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Escherichia coli O9:K38 capsular antigen: another ribofuranose-containing glycan

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ABSTRACT

The primary structure of the *O*-deacetylated acidic capsular antigen of *Escherichia coli* O9:K38 was shown by glycose analysis, methylation analysis, and one- and two-dimensional ^{1}H- and ^{13}C-n.m.r. spectroscopy to be composed of repeating linear pentasaccharide units having the structure:

→4)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→2)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1-

This is only the second *E. coli* capsular polysaccharide, belonging to the Group I antigens, which contains ribofuranose in its repeating unit.

INTRODUCTION

The structures of 51 *Escherichia coli* capsular antigens have been reported; of these, K6[1], K13[1], K16[2], K18–20[1], K22[1], K23[1], K57[3], K74[1], K95[1], and K100[1] contain ribofuranose in their repeating units. Serotype K57[3] is the only antigen among these which belongs to the Group I antigens[4], the rest belong to the Group II antigens[4]. The former antigens tend to be heat stable and are most often co-expressed with O8 and O9 antigens; the latter have lower molecular weights and are thermolabile. The capsular antigen of *E. coli* O9:K38, whose structure we now describe, is only the second Group I antigen which has been found to contain ribose.

RESULTS AND DISCUSSION

Isolation, composition, and linkage analysis of the capsular antigen.—E. coli O9:K38 bacteria were grown on Mueller–Hinton agar, and the acidic capsular polysaccharide was isolated and purified by precipitation with cetyltrimethylammonium bromide. Further purification of the polysaccharide was effected by treatment with RNAse and DNAse followed by gel-permeation chromatography on Sephacryl S400-HR. The purified polysaccharide showed a broad distribution of molecular weights in gel-permeation chromatography on Sephacryl S500 with an average M_r at 10^7.

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G.l.c. examination of the derived alditol acetates showed the polysaccharide to be composed of Rib, Gal, GalA, GalN, and GlcN. The constituent sugars were shown to have the D configuration by g.l.c. of the derived acetylated (−)-2-octyl glycosides[5]. The ^{1}H-n.m.r. spectrum of the purified polysaccharide was complex because of the presence of numerous partial resonances resulting from variable *O*-acetylation. Therefore, the polymer was treated with base and all subsequent n.m.r. experiments were performed on the *O*-deacetylated polysaccharide. The ^{1}H-n.m.r. spectrum (Fig. 1) contained H-1 signals at δ 5.40 ($^3J < 1$ Hz), 5.31 (^3J 3.5 Hz), 4.97 (^3J 3.0 Hz), 4.63 (^3J 8.0 Hz), and 4.48 (^3J 7.8 Hz), and a signal for the methyl protons of two NAc groups at δ 2.05 (6 H). The ^{13}C-n.m.r. data complemented the ^{1}H-n.m.r. results and confirmed the pentasaccharide repeating unit for the polysaccharide, with signals for C-1 at 107.84, 105.74, 102.24, 98.92, and 98.39 p.p.m., and a signal at 23.00 p.p.m. for the methyl carbons of the NAc groups. In addition, signals for carbonyl carbons occurred at 175.64, 175.22, and 172.66 p.p.m. The C-1 signal at 107.84 p.p.m. indicated the presence of a furanoside.

Methylation analysis of the polysaccharide gave 3,5-di-*O*-methylribose, 2,3,6-tri-*O*-methylgalactose, 2-deoxy-3,6-di-*O*-methyl-2-methylacetamidoglucose, 2-deoxy-4,6-di-*O*-methyl-2-methylacetamidogalactose, and 2,3-di-*O*-methylgalactose (after carboxyl reduction). These results accord with a linear pentasaccharide repeating unit for the polysaccharide.

