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Note

The structure of the O-polysaccharide from the lipopolysaccharide of *Providencia stuartii* O47

Olga G. Ovchinnikova,[a,*] Nina A. Kocharova,[a] Leon V. Bakinovskiy,[a] Agnieszka Torzewska,[b] Alexander S. Shashkov,[a] Yuriy A. Knirel[a] and Antoni Rozalski[b]

[a]*N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 119991 Moscow, Russian Federation*
[b]*Department of Immunobiology of Bacteria, Institute of Microbiology and Immunology, University of Lodz, PL 90-237 Lodz, Poland*

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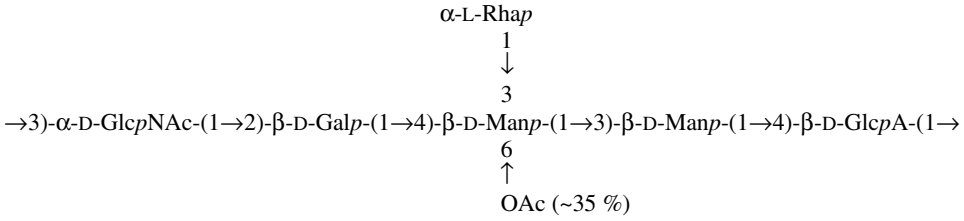
Abstract—The O-polysaccharide was obtained by mild acid degradation of the lipopolysaccharide of *Providencia stuartii* O47:H4, strain 3646/51. Studies by sugar and methylation analyses along with Smith degradation and ^{1}H and ^{13}C NMR spectroscopy, including two-dimensional $^{1}H,^{1}H$ COSY, TOCSY, ROESY and H-detected $^{1}H,^{13}C$ HSQC and HMBC experiments, showed that the polysaccharide has a branched hexasaccharide repeating unit with the following structure:



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

Gram-negative bacteria of the genus *Providencia* are facultative pathogens, which are able to invade intestinal mucosa and other cell types and may cause intestinal infections. They may cause also urinary tract infections. Particularly, *Providencia stuartii* is a well recognized pathogen that causes urinary tract infections in patients with chronic indwelling urinary catheters.[1] Currently, the genus *Providencia* is subdivided into five species: *P. alcalifaciens*, *P. rustigianii*, *P. heimbache*, *P. rettgerii* and *P. stuartii*. The serological O-specificity of *Providencia* is defined by the structure of the O-polysaccharide chain (O-antigen) of the lipopolysaccharide, which is considered also as a virulence factor of these bacteria. The serological classification scheme of *P. alcalifaciens*, *P. rustigianii* and *P. stuartii* includes 62 O-serogroups.[2] Immunochemical studies of *Providencia* O-antigens aim at the creation of the molecular basis for the serological classification and cross-reactivity of *Providencia* strains and related bacteria, including *Proteus*. Recently, the O-polysaccharides structures of *P. stuartii* O4,[3] O18,[4] O33[5] and O49[6] have been established. Now we report on the structure of the O-polysaccharide of *P. stuartii* O47.

A high-molecular-mass polysaccharide was isolated by mild acid degradation of the lipopolysaccharide of *P. stuartii* O47 followed by GPC of the carbohydrate

* Corresponding author. Tel.: +7 095 9383613; fax: +7 095 1355328; e-mail: olgao@hotmail.ru

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portion on Sephadex G-50. Sugar analysis using GLC of the acetylated alditols indicated the presence of Rha, Man, Gal and GlcN in the ratios ~1:2:0.7:0.5. In addition, glucuronic acid (GlcA) was identified by anion-exchange chromatography using a sugar analyzer. The L configuration of Rha and the D configuration of all other monosaccharides were determined by GLC of the acetylated glycosides with (+)-2-octanol.[7]

GLC–MS of the partially methylated alditol acetates derived from the methylated polysaccharide showed the presence of nonsubstituted Rha*p*, 3-substituted Man*p*, 3,4-disubstituted Man, 2-substituted Gal*p* and 3-substituted Glc*p*N. In addition to these monosaccharides, similar analysis after carboxyl reduction of the methylated polysaccharide pointed to the presence of 4,6-disubstituted Glc, which was evidently derived from 4-substituted GlcA. The pyranose form of the 4-substituted monosaccharides was deduced by NMR from spectroscopic data (see below).

