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Structure of the O-specific polysaccharide chain of *Shigella boydii* type 5 lipopolysaccharide: a repeated study

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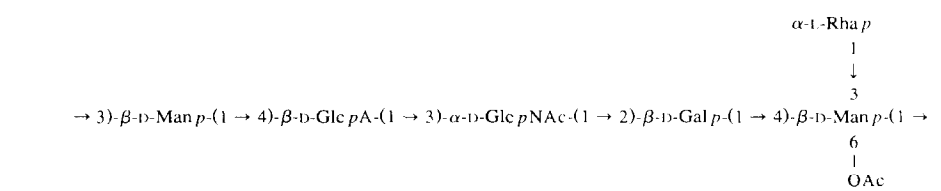
Abstract

An acidic, partially *O*-acetylated O-specific polysaccharide was obtained by mild acid degradation of *Shigella boydii* type 5 lipopolysaccharide and studied by ^{1}H and ^{13}C NMR spectroscopy, including 2D COSY, ^{13}C–^{1}H heteronuclear COSY, 1D NOE, and 2D ROESY experiments, and chemical methods (sugar and methylation analysis, *O*-deacetylation, carboxyl reduction, solvolysis with anhydrous HF, partial acid hydrolysis, Smith degradation). It was concluded that the polysaccharide has a hexasaccharide repeating unit of the following structure:



with the degree of *O*-acetylation varying over 30–50%. The established structure differs from that

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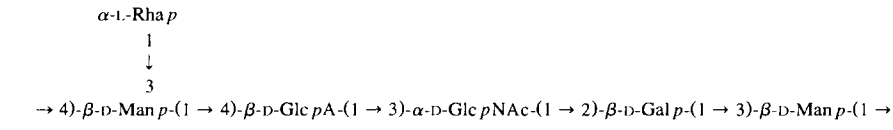
proposed recently for the O-specific polysaccharide of the same *S. boydii* serotype [M.J. Albert et al., *Carbohydr. Res.*, 265 (1994) 121–127].

Keywords: O-Antigen; Polysaccharide; Lipopolysaccharide; Enterobacteria; *Shigella boydii*; NMR spectroscopy

1. Introduction

Enterobacteria *Shigella* cause intestinal diseases, including dysentery. Structures of O-specific polysaccharide chains of outer-membrane lipopolysaccharides have been elucidated for a number of, but not all, serotypes of *Shigella boydii* [1]. Recently [2], the structure **1** has been proposed for the *S. boydii* type 5 O-specific polysaccharide, which also contains an *O*-acetyl group at an unknown primary position.



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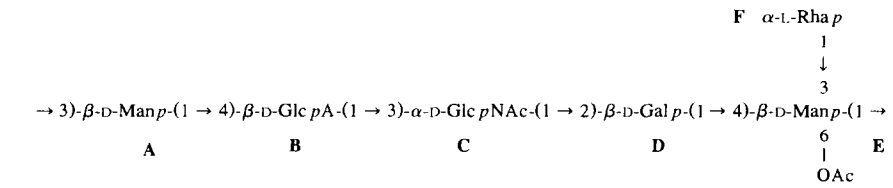
We now report the structure of the O-specific polysaccharide of the same *S. boydii* serotype, which differs from the structure **1** and is shown to be structure **2**.



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2. Results and discussion

The lipopolysaccharide was isolated from dry cells by phenol–water extraction [3] and cleaved with aq 2% acetic acid to give an O-specific polysaccharide isolated by GPC on Sephadex G-50. As judged by paper electrophoresis, the polysaccharide was acidic. Sugar analysis revealed the presence of mannose, galactose, and rhamnose in the ratio ca. 2:1:1, as well as glucuronic acid and 2-amino-2-deoxyglucose. The D configuration of the hexoses and GlcN and the L configuration of Rha were determined on the basis of the optical rotation values measured after their isolation by PC and, for GlcN, paper electrophoresis. The D configuration of GlcA was determined using D-glucose oxidase after carboxyl reduction.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1a) pointed to an irregular structure, most probably because of non-stoichiometric *O*-acetylation [there were signals

