L-671,329, A NEW ANTIFUNGAL AGENT

I. FERMENTATION AND ISOLATION

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(Received for publication August 22, 1988)

In screening for new antifungal agents from fungi, a new lipopeptide antifungal agent, L-671,329, similar to echinocandin B, has been isolated from *Zalerion arboricola*. Studies indicate that L-671,329 is produced under both solid and liquid fermentation conditions.

Echinocandins/aculeacins are lipopeptide antifungal agents. The polar, cyclic hexapeptide portion of these compounds is structurally unusual in that all the amino acids contain one or more hydroxyl groups. In sharp contrast to the peptide portion is the extremely non-polar, fatty side chain, which is amide-linked to the amino group of an ornithine-derived amino acid residue[1]. Variation has been observed in both the cyclic peptide (hydroxylation and amino acid substitution patterns)[2,3] and in the lipophilic side chain (length, branching, and degrees of unsaturation)[3,4].

The antibiotic activity of echinocandins appears to be limited to yeasts, especially *Candida* sp.[5]. While both natural[2~4] and chemical[6] variation of the echinocandin structure have shown that the biological activity can be manipulated *via* structural modification, enzymatic removal of the fatty side chain has shown that the cyclic peptide is inactive alone[7], as is the fatty acid. LY121019, a semisynthetic derivative of echinocandin B which showed good *in vitro* and *in vivo* anti-*Candida* activity, was reported to be at least 20-fold less toxic than amphotericin B and, therefore, promises to be important in treating these types of infections in the clinic[8].

In this paper the fermentation and isolation of L-671,329, a new echinocandin, will be discussed; in the following papers the structural elucidation[9] and biological activities[10] of this compound will be described.

Materials and Methods

The methods of detection utilized in the following studies and isolations were antifungal activity as measured *via* zones of inhibition in a disc diffusion assay on agar plates seeded with *Candida albicans* (MY 1028), Merck Culture Collection, HPLC on C18 reversed-phase, or both.

Antifungal Assays

Seeded agar plates were prepared as follows. Potato dextrose broth (50 ml, Difco) was inoculated with a lyophilized pellet of *C. albicans*, and the culture was incubated for 24 hours at 28°C, with agitation at 220 rpm. This culture was used as a 1% inoculum to seed potato dextrose agar (PDA, Difco). Assay samples (20 μl) were applied to 6.2 mm-filter discs and air dried at room temperature before being placed on the seeded assay plates. After incubation at 28°C for 24 hours, zones of inhibition were measured *via* image analysis from the extreme edges of clearing against the background lawn. A standard curve was generated using compound L-671,329. The critical variation of standard concentrations ranged from 3~8%.

Table 1. Components of the solid fermentation medium F204.

Component	Concentration (per flask[a])
Millet	15.0 g
Yeast extract	0.5 g
Sodium tartrate	0.1 g
Ferrous sulfate	0.01 g
Monosodium glutamate	0.1 g
Corn oil	0.1 ml
Distilled water	15.0 ml
pH not adjusted	

Autoclave 20 minutes, then add 10 ml distilled water. Autoclave 20 minutes more.

[a] 250-ml Erlenmeyer flask.

Table 2. Components of the liquid fermentation medium.

Component	PB9	PBM
Glycerol	85.0 g	75.0 g
Citrus pectin	10.0 g	
Peanut meal	4.0 g	
Peptonized milk	4.0 g	
Tomato paste	4.0 g	5.0 g
Corn steep liquor	4.0 g	5.0 g
Lard water	4.0 g	5.0 g
Glycine	2.0 g	
KH_2PO_4	2.0 g	
$(NH_4)_2SO_4$		2.0 g
$CoCl_2 \cdot 6H_2O$		10.0 mg
P-2000		2.0 ml
Cod liver oil		2.0 ml
Sodium citrate		2.0 g
Distilled water	1,000 ml	1,000 ml
Presterile pH	7.0	7.0

