Antimutagenic Activity of Isoflavone from *Pueraria lobata*

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A methanol extract from *Pueraria lobata* showed a suppressive effect on *umu* gene expression of the SOS response in *Salmonella typhimurium* TA1535/pSK1002 against the mutagen 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (furylfuramide). The methanol extract from *P. lobata* was re-extracted with hexane, dichloromethane, ethyl acetate, butanol, and water, respectively. A suppressive compound in the dichloromethane and ethyl acetate extract fractions was isolated by SiO_2 column chromatography and identified as tectorigenin (**1**) by EI-MS and ^1H and ^{13}C NMR spectroscopy. Compound **1** and its methylated derivative [7,4′-di-*O*-methyltectorigenin (**2**)] had the suppressive effects on *umu* gene expression of the SOS response in *Salmonella typhimurium* TA1535/pSK1002 against furylfuramide, 4-nitroquinoline-1-oxide, *N*-methyl-*N*′-nitrosoguanidine, and activated Trp-P-1, which do not require live metabolic activation by S9. These compounds also showed suppression of SOS-inducing activity against Trp-P-1 and AfB_1, which requires liver metabolizing enzymes. In addition to the antimutagenic activities of these compounds against furylfuramide, Trp-P-1 and activated Trp-P-1 were also assayed by an Ames test using *S. typhimurium* TA100.

Keywords: *Pueraria lobata; tectorigenin; antimutagenic activity; umu test; Ames test*

INTRODUCTION

Pueraria lobata (Will.) Ohwi (Leguminosae) has been used for the treatment of flatulence as a folk medicine in China, Korea, Taiwan, and Japan. It has been reported that irisolidone-7-*O*-gulcoside from Japanese *Pueraria* flowers [*P. lobata* (Will.) Ohwi)] (Kubo et al., 1973) has been isolated. In addition, the essential oily components from the fresh flowers of *P. lobata* (Will.) Ohwi (Kurihara et al., 1973) were isolated.

In the evaluation of the carcinogenicity or mutagenicity of environmental chemicals, it is important to determine factors present in the environment that may affect these activities. With the development of laboratory techniques for the detection of possible environmental carcinogens and mutagens (Ames et al., 1975), it has been shown that ordinary human diets contain several mutagens and antimutagens. Ishii et al. (1984) reported on the screening of the bioantimutagenic capacities of plant extracts with criteria of suppressing UV-induced mutations in *Escherichia coli* B/r Wp2 (*trp*) and the mutator activity in *Bacillus subtilis* NIG 1125 (*his met*). Protoanemonin was identified as the factor responsible for the antimutagenicity of ranunculus and anemone plants against UV- and *N*-methyl-*N*′-nitrosoguanidine (MNNG)-induced *E. coli* B/r Wp2 *trp* (Minakata et al., 1983). In particular, the *umu* test system was developed to evaluate the genotoxic activities of a wide variety of environmental carcinogens and mutagens (Oda et al., 1985; Nakamura et al., 1987). The results of this test are in agreement with the results of the Ames test and may be useful with respect to simplicity, sensitivity, and rapidity (Reifferscheid et al., 1996). The *umu* test detects the induction of the SOS response following treatment of *Salmonella typhimurium* strain TA1535 with test compounds. This strain carries the plasmid pSK1002 in which the *umu*C′ gene is fused inflame to the *lac*Z′ gene. The SOS-inducing potency of test compounds would therefore be estimated by the measurement of the induction of level of *umu* operon in terms of intracellular β-galactosidase activity. Furylfuramide was one of the nitrofuran derivatives that had been widely used as a food preservative in Japan. It genetic effects were reported by many researchers, which led to its classification as a potent mutagen (Tazima et al., 1975; Ohta et al., 1983). Likewise, 4-nitroquinoline-1-oxide (4NQO), a nitroheterocyclic compound, and MNNG, a direct-acting alkylating agent, were classified as potent mutagens (Olive et al., 1983, 1984; Watanabe et al., 1990; Haggery et al., 1990). Alternatively, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) (a heterocyclic amine derived from protein pyrolysis) and aflatoxin B_1 (a difurofuran ring fused to a substituted coumarin moiety) were shown to be highly mutagenetic following their activation by the enzymes contained in the liver S9 fraction (Ishikawa et al., 1979; Groopman et al., 1990; Selkirk et al., 1982).

