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NMR reinvestigation of two *N*-acetylneuraminic acid-containing O-specific polysaccharides (O56 and O24) of *Escherichia coli*

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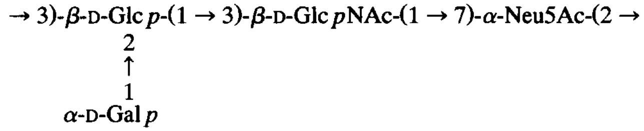
Abstract

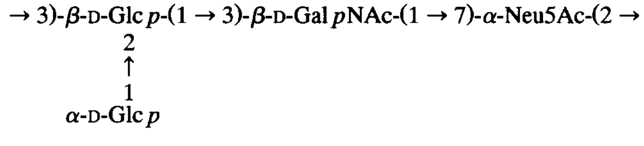
Structures for the *N*-acetylneuraminic acid (Neu5Ac)-containing O56 and O24 polysaccharides of *Escherichia coli* have been reported previously. During these studies unusual chemical shifts had been observed for the NMR signals for H-3eq and C-3 of the Neu5Ac residues of both polysaccharides. In further pursuing this phenomenon, we have reinvestigated the O56 and O24 polysaccharides as well as derived oligosaccharides by one- and two-dimensional NMR spectroscopy. The results showed that structures of both polysaccharides (PSs) had to be modified and formulated as

O56 PS:



$$\rightarrow 3)\text{-}\beta\text{-D-}\underset{\begin{array}{c}2\\ \uparrow\\ 1\\ \alpha\text{-D-Gal}p\end{array}}{\text{Glc}}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 7)\text{-}\alpha\text{-Neu5Ac-}(2\rightarrow$$

O24 PS:



$$\rightarrow 3)\text{-}\beta\text{-D-}\underset{\begin{array}{c}2\\ \uparrow\\ 1\\ \alpha\text{-D-Glc}p\end{array}}{\text{Glc}}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow 7)\text{-}\alpha\text{-Neu5Ac-}(2\rightarrow$$

2D ROESY spectra revealed a strong NOE between H-3eq of Neu5Ac and the protons of the side-chain sugar (H-3 and H-5 of α-D-Gal*p* in the O56 PS and H-3 of α-D-Glc*p* in the O24 PS) and also between H-3ax of Neu5Ac and H-3 of β-D-Glc*p* in the main chain. This indicated a

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close spatial apposition of the seven-linked α-Neu5Ac and the side-chain residues α-D-Gal*p* (O56 PS) and α-D-Glc*p* (O24 PS), respectively. The strong long-range spatial contacts caused the unusual chemical shifts of H-3eq and C-3 of Neu5Ac.

Keywords: *Escherichia coli*; Polysaccharide structure; O24 and O56 antigens; NMR spectroscopy

1. Introduction

N-Acetylneuraminic acid (Neu5Ac) is not only a widespread component of complex carbohydrates in eukaryotes but also occurs in a number of bacterial polysaccharides [1–3]. In capsular polysaccharides and cell wall lipopolysaccharides (LPSs) [3–6], Neu5Ac may be present as a side-chain substituent or within the polysaccharide backbone. We have recently reported on the structures of the O-specific polysaccharide moieties of the O24 and O56 antigens (LPSs) from *Escherichia coli* that contain seven-linked Neu5Ac [7,8]. The O56 polysaccharide was reported to have a branched structure consisting of a backbone of → 3)-β-D-Glc*p*NAc-(1 → 3)-β-D-Glc*p*-(1 → 7)-α-Neu5Ac-(2 →, with α-Gal linked to the 2 position of the Glc residue [7]. The O24 polysaccharide contained GalNAc instead of GlcNAc in the main chain and Glc instead of Gal as a side-chain substituent [8]. NMR analysis of both polysaccharides had revealed unusual chemical shifts for H-3eq (δ 2.51 instead of δ 2.7–2.8) and for C-3 (δ 37.45 instead of δ 40) of α-Neu5Ac [9]. To explain this phenomenon we started further investigations, in the course of which it became evident that the previously reported structures were not correct. There were two causes for misinterpretations which led to these erroneous results. There was an overlap of signals from H-1, H-2, and H-3 of β-Glc*p* and β-Glc*p*NAc in the ^{1}H NMR spectrum of the O56 polysaccharide. This made an assignment of NMR signals quite difficult. Therefore, in the assignments, use was made of sequence data obtained by partial acid hydrolysis and Smith degradation of the polysaccharides [7,8]. As we found later, the oligosaccharides obtained were not homogeneous and were poorly separated. As a consequence the interpretation of the NMR spectra [7,8] was incorrect. In subsequent studies, originally designed to understand the unusual chemical shifts of H-3eq and C-3 of the sialyl residues, we tested the hydrolysis conditions more closely and purified the oligosaccharides more carefully. Data obtained with these oligosaccharides are now the basis of the present NMR investigations. We also ran several spectra at pD 2, where signal overlap was not observed. The results of the reinvestigation showed that in both polysaccharides the sugar sequence in the main chain had to be corrected. The data now provide a better understanding of the unusual chemical shifts of H-3eq and of C-3 of the sialyl residues in both polysaccharides.

2. Results and discussion

Isolation of the polysaccharides.—The polysaccharides were obtained from the lipopolysaccharides [7,10] by mild acid hydrolysis and purified by gel permeation

Table 1
Sugar composition of the O56 and O24 polysaccharides

Residue	Composition (%)	
	O56 PS	O24 PS
Neu5Ac	34.5	35.0
GalNAc	–	20.1
GlcNAc	24.0	–
Gal	18.6	–
Glc	18.3	36.5

chromatography (GPC). The O56 polysaccharide consisted of equimolar amounts of D-glucose (Glc), D-galactose (Gal), *N*-acetyl-D-glucosamine (GlcNAc), and *N*-acetylneuraminic acid (Neu5Ac), and the O24 polysaccharide contained Glc, *N*-acetylgalactosamine (GalNAc), and Neu5Ac in the molar ratios 2 : 1 : 1 (Table 1). The absolute configurations of the hexoses and *N*-acetylhexosamines have been determined [7,8].

Isolation of O56-derived oligosaccharides (**I–IV**).—The polysaccharide backbone was stable to periodate oxidation, the only change being the conversion of the seven-linked Neu5Ac into its C-8 analogue, seven-linked 5-acetamido-3,5-dideoxyoctulosonic acid (8Neu5Ac). The side-chain galactose was therefore removed by a sequence of periodate oxidation, borohydride reduction, and mild acid hydrolysis (Smith degradation), with concomitant cleavage of the sialyl bond in the hydrolysis step. The optimal hydrolysis conditions were found to be 2% (0.33 M) acetic acid/100°C/30 min. From the neutralised hydrolysate, trisaccharide **I** and hexasaccharide **II** were obtained by repeated GPC. The purity of the main fractions was ascertained by thin-layer chromatography (TLC).

For the isolation of tetrasaccharide **III** and octasaccharide **IV**, the polysaccharide was directly subjected to hydrolysis in 2% (0.33 M) acetic acid for 6 h at 100°C. **III** and **IV** were purified by repeated GPC and their purity was checked by TLC.

