Carbohydrate Research, 82 (1980) 175–184

MEASUREMENT OF CARBON-13–PROTON COUPLING-CONSTANTS IN OLIGOSACCHARIDES BY TWO-DIMENSIONAL CARBON-13 N.M.R. SPECTROSCOPY*

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(Received January 11th, 1980; accepted for publication, January 30th, 1980)

ABSTRACT

A two-dimensional, n.m.r. experiment, ^{13}C two-dimensional *J* spectroscopy, has been used to facilitate the measurement of ^{1}H–^{13}C couplings in spectra of oligosaccharides. One-bond, ^{13}C–^{1}H coupling-constants ($^{1}J_{CH}$) have been determined for all of the ^{13}C resonances of raffinose [*O*-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl β-D-fructofuranoside], and for the anomeric carbon atoms of a single tetrasaccharide repeating-unit [*O*-α-D-galactopyranosyl-(1→2)-*O*-α-L-rhamnopyranosyl-(1→3)-*O*-β-L-rhamnopyranosyl-(1→4)-L-rhamnopyranose] of the cell-wall polysaccharide of *Klebsiella* type K32 bacteria.

INTRODUCTION

It has been shown[1,2] that the one-bond, ^{13}C–^{1}H coupling-constants of the anomeric carbon atoms in pyranoid sugars show a strong stereospecific dependence, that can be used to determine configuration at anomeric centres. For monosaccharides, these couplings may simply be measured directly from the proton-coupled ^{13}C spectrum, but in more-complex systems, the presence of overlap between multiplets may prevent accurate measurements. Two-dimensional, n.m.r. spectroscopy[3,4] offers a general method of overcoming problems of overlap, by separating the effects of chemical shifts and multiplet structure into orthogonal frequency-dimensions. The appropriate, two-dimensional experiment for analyzing proton-coupled ^{13}C spectra is known as carbon-13 two-dimensional *J* spectroscopy[4–9], in which the second halves of modulated, ^{13}C spin-echoes are accumulated for a range of pulse spacings, and are then subjected to a double Fourier-transformation with respect both to time and to interpulse spacing. The result is a spectrum in two dimensions, showing signal strength as a function of chemical shift in one frequency-

*Dedicated to Professor Stephen J. Angyal on the occasion of his retirement.

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dimension, and as a function of coupling constant in the other, the net effect being to produce a separate, proton-coupled, ^{13}C multiplet for each decoupled carbon resonance. In order to illustrate the potential utility of ^{13}C two-dimensional *J* spectroscopy in carbohydrate chemistry, two-dimensional spectra have been measured for solutions of raffinose (**1**) and of a single tetrasaccharide repeating-unit (**2**) of the *Klebsiella* K32 bacterial cell-wall polysaccharide.

EXPERIMENTAL

The principles and experimental practice of ^{13}C two-dimensional *J* spectroscopy have been extensively discussed elsewhere[4-10] and will not be dealt with in detail here. The pulse sequence of Fig. 1 was used to collect a series of free-induction decays $S(t_2)$ for a set of equally spaced values of the delay* t_1. The resultant matrix $S(t_1,t_2)$ was subjected to a double Fourier-transformation, once with respect to t_2 and once with respect to t_1, to yield a two-dimensional spectrum $S(f_1,f_2)$. Stacked plots of the two-dimensional spectra obtained for the two compounds studied were produced by using absolute-value display, with signal-bearing cross-sections through the f_1 domain being replotted in phase-sensitive mode; zero-filling prior to the second Fourier transformation was used in producing these cross-sections in order to make best use of the digitized data.

Raffinose (**1**, Pfanstiehl) was studied as a 10% solution by weight in D_2O, with 3 drops of 1,4-dioxane (Fisher) added as the chemical-shift reference, 1,4-dioxane being taken as 67.40 p.p.m. from external tetramethylsilane. The tetrasaccharide repeating-unit (**2**) of K32 polysaccharide kindly provided by Dr. G. G. S.