Antimutagenic effects of naturally occurring compounds against mutagens have been investigated; *S*-(*N*,*N*-diethyldithiocarbamoyl)-*N*-acetyl-L-cysteine (Lee et al., 1997a), α-pinene-7β-*O*-β-D-2,6-diacetylglucopyranoside (Ragasa et al., 1997), cinnamic acid (Mitscher et al., 1996), and palmitic acids (Bakalinsky et al., 1996) are some of them. In the case of resveratrol, a stilbene derivative was shown to suppress the Trp-P-1-induced SOS as measured by the *umu* test and also to suppress its mutagenic potential as measured by the Ames test (Uenobe et al., 1997), and Jang et al. (1997) have also shown its ability to act as a potent cancer chemopreventive agent.

In our search for new naturally occurring antimutagenic compounds in plants, which have a history of

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safe use as Chinese crude drugs (Miyazawa et al., 1995, 1996, 1997), we found that the methanol extract of *P. lobata* (kakka in Japanese) exhibited suppression of the SOS-inducing activity of furylfuramide. This study reports the isolation and identification of the antimutagenic compound contained in *P. lobata*.

MATERIALS AND METHODS

General Procedure. Electron-impact mass spectra (EI-MS) were obtained on a Hewlett-Packard 5972A mass spectrometer. IR spectra were determined with a Perkin-Elmer 1760-x infrared Fourier transform spectrometer. Nuclear magnetic resonance (NMR) spectra (δ, J in hertz) were recorded on a JEOL GSX 270 NMR spectrometer. Tetramethylsilane (TMS) was used as the internal reference (δ 0.00) for ^{1}H NMR spectra measured in DMSO-d_6 and acetone-d_6. This solvent was used for ^{13}C NMR spectra.

Materials. A commercially available air-dried rhizome of *P. lobata* was purchased from Takasago Yakugiyo Co. (Osaka, Japan). Rhizomes for use as a crude drug were collected in 1994 from plants cultivated in Nagano prefecture in Japan. Furylfuramide, 4NQO, and Trp-P-1 were purchased from Wako Pure Chemicals Co. (Osaka, Japan). MNNG was purchased from Aldrich Chemical Co. (Milwakukee, WI), and aflatoxin B_1 (AfB_1) was from Sigma Chemical Co. (St. Louis, MO). S9 fractions prepared from the liver of male rats that had been pretreated with phenobarbital or 5,6-benzoflavone were 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. (1977).

***Umu* Test.** The *umu* test is based upon the abilities of carcinogens and mutagens to induce expression of an *umu* gene in *Salmonella typhimurium* TA1535/pSK1002 (Oda et al., 1985), in which a plasmid (pSK1002) carrying a fused gene (*umu*C′-*lac*Z) had been introduced. The SOS-inducing potency is estimated by the measurement of the level of *umu* operon expression in terms of cellular β-galactosidase activity. The SOS response appears after DNA damage or interference with DNA replication (Little et al., 1982; Walker, 1984). The overnight culture of the tester bacterial strain in Luria broth (1% Bactotryptone, 0.5% NaCl, and 0.2% glucose; supplemented with 20 mg/L ampicillin) was incubated at 37 °C until the bacterial density at 600 nm reached 0.25−0.30. The culture was divided into 2.3 mL portions in test tubes. The test compound (50 μL, dissolved in DMSO), 0.1 M phosphate buffer (300 μL, pH 7.4), and furylfuramide (50 μL, 1 μg/mL in DMSO) were added to each tube. In the case of Trp-P-1 (50 μL, 40 μg/mL in DMSO), 300 μL of S9-metabolizing enzyme instead of 0.1 M phosphate buffer mixture was added. After 2 h of incubation at 37 °C with shaking, the culture was centrifuged to collect cells, which were resuspended in 25 mL of PBS; the cell density was read at 600 nm with one portion (1.0 mL) of the suspension. Using the other portion (0.25 mL), the level of β-galactosidase activity in the cell was assayed according to the method of Miller (1972).

