[Chem. Pharm. Bull. 33(11)4950—4956(1985)]

# Structure–Function Relationship of Antitumor β-1,3-Glucan Obtained from Matted Mycelium of Cultured *Grifola frondosa*

KAZUYOSHI IINO,[a] NAOHITO OHNO,[a] IWAO SUZUKI,[a]
KICHIRO SATO,[b] SHOZO OIKAWA,[b]
and TOSHIRO YADOMAE*,[a]

*Tokyo College of Pharmacy,[a] Horinouchi, Hachioji, Tokyo 192–03, Japan*
*and Nippon Beet Sugar Mfg. Co., Ltd.,[b]*
*Chuo-ku, Tokyo 104, Japan*

(Received March 26, 1985)

The structure–function relationship of the antitumor 6-branched β-1,3-glucan (grifolan NMF-5N) extracted from the matted mycelium of *Grifola frondosa* was examined by using various chemical modification procedures (periodate oxidation, Smith degradation, and enzymic hydrolysis). The antitumor activity of grifolan NMF-5N was not lowered by periodate oxidation. Partial removal of branches by mild hydrolysis did not affect the activity. However, the linear β-1,3-glucan obtained by Smith degradation lacked antitumor activity. From the results of enzymic hydrolysis of grifolan NMF-5N, this glucan contains a β-1,3-glucanase-resistant core structure. The core structure is present randomly in this glucan, and does not itself show antitumor activity. These results suggest that some of the branches at C-6 in grifolan NMF-5N are important for the antitumor activity, and that the activity requires a relatively high molecular weight.

**Keywords**——*Grifola frondosa*; antitumor activity; β-1,3-glucan; chemical modification; matted mycelium

The polysaccharides extracted from the fruit bodies of Basidiomycotina and Ascomycotina are potent antitumor agents. It is well known that the antitumor activity of these fungal extracts is due to β-1,3-glucans. *Grifola frondosa* is a fungus belonging to the Basidiomycetes, Aphyllophorales, Polypolaceae. Recently, we reported that the cultured fruit body and the matted mycelium of *G. frondosa* contained antitumor β-1,3-glucans(grifolan).[1)] The primary structure of grifolan is a branched β-1,3-glucan possessing a β-glucosyl branch at position C-6 of every third 3-substituted β-glucosyl unit.[1c,d)] Antitumor activity of the β-1,3-glucan is due to the activation of certain immune systems. Many kinds of polysaccharides are known to show host-mediated antitumor activity, but the molecular basis for this (*e.g.* the active site of glucan, the earliest biochemical events of the action, and the nature of the receptor protein) is not clear yet.

In this work, we examined the structural factors required for antitumor activity of grifolan NMF-5N by using periodate oxidation and enzymic hydrolysis methods.

## Materials and Methods

**Periodate Oxidation and Smith Degradation**——Grifolan NMF-5N (201 mg) was oxidized with 10 mM sodium metaperiodate (700 ml) at 4 °C in the dark. When the reaction was complete (162 h), excess periodate was destroyed with ethylene glycol, and the mixture was dialyzed against tap water for 2 d, then distilled water for 1 d. The non-dialyzable fraction was reduced with sodium borohydride (875 mg) at 4 °C for 48 h. After acidification with acetic acid, the mixture was dialyzed and lyophilized to give grifolan NMF-5N(I/B) (180.64 mg).

A portion (40 mg) of grifolan NMF-5N(I/B) was partially hydrolyzed with 0.1 N sulfuric acid (47 ml) at 25 °C for 24 h. Another portion (40 mg) was partially hydrolyzed with 0.2 N sulfuric acid (47 ml) at 90 °C for 1 h. Each mixture,

after neutralization, was dialyzed against distilled water for 2 d. The non-dialyzable fraction was methylated, and then analyzed by gas liquid chromatography (GLC).

