# Chlovalicin, a New Cytocidal Antibiotic Produced by *Sporothrix* sp. FO-4649

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

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Selective growth inhibition against IL-6 dependent cells was detected in fermentation extracts of a fungal strain FO-4649 which was characterized as *Sporothrix* species. An active metabolite (**1**) termed chlovalicin was isolated together with ovalicin and two other ovalicin derivatives (compounds **3** and **4**). Chlovalicin, a ovalicin derivative with a chlorinated methylene moiety at the C-1 position of the cyclohexane ring, dose-dependently inhibited the growth of IL-6 dependent MH60 cells ($IC_{50}$, 7.5 $\mu$M) in the presence of 0.2 U/ml IL-6 and, to a lesser extent, the growth of B16 melanoma cells ($IC_{50}$, 38 $\mu$M). Among the other three compounds, only ovalicin showed inhibitory activity ($IC_{50}$, 27 $\mu$M) against MH60 cells. These four compounds did not show any antimicrobial activity at a concentration of 1000 $\mu$g/ml.

In the course of a screening program for new types of antibiotics showing cytocidal activity, four structurally related compounds were discovered from the fermentation broth of *Sporothrix* sp. FO-4649, which was isolated from a soil sample. One appeared to be a new compound and was designated as chlovalicin (**1**), and the other compounds were identified as ovalicin (**2**) and its derivatives (**3**, **4**)[1,2] (Fig. 1). The present paper outlines taxonomic studies of the producing strain, and the production, isolation and biological activity of the new compound, chlovalicin.

## Materials and Methods

### General Experimental Procedures

The fungal strain FO-4649, isolated from a soil sample, was used for production of chlovalicin (**1**). DC-Alufolien Kieselgel $60F_{254}$ (Merck) was used for TLC analysis. HPLC was carried out using the HITACHI D-2000 system and an ODS packed column (Senshu Pak Pegasil ODS, 5 $\mu$m, i.d. 20 × 250 mm).

### Taxonomic Studies

The fungal strain FO-4649 was originally isolated from a soil sample. For identification of the fungi, potato-dextrose agar, malt extract agar, CZAPEK's agar and LCA (MIURA's medium) agar were used. Morphology was observed by microscopy (Olympus Vanox-S AH-2).

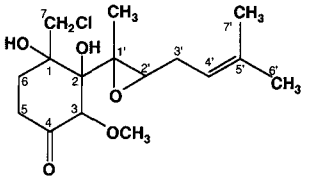
### Antimicrobial Activity

Antimicrobial activity was tested using paper disks (i.d. 6 mm, ADVANTEC). Bacteria were grown on Mueller-Hinton agar medium (Difco), and fungi and yeasts were grown on potato broth agar medium. Antimicrobial activity was observed after 24-hour incubation at 37°C for bacteria and after 48-hour incubation at 27°C for fungi.

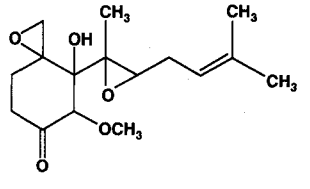
### Cell Lines

P388/S (mouse leukemia), P388/ADM (adriamycin resistant cell line), L929 (mouse fibroblast), HeLa (human cerical carcinoma), and B16 (mouse melanoma)

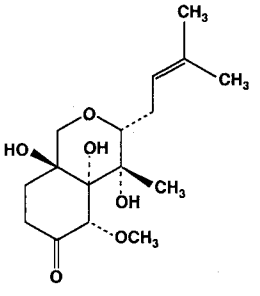
Fig. 1. Structures of chlovalicin (**1**), ovalicin (**2**) and related compounds (**3**, **4**).



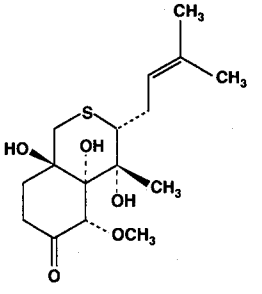
Chlovalicin (**1**)



Ovalicin (**2**)



Compound **3**



Compound **4**

cell lines were kindly provided by Dr. INABA, Cancer Research Institute. IL-6 dependent MH60 cells were kindly provided by Prof. OH-ISHI, Kitasato University.

Cytotoxic Activity Tests

Five cell lines were maintained in monolayers or in suspension in EAGLE's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) or RPMI 1640 medium supplemented with 10% FCS, respectively. To determine the cytotoxicity of chlovalicin, cells suspended in 200 $\mu$l of the medium ($1 \sim 2 \times 10^4$ cells/ml) were plated in a 96-well culture plate (Corning) and incubated for 24 hours at 37°C in a 5% $CO_2$-95% air atmosphere. To each well was added 5 $\mu$l of medium containing different concentrations of chlovalicin. After 72 hour incubation, the cell growth was measured colorimetrically by the tetrazolium salt (MTT) method[3].

