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A New Mycotoxin produced by *Aspergillus clavatus*[1]

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Extensive investigation of mycotoxin-producing fungi on and in Japanese foods has been carried out in our institute. About fifteen hundred strains were isolated from more than five hundred samples of various kinds of foods. Two strains of them were proved to produce aflatoxins. New metabolite was isolated from one strain of *Aspergillus clavatus* (WF-38-11) found in wheat flour and named ascladiol.

Recently the toxicities of the metabolites produced by fungi have attracted the attention of many researchers and numerous mycotoxins have been reported from many laboratroies, since in England very strong carcinogenic substances, aflatoxins, had been found in moist peanuts imported from Argentina, 1960.

Extensive investigation of mycotoxin-producing fungi on and in Japanese foods has been carried out in the laboratory of mycology in cooperation with our laboratory (Dept. of Foods) in our institutes.

Already more than five hundred samples of various foods such as flour, rice, beans have been investigated and about fifteen hundred strains were isolated. Two strains of these isolates (*Aspergillus flavus*) were proved to produce aflatoxins. This was the first finding of this kind of fungi in Japan. Among these isolates, liquid cultures or methanol extracts from solid cultures of one hundred and seventy strains were applied to bioassay for their toxicities and thirty one strains were found to be considerably toxic to mice.[3]

One strain of *Aspergillus clavatus* (WF-38-11) isolated from wheat flour had strong toxicity. Since *A. clavatus* has been known to produce patulin, strong toxic metabolite, for the purpose of identification of patulin in the culture, its isolation was carried out in general way, *i.e.* adsorption of the toxin on activated charcoal, elution of it by column chromatography.

During this purification, a new toxic material was obtained in slightly more polar fraction than that of patulin.

The new toxin was named ascladiol. Acute toxicity (*i.p.*) of this compound was about 1/4 times as strong as that of patulin.

Very hygroscopic crystalline material obtained from the last fraction was recrystallized from the mixture of chloroform and acetone. The absorption bands at both of 1735 cm^{-1} and 1750 cm^{-1} and 3300 cm^{-1} in the infrared (IR) spectrum (KBr tab.) of the compound indicate the presence of 5 membered lactone-ring and associated hydroxyl groups in the molecule, respectively. The ultraviolet (UV) spectrum, measured in ethanol showed maximum absorption at 271 mμ while that of patulin is at 277 mμ. This shift by 6 mμ toward shorter wave length seemed to suggest the lack of one exo double bond in the new compound in comparison with patulin.

The nuclear magnetic resonance (NMR) spectrum (Fig. 1) in deuterioacetone solution showed a absorption at 6.29 ppm (quartet) and 5.87 ppm (multiplet), each indicating the

1) This work was reported at the 16th Annual Meeting of the Japan Hygienic Society (Niigata).
2) Location: *18-1, Kamiyoga 1-chome, Setagaya-ku, Tokyo.*
3) H. Kurata and M. Ichinoe, *J. Food Hyg. Soc. Japan*, **8**, 237, 247 (1967); H. Kurata, S. Udagawa, M. Ichinoe, Y. Kawasaki, M. Takada, M. Tazawa, A. Koizumi and H. Tanabe, *J. Food Hyg. Soc. Japan*, **9**, 23 (1968).

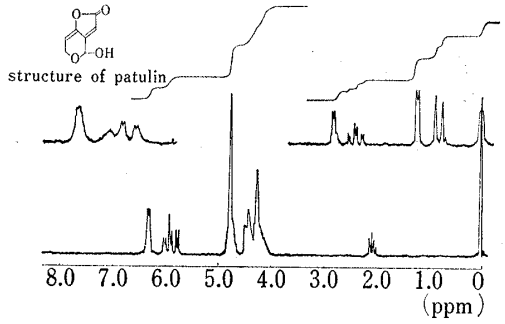

Fig. 1. NMR Spectra of New Toxicant Isolated from *Aspergillus clavatus* (WF-38-11)

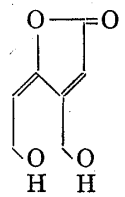

Fig. 2

presence of different one vinyl proton. Broad single absorption was also observed at 4.74 ppm, indicating the presence of 4 protons. Since two of these protons were disappeared by addition of D_2O into sample solution, it seemed that two hydroxy protons and two methylene protons overlapped each other. Other two methylene protons were observed at 4.30 ppm. This compound seemed not have aldehyde or ketone groups in the molecule, since it did not react with phenylhydrazine.

According to these data mentioned above, the chemical structure of the new toxin was presumed to be as follows (Fig. 2).

Finally, the structure was confirmed by the comparison of physical data with authentic sample synthesized from patulin by reducing it in ethanol with $NaBH_4$ under very mild condition (at 0—2°, for 10 min).

Experimental

Isolation of Ascladiol——*A. clavatus* (WF-38-11) obtained from wheat flour was used for the isolation of the new toxicant. Status cultures were maintained at 25° for 10 days on modified Czapec medium.

Liquid culture (5 l.) of *A. clavatus* isolated from wheat flour was filtered and the broth obtained was shaken with activated charcoal (50 g) overnight. The charcoal was centrifuged, washed with small amount of water and then extracted with several portions (each 200 ml) of the mixture of acetone and water (4:1) to elute patulin adsorbed.

The extracts were combined and concentrated under suction about 200 ml. The residual solution was applied to continuous extraction with ether for 20 hr. The ether extract was evaporated to dryness, and the residue was purified by silica gel column chromatography. The residue was dissolved in small amount of chloroform, mixed with silica gel (5 g) and then subjected to silica gel (30 g) column chromatography. Elution was carried out with three portions of the mixture of chloroform and acetone (50:1) (fraction 1 and 2, 300 ml and fraction 3400 ml) and then with three portions of another mixtures of chloroform and acetone (50:2) (fraction 4, 300 ml, fraction 5400 ml and fraction 6, 1500 ml).

Fraction 1 and 2 gave an oily material (250 mg), which has been under investigation, along with the crystalline substance (360 mg) which was proved to be patulin by IR-spectroscopy, thin-layer chromatography and depression test of mp on admixture with authentic sample.

From the last fraction, when concentrated and treated with acetone and chloroform, a very hygroscopic crystalline compound was obtained. This was purified by dissolving it in small amount of acetone followed by addition of chloroform, until the solution became turbid, and allowed to stand overnight in a refrigerator.

The crystal obtained was recrystallized from mixture of chloroform and acetone again as described above, mp 65—66°. *Anal.* Calcd. for $C_7H_8O_4 \cdot 1/10\ H_2O$: C, 53.23; H, 5.23. Found: C, 53.01, 53.40; H, 5.41, 5.29. IR ν_{max}^{KBr} cm^{-1}: 1735, 1750, and 3300. UV λ_{max}^{EtOH} $m\mu$: 271. NMR δ (ppm): 6.29 (1H), 5.87 (1H), 4.74 (4H), and 4.30 (2H).

Reduction of Patulin with $NaBH_4$——To a solution of patulin (500 mg) in ethanol (15 ml) was added a solution of $NaBH_4$ (120 mg) in ethanol (20 ml) under ice-cooling during 10 min. and allowed to stand for 10min. After decomposition of the excess reagent with acetic acid the reaction mixture was diluted with water (150 ml) and added activated charcoal (2.0 g). The activated charcoal was collected by centrifugation and organic materials were eluted by the mixed solution of acetone and water (80:20). The extract was evaporated to dryness under suction, and the residue was chromatographed on silica gel (2.0 g). Elution with chloroform

and acetone (90:10) gave an oil (68 mg), which was crystallized from chloroform and acetone to yield colorless needles, mp 65—66°.

This product was identical with natural ascladiol in IR (KBr), UV and in retention time in gas–liquid chromatography (as a trimethylsilyl derivative).

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