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Note

Structure of an acidic O-specific polysaccharide from marine bacterium *Shewanella fidelis* KMM 3582^T containing N^{ε}-[(*S*)-1-carboxyethyl]-N^{α}-(D-galacturonoyl)-L-lysine ☆

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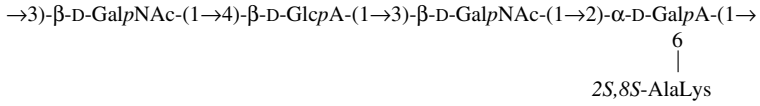
Abstract—The O-specific polysaccharide was isolated by mild acid degradation of the lipopolysaccharide of the marine bacterium *Shewanella fidelis* type strain KMM 3582^T and studied by sugar analysis along with ^{1}H and ^{13}C NMR spectroscopy including one-dimensional NOE in difference mode and two-dimensional experiments. The polysaccharide was found to consist of linear tetrasaccharide repeating units containing N^{ε}-[(*S*)-1-carboxyethyl]-N^{α}-(D-galacturonoyl)-L-lysine and having the following structure:



The amide of D-galacturonic acid with N^{ε}-[(*S*)-1-carboxyethyl]-L-lysine ('alaninolysine', 2*S*,8*S*-AlaLys) was found for the first time in nature as a component of the O-specific polysaccharide of *Providencia rustigianii* O14 (*Carbohydr. Res.* **2003**, *338*, 1009–1016).

Keywords: *Shewanella fidelis*; Lipopolysaccharide; Structure; NMR spectroscopy; Bacterial polysaccharide; Marine bacterium; Alaninolysine

The genus *Shewanella* is comprised of a group of Gram-negative, facultatively anaerobic, readily cultivated γ-*Proteobacteria* mainly associated with aquatic (e.g., cold adapted, high pressure and deep-sea, etc.) habitats.[1,2] In the last few years *Shewanella* bacteria have been under intensive investigation due to their capability for co-metabolic bioremediation of halogenated organic pollutants,[3] destructive oxidation of crude petroleum,[4] the dissimilatory reduction of magnesium and iron oxides[5] and the production of high proportions of polyunsaturated fatty acids (PUFA).[6] As a result, several novel species of this genus have been described and have recently been reviewed.[7] Another novel species, *S. fidelis*, was isolated from a sediment sample in the South China Sea. *S. fidelis* type strain KMM 3582^T had all the characteristics typical for other members of the genus, that is colonies were slightly pinkish, cells were

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Gram-negative, polarly flagellated with facultatively anaerobic metabolism, mesophilic (with temperature range from 4 to 30 °C), neutrophilic, haemolytic and were able to degrade alginate, gelatin and DNA.[8] Comparative 16S rDNA sequence-based phylogenetic analysis placed this strain into a separate branch of the γ-*Proteobacteria* within members of the genus *Shewanella* since the description of new organisms along with phylogenetic evidence needs to be supported by a number of phenotypic characteristics and, more importantly, chemotaxonomic features, which might be a reflection of phylogenetic relatedness among bacteria of different taxa.

Recently, we have determined a structure of an acidic antigenic polysaccharides from clinical isolates of *S. putrefaciens*[9,10] and *S. algae*.[11] In the present study, we report the results of structural analysis of the O-specific polysaccharide from newly described species *S. fidelis* type strain KMM 3582^T containing the unusual amino acid N^{ε}-[(*S*)-1-carboxyethyl]-L-lysine.

Lipopolysaccharide was extracted with aq 45% phenol, recovered from the aqueous phase and degraded with dilute acetic acid to give high-molecular weight polysaccharide. Analysis of the sugar composition of the polysaccharide using GLC of acetylated methyl glycosides identified glucuronic acid (GlcA) and 2-amino-2-deoxygalactose (GalN). When the polysaccharide was hydrolysed with 2 M TFA before methanolysis, galacturonic acid (GalA) was identified in addition to the sugar mentioned above.

