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Structure of the O-specific polysaccharide of *Salmonella enterica* ssp. *arizonae* O50 (*Arizona* 9a,9b)

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Abstract

On the basis of sugar and methylation analysis, selective removal of 3,6-dideoxy-L-*xylo*-hexose (colitose, Col), ^{1}H and ^{13}C NMR spectroscopy, including 1D NOE, 2D COSY, and 2D H-detected $^{1}H,^{13}C$ heteronuclear multiple-quantum coherence (HMQC), the following structure of the repeating unit of the O-specific polysaccharide of *Salmonella enterica* ssp. *arizonae* O50 (*Arizona* 9a,9b) was established:

α-Col*p*-(1→2)-β-D-Gal*p*
1
↓
3
→3)-β-D-Glc*p*NAc-(1→6)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→

The O-antigen studied includes a trisaccharide fragment α-Col *p*-(1 → 2)-β-D-Gal *p*-(1 → 3)-β-D-Glc *p*NAc, which is a colitose ('3-deoxy-L-fucose') analogue of the Lewis^{d} (precursor) blood group antigen. © 1997 Elsevier Science Ltd.

Keywords: O-Antigen; Bacterial polysaccharide, structure; Lipopolysaccharide; Enterobacteria; 3,6-Dideoxy-L-*xylo*-hexose (colitose); *Salmonella enterica* ssp. *arizonae*

1. Introduction

Strains of *Salmonella enterica* ssp. *arizonae* (IIIa and IIIb) are distributed among more than 30 *Salmonella* O-serogroups [1]. The serological O-specificity of the strains is defined by the polysaccharide chain of the outer-membrane lipopolysaccharide (O-antigen). Structures of the *S. enterica* ssp. *arizonae* O-antigens have been established for only a few serogroups [2–7], and most of them were found to be acidic [4–7]. We now report the structure of a

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new neutral O-specific polysaccharide of *S. enterica* ssp. *arizonae* O50 (*Arizona* 9a,9b).

2. Results and discussion

The lipopolysaccharide was isolated from dry bacterial cells by extraction with hot aqueous phenol [8] and degraded with sodium acetate buffer at pH 4.5 at 100 °C to cleave the lipid moiety. The subsequent fractionation of a mixture of poly- and oligo-saccharides obtained by GPC on Sephadex G-50 afforded an O-specific polysaccharide (PS-I) that eluted shortly after the void volume of the column as a wide peak.

Sugar analysis of PS-I revealed a 3,6-dideoxy-*xylo*-hexose (abequose or colitose) and galactose, identified using a sugar analyser after hydrolysis with 0.5 M trifluoroacetic acid (100 °C, 1 h), and 2-amino-2-deoxyglucose, identified using an amino acid analyser after hydrolysis with 2 M trifluoroacetic acid (120 °C, 2 h). Determination of absolute configurations by GLC of acetylated glycosides with (+)-2-butanol according to a modified method [9] showed that GlcN has the D configuration; similar analysis of acetylated glycosides with (+)-2-octanol proved that Gal has the D configuration, whereas 3,6-dideoxy-*xylo*-hexose has the L configuration and is, thus, colitose (Col).

The ^{13}C NMR spectrum of PS-I (Fig. 1a) contained signals for five anomeric carbons in the region δ 100.0–104.7, four CH_2O groups (C-6 of Gal and GlcN), of which three were nonsubstituted (δ 61.9–62.3) and the fourth was substituted (δ 69.3, data of attached-proton test [10]), methyl and methylene groups (C-6 and C-3 of Col) at δ 16.8 and 34.2, respectively, two carbons bearing nitrogen (C-2 of GlcN) at δ 55.2 and 56.1, and other sugar ring carbons in the region δ 64.5–80.6, as well as two *N*-acetyl groups (CH_3 at δ 23.6 and 23.8, CO at δ 174.9 and 175.2).

