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Note

Structural analysis of soluble D-glucans from strains of *Streptococcus mutans* by ^{13}C-nuclear magnetic resonance spectrometry

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INTRODUCTION

This series of articles has previously examined, in terms of type of branching and degree of linearity, a variety of extracellular dextrans from *Leuconostoc mesenteroides* and related genera. It has been established that more-precise, structural descriptions of these dextrans can be obtained by considering the dextran in terms of variously *O*-substituted residues rather than in terms of various types of intersaccharide linkages[2,3]. We have observed three different classes of dextrans which principally differ from linear dextran by their content of (1→3)-α-D-linkages, and that have been defined[1] as (*a*) class I ($m = 3$) dextrans, which contain 3,6-di-*O*-substituted α-D-glucopyranosyl residues; (*b*) class II dextrans, which contain significant proportions of non-contiguously linked, 3-mono-*O*-substituted α-D-glucopyranosyl residues; and (*c*) class III dextrans, which have an i.r. band at 822 cm^{-1} and are presumed to contain significant proportions of contiguously linked, (1→3)-α-D-linked D-glucopyranosyl residues. It is possible to distinguish these (1→3)-linkage-containing dextrans from dextrans containing (1→2)- and (1→4)-linkages by (*a*) methylation-fragmentation analysis[4], (*b*) periodate-oxidation analysis[5], (*c*) ^{13}C-n.m.r. spectrometry[6,7], and, to a lesser degree, (*d*) ^{1}H-n.m.r. spectrometry[8,9] and Fourier-transform, infrared difference-spectrometry[10].

However, it is more difficult to differentiate among the three classes of dextrans that contain significant percentages of (1→3)-linkages. Periodate-oxidation analysis

*Unusual Dextrans, Part XIV. For Part XIII, see ref. 1.

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cannot differentiate among class I ($m = 3$) dextrans, class II dextrans, or class III dextrans[5,11]. ^{1}H-N.m.r. spectrometry at 100 MHz of the compounds in aqueous solution at 90° cannot differentiate between class I ($m = 3$) dextrans and class II dextrans (we currently have no comparison data for class III dextrans). However, with aqueous dimethyl sulfoxide as the solvent, ^{1}H-n.m.r. spectrometry can differentiate between soluble D-glucans and insoluble D-glucans[8] [which have structural features similar, or identical, to those of class I ($m = 3$) dextrans and class III dextrans]. Methylation-fragmentation analysis can distinguish class I ($m = 3$) dextrans from class II and class III dextrans, but cannot differentiate between the latter two classes. Fourier-transform, infrared difference-spectrometry can distinguish among class I ($m = 3$) dextrans, class II dextrans, and class III dextrans; however, only limited data are available for this technique.

^{13}C-N.m.r. spectrometry, at 25 MHz, can distinguish among all three classes of dextrans containing (1→3)-α-D-linkages. However, (*a*) due to the closely placed, diagnostic chemical-shifts for class I ($m = 3$) dextrans and class III dextrans, it is difficult to identify small percentages of the structural feature of class I ($m = 3$) dextran or of class III dextran when large percentages of the complementary structural feature are present[1], and (*b*) it is possible that solubility–structure relationships prevent a dextran containing significant percentages of the structural features of class III dextran from being soluble, so that it cannot be analyzed under current ^{13}C-n.m.r. conditions[1].

We have previously noted that bacterial strains from the species *L. mesenteroides* and *S. mutans* have a number of similarities, both in the characteristics of the bacteria and in the structures of the extracellular D-glucans which these strains can produce[1]. Although strains from both species can engender extracellular D-glucans *in vivo* (and such polysaccharides from *L. mesenteroides* have been those that have been studied in previous parts of this "Unusual Dextran" series), it is possible, and sometimes more convenient, to employ dextransucrase systems from specific, bacterial strains for *in vitro* production of these D-glucans. Such *in vitro* formation was initially employed for strains of *L. mesenteroides*[12], and is the method commonly employed for obtaining analytical D-glucans from strains of *S. mutans*. It has proved possible to fractionate the D-glucans elaborated by specific strains of *S. mutans* into fractions of different solubility properties[8], described as S (soluble) and A and B (insoluble), by precipitation techniques similar to the fractionation methods employed for analytical fractionation of the native dextrans produced by *L. mesenteroides*[13].

