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Note

A similarity in the O-acetylation pattern of the O-antigens of *Shigella flexneri* types 1a, 1b, and 2a

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ABSTRACT

Shigella flexneri type 2a is the first, and type 1b is the second, most prevalent isolates from patients with shigellosis in Russia. The O-specific polysaccharides (OPSs, O-antigens) of *S. flexneri* types 1–5 possess a common →2)-α-L-Rha*p*III-(1→2)-α-L-Rha*p*II-(1→3)-α-L-Rha*p*I-(1→3)-β-D-Glc*p*NAc-(1→ backbone and differ from each other in its glucosylation or/and O-acetylation at various positions, the modifications being responsible for various O-factors. It was suggested that O-factor 6 expressed by type 1b is associated with O-acetylation of RhaI at position 2 but more than one *O*-acetyl group has been detected in the type 1b OPS [Kenne, L. et al. *Eur. J. Biochem.* **1978**, *91*, 279–284]. In this work, O-acetylation of Rha*p*I in the type 1b OPS was confirmed by NMR spectroscopy and location of an additional *O*-acetyl group at position either 3 (major) or 4 (minor) of Rha*p*III was determined. Type 1a differs from type 1b in the lack of O-acetylation of Rha*p*I only. In type 2a, in addition to two reported major *O*-acetyl groups at position 6 of GlcNAc and position 3 of Rha*p*III [Kubler-Kielb, J. et al. *Carbohydr. Res.* **2007**, *342*, 643–647], a minor *O*-acetyl group was found at position 4 of RhaIII. Therefore, Rha*p*III is O-acetylated in the same manner in all three *S. flexneri* serotypes studied.

Shigella flexneri is the causative agent of shigellosis or bacillary dysentery, especially in developing countries. Based on O-antigens (O-specific polysaccharides, OPSs), strains of *S. flexneri* are classified into 14 types. From them, the first and the second most prevalent isolates from patients in Russia are types 2a and 1b, respectively. The OPSs are important for virulence of *S. flexneri*[1–4], and are promising components of vaccines for prophylaxis of shigellosis.[5–7] A knowledge of the fine structures of the OPSs is necessary for substantiation of the serospecificity of strains on the molecular level, including serological cross-reactivity between various bacterial clones,[8] and for a better understanding of the role of the OPSs in pathogenesis of shigellosis and in the final vaccine formulation.

The OPSs of *S. flexneri* types 1–5 possess a common backbone consisting of one β-D-Glc*p*NAc and three α-L-Rha*p* residues (Rha*p*I–Rha*p*III), which is encoded by a common O-antigen gene cluster.[9] The differences between the types are conferred by phage modifications, including addition to the basic structure of α-D-glucosyl or/and *O*-acetyl groups.[10] Various immunodeterminants (O-factors) within *S. flexneri* O-antigens are associated with these groups.[11,12]

S. flexneri types 1 and 2 are distinguished by the presence of α-D-Glc*p* at position 4 of Glc*p*NAc or Rha*p*I giving rise to O-factors I and II, respectively. The OPSs of types 1a, 1b, and 2a are also modified by O-acetylation.[11] O-Factor 6 expressed by *S. flexneri* type 1b was suggested to be defined by an *O*-acetyl group at position 2 of Rha*p*I.[13] *S. flexneri* types 1a and 2a possess O-factors 4 and 3,4, respectively, whose molecular nature remains obscure. Recently, in type 2a, two sites of non-stoichiometric O-acetylation have been established at position 6 of Glc*p*NAc and position 3 of RhaIII.[14] By now, no detailed studies of O-acetylation pattern in types 1a and 1b have been undertaken. In this work, we found that in the OPSs of *S. flexneri* types 1a, 1b, and 2a, Rha*p*III is similarly O-acetylated at position either 3 (major site) or 4 (minor site). We also confirmed O-acetylation of Rha*p*I at position 2 in the type 1b OPS.

The OPSs were obtained by mild acid degradation of the lipopolysaccharides that were isolated from bacterial cells of *S. flexneri* types 1a, 1b (two strains), and 2a (two strains) by the phenol–water procedure.[15] All OPSs were O-acetylated as followed from the presence of signals at δ_H 2.16–2.21 and δ_C 21.7–22.1 for methyl groups of *O*-acetyl groups in the ^{1}H and ^{13}C NMR spectra.