NMR analysis of the O56-derived oligosaccharides **I** *and* **II**.—According to the NMR data shown in Table 2, oligosaccharide **I** proved to be a trisaccharide containing β-D-Glc*p*, β-D-Glc*p*NAc, and 8Neu5Ac, the latter being at the reducing end and thus in the β form. Assignments of the proton signals (Table 2) were obtained with 2D COSY, one- and two-step H-relayed coherence transfer (COSYRCT) [11,12]. The sequence and type of linkages were established (Table 3) in a 1D-NOE experiment with preirradiation of the anomeric protons of Glc (residue **A**) and GlcNAc (residue **B**). The results (Table 3) indicated that **I** was the trisaccharide

$$\textbf{I}\quad \overset{\textbf{A}}{\beta\text{-D-Glc}p}\text{-}(1\rightarrow 3)\text{-}\overset{\textbf{B}}{\beta\text{-D-Glc}p\text{NAc}}\text{-}(1\rightarrow 7)\text{-}\overset{\textbf{C}}{\beta\text{-[8Neu5Ac]}}$$

The assignments of the signals in the ^{13}C NMR spectrum (Table 2) were not hampered by signal overlaps and broadening, as had been the case with the O56 polysaccharide in the previous study [7]. This holds for all oligosaccharides described here. The assignments were done with a 2D heteronuclear ^{13}C/^{1}H COSY spectrum. They are in agreement with the formulation of **I**.

Table 2
Assignments of the signals in the ^{1}H and ^{13}C NMR spectra (pD 2) of the O56-derived trisaccharide **I**

Residue	Proton	δ	$J_{H,H}$	Hz	Carbon	δ
β-D-Glc-(1 →	H-1	4.45	1,2	7.5	C-1	104.35
(**A**)	H-2	3.29	2,3	9.0	C-2	74.2
	H-3	3.47	3,4	9.0	C-3	76.8
	H-4	3.49	4,5	9.0	C-4	70.0
	H-5	3.44	5,6a	< 2	C-5	77.2
	H-6a	3.87	5,6b	5.5	C-6	61.9
	H-6b	3.68	6a,6b	12.0		
→ 3)-β-D-GlcNAc-(1 →	H-1	4.79	1,2	7.5	C-1	102.3
(**B**)	H-2	3.72	2,3	10.0	C-2	56.3
	H-3	3.82	3,4	10.0	C-3	84.1
	H-4	3.49	4,5	10.0	C-4	69.9
	H-5	3.75	5,6a	< 2	C-5	76.5
	H-6a	3.93	5,6b	< 2	C-6	62.2
	H-6b	3.73	6a,6a	12.0		
→ 7)-β-[8Neu5Ac]					C-1	174.4
(**C**)					C-2	96.7
	H-3ax	1.84	3ax,3eq	13.5	C-3	40.0
			3ax,4	12.0		
	H-3eq	2.14	3eq,4	5.0		
	H-4	4.07	4,5	10.0	C-4	67.85
	H-5	3.75	5,6	10.0	C-5	54.35
	H-6	4.10	6,7	1.5	C-6	71.05
	H-7	3.94	7,8	6.0	C-7	79.1
	H-8a,8b	3.64			C-8	61.9

The NMR analysis of oligosaccharide **II** (Table 4) showed that it was a hexasaccharide composed of two trisaccharide parts and joined through an α-8Neu5Ac-(2 → 3)-Glc linkage.

A **B**
II β-D-Glc*p*-(1 → 3)-β-D-Glc*p*NAc-(1 → 7)-α-[8Neu5Ac]-(2
A′ **B′** **C′**
→ 3)-β-D-Glc*p*-(1 → 3)-β-D-Glc*p*NAc-(1 → 7)-β-[8Neu5Ac]

Table 3
NOE data for the O56-derived trisaccharide **I**

NOE observed on		Pre-irradiated proton	
Residue	Proton	**A**, H-1	**B**, H-1
β-D-Glc-(1 →	H-2	+	
(**A**)	H-3	+	
	H-5	+	
→ 3)-β-D-GlcNAc-(1 →	H-2		+
(**B**)	H-3	+	+
	H-5		+
→ 7)-β-[8Neu5Ac]	H-7		+
(**C**)	H-8a,8b		+[a]

[a] Minor signal.

Table 4
Assignments of the signals in the ^{1}H and ^{13}C NMR spectra (pD 6) of the O56-derived hexasaccharide **II**

Residue	Proton	δ	$J_{\mathrm{H,H}}$	Hz	Carbon	δ
β-D-Glc-(1 →	H-1	4.47	1,2	7.5	C-1	104.6
(**A**)	H-2	3.31	2,3	9.0	C-2	74.3
	H-3	3.50	3,4	9.0	C-3	76.9
	H-4	3.42	4,5	9.0	C-4	70.85
	H-5	3.48	5,6a	< 2	C-5	77.3
	H-6a	3.90			C-6	61.9 [a]
	H-6b	3.67				
→ 3)-β-D-GlcNAc-(1 →	H-1	4.75	1,2	7.5	C-1	102.75
(**B**)	H-2	3.76	2,3	10.0	C-2	56.4
	H-3	3.79	3,4	10.0	C-3	84.4
	H-4	3.54	4,5	10.0	C-4	70.0
	H-5	3.46			C-5	76.6
	H-6a	3.92			C-6	62.1 [a]
	H-6b	3.70				
→ 7)-α-[8Neu5Ac]-(2 →					C-1	175.0
(**C**)					C-2	101.15
	H-3ax	1.88	3ax,3eq	13.5	C-3	40.1
			3ax, 4	12.0		
	H-3eq	2.72	3eq, 4	5.0		
	H-4	3.82	4,5	10.0	C-4	69.2
	H-5	3.92	5,6	10.0	C-5	53.5
	H-6	3.90			C-6	73.9
	H-7	3.94			C-7	78.4
	H-8a,8b	3.68			C-8	62.4
→ 3)-β-D-Glc-(1 →	H-1	4.57	1,2	7.5	C-1	103.8
(**A′**)	H-2	3.30	2,3	9.0	C-2	73.2
	H-3	3.99	3,4	9.0	C-3	81.3
	H-4	3.44	4,5	9.0	C-4	69.6
	H-5	3.50			C-5	77.0
					C-6	62.0 [a]
→ 3)-β-D-GlcNAc-(1 →	H-1	4.75	1,2	7.5	C-1	102.1
(**B′**)	H-2	3.76	2,3	10.0	C-2	56.4
	H-3	3.84	3,4	10.0	C-3	83.4
	H-4	3.54	4,5	10.0	C-4	69.95
	H-5	3.47			C-5	76.7
	H-6a	3.92			C-6	62.2
	H-6b	3.70				
→ 7)-β-[8Neu5Ac]					C-1	175.0
(**C′**)					C-2	96.7
	H-3ax	1.66	3ax,3eq	13.5	C-3	40.1
			3ax,4	12.0		
	H-3eq	2.29	3eq,4	5.0		
	H-4	4.18	4,5	10.0	C-4	67.9
	H-5	3.81	5,6	10.0	C-5	54.6
	H-6	4.23	6,7	1.5	C-6	71.0
	H-7	3.98	7,8	6.0	C-7	79.4
	H-8a,8b	3.65			C-8	62.4

[a] Interchangeable values.

