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GLYCAN STRUCTURES OF GANODERANS B AND C, HYPOGLYCEMIC GLYCANS OF *GANODERMA LUCIDUM* FRUIT BODIES*

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Key Word Index—*Ganoderma lucidum*; Polyporaceae; peptidoglycans; structure; hypoglycemic activity.

Abstract—Two hypoglycemic principles, ganoderans B and C, isolated from the fruit bodies of *Ganoderma lucidum* were shown to be peptidoglycans with M_rs of 7400 and 5800, respectively. Physico-chemical and chemical studies demonstrated that the backbone and side chains of ganoderan B contain D-glucopyranosyl β-1 → 3 and β-1 → 6 linkages while those of ganoderan C contain D-glucopyranosyl β-1 → 3 and β-1 → 6 linkages and a D-galactopyranosyl α-1 → 6 linkage.

INTRODUCTION

The crude drug 'reishi' (fruit bodies of the mushroom *Ganoderma lucidum* Karsten), together with ginseng, has long been employed as an elixir in Oriental medicine.

Because the hypoglycemic activity of this crude drug has been clinically observed, we have recently carried out a phytochemical examination of this crude drug, produced in Kanagawa, Japan, for hypoglycemic principles and have isolated two active peptidoglycans, ganoderans A and B [1]. We then became interested in the constituents of the same crude drug, produced in Kyoto, Japan, from the viewpoint of a location–composition relationship and performed an analysis for constituents possessing hypoglycemic activity.

This paper reports a structural study on two active peptidoglycans from the drug produced in Kyoto.

RESULTS AND DISCUSSION

A water extract of *G. lucidum* significantly lowered the blood sugar level on i.p. injection (10 g crude drug equivalent/kg) to normal mice (7 hr: 72%; 24 hr: 83% of the control).

The extract was then fractionated on the basis of its hypoglycemic activity to give two constituents, each of which gave a single spot on glass-fibre paper electrophoresis, and a clear single band on PAGE, respectively. In each case, both the periodate–Schiff reagent and the Coomassie blue reagent visualized a band in the same position. In addition, each gave a single peak on gel chromatography with Sephacryl S-200. Gel chromatography using standard pullulans gave values of 7400 and 5800 for the M_rs of ganoderans B and C, respectively. Because the above properties of the constituent possessing the M_r of 7400 were the same as those of ganoderan B, it was concluded to be identical with ganoderan B. Although the following properties of the present ganoderan B are somewhat different from those of the previous ganoderan B [1], the discrepancy is considered to be due to a difference of the magnitude of purification, the present ganoderan B being more rigorously purified. The other constituent having the M_r of 5800 is apparently a novel substance which we named ganoderan C. Quantitative analyses showed that ganoderan B was composed of a glucan (55.1%) and a peptide moiety (44.4%), and that ganoderan C was composed of a polysaccharide (72.5%) and a peptide moiety (25.5%). The polysaccharide moiety of the latter was composed of D-glucose (69.6%) and D-galactose (2.9%). The amino acid composition of ganoderan B was as follows (mol %): Gly (13.6), Asp (12.3), Ala (10.9), Glu (10.3), Thr (9.4), Ser (9.1), Val (6.6), Tyr (5.5), Leu (4.1), Pro (4.0) and other minor amino acids. In ganoderan C, the amino acid composition was as follows (mol %): Gly (12.7), Ala (12.6), Ser (11.5), Thr (11.1), Asp (9.2), Glu (7.6), Val (6.6), Tyr (5.5), Pro (5.2), Leu (4.0) and other minor amino acids.

Ganoderan B exhibited a negative specific rotation ($[\alpha]_D^{27} - 25.8°$) and its ^{1}H NMR spectrum showed two anomeric hydrogen signals at δ4.52 (*d*, J = 7 Hz) and 4.75 (*d*, J = 7 Hz). These data suggest that the D-glucose residues are β-linked. Among the eight signals at δ63.46, 71.63, 72.28, 75.76, 77.57, 78.24, 87.26 and 105.25 observed in the ^{13}C NMR spectrum of ganoderan B, it was evident that those at δ63.46 and 71.63 were attributable to the C-6 carbons, those at δ78.24 and 87.26 to the C-3 carbons, and that at δ105.25 to the C-1 carbons. Therefore, it was concluded that the β-D-glucose units are linked at the 1, 3 and 6-positions in ganoderan B.

