Antimutagens in Gaiyou (*Artemisia argyi* Levl. et Vant.)

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Antimutagens from gaiyou (*Artemisia argyi* Levl. et Vant., Compositae) were examined. The methanol extract prepared from aerial parts of this plant strongly reduced the mutagenicity of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), when *Salmonella typhimurium* TA98 was used in the presence of the rat liver microsomal fraction. The antimutagens were purified chromatographically while monitoring the antimutagenic activity against Trp-P-2 with a modified Ames test employing a plate method. This purification resulted in the isolation of four strong antimutagens, 5,7-dihydroxy-6,3′,4′-trimethoxyflavone (eupatilin), 5,7,4′-trihydroxy-6,3′-dimethoxyflavone (jaceosidin), 5,7,4′-trihydroxyflavone (apigenin) and 5,7,4′-trihydroxy-3′-methoxyflavone (chrysoeriol) from the methanol extract. These antimutagenic flavones exhibited strong antimutagenic activity against not only Trp-P-2 but also against other heterocyclic amines, such as 3-amino-1,4-dimethyl-5*H*-pyrido-[4, 3-*b*]indole (Trp-P-1), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole ($MeA_{\alpha}C$) in *S. typhimurium* TA98. In contrast, they did not exhibit antimutagenic activity against benzo[*a*]pyrene (B[*a*]P), 4-nitroquinoline-1-oxide (4-NQO), 2-aminofluorene (2-AF), 2-nitrofluorene (2-NF) or furylfuramide (AF-2) in *S. typhimurium* TA98, or B[*a*]P, 4-NQO, 2-NF, AF-2, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) or sodium azide (SA) in *Salmonella typhimurium* TA100, whereas they decreased the mutagenicity caused by aflatoxin B_1 (AFB_1) and 2-aminoanthracene (2-AA) in both of these tester strains. Regarding the structure−activity relationship, the tested flavones had distinct differences in the intensities of their antimutagenic activities according to the differences of their substitution patterns. Namely, the intensity of antimutagenic activities against Trp-P-2 decreased in the order of: 5,7,3′,4′-tetrasubstituted flavones (IC_{50}: <0.1 mmol/plate), 5,7,4′-trisubstituted flavones (IC_{50}: 0.120−0.260 mmol/plate), 5,6,7,3′,4′-pentasubstituted flavones (IC_{50}: 0.440−0.772 mmol/plate). The four isolated flavones were also studied regarding their antimutagenic mechanisms with preincubation methods of the modified Ames test and emission spectroscopic analysis. The results suggested that all isolated flavones were desmutagens which directly inactivated Trp-P-2 or inhibited its metabolic activation.

Keywords: *Artemisia argyi; eupatilin; jaceosidin; apigenin; chrysoeriol; Trp-P-2; desmutagen*

INTRODUCTION

Cancer is currently one of the most dreaded diseases, although methods for its detection at early stages and therapeutic remedies have greatly advanced. In Japan, cancer has been the leading cause of mortality since 1981 (Namiki, 1994). Kee (1983) reported that the causes of cancer exist in the environment, especially in the diet and tobacco. Cancer is initiated by DNA damage, which is caused by natural and man-made chemical substances (mutagens) in the environment (Shinohara, 1993).

B[*a*]P and heterocyclic amines, which exist in the tar of tobacco and in charred fish and meat, respectively, are well-known mutagens (Shinohara, 1993). After these mutagens undergo metabolic activation by P-450 enzymes, especially the CYP1A enzyme family, the resultant ultimate carcinogenic metabolites form covalent bonds with DNA and thereby cause mutations (Hamilton and Teel, 1996; Shimada, 1992).

On the other hand, phytochemicals (antimutagens), which can strongly inhibit mutations caused by mutagens such as B[*a*]P and heterocyclic amines, have been found in edible plants. For example, Tanaka (1994) reported that quercetin inhibited the mutagenicity caused by benzo[*a*]pyrene and heterocyclic amines. Kanazawa et al. (1995) reported that galangin and quercetin in oregano were specific desmutagens against Trp-P-2. Samejima et al. (1995) reported that luteolin, which is contained in peppermint, sage, and thyme, was a strong antimutagen against Trp-P-2. We also reported that antimutagenic flavones, such as salvigenin and cirsimaritin in rosemary (1996), and apigenin, hispidulin, genkwanin, and cirsimaritin in carqueja (1998), were strong antimutagens against Trp-P-2. In addition, we recently found that methanol extracts from *Artemisia argyi* Levl. et Vant., whose leaves are called gaiyou, have strong antimutagenic activities against Trp-P-2. Gaiyou are used to treat eczema, inflammation, hemostasis, menstruation-related problems and tuberculosis in Chinese medicine (Otsuka et al., 1992). We thought that this plant might be usuful as a cancer chemopreventive agent and were interested in the yet unidentified antimutagens in *A. argyi* Levl. et Vant.

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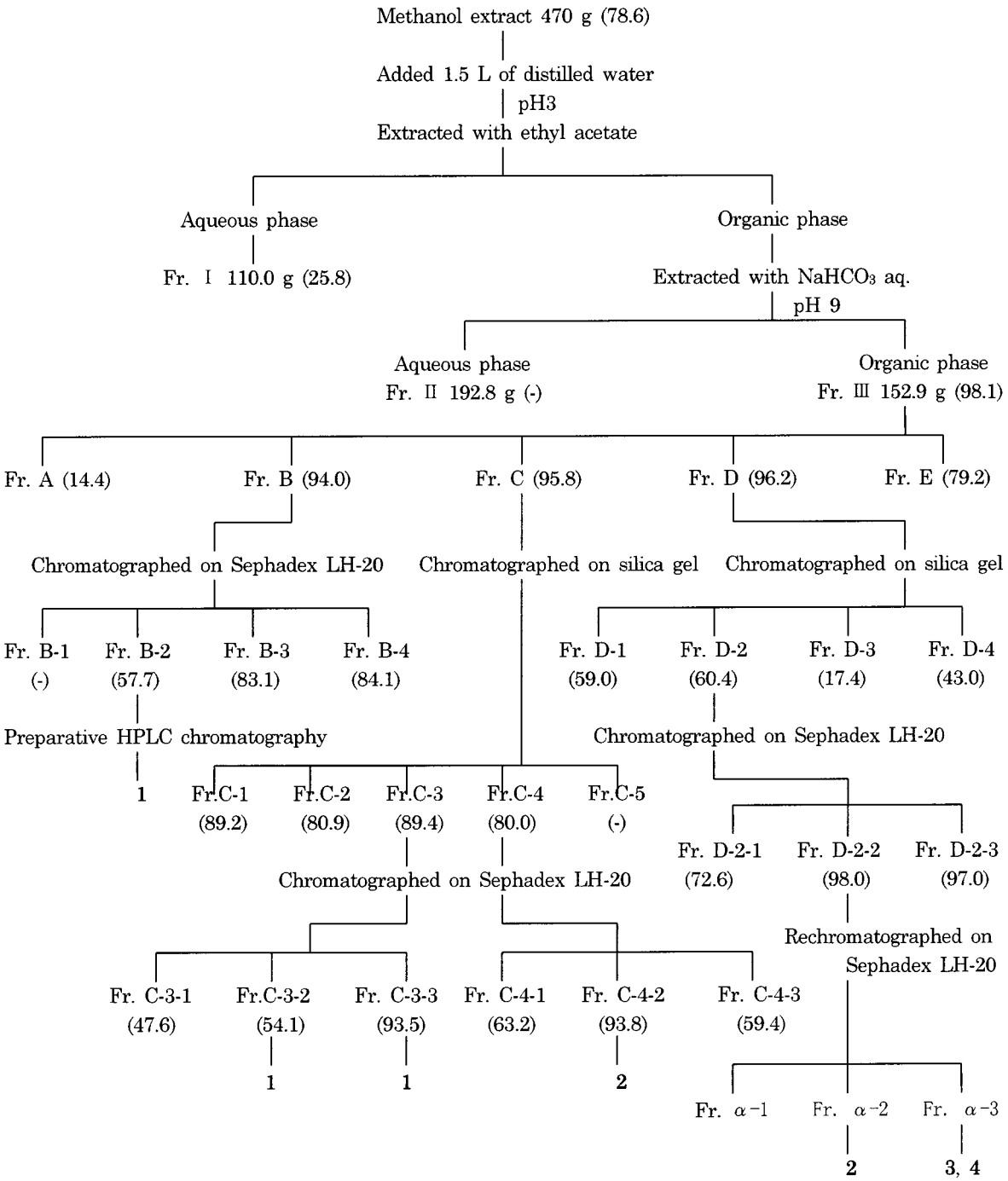

Figure 1. Isolation of compounds **1**−**4**. The antimutagenicity tests in the isolation process were done with the plate method of the Ames test. The values in parentheses are the antimutagenic activities (% inhibition) to control with 0.15 μg of Trp-P-2 per plate, respectively. Each sample was tested at the dose of 0.5 mg per plate. (−): inactive.