**Enzymic Hydrolysis**——Grifolan NMF-5N (100 mg) was dissolved in 0.03 N McIlvaine buffer (pH 4.9) (100 ml) and *exo*-$\beta$-1,3-glucanase (57 mg, 50 ml) was added. The mixture was shaken at 37 °C for 24 h, and the reaction was terminated by heating at 100 °C for 15 min. The resulting solution was dialyzed against tap water for 2 d, and distilled water for 1 d. The non-dialyzable fraction (L-grifolan NMF-5N) was methylated, and then analyzed by GLC.

**Complex Formation with Congo Red**——The change of absorption maximum of Congo Red (Wako Pure Chemical Co., Ltd.) in the presence of glucans or glucan-polyalcohols was followed by the procedure of Ogawa *et al.*[2] Glucan or glucan-polyalcohol solutions (1 mg/ml) and $2.16 \times 10^{-5}$ M Congo Red were mixed in equal volumes, and $\lambda_{max}$ was measured using a Hitachi 557 spectrophotometer.

**Viscosity Measurement**——The viscosity was determined with a Ostwald-type viscometer (capillary 1 mm) at 25 °C in a water bath. All measurements were performed after equilibration of the solution (1 mg/ml, 15 ml) at 25 °C. Under these conditions, the influence of alkali on the glucans was negligible.

**General Methods**——Purification of grifolan NMF-5N, evaluation of antitumor activity, methylation analysis, and other physicochemical methods were performed as described previously.[1]

## Results

### Structure and Antitumor Activity of Modified Grifolan NMF-5N Prepared by Enzymic Hydrolysis

In previous papers, we reported that about 50% of grifolan NMF-5N was digested with *exo*-$\beta$-1,3-glucanase. This result suggested that enzyme-resistant structure might exist in grifolan NMF-5N. Thus, we studied the structure and antitumor activity of the enzyme-resistant part.

When the enzyme-resistant fraction (L-grifolan NMF-5N) was applied to a column of Sepharose CL-4B, the elution profile was broad, as shown in Fig. 1, and two major fractions (4B-1, 4B-2) were obtained. From the results of methylation analysis, the structure of 4B-1 was concluded to be similar to that of native grifolan NMF-5N. On the other hand, 4B-2 showed different molar ratios: 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri-, and 2,4-di-*O*-methyl-D-glucose 1.00 : 1.32 : 1.55 : 1.33 (Table I). The results suggest that 4B-2 was a highly branched part, and that an enzyme-resistant, irregular basic structure (core part) existed in grifolan NMF-5N. Further, the core part may exist randomly in the extracted polysaccharides because the molecular weight of enzymic hydrolysate by *exo*-$\beta$-1,3-glucanase was broadly dispersed.

Further, we measured the absorption maximum ($\lambda_{max}$) of glucan–Congo Red complex (Table II). $\lambda_{max}$ of 4B-1–Congo Red complex was shifted to longer wavelength (506 nm), and returned reversibly to $\lambda_{max}$ of Congo Red (481 nm) at higher alkali concentration. This result suggests that 4B-1 formed helix structure. On the other hand, the shift with 4B-2 was irreversible. It appears that the ultrastructure of 4B-2 is quite different from that of native grifolan NMF-5N.

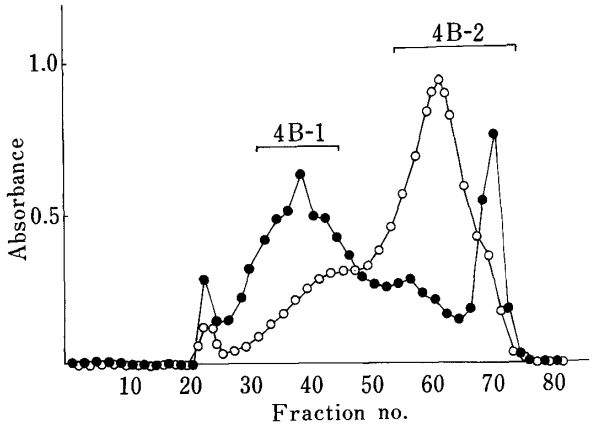


Fig. 1. Elution Profiles of L-Grifolan NMF-5N from a Column of Sepharose CL-4B

The column (1.8 × 90 cm) was equilibrated with 0.2 N NaOH–8 M urea, and 100 mg of L-grifolan NMF-5N was applied. Fractions of 2.4 ml were collected and carbohydrate and protein were assayed by the phenol–sulfuric acid method (carbohydrate, 490 nm; —●—), and by ultraviolet absorption measurement (protein, 280 nm; —○—), respectively.

