L-696,474, A NOVEL CYTOCHALASIN AS AN INHIBITOR OF HIV-1 PROTEASE

I. THE PRODUCING ORGANISM AND ITS FERMENTATION

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A novel cytochalasin, L-696,474, (18-dehydroxy cytochalasin H) that inhibits HIV-1 protease was discovered in fermentations of a bark-inhabiting Ascomycete, *Hypoxylon fragiforme*. The product was first identified from extracts of an agar medium. Fermentation studies on a number of media indicated that the product can be made on several solid and liquid media. Optimum production was obtained from growth in a complex medium composed of glycerol, glucose, citrate, Ardamine, soybean meal, tomato paste, and inorganic salts. Other *Hypoxylon* spp., related species of Xylariales, and other fungi known to produce cytochalasins, were also surveyed for their ability to make L-696, 474. Only one other *Hypoxylon fragiforme* isolate was found to make this novel cytochalasin; none of the other cultures surveyed made L-696,474 or any other compounds which inhibit HIV-1 protease.

The human immunodeficiency virus (HIV-1) is a retrovirus which causes acquired immunodeficiency syndrome (AIDS). HIV-1 protease has been identified as a potential therapeutic target to block the formation of infectious virus particles[1]. During our screening of fungi for novel metabolites, an isolate of *Hypoxylon fragiforme* from Frost Valley, New York was observed to produce a novel cytochalasin which inhibits the HIV-1 protease. The purified compound, L-696,474, whose structure is shown in the accompanying paper[2], has a molecular weight of 477 and an empirical formula of $C_{30}H_{39}NO_4$.

The cytochalasins are a group of fungal secondary metabolites which inhibit cell division and motility. These compounds are characterized by a highly substituted isoindolinone system, to which is fused a macrocyclic ring. The latter is either carbocyclic, a lactone or a cyclic carbonate. The cytochalasins have a phenyl group at position 10 while other similar compounds have indoyl or isopropyl groups at this position. Cytochalasins are distributed among many groups of Ascomycotina and/or their anamorphic stages, including the Xylariales, but are not found in bacteria or plants. These compounds have been extensively reviewed[3,4].

We describe here the recovery from nature of the organism which produces the novel cytochalasin L-696,474, its identification, and comparative fermentation studies designed to optimize for production of this compound. We also report the results of a survey of other organisms, to determine if other cultures produce L-696,474 or similar cytochalasins, from among selected species of the Xylariales and other known cytochalasin-producing fungi. Details of the isolation, structure determination and biological properties of L-696,474 are considered in the following papers by ONDEYKA *et al.*[2] and LINGHAM *et al.*[5].

Materials and Methods

Isolation of *Hypoxylon fragiforme*

One isolate (MF5511; ATCC 20995) was obtained by collecting stromata from the bark of a recently dead American beech (*Fagus grandifolia*) on September 10, 1989, Frost Valley, New York. The stromata were actively discharging ascospores in the forest as evidenced by the black halo of ascospores on the bark surrounding the stromata. Pieces of bark with stromata were fixed to the tops of Petri dishes with stop-cock grease. Ascospores were discharged directly onto YM agar. Ascospores were observed until they germinated to ensure culture development would indeed be the *Hypoxylon*. Mycelial fragments were then transferred to YME slants.

A second isolate (MF5510; ATCC 20994) of *H. fragiforme* was obtained from stromata collected on a recently dead American beech near the New River, Fayette Co., West Virginia, on February 18, 1990. Because this time the fungus was not actively discharging ascospores, ascospores were dissected from the perithecia within the stromata and were then transferred to the isolation medium with an insect pin. Mycelial growth derived from germination of a mass of ascospores was then transferred to YME slants.

Ascospore cultures were initiated on YM agar: 10 g malt extract, 2 g yeast extract, 20 g agar, with 50 mg streptomycin sulfate and chlorotetracyline per liter of distilled water. The initial fermentations were carried out by inoculating mycelium from the initial multi-ascospore isolates onto 15 ml of yeast - malt extract agar (Difco, 41 g/liter) slanted in 50 ml polypropylene centrifuge tubes sealed with a cotton plug. The cultures were grown at 25°C in continuous fluorescent light for 21 days.

