

## STUDIES ON WF-3161, A NEW ANTITUMOR ANTIBIOTIC

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WF-3161 is an antitumor antibiotic produced by a strain of fungus, *Petriella guttulata*. The antibiotic was purified by solvent extraction and a combination of silica gel and reverse phase column chromatography. The chemical structure of the antibiotic ( $C_{31}H_{44}N_4O_6$ , mp 181~183°C) was found to be a cyclic tetrapeptide consisting of phenylalanine, leucine, pipercolinic acid and 2-amino-8-oxo-9,10-epoxydecanoic acid. WF-3161 inhibited the growth of *Trichophyton asteroides*. It prolonged survival period of mice bearing leukemia P-388 with a high therapeutic index.

In the course of our screening for new antitumor antibiotics one tentatively designated WF-3161 was isolated from the culture filtrate of *Petriella guttulata* No. 3161, from a soil sample collected at Kamakura City, Kanagawa Prefecture. This paper deals with taxonomic studies on the producing strain, the production and isolation procedures, and physicochemical and biological properties of WF-3161.

### Methods

#### Fermentation

The growth of *Petriella guttulata* No. 3161 on mature slant cultures was used to inoculate eight 500-ml flasks containing 100 ml each of sterile seed medium consisting of 2% corn starch, 0.5% glucose, 1.0% soy bean meal, 1% dried yeast and 1.0% corn steep liquor (pH 6.2). The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) for 3 days at 30°C. The content of the flasks was used to inoculate 160 liters of fermentation medium in a stainless steel fermentor. The composition of the medium was as follows: 1.5% soluble starch, 0.25% cotton seed meal, 0.25% gluten meal, 0.25% wheat germ, 0.25% dried yeast and 0.2%  $CaCO_3$ . Fermentation was allowed to proceed for 3 days at temperature of 30°C, with air flow of 160 liters per minute and agitation of 250 rpm.

#### Detection of the Antibiotic

The antibiotic present in the fermentation broth or in preparations obtained during the purification process was detected by standard disc-agar diffusion assay using *Trichophyton asteroides* as a test organism.

#### Antitumor Activity

The antitumor activity of WF-3161 was determined in experimental tumor system on mice. Lymphocytic leukemia P-388 was implanted intraperitoneally into BDF<sub>1</sub> mice at an inoculum size of  $1 \times 10^8$  cells per mouse. Twenty-four hours after the implantation of tumor cells, graded doses of the antibiotic which was suspended in 0.5% methylcellulose were administered to mice intraperitoneally. Treatments were given on day 1, 2, 3, and 4. Adriamycin was comparatively tested as reference compound. Death or survival of the treated and non-treated (control) animals was recorded daily. T/C is the ratio of the median survival time of the test group (T) divided by that of the control group (C).

### Assay for the Antimicrobial Activity

Antimicrobial activity was determined by the serial agar dilution streak method. One loopful of an overnight culture of each test organism in an appropriate medium was streaked on plates containing graded a concentration of the drug and the minimal inhibitory concentration (MIC) was expressed in terms of  $\mu\text{g/ml}$  after incubation at  $37^\circ\text{C}$  for 18 hours for bacteria, and 48 to 72 hours at  $30^\circ\text{C}$  for yeast and fungi.

