Fusacandins A and B; Novel Antifungal Antibiotics of the Papulacandin Class from *Fusarium sambucinum*

I. Identity of the Producing Organism, Fermentation and Biological Activity†

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The fusacandins, antifungal agents of the papulacandin class, are produced by a strain of *Fusarium sambucinum*. Fermentation yielded 60 mg/liter of fusacandin A and minor amounts of fusacandin B. As expected, the fusacandins inhibit (1,3)-β-glucan synthesis. Fusacandin A is slightly less active than papulacandin B against *Candida albicans* and, like papulacandin, loses activity in the presence of serum.

The incidence of fungal infections has increased sharply during the past ten years. At greatest risk are hospital patients, especially immunocompromised, surgical and burn patients[1]. The main therapy for fungal infections is amphotericin B, a drug with many severe side-effects. Long term treatment of oral candidiasis and other fungal infections in AIDS patients with current antifungal agents, such as the azoles, has led to the emergence of resistant strains. The need for new antifungal therapies is clear, but the problem of identifying targets with properties distinctly different from humans is more difficult than it has been for antibacterial therapy. The fungal cell wall offers a structure not present in human cells[2], and enzymes which synthesize cell wall polymers are ideal targets for selective antifungal agents. The fusacandins are antifungal agents which inhibit growth of *Candida albicans* by inhibiting (1,3)-β-glucan synthesis. The (1,3)-β-glucan polymer is an essential component of the fungal cell wall and is synthesized by the plasma-membrane bound enzyme, (1,3)-β-glucan synthase (EC 2.4.1.34). In this paper we describe the producing microorganism, the production of fusacandin by fermentation, the antifungal activity of fusacandins A and B and the mode-of-action of fusacandin A. The isolation and structural elucidation of fusacandins A and B are described in a companion paper[3].

Materials and Methods

Microorganisms

The producing fungus, strain AB 1900A-1314, was isolated from a polypore fruitbody obtained in Piatt County, IL, U.S.A. A subculture of the microorganism has been deposited in the permanent collection of the National Center for Agricultural Utilization Research, United States Department of Agriculture, 1815 North University Street, Peoria, IL, 61604, U.S.A., where its accession number is NRRL 21252.

The microorganisms used in the bioactivity evaluations and in the mode-of-action studies were obtained from the American Type Culture Collection or from our laboratory culture collection.

Fermentation

Fusacandin was first isolated from a solid state fermentation similar to the fermentation we described for the aselacins[4]. Studies in shaken flasks and small fermentors led to the large-scale fermentation described here. The seed medium consisted of soy flour 2% and mannitol 2% as reported by TRAXLER *et al.* for the inoculum preparation for papulacandin production[5]. The production medium was a modification of a medium used by VANMIDDLESWORTH *et al.*[6] and consisted of glucose monohydrate 5.5%, mannitol 1%, glycine 0.2%, dried lard water 0.5%, soybean meal 0.5%, sodium citrate 0.2%, KH_2PO_4 0.2% and $CoCl_2 \cdot 6H_2O$ 0.001%. The pH was 6.4 after sterilization. XFO-371, a silicone

† Dedicated to Professor SATOSHI ŌMURA on the happy occasion of his 60th birthday.

antifoam, was used at 0.01% for all stages of inoculum preparation and for fermentation.

Inoculum was prepared in the seed medium in a series of steps commencing with the inoculation of 100 ml of medium in a 500-ml Erlenmeyer flask with 1 ml of frozen vegetative mycelium. The resulting growth was inoculated at 5% into 1 liter of seed medium in a 4-liter Erlenmeyer flask. Both flasks were incubated for 48 hours at 28°C on a rotary shaker operating at 225 rpm. A 1,000-liter seed tank containing 300 liters of the seed medium (sterilized at 121~125°C for 1 hour) was inoculated with 1 liter of second stage flask growth. The seed tank was maintained at 25°C, 150 rpm for 48 hours with an air flow of 300 liters per minute.