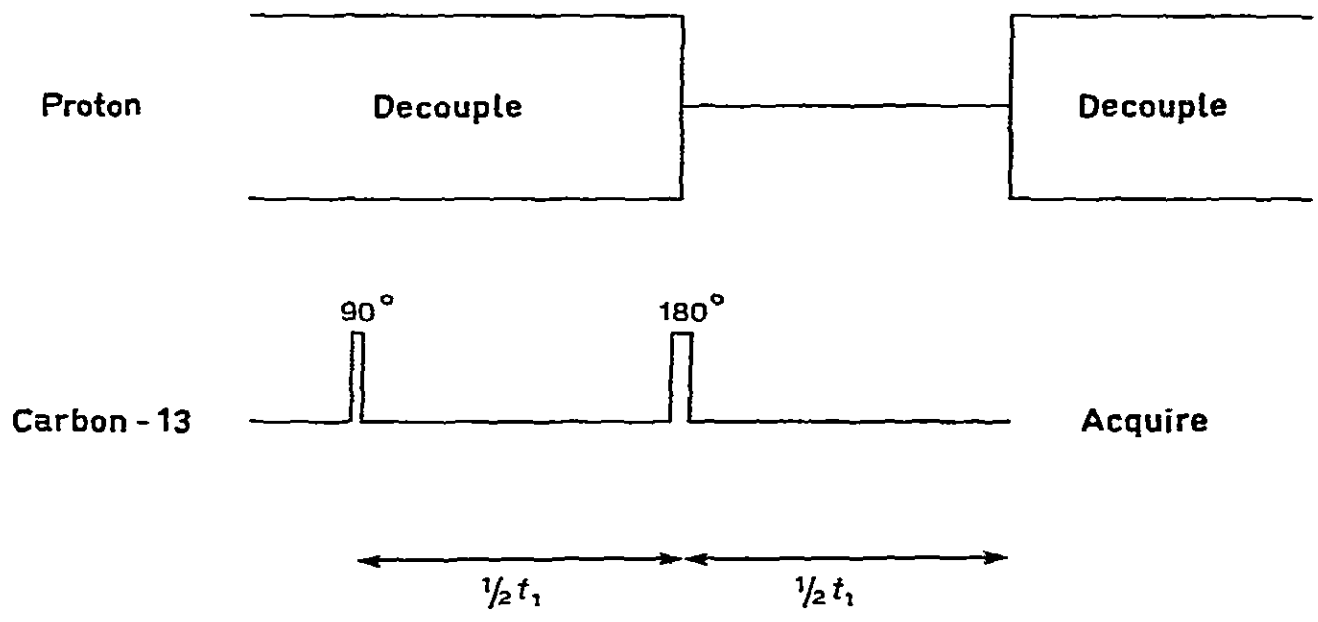

Fig. 1. Pulse sequence used in measuring the ^{13}C two-dimensional *J* spectra of Figs. 2, 3, and 5; this is the "gated decoupler" pulse-sequence of refs. 6 and 7.

*A referee expressed concern that the pulse spacings in Fig. 1 are given in units of $(1/2)t_1$. In two-dimensional n.m.r., t_1 is the interval varied in successive experiments; in *J* spectroscopy, the 180°, refocussing pulse is applied at the midpoint of the t_1 period.

Dutton of this department was used as a solution of 46 mg in 1 mL of D_2O, together with one drop of 1,4-dioxane reference, the sample volume in the 10-mm tube used being restricted with the aid of Teflon anti-vortex plugs (Wilmad) to optimize sensitivity.

Experiments were performed with a home-built superconducting spectrometer operating at 67.89 MHz for ^{13}C, based on a modified Bruker–Nicolet TT-23 console and an Oxford Instruments solenoid. Data acquisition and processing were performed by the standard NTCFT control programme of a Nicolet 1180/293A′ computer system, equipped with a Diablo Model 31 disc unit for data storage. Data-acquisition times for experimental spectra ranged from about one min for a conventional decoupled spectrum of **1** to 16 h for the two-dimensional J spectrum of **2**.

