Aflastatin A, a Novel Inhibitor of Aflatoxin Production by Aflatoxigenic Fungi

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Aflastatin A, a novel inhibitor of the production of aflatoxin by aflatoxigenic fungi, has been isolated from the solvent extract of mycelial cake of *Streptomyces* sp. and its molecular formula was determined as $C_{62}H_{115}NO_{24}$.

Aflastatin A completely inhibited aflatoxin production by *Aspergillus parasiticus* NRRL 2999 in liquid medium or on agar plate at a concentration of 0.5 μg/ml. The mycelial growth of this fungus was not affected in the liquid medium at the same concentration, while the hyphal extension rate was reduced on the plate together with some morphological changes. The growth of the fungus was not completely inhibited even at a concentration of 100 μg/ml. Aflastatin A exhibits antimicrobial activity against some bacteria, yeasts and fungi as well as antitumor activity.

Aflatoxin is the most well-known mycotoxin for its potent carcinogenicity. It is produced by some strains of *A. parasiticus*, *A. flavus* and *A. nomius*. Since discovery of the potent carcinogenicity of aflatoxin in the 1960s and subsequent detection of the toxin in a wide variety of food commodities, control and management of this toxin has been the issues of concern[1]. Many substances including pesticides[2], spices[3] and the extract of plants[4] were screened for prevention of aflatoxin contamination in foods and feeds, but none of them has practically been used. Among such substances, dichlorvos, an organophosphorous insecticide having the choline esterase inhibitory activity, was found to show unique effect on aflatoxin production. It inhibits some esterases involved in the biosynthetic pathway of aflatoxin to result in the inhibition of aflatoxin production[5].

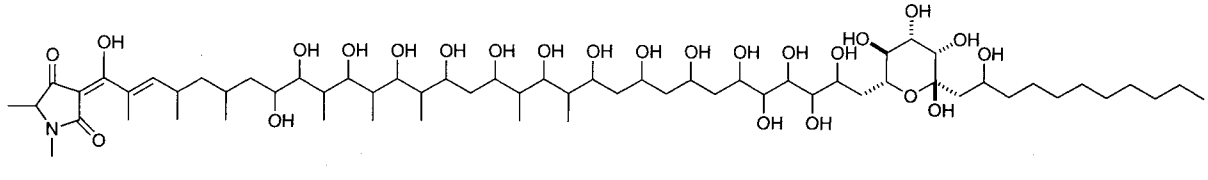
On the other hand, emerging resistant strains against some kind of antibiotics is becoming serious problem. This phenomenon is primarily caused by selection of the antibiotic-resistant mutant by the antibiotic itself. In this context, new program was adapted to the screening for the inhibitors of aflatoxin production. Since the production of aflatoxin doesn't seem to be essential for the growth of its producing strains, a specific inhibitor of its production may not select the resistant strain against such an agent. Then, a specific inhibitor of aflatoxin production may become the substance that prevents aflatoxin contamination of agricultural commodities and minimizes the risk of pervasion of drug-resistant strains. In the course of our screening search based on the above strategy, *Streptomyces* sp. MRI 142 was found to produce a novel inhibitor of aflatoxin production by *A. parasiticus* and named aflastatin A (Fig. 1).

This paper deals with the taxonomy of the producing strain as well as the fermentation, isolation, physicochemical and biological properties of aflastatin A. The elucidation of the structure and biosynthesis of aflastatin A has been reported in a preliminary communication[6].

Taxonomic Studies

Strain MRI 142 was isolated from a soil sample

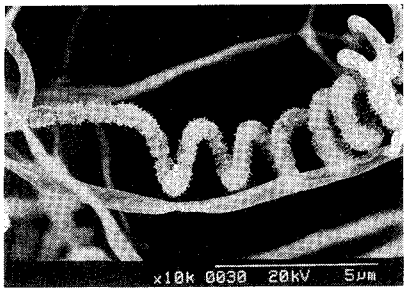
Fig. 1. Structure of aflastatin A.



collected in Zushi-shi, Kanagawa Prefecture, Japan.