Products of such *in vitro* syntheses, employing various strains of *S. mutans*, have been studied in conjunction with controlled, specific, enzymic degradations, by (*a*) ^{1}H-n.m.r. spectrometry employing dimethyl sulfoxide–water as the solvent[8], (*b*) methylation-fragmentation analysis[14], and (*c*) ^{13}C-n.m.r. spectrometry, employing[6] solutions having a high pD. The results of these investigations agree in showing that the D-glucans produced by strains of *S. mutans* contain only (1→6)-α-D- and (1→3)-α-D-linked residues, and support the concept, originally proposed by Ebisu *et al.*[15], that the insoluble D-glucans contain contiguously linked, (1→3)-α-D-

linked D-glucopyranosyl residues. In addition, the controlled, enzymic degradations[8] indicated that most, if not all, of these contiguously linked, (1→3)-α-D-linked D-glucopyranosyl residues are located in the backbone chains of these insoluble D-glucans produced by strains of *S. mutans*. The methylation-fragmentation analysis data of Hare *et al.*[14] show, with regard to D-glucans produced by strain OMZ 176 of *S. mutans*, that the soluble fraction contains a high percentage of 3,6-di-*O*-substituted D-glucopyranosyl residues, and a very low percentage of 3-mono-*O*-substituted D-glucopyranosyl residues, whereas the ratio of the proportions of such residues was reversed for the insoluble fraction. In a similar way, it was demonstrated, by use of high-field (68 MHz) ^{13}C-n.m.r. spectrometry, that, at pD 14, the anomeric resonance associated with the (1→3)-α-D-linked residues (~102.3 p.p.m.) is partially resolved into resonances at 102.8 and 102.3 p.p.m., which were assigned, respectively, to the 3,6-di-*O*-substituted and the 3-mono-*O*-substituted residues[6].

RESULTS AND DISCUSSION

We report high signal-to-noise, ^{13}C-n.m.r. spectra, recorded at 25 MHz and 90°, for solutions in deuterium oxide, of soluble D-glucan fractions that are the result of the analytical fractionation, of D-glucans produced *in vitro*, by α-D-glucosyltransferase systems obtained from five strains of *S. mutans*. The spectra of these D-glucan fractions from the five strains (AHT, FA-1, Ingbritt, OMZ 176, and LM-7), which represent five of the principal serotypes[16] of the strains of *S. mutans*, are shown in Figs. 1 and 2, and the chemical shifts of the resonances in these spectra are given in Table I.

Each of the fractions S dissolved to give an opalescent solution, at ~50 mg/mL, of low viscosity. Attempts to dissolve, or solubilize, the complementary, insoluble fractions [D-glucan OMZ 176 fraction B, D-glucan LM-7 fraction A, and a serotype *a* D-glucan HS-6 fraction A (a serotype *a* strain corresponding to AHT)] under conditions identical to those used for the S fractions, yielded solvents filled with flocculent precipitate, and such samples gave no ^{13}C-n.m.r. signals at ~10,000 acquisitions. However, a D-glucan Ingbritt fraction A sample (at a concentration of ~50 mg/mL), although also giving a solution of physical appearance similar to that of the other insoluble D-glucans, yielded a ^{13}C-n.m.r. spectrum similar to that of the corresponding S fraction (see Fig. 2). It is possible that this fraction A from D-glucan Ingbritt had been less well fractionated than the other insoluble D-glucan fractions, and thus retained a significant proportion of fraction S material.

Examples of expanded spectra for the data sets plotted in Fig. 1 are shown in Fig. 3 for the 69–76-p.p.m. region in the spectra of the fractions S of D-glucan AHT, D-glucan FA-1, and D-glucan LM-7. All spectra plotted here were recorded at a relatively high signal-to-noise ratio, and the resulting data sets were therefore processed by convolution-difference resonance-enhancement (c.d.r.e.)[17], an operation that narrows the line-width of the resonances, allowing better visual display and improving visual discrimination of minor spectral features. The spectra shown in