Table 5
Assignments of the signals in the ^{1}H and ^{13}C NMR spectra (pD 6) of the O56-derived tetrasaccharide **III**

Residue	Proton	δ	$J_{H,H}$	Hz	Carbon	δ	DGE [a]
→ 2)-β-D-Glc-(1 →	H-1	4.63	1,2	7.0	C-1	102.9	−1.0
(A)	H-2	3.49	2,3	9.0	C-2	78.3	−1.8
	H-3	3.57	3,4	9.0	C-3	76.1	
	H-4	3.52	4,5	9.0	C-4	69.8	
	H-5	3.47	5,6a	< 2	C-5	77.2	
	H-6a	3.92	6a,6b	12.0	C-6	62.2 [b]	
	H-6b	3.74	5,6b	5.5			
→ 3)-β-D-GlcNAc-(1 →	H-1	4.90	1,2	8.0	C-1	101.9	
(B)	H-2	3.60	2,3	10.0	C-2	57.7	
	H-3	4.12			C-3	80.1	−4.5
	H-4	3.52	4,5	10.0	C-4	70.9	
	H-5	3.56	5,6a	< 2	C-5	76.6	
	H-6a	3.99	6a,6b	12.0	C-6	62.5 [b]	
	H-6b	3.76	5,6b	5.5			
→ 7)-β-Neu5Ac					C-1	176.0	
(C)					C-2	97.7	
	H-3ax	1.86	3ax,3eq	13.0			
			3ax,4	12.0			
	H-3eq	2.16	3eq,4	5.0	C-3	40.6	
	H-4	4.23	4,5	10.0	C-4	67.9	
	H-5	3.81	5,6	10.0	C-5	55.2	
	H-6	4.38	6,7	1.0	C-6	70.45	
	H-7	3.87	7,8	7.5	C-7	79.4	
	H-8	3.82	8,9a	3.0	C-8	72.6	
	H-9a	3.77	9a,9b	12.0	C-9	63.9	
	H-9b	3.51	8a,9b	7.0			
α-D-Gal-(1 →	H-1	5.37	1,2	4.0	C-1	99.0	−0.1
(D)	H-2	3.85	2,3	10.0	C-2	69.8	
	H-3	3.91	3,4	2.5	C-3	70.6	
	H-4	4.02	4,5	1.5	C-4	70.45	
	H-5	4.23	5,6 [c]	5.0	C-5	72.0	
	H-6a,6b	3.73			C-6	62.0 [b]	

[a] Deviation from additivity of glycosylation effects GE (refs. [13,14]).

[b] Interchangeable values.

[c] $1/2(J_{5,6a} + J_{5,6b})$.

It is noteworthy that the chemical shifts of C-3 and H-3eq of the internal 8Neu5Ac (residue **C**) were normal (at δ 40.1 and δ 2.72) [13]. This indicated that the unusual chemical shifts observed in the NMR spectra of the O56 polysaccharide [7] may be caused by the presence of a branching point close to the anomeric center of the Neu5Ac residue. The α-(2 → 3) linkage (**C** → **A′**) between the two trisaccharide subunits was apparent from the chemical shift of C-3 of residue **A′** and from the NOE contact between H-3ax of residue **C** and H-3 of residue **A′**.

NMR analysis of the O56-derived oligosaccharides **III** *and* **IV**.—The NMR data (Tables 5–7) showed that **III** was the tetrasaccharide repeating unit of the O56 polysaccharide and that **IV** was its dimer joined through an α-Neu5Ac-(2 → 3)-Glc linkage. The assignments of the signals in the ^{1}H and ^{13}C NMR spectra of **III** (Table 5)

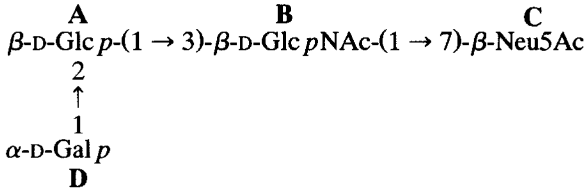
Table 6
NOE data for the O56-derived tetrasaccharide **III**

NOE observed on		Pre-irradiated proton		
Residue	Proton	**A**, H-1	**B**, H-1	**D**, H-1
→ 2-β-D-Glc-(1 →	H-2	+		+
(**A**)	H-3	+		
	H-5	+		
→ 3)-β-D-GlcNAc-(1 →	H-2		+	
(**B**)	H-3	+	+	
	H-5		+	
→ 7)-β-Neu5Ac	H-7		+	
(**C**)	H-8		+[a]	
α-D-Gal-(1 →	H-2			+
(**D**)				

[a] Minor signal.

were achieved using 2D-^{1}H–^{1}H-COSY, COSYRCT, and ^{13}C–^{1}H COSY experiments. The sequence of sugar residues and the types of substitution were proved with a 1D-NOE experiment (Table 6). The results showed that **III** has the structure

III



$$\begin{array}{lll} \mathbf{A} & \mathbf{B} & \mathbf{C} \\ \beta\text{-D-Glc}p\text{-}(1 \rightarrow & 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1 \rightarrow & 7)\text{-}\beta\text{-Neu5Ac} \\ 2 & & \\ \uparrow & & \\ 1 & & \\ \alpha\text{-D-Gal}p & & \\ \mathbf{D} & & \end{array}$$

An analysis of the ^{13}C NMR spectrum of **III** (Table 5) showed unusual high-field chemical shifts and small glycosylation effects for C-1 and C-2 of → 2-β-D-Glc*p*-1 → (residue **A**) and for C-3 of → 3-β-D-Glc*p*NAc (residue **B**), when compared with the values of the corresponding disaccharides (**D–A**, or **A–B**). This phenomenon, the deviation [13] of glycosylation effects from additivity [18], is characteristic of branched trisaccharides with vicinal substitution in the reducing sugar and of linear oligosaccharides with a 1 → 2 linkage at the non-reducing end [14]. It is usually a sign of spatial interaction (crowding) of the residues at the vicinal hydroxyl groups. The deviation from additivity observed in the spectrum of **III** indicated an α-D-Gal*p*-(1 → 2)-β-D-Glc*p*-(1 → linkage [**D**-(1 → 2)-**A**]. The 1 → 7 linkage in **III** was evident from chemical shifts (Table 5) and from NOE interactions (Table 6), and they are in accord with previously reported GC-MS data [7] of the methylated sialyl residue in the O56 polysaccharide.

The assignments of the signals from the proton NMR spectrum of **IV** (Table 7) were obtained in the same manner as for **III**. In addition, 1D HOHAHA (excitation of the anomeric protons of the hexoses and of H-3eq of both α- and β-Neu5Ac residues) was used. The sequence of sugar residues and the types of linkages in **IV** were determined with 2D ROESY spectra [17] (Fig. 1). The following interresidue contacts were taken into account: H-1 of **D'** with H-2 of **A'**, H-1 of **D** with H-2 of **A**, H-1 of **B'** with H-7 of **C'**, H-1 of **B** with H-7 of **C**, H-1 of **A** with H-3 of **B**, H-1 of **A'** with H-3 of **B'**, and H-3ax of **C** with H-3 of **A'**. Due to the closeness of the signals from H-7 and H-8 of **C**,

Table 7
Assignments of the signals in the ^{1}H and ^{13}C NMR spectra (pD 6) of the O56-derived spectra of octasaccharide **IV**

