Moscatilin from *Dendrobium nobile*, a Naturally Occurring Bibenzyl Compound with Potential Antimutagenic Activity

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A bibenzyl compound that possesses antimutagenic activity was isolated from the storage stem of *Dendrobium nobile*. The isolated compound suppressed the expression of the *umu* gene following the induction of SOS response in *Salmonella typhimurium* TA1535/pSK1002 that have been treated with various mutagens. The suppressive compound was mainly localized in the *n*-hexane extract fraction of the processed *D. nobile*. This *n*-hexane fraction was further fractionated by silica gel column chromatography, which resulted in the purification and subsequent identification of the suppressive compound. EI-MS and ^{1}H and ^{13}C NMR spectroscopy were then used to delineate the structure of the compound that confers the observed antimutagenic activity. Comparison of the obtained spectrum with that found in the literature indicated that moscatilin is the secondary suppressive compound. When using 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (furylfuramide) as the mutagen, moscatilin suppressed 85% of the *umu* gene expression compared to the controls at <0.73 μmol/mL, with an ID_{50} value of 0.41 μmol/mL. Additionally, moscatilin was tested for its ability to suppress the mutagenic activity of other well-known mutagens such as 4-nitroquinoline-1-oxide (4NQO), *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), UV irradiation, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3*b*]indole (Trp-P-1), benzo[*a*]pyrene (B[a]P), and aflatoxin B_1 (AFB_1). With all of the aforementioned chemicals or treatments, moscatilin showed a dramatic reduction in their mutagenic potential. Interestingly, moscatilin almost completely suppressed (97%) the AFB_1-induced SOS response at concentrations <0.73 μmol/mL, with an ID_{50} of 0.08 μmol/mL. Finally, the antimutagenic activities of moscatilin against furylfuramide and Trp-P-1 were assayed by the Ames test using the *S. typhimurium* TA100 strain. The results those experiments indicated that moscatilin demonstrated a dramatic suppression of the mutagenicity of only Trp-P-1 but not furylfuramide.

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

INTRODUCTION

Dendrobium nobile (Orchidaceae) is a plant that is widely distributed in China. The cultivated storage stem derived from this plant is commonly used for treatment of anorexia and several gastrointestinal disorders. The *Dendrobium* species have been demonstrated to produce a wide variety of potentially useful chemical compounds that include alkaloids (Suzuki et al., 1973), fluorenones, sesquiterpenoids (Talapatra et al., 1985, 1992), bibenzyls, and phenanthrenes (Majumder et al., 1992, 1993). These chemical species have been shown to be the active ingredients that confer the observed medicinal properties of the crude plant extracts. For example, dendrobine, an alkaloid, was isolated from *D. nobile* as an antagonist of β-alanine, taurine, and presynaptic inhibitor of the frog's spinal cord (Kubo et al., 1983). Moreover, moscatilin and moscatin, a bibenzyl and phenanthrene, respectively, were isolated as antiplatelet aggregation principles of *D. loddigesii* (Chen et al., 1994). Finally, 4,7-dihydroxy-2-methoxy-9,10-dihydrophenanthrene, which was isolated from *D. noble*, showed antitumorgenic activity in ICR mice (Lee et al., 1995).

With the development of labortory 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. In particular, the *umu* test system was developed as a simple, but sensitive, tool to evaluate the genotoxic activities of a wide variety of environmental carcinogens and mutagens (Oda et al., 1985; Nakamura et al., 1987). The *umu* test detects the induction of the SOS response following treatment of the *Salmonella typhimurium* strain TA1535 with test compounds. This strain carries the plasmid pSK1002 in which the *umuC*′ gene is fused inframe to the *lac*Z′ gene. The SOS-inducing potency of test compounds would therefore be estimated by the measurement of induction of the 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. Its genetic effects were reported by many researchers, which led to its classification as a potent

