Assignment of the ^{13}C N.M.R. Spectrum of the *Klebsiella* K3 Serotype Polysaccharide by COSY Spectroscopy

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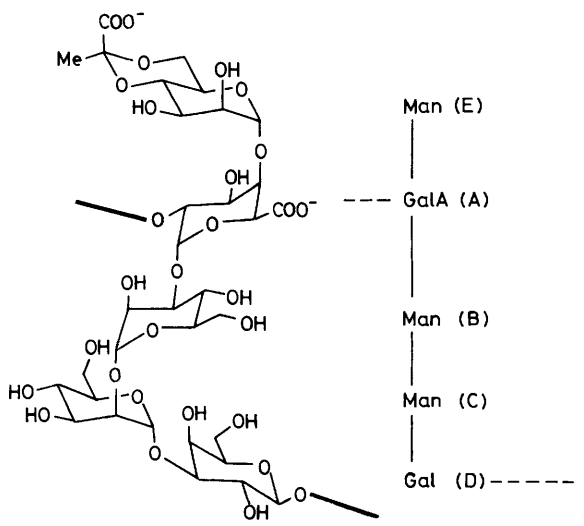
Klebsiella K3 serotype polysaccharide has been biosynthetically enriched by growth on [$^{13}C_6$]glucose and its ^{13}C n.m.r. spectrum has been fully assigned using a phase-sensitive double-quantum-filtered COSY spectrum; analysis of the isotopomer distribution in the COSY cross-peaks shows that some of the glucose is established through the phosphogluconate pathway before incorporation into the polymer.

We present an approach for the complete and unambiguous assignment of the ^{13}C n.m.r. spectra of microbial natural products, using the *Klebsiella* K3 serotype polysaccharide (**1**)[1] as an example. The repeating unit of this polymer contains five similar, but distinct, hexose moieties, resulting in a large number of tightly-grouped carbon resonances; clearly, assignments based on chemical shift precedents will not be adequate in such a system. A two-dimensional C–H correlation led to reliable assignments of only the anomeric carbons.[2] We now show that the combination of biosynthetic enrichment with two-dimensional COSY spectrum provides a general solution to this assignment problem. Furthermore, we show that the COSY cross-peak fine structure contains detailed information on the catabolic and biosynthetic pathways involved.



(**1**)

[99%-$^{13}C_6$]-α-D-glucose was diluted four-fold with natural abundance material, so that approximately 20% of the molecules were fully labelled; *Klebsiella rhinoscleramatis*, capsular serotype K3 (NCTC 5046), was grown on a solid medium containing this labelled glucose as the sole carbon source. The culture was harvested and diluted with D_2O and phosphate-buffered saline, pH 7.0, before 100 MHz ^{13}C spectra were acquired. In our experience, high-quality spectra are obtained under these near-physiological conditions; the organism over-produces extra-cellular polysaccharide to such an extent that the resonances of this molecule dominate the spectrum. Any attempt at isolation or purification generally leads to a degradation in spectral quality.

The anomeric region of the spectrum (Figure 1) shows the expected five anomeric carbons and the pyruvate acetal

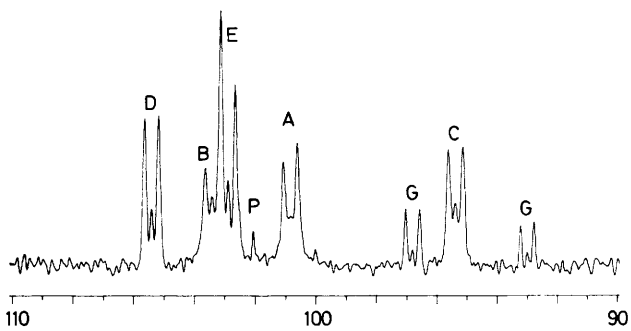


Figure 1. Anomeric region of the 100 MHz ^{13}C n.m.r. spectrum of a culture of *K. rhinoscleramatis* K3 grown on 20% [$^{13}C_6$]glucose. A—E are the anomeric carbons of (**1**); G is free glucose; P is pyruvate acetal carbon. 512 transients were acquired.

