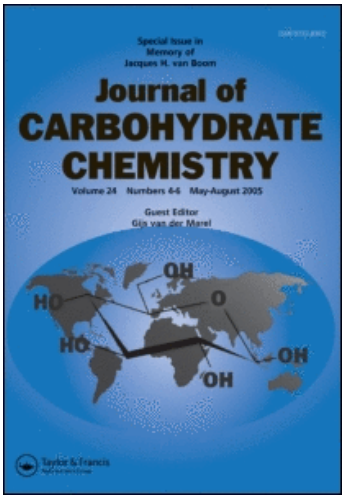

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REVIEW ARTICLE

STRUCTURAL STUDY OF O-SPECIFIC POLYSACCHARIDES OF *PROTEUS*

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Table of Contents

1. Introduction

Proteus bacteria are important human opportunistic pathogens which frequently cause urinary tract infections. According to *Bergey's Manual of Systematic Bacteriology*,[1] this genus includes three species: *P. mirabilis*, *P. vulgaris*, and *P. myxofaciens*. A novel species of *P. penneri* has been recently proposed[2,3] for strains formerly called *P. vulgaris* biogroup I. *Proteus* is an antigenically heterogeneous group of bacteria, and this is mainly associated with diverse composition and structures of O-specific polysaccharide chains of outer-membrane lipopolysaccharides (O-antigens). The Kauffman-Perch serological classification[4] of *P. mirabilis* and *P. vulgaris* includes 49 O-serogroups. However, a number of S-strains remain unclassified,[5] including strains of *P. penneri*.

Chemical studies of *P. mirabilis* and *P. vulgaris* lipopolysaccharides, undertaken from the late 1960s to the 1980s (review[6]), led to identification of glucose, galactose, L-*glycero*-D-*manno*-heptose and D-*glycero*-D-*manno*-heptose, glucosamine, galactosamine, glucuronic, galacturonic, and 3-deoxyoctulosonic acids as being among the carbohydrate units of the core oligosaccharide or, except for heptoses and 3-deoxyoctulosonic acid, as being part of both the core and O-specific polysaccharide units. Additionally, rhamnose, ribitol, lysine, and phosphate were identified as constituents of O-antigens. Lipopolysaccharides of *P. penneri* were shown to be similar to those of *P. mirabilis* and *P. vulgaris* in chemical composition.[7] More detailed data on O-specific polysaccharides of *Proteus* were restricted to partial structures of two non-serotyped strains of *P. mirabilis* 1959[8,9] and D52.[10]

Our systematic chemical and immunochemical studies of *Proteus* lipopolysaccharides, in particular those having medical importance, aim at correlation of structures of their O-specific polysaccharide chains with immunospecificity of strains. We describe in this review the composition and

structures of seventeen O-antigens of *P. mirabilis*, *P. vulgaris*, and *P. penneri*.[11-21]

2. NMR Studies of O-Specific Polysaccharides

NMR spectroscopy was widely used in our studies of both composition and structures of *Proteus* O-antigens. Primary analysis of the ^{13}C NMR spectra of these heteropolysaccharides allowed us to determine the total number of monosaccharide residues in the repeating unit, their structures, and the structures of the non-sugar components. For example, appearance of characteristic signals in the spectra indicated the presence of amino sugars (C-2 or C-3 linked to N at 49-58 ppm), 6-deoxy sugars (C-6 at 16-19 ppm), amides of uronic acids with amino acids (C-6 of the former at 170-172 ppm, C-1 of the latter at 176-178 ppm), *O*-acetyl groups (CH_3 at 21-22 ppm), a residue of ether-linked lactic acid (CH_3 at 19-21 ppm, COOH at 180-183 ppm), 2-aminoethyl phosphate (C-1 at 63-64 ppm, C-2 at 41-42 ppm) etc.

Non-stoichiometric *O*-acetylation, occurring in several O-antigens, masked their regularity and complicated interpretation of the ^{13}C NMR data. *O*-Acetyl groups could easily be removed by treatment with aqueous ammonia to give the corresponding *O*-deacetylated polysaccharides (see **1** to **2** below) used in further structural analysis [cf., e.g., the ^{13}C NMR spectrum of the O-specific polysaccharide of *P. mirabilis* 3/6 (**1**) containing partially *O*-acetylated residues of GlcNAc and GalA and that of the corresponding *O*-deacetylated polysaccharide (**2**), FIG. 1].

Unambiguous assignment of the signals in the ^1H NMR spectra of the O-antigens allowed identification of the constituent sugar residues (including their anomeric configuration) on the basis of coupling constants values and made possible application of NOE spectroscopy for linkage and sequence analysis. The majority of the spectra were resolved well enough to allow assignment by using sequential, selec-

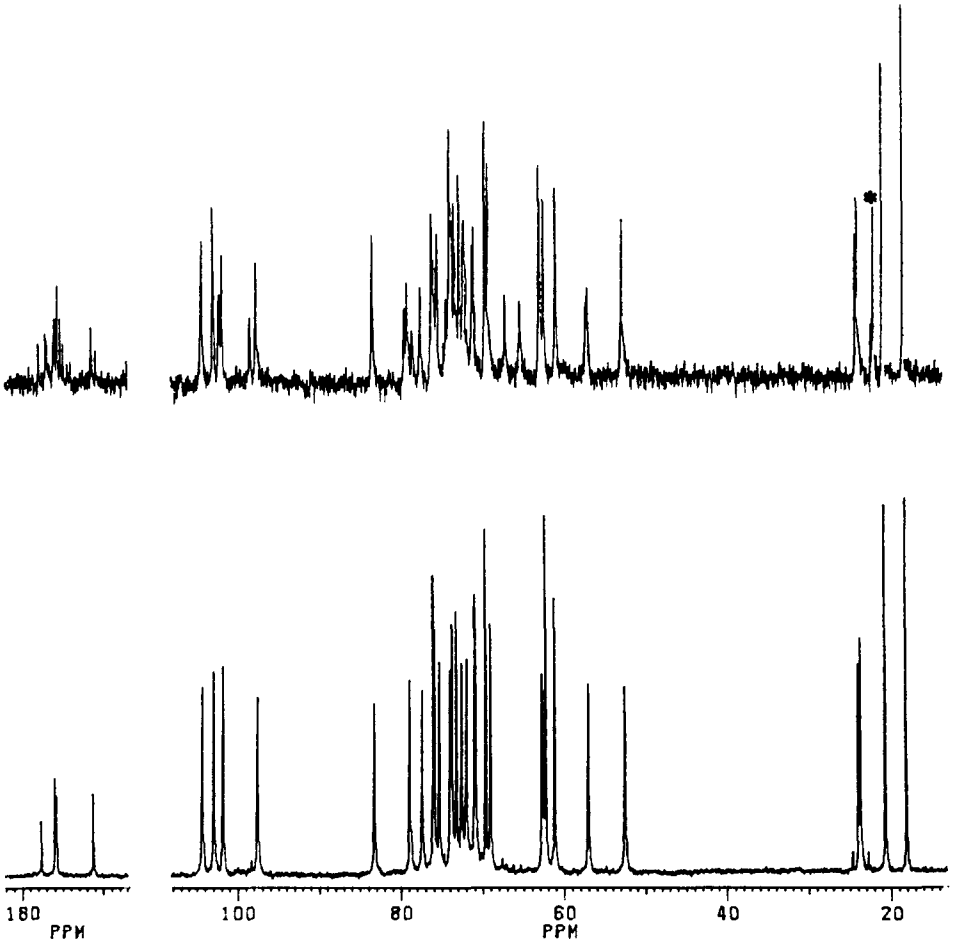
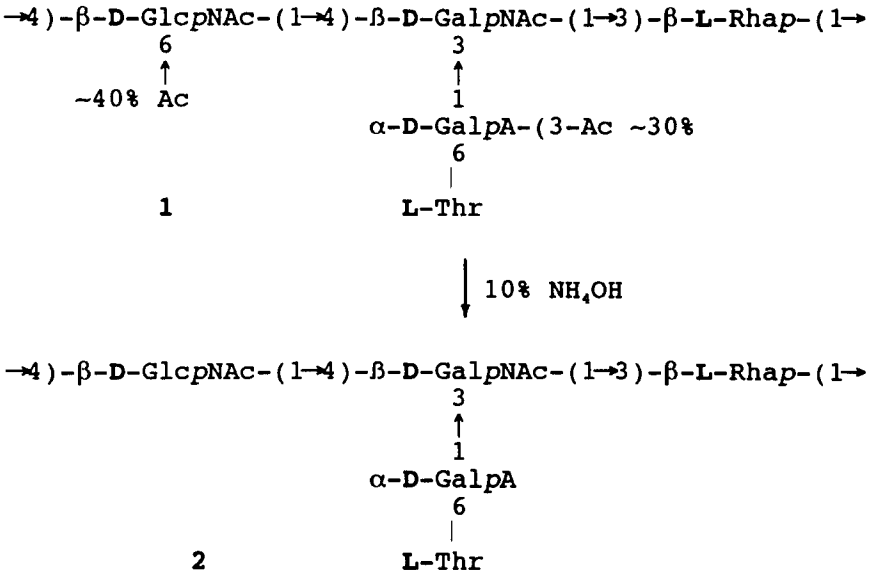


FIG. 1. 75 MHz ^{13}C NMR spectra of the *P. mirabilis* 3/6 O-specific polysaccharide (structure **1**, top curve) and the corresponding *O*-deacetylated polysaccharide (structure **2**, bottom curve). The signal for methyl of the *O*-acetyl group is marked with an asterisk.

tive spin-decoupling, 1D total correlation (TOCSY) and 2D shift-correlated (COSY) spectroscopy.

