Antimutagenic Activity of Gigantol from *Dendrobium nobile*

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A methanol extract from *Dendrobium nobile* 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 *D. nobile* was re-extracted with *n*-hexane, dichloromethane, 1-butanol, and water, respectively. A suppressive compound in the *n*-hexane extract fraction was isolated by SiO_2 column chromatography and chemical fractionation and was identified as gigantol by EI-MS and ^1H and ^{13}C NMR spectroscopy. Gigantol suppressed the SOS-inducing activity of furylfuramide in the *umu* test. Gene expression was suppressed 90% at <0.73 μmol/mL, and the ID_{50} value was 0.35 μmol/mL. Gigantol was also assayed with the mutagen 3-amino-1,4-dimethyl-5*H*-pyrido[4,3*b*]indole (Trp-P-1), which requires liver metabolizing enzymes, and it suppressed the SOS-inducing activity of Trp-P-1 in the *umu* test. Gene expression was suppressed 91% at <0.73 μmol/mL, and the ID_{50} value was 0.32 μmol/mL. In addition, gigantol was assayed for suppressive effect on UV irradiation in the *umu* test, where it showed suppression of the SOS-inducing activity caused by UV irradiation. Gene expression was suppressed 84% at <0.36 μmol/mL, and the ID_{50} value was 0.17 μmol/mL. The antimutagenic activities of gigantol against furylfuramide and Trp-P-1 were assayed by an Ames test using *S. typhimurium* TA100, which indicated that gigantol suppressed each of the mutagens.

Keywords: *Dendrobium nobile; gigantol; Orchidaceae; antimutagenic activity; umu test; Ames test*

INTRODUCTION

Dendrobium nobile (Orchidaceae) is widely distributed in China. The cultivated storage stalk is used for treatment as a roborant and for anorexia and gastrointestinal disorders. *Dendrobium* species are known to produce alkaloids (Suzuki et al., 1973), fluorenones and sesquiterpenoids (Talapatra et al., 1985, 1992), and bibenzyls and phenanthrenes (Majumder et al., 1989, 1992).

With the development of techniques for detecting possible environmental carcinogens and mutagens (Ames et al., 1975), it has been shown that ordinary diets contain many kinds of 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*′-nitro-*N*-nitrosoguanidine-induced *E. coli* B/r WP2 *trp* (Minakata et al., 1983).

The *umu* test system was developed to evaluate the genotoxic activities of a wide variety of environmental carcinogens and mutagens, using the expression of one of the SOS genes to detect DNA-damaging agents (Oda et al., 1985; Nakamura et al., 1987). In our search for new naturally occurring antimutagenic compounds in plants that have a history of safe use as Chinese crude drugs (Miyazawa et al., 1995a−c), we found that the methanol extract of *D. nobile* (sekkoku in Japanese) exhibited suppression of the SOS-inducing activity of furylfuramide. In this paper, we report the isolation and identification of the antimutagenic compounds contained in *D. nobile*.

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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 ^1H NMR spectra measured in $CDCl_3$. This solvent was used for ^{13}C NMR spectra. Specific rotation was determined with a JASCO DIP-140 digital polarimeter.

Materials. Commercially available air-dried rhizome of *D. nobile* was purchased from Takasago Yakugiyo Co. (Osaka, Japan). The rhizomes for use as a crude drug were collected in 1994 from plants cultivated in Nagano prefecture in Japan. Furylfuramide [2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide], Trp-P-1 (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole), and isopropyl β-D-galactopyranoside (IPTG) were purchased from Wako Pure Chemical Ltd. S9 microsomal preparation was purchased from Oriental Yeast Co.

***umu* Test.** The *umu* test for detecting the SOS-inducing activity of chemicals was carried out essentially as described by Oda et al. (1985) using *Salmonella typhimurium* TA1535/pSK1002 whose plasmid pSK1002 carries a *umuC*′-*lacZ*′ fused gene. The SOS-inducing potency is estimated by the measurement of the level of *umu* operon expression in terms of cellular β-galactosidase activity (Miller, 1972).