The type 1a OPS was O-deacetylated with aqueous ammonia, and the resultant polysaccharide was studied by 2D NMR spectroscopy (for ^{13}C NMR spectrum, see Fig. 1A). Its ^{1}H and ^{13}C NMR spec-

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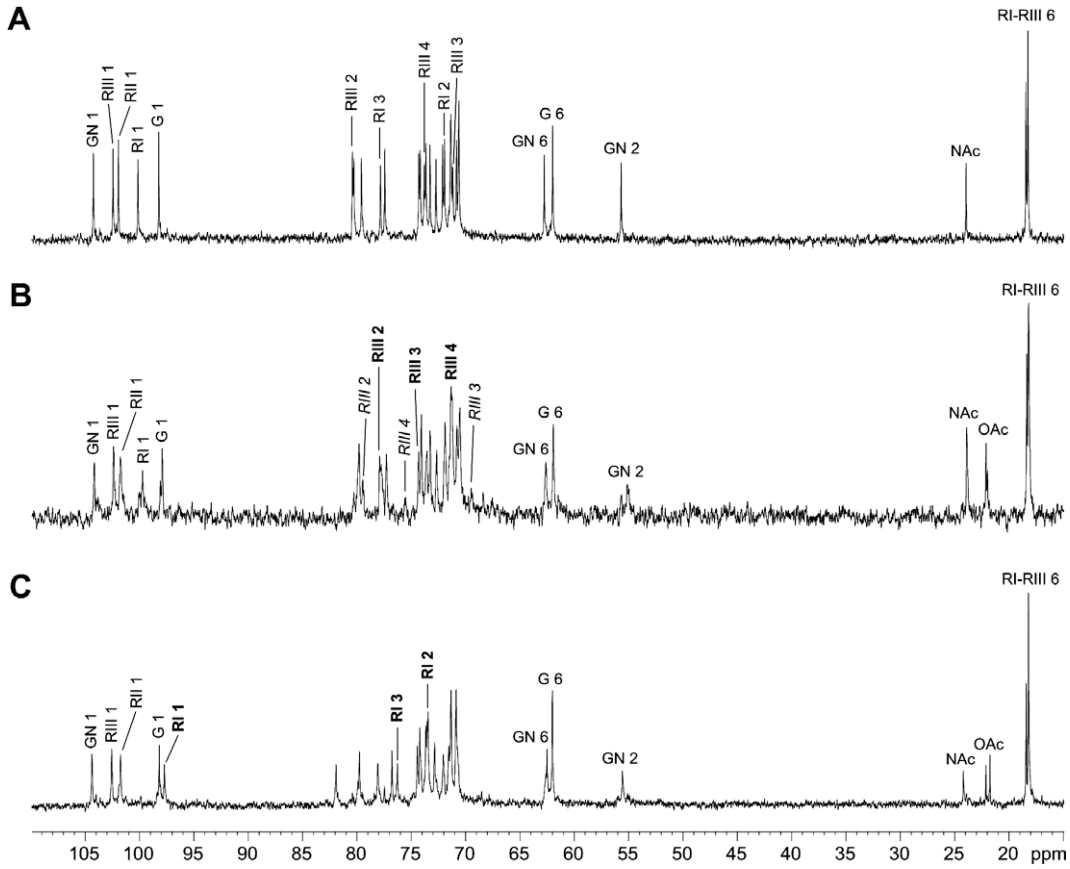


Figure 1. ^{13}C NMR spectra of the O-deacetylated OPS from *S. flexneri* type 1a (A), OPS of *S. flexneri* type 1a (B) and OPS of *S. flexneri* type 1b (C). Numbers refer to carbons in sugar residues denoted as followed: G, Glc*p*; GN, Glc*p*NAc, RI-RIII, Rha*p*I-Rha*p*III. Peak annotations for 2-O-acetylated Rha*p*I and 3-O-acetylated Rha*p*III are shown in boldface, and those for 4-O-acetylated Rha*p*III are shown in italics.

tra were assigned using 2D COSY, TOCSY, ROESY, and ^{1}H–^{13}C HSQC experiments (Table 1). Based on ^{1}H–^{1}H correlations and ^{3}J coupling constant values estimated from the COSY and TOCSY spectra, spin systems were assigned to five residues, including GlcNAc, RhaI–RhaIII, and Glc, all being in the pyranose form. A $J_{1,2}$ coupling constant of 3 Hz showed that Glc*p* is α-linked and that of 7 Hz indicated that Glc*p*NAc is β-linked. The position of the signals for C5 at δ 70.6–71.4 indicated that Rha*p*I–Rha*p*III are α-linked (compare published data for α- and β-Rha*p*[16]).

