ISOLATION IN HIGH YIELD OF CITRININ FROM *PENICILLIUM ODORATUM* AND OF MYCOPHENOLIC ACID FROM *PENICILLIUM BRUNNEO-STOLONIFERUM*[1]

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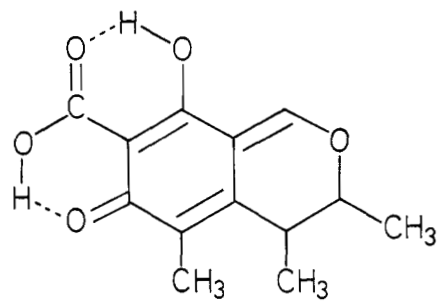
In screening various fungi for antimicrobial metabolites, the authors isolated oosponol and oospolactone as antifungal constituents of *Gloeophyllum sepiarium* (Wulf. ex Fr.) Karsten (1, 2). Further investigation of this type led us to the finding that *Penicillium odoratum* Christensen & Backus produced an antibacterial metabolite in its culture broth and also that *Penicillium brunneo-stoloniferum* Abe produced an antifungal metabolite in its culture broth and mycelium. These two compounds were identified as citrinin (**1**) and mycophenolic acid (**2**), respectively. The yields of these two



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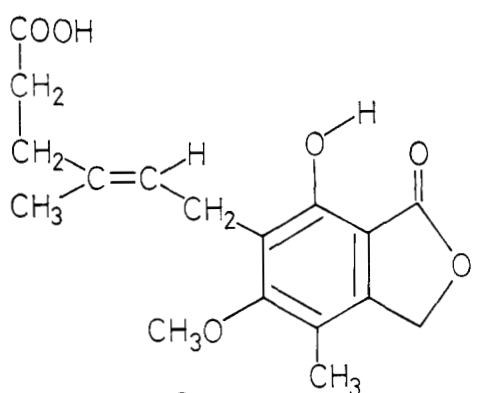

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antimicrobial compounds were excellent as compared with those from other fungi reported in the literature (3, 4).

[1]Part 5 in the series "Studies on Fungal Products." For Part 4 see LITERATURE CITED 2.

EXPERIMENTAL[2]

CULTURES.—*Penicillium odoratum* Christensen and Backus (IFO 7741) and *Penicillium brunneo-stoloniferum* Abe (IFO 5727) were obtained from the Institute for Fermentation, Osaka, Japan, and were maintained on potato extract slants containing 0.03 g per ml of sucrose, 0.03 g per ml of sodium chloride, and 0.02 g per ml of agar. Each strain was grown in stationary culture for 20 days at 27° in a medium containing: sucrose, 30 g; $NaNO_3$, 6 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; corn steep liquor, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; biotin and vitamin B_1, trace; water, 1 liter; and NaOH to bring the pH to 5.5. Growth of the fungus was started in 200 ml of this medium contained in a Roux flask by innoculation with a heavy spore suspension obtained by the addition of sterile water to cultures on potato extract agar slants. One hundred and sixty flasks were innoculated for each strain.

ANTIMICROBIAL TEST.—The antimicrobial activity was determined by the cylinder-agar plate assay with *Bacillus subtilis* (IFO 3108), *Escherichia coli* (IFO 3044), *Giberella zeae* (IFO 4474), *Neurospora sitophila* (IFO 4596) and *Alternaria maritima* (IFO 8618) as test organisms. The medium for growing bacteria was prepared by dissolving 1.75 g of antibiotic medium (Difco)[3] and 1.6 g of agar in 100 ml of water. Fungi were cultivated in Czapek-Dox medium containing 1.6 w/v % agar. In the preliminary assay, 1 ml of a 5-day-old mycelial suspension or 1-day-old bacterial suspension and 19 ml of the corresponding above-mentioned medium were combined to prepare the assay plates. Paper discs (0.6 cm diameter) were dipped into acetone solutions prepared either from column eluate fractions or from crystalline material and placed on the assay plates. Zones of inhibition were recorded after 24 hours' incubation at 37° in case of bacteria, and after 72 hours' incubation at 27° in case of fungi.

To quantitate the activity, the minimum inhibitory concentration (MIC) was determined by a two-fold dilution method. To prepare the individual assay plates, the original acetone solution, 8 mg in 1 ml, was diluted two-fold serially with acetone, and 0.5 ml of each of the serial two-fold dilutions was combined with 19.5 ml of medium at 45° and then cooled. In the antibacterial assay, the bacterial suspension was streaked; whereas in the antifungal assay, a round disc of the agar medium, 0.6 cm in diameter and 0.5 cm in depth, on which the test organism was grown was placed upside down, on the assay plate. After cultivation for the same period as in the preliminary test, the lowest concentration to inhibit completely the growth of microbes was determined by visual examination.

ISOLATION OF CITRININ (**1**) FROM *P. odoratum*.—The culture of *P. odoratum* was harvested by filtration through cheesecloth to remove mycelia. The filtrate was acidified to pH 2 with hydrochloric acid, producing a precipitatation of a yellow powder. The resulting suspension was extracted with one half of its volume of methylene chloride. The resulting extractive was dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo*. The sticky brown solid, amounting to 45 g, thus obtained became crystalline after 12 hours. Purification by recrystallization from ethanol gave yellow needles of mp 175°; *m/e* 250 (M^+). The identity of this compound with citrinin (**1**) was established by noting that the mixed melting point with an authentic sample was not depressed.

Ergosterol (3.2 g) was isolated from the dried and pulverized mycelium (250 g) by extraction for 135 hours with ether in an Asahina-type extractor (5). It was identified with an authentic sample[4] by mixed fusion.

ISOLATION OF MYCOPHENOLIC ACID (**2**), MANNITOL AND BREVIANAMIDE A FROM *P. brunneo-stoloniferum*.—Extraction from the culture broth of *P. burunneo-stoloniferum* was carried out with methylene chloride in the manner described for *P. odoratum*. The yellowish crystalline extract was mostly soluble in 10% sodium bicarbonate solution. The small insoluble fraction was separated by filtration, and the filtrate was acidified to give 65 g of crude crystals, which were purified by recrystallization from benzene to give colorless feather-like crystals of mp 140°; *m/e* 320 (M^+). The identity of this compound with mycophenolic acid (**2**) was established by mmp determination with an authentic sample.

Brevianamide A was obtained from the bicarbonate insoluble fraction (1.7 g), which was washed with water and dried. A portion (20 mg) was dissolved in benzene and poured onto a column of silica gel[5] (1.8×5 cm) and chromatographed with benzene-acetone (5:1) as the eluting solvent. After removal of the solvent, the 53rd and 54th 50 ml fractions gave yellow cubes, which melted at 254° after purification by recrys-

[2]Melting points were not corrected. The ir spectra were obtained on a Hitachi model 215 recording spectrophotometer, in KBr pellets. The proton nmr spectra were obtained in deuterated solvents on a JEOL model FX-100 (100 MHz) spectrometer with TMS as internal standard. Electron impact mass spectra were obtained on a Hitachi model RMS-4 instrument. The uv spectra were obtained on a Hitachi model 124 spectrophotometer.

[3]Sankyo Junyaku Co. Ltd.

[4]Tokyo Kasei Kogyo Co. Ltd.

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

tallization from chloroform. The yield was 6 mg. *Anal.* Calcd for $C_{21}H_{23}O_3N_3 \cdot CHCl_3$: C, 54.44; H, 5.08; N, 8.47. Found: C, 54.22; H, 4.96; N, 8.70. *M/e* 365. This compound (500 mg) was converted to the monoacetate (a new derivative) by refluxing for two hours with acetic anhydride, followed by recrystallization from methanol, yielding 420 mg of yellow needles of mp 241°. Anal. Calcd. for $C_{25}H_{25}N_3O_4$: C, 67.16; H, 5.96. Found: C, 67.79; H, 6.18. It showed uv λ max (EtOH) 235, 256, 405 nm (ψ-indoxyl structure); *m/e* 407 (M^+). These and various spectral (uv, ir and nmr) data identified this metabolite, which had no antimicrobial activity, as brevianamide A (6, 7).

Mannitol was obtained when the dried and pulverized mycelium was extracted in an Asahina-type extractor (5) for 140 hours with ether and subsequently for 160 hours with acetone. The ethereal extract (2.1 g) was dissolved in benzene and chromatographed over silica gel[4] (90 g) using benzene-acetone (10:1) as the eluting solvent. The 12th and 13th 100-ml-fractions gave mycophenolic acid (50 mg). The acetone extract (6.4 g) was mixed with 100 ml of water and filtered. The residual solid (1.0 g), after removal of water, was acetylated by boiling for 40 minutes with 3 ml of acetic anhydride in the presence of a few drops of sulfuric acid. After removal of the solvent *in vacuo*, the residue was purified by recrystallization from ethanol yielding colorless leaves, mp 121.5°. This derivative was identified as mannitol hexaacetate by mmp determination with the mannitol hexaacetate made from authentic mannitol.[4]

TIME-COURSE VARIATION IN PRODUCTION OF CITRININ AND MYCOPHENOLIC ACID.—The time-course change in the amount of citrinin and mycophenolic acid in the culture fluid of each fungus was determined spectrophotometrically. Czapek-Dox medium, pH 5.5, was sterilized in an Erlenmeyer flask. A volume of 15 ml was taken out as a control solution. The main part was innoculated with *P. odoratum* or *P. brunneo-stoloniferum* and kept as stationary cultures. At every 2 days' interval, 10 ml of culture fluid was pipetted out and was extracted twice with 30 ml of chloroform[5]. The combined chloroform extracts in each case were washed with 10 ml of water, dried over a small amount of anhydrous sodium sulfate, concentrated to 5 ml, and the volumes adjusted to exactly 10 ml[6]. The uv absorption was measured at 332 nm in case of citrinin and 306 nm in case of mycophenolic acid. The control solution was treated in the same way. The time-course variation in the amount of the two antimicrobial substances was calculated from the respective standard curves prepared with the respective authentic samples.

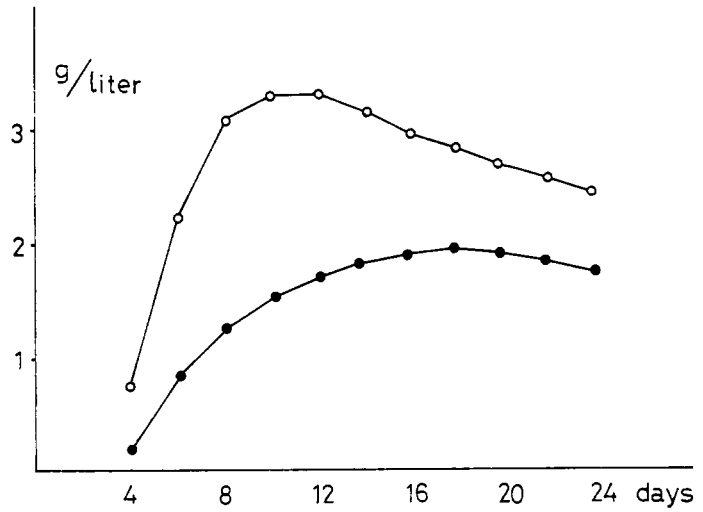

FIG. 1. Time-course variation in the amount of citrinin (●——●) in *Penicillium odoratum*, and mycophenolic acid (○——○) in *Penicillium brunneo-stoloniferum*, in the culture broth.

RESULTS AND DISCUSSION

In a preliminary test, it was shown that the culture fluid of *P. odoratum* possessed antibacterial activity. On acidification of the culture filtrate, precipitation of a yellow powder oc-

[5]Grade sufficient for spectrophotometric use: Kanto Kaguku Co., Ltd.

curred. This substance, mp 175°, was demonstrated to be responsible for the antibacterial activity of the culture broth, completely inhibiting the growth of *Bacillus subtilis* at a minimum concentration of 25 mcg per ml. No other active materials were detected. This compound rapidly absorbed one mole of hydrogen in the presence of a catalyst in methanol, yielding a colorless dihydro compound, mp 171°. In contrast to the parent compound, this derivative was positive in the ferric chloride test indicating that the parent compound is of a quinoid structure. These and the various spectral data (uv, ir, nmr and mass) confirmed this compound to be citrinin (**1**).

A preliminary test showed that the culture fluid of *P. brunneo-stoloniferum* possessed antifungal activity. Colorless feather-like crystals of mp 140° were isolated from the culture fluid as the sole substance responsible for the activity. This substance inhibited the growth of *Giberella zeae* and *Alternaria maritima* completely at a minimum concentration of 200 mcg per ml. No other metabolites with antifungal activity could be detected. This compound was shown to have properties of a phenol by color formation with ferric chloride. The spectral (uv, ir, nmr and mass) and analytical data unequivocally demonstrated that this compound is mycophenolic acid (**2**).

A survey of the literature revealed that a maximum yield of citrinin of 2.4 g per liter of the culture broth was reported by Terui *et al.* in *Penicillium velutinum* (3). However, the original strain used in the earlier work is no longer available (Terui, personal communication) and *Penicillium velutinum* van Beyma strain No. 6064 obtained from the Institute for Fermentation, Osaka, Japan, had apparently lost the ability to produce citrinin. As seen in figure 1, *P. odoratum* used in the present work could produce this compound at a level of 1.9 g per liter in a synthetic medium. The yield of mycophenolic acid with *P. brunneo-stoloniferum* reached 3.25 g per liter. This amount is more than seven times higher than the highest yield ever reported in a species of *Penicillium* (4).

Brevianamide A was also obtained from *P. brunneo-stoloniferum*. This same compound was obtained by Dr. J. E. Robbers, from *Penicillium ochraceum* Bainier ex Thom ATCC 10112, a strain isolated from "blue-eye corn" (7).

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