Polysaccharides in Fungi. XXXIV.¹⁾ A Polysaccharide from the Fruiting Bodies of *Amanita muscaria* and the Antitumor Activity of Its Carboxymethylated Product

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A water-insoluble, alkali-soluble, glucan (AM-APP), $[\alpha]_D +160^\circ$ in 0.4 M NaOH, was isolated from the alkaline extract of the fruiting bodies of *Amanita muscaria*. The results of chemical and spectroscopic investigations indicate that AM-APP is a linear (1→3)- α -D-glucan with a molecular weight estimated by gel chromatography of about 42000. Its carboxymethylated product (AM-APP-CM) showed potent antitumor activity against sarcoma 180 in mice, although the native polysaccharide (AM-APP) had little effect. The distribution of carboxymethyl groups in the molecule was analyzed by gas chromatography and mass spectrometry. The degree of substitution of carboxymethyl groups was 0.95 and the substituents were located at O-2, at O-4, at O-6, at O-2 and O-6, and at O-4 and O-6 on glucose.

Keywords polysaccharide; (1→3)- α -D-glucan; *Amanita muscaria*; antitumor activity; carboxymethyl group

Previously, we reported on the structure and antitumor activity of a branched (1→3)- β -D-glucan (AM-ASN)²⁾ from the alkaline extract of the fruiting bodies of *Amanita muscaria* (Fr.) HOOKER. In addition to AM-ASN, a water-insoluble polysaccharide (AM-APP) has been isolated from the same alkaline extract. Since we had found that the antitumor effect of a water-insoluble, linear (1→3)- α -D-glucan (AG-AL)³⁾ from the fruiting bodies of *Agrocybe cylindracea* against sarcoma 180 tumor in mice was activated by carboxymethylation of AG-AL, we examined the antitumor activity of the carboxymethylated product (AM-APP-CM) of AM-APP. The present paper deals with the structural features and antitumor activity of AM-APP and AM-APP-CM against sarcoma 180 tumor. In addition, we describe the distribution of carboxymethyl groups in AM-APP-CM.

MATERIALS AND METHODS

Materials The fruiting bodies of *Amanita muscaria* (Fr.) HOOKER were harvested in Hokkaido, Japan. Standard dextrans (dextran T-40, T-70, T-110) and Toyopearl HW-65F were obtained from Pharmacia Fine Chemicals and Tosoh Industry Co., respectively. The carboxymethylated glucan (CMPS) was kindly provided by Takeda Chemical Industries Ltd.

General All evaporations were conducted under reduced pressure at a bath temperature under 40 °C. Specific rotation was measured with a JASCO DIP-4 automatic polarimeter. Infrared (IR) spectra were recorded on a JASCO A-120 spectrometer. Gas chromatography (GC) was performed on a Shimadzu GC-4CM instrument fitted with a packed column of 3% ECNSS-M on Gaschrom Q, and also on a Shimadzu GC-15A instrument fitted with a CP-Sil 88 FS-WCOT fused capillary column; GC-mass (MS) spectrometry was carried out using a JEOL JMS-D 300 instrument equipped with a 3% ECNSS-M column, as previously described.⁴⁾

Isolation of the Polysaccharide After the air-dried cap-portion (180 g) of the fruiting bodies was successively extracted with methanol, 0.9% NaCl, hot water, 5% Na₂CO₃, and 1 M NaOH (1 l) containing sodium borohydride (200 mg) for 24 h at room temperature, the final extract was neutralized with 1 M HCl and then dialyzed against distilled water. A branched (1→3)- β -D-glucan (AM-ASN) was obtained from the water-soluble fraction of the non-dialyzable suspension, as previously described.²⁾ Its water-insoluble product was dissolved in 0.5 M NaOH, and the solution was neutralized with acid, then dialyzed against distilled water. The insoluble material in the non-dialyzable fraction obtained by centrifugation was suspended in water, and lyophilized to give the polysaccharide (AM-APP).

