Formation of a New Toxic Compound, Citrinin H1, from Citrinin on Mild Heating in Water

Alka Ben Trivedi,[a] Mitsuru Hirota,[b] Etsushiro Doi[a] and Naofumi Kitabatake*[,a]
[a] *Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan*
[b] *Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, 8304 Minami-minowa, Kami-ina, Nagano 399-45, Japan*

Citrinin, a relatively weak mycotoxin, can be detoxified by heating; however, a compound having high toxicity was formed on heating at 140 °C in the presence of water. The toxic compound was isolated from heated citrinin and its structure was determined. Its toxicity, evaluated by cytotoxicity assay, was 10-fold higher on a weight basis than that of citrinin. This new compound was named citrinin H1, which was also formed by heating citrinin at 100 °C for 30 min.

Mycotoxins, toxic metabolites of fungi, can contaminate food and agricultural products. Several highly toxic mycototoxins, *e.g.*, aflatoxin, can be eliminated by strict surveillence, while weakly toxic mycotoxins are not always analysed for. Citrinin, one such mycotoxin, has a polyketide structure,[1-4] and is both nephrotoxic[5] and carcinogenic.[6]

Citrinin decomposes at 175 °C and concurrently detoxifies at this temperature under anhydrous conditions.[7,8] Under semi-moist conditions, citrinin can be detoxified by being heated at 140 °C.[7] However, a previous study[8] showed that the toxicity of citrinin increased during heating at 140 °C in the presence of water and this suggested the formation of some toxic compound(s).

There are many reports concerning the change in structure and activity of mycotoxins during heating. Aflatoxin is degraded when heated with ammonia and is subsequently detoxified.[9] Ochratoxin A is decomposed and detoxified during heating under alkaline conditions.[10] Changes in structure and toxicity occurred not only by physical treatment but also by biological treatment.[11] Aflatoxin B_1 is metabolized to aflatoxin B_1 2,3-epoxide by the action of the cytochrome *P*-450 system in the endoplasmic reticulum of the liver.[12] This means that aflatoxin exhibits a potentially strong toxicity. Similar to this effect, heating as occurs in ordinary cooking may also produce such an effect, which has been rarely reported.

Here we show that a weak mycotoxin, citrinin, changed to a more toxic compound during heating. We isolated this new toxin, determined its structure, and examined its toxicity.

Results and Discussion

When citrinin was heated to 140 °C in the presence of water, it decomposed and various spots and peaks were found by TLC and HPLC. In the preliminary experiment, the heated sample was separated into less polar compounds (Group I) and polar compounds (Group II) by TLC. Group I was found to be more toxic than Group II from a toxicity assay (Fig. 1), and many peaks were observed during HPLC analysis (Fig. 2, upper). When the toxicity of each fraction collected from the HPLC eluate was examined using HeLa cells, three main toxic peaks (a, b, and c) were observed (Fig. 2, lower) which correspond to the three main HPLC peaks (A, B and C) observed on 254 nm detection. Since peak C contained a comparatively stable and sufficient amount of product for purification, we started to isolate the toxic compound corresponding to peak C.

Peak C [t_R 64.2 min in HPLC, R_f 0.27 in TLC (ethyl acetate–toluene, 1:2)] was purified by preparative HPLC to give the toxic compound which we named citrinin H1 **1** (4 mg) from

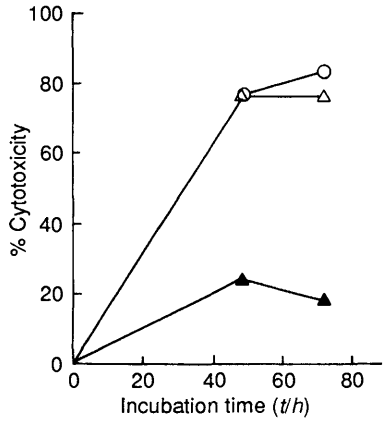


Fig. 1 Cytotoxicity of Groups I and II from heated citrinin to HeLa cells. Groups I and II were prepared by TLC from heated citrinin (2.5 μg). Cells were incubated in a well of a 96-well plate with citrinin 2.5 μg (○), Group I (△), or Group II (▲). The number of cells was assayed by the MTT method at the test wavelength of 540 nm, and the % cytotoxicity was calculated by the following equation: % cytotoxicity = [1 − (Absorbance$_{540nm}$ of the treated well/Absorbance$_{540nm}$ of the control well)] × 100.