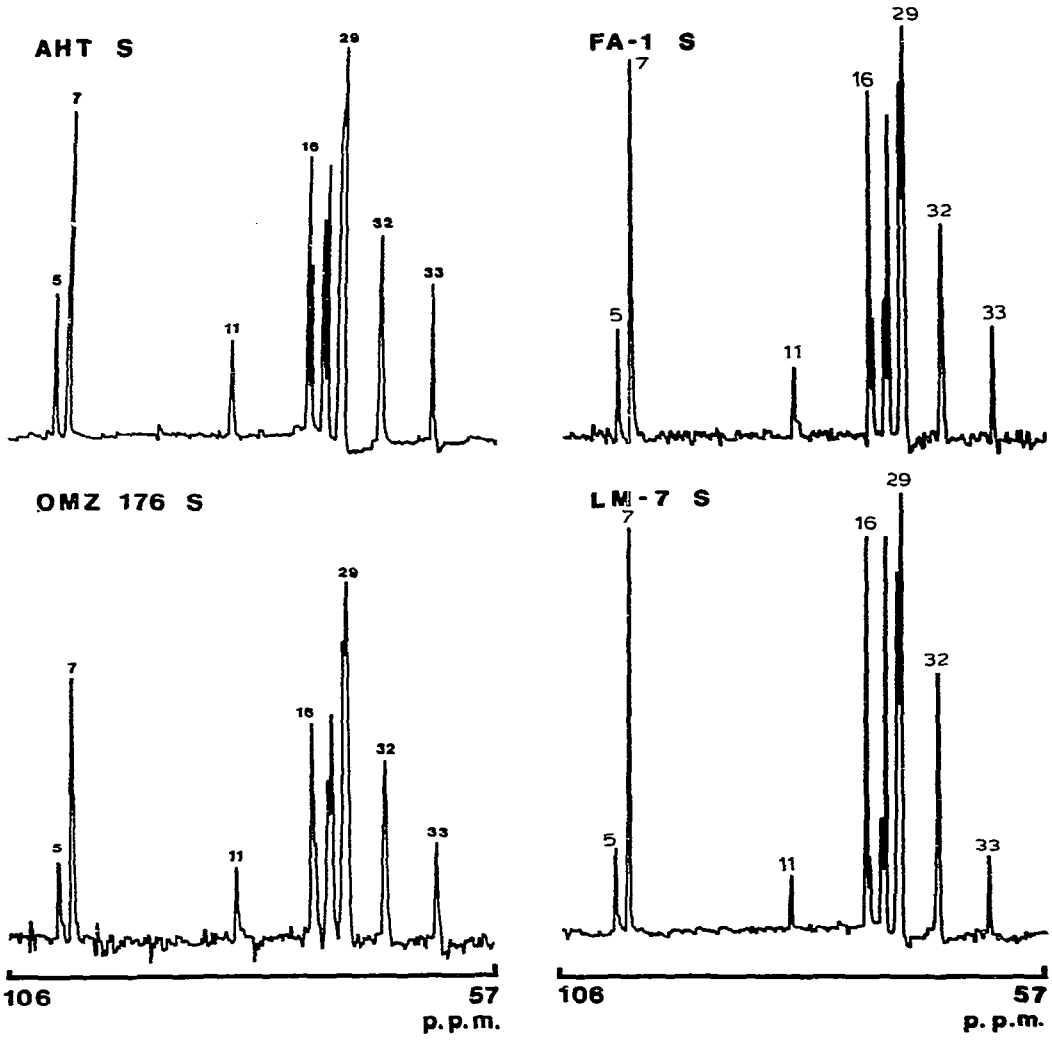

Fig. 1. ^{13}C-N.m.r. spectra at 90° for D-glucan AHT fraction S, D-glucan FA-1 fraction S, D-glucan OMZ 176 fraction S, and D-glucan LM-7 fraction S. (The identifying resonance-numbers are referenced to the resonances in Table I, and the number of acquisitions for each spectrum is also given in Table I. All data were processed by c.d.r.e. before plotting.)

Figs. 1–3, and the corresponding peak maxima as established by digital slope-detection and cross-referenced to Table I (see ref. 7 for the assignment of the reference numbers), indicate the following relationships for these D-glucans from strains of *S. mutans*.