The procedure of selective spin-decoupling performed in the difference mode[22] was specially modified by us for complex polysaccharides using a decoupling power less by 5-10 times that necessary for full spin decoupling in order to increase



the selectivity. As a result, only the initial (but not decoupled) view of the signals of protons coupled with the irradiated signal was clearly observed in the difference spectrum.

In TOCSY spectroscopy selective excitation of each of the anomeric protons with migration of magnetization through the entire coupled spin system[23] led to isolation of the subspectra of the corresponding monosaccharides. Magnetization, however, could not be transferred when a coupling constant was too small. Thus, subspectra of the residues with the *gluco* configuration contained the signals for all protons whereas those of the sugar units with the *galacto* configuration lacked the signals for H-5 and H-6 owing to a small $J_{4,5}$ (<1 Hz).

Another problem associated with TOCSY spectroscopy was assignment of similar multiplets, for example, those of H-3 and H-4 in D-glucopyranose or H-2 and H-3 in α-D-galactopyranose. These protons were distinguished by simple tracing connectivities using COSY experiments. When a number of correlation peaks of non-anomeric protons were close to the diagonal and complicated or made impossible interpretation of

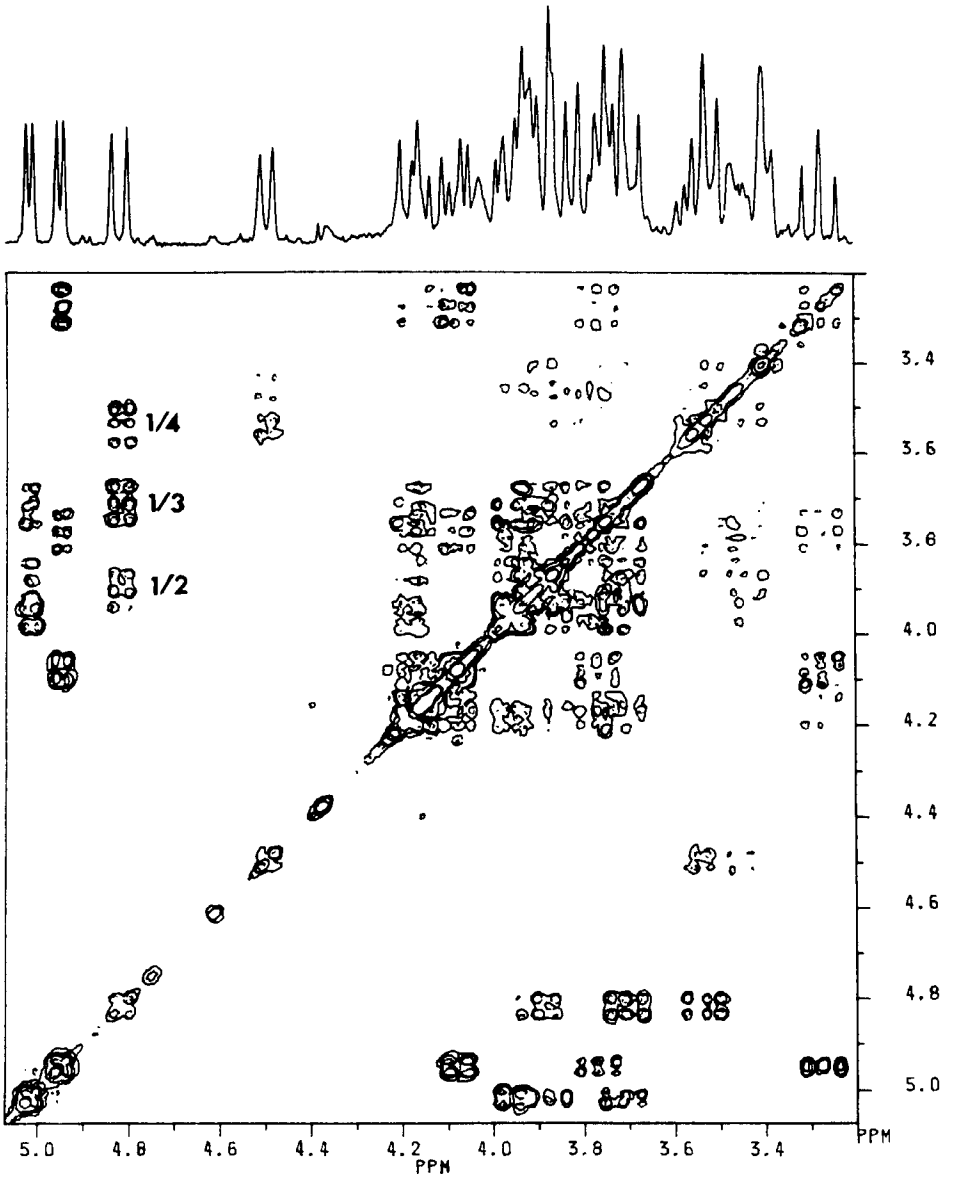


FIG. 2. 250 MHz 2D shift-correlated (COSY) spectrum of the *O*-deacetylated polysaccharide from *P. vulgaris* 5/43 (structure **26**). The respective 1D spectrum is displayed along the F_2 axis. As an example, cross-peaks showing the connectivities of H-1 and several other protons of the ß-D-Glc*p*NAc residue are labeled in the 2D spectrum.

the COSY spectra, COSY with one- or two-step relayed coherence transfer (COSY-RCT) was applied and allowed correlation of H-1 to non-coupled H-3 or both H-3 and H-4 using H-2 or H-2 and H-3, respectively, as mediators.[23] Information could thus be transferred from the overloaded region of the 2D spectrum close to the diagonal into the rather free region of H-1 correlations (for example, the two-step COSYRCT spectrum of the *O*-deacetylated O-antigen of *P. vulgaris* 5/43 is shown in FIG. 2).

Remaining uncertainties in assignment of the ^{1}H NMR spectra, like those associated with the position of H-5 and H-6 of galactose residues or with exact coincidence of signals of some coupled protons within the same sugar unit, were eliminated by application of 2D heteronuclear $^{13}C/^{1}H$ shift-correlated (COSY) spectroscopy, which, with the ^{1}H NMR spectrum assigned, was also used for unambigous assignment of ^{13}C NMR spectrum.

Finally, NOE spectroscopy, carried out in 1D or 2D rotating-frame (ROESY) version, also contributed to solving the problem of proton assignment. In particular, it was useful for determination of the H-5 signal in sugars with the ß-*galacto* configuration and for its confirmation in other ß-linked monosaccharides by observation of H-5 signal in the NOE spectra obtained on pre-irradiation of H-1 of the same sugar residue.

Appearance of the inter-residue NOE contacts between the anomeric and transglycosidic protons served as a basis for determination of both the substitution pattern of sugar residues and their sequence in the repeating unit. NOE experiments were also used to determine the site of attachment of the ether-linked lactic acid residue. Pre-irradiation of the lactic acid H-2 induced a NOE response on the corresponding sugar proton.

Some uncertainty arose from dependence of NOE's on proton proximity rather than on linkage position but could be eliminated by application of conformational analysis[24] which allowed prediction as to when NOE contacts other than trans-

glycosidic could be expected. Moreover, such analysis revealed the steric conditions required for additional interresidue NOE's and was helpful in determination of relative absolute configurations of the constituent monosaccharides.

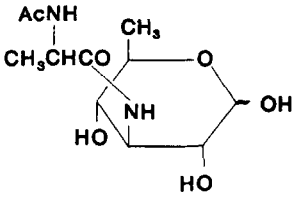
Sites of glycosylation (as well as of etherification) were confirmed by low-field displacements of the ^{13}C signals for polysaccharide transglycosidic carbons as compared with their positions in the spectra of the corresponding free monosaccharides. Analysis of the values of these α-effects of glycosylation and the ß-effects on the neighboring carbons of the glycosylated sugar residues, taking into account the regularities found by us previously,[25,26] allowed determination of relative absolute configuration of the constituent monosaccharides.