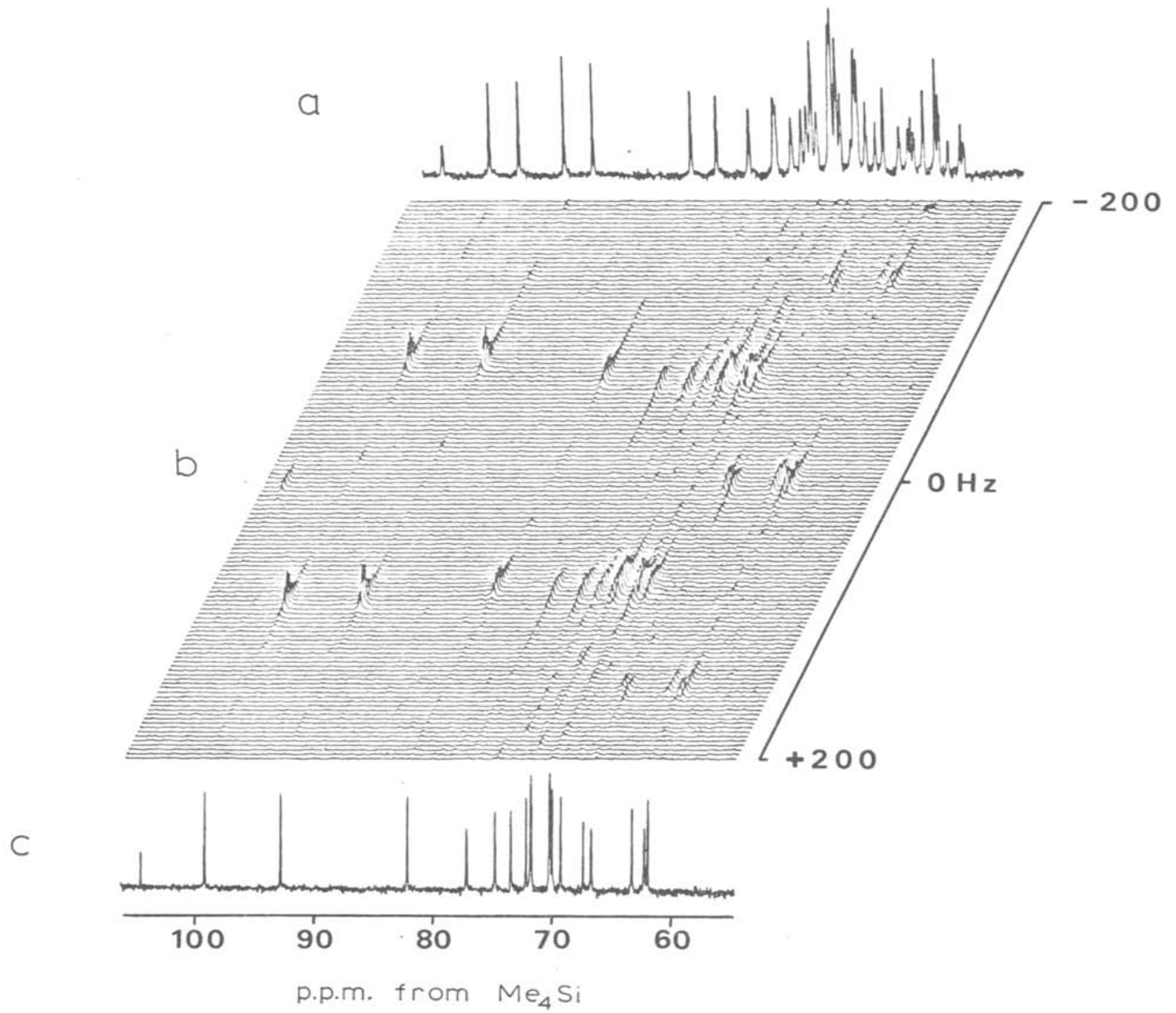

Fig. 2. ^{13}C Spectra of raffinose (**1**): (a) the conventional proton-coupled spectrum with Overhauser enhancement; (b) the two-dimensional J spectrum obtained by using the pulse sequence of Fig. 1; and (c) the conventional decoupled spectrum.

RESULTS

The spectra of raffinose shown in Fig. 2 illustrate the advantages of two-dimensional n.m.r. over conventional methods. The fully proton-decoupled ^{13}C spectrum of Fig. 2c shows all but two of the 18 ^{13}C signals of **1** resolved, several of the resonances being sufficiently shifted from their neighbours for the multiplet structure in the Overhauser-enhanced, proton-coupled spectrum[11] of Fig. 2a to be clearly visible. The majority of the resonances, however, give rise to a complex set of overlapping multiplets that are difficult to assign with certainty. In contrast, the ^{13}C two-dimensional J spectrum of Fig. 2b effects a complete separation of chemical-shift and multiplet structure, allowing measurement of all one-bond ^{1}H–^{13}C coupling-constants ($^{1}J_{CH}$).

