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Note

The structure of the O-polysaccharide from the lipopolysaccharide of *Providencia stuartii* O57 containing an amide of D-galacturonic acid with L-alanine

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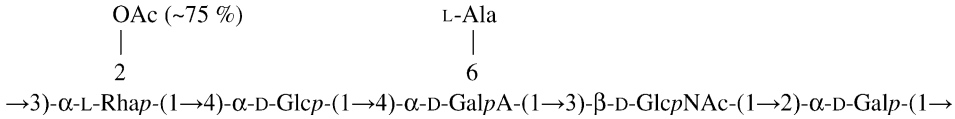
Abstract—The O-polysaccharide (O-antigen) was obtained by mild acid degradation of the lipopolysaccharide of *Providencia stuartii* O57:H29. Studies by sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy, including two-dimensional $^{1}H,^{1}H$ COSY, TOCSY, ROESY, H-detected $^{1}H,^{13}C$ HSQC, and HMBC experiments, showed that the polysaccharide contains an amide of D-galacturonic acid with L-alanine and has the following pentasaccharide repeating unit:



Keywords: *Providencia stuartii*; O-antigen; Lipopolysaccharide; Polysaccharide structure

Providencia is a genus of Enterobacteriaceae, which, together with genera *Proteus* and *Morganella*, is included in the tribe *Proteae*. Strains of three *Providencia* species, *P. alcalifaciens*, *P. rustigianii*, and *P. stuartii*, are classified into 63 O-serogroups. Bacteria *Providencia* are facultative pathogens, which under favorable conditions may cause various infections, mainly urinary tract infections, wound infections, and enteric diseases.[1,2] The serological O-specificity of *Providencia* is defined by the structure of the O-polysaccharide chain (O-antigen) of the lipopolysaccharide. Immunochemical studies of *Providencia* O-antigens aim at creation of the molecular basis for the serological classification and cross-reactivity of *Providencia* strains and related bacteria, including *Proteus*. Recently, the O-polysaccharide structures of a number of *Providencia* O-serogroups, including *P. stuartii* O4,[3] O18,[4] O33,[5] O47,[6] and O49,[7] have been established. Now we report on the structure of the O-polysaccharide of *P. stuartii* O57.

A high-molecular-mass polysaccharide was isolated by mild acid degradation of the lipopolysaccharide of *P. stuartii* O57 followed by GPC of the carbohydrate portion on Sephadex G-50. Sugar analysis using GLC of the acetylated alditols revealed Rha, Glc, Gal, and GlcN in the ratios 0.75:1:0.7:0.9, respectively. In addition, galacturonic acid (GalA) was identified by anion-exchange chromatography using a sugar analyzer. Amino acid analysis revealed Ala and GlcN in the ratio ~1:1. An enzymatic assay with D-glucose oxidase showed that the O-polysaccharide contains D-Glc. The

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D configuration of Gal, GalA, and GlcN, and the L configuration of Rha and Ala were determined by GLC of the acetylated glycosides with (+)-2-octanol[8] (for sugars) or the acetylated (+)-2-octyl ester (for Ala).

GLC–MS of the partially methylated alditol acetates derived from the methylated polysaccharide revealed 3-substituted Rha*p*, 2,3-disubstituted Rha*p* (from 3-substituted 2-*O*-acetylrhamnose, see below), 4-substituted Glc, 2-substituted Gal*p*, and 3-substituted Glc*p*N. In addition to these, similar analysis after carboxyl-reduction of the methylated polysaccharide showed a small amount of 4,6-disubstituted Gal, which was evidently derived from non-amidated 4-substituted GalA. The pyranose form of the 4-substituted monosaccharides was demonstrated by NMR spectroscopy (see below).