The ^{13}C NMR spectrum of the polysaccharide showed a heterogeneity, which, most likely, was caused by non-stoichiometric *O*-acetylation. The ^{13}C NMR spectrum of the *O*-deacetylated polysaccharide (Fig. 1) contained signals for six sugar residues, including those for six anomeric carbons at δ 96.4–103.6, one nitrogen-bearing carbon at δ 53.4 (C-2 of GlcNAc), one methyl group (C-6 of Rha) at δ 17.5, one *N*-acetyl group at δ 23.3 (Me) and δ 175.2 (CO) and one carboxyl group (C-6 of GlcA) at δ 175.5. The ^{1}H NMR spectrum of the polysaccharide contained, inter alia, signals for six anomeric protons at δ 4.52–5.37, one methyl group (H-6 of Rha) at δ 1.32 and one *N*-acetyl group at δ 2.09.

The 2D TOCSY spectrum of the *O*-deacetylated polysaccharides revealed spin systems for six monosaccharide residues designated as **A**–**F** according to their sequence in the repeating unit established later (see below), and the COSY spectrum enabled differentiation between protons within each spin system (Table 1). The spin systems were ascribed to particular monosaccharides by characteristic coupling constant values estimated from the 2D NMR spectra, and that of GlcNAc was identified by correlation of proton at the nitrogen-bearing carbon (H-2) to the corresponding carbon (C-2) in the 2D H-detected ^{1}H,^{13}C HSQC spectrum. The latter was used also for assignment of the ^{13}C NMR spectrum of the *O*-deacetylated polysaccharide (Table 2).

The configurations of the glycosidic linkages were established by $J_{1,2}$ coupling constant values of ~3 Hz for α-GlcNAc (unit **B**) and 7.3–7.5 Hz for β-Gal and β-GlcA (units **C** and **F**, respectively). The configuration of α-Rha (unit **A**) and both β-Man residues (units **D** and **E**) were determined by typical positions of the H-5 and C-5 signals, which are known to differ most significantly in α- and β-pyranosides (Tables 1 and 2). The anomeric configuration of the β-linked monosaccharides was confirmed by correlation between H-1,H-3 and H-1,H-5 pairs of the 1,3-diaxial protons in the 2D ROESY spectrum of the *O*-deacetylated polysaccharide.

The relatively low-field position of the signals for C-3 of units **B** at δ 81.1 and **E** at δ 80.4 and C-4 of unit **F** at δ 81.1 demonstrated the mode of substitution of the monosaccharides (compare the chemical shifts δ 71.74, 74.03 and 72.69 of the corresponding signals in the nonsubstituted α-GlcNAc, β-Man and β-GlcA, respectively).[8] In 3,4-disubstituted Man (unit **D**) and 2-substituted Gal (unit **C**), the positive α-effects on the linkage carbons are small owing to a steric hindrance in the GlcNAc-(1→2)-Gal-(1→4)-[Rha-(1→3)]-Man branched tetrasaccharide fragment (compare the chemical shifts for C-3 and C-4 of β-Man and C-2 of β-Gal δ 74.8, 70.1 and 73.8 in the polysaccharide with δ 74.03, 67.69, 72.96 in the corresponding free monosaccharides,[8] respectively). In Man (unit **D**) the positive α-effects on C-3 and C-4 are also reduced by a negative β-effects on the neighbouring carbons.[8] The C-2–C-6 chemical shifts of unit **A** were similar to those in α-rhamnopyranose.[9]

The ROESY spectrum of the polysaccharide (Fig. 2) showed interresidue cross-peaks between the anomeric protons and protons at the linkage carbons, which, taking into account the positions of glycosylation of the

