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Note

The structure of the O-polysaccharide isolated from the lipopolysaccharide of *Salmonella* Dakar (serogroup O:28)

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Abstract—The structure of the O-antigenic part of the lipopolysaccharide (LPS) of *Salmonella* Dakar was analysed using chemical methods, NMR spectroscopy and mass spectrometry. The following structure for the repeating unit of the O-polysaccharide was determined:

β-D-Glc*p*
↓4
→4)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→4)-α-D-Qui*p*3NAc-(1→3)-α-L-Rha*p*-(1→

where Qui*p*3NAc is 3-acetamido-3,6-dideoxyglucose. This is the first published structure of the O-polysaccharides from 101 serotypes of *Salmonella* bacteria belonging to serogroup O:28 (formerly M) in the Kauffmann–White scheme.

Keywords: *Salmonella* Dakar; Lipopolysaccharide; O-Polysaccharide structure; Serogroup O:28; NMR; Mass spectrometry

The genus *Salmonella*, a typical member of the family *Enterobacteriaceae*, has been divided into two genomic species, *S. enterica* and *S. bongori*, and more than 2500 serologically distinct types of *Salmonella* listed in the Kauffmann–White scheme, later modified by Popoff and Le Minor.[1] These microorganisms figure predominantly as one of the leading causes of bacterial food-borne disease,[2,3] and they are also responsible for extra-intestinal infections such as bacteraemia, meningitis, pneumonia, urinary track infection and abscesses.[4]

The *Salmonella* serotype is based on the immunoreactivity of three different types of antigens: somatic O, flagellar H and capsular Vi.[5] The serological O-specificity of *Salmonella* is defined by the structure of the O-antigen (O-polysaccharide, OPS), being a part of the lipopolysaccharide (LPS), one of the major components of the outer surface of smooth-type Gram-negative bacteria.

In this paper, we report on the structure of the *Salmonella* Dakar O-polysaccharide. This bacterium was identified in 1954[6] and classified into the serogroup O:28 (formerly M) in the Kauffmann–White scheme. Strains from this serogroup contain only the O28 epitope, which was divided into three subfactors—$O28_1$, $O28_2$ and $O28_3$—without structural differences being ascribed.[5] *S.* Dakar has the subfactors $O28_1$ and $O28_3$, whereas *S.* Telaviv from the same serogroup has $O28_1$ and $O28_2$. As yet, none of their O-antigens have been structurally assigned. *S.* Dakar cross-reacts with *S.* Champaing, *S.* Frankfurt, and with *Citrobacter freundii* 8090 and *C. freundii* 869.[7]

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The *Salmonella* Dakar LPS was isolated from the bacterial membrane by hot phenol-aqueous extraction and recovered from the aqueous phase. Mild acid hydrolysis of the LPS afforded a lipid sediment and a water-soluble carbohydrate portion, which was fractionated by gel permeation chromatography (GPC) on Bio-Gel P-10 column to yield a high-molecular-mass O-polysaccharide (OPS). Sugar analysis of the OPS revealed five sugars: rhamnose, 3-amino-3,6-dideoxyhexose, glucose, galactose and galactosamine, in the ratios of 0.8:0.9:1.0:1.1:0.7. NMR studies of the O-polysaccharide showed that 3-amino-3,6-dideoxyhexose was 3-amino-3,6-dideoxyglucose (Qui3N) ($^3J_{H\text{-}1,H\text{-}2} = 3.6$ Hz, $^3J_{H\text{-}2,H\text{-}3} = 10.4$ Hz, $^3J_{H\text{-}3,H\text{-}4} = 10.2$ Hz, $^3J_{H\text{-}4,H\text{-}5} = 9.8$ Hz, $^3J_{H\text{-}5,H\text{-}6} = 6.8$ Hz). This was in agreement with the result of Lüderitz et al.,[7] who identified Qui3N in the LPS *S.* Dakar. The absolute configuration of all monosaccharides, except Qui3N, was determined by GLC of the acetylated glycosides with (*S*)-2-butanol.[8,9] These analyses demonstrated the D configuration of Glc, Gal and GalN and the L configuration of Rha. The D configuration of Qui3N was established by NMR studies (see below).

GLC–MS of the partially methylated alditol acetates derived from the methylated O-polysaccharide by acid hydrolysis revealed the presence of a 3-substituted Rha, terminal Glc, 4-substituted Gal, 4-substituted Qui3N and 3,4-disubstituted GalN in the molar ratios of 1.0:1.0:1.0:0.5:0.5.

