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COMPLETE ASSIGNMENT OF THE ^{1}H-NMR SPECTRUM OF STACHYOSE BY TWO-DIMENSIONAL NMR SPECTROSCOPY

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ABSTRACT.—The ^{1}H-nmr spectrum of the tetrasaccharide stachyose [*O*-α-D-galactopyranosyl-(1↦6)-*O*-α-D-galactopyranosyl-(1↦6)-*O*-α-D-glucopyranosyl-(1↦2)-β-D-fructofuranoside] has been completely assigned on the basis of 2D COSY, relayed, double relayed, and triple relayed coherence transfer COSY, and ^{13}C-^{1}H correlation experiments. All ^{1}H-nmr signals could be assigned unambiguously despite the fact that most of the signals fall in the relatively narrow range of δ_H 3.53 to 4.14 ppm. All two-bond proton-proton coupling constants were obtained from a phase-sensitive COSY spectrum.

The T_1 relaxation times for all carbons were also obtained at 80° and are interpreted in terms of different correlation times of the various saccharide rings in this molecule.

The tetrasaccharide stachyose [*O*-α-D-galactopyranosyl-(1↦6)-*O*-α-D-galactopyranosyl-(1↦6)-*O*-α-D-glucopyranosyl-(1↦2)-β-D-fructofuranoside] is widely distributed in nature. It is found in many species of plants, notably beans and other legumes. Due to the degradation of stachyose and the related trisaccharide raffinose [*O*-α-D-galactopyranosyl-(1↦6)-*O*-α-D-glucopyranosyl-(1↦2)-β-D-fructofuranoside] into CO_2, CH_4, and H_2, these sugars are responsible for the flatulence associated with the consumption of such foods (1,2).

^{13}C- and ^{1}H-nmr spectroscopy have proven to be valuable tools for the study of oligosaccharides, but the major difficulty, due to signal overlap, has been the complete assignment of the ^{1}H spectra. Although the anomeric ^{1}H signals are generally well resolved, most of the non-anomeric ^{1}H signals fall into the narrow chemical shift range of δ_H 3.4 to 4.0 ppm (3); thus, assignments based solely on conventional 1D spectra are usually difficult if not impossible due to excessive signal overlap. As the carbon spectra of such molecules are, in many cases, easily assigned by a variety of techniques (4), the use of 2D heteronuclear ^{13}C-^{1}H correlation spectra, obtained from carbon-detected HETCOR experiments (5), or proton-detected HMQC experiments (6), may be of considerable assistance in the assignment of ^{1}H spectra. However, overlap of resonances in the region δ_C 68–75 ppm, which arise from non-anomeric ring carbons, may lead to difficulties using this approach in the absence of other information. Conventional 2D ^{1}H COSY spectra are very helpful in this regard, but even with the aid of phase-sensitive spectra, complete ^{1}H assignments are frequently difficult to obtain by this route alone due to overlapping cross peaks near the diagonal.

Relayed coherence transfer COSY spectra, in which the magnetization is relayed starting from the anomeric proton to the other protons on each individual saccharide ring, has proven to be of considerable utility in the assignment of ^{1}H spectra (7,8). By the use of standard COSY, relay COSY, multiple relay COSY, and, if necessary, a HETCOR spectrum, it is possible to assign all protons in spectra of most oligosaccharides. Here we have used this procedure to completely assign the ^{1}H spectrum of the tetrasaccharide stachyose, which to our knowlege has not been reported in the literature. In one of the earliest applications of HETCOR spectroscopy, the ^{1}H-nmr spectrum of raffinose has been assigned (9). The chemical shifts of the glycosidic protons of stachyose have been recorded (10).

RESULTS AND DISCUSSION

ASSIGNMENT OF THE ^{1}H-NMR SPECTRUM OF STACHYOSE.—The triple-relay COSY spectrum of stachyose is presented in Figure 1, the phase sensitive COSY spectrum in Figure 2, and the HETCOR spectrum is shown in Figure 3. The complete assignments of the ^{1}H and ^{13}C spectra, the proton-proton coupling constants, and the ^{13}C T_1 relaxation times (see below) are tabulated in Table 1.