Table I. Gas Liquid Chromatography of Alditol Acetates Derived from the Methylated Polysaccharides

| Alditol acetate of | Grifolan NMF-5N | L-Grifolan NMF-5N 4B-1[a] | L-Grifolan NMF-5N 4B-2[a] |
|---|---|---|---|
| 2,3,4,6-$Me_4$-Glc | 1.0 | 1.0 | 1.0 |
| 2,4,6-$Me_3$-Glc | 1.97 | 1.93 | 1.32 |
| 2,3,4-$Me_3$-Glc | 0.01 | 0.02 | 1.55 |
| 2,3,6-$Me_3$-Glc | 0.02 | 0.0 | 0.04 |
| 2,6-$Me_2$-Glc | 0.0 | 0.0 | 0.31 |
| 3,4,6-$Me_3$-Glc | 0.05 | 0.04 | 0.0 |
| 2,4-$Me_2$-Glc | 1.22 | 1.07 | 1.33 |
| 2,3-$Me_2$-Glc | 0.0 | 0.0 | 0.05 |
| 3,6-$Me_2$-Glc | 0.0 | 0.0 | 0.39 |

*a*) Lysing enzyme-treated grifolan NMF-5N.

Table II. Absorption Maximum of Congo Red in Alkaline Solution in the Presence and in the Absence of Polysaccharides

| Sample | $\lambda_{max}$ nm | | |
|---|---|---|---|
| | 0.1 N NaOH | 0.2 N NaOH | 0.3 N NaOH |
| Congo Red only | 482 | 479 | 480 |
| Congo Red + amylopectin | 482 | 479 | 481 |
| Congo Red + Islandican | 479 | 480 | 480 |
| Congo Red + laminaran | 481 | 478 | n.d.[a] |
| Congo Red + curdlan | 500 | 488 | 480 |
| Congo Red + grifolan NMF-5N | 503 | 483 | 481 |
| Congo Red + grifolan NMF-5N (I/B) | 503 | 500 | 481 |
| Congo Red + SD(1)-grifolan NMF-5N (I/B)[b] | 508 | 501 | 481 |
| Congo Red + SD(3)-grifolan NMF-5N (I/B)[c] | 492 | 492 | 492 |
| Congo Red + L-grifolan NMF-5N 4B-1 | 506 | 502 | 481 |
| Congo Red + L-grifolan NMF-5N 4B-2 | 501 | 501 | 501 |

*a*) Not determined. *b*) Grifolan NMF-5N (I/B) was treated with 0.1 N $H_2SO_4$ at 25 °C for 24 h. *c*) Grifolan NMF-5N (I/B) was treated with 0.2 N $H_2SO_4$ at 90 °C for 1 h.

As shown in Table III, 4B-1 showed antitumor activity, but 4B-2 showed no activity. These results suggest that the core structure does not participate in antitumor activity. The core structure may exist at the terminal portion of the sugar chains.

**Glucan-Polyalcohols Obtained from Grifolan NMF-5N by Periodate Oxidation and Smith Degradation**

As shown in Fig. 2, the carbon-13 nuclear magnetic resonance ($^{13}$C-NMR) spectrum of grifolan NMF-5N(I/B) obtained by periodate oxidation and sodium borohydride reduction exhibits characteristic signals (61.35 and 62.11 ppm) of several alcohol groups. Further, this spectrum is similar to that of water-insoluble glucans derived from *Auricularia auricula-judae*.[3] $Me_4$-Glucose was not detected in methylation analysis. This result suggested that all of the non reducing-terminal glucose residues of side chain were cleaved. The polyalcohol gave two peaks corresponding to 2,4,6-tri- and 2,4-di-*O*-methyl-D-glucose, in a molar ratio of 1.00 : 0.55. This ratio is consistent with that of the native glucan (Table IV).

