# NF00659$A_1$, $A_2$, $A_3$, $B_1$ and $B_2$, Novel Antitumor Antibiotics Produced by *Aspergillus* sp. NF 00659

## I. Taxonomy, Fermentation, Isolation and Biological Activities

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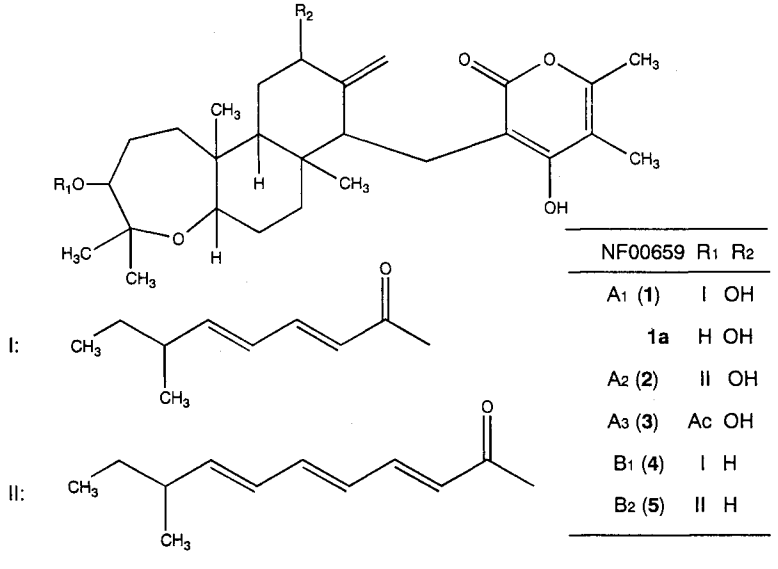
Five novel cytotoxic antibiotics, NF00659$A_1$ (**1**), $A_2$ (**2**), $A_3$ (**3**), $B_1$ (**4**) and $B_2$ (**5**) were discovered. They were isolated from a culture mycelium of *Aspergillus* sp. These compounds were proved to have 4,5-*seco*-tricyclic diterpene α-pyrone structure by spectroscopic analyses. They showed potent antitumor activities against human ovarian carcinoma A2780 and human colorectal adenocarcinoma SW480 cells, but did not show any antimicrobial activities at 1,000 μg/ml against Gram-positive and Gram-negative bacteria, yeasts and fungi.

In our continuing search for new pesticidal compounds from microbial metabolites, *Aspergillus* sp. NF 00659 (FERM P-13834) was found to produce new antibiotics with potent insecticidal activities against the larvae of mosquito, *Culex pipiens molestus*, and the larvae of diamondback moth, *Plutella xylostella*. NF00659$A_1$ (**1**), $A_2$ (**2**), $A_3$ (**3**), $B_1$ (**4**) and $B_2$ (**5**) were isolated from the mycelium with organic solvents and were purified by chromatographies. The structural studies by spectroscopic analysis revealed that they have a new tricyclic diterpene α-pyrones skeleton (Fig. 1).

NF00659s have showed potent growth inhibitory activities against human ovarian carcinoma A2780 and human colorectal adenocarcinoma SW480 cells as well as insecticidal activities. They did not show any inhibitory activities against bacteria, yeasts and fungi at 1,000 μg/ml.

In this paper, we describe the taxonomy of the producing strain, fermentation, isolation, and biological activities of these metabolites. The structural elucidation will be described in the following paper[1].

Fig. 1. Structures of NF00659$A_1$ (**1**), $A_2$ (**2**), $A_3$ (**3**), $B_1$ (**4**) and $B_2$ (**5**).




| NF00659 | R1 | R2 |
|---|---|---|
| A1 (**1**) | I | OH |
| **1a** | H | OH |
| A2 (**2**) | II | OH |
| A3 (**3**) | Ac | OH |
| B1 (**4**) | I | H |
| B2 (**5**) | II | H |

## Materials and Methods

### Producing Organism and Taxonomic Studies

The producing fungal strain NF 00659 was isolated from a soil. Mycological observations were made by using the culture grown at 25°C for 7 days on potato dextrose agar (PDA; Nissui), malt extract agar (MEA; malt extract 2.0%, pepton 0.1%, dextrose 2.0% and agar 2.0%), Czapek-Dox agar (CZA; $K_2HPO_4$ 0.1%, $NaNO_3$ 3.0%, KCl 0.5%, $MgSO_4 \cdot 7H_2O$ 0.5%, $FeSO_4 \cdot 7H_2O$ 0.01%, sucrose 3.0% and agar 1.5%) and Czapek yeast extract agar (CYA; CZA added yeast extract 0.5%). Morphological observations were made with light microscope (Olympus BH2) and scanning electron microscope (Hitachi S5520N).

