DORRIGOCINS: NOVEL ANTIFUNGAL ANTIBIOTICS THAT CHANGE THE MORPHOLOGY OF *ras*-TRANSFORMED NIH/3T3 CELLS TO THAT OF NORMAL CELLS

I. TAXONOMY OF THE PRODUCING ORGANISM, FERMENTATION AND BIOLOGICAL ACTIVITY

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The dorrigocins are new secondary metabolites produced by submerged fermentation of a streptomycete which was isolated from a soil sample collected in Australia. The dorrigocins show moderate antifungal activity and reverse the morphology of *ras*-transformed NIH/3T3 cells from a transformed phenotype to a normal one. The producing culture was identified as *Streptomyces platensis* subsp. *rosaceus* strain AB1981F-75.

The dorrigocins are novel glutarimide antifungal antibiotics discovered in the fermentation broth and mycelium of *Streptomyces platensis* subsp. *rosaceus* strain AB 1981F-75. This paper describes the taxonomy of the producing strain and the fermentation, antifungal and antitumor activity of the dorrigocins. The isolation, structural elucidation, biological properties and mechanism of action of these compounds are described in accompanying publication[1,2].

Materials and Methods

Microorganisms

Strain AB 1981F-75 was isolated from soil collected on the Dorrigo plateau in New South Wales, Australia. A subculture of the microorganism was deposited at the National Center for Agricultural Utilization Research, United States Department of Agriculture, 1815 North University Street, Peoria, Illinois, 61604 U.S.A. The accession code at this depository is NRRL 18993. *Streptomyces platensis* ATCC 13865 was obtained from the American Type Culture Collection (ATCC). The fungi used for bioassays were from the stock culture collection in our laboratory, the ATCC and the National Center for Agricultural Utilization Research (NRRL). The tumor cell lines were obtained from the ATCC.

Taxonomic Studies

Most of the cultural and physiological characteristics of strain AB 1981F-75 were examined using the methods and media described by SHIRLING and GOTTLIEB[3], WAKSMAN[4] and GORDON *et al.*[5]. Incubation for cultural characteristics was at 28°C for 21 days. The techniques of KORN-WENDISCH and KUTZNER[6] were used to observed reduction of nitrate and tolerance to NaCl. Cultures were tested for H_2S production on ISP-6 medium slants by method 2 of SMIBERT and KRIEG[7]. Analysis of the whole-cell diaminopimelic acid isomer was done by the method of HASEGAWA *et al.*[8]. Color names were assigned to the mycelial and diffusible pigments on the basis of the Inter-Society Color Council-National Bureau of Standards

(ISCC-NBS) Centroid Color Charts.

Fermentation

Dorrigocins were produced by fermentation in a 22-liter LH Fermentation stainless steel stirred vessel. Frozen vegatative mycelium of *S. platensis* subsp. *rosaceus* was used at 0.5% to inoculate 600 ml of seed medium in a 2-liter Erlenmeyer flask. The seed medium consisted of glucose monohydrate 1.5%, soy flour 1.5%, yeast extract 0.1%, NaCl 0.1% and $CaCO_3$ 0.1%. The seed flasks were incubated for 72 hours at 28°C on a rotary shaker operating at 225 rpm (5.6 cm stroke). The fermentor was charged with 15 liters of a medium consisting of glucose monohydrate 2%, soy flour 2% yeast extract 0.1% and $CaCO_3$ 0.2%. Amberlite XAD-16 resin was added to the medium at 10%. The medium-resin mixture was sterilized in the fermentor at 121°C for 1 hour. The fermentor was inoculated at 5% with the seed flask growth. During fermentation the temperature was controlled at 28°C. The agitation rate was 250 rpm and the air flow was 0.7 v/v/minute. Antifoam (XFO 371) was added initially at 0.01% and was available on demand. The fermentation was terminated at 136 hours.