Firstly, the prominent resonances of linear dextran (resonances A through F)[18] dominate the ^{13}C-n.m.r. spectra of these D-glucans. Secondly, the chemical shifts of the diagnostic resonances (resonances 5, 11, 17, 21, 22, 28, and 33) of all of the spectra in Table I are essentially identical, and are identical to the diagnostic reso-

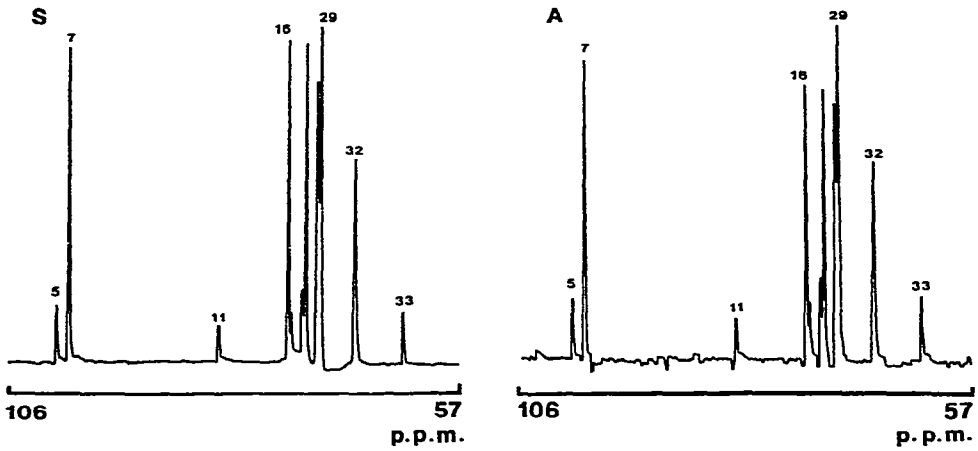

Fig. 2. ^{13}C-N.m.r. spectra at 90° for the fractions A and S of the D-glucan produced by the Ingbritt strain. (The identifying resonance numbers are referenced to the resonances in Table I, and the number of acquisitions for each spectrum is also given in Table I. All data were processed by c.d.r.e. before plotting.)

TABLE I

CHEMICAL SHIFTS FOR ^{13}C-N.M.R. SPECTRA AT 90° OF D-GLUCAN FRACTIONS FROM VARIOUS STRAINS OF *Streptococcus mutans*[a]

		D-*Glucan* *AHT*	*FA-1*	*Ingbritt*	*Ingbritt*	*OMZ 176*	*LM-7*
		Fraction *S*	*S*	*S*	*A*	*S*	*S*
		Serotype *a*	*b*	*c*	*c*	*d*	*e*
		Acquisitions[b] *8*	*27*	*10*	*8*	*27*	*20*
	5[c]	100.89	100.86	100.85	100.90	100.91	100.86
A[d]	7	99.51	99.51	99.47	99.51	99.51	99.50
	11	82.84	82.85	82.81	82.85	82.85	82.80
B	16	75.14	75.14	75.13	75.14	75.17	75.13
	17	74.48	74.78	74.77	74.78	74.83	74.78
	21	73.60	73.60	73.60	73.60	73.55	73.61
	22	73.51	73.51	73.50	73.51		73.51
C	24	73.15	73.15	73.15	73.15	73.18	73.15
D	27	71.92	71.97	71.93	71.97	71.97	71.97
	28	71.80	71.80				
E	29	71.57	71.57	71.57	71.57	71.60	71.57
F	32	67.76	67.76	67.76	67.76	67.76	67.76
	33	62.45	62.45	62.41	62.45	62.45	62.45

[a]The shifts are expressed in p.p.m. relative to tetramethylsilane. [b]Acquisitions in thousands. [c]These numbers were assigned in ref. 7, and are referenced to resonances in Figs. 1–3. [d]The letters A–F designate the major resonances of linear dextran (see ref. 18).

