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Lipopolysaccharides from *Serratia marcescens* Possess One or Two 4-Amino-4-deoxy-L-arabinopyranose 1-Phosphate Residues in the Lipid A and D-*glycero*-D-*talo*-Oct-2-ulopyranosonic Acid in the Inner Core Region**

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Abstract: The carbohydrate backbones of the core-lipid A region were characterized from the lipopolysaccharides (LPSs) of *Serratia marcescens* strains 111R (a rough mutant strain of serotype O29) and IFO 3735 (a smooth strain not serologically characterized but possessing the O-chain structure of serotype O19). The LPSs were degraded either by mild hydrazinolysis (de-*O*-acylation) and hot 4 M KOH (de-*N*-acylation), or by hydrolysis in 2% aqueous acetic acid, or by deamination. Oligosaccharide phosphates were isolated by high-performance anion-exchange chromatography. Through the use of compositional analysis, electrospray ionization Fourier transform mass spectrometry, and ^{1}H and ^{13}C NMR spectroscopy applying various one- and two-dimensional experiments, we identified the structures of the carbohydrate backbones that contained D-*glycero*-D-*talo*-oct-2-ulopyranosonic acid and 4-amino-4-deoxy-L-arabinose 1-phosphate residues. We also identified some truncated structures for both strains. All sugars were D-configured pyranoses and α-linked, except where stated otherwise.

Keywords: carbohydrates · lipopolysaccharides · mass spectrometry · NMR spectroscopy · structure elucidation

Introduction

The Gram-negative bacillus *Serratia marcescens* of the family *Enterobacteriaceae* is common in soil and water and also found in food.[1] Usually not considered as highly pathogenic, it represents an opportunistic pathogen that can be responsible for serious infections and occasional fatalities in compromised patients, in particular in hospitals. The epidemiology of such infections can be investigated by typing techniques, of which serotyping, based in particular on the O-antigens of lipopolysaccharides (LPSs) or the K-antigens of capsules, is highly useful.[2–6] Currently, 29 different O-serotypes of *S. marcescens* are known, of which most of the O-specific polysaccharides structures have been characterized.[7,8] Also, *S. marcescens* is one of the few known bacterial pathogens of *Drosophila melanogaster* and other flies.[9–11]

Lipopolysaccharides (LPSs) are the endotoxins of Gram-negative bacteria and known for their immunological, pharmacological and pathophysiological effects displayed in eucaryotic cells and organisms.[12] The lipid part of LPSs, lipid A, was proven to represent the toxic principle of endotoxin. However, lipid A toxicity depends strongly on its structure, and is influenced by a second region of LPSs, the core region,[13,14] that is covalently linked to lipid A. The core region also possesses immunogenic properties and is involved in maintaining membrane stability.[15] Therefore, complete structural analyses of the core region and the comparison of its structures with biological features of LPSs are

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of high importance for a better understanding of LPSs action. In the smooth-form (S-form) LPSs, the O-specific polysaccharide is linked to the core region, which in turn is bound to the lipid A.

The core regions identified so far possess at least one residue of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) that links this region to the lipid A (KdoI). A second characteristic component of the core region is L-*glycero*-D-*manno*-heptose (Hep). In addition to Hep, a few LPSs contain D-*glycero*-D-*manno*-heptose (D,D-Hep), which was identified as the biosynthetic precursor of Hep.[16–19] However, there are also some LPSs that contain only D,D-Hep or even lack heptose. Either the Kdo that links the core region to lipid A (KdoI, in *Acinetobacter*)[20–22] or the branching Kdo attached to it (KdoII, in *Burkholderia cepacia* and *Yersinia pestis*)[23–29] may be replaced by the stereochemically similar sugar, D-*glycero*-D-*talo*-oct-2-ulopyranosonic acid (Ko).[30] This sugar was also detected in the LPSs of *B. caryophylli*; however, its location could not be determined.[31] The biosynthesis of Ko and the regulation of the exchange between Kdo and Ko have not been elucidated so far.

In those cases in which Hep is present, KdoI is mostly substituted by a α-Hep residue at O-5, regardless of the number of Kdo residues in the structure, and elongation of the core occurs from this Hep residue. The only exception known to date is the structure of the core regions from the LPSs of *Shewanella algae* BrY and *S. oneidensis* MR-1, in which a α-D,D-Hep is linked to the O-5 position of an 8-amino-3,8-dideoxy-D-*manno*-oct-2-ulosonic acid.[32,33] Still, the presence of one α-Hep-(1→5)-Kdo moiety is a characteristic feature of heptose-containing core regions of LPSs. KdoI may further be substituted at O-4 by a second Kdo residue (KdoII, for example, in *Salmonella enterica* and *Escherichia coli*). In a few cases, KdoII is substituted by neutral sugar residues, for example, L-rhamnose or D-galactose in particular *E. coli* strains. Regarding the core structures of LPSs from *Serratia marcescens*, one structural investigation was published earlier for strain 28b.[34]

We investigated the structures of the core-lipid A regions from LPSs of two other *S. marcescens* strains and now present the complete structures of their carbohydrate backbones, establishing a novel LPSs core region that combines features of the core regions of LPSs from *Yersinia pestis*, *Proteus mirabilis*/*P. penneri*, *Klebsiella pneumoniae* and *Burkholderia cepacia*/*B. pseudomallei*.

Results

Isolation and characterization of the LPSs: By extracting dried bacteria of *S. marcescens* strain 111R (28 g) with phenol/chloroform/light petroleum, we obtained LPSs in the yield of 1.69 g (6%). Compositional analyses revealed the presence of D-GlcN, D-Glc, D-GalA, Hep, D,D-Hep, Kdo, and organic-bound phosphate and of the fatty acids 12:0, 14:0, and 14:0(3-OH). No rhamnose was detected, proving that no S-form LPSs were present.[35] D-*glycero*-D-*talo*-Oct-2-ulopyranosonic acid (Ko) was not determined chemically. We isolated 4-amino-4-deoxy-arabinose from the LPSs by acid hydrolysis and cation-exchange chromatography. We established its L configuration by the determination of the optical rotation. By extracting dried bacteria of *S. marcescens* strain IFO 3735 (60 g) with hot phenol/water, we obtained LPSs in the yield of 2.22 g (3.7%).

