Carbohydrate Research, 231 (1992) 1–11
Elsevier Science Publishers B.V., Amsterdam

The structure of the O-specific polysaccharide chain of the lipopolysaccharide of *Salmonella arizonae* O61

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(Received August 5th, 1991; accepted September 12th, 1991)

ABSTRACT

The O-specific polysaccharide was obtained by mild degradation of the *Salmonella arizonae* O61 lipopolysaccharide with acid. It contained 2-acetamido-2-deoxy-D-glucose, 2-acetamidino-2,6-dideoxy-L-galactose (FucAm), and 7-acetamido-3,5,7,9-tetradeoxy-5-[(*R*)-3-hydroxybutyramido]-D-*glycero*-L-*galacto*-nonulosonic acid (Sug). On the basis of partial acid hydrolysis with 0.1 M HCl, solvolysis with anhydrous HF in methanol, and ^{1}H- and ^{13}C-NMR analysis (including $^{1}H/^{13}C$ inversely correlated spectroscopy for localisation of *N*-acyl substituents), it was concluded that the O-specific polysaccharide had the following structure.

→ 3)-α-L-FucAm-(1 → 3)-α-D-GlcNAc-(1 → 8)-β-Sug-(2 →

The O-antigen of *S. arizonae* O61 is structurally related to that of *Pseudomonas aeruginosa* O12, thus explaining the known serological cross-reactivity between these micro-organisms.

INTRODUCTION

The structures of the O-antigens of *S. arizonae* O59, O63, and O64 have been established[1–3] and that of serogroup O59 was found to be serologically[4] and structurally[1,5] related to the O-antigen of *P. aeruginosa* O11 (Lányi–Bergan classification[6]). We report now the structure of the O-specific polysaccharide chain of the lipopolysaccharide of *S. arizonae* O61 (*Arizona* O26), which is known[4] to cross-react serologically with *P. aeruginosa* O12.

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RESULTS AND DISCUSSION

The lipopolysaccharide of *S. arizonae* O61 was isolated from the dry bacterial cells by the Westphal and Jann procedure[7] and cleaved with dilute acetic acid to give an O-specific polysaccharide (PS) that was isolated by gel-permeation chromatography on Sephadex G-50 followed by anion-exchange chromatography and had $[\alpha]_D$ + 22° (water).

The ^{13}C-NMR spectrum (Fig. 1, Table I) of the PS indicated a trisaccharide repeating unit (signals for three anomeric carbons at 94.6, 96.6, and 104.7 ppm), with one of the components being a keto sugar (data of the attached-proton test). There were also present signals for four carbons bearing nitrogen (52.4–54.6 ppm), one CH_2OH group of a hexopyranose (61.6 ppm), two Me groups (15.3 and 16.9 ppm) and one CH_2 group (42.5 ppm) belonging to deoxy sugars, one COOH group (173.2 ppm), other sugar carbons (in the region 67.4–77.2 ppm), one *N*-acetimidoyl group (Me at 20.4 ppm) and >C=N at 167.6 ppm (cf. ref. 8), one *N*-(3-hydroxybutyryl) group (C-1 at ~ 175 ppm, C-2,3 at 46.5 and 66.1 ppm, respectively, and C-4 at ~ 23 ppm, cf. ref. 9), and two NAc groups (Me at ~ 23 ppm, CO at ~ 175 ppm). The spectrum also contained a number of signals having much smaller intensities, which may belong to a minor type of repeating unit or to a contaminating polysaccharide.

Hydrolysis of the PS with 4 M HCl revealed the presence of 2-amino-2-deoxyglucose and 2-amino-2,6-dideoxygalactose in the ratio ~ 1:1, which were identified conventionally by GLC of the alditol acetates. The $[\alpha]_D$ values showed the D configuration of the former sugar and the L configuration of the latter. The