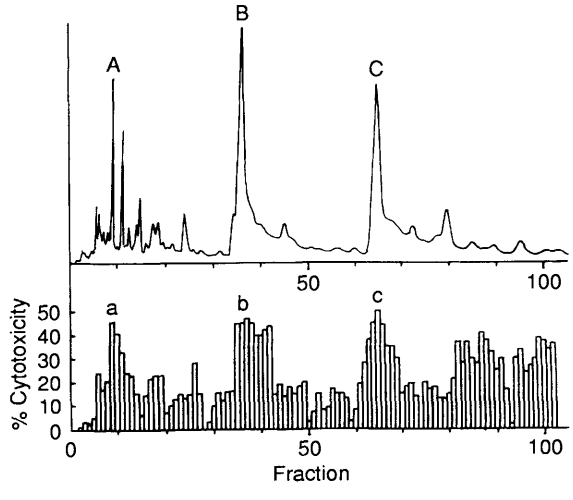


Fig. 2 HPLC of citrinin heated at 140 °C in water. Eluate was monitored by UV absorption at 254 nm (upper) and cytotoxicity (lower). Column ODS C_{18} (Cosmosil $5C_{18}$-AR, Nacalai tesque, 20 mm × 250 mm), eluent acetonitrile–water (1:1), flow rate 7 cm^3 min^{-1}, detection 254 nm.

Table 1 ^1H and ^{13}C NMR assignments of citrinin H1 **1** in $CDCl_3$

Position	^{13}C		^1H
1	61.5	CH	5.34 s
3	79.8	CH	4.13 qd, *J* 6.2, 4.8 Hz
4	37.9	CH	2.92 qd, *J* 7.3, 4.4 Hz
4a	140.4	C	
5	120.1	C	
6	155.0	C	
7	100.6	CH	6.57 s
8	145.0	C	
8a	111.4	C	
9	21.2	CH_3	1.32 d, *J* 6.2 Hz
10	22.7	CH_3	1.31 d, *J* 7.3 Hz
11	10.6	CH_3	2.16 s
1′	180.2	C	
2′	142.4	C	
3′	143.9	C	
4′	185.5	C	
5′	115.1	C	
6′	149.3	C	
7′	40.4	CH	3.10 qd, *J* 9.9, 7.3 Hz
8′	15.5	CH_3	1.30 d, *J* 7.3 Hz
9′	73.0	CH	5.49 qd, *J* 8.1, 5.9 Hz
10′	19.1	CH_3	1.39 d, *J* 5.9 Hz
11′	12.3	CH_3	2.14 s
12′	160.2	CH	7.83 s
		OH	5.25 s

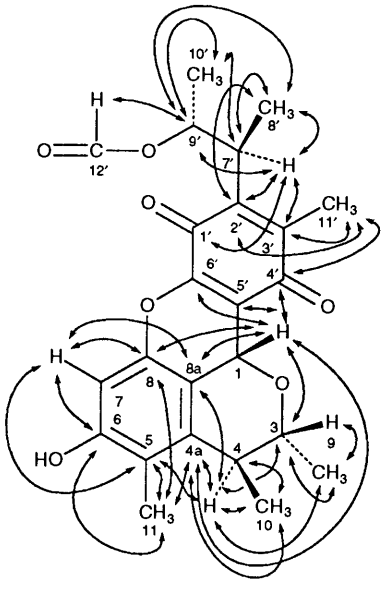


Fig. 3 Correlations ascribed by the HMBC experiment on citrinin H1 **1**

citrinin (500 mg). A separate quantitative analysis of heated citrinin with HPLC showed that compound **1** (6 mg) was formed from citrinin (100 mg).

