5-*N*-ACETYLARDEEMIN, A NOVEL HETEROCYCLIC COMPOUND WHICH REVERSES MULTIPLE DRUG RESISTANCE IN TUMOR CELLS

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

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The ardeemins are a new family of secondary metabolites produced by submerged fermentation of a fungus which was isolated from a soil sample collected in Brazil. Based on taxonomic studies, the producing culture was identified as *Aspergillus fischeri* var. *brasiliensis* strain AB 1826M-35. 5-*N*-Acetylardeemin potentiated the cytotoxicity of the anticancer agent vinblastine in multidrug resistant human tumor cells.

Multidrug resistance (MDR) is characterized by the development of resistance to several structurally unrelated anticancer agents and is a major cause of failure of cancer chemotherapy. The appearance of a specific glycoprotein, P-170, is generally associated with resistant cells[1]. P-170 is a membrane associated glycoprotein thought to actively export cytotoxic compounds such as anthracyclines and Vinca alkaloids from resistant tumor cells[2]. Although several compounds are known to reverse MDR, none are used clinically because of adverse side effects[3]. In the course of screening for modulators of MDR, we discovered a family of novel compounds which we have called ardeemins. 5-*N*-Acetylardeemin appears to be the most efficacious of these compounds in the reversal of MDR. The compounds are produced in the fermentation broth of *Aspergillus fischeri* var. *brasiliensis* strain AB 1826M-35. This paper describes the taxonomy and the fermentation of the producing microorganism and the biological activity of ardeemin and 5-*N*-acetylardeemin. The isolation and structural elucidation of these and other congeners are described in an accompanying publication[4].

Materials and Methods

Microorganisms

Strain AB 1826M-35 was isolated from soil collected in Brazil. 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 18896. *A. fischeri* ATCC 1020 and *A. fischeri* var. *spinosus* ATCC 16898 were obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A.

Taxonomic Studies

The producing culture was identified following the classification procedures described by RAPER and FENNEL[5]. Morphological observations were made after incubation for 14 days at 24°C and 37°C. Color names and numbers are from the Inter-Society Color Council-National Bureau of Standards

(ISCC-NBS) Centroid Color Charts[6].

Fermentation Studies

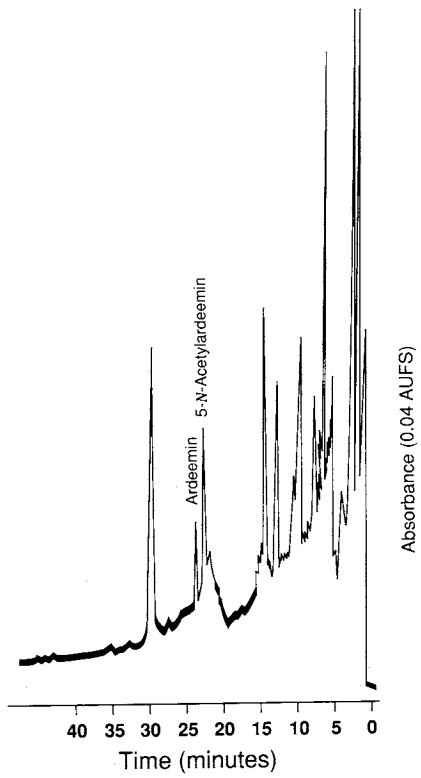
Inoculum for the production of ardeemins was initiated from vegetative mycelium, preserved at −75°C. Two-liter seed flasks containing 600 ml of medium were inoculated at 0.4%. The seed medium consisted of corn steep powder (Roquette Corporation) 0.25%, glucose monohydrate 1%, oat flour (National Oats Company) 1%, tomato paste (Contadina Foods, Inc.) 4%, $CaCl_2 \cdot 2H_2O$ 1% and 10 ml per liter of a trace element solution. The trace element solution contained $FeSO_4 \cdot 7H_2O$ 0.1%, $MnCl_2 \cdot 4H_2O$ 0.1%, $CuCl_2 \cdot 2H_2O$ 0.0025%, $CaCl_2 \cdot 2H_2O$ 0.01%, H_3BO_3 0.056%, $(NH_4)_6MoO_2 \cdot 4H_2O$ 0.0019% and $ZnSO_4 \cdot 7H_2O$ 0.02% in distilled water. The seed flasks were incubated for 72 hours at 28°C on a rotary shaker operating at 225 rpm (5.08 cm stroke).