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mutagen (Tazima et al., 1975; Ohta et al., 1983). Likewise, 4-nitroquinoline-1-oxide (4-NQO), a nitroheterocyclic compound, and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), a direct-acting alkylating agent, were classified as potent mutagens (Olive et al., 1983, 1984; Watanabe et al., 1990; Haggerty 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), benzo[*a*]pyrene (B[a]P) (a polycyclic aromatic hydrocarbon), and aflatoxin B_1 (AFB_1) (a difurofuran ring fused to a substituted coumarin moiety) were shown to be highly mutagenic 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., 1997), α-pinene-7*β*-*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 novel naturally occurring antimutagenic compounds derived from plants that have a history of safe use as Chinese crude drugs (Miyazawa et al., 1995, 1996a,b), we reported the antimutagenic activity of gigantol, a bibenzyl compound derived from *D. nobile* (Miyazawa et al., 1997). This compound suppressed furylfuramide, Trp-P-1, and UV-induced SOS responses in the *umu* test. Moreover, gigantol suppressed the mutagenicity of furylfuramide and Trp-P-1 when the *S. typhimurium* TA100 strain was used in the Ames test.

Additional investigations demonstrated that the *n*-hexane extract fraction of *D. nobile* had secondary suppressive properties toward the furylfuramide-induced SOS response. In this paper, we report the isolation by chemical extraction and silica gel column chromatography and identification by mass spectrometry and NMR of this secondary antimutagenic compound contained in *D. nobile*.

MATERIALS AND METHODS

General Procedures. 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 and ^{13}C NMR spectra measured in $CDCl_3$. Specific rotation was determined with a JASCO DIP-140 digital polarimeter.

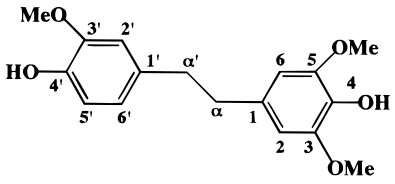
Materials. Commercially available air-dried stem of *D. nobile* was purchased from Takasago Yakugiyo Co. (Osaka, Japan). Furylfuramide, 4NQO, B[a]P, and Trp-P-1 were purchased from Wako Pure Chemical Co.(Osaka, Japan). MNNG was purchased from Aldrich Chemical Co. (Milwaukee, WI), and AFB_1 was from Sigma Chemical Co. (St. Louis, MO). S9 fractions prepared from livers of male rats that have 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.** An overnight culture of the tester bacterial strain (*S. typhimurium* TA1535/pSK1002) in Luria broth (1% Bactotryptone, 0.5% NaCl, and 0.5% yeast extract; supplemented with 20 μg/mL ampicillin) was diluted 50-fold with fresh TGA medium (1% Bactotryptone, 0.5% NaCl, and 0.2% glucose; supplemented with 20 μg/mL ampicillin) and incubated at 37 °C until the optical density at 600 nm of the culture reached 0.25−0.30. The culture was then aliquoted into 2.3 mL portions in test tubes, and the test compound (50 μL, diluted 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 mixture including the cofactors were added instead of the phosphate buffer. As a positive control an equivalent volume of DMSO was added instead of test compound, whereas with negative control an equivalent volume of DMSO was added instead of both the test compound and the mutagen. After a 2 h incubation at 37 °C with shaking, the cultures were centrifuged to sediment the cells, and then the cells were resuspended in 2.5 mL of PBS. The optical density of the suspensions at 600 nm was recorded with one portion (0.25 mL); the rest of the cell suspensions were used to measure the level of intracellular *β*-galactosidase activities using the method of Miller (1972).

UV Irradiation. Briefly, an overnight culture of the tester bacterial strain (*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 (4.0 J/m^2) for 20 s using a germicidal lamp.

Ames Test. The mutation test was carried out using a modification (Yahagi et al., 1977) of the original method (Ames et al., 1975).

Purification of the Suppressive Compound 1. The dry powder (9 kg) of stem of *D. nobile* was refluxed with methanol for 12 h to generate a methanol extract (545 g). This extract



Compound 1

was then suspended in water (2 L) and re-extracted with *n*-hexane (1 L), dichloromethane (1 L), *n*-butanol (1 L), and water successively. Each of the organic fractions was concentrated under reduced pressure to give *n*-hexane (83 g), dichloromethane (182 g), *n*-butanol (153 g), and water (127 g) fractions. To identify the compounds responsible for the suppression of the SOS-inducing activity, each of the individual fractions was tested. The *n*-hexane fraction had positive activity, whereas the dichloromethane, *n*-butanol, and water fractions did not show any suppressive effects. The *n*-hexane fraction was then further fractionated as described in Figure 1, which resulted in the isolation of suppressive compound **1** (180 mg).