Effect of Suppressive Compounds on mRNA Synthesis Induced by IPTG. The test strain *E. coli* CSH 26T/F*lac*$^+$, which was kindly supplied by Dr. Yoshimitsu Oda (Osaka Prefectural Institute of Public Health, Japan), was used to investigate the effect on mRNA synthesis in the *umu* test; this strain induces β-galactosidase activity by addition of IPTG instead of mutagens. The affect on β-galactosidase synthesis

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Table 1. Suppression of Furylfuramide[a]-Induced SOS Response by *D. nobile* Fractions in *S. typhimurium* TA1535/pSK1002

		dose response[c]			
sample	control[b]	200 μg/mL	100 μg/mL	50 μg/mL	0 μg/mL
MeOH extract[d,e]	240.3	510.2	635.2	716.8	921.1
n-hexane fraction[e]	226.4	764.1	793.5	800.1	1037.2
CH_2Cl_2 fraction	226.4	884.9	947.8	1053.7	1037.2
1-BuOH fraction	226.4	1067.9	1017.3	1005.2	1037.2
water fraction	226.4	1099.8	1028.6	1052.4	1037.2
fraction 1	244.7	579.8	632.0	670.6	720.9
fraction 2[e]	244.7	477.8	597.6	734.9	720.9
fraction 3	244.7	589.3	652.1	717.8	720.9
fraction 4	166.3	529.2	596.5	708.2	787.5
fraction 5	155.3	557.4	604.9	735.3	787.5
fraction 6[e]	166.3	278.2	489.6	791.4	787.5
fraction 7[e]	166.3	388.8	539.0	740.7	787.5
fraction 8[e]	254.4	296.3	542.7	732.5	956.8
fraction 9	254.4	782.3	821.1	975.6	956.8

[a] Furylfuramide (1 μg/mL in DMSO) was added at 50 μL. [b] Control was exposed to DMSO. [c] β-galactosidese activity (units). [d] MeOH extract was assayed at concentrations of 1000, 500, and 200 μg/mL. [e] Suppressive fraction.

by suppressive compound was assayed as follows: An overnight culture of the tester bacterial strain (*E. coli* CSH 26T/F*lac*$^+$) diluted 50-fold with TG medium (1% Bactotryptone, 0.5% NaCl, and 0.2% glucose) was incubated at 37 °C until the bacterial density at 600 nm reached 0.25−0.30. The culture was divided into 1.9 mL portions in test tubes. The test compound (50 μL, diluted with DMSO), 0.1 M phosphate buffer (350 μL, pH 7.4), and 0.01 M IPTG (200 μL) were added to each tube. After 60 min of incubation at 37 °C with shaking, the culture was centrifuged to collect cells, which were resuspended in 2.5 mL of PBS; the cell density was read at 600 nm with one portion (1.0 mL) of the suspension. Using the other portion (50 μL), the level of β-galactosidase activity in the cell was assayed according to the method of Miller (1972).

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

UV Irradiation. Briefly, an overnight culture of the tester bacterial strain in Luria broth (1% Bactotryptone, 0.5% NaCl, and 0.5% yeast extract) was diluted 50-fold with fresh TGA medium (1% Bactotryptone, 0.5% NaCl, and 0.2% glucose; supplemented with 20 mg/L ampicillin) and incubated at 37 °C until the bacterial density at 600 nm reached 0.25−0.30. The cultured cells were centrifuged for collection and then suspended with 5 mL of 0.1 M phosphate buffer. They were removed into a Petri dish (4 cm) and UV irradiated for 20 s (4.0 J/m^2) with a germical 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).

