

Isolation of an Antitumor Compound from *Agaricus blazei* Murill and Its Mechanism of Action¹

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ABSTRACT

The Basidiomycete fungus *Agaricus blazei* Murill has traditionally been used as a health food for the prevention of cancer, diabetes, hyperlipidemia, arteriosclerosis and chronic hepatitis. In the present study, we examined the antitumor activities of various substances isolated from the lipid fraction of *A. blazei*. Tumor growth was retarded by the oral administration of the lipid fraction extracted from *A. blazei* with a chloroform/methanol mixture in sarcoma 180-bearing mice. The substance with the antitumor activity in the lipid fraction was isolated via silica gel column chromatography, eluted with an acetonitrile/methanol (3:2) mixture and identified as ergosterol by direct comparison of the ¹H NMR and mass spectrometry spectral data of an authentic sample. The oral administration of ergosterol to sarcoma 180-bearing mice significantly reduced tumor growth at doses of 400 and 800 mg/kg administered for 20 d without side effects, such as the decreases in body, epididymal adipose tissue, thymus, and spleen weights and leukocyte numbers induced by cancer chemotherapy drugs. Ergosterol had no cytotoxicity against tumor cells. To clarify the antitumor activity of ergosterol, we examined the effects of ergosterol on tumor-induced angiogenesis using two in vivo models. Intraperitoneal administration of ergosterol at doses of 5, 10 and 20 mg/kg for 5 consecutive d inhibited the neovascularization induced by Lewis lung carcinoma cell-packed chambers, suggesting that either ergosterol or its metabolites may be involved in the inhibition of tumor-induced neovascularization. Therefore, we further examined the inhibitory effects of ergosterol on Matrigel-induced neovascularization. Female C57BL/6 mice were subcutaneously inoculated with Matrigel containing acidic fibroblast growth factor and heparin with or without ergosterol. Ergosterol inhibited the Matrigel-induced neovascularization, suggesting that ergosterol directly inhibits Matrigel-induced neovascularization. From these results, it seems likely that the antitumor activity of ergosterol might be due to direct inhibition of angiogenesis induced by solid tumors. This is the first report of ergosterol as an antiangiogenic substance.

Key Words: *Agaricus blazei* • antitumor activity • antiangiogenic activity • antitumor substance • mice

INTRODUCTION

The Basidiomycete fungus *Agaricus blazei* Murill (Japanese name: Himematsutake or Agarikusutake) has been traditionally used as a health food source in Brazil for the prevention of

cancer, diabetes, hyperlipidemia, arteriosclerosis and chronic hepatitis. It has been reported that 100,000–300,000 kg of the dried body of *A. blazei* is produced every year in Japan. *A. blazei* is used by 300,000–500,000 persons for the prevention of cancer and/or as an adjuvant with cancer chemotherapy drugs after the removal of a malignant tumor. The intake of the water extract of *A. blazei* is 3–5 g three times daily. The hot water extract of *A. blazei* has potent antitumor activity in sarcoma 180-bearing mice (1, 2, 3, 4), and the antitumor substance was postulated to be the β -(1–6)-glucan fraction. However, the antitumor effects of lipid fractions have not been well studied. We examined the antitumor activities of various substances isolated from the lipid fraction of *A. blazei* via oral or intraperitoneal administration to identify the active substances. We first isolated ergosterol as an antitumor substance from the lipid fraction of *A. blazei*. Ergosterol is contained in various mushrooms, such as *Lentinus edodes* (Berk.) Sing. (Japanese name: Shiitake) and *Polyporus umbellatus* Fries (Japanese name: Chorei) (5), and ergosterol is a precursor of ergocalciferol. In addition, we studied the side effects (e.g., myelotoxicity, immunotoxicity and reduction in body weight) of these substances from *A. blazei*. To clarify the mechanism of antitumor activity by an isolated active substance (ergosterol), we examined the effects of ergosterol on splenic immunofunction and tumor-induced neovascularization.