Gel Filtration and Estimation of Molecular Weight Gel filtration of AM-APP (1.2 mg/0.5 ml) on a column (95 × 1.5 cm) of Toyopearl HW-65F in 0.5 M NaOH was performed and the molecular weight was estimated from a calibration curve constructed using standard dextrans, as previously described.⁵⁾

Analysis of Component Sugars The polysaccharide was hydrolyzed with 90% formic acid for 5 h at 100 °C, and then with 2 M trifluoroacetic acid and 6 M HCl (v/v, 1:1) for 6 h at 100 °C. The hydrolysate was evaporated to remove the acid, and the alditol acetates analyzed by GC using a packed column.²⁾

Methylation Analysis AM-APP (5.2 mg) was methylated 3 times by the Hakomori method, as previously described.⁴⁾ The fully methylated product was heated successively with 90% formic acid for 7 h at 100 °C, and then with 2 M trifluoroacetic acid for 5 h at 100 °C. The resulting partially methylated alditol acetates were analyzed by GC and GC-MS spectra.

Carbon-13 Nuclear Magnetic Resonance (¹³C-NMR) The ¹³C-NMR spectra were recorded on a JEOL-GX 270 FT NMR spectrometer using solutions in DMSO-*d*₆ (21 mg/0.5 ml) at 60 °C.

Carboxymethylation The polysaccharide was carboxymethylated according to the procedure of Sasaki *et al.*⁶⁾ A suspension of AM-APP (100 mg) in 2-propanol (2.8 ml) was stirred for 30 min at room temperature. 30% Sodium hydroxide solution (0.28 ml) was added, with stirring, over 60 min, and the mixture was stirred vigorously for 90 min. Monochloroacetic acid (127 mg) was then added and the mixture was stirred for 5 h at 60–70 °C. The suspended product was recovered by filtration, and successively washed with methanol–acetic acid (7:3), methanol–water (4:1), methanol and acetone. The product was suspended in water, and dialyzed against distilled water at 4 °C. The non-dialyzable fraction was centrifuged, and the supernatant was lyophilized to give the water-soluble, carboxymethylated polysaccharide (AM-APP-CM) (84 mg).

Distribution of Carboxymethyl Groups The distribution of the substituents in the carboxymethylated polysaccharide was determined by our previously reported method.^{7,8)} The carboxymethylated polysaccharide was reduced with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and sodium borohydride to the hydroxyethyl polysaccharide. The product was then hydrolyzed with acid, and hydrolysate was reduced with sodium borohydride, and acetylated with acetic anhydride–pyridine (1:1) for 2 h at 100 °C. The resulting derivatives were analyzed by GC and GC-MS, using a glass column (1.5 m × 0.3 cm) packed with 2% EGSS-X on Chromosorb W (60–80 mesh), as previously described.^{7,8)}

Antitumor Activity Sarcoma 180 ascites cells (2×10^6 cells) were transplanted subcutaneously into the groin of male, ddY mice (5 weeks old) (Japan SLC). The saline suspension or solution was sterilized for 20 min at 120 °C, and then injected intraperitoneally (i.p.) daily for 10 d, starting 24 h after the transplantation. After 30 d, the mice were sacrificed, and the tumors were extirpated and weighed. The inhibition ratios (%) were given by $[(A-B)/A] \times 100$, where *A* is the average tumor weight of the control group, and *B* is that of the test group. The significance of the difference between the means was evaluated using Student's *t*-test.

RESULTS AND DISCUSSION

A water-insoluble polysaccharide (AM-APP), $[\alpha]_D^{+160} (c=0.11, 0.4\text{M sodium hydroxide})$, was obtained from the alkaline extract of the fruiting bodies of *A. muscaria* in a yield of 0.08%, in addition to a branched (1→3)-β-D-glucan (AM-ASN). The former showed a symmetrical elution pattern during gel filtration on Toyopearl HW-65F with 0.5 M sodium hydroxide, and its molecular weight was estimated by gel chromatography to be about 42000. GC analysis of the hydrolysate with acid showed it to be composed of D-glucose together with a slight amount of D-mannose.