The pyranose form of all monosaccharides was assigned from NMR spectroscopic data. The absence of signals at 84–88 ppm in the ^{13}C NMR spectrum of the OPS (Fig. 1) indicated six-membered rings in all the sugars.[10] In addition, methylation analysis of the OPS confirmed the pyranose ring of Rha and Glc.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained signals for five anomeric carbons (δ 96.87, 98.58, 103.19, 103.93 and 106.22), three HO*C*H$_2$–C groups (C-6 of Gal, Glc and GalN) at δ 61.9–62.9, two *C*H$_3$–C groups (C-6 of Rha and Qui3N) at δ 17.96 and 19.27, two nitrogen-bearing carbons of amino sugars (C-2 of GalN and C-3 of Qui3N) at δ 49.80 and 55.09, 18 oxygen-bearing sugar-ring carbons at δ 68.0–78.4 and two *N*-acetyl groups (CO at δ 174.54 and 175.72, and CH$_3$ at δ 23.76 and 23.45).

The ^{1}H NMR spectrum (Fig. 2), supported by the ^{1}H,^{13}C HSQC spectrum of the OPS (data not shown), showed anomeric resonances at δ 5.24, 5.14, 5.06, 4.82 and 4.44, at almost equal relative signal intensities. Besides the ring proton region at δ 3.3–4.5, other characteristic signals were present: at δ 1.34 and 1.32 for two methyl groups (H-6 of Rha and Qui3N), and at δ 1.98 and 2.03 for two *N*-acetyl groups. These data indicate that the pentasaccharide repeating unit of *S.* Dakar polysaccharide consists of D-Glc, D-Gal, D-GalNAc, L-Rha and D-Qui3NAc.

The complete structural characterisation of the OPS was retrieved from the results of 2D NMR analysis involving ^{1}H,^{1}H COSY, TOCSY, NOESY and ^{1}H,^{13}C HSQC and HMBC experiments, which were used to assign the chemical shift spin systems of the five sugar residues present in the repeating unit (Table 1) according to the published methodology.[11,12]

The spin systems of GalNAc and Qui3NAc were distinguished from the other sugars using the characteristic chemical shifts of the protons attached to the nitrogen-bearing carbons at δ 4.49/49.80 (H-2/C-2) of GalNAc and at δ 4.33/55.09 (H-3/C-3) of Qui3NAc, respectively. The spin system of Qui3NAc was distinguished from that of GalNAc by correlation of the proton H-1 (Qui3NAc) to H-6 at δ 1.32 in the TOSCY spectrum. The spin system of Rha was identified by correlation of the proton H-1 (δ 5.14) to the H-6 characteristic of a 6-deoxysugar (δ 1.34). The Glc and Gal spin systems were distinguished on the basis of characteristic chemical shifts and $^3J_{H,H}$ coupling constants of the sugar-ring protons; they were in agreement with published data of the respective pyranosides.[10,13–15]

The TOCSY spectrum clearly showed correlations from H-6 to all the protons of Qui3NAc and Rha, from H-1 to H-6 of Glc and from H-1 to H-4 of GalNAc and Gal. The COSY spectrum showed most correlations be-

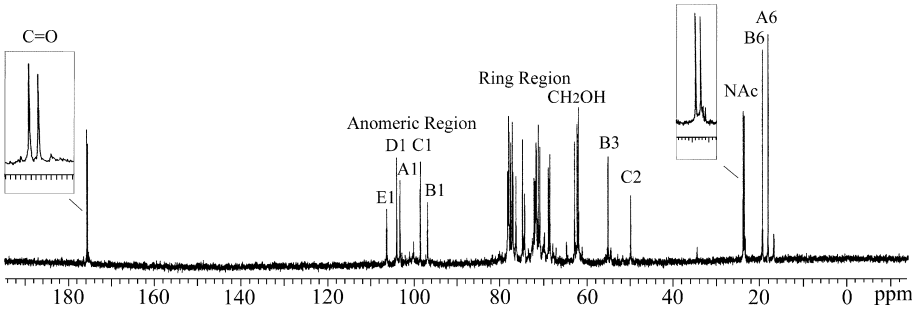


Figure 1. ^{13}C NMR spectrum of the O-polysaccharide of *Salmonella* Dakar lipopolysaccharide. A1, B1, C1, D1 and E1 correspond to the anomeric carbons of individual sugar residues (**A** Rha, **B** Qui3NAc, **C** GalNAc, **D** Glc and **E** Gal, respectively). The Arabic numerals refer to the atoms in the sugar residues denoted by capital letters, as shown in Table 1.