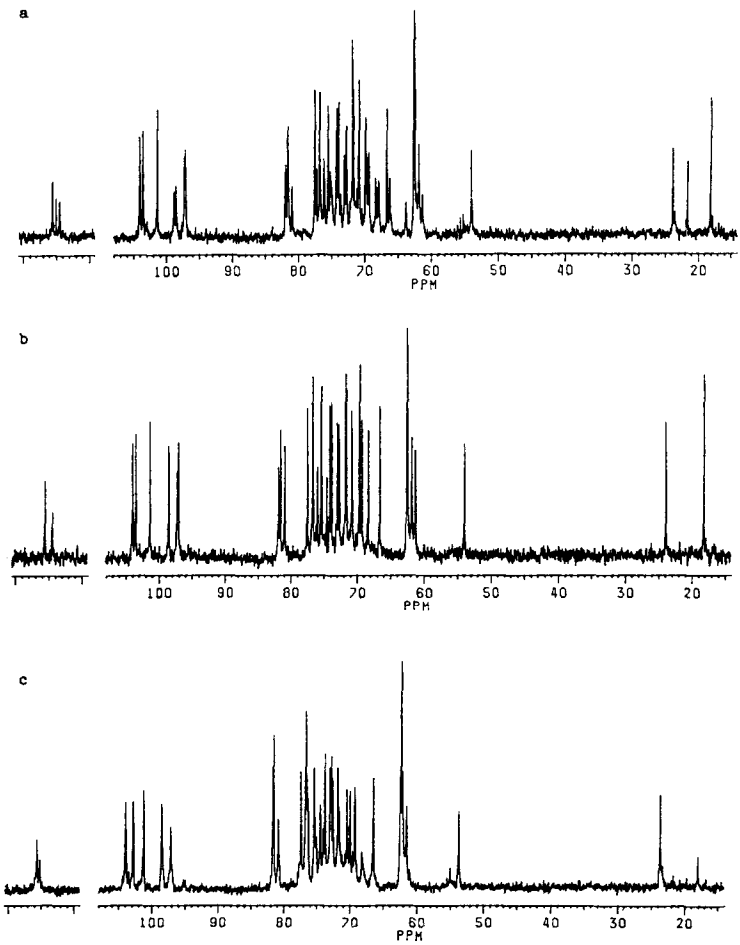

Fig. 1. ^{13}C NMR spectra of (a) O-specific polysaccharide, (b) *O*-deacetylated polysaccharide, and (c) polysaccharide treated with anhydrous HF.

for an *O*-acetyl group at δ 21.5 (Me) and 174.9 (CO)]. After *O*-deacetylation with aq ammonia, the ^{13}C NMR spectrum (Fig. 1b, Table 1) became typical of a regular polysaccharide: it contained signals for six anomeric carbons at δ 97–104, two methyl groups at δ 18.0 (C-6 of Rha) and 23.7 (NAc), two carbonyl groups at δ 174.4 (C-6 of GlcA) and 175.5 (NAc), four $HOCH_2$ groups at δ 61–63 (C-6 of 2 Man, Gal, and GlcN), one carbon-bearing nitrogen at δ 53.8 (C-2 of GlcN), and 23 other sugar ring carbons at δ 66–82. The ^1H NMR spectrum of the *O*-deacetylated polysaccharide contained, inter alia, signals for six anomeric protons at δ 4.5–5.4 and two methyl groups at δ 1.31 (d, H-6 of Rha) and 2.08 (s, NAc). Therefore, the polysaccharide has a hexasaccharide repeating unit containing two residues of D-Man, one residue each of D-Gal, L-Rha, D-GlcA, and D-GlcNAc, as well as an *O*-acetyl group.

Methylation analysis [4,5] of the *O*-deacetylated polysaccharide resulted in identifica-