Table 1. ^{13}C assignments for the *Klebsiella* K3 serotype polysaccharide.[a]

Atom	α-GalA-(A)	α-Man-(B)	α-Man-(C)	β-Gal-(D)	α-Man-(E)	Pyr
C-1	100.8	103.3	95.8	105.4	102.8	176.2
C-2	79.3	70.5	80.4	70.5	71.8	102.7
C-3	68.5	79.6	71.0	77.6	68.9	25.9
C-4	80.2	67.2	68.0	65.4	74.7	
C-5	72.1	74.2	73.6	76.1	64.5	
C-6	175.5	62.1	61.9	61.9	65.1	

[a] Shifts are given in p.p.m. relative to internal acetone (δ 31.05 p.p.m.).

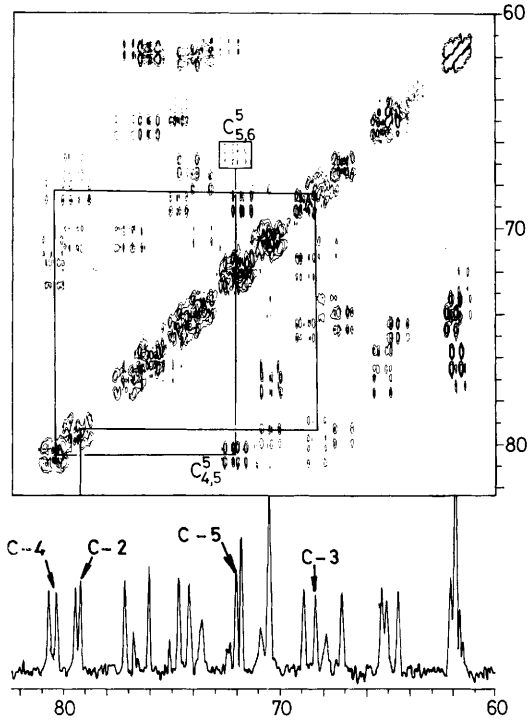


Figure 2. Ring carbon region of the phase-sensitive double-quantum-filtered COSY spectrum of the same sample as in Figure 1. The spectral width in both dimensions was 5494 Hz. In f_2, the acquisition time was 0.4 s, giving a total of 4096 real points. In f_1, the evolution time t_1 was incremented in 256 equal steps to a maximum value of 0.0233 s. A total of 512 transients were acquired per increment, with a 0.2 s relaxation delay between transients. The data were zero-filled to 512 points and treated with a mild Lorentzian-to-Gaussian apodization before Fourier transformation. Correlation lines connect the resonances of the GalA(A) residue.