Purification of the Suppressive Compound 1. The dry powder (9 kg) of *D. nobile* was refluxed with methanol for 12 h to give a methanol extract (545 g). This extract was suspended in water (2 L) and partitioned between *n*-hexane (1 L), dichloromethane (1 L), 1-butanol (1 L), and water successively. Each soluble fraction was concentrated under reduced pressure to give *n*-hexane (83 g), dichloromethane (182 g), 1-butanol (153 g), and water (127 g) fractions. To identify the compound responsible for the suppression of the SOS-inducing activity, the fractions were tested. As shown in Table 1, the *n*-hexane fraction had positive activity, whereas the dichloromethane, 1-butanol, and water fractions did not show activity. To prepare the suppressive fraction, fractionation of the *n*-hexane fraction was carried out as described in Figure 1. Finally, suppressive compound **1** (240 mg) was isolated.

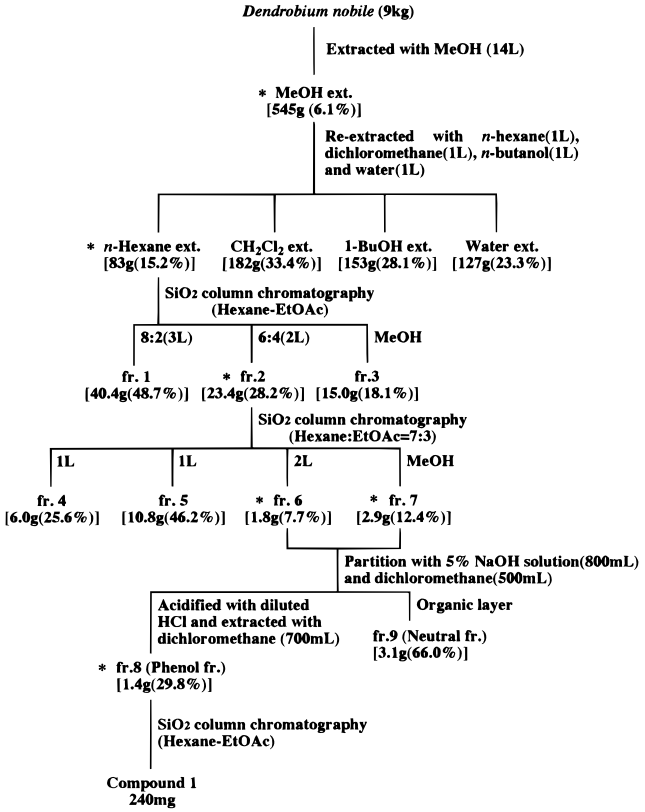
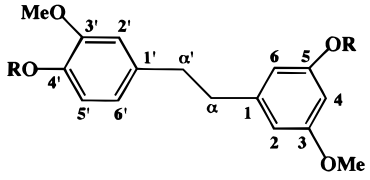


Figure 1. Isolation scheme for the suppressive compound from *D. nobile*.

Compound 1. Compound **1** was an oil; $[\alpha]^{20}_D$ −4.19 ($CHCl_3$, *c* 1.0); MS, *m*/*z* 274 [M]$^+$, 137 (base peak); IRγ_{max}^{KBr} 3480, 2930, 1700, 1600, 1510, 1450, 1270, 1230, 1200, 1150 cm^{-1}. The ^{1}H NMR spectrum of **1** confirmed the presence of methoxy groups at δ 3.75 and 3.84, and the signals at δ 5.05 and 5.50 could be assigned to two hydroxy groups. The signal at δ 2.80 could be assigned to four ethane hydrogens. The ^{13}C NMR of **1** was identical to that of gigantol (Juneja et al., 1985, 1987). Compound **1** was identified as gigantol [5-[2-(3-hydroxy-5-methoxyphenyl)ethyl]-2-methoxyphenol] from these spectral data and physical properties.



Acetylation of 1. The acetylate of **1** (compound **1A**) was obtained by the reaction with acetic anhydride and pyridine. Compound **1A** was an oil; MS, *m*/*z* 358 [M]$^+$, 316, 274, 137 (base peak). The ^{1}H NMR spectrum of **1A** confirmed the presence of the acetate methyl group at δ 2.28 and 2.30, and the signals at δ 3.75 and 3.78 could be assigned to two methoxy groups. Compound **1A** was identified with that of the diacetate of gigantol (Juneja et al., 1985; Majumder et al., 1993). Compound **1A** was identified as diacetoxygigantol from these spectral data.

