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Note

The structure of the O-polysaccharide from the lipopolysaccharide of *Providencia alcalifaciens* O36 containing 3-deoxy-D-*manno*-oct-2-ulosonic acid

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Dedicated to the memory of Professor Nikolay K. Kochetkov

Abstract—An oligosaccharide that corresponds to the repeating unit of the O-polysaccharide was obtained by mild acid degradation of the lipopolysaccharide of *Providencia alcalifaciens* O36. Structural studies of the oligosaccharide and O-deacylated lipopolysaccharide were performed using sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy, including 2D $^{1}H,^{1}H$ COSY, TOCSY, ROESY, and H-detected $^{1}H,^{13}C$ HSQC and HMBC experiments. It was found that the O-polysaccharide is built up of linear trisaccharide repeating units containing 2-acetamido-2-deoxyglucose, 6-deoxy-L-talose (L-6dTal), and 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and has the following structure.

$$[\rightarrow 3)\text{-}\alpha\text{-L-6dTal}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow 7)\text{-}\beta\text{-Kdo}p\text{-}(2\rightarrow]_n$$
$$\begin{array}{c} 2 \\ | \\ \text{OAc (}\sim 70\ \%\text{)} \end{array}$$

Keywords: *Providencia alcalifaciens*; O-Antigen; Lipopolysaccharide; Polysaccharide structure; 6-Deoxy-L-talose; Kdo

Gram-negative bacteria of the genus *Providencia* are facultative pathogens, which under favorable conditions may cause various infections, mainly urinary tract infections, wound infections, and enteric diseases.[1,2] Strains of three *Providencia* species, viz, *P. alcalifaciens*, *P. rustigianii*, and *P. stuartii*, are classified into 63 O-serogroups.[1] The serological O-specificity of *Providencia* is defined by the structure of the O-polysaccharide chain (O-antigen) of the lipopolysaccharide (LPS), which is considered also as a virulence factor of these bacteria. 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*. At present, 25 O-polysaccharide structures have been established. In this paper, we report on the structure of a new acidic O-polysaccharide isolated from *P. alcalifaciens* O36.

Mild acid degradation of the LPS followed by GPC of the carbohydrate portion on Sephadex G-50 resulted in an oligosaccharide (OS), thus indicating the presence of an acid-labile linkage in the polysaccharide chain. Sugar analysis of the OS and O-deacylated LPS using GLC of the acetylated alditols demonstrated the presence of 6-deoxytalose (6dTal) and 2-amino-2-deoxyglucose in

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the ratio ~1.0:0.9. The absolute configurations of the monosaccharides were determined by GLC of the acetylated (*S*)-2-octyl glycosides as L for 6dTal and D for GlcN. GLC–MS analysis of the acetylated methyl glycosides confirmed 6dTal and GlcN and revealed the presence of a 3-deoxyoct-2-ulosonic acid. Further NMR studies showed that this monosaccharide is 3-deoxy-*manno*-oct-2-ulosonic acid (Kdo).

GLC–MS of the partially methylated alditol acetates derived from the methylated O-deacylated LPS revealed 3-substituted GlcN and 6dTal, whereas similar analysis of the OS demonstrated the presence of 3-disubstituted GlcN and terminal 6dTal. When the OS was reduced with $NaBH_4$ and carboxyl-reduced prior to methylation, 7-substituted Kdo was identified.

In addition to low-intense signals of the core and lipid A moieties, the ^{13}C NMR spectrum of the O-deacylated LPS contained signals for three anomeric carbons at δ 100.1–102.6, one C–*C*H_2–C group (Kdo C-3) at δ 36.0, one *C*H_3–C group (6dTal C-6) at δ 16.7, two HO*C*H_2–C groups (GlcN C-6 and Kdo C-8) at δ 61.9 and 64.3 [data of an attached-proton test (APT) experiment, Fig. 1], 12 sugar ring carbons in the region δ 66.8–79.4, and one *N*-acetyl group at δ 23.1 (CH_3) and 174.9 (CO). As judged by the absence of signals for non-anomeric sugar carbons within the region δ 82–88, all sugar residues are in the pyranose form.[3]

The ^1H NMR spectrum of the O-deacylated LPS contained signals for two anomeric protons (6dTal and GlcN H-1) at δ 4.94 and 4.96, one C–CH_2–C group of Kdo (H-3ax at δ 1.84 and H-3eq at δ 2.49), one CH_3–C group of 6dTal (H-6) at δ 1.12 and one *N*-acetyl group at δ 2.00.

