Carbohydrate Research, 224 (1992) 237–243
Elsevier Science Publishers B.V., Amsterdam

Structure and antitumor activity of a branched (1→3)-β-D-glucan from the alkaline extract of *Amanita muscaria**

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(Received March 12th, 1991; accepted for publication July 26th, 1991)

ABSTRACT

A β-(1→6)-branched (1→3)-β-D-glucan(AM-ASN) was isolated from the alkaline extract of the fruiting bodies of *Amanita muscaria*. AM-ASN had $[\alpha]_D$ −11° in 0.5M sodium hydroxide. Its estimated molecular weight was 95,000 in this alkaline solution and 260,000 in a neutral solution. The branches in the glucan were primarily single, (1→6)-linked D-glucopyranosyl groups, two for every seven residues in the (1→3)-linked main chain. AM-ASN exhibited significant antitumor activity against Sarcoma 180 in mice, and a mixture of AM-ASN with mitomycin C was more effective against the tumor than mitomycin C only.

INTRODUCTION

Amanita muscaria (Fr.) Hooker is a toxic mushroom belonging to the Amanitaceae. Although the fruiting body contains muscarine, a toxic alkaloid, following its removal the mushroom is eaten in the Hokkaido area of Japan. We isolated an antitumor glucan (AM-ASN) from the cap portion of the fruiting bodies. The present paper deals with the structural characterization of the glucan and its antitumor activity in mice. Furthermore, in the hope of diminishing the side effects of the antineoplastic agent mitomycin C (MMC) we prepared a mixture of AM-ASN and MMC, and tested its antitumor activity.

RESULTS AND DISCUSSION

The cap portion of the fruiting bodies was washed with methanol, and successively extracted with saline solution, with water in a boiling water bath, then with 5% sodium carbonate. The final residue was extracted with M sodium hydroxide containing sodium borohydride for 24 h at room temperature. The alkaline extract was neutralized, and dialyzed against distilled water. The soluble material (AM-AS) in the non-dialyz-

* Polysaccharides in Fungi, Part XXVII. For Part XXVI, see ref. 1.
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0008-6215/92/$05.00

able fraction was deproteinized by protease digestion and the Sevag procedure[2], then purified by anion-exchange chromatography. A neutral polysaccharide (AM-ASN) was obtained from the non-ionic fraction in 0.34% yield. AM-ASN had $[\alpha]_D$ −11° (*c* 0.31, 0.5M NaOH) and showed a symmetrical elution pattern in gel filtration on Toyopearl HW-65 in 0.5M sodium hydroxide. Its molecular weight was estimated from a calibration curve prepared with standard dextrans to be about 95,000. AM-ASN was composed solely of D-glucose, as shown by paper chromatography (p.c.) and gas-liquid chromatography (g.l.c.) of the hydrolyzate.

TABLE I

G.l.c. and g.l.c.–m.s. data for the alditol acetates derived from the methylated polysaccharide

Methylated sugar (as alditol acetate)	T[a]	*Main mass-fragments* (m/z)	*Molar ratio*	*Mode of linkage*
2,3,4,6-Me_4-Glc	**1.00**	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	**1.0**	Glc*p*-(1→
2,4,6-Me_3-Glc	2.08	43, 45, 87, 101, 117, 129, 161, 233	2.4	→3)-Glc*p*-(1→
2,3,4-Me_3-Glc	2.52	43, 87, 99, 101, 117, 129, 161, 189, 233	0.2	→6)-Glc*p*-(1→
2,4-Me_2-Glc	5.57	43, 87, 117, 129, 189	0.9	→3,6)-Glc*p*-(1→

[a] Relative retention time with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on 3% ECNSS-M on Gaschrom Q at 170°.

Methylation analysis of the glucan gave the partially *O*-methylated alditol acetates listed in Table I. The results indicated the presence of nonreducing terminal D-glucopyranosyl, (1→3)-linked D-glucopyranosyl, and (1→3,1→6)-linked (branch-point) D-glucopyranosyl residues, together with small amounts of (1→6)-linked, interior D-glucopyranosyl units. On periodate oxidation, AM-ASN consumed 0.52 mol of periodate per mol of glucosyl residues, and Smith degradation yielded 1.0 mol of glycerol and 2.74 mol of glucose. The results accord with the data obtained from the methylation analysis. Methylation analysis of the product obtained by controlled Smith degradation (sequential borohydride reduction of the periodate-oxidation product and partial acid hydrolysis) revealed that it was composed entirely of (1→3)-linked glucopyranosyl residues. Its molecular weight was about 30,000.