Residue	Proton	δ	$J_{H,H}$	Hz	Carbon	δ
→ 2)-β-D-Glc-(1 →	H-1	4.64	1,2	7.5	C-1	103.1
(A)	H-2	3.51	2,3	9.0	C-2	77.8
	H-3	3.58	3,4	9.0	C-3	76.0
	H-4	3.50	4,5	9.0	C-4	69.85
	H-5	3.45			C-5	77.3
	H-6a	3.92			C-6	62.4 [a]
	H-6b	3.73				
→ 3)-β-D-GlcNAc-(1 →	H-1	4.77	1,2	8.0	C-1	102.9
(B)	H-2	3.67	2,3	10.0	C-2	57.1
	H-3	4.07			C-3	80.7
	H-4,5	3.56			C-4	70.5
					C-5	76.7
	H-6a	4.02			C-6	62.7 [a]
	H-6b	3.81				
→ 7)-α-Neu5Ac-(2 →					C-1	176.0
(C)					C-2	101.6
	H-3ax	2.0	3ax,3eq	13.0	C-3	37.4
			3ax,4	12.0		
	H-3eq	2.51	3eq,4	5.0		
	H-4	4.02			C-4	68.8
	H-5	3.99			C-5	54.2
	H-6	3.84			C-6	73.1
	H-7	3.70			C-7	79.45
	H-8	3.83			C-8	73.0
	H-9a	3.76			C-9	63.8 [a]
	H-9b	3.60				
α-D-Gal-(1 →	H-1	5.38	1,2	4.0	C-1	98.6
(D)	H-2	3.86	2,3	10.0	C-2	69.9
	H-3	3.92	3,4	2.5	C-3	70.7
	H-4	4.03	4,5	1.5	C-4	70.5
	H-5	4.23			C-5	72.1
	H-6a,6b	3.82			C-6	62.3 [a]
→ 3)-β-D-Glc-(1 →	H-1	4.71	1,2	7.5	C-1	103.2
2	H-2	3.66	2,3	9.0	C-2	73.1
(A′)	H-3	4.18			C-3	80.7
	H-4	3.51			C-4	69.6
	H-5	3.45			C-5	76.0
	H-6a	3.98			C-6	62.0
	H-6b	3.81				
→ 3)-β-D-GlcNAc-(1 →	H-1	4.87	1,2	8.0	C-1	102.6
(B′)	H-2	3.66			C-2	57.0
	H-3	4.14			C-3	80.8
	H-4	3.56			C-4	70.9
	H-5	3.48			C-5	76.7
	H-6a	4.01			C-6	62.6 [a]
	H-6b	3.86				

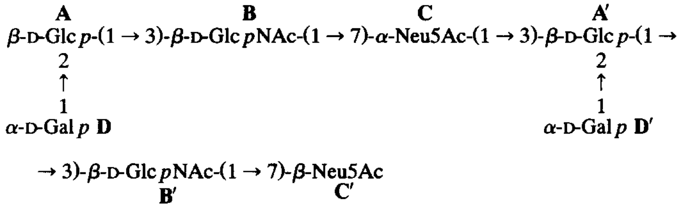
Table 7 (continued)

Residue	Proton	δ	$J_{H,H}$	Hz	Carbon	δ
→ 7)-β-Neu5Ac					C-1	176.0
(**C′**)					C-2	96.8
	H-3ax	1.87	3ax,3eq	13.0	C-3	40.1
			3ax,4	12.0		
	H-3eq	2.17	3eq,4			
	H-4	4.25			C-4	66.9
	H-5	3.81			C-5	55.5
	H-6	4.41			C-6	70.5
	H-7	3.88			C-7	79.45
	H-8	3.84			C-8	72.6
	H-9a	3.77			C-9	63.7 [b]
	H-9b	3.51				
α-D-Gal-(1 →	H-1	5.55	1,2	4.0	C-1	97.0
(**D′**)	H-2	3.82	2,3	10.0	C-2	69.8
	H-3	3.91	3,4	2.5	C-3	70.7
	H-4	3.99			C-4	70.5
	H-5	4.13			C-5	72.1
	H-6a,6b	3.68			C-6	62.3 [a]

[a,b] Interchangeable values.

the **B**1/**C**7 cross-peak could in principle also be read as **B**1/**C**8. However, the chemical shift of **C**7 (Table 6) and the mass spectrometric data obtained previously [7] showed that **C** was substituted at position 7. These results showed that **IV** has the structure

IV



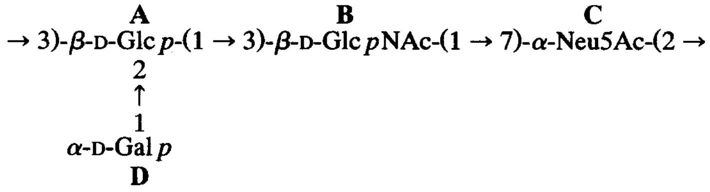
$$\underset{\substack{2\\ \uparrow\\ 1\\ \alpha\text{-D-Gal}p\ \mathbf{D}}}{\overset{\mathbf{A}}{\beta\text{-D-Glc}p}}\text{-}(1\to 3)\text{-}\overset{\mathbf{B}}{\beta\text{-D-Glc}p\text{NAc}}\text{-}(1\to 7)\text{-}\overset{\mathbf{C}}{\alpha\text{-Neu5Ac}}\text{-}(1\to 3)\text{-}\underset{\substack{2\\ \uparrow\\ 1\\ \alpha\text{-D-Gal}p\ \mathbf{D}'}}{\overset{\mathbf{A}'}{\beta\text{-D-Glc}p}}\text{-}(1\to$$

$$\to 3)\text{-}\underset{\mathbf{B}'}{\beta\text{-D-Glc}p\text{NAc}}\text{-}(1\to 7)\text{-}\underset{\mathbf{C}'}{\beta\text{-Neu5Ac}}$$

The interresidue correlation peak **D**1/**B**4 (Fig. 1a) is also a sign of the above-mentioned spatial interaction of the residues **D** and **B** at the vicinal hydroxyl group of residue **A**.

NMR analysis of the O56 polysaccharide.—The NMR data obtained for the O56 polysaccharide (Table 8 and Fig. 2) were very similar to those for **IV**. In particular, the ROESY spectrum (Fig. 2) showed interresidue contacts which were also observed for **IV** (H-1 of **D** with H-2 of **A**, H-1 of **A** with H-3 of **B**, H-1 of **B** with H-7 of **C**, and H-3ax of **C** with H-3 of **A**). The results indicated that the O56 polysaccharide has structure **V**:

V



$$\to 3)\text{-}\underset{\substack{2\\ \uparrow\\ 1\\ \alpha\text{-D-Gal}p\\ \mathbf{D}}}{\overset{\mathbf{A}}{\beta\text{-D-Glc}p}}\text{-}(1\to 3)\text{-}\overset{\mathbf{B}}{\beta\text{-D-Glc}p\text{NAc}}\text{-}(1\to 7)\text{-}\overset{\mathbf{C}}{\alpha\text{-Neu5Ac}}\text{-}(2\to$$