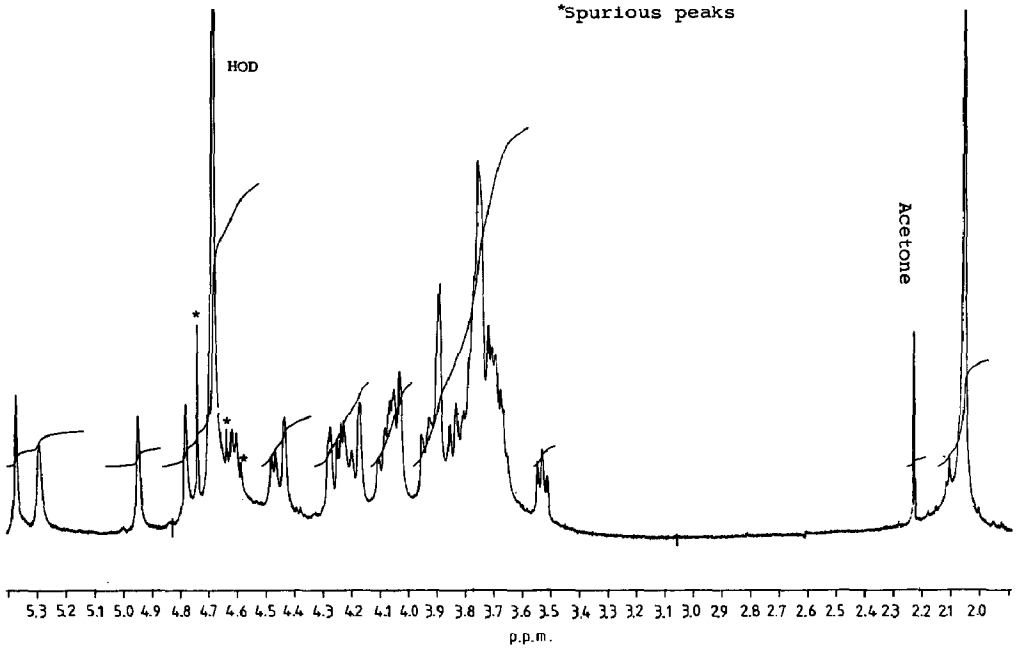

Fig. 1. 500-MHz ^{1}H-n.m.r. spectrum of the *E. coli* K38 capsular polysaccharide at 33°.

2D-N.m.r. studies of the E. coli *K38 polysaccharide.* — The sequence of the residues in the repeating unit was established by 2D-n.m.r. experiments, which also confirmed the glycosylation sites in the polysaccharide. Most of the ^1H resonances of the five sugar residues in the repeating unit were established from COSY[6] and one- and two-step relay COSY experiments[7]. The residues in the repeating unit were labelled **a**–**e** in order of decreasing chemical shift of their anomeric protons (Fig. 2). All the ^1H resonances of residues **a**, **b**, and **c**, together with H-1 to H-4 of residues **d** and **e**, were assigned by following the cross-peaks in the contour maps of the COSY (Fig. 2) and relay COSY experiments (Fig. 3 shows the one-step relay COSY contour map). The resonances for H-5 of residues **d** and **e** were assigned from the intramolecular n.O.e.'s observed between H-1 and H-3, and H-1 and H-5, of residues **d** and **e** in the NOESY[8] experiment (see below). Cross-peaks between H-5 and H-6a and H-6b for residues **d** and **e** could not be distinguished because of the proximity of these resonances.