Linkage and sequence analyses of the O-deacetylated OPS were performed using a ROESY experiment, which showed interresidue correlations between the following anomeric protons and protons at the linkage carbons: Rha*p*III H-1, Rha*p*II H-2; Rha*p*II H-1, Rha*p*I H-3; Rha*p*I H-1, Glc*p*NAc H-3; Glc*p*NAc H-1, Rha*p*III H-2 and Glc*p* H-1, Glc*p*NAc H-4 at δ 5.18/4.05, 5.20/3.79, 5.09/4.11, 4.77/4.13 and 5.39/3.98, respectively. These data are in agreement with the type 1 OPS carbohydrate backbone structure that was established earlier[11] with a lateral α-D-Glc*p* residue attached at position 4 of GlcNAc (Chart 1).

The ^{13}C NMR spectrum of the initial type 1a OPS (Fig. 1B) was much more complex. Its ^{1}H–^{13}C HSQC spectrum (Fig. 2A) compared with the spectrum of the O-deacetylated OPS showed a marked weakening of the Rha*p*III H-3,C-3 cross-peak at δ 3.86/71.2 and appearance of an intense cross-peak at δ 5.08/74.2 as well as a less intense cross-peak at δ 4.82/75.6 (Fig. 2, Table 1). Using 2D ^{1}H–^{1}H COSY, TOCSY (Fig. 3A) and ROESY experiments, the new cross-peaks were assigned unambiguously to Rha*p*III H-3, C-3 and Rha*p*III H-4, C-4 correlations, respectively. Such significant downfield displacements of these cross-peaks in both dimensions in the H–^{13}C HSQC spectrum of the OPS were evidently due to a deshielding effect of O-acetylation at position either 3 or 4 of Rha*p*III. As judged by the ratios of intensities of ^{1}H NMR signals for O-acetylated and non-acetylated Rha*p*III, as well as those of the major and minor O-acetyl groups at δ 2.22 and 2.18, the degree of O-acetylation at positions 3 and 4 is ~65% and ~25%, respectively (Chart 1).

The OPS of *S. flexneri* type 1b strain G1662 was studied by 2D NMR spectroscopy, including ^{1}H–^{1}H COSY, TOCSY (Fig. 3B), ROESY, ^{1}H–^{13}C HSQC (Fig. 2B), and HMBC experiments (Table 1), and found to have the same carbohydrate backbone and the same O-acetylation pattern of Rha*p*III as the type 1a OPS, the degree of O-acetylation at positions 3 and 4 being ~70% and ~15%, respectively. In addition, only a minor Rha*p*I H-2,C-2 cross-peak was observed in the ^{1}H–^{13}C HSQC spectra of the type 1b OPS. Instead, a new major cross-peak appeared at δ 5.23/73.5, which was assigned to the H-2, C-2 correlation of 2-O-acetylated Rha*p*I. The degree of O-acetylation at this position was estimated as ~80%. A higher degree of O-acetylation at the major sites and a lower degree of O-acetylation at the minor site seem to be responsible for a less complex view of the ^{13}C NMR spectrum of the type 1b OPS (Fig. 1C) as compared with that of the type 1a OPS (Fig. 1B).

These data indicate that the OPS of *S. flexneri* type 1b has the structure shown in Chart 1 and differs from the OPS of type 1b in the presence of an additional *O*-acetyl group at position 2 of Rha*p*I only. Therefore, it is confirmed that O-factor 6 characteristic of *S. flexneri* type 1b, but not type 1a, is associated with O-acetylation at this position.[13]

The ^{1}H and ^{13}C NMR spectra of the deacetylated OPS of *S. flexneri* type 2a strain G1663 were assigned (Table 1), and linkage and sequence analyses were performed as described above for

Table 1
^{1}H and ^{13}C NMR chemical shifts (δ, ppm) and interresidue correlations for the anomeric protons in the 2D ROESY and ^{1}H–^{13}C HMBC spectra of the OPSs of *S. flexneri* types 1a, 1b, and 2a and O-deacetylated OPSs from types 1a and 2a