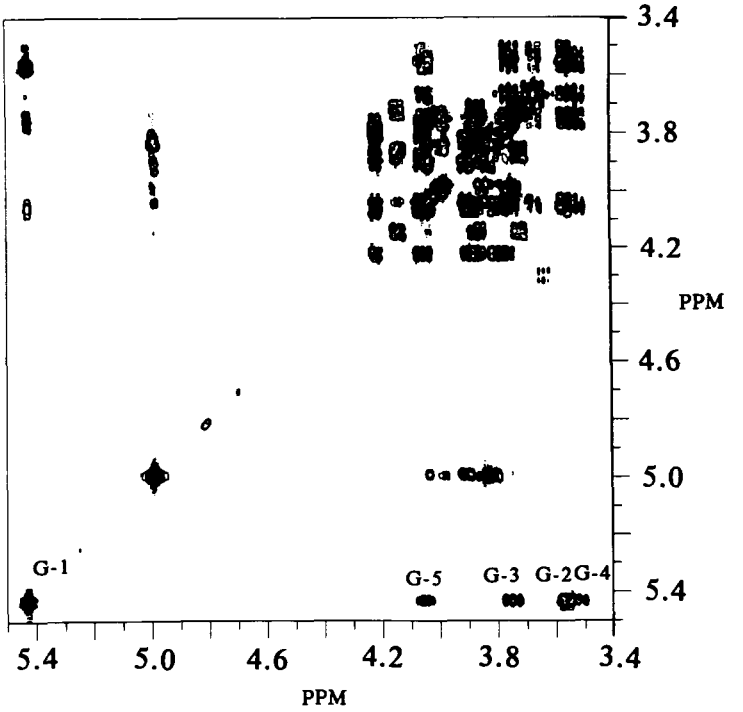

FIGURE 1. Triple relay COSY ^{1}H-nmr spectrum of stachyose in D_2O at 25°, obtained with presaturation of the residual HDO signal (at 4.82 ppm). This spectrum was obtained in the magnitude mode and has been background-subtracted. Cross peaks and the diagonal peak involving the galactose anomeric proton are labeled.

The assignment of the ^{1}H spectrum from the relayed-COSY and HETCOR spectra was straightforward. Starting from the glucose anomeric proton at 5.43 ppm (9), the chemical shifts of H-2, H-3, H-4, and H-5 were obtained from newly arising cross peaks with the anomeric proton in the COSY, relay COSY, double relay COSY, and triple relay COSY spectra, respectively (Figure 1). Assignment of the H-6 signal required the use of the HETCOR spectrum (Figure 3) or the phase-sensitive COSY spectrum (Figure 2); the greater resolution in the phase-sensitive spectrum allowed us to locate the H-5–H-6 crosspeaks unambiguously.

The galactose signals were assigned in a similar manner, starting from the anomeric protons at 4.99 ppm (9); however, two complications arise. One problem is that the small (1 Hz) coupling constant between H-4 and H-5 resulting from the axial-equatorial relationship of these protons makes it difficult to relay from H-3 to H-5, resulting in low cross-peak intensity. The required mixing time needed to optimize this transfer, which is in the neighborhood of 500 msec for a 1.0 Hz coupling (11), is prohibitive due to T_2 relaxation effects. This problem does not appear with glucans, where all the ring

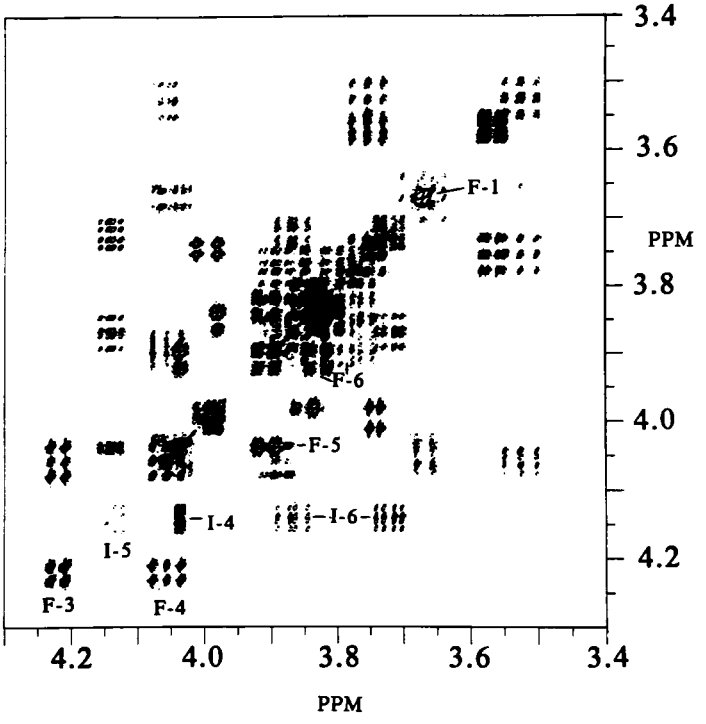

FIGURE 2. Phase-sensitive COSY spectrum of the non-anomeric protons of stachyose in D_2O at 25°, obtained with presaturation of the residual HDO signal. Various fructose cross peaks and diagonal peaks are labeled.