The fermentation medium consisted of lactose 3%, glucose monohydrate 2%, ammonium acetate 0.5%, sodium acetate 0.41%, KH_2PO_4 0.2%, $FeSO_4 \cdot 7H_2O$ 0.02%, $CuSO_4 \cdot 5H_2O$ 0.0005% and $ZnSO_4 \cdot 7H_2O$ 0.002%. The medium was prepared in distilled water and adjusted to pH 6.2 prior to sterilization. The lactose and glucose were sterilized separately. The fermentation was conducted in a New Brunswick 150-liter fermentor charged to a volume of 100 liters. Sterilization was at 121°C and 1.05 kg/cm^2 for 1 hour. Antifoam (XFO-371, Ivanhoe Chemical Co.) was added at 0.01% initially and was available on demand. The fermentor operation was carried out for nine days at 22°C with an agitation rate of 250 rpm, an aeration rate of 0.7 vol/vol/minute and a head pressure of 0.35 kg/cm^2. Temperature was controlled at 22°C. After nine days of incubation 48 liters of broth were harvested. The pH, not controlled during fermentation, was 8.3.

Fermentation Analyses

The fermentation was monitored daily for growth, pH, carbohydrate consumption and accumulation of ardeemins. Growth was evaluated by determining packed cell volume after centrifugation in a conical tube at 600 × g for 30 minutes. Carbohydrate was determined by DUBOIS' phenol-sulfuric acid method[7]. The production of ardeemins was determined by HPLC. Samples were prepared for HPLC by adjusting 10 ml of whole fermentation broth to pH 7, adding 3 ml acetone and extracting 3 times with 5 ml ethyl acetate using a 20 ml Mixxor extractor (Thomas Scientific). The ethyl acetate extracts were combined, solvent was removed by evaporation in a Speedvac concentrator and the residue was reconstituted to 1 ml in CH_3CN. This was a 10-fold concentration of the original broth sample. In the final two days of the fermentation a 4-fold concentration was also chromatographed due to the increased potency of 5-*N*-acetylardeemin. Analytical chromatograms were obtained with the following components of a Perkin-Elmer HPLC system: an ISS 100 autosampler for injections, a Series 4 pump operating at 1 ml/minute for solvent delivery. The UV response was measured at 270 nm with an LC-85 spectrophotometric detector. An example of a chromatogram of an extract from a typical fermentation is shown in Fig. 1.

Fig. 1. HPLC chromatogram of the extract of the sample taken on day 7.



Column (Waters): μ-Bondapak phenyl (3.9 × 300 mm) 5 μ. Mobile phase (nonlinear gradient program): MeOH - H_2O (30 : 70) to (70 : 30) and then to (90 : 10) over two 15 minutes intervals (convex curve 0.5 in each case), followed by a 15 minutes hold. Injected 10 μl of the 10-fold preparation.