The fermentation was conducted in a 7,500-liter fermentor charged with 5,000 liters of the fermentation medium, which was batch sterilized in the fermentor at 121~125°C for 1 hour. The fermentor was inoculated with 300 liters of culture from the described seed tank. Temperature was controlled at 25°C. To maintain the culture in its mycelial form, dissolved oxygen was controlled at 8 to 13% with variable agitation rate. Air was supplied at 2,000 liters per minute. The fermentor was harvested at 114 hours.

Fermentation Analysis

The production of fusacandin A was followed by HPLC with Rainin software using an external standard method. The chromatograph consisted of a Perkin-Elmer Series 4 pump, a Perkin-Elmer ISS-100 autosampler and a Thermo Separation Products spectroMonitor 5000 diode array detector. Fermentation samples were prepared for analysis by treating 3 ml of broth with 6 ml methanol. To extract fusacandin from the mycelium, the mixture was shaken for 1 hour on a rotary shaker operating at 225 rpm. The samples were centrifuged at $280 \times g$ for 10 minutes. The solvent rich supernatants were assayed by injecting 10 μl onto a Spherisorb C-8 column (5 μ, 4.6 × 150 mm) equilibrated in CH_3CN - H_2O (44 : 56) at a flow rate of 1 ml/minute. Data were collected at a detector setting of 225 nm. The robustness of the method was enhanced by changing the isocratic system to 100% CH_3CN 15 minutes into the run (after elution of the fusacandins) and holding at that solvent strength for 15 minutes to clean the column of residuals. Equilibration between runs took 10 minutes.

The consumption of glucose was followed by the colorimetric glucose oxidase assay in the IBI Biolyzer Rapid Analysis System, manufactured by Eastman Kodak Company, New Haven. Carbon dioxide in fermentor exhaust gases was analyzed by a mass spectrometer.

Biological Activity

Minimal inhibitory concentrations (MIC) were determined using microtiter broth dilution in Yeast Nitrogen Base (YNB) (Difco) containing 1% glucose (YNBG). 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[7]. Plates were incubated at 35°C for 24 hours, and the MICs were defined as the lowest concentration of drug completely inhibiting visible growth.

Mode-of-Action Studies

The inhibition of (1,3)-β-glucan synthase was determined with the method previously described[8]. The sorbitol protection and cell morphology assay has also been described[9].

Cell Labeling Studies: *C. albicans* CCH 442 cells were grown overnight in YNB plus 0.5% glucose with 0.8 M sorbitol at 30°C with shaking at 200 rpm (OD_{420} 2.5~3.5). Undiluted cells were then pretreated with inhibitor (fusacandin A 10 μg/ml, papulacandin B 10 μg/ml or cilofungin 7.5 μg/ml) for 30 minutes and then incubated with appropriate radiolabeled precursor added for 30 minutes. An untreated control was determined for each radiolabeled analysis. A 1 ml cell aliquot was used to determine the labeling of each macromolecular constituent, and the reactions were terminated with an equal volume of 10% cold TCA. Protein and nucleic acid synthesis were measured using L-[4,5-^{3}H]leucine (167 Ci/mmol) and [8-^{14}C]adenine (53 mCi/mmol), both at 0.04 μCi/ml for the labeling reaction[10]. The effect of inhibitors on lipid synthesis was assessed by the incorporation of [1-^{14}C]acetic acid, sodium salt (67 mCi/mmol) at 0.05 μCi/ml. TCA-stopped reactions were washed 4 times with equal volumes of deionized distilled water, and cell lipids were extracted by the modified Folch method[11]. Mannan, (1,6)-β-glucan and (1,3)-β-glucan syntheses were monitored with D-[*U*-^{14}C]glucose (293 mCi/mmol) at 0.05 μCi/ml, while chitin was labeled with the *N*-acetyl-D-[1-^{3}H]glucosamine (7.0 Ci/mmol) at 0.05 μCi/ml. Prior to fractionating the different cell wall polymers, TCA-washed pellets from each radiolabeled reaction were delipidated with 1 ml ethanol - water - diethyl ether - pyridine - NH_4OH (15 : 15 : 5 : 1 : 0.018) for 1 hour at 60°C. Cells were pelleted and then extracted with 1 ml of 6% KOH at 80°C for 90 minutes. The extracts were centrifuged, and alkali-solubles were precipitated with Fehling's reagent to determine mannan levels[12]. The alkali-insolubles (β-glucans and chitin) were washed with 1 ml of 50 mM acetate buffer (pH 5.4) and digested overnight at 30°C in 0.5 ml of 50 mM acetate buffer containing 0.5 mg/ml chitinase (Chitinase-GODO, Seikagaku Kogyo Co.). Samples were centrifuged, and the supernatant was counted as a measurement of radiolabeled chitin from *N*-acetyl-D-[1-^{3}H]glucosamine. Chitinase treatment was necessary for β-glucan determinations since glucose can eventually label chitin. The precipitates were washed with 10 mM Tris-HCl, pH 7.5 and digested overnight with 5 units of β-glucanase (Quantazyme ylg, Quantum Biotechnologies, Montreal, Quebec) at 30°C with agitation in 0.5 ml 10 mM Tris-HCl and 40 mM β-mercaptoethanol. The (1,6)-β-glucan was

