Biotransformation of Imperatorin by *Aspergillus flavus*

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Imperatorin (**1**) was metabolized by *Aspergillus flavus*, in growth media, to give five metabolites. On the basis of their physical data, the structures of the five metabolites were elucidated as xanthotoxol (**2**), *E*-trichoclin (**3**), *Z*-trichoclin (**4**), *E*-imperatorin acid (**5**), and *Z*-imperatorin acid (**6**); among these, **4**, **5**, and **6** were characterized as new coumarins. The five metabolites **2**−**6** were tested for anti-hepatitis B virus activity in vitro and found to be less active than the parent compound **1**.

Imperatorin (**1**), a constituent of the fruit peel of shaddock (*Citrus maxima* (Burm. f.) Merr. form. Buntan (Hay.) Hort.), is known to exhibit a wide spectrum of biological activities. It possesses cytotoxic, antibacterial, insecticidal, and antifungal activities,[1,2] as well as phototoxic activity.[3,4] It has been shown to significantly inhibit contact dermatitis in mice and passive cutaneous anaphylaxis reaction in guinea pigs[5] and also strong antiplatelet aggregation activity in vitro.[6] Recently, it has been reported that **1** induces a vasorelaxing effect on rabbit corpus cavernosum.[7]

Biotransformation, with fungi, has been one of the major goals of our studies in recent years. In an ongoing program, biotransformation of **1** was performed to generate metabolites and to assess their biological activity. Herein we describe the metabolism of **1** by *Aspergillus flavus*. The fermentation methods, isolation, and structural identification of the metabolites are reported. The anti-hepatitis B virus (anti-HBV) activities of **1** and its metabolites were evaluated.

Results and Discussion

For the biotransformation of imperatorin (**1**), 10 microorganisms isolated from the moldy fruit peel of *C. maxima* were subjected to screening experiments. *A. flavus* showed the best results and was able to convert **1** into several metabolites (Figure 1). This reaction was scaled up to afford five metabolites, **2**−**6**. Compound **2** was identified as xanthotoxol by comparison of its spectroscopic data with those of xanthotoxol,[8] which is the hydrolysis product of **1**.

Metabolites **3** and **4** had the same molecular formula, $C_{16}H_{14}O_5$, as deduced by HREIMS and ^{1}H and ^{13}C NMR, 16 amu more than **1**. The ^{1}H and ^{13}C NMR spectroscopic data of **3** and **4** were similar. Their ^{1}H NMR spectra indicated that compounds **3** and **4** also possessed a basic linear furanocoumarin structure, as does **1**. In comparison with the ^{1}H NMR spectrum of **1**, the spectra of **3** and **4** indicated the appearance of a CH_2OH group and the disappearance of a methyl group. This clearly indicated that compounds **3** and **4** were produced by the hydroxylation of **1**. The ^{1}H NMR spectrum of compound **3** was similar to that of *E*-trichoclin.[9] In NOE experiments, compound **4** showed an NOE correlation between H-12 (δ 5.67) and H_3-14 (δ 1.90). Consequently, the structure of **4** was concluded to be *Z*-trichoclin.

The ^{1}H and ^{13}C NMR spectra of metabolites **5** and **6** were also similar to each other. From the ESIMS and ^{1}H and ^{13}C NMR data, the molecular formulas of **5** and **6** were deduced to be $C_{16}H_{12}O_6$, 30 amu more than **1**. Comparison of the ^{13}C NMR spectroscopic data of **5** and **6** with **4** showed 11 similar chemical shifts (deviation <3 ppm), of which 10 were assigned to the linear furanocoumarin and the other one carbon was assigned to the oxymethylene at C-11. The remaining four ^{13}C chemical shifts included one methyl carbon (δ_C 11.9 in **5**; 13.1 in **6**), one methine carbon (δ_C 131.3 in **5**; 132.5 in **6**), one quaternary carbon (δ_C 135.5 in **5**; 136.7 in **6**), and one carboxylic carbon (δ_C 169.3 in **5**; 170.5 in **6**). The upfield shift of the olefinic C-13 quaternary carbon and the downfield shift of the olefinic C-12 carbon coupled with the carboxylic carbon (C-14 or C-15) in the ^{13}C NMR spectra showed the presence of an α,β-conjugated carboxylic group in both **5** and **6**. The ^{1}H NMR spectra of **5** and **6** compared to **4** showed that the CH_2OH functionalities in **5** and **6** disappeared and that the olefinic proton of H-12 (δ_H 7.01 in **5**; 6.66 in **6**) shifted downfield, consistent with a β-effect of the carboxylic group. Irradiating H-12 (δ 6.66) of metabolite **6** caused enhancement of H_3-14 (δ 1.90). Thus, metabolite **5** was characterized as *E*-imperatorin acid, and the metabolite **6** was characterized as *Z*-imperatorin acid.