In Vitro Activity

Human tumor cell lines, KB3-1 (normal) and KBV-1 (multidrug resistant), were both obtained from ATCC and maintained on RPMI 1640 with 10% fetal bovine serum (FBS). Cells were routinely cultured in 25 cm^2 flasks containing 8 ml of medium. KBV-1 cells were cultured in vinblastine (1 μg/ml) each week to maintain a high level of drug resistance. *In vitro* cell cytotoxicity was determined according to the procedure described by BRADLEY *et al.*[8]. Briefly, cells were seeded at 1×10^3 cells per ml of medium per well in 24 well microplates containing 2-fold serially diluted cytotoxic compound in 23 wells. The remaining well contained only cells. 5-*N*-Acetylardeemin was tested at 10 μM in the plate containing KBV-1 cells and serially diluted vinblastine. After a 7~10 days incubation, at 37°C in 4% CO_2 in humidified air, the cell colonies were stained with methylene blue. Typically, wells containing a low level of cytotoxic compound showed a high density of colonies similar to the control well. Inhibition of cell growth was noted over a narrow range of concentration of the cytotoxic compound. Resistance and reversal were calculated by comparing the concentration of the cytotoxic compound required to reduce plating efficiency to <10% of the control well in each cell line.

Results and Discussion

Taxonomy

Culture Identification

Colonies of strain AB 1826M-35 on Czapek Dox agar are cottony, white (263), produce a clear exudate and grow rapidly to 13~15 cm in diameter. The reverse is pale yellowish pink (31). Cleistothecia are absent on media prepared with 3 and 30% sucrose but are moderately produced on a 0.3% sucrose medium. Conidiophores are absent on 0.3 and 3% sucrose media, but moderate formation occurs with 30% sucrose and are more numerous at colony margins. The conidiophores measure 30~160 μm × 7~10 μm. Vesicles are 10~12 μm in diameter, flask shaped, smooth to delicately roughened and colorless. Conidial heads are 30~400 μm × 15~25 μm, columnar but diverging somewhat near the terminus. As shown in Fig. 2, sterigmata are uniseriate. They are crowded and bottle-shaped and fertile over the upper half of the conidial head. The conidia are smooth to slightly roughened, globose to subglobose in shades of grayish green (150) to light grayish olive (109) with age and are 1.4~1.7 μm × 1.7~2.0 μm in diameter.

On BLAKESLEE's malt extract agar colonies also grow rapidly to 13~15 cm in diameter. The aerial mycelium is felted, and there is a tough basal mycelium. The colonies are yellowish white (92) with a continuous layer of cleistothecia. The reverse is pale greenish yellow (104) with no exudate. Conidiophore

Fig. 2. Scanning electron micrograph of a conidial head of strain AB 1826M-35 grown on corn meal agar.

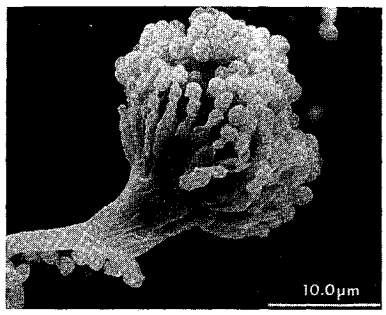


Fig. 3. Scanning electron micrograph of ascospores of strain AB 1826M-35 grown on corn meal agar.

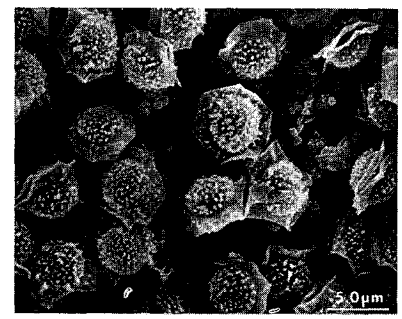


Table 1. Differential characteristics of *Aspergillus fischeri* var. *brasiliensis* strain AB 1826M-35, *A. fischeri* ATCC 1020 and *A. fischeri* var. *spinosus* ATCC 16898.

