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A ^{13}C-N.M.R.-SPECTRAL STUDY OF A GEL-FORMING, BRANCHED (1→3)-β-D-GLUCAN, (LENTINAN) FROM *Lentinus edodes*, AND ITS ACID-DEGRADED FRACTIONS. STRUCTURE, AND DEPENDENCE OF CONFORMATION ON THE MOLECULAR WEIGHT

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ABSTRACT

The structure and conformation of lentinan, an anti-tumor, branched (1→3)-β-D-glucan from *Lentinus edodes*, and its acid-degraded, lower molecular-weight fractions have been investigated by ^{13}C-n.m.r. spectroscopy. It is found that their ^{13}C-n.m.r. spectra are considerably changed, depending on the molecular weight. The conformational behavior as studied by ^{13}C-n.m.r. spectroscopy is consistent with that revealed by a study of the shift in the absorption maximum of Congo Red complexed with lentinan and its acid-degraded fractions. It is found that the water-soluble fraction II (mol. wt. 3,640) gives rise to well-resolved ^{13}C-n.m.r. spectra; the ^{13}C-signals are assigned to (1→3)-β-D-glucan and branch points at C-6. The branched structure is also confirmed by examination of the ^{13}C-n.m.r. spectra of the compounds in dimethyl sulfoxide. For the gel state of the fractions of higher molecular-weight, lentinan (mol. wt. 1,000,000) and fraction IV (mol. wt. 16,200), however, ^{13}C-n.m.r. spectra of considerably attenuated signal-amplitude are observed. The fact that the ^{13}C-signals of the β-D-(1→3)-linked main chain and side chains are completely suppressed is explained as a result of immobilization caused by their taking an ordered conformation. The ^{13}C-resonances observed in the gel state, which are assigned to β-D-(1→6)-linkages, are unequivocally assigned to the side chains (of disordered conformation). Finally, the ordered conformation of both the β-D-(1→3)-linked main chain and side chains is identified as the single-helix conformation, which tends to form multiple helixes as junction zones for gel structure.

INTRODUCTION

In very small dosages against certain allogeneic tumors, particularly Sarcoma-180 in mice, lentinan (mol. wt. ~ 1,000,000) isolated from *Lentinus edodes*, an edible mushroom popular in Japan, has a prominent growth-inhibitory activity which seems to be elicited by the stimulation of cell-mediated response[1–5]. Recently, Sasaki and Takasuka reported[6] that lentinan has a branched (1→3)-β-D-glucan structure with side-chains of both β-D-(1→3)- and β-D-(1→6)-linked D-glucose residues, this was shown by periodate oxidation, Smith degradation, methylation analysis, and bioassay[6] Furthermore it was reported that only higher molecular-weight fractions (mol wt >16,000) from lentinan partially hydrolyzed with formic acid showed antitumor activity[7] This finding is interesting, because it was also determined that those fractions take an ordered conformation, by a study of the shift in the absorption maximum[8] of Congo Red complexed with them[7] Accordingly, identification of the ordered conformation, as well as further confirmation of detailed structure, seemed important as regards understanding the mode of action of this antitumor-active polysaccharide

Although the ^{13}C-n m r -spectral method has proved a valuable tool for determination of the composition of and sequence in polysaccharides[9–26], very few studies have been directed to elucidation of the conformation This is perhaps due to supposition that ^{13}C chemical shifts are rather insensitive to conformational change In this regard, it is interesting that Colson *et al* [14] observed substantial, downfield ^{13}C-shifts of the linked carbon atoms, C-1 and C-4, of cyclohexaamyloses compared with those of linear (1→4)-α-D-glucans, suggesting the existence of conformation-dependent, ^{13}C-chemical shifts Furthermore, we have demonstrated that downfield displacements of C-1 and C-3 resonances with respect to those of acid-degraded fractions of smaller molecular weight in a resilient gel, and in dilute alkaline solution, of a curdlan-type polysaccharide 13140, a linear (1→3)-β-D-glucan from *Alcaligenes faecalis* var *myxogenes* IFO 13140, are characteristic of the helical conformation in addition, there is a substantial loss of peak areas[27] The observation of these signals was found to correlate with that of the shift of the absorption maximum of Congo Red complexes[27] A similar phenomenon was noted for a branched (1→3)-β-D-glucan [A_3 from *Pleurotus ostreatus* (Fr) Quel] in the gel state[28] the ^{13}C-signal due to β-D-(1→3)-linked main-chains is completely lost, while ^{13}C-resonance signals of the side-chains are clearly observed In this paper, the conformational behavior of lentinan and its acid-degraded fractions as a function of molecular weight is examined by ^{13}C-n m r spectroscopy and compared with the result of the shift in the absorption maximum of Congo Red The relative mobility of both β-D-(1→3)- and β-D-(1→6)-D-glucose residues is also described