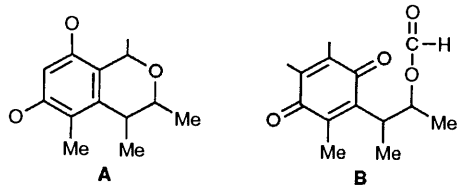
Structure of Citrinin H1 **1**.—The appearance of 24 carbons in the ^{13}C NMR spectrum and the high-resolution mass spectrum of compound **1** (a molecular ion peak at *m/z* 426.1676) confirmed the molecular formula $C_{24}H_{26}O_7$ and 12 units of unsaturation, suggesting that two molecules of citrinin had combined with a loss of 2 carbon units.

An off-resonance decoupling experiment and chemical shift analysis of the ^{13}C NMR spectrum indicated the presence of a formyl ester (δ_C 160.2), two carbonyls (δ_C 185.5, 180.2), and 10 olefinic carbons (δ_C 100.6–155.0), which also suggested that citrinin H1 **1** is composed of two conjugated ring systems derived from the citrinin molecule. The ^1H NMR spectrum in the presence of D_2O showed one exchangeable singlet, at δ 5.25, ascribable to a hydroxy group.

The IR spectrum of compound **1** indicated the presence of both a hydroxy group (3410 cm^{-1}) and an ester group (1715 and 1186 cm^{-1}). Also, the IR absorption band at 1670 cm^{-1} and those at 1651 and 1638 cm^{-1} suggested a benzene ring and a quinone ring,[13] respectively.

The UV absorption maximum at 271 nm supported the presence of the quinone ring, along with the absorptions at 322 and 423 nm.[14]

The two-dimensional C/H COSY NMR spectrum of citrinin H1 **1** showed assignment of protons and gave the correlations between C and H atoms as shown in Table 1. The COSY NMR spectrum of compound **1** showed the coupling of a methyl (9-H_3) with an oxygen-bearing methine (3-H); 3-H with 4-H; 4-H with 10-H_3, indicating the existence of a 2-oxybutane moeity. The coupling of methyl 10′-H_3 with 9′-H; 9′-H with 7′-H; and 7′-H with 8′-H_3 was also observed. These data showed the existence of two sets of 2-oxybutane moieties, indicating that the citrinin H1 molecule is composed of two parts (partial structures **A** and **B**), each of which was from one citrinin molecule. The ^1H