separated from the digested β-(1,3) glucan products by passing the reaction products through a Microcon-10 membrane (MW cut off-10,000 daltons, Amicon Co.). The retained material [(1,6)-β-glucan] in the Microcon-10 was washed with buffer and reconcentrated. The pass-through materials of both runs were combined and counted [(1,3)-β-glucan)]. Effects of inhibitors on macromolecular constituents from cells were calculated on cpm/cell dry wt, and the data were expressed as % of untreated cells. Cells from a 1 ml aliquot of each labeling reaction were washed 4 times with deionized distilled water and dried to determine mass.

Results and Discussion

Identification and Description of the Producing Strain

Strain AB 1900A-1314 was identified as *Fusarium sambucinum* Fuckel at the Pennsylvania State University Fusarium Research Center. Growth characteristics of strain AB 1900A-1314 incubated at 25°C for seven days on Potato Dextrose agar (Difco) are given below. The colors and numbers in parenthesis in the strain description follow the ISCC-NBS Centroid Color Charts[13].

The colonies were dense, floccose and moderate yellowish pink (29) in color. The culture grew rapidly to 75~80 mm in diameter and produced a clear exudate. The aerial mycelium was pale pink (7) and the colony reverse light orange (52) to moderate orange (53). Spores were not found on this medium at seven days. As the culture aged, many raised, round, light orange (52) structures, 1~3 mm in diameter, developed on the agar surface. These aggregates were hard but could be broken apart by moderate pressure with an inoculating loop. When mounted in lactophenol and viewed by light microscopy, fragments of the structures consisted of flattened, irregular cells mixed with a few strands of mycelia. The aggregates appear to be similar to the perithecia-bearing stromata that *Fusarium sambucinum* (*Gibberella pulicaris*) can form on woody host tissue[14]. We did not, however, observe perithecia or asci.

Strain AB 1900A-1314 produces characteristic sickle-shaped macroconidia and 1~2 septate microconidia on Cornmeal agar (Difco) when incubated under the same conditions. Colonies grew rapidly and attained a diameter of 75~80 mm in one week on this medium. The colonies had white (263), wispy aerial mycelia and the reverse was colorless. Macroconidia were produced abundantly, measuring 37.5~70×5~7 μm. Microconidia measured 20~37.5×3.75~7.5 μm. The culture developed medium orange (53) colored sporodochia at 14 days at 25°C on Cornmeal agar. Fig. 1 is a scanning

Fig. 1. Scanning electron micrograph of macroconidia of *Fusarium sambucinum* AB 1900A-1314 grown on corn meal agar for 21 days at 25°C.