MATERIALS AND METHODS

General experimental procedures.

Melting points, which were determined with a Yamato MO-21 capillary apparatus (Yamato Science, Tokyo, Japan), are uncorrected. Infrared and ultraviolet spectra were measured with a Shimadzu IR-400 spectrometer (Kyoto, Japan) and a JASCO ORD/UV-5 spectrometer (Tokyo, Japan), respectively. The ¹H NMR (499.83 MHz) spectra were recorded in CDCl₃ with a Varian Unity Inova 500 spectrometer (TOSO, Tokyo, Japan). Mass spectra were measured with an Hitachi M-4000 H spectrometer (Tokyo, Japan). Column chromatography was performed using silica gel 60 (70–230 mesh; ASTM, Merck, Germany). Other chemicals were of reagent grade.

Natural materials and isolation of antitumor substances from *A. blazei*.

A. blazei was supplied by Bizen Chemical (Okayama, Japan). Voucher samples are stored at the Second Department of Medical Biochemistry, School of Medicine, Ehime University, Japan. The dried fungal bodies of *A. blazei* (1 kg) were directly extracted with chloroform/methanol (1:1, v/v) (2 L x 3) for 3 h under reflux. The chloroform/methanol extract was concentrated under reduced pressure to provide a brown extract (250 g). The chloroform/methanol extract (200 g) was divided into acetone-soluble (160 g) and -insoluble (40 g) fractions. The acetone-soluble fraction (35 g) was further divided into *n*-hexane-insoluble (16 g) and -soluble (14 g) fractions. The *n*-hexane-insoluble fraction (15 g) was chromatographed on a silica gel column, and ergosterol (2.4 g) was isolated as an active substance. Each fraction was tested for antitumor action in sarcoma 180-bearing mice.

Cells.

The highly metastatic, drug-resistant mouse Lewis lung carcinoma (LLC)³ cells were obtained from Riken Gene Bank (Tukuba, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (1 x 10⁵ U/L), streptomycin (100 mg/L) and amphotericin B (0.25 mg/L). Sarcoma 180 cells were also maintained in the laboratory of the Second Department of Medical Biochemistry, School of Medicine, Ehime University.

Materials.

Mouse lymphocyte separation medium (Lympholytes-Mouse) was purchased from Dainippon Pharmacy Ltd. (Osaka, Japan), and fluorescein isothiocyanate (FITC)-labeled anti-mouse CD4 and CD8 and phycoerythrin-labeled anti-mouse NK1.1 antigen were purchased from Serotec (Oxford, U.K.). Matrigel basement membrane (reduced growth factor) was obtained from Becton Dickinson Labware (Bedford, MA). DMEM were obtained from Nissui Pharmaceutical Ltd. (Tokyo, Japan) and used as culture medium. Antibiotic and antimycotic solutions were purchased from Sigma Chemical (St. Louis, MO). Fetal bovine serum was purchased from ICN Biochemicals (Aurora, OH). Round nitrocellulose membrane chambers (pore size 0.45 μm) were purchased from Millipore (Bedford, MA). Culture plates were purchased from Corning Glass Works (Corning, NY). Other chemicals were of reagent grade. β -Cyclodextrin was supplied by Ensui Sugar Refining Ltd. (Yokohama, Japan). Ergosterol was suspended in 9 g NaCl/L solution or distilled water containing 20 g β -cyclodextrin/L.

Animals.

Male ICR strain mice (6 wk old) and female C57BL/6 strain mice (5 wk old) were obtained from Clea Japan (Osaka, Japan). They were housed for 1 wk in a room maintained at $25 \pm 1^\circ\text{C}$ with 60% humidity and had free access to standard nonpurified diet (8 g water, 51.3 g crude carbohydrate, 24.6 g crude protein, 5.6 g crude lipid, 3.1 g crude fiber, 6.4 g mineral mixture and 1 g vitamin mixture per 100 g diet; Oriental Yeast Ltd., Osaka, Japan) and water. The room was illuminated for 12 h/d starting at 0700 h. Animals were treated according to the ethical guidelines of the Animal Center, School of Medicine, Ehime University. The experimental protocol was approved by the Animal Studies Committee of Ehime University.