The growth curve of *A. flavus* is shown in Figure 2. The results showed that *A. flavus* grew rapidly and the whole growth period included three phases: (1) a linear phase (0−2nd day), (2) a stationary phase (2−13th day), (3) a death phase (15th day). Imperatorin was administrated on the third day.

The yields of the metabolites *E*- and *Z*-imperatorin acid (**5**, **6**) were too low to quantify. The contents of metabolites **2**−**4** and substrate **1** in broth and mycelium during the 14 days after administration are shown in Table 1. Incubation of **1** with *A. flavus* afforded three metabolites, **2**−**4**, in 3, 6, and 10% yield, respectively, based on the weight relative to **1**, 14 days after administration. In broth, the contents of metabolites increased, except **2**, with the incubation days until 6 days after administration. In mycelium, the changes of metabolite **2** varied slightly during the incubation; however, metabolites **3** and **4** dramatically increased after 14 days administration. At day 14, we detected the formation of metabolites **5** and **6** using HPLC analysis (Figure 1).

The proposed biotransformation pathway is illustrated in Scheme 1. Imperatorin (**1**) could be specifically oxidized at the C-14 and C-15 positions in addition to the cleavage

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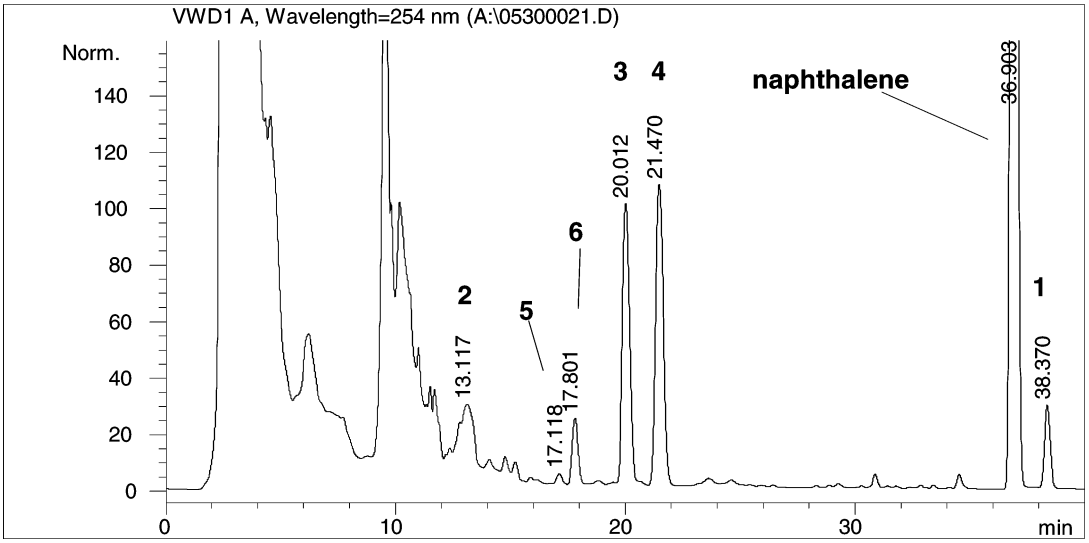

Figure 1. HPLC chromatogram of metabolites formed by *A. flavus*, 14 days after adminstration in broth.