Bar = 10 μm.

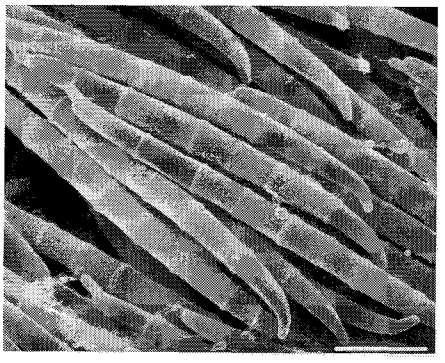


Fig. 2. HPLC chromatogram of the extract of the 114-hour fermentation sample showing separation of fusacandins A and B.

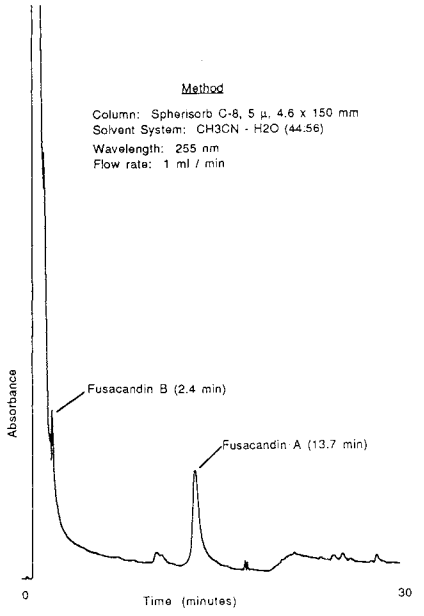
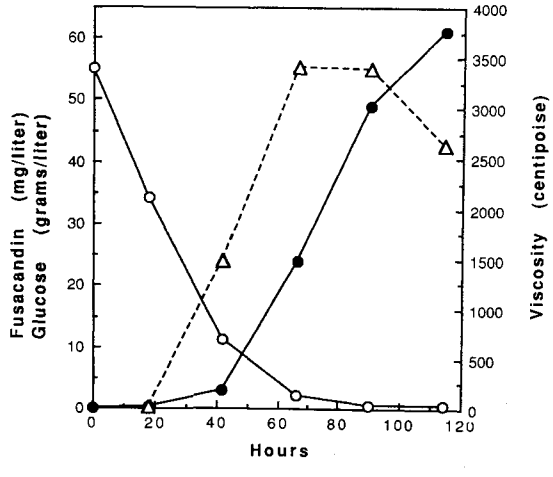


Fig. 3. Time course of the fusacandin fermentation.

● Fusacandin, ○ glucose, △ viscosity.



electron micrograph of the macroconidia.

Fermentation

The chromatogram (Fig. 2) illustrates the HPLC assay, used to determine concentration of fusacandin A in fermentation samples, as well as the separation of fusacandins A and B. A time course of the fermentation is shown in Fig. 3. Growth, as evaluated by viscosity, followed the consumption of glucose. Production of fusacandin A appears to be dependent on near depletion of glucose. Fig. 4 illustrates the change in agitation rate required to control dissolved oxygen at 10%. Changes in pH and CO_2 evolution in exhaust gases during fermentation are shown in Fig. 5. A yield of 60 mg/liter was achieved. Fusacandin B, produced in minor amounts, was found only on scale-up to kiloliter fermentations.