EXPERIMENTAL

Materials — Lentinan was provided by Dr G Chihara of this Institute Low molecular-weight fractions of lentinan were prepared with 80% or 90% formic acid by the method previously reported[6 7] Glucan GE-3, a (1→6)-β-D-glucan from[29] *Gyrophora esculenta* MIYOSHI, was supplied by Dr Y Nishikawa of Kanazawa University Schizophyllan, a branched (1→3)-β-D-glucan from *Schizophyllum commune*, having a single D-glucosyl group as a branch, was a gift of Dr S Kikumoto of Taito Company

^{13}C-N m r spectrocopy — ^{13}C-N m r spectra were recorded with a JEOL

PFT-100/EC-100 spectrometer operating at 25 03 MHz in the pulsed, Fourier-transform mode Free-induction decays were accumulated with a 90° pulse (22 μs) and a repetition time of 0 6 s A phase-alternating pulse-sequence was used, in order to minimize base-line aberrations All spectra were recorded using 4 K data points and a spectral width of 4 kHz ^{13}C-Chemical shifts were expressed in p p m down-field from external tetramethylsilane Spin–lattice relaxation-times were obtained by using the pulse sequence of 180°-t-90°, and nuclear Overhauser enhancements (nOe values, the maximum possible nOe being 2 988) were obtained from the ratio of the intensity of fully decoupled spectra to the intensity of spectra in which the proton noise-decoupler was gated off to remove the nOe All n m r measurements were conducted at 28°

Interaction with Congo Red — The complex-formation of lentinan and its acid-degraded fractions (1 mg/ml) with Congo Red (38μM) was evaluated from the shift in the visible absorption of Congo Red induced by the presence of lentinan or acid-degraded fractions in 0 1M sodium hydroxide at 20°

RESULTS AND DISCUSSION

A Molecular-weight Dependence of ^{13}C Spectra of Lentinan

It has been demonstrated that the peak intensities, line widths, and peak positions of the linear, (1→3)-β-D-glucan, curdlan-type polysaccharide PS 13140, vary with the molecular weight[27] This trend was found to be consistent with the results obtained from the absorption maximum of Congo Red complexed with this polysaccharide The difference in the spectral pattern was interpreted by the theory that only higher molecular weight D-glucans are capable of forming helix structures In order to clarify the conformational behavior of the branched (1→3)-β-D-glucan we compared the molecular-weight dependence of ^{13}C-spectra of lentinan and its low molecular-weight fractions with that of the shift in the absorption maximum of Congo Red In Fig 1 is shown a plot of the shift in the absorption maximum of Congo Red complexed with lentinan and its low molecular-weight fractions against molecular weight Fig 1 clearly shows that fractions of molecular weight higher than 20,000 (exhibiting anti-tumor activity) are capable of forming a complex with Congo Red as a result of the ordered conformation of the polymer chains On the other hand, it is seen that the shift in the absorption maximum of Congo Red does not occur with fractions of smaller molecular weight (<4,000) (exhibiting no anti-tumor activity), because of their taking a disordered conformation Fractions of molecular weight between 4,000 and 20,000 were found to interact partially with Congo Red The observation of the region where there is a gradual increase of the shift in the absorption maximum with molecular wieght is in contrast to that of the linear D-glucan PS 13140, in which the disordered form is abruptly assumed[28] at $\mathrm{dp}_n \sim 25$ Accordingly, the behavior shown in Fig 1 is characteristic of a branched (1→3)-β-D-glucan Among the materials, fraction II (mol wt 3,640), fraction IV (mol wt 16,200), and lentinan (mol wt 1,000,000) were chosen as typical examples for ^{13}C-n m r -spectral studies

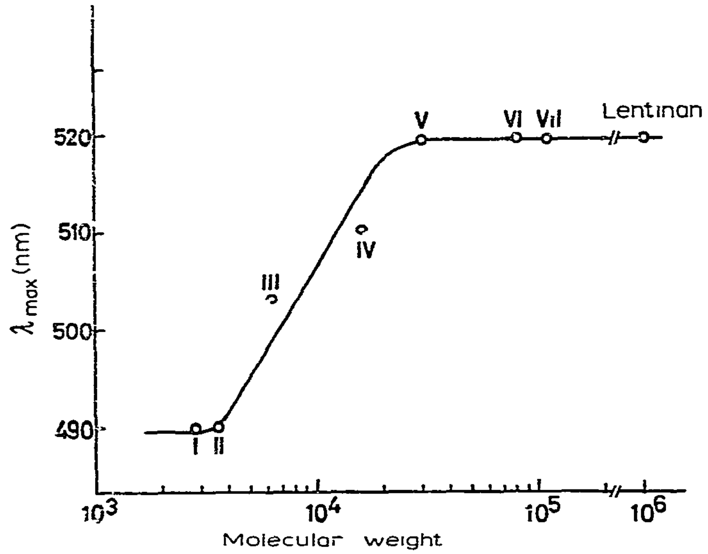


Fig 1 Changes in the absorption maximum of a solution of each of fractions I–VII (prepared from lentinan by hydrolysis with formic acid) and Congo Red in 0 1M NaOH at 20°