Table 1
^{13}C NMR data (δ in ppm)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	CH_3CON	CH_3CON
O-Deacetylated polysaccharide **3**								
→ 3)-β-D-Man*p*-(1 → **A**	101.3	69.2	80.7	66.5	77.5	62.4		
→ 4)-β-D-Glc*p*A-(1 → **B**	104.0	74.1	75.3	81.4	76.0	174.4		
→ 3)-α-D-Glc*p*NAc-(1 → **C**	97.1	53.8	81.8	69.7	72.6	61.7	23.7	175.5
→ 2)-β-D-Gal*p*-(1 → **D**	103.4	74.9	72.9	70.7	76.7	62.5		
→ 3,4)-β-D-Man*p*-(1 → **E**	98.5	68.2	75.3	70.7	76.7	61.2		
α-L-Rha*p*-(1 → **F**	96.9	71.5	71.7	73.7	69.6	18.0		
Disaccharide-erythritol **4**								
β-D-Man*p*-(1 → **E**	98.1	72.1	74.2	68.2	77.2	62.4		
→ 3)-β-D-Man*p*-(1 → **A**	101.2	69.0	80.4	66.5	77.7	62.4		
→ 2)-D-Ery-ol	62.6	82.3	72.1	63.5				
Disaccharide-galactitol **6**								
β-D-Glc*p*A-(1 → **B**	104.0	73.9	76.6	72.8	76.4			
→ 3)-α-D-Glc*p*NAc-(1 → **C**	97.8	53.8	81.7	69.7	73.7	61.8	23.3	175.5
→ 2)-D-Gal-ol **D**	62.4	77.4	70.5	70.6	71.4	64.5		

The spectrum of *O*-deacetylated polysaccharide **2** was assigned using ^{1}H–^{13}C heteronuclear COSY; the spectra of oligosaccharides **3** and **5** were tentatively assigned using the published data [11,12].

tion of 2,3,4-tri-*O*-methylrhamnose, 3,4,6-tri-*O*-methylgalactose, 2,4,6-tri-*O*-methylmannose, 2,6-di-*O*-methylmannose, and 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylamino)glucose. When the polysaccharide was carboxyl reduced [6] prior to methylation, in addition to the above-mentioned, partially methylated monosaccharides, 2,3,6-tri-*O*-methylglucose was identified, which was derived from GlcA. These data showed that the polysaccharide is branched, one of the Man residues (unit **A**) and GlcNAc (unit **C**) are 3-substituted, GlcA (unit **B**) is 4-substituted, Gal (unit **D**) is 2-substituted, the second Man residue (unit **E**) is the branching point and substituted at positions 3 and 4, and Rha (unit **F**) is the terminal sugar residue.

Most of Rha (up to 80%) was selectively removed from the *O*-deacetylated polysaccharide by treatment with anhydrous HF at −78°C. This followed from sugar analysis of the modified polysaccharide (the ratios Man:Gal:Rha ca. 2:1:0.2) and from its ^{13}C NMR (Fig. 1c) and ^{1}H NMR (not shown) spectra, where the intensities of the signals for residual Rha were 4–5 times as low as those for the other monosaccharides. Methylation analysis of the modified polysaccharide revealed a significant decrease in the content of 2,3,4-tri-*O*-methylrhamnose and 2,6-di-*O*-methylmannose and the appearance of 2,3,6-tri-*O*-methylmannose. Therefore, the modified repeating unit is linear, and Rha **F** is attached directly to the branching point Man **E** at position 3.

The ^{1}H NMR spectrum of the *O*-deacetylated polysaccharide was assigned using sequential, selective spin decoupling, and 2D COSY (Table 2). As judged by the $J_{1,2}$ coupling constant values, GlcA **B** and Gal **D** are β-linked ($J_{1,2}$ 7.5 Hz for both) and GlcNAc **C** is α-linked ($J_{1,2}$ 3.5 Hz). The appearance of an NOE on H-5 of both Man **A** and **E** on pre-irradiation of H-1 of the same sugar residue showed that they are β-linked, while the absence of such an NOE indicated that Rha **F** is α-linked.

Sequence analysis of the *O*-deacetylated polysaccharide was carried out using 1D