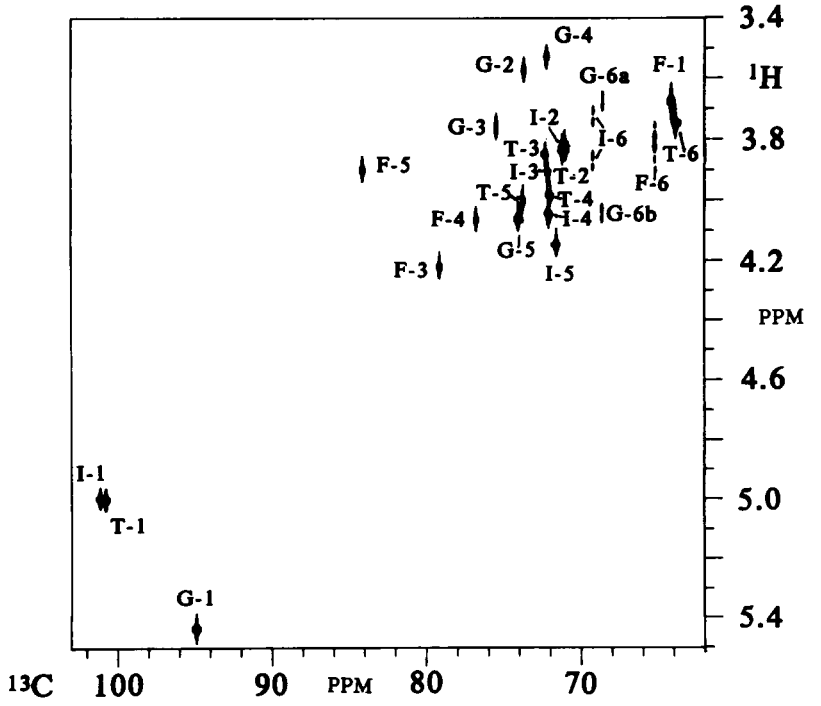

FIGURE 3. HETCOR nmr spectrum of stachyose in D_2O at 25°. Cross peaks are labeled according to the following: F, fructose; G, glucose; I, internal galactose; T, terminal galactose.

TABLE 1. Chemical Shift Assignments (δ, ppm, referred to TSP), Proton-Proton Coupling Constants (Hz) and ^{13}C Relaxation Times (sec) for Stachyose in D_2O Solution.

Sugar	Atom	δ_H (ppm) 25°	Coupling constant (Hz) 25°	δ_C (ppm) 25°	δ_C (ppm) 80°	T_1 (sec) 80°
Terminal	1	4.99	$J_{1,2}=4$	100.60	101.00	1.12
Galactose	2	3.81	$J_{2,3}=10$	71.08	71.16	0.84
	3	3.84	$J_{3,4}=3$	72.28	72.56	0.78
	4	3.98	$J_{4,5}=1$	72.03	72.13	0.95
	5	4.00	$J_{5,6a}=7, J_{5,6b}=7$	73.75	73.73	0.90
	6	3.74, 3.74	$J_{6a,6b}=13$	63.94	63.91	0.98
Internal	1	4.99	$J_{1,2}=4$	101.15	101.26	0.79
Galactose	2	3.83	$J_{2,3}=10$	71.23	71.27	0.73
	3	3.91	$J_{3,4}=3$	72.14	72.30	0.73
	4	4.04	$J_{4,5}=1$	72.14	72.21	0.71
	5	4.14	$J_{5,6a}=5, J_{5,6b}=8$	71.58	71.66	0.69
	6	3.72, 3.87	$J_{6a,6b}=11$	69.27	69.47	0.46
Glucose	1	5.42	$J_{1,2}=4$	94.88	94.90	0.82
	2	3.57	$J_{2,3}=10$	73.75	73.85	0.71
	3	3.75	$J_{3,4}=9$	75.51	75.65	0.72
	4	3.53	$J_{4,5}=10$	72.28	72.43	0.90
	5	4.06	$J_{5,6a}=9, J_{5,6b}=4$	74.07	74.14	0.69
	6	3.67, 4.05	$J_{6a,6b}=13$	68.66	69.10	0.46
Fructose	1	3.67, 3.67		64.23	64.73	0.53
	2	—		106.58	106.60	5.8
	3	4.22	$J_{3,4}=9$	79.14	79.77	0.85
	4	4.06	$J_{4,5}=9$	76.80	76.79	0.94
	5	3.89	$J_{5,6a}=7, J_{5,6b}=4$	84.13	84.17	0.82
	6	3.77, 3.83	$J_{6a,6b}=12$	65.26	65.24	0.64

