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A reverse approach to ^{1}H-n.m.r. assignments of bacterial polysaccharides

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ABSTRACT

A new approach is reported for obtaining the assignment of the ^{1}H-n.m.r. spectra of bacterial polysaccharides which are not amenable to analysis using conventional strategies. An unambiguous assignment of the ^{13}C-n.m.r. spectrum is made by ^{13}C-COSY of the polysaccharide labelled to a high level with ^{13}C. Assignment of the ^{1}H-n.m.r. spectrum is then made by proton-detected CH-correlation spectroscopy of material that is labelled at a low level. Using this approach, virtually complete assignments have been made for the *Klebsiella* K3 serotype polysaccharide under physiological conditions of temperature and pH.

INTRODUCTION

The assignment of the ^{1}H-n.m.r. spectra of complex bacterial polysaccharides under physiological conditions is a prerequisite for the determination of conformations in solution from n.O.e. data. For many biomolecules, these assignments are achieved by the use of ^{1}H–^{1}H correlation techniques such as COSY or TOCSY. Such methodology is now used widely in the study of protein structures where the diverse functionalities and the shifts due to the aromatic ring currents give rise to relatively well dispersed ^{1}H-n.m.r. spectra[1]. However, the study of bacterial polysaccharides is a different proposition. The relatively few different chemical species present in such molecules gives rise to severely overlapped ^{1}H-n.m.r. spectra with the majority of signals often concentrated in a region of only 1 p.p.m. (Fig. 1). For some polysaccharides, this overlap does not pose any problems in making assignments using conventional methodology[2–4]. However, for many high-molecular-weight polysaccharides, this overlap remains a problem even in 2D-n.m.r. spectra and, combined with the rapid nuclear spin–lattice relaxation rates, limits the utility of ^{1}H–^{1}H correlation techniques. Sometimes, these problems can be overcome, in part, by recording the n.m.r. spectra of purified polysaccharides at elevated temperatures. The effects of rapid spin–spin relaxation processes are reduced and there is greater resolution of individual multiplets, but this is not a general solution to the problem.

As part of a study of molecular interactions, we have been interested in determining the conformations in solution of native unextracted polysaccharides under physio-

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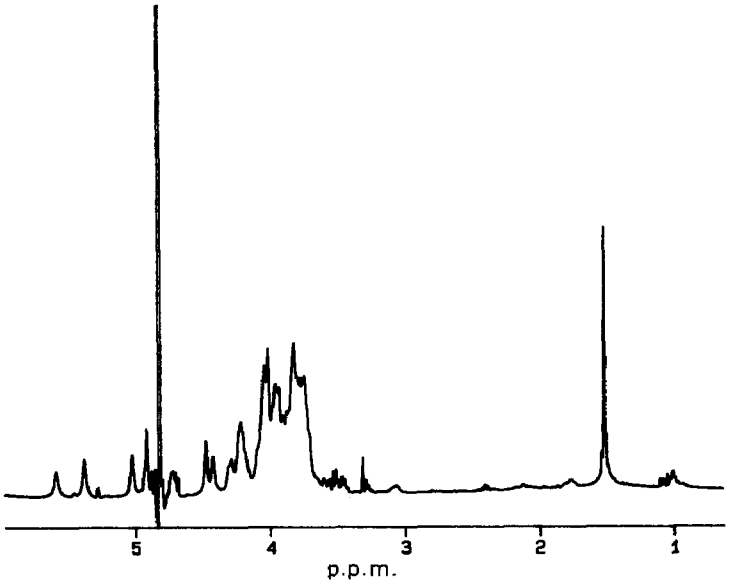


Fig. 1. ^{1}H-N.m.r. spectrum (400 MHz) of the *Klebsiella* K3 polysaccharide, obtained at ambient temperature on a lyophilised whole culture.

logical conditions. In order to prevent degradation of the sample at physiological pH, all spectra must be recorded at $<40°$. Under these conditions, the assignment of the ^{1}H-n.m.r. spectrum shown in Fig. 1 is virtually impossible using the conventional approach. Consequently, an approach has been adopted which exploits the greater effective resolution available in the ^{13}C-n.m.r. spectrum in order to obtain a complete assignment of the ^{1}H-n.m.r. spectrum.