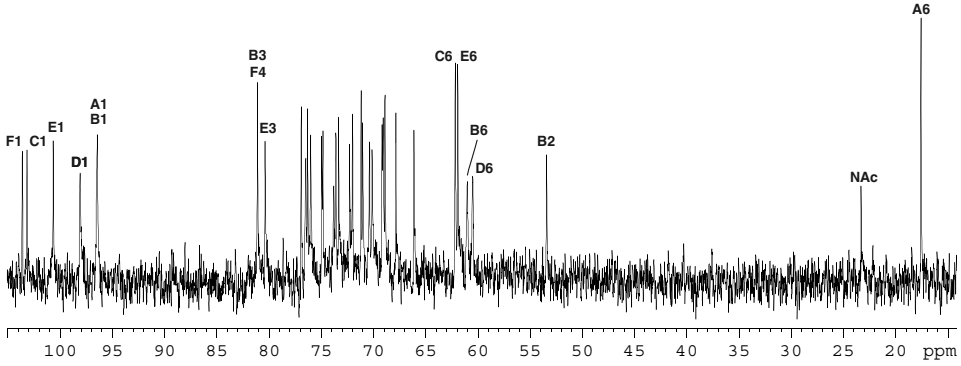

Figure 1. Part of a ^{13}C NMR spectrum of the *O*-deacetylated polysaccharide of *P. stuartii* O47 (region of CO resonances is not shown).

Table 1. ^{1}H NMR data

Sugar residue		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
O-Deacetylated polysaccharide								
α-L-Rha*p*-(1→	**A**	4.96	3.99	4.02	3.40	4.37	1.32	
→3)-α-D-Glc*p*NAc-(1→	**B**	5.37	4.11	3.98	3.65	4.08	3.83	3.80
→2)-β-D-Gal*p*-(1→	**C**	4.57	3.67	3.72	3.93	3.66	3.85	3.83
→3,4)-β-D-Man*p*-(1→	**D**	4.84	4.25	3.88	4.17	3.42	3.89	3.74
→3)-β-D-Man*p*-(1→	**E**	4.66	4.17	3.87	3.70	3.42	3.94	3.75
→4)-β-D-Glc*p*A-(1→	**F**	4.52	3.40	3.66	3.77	3.81		
Oligosaccharide **2**								
→3)-α-D-Glc*p*NAc-(1→	**B**	5.22	3.95	3.79	3.52	3.86	3.76	
→2)-β-D-Gal*p*-(1→[a]	**C**	5.06	3.95	3.80	3.86	3.92	3.86	3.80
→4)-β-D-Man*p*-(1→	**D**	4.87	4.09	3.81	3.89	3.47	3.96	3.76
3)-β-D-Man*p*-(1→	**E**	4.84	4.25	3.96	3.725	3.45	3.71	3.67
→3)-erythronic acid	**F**		4.23	4.13	3.68[b]			
Oligosaccharide **1**								
β-D-Man*p*-(1→	**D**	4.87	4.07	3.69	3.60	3.41	3.94	3.74
→3)-β-D-Man*p*-(1→	**E**	4.85	4.26	3.97	3.72	3.46	3.77	3.68
→3)-erythronic acid	**F**		4.23	4.14	3.68[b]			
Monosaccharides[8]								
α-L-Rha*p*		5.12	3.92	3.81	3.45	3.86	1.28	
β-L-Rha*p*		4.85	3.93	3.59	3.38	3.39	1.30	
α-D-Man*p*		5.18	3.94	3.86	3.68	3.82	3.74	3.86
β-D-Man*p*		4.89	3.95	3.66	3.60	3.38	3.75	3.91

Additional chemical shift for *N*-acetyl group is δ_H 2.09.
[a] The oxidized residue (Gal* in Fig. 3).
[b] H-4a; H-4b at δ 3.79.