Table 1. Quantitative Analysis of **1** and Its Metabolites **2**−**4** by HPLC

sampling time	**1** μg/flask	**2** μg/flask	**3** μg/flask	**4** μg/flask
0 day	4943[a] ± 20 (100)[b]			
2nd day	4681 ± 76 (95)	101 ± 4.8 (2)	74 ± 3.5 (2)	73 ± 1.1 (1)
4th day	4123 ± 63 (83)	119 ± 4.2 (2)	132 ± 10.2 (5)	141 ± 8.4 (3)
6th day	4022 ± 210 (81)	169 ± 9.3 (3)	194 ± 19.0 (4)	214 ± 51.8 (4)
8th day	3215 ± 30 (65)	216 ± 38.5 (4)	212 ± 16.0 (4)	248 ± 12.8 (5)
10th day	3147 ± 43 (64)	164 ± 12.3 (3)	223 ± 53.9(5)	258 ± 48.2 (5)
12th day	3083 ± 38 (62)	219 ± 22.7 (4)	213 ± 9.1 (4)	287 ± 0.2 (6)
14th day	2887 ± 113 (58)	ND[c]	306 ± 59.8 (6)	509 ± 44.0 (10)

[a] Value is the sum of contents in broth and mycelium. [b] The value in parentheses denotes the yield rate based on weight relative to starting material **1** (5 mg), every other day after administration (zero time). [c] ND: not determined; the peak is overlapped by other peaks.

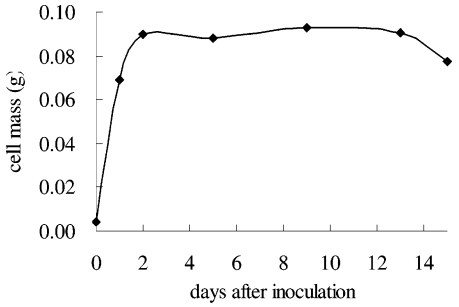

Figure 2. Growth curve of *A. flavus*.

of the isoprenyl side chain. Moreover, at the death phase of the fungus, the primary alcohol functionalities in **3** and **4** could be further oxidized to the carboxylic acid.

The anti-HBV effects of the substrate **1** and metabolites **2**−**4** and **6** on the MS-G2 cell line (metabolite **5** was too minor to analyze) are shown in Table 2. Compound **1** showed the best anti-HBV activity at the noncytotoxic concentrations of 50 and 100 μM and suppressed both HBV surface and e antigen production, with the highest inhibition percentages of 55.3%, 68.4% and 22.5%, 34.3%, respectively. Metabolite **3** showed medium inhibition of HBV surface antigen production, with 61.3% inhibition at 100 μM. Metabolite **6**, at 100 μM, only slightly suppressed HBV surface antigen production. Among the compounds tested, the biotransformation products that underwent isoprenyl group cleavage and methyl group oxidation exhibited less potent activity of anti-HBV surface antigen production than its corresponding precursors and showed no effect on anti-HBV e antigen.

Experimental Section

General Experimental Procedures. Melting points were recorded on a Yanaco MP-I3 micro melting point apparatus and are uncorrected. NMR spectra were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C on a Varian Unity Inova-500 spectrometer. Coupling constants are reported in Hz. EIMS and HREIMS spectra were obtained using Finnigan MAT GCQ and Finnigan MAT 95S spectrometers, respectively. ESIMS data were recorded on a Finnigan LCQ spectrometer. Reversed-phase HPLC was performed on a Hewlett-Packard series 1100 pump system (Hewlett-Packard) equipped with a Hewlett-Packard UV/vis detector.

Plant Material. The buntan shaddock was cultivated at Hwa-Lien District Agricultural Improvement Station, Hwa-Lien County, Taiwan. The fruit peels were collected in October 2001, dried at 45 °C, and pulverized. A voucher specimen was deposited in the Laboratory of Plant Nutrition, Department of Agricultural Chemistry, National Taiwan University, Taiwan.