Fermentation Analyses

The fermentation was monitored on-line for changes in pH and dissolved oxygen. The fermentor was sampled daily to evaluate growth, glucose consumption and production of biological activity. Growth was estimated from the packed cell volume obtained after centrifugation in a conical tube at $600 \times g$ for 20 minutes. Glucose concentration was analyzed enzymatically with glucose oxidase (Sigma Diagnostics kit No. 510-A). Fermentation samples were prepared for the bioassays by adding to 10 ml of whole culture broth an additional 2 ml of XAD-16 resin and mixing for 2.5 hours at room temperature. The mixture was centrifuged at $600 \times g$ for 40 minutes. The supernatant was discarded and the mixture of mycelium and resin was washed with 10 ml of water. The resin was eluted with 5 ml methanol (1.5 hours contact). The solvent was removed under vacuum. The residue was partitioned between methanol, water and chloroform 1 : 1 : 1. The chloroform layer was collected and the chloroform removed under vacuum. The resulting residue was suspended in 0.5 ml dimethyl sulfoxide for the bioassays. To assess accumulation of antifungal activity, the prepared samples (20 μl) were applied to 6.35 mm filter disks and placed on Sabouraud dextrose agar plates seeded with *Aspergillus niger* ATCC 16404. After incubation of the plates at 32°C for 24 hours, the diameter of the inhibition zones was measured. The reversion of *ras*-transformed cells by the fermentation samples was monitored by the morphology test described by KADAM & MCALPINE[2].

In Vitro Antitumor and Antifungal Activities

In vitro cytotoxicity tests were performed using the MTT colorimetric assay[9] with murine leukemia P388, human lung sarcoma A549 (ATCC CCL 185), human adenocarcinoma HT-29 (ATCC HTB 38) and murine melanoma B16F10 cell lines. The cells were seeded at 2.5×10^2 cells per well in a 96-well micro tray and incubated for 72 hours at 37°C in a humidified atmosphere at 5% CO_2 after addition of test compounds. All cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum.

Minimal inhibitory concentrations (MICs) were determined by an agar dilution method. The test compounds were serially diluted in MeOH, and 0.2 ml portions were mixed with 20 ml of molten, cooled Sabouraud dextrose agar (Difco). Yeast cell inoculum was prepared by growing cultures on Sabouraud dextrose agar for 18 hours at 32°C and suspending the cells in phosphate buffered saline. Filamentous fungi were grown under the same conditions for 4 days to obtain spores. The inoculum level for all cultures was adjusted to 10^4 cells using a Petroff-Hauser cell counter. The glutarimide antifungal compound cycloheximide was used as a control. Inoculated test plates were incubated at 32°C and examined after 20 hours.

Results

Taxonomy

The vegetative mycelium of strain AB 1981F-75 is well developed and does not fragment. The aerial

Table 1. Cultural characteristics of strain AB 1981F-75 and *Streptomyces platensis* ATCC 13865.

Medium		AB 1981F-75	ATCC 13865
Yeast extract - malt extract agar (ISP 2)	G[a]:	Abundant	Abundant
	AM:	Pinkish white (9)[b] and purplish gray (233); sporulated	Brownish gray (64); sporulated
	R:	Dark reddish brown (44)	Deep yellowish brown (75)
	SP:	Light reddish brown (42)	Light reddish brown (42)
Oatmeal agar (ISP 3)	G:	Moderate	Moderate
	AM:	Dark yellowish brown (78) and purplish gray (233) with brownish black (65) moist specks; sporulated	Dark yellowish brown (78); moist; sporulated
	R:	Dark orange yellow (72)	Light grayish reddish brown (45)
	SP:	Light yellowish brown (76)	Light yellowish brown (76)
Inorganic salts - starch agar (ISP 4)	G:	Abundant	Abundant
	AM:	Pinkish white (9) and purplish gray (233); sporulated	Light grayish reddish brown (45) to brownish black (65); sporulated
	R:	Grayish reddish brown (46)	Grayish brown (61)
	SP:	Light yellowish brown (76)	Light yellowish brown (76)
Glycerol - asparagine agar (ISP 5)	G:	Poor	Poor
	AM:	Pinkish white (9); not sporulated	Pinkish white (9); not sporulated
	R:	Yellowish white (92)	Yellowish white (92)
	SP:	Absent	Absent
Peptone - yeast extract - iron agar (ISP 6)	G:	Moderate	Moderate
	AM:	Purplish white (231)	Purplish white (231)
	R:	Light orange yellow (70)	Light orange yellow (70)
	SP:	Absent	Absent
Tyrosine agar (ISP 7)	G:	Moderate	Moderate
	AM:	Purplish white (231)	Purplish white (231)
	R:	Yellowish white (92)	Yellowish white (92)
	SP:	Absent	Absent
BENNETT's agar	G:	Abundant	Abundant
	AM:	Purplish gray (233)	Light grayish reddish brown (45)
	R:	Dark reddish brown (44)	Moderate reddish brown (43)
	SP:	Light yellowish brown (76)	Light yellowish brown (76)
Sucrose nitrate agar	G:	Moderate	Moderate
	AM:	Pale pink (7) to grayish red (19)	Dark reddish gray (22)
	R:	Pale pink (7) and to grayish red (19)	Light grayish reddish brown (45)
	SP:	Pale orange yellow (73)	Absent
Glucose - asparagine agar	G:	Moderate	Moderate
	AM:	Grayish pink (8)	Grayish pink (8)
	R:	Moderate brown (58)	Pale orange yellow (73) to moderate brown (58)
	SP:	Absent	Absent
Nutrient agar	G:	Moderate	Moderate
	AM:	Pale pink (7)	Dark orange yellow (72)
	R:	Pale yellow (89)	Pale orange yellow (73)
	SP:	Absent	Absent