The method described by SHIRLING and GOTTLIEB[7] was employed for characterization of the strain. The color names used in this study were based on Hyoujyun Shikihyou (Nihon Kikaku Kyoukai). Morphological observations were made on the cultures grown at 27°C for 14 days on inorganic salts-starch agar and sucrose-nitrate agar. Mature spore chains had 30 or more spores in the form of *Spira*. The spores were cylindrical or oval and $0.5 \sim 0.7 \times 0.8 \sim 1.2\ \mu m$ in size. Spore surfaces were spiny (Fig. 2). Cultural characteristics after incubation for 14 days at 30°C on various media are shown in Table 1. Colonies with appearance of the gray color series were observed on most of the media used and no soluble pigment was produced. Physiological properties are summarized in Table 2. Whole-cell analysis was done according to BECKER *et al.*[8]. Strain MRI 142 contained LL-diaminopimelic acid in its hydrolyzate. According to these results, strain MRI 142 was judged to be similar to *Streptomyces griseochromogenes* among the species described in "BERGEY's Manual of Determinative Bacteriology" 8th Ed.[9] and ISP reports[10~13]. A simultaneous comparison of carbon utilization test of strain MRI 142 and the type strain (IFO 13413) of *S. griseochromogenes* revealed that strain MRI 142 differed from the type strain (Table 3). Further taxonomic studies will be done for the final identification of strain MRI 142.

Fig. 2. Electron micrograph of aflastatin A-producing strain.



Fermentation

Erlenmeyer flasks (500 ml) containing 100 ml of the production medium were inoculated with spores from a slant culture of strain MRI 142 and incubated at 27°C on a rotary shaker with a 5 cm throw at 160 rpm. The production medium consisted of glucose 3%, soy bean meal 1.5%, K_2HPO_4 0.08%, $MgSO_4 \cdot 7H_2O$ 0.02%, $CaCO_3$ 0.4%, pH 7.20. The activity appeared 2 days after inoculation and reached a maximum after 6 days of cultivation. No distinctive loss of the activity occurred during prolonged cultivation. A profile of the fermentation is shown in Fig. 3.

Production of aflastatin A in jar fermenter was carried out using the same medium as that of the flask fermentation. Ten-liter fermenter with 5 liters of the medium was inoculated with 5% volume of the seed culture, precultured for 44 hours by shaking in the same medium at 27°C. The fermentation was carried out with the supply of air at a rate of 1/1 volume per minute, with agitation of 400 rpm and at 27°C for 166 hours.

Isolation and Purification of Aflastatin A

Since the most activity was present in the mycelium, 4.3 liters of the culture broth was filtered to collect the mycelial cake, which was washed with 1 liter of deionized water and extracted three times with 1.2 liters of methanol at 65°C with vigorous stirring. The methanol

Table 1. Cultural characteristics of strain MRI 142.

Medium	Growth	Aerial mycelium	Reverse side of colony	Soluble pigment
Tryptone-yeast extract agar	Moderate	Scant, white	Yellowish white 7.5y9/2	None
Yeast-malt extract agar	Good	Thin, white	Dull yellow 5y7/8	None
Oatmeal agar	Good	Good, grayish yellow-brown 2.5y4/2	Colorless	None
Inorganic salts-starch agar	Good	Good, olive gray 10y5/2	Dull yellow 10y6/6	None
Glycerol-asparagine agar	Good	Thin, yellowish white 5y9/2	Yellowish white 7.5y9/2	None
Peptone-yeast-iron agar	Moderate	None	Light olive gray 5y7/3	Brown 2.5yr4/2
Tyrosine agar	Good	Thin, white	Olive gray 7.5y6/6	Light olive 5y5/6
Sucrose-nitrate agar	Poor	Thin, light olive gray 5y7/2	Colorless	None
Glucose-asparagine agar	Good	Scant, white	White 10y9/1	None
Nutrient agar	Good	None	Pale olive 7.5y8/6	None

Table 2. Physiological properties of strain MRI 142.