The polysaccharide was fully methylated the methylated product was hydrolyzed with acid, then the sugars were converted into the partially *O*-methylated alditol acetates. The derivative of 2,4,6-tri-*O*-methyl-D-glucose (primary mass fragments (*m/z*): 45, 117, 161, 233) and a trace of 2,3,4,6-tetra-*O*-methyl-D-glucose (primary mass fragments (*m/z*): 45, 117, 161, 205) was identified by GC and

GC-MS.⁹⁾ The ¹³C-NMR spectrum of AM-APP in DMSO-*d*₆ is shown in Fig. 1. The resonance at 99.62 ppm was assigned¹⁰⁾ to the anomeric carbon atoms of (1→3)-linked α-D-glucopyranosyl residues, and other signals at 82.95, 71.92, 70.78, 69.43, and 60.25 ppm were attributable to the C-3, C-5, C-2, C-4, and C-6 of the residues. The α-D-configurations of the glucopyranosyl residues were supported by the ¹*J*_{CH} value¹¹⁾ of 168.4 Hz.

The data obtained indicate that the water-insoluble, alkali-soluble, polysaccharide (AM-APP) from the alkaline extract of the fruiting bodies of *A. muscaria* is a linear (1→3)-α-D-glucan. The molecular weight (42000) of AM-APP is smaller than that (560000) of AG-AL³⁾ (a linear (1→3)-α-D-glucan) from *Agrocybe cylindracea*. AM-APP differs from (1→3)-linked α-D-glucans¹²⁾ isolated from other fungi in that minor sugar components or α-(1→4)-linked D-glucopyranosyl residues are present.

Since we previously reported that carboxymethylation of AG-AL increased its solubility and antitumor potency, we applied a similar carboxymethylation to AM-APP. The carboxymethylation of AM-APP afforded a water-soluble carboxymethylated (1→3)-α-D-glucan (AM-APP-CM) in good yield together with a small amount of a gelatinous product; similar treatment of a water-insoluble AG-AL³⁾ gave a water-soluble AG-AL-CMS and a gelatinous AG-AL-CMI in the ratio of 3:1. The difference in the ratio of water-soluble to gelatinous products following

Fig. 1. ¹³C-NMR Spectrum of AM-APP in DMSO-*d*₆ at 60 °C

TABLE I. Antitumor Activity of AM-APP and AM-APP-CM against Sarcoma 180

Sample (<i>n</i> =7)	Dose (mg/kg/d × 10)	Mean tumor wt. ± S.D. (g)	Inhibition ratio (%)
Exp-1			
AM-APP ^{a)}	1	7.95 ± 2.33	41
	10	10.74 ± 4.73	20
Control	—	13.47 ± 5.13	—
Exp-2			
AM-APP-CM ^{b)}	1	2.38 ± 1.86 ^{d)}	58
	10	1.07 ± 0.60 ^{e)}	81
CMPS ^{c)}	10	0.59 ± 0.61 ^{e)}	90
Control	—	5.60 ± 0.61	—

a) Water-insoluble polysaccharide of the alkaline extract from *Amanita muscaria*.
b) Carboxymethylated product of AM-APP. c) Carboxymethylated (1→3)-β-D-glucan. Significantly different from the control, d) *p* < 0.01, e) *p* < 0.001.

