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Proton and carbon chemical-shift assignments for 6-kestose and neokestose from two-dimensional n.m.r. measurements*

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ABSTRACT

The proton and carbon chemical shift assignments of the simplest levan, 6-kestose, [O-β-D-fructofuranosyl-(2→6)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside], along with another trisaccharide, neokestose, [O-β-D-fructofuranosyl-(2→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside], were determined using two-dimensional (2D) homonuclear and heteronuclear n.m.r. methods. The ^{1}H peak of H-1 of the glucose residue was determined by its chemical shift. Using H–H coupling information to this proton, the chemical shifts of most of the proton signals of the glucose moiety were determined. Though the signals from the two fructose residues were very close, a NOESY experiment and long-range C–H correlation experiments allowed their complete carbon and proton assignment. This work completes and corrects literature assignments.

INTRODUCTION

The kestoses are the three fructan trisaccharides which have a fructofuranose attached to sucrose, and they differ only by the linkage of the second fructose residue to the sucrose. 6-Kestose (**1**), is the simplest levan, one of the important fructan polymers. Neokestose (**2**) is the simplest polymeric fructan in some Liliaceae. The third kestose is 1-kestose, [O-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside]. Both **1** and **2** were first observed during the action of yeast invertase preparations on concentrated sucrose solutions[1,2]. Fructan sugars occur in numerous plants[3]. While it is now clear that fructan is a critical carbohydrate source for many plants, the reasons for its importance have not been explained. Current postulates of the mechanism for its action involve freezing point depression, and the provision of a sink for the products of photosynthesis when pyranose enzymes are slowed by low temperatures[4]. As a result, plants that produce fructan sugars have growth under cool-temperature conditions.

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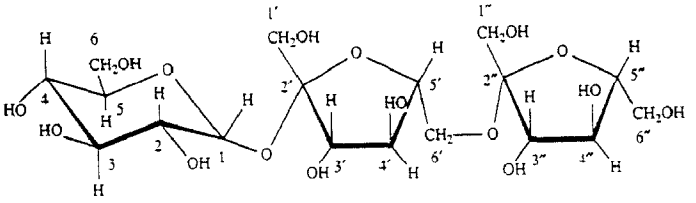
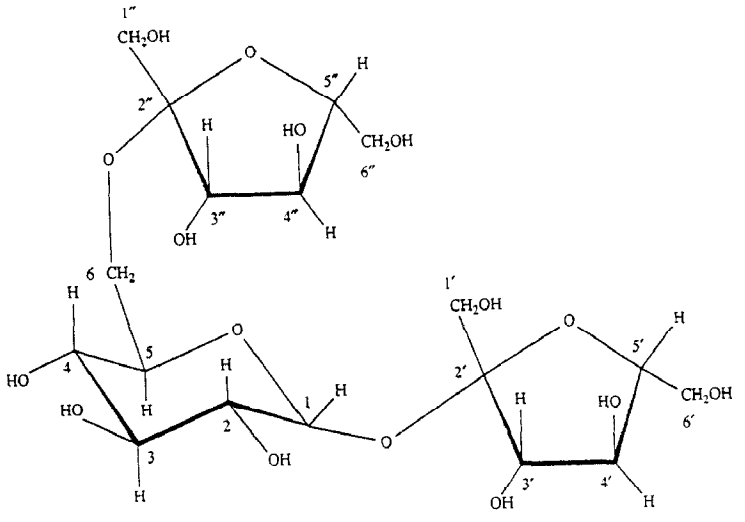


6-Kestose (**1**)



Neokestose (**2**)

While n.m.r. spectroscopy is used extensively to identify plant fructans[5–7], to date, only partial ^{13}C and/or ^{1}H spectral assignment of **1** (ref. 8) and **2** (refs. 7 and 8) have been reported. The assignment of chemical shifts is a basis for analyzing molecular conformation from experimental n.m.r. data, and the conformation is very important in understanding the biological activity of fructans in plants. A complete assignment of 1-kestose ^{1}H- and ^{13}C-spectra has been reported[9].

RESULTS AND DISCUSSION

The *J*-resolved spectra of **1** and **2** are shown in Figs. 1 and 2 and Table I and II shows the chemical shift assignments of **1** and **2**, respectively.

Proton spectral assignments. — The complete ^{1}H (and ^{13}C) chemical shift assignments of **1** and **2** depended on the unambiguous assignment of H-1. H-1 is the only signal in the ^{1}H spectrum which can be easily identified, because it is the only proton connected to a carbon atom bearing two oxygens. The key to completing the assignment was distinguishing the signals from the different D-fructosyl units.