Initially, the ^{13}C-n.m.r. spectrum of the polysaccharide was assigned using a ^{13}C-COSY spectrum of polysaccharide that had been labelled biosynthetically with 99% uniformly ^{13}C-labelled D-glucose diluted with natural abundance material. This strategy yielded a high concentration of coupled ^{13}C-pairs[5]. Assignments made using this approach are unambiguous, as they do not rely on chemical shift arguments other than to provide a few reliable starting points. Moreover, the spectrum will often contain readily identifiable, independent starting points such as the resonances of the carboxyl groups of uronic acid residues. In addition to providing a complete ^{13}C shift assignment, it was found that an analysis of the multiplet patterns within the cross-peaks of this COSY spectrum allowed determination of the biosynthetic pathways involved in the catabolism of the glucose prior to its incorporation into the polysaccharide[6].

The subsequent assignment of the ^{1}H-n.m.r. spectrum was made by proton-detected CH-correlation spectroscopy. This technique places the crowded ^{1}H-n.m.r. spectrum in the f_2 dimension where it can be digitised finely and leaves the better-resolved ^{13}C-n.m.r. spectrum in the less finely digitised f_1 dimension. The sensitivity of the experiment was enhanced by labelling the polysaccharide biosynthetically with 14%

uniformly ^{13}C-labelled D-glucose. This strategy gives improved detection of ^{13}C satellites without incurring complications from ^{13}C–^{13}C coupling.

EXPERIMENTAL

Sample preparation. — A culture of *Klebsiella rhinoscleramatis*, serotype K3, was obtained from Dr. I. Ørskov (Copenhagen). The bacteria were grown in Brain Heart Infusion broth (Oxoid Ltd.) (9 mL, 24 h, 32°) and the resulting culture was used to inoculate 5 agar plates (9 mm diam.) that contained yeast extract (2 g), magnesium sulphate (0.25 g), sodium chloride (2 g), potassium sulphate (1 g), agar (15 g), and 14% uniformly ^{13}C-labelled D-glucose (4 g) per litre of culture medium. The resulting cultures from three plates were combined, harvested, and resuspended in physiological buffered saline solution at pH 7.0 (5 mL). The sample was then exchanged three times with D_2O (99 atom %) (5 mL) before running the n.m.r. spectra. The final concentration of the polysaccharide was in the range 10–15 mg.mL^{-1} as measured by the phenol–sulphuric acid method[7].

N.m.r. methods. N.m.r. spectra were recorded on a Bruker AM 400 spectrometer with a 5-mm proton-observe broad band-decouple probe. For the CH-correlation, a spectral width of 2336 Hz and an acquisition time of 0.9 s were used in the f_2 dimension, giving a total of 4096 real data points. The f_1 spectral width was 10 638 Hz and t_1 was incremented in 1024 equal steps to a maximum value of 0.021 s, 480 scans were recorded per t_1 increment, and two dummy scans were inserted prior to the acquisition of each increment. The resulting data were zero filled to 2048 points prior to Fourier transformation using mild Lorentzian to Gaussian apodization in both dimensions.

RESULTS AND DISCUSSION

The *Klebsiella* K3 serotype polysaccharide (**1**, see page 20) consists[8] of a tetrasaccharide backbone unit composed of an α-D-galacturonic acid residue (A), two α-D-mannose residues (B and C), and a β-D-galactose residue (D). In addition, A is 4-substituted with a 4,6-pyruvylated α-D-mannose residue (E).

The CH-correlation spectrum was recorded using the original pulse sequence proposed by Müller[9] in conjunction with the double-difference phase-cycling procedure proposed by Cavanagh and Keeler[10]. In the resulting spectrum, the ^{1}H signals appeared as doublets in f_2 because the spectrum was acquired without heteronuclear decoupling. Consequently, the spectrum contained the extra information of the magnitude of the $^1J_{C,H}$ values.

In the region of the spectrum for the signals of anomeric protons, correlations were found for each H-1 of the polysaccharide[11]. A comparison of the published chemical shifts for the resonances of H-1 and C-1 of the α-mannose (E) and α-galacturonic acid (A) residues[8] for **1** led to the interchange of this pair of C-1 assignments[11]. These reassignments had been confirmed by an assignment of the ^{13}C-n.m.r. spectrum by ^{13}C-COSY which contained independent, non-anomeric starting points[5].