The ^1H NMR spectrum of PS-I, a part of which is shown in Fig. 2, contained two series of signals in the

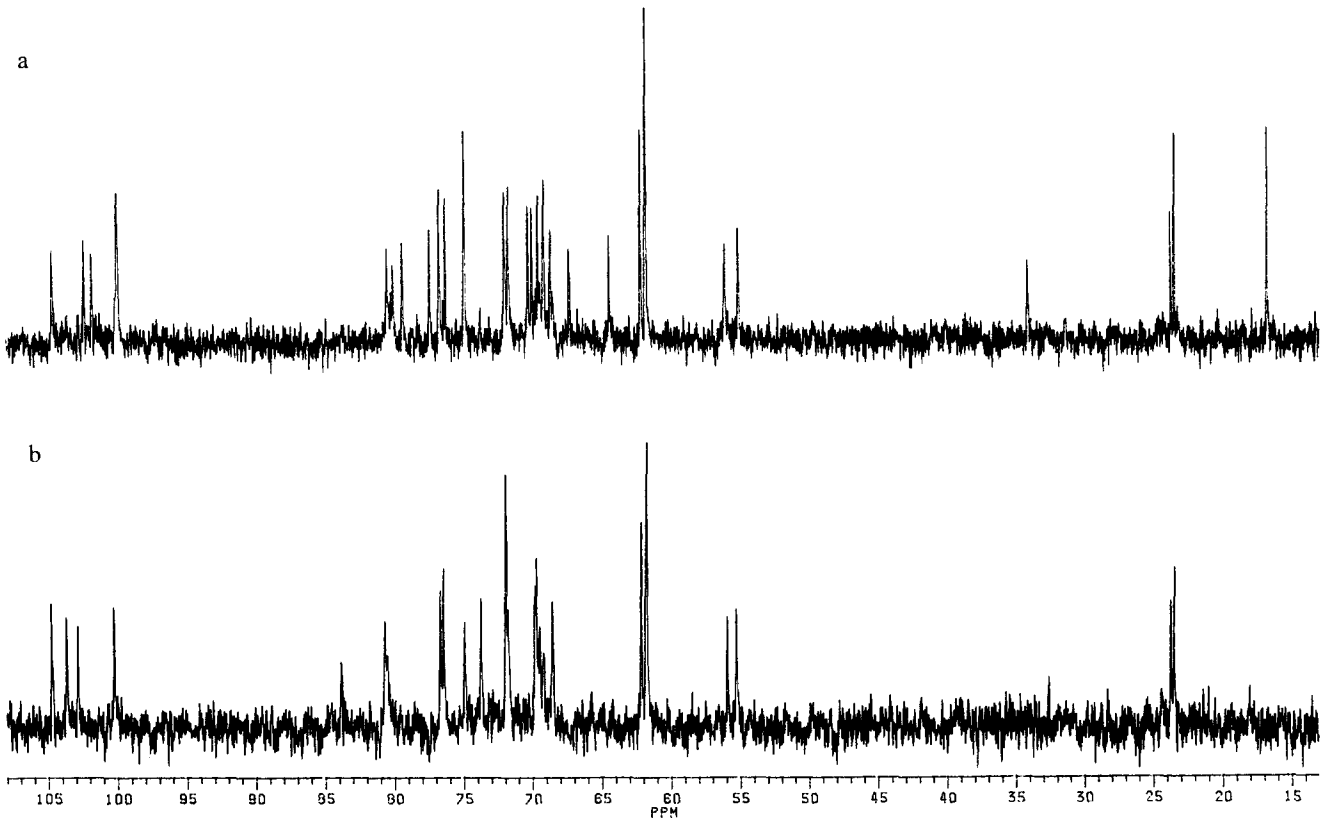


Fig. 1. ^{13}C NMR spectra of (a) O-specific polysaccharide (PS-I) and (b) colitose-free polysaccharide (PS-II). Signals for CO groups are not shown.

ratio ca. 5:1, thus indicating structural heterogeneity. The major series included inter alia signals for five anomeric protons in the region δ 4.60–5.46, methyl and methylene groups of Col at δ 1.19 (d, 3 H, $J_{5,6}$ 7 Hz, H-6), 1.81 ($J_{2,3ax}$ 12, $J_{3ax,3eq}$ 13, $J_{3ax,4}$ 4 Hz, H-3ax), and 2.06 ($J_{2,3eq}$ 5, $J_{3eq,4}$ < 2 Hz, H-3eq), and two *N*-acetyl groups at δ 2.05 and 2.07 (s, each 3 H).