AM-ASN showed characteristic absorbance at 890 cm^{-1} in the i.r. spectrum, indicating the presence of β-D-glycosidic linkages. The ^{13}C-n.m.r. spectrum (Fig. 1) characteristically showed a triplet signal (86.60, 86.11, and 85.87 p.p.m.) due to C-3 of β-(1→3)- and β-(1→3,1→6)-linked glucopyranosyl units and was similar to the spectra of other β-(1→6)-branched (1→3)-β-D-glucans[1,3,4]. The signals at 102.87, 76.18, 72.70, 68.30, and 60.78 p.p.m. were assigned to C-1, C-5, C-2, C-4, and C-6 of β-(1→3)-linked glucopyranosyl units. The other major signals, at 76.54, 75.93, 73.54, 70.02, and 60.98 p.p.m., were attributable to C-3, C-5, C-2, C-4, and C-6 of nonreducing terminal

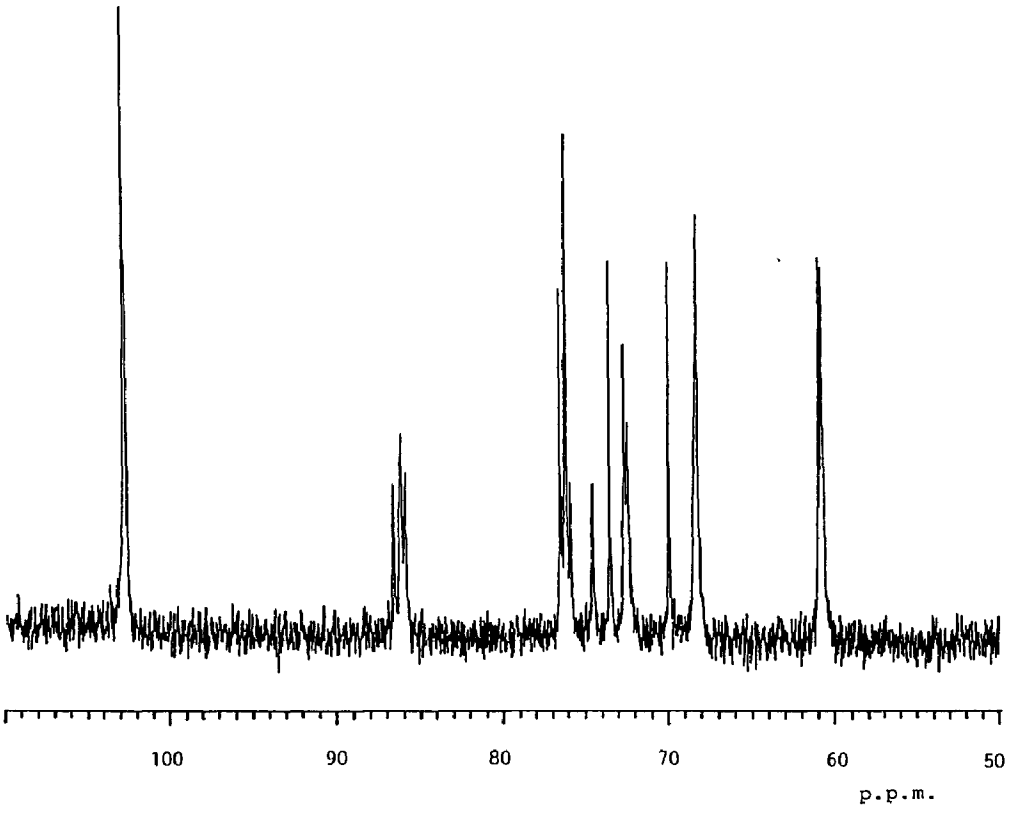
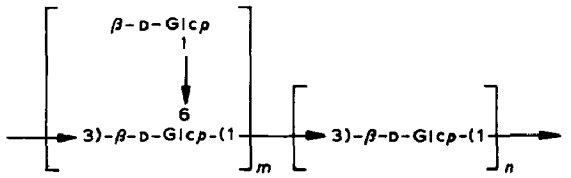


Fig. 1. ^{13}C-N.m.r. spectrum of AM-ASN in Me_2SO-d_6 at 60°.

β-D-glucopyranosyl units. The β-D-configurations of the glucopyranosyl residues were substantiated by the $^1J_{C,H}$ value[5] of 163.0 Hz.

The foregoing data indicated that the glucan AM-ASN from the fruiting bodies of *A. muscaria* has a main chain composed of β-(1→3)-linked D-glucopyranosyl residues and side chains of single, β-(1→6)-linked D-glucopyranosyl groups attached, on the average, to two out of every seven residues of the main chain. The glucan also contains small amounts of nonterminal β-(1→6)-linked D-glucopyranosyl residues, but whether these are in the side branches or in the main chain was not determined. It is suggested that AM-ASN has the average repeating unit shown in formula **1**.



