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STRUCTURAL EXAMINATION OF ANTITUMOUR, WATER-SOLUBLE GLUCANS FROM *Grifora umbellata* BY USE OF FOUR TYPES OF GLUCANASE*

TOSHIO MIYAZAKI, NAOKO OIKAWA, HARUKI YAMADA, AND TOSHIRO YADOMAE
Department of Microbial Chemistry, Tokyo College of Pharmacy, Hachioji, Tokyo 192-03 (Japan)
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ABSTRACT

Antitumour glucans (GU) from the fungus *Grifora umbellata* have been subjected to periodate oxidation, Smith degradation, methylation analysis, and treatment with endo-(1→6)-β-D-, endo-(1→3)-β-D-, and exo-(1→3)-β-D-glucanases, and alpha-amylase; the following structural features were revealed. GU-2 contains a backbone involving (1→6)-β- and (1→3)-β linkages, and two kinds of branches involving (1→6)-β and (1→4)-α linkages. GU-3 has a (1→3)-β-linked backbone and branches involving (1→6)-β linkages or (1→4)-α and (1→6)-β linkages. GU-4 also contains a (1→3)-β-D-glucan backbone and a small number of (1→6)-β-linked branches. Probable structural units of these glucans are proposed.

INTRODUCTION

Miyazaki *et al.* have reported that a water-soluble glucan[2] from *Grifora umbellata* markedly inhibited the growth of subcutaneously implanted Sarcoma 180 in mice[3]. We now report structural examinations of water-soluble polysaccharides isolated from *G. umbellata*; the endo-(1→6)-β-D- and endo-(1→3)-β-D-glucanases from *Mucor hiemalis*[4,5], exo-(1→3)-β-D-glucanase from *Alternaria solani*[6], and alpha-amylase from *Bacillus subtilis*[7] were used in these examinations.

MATERIALS AND METHODS

Isolation of water-soluble polysaccharide from Grifora umbellata. — Sclerotium of *G. umbellata* (1.2 kg) was extracted with hot water (3 × 5 vol.). The extract was dialysed against running water through Visking cellulose, and the residual solution was concentrated to a small volume. Addition of ethanol then gave a precipitate

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(GU-0) that was dried by washing with acetone and then ether. GU-0 was extracted with aqueous 0.4% sodium borate, and the residue was collected by centrifugation, suspended in water, acidified weakly with acetic acid, dialysed against running water, and lyophilised to give fraction BP. The extract was treated with aqueous 10% cetyltrimethylammonium bromide[8] (Cetavlon). The precipitate was collected by centrifugation, and dissolved in aqueous 10% sodium chloride, and the solution was weakly acidified with acetic acid, dialysed against running water, and then lyophilised to give fraction CP. To the Cetavlon supernatant was added ethanol (3 vol.), the precipitate was collected by centrifugation and washed with ethanol, and a solution in water was weakly acidified with acetic acid, dialysed against running water, concentrated, and diluted with ethanol (3 vol.). The precipitate was collected by centrifugation, washed with ethanol and acetone, and dried *in vacuo* to give fraction GU-1 (0.11% of dry weight of sclerotium).

A solution of GU-1 (200 mg) in water (4 ml) was applied to a column (2.6 × 89 cm) of Sepharose 2B. Elution with water gave first GU-2 (0.03%, molecular size larger than that of Dextran T-500), and then GU-3 (0.04%, molecular size smaller than that of GU-2). The column was calibrated with the following dextrans: T-2000 (mol. wt. 2,000,000), T-500 (500,000), T-250 (250,000), and T-110 (110,000) (Seikagaku Kogyo Co. Ltd., Japan).

Fraction BP was extracted with 500 vol. of hot water (50°), the extract was centrifuged at 10,000 r.p.m., and the supernatant solution was lyophilised to give fraction GU-4 (0.12%).