Methylation of 1. The methylated derivative of **1** (compound **1M**) was obtained by reaction with CH_2N_2. Compound **1M** was an oil; MS, *m*/*z* 302 [M]$^+$, 151 (base peak). The ^{1}H NMR spectrum of **1M** confirmed the presence of the methoxy groups at δ 3.76 (*s*, 6H), 3.85 (*s*, 3H), and 3.86 (*s*, 3H). Compound **1M** was identical with 3,5,4′,5′-tetramethoxydihydrostilbene (Juneja et al., 1985).

Table 2. Suppression of Furylfuramide[a] and Trp-P-1[b]-Induced SOS Response by 1, 1A, and 1M in *S. typhimurium* TA1535/pSK1002

chemical	furylfuramide	Trp-P-1	control[c]	dose response[d] 0.73 μmol/mL	0.37 μmol/mL	0.15 μmol/mL
1	469.0		113.5	150.1	280.6	369.5
1A	469.0		113.5	365.9	394.3	419.2
1M	469.0		113.5	344.6	362.4	390.8
1		468.8	173.4	200.6	315.2	389.0
1A		468.8	173.4		194.1	244.3
1M		468.8	173.4		176.4	208.8

[a] Furylfuramide (1 μg/mL in DMSO) was added at 60 μL. [b] Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL. [c] Control was exposed to DMSO. [d] β-galactosidase activity (units).

RESULTS

Fractionation of the Extract from *D. nobile* and Isolation of Compound 1. 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 200, 100, and 40 μg/mL. As shown in Table 1, the methanol extract from *D. nobile* 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 Figure 1 and Table 1. Finally, suppressive compound **1** (240 mg) was isolated.

Structure Determination of 1. The identity of compound **1** as gigantol was established by comparison of spectral data and physical constants with literature data.

Suppression of 1, 1A, and 1M on the SOS-Inducing Activity. The suppressive effects of **1**, **1A**, and **1M** were determined in the *umu* test. As shown in Table 2, **1**, **1A**, and **1M** inhibited the SOS induction of furylfuramide. Compound **1** suppressed 90% of the SOS-inducing activity at concentrations <0.73 μmol/mL, and the ID_{50} value was 0.33 μmol/mL. This result includes the possibilities that **1** suppressed the furylfuramide-induced β-galactosidase activity. In the case of addition of **1** into the test strain, which was added with furylfuramide, at 20 min intervals after incubation of 2 h, the furylfuramide-induced SOS responses were measured, and the concentration of test compounds was 0.73 μmol/mL (Figure 2). These results suggested that mutagen-induced SOS response was effected by suppressive compounds, although suppressive compounds did not affect β-galactosidase activity. Mechanisms for the inhibition of mutagen-induced SOS response by **1** include the possibilities that suppressive compounds affect the *lexA-recA* regulation of the *umu* operon. Compound **1** was assayed for its effects on mRNA synthesis using *E. coli* CSH26T/F*lac*$^+$, which produces β-galactosidase without mutagens. From the assay using *E. coli* with suppressive compound, **1** did not affect β-galactosidase activity (Figure 3). Therefore, it suggested that **1** had the potency of suppressive effect on the mutagen-induced SOS response. Compound **1** also showed suppression of the SOS-inducing activity of Trp-P-1, which requires metabolic activation. Compound **1** suppressed 91% of the SOS-inducing activity at concentrations <0.35 μmol/mL, and the ID_{50} value was 0.32 μmol/mL. Compound **1** similarly suppressed both mutagens. In addition, **1** was assayed for the ability of