In this paper, the isolation and antimutagenicity of the antimutagens from gaiyou are described.

MATERIALS AND METHODS

Materials. Dried gaiyou was purchased from Nippon Funmatsu Yakuhin & Co. (Osaka, Japan). Bacto agar and nutrient broth were purchased from Difco (Detroit, MI). S-9 (which was prepared from the livers of 7-week-old male Sprague−Dawley rats treated with phenobarbital and 5,6-benzoflavone) and cofactor I, used for the activation of mutagens, were purchased from Oriental Yeast Co. (Tokyo, Japan). Trp-P-1 and Trp-P-2 (both as the acetate form), IQ, MeIQ, MeIQx, $MeA_{\alpha}C$, B[*a*]P, 2-AA, 4-NQO, AF-2, and sodium azide were purchased from Wako Pure Chemicals (Osaka, Japan). AFB_1 was purchased from Sigma (St. Louis, MO). 2-Aminofluorene and 2-nitrofluorene were purchased from Aldrich Chem. Co. (Milw., WI). MNNG was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Tester strains *S. typhimurium* TA98 and TA100 were obtained from Institute For Fermentation Osaka (IFO). Methoxy resorufin and ethoxy resorufin for emission spectroscopic analysis were purchased from Sigma. Silica gel (Silicagel 60, No. 9385, 230−240 mesh) and Sephadex LH-20 for chromatography were purchased from Merck (Darmstadt, Germany) and Pharmacia Biotech (Uppsala, Sweden), respectively.

Isolation of Active Substances. First, 4.4 kg of dried leaves were extracted for 3 days with 32.0 L of methanol at room temperature. The methanol extract was filtered through filter paper No. 2 (Advantec Toyo, Tokyo, Japan). The residue was similarly extracted with an equal amount of methanol, and the methanol extract was filtered. Both filtrates (16.344 and 14.490 Ls) were combined, and the solution was concen-

trated under reduced pressure to 480.4 g. Four hundred seventy grams of this concentrate was used for the purification of the antimutagens whose antimutagenic activities were tested against Trp-P-2 (Figure 1) as follows: The concentrate was suspended in 1.5 L of distilled water, brought to pH 3 with 5% hydrochloric acid, and partitioned three times with 1.5 L of ethyl acetate. The aqueous phase was neutralized and concentrated to 110.0 g (fraction I). On the other hand, the organic phase was repartitioned to a $NaHCO_3$ phase and organic phase with an equal volume of 5% $NaHCO_3$ solution. After the $NaHCO_3$ phase was neutralized, the salts were removed, and the neutral solution was concentrated to 192.8 g (fraction II). The organic phase washed with $NaHCO_3$ was concentrated to 152.9 g (fraction III). Fraction III, which exhibited strong antimutagenicity, was further purified as follows: One hundred fifty grams of fraction III was chromatographed on silica gel (glass column with ∅ 4.5 cm × length 60 cm) with a chloroform−methanol gradient with increasing methanol content, and was fractionated into fractions A, B, C, D,and E (yields: 2.0, 62.846, 17.915, 61.280, and 3.929 gs, respectively). Since fractions C and D showed strong antimutagenic activities, they were rechromatographed on silica gel (glass column with ∅ 4.5 cm × length 60 cm) with a chloroform−methanol gradient with increasing methanol content. Chromatography yielded subfractions C-1 (0.334 g), C-2 (4.329 g), C-3 (2.294 g), C-4 (5.164 g) and C-5 (3.989 g), and D-1 (2.4 g), D-2 (31.905 g), D-3 (4.4 g), and D-4 (7.9 g) (Figure 1). Subfractions C-3 and C-4 were rechromatographed on Sephadex LH-20 with methanol as eluent and yielded subfractions C-3-1 (1.017 g), C-3-2 (0.813 g) and C-3-3 (0.170 g), and C-4-1 (3.257 g), C-4-2 (0.611 g) and C-4-3 (70.3 mg), respectively. Subfraction C-3-3 yielded **1** by recrystallization from methanol (115.0 mg). **1** was also isolated from subfractions C-2 and C-3-2 by rechromatography on Sephadex LH-20 (yields: 106.3 and 36.9 mg). Subfraction C-4-2 yielded **2** by recrystallization from methanol (yield: 100.7 mg). Subfraction D-2 yielded subfractions D-2-1 (25.558 g), D-2-2 (1.592 g) and D-2-3 (0.786 g), and three subfractions of fraction D-2-2, namely subfractions α-1 (0.265 g), α-2 (0.876 g) and α-3 (0.279 g) by repeated chromatography on Sephadex LH-20. Subfraction α-2 yielded **2** by recrystallization from methanol (yield: 5.0 mg). Subfraction α-3 yielded **3** and **4** by rechromatography on Sephadex LH-20 and recrystallization from methanol (yields: 81.6 mg and 2.0 mg). On the other hand, because fraction B also showed a strong antimutagenic activity, it was rechromatographed on Sephadex LH-20. Chromatography yielded subfractions B-1 (6.465 g), B-2 (21.433 g), B-3 (16.545 g) and B-4 (16.114 g). Subfraction B-2 yielded **1** by rechromatography on Sephadex LH-20 and preparative HPLC chromatography (yield: 2.0 mg).

Instrumental Analysis of the Isolated Compounds. Four compounds isolated chromatographically were analyzed with the following instruments. The melting point (mp) was obtained with a melting point measurement instrument (Yanagimoto MP, Yanaco, Suita, Japan). The ultraviolet (UV) and infrared (IR) spectra were determined with a UV-240 spectrometer (Shimadzu, Kyoto, Japan) and FT-IR 8020D spectrometer (Shimadzu), respectively. Electron impact mass spectra (EI-MS) were obtained using a mass spectrometer (Shimadzu GC-MS 9100MK). Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded at 270 MHz using a JNM-EX 270 spectrometer (JEOL, Japan).

Preparation for Bacterial Assays. The preparations of a semi-enriched agar medium (MBB medium)(Natake, 1989), of the S-9 mix which was used for metabolic activation of heterocyclic amines and the other indirectly acting mutagens, and of suspension cultures of tester strains for the antimutagenicty tests were described previously (Nakasugi et al., 1996). Composition of MBB medium is as follows: 15 g of bacto agar (Difco), 1 g of $(NH_4)_2SO_4$, 10 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of trisodium citrate, 100 mg of bacto nutrient broth powder (Difco), 6.67 g of glucose and 0.1 g of biotin per a liter of MBB medium. *S. typhimurium* TA98 for the suspension for the desmutagenicity test and bio-antimutagenicity test was grown at 37 °C overnight in nutrient broth medium. Trp-P-2 was dissolved in distilled water at a concentration of 1.5 μg/mL in the antimutagenicity test, and 2.0 μg/mL in the desmutagenicity test and bio-antimutagenicity test. Similarly, MeIQ, IQ, MeIQx, Trp-P-1, sodium azide and $MeA_\alpha C$ were prepared at concentrations of 0.1, 0.5, 0.5, 2.0, 10, and 200 μg/mL, respectively. These mutagenic solutions were then sterilized through membrane filters (cellulose acetate, 0.45 μm, Advantec Toyo, Dismic 25CS). The other mutagens, AF-2, 4-NQO, AFB_1, MNNG, 2-AA, 2-aminofluorene, 2-nitrofluorene, and B[*a*]P, were dissolved in dimethyl sulfoxide at the concentrations of 1, 2, 40, 50, 100, 100, 100, and 200 μg/mL, respectively. Similarly, each test sample was dissolved in dimethyl sulfoxide at various concentrations. The mutagenic solutions and each test sample or dimethyl sulfoxide as control were then sterilized through membrane filters (PTFE, 0.2 μm, Sartorius, Minisart SRP 15, Gottingen, Germany). *N*-OH-Trp-P-2 was prepared for the desmutagenicity test as follows: 0.1 mL of Trp-P-2 (30 μg/mL) was mixed with 0.5 mL of S-9 mix and incubated for 20 min at 37 °C with shaking. The reaction was stopped by the addition of 0.6 mL of cold acetone and the resultant mixture was allowed to stand on ice for 15 min. After centrifugation of the mixture at 1,200 × *g* for 15 min, the supernatant was retained and evaporated to remove acetone under reduced pressure. The concentrate was then diluted in 0.1 mL of distilled water and sterilized through a membrane filter (cellulose acetate, 0.45 μm).