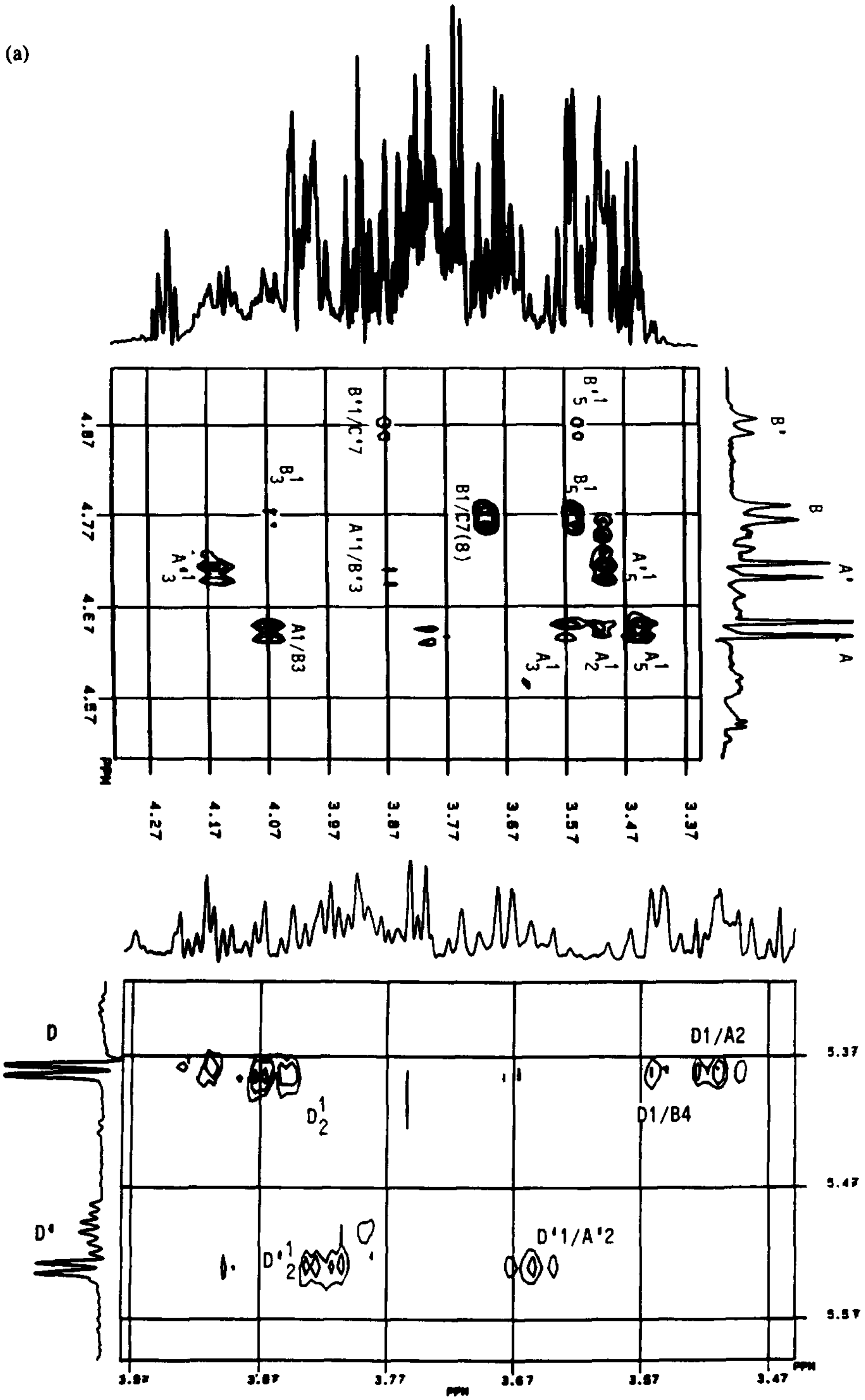


Fig. 1 (continued).

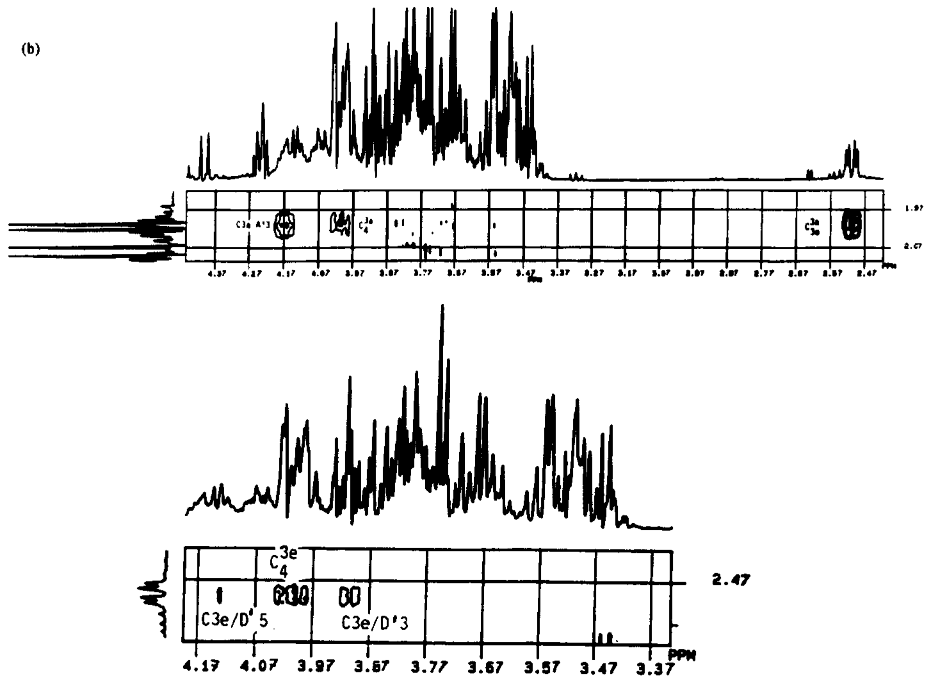


Fig. 1. Parts of the ROESY spectrum of the O56-derived octasaccharide **IV**. (a) Two regions of the responses of anomeric protons; the correlation **B**1/**C**7 (**C**8) was due to the closeness of H-7 and H-8 of residue **C** in the ^{1}H NMR spectrum (Table 5). (b) Two regions for the responses of H-3eq and H-3ax of residue **C**. Arabic numerals refer to the protons in the respective sugar residues, which are denoted by capital letters as in formula **IV**.

Glycosidically linked α-Neu5Ac generally exhibits an H-3eq signal at δ 2.75–2.8 and a C-3 signal at about δ 40 (refs [13–16]). To explain the unusual chemical shifts observed for H-3eq and C-3 of the Neu5Ac residue in both the octasaccharide and the O56 polysaccharide, long-range connectivities of the octasaccharide were analysed in ROESY (Figs 1 and 2). In both cases, NOEs were observed between H-3eq of Neu5Ac with H-3 and (to a lesser extent) H-5 of the side-chain galactose (residue **D**). We propose that the unusual positions of the H-3eq and C-3 signals of Neu5Ac in the NMR spectra of the polysaccharide and the octasaccharide indicated a spatial closeness of the Neu5Ac (**C**) and Gal (**D**) residues. This would result in a crowding around the ketosidic bond of Neu5Ac.