Table 2. ^{13}C NMR data

Sugar residue		C-1	C-2	C-3	C-4	C-5	C-6
O-Deacetylated polysaccharide							
α-L-Rha*p*-(1→	**A**	96.4	71.2	71.0	73.6	69.2	17.5
→3)-α-D-Glc*p*NAc-(1→	**B**	96.4	53.4	81.1	69.1	72.0	61.0
→2)-β-D-Gal*p*-(1→	**C**	103.2	73.8	72.3	70.4	76.3	62.1
→3,4)-β-D-Man*p*-(1→	**D**	98.1	67.8	74.8	70.1	76.0	60.5
→3)-β-D-Man*p*-(1→	**E**	100.6	68.9	80.4	66.1	76.9	61.9
→4)-β-D-Glc*p*A-(1→	**F**	103.6	73.3	74.9	81.1	76.5	175.5
Oligosaccharide **2**							
→3)-α-D-Glc*p*NAc-(1→	**B**	98.2	55.0	72.1	71.2	73.4	62.3[a]
→2)-β-D-Gal*p*-(1→[c]	**C**	103.5	80.3	61.7[b]	61.6[b]	79.1	61.6[b]
→4)-β-D-Man*p*-(1→	**D**	98.0	72.0	73.5	74.0	76.5	62.4[a]
→3)-β-D-Man*p*-(1→	**E**	99.8	69.1	81.6	66.6	77.2	62.5[a]
→3)-erythronic acid	**F**	[d]	73.3	82.5	62.3		
Oligosaccharide **1**							
β-D-Man*p*-(1→	**D**	98.3	72.2	74.3	68.3	77.7	62.5
→3)-β-D-Man*p*-(1→	**E**	100.0	69.3	80.6	66.7	77.3	62.5
→3)-erythronic acid	**F**	[d]	73.5	82.7	62.5		
Monosaccharides[8]							
α-L-Rha*p*		94.84	71.81	71.00	73.19	69.12	17.67
β-L-Rha*p*		94.37	72.23	73.76	72.83	72.83	17.61
α-D-Man*p*		94.94	71.69	71.25	67.94	73.34	61.99
β-D-Man*p*		94.55	72.13	74.03	67.69	77.00	61.99

Additional chemical shifts for the *N*-acetyl group are δ_C 23.3 (CH_3) and 175.2 (CO).
[a,b] Assignment could be interchanged.
[c] The oxidized residue (Gal* in Fig. 3).
[d] The signal of the carboxyl group was not found owing to its relatively low intensity.

monosaccharides determined earlier, could be interpreted as follows: α-GlcNAc H-1, β-Gal H-2 at δ 5.37/3.67, β-Gal H-1, β-Man (**D**) H-4 at δ 4.57/4.17, α-Rha H-1, β-Man (**D**) H-3 at δ 4.96/3.88, β-Man (**D**) H-1, β-Man (**E**) H-3 at δ 4.84/3.87, β-Man (**E**) H-1, β-GlcA H-4 at δ 4.66/3.77 and β-GlcA H-1, α-GlcNAc H-3 at

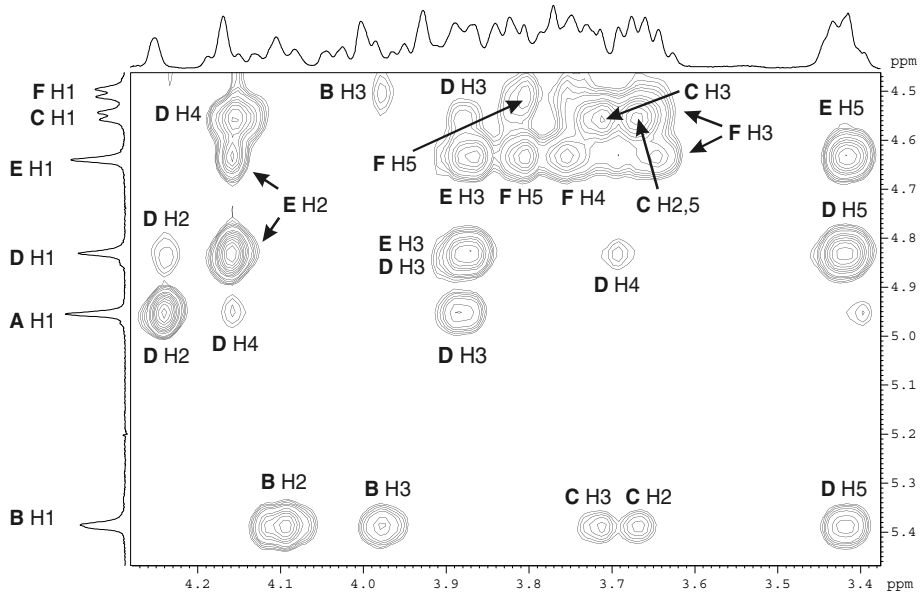

Figure 2. Part of a 2D ROESY spectrum of the *O*-deacetylated polysaccharide of *P. stuartii* O47. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Arabic numerals refer to atoms in sugar residues denoted by letters as shown in Tables 1 and 2.

