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A structural investigation of the capsular polysaccharide of *Escherichia coli* O9:K57:H32

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ABSTRACT

The primary structure of the acidic capsular polysaccharide of *Escherichia coli* K57 was elucidated by methylation analysis and 1D- and 2D-n.m.r. spectroscopy. The repeating unit was identified as a linear tetrasaccharide having the structure shown.
→2)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→

INTRODUCTION

The capsular polysaccharide antigens of *Escherichia coli* have been subdivided into two main groups[1]. Group I antigens have high molecular weights and low electrophoretic mobilities, are heat stable at pH 5–6, are most often co-expressed with O8 and O9 antigens, and may contain amino sugars. Group II antigens have lower molecular weights and higher electrophoretic mobilities, are heat labile at pH 5–6, and are co-expressed with many O antigens but not O8 and O9. The structures of 49 *E. coli* capsular antigens have been reported[2]; of these, serotypes K6, K13, K18–K20, K22, K23, K74, K95, and K100 contain ribofuranose and belong to the Group II antigens. Serotypes K18, K22, and K100 incorporate ribitol phosphate, while the rest contain Kdo in their repeating units. The capsular antigen of *E. coli* O9:K57:H32[3], whose structure we now describe, is the first ribose-containing antigen in the *E. coli* series which is not associated with ribitol phosphate or Kdo and which belongs to the Group I antigens.

RESULTS AND DISCUSSION

Isolation, composition, and linkage analysis. — *E. coli* O9:K57:H32 bacteria were grown on Mueller–Hinton agar, and the capsular polysaccharide was isolated and purified as previously described[4]. The polysaccharide showed a single peak at M_r 250 000 in gel-permeation chromatography on Sepharose 4B CL.

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G.l.c. examination of the derived *O*-methyloxime acetates[5] confirmed the presence of equimolar proportions of GlcN, GalA, Gal, and Rib in the polysaccharide. The sugar residues were found to be D by measurement of the optical rotation of the individual sugars isolated after paper chromatography. The ^{1}H-n.m.r. spectrum of the polysaccharide (Fig. 1) contained H-1 signals at δ 5.39, 5.24 (^3J 3.5 Hz), 4.93 (^3J 2.7 Hz), and 4.46 (^3J 7.8 Hz), and a signal for NAc at δ 2.11 (3 H). In addition, signals for ring protons occurred at δ 4.51 ($^3J \sim 1$) and 4.38 (^3J 2.5 and 1.0 Hz). The ^{13}C-n.m.r. data confirmed a tetrasaccharide repeating-unit for the polysaccharide, with C-1 signals at 107.95, 104.25, 100.07, and 98.72 p.p.m., and a signal at 23.24 p.p.m. for the methyl carbon of an NAc group. In addition, a signal for a carbonyl carbon occurred at 175.75 p.p.m. The C-1 signal at 107.95 p.p.m. is indicative[6] of the presence of a furanoside in the repeating unit.