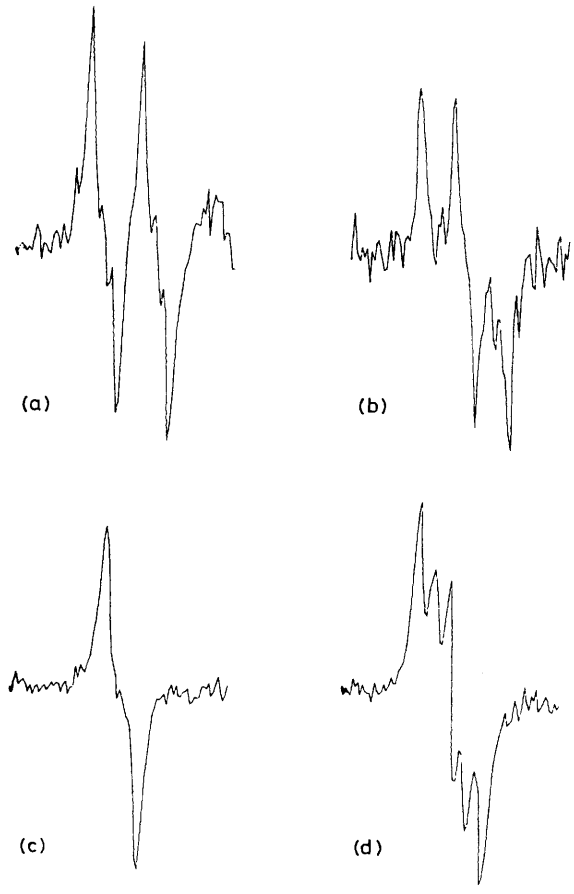


Figure 3. Cross-sections of selected multiplets from the COSY spectrum: (a) $C^5_{4,5}$, (b) $C^5_{5,6}$, (c) $C^1_{1,2}$, (d) $C^2_{1,2}$. Each trace is a 250 Hz section parallel to f_2.

carbon of (**1**), together with smaller signals due to the two anomers of free glucose. The level of label incorporation is very high, all the anomeric carbons appearing as doublets by virtue of coupling to C-2. Small natural abundance singlets are visible in the centre of the doublets.

Part of the phase-sensitive double-quantum-filtered COSY spectrum[3] of the sample is shown in Figure 2. Strong correlations were obtained from each of the anomeric carbons to their C-2 neighbours. Using these anomeric resonances as secure starting points, we were able to trace the connectivities throughout each residue to C-6, giving a complete assignment.† There are no ambiguities in the assignments (Table 1), and no signals remain unassigned. The appearance of the pyruvate methyl resonance at 26 p.p.m. defines the acetal

† The folded C-6 of GalA-(A) (Figure 2) provided a confirmatory starting point for that residue, while C-6 of Man-(E) and the pyruvate-C-2 had already been assigned by a *J*-modulated spin-echo spectrum.[5] The C-2 resonances of Man-(B) and Gal-(D) are coincident at 70.5 p.p.m.; this does not create C-3 assignment difficulties as all the relevant COSY cross-peaks for Man-(B) show $J_{1,2}$ of 46 Hz while those for Gal-(D) show a $J_{1,2}$ of 48 Hz.

configuration as shown, with the methyl group equatorial.[4]‡

Fine structure in phase-sensitive COSY cross-peaks contains both useful clues for assignment and novel biosynthetic information. Lines separated by the active§ coupling appear antiphase, while those separted by a passive coupling appear in-phase. This effect is particularly useful when the two couplings are characteristically different; *e.g.* $J_{5,6}$ in GalA-(A) is 59 Hz, while $J_{4,5}$ is 40 Hz. So, the $C_{4,5}$ cross-peak at the C-5 shift in f_2 ($C^5_{4,5}$) shows alternating line phases while the $C_{5,6}$ cross-peak at the same f_2 shift ($C^5_{5,6}$) shows pairs of lines in antiphase (Figures 3a,b). Similarly, $C^1_{1,2}$ is a simple doublet (Figure 3c) while $C^2_{1,2}$ should be a double doublet with $J_{1,2}$ = 48 and $J_{2,3}$ = 38 Hz. The actual $C^2_{1,2}$ cross-section (Figure 3d) shows that some molecules have lost the label at C-3, giving a simple antiphase doublet superimposed on the four-line pattern; this effect is observed in all five $C^2_{1,2}$ responses. Several other sets of responses, including the pyruvate-C-2, show the same effect. Analysis of the cross-peaks, supported by simulations, shows that approximately 30% of the glucose is catabolised *via* the phosphogluconate pathway[6] before incorporation into the polysaccharide.

Details of these biosynthetic results, together with the studies of conformation and mobility under physiological conditions that are made possible by complete assignment of the spectrum, will be given elsewhere. More importantly, the way is now open to detailed spectroscopic and biosynthetic studies on any polysaccharide or biopolymer that can be biosynthetically labelled to very high levels.

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‡ The acetal configuration shown in Ref. 2 is incorrect.

§ If A, M and X are mutually-coupled, then the cross-peak between A and M has J_{AM} as the active coupling and J_{AX} or J_{MX} as the passive couplings.

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