The two-dimensional spectrum of Fig. 2b is presented with the chemical-shift axis (f_2) horizontal, in order to facilitate comparison with the conventional one-dimensional spectra of Figs. 2a and c. The choice of display-mode is dictated purely by convenience; the same experimental data have been replotted in Fig. 3 with the chemical-shift axis vertical in order to make the range of coupling constants encountered more accessible. The time taken to produce stacked plots of such full two-dimensional spectra as Figs. 2b and 3 (30–120 min) is sufficient that, for routine use, the most satisfactory data-presentation method is simply to plot only those traces through the f_1 domain of the two-dimensional spectrum that actually show signals, as has been done for **1** at the left hand side of Fig. 3. These traces, obtained by taking cross-sections through the two-dimensional spectrum at the chemical-shift frequencies in f_2, afford a concise summary of the information content of the two-dimensional spectrum. This also allows zero-filling of f_1 data to be carried out without requiring exorbitant amounts of data storage. The coupling constants obtained from the cross-sections of Fig. 3 are summarized in Table I, the values for the anomeric carbon atoms 1′ and 1″ of **1** being typical of α linkages.

In more-complex oligosaccharides, even the anomeric region of the proton-coupled ^{13}C spectrum becomes too crowded for easy interpretation, as the conventional spectra of Fig. 4 illustrate*. These are low-field portions of (a) the proton-coupled and (b) the proton-decoupled ^{13}C spectra of the tetrasaccharide repeating-unit [*O*-α-D-galactopyranosyl-(1→2)-*O*-α-L-rhamnopyranosyl-(1→3)-*O*-β-L-rhamnopyranosyl-(1→4)-L-rhamnopyranose] (**2**) of *Klebsiella* K32 bacterial cell-wall polysaccharide. The measurement of the five anomeric ^{13}C–^{1}H coupling-constants (the terminal rhamnose residue is present in both α and β forms) is greatly facilitated by obtaining the two-dimensional J spectrum, the anomeric portion of which is shown in Fig. 5, signal-bearing traces once more being replotted to the left of the full spectrum.

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*A referee queried the inclusion of this as a useful example because the anomeric region of the proton-coupled ^{13}C spectrum can be interpreted directly. In fact, this example was selected because (*a*) the direct interpretation is on the borderline of credibility, whereas the two-dimensional result is completely clear-cut, and (*b*) it is important to show that two-dimensional n.m.r. experiments are viable with small quantities of oligosaccharides.