Antimutagenicity Test. The antimutagenicity test was done according to the method of Ames et al. (1975). The details of the antimutagenicity test are as follows: In the case of the mutagens that needed metabolic activation such as Trp-P-1, Trp-P-2, IQ, MeIQ, MeIQx, $MeA_\alpha C$, B[*a*]P, AFB_1, 2-AA, 2-aminofluorene and 2-nitrofluorene, 0.1 mL of sample solution or dimethyl sulfoxide, 0.1 mL of the bacterial suspension, 0.1 mL of each mutagenic solution and 0.5 mL of S-9 mix were added to 3 mL of molten soft agar. The reagent mix was briefly shaken by hand and poured onto an MBB agar plate. This plate was incubated at 37 °C for 3 days, and then the number of revertant colonies was counted. On the other hand, in the case of directly acting mutagens, the reagent mix except for the S-9 mix was tested by the above method. In addition, the number of surviving tester strain cells was also counted in order to examine the cytotoxicity of the test samples against tester strains TA98 or TA100 as follows: 0.1 mL of the bacterial suspension diluted 10^5-fold with phosphate buffer and 0.1 mL of sample solution or 0.1 mL of dimethyl sulfoxide was added to 3 mL of molten soft agar, and then the mixed solution was poured onto an MBB agar plate. After incubation at 37 °C for 3 days, the number of surviving cells was counted.

Desmutagenicity Test. The desmutagenicity and bio-antimutagenicity tests were done with the preincubation method of Watanabe et al. (1989). The desmutagenic effects of test samples against Trp-P-2, activated *N*-OH-Trp-P-2 and S-9 mix were examined as follows: First, in the case of the desmutagenic effect against Trp-P-2 (desmutagenicity test 1), 0.1 mL of test sample was preincubated with 0.1 mL of Trp-P-2 for 30 min at 37 °C. Subsequently, 0.5 mL of S-9 mix and 0.1 mL of bacterial suspension were added to the above mixture and incubated for 20 min at 37 °C. After the addition of 3 mL of soft agar, the mixture was poured onto an MBB agar plate as described above for the antimutagenicity test. Next, to test the desmutagenic effect against the S-9 mix (desmutagenicity test 2), 0.1 ml of test sample was preincubated with 0.5 mL of S-9 mix for 30 min at 37 °C. Subsequently, 0.1 mL of Trp-P-2 and 0.1 mL of bacterial suspension were added to the above mixture, and incubated for 20 min at 37 °C. After the addition of 3 mL of soft agar, the mixture was treated as described for desmutagenicity test 1. In the case of the desmutagenic effect on the activated *N*-OH-Trp-P-2 (desmutagenicity test 3), 0.1 mL of test sample was preincubated with 0.1 mL of *N*-OH-Trp-P-2 solution, 0.1 mL of bacterial suspension and 0.5 mL of phosphate buffer for 20 min at 37 °C. After the addition of 3 mL of soft agar, the mixture was treated as described for desmutagenicity test 1. After MBB agar plates

Table 1. Effects of Four Isolated Flavones and Related Compounds on Frameshift Mutations Caused by Trp-P-2 (3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole) with Preincubation Method in *Salmonella typhimurium* TA98[a]

sample	no. of revertant colonies/ plate	anti-mutagenic activity (% inhibition)	no. of surviving cells/plate	IC_{50} value (mmol)
control	3000	0	481	
eupatilin (**1**)	757	74.8	469	0.504
jaceosidin (**2**)	506	83.1	430	0.440
apigenin (**3**)	420	86.0	449	0.120
chrysoeriol (**4**)	220	92.7	409	0.096
acacetin (**5**)	422	85.9	350	0.260
chrysin (**6**)	984	67.2	471	0.902
diosmetin (**7**)	181	94.0	453	0.096
eupatorin (**8**)	848	71.7	437	0.772
eupatorin-5-methyl ether (**9**)	1124	62.5	406	0.552
genkwanin (**10**)	299	90.0	546	0.159
luteolin (**11**)	113	96.2	511	0.089

[a] Dimethyl sulfoxide was used as a control solution in the presence of 0.15 μg of Trp-P-2 and 0.5 mL of S-9 mix. The number of spontaneously mutated colonies was 42/plate. Dose of each sample: 100 μmol/plate. The numbers of revertants, including spontaneously mutated colonies, and surviving cells are the means from three plates.

for each desmutagenicity test were incubated at 37 °C for 3 days, the number of revertant colonies were counted.

Method of Bio-antimutagenicity Test. To produce mutations in TA98 cells, 0.1 mL of bacterial suspension was preincubated with 0.1 mL of Trp-P-2 and 0.5 mL of S-9 mix for 20 min at 37 °C. The mixture was centrifuged at 1500 × *g* for 15 min, washed three times with 13.8 mL of phosphate buffer (to remove the mutagen) and then resuspended in 0.7 mL of phosphate buffer. Subsequently, 0.7 mL of the mutated bacterial suspension was incubated with 0.1 mL of each test sample for 40 min at 37 °C. Three milliliters of molten soft agar was added to this mixture, and then poured onto an MBB agar plate. After incubation for 3 days at 37 °C, the number of revertant colonies was counted.

Calculation of the Antimutagenic Activities. The reversion and cell toxicity estimations were done in triplicate. Antimutagenic activity (inhibition %) = $[(C - N) - (S - A)] \times 100/(C - N)$; where C is the number of His^+ revertant colonies of the control solution in the presence of Trp-P-2; S is the number of His^+ revertant colonies of each sample solution in the presence of Trp-P-2; N is the number of spontaneous His^+ revertant colonies of the control solution; and A is the number of His^+ revertant colonies of each sample solution in the absence of Trp-P-2. C, N, S, and survival are expressed as the means from three plates. The IC_{50}, which is the concentration of a substance required for 50% inhibition of the mutagenicity of 0.15 μg of Trp-P-2, of the isolated antimutagens was determined by plotting the antimutagenicity at four different doses with the probit method.

The Assay of EROD and MROD Activities. The ethoxy resorufin-*O*-deethylase (EROD) and methoxy resorufin-*O*-demethylase (MROD) activities were determined as follows: 100 μL of ethoxy resorufin or methoxy resorufin (5 μM in 0.5% dimethyl sulfoxide), 100 μL of each sample, and 300 μL of S-9 mix were added to 500 μL of phosphate buffer, and the mixture was incubated for 30 min at 37 °C with gentle shaking. The reaction was initiated by the addition of S-9 mix, and was terminated by the addition of 1 mL of methanol. Subsequently, after the mixture was vortexed and centrifuged at 8000 × *g* for 10 min, the fluorescence of the supernatant was measured at λ_{ex} 550 and λ_{em} 586 nm with a Hitachi Fluorescence Spectrometer F-3010. The control tubes contained no test samples.

Inhibitory Effect of the Isolated Antimutagens against Trp-P-2. The direct effects on Trp-P-2 of the isolated antimutagens were examined as follows: One hundred microliters of Trp-P-2 (1.2 mM), 100 μL of test sample, 500 μL of phosphate buffer and 400 μL of distilled water were mixed, and the fluorescence of the mixture was measured at λ_{ex} 265 and λ_{em} 403 nm as described above.

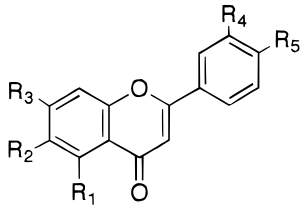

Figure 2. Structures of four isolated flavones **1**−**4** and related compounds **5**−**11**: (**1**) $R_1 = R_3 = OH$, $R_2 = R_4 = R_5 = OCH_3$, eupatilin; (**2**) $R_1 = R_3 = R_5 = OH$, $R_2 = R_4 = OCH_3$, jaceosidin; (**3**) $R_1 = R_3 = R_5 = OH$, $R_2 = R_4 = H$, apigenin; (**4**) $R_1 = R_3 = R_5 = OH$, $R_4 = OCH_3$, $R_2 = H$, chrysoeriol; (**5**) $R_1 = R_3 = OH$, $R_5 = OCH_3$, $R_2 = R_4 = H$, acacetin; (**6**) $R_1 = R_3 = OH$, $R_2 = R_4 = R_5 = H$, chrysin; (**7**) $R_1 = R_3 = R_4 = OH$, $R_5 = OCH_3$, $R_2 = H$, diosmetin; (**8**) $R_1 = R_4 = OH$, $R_2 = R_3 = R_5 = OCH_3$, eupatorin; (**9**), $R_4 = OH$, $R_1 = R_2 = R_3 = R_5 = OCH_3$, eupatorin-5- methyl ether; (**10**) $R_1 = R_5 = OH$, $R_3 = OCH_3$, $R_2 = R_4 = H$, genkwanin; (**11**) $R_1 = R_3 = R_4 = R_5 = OH$, $R_2 = H$, luteolin.