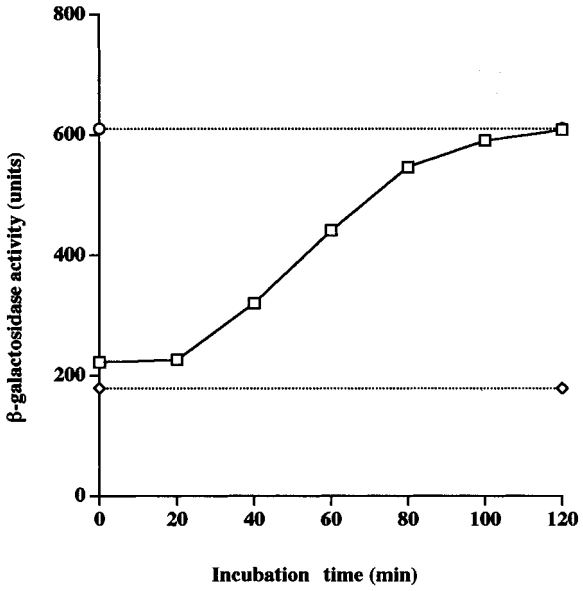


Figure 2. Effect of **1** on β-galactosidase activity in *S. typhimurium* TA1535/pSK1002. Compound **1** was added into each tube at 20 min intervals from the start of the incubation of 2 h. The concentration of **1** was 0.73 μmol/mL.

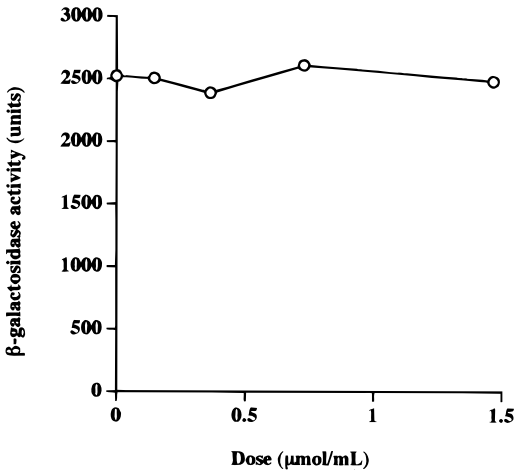


Figure 3. Effect of **1** on mRNA synthesis induced by IPTG in *E. coli* CSH26T/F*lac*+. IPTG (10^{-2} M) was added at 200 μL.

suppressive effect on activated Trp-P-1-induced SOS response (Figure 4). From this result, **1** suppressed the activated Trp-P-1-induced SOS response; it did not suggest that suppression of activated Trp-P-1-induced SOS response by **1** was caused by prevention of metabolic activation. Compound **1A** suppressed 29% of the SOS-inducing activity of furylfuramide at concentrations <0.73 μmol/mL, and **1M** also suppressed 35% of the SOS-inducing activity of furylfuramide at concentrations <0.73 μmol/mL. In addition, compound **1A** suppressed 93% of the SOS-inducing activity of Trp-P-1 at concentrations <0.37 μmol/mL, and the ID_{50} value was 0.30 μmol/mL. Compound **1M** almost suppressed the SOS-inducing activity of Trp-P-1 completely at concentrations <0.37 μmol/mL, and the ID_{50} value was 0.26 μmol/mL.

Suppressive Effect of 1, 1A, and 1M on UV Irradiation. Compounds **1**, **1A**, and **1M** showed suppressive effects on *umu* gene expression of the SOS responses against both furylfuramide and Trp-P-1 (Table 2). In addition, these compounds were tested for suppressive effects on UV-irradiation-induced SOS response

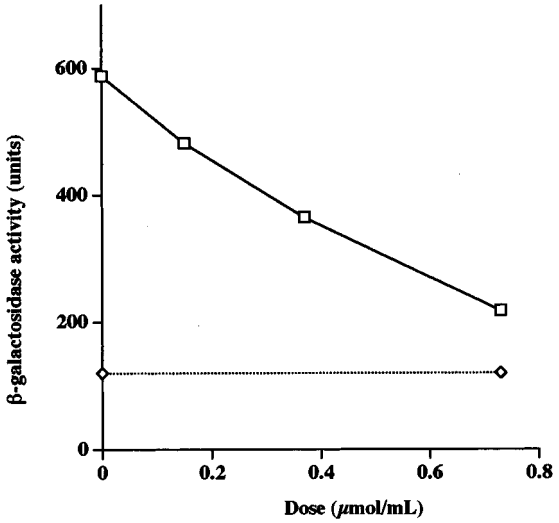


Figure 4. Suppression of activated Trp-P-1-induced SOS response by **1** in *S. typhimurium* TA1535/pSK1002.