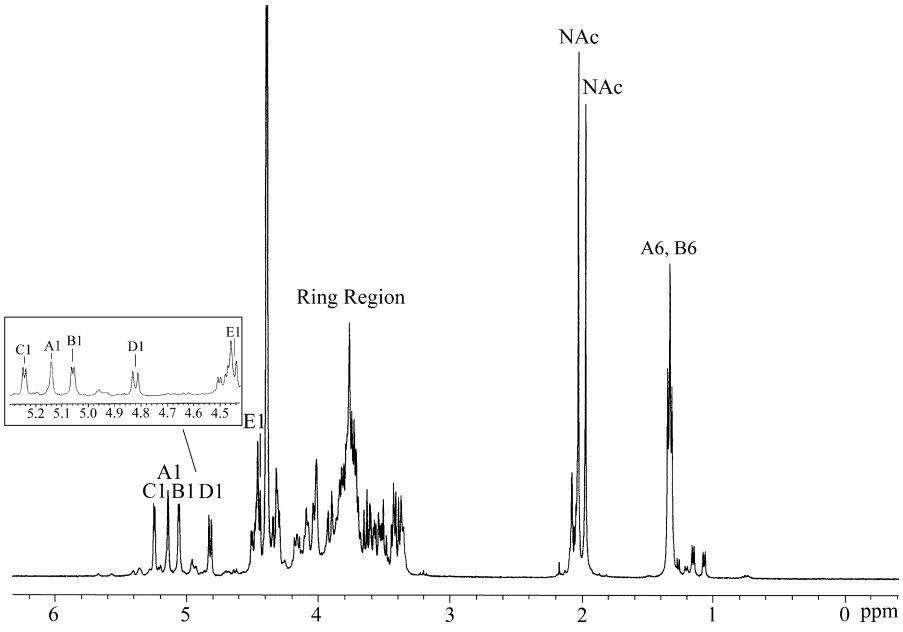


Figure 2. ^{1}H NMR spectrum of the O-polysaccharide of *Salmonella* Dakar. A1, B1, C1, D1 and E1 correspond to the anomeric protons of individual sugar residues (**A** Rha, **B** Qui3NAc, **C** GalNAc, **D** Glc and **E** Gal, respectively). The Arabic numerals refer to the atoms in the sugar residues denoted by capital letters, as shown in Table 1.

Table 1. ^{1}H and ^{13}C NMR chemical shifts (δ) for the O-polysaccharide of *Salmonella* Dakar

Sugar residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6
A →3)-α-L-Rha*p*-(1→	5.14 103.19	4.31 68.84	3.84 78.26	3.64 71.71	3.78 70.78	1.34 17.96
B →4)-α-Qui*p*3NAc-(1→	5.06 96.87	3.60 71.60	4.33 55.09	3.51 78.19	4.16 68.52	1.32 19.27
C →3,4)-α-D-Gal*p*NAc-(1→	5.24 98.58	4.49 49.80	4.03 77.98	4.46 77.00	4.09 72.19	3.79,[a] 3.73[b] 61.93
D β-D-Glc*p*-(1→	4.82 103.93	3.38 74.85	3.43 77.55	3.41 71.15	3.35 77.18	3.75,[a] 3.91[b] 62.25
E →4)-β-D-Gal*p*-(1→	4.44 106.22	3.56 71.88	3.76 74.38	4.01 77.98	3.71 76.31	3.75 62.86

The ^{1}H NMR spectrum was recorded at 400 MHz and at 62 °C. The ^{13}C NMR spectrum was recorded at 100 MHz on a 400-MHz instrument at the same temperature. Values are referred to an internal acetone standard (^{1}H 2.225, ^{13}C 31.45 ppm).

There are additional chemical shifts for NAc in the ^{1}H NMR spectrum at δ 2.03 and 1.98 for CH_3, in the ^{13}C NMR spectrum at δ 23.76 and 23.45 for CH_3, and at 175.72 and 175.54 ppm for C=O, respectively.

[a] Chemical shift for H-6a.

[b] Chemical shift for H-6b.