Fermentation Conditions

Solid fermentation: An agar slant of the culture was used to prepare FVM (frozen vegetative mycelia), by aseptically transferring a portion of the slant growth into YME seed medium and incubating at 25°C, 220 rpm for 3~4 days; the grown seed was frozen in 10~15% glycerol at −75°C until used. To grow the culture, YME seed flasks were inoculated with 1.0 ml of the FVM source and incubated on a gyratory shaker (220 rpm; 5.1 cm throw) for 4 days at 25°C. The culture grew as a mycelial mass. In order to break up the mass and facilitate inoculation of the production medium, small sterile ceramic balls and cylinders were added to the flask which was incubated on a gyratory shaker for 30 minutes. A portion of the seed (24 ml) was used to inoculate each 4-liter roller jar production vessel. Medium-1 production roller jars were incubated on a roller machine at 22 to 25°C for 14~21 days.

Liquid media screen: An FVM was used to inoculate a YME seed flask (1 ml per flask); incubation was at 25°C, 220 rpm for 3 days. A portion of this seed was used to inoculate second stage YME seed flasks (1 ml of the grown first stage seed per flask); incubation was at 25°C, 220 rpm for 2 days. The second stage YME seed was used to inoculate the production flasks (1 ml seed per production flask). The production flasks were incubated 25°C, 220 rpm, and samples taken at various time points for HPLC analysis[2].

Fungi surveyed for HIV protease activity: Cultures were grown from FVM, from agar plugs taken from plates (5 mm plugs), or from a lyophilized culture source. Seed cultures were started in YME seed flasks, and incubated at 25°C, 220 rpm. The time period varied, depending on the culture; most seed cultures were well grown in 3~4 days. A 1.0 ml portion of the seed was used to inoculate the liquid PBGG1 production flasks; for the larger solid production flasks (PBGG1 and Medium-1), a 5.0 ml inoculum was used. The liquid production flasks were incubated at 25°C, 220 rpm, 15 days; the solid production flasks were incubated at 25°C, 21 days, under static conditions.

Media

All media flasks were prepared with distilled water, adjusted to the appropriate pH prior to sterilization, closed with cotton plugs and autoclaved at 121°C.

Seed medium YME: Yeast extract 0.4%, malt extract 1.0%, and glucose 0.4% (pH 7.0). Dispensed as 50 ml/250-ml Erlenmeyer flasks.

Production media:

A. For solid fermentation:

Medium-1: Glucose 1.0%, fructose 1.5%, sucrose 4.0%, NZ amine Type E 0.4%, urea 0.4%, K_2HPO_4 0.05%, KCl 0.025%, $MgSO_4 \cdot 7H_2O$ 0.025%, $ZnSO_4 \cdot 7H_2O$ 0.09%, and $CaCO_3$, 0.8%.

(pH was adjusted to 7.0 prior to the addition of $CaCO_3$). The medium was dispensed at 425 ml per 1-liter Erlenmeyer flask. The solid fermentation was performed in 4-liter roller jars, containing 1,250 ml of vermiculite. These were closed with latex closures and sterilized separately for 60 minutes at 121°C. The liquid portion of the medium was mixed with the seed culture and the mixture added to the vermiculite-containing roller jar. The combination was mixed vigorously to coat the vermiculite.