Therefore, PS-I consists mainly of pentasaccharide repeating units containing two residues each of D-Gal

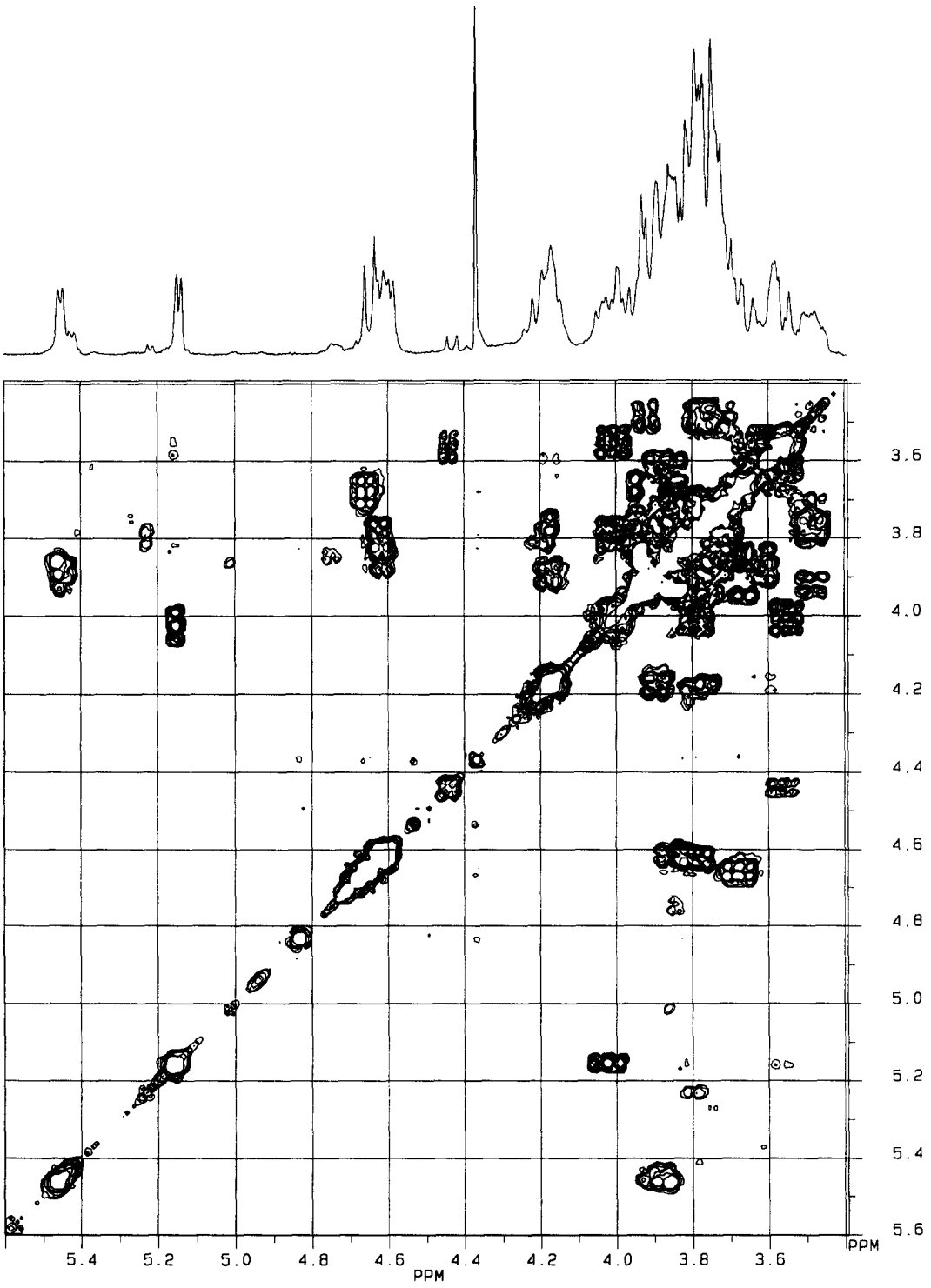


Fig. 2. Part of a 2D COSY spectrum of the O-specific polysaccharide (PS-I). The corresponding part of the ^{1}H NMR spectrum is displayed along the horizontal axis.