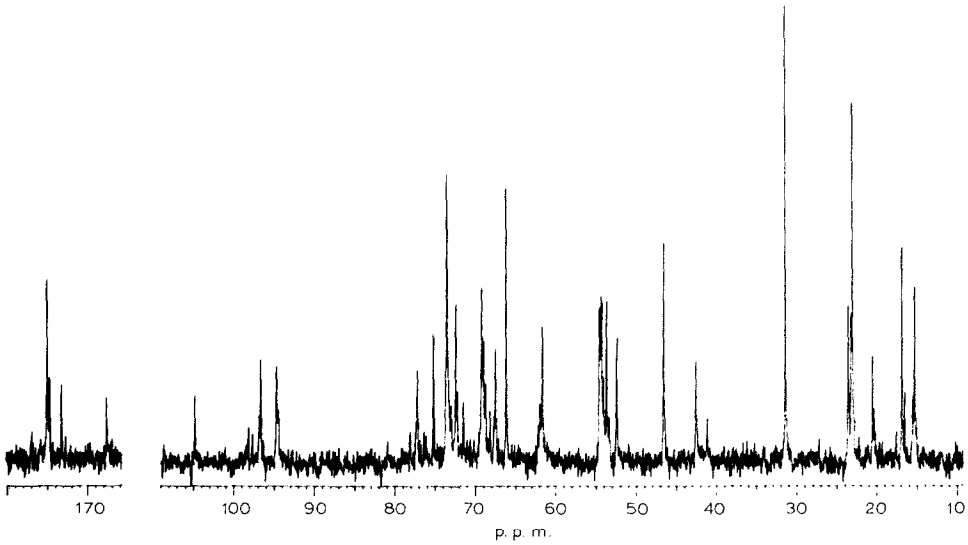


Fig. 1. ^{13}C-NMR spectrum of *S. arizonae* O61 O-specific polysaccharide.

TABLE I

Chemical shifts in the ^{13}C-NMR spectra (δ in ppm) [a]

Compound	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
Unit I (5,7-diamino-3,5,7,9-tetradeoxy- D-glycero-L-galacto-*nonulosonic acid)*									
1	[b]	104.5	41.7	68.2	53.4	74.1	54.5	73.5	15.3
2 [c]	173.1	104.4	42.3	69.4	53.8	73.5	54.5	73.5	15.3
3	174.3	96.6	40.7	68.1	53.8	71.6	54.0	73.0	15.6
5	174.5	96.7	40.7	68.0	53.8	71.5	54.0	72.6	15.4
6	173.2	104.7	42.5	69.2	53.6	73.5	54.4	73.5	15.3
7 [c]	173.3	104.6	42.3	69.5	53.8	73.5	54.5	73.5	15.0
*Unit II (α-*D*-GlcN or α-*D*-QuiN)*									
1	94.7	54.6	72.4	70.4	73.3	61.2			
2 [c]	94.6	54.9	72.6	75.4	69.2	17.9			
3	94.4	54.6	72.3	71.0	73.5	61.8			
5	94.2	54.4	76.8	69.1	73.4	61.7			
6	94.6	54.6	77.2	69.1	73.5	61.6			
7 [c]	94.2	54.5	76.8	74.6	69.2	18.0			
*Unit III (α-*L*-FucN)*									
1	98.3	52.4	76.7	72.4	67.0	16.8			
2 [c]	98.1	52.6	76.5	72.3	67.1	16.9			
4	98.2	54.3	69.4	72.2	67.8	16.5			
5	96.7	54.1	68.9	72.1	68.0	16.6			
6	96.6	52.4	75.2	72.4	67.4	16.9			
7 [c]	96.6	52.3	75.3	72.4	67.3	16.9			
N-*(3-Hydroxybutyryl)*									
1	[b]	46.5	66.2	23.0					
3	175.1	46.7	66.2	22.9					
5	174.9	46.8	66.2	22.9					
6	175.0	46.5	66.1	23.1					

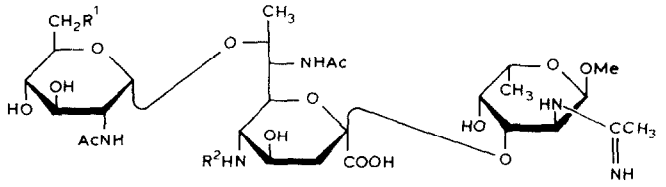
[a] Assignment of the spectrum of **5** was made with the help of 2D heteronuclear ^{1}H/^{13}C shift-correlated (XHCORRD) spectroscopy. Tentative assignments of the spectra of **1**, **3**, **4**, and **6** were based on their comparison with the spectra of **2**, **5**, and **7**. Additional signals: NAc at 23.0–23.6 (Me), 174.7–175.6 (CO) ppm; *N*-acetimidoyl at 20.0–20.5 (Me) and 167.2–167.8 (>C=NH) ppm; MeO at 56.4–56.5 ppm. [b] Not found. [c] Data from ref. 8.

