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Structure of the capsular antigen of *Escherichia coli* O8 : K50 : H⁻

M. Ruth Grue, Haralambos Parolis *, Lesley A.S. Parolis

School of Pharmaceutical Sciences, Rhodes University, Grahamstown 6140, South Africa

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Abstract

The primary structure of the acidic capsular antigen of *Escherichia coli* O8 : K50 : H^- was shown by glycose analysis, methylation analysis, and one- and two-dimensional ^1H and ^{13}C NMR spectroscopy to be composed of repeating linear tetrasaccharide units having the structure:

→ 3)-β-L-Rha*p*-(1 → 4)-α-D-Glc*p*NAc-(1 → 2)-β-D-Man*p*-(1 → 3)-α-D-Man*p*NAc-(1 →
4 \ / 6
Pyr (*S*)

1. Introduction

E. coli capsular (K) antigens are generally acidic polysaccharides and have been subdivided into two groups on the basis of their physical, chemical, and microbiological characteristics [1]. Structures have been reported for 60 of the 74 known *E. coli* polysaccharide capsular antigens. The *E. coli* K50 capsular antigen is co-expressed with O-group 8 [2] and contains amino sugars, thereby placing it in the Group I polysaccharides [1]. *E. coli* K50 has been implicated in appendicitis peritonitis [2]. It is interesting to note that the polysaccharide has no uronic acid, its only acidic function being pyruvic acetal. To date, only two other polysaccharides of this type have been isolated, from *E. coli* K37 [3] and K47 [4].

* Corresponding author.

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2. Results and discussion

Isolation, composition, and linkage analysis of the capsular antigen.—*E. coli* O8 : K50 : H^- bacteria were grown on Mueller–Hinton agar, and the acidic capsular polysaccharide (**PS**) was isolated and purified by precipitation with cetyltrimethylammonium bromide. The polysaccharide was further purified by ion-exchange chromatography on DEAE-Sepharose CL-6B. The average molecular weight of the **PS** was found to be M_r 2.63×10^6 on a dextran-calibrated column of Sephacryl S-500. Hydrolysis of the **PS** followed by GLC–MS examination of the derived alditol acetates showed that Rha, Man, GlcN, and ManN were present. Methanolysis of the **PS**, treatment of the products with $NaBH_4$ to effect carboxyl reduction, followed by hydrolysis, and GLC–MS examination of the derived alditol acetates gave the same result as before, indicating the absence of a uronic acid in the polymer. GLC analysis of the derived acetylated (−)-2-octyl glycosides [6] of the **PS** showed the configuration of Rha to be L and that of the other constituent sugars to be D.

The ^1H NMR spectrum of the **PS** in D_2O contained H-1 signals at δ 5.12 ($J_{1,2}$ 3.7 Hz), 5.05, and 4.89 (2 H), signals for the methyl protons for two NAc groups at δ 2.07 and 2.08, a signal for H-6 of a deoxy sugar at δ 1.33 ($J_{5,6}$ 5.4 Hz), and a signal for the methyl protons of the pyruvic acetal at δ 1.56. The ^{13}C NMR data complemented the ^1H NMR results and confirmed a pyruvated tetrasaccharide repeating unit for the **PS**, with signals at 96.18, 97.86, 99.43, 100.76, and 101.57 ppm in the anomeric region (95–105 ppm), one being that of the acetal carbon of the pyruvic group. Signals for carbonyl carbons occurred at 175.46, 174.89, and 174.07 ppm, while signals at 54.96 and 49.79 ppm indicated the presence of two C–N bonds. The origins of the additional minor signals in the ^{13}C spectrum, indicating heterogeneity, were not investigated further. The ^{13}C and ^1H NMR spectra are shown in Fig. 1.

Methylation of the polysaccharide followed by GLC and GLC–MS analysis of the permethylated alditol acetates derived from the products of an acid hydrolysate showed the presence of 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose, 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)mannose, 2,4-di-*O*-methylrhamnose, and 3-*O*-methylmannose. These results indicated the presence of 4-substituted GlcNAc, 3-substituted ManNAc, 3-substituted Rha, and 3,4,6-substituted Man in a linear tetrasaccharide repeating unit with the pyruvic group attached to the Man residue.