Identification of Suppressive Compound 1. Compound **1** had the appearance of colorless needles; $[\alpha]^{20}_D$ −1.19 ($CHCl_3$; *c* 1.0); MS *m*/*z* 304 $[M]^+$, 167, 137; IR γ^{KBr}_{max} 3470, 2922, 1700, 1600, 1510, 1450, 1270, 1230, 1200, 1150 cm^{-1}. The ^{1}H NMR spectrum of compound **1** confirmed the presence of three methoxy groups at δ 3.84, and the signals at δ 5.38 and 5.48 could be assigned to two hydroxy groups. The signal at δ 2.82 could be assigned to four ethane hydrogens. The ^{13}C NMR of compound **1** was identical with that of moscatilin (Majumder et al., 1987). From the above spectral and physical properties, compound **1** was identified as moscatilin (4,4′-dihydroxy-3,3′,5-trimethoxybibenzyl).

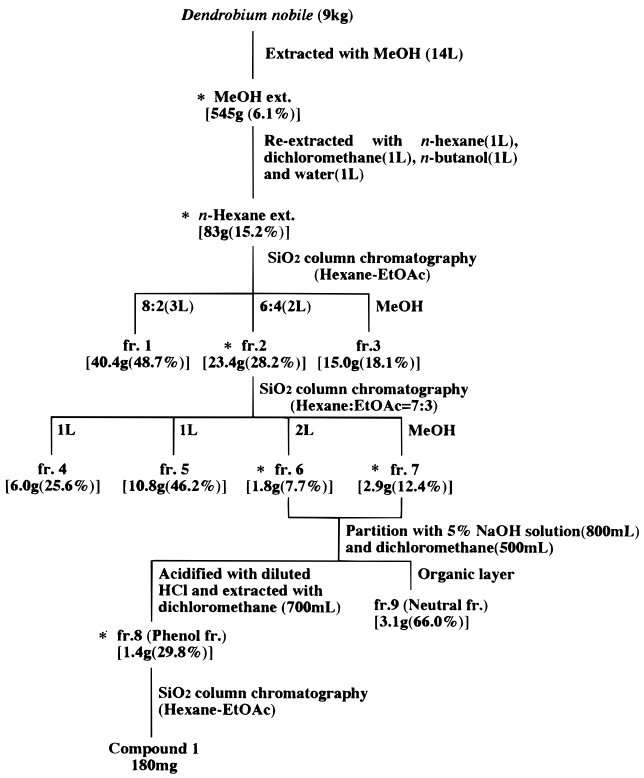


Figure 1. Schematic diagram showing the steps used to isolate suppressive compounds from the dried stem of *D. nobile*.

RESULTS AND DISCUSSION

Fractionation of the Extract from *D. nobile* and Isolation of Compound 1. The initial methanol extract of *D. nobile* was further fractionated to identify suppressive compounds using the *umu* test as a guide. To obtain dose−response data for purification of the suppressive compound, test samples were evaluated at dose levels of 200, 100, and 40 μg/mL (data not shown). The initial methanol extract from *D. nobile* (1000 mg/mL) suppressed 60% of the *umu* gene expression in *S. typhimurium* TA1535/pSK1002 against furylfuramide (data not shown). To further purify the suppressive fraction, the methanol extract was fractionated as described in Figure 1. That purification scheme resulted in the isolation of 180 mg of a suppressive substance (compound **1**).

Structure Determination of Compound 1. The identity of compound **1** as moscatilin was established by comparison of spectral data and physical constants with those derived from the literature.