RESULTS

Identification of the Isolated Compounds. Compound **1** consisted of pale yellow crystals with mp 234.5−234.7 °C. The UV spectrum in methanol gave peaks at λ_{max} 274 and 339 nm. The addition of aluminum chloride shifted these values to 287 and 363 nm. The addition of hydrochloric acid to the above solution did not affect band I. The addition of sodium methylate shifted band I to 370 nm, while the addition of sodium acetate did not shift band II. The IR spectrum in KBr showed absorbances at 2945, 2841, 1655, 1622, 1582, 1510, 1462, and 1425 cm^{-1}. The EI/MS spectrum gave a molecular ion peak at *m/z* 344 and fragment ion peaks at *m/z* 329, 326, 301 and 167. The ^1H NMR spectrum in dimethyl sulfoxide-d_6 gave signals at δ (ppm) 3.85 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 3.97 (3H, s, OCH_3), 6.72 (1H, s, 3-H), 7.01 (1H, s, 8-H), 7.21 (1H, d, $J = 8.5$ Hz, 5′−H), 7.64 (1H, d, $J = 2.25$ Hz, 2′−H), 7.76 (1H, dd, $J = 2.25$ Hz, 8.5 Hz, 6′-H) and 12.95 (1H, s, 5-OH). The ^{13}C NMR spectrum in dimethyl sulfoxide-d_6 gave signals at δ (ppm) 182.0 (C-4), 163.2 (C-2), 157.3 (C-7), 152.6 (C-5), 152.3 (C-9), 152.1 (C-4′), 149.0 (C-3′), 131.3 (C-6), 122.9 (C-1′), 119.9 (C-6′), 111.7 (C-5′), 109.5 (C-2′), 104.0 (C-10), 103.3 (C-3), 94.3 (C-8), 59.8 (OCH_3), 55.8 (OCH_3) and 55.7 (OCH_3). Compound **1** was identified as eupatilin because its instrumental analysis data corresponded with the reference data for eupatilin (Kupchan et al., 1969; Zhong and Cui, 1992).

Compound **2** consisted of pale yellow crystals with mp 224−226 °C. The UV spectrum in methanol gave peaks at 273 and 343 nm. The addition of aluminum chloride shifted these values to 261 nm (sh), 282 and 373 nm. The addition of sodium methylate shifted band I to 408 nm, and the addition of sodium acetate shifted band I to 394 nm, but did not shift band II. The IR spectrum in KBr showed absorbances at 3491, 3186, 3088, 1655, 1618, and 1578 cm^{-1}. The EI/MS spectrum gave a molecular ion peak at *m/z* 330 (M^+) and fragment ion peaks at *m/z* 315, 312, 287 and 167. The ^1H NMR spectrum in dimethyl sulfoxide-d_6 gave signals at δ (ppm) 3.85 (3H, s, OCH_3), 3.98 (3H, s, OCH_3), 6.69 (1H, s, H-8), 6.94 (1H, s, H-3), 7.02 (1H, d, $J = 9.0$ Hz, H-5′), 7.63 (1H, d, $J = 2.0$ Hz, H-2′), 7.63 (1H, dd, $J = 9.0$ Hz, 2.0 Hz, H-6) and 13.04 (1H, s, 5-OH). The ^{13}C NMR spectrum in dimethyl sulfoxide-d_6 gave signals at δ (ppm) 182.0 (C-

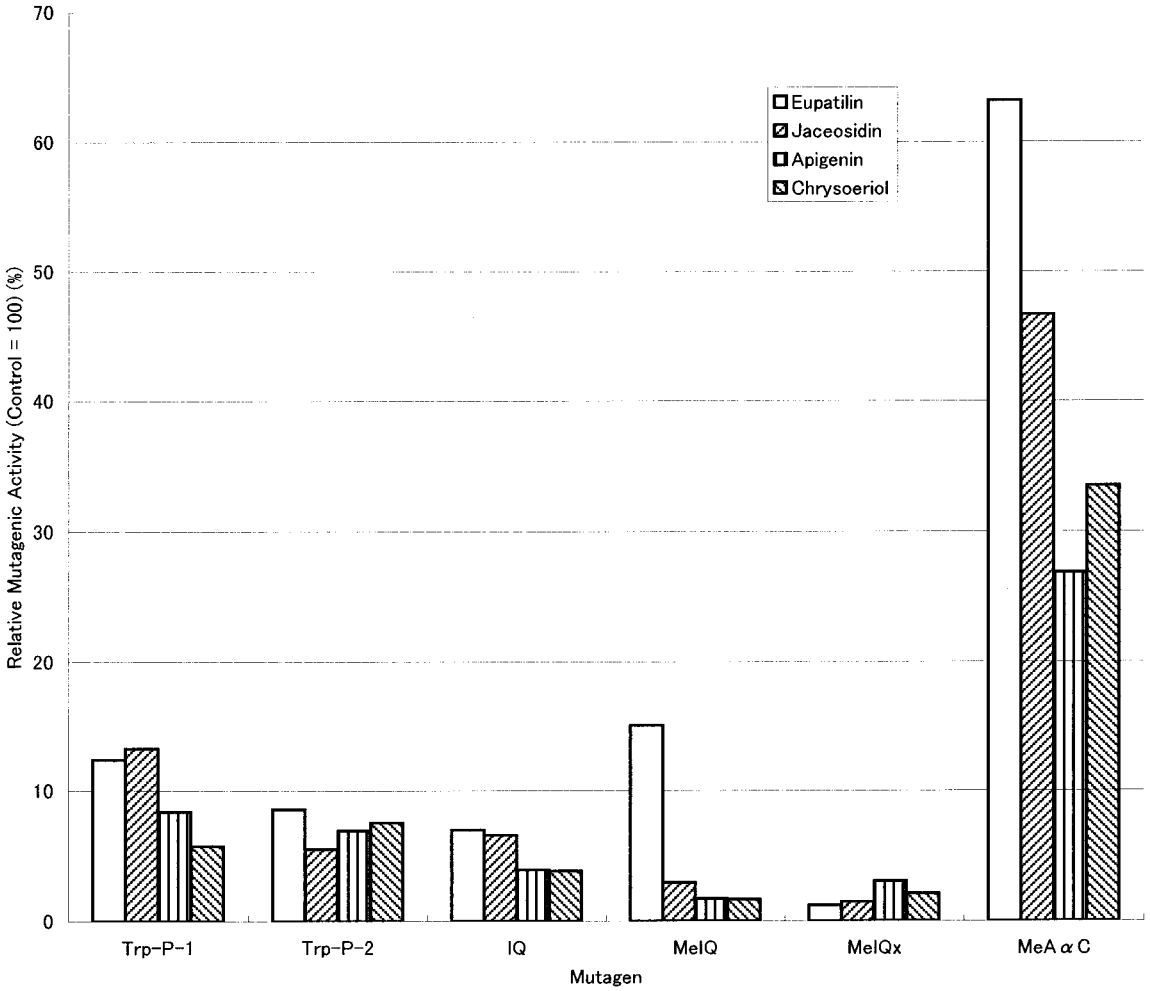

Figure 3. Effects of the four isolated flavones on mutation caused by heterocyclic amines in *Salmonella typhimurium* TA98. The mutagenic activities are expressed relative to control (100%). Each test sample was tested at 50 μg/plate. Doses and revertant colony counts for the control (100%) of each mutagen used were as follows: Trp-P-1 (0.20 μg/plate; 706 rev.), Trp-P-2 (0.15 μg/plate; 522 rev.), IQ (0.05 μg/pate; 1045 rev.), MeIQ (0.01 μg/plate; 3446 rev.), MeIQx (0.05 μg/plate; 1405 rev.), and $MeA_{\alpha}C$ (20.0 μg/plate; 676 rev.).

4), 163.7 (C-2), 157.3 (C-7), 152.6 (C-5), 152.3 (C-9), 150.7 (C-4′), 148.0 (C-3′), 131.3 (C-6), 121.5 (C-1′), 120.3 (C-6′), 115.7 (C-5′), 110.3 (C-2′), 104.0 (C-10), 102.7 (C-3), 94.2 (C-8), 59.8 (OCH_3-7) and 55.9 (OCH_3-6). Compound **2** was identified as jaceosidin because its instrumental analysis data corresponded with the reference data for jaceosidin (Chari et al., 1981; Voirin, 1983; Liu and Mabry, 1981).