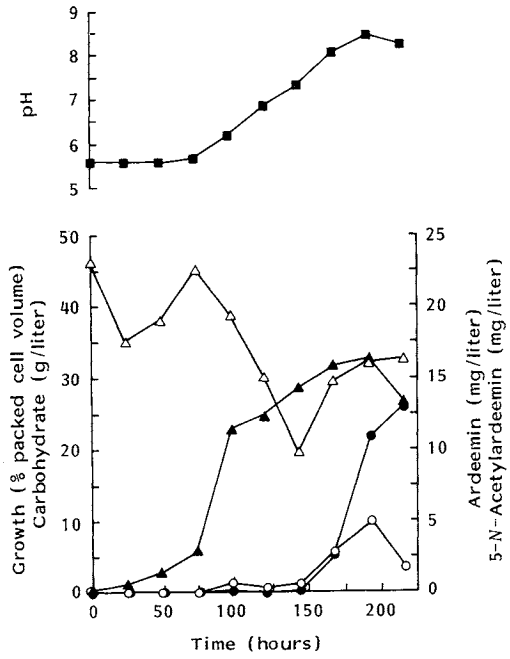
Culture	Colony color on malt extract agar (37°C)	Colony texture/ color on Czapek Dox agar (24°C)	Conidia formation		Ascospore ornamentation
			Malt extract agar (24°C)	Czapek Dox agar (24°C)	
Aspergillus fischeri var. *brasiliensis* AB 1826M-35	Yellowish white (92)[a]	Cottony, white (263)	Sparse	Absent	Spines
A. fischeri ATCC 1020	Olive gray (113)	Loose, cottony, white (263)	Abundant	Moderate	Smooth
A. fischeri var. *spinosus* ATCC 16898	Light grayish olive (109)	Felted to floccose, variable, pinkish white (9)	Moderate	Moderate	Spines

[a] Color and number in parenthesis follow ISCC-NBS Centroid Color Charts.

formation is sparse at 24°C and does not influence colony appearance. Conidiophores are more abundant at 37°C, and some are up to 450 μm long. Cleistothecia are produced within a cottony mass of mycelium. At 24°C they are globose to subglobose, 250~500 μm in diameter and yellowish white (92). Walls are thin and delicate, consisting of flattened mycelial cells. At 37°C cleistothecia are smaller (50~175 μm in diameter), globose or nearly so, delicate and are colored white (263) to yellowish white (92). Asci are 8~12 μm in diameter containing eight spores. Ascospores are uncolored and nearly globose. The spore bodies are 4.5~5 μm in diameter with two flexuous equatorial bands about 1 μm apart. In face view, bands are 6~8 sided polygons (Fig. 3). The convex surfaces of the spores are covered by spiny projections less than 0.5 μm in length. Structures are generally larger on malt extract than on Czapek Dox or potato dextrose agars.

Fig. 4. Time course of the fermentation of ardeemin and 5-*N*-acetylardeemin.

■ pH, ▲ growth, △ carbohydrate, ○ ardeemin, ● 5-*N*-acetylardeemin.



On potato dextrose agar colonies grow rapidly to 13~15 cm in diameter. They are white (263) with felted mycelia and produce a clear, pale yellow (89) exudate. The reverse is dark reddish gray (23) at colony center to light grayish brown (60) at the margins. Cleistothecia are white (263) to yellowish white (92). Cleistothecia formation is moderate and they range in size from 150 to 500 μm in diameter at 24°C but are smaller and more abundant at 37°C. Conidiophore production is very limited, and they are found primarily at colony margins.

Culture AB 1826M-35 produces conidia on swollen flask-shaped vesicles arising from characteristic foot cells indicative of the genus *Aspergillus*. Examination of published descriptions of *Aspergillus*

species[5,9] indicated that our isolate closely resembled *A. fischeri* and *A. fischeri* var. *spinosus*. A laboratory comparison of strain AB 1826M-35 and these two cultures indicated they were all, in fact, quite similar and differed in minor characteristics as indicated in Table 1. Because of its close similarity to *A. fischeri*, we consider strain AB 1826M-35 another variety of this culture. We have designated it *Aspergillus fischeri* var. *brasiliensis* by virtue of its place of origin. The teleomorphic phase of *A. fischeri* is *Neosartorya fischeri*[10]. The ascigerious state of strain AB1826M-35 is consistent with the description of the teleomorph.