Suppression of Mutagen-Induced Responses by Moscatilin. The suppressive effects of moscatilin on mutagen-induced SOS responses were determined using the *umu* test. Moscatilin was evaluated at dose levels of 0.73, 0.37, and 0.15 μmol/mL to obtain dose−response data. As shown in Figure 2, compound **1** suppressed 85% of the SOS-inducing activity due to furylfuramide at concentrations <0.73 μmol/mL, with an ID_{50} value of 0.41 μmol/mL. This compound suppressed 86% of the SOS-inducing activity due to Trp-P-1 at concentrations <0.73 μmol/mL. The ID_{50} value was 0.14 μmol/mL. Moscatilin was also assayed for its ability to suppress the SOS-inducing response caused by 4NQO, MNNG, B[a]P, and AFB_1. As shown in Figure 3, moscatilin

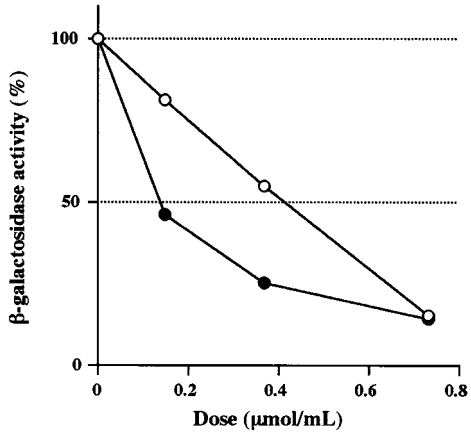


Figure 2. Suppression of furylfuramide and Trp-P-1-induced SOS responses by compound **1** in *S. typhimurium* TA1535/pSK1002: (○) effect of compound **1** on furylfuramide; (●) effect of compound **1** on Trp-P-1. Furylfuramide (1 mg/mL) was added at 60 μL.

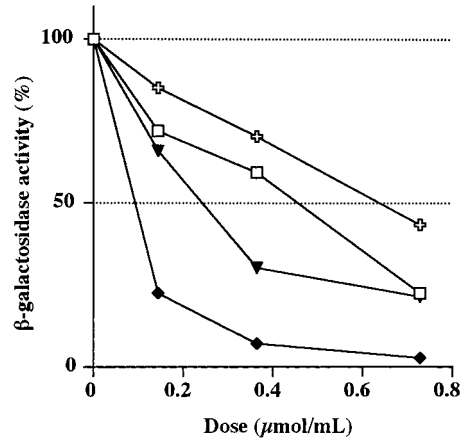


Figure 3. Suppression of MNNG-, 4-NQO-, AFB_1-, and B[a]P-induced SOS responses by compound **1**: (□) effect of compound **1** on MNNG; (✣) effect of compound **1** on 4-NQO; (◆) effect of compound **1** on AFB_1; (▼) effect of compound **1** on B[a]P. MNNG (100 mg/mL in DMSO) was added at 50 mL. 4-NQO (10 mg/mL in DMSO) was added at 50 mL. AFB_1 (2 mg/mL in DMSO) was added at 50 mL. B[a]P (60 mg/mL in DMSO) was added at 50 mL.

demonstrated suppressive activities toward the mutagen-induced SOS responses. Notably, moscatilin almost completely (97%) suppressed the SOS response of the cells following AFB_1 treatment at concentrations <0.73 μmol/mL, with an ID_{50} of 0.08 μmol/mL.

Suppressive Effects of Moscatilin on UV Irradiation. UV irradiation of *S. typhimurium* cells followed by moscatilin treatment was used to investigate the ability of this compound to suppress the effects of a directly acting mutagen such as UV. As shown in Figure 4, moscatilin suppressed 55% of the *umu* gene expression at concentrations <0.37 μmol/mL, and the ID_{50} value was 0.31 μmol/mL. These results indicated that the mechanisms for inhibition of the SOS-inducing activity by moscatilin may include the possibility that it not only acts directly on the mutagens but also is involved with cellular repair systems within the cell.

Antimutagenic Activity of Moscatilin in the Ames Assay. The antimutagenic activity of moscatilin against furylfuramide and Trp-P-1 was also demonstrated by the Ames test using *S. typhimurium* TA100. Moscatilin was evaluated at dose levels of 0.91, 0.36,

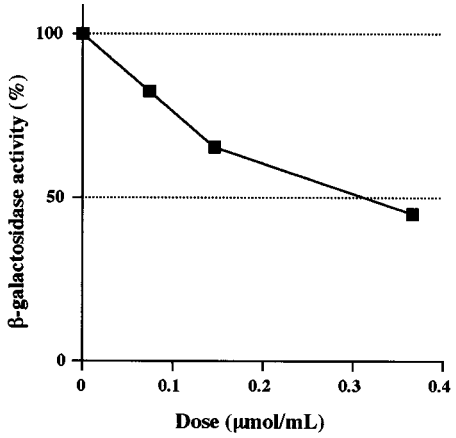


Figure 4. Suppression of UV-induced SOS response by compound **1** using *S. typhimurium* TA1535/pSK1002. The cells were exposed to UV light (4.0 J/m^2) with a germicidal lamp at room temperature.

