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Note

The structure of the core–O-chain linkage region of the lipopolysaccharide from *Bordetella hinzii*

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Dedicated to the memory of Professor Nikolay K. Kochetkov

Abstract—Linkage region between core and the O-chain of the lipopolysaccharide from *Bordetella hinzii* has been analyzed by NMR and MS analysis of the products, obtained by anhydrous HF treatment or consecutive ammonia and AcOH treatment of the LPS. The following structure of this region was deduced from the experimental results:

PS-4-β-ManNAc3NAcAN-4-β-GlcNAc3NAcAN-4-α-GalNAc-4-β-ManNAc3NAcA-3-β-FucNAc4N-6-α-GlcN-inner core

This structure is identical to the structure of the respective region of *Bordetella parapertussis* LPS. Polysaccharide part (PS) consists of not more than 15 2,3-diacetamido-2,3-dideoxyhexuronamides, methylated at the only hydroxyl group of the non-reducing terminal monosaccharide.

Keywords: LPS; Structure; *Bordetella*; *Bordetella hinzii*; NMR

Bacteria of the genus *Bordetella* are Gram-negative aerobic coccobacilli, belonging to the family Alcaligenaceae. They are found only in warm-blooded animals. *Bordetella pertussis* and *Bordetella parapertussis* are human respiratory tract pathogens, *Bordetella bronchiseptica* and *Bordetella hinzii* are animal pathogens, but are increasingly found in AIDS and cystic fibrosis patients.[1–5] Lipopolysaccharide is an important component of bacterial surface, actively interacting with the host defense. The structures of the core and polysaccharide parts of *B. hinzii* LPS have been determined.[6,7] However the linkage between these fragments was not analyzed. Recently we developed methods for the analysis of this part of the molecule and determined structures of the linkage region in *B. bronchiseptica* and *B. parapertussis*.[8] Here we present data on the structure of the core–O-chain linkage region of *B. hinzii*.

LPS from *B. hinzii* was treated with anhydrous HF for 24 h at 25 °C, and the polysaccharide was isolated using size-exclusion chromatography. The product was analyzed by NMR spectroscopy (Fig. 1, Table 1). The signals of previously described polymeric structure with repeating trisaccharide units and methylated terminal monosaccharide were identified[7] (Scheme 1). Additionally, spectra contained signals of the fragment Z–MM–X–Y, identified previously during the analysis of LPS from *B. bronchiseptica* and *B. parapertussis*.[8] The polymer consisted of amides of 2,3-diacetamido-2,3-dideoxyhexuronic acids and had a GalNAc residue at the reducing end. Most of the repeating units gave overlapped signals, but monosaccharides A (terminal) and F could be identified. Repeating structure was linked

Abbreviations: LPS, lipopolysaccharide; HMBC, heteronuclear multiple bond connectivity; FucNAc4N, 2-acetamido-4-amino-2,4,6-trideoxy-galactose; GalNA, galactosaminuronic acid; GlcA, glucuronic acid; GlcN, glucosamine; Hep, L-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-octulosonic acid; ManNAc3NAcA, 2,3-diacetamido-2,3-dideoxy-mannuronic acid; GalNAc3NAcA, 2,3-diacetamido-2,3-dideoxy-galacturonic acid; GlcNAc3NAcA, 2,3-diacetamido-2,3-dideoxy-glucuronic acid

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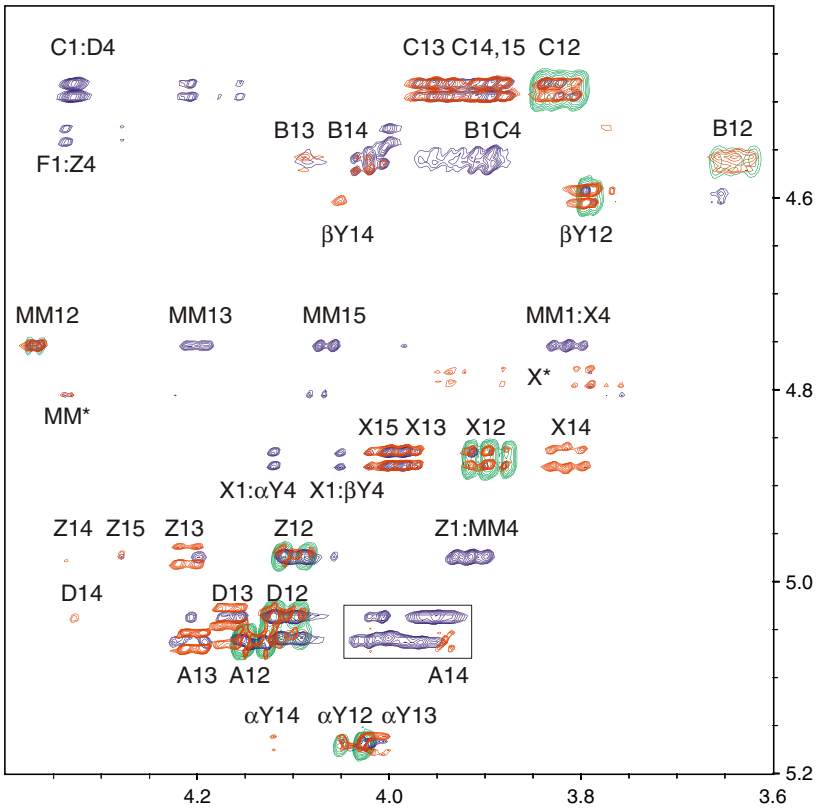