TABLE II. GC and GC-MS of D-Glucose Derivatives from the Hydrolysate of Reduced AM-APP-CM

Sugar derivative ^{a)} (as acetate)	Relative retention time ^{b)}	Prominent mass-fragments (<i>m/z</i>)	Molar percent
1,2- <i>O</i> -etn- α -D-Glc ^f	0.62	43, 73, 127, 170, 187, 273	0.5
1,2- <i>O</i> -etn- α -D-Glc ^p	0.76	43, 73, 86, 157, 170, 199, 230, 272	3.1
D-Glc-ol	1.00	43, 73, 145, 217, 289, 361, 375	33.8
1,2- <i>O</i> -etn- β -D-Glc ^p	1.51	43, 73, 86, 157, 170, 199, 230, 272	3.8
1,2- <i>O</i> -etn-6- <i>O</i> -he- α -D-Glc ^f	1.96	43, 73, 87, 127, 187	8.9
1,2- <i>O</i> -etn-6- <i>O</i> -he- α -D-Glc ^p	2.34	43, 73, 86, 157, 170, 199, 259, 317	5.2
6- <i>O</i> -he-D-Glc-ol	2.79	43, 87, 117, 170, 375	20.4
2- <i>O</i> -he-D-Glc-ol	3.38	43, 73, 87, 189, 375, 405	7.7
4- <i>O</i> -he-D-Glc-ol	4.43	43, 87, 145, 261, 333, 375	2.5
1,2- <i>O</i> -etn-6- <i>O</i> -he- β -D-Glc ^p	5.42	43, 73, 86, 157, 170, 199, 259, 316	3.9
4,6- <i>O</i> -dihe-D-Glc-ol	7.02	43, 73, 87, 117, 305, 333, 419	2.7
2,6(2,4)- <i>O</i> -dihe-D-Glc-ol ^{c)}	8.79	43, 73, 87, 115, 117, 189, 261, 375	7.7

a) etn = ethylene, he = hydroxyethyl, dihe = dihydroxyethyl, Glc^f = glucofuranose, Glc^p = glucopyranose, Glc-ol = Glucitol. b) Relative to hexa-*O*-acetyl-D-glucitol on a glass column of 2% EGSS-X at 215 °C. c) Overlapping peak.

TABLE III. Location of Carboxymethyl Groups on Glucose

Sample	Percentage of total						DS ^{b)}
	Glc	2-CM-Glc ^{a)}	4-CM-Glc	6-CM-Glc	2,6-di-CM-Glc	4,6-di-CM-Glc	
AM-APP-CM	33.6	15.1	2.5	20.4	25.7 ^{d)}	2.7	0.95
AG-AL-CMS ^{c)}	39.1	19.7	7.3	14.8	19.4 ^{d)}	—	0.80

a) 2-CM-Glc = 2-*O*-carboxymethyl-D-glucose. b) Degree of substitution per glucose. c) A soluble, carboxymethylated product of AG-AL from *Agrocybe cylindracea* (see ref. 3). d) Contains small amounts of 2,4-di-CM-Glc.

carboxymethylation could be attributable to the difference in molecular size between AM-APP (M.W. = 42000) and AG-AL (M.W. = 560000). Although a water-insoluble, native polysaccharide (AM-APP) had little effective on sarcoma 180 in mice when administered daily by the i.p. route for 10 d, a water-soluble, carboxymethylated product (AM-APP-CM) exhibited potent antitumor activity, as shown in Table I. The antitumor activity of AM-APP-CM was more similar to that of the carboxymethylated (1 $\rightarrow$ 3)- α -D-glucan (AG-AL-CMS). The degree and location of the substitution of the carboxymethyl groups in AM-APP-CM were examined by a method developed in our own laboratory.^{7,8)} The procedure involves the reduction of the *O*-carboxymethyl groups to *O*-hydroxyethyl groups, hydrolysis of the resulting *O*-hydroxyethyl polysaccharide, and GC-MS analysis of the hydrolysate as the alditol acetates and 1,2-ethylene-D-glucose derivatives. The results of the GC and GC-MS analyses of the carboxymethyl groups are listed in Table II. The de-

