sodium citrate buffer, pH 4.2, and 0.35 or 0.7 M sodium citrate buffer, pH 5.3. An amino component was isolated from the hydrolysate (from 5 mg polysaccharide) by cation-exchange chromatography on the same column in 0.7 M sodium citrate buffer, pH 5.3 (retention time 1.74 related to ammonia), desalted using sequentially 1-mL columns of a Dowex AG1 × 2 anion-exchange resin ($HCO_3^-$-form, elution with water) and a Dowex 50 × 2 cation-exchange resin ($H^+$-form), from which amino compounds were eluted with 0.5% aq ammonia.

Glycoside **2** (5 mg) was deaminated with a solution of $NaNO_2$ (15 mg) in aq 10% HOAc (0.5 mL) for 2 h at 18 °C, the product was isolated by GPC on Sephadex G-25 and applied to a 1-mL column of Dowex 50 × 2 ($H^+$-form). The desired neutral compound (2.1 mg) eluted with water was hydrolyzed with 4 M $CF_3CO_2H$ (110 °C, 2 h), a portion of the hydrolysate was analyzed using an amino acid analyzer in 0.35 M sodium citrate buffer, pH 5.3, to reveal quinovosamine. Another portion was N-acetylated with $Ac_2O$ (30 μL) in a mixture of aq 0.05% ammonia (40 μL) and methanol (10 μL) for 2 h at 20 °C to give *N*-acetylquinovosamine, which was converted into acetylated (*R*)-2-octyl glycosides[23,24] and analyzed by GLC using reference samples of the corresponding (*R*)- and (*RS*)-2-octyl glycosides.

Glycoside **2** (3 mg) was oxidized using 2,2,6,6-tetramethyl-1-piperidinyloxy radical (TEMPO) as described.[25] After hydrolysis with 2 M $CF_3CO_2H$ (0.2 mL, 100 °C, 1 h), ribonic acid was esterified with (*R*)-2-octanol (0.2 mL) in the presence of $CF_3CO_2H$ (30 μL) at 100 °C for 16 h, acetylated with a mixture (1:1, v/v) of acetic anhydride and pyridine (0.2 mL, 100 °C, 1 h), and analyzed by GLC as described above using reference samples derived from D-ribonic acid and (*R*)- and (*RS*)-2-octanol.

### 3.5. NMR spectroscopy and mass spectrometry

NMR spectra were recorded with a Bruker DRX-500 spectrometer for solutions in $D_2O$ at 30 °C using internal acetone ($\delta_H$ 2.225, $\delta_C$ 31.45) as reference. Standard

Bruker software (XWINNMR 2.6) was used to acquire and maintain the NMR data. A mixing time of 200 and 100 ms was used in 2D TOCSY and ROESY experiments, respectively.

Positive ion MALDI-TOF mass spectrometry was performed on an Ultra Flex instrument (Bruker Daltonics, Germany) using 3,5-dihydroxybenzoic acid as matrix.

### 3.6. Rabbit antiserum and serological assays

Polyclonal O-antiserum against *P. vulgaris* TG 276-1 was obtained by immunization of rabbits with bacterial suspension according to the published procedure.[26] Passive immunohemolysis, enzyme immunosorbent assay, absorption experiments, SDS-PAGE (using 12% acrylamide), electrotransfer of LPS from gel to nitrocellulose sheets and immunostaining were performed as described.[27]

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