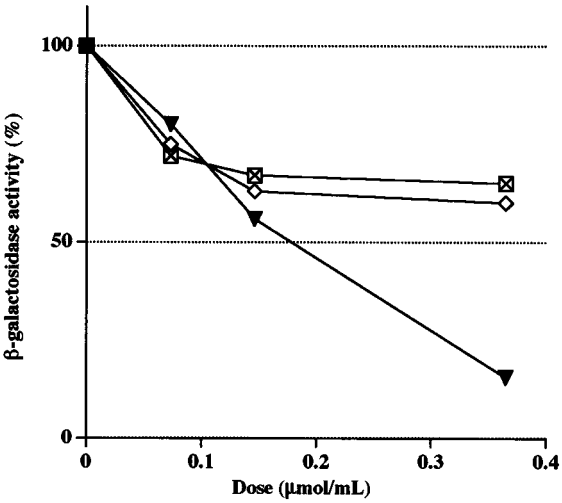


Figure 5. Suppression of UV-induced SOS response by **1**, **1A**, and **1M** in *S. typhimurium* TA1535/pSK1002: (▼) effect of **1** on UV irradiation; (crossed box) effect of **1A** on UV irradiation; (◇) effect of **1M** on UV irradiation.

using *S. typhimurium* TA1535/pSK1002. Compounds **1**, **1A**, and **1M** were tested for their suppressive effects on UV irradiation. As shown in Figure 5, compounds **1**, **1A**, and **1M** suppressed the SOS-inducing activity of UV irradiation. Compound **1** suppressed 84% of the SOS-inducing activity at concentrations <0.37 μmol/mL, with an ID_{50} of 0.19 μmol/mL. On the other hand, compounds **1A** and **1M** suppressed 35 and 40% of the SOS-inducing activity at <0.37 μmol/mL, respectively.

Antimutagenic Activity of 1, 1A, and 1M. The antimutagenic activity of these compounds against furylfuramide and Trp-P-1 was also demonstrated by Ames test using *S. typhimurium* TA100. As shown in Figure 6, compound **1** suppressed 38% of the mutagenicity of furylfuramide at <0.91 μmol/plate. Compound **1** also suppressed 56% of the mutagenicity of Trp-P-1 at <0.91 μmol/plate, within an ID_{50} of 0.79 μmol/plate. Compounds **1A** and **1M** suppressed 42 and 44% of the mutagenicity of furylfuramide at <0.91 μmol/plate, respectively. Compound **1A** suppressed 83% of the mutagenicity of Trp-P-1 at <0.91 μmol/plate, within an ID_{50} of 0.30 μmol/plate. Compound **1M** suppressed 83% of the mutagenicity of Trp-P-1 at <0.91 μmol/plate, with an ID_{50} of 0.26 μmol/plate.