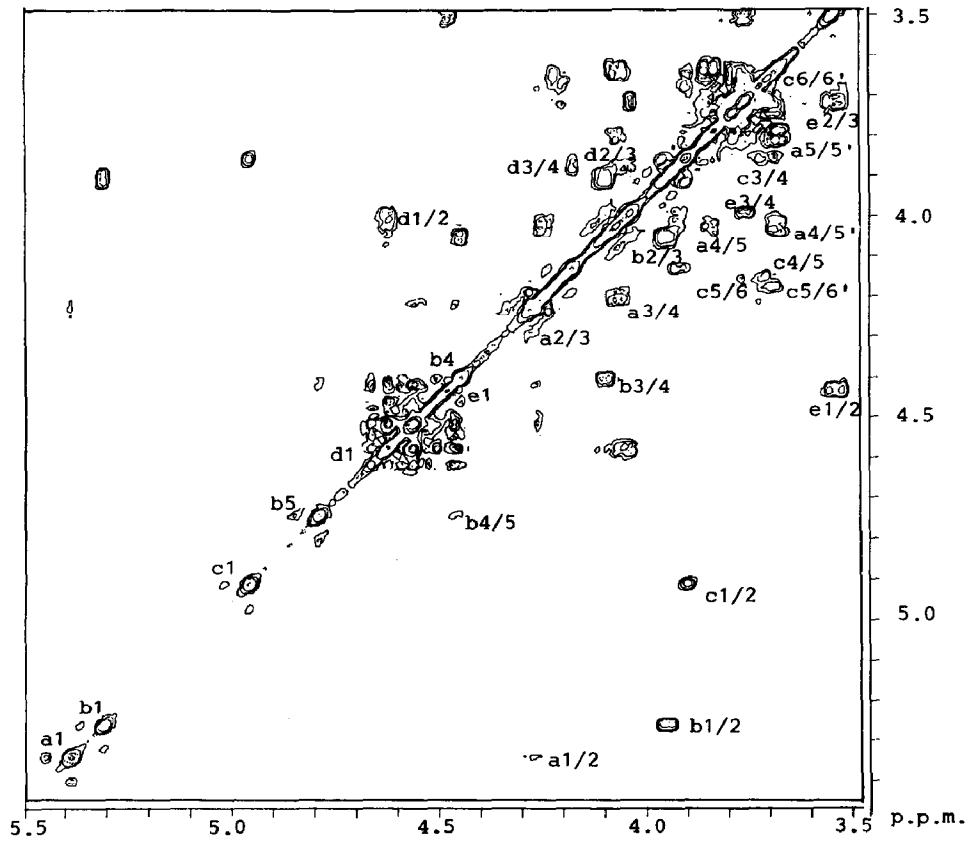

Fig. 2. COSY contour plot of the region δ 3.5–5.5 for the *E. coli* K38 capsular polysaccharide. The ^1H resonances of the *J*-coupled spin systems are labelled **a**–**e**; **a1** connotes H-1 of residue **a**, and **a1/2** connotes the cross-peak between H-1 and H-2 of residue **a**, *etc.*

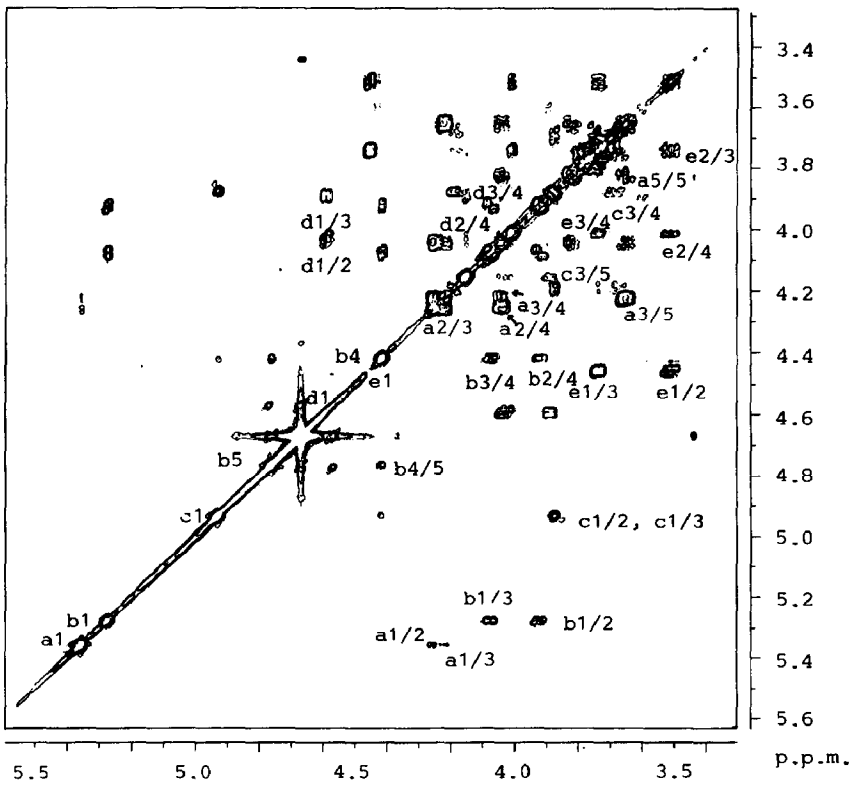

Fig. 3. One-step relay COSY contour plot of the region δ 3.3–5.6 for the *E. coli* K38 capsular polysaccharide.

The ^1H resonances for each residue were then compared with data obtained from a ^1H–^{13}C shift-correlated experiment[9] (HETCOR) (Fig. 4 and Table I). In this way, all the ^{13}C resonances of residues **a**–**c** and C-1 to C-5 of residues **d** and **e** could be assigned. The two remaining sets of $^{13}C/^1H$ resonances from the HETCOR experiment at 61.67 p.p.m./δ 3.81 and 62.02 p.p.m./δ 3.77 were assigned to C-6/H-6a and H-6b of residues **d** and **e**, respectively (see below for a discussion of these assignments).

