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Structural Analysis of a Novel Antimutagenic Compound, 4-Hydroxypanduratin A, and the Antimutagenic Activity of Flavonoids in a Thai Spice, Fingerroot (*Boesenbergia pandurata* Schult.) against Mutagenic Heterocyclic Amines

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Six compounds were isolated from fresh rhizomes of fingerroot (*Boesenbergia pandurata* Schult.) as strong antimutagens toward 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) in *Salmonella typhimurium* TA98. These compounds were 2′,4′,6′-trihydroxychalcone (pinocembrin chalcone; **1**), 2′,4′-dihydroxy-6′-methoxychalcone (cardamonin; **2**), 5,7-dihydroxyflavanone (pinocembrin; **3**), 5-hydroxy-7-methoxyflavanone (pinostrobin; **4**), (2,4,6-trihydroxyphenyl)-[3′-methyl-2′-(3″-methylbut-2″-enyl)-6′-phenylcyclohex-3′-enyl]methanone (**5**), and (2,6-dihydroxy-4-methoxyphenyl)-[3′-methyl-2′-(3″-methylbut-2″-enyl)-6′-phenylcyclohex-3′-enyl]methanone (panduratin A; **6**). Compound **5** was a novel compound (tentatively termed 4-hydroxypanduratin A), and **1** was not previously reported in this plant, whereas **2**−**4** and **6** were known compounds. The antimutagenic IC_{50} values of compounds **1**−**6** were 5.2 ± 0.4, 5.9 ± 0.7, 6.9 ± 0.8, 5.3 ± 1.0, 12.7 ± 0.7, and 12.1 ± 0.8 μM in the preincubation mixture, respectively. They also similarly inhibited the mutagenicity of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). All of them strongly inhibited the N-hydroxylation of Trp-P-2. Thus, the antimutagenic effect of compounds **1**−**6** was mainly due to the inhibition of the first step of enzymatic activation of heterocyclic amines.

Keywords: *Antimutagen; fingerroot (Boesenbergia pandurata Schult.); Ames test; Trp-P-1; 4-hydroxypanduratin A; Thailand; JIRCAS*

INTRODUCTION

In Asian countries, various spices, herbs, and condiments have been utilized as important ingredients to prepare unique gastronomic dishes since ancient times. Many of the commonly consumed spices in the world, such as ginger, cinnamon, nutmeg, clove, and garlic, originated from Asia. Inhabitants of countries in each part of Asia selected indigenous plants to fulfill their art of cooking, in either dehydrated or fresh form. Spices and herbs are used not only to flavor food but also to maintain health and promote wellness. For example, the cancer chemopreventive effect of herbal diets was established by numerous epidemiological studies as well as animal experiments (*1*−*3*). The incidence rate of cancer in Asian countries, where a large quantity of fruits and vegetables is consumed, is much lower than in northern Europe and North America (*4*, *5*).

In Thai cuisines, the main spices are fresh chilli, shallot, garlic, fingerroot, galanga, lemon grass, young pepper, kaffir lime leaf, and turmeric. Some of these spices are common in other countries; therefore, there are intensive studies on their health benefits. Antitumor effect and antimutagenicity in some of these plants have been elucidated by various methods using bacteria (Ames test), animal cell lines, and rats (*6*−*10*).

Fingerroot (*Boesenbergia pandurata* Schult.) is a member of the ginger family (Zingiberaceae). Fresh rhizomes have a characteristic aroma and slightly pungent taste. The rhizomes are used as a food ingredient, as a folk medicine for the treatment of colic disorder, and as an aphrodisiac in Southeast Asia. The constituents of the extract of fingerroot were studied from the pharmacological aspects (*11*−*14*). Mongkolsuk and Dean isolated pinostrobin (5-hydroxy-7-methoxyflavanone) and alpinetin (5-methoxy-7-hydroxyflavanone) from a diethyl ether extract of dried rhizomes (*11*). Jaipetch et al. identified pinocembrin (5,7-dihydroxyflavanone), 2′,6′-dihydroxy-4′-methoxychalcone, cardamonin (2′,4′-dihydroxy-6′-methoxychalcone), and a new chalcone, (±)-(*E*)-1-[7′-hydroxy-5′-methoxy-2′-methyl-2′-(4″-methylpent-3″-enyl)-2′*H*-chromen-8′-yl]-3-phenylprop-