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In studies on the conformation of glucans using Congo Red[6,7] it was shown that the value of λ_{max} (487 nm) of Congo Red in the presence of the glucan was shifted to longer wavelength (511 nm) in base at low concentration (0.1M sodium hydroxide), but not at higher concentration (0.25M sodium hydroxide). This phenomenon was shown by glucan AM-ASN. Furthermore, the molecular weight of AM-ASN in 0.1M sodium chloride was estimated by gel filtration at about 260,000, a value some three times that determined in 0.5M sodium hydroxide. These results suggest that the glucan has a triple helical structure in neutral and weakly alkaline solution.

Among antitumor, β-(1→6)-branched (1→3)-β-D-glucans such as lentinan[8] and schizophyllan[9], a glucan having a branching ratio similar to that of AM-ASN is T-5-N ($[\alpha]_D$ +28.7° in water), isolated from the fruiting bodies of *Dictyophora indusiata*[7,10]. However, T-5-N contains a few glucosyl residues linked at O-2 and O-3, and its molecular weight (330,000 in alkaline solution and 1,000,000 in neutral solution) is larger than that of AM-ASN.

The antitumor activities of AM-AS and AM-ASN against Sarcoma 180 in mice were determined in experiment 1, summarized in Table II. Both showed significant activity by intraperitoneal (i.p.) administration. Next, we tested the effect on the tumor of the mixture with MMC. MMC is widely used in cancer chemotherapy, but shows the side effects of severe bone marrow suppression and gastrointestinal complications. As shown under experiment 2 in Table II, a mixture of MMC and AM-ASN at a dose of 1 mg + 5 mg per kg per day, × 7, i.p., showed higher activity against Sarcoma 180 than MMC alone, although AM-ASN had little effect on the tumor at a dose of 5 mg per kg per day, × 7. Since it has been known that antitumor substances such as MMC reduce

TABLE II

Antitumor activities of the polysaccharides and MMC against sarcoma 180

Sample	*Dose (mg/kg/day × no. of days)*	*Mean tumor wt. (g ± s.d.)*	*Inhibition ratio (%)*[a]	*Complete regression*
Experiment 1				
AM-AS	1 × 10	3.34 ± 2.95[b]	71	0/6
	5 × 10	3.50 ± 2.73[b]	70	0/6
AM-ASN	1 × 10	1.38 ± 1.68[b]	88	1/6
	5 × 10	6.19 ± 2.73[c]	47	0/6
PSK[d]	200 × 10	1.34 ± 2.22[b]	89	1/6
Control	–	11.67 ± 2.97	–	0/7
Experiment 2				
AM-ASN	5 × 7	8.63 ± 4.67	30	0/6
AM-ASN + MMC	(5 + 1) × 7	0.99 ± 0.90[b]	92	1/6
MMC	1 × 7	3.06 ± 2.48[b]	75	0/6
Control	–	12.40 ± 4.57	–	0/6

[a] The inhibition ratio (%) is given by $[(A - B)/A] \times 100$, where A is the average tumor weight in the control group and B is that in test group. [b] Significant difference from the control, $p < 0.001$. [c] $p < 0.005$. [d] Polysaccharide preparation from *Coriolus versicolor* (Kureha Chemical Ind., Japan).

the number of leukocytes in the peripheral blood of animals[11], the numbers were determined for mice injected once, i.p., with MMC (4 mg/kg), with AM-ASN (20 mg/kg), and with a mixture of MMC (4 mg/kg) and AM-ASN (20 mg/kg). The values (cells/mm^3, mean ± s.e.) at 4 days after injection were 3179 ± 423 with MMC, 7548 ± 499 with AM-ASN, 5670 ± 944 with the mixture, and 7952 ± 608 with control. Thus, MMC reduced the number of leukocytes, but the population was fairly well restored by the addition of AM-ASN.

The biological results indicated that the glucan AM-ASN has significant activity against sarcoma 180 in mice, and that a mixture of MMC and the glucan is effective against the tumor. Moreover, the addition of AM-ASN reduced the side effects of MMC without reduction of its antitumor activity. Many β-(1→6)-branched (1→3)-β-D-glucans are sparingly soluble in water, but AM-ASN, as well as T-5-N, is rather soluble, having a branching ratio of 2:7. The solubility may contribute to the activity.