After de-*O*-acylation, we subjected the native LPSs of both strains to high-resolution ESI Fourier transform mass spectrometric analysis (ESI FT-MS), which led to the identification of compounds **1**–**15** (Table 1). The charge deconvoluted mass spectra obtained in the negative ion mode exhibited different molecular species, reflecting the heterogeneity of the isolated samples.

Based on the results of the chemical analysis, the composition of the most abundant molecular peak of de-*O*-acylated LPSs from strain 111R (Figure 1A) with a mass of 3084.17 u could be assigned to a molecule consisting of 2 HexN, 2 *P*, 2 (OH)-C14:0, 2 Kdo, 5 Hep, 1 Hex, 1 HexA, and 3 Ara4N (compound **9** in Table 1) with a calculated monoisotopic mass of 3084.161 u. Other abundant species corresponded to this molecule missing one or two Hep (2892.13 u

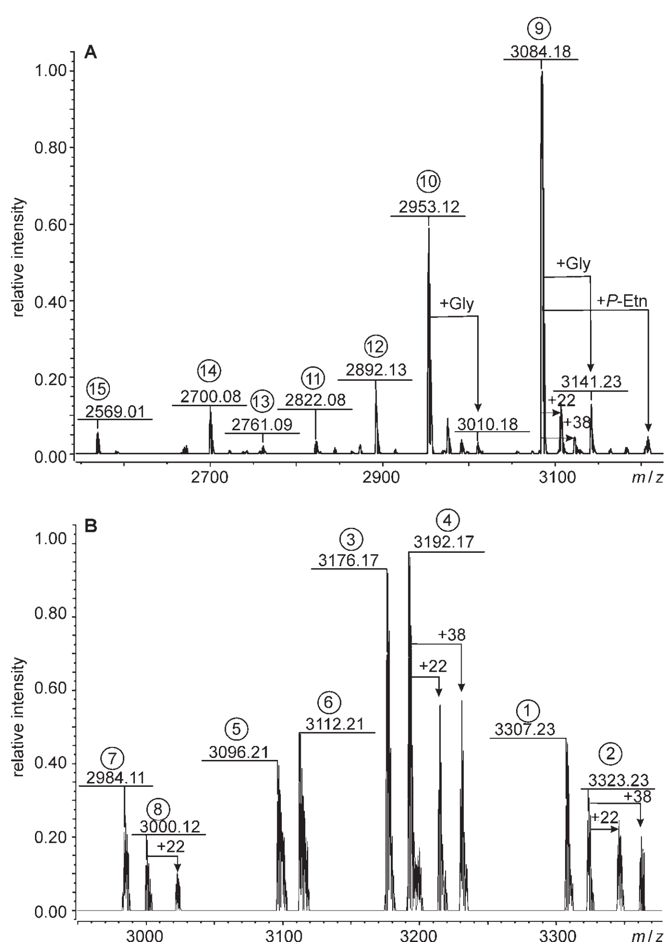

Figure 1. ESI FT-MS mass spectrum of negative ions of the de-*O*-acylated LPS from *S. marcescens* strain 111R (A) and of the de-*O*-acylated LPSs from *S. marcescens* strain IFO 3735 (B). The spectra are charge deconvoluted and the mass numbers refer to monoisotopic molecular masses.

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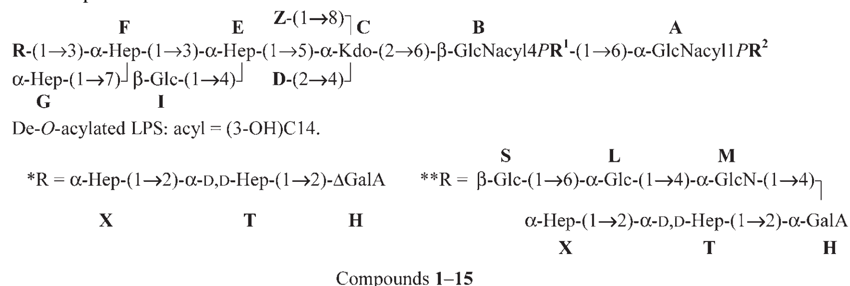
Table 1. Structures of the carbohydrate backbones of the lipid A core regions of de-*O*-acylated LPSs from *S. marcescens*. The observed masses were consistent with the observed molecular species in ESI FT-MS.[a]



Compounds **1–15**

Compound	Strain	R	**D**	**Z**	R1	R2	Calculated mass [u]	Observed mass [u]
1	IFO 3735	**	Kdo	H	Ara4N	H	3307.219	3307.23
2	IFO 3735	**	Ko	H	Ara4N	H	3323.213	3323.23
3	IFO 3735	**	Kdo	H	H	H	3176.160	3176.17
4	IFO 3735	**	Ko	H	H	H	3192.155	3192.17
5	IFO 3735	compound **3**–*P*					3096.194	3096.21
6	IFO 3735	compound **4**–*P*					3112.189	3112.21
7	IFO 3735	compound **3**–Hep					2984.097	2984.11
8	IFO 3735	compound **4**–Hep					3000.094	3000.12
9	111R	*	Kdo	Ara4N	Ara4N	Ara4N	3084.160	3084.18
10	111R	*	Kdo	Ara4N[b]	Ara4N	H[b]	2953.102	2953.12
11	111R	*	Kdo	H	Ara4N	H	2822.044	2822.08
12	111R	D,D-Hep-(1→2)-GalA	Kdo	Ara4N	Ara4N	Ara4N	2892.097	2892.18
13	111R	D,D-Hep-(1→2)-GalA	Kdo	Ara4N[b]	Ara4N	H[b]	2860.996	2761.09
14	111R	GalA	Kdo	Ara4N	Ara4N	Ara4N	2700.033	2700.08
15	111R	GalA	Kdo	Ara4N[b]	Ara4N	H[b]	2569.012	2569.01

