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The structure of the O-specific polysaccharide of *Salmonella arizonae* O21 (*Arizona* 22) containing *N*-acetylneuraminic acid

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Abstract

The O-specific polysaccharide of *S. arizonae* O21 was found to contain 2-acetamido-2-deoxy-D-glucose, 2-acetamidino-2,6-dideoxy-L-galactose, *N*-acetylneuraminic acid, and *O*-acetyl groups. On the basis of ^{1}H and ^{13}C NMR studies of the intact and *O*-deacetylated polysaccharide and oligosaccharide fragments obtained by solvolysis with anhydrous hydrogen fluoride, partial methanolysis and partial hydrolysis, it was concluded that the O-specific polysaccharide has the following structure:

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→ 3)-α-L-FucpN-(1 → 3)-β-D-GlcpNAc-(1 → 7)-α-Neup5Ac-(2 →
            2|                    6|
       CH3C=NH       ~70%    OAc
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1. Introduction

The O-antigens of *Salmonella arizonae* are neutral or acidic polysaccharides containing various amino sugars (ref 1 and refs cited therein). In continuation of

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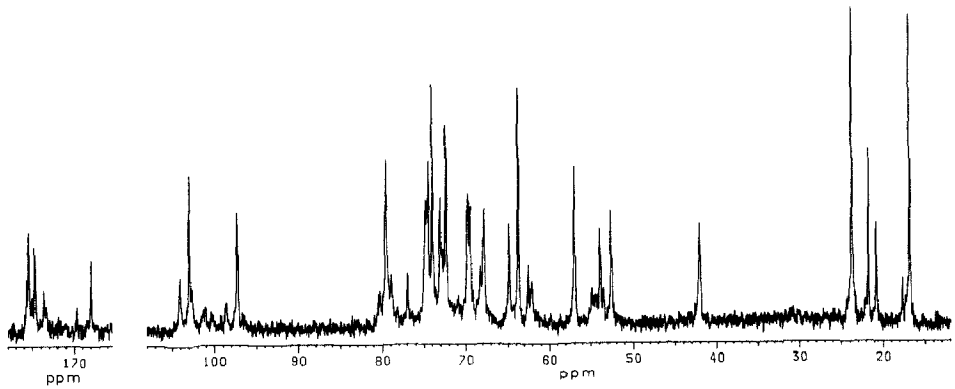

Fig. 1. 75-MHz ^{13}C NMR spectrum of the O-specific polysaccharide (PS-I).

our studies of the O-specific polysaccharides of this species, we now describe the structural determination of a new acidic O-antigen of *S. arizonae* O21 (*Arizona* 22) which contains *N*-acetylneuraminic acid and 2-acetamidino-2,6-dideoxy-L-galactose. A part of this work has been published as a preliminary communication [2].

2. Results and discussion

An acidic polysaccharide (PS-I) was obtained by mild acid degradation of the lipopolysaccharide isolated from dry bacterial cells of *S. arizonae* O21 by phenol–water extraction [3], and purified by anion-exchange chromatography on DEAE-Toyopearl 650M. Sugar analysis of PS-I revealed the presence of 2-amino-2-deoxy-D-glucose, 2-amino-2,6-dideoxy-L-galactose (L-FucN), and neuraminic acid.

The ^{13}C NMR spectrum of PS-I (Fig. 1) contained, inter alia, the characteristic signals for C-1 of GlcN and FucN at 97.2 and 102.9 ppm, C-2 of Neu at 104.1 ppm (the APT experiment revealed no proton attached to this carbon), C-6 (Me) of FucN at 16.7 ppm, C-3 (CH_2) of Neu at 42.0 ppm, C-2 of GlcN and FucN and C-5 of Neu carrying nitrogen at 52.6, 53.9, and 57.0 ppm, *N*-acetyl groups (Me at 23.7 ppm), *O*-acetyl group (Me at 21.7 ppm), and *N*-acetimidoyl group (Me at 20.7, C=N at 168.0 and 169.7 ppm). The spectrum appeared complex, most probably because of nonstoichiometric *O*-acetylation, existence of the *N*-acetimidoyl group in the *E* and *Z* forms (cf. the published data [4]), and the presence of some contaminants.