Comparison of the ^1H- and ^{13}C-n.m.r. data for residues **a**–**e** with literature values for methyl glycosides[10–12] permitted the residues in the repeating unit to be identified, as indicated in Table I, and their linkage positions to be established. In agreement with the results of methylation analysis, C-2 of **a**, C-4 of **b**, C-4 of **c**, C-3 of **d**, and C-4 of **e** experienced significant deshielding.

The sequence of the residues **a**–**e** in the repeating unit was established by a NOESY[8] experiment. The inter- and intra-residue n.O.e. contacts are listed in Table II. The α-linked pyranoside residues **b** and **c** showed characteristic intramolecular n.O.e.'s between H-1 and H-2 while the β-linked pyranoside residues showed the expected n.O.e.'s from H-1 to H-3 and H-5. Residue **b** also showed intraresidue n.O.e.'s between

TABLE I

N.m.r. data for *E. coli* K38 polysaccharide [a]

Atom	*Residue*							
	a →2)-β-Rib		**b** →4)-α-GalA		**c** →4)-α-GlcNAc	**d** →3)-β-GalNAc	**e** →4)-β-Gal	
H-1	5.40	(5.39)[b]	5.31	(5.24)	4.97	4.63	4.48	(4.46)
C-1	107.84	(107.95)	98.39	(98.72)	98.92	102.24	105.74	(104.25)
H-2	4.29	(4.28)	3.94	(3.93)	3.92	4.07	3.54	(3.52)
C-2	80.73	(80.74)	68.32	(68.90)	53.98	52.23	71.46	(71.56)
H-3	4.27	(4.24)	4.12	(4.10)	3.92	3.92	3.79	(3.79)
C-3	70.76	(70.96)	69.02	(69.68)	70.07	80.56	73.56	(73.69)
H-4	4.09	(4.07)	4.44	(4.38)	3.75	4.20	4.05	(4.05)
C-4	83.35	(83.89)	78.98	(80.93)	79.68	68.67	77.23	(77.22)
H-5a	3.69	(3.67)	4.80	(4.51)	4.22	3.73	3.77	(3.78)
H-5b	3.88	(3.83)						
C-5	63.25	(63.46)	70.94	(72.28)	71.11	75.14	75.66	(75.19)
H-6a					3.71	3.81	3.77	(3.78)
H-6b					3.81	3.81	3.77	(3.78)
C-6					60.27	61.67	62.02	(62.01)

[a]Chemical shifts with acetone as internal reference, δ 2.23 and 31.07 p.p.m. for ^{1}H and ^{13}C, respectively. [b]Values in parentheses are those reported[3] for *E. coli* K57 capsular polysaccharide.