In the region of the spectrum that corresponded to the resonances of the ring carbons and protons (Fig. 2), the proton signals were now well resolved by extension into the already well-resolved ^{13}C dimension. The only part of the spectrum where overlap remained a significant problem was that for the C-6 resonances where three similar CH_2 groups gave rise to the large unresolved set of responses at the lower-

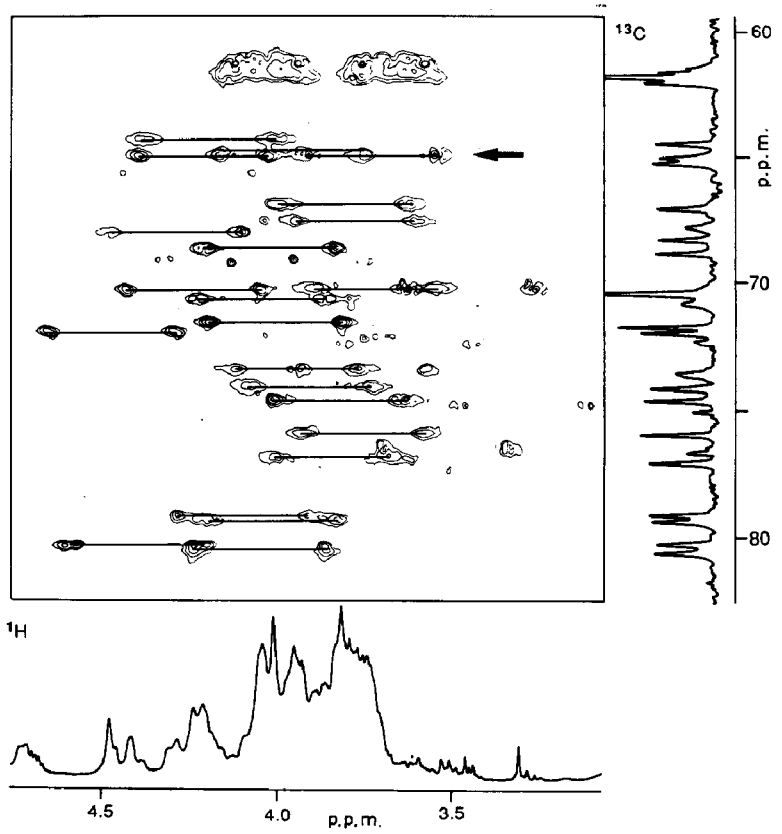


Fig. 2. Part of the proton-detected CH-correlation spectrum of the *Klebsiella* K3 polysaccharide, showing the correlations for the ring carbons and protons.

TABLE I

^{1}H-N.m.r. assignments for the *Klebsiella* K3 serotype polysaccharide

Atom	*α-GalA (A)*	*α-Man (B)*	*α-Man (C)*	*β-Gal (D)*	*α-Man (E)*
H-1	5.59	5.03	5.37	4.72	4.88
H-2	4.11	4.26	4.07	3.74	4.03
H-3	4.33	4.03	4.07	3.86	4.04
H-4	4.42	3.82	3.84	4.23	3.84
H-5	4.48	3.92	3.96	3.78	4.20
H-6*a*					3.75
H-6*e*					3.97

frequency end of f_1. Nevertheless, it was found that neither ^{1}H-COSY at 500 MHz nor carbon-detected CH-correlation at 150 MHz (14 Tesla) could provide spectra with the resolution obtained in this spectrum.

The resonances of the remaining ring protons could now be assigned completely (Table I) and the only region of ambiguity, other than the methylene region mentioned above, corresponded to the H-6,6 of α-D-mannose (E) and H-4 of β-D-galactose (D) which were almost coincident at 65.4 p.p.m. in f_1. The signals centred at 4.23 p.p.m. were ~10 Hz to higher frequency in the f_1 dimension, just at the limits of digital resolution.

Tentative stereospecific assignments of the H-6,6 resonances of the α-mannose (E) were made following consideration of the stereochemistry of the C-6–O-6 bond (Fig. 3). H-6*e* is *gauche* to both lone pairs of O-6, whereas H-6*a* is *gauche* to one lone pair and *anti* to the other. The effect of orientations of lone pairs on the magnitude of $^1J_{C,H}$ values is well documented[12]; *gauche* interactions increase and *anti* interactions decrease the magnitude. A cross-section parallel to f_2 at the chemical shift of the resonance of C-6 of

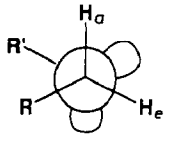


Fig. 3. Newman projection of the C-6–O-6 bond of the α-D-mannose (E) residue, showing the orientation of the axial and equatorial protons relative to the lone pairs of the oxygen.