Treatment of PS-I with 0.1 M hydrogen chloride in methanol resulted in removal of the *O*-acetyl group but did not affect the *N*-acetimidoyl group. The ^{13}C NMR spectrum of PS-II thus obtained was, however, almost as complex as that of PS-I.

Solvolysis of PS-I with anhydrous hydrogen fluoride at 20°C cleaved selectively the glycosidic linkages of FucN and Neu to give the disaccharide **1** containing

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

Compound	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
2-Acetamidino-2,6-dideoxygalactose (FucN) $CH_3C{=}NH$									
PS-I	97.2	52.6	74.5	73.0	67.8	16.7			
PS-II	97.8	52.1	74.5	72.7	67.6	16.5			
2	96.8	54.7	68.9	72.1	68.1	16.4			
3	97.2	54.5	69.4	72.6	68.5	16.8			
	97.5								
4	96.6	54.7	68.4	71.7	67.6	16.0			
2-Acetamido-2-deoxyglucose (GlcNAc)									
PS-I [b]	102.9	57.0	79.8	69.7	74.8	64.8			
	102.9	57.0	79.8	69.7	77.0	62.5			
PS-II	103.0	56.5	80.4	69.5	76.6	61.9			
1	103.7	56.9	74.9	71.5	77.0	62.7			
2	99.3	54.8	72.4	71.2	72.9	61.8			
3 α	92.6	55.5	77.5	70.0	73.2	62.3			
β	96.7	58.2	80.1	70.0	77.0	62.3			
4	102.4	56.7	79.2	69.3	76.3	61.8			
N-Acetylneuraminic acid (Neu5Ac)									
PS-I	[c]	104.1	42.0	72.4	53.9	74.0	79.6	66.4	63.7
PS-II	[c]	[d]	41.3	72.4	53.8	73.1	79.7	66.8	63.3
1	173.1	96.4	35.7	66.8	51.2	77.2	72.3	71.5	61.5
4 [e]	176.4	97.0	39.9	67.3	53.7	70.0	79.1	72.3	63.3

[a] Additional signals: *N*- and *O*-acetyl groups at 23.1–23.9 and 21.7 ppm (Me), respectively, 173.7–176.5 ppm (CO); *N*-acetimidoyl group at 19.9–20.7 ppm (Me), 167.0–168.0 and 169.7 ppm (C=N); OMe in **2** at 56.4 ppm.
[b] The data for the *O*-acetylated unit are listed first.
[c] The signal lay in the region 173.7–175.6 ppm.
[d] The signal was not found.
[e] The data for the predominant β anomer.

N-acetyl-2,8-anhydroneuraminic acid. Structural elucidation of **1** using methylation analysis and ^1H NMR spectroscopy, including 2D shift-correlated (COSY) and 1D NOE spectroscopy, has been already described [2] and the ^{13}C NMR data of **1**, which were interpreted with the aid of heteronuclear $^{13}C/^1H$ shift-correlated (COSY) spectroscopy, are given in Table 1. Since the *N*-acetimidoyl group is known [4] to be stable towards anhydrous hydrogen fluoride, the presence in **1** of two *N*-acetylated amino sugars (GlcNAc and Neu5Ac) indicated that the *N*-acetimidoyl group was attached to the third amino sugar, namely to FucN.

β-D-Glc*p*NAc-(1 → 7)-2,8anhNeu*p*5Ac

1

Methanolysis of PS-I in the presence of 0.5 M hydrogen chloride led to the methyl bioside **2** isolated, together with methyl 2-acetamido-2-deoxy-α-D-gluco-

pyranoside and methyl (methyl 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranosidonate) [5], by HPLC on reversed-phase C18. Partial hydrolysis of the lipopolysaccharide with 0.1 M trifluoroacetic acid afforded a mixture of products from which the disaccharide **3** and the major oligosaccharide **4** were isolated by HPLC on the same phase. The structures of **2** and **3** were established on the basis of their ^{13}C NMR spectra (Table 1), which were interpreted using the chemical shifts for methyl 2-acetamidino-2,6-dideoxy-L-galactopyranoside [6], the bioside α-L-Fuc*p*NAc-(1 → 3)-β-D-Glc*p*NAc-(**1** → 2)-Gro [7], and the data on glycosylation effects [8]. One may conclude from the structures of **2** and **3** that the FucN residue was α and linked to the GlcN residue at position 3.

