STUDY ON THE TOXIC METABOLITES OF PENICILLIUM ISLANDICUM*

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It was known that there are three kinds of poisonous yellowish rice. They are the citrinum yellowish rice[1], caused by Penicillium citrinum; the so called yellowish rice[2], caused by Penicillium toxicarium; and the islandia yellowish rice[3], caused by Penicillium islandicum Sopp. The poisonous substances of the first two were found to be citrinin and $C_{27}H_{36}O_7$[5], in the form of a yellow pigment of polyene[6], respectively. The toxic substances of the last one have been identified as islanditoxin[7,8], luteoskyrin[9] and malonic acid[10,11].

In this report it will be reported that there are three compounds isolated in crystalline form from Penicillium islandicum when cultured in the koji juice media. Two of the new crystals are obtained from the cultured mycelium and both possess fluorescence when viewed under ultraviolet light. One of the crystals, the kojic acid, was obtained from the cultured broth. The method of isolation and some properties of the compounds will be described in the following.

EXPERIMENTAL

Organism and Cultured condition:

Penicillium islandicum JC (4209), obtained through the courtesy of Dr. C. T. Hsu (Dean of Taipei Medical College), was used throughout this work.

Instead of Czapeck's slant media, koji juice slant media was used for the growth of Penicillium islandicum. After the incubation at 30°C for 7 days, the spores were transferred to a sterilized 1 l. Erlenmeyer flask (treated in an autoclave at 15 lb./sq. in. for 15 mins.) containing 150 ml of koji juice. During the incubation the white mycelium formed on the 2nd day and green spores appeared on the 4th day. The growth of the fungus achieved a maximum on the 12th day.

Method for the preparation of koji juice media:

Aspergillus oryzae E. B., a gift of Dr. C. C. Tung (Director of the Research Division, Wei-Chuan Foods Corporation), was inoculated on steamed rice, then was incubated at room temperature with occasional stirring. White mycelium formed on the 2nd day and green spores appeared on the 3rd day. On the 6th day the fungus reached a maximum growth. Then distilled water was added and the incubation at 60°C continued until the iodine test was negative. After filtration, a yellow koji juice was obtained.

Method of isolation of the compounds:

The Penicillium islandicum grown in 75 Erlenmeyer flasks was all transferred into a Waring blender and was blended well. After filtration we obtained the residues (cultured mycelium) and the filtrate (cultured broth).

(1) The Residues: About 3 kg. of the residues were defatted with *n*-hexane for 3 days, then extracted with chloroform for 4 days. After filtration, the extracts were concentrated under reduced pressure to ca. 30 ml. This concentrate was passed through a silica gel column (Wako-gel C-200, 3 cm × 45 cm). Chloroform was used as an eluant and the first

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yellowish green (ca. 350 ml) and the second pink (ca. 320 ml) fluorescent fractions were collected. After concentration of these fractions under reduced pressure and recrystallization of them from 95% ethyl alcohol, a white needle and a pale brown needle crystals could be obtained. We named them "Compound G" and "Compound P" respectively. The yield of the "Compound G" was about 0.003% and that of the "Compound P" was about 0.004%.

(2) The Filtrate: About 10 l. of the cultured broth (yellowish brown color) was concentrated under reduced pressure to ca. 500 ml. and acetone was added for the precipitation of carbohydrates and inorganic salts[12]. After filtration, the filtrates (reddish brown color) were concentrated until acetone free. Then the concentrate was extracted with ethylacetate (200 ml × 4), this extract was concentrated and put into the refrigerator. A pale yellow needle crystal was obtained. After recrystallization from acetone we obtained white prismatic needleshaped crys tals. This crystal was named as "Compound E", the yield was about 0.03%.

Thin layer chromatography:

A layer of Wako-gel B-5 of 250 micron thickness was applied on a glass plate (10 cm × 20 cm) with an applicator. The plate was dried overnight at room temperature and activated at 105°C when it was used. The sample was applied to the plate and developed in 2% methanolic chloroform at room temperature for 40 minutes. The plate was dried in air and viewed under U. V. light.

Toxicity study:

Male mice of albino strain were used as experimental animals, each weighing from 16 to 20 grams. They were fed with "Ta Di Brand Chick Diet" and tap water ad libitum. The animals were divided into 11 groups, 8 in each group.

Group I to V were injected intraperitoneally with 0.2 ml. "Compound G" in propylene glycol solution. The dosages were 400, 200, 100, 50 and 25 γ per 10 gm. body weight respectively. Group VI to X were treated as above except the injected substance was "Compound P". Group XI was used as experimental control and was injected with 0.2 ml. propylene glycol only.

After the experimental mice died, they were dissected. The brains, lungs, hearts, livers and kidneys were taken out for pathological examination.

RESULTS

"Compound G" and "Compound P" show yellowish green and pink fluorescent spot on the Wako-gel B-5 chromatogram; they possess the R_f values of 0.6 and 0.5 respectively. When the spots of "Compound G" and "Compound P" on the Wako-gel B-5 chromatogram are sprayed with 0.5% methanolic magnesium acetate solution[13], no color reaction develops.

The melting point (Thomas Hoover Capillary melting point apparatus) of these compounds are as follows:

Compound G: 133-135°C,

Compound P: 240-241°C (decomp.)

Compound E: 152-153°C

The U. V. absorption spectra (Hitachi Perkin-Elmer UV-VIS Spectrophotometer) are

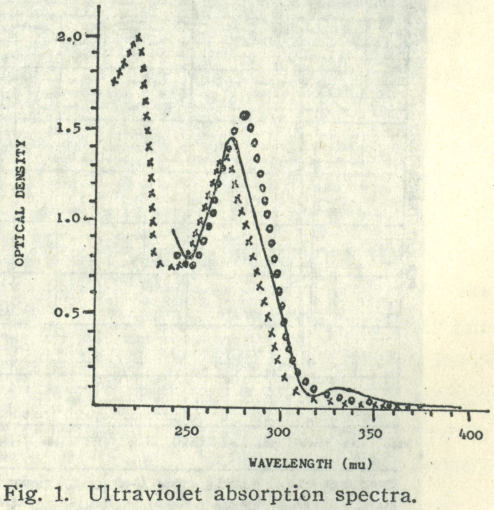

Fig. 1. Ultraviolet absorption spectra.
——— "Compound G" in chloroform
○○○○○ "Compound P" in chloroform
×××××× "Compound E" in water

shown in Fig. 1 which indicate that "Compound G" and "Compound P" have a peak of absorption at 275 mμ and 280 mμ respectively, "Compound E" has two peaks, one at 220 mμ and the other at 270 mμ.

The fluorescence spectra are shown in Figs. 2 and 3, which indicate that "Compound G" has the excitation and emission maximum at 335 mμ and 680 mμ respectively, "Compound P" has the excitation and emission maximum at 295 mμ and 480 mμ respectively.

The infrared absorption spectra, measured on Hitachi Grating Infrared Spectrophotometer as Nujol mull, are shown in Figs. 4, 5 and 6 as "Compound G", "Compound P" and "Compound E" respectively.

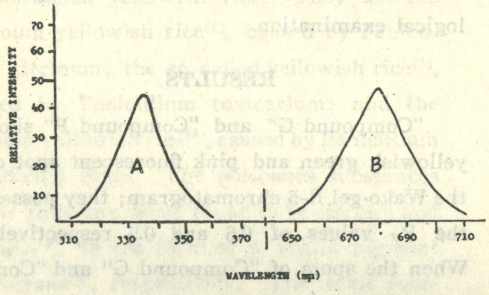

Fig. 2. Fluorescence spectrum of "Compound G" in chloroform (determined by Aminco-Bowman spectrofluorometer, meter multiplier=0.001, sensitivity=40)
A: excitation spectrum at emission of 680 mμ
B: emission spectrum at excitation of 335 mμ

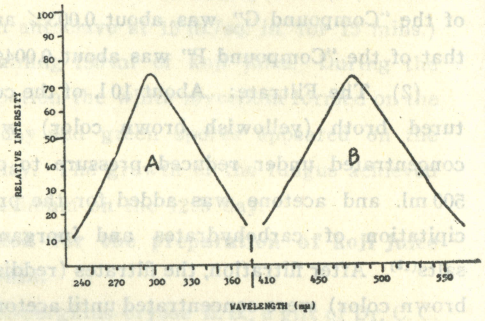

Fig. 3. Fluorescence spectrum of "Compound P" in chloroform (determined by Aminco-Bowman spectrofluorometer, meter multiplier=0.001, sensitivity=40)
A: excitation spectrum at emission of 480 mμ
B: emission spectrum at excitation of 295 mμ

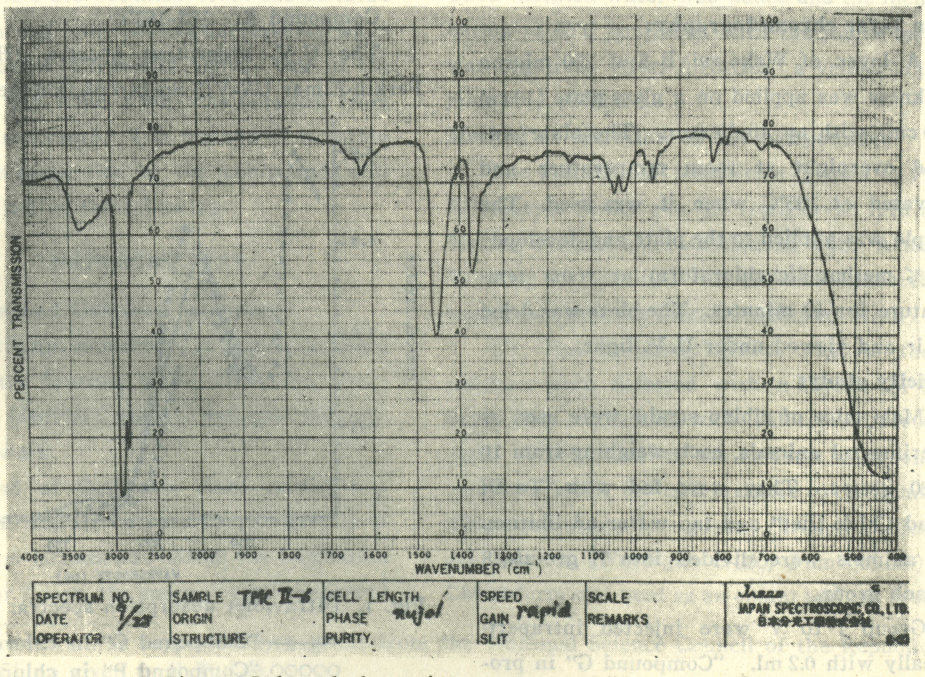

Fig. 4. Infrared absorption spectrum of "Compound G"

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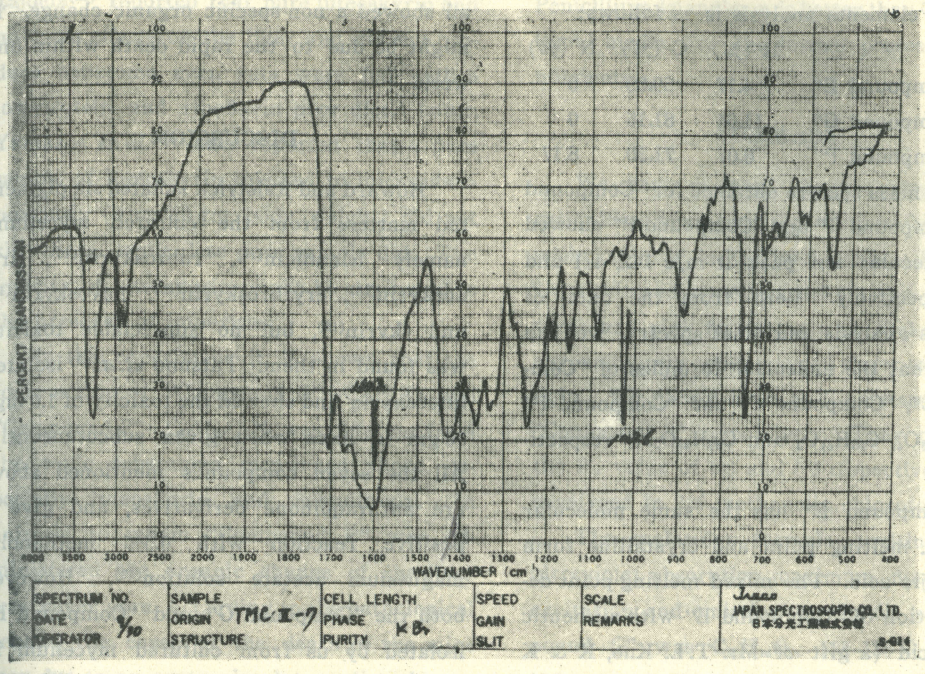

Fig. 5. Infrared absorption spectrum of "Compound P"

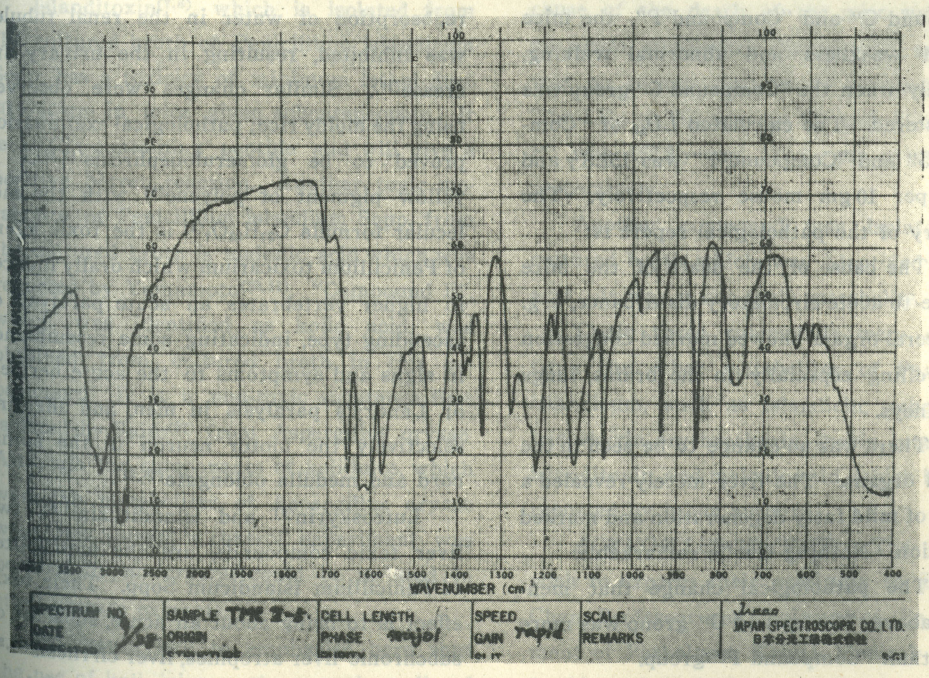

Fig. 6. Infrared absorption spectrum of "Compound E"

The elements analysis is as follows:

	H (%)	C (%)	N (%)
"Compound E"	4.17	50.59	0
"Compound G"	11.04	81.15	0
"Compound P"	6.07	71.30	8.17

The mass spectra obtained for "Compound E", "Compound G" and "Compound P" showed the molecular ion peak at m/e 142, 411 and 336 respectively. Both from the data of elements analysis and mass spectra, we can expect that the molecular formulas of "Compound E", "Compound G" and "Compound P" are $C_6H_6O_4$, $C_{28}H_{44}O_2$ and $C_{20}H_{20}O_3N_2$ respectively.

"Compound E" has the same molecular formula, melting point and crystalline form with kojic acid. The mixed melting point determination of "Compound E" with authentic kojic acid (a gift of Mr. T. L. Kuo, K & K Laboratories Inc.) showed no depression. "Compound E" also showed the same ultraviolet and infrared absorption spectrum with authentic kojic acid. Thus "Compound E" was identified as kojic acid.

After the injection of the mice with "Compound G" and "Compound P", the mice revealed weakness and abnormal walking, especially when the "Compound G" group was administered. The calculated LD_{50} of "Compound G" and "Compound P" was 152.8 γ and 114.6 γ per 10 gm mouse respectively. The summary of the pathological report is:

1. The cause of the death of the mice was due to acute circulatory disturbance, e. g. the visceral organs showed marked congestion and swelling especially in the brains, lungs and kidneys.

2. There was no change to be seen in the visceral organs. The liver merely revealed a picture of mild fatty degeneration and marked congestion. Nothing else is remarkable.

3. The pathological change that mentioned above, "Compound G" group was more severe than "Compound P" group.

4. In all the cases, the visceral organs all showed a non-specific organic change. It might be due to the rapid death within one week.

DISCUSSION

Howard and Raistrick isolated six pigmented matters from the cultured mycelium, namely islandicin[14], rubroskyrin[15], iridoskyrin[15], erythroskyrin[15], flavoskyrin[16] and skyrin[16], but no appreciable toxicity was found in them. Tatsuno *et. al.*[9] isolated luteoskyrin and showed the toxicity of 1.0 mg/10 gm in mice when given subcutaneously. All the seven compounds mentioned above are anthraquinone derivatives, and give a positive reaction with 0.5% methanolic magnesium acetate solution[13]. However both the "Compound G" and "Compound P" isolated by us from cultured mycelium in this experimental condition gave a negative reaction.

Citrinin[4], the toxic pigment of Penicillium citrinum, with LD_{50} 0.6 mg/10 gm in mice subcutaneously and orally, caused acute and chronic damage in the kidney, where the reabsorption of water in the renal tubules was inhibited, resulting in the urinary increase[17]. Similar changes were observed using the moldy rice; pathological examination proved to be glomerulonephrosis[18,19]. A yellow pigment of polyene[6] with the molecular formula $C_{27}H_{36}O_7$[5] is the toxic agent of Penicillium toxicarium which orally induces in higher vertebrates an acute paralysis of ascending type, indicating some resemblance in signs and symptoms to the acute beriberi or Landry's paralysis in men, and affecting selectively the motor neuron in the spinal cord and medulla oblongata[20,21].

Luteoskyrin[9] and islanditoxin[7,8], two liver toxic compounds, are the metabolites of Penicillium islandicum Sopp. Their toxic effect is to bring about an acute, subacute or subchronic liver atrophies, liver cirrhosis[22,23] or liver carcinoma[24]. However Buu-Hoi and

Zajdela[25] reported that pure luteoskyrin had no toxic effects on the liver and did not produce hepatoma even rats were fed with a low-protein and riboflavin poor diet. Later, Yamazoe *et. al.*[26] also reported that a pure sample of luteoskyrin injected to mice subcutaneously (3 mg/10 gm body weight) did not kill them, while less pure sample did. The toxicity of luteoskyrin might be attributed to the presence of impurities.

In this experiment it is shown that the toxic action of "Compound G"—$C_{28}H_{44}O_2$ and "Compound P"—$C_{20}H_{20}O_8N_2$ was different from the toxic substances mentioned above, especially luteoskyrin and islanditoxin. In animals to whom "Compound P" was administered the organic change of the liver was never seen. According to the pathological report, the cause of the death of the mice was due to an acute circulatory disturbance, and there was nonspecific change seen in the visceral organs. A toxicological study should be performed to investigate the toxic mechanism of "Compound G" and "Compound P"

Islanditoxin[7,8], which is isolated from the cultured broth of Penicillium islandicum Sopp, has a toxicity of minimum lethal dose 30 γ/10 gm in mice given subcutaneously. It is a Cl—containing peptide and will give a positive biuret reaction. Another toxic metabolite of Penicillium islandium Sopp. from the cultured broth is malonic acid which was reported by Yamamoto[10,11]. "Compound E", which was identified as kojic acid, was obtained from cultured broth in this experiment. It is a non-toxic substance first isolated by Saito[27] from Aspergillus oryzae Although the components of koji juice, which were the metabolites of Aspergillus oryzae were not known, a control experiment was performed; no crystal could be isolated in ethyl acetate extracts.

We would like to emphasize here that the use of koji juice as the growth media of Penicillium islandicum is better than Czapeck's liquid media, for the former possesses more nutrients.

SUMMARY

We have isolated three compounds in crystalline form from Penicillium islandicum when cultured in koji juice media. Two were from cultured mycelium and showed yellowish green and pink fluorescence on Wako-gel B-5 chromatogram when viewed under ultraviolet light. We named them as "Compound G" and "Compound P" respectively. They possessed the R_f value of 0.6 and 0.5 respectively when developed in 2% methanolic chloroform.

The molecular formula, melting point, ultraviolet absorption maximum, fluorescence excitation and emission maximum of "Compound G" was $C_{28}H_{44}O_2$, 133–135°C, 275 mμ, 335 mμ and 680 mμ respectively. Those of "Compound P" was $C_{20}H_{20}O_8N_2$, 240–241°C (decomp.), 280 mμ, 295 mμ and 480 mμ respectively.

The toxicity of these two compounds was proved by pathological examination as the cause of the death of the mice was due to an acute circulatory disturbance, and there was no change seen in the visceral organs. The LD_{50} of "Compound G" and "Compound P" was 152.8 γ and 114.6 γ per 10 gm mouse respectively.

Another compound was isolated from cultured broth which was identified as kojic acid. It was a non-toxic metabolite.

These three compounds were different from the other metabolites of Penicillium islandicum, such as islandicin, rubroskyrin, iridoskyrin, erythroskyrin, skyrin, luteoskyrin, islanditoxin and malonic acid, which had been reported by other authors.

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Study on the Toxic Metabolites of Penicillium Islandicum

青黴菌 (Penicillium islandicum) 毒代謝產物之研究

李 宏 圖 董 一 致

(1971年8月10日受理)

將青黴菌 (Penicillium islandicum) 培養於麵汁培養基中，我們可以分離出三種結晶物質。二種爲由培養菌絲證分離得到，在 Wako-gel B-5 層析圖上以紫外線照射時，分別具有黃綠色與粉紅色之螢光，吾將它們分別命名爲 "化合物 G" 與 "化合物 P"；當在2%甲醇氯仿溶液中展開時，它們之 R_f 值分別爲0.6和0.5。

"化合物G"之分子式，融點，紫外線最大吸收，螢光之最大激發和發射波長分別是 $C_{28}H_{44}O_2$，133-135°C，275 mμ，335 mμ 和 680 mμ。"化合物 P" 者則分別爲 $C_{20}H_{20}O_3N_2$，240-241°C (分解)，280 mμ，295 mμ 和 480 mμ。

這二種化合物之毒性由病理檢查之證實，小白鼠之致死因是由於急性循環障害所致，內臟器官並無特殊之器官變化。"化合物 G" 和 "化合物 P" 對於10克小白鼠之 LD_{50} 分別爲152.8γ 和 114.6γ。

另一種化合物質爲由培養之肉湯分離得到，它經證實是蟻酸，爲一種無毒性之代謝產物。

這三種化合物質是異於已被許多作者報告之青黴菌代謝產物，如：Islandicin, rubroskyrin, iridoskyrin, erythroskyrin, flavoskyrin, skyrin, luteoskyrin, islanditoxin 和 malonic acid.

臺北醫學院生化學科