### Fermentation

A loopful of the slant culture of strain NF 00659 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium consisting of glucose 1%, sucrose 2%, soy bean meal 1.5%, pepton 0.3%, yeast extract 0.2%, $KH_2PO_4$ 0.1%, $MgSO_4$ 0.025%, $FeSO_4 \cdot 7H_2O$ 0.00011%, $CuSO_4 \cdot 5H_2O$ 0.00064%, $ZnSO_4 \cdot 7H_2O$ 0.00015%, $MnCl_2 \cdot 4H_2O$ 0.00079% and antiform (Pronal ST-1) 0.01% at pH 6.5. The inoculated medium was incubated at 25°C for 48 hours on a rotary shaker at 220 rpm. The seed culture (2 ml) was transferred into a 500-ml Erlenmeyer flask containing 100 ml of the production medium consisting of glucose 1%, sucrose 2%, soy bean meal 2%, $KH_2PO_4$ 0.1%, $MgSO_4$ 0.025%, $FeSO_4 \cdot 7H_2O$ 0.00011%, $CuSO_4 \cdot 5H_2O$ 0.00064%, $ZnSO_4 \cdot 7H_2O$ 0.00015%, $MnCl_2 \cdot 4H_2O$ 0.00079% and antiform 0.01% at pH 6.5. The fermentation was carried out at 25°C for 7 days on a rotary shaker at 220 rpm.

### Insecticidal Activity

The insects used in this study, which are sensitive strains against organophosphate, *Culex pipiens molestus* (mosquito) and *Plutella xylostella* (diamondback moth) were collected at Ageo, Saitama, Japan and maintained in the Ageo Research Laboratories for more than ten years.

*Culex pipiens molestus*: Five third-instar larvae of mosquito were transferred into a 6-well tissue culture plate containing a solution of the test material (0.1 ml) and distilled water (10 ml). The plate was kept at 25°C under fluorescent light. Evaluation of the insecticidal activities was carried out by counting the number of living larvae after 72 hours.

*Plutella xylostella*: A cabbage leaf, 4 cm in diameter, was dipped into the solution of the test material for a few seconds. After drying, the leaf was transferred into a plastic cup (9 cm in diameter). Five third-instar larvae of diamondback moth were transferred onto the leaf. The cup was kept at 25°C under fluorescent light. Evaluation of the insecticidal activity was carried out by counting the number of living larvae after 72 hours.

### *In Vitro* Cytotoxicity

The A2780 human ovarian carcinoma cell line was obtained from the Cancer Chemotherapy Center, Tokyo, Japan. The SW480 human colorectal adenocarcinoma cell line (ATCC CCL228) was purchased from Dainippon Pharmaceutical Co., Ltd., Japan.

The A2780 cells were grown in RPMI-1640 medium contained with L-glutamine and $NaHCO_3$ (Nikken Bio Medical Laboratory) supplemented with fetal bovine serum (FBS, 10%), streptomycin (100 μg/ml) and benzylpenicillin (100 U/ml) at 37°C under humidified atmosphere in a 5% $CO_2$ incubator. The SW480 cells were grown in L-15 medium (JRH BIOSCIENCES) supplemented with FBS (10%), L-glutamine (2.05 mM), streptomycin (100 μg/ml) and benzylpenicillin (100 U/ml) under the same conditions as described above. Exponentially growing cells of both cell lines were harvested, counted and suspended in the culture media at $2.0 \times 10^4$ and $5.0 \times 10^4$ cells/ml, respectively. After planting these cells to each well of a 96-well tissue culture plate, they were incubated for 24 hours in the $CO_2$ incubator. The solutions of the test materials were added to the wells and the plates were successively incubated for 72 hours under the same conditions. Evaluation of the antitumor activities was carried out by the MTT method. That is; after adding MTT solution to each well, the tissue culture plate was incubated for 3 hours in the same incubator, and then DMSO (100 μl) was added to each well after removing the medium from the wells, and the absorbancies were measured at 540 nm.

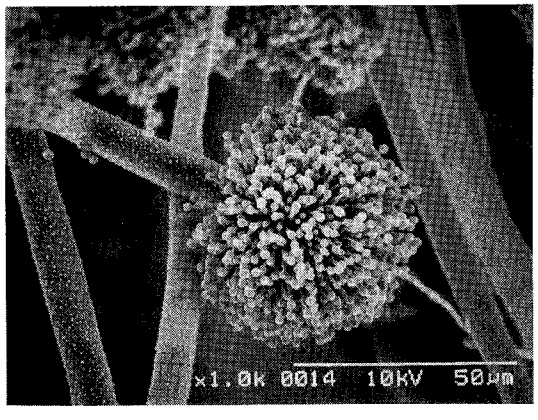
## Results and Discussion

### Taxonomy of Strain NF 00659

Cultural and morphological characteristics of the producing strain NF 00659 were described below. A scanning micrograph of the strain was displayed in Fig. 2. Colonies on PDA grew rapidly, attending a diameter

Fig. 2. Scanning electron micrograph of *Aspergillus* sp. NF00659 grown on potato dextrose agar medium.