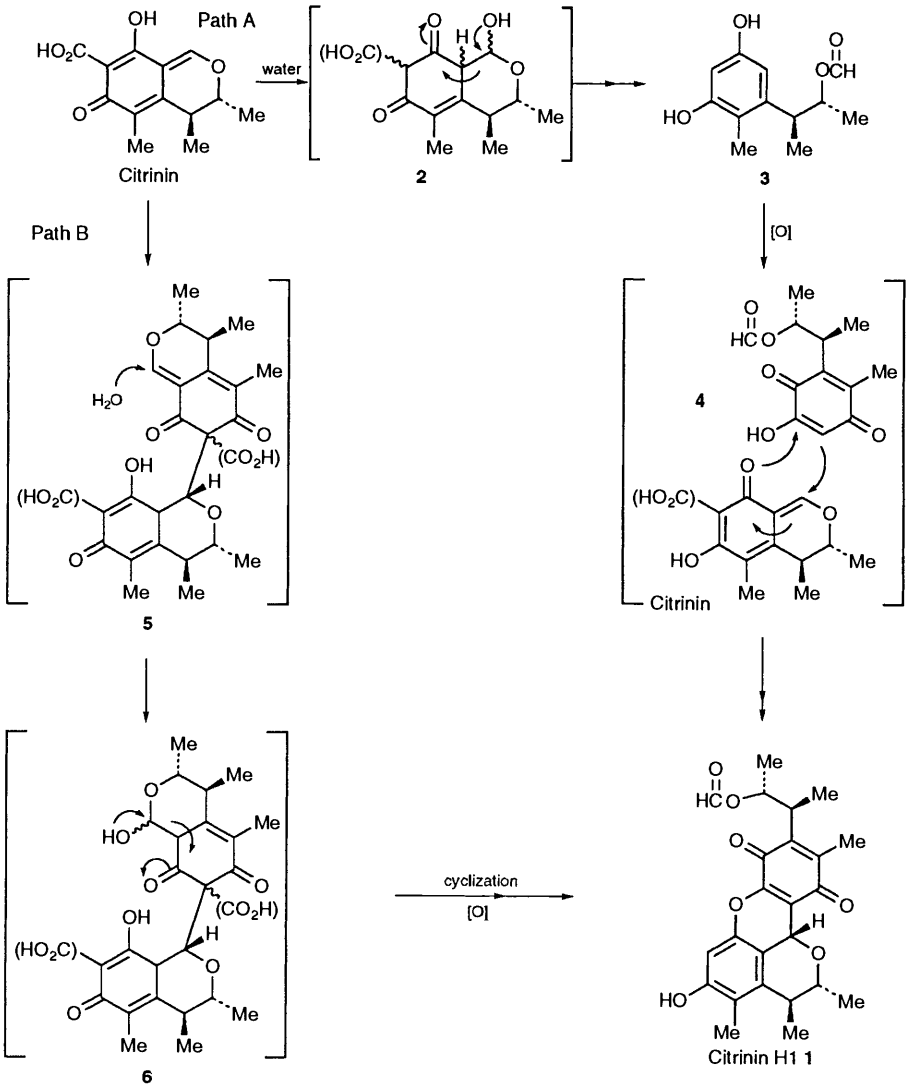


A **B**

NMR data also revealed the presence of an aromatic methine (7-H; δ_C for C-7, 100.6) adjacent to an oxygen-substituted aromatic carbon. From the COSY experiment, no information about the substitution pattern of the partial structure **A** was obtained. The long-range coupling obtained by 2D heteronuclear multiple bond coherence (HMBC) and homonuclear Hartmann–Hahn spectroscopy (HOHAHA) experiments of compound **1** was valuable for confirming substitutions as shown in Fig. 3. The HMBC experiment on compound **1** revealed that 1-H correlates to C-3, -4a, -8 and -8a; 3-H has coupled with C-9; 4-H with C-3, -4a, -5, -6, -8a, -9 and -10; 7-H with C-5, -6, -8, and -8a; 11-H_3 with C-4a, -5, -6 and -8. The long-range H/H couplings obtained from the HOHAHA experiment also supported the partial structure **A**; that is, couplings were observed between 7-H and 11-H_3, and between 7-H and 1-H.

The partial structure **B** was also deduced from a parallel set of NMR signals. As previously mentioned, COSY showed the presence of another 2-oxybutane group. The H/H long-range coupling obtained from the HOHAHA experiment showed a correlation between the formyl proton at δ 7.83 and the methyl (10′-H_3), ascertaining the position of the formyl ester group. Two carbonyl carbons along with four olefinic carbons (δ_C 149.3, 143.9, 142.4 and 115.1), except those of the benzene ring of partial structure **A**, are ascribable to quinone ring carbons. The quinone ring structure was unambiguously ascertained from the C/H long-range correlation obtained from the HMBC spectrum (Table 2). The HMBC spectrum also revealed the presence of binding between the quinone ring and the 2-formyloxybutane group. The most important correlations are as follows: methyl protons 11′-H_3 correlate to the quaternary carbons in quinone ring C-2′, -3′ and -4′; and 7′-H with C-1′, -2′ and -3′ carbons in the quinone ring.

