Vol. 10 (1962)

Notes

UDC 615.779.932

Naoki Inagaki : Isolation and Properties of Helmintin, an Antifungal Substance produced by *Helminthosporium siccans*.

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In the past literature, many fungi were reported to produce antifungal substances, such as alternaric acid,[1] griseofulvin,[2] viridin,[3] trichothecin,[4] variotin,[5] etc. Also several kind of antifungal substances from Helminthosporia have been reported in the literature. Nakamura, *et al.*[6] isolated ophiobolin from the culture filtrate of *Ophiobolus miyabeanus* ITO et KURIBAYASHI, and Orsenigo[7] obtained cochliobolin from *Helminthosporium oryzae* BREDA DE HAAN, and Ishibashi[8] obtained pyrenopholin from *Helminthosporium avenae* EIDAM. Recently, zizanin and siccanin were obtained from the culture filtrate of *Helminthosporium zizaniae* NISHIKADO and *Helminthosporium siccans* DRECHSLER, respectively.[8]

The present report deals with the isolation and general properties of helmintin, an antifungal substance obtained from the culture filtrate of *Helminthosporium siccans* DRECHSLER, isolated from a leaf-spot of Italian rye-grass.

Crystalline helmintin is a colorless, neutral compound whose analysis corresponds to an empirical formula of $C_{11}H_{18}O_2N_2$. Absence of sulfur and halogens was demonstrated. It is interesting that helmintin contains nitrogen in the molecule, while ophiobolin, pyrenopholin, cochliobolin, siccanin, and zizanin contain only carbon, hydrogen, and oxygen in their molecule.

The ultraviolet absorption spectrum of helmintin, measured in ethanolic solution (concn. 8 mg./100 cc.), shows no characteristic maximum in the region of 215~340 mμ. From this spectrum, it is considered that helmintin has no conjugated system in its structure.

Helmintin is highly soluble in chloroform. It is also soluble in benzene, ethanol, methanol, butanol, acetone, ethyl acetate, and carbon tetrachloride, and slightly soluble in ether, but nearly insoluble in water and petroleum ether. Helmintin is stable in acidic, neutral, and basic solutions.

The infrared absorption spectrum of helmintin in KBr tablet is shown in Fig. 1. A chloroform solution of helmintin has characteristic bands at 3385, 1670, 1420, 1350, 1308, 1157, 995, and 915 cm^{-1}. A Nujol mull of helmintin has characteristic bands at 3270, 1668, 1633, 1430, 1300, 1157, 1128, 992, 916 and 705 cm^{-1}. From these spectra, it is considered that helmintin is a non-aromatic substance.

Helmintin has a specific rotation of $[\alpha]_D^{25}$ $-158°$ (c=2.6 in chloroform); it gives a positive Bayer test and negative Ninhydrin, 2,4-dinitrophenylhydrazine, ferric chloride, and Molish reactions.

The activity of helmintin against microörganisms was examined by the agar streak-

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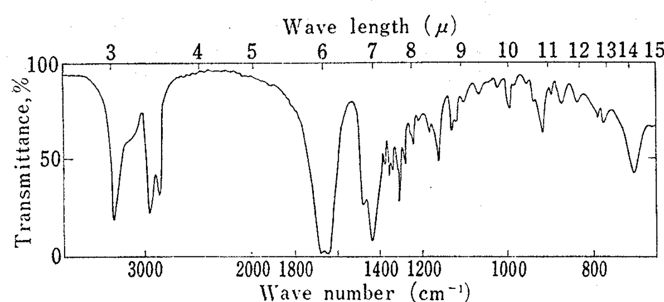

Fig. 1.

Infrared Absorption Spectrum of Helmintin (in Nujol mull)

dilution method using glucose-peptone or bouillon medium. Minimal concentrations necessary for complete inhibition against series of organisms are shown in Table I. Helmintin was the most active to Trichophyton spp. among the fungi tested, but no inhibition of growth was noted in bacteria and yeast.

Table I. Antimicrobial Activity of Helmintin

Test organism	Inhibition concentration (γ/cc.)	Test organism	Inhibition concentration (γ/cc.)
Trichophyton mentagrophytes	5.0	*Chaetomium globosum*	50
Trichophyton interdigitales	5.0	*Aspergillus niger*	50
Microsporum gypseum	10.0	*Aspergillus fumigatus*	50
Rhizopus nigricans	10.0	*Penicillium chrysogenum*	50
Piricularia oryzae	25	*Penicillium islandicum*	50
Fusarium moniriforme	25	*Torula utilis*	>100
Cladosporium herbarum	25	*Saccharomyces cerevisiae*	>100
Helminthosporium oryzae	30	*Candida albicans*	100
Alternaria kikuchiana	30	*Staphylococcus aureus*	100
Gibberella fujikuroi	30	*Bacillus subtilis*	>100
Gromella lagenaria	30	*Escherichia coli*	>100
Macrosporium bataticola	30	*Mycobacterium sp.* 607	>100

Experimental

Assay——Helmintin is bioassayed by the agar diffusion method using filter paper discs against *Rhizopus nigricans* and *Trichophyton mentagrophytes*.

Organism and Culture——No. B-34 isolated strain of *Helminthosporium siccans* Drechsler was used in this work. Cultural characteristics of this strain are as follows: Colonies on potato-glucose agar at 25°, growing broadly, attaining a diameter of 50~60 mm. in 5 days, 75~85 mm. in 7 days (35~45 mm. at 30°; seldom growing at 37°); generally floccose, plane, zonate in grayish olive-green; reverse side, greenish black.

The optimum condition for production of helmintin in shake culture was examined. As a result of preliminary cultural examination, the medium consisted of 2% glucose, 0.5% peptone, 0.5% KH_2PO_4; 0.25% $MgSO_4 \cdot 7H_2O$, and 10% potate decoction (pH 5.0). Fifty cc. of this medium in a 200-cc. Erlenmeyer flask was inoculated with *Helminthosporium siccans* B-34 and incubated for 4~5 days at 25° on a reciprocating shaker. On the 4th to 5th day, the mycelia became greenish black and the solution also turned dark (pH 4.8~5.0).

Isolation and Purification of Helmintin——At the end of 5 days, when the concentration of helmintin in the culture had reached a maximum, the culture was filtered through cloth to separate from the mycelium and the combined filtrate was extracted three times with 1/3 volume of $CHCl_3$. $CHCl_3$ extract was evaporated to dryness *in vacuo* and the brown gummy residure was extracted with Et_2O. This Et_2O solution was concentrated *in vacuo* and the resulting Et_2O solution was left in a refrigerator. Crude crystals crystallized from Et_2O solution were dissolved in benzene and poured through a column filled with silica gel. The silica gel column was eluted successively with petr. ether, benzene, Et_2O, and $CHCl_3$, and the $CHCl_3$ effluent was evaporated *in vacuo*. Colorless syrup thus obtained was recrystallized several times from Et_2O. About 300 mg. of crystalline substance (m.p. 158~159°) was obtained from 10 L. of filtrate, corresponding to about 0.0025% of the cultural medium. *Anal.* Calcd. for $C_{11}H_{18}O_2N_2$: C, 62.83; H, 8.63; O, 15.22; N, 13.32; mol. wt., 210.3. Found: C, 62.65; H, 8.46; O, 15.07; N, 13.82; mol. wt. (Rast), 219.

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The author expresses his gratitude to Dr. H. Kurata of this Laboratory for his kind encouragement and guidance. Thanks are due to Dr. S. Sakai and Dr. I. Kitagawa of the Faculty of Pharmaceutical Sciences, University of Tokyo, for elementary analyses, and to Dr. T. Oba of this Institute for spectral measurements.

(Received September 24, 1960)

UDC 547.747

Takuo Kosuge and Hiroko Kamiya : L-Leucyl-L-proline from Peptone.

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During investigation of antifungal principles derived from culture of a *Bacillus subtilis* strain, a crystalline matter was obtained as one of the by-products. It was necessary to decide whether this substance was a metabolite of the organism or originally contained in peptone used as a dietary essential. The same procedure employed as above afforded the same crystals from commercial peptone, Mikuni Pepton, proving the crystals as a component of peptone. This peptone was extracted with hot chloroform and chromatography over silica gel column gave a crystalline substance.

Purification was effected by recrystallization from benzene-petroleum ether mixture. The purified colorless crystals needles melted at 159.5° and were readily soluble in alcohols, chloroform, and benzene, and insoluble in water, ether, and hydrocarbons.

Elemental analysis and molecular weight measurement by the Rast method indicated molecular formula of $C_{11}H_{18}O_2N_2$. The infrared spectrum showed absorptions at 3260, 1670, and 1630 cm^{-1}, no absorption at 1550 cm^{-1} region, which is characteristic of cyclic amides. Significant optical rotation in methanol solution was observed at $[\alpha]_D$ $-132.8°$.

Quantitative analysis of the amino acids was carried out by paper chromatographic method. Hydrolysis with 6*N* hydrochloric acid in sealed tube at 110° for 20 hours yielded amino acids and the quantitative analysis by paper chromatography indicated equimolar quantities of L-proline and L-leucine.

All data are in agreement for cyclic dipeptide composed of two amino acids, L-proline and L-leucine. The isolated dipeptide was identical with synthesized L-leucyl-L-proline[1] in mixed melting point and infrared spectrum. This would be identical with the dipeptide reported by Abderhalden[2] as probably L-prolyl-L-leucine anhydride obtained from a tryptic digest of gliadin. The same isolation procedure was employed on milk casein, but the same substance was not obtained.

Experimental

Extraction of Dipeptide——To 100 g. of peptone 300 cc. of water was added and the mixture was extracted with three 150-cc. portions of $CHCl_3$. $CHCl_3$ extract was dried over anhyd. Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was submitted to column chromatography on silica gel with $CHCl_3$. A part of $CHCl_3$ eluates contained crude dipeptide. Its recrystallization from benzene-petr. ether mixture gave 100 mg. of a pure compound, m.p. 159.5°; mol. wt., 215 (camphor). *Anal.* Calcd. for $C_{11}H_{18}N_2O_2$: C, 62.85; H, 8.56; N, 13.32. Found : C, 62.79; H, 8.59; N, 13.16.

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