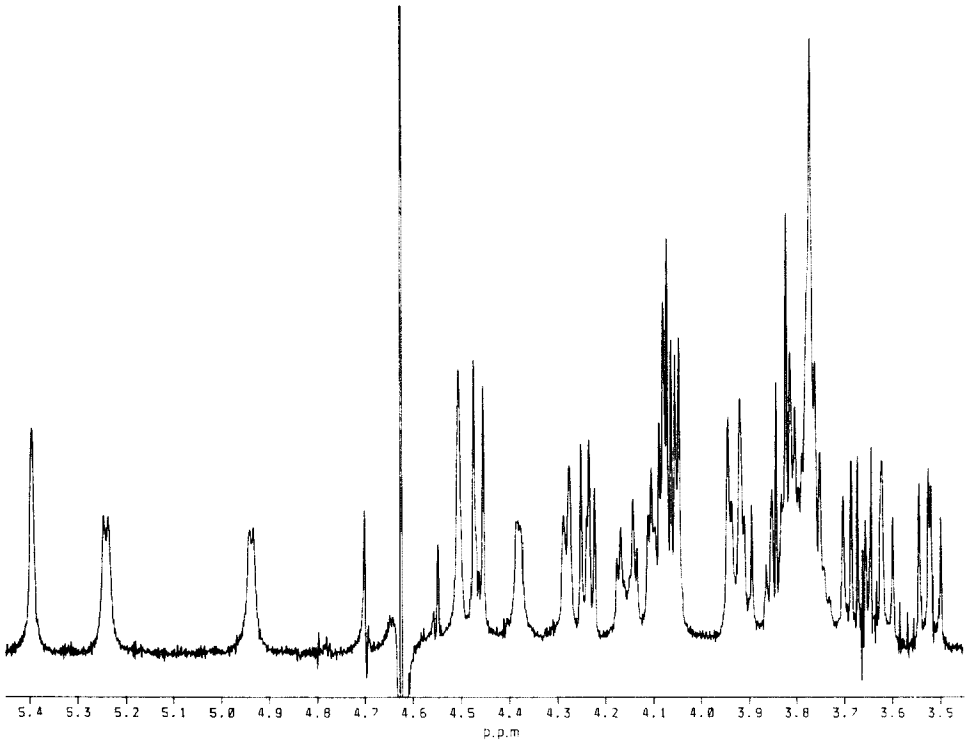


Fig. 1. Resolution-enhanced ^{1}H-n.m.r. spectrum (400 MHz) of the *E. coli* K57 capsular polysaccharide.

Methylation analysis of the polysaccharide gave 2,3,6-tri-*O*-methylgalactose, 2-deoxy-4,6-di-*O*-methyl-2-methylacetamidoglucose, 3,5-di-*O*-methylribose, and 2,3-di-*O*-methylgalactose (after carboxyl reduction).

2D-N.m.r. studies on the K57 polysaccharide. — The ^{1}H resonances of the sugar residues in the repeating unit of the polysaccharide were established (Table I) mainly

from COSY[7] and RELAY COSY[8] experiments. The H-1 resonances of the residues in the repeating unit were arbitrarily labelled **a**–**d** in order of decreasing chemical shift (Fig. 2). Following the cross-peaks in the contour plot, all of the resonances of residues **a** and **c** were readily assigned. In the case of residue **b**, the cross-peak between H-4 and H-5 was not observed in the COSY experiments. The chemical shift for H-5 of **b** was established from the intra-residue n.O.e. contacts observed for this unit in a NOESY[9] experiment (see later). Cross-peaks between H-4/H-5, H-5/H-6, H-5/H-6′, and H-6/H-6′ of residue **d** were also not observed; however, the chemical shifts for H-5 and H-6,6′ could be assigned from the ^1H–^{13}C correlation data (see later). The ^1H coupling constants (Table I) were obtained from the 1D spectrum of the polysaccharide (Fig. 1). The ^1H resonances for each residue in the polysaccharide were compared with the ^1H–^{13}C correlation data (Table I, Fig. 3) obtained from a heteronuclear correlated experiment (HETCOR)[10]. In this way, all of the ^1H resonances for residues **a**–**c** and H-1 to H-4 of residue **d**