protons are axial, due to the larger (typically 10 Hz) coupling constants involved. Alternatively, it was possible to initiate the assignments from H-6 in the phase-sensitive COSY spectrum. In order to distinguish which set of signals belonged to the internal galactose versus the terminal galactose sugars, the downfield shift of the H-4, H-5, and one of the H-6 protons was used. In the terminal galactose, these shifts were similar to those observed in the monosaccharide (9).

The other difficulty encountered when making these assignments was that the chemical shifts of the anomeric protons on the two galactose rings are almost identical, at 4.99 ppm, as are the H-2 protons (3.81 and 3.83 ppm) and the H-3 protons (both at 3.91 ppm). Therefore, the cross peaks between the anomeric protons and H-2 in the COSY spectrum and between the anomeric protons and H-3 in the relay COSY spectrum overlap. Inspection of the phase-sensitive COSY spectrum revealed a small (0.005 ppm) difference in chemical shift between the anomeric protons, as revealed in the H-2 cross peak (data not shown). Due to the relatively low resolution and consequent loss of fine structure in the cross peaks of the magnitude-mode spectra, the cross peaks merged in these spectra. From the phase-sensitive COSY and relayed COSY spectra, it was then possible to assign the remainder of the galactose signals, in the same manner that the glucose signals were assigned.

The H-1 signals of the fructose moiety, which do not couple with other protons, were assigned from the HETCOR spectrum, using the known ^{13}C chemical shift. The remainder were assigned from the multiple relay COSY spectra, beginning with H-3 at 4.22 ppm, which was assigned on the basis of its unique chemical shift (9).

^{13}C-NMR SPECTRA AND RELAXATION TIMES OF STACHYOSE.—The ^{13}C chemical shifts obtained in this study and checked against the independently derived ^{1}H assignments were in fairly close agreement with the published values (12–14) considering their temperature dependence. For this reason, we have reported the chemical shifts at two different temperatures, 25° and 80°. At intermediate temperatures, the chemical shifts varied approximately linearly with temperature. The signals with the largest temperature effect on the chemical shift were C-1 and C-3 of the fructose ring, C-6 in the glucose ring, and the anomeric carbon of the terminal galactose ring. In many cases, changing the temperature is useful in the resolution of overlapping signals, and this effect has been of some use in the assignment of ^{13}C spectra of carbohydrates (15). In the case of stachyose, the complex series of overlapping signals around δ_C 72 ppm could only be resolved upon heating to 80°; thus, the T_1 data were obtained at this temperature.

One-bond carbon-proton coupling constants for the three pyranose anomeric carbons were all 170 Hz, which confirmed that all (1→6) linkages were, in fact, α (16).

The relaxation times (T_1) measured for the stachyose carbons at 80°, with the exception of the quaternary carbon of the fructose ring, ranged from 0.46 to 1.12 sec (Table 1). The shortest relaxation times were observed for C-6 in the glucose and the internal galactose units. The relaxation times for all the carbons in the terminal galactose ring are longer than those for the internal ring.

CONCLUSIONS

The complete ^{1}H-nmr spectrum of the tetrasaccharide stachyose has been assigned with the assistance of COSY, multiple relayed COSY, and carbon-proton correlation spectra. In the main, the proton chemical shifts and coupling constants were unexceptional for a molecule of this nature. As one would expect, the shifts for the protons in the 1, 2, 3, and 4 positions for the two galactose rings were quite similar for both rings, with the exception that, for H-4, the internal galactose signal was 0.05 ppm to low field of the corresponding terminal galactose signal. In the case of the H-6 signals for these galactose residues, the diastereomeric protons had similar chemical shifts in the case of the terminal galactose ring, but they differed by δ_H 0.12 ppm in the case of the internal galactose.