Bar represents 50 μm.



of 51~52 mm and were floccose to cottony, white to yellow in color. On the reverse side, the colonies were whitish yellow to brownish yellow. Colonies on MEA grew similarly to those on PDA, attending a diameter of 49~50 mm. Colonies on CZA were 22~23 mm in diameter, floccose and white to pale yellow in color. On the reverse side, the colonies were whitish yellow. Colonies on CYA grew rapidly. They attained a diameter of 56~57 mm, were floccose, spread zonally, and were white to yellow in color. On the reverse side, the colonies were whitish yellow. The optimum growth temperature was 25°C but the growth was not observed at 37°C. The pH range for growth was 2 to 8 and the optimum pH was 6.0.

Morphological observations were made with the culture grown on CYA. Conidial heads on CYA were sparse with loose columns. They were colorless to yellow color and had a length of 200~300 $\mu$m. Conidiophores were colorless to pale yellow coarsely roughened, 1~2 mm in length, 7~12 $\mu$m in diameter. Vesicles were elliptic to lageniform and 10~16 $\mu$m in diameter. Metulae were cylindrical, 3.4~4.4 × 1.7~2.5 $\mu$m. Phialoconidia were globose to subglobose, colorless to pale yellow in color with rough-walls and were 1.7~2.5 $\mu$m in diameter.

On the basis of the above characteristics, the strain NF 00659 was identified as belonging to the genus *Aspergillus*[2~4], and named *Aspergillus* sp. NF 00659. This strain was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan as FERM P-13834.

## Isolation

Isolation was performed by measuring the insecticidal activities against the larvae of mosquito and diamondback moth. The culture filtrate did not show insecticidal activities. The active components were extracted from the mycelial cake of culture broth (5 liters) twice with 3 liters of acetone-methanol (1:1).

The extract was concentrated *in vacuo* to remove the organic solvents. The remaining aqueous solution (1.2 liters) was extracted with ethyl acetate (2.25 liters). The extract was concentrated *in vacuo* to give a brown greasy residue (11.2 g). The residue was applied onto a silica gel column (Silica gel 60, 70~230 mesh, Merck, 60 g) and chromatographed by stepwise elution with *n*-hexane-acetone mixture. The active fractions were collected and evaporated to dryness *in vacuo*. The active material (956 mg) was successively rechromatographed on a silica gel column (Micro Bead Silica gel MB4B, 30~50 $\mu$m, Fuji Silysia Chemical Ltd., 72 g) with *n*-hexane-diethylether-methanol (50:50:1). Three active fractions were obtained and concentrated *in vacuo*. The first fraction (135 mg) was applied onto a low pressure column (Diaion CHP-20, Mitsubishi Kasei, 1.2 × 45 cm) with a linear gradient elution system of acetonitrile (10~90%) in 5 mM ammonium acetate (pH 4.0) by monitoring UV absorption at 254 nm. NF00659$A_1$ (**1**) was obtained as white powder (21 mg), $A_2$ (**2**) was as white powder (9 mg). The second fraction (291 mg) was applied onto the same column (CHP-20) under the same conditions as described above. NF00659$B_1$ (**4**) was obtained as white powder (22 mg) and $B_2$ (**5**) was as white powder (27 mg). The third (128 mg) was rechromatographed on a silica gel column (MB4B, 10 g) with *n*-hexane-ethyl acetate (2:1). The active fraction (82 mg) was successively applied onto a Sephadex LH-20 column with methanol. NF00659$A_3$ (**3**) was obtained as colorless crystal (49 mg).

## Biological Activities

The insecticidal activities of **1**, **2**, **3**, **4** and **5** against the larvae of mosquito and diamondback moth are summarized in Table 1. NF00659s were tested for cytotoxicities *in vitro* against human ovarian and colorectal tumor cells. The results are summarized in Table 2.