Ganoderan C also showed a negative specific rotation ($[\alpha]_D^{27} - 20.1°$) and its ^{1}H NMR spectrum exhibited three anomeric hydrogen signals at δ4.52 (*d*, J = 7 Hz), 4.74 (*d*, J = 7 Hz) and 5.19 (*d*, J = 3 Hz). The ratio of their integrals was *ca* 15:10:1. These results suggest that all the D-glucose units are β-linked, while the D-galactose unit is α-linked in ganoderan C.

In the ^{13}C NMR spectrum of ganoderan C, nine signals at δ63.43, 70.82, 72.28, 75.82, 78.24, 78.52, 87.01, 98.24 and 105.23 were visible. It was apparent that those at δ63.43

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and 70.82 were attributable to the C-6 carbons, those at δ78.52 and 87.01 to the C-3 carbons, that at 98.24 to the C-1 carbon of α-D-galactose, and that at 105.23 to the C-1 carbons of β-D-glucose residues, indicating that the component hexose units are linked at the 1, 3 and 6-positions in ganoderan C.

In each substance, a carbohydrate-rich moiety was isolated by treatment with a protease followed by gel chromatography on Sephadex G-25 and Sephacryl S-200. The fraction obtained from ganoderan B was composed of 59.6% carbohydrate and 40.4% peptide, and the fraction from ganoderan C contained 81.5% carbohydrate and 18.5% peptide.

These fractions were methylated with methylsulphinyl carbanion and methyl iodide in dimethyl sulphoxide [2]. The methylated products were hydrolysed, then converted into the partially methylated alditol acetates. GC/MS [3] showed the presence of 2,3,4,6-tetra-*O*-methyl glucose, 2,4,6-tri-*O*-methyl glucose, 2,3,4-tri-*O*-methyl glucose and 2,4-di-*O*-methyl glucose as the products from ganoderan B in a molar ratio of 1.0:1.1:2.0:1.1. Ganoderan C gave 2,3,4,6-tetra-*O*-methyl glucose, 2,4,6-tri-*O*-methyl glucose, 2,3,4-tri-*O*-methyl glucose, 2,3,4-tri-*O*-methyl galactose and 2,4-di-*O*-methyl glucose in a molar ratio of 1.0:1.0:2.1:0.2:1.1.

Further, ganoderans B and C were oxidized with periodate, and the products were reduced [4], hydrolysed and analysed. The residual sugar was glucose in both samples, and the yields were 19.5% in ganoderan B and 25.6% in ganoderan C.

Based on the evidence described above, it can be concluded that ganoderan B contains a backbone and side chains involving D-glucopyranosyl β-1 → 3 and β-1 → 6 linkages, and that ganoderan C has a backbone and side chains involving D-glucopyranosyl β-1 → 3 and β-1 → 6 linkages and D-galactopyranosyl α-1 → 6 linkages.

Recently, several polysaccharides having antitumour activity were obtained from fruit bodies of *G. lucidum* [5–7], and the presence of a backbone chain composed of β-1 → 3-linked glucan was reported. These results suggest that 1 → 6-linked side chains are attached to the 1 → 3-linked backbone in ganoderans B and C (Fig. 1). The glucans already reported [5–7] have a backbone chain of β-1 → 3-linked D-glucose residues which is substituted at the O-6 positions with single glucosyl units. Thus, the presence of β-1 → 6-linked D-glucosyl chains in ganoderans B and C is unique as compared with the other glucans from this crude drug.

When administered i.p. (10, 30, 100 mg/kg), both ganoderans reduced the blood glucose concentration dose dependently (ganoderan B, after 7 hr: 83, 63, 59%, after 24 hr: 90, 86, 79% of the control; ganoderan C, after 7 hr: 86, 76, 59%, after 24 hr: 105, 87, 82% of the control). The hypoglycemic potency of ganoderan B was approximately the same as a previous sample [1]. I.p. injection of ganoderan C to alloxan-induced hyperglycemic mice also lowered the blood glucose level but the activity was less than that of ganoderans A and B [1] (after 7 hr: 75%; after 24 hr: 112% of the control).