NMR analysis of the O24-derived trisaccharide **VI**.—**VI** was obtained from the O24 polysaccharide by periodate oxidation, followed by reduction with sodium borohydride and acid hydrolysis, as described for the O56-derived trisaccharide **I**. The signal assignments of the ^{1}H and ^{13}C NMR spectra are shown in Table 9. The 1D NOE differential spectra with preirradiation of the anomeric protons of β-D-Glc*p*-1 → (residue **A**) and β-D-Gal*p*NAc-1 → (residue **B**) showed major responses of H-3 of residue **B** and H-7 of β-[8Neu5Ac] (residue **C**), respectively (not shown). This agreed with the

Table 8
Assignments of the signals in the ^{1}H and ^{13}C NMR spectra (pD 2) of the O56 polysaccharide **V**

Residue	Proton	δ	$J_{H,H}$	Hz	Carbon	δ
→ 3)-β-D-Glc-(1 →	H-1	4.70	1,2	7.5	C-1	103.3
2	H-2	3.66	2,3	9.0	C-2	73.6
(A)	H-3	4.15			C-3	78.7
	H-4	3.54			C-4	69.9
	H-5	3.50			C-5	76.75
	H-6a	3.90			C-6	62.4
	H-6b	3.73				
→ 3)-β-D-GlcNAc-(1 →	H-1	4.74	1,2	8.0	C-1	102.2
(B)	H-2	3.68	2,3	10.0	C-2	57.3
	H-3	4.08	3,4	10.0	C-3	80.8
	H-4	3.52			C-4	70.6
	H-5	3.53			C-5	76.3
	H-6a	4.00			C-6	62.45
	H-6b	3.79				
→ 7)-α-Neu5Ac-(2 →					C-1	173.9
(C)					C-2	102.4
	H-3ax	2.0			C-3	37.75
	H-3eq	2.51				
	H-4	4.05			C-4	69.6
	H-5	3.91			C-5	69.6
	H-6	3.88			C-6	72.5
	H-7	3.72			C-7	78.9
	H-8	3.78			C-8	73.0
	H-9a	3.80			C-9	63.7
	H-9b	3.50				
α-D-Gal-(1 →	H-1	5.52	1,2	3.6	C-1	97.2
(D)	H-2	3.83	2,3	10.0	C-2	69.6
	H-3	3.92	3,4	2.8	C-3	70.6
	H-4	4.00	4,5	1.5	C-4	70.5
	H-5	4.13			C-5	71.55
	H-6a,6b	3.75			C-6	62.4

sequence which can be deduced from the data of Table 9 and it indicated that the O24-derived trisaccharide **VI** has the structure

A **B** **C**
VI β-D-Glc*p*-(1 → 3)-β-D-Gal*p*NAc-(1 → 7)-β-[8Neu5Ac]

Thus, the sequence in the main chain of the O24 polysaccharide is Glc–GalNAc–Neu5Ac.

NMR analysis of the O24 polysaccharide.—The assignments of the NMR signals are shown in Table 10 and the results of the 2D ROESY experiment are shown in Table 11. They are in agreement with the formulation of the O24 polysaccharide as **VII**

A **B** **C**
VII → 3)-β-D-Glc*p*-(1 → 3)-β-D-Gal*p*NAc-(1 → 7)-α-Neu5Ac-(2 →
2
↑
1
α-D-Glc*p*
D

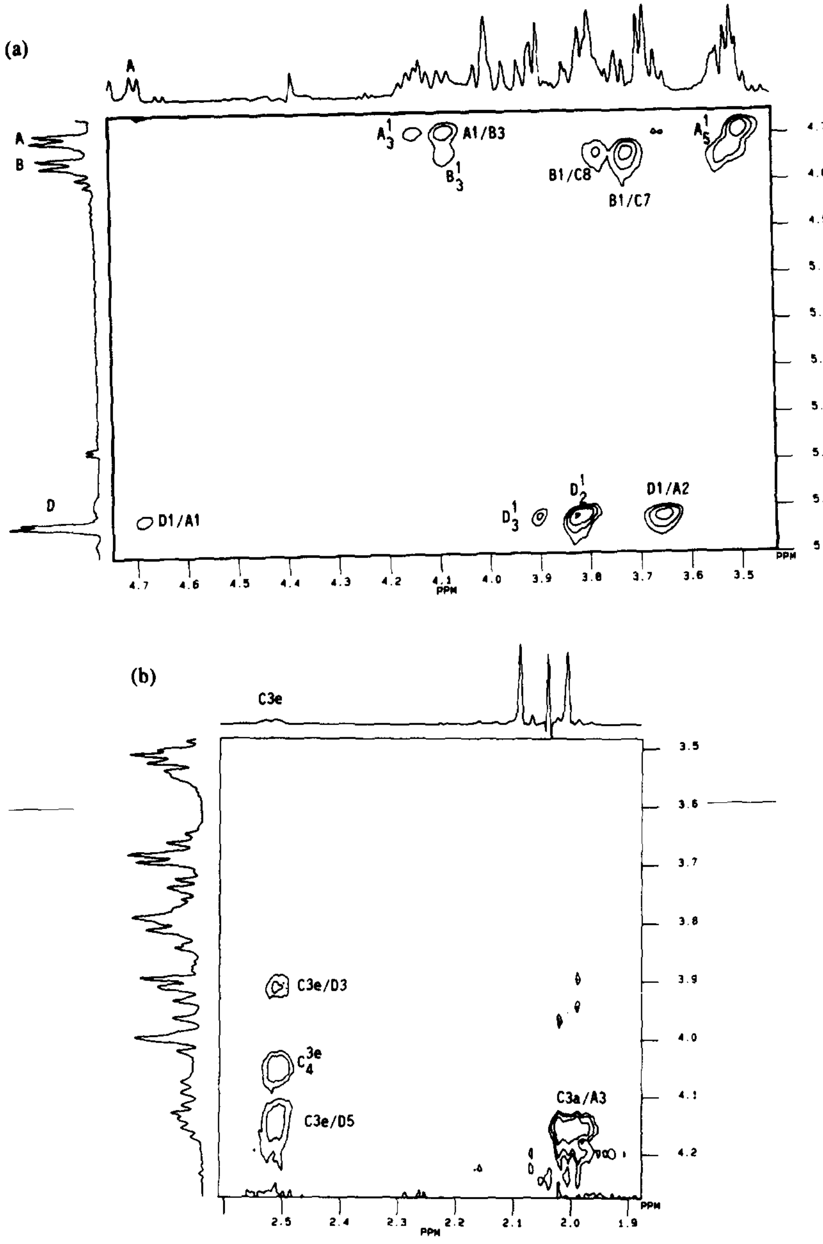
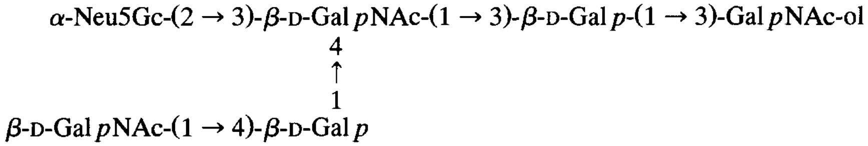


Fig. 2. Parts of the ROESY spectrum of the O56 polysaccharide V. (a) Region for responses of the anomeric protons; (b) region of the responses of H-3eq and H-3ax of residue C. Arabic numerals refer to the protons of the respective sugar units, which are denoted by capital letters as in formula V.

The 2D ROESY experiment (Fig. 3) showed that H-3eq of the Neu5Ac residue exhibited an NOE with H-3 of the side-chain glucose. Thus, the Neu5Ac residue of the O24 polysaccharide is in a close spatial contact with the side-chain glucose. This indicated that, in the O56 and O24 polysaccharides, the main-chain α-Neu5Ac and the side-chain sugar have comparable spatial arrangements. The same holds also for the internal Neu5Ac (residue **C**) in the O56-derived octasaccharide **IV**, but not for the corresponding hexasaccharide **II** that does not contain the side-chain galactose (Tables 7 and 4). A vicinal substitution of a branch-point sugar by sialic acid and a hexose (hexosamine) is also found, for example, in the (reduced) oligosaccharide (Table 9 of ref. [9]) having the following structure.



$$\begin{array}{l} \alpha\text{-Neu5Gc-}(2 \rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1 \rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1 \rightarrow 3)\text{-Gal}p\text{NAc-ol} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 4 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \\ \beta\text{-D-Gal}p\text{NAc-}(1 \rightarrow 4)\text{-}\beta\text{-D-Gal}p \end{array}$$

In this oligosaccharide, H-3eq of Neu5Gc has a chemical shift of δ 2.554. In the O56 and O24 polysaccharides, this spatial arrangement may have caused the unusual chemical shifts.