In elucidating the binding of the partial structures **A** and **B**, long-range C/H correlations from the HMBC spectrum are informative. The proton 1-H is coupled with carbonyl C-4′ and quaternary carbons C-5′ and -6′ in the quinone ring. This long-range correlation between two partial structures **A** and **B** implies a bond between C-1 and C-5′. Obviously, the quaternary carbon C-6′ connects to the oxygen at C-8, making a six-membered ring. This structure satisfied the 12 units of unsaturation of citrinin H1 **1**.



Scheme 1 Formation of citrinin H1 **1** from citrinin

Table 2 Long-range correlations revealed by HMBC and HOHAHA experiments on compound **1**

HMBC	HOHAHA
1-H C-3, C-4a, C-4′, C-5′, C-6′, C-8, C-8a	7-H
3-H C-9	4-H, 9-H_3
4-H C-3, C-4a, C-5, C-8a, C-9, C-10	3-H, 10-H_3
7-H C-5, C-6, C-8, C-8a	1-H, 11-H_3
9-H_3 C-3	3-H
10-H_3 C-4, C-4a	4-H
11-H_3 C-4a, C-5, C-6, C-8	7-H
7′-H C-1′, C-2′, C-3′, C-8′, C-9′	8′-H_3, 9′-H, 10′-H_3
8′-H_3 C-2′, C-7′, C-9′	7′-H, 9′-H
9′-H	7′-H, 10′-H_3, 8′-H_3
10′-H_3 C-7′, C-9′	12′-H, 9′-H, 7′-H
11′-H_3 C-2′, C-3′, C-4′	
12′-H C-9′	10′-H_3

The stereochemistry of compound **1** was investigated by using two-dimensional nuclear Overhauser effect spectroscopy (NOESY). A strong NOE between 1-H and 3-H indicated that these protons are oriented to the same face of the molecule. The configuration of C-3 and C-4 was the same as that of citrinin.[15] The configuration of C-7′ and C-9′ should be the same as that of the corresponding positions C-4 and C-3, respectively.

From the structure **1**, citrinin H1 might be formed through decarboxylation and dehydration of citrinin. Barber *et al.* reported that heating of citrinin under alkaline conditions led to the formation of 3-(3,5-dihydroxy-2-methylphenyl)butan-2-ol[16,17] and that citrinin existed as a hydrate form in water at pH 7.4.[2] The formyl ester carbon of citrinin H1 may be derived from C-1 citrinin. Two plausible mechanisms for the formation of citrinin H1 from citrinin are as follows (Scheme 1). Citrinin changes to a resorcinol intermediate **3** in the reaction with water (*via* species **2**), followed by ring opening and decarboxylation (path A). Then, the intermediate **3** is oxidized, presumable by air, to become a *para*-quinone-type compound **4**. It reacts with another citrinin molecule *via* a hetero-Diels–Alder-type reaction to make a six-membered ring.[18] Subsequent decarboxylation and dehydration led to the formation of citrinin H1.

On the other hand, two molecules of citrinin combine together at positions C-1 and C-7 to make an intermediate **5** (path B), which undergoes attack of water to yield an intermediate **6**. The intermediate **6** changes to citrinin H1 through several steps including ring opening, a new ring formation, dehydration, and decarboxylation. Recently, we isolated the intermediate **3**, which we have named citrinin H2, as a detoxified compound from mild heating of citrinin in water.[19] The former route (path A) may therefore be the more probable route to citrinin H1.