The TOCSY spectrum of the O-deacylated LPS revealed spin systems for three monosaccharide residues designated as **A**–**C** 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 ^{13}C NMR spectrum of this sample was assigned using an $^1H,^{13}C$ HSQC experiment (Fig. 2, Table 2).

The α-linkage of GlcN was established from the C-2 chemical shift at δ 55.1 (compare published data[4] δ 55.3 and 57.0 for α- and β-GlcN, respectively). An intense 6dTal H-1,H-2 cross-peak and the absences of 6dTal H-1,H-3 and H-1,H-5 cross-peaks in the ROESY spectrum of the O-deacylated LPS demonstrated the equatorial position of H-1 and, hence, the α-linkage of 6dTal. The β-linkage of Kdo was inferred based on the difference between ^1H NMR chemical shifts for H-3ax and H-3eq of 0.65 ppm (compare published data[5] 0.27 and 0.64 ppm for methyl esters and methyl glycosides of α- and β-Kdo*p*, respectively). In the OS (the major form, see below), this difference is 0.11 ppm and, hence, Kdo has the α-configuration, which is favored by the equatorial orientation of the bulky carboxyl group.

Relatively low-field positions in the ^{13}C NMR spectrum of the signals for 6dTal C-3, GlcN C-3, and Kdo C-7 at δ 69.8, 79.4, and 78.4, respectively, as compared with their positions in the corresponding non-substituted

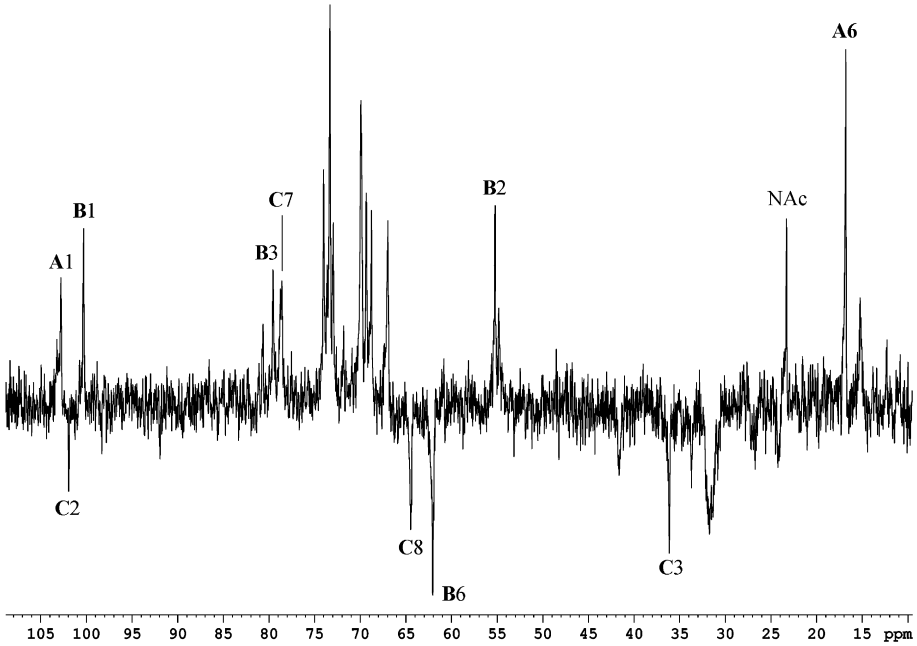


Figure 1. ^{13}C NMR APT spectrum of the O-deacylated LPS of *Providencia alcalifaciens* O36 (region of CO resonances is not shown). Arabic numerals refer to carbons in sugar residues denoted by letters as shown in Tables 1 and 2.

