CALBISTRINS, NOVEL ANTIFUNGAL AGENTS PRODUCED BY *Penicillium restrictum*

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

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A novel antibiotic complex, named the calbistrins, has been discovered in the culture broth of a soil fungus. The producing organism, designated AB 1875C-28, was identified as a strain of *Penicillium restrictum*. Calbistrin A, the most potent of the 4-membered complex, has MICs of 0.78 μg/ml against *Candida albicans*. Only poor activity is observed against non-candida yeasts, filamentous fungi and bacteria.

The calbistrins are a new complex of compounds isolated from the culture broth of a soil microorganism, identified as a strain of *Penicillium restrictum* Gilman & Abbott. This paper describes the taxonomy of the producing organism, production of the complex by fermentation and the biological activity of the calbistrins. The isolation and structure elucidation are described in a companion paper[1].

Materials and Methods

Microorganisms

Strain AB 1875C-28 was isolated from soil collected in Brazil. A subculture of the microorganism was deposited at the National Center for Agricultural Utilization Research in Peoria, Illinois 61604, U.S.A., where it was assigned the accession number NRRL 18926. *Penicillium restrictum* ATCC 11257 and *Penicillium dimorphosporum* ATCC 22783 were obtained from the American Type Culture Collection (ATCC). Microorganisms used in the biological activity evaluation were obtained from the ATCC or the clinical culture collection in our laboratory.

Taxonomic Studies

The producing culture was identified following the classification procedures described by PITT[2]. Growth on tap water agar was included in the description. Morphological observations were made after incubation for 7 days at 25°C unless stated otherwise. Color names and numbers are from the Inter-Society Color Council-National Bureau of Standards (ISCC-NBS) Centroid Color Charts[3].

Fermentation

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 production medium consisted of glycerol 1.5%, molasses (Chicago Sweetners) 1.5%, peptone (Difco) 0.6%, yeast extract 0.15%, NaCl 3%, KH_2PO_4 0.06%, $MgSO_4 \cdot 7H_2O$ 0.5%, $CuSO_4 \cdot 5H_2O$ 0.0001% and $FeSO_4 \cdot 7H_2O$ 0.0003%.

Penicillium restrictum AB 1875C-28 was maintained at −70°C as frozen vegetative inoculum. Inoculum for fermentation was prepared by seeding 2-liter flasks containing 600 ml of the seed medium at 0.4% with stock vegetative mycelium. The seed flasks were incubated for 72 hours at 28°C on a rotary shaker operating at 225 rpm (5.08-cm stroke). The antibiotic was produced in a 150-liter New Brunswick fermenter charged with 100 liters of the production medium. The medium was prepared with distilled water. The pH was 5.5 after sterilization. The antifoam (XFO-371) was added at 0.01% initially and was available on demand during the fermentation. The temperature was controlled at 22°C. The agitation rate was 200 rpm, the air rate was 0.7 vol/vol/minute, and the head pressure was 0.35 kg/cm^2. Ninety liters of broth were harvested after seven days of fermentation.

Fermentation Analyses

Growth was evaluated as packed cell volume by centrifuging the fermentation broth in a graduated conical tube at $600 \times g$ for 20 minutes. Residual carbohydrate was monitored by DUBOIS' phenol-sulfuric acid method[4]. Glycerol was not analyzed. Calbistrins were determined by an agar diffusion assay with *Candida albicans* 579a grown in Yeast Nitrogen Base agar supplemented with 1% glucose (YNBG). The *Candida* assay plates were incubated at 32°C for 24 hours.

Biological Activity

Minimal inhibitory concentrations (MIC) were determined by microtiter broth dilution testing in Yeast Nitrogen Base (Difco) containing 0.05% glucose[5]. Frozen spore suspensions of filamentous fungi or growth from an overnight plate culture (yeast) were prepared in YNBG, and wells were inoculated to a final concentration of 5×10^4 cfu/ml. Plates were incubated at 35°C for 24~48 hours and the MICs defined as the lowest concentration of drug completely inhibiting visible growth.

The acute toxicity (LD_{50}) was determined in mice by intraperitoneal injection.

Results and Discussion

Taxonomy

Culture Identification

The formation of penicilli by the calbistrin producer distinguished it as a member of the genus *Penicillium*. For further identification, strain AB 1875C-28 was grown on the three media recommended by PITT[2] for characterization of *Penicillium* species. Because reproductive structures were not observed on any of these media, tap water agar was added for characterization of the penicilli. Descriptions of the culture on these media are as follows:

Colonies on CZAPEK yeast extract agar are white (263), floccose, raised, sulcate, 20~23 mm with a clear pale yellow (89) exudate. Margins sunken, distinct and smooth with slight medium buckling. Reverse pale yellow (89), no soluble pigments. At 37°C, colonies white (263), floccose, strongly sulcate and convoluted, often raising the colony off the agar surface, 12~15 mm with a clear pale yellow (89) exudate. Margins distinct and smooth, no soluble pigments. Penicilli not found at either temperature. No germination at 5°C.