Cytotoxicity of Citrinin H1 **1**.—The cytotoxic effect of

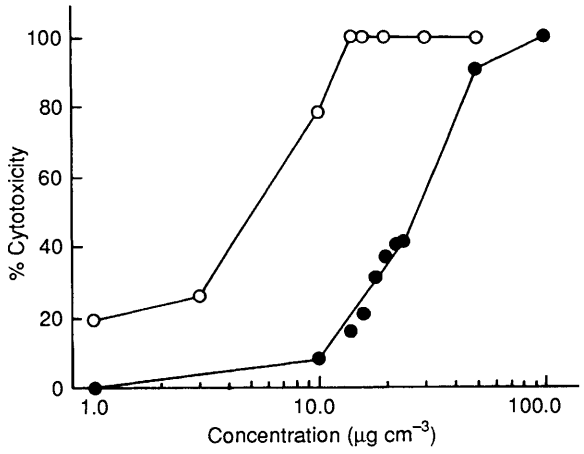


Fig. 4 Dose–response curves of citrinin H1 **1** and citrinin. HeLa cells were incubated with citrinin H1 **1** (○) and citrinin (●) for 24 h

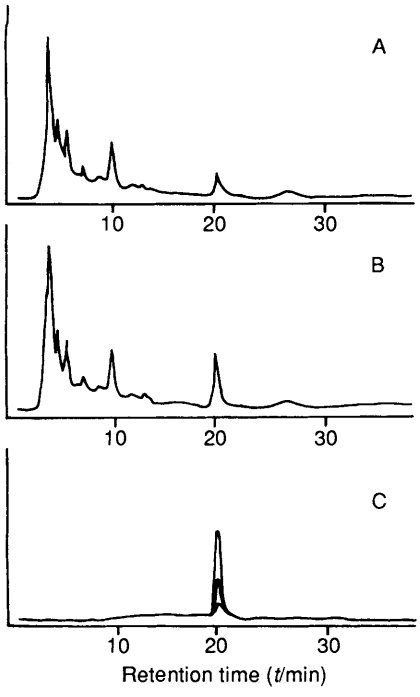
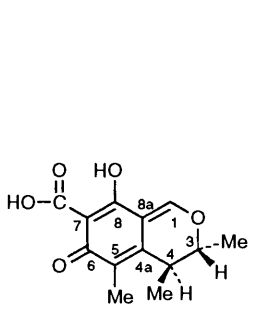


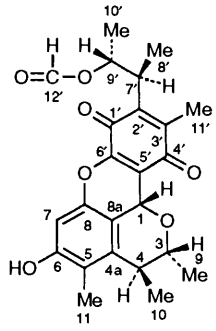
Fig. 5 HPLC of citrinin heated at 100 °C for 30 min. Column ODS C_{18} (Cosmosil $5C_{18}$-AR, Nacalai tesque, 4 mm × 250 mm), eluent acetonitrile–water (1:1), flow rate 1 cm^3 min^{-1}, detection 280 nm. (A) Heated citrinin (50 μg); (B) a mixture of heated citrinin (50 μg) and compound **1** (1 μg); (C) compound **1** (0.5, 1.0 and 1.5 μg) applied to HPLC.

citrinin H1 **1** was examined by using HeLa cells (Fig. 4). HeLa cells were incubated with the compound for 72 h at 37 °C and, from time to time, the toxicity was observed. At 24 h of incubation, citrinin showed 90% cytotoxicity for a dose of 100 μg cm^{-3} and 43% for a dose of 25 μg cm^{-3}. Citrinin H1 showed 100% cytotoxicity for 10 μg cm^{-3} and 73% for a 5 μg cm^{-3} dose, indicating that the toxicity of citrinin H1 is about 10 times that of citrinin on a weight basis, and 20 times higher on a molar basis. Citrinin has an α,β-unsaturated carbonyl group which might be reactive towards an electron-rich group, such as an amino group or thiol in macromolecules.