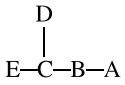
tween the neighbouring protons within each spin system. The signals of H-5 and H-6 protons of Gal and GalNAc were assigned using 2D COSY, HMBC and ^{1}H,^{13}C HSQC experiments. The H-5 and H-6 resonances for Gal were found by correlations between H-4, H-5 and H-6 in the COSY spectrum, by those of H-5 with C-6 in the HMBC spectrum, and between the carbons and the attached protons in the ^{1}H,^{13}C HSQC spectrum. H-5 and H-6a,6b protons for GalNAc were determined by correlations of H-5 with C-4 and C-6 in the HMBC spectrum, and H-5/H-6a,6b cross-peaks in the COSY spectrum.

The relatively low-field positions of the carbon signals for C-3 of Rha (δ 78.26), C-4 of Qui3NAc (δ 78.19), C-4 of Gal (δ 77.98) and C-3 and C-4 of GalNAc (δ 77.98 and 77.00, respectively) demonstrated the glycosylation pattern of the sugars.[10] These results concur with the results of the methylation analysis of the OPS.

The $^{3}J_{\text{H-1,H-2}}$ coupling constants, determined from the ^{1}H NMR spectrum—4.0 Hz for GalNAc and 3.6 Hz for

Qui3NAc—exhibited α configurations for these monosaccharides, whereas $^{3}J_{H-1,H-2}$ values of 8 Hz indicated β-linked Glc and Gal. The α configuration of Rha was established from the chemical shift of the C-5 signal at δ 70.78 (δ 70.0 and 73.2 for C-5 in α-Rha*p* and β-Rha*p*,[14,16] respectively; Table 1). In addition, these conclusions were confirmed by the $^{1}J_{H-1,C-1}$ coupling constants determined from the coupled $^{1}H,^{13}C$ HSQC spectrum. The $^{1}J_{H-1,C-1}$ values of 171.7 Hz for Qui3NAc, 177.7 Hz for GalNAc and 174.6 for Rha again indicated α-linked sugar residues, whereas the $^{1}J_{H-1,C-1}$ of 164.1 Hz for Gal substantiated the β anomeric configuration of this monosaccharide. Unexpectedly, the high value of $^{1}J_{H-1,C-1}$ for Glc (173.1 Hz) did not tally with the previously proposed β-anomeric configuration of Glc ($^{3}J_{H-1,H-2}$ 8 Hz, H-1 at δ 4.82, C-1 at δ 103.93).[12,14] Determination of the anomeric configuration of Glc was supported by NOESY data. In the NOESY spectrum intra-residual NOE correlations were observed between H-1/H-3 and H-1/H-5 resonances for Glc, which proved the β anomeric configuration of this sugar residue.

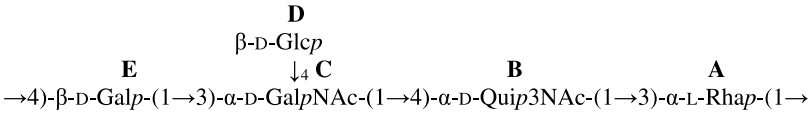
The monosaccharide sequence within the repeating unit was established by NOESY and HMBC experiments. In the NOESY spectrum (data not shown), the following inter-residual NOE correlations were observed: **A**1–**E**4, **B**1–**A**3, **C**1–**B**4, **D**1–**C**4, **E**1–**C**3. The HMBC spectrum (Fig. 3) showed inter-residual cross-peaks between the following anomeric protons and linkage carbons: **A** H-1, **E** C-4, **C** H-1, **B** C-4, **D** H-1, **C** C-4, **E** H-1, **C** C-3, and between the protons at the linkage and anomeric carbons: **B** H-4, **C** C-1, **C** H-4, **D** C-1, **E** H-4, **A** C-1. These results determined the monosaccharide sequence within the repeating unit of the *S.* Dakar polysaccharide:



Determination of the monosaccharide sequence and absolute configuration of Rha allowed to establish the configuration of Qui3NAc by analysis of glucosylation effect on the ^{13}C chemical shifts using the published rules.[17,18] An experimental ^{13}C chemical shift for C-1 of Qui*p*3NAc and for all carbons of Rha*p* (Table 1), compared with those calculated for the α-D-Qui*p*3-NAc-(1→3)-L-Rha*p*- and the α-L-Qui*p*3NAc-(1→3)-L-Rha*p*-fragment, clearly indicated the D-configuration of Qui3NAc.

MALDI-TOF-MS analysis of the whole *S.* Dakar O-polysaccharide was also performed (Fig. 4). The MALDI spectrum showed several ions up to *m*/*z* 9000, with differences of ~860 between their *m*/*z* values and confirmed perfectly that the repeating unit has a molecular mass of 860 Da.