UV Irradiation. Briefly, an overnight culture of the tester bacterial strains (*S. typhimurium* TA1535/pSK1002) in Luria broth was diluted 50-fold with fresh TGA medium and incubated at 37 °C until an optical density at 600 nm of 0.25−0.30 was reached. The cultures were then collected by centrifugation and suspended in 5 mL of 0.1 M phosphate buffer. The cell suspensions were then poured into Petri dishes and exposed to UV light (2.0 J/m^2) for 5 s using a germicidal lamp at room temperature.

Ames Test. The mutation test was carried out according to the preincubation method (Yahagi et al., 1977), which is a modification of the Ames method (Ames et al., 1975). The test compound (50 μL), Trp-P-1 (50 μL, 20 μg/mL in DMSO), and 500 μL of S9-metabolizing enzyme mixture instead of 0.1 M phosphate buffer were added to each tube. In the case of furylfuramide, 0.1 M phosphate buffer (500 μL) and furylfuramide (50 μL, 0.5 μg/mL in DMSO) were added. The culture of the tester bacterial strain (S. *typhimurium* TA100) was divided into 100 μL portions into the test tube. The mixture was preincubationed at 37 °C for 20 min, mixed with 2.0 mL of top agar at 45 °C, and poured onto a minimal glucose agar plate. After incubation for 2 days at 37 °C, the colonies on the plate were counted.

Table 1. Suppression of Furylfuramide[a]-Induced SOS Responses by *P. lobata* Fractions in *S. typhimurium* TA1535/pSK1002

sample	control[b]	dose response[c] 200 μg/mL	100 μg/mL	40 μg/mL	0 μg/mL
MeOH extract[d]	260	487	585	708	765
hexane fraction	260	620	582	661	765
CH_2Cl_2 fraction[d]	260	306	432	597	765
EtOAc fraction[d]	260	432	482	593	765
BuOH fraction	260	726	734	750	765
water fraction	260	719	708	716	765
fraction 1	269	539	563	600	643
fraction 2[d]	269	363	388	473	643
fraction 3[d]	269	365	446	498	643
fraction 4	269	427	429	539	634
fraction 5	269	572	587	633	643
fraction 6	240	619	720	768	770
fraction 7[d]	240	494	601	687	770
fraction 8	240	682	687	762	770

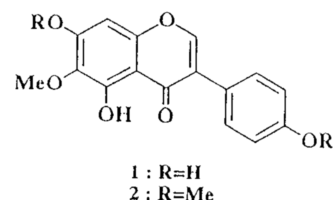
[a] Furylfuramide (1 μg/mL in DMSO) was added at 50 μL. [b] Control was exposed to DMSO. [c] β-Galactosidase activity (units). [d] Suppressive fraction.

Preparation of Activated Trp-P-1. Preparation of activated Trp-P-1 was carried out according to method of Arimoto et al. (1980).

Purification of the Suppressive Compound 1. The dry powder (4 kg) of *P. lobata* was refluxed with methanol for 12 h to give a methanol extract (593 g). This extract was suspended in water (2 L) and partitioned between hexane (1 L), ethyl acetate (1 L), butanol (1 L), and water successively. Each soluble fraction was concentrated under reduced pressure to give hexane (65.7 g), dichloromethane (19.3 g), ethyl acetate (38 g), butanol (230 g), and water (240 g) fractions. To pursue the compound responsible for the suppression of the SOS-inducing activity, these fractions were tested. As shown in Table 1, the dichloromethane and ethyl acetate fractions had positive activity, whereas the hexane, butanol, and water fractions did not show activity. To prepare the suppressive fraction, fractionation of the dichloromethane and ethyl acetate fractions was carried out as described in Table 1 and Figure 1. Finally, suppressive compound **1** (108 mg) was isolated.

Methylation of Compound 1. The methylated derivative of compound **1** (compound **2**) was obtained by reaction with CH_2N_2. This structure was identified by GC-MS, IR, and ^{1}H and ^{13}C NMR.

Compound 1. Compound **1** was yellow plates: MS, *m*/*z* 300 [M]$^+$, 285, 257 (base peak), 69; IR γ_{max} KBr, 3390, 1615, 1515, 1460 cm^{-1}; ^{1}H NMR and ^{13}C NMR identified with those of tectorigenin (Hanawa et al., 1991). Compound **1** was identified as tectorigenin [4*H*-benzopyran-4-one-5,7-dihydroxy-6-methoxy-3-(4-hydroxyphenyl)] from these spectral data and physical properties.