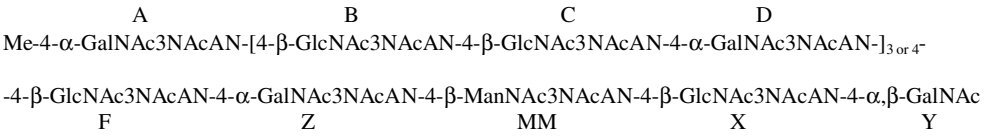
Figure 1. Fragment of overlapped COSY (green), TOCSY (red) and NOESY (blue) spectra of the HF-released *B. hinzii* O-specific polysaccharide, containing correlations from anomeric protons. Blue signals inside the box are NOE correlations from α-diacetamidogalacturonic acid A and D to B4, B5 and C3. Spin system of the residue F is not visible because the intensity of spectra was lowered to clarify the picture.

Table 1. NMR data for HF released polysaccharide (D_2O, 35 °C)

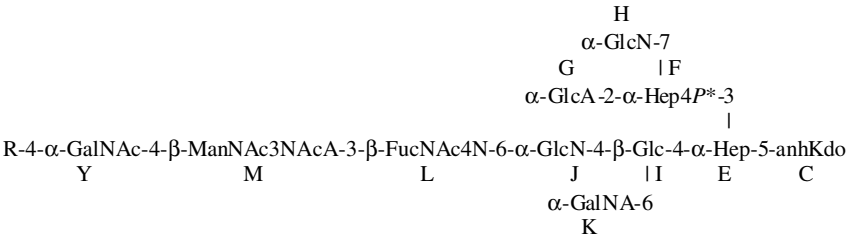
Unit	H/C 1	H/C 2	H/C 3	H/C 4	H/C 5	H/C 6
A α-GalNAc3NAcAN4Me	5.06	4.12	4.19	3.94	4.22	
	98.7	48.3	49.3	78.9	71.8	173.8–174.5
B β-GlcNAc3NAcAN	4.56	3.64	4.08	3.95–4.05	3.89	
	103.1	55.5	54.5	76.4–76.7	76.3	172.2
C β-GlcNAc3NAcAN	4.49	3.82	3.94	3.90–3.93	3.89	
	103.3	54.2	53.9	78.3	76.3	172.2
D α-GalNAc3NAcAN	5.04	4.12	4.18	4.33	4.22	
	98.7	48.3	48.3	75.7	71.8	173.8–174.5
F β-GlcNAc3NAcAN	4.53	3.78				
	102.9	55.4				
Z α-GalNAc3NAcAN	4.97	4.12	4.18	4.33	4.28	
	99.3	48.3	48.3	75.7	71.8	174.0
MM β-ManNAc3NAcAN	4.75	4.36	4.20	3.92	4.07	
	100.9	51.9	53.4	74.5	77.3	172.5
X β-GlcNAc3NAcAN	4.87	3.89	3.99	3.81	3.99	
	103.2	54.3	54.5	79.9	75.5	173.6
α-Y α-GalNAc	5.16	4.02	4.02	4.12	4.07	3.74
	92.4	51.7	68.8	77.2	71.3	62.3
β-Y β-GalNAc	4.59	3.79	3.79	4.05	3.65	3.71
	96.8	54.9	72.6	76.3	75.6	62.4

Methyl group signals 3.42/62.9 ppm ($^1H/^{13}C$). Acetate groups: 1.87–2.02/23.2 ppm (Me), 175.7 (CO).

