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Heteronuclear NMR studies of ^{13}C-labeled yeast cell wall β-glucan oligosaccharides

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SUMMARY

The structures of uniformly ^{13}C-labeled β-glucan octa- and undeca-oligosaccharides enzymatically prepared by the yeast cell wall glucanosyl transferase of *Candida albicans* were characterized by using a combination of HCCH-COSY, HCCH-TOCSY, and HMBC experiments. The oligosaccharide structures indicate that the cell wall glucanosyl transferase cleaves two glucosyl units from the reducing end of the initial linear β(1→3) penta-oligosaccharide and subsequently transfers the remainder to another oligosaccharide at the nonreducing end via a β(1→6) linkage. These results indicate that the combined action of cell wall glucanase and glucanosyl transferase activities could not only introduce intrachain β(1→6) linkages within a single glucan strand, but also result in cross-linking of two initially separate glucan strands with concurrent introduction of intrachain β(1→6) linkages. Since isolated fungal membranes only synthesize linear β(1→3) glucan strands, wall-associated enzymes probably participate in the assembly of the final wall glucan structure during cell growth and division.

INTRODUCTION

Carbohydrates have been shown to play an important role in cell recognition and adherence, modulating protein function and dynamics, and controlling cellular shape and support (Bush, 1992). Although structural elucidation of carbohydrates by NMR has mostly relied upon conventional 1D and 2D NMR methods (Byrd et al., 1987; Homans et al., 1987; Lerner and Bax, 1987; Tsui et al., 1988; Skelton et al., 1991; Miller et al., 1992; Poppe et al., 1992; Riviere and Puzo, 1992; Shibata et al., 1992; Van Halbeek and Poppe, 1992; Whitfield et al., 1992), homonuclear and heteronuclear 3D NMR experiments (Fesik et al., 1989; De Waard et al., 1992; Homans, 1992; Rutherford and Homans, 1992) have recently been applied in studies of oligosaccharides.

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However, due to the low natural abundance of ^{13}C, heteronuclear multidimensional experiments involving ^{13}C–^{13}C coherence transfers cannot be readily applied to the studies of unlabeled sugars.

Here we demonstrate the usefulness of ^{13}C-labeled sugars in the structure determination of β-glucan octa- and undeca-oligosaccharides formed by the cell wall glucanosyl transferase (Hartland et al., 1991). β-Glucan, a homopolymer of glucose, is the major structural component of the cell wall in fungi and bacteria. Linear β-glucan is synthesized at the plasma membrane by a process of vectorial extrusion through a UDP-glucose-dependent glucan synthetase (Cabib et al., 1988). Branching and cross-linking of the synthesized linear β-glucan is proposed to occur in the cell wall. Recently, a cell wall glucanosyl transferase has been isolated from an important pathogenic yeast, *Candida albicans* (Hartland et al., 1991). This enzyme specifically transforms the initial linear β(1→3) glucan into a β(1→3) glucan with β(1→6) linkages by cleaving two glucosyl units from the reducing end of the linear β(1→3) glucan and transferring the remaining sugar residues to another linear β(1→3) glucan. Although the types of linkages have been previously determined, based on an analysis of 1D ^{1}H and ^{13}C NMR spectra of the glucanosyl transferase products by comparison to the chemical shifts of other sugars (Hartland et al., 1991), the exact location of the β(1→6) linkage site on the linear β(1→3) glucan has not been identified. This structural information is important for gaining insights into the mechanism of the glucanosyl transferase and understanding how the branched and cross-linked glucan in the cell wall is biologically synthesized. By using ^{13}C-labeling techniques, the structures of these ^{13}C-labeled β-glucan oligosaccharides were obtained from recently developed heteronuclear NMR techniques, which are widely used in NMR studies of ^{13}C-labeled proteins. The NMR techniques used in this study have been optimized for the study of sugars to enable high-resolution spectra to be recorded, which is important for resolving the poorly dispersed signals of the β-glucan oligosaccharides.

MATERIALS AND METHODS

Preparation of uniformly ^{13}C-labeled β-glucan oligosaccharides G8 and G11

Candida albicans strain ATCC 10261 (obtained from the American Type Culture Collection, Rockville, MD) was grown at 37 °C to stationary phase in yeast nitrogen base media (Difco) containing 0.125% (w/v) uniformly labeled ^{13}C-glucose (Cambridge Isotopes). Cells were washed in H_2O and broken with glass beads in an MSK homogenizer. Cell walls were isolated by centrifugation, washed with H_2O, and extracted with 1% SDS in H_2O at 90 °C for 10 min. Cell walls were then washed with H_2O and lyophilized (360 mg dry weight). Walls suspended in 45 ml 0.01 M Na/K phosphate buffer, pH 7.5, were then digested at 37 °C for 15 h with laminarapentaohydrolase (388 μg, 0.4 A_{280} units) prepared from Zymolyase 100T (Seikagaku Inc., Rockville, MD) as described previously (Kitamura et al., 1974). The digest was heated to 65 °C for 10 min to inactivate the enzyme, centrifuged at 10 000 × g for 10 min, and then the supernatant was

Abbreviations: G5, β(1→3) laminarapentose or penta-oligosaccharide; G8, octa-oligosaccharide; G11, undeca-oligosaccharide; UDP, uridine diphosphate; 1D, one-dimensional; 2D, two-dimensional; 3D, three-dimensional; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; HCCH, proton–proton correlation via carbon–carbon coupling; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence; HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate.

lyophilized. The sample was dissolved in 5 ml H_2O and 2.5 ml aliquots were applied to a column of Sephadex G25 (2.0 × 145 cm) equilibrated with H_2O. The column was eluted with H_2O and 5 ml fractions were collected and assayed for sugar by the phenol sulfuric acid method (Dubois et al., 1956). Material collected from the included volume of the column (laminaraoligosaccharides) was pooled, lyophilized (93 mg dry weight), dissolved in 5 ml H_2O, and rerun on Sephadex G25. This sample was eluted in the included volume with a slight trailing edge which was not pooled in the final sample (55 mg dry weight).