Compound **3** consisted of pale yellow crystals with mp > 300 °C. The UV spectrum in methanol gave peaks at 267 and 335 nm. The addition of aluminum chloride resulted in four bands at 275, 301, 346, and 383 nm. The addition of sodium methylate shifted the peaks of the UV spectrum in methanol to 275, 325, and 390 nm. The IR spectrum in KBr showed absorbances at 3296, 3094, 3011, 2947, 1651, 1614, and 1607 cm^{-1}. The EI/MS spectrum gave a molecular ion peak at *m/z* 270 (M^+) and fragment ion peaks at *m/z* 242 and 153. The spectrum of ^1H NMR in dimethyl sulfoxide-d_6 gave signals at δ (ppm) 6.17 (1H, d, J = 2.0 Hz, H-6), 6.45 (1H, d, J = 2.0 Hz, H-8), 6.73 (1H, s, H-3), 6.92 (2H, s, H-3′ and H-5′), 7.91 (2H, d, J = 9.0 Hz, H-2′ and H-6′) and 12.93 (1H, s, 5-OH). The ^{13}C NMR spectrum in dimethyl sulfoxide-d_6 gave signals at δ (ppm) 181.5 (C-4), 164.8 (C-7), 163.5 (C-2), 161.3 (C-5), 161.1 (C-4′), 157.3 (C-9), 128.3 (C-2′ and C-6′), 121.1 (C-1′), 115.9 (C-3′ and C-5′), 103.3 (C-10), 102.7 (C-3), 98.9 (C-6) and 93.9 (C-8). Compound **3** was identified as apigenin because its instrumental analysis data corresponded with the reference data for apigenin and the data of an authentic sample (Loo and Bruyn, 1986; Kuroyanagi et al., 1985).

Compound **4** was a pale yellow crystal which was analyzed only with UV, IR, EI-MS, ^1H NMR and ^{13}C NMR because it was obtained only in small amounts. The IR spectrum in KBr showed absorbances at 3354, 3128, 3086, 1651, 1626, 1599, 1564, and 1514 cm^{-1}. The EI/MS spectrum gave a molecular ion peak at *m/z* 300 (M^+) and fragment ion peaks at *m/z* 272, 257, 229, and 153. The ^1H NMR spectrum in dimethyl sulfoxide-d_6 gave signals at δ (ppm) 3.89 (3H, s, OCH_3), 6.17 (1H, d, J = 1.75 Hz, H-6), 6.48 (1H, d, J = 1.75 Hz, H-8), 6.84 (1H, s, H-3), 6.94 (1H, d, J = 9.0 Hz, H-5′), 7.54 (1H, d, J = 9.0, 2.0 Hz, H-6′), 7.54 (1H, dd, J = 9.0 Hz, H-2′) and 12.93 (1H, s, 5-OH). The ^{13}C NMR spectrum in dimethyl sulfoxide-d_6 gave signals at δ (ppm) 181.6 (C-4), 164.7 (C-7), 163.5 (C-2), 161.3 (C-5), 157.3 (C-9), 150.8 (C-3′), 148.0 (C-4′), 121.4 (C-6′), 120.3 (C-1′), 115.8 (C-5′), 110.3 (C-2′), 103.4 (C-10), 103.1 (C-3), 98.9 (C-6), 94.1 (C-8) and 55.9 (C-3′).

Compound **4** was identified as chrysoeriol because its instrumental analysis data corresponded with the reference data for chrysoeriol and the data of an authentic sample (Wagner and Chari, 1976).

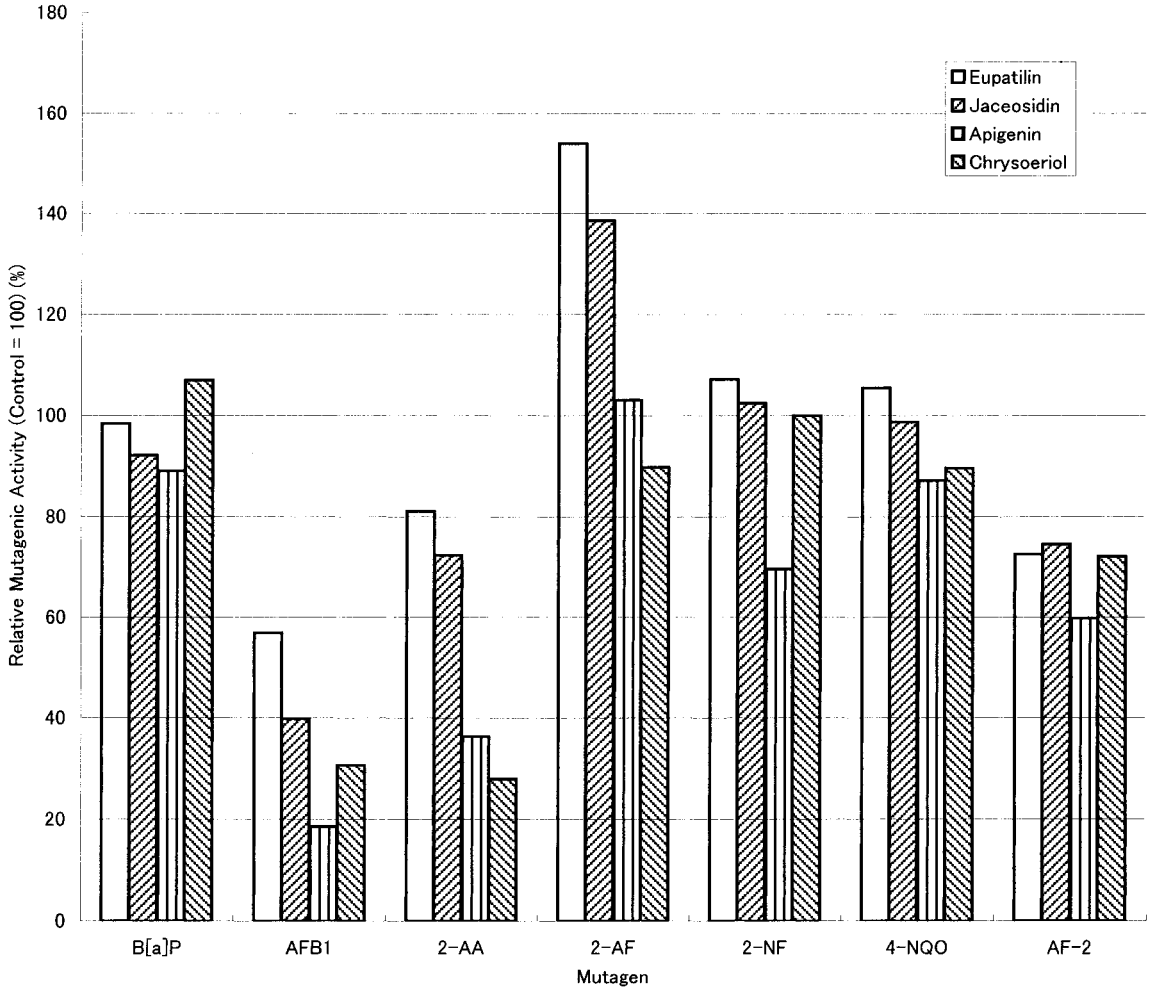

Figure 4. Effects of the four isolated flavones on mutation caused by various mutagens in *Salmonella typhimurium* TA98. Mutagenic activities are expressed relative to control (100%). Each test sample was tested at 50 μg/plate. Doses and revertant colony counts for the control (100%) of each mutagen used were as follows: B[*a*]P (20.0 μg/plate; 157 rev.), AFB_1 (4.0 μg/plate; 2673 rev.), 2-AA (10.0 μg/plate; 1893 rev.), 2-AF (10.0 μg/plate; 2624 rev.), 2-NF (10.0 μg/plate, 1523 rev.), 4-NQO (0.2 μg/plate; 187 rev.) and AF-2 (0.1 μg/plate, 231 rev.).

The structures of compounds **1**−**4** are shown in Figure 2.