TABLE I

^1H- and ^{13}C-n.m.r. data[a] for the K57 polysaccharide

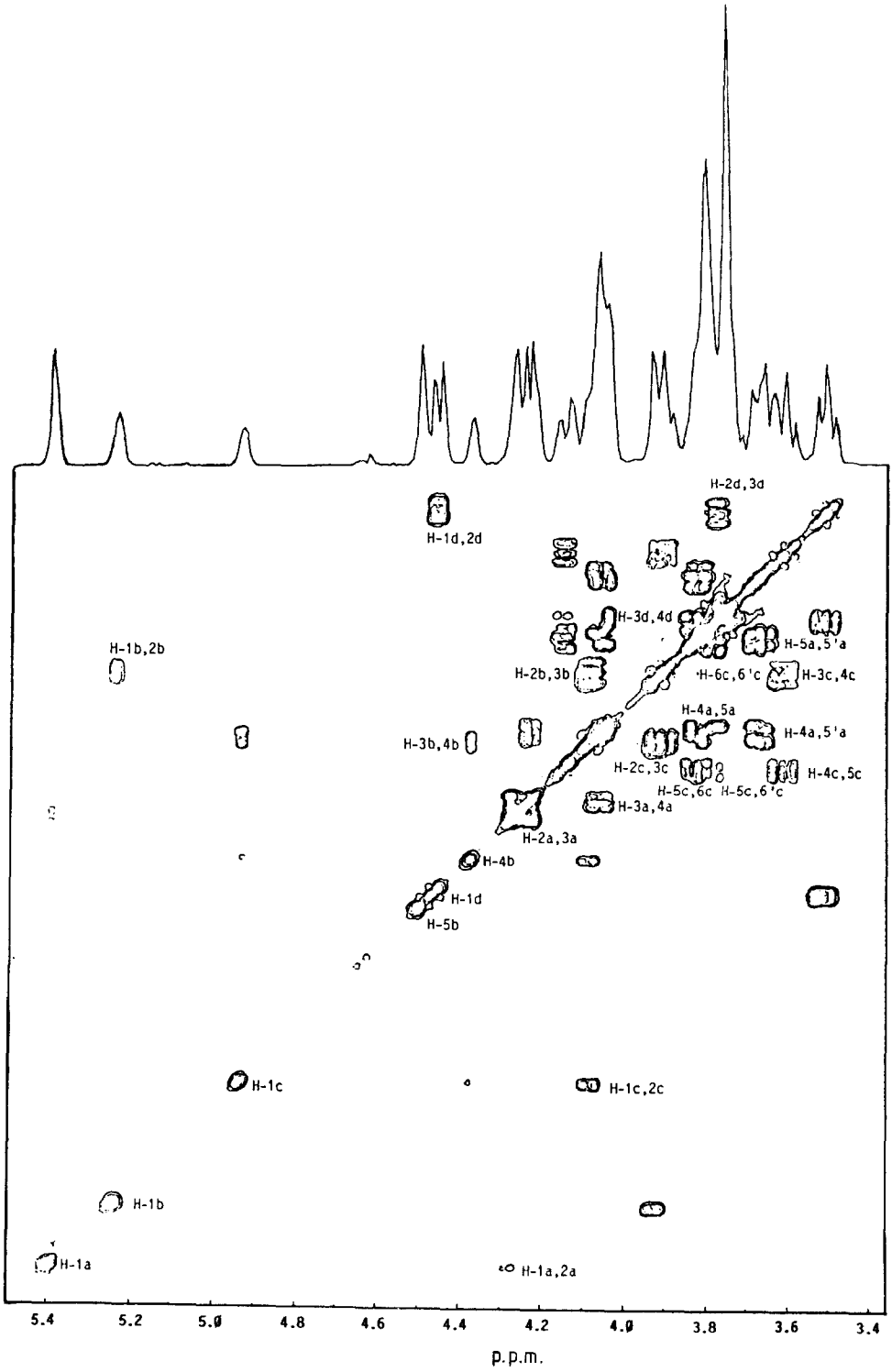
Residue		*Proton or carbon* 1	2	3	4	5	5′	6	6′
→2)-β-Rib	*H*	5.39	4.28	4.24	4.07	3.67	3.83		
(**a**)	*J*(Hz)	<1 (1,2)	4.8 (2,3)	6.72 (3,4)	6.7 (4,5)	12.1 (5,5′)			
	C	107.95	80.74	70.96	83.89	63.46			
→4)-α-GalA	*H*	5.24	3.93	4.10	4.38	4.51			
(**b**)	*J*	3.5 (1,2)	10.0 (2,3)	2.5 (3,4)	1.0 (4,5)				
	C	98.72	68.90	69.68	80.93	72.28			
→3)-α-GlcNAc	*H*	4.93	4.09	3.92	3.62	4.16		3.85	3.78
(**c**)	*J*	2.7 (1,2)	9.4 (2,3)	9.2 (3,4)	9.1 (4,5)				
	C	100.07	53.36	80.85	69.21	72.76		61.16	
→4)-β-Gal	*H*	4.46	3.52	3.79	4.05	3.78		3.78	3.78
(**d**)	*J*	7.8 (1,2)	9.9 (2,3)						
	C	104.25	71.56	73.69	77.22	75.19		62.01	

[a]Chemical shifts in p.p.m. downfield from the signal for acetone at δ 2.23 and 31.07 for ^1H and ^{13}C, respectively.