R configuration of 3-hydroxybutyric acid, obtained by a milder hydrolysis of the PS with 2 M CF_3COOH, was established by GLC of the corresponding (*R*)-2-octyl ester according to the method reported[10], except that the *O*-acetyl derivative was used instead of the *O*-methyl derivative. The keto sugar, which seemed to be the acidic component of the polysaccharide, was not detected after hydrolysis, probably due to its lability.

Solvolysis of the PS with anhydrous HF in methanol gave the oligosaccharide **1** as the main product. As judged by the ^{13}C-NMR data, **1** contained all the components of the repeating unit. The spectrum showed that **1** was a methyl glycoside (MeO at 56.4 ppm) of a trisaccharide similar to, but not identical with, the oligosaccharide **2**, which was obtained[8] by the analogous solvolysis of the O-antigen of *P. aerugonosa* O12 and consisted of 2-acetamido-2,6-dideoxy-D-glu-

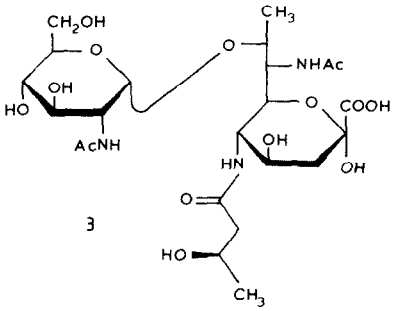
cose, 2-acetamidino-2,6-dideoxy-L-galactose, and 5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-L-*galacto*-nonulosonic acid[8]. The differences between the spectra of **1** and **2** were associated with the replacement of the residue of 2-amino-2,6-dideoxyglucose (C-4,5,6 at 75.4, 69.2, and 17.9 ppm) and one of the NAc groups (C-2 in the region 23.0–23.3 ppm) in **2** by the GlcN residue (C-4,5,6 at 70.4, 73.3, and 61.2 ppm) and the 3-hydroxybutyryl group (C-2,3,4 at 46.5, 66.2, and 23.0 ppm), respectively, in **1** (Table I).

Thus, it is proposed that the third sugar component of the *S. arizonae* O61 polysaccharide (PS) is 5,7-diamino-3,5,7,9-tetradeoxy-D-*glycero*-L-*galcto*-non-



1 $R^1 = OH$, $R^2 = CH_3CHCH_2CO$ (OH)

2 $R^1 = H$, $R^2 = Ac$



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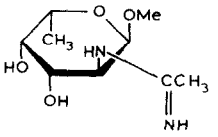

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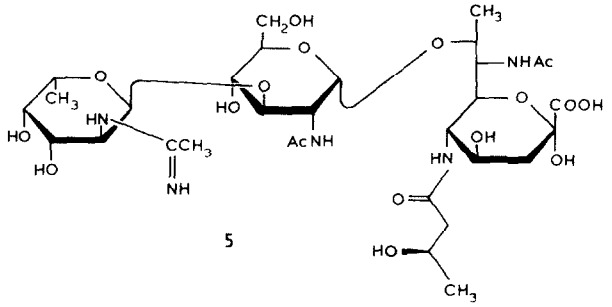