The ^{13}C-laminarapentose preparation (30 mg) was dissolved in 120 µl sodium acetate buffer (200 mM), pH 5.6, mixed with 1 ml (30 µg) of glucanosyl transferase isolated from *Candida albicans* (Hartland et al., 1991), and incubated for 45 h at 37 °C. The progress of the reaction was monitored by HPLC analysis as described (Hartland et al., 1991), and the G8 and G11 products were isolated by preparative HPLC using a TSKHW-40CS column (25–40 µm; 177 × 16 mm) run with H_2O at 0.1 ml/min and monitored by refractive index. The G8 and G11 products were lyophilized and dissolved in D_2O for the NMR studies.

NMR measurements

All NMR spectra were collected at 30 °C on a Bruker AMX 500 (500 MHz) NMR spectrometer. The 2D HCCH-TOCSY (Bax et al., 1990; Fesik et al., 1990) spectra were acquired using a pulse sequence that has been modified by including a constant time evolution period (Santoro and King, 1992; Van De Ven and Philippens, 1992; Vuister and Bax, 1992) and by concatenation of 180° pulses (Kay et al., 1991) as shown in Fig. 1A. The data sets contained 384 t_1 (^{13}C) × 1024 t_2

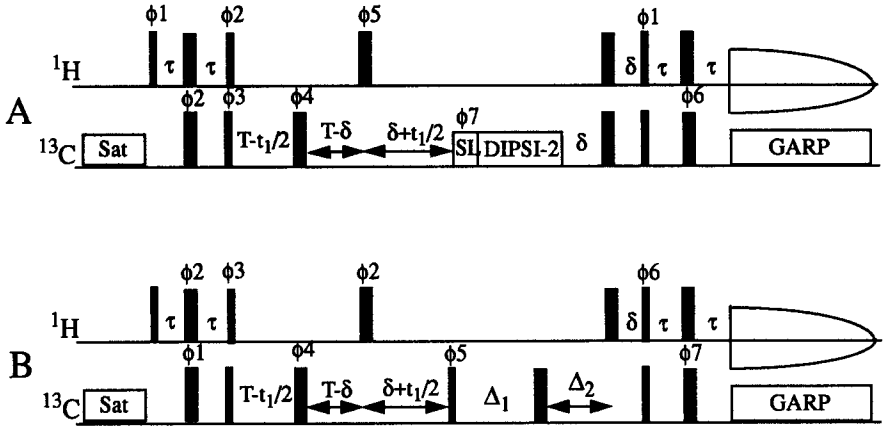

Fig. 1. Pulse sequences of the 2D constant time HCCH-TOCSY (A) and HCCH-COSY (B) experiments. The narrow and wide pulses have a flip angle of 90° and 180°, respectively. Pulses for which the phase is not indicated are applied along the x axis. The phase cycling for the HCCH-TOCSY sequence is: ϕ1 = 2(x), 2(−x); ϕ2 = y, −y; ϕ3 = 8(x), 8(−x); ϕ4 = 2(x), 2(y), 2(−x), 2(−y); ϕ5 = 4(x), 4(y), 4(−x), 4(−y); ϕ6 = 4(x), 4(y); ϕ7 = x, −x; and receiver = 2(x, 2(−x), x), 2(−x, 2x, −x). All pulses of the DIPSI-2 sequence are applied along the ± y axis. The phase cycling for the HCCH-COSY sequence is: ϕ1 = x, −x; ϕ2 = 4(x), 4(y), 4(−x), 4(−y); ϕ3 = y, −y; ϕ4 = 2(x), 2(y), 2(−x), 2(−y); ϕ5 = 4(x), 4(−x); ϕ6 = 8(x), 8(−x); ϕ7 = 2(x), 2(−x); and receiver = x, 2(−x), x, 2(−x, 2x, −x), x, 2(−x), x. The ^{13}C signals are initially saturated by applying a series of 90° pulses followed by short delays of 20 to 100 µs for a total time of 1.3 ms. Quadrature in t_1 was obtained using the States-TPPI method (Marion et al., 1989) by phase cycling the first 90° carbon pulse. ^{13}C decoupling during data acquisition was accomplished using a GARP decoupling scheme (Shaka et al., 1985).

(^{1}H) complex points with spectral widths of 7692 Hz (^{13}C) and 2000 Hz (^{1}H). The total constant time period (2T) was set to 50 ms, in order that $\cos(2\pi J_{CC}T) = 1$ for a J_{CC} value of 40 Hz, which is representative of the J_{CC} values (39–46 Hz) typically observed in saccharides. The delay τ was set to 1.5 ms, and the delay δ to 1.2 ms; this choice should result in optimal magnetization transfer for methine and methylene groups simultaneously. The spin-locking pulse (SL) was applied for a duration of 1.0 ms. DIPSI-2 (Shaka et al., 1988) mixing times of 14 and 25 ms were employed with a radiofrequency field strength of 8.1 kHz. The ^{1}H carrier was set on the water frequency. Water suppression was accomplished by applying a spin-locking purge pulse of 1.0 ms duration just before data acquisition. A total of 32 transients per t_1 complex point were accumulated with 1.5 s relaxation delay between scans.

The 2D HCCH-COSY (Kay et al., 1990) spectra were acquired using a pulse sequence that was also modified by incuding a constant time evolution period (Ikura et al., 1991) and by concatenation of 180° pulses as shown in Fig. 1B. The HCCH-COSY spectra were collected with a total constant time period (2T) set to 42 ms. This time period was chosen to allow $2\pi J_{CC}T = n\pi/4$, where n is equal to 7. The total constant time period of 42 ms was chosen in order to obtain high-resolution spectra in the ^{13}C dimension, which is important for resolving the poorly dispersed signals of the sugars. Unlike with proteins, the loss in sensitivity due to relaxation decay during evolution time is relatively small. The delays τ and δ were set to 1.5 and 1.2 ms, respectively. The refocusing delays between the second and third carbon 90° pulses were set to 3.0 ms ($1/8J_{CC}$) for Δ_1 and 1.8 ms ($1/8J_{CC}-\delta$) for Δ_2. The other relevant parameters were identical to those used in acquiring the HCCH-TOCSY spectra.