On the basis of the data obtained, it was concluded that the repeating unit of the *Salmonella* Dakar O-polysaccharide has the following structure:



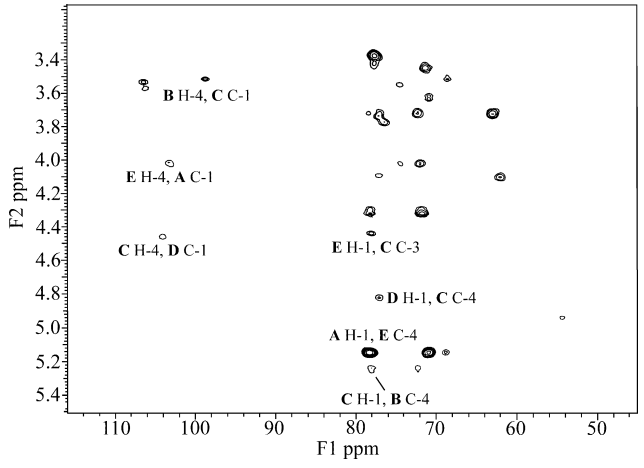


Figure 3. Part of the HMBC spectrum of the O-polysaccharide of *Salmonella* Dakar. The Arabic numerals refer to atoms in sugar residues denoted by capital letters as shown in Table 1.

This is the first established structure of the O-specific polysaccharide chain from 101 serotypes of bacteria *Salmonella* classified in the serogroup O:28 (formerly M). In

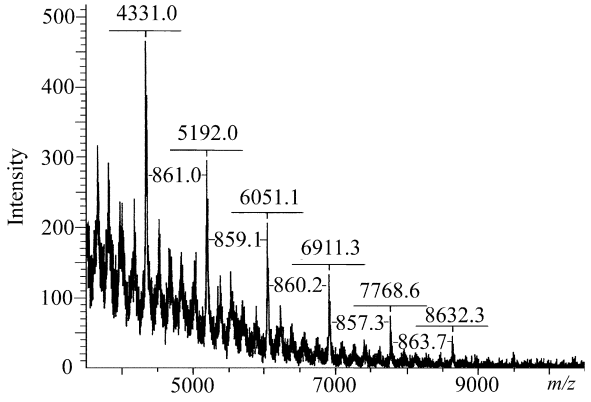


Figure 4. MALDI mass spectrum of the *Salmonella* Dakar O-polysaccharide.

the future, we would like to determine the size of the O28 epitope and explain the molecular basis for subdividing it into subfactors $O28_1$ and $O28_3$.

1. Experimental

1.1. Bacterial culture and growth conditions; isolation of the lipopolysaccharide

Salmonella enterica subsp. *enterica* serovar Dakar (I 28:a:-,1,6), strain KOS no. 1417, was obtained from the National Salmonella Centre of Poland, KOS collection, Gdańsk, Poland. The bacteria were cultivated on an enriched agar medium for 24 h at 37 °C, as described previously.[19] The isolated bacteria were washed from the agar with 0.85% NaCl, killed with acetone and dried. The lipopolysaccharide (LPS) was obtained from the dry bacterial mass (15.1 g) by the hot phenol-aqueous extraction procedure described by Westphal and Jann.[20] The top aqueous solution layers were collected, dialysed against distilled water, concentrated and treated with ethanol, first at pH 4.5 to obtain a 40% ethanolic solution for the removal of nucleic acids, then at pH 7.0 to obtain an 80% ethanolic solution for the precipitation of the lipopolysaccharide. The precipitated gel was dissolved in water, dialysed against distilled water and lyophilised to produce 0.557 g pure LPS (3.7% of dry bacterial weight).

1.2. Separation of the O-polysaccharide chain from the *S.* Dakar lipopolysaccharide

The *S.* Dakar LPS was hydrolysed with aq 1% HOAc (1 mL 1% acetic acid/10 mg of LPS, 100 °C, 2.5 h) and after removal of the lipid sediment, the carbohydrate-containing material (82% of the LPS weight) was fractionated by GPC on a Bio-Gel P-10 (200–400 mesh, BioRad, Richmond, USA) column (100×0.9 cm) with water as eluent at a flow rate of 5 mL h^{-1}. The fractions were monitored with a differential refractometric detector (RIDK 101, Prague, Czech Republic).

