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Note

# Structure of the O-specific polysaccharide of the lipopolysaccharide of *Rahnella aquatilis* 95 U003

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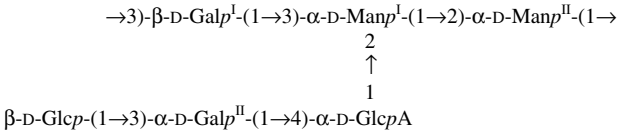
ABSTRACT

The O-polysaccharide of *Rahnella aquatilis* 95 U003 was obtained by mild acid degradation of the lipopolysaccharide and studied by sugar and methylation analyses, Smith degradation and $^{1}H$ and $^{13}C$ NMR spectroscopy, including 2D $^{1}H,^{1}H$ COSY, TOCSY, ROESY, H-detected $^{1}H,^{13}C$ HSQC and HMQC-TOCSY experiments. The O-polysaccharide was found to have a branched hexasaccharide repeating unit of the following structure:






In spite of the significant progress in investigation of lipopolysaccharides (LPS) of pathogenic Gram-negative bacteria, LPS of saprophytic and opportunistic microbes have been studied scarcely. At the same time, their role in pathogenesis of infectious diseases has increased recently as a result of a process of an evolutionary adaptation of microorganisms, which was intensified by large use of antibiotics, and change for the worse of ecological conditions. One of such bacteria is *Rahnella aquatilis*, a complex heterogeneous species whose representatives are similar in their phenotypic properties but different in genotypic characteristics. Accordingly, the species is divided into genomospecies.[1] Therefore, the genotypic differences seem to be essential but not so crucial for classification of these microorganisms as the individual species.

*R. aquatilis* was isolated from various sources: open water reservoirs, soil (mostly rhizosphere of cereals,[2,3] where they display the ability to fix atmospheric nitrogen) and from clinical material.[1,4] As a result, some scientists believe that *R. aquatilis* are phytotrophic microorganisms, whilst others point to their ability to cause diseases in mammals.[1–4] So far as *R. aquatilis* are opportunistic pathogens, they are characterized by wide clinical spectrum of diseases, which they are able to cause. An important role in pathogenesis of diseases caused by Gram-negative bacteria belongs to the lipopolysaccharide. Fine structures of the O-specific polysaccharide (OPS) chains of the lipopolysaccharide serve as a molecular basis for classification of strains. No serological classification scheme has been elaborated for *R. aquatilis*. Earlier, we have established the OPS structures of two *R. aquatilis* strains.[5,6] In this paper, we report on the structure of the OPS of *R. aquatilis* 95 U003.

Mild acid degradation of the lipopolysaccharide of *R. aquatilis* 95 U003 resulted in a high-molecular-mass OPS, which was isolated by GPC on Sephadex G-50. Sugar analysis by GLC of the alditol acetates derived after acid hydrolysis (2 M $CF_3COOH$, 2 h, 120 °C) of the OPS revealed the presence of glucose, mannose and galactose in the ratios ~0.6:1:1. In addition, glucuronic acid (GlcA) was identified by anion-exchange chromatography of the hydrolysate using a sugar analyzer. Determination of the absolute configuration by GLC of the acetylated glycosides with (*S*)-2-octanol indicated that all monosaccharides have the D configuration.

Methylation analysis of the OPS, including GLC of the partially methylated alditol acetates revealed derivatives of 2,3,4,6-tetra-*O*-methylglucopyranose, 3,4,6-tri-*O*-methylhexopyranose,