Measurements of antitumor activities and side effects of various fractions and active substances isolated from *A. blazei* in sarcoma 180- and LLC-bearing mice.

Solid-type sarcoma 180 was prepared through the subcutaneous transplantation of 1.0×10^6 or 2.5×10^6 cells into the right abdomen of mice on d 0. Various lipid fractions such as chloroform/methanol extract, acetone-soluble and -insoluble fractions and *n*-hexane-soluble and -insoluble fractions were suspended in water containing 50 g gum arabic/L through sonication. These lipid fractions were administered orally for 20 consecutive d at a dose of 800 mg/kg, starting 12 h after the implantation of tumor cells. Ergosterol was administered intraperitoneally at doses of 10, 50, 100 or 200 mg/kg or orally at doses of 100, 200, 400 or 800 mg/kg for 20 consecutive d. Control mice were also fed 9 g NaCl/L or water containing 50 g gum arabic/L alone on the same schedule.

The tumor volume was determined through direct measurement with calipers and calculated by the formula $[\text{length (mm)} \times \text{width (mm)}^2]/2$ every 2–3 d. On d 21, blood was obtained via venipuncture in mice with diethyl ether anesthesia, and then the tumor, epididymal adipose tissue, spleen and thymus were removed and weighed for evaluation of antitumor activity and side effects. The blood samples were chilled in test-tubes containing heparin, and the number of leukocytes was measured using a Coulter Counter (Japan Scientific Instruments Ltd., Tokyo, Japan).

Solid-type LLC cells were also prepared through subcutaneous transplantation of 5×10^5 cells (1 mL) into the right abdomen of mice on day 0. Ergosterol (50, 200 or 800 mg/kg) was administered orally once daily for 24 consecutive d, starting 12 h after the implantation of tumor cells. Control mice were administered distilled water alone on the same schedule. On d 25, the mice were killed by cervical dislocation, and their spleens, thymus and lungs were quickly removed and weighed.

Measurement of lymphocyte number and T-cell population (CD4+, CD8+ and NK1.1+ T cells) in LLC-bearing C57BL/6 mice.

The spleen was gently teased to release cells through dissection in cold PBS. The cell suspension (5 mL) was layered onto 5 mL of Lympholytes-Mouse and centrifuged at 1500 x g for 30 min. The lymphocyte band at the interface was recovered, and the cells were rinsed three times with PBS (pH 7.4). The number of lymphocytes was measured using a Coulter Counter. The cell concentration was adjusted to 2 x 10¹⁰ cells/L, and then 10 µL of FITC-labeled anti-mouse CD8, FITC-labeled anti-mouse CD4 or phycoerythrin-labeled anti-mouse NK1.1 was added to 100 µL of the cell suspension. After incubation for 30 min at 4°C, lymphocytes were rinsed three times with 1 mL of PBS and centrifuged at 700 x g for 5 min. Then CD4+, CD8+ and NK1.1+ T-cell populations were analyzed by flow cytometry with an FACS Calibur (Becton Dickinson, Mountain View, CA).

Measurement of neovascularization induced by tumor cells.

In vivo tumor-induced neovascularization was assayed according to the dorsal air-sac method. Briefly, 1.5 x 10⁶ cultured LLC cells were suspended in DMEM, packed into a round nitrocellulose membrane chamber with a diameter of 14 mm and implanted into dorsal air-sac mice on day 0. Ergosterol (5, 10 or 20 mg/kg) was administered once daily on d 1–5. The mice were killed on d 6, and the hair on the skin in contact with the chamber was carefully shaved off. The formation of new blood vessels in the subcutaneous region was photographed.