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2-enone (boesenbergin A) as the components of fingerroot (*12*). Subsequently, Tuntiwachwuttikul et al. discovered a new chalcone derivative, 2,6-dihydroxy-4-methoxyphenyl-[3′-methyl-2′-(3″-methylbut-2″-enyl)-6′-phenylcyclohex-3′-enyl]methanone (panduratin A), from this plant (*13*). Mahidol et al. isolated another new compound, (±)-(*E*)-1-[5′-hydroxy-7′-methoxy-2′-methyl-2′-(4″-methylpent-3″-enyl)-2′*H*-1-benzopyran-6′-yl]-3-phenylprop-2-en-1-one (boesenbergin B) from fingerroot (*14*).

Several biological functions of the components of fingerroot have also been revealed (*7*, *10*). For example, Murakami et al. found that an extract of fingerroot exhibited in vitro antitumor-promoting activity by the tumor promoter-induced Epstein−Barr virus activation assay, and the active principle was identified as cardamonin (*7*). In a previous paper (*10*), we demonstrated that methanolic extract of rhizomes of fingerroot showed a strong antimutagenic effect toward Trp-P-1 in the Ames test. The methanolic extract contained at least six active compounds that were suspected to be chalcones and flavanones on the basis of their UV absorption spectra and liquid chromatography mass spectrometry. We now report the total chemical structures and the antimutagenic properties of these compounds.

MATERIALS AND METHODS

Materials. Rhizomes of fingerroot produced in Thailand were purchased from a local market in Japan. Mutagens, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), were products of Wako Pure Chemicals (Osaka, Japan). S9 mix (rat liver) for the Ames test was purchased from Kikkoman (Noda, Japan). CD_3OD and DMSO-d_6 (NMR grade) were products of Sigma-Aldrich Chemical Co., Ltd. (St. Louis, MO). All other reagents were of analytical grade.

Determination of Antimutagenic Activities. The antimutagenic effect of fingerroot constituents toward Trp-P-1 in *Salmonella typhimurium* TA98 was assayed by the preincubation method (*15*, *16*) with some minor modifications and using an S9 mix as previously described (*10*). The activity was calculated using the formula defined by Kanazawa et al. (*17*). The bio-antimutagenic activity was examined according to the method of Kanazawa et al. (*17*).

Extraction and Fractionation of Antimutagenic Compounds. Fresh rhizomes (1.0 kg) of fingerroot were homogenized in methanol (3.0 L) and further extracted by standing at room temperature for 24 h. The extract was evaporated at 35 °C, and the syrup obtained was partitioned twice with diethyl ether (250 mL) and water (100 mL). The diethyl ether layer, which showed the antimutagenic activity, was subjected to chromatography on a column of Wakogel LP-40 C18 (5.0 × 20 cm). The column was eluted by a stepwise gradient: 40, 50, 60, and 70% of ethanol/water, acidified with 0.1% formic acid, 1.5 L each step. The effluents were analyzed by an HPLC (PX-8020 system equipped with a photodiode array detector, Tosoh) with a TSK gel super-ODS column (4.6 × 100 mm, Tosoh) maintained at 40 °C. The mobile phase system was a linear gradient using 0.5% formic acid and acetonitrile. Successive fractions that showed activity were combined and then subjected to preparative HPLC (TSK gel ODS-80Ts, 20 × 250 mm, Tosoh) for further purification. The active fractions 1−6 were then recrystallized in aqueous methanol (60−80%). Finally, compounds **1**−**6** were purified to homogeneity (>99% by HPLC) and identified as antimutagens. On the basis of their chromatographic behaviors, UV absorption spectra, and molecular weights, compounds **1**, **2**, **3**, **4**, **5**, and **6** corresponded to compounds FR1, FR3, FR2, FR4, FR5, and FR6 in the previous paper (*10*).

Instrumental Analysis. The UV absorption spectra of the compounds in methanol were recorded on a UV-1200 spectrophotometer (Shimadzu). The mass spectra were determined on an SX-102 spectrometer (JEOL) by fast-atom bombardment (FAB) ionization. The molecular formula of **5** was verified by high-resolution mass mesurement using an electrospray ionization Fourier transform ion cyclotron resonance mass spectrometer (ESI-FTICRMS, ApexII70e, Bruker Daltonics) within an error of 4×10^{-4} (0.4 ppm) in mass assignment on the sample. The ^{1}H nuclear magnetic resonance (NMR) spectra at 600.05 MHz and the ^{13}C NMR at 150.80 MHz were recorded on a DRX 600 spectrometer (Bruker).