These regularities were put also in the background of an alternative ^{13}C NMR-based method of structural elucidation of polysaccharides, which is described in published works.[26,27] It consists in calculation of chemical shifts for all possible structures of a polysaccharide with the given monosaccharide composition and searching for the structure with the best fit of the calculated and experimental data. This method was successfully applied to a number of *Proteus* O-antigens, in particular, to the *O*-deacetylated *P. mirabilis* O3[15] and dephosphorylated *P. vulgaris* 5/43[18] O-antigens and gave in each case one or two structures consistent with the experimental ^{13}C NMR spectrum. The real structure was selected with the help of methylation analysis and/or a selective chemical cleavage, e.g., Smith degradation (see below).

3. Chemical Degradations of O-Specific Polysaccharides

Independent confirmation of composition and structures elucidated by NMR spectroscopy was obtained by sugar analysis, methylation analysis, modifications of polysaccharides or their selective chemical degradations to oligosaccharides. Common monosaccharides and amino acids were identified after conventional acid hydrolysis. Neutral sugars and uronic

acids were identified with the help of anion-exchange chromatography in borate buffer and amino sugars and amino acids using an amino acid analyzer. Amino sugars with unusual *N*-acyl substituents were isolated after solvolysis with anhydrous HF.[28] These amino sugars include 3,6-dideoxy-3-[(*R*)-3-hydroxybutyryl]amido-D-galactose (**3**) and 3-(*N*-acetyl-D-alanyl)amido-3,6-dideoxy-D-glucose (**4**) from the O-antigens of *P. penneri* 14[19] and 16,[17] respectively; amides of uronic acid with amino acids such as D-galacturonoyl-L-alanine (**5**) and D-galacturonoyl-L-lysine (**6**) from the polysaccharides of *P. mirabilis* 1959[11] and *P. penneri* 14,[19] respectively; ethers of monosaccharides with lactic acid, for example, *N*-acetylisomuramic acid (**7**) from the O-antigen of *P.*



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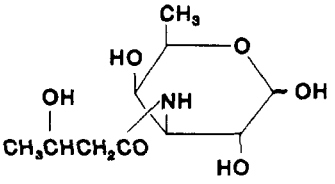

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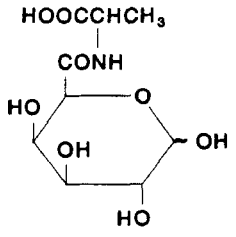

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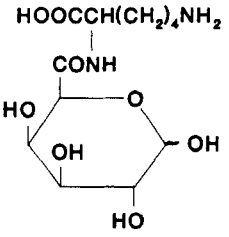

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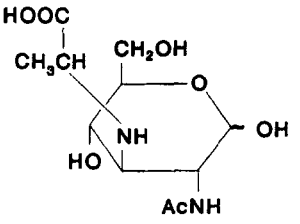

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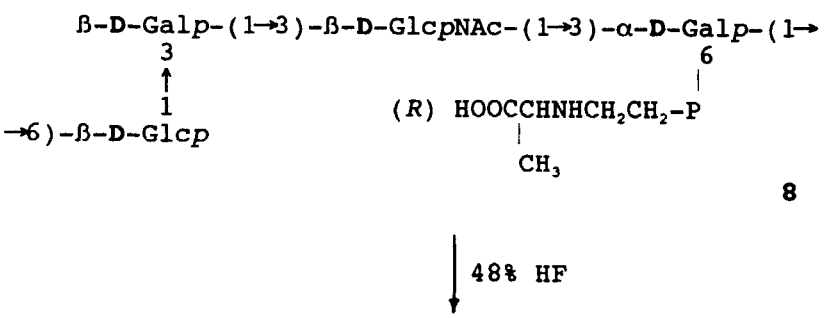
penneri 62.[21] HF cleaved the glycosidic linkages without destruction of the free monosaccharides and without splitting the amide linkages. Identification of the isolated new components was carried out by interpretation of NMR spectra and MS experiments on the corresponding alditol acetates.

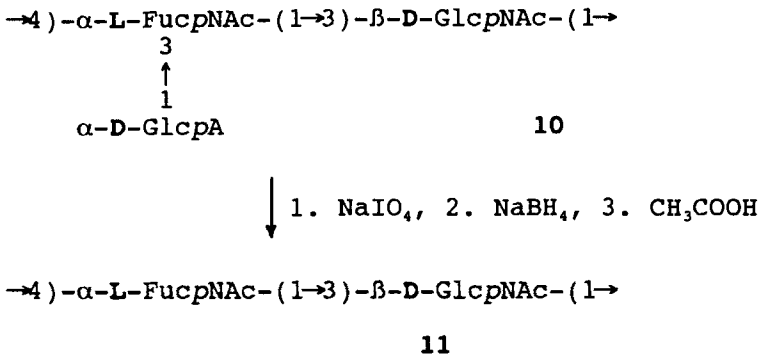
Apart from the above-mentioned *O*-deacetylation, a useful modification consisted in *O*-dephosphorylation of phosphate-containing O-antigens with 48% HF. Conversion of these O-antigens into the corresponding dephosphorylated polysaccharides without depolymerization proved that, unlike in teichoic acids, phosphate and hence non-sugar components attached by phosphate do not enter the main chain. Dephosphorylation of the O-antigen of *P. mirabilis* O3[15] (**8**) led to structural elucidation of the dephosphorylated polysaccharide (**9**) using a ^{13}C NMR-based computer-assisted method[26,27] and determination of the location of the 2-[(*R*)-1-carboxyethylamino]ethyl phosphate group by comparison of the ^{13}C NMR spectra of the initial and modified O-antigens.



Similar comparison of the ^{13}C NMR spectra of the initial and *O*-deacetylated polysaccharides (e.g., **1** and **2**), taking into account α- and ß-effects of *O*-acetylation,[29] revealed location of *O*-acetyl groups.

Smith degradation was applied both for modification of the polysaccharides and for preparation of the oligosaccharide fragments. Periodate oxidation of branched polysaccharides with the main chain stable to oxidation, such as that from *P. mirabilis* O33 (**10**), led to degradation of the side

chain. A comparative study of the initial O-antigen and the modified polymer (**11**) using ^{13}C NMR spectroscopy resulted in determination of the site of attachment of the lateral monosaccharide (GlcA).

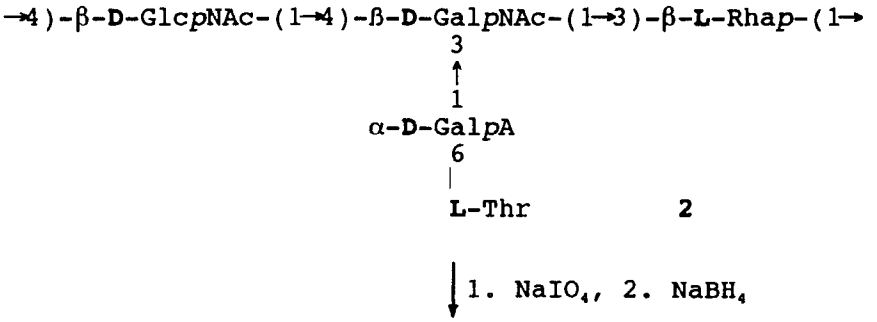


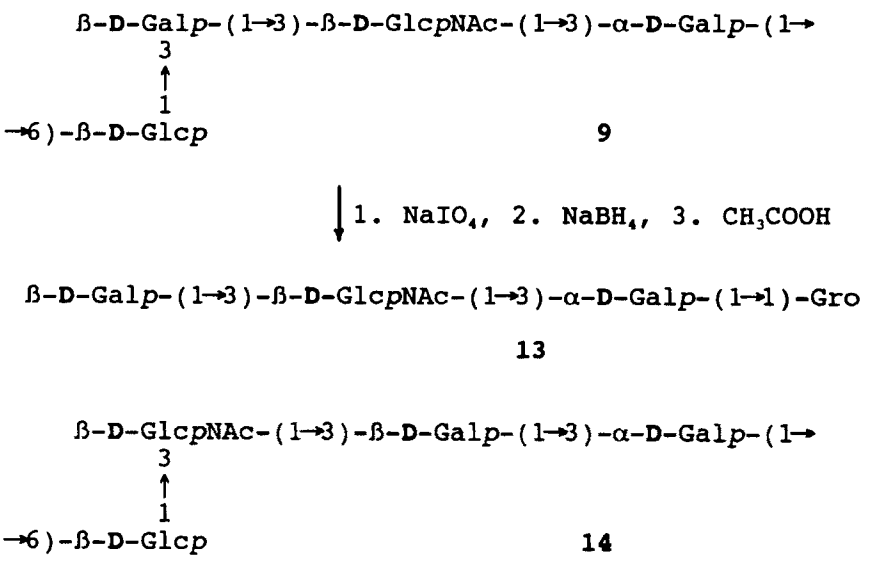
The same information as for the *P. mirabilis* O33 O-antigen was obtained by comparison of the ^{13}C NMR spectrum of the modified polysaccharide of *P. mirabilis* 3/6 (**12**), still containing the oxidized **D**-galacturonamide residue, with that of the initial *O*-deacetylated O-antigen (**2**).

