ISOLATION OF RADICICOL FROM *PENICILLIUM LUTEO-AURANTIUM*, AND MELEAGRIN, A NEW METABOLITE, FROM *PENICILLIUM MELEAGRINUM*[1]

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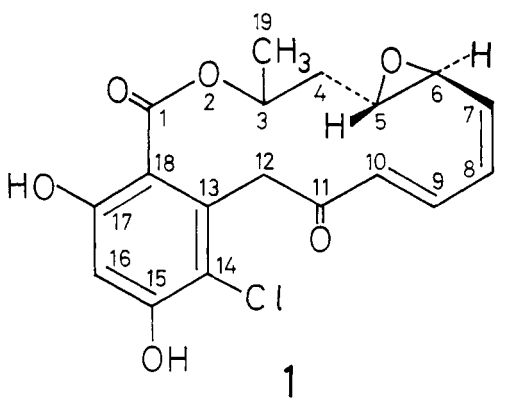
ABSTRACT.—Antifungal principle of *Penicillium luteo-aurantium* was isolated and identified as radicicol (**1**). A new metabolite of the molecular formula $C_{23}H_{23}N_5O_4$, designated meleagrin, was isolated from *Penicillium meleagrinum*. It was clarified that meleagrin has a structural similarity with tremorgenic mycotoxins, such as fumitremorgin A and B (synonymous to lanosulin), verruculogen, and roquefortin C and D.

In screening *Penicillium* species for antimicrobial metabolites, we found that the culture broth of *Penicillium luteo-aurantium* Smith showed strong antifungal activity. The active principle was isolated and disclosed to be radicicol (**1**) (synonymous to monorden), which is known as a macrolide antibiotic and a potent tranquilizer of remarkably low toxicity (2). While there are two reports so far on the isolation of this antibiotic, one from *Monosporium bonorden* (2) and the other from *Cylindrocarpon radicicola* (3), this is the first report of its isolation from a mold of *Penicillium* species.



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The present authors found also that the culture broth of *Penicillium meleagrinum* Biourge had antibacterial activity. While no active principle has been successfully isolated, a new yellow pigment of the molecular formula $C_{23}H_{23}N_5O_4$, which we named "meleagrin," was isolated. It was demonstrated that meleagrin is the compound formed from two nitrogen-containing amino acids with one isoprenyl side chain in its structure. It is obvious that meleagrin belongs to the category of the tremorgenic mycotoxins, all of which were recently found, such as fumitremorgin A (4, 5, 6), fumitremorgin B (synonymous to lanosulin) (4, 7, 8), verruculogen (9), roquefortin C (10, 11) and roquefortin D (12).

There are no reports published on the isolation of any metabolites from these two molds of *Penicillium* species.

[1]Part 6 in the series "Studies on Fungal Products." For Part 5 see LITERATURE CITED 1.

EXPERIMENTAL[2]

ORGANISMS.—*Penicillium luteo-aurantium* Smith (IFO8176) and *Penicillium meleagrinum* Biourge (IFO 8143) were obtained from the Institute for Fermentation, Osaka, Japan. The organisms were maintained on slants of malt extract agar containing 3% malt extract powder, 0.1% peptone, and 2% agar.

FERMENTATION.—The organism was grown in a medium containing 3% sucrose, 0.6% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.01% cornsteep liquor, 0.001% $FeSO_4 \cdot 7H_2O$, and a trace of biotin and vitamin B_1 in water. The pH of the medium was adjusted to 5.5 by adding dilute NaOH solution. The medium was distributed into Roux bottles (200 ml per each bottle) which were stoppered with cotton plugs and autoclaved at 121°C, 15 psi for 30 minutes. Each bottle was inoculated and cultured. In the case of *P. luteo-aurantium*, 1200 Roux bottles containing a total of 240 liters of the culture medium were used for stationary cultivation at 27° for 21 days. And in case of *P. meleagrinum*, 240 bottles, or 48 liters, of the medium were used for stationary cultivation at 27°C for 17 days.

ANTIMICROBIAL TESTS.—*Bacillus subtilis* (IFO 3108) and *Escherichia coli* (IFO 3044) were used for antibacterial testing. And *Giberella zeae* (IFO 4474) and *Neurospora sitophila* (IFO 4956) were used to test for antifungal activity. Small portions of the culture filtrate of each mold were extracted with methylene chloride, or with ethyl acetate at various pH between 2.0 to 10.0, and the activity was examined with each of the extracts. Activated charcoal, 2 g per 100 ml, was added to the culture filtrate after extraction and stirred for 12 hours. The charcoal collected by filtration was washed with a small amount of water, dried and extracted with acetone. Antimicrobial activity was tested also with this acetone extract. The methodology for both the qualitative and quantitative assay was the same as those reported in the preceding paper (1).

ISOLATION OF RADICICOL (**1**) FROM *Penicillium luteo-aurantium*.—Antifungal activity against *Neurospora sitophila* and *Giberella zeae* was demonstrated with the culture filtrate in the preliminary test of antimicrobial activity. And the substance responsible for the activity was found to be present only in the extract from the acidified cultures. Thus the culture filtrate (240 liters) was acidified to pH 2 with concentrated hydrochloric acid and extracted (usually in 3 liter portions) with one-third of its volume of methylene chloride. The extract was dried over anhydrous sodium sulfate and the solvent evaporated *in vacuo* to leave a crude brown solid (2.3 g). The solid was dissolved in 30 ml of benzene and poured onto the column (3.2×60 cm) of silica gel[3] and eluted with a mixed solvent of benzene-acetone (20:1). By use of a fraction collector,[4] sixteen-ml fractions were collected. Fractions 84–107 showed strong antifungal activity, and these portions were combined and the solvent was removed by evaporation *in vacuo* to give 1.6 g of crude crystalline substance. This was purified by recrystallization from benzene to yield colorless needles, mp 191°. *Anal.* Calcd for $C_{15}H_{17}O_6Cl$: C, 59.11; H, 4.29. Found: C, 59.39; H, 4.71. $[\alpha]D+203°$ (c=0.45, $CHCl_3$) [literature; +216° (13, 14), +203° (15)]. Cmr (deuterated acetone) δ 18.49 (C-19, q), 36.69 (C-4, t), 46.44 (C-12, t), 55.60 (C-5 or 6, d), 55.95 (C-6 or 5, d), 71.74 (C-3, d), 103.68 (C-16, d), 110.00 (C-18, s), 115.59 (C-14, s), 130.10 (C-7, 8, 9 or 10, d), 130.86 (C-7, 8, 9 or 10, d), 135.56 (C-7, 8, 9 or 10, d), 136.35 (C-13, s), 138.91 (C-7, 8, 9 or 10), 157.87 (C-17, s), 160.92 (C-15, s), 168.73 (C-1, s), 196.79 (C-11, s).

Upon continuous extraction of mycelium (350 g) with hexane in an Asahina-type extractor (16), a yellow-colored sticky liquid remained after removal of solvent. It was washed with a small amount of methanol and recrystallized from ethanol to give colorless needles, mp 161° (1.6 g), which was identified as ergosterol by mixed fusion with the authentic sample.[5]

ISOLATION OF MELEAGRIN FROM *Penicillium meleargrinum*.—The culture filtrate (pH 9), 48 liters in all, was adjusted to pH 7 by adding hydrochloric acid, and then extracted with one-third of its volume of ethylene chloride. The crude extract (1.6 g) obtained after removal of the solvent was purified by repeated crystallization from chloroform to give thin yellow leaves of meleagrin, which melted at 250° under decomposition (800 mg). *Anal.* Calcd for $C_{25}H_{23}N_5O_4$:

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[2]Melting points were not corrected. The ir spectrum was obtained in a KBr pellet on a Hitachi model 215 spectrophotometer. The uv spectrum was determined in ethanol on a Hitachi model 124 spectrophotometer. Mass spectra were obtained on a Hitachi RMS-4 spectrometer. High resolution mass spectra were measured on a JMS-01SG-2 spectrometer. The pmr and cmr spectra were obtained at room temperature on a JEOL FX-100 spectrometer, at 100 MHz and 25.05 MHz, respectively, with tetramethylsilane as internal standard. Sample concentration and number of scans at cmr were 0.17 mmol per ml, and 20000, respectively. Off resonance technique was used for decoupling at cmr.

[3]Kanto Kagaku Co. Ltd., 100 mesh.

[4]Toyo SF-160K rectangular balance-operated fraction collector.

[5]Tokyo Kasei Kogyo Co. Ltd.

C, 63.73; H, 5.35. Found: C, 63.09; H, 5.41. $[\alpha]D-116°$ (c=0.088, $CHCl_3$). Uv λ max (EtOH) 232 (log ε 4.39), 285 shoulder (3.92), 349 nm (4.44); uv λ max (EtOH–HCl) 232 (log ε 4.44), 285 shoulder (4.04), 330 (4.44), 344 nm shoulder (4.38); ir ν max (KBr) 3420, 3180, 3075, 2975, 2940, 1700, 1670, 1655, 1623, 1584, 1458, 1440, 1390, 1352, 1316, 1250, 1228, 1110, 1030, 982, 910, 822, 764, 690; *m/e* 433.1796 (required for $C_{23}H_{23}N_5O_4$ 433.1750, M^+), 402, 365, 364 (base peak), 334, 318, 305, 290, 277, 262, 176, 162, 108, 86, 84, 69.

The mycelium was dried (30 g), pulverized, and extracted continuously for 6 days with hexane, then 4 days with methylene chloride. From the hexane extract was obtained ergosterol (0.8 g). After removal of the solvent, the methylene chloride extract was washed with 10 ml of hexane and dissolved in chlorform. From this solution an additional 20 mg of meleagrin crystallized.

PREPARATION OF MELEAGRIN MONOACETATE.—Meleagrin (50 mg) was reacted with 1 ml of acetic anhydride in the presence of 0.5 ml of pyridine at room temperature for 48 hours. Water (25 ml) was then added. The precipitate, which was collected by filtration, was washed with water, dried and crystallized from benzene-petroleum ether to give meleagrin monoacetate as a yellow crystalline powder, melting at 247° (dec), *m/e* 475.1871 (required for $C_{25}H_{25}N_5O_5$: 475.1856, M^+), 444, 433, 407, 375, 365, 364, 334, 318, 305, 290, 277, 262, 196, 149, 108, 83, 70, 60, 55, 43; ir ν max (KBr) 3400, 2980, 2950, 2820, 1770, 1708, 1690, 1640, 1590, 1460, 1405, 1370, 1350, 1307, 1197, 1112, 1090, 1030, 1006, 986, 922, 830, 760.

RESULTS AND DISCUSSION

Colorless needles (mp 191°) which were isolated from the culture filtrate and the mycelia of *P. luteo-aurantium* constituted the sole substance responsible for the strong antifungal activity. This compound, soluble in sodium carbonate but not soluble in sodium bicarbonate solution, was thought to be phenolic in view of the blue color resulting from the ferric choloride test. The presence of a chlorine atom in the structure was demonstrated in the mass spectrum where the relative intensity ratio of M^+ to M^+-2 was three to one. High resolution mass spectrometry showed a molecular ion at *m/e* 366.0676 with a comparison of $C_{18}H_{17}O_6{}^{37}Cl$, and *m/e* at 364.0722 with a comparison of $C_{18}H_{17}O_6{}^{35}Cl$. There are two hydroxyls, one chelated showing pmr singlets at 11.07 and 6.29 ppm both exchangeable with deuterium oxide. A benzene nucleus is penta-substituted, as judged from the pmr spectrum in which only one aromatic proton signal exists at 6.64 ppm. In addition to these data, all the analytical (elemental analysis and [α]D) and the spectral (uv, ir, pmr, and mass) data indicated this compound to be radicicol (**1**) conforming to the data presented by R. N. Mirrington *et al.* (13, 14) and F. McCapra *et al.* (15). In addition, the cmr spectra were examined, and all the signals were assigned to their appropriate carbons exactly as expected for the structure of radicicol, thus re-confirming the structure presented by the preceding authors (13, 14, 15).

A new nitrogen-containing metabolite, designated meleagrin, was isolated from *P. meleagrinum*. The molecular formula was determined as $C_{23}H_{23}N_5O_4$ by high-resolution mass spectrometry and elemental analysis. Upon acetylation with acetic anhydride, it gave a monoacetate. A methoxy group is present in the structure, as seen from the three-proton signal at 3.73 ppm in the pmr, and a quartet signal at 64.91 ppm in the cmr spectrum. Two methyls in geminal position are also present, because there are two three-proton singlets at 1.24 and 1.35 ppm in the pmr spectrum and a quartet signal at 23.88 ppm in the cmr spectrum. These geminal methyls suggest the presence of one isoprenyl unit in the molecule. This assumption was strongly supported by the mass spectrum, where the primary fragmentation was the loss of a C_5H_9 radical (mass 69) to give a prominent fragment at 364 (base peak) (11). The ir absorption at 3180, 1655, and 1670 cm^{-1} could be assigned to amide groups, and in view of the lack of the amide II band near 1550 cm^{-1}, meleagrin was suggested to have a diketopiperazine structure (8, 12). The two carbonyl signals at 159.93 and 166.66 in the cmr spectrum con-

clusively ascertained this assumption (11). It can be said that in view of the presence of the diketopiperazine ring in the molecule this compound is formed from two amino acids. Very recently, various tremorgenic toxins were isolated from mold origins. They contain a diketopiperazine ring and are all formed from two amino acids, and have 1 to 3 isoprenyl side chains. Fumitremorgin A ($C_{32}H_{41}N_3O_7$) is the compound formed from 6-methoxytryptophan and proline, and has 3 isoprenyl side chains (4, 5, 6). Fumitremorgin B (synonymous to lanosulin) ($C_{27}H_{33}N_3O_5$) (4, 7, 8) and verruculogen ($C_{27}H_{33}N_3O_7$) (9) are both from 6-methoxytryptophan, proline, and 2 isoprenyl moieties. Roquefortin C ($C_{22}H_{23}N_5O_2$) (10, 11) and D ($C_{22}H_{25}N_5O_2$) (12) are from tryptophan, histidine, and 1 isoprenyl moeity. Brevianamide A ($C_{23}H_{25}N_3O_4$) (17, 18) and austamide ($C_{21}H_{21}N_3O_3$) (19) are both from tryptophan, proline and 1 isoprenyl unit. Thus we consider that meleagrin is a new addition to this same category, having two nitrogen-containing amino acids and one isoprenyl side chain. A toxic property might be expected. Further study on the biological activity and the structure is in progress.

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