Properties observed	Characteristics
Temperature range for growth	20~37°C
Optimum temperature	27~37°C
Nitrate reduction	Doubtful
Starch hydrolysis	Positive
Milk coagulation	Negative
Milk peptonization	Positive
Melanin production	Positive
Gelatin liquefaction	Negative
H_2S production	Positive
Cellulose hydrolysis	Negative
NaCl tolerance	<7%

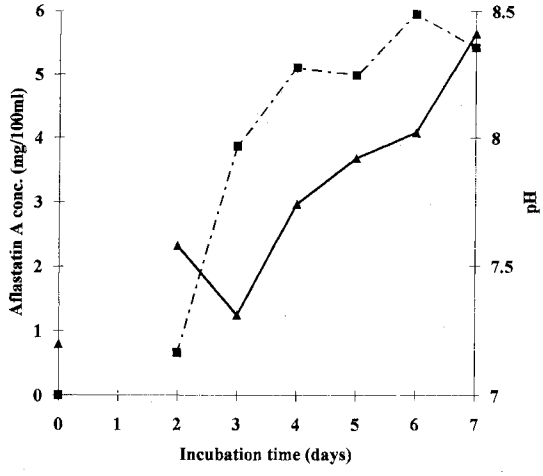
Table 3. Carbon utilization of strain MRI 142 and *Streptomyces griseochromogenes* IFO 13413.

	MRI 142	IFO 13413
L-Arabinose	+	+
D-Fructose	+	+
D-Glucose	+	+
D-Galactose	+	+
Inositol	+	+
Sucrose	-	+
D-Mannitol	+	+
L-Rhamnose	-	-
Raffinose	+	+
D-Xylose	+	+
Salicin	+	-

Symbols: +; Utilization, -; No utilization

Fig. 3. Fermentative production of aflastatin A.

■ Aflastatin A, ▲ pH.



extract was evaporated *in vacuo* to give a 29.3 g of slurry, which was dissolved in 600 ml of butanol saturated with water and the solution was washed twice with 300 ml of 0.5% $NaHCO_3$ and once with 300 ml of deionized water successively. The organic layer was evaporated *in vacuo* to give a 21.9 g of a brownish oily material. Tetrahydrofuran (500 ml) was poured onto this oily material and stirred vigorously, and then incubated statically in the dark at room temperature overnight. An amorphous precipitate (800 mg) was obtained. After filtration, the precipitate was washed with a small amount of tetrahydrofuran, and then suspended into 200 ml of chloroform - methanol (2 : 1). The insoluble matter was washed with a small amount of methanol and dried in a desiccator. Crude aflastatin A (632 mg), whose purity was estimated at 98% based on the UV absorption at 300 nm, was further purified by using HPLC on a C_{18} column (Capcell Pak C_{18} AG120, 250 mm × 15 mm i.d., Shiseido). Elution was carried out with methanol - 0.5% diethylamine in water (65 : 35) and a flow rate of 5.0 ml/minute.

Because of poor solubility of aflastatin A in the mobile phase (0.6 ~ 0.9 mg/ml), this chromatography was repeated and the active fractions were combined. Lyophilization of the combined fraction gave 17 mg of aflastatin A from a 20 mg of crude aflastatin A.

Physico-chemical Properties of Aflastatin A

The physico-chemical properties of aflastatin A are summarized in Table 4. Aflastatin A was soluble in dimethyl sulfoxide, slightly soluble in methanol and aqueous ethanol and practically insoluble in water and other organic solvents.