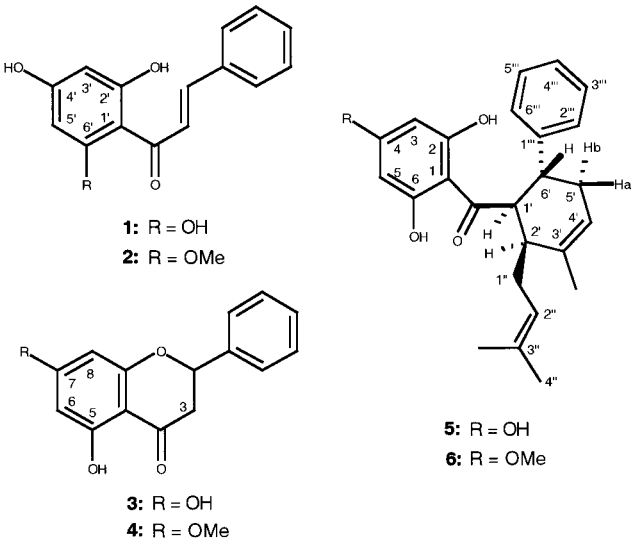

Figure 1. Chemical structures of antimutagens (**1**, pinocembrin chalcone; **2**, cardamonin; **3**, pinocembrin; **4**, pinostrobin; **5**, 4-hydroxypanduratin A; **6**, panduratin A) from fingerroot.

Analysis of Enzymatic N-Hydroxylation of Trp-P-2. The effect of compounds **1**−**6** on *N*-hydroxylation of Trp-P-2 was determined by HPLC equipped with an electrochemical detector (*18*). Briefly, the reaction mixture, with a total volume of 500 μL, consisted of 40 nmol of Trp-P-2 (dissolved in 20 μL of DMSO) and 25 μL of S9 mix in 0.1 M potasium phosphate buffer (pH 7.0). Compounds **1**−**6** were dissolved in methanol (10 μL) and added to the reaction mixture. The reaction was initiated by adding 25 μL of S9 mix. After incubation at 37 °C for 15 min, 500 μL of acetonitrile was added to stop the reaction. Then, 20 μL of the mixture was analyzed by HPLC (TSKgel super-ODS column 4.6 × 100, isocratic elution with 20% acetonitrile in 20 mM KH_2PO_4 and 0.1 mM EDTA-2Na).

Preliminary Quantification of Compounds 1—6 in Fingerroot. The content of compounds **1**−**6** in fingerroot was determined by HPLC analysis using the same analytical HPLC system as described above, equipped with a TSKgel super-ODS column (4.6 × 100 mm). The mobile phase system was a linear gradient using 0.5% formic acid and acetonitrile, operating at a flow rate of 1.0 mL/min. Fresh fingerroot (10 g) from 16 individual samples was extracted by 90 mL of methanol for 30 min. A part of the extracts was filtered (0.22 μm membrane filter), and the filtrate was used for analysis. The injection volume was 10 μL, and the effluent was monitored at 280 and 340 nm. The purified compounds **1**−**6** were used as standards. Quantification was calculated from the calibration curve of compounds **1**−**6** based on the concentrations calculated by their molecular extinction coefficients.

RESULTS

Identification of Compounds 1—4 and 6. Final yields of antimutagenic compounds **1**−**6** from 1.0 kg of fresh rhizomes were 249 mg (43.4% of the crude content), 801 mg (52.8%), 270 mg (46.2%), 816 mg (52.6%), 39 mg (57.4%), and 157 mg (55.7%), respectively. The physicochemical properties and the structures of compounds **1**—**4** and **6** (Figure 1)were as follows.

Compound **1** was red prisms: MS, *m*/*z* 257 ($M + H^+$); UV absorption λ_{max} 254 and 341 nm (methanol, log ϵ = 4.6 at 341 nm). The ^1H NMR and ^{13}C NMR spectra of compound **1** were compared with those of 2′,4′,6′-trihydroxychalcone (pinocembrin chalcone) (*19*, *20*). From these spectral data, compound **1** was identified as pinocembrin chalcone. Pinocembrin chalcone is unstable in methanol solution, slowly being converted into the isomeric flavanone, pinocembrin (*21*). Therefore, the solution of compound **1** for the antimutagenic assay was freshly prepared before use.