The polyalcohol was partially hydrolyzed by diluted acid (SD(1) : 0.1 N $H_2SO_4$, 25 °C,

TABLE III. Antitumor Effect of Polysaccharide Fractions from the Matted Mycelium of Cultured *G. frondosa*[a]

| Sample | Dose × 10 (μg/mouse) | Tumor weight[c] (g, mean ± S.D.) | Inhibition[b] ratio (%) | C.R.[b] |
|---|---|---|---|---|
| Grifolan NMF-5N | 4 | 1.5 ± 1.6[e] | 63 | 1/10 |
| | 20 | 0.2 ± 0.7[f] | 95 | 9/10 |
| | 100 | 0.1 ± 0.2[f] | 98 | 1/10 |
| L-Grifolan NMF-5N 4B-1 | 4 | 3.4 ± 2.7 | 15 | 0/9 |
| | 20 | 1.3 ± 2.3[e] | 68 | 4/9 |
| | 100 | 0.4 ± 0.8[f] | 90 | 3/10 |
| L-Grifolan NMF-5N 4B-2 | 4 | 3.0 ± 2.3 | 25 | 0/7 |
| | 20 | 5.2 ± 2.8 | −30 | 0/8 |
| | 100 | 6.4 ± 3.1 | −60 | 0/10 |
| Lysing enzymes | 100 | 3.9 ± 2.0 | 3 | 0/10 |
| | 400 | 2.2 ± 1.5[d] | 45 | 0/10 |
| Control | — | 4.0 ± 2.8 | — | 0/20 |

*a*) Sarcoma 180 tumor cells ($5 \times 10^6$) were inoculated subcutaneously. Each sample was administered as saline solution by intraperitoneal injection. *b*) Inhibition ratio and complete regression (C.R.) were determined at 35 d after tumor inoculation. *c*) The significance of differences was evaluated according to Student's *t*-test. Significant difference from the control (*d*) $p<0.02$, *e*) $p<0.01$, *f*) $p<0.001$).

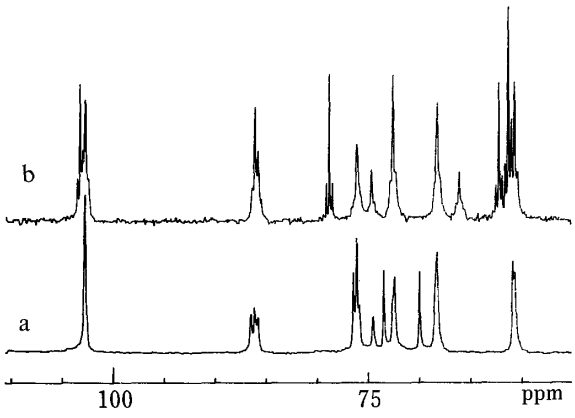


Fig. 2. $^{13}$C-NMR Spectra of Polysaccharide Fractions in DMSO-$d_6$ at 60 °C

a, grifolan NMF-5N; b, grifolan NMF-5N (I/B).