5

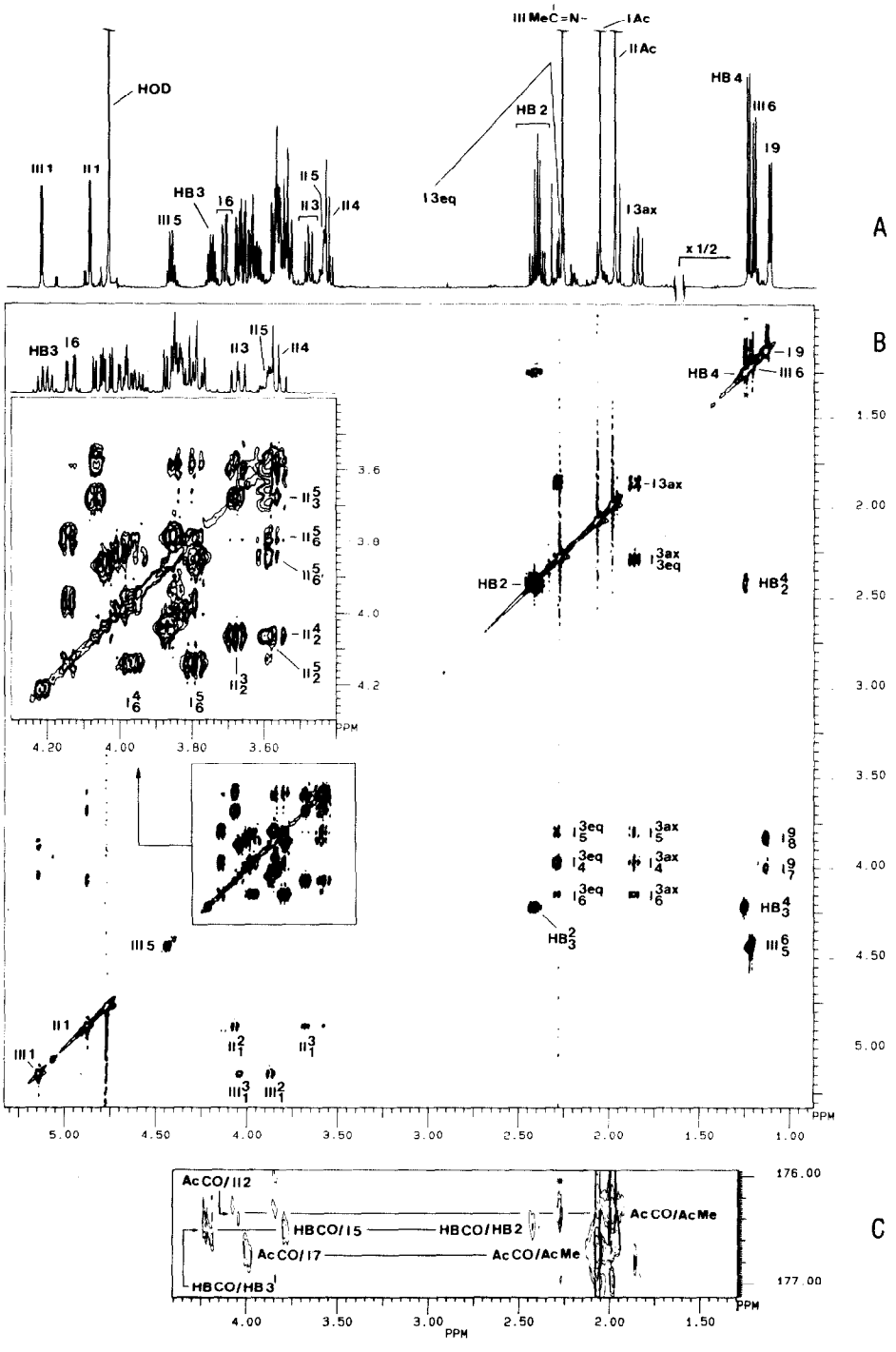
ulosonic acid. Moreover, the similar positions of the signals for C-6 (δ 74.1 and 73.5) and C-9 (δ 15.3 and 15.3) of this monosaccharide in the ^{13}C-NMR spectra of **1** and **2**, respectively, proved that (*a*) the ulosonic acid in **1** has the same β configuration as in **2** (the chemical shift for C-6 in ulosonic acids is known[11] to depend significantly on the orientation of the C-1 carboxyl group), and (*b*) in both **1** and **2**, the ulosonic acid was glycosylated at O-8 (C-9 of the unsubstituted sugar resonated[8] at ~ 20 ppm).

Partial hydrolysis of **1** with 0.1 M HCl cleaved the acid-labile glycosidic linkage of the ulosonic acid and gave the disaccharide **3** and methyl 2-acetamidino-2,6-dideoxy-α-L-galactopyranoside (**4**). Compound **4** was identified on the basis of the ^{1}H- and ^{13}C-NMR spectra (Tables I and II). Its formation proved that, in **1**, as in **2**, the FucN residue carried the *N*-acetimidoyl group. The displacement of the signal for C-2 of this monosaccharide from 54.3 ppm in the ^{13}C-NMR spectrum of **4** to 52.4 ppm in the spectrum of **1** was due to the β-effect of glycosylation[12] and proved 3-substitution of the FucAm residue in **1**.