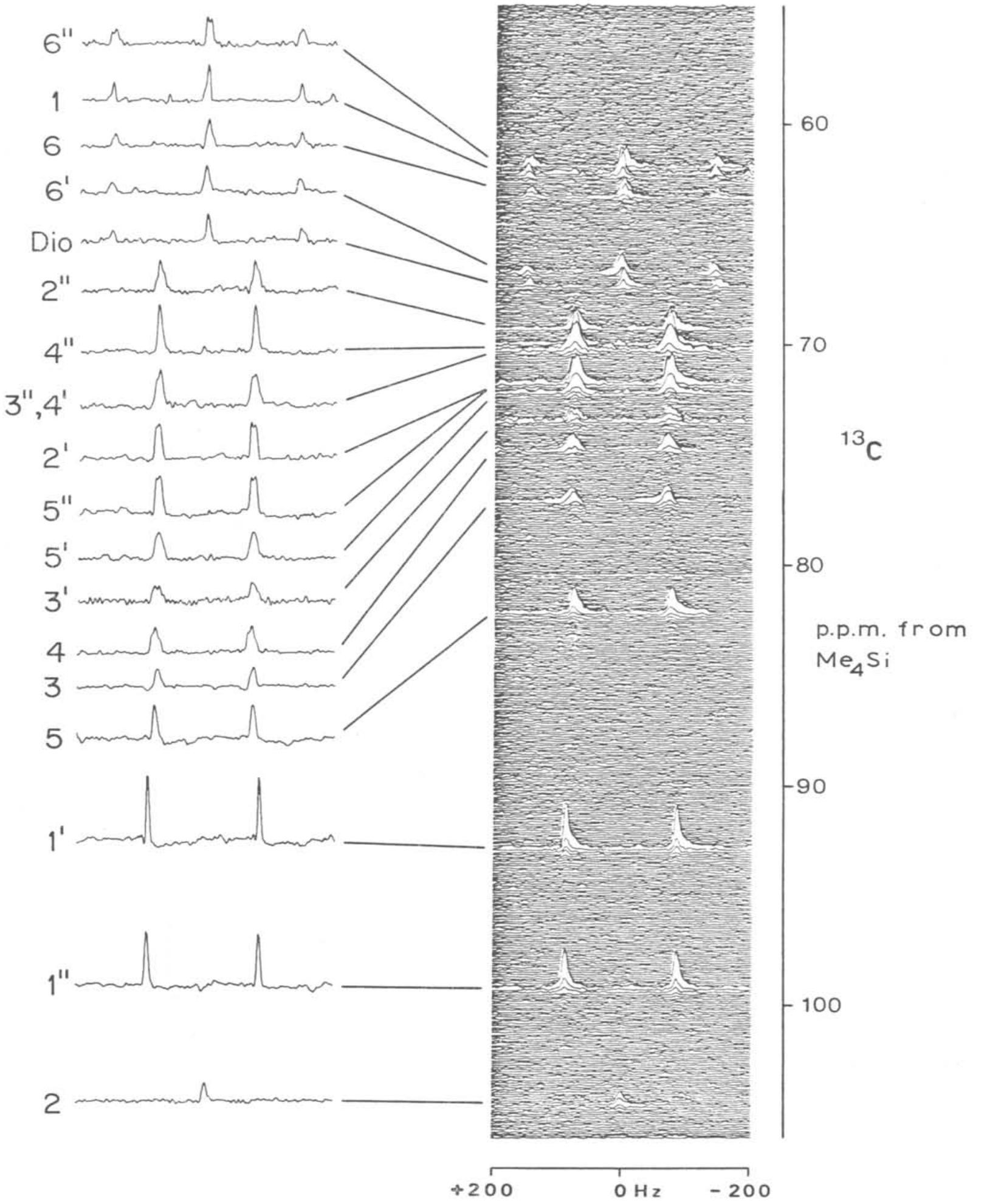

Fig. 3. The ^{13}C two-dimensional J spectrum of Fig. 2b replotted with the f_1 (multiplet structure) axis horizontal. The full two-dimensional spectrum is plotted in absolute-value mode at the right, whereas to the left, individual signal-bearing traces have been replotted in phase-sensitive mode. Dio = 1,4-dioxane.

TABLE I

CARBON-13 CHEMICAL-SHIFTS AND ONE-BOND CARBON–PROTON COUPLING-CONSTANTS FOR RAFFINOSE (1)

Residue	*Value*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Galactopyranosyl	δ_C[a]	99.29	69.30	70.25	70.03	71.83	61.94
	J_{CH}[b]	171	147	146	144	146	144
Glucopyranosyl	δ_C[a]	92.90	71.77	73.48	70.25	72.21	66.72
	J_{CH}[b]	170	146	145	144	144	144
Fructofuranoside	δ_C[a]	62.23	104.60	77.16	74.81	82.15	63.27
	J_{CH}[b]	145	—	147	147	150	143

[a]Expressed to ±0.02 p.p.m., with respect to 1,4-dioxane at 67.40 p.p.m. from external tetramethylsilane. [b]Given to ±2 Hz.