δ 4.52/3.98. In addition, the ROESY spectrum contained α-GlcNAc H-1, β-Man (**D**) H-5 cross-peak at δ 5.37/3.42 due to a spatial proximity of these protons of the nonlinked monosaccharide residues. Most correlations between protons of the linked monosaccharides were confirmed by an ^{1}H,^{13}C HMBC experiment, which showed the following cross-peaks: β-GlcA H-1, α-GlcNAc C-3 at δ 4.52/81.1, β-Man (**E**) H-1, α-GlcA C-4 at δ 4.66/81.1, β-Man (**D**) H-1, β-Man (**E**) C-3 at δ 4.84/80.4, α-Rha H-1, β-Man (**D**) C-3 at δ 4.96/74.8, α-GlcNAc H-1, β-Gal C-2 at δ 5.37/73.8, as well as β-GlcA C-1,α-GlcNAc H-3 at δ 103.6/3.98, β-Gal C-1, β-Man (**D**) H-4 at δ 103.2/4.17, β-Man (**E**) C-1, β-GlcA H-4 at δ 100.6/3.77. These data define the monosaccharide sequence in the branched repeating unit of the polysaccharide, which, therefore, has the structure shown in Chart 1.

Comparison of the ^{13}C NMR spectra of the *O*-deacetylated and initial polysaccharides showed a displacement of part of the signal for C-6 of β-Man (**D**) from δ 60.5 to δ 63.0, which is caused by an α-effect of *O*-acetylation of Man (**D**) at C-6. Accordingly, the signal for C-1 of Man (**D**) was split at δ 97.8 and 98.2. As judged by the ratio of the integral intensities of the signal for the *O*-acetylated and nonacetylated residues, the average degree of *O*-acetylation of β-Man (**D**) is ~35%.

α-L-Rha*p*
1
↓
3
→3)-α-D-Glc*p*NAc-(1→2)-β-D-Gal*p*-(1→4)-β-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Glc*p*A-(1→
6
↑
OAc (~35 %)

β-D-Man*p*-(1→3)-β-D-Man*p*-(1→3)-erythronic acid **1**

α-D-Glc*p*NAc-(1→2)-β-D-Gal*-(1→4)-β-D-Man*p*-(1→3)-β-D-Man*p*-(1→3-erythronic acid **2**

β-D-Gal* =

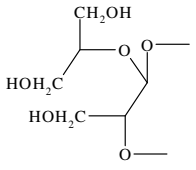

Chart 1. Structures of the O-polysaccharide of *P. stuartii* O47 and the oligosaccharides derived by Smith degradation.

For an independent structure confirmation, the *O*-deacetylated polysaccharide was Smith degraded to give the expected oligosaccharide (**1**) and, in addition, a larger oligosaccharide (**2**) resulted from incomplete cleavage of the linkage of the destroyed Gal residue during mild acid hydrolysis of the degraded polysaccharide. After separation by GPC, the structures of both oligosaccharides were established by NMR spectroscopy as described above for the *O*-deacetylated polysaccharide (for the ^{1}H and ^{13}C NMR chemical shifts see Tables 1 and 2). Specifically, the presence of a degraded unit **C** (galactose) in oligosaccharide **2** was inferred from the presence in the ^{13}C NMR spectrum of additional signals for three HO*C*H_2–C groups at δ 61.6–61.7, whereas signals for a nitrogen-bearing carbon at δ 55.0 and an *N*-acetyl group at δ 23.3 (Me) confirmed the presence of GlcNAc. The NMR data showed that **1** is a linear disaccharide composed of terminal Man (**D**) and 3-substituted Man (**E**) linked to erythronic acid (from GlcA). Oligosaccharide **2** consists of the same fragment and, in addition, contains a terminal GlcNAc residue linked to O-4 of Man (**D**) via a destroyed Gal residue. These data finally confirmed the structure of the O-polysaccharide of *P. stuartii* O47.