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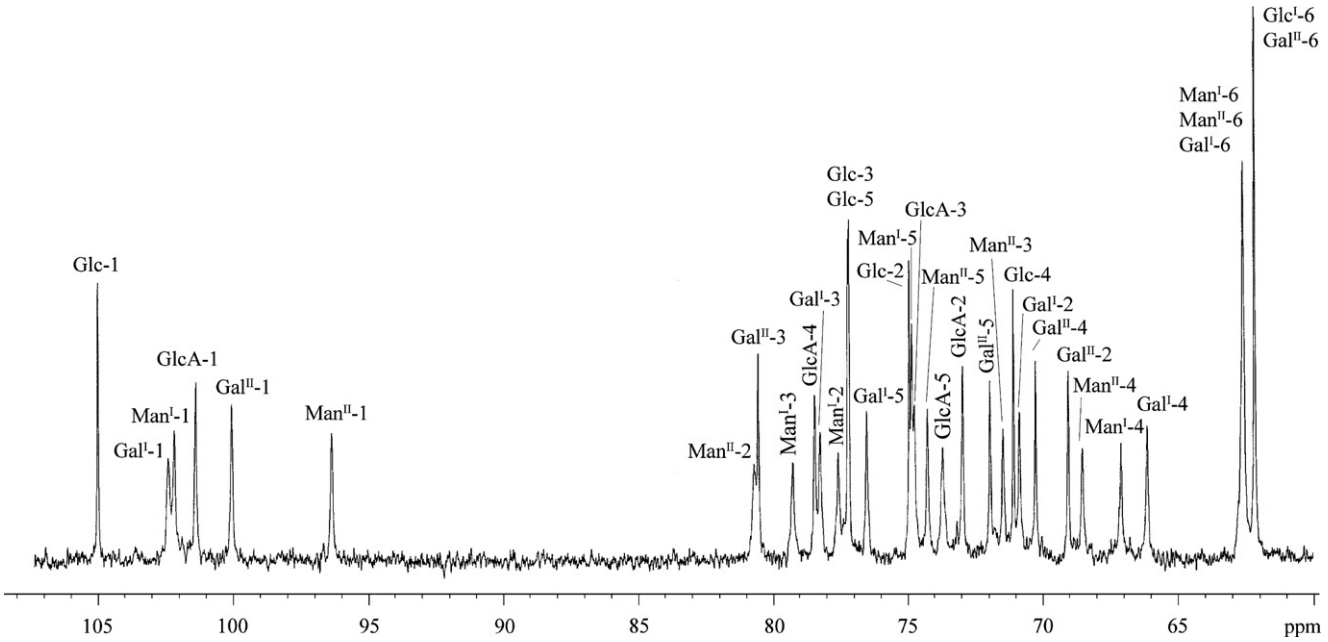



**Figure 1.** $^{13}$C NMR spectrum of the O-polysaccharide of *R. aquatilis* 95 U003. Signal of the carboxyl group (GlcA C-6) is not shown.

2,4,6-tri-*O*-methylhexopyranose and 4,6-di-*O*-methylhexopyranose. In addition to these monosaccharides, the same procedure after carboxyl reduction of the methylated polysaccharide showed a 2,3-di-*O*-methylhexose, which was evidently derived from a 4-substituted GlcA residue. Therefore, the OPS is branched and glucose is the terminal sugar residue of the side chain.

The $^{13}$C NMR spectrum of the OPS (Fig. 1) demonstrated a regular structure. It contained signals for six anomeric carbons at