TABLE II

N.O.e. contacts for the K57 polysaccharide

Proton	*N.O.e. contact to*
a, H-1	5.24 (**b**, H-1), 4.05 (**d**, H-4)
b, H-1	5.39 (**a**, H-1), 3.93 (**b**, H-2)
b, H-5	4.38 (**b**, H-4), 4.10 (**b**, H-3)
b, H-4	4.10 (**b**, H-3)
c, H-1	4.38 (**b**, H-4), 4.09 (**c**, H-2)
d, H-1	3.92 (**c**, H-3), ~3.78 (**d**, H-3/or H-5)


H-2d,3d
H-1d,2d
H-3d,4d
H-5a,5'a
H-1b,2b
H-2b,3b
H-6c,6'c
H-3c,4c
H-4a,5a
H-3b,4b
H-4a,5'a
H-2c,3c
H-4c,5c
H-5c,6c
H-5c,6'c
H-3a,4a
H-2a,3a
H-4b
H-1d
H-5b
H-1c
H-1c,2c
H-1b
H-1a
H-1a,2a
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
p.p.m.

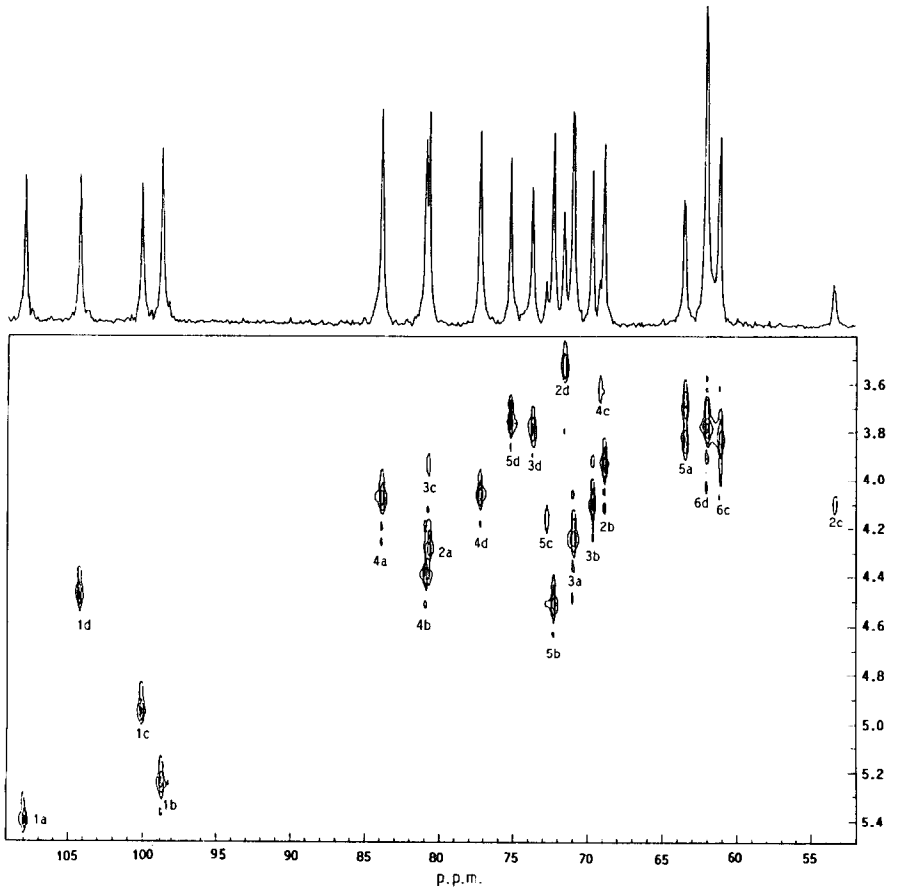


Fig. 3. ^{1}H–^{13}C Shift-correlation map of the spectral region F_2 (109–52 p.p.m.) and F_1 (5.5.–3.4 p.p.m.). The ^{13}C projection is displayed along the F_2 axis. The F_1 axis represents the ^{1}H resonances. The correlated resonances are labelled **a**–**d**.

were correlated with ^{13}C resonances. The two unassigned ^{13}C signals at 75.19 and 62.01 p.p.m., which arose from **d**, may now be confidently assigned to C-5 and C-6, respectively, of this residue. Both signals correlate with ^{1}H resonances at δ 3.78, which explains why cross-peaks were not observed between H-5/H-6, H-5/H-6′, and H-6/H-6′ of **d**.

