L-657,398, A NOVEL ANTIFUNGAL AGENT: FERMENTATION, ISOLATION, STRUCTURAL ELUCIDATION AND BIOLOGICAL PROPERTIES

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L-657,398 is a broad spectrum antifungal agent isolated from solid fermentation or from the mycelium of the liquid fermentation of *Aspergillus ochraceus*. Structurally, the compound is a novel pyrollidine related to anisomycin.

Various isolates of *Aspergillus ochraceus* produce ochratoxin, xanthomegnin, mellein, vionellein, trypacidin, aspyrone, ergosta-4,6,8(14),22-tetraene-3-one, flavacol, penicillic acid and two modified asperigillic acids[1]. The subject of this paper is the production of a new secondary metabolite, the pyrollidine L-657,398 (**1**), from the fermentation of *A. ochraceus* ATCC 22947. L-657,398 is structurally related to anisomycin (**3**), an anti-protozoan and anti-yeast compound isolated from *Streptomyces griseolus* and *Streptomyces roseochromogenes*[2~4]. Anisomycin is reported to be a potent and reversible inhibitor of protein synthesis in certain yeasts and mammalian cells[5]. The fermentation, isolation, structural elucidation and antifungal activity of L-657,398 are reported in this paper.

Materials and Methods

Isolations and fermentations were monitored initially *via* zones of inhibition in an agar-disc diffusion assay using *Candida albicans* (MY992, Table 3) and later with TLC, E. Merck, Kieselgel 60 F_{254}, EtOAc, Rf 0.21, iodine visualization.

Flask Fermentation

The seed medium for growth of *A. ochraceus* in flasks consisted of corn steep liquor 5.0 g/liter, tomato paste 40.0 g/liter, oat flour 10.0 g/liter, glucose 10.0 g/liter and trace element mix 10 ml/liter. Trace element mix contained $FeSO_4 \cdot 7H_2O$ 1 g/liter, $MnSO_4 \cdot 4H_2O$ 1 g/liter, $CuCl_2 \cdot 2H_2O$ 25 mg/liter, $CaCl_2$ 100 mg/liter, H_3BO_3 56 mg/liter, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 19 mg/liter and $ZnSO_4 \cdot 7H_2O$ 200 mg/liter. The medium was adjusted to pH 6.8 with NaOH prior to sterilization. *A. ochraceus* ATCC 22947 was inoculated into 50 ml of this sterilized growth medium and cultured for 3 days on a rotary shaker (212 rpm, 5 cm throw) at 28°C.

Two ml of the seed medium culture was transferred, after growth, to a 250-ml unbaffled Erlenmeyer flask containing of cracked corn 10.0 g, yeast hydrolysate (Ardamine pH) 2.0 mg, KH_2PO_4 1.0 mg, $MgSO_4 \cdot 7H_2O$ 1.0 mg, sodium tartrate 1.0 mg, $FeSO_4 \cdot 7H_2O$ 0.1 mg, $ZnSO_4 \cdot H_2O$ 0.1 mg and 25.0 ml of distilled water. After inoculation flasks were incubated for 14 days on a rotary shaker (220 rpm, 5 cm throw) at 25°C.

Fermentor Fermentation

The constituents of the seed medium for the fermentor fermentation were the same as the seed medium described for the flask fermentation above. A lyophilized culture of *A. ochraceus* ATCC

22947, was used to inoculate a 250-ml Erlenmeyer flask containing 50 ml of seed medium. This seed flask was incubated at 28°C for 48 hours on a rotary shaker at 220 rpm. Two % inoculum was used to inoculate 2-liter unbaffled Erlenmeyer flasks containing 500 ml of the seed medium previously described. The second stage seed flasks were incubated at 28°C for 24 hours on a rotary shaker at 200 rpm.