Compound 2. Compound **2** was yellow plates: MS, *m*/*z* 328 [M]$^+$, 313, 285; IR γ_{max} KBr, 3370, 1612, 1510, 1444 cm^{-1}; ^{1}H

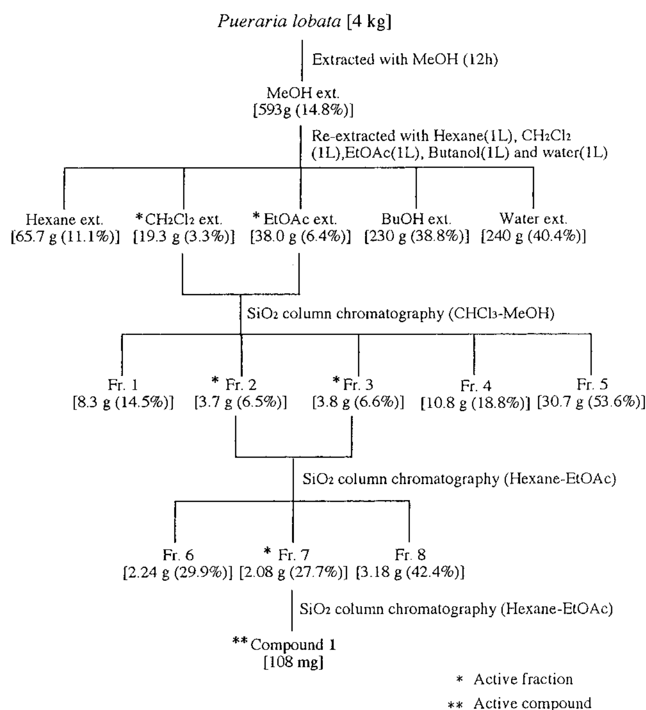


Figure 1. Isolation scheme for the suppressive compound from *P. lobata*.

NMR and ^{13}C NMR compared with those of tectorigenin (Hanawa et al., 1991). Compound **2** was identified as 7,4′-di-*O*-methyltectorigenin [4*H*-benzopyran-4-one-5-dihydroxy-6,7-methoxy-3-(4-methoxyphenyl)] from these spectral data and physical properties.

RESULTS

Fractionation and Isolation of Suppressive Compound from *P. lobata*. The methanol extract was fractionated to search for suppressive compounds using the *umu* test as a guide. To obtain dose−response data, test samples were evaluated at dose levels of 0.2, 0.1, and 0.04 mg/mL. As shown in Table 1, the methanol extract from *P. lobata* showed a suppressive effect on *umu* gene expression of the SOS responses in *S. typhimurium* TA1535/pSK1002 against furylfuramide. To prepare the suppressive fraction, fractionation of the methanol extract was carried out as described in Table 1 and Figure 1. Finally, suppressive compound **1** (108 mg) was isolated. The identity of compound **1** as tectorigenin was established by comparison of spectral data and physical constants with literature data.

Suppression of Mutagen-Induced Responses by Compounds 1 and 2. The suppressive effects of compounds **1** and **2** on mutagen-induced SOS responses were determined using the *umu* test. Compounds **1** and **2** were evaluated at dose levels of 0.67, 0.33, 0.13, and 0.07 μmol/mL to obtain dose−response data. As shown in Table 2 and Figure 2, compound **1** suppressed 64.7, 85.5, and 95.0% of the SOS-inducing activity due to furylfuramide, 4NQO, and MNNG at concentrations <0.67 μmol/mL, with a IC_{50} values of 0.44, 0.11, and 0.07 μmol/mL, respectively. Compound **2** also suppressed 32.4, 31.2, and 19.9% of the SOS-inducing activity due to furylfuramide, 4NQO, and MNNG at concentrations <0.67 μmol/mL, respectively. In addition, compounds **1** and **2** were also assayed with Trp-P-1 and AfB_1, which require liver metabolic activation. As shown in Table 3 and Figure 3, compound **1** suppressed 84.2 and 98.7% of the SOS-inducing activity due to Trp-P-1 and AfB_1 at concentrations <0.67μmol/mL, with a IC_{50} values of 0.12 and 0.11 μmol/mL, respectively. Compound **2** also suppressed 71.8 and 57.3% of the SOS-inducing activity due to Trp-P-1 and AfB_1 at concentrations <0.67μmol/mL, with a IC_{50} values of 0.13 and 0.51 μmol/mL, respectively. Furthermore, compounds **1** and **2** were assayed for suppressive effects on activated Trp-P-1-induced SOS responses. Compounds **1** and **2** suppressed 66.9 and 25.3% of the SOS-inducing activity due to activated Trp-P-1 at concentrations <0.67 μmol/mL, respectively. The IC_{50} value of compound **1** against activated Trp-P-1 was 0.42 μmol/mL. These results suggest that the inhibition of SOS induction of Trp-P-1, which was caused by compound **2**, is due to the inhibition of metabolic activation by S9.