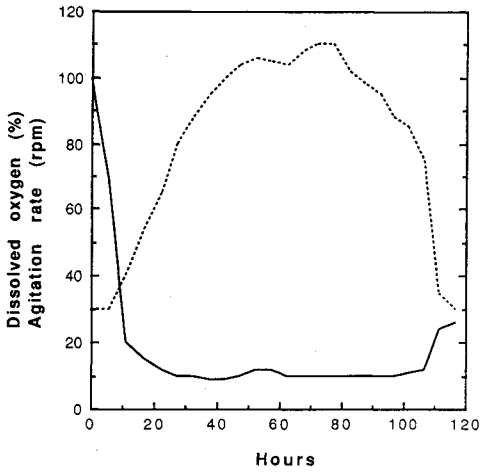
Biological Activity

The minimum inhibitory concentrations (MICs) of fusacandins A and B are shown in Table 1 where they are compared with the MICs of papulacandin B and amphotericin B against a number of strains of *Candida albicans* and several other fungi. Fusacandin A is considerably more active than fusacandin B. Fusacandin A is slightly less active than papulacandin B and 2 to 8-fold less active than amphotericin B against the strains tested. When 50% mouse serum (Table 1) is added to the medium the MICs of both fusacandin A and papulacandin B are greatly increased. Fusacandin A has no significant antibacterial activity. MICs against a panel of 15 Gram-positive bacteria ranged from 12.5 to $>100\,\mu g/ml$ and $>100\,\mu g/ml$ against 15 Gram-negative bacteria.

Fusacandin A was shown (Fig. 6) to inhibit (1,3)-β-

Fig. 4. Fusacandin fermentation.

—— Dissolved oxygen, ------ agitation rate.



Agitation used to maintain dissolved oxygen at 10%.

Fig. 5. Fusacandin fermentation.

—— CO_2 evolution rate, ------ pH.

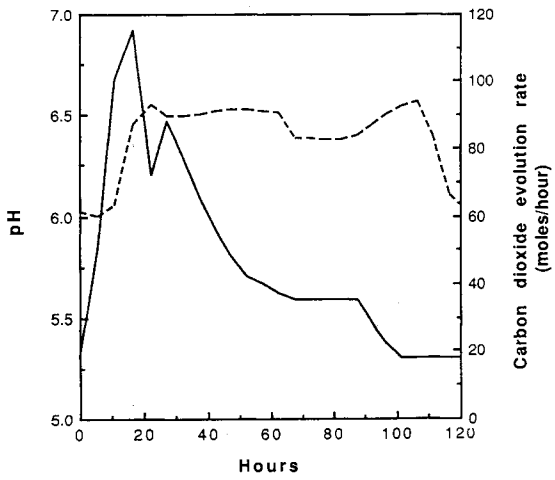


Table 1. Comparison of MICs of fusacandin A, fusacandin B, papulacandin B and amphotericin B against yeasts and *Aspergillus niger* in yeast nitrogen base medium with glucose. Effect of 50% mouse serum on the MIC of fuascandin A and papulacandin B.

Microorganism	MIC, $\mu g/ml$				With 50% mouse serum	
	Fusacandin A	Fusacandin B	Papulacandin B	Amphotericin B	Fusacandin A	Papulacandin B
Candida albicans ATCC 10231	3.12	>100	1.56	1.56	>100	>100
C. albicans 579a	3.12	50	3.12	1.56	>100	>100
C. albicans CCH 442	3.12	50	1.56	3.12	>100	>100
C. albicans ATCC 38247	>100	>100	1.56	25.0	>100	>100
C. albicans ATCC 62376	6.25	>100	1.56	1.56	>100	>100
C. tropicalis NRRL Y-112	6.25	50	6.25	0.78	>100	>100
C. kefyr ATCC 28838	6.25	100	3.12	3.12	>100	>100
Torulopsis glabrata ATCC 15545	3.12	100	6.25	3.12	>100	>100
Cryptococcus albidus ATCC 34140	>100	>100	6.25	1.56	>100	>100
Saccharomyces cerevisiae GS1-36	3.12	50	6.25	1.56	>100	>100
Aspergillus niger ATCC 16404	6.25	>100	12.5	0.78	>100	>100

glucan synthase (GS). It is as effective as cilofungin but is less effective as an inhibitor of GS than the structurally similar papulacandin B.