α-L-Fuc*p*N-(1 → 3)-α-D-Glc*p*NAc-(1 → OMe
2|
CH_3C=NH

2

α-L-Fuc*p*N-(1 → 3)-D-Glc*p*NAc
2|
CH_3C=NH

3

α-L-Fuc*p*N-(1 → 3)-β-D-Glc*p*NAc-(1 → 7)-β-Neu*p*5Ac
2|
CH_3C=NH

4

The ^1H NMR spectrum of **4** was completely assigned by 2D COSY and relayed coherence transfer COSY (COSYRCT) (Table 2). The coupling constants $J_{1,2}$ 4 and 8.5 Hz indicated that the FucN and GlcN residues were glycosidically linked and had the α and β configuration, respectively. The reducing Neu residue existed predominantly as the β anomer ($\delta_{H\text{-}3e}$ 2.11 ppm, cf. the published data [5]) with a small proportion of the α anomer ($\delta_{H\text{-}3e}$ 2.59 ppm, the data are not shown in Table 2). Linkage and sequence analysis of **4** was carried out with the help of 1D NOE spectroscopy. On preirradiation of H-1 of the FucN residue, an interresidue NOE was observed on H-3 of the GlcN residue. Preirradiation of H-1 of the GlcN residue caused an interresidue NOE on H-7 of the Neu residue. The structure of **4** thus established was confirmed independently by the ^{13}C NMR spectrum, which was assigned using heteronuclear $^{13}C/^1H$ shift-correlated (COSY) spectroscopy (Table 1) and accorded with the structures of the disaccharides **1–3**.

Therefore, **4** represented a trisaccharide repeating unit of PS-II, which resulted from selective cleavage of the glycosidic linkage of Neu.

Comparison of the ^{13}C NMR spectra of **4** and PS-II (Table 1) revealed the displacements of the signals for C-3 and C-2 of FucN from 68.4 and 54.7 ppm to

Table 2
^{1}H NMR data for trisaccharide **4** (δ in ppm, $^3J_{H,H}$ in Hz)

H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9
2-Acetamidino-2,6-dideoxygalactose			(FucN)					
			$CH_3C{=}NH$					
5.08	3.77	3.97	3.78	4.38	1.12			
$J_{1,2}$ 4	$J_{2,3}$ 10.5	$J_{3,4}$ 3	$J_{4,5}$ <1	$J_{5,6}$ 6.5				
2-Acetamido-2-deoxyglucose (GlcNAc)								
4.50	3.80	3.56	3.44	3.44	3.93			
					3.67			
$J_{1,2}$ 8.5	$J_{2,3}$ 11.5				$J_{6a,6b}$ 11.5			
N-Acetylneuraminic acid (Neu5Ac) [a]								
		1.77 [b]	4.18	3.77	4.29	3.76	3.69	3.69
		2.11 [c]						3.38
		$J_{3a,4}$ 11.5	$J_{3e,4}$ 5	$J_{4,5}$ 10	$J_{5,6}$ 10.5	$J_{6,7}$ <1	$J_{9a,9b}$ 12	
		$J_{3a,3e}$ 12						

[a] The data for the predominant β anomer. [b] H-3*a*. [c] H-3*e*.

74.5 and 52.1 ppm, respectively. These shifts are characteristic for α- and β-effects of glycosylation [8] and indicated that FucN in PS-II is substituted at position 3. The chemical shift for C-6 of Neu (73.1 and 74.0 ppm in PS-II and PS-I, respectively) was close to that (73.8-74.0 ppm) for methyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosidonic acid and differed from that (71.2–71.4 ppm) for the corresponding β anomer [5]; hence, the Neu residue in the polysaccharides is α-linked.

For GlcN in the ^{13}C NMR spectrum of PS-I, there were present two series of the signals belonging to the *O*-acetylated and nonacetylated sugar unit, the chemical shifts for the latter series being similar to those for the corresponding signals in the spectrum of PS-II (Table 1). The most significant displacements caused by *O*-acetylation were observed for C-5 and C-6 of GlcN (from 77.0 and 62.5 ppm to 74.8 and 64.8 ppm, respectively) and were indicative [9] of the presence of the *O*-acetyl group at position 6 of this monosaccharide residue. As judged by the relative intensities of the signals of the two series in the spectrum of PS-I, ca. 70% of the total of the GlcN residues are *O*-acetylated.

