

MYROCIN C, A NEW DITERPENE ANTITUMOR ANTIBIOTIC
FROM *MYROTHECIUM VERRUCARIA*

I. TAXONOMY OF THE PRODUCING STRAIN,
FERMENTATION, ISOLATION AND
BIOLOGICAL PROPERTIES

MASAHIRA NAKAGAWA, YUAN-HSUN HSU, AKIRA HIROTA,
SHOJI SHIMA and MITSURU NAKAYAMA*

Department of Agricultural Chemistry, College of Agriculture,
University of Osaka Prefecture,
Sakai, Osaka 591, Japan

(Received for publication July 6, 1988)

A new diterpene antitumor antibiotic, myrocin C, has been isolated from the culture filtrate of a soil fungus, *Myrothecium verrucaria* strain No. 55. The antibiotic was effective against Gram-positive bacteria, fungi and yeasts, and prolonged the life span of mice bearing Ehrlich ascites carcinoma.

During the course of our screening program for novel antitumor antibiotics, we have recently isolated a new pimarane-type antitumor antibiotic named myrocin C (**1**) from the culture filtrate of a soil fungus, *Myrothecium verrucaria* strain No. 55, in addition to three known macrocyclic trichothecenes, verrucarins A, roridins D and A. In continuation of our work on the metabolites of the same strain, we have found one more new antibiotic, a myrocin C analogue, which was named myrocin B.¹⁾

The new antibiotic, myrocin C, showed antimicrobial activity against various Gram-positive bacteria, fungi and yeasts, and *in vivo* inhibitory activity against Ehrlich ascites carcinoma. In this paper, we report the taxonomy of the producing organism and the fermentation, isolation and biological properties of **1**. The structure determination of **1** as well as its physico-chemical properties will be described in the following paper.²⁾

Taxonomy

The myrocin C producing organism, strain No. 55, was freshly isolated from a soil sample collected at Takatsuki-city in Osaka Prefecture. The strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Tsukuba, Japan, under accession No. FERM P-8880.

Morphological Characteristics

Morphological observations of the strain grown on potato - glucose agar were made under an optical microscope.

From the facts that strain No. 55 had no sexual reproductive organ and did not form any pycnidia and acervuli, it was decided that the microorganism belongs to the Hyphomycetes.

Conidiophores which directly arose from the knot of basal hyphae, the sporodochium, were colorless, septate and hyaline. Cells were 10.0~14.0×1.5~2.0 μm in size. Generally they were re-

peatedly branched, forming 2~4 branches at each interval. Each branch bore phialides at the terminus. The conidia fusiform, single celled, green but appeared dark olive to dark green in mass, cells $2.0 \sim 3.0 \times 7.0 \sim 8.0 \mu\text{m}$ (Fig. 1).

Phialides were 3~6 in a whorl, hyaline, columnar in shape, $1.5 \sim 2.0 \times 11.0 \sim 15.0 \mu\text{m}$ in size. The hyphae were somewhat branched, smooth and septate, thin-walled, colorless, $1.5 \times 3.0 \mu\text{m}$.

Cultural Characteristics

The characteristics of the culture on potato - glucose agar and malt extract agar were determined after 2 weeks of incubation at 30°C.

The colonies on potato - glucose agar grew fairly well. The diameter reached about 40~50 mm and the surface was white and flocculent, while the reverse was brownish yellow to light brown. The growth on malt extract agar was slightly repressed and conidial formation was very limited. The surface of the colonies was white and flocculent and the reverse was light yellow to rose-buff.

Growth Conditions

The organism can grow at a temperature range of 8 to 40°C on potato - glucose agar and the optimum temperature for growth was around 30°C.

The taxonomic characterization mentioned above indicated that strain No. 55 belongs to *M. verrucaria* referring to the descriptions of UDAGAWA *et al.*⁹⁾

Fermentation

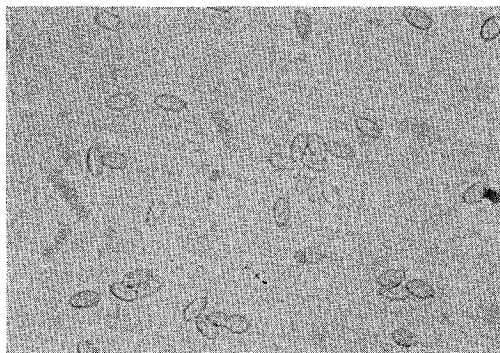
A few loopfuls from a well sporulated slant culture were inoculated into a test tube (18×180 mm) containing 6 ml of seed medium having the composition of glucose 40 g, soybean meal 2.5 g, yeast extract 1 g, KH_2PO_4 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1 g, NaCl 0.5 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.5 g, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 2.0 mg and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 3.0 mg in 1 liter of deionized water. The pH of the medium was adjusted to 5.5 with 2 N HCl before sterilization. The test tube was incubated at 30°C for 48 hours with reciprocal shaking (100 strokes/minute). The resulting vegetative culture (6 ml) was used for inoculating a 5-liter Erlenmeyer flask containing 1.8 liters of the same medium to start antibiotics production. Fermentation was carried out at 30°C for 120 hours on a reciprocal shaker with 5 cm-radius at 200 rpm.