TABLE IV. Gas Liquid Chromatography of Alditol Acetates Derived from the Methylated Polysaccharides

| Alditol acetate of | Grifolan NMF-5N | Grifolan NMF-5N (I/B) | SD(1)–grifolan NMF-5N (I/B) | SD(3)–grifolan NMF-5N (I/B) |
|---|---|---|---|---|
| 2,3,4,6-$Me_4$-Glc | 0.51 | 0.0 | 0.0 | 0.0 |
| 2,4,6-$Me_3$-Glc | 1.0 | 1.0 | 1.0 | 1.0 |
| 2,4-$Me_2$-Glc | 0.62 | 0.55 | 0.22 | 0.01 |

24 h; SD(3) : 0.2 N $H_2SO_4$, 90 °C, 1 h). Only glycerol was detected in the dialyzable fraction of each hydrolyzate (data not shown). From the methylation analysis of the non-dialyzable fraction, SD(1)-grifolan NMF-5N(I/B) gave 2,4,6-tri-, and 2,4-di-*O*-methyl-D-glucitol in a molar ratio of 1.00 : 0.22. On the other hand, SD(3)-grifolan NMF-5N(I/B) gave 2,4,6-tri-, and 2,4-di-*O*-methyl-D-glucitol in a molar ratio of 1.00 : 0.01. SD(3)-grifolan NMF-5N(I/B) seems to consist largely of linear β-1,3 linkages (Table IV).

Further, we measured the absorption maximum of Congo Red in the presence of chemically modified glucans (Table II): the values of $\lambda_{max}$ of grifolan NMF-5N(I/B) and SD(1)-grifolan NMF-5N(I/B)–Congo Red complex were shifted to longer wavelength, and

Table V. Antitumor Effect of Polysaccharide Fractions from the Matted Mycelium of Cultured *G. frondosa*[a]

| Sample | Dose × 10 (μg/mouse) | Tumor weight[b] (g, mean ± S.D.) | Inhibition[a] ratio (%) | C.R.[a] |
|---|---|---|---|---|
| Grifolan NMF-5N | 4 | 1.8 ± 1.5[d] | 73 | 1/8 |
| | 7.5 | 0.5 ± 0.8[d] | 92 | 3/9 |
| | 20 | 0.4 ± 0.6[d] | 94 | 1/10 |
| Grifolan NMF-5N (I/B) | 4 | 1.7 ± 2.3[d] | 74 | 2/9 |
| | 20 | 0.4 ± 0.7[d] | 94 | 2/10 |
| | 100 | 1.0 ± 0.8[d] | 85 | 0/8 |
| SD(1)-grifolan NMF-5N (I/B) | 4 | 2.8 ± 3.9[c] | 58 | 1/10 |
| | 20 | 0.4 ± 0.8[d] | 94 | 2/10 |
| | 100 | 1.1 ± 1.2[d] | 83 | 0/10 |
| SD(3)-grifolan NMF-5N (I/B) | 4 | 5.5 ± 4.0 | 17 | 0/10 |
| | 20 | 5.3 ± 3.3 | 20 | 0/8 |
| | 100 | 6.9 ± 4.0 | −4.5 | 0/10 |
| Control | — | 6.6 ± 3.7 | — | — |

*a*) See Table III. *b*) The significance of differences was evaluated according to Student's *t*-test. Significant difference from the control (*c*) $p<0.05$, *d*) $p<0.001$).