Isolation of Imperatorin (1). Dried fruit peels (2 kg) were extracted by SF−CO_2 at 4000 psi and 50 °C. The SF−CO_2 extract (33.07 g) was subjected to a silica gel column and successively eluted with *n*-hexane−EtOAc (1:0, 15:1, 10:1, 5:1, 2:1, 1:1, 0:1, v/v) to yield compound **1** (2.53 g; relative retention time, Rt_R 1.04 in HPLC). The physical and ^{1}H NMR spectroscopic data of **1** were in agreement with the reported data for imperatorin.[10]

Preparative Scale Conversion. A culture of *A. flavus* (authenticated by Bioresources Collection and Research Center, Hsin-Chu, Taiwan, ROC) was maintained on potato dextrose agar slants and stored at 4 °C. Seed culture was grown in five 125 mL Hinton flasks containing 25 mL of potato-dextrose broth medium. After incubation at room temperature on a 100 rpm rotary shaker for 3 days, the whole culture was

Scheme 1. Metabolites of Imperatorin (**1**) by *A. flavus*

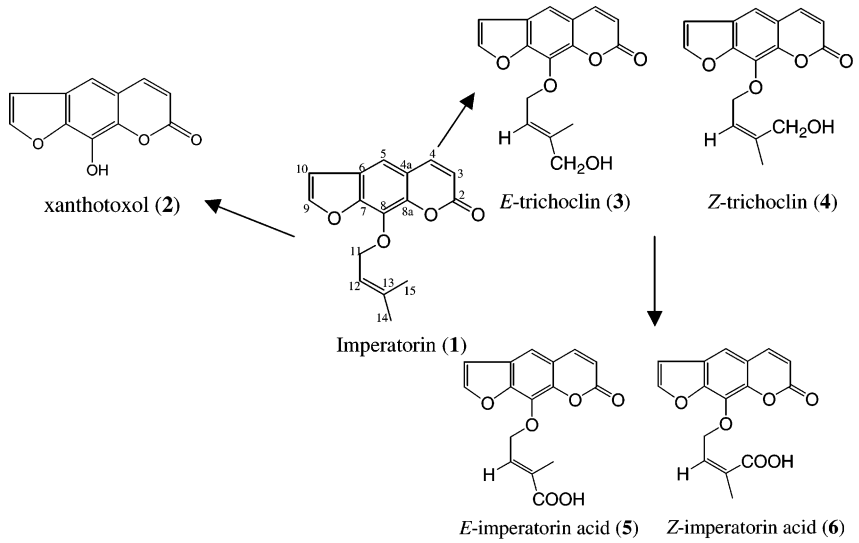

Table 2. Anti-HBsAg and Anti-HBeAg Effects of **1** and Its Metabolites **2**−**4** and **6** on HBV-Producing Cell Line MS-G2 at 48 h

	conc (μM)	HBsAg (inhibition %)[a]	HBeAg (inhibition %)[a]	MTT (inhibition %)[a]
DMSO	2.5 μL/mL	11.5	3.1	6.1
1	100	68.4	34.3	−8.7
	50	55.3	22.5	8.9
	10	14.9	3.8	11.0
2	100	19.8	0.7	17.0
	50	10.5	2.7	12.2
	10	5.2	−5.0	7.8
3	100	61.3	29.0	10.3
	50	37.4	16.5	3.4
	10	20.9	8.0	13.0
4	100	45.2	12.4	12.6
	50	34.6	11.9	15.0
	10	13.6	8.2	18.0
6	100	27.0	12.5	14.7
	50	12.3	1.6	18.4
	10	13.2	−0.7	11.7

[a] The value is the average of three replications.