Antimutagenicity of Each Fraction and Isolated Compounds. The antimutagenicity tests of the methanol extract and chromatographic fractions were done at the doses of 1.0 and 0.5 mg/plate, and only the results at the dose of 0.5 mg/plate are shown in Figure 1. The methanol extract reduced the mutagenic activity of Trp-P-2 by 78.6%, and exhibited some cytotoxicity against the tester strain cells. After partitioning with ethyl acetate, the antimutagenic activity was found in Fraction III (98.1%), which was accompanied with slightly cytotoxicity (12.2%). Fraction I and II had no cytotoxicities. Silica gel column chromatography of Fraction III yielded highly active fractions B (94.0%), C (95.8%), and D (96.2%). Fractions A−E did not exhibit the cytotoxicity. Chromatography of subfraction C yielded strong antimutagenic subfractions C-1 (89.2%), C-2 (80.9%), C-3 (89.4%), and C-4 (80.0%). The cytotoxicities of subfractions C-1, C-2, C-3, C-4, and C-5 were 23.1%, 17.1%, 24.7%, 8.4%, and 5.2%, respectively. Sephadex LH-20 column chromatography of subfractions C-3 and C-4 yielded highly antimutagenic subfractions C-3-3 (93.5%) and C-4-2 (93.8%). The cytotoxicity of C-3-1 was 29.3%. The other subfractions of fraction C-3 have no cytotoxicities. Chromatography of subfraction D yielded antimutagenic subfractions D-1 (59.0%) and D-2 (60.4%). The subfractions of fraction D had no cytotoxicity. Subsequently, subfraction D-2 yielded highly antimutagenic subfractions D-2-2 (98.0%) and D-2-3 (97.0%). Three subfractions of fraction D-2 and four subfractions of fraction B were also no cytotoxicity. The antimutagenic activities and IC_{50} values of eupatilin (**1**), jaceosidin (**2**), apigenin (**3**) and chrysoeriol (**4**), which were isolated from *A. argyi*, are shown in Table 1, together with those of related flavones **5**−**11**. All tested flavones exhibited strong antimutagenic activity with almost no cytotoxicity against the tester strain at the dose of 100 μmol/plate. Especially, chrysoeriol (**4**) had strong antimutagenic activity, comparable to that (IC_{50} value: 0.096 mmol/plate) of luteolin (**11**), which is a strong desmutagen found in peppermint, sage and thyme (Samejima et al., 1995), and that of diosmetin (**7**). Although apigenin (**3**) also had strong antimutagenic activity, it was effective only at higher doses than the above three flavones. On the other hand, the antimutagenic effects of eupatilin (**1**) and jaceosidin (**2**) were weaker than those of chrysoeriol (**4**) and apigenin (**3**), and their IC_{50} values were 4.2 times and 3.67 times higher than the IC_{50} value of apigenin (**3**), respectively. On the other hand, chrysin (**6**), which has no functional group on the B ring, exhibited the weakest antimutagenic activity among the 11 flavones tested as indicated by the IC_{50} value: 10.13 times of luteolin and 7.52

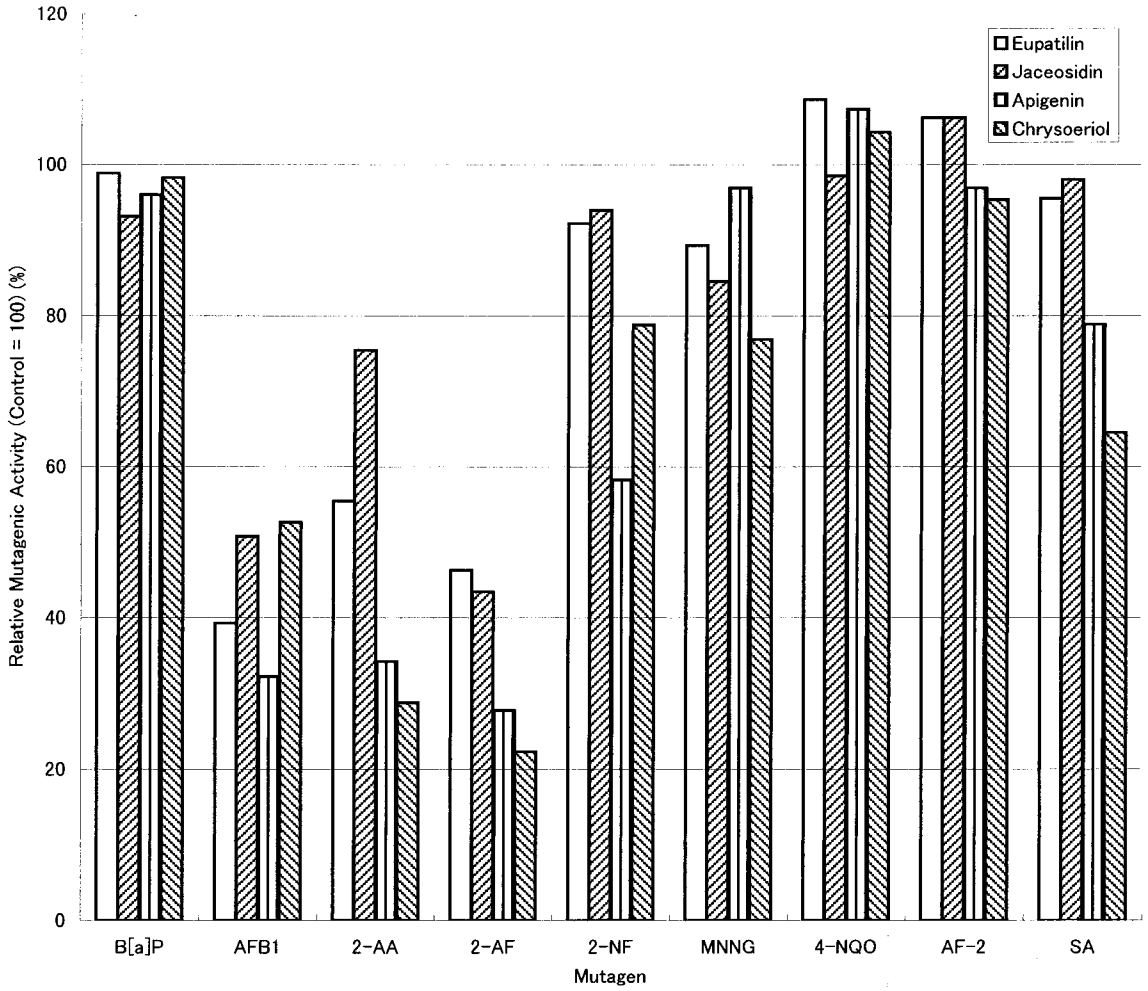

Figure 5. Effects of the four isolated flavones on mutation caused by typical mutagens in *Salmonella typhimurium* TA100. Mutagenic activities are expressed relative to control (100%). Each test sample was tested at 50 μg/plate. Doses and revertant colony counts for the control mutagen used were as follows: B[*a*]P (20.0 μg/plate; 320 rev.), AFB_1 (4.0 μg/plate; 1859 rev.), 2-AA (10.0 μg/plate; 1786 rev.), 2-NF (10.0 μg/plate; 1385 rev.), 2-AF (10.0 μg/plate; 1605 rev.), MNNG (5.0 μg/plate; 565 rev.), 4-NQO (0.2 μg/plate; 611 rev.), AF-2 (0.1 μg/plate, 389 rev.), and SA (1.0 μg/plate; 732 rev.).

times of apigenin. The isolated flavones also exhibited strong antimutagenicity against not only Trp-P-2 but also against other heterocyclic amines, such as Trp-P-1, IQ, MeIQ, MeIQx, and $MeA_{\alpha}C$, in tester strain TA98 (Figure 3). In addition, these flavones exhibited antimutagenic activity against aflatoxin B_1 and 2-aminoanthracene, but no antimutagenic activities against 2-aminofluorene and directly acting mutagens such as 2-nitrofluorene, 4-NQO and AF-2 in tester strain TA98 (Figure 4). Similarly, in assays using tester strain TA100, although these flavones exhibited strong antimutagenicity against aflatoxin B_1, 2-aminoanthracene and 2-aminofluorene, they exhibited no antimutagenic activity against 2-nitrofluorene, MNNG, 4-NQO, AF-2 or sodium azide (Figure 5).

Desmutagenicity of Isolated Compounds. The four isolated flavones exhibited strong desmutagenic effects on Trp-P-2 (Table 2) and the S-9 mix (Table 3). Especially, apigenin and chrysoeriol exhibited strong desmutagenicity. However, these flavones exhibited no desmutagenic effect on activated *N*-OH-Trp-P-2 (Table 4).

Bio-antimutagenicity of Isolated Compounds. The four isolated flavones did not affect TA98 cells previously mutated by Trp-P-2 (Table 5).