Table 2
^{1}H NMR data (δ in ppm) at pD 5

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	CH_3CON
O-Deacetylated polysaccharide **3** [a]								
→ 3)-β-D-Man*p*-(1 → **A**	4.67	4.17	3.87	3.72	3.42	3.93	3.75	
→ 4)-β-D-Glc*p*A-(1 → **B**	4.56	3.42	3.68	3.83	3.98			
	(4.58)	(3.43)	(3.70)	(3.86)	(4.06)			
→ 3)-α-D-Glc*p*NAc-(1 → **C**	5.37	4.12	3.98	3.65	4.11	3.82		2.08
→ 2)-β-D-Gal*p*-(1 → **D**	4.56	3.66	3.73	3.93	3.68	3.80		
→ 3,4)-β-D-Man*p*-(1 → **E**	4.82	4.24	3.87	4.17	3.41	3.93	3.78	
α-L-Rha*p*-(1 → **F**	4.95	4.00	4.03	3.41	4.34	1.31		
Disaccharide-erythritol **4**								
β-D-Man*p*-(1 → **E**	4.80	4.09	3.71	3.63	3.42	3.96	3.79	
→ 3)-β-D-Man*p*-(1 → **A**	4.87	4.27	3.96	3.75	3.46	3.92	3.76	
Trisaccharide **5** [b]								
β-D-Glc*p*A-(1 → **B**	4.59	3.37	3.54	3.62	4.02			
→ 3)-α-D-Glc*p*NAc-(1 → **C**	5.24	4.11	3.94	3.62				2.06
→ 2)-β-D-Gal*p* **D**	4.71	3.52	3.72					
Disaccharide-galactitol **6**								
β-D-Glc*p*A-(1 → **B**	4.57	3.39	3.55	3.62	3.90			
→ 3)-α-D-Glc*p*NAc-(1 → **C**	5.18	4.16	3.94	3.62				2.06

[a] The data at pD 1 are given in parentheses.
[b] The data given are for the predominant isomer with β-D-Gal*p*.

NOE experiments performed in the difference mode with selective pre-irradiation of the anomeric protons at pD 5, and a 2D ROESY experiment at pD 1. Two interresidue NOEs on H-4 **E** and H-3 **C** (both triplets) were observed on simultaneous pre-irradiation of H-1 **B** and H-1 **D** both resonating at pD 5 at δ 4.56 (Fig. 2a). At pD 1 the signal for H-1 **B** shifted to δ 4.58 and the correlation peaks H-1 **B**/H-3 **C** and H-1 **D**/H-4 **E** were unambiguously recognized in the ROESY spectrum (not shown) at δ 4.58/3.98 and 4.56/4.17, respectively. These data were in accord with substitution of unit **C** at position 3 and unit **E** at position 4, followed from methylation analysis, and suggested the sequences **B** → **C** and **D** → **E**. Pre-irradiation of H-1 **A** resulted in an intense NOE on H-4 **B** at δ 3.83 (triplet), together with a less intense NOE on H-3 **B** and those on some other non-anomeric protons of units **A** and **B** (Fig. 2b). Therefore, unit **A** is attached to unit **B**. The main NOE on H-4 **B** is consistent with substitution of this unit at position 4 (see above), those on H-2,3,5 **A** are typical of a β-linked pyranoside (β-Man*p*), while other minor responses in this spectrum and all other 1D NOE spectra may be accounted for, at least partially, by spin diffusion characteristic for polymers.

Attachment of unit **E** to unit **A** and unit **F** to unit **E** followed from the appearance of comparable NOEs on H-2 **A** (doublet) and H-3 **A** (doublet of doublets) on pre-irradiation of H-1 **E** at δ 4.82 (Fig. 2c), and of those on H-2 **E** (doublet) and H-3 **E** (doublet of doublets) on pre-irradiation of H-1 **F** at δ 4.95 (Fig. 2d). Methylation analysis showed that none of units **A** and **E** is 2-substituted, but such NOE pattern is typical of β-(1 → 3) linkage between the sugar residues having the same absolute configuration (D-Man → D-Man) and of α-(1 → 3) linkage between the sugar residues having different absolute