Smith degradation of the dephosphorylated O-antigen of *P. mirabilis* $O3^{15}$ (**9**) led to the triosyl glycerol (**13**) terminated with a galactose residue. This finding allowed a choice between two alternative structures of the polysaccharide (**9**) and (**13**) proposed on the basis of methylation and ^{13}C NMR-based computer-assisted analysis (see above).

Partial acid hydrolysis with 0.1M HCl and solvolysis with anhydrous HF were methods of choice for isolation of oligosaccharide fragments of a number of the *Proteus* polysaccharides. These methods are rather sensitive to particular monosaccharides and allow conditions of reaction to be varied over a wide range making the methods applicable practically to any heteropolysaccharide.

In many cases solvolysis with HF proceeded with high selectivity to afford a good yield of an oligosaccharide (or oligosaccharides). Thus, the only oligosaccharide product,

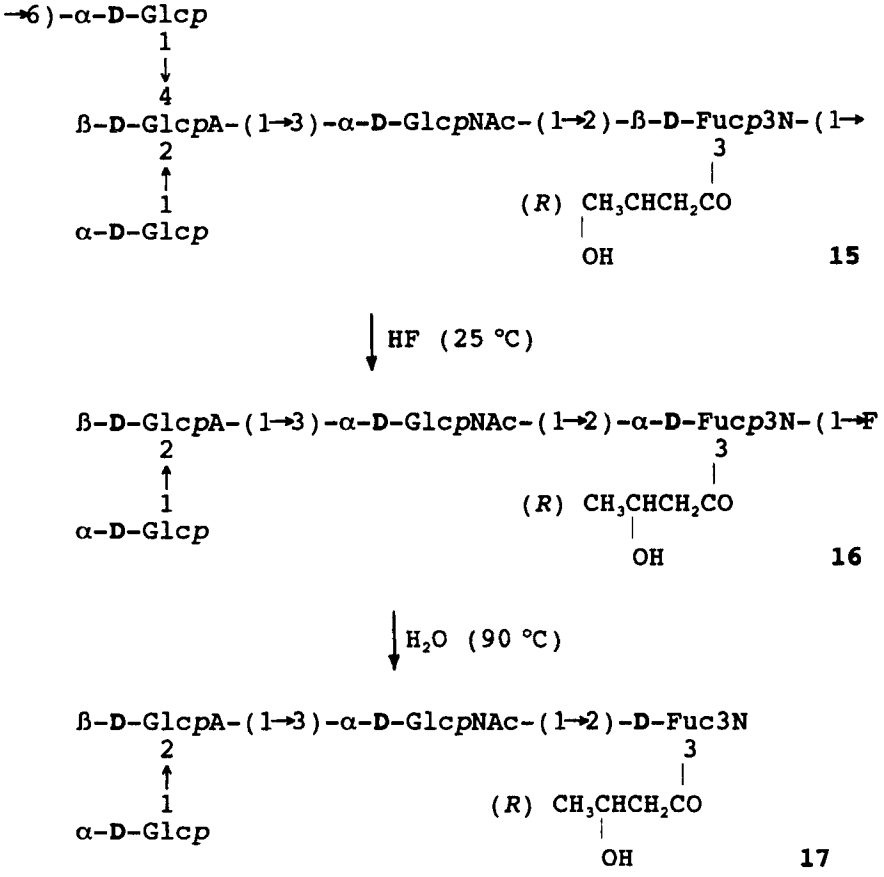




the tetrasaccharide (**17**), was isolated on HF treatment of the *O*-deacetylated O-antigen of *P. penneri* 16[17] (**15**).

The α-oligosyl fluoride (**16**) isolated after treatment of the reaction mixture with water was stable, probably, due to substitution of the glycosyl fluoride moiety at position 2. The structure of fluoride **16** was determined from NMR basing on characteristic chemical shifts and coupling constants

(*inter alia* that for H-1 at 5.79 ppm, dd, $J_{H,F}$ 54 Hz; and C-1 at 105.1 ppm, d, $J_{C,F}$ 226 Hz). The oligosyl fluoride **16** was converted into the free tetrasaccharide **17** by hydrolysis with water at 90 °C.

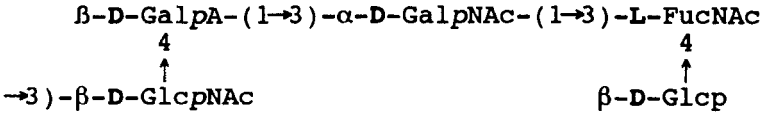


Solvolysis of the dephosphorylated *P. penneri* 8 O-antigen (**18**) with HF also proceeded with the loss of one of the glucose residues to give the pentasaccharide (**19**). As expected, the α-glucosidic linkage was less stable towards HF than the ß one.[28]

HF treatment of the *P. mirabilis* O27 antigen[14] (**20**) resulted in selective cleavage of the glycosidic linkage of one or both of the amino sugar residues (directed solvolysis), whereas the linkages of both uronic acid derivatives

18

HF (25 °C)



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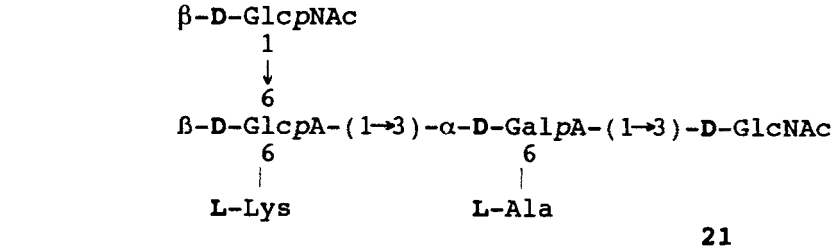
were stable. Solvolysis was accompanied by complete dephosphorylation but did not affect the amide-linked amino acids (alanine and lysine) and led mainly to the tetrasaccharide (**21**) at 0 °C and the trisaccharide (**22**) at 20 °C.

Partial hydrolysis with 0.1M HCl (100 °C) of the O-antigen of *P. penneri* 14[19] (**23**) gave two oligosaccharides (**24** and **25**) containing all constituents of the repeating units including non-sugar ones (L-alanine, *N*-acetyl and *N*-acetyl-D-alanyl groups). Therefore, like solvolysis with HF, such a mild hydrolysis did not affect the amide linkages.

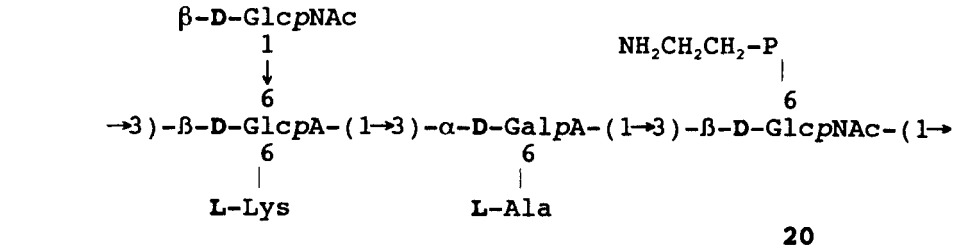
Combination of both degradation methods was used in some cases and led to different sets of oligosaccharides. For example, HF treatment of the *P. vulgaris* 5/43 O-antigen[18] (**26**) gave the disaccharide (**27**), whereas partial hydrolysis afforded a mixture of the trisaccharides (**28**) and (**29**) and the tetrasaccharide (**30**), the latter representing a repeating unit of the polysaccharide.