Table 1. ^{1}H NMR data (δ, ppm)

Sugar residue		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	
O-Deacylated LPS									
→3)-α-L-6dTal*p*-(1→	**A**	4.94	3.69	4.27	3.59	4.24	1.12		
→3)-α-D-Glc*p*NAc-(1→	**B**	4.96	4.02	3.77	3.50	3.84	3.77	3.87	
		H-3ax	H-3eq	H-4	H-5	H-6	H-7	H-8a	H-8b
→7)-β-Kdo*p*-(2→	**C**	1.84	2.49	3.74	3.79	3.64	3.88	3.79	3.98
		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	
O-Deacetylated OS									
α-L-6dTal*p*-(1→		4.99	3.75	3.87	3.67	4.30	1.21		
→3)-α-D-Glc*p*NAc-1→		5.09	4.09	3.83	3.55	3.91	3.81	3.87	
		H-3ax	H-3eq	H-4	H-5	H-6	H-7	H-8a	H-8b
→7)-α-Kdo*p*		1.90	2.01	4.09	3.95	3.89	3.91	3.86	3.90

Additional chemical shifts for the *N*-acetyl group are δ_H 2.00 in the O-deacylated LPS and δ_H 2.01 in the O-deacetylated OS.

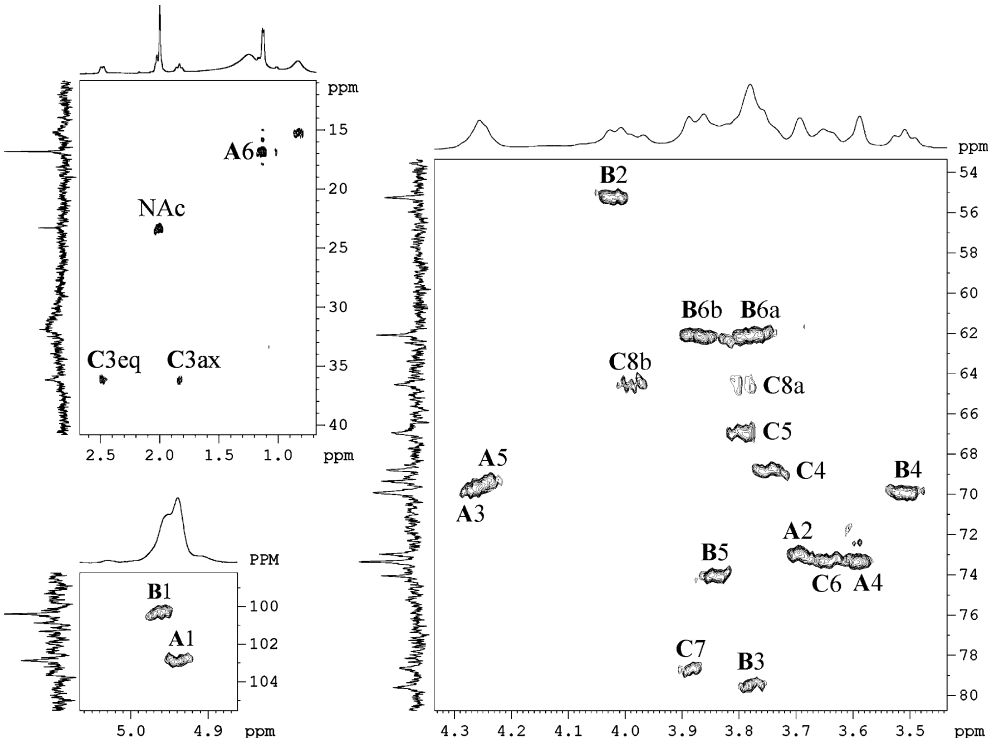


Figure 2. Parts of an H-detected ^{1}H,^{13}C HSQC spectrum of the O-deacylated LPS of *Providencia alcalifaciens* O36. The corresponding parts of the ^{1}H and ^{13}C NMR spectra are shown along the horizontal and vertical axes, respectively. Arabic numerals refer to atoms in sugar residues denoted by letters as shown in Tables 1 and 2.

monosaccharides at δ 66.3,[6] 72.0,[4] and 70.5,[7] confirmed the modes of glycosylation of the monosaccharides, determined by methylation analysis.