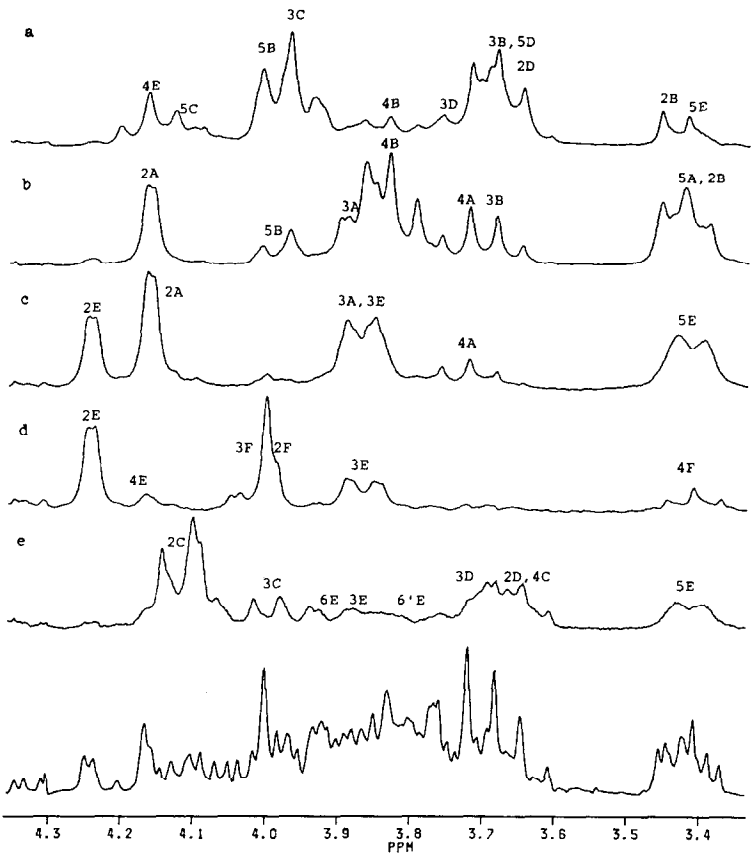

Fig. 2. Parts of 1D NOE difference spectra of *O*-deacetylated polysaccharide obtained on selective pre-irradiation of H-1 of GlcA **B** and Gal **D** (a), Man **A** (b), Man **E** (c), Rha **F** (d), and GlcNAc **C** (e). The corresponding part of the ^{1}H NMR spectrum is shown at the bottom. Arabic numbers refer to the protons in the sugar residues denoted by letters.

configurations (L-Rha → D-Man) [7]. While H-3 **A** and H-3 **E** have the same chemical shift (δ 3.87), H-2 **A** and H-2 **E** have different chemical shifts (δ 4.17 and 4.24, respectively), and it is the NOEs on H-2 that are of diagnostic value for the sequence determination.

Finally, pre-irradiation of H-1 **C** at δ 5.37 resulted in interresidue NOEs on H-2 and H-3 of unit **D** and on some protons of unit **E** (Fig. 2e). As followed from the structure of oligosaccharide **4** (see below) and methylation analysis data (see above), unit **C** is attached to unit **D** at position 2. The additional NOEs on unit **E** were, probably, due to long-range space contacts, which may occur in the trisaccharide fragment **C** → **D** → **E**, where unit **D** is 2-substituted.

The interpretation of the NOE data was consistent with the complete assignment of the ^{13}C NMR spectrum of the *O*-deacetylated polysaccharide (Table 1), and revealed the sequence of the sugar units in this polysaccharide, which, thus, has the structure **3**.

F α-L-Rha*p*
1
↓
3
→ 3)-β-D-Man*p*-(1 → 4)-β-D-Glc*p*A-(1 → 3)-α-D-Glc*p*NAc-(1 → 2)-β-D-Gal*p*-(1 → 4)-β-D-Man*p*-(1 →
A B C D E

3

The structure **3** was confirmed by selective cleavages. Smith degradation of the carboxyl-reduced polysaccharide resulted in a disaccharide-erythritol **4**. Its structure was elucidated by sugar analysis, which revealed Man and erythritol (Ery-ol), and ^{1}H and ^{13}C NMR data (Tables 1 and 2). In particular, the 1 → 3 linkage between the monosaccharides was established by NOEs on H-2,3 of Man **A** caused by pre-irradiation of H-1 of Man **E** and by a low-field position of the signal for C-3 of Man **A** at δ 80.4 compared with that of Man **E** at δ 74.2.

β-D-Man*p*-(1 → 3)-β-D-Man*p*-(1 → 2)-D-Ery-ol
E A

4

Partial hydrolysis of the *O*-deacetylated polysaccharide with 0.25 M HCl followed by anion-exchange chromatography afforded a trisaccharide and a mixture of two tetrasaccharides. The trisaccharide contained GlcA **B**, GlcNAc **C**, and Gal **D**; on borohydride reduction, the last sugar was converted into galactitol and was thus at the reducing end. The trisaccharide and the derived disaccharide-galactitol were studied by ^{1}H and ^{13}C NMR spectroscopy (Tables 1 and 2), including 1D NOE experiments with selective pre-irradiation of H-1 **B** and H-1 **C**, which resulted in intense NOEs on H-3 **C** and H-2 **D**, respectively. Based on these data, it was concluded that the studied compounds have the structures **5** and **6**. A similar study of the mixture of tetrasaccharides before and after borohydride reduction showed that they contain an additional Man residue at the reducing (**E**) or non-reducing end (**A**) and have the structures **7** and **8**, respectively, the former being twice as predominant as the latter.