EROD and MROD Activities of the Isolated Antimutagens. All four flavones exhibited strong inhibitory activities against ethoxy resorufin-*O*-deethylase and methoxy resorufin-*O*-demethylase at concentrations higher than 100 μmol/mL. However, these flavones showed distinct differences in their inhibitory effects against these enzymes at concentrations less than 50 μmol/mL. Namely, these flavones caused the markedly decreased inhibitory activities against methoxy resorufin-*O*-demethylase at 20 μmol/mL, whereas they exhibited strong inhibitory activities against ethoxy

Table 2. Desmutagenic Effects of the Four Isolated Flavones against Trp-P-2 (3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole) with Preincubation Method in *Salmonella typhimurium* TA98[a]

sample	no. of revertant colonies/plate	desmutagenic activity (% inhibition)
control	4641	0
eupatilin (**1**)	2317	50.1
jaceosidin (**2**)	1145	75.3
apigenin (**3**)	401	91.4
chrysoeriol (**4**)	323	93.1

[a] Dimethyl sulfoxide was used as a control solution in the presence of 0.2 μg of Trp-P-2 and S-9 mix. The number of spontaneously mutated colonies was 43/plate. Dose of each sample: 0.1 mg/plate. The numbers of revertants, including spontaneously mutated colonies, are the means from three plates. Desmutagenic activity was calculated as described for the antimutagenicity test.

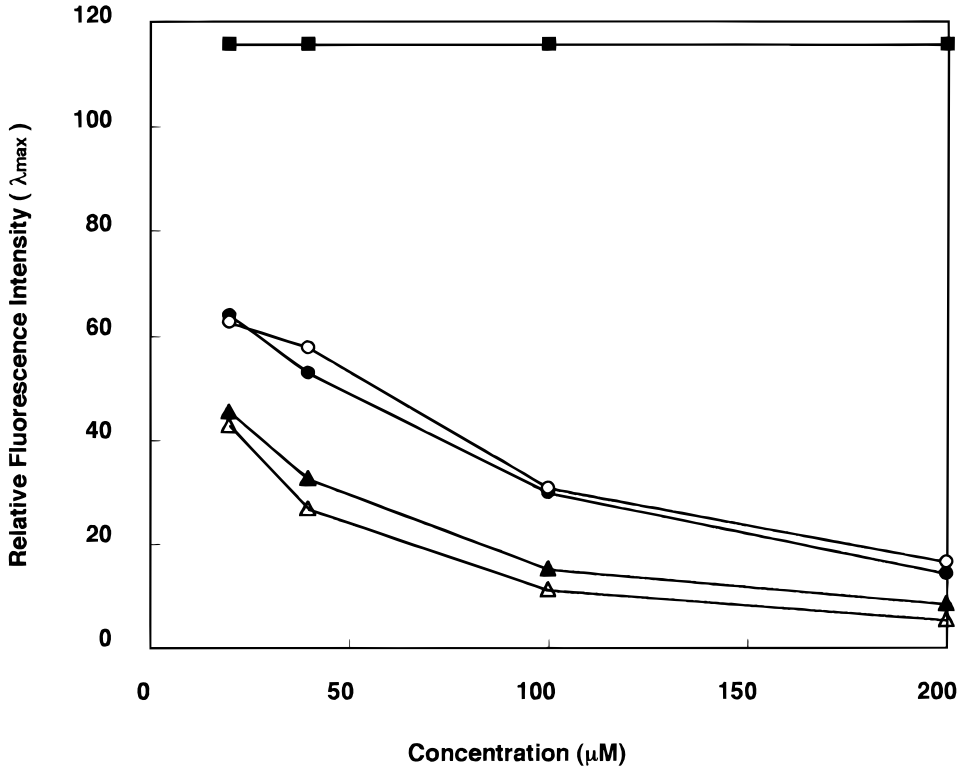

Figure 6. Inhibitory effects of the four isolated flavones on EROD activity: ■ control, ● eupatilin, ○ jaceosidin, ▲ apigenin, △ chrysoeriol.

Table 3. Desmutagenic Effects of the Four Isolated Flavones against S-9 Mix on Frameshift Mutation Caused by Trp-P-2 (3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole) with Preincubation Method in *Salmonella typhimurium* TA98[a]

sample	no. of revertant colonies/plate	desmutagenic activity (% inhibition)
control	3529	0
eupatilin (**1**)	1395	60.5
jaceosidin (**2**)	724	79.5
apigenin (**3**)	258	92.7
chrysoeriol (**4**)	300	91.5

[a] Dimethyl sulfoxide was used as a control solution in the presence of 0.2 μg of Trp-P-2 and S-9 mix. The number of spontaneously mutated colonies was 42/plate. Dose of each sample: 0.1 mg/plate. The numbers of revertants, including spontaneously mutated colonies, are the means from three plates. Desmutagenic activity was calculated as described for the antimutagenicity test.

Table 4. Desmutagenic Effects of the Four Isolated Flavones on Frameshift Mutation Caused by *N*-OH-Trp-P-2 (3-Hydroxyamino-1-methyl-5*H*-pyrido[4,3-*b*]indole) with Preincubation Method in *Salmonella typhimurium* TA98[a]

sample	no. of revertant colonies/plate	desmutagenic activity (% inhibition)
control (**1**)	267	0
eupatilin (**2**)	226	15.4
jaceosidin (**3**)	193	27.9
apigenin (**4**)	247	7.6
chrysoeriol (**5**)	218	18.4

[a] Dimethyl sulfoxide was used as a control solution. The number of spontaneously mutated colonies was 43/plate. Dose of each sample: 0.1 mg/plate. The numbers of revertants, including spontaneously mutated colonies, are the means from three plates. Desmutagenic activity was calculated as described for the antimutagenicity test.

resorufin-*O*-deethylase at the same concentration. Especially, eupatilin and jaceosidin exhibited no inhibitory activities against the methoxy resorufin demethylase.

Inhibitory Effect of the Isolated Antimutagens on Trp-P-2. All of the four flavones dose dependently decreased the fluorescence caused by Trp-P-2. Especially, apigenin and chrysoeriol strongly reduced the fluorescence caused by Trp-P-2.

Table 5. Bio-antimutagenic Effects of the Four Isolated Flavones in *Salmonella typhimurium* TA98[a] Cells Mutated by Trp-P-2 (3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole)

sample	no. of revertant colonies/plate	bio-antimutagenic activity (% inhibition)
control	3251	0
eupatilin (**1**)	3475	inactive
jaceosidin (**2**)	3268	inactive
apigenin (**3**)	3033	6.7
chrysoeriol (**4**)	2943	9.5

[a] Dimethyl sulfoxide was used as a control solution. TA98 cells were mutated with 0.2 μg of Trp-P-2 in the presence of S-9 mix. The number of spontaneously mutated colonies was 43/plate. Dose of each sample: 0.1 mg/plate. The numbers of revertants, including spontaneously mutated colonies, are the means from three plates. Bio-antimutagenic activity was calculated as described for the antimutagenic activity.

DISCUSSION

This study demonstrated that all four flavones, namely eupatilin (**1**), jaceosidin (**2**), apigenin (**3**), and chrysoeriol (**4**), isolated from *Artemisia argyi* Levl. et Vant., were strong desmutagens against Trp-P-2. Because eupatilin (**1**) and jaceosidin (**2**) are relatively abundant (namely 309 and 305 mgs, respectively) compared with apigenin (**3**) and chrysoeriol (**4**) (98 and 2.4 mgs, respectively) in 4.4 kg of plant material, it is likely that these components are the main contributors to the antimutagenicity of gaiyou.