[a] Hep is L-*glycero*-D-*manno*-heptose, D,D-Hep is D-*glycero*-D-*manno*-heptose, GalA is galacturonic acid, Kdo is 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid, Ko is D-*glycero*-D-*talo*-oct-2-ulopyranosonic acid, *P* is phosphate, and Ara4N is 4-amino-4-deoxy-L-arabinose. All sugars were D-configured pyranoses and α-linked, except where stated otherwise. [b] Assignment interchangeable.

and 2700.08 u) and/or one and two Ara4N residues (2953.12 u and 2822.08 u, 2761.09 u, and 2569.01 u, respectively). Besides sodium and potassium adduct products, the mass spectrum comprised additional molecular species (3141.20 u and 3207.20 u), although with low abundance, that may be assigned to molecules carrying either a *P*-Etn ($\Delta m = 123$ u) moiety (Etn = 2-amino-ethanol) or the amino acid glycin ($\Delta m = 57$ u), as described for LPSs of *Yersinia pestis*.[26]

To obtain further structural information on the de-*O*-acylated LPSs, we subjected samples to collision-induced dissociation (CID), as well as to capillary skimmer dissociation (CSD), which generates corresponding Y and B fragments resulting from the rupture of the lipid A/Kdo linkage.[36] As an example, the mass spectrum, obtained by charge deconvoluted CID-MS/MS, of the triply charged parent ion of compound **9** (3084.14 u) is depicted in Figure 2, showing the corresponding de-*O*-acylated lipid A (1214.54 u) and core oligosaccharide (1869 u) fragments that sum up to the parent mass. The mass of the de-*O*-acylated lipid A ion was consistent with the composition of 2 HexN, 2 (OH)-C14:0, 2 *P*, and 2 Ara4N (1214.548 u), thus proving that the lipid A of the most prominent LPSs species carried two Ara4N residues, and the core only one. Further fragment ions resulted from the cleavage of Kdo ($\Delta m = 220$ u, 264 u), of Ara4N ($\Delta m = 131$ u) and the glycosidic phosphate ($\Delta m = 80$ u, 98 u).

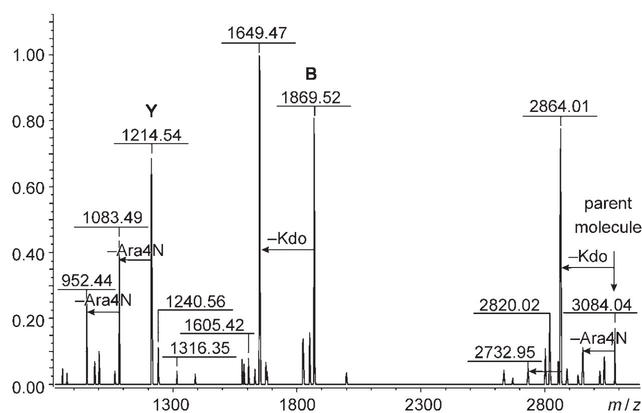


Figure 2. CID-MS/MS mass spectrum of triply charged parent ion of the most intensive molecular species the de-*O*-acylated LPSs from *S. marcescens* strain 111R (compound **9**, 3084.18 mass units). The spectrum is charge deconvoluted.

We measured the monoisotopic masses of 3176.17 u and 3192.17 u for the most prominent species in the spectrum of de-*O*-acylated LPSs of strain IFO 3735 (Figure 1B). The first one was in excellent agreement with a molecule (compound **3** in Table 1) consisting of 3 HexN, 2 *P*, 2 (OH)-C14:0, 2 Kdo, 5 Hep, 3 Hex, and 1 HexA residues, with a calculated mass of 3176.160 u. The second species was assigned to a molecule in which 1 Kdo group was exchanged to Ko (com-

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pound **4** in Table 1), while the other components were unchanged (calculated mass 3192.16 u). The same heterogeneity was observed for the other molecular species carrying one additional Ara4N moiety (3307.23 u and 3323.23 u) or missing either one phosphate group (3096.21 u and 3112.21 u) or one Hep residue (2984.11 u and 3000.12 u). Further species within the spectrum were due to sodium and potassium adduct products of the Ko-containing de-*O*-acylated LPSs molecules (Δm = 22 u and 38 u, respectively).

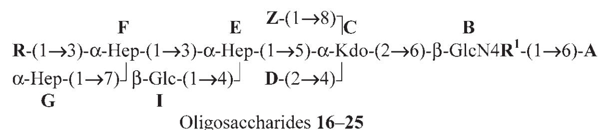
Preparation of oligosaccharides: Through the use of mass spectrometric analysis of de-*O*-acylated LPSs, we showed that the two LPSs isolated from strains IFO 3735 and 111R differed, inter alia, by the substitution of the glycosidic phosphate in lipid A with L-Ara*p*4N. Thus, from alkaline deacylation of the LPSs of *S. marcescens* smooth strain 3735 (which possessed no such substitution), a complex mixture of oligosaccharide phosphates could be obtained; this mixture was separated by preparative high-performance anion-exchange chromatography (HPAEC) into compounds **16–23** (Table 2). However, alkaline deacylation of the LPSs from the rough strain 111R, which possessed a substitution of the glycosidic phosphate in lipid A with L-Ara*p*4N, resulted in the destruction of the lipid A disaccharide, since this phosphodiester was cleaved, yielding a free reducing end of GlcN I of lipid A. To avoid such destruction, the LPSs of this strain were first dephosphorylated with 48% aqueous HF, followed by reduction with $NaBH_4$ of the thus unsubstituted reducing GlcN. After alkaline deacylation of LPSs that had been dephosphorylated and reduced in this way, we isolated the two main oligosaccharides **24** and **25** (Table 2) by HPAEC.