Because almost all the signals fall in a small range of ~0.8 p.p.m. in the ^{1}H

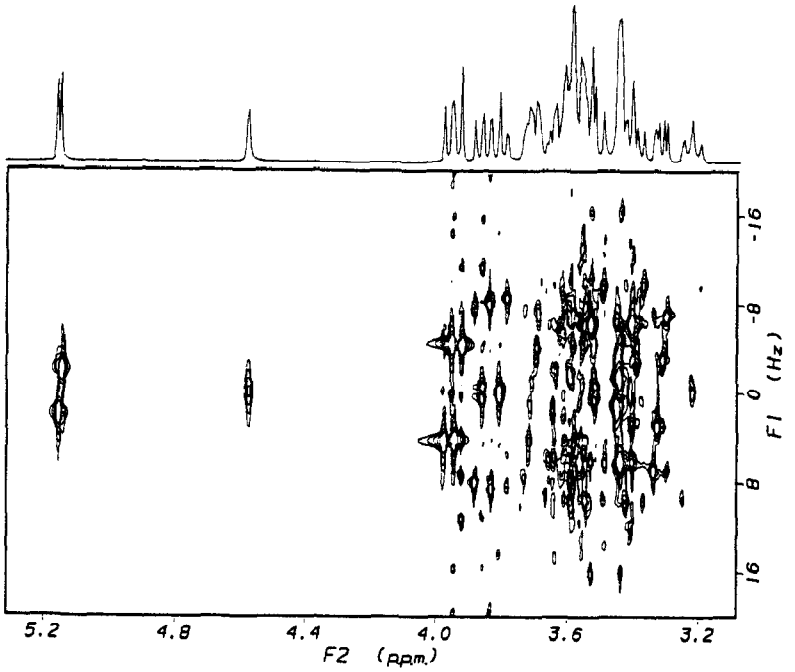


Fig. 1. Homonuclear (H–H) *J*-resolved spectrum of 6-kestose (**1**).

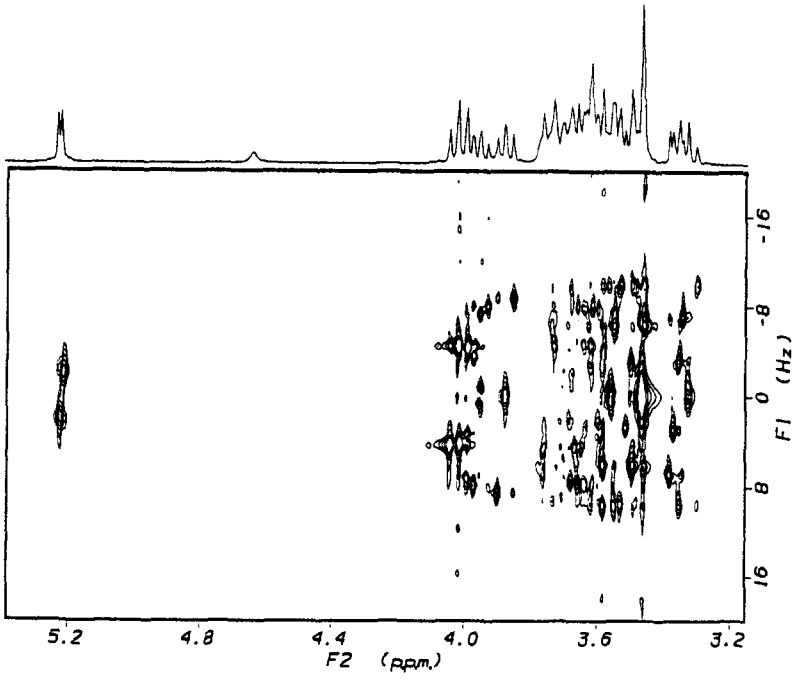


Fig. 2. Homonuclear (H–H) *J*-resolved spectrum of neokestose (**2**).