The ^{13}C NMR spectrum (Fig. 1, Table 1) of the polysaccharide showed four signals in the anomeric

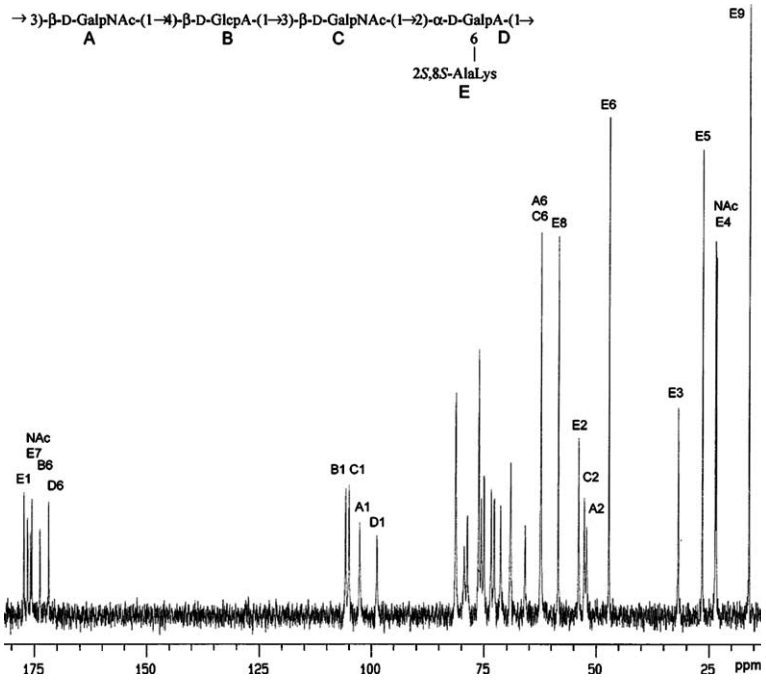


Figure 1. 100 MHz ^{13}C NMR spectrum of the polysaccharide of *S. fidelis* KMM 3582^T.

Table 1. 100 MHz ^{13}C NMR chemical shifts for the polysaccharide of *S. fidelis* KMM 3582^T (δ, ppm)

Sugar or amino acid residue	C-1 C-1	C-2 C-2	C-3 C-3	C-4 C-4	C-5 C-5	C-6 C-6	C-7	C-8	C-9
→3)-β-D-GalNAc^I-(1→	102.6	52.4 (−1.3)	81.5 (+7.1)	66.1 (−3.2)	76.3	62.5			
→4)-β-D-GlcA-(1→	105.5 (+8.1)	73.75	75.3 (−1.9)	79.7 (+7.0)	75.8 (−1.6)	173.5			
→3)-β-D-GalNAc^{II}-(1→	104.6	53.0 (−0.8)	81.3 (+8.9)	69.4 (0)	76.3	62.5			
→2)-α-D-GalA-(1→	98.8 (+5.2)	78.6 (+9.9)	69.4	71.4	72.9	171.6			
AlaLys	177.1	56.1	32.0	23.4	26.5	47.3	175.7	58.8	16.1

Glycosylation effects are given in parenthesis.
Chemical shifts for NAc groups are δ 23.8 (CH_3) and δ 176.35 and 175.6 (CO) (for GalNAc^I and GalNAc^{II}, respectively).

region at δ 98.8–105.5. This spectrum also contained two sugar signals for nitrogen-bearing carbons at δ 52.4 and 53.0; one signal of double intensity for two HO*C*H_2–C groups (C-6 of two GalN residues) at δ 62.5 (data from the DEPT experiment); two signals for C–*C*OOH groups (C-6 of uronic acids, data from the HMBC experiment) at δ 171.4 and 173.4; two signals for *N*-acetyl groups at δ 176.35 and 175.6 (CO) and δ 23.8 (Me). Therefore, the polysaccharide contains two residues of D-GalNAc, and one each of D-GalA and D-GlcA. The pyranoid form of all sugar residues was demonstrated by the absence from the ^{13}C NMR spectrum of any signals for nonanomeric sugar carbons at a lower field than δ 82.[12] Moreover, the DEPT spectrum showed four H-bearing carbons at δ 47.3, 32.0, 26.5 and 23.4. These signals, in combination with two additional low-field signals at δ 175.6 and 177.1, one C–*C*H_3 group at δ 16.1 and two signals at δ 56.1 and 58.8, demonstrated the presence of the unusual amino acid N^{ε}-(1-carboxyethyl)lysine (AlaLys).[13]