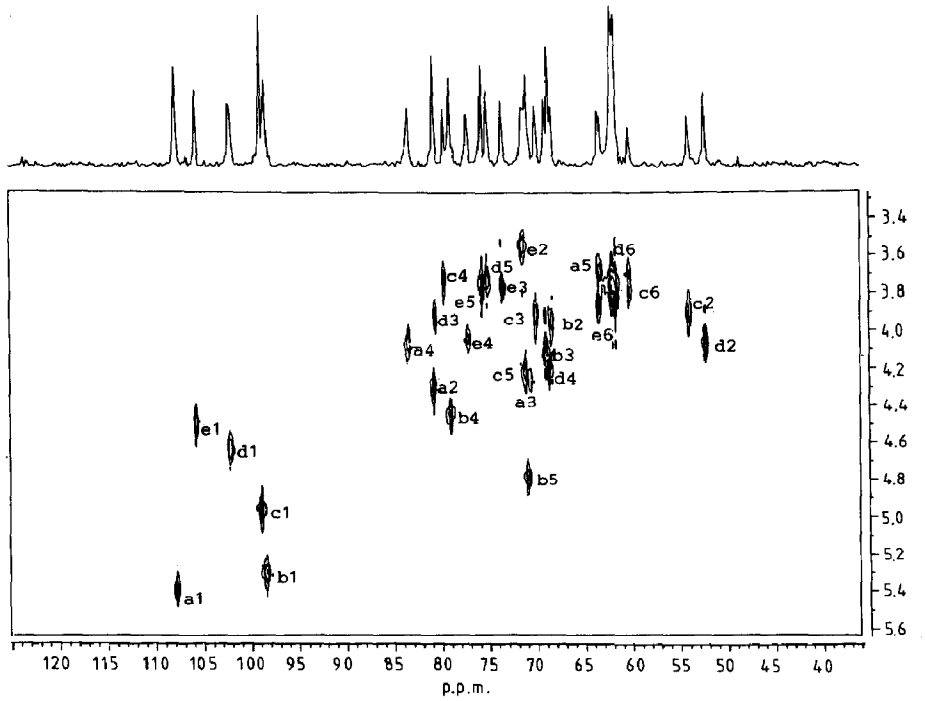

Fig. 4. ^{1}H–^{13}C shift correlation map of the spectral region f_2 125–36 p.p.m., and f_1 5.6–3.3 p.p.m. The ^{13}C projection is displayed along the f_2 axis. The f_1 axis represents the ^{1}H resonances. The correlated resonances are labelled **a**–**e**.

H-3 and H-4, H-4 and H-5, and H-3 and H-5. These contacts are further confirmation that residue **b** has the *galacto* configuration. Intense interresidue n.O.e.'s between the anomeric protons and the protons across the glycosidic linkages were observed for residues **a**, **b**, and **c**, while less intense n.O.e.'s were observed for **d** and **e**. These interresidue n.O.e.'s establish the sequence

→4)**c**(1→4)**b**(1→2)**a**(1→4)**e**(1→3)**d**(1→

for the repeating unit of the polysaccharide. An intense interresidue n.O.e. was also noted between the anomeric protons of residues **a** and **b**. A similar n.O.e. was reported[3] to occur between these residues in the capsular polysaccharide of *E. coli* K57 and has also been observed[13–15] for other α-D-hexopyranose residues glycosylated at position 2. A further, but less intense, n.O.e. was noted between the anomeric proton of residue **a** and a proton resonating at δ 3.77, which could either be H-5 or H-6a and H-6b of residue **e**. Inspection of a molecular model of the repeating unit of the polysaccharide suggests that the n.O.e. effect from the anomeric proton of residue **a** is to H-6a and/or H-6b rather than to H-5 of residue **e**. This unexpected n.O.e. permitted the assignment of the set of $^{13}C/^{1}H$ resonances at 62.02 p.p.m./δ 3.77 to C-6 and H-6a and H-6b of residue **e**, and thus the set at 61.67 p.p.m./δ 3.81 was assigned to C-6 and H-6a and H-6b of residue **d**.

TABLE II

N.O.e. contacts for the K38 polysaccharide

Proton	*N.O.e. contact to*
a, H-1	5.31 (**b**, H-1), 4.29 (**a**, H-2), 4.05 (**e**, H-4), 3.77 (**e**, H-6a, 6b)
b, H-1	4.29 (**a**, H-2), 3.94 (**b**, H-2)
b, H-5	4.44 (**b**, H-4), 4.12 (**b**, H-3)
b, H-4	4.12, (**b**, H-3)
c, H-1	4.44 (**b**, H-4), 3.92 (**c**, H-2)
d, H-1	3.92 (**d**, H-3), 3.73 (**d**, H-5), 3.75 (**c**, H-4)
e, H-1	3.79 (**e**, H-3), 3.77 (**e**, H-5), 3.92 (**d**, H-3)

The combined chemical and n.m.r. data permit the structure of the pentasaccharide repeating unit of *E. coli* K38 to be written as

c **b** **a** **e** **d**

→4)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→2)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→

The above repeating unit closely resembles that determined[3] for the capsular polysaccharide of *E. coli* K57, which is shown below.

→3)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→2)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→

Comparison of the n.m.r. data for the two repeating units reveals that the ^{1}H and ^{13}C chemical shifts for the α-GalA, β-Rib, and β-Gal residues are almost identical (the values in parentheses in Table I are those previously reported for the K57 repeating unit). The principal deviations noted are for H-4/C-4 and H-5/C-5 of the α-GalA. These deviations are readily accounted for by the different pH conditions under which the two sets of n.m.r. data were acquired; the sodium salt of the K57 capsular polysaccharide was used, whereas the acid form of the K38 polysaccharide was employed.