Fraction II is water-soluble, and gives a well-resolved ^{13}C-n m r spectrum, as shown in Fig 2A The ^{13}C-peak positions of fraction II are found to be exactly the same as those of linear (1→3)-β-D-glucans, except for a peak designated as C-6′ (see Fig 2D) Because the extent of occurrence of β-D-(1→6)-linked D-glucose residues is very small (8 % from g l c analysis[6]), and the peak separation between β-D-(1→3)- and β-D-(1→6) linked D-glucose residues is not so large (see Table I), compared with the line-widths, the peaks of β-D-(1→6)-linkages might be buried under the intense signals of β-D-(1→3)-linkages Naturally, the peaks due to the β-D-(1→3)-linked side-chains are collapsed into the peaks that are due to the backbone In view of the peak assignments of branched (1→3)-β-D-glucans in dimethyl sulfoxide (as described later), the C-6′ peak is unequivocally ascribed to C-6′ of the branch point (see formula 1)

Conversely, ^{13}C-signals of lentinan in the gel state are extremely suppressed, because of apparent loss of the peak areas, as shown in Fig 2B The highermost peak of low intensity, at 61 9 p p m , is clearly ascribed to C-6 of β-D-(1→3)-linked D-glucose residues[14 27] The other signals of this linkage (C-1–C-5), however, seem to be almost completely suppressed Similar behavior of the peak areas, that is, the difference between the peak areas of C-6 and of the others. was also noted[27] in the ^{13}C-n m r spectra of the resilient gel of the linear (1→3)-β-D-glucan (PS 13140) A possible, internal rotation of the hydroxymethyl groups is clearly responsible for the higher peak-amplitude of the C-6 signal The peak areas of C-1–C-5 are partially recovered in the presence of 0 03M sodium hydroxide, as described later Thus, the broad resonance peaks (other than the peak at 61 9 p p m observed in Fig 2B)