The ^{13}C (Fig. 1) and ^{1}H NMR spectra of the polysaccharide showed the absence of the strict regularity owing to non-stoichiometric O-acetylation (a peak for CH_3 of an O-acetyl group was present at δ_C 22.2 and δ_H 2.27). O-Deacetylation with aqueous ammonia resulted in a modified polysaccharide with NMR spectra typical of a regular polymer (Tables 1 and 2). The ^{1}H NMR spectrum of the O-deacetylated polysaccharide contained signals for five anomeric protons at δ 4.62, 4.88, 4.93, 5.32, and 5.56, other sugar-ring protons in the region 3.4–4.5, two CH_3–C groups at δ 1.40 (H-3 of Ala) and 1.28 (H-6 of Rha), and one CH_3CON group at δ 1.90. The ^{13}C NMR spectrum contained signals for five sugar residues, including those for five anomeric carbons at δ 105.0, 101.8, 101.1, 100.7, and 97.0, three unsubstituted CH_2OH groups at δ 61.3–62.3, one nitrogen-bearing carbon at δ 55.6, two CH_3–C groups at δ 18.1 (C-6 of Rha) and 19.1 (C-3 of Ala), one *N*-acetyl group at δ 23.6 (CH_3) and δ 176.5 (CO), one carboxamide group at δ 170.5 (C-6 of GalA) and one carboxyl group at δ 180.9 (C-1 of Ala). The CO signals were assigned by their correlations in the ^{1}H,^{13}C HMBC spectrum with the CH_3 signals of NAc and Ala at 176.5/1.90 and 180.9/1.40, respectively. The position of the C-6 signal of GalA at δ 170.5 is typical of galacturonamides (e.g., compare published data[9] δ 170.1 for an amide of GalA with Ala in the O-polysaccharide of *Proteus vulgaris* O44). A minor amount of free GalA revealed in methylation analysis may indicate an incomplete amidation in *P. stuartii* O57. As judged by the absence from the ^{13}C NMR spectrum of signals at δ 82–88 that are typical of furanosides,[10] all sugar residues are in the pyranose form.

The ^{1}H NMR spectrum of the O-deacetylated polysaccharide was assigned using ^{1}H,^{1}H COSY, TOCSY, and ROESY experiments (Table 1, Fig. 2), and the ^{13}C NMR spectrum was assigned using an ^{1}H,^{13}C HSQC experiment (Table 2, Fig. 3). The signals of Ala were found by a typical H-3,H-2 correlation at δ 1.40/

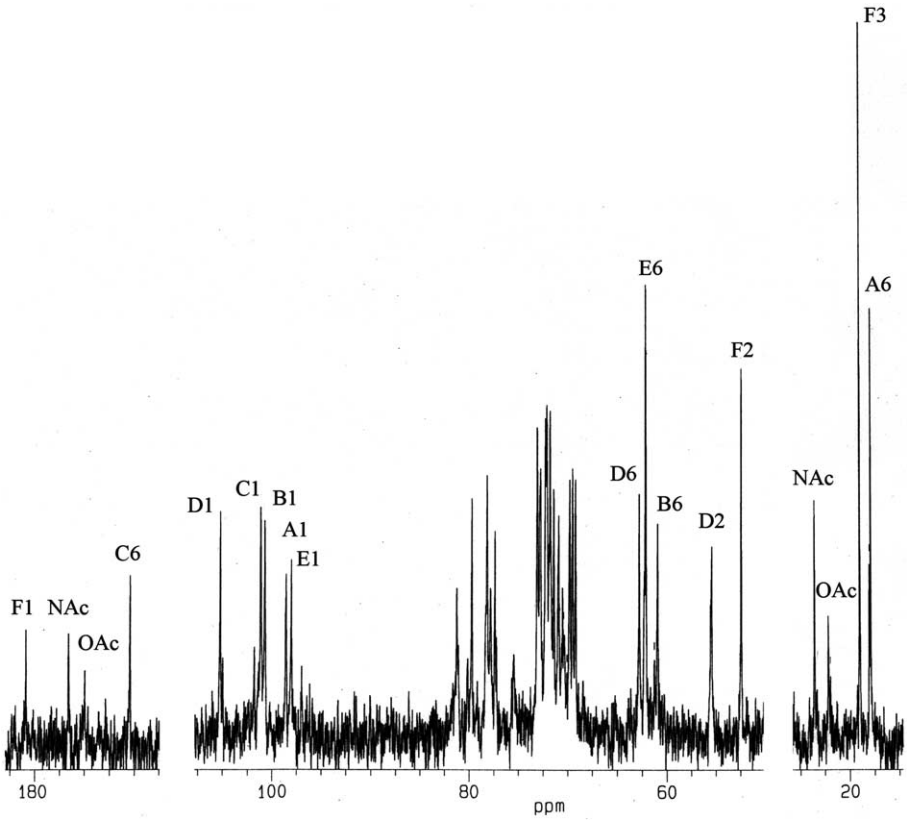


Figure 1. ^{13}C NMR spectrum of the O-polysaccharide of *P. stuartii* O57.