EXPERIMENTAL

Isolation of ganoderans B and C. The crude drug 'reishi' (*G. lucidum* fruit bodies collected in Kyoto, Japan) (10 kg) was extracted with H_2O (100 l. × 3) at 95° for 2 hr (each extraction) (yields 6.5%). The H_2O extract (100 g) was dissolved in H_2O (2 l.) to which EtOH (10 l.) was then added. The resultant ppt. (17.4 g) was chromatographed over DEAE-Toyopearl 650 M (2.2 i.d.

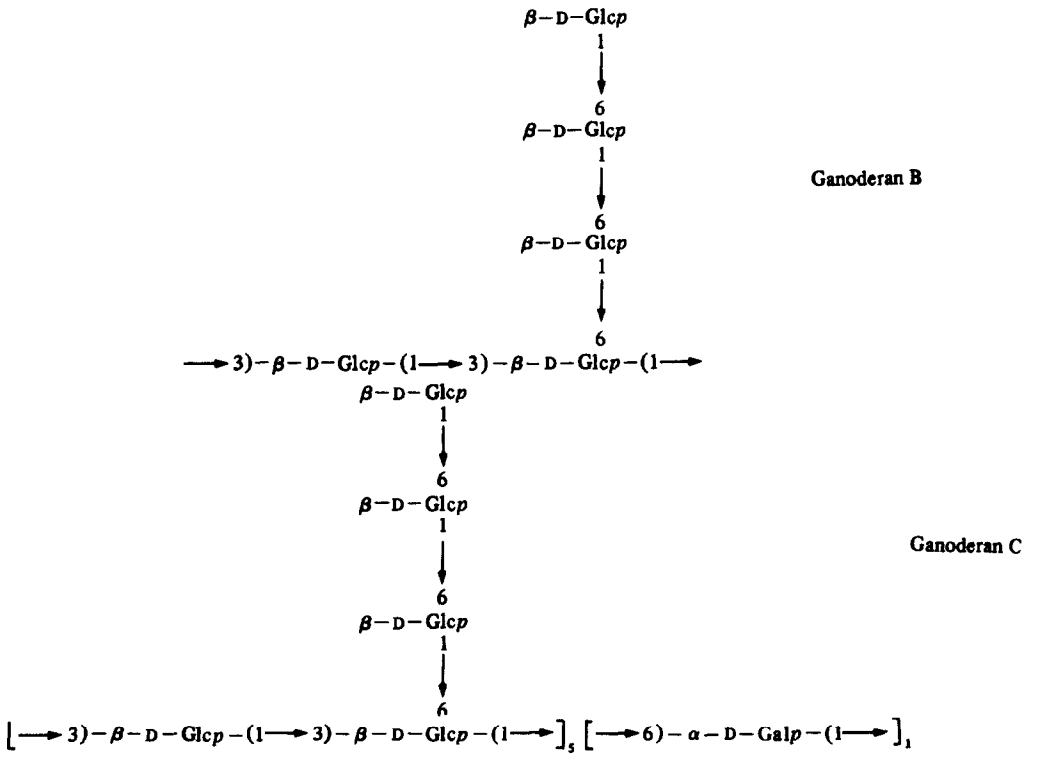

Fig. 1. Possible structural fragments of the polysaccharide moiety.

× 45 cm) to afford a H_2O eluate and a 1.0 M NaCl eluate. Each fraction was chromatographed over Sephacryl S-200 (4.0 i.d. × 95 cm) with 0.1 M Tris–HCl buffer (pH 7.0) containing 0.5 M NaCl to give a main fraction, 50 mg of which was dissolved in H_2O (2 ml) and applied to a column (5 i.d. × 85 cm) of Sephadex G-50. The column was eluted with H_2O and fractions of 20 ml were collected and analysed by the PhOH–H_2SO_4 method [8]. The eluates obtained from tubes 27–41 in the case of ganoderan B, and from tubes 27–38 in the case of ganoderan C, were combined, concentrated and lyophilized to give 25 mg ganoderan B and 15 mg ganoderan C, respectively.