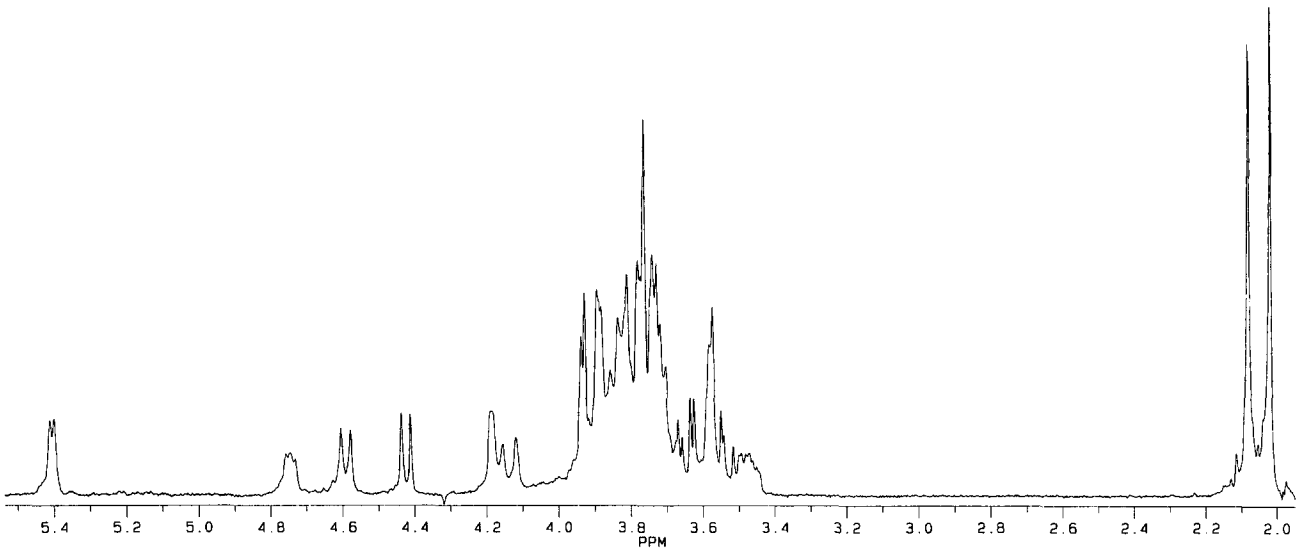


Fig. 3. ^{1}H NMR spectrum of the colitose-free polysaccharide (PS-II).

and D-GlcNAc and one residue of Col. The minor repeating units lacked Col (see below).

To obtain a colitose-free polysaccharide, the lipopolysaccharide was degraded with 2% acetic acid, and the resulting polysaccharide (PS-II) was isolated by GPC on Sephadex G-50.

The ^{1}H and ^{13}C NMR spectra of PS-II (Figs. 3 and 1b, Tables 1 and 2, respectively) were typical of a regular polysaccharide. Particularly, they contained signals for four anomeric atoms (δ_H 4.42–5.41, δ_C 100.3–104.8) and no signals for Col.

Therefore, in PS-I, Col is a lateral sugar residue, and PS-II has a tetrasaccharide repeating unit containing two residues each of D-Gal and D-GlcNAc.

Methylation analysis (Table 3) demonstrated that PS-II is branched with a lateral Gal residue and a 3,6-disubstituted GlcNAc residue at the branching point, the second Gal and the second GlcNAc residues

Table 1
^{1}H NMR chemical shifts (in ppm). Chemical shifts for the NAc groups are δ 2.05 and 2.07