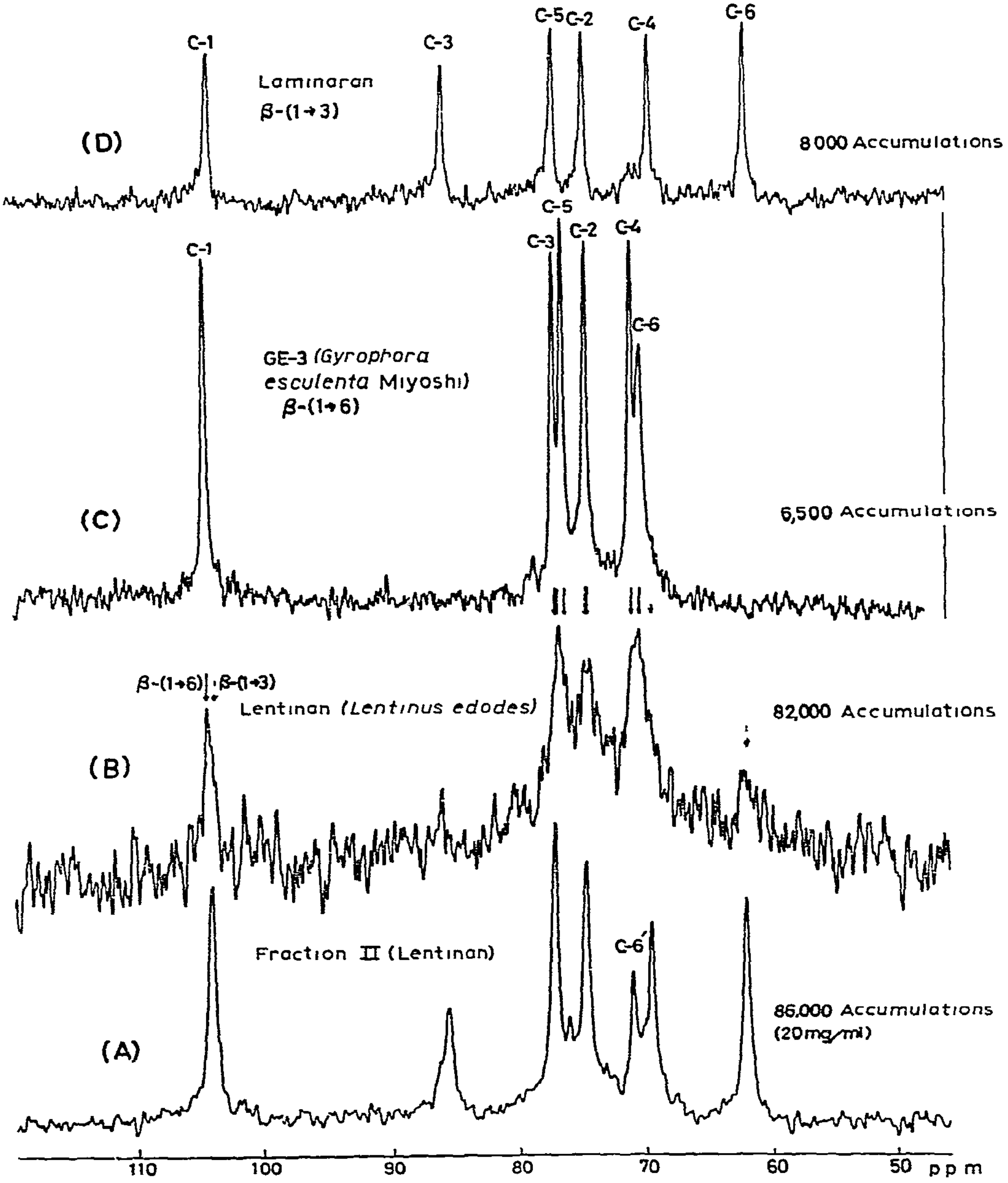


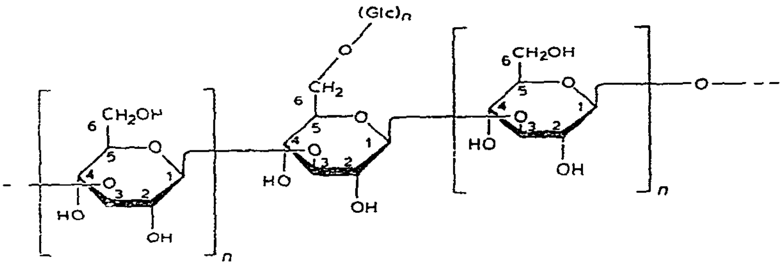
Fig 2 ^{13}C-N m r spectra of lentinan and fraction II, together with their components (in D_2O, pD 7, at 100 mg/ml, except for A) [A Fraction II (20 mg/ml), 86,000 accumulations B Lentinan (in the gel state), 82,000 accumulations C (1→6)-β-D-Glucan, GE-3 from *Gyrophora esculenta* MIYOSHI, 6,500 accumulations D (1→3)-β-D-Glucan, laminaran, 8,000 accumulations]

TABLE I

^{13}C-CHEMICAL SHIFTS OF β-D-(1→3)-, β-D-(1→6)-LINKED-D-GLUCANS, FRACTION II, AND FRACTION IV IN AQUEOUS SOLUTION (pD 7)

Carbon atom	*β-D-(1→3)*[a] *(Laminaran)*	*β-D-(1→6)*[b] *(GE-3)*	*Fraction II [β-D-(1→3)]*	*Fraction IV*	
				β-D-(1→6)	*β-D-(1→3)*
C-1	103 7	104 2	103 8	104 2	n o [c]
C-2	74 5	74 2	74 4	74 4	74 4
C-3	85 5	76 8	85 3	76 9	n o
C-4	69 3	70 7	69 3	70 4	69 3
C 5	76 8	76 1	76 8	76 1	76 9
C-6	61 9	70 0	61 9	69 8	61 9
C-6′[d]			70 8		70 9

[a] Assignment made by Colson *et al* [14] [b] Assignment based on that of gentiobiose[9] [c] Not observed [d] C-6 at the branch point of the β-D-(1→3)-glucosidic linkage



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The numbers of the carbon atoms of a D-glucopyranosyl group containing a branch at C-6 are primed

can be unequivocally assigned to peaks of β-D-(1→6)-linked D-glucose residues, in comparison with those of the (1→6)-β-D-glucan GE-3 (from *Gyrophora esculenta* MIYOSHI) The observation of D-glucose residues occurring in low proportion is made possible only in the gel state, due to the complete loss of the major components A similar situation had been encountered with A_3 (from *Pleurotus ostreatus*), namely, that only α-D-(1→4)-linked side-chains are clearly seen in the gel phase, and the ^{13}C-resonance of the β-D-(1→3)-linked main-chain is completely lost[28] By comparing the peak intensities in Fig 2B with those in spectra taken at pD 13 7 (with the nOe suppressed), the proportion of β-D-(1→6)-linked D-glucose residues was estimated as 8%, in agreement with that from g l c analysis (8%) This result indicates that the intensities of β-D-(1→6)-linked D-glucose residues are fully observed, and that the location of these linkages should be in the side chains having a disordered conformation The β-D-(1→3)-glucosidic linkages of both the backbone and the side chains might be taking an ordered conformation, as manifested by complete loss of the peak areas