Time course of antibiotics production was followed by the paper-disc agar diffusion method using *Bacillus subtilis* IFO 12210 and *Candida albicans* IFO 0579 as test organisms. The production of the antibiotics reached maximum after 120 hours of fermentation.

Isolation

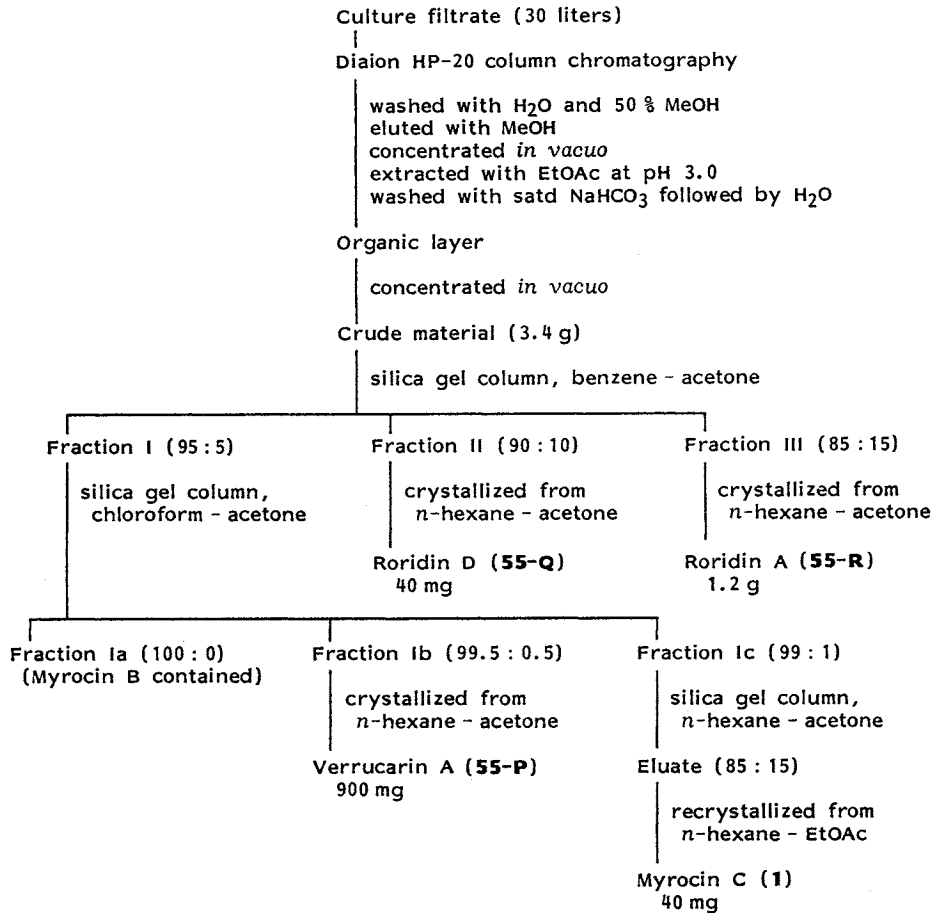
The isolation procedure for the antibiotics is outlined in Scheme 1. Fermentation beer (30 liters) was mixed with Celite (2 kg, Manville) and filtered. The filtrate was passed through a column of Diaion HP-20 (500 ml, Mitsubishi Chemical Industries Limited), washed with 4.5 liters of H_2O followed by 3 liters of 50% aqueous methanol and the active principles were eluted with 3 liters of methanol. The eluate was concentrated into a small volume and extracted with ethyl acetate at pH 3.0.

Fig. 1. Conidia of strain No. 55 on potato - glucose agar (incubated for 14 days at 30°C).



The organism was observed with an optical microscope ($\times 600$).

Scheme 1. Isolation and purification of each antibiotic.