1.3. Monosaccharides analysis

Neutral and amino sugars were analysed by gas–liquid chromatography (GLC) as alditol acetates following hydrolysis of the O-polysaccharide (0.6 mg) with 2 M trifluoroacetic acid (300 μL, 2 M TFA) (Lancaster, Germany) at 120 °C for 2 h, reduction with $NaBH_4$ and acetylation (300 μL Ac_2O, 1 mg AcONa, 120 °C, 2 h). GLC was performed on a GC 8000 TOP (CE Instruments) gas chromatograph equipped with an Rtx-1 capillary column (30 m, 0.25 mm I.D., 0.25 μm film thickness, Restek Co. USA) using a linear temperature programme from 140 to 240 °C at 1 °C min^{-1}. For sugar identifications, GLC analyses with standard co-injections and mass spectra were employed. Gas–liquid chromatography–mass spectrometry (GLC–MS) analysis was performed using a TRIO-2000 instrument (VG Biotech, UK) with an electron impact ionisation energy of 70 eV, coupled to a Hewlett-Packard 5890 gas chromatograph.

For determination of the absolute configuration of the monosaccharides, the polysaccharide was hydrolysed with 2 M TFA as above, N-acetylated (400 μL 1% aq $NaHCO_3$, 100 μL Ac_2O, 20 °C, 1 h), subjected to (*S*)-(+)-2-butanolysis (450 μL (*S*)-(+)-2-butanol, 35 μL TFA, 105 °C, 6 h),[8,9] acetylated and analysed by GLC. GLC was performed on a DB-23 capillary column (60 m, 0.3 mm I.D.) using a temperature programme from 120 to 256 °C at 2 °C, and then at 256 °C for 30 min. The GLC peaks were identified by GLC co-injection with standards.

1.4. Methylation analysis

Methylation of the polysaccharide was performed according to the Hakomori procedure.[21] The permethylated products were purified by dialysis on a microscale against distilled water, then recovered using a C18 Sep-Pak cartridge.[22] Partially methylated monosaccharides were derived by hydrolysis with aq 2 M TFA, converted into the alditol acetates as above, and analysed by GLC and GLC–MS using an ECONO-CAP EC-1 ($30\ m \times 0.25$ nm I.D., 0.25 μm film thickness, Alltech) capillary column and a temperature programme from 100 to 260 °C at 2 °C min^{-1}.

1.5. MALDI mass spectrometry

MALDI mass spectrum of the *S.* Dakar OPS was recorded for the ethanolic solution of the sample deposited on a DHB matrix (2,5-dihydroxy benzoic acid) with a BIFLEX III MALDI TOF mass spectrometer (Bruker, Germany) equipped with a nitrogen laser ($\lambda = 337$ nm). The spectrum was recorded in positive mode with a pulse width of 3 ns and an energy density of 10^6–10^7 W cm^{-2}. The mass spectrum was recorded from m/z 200 to 12,000 amu and presented as averages of 450 acquisitions.

1.6. NMR spectroscopy

The sample was freeze-dried twice from D_2O solution, then dissolved in 0.7 mL of 99.95% D_2O (Deutero GmbH, Kastellaun, Germany). Spectra were recorded for the native O-polysaccharide of *S.* Dakar (22.2 mg) at 62 °C on a Varian Mercury 400 MHz spectrometer. ^1H NMR experiments were carried out using a spectral width of 2.5 kHz for a 32 K data set. Proton decoupled ^{13}C NMR spectrum was recorded at 100 MHz for a

32 K data set. Chemical shifts were expressed relative to internal acetone (δ_H 2.225 ppm, δ_C 31.45 ppm).

Standard pulse sequences from the Varian were used to acquire 2D COSY, TOCSY and NOESY spectra. A spectral width of 2.5 kHz was applied. The time-domain data matrix containing 2048×2048 data set points was zero-filled in both dimensions and multiplied by a shifted sine-bell window function prior to Fourier transformation. The TOCSY experiment was measured with a MLEV17 sequence with a mixing time of 100 ms; the NOESY mixing time was 300 ms.

The $^{1}H,^{13}C$ single quantum coherence experiment (HSQC) was performed with spectral widths of 2.5 kHz in ^{1}H and 16 kHz in ^{13}C. The relaxation delays were 1.0 s for both coupled and non-coupled spectra. The heteronuclear multiple-bond correlation spectrum (HMBC) was recorded with a spectral window of 2.5 kHz in the f_2 dimension (^{1}H) and 25 kHz in the f_1 dimension (^{13}C) and optimised for long-range correlations (delay 60 ms).

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