Properties of GU-2, GU-3, and GU-4. — Hexose content was determined by the phenol–sulphuric acid method[17], with D-glucose as the standard substance. I.r. spectra and $[\alpha]_D$ values were measured with a Hitachi Grating Infrared Spectrophotometer Model 215 and a Jasco DIP-181 Digital Polarimeter, respectively. Paper electrophoresis was carried out in aqueous 1% sodium borate (pH 9.2) for 1.5 h at 1 mA/cm, and detection with the periodate–Schiff[9] reagent. Sedimentation analysis was carried out with a Hitachi Ultracentrifuge Model 282 at 60,000 r.p.m. on 1 or 0.5% solutions in distilled water at 23° (50° for GU-4), and photographed at intervals of 15 or 12 min after reaching full speed. P.m.r. spectra (100 MHz) of solutions of the glucans and their enzyme-treated samples in D_2O (internal 2,2,3,3-tetradeuterio-3-trimethylsilylpropionate) were recorded with a JNM-4H-100 spectrometer at 84°. The spectrum of GU-4 was recorded at 23° in $CDCl_3$ after methylation. Paper chromatography of hydrolysates (0.5M H_2SO_4 for 5 h, and then 4M HCl at 100° for 5 h) was performed on Toyo Roshi No. 50 paper with ethyl acetate–pyridine–water (10:4:3) and detection with alkaline silver nitrate[10] and *p*-anisidine[11].

Periodate oxidation. — Solutions of samples (10 mg) in 7.6mM sodium periodate (5.9 ml) were stored at 22° in the dark. Consumption of oxidant and formation of formic acid were determined by the methods of Avigad[12] and Whistler[13]. Smith degradation[14] was carried out after 48 h, when the oxidation was complete. The oxidised glucan was reduced with borohydride, and hydrolysed (0.5M H_2SO_4, 100°, 5 h). After neutralisation of the acid, the products were reduced with borohydride,

and then treated with acetic anhydride–pyridine[14] (1:1). G.l.c. of the resulting alditol acetates was carried out with a Shimadzu GC-6A unit, equipped with a flame-ionization detector, using a glass column (200 × 0.3 cm) packed with 3% of ECNSS-M on Gas Chrom Q (100–200 mesh): column temperature, 130–190°, 5°/min; N_2 flow-rate, 60 ml/min.

Methylation analyses. — Each glucan was fully methylated by the Hakomori method[15], and the product was hydrolysed with 90% formic acid at 100° for 10 h and then 0.5M H_2SO_4 at 100° for 5 h. Methylated sugars were converted into their alditol acetates, and analysed by g.l.c.–m.s.[16] using a Hitachi RMU-7L mass spectrometer and the column described above.

Enzymes. — Endo-(1→6)-β-D-glucanase and endo-(1→3)-β-D-glucanase were prepared[4,5] from *Mucor hiemalis*, and exo-(1→3)-β-D-glucanase from *Alternaria solani*[6]. alpha-Amylase from *Bacillus subtilis*, saccharifying type, was purchased from Seikagaku Kogyo Co. Ltd., Japan.

Degradation of the glucans by glucanases. — (*a*) *Endo-(1→6)-β-D-glucanase.* A ratio of 6.4 units of enzyme per 10 mg of the glucan dissolved in 3mM acetate buffer (pH 4.5) was used for 48 h at 55°. Each mixture was dialysed against distilled water after inactivation of the enzyme at 100° for 5 min. The procedure was repeated on the non-dialysable fraction. The combined dialysable fractions were concentrated to a small volume, passed through a column of Dowex 50W X8 resin, and lyophilised.

(*b*) *Other enzymes.* These enzymes were incubated under the following optimum conditions, and the mixtures were then processed as described above: endo-(1→3)-β-D-glucanase, pH 5.0, 50°; exo-(1→3)-β-D-glucanase, pH 5.0, 55°; and alpha-amylase, pH 5.3, 40°.

Analysis of the dialysable and the non-dialysable fractions obtained after the enzyme treatment. — The ratio of sugar contents of the dialysable fraction and the glucan was determined by the phenol–sulphuric acid method[17]. After deionization with Dowex 50W resin and lyophilisation, the dialysable fraction was chromatographed on a column of Biogel P-4, or on Whatman 3MM paper by multi-development with 1-propanol–ethyl acetate–water (6:1:3). The non-dialysable fraction was examined by methylation, p.m.r. spectroscopy, and gel filtration on Sepharose 2B.

RESULTS AND DISCUSSION

Many antitumour polysaccharides are known[18], including the water-soluble glucan from the sclerotium of *Grifora umbellata*. Some data on the glucan fraction GU-1, extracted with hot water from the fungus, have been reported[2,3]. In order to examine the relationship between chemical structure and antitumour activity, the antitumour-active fraction, GU-1, and the other active fraction, GU-4, have been subjected to detailed investigation.