Table 9
Assignments of the signals in the ^{1}H and ^{13}C NMR spectra (pD 6) of the O24-derived trisaccharide **VI**

Residue	Proton	δ	$J_{H,H}$	Hz	Carbon	δ
β-D-Glc-(1 →	H-1	4.50	1,2	7.5	C-1	105.75
(**A**)	H-2	3.30	2,3	9.0	C-2	74.3
	H-3	3.47	3,4	9.0	C-3	76.9
	H-4	3.40	4,5	9.0	C-4	70.8
	H-5	3.44			C-5	77.1
	H-6a	3.89	6a,6b	12.0	C-6	61.9
	H-6b	3.73	5,6a	5.0		
→ 3)-β-D-GalNAc-(1 →	H-1	4.68	1,2	7.5	C-1	103.25
(**B**)	H-2	3.94	2,3	10.0	C-2	53.1
	H-3	3.89	3,4	3.0	C-3	81.6
	H-4	4.17	4,5	1.0	C-4	69.3
	H-5	3.72	5,6 [a]	4.0	C-5	76.1
	H-6a,6b	3.83			C-6	62.0
→ 7)-β-[8Neu5Ac]					C-1	175.8
(**C**)					C-2	96.7
	H-3ax	1.87	3ax,3eq	13.0	C-3	40.2
			3ax,4	12.0		
	H-3eq	2.29	3eq,4	5.0		
	H-4	4.30	4,5	10.0	C-4	67.3
	H-5	3.74	5,6	10.0	C-5	55.0
	H-6	4.33	6,7	1.5	C-6	70.8
	H-7	3.99	7,8a	6.0	C-7	79.4
	H-8a,8b	3.66			C-8	62.65

[a] $1/2(J_{5,6a} + J_{5,6b})$.

3. Experimental

Isolation of the polysaccharides.—The LPSs were isolated from *Escherichia coli* strains Su 3684-41 (O56 : K$^-$: H$^-$, Freiburg collection Nr. 2405) and E41a (O24 : K : H$^-$, Freiburg collection Nr. 21735) with a combination of phenol–water extraction and ultracentrifugation [10] and precipitation with CTAB as described previously [8]. A solution of the LPS (200 mg) was hydrolysed in 25 mL of 2% (0.33 M) AcOH (100°C, 20 min). The sediment (lipid A) was removed by centrifugation and the supernatant solution was concentrated in vacuo. The polysaccharide was obtained by chromatography on Sephadex G50 in the void volume.

Isolation of oligosaccharides **I** *and* **II**.—The O56 polysaccharide (200 mg) was incubated in 0.1 M NaIO$_4$ (25 mL) at room temperature in the dark. After reduction with

Table 10
Assignments of the signals in the ^{1}H and ^{13}C NMR spectra (pD 2) of the O24 polysaccharide **VII**

Residue	Proton	δ	$J_{H,H}$	Hz	Carbon	δ
→ 3)-β-D-Glc-(1 →	H-1	4.74	1,2	8.0	C-1	104.7
2	H-2	3.63	2,3	10.0	C-2	73.1
(**A**)	H-3	4.15	3,4	10.0	C-3	78.75
	H-4	3.52	4,5	10.0	C-4	70.5
	H-5	3.54			C-5	76.5
	H-6a	3.77			C-6	62.2
	H-6b	3.65				
→ 3)-β-D-GalNAc-(1 →	H-1	4.61	1,2	8.0	C-1	103.1
(**B**)	H-2	3.94	2,3	10.0	C-2	53.2
	H-3	4.04	3,4	3.0	C-3	79.8
	H-4	4.15	4,5	2.0	C-4	69.4
	H-5	3.75			C-5	75.6
	H-6a,6b	3.84			C-6	62.9
→ 7)-α-Neu5Ac-(2 →					C-1	173.9
(**C**)					C-2	102.35
	H-3ax	1.99	3ax,3eq	12.0	C-3	37.9
			3ax,4	12.0		
	H-3eq	2.59	3eq,4	6.0		
	H-4	4.15	4,5	10.0	C-4	68.9
	H-5	3.77	5,6	10.0	C-5	54.7
	H-6	4.04	6,7	2.0	C-6	72.3
	H-7	3.74	7,8	9.0	C-7	78.9
	H-8	3.81			C-8	73.1
	H-9a	3.80			C-9	63.6
	H-9b	3.56				
α-D-Glc-(1 →	H-1	5.44	1,2	3.5	C-1	96.7
(**D**)	H-2	3.49	2,3	10.0	C-2	72.95
	H-3	3.85	3,4	10.0	C-3	74.3
	H-4	3.30	4,5	10.0	C-4	71.4
	H-5	3.88			C-5	72.65
	H-6a	3.85			C-6	62.0
	H-6b	3.70				

NaBH$_4$, the mixture was dialysed and the material was obtained by lyophilisation. The residue was hydrolysed in 2% (0.33 M) AcOH for 30 min at 100°C. The resulting mixture of oligosaccharides was resolved by chromatography on a column (90 × 1.6 cm) of TSK SW 40(S) in 0.1 M AcOH. The eluate was monitored with a refractometer (Waters) at 240 nm. The purity of **I** and **II** in the main fractions was demonstrated by chromatography on TLC plates coated with Silica Gel 60 (Merck) with 100 : 10 : 10 : 30 : 3 EtOH–butan-1-ol–pyridine–water–AcOH as eluent [19].

Isolation of oligosaccharides **III** *and* **IV**.—The O56 polysaccharide was hydrolysed (0.33 M AcOH, 6 h, 100°C) and the oligosaccharides were then subjected to GPC and TLC [19].

Analytical methods.—Glucose and galactose were determined as their alditol acetates by GLC on ECNSS-M at 170°C as well as with D-glucose oxidase and D-galactose oxidase, respectively. Glucosamine and galactosamine were determined [20] as alditol acetates by GLC on PolyA 103 at 220°C. For the identification of Neu5Ac, the polysaccharides were hydrolysed, and the liberated neuraminic acid was re-*N*-acetylated with Ac$_2$O–NaHCO$_3$ and treated with Neu5Ac aldolase–lactic acid dehydrogenase–NADH [21].

The NMR spectra were recorded in D$_2$O with a Bruker WM 300 spectrometer at 300 MHz (^{1}H NMR) or at 75 MHz (^{13}C NMR) with acetone as the internal standard (δ_H 2.225; δ_C 31.45). Standard Bruker software was used for 2D COSY, COSYRCT and heteronuclear ^{13}C–^{1}H COSY (HXCOOR). 1D-NOE experiments were performed in the differential mode using the Bruker NOEMULT program. For ROESY spectra with a

Table 11
NOE data for the O24 polysaccharide **VII**

NOE observed on		Pre-irradiated protons			
Residue	Proton	**A**, H-1	**B**, H-1	**D**, H-1	C, 3eq
→ 3)-β-D-Glc-(1 →	H-1			+	
2	H-2	+		+	
(**A**)	H-3	+			
	H-5	+			
→ 3)-β-D-GalNAc-(1 →	H-2	+[a]	+		
(**B**)	H-3	+	+		
	H-4		+[a]		
	H-5		+		
→ 7)-α-Neu5Ac-(2 →	H-4				+
(C)	H-5				+
	H-7		+		
α-D-Glc-(1 →	H-2			+	
(**D**)	H-3				+
	H-4			+[a]	

[a] Minor signal, probably due to spin diffusion.

mixing time of 0.2 s, the Rance pulse sequence [22] was applied. 1D HOHAHA spectra were run with variable mixing times during accumulation [23]. The DANTE pulse sequence [24] was used for selective excitation.