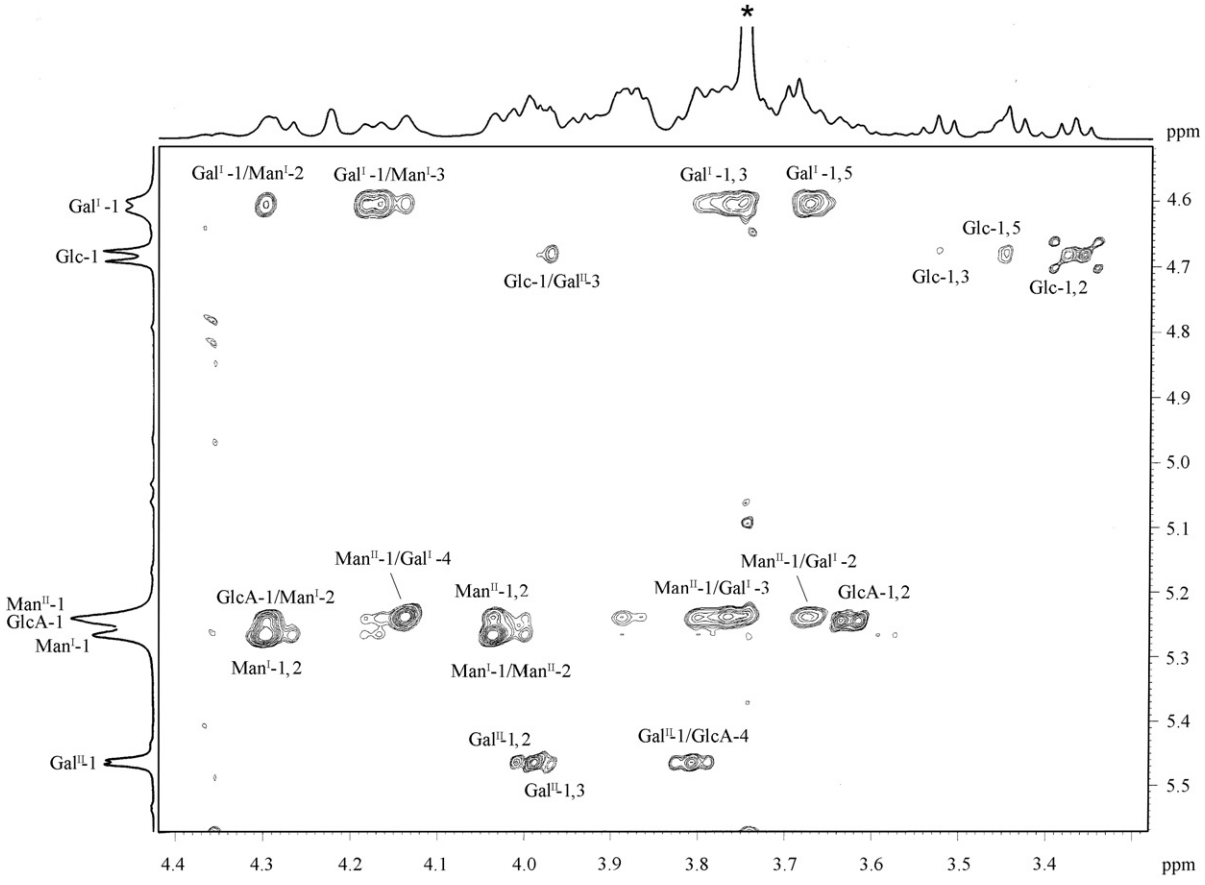



**Figure 2.** Part of a NOESY spectrum of the O-polysaccharide of *R. aquatilis* 95 U003. The corresponding parts of the $^{1}$H NMR spectrum are displayed along the axes. Asterisk indicates a signal of an unknown contamination.

**Table 1**
500-MHz $^1$H NMR data of the OPS and oligosaccharides obtained after Smith degradation of the OPS ($\delta$ in ppm)

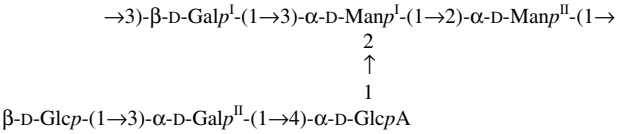
| Sugar residue | 1 | 2(2a/2b) | 3(3a/3b) | 4(4a/4b) | 5 | 6(6a) | 6b |
|---|---|---|---|---|---|---|---|
| *OPS* | | | | | | | |
| →3)-α-D-Gal*p*$^{II}$-(1→ | 5.46 | 3.99 | 3.97 | 4.23 | 3.94 | 3.70 | 3.70 |
| →2,3)-α-D-Man*p*$^{I}$-(1→ | 5.28 | 4.30 | 4.18 | 3.89 | 3.79 | 3.91 | 3.80 |
| →4)-α-D-Glc*p*A-(1→ | 5.24 | 3.63 | 4.00 | 3.81 | 4.27 | | |
| →2)-α-D-Man*p*$^{II}$-(1→ | 5.23 | 4.04 | 4.01 | 3.71 | 3.88 | 3.87 | 3.76 |
| β-D-Glc*p*-(1→ | 4.69 | 3.37 | 3.53 | 3.43 | 3.46 | 3.89 | 3.74 |
| →3)-β-D-Gal*p*$^{I}$-(1→ | 4.61 | 3.67 | 3.76 | 4.14 | 3.68 | 3.87 | 3.78 |
| *Oligosaccharide* **1** | | | | | | | |
| →3)-β-D-Gal*p*$^{I}$ | 4.54 | 3.61 | 3.69 | 3.93 | 3.71 | 3.75 | |
| →2,3)-α-D-Man*p*$^{I}$ | 5.14 | 4.21 | 4.11 | 3.82 | 3.91 | 3.75 | 3.89 |
| Gro | 5.14 | 3.93 | 3.86/3.77 | | | | |
| *Oligosaccharide* **2** | | | | | | | |
| →3)-β-D-Gal*p*$^{I}$ | 4.54 | 3.61 | 3.69 | 3.93 | 3.71 | 3.75 | |
| →2,3)-α-D-Man*p*$^{I}$ | 5.12 | 4.22 | 4.06 | 3.82 | 3.91 | 3.75 | 3.89 |
| →2)-α-D-Man*p*$^{IIa}$ | 3.71 | 3.86/3.77 | 5.13 | 3.63/3.72 | 4.30 | 3.81/4.03 | |
| *Oligosaccharide* **3** | | | | | | | |
| α-D-Gal*p*$^{II}$-(1→ | 5.19 | 3.80 | 3.89 | 4.01 | 4.09 | 3.73 | |
| EryA | | 4.32 | 4.02 | 3.73/3.79 | | | |
| *Oligosaccharide* **4** | | | | | | | |
| α-D-Gal*p*$^{II}$-(1→ | 5.12 | 3.86 | 3.92 | 3.99 | 4.17 | 3.75 | |
| EryA 1,4-lactone | | 4.68 | 4.80 | 4.45/4.54 | | | |