β-D-Glc*p*A-(1 → 3)-α-D-Glc*p*NAc-(1 → 2)-D-Gal
B C D

5

β-D-Glc*p*A-(1 → 3)-α-D-Glc*p*NAc-(1 → 2)-D-Gal-ol
B C D

6

β-D-Glc*p*A-(1 → 3)-α-D-Glc*p*NAc-(1 → 2)-β-D-Ga*p*-(1 → 4)-D-Man
B C D E

7

β-D-Man*p*-(1 → 4)-β-D-Glc*p*A-(1 → 3)-α-D-Glc*p*NAc-(1 → 2)-D-Gal
A B C D

8

Compared with the ^{13}C NMR spectrum of the *O*-deacetylated polysaccharide, the spectrum of the intact polysaccharide exhibited a new signal for a CH_2-O group at δ 63.6, while the signal at δ 61.2, which belongs to C-6 of Man **E** (Table 1), became

weaker. These changes caused by *O*-acetylation [8] suggested that the *O*-acetyl group is attached at position 6 of Man **E**. As judged by the relative intensities of the signals at δ 63.6 and 61.2, the degree of *O*-acetylation varies over 30–50% for polysaccharide preparations isolated from different batches of bacterial cells. In one of the preparations, acetylation at O-6 of Man **E** could not account for all changes in the ^{13}C NMR spectrum (Fig. 1a), and the presence of an additional *O*-acetyl group could not be excluded.

On the basis of the data obtained, it was concluded that the O-specific polysaccharide of *S. boydii* type 5 has the structure **2**.

The ^{1}H and ^{13}C NMR spectra of the *O*-deacetylated polysaccharide run at pD 5 were practically identical to the corresponding spectra reported recently for the same *S. boydii* serotype [2]. However, the structure **2** of the *O*-deacetylated polysaccharide established in this work differs from the structure **1** proposed previously [2] in the sequence of the mono- and di-glycosylated mannose residues.

3. Experimental

General methods.—The ^{1}H and ^{13}C NMR spectra were obtained with Bruker WM-250 and AM-300 instruments, respectively, in D_2O at 60°C. Acetone was used as an internal standard (δ_H 2.23, δ_C 31.45). Selective spin decoupling was performed in the difference mode by the modified procedure [9]. One-dimensional NOE, 2D COSY, COSYRCT, and heteronuclear ^{13}C–^{1}H COSY (XHCORRD) experiments were performed using standard Bruker software. A mixing time of 1 s was used in 1D NOE experiments. Two-dimensional rotating frame NOE spectrum (ROESY) was obtained using the Rance pulse sequence [10] with DANTE pulse of 230 ms (20° pulse and 54 μs delay) for spin locking. The carrier frequency was replaced from the centre (4.3 ppm) to the left side of the spectrum (5.5 ppm) during spin-lock time in order to minimize HOHAHA-type magnetization transfer. HDO signal was suppressed by pre-saturation during 1 s. The spectral window provided resolution of 2 Hz/point in the F2 dimension and 4 Hz/point in the F1 dimension.

Optical rotations were measured with a Jasco DIP 360 polarimeter in water at 25°C. GPC was carried out on a column (68 cm × 2.6 cm) of Sephadex G-50 in pyridine acetate buffer (pH 4.5) or a column (1.3 cm × 75 cm) of TSK HW-40 (S) in water with monitoring by the phenol–H_2SO_4 reaction or using a Knauer differential refractometer, respectively. PC was performed on Whatman No. 1 paper using a system of 1-butanol–pyridine–water (6:4:3, v/v). Electrophoresis was performed on Whatman No. 1 paper in 0.25 M pyridine–acetic acid buffer (pH 5.5) at 28 V/cm. Substances were detected on paper with the alkaline silver nitrate reagent. GLC was performed using a Hewlett–Packard 5890 instrument equipped with a glass capillary column (25 m × 0.2 mm) coated with OV-1 stationary phase. GLC–MS was performed using a Varian MAT 311 instrument operating at the ionization potential 70 eV under the same chromatographic conditions as in GLC.