On BLAKESLEE's malt extract agar, colonies are white (263), plain to umbonate, floccose, raised, 17~20 mm, no exudate. Margins distinct, fimbriate; reverse pale yellow (89), no soluble pigments. Penicilli not found.

Colonies on 25% glycerol nitrate agar are white, raised, floccose and sulcate, 14~17 mm, no exudate. Margins distinct, sunken and smooth; reverse clear pale yellow (89) to light yellow brown (76), no soluble pigments. No reproductive structures found.

On tap water agar, colonies are white (263), mycelium floccose, appressed and very limited, 2~4 mm, no exudate. Margins fimbriate. Stipes borne on substrate mycelium, smooth and short,

Table 1. Comparison of *Penicillium restrictum* strain AB 1875C-28, *P. restrictum* ATCC 11257 and *Penicillium dimorphosporum* ATCC 22783.

Culture	Colony characteristics on malt extract agar	Penicilli on malt extract agar	Growth on tap water agar	Length of collula
Penicillium restrictum strain AB 1875C-28	White (263)[a], umbonate, floccose, margins distinct	Absent	Poor, light sporulation	About 1 μm
P. restrictum ATCC 11257	White (263), umbonate, floccose, margins distinct	Present, sparse	Poor, light sporulation	About 1 μm
P. dimorphosporum ATCC 22783	White (263) at margins, center light greenish gray (154), appressed, fimbriate, margins indistinct	Present, moderate	No growth	3~4 μm

[a] Color and number in parenthesis follow ISCC-NBS Centroid Color Charts[3].

Fig. 1. Scanning electron micrograph of a penicillus of *Penicillium restrictum* strain AB 1875C-28 grown on tap water agar.

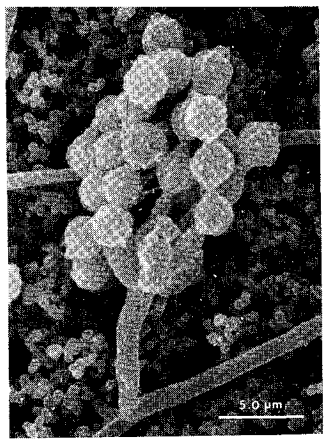
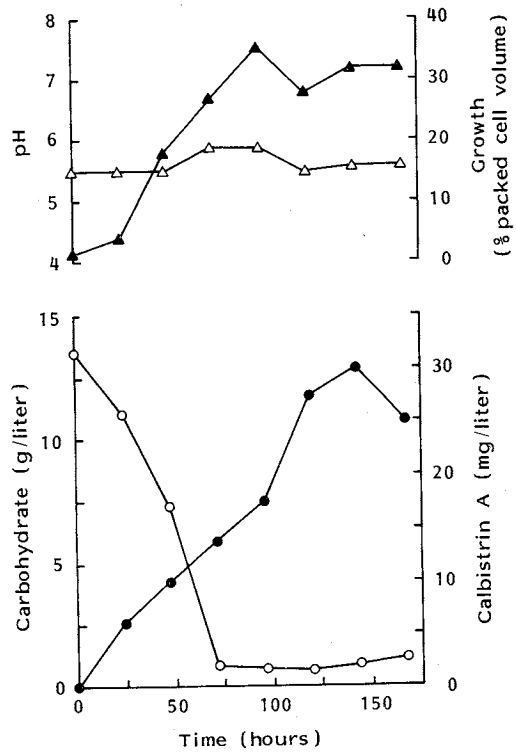


Fig. 2. Time course of the calbistrin fermentation.
△ Growth, ▲ pH, ○ carbohydrate, ● calbistrin A.



12.5~17 μm × 2.5~2.8 μm. Penicilli are nonvesiculate, strictly monoverticillate; phialides 1~4 per verticil, terminal to subterminal, ampulliform, primarily short, 5~7.5 μm × 2.5~2.8 μm, up to 7 μm when long collula formed, some producing a distinct collar near terminus. Conidia spheroidal to ellipsoidal, 3~3.5 μm × 3~3.2 μm bearing longitudinal ridges and prominent disjunctors. Fig. 1 shows a typical penicillus.

Based on the observed characteristics, strain AB 1875C-28 belongs to Subgenus *Aspergilloides* Section *Exilicaulis* Series *Restrictum*. A laboratory comparison was done between strain AB 1875C-28 and two cultures in this group that it most closely resembled, *P. restrictum* ATCC 11257 and *P. dimorphosporum* ATCC 22783. Several characteristics of the three strains are compared in Table 1. Strain

AB 1875C-28 differs from *P. dimorphosporum* ATCC 22783 in colony characteristics and penicilli formation on BLAKESLEE's malt extract agar, in growth on tap water agar and in length of the collula. Our strain is identical to *P. restrictum* ATCC 11257 except for its inability to form penicilli on BLAKESLEE's malt extract agar. Therefore, we have identified AB 1875C-28 as *Penicillium restrictum* Gilman & Abbott.