2D NMR studies of the E. coli *K50 polysaccharide.*—The sequence of the residues in the repeating unit of the **PS** was established by 2D NMR experiments, which also confirmed the glycosylation sites in the polysaccharide. Carbon and proton resonances were established from COSY [6], 2D Homonuclear Hartmann–Hahn (HOHAHA) [7], HMQC [8], and HMQC-TOCSY [9] experiments. The residues in the repeating unit were labelled **a**–**d** in order of decreasing chemical shift of their anomeric protons.

Residue **a** *[→4)-D-Glc*p*NAc].*—The H-1/H-2 cross-peak was the only one clearly identifiable for this residue in the COSY spectrum. The resonances for C-1

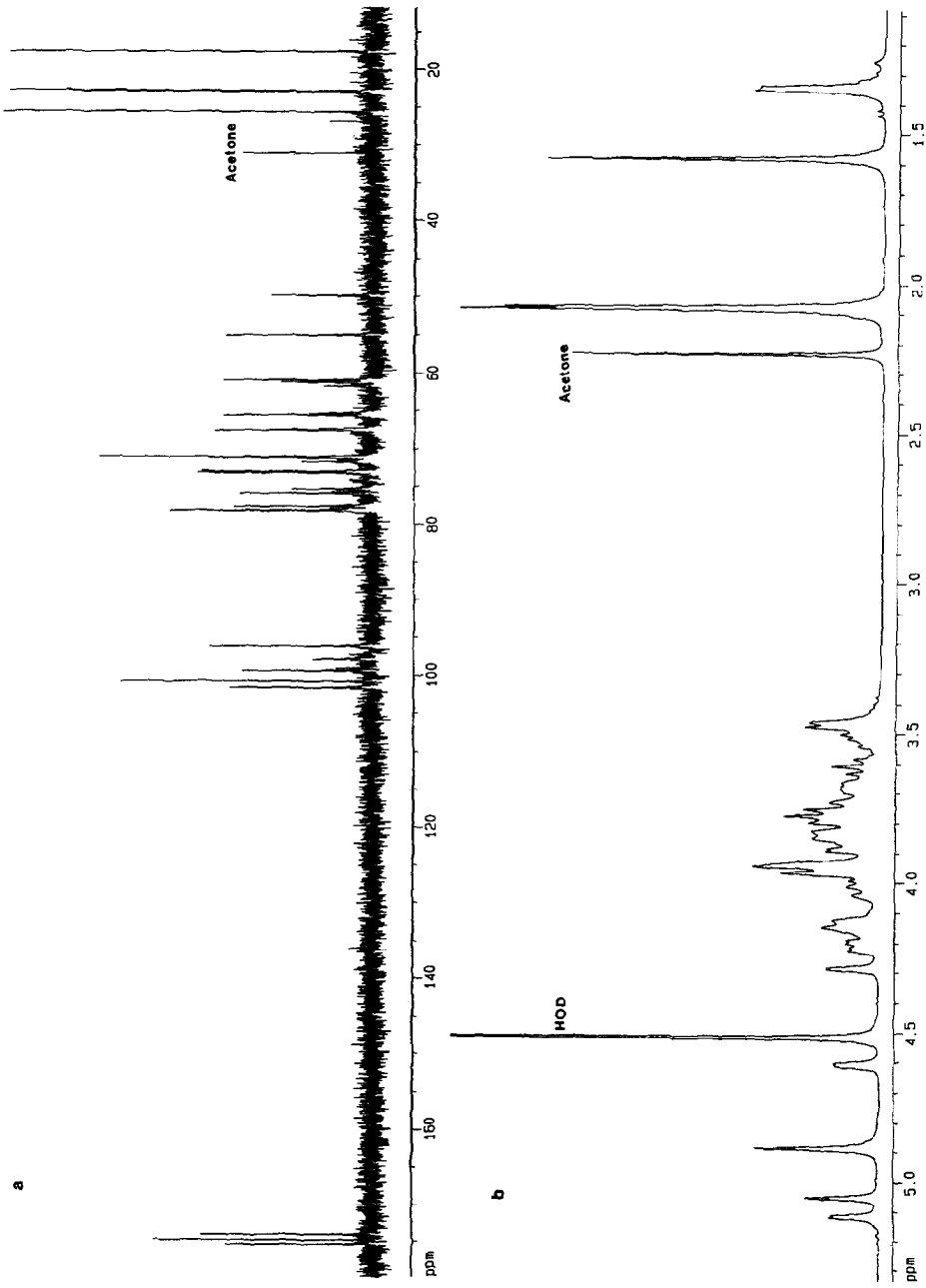

Fig. 1. (a) ^{13}C and (b) ^{1}H NMR spectra for K50 PS at 50°C.