Interestingly, the ^{13}C-n m r -spectral patterns of fraction IV vary with the concentration, in contrast to those of fraction II, lentinan, and other (1→3)-β-D-glucans[27 28] Fraction IV is water-soluble under the conditions of isolation, at 80 mg/ml, however, fraction IV is not soluble in water, but forms a soft gel similar to that of lentinan The ^{13}C-n m r spectrum of fraction IV at this concentration is, therefore, very similar to that of lentinan (see Fig 3A) However, the spectrum recorded at 40 mg/ml shows rather narrow line-widths, as seen in Fig 3B As shown in Table I, the lowermost sharp signal of fraction IV is not assigned to C-1 of β-D-(1→3)-linked, but to that of β-D-(1→6)-linked, D-glucose residues Hence, C-1 and C-3 signals due to β-D-(1→3)-linked D-glucose residues are still completely lost in this sample Furthermore the C-4 signal of β-D-(1→3)-linked D-glucose residues is partially lost, because the peak intensity is about half that of the corresponding C-6 of β-D-(1→3)-linked D-glucose residues This result is again consistent with the aforementioned finding about lentinan, namely, that the β-D-(1→3)-linked main chain and side chains are in a rigid conformation that produces very wide line-widths

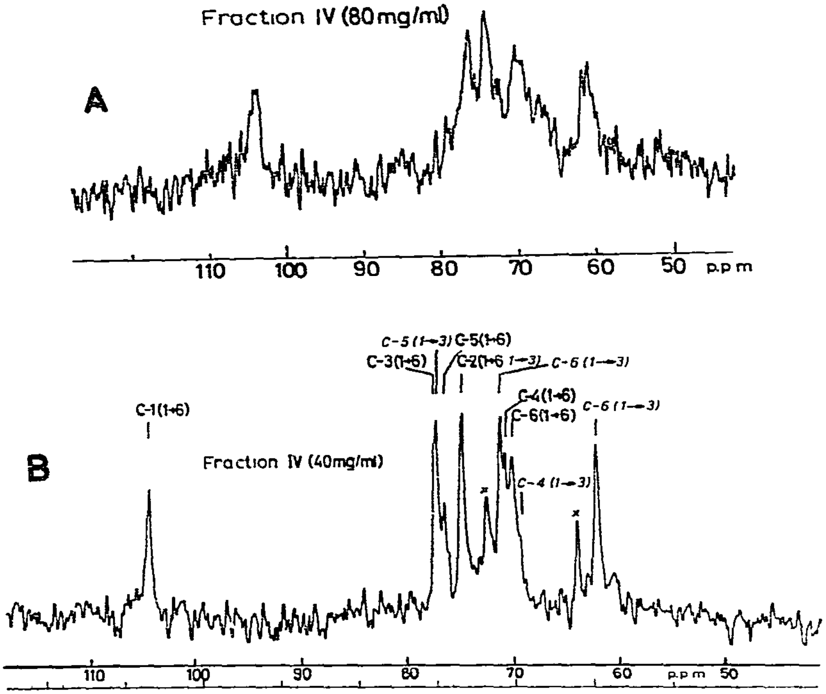

Fig 3 ^{13}C-N m r spectra of fraction IV at various concentrations [A Gel state (80 mg/ml) B Viscous solution (40 mg/ml) The resonances marked × are extraneous, due to impurities or products of degradation, or both] (beyond observation by the conventional, high-resolution spectrometer), while the β-D-(1→6)-glucosidic linkages are located at such positions as to gain molecular mobility It is recognized that chain lengths of intermediate size require, as seen in the study with Congo Red, a high concentration in order to achieve gel structure, as with fraction IV The β-D-(1→6)-linked D-glucose residues in side chains are likely to occur as long blocks, as no further splitting of peaks[11,14] due to involvement of other linkages is observed