[a] A dioxolan derivative from oxidized Man*p*$^{II}$.

$\delta$ 96.4–105.1, five hydroxymethyl groups (C-6 of Glc, Man, Gal) at $\delta$ 62.2–62.7, one carboxyl group of GlcA at $\delta$ 175.6 and non-anomeric sugar ring carbons in the region $\delta$ 66.2–80.6. Accordingly, the $^1$H NMR spectrum of the OPS (Fig. 2) contained signals for six anomeric protons at $\delta$ 4.61–5.46 and other sugar protons at $\delta$ 3.03–4.23. As judged by the absence of signals in the range of $\delta$ 83–88 in the $^{13}$C NMR spectrum, all sugar residues are in the pyranose form.[7]

The spin systems for the sugar residues were assigned by a combined analysis of 2D COSY, TOCSY and NOESY spectra based on $^3J_{H,H}$ coupling constants. The spin system of Glc*p*A was distinguished by the absence of any H-6 signal and a corresponding shape of the H-5 signal. The assigned $^1$H NMR chemical shifts are tabulated in Table 1. Relatively large $J_{1,2}$ coupling constants of ~8 Hz indicated that glucose and one of the galactose residues (Gal*p*$^{I}$) are β-linked. The α-linkage of Glc*p*A, Gal*p*$^{II}$ and both mannose residues (Man*p*$^{I}$ and Man*p*$^{II}$) were determined by a comparison of the C-5 chemical shifts with published data of the corresponding α- and β-pyranosides.[7] The configurations of the glycosidic linkages were confirmed by H-1,H-5 correlations for the β-linked sugars and H-1,H-2 correlations for the α-linked sugars, which were observed in the NOESY spectrum.

The $^{13}$C NMR spectrum of the OPS was assigned using an H-detected $^1$H,$^{13}$C HSQC experiment (Table 2). Low-field displacements of the signals for C-2 of Man$^{II}$ to $\delta$ 80.7, C-2 and C-3 of Man$^{I}$ to $\delta$ 77.6 and 79.3, respectively, C-3 of Gal$^{I}$ and Gal$^{II}$ to $\delta$ 78.3 and 80.6, respectively, and C-4 of GlcA to $\delta$ 78.6, as compared with their positions in the corresponding non-substituted hexopyranosides at *$\delta$ 70–73*,[7] demonstrated the positions of substitution of the constituent monosaccharides, which were in agreement with methylation analysis data. The NOESY spectrum (Fig. 2) showed the following interresidue correlations between the anomeric protons and protons at the linkage carbons: Glc H-1,Gal$^{II}$ H-3 at $\delta$ 4.69/3.97, Gal$^{II}$ H-1,GlcA H-4 at $\delta$ 5.46/3.81, GlcA H-1,Man$^{I}$ H-2 at $\delta$ 5.24/4.30, Man$^{I}$ H-1,Man$^{II}$ H-2 at $\delta$ 5.28/4.04, Man$^{II}$ H-1,Gal$^{I}$ H-3 at $\delta$ 5.23/3.76 and Gal$^{I}$ H-1,Man$^{I}$ H-3 at $\delta$ 4.61/4.18, respectively. In addition, Man$^{I}$ H-1,GlcA H-5 and Man$^{II}$ H-1,Man$^{I}$ H-5 cross-peaks were observed at $\delta$ 5.28/4.27 and 5.23/3.79, which confirmed the α-GlcA-(1→2)-α-Man$^{I}$ and α-Man$^{I}$-(1→2)-α-Man$^{II}$ linkages, respectively. These data confirmed the positions of substitution of the monosaccharides and defined their sequence in the OPS repeating unit, which thus have the following structure:



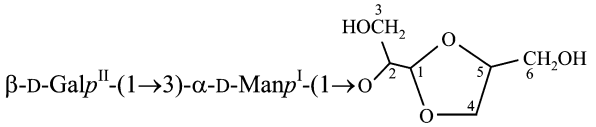

To confirm the structure, Smith degradation was applied to the OPS. The products of the subsequent mild acid hydrolysis were fractionated by GPC on TSK HW-40 to give two oligosaccharide fractions, which were analyzed by NMR spectroscopy as described above for the OPS (the assigned $^1$H and $^{13}$C NMR chemical shifts are given in Tables 1 and 2).

**Table 2**
125-MHz $^{13}$C NMR data of the OPS and oligosaccharides obtained after Smith degradation of the OPS ($\delta$ in ppm)

| Sugar residue | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| *OPS* | | | | | | |
| →3)-α-D-Gal*p*$^{II}$-(1→ | 100.3 | 69.0 | 80.6 | 70.3 | 72.1 | 62.2 |
| →2,3)-α-D-Man*p*$^{I}$-(1→ | 102.2 | 77.6 | 79.3 | 67.2 | 74.9 | 62.7 |
| →4)-α-D-Glc*p*A-(1→ | 101.4 | 72.9 | 74.8 | 78.6 | 73.3 | 175.6 |
| →2)-α-D-Man*p*$^{II}$-(1→ | 96.4 | 80.7 | 71.5 | 68.6 | 74.3 | 62.6 |
| β-D-Glc*p*-(1→ | 105.1 | 75.0 | 77.2 | 71.1 | 77.3 | 62.2 |
| →3)-β-D-Gal*p*$^{I}$-(1→ | 102.5 | 70.9 | 78.3 | 66.2 | 76.6 | 62.7 |
| *Oligosaccharide* **1** | | | | | | |
| →3)-β-D-Gal*p*$^{I}$ | 102.5 | 72.3 | 74.2 | 70.1 | 76.7 | 62.4 |
| →2,3)-α-D-Man*p*$^{I}$ | 100.3 | 69.1 | 78.8 | 66.6 | 74.4 | 60.9 |
| Gro | 90.5 | 77.8 | 62.6 | | | |
| *Oligosaccharide* **2** | | | | | | |
| →3)-β-D-Gal*p*$^{I}$ | 102.5 | 72.3 | 74.2 | 70.1 | 76.7 | 62.4 |
| →2,3)-α-D-Man*p*$^{I}$ | 100.7 | 69.2 | 78.8 | 66.6 | 74.4 | 60.9 |
| →2)-α-D-Man*p*$^{IIa}$ | 81.2 | 62.6 | 104.2 | 63.4 | 78.7 | 67.9 |
| *Oligosaccharide* **3** | | | | | | |
| α-D-Gal*p*$^{II}$-(1→ | 100.5 | 70.1 | 70.8 | 70.6 | 73.9 | 62.6 |
| EryA | n.d. | 74.4 | 82.2 | 61.8 | | |
| *Oligosaccharide* **4** | | | | | | |
| α-D-Gal*p*$^{II}$-(1→ | 97.8 | 69.5 | 70.7 | 70.7 | 72.9 | 62.7 |
| EryA 1,4-lactone | n.d | 70.3 | 74.4 | 71.6 | | |

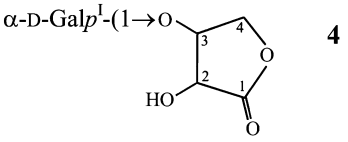
n.d., not determined.
[a] A dioxolan derivative from oxidized Man*p*$^{II}$.