Comparison of the ^{1}H- and ^{13}C-n.m.r. data for residues **a**–**d** with literature values for methyl glycosides[6,11,12] permitted the residues in the repeating unit to be identified, as indicated in Table I, and their linkage positions to be established. In accordance with the results of methylation analysis, C-2 of **a**, C-4 of **b**, C-3 of **c**, and C-4 of **d** experienced significant deshielding.

Fig. 2. COSY contour plot of the region 5.5–3.4 p.p.m. of *E. coli* K57 capsular polysaccharide. A low-resolution one-dimension projection is displayed along the F_2 axis. The ^{1}H resonances of the *J*-coupled spin systems are labelled **a**–**d**.

The sequence of the residues **a**–**d** in the repeating unit of the polysaccharide was established by a NOESY experiment[9]. The inter- and intra-residue n.O.e. contacts are presented in Table II. The α-linked pyranoside residues **b** and **c** showed characteristic intramolecular n.O.es between H-1 and H-2, while the β-linked residue **d** showed n.O.e.(s) from H-1 to H-3 and/or H-5. Residue **b** also showed three further intramolecular n.O.es. between H-3 and H-4, H-4 and H-5, and H-3 and H-5. These n.O.e. contacts permitted the chemical shift for H-5 of **b** to be established. Inter-residue n.O.es. between the anomeric protons and the relevant protons of the adjacently linked residues were clearly observed for residues **a**, **b**, and **d**, and permit the following sequence to be written.

a(1→4)**d**(1→3)**c**(1→4)**b**

The expected n.O.e. between **b** and **a** was not observed. Instead, an intense cross-peak was noted between the anomeric protons of these residues. Such uncommon n.O.es. have been observed previously[13–15] for α-D-hexose residues substituted by a glycosyl group at O-2.

The combined n.m.r. and methylation data permit the structure of the tetrasaccharide repeating-unit of the capsular polysaccharide of *E. coli* K57 to be written as

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

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

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 the carrier gas. A J + 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) and partially methylated methyl glycoside acetates (programme II). A J + 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 *O*-methyloxime acetates[5] (programme III). The temperature programmes used were I, 180° for 1 min, then 3°.min^{-1} to 240°; II, 180° for 30 min, then 2°.min^{-1} to 240°; and III, 80° for 1 min, then 20°.min^{-1} to 180°, hold for 1 min, then 2°.min^{-1} to 210°, hold for 1 min, then 10°.min^{-1} to 230°. The identities of all derivatives were confirmed by g.l.c.–m.s. on a Hewlett–Packard 5988A g.l.c.–mass spectrometer using the appropriate column. G.p.c. of K57 polysaccharide was performed on a dextran-calibrated column (1.6 × 65 cm) of Sepharose 4B CL with M sodium chloride as eluent.

Samples were hydrolysed with 4M trifluoroacetic acid for 1 h at 120°. Acid hydrolysates were co-evaporated with water under reduced pressure at bath temperatures ≤40°. Alditol acetates were prepared by reduction of aqueous solutions of hydrolysates with sodium borohydride followed by acetylation with 1:1 acetic anhydride–pyridine at 100° for 1 h. Samples were methanolysed by treatment with refluxing methanolic 3% hydrogen chloride for 16 h. *O*-Methyloxime acetates were

prepared as described[5]. Methylated K57 polysaccharide was carboxyl-reduced with lithium aluminium hydride in tetrahydrofuran. Methylations were carried out on the acid form of the polysaccharide, using potassium dimsyl[16] and methyl iodide in dimethyl sulphoxide.

Preparation of the K57 polysaccharide. — An authentic culture of *E. coli* O9:K57:H32 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 purified by precipitation with cetyltrimethylammonium bromide.