Table 1. ^{1}H NMR chemical shifts for the initial and O-deacetylated polysaccharides (δ, ppm)

Residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→3)-α-L-Rha*p*-(1→ **A**	4.93	4.25	3.82	3.55	4.07	1.28	
	(4.97)	(5.22)	(4.00)	(3.58)	(4.13)	(1.29)	
→4)-α-D-Glc*p*-(1→ **B**	4.88	3.45	3.73	3.59	4.23	3.81	3.73
	(4.88)	(3.44)	(3.73)	(3.58)	(4.23)	(3.85)	(3.80)
→4)-α-D-Gal*p*A-(1→ **C**							
6	5.56	3.96	3.96	4.40	4.29		
|	(5.55)	(3.95)	(3.98)	(4.40)	(4.27)		
L-Ala **F**		4.19	1.40				
→3)-β-D-Glc*p*NAc-(1→ **D**	4.62	3.86	3.83	3.68	3.52	3.94	3.72
	(4.48)	(3.74)	(3.78)	(3.54)	(3.49)	(3.97)	(3.68)
→2)-α-D-Gal*p*-(1→ **E**	5.32	3.85	3.94	3.98	4.17	3.96	3.76
	(5.26)	(3.78)	(3.91)	(3.98)	(4.18)	(3.74)	(3.69)

Data of the O-acetylated repeating unit are given in parentheses. Additional chemical shifts for CH_3 groups are δ 1.90 (NAc) and δ 2.27 (OAc).

Table 2. ^{13}C NMR chemical shifts for the initial and O-deacetylated polysaccharides (δ, ppm)

Residue	C-1	C-2	C-3	C-4	C-5	C-6
→3)-α-L-Rha*p*-(1→ **A**	101.8	68.5	77.7	71.8	70.5	18.1
	(98.6)	(71.6)	(75.8)	(71.9)	(70.7)	(17.9)
→4)-α-D-Glc*p*-(1→ **B**	100.7	73.1	73.1	78.4	72.3	61.3
	(100.7)	(73.1)	(73.0)	(77.9)	(72.2)	(61.0)
→4)-α-D-Gal*p*A-(1→ **C**						
6	101.1	69.6	69.9	78.1	72.1	170.5
|	(101.2)	(69.3)	(69.7)	(78.2)	(72.3)	170.5
L-Ala **F**	180.9	52.5	19.1			
→3)-β-D-Glc*p*NAc-(1→ **D**	105.0	55.6	81.1	72.2	77.2	62.3
	(105.3)	(55.4)	(81.3)	(72.8)	(77.4)	(62.8)
→2)-α-D-Gal*p*-(1→ **E**	97.0	80.15	69.2	71.0	71.9	62.2
	(98.0)	(80.0)	(69.4)	(71.1)	(72.0)	(62.2)

Data of the O-acetylated repeating unit are given in parentheses. Additional chemical shifts for the *O*-acetyl group are δ 22.2 (CH_3) and 175.0 (CO), for the *N*-acetyl group δ 23.6 (CH_3) and 176.5 (CO).

4.19 in the COSY spectrum. The spin system of Rha*p* was distinguished by correlations between all neighboring protons in the COSY spectrum and confirmed by correlations between H-6 and H-5,4,3 in the TOCSY spectrum. The chemical shift of δ 70.5 for C-5 indicated the α configuration of Rha*p*.[11]

An $^{1}H,^{13}C$ HSQC correlation between the nitrogen-bearing carbon and the attached proton at δ 55.6/3.86 enabled identification of the spin system for Glc*p*NAc. This was assigned using the COSY spectrum, which showed correlations between all neighboring protons of the sugar residue. The $J_{1,2}$ value of 7.5 Hz for the H-1 signal at δ 4.62 indicated the β configuration of Glc*p*NAc. The signal assignment and the anomeric configuration of β-Glc*p*NAc were confirmed by the ROESY spectrum, which showed an intra-residue H-1,H-5 cross-peak at δ 4.62/3.52.

