Notes

Biotransformation of Nobiletin by *Aspergillus niger* and the Antimutagenic Activity of a Metabolite, 4′-Hydroxy-5,6,7,8,3′-pentamethoxyflavone

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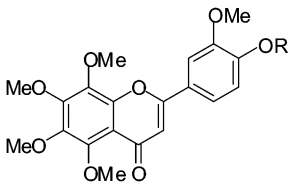
Biotransformation of nobiletin (**1**) by *Aspergillus niger* has been investigated, and the product obtained was determined as 4′-hydroxy-5,6,7,8,3′-pentamethoxyflavone (**2**). Antimutagenic activity of compound **2** was found, which showed suppressive effects on *umu* gene expression of the SOS response to DNA damage in *Salmonella typhimurium* TA1535/pSK1002, induced by the chemical mutagens furylfuramide, MeIQ, and Trp-P-1.

Flavonoids are some of the most common secondary metabolites of fruits, vegetables, nuts, wine, and tea, and the human dietary intake of these natural products is estimated to be about 1 g/day of mixed flavonoids. They display a wide range of biological and pharmacological activity, including antioxidant,[1] cytotoxic,[2] and antimutagenic effects.[3] Nobiletin (**1**) is a polymethoxyflavonoid, 5,6,7,8,3′,4′-hexamethoxyflavone, occurring in *Citrus* fruits, and has been reported to exhibit antiproliferative activity toward a human squamous cell carcinoma cell line[4] and inhibitory activity on HL-60 cell differentiation,[5] to inhibit tyrosinase activity,[6] and to exhibit antimutagenic activity.[7] Recently, we reported the inhibition of the SOS response and antimutagenic activity of nobiletin (**1**) against chemical mutagens.[8]

The biotransformation of flavonoids by fungi has been investigated. For example, Ibrahim and Abul-Hajj reported the C-4′ hydroxylation of three ring-A monohydroxy flavones by *Streptomyces fulvissimus*[9] and the sulfation of flavanones by *Cunninghamella elegans.*[10,11] However, there has been no report showing the biotransformation of polymethoxyflavonoids by fungi. In this study, the biotransformation of nobiletin (**1**) by *Aspergillus niger* and the biological activity of metabolite **2** are reported.



R
1 Me
2 H

The transformation of nobiletin (**1**) by *A. niger* was examined and was metabolized with compound **2** generated by static cultivation. The time-course of the metabolic product was observed by TLC and FID/TLC analysis (Figure S1). Metabolite **2** was produced gradually and increased in proportion to the decline in compound **1**. To isolate the metabolic product, cultivation media were combined after 3 days of incubation and extracted with EtOAc. The extract was chromatographed on silica gel repeatedly, and compound **2** was isolated. Other metabolic compounds were not detected after 7 days of incubation. The structure of metabolite **2** was determined as 4′-hydroxy-5,6,7,8,3′-pentamethoxyflavone by spectral data comparison with literature values.[12−14] Biotransformation of nobiletin (**1**) was reported by Murakami et al., who showed that this compound was metabolized to 3′-demethylnobiletin by a rat liver S9 mixture.[15] Nielsen et al. reported the in vivo and in vitro biotransformation of the related polymethoxyflavonoid, tangeretin.[16,17] The main metabolite was the demethylated compound at the 4′ position of ring-B (*para*-position) in both these studies. Previously, we reported that polymethoxylignans may be demethylated at the *para*-methoxy group by *A. niger*.[18] From these results, it is thought that a methoxy group at the *p*-position is much more subject to demethylation than at other positions.