TABLE I

N.m.r. data for 6-kestose[a]

Atom No.	*^{13}C Shift (p.p.m.)*	*^{1}H Shift (p.p.m.)*	*Assigned ^{1}H*	
			All COSY[b] cross-peaks	*Long-range C–H correl. cross-peaks*
1	91.72	5.15	2	
2	70.65	3.33	1, 3	1, 3
3	72.19	3.53	2, 4	1, 2, 4
4	68.88	3.25	3, 5	3, 6
5	71.99	3.60	4	1, 6
6	59.94	3.61	5	4
1′	60.88	3.46		
2′	103.35			1, 1′, 3′, 4′, 5′
3′	75.81	3.97	4′	1′, 4′
4′	74.01	3.82	3′, 5′	3′
5′	79.86	3.72	4′, 6′	4′, 6′
6′	62.58	3.55	5′	4′
		3.73		
1″	59.48	3.46		
		3.53		
2″	103.35			1″, 3″, 4″, 5″
3″	76.20	3.94	4″	1″, 4″
4″	74.35	3.87	3″, 5″	3″
5″	80.77	3.65	4″, 6″	6″
6″	62.20	3.40	5″	4″
		3.61		

[a] With an internal standard of acetone at 2.04 p.p.m. for ^{1}H and 29.8 p.p.m. for ^{13}C. [b] Includes ZQCOSY, DQCOSY, and COSY experiments.

spectrum, a normal COSY[10] spectrum is not the most suitable experiment to be used for proton correlation due to the signal–signal and signal–diagonal peak overlap. Two COSY-related techniques, namely zero-quantum and double-quantum COSY (ZQCOSY[11,12] and DQCOSY[12]), were employed in this work. These two experiments are complementary to the COSY experiment and particularly useful when there is extensive overlap of cross-peaks with the diagonal peaks.

The glucose ^{1}H signals, H-2, H-3, H-4, and H-5, were assigned using the DQCOSY and ZQCOSY spectra for both **1** and **2**, starting from H-1 and following the correlations, and these assignments were further confirmed by the normal COSY experiment. Starting from the H-3 signals of D-fructosyl units, the only doublets remaining, the connectivity between the 3,4,5, and 6 positions of the two D-fructosyl units were also determined by ZQCOSY and DQCOSY spectra. However, these rings could not be distinguished. In order to do so, two different approaches were applied to the two compounds.

For compound **2**, long-range coupling between H-1 and C-2′, was observed. Then by the long-range couplings of C-2′ to H-1′, H-1′ to C-3′, and C-3′ to H-4′, one could differentiate the proton signals from two D-fructosyl units, leading to the completion of proton spectrum assignments of **2**.

TABLE II

N.m.r. data for neokestose[a]

Atom No.	*^{13}C Shift (p.p.m.)*	*^{1}H Shift (p.p.m.)*	*Assigned ^{1}H*	
			All COSY[b] cross-peaks	*Long-range C–H correl. cross-peaks*
1	91.56	5.22	2	
2	70.58	3.37	1, 3	1, 3
3	72.03	3.56	2, 4	1, 2, 4
4	68.81	3.34	3, 5	3
5	71.11	3.77	4	4
6	59.95	3.50	5	4
		3.66		
1′	61.13	3.46		
2′	103.29			1, 1′
3′	75.94	4.04	4′	1′, 4′
4′	73.57	3.89	3′, 5′	3′
5′	80.70	3.69	4′, 6′	3′
6′	61.96[c]	3.53	5′	4′
		3.63		
1″	59.89	3.52		
		3.64		
2″	103.24			1″
3″	76.47	4.00	4″	1″, 4″
4″	74.01	3.97	3″, 5″	3″
5″	80.90	3.71	4″, 6″	3″
6″	61.93[c]	3.54	5″	4″
		3.63		

[a] With an internal standard of acetone at 2.04 p.p.m. for ^{1}H and 29.8 p.p.m. for ^{13}C. [b] Includes ZQCOSY, DQCOSY, and COSY experiments. [c] Assignments may be interchanged.

In **1** the chemical shift of C-2′ and C-2″ are identical, and the above method of using long-range C–H coupling cannot be applied to distinguish the two fructosyl units. Two-dimensional *n*uclear *O*verhauser *e*nhancement *s*pectroscop*y* (NOESY)[13] was used to accomplish this task. This experiment gives through-space couplings between ^{1}H signals. The first D-fructosyl unit is closer to H-1 on the glucose unit compared to the second D-fructosyl unit; therefore, the n.O.e. between H-1 to any one of the ^{1}H signals from D-fructose rings will be the key in discriminating the D-fructose units. In the NOESY spectrum of **1**, the cross-peak between H-1 and the proton signal at 3.84 p.p.m. indicated that this signal could be assigned as H-4′ and thus, the signal at 3.89 p.p.m. was assigned to H-4″. These assignments, combined with the results from the correlation experiments, allow us to distinguish H-3, H-5, and H-6 of the two D-fructosyl units of **1**. H-1′ and H-1″ were assigned based on the H-1′ to C-3′ and H-1″ to C-3″ long-range couplings.