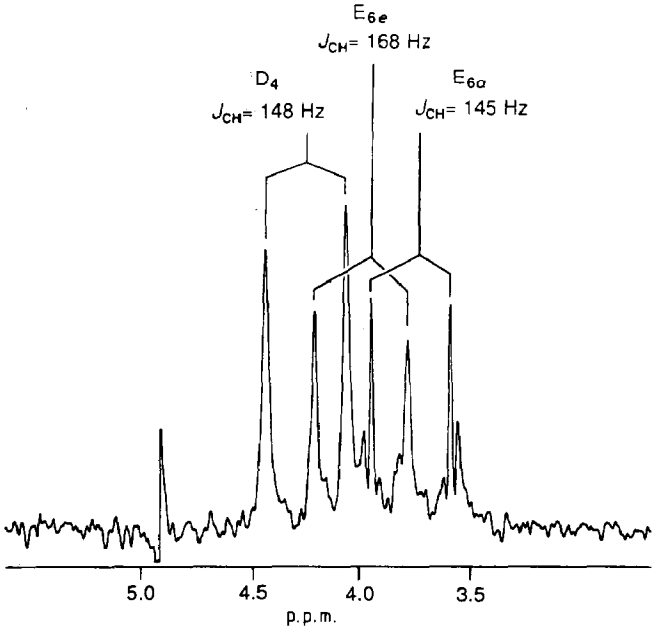
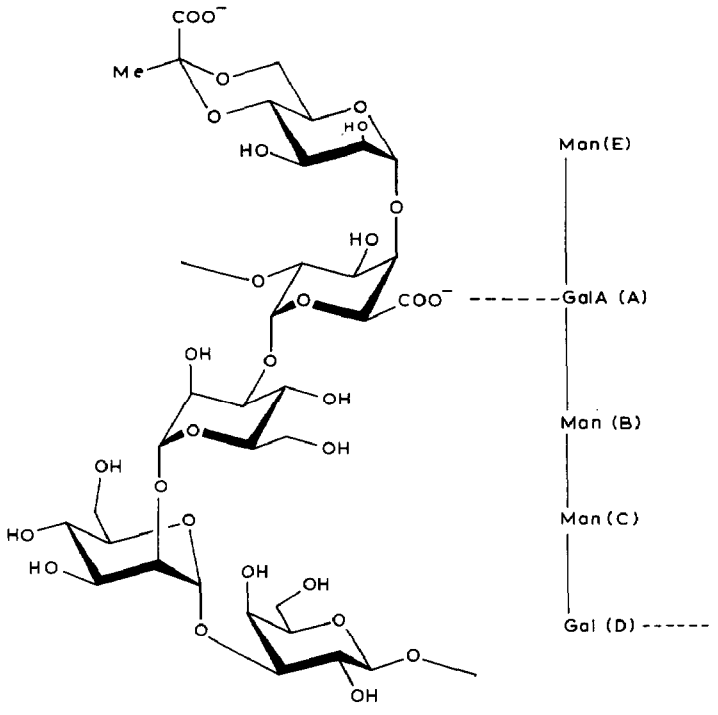


Fig. 4. Cross-section parallel to f_2 at the chemical shift in f_1 of the C-6 resonance of the α-D-mannose (E) residue marked with an arrow in Fig. 2. The pair of signals centred at 4.23 p.p.m. are from the C-4 of β-D-galactose (D).



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α-D-mannose (E) (Fig. 4) showed three signals with $^1J_{CH}$ values of 148, 145, and 168 Hz. The signal centred at 4.23 p.p.m. was the tail of the H-4 resonance of β-D-galactose (D). Thus, the resonance centred at 3.75 p.p.m. ($^1J_{CH}$ 145 Hz) was assigned to H-6*a* and that centred at 3.97 p.p.m. ($^1J_{CH}$ 168 Hz) to H-6*e*.

Lone-pair orientation effects have been used to explain the well-known stereospecificity of $^1J_{C\text{-}1,H\text{-}1}$ values[12]. Generally, the values for H-1*a* are ~10 Hz smaller than those for H-1*e*. The enhanced magnitude of the $^1J_{C\text{-}1,H\text{-}1}$ values compared to those of the other ring carbons is attributed to the fact that C-1 is attached to two oxygens. Thus, the value of $^1J_{CH}$ for H-6*e* of α-D-mannose (E) would be expected to be only 10 Hz greater than that of H-6*a*. No explanation is available at present for the large differences in $^1J_{CH}$ observed for H-6,6 of α-D-mannose (E).

The above reverse approach for assigning the ^{1}H-n.m.r. spectra is most useful for those polysaccharides which are so complex that they preclude the use of conventional strategies. The strategy outlined is dependent on the availability of reliable ^{13}C assignments, but this should not be a problem for microbial polysaccharides which can be enriched with ^{13}C biosynthetically to a high level. The ^{1}H assignments obtained have been used to determine the conformation in solution of the polysaccharide by n.O.e. spectroscopy[13].

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