On the basis of these data, it was concluded that the O-specific polysaccharide of *S. arizonae* O21 (*Arizona* 22) has the following structure:

$$\rightarrow 3)\text{-}\alpha\text{-L-Fuc}p\text{N-}(1 \rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1 \rightarrow 7)\text{-}\alpha\text{-Neu}p\text{5Ac-}(2 \rightarrow$$

2 | $CH_3C{=}NH$ (at FucN)   ~70% 6 | OAc (at GlcNAc)

PS-I

This O-antigen is structurally related to the O-antigen of *S. arizonae* O61 [6], which also has a trisaccharide repeating unit that contains L-FucN carrying an

N-acetimidoyl group and D-GlcNAc, in common with the O-antigen of *S. arizonae* O21, and a higher sialic acid-like sugar, namely 7-acetamido-3,5,7,9-tetradeoxy-5-[(*R*)-3-hydroxybutyramido]-D-*glycero*-L-*galacto*-nonulosonic acid.

3. Experimental

General methods.—^{1}H NMR and ^{13}C NMR spectra were run on a Bruker AM-360 (for oligosaccharides) or Bruker AM-300 (for polysaccharides) spectrometer in D_2O at 20 or 60°C, respectively, using acetone as an internal standard (δ_H 2.225 ppm, δ_C 31.45 ppm). GLC, gel-permeation chromatography, anion-exchange chromatography, and HPLC were carried out as described [6].

Bacterial strain, growth, and isolation of lipopolysaccharide and O-specific polysaccharide.—The strain 40027 of *S. arizonae* O21:z_{29}:- (*Arizona* 22:16, 17, 18:-; Sol. 16 — NCTC 7347 — NCTC 1967, P.R. Edwards, Lexington, Kentucky, USA) was obtained from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest). The bacterium was cultivated on a solid medium at 37°C as described [10,11], and the cells were dried with acetone and extracted with aq 45% phenol [3]. The lipopolysaccharide obtained was degraded with aq 2% AcOH at 100°C for 1.5 h and the O-specific polysaccharide (PS-I) was isolated as described previously [12].

Sugar analysis.—The samples were hydrolysed with 2 M CF_3CO_2H at 120°C for 3 h, and the hydrolysates were evaporated in vacuo and converted conventionally into alditol acetates. For identification of neuraminic acid, the samples were methanolysed (0.5 M HCl–MeOH, 100°C, 3 h) and acetylated. Both types of the derivatives were analysed by GLC. Absolute configurations of the monosaccharides, including neuraminic acid, were determined by GLC according to the published [13,14] and modified [15] methods using (*R*)- and (*S*)-2-butanol.

O-Deacetylation.—PS-I (60 mg) was kept in 0.1 M HCl–MeOH (5 mL, 20°C, 16 h), the solution was evaporated, and PS-II (52 mg) was isolated by gel-permeation chromatography on Sephadex G-50 in pyridine-acetate buffer (pH 4.5).

Solvolysis with anhydrous HF.—PS-I (50 mg) was treated with anhyd HF (ca. 3 mL) under stirring at 20°C for 2 h, HF was removed in vacuo over solid NaOH, the products were separated by gel-permeation chromatography on TSK HW-40 (S) in water, and the disaccharide **1** (11 mg) was isolated from the oligosaccharide fraction by HPLC on Nucleosil C18 in 0.05% CF_3CO_2H.

Solvolysis, under the same conditions, of *N*-acetylneuraminic acid or methyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosidonic acid led to *N*-acetyl-2,7-anhydroneuraminic acid [2] as the only product.

Partial methanolysis.—PS-I (40 mg) was heated with 0.5 M HCl–MeOH (5 mL, 60°C, 1 h), the solution was evaporated, and the bioside **2** (7 mg) and methyl glycosides of monosaccharides were isolated by HPLC as described above.

Partial hydrolysis.—The lipopolysaccharide (200 mg) was hydrolysed with 0.1 M CF_3CO_2H (8 mL, 100°C, 3 h), the precipitate of lipid was removed by centrifugation, the supernatant solution was evaporated, the products were separated by

HPLC as described above, and the disaccharide **3** (6 mg) and the trisaccharide **4** (17 mg) were additionally purified by gel-permeation chromatography on TSK HW-40 (S).

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