Extraction of the sclerotium of *G. umbellata* with hot water gave glucan GU-0, part of which (BP) was insoluble in 0.4% sodium borate. The supernatant solution was fractionated with Cetavlon, to give glucan GU-1, which gave a single spot on

paper electrophoresis but a wide distribution pattern on gel filtration with Sepharose 2B. Two fractions were obtained, namely, GU-2 (molecular size >500,000) and GU-3 (molecular size smaller than that of GU-2). Glucan GU-4 was obtained by extraction of fraction BP with hot water.

Glucan GU-2, $[\alpha]_D$ +58° (water), gave only glucose on acid hydrolysis, and contained 98.6% of total sugar as glucose. The p.m.r. spectrum contained signals at τ 4.57, 4.81, and 5.42, consistent with anomeric protons due to (1→6)-β-, (1→3)-β-, and (1→4)-α-D-glucosidic linkages[19]. GU-2 consumed 1.18 mol of periodate and released 0.3 mol of formic acid per glucosyl residue during 102 h, and Smith degradation gave glycerol, erythritol, and glucitol. G.l.c.–m.s. of the methylated alditol acetates derived from GU-2 revealed 2,3,4,6-tetra-*O*-methyl-, 2,4,6-, 2,3,4-, and 2,3,6-tri-*O*-methyl-, and 2,3- and 2,4-di-*O*-methyl-glucitol acetates in the molar ratios 1.0:0.7:4.2:1.0 for 2,3,4,6:2,4,6:2,3,4+2,3,6:2,3+2,4.

When GU-2 was incubated with amylase, 23.8% of the glucan was released as dialysable material (glucose, 5.7%; maltose, 9.8%; unidentified higher oligomers, 8.3%). The non-dialysable fraction had $[\alpha]_D$ +13°, and its gel-filtration pattern on Sepharose 2B was not significantly different from that of GU-2 (Fig. 1), indicating a similar molecular shape. The broad distribution pattern indicates a mixture of molecular species. Methylation analysis of the non-dialysable fraction gave 2,3,4,6-tetra-*O*-methyl-, 2,4,6-, 2,3,4-, and 2,3,6-tri-*O*-methyl-, and 2,3- and 2,4-di-*O*-methyl-glucitol acetates in the molar ratios 3.0:2.4:7.8:3.0 for 2,3,4,6:2,4,6:2,3,4+2,3,6:2,3+2.4.

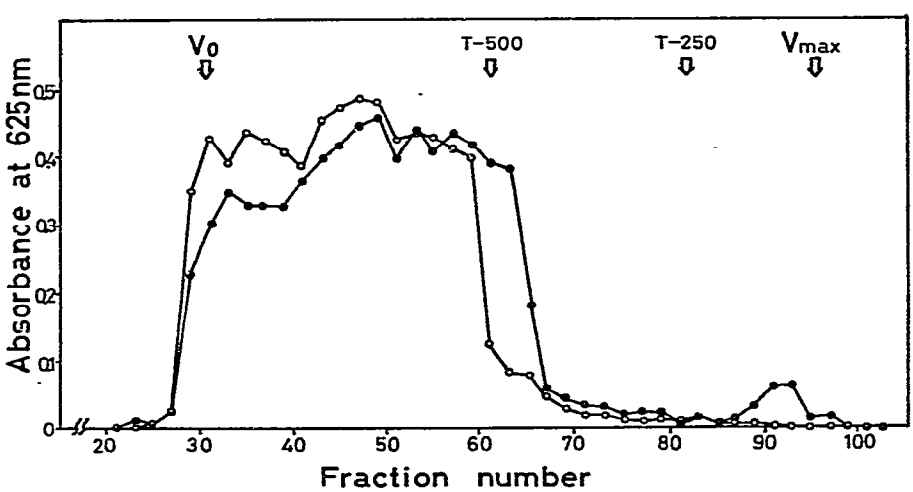


Fig. 1. Gel filtration of GU-2 (—○—) and α-amylase-treated GU-2 (—●—) on a column (1.9 × 88 cm) of Sepharose 2B. Amylase-treated GU-2 (30 mg) in distilled water (2 ml) was eluted with distilled water, and fractions (3 ml) were assayed with anthrone–H_2SO_4: V_0, void volume; T-500, elution volume of Dextran T-500 (mol. wt. 500,000); T-250, elution volume of Dextran T-250 (mol. wt. 250,000); V_{max}, maximum elution volume.