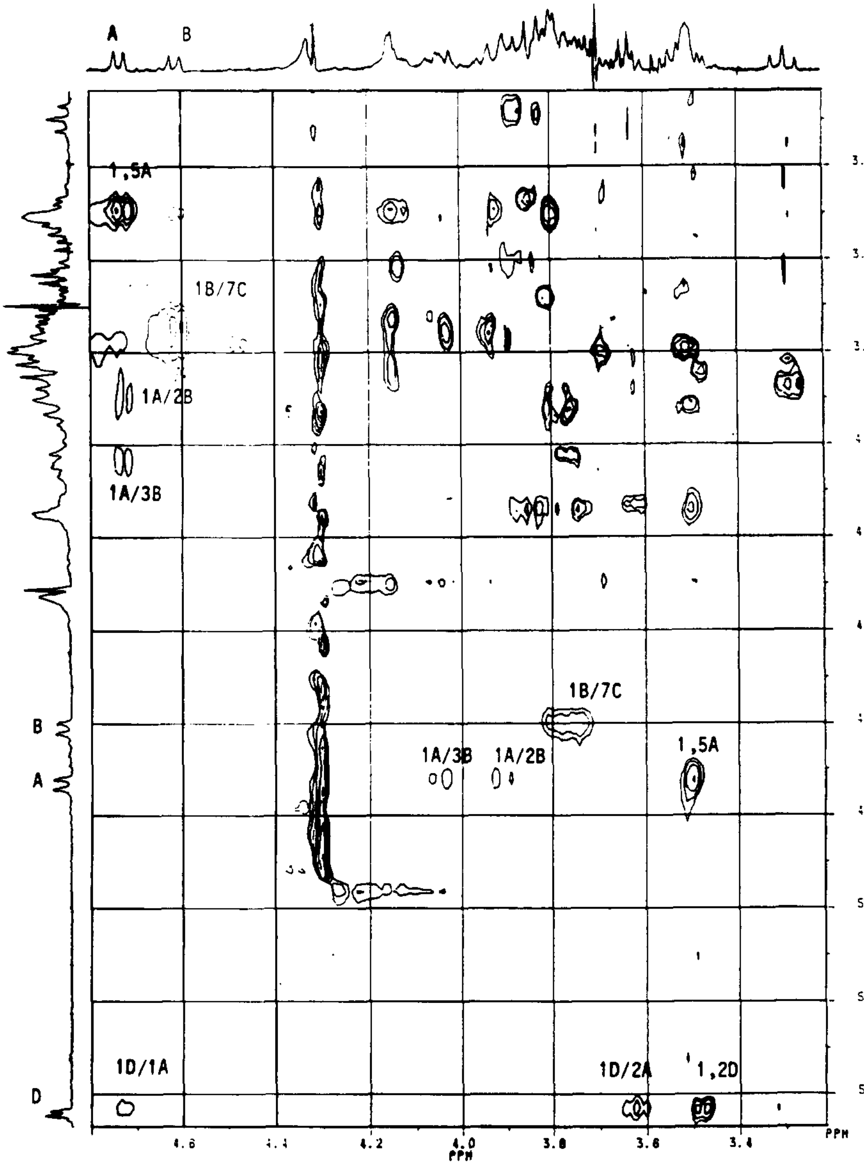


Fig. 3. ROESY spectrum of the O24 polysaccharide **VII**. Arabic numerals refer to the protons of the respective sugar units, which are denoted by capital letters as in formula **VII**.

Acknowledgment

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References

[1] J. Roth, U. Rutishauser, and F.A. Troy (Eds.), *Polysialic Acid*, Birkhäuser, Basel, 1993.

[2] B. Jann and K. Jann, *Curr. Top. Microbiol. Immunol.*, 150 (1990) 19–42.

[3] H.J. Jennings, *Curr. Top. Microbiol. Immunol.*, 150 (1990) 97–127.

[4] G.G.S. Dutton, H. Parolis, and L.A.S. Parolis, *Carbohydr. Res.*, 170 (1987) 193–206.

[5] G. Kogan, B. Jann, and K. Jann, *FEMS Microbiol. Lett.*, 91 (1992) 135–140.

[6] J.H. Krauss, K. Himmelspach, G. Reuter, R. Schauer, and H. Mayer, *Eur. J. Biochem.*, 204 (1992) 217–223.

[7] G. Kogan, A.S. Shashkov, B. Jann, and K. Jann, *Carbohydr. Res.*, 238 (1993) 261–270.

[8] G. Kogan, B. Jann, and K. Jann, *Carbohydr. Res.*, 238 (1993) 335–338.

[9] J.F.G. Vliegenthart, L. Dorland, H. van Halbeek, and J. Haverkamp, in R. Schauer (Ed.), *Sialic Acids, Chemistry, Metabolism and Function*, Springer, Wien, 1982, pp 127–172.

[10] O. Westphal and K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–91.

[11] J. Jeener, B.H. Meier, P. Bachmann, and E.E. Ernst, *J. Chem. Phys.*, 71 (1979) 4546–4553.

[12] J. Dabrowski, *Methods Stereochem. Anal.*, 9 (1987) 349–386.

[13] G.M. Lipkind, A.S. Shashkov, Y.A. Knirel, E.V. Vinogradow, and N.K. Kochetkov, *Carbohydr. Res.*, 175 (1988) 59–75.

[14] A.S. Shashkov, E.V. Vinogradov, Y.A. Knirel, N.E. Nifant'ev, N.K. Kochetkov, J. Dabrowski, E.V. Kholodkova, and E.S. Stanislawsky, *Carbohydr. Res.*, 241 (1993) 177–178.

[15] S. Prytulla, J. Lauterwein, M. Klessinger, and J. Thiem, *Carbohydr. Res.*, 215 (1991) 345–349.

[16] V. Pavlik, J.R. Brisson, F. Michon, D. Uhrin, and H.J. Jennings, *J. Biol. Chem.*, 268 (1993) 14146–14152.

[17] L. Poppe and J. Dabrowski, *Biochem. Biophys. Res. Commun.*, 159 (1989) 618–623.

[18] A.S. Shashkov, G.M. Lipkind, Y.A. Knirel, and N.K. Kochetkov, *Magn. Reson. Chem.*, 26 (1988) 735–747.

[19] R. Schauer and A.P. Corfield, in R. Schauer (Ed.), *Sialic Acids, Chemistry, Metabolism and Function*, Springer, Wien, 1982, pp 77–94.

[20] W. Niedermayer and M. Thomana, *Anal. Biochem.*, 57 (1974) 363–368.

[21] P. Brunetti, A. Swanson, and S. Roseman, *Methods Enzymol.*, 6 (1963) 465–473.

[22] M. Rance, *J. Magn. Reson.*, 74 (1985) 557–564.

[23] D.G. Davis and A. Bax, *J. Am. Chem. Soc.*, 107 (1985) 2820-2821.

[24] G.A. Morris and R. Freeman, *J. Magn. Reson.*, 29 (1978) 433–462.