EXPERIMENTAL

General methods. — Analytical g.l.c. was performed with a Hewlett–Packard 5890A gas chromatograph, fitted with flame-ionisation detectors and a 3392A recording integrator, with helium as carrier gas. A J and W Scientific fused-silica DB-17 bonded-phase capillary column (30 m × 0.25 mm) having a film thickness of 0.25 μm was used for separating partially methylated alditol acetates (programme I), alditol acetates (programme II), and octyl glycoside acetates (programme III). A J and W Scientific fused-silica DB-Wax bonded-phase capillary column (30 m × 0.25 mm) having a film thickness of 0.15 μm was used for separating alditol acetates of ManNAc, GlcNAc, and GalNAc (240° isothermal). The temperature programmes used were I, 180° to 240° at 3°.min^{-1}; II, 180° for 1 min, then 3°.min^{-1} to 250°; and III, 180° for 2 min,

then 3°.min^{-1} to 240°. The identities of all derivatives were confirmed by g.l.c.–m.s. on a Hewlett–Packard 5988A instrument, using the appropriate column. G.p.c. of K38 polysaccharide was performed on dextran-calibrated columns (1.6 × 65 cm) of Sephacryl S500 and Sephacryl S400 HR, using 0.1M sodium acetate buffer (pH 5.00) as eluent. Semi-preparative g.p.c. was performed on a column (2.6 × 65 cm) of Sephacryl S400 HR, using the same eluent.

Samples were hydrolysed with 4M trifluoroacetic acid for 1 h at 125°. Alditol acetates were prepared by reduction of the products in aqueous solutions of hydrolysates with sodium borohydride followed by acetylation with 1:1 acetic anhydride–pyridine for 1 h at 100°. Samples were methanolysed by treatment with refluxing methanolic 3% hydrogen chloride for 16 h. Native and methylated polysaccharides were carboxyl-reduced with $NaBH_4$ in dry methanol after methanolysis. Methylations were carried out on the acid form of the polysaccharide, using potassium dimsyl[16] and methyl iodide in dimethyl sulphoxide.

Preparation of the K38 polysaccharide. — An authentic culture of *E. coli* O9:K38 was obtained from Dr. I. Ørskov (Copenhagen), and the bacteria were grown on Mueller–Hinton agar. The capsular polysaccharide was separated from the cells by ultracentrifugation and isolated by precipitation with cetyltrimethylammonium bromide. The polysaccharide was purified by treatment with RNAse and DNAse followed by g.p.c. on Sephacryl S400 HR. *O*-Deacetylation was effected by heating the polysaccharide for 2 h at 40° in 0.1M sodium hydroxide. The *O*-deacetylated polysaccharide was purified by dialysis and recovered by freeze-drying.

N.m.r. spectroscopy. — Samples were deuterium-exchanged several times with D_2O, and then examined as solutions in 99.99% D_2O (0.45 mL) containing a trace of acetone as internal standard (δ 2.23 for ^1H and 31.07 p.p.m. for ^{13}C). Spectra were recorded on a Bruker WM-500 spectrometer, using standard Bruker software. All two-dimensional experiments were carried out at 33°. A COSY[6] experiment was performed using a spectral width of 2024 Hz. A data matrix of 256 × 1024 data points was collected for 32 scans per t_1 value. The initial matrix was zero-filled to 512 data points in the t_1 dimension and was transformed and symmetrised after resolution enhancement in both domains with a non-shifted sine-bell window function. Digital resolution in the f_1 domain was 3.95 Hz per point. One-step and two-step relay COSY[7] and NOESY[8] experiments were carried out using a spectral width of 2500 Hz; initial data matrices of 512 × 2048 data points were zero-filled to 1024 × 2048 data points to provide a digital resolution of 2.44 Hz per point in the f_1 domain. Resolution enhancement and Fourier transformation was as for the COSY experiment. Relaxation delays of 1.1–1.2 s were used. For the relay COSY experiments, fixed delays of 0.036 s were used. The mixing delay in the NOESY experiment was 0.3 s.

A ^{13}C–^1H shift-correlated (HETCOR)[9] experiment was recorded using a spectral width in f_2 of 13,513 Hz (108.1 p.p.m.), and 1440 Hz (2.88 p.p.m.) in f_1. The initial matrix of 256 × 2048 was zero-filled to 512 × 2048 data points and processed with Gaussian functions. Digital resolution in f_2 was 13.2 Hz per point and in f_1 5.6 Hz per point. A recycle delay of 1.5 s was employed and 1600 scans per f.i.d. were collected.

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