Sugar residue	H-1	H-2	H-3 (H-3ax, H-3eq)	H-4	H-5	H-6 (H-6a, H-6b)
Colitose-free polysaccharide (PS-II)						
→ 3)-β-D-Glc*p*NAc-(1 → **(A)**	4.59	3.84	3.77	3.73	3.47	3.91, 3.76
→ 6)-β-D-Glc*p*NAc-(1 → **(B)**	4.76	3.81	3.83	3.57	3.57	4.15, 3.87
3 ↑						
→ 3)-β-D-Gal *p*-(1 → **(C)**	5.41	3.89	3.83	4.18	3.88	3.75
β-D-Gal *p*-(1 → **(D)**	4.42	3.55	3.66	3.93	3.73	3.8
O-Specific polysaccharide (PS-I)						
→ 3)-β-D-Glc *p*NAc-(1 → **(A)**	4.60	3.84	3.73	3.73	3.48	3.92, 3.76
→ 6)-β-D-Glc*p*NAc-(1 → **(B)**	4.61	3.80	4.00	3.57	3.60	4.18, 3.86
3 ↑						
→ 3)-β-D-Gal *p*-(1 → **(C)**	5.46	3.88	3.75	4.18	3.86	3.78
→ 2)-β-D-Gal *p*-(1 → **(D)**	4.65	3.66	3.84	3.92	3.71	3.8
α-Col *p*-(1 → **(E)**	5.14	4.02	1.81, 2.06	3.80	4.21	1.19

Table 2
^{13}C NMR chemical shifts (in ppm). Chemical shifts for the NAc groups are δ 23.6 and 23.8 (CH_3), and δ 174.9 and 175.2 (CO)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
Colitose-free polysaccharide (PS-II)						
→ 3)-β-D-Glc*p*NAc-(1 → (**A**)	102.9	55.4	80.7	72.0	76.8	61.9
→ 6)-β-D-Glc*p*NAc-(1 → (**B**)	103.7	56.0	83.9	69.3	75.0	69.6
3 ↑						
→ 3)-α-D-Gal*p*-(1 → (**C**)	100.3	68.6	80.8	69.9	71.9	62.2
β-D-Gal*p*-(1 → (**D**)	104.8	72.0	73.8	69.8	76.5	61.9
O-Specific polysaccharide (PS-I)						
→ 3)-β-D-Glc*p*NAc-(1 → (**A**)	102.4	55.2	80.6	72.1	76.8	61.9
→ 6)-β-D-Glc*p*NAc-(1 → (**B**)	104.7	56.1	79.4	69.3	75.0	69.3
3 ↑						
→ 3)-α-D-Gal*p*-(1 → (**C**)	100.0	68.7	80.1	70.1	71.8	62.3
→ 2)-β-D-Gal*p*-(1 → (**D**)	101.9	77.5	75.0	70.4	76.4	61.9
α-Col*p*-(1 → (**E**)	100.0	64.5	34.2	69.7	67.4	16.8

being 3-substituted. Methylation analysis of PS-I confirmed that Col is the terminal residue and showed that most of Gal in the side-chain is substituted with Col at O-2. A low amount of methylated Col derived from PS-I is accounted for partially by its removal during delipidation of the lipopolysaccharide at pH 4.5 and partially by its destruction during acid hydrolysis of the methylated polysaccharide. As judged by the amount of 2,3,4,6-tetra-*O*-methylgalactose derived from PS-I, the minor, Col-lacking repeating units constitute about 1/4 of the total.

The ^1H NMR spectra of PS-I and PS-II were assigned using sequential, selective spin-decoupling, 2D COSY (Fig. 2), and H,H-relayed COSY (Table 1). The full spin-systems were identified for both GlcNAc residues, distinguished by the coupling constant values $J_{2,3} \approx J_{3,4} \approx J_{4,5}$ 9 Hz, and for Col. For two Gal residues, characterised by the coupling constant values $J_{2,3}$ 10, $J_{3,4}$ 3, $J_{4,5} < 2$ Hz, the signals for H-1,2,3,4 were assigned only, while the positions of the signals for H-5,6 were determined from H-detected $^1H,^{13}C$ HMQC experiments used also for the assignment of the ^{13}C NMR spectra of PS-I and PS-II (Table 2). The assignment of the H-5 signal of β-Gal was confirmed by a NOE experiment with selective preirradiation of H-1 resulting in a response on H-5 of the same sugar residue.

