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Chemical structure of aeromonas gum—extracellular polysaccharide from *Aeromonas nichidenii* 5797

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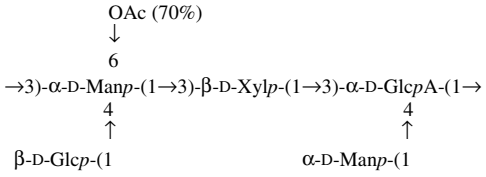
Abstract—Aeromonas (A) gum, an extracellular heteropolysaccharide produced by the bacterium *Aeromonas nichidenii* strain 5797, was studied by ^{1}H and ^{13}C NMR spectroscopy including 2D COSY, TOCSY, ^{1}H, ^{13}C HMQC, HMBC and ROESY experiments after O-deacetylation and Smith degradation. These investigations revealed the presence of an O-acetylated pentasaccharide repeating unit composed of mannose, glucose, xylose and glucuronic acid, and it has the following structure:



Keywords: Aeromonas gum; Polysaccharide structure; NMR; *Aeromonas nichidenii*

1. Introduction

Gram-negative *Aeromonas* bacteria are facultatively anaerobic and some species of the genus have been epidemiologically incriminated as enteropathogens.[1,2] They are autochthonous inhabitants of aquatic environments and their presence in several foodstuffs,[3,4] including drinking water,[5] is well documented. The genus is taxonomically complex and includes at least 16 species that can be differentiated only on the basis of such genetic methods as DNA–DNA hybridization.[6] At present, much research on *Aeromonas* bacteria is focused on epidemiology[1,2] and immunology.[7–9] However, since polysaccharides from many different bacteria are important in the manufacture, distribution, storage and consumption of food products,[10] cosmetics and paints, *Aeromonas* polysaccharides are also attracting attention for such uses.

Aermonas gum, an acidic polysaccharide composed of mannose (Man), glucose (Glc), xylose (Xyl) and glucuronic acid (GlcA), is produced by the bacterium *Aeromonas nichidenii* strain 5797.[11] This gum exists as aggregates in aqueous solution.[12–14] It shows very high viscosity in aqueous solution and is used as a food-thickening agent in Japan. In addition, it exists as semi-flexible single chains in cadoxen and dimethyl sulfoxide (Me_2SO) solutions at room temperature.[12,15,16] Apart

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from the sugar composition reported in the patent,[11] there has been no detailed information on the chemical structure of the repeating unit. Such an analysis may provide a basis for a better understanding of its use as a gelling agent for food products and for other uses. In this paper, we report on the structure determination of the extracellular polysaccharide of *A. nichidenii* strain 5797.

2. Results and discussion

The monosaccharide composition was determined to be mannose, glucose, xylose and glucuronic acid using gas chromatography (GC) of the alditol acetates.

The ^{13}C NMR spectrum of the original polysaccharide (A gum) showed a signal for the methyl group of *O*-acetyl (CH_3COO) at δ 21.8. The spectrum was exceedingly complex and therefore the O-deacetylated polysaccharide (**PS I**) (Fig. 1) was used for further investigations. This showed three anomeric signals at δ 104.4, 103.2 and 102.0 (triple intensity, Table 1). There were three signals present in the CH_2OH region at δ 61.0, 61.9 and 62.5, one signal for CH_2CH at δ 66.1 (confirmed by the DEPT), a *C*OOH group of an uronic acid at 176.2 ppm, and signals for ring carbons between 67.75 and 83.8 ppm. The attached proton test (APT) showed that there was no non-anomeric signals lower field than δ 82, demonstrating that all sugars present were in their pyranoid form.[17]

The ^1H NMR spectrum of **PS I** (Fig. 2, Table 1) contained five signals in the anomeric region at δ 5.30, 5.20 (double integral intensity), 4.78 and 4.70. 2D COSY, TOCSY, ROESY (Fig. 4), HSQC, HMQC-TOCSY and HMBC spectra, as well as 1D NOE spectra in difference mode were acquired for **PS I**. Assignment of the signals was difficult due to overlapping of the two anomeric proton signals in the ^1H NMR spectrum and