The determination of the multiplicities of the ^{1}H signals were usually complicated by overlapping or unresolved peaks, but the problem was overcome by employing the homonuclear *J*-resolved experiment[14]. The experiment disperses the coupling pattern in

the F1 dimension, enabling not only the determination of signal multiplicity and precise chemical shifts, but also their coupling constants for first-order couplings, even for overlapped signals.

Carbon spectral assignments. — Based on the above ^{1}H assignments, ^{13}C assignments can be made with a one-bond heteronuclear (C–H) shift correlation spectrum[15]. This experiment was carried out to correlate the above ^{1}H assignments with the carbon signals. Most of these assignments were confirmed by the long-range heteronuclear (C–H) shift-correlation spectrum. For C–H correlation experiments, both ^{13}C and ^{1}H detetcted were performed to obtain higher resolution in the ^{13}C and ^{1}H dimensions alternately. The ^{13}C detected long-range experiment was one developed by Martin and coworkers which uses a BIRD pulse to suppress one-bond correlation peaks, and in our hands provides the highest signal/noise ratio for this type of experiment[16].

In the long-range C–H correlation spectra of **2**, the cross-peak between H-1 and ^{13}C signal at 102.14 p.p.m. gives the key to differentiate the two D-fructosyl units. The peak at 102.14 p.p.m. thus was assigned to C-2′ and the one at 102.11 to C-2″. Along C-2′ to H-1′ and C-2″ to H-1″ cross-peaks C-3′ and C-3″ can be assigned. The cross-peak between H-4 and ^{13}C signal at 58.81 give us the C-6 assignment. All signals except C-6′ and C-6″ could be unambiguously assigned, but their small separation (1 Hz) precluded distinguishing them.

EXPERIMENTAL

N.m.r. spectra. — One hundred milligrams of **1** or **2** was dissolved in 1.5 mL of D_2O and transferred to a 5-mm n.m.r. tube.

Nuclear magnetic resonance spectra, 1D and 2D, were recorded using a Bruker AF 200 narrow-bore spectrometer with a broad-band probe operating at 200.13 MHz for ^{1}H and 50.32 MHz for ^{13}C and a Bruker AMX 360 MHz wide-bore spectrometer with an inverse broad band probe operating at 360.13 MHz for ^{1}H and 90.56 MHz for ^{13}C.

COSY, ZQCOSY and DQCOSY. — These experiments were recorded using the AF 200 instrument with a sweep width of 593.824 Hz in the F2 dimension and 400 Hz in the F1 dimension for ZQCOSY and DQCOSY. Each spectrum was composed of 256 experiments and 16 scans for each experiment acquiring 2K complex data points. A water-suppression routine, by T2-relaxation, was incorporated in these experiments to eliminate the water peak. The total experiment time for each spectrum was about 6 h.

Nuclear Overhauser enhancement spectroscopy (NOESY). — This determination was carried out using the AMX 360 instrument with a sweep width of 1865.67 Hz for both dimensions and 512 experiments and 32 scans for each experiment. The mixing time was optimized to 750 ms. A sine window with a shift of $\pi/2$ was multiplied to both dimensions prior to phase-sensitive Fourier transform.

Homonuclear (H–H) J-resolved spectra. — These spectra were recorded using the AMX 360 instrument at a sweep width of 1865 Hz in the F2 dimension and 30 Hz in the F1 dimension. The spectra required 40 experiments and 32 scans for each experiment.

The total experiment time was about 30 min.

One-bond C–H correlation and long range C–H coupling spectra. — These experiments were carried out using both the AF 200 machine (for ^{13}C detection) and the AMX 360 machine (for ^{1}H detection).

Both of the C–H shift-correlation spectra (^{13}C detection) were comprised of 128 experiments, each having 2K complex data points with the sweep width of 2958.58 Hz in the F2 dimension and 296.92 Hz in the F1 dimension. The one-bond C–H correlation spectrum was recorded using 200 scans per experiment requiring a total experimental time of 11 h, while the long-range C–H correlation experiment was recorded using 400 scans and requiring a total experimental time of 18 h.

The ^{1}H-detected correlation spectra[17] were recorded with sweep widths of 1260 Hz in the F2 dimension and 6250 Hz in the F1 dimension. The one-bond C–H correlation spectra were recorded using 256 experiments with 32 scans per experiment, requiring a total experimental time of 1.5h, while the long-range C–H correlation experiments were recorded using 128 experiments with 192 scans per experiment and requiring a total time of 10 h.

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