The constant time 2D HSQC (Santoro and King, 1992; Van De Ven and Philippens, 1992; Vuister and Bax, 1992) spectra were collected with a constant time period (2T) of 50 ms. The refocused 2D HMBC spectra were acquired as described previously (Bax and Summers, 1986) by using a delay of 60 ms for the generation of antiphase proton magnetization. A total of 32 and 24 scans per t_1 complex point were collected for the HSQC and HMBC spectra, respectively. A total of 150 t_1 (^{13}C) increments were collected in the HMBC experiments. The residual water line in the HMBC spectra was not saturated. The other relevant parameters were identical to those used in acquiring the HCCH-TOCSY spectra.

NMR spectra were processed and analyzed using in-house written software on Silicon Graphics computers. Linear prediction was employed to improve the resolution in the indirect dimension as described previously (Olejniczak and Eaton, 1990) for all the constant time spectra. Typically, the time domain data was extended by 64 points.

RESULTS AND DISCUSSION

Assignment strategy

The ^{1}H and ^{13}C chemical shifts of G8 and G11 homo-oligosaccharides cover a relatively small region from 3.3 to 5.3 ppm for ^{1}H and 63 to 106 ppm for ^{13}C. Owing to the severe overlap of the ^{1}H and ^{13}C resonances within this small spectral region, it would be very difficult to make the complete assignments of these large homo-oligosaccharides by using conventional NMR methods. In order to assign the ^{1}H and ^{13}C resonances of G8 and G11, we prepared uniformly ^{13}C-labeled G8 and G11 and applied recently developed heteronuclear NMR techniques (Bax et al., 1990; Fesik et al., 1990; Kay et al., 1990; Ikura et al., 1991) that were optimally performed for

the study of oligosaccharides. By uniformly ^{13}C-labeling these large homo-oligosaccharides, we were able to utilize NMR experiments that involve ^{13}C–^{13}C coherence transfer (Bax et al., 1990; Fesik et al., 1990; Kay et al., 1990; Ikura et al., 1991), which is widely used in the NMR studies of ^{13}C-labeled proteins (Clore and Gronenborn, 1991). Since one bond carbon–carbon coupling constants are much larger and more uniform than three-bond proton–proton coupling constants in sugar residues, the coherence transfer through the ^{13}C network is much more efficient and predictable. Furthermore, the experiments utilize the resolution of both the ^{1}H and ^{13}C signals.

In these ^{13}C–^{13}C coherence transfer experiments, carbon–carbon coherence transfer can be accomplished by one-bond transfer (HCCH-COSY) (Kay et al., 1990; Ikura et al., 1991) or through multiple bonds by using an isotropic mixing sequence (HCCH-TOCSY) (Bax et al., 1990; Fesik et al., 1990). For ^{13}C-labeled proteins, the HCCH-COSY and HCCH-TOCSY data sets are typically acquired as 3D experiments with t_1 (^{13}C) × t_2 (^{1}H) × t_3 (^{1}H) and analyzed as 2D ^{1}H,^{1}H contour plots extracted at the individual ^{13}C frequencies. However, for the NMR studies of oligosaccharides, very high resolution is required which results in long data acquisition time for the 3D versions of these experiments. In order to obtain the necessary resolution, which is the key

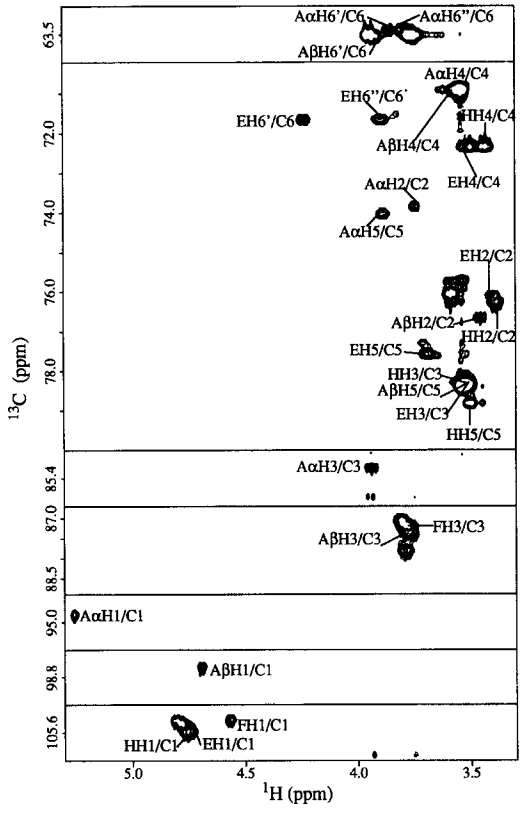

Fig. 2. 2D ^{1}H/^{13}C HSQC correlation spectrum of β-glucan oligosaccharide G8. The glucose residues in G8 are named alphabetically A through H for G8, starting from the reducing end. The carbon positions within each glucose unit are named C1, C2, C3, C4, C5, and C6, respectively. The proton positions within each glucose unit are named H1, H2, H3, H4, H5, H6', and H6". The α and β forms of the reducing end of glucose unit A are designated as Aα and Aβ.