Bacterium, isolation of lipopolysaccharide and polysaccharide.—The strain of *S. boydii* serotype 5 was from the collection of the I.I. Mechnikov Institute of Vaccines and Sera (Moscow). Cultivation of broth culture was performed on a slightly alkaline

agar medium at 37°C. The lipopolysaccharide was isolated in a yield of 7.4% from dried bacterial cells of *S. boydii* type 5 by phenol–water extraction and purified by precipitation with Cetavlon [3]. The O-specific polysaccharide was obtained in a yield of 35.5% by degradation of the lipopolysaccharide with aq 2% AcOH (100°C, 2 h) followed by GPC of the water-soluble portion on Sephadex G-50.

Sugar analysis.—Polysaccharide (2 mg) was hydrolysed with 2 M CF_3COOH (121°C, 2 h), and the hydrolysate was evaporated to dryness. Neutral sugars and uronic acid were analysed with a Biotronik LC-2000 sugar analyser using a column (15 cm × 0.37 cm) of Dionex DAX8-11 resin in 0.5 M sodium borate buffer (pH 8.0) at 65°C and 0.04 M sodium phosphate buffer (pH 2.4) at 70°C, respectively. Amino sugar was analysed with a Biotronik LC-2000 amino acid analyser using a column (28 cm × 0.38 cm) of Chromex UAX8 in 0.35 M sodium citrate buffer (pH 5.28) at 55°C. A portion of the hydrolysate was conventionally converted into alditol acetates and analysed by GLC. Oxidation of glucose was conventionally performed with D-glucose oxidase (Glucotest, Boehringer).

Methylation analysis.—Methylation of polysaccharides (5 mg of each) was performed according to the Hakomori procedure [4]; the methylated substance was hydrolysed with 1 M CF_3COOH (121°C, 1 h), and the partially methylated monosaccharides obtained were conventionally converted into alditol acetates and analysed by GLC–MS.

O-Deacetylation.—The polysaccharide (180 mg) was treated with 0.2% solution of $NaBH_4$ in aq concd ammonia (2 mL, 37°C, 16 h), the solution acidified with concd HCl, and desalted by GPC on Sephadex G-50. The yield of *O*-deacetylated polysaccharide was 155 mg.

Carboxyl reduction.—The *O*-deacetylated polysaccharide (40 mg) in dimethylsulfoxide (4 mL) was esterified with an excess of ethereal CH_2N_2 for 15 min, ether evaporated, the solution dialysed against distilled water, reduced with $NaBH_4$ in aq boric acid at pH 7–7.5, and carboxyl-reduced polysaccharide (32 mg) was isolated by GPC on Sephadex G-50.

Solvolysis with anhydrous HF.—The *O*-deacetylated polysaccharide (45 mg) was dried over P_2O_5 at 37°C, treated with liquid HF for 10 min at −78°C, the mixture poured into ether–hexane (1:1) cooled to −78°C, filtered with a steel filter, the residue washed twice with cold ether, and the modified polysaccharide (11 mg) was isolated by GPC on Sephadex G-50.

Smith degradation.—The carboxyl-reduced polysaccharide (30 mg) was oxidized with aq 0.05 M $NaIO_4$ (3 mL, 6°C, 72 h, in dark), the excess of $NaIO_4$ destroyed with ethylene glycol, the polysaccharide reduced with $NaBH_4$ (20 mg), the solution acidified with 0.5 M HCl, and desalted by GPC on Sephadex G-50. The polymeric product was hydrolysed with aq 1% AcOH (1.5 mL, 100°C, 1.5 h), and disaccharide-erythritol **4** was isolated by GPC on TSK HW-40.

Partial acid hydrolysis.—The *O*-deacetylated polysaccharide (60 mg) was hydrolysed with 0.25 M HCl (10 mL, 100°C, 1 h), the solution concentrated in vacuum and fractionated on a column [(20 cm × 0.8 cm) of Amberlite AG1 × 8 (AcO^- form] in a linear gradient of aq AcOH (0 → 5%), with control by paper electrophoresis, to give trisaccharide **5** and a mixture of tetrasaccharides **7** and **8**.

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