β-D-Gal*p*$^{II}$-(1→3)-α-D-Man*p*$^{I}$-(1→2)-Gro-al **1**




**2**

α-D-Gal*p*$^{I}$-(1→3)-EryA **3**




**4**

**Chart 1.** Structures of the Smith degradation products from the O-polysaccharide of *R. aquatilis* 95 U003.

A fraction eluted from the gel earlier was found to be a mixture of two compounds having structures **1** and **2**, which were evidently derived from the main chain of the OPS as a result of oxidation of the 2-substituted Man$^{II}$ followed by hydrolysis of the acetal linkage of the oxidized sugar and elimination of the side chain. Oligosaccharide **1** includes the expected hydrated glyceraldehyde aglycon (C-1 at $\delta$ 90.5) derived from the oxidized Man$^{II}$, whereas an unwanted dioxolan aglycon in oligosaccharide **2** resulted from an intraresidue reacetalation in the oxidized Man$^{II}$ (Chart 1). A fraction eluted next was a mixture of two glycosides of α-D-Gal*p*$^{II}$ **3** and **4** derived from the side chain of the OPS by oxidation of the terminal Glc and 4-substituted GlcA followed by hydrolysis of the acetal linkages of the oxidized sugars. The aglycon in **3** is erythronic acid (EryA) derived from the oxidized GlcA and that in **4** is an 1,4-lactone of EryA (Chart 1). Therefore, Smith degradation confirmed the OPS structure of *R. aquatilis* 95 U003 established by methylation analysis and NMR spectroscopy.

## 1. Experimental

### 1.1. Growth of bacteria and isolation of the lipopolysaccharide and OPS

*R. aquatilis* strain 95 U003 isolated from excrements of a patient with diarrheal disease was kindly provided by Dr. S. Pokhil (Institute of Microbiology and Immunology of Medical Academy of Sciences of Ukraine, Kharkov, Ukraine). It was grown at 28 °C for 24 h on a beef-extract agar medium. Cells were separated by centrifugation and dried with acetone and ether. The lipopolysaccharide was isolated by the phenol–water procedure[8] followed by removal of nucleic acids by ultracentrifugation at 105,000*g*.

The OPS was obtained by degradation of the LPS with aq 3% HOAc for 6 h at 100 °C followed by GPC on a column (70 × 3.0 cm) of Sephadex G-50 (S) using 0.05 M pyridinium acetate (pH 4.5) as eluent and monitoring of carbohydrates by the phenol–sulfuric reaction.

### 1.2. Sugar analysis

Hydrolysis was performed with 2 M $CF_3CO_2H$ (120 °C, 2 h), the monosaccharides were analyzed by GLC as the alditol acetates on an Ultra 2 capillary column using a Hewlett–Packard 5880 instrument and a temperature program from 180 °C (1 min) to 290 °C at 10 °C min$^{-1}$. The absolute configurations of the monosaccharides were determined by GLC of the acetylated glycosides with (*S*)-2-octanol as described.[9]

### 1.3. Methylation analysis

Methylation was performed according to the Hakomori procedure,[10] the products were recovered using a Sep-Pak cartridge and divided into two parts, one of which was reduced with $LiBH_4$ in aq 70% 2-propanol (20 °C, 2 h). The partially methylated monosaccharides were obtained by hydrolysis of the permethylated polysaccharide with 10 M HCl for 30 min at 80 °C and converted into the alditol acetates. Analysis was performed by GLC–MS using a Hewlett–Packard 5971 A system equipped with an HP-1 glass capillary column (0.2 mm × 12 m) and a temperature program from 150 to 270 °C at 8 °C min$^{-1}$.

### 1.4. NMR spectroscopy

Samples were deuterium-exchanged by freeze-drying from $^2H_2O$. $^1H$ and $^{13}C$ NMR spectra were recorded on a Bruker DRX-500 spectrometer for solutions in $^2H_2O$ at 27 °C. Chemical shifts are reported with internal acetone ($\delta_H$ 2.225, $\delta_C$ 31.45). A mixing time of 200 and 150 ms was used in TOCSY and NOESY experiments, respectively.

## Acknowledgement

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