The ROESY spectrum of the O-deacylated LPS (Fig. 3) showed interresidue cross-peaks between the following anomeric protons and protons at the linkage carbons: 6dTal H-1,GlcN H-3 at δ 4.94/3.77 and GlcN H-1,Kdo H-7 at δ 4.96/3.88. These data are consistent with the glycosylation pattern and defined the sequence of the monosaccharides in the repeating unit.

The ^{13}C NMR spectrum of the OS showed heterogeneity owing to non-stoichiometric O-acetylation (there were signals for *O*-acetyl groups at δ 22.3 and 24.2) and the occurrence of Kdo at the reducing end in multiple forms. O-Deacetylation of the OS eliminated the heterogeneity of the former type.

The structure of the O-deacetylated OS was established by NMR spectroscopy as described above for the O-deacylated LPS (for the ^{1}H and ^{13}C NMR chemical shifts, see Tables 1 and 2). The comparison of the

Table 2. ^{13}C NMR data (δ, ppm)

Sugar residue		C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
O-Deacylated LPS									
→3)-α-L-6dTal*p*-(1→	**A**	102.6	72.8	69.8	73.2	69.2	16.7		
→3)-α-D-Glc*p*NAc-(1→	**B**	100.1	55.1	79.4	69.8	73.8	61.9		
→7)-β-Kdo*p*-(2→	**C**	[a]	101.9	36.0	68.6	66.8	73.2	78.4	64.3
O-Deacetylated OS									
α-L-6dTal*p*-(1→		103.3	73.6	66.9	71.8	69.0	16.9		
→3)-α-D-Glc*p*NAc-(1→		100.0	54.8	80.7	69.9	74.0	61.9		
→7)-α-Kdo*p*[b]		[a]	99.5	35.1	70.9	68.4	70.0	78.8	62.5

Additional chemical shifts for the *N*-acetyl group are δ_C 23.1 (CH_3) and 174.9 (CO) in the O-deacylated LPS, δ_C 23.4 (CH_3) and 175.6 (CO) in the O-deacetylated OS.

[a] The signal of the carboxyl group was not found owing to its low intensity.

[b] The major form.

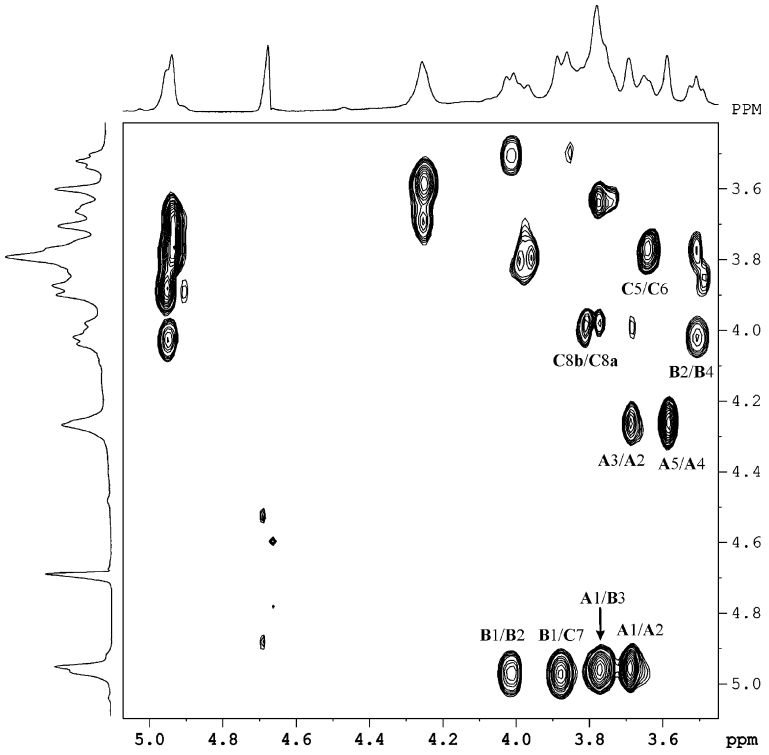
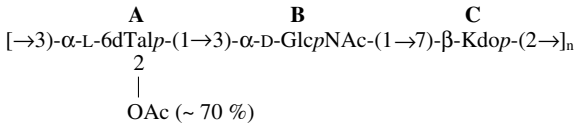