The production medium was adjusted to pH 7.0 prior to sterilization in a nutrient sterilizer. Four sterile New Brunswick fermentors were charged with sterile medium consisting of glucose 10.0 g/liter, glycerol 10.0 g/liter, corn steep liquor 5.0 g/liter, $(NH_4)_2SO_4$ 2.0 g/liter, corn meal 10.0 g/liter, $CoCl_2 \cdot 6H_2O$ 10.0 mg/liter, soybean meal 5.0 g/liter, glycine 2.0 g/liter and P-2000 antifoam (Dow) 3.0 ml/liter in distilled water. Five % inoculum was then used to inoculate each 14-liter scale fermentor containing 10 liters of production medium. The fermentations were carried out at 28°C under a range of conditions for airflow (2.0 to 5.0 liters/minute) and agitation rate (200 to 500 rpm) for 90 hours.

Isolation Flask Fermentation

The solid fungal fermentation consisted of thirty-four 250-ml flasks (initially containing 35 g of corn based medium per flask) which were extracted by adding 100 ml of MeOH, stirring to break up the fungal/medium cake, steeping overnight at room temperature and filtering to yield 2,600 ml of filtrate. The spent mycelium was extracted again with MeOH and filtered to yield 2,500 ml of filtrate.

The first extraction (2,600 ml) was partitioned into two layers by adding 650 ml of water and 3,250 ml of CH_2Cl_2 to make the mixture 4 : 1 : 5, MeOH extract - H_2O - CH_2Cl_2. The lower CH_2Cl_2 - MeOH layer (3,300 ml), contained all the antifungal activity and only 20% of the solids, while the upper MeOH layer is 3,050 ml. A similar procedure was performed on the second MeOH extract (2,500 ml).

The two CH_2Cl_2 - MeOH lower layers from the partitioning steps described above were combined, concentrated to dryness and taken up in 100 ml of EtOAc. This concentrate was chromatographed on 1 liter of silica gel (E. Merck, Grade 62, 60～200 mesh) using EtOAc as the eluant, and the rich cuts from this chromatography were rechromatographed on 200 ml silica gel (EM Science, Kieselgel 60, 230～400 mesh) using EtOAc as the eluting solvent.

The rich cuts from the second silica gel chromatography were combined, concentrated to dryness, diluted to 2 ml with hexane - CH_2Cl_2 - MeOH (10 : 10 : 1) and chromatographed on 100 ml Sephadex LH-20 in the same solvent system. The rich cuts from this chromatography were combined, concentrated and weighed to give 18.9 mg of pure L-657,398 (**1**).

Isolation Fermentor Fermentation

Four 14-liter scale fermentations of *A. ocraceus* ATCC 22947 were filtered and the mycelia extracted with 2 liters CH_2Cl_2 - MeOH (1 : 1) (2×) and 1 liter CH_2Cl_2 - MeOH (1 : 1) (1×). The first extraction yielded two layers due to the water present in the mycelium. The lower CH_2Cl_2 - MeOH layer contained L-657,398. The second 2 liters extraction and third 1 liter extraction resulted in a single phase, but the addition of 200 ml of H_2O to each extract resulted in two layers, the lower (CH_2Cl_2 - MeOH) of which, again, contained L-657,398. All the CH_2Cl_2 - MeOH layers were combined, concentrated to a small volume and rediluted to 500 ml with EtOAc.

This concentrate was chromatographed on 5 liters of silica gel (E. Merck, Grade 62, 60～200 mesh) using EtOAc as the eluting solvent and a flow rate of 100 ml/minute. Active fractions, detected *via* antifungal bioassay, were combined, concentrated to 60 ml *in vacuo* and diluted to 200 ml with CH_2Cl_2 - hexane - MeOH (10 : 10 : 1). This preparation was then chromatographed on 1.5 liters Sephadex LH-20 in the same CH_2Cl_2 - hexane - MeOH (10 : 10 : 1) solvent system.

The rich cut from the Sephadex LH-20 separation was then chromatographed on 2 liters silica gel (E. Merck, Grade 62, 60～200 mesh) using a step gradient of EtOAc - hexane (2 : 8) followed by EtOAc - hexane (1 : 1) and a flow rate of 30 ml/minute. The active fractions were then combined, concentrated and chromatographed on 200 ml silica gel (EM Science, Kieselgel 60, 230～400 mesh) using a step gradient of EtOAc - hexane (35 : 65) followed by EtOAc - hexane (1 : 1) and a flow rate of 4 ml/minute. In both cases the active constituent eluted with the EtOAc - hexane (1 : 1) solvent system.