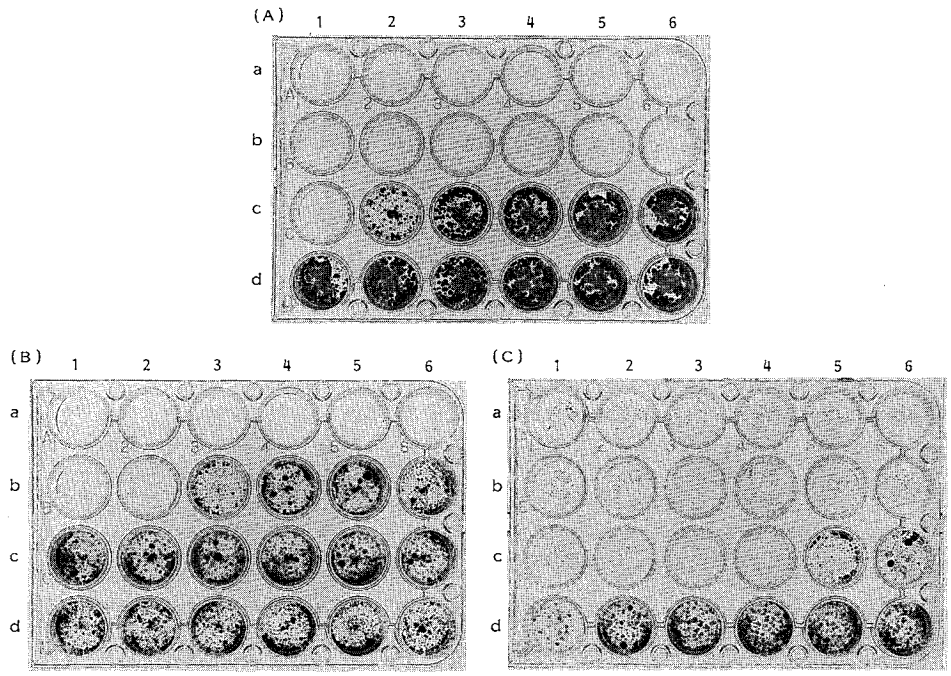
Fermentation

The time course of the fermentation is shown in Fig. 4. The yield of 5-*N*-acetylardeemin was 13 mg/liter at harvest, although the fermentation appears to have been terminated prior to the maximum yield. An examination of the curves for ardeemin and 5-*N*-acetylardeemin suggests that the latter compound may result from the acetylation of ardeemin. The intermittent increases in the carbohydrate concentration suggest that the culture is producing sugars or polysaccharides during the fermentation.

Biological Activity of 5-*N*-Acetylardeemin

Results of the *in vitro* drug sensitivity assay are shown in Fig. 5. The highest concentration of vinblastine sulfate, 1 μg/ml for KB3-1 cells and 50 μg/ml for KBV-1 was in wells a1 of each plate. In

Fig. 5. Drug sensitivity assay in 24-well tissue culture trays.



(A), Drug-sensitive KB3-1 cell line. (B), (C) drug-resistant KBV-1 cells in the absence and presence of 10 μM 5-*N*-acetylardeemin respectively. Two-fold serial dilutions of vinblastine sulfate were added in each well beginning from well a1 and continuing left to right in each row. The starting concentration of vinblastine sulfate in plate (A) was 1.0 μg/ml and 50 μg/ml in plates (B) and (C). Well d6 in each plate contained cells in growth medium without vinblastine sulfate except plate (C) where d6 had 10 μM 5-*N*-acetylardeemin.