Measurement of Matrigel-induced neovascularization.

In vivo Matrigel-induced neovascularization was assayed according to the methods of Passaniti et al. (6). Briefly, female C57BL/6 mice were each injected subcutaneously with 0.5 mL of Matrigel containing 1 mg of acidic fibroblast growth factor (aFGF) and 64 x 10³ U of heparin per L in the presence or absence of ergosterol (400 or 800 mg/L). The mice were killed on d 5 with an overdose of pentobarbital, and the gels were removed and weighed. Then the hemoglobin contents in the gels were determined using Hemoglobin-Test kits (Wako Pure Chemical, Osaka, Japan).

Data and statistical analyses.

All values are expressed as means ± SEM. Data were analyzed by one-way ANOVA, and then differences in means among groups were analyzed using Dunnett's test or Fisher's protected LSD multiple comparison test (significantly different at $P < 0.05$).

The structure of the isolated substance.

The isolated substance formed colorless needles with a melting point of 157°C. It was reddish violet with concentrated H₂SO₄ and CH₃COOH [fast atom bombardment–mass spectrometry m/z 489 (M+ H⁺)]. The isolated substance was identified as ergosterol through direct comparison of the ¹H NMR spectral data of an authentic sample. The yield was □1.5 g/kg dried body of *A. blazei*.

RESULTS

Antitumor and side effects of lipid fractions prepared from *A. blazei* in sarcoma 180-bearing mice.

The chloroform/methanol extract (800 mg/kg x 20 d) strongly inhibited tumor growth (Table 1□). Moreover, the oral administration of the acetone-soluble fraction isolated from the

chloroform/methanol extract significantly inhibited tumor growth at a dose of 800 mg/kg during the 20-d treatment. On the other hand, tumor growth was not affected by the oral administration of the acetone-insoluble fraction on d 20. Therefore, the acetone-soluble fraction, which had higher antitumor activity, was divided into two fractions through treatment with *n*-hexane. The *n*-hexane-soluble and -insoluble fractions (800 mg/kg x 20 d) also inhibited the tumor growth, and their inhibitory ratios were 80.2 and 90.1%, respectively.

Table 1. Effects of various lipid fractions of chloroform/methanol extract (1:1, v/v), acetone-soluble and -insoluble fractions and *n*-hexane-soluble and -insoluble fractions on tumor volume at 20 d and tumor weight at 21 d in sarcoma 180-bearing mice¹

| | Tumor volume ² | Inhibition ² | Tumor weight ³ | Inhibition ³ |
|--|---------------------------|-------------------------|---------------------------|-------------------------|
| | <i>mm</i> ³ | % | <i>mg</i> | % |
| Chloroform/methanol extract | | | | |
| Control | 4826.9 ± 1150.6a | — | 4470.0 ± 870.6a | — |
| Chloroform/methanol extract | 1087.3 ± 567.6b | 77.5 | 844.2 ± 425.1b | 81.1 |
| Acetone soluble and insoluble fractions | | | | |
| Control | 928.8 ± 250.9a | — | 827.7 ± 381.2a | — |
| Acetone-soluble fraction | 124.1 ± 83.6b | 86.6 | 108.6 ± 83.4b | 86.8 |
| Acetone-insoluble fraction | 649.1 ± 140.9ab | 30.1 | 490.5 ± 382.3ab | 40.7 |
| <i>n</i> -Hexane soluble and insoluble fractions | | | | |
| Control | 766.9 ± 302.9a | — | 812.0 ± 277.2a | — |
| <i>n</i> -Hexane-soluble fraction | 152.0 ± 74.6b | 80.2 | 163.9 ± 150.7b | 79.8 |
| <i>n</i> -Hexane-insoluble fraction | 75.7 ± 24.6b | 90.1 | 54.6 ± 21.4b | 93.3 |

1 Various lipid fractions (800 mg/kg) were orally administered for 20 d in sarcoma 180-bearing mice.

2 At 20 d.

3 At 21 d. The inhibition ratio (%) was measured as tumor volume or tumor weight of various lipid fraction-treated mice/tumor volume or tumor weights of control mice. Each value represents the mean ± SEM, *n* = 10. Those not sharing a letter are significantly different, *P* < 0.05.