The final purification step was chromatography on 200 ml Sephadex LH-20 CH_2Cl_2 - hexane - MeOH (10 : 10 : 1) with a flow rate of 2 ml/minute to yield, upon concentration, 619 mg of L-657,398 (**1**) as a waxy solid.

Spectroscopic Methods

^1H and ^{13}C NMR spectra were obtained at 400 and 100 MHz, respectively, on a Varian XL-400 spectrometer at ambient room temperature. Electron impact mass spectra (EI-MS) data were obtained on a Finnigan MAT-212 mass spectrometer; fast atom bombardment (FAB) data were obtained on a Finnigan MAT-731 mass spectrometer. UV spectra were obtained on a Beckman DU-70 spectrophotometer.

L-657,398 (**1**)

The molecular weight of L-657,398 was determined to be 317 by FAB-MS (observed $(M+H)^+$ at *m/z* 318). However, EI-MS of L-657,398 disclosed an apparent molecular ion at *m/z* 316 which corresponds to $(M-H)^+$. Abundant $(M-H)^+$ ions are frequently observed in cyclic amines under EI-MS conditions[6]. The molecular formula $C_{21}H_{35}NO$ (calcd for $C_{21}H_{34}NO$ 316.2639, found 316.2635, $(M-H)^+$) was assigned to L-657,398 based upon the FAB and EI data.

The UV spectrum in MeOH showed a peak at 203 nm, $E^{1\%}_{1cm}$ 407. ^{13}C and ^1H NMR data are listed in Tables 1 and 2, respectively, of the Results and Discussion section.

L-657,398 Acetate (**2**)

L-657,398, 16.6 mg, was dissolved in pyridine and acetic anhydride was added at room temperature to produce the monoacetate derivative, **2** (16.8 mg), of L-657,398. $C_{23}H_{37}NO_2$ (calcd *m/z* 358.2746, found *m/z* 358.2705, for M^+-H) *via* high resolution (HR)-MS. ^{13}C NMR data for this compound is reported in Table 1 of the Results and Discussion section.

Anisomycin (**3**)

Authentic anisomycin was obtained from Sigma, St. Louis, Mo., catalog number A 9789, lot number 56F-4026.

Antifungal Assays

The microbial strains used are listed in Table 3. Stock cultures of filamentous fungi were maintained on potato dextrose agar (Difco, Detroit, Mich.) and were transferred serially at 2 weeks intervals using standard microbiological techniques. Stock yeast strains, *Streptomyces* sp. (MA4798) and *Acholeplasma laidlawii* (MB4558) were maintained frozen at $-80°C$ in 20% aqueous glycerol.

Seeded agar assay plates were prepared according to the type of assay strain. Inoculum for filamentous fungi was prepared by scraping the surface of stock plates with a moistened sterile dacron swab. The spores and mycelia were then suspended in 10 ml of sterile potato dextrose broth (PDB) and adjusted to 70% transmittance (T) at 660 nm. Inoculum for yeasts and bacterial strains was prepared from overnight broth cultures. With the exception of *A. laidlawii*, which required specialized preparation, cultures were then diluted into PDB to a final concentration of either 40 or 70% T at 660 nm (see Table 3). *A. laidlawii* was suspended in brain heart infusion broth supplemented with 2.5% yeast extract and 20% heat inactivated horse serum and adjusted to a final concentration of 60% T at 660 nm. Assay plates were prepared by diluting the inoculum into appropriate molten agar medium, cooled to 45°C, to yield a final concentration of 4%.

Samples were applied to 6.2 mm filter paper discs (25 μl/disc) and air dried at 24°C. The discs were then applied to seeded assay plates with sterile forceps, and rewetted with 25% sterile aq DMSO. The assay plates were then incubated at either 28 or 37°C for 24 hours (see Table 3).

Following incubation, inhibition zones were measured and recorded. Measurement was from the extreme edge of any zone where the growth differs from the background lawn. Inhibition zones were further qualified as follows: fuzzy (F) - a zone that had a fuzzy edge and clear center surrounding the disc, hazy (H) - a zone that was hazy throughout, slightly hazy (S) - a zone in which low levels of growth were discernible throughout the inhibition zone, and very hazy (V) - a zone in which the differences between the background lawn and inhibition zone were barely discernable. Zones without a qualifier were clear throughout.

Table 1. ^{13}C NMR assignments of L-657,398 (**1**) and acetate (**2**)[a].

Assignment	**1**[b]	**1**[c]	**2**[b]
C-14	14.1	14.4 q	14.1 q
$COCH_3$			21.3 q
C-13	22.7 t	23.5 t	22.7 t
C-7	26.3 t	27.1	26.5 t
C-8[d]	29.3 t	30.2 t	29.3 t
C-9[d]	29.58 t	30.2 t	29.5 t
C-10[d]	29.65 t	30.3 t	29.6 t
C-11[d]	29.9 t	30.4 t	29.7 t
C-1	31.9 t	31.2 t	31.8 t
C-6	33.6 t	32.2 t	33.9 t
C-12	34.8 t	32.8 t	34.6 t
NCH_3	38.6 q	33.8 q	37.9 q
C-4	39.3 t	38.4 t	39.3 t
C-5	66.0 d	70.2 d	66.2 d
C-3	70.4 d	69.8 d	71.7 d
C-2	73.7 d	75.6 d	73.3 d
C-4′	126.1 d	127.9 d	126.2 d
C-3′ (2×)	128.4 d	129.7 d	128.4 d
C-2′ (2×)	129.4 d	130.2 d	128.9 d
C-1′	139.4 s	137.45 s	138.9 s
$COCH_3$			170.7 s

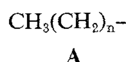
[a] In ppm downfield of TMS at ambient room temperature.
[b] In $CDCl_3$.
[c] In CD_3COOD.
[b] Assignments may be interchanged.

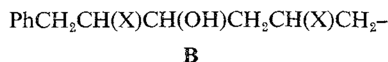
Table 2. ^1H NMR assignments of L-657,398 (**1**) in CD_3COOD[a].

Assignment	**1**	Comments
1-H_a	3.14 dd (5, 13.5)	$J_{1a,2}=5$, $J_{1a,1b}=13.5$
1-H_b	3.29 dd (10, 13.5)	$J_{1b,2}=10$
2-H	3.47 dt (10, ~4.5)	
3-H	4.33 ddd (1.5, 4.3, 6.8)	$J_{2,3}=4.3$, $J_{3,4b}=6.8$, $J_{3,4a}=1.5$
4-H_a	1.93 (1.5, 7.2, 14.8)	$J_{4a,5}=7.2$, $J_{4a,4b}=14.8$
4-H_b	2.65 ddd (6.8, 9.8, 14.8)	$J_{4b,5}=9.8$
5-H	~3.31 obsc	
6-H_a	~2.03 m	
6-H_b	~1.76 m	
$(CH_2)_7$	~1.28 m	
CH_3	0.88 t (6.8)	

[a] Chemical shifts are in ppm downfield of TMS using the solvent peak at δ 2.03 as reference. Coupling constants in Hz are given in parentheses.
obsc: Obscured (overlapping signals).

Fig. 1. Partial structures **A** and **B**.





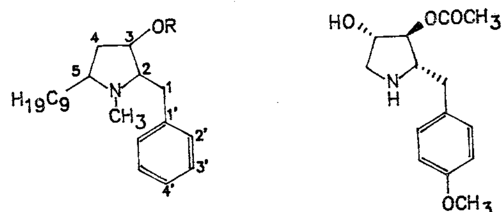
A **B**

Results and Discussion

Structure of L-657,398

HR-MS of the compound gave an empirical formula of $C_{21}H_{35}NO$ and forms a monoacetate derivative $C_{23}H_{37}NO_2$ on acetylation. The molecular formula was confirmed by ^{13}C NMR analysis including APT[7] spectra in $CDCl_3$, which indicated 21 carbons comprising $1\times CH_3$, $10\times CH_2$, $1\times CH_3N$, $3\times CHX$ (X=O or N), $5\times CH=$ and $1\times C=$ (see Table 1) implicating 34 carbon bound protons. ^{13}C NMR assignments were made based on a HETCOR experiment in CD_3COOD (Table 1). The molecule has 5 units of unsaturation and/or rings and must, therefore, have an extra ring in addition to the phenyl ring suggested by the ^{13}C NMR data. ^1H-^1H connectivity experiments, including 1D double irradiation and 2D correlation spectroscopy (COSY) were carried out in a variety of solvents ($CDCl_3$, benzene-d_6,

Fig. 2. The structure of L-657,398 (**1**), L-657,398 monoacetate (**2**) and anisomycin (**3**).



L-657,398 (**1**) R=H
L-657,398 monoacetate (**2**) R=Ac
Anisomycin (**3**)

Table 3. Activity of L-657,398 and anisomycin against a panel of bacteria, filamentous fungi, and yeasts.

Culture number	Media[b]	Temp (°C)	Strain designation	Zone diameter[a] (mm)												
				Anisomycin (μg/ml)						L-657,398 (μg/ml)						
				1,000	500	250	125	62	31	1,000	500	250	125	62	31	16
MA4798	YED	28	*Streptomyces* sp.	0	0	0	0	0	0	13S	12H	10V	0	0	0	0
MY34	YED	28	*Saccharomyces cerevisiae*	25F	22F	20F	16F	14S	11S	20S	17S	15S	11S	9H	0	0
MY992	YED	28	*Candida albicans*	11S	8H	0	0	0	0	12H	10H	9V	0	0	0	0
MF4626	PDA	28	*Cochliobolus miyabeanus*	17F	13F	10H	8V	0	0	40F	36S	32S	31S	27S	21S	17S
MF442	YED	28	*Aspergillus niger*	8V	0	0	0	0	0	13S	10H	8V	0	0	0	0
MF5014	PDA	28	*Penicillium* sp.	23F	18F	14F	11H	9V	0	11H	8V	0	0	0	0	0
MF5016	PDA	28	*Penicillium* sp.	23F	17F	14S	10S	9H	0	9H	7V	0	0	0	0	0
MF5020	PDA	28	*Penicillium* sp.	13H	9H	0	0	0	0	7V	0	0	0	0	0	0
MF11	PDA	28	*A. niger*	14S	10H	0	0	0	0	12S	10S	8H	0	0	0	0
MF4064	PDA	28	*Trichoderma* sp.	10V	8V	0	0	0	0	9H	7V	0	0	0	0	0
MF4332	PDA	28	*Phoma* sp.	0	0	0	0	0	0	13S	9H	0	0	0	0	0
MF3560	PDA	28	*Trichoderma lignorum*	9V	7V	0	0	0	0	19H	16H	13V	0	0	0	0
MF4014	PDA	28	*Fusarium oxysporum*	9H	8V	8V	0	0	0	0	0	0	0	0	0	0
MF1996	PDA	28	*Ustilago zeae*	22H	19H	14H	10H	8V	0	27S	24S	20S	17S	10H	8H	0
MF4042	PDA	28	*Ceratocystis ulmi*	10S	9H	8V	0	0	0	23F	20S	17H	14H	8V	0	0
MF3550	PDA	28	*Alternaria solani*	0	0	0	0	0	0	32F	30F	29S	28H	19H	18H	13H
MF3794	PDA	28	*Verticillium serrae*	0	0	0	0	0	0	21F	17S	13H	10H	0	0	0
MF3587	PDA	28	*Botrytis allii*	26S	22S	17H	12H	10V	0							
MF3769	PDA	28	*Scopulariopsis communis*	0	0	0	0	0	0	0	0	0	0	0	0	0
MF4641	PDA	28	*Cephalosporium* sp.	0	0	0	0	0	0	9H	8V	0	0	0	0	0
MF4608	PDA	28	*Cercospora beticola*	10H	8V	0	0	0	0	20H	18H	15H	12H	12V	8V	0
MY1012	SDA	37	*Candida tropicalis*	30F	26F	22F	15S	11H	9V	25F	21F	18F	15S	11S	8H	0
MY1022	SDA	37	*Candida rugosa*	9V	0	0	0	0	0	8H	0	0	0	0	0	0
MY1028	SDA	37	*C. albicans*	13H	9V	0	0	0	0	10H	8V	0	0	0	0	0
MB4558	BHI	37	*Acholeplasma laidlawii*							0	0	0	0	0	0	0
MY315	SDA	28	*Brettanomyces bruxellensis*	12V	8V	0	0	0	0	0	0	0	0	0	0	0
MY321	SDA	28	*Torulospora hansenii*	15H	10V	8V	0	0	0	14S	12S	9H	0	0	0	0
MY410	PDA	28	*S. cerevisiae*	21S	16S	13S	9H	9V	0	11S	8S	0	0	0	0	0
MY1019	SDA	37	*Candida guilliermondii*	0	0	0	0	0	0	8V	0	0	0	0	0	0
MY1074	SDA	28	*Cryptococcus laurentii*	17V	12V	0	0	0	0	22S	17S	15S	11S	0	0	0
MY1099	SDA	37	*C. albicans*	10V	8V	0	0	0	0	9V	0	0	0	0	0	0
MY1100	SDA	37	*Candida pseudotropicalis*	21F	16F	13F	9H	0	0	16S	13S	8S	7V	0	0	0
MY1062	SDA	37	*Torulopsis glabrata*	8H	0	0	0	0	0	0	0	0	0	0	0	0
MY1070	SDA	28	*Cryptococcus albidus*	0	0	0	0	0	0	12H	9V	0	0	0	0	0
MY1073	SDA	28	*Cryptococcus laurentii*	14V	9V	0	0	0	0	19H	15H	13H	8V	0	0	0
MY1077	SDA	28	*C. laurentii*	8V	0	0	0	0	0	17H	15H	12H	8V	0	0	0
MY1113	SDA	28	*Kluyveromyces fragilis*	14F	10S	9H	8V	0	0	14S	10S	8H	0	0	0	0
MY1029	SDA	28	*C. albicans*	9V	0	0	0	0	0	8V	0	0	0	0	0	0
MY1055	SDA	28	*C. albicans*	8V	0	0	0	0	0	9V	8V	0	0	0	0	0
MF4784	PDA	37	*Rhizomucor miehei*	0	0	0	0	0	0	9H	7V	0	0	0	0	0
MF383	PDA	28	*Aspergillus flavus*	0	0	0	0	0	0	15S	12S	9H	0	0	0	0
MF4839	PDA	28	*Aspergillus fumigatus*	8V	0	0	0	0	0	14S	8H	0	0	0	0	0

[a] F: Fuzzy edge, S: slightly hazy, H: hazy, V: very hazy.

[b] Difco PDA: Potato dextrose agar, SDA: SABOURAUD dextrose agar, YED: yeast extract dextrose, BHI: brain heart infusion.

CD_3COOD) of which CD_3COOD proved to be optimal in terms of chemical shift dispersion.

The data (see Table 2) suggested partial sequences **A** and **B** (Fig. 1) which on the basis of the empirical formula and ^{13}C NMR data can be formulated into the *N*-methyl pyrrolidine structure **1** (Fig. 2). The ^1H NMR spectrum of the acetate derivative **2** confirms the assignment of the OH group by the characteristic downfield shift of 3-H from δ 4.33 to 5.13. Confirmation was obtained by MS fragmentation which is directed by the ring nitrogen, resulting in a base peak at m/z 226.2165 (calcd for $C_{14}H_{28}NO$, m/z 226.2170) due to loss of tropylium ion (m/z 91) and a weaker ion at m/z 190.1224 (calcd for $C_{12}H_{16}NO$, m/z 190.1231) corresponding to the loss of the C_9 alkyl side chain. The corresponding ions in the *O*-acetate derivative **2** are greater by 42 mass units. The MS, ^1H NMR (see Table 2) and ^{13}C NMR (see Table 1) are, therefore, fully consistent with the pyrrolidine structure **1** for L-657,398 shown in Fig. 2. However, the relative stereochemistry could not be unambiguously assigned based on ^1H NMR.

Antifungal Activity

The antifungal activity of L-657,398 was determined by the disk diffusion method (as described in the Materials and Methods section) and compared to anisomycin at a variety of concentrations ranging from 1 mg/ml to 16 μg/ml. The results of this evaluation indicated that the antifungal spectrum of L-657,398 was broad, with activity against both filamentous fungi and yeasts, and significantly broader than anisomycin.

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