B Observation of Single-helix Chains

In the foregoing section, we have demonstrated that all of the peaks due to β-D-(1→3)-glucosidic linkages (except for low-intensity C-6) are completely lost in the gel state However, it was shown[27] that 20–30% of the total gel (and 60% from C-6) were clearly observed in the resilient gel of a linear (1→3)-β-D-glucan (PS 13140) Presumably, this discrepancy might be caused by immobilization of the single-helical region, which is visible by ^{13}C-n m r spectroscopy for the latter, due to the side-chain participation in interchain connections in the former This view had been suggested[28] for interpretation of the peak areas of A_3 from *Pleurotus ostreatus* In Fig 4B is shown the ^{13}C-n m r spectrum of lentinan in the presence of 0 03M sodium hydroxide (pD 12 5) From the shift in the absorption maximum of Congo Red complexed with lentinan, and also from the titration study of ^{13}C-n m r spectra, it is seen that the ordered conformation is retained[31] up to a concentration of sodium hydroxide of 0 20M On comparing Fig 4B with Fig 4A (lentinan gel) and Fig 4C (gel of PS 13140), it is clearly seen that there are broad signals (line-width ~150 Hz) for C-1 (105 3 p p m) and C-3 (88 5 p p m), the positions of which are in good agreement with those[27] of *PS 13140, these appear in addition to the considerable increase of the peak height of C-6 in the β-D-(1→3)-glucosidic linkages in the presence of dilute alkali Broad envelopes of C-5, C-2, and C-4 of β-D-(1→3)-linkages are likely to be superimposed in the region of C-3, C-5, C-2, C-4, and C-6 of β-D-(1→6)-linked D-glucose residues originally observed in the gel state Moreover, in accordance with the previous observation[27], the peak positions of C-1 and C-3 of lentinan are found to be unchanged up to 0 19M concentration of sodium hydroxide, at which, full peak-areas are observed as a result of helix–coil transition[31] Therefore, those signals are ascribed to the single-helix conformation of (1→3)-β-D-glucans These results indicate that regions of single-helix chains exist in the branched (1→3)-β-D-

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*It was reported that the ^{13}C chemical-shifts of C-1 and C-3 of the resilient gel of PS 13140 are 106 5 and 88 7 p p m , respectively[27] With respect to those of laminaran (see Table I), downfield displacements of the C-1 and C-3 signals of PS 13140 gel are found to amount to 2 8 and 3 2 p p m , respectively Similarly, downfield displacement of C-1 and C-3 of lentinan (in 0 03M NaOH) are 1 6 and 3 0 p p m , respectively Interestingly, such substantial, downfield displacements occur at carbon atoms participating in the D-glucosidic linkages of (1→3)-β-D-glucans Those downfield displacements were explained by the presence of the fixed conformation having the favored dihedral angle, in which internal rotations around the D-glucosidic bonds are not allowed[27] Combined with the results of theoretical prediction and the downfield shift of the C-4 signal, which is consistent with the presence of an O-4 –O-5 intramolecular hydrogen-bond, those downfield displacements of the signals were ascribed to the presence of a helical conformation[27]