Oral administration of the chloroform/methanol extract and the various fractions prepared from the chloroform/methanol extract had no effect on food intakes and body weight gain for 20 consecutive d in sarcoma 180-bearing mice (data not shown).

Antitumor activity and side effects of ergosterol in sarcoma 180- and LLC-bearing mice.

Oral administration of ergosterol for 20 d reduced tumor volume (**Fig. 1**). The inhibition ratios for tumor growth with oral administration of ergosterol at the doses of 100, 200, 400 and 800 mg/kg x 20 d were 0.0 ± 30.6 , 62.3 ± 8.8 , 70.9 ± 10.8 and $85.5 \pm 4.7\%$, respectively. Intraperitoneal injection of ergosterol also inhibited tumor growth at doses of 10, 50, 100 and 200 mg/kg for 20 d, with inhibition ratios of 20.6 ± 20.5 , 57.1 ± 6.5 , 65.8 ± 4.7 and $84.7 \pm 4.2\%$, respectively.

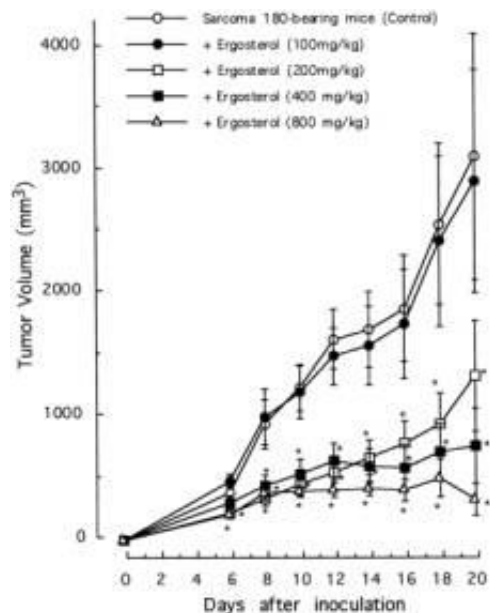


Figure 1. Effects of oral administration of ergosterol isolated from *Agaricus blazei* for 20 d on tumor volume in sarcoma 180-bearing mice. Solid-type sarcoma 180 was prepared by subcutaneous transplantation of 2.5×10^6 cells into the right abdomen of mice on d 0. The indicated amounts of ergosterol were administered orally for 20 consecutive d, starting 12 h after the implantation of tumor cells. Control mice were also administered water containing 50 g gum arabic/L alone on the same schedule. The tumor volume was determined by direct measurement with calipers and calculated by the formula $[\text{length (mm)} \times \text{width (mm)}^2]/2$ every 2–3 d. Results are expressed as means \pm SEM, $n = 10$. Those at a time not sharing a letter are significantly different, $P < 0.05$.

Tumor weights were significantly reduced with both intraperitoneal and oral administration of ergosterol (Table 2). Neither oral nor intraperitoneal administration of ergosterol caused side effects such as a reduction in body or epididymal adipose tissue, myelotoxicity (reduction in leukocyte number) or immunotoxicity (reduction in thymus and spleen weights) (data not shown). In addition, ergosterol reduced final tumor weight and tumor growth only at the dose of 800 mg/kg on d 20–23 in LLC-bearing mice (data not shown).