As a rule, the glycosidic linkage of 6-deoxy sugars is more labile towards HF solvolysis than that of other monosaccharides.[28] The same is also believed to be the case for partial hydrolysis. Unexpectedly, in the O-antigens of *P. vulgaris* 5/43[18] (**26**) and *P. penneri* 14[19] (**23**) the most labile linkage was that of one of the GlcNAc residues, which was



21

HF (0 °C)



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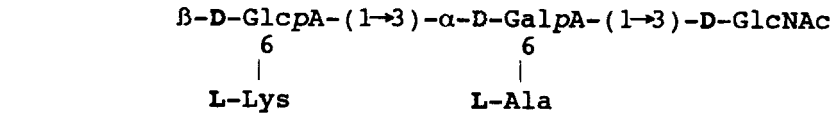
HF (20 °C)



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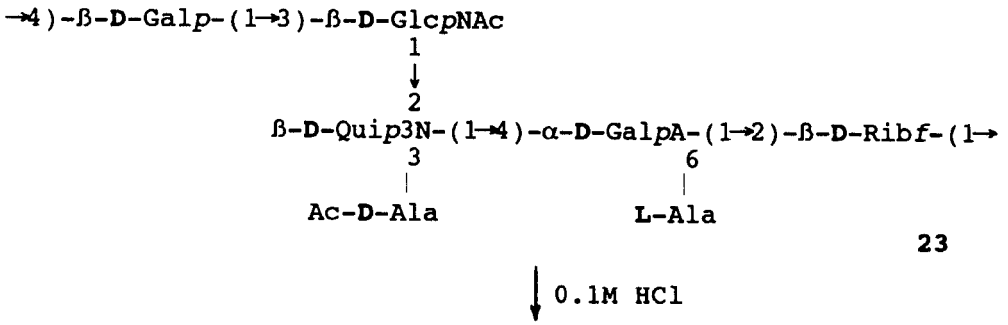
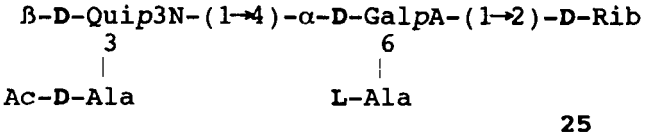


23

0.1M HCl

ß-D-Gal*p*-(1→3)-D-GlcNAc

24



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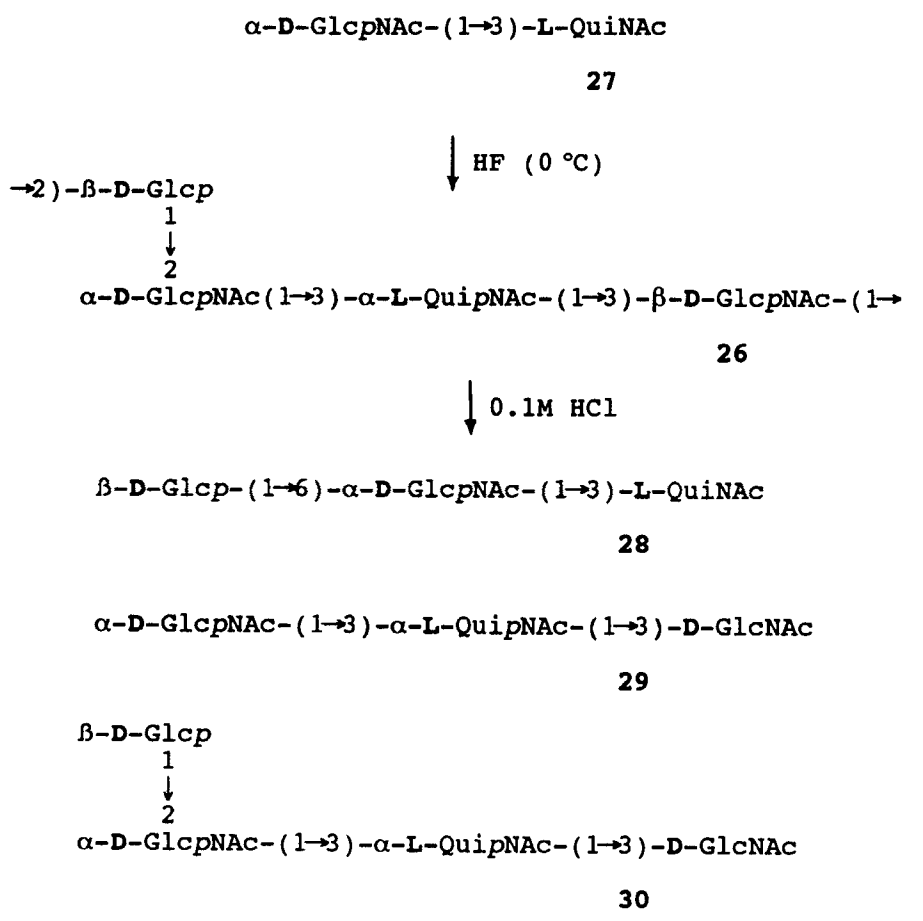


split by dilute HCl more easily than was the glycosidic linkage of derivatives of 6-deoxyamino sugars (QuiN and Qui3N, respectively). Probably, this lability is accounted for by attachment of the GlcNAc residues in both polysaccharides by a strained 1,2-linkage.

The oligosaccharide fragments thus obtained were structurally elucidated mainly by NMR-spectroscopy, including NOE spectroscopy and ^{13}C NMR-based computer-assisted analysis, as described above for the polysaccharides. The reducing oligosaccharides were converted, prior to analysis, into the corresponding oligosyl alditols, which facilitated the NMR study and allowed identification of the monosaccharide at the reducing end. Methylation followed by MS analysis was also

applied in order to determine either sites of glycosylation or distribution of amide-linked amino acids.

4. Composition and Structures of the O-Antigens and Their Biological Significance.

Distribution of monosaccharides and non-sugar components and structures of seventeen *Proteus* O-antigens established by us so far are given in Tables 1-3. The most frequently occurring monosaccharide in these O-specific polysaccharides is **D**-glucosamine, in all the polysaccharides but one. Other typical sugar constituents, widespread in nature, are **D**-glucose, **D**-galactose, **D**-glucuronic acid, **D**-galacturonic acid, and **D**-galactosamine as well as less commmon **L**-fucos-amine (FucN). **D**-Ribose and **L**-rhamnose are components of two O-antigens each, and three polysaccharides contain 6-deoxy-amino sugars rarely occurring in nature: **L**-quinovosamine (QuiN, *P. vulgaris* 5/43[18]), 3-amino-3,6-dideoxy-**D**-glucose (Qui3N, *P. penneri* 14[19]), and 3-amino-3,6-dideoxy-**D**-galactose (Fuc3N, *P. penneri* 16[17]).

The amino group of 2-amino-2-deoxyhexoses is always acetylated and that of 3-amino-3-deoxyhexoses is acylated by the *N*-acetyl-**D**-alanyl group (Qui3N), which was identified in the *P. penneri* 14 O-antigen[19] for the first time in nature, or by the (*R*)-3-hydroxybutyryl group (Fuc3N) in the *P. penneri* 16 O-antigen.[17,31] In some polysaccharides, various amino acids (**L**-alanine, **L**-serine, **L**-threonine or **L**-lysine) are attached to the carboxyl group of **D**-glucuronic acid or, more often, **D**-galacturonic acid, to form the corresponding amides, lysine being linked by its α-amino group.[32]

Several polysaccharides include amino components of another type, namely 2-aminoethanol and *N*-(2-aminoethyl)-**D**-alanine, which are bound by the phosphodiester linkage at position 6 of *N*-acetylglucosamine or galactose, respectively. Like uronamides, phosphate is not common for O-antigens. 2-Aminoethyl phosphate is well known as a component of the lipopolysaccharide core of many gram-negative bacteria,[33] but

TABLE 1. Distribution of Monosaccharides in O-antigens of *Proteus*

Monosaccharide[a]	O-Serogroup or strain																	
	P. mirabilis								*P. vulgaris*			*P. penneri*						
	O3	O23	O27	O28	O33	O43	1959	3/6	O19	O25	5/43	8	14	16	42	52	62
D-Rib (2)										+			+				
D-Glc (8)	+					+	+			+	+	+		+	+		
D-Gal (6)	+	+		+					+				+				+
L-Rha (2)								+		+							
D-GlcA (4)		+	+		+		+										
D-GalA (10)			+	+		+	+	+				+	+	+	+	+	
D-GlcN (16)	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+
D-GalN (6)							+	+	+	+		+				+	
L-QuiN (1)									+								
L-FucN (4)		+			+				+			+					
D-Qui3N (1)													+				
D-Fuc3N (1)														+			

a. A total number of polysaccharides containing the given monosaccharide is indicated in parentheses.