Table 1
NMR data [a] for *E. coli* K50 polysaccharide

Residue		Proton or Carbon											
		1	2	3	4	5	6a	6b	NAc CH_3	NAc C=O	Pyr CH_3	Pyr COOH	Pyr C-2
a	H	5.12	3.87	3.97	3.76	4.14	4.03	3.72	2.07				
→ 4)-α-D-Glc*p*NAc	C	99.43	54.96	71.17	78.16	70.97	61.30		22.84 [b]	175.46			
b	H	5.05	4.61	4.21	3.61	3.95	3.84	3.84	2.08				
→ 3)-α-D-Man*p*NAc	C	96.18	49.79	75.89	65.55	73.22	61.00		22.99 [b]	174.89			
c	H	4.89	4.29	3.67	3.47	3.45	1.33						
→ 3)-β-D-Rha*p*	C	101.57	67.52	77.60	70.97	73.02	17.61						
d	H	4.89	3.93	3.96	3.78	3.52	4.15	3.80					
→ 2)-β-D-Man*p*	C	97.86	78.16	71.69	75.34	67.50	65.32						
4,6 Pyr													
Pyr	H										1.56		
	C										25.55	174.07	100.76

[a] Chemical shifts in ppm with acetone as internal standard, δ 2.23 and 31.07 ppm for ^{1}H and ^{13}C, respectively.
[b] These values are interchangeable.