Table 2. Effects of intraperitoneal (IP) administration and oral administration ergosterol on tumor weight at 21 d in sarcoma 180-bearing mice¹²

| | |
|----------------|--------------|
| | Tumor weight |
| | mg |
| IP dose, mg/kg | |

| | |
|-----------------------------|------------------|
| Intraperitoneal dose, mg/kg | |
| Control | 2923.5 ± 590.2a |
| 10 | 2320.4 ± 599.3a |
| 50 | 1254.3 ± 192.0b |
| 100 | 998.4 ± 136.9bc |
| 200 | 447.4 ± 124.0c |
| Oral dose, mg/kg | |
| Control | 2728.2 ± 913.9a |
| 100 | 2748.8 ± 835.3a |
| 200 | 1028.7 ± 241.4ab |
| 400 | 793.1 ± 293.3bc |
| 800 | 394.1 ± 127.9c |

1 Ergosterol was intraperitoneally or orally administered for 20 d in sarcoma 180-bearing mice.

2 Each value represents the means ± SEM, $n = 10$. Those not sharing a letter are significantly different, $P < 0.05$.

Effects of ergosterol on immunofunction and metastasis to the lung in LLC-bearing mice, and cytotoxicity against sarcoma 180 and LLC cells.

Ergosterol had no effect on the numbers of splenic lymphocytes or CD4+, CD8+ or NK1.1+ T cells (data not shown). Colony numbers in the lungs of LLC-bearing mice (control) were $4.80 \pm 1.24 \times 10^6$. The oral administration of ergosterol had no effect on metastasis to the lung at the doses of 50, 200 and 800 mg/kg for 23 d, with tumor colony numbers ($\times 10^6$) of 3.80 ± 1.62 , 4.00 ± 0.58 and 5.33 ± 0.67 , respectively. Ergosterol had no cytotoxicity against sarcoma 180 and LLC cells (data not shown).

Effects of ergosterol on LLC-induced neovascularization.

At 5 d after implantation of the LLC cells, which were packed into the membrane chamber, neovascularization was evident in the region in contact with the chamber containing LLC cells. Intraperitoneally administered ergosterol (20 mg/kg) prevented neovascularization induced by LLC cells (Fig. 2).

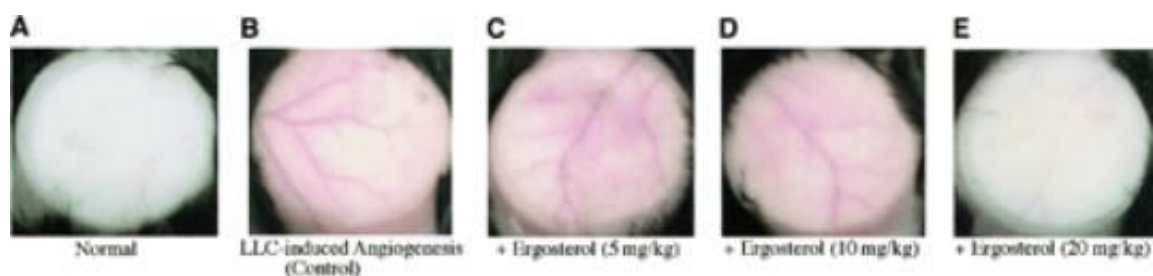


Figure 2. Effects of ergosterol on neovascularization in C57BL/6 mice bearing LLC cell-packed chambers. Chambers packed with Lewis lung carcinoma (LLC) cells were subcutaneously implanted into a dorsal air-sac of C57BL/6 mice on day 0. Dulbecco's modified Eagle's medium alone (normal, A). LLC cell-packed chamber (control, B) or 5 (C), 10 (D) or 20 (E) mg

alone (normal, -), 220 cell packed chamber (control, -) or 0 (-), 10 (-) or 20 (-) mg ergosterol/kg was intraperitoneally administered from d 1 to 5. The mice were killed on d 6, and the hair of skin in contact with the chamber was carefully shaved. The formation of new blood vessels in the subcutaneous region was photographed.

Effects of ergosterol on Matrigel-induced neovascularization.