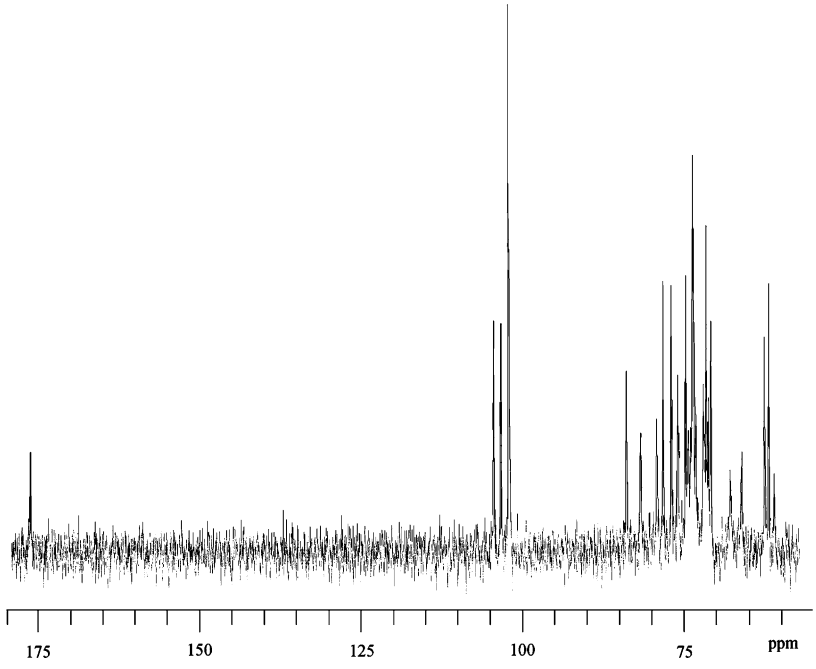

Figure 1. 125 MHz ^{13}C NMR partial spectrum of **PS I** at 60 °C.

Table 1. ^1H NMR data (δ, ppm) for the polysaccharide

Residue		H-1	H-2	H-3	H-4	H-5	H-5′,6	H-6′
PS I								
β-D-Glc*p*-(1→	**D**	4.55	3.31	3.51	3.32	3.51	3.98	3.72
→3,4)-α-D-Man*p*-(1→	**A**	5.20	4.19	4.03	4.20	4.12	3.91	3.89
→3)-β-D-Xyl*p*-(1→	**B**	4.78	3.41	3.58	3.81	3.96	3.29	
→3,4)-α-D-Glc*p*A-(1→	**C**	5.20	3.79	4.13	3.83	4.11		
α-D-Man-(1→	**E**	5.30	4.05	3.76	3.71	3.70	3.82	3.78
PS II								
→3)-α-D-Man*p*-(1→	**A**	5.21	4.19	3.98	3.91	4.03	3.85	3.80
→3)-β-D-Xyl*p*-(1→	**B**	4.70	3.43	3.62	3.78	3.96	3.31	
→3)-α-D-Glc*p*A-(1→	**C**	5.27	3.80	4.00	3.64	4.26		

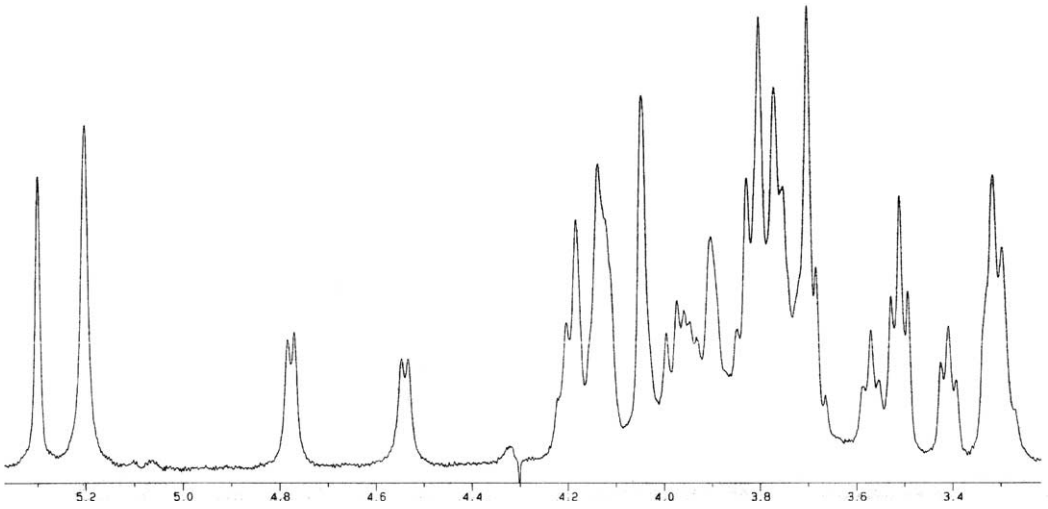

Figure 2. 500 MHz ^{1}H NMR partial spectrum of **PS I** at 60 °C.