Analogous mild hydrolysis of the PS with acid resulted in essentially a single product, viz., the oligosaccharide **5**. According to the ^{13}C-NMR spectrum (Table I), **5**, like the oligoside **1**, contained all the components of the PS. The relatively low-field position (76.8 ppm) of the signal for C-3 of the GlcN residue, compared to that for C-3 in the spectrum of the corresponding unsubstituted sugar (e.g., 72.0 ppm for 2-acetamido-2-deoxyglucose[12] or 72.4 ppm for **1**), showed 3-substitution of this sugar residue in **5**.

The ^{1}H-NMR spectrum of **5** (Fig. 2A) was assigned completely with the use of total correlation (TOCSY)[13] homonuclear Hartmann–Hahn spectroscopy (HOHAHA)[14] (Fig. 2B, Table II). The data proved that the FucN residue (unit III) and the GlcN residue (unit II) were α-linked (the signals for H-1 at 4.877 and 5.142 ppm, $J_{1,2}$ 3.7 and 4.0 Hz, respectively) and, hence, the residue of the ulosonic acid (unit I) occupied the reducing terminus in **5**. The coupling constants for the residue of the ulosonic acid in **2** and **5** were very similar (Table II), thus confirming again the identity of the general configurations of these higher sugars.

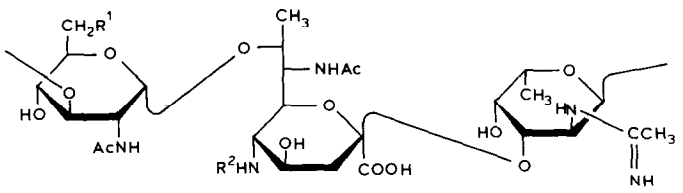
The location of the 3-hydroxybutyryl group in **5** was determined with the aid of inversely correlated ^{1}H/^{13}C spectroscopy. Since the three carbonyl resonances were nearly coincident, the pertinent region of the spectrum was recorded with a high digital resolution in order to discriminate unambiguously the ^{13}C co-ordinates of the relevant correlation cross-peaks (Fig. 2C). One of these resonances (δ 176.50 ppm) correlated with both the methylene and methine proton resonances of the 3-hydroxybutyryl residue, and was thus assigned unequivocally to C-1 of this group. At the same time, it showed a strong cross-peak at the ^{1}H co-ordinate of H-5 of unit I (δ 3.791), thus pointing to the localisation of the 3-hydroxybutyryl substituent at position 5 of unit I. Although the chemical shift for H-6 of unit II was almost identical (δ 3.786), an alternative localisation was unlikely since no correlation with H-6′ was detectable. The correlations of the two acetyl carbonyl


A
B
C
HOD
HB3
HB2
HB4
13eq
13ax
x 1/2
PPM
AcCO/I12
HBCO/I5
HBCO/HB2
AcCO/I7
AcCO/AcMe
HBCO/HB3

resonances (δ 176.37 and 176.72) with the respective sugar protons were self-explanatory.

From these data, it followed that the ulosonic acid carried the 3-hydroxybutyryl group at N-5 and, therefore, the acetyl groups were located at N-7 of this sugar and at N-2 of the GlcN residue.

Finally, comparison of the ^{13}C-NMR spectra of the O-antigens of *S. arizonae* O61 and *P. aeruginosa* O12 (Table I) indicated that they differ in the same manner as those of trisaccharides **1** and **2**. This conclusion, together with the data described above, permitted the structure of the *S. arizonae* O61 O-specific polysaccharide to be deduced as **6**.



6 R^1 = OH, R^2 = $CH_3CH(OH)CH_2CO$ (*S. arizonae* O61)

7 R^1 = H, R^2 = Ac (*P. aeruginosa* O12)

It is noteworthy that structure **6** contains both acidic (carboxyl) and basic (acetamidino) functions, whereas all of the O-specific polysaccharides of the genus *Salmonella* studied before[15], including *Salmonella arizonae*[1–3], did not contain any charged groups. The O-antigen of *S. arizonae* O61 is the third bacterial polysaccharide found to contain a derivative of 5,7-diamino-3,5,7,9-tetradeoxy-D-*glycero*-L-*galacto*-nonulosonic acid and the first in which this higher sugar carries the 3-hydroxybutyryl group. The di-*N*-acetyl derivative of this sugar has been identified as a component of the O-antigens of *P. aeruginosa* O12 (ref. 8) and *Vibrio alginolyticus* strain 945-80 (ref. 16). The *N*-acetimidoyl group also occurs in natural carbohydrates very rarely; to the best of our knowledge, it has been found hitherto as a component of O-antigens of *P. aeruginosa* O2 (ref. 17), O12 (ref. 8), and *Vibrio cholerae* O2 (ref. 18).