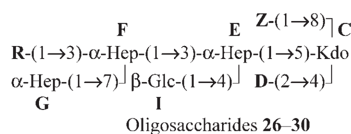
Through acetic acid hydrolysis of the LPSs from *S. marcescens* smooth strain 3735, followed by successive gel-permeation and anion-exchange chromatography, we obtained oligosaccharides **26** and **27** (Table 2). Through acetic acid hydrolysis of the LPSs from rough strain 111R, we obtained three major products **28–30** (Table 2), which could be separated by HPAEC.

We deaminated the LPSs from *S. marcescens* strain 3735 with nitrous acid, producing a polysaccharidic and the oligosaccharide β-Glc-(1→6)-Glc-(1→4)-*anh*Man **31** (residues **S**, **L**, **M**).

Structural analysis of the oligosaccharides: We established the structures of the oligosaccharides **16–25** by ^{1}H and ^{13}C NMR spectroscopy (Figure 3, Table 3). Utilizing COSY, TOCSY, NOESY, HMQC, and HMBC experiments, we assigned chemical shifts. On the basis of these shifts and the $J_{1,2}$ values, which were determined from the 1D ^{1}H spectra, we assigned anomeric configurations. We also compared NMR data with data published for deacylated oligosaccharides isolated from the LPSs of *Klebsiella* and *Proteus*.[37–39] We determined the monosaccharide sequence on the basis of NOE and HMBC data, in which all transglycosidic correlations were observed (Table 3). Oligosaccharides **16–25** possessed a common structural element built up of the monosaccharide units **B**, **C**, **E**, **F**, **G**, **I** (monosaccharide units are as shown in Table 2). All oligosaccharides isolated from strain 3735 (**16–23**) possessed a α-GlcN1*P* residue at the reducing end (H-1, δ = 5.63 ppm; C-1, δ = 91.5 ppm), originating from the lipid A backbone. This residue was glucosaminitol in oligosaccharides **24** and **25**, obtained from strain 111R, due to dephosphorylation and reduction of the de-*O*-acylated LPSs with $NaBH_4$. The second residue of the lipid A backbone, β-GlcN **B** (H-1, δ = 4.85 ppm, C-1, δ = 100.5 ppm), was not phosphorylated in oligosaccharides **16–19**, **24**, and **25**; however, in oligosaccharides **20–23** it was phosphorylated at the O-4 position (H-1, δ = 4.80 ppm; C-1,

Table 2. Structures of oligosaccharides **16–25** isolated by HPAEC from deacylated LPSs and of oligosaccharides **26–30** isolated after acetic acid treatment from *S. marcescens* strains IFO 3735 and 111R.





***R = α-Hep-(1→2)-α-D,D-Hep-(1→2)-α-GalA

X T H

Oligosaccharide	Strain	R	**A**	**D**	R1	**Z**
16	IFO 3735	H	α-GlcN1*P*	Kdo	H	H
17	IFO 3735	H	α-GlcN1*P*	Ko	H	H
18	IFO 3735	*[a]	α-GlcN1*P*	Kdo	H	H
19	IFO 3735	*	α-GlcN1*P*	Ko	H	H
20	IFO 3735	H	α-GlcN1*P*	Kdo	P	H
21	IFO 3735	H	α-GlcN1*P*	Ko	P	H
22	IFO 3735	*	α-GlcN1*P*	Kdo	P	H
23	IFO 3735	*	α-GlcN1*P*	Ko	P	H
24	111R	***	GlcN-ol	Kdo	H	β-Ara4N
25	111R	***	GlcN-ol	Kdo	H	H
26	IFO 3735	**		H		H
27	IFO 3735	**		Ko		H
28	111R	*		H		β-Ara4N
29	111R	*		H		H
30	111R	*		Ko		β-Ara4N

[a] For the structure of *, see Table 1.

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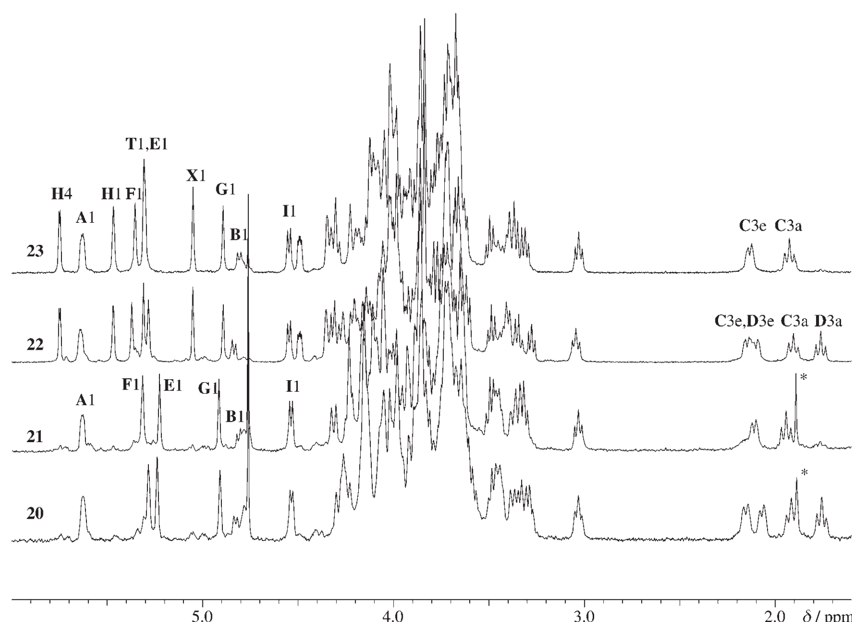

Figure 3. ^{1}H NMR spectra of oligosaccharides **20–23**, obtained from LPSs of *S. marcescens* strain IFO 3735 after hydrazine/KOH deacylation.