Figure 3. Part of a 2D ROESY spectrum of the O-deacylated LPS of *Providencia alcalifaciens* O36. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Arabic numerals refer to atoms in sugar residues denoted by letters as shown in Tables 1 and 2.

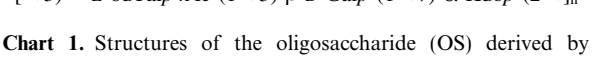
NMR spectra of the O-acetylated and O-deacetylated OS resulted in the location of the acetyl substituents. A low-field position at δ 4.84 of the major part of the signal for 6dTal H-2 in the initial OS, as compared with its position at δ 3.75 in the O-deacetylated OS, was evidently caused by a deshielding effect of the *O*-acetyl group and demonstrated O-acetylation of 6dTal at position 2. This was confirmed by an up-field shift to δ 100.6 of the signal for 6dTal C-1 in the OS from its position at δ 103.3 in the O-deacetylated OS. In addition, there was a minor *O*-acetyl group at position 4 of 6dTal. This followed from a low-field position at δ 5.13 of a minor signal for 6dTal H-4 (compare with its position at δ 3.67 in the O-deacetylated OS, Table 1), which was assigned by minor 6dTal H-6,C-4 and H-4,C-4 correlations at δ 1.09/74.5 and 5.13/74.5 in the ^{1}H,^{13}C HMBC and ^{1}H,^{13}C HSQC spectra of the OS, respectively. As judged by relative signal intensities of the O-acetylated and non-acetylated 6dTal residues, the average degree of O-acetylation of this monosaccharide at position 2 was ~70% and at position 4, it was ~20%. The occurrence of the minor 4-*O*-acetyl group may result from migration of the 2-*O*-acetyl group upon mild acid hydrolysis of the LPS (compare published data[8]).

The structure of the O-deacetylated OS was confirmed by negative ion electrospray ionization MS. The mass

α-L-6dTal*p*-(1→3)-α-D-Glc*p*NAc-(1→7)-α-Kdo*p* **1**



2



3

Chart 1. Structures of the oligosaccharide (OS) derived by acid hydrolysis of the LPS of *Providencia alcalifaciens* O36 (**1**), the O-polysaccharide of *Providencia alcalifaciens* O36 (**2**) and the polysaccharide from *Pseudoalteromonas flavipulchra* NCIMB 2033^{T10} (**3**).

spectrum contained an intense peak for a $[M-H]^-$ pseudomolecular ion at *m*/*z* 586.20 (the calculated monoisotopic molecular mass is 587.21 Da) as well as peaks at *m*/*z* 568.19 and 440.14 for $[M-H_2O-H]^-$ and $[M-6dTal-H]^-$ ions.

Therefore, the OS has structure **1** and the O-polysaccharide of *P. alcalifaciens* O36 has structure **2** as shown in Chart 1.

Although less common than L-rhamnose and L-fucose, 6-deoxy-L-talose occurs in a number of bacterial polysaccharides and often is O-acetylated.[9] Kdo is a rare component of O-polysaccharide chains of bacterial LPS (O-antigens). However, Kdo is a characteristic constituent of the LPS core, providing the linkage between lipid A and the carbohydrate moiety of LPS. Kdo is also known as a component of some capsular polysaccharides and exopolysaccharides (see Bacterial Carbohydrate Structure Database at http://www.glyco.ac.ru/bcsdb/).

It should be noted that structure **3** of the acidic polysaccharide from marine bacteria *Pseudoalteromonas flavipulchra* NCIMB 2033^{T10} resembles that of the O-polysaccharide of *P. alcalifaciens* O36 studied in this work, differing in one sugar residue, the position of O-acetylation of 6dTal and the anomeric configuration of Kdo (Chart 1).