Compound **2** was yellow prisms: MS, *m*/*z* 271 ($M + H^+$); UV, 288 and 340 nm (methanol, log ϵ = 4.31 at 340 nm). The ^1H NMR and ^{13}C NMR spectra of compound **2** were compared with those of 2′,4′-dihydroxy-6′-methoxychalcone (cardamonin) (*12*). Compound **2** was identified as cardamonin from these spectral data.

Compound **3** was yellowish white crystrals: MS, *m*/*z* 257 ($M + H^+$); UV, 288 nm (methanol, log ϵ = 4.16). The ^1H NMR and ^{13}C NMR spectra of compound **3** were identical to those of 5,7-dihydroxyflavanone (pinocembrin) (*12*). Thus, compound **3** was identified as pinocembrin.

Compound **4** was white hexagonal plates: MS, *m*/*z* 271 ($M + H^+$); UV, 288 nm (methanol, log ϵ = 4.21). The ^1H NMR and ^{13}C NMR spectra of compound **4** were compared with those of 5-hydroxy-7-methoxyflavanone (pinostrobin) (*12*). From these data, compound **4** was assigned as pinostrobin.

Compound **6** was yellow needles: MS, *m*/*z* 407 ($M + H^+$); UV, 340 nm (methanol, log ϵ = 4.28). The ^1H NMR and ^{13}C NMR spectra of compound **6** were compared with those of 2,6-dihydroxy-4-methoxyphenyl-[3′-methyl-2′-(3″-methylbut-2″-enyl)-6′-phenylcyclohex-3′-enyl]-methanone (panduratin A) (*13*). These spectral data of compound **6** were the same as those of panduratin A.

Structural Determination of Compound 5. Compound **5** was yellow crystals: MS, *m*/*z* 393 ($M + H^+$); high-resolution FT-MS calculated for $C_{25}H_{28}O_4$ ($[M + H]^+$) 393.20604, found 393.20550; UV, 338 nm (methanol, log ϵ = 4.27); ^1H NMR chemical shifts (CD_3OD) δ 1.51 (6H, bs, $2CH_3$), 1.77 (3H, ddd, J = 1.9, 1.9, and 1.9 Hz, 3′-Me), 1.98 (1H, ddddq, J = 1.6, 1.9, 2.8, 10.6, and 18.1 Hz, H_b5'), 2.05 (1H, ddd, J = 4.5, 7.1, and 15.2 Hz, H_b1''), 2.25 (1H, ddd, J = 7.1, 7.2, and 15.2 Hz, H_a1''), 2.33 (1H, dddq, J = 1.9, 4.5, 6.4, and 18.1 Hz, H_a5'), 2.64 (1H, dddd, J = 1.6, 4.5, 4.7, and 7.2 Hz, H2′), 3.36 (1H, ddd, J = 6.4, 10.6, and 11.6 Hz, H6′), 4.75 (1H, dd, J = 4.7 and 11.6 Hz, H1′), 4.90 (1H, dd, J = 7.1 and 7.1 Hz, H2″), 5.41 (1H, ddd, J = 1.9, 2.8, and 4.5 Hz, H4′), 5.76 (2H, s, H3 and H5), 7.05 (1H, m, 4‴), 7.17 (4H, m, 4 aromatic H); ^{13}C NMR chemical shifts (DMSO-d_6) δ 17.6 (3′-Me), 22.6 (3′-Me), 25.5 (C4″), 28.4 (C1″), 35.7 (C5′), 36.4 (C6′), 42.1 (C2′), 52.8 (C1′), 94.8 (C3, C5), 104.7 (C1), 120.9 (C4′), 124.3 (C2″), 125.3 (C4‴), 126.9 (C2‴, C6‴), 128.1 (C3‴, C5‴), 130.6 (C3″)7.1 (C1‴), 164.1 (C2, C6), 164.3 (C4), 205.7 (C═O). The chemical shifts of these signals closely resembled those of panduratin A (2,6-dihydroxy-4-methoxyphenyl)-[3′-methyl-2′-(3″-methylbut-2″-enyl)-6′-phenylcyclohex-3′-enyl]methanone), which was also isolated from fingerroot (*13*). A notable difference between the NMR spectra of compound **5** and panduratin A was the absence of a signal corresponding to 4-OCH_3 (3.67 ppm in the ^1H NMR and 55.5 ppm in the ^{13}C NMR). The coupling constants $J_{1',6'}$ (11.0 Hz) and $J_{1',2'}$ (4.7 Hz) observed in the spectrum of compound **5** were similar to those of panduratin A, therefore, H1′ and H2′ must be cis-oriented, and H1′ and H6′ must have a trans relationship. In addition, the difference in molecular weights of panduratin A (406) and compound **5** (392) was 14, corresponding to substitution by −OH rather than $-OCH_3$. Thus, compound **5** was identified as 2,4,6-trihydroxyphenyl-[3′-methyl-2′-(3″-methylbut-2″-enyl)-6′-phenylcyclohex-3′-enyl]methanone (Figure 1), and this novel compound is therefore given the trivial name 4-hydroxypanduratin A.