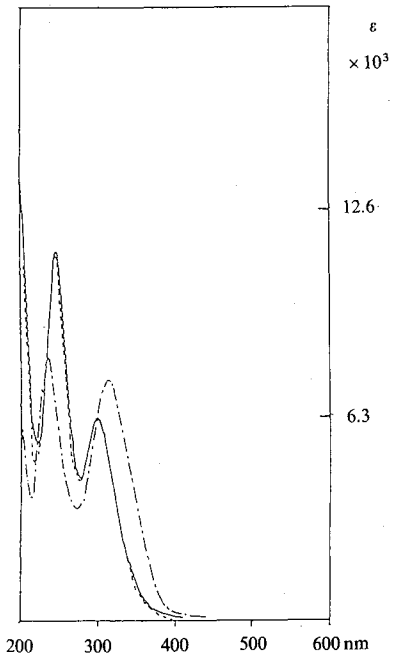
Color reactions were as follows: positive in iodine vapor and ferric chloride and negative in ninhydrin test. The molecular formula was determined from the data of elementary analysis as well as FAB-MS and NMR spectra. The UV spectra of aflastatin A are shown in Fig. 4. The IR, ^1H and ^{13}C NMR spectra of aflastatin A are shown in Figs. 5, 6 and 7, respectively. The UV and IR spectra of aflastatin A were very similar to those of blasticidin A[14]. To examine whether they are identical or not, the sample of blasticidin A was isolated

Table 4. Physico-chemical properties of aflastatin A.

Appearance	White powder
Analysis	Calcd. for $C_{62}H_{115}NO_{24}.7H_2O$: C 53.78, H 9.39, N 1.04, O 35.82 Found: C 53.96, H 8.95, N 1.08, O 35.77
Mass Spectrometry	HR-FAB-MS (positive, glycerol matrix) m/z 1280.7749 $(M+Na)^+$ Calcd. for: $C_{62}H_{115}NO_{24}$ FAB-MS (negative, glycerol matrix) m/z 1256 $(M-H)^-$
Optical Rotation	$[\alpha]^{19}_D$; -2.6° (*c* 0.545, DMSO)
Molecular Formula	$C_{62}H_{115}NO_{24}$
UV Spectrometry	λ_{max} (MeOH/H_2O, 1:1), nm (ε); 299 (6,200), 247 (11,000) λ_{max} (MeOH/0.01N-NaOH), nm (ε); 299 (6,200), 247 (11,000) λ_{max} (MeOH/0.01N-HCl), nm (ε); 314 (7,300), 237 (7,900)
IR Spectrometry	ν_{max} (KBr wafer), cm^{-1}; 3380, 2930, 2850, 1600, 1450, 1380, 1310, 1275, 1060, 960, 845

Fig. 4. UV spectra of aflastatin A.

—— MeOH - H_2O (1 : 1), ··· MeOH - 0.01 N-NaOH (1 : 1), —·— MeOH - 0.01 N-HCl (1 : 1).



from *Streptomyces griseochromogenes* IFO13413 according to the method of KONO *et al.*[14]. The comparison experiment was done by using HPLC on a C_{18} column (Capcell Pak C_{18} AG120, 150 mm × 4.6 mm i.d., Shiseido). Elution was carried out with methanol - 0.5% diethylamine in water (63 : 37) and a flow rate of 0.5 ml/minute. The result of co-elution experiment on HPLC with aflastatin A and blasticidin A revealed that they are not identical, indicating retention times of 8.8 and 6.5 minutes, respectively.

Biological Properties

The effect of aflastatin A on aflatoxin production was examined by both serial broth and agar dilution methods. At a concentration of 0.5 μg/ml, aflastatin A completely inhibited aflatoxin production by *A. parasiticus* in both methods. In the case of broth dilution method, aflastatin A showed no effect on the weight increase of the fungus at this concentration (Fig. 8). On the other hand, hyphal extension rate was reduced in the dose dependent manner by agar dilution method. It was reduced by about 30% of control at a concentration of 0.5 μg/ml (Fig. 9). The cultural characteristics on the agar plate revealed that the colony of control group was spreading toward the edge, but the colonies of treated group were dense and elevated in proportion to the concentration of aflastatin A. The growth of *A. parasiticus* NRRL 2999 was not completely inhibited even at a concentration of 100 μg/ml on the agar plate (Table 5), but the hyphal extension rate was significantly reduced (by 70% of control) at the same concentration. These biological activities suggest that aflastatin A can inhibit the aflatoxin production with some morphological changes of the producing organism, but without inhibition of the growth.