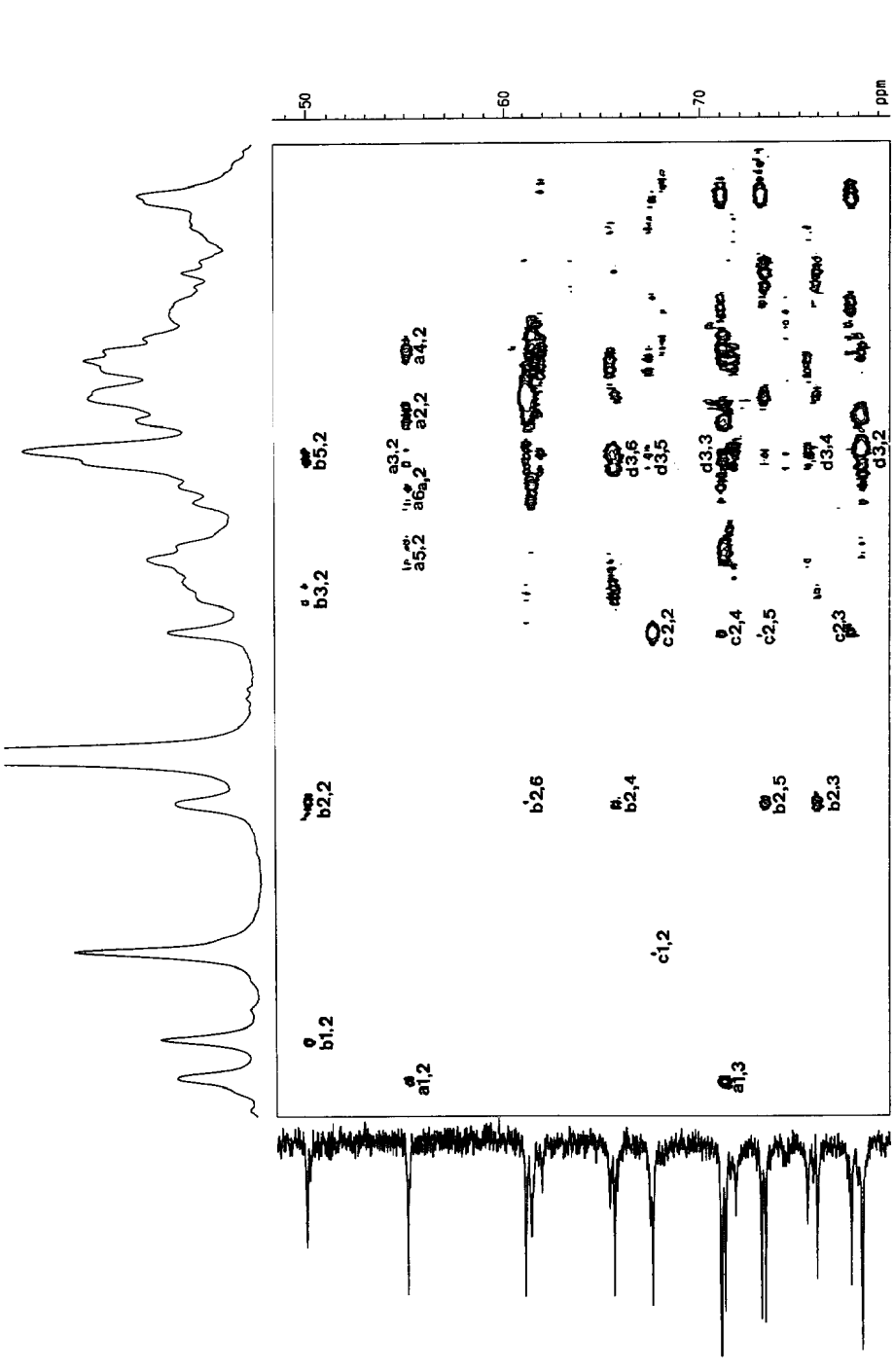

Fig. 2. HMQC-TOCSY contour plot for K50 **PS** showing the region δ 3.4–5.2 for f_2 and 49–80 ppm for f_1; **a**1,3 connotes the cross-peak between H-1 and C-3 of residue **a**, etc. The 1D ^1H and ^{13}C spectra are projected along the f_2 and f_1 axes, respectively. (See Table 1 for identification of **a**–**d**.)

and C-2 of this residue could be established from the H-1/C-1 and H-1/C-2 cross-peaks, respectively, in the HMQC-TOCSY experiment. The signal for H-2 could then be identified by correlation with the C-2 resonance from the HMQC spectrum, allowing assignment of the H-2/H-3 cross-peak in the COSY spectrum. The remaining proton resonances could then be assigned from the COSY and HOHAHA spectra. Carbon resonances were assigned by comparing the ^{1}H assignments with the ^{1}H–^{13}C correlation data from the HMQC experiment.

Residue **b** *[→3)-D-Man*p*NAc].*—The ^{1}H resonances for residue **b** were traced readily via their cross-peaks in the COSY and HOHAHA spectra. Magnetism relayed well through this spin system in the HOHAHA experiment and all cross-peaks were clearly visible. The ^{13}C resonances were assigned from the HMQC spectrum as for residue **a**.

Residue **c** *[→3)-L-Rha*p*].*—Since the H-1 resonances of residues **c** and **d** exactly coincide (Table 1), the H-6 resonance at δ 1.33 was used as the starting point for tracing the ^{1}H resonances for residue **c** in the COSY and HOHAHA spectra. The ^{13}C resonances for residue **c**, with the exception of C-1, were then assigned from the ^{1}H–^{13}C correlation data obtained in the HMQC experiment.

Residue **d** *[→2,4,6)-D-Man*p*].*—The ^{1}H resonances for residue **d** were traced via their cross-peaks in the COSY spectrum. The connectivities were confirmed from the H-3 track in the HMQC-TOCSY spectrum (Fig. 2), which showed relays to all the carbon signals for residue **d**, with the exception of C-1, and from the C-3 track which showed relays to H-2/6. The ^{13}C resonances thus obtained were confirmed from the HMQC spectrum.

The anomeric configurations were assigned by measuring the C-1/H-1 coupling constants obtained from a proton-coupled HMQC experiment. The values obtained were 175.7 Hz for residue **a**, 173.3 Hz for residue **b**, 163.6 Hz for residue **c**, and 162.8 Hz for residue **d**, indicating α configurations for residues **a** and **b**, and β configurations for residues **c** and **d**.