## Results and Discussion

### Taxonomic Studies on Strain No. 3161

Cultural characteristics of strain No. 3161 were as follows. On potato - dextrose agar, the colonies reached a diameter of  $6\sim 8$  cm in 4 weeks. Colonies were white at first, turning pale grayish white with the production of the *Sporotrichum* stage and then becoming blackish in patches with the formation of the *Graphium* coremia in clumps. Growth was irregularly zonate. Perithecia were produced abundantly, at first in the aerial hyphae, later in and on the agar, especially to the outside of the colonies in older cultures. Perithecia were black, carbonaceous and subglobose to pyriform on yeast phosphate soluble starch agar and oatmeal agar. Perithecia were  $100\sim 250$   $\mu\text{m}$  in diameter with papillate ostiole. Hairs formed a dense mat round the perithecium and these were usually short, septate, pigmented, smooth and about  $100\times 1.5$   $\mu\text{m}$  in diameter. Asci were avoid to broadly clavate, and  $18\sim 30\times 12\sim 18$   $\mu\text{m}$  in diameter (Plate 1). Asci were irregularly disposed and walls of asci remained till maturity of the ascospores. Ascospores were plano-convex to concavo-convex in one view, ellipsoid in other view, single-celled, red-brown in color,  $8.5\sim 10\times 4.5\sim 5.5\times 4.0\sim 4.5$   $\mu\text{m}$  in size (Plate 2). Ascospores contained a few large oil droplets and several smaller oil droplets and extruded at maturity as a ball at the mouth of the perithecium. Conidial stages were *Sporotrichum* (Plate 3) and *Graphium* (Plate 4). Both conidial phases were produced abundantly on potato - dextrose agar. *Sporotrichum* stage arised

Plate 1. Asci of strain No. 3161.

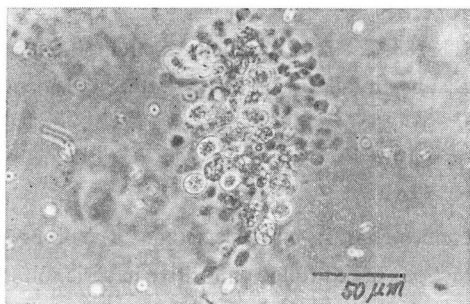


Plate 2. Ascospores of strain No. 3161.

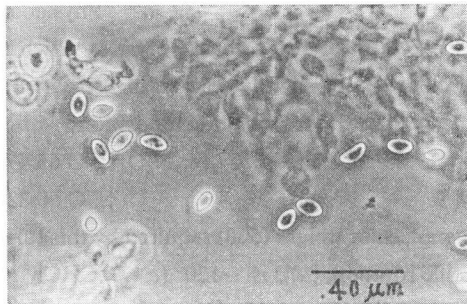


Plate 3. *Sporotrichum* stage of strain No. 3161.

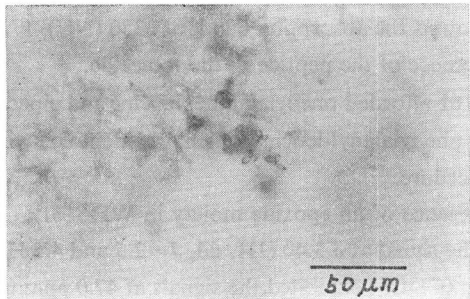


Plate 4. *Graphium* stage of strain No. 3161.



at first with the production of numerous conidiophores from the vegetative hyphae and aerial strands. Conidiophores were simple, hyaline, tapering, up to 30  $\mu\text{m}$  long, bearing a number of conidia at the apical end. Conidia readily displaced and were hyaline, variable in shape from pyriform to long cylindrical or wedge-shaped, 5~10 $\times$ 2~4  $\mu\text{m}$  in size. *Graphium* stage was evident. Vegetative hyphae grew at right angles to the substrate hyphae. Anastomose and corticate formed coremia which were dark-brown colored, up to 1 mm high, 6~25  $\mu\text{m}$  in diameter. Coremia were produced single or in clusters. Conidia were similar in shape and size to the *Sporotrichum* type but averaging slightly larger and more consistently long-cylindrical in shape.

Based on the taxonomical studies, strain No. 3161 was compared with known species described in the report by BARRON *et al.*<sup>1)</sup> As the result, strain No. 3161 showed a close resemblance to *Petriella guttulata* and *Petriella sordida* in conidial and cultural characters. But *P. sordida* differs in having an extremely hairy, papillate perithecium and distinctive ascospores.