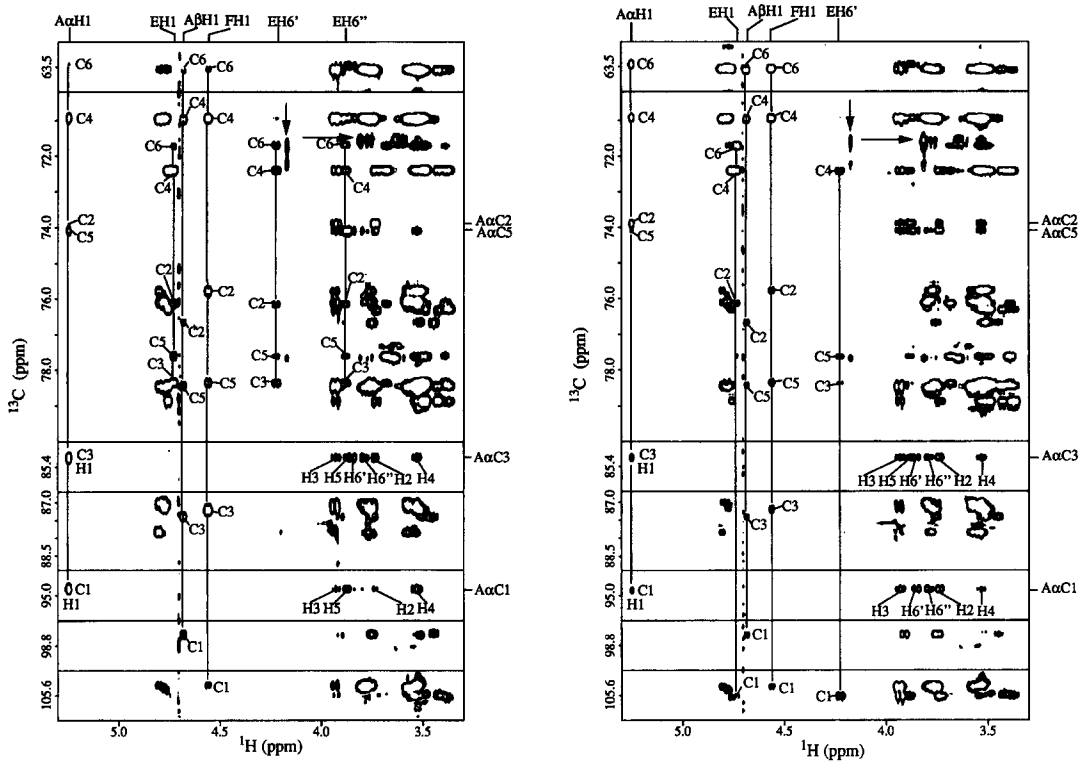

Fig. 3. (left) 2D constant time HCCH-TOCSY spectrum of β-glucan oligosaccharide G8. Coherence transfer mixing time is equal to 14 ms. The ^{1}H positions for those cross peaks whose ^{13}C assignments are labeled are indicated along the top of the contour plots. The ^{13}C positions for those cross peaks whose ^{1}H assignments are labeled are indicated along the right side of the contour plots. Arrows indicate the cross peaks corresponding to impurities in the sample.

Fig. 4. (right) 2D constant time HCCH-TOCSY spectrum of β-glucan oligosaccharide G8 acquired with a coherence transfer mixing time of 25 ms. The ^{1}H positions for those cross peaks whose ^{13}C assignments are labeled are indicated along the top of the contour plots. The ^{13}C positions for those cross peaks whose ^{1}H assignments are labeled are indicated along the right side of the contour plots. Arrows indicate the cross peaks corresponding to impurities in the sample.

for assigning these ^{13}C-labeled sugars, 2D versions of the HCCH-COSY and HCCH-TOCSY pulse sequences were employed that have been optimized (Fig. 1) by including a constant time ^{13}C evolution period to decouple the ^{13}C-^{13}C coupling (Santoro and King, 1992; Van De Ven and Philippens, 1992; Vuister and Bax, 1992), concatenating 180° pulses for shortening total delays (Kay et al., 1991), presaturating the ^{13}C signals before the start of the pulse sequence to insure the same initial magnetization, and setting optimal delays in the pulse sequences (see Materials and Methods) for studies of oligosaccharides. Using these two experiments and a ^{1}H/^{13}C HSQC spectrum, we were able to unambiguously correlate the ^{1}H and ^{13}C resonances of individual sugar residues of G8 and G11. In order to make the sequential assignments, the terminal sugar residues that were assigned based upon their characteristic chemical shifts were used as the starting points. The remaining sugar residues were then linked together and assigned from the interresidue ^{1}H/^{13}C three-bond correlations observed in the HMBC spectra of the ^{13}C-labeled oligosaccharides.

Oligosaccharide G8

The constant time $^{1}H/^{13}C$ HSQC spectrum of G8 is shown in Fig. 2. For a better representation of the data, only regions of the spectrum are shown that contain the $^{1}H/^{13}C$ cross peaks. The ^{1}H and ^{13}C assignments that are given in the spectrum refer to glucose residues of G8 that have been alphabetically named A through H, starting at the reducing end of the oligosaccharide. The first step of the assignment procedure involved the correlation of the individual ^{1}H and ^{13}C spins of each glucose unit in the 2D HCCH-COSY and HCCH-TOCSY spectra. For example, the characteristic upfield shifted anomeric carbon (C1) of the α form of the reducing terminal glucose residue A (Aα) (Heyraud et al., 1979) at 94.87 ppm was correlated to its attached anomeric proton (H1) at 5.248 ppm in the HSQC spectrum (Fig. 2). The ^{13}C chemical shifts of all the other carbons in Aα are found in the HCCH-TOCSY spectra along the ^{13}C dimension at the AαH1 proton chemical shift as labeled C2 through C6 (Figs. 3 and 4). In addition, the ^{1}H chemical shifts belonging to the individual sugar spin systems can be located in the HCCH-TOCSY spectra along the ^{1}H dimension as illustrated at the AαC1 and AαC3 carbon chemical shifts (Figs. 3 and 4). Since the coherence transfer efficiency is time dependent, recording the HCCH-TOCSY spectra at more than one mixing time is helpful for unambiguous identification of the ^{1}H and ^{13}C spin systems. For instance, the AαH1/C6 cross peak for the α form of residue A is hardly visible in the spectrum collected with a 14-ms mixing time (Fig. 3). However, this same cross peak is clearly present in the spectrum acquired with a 25 ms mixing time (Fig. 4).