The signal at 100.76 ppm in the ^{13}C spectrum, which was absent from the HMQC spectrum, could now be assigned to the acetal carbon of the pyruvic group. Comparison of the chemical shift data for residues **a**–**d** in Table 1 with those reported for methyl glycosides [10–12] permitted identification of residue **a** as 4-substituted α-D-GlcNAc, residue **b** as 3-substituted α-D-ManNAc, residue **c** as 3-substituted β-D-Rha, and residue **d** as 2,4,6-substituted β-D-Man. These data are in agreement with the methylation results for **PS**.

The sequence of the residues in the repeating unit was established by a heteronuclear multiple bond correlation (HMBC) experiment [13]. The first easily identifiable correlation was between C-1 of GlcNAc and H-2 of Man. A second correlation was visible between H-4 of GlcNAc and C-1 of either residue **c** or **d**. Since a link between GlcNAc and Man (residue **d**) had already been established, it was clear that this carbon signal belonged to residue **c**, thus allowing assignment of the C-1 resonances for residues **c** and **d**. Other relevant correlations identified were: H-1 of ManNAc to C-3 of Rha, H-1 of Man to C-3 of ManNAc, C-1 of Man to H-3 of ManNAc, and H-6 and H-4 of Man to the acetal carbon of the pyruvic group. It was also possible to assign the carbonyl signals to appropriate residues,

Table 2
Two- and three-bond $^{1}H-^{13}C$ correlations for the **PS**

Residue		Proton	Correlation to
a	→ 4)-α-D-Glc*p*NAc	H-1	78.16 (**d**; C-2), 71.17 (**a**; C-3), 70.97 (**a**; C-5)
		H-2	99.43 (**a**; C-1), 175.46 (NAc C=O)
		H-3	54.96 (**a**; C-2)
		H-4	101.57 (**c**; C-1), 70.97 (**a**; C-5)
b	→ 3)-α-D-Man*p*NAc	H-1	77.60 (**c**; C-3), 75.89 (**b**; C-3), 73.22 (**b**; C-5)
		H-2	96.18 (**b**; C-1), 75.89 (**b**; C-3), 65.55 (**b**; C-4), 174.89 (NAc C=O)
		H-3	97.86 (**d**; C-1), 65.55 (**b**; C-4)
c	→ 3)-β-D-Rha*p*	H-1	78.16 (**a**; C-4), 77.60 (**c**; C-3), 67.52 (**c**; C-2)
		H-2	77.60 (**c**; C-3), 70.97 (**c**; C-4)
		H-4	73.02 (**c**; C-5)
d	→ 2)-β-D-Man*p*	H-1	75.89 (**b**; C-3), 71.69 (**d**; C-3), 67.50 (**d**; C-5)
	4 6	H-2	99.43 (**a**; C-1), 71.69 (**d**; C-3), 75.34 (**d**; C-4)
	Pyr	H-4	100.76 (Pyr; C-2), 78.16 (**d**; C-2)
		H-6a,b	100.76 (Pyr; C-2)
Pyr		CH_3	174.07 (Pyr; C=O)

since correlations were visible from H-2 of GlcNAc to the carbonyl resonance at 175.46 ppm, from H-2 of ManNAc to the carbonyl resonance at 174.89 ppm, and from the methyl protons of the pyruvic group to the carbonyl signal at 174.07 ppm. HMBC correlations are listed in Table 2.

The chiral carbon of the pyruvic acetal was assigned the *S* configuration based on the ^{1}H and ^{13}C chemical shifts of its methyl group [14].

The combined chemical and NMR data permit the structure of the tetrasaccharide repeating unit of the *E. coli* K50 capsular polysaccharide to be written as:

c **a** **d** **b**
3)-β-L-Rha*p*-(1 → 4)-α-D-Glc*p*NAc-(1 → 2)-β-D-Man*p*(1 → 3)-α-D-Man*p*NAc-(1 →
4 6
Pyr (*S*)

This is only the third *E. coli* capsular polysaccharide found to contain a pyruvic acetal as the sole acidic function [3,4]. Four capsular polysaccharides having phosphate groups as their sole acidic function have also been reported [15–18]. In addition, it is only the second capsular polysaccharide in the *E. coli* series to date found to contain a ManNAc residue, the other being *E. coli* K84 [19].