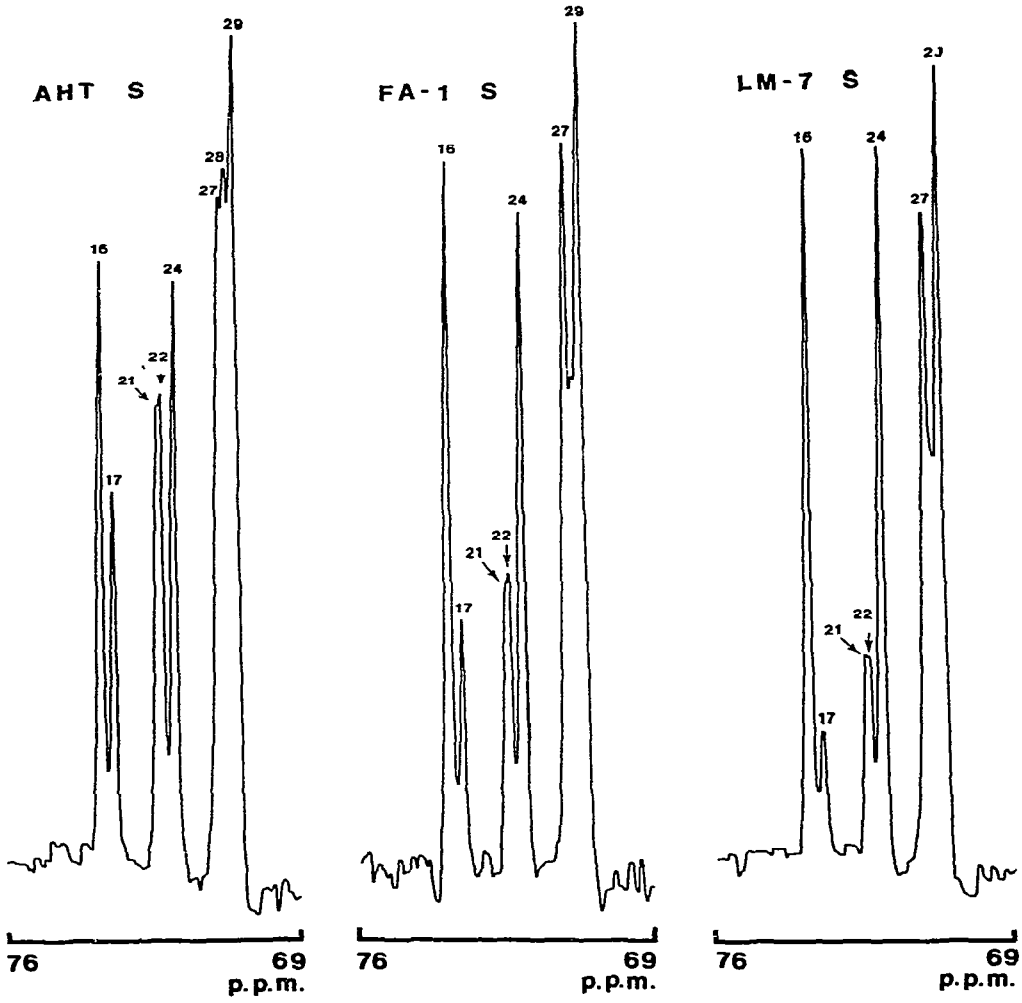

Fig. 3. The expanded, 69–76-p.p.m. region of the data presented in Fig. 1 for D-glucan AHT fraction S, D-glucan FA-1 fraction S, and D-glucan LM-7 fraction S.

nances observed for class I ($m = 3$) dextrans produced by strains of *L. mesenteroides* (see Table II of ref. 1). The expanded, 69–76-p.p.m. regions of the spectra of D-glucans from *S. mutans*, shown in Fig. 3, also indicate no observable minor resonances in addition to those listed in Table I, and these spectral regions are identical, in terms of chemical shifts, to similar, expanded spectral-regions for class I ($m = 3$) dextrans (see Fig. 3, ref. 1). The absence of additional resonances in the spectra of these D-glucans produced by strains of *S. mutans* indicates the absence (to a maximum permissible level of a few percent) of (1→2)-α-D- or (1→4)-α-D-linkages in any of these polysaccharides, and confirms such findings made by alternative means on these[6], and similar[14], soluble fractions. The spectra herein also preclude the presence of any significant ($> \sim 2\%$) proportion of non-consecutively linked, 3-mono-*O*-substituted