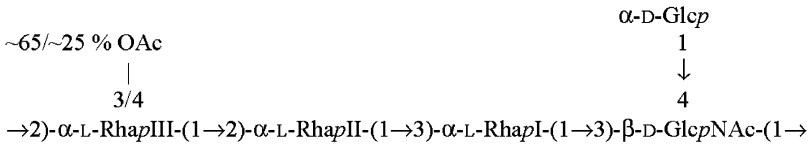
Sugar residue		1	2	3	4	5	6 (6a, 6b)	NAc	OAc	NOE (ROESY)	$^{3}J_{H,C}$ (HMBC)
S. flexneri type 1a O-deacetylated OPS											
→3,4)-β-D-Glc*p*NAc-(1→	^{1}H	4.77	3.97	4.11	3.98	3.75	3.82, 3.85	2.04		H-2 Rha*p*III	
	^{13}C	104.4	55.7	80.4	72.2	77.5	62.8	23.9, 175.7			
→3)-α-L-Rha*p*I-(1→	^{1}H	5.09	3.96	3.79	3.59	3.77	1.27			H-3 Glc*p*NAc	
	^{13}C	100.3	72.0	77.9	72.8	71.4	18.2				
→2)-α-L-Rha*p*II-(1→	^{1}H	5.20	4.05	3.93	3.48	3.76	1.32			H-3 Rha*p*I, H-5 Rha*p*III	
	^{13}C	102.1	79.7	71.4	73.7	70.6	18.4				
→2)-α-L-Rha*p*III-(1→	^{1}H	5.18	4.13	3.86	3.35	3.68	1.25			H-2 Rha*p*II	
	^{13}C	102.6	80.5	71.2	73.9	70.6	18.2				
α-D-Glc*p*-(1→	^{1}H	5.39	3.57	3.70	3.45	3.64	3.76, 3.86			H-3,4 Glc*p*NAc	
	^{13}C	98.3	73.3	74.4	70.9	74.2	62.0				
S. flexneri type 1a OPS											
→3,4)-β-D-Glc*p*NAc-(1→[a]	^{1}H	4.59	4.04	4.14	4.03	3.72	3.84; 3.84	2.09		H-2 Rha*p*III	
	^{13}C	104.3	55.0	80.2	71.5	77.4	62.6	23.7, 175.5			
→3)-α-L-Rha*p*I-(1→[a]	^{1}H	5.12	4.00	3.81	3.55	3.78	1.28			H-3 Glc*p*NAc	
	^{13}C	100.0	71.9	77.8	72.6	71.3	18.0				
→3)-α-L-Rha*p*II-(1→[a]	^{1}H	5.26	4.09	3.95	3.51	3.78	1.34			H-3 Rha*p*I, H-5 Rha*p*III	
	^{13}C	101.8	79.9	71.3	73.6	70.6	18.2				
→2)-α-L-Rha*p*III3Ac-(1→	^{1}H	5.17	4.27	5.08	3.56	3.79	1.29		2.21	H-2 Rha*p*II	
	^{13}C	102.6	78.1	74.2	71.5	70.5	18.1		21.9, 174.8		
→2)-α-L-Rha*p*III4Ac-(1→	^{1}H	5.22	4.23	4.11	4.82	3.87	1.16		2.17	H-2 Rha*p*II	
	^{13}C	102.0	79.6	69.4	75.6	68.4	17.9		21.8, 174.8		
α-D-Glc*p*-(1→[a]	^{1}H	5.42	3.60	3.69	3.45	3.65	3.78; 3.86			H-3,4 Glc*p*NAc	
	^{13}C	98.2	73.3	74.3	70.7	74.2	61.9				
S. flexneri type 1b OPS											
→3,4)-β-D-Glc*p*NAc-(1→[a]	^{1}H	4.57	3.98	4.15	4.00	3.63	3.81, 3.85	2.08		H-2 Rha*p*III	C-2 Rha*p*III
	^{13}C	104.4	55.5	82.0	72.0	76.8	62.5	24.2, 175.4			
→3)-α-L-Rha*p*I2Ac-(1→[a]	^{1}H	5.17	5.23	3.98	3.61	3.87	1.29		2.18	H-3 Glc*p*NAc	C-3 Glc*p*NAc
	^{13}C	97.6	73.5	76.3	73.6	71.3	18.2		21.7		
→2)-α-L-Rha*p*II-(1→[a]	^{1}H	5.20	4.01	3.74	3.50	3.70	1.31			H-3 Rha*p*I, H-5 Rha*p*III	C-3 Rha*p*I
