INTRINSIC FLUORESCENT COMPOUNDS IN *ARMILLARIA MELLEA;* HYPHAE AND RHIZOMORPH

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Abstract

The organic solvent extraction of *Armillaria mellea* showed different highly intrinsic fluorescent compounds either in rhizomorph or in hyphae (F_1, F_2, F_3, MF_1, MF_2 and MF_3). The characterization of ultraviolet and fluoro-spectrophotometry of these compounds has been exhibited. Thin layer chromatography and fluorescent spectrum of hyphae and rhizomorph indicated an additional compound (FH) in hyphae. The compound probably plays a role in differentiation from hyphae to rhizomorph.

Introduction

Armillaria mellea, a pathogenic basidiomycete, is the causal agent of root disease in deciduous and evergreen trees, and also exists as a saprophyte, particularly on tree stumps [1]. The biologically active sesquiterpene esters isolated from different strains of the fungus comprise two major structural types, represented by armillyl orsellinate and melleolide [2, 3, 4].

Three sesquiterpene aryl esters have been isolated from a mycelial extract of *A. mellea* [5]. In China, tablets containing artificially cultured mycelium of *A. mellea* (vahl Ex. Fr...) Quel, (Tricholomataceae) are used for the treatment of dizziness, headaches, neurasthenia, insomnia, numbness of the limbs and infantile convulsions [6]. Cultured Mi Huan jun *A. mellea* mycelia are used in the form of tablets to treat geriatric patients with palsy. Recently a novel N^6-substituted adenosine has been isolated from Mi Huan jun as a cerebral protecting compound [7]. We previously reported, in the Knop-Heller medium, that serine promotes differentiation from hyphae to rhizomorph [8].

Materials and Methods

A. mellea was obtained from *Quercus sativus* from the forests of Karasang, Amol, Iran. Each culture was produced by growing segments of fungi stalk (5 mm thick) on malt agar and incubating them at 25°C in the dark. The clones were maintained on malt agar at 4°C and later transferred to other media.

In the Knop-Heller* medium plus peptone (g lit^{-1}) under the anaerobic condition only the mycelium was produced without differentiation to rhizomorph. In ammoniacal Knop-Heller (NO_3^- replaced by NH_4^+) under anaerobic and aerobic conditions the mycelium was produced without rhizomorph [8].

Vegetative structures from the above cultures were dried for 24 h at 60°C, ground and extracted in a soxhlet apparatus for 2 days with chloroform. The chloroform extract was filtered, evaporated and dissolved in chloroform (10 $mgml^{-1}$). One kilogram of dried and powdered mycelia gives 45 g of brown oil extract.

The extract was chromatographed on a column of silica gel, (0.45 mm) using hexane-ethyl acetate-

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* Knop-Macroelement; $(NO_3)_2Ca$, $4H_2O$ 1g. lit^{-1}, NO_3K 250 mg.lit^{-1}, SO_4Mg,$7H_2O$ 250 mg.lit^{-1} PO_4H_2K 250 mg lit^{-1}. Heller - Microelement; Cl_3Fe, $6H_2O$ 1 mg.lit^{-1}, SO_4Z_n, $7H_2O$ 1 mg. lit^{-1}, BO_3H_3 1 mg. lit^{-1}, SO_4Mn, $4H_2O$ 0.1 mg. lit^{-1} SO_4Cu, $5H_2O$ 0.03 mg. lit^{-1}, Cl_3Al 0.03 mg.lit^{-1}, Cl_3Ni, $6H_2O$ 0.03 mg.lit^{-1}, Ik 0.01 mg.lit^{-1}, MO_3 10^{-10} g. lit^{-1}.

methanol (80: 20: 1) and purified further by prep. TLC (PLC) using the same solvent. Absorption spectra were recorded on a Shimadzu - 160 UV-VS spectrophotometer.

Corrected fluorescence excitation and emission spectra were obtained by use of a RE - 500 spectrofluorometer. Fluorescence spectra of TLC were recorded by a CS-9000 densitometer (Shimadzu).

Results and Discussion

The intrinsic fluorescent metabolites (F) were purified by silica gel column and PLC and visualized by 366 nm in the UV-cabinet (Fig. 1). Six compounds were noticed with rhizomorph extract (F_1, F_2, F_3, MF_1, MF_2, MF_3), which had vivid blue flourescence in TLC.

R_F of these compounds with their TLC and UV spectrum characteristics are presented in Table 1. The hyphae extract showed another band (F_H) with vivid violet fluorescence (Fig. 1, 2). These compounds also exist in culture media of rhizomorph and hyphae and there are many similarities between them (Fig. 2, 3).

The fluorescent spectra of the compounds depend on the solvent, and differ in methanol and tris - HCl pH 7.4.

Table 1. R_F and UV spectra of F compounds

F compounds*	R_F	Characterization of UV MeOH λ_{max} (nm),...
F_1	0.06	260 , 210
F_2	0.12	215 , 268.8
F_3	0.56	210 , 348
MF_1	0.37	269 , 8 , 280 , 292
MF_2	0.40	259 , 269 , 8 , 280 , 293
MF_3	0.42	210 , 220 , 240
F_H	0.64	226 , 340 , 360 , 381

* F_1 , F_2 , F_3 shows high flourescence and MF_1 , MF_2 , MF_3 moderate fluorescence in plate.

The λexcitation (λex), λemission (λem) and fluorescence intensity of the compound are shown in Table 2. As we observe, F_3 has the greatest intensity.

For determination of fluorescence intensity of the compounds in the aqueous media, stock solutions of these compounds were obtained by dissolution in dimethyl sulfoxide (DMSO) with a concentration of 1 mg/200 µl. These solutions were stored at -20° C. Further experiments were performed with 200 µl of stock solution diluted with 4 ml tris - HCl pH 7.4 (Table 3). In

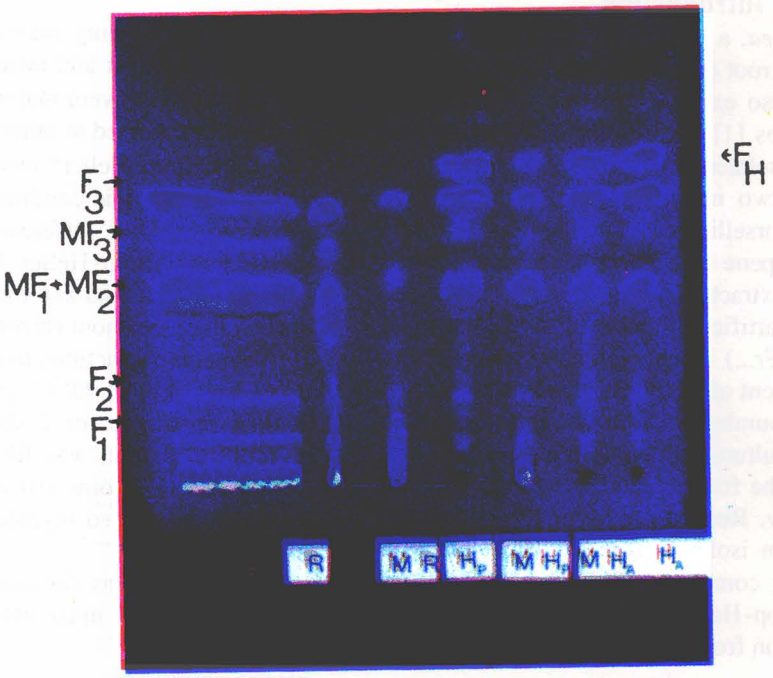

Figure 1. The chromatogram of TLC
R = Rhizomorph MR=Medium of rhizomorph
H_P = Hyphae in peptone medium
MH_P = Medium of hyphae
MH_A =Ammoniacal medium of hyphae
H_A = Hyphae from ammoniacal medium

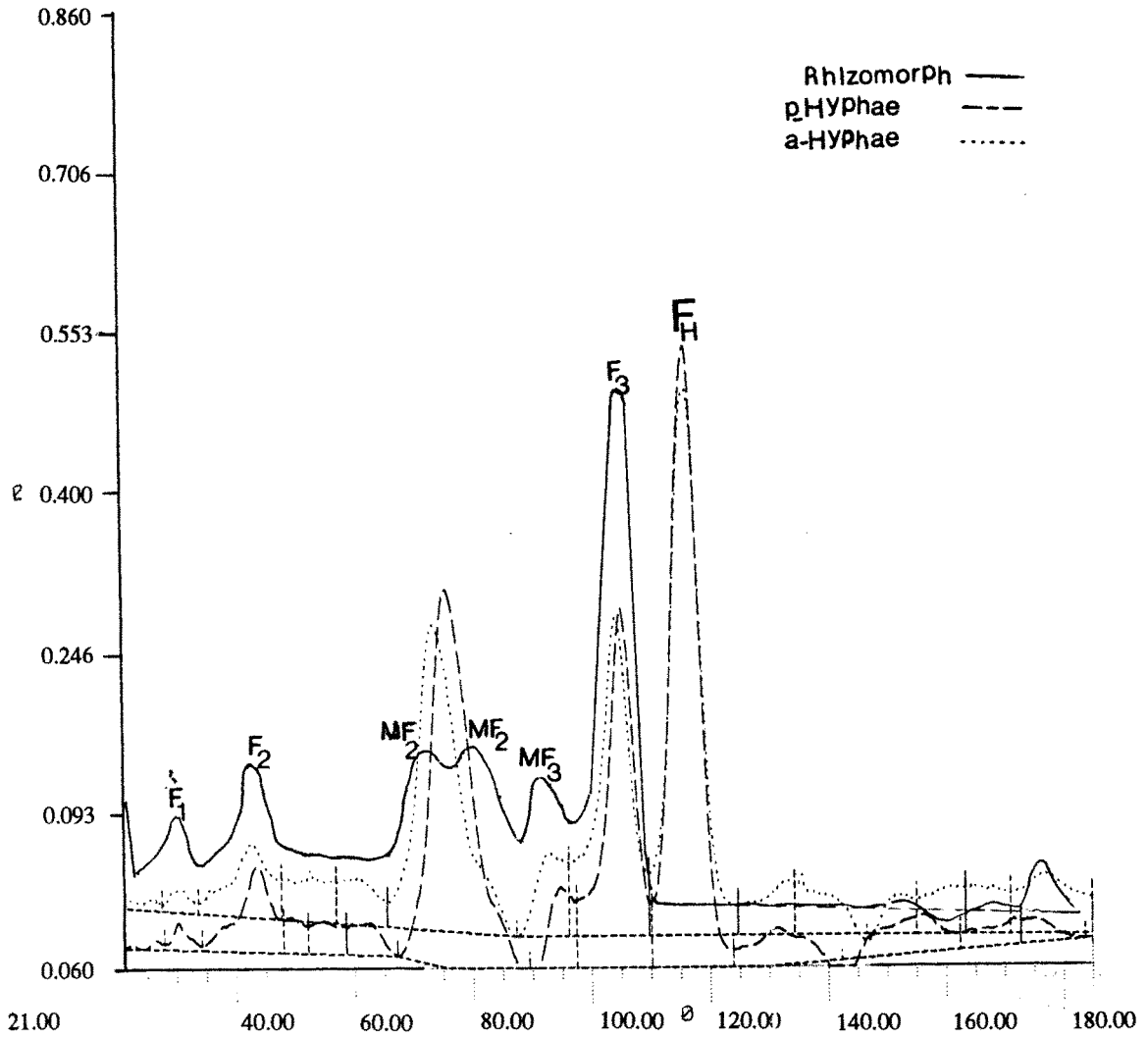

Figure 2. Fluoro-densitometric spectrum of rhizomorph ——
Hyphae from peptone media – – – –
Hyphae from ammoniacal media .....
PKF (Peak find filter) = 30
DF (Drift line) = -0.04
= 366 nm

Table 2. Fluorescence intensity of F-metabolite in methanol

Fluorescent compound	λex (nm)	λem (nm)	Fluorescence intensity in $mg.ml^{-1}$
F_1	365	443.2	2.614
F_2	356	432	1.576
F_3	356	430.4	17.02
MF_1	372	460.8	1.40
MF_2	356	430	1.68
MF_3	366	440	1.41
F_H	359.0	428.8	16.51

Table 3. The characterization of F compounds in tris - HCl pH 7.4

Fluorescent compound (F)	λex (nm)	λem (nm)	Fluorescence intensity in $mg.ml^{-1}$
F_1	356	476.8	119.65
F_2	365	473.6	81.41
F_3	355	470.4	1.73
MF_1	350	460	9.03
MF_2	350	460.8	3.63
MF_3	340	388.8	8.8
F_H	359.0	440.0	140.1

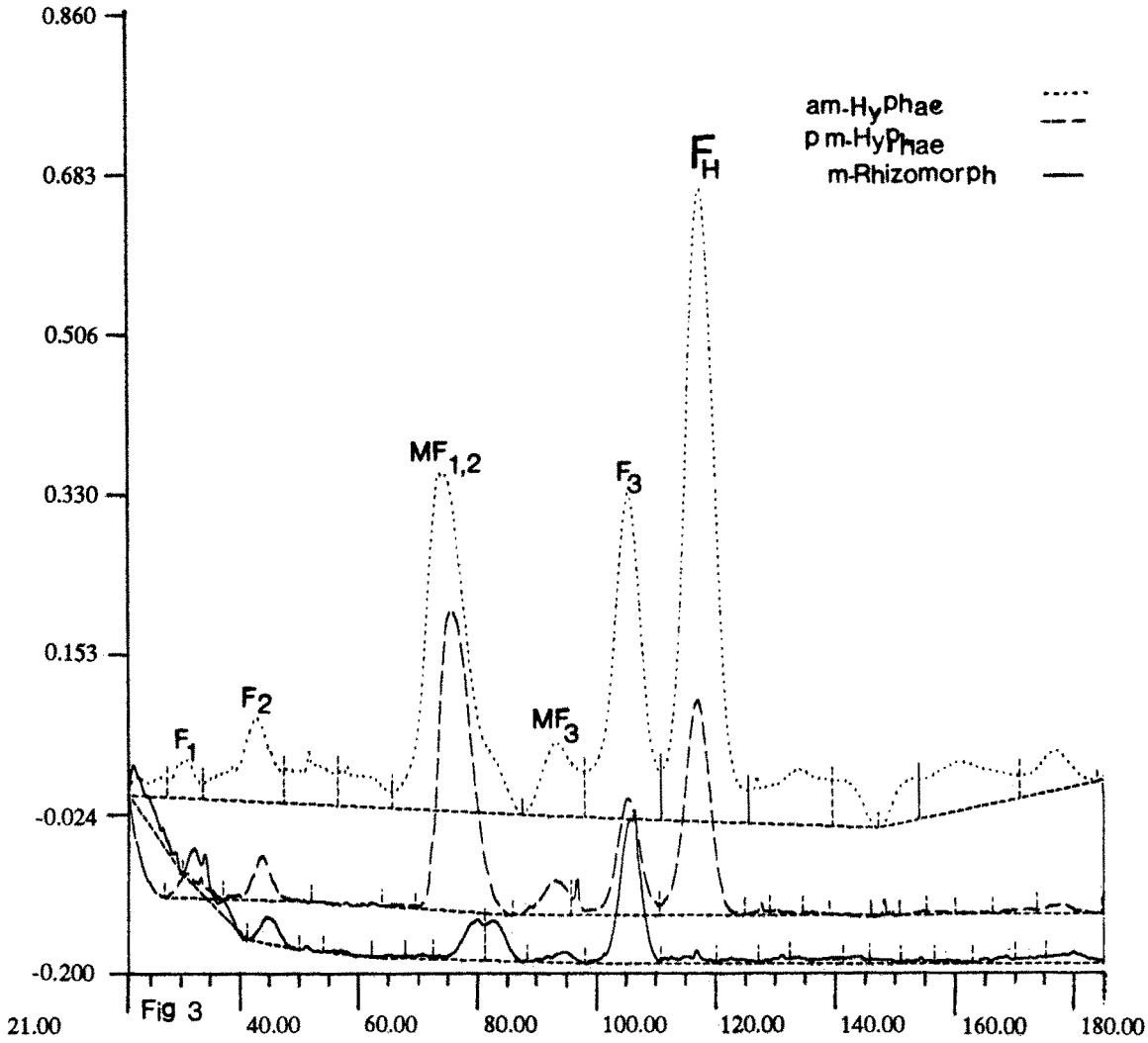

Figure 3. Fluoro-densitometric spectrum of F-compounds in
media of rhizomorph ——
Peptone media of hyphae – –
Ammoniacal media of hyphae .....
PKF (Peak find filter) = 30
DF (Drift line) = -0.06
= 366 nm

this condition, λex and λem change with the solvent, and although F_H preserves its first rank, F_1 instead of F_3 holds second place.

The results of fluorescence scanning densitometry of the compounds showed the percent of each metabolite in the rhizomorph and hyphae (Tables 4 and 5). Recordings from this experiment show that the extracts obtained from the rhizomorph and hyphae differ not only by the kind of compounds but also by relative amounts.

Table 4. F-compound percent in the rhizomorph

Fluorescent metabolite (F)	%
F_1	13.781
F_2	5.742
F_3	39.311
MF_1	21.069
MF_2	10.457
MF_3	4.71

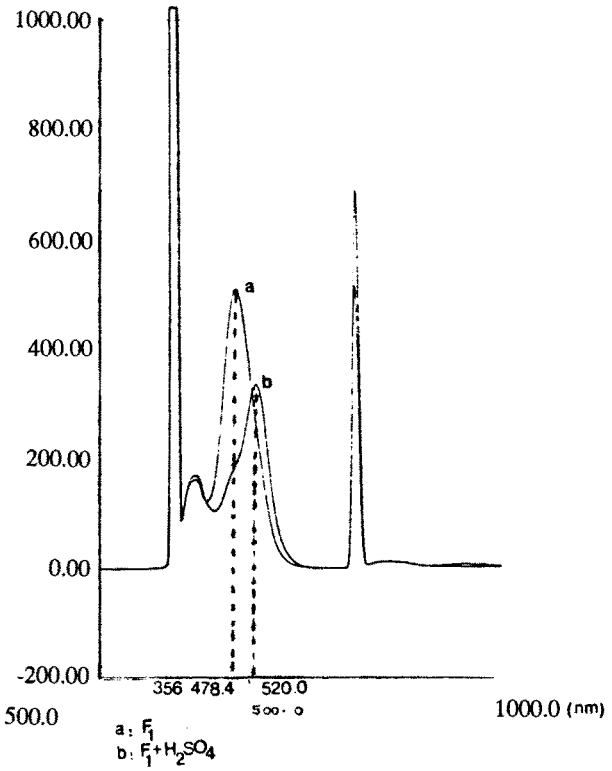

Figure 4. 1 ml of stock solution of F_3 4 ml tris-HCl pH 7.4 and 50 µl concentrated H_2SO_4

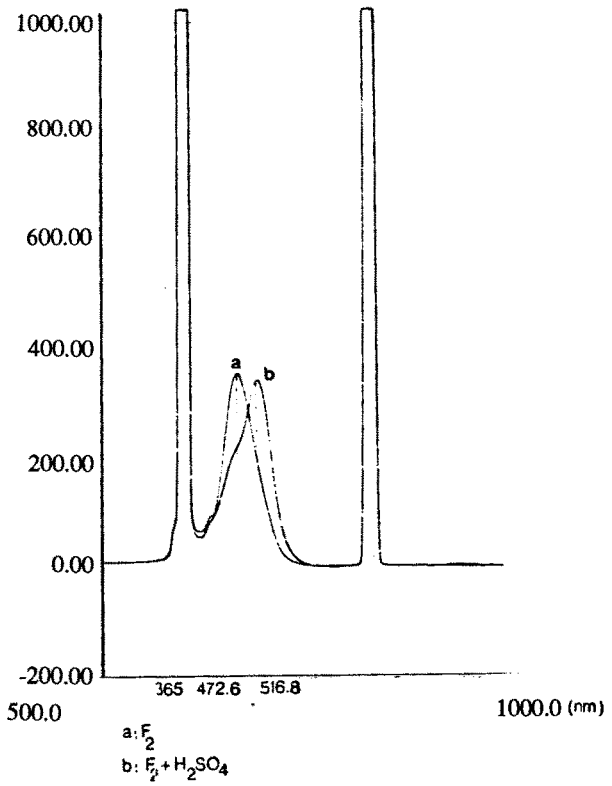

Figure 5. 1 ml of stock solution of F_1 4 ml tris-HCl pH 7.4 and 50 µl concentrated H_2SO_4

Band Width (nm)
EX = 5.0
EM = 10.0
Response (sec): Auto
Sensitivity; High
Shutter Cont: Manual

Table 5. F-compound percent in the hyphae

Fluorescent metabolite (F)	%
F_1	3.077
F_2	5.655
F_3	13.963
$MF_1 + MF_2$	49-64.5
MF_3	4.06
F_H	21.09

The mixture of concentrated H_2SO_4 and these metabolites showed a vivid green fluorescence in UV light. Spectrofluorometry of these mixtures showed a λem shift to 520 nm. The reaction is useful for characterization of $C_{19}O_3$ steroids [11]. Only three compounds showed a λem shift (Table 6 and Figs. 4, 5, 6).

Table 6. The mixture of concentrated H_2SO_4 and F-compounds*

Fluorescent compound (F)	λex (nm)	λem (nm)	λem (nm) (New)	Δλem
F_1	356	478.4	520.0	41.6
F_2	365	472.6	516.8	44.2
F_3	355	470.4	520.0	49.6

* 1 ml of stock solution, 4 ml tris - HCl pH 7.4 and 50 µl concentrated H_2SO_4

Here overall results from our experiments are given:

1. Such fluorescent compounds have not previously been reported in *A. mellea.*
2. Some of these metabolites, for example F_1 (probably $C_{19}O_3$ steroid) show remarkable fluorescent spectrum in the aqueous media (tris HCl pH 7.4). This feature is usually used in biochemical and biophysical methods, for tracing and interaction studies, for example interaction of a fluorescent ligand like actino-

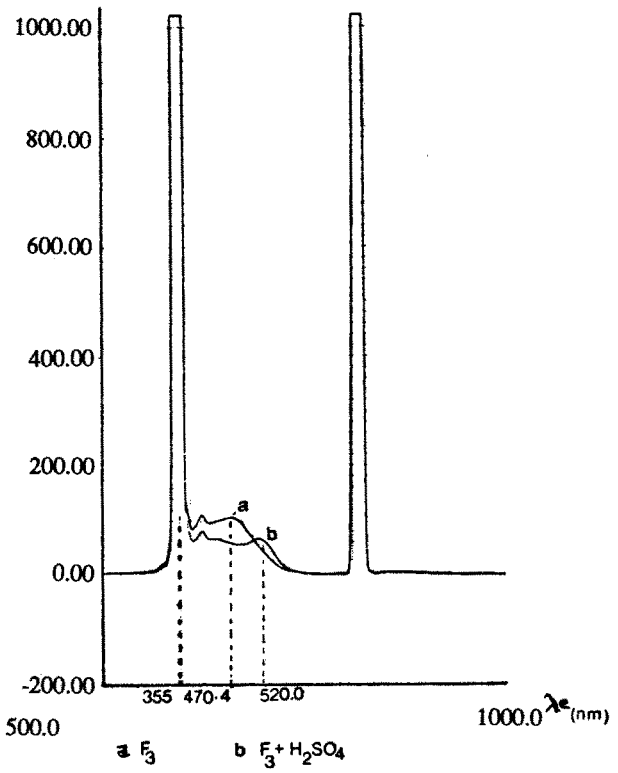

Figure 6. 1 ml stock solution of F_2 , 4 ml tris-HCl pH 7.4, 50 µl concentrated H_2SO_4

mycin and DNA [12]. In our laboratory such investigations, for example the interaction of these compounds and E. coli DNA, calf thymus DNA and plant DNA, are being continued.

3. Some of the metabolites, such as F_H, probably act as a repressor agent in differentiation of hyphae (mycelium) to rhizomorph, and prevent the attack of parasite to host.

4. Strongly, according to the Rex. M. C. Dawson experiment 1986 [11], some of these compounds such as F_1, F_2, F_3 are $C_{19}O_3$ steroid. Purification of these is now underway.

5. Many of these fluorescent compounds when secreted into a medium have very strong antibacterial and fungicide activity.

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