Table 1. Inhibitory Effects of Isolated Compounds from Fingerroot toward Mutagenesis Induced by Trp-P-1, Trp-P-2, and PhIP in *S. typhimurium* TA98[a,b]

compound (0.1 μmol/test)	% inhibition of mutagenesis		
	Trp-P-1 (50 ng/test)	Trp-P-2 (20 ng/test)	PhIP (250 ng/test)
1	93	83	82
2	98	97	88
3	94	88	85
4	95	86	86
5	91	88	82
6	87	89	81

[a] Compounds **1**−**6** were dissolved in methanol (50 μL) before use. [b] Average of four independent experiments.

Table 2. Inhibitory Effects of Fingerroot Constituents toward the N-Hydroxylation of Trp-P-2[a,b]

compound (100 nmol/test)	*N*-hydroxy-Trp-P-2 formed (%)
none (control)	100.0
1	6.3 ± 0.7
2	4.2 ± 0.9
3	5.8 ± 0.6
4	5.4 ± 0.7
5	6.8 ± 0.6
6	7.2 ± 0.8

[a] Methanol (10 μL) was used for control. [b] Average of four independent experiments.

Antimutagenic Properties of Compounds 1—6. The IC_{50} values, which are concentrations in 1 mL of the preincubation mixture for 50% inhibition against mutagenesis in the presence of 50 ng of Trp-P-1, of compounds **1**−**6** were 5.2 ± 0.4, 5.9 ± 0.7, 6.9 ± 0.8, 5.3 ± 1.0, 12.7 ± 0.7, and 12.1 ± 0.8 μM, respectively. Compounds **1**−**6** also showed strong inhibitory effects against the other cooked food mutagens, Trp-P-2 and PhIP (Table 1).

Inhibitory Effects of Compounds 1—6 on N-Hydroxylation of Trp-P-2. All isolated compounds strongly inhibited the *N*-hydroxylation of Trp-P-2 as indicated in Table 2. The *N*-hydroxylation reaction is considered to be the key reaction in chemical mutagenesis catalyzed by cytochromes P450 1A1 and 1A2. In the Ames test system, *N*-hydroxy-Trp-P-2 is transported into bacterial cells and then converted to the acetyl ester. *N*-Acetyl-Trp-P-2 may react with DNA and induces mutation of the gene. If the antimutagenic compounds act on DNA repair processes, the inhibitory effect would be observed in the bioantimutagenic activity assay. However, no considerable effect was observed for any of the six compounds (data not shown). Therefore, it is suggested that the antimutagenic effect of these compounds is mainly based on inhibition of *N*-hydroxylation of Trp-P-2 (desmutagenicity).