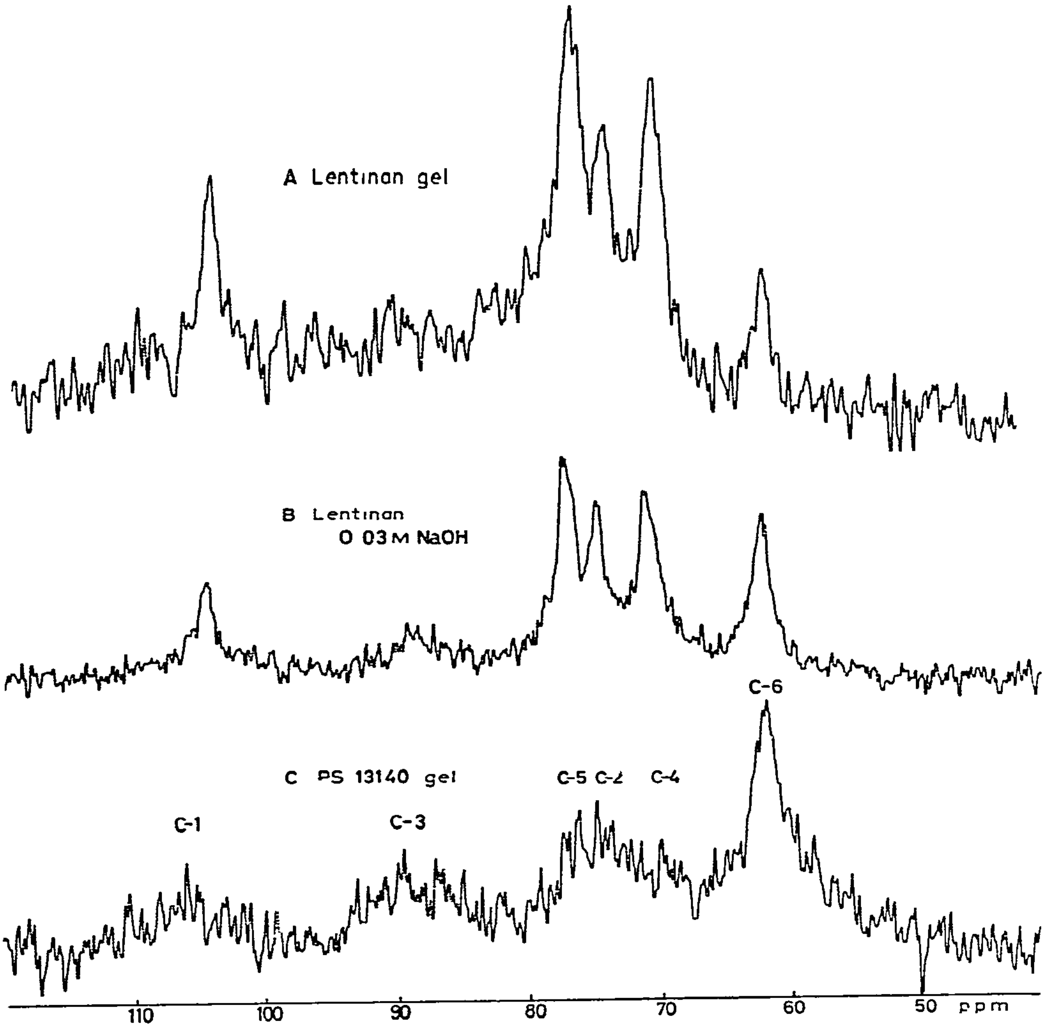

Fig 4 Change of ^{13}C-n m r spectra in the presence of a low concentration of alkali (0 03M NaOH) [A Lentinan gel (neutral) B Lentinan in the presence of 0 03M NaOH C Gel of PS 13140 (ref 27)]

glucan, in addition to the multi-helix junction-zones which are still not observed at that concentration Considerable losses of peak areas are well documented for various cases, including gels[27 28 32–34], solid rubber, and amorphous polymers above the glass transition[35] Estimation of the size of peak areas of C-1, (C-3), and C-6 compared to those at pD 13 7 gives values of 30–40% and 60%, respectively, these values are in good agreement with those[27] of the resilient gel of PS 13140 To observe these signals, however, it was necessary to remove side-chain interaction (which is rather weak) Accordingly, it is likely that the mode of forming the gel network is essentially identical for linear and branched glucans The line-broadening of ^{13}C-signals of ordered regions has been explained in terms of a broad distribution of correlation times[36] Hence, peak loss of the single-helix conformation in the gel state may be attributable to wider distribution of correlation times, especially the presence, at the lower-frequency end, of correlation times due to side-chain interactions

C Confirmation of Branched Structure

As already mentioned, the conformation both of linear and of branched (1→3)-β-D-glucans is converted into the random coil or, at least, into a coil of shorter helical chains, in dimethyl sulfoxide and in alkaline solution (pD >13 4) ^{13}C-N m r spectra of lentinan and fraction IV are given in Fig 5, together with those of A_3 and schizophyllan Regardless of differences in the types of the side chains attached to the backbone of (1→3)-β-D-glucans, the ^{13}C-n m r spectra shown in Fig 5 are very similar to each other, suggesting that the peaks are ascribable to the β-D-(1→3)-linked main-chains, which are common to all of the branched (1→3)-β-D-glucans containing branch points at C-6′ Side chains, β-D-(1→6)-linked D-glucose (lentinan), and D-glucose residues (schizophyllan) are obviously buried under the peaks of β-D-(1→3)-linked D-glucose residues Based on the assignments of the peaks of A_3 previously reported, the assignment of peaks (as marked by arrows) is straightforward, namely, to C-5′ and C-6′ of the branch points, as the chemical shifts of these materials are in good agreement (see Table II)

Furthermore, the nOe of C-6′ (1 1) is found to be smaller than that of C-6 (1 5) The other nOe values are 1 5 (C-1), 1 6 (C-2), 1 4 (C-3), 1 5 (C-4), and 1 2 (C-5) The decrease of the nOe of C-6′ compared to that of C-6 is, therefore, explained in terms of the lessened mobility due to glucosidic bond-formation at C-6′ This observation is consistent with that[28] for the similar, branched (1→3)-β-D-glucan A_3 The peak height of C-3 is apparently lowered with respect to that of other signals The nOe values described, and the spin–lattice relaxation-times are found to be

TABLE II

^{13}C-CHEMICAL SHIFTS (IN P P M) OF BRANCHED (1→3)-β-D-GLUCANS IN $Me_2SO\text{-}d_6$

	Linear β-D-(1→3) PS 13140	*Branched β-D-(1→3)*				*β-D-(1→6) (GE-3)*
		Lentinan[a]	*Fraction IV*[a]	A_3[b c]	*Schizophyllan*[d]	
C-1	104 5	104 6	104 6	104 5	104 5	104 9
C-2	74 3	74 3	74 2	74 3	74 3	75 0
C-3	87 5	87 7	87 7	87 8	87 9	78 1
C-4	69 9	70 2	70 2	69 9	70 2	71 4
C-5	77 7	77 9	77 8	77 6	77 8	77 1
C-6	62 4	62 5	62 5	62 4	62 5	70 0
C-5′[e]		75 3	75 3	75 3	75 4	
C-6′[e]		71 5	71 6	71 6	71 5	