From the above-mentioned informations, strain No. 3161 is considered to be *Petriella guttulata*.

#### Isolation of WF-3161

Fermentation broth (160 liters) was filtered with the aid of filter aid (Radiolite). The filtrate was concentrated *in vacuo* to 10 liters, and was extracted twice with 10 liters of ethyl acetate after acidification (pH 2.0) with 6 N HCl. The extract was concentrated *in vacuo* and the crude oily matter obtained was applied to a 1 liter silica gel column. The column was washed with 3 liters of *n*-hexane and 2 liters of a mixture of *n*-hexane - ethyl acetate (1:1), and eluted with ethyl acetate. The fractions containing WF-3161 were combined and concentrated to dryness. The dry residue was dissolved in 50 ml of methanol and applied to a column packed with 1.4 liters NS gel (Nihon Seimitsu Kagaku Co.). When the column was developed with 1.5 to 2.0 liters of methanol, the antibiotic was eluted. After concentration of the active fraction *in vacuo* the crude solid obtained was crystallized from hot ethanol yielding 420 mg of the antibiotic as colorless needles.

#### Physicochemical Properties

WF-3161 forms colorless crystals which melt at 181~183°C. It is soluble in methanol, ethanol and chloroform, slightly soluble in benzene and hexane, substantially insoluble in water. Elemental analysis gave the following composition:

*Anal.* Calcd. for  $\text{C}_{31}\text{H}_{44}\text{N}_4\text{O}_8$ : C 65.47, H 7.80, N 9.85.

Found: C 65.33, H 7.99, N 9.59.

The molecular weight (568) required by this formula was confirmed by its mass spectrum. The observed specific rotation,  $[\alpha]_D^{25}$  is  $-120^\circ$  ( $c$  1.0,  $\text{CHCl}_3$ ). Color reactions are as follows: positive in ceric sulfate, sodium thiosulfate and iodine, negative in ferric chloride and ninhydrin. The UV spectrum showed end absorption. The  $R_f$  value in thin-layer chromatography on a silica gel plate (Kiesel gel 60 F254, Merck) is shown in Table 1. Its IR spectrum (Fig. 1) showed the absorption bands at 3350 (NH), 1710 (CO), 1655 (CO) and 1635 (CO)  $\text{cm}^{-1}$  suggesting the presence of the peptide in the molecule.

Acid hydrolysis (6 N HCl, 110°C, 15 hours) of WF-3161 afforded phenylalanine, leucine and pipercolinic acid established as their dansyl derivatives, and also phenylalanyl-leucine which was identified with L-phenylalanyl-L-leucine prepared by a conventional procedure.

A positive sodium thiosulfate test supported the presence of an epoxide moiety in WF-3161. In addition,  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ ) (Fig. 2) revealed the signal at  $\delta$  3.40 (1H, dd,  $J=2.5$  and 4.5 Hz) due to the epoxide methine proton.  $^{13}\text{C}$  NMR spectrum ( $\text{CDCl}_3$ ) exhibited the signals at 47.0 ppm (d)

Fig. 1. IR spectrum of WF-3161 (KBr).