The observation that the terminal galactose carbons have longer relaxation times than the corresponding carbons in the internal galactose can best be explained by the fact that, due to the dominance of the dipole-dipole relaxation mechanism in molecules of this nature, a shorter correlation time for the terminal galactose ring relative to the internal galactose ring (due to greater mobility) would result in longer relaxation times for the carbons on the terminal ring.

Application of the techniques described herein permits the complete assignment of medium-sized carbohydrates. In fact, because a combination of different nmr techniques was used, there is a certain redundancy in the assignments, which gives more confidence in their correctness. One problem which should be stressed is that small (<2 Hz) coupling constants make relayed coherence transfer experiments less useful. In such cases, it is profitable to take the approach of identifying the C-6 protons from a carbon-proton shift correlation experiment, and then tracing the connectivities from these protons with the help of COSY and relay COSY spectra. In the case of larger carbohydrates with longer correlation times, the multiple relay COSY experiments are generally less useful because of effective T_2 relaxation during the mixing times. In these cases, the use of total correlation experiments such as HOHAHA (17–20) or TOCSY (21) may prove to be more useful because of the shorter mixing times that can be used in these experiments (22).

EXPERIMENTAL

The sample of stachyose used in this investigation was obtained from the Sigma Chemical Company, St. Louis, Missouri and was used as received. All spectra were obtained from D_2O solutions and are referred to internal sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP). Sample concentrations used were typically 5% w/v for ^{1}H spectra, and 10% w/v for ^{13}C spectra.

All spectra were obtained using a Bruker AM-400 nmr spectrometer operating in the FT mode at 400.1 MHz for ^{1}H and 100.6 MHz for ^{13}C. Standard Bruker software, with modified phase cycling for relay COSY experiments, was used throughout. In all ^{1}H spectra, the residual HDO signal at δ_H 4.82 ppm was suppressed by the use of presaturation. ^{13}C spectra were broadband proton-decoupled by the use of composite pulse decoupling which was employed in a bilevel scheme, 2 W during acquisition and 0.04 W during the relaxation delay. In the case of the magnitude mode COSY and relay COSY spectra, the original 256 × 1024 data matrix was zero-filled in both the F1 and F2 dimensions to yield a 1024 × 2048 data matrix, giving a final digital resolution of 1.2 Hz/point. The total mixing time in all relay COSY experiments was 60 msec, which is the optimum value (13) for the 8–10 Hz couplings commonly observed between axial protons in pyranose rings. The number of scans for each t_1 increment, using a 1250 Hz spectral width, was 4, 8, 16, and 64 for the COSY, relay COSY, double relay COSY, and triple relay COSY spectra, respectively. All COSY spectra were apodized with a standard sine-bell function in both F1 and F2 and are presented in the magnitude mode, with the exception of the phase-sensitive COSY spectrum. This spectrum, obtained as a 1024 × 2048 data matrix using a 1000 Hz spectral width, was zero-filled to 2048 × 4098 to give a final digital resolution of 0.5 Hz/point in the F2 dimension. Coupling constants were determined by measurement parallel to the F2 axis of the active coupling in the cross peaks of expanded plots of the spectrum.

In the case of the HETCOR experiment, vicinal proton decoupling was employed (23) with phase cycling as suggested by Wilde and Bolton (24), using the Bruker microprogramme XHCORRD.AU. This sequence is:

^{1}H: D1-90-D0-90-D3-180-D3-90-D0-D3-90-Decouple
^{13}C: D1 180 90-D4-FID

where D0 is the incremental delay, D1 the relaxation delay (1.5 sec), D3 is 1/(2J X-H), and D4 is 1/(4J X-H). A value of 140 Hz was assumed for J X-H. The 256 × 1024 data matrix was zero-filled to 2048 × 2048, giving a digital resolution of 1.2 Hz/point in F1 and 11.7 Hz/point in F2. The spectral width in F1 (^{1}H) was 1250 Hz; in F2 (^{13}C), it was 6000 Hz; 128 scans were used per t_1 increment. The resulting matrix was apodized with a Lorentz-Gauss function in both F1 (line broadening = −1.0 Hz, Gaussian broadening fraction = 0.2) and F2 (line broadening = 5.0 Hz, Gaussian broadening fraction = 0) prior to transformation. The contour display is presented in the magnitude mode.

Spin-lattice relaxation times (T_1) were measured for the ^{13}C spectra of stachyose using the inversion-recovery method with a recycle time of 30 sec, 10 delay values, and delay list cycling. The resulting data were processed with standard Bruker software. Typical standard deviations for the T_1 values were in the range 0.02 to 0.04.

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