transferred into 25 1 L Hinton flasks containing 250 mL of PDB and incubated for 2 days under aeration by shaking. Substrate **1** (1.25 g) in 12.5 mL of DMSO was dispersed into the flasks. After a further 14 days, the fermentation was harvested. The mycelium was filtered off, washed with H_2O, ground to a powder to destroy the cell wall using liquid nitrogen, and extracted with MeOH. The broth filtrate was extracted three times with EtOAc, and the organic layers were combined. The extracts of mycelium (3.15 g) and broth (3.39 g) were combined and subjected to column chromatography on Si gel. The column was eluted with CH_2Cl_2−MeOH (30:1, 25:1, 15:1, 10:1, 5:1, v/v) gradients, and the CH_2Cl_2−MeOH (15:1, 10:1, and 5:1) fractions were further separated with a semipreparative HPLC system. A linear gradient of 85 to 40% H_2O−acetonitrile (v/v) over 40 min at 2.0 mL/min was used as a mobile phase on a 10 × 250 mm, C18 reversed-phase column (Waters, 5C 18-MS-II column). Compounds **2** (8.1 mg; Rt_R 0.39 in HPLC), **3** (10.0 mg; Rt_R 0.56), **4** (22.5 mg; Rt_R 0.60), **5** (1.0 mg; Rt_R 0.43), and **6** (4.1 mg; Rt_R 0.48) were then obtained.

***Z*-Trichoclin (4):** colorless prisms ($CHCl_3$); mp 162−163 °C; UV (MeOH) λ_{max} (log ϵ) 308 (3.90), 258 (3.98), 220 (4.18), 202 (4.20) nm; ^{1}H NMR ($CDCl_3$) δ 1.90 (3H, s, H-14), 4.18 (2H, s, H_2-15), 5.01 (2H, d, J = 6.8 Hz, H-11), 5.67 (1H, t, J = 6.8 Hz, H-12), 6.30 (1H, d, J = 9.6 Hz, H-3), 7.04 (1H, d, J = 2.4 Hz, H-10), 7.14 (1H, s, H-5), 7.58 (1H, d, J = 2.4 Hz, H-9), 8.12 (1H, d, J = 9.6 Hz, H-4); ^{13}C NMR ($CDCl_3$) δ 21.4 (C-15), 61.9 (C-14), 68.8 (C-11), 94.4 (C-5), 104.9 (C-10), 107.4 (C-4a), 112.7 (C-3), 114.1 (C-6), 122.0 (C-12), 139.4 (C-4), 141.4 (C-13), 145.1 (C-9), 148.6 (C-8), 152.6 (C-8a), 158.10 (C-7), and 161.3 (C-2); EIMS m/z 286 $[M]^+$ (29), 202 (100); HREIMS m/z 286.0843 (calcd for $C_{16}H_{14}O_5$, 286.0836).

***E*-Imperatorin acid (5):** colorless powder (MeOH); ^{1}H NMR (CD_3OD) δ 1.91 (3H, s, H-15), 5.26 (2H, d, J = 6.0 Hz, H-11), 6.32 (1H, d, J = 10 Hz, H-3), 7.01 (1H, t, J = 6.0 Hz, H-12), 7.18 (1H, d, J = 2.0 Hz, H-10), 7.23 (1H, s, H-5), 7.82 (1H, d, J = 2.0 Hz, H-9), 8.30 (1H, d, J = 10 Hz, H-4); ^{13}C NMR (CD_3OD) δ 11.9 (C-15), 69.6 (C-11), 94.0 (C-5), 104.8 (C-10), 107.3 (C-4a), 112.3 (C-3), 114.3 (C-6), 131.3 (C-13), 135.5 (C-12), 139.9 (C-4), 146.0 (C-9), 148.7 (C-8), 152.7 (C-8a), 158.6 (C-7), 161.9 (C-2), and 169.3 (C-14); ESIMS m/z 299 $[M - H]^-$ (56), 201 $[M - C_5H_6O_2 - H]^-$ (100).