B. For liquid media study screen (all dispensed at 50 ml/250-ml Erlenmeyer flask):
Medium-1: Recipe as above.
Medium-2: Glucose 1.0%, glycerol 2.0%, malt extract 3.0%, yeast extract 0.1%, $NaNO_3$ 0.1%, monosodium glutamate 0.3%, Na_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CaCl_2$ 0.05%, and trace elements 1.0 ml/liter (containing in g/liter: $FeCl_3 \cdot 6H_2O$ 5.8, $MnSO_4 \cdot H_2O$ 0.1, $CoCl_2 \cdot 6H_2O$ 0.02, $CuSO_4 \cdot 5H_2O$ 0.015, $NaMoO_4 \cdot 2H_2O$ 0.012, $ZnCl_2$ 0.02, $SnCl_2 \cdot 2H_2O$ 0.005, H_3BO_3 0.01, KCl 0.02, HCl (concentrated) 2.0 ml/liter). pH to 7.0.
KF: Corn steep liquor 0.5%, tomato paste 4.0%, oat flour 1.0%, glucose 1.0%, and trace elements solution 10.0 ml/liter (containing in 0.6 N HCl in g/liter: $FeSO_4 \cdot 7H_2O$ 1.0, $MnSO_4 \cdot 4H_2O$ 1.0, $CuCl_2 \cdot 2H_2O$ 0.025, $CaCl_2 \cdot H_2O$ 0.1, H_3BO_3 0.056, $(NH_4)_6MoO_{24} \cdot 4H_2O$ 0.019, $ZnSO_4 \cdot 7H_2O$ 0.2). The medium was adjusted to pH 6.8.
PBGG1: Glycerol 7.5%, glucose 1.0%, Ardamine PH 0.5%, $(NH_4)_2SO_4$ 0.2%, soybean meal 0.5%, tomato paste 0.5%, sodium citrate 0.2%, and polyglycol 2000 0.2% (pH to 7.0).
RLM-7: Glycerol 7.5%, glucose 1.0%, KH_2PO_4 0.2%, lard water 0.5%, $CoCl_2 \cdot 6H_2O$ 0.001%, glycine 0.2%, soybean meal 0.5%, sodium citrate 0.2%, and polyglycol 2000 0.2% (pH to 7.0).

C. For survey of fungi for HIV-1 protease inhibitors:
Medium-1: Recipe as above. The medium was dispensed at 125 ml per 250-ml Erlenmeyer flask, closed with cotton plugs. The solid fermentation was in 1-liter Erlenmeyer flasks. First 35.0 g of vermiculite were added, and the flasks were closed with cotton closures and sterilized for 60 minutes at 121 °C. Then the liquid portion of the medium was mixed with the seed culture and the mixture added to the vermiculite-containing 1-liter flask. The combination was mixed vigorously to coat the vermiculite.
PBGG1: Recipe as above. The medium was dispensed at 50 ml medium/250-ml Erlenmeyer flask for liquid fermentation. For solid PBGG1 fermentation, the procedure was as above for Medium-1.

Source of Materials

Yeast extract and malt extract were obtained from Difco; NZ amine Type E was from Sheffield Products. Ardamine PH was from Champlain Industries, lard water from Inland Molasses Co., soybean meal from BioServ, tomato paste from Hunt's, oat flour (#36) from Quaker Oats, corn steep liquor from Sigma, and polyglycol 2000 from Dow Chemical. All other ingredients were reagent grade.

Assay Method

The HPLC assay used to follow the fermentation process and measure the level of L-696,474, is described in the accompanying paper by ONDEYKA *et al.*[2].

Isolation and Structure Determination

The isolation and structure determination of L-696,474 is described in the accompanying paper by ONDEYKA *et al.*[2].

Biological Activity

The biological activity of L-696,474 is described in an accompanying paper by LINGHAM *et al.*[5].

Results and Discussion

Origin and Description of Producing Strains

The producing organism is *Hypoxylon fragiforme* (Scopoli) J. Kickx (Ascomycotina, Xylariales). The species is best known by its conspicuous stromatic stage which develops on the bark of debilitated and

recently dead hardwood limbs and trunks, especially the bark of *Fagus* spp. (beech)[6]. However, the species is a common endophyte, growing in apparently healthy living tissues, in a wide variety of vascular plants[7,8]. The habitat, stromata, conidial stage, and culture morphology were identical in both isolates. They both agreed well with published descriptions[7,9,10]. In the following description of our producing strains, capitalized color names are from RIDGWAY[11].