In many cases, the identification of the ^{1}H and ^{13}C signals to a particular position on the sugar ring could be accomplished on the basis of characteristic ^{13}C chemical shifts. Any ambiguities were resolved from the HCCH-COSY spectra. For example, in the HCCH-COSY spectrum (Fig. 5), H2 of the α form of sugar A was unambiguously found at 3.736 ppm along the ^{1}H dimension at the AαC1 chemical shift, and the C2 was identified at 73.88 ppm along the ^{13}C dimension at the AαH1 chemical shift. Similarly, the H4 proton can be unambiguously identified at 3.534 ppm along the ^{1}H dimension at AαC3 ^{13}C chemical shift (Fig. 5). Thus, the complete ^{1}H and ^{13}C chemical shifts were obtained for the α form of the reducing terminal glucose unit A as listed in Tables 1 and 2.

TABLE 1
^{1}H CHEMICAL SHIFTS OF β-GLUCAN OLIGOSACCHARIDES G8 AND G11

Sugar residues		Positions						
G8	G11	H1	H2	H3	H4	H5	H6'	H6"
Aα	Aα	5.248	3.736	3.930	3.534	3.873	3.851	3.785
Aβ	Aβ	4.685	3.448	3.752	3.516	3.516	3.909	3.745
B	B	4.770	3.575	3.792	3.528	3.518	3.933	3.753
C	C	4.802	3.575	3.792	3.528	3.518	3.933	3.753
D	D	4.802	3.573	3.777	3.528	3.518	3.933	3.753
E	E,H	4.730	3.385	3.509	3.509	3.679	4.223	3.883
F	F,I	4.562	3.526	3.761	3.529	3.508	3.933	3.754
	G	4.775	3.570	3.766	3.528	3.518	3.933	3.753
G	J	4.775	3.575	3.780	3.528	3.518	3.933	3.753
H	K	4.758	3.370	3.507	3.414	3.489	3.928	3.728

In a similar fashion, the β form of the reducing terminal glucose A was assigned. The characteristic upfield shifted anomeric carbon (C1) (Heyraud et al., 1979) at 98.54 ppm was first correlated to its attached anomeric proton (H1) at 4.685 ppm in the HSQC spectrum (Fig. 2). The complete ^{1}H and ^{13}C chemical shifts were identified for Aβ by using the HCCH-TOCSY and HCCH-COSY experiments (see Figs. 3–5). The chemical shifts of the β form, as might have been expected, resemble those of normal β(1→3)-linked glucose residues more than those of the α form (see Tables 1 and 2).

Other unique signals were the anomeric H1 ^{1}H resonance of residue F at 4.562 ppm, which was correlated to its C1 at 105.39 ppm (cross peak FH1/C1, Fig. 2), and the resolved ^{1}H peak at 4.223 ppm, belonging to residue E, which was correlated with a carbon of an apparent CH_2 group at 71.72 ppm (cross peak EH6'/C6, Fig. 2). This CH_2 group is confirmed by identifying two cross peaks with identical ^{13}C chemical shifts at their CH_2 proton chemical shifts of 4.223 and 3.883 ppm (Fig. 3). The complete ^{1}H and ^{13}C chemical shifts for the residues E and F were obtained using the methods discussed above (see Figs. 3–5 and Tables 1 and 2).

The ^{1}H and ^{13}C chemical shifts of the other glucose residues B, C, D, G, and H in G8 are severely overlapped. However, we were still able to identify these spin systems completely by using the high-resolution HCCH-TOCSY and HCCH-COSY spectra. The HCCH-TOCSY connectivities for the residues D and H are shown in Fig. 6A. The ^{1}H and ^{13}C chemical shifts for these residues are listed in Tables 1 and 2.

In the next step of the assignment procedure, the sugar residues are linked together using the three-bond $^{1}H/^{13}C$ correlation across the glycosidic linkages present in the HMBC spectrum. The sugar residue A in G8 is the reducing terminal residue. The observation of a strong cross peak between the anomeric H1 proton of the residue B and the C3 carbon of the α-form of reducing terminal residue A (i.e., cross peak BH1/AαC3) in the HMBC spectrum (Fig. 7) uniquely assigns residue B as being linked to residue A via a β(1→3) linkage. Similarly, the HMBC cross peaks FH1/EC6, EH1/DC3, GH1/FC3, and HH1/GC3 (Fig. 7) indicate that residues F/E are linked via a β(1→6) linkage and residues E/D, G/F and H/G are connected via β(1→3) linkages. The sugar residue H is determined to be the terminal sugar residue since its C2, C3, C4, C5, and C6 ^{13}C

TABLE 2
^{13}C CHEMICAL SHIFTS OF β-GLUCAN OLIGOSACCHARIDES G8 AND G11

Sugar residues		Positions					
G8	G11	C1	C2	C3	C4	C5	C6
Aα	Aα	94.87	73.88	85.16	70.95	74.06	63.42
Aβ	Aβ	98.54	76.66	87.43	70.98	78.41	63.59
B	B	105.47	76.08	87.09	70.96	78.44	63.54
C	C	105.39	76.10	87.03	70.96	78.44	63.54
D	D	105.39	75.77	87.85	70.96	78.44	63.54
E	E,H	105.64	76.13	78.32	72.42	77.57	71.72
F	F,I	105.39	75.77	87.24	70.95	78.34	63.54
	G	105.42	75.80	87.93	70.96	78.44	63.54
G	J	105.47	76.08	87.18	70.96	78.44	63.54
H	K	105.67	76.29	78.42	72.42	78.84	63.54