Accordingly, the ^{1}H NMR (Fig. 2) spectrum showed three signals in the anomeric region at δ 5.37, 4.70 and 4.57 (double intensity). Signals characteristic for AlaLys[13] were seen in this spectrum as well as two peaks for *N*-acetyl groups at δ 2.01 and 1.90 in the high-field region. The ^{1}H NMR spectrum of the polysaccharide was assigned using 2-D COSY and TOCSY experiments (Table 2). Additionally, the monosaccharide composition was confirmed by 1-D NOE experiments in difference mode. This latter experiment allows the observation of the shape and splitting of signals in the individual residues. Thus, the irradiation of H-3 of GlcA revealed that H-5 of the same residue is a doublet, with a coupling constant of 8.8 Hz, which designates the sugar

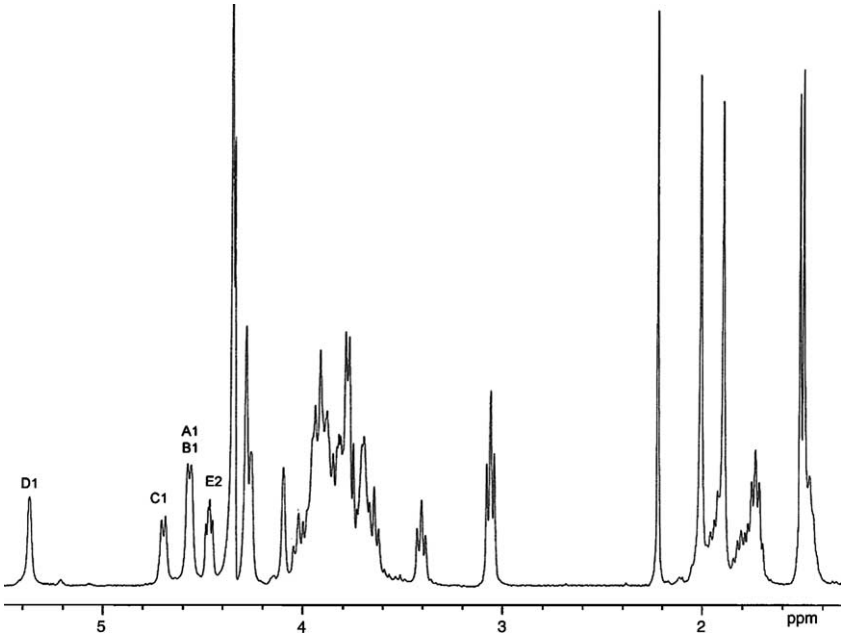


Figure 2. 400 MHz ^{1}H NMR spectrum of the polysaccharide of *S. fidelis* KMM 3582^T.

Table 2. 400 MHz ^{1}H NMR chemical shifts for the polysaccharide of *S. fidelis* KMM 3582^T (δ, ppm)

Sugar or amino acid residue	H-1 H-1	H-2 H-2	H-3 H-3	H-4 H-4	H-5 H-5	H-6a,6b H-6	H-7	H-8	H-9
→3)-β-D-GalNAc^I-(1→	4.57 [$J_{1,2}$ 7.1]	3.97	3.84 [$J_{3,4}$ 7.8]	4.28	3.70	3.78 [$J_{6,5}$ 5.6]			
→4)-β-D-GlcA-(1→	4.57 [$J_{1,2}$ 7.8]	3.41 [$J_{2,3}$ 8.45]	3.64 [$J_{3,4}$ 8.8]	3.82 [$J_{4,5}$ 3.7]	3.92 [$J_{5,4}$ 8.8]				
→3)-β-D-GalNAc^{II}-(1→	4.70 [$J_{1,2}$ 8.1]	4.02 [$J_{2,3}$ 10]	3.92 [$J_{3,4}$ 12.4]	4.10	3.70	3.78 [$J_{6,5}$ 5.6]			
→2)-α-D-GalA-(1→	5.37	3.90	3.95	4.26	4.28				
AlaLys		4.56	1.94[a]	1.47	1.74 [$J_{5,4}$ 7.2]	3.06 [$J_{6,5}$ 7.7]		3.77	1.51 [$J_{9,8}$ 7.3]

Coupling constant values (J, Hz) are given in square brackets.
Chemical shifts for NAc groups are δ 1.90 and 2.01 (for GalNAc^I and GalNAc^{II}, respectively).
[a]H-3a; H-3b at δ 1.77.

as an uronic acid. The splitting of signals of the other sugar residues was observed after irradiation of their anomeric protons.

The ^{13}C NMR spectrum of the polysaccharide was then assigned using an H-detected $^{1}H,^{13}C$ HMQC experiment (Table 1). Two GalNAc residues ($GalNAc^{I}$ and $GalNAc^{II}$) were distinguished from uronic acids by correlations of the protons at nitrogen-bearing carbons (H-2) to the corresponding carbons (C-2) at δ 3.97/52.4 and δ 4.02/53.0, respectively, which were revealed by a $^{1}H,^{13}C$ HMQC experiment. The signals for C-6 of GalA and GlcA were assigned by H-4/C-6 and H-5/C-6 correlations demonstrated by an HMBC experiment. The $J_{1,2}$ coupling constant values of ~3 Hz indicated that the GalA residue is α-linked, whereas the $J_{1,2}$ values of ~8 Hz showed that other three sugar residues are β-linked. The down-field chemical shift of C-3 of both GalNAc residues, C-4 of GlcA and C-2 of GalA, as compared to their positions in the spectra of the corresponding nonsubstituted monosaccharides,[14] showed the linkage sites of these residues.

The 2-D ROESY spectrum revealed the direct assignment of inter-residue cross-peaks: H-1 $GalNAc^{I}$/H-4 GlcA; H-1 GlcA/H-3 $GalNAc^{II}$, H-1 $GalNAc^{II}$/H-2 GalA and H-1 GalA/H-3 $GalNAc^{I}$. The glycosylation pattern was confirmed by the data of the HMBC spectrum (Fig. 3, Table 3). In addition, the correlation peak of H-2 AlaLys/C-6 GalA revealed the location of AlaLys. As the HMBC experiment displays heteronuclear connectivities through three bonds, an amidic linkage is inferred. This acylation at N-2 is further confirmed by the down-field shift of H-2 of lysine to δ 4.56 from its position at δ 3.78 in the free amino acid.[15] Concomitantly, the signal for C-6 of GalA at δ 171.6 is characteristic of hexuronamides and compares favourably with that of $\delta_{C\text{-}6}$ 171.5 for an amide of GalA with threonine.[16]

Examination of the glycosylation effects (Table 1) showed that all the residues have the same relative configuration.[17,18] GLC of the acetylated derivatives of the polysaccharide with *R*-(−)-2-octanol and comparison with a D-GalNAc standard determined that the absolute configuration of the GalNAc residue was D. Hence, all sugar residues have the D configuration. In order to determine the absolute configuration of AlaLys the polysaccharide was hydrolysed and amino acid (**1**), was isolated from the monosaccharides by gel-permeation chromatography. The ^{13}C NMR spectrum of **1** showed signals for lysine and alanine, but the signals for C-6 of lysine and C-2 of alanine were shifted down field to δ 47.3 and 58.8, as compared to their positions at δ

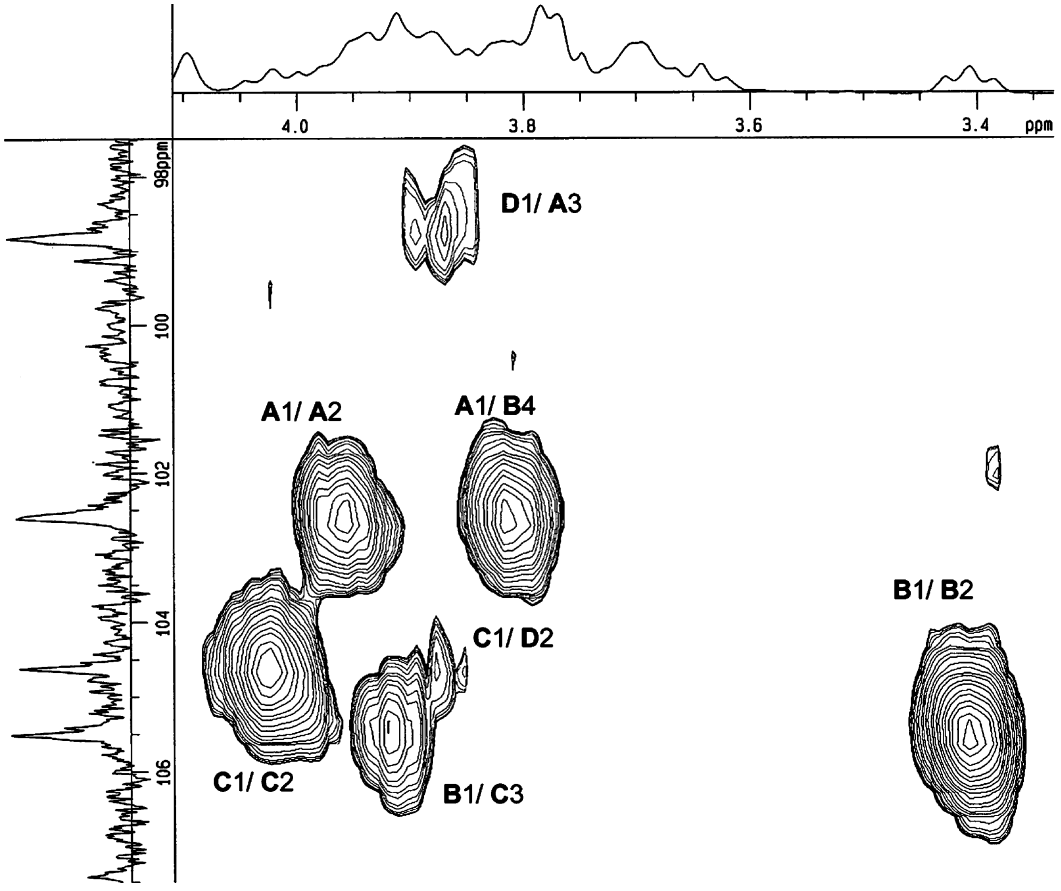


Figure 3. Part of 400 MHz $^{1}H,^{13}C$ HMBC spectrum of the polysaccharide of *S. fidelis* KMM 3582^{T}.

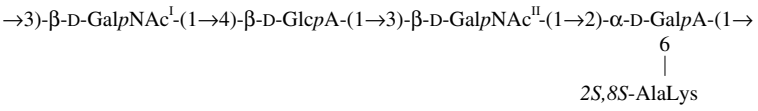
Table 3. 400 MHz HMBC data for inter-residue linkages (δ, ppm) for the anomeric atoms in the polysaccharide of *S. fidelis* KMM 3582^T

Sugar residue	δ_{H-1}	δ_{C-1}	δ_H	δ_C	Connectivity to
→3)-β-D-GalNAc^I-(1→	4.57			79.7	GlcA C-4
		102.6	3.82		GlcA H-4
→4)-β-D-GlcA-(1→	4.57			81.3	GalNAc^{II} C-3
		105.5	3.92		GalNAc^{II} H-3
→3)-β-D-GalNAc^{II}-(1→	4.70			78.6	GalA C-2
		104.6	3.90		GalA H-2
→2)-α-D-GalA(1→	5.37			81.5	GalNAc^I C-3
		98.8	3.84		GalNAc^I H-3
Amino acid	δ_{H-2}			δ_C	
AlaLys	4.56			171.6	GalA C-6

40.6 and 51.6, respectively, in the corresponding free amino acids. Hence, it was confirmed that **1** was N^{ε}-(1-carboxyethyl)lysine.

A positive optical rotation value of **1**, $[\alpha]_D$ +10.8 (*c* 0.4, water), demonstrated that the lysine residue has the L configuration (compare published data:[19] $[\alpha]_D$ +9.7 and +11.6 (water) for N^{ε}-[(*R*)-1-carboxyethyl]-L-lysine (2*S*,8*R*-AlaLys) and N^{ε}-[(*S*)-1-carboxyethyl]-L-lysine (2*S*,8*S*-AlaLys), respectively.) The ^{13}C NMR spectrum of a mixture of **1** and the authentic sample of 2*S*,8*S*-AlaLys were indistinguishable from the spectra of the individual compounds, whereas two series of signals were present in the spectrum of a mixture of **1** and 2*S*,8*R*-AlaLys, the largest difference between the spectra of the isomers being observed for C-4 (~0.05 ppm; compare published data[20]). Therefore, **1** is N^{ε}-[(*S*)-1-carboxyethyl]-L-lysine (Fig. 4).

On the basis of these data, it can be concluded that the chemical structure of the repeating unit of the O-specific polysaccharide of *S. fidelis* type strain KMM 3582^T has the following structure:



The occurrence of amides of AlaLys with galacturonic and glucuronic acids has been reported previously only for the O-specific polysaccharides from a number of members of *Enterobacteriaceae* family: *Providencia rustigianii* O14[13] (N^{ε}-[(*S*)-1-carboxyethyl]-N^{α}-(D-galacturonoyl)-L-lysine, (**2**)), *Providencia alcalifaciens* O23[20] and *Proteus myxofaciens*[21] (N^{ε}-[(*R*)-1-carboxyethyl]-N^{α}-(D-glucuronoyl)-L-lysine, (**4**)) and *Proteus mirabilis* O13[22] (N^{ε}-[(*R*)-1-carboxyethyl]-N^{α}-(D-galacturonoyl)-L-lysine, (**3**)), respectively (Fig. 5). Now we have found for the first time a similar amide in the O-antigenic polysaccharide of a bacterium from a different taxon, namely *Shewanella* sp. In our opinion, the presence of such characteristic amides might be an indication of a common ancestor for bacteria of these genera as members of the γ-subclass of *Proteobacteria*.[7,23]

Uronic acids and their amides are often the immunodominant sugars in specific epitopes and this is especially true when they are amidated by amino acids.[24] Although substitution of microbial polysaccharides with amino acids is relatively rare, this phenomenon is not irregular in genus *Proteus*[24] and in each case the amino acid was shown to be important in manifesting immunospecificity,[25–28] except for one instance, where it was found to be of minor importance.[16] Despite the fact that serological studies have not been carried out on this occasion, it may be demonstrated from previous studies involving the amides of alaninolysine with galacturonic and glucuronic acids that AlaLys is important in immunospecificity and cross-reactivity.[13,21] Further, the configuration of AlaLys or the amidated uronic acid appears not to be significant in antibody recognition.

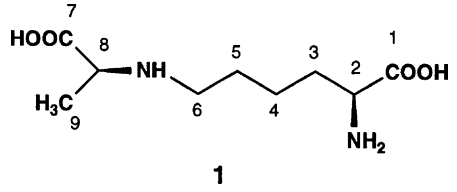


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Figure 4. Structure and numeration of carbons of N^{ε}-[(*S*)-1-carboxyethyl]-L-lysine (**1**).

1. Experimental

1.1. Bacterial growth, isolation and degradation of lipopolysaccharide

These were performed as described.[29] LPS was hydrolyzed with aq 2% HOAc (100 °C, 2 h), lipid A precipitate (11%) was removed by centrifugation, a water-soluble

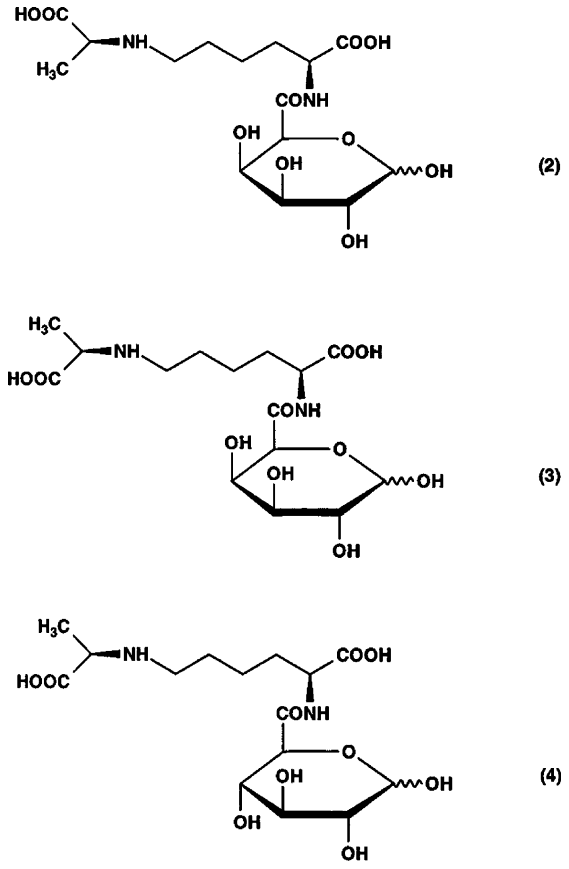

Figure 5. Amides of uronic acids with N^{ε}-(1-carboxyethyl)-L-lysine found in bacterial polysaccharides where (**2**) is N^{ε}-[(*S*)-1-carboxyethyl]-N^{α}-(D-galacturonoyl)-L-lysine, (**3**) is N^{ε}-[(*R*)-1-carboxyethyl]-N^{α}-(D-galacturonoyl)-L-lysine and (**4**) is N^{ε}-[(*R*)-1-carboxyethyl]-N^{α}-(D-glucuronoyl)-L-lysine.

portion was concentrated and then fractionated by GPC on a column (2.5×90 cm) of TSK-50 (F) in 0.3% HOAc to give high-molecular weight polysaccharide.

1.2. Acid hydrolysis and amino acid identification

The polysaccharide (40 mg) was hydrolysed with 2 M CF_3COOH (120 °C, 2 h). The hydrolysate was fractionated by GPC on TSK HW-40 (S) to give amino acid **1** (4.8 mg), which was converted into the ammonium salt using a column (3×1 cm) of an IRA-120 (H^+-form) resin and elution with aq 5% ammonia. **1** was identified by ^1H and ^{13}C NMR spectroscopy and the specific optical rotation measured on a PU-5 polarimeter (Russian Federation) at 20 °C. Authentic samples of 2*S*,8*R*-AlaLys and 2*S*,8*S*-AlaLys were synthesised as described.[19,20]

1.3. Chemical analysis

Methanolysis of the polysaccharide (1 mg) was carried out using 1 M HCl–MeOH (85 °C, 16 h), followed by acetylation with Ac_2O in pyridine (120 °C, 30 min) and subsequently analysed by GLC. The absolute configurations of the amino sugars were determined by GLC of acetylated (*R*)-(−)-2-octyl glycosides according to published methods.[30–32] GLC was performed using a Varian 3900 equipped with a HP-5 capillary column using a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C/min.

1.4. NMR spectroscopy

Samples were deuterium exchanged by freeze drying three times from D_2O and then examined in solutions of 99.97% D_2O, using internal acetone as reference (δ_H 2.225, δ_C 31.45). NMR spectra were recorded at 70 °C on a JEOL Lambda 400 MHz spectrometer equipped with a DEC AXP 300 computer workstation. The mixing time for the ROESY was 250 ms. For the TOCSY the duration of the MLEV17 spin-lock was 80 ms. Other 2-D parameters were essentially the same as previously described.[29]

Acknowledgements

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