Table 2. ^{13}C NMR data (δ, ppm) for the polysaccharide (glycosylation effects in parentheses)

Residue		C-1	C-2	C-3	C-4	C-5	C-6
PS I							
β-D-Glc-(1→	**D**	103.2 (+6.5)	74.65	76.8	71.4	78.1	62.5
→3,4)-α-D-Man*p*-(1→	**A**	102.0	71.9	79.1	73.1	73.5	61.0 (+0.1)
→3)-β-D-Xyl*p*-(1→	**B**	104.4	73.5	83.8	70.8	66.1	
→3,4)-α-D-Glc*p*A-(1→	**C**	102.0	73.4	81.65	75.85	74.3 (−2.6)	176.2
α-D-Man*p*-(1→	**E**	102.0 (+7.0)	71.15	71.8	67.75	73.9	61.9
PS II							
→3)-α-D-Man*p*-(1→	**A**	102.0 (+7.0)	71.5 (+0.2)	80.5 (+9.2)	67.1 (−0.9)	74.15	62.0
→3)-β-D-Xyl*p*-(1→	**B**	104.7 (+7.2)	73.3 (−1.8)	83.1 (+6.3)	70.9 (+0.7)	66.2	
→3)-α-D-Glc*p*A-(1→	**C**	102.0 (+9.1)	72.6 (+0.4)	81.9 (+8.1)	71.3 (−1.7)	73.3	175.0

Chemical shifts for NAc are δ 23.3 (Me) and 174.4 and 175.5 (assignment could be interchanged) (CO).

of the three anomeric carbons in the ^{13}C NMR spectrum. Therefore, **PS I** was subjected to Smith degradation in order to simplify the spectrum.

The ^{13}C NMR spectrum of **PS I** after Smith degradation (**PS II**) was typical of a regular polysaccharide and contained three signals for anomeric carbons at δ 104.7 and 102.0 (double integral intensity, Table 2). It also showed two CH_2 signals at δ 62.0 and 66.2 (confirmed by DEPT), one *C*OOH signal at 175.0 ppm and other ring carbon signals in the region δ 67.0–83.1.

The ^{1}H NMR spectrum of **PS II** showed three signals for anomeric protons at δ 5.27, 5.21 and 4.70. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) of **PS II** were assigned using 2D COSY, TOCSY (Fig. 3), HSQC, HMBC and ROESY experiments. The monosaccharide composition of the repeating units of **PS II** was found to be α-Glc*p*A (**C**), α-Man*p* (**A**) and β-Xyl*p* (**B**) based on coupling constants values and chemical shift data.[17,18] An analysis of the glycosylation effects in the ^{13}C NMR chemical shifts revealed a significant downfield chemical shift at C-3 of all three residues in comparison with corresponding nonsubstituted monosaccharides, indicating substitution at position 3.[18,19] The ROESY spectrum of **PS II** (Table 3) showed strong inter-residue cross-peaks between the following transglycosidic protons: **A** H-1/**B** H-3 at δ 5.21/3.62; **B** H-1/**C** H-3 at δ 4.70/4.00, and **C** H-1/**A** H-3 at δ 5.27/3.98, respectively. These data defined the following monosaccharide sequence in the repeating unit:

→ 3)-α-Man*p*-(1 → 3)-β-Xyl*p*-(1 → 3)-α-Glc*p*A-(1 →

The sequence was confirmed by the HMBC spectrum (Table 3) where the following inter-residue correlation peaks were observed: **A** H-1/**B** C-3 at δ 5.21/83.1; **B** H-1/**C** C-3 at δ 4.70/81.9; **C** H-3/**B** C-1 at δ 4.00/104.7, and **B** H-3/**A** C-1 at δ 3.62/102.0.