$\delta = 100.4$ ppm). In all oligosaccharides obtained, the core region was linked to the lipid A backbone through an α-Kdo residue (**C**: H-3*ax*, $\delta = 1.89$–1.93 ppm; H-3*eq*, $\delta = 2.12$–2.17 ppm; C-3, $\delta = 35.1$–35.3 ppm). In oligosaccharides **16**, **18**, **20**, **22**, **24**, and **25**, this residue was substituted at O-4 by another α-Kdo (residue **D**: H-3*ax*, $\delta = 1.76$ ppm; H-3*eq*, $\delta = 2.16$ ppm; C-3, $\delta = 35.2$ ppm), which was replaced by α-Ko in the oligosaccharides **17**, **19**, **21** and **23** (H-3, $\delta = 4.06$ ppm; C-3, $\delta = 72.5$ ppm). We confirmed the identification of the α-Ko residue by comparing the NMR data with published data.[21,22,30] The Kdo **C** group was further substituted at the O-3 position by an α-Hep residue (**E**: H-1, $\delta = 5.30$ ppm; C-1, $\delta = 99.4$ ppm), to which a second α-Hep residue (**F**: H-1, $\delta = 5.25$–5.35 ppm; C-1, $\delta = 102.2$–102.9 ppm) was attached, also in the O-3 position. In all oligosaccharides, **F** was substituted at the O-7 position by a third α-Hep residue (**G**: H-1, $\delta = 4.90$ ppm; C-1, $\delta = 102.2$ ppm). In oligosaccharides **16**, **17**, **20**, and **21**, heptose **F** carried no substituent at the O-3 position. However, in oligosaccharides **24** and **25**, this position was substituted by the trisaccharide α-Hep-(1→2)-α-D,D-Hep-(1→2)-α-GalA (residues **X**, **T**, and **H**), which was present in **18**, **19**, **22**, and **23** as α-Hep-(1→2)-α-D,D-Hep-(1→2)-ΔGalA (ΔGalA = β-L-*threo*-hex-4-enuronopyranosyl), due to β-elimination at the O-4 position of the GalA residue during treatment with strong alkali. We determined the positions of the phosphate groups through ^{1}H,^{31}P HMQC experiments (data not shown). In oligosaccharides **16–19**, only one phosphate group resonating at $\delta \sim 2.7$ ppm was present at the O-1 position of GlcN **A**, and in oligosaccharides **20–23** an additional phosphate group resonating at $\delta \sim 4.3$ ppm was identified at the O-4 position of GlcN **B**. Oligosaccharides **24** and **25** were not phosphorylated.

We isolated oligosaccharides **26** and **27** (from LPSs of strain 3735) and **28–30** (from LPSs of strain 111R) after mild acid hydrolysis of the LPSs. These oligosaccharides contained a common part built up of the monosaccharide residues **C**, **E**, **F**, **G**, **I**, **H**, **T**, **X** (see Table 2). Oligosaccharide **29** from LPSs of strain 111R possessed no additional components, whereas **28** contained a residue of β-linked 4-amino-4-deoxy-L-arabinopyranose (Ara4N, residue **Z**: H-1, $\delta = 4.95$ ppm; C-1, $\delta = 99.1$ ppm), substituting the at the O-8 position of Kdo **C**. The minor product **30** also contained this residue at O-8 of Kdo **C**, and in addition, Ko (residue **D**) was linked to the O-4 position of **C**. In oligosaccharides **26** and **27**, obtained from LPSs of strain 3735, the trisaccharide β-Glc-(1→6)-α-Glc-(1→4)-α-GlcN-(1→ substituted O-4 of α-GalA **H**. Oligosaccharide **27** also contained Ko **D** at the O-4 position of Kdo **C**. We determined the structures of the oligosaccharides **26–30** on the basis of NMR data as described for oligosaccharides **1–10**. Data from investigations on the structures of LPSs core regions from *Proteus vulgaris* and *P. penneri* was used for comparison.[38,39]

Mass spectrometric analysis of oligosaccharides **16–30** revealed molecular ions that were in agreement with the proposed structures (data not shown). The mass spectra of oligosaccharides **26** and **27** additionally contained ions with minor intensities that corresponded to variants missing one heptose residue.

Although the Ko-containing oligosaccharide **30** was isolated after acetic acid degradation of the LPSs, its parent ions were not visible in the mass spectrum of de-*O*-acylated LPSs, probably because of the low concentration. Other ions with minor intensities could be attributed to the structures **5–8** and **12–15**; however, corresponding products could not be isolated, neither after alkaline deacylation, nor after acetic acid hydrolysis.

By the use of monosaccharide analysis (GLC of alditol acetates), we confirmed the composition of the isolated compounds. The absolute configuration of Gal, Glc, L-*glycero-manno*-Hep, D-*glycero-manno*-Hep and GlcN was determined as D.

Discussion

Based on the structures that have been identified in enterobacterial LPSs so far, two types of core regions may be dis-

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Table 3. ^{1}H and ^{13}C NMR chemical shifts (δ) of sugar residues of core oligosaccharides. NMR spectra of oligosaccharides and oligosaccharide phosphates were recorded from solutions in D_2O. Measurements were taken at 27°C.