***Z*-Imperatorin acid (6):** colorless powder (MeOH); ^{1}H NMR (CD_3OD) δ 1.90 (3H, s, H-14), 5.19 (2H, d, J = 6.5 Hz, H-11), 6.31 (1H, d, J = 9.5 Hz, H-3), 6.66 (1H, t, J = 6.5 Hz, H-12), 7.21 (1H, d, J = 2.0 Hz, H-10), 7.22 (1H, s, H-5), 7.80 (1H, d, J = 2.0 Hz, H-9), 8.34 (1H, d, J = 9.5 Hz, H-4); ^{13}C NMR (CD_3OD) δ 13.1 (C-14), 70.7 (C-11), 95.1 (C-5), 106.0 (C-10), 108.4 (C-4a), 113.5 (C-3), 115.4 (C-6), 132.5 (C-13), 136.7 (C-12), 141.11 (C-4), 147.1 (C-9), 149.8 (C-8), 153.8 (C-8a), 159.7 (C-7), 163.1 (C-2), and 170.5 (C-15); ESIMS m/z 301 $[M + H]^+$ (10), 203 $[M - C_5H_6O_2 + H]^+$ (100).

Time Course of Biotransformation and Quantification of Metabolites. In a separate study for quantitative analysis of **1** and metabolites, 5 mg of **1** in 0.1 mL of dimethyl sulfoxide (DMSO) was added to a 72 h old culture. Samples of each flask were taken every other day for two weeks following substrate addition. Each sample was filtrated into broth and mycelium and freeze-dried for 3 days, and the dry fungus was weighed and further redissolved in MeOH. The controls were subjected to the same analysis. The flasks containing media and **1** showed only the presence of the substrate. The chromatograms of the cultures without **1** were also analyzed, and the cell growth curve was evaluated. Each treatment was replicated three times. The same volume of 1% naphthalene (internal standard, Rt_R 1.00) was added to all filtrates, which were then subjected to HPLC. The mobile phase and detector used were the same as above, but the column was 4.6 × 250 mm and the flow rate was 1.0 mL/min. The contents of these compounds were calculated by means of the respective calibration curves.

Anti-HBV Tests. Antiviral analyses were performed as previously described.[11] The results of three replicates were expressed as the mean ± standard deviation of the mean (SDM). Inhibition between 20 and 35% was defined as slight, 35−50% as medium, and 50−65% as strong, while anything over 65% was defined as very strong inhibition. Cell damage was assessed using the MTT assay.

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References and Notes

(1) Kumar, V.; Bulumulla, H. N. K.; Wimalasiri, W. R.; Reisch, J. *Phytochemistry* **1994**, *36*, 879−881.
(2) Kwon, Y. S.; Kobayashi, A.; Kajiyama, S. I.; Kawazu, K.; Kanzaki, H.; Kim, C. M. *Phytochemistry* **1997**, *44*, 887−889.
(3) Averbeck, D. *Photochem. Photobiol.* **1989**, *50*, 859−882.
(4) Colombain, M.; Goll, V.; Muyard, F.; Girard, C.; Bevalot, F.; Richert, L. *Planta Med.* **2001**, *67*, 644−646.
(5) Yamahara, J.; Kozuka, M.; Sawada, T.; Fujimura, H.; Nakano, K.; Tomimatsu, T.; Nohara, T. *Chem. Pharm. Bull.* **1985**, *33*, 1676−1680.
(6) Chen, I. S.; Chang, C. T.; Sheenm, W. S.; Teng, C. M.; Tsai, I. L.; Duh, C. Y.; Ko, F.-N. *Phytochemistry* **1996**, *41*, 525−530.
(7) Chiou, W. F.; Huang, Y. L.; Chen, C. F.; Chen, C. C. *Planta Med.* **2001**, *67*, 282−284.
(8) Harkar, S.; Razdan, T. K.; Waight, E. S. *Phytochemistry* **1984**, *23*, 419−426.
(9) Miyakado, M.; Ohno, N.; Yoshioka, H.; Mabry, T. J. *Phytochemistry* **1978**, *17*, 143−144.
(10) Masuda, T.; Takasugi, M.; Anetai, M. *Phytochemistry* **1998**, *47*, 13−16.
(11) Huang, R. L.; Chen, C. C.; Huang, H. L.; Chang, C. G.; Chen, C. F.; Chang, C. M.; Hsieh, M. T. *Planta Med.* **2000**, *66*, 694−698.

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