HPLC Assays

HPLC analysis was performed on a system consisting of a Spectraphysics 8700 pump, a Spectraphysics 8780 autoinjector or a Waters U6K manual injector, a DuPont Zorbax ODS 4.6 i.d. $\times$25 cm column, an LKB 2151 variable wavelength UV detector equipped with a 10-mm pathlength cell and a Spectraphysics 4200 integrator. Column temperature was maintained constant at 40°C *via* jacketing and a constant temperature bath. The flow rate was 1 ml/minute, the isocratic solvent system was 50% CH_3CN - 50% H_2O and the UV of the effluent was monitored at 210 nm and an attenuation of 0.64 aufs. Under these conditions the retention time of L-671,329 was up to 10.7 minutes.

Fermentation

The microorganism, which produced L-671,329, was isolated from a water sample at the MSDRL CIBE Laboratories, Madrid, Spain. This culture has been identified as *Zalerion arboricola* by Centraalbureau voor Schimmelcultures and has been deposited at the American Type Culture Collection under the accession No. ATCC 20868.

The morphology of ATCC 20868 was observed for 30 days at 25°C on PDA, Sabouraud maltose agar (SMA), corn meal agar (CMA) and yeast malt extract agar (YME), supplied by Difco.

Vegetative growth of the culture was prepared by inoculating a lyophilized pellet of ATCC 20868 into a 250-ml unbaffled Erlenmeyer flask containing 54 ml of seed medium. The seed medium consisted of; corn steep liquor 0.5%, tomato paste 4.0%, oat flour 1.0%, glucose 1.0% and trace elements 1.0% in distilled water at pH 6.8. The trace element mixture contained per liter; $FeSO_4 \cdot 7H_2O$ 1 g, $MnSO_4 \cdot 4H_2O$ 1 g, $CuCl_2 \cdot 2H_2O$ 25 mg, $CaCl_2$ 100 mg, H_3BO_3 56 mg, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 19 mg and $ZnSO_4 \cdot 7H_2O$ 200 mg.

Seed flasks were incubated from 3 to 6 days at 25°C with agitation at 220 rpm and a 5-cm throw on a rotary shaker. Two ml of the resulting culture growth were used to inoculate either a solid or liquid production medium (Tables 1 and 2). The solid production medium F204 was incubated in 250 ml unbaffled Erlenmeyer flasks under static conditions at 25°C and 50% relative humidity for the fermentation studies. Eight times the amount of production medium used in the 250-ml Erlenmeyer was charged in 2 liters unbaffled Erlenmeyer flasks to provide material for the isolation of L-671,329 described below. Forty five ml of liquid production medium, PB9 or PBM, were distributed into 250 ml unbaffled Erlenmeyer flasks. Flasks containing PB9 or PBM were incubated at 25°C with agitation at 220 rpm on a rotary shaker with a 5-cm throw in a room at 50% relative humidity. Production media were incubated for 3 to 24 days. At harvest, the cultures were mixed with equal volumes of 100% methanol, or 100% methyl ethyl ketone, and extracted at room temperature for 24 hours. After extraction, samples were centrifuged for 20 minutes at 3,000 rpm. The supernatant was filtered through a 0.2-μm syringe filter. These samples were assayed for L-671,329 *via* antifungal assays,

HPLC assays or both.

Optimization of production media used the experimental protocol of PLACKETT and BURMAN[11]. The following media ingredients were used during optimization studies: Ardamine pH (Yeast Products, Inc.), corn steep liquor (Grain Processing Corp.), lard water (Inland Molasses Co.), citrus pectin (BioServ., Inc.), soybean meal (Centra Soya Co.), P-2000 (Dow Chemical), cod liver oil (BioServ., Inc.), tomato paste (Hunts), soy flour (50% protein) (BioServ., Inc.), peanut meal (BioServ., Inc.), peptonized milk (Sheffield) and distillers solubles (Grain Processing Co.). Glucose, glycerol, KH_2PO_4, $(NH_4)_2SO_4$, $CoCl_2 \cdot 6H_2O$, sodium citrate and glycine were obtained from Fisher.