Incubation of GU-2 with endo-(1→6)-β-D-glucanase released 26% of dialysable material that gave two major fractions on gel filtration on Biogel P-4. The fraction of smaller size was analysed by p.c., and by g.l.c. after methylation; gentiobiose (4.8%), gentiotriose (1.6%), and glucose (2.4%) were detected. The fraction of larger size could not be analysed. The non-dialysable fraction had $[\alpha]_D$ +109° and gave a narrow band when eluted from Sepharose 2B (Fig. 2), suggesting that it was a simpler molecular species than GU-2. Methylation analysis gave 2,3,4,6-tetra-, 2,4,6-, 2,3,4-,

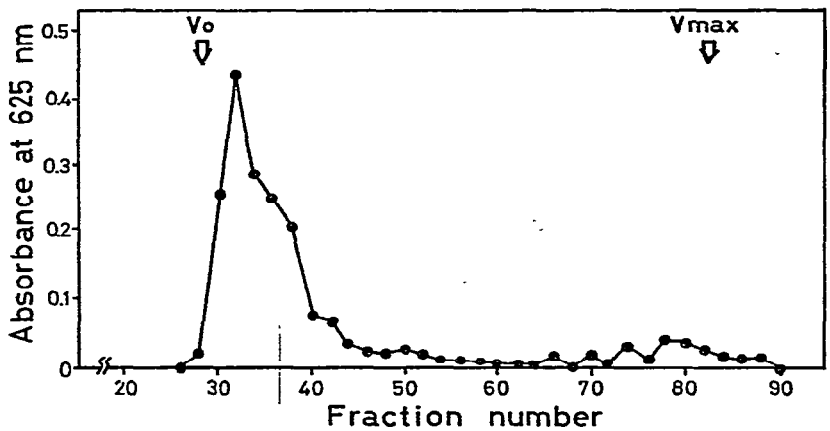


Fig. 2. Gel filtration of endo-(1→6)-β-D-glucanase-treated GU-2 on a column (1.9×41 cm) of Sepharose 2B. Glucan (5 mg) in distilled water (1 ml) was eluted with distilled water, and fractions (1.5 ml) were assayed with anthrone–H_2SO_4: V_o, void volume; V_{max}, maximum elution volume. See Fig. 1, for elution pattern of GU-2.

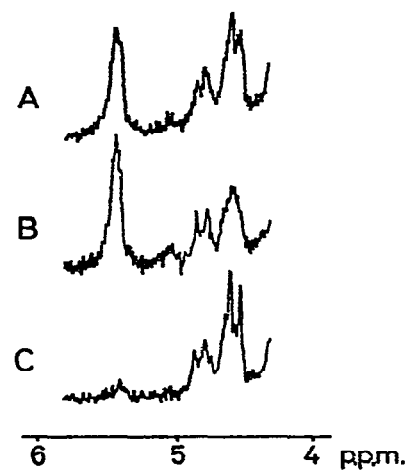


Fig. 3. P.m.r. spectra of GU-2 (A), endo-(1→6)-β-D-glucanase-treated GU-2 (B), and alpha-amylase-treated GU-2 (C).

and 2,3,6-tri-, and 2,4- and 2,3-di-*O*-methylglucitol acetates in the molar ratios of 2.0:1.6:9.3:2.0 for 2,3,4,6:2,4,6:2,3,4+2,3,6:2,3+2,4. The decrease of (1→4)-α and (1→6)-β linkages by the glucanase treatments was confirmed by p.m.r. spectroscopy (Fig. 3).

Endo-(1→3)-β-D-glucanase had no effect on GU-2. However, the non-dialysable fraction (Fig. 2) produced by successive enzyme treatments of GU-2 may be a single molecular species, and probable structural units are shown in Fig. 4. These units will also be present in GU-2.

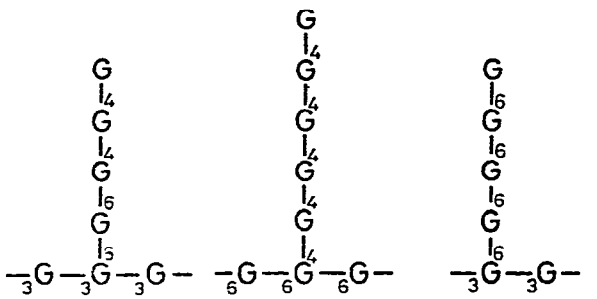


Fig. 4. Probable structural units of GU-2.