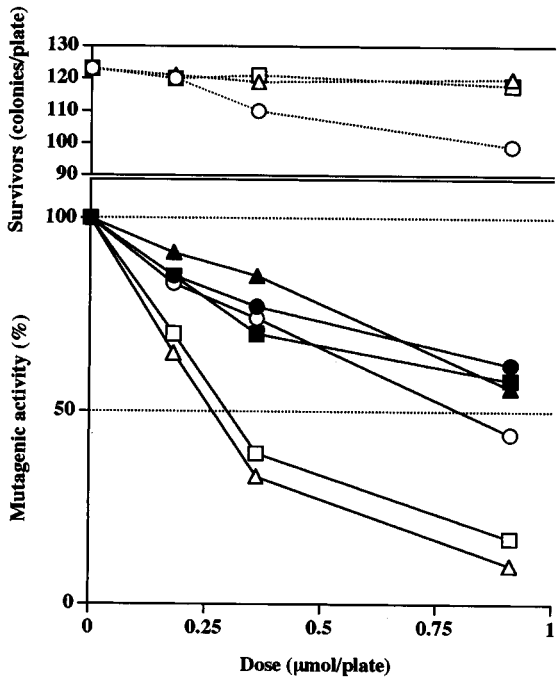


Figure 6. Suppression of furylfuramide and Trp-P-1-induced SOS response by **1**, **1A**, and **1M** in *S. typhimurium* TA100: (●) effect of **1** on furylfuramide; (○) effect of **1** on Trp-P-1; (■) effect of **1A** on furylfuramide; (□) effect of **1A** on Trp-P-1; (▲) effect of **1M** on furylfuramide; (△) effect of **1**-OMe on Trp-P-1. Furylfuramide (0.5 μg/mL in DMSO) was added at 20 μL/plate. Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL/plate.

DISCUSSION

The antimutagenic compound in *D. nobile* was clearly identified as **1**. This compound had a suppressive effect on *umu* gene expression of the SOS response in *S. typhimurium* TA1535/pSK1002 against furylfuramide, Trp-P-1, and UV irradiation. As shown in Table 2, **1** suppressed the SOS-inducing activity of furylfuramide and also of Trp-P-1 with an activity nearly equal to that of furylfuramide. However, **1** showed inhibition of the SOS induction due to UV irradiation at a lower concentration than those of chemical mutagens (Figure 4). The SOS regulatory system involves the action of two proteins: the LexA protein, which respresses a set of unlinked genes, and the RecA protein, which is activated as a protease by an inducing signal and specifically inactivates the repressor. The SOS response is activation of the protease, and the later manifestations of the SOS response are a secondary consequence of these events (Little et al., 1982). Mechanisms for the inhibition of mutagen-induced SOS response by **1** include the following possibilities: (i) suppression of inactivation of the LexA repressor by the RecA protease, (ii) suppression of the transcription of the *recA* gene, and (iii) suppression of RecA protein synthesis. Since the expression of the *umuC* gene is known to be regulated by the *recA* and *lexA* gene products (Shimagawa et al., 1983; Walker, 1984), the present data with *E. coli* may exclude the possibility that **1** suppresses the *lexA-recA* regulation of the *umu* operon.

From the results of the Ames test, **1** did not suppress the mutagen-induced SOS responses but showed suppression of mutagenicity caused by mutagens. This result clearly indicated that **1** had potency as an antimutagenic for suppression of DNA damage.

Recently, it was reported that curucumin suppressed UV-induced SOS response (Oda, 1995). Shimoi et al.

(1985) also reported that tannic acid suppressed mutagenesis in *E. coli* B/r WP2 (*trp*$^-$) induced by UV or 4-nitroquinoline 1-oxide (4NQO). Paeonol suppressed the mutagenesis in *E. coli* WP2s (*trp*$^-$, *uvrA*$^-$) induced by 4NQO (Fukuhara et al., 1987). In our previous paper, we reported that paeonol also suppressed the SOS-inducing activity of furylfuramide, using *S. typhimurium* TA1535/pSK1002 in the *umu* test, and mutagenicity of furylfuramide, using *S. typhimurium* TA100 (Miyazawa et al., 1996). In addition, Wall et al. (1990) reported on antimutagenic compounds in plants, some of which were phenolic compounds. Thus, various phenolic compounds have been isolated from plant extracts as antimutagens, and **1** is classified in this group.

Various bibenzyl and phenanthrene derivatives have been isolated from Orchidaceae plants. Compound **1** was isolated with other derivatives from Orchidaceae; Juneja et al. (1987) reported on isolation of bibenzyl and 9,10-dihydrophenanthrene derivatives together with compound **1** from *Cymbidium aloifolium*. In this paper, we have shown that the principal antimutagenic component in *D. nobile* is **1** and that **1** also suppressed the SOS-inducing activity of chemical mutagens and UV irradiation.

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