TABLE 2. Distribution of Non-Sugar Components in O-antigens of *Proteus*

Component	O-Serogroup or strain																
	P. mirabilis								*P. vulgaris*			*P. penneri*					
	O3	O23	O27	O28	O33	O43	1959	3/6	O19	O25	5/43	8	14	16	42	52	62
N-Linked																	
Ac	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
(*R*) CH_3CHCH_2CO | OH														+			
Ac-**D**-Ala													+				
O-Linked																	
Ac	+							+			+			+			+
(*R*) $CH_3CHCOOH$ |										+							
(*S*) $CH_3CHCOOH$ |																	+
$NH_2CH_2CH_2$-P			+									+					
(*R*) $CH_3CHCOOH$ | $HNCH_2CH_2$-P	+																
Carboxyl-linked																	
L-Ala			+										+				
L-Ser				+													
L-Thr								+									
L-Lys			+	+			+										

TABLE 3. Structures of O-Specific Polysaccharides of *Proteus*

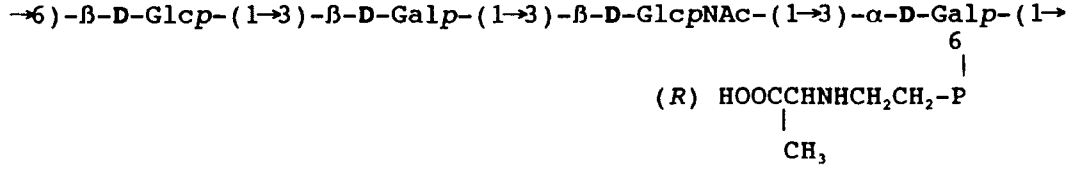
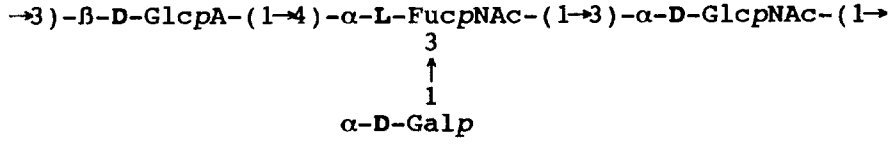
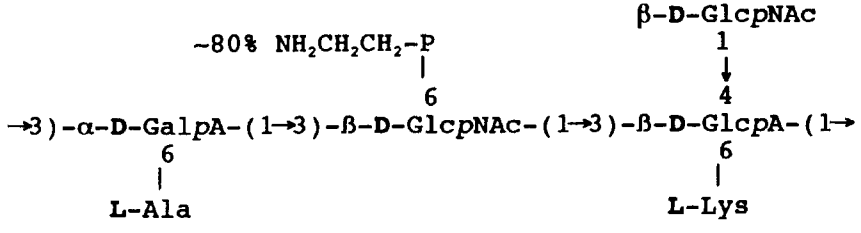
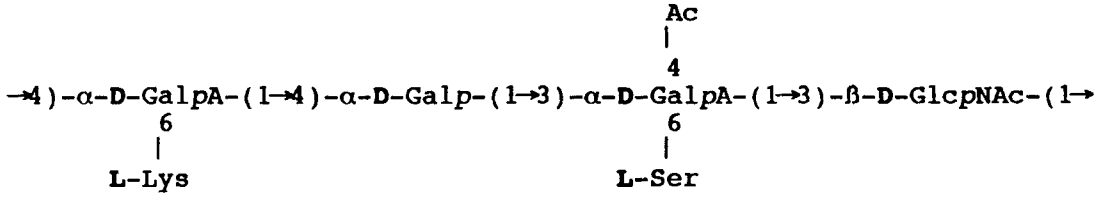
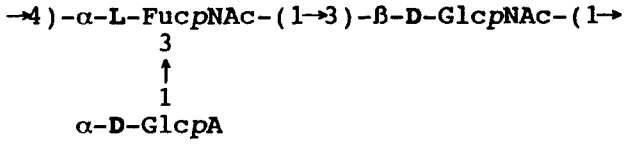
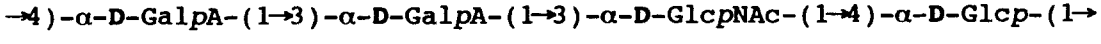
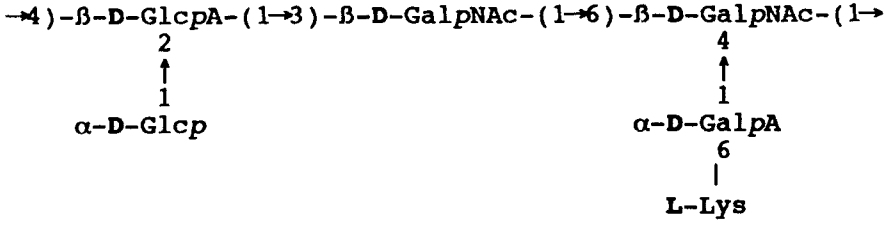
O-serogroup or strain	Structure of repeating unit	References
P. mirabilis		
O3		15
O23		20
O27		14

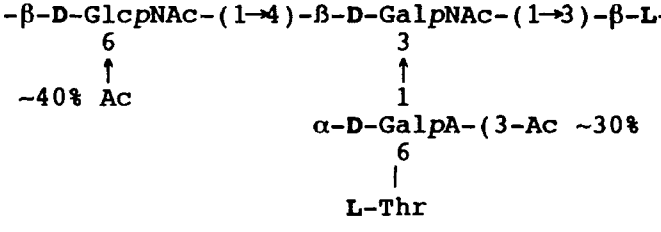
TABLE 3 (continued)

028		37
033		30
043		20
1959		11,12

(continued)

TABLE 3 (continued)

3/6 →4)-β-D-Glc*p*NAc-(1→4)-ß-D-Gal*p*NAc-(1→3)-β-L-Rha*p*-(1→ 20



P. vulgaris

O19 →4)-α-D-Gal*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-ß-D-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→ 13,16

O25 →4)-α-D-Gal*p*NAc-(1→3)-ß-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→2)-ß-D-Rib*f*-(1→ 20

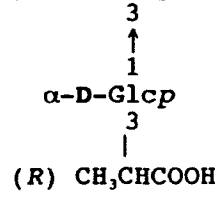
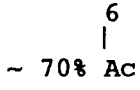


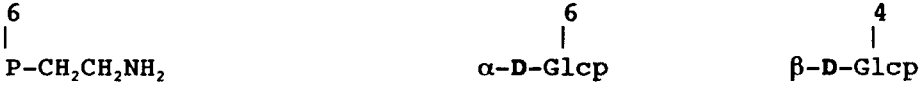
TABLE 3 (continued)

5/43 (OX19) →2)-β-D-Glc*p*-(1→6)-α-D-GlcpNAc-(1→3)-α-L-Quip*N*Ac-(1→3)-β-D-Glc*p*NAc-(1→ — 18,20



P. penneri

8 →3)-β-D-Glc*p*NAc-(1→4)-β-D-Gal*p*A-(1→3)-α-D-Gal*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→



14 →4)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→2)-β-D-Quip3N-(1→4)-α-D-Gal*p*A-(1→

→2)-β-D-Rib*f*-(1→ — 19

16 →6)-α-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→3)-α-D-Glc*p*NAc-(1→2)-β-D-Fuc*p*3N-(1→ — 17,31

(continued)

TABLE 3 (continued)

42	→2)-ß-D-Glc*p*-(1→4)-ß-D-Glc*p*-(1→3)-ß-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→	20
52	→4)-ß-D-Gal*p*A-(1→3)-ß-D-Glc*p*NAc-(1→4)-ß-D-Gal*p*NAc-(1→3)-α-D-Gal*p*NAc-(1→ 3 ↑ 1 ß-D-Glc*p*NAc	20
62	→3)-α-D-Gal*p*-(1→3)-ß-D-Glc*p*NAc-(1→6)-ß-D-Glc*p*NAc-(1→ 6 3 | | ~70% Ac (*S*) $CH_3CHCOOH$	20,21

has never been identified in a lipopolysaccharide side chain of bacteria other than *Proteus*. Besides the polysacharides from *P. mirabilis* O27[14] and *P. penneri* 8, it was found in the O-antigen of *P. mirabilis* D52,[10] together with another phosphate-containing substituent, D-ribitol-1-phosphate. As for *N*-(2-aminoethyl)-D-alanine, the new component of the *P. mirabilis* 03 O-antigen,[15] it has not been found hitherto in bacterial polysaccharides.

And, finally, unusual acidic components of another type were found in the O-antigens of *P. vulgaris* O25[20] and *P. penneri* 62[21] and identified as ethers of D-glucose with (*R*)-lactic acid and of *N*-acetyl-D-glucosamine with (*S*)-lactic acid, respectively. In both ethers the residue of lactic acid is attached at position 3, and the latter one, so called *N*-acetylisomuramic acid, was known hitherto as a synthetic but not natural compound.

O-Acetylation is rather typical for bacterial polysaccharides[34] and the O-specific polysaccharides of *Proteus* are not an exception. As a rule, *O*-acetylation at each position is only partial (20 to 70%) except for the O-antigen of *P. mirabilis* O28,[35] where the *O*-acetyl group is present in nearly stoichiometric amount.