Ganoderan B. $[\alpha]_D^{27}$ −25.8° (*c* 0.29; H_2O); ^{1}H NMR (D_2O): δ4.52 (*d*, *J* = 7 Hz), 4.75 (*d*, *J* = 7 Hz); ^{13}C NMR (D_2O): δ63.46, 71.63 (C-6), 72.28 (C-4), 75.76 (C-2), 77.57 (C-5), 78.24, 87.26 (C-3), 105.25 (C-1, β).

Ganoderan C. $[\alpha]_D^{27}$ −20.1° (*c* 0.19; H_2O); ^{1}H NMR (D_2O): δ4.52 (*d*, *J* = 7 Hz), 4.74 (*d*, *J* = 7 Hz), 5.19 (*d*, *J* = 3 Hz, α-Gal); ^{13}C NMR (D_2O): δ63.43, 70.82 (C-6), 72.28 (C-4), 75.82 (C-2), 78.24 (C-5), 78.52, 87.01 (C-3), 98.24 (C-1, α-Gal), 105.23 (C-1, β-Glc).

Glass-fibre paper electrophoresis was performed on Whatman GF 83 glass-fibre paper (12 i.d. × 38 cm) with 0.1 M NaOH–0.025 M $Na_2B_4O_7 \cdot 10H_2O$ (1:10, pH 9.3) at 570 V for 1 hr [9]. Ganoderan B, single spot 7.8 cm towards cathode; ganoderan C, one spot, 9.2 cm towards cathode.

PAGE. This was carried out in apparatus equipped with gel [10] tubes (0.5 i.d. × 13.5 cm) and 0.05 M Tris–glycine buffer (pH 8.3) at 5 mA/tube for 50 min. Gels were stained for carbohydrate using the PAS procedure, and stained for protein with Coomassie blue.

Determination of M_r. The sample (5 mg) dissolved in 0.1 M Tris–HCl buffer (pH 7.0) was applied to a column (2.6 i.d. × 80 cm) of Sephacryl S-200 pre-equilibrated and developed with the same buffer. Fractions of 5 ml were collected and analysed by the PhOH–H_2SO_4 method. The M_r was calculated by comparison of the V_e of the sample with the V_es of standard pullulans.

Determination of the components. The sample was hydrolysed with 1 M H_2SO_4 at 100° for 6 hr and analysed by cellulose TLC [11], then the product was reduced, acetylated and analysed for sugar components by GC [12]: 3% OV 225 on Gaschrom Q (100–120 mesh; 0.3 i.d. × 200 cm, spiral glass), 220°, He 50 ml/min. Quantitative determination of Glc was performed by the chromotropic acid method [13]. The ratio of Glc:Gal was calculated by GC.

Determination of protein was conducted by the method of Lowry *et al.* [14]. The amino acid composition was determined with an amino acid analyser after hydrolysis with 6 M HCl at 110° for 24 hr.

Treatment with protease. The sample (100 mg) was dissolved in H_2O (5 ml) and 0.2 M Tris–HCl buffer containing 0.004 M $CaCl_2$ (pH 7.8, 5 ml) was added. Actinase E (Kaken Seiyaku Co., 5 mg) was added to the resulting soln, which was then incubated at 40° with a few drops of toluene for 48 hr. After addition of more Actinase E (2.5 mg), the incubation was continued for a further 24 hr under the same conditions. The soln was applied twice to a column (5 i.d. × 87 cm) of Sephadex G-25. The column was eluted with H_2O, and fractions of 20 ml were collected and analysed by the PhOH–H_2SO_4 method. The eluates obtained from tubes 30–35 were combined, concentrated and lyophilized. One quarter of the product (22 mg from ganoderan B; 20 mg from ganoderan C) obtained was dissolved in 6 M urea (0.5 ml) and applied to a column (2.6 i.d. × 83 cm) of Sephacryl S-200. The column was eluted with 0.5 M NaCl and fractions of 10 ml were collected. The eluates obtained from tubes 25–35 were combined and dialysed against H_2O. After concn, the soln was applied twice to a column (2.6 i.d. × 93 cm) of Sephadex G-25. The column was eluted with H_2O and fractions of 10 ml were collected. The eluates obtained from tubes 19–21 were combined, concentrated and lyophilized. Yield: 16 mg.