3. Experimental

General methods.—Analytical GLC was performed with a Hewlett–Packard 5890A gas chromatograph, fitted with flame-ionization detectors and a 3392A recording integrator, with helium as carrier gas. A J&W Scientific fused-silica DB-17 bonded-phase capillary column (30 m × 0.25 mm; film thickness, 0.25 μm) was used for separating partially methylated alditol acetates (programme I), and

alditol acetates and acetylated octyl glycosides (programme II). A J&W Scientific fused-silica DB-Wax bonded-phase capillary column (30 m × 0.25 mm; film thickness, 0.15 μm) was used for separating alditol acetates of ManNAc, GlcNAc, and GalNAc (130 kPa, 240°C isothermal). A J&W Scientific DB-225 bonded-phase capillary column (30 m × 0.25 mm; film thickness, 0.25 μm) was also used for separating acetylated octyl glycosides (130 kPa, 240°C isothermal). The temperature programmes used were: I, 180°C for 2 min, then 3°C min^{-1} to 240°C, 100 kPa; II, 180°C for 2 min, then 2°C min^{-1} to 240°C, 100 kPa. The identities of all derivatives were determined by comparison with authentic standards and confirmed by GLC–MS on a Hewlett–Packard 5988A instrument, using the appropriate column. Spectra were recorded at 70 eV and an ion-source temperature of 200°C. Gel permeation chromatography was performed on a dextran-calibrated column (1.6 × 65 cm) of Sephacryl S500, using 0.1 M NaOAc buffer (pH 5.00) as eluent. Gel permeation–ion-exchange chromatography was performed on a DEAE-Sepharose CL-6B column (2.6 × 27 cm), using gradient elution with 0–1 M NaCl in 0.01 M Tris-HCl (pH 8.50). Fractions were assayed for carbohydrate by using the phenol–H_2SO_4 reagent [20].

Polysaccharide samples were hydrolysed with 4 M CF_3CO_2H for 1 h at 125°C. Alditol acetates were prepared by reduction of the products in aqueous solutions of hydrolysates with $NaBH_4$ for 1 h followed by acetylation with 2:1 Ac_2O–pyridine for 1 h at 100°C. Samples were methanolysed by refluxing with methanolic 3% HCl for 16 h. Native and methylated polysaccharides were carboxyl-reduced with $NaBH_4$ in dry MeOH after methanolysis. Methylations were carried out on the acid form of the polysaccharide, using potassium dimsyl [21] and MeI in Me_2SO, followed by a 48-h Kuhn methylation in DMF with Ag_2O and MeI [22].

Preparation of the K50 polysaccharide.—An authentic culture of *E. coli* O8:K50:H^- was obtained from Dr. I. Ørskov (Copenhagen) and propagated on Mueller–Hinton agar (9 trays, 30 × 60 cm; each inoculated with 10 mL of liquid culture). The capsular polysaccharide was extracted with aq 1% phenol, separated from the cells by ultracentrifugation, and purified by precipitation with cetyltrimethylammonium bromide, followed by gel permeation–ion-exchange chromatography on DEAE-Sepharose CL-6B (467 mg of capsular polysaccharide obtained).

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying several times from D_2O and then examined as solutions in 99.99% D_2O containing a trace of acetone as internal standard (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). Spectra were recorded at 50°C on a Bruker AMX-400 spectrometer equipped with an X32 computer. The parameters used for 2D experiments were as follows. COSY [256 × 2048 data matrix, zero-filled to 1024 data points in t_1; 80 scans per t_1 value; spectral width, 2007.9 Hz; recycle delay, 1.0 s; unshifted sine-bell filtering in t_1 and t_2]. HOHAHA [256 × 4096 data matrix, zero-filled to 1024 data points in t_1; 96 scans per t_1 value; spectral width, 2007.9 Hz; recycle delay, 1.0 s; mixing time, 89 ms; shifted sine-squared filtering in t_1 and t_2]. HMQC, HMQC-TOCSY, and HMBC [512 × 4096 data matrix, zero-filled to 1024 data points in t_1; 52, 56, or 64 scans per t_1 value; recycle delay, 1.0 s; fixed delay, 3.45 ms; mixing time, 89 ms

(HMQC-TOCSY); spectral width in t_1, 11 068 Hz (HMQC and HMQC-TOCSY) and 20 828 Hz (HMBC), and in t_2, 2008.03 Hz (HMQC and HMQC-TOCSY) and 2016.13 Hz (HMBC); shifted sine-squared filtering in t_1 and t_2].

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