[a] Abbreviations: G, growth; AM, aerial mycelium; R, reverse; SP, soluble pigment.

[b] Color and number in parenthesis follow the color standard in KELLY, K. L. and D. B. JUDD: ISCC-NBS Color-Name Charts Illustrated with Centroid Colors. U.S. Dept. of Comm. Suppl. to Cir. 553, Washington, D.C. (1976).

mycelium is monopodially branched and forms clusters of tightly coiled spore chains. The spore chains usually have 4 to 5 turns and contain more than 10 spores. This morphology is seen on yeast extract - malt extract, oatmeal and inorganic salts - starch agars. No spores were observed on glycerol - asparagine agar.

Fig. 1. Scanning electron micrograph of spore chains of strain AB 1981F-75 grown on ISP-4 agar for 10 days at 28°C.

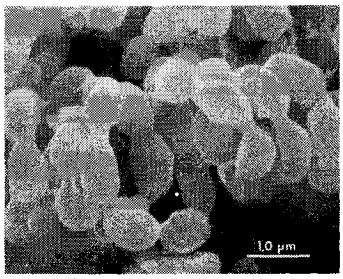


Fig. 2. Scanning electron micrograph of coalesced masses formed in the mycelial mat of strain AB 1981F-75 grown on ISP-4 agar for 10 days at 28°C.

Bar represents 10 μm.

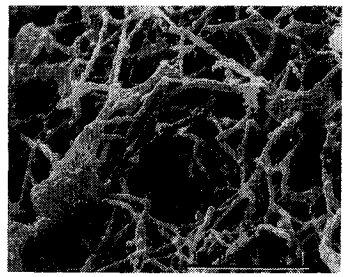


Table 2. Utilization of carbon sources by strain AB 1981F-75 and *Streptomyces platensis* ATCC 13865.

Carbon source	AB 1981F-75	ATCC 13865	Carbon source	AB 1981F-75	ATCC 13865
Adonitol	−	−	D-Melezitose	++	++
L-Arabinose	+	+	D-Melibiose	++	++
Dulcitol	−	−	D-Raffinose	++	++
D-Fructose	++	++	L-Rhamnose	−	−
D-Galactose	++	++	Salicin	+	+
D-Glucose	++	++	Sucrose	++	++
meso-Inositol	++	++	Xylitol	++	++
D-Mannitol	++	++	D-Xylose	++	+

++: Good utilization, +: poor utilization, −: did not utilize.

Table 3. Physiological characteristics of strain AB 1981F-75 and *Streptomyces platensis* ATCC 13865.

Text	AB 1981F-75	ATCC 13865
Decomposition of:		
Casein	+	+
Hypoxanthine	+	+
L-Tyrosine	+	+
Xanthine	+	+
Adenine	+	+
Starch hydrolysis	+	+
Nitrate reduction	+	+
Milk peptonilzation	+	−
Milk coagulation	−	−
Gelatin (4%) liquefaction	+	−
H_2S production	−	−
Temperature range for growth (on ISP 2)	10~32°C; no growth at 4 and 37°C	15~37°C; no growth at 10 and 42°C
NaCl tolerance	Growth at 10% but not 13%	Growth at 10% but not 13%
Melanoid pigment:		
Peptone-yeast extract-iron agar (ISP-6)	−	−
Tyrosine agar (ISP 7)	−	−
Tryptone-yeast extract broth (ISP 1)	−	−

Scanning electron microscopy showed that the spores have a smooth surface, are elliptical to half-moon or crescent-shaped (Fig. 1) and typically measure 0.7 μm × 1.0 μm. The culture forms unusual coalesced masses that appear to be tangles of mycelia on some media (Fig. 2). No sporangia or zoospores were observed.