Suppressive Effects of Compounds 1 and 2 on UV Irradiation. UV irradiation of *S. typhimurium* cells followed by compound **1** and **2** treatment was used to investigate the ability of these compounds to suppress the effect of a directly acting mutagen such as UV. As shown Table 4 and Figure 4, compounds **1** and **2** suppressed 53.4 and 31.0% of the *umu* gene expression at concentrations <0.67 μmol/mL. The IC_{50} value of compound **1** against UV irradiation was 0.49 μmol/mL.

Antimutagenic Activities of Compounds 1 and 2 in the Ames Assay. The Ames test using *S. typhimurium* TA100 also demonstrated the antimutagenic activities of these compounds against furylfuramide and Trp-P-1. As shown in Figure 5, compounds **1** and **2** suppressed 42.4 and 42.8% of the mutagenicity of furylfuramide at <1.67 μmol/plate. Compounds **1** and **2** also suppressed 78.0 and 86.9% of the mutagenicity of Trp-P-1 at <1.67 μmol/plate, with IC_{50} values of 0.31 and 0.26 μmol/plate, respectively. Additionally, compounds **1** and **2** suppressed 55.1 and 25.9% of the mutagenicity of activated Trp-P-1 at <1.67 μmol/plate. The IC_{50} of compound **1** against activated Trp-P-1 was 1.55 μmol/plate.

DISCUSSION

The antimutagenic compound in *P. lobata* was clearly identified as compound **1**. This compound had a suppressive effect on *umu* gene expression of the SOS response in *S. typhimurium* TA1535/pSK1002 against furylfuramide, 4NQO, MNNG, Trp-P-1, AfB_1, activated Trp-P-1, and UV irradiation. As shown in Tables 2−4 and Figures 2−4, compound **1** showed a greater suppressive potency against furylfuramide, 4NQO, MNNG, activated Trp-P-1, and AfB_1, which requires liver metabolizing enzymes and UV irradiation, than compound **2**. However, SOS induction of Trp-P-1 was suppressed by compound **2** as well as by compound **1**. From these results, the presence of hydroxyl groups at the 7- and 4′-positions is important for suppression of SOS-inducing activity against against furylfuramide, 4NQO, MNNG, Trp-P-1, AfB_1, activated Trp-P-1, and UV irradiation, and the suppressive effect against Trp-P-1 by compound **2** also depends on inhibition of the metabolic activation of Trp-P-1 by S9. In addition, the finding that compounds **1** and **2** showed suppressive effects on UV-irradiation-inducing SOS response indicates that the mechanisms for inhibition of SOS-inducing activity by these compounds may include the possibility that it not only acts directly on the mutagens but also is involved with cellular repair systems within the cell.

Table 2. Suppressive Effect of Compounds 1 and 2 on Furylfuramide,[a] 4NQO,[b] and MNNG[c] Using *S. typhimurium* TA1535/pSK1002

compd	furylfuramide	4NQO	MNNG	control	dose response[d] 0.67 μmol/mL	0.33 μmol/mL	0.13 μmol/mL	0.07 μmol/mL
1	908			238	475	623	726	819
2	623			197	485	493	526	536
1		385		123	161	217	219	343
2		390		153	316	325	376	388
1			325	123	133	165	177	218
2			465	153	403	441	456	458

[a] Furylfuramide (1 μg/mL in DMSO) was added at 50 μL. [b] 4NQO (10 μg/mL in DMSO) was added at 50 μL. [c] MNNG (200 μg/mL in DMSO) was added at 50 μL. [d] β-Galactocidase activity (units).