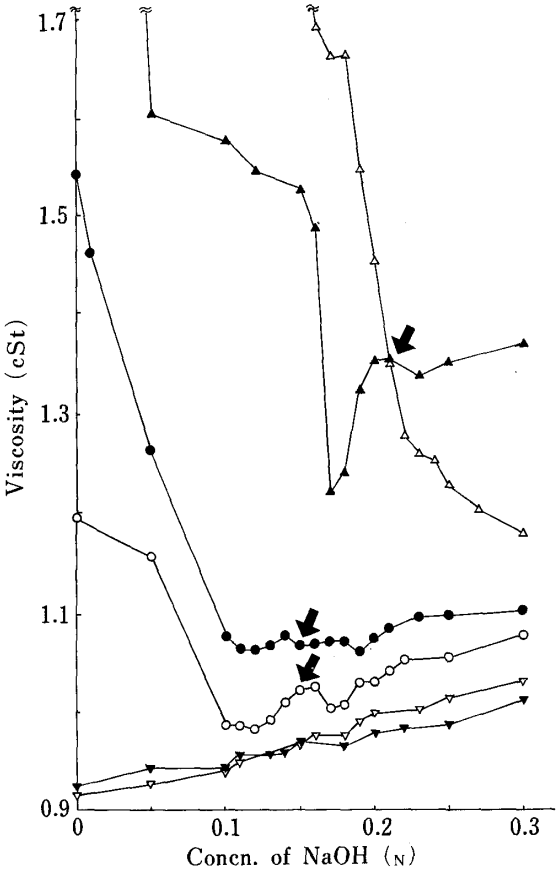


Fig. 3. Dependence of the Viscosity of Glucan Solution on Alkali Concentration

—●—, grifolan NMF-5N; —○—, grifolan-7N; —▲—, curdlan; —△—, islandican; —▼—, laminaran; —▽—, amylopectin. Arrows indicate the stationary states.

returned reversibly to 481 nm in higher alkali concentration, but the shift of SD(3)-grifolan NMF-5N(I/B) was irreversible. The ultrastructure of this glucan may be different from that of native grifolan NMF-5N.

Table V showed the antitumor activity of the above glucans. Grifolan NMF-5N(I/B) showed potent antitumor activity. The activity was not lowered by periodate oxidation and

partial acid hydrolysis (SD(1)-grifolan NMF-5N(I/B)). However, complete loss of branching points caused disappearance of the antitumor activity. These results suggest that β-glucose at C-6 is not required for antitumor activity, but that the existence of a certain proportion of substituent groups is necessary for antitumor activity, and that the antitumor activity depends on a specific ultrastructure of β-glucans.

**Gel Strength of Grifolan NMF-5N**

Under physiological conditions, β-1,3-glucan forms helical conformation and produces a soft gel.[4] The gel is cleaved by the addition of alkali or urea. It was reported that viscosity measurement was useful for comparing the strength of glucan-gel formation.[5] Figure 3 shows the dependence of viscosity of glucan solutions on the alkali concentration. Curdlan and grifolan, both of which formed helical conformation, showed a stationary state (arrows in Fig. 3), but amylopectin, islandican, and laminaran, none of which formed helical conformation, did not show this phenomenon. The alkali concentration at the stationary state was 0.22 N NaOH for curdlan. In the case of grifolan (NMF-5N, 7N), the state appeared weakly at about 0.15 N NaOH. These results suggested that a helix-random coil rearrangement of curdlan occurred in 0.22 N NaOH, while that of grifolan occurred in 0.15 N NaOH. It was reported that the rearrangement of lentinan occurs in 0.19 N NaOH.[6] Thus, it appears that the strength of gel formation of grifolan is weaker than that of curdlan or lentinan.

## Discussion

In previous papers,[1] we reported the purification of antitumor 6-branched β-1,3-glucan (grifolan) extracted from the fruit body and the matted mycelium of *Grifola frondosa*. The primary structure of grifolan was similar to those of other polysaccharides obtained from Basidiomycotina and Ascomycotina (*e.g.*, lentinan, scleroglucan, and schizophillan). In this work, we investigated the structure-function relationship of the antitumor β-1,3-glucan (grifolan NMF-5N) extracted from the matted mycelium of *G. frondosa* by utilizing several chemical modification procedures.

From the results of enzymic hydrolysis, it was concluded that grifolan NMF-5N contains a β-1,3-glucanase-resistant core structure. The methylation data suggest that the core part contained more branching points than the unit structure (Table I), and the core part did not participate in the antitumor activity (Table III). The molecular weight of grifolan NMF-5N is 750000 (distributed from 20000000 to 8000),[1c] and the core part is dispersed uniformly throughout the molecular-weight range (data not shown).

Enzyme-digested polysaccharide (4B-1, 4B-2) contained core structure at non-reducing regions of the main chain. 4B-1 was composed of unit structure in addition to a small portion of core structure, and showed antitumor activity (Table III). These results suggest that the inner part is necessary for the antitumor activity, and that the terminal group of sugar chains is not necessary. This is different from the situation in the case of oligosaccharide chains in glycoproteins.