As for the structure−activity relationships, the 11 tested flavones had distinctly different antimutagenic activities against Trp-P-2, according to their substitution paterns. For example, chrysoeriol (IC_{50}: 0.096

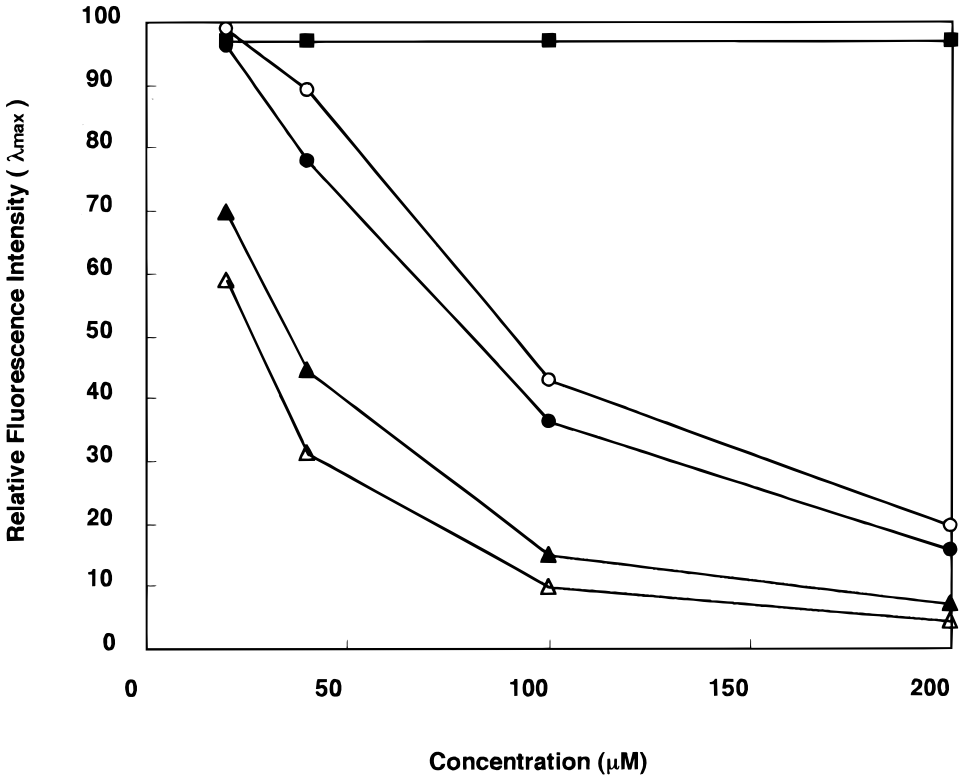

Figure 7. Inhibitory effects of the four isolated flavones on MROD activity: ■ control, ● eupatilin, ○ jaceosidin, ▲ apigenin, △ chrysoeriol.

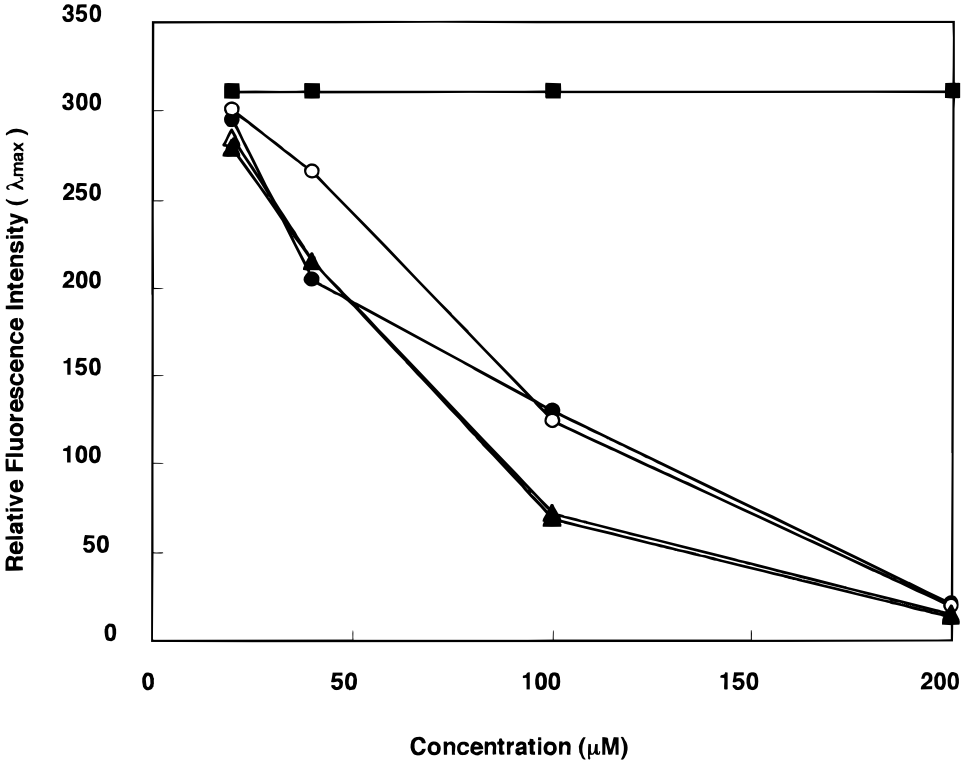

Figure 8. Change of Trp-P-2 in the presence of the four isolated flavones: ■ control, ● eupatilin, ○ jaceosidin, ▲ apigenin, △ chrysoeriol.

mmol/plate) exhibited the stronger antimutagenic activity than apigenin (IC_{50}: 0.120 mmol/plate), although only a functional group on the 3′-position is different. Same tendency was found between 5,7,3′,4′-tetrasubstituted flavones (such as luteolin and diosmetin, IC_{50}: 0.089 and 0.096 mmols/plate, respectively) and 5, 7, 4′-trisubstituted flavones (such as acacetin and genkwanin, IC_{50}: 0.260 and 0.159 mmols/plate, respectively). The above results indicate that the 3′, 4′-*ortho*-functional group on B-ring are most important for the antimutagenic activity. In addition, jaceosidin (IC_{50}: 0.440 mmol/plate) exhibited the weaker antimutagenic activity than chrysoeriol as well as the other 5,6,7,3′,4′-pentasubstituted flavones (such as eupatilin, eupatorin

and eupatorin-5-methyl ether, IC_{50} values: 0.504, 0.772, and 0.552 mmols/plate, respectively), although only a functional group on 6-position is different. It was therefore deduced that the presence of 6-methoxyl group caused the decreasing of the antimutagenic activity. In addition, tested flavones exhibited the decreasing tendency of the antimutagenic activity, when the hydroxyl group was replaced by the methoxyl group, for example, between jaceosidin and eupatilin or between luteolin and chrysoeriol or between apigenin, acacetin and genkwanin. It was therefore found that hydroxyl group was important for the antimutagenic activity.

As for the antimutagenic mechanisms, it was found that **1**−**4** were strong desmutagens, which directly inactivate Trp-P-2 by the formation of adducts or inhibit the metabolic activation to *N*-OH-Trp-P-2, based on the results of the assays with the preincubation method. In addition, the above flavones did not exhibit the bio-antimutagenicity. In general, the antimutagens are divided into desmutagens and bio-antimutagens according to differences in their modes of action. Desmutagens are antimutagens that inactivate the mutagens before the mutagens are incorporated into the bacterial cells and include antimutagens that act directly on mutagens or on activated mutagens. Other desmutagens inhibit the action of P-450 enzymes in the metabolic activation of mutagens. Bio-antimutagens are antimutagens that act on the DNA repair system after the mutagen is incorporated into DNA. Thus, the above result means that the isolated flavones were the antimutagens which did not affect the DNA repair system after DNA damage. We therefore believe that the isolated desmutagenic flavones serve to avoid the DNA damage in normal cells, which is a cause of carcinogenesis. These conclusions were also supported by the emission spectroscopic analysis. Namely, the four flavones dose dependently decreased the fluorescence caused from Trp-P-2 (Figure 8). This result suggests that these flavones could form inactive adducts with Trp-P-2. In addition, the four flavones strongly inhibited the deethylation of ethoxyresorufin (Figure 6), which is specific for CYP1A1, and demethylation of methoxyresorufin (Figure 7), which is specific for CYP1A2 (Hamilton and Teel, 1996). It is known that Trp-P-2 is metabolically activated by the CYP1A family, especially CYP1A2 (Shimada, 1992; Degawa, 1995). Accordingly, the above results suggest that these flavones can interfere with the metabolic activation from Trp-P-2 to *N*-OH-Trp-P-2 by inhibition of the CYP1A family. This is also similar to the conclusion drawn about the antimutagenicity of flavones and flavonols by Kanazawa et al. (1998). The inhibition of the metabolic activation was also closely related to the results of the antimutagenicity test of **1**−**4** with the various mutagens. Namely, these flavones exhibited strong antimutagenic activity only against indirectly acting mutagens such as heterocyclic amines, AFB_1, 2-AA and 2-NF, which are metabolically activated by the CYP1A family, although it is unknown why these flavones did not affect the antimutagenicity against B[*a*]P. Accordingly, it appears that the inhibition of the CYP1A family is involved in the mechanisms of action of these mutagens.

We expect that the antimutagenic flavones (**1**−**4**) isolated from *A. argyi* Levl. et Vant. will be useful cancer chemo-preventive agents. However, these flavones may not exhibit their expected effects in vivo if they are adversely affected by factors such as absorption, bio-disposition and metabolism after they are incorporated into the human body. Further studies with mammalian cells in vitro or in vivo are needed to determine the efficacy of these flavones for the prevention of human cancer.

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