	^{13}C	101.8	79.8	71.2	73.5	70.9	18.4				
→2)-α-L-Rha*p*III3Ac-(1→	^{1}H	5.14	4.24	5.06	3.53	3.77	1.28		2.20	H-2 Rha*p*II	C-2 Rha*p*II
	^{13}C	102.6	78.1	74.1	71.5	70.7	18.2		22.1		
→2)-α-L-Rha*p*III4Ac-(1→	^{1}H	5.18	4.19	4.08	4.80	3.84	1.16		2.16	H-2 Rha*p*II	C-2 Rha*p*II
	^{13}C	101.9	79.5	69.3	75.6	68.4	18.0		22.0		
α-D-Glc*p*-(1→[a]	^{1}H	5.45	3.58	3.65	3.45	3.61	3.76, 3.89			H-3,4 Glc*p*NAc	
	^{13}C	98.2	72.7	74.3	70.7	73.6	61.9				
S. flexneri type 2a O-deacetylated OPS											
→3)-β-D-Glc*p*NAc-(1→	^{1}H	4.72	3.85	3.63	3.55	3.45	3.76, 3.92	2.07		H-2 Rha*p*III	C-2 Rha*p*III
	^{13}C	103.7	57.1	82.8	69.7	77.3	62.1	23.8, 175.8			
→3,4)-α-L-Rha*p*I-(1→	^{1}H	4.84	3.94	3.92	3.79	4.16	1.34			H-3 Glc*p*NAc	C-3 Glc*p*NAc
	^{13}C	102.4	72.2	80.7	76.1	70.5	19.3				
→2)-α-L-Rha*p*II-(1→	^{1}H	5.02	4.10	3.88	3.48	3.80	1.29			H-3 Rha*p*I, H-5 Rha*p*III	C-3 Rha*p*I
	^{13}C	103.1	80.7	71.2	73.6	70.8	18.0				
→2)-α-L-Rha*p*III-(1→	^{1}H	5.13	4.15	3.88	3.33	3.76	1.29			H-2 Rha*p*II	C-2 Rha*p*II
	^{13}C	102.8	80.1	71.2	73.8	70.8	18.3				
α-D-Glc*p*-(1→	^{1}H	5.20	3.54	3.73	3.42	3.97	3.80, 3.89			H-4,6 Rha*p*I	C-4 Rha*p*I
	^{13}C	98.9	72.7	74.1	71.2	73.2	62.1				
S. flexneri type 2a OPS											
→3)-β-D-Glc*p*NA6Ac-(1→[a]	^{1}H	4.54	3.85	3.66	3.42	3.63	4.21, 4.33	2.07, 2.11	2.15	H-2 Rha*p*III	
	^{13}C	103.7	57.0	82.5	71.2	74.6	64.8	23.8, 175.6	22.0[b]		
→3,4)-α-L-Rha*p*I-(1→[a]	^{1}H	4.88	3.94	3.94	3.79	4.16	1.34			H-3 Glc*p*NAc	
	^{13}C	102.2	72.2	80.5	76.1	70.5	19.3				
→2)-α-L-Rha*p*II-(1→[a]	^{1}H	5.06	4.09	3.88	3.49	3.81	1.30			H-3 Rha*p*I	
	^{13}C	102.8	81.0	71.2	73.6	70.7	18.0				
→2)-α-L-Rha*p*III3Ac-(1→	^{1}H	5.15	4.26	5.09	3.50	3.82	1.32		2.21	H-2 Rha*p*II	
	^{13}C	102.8	78.2	74.1	71.6	70.8	18.3		21.7[b]		
→2)-α-L-Rha*p*III4Ac-(1→	^{1}H	5.10	4.23	4.10	4.80	3.92	1.18		2.18		
	^{13}C	102.8	79.3	69.7	75.8	68.6	18.2		22.1		
α-D-Glc*p*-(1→[a]	^{1}H	5.20	3.54	3.73	3.42	3.97	3.80, 3.89			H-4,6 Rha*p*I	
	^{13}C	98.9	72.7	74.1	71.2	73.2	62.1				