Residue	Nucleus	1	2 (3*ax*)	3 (3*eq*)	4	5	6a	7a (6b)	8a (7b)	8b	NOE from H-1 to proton	HMBC from H-1 to carbon
α-GlcN1*P* **A**, **16–23**	^{1}H	5.63	3.38	3.90	3.68	4.15	3.78	4.32				
	^{13}C	91.5	55.3	70.4	70.2	72.7	70.6					
β-GlcN **B**, **16–19**	^{1}H	4.85	3.10	3.63	3.46	3.62	3.53	3.53				
	^{13}C	100.5	56.9	73.5	71.2	75.6	62.7					
β-GlcN4*P* **B**, **20–23**	^{1}H	4.80	3.03	3.82	3.64	3.70	3.45	3.45				
	^{13}C	100.4	56.3	73.4	74.0	74.8	63.3					
α-Kdo **C**, **16**, **18**, **20**, **22**, **25**	^{1}H		1.89	2.12	4.15	4.22	3.69	3.81	3.69	3.87		
	^{13}C			35.1	70.9	70.1	72.6	71.9	64.1			
α-Kdo **C**, **17**, **19**, **21**, **23**	^{1}H		1.93	2.14	4.12	4.23	3.73	3.85	3.64	3.88		
	^{13}C			35.2	71.4	69.5	72.5	71.0	64.1			
α-Kdo **C**, **24**	^{1}H		1.91	2.17	4.16	4.26	3.64	4.07	3.69	3.90		
	^{13}C			35.3	71.4	70.3	73.8	69.0	72.0			
α-Kdo **C**, **28**	^{1}H			1.79	4.10	4.17	3.82	3.92	3.60	3.89		
	^{13}C			34.5	66.3	75.4	71.3	67.9	68.9			
α-Kdo **C**, **30**	^{1}H			1.93	4.11	4.27	3.95	3.94	3.56	3.86		
	^{13}C			34.2	71.7	70.4	71.8	68.1	68.8			
α-Kdo **D**, **16**, **18**, **20**, **22**, **24**, **25**	^{1}H		1.76	2.16	4.07	4.04	3.61	3.86	3.74	4.02		
	^{13}C			35.2	67.0	67.2	72.7	71.1	63.7			
α-Ko **D**, **17**, **19**, **21**, **23**, **30**	^{1}H			4.06	3.91	4.13	3.62	4.11	3.75	4.01		
	^{13}C			72.5	66.9	69.1	72.8	71.3	63.7			
α-Hep **E**, **16–27**, **30**	^{1}H	5.30	4.05	4.09	4.31	4.10	4.09	3.77	3.94		**C**5 s, **C**7 s	**C**5
	^{13}C	99.4	71.1	74.8	73.3	71.3	69.5	64.6				
α-Hep **E**, **28**	^{1}H	5.08	4.07	4.24	4.22	4.11	4.09	3.67	3.72		**C**5 s, **C**7 s	**C**5
	^{13}C	101.3	70.4	75.7	74.4	71.7	69.1	63.3				
α-Hep **E**, **29**	^{1}H	5.12	4.05	4.23	4.23	4.17	4.11	3.67	3.73		**C**5 s, **C**7 s	**C**5
	^{13}C	100.8	70.5	76.3	74.3	71.6	69.1	63.3				
α-Hep **F**, **16**, **17**, **20**, **21**	^{1}H	5.25	4.15	3.86	3.86	3.65	4.10	3.67	3.73		**E**2 s, **E**3 s	**E**3
	^{13}C	102.9	70.8	71.7	67.3	72.9	69.1	63.3				
α-Hep **F**, **18**, **19**, **22–27**	^{1}H	5.35	4.35	4.07	3.95	3.66	4.19	3.69	3.78		**E**2 s, **E**3 s	**E**3
	^{13}C	102.2	69.3	81.2	66.0	72.9	69.1	71.7				
α-Hep **F**, **28–30**	^{1}H	5.28	4.11	4.00	3.95	3.71	4.16	3.74	3.78		**E**2 s, **E**3 s	**E**3
	^{13}C	101.0	70.2	79.8	66.1	73.1	68.8	70.2				
α-Hep **G**, **16–30**	^{1}H	4.90	3.99	3.86	3.86	3.66	4.01	3.77	3.94		**F**7w	**F**7
	^{13}C	102.2	70.7	71.0	66.8	72.7	69.7	64.6				
ΔGalA **H**, **18**, **19**, **22**, **23**	^{1}H	5.47	3.98	4.50	5.76						**F**2 s, **F**3 s	**F**3
	^{13}C	99.2	76.1	65.1	107.3							
α-GalA **H**, **24–30**	^{1}H	5.42	4.01	4.08	4.32	4.43					**F**2 s, **F**3 s	**F**3
	^{13}C	98.6	73.1	68.2	71.3	72.7						
β-Glc **I**, **16–30**	^{1}H	4.55	3.31	3.50	3.37	3.40	3.74	3.86			**E**4 s, **E**6 s	**E**4
	^{13}C	102.5	74.5	75.9	70.6	77.0	61.9					
α-GlcN **M**, **26**, **27**	^{1}H										**H**4 s, **H**5 m	**H**4
	^{13}C											
α-D,D-Hep, **T**, **18**, **19**, **22–30**	^{1}H	5.31	4.02	3.93	3.79	3.85	4.07	3.68	3.79		**H**1 s,**H**2 s, **X**1 s, **X**5 m	**H**4
	^{13}C	98.0	79.1	70.7	68.0	74.1	72.8	62.7				
α-Hep **X**, **18**, **19**, **22–30**	^{1}H	5.05	4.03	3.83	3.86	3.67	4.01	3.73	3.73		**T**1 m, **T**2 s	**T**2
	^{13}C	102.6	70.7	71.0	66.7	72.8	69.6	63.6				
β-Ara4N **Z**, **28**	^{1}H	4.95	3.78	4.03	3.39	3.61	4.02				**C**8 m	**C**8
	^{13}C	99.1	68.6	68.0	51.5	60.9						
β-Ara4N **Z**, **30**	^{1}H	4.96	3.71	4.16	3.65	4.12	3.68				**C**8 m	**C**8
	^{13}C	98.5	68.6	66.2	52.3	58.6						
2,5-*anh*Man, **M**, **31**	^{1}H	5.08	3.77	4.37	4.17	4.12	3.73	3.77				
	^{13}C	90.2	86.3	77.6	84.1	83.6	61.9					
α-Glc **L**, **26**, **27**, **31**	^{1}H	5.15	3.56	3.67	3.50	3.77	3.85	4.15			**M**4 s	**M**4
	^{13}C	99.0	72.0	73.7	70.0	72.3	69.2					
β-Glc **S**, **26**, **27**, **31**	^{1}H	4.47	3.30	3.48	3.37	3.43	3.71	3.90			**L**6 m	**L**6
	^{13}C	103.6	74.1	76.8	70.6	77.0	61.5					

tinguished, namely the *S. enterica* type core region and those that are different to this.[13,14] The *S. enterica* type core structures contain as a common structural element Hep-(1→7)-Hep-(1→3)-Hep-(1→5)-α-Kdo (HepIII-HepII-HepI-KdoI), which is substituted at the O-3 position of HepII by D-Glc. The heptose I and II residues are phosphorylated, and the O-4 position of HepI is not substituted by a saccharide. In the second type, the common partial structure HepIII-HepII-HepI-KdoI is not substituted at the O-3 position of HepII by D-Glc, and heptose residues are not generally