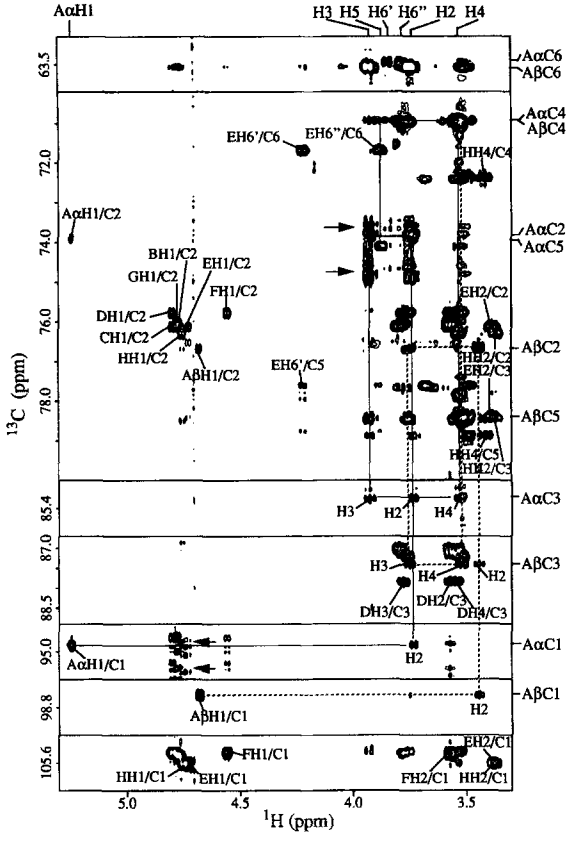

Fig. 5. 2D constant time HCCH-COSY spectrum of β-glucan oligosaccharide G8. Both positive and negative contour levels are plotted. The total constant evolution time (2T) used for acquiring this spectrum is equal to 42 ms. The ^{13}C positions for both the α and β forms of the terminal residue A are labeled along the right side of the contour plots. The ^{1}H positions for the α form of the terminal residue A are labeled along the top of the contour plots. The spin systems for the α form (solid lines) and the β form (dotted lines) of the terminal residue A are partially traced as an example of how to find the spin connectivity. Arrows mark the irregular peaks derived from the experimental artifacts. It was found that these artifacts are introduced in the HCCH-COSY spectra which have been acquired with a relatively long constant evolution time (2T). Thus, resolution enhancement in the ^{13}C dimension by using a long constant time period is compromised to some degree by the presence of some artifacts and small loss in the sensitivity due to the relaxation decay during the evolution time. The phases, i.e. positive or negative, of the cross peaks in the HCCH-COSY spectra, as expected, are dependent upon the length of the constant evolution period.

chemical shifts are characteristic of free OH groups. The C3 carbon of residue E also has a characteristic upfield shifted ^{13}C chemical shift (~ 9 ppm), clearly indicating that this residue has a free OH group at the C3 position. Therefore, these results dictate that the sugar residue E in G8 must be the terminal residue located at the nonreducing end and that the attachment of the trisaccharide to the linear β(1→3) penta-oligosaccharide chain must occur at the nonreducing end via a β(1→6) linkage to residue F.

The only remaining residue C in G8 is then assigned by elimination. The C3 ^{13}C resonances of the residues B and C and the anomeric H1 ^{1}H resonances of the residues C and D are severely

overlapped (Tables 1 and 2). Thus, the DH1/CC3 cross peak between residues D and C and the CH1/BC3 cross peak between residues C and B are not resolved in the HMBC spectrum. This overlapping of the resonances of the linkage groups could potentially limit the sizes of the oligosaccharides for sequence-specific assignments.

From an analysis of the NMR data, the structure of the oligosaccharide G8 was determined as shown in Fig. 8. The structure is consistent with the observed chemical shifts. For example, the upfield shifted signal at 4.562 ppm of the anomeric proton of residue F is consistent with the β(1→6) linkage to residue E. The sugar residue E has significantly downfield shifted C6 ($\Delta\delta = \sim 8$ ppm), H6' ($\Delta\delta = \sim 0.3$ ppm) and H6" ($\Delta\delta = \sim 0.1$ ppm) when compared to the C6, H6' and H6" of the other residues. Again, these unique shifts are in agreement with the structure, since the CH_2 group of residue E has been linked via a β(1→6) linkage while the other residues have free OH groups at this position. The determination of β(1→6) linkage from this study is consistent with the previous proposal from the comparison of 1D NMR spectra of known sugars (Hartland et al., 1991). Moreover, the determined structure of G8 clearly indicates that the cell wall glucanosyl transferase works by cleaving two glucose units from the reducing end of the initial linear β(1→3) G5 and then transferring the remaining three residues to another G5 at the nonreducing end via a β(1→6) linkage to form the product G8.

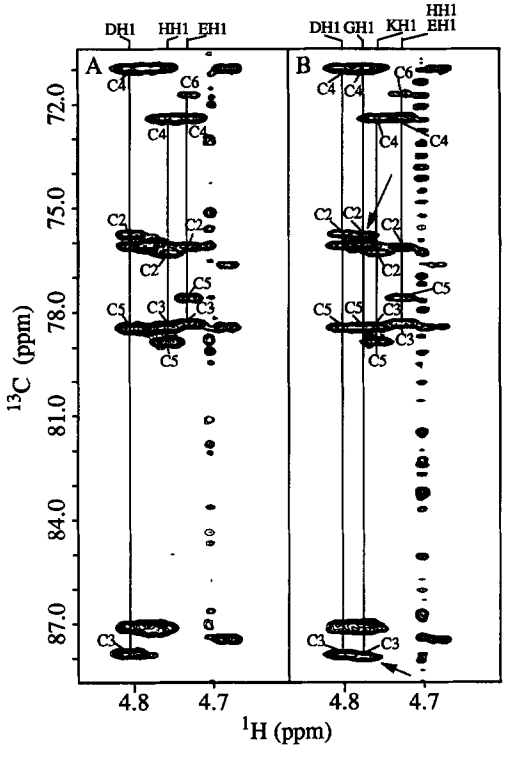

Fig. 6. Comparison of regions of the 2D constant time HCCH-TOCSY spectra of β-glucan oligosaccharides G8 (A) and G11 (B). The coherence transfer mixing times used are 14 ms. Arrows mark the additional cross peaks present in the G11 oligosaccharide. The ^{1}H positions for those cross peaks, whose ^{13}C assignments are labeled, are indicated along the top of the contour plots.