[a] Given are the data of the major signals for the O-unit containing Rha*p*III3Ac.
[b] Assignment could be interchanged.

the type 1a O-deacetylated OPS. As a result, the type 2a OPS carbohydrate backbone structure with a lateral α-D-Glc*p* residue at position 4 of Rha*p*I[11] was confirmed (Chart 1).

The 2D NMR spectra of the initial OPS (for TOCSY and ^{1}H–^{13}C HSQC spectra see Figs. 3C and 2C, respectively) showed series of signals for five non-O-acetylated and three mono-O-acetylated monosaccharide residues. From the latter, two major belonged, as reported,[14] to 6-O-acetylated Glc*p*NAc and 3-O-acetylated Rha*p*III (signals for H-6a, 6b of Glc*p*NAc and H-3 of Rha*p*III were shifted significantly downfield from δ 3.76, 3.92 to δ 4.21, 4.33 and from δ 3.88 to δ 5.09, respectively; Fig. 2C). In addition, there was a minor series for a 4-O-acetylated rhamnose residue, which could be clearly traced from H-4 at δ 4.80 to H-1, 2, 3, 5, 6 and from H-6 at δ 1.18 to H-5, 4, 3, 2 (Fig. 3C). The ^{1}H and ^{13}C NMR chemical

A



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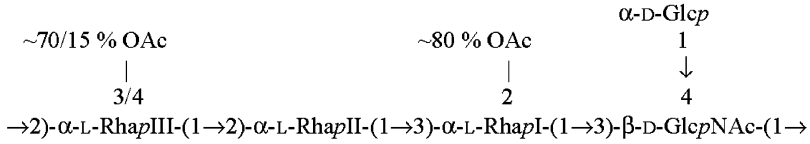

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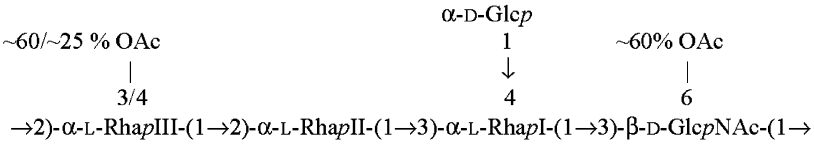

Chart 1. Structures of the OPSs of *S. flexneri* types 1a (A), 1b (B), and 2a (C).

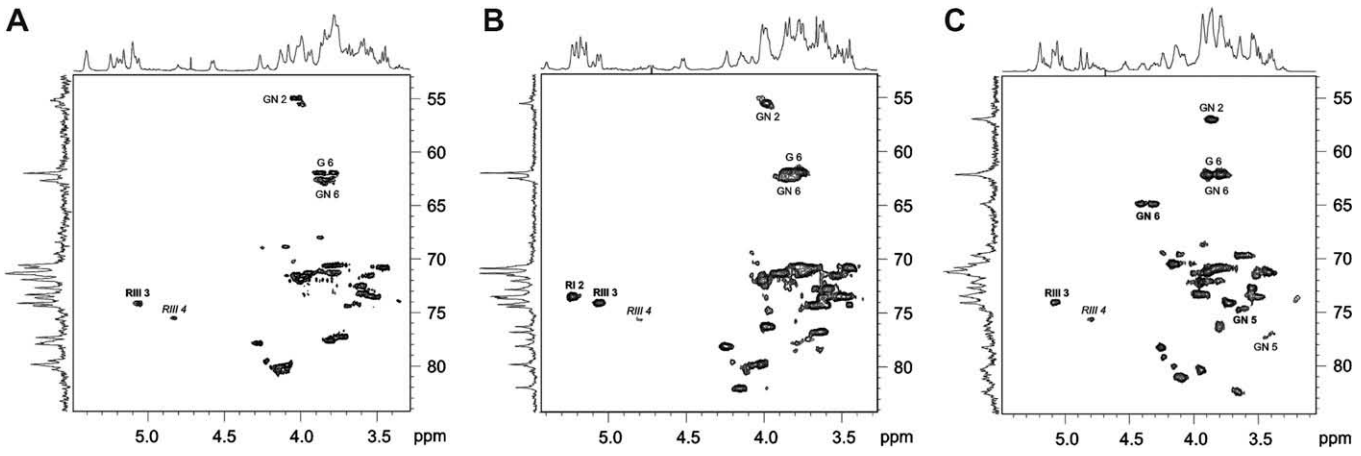

Figure 2. Parts of 2D $^{1}H–^{13}C$ HSQC spectra of the OPSs of *S. flexneri* types 1a (A), 1b (B), and 2a (C). The corresponding parts of the ^{1}H and ^{13}C NMR spectra are displayed along the horizontal and vertical axis, respectively. Numbers refer to H/C pairs in sugar residues denoted as follows: G, Glc*p*; GN, Glc*p*NAc; RI, Rha*p*I; RIII, Rha*p*III. Peak annotations for 6-O-acetylated Glc*p*NAc, 2-O-acetylated Rha*p*I, and 3-O-acetylated Rha*p*III are shown in boldface, and those for 4-O-acetylated Rha*p*III are shown in italics.