Glucan GU-3 had $[\alpha]_D$ −11°, appeared homogeneous on ultracentrifugation, gave glucose on acid hydrolysis (0.5M H_2SO_4, 100°, 5 h), and had a sugar content of 95.5%. The p.m.r. spectrum contained signals at τ 4.57, 4.81, and 5.41 for anomeric protons associated with (1→6)-β-, (1→3)-β-, and a small proportion of (1→4)-α-D linkages. On treatment with periodate, GU-3 consumed 1.34 mol of oxidant and released 0.47 mol of formic acid per glucosyl residue during 47 h, and Smith degradation gave erythritol and glucitol in the molar ratio 1:3.5, together with a significant quantity of glycerol. Methylation analysis of GU-3 gave 2,3,4,6-tetra-, 2,4,6-, 2,3,4-, and 2,3,6-tri-, and 2,4-di-*O*-methyl-glucitol in the molar ratios 1.0:1.2:2.8:1.0 for 2,3,4,6:2,4,6:2,3,4+2,3,6:2,4.

On treatment with (1→6)-β-D-glucanase, 41.9% of GU-3 was released as dialysable material (glucose, 5.9%; gentiobiose, 10.2%; gentiotriose, 5.5%; and unidentified higher oligosaccharide, 5.5%). The non-dialysable material was separated into two fractions on Sepharose 6B (Fig. 5), the major (F-1) being eluted between the void volume and the elution volume of Dextran T-110, and the minor (F-2), corresponding to 11.8% of the non-dialysable fraction, emerged at maximum elution volume.

As shown in Fig. 5, F-1 appears to have a molecular size larger than that of GU-3, and showed a wide distribution pattern. It is possible that the enzymic treatment of GU-3 caused a change in the molecular conformation or association of the molecule by the elimination of (1→6)-β-linked branches. Methylation analysis yielded 2,3,4,6-tetra-*O*-methyl-, 2,4,6-tri-*O*-methyl-, 2,3,4- and 2,3,6-tri-*O*-methyl-,

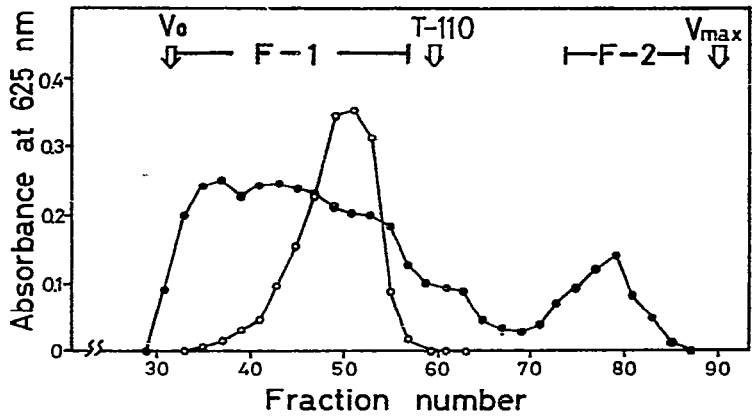
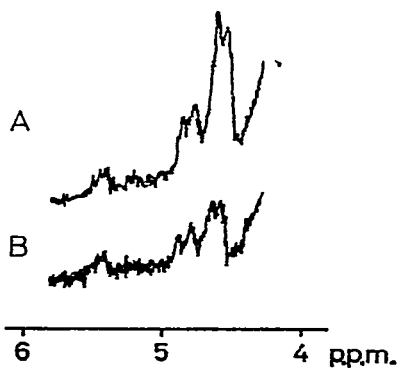


Fig. 5. Gel filtration of GU-3 (—○—) and endo-(1→6)-β-D-glucanase-treated GU-3 (—●—) on a column (1.9×40 cm) of Sepharose 6B. Glucans (16 mg) in distilled water (1 ml) were eluted with distilled water, and fractions (1.5 ml) were assayed with anthrone–H_2SO_4; V_0, void volume; V_{max}, maximum elution volume; T-110, elution volume of Dextran T-110 (mol. wt. 110,000).

and 2,4-di-*O*-methyl-glucitol acetates in the molar ratios 1.0:1.0:2.2:1.0 for F-1, and 1.0:1.7:2.9:1.0 for F-2. Further, the decrease of (1→6)-β linkages in the non-dialysable fraction was shown by the p.m.r. spectrum (Fig. 6). The ratio of yields of F-1 and F-2 was 7.5:1. Treatments with alpha-amylase and exo-(1→3)-β-D-glucanase had no effect on GU-3. From these data, the probable partial units of GU-3 shown in Fig. 7 can be deduced.