Table 3. Suppressive Effect of Compounds 1 and 2 on Trp-P-1,[a] Activated Trp-P-1, and AfB_1[b] Using *S. typhimurium* TA1535/PSK1002

compd	Trp-P-1	activated Trp-P-1	AfB_1	control	dose response[c] 0.67 μmol/mL	0.33 μmol/mL	0.13 μmol/mL	0.07 μmol/mL
1	711			165	251	390	622	702
2	510			171	267	338	340	386
1		444		118	226	301	340	345
2		620		174	507	520	564	576
1			350	90	94	165	201	272
2			394	123	241	283	331	355

[a] Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL. [b] AfB_1 (2 μg/mL in DMSO) was added at 50 μL. [c] β-Galactocidase activity (units).

Table 4. Suppressive Effect of Compounds 1 and 2 on UV Irradiation[a] Using *S. typhimurium* TA1535/PSK1002

compd	UV irradiation	control	dose response[b] 0.67 μmol/mL	0.33 μmol/mL	0.17 μmol/mL	0.07 μmol/mL
1	1044	178	580	639	682	756
2	1081	204	809	856	862	914

[a] The cells were exposed to UV light (2.0 J/m^2) with a germicidal lamp at room temperature. [b] β-Galactocidase activity (units).

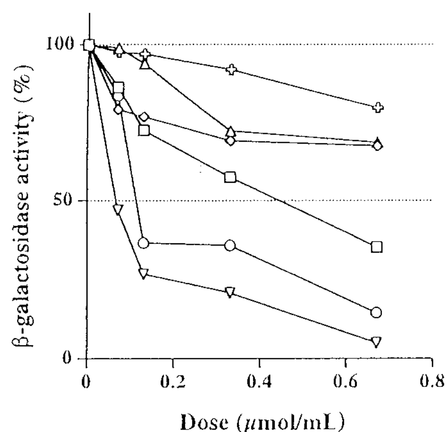


Figure 2. Suppression of furylfuramide-, 4NQO-, and MNNG-induced SOS responses by compounds **1** and **2** in *S. typhimurium* TA1535/pSK1002: (□) effect of **1** on furylfuramide; (◇) effect of **2** on furylfuramide; (○) effect of **1** on 4NQO; (△) effect of **2** on 4NQO; (▽) effect of **1** on MNNG; (✙) effect of **2** on MNNG. Furylfuramide (1 μg/mL in DMSO) was added at 50 mL. 4NQO (10 μg/mL in DMSO) was added at 50 mL. MNNG (200 μg/mL in DMSO) was added at 50 mL.

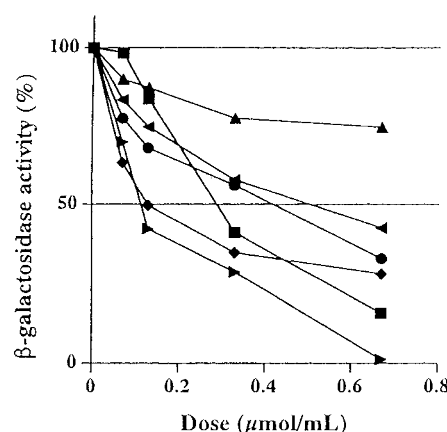


Figure 3. Suppression of Trp-P-1-, activated Trp-P-1-, and AfB_1-induced SOS responses by compounds **1** and **2** in *S. typhimurium* TA1535/pSK1002: (■) effect of **1** on Trp-P-1; (◆) effect of **2** on Trp-P-1; (●) effect of **1** on activated Trp-P-1; (▲) effect of **2** on activated Trp-P-1; (◭) effect of **1** on AfB1; (◭) effect of **2** on AfB1. Trp-P-1 (40 μg/mL in DMSO) was added at 50 mL. AfB_1 (2 μg/mL in DMSO) was added at 50 mL.