1. Experimental

1.1. Bacterial strain and isolation of the LPS

P. alcalifaciens O36 strain 1757/7 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 LPS was isolated in a yield of 4.9% of dry bacterial weight by phenol–water extraction[11] followed by dialysis of the extract without layer separation and freed from insoluble contaminations by centrifugation. The resulting solution was purified by treatment with cold aq 50% CCl_3CO_2H; after centrifugation the aqueous layer was dialyzed and freeze-dried.

1.2. Degradation and modification of the LPS

A portion of the LPS (100 mg) was heated with 2% AcOH for 5 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, pH 4.5. OS was isolated in a yield of 27% of the LPS weight after gel-filtration on a column of TSK HW-40 (80 × 1.5 cm) in 1% AcOH.

For O-deacylation, the LPS (95 mg) was heated with aq 12% ammonia at 37 °C for 16 h and the supernatant was fractionated on a column of Sephadex G-50. The yield of the O-deacylated LPS was 56.4 mg.

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

1.3. Chemical methods

For sugar analysis, the O-deacylated LPS was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h), the products were reduced with an excess of $NaBH_4$ (20 °C, 2 h), acetylated with an Ac_2O–pyridine mixture (1:1, 100 °C, 1 h) and analyzed by GLC on a Hewlett–Packard HP 5890 chromatograph equipped with an Ultra-2 column (Hewlett–Packard) using a temperature gradient of 10 °C min^{-1} from 180 to 290 °C.

The OS was subjected to methanolysis (2 M HCl/MeOH, 85 °C, 16 h) followed by acetylation (Ac_2O–pyridine, 85 °C, 20 min) and analyzed by GLC–MS on a ThermoQuest Finnigan model Trace series GC 2000 instrument equipped with an EC-1 column (0.32 mm × 30 m) using a temperature gradient from 150 °C (2 min) to 250 °C at 10 °C min^{-1}.

For determination of the absolute configurations of the monosaccharides, the OS was hydrolyzed with 2 M CF_3CO_2H as above, N-acetylated (400 μL satd aq $NaHCO_3$, 60 μL Ac_2O, 0 °C, 1 h), subjected to (+)-2-octanolysis[12] (100 μL (+)-2-octanol, 15 μL CF_3CO_2H, 120 °C, 16 h), acetylated and analyzed by GLC.

Methylation of the OS and O-deacylated LPS was performed according to the Hakomori procedure,[13] the products were recovered using a Sep-Pak cartridge. Partially methylated monosaccharides were derived by hydrolysis with 2 M CF_3CO_2H, converted into the alditol acetates and analyzed by GLC–MS for 6dTal and GlcNAc as above.

Alternatively, the OS was reduced with $NaBH_4$ (20 °C, 2 h), esterified with diazomethane at room temperature, reduced again with $NaBH_4$, methylated,[13]

subjected to methanolysis with 2 M HCl/MeOH (85 °C, 4 h), acetylated and analyzed by GLC–MS for Kdo.

1.4. Electrospray ionization MS and NMR spectroscopy

Negative ion electrospray ionization mass spectrum was obtained on a Fourier transform ion-cyclotron resonance mass spectrometer (ApexII, Bruker Daltonics, USA) equipped with a 7 T actively screened magnet and ion source. The sample was dissolved in a 2-propanol–water–Et_3N mixture (30:30:0.01 by vol.) at a concentration of $\sim$20 ng μL^{-1} and sprayed with a flow rate of 2 $\mu L\ min^{-1}$.

The samples were freeze-dried twice from a ^2H_2O soln and dissolved in 99.96% ^2H_2O with internal TSP (δ_H 0) and external acetone (δ_C 31.45) as references. ^1H and ^{13}C NMR spectra were recorded at 30 °C using Bruker DRX-500 NMR instrument and XwinNMR software on a SGI Indy/Irix 5.3 workstation. Mixing time of 300 ms and spin-lock time of 30 ms were used in ROESY and TOCSY experiments, respectively. Other NMR experimental parameters were essentially as previously described.[14]

Acknowledgments

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