Stromata erumpent through the bark, gregarious to confluent, often converging over extensive areas of the bark surface, hemispherical to pulvinate, with papillate ostioles, dry, dull, pinkish cinnamon to brick-red when young, Fawn Color, Avellaneous, Vinaceous-Russet, becoming dull reddish brown or grayish brown in age Wood Brown, Army Brown, Sorghum Brown, Pecan Brown, finally developing some blackish colors from deposit of discharged ascospores and decomposition of outer surface, occasionally with minute tufts of *Nodulisporium* conidiophores. Stromatal tissue extremely brittle, carbonaceous, purplish black to black. Perithecia 0.1 ~ 0.3 mm in diameter, pyriform to subglobose, with papillate ostioles. Asci 8-spored, uniseriate, narrowly cylindrical, stipitate, 110 ~ 175 × 6 ~ 9 μm, with an amyloid apical ring. Ascospores purplish black in mass, olive-brown to olive gray in 3% KOH, ellipsoid-inequilateral with narrowly rounded ends, 10 ~ 14.5 × 5.5 ~ 7.5 μm, with 1 ~ 5 guttulae.

In culture, colonies attaining 55 ~ 60 mm on potato - dextrose agar (Difco) in one week at 20°C. Colonies with relatively sparse aerial hyphae and abundant submerged hyphae, somewhat transparent, downy to thinly tomentose, with surface developing a mealy, granulose, or pustulate texture in age, hyaline at first but soon becoming pale grayish cream, grayish buff, developing patches of buff or cinnamon, Pale Ochraceous Buff, Light Ochraceous Buff, Light Pinkish Cinnamon, Pinkish Cinnamon, Cinnamon where conidial development occurs. Reverse becoming deeply pigmented due to exudate in the medium, ranging from pale yellow, yellowish green, Barium Yellow, Napthalene Yellow, Citron Yellow, Yellowish Citron, to dark green, blackish green, or black, Serpentine Green, Dull Blackish Green.

A conspicuous *Nodulisporium* conidial stage is formed in culture, as well as on the stromata in nature. Conidiophores (on corn-meal agar, Difco) macronematous, or occasionally micronematous, more or less erect, rigid, 240 ~ 500 μm tall, 3.5 ~ 6 μm in diameter, generally without a well-defined axis, branching 1 ~ 8 times in a sympodial pattern, occasionally verticillately branched in the terminal branches, with walls smooth to minutely verruculose, hyaline to pale olive-brown in 3% KOH. Conidiogenous cells borne as terminal branches singly, or in groups of 2 ~ 3, or as lateral branches originating from subtending septa, cylindrical or slightly clavate, polyblastic, with minute denticles remaining after conidial dehiscence. Conidia 5 ~ 6 × 3 ~ 4.5 μm, subpyriform, obovate, or ellipsoid-equilateral, dry, hyaline, smooth, slightly truncate because of the basal scar, in groups of 4 ~ 10 at the terminus of the conidiogenous cell.