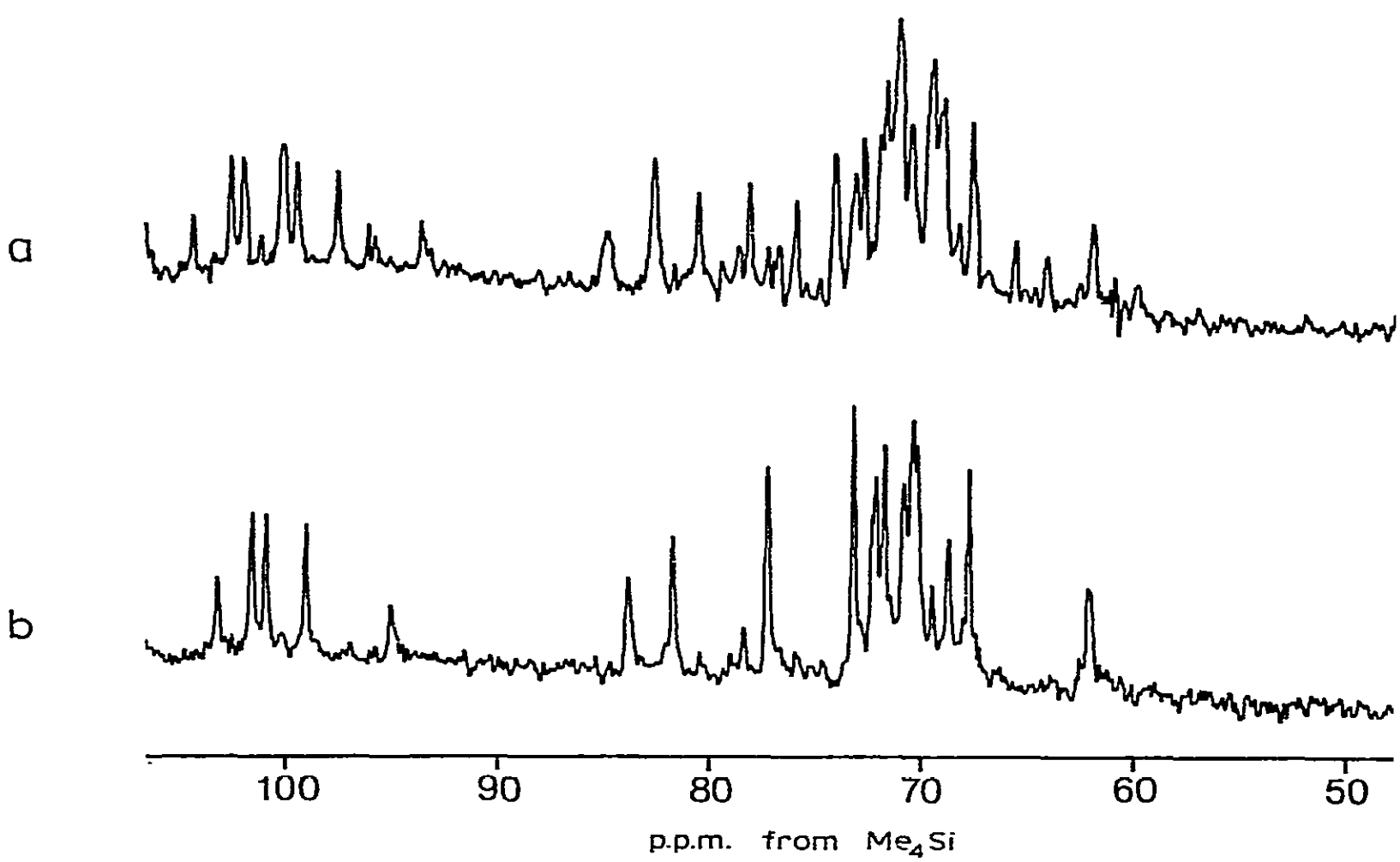

Fig. 4. (a) Proton-coupled and (b) -decoupled ^{13}C spectra of the tetrasaccharide repeating-unit (2) of *Klebsiella* K32 capsular polysaccharide. Total data-acquisition times were ~10 min for (b) and ~120 min for (a).

The coupling constants measured from cross-sections through the two-dimensional spectrum are given in Table II, together with assignments made on the basis of the coupling constants and chemical shifts measured. These assignments differ slightly from those proposed earlier for the native polysaccharide[12].