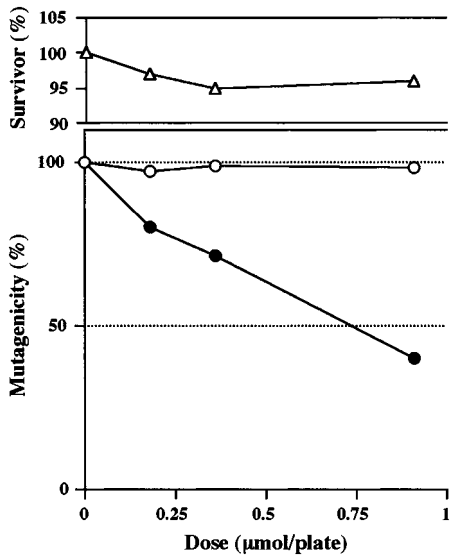


Figure 5. Effect of compound **1** on the mutagenicity of furylfuramide and Trp-P-1 using *S. typhimurium* TA100: (○) effect of compound **1** on the mutagenicity of furylfuramide; (●) effect of compound **1** on the mutagenicity of Trp-P-1; (△) effect of compound **1** on survival for *S. typhimurium* TA100. Furylfuramide (0.5 mg/mL in DMSO) was added at 20 mL/plate. Trp-P-1 (40 mg/mL in DMSO) was added at 40 mL/plate.

and 0.18 μmol/plate. As shown in Figure 5, the survival curve showed a slight decease at low concentrations of moscatilin. However, the survival curve did not show a dose-dependent decrease at higher concentrations of moscatilin. Therefore, the observed suppressive effects of moscatilin on the mutagenicity of Trp-P-1 are not caused by the decrease in the cell number. Moscatilin suppressed 61% of the mutagenicity of Trp-P-1 at <0.91 μmol/plate, with an ID_{50} value of 0.73 μmol/plate. However, moscatilin did not show any suppressive effects on the mutagenicity of furylfuramide.

Previously, gigantol was isolated as a suppressive compound on mutagen-induced SOS response with potential antimutagenic activity from *D. nobile* (Miyazawa et al., 1997). Gigantol, also a bibenzyl compound, showed suppression of SOS-inducing activity of furylfuramide, Trp-P-1, and UV in the *umu* test and suppressive effects of the mutagenicity of furylfuramide and Trp-P-1 in the Ames test. Similarly, moscatilin suppressed furylfuramide-, Trp-P-1-, and UV-induced SOS responses in the *umu* test. However, moscatilin did not show suppressive effect on the mutagenicity of furylfuramide when using the Ames test. The two compounds share the basic bibenzyl chemical structure but have different functional groups. Gigantol has two hydroxy and two methoxy groups (5,4′-dihydroxy-3,3′-dimethoxybibenzyl), whereas moscatilin has two hydroxy and three methoxy groups. The differences in the structures between the two compounds may explain the observed differences in the mutagenicity suppression due to furylfuramide.

Benzaldehyde derivatives showed different suppressive effects on mutagenicity depending upon the difference of the binding position between hydroxyl group and methoxy group using *Escherichia coli* WP2s (Watanabe et al., 1989). Such results suggest that these bibenzyl compounds show different suppressive effects depending on the binding position between the hydroxyl and methoxy moieties of the molecule. In this paper, we have shown that moscatilin is also identified as a naturally occurring antimutagenic bibenzyl compound, and this compound suppressed furylfuramide-, Trp-P-1-, 4NQO-, MNNG-, B[a]P-, AFB_1-, and UV-induced SOS response and suppressed the mutagenicity of Trp-P-1.

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