The cultural characteristics of strain AB 1981F-75 on various media are given in Table 1. The aerial mycelium was light pink to purplish gray on yeast extract-malt extract and inorganic salts-starch agars. Brown to black hygroscopic patches were formed on oatmeal agar. The reverse was dark brown modified by red on ISP-2 and ISP-4, dark orange yellow on ISP-3 and yellowish white on ISP-5 agar. The reverse mycelium pigments were not pH sensitive. A reddish brown soluble pigment, which was also not pH sensitive, was produced on yeast extract-malt extract agar. Light yellow orange or brown pigments were formed on several other media. Melanoid pigments were not produced in tryptone-yeast extract broth or peptone-yeast extract-iron and tyrosine agar agars.

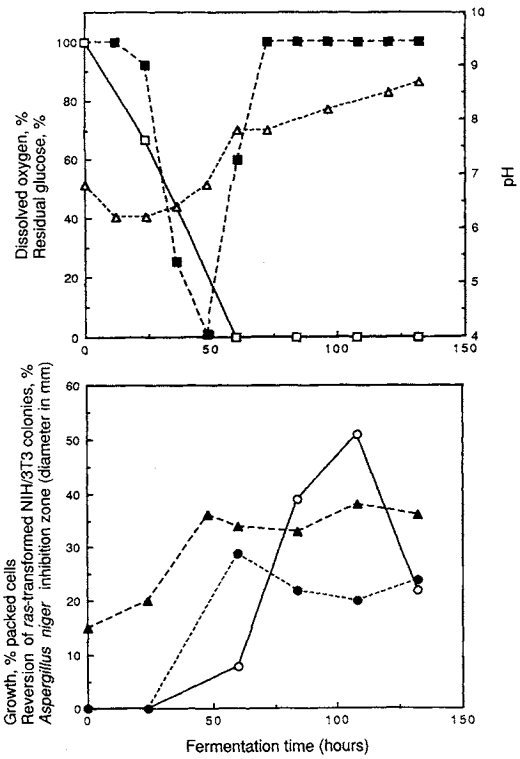
Analysis of whole cell hydrolysates of strain AB 1981F-75 revealed the presence of major amounts of LL-diaminopimelic acid.

The utilization of carbon sources and physiological properties of strain AB 1981F-75 are give in Tables 2 and 3, respectively.

The morphology and cell wall analysis of strain AB 1981F-75 clearly places it in the genus *Streptomyces*. A review of the published descriptions[10~15] of members of the genus indicated that this isolate was most similar to *S. platensis*. A direct laboratory comparison of AB 1981F-75 and the type strain, *S. platensis* ATCC 13865 (NRRL 2364), was then performed. The results are in Tables 1, 2 and 3.

Fig. 3. Time course of dorrigocin fermentation in a 22-liter fermentor.

■ Dissolved oxygen, □ residual glucose, △ pH, ▲ growth, ○ reversion of *ras*-transformed cells, ● *A. niger* inhibition.



Fermentation

The time course of the fermentation is shown in Fig. 3. The packed cell volumes and the dissolved oxygen profile indicate that growth occurs during the first 48 hours and ends as the glucose is depleted. The production of antifungal activity parallels growth but the major production of the reversion of *ras*-transformed cells occurs later, during stationary phase. While the dorrigocins have inhibitory activity

Table 4. Antitumor activity of dorrigocins A and B.

Cell lines	IC_{50} (μg/ml)		
	A	B	Adriamycin
A549 (Lung carcinoma)	>100	60.5	0.011
HT-29 (Adenocarcinoma)	>100	33.7	0.111
B16F10 (Melanoma)	94.6	48.3	0.0018
P388 (Leukemia)	81.5	16.8	0.0011

Table 5. Agar dilution *in vitro* antifungal activity of dorrigocins A and B.