Preliminary Quantification of Compounds 1—6 in Fingerroot. Figure 2 shows the typical HPLC chromatogram of a freshly prepared methanol extract of fingerroot. The tested samples had a rather big

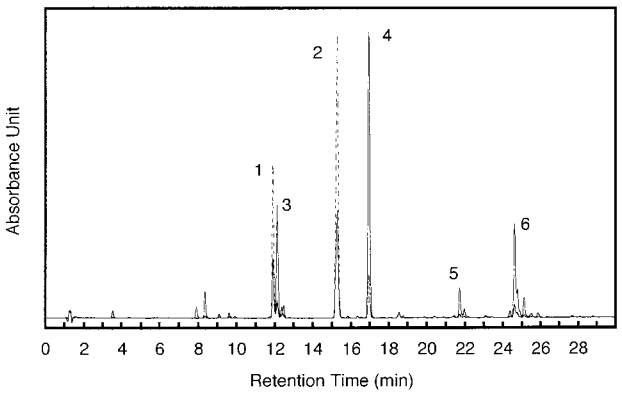

Figure 2. HPLC chromatogram of methanol extract of fingerroot: solid line, chromatogram at 280 nm; broken line, at 340 nm. The analytical condition was described under Materials and Methods. The mobile phase was a linear gradient of 0.5% formic acid and acetonitrile from 20 to 75% in 25 min.

difference in the composition of antimutagenic compounds among samples that were produced in different places. The average contents of each compound were as follows: **1**, 28.8 ± 18.3; **2**, 78.7 ± 52.2; **3**, 55.9 ± 15.6; **4**, 118.1 ± 52.8; **5**, 7.8 ± 4.13; and **6**, 29.5 ± 15.6 mg/100 g of fresh weight, respectively.

DISCUSSION

This study demonstrated that strong antimutagens against Trp-P-1, Trp-P-2, and PhIP in fingerroot were flavonoids and their derivatives, which were pinocembrin chalcone, pinocembrin, cardamonin, pinostrobin, a novel compound (4-hydroxypanduratin A), and panduratin A. These compounds were detected as major components in the crude methanolic extract (*10*). All six compounds **1**−**6** exhibited a strong antimutagenic activity against mutagenic heterocyclic amines. The antimutagenic IC_{50} values for all them were quite low, ranging from 10^{-5} to 10^{-6} M, which were comparable to that of luteolin or apigenin (*22*) and, therefore, contributed significantly to the antimutagenicity of fingerroot extract.

Four compounds, pinocembrin, cardamonin, pinostrobin, and panduratin A, of the six compounds identified in this study have already been found in fingerroot (*11*, *12*). However, pinocembrin chalcone and 4-hydroxypanduratin A have not been reported. According to results of the preliminary quantitative analysis of compounds **1**−**6** in various samples of fingerroot, there was a significant difference in composition by samples that were produced in different areas. It was found that some samples contained only small amounts of pinocembrin chalcone and 4-hydroxypanduratin A. In addition, pinocembrin chalcone was easily converted to pinocembrin in methanol solution. Therefore, pinocembrin chalcone and 4-hydroxypanduratin A may not have been found in fingerroot despite many previous studies. Compound **5** (4-hydroxypanduratin A) was a Diels−Alder adduct of chalcone derivative with a cyclohexene moiety analogous to compound **6**. This type of compound occurs in mulberry as albanins F and G (*23*) and sanggenon C (*24*).

In addition, there are some studies on the various biological functions of the compounds identified in this study. Pinocembrin chalcone was found in *Helichrysum trilineatum* DC., an African folk medicine, and was shown to have an antibacterial activity (*20*). Pinocembrin, which occurs in propolis, showed antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* (*25*). Antimutagenicity of pinocembrin on ofloxacin-induced bleaching (mutagenicity) of *Euglena gracilis* was also reported (*26*).

Kato et al. reviewed the metabolic activation of carcinogenic heterocyclic amines. In the Ames test, the 2-amino groups in Trp-P-1 and Trp-P-2 are hydroxylated by P450 monooxygenase systems in rat liver microsomes (*27*). The *N*-hydroxyl derivative is then transported into cells of *S. typhimurium* TA98, and it is acetylated by acetyl-CoA:*N*-hydroxyarylamine *O*-acetyl transferase in the cytoplasm (*28*). *N*-Acetylamide-Trp-P-1/Trp-P-2, or some other unknown metabolites, would react with DNA to form a covalent bond, inducing mutation of the gene. In this study, all of the compounds **1**−**6** inhibited the *N*-hydroxylation of Trp-P-2, whereas they exhibited a weak inhibition in the bioantimutagenicity assay (data not shown). Therefore, it is suggested that the antimutagenic effects of compounds **1**−**6** are due to the inhibition of *N*-hydroxylation by P450 monooxygenase systems in S-9. This is supported by the fact that some flavonoids show inhibitory effects on monooxygenase activities in human and rat liver microsomes (*29*−*31*).

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