Antitumor activity of grifolan NMF-5N was not lowered by periodate oxidation. This result suggests that the cleavage of glucose units at position C-6 did not influence the antitumor activity. Furthermore, the activity was not lowered by decreasing the branching at position C-6 (SD(1)-grifolan NMF-5N(I/B)). However, completely de-branched β-1,3-glucan (SD(3)-grifolan NMF-5N(I/B)) showed no activity. De-branched glucan had a quite different ultrastructure from native grifolan NMF-5N. It was suggested that branching at position C-6 is generally required for antitumor activity,[3] though curdlan (linear β-1,3-glucan) shows antitumor activity. We suggest that, in the case of grifolan, not only a small amount of branching but also an aggregated macromolecular structure (gel formation) are necessary for

antitumor activity.

Further, the gel rigidity of grifolan NMF-5N was found to be weaker than that of lentinan and curdlan from viscosity measurement (Fig. 3), $[\alpha]_D$, and circular dichroism spectroscopy (data not shown) in alkaline solutions. The core structure in grifolan NMF-5N presumably affects the strength of the gel (lentinan is known to consist completely of repeating-unit structure, but grifolan NMF-5N would not be able to form a complete repeating-unit structure because of the presence of the core structure). Recently, we reported that the hot water extract of *G. frondosa* (GF-1) showed a different mode of antitumor action from other antitumor glucans.[7] In this paper, we have clarified that aggregated macromolecular structure (gel formation) is necessary for antitumor activity. Hence it would be interesting to examine whether the difference of rigidity of this glucan is related to the mode of antitumor action.

**Acknowledgments** The authors thank Mr. T. Takeyama, Mr. J. Saito, Mr. M. Matsumura, and Mr. Y. Adachi for their technical assistance.

## References

1) *a*) N. Ohno, I. Suzuki, S. Oikawa, K. Sato, T. Miyazaki, and T. Yadomae, *Chem. Pharm. Bull.*, **32**, 1142 (1984); *b*) N. Ohno, K. Iino, I. Suzuki, S. Oikawa, K. Sato, T. Miyazaki, and T. Yadomae, *ibid.*, **33**, 1181 (1985); *c*) N. Ohno, K. Iino, T. Takeyama, I. Suzuki, K. Sato, S. Oikawa, T. Miyazaki, and T. Yadomae, *ibid.*, **33**, 3395 (1985); *d*) K. Iino, N. Ohno, I. Suzuki, T. Miyazaki, T. Yadomae, S. Oikawa, and K. Sato, *Carbohydr. Res.*, **141**, 111 (1985).
2) K. Ogawa, J. Tsurugi, and T. Watanabe, *Chem. Lett.*, **1972**, 689.
3) A. Misaki, M. Kakuta, T. Sasaki, M. Tanaka, and H. Miyaji, *Carbohydr. Res.*, **92**, 115 (1981).
4) *a*) Y. Deslandes, R. H. Marchessalt, and A. Sarko, *Macromolecules*, **13**, 1466 (1980); *b*) T. L. Bluhm, Y. Deslandes, R. H. Marchessalt, S. Perez, and M. Rinaudo, *Carbohydr. Res.*, **100**, 117 (1982); *c*) T. L. Bluhm and A. Sarko, *Can. J. Chem.*, **55**, 293 (1977).
5) K. Ogawa, T. Watanabe, J. Tsurugi, and S. Ono, *Carbohydr. Res.*, **23**, 399 (1972).
6) H. Saito, *Yuki Gosei Kagaku Kyokai Shi*, **37**, 935 (1979).
7) *a*) I. Suzuki, T. Itani, N. Ohno, S. Oikawa, K. Sato, T. Miyazaki, and T. Yadomae, *J. Pharmacobio-Dyn.*, **7**, 492 (1984); *b*) *Idem, ibid.*, **8**, 217 (1985).