The extract was successively washed with saturated aqueous sodium bicarbonate and water, and was then evaporated to dryness to give 3.4 g of oily residue. The oily residue was found to contain four active components which were monitored by TLC on a silica gel plate (Kieselgel 60 GF₂₅₄, Merck) using a solvent system composed of benzene - ethyl acetate - methanol (85:10:5). Components were detected by UV lamp at 254 nm and assayed by the agar diffusion method using *B. subtilis* IFO 12210 and/or *C. albicans* IFO 0579 as test organisms. This oily residue was consequently subjected to a silica gel chromatography column (175 g, Fusica gel, BM820MW) packed with benzene and eluted with a gradient system of benzene - acetone (100:0~80:20).

Three active fractions (I, II and III) were obtained.

Fractions II (90:10) and III (85:15), active against *C. albicans* IFO 0579 and not *B. subtilis* IFO 12210, were concentrated to dryness *in vacuo* and crystallized from the *n*-hexane - acetone (3:1 for the former compound and 2.5:1 for the latter compound) to give 40 mg of colorless needles (compound **55-Q**) and 1.2 g of colorless prisms (compound **55-R**), respectively.

The fraction I (95:5) containing **1** and other antimicrobial substances was rechromatographed on a silica gel column (90 g, Fusica gel, BM820MW) and eluted with a solvent system of chloroform - acetone to give three active fractions (Ia, Ib and Ic).

Table 1. Antimicrobial spectrum of myrocin C (1).

Test organism	MIC ^a (μ g/ml)
<i>Escherichia coli</i> K-12 IFO 3301	>200
<i>Pseudomonas putida</i> IFO 3738	>200
<i>Serratia marcescens</i> IFO 12648	>200
<i>Alcaligenes faecalis</i> IFO 12699	>200
<i>Bacillus subtilis</i> IFO 12210	50
<i>B. brevis</i> IFO 3331	50
<i>Arthrobacter globiformis</i> IFO 12140	100
<i>Corynebacterium xerosis</i> IFO 12684	100
<i>Micrococcus roseus</i> IFO 3768	50
<i>Staphylococcus aureus</i> IFO 3060	50
<i>Aspergillus niger</i> IFO 4416	100
<i>Penicillium chrysogenum</i> IFO 4897	50
<i>Fusarium oxysporum</i> IFO 5880	100
<i>Saccharomyces cerevisiae</i> IFO 0251	50
<i>Candida albicans</i> IFO 0579	50

^a The MIC values were determined by a serial agar dilution method with bouillon agar for bacteria and CZAPEK's agar for fungi and yeasts.

was isolated as previously reported.¹⁾

The fraction 1b (99.5:0.5), active against *C. albicans* IFO 0579 and not *B. subtilis* IFO 12210, was concentrated *in vacuo* and recrystallized from *n*-hexane - acetone (1:1) to give 900 mg of colorless needles (compound 55-P).

Compounds 55-P, 55-Q and 55-R were identified to be verrucarins A, roridin D and roridin A on the basis of their spectroscopic data and physico-chemical properties,⁴⁾ which were identical with the results as reported by TAMM *et al.*⁵⁻⁸⁾

Fraction 1c, eluted with chloroform - acetone (99:1), was concentrated to dryness *in vacuo* followed by silica gel column chromatography (35 g, Fusica gel, BM820MW) using the solvent system of *n*-hexane - acetone (85:15) as eluent. Recrystallization from *n*-hexane - ethyl acetate (2:3) gave 40 mg of 1 as colorless needles.

Biological Properties

Antimicrobial Activity

The antimicrobial activity of 1 was determined by the conventional agar dilution method using bouillon agar for Gram-positive and Gram-negative bacteria, and CZAPEK's agar for fungi and yeasts. The MIC (μ g/ml) was measured after incubation of each test organism at 30°C for 18 hours for bacteria except for *Micrococcus roseus* IFO 3768, fungi and yeasts, in which MIC was determined after 40 hours of incubation at 30°C. The results are given in Table 1.

1 had inhibitory activity mainly against Gram-positive bacteria, and also active against some fungi and yeasts including *Aspergillus niger* IFO 4416 and *C. albicans* IFO 0579. On the other hand, 1 exhibited no activity against all the Gram-negative bacteria tested.

Antitumor Activity

The antitumor activity of 1 was examined in Ehrlich ascites tumor-bearing mice. Ehrlich ascites

Table 2. Antitumor activity of myrocin C (1) against mouse Ehrlich ascites carcinoma.

Dose (mg/kg/day)	T/C (%) ^a
Myrocin C 8.5	21
4.3	33
1.6	130
1.0	106
0.5	88
Mitomycin C 1.1	247

^a Prolongation rate (T/C, %) = mean survival period of treated/mean survival period of controls. 2×10^9 of Ehrlich ascites carcinoma cells were inoculated intraperitoneally into each IRC mouse (female, 5 weeks old) and myrocin C (1) was administered intraperitoneally once a day for 40 days starting on the day of the tumor cell inoculation.