Fig. 2. NMR spectra of the trisaccharide **5**. A, The resolution-enhanced 1D, 500-MHz, ^{1}H-NMR spectrum [arabic numerals refer to the protons in the sugar residues denoted by the Roman numerals (see formula **5**); HB, 3-hydroxybutyryl]. B, The TOCSY–HOHAHA spectrum [diagonal peaks are labelled as in A; for the correlation cross-peaks, the horizontal (F2) and vertical (F1) axes give the chemical shifts of the protons designated by the superscript and subscript, respectively]. C, Part of the inversely correlated ^{1}H/^{13}C spectrum (the ordinate of each cross-peak defines the ^{13}C chemical shift of the relevant carbonyl carbon and the abscissa corresponds to the chemical shift of the proton coupled with this carbon; the undesirable resonances were folded outside of the spectral region shown except for the >C=N resonance, marked with the asterisk, which was folded into this window).

TABLE II

^{1}H-NMR data[a] (δ in ppm, J in Hz)

	H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9
Methyl 2-acetamidino-2,6-dideoxy-α-L-galactopyranoside **(4)**									
δ	4.84	3.87	4.01	3.83	4.08	1.24			
J	$J_{1,2}$ 3.6	$J_{2,3}$ 10.7	$J_{3,4}$ 3.3	$J_{4,5}$ < 1	$J_{5,6}$ 6.5				
Oligosaccharide **5**									
Unit I (5,7-diamino-3,5,7,9-tetradeoxy- D-glycero-L-galacto-*nonulosonic acid)*									
δ			1.862 [b] 2.288 [c]	3.968	3.791	4.140	3.997	3.827	1.130
J [d]			$J_{3ax,4}$ 11.5 (~12)	$J_{3eq,4}$ 4.9 (4.2)	$J_{4,5}$ 10.1 (~10)	$J_{5,6}$ 10.3 (~10)	$J_{6,7}$ 2.2 (1.5)	$J_{7,8}$ 9.5 [e]	$J_{8,9}$ 6.1 (~6)
Unit II (α-D-GlcN)									
δ	4.877	4.057	3.677	3.565	3.597	3.786 (H-6)[f] 3.849 (H-6′)			
J	$J_{1,2}$ 3.7	$J_{2,3}$ 10.3	$J_{3,4}$ 8.3	$J_{4,5}$ 9.9	$J_{5,6}$ 4.4	$J_{5,6'}$ 2.1			
Unit III (α-L-FucN)									
δ	5.142	3.868	4.039	3.851	4.433	1.217			
J	$J_{1,2}$ 4.0	$J_{2,3}$ 10.5	$J_{3,4}$ 3.2	$J_{4,5}$ 1.1	$J_{5,6}$ 6.6				
N-*(3-hydroxybutyryl)*									
δ		2.403 2.431	4.211	1.249					
J		$J_{2a,3}$ 7.3 $J_{2a,2b}$ 14.1	$J_{2b,3}$ 5.9	$J_{3,4}$ 6.3					

[a] Additional signals: NAc at 1.987 and 2.070 ppm; *N*-acetamidoyl at 2.27 ppm in **4** and 2.278 ppm in **5**; OCH_3 at 3.37 ppm. [b] H-3*ax*, $J_{3ax,3eq}$ 13.1 Hz. [c] H-3*eq*. [d] In parentheses are given the data[8] for 5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-L-*galacto*-nonulosonic acid. [e] Not determined. [f] $J_{6,6'}$ 12.2 Hz.

The similarity of the structure **7** of the O-antigen of *P. aeruginosa* O12 to the structure **6** explains the known[4] serological cross-reactivity of this micro-organism and *S. arizonae* O61.

EXPERIMENTAL

General methods.—Optical rotations were measured with a Jasco DIP 360 polarimeter at 25°. Solutions were freeze-dried or evaporated in vacuo at 40°. The growth[5] of bacteria and isolation[7,8] of the lipopolysaccharide and the O-specific polysaccharide (PS) were performed as described.