The antimicrobial activity of aflastatin A was tested by agar dilution method. As shown in Table 5, aflastatin A is active against Gram-positive bacteria, yeasts and filamentous fungi. Aflastatin A showed no effect on the Gram-negative bacteria.

Fig. 5. IR spectrum of aflastatin A (KBr wafer).

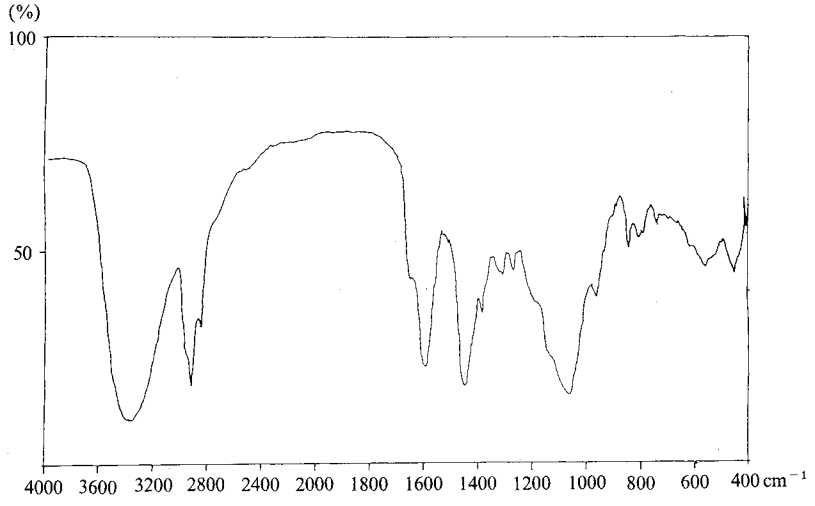


Fig. 6. ^{1}H NMR spectrum of aflastatin A (500 MHz, in DMSO-d_6).

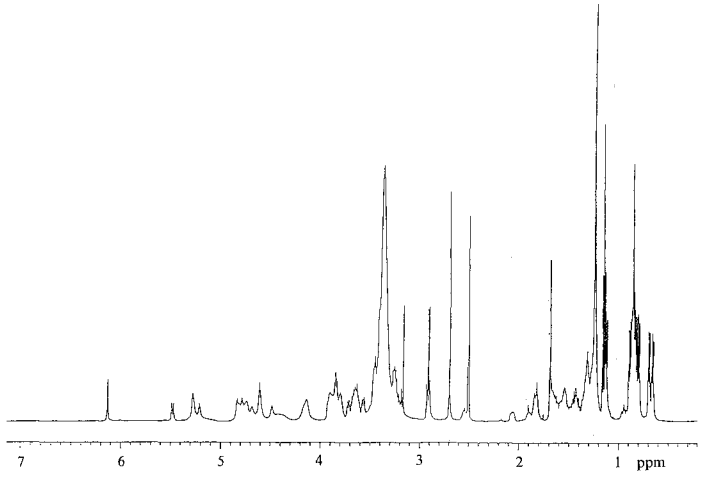


Fig. 7. ^{13}C NMR spectrum of aflastatin A (125 MHz, in DMSO-d_6).

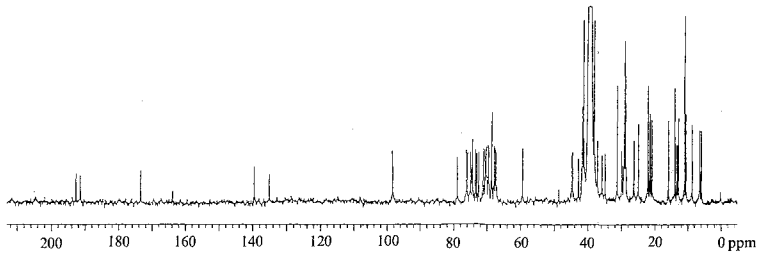


Fig. 8. Inhibitory effect of aflastatin A on AF production in liquid medium.