HF-released polysaccharide:



Products of the LPS degradation with NH_3 (β-elimination) and then AcOH (lipid A release):



R = Δ-β-GlcNAc3NAcAN- **1a**
X

R = Δ-β-ManNAc3NAcAN-4-β-GlcNAc3NAcAN- **1b**
MM X

R = Δ-α-GalNAc3NAcAN-4-β-ManNAc3NAcAN-4-β-GlcNAc3NAcAN- **1c**
Z MM X

Scheme 1. Structures of the isolated compounds. Amides of the uronic acids are indicated by addition of 'N' at the end of abbreviated monosaccharide name. 'Δ' before monosaccharide name stands for 4,5-β-elimination products (2,3-diacetamido-2,3,4-trideoxy-hex-4-enuronopyranosyl).

to the GalNAc3NAcAN residue Z through the β-GlcNAc3NAcAN residue F. Minor signals of the variant of MM-X fragment were observed (shown on Fig. 1), which may be due to incomplete amidation of some of these monosaccharides.

MALDI mass spectrum of the HF-released polysaccharide (Fig. 2) contained one major peak at 3836 Da, corresponding to the structure with 14 residues of HexNAc3NAcAN, HexNAc, and methyl group. Minor signals differing by approx. 257 Da (amide of diacetamidohexuronic acid) were observed on both sides of the main signal. Shorter structures may originate from partial HF cleavage or incomplete biosynthesis. The most extended structure contained 17 diacetamidohexuronic acid amides.

Considering that without the residues MM and X, mass spectral data show the presence of 12 diacetamidouronic acids in the structure, it should contain four repeating units. However residues A and Z at the ends of the repeating structure both have *galacto*-configuration, which is inconsistent with the presented structure of repeats. Thus, probably there are only three 'correct' repeats, and one of the *gluco*-sugars at the reducing or non-reducing terminal repeating unit is replaced by a *galacto*-one.

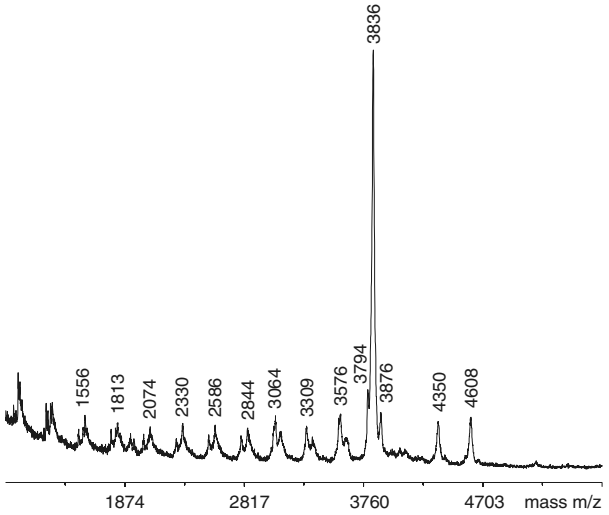


Figure 2. Positive mode MALDI mass spectrum of the HF-released *B. hinzii* O-specific polysaccharide.

In order to find how the above described polymer is linked to the rest of the LPS, compounds **1a**–**c** were prepared and analyzed. Treatment of the LPS with ammonia causes O-chain depolymerization due to