The $J_{1,2}$ values <3 Hz for the three remaining H-1 signals at δ 4.88, 5.32, and 5.56 showed the α configuration of Glc*p*, Gal*p*, and Gal*p*A. The spin system with the H-1 signal at δ 4.88 was assigned by tracing all connectivities in the COSY spectrum and correlations between H-1 and H-2,3,4,5 in the TOCSY spectrum. The ROESY spectrum showed a strong intra-residue H-2,H-4 cross-peak, which excluded the *galacto* configuration. Hence, this sugar is α-Glc*p*.

The COSY spectrum revealed stepwise connectivities from the H-1 signal at δ 5.56 to H-5 at δ 4.29, but there was no H-5,H-6 correlation and the H-5 signal appeared as a singlet ($J_{4,5} < 2$ Hz). Therefore, this sugar is α-Gal*p*A, and the remaining sugar with the H-1 signal at δ 5.32 can be only α-Gal*p*. The signals for α-Gal*p* were assigned by H-1,H-2; H-2,H-3; and H-5,H-6 correlations in the COSY spectrum, an H-3,H-5 correlation in the ROESY spectrum, and a C-4,H-4 correlation at δ 71.0/3.98 between the last remaining unassigned sugar carbon signal and the attached proton in the $^{1}H,^{13}C$ HSQC spectrum.

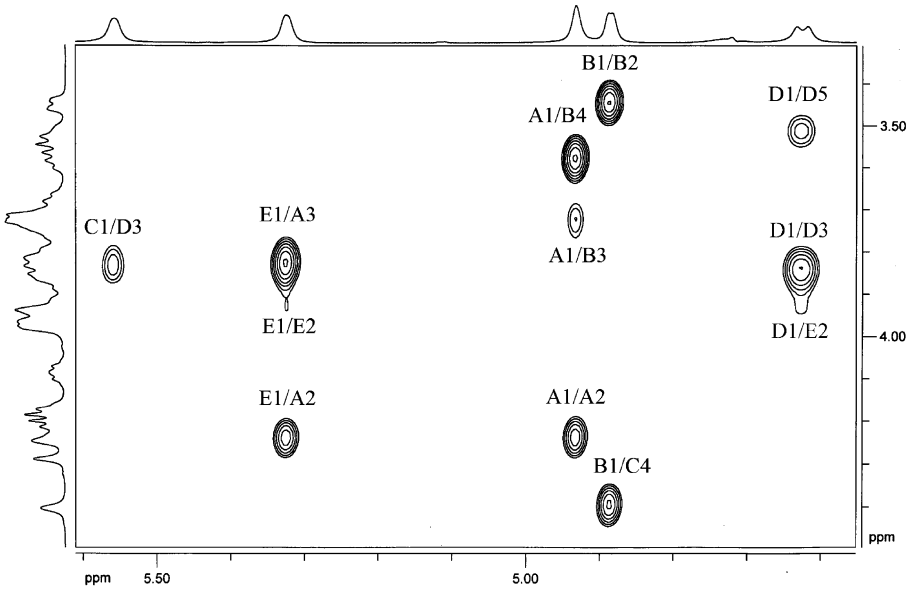


Figure 2. Part of a ROESY spectrum of the O-deacetylated polysaccharide of *P. stuartii* O57. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Arabic numerals refer to atoms in sugar residues denoted by capital letters as shown in Tables 1 and 2.