The assignment of the signals in both ^{1}H and ^{13}C NMR spectra of **PS II** allowed the establishment of the structure of **PS I** despite the aforementioned overlapping peaks. Thus, comparison of COSY, TOCSY and HSQC spectra of the two polysaccharides revealed that **PS I** contained two additional terminal residues, namely β-Glc*p* (**D**) and another α-Man*p* (**E**) according to coupling constants values along with chemical shift data.

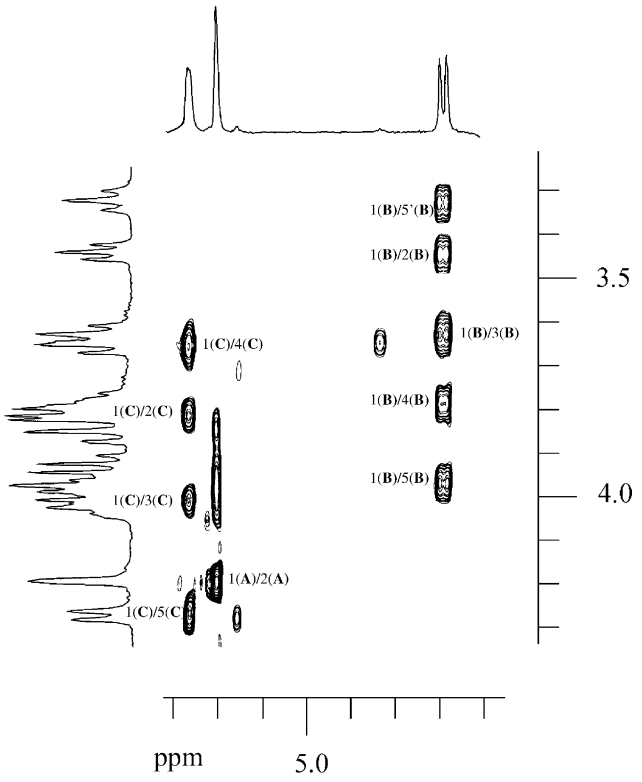

Figure 3. Partial 2D TOCSY spectrum showing the anomeric region of **PS II** at 60 °C.

Table 3. Homonuclear (ROESY) and heteronuclear (^{1}H, ^{13}C HMBC) interresidue connectivities for the anomeric atoms in the O-deacetylated polysaccharide (**PS I**) and Smith-degraded product (**PS II**)

Residue	δ_{H-1}	δ_{C-1}	δ_H	δ_C	Connectivity to
PS I					
HMBC data					
D					α-Man C-4
					α-Man H-4
A	5.20			83.8	β-Xyl C-3
		102.0	3.58		β-Xyl H-3
B	4.78			81.65	α-GlcA C-3
		104.4	4.13		α-GlcA H-3
C	5.20			79.1	α-Man C-3
		103.2	4.20		α-Man H-3
E	5.30			75.85	α-GlcA C-4
		102.0	3.83		α-GlcA H-4
PS II					
ROESY data					
A	5.21		3.62		β-Xyl H-3
B	4.70		4.00		α-GlcA H-3
C	5.27		3.98		α-Man H-3
HMBC data					
A	5.21			83.1	β-Xyl C-3
		102.0	3.62		β-Xyl H-3
B	4.70			81.9	α-GlcA C-3
		104.7	4.00		α-GlcA H-3

The location of the additional residues **D** and **E** at positions 4 of both residues **A** and **C** was determined due to downfield shift of the corresponding signals in comparison with that in the ^{13}C NMR spectra of **PS II** (Table 2). The ROESY spectrum of **PS I** (Fig. 4) contained intensive inter-residue correlation peaks **D** H-1/**A** H-4, 6 and **E** H-1/**C** H-4. The inter-residue correlation peaks for the sugars of the backbone were the same as those observed in the ROESY spectra of **PS II**. Thus, the structure of **PS I** is as following:

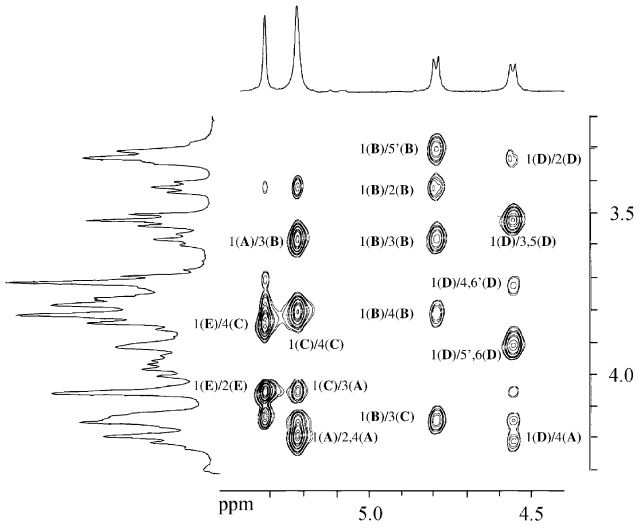

Figure 4. Partial 2D ROESY spectrum showing the anomeric region of **PS I** at 60 °C.

β-Glc*p*-(1 α-Man*p*-(1
↓ ↓
4 4
→3)-α-Man*p*-(1→3)-β-Xyl*p*-(1→3)-α-Glc*p*A-(1→

The proposed structure was confirmed by the HMBC spectrum (Table 3) where the following inter-residue correlation peaks were observed: **A** H-1/**B** C-3 at δ 5.20/83.8; **B** H-1/**C** C-3 at δ 4.78/81.65; **C** H-1/**A** C-3 at δ 5.20/79.1; **E** H-1/**C** C-4 at δ 5.30/75.85; **D** H-1/**A** C-4 at δ 4.55/73.1; **C** H-3/**B** C-1 at δ 4.13/104.4; **B** H-3/**A** C-1 at δ 3.58/102.0; **A** H-4/**D** C-1 at δ 4.20/103.2 and **C** H-4/**E** C-1 at δ 3.83/102.0.

A comparison of the ^{13}C NMR spectra of the initial O-acetylated polysaccharide with that of **PS I** (Fig. 1) showed the absence of the signals for the *C*H$_3$COO group (at δ 21.8 and 175.4). The presence of a small peak at δ 61.1 in the ^{13}C NMR spectrum of the initial polysaccharide demonstrated O-acetylation in this position and may be estimated as approximately 70%, taking into account the ratio of peaks at δ 61.1 (C-6 of non-O-acetylated **A**) and at δ 64.0 (C-6 of O-acetylated **A**).

In order to ascertain the relative absolute configurations of the sugar residues their glycosylation effects[19,20] were examined (Table 2). **PS II** was examined first in order to remove the complicating effects of the branch-

ing residues. The determining effect for the α-(1→3) linked residues of **A** and **B** was a *β* effect on C-4. If the absolute configurations were different, a relatively large absolute value negative effect would be expected. However, a value of +0.7 ppm indicated that they had identical absolute configurations. A relatively large absolute value negative *β* effect is expected for the β-(1→3) linked residues of **B** and **C** on C-4 of residue **C** in the case of the same absolute configurations. The value of −1.7 ppm confirms this. Therefore, it can be concluded that the relative absolute configurations of residues **A**, **B** and **C** are all the same.

The glycosylation effects for the branched **PS I** were then examined. For residues **D** and **E**, the *β* effect on C-5 and the *α* effect on C-1′ could only be considered due to the interference of the other substitutions on residues **A** and **C**. The *α* and *β* effects of +7.0 and −2.6 ppm, respectively, for the α-(1→4) linked sugars **E** and **C** imply that the relative absolute configurations are the same. Values of +0.1 and +6.5 ppm for the *β* and *α* effects for β-(1→4) linked residues **D** and **A** also suggest identical absolute configurations. In conclusion, all the residues of the polysaccharide repeating unit have the same relative absolute configuration.

The absolute configurations of all constituent residues of the polysaccharide were concluded to be D following GC analysis of the glycosides with a chiral alcohol, concurring with the analysis of NMR data as above.