Chromatography.—Ascending PC was carried out on Whatman 3MM paper in pyridine–EtOAc–acetic acid–water (5:5:1:3). Substances were detected with alkaline silver nitrate. GLC was performed with a Hewlett–Packard 5890 instrument equipped with a flame-ionisation detector and a glass capillary column (0.2 mm × 25 m) coated with Ultra 1 stationary phase. Gel-permeation chromatography was performed on a column (70 × 3.5 cm) of Sephadex G-50, using a pyridine acetate buffer (pH 5.5), with monitoring by the orcinol–H_2SO_4 reaction with the help of a Technicon sugar analyser or a column (80 × 1.6 cm) of TSK HW 40 (S) in water with monitoring by a Knauer differential refractometer. Anion-exchange chromatography was carried out on a column (20 × 2 cm) of DEAE-Toyopearl 650M eluted with a linear gradient of NaCl (0 → 0.5 M) in water and monitored by a Knauer variable wavelength monitor at 220 nm. HPLC was performed on a column (30 × 0.8 cm) with Silasorb SPH C18 eluted with 0.05% of trifluoroacetic acid in 2–4% aq MeOH and monitored as in anion-exchange chromatography.

NMR spectroscopy.—The ^{13}C-NMR spectra were recorded on a Bruker AM-300 spectrometer for solutions in D_2O at 60° for the polysaccharide, or 30° for oligosaccharides (internal acetone, δ 31.45). The standard Bruker software was used to obtain the 2D heteronuclear $^{13}C/^{1}H$ shift-correlated (XHCORRD) spectrum of **5** (for details see ref. 19).

The ^{1}H-NMR spectra of **4** and **5** were recorded with a Bruker WM-250 or AM-500 spectrometer, respectively, for solutions in D_2O at 30° (internal acetone, δ 2.23). The TOCSY[13]–HOHAHA[14] spectrum of **5** was obtained under the following conditions: the spectral width was 2200 Hz and the spectral size in the time domain was 2048 (F_2) × 512 (F_1). For each t_1, 64 transients were accumulated. The matrix was zero-filled in both dimensions, multiplied by a phase-shifted ($\pi/2$) squared sine-bell window function, and Fourier-transformed in the magnitude mode.

The inversely correlated $^{1}H/^{13}C$ spectrum of **5** was obtained as described[20,21]. The preparatory sequence by Brühwiler and Wagner[21] was applied, and the experiment was optimised for a long-range $^{1}H/^{13}C$ coupling constant of 7 Hz. The spectral width for ^{1}H (F_2) was 3000 Hz, and 2048 data points were used in this dimension. In order to increase the digital resolution for ^{13}C (F_1), a narrow spectral window of 1888 Hz in this dimension was registered, with the carrier

placed in this window's center at 175 ppm. For each of the 256 incremented t_1 values, 128 transients were accumulated, and the spectrum was transformed as above, except that zero-filling was made in t_1 only.

Complete acid hydrolysis.—The PS was hydrolysed in sealed ampoules with 2 M CF_3COOH (120°, 2 h) for liberation of 3-hydroxybutyric acid, or 4 M HCl (100°, 3 h) for isolation of the amino sugars. 3-Hydroxybutyric acid was extracted with EtOAc and converted into the (*R*)-2-octyl ester by heating (140°, 3 h) with (*R*)-2-octanol in the presence of a drop of CF_3COOH. The product was *O*-acetylated conventionally and analysed by GLC. Monosaccharides were separated by preparative PC, and individual amino sugars were converted with dilute HCl into the corresponding hydrochlorides.

Solvolysis with anhydrous HF.—Methanol (3 mL) and anhydrous HF (~ 10 mL) were added to the PS (70 mg), and the mixture was stirred for 2 h at room temperature and then poured into cold ether (200 mL). The precipitate was separated with a stainless-steel filter, washed several times with cold ether, and dissolved in water; **2**, $[\alpha]_D$ + 95° (*c* 0.6), was isolated by gel-permeation chromatography on TSK HW 40 (S) followed by HPLC.

Partial acid hydrolysis.—The PS (80 mg) was heated with 0.1 M HCl (3 mL) for 5 h at 100°, the hydrolysate was evaporated, and **5**, $[\alpha]_D$ – 5.4° (*c* 1), was isolated by gel-permeation chromatography on TSK HW 40 (S). Analogous hydrolysis of **1** gave **3**, $[\alpha]_D$ + 86° (*c* 0.6), and **4**, $[\alpha]_D$ – 77° (*c* 0.8), which were isolated by HPLC.

ACKNOWLEDGMENT

J. D. was supported by the Fritz Thyssen Stiftung.

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