Isolation

Five hundred ml of MeOH was added to each of five 2-liter flasks of solid fermentation; the contents of these flasks were then combined, stirred and filtered. The spent cake was re-extracted with an additional 2,500-ml MeOH, stirred and filtered. The spent cake was then discarded and the filtrates combined and concentrated to 500 ml.

This concentrate was extracted two times with 500 ml hexane, followed by two 500-ml EtOAc extractions. No antifungal activity was observed in the hexane extracts, which were discarded. Most of the antifungal activity was found in the EtOAc extracts; however, a significant amount of the active constituent remained in the resulting spent aqueous. This spent aqueous was adsorbed onto Diaion HP-20 and eluted with 100% MeOH. The Diaion HP-20 eluate, which contained the remainder of the antifungal component, and the EtOAc extracts were combined, concentrated, and reconstituted to 7 ml with CH_2Cl_2 - hexane - MeOH (5 : 5 : 2) and chromatographed on a 200-ml Sephadex LH-20 column (CH_2Cl_2 - hexane - MeOH, 5 : 5 : 2).

The active fractions from the Sephadex LH-20 chromatography were combined, concentrated and chromatographed on 200 ml silica gel (EM Science, Kieselgel 60, 230~400 mesh) using an EtOAc - MeOH step gradient (100% EtOAc; MeOH - EtOAc (10 : 90), MeOH - EtOAc (25 : 75), 100% MeOH); the antifungal activity was eluted in the 100% MeOH step. The active fractions from this chromatography were combined, concentrated and chromatographed on a 200-ml silica gel (EM Science, Kieselgel 60, 230~400 mesh) column using an MeOH - EtOAc (25 : 75) isocratic system. The final separation step was a 100-ml Sephadex LH-20 chromatography using MeOH as the eluting solvent to yield 95 mg of purified L-671,329. Pure L-671,329 was an amorphous, white solid which decomposed in the range 206~214°C.

In subsequent, larger scale isolations the Diaion HP-20 adsorption/elution and both Sephadex LH-20 steps were eliminated. The Diaion HP-20 step was replaced with an additional EtOAc extraction (for a total of three). As a result of the elimination of the first Sephadex LH-20 step, it was necessary to dissolve the sample in MeOH and dry it onto silica gel in preparation of the sample for the first silica gel chromatography, since, in this less purified state, it would not dissolve in the first mobile phase of the step gradient.

It was also necessary to add an additional purification step, that is, preparative, reversed-phase HPLC. The preparative HPLC system consists of a Rainin Rabbit HPX pump equipped with a 25 ml/minute head, a Waters U6K injector, a DuPont Zorbax ODS 21.2 mm i.d. × 25 cm column, a Gilson Model 116 UV detector equipped with a 0.05-mm pathlength cell, a Spectraphysics 4200 integrator and a Pharmacia Frac-300 fraction collector. Column temperature was maintained constant at 40°C *via* jacketing and a constant temperature bath. Two hundred mg of the mixture to be separated was dissolved in 2 ml MeOH and chromatographed at 20 ml/minute, using a solvent system of H_2O - CH_3CN (60 : 40). The separation was monitored *via* UV at 210 nm, and fractions were taken every 20 ml after an initial 30-ml void volume was taken and discarded.

Results and Discussion

Fermentation

Culture ATCC 20868 has been identified as *Z. arboricola*. When incubated at 25°C for 10 days on a variety of substrates, such as PDA, SMA, CMA and YME, the organism produces a dark green

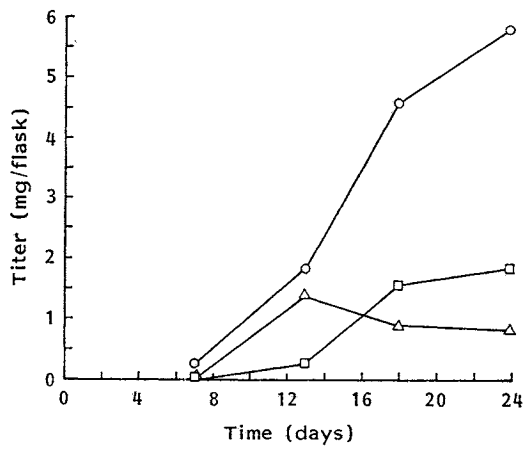
Table 3. Effect of fermentation ingredients on production of L-671,329.