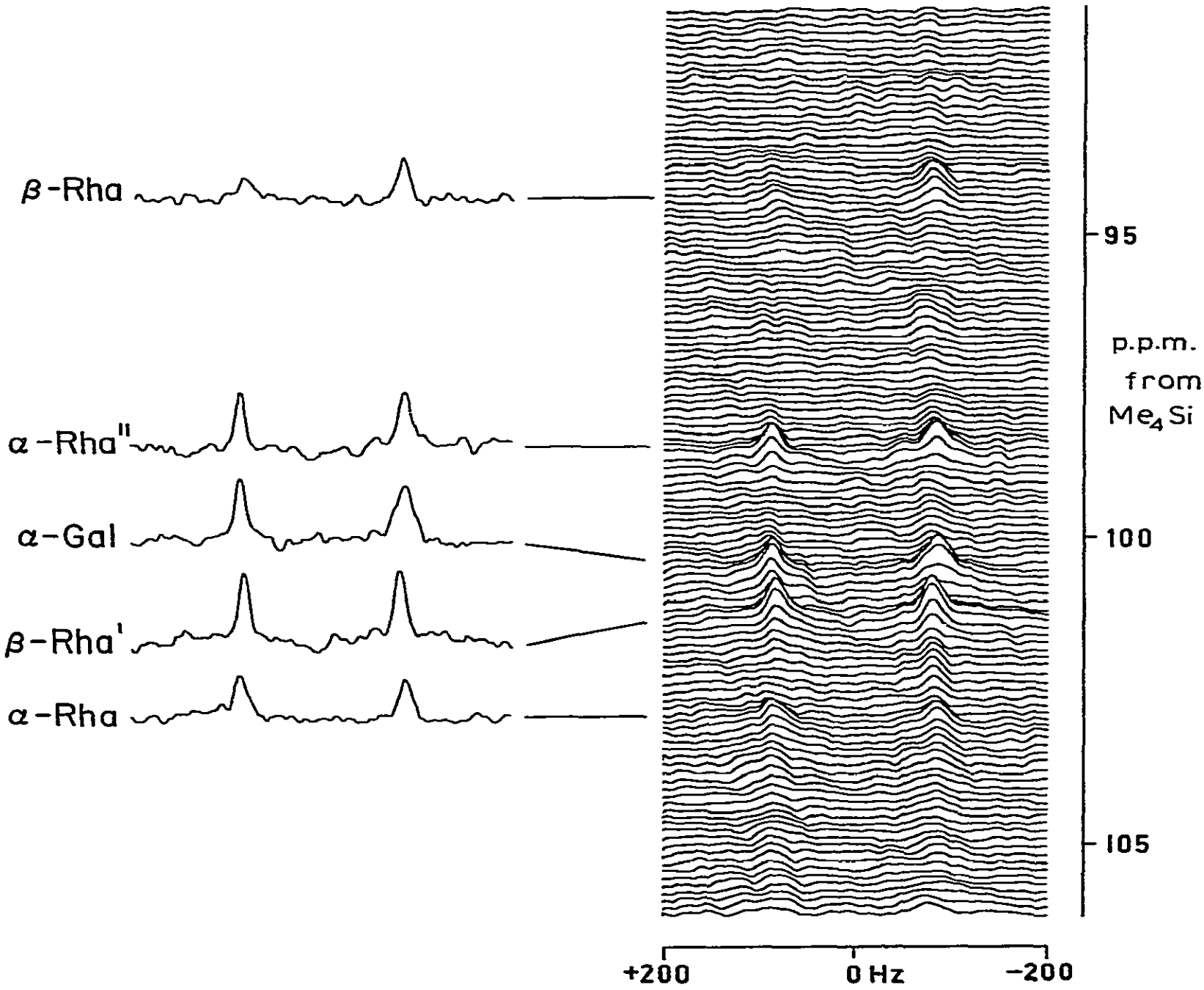

Fig. 5. Anomeric region of the ^{13}C two-dimensional J spectrum of *Klebsiella* K32 repeating-unit (**2**), signal-bearing traces again being replotted to the left of the two-dimensional spectrum. Total data-acquisition time was ~16h.

TABLE II

CARBON-13 CHEMICAL-SHIFTS AND ONE-BOND CARBON–PROTON COUPLING-CONSTANTS FOR THE ANOMERIC-CARBON RESONANCES OF α-D-Gal-(1→2)-α-L-Rha″-(1→3)-β-L-Rha′-(1→4)-L-Rha (**2**)

Residue	δ_C[a]	J_{CH}[b]
α-Gal	100.4	172
α-Rha″	98.6	171
β-Rha′	101.1	162
α-Rha	102.7	172
β-Rha	94.5	167

[a]Expressed to ±0.1 p.p.m., with respect to 1,4-dioxane at 67.40 p.p.m. from external tetramethylsilane. [b]Given to ±2 Hz.