EXPERIMENTAL

Isolation of the polysaccharide. — Fruiting bodies of *Amanita muscaria* (Fr.) Hooker were harvested in Ishikari county, Hokkaido, Japan, in October 1988. The air-dried cap portion (180 g) of the fruiting bodies was pulverized and extracted with methanol (1 L) in a Soxhlet apparatus for 70 h at 80°. The residue (100 g) was successively extracted once with 0.9% sodium chloride (saline) solution (1 L) for 24 h at 4°, 27 times with water (500 mL) in a boiling water bath, then 15 times with 5% (0.47M) sodium carbonate (500 mL) for 40 h at 4°. The final residue was extracted 3 times with M sodium hydroxide (1 L) containing sodium borohydride (200 mg) for 24 h at room temperature, and the extract collected by centrifugation was neutralized with M hydrochloric acid. The solution was dialyzed against distilled water, and the non-dialyzable suspension was centrifuged (2,500 × g, 20 min). Supernatant and precipitate were lyophilized to afford AM-AS as the water-soluble product in 1.7% yield and AM-AP as the water-insoluble product in 0.7% yield. AM-AS was deprotenized by protease (Actinase E, Kaken Chemical Co., Tokyo) digestion and the Sevag procedure, and further purified by anion-exchange chromatography on DEAE-Toyopearl 650M (phosphate form), as previously described[12]. The non-ionic fraction was dialyzed and lyophilized to give a neutral polysaccharide (AM-ASN) in 0.34% yield.

Gel filtration. — The sample (~ 2 mg) was dissolved in 0.1M sodium chloride or 0.5M sodium hydroxide, applied to a column (1.5 × 94 cm) of Toyopearl HW-65, and eluted with the same solution. Gel filtration of the product of controlled Smith degradation on a column (1.5 × 95 cm) of Toyopearl HW-55 was performed with 0.5M sodium hydroxide as the eluent. Fractions of 4 mL were collected, and analyzed by the phenol–sulfuric acid method[13]. The molecular weight was estimated from a calibration curve constructed by the use of standard dextrans as previously described[12].

Analysis of component sugars. — The sample was heated successively with 90% formic acid for 5 h at 100°, then 2M trifluoroacetic acid (TFA) for 5 h at 100°. The hydrolyzate was evaporated to remove the acid, and analyzed by p.c. and g.l.c. of its alditol acetates, as previously described[14].

Methylation analysis. — The sample was methylated 3 times by the Hakomori method[14] as previously described[1]. The partially *O*-methylated sugars obtained by hydrolysis of the fully *O*-methylated glucan were converted into their alditol acetates, and analyzed by g.l.c. and g.l.c.–mass spectrometry (g.l.c.–m.s.) using a glass column packed with 3% of ECNSS-M on Gaschrom Q, as previously described[1].

Periodate oxidation and Smith degradation. — The sample (10 mg) was oxidized with 20mM sodium metaperiodate in 0.5M sodium chloride for 8 days at 4°, in the dark, and the periodate consumption was determined at time intervals as previously described[1]. The oxidized glucan was reduced with sodium borohydride to give the polyalcohol. The hydrolyzate (90% formic acid and 2M TFA) of the polyalcohol was analyzed as alditol acetates by g.l.c., using a programmed rise in temperature, as previously described[1].

Controlled Smith degradation. — The polyalcohol (12 mg) was partially hydrolyzed with 0.1M sulfuric acid for 24 h at room temperature, and the insoluble Smith degradation product (10 mg) was collected by centrifugation. This product was subjected to gel filtration over Toyopearl HW-55 in 0.5M sodium hydroxide. For methylation analysis, the product (3.3 mg) was dissolved in 4-methylmorpholine 4-oxide (400 mg) at 120°, dimethyl sulfoxide (2 mL) was added to the solution, then the sample was fully methylated in the presence of methylsulfinyl carbanion[15].

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra were recorded with a JEOL-GX 270 spectrometer in the Fourier-transform mode for solutions in Me_2SO-d_6 (30 mg/0.5 mL) at 60°.

Interaction with Congo Red. — The sample (0.2 mg/mL) was dissolved in sodium hydroxide (0.10 and 0.25M) containing Congo Red (0.1mM), and the visible absorption spectra were recorded with a Hitachi 323 spectrophotometer.

Antitumor activity. — Male ddY mice (4-week-old) were obtained from Japan SLC, Inc., Hamamatsu, Japan. Sarcoma 180 ascites cells (2×10^6 cells) were transplanted subcutaneously into the right groin of mice. Starting 24 h after the transplantation, AM-ASN and/or MMC (a kind of gift from Kyowa Hakko Co. Ltd., Japan) dissolved in saline was administered i.p. daily for 10 days or 7 days. After 30 days, the mice were sacrificed, and tumors were weighed.

Determination of the number of leukocytes in mice. — Saline or test sample dissolved in saline was injected i.p. into each of a group of five male ddY mice (4-week-old), and 20 μL of blood was taken from the orbital vein 4 days after injection. The blood was diluted with ISOTON II (Coulter Scientific, Japan), hemolyzed with ZAP-OGLOBIN II (Coulter Scientific, Japan), and leukocytes were counted by a Coulter counter (Coulter Electronics).

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