shifts as well as the ROESY correlation pattern of this residue were essentially the same as those of 4-O-acetylated Rha*p*III in the OPS of types 1a and 1b. As judged by the ratio of the integral intensities of H-1 signals of Rha*p*I at δ 4.88 and 4.84 (when linked to Glc*p*NAc6Ac and Glc*p*NAc, respectively), the degree of O-acetylation at position 6 of Glc*p*NAc is ~60%. Based on relative intensities of signals for various Rha*p*III forms, the degree of O-acetylation at positions 3 and 4 is ~60% and ~25%, respectively. Therefore, the OPS of *S. flexneri* type 2a has the structure shown in Chart 1.

Similar studies of the OPS from other *S. flexneri* types 1b and 2a strains (1605 and 1818) revealed essentially the same structures as in strains G1662 and G1663, respectively, but the degree of O-acetylation ~1.5 times lower at position 3 of Rha*p*III in type 1b and at each position in type 2a. These minor distinctions may be due to different storage conditions or cultivation of strains on different media.

The data that are obtained demonstrate that the OPSs of all three *S. flexneri* serotypes studied are characterized by the same O-acetylation pattern of Rha*p*III. The O-acetylation at both position 3 (major) and position 4 (minor) suggests that O-acetyl transferase for Rha*p*III is not strictly regiospecific, though favouring position 3. Alternatively, there are two O-acetyl transferases for Rha*p*III, from which the transferase that adds the *O*-acetyl group to position 3 is more active.

No known O-factor is linked to O-acetylation of Rha*p*III and on the other hand, no OPS domain was found to be associated with O-factors 4 and 3,4 expressed by types 1a and 2a, respectively. Further detailed studies of the lipopolysaccharides of other *S. flexneri* serotypes that express these O-factors may shed lights on their chemical nature.

1. Experimental

1.1. Bacterial strains, cultivation, and isolation of lipopolysaccharides

S. flexneri types 1a, 1b, and 2a, strains G1661-G1663, respectively, were obtained from the Institute of Medical and Veterinary Science, Adelaide, Australia (IMVS). *S. flexneri* type 2a, strain 1605 and type 1b, strain 1818 were from the L. A. Tarasevich State Research Institute for Standardization and Control of Medical Biological Preparations, Moscow, Russia. Strains G1661–G1663 were grown to late log phase in 8 L Luria broth using a 10-L fermentor (BIOSTAT C-10, B. Braun Biotech International, Germany) under constant aeration at 37 °C and pH 7.0. Strains 1605 and 1818 were cultivated in semi-synthetic neutral medium under the same conditions. Bacterial cells were washed and dried as described.[17]