As one can see from Table 3, the O-specific polysaccharides of *Proteus* are built up of linear or branched tri- to penta- and, in the case of *P. penneri* 8, even hexa-saccharide repeating units. Most of these polysaccharides contain one or two acidic functions (carboxyl or phosphate groups) or both acidic and basic (amino) functions. Such a high concentration of charged groups (up to five per repeating unit in the O-antigen of *P. mirabilis* O27[14]) endows the polysaccharides with amphoteric properties that may be important for adaptation of microorganisms to grow under different pH conditions.

A frequent occurrence[5] in clinical isolates of *P. mirabilis* serogroups O3, O27, and O28, which possess amphoteric O-antigens, is in favour of this suggestion. The O3 serogroup, isolated from diarrhea, urinary tract infections, and bacteraemia,[5] is the most widespread among all *Proteus*

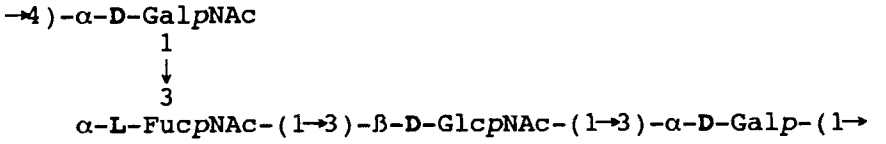
strains. One can speculate that the new non-sugar component of its O-specific polysaccharide, *N*-(2-aminoethyl)-D-alanine phosphate, contributes to the high virulence associated with the O3-antigen, for example, by improvement of adhesion of bacterial cell by binding to a specific receptor.

A mutant strain *P. mirabilis* 3/6, derived from *P. mirabilis* O3 by the transposon Tn5phoA transfer, differs significantly from the parent strain both in biological properties (biochemical reactions, antibiotic sensitivity, hemolytical activity, binding the complement and rate of PMN's phagocytosis[36]) and in the structure of the O-antigen (Table 3), particularly, in the absence of any phosphate-containing substituent. Therefore, the gene(s) controlling biosynthesis of O-antigen is (are) among those affected by such types of induced mutation.

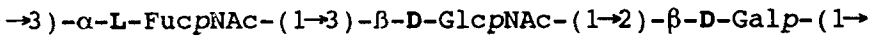
Another mutant strain, *P. mirabilis* R14, derived from *P. mirabilis* 1959, represented a transient form between the S- and R-forms. It was found to produce a lipopolysaccharide with a changed side-chain polysaccharide (T-antigen). Surprisingly, the structure of the T-antigen turned out to be identical to the structure of the O-antigen of *P. penneri* 42 (Table 3).

We were interested in whether the O-antigens of *Proteus* contain any fragment (or fragments) in common, which serve as a base for their serological relationship. One can see from Table 3 that all polysaccharides which contain *N*-acetyl-L-fucosamine (those of *P. mirabilis* O23, O33, *P. vulgaris* O19, and *P. penneri* 8) include also the disaccharide fragment α-L-Fuc*p*NAc-(1→3)-D-Glc*p*NAc. This feature does not involve necessarily cross-reactivity of the corresponding strains with each other, that might be accounted for by the position of Fuc*p*NAc at the branching point with different side-chain monosaccharides attached in three of four polysaccharides and/or by a different anomeric configuration of the neighbouring Glc*p*NAc residue. However, *P. vulgaris* O19[13] (**31**) does not cross-react with *Salmonella arizonae* O59 [the structure (**32**) has been established[37] for the O-antigen of the latter]

despite that they are both linear and contain the disaccharide fragment α-L-Fuc*p*NAc-(1→3)-ß-D-Glc*p*NAc in common. Thus, this fragment does not seem to contribute significantly in manifestation of O-specificity of these strains.



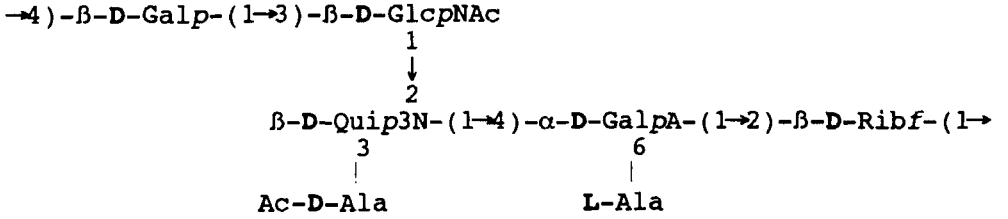
31 (*P. vulgaris* O19)



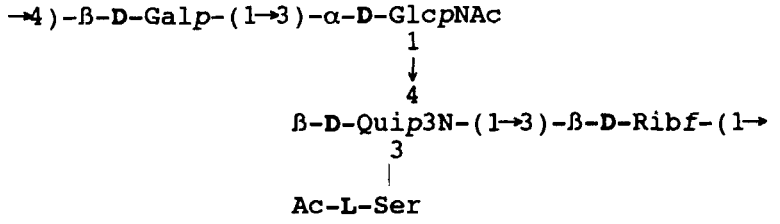
32 (*S. arizonae* O59)

Another fragment found in more than one strain is the amide of D-galacturonic acid with L-lysine, which is a component of the O-antigens of *P. mirabilis* O28 and 1959. It plays the immunodominant role both in the latter polysaccharide,[8] where this uronamide is attached as a side chain, and in the former one,[35] where it occupies a position in the main chain (Table 3). On the contrary, the amide of D-glucuronic acid with L-lysine in the main chain of the *P. mirabilis* O27 O-antigen is not the immunodominant component.[14]

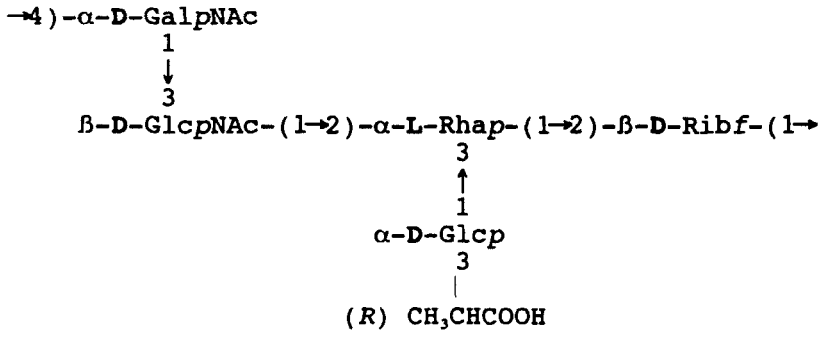
In contrast to the previous observation,[38] the O-antigen of *P. mirabilis* O3, which cross-reacts[6] with *P. mirabilis* O27, does not contain lysine in the repeating unit of the O-antigen, and the serological relationship is supposed[15] to be based on the presence of the phosphate-containing non-sugar constituents (Table 3). Noteworthy, only a one-way cross-reaction is observed between these two O-serogroups (anti-O27 serum does not react with the O3 antigen). The reason seems to be the occurrence of the different immunodominant components: 2-[(*R*)-1-carboxyethylamino]ethyl phosphate in the O3 serogroup but the lateral GlcNAc residue in the O27 serogroup.[39]



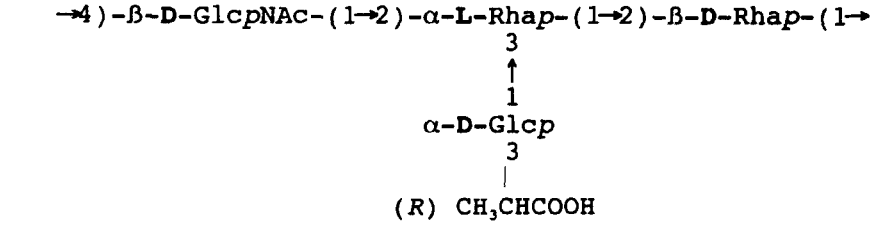
23 (*P. penneri* 14)



33 (*E. coli* O:114)



34 (*P. vulgaris* O25)



35 (*A. haloplanktis* KMM 156)

Serological cross-reactions between *Proteus* and many other bacteria are known (review[5]). They may give rise to diagnostic problems but can be useful in particular cases, like, for example, in the Weil-Felix test for serological diagnosis of epidemic typhus caused by *Rickettsia*.[40] This test is based on cross-reactivity between *P. vulgaris* OX19 group and *Rickettsia* associated with their polysaccharide antigens. The structure of the O-antigen of one of the representatives of the OX19 group, *P. vulgaris* 5/43, is now established (Table 3), and structural elucidation of the rickettsial antigen must be the next step in order to reveal a fragment in common responsible for the serological relationship.