In the case of the Ames test using *S. typhimurium* TA100, compounds **1** and **2** suppressed the mutagenicity of Trp-P-1 and activated Trp-P-1 similar to the *umu* test. In these results of antimutagenicity against furylfuramide, compound **2** showed suppression similar to that of the *umu* test, whereas the antimutagenic activity of compound **1** was weaker than in the case of the *umu* test. The antimutagenic factors are divided into the two main classes: one type of factor is desmutagen, (i) which inhibits the formation of mutagens out of the cell or takes the mutagens into the cellular out of the cell or (ii) which inactivates or destroys mutagens directly or indirectly out of the cell; and another type of factor is called bioantimutagen, which suppresses the process of mutagenesis itself in the cell, for example, eliminates the radical or enhances DNA repair systems. Compounds **1** and **2** inhibited the SOS-inducing activity by UV irradiation, which physical mutagen (Figure 4). This

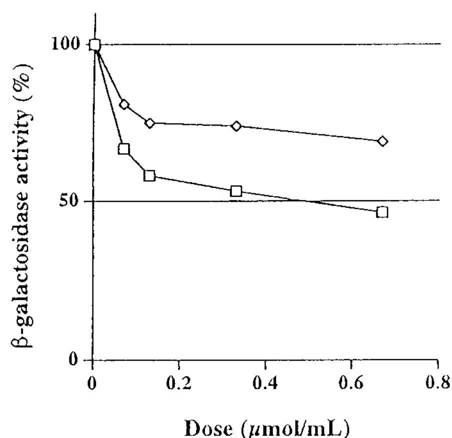


Figure 4. Suppression of UV-induced SOS responses by compounds **1** and **2** in *S. typhimurium* TA1535/pSK1002: (□) effect of **1** on UV; (◇) effect of **2** on UV. The cells were exposed to UV light (2.0 J/m^2) with a germicidal lamp at room temperature.

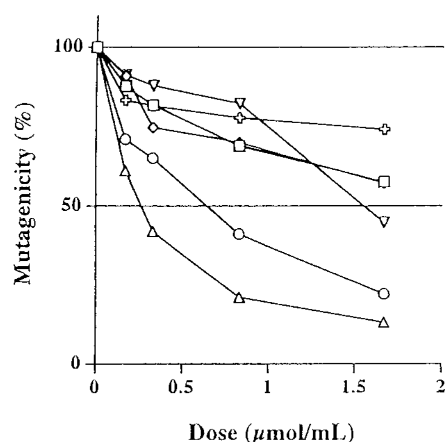


Figure 5. Effect of compounds **1** and **2** on the mutagenicity of furylfuramide, Trp-P-1, and activated Trp-P-1 in *S. typhimurium* TA100: (□) effect of **1** on furylfuramide; (◇) effect of **2** on furylfuramide; (○) effect of **1** on Trp-P-1; (△) effect of **2** on Trp-P-1; (▽) effect of **1** on activated Trp-P-1; (✙) effect of **2** on activated Trp-P-1. Furylfuramide (0.5 μg/mL in DMSO) was added at 50 mL/plate. Trp-P-1 (20 μg/mL in DMSO) was added at 50 mL/plate.

result suggests that compounds **1** and **2** might have the potency of bioantimutagens.

Previously many antimutagenic compounds were isolated from plant extracts. Nakamura et al. (1993) reported on the antimutagenic activity of *S*-methylmethanethiosulfonate front cauliflower (*Bassica oleracea* L. var. *botrytis*). Wall et al. (1988a,b) reported on antimutagenic homoisoflavones (intricatin and intricatinol) from the roots of *HoBhanosseggia iniricata* Brandegee. Okuda et al. (1984) reported on inhibitory effects of tannins included in *Germium thunbergii*. Many antimutagenic compounds have various skeletons, and these suppressive compounds were examined to elucidate the mechanism of suppressive effects in various assay systems. Compound **1** was isolated from several other plants, for example, *Iris crocea* (Shawl et al., 1992) and *Belamcanda chinensis* (Yamaki et al., 1990). However, no reports on the antimutagenicity of tectorigenin have appeared. In this paper, tectorigenin (**1**) was isolated and identified as the primary antimutagenic compound in *P. lobata.* 7,4′-Di-*O*-methyltectorigenin (**2**), which was derivative of compound **1**, also has potency as an antimutagen, and these compounds also suppressed the SOS-inducing activity of chemical mutagens and UV irradiation.

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