N.m.r. spectroscopy. — Samples were deuterium-exchanged by freeze-drying solutions in D_2O and then dissolved in 99.99% D_2O (0.45 mL) containing a trace of acetone as internal reference (δ 2.23 for ^1H and 31.07 p.p.m. for ^{13}C). Spectra were recorded at 40° with a Bruker WH-400 or AM-400 spectrometer, equipped with an Aspect 3000 computer and an array processor, using standard Bruker software.

^1H-N.m.r. spectra at 400 MHz were recorded using a spectral width of 2400 Hz and a 16k data set for a digital resolution of 0.29 Hz/point.

^1H-Homonuclear shift-correlated experiments (COSY[7] and one-step RELAY COSY[8]) and homonuclear dipolar-correlated (NOESY[9]) experiments were performed using a spectral width of 1448 Hz. Data matrices of 256 × 1024 data points were collected for 48 or 112 transients for each t_1 delay. The matrices were zero-filled in the t_1 dimension and transformed in the magnitude mode by use of a non-shifted sine-bell window function in both dimensions and symmetrised. Digital resolution in the resulting 512 × 1024 matrices was 2.8 Hz per point. Relaxation delays of 1.2 to 1.5 s were used. For the RELAY COSY experiment, a fixed delay of 0.036 s was used. The mixing delay in the NOESY experiments was varied between 0.2 and 0.3 s.

A ^{13}C–^1H shift-correlated (HETCOR)[10] experiment was recorded using a spectral width in F_2 of 10,000 Hz (99.4 p.p.m.) and 1500 Hz (3.75 p.p.m.) in F_1. The initial matrix of 256 × 2048 data points was transformed to 512 × 2048 points and processed with Gaussian functions. Digital resolution in F_2 was 9.8 Hz/point and in F_1 5.9 Hz/point. A recycle delay of 1.5 s was employed and 1600 transients per f.i.d. were collected.

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REFERENCES

1 K. Jann and B. Jann, *Rev. Infect. Dis.*, 9 (1987) S517–S526.
2 G. G. S. Dutton and L. A. S. Parolis, in V. Crescenzi, I. C. M. Dea, and S. S. Stivala (Ed.), *Proc. Int.*

Workshop on Recent Developments in Industrial Polysaccharides: Biomedical and Biotechnological Advances, 3rd, Gordon and Breach, New York, 1989, to be published.
3 I. Ørskov, F. Ørskov, B. Jann, and K. Jann, *Bacteriol. Rev.*, 41 (1977) 667–710.
4 H. Parolis, L. A. S. Parolis, and S. M. R. Stanley, *Carbohydr. Res.*, 175 (1988) 77–83.
5 J. -R. Neeser and T. F. Schweizer, *Anal. Biochem.*, 142 (1984) 58–67.
6 K. Bock and H. Thϕrgersen, *Annu. Rep. N.M.R. Spectrosc.*, 13 (1982) 1–57.
7 A. Bax and R. Freeman, *J. Magn. Reson.*, 44 (1981) 542–561.
8 A. Bax and G. Drobny, *J. Magn. Reson.*, 61 (1985) 306–320.
9 R. Baumann, G. Wider, R. R. Ernst, and K. Wüthrich, *J. Magn. Reson.*, 44 (1981) 402–406.
10 A. Bax and G. Morris, *J. Magn. Reson.*, 42 (1981) 501–505.
11 B. Matsuhiro, A. B. Zanlungo, and G. G. S. Dutton, *Carbohydr. Res.*, 97 (1981) 11–18.
12 K. Izumi, *Carbohydr. Res.*, 170 (1987) 19–25.
13 G. G. S. Dutton, S. K. Ng, L. A. S. Parolis, H. Parolis, and A. K. Chakraborty, *Carbohydr. Res.*, 193 (1989) 147–155.
14 J. Dabrowski, M. Hauck, E. Romanowska, and A. Gamian, *Carbohydr. Res.*, 180 (1988) 163 – 174.
15 J. -R. Brisson and J. P. Carver, *J. Biol. Chem.*, 258 (1983) 1431–1434.
16 L. R. Phillips and B. A. Fraser, *Carbohydr. Res.*, 90 (1981) 149–152.