Methylation analysis. NaH (15 mg) was mixed with DMSO (3 ml) in an ultrasonic bath for 30 min. The mixture was stirred at 70° for 1 hr and added to the product (12 mg) obtained by protease treatment in DMSO (1 ml). After stirring at room temp. for 4 hr, MeI (3 ml) was added, and the entire mixture was stirred overnight at room temp. All procedures were carried out under N_2. The reaction mixture was poured into H_2O (20 ml) and extracted (× 5) with $CHCl_3$ (20 ml each). The combined extract was washed (× 5) with H_2O (100 ml each), then dried (Na_2SO_4), and the filtrate was concentrated to dryness. The residue was methylated (× 3) under the same conditions. The final residue was dissolved in $CHCl_3$–MeOH (2:1), and applied to a column (1 i.d. × 18 cm) of Sephadex LH-20. The column was eluted with the same solvent and fractions of 1 ml were collected. The eluates obtained from tubes 5–8 were combined and concentrated to dryness. The final product (11 mg) showed no hydroxyl absorption in its IR spectrum.

The product was hydrolysed with dilute H_2SO_4 in HOAc, then reduced and acetylated in the manner described in ref. [15]. The partially methylated alditol acetates obtained were analysed by GC/MS using the GC conditions described above, except column temp. 200°, He 60 ml/min. The RR_ts of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and their main fragments in the mass spectra are shown in Table 1.

Periodate oxidation followed by Smith degradation. The sample (22 mg) was dissolved in H_2O (5 ml) and after addition of 0.1 M $NaIO_4$ (5 ml) the reaction mixture was kept at 5° in the dark. Periodate consumption was measured by a spectrophotometric method [16, 17]. The oxidation was completed after 6 days, and the reaction mixture was treated successively with ethylene glycol (0.1 ml) at 5° for 1 hr and $NaBH_4$ (60 mg) at 5° for 16 hr, then adjusted to pH 5 by addition of HOAc. The soln was concentrated and applied to a column (5 i.d. × 85 cm) of Sephadex G-25. The column was eluted with H_2O and fractions of 20 ml were collected. The eluates obtained from tubes 29–33 were combined, concentrated and applied to a column (2.6 i.d. × 91 cm) of Sephadex G-25. The column was eluted with H_2O

Table 1. RR_ts on GC and main fragments in the mass spectra of partially methylated alditol acetates

Acetate	RR_t	Main fragments (*m/z*)
1,5-Ac-2,3,4,6-Me-D-Glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,3,5-Ac-2,4,6-Me-D-Glucitol	1.72	43, 45, 87, 101, 117, 129, 161
1,5,6-Ac-2,3,4-Me-D-Glucitol	2.05	43, 87, 99, 101, 117, 129, 161, 189
1,5,6-Ac-2,3,4-Me-D-Galactitol	2.44	43, 87, 99, 101, 117, 129, 161, 189
1,3,5,6-Ac-2,4-Me-D-Glucitol	3.60	43, 87, 117, 129, 189

and fractions of 10 ml were collected. The eluates obtained from tubes 19–22 were combined and lyophilized. The product was hydrolysed with 1 M H_2SO_4 containing D-allose as internal standard at 100° for 6 hr. The hydrolysate was reduced and acetylated as described above, and the resulting alditol acetate mixture was analysed by GC.

Measurement of hypoglycemic activity. Male mice (Std:ddY strain, 25–30 g) were used in groups of five and given food and drinking water *ad libitum*. The ganoderans were dissolved in physiological saline soln and injected (i.p.) into normal mice or into alloxan-induced hyperglycemic mice pretreated with alloxan (35 mg/kg) 5 days prior to sample administration. Blood was drawn periodically from the orbital sinus by micro-haematocrit tubes. The glucose level of plasma obtained by centrifugation of blood was measured with a glucose analyser by the glucose oxidase method. Data are expressed as mean ± s.e. One-way analysis of variance was used to evaluate the results.

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