

Suppression of Chemical Mutagen-Induced SOS Response by Alkylphenols from Clove (*Syzygium aromaticum*) in the *Salmonella typhimurium* TA1535/pSK1002 *umu* Test

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A methanol extract from clove (*Syzygium aromaticum*) showed a suppressive effect of the SOS-inducing activity on the mutagen 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (furylfuramide) in the *Salmonella typhimurium* TA1535/pSK1002 *umu* test. The methanol extract was re-extracted with hexane, dichloromethane, ethyl acetate, butanol, and water. The hexane fraction showed a suppressive effect. Suppressive compounds in the hexane fraction were isolated by silica gel column chromatography and identified as *trans*-isoeugenol (**1**) and eugenol (**2**) by GC, GC-MS, IR, and ^1H and ^{13}C NMR spectroscopy. Compounds **1** and **2** suppressed the furylfuramide-induced SOS response in the *umu* test. Compounds **1** and **2** suppressed 42.3 and 29.9% of the SOS-inducing activity at a concentration of 0.60 $\mu\text{mol/mL}$. These compounds were assayed with other mutagens, 4-nitroquinoline 1-oxide (4NQO) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). In addition, compounds **1** and **2** were assayed with aflatoxin B₁ (Afb₁) and 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), which require liver metabolizing enzymes. These compounds showed suppressive effects of the SOS-inducing activity against furylfuramide, 4NQO, Afb₁, and Trp-P-1. To research the structure–activity relationship, methyl esters of **1** and **2** (**1Me** and **2Me**) and *o*-eugenol (**3**), as compounds similar to **2**, were also assayed with all mutagens. Compounds **1Me**, **2Me**, and **3** showed weak suppressive effects of the SOS-inducing activity against furylfuramide.

Keywords: Clove; *Syzygium aromaticum*; *trans*-isoeugenol; eugenol; SOS response; *umu* test; *Salmonella typhimurium* TA1535/pSK1002

INTRODUCTION

Several short-term tests for screening of environmental mutagens and carcinogens have been developed and used widely in many laboratories (1, 2). The Ames test is a convenient method to evaluate the mutagenic activities of these chemicals (1), and several lines of evidence have suggested that the mutagenic activities of a number of chemicals correlate well with the carcinogenic activities reported so far (3, 4).

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 (5, 6). The results of this test are in agreement with the results of the Ames test and may be more useful with respect to simplicity and rapidity (7).

The dried flower buds of clove ("tyouji" in Japanese; Myrtaceae) are used as an oriental drug, which has been used as a vermifuge, as an antibacterial agent, and to treat toothache (8). Various compounds such as tannins and triterpenoids were isolated and identified from this plant (9, 10). It is well-known that clove possesses a phenolic compound, 4-allyl-2-methoxyphenol, commonly called eugenol. Eugenol acts as an antioxidant on oleaginous foods, as an anticarcinogenic, antispasmodic, and antiseptic in pharmacy, and also as an antimicro-

bial agent (11–13). In our search for new naturally occurring antimutagenic compounds in plants, with a history of safe use as Chinese crude drugs (14, 15), we found that the methanol extract of clove exhibited a suppression of the furylfuramide-induced SOS response. In this paper, we report the isolation and identification of the suppressive compounds on SOS response against mutagens in clove.

MATERIALS AND METHODS

General Procedure. Gas chromatography (GC) was performed on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector (FID). GC-MS was performed on a Hewlett-Packard 5972 series mass spectrometer interfaced with a Hewlett-Packard 5890 gas chromