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Note

A Major Decomposition Product, Citrinin H2, from Citrinin on Heating with Moisture

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Citrinin is one of the mycotoxins produced by *Penicillium citrinum*. We examined the decomposition products after heating citrinin in water at 140°C and isolated a major product, citrinin H2 (3-(3,5-dihydroxy-2-methylphenyl)-2-formyloxy-butane). Citrinin H2 did not show significant cytotoxicity to HeLa cells up to a concentration of 200 μg/ml (% cytotoxicity: 39%) in 63 h of incubation, but citrinin showed severe toxicity at a concentration of 25 μg/ml (% cytotoxicity: 73%). HPLC analysis of citrinin after heating under various conditions indicates that citrinin H2 is mainly yielded from citrinin.

Key words: citrinin; detoxified product; HeLa cells; cytotoxicity

Citrinin is one of the mycotoxins produced by fungi belonging to *Penicillium* or *Aspergillus* species.[1] It is toxic, especially to the kidneys, and is suspected to be a carcinogen causing renal tumors.[2] Contamination by citrinin in several foods and feed has been reported.[3–6] To avoid the direct intake of citrinin or indirect intake through livestock, it is important to develop detoxification methods for citrinin during food processing. Vail *et al.* reported a sensitive detection method for citrinin using HPLC.[7] However, there are few reports on the detoxification of citrinin. Recently, Kitabatake *et al.* found that heating of citrinin at 130°C in the presence of a small amount of water caused a significant decrease in the toxicity of citrinin to HeLa cells.[8] However, the citrinin heated at 140°C or 150°C showed strong cytotoxicity. This indicates that heating of citrinin in water at 140°C or 150°C caused formation of a highly toxic compound(s).[9]

We were interested in clarifying the degradation compounds of citrinin and tried to purify the degradation products and obtained a product, citrinin H1, with potent cytotoxic activity.[10] Further examination of the decomposition products from the heated citrinin led to the isolation of another product, citrinin H2. In this paper, we describe the structure of citrinin H2, its lower toxicity to HeLa cells, and the course of citrinin H2 formation from heated citrinin.

The structure of citrinin H2 was analyzed from the spectral data and identified as 3-(3,5-dihydroxy-2-methylphenyl)-2-formyloxybutane by comparison to the reference data of Curtis *et al.*[11] They isolated it from a cultivation medium of *Penicillium citrinum* and proposed that citrinin H2 might be formed biosynthetically from citrinin. Barber *et al.* reported that citrinin is present in a hydrate form at the C-1 of citrinin in aqueous solution at pH 7.4.[12] Citrinin H2 is probably produced through the addition of water at the C-1 position of citrinin followed by decarboxylation and ring opening (Fig. 1).

The cytotoxic effect of citrinin H2 on HeLa cells was evaluated by the MTT method.[13] HeLa cells were incubated in the presence of test compounds for 63 h at 37°C. Citrinin H2 showed only 39% cytotoxicity at a concentration of 200 μg/ml and 23% at a concentration of 100 μg/ml, whereas citrinin showed 90% cytotoxicity at a concentration of 100 μg/ml and 73% at a concentration of 25 μg/ml (Table 1). The cytotoxicity of citrinin H2 is much weaker than that of citrinin.

Citrinin H2 has a phenolic hydroxyl group and a formyl group. To examine the role of the functional groups for cytotoxicity, we prepared derivatives of citrinin H2 and examined their cytotoxicity (Fig. 2). Compound **1** is a diacetate of citrinin H2. Compound **2** is the hydrolyzed form of citrinin H2. Compound **3** is a dimethyl ether of compound **2**. As shown in Table 1, these compounds showed only weak cytotoxic activity at a concentration of 200 μg/ml, like that of citrinin H2. The potency of the cytotoxic activity of these compounds is weak, similar to that of citrinin H2. The weak cytotoxic activity of these

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Abbreviations: MTT, 3(4,5-dimethyl-thiazoyl-2-yl)2,5-diphenyltetrazolium bromide; FBS, fetal bovine serum; MEM, minimum essential medium eagle; HPLC, high-pressure liquid chromatography

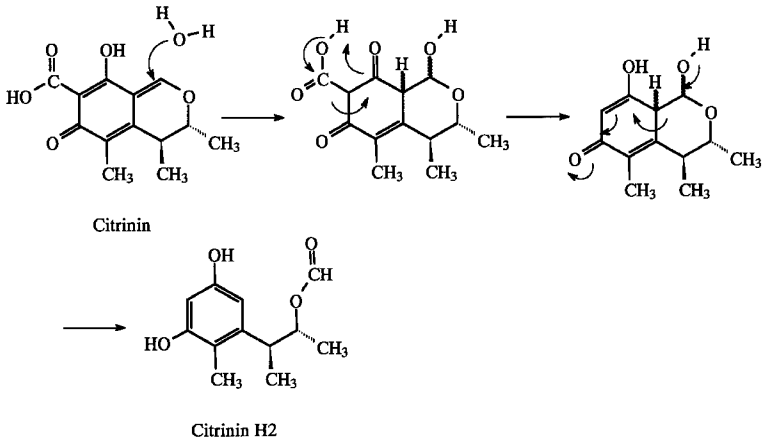

Fig. 1. Possible Mechanisms of Formation of Citrinin H2 from Citrinin by Heating.

Table 1. Cytotoxicity of Citrinin, Citrinin H2, and Citrinin H2 derivatives to HeLa Cells

Tested compound	Concentration (μg/ml)			
	25	50	100	200
Citrinin	0.150*(73%)	0.086(84%)	0.055(90%)	NT
Citrinin H2	NT	0.490(12%)	0.425(23%)	0.337(39%)
Compound **1**	NT	0.453(18%)	0.427(23%)	0.301(46%)
Compound **2**	NT	0.486(12%)	0.426(23%)	0.324(42%)
Compound **3**	NT	0.402(27%)	0.380(31%)	0.362(35%)

* Absorbance at 540 nm: control without test compound: 0.554. % Cytotoxicity in parentheses. % Cytotoxicity = (control-test sample)/control $\times$ 100. Data are means of triplicate examinations.

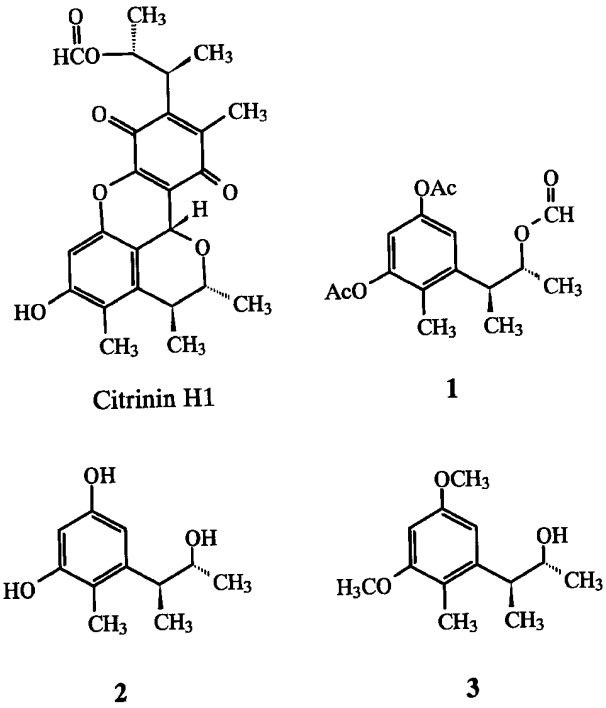

Fig. 2. Structures of Citrinin H1 and Citrinin H2-derivatives.

compounds at high concentrations is non-significant.

To confirm the time course and temperature dependency of citrinin H2 formation, we attempted HPLC analysis of citrinin after heating at various temperatures and heating times. Two hundred μg of citrinin was heated in water, and the residue was then dissolved in acetonitrile and analyzed by HPLC. The HPLC analysis of citrinin H2 showed a peak at t_R 7.2 min by the conditions as indicated in the Experimental section.

As shown in Fig. 3, heating at 140°C–160°C immediately converted 200 μg of citrinin to 30–45 μg of citrinin H2. About 20% of citrinin was changed to citrinin H2 by heating under these conditions. Prolonged heating times up to 60 min did not have any effect on the conversion ratio (*ca.* 20%). Heating of citrinin at 120°C or 130°C required 20 min to reach a similar conversion ratio and heating of citrinin at 100°C required 40 min–60 min. Heating at 80°C for 60 min yielded only 12 μg of citrinin H2 from 200 μg of citrinin.

Every conditions that we tested, except for heating at 80°C, finally produced a similar amounts of citrinin H2. Heating at higher temperature or for a prolonged time did not increase the amount of citrinin H2. These results suggest that citrinin H2 formed under these conditions is not the non-toxic end product and further changes to different compounds. Other reactions might occur to decrease the amounts of citrinin H2. These results correlate with previous reports relating to cytotoxity tests of citrinin, heated under various conditions, toward HeLa cells.[9]

Citrinin is unstable and thermolabile in water. It degrades on heating above 80°C under aqueous conditions. A major degradation product, citrinin H2, which we isolated, is considered to be non-cytotoxic to HeLa cells under physiological conditions. The experiments on heating citrinin under various conditions also indicated that citrinin H2 is a major decomposition product. Under selective heating conditions, citrinin decomposed to citrinin H2, and the

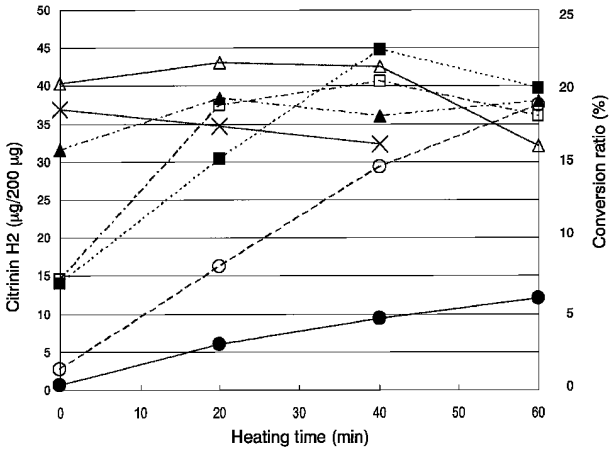

Fig. 3. Course of Citrinin H2 Formation in Citrinin Heated with Water at Various Temperatures.

Heating at 80°C (●), 100°C (○), 120°C (■), 130°C (□), 140°C (▲), 150°C (△), and 160°C (×).

citrinin H2 content reached at a plateau level. At that level, about 20% of the citrinin was changed to citrinin H2. Probably, citrinin H2 decomposed further to form other complex compounds. Kitabatake demonstrated that citrinin decomposed to a nontoxic compound on heating in water only under selective conditions.[14] Citrinin H2, as well as citrinin H1, might be markers of citrinin decomposition by heating in water.

Experimental

Instruments. Melting points were measured on a hot stage and are uncorrected. The following instruments were used; a Bruker AC250 FT-NMR spectrometer for ^{1}H- and ^{13}C-NMR spectra using tetramethylsilane as internal standard, a Hitachi M80B and a JEOL JMS700 mass spectrometers for mass spectra, and a JASCO IR spectrometer for IR spectra. The HPLC analysis was done on Hitachi HPLC equipment: Model L-6200 pump and L-4000 UV detector.

Materials. Citrinin was isolated from a cultivated medium of *Penicillium citrinum*, as described in the reference.[10]

Heating of citrinin. Citrinin was heated at 140°C as described in our previous paper.[10] Briefly, citrinin (500 mg) and water (375 ml) were distributed in fifty 10-ml glass vials. After the vials were sealed, the mixture was heated in a thermo-regulated pressure vessel from room temperature to 140°C at the rate of 3°C/min. After cooling to room temperature, the heated citrinin was collected and extracted with chloroform. The chloroform layer was washed with water, dried over sodium sulfate, and evaporated, to give a dark brown residue (420 mg).

Isolation of citrinin H2. The brown residue was put on a silica gel column (Wako gel C300, 15 g) with benzene, and eluted with toluene (50 ml) followed by a mixture of ethyl acetate: toluene (1:2). The eluate (each 5 ml) was collected to give 50 fractions. TLC analysis showed that the fractions No. 7–12 contained less polar compounds than citrinin. The fractions were combined and concentrated. The resultant residue thus obtained was rechromatographed on silica gel (Wako gel C300, 15 g) with diethyl ether-hexane mixture (0 to 80% diethyl ether). Each fraction (7 ml) was collected, and fractions No. 40–60 containing the less polar compounds, were combined and concentrated to obtain an oily residue (159 mg). The residue was dissolved in acetonitrile and put on preparative HPLC (column, Nacalai Tesque; eluant, acetonitrile: water (1:1); flow rate, 7 ml/min; detection, UV 254 nm). The peaks corresponding to t_R 9–12 min were corrected and concentrated to yield a brown residue (38 mg). The residue (19 mg) was further purified on silica gel (Wako gel C300, 2 g) with toluene-ethyl acetate mixtures with the 10% stepwise-elution method (0, 10, 20, 30, 40, and 50% ethyl acetate in toluene; each fraction, 10 ml). The 20% EtOAc eluate was evaporated to give citrinin H2 (13 mg), which was crystallized from chloroform to give pale yellow prisms, mp 139.5–140.0°C (mp 131–133°C[11]). IR ν_{max} (KBr) cm^{-1}: 3380 (OH), 3200, 2900, 1700 (C=O), 1620, 1600, 1510, 1230, 1140, 1130, 1105, 1100, 1010, 830; UV ν_{max} (EtOH) nm (ε): 203 (2.2×10^4), 223sh. (2.1×10^3), 282 (1.2×10^3); ^{1}H-NMR ($CDCl_3$) δ_H: 7.92 (s, 1H), 6.34 (d, J=2.4 Hz, 1H), 6.21 (d, J=2.4 Hz, 1H), 5.29 (br.s, OH), 5.20 (dq, J=7.2, 6.3 Hz, 1H), 3.26 (dq, J=7.2, 7.1 Hz, 1H), 2.16 (s, 3H), 1.26 (d, J=6.3 Hz, 3H), 1.20 (d, J=7.1 Hz, 3H); ^{13}C-NMR ($CDCl_3$) δ_C: 160.8, 154.5, 154.0, 143.8, 114.7, 105.9, 101.0, 74.0, 39.3, 17.3, 16.5, 10.5; EIMS m/z: 224 (M, 36%), 195 (1%), 178 (17%), 164 (15%), 151 (base peak, 100%), 137 (19%), 123 (17%); HREIMS m/z (M^+): calcd. for $C_{12}H_{16}O_4$, 224.1048; found, 224.1014.

Acetylation of citrinin H2. Citrinin H2 (2 mg) was dissolved in dry pyridine (0.5 ml) and acetic anhydride (0.25 ml) and the reaction mixture was left at room temperature overnight. After the usual workup, followed by silica gel column chromatography (Wako gel C300, 0.3 g) with toluene-ethyl acetate mixtures, citrinin diacetate was obtained (**1**. 1.9 mg). ^{1}H-NMR ($CDCl_3$) δ_H: 7.95 (s, 1H), 6.89 (d, J=2.3 Hz, 1H), 6.76 (d, J=2.3 Hz, 1H), 5.18 (m, J=6.6 Hz, 1H), 3.33 (m, J=7.0 Hz, 1H), 2.32 (s, 3H), 2.27 (s, 3H), 2.15 (s, 3H), 1.26 (d, J=7.0 Hz, 3H), 1.24 (d, J=6.5 Hz, 3H); HREIMS m/z (M^+): calcd. for $C_{16}H_{20}O_6$, 308.1257; found, 308.1240.

Alkaline degradation of citrinin. Alkaline degrada-

tion was done by the method of Barber *et al.*[15] Citrinin (42 mg) was dissolved in 10% aqueous sodium hydroxide, and the solution was heated to 120°C for 4 h. After cooling to room temperature, the reaction mixture was treated with barium hydroxide and filtered. The filtrate was neutralized with 2 N HCl and extracted with ethyl ether. The ethyl ether layer was dried over magnesium sulfate and evaporated to give a residue, which was chromatographed on silica gel (Wako gel C300, 500 mg) to give decarboxylated compound **2** (8 mg). ^{1}H-NMR (pyridine-d_5) δ_H: 7.07 (d, J=2.3 Hz, 1H), 6.93 (d, J=2.3 Hz, 1H), 4.29 (m, J=6.2 Hz, 1H), 3.45 (m, J=7.0 Hz, 1H), 2.57 (s, 3H), 1.41 (d, J=7.0 Hz, 3H), 1.39 (d, J=6.2 Hz, 3H); ^{13}C-NMR (pyridine-d_5) δ_C: 158.0, 157.6, 146.4, 115.0, 106.6, 101.9, 71.5, 43.4, 21.0, 17.5, 12.0. HREIMS m/z (M^+): calcd. for $C_{11}H_{16}O_3$, 196.1098; found, 196.1062.

Methylation of compound 2. Compound **2** (4 mg) was dissolved in dry acetone and treated with dimethyl sulfate (2 ml) and dry potassium carbonate (37 mg). The reaction mixture was refluxed for 20 h and filtered. The solid was washed with dry acetone. The filtrate and the washing were combined and evaporated to give a residue, which was dissolved in ethyl acetate. The ethyl acetate solution was washed with water and sodium bicarbonate, then dried over magnesium sulfate and filtered. The filtrate was evaporated to give a dark brown residue. The residue was chromatographed on silica gel (Wako gel C300, 0.5 g) with benzene-ethyl acetate mixture to give compound **3** (3 mg). ^{1}H-NMR ($CDCl_3$) δ_H: 7.26 (s, 1H), 6.43 (d, J=2.4 Hz, 1H), 6.36 (d, J=2.4 Hz, 1H), 3.90 (dq, J=8.2, 6.2 Hz, 1H), 3.81 (s, 3H), 3.80 (s, 3H), 3.05 (dq, J=8.2, 7.0 Hz, 1H), 2.15 (s, 3H), 1.27 (d, J=6.2 Hz, 3H), 1.18 (d, J=7.0 Hz, 3H); HREIMS m/z (M^+): calcd. for $C_{13}H_{20}O_3$, 224.1411; found, 224.1425.

Cytotoxicity test. Cytotoxicity of compounds to HeLa cells was examined using the MTT method as previously described.[13,16] Briefly, each test sample (1.15 mg) was dissolved in FBS (22 μl) and diluted with MEM (208 μl) to obtain the original test solution (5.0 mg/ml). The solution was further diluted with MEM containing 10% FBS to 400 μg/ml, 200 μg/ml and 100 μg/ml (50 μg/ml in the case of citrinin) for the cytotoxicity test. HeLa cells (3×10^3 cells/well) were incubated with test samples at 37°C under a 5% carbon dioxide atmosphere for 63 h. The cells were further incubated for 4 h after the addition of MTT, and the resultant blue formazan was dissolved in acidic isopropanol. Cytotoxicity was evaluated with the absorption of isopropanol solution at 540 nm.

Quantitative analysis. To each pressure vial, 200 μg of citrinin was added to 150 μl of water. The sealed vials were heated under various heating conditions from 80°C to 160°C. After heating for 0 min to 60 min the vials were cooled at room temperature. To each vial, acetonitrile (100 μl) was added and the dissolved sample was analyzed by HPLC (conditions: column, TOSO TSK gel C18, 4 mm i.d. × 20 cm; flow rate, 0.7 ml/min; eluent, 45% acetonitrile in water; detection at 280 nm).

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