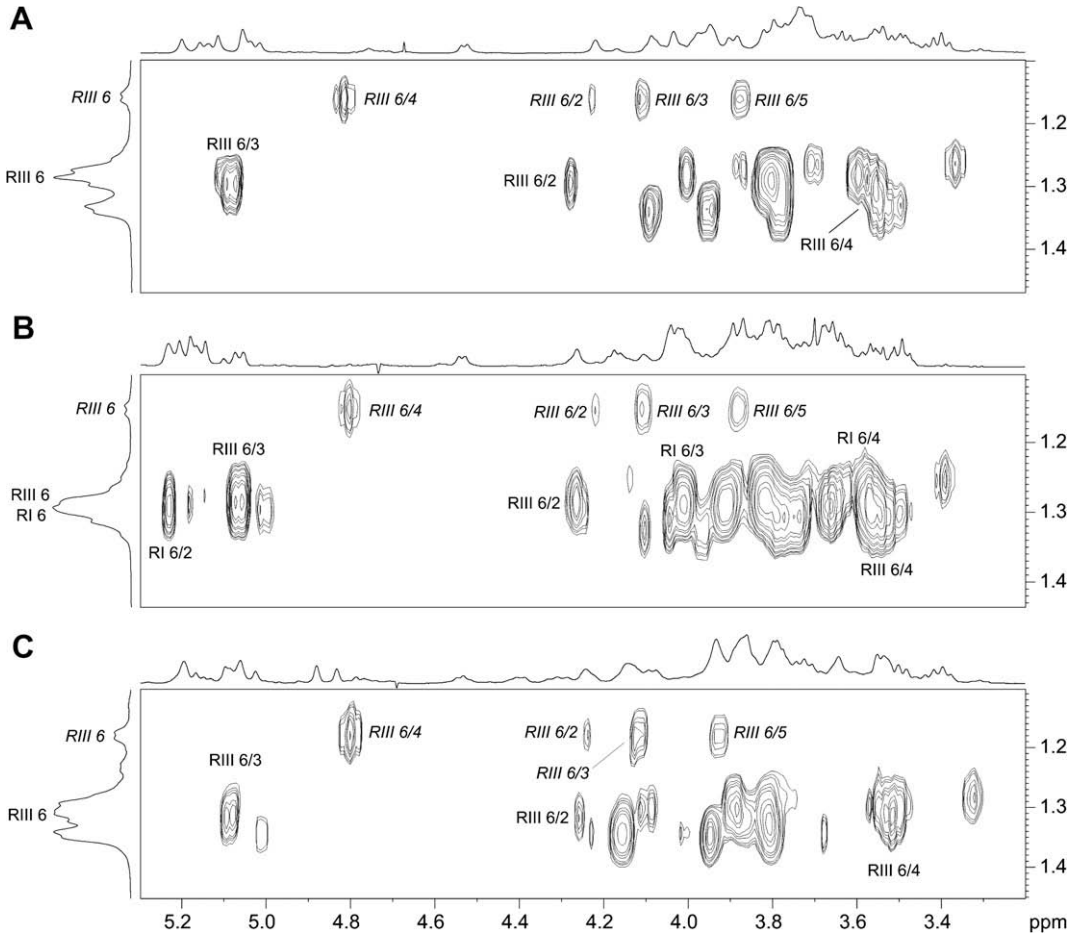

Figure 3. Parts of 2D TOCSY spectra of the OPSs of *S. flexneri* types 1a (A), 1b (B), and 2a (C). The corresponding parts of the ^{1}H NMR spectrum are displayed along the axes. Numbers refer to protons in the O-acetylated Rha*p*I and Rha*p*III residues denoted as RI and RIII, respectively. H-6 of the 4-O-acetylated Rha*p*III resonates in a higher field than H-6 of the other rhamnose residues and displays clear correlations with ring protons of this residue (shown in italics).

Lipopolysaccharides were isolated in yields 8–14% from dried cells by the phenol–water method[15] and purified by precipitation of nucleic acids and proteins with aq 50% trichloroacetic acid.[18]

1.2. Preparation and O-deacetylation of O-specific polysaccharides

Delipidation of the lipopolysaccharides (100–120 mg) was performed with aq 2% HOAc at 100 °C until the precipitation of lipid A (2–3 h). The precipitate was removed by centrifugation (13,000*g*, 20 min), and the supernatant was fractionated by GPC on a column (56 × 2.6 cm) of Sephadex G-50 (S) (Amersham Biosciences, Sweden) in 0.05 M pyridinium acetate buffer, pH 4.5, monitored by a differential refractometer (Knauer, Germany). High-molecular mass OPSs were obtained in yields of 28–32% of the lipopolysaccharide weight.

The OPSs of types 1a and 2a (20 mg each) were treated with aq 12.5% ammonia at 37 °C for 16 h, ammonia was removed with a stream of air, and the O-deacetylated OPSs were isolated by GPC on a column (90 × 2.5 cm) of TSK HW-40 (S) (Merck, Germany) in water.

1.3. NMR spectroscopy

Samples were deuterium exchanged by freeze drying twice from 99.9% D_2O, and then examined as solutions in 99.96% D_2O at 30 °C or for the O-deacetylated OPS from *S. flexneri* type 1a, at 35 °C. NMR spectra were recorded on a Bruker DRX-500 spectrometer (Germany) using internal TSP (δ_H 0.00) and acetone (δ_C 31.45) as references. 2D NMR spectra were obtained using standard BRUKER software, and BRUKER XWINNMR 2.6 program was used to acquire and process the NMR data. Mixing times of 200 and 100 ms were used in TOCSY and ROESY experiments, respectively.

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