residues in these D-glucans from strains of *S. mutans*, because of the absence of resonances at ~83.3 and ~101.04 p.p.m. In addition, although with a lesser degree of confidence, owing to the close spacing of possible diagnostic resonances, these spectra preclude the presence of significant proportions of contiguously linked, 3-mono-*O*-substituted residues, as such residues are associated with resonances at ~101.0 and ~82.5 p.p.m. [but it is possible that a trace of such 3-mono-*O*-substituted residues is evidenced by the small, up-field shoulder on resonance 11 in the spectrum of D-glucan FA-1 fraction S (see Fig. 1)]. In general, the relatively narrow line-widths of these resonances, and the simplicity of the spectra, preclude significant spectral-contributions from branching features other than those from the 3,6-di-*O*-substituted α-D-glucopyranosyl residues.

The foregoing indicates an essential spectral identity, in terms of the results of 25-MHz, ^{13}C-n.m.r. spectrometry of aqueous solutions at 90°, between these soluble D-glucans from strains of *S. mutans* and the extracellular dextrans from strains of *Leuconostoc* and related genera, which have been defined as class I ($m = 3$) dextrans, and which can be described by the generalized structure **1**, where Glc*p* represents a D-glucopyranosyl residue or group. In addition, these polysaccharides of microbial

$$-\{-[-\alpha\text{-Glc}p\text{-}(1\rightarrow6)\text{-}]_n\text{-}\alpha\text{-Glc}p\text{-}(1\rightarrow6)\text{-}\}_x\text{-}$$
$$\begin{array}{c} 3 \\ \uparrow \\ 1 \\ \alpha\text{-Glc}p \end{array}$$

1

origin give, at 34°, 25-MHz spectra that are identical to those of synthetically produced dextrans that contain 3,6-di-*O*-substituted residues[19]. The spectra of the D-glucans presented herein differ from one another only in the relative intensities of the diagnostic resonances, in comparison to the corresponding resonances A–F of linear dextran. Although differing somewhat in relative intensity, and, consequently, indicating somewhat differing degrees of linearity for the D-glucans produced by the different strains of *S. mutans*, the spectra presented in Figs. 1 and 2 show a relatively narrow range of differing degrees of linearity for the polysaccharides, in contrast to the considerable variation for such class I ($m = 3$) dextrans from strains of *Leuconostoc* and related genera (see Figs. 1 and 2, ref. 1). In general, the spectra of all D-glucans presented herein are similar, in terms of the relative peak-heights of the resonances associated with branching, to the spectrum of dextran B-1191, which can be described by **1** where n is ~3.

The essential identity, spectroscopically, of these D-glucans produced by strains representing five different serotypes of *S. mutans* indicates that (*a*) the immunochemical responses, on which these serotypes are based, are not dependent on the structure of the microbial, extracellular polysaccharide (in contrast to the correlations of immunochemical response to structure for extracellular dextrans from *L. mesenteroides*[3,20]), or (*b*) such structural differences are too subtle, or involve too small

mole-percentages of specific, different residues, to be observed under the ^{13}C-n.m.r.-spectrometric conditions currently employed, or (c) the *in vitro* production of D-glucans by use of α-D-glucosyltransferases from strains of *S. mutans* fails to duplicate all of the structural details of these D-glucans produced *in vivo*. The last possibility is a viable alternative, as, in one of the relatively few structural analyses on a broth polysaccharide from *S. mutans* (strain E49, a serotype *a* strain[16], alternatively identified as OMZ 49), Lewicki *et al.*[21], employing methylation-fragmentation analysis, observed the presence of (1→4)-linkages in the 4–8% range of content thereof.

High-temperature, ^{13}C-n.m.r. spectrometry of aqueous solutions therefore provides a useful alternative to methods previousiy employed for structural determinations on these soluble D-glucans, as it allows discrimination amongst a wide variety of structural features possible. In addition, such ^{13}C-n.m.r. spectrometry is complementary to methylation-fragmentation analysis, because of the problem associated with the resistance of the 3-hydroxyl group of the α-D-glucopyranosyl residue to complete methylation. Methylation-fragmentation analysis can provide an overestimate of the (1→3)-linkage content of D-glucans (see ref. 22, and references cited therein).