It has been known that carcinogenicity and mutagenicity are caused by environmental chemicals, and it is important to determine materials that provide activity or inhibition against these actions. The SOS response appears to be induced by an alteration in DNA synthesis, either directly by DNA damage blocking to the replication fork or indirectly by antibiotics, such as novobiocin, that inhibit DNA synthesis. The *umu* test system was developed to evaluate the genotoxic activity of a wide variety of environmental carcinogens and mutagens, using the expression of one of the SOS genes to detect DNA-damaging agents.[19] The biological and pharmacological activity of metabolite **2** has not been reported. The suppressive effect of metabolite **2** was determined in the *umu* test. As shown in Table 1 and Figure S2A, compound **2** exhibited inhibition of the SOS induction of furylfuramide and suppressed 28.7% of the SOS-inducing activity at a concentration of 0.6 mM. This metabolite was also assayed with other mutagens, MeIQ and Trp-P-1, which require liver metabolic activation (Table 1 and Figures S2B, S2C). Compound **2** suppressed 80.9% of the MeIQ-induced and 72.4% of the Trp-P-1-induced SOS response at a concentration of 0.6 mM. The

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Table 1. Suppression of the Mutagen-Induced SOS Response by Compound **2** Using *S. typhimurium* TA1535/pSK1002

concentration (mM)	β-galactosidase activity (units) furylfuramide (0.02 μg/mL)	UV (0.1 J/m²)	MeIQ (0.2 μg/mL)	activated MeIQ[a] (1.0 μg/mL)	Trp-P-1 (0.8 μg/mL)	activated Trp-P-1[a] (2.0 μg/mL)
control[b]	147.7	72.4	78.8	78.7	77.3	69.1
0.6	443.7	495.7	163.9	224.2	168.9	118.5
0.3	489.9	563.5	195.3	243.5	219.1	182.4
0.1	511.2	551.9	232.6	376.3	285.4	233.2
0.05	551.2	574.1	267.7	394.0	299.6	283.7
0	562.2	560.6	523.9	426.9	409.2	278.6

[a] Activated heterocyclic amines were treated with S9 before the *umu* test. [b] Control was treatment without mutagen and compound.

ID_{50} values of compound **2** against MeIQ and Trp-P-1 were 0.04 and 0.16 mM, respectively. In addition, compound **2** was assayed with activated MeIQ and Trp-P-1 and suppressed 58.2% of the activated MeIQ-induced and 76.4% of the activated Trp-P-1-induced SOS response at a concentration of 0.6 mM. The ID_{50} values of compound **2** against activated MeIQ and activated Trp-P-1 were 0.30 and 0.34 mM. In the case of UV irradiation, suppressive effects of this compound were not regarded as significant against a positive control at a concentration of 0.6 mM. Nobilrtin (**1**) and the metabolite **2** showed similar suppressive effects against furylfuramide, UV, Trp-P-1, and MeIQ, but compound **2** exhibited greater suppressive effects than compound **1** against activated Trp-P-1 and MeIQ.[8]

Experimental Section

General Experimental Procedures. IR spectra were determined with a JASCO FT/IR-470 plus Fourier transform infrared spectrometer. UV spectra were obtained with a Hitachi U-2000A spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained with a JEOL FX-500 (500.00 MHz, ^{1}H; 125.65 MHz, ^{13}C) spectrometer. EIMS were obtained on a JEOL JMS-700 TKM Tandem MS station. TLC/FID analysis was carried out using an Iatroscan MK5 (Iatron Laboratories, Inc., Tokyo, Japan). The peak areas were integrated with a Chromatocorder 21 (System Instruments Co., Ltd., Tokyo, Japan).