Other bacterial polysaccharide gums used in the food industry are xanthan gum from *Xanthomonas campestris*,[21] alginates from *Pseudomonas aeruginosa*[22] and *Azo-bacter vinelandii*,[23] and gellan gum from *Sphingmonas paucimobilis*[24] (Fig. 5). Acetylation at position 6 of mannose and the presence of glucose and glucuronic acid are the only structural similarities of the xanthan and aeromonas gums. Gellan gum also has the sugars glucose and glucuronic acid, although the O-acetylation at position 6 is on a glucose residue.[24]

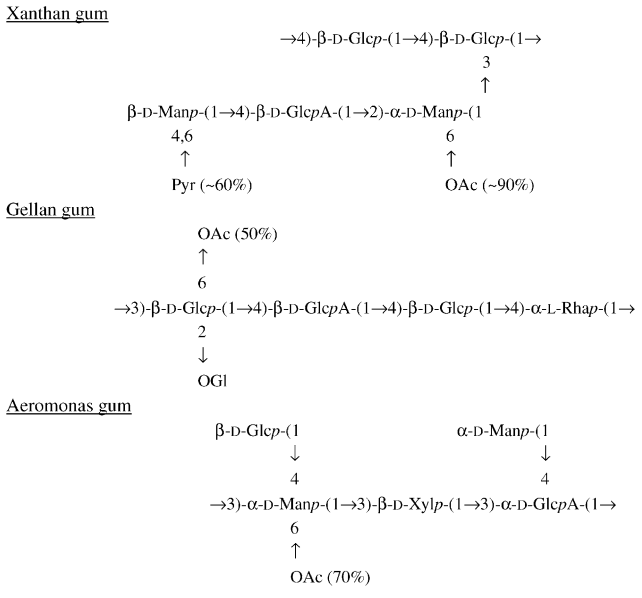

Figure 5. Structures of xanthan, gellan and aeromonas gums.

3. Experimental

3.1. Purification and degradation of the aeromonas (A) gum

An original gum sample A from *A. nichidenii* 5797 was kindly provided by Asahi Chemical Industry Co., Japan. Crude A was dissolved in distilled water and stirred overnight (turbid solution) and the water-insoluble gum was removed by centrifugation to obtain a clear solution, which was then concentrated by rotary evaporation under reduced pressure at room temperature. Finally, the concentrated solution was precipitated by EtOH. The precipitates were washed with sufficient EtOH and vacuum-dried for one week to obtain the pure powder sample. The powder was dissolved in ultrapure H_2O and depolymerized in an Ultrawave ultrasonic bath at 50 °C for 24 h. O-Deacetylation of the polysaccharide was performed with 12.5% ammonia (16 h, 37 °C) followed by GPC on a TSK-40 column (70×2.0 cm) in aq 1% HOAc.

3.2. Sugar analysis

Monosaccharides were analyzed as their alditol acetates by GC after hydrolysis and derivatization, essentially as described by Englyst et al.[25]

The absolute configurations of the monosaccharides and the D sugar standards were determined by GC of the alditol derivatives of the octan-2-ol glycosides prepared according to the method of Leontein et al.[26]

Gas chromatography was performed on a Hewlett–Packard model 6890 instrument equipped with a FID detector, using an Alltech DB-225 capillary column (15 m×0.25 mm) programmed from 180 to 220 °C at 4 °C/min and held at 220 °C for 30 min. Two microlitres of CH_2Cl_2 solution of each sample was injected, and helium was used as a carrier gas.

3.3. Smith degradation

The O-deacetylated polysaccharide (**PS I**) (12.8 mg) was oxidized with 0.05 M $NaIO_4$ in the dark for 72 h at 4 °C. After adding an excess of ethylene glycol, $NaBH_4$ reduction was carried out followed by neutralization with AcOH and desalting on a column (80×1.6 cm) of TSK HW-40 (S) in 1% AcOH. The product was then hydrolyzed with aq 2% AcOH for 2 h at 100 °C and fractionated on TSK HW-40 (S) in 1% AcOH to give **PS II** (5.8 mg).

3.4. NMR spectroscopy

Samples were deuterium-exchanged by freeze-drying three times from D_2O and then examined as solutions in 99.97% D_2O. All spectra were recorded at a temperature of 60 °C, using internal acetone (δ_H 2.225, δ_C 31.45) as a reference, on a Bruker DRX-500 MHz spectrometer equipped with an SGI INDY workstation or on a Jeol Lambda 400 MHz spectrometer equipped with a DEC AXP 300 computer workstation, where data were acquired and performed using XWINNMR 1.2 version software. In the TOCSY and HMQC-TOCSY experiments the duration of the spin-lock was 200 ms. The HMBC experiment was optimized for a coupling constant of 8 Hz. A mixing time of 100 ms was used in the 2D ROESY experiments. Other 2D parameters were essentially the same as previously described.[27]

Acknowledgements

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