EXPERIMENTAL

The production and fractionation of the D-glucans from strains of *S. mutans* have been described[8]. The sample of OMZ 176 fraction S employed herein was prepared by a slightly modified procedure[6], and resulted from an independent production. The ^{13}C-n.m.r. recording techniques and sample conditions have been described[2,7,18]. In general, experiments were performed at 25.2 MHz with a Varian XL-100-15A spectrometer equipped with a Varian variable-temperature probe and a Nicolet TT-100 pulse, Fourier-transform data-system. The chemical shifts are expressed in p.p.m. relative to external tetramethylsilane, but were actually calculated by reference to the internal, deuterium lock-signal. The D-glucans were dissolved, or partially dissolved (in the case of D-glucan Ingbritt fraction A), at 50 mg/mL of deuterium oxide.

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REFERENCES

1 F. R. SEYMOUR AND R. D. KNAPP, *Carbohydr. Res.*, 81 (1980) 105–129.
2 F. R. SEYMOUR, R. D. KNAPP, S. H. BISHOP, AND A. JEANES, *Carbohydr. Res.*, 68 (1979) 123–140.

3 F. R. SEYMOUR, R. D. KNAPP, E. C. M. CHEN, S. H. BISHOP, AND A. JEANES, *Carbohydr. Res.*, 74 (1979) 41–62.
4 F. R. SEYMOUR, M. E. SLODKI, R. D. PLATTNER, AND A. JEANES, *Carbohydr. Res.*, 53 (1977) 153–166.
5 A. JEANES AND F. R. SEYMOUR, *Carbohydr. Res.*, 74 (1979) 31–40.
6 P. COLSON, H. C. JARRELL, B. L. LAMBERTS, AND I. C. P. SMITH, *Carbohydr. Res.*, 71 (1979) 265–272.
7 F. R. SEYMOUR AND R. D. KNAPP, *Carbohydr. Res.*, 81 (1980) 67–103.
8 T. S. MEYER, B. L. LAMBERTS, AND R. S. EGAN, *Carbohydr. Res.*, 66 (1978) 33–42.
9 F. R. SEYMOUR, R. D. KNAPP, AND S. H. BISHOP, *Carbohydr. Res.*, 74 (1979) 77–92.
10 F. R. SEYMOUR AND R. L. JULIAN, *Carbohydr. Res.*, 74 (1979) 63–75.
11 A. JEANES, W. C. HAYNES, C. A. WILHAM, J. C. RANKIN, E. H. MELVIN, M. J. AUSTIN, J. E. CLUSKEY, B. E. FISHER, H. M. TSUCHIYA, AND C. E. RIST, *J. Am. Chem. Soc.*, 76 (1954) 5041–5046.
12 A. JEANES, *Methods Carbohydr. Chem.*, 5 (1965) 127–132.
13 C. A. WILHAM, B. H. ALEXANDER, AND A. JEANES, *Arch. Biochem. Biophys.*, 59 (1975) 61–75.
14 M. D. HARE, S. SVENSSON, AND G. J. WALKER, *Carbohydr. Res.*, 66 (1978) 245–264.
15 S. EBISU, A. MISAKI, K. KATO, AND S. KOTANI, *Carbohydr. Res.*, 38 (1974) 274–381.
16 B. PERCH, E. KJEMS, AND T. RAUN, *Acta Pathol. Microbiol. Scand., Sect. B*, 82 (1974) 357–370.
17 I. D. CAMPBELL, C. M. DOBSON, R. J. P. WILLIAMS, AND A. V. XAVIER, *J. Magn. Reson.*, 11 (1973) 172–181.
18 F. R. SEYMOUR, R. D. KNAPP, AND S. H. BISHOP, *Carbohydr. Res.*, 51 (1976) 179–194.
19 H. ITO AND C. SCHUERCH, *J. Am. Chem. Soc.*, 101 (1979) 5797–5806.
20 F. R. SEYMOUR, R. D. KNAPP, E. C. M. CHEN, A. JEANES, AND S. H. BISHOP, *Carbohydr. Res.*, 71 (1979) 231–250.
21 W. J. LEWICKI, L. W. LONG, AND J. R. EDWARDS, *Carbohydr. Res.*, 17 (1971) 175–182.
22 F. R. SEYMOUR, E. C. M. CHEN, AND S. H. BISHOP, *Carbohydr. Res.*, 68 (1979) 113–121.