Glucan GU-4 was homogeneous on ultracentrifugation, gave glucose on acid hydrolysis (0.5M H_2SO_4, 100°, 5 h, and then 4M HCl, 100°, 5 h), and had a sugar content of 97.3%. On treatment with periodate, GU-4 consumed 0.47 mol of oxidant and released 0.17 mol of formic acid per glucosyl residue. On Smith degradation,



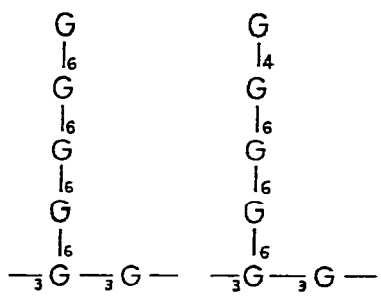


Fig. 6 (left). P.m.r. spectra of GU-3 (A) and endo-(1→6)-β-D-glucanase-treated GU-3 (B).

Fig. 7 (right). Probable structural units of GU-3.

~40% of GU-4 remained as an insoluble precipitate, and the supernatant solution contained glucitol and glycerol in the molar ratio of 3:4 (g.l.c. analysis). Methylation analysis of the insoluble material gave 2,4,6-tri-*O*-methyl-, 2,3,4,6-tetra-*O*-methyl-, and 2,4-di-*O*-methyl-glucitol acetates in the molar ratios 47.4:1:1. Methylation analysis of GU-4 gave 2,3,4,6-tetra-*O*-methyl-, 2,4,6-tri-*O*-methyl-, 2,3,4-tri-*O*-methyl-, and 2,4-di-*O*-methyl-glucitol acetates in the molar ratios 6:15:1:6. In the p.m.r. spectrum of methylated GU-4, the signal for the anomeric proton due to (1→3)-*β* linkages was observed at τ 4.78.

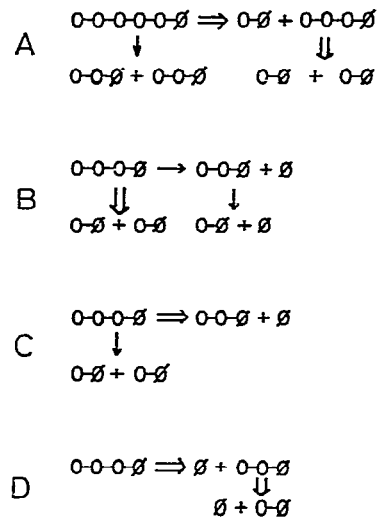


Fig. 8. Mode of action of enzymes: A, endo-(1→6)-*β*-D-glucanase; B, endo-(1→3)-*β*-D-glucanase; C, alpha-amylase; D, exo-(1→3)-*β*-D-glucanase; ⇒, main route; →, minor route; O, D-glucose residue; ∅, reducing end.

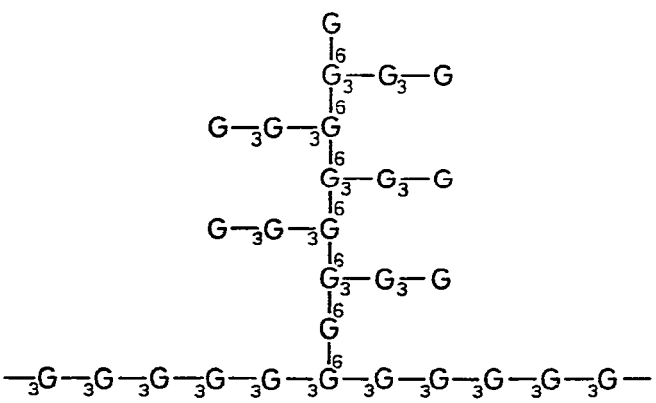


Fig. 9. Probable structural unit of GU-4.

In the enzymic experiments, GU-4 was not hydrolysed by the endo-(1→6)-β-D- and endo-(1→3)-β-D-glucanases or by the exo-(1→3)-β-D-glucanase. These results suggest that the number of glucose residues in the terminal side-chains was less than two (Fig. 8), and the main structural unit was presumed to be that shown in Fig. 9.

The foregoing data indicate that the antitumour glucans from *G. umbellata*, GU-2, GU-3, and GU-4 have complicated structures.

In the studies of antitumour effect against Sarcoma 180, the complete regressions effected by GU-2, GU-3, and GU-4 were 7/10 (1 mg/kg), 9/10 (1 mg/kg), and 6/10 (5 mg/kg), respectively, at the end of 6 weeks after the tumour implantation in ICR/JCL female mice. Further details of the relationship between chemical structure and the antitumour effect of these glucans will be reported elsewhere.

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