[a] Side chain β-D-(1→3), and β-D-(1→6) [b] Side chain α-D-(1→4) [c] C-1 and C-4 of α-D-(1→4)-glucosidic linkages are at 101 9 and 80 7 p p m , respectively [d] Side chain D-glucose [e] Branch point of β-D-(1→3)-linked main-chain

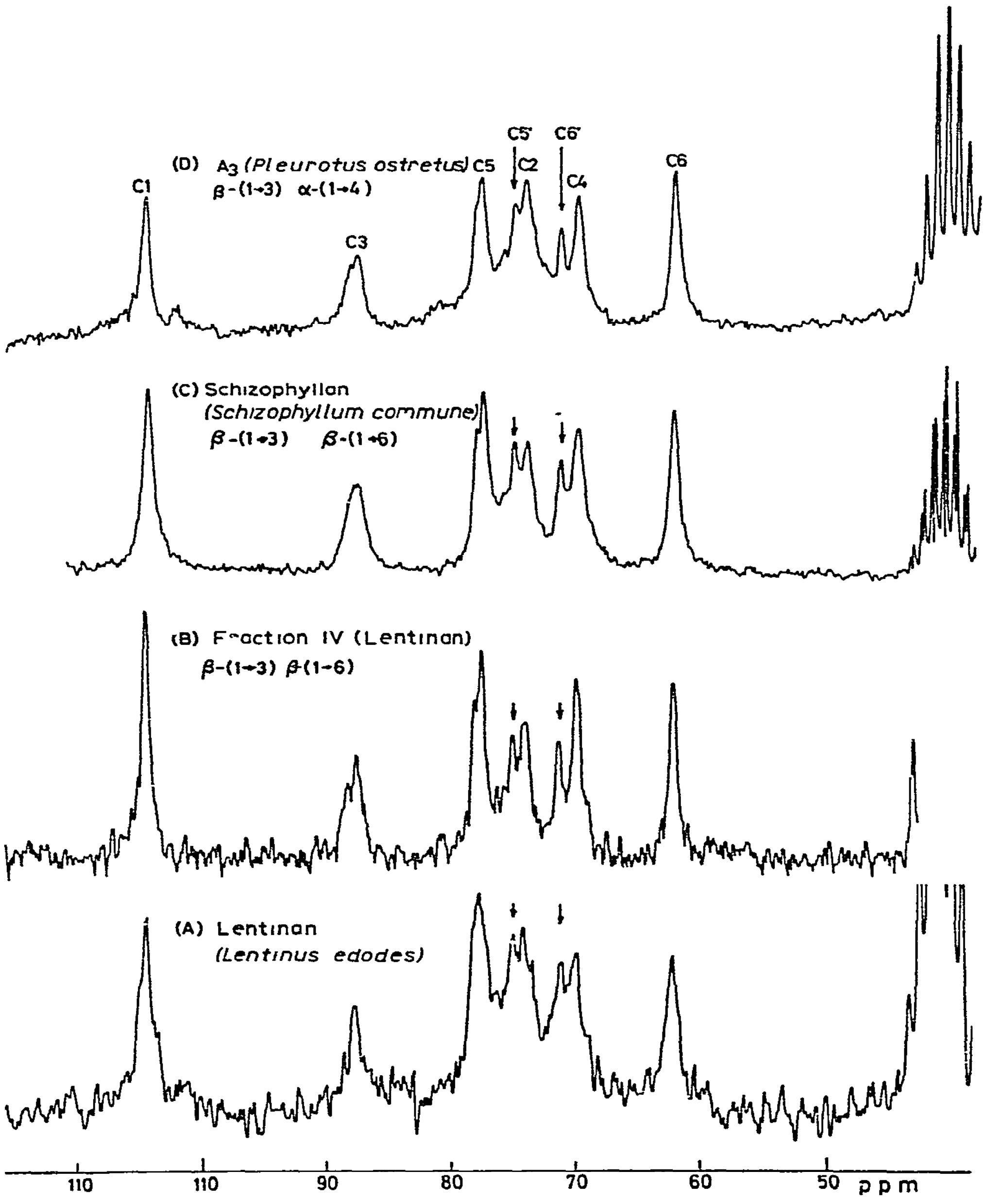

Fig 5 ^{13}C-N m r spectra of various types of branched (1→3)-β-D-glucans in Me_2SO-d_6 {A Lentinan [side chains, β-(1→3), β-(1→6)] B Fraction IV [side chains, β-(1→3), β-(1→6)] C Schizophyllan (D-glucose) D A_3 [α-(1→4)]} almost the same among the atoms C-1 to C-5 (60–80 msec for T_1 values) Presumably, a slight displacement of the shift due to the glucosidic bond at C-6′ is responsible for the broadening of the C-3 signals that is common to all branched D-glucans containing a similar proportion of branch points

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