Apart from the O-antigens of *P. vulgaris* O19 and *S. arizonae* O59 discussed above, another pair of the O-antigens of *P. penneri* 14[19] (**23**) and *Escherichia coli* O:114[41] (**33**) exhibit a structural similarity. Only a weak cross-reaction was observed between these two antigens that may be accounted for by the absence of galacturonic acid and alanine and a smaller-size repeating unit in the latter polysaccharide.

The O-specific polysaccharide of *P. vulgaris* 025[20] (**34**) and the capsular polysaccharide of *Alteromanas haloplanktis* KMM 156[42] (**35**) also possess a common trisaccharide fragment, which includes **L**-rhamnose, *N*-acetyl-**D**-glucosamine, and the ether of **D**-glucose with (*R*)-lactic acid. However, no data about serological cross-reactivity between these two microorganisms are available.

5. Summary

Structures of seventeen O-specific polysaccharides of *P. mirabilis*, *P. vulgaris*, and *P. penneri* were elucidated with the help of 1D and 2D NMR spectroscopy (including 1D TOCSY and NOE, 2D homonuclear and $^{13}C/^{1}H$ heteronuclear COSY and ROESY) and chemical degradations (*O*-deacetylation with NH_4OH, *O*-dephosphorylation with 48% HF, partial hydrolysis with 0.1M HCl, solvolysis with anhydrous HF, Smith degradation). To-

gether with aldoses and *N*-acylated amino sugars, the polysaccharides typically contain acidic or both acidic and basic components, such as **D**-glucuronic and **D**-galacturonic acids, their amides with various amino acids (**L**-alanine, **L**-serine, **L**-threonine, **L**-lysine), ethers of sugars with (*R*)- and (*S*)-lactic acid, 2-aminoethyl and 2-[(*R*)-1-carboxyethylamino]-ethyl phosphate. The charged substituents and some common fragments contribute to serological specificity of the *Proteus* O-antigens.

The data on the structures of the O-antigens can serve as a basis for serological classification of *Proteus* strains. They showed that each of the serologically distinguishable strains of *Proteus*, both classified and unclassified, possesses an O-antigen with a unique structure. Thus, the unclassified strains studied, including the strains of *P. penneri*, should be placed into the classification scheme of *Proteus* as individual O-serogroups. Further study can answer the question how the genetic and metabolic differences of three *Proteus* species are reflected in composition and structures of their O-antigens.

6. Experimental Procedures Employed

General Procedures. Ascending paper chromatography was carried out on FN-11 paper in a system of pyridine/ethyl acetate/acetic acid/water (5:5:1:3, by vol.); substances were detected by ninhydrin or the alkaline silver nitrate reagent. GLC was carried out with a Hewlett-Packard 5890 instrument equipped with a glass-capillary column coated with Ultra 1 stationary phase. Gel-permeation chromatography was done on Sephadex G-50 in pyridine-acetic acid, pH 4.5, or TSK HW 40 (S) in water or 1% acetic acid and monitored by the phenol-sulfuric acid reagent or with a Knauer differential refractometer, respectively. Anion-exchange chromatography was carried out on an amino acid analyzer Microtechna T339 and a sugar analyzer Biotronic LC-2000 equipped with a Durrum DAx8 column in borate buffer as described.[17] HPLC was per-

formed on analytical columns of Silasorb SPH C18 eluted with 0.1-0.4% trifluoroacetic acid in 0-4% aqueous methanol or DEAE-TSK in 2% acetic acid and monitored by a Knauer differential refractometer or a Knauer variable wavelength monitor at 220 nm. MS was performed with a Varian MAT 311 instrument. Optical rotations were measured with a Jasco DIP 360 polarimeter in water at 25 °C.

NMR Spectroscopy. ^{1}H NMR spectra were recorded with a Bruker WM-250 or Bruker AM-500 instrument in D_2O at 30 °C for oligosaccharides and an elevated temperature (50-80 °C) for polysaccharides. ^{13}C NMR spectra were recorded with a Bruker AM-300 spectrometer in D_2O at 30 °C for oligosaccharides and 60 °C for polysaccharides. Acetone was used as an internal standard (δ_H 2.225 ppm, δ_C 31.45 ppm). Selective spin-decoupling, 1D TOCSY-HOHAHA and NOE, 2D homonuclear and heteronuclear $^{13}C/^{1}H$ shift-correlated (COSY) and rotating-frame NOE (ROESY) spectroscopy were performed as described.[17,23,43]

Bacterial Strains, Growth of Bacteria, Isolation of Lipopolysaccharides and O-Specific Polysaccharides. *P. mirabilis* and *P. vulgaris* strains were derived from Czechoslovak National Collection of Type Cultures of the Institute of Epidemiology and Microbiology, Prague. *P. mirabilis* strain 1959 came from the strains collection of the Institute of Microbiology, University of Lodz. *P. penneri* strains were kindly provided by Prof. D. J. Brenner (Center for Disease Control, Atlanta, U.S.A.). Cultivation of bacteria was carried out in nutrient broth (Warsaw Serum and Vaccine Laboratory) with 1% glucose added. The bacteria were harvested at the end of logarithmic growth phase, centrifuged, washed with saline or distilled water, and freeze-dried.

Lipopolysaccharides were isolated by phenol-water extraction[44] and purified by ultracentrifugation followed by digestion with nucleases.[10] O-Specific polysaccharides were obtained by treatment of lipopolysaccharides with 1% CH_3COOH (100 °C) followed by gel chromatography on Sephadex G-50.

Sugar, Amino Acid, and Phosphate Analysis. Polysaccharides were hydrolyzed with 2M CF_3COOH (120 °C). Neutral sugars

and uronic acids were identified by using anion-exchange chromatography in borate buffer with the help of a sugar analyzer. Amino sugars, amino acids, and ethanolamine were identified by use of an amino acid analyzer under standard conditions. Neutral and amino sugars were identified also by GLC of fully acetylated alditols. Absolute configurations of monosaccharides and amino acids were determined by GLC of acetylated (*R*)-2-butyl or (*R*)-2-octyl glycosides[45,46] and (*R*)-2-butyl esters, respectively; for monosaccharides the known regularities in the glycosylation effects in ^{13}C NMR spectra[25,26] were used as well.

In order to isolate *N*-acylated amino sugars and amides of uronic acids with amino acids on a preparative scale, polysaccharides were solvolyzed with anhydrous HF[28] and the products were separated by PC, HPLC on a reversed phase C18 or an anion-exchanger DEAE-TSK. The isolated components were identified on the basis of specific optical rotation values, ^{1}H and ^{13}C NMR spectroscopic data and MS data from acetylated or methylated derivatives.

Phosphate content was determined by the colorimetric method.[47]

Methylation Analysis. Methylation of polysaccharides was carried out under the Hakomori conditions,[48] methylated polysaccharides were recovered using a Sep-Pak cartridge, cleaved with 2M CF_3COOH (100 °C). Partially methylated monosaccharides were analyzed by GLC as alditol acetates. For analysis of uronic acids methylated polysaccharides were reduced with $LiBH_4$ prior to hydrolysis.

Modifications and Selective Degradations of Polysaccharides.

a) *O-Deacetylation.* A polysaccharide was heated with 10-12% NH_4OH at 25 or 60 °C, the aqueous solution concentrated to dryness to give an *O*-deacetylated polysaccharide, which was purified, if necessary, by gel-permeation chromatography on TSK HW-40.

b) *Dephosphorylation.* A polysaccharide was treated with 48% HF (10 °C), the solution was concentrated in a vacuum

desiccator over solid NaOH and a dephosphorylated polysaccharide was isolated by gel-permeation chromatography on TSK HW-40.

c) *Smith degradation.* A solution of a polysaccharide in 0.1M $NaIO_4$ was kept overnight at room temperature, then treated with several drops of ethylene glycol and an excess of $NaBH_4$, acidified with glacial CH_3COOH and the Smith-degraded polysaccharide recovered by gel-permeation chromatography on TSK HW-40.

The Smith-degraded polysaccharide was hydrolyzed with 1% CH_3COOH (100 °C), the solution concentrated, the residue treated with $NaBH_4$, and the product subjected to gel-permeation chromatography on TSK HW-40 to give a modified polysaccharide or an oligosaccharide.

d) *Partial acid hydrolysis.* A polysaccharide was hydrolyzed with 0.1M HCl (100 °C) and the products were separated by gel-permeation chromatography on TSK HW-40 to give an oligosaccharide fragment (or fragments), which was purified, if necessary, by HPLC on a reversed phase.

e) *Solvolysis with anhydrous HF.* A polysaccharide was treated with anhydrous HF (0 or 25 °C), the reagent was removed under diminished pressure and absorbed by solid NaOH and the product (or products) isolated as in partial acid hydrolysis. In some cases oligosaccharides were reduced with $NaBH_4$ or *N*-acetylated before or after separation on gel.

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