The fraction 1a (100:0), active against both *C. albicans* IFO 0579 and *B. subtilis* IFO 12210, was further purified by silica gel column chromatography and finally a new antibiotic myrocin B

carcinoma cells were inoculated intraperitoneally into IRC mice (female, 5 weeks old) at an inoculum size of 2×10^6 cells per mouse. From the day after tumor inoculation (day 1), graded doses of **1** were administered to mice intraperitoneally once a day for 40 days. Five mice were used in each test group. Antitumor activity was evaluated by the mean survival time of a group and was expressed by the T/C value (%).

The antitumor activity of **1** against Ehrlich ascites carcinoma was compared with that of mitomycin C (MMC). As shown in Table 2, **1** had low but significant therapeutic effect on mouse against Ehrlich ascites carcinoma with the prolongation rate (T/C) of 130% at a dose of 1.6 mg/kg/day.

Discussion

Myrococin C (**1**) had a wide antimicrobial spectrum of rather weak activity against Gram-positive bacteria, fungi and yeasts, and moderately increased the life span of Ehrlich ascites tumor-bearing mice. As will be described in the next paper,²⁾ **1** is a novel pentacyclic diterpenoid possessing a unique cyclopropyl group and structurally related to LL-S491 β and γ , two pimarane diterpenes produced by *Aspergillus chevalieri*.⁹⁾ The latter has recently been reisolated as a phytotoxin from the culture filtrate of a parasitic fungus, *Phomopsis* sp.¹⁰⁾ To the best of our knowledge, however, this is the first report of a pimarane-type diterpene being discovered as the secondary metabolite from the genus *Myrothecium*.

Acknowledgments

The authors are grateful to Professor S. FUKUSHIMA of Shizuoka College of Pharmacy for antitumor studies.

References

- 1) HSU, Y.-H.; M. NAKAGAWA, A. HIROTA, S. SHIMA & M. NAKAYAMA: Structure of myrococin B, a new diterpene antibiotic produced by *Myrothecium verrucaria*. Agric. Biol. Chem. 52: 1305~1307, 1988
- 2) HSU, Y.-H.; A. HIROTA, S. SHIMA, M. NAKAGAWA, T. ADACHI, H. NOZAKI & M. NAKAYAMA: Myrococin C, a new diterpene antitumor antibiotic from *Myrothecium verrucaria*. II. Physico-chemical properties and structure determination. J. Antibiotics 42: 223~229, 1989
- 3) UDAGAWA, S.; K. TSUBAKI, G. HORIE, K. KIMURA, K. MINOURA, M. YAMAZAKI, T. YOKOYAMA & S. WATANABE: Kinrui Zukan. II. pp. 1071~1072, Kodansha, Tokyo, 1978
- 4) COLE, R. J. & R. H. COX: Handbook of Toxic Fungal Metabolites. pp. 230~238, 247~252, Academic Press, New York, 1981
- 5) HARRI, E.; W. LOEFFLER, H. P. SIGG, H. STAHELIN, CH. STOLL, CH. TAMM & D. WIESINGER: Über die Verrucarine und Roridine, eine Gruppe von cytostatish hochwirksamen Antibiotica aus *Myrothecium* arten. Helv. Chim. Acta 45: 839~853, 1962
- 6) TAMM, CH. & J. GUTZWILLER: Über die Verrucarine und Roridine. Partialstruktur von Verrucarin A. Helv. Chim. Acta 45: 1726~1731, 1962
- 7) GUTZWILLER, J. & CH. TAMM: Über die Struktur von Verrucarin A. Helv. Chim. Acta 48: 157~176, 1965
- 8) BOHNER, B. & CH. TAMM: Die Konstitution von Roridin A. Helv. Chim. Acta 49: 2527~2546, 1966
- 9) ELLESTAD, G. A.; M. P. KUNSTMANN, P. MIRANDO & G. O. MORTON: Structures of fungal diterpene antibiotics LL-S491 β and γ . J. Am. Chem. Soc. 94: 6206~6208, 1972
- 10) MOROOKA, N.; T. TATSUNO, H. TSUNODA, K. KOBAYASHI & T. SAKURAI: Chemical and toxicological studies of the phytotoxin, 6 α ,7 β ,9 α -trihydroxy-8(14),15-isopimaradiene-20,6- γ -lactone, produced by a parasitic fungus, *Phomopsis* sp., in wilting pine trees. Agric. Biol. Chem. 50: 2003~2007, 1986