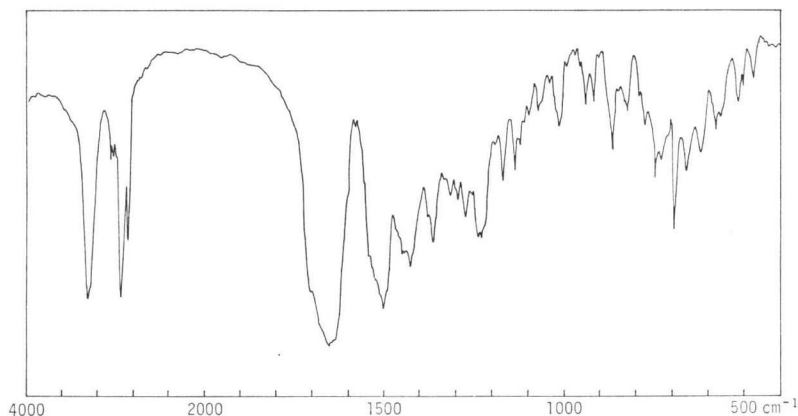
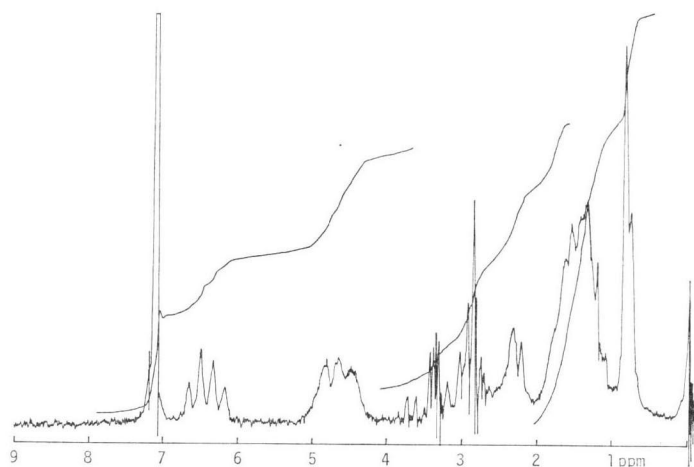
Fig. 2.  $^1\text{H}$  NMR spectrum of WF-3161 ( $\text{CDCl}_3$ ).

Table 1. Chromatographic properties of WF-3161.

System	Rf
TLC, silica gel (Merck)	
Ethyl acetate	0.55
Chloroform - methanol (10: 1)	0.78

and 46.4 ppm (t) attributable to the carbons of the epoxide ring attached to the carbonyl group (208.1 ppm). Further, the  $^1\text{H}$  NMR spectrum of WF-3161 exhibited signals at  $\delta$  0.83 (6H, d,  $J=5$  Hz) due to secondary methyl groups of leucine, at  $\delta$  4.2~5.1 (5H, m) assigned to four  $\alpha$ -methine protons of amino acids (phenylalanine, leucine, pipercolinic acid and 2-amino-8-oxo-9,10-epoxydecanoic acid) and a proton of a methylene of pipercolinic acid, and at  $\delta$  7.13 (5H, s) for a phenyl group of a phenylalanine unit. In the  $^1\text{H}$  NMR spectrum a signal pattern of WF-3161 for methylene and methine protons in the region of  $\delta$  2~5 was found to closely resemble that of Cyl-2 except for aromatic methoxyl protons. Cyl-2 is a plant growth regulator which was isolated by TAMURA *et al.*<sup>2)</sup> from a culture filtrate of *Cylindrocladium scoparium*. The structure was deduced by HIROTA *et al.*<sup>3)</sup> as shown in Fig. 3. The mass spectrum of WF-3161 revealed peaks at  $m/z$  568 ( $\text{M}^+$ ), 442, 399, 329, 170, 120, 86 and 84 (base). The fragmentation pattern in the mass spectrum of WF-3161 resembled that of Cyl-2.<sup>4)</sup>

On the basis of these results WF-3161 has structure as shown in Fig. 4.

Fig. 3. Structure of Cyl-2.

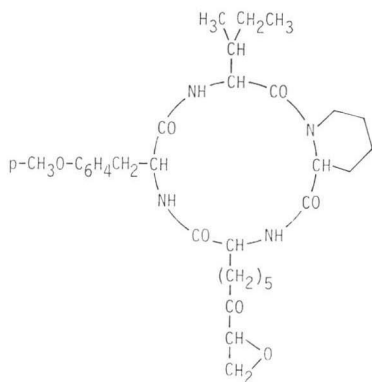


Fig. 4. Structure of WF-3161.