▲: Mycelial dry weight, ■: AF concentration. Each value represents mean of triplicated experiments and vertical bar represents standard deviation.

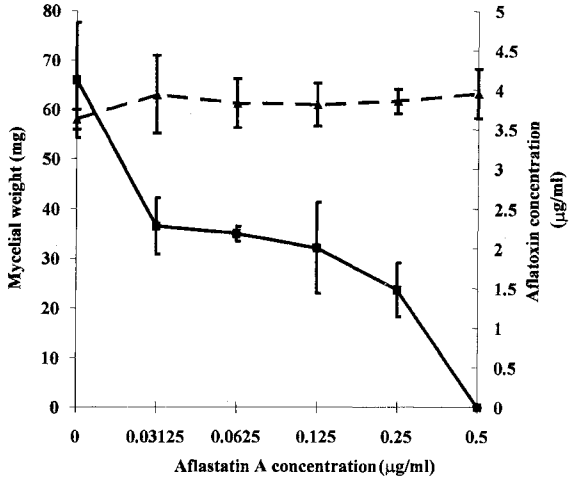


Fig. 9. Inhibitory effect of aflastatin A on AF production on agar plate.

▲: Colony diameter, ■: AF concentration. Each value represents mean of triplicated experiments and vertical bar represents standard deviation.

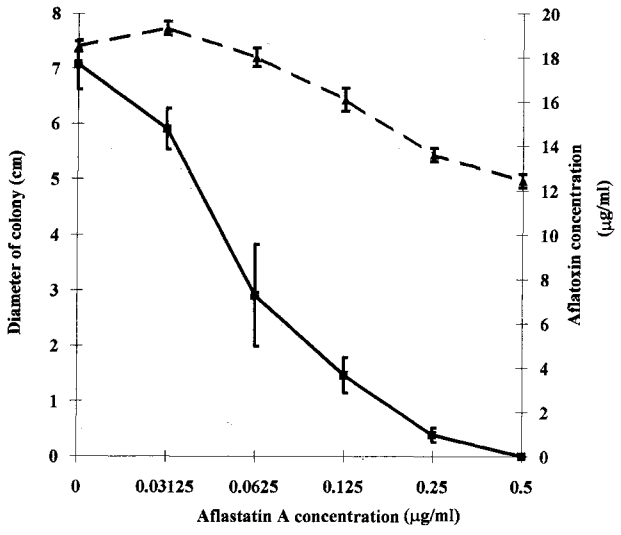


Table 5. Antimicrobial activity of aflastatin A.

Organisms	MIC(μg/ml)	Medium*	Period (hrs)
Bacillus subtilis IAM 1145	0.39	A	24
Staphylococcus aureus IFO 13276	0.78	A	24
Escherichia coli IFO 14249	>100	A	24
Pseudomonas aeruginosa IAM 1514	>100	A	24
Candida albicans IFO 1594	0.39	B	48
Saccharomyces cerevisiae IFO 0250	<0.19	B	48
Trichophyton mentagrophytes IFO 5809	0.39	B	48
Aspergillus parasiticus NRRL 2999	>100	C	48
Fusarium graminearum ATCC 34909	>100	C	48
Pyricularia oryzae IFO 30732	<0.19	C	48

*: A; Nutrient agar, B; Sabouraud agar, C; Potato dextrose agar.

Aflastatin A was suspended in sterilized water containing 0.25% carboxymethylcellulose was administered to mice to determine its toxicity. The LD_{50} values were 6.17 mg/kg and more than 1000 mg/kg by intraperitoneal and oral administrations, respectively.

The effect of aflastatin A on the growth of adenocarcinoma 755 is shown in Table 6. Aflastatin A showed a dose-related inhibition of the tumor growth. When aflastatin A was administered at a dose of 1.0 mg/kg, the tumor growth was reduced by more than 75%.

Table 6. Antitumor activity of aflastatin A.