Fermentation

The time course of the calbistrin fermentation is shown in Fig. 2. The medium described, which supported yields of 25 to 30 mg/liter, was used for the fermentations from which the calbistrins were

Fig. 3. Effect of NaCl on yield of calbistrin A.

● Calbistrin A, △ growth.

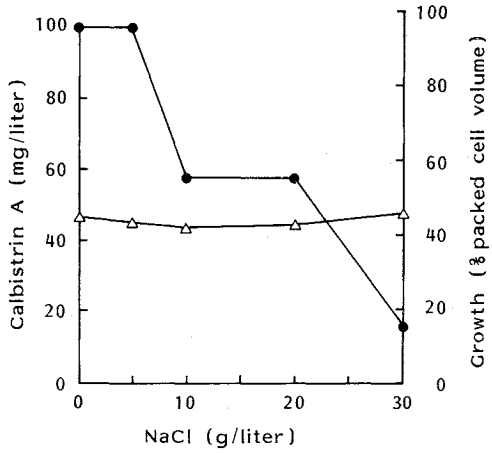


Table 2. Minimal inhibitory concentration of calbistrins A, B and C against yeasts.

Organism	MIC (μg/ml)			
	Calbistrin A	Calbistrin B	Calbistrin C	Amphotericin B
Candida albicans				
ATCC 10231	0.78	3.12	50	1.56
579a	0.78	3.12	50	1.56
CCH 442	0.78	3.12	50	1.56
ATCC 38247	—	0.78	—	50
ATCC 62376	0.78	3.12	50	1.56
C. tropicalis NRRL-Y112	1.56	3.12	50	1.56
C. kefyr ATCC 28838	25	50	>100	1.56
C. glabrata ATCC 15545	100	100	>100	1.56
Cryptococcus albidus ATCC 34140	>100	>100	>100	1.56
Saccharomyces cerevisiae GS1-36	100	100	>100	0.39

Table 3. Minimal inhibitory activity of calbistrin A against a variety of *Candida* strains.

Candida strains	MIC (μg/ml)		*Candida* strains	MIC (μg/ml)	
	Calbistrin A	Amphotericin B		Calbistrin A	Amphotericin B
C. albicans AC3401	0.78	0.78	*C. krusei* AC3418	6.25	0.78
C. albicans AC3408	0.78	0.78	*C. krusei* AC3419	>100	1.56
C. albicans AC3409	0.78	0.78	*C. stellatoidea* AC3423	0.39	0.78
C. albicans AC3410	0.78	0.78	*C. stellatoidea* AC3458	0.78	0.78
C. albicans AC3411	0.78	1.56	*C. lusitaniae* AC3424	1.56	1.56
C. albicans AC3412	>100	1.56	*C. lusitaniae* AC3425	1.56	0.78
C. albicans AC3413	0.78	1.56	*C. lusitaniae* AC3457	100	0.78
C. albicans AC3414	0.78	0.78	*C. parapsilosis* AC3426	3.12	0.78
C. kefyr AC3420	25	1.56	*C. parapsilosis* AC3427	3.12	1.56
C. kefyr AC3421	100	1.56	*C. parapsilosis* AC3428	1.56	1.56
C. kefyr AC3422	50	1.56	*C. tropicalis* AC3429	1.56	1.56
C. krusei AC3417	>100	1.56	*C. tropicalis* AC3430	1.56	1.56

isolated. This formulation had been the screening medium for discovery of the activity. In subsequent yield improvement studies, we found that NaCl, while having little effect on growth, was quite suppressive on calbistrin production. Fig. 3 shows growth and production of calbistrin A at several levels of NaCl with an ultra-violet isolate of AB 1875C-28.

Biological Activity

The activities of calbistrins A, B and C are shown in Table 2. Calbistrin A was the most active of the three compounds and comparable to amphotericin B against *Candida albicans*. Interestingly, the activity was superior against *C. albicans* and *C. tropicalis* compared to other yeasts and this trend was consistent for all three calbistrins. Testing of *Candida* species clinical isolates confirmed the preferential activity against *C. albicans* and indicated that some strains of *Candida* species were relatively insensitive to the calbistrins (Table 3). *Aspergillus* species were found to be generally insensitive to calbistrins (Table 4) although *A. fumigatus* was more sensitive than other *Aspergillus* species. None of the calbistrins showed appreciable antibacterial activity.

Table 4. Activity of calbistrin A and amphotericin B against several strains of *Aspergillus*.

Organism	MIC (μg/ml)	
	Calbistrin A	Amphotericin B
Aspergillus niger		
AC3435	>100	1.56
AC3436	>100	1.56
AC3437	>100	3.12
AC3252	>100	3.12
ATCC 16404	>100	3.12
Aspergillus flavus		
AC3438	>100	3.12
AC3439	>100	>100
AC3440	>100	6.25
AC3441	>100	6.25
Aspergillus fumigatus		
AC3446	50	1.56
AC3447	50	3.12
AC3448	50	3.12
AC3449	50	3.12

The acute LD_{50}s of calbistrins A and B were 1.5 mg/kg and 7.8 mg/kg, respectively.

Acknowledgments

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