The antimicrobial activities of **1**, **2**, **3**, **4** and **5** were determined by the paper disk agar-diffusion method. No antimicrobial activity was detected against the following organisms at a concentration of 1,000 $\mu$g/ml. The tested organisms were *Bacillus subtilis* IFO-3007, *Staphylococcus aureus* FAD 209 P, *Escherichia coli* K-12,

Table 1. Insecticidal activities of NF00659$A_1$ (**1**), $A_2$ (**2**), $A_3$ (**3**), $B_1$ (**4**) and $B_2$ (**5**).

| | $LD_{50}$ ($\mu$g/ml)[a] | | | | |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** |
| Mosquito | 2.5 | > 5.0 | 1.0 | 0.5 | 4.0 |
| Diamondback moth | 25 | >100 | 50 | 10 | 100 |

[a] Concentration causing 50% lethal of insect.

Table 2. *In vitro* cytotoxicities of NF00659$A_1$ (**1**), $A_2$ (**2**), $A_3$ (**3**), $B_1$ (**4**) and $B_2$ (**5**).

| | $IC_{50}$ ($\mu$g/ml)[a] | | | | |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** |
| A2780 | 2.5 | 2.0 | 26.7 | 0.9 | 0.6 |
| SW480 | 0.003 | 0.003 | 0.28 | 0.002 | 0.003 |

[a] Concentration causing 50% inhibition of cell growth.

*Xanthomonas oryzae* IFO-3510, *Saccharomyces cerevisiae* IFO-0251, *Candida albicans* IFO-1385, *Mucor racemosus* IFO-4581, *Botorytis cinerea*, and *Pyricularia oryzae*.

The preliminary acute toxicity of NF00659$A_1$ was tested by intraperitoneal (ip) or oral (po) administrations into mice. The mice received 8.0 mg/kg (ip) or 37.5 mg/kg (po) were survived, and the mice received 12.5 mg/kg (ip) or 75.0 mg/kg (po) died.

NF00659s having insecticidal and antitumor activities were classified as diterpene α-pyrones. It was already reported that viridoxins from fungus[5], and stypotriol, epitandiol and its derivatives from sponge[6] have diterpene pyrone structure with insecticidal activities. Colletotricin[7] isolated as a phytotoxin from the culture broth of phytopathogenic fungus *Colletotrichum capisici* is classified as a labdane type diterpene γ-pyrone. Colletotricin inhibits an oxidation of NADH and succinate in mitochondria prepared from rat liver cells[8,9]. Antimycins exhibiting strong insecticidal, fungicidal and antitumor activities also inhibit electron transfer system[10] as well as colletotricin. However, there is no description showing cytotoxicity of colletotricin against mammalian cells. NF00659s exhibit the cytotoxities against insects and human tumor cells without antimicrobial activity. The antitumor effect of NF00659s in mice and the mode of action are currently under investigation.

## References


1) SUZUKI, K.; A. KUWAHARA, T. NISHIKIORI & T. NAKAGAWA: NF00659$A_1$, $A_2$, $A_3$, $B_1$ and $B_2$, novel antitumor antibiotics produced by *Aspergillus* sp. NF 00659. II. Structural elucidation. J. Antibiotics 50: 318～324, 1997
2) RAPER, K. B. & D. I. FENNELL: The Genus Aspergillus. The Williams & Wilkins Company, Baltimore, 1965
3) DOMSCH, K. H.; W. GAMS & T. H. ANDERSON: Compendium of Soil Fungi. Vol. 1, pp. 76～124, Academic Press, London, 1993
4) BARRON, G. L.: The Genera of Hypomycetes from Soil. The Williams & Wilkins Company, Baltimore, 1968
5) GUPTA, S.; S. B. KRASNOFF, J. A. A. RENWICK, D. W. ROBERTS, J. R. STEINER & J. CLARDY: Viridxins A and B: novel toxins from the fungus *Metarhizium flavoviride*. J. Org. Chem. 58: 1062～1067, 1993
6) ROVIROSA, J.; M. SEPULVWDA, E. QUEZADA & A. SAN-MARTIN: Isoepitandiol, a diterpenoid of *Stypopodium flabelliform* and the insecticidal activity of stypotriol, epitandiol and derivatives. Phytochemistry 31: 2679～2681, 1992
7) GROVE, J. F.; R. N. SPEAKE & G. WARD: Metabolic products of *Colletotrichum capsici*, isolation and characterization of acetyl colletotrichin and colletodiol. J. Chem. Soc. (C) 1966: 230～234
8) FOUCHER, B.; J. B. CHAPPELL & J. D. MCGIVAN: The effects of acylcolletotrichin on the mitochondrial respiratory chain. Biochem. J. 138: 415～423, 1974
9) HALESTRAP, A. P.: The pathway of electron flow through ubiquinol, cytochrome *c* oxidoreductase in the respiratory chain. Biochem. J. 204: 49～59, 1982
10) HATEFI, Y.; A. G. HAAVIK & D. E. GRIFFITHS: Studies on the electron transfer system. J. Biol. Chem. 237: 1681～1685, 1962