MH60 cells ($5 \times 10^3$ cells) suspended in 100 $\mu$l of RPMI 1640 medium supplemented with 10% FCS were plated in a 96-well culture plate and incubated with 5 $\mu$l of test samples for 72 hours at 37°C in the presence of 0.02 U rhIL-6 (100 $\mu$l) in a 5% $CO_2$-95% air atmosphere. The cell growth was evaluated by the MTT method.

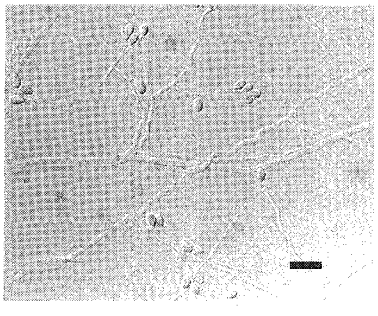
## Results and Discussion

Taxonomy of Producing Organism

The fungal strain FO-4649 was isolated from a soil sample. For identification of the fungi, potato-dextrose agar, malt-extract agar, CZAPEK's agar and LCA (MIURA's medium) agar were used. This strain grew moderately to form oyster white to light brownish gray colonies with a diameter of 20 ~ 40 mm after inoculation for 7 days at 25°C. The colony surface was floccose on various agar media. The reverse color was pale yellow to grayish white. Morphological observation was done under a microscope (Olympus Vanox-S AH-2). When the strain FO-4649 was grown on potato-dextrose agar at 25°C for 7 days, the conidia were formed by blastoconidia from direct substrate hyphae and sympodioconidia from conidiophores. Conidia were obovoid or obpyriform, smooth, hyaline to slightly colored, unicellular, $5 \sim 12 \times 3 \sim 6$ $\mu$m in diameter. The temperature permitting growth of the strain was 12 to 35°C, and optimum temperature for growth was 15 to 30°C. A light micrograph of this strain is shown in Fig. 2.

Fig. 2. Photomicrograph of strain FO-4649 on potato-dextrose agar.

Bar represents 20 $\mu$m.



From the above characteristics, strain FO-4649 was identified as a member of the genus *Sporothrix*[4,5], and named *Sporothrix* sp. FO-4649. It was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology Japan, as FERM P-14796.

Fermentation

The stock culture of the producing organism was inoculated into a test tube (20 × 200 mm) containing 10 ml of a seed medium [glucose 2%, yeast extract 0.2%, polypeptone 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, $KH_2PO_4$ 0.1% and agar 0.1% (adjusted to pH 6.0 before sterilization)]. The test tube was incubated at 27°C for 72 hours on a reciprocal shaker. The resulting culture (20 × 10 ml) was transferred to 100 ml of production medium (100 × 100 ml) consisting of glucose 1.0%, tryptone 0.5%, yeast extract 0.3%, maltose 0.3%, and agar 0.1%, (adjusted to pH 6.5 before sterilization) in a 500-ml Erlenmeyer flasks, and the fermentation was carried out at 27°C for 6 days with an agitation rate of 200 rpm. Detection of the chlovalicin in the fermentation broth was followed by growth inhibition on the IL-6 responsive cell system. After 6 days of fermentation, the amount of chlovalicin in the broth filtrate reached a maximum (0.35 $\mu$g/ml).

Extraction and Isolation of Active Compounds

The culture broth (10 liters) of *Sporothrix* sp. FO-4649 was filtered, the resulting supernatant was extracted with EtOAc (10 liters × 2) and then the organic layer was dried with $Na_2SO_4$ anhydride. The combined EtOAc layers were concentrated under reduced pressure to give a brown oil (1.75 g). The oily residue was applied to silica gel column chromatography (E. Merck, Kieselgel 60, 70 ~ 230 mesh). The materials were eluted with a step wise gradient of $CHCl_3$-$CH_3OH$ (100 : 0, 100 : 1, 50 : 1, 20 : 1, 10 : 1, each 500 ml). Active fractions ($CHCl_3$-$CH_3OH$, 50 : 1) were concentrated *in vacuo* to yield an oily material (100 mg).

Further purification of chlovalicin was carried out by HPLC using a preparative ODS column (PEGASIL ODS (i.d. 20 × 250 mm); Senshu Science Co. LTD., Japan; solvent: 60% aq $CH_3CN$: UV 210 nm, flow rate 7 ml/minute) to give a colorless oil (30.2 mg) (mixture of

Fig. 3. Effect of chlovalicin (1) on the growth of various mammalian cells.

●: MH60 (IL-6 dependent), ▲: P388C, △: P388/ADM, ○: L929, ■: HeLa, □: B16.