Ingredient	Effect	Significance (%)[a]
P-2000	Titer increase	95
Glycine	Titer increase	95
Soy flour (50% protein)	Titer increase	93
Lard water	Titer increase	92
Peanut meal	Titer increase	86
KH_2PO_4	Titer increase	80
$CoCl_2 \cdot 6H_2O$	Titer increase	80
Cod liver oil	Titer decrease	68
Corn steep liquor	Titer decrease	68
Glucose	Titer decrease	91
Citrus pectin	Titer decrease	95

[a] Student's t-test, fermentation 13 days, 25°C, 220 rpm.

Fig. 1. Comparison of media for production of L-671,329.

○ PBM, △ PB9, □ F204.



pigmented substrate and aerial hyphae. As the culture is further incubated at 25°C, the dark green pigmented hyphae turn black by 25 days. Microscopic examination of this culture revealed reproductive structures and conidia by 30 days incubation. The hyphae were approximately 3.6 μm in diameter, septate and pigmented. Conidia of approximately 5.0 to 6.0 μm in diameter were found associated with the mycelium on all above media. The culture is capable of growth at 28, 25 and 20°C; no growth was observed at 37°C.

The experimental protocol of PLACKETT and BURMAN was selected for optimization of L-671,329 production in liquid media. Ingredients that aided production of antifungal activity in liquid agitated fermentation included P-2000, glycine, soy flour (50% protein), lard water, peanut meal, KH_2PO_4 and $CoCl_2 \cdot 6H_2O$ (Table 3). Two media, PBM and PB9, were found to support high titers in the above experiments and were, therefore, compared to the solid medium F204 (Fig. 1). When growing culture ATCC 20868 in medium PBM, production of compound L-671,329 increased steadily over 24 days. PBM produced the highest titer of the 3 media, at all the time periods tested, reaching a maximum of 5.8 mg/flask. In solid medium F204, a steady increase is also observed in production of L-671,329, reaching 1.8 mg/flask at 24 days. Production of L-671,329 in medium PB9, on the other hand, peaked after 13 days incubation at a titer of 1.4 mg/flask.

Isolation

The main problems encountered during the isolation of L-671,329 were solubility difficulties. As a result of limited solubility in ethyl acetate, the lipopeptide could never be quantitatively extracted with this solvent from the methanol concentrate (mostly H_2O). However, ethyl acetate extraction was the preferred method since it resulted in a preparation that was significantly cleaner than the Diaion HP-20 eluate, even though Diaion HP-20 removed the antifungal agent quantitatively.

Silica gel chromatography yielded a significant purification in the overall isolation scheme, even though L-671,329 streaks badly on silica. It was however, always difficult applying the mixture to be separated onto the column because of the limited solubility in solvent mixtures that were necessary for a good separation. Hence, the mixtures were solubilized in methanol (the solvent of choice for

these types of compounds), dried onto silica and the dried silica preparation applied to the head of the column.

The Sephadex LH-20 separation steps were eliminated because they were very sensitive to overloading, and even under the best of conditions, yielded poor purifications.

Another problem involved solubilizing the mixtures in the preferred mobile phase for the reversed-phase preparative HPLC separation, that is, CH_3CN - H_2O. The only solvent in which these mixtures could be sufficiently solubilized was methanol. Fortunately, this amount of methanol (2 ml) did not interfere with the reversed-phase HPLC separation of this particular compound.

Acknowledgments

The authors would like to thank Dr. S. HERNANDEZ (CIBE, Madrid, Spain) for isolating the producing culture, and Dr. J. ONISHI and J. MILLIGAN for providing assay support, and Dr. SYBREN GSDE HOOG (Centraalbureau voor Schimmelcultures) for culture identification.

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