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phosphorylated. The O-4 position of HepI is substituted by a hexose residue or an oligosaccharide.

The structures of the core regions from the LPSs of two strains of *S. marcescens* that are reported in this communication prove that these core types integrate into the second core type. An earlier investigation on the core structure from LPSs of *S. marcescens* strain 28b confirms this.[34] Moreover, the core oligosaccharide structures reported here, which represent the first complete structures to be determined in *Serratia* LPSs, include particular structural fragments that were also found in core regions of LPSs from *Klebsiella*,[37,40–42] *Proteus*,[38,39,43–53] *Yersinia pestis* (all *Enterobacteriaceae*),[25,26–29] and *Burkholderia* (*Pseudomonadaceae*).[23,24,54] As in the core structures of *Serratia* LPSs, the core regions of LPSs from *Klebsiella* and *Proteus* contain the partial structure HepIII-HepII-HepI-KdoI, substituted at the O-3 position of HepII by α-D-GalA (residue **H** in this presentation), which in turn is substituted at the O-4 position by an amino sugar, namely only GlcN (*Klebsiella*), or either GlcN or GalN (*Proteus*). This same α-D-GalA residue also carries the disaccharide α-Hep-(1→2)-α-D,D-Hep- in the core regions of several *Proteus* LPSs at the O-2 position. Furthermore, substitution at the O-8 position of Kdo **C** by L-Ara*p*4N was frequently found among *Proteus* LPSs core structures. As in the core regions of LPSs from *Y. pestis* and *Burkholderia*, the core regions of *S. marcescens* LPSs possessed one Ko residue, which exchanged KdoII in nonstoichiometric amounts. This is the second case in which Ko was identified in an enterobacterial LPSs core region. Ko was first found in LPSs of *Acinetobacter*, in which it replaced KdoI in nonstoichiometric amounts,[20–22] a substitution that is specific for this genus. Later, the non-stoichiometric replacement of KdoII by Ko was identified in LPSs of *B. cepacia* and *B. pseudomallei*,[23,24]. This novel partial core structure was considered useful to help distinguish between the genera *Burkholderia* (RNA group II of *Pseudomonadaceae*) and *Pseudomonas* (RNA group I of *Pseudomonadaceae*). However, as shown in the LPSs of *Y. pestis* and in this presentation, the replacement of KdoII by Ko may occur more often than is currently known. Also, as in the LPSs of *Y. pestis* and *Proteus mirabilis* O28,[26,75] the presence of small amounts of glycin was identified in the LPSs of *S. marcescens* strains 111R and IFO 3735. However, the exact location within the core oligosaccharide could not be established.

Resistance of Gram-negative enterobacteria to the polycationic antibiotic polymyxin B is thought to be associated with a shift towards a less anionic net charge achieved by a higher level of substitution of phosphate residues by 2-aminoethanol phosphate and L-Ara*p*4N.[55–60] Also, myristoylation of lipid A appears to be a prerequisite for polymyxin B resistance.[61] In particular, the esterification of the 4′-phosphate group of lipid A with L-Ara*p*4N results in resistance against polymyxin B, whereas a similar substitution of the glycosidic phosphate seems to be less important. Thus, a phosphate monoester at the O-4′ position of lipid A could represent a region of the receptor site for polymyxin B. Earlier, by comparison of the S-form LPSs of a PMB-sensitive and a PMB-resistant variant of wild-type *S. marcescens* O:29, it was shown that the latter LPSs bound significantly less PMB than the former did and that these contained significantly higher amounts of Ara4N than the former.[57] In the present investigation, we proved that three different L-Ara*p*4N residues were present in the lipid A core region of strain 111R, one bound to the glycosidic phosphate group of GlcNI of lipid A, the second substituting the phosphate group at the O-4 position of GlcNII of lipid A, and the third substituting at the O-8 position of KdoII of the core region. All three residues were present in nonstoichiometric amounts. It is not known to what extent the last substitution contributed to PMB resistance of strain 111R. *Serratia marcescens* strain IFO 3735 possessed one L-Ara*p*4N residue in nonstoichiometric amounts linked to the 4′-phosphate group of lipid A in its LPSs and was more resistant to PMB than IFO 3736, the LPSs of which did not contain any Ara4N.[76]

Investigations on the biosynthesis of the attachment of L-Ara*p*4N to lipid A in LPSs of *Salmonella enterica* and *Escherichia coli* gave a pathway,[62–66], which inter alia integrates an inner membrane enzyme (ArnT) that was expressed in polymyxin-resistant mutants and which adds one or two L-Ara*p*4N residues to lipid A or its precursors. In *E. coli*, biosynthesis of the core region begins with the attachment of two Kdo residues to precursor IV_A, the tetraacylated and bisphosphorylated GlcN disaccharide; the attachment is performed by one Kdo-transferase WaaA (KdtA) and results in Kdo_2–lipid IV_A.[19,67–69] Completion of lipid A employs two additional acylation steps of Kdo_2–lipid IV_A, after which the Kdo residue that is attached to lipid A is substituted at O-5 by Hep*p*, followed by further steps of core biosynthesis. Most interestingly, ArnT adds two L-Ara*p*4N residues to Kdo_2–lipid IV_A and to Kdo_2–lipid A (Re-LPSs).[62] To precursor IV_A it adds only one L-Ara*p*4N residue to the glycosidic phosphate group. Although the biosynthesis of the LPSs of *S. marcescens* has not yet been elucidated, it may be speculated that the two L-Ara*p*4N residues of lipid A are transferred to Kdo_2–lipid IV_A and/or to Kdo_2–lipid A. This could also be true for the third L-Ara*p*4N residue that is linked to KdoII; however, nothing is known about the biosynthesis of this linkage.