Chemicals. Nobiletin (**1**) was isolated from *Citrus aurantium* as an antimutagenic compound.[8] Furylfuramide, 3-amino-14-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), and 4-dimethyl-3*H*-imidazo[4,5-*f*]quinolin-2-amine (MeIQ) were purchased from Wako Pure Chemical Co. (Osaka, Japan). The S9 fraction, prepared from the livers of male rats that were pretreated with phenobarbital or 5,6-benzoflavone, was purchased from Oriental Yeast Co. (Osaka, Japan). The cofactors (G-6-P, NADH, and NADPH) for the S9 mix were prepared according to the detailed methods of Yahagi et al.[20]

Microorganism and Culture Conditions. Spores of *A. niger* IFO 4414 (purchased from the Institute of Fermentation of Osaka) maintained on nutrient agar slants at 4 °C were inoculated into the autoclaved culture medium (250 mL in a 500 mL conical flask): sucrose 15 g, glucose 15 g, polypeptone 5 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, K_2HPO_4 1 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, H_2O 1 L. The culture was maintained for 2 days in the incubator (28 °C under shaking). Mycelia were then transplanted to the culture medium (15 mL in a 50 mL Petri dish) and incubated for 36−48 h (until mycelia occupied 60−80% of the surface area of a culture medium) under the same conditions. Substrate was added (3 mg/plate) as a solution in DMSO (3 mg/mL). Petri dishes were incubated at 28 °C under static cultivation, together with two controls, which contained either mycelia with medium or substrate dissolved in DMSO with medium.

Time-Course of Substrate and Metabolic Compounds. Cultivation media from Petri dishes were acidified to pH 2 with 1 M HCl and extracted with EtOAc several times at various intervals. Metabolic products of substrate were confirmed by preliminary examination. EtOAc extracts (2 mg) were dissolved in 200 μL of acetone and 5 μL of each solution applied to silica gel-coated rods. The rods were dried and then developed in CH_2Cl_2−acetone (8:2) to a distance of 10 cm from the point of application. The rods were dried again and subjected to TLC/FID analysis. The conditions for analysis were as follows: H_2, flow rate 160 mL/min, air, flow rate 2 L/mL, and scan speed, 200 mm/min. The ratio between the substrate and metabolic products was quantified and is shown in Figure S1.

Isolation of Metabolite of Nobiletin (1). After 3 days of cultivation of nobiletin (**1**) (50 mg), the culture medium was collected and acidified to pH 2 with 1 M HCl and saturated with NaCl. The culture was then extracted with EtOAC several times, dried (anhydrous Na_2SO_4), and evaporated under reduced pressure. The EtOAc extract (132 mg) was chromatographed on silica gel repeatedly, and the metabolite (**2**) (35 mg) was isolated.

4′-Hydroxy-5,6,7,8,3′-pentamethoxyflavone (2): white crystals; mp 140−142 °C; exhibited spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, EIMS) data comparable to literature values.[13−15]

***Umu* Test.** The method of the *umu* test for detecting the SOS-inducing activity of chemicals was carried out according to Oda et al.[19] using *S. typhimurium* TA1535/pSK1002, whose plasmid pSK1002 carries an *umuC′-′lacZ* fused gene. The β-galactosidase activity of the unit was calculated according to Miller.[21]

Preparation of Activated Trp-P-1 and MeIQ. Preparation of activated Trp-P-1 and MeIQ was carried out according to the method of Arimoto et al.[22]

UV Irradiation. The cells, cultivated overnight (*Salmonella typhimurium* TA1535/pSK1002), were diluted 50-fold with fresh TGA medium and incubated at 37 °C to the appropriate bacterial density (absorbance at 600 nm: 0.25−0.30). The cultured cells were centrifuged at 3000 rpm for 10 min. The precipitated cells were suspended with 5 mL of 0.1 M phosphate buffer. Cells were removed into a Petri dish (4 cm) and irradiated with UV light for 20 s (0.1 J/m²) with a germicidal lamp at room temperature, then the TGA medium was added to the appropriate bacterial density (absorbance at 600 nm: 0.25−0.30).

Supporting Information Available: IR, UV, NMR, and EIMS data for 4′-hydroxy-5,6,7,8,3′-pentamethoxyflavone. Figures showing the time-course of nobiletin (**1**) metabolism after incubation with *A. niger* and suppression of mutagen-induced SOS response by **2**. This information is available free of charge via the Internet at http://pubs.acs.org.

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