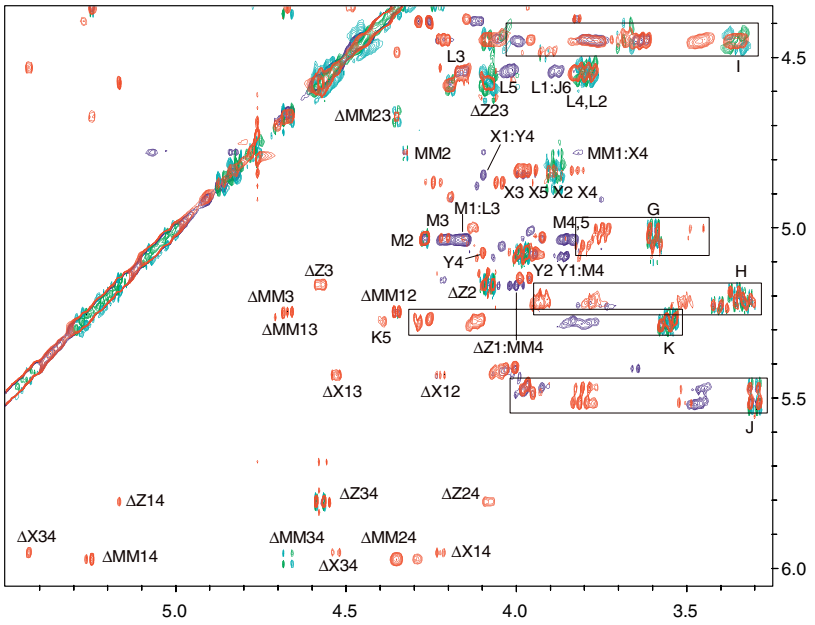


Figure 3. Fragment of overlapped COSY (green, cyan), TOCSY (red) and NOESY (blue) spectra of the mixture of compounds **1a**–**c**. Single number labeled correlations are from H-1 of the respective residue. Core monosaccharide signals are boxed. Signals of the fragment X–Y–M–L–J are clearly visible, as well as three products of β-elimination from residues MM, Z, and X.

β-elimination of the amides of uronic acids. After lipid A cleavage by mild acid hydrolysis, the products **1a**–**c** containing full core with fragments of PS linking region were obtained (Scheme 1). Ammonia cleavage was not complete, and three products ending at elimination derivatives of units X, MM, or Z were identified based on NMR (Fig. 3) and MS data. ESI masses of 2331, 2588, and 2846 Da for **1a**–**c**, respectively (structures without phosphate at Hep F; corresponding masses at +80 Da with lower intensity were also present), were observed. NMR data showed that GalNAc Y has the α-configuration within the LPS and is linked to the fragment ManNAc3NAcA-3-β-FucNAc4N- (M–L), connected to O-6 of the core GlcN J. Thus overall the structure of the region between repeating units and the core is identical to that of *B. parapertussis*.[8]

O-Chain of *B. hinzii* LPS, in contrast to homopolymeric O-chains of LPS of *B. bronchiseptica* and *B. parapertussis*, consists of trisaccharide repeating units. Still this polysaccharide is short, and has the length similar to the length of the *B. bronchiseptica* and *B. parapertussis* polysaccharides. Polysaccharide is linked to the core part through a specific oligosaccharide, common for all analyzed *Bordetella* LPS. This oligosaccharide is not a part of the core, and is not present in natural LPS variants without O-chain. O-Chain in *Bordetella* is polymerized by a processive glycosyltransfer mechanism, i.e. sequential addition of monosaccharides to the nonreducing end of the growing chain. Polymer growth starts on a 'primer' sugar, which is then together with O-chain transferred to the core. Still it is not clear which part of the oligosaccharide, described here, serves as a primer.

1. Experimental

B. hinzii strain ATCC 51730 was originally isolated from blood of AIDS patients. Cells were grown and LPS were isolated as described.[9]

1.1. NMR spectroscopy

NMR spectra were recorded at 35 °C in D_2O on Varian UNITY INOVA 500 instrument, using acetone as a reference for proton (2.225 ppm) and carbon (31.5 ppm) spectra. Varian standard programs, COSY, NOESY (mixing time of 200 ms), TOCSY (spinlock time of 120 ms), HSQC, and gHMBC (long-range transfer delay of 100 ms) were used.

1.2. MALDI

MALDI mass spectra were obtained using Perseptive Biosystems Voyager DE STR spectrometer with 2,4-dihydroxybenzoic acid (DHB) as a matrix.

1.3. Preparation of the compounds 1a–c

LPS (100 mg) was heated at 50 °C for 24 h in 12% NH_4OH (4 mL) containing $NaBH_4$ (25 mg), lyophilized, acidified with 2 M HCl, and separated on a Sephadex G50 SF gel (Pharmacia) column (2.5 × 80 cm) using pyridine-acetic acid buffer (4 mL of pyridine and 10 mL of AcOH in 1 L of water). The fraction eluted with solvent front was hydrolyzed by 2% AcOH (100 °C, 3 h), the precipitate was removed by centrifugation, and the soluble

products were separated on a Sephadex G50 SF gel to give oligosaccharide core fraction. It was additionally cleaned by passing through Seppak C18 cartridge in water.

1.4. Preparation of the polysaccharide

LPS (100 mg) was dissolved in anhydrous HF (about 7 mL) and kept for 24 h in closed polypropylene tube (50 mL vol), then opened under fume hood. After HF evaporated, the product was dissolved in water, neutralized by 24% ammonia, clarified by centrifugation, and separated on a Sephadex G50 SF gel to give clean polysaccharide (30 mg).

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