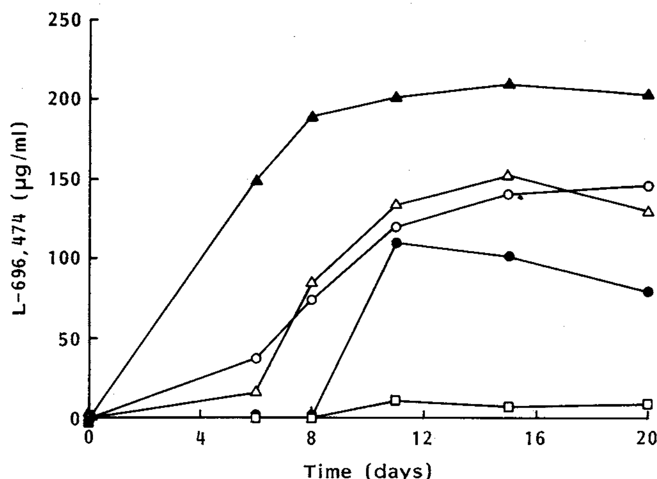
Fermentation Studies

Media Screen

The original production conditions for MF5511 *H. fragiforme* were on an agar slant, then on a vermiculite-based solid fermentation (Medium-1). This was converted initially to a liquid fermentation by using the liquid portion of Medium-1. This medium yielded L-696,474, but the fermentation was inconsistent, with low levels of the product usually being observed. A survey of 20 other liquid media was undertaken to find a better-producing and more consistent medium. The production levels varied dramatically among these media (data not shown), with yields varying from 0 to over 100 μg/ml. The best of the 20 media and Medium-1 liquid production medium were chosen for a more extended time study (Fig. 1). Note that the

Fig. 1. Production of L-696,474 (μg/ml) by *Hypoxylon fragiforme* (MF5511) over time (days) on five different liquid fermentation media.

● KF, □ Medium-1, ○, Medium-2, △ RLM-7, ▲ PBGG1.



See Methods for media and fermentation, procedures. Cultures were grown at 25°C, 220 rpm. Fermentation titers were measured by HPLC analysis described in ONDEYKA *et al.*[2]).

production levels vary among the five media shown here, with PBGG1 yielding the best titers, over 200 μg/ml. It is interesting to note that, even though it is not used as a sole source of carbon, media with high levels of glycerol yielded the most consistent high-titer liquid fermentations. Following these experiments, PBGG1 was chosen for scale-up of MF5511.

Systematic Screening of Potential Cytochalasin-producing Fungi

In the same screening program that discovered L-696,474, an isolate of *Drechslera dermatioidea* was found to produce cytochalasin A, which also inhibits the HIV-1 protease[5]). This observation, along with the discovery of L-696,474 in fermentations of *H. fragiforme*, led us to examine cultures of other Xylariaceous fungi or known cytochalasin-producing fungi, for HIV-1 protease inhibitors. Soon after the discovery of the original *H. fragiforme* culture (MF5511), we obtained a second isolate of *H. fragiforme* from West Virginia (MF5510) that also produced the novel cytochalasin L-696,474. Our screen for HIV-1 protease inhibitors included other *Hypoxylon* cultures, as well as *Nodulisporium*, *Geniculosporium*, *Curvularia*, *Phoma*, *Metarrhizium*, and others. Approximately 45 cultures were examined. Representatives of some of those groups are shown in Table 1. None of the other *Hypoxylon spp.* or other fungi that we screened, produced L-696,474 or any other HIV-1 protease inhibitor, that could be detected under the conditions used here. The levels of L-696,474 produced by *Hypoxylon fragiforme* MF5511 even varied from one experiment to the other, as shown in two separate experiments in Table 1. In addition, our *H. fragiforme* isolates did not produce sufficient amounts of L-696,474 on some of the media, for detection by HPLC or to be considered active by the HIV-1 protease inhibition assay. (The protease inhibition assay is based on 25 ~ 50 μl WBE/ml (whole broth equivalents/ml)). Neither could we detect significant amounts of L-696,474 in extracts of field-collected stromata of *H. fragiforme* (data not shown).

Table 1. Survey of some representative Xylariaceous and cytochalasin-producing fungi.

Isolate, origin	L-696,474 (μg/ml)			% HIV-1 protease inhibition		
	PBGG1 liquid medium	PBGG1 solid medium	Medium-1 solid medium	PBGG1 liquid medium	PBGG1 solid medium	Medium-1 solid medium
Hypoxylon fragiforme, MF5510, *Fagus grandifolia*, New River Gorge, West Virginia	6	—	186	32	12	83
Hypoxylon tinctor, GB1326, *Magnolia virginiana*, Roselle, New Jersey	—	—	—	45	0	0
Nodulisporium sp. L-6, endophyte, live foliage, *Chamaecyparis thyoides*, Ocean Co., New Jersey	—	—	—	11	11	18
Geniculosporium sp., D-10, dead foliage, *Chamaecyparis thyoides*, Ocean Co., New Jersey	—	—	—	32	10	6
Phoma cirsii, MF3750, Instituto Oswaldo Cruz	—	—	—	43	0	0
Metarrhizium anisopliae, MF5430, from ATCC 26852	—	—	—	3	0	0
Curvularia lunata, MF4750, from ATCC 26425	—	—	—	28	2	0
Hypoxylon deustum, DF78, endophyte, *Fagus grandifolia*, Hughes River, West Virginia	—	—	—	0	51	14
Hypoxylon fragiforme, MF5511, *Fagus grandifolia*, Frost Valley, New York						
exp 1	232	68	150	78	66	88
exp 2	100	36	167	84	38	87