The presence of sorbitol, an osmotic protecting agent, antagonized the inhibition of *Candida albicans* by fusacandins A and B, papulacandin B and cilofungin (Table 2). It did not protect *C. albicans* from inhibition by a non-cell wall synthesis inhibitor (amphotericin B). The morphology of the inhibitor-treated cells was examined by phase contrast microscopy (400×). Untreated cells in the presence and absence of sorbitol are rounded and nonaggregated (4~6 μm dia.). At 2×MIC, in the absence of sorbitol, papulacandin B-treated cells were round, enlarged (5~10 μm diameter), distorted and aggregated (50~125 cells/clump). In the presence of sorbitol, the cell morphology of cells treated with 500~1,000 μg/ml papulacandin B remained the same but the size range increased to 7.5~20 μm in diameter. Cells treated with cilofungin had similar morphology to papulacandin B-treated cells but were slightly larger in diameter. In contrast, fusacandin A and B-treated cells at 2×MIC without sorbitol were similar to normal cells. However, in the presence of sorbitol fusacandin-treated cells were round and enlarged in size (7.5~10 μm dia.) but did not aggregate.

The whole cell labeling studies of macromolecular cell components showed that fusacandin A, like papulacandin B and cilofungin, inhibited the eventual formation of β-glucans (Table 3). Interestingly, all three compounds inhibited synthesis of both (1,3)-β and (1,6)-β-glucans. Other researchers have also noted that papulacandin B and some lipopeptides inhibit the labeling of both glucans[15,16].

Fig. 6. Comparison of glucan synthase (GS) inhibition activity of fusacandins A and B, papulacandin B and cilofungin.

○ Fusacandin A, □ fusacandin B, ◇ cilofungin, △ papulacandin B.

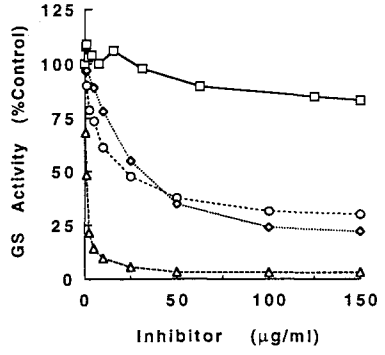


Table 2. Effect of sorbitol on the inhibition of *Candida albicans* CCH442 by antifungal agents in the microtiter broth dilution assay.

	MIC, μg/ml at 2 days		MIC, μg/ml at 7 days	
	No sorbitol	With sorbitol	No sorbitol	With sorbitol
Amphotericin B	0.97	0.97	0.97	0.97
Cilofungin	0.97	500	1.95	>1000
Fusacandin A	0.97	>1000	1.95	>1000
Fusacandin B	15.6	>1000	31.2	>1000
Papulacandin B	0.97	500	0.97	>1000

Conclusion

The producing fungus was characterized as a strain of *Fusarium sambucinum*. The fermentation was scaled up to produce 100-gram quantities of fusacandin A and to permit the detection of fusacandin B as a minor congener. Fusacandin A was shown by two methods to be an inhibitor of (1,3)-β-glucan synthesis, namely, direct inhibition of the *in vitro* GS enzyme and reduced incorporation of radiolabel in (1,3)-β-glucan in whole cell studies. The protection of *C. albicans* cells by an osmotic protecting agent (sorbitol) further implicates the fungal cell wall as the target of fusacandin activity.

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Table 3. Effect of inhibitors on the incorporation of radioactive precursors into macromolecules in *Candida albicans* CCH442.

	Macromolecular constituents (% control)						
	Protein	Nucleic acids	Lipid	Chitin	Mannan	(1,3)-β-Glucan	(1,6)-β-Glucan
Fusacandin A	117	86	118	98	87	14	21
Papulacandin B	98	110	87	90	105	6	13
Cilofungin	120	96	99	62	170	7	30

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