Table 3
Data of methylation analysis

Methylated sugar	T_R [a]	Relative detector response for compound	
		PS-II	PS-I
2,4-MeCol	0.46		0.15
2,3,4,6-MeGal	1.00	1.2	0.25
2,4,6-MeGal	1.31	1.35	+ [b]
3,4,6-MeGal	1.31		+ [b]
4,6-MeGlcNMeAc	2.57	1	1
4-MeGlcNMeAc	3.10	0.65	0.8

[a] Retention time in GLC of the corresponding alditol acetate referenced to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol (2,3,4,6-MeGal).
[b] Relative detector response for 2,4,6-MeGal + 3,4,6-MeGal was 1.9.

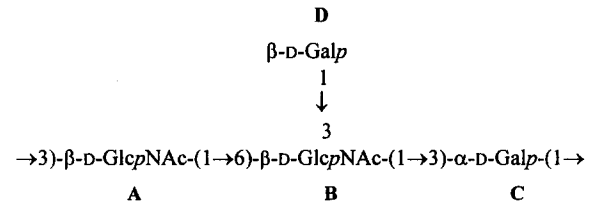
Relatively large coupling constant values, $J_{1,2}$ 8 Hz, determined from the spectrum of PS-I, showed that both GlcNAc residues ($\delta_{H\text{-}1}$ 4.60 and 4.61, units **A** and **B**, respectively) and one of the Gal residues ($\delta_{H\text{-}1}$ 4.65, unit **D**) were β-linked, while a much smaller $J_{1,2}$ value of 4 Hz proved that the second Gal residue ($\delta_{H\text{-}1}$ 5.46, unit **C**) and Col ($\delta_{H\text{-}1}$ 5.14, unit **E**) were α-linked.

Downfield displacements of the signals for C-3 of units **A** and **C** and C-3 and C-6 of unit **B** to 80.7, 80.8, 83.9, and 69.2, respectively, i.e. by 7–11 ppm as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides [11], confirmed the substitution pattern of PS-II determined by methylation analysis.

For sequence analysis, 1D NOE experiments with sequential, selective preirradiation of anomeric protons were carried out in the difference mode. In addition to intraresidue NOEs on H-2 (strong) and H-3 (weak), preirradiation of H-1 of α-linked unit **C** at δ 5.41 resulted in interresidue NOEs on H-3 (strong) and H-4 (weak) of unit **A** at δ 3.77 and 3.73, respectively. Preirradiation of H-1 of β-linked unit **A** at δ 4.59 caused intraresidue NOEs on H-2,3,4,5 as well as interresidue NOEs on H-5,6a,6b of unit **B** at δ 3.57, 4.15, and 3.87, respectively. Similarly, intraresidue NOEs on H-2,3,5 and an interresidue NOE on H-3 of unit **B** at δ 3.83, and probably also a NOE on H-2 of unit **B** at the same frequency, appeared on preirradiation of H-1 of β-linked unit **D** at δ 4.42. Most likely, NOEs on protons adjacent to the transglycosidic protons were due to spin-diffusion typical of polymers (e.g., see published data [5]).

The data obtained were consistent with the substitution pattern and demonstrated the sequence **C**–**A**–(**D**–)**B**– in the repeating unit of the polysaccharide. As for the site of attachment of unit **B**, it could not be unambiguously determined from the NOE experiment with selective preirradiation of H-1 of unit **B** at δ 4.76 owing to the coincidence of the expected interresidue response on H-3 of unit **C** at δ 3.83 and the intraresidue response on H-2. However, the attachment of unit **B** to unit **C** followed from the substitution of the latter at position 3 established earlier.

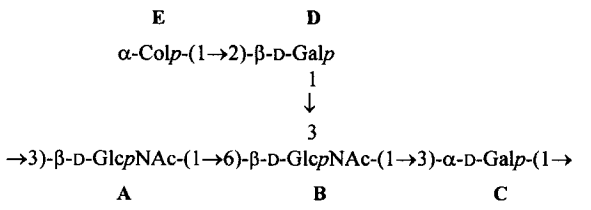
Therefore, PS-II has the following structure:



PS-II

In accordance with the methylation analysis data, the signal for C-2 of unit **D** shifted from δ 72.0 in the ^{13}C NMR spectrum of PS-II to δ 77.5 in the spectrum of PS-I. Attachment of Col at O-2 of β-Gal was confirmed also by a NOE on H-2 of unit **D** at δ 3.66 which, together with the intraresidue NOE on H-2, appeared on preirradiation of H-1 of unit **E** at δ 5.14.