Microorganism	MIC (μg/ml)		
	A	B	Cycloheximide
Candida albicans ATCC 10231	>100	>100	>100
C. albicans 579A	>100	>100	>100
C. albicans ATCC 38247	>100	>100	>100
C. tropicalis NRRL-Y-112	>100	>100	0.4
Torulopsis glabrata ATCC 15545	>100	>100	0.4
Saccharomyces cereviseae GS1-36	>100	>100	<0.05
Aspergillus niger ATCC 16404	25	12.5	1.6
A. fumigatus ATCC 26430	25	12.5	1.6
A. flavus NRRL 6541	>100	100	50
A. parasiticus NRRL 13539	>100	100	25
Fusarium moniliforme AARC 0397	100	50	0.8
F. solani AARC 0353	25	25	0.8

against *A. niger* (Table 4), the dissimilarity of the accumulation rates of bioactivity against *A. niger* and the reversion of *ras*-transformed cells indicate that other antifungal substances are produced by *S. platensis* subsp. *rosaceus*.

Antitumor and Antifungal Activity

The dorrigocins have low activity against the tumor cell lines shown in Table 4. The MICs of dorrigocins A and B for several fungi are listed in Table 5. The dorrigocins have moderate activity against some *Aspergillus* and *Fusarium* species but none against yeasts. Dorrigocin B is more potent than dorrigocin A against both fungi and tumor cells.

Discussion

Based on the *Streptomyces* keys presented in the 8th Edition of BERGEY's Manual[14], strain AB 1981F-75 belongs either to the red or gray color series in the subsets that form spiral spore chains (*Spira*), do not produce melanoid pigments and have smooth-walled spores. There were no cultures in the red series that resembled strain AB 1981F-75. Morphological and physiological characteristics of AB 1981F-75 indicated, however, that it had many features in common with the *S. hygroscopicus*-like cultures in the gray series. TRESNER *et al.*[16] and DIETZ[17] divided hygroscopic cultures that form tightly coiled spore chains from this gray series subset into two groups. TRESNER *et al.*[16] considered cultures that had short, cylindrical, phalangiform spores to be members of the species *S. hygroscopicus*. *Streptomyces* with elliptical spores were assigned either to *S. platensis* or other species. They also noted that these cultures could be differentiated based on physiological characteristics. Phalangiform spored cultures usually utilized L-rhamnose and reduce nitrates and could tolerate 7% but not 10% NaCl. Strains with elliptical spores did not utilized L-rhamnose, could not reduce nitrate but could tolerate 10% NaCl. DIETZ[17] also designated cultures with phalangiform spores as *S. hygroscopicus* and provided another feature that could be used to separate these hygroscopic cultures. She pointed out that her transmission and scanning electron microscopy studies showed that the spores of cultures in this group actually had a rugose surface while the elliptical spores were indeed smooth. She placed *Streptomyces* with elliptical spores, including strains of *S. platensis*, into a new species called "*S. neohygroscopicus*".

Based on the criteria presented in these publications it was obvious that strain AB 1981F-75 is similar to *S. platensis* Tresner and Backus[18]. Tables 2 and 3 show that both strains have the same carbon utilization pattern and differ only slightly in growth temperature range, milk peptonization and gelatin liquefaction among the physiological characteristics examined. Although TRESNER *et al.* found that elliptical spored cultures did not reduce nitrate, this test has been performed many different ways with varying results. In

this study, both cultures gave a positive reaction when tested by the KORN-WENDISCH-KUTZNER[5] technique. A striking difference between the two strains is the color of the aerial mycelia on yeast extract - malt extract, inorganic salts - starch and sucrose nitrate agar media. The aerial mycelia of *S. platensis* ATCC 13865 is dark brown or gray while strain AB 1981F-75 forms much lighter pink and purplish gray pigments on these media. Stran AB 1981F-75 has been designated *Streptomyces platensis* subsp. *rosaceus* subsp. nov. (L. adj. *rosaceus* pink) in recognition of the pink color of its aerial mycelium.

The dorrigocins are similar structurally to another antifungal antibiotic, S-632-C[19]. Interestingly, however, these compounds differ in their antifungal activity. Whereas the dorrigocins inhibit aspergilli and have no activity against yeasts, S-632-C has moderate activity against *Saccharomyces* spp. but none against *Aspergillus* spp.

The fermentation yield of the dorrigocins is quite low. A specific assay was not developed, but production of these compounds appeared to be stabilized by the presence of resin during the fermentation.

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