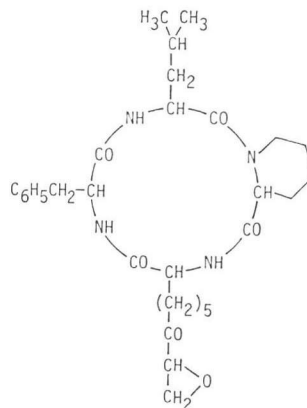


Fig. 5. Structure of chlamydocin.

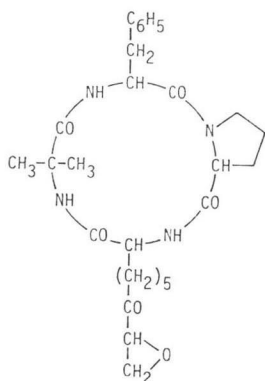
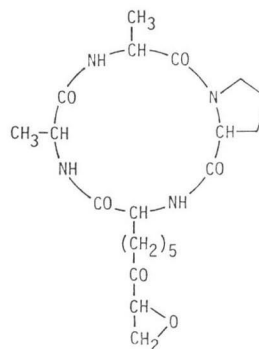


Fig. 6. Structure of HC-toxin.



Structurally related antibiotics, chlamydocin<sup>5,6)</sup> (Fig. 5) and HC-toxin<sup>7)</sup> (Fig. 6) were isolated from cultures of fungal strains. It is very interesting that these cyclic peptides have a unique amino acid, 2-amino-8-oxo-9,10-epoxy-decanoic acid in their molecule.

#### Biological Characteristics

The antimicrobial spectrum of WF-3161 is shown in Table 2. The antibiotic showed activity against *Trichophyton asteroides*, but was devoid of any significant antimicrobial activity against other microorganisms tested.

Antitumor activity of WF-3161 on P-388 leukemia is shown in Table 3. Mice that were implanted with  $1 \times 10^6$  P-388 cells intraperitoneally, 24 hours later received various doses of WF-3161 daily for 4 days after the inoculation. The administration of WF-3161, 12.5~100 mg/kg/day, daily for 4 days prolonged the survival period. The administration of 100 mg/kg/day daily for 4 days caused reduction in body weight.

Table 2. Antimicrobial properties of WF-3161.

Organism	MIC ( $\mu$ g/ml)
<i>Staphylococcus aureus</i> 209 P	>1,000
<i>Bacillus subtilis</i> ATCC 6633	>1,000
<i>Escherichia coli</i> NIHJ JC-2	>1,000
<i>Aspergillus niger</i>	>1,000
<i>Penicillium chrysogenum</i>	>1,000
<i>Candida albicans</i>	>1,000
<i>Saccharomyces cerevisiae</i>	>1,000
<i>Trichophyton asteroides</i>	3

Agar dilution method (Nutrient agar for bacteria, Sabouraud dextrose agar for fungi and yeast).

Table 3. Antitumor activity of WF-3161 against leukemia P-388.

Material	Treatment	Dose (mg/kg/day)	MST (days)	T/C (%)	Survivors (Day 5)
WF-3161	q.d. 1→4	100	17.5	175	10 / 10
		50	14.1	141	10 / 10
		25	15.0	150	10 / 10
		12.5	13.3	133	10 / 10
		6.3	10.0	100	10 / 10
Adriamycin	q.d. 1→4	0.2	18.5	185	10 / 10
Control		0.5% methyl- cellulose	10.0	—	10 / 10

Tumor inoculum:  $1 \times 10^8$  ascites cells implanted i.p. on day 0.

Host : 7-week-old BDF<sub>1</sub> mice (♀).

Evaluation : MST=median survival time.

T/C % : MST treated/MST control  $\times 100$ .

Survivors : Number of survivor/treated.

Criteria : T/C $\geq 125$  considered significant antitumor effect.

The LD<sub>50</sub> of WF-3161 was estimated to be 200 mg/kg in ddY mice by i.p. route.

#### Acknowledgment

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