Experimental Section

Bacteria and bacterial LPSs: The *Serratia marcescens* strain 111R was isolated as a spontaneously occurring rough mutant of a polymyxin B (PMB)-resistant variant of the wild-type strain *S. marcescens* 111 (serotype O:29).[35,57] The bacteria were cultivated, harvested, and dried as described.[57] The LPSs were extracted by utilizing the phenol/chloroform/light petroleum method.[70] *Serratia marcescens* strain IFO 3735 was obtained from the strain collection of the Institute of Fermentation, Osaka (Japan). The bacteria were cultivated under aerobic conditions in a tryptose–tryptone medium (pH 7.2) at 37 °C and were harvested at the beginning of the stationary phase and dried. Dry cells (60 g) were extracted with hot phenol/water,[71] and the LPSs were purified by repeated ultracentrifugation (105.000 × g; 4 h) to give 2.22 g (3.7 % of the bacterial dry mass) of LPSs.

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Isolation of oligosaccharides: The LPSs (100 mg) or the de-*O*-acylated,[72] dephosphorylated and reduced LPSs (50 mg) of strain 111R were treated with 4 M NaOH (4 mL, 100 °C, 4 h), cooled, and neutralized with 2 M HCl (7.5 mL). The precipitate was removed by centrifugation and the products were isolated by gel-permeation chromatography on a column (2.5 × 80 cm) of Sephadex G-50 SF (Pharmacia), which was eluted with a pyridine/acetic acid buffer (4 mL of pyridine and 10 mL of AcOH in 1 L of water). Thus, we obtained a mixture of oligosaccharides **16–23** (30 mg) and a mixture of oligosaccharides **24** and **25** (15 mg). Individual compounds were isolated by HPAEC on a column (250 × 9 mm) of Carbopac PA1 that was linearly eluted with a gradient of 10 to 80 % of 1 M sodium acetate in 0.1 M NaOH at a rate of 3 mL min^{-1} over 60 min. After desalting, oligosaccharides **16–25** were isolated as single compounds in yields of 0.5–3 mg.

The LPSs were hydrolyzed from strains IFO 3735 and 111R (80 mg each) with 2 % acetic acid (100 °C, 4 h). The resulting precipitate was removed by centrifugation, and the supernatant was separated on Sephadex G50 (as above), resulting in a mixture of core oligosaccharides (20 mg from strain 3735 and 25 mg from strain 111R, 25 and 31 %, respectively, of the LPSs). Oligosaccharides **26** and **27** from LPSs of strain 3735, as well as a number of minor products, were separated by anion-exchange chromatography on a column of Hitrap (5 mL, Pharmacia) that was linearly eluted with a gradient of 0 to 100 % of 1 M NaCl over 60 min.

Oligosaccharides **28–30** were isolated from LPSs of strain 111R by HPAEC on a column (250 × 9 mm) of Carbopac PA1 that was linearly eluted with a gradient of 10 to 80 % of 1 M sodium acetate in 0.1 M NaOH at a rate of 3 mL min^{-1} over 60 min.

Deamination of the LPSs and isolation of oligosaccharide **31** were performed as described in the literature.[46]

Mass spectrometry: ESI FT-MS analyses of de-*O*-acylated LPSs were performed in the negative ion mode by using an APEX II instrument (Bruker Daltonics, Billerica, MA, USA) equipped with an actively shielded 7 T magnet and an Apollo II ESI source. Mass spectra were acquired by using standard experimental sequences as provided by the manufacturer. Samples (~10 ng µL^{-1}) were dissolved in a 50:50:0.001 (v/v/v) mixture of 2-propanol, water, and triethylamine. The pH was controlled to ensure that it should not exceed pH 8.5. The samples were sprayed at a flow rate of 2 µL min^{-1}. The capillary entrance voltage was set to 3.8 kV and the drying gas temperature to 150 °C. Argon was used as the collision gas for sustained off resonance irradiation collision-induced dissociation (SORI-CID) at a peak pressure of ~10^{-6} mbar. CSD was induced by increasing the capillary exit voltage from 100 V to values between 250 and 350 V. The spectra were charge-deconvoluted, using the XMASS-6.1 software, and mass numbers given refer to monoisotopic molecular masses. Mass scale was calibrated externally with LPSs from *E. coli* R-mutants of known structure.

General: Neutral sugar and uronic acid analyses, fatty acid analyses, determination of organic-bound phosphate, and Kdo and GlcN quantification were performed as described in the literature,[73,74] and likewise the determination of the absolute configurations of the sugars. The NMR spectra of oligosaccharides (COSY, TOCSY, NOESY, $^{1}H,^{13}C$ HMQC, and gHMBC) were recorded for solutions in D_2O at 27 °C on a Bruker AMX 600 instrument (operating frequency 600.13 MHz for ^{1}H and 150 MHz for ^{13}C) by using standard Bruker software with a digital resolution of <2 Hz in the *F*2 dimension. ^{31}P and $^{1}H,^{31}P$ HMQC spectra were obtained on a Bruker DMX 250 spectrometer. Chemical shifts are presented relative to acetone signals (^{1}H, $\delta = 2.225$ ppm; ^{13}C, $\delta = 31.5$ ppm) or 85 % H_3PO_4 (0 ppm).

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