The gels that formed after subcutaneous implantation of Matrigel alone were readily distinguished from surrounding tissue and produced little or no local reaction or angiogenic response (Fig. 3A). However, Matrigel supplemented with 1 mg aFGF and 64,000 U heparin per L produced gels that showed an angiogenic reaction (Fig. 3B). The Matrigel/aFGF/heparin mixture significantly increased the weight of the gel and the hemoglobin contents in the gels at 6 d after implantation compared with mice treated with Matrigel alone (Table 3). Ergosterol (400 and 800 mg/L) inhibited increases in the weight and hemoglobin concentration of the gels (Fig. 3, Table 3).



Figure 3. Photographs of Matrigel gel 5 d after subcutaneous injection with 0.5 mL of Matrigel alone (A), Matrigel supplemented with 1 mg/L acidic fibroblast growth factor and 64,000 U/L heparin (B) or Matrigel/acidic fibroblast growth factor/heparin mixture at 400 (C) or 800 (D) mg/L.

Table 3. Effects of ergosterol on the weights and hemoglobin contents in the gels 5 d after implantation into mice of Matrigel supplemented with acidic fibroblast growth factor (aFGF) and heparin

| Treatment | Matrigel weight | Hemoglobin content |
|---|-----------------|--------------------|
| | mg | mg/Matrigel |
| Matrigel alone | 103.16 ± 10.15b | 2.6 ± 0.68b |
| Matrigel+ aFGF (1 mg/L)+ heparin (64,000 U/L) (Control) | 371.60 ± 39.75a | 21.0 ± 4.00a |
| Matrigel+ aFGF+ heparin+ ergosterol (400 mg/L) | 105.50 ± 11.10b | 2.4 ± 0.68b |
| Matrigel+ aFGF+ heparin+ ergosterol (800 mg/L) | 103.16 ± 10.15b | 2.6 ± 0.68b |

| | | |
|--|-----------------------------|-------------------------|
| Matrigel, aFGF, heparin+ ergosterol (400 mg/L) | 185.58 ± 44.40 ^a | 6.4 ± 1.86 ^b |
| Matrigel, aFGF, heparin+ ergosterol (800 mg/L) | 108.84 ± 9.69 ^b | 3.8 ± 0.58 ^b |

1 C57BL/6 mice were each injected subcutaneously with 0.5 mL of Matrigel supplemented 1 mg aFGF/L and 64 x 10 U heparin/L in the absence or presence of ergosterol (400 or 800 mg/L).

2 Each value represents the means ± SEM, $n = 5$. Those not sharing a letter are significantly different, $P < 0.05$.

DISCUSSION

A. blazei has been used by 300,000–500,000 persons for the prevention of cancer and/or as an adjuvant with cancer chemotherapy drugs after the removal of a malignant tumor. There have been a number of reports that various Basidiomycetes have antitumor activity in sarcoma 180-bearing mice. Chihara (7) reported a study in which the assessments of antitumor activity were performed by the intraperitoneal injection of water extracts of various Basidiomycetes. Mizuno et al. (1) also evaluated the antitumor activity in sarcoma 180-bearing mice by intraperitoneal injection of a water extract of *A. blazei*. Recently, Ito et al. (8) reported that the intraperitoneal or oral administration of a polysaccharide/protein complex of *A. blazei* had antitumor activity in tumor-bearing mice. However, the antitumor activities of lipid fractions are not yet known. In the present study, we found that the lipid fraction, as well as the β -glucan fraction, inhibited tumor growth. We isolated the antitumor substance ergosterol from the lipid fraction through repeated silica gel column chromatography. Furthermore, we found several compounds in addition to ergosterol. Studies of the isolated unknown compounds are in progress to determine their structures and to examine their effects on tumor growth. Intraperitoneal and oral administration of ergosterol inhibited the tumor growth without causing any of the side effects that are usually caused by cancer chemotherapy drugs. Ergosterol had no cytotoxic effect against sarcoma 180 cells in vitro (data not shown). Ergosterol is a precursor of ergocalciferol. It has been reported that cholecalciferol inhibits angiogenesis (9 10 11)