Therefore, on the basis of the data obtained, it was concluded that PS-I has the following structure:



PS-I

As expected, the O-antigen of *S. enterica* ssp. *arizonae* O50 (*Arizona* 9a,9b) is structurally similar to that of *S. enterica* serovar greenside [12], another representative of *Salmonella* serogroup O50 (group Z). Their O-specific polysaccharides differ only in the replacement of the 3-substituted β-GlcNAc (unit **A**) in the former with the 3-substituted β-GalNAc in the latter. The O-specific polysaccharide of one more strain of this group classified as *Arizona* 9a,9c, has the same sugar composition [13] and seems to have the same structure as that of *S. enterica* serovar greenside.

It is worthy to note that the *Salmonella* serogroup O50 antigens include a trisaccharide fragment α-Col*p*-(1 → 2)-β-D-Gal*p*-(1 → 3)-β-D-Glc*p*NAc, which is a colitose ('3-deoxy-L-fucose') analogue of the Lewis^d (precursor) blood group antigen α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 3)-β-D-Glc*p*NAc. Structurally defined O-antigens of some other *S. enterica* serogroups have been found to contain oligosaccharide fragments in common with or similar to A, B, and Lewis blood group antigens [5,14,15] that is considered as bacterial mimicry aiming at downregulation of the immune response to infection.

3. Experimental

NMR spectroscopy.—The ^1H and ^{13}C NMR spectra were obtained with a Bruker AM-300 instrument in D_2O at 55 °C. Acetone was used as internal standard (δ_H 2.23, δ_C 31.45). Selective spin-decoupling was carried out by a modified method [16]; the HDO signal was suppressed during acquisition by presaturation for 1 s. 2D correlation experiments were performed using standard Bruker software. A mixing time of 1 s was used in 1D NOE experiments. A BSV-3 generator was applied to perform HMQC experiments in inverse mode [17].

Chromatography and mass spectrometry.—GPC was carried out on a column (68 cm × 2.6 cm) of

Sephadex G-50 in pyridinium acetate buffer (pH 4.5), monitored using a Knauer differential refractometer. Neutral sugars were identified using a Biotronik LC-2000 sugar analyser and a column of Dionex A × 9-11 anion-exchange resin in 0.5 M sodium borate buffer (pH 8.0) at 65 °C. Amino sugars were analysed using a Biotronik LC-2000 amino acid analyser and standard 0.35 M sodium citrate buffer (pH 5.28).

GLC was performed on a Hewlett–Packard 5890 instrument equipped with a glass capillary column (13 m × 0.25 mm) coated with DB-5 stationary phase using a temperature program of 160 °C (3 min) to 250 °C at 10 °C/min. GLC–MS (EI) was carried out with the same chromatograph equipped with a Hewlett–Packard 5970 mass spectrometer.

Bacterial strain, isolation and degradation of lipopolysaccharide.— The bacterium *Arizona* 9a,9b:13,15:-, strain N99 was obtained from Dr. Rohde (Institute of Hygiene, Hamburg) and cultivated by a published procedure [18]. Lipopolysaccharide was isolated by phenol–water extraction and purified as described [8]. Degradation of the lipopolysaccharide was performed in 0.1 M NaOAc buffer (pH 4.5) at 100 °C for 4 h or in aq 2% HOAc at 100 °C for 2 h, and water-soluble products were fractionated by GPC on Sephadex G-50 to give high-molecular-mass PS-I or PS-II, respectively.

Sugar and methylation analysis.—For sugar analysis, a polysaccharide (1 mg) was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h), then the soln was concd to dryness and the residue was analysed using sugar and amino acid analysers. A portion of the hydrolysate was conventionally reduced with $NaBH_4$ or converted into (*S*)-2-butyl or (*S*)-2-octyl glycosides as described [9], acetylated, and analysed by GLC.

Methylation was performed with CH_3I in Me_2SO in the presence of lithium methylsulfinylmethanide [19]. After hydrolysis, reduction with $NaBD_4$, and acetylation as in the sugar analysis, the partially methylated alditol acetates were identified by GLC–MS using published procedures [20,21].

Acknowledgements

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