Conditions: 25°C, 220 rpm 15 days for liquid, static 21 days for solids. —: None detected.

Note: HIV-1 protease activity measured at 25 μl WBE/ml, except for cultures MF3750, MF5430, MF4750, DF78 which were measured at 50 μl WBE/ml. (Active is >60%).

Medium Development

In medium development for the production of the novel cytochalasin L-696,474, by culture MF5511, various carbon sources were tested on Czapek-Dox agar plates (Difco) with washed cells. From among the 20 carbon sources tested (Table 2), several supported good growth, and were chosen to test in the PBGG1 fermentation medium, in the place of glucose. The effect of 50 mM MES buffer with these carbon sources was also tested, to determine if buffering the medium would improve the productivity. The cultures were grown 14 days, 25°C, 220 rpm in liquid PBGG1 medium, with carbon substitutes for glucose. (Note that carbon substitutions were only for glucose, not for glycerol. Glycerol was included as usual in PBGG1, although it probably does not contribute to the growth or fermentation, based on lack of growth on glycerol in the plate experiments). Table 3 shows that sucrose appeared to yield a higher level of the compound than glucose. MES did not improve the titers. Titers over 400 μg/ml were observed with sucrose in place of glucose in PBGG1 in this experiment. However, the glucose control was also higher in this experiment (339 μg/ml), compared to the 200 μg/ml in the media screen above.

Several nitrogen sources were also tested as a means to improve the fermentation. The results showed

Table 2. Growth of MF5511 *Hypoxylon fragiforme* on various carbon sources.

Carbon source	Amount of growth
Glucose	++++
Fructose	++++
Mannose	++++
Maltose	++++
Dextrin	++++
Sucrose	++++
Lactose	++
Sorbitol	++
Ribose	++
Mannitol	++
Galactose	++
Raffinose	++
Glycerol	−
Sorbose	−
Fucose	−
Lactate	−
Succinate	−
Malonate	−
Citrate	−
Glutamate	−

++++, good growth; ++, fair growth; −, no growth.

Table 3. Effect of carbon source on L-696,474 production in MF5511.

Carbon source	MES	L-696,474 (μg/ml)
Glucose	−	339
Starch	−	109
Sucrose	−	437
Dextrin	−	280
Glucose	+	292
Starch	+	160
Sucrose	+	396
Dextrin	+	364

The medium is PBGG1 with carbon source substitutions, as indicated in the table.

that the Ardamine PH used in the PBGG1 medium yielded the best titers (data not shown).

Fungi of the Xylariales provide a rich source of secondary metabolites[12]. Several members of the order are known to produce cytochalasins, *e.g. Hypoxylon terricola* J. H. Miller[13], *Xylaria* spp.[14,15], and *Rosellinia necatrix*[16]. *Hypoxylon fragiforme* has been found here to produce L-696,474, a novel cytochalasin (18-dehydroxy cytochalasin H), which inhibits the HIV-1 protease. The culture is able to make the compound in fermentation on solid medium, as well as on a number of liquid media surveyed here. The best medium was optimized to yield levels of L-696,474 over 400 μg/ml. It is noteworthy that our limited survey of a group of related organisms did not reveal any which produce L-696,474 or other HIV-1 protease inhibitors. A more extensive screen might have identified such compounds.

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