1. Experimental

1.1. Bacterial strain, isolation and degradation of the lipopolysaccharide

Providencia stuartii O47:H4 (strain 3646/51) obtained 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 5.3% of dry bacterial weight was isolated by phenol–water extraction and purified by ultracentrifugation.[4]

A portion of the lipopolysaccharide (200 mg) was heated with 2% AcOH (4 mL) for 2 h at 100 °C and the carbohydrate-containing supernatant was fractionated on a column of Sephadex G-50 (60 × 2.5 cm) in 0.05 M pyridinium acetate buffer. The yield of the polysaccharide was 16% of the lipopolysaccharide weight.

1.2. *O*-Deacetylation and Smith degradation of the polysaccharide

O-Deacetylation of the polysaccharide (25 mg) was carried out with aq 12% ammonia (20 °C, 16 h).

For Smith degradation, the polysaccharide (20 mg) was oxidized with 0.1 M sodium metaperiodate in the dark for 72 h at 20 °C; after adding an excess of ethylene glycol, the product was reduced by an excess of $NaBH_4$ and desalted on a column of TSK HW-40 in 1% AcOH. The resulting modified polysaccharide was hydrolyzed with 2% AcOH for 2 h at 100 °C, and the products were fractionated on a TSK HW-40 gel column in 1% AcOH. The yield of oligosaccharides **2** and **1** were 1.7 and 2.2 mg, respectively.

1.3. Monosaccharide analysis

For sugar analysis, the polysaccharide (0.3 mg) was hydrolyzed with 2 M CF_3CO_2H for 2 h at 120 °C. 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 a Ultra-2 column (Hewlett–Packard) using a temperature gradient of 10 °C min^{-1} from 180 to 290 °C.

For determination of the absolute configuration, the O-polysaccharide (0.5 mg) was hydrolyzed with 2 M CF_3CO_2H as above, N-acetylated (400 μL $NaHCO_3$, 60 μL Ac_2O, 0 °C, 1 h), subjected to 2-octanolysis[7] [100 μL (*S*)-2-octanol, 15 μL CF_3CO_2H, 120 °C, 16 h], acetylated and analyzed by GLC as above.

Uronic acids were analyzed using a Biotronik LC-2000 sugar analyzer as described.[10] For determination of the absolute configuration of GlcA, the polysaccharide was subjected to methanolysis (1 mL MeOH, 0.1 mL AcCl, 16 h, 80 °C) followed by 2-octanolysis, acetylation and analysis by GLC.

1.4. Methylation analysis

Prior to methylation, the polysaccharide (1.2 mg) was treated with Amberlite IR-120 (H^+) and lyophilized. Methylation of the polysaccharide was performed according to the Hakomori procedure,[11] the products were recovered by Sep-Pak and divided into two parts, one of which was reduced with $LiBH_4$ in aq 70% 2-propanol (20 °C, 2 h). Partially methylated monosaccharides were obtained by hydrolysis with 10 M HCl for 30 min at 80 °C, converted into alditol acetates and analyzed by GLC–MS on a Hewlett–Packard HP 5989A instrument equipped with a 30-m HP-5MS column (Hewlett–Packard) using a temperature gradient of 150 °C (3 min) → 320 °C at 5 °C min^{-1}.

1.5. NMR spectroscopy

Samples were freeze-dried twice from a $^{2}H_2O$ solution and dissolved in 99.96% $^{2}H_2O$ with internal TSP (δ_H 0) and external acetone (δ_C 31.45) as references. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer at 40 and 60 °C. 2D NMR experiments were performed using standard Brucker

software. Mixing time of 100 ms and spin-lock time of 30 ms were used in ROESY and TOCSY experiment, respectively.

Acknowledgements

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