rivatives of 1,2-*O*-ethylene (etn)- α -D-glucofuranose(Glc^f), 1,2-*O*-etn- α -D-glucopyranose(Glc^p), 1,2-*O*-etn- β -D-Glc^p, and 2-*O*-hydroxyethyl(he)-D-glucitol(Glc-ol) indicate substituents at *O*-2, and those of 1,2-*O*-etn-6-*O*-he- α -D-Glc^f, 1,2-*O*-etn-6-*O*-he- α -D-Glc^p, 1,2-*O*-etn-6-*O*-he- β -D-Glc^p, and 2,6-*O*-dihydroxyethyl(dihe)-D-Glc-ol indicate substituents at *O*-2 and *O*-6. The location of carboxymethyl groups and the degree of substitution (DS) per glucosyl residue are shown in Table III. The DS of AM-APP-CM (0.95) was slightly higher than that of AG-AL-CMS (0.80). AM-APP-CM had more substituents at *O*-6, at *O*-2 and *O*-6, and at *O*-4 and *O*-6 than AG-AL-CMS, compared with the amounts of substituents at *O*-2, at *O*-4, and unsubstituted glucose. The carboxymethylated product (CMPS) of (1 $\rightarrow$ 3)- β -D-glucan (curdlan type, PS) is not substituted at *O*-2, although both AM-APP-CM and AG-AL-CMS have the glucosyl residues substituted at *O*-2. This finding could be due to the difference in conformation between β -(1 $\rightarrow$ 3)-linked and α -(1 $\rightarrow$ 3)-linked glucans, as previously reported.⁸⁾

It was confirmed from the results of the antitumor testing of AM-APP-CM that carboxymethylation of the polysaccharide consisting of (1 $\rightarrow$ 3)-linked α -D-glucopyranosyl residues produces an active antitumor agent. We suggest from our work on the antitumor activity of the conjugate of mitomycin C with CMPS¹³⁾ that the carboxymethylated polysaccharide (AM-APP-CM) could also be a useful polymer for a carrier in a drug delivery system.

REFERENCES

- 1) Polysaccharides in Fungi XXXIII: T. Kiho, Y. Tsujimura, M. Sakushima, S. Usui, S. Ukai, *Yakugaku Zasshi*, **114**, 308 (1994).
- 2) T. Kiho, M. Katsuragawa, K. Nagai, S. Ukai, M. Haga, *Carbohydr. Res.*, **224**, 237 (1992).
- 3) T. Kiho, I. Yoshida, K. Nagai, S. Ukai, *Carbohydr. Res.*, **189**, 273 (1989).
- 4) T. Kiho, Y. Shiose, K. Nagai, M. Sakushima, S. Ukai, *Chem. Pharm. Bull.*, **40**, 2212 (1992).
- 5) T. Kiho, M. Ito, K. Nagai, C. Hara, S. Ukai, *Chem. Pharm. Bull.*, **35**, 4286 (1987).
- 6) T. Sasaki, N. Abiko, K. Nitta, N. Takasuka, Y. Sugino, *Eur. J. Cancer*, **15**, 211 (1979).
- 7) S. Ukai, A. Honda, K. Nagai, T. Kiho, *J. Chromatogr.*, **513**, 338 (1990).
- 8) S. Ukai, I. Yoshida, A. Honda, K. Nagai, T. Kiho, *Carbohydr. Res.*, **224**, 201 (1992).
- 9) H. Björndal, B. Lindberg, S. Svensson, *Carbohydr. Res.*, **5**, 433 (1967).
- 10) H. M. Davis, W. J. Boyko, J. R. Edwards, *Carbohydr. Res.*, **152**, 279 (1986).
- 11) K. Bock, I. Lundt, C. Pedersen, *Tetrahedron Lett.*, **1973**, 1037.
- 12) E. Barreto-Bergter, P. A. J. Gorin, *Adv. Carbohydr. Chem. Biochem.*, **41**, 68 (1983).
- 13) K. Nagai, J. Tanaka, T. Kiho, S. Ukai, *Chem. Pharm. Bull.*, **40**, 986 (1992).