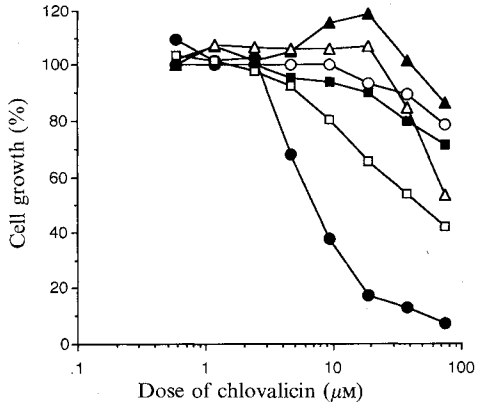
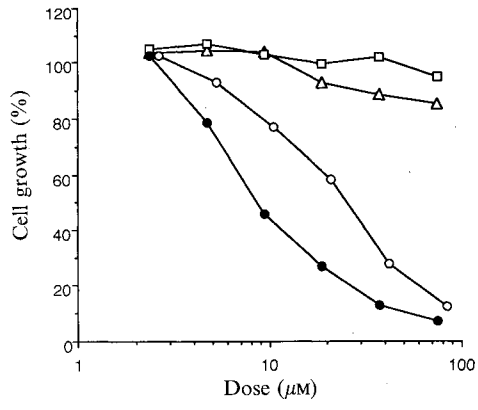



Fig. 4. Effect of chlovalicin and other compounds on the growth of IL-6-dependent MH60 cells.

●: Chlovalicin (1), ○: ovalicin (2), △: compound 3, □: compound 4.




chlovalicin and its derivatives). Final purification was carried out by HPLC using a preparative ODS column (PEGASIL ODS H-5251 (i.d. 20×250 mm); Senshu Science Co. LTD., Japan; solvent: 50% aq $CH_3CN$: UV 210 nm, flow rate 7 ml/minute). Pure chlovalicin (**1**) (3.5 mg), ovalicin (**2**) (12.3 mg), compound **3** (2.5 mg) and compound **4** (2.6 mg) were obtained.

Structures of chlovalicin and the related compounds are shown in Fig. 1. Studies on the structural determination of chlovalicin will be reported in a separate paper.

## Biological Activity

Growth inhibitory activity of chlovalicin was examined using various mammalian cells *in vitro* (Fig. 3). The antibiotic markedly inhibited growth of IL-6 dependent MH60 cells in a dose dependent manner. The $IC_{50}$ values for MH60 and B16 melanoma were 5.7 μM and 38 μM, respectively. While the $IC_{50}$ values for P388/S leukemia, P388 leukemia/ADM, HeLa S3 carcinoma and L929 fibroblast were all more than 75 μM.

The cytotoxicities of chlovalicin, ovalicin and other related compounds against IL-6 dependent MH60 cells were further examined. MH60 cells were incubated with chlovalicin, ovalicin, compound **3**, or compound **4** for 72 hours in the presence of 0.2 U/ml of IL-6. As shown in Fig. 4, chlovalicin showed higher activity about 3 times of ovalicin ($IC_{50}$, 27 μM) but compound **3** and compound **4** were not active.

These four antibiotics showed no antimicrobial activities at a concentration of 1000 μg/ml against *Staphylococcus aureus* KB34 (FDA 209p), *Micrococcus luteus* KB40 (PCI 1001), *Bacillus subtilis* KB27 (PCI 219), *Mycobacterium smegmatis* KB46 (ATCC 607), *Escherichia coli* KB 8 (NIHJ), *Pseudomonas aeruginosa* KB 105 (P-3), *Xanthomonas oryzae* KB 88, *Candida albicans* KF1, *Saccharomyces sake* KF26, *Aspergillus niger* KF 103 (ATCC-6725), *Piricularia oryzae* KF 180, *Mucor racemosus* KF 223, *Clostridium perfringens* KB 129, *Bacteroides fragilis* KB 169, and *Acholeplasma laidlawii* PG-8.

Chlovalicin dose-dependently inhibited the growth of IL-6 dependent MH60 cells ($IC_{50}$, 7.5 μM) in the presence of IL-6. MH60 cells seemed the most sensitive among these cells lines, so it is not clear whether chlovalicin selectively inhibits the IL-6 activity . Further examinations are necessary to clarify the mechanism of chlovalicin and the specificity for other cytokines.

It has been suggested that abnormal IL-6 production is closely related to the progression of cancer cachexia[6~8] and hormone-dependent hypercalcemia[9,10], and to the development of multiple myeloma[11,12] and rheumatoid arthritis[13,14]. Only a few substances are known to suppress IL-6 activity, *i.e.*, suramin[15] and monoclonal antibodies[16]. Therefore, IL-6 inhibitors will be useful in studying its role in disease.

Besides our present report concerning the inhibitory effect of chlovalicin on IL-6-dependent cell growth, recently, a new activity of chlovalicin as IgE production inhibitor was reported[17]. Therefore, chlovalicin is expected to be a new type anti-allergic drug possessing the inhibitory effects to both IgE production and IL-6 activities.

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