DISCUSSION

As the two-dimensional spectra of Figs. 2–4 illustrate, ^{13}C two-dimensional J spectroscopy allows the measurement of a separate proton-coupled ^{13}C multiplet for each resolved chemical-shift in a decoupled ^{13}C spectrum. In very round terms, this increases by about an order of magnitude the limiting size of system for which clearly resolved, proton-coupled multiplets may be obtained. The price paid for this improvement is first that the time taken to measure a two-dimensional spectrum is about one order of magnitude more than that needed to obtain a normal coupled spectrum, and second, that the organization of the experiment and associated data-processing is considerably more complex. In practice, the latter problem is less severe than it might at first appear, as a number of spectrometer manufacturers now offer software packages for two-dimensional, n.m.r. spectroscopy. The only other experimental technique (apart from ^{13}C labelling) that offers separated proton-coupled ^{13}C multiplets is the combination of gated proton-decoupling with selective excitation[13–15]. This is a very simple technique to implement and perform, but requires a separate experiment for each carbon site in a molecule. Consequently, for systems containing more than about ten carbon sites, the experiment of choice is ^{13}C two-dimensional J spectroscopy, if a full set of multiplets is desired.

Three main potential uses for two-dimensional J spectroscopy in polysaccharides may be distinguished. First, in very crowded ^{13}C-spectra it is frequently important to make a simple determination of multiplicity for each decoupled resonance in order to aid assignment. The techniques currently available for this purpose are off-resonance decoupling[16–18], J-scaling[19,20], selective excitation, and two-dimensional J spectroscopy. The technique of J-scaling is closely related to J spectroscopy and is rather less sensitive in general, although it requires less data-processing and storage. The most sensitive technique is usually off-resonance decoupling, but unfortunately this method frequently suffers from overlapping multiplets and from distortion of multiplets through strong coupling-effects[21] and through inhomogeneity of the decoupling field[22]. Second, the measurement of one-bond ^{1}H–^{13}C coupling-constants for anomeric resonances is, as has already been discussed, a well-established assignment tool. Third, in some systems it is possible to resolve longer-range (particularly three-bond) couplings, which may have considerable diagnostic potential.

The three uses discussed require slightly different experimental conditions, as the measurement of fine coupling-structure necessitates much better digital resolution in the f_1 domain of the two-dimensional spectrum than the determination of multiplicity. The f_1 digitization of the two-dimensional spectrum depends on the number of different values of t_1 (see Fig. 1) for which spin echoes are measured, and hence governs the time required for the experiment. A multiplicity determination could be quite satisfactorily carried out with only 16 or 32 t_1 samples, whereas the experiments illustrated in Figs. 2–4 used 128 samples in t_1 in order to obtain good values for $^{1}J_{CH}$. Consequently, the sensitivity obtained when using ^{13}C two-dimensional J spectroscopy for examining multiplet fine-structure, requiring even better digitization,

is not good; the compensations are that fully separated multiplets are obtainable, even in crowded spectra, and that contributions of static magnetic-field inhomogeneity to multiplet linewidths are largely suppressed.

Two-dimensional n.m.r. spectroscopy greatly increases the amount of information that it is possible to obtain through n.m.r. experiments on complex systems. The n.m.r. spectroscopy of carbohydrates exhibits many problems potentially amenable to study through two-dimensional n.m.r., and a significant amount of work has already been done in this area by using homonuclear, ^{1}H two-dimensional J spectroscopy[23–29] and ^{13}C–^{1}H chemical-shift correlation[27,28,30,31]. Carbon-13 two-dimensional J spectroscopy, the heteronuclear analogue of the experiment described in refs. 23–29, also appears to offer considerable scope in carbohydrate systems, as the very preliminary results presented here illustrate.

ACKNOWLEDGMENTS

This work was supported by an operating grant from the National Research Council of Canada (A1905 to L.D.H.), and by a postdoctoral research fellowship from the Izaak Walton Killam Foundation (to G.A.M.). The technical assistance of Tom Markus in the construction of the spectrometer used is warmly appreciated. The sample of phage-degraded, *Klebsiella* K32 repeating-unit was very kindly made available by Prof. G. G. S. Dutton of this department.

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