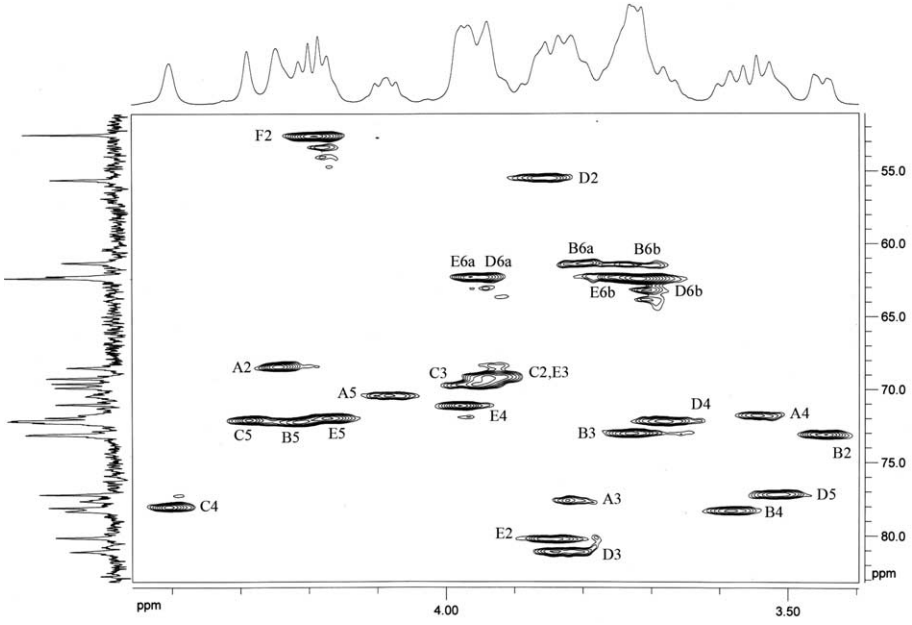


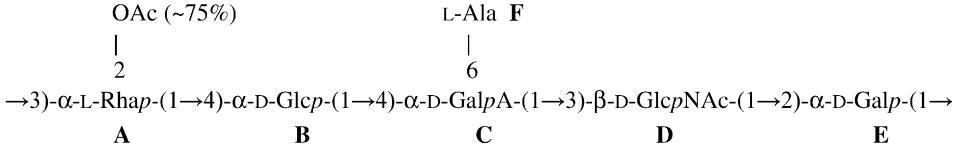
Figure 3. Part of an ^{1}H,^{13}C HSQC spectrum of the O-deacetylated polysaccharide of *P. stuartii* O57. The corresponding parts of the ^{1}H and ^{13}C NMR spectra are shown along the horizontal and vertical axes, respectively. Arabic numerals refer to atoms in sugar residues denoted by capital letters as shown in Tables 1 and 2.

Significant downfield displacements of the ^{13}C NMR resonances from δ 69.6 to 80.2 for C-2 of α-Gal*p*, from δ 71.3 and 75.1 to 77.7 and 81.1 for C-3 of Rha*p* and α-Glc*p*NAc, from δ 70.9 and 71.7 to 78.4 and 78.1 for C-4 of α-Glc*p* and α-Gal*p*A, respectively, as compared with the corresponding chemical shifts of the non-substituted monosaccharides,[12] demonstrated the substitution pattern in the repeating unit of the polysaccharide. The ROESY spectrum confirmed the substitution pattern and defined the sequence of the monosaccharide residues in the repeating unit by the following inter-residue cross-peaks between the anomeric protons and the protons at the linkage carbons: α-Gal*p*A H-1,β-Glc*p*NAc H-3 at δ 5.56/3.83; β-Glc*p*NAc H-1,α-Gal*p* H-2 at δ 4.62/3.85; α-Gal*p* H-1,α-Rha*p* H-3 at δ 5.32/3.82; α-Rha*p* H-1,α-Glc*p* H-4 at δ 4.93/3.59; and α-Glc*p* H-1,α-Gal*p*A H-4 at δ 4.88/

4.40. An additional cross-peak between α-Gal*p* H-1 and α-Rha*p* H-2 at δ 5.32/4.25 is typical of α-(1→3)-linked Rha disaccharides with different absolute configurations of the constituent monosaccharides.[13]

Comparison of the $^{1}H,^{13}C$ HSQC spectra of the O-deacetylated and initial polysaccharides revealed a significant shift of the α-Rha*p* C-2,H-2 cross-peak from δ 4.25/68.5 to δ 5.22/71.6, respectively, which was evidently caused by a deshielding effect of the *O*-acetyl group. The O-acetylation of α-Rha*p* at position 2 was confirmed by displacements of α-Rha*p* C-1,H-1 and C-3,H-3 cross-peaks from δ 101.8/4.93 and 77.7/3.82 to 98.6/4.97 and 75.8/4.00, respectively (compare published data on effects of O-acetylation on ^{13}C NMR chemical shifts[12]) whereas no other cross-peaks shifted by more than δ 0.2 in the ^{1}H dimension and δ 0.3 in the ^{13}C dimension (Tables 1 and 2). As judged by the ratio of the integral intensities of the C-1 signals for α-Rha*p*2Ac and α-Rha*p* in the ^{13}C NMR spectrum of the initial polysaccharide, the average degree of O-acetylation of the rhamnose residue is ~75%. No signals for the non-amidated GalA residue (see above) were observed in the NMR spectra, which allowed an estimation of its content as less than 0.1 related to the amidated GalA.