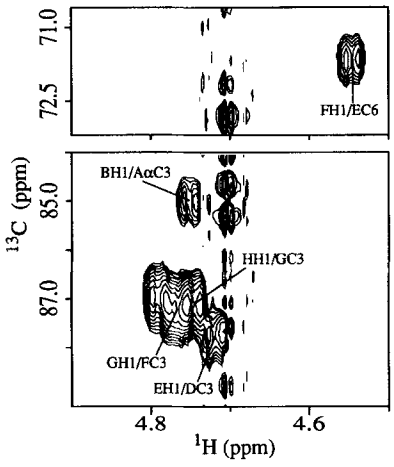

Fig. 7. 2D HMBC spectrum of β-glucan oligosaccharide G8. Residue solvent line was not saturated when acquiring this spectrum.

Oligosaccharide G11

The glucose residues in the oligosaccharide G11 are named alphabetically A through K, starting from the reducing end with residue A. Based on the ^{1}H and ^{13}C assignments of the oligosaccharide G8, G11 was readily assigned simply by inspecting the similarities and differences of the 2D HCCH-TOCSY, HCCH-COSY, and HSQC spectra between G8 and G11. Sugar residues A, B, C, D, J and K in G11 were found to correspond to the residues A, B, C, D, G and H in G8, respectively, since they have the identical cross peaks in these spectra. The integrated intensities of the ^{1}H resonances at 4.562 and 4.223 ppm in the 1D ^{1}H NMR spectrum of G11 correspond to two protons. Thus, from the identical cross peaks observed at these frequencies in these spectra, it was concluded that sugar residues E and H in G11 correspond to the residue E in G8, and residues F and I in G11 correspond to the residue F in G8.

Although the ^{1}H/^{13}C HSQC spectrum of G11 is similar to that of G8, some differences between G11 and G8 were observed in the HCCH-COSY and HCCH-TOCSY spectra. These differences are attributed to a residue, designated as G in G11 (Fig. 6B, marked by arrows). The ^{13}C spin systems for residues D, G, K, E and H in G11 are shown in Fig. 6B. The complete assignments of the ^{1}H and ^{13}C resonances in G11 are listed in Tables 1 and 2.

The HMBC spectrum of G11 is similar to that of G8. The integrated two proton intensities at 4.562 ppm in G11 and the identical cross peaks to the carbons at 71.72 ppm in the HMBC spectrum indicate that there are two β(1→6) linkages present in G11. This result, along with the assigned ^{1}H and ^{13}C chemical shifts, clearly indicates that residues E and H in G11 are the terminal residues at the nonreducing ends and that the β(1→6) linkages in G11 must occur between these nonreducing terminal residues E and H and residues F and I, respectively. The C3 carbon chemical shifts of the residues D and G in G11 are severely overlapped and the anomeric H1 proton chemical shifts of the residues E and H are degenerate. Thus, the cross peaks EH1/DC3 and HH1/GC3 in the HMBC spectrum of G11 are not resolved. Similarly, the cross peaks JH1/IC3 and GH1/FC3 are not resolved. However, since the residues D and J in G11 have chemical shifts identical to those of the assigned residues D and G in G8, residue G in G11 must be located between residues H and F. Therefore, the structure of the oligosaccharide G11 is

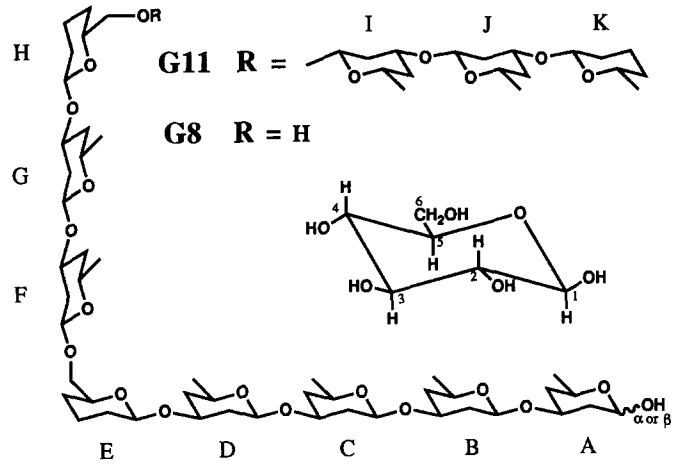

Fig. 8. The structures of β-glucan oligosaccharides G8 and G11. The glucose residues are named alphabetically A through H in G8 and A through K in G11.

determined as shown in Fig. 8. The structure of G11 clearly indicates that it is formed by the cell wall glucanosyl transferase by transferring three residues to the above determined product G8 at the nonreducing end via a β(1→6) linkage. The ^{13}C chemical shifts of residue G in G11 are noted to be more similar to those of residue D in both G8 and G11, which might be expected from the similar chemical environment for these two residues (Fig. 8).