Occurrence of Citrinin H1 **1** *by Heating at/below* 100 °C *for* 30 *min.*—Until now, heating had been done at a rate of 3 °C min^{-1} up to 140 °C. The heated citrinin sample was then cooled to room temperature. Since the reaction depends on both the temperature and the heating time, it may be possible that citrinin H1 **1** is formed even at a temperature lower than 140 °C if heating was prolonged. Fig. 5 shows the HPLC pattern of citrinin heated at 100 °C for 30 min. The peak corresponding to citrinin H1 **1** (t_R 19.8 min) was observed. By NMR analysis, it was confirmed that this peak contained the same compound as that already purified, and TLC of this peak showed the same R_f-value as that of previously prepared compound **1**. From Fig. 5, we calculate that compound **1** (1.2 mg) was formed from citrinin (100 mg) under these conditions. In another experiment, we found that compound **1** was also formed by heating of citrinin at 90 °C for 30 min. This result indicated that citrinin can be changed to a more toxic compound by ordinary cooking and/or food processing in water.



Citrinin



Citrinin H1

The degradation and detoxification of aflatoxin have been well studied. Although aflatoxin B1 is heat stable, the autoclaving of moist ground nuts at 120 °C for 4 h reduced the amount of aflatoxin and accordingly the toxicity.[20] Dollear *et al.*[9] made a detailed study of the effect of heat and moisture on the degradation of aflatoxin. They found that the degradation rate increased with increasing moisture or time of heating. However, with citrinin, its degradation by heating in the presence of water does not result in detoxification, but instead forms a more toxic compound. The present study reveals the necessity to reexamine the effect of heating on the toxicity of mycotoxins. That is, the decomposition of mycotoxins may not always result in detoxification. Citrinin is considered to be a weak toxin but the formation of new toxic compounds by heating is strong recommendation for a reassessment of the health hazards due to citrinin, and it is necessary to consider whether the mycotoxins can be chemically converted into materials of high toxicity.

Experimental

Organic reagents of extra-pure reagent grade were used. The cell-culture reagents were purchased from Sigma Chemical Co, Ltd, (St Louis, MO, USA), Gibco/BRL Life Technologies (Gaithersburg, MD, USA) or Whitaker Bioproducts. Inc. (Walkersville, MD, USA). The HeLa cell line was obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). Silica gel column chromatography was performed on Wakogel C-300 manufactured by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HPLC analysis was performed on Hitachi HPLC equipment; Model L-6200 pump, L-4000 UV detector and D-2500 chromato integrator (Tokyo, Japan). Preparative HPLC was done with a Cosmosil packed column 5C18-AR (20 i.d. × 250 mm) with a C_{18} guard column (4.6 i.d. × 10 mm, Nacalai Tesque, Kyoto, Japan). NMR spectra were recorded by using a JEOL JNM-GX (400 MHz) and a GE-Omega 500 (500 MHz) spectrometer, with tetramethylsilane as internal standard. Mass spectra were taken on a JEOL JMS-DX 300 mass spectrometer. IR spectra were recorded on a Shimadzu FTIR-4200 spectrometer.

Preparation of Citrinin.—Citrinin was prepared from the toxigenic strain of *Penicillium citrinum* isolated during a survey of toxigenic fungi from food materials,[21] and this strain was independently confirmed by the Commonwealth Mycological Institute at Kew, United Kingdom (accession number IMI 309573). Culture was maintained on PDA (potato dextrose agar) slant and stored at −4 °C. The spores from 6-day-old cultures on PDA were inoculated into YES broth (2% yeast extract and 15% sucrose) and statically incubated at room temperature. Every third day the broth medium was replaced by fresh medium and citrinin was extracted three times with chloroform. All chloroform fractions were pooled, and concentrated under reduced pressure. The resultant residue was purified by silica gel column chromatography [(95 : 5) benzene–ethyl acetate]. Citrinin was crystallized from ethanol to give pale yellow prisms.