plate C, all wells also contained 5-*N*-acetylardeemin. Serially diluted vinblastine inhibited the growth of KB3-1 cells to $<10\%$ of control at a concentration of 1.2×10^{-4} μg/ml (Fig. 5A). In plates containing KBV-1 cells similar inhibition occured at 2×10^{-1} μg/ml (Fig. 5B). The KBV-1 cells were therefore 1,600-fold resistant to vinblastine compared with KB3-1. In the presence of 10 μM 5-*N*-acetylardeemin, however, KBV-1 cells were sensitized significantly (Fig. 5C). Their resistance to vinblastine was reduced from 1,600-fold to 6-fold. Unlike human tumors which become refractory at $2\sim3$ fold drug resistance, the cell lines used for cytotoxicity tesiting are highly resistant. In *in vitro* experiments these cells are rarely fully sensitized[11]. Additionally, the continuous exposure to vinblastine in these cells may cause other alterations which could account for the low level of vinblastine resistance in chemosensitized cells. Compared with verapamil, a well-characterized modulator of MDR, 5-*N*-acetylardeemin was 10-fold more effective in chemosensitizing KBV-1 cells (data not shown). The biological activity data presented here suggests that 5-*N*-acetylardeemin may overcome MDR. Ardeemin itself did not reverse MDR in KBV-1 cells. Another ardeemin, 15b-β-hydroxy-5-*N*-acetylardeemin, was also isolated from fermentation broths in small quantities as noted in the accompanying publication[4]. Preliminary studies indicate that this compound also reverses MDR. None of the ardeemins mentioned here exhibited antimicrobial activity when tested against a broad spectrum of common human pathogenic fungi and bacteria.

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References

1) JULIANO, R. L. & V. LING: A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim. Biophys. Acta 455: 152~162, 1976
2) KARTNER, N.; J. R. RIORDAN & V. LING: Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. Science 221: 1285~1288, 1983
3) OZOLS, R. F.; R. E. CUNNION, R. W. KLECKER, T. C. HAMILTON, Y. OSTCHEGA, J. E. PARRILLO & R. C. YOUNG: Verapamil and adriamycin in the treatment of drug-resistant ovarian cancer patients. J. Clin. Oncol. 5: 641~647, 1987
4) HOCHLOWSKI, J. E.; M. M. MULLALLY, S. G. SPANTON, D. N. WHITTERN, P. HILL & J. B. MCALPINE: 5-*N*-Acetylardeemin, a novel heterocyclic compound which reverses multiple drug resistance in tumor cells. II. Isolation and elucidation of structures. J. Antibiotics 46: 380~386, 1993
5) RAPER, K. B. & D. I. FENNEL (*Ed.*): The Genus *Aspergillus*. The Williams & Wilkins Co., Baltimore, 1965
6) KELLY, K. L. & D. B. JUDD (*Ed.*): ISCC-NBS Color-name Charts Illustrated with Centroid-colors. U.S. Dept. of Comm. Suppl. to Cir. No. 553, Washington, D.C., 1976
7) DUBOIS, M.; K. A. GILLES, J. K. HAMILTON, P. A. REBERS & F. SMITH: Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350~356, 1956
8) BRADLEY, G.; M. NAIK & V. LING: P-glycoprotein expression in multidrug-resistant human ovarian carcinoma cell lines. Cancer Res. 49: 2790~2796, 1989
9) SAMSON, R. A.: A compilation of the Aspergilli described since 1965. *In* Studies in Mycology. No. 18. Centraalbureau voor Schimmelcultures, Baarn, 1979
10) MALLOCH, D. & R. F. CAIN: The Trichocomaceae (Ascomycetes): Synonyms in recent publications. Can. J. Bot. 51: 1647~1648, 1972
11) KADAM, S.; M. MAUS, J. PODDIG, S. SCHMIDT, R. RASMUSSEN, E. NOVOSAD, J. PLATTNER & J. MCALPINE: Reversal of multidrug resistance by two novel indole derivatives. Cancer Res. 52: 1~6, 1992