Dose (mg/kg) *	Weight of tumor (m ± S.E.: mg)**
Control (0)	1617.8 ± 179.07
0.3	680.2 ± 176.63
1.0	392.8 ± 87.07
3.0	-***

BDF1 mice inoculated sc. with 5 x 10^5 cells of adenocarcinoma 755.
*: Administered i.p. from day 1 to day 4 (once a day).
**: Wet weight of tumor on day 14 (n=6).
***: All mice died by day 3 due to toxicity.

Experimental

Screening for Inhibitors of Aflatoxin Production

A. parasiticus NRRL 2999 was used as the aflatoxin producer. *Bacillus megaterium* ATCC 25848, a strain sensitive to aflatoxin[15], was used as indicator of aflatoxin production. The assay was done by the following method. The culture broths of streptomycetes were sterilized by filtration. The filtrate (2 ml) was added to a 100 ml-Erlenmeyer flask containing autoclaved 10/8 concentrated modified -YES medium (8 ml) which is suitable for aflatoxin production[16]. A spore suspension (0.1 ml) of *A. parasiticus* (prepared from the growth on a PDA slant incubated at 26°C for 7 days) was used as inoculum (about 2.5×10^5 CFU per flask). The flasks were incubated at 27°C for 7 days in static culture. After incubation, the growth of *A. parasiticus* was visually observed and the aflatoxin content of the culture broth was determined. The culture broth of *A. parasiticus* (20 μl) was spotted onto a paper disk (6 mm in diameter), which was placed onto the agar plate prepared as follows. *B. megaterium* ATCC 25848 was incubated overnight in nutrient broth at 30°C. The culture broth (0.1 ml) was added to 100 ml of sterilized plate count agar (Difco) at 60°C and the agar medium (30 ml) was poured into a square dish (13.5 cm × 9.5 cm) to make the agar plate. The plate was incubated at 30°C for 24 hours and the diameter of the inhibitory zone around the paper disk was measured. Streptomycetes, whose culture broths exhibited no growth-inhibiting activity against *A. parasiticus* and no zone around the disk, were chosen for further studies. The diameter of the inhibitory zone was linearly related to the logarithm of the concentration of aflatoxin in the culture broth in the range of 100 μg/ml to 400 μg/ml.

Evaluation of the Effect of Aflastatin A

To evaluate the effect of aflastatin A on the growth and the aflatoxin production of aflatoxigenic fungi, both broth and agar dilution methods were employed. Aflastatin A was dissolved in dimethyl sulfoxide at appropriate concentrations and added to Potato dextrose broth (Difco Co.) for broth dilution method and to PDA medium (Nissui Co.) of below 60°C for agar dilution method. The dimethyl sulfoxide solution was added to give a final concentration of 0.1% (v/v) in broth or agar. As for control, only dimethyl sulfoxide was added to give the same final concentration as treated. In the case of broth dilution method, 10 ml of the broth was placed in a 100 ml-Erlenmeyer flask. In the case of agar dilution method, 10 ml of the agar medium was used to make each plate (9 cm internal diameter). The spore suspension (10 μl) of *A. parasiticus* was inoculated into the flask or at the center of the plate. The inoculum was 2.5×10^4 CFU per flask or plate. These flasks or plates were incubated at 27°C for 7 days. In the case of broth dilution method, the mycelial cake was harvested by filtration and washed with 25 ml of distilled water. The collected mycelial cake was dried at 103°C for 3 hours and weighed. The culture filtrate and washings were combined and extracted with 25 ml of chloroform (2 times). In the case of agar dilution method, the diameter of colony was measured and whole agar plate was scraped off the dish. The agar plate was extracted with chloroform (80 ml) by using a Waring blender. The extracts from both broth and agar dilution methods were subjected to aflatoxin analysis[17]. HPLC analysis of aflatoxin was carried out on a phenylated silica gel column (COSMOSIL 5-ph, 150 mm × 4.6 mm i.d., Nacalai Co.) with the mobile phase of tetrahydrofuran - H_2O (20 : 80).

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