Heating of Citrinin.—Citrinin was dissolved in chloroform and the solution was divided into 50 10 cm^3 glass vials (V-10B, Nichiden Rika Glass Co. Ltd., Tokyo), each vial containing citrinin (10 mg). The chloroform was completely evaporated off under dry N_2. Doubly distilled water (7.5 cm^3) was then added to each vial. Each vial was sealed with an aluminium cap having a Teflon packing. Heating was done in a thermoregulated pressure vessel (type TEM-V, Taiatsu Scientific Glass Co., Ltd., Tokyo, Japan). Samples were heated to 140 °C at the rate of 3 °C min^{-1}. After being cooled to room temperature, the samples were collected in a separatory funnel and extracted with chloroform. The resultant chloroform layer was evaporated to give a brown residue.

Toxicity Assay.—The toxicity was checked by using a HeLa cell line by the MTT assay method as described elsewhere.[7,8]

Preliminary Experiment.—The heated citrinin (40 mg) was applied to a preparative TLC plate (10 × 10 cm. Empore™, 3M, St. Paul, MN, USA), and developed in ethyl acetate. Two groups of spots separated; those between R_f 0.43–0.93 were called Group I and those between R_f 0.00–0.14 were called Group II. Group I, showing high cytotoxicity, which was assayed using HeLa cells, was applied to preparative HPLC in acetonitrile. Elution was with (1 : 1) acetonitrile–water at a flow rate of 7 cm^3 min^{-1} and the eluate was monitored at 254 nm. Three major peaks (A, B and C) were observed (Fig. 2). Fractions were collected every minute. All fractions were lyophilized, subjected to the toxicity assay, and confirmed by TLC. Fractions 9–12 (peak a, t_R 9–12 min), 35–42 (peak b, t_R 35–42 min) and 63–69 (peak c, t_R 63–69 min) showed high cytotoxicity.

Isolation of Citrinin H1 **1**.—Citrinin (500 mg) was heated with water (375 cm^3). To separate Groups I and II, silica gel column chromatography was used in place of preparative TLC. The heated citrinin, a brown residue, was applied to the silica gel column (Wakogel C300, 15 g) and eluted with toluene (50 cm^3) followed by a mixture of (1 : 2) ethyl acetate–toluene. Fractions (5 cm^3) were collected to give a total of 50 fractions. Fractions 7–12 contained Group I, which was checked by TLC, and fractions were combined and concentrated. The resultant resin thus obtained was chromatographed again on silica gel (Wakogel C300, 15 g) with an diethyl ether–hexane mixture (0–80% diethyl ether). Each fraction (7 cm^3) was collected and fractions 40–60 containing Group I were combined and concentrated. The solvent could not be removed completely. An oily residue (159 mg) was obtained. The residue was dissolved in acetonitrile and subjected to preparative HPLC. Elution was with (1 : 1) acetonitrile–water, and detection at 254 nm. The flow rate was 7 cm^3 min^{-1}. Each fraction (7 cm^3) was collected. Toxicity of each fraction was checked and fractions 48–67 showing a single spot on TLC [ethyl acetate–toluene (1 : 2), R_f 0.27] and high toxicity were combined, and evaporated *in vacuo*. The sample obtained was applied to HPLC again under the same conditions as those used above to remove traces of impurity. The pure compound (4 mg), which we named citrinin H1, was obtained.

Citrinin H1 **1**. λ_{max}(EtOH)/nm 271 (ε 1.38 × 10^4 dm^3 mol^{-1} cm^{-1}) 322 (ε 1.95 × 10^3) and 423 (ε 1.34 × 10^3); ν_{max}(film)/cm^{-1} 3410, 2974, 1715, 1670, 1651, 1638, 1593, 1260, 1186, 1148, 1113 and 677; δ_H($CDCl_3$) and δ_C($CDCl_3$): see Table 1; *m/z* 428 (29%), 426 (M^+, 43), 425 (50), 411 (2), 397 (11), 382 (100), 353 (68) and 311 (62) (Found: M^+, 426.1676. $C_{24}H_{26}O_7$ requires M, 426.1671).

Acknowledgements

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