CONCLUSIONS

Using ^{13}C-labeled oligosaccharides and recently developed heteronuclear NMR techniques modified for the study of oligosaccharides, the ^{1}H and ^{13}C chemical shifts of two oligosaccharide products from a fungal wall glucanosyl transferase were sequence-specifically assigned, and the structures of these oligosaccharides were determined. The determined structures of both G8 and G11 oligosaccharides indicate that the attachment of sugars to the linear β(1→3) oligosaccharides, catalyzed by this glucanosyl transferase, occurs at the nonreducing terminal residue via β(1→6) linkages (Fig. 8). Although the precise role of the glucanosyl transferase activities in cell wall assembly is unknown, this enzyme plus wall-associated glucanases can introduce intrachain β(1→6) linkages and cross-link two different β(1→3) glucan strands.

Thus far, glucanosyl transferase reactions have only been examined using small molecular weight β(1→3) oligosaccharides as substrates. It is conceivable that a true internal branch may be formed with a larger glucan substrate. Another possibility for obtaining true internal branch points is through the action of an as yet unidentified glucanosyl transferase, analogous to the xyloglucan transferase of plants (Frey et al., 1992; Nishitani and Tominaga, 1992), which would catalyze the transfer of glucan to the C3 position of the nonreducing end residues E and/or H (Fig. 8). Such extracellular cell wall assembly enzymes in fungi are potentially attractive targets for antifungal agents.

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REFERENCES

Bax, A. and Summers, M.F. (1986) *J. Am. Chem. Soc.*, **108**, 2093–2094.
Bax, A., Clore, G.M. and Gronenborn, A.M. (1990) *J. Magn. Reson.*, **88**, 425–431.
Bush, C.A. (1992) *Curr. Opin. Struct. Biol.*, **2**, 655–660.
Byrd, R.A., Egan, W. and Summers, M.F. (1987) *Carbohydr. Res.*, **166**, 47–58.
Cabib, E., Bowers, B., Sburlati, A. and Silverman, S.J. (1988) *Microbiol. Sci.*, **5**, 370–375.
Clore, G.M. and Gronenborn, A.M. (1991) *Science*, **252**, 1390–1399.
De Waard, P., Leeflang, B.R., Vliegenthart, J.F.G., Boelens, R., Vuister, G.W. and Kaptein, R. (1992) *J. Biomol. NMR*, **2**, 211–226.
Dubois, M, Grilles, K.A., Hamilton, J.K. and Smith, F. (1956) *Anal. Chem.*, **28**, 350–356.
Fesik, S.W., Gampe Jr., R.T. and Zuiderweg, E.R.P. (1989) *J. Am. Chem. Soc.*, **111**, 770–772.
Fesik, S.W., Eaton, H.L., Olejniczak, E.T., Zuiderweg, E.R.P., McIntosh, L.P. and Dahlquit, F.W. (1990) *J. Am. Chem. Soc.*, **112**, 886–888.
Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K. and Mathews, K.J. (1992) *Biochem. J.*, **282**, 821–828.
Hartland, R.P., Emerson, G.W. and Sullivan, P.A. (1991) *Proc. R. Soc. Lond. B*, **246**, 155–160.
Heyraud, A., Rinaudo, M. and Vignon, M. (1979) *Biopolymers*, **18**, 167–185.
Homans, S.W. (1992) *Glycobiology*, **2**, 153–159.
Homans, S.W., Dwek, R.A., Boyd, J., Soffe, N. and Rademacher, T.W. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1202–1205.
Ikura, M., Kay, L.E. and Bax, A. (1991) *J. Biomol. NMR*, **1**, 299–304.
Kay, L.E., Ikura, M. and Bax, A. (1990) *J. Am. Chem. Soc.*, **112**, 888–889.
Kay, L.E., Ikura, M. and Bax, A. (1991) *J. Magn. Reson.*, **91**, 84–92.
Kitamura, K., Kaneko, K. and Yamamoto, Y. (1974) *J. Gen. Appl. Microbiol.*, **20**, 323–344.
Lerner, L. and Bax, A. (1987) *Carbohydr. Res.*, **166**, 35–46.
Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989) *J. Magn. Reson.*, **85**, 393–399.
Miller, K.E., Mukhopadhyay, C., Cagas, P. and Bush, C.A. (1992) *Biochemistry*, **31**, 6703–6709.
Nishitani, K. and Tominaga, R. (1992) *J. Biol. Chem.*, **267**, 21058–21064.
Olejniczak, E.T. and Eaton, H.L. (1990) *J. Magn. Reson.*, **87**, 628–632.
Poppe, L., Stuike-Prill, R., Meyer, B. and van Halbeek, H. (1992) *J. Biomol. NMR*, **2**, 109–136.
Riviere, M. and Puzo, G. (1992) *Biochemistry*, **31**, 3575–3580.
Rutherford, T.J. and Homans, S.W. (1992) *Glycobiology*, **2**, 293–298.
Santoro, J. and King, G.C. (1992) *J. Magn. Reson.*, **97**, 202–207.
Shaka, A.J., Barker, P.B. and Freeman, R. (1985) *J. Magn. Reson.*, **64**, 547–552.
Shaka, A.J., Lee, C.J. and Pines, A. (1988) *J. Magn. Reson.*, **77**, 274–293.
Shibata, N., Hisamichi, K., Kikuchi, T., Kobayashi, H., Okawa, Y. and Suzuki, S. (1992) *Biochemistry*, **31**, 5680–5686.
Skelton, M.A., Van Halbeek, H. and Cherniak, R. (1991) *Carbohydr. Res.*, **221**, 259–268.
Tsui, F.-P., Egan, W., Summers, M.F. and Byrd, R.A. (1988) *Carbohydr. Res.*, **173**, 65–74.
Van De Ven, F.J.M. and Philippens, M.E.P. (1992) *J. Magn. Reson.*, **97**, 637–644.
Van Halbeek, H. and Poppe, L. (1992) *Magn. Reson. Chem.*, **30**, S74–S86.
Vuister, G.W. and Bax, A. (1992) *J. Magn. Reson.*, **98**, 428–435.
Whitfield, D.M., Choay, J. and Sarkar, B. (1992) *Biopolymers*, **32**, 585–596.