On the basis of the data obtained, it was concluded that the O-polysaccharide of *P. stuartii* O57 has the repeating unit with the following structure:



1. Experimental

1.1. Bacterial strain, isolation, and degradation of the lipopolysaccharide

P. stuartii O57:H29, strain 2615/53, from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest) was cultivated under aerobic conditions in tryptic soy broth supplemented with 0.6% yeast extract. The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with distilled water, and lyophilized. The lipopolysaccharide, in a yield of 8.4% of dry bacterial weight, was isolated by phenol–water extraction followed by dialysis of the extract without layer separation and purification by ultracentrifugation.

A portion of the lipopolysaccharide (150 mg) was heated with 2% AcOH (4 mL) for 70 min at 100 °C, and the carbohydrate-containing supernatant was fractionated on a column (60 × 2.5 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer pH 4.5. The yield of the polysaccharide was 33% of the lipopolysaccharide weight.

1.2. Chemical methods

O-Deacetylation of the polysaccharide (37 mg) was carried out with aq 12% ammonia (20 °C, 16 h).

For sugar analysis, the polysaccharide was hydrolyzed with 10 M HCl for 30 min at 85 °C, the alditol acetates were prepared by reduction with an excess of $NaBH_4$ (20 °C, 2 h) followed by acetylation (0.2 mL Ac_2O, 0.2 mL pyridine, 100 °C, 1 h) and analyzed by GLC on a Hewlett–Packard HP 5880 chromatograph equipped with an Ultra-2 column (Hewlett–Packard) using a temperature gradient of 10 °C min^{-1} from 180 to 290 °C. Uronic acid was analyzed using a Biotronik LC-2000 sugar analyzer as described.[14] Amino components were analyzed on a Biotronik LC-2000 amino acid analyzer using standard sodium citrate buffers.

For determination of the absolute configurations of neutral and amino sugars by GLC, the O-polysaccharide was hydrolyzed with 10 M HCl as above, N-acetylated (400 μL $NaHCO_3$, 60 μL Ac_2O, 0 °C, 1 h), subjected to (+)-2-octanolysis[8] [100 μL (+)-2-octanol, 15 μL CF_3CO_2H, 120 °C, 16 h], and acetylated. A portion of the hydrolysate was treated with D-glucose oxidase, reduced with $NaBH_4$, and acetylated. For determination of the absolute configuration of GalA, the polysaccharide was subjected to methanolysis (1 mL MeOH, 0.1 mL AcCl, 16 h, 80 °C) followed by (+)-2-octanolysis and acetylation. For determination of the absolute configuration of alanine, the polysaccharide was hydrolyzed with 10 M HCl (30 min, 85 °C), the products were N-acetylated and subjected to (+)-2-octanolysis as described above.

Methylation of the polysaccharide was performed according to the Hakomori procedure.[15] For a better solubility in Me_2SO, the polysaccharide was treated with Amberlite IR-120 (H^+) and lyophilized prior to methylation. The methylated product was recovered using a Sep-Pak cartridge, and a portion was reduced with $LiBH_4$ in aq 70% 2-propanol (20 °C, 2 h). Partially methylated monosaccharides were derived by hydrolysis with 10 M HCl (30 min, 85 °C), converted into the alditol acetates and analyzed by GLC–MS using a Hewlett–Packard 5971A system with an HP-1 glass capillary column (0.2 mm × 12 m) and a temperature program of 150–270 °C at 8 °C min^{-1}.

1.3. NMR spectroscopy

Prior to the measurements, the samples were freeze-dried twice from a ^2H_2O soln and dissolved in 99.96% ^2H_2O. Internal TSP (δ_H 0) and external acetone (δ 31.45) were used as references. ^1H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer at 30 °C. 2D NMR experiments were performed using standard Bruker software. Mixing times of 100 and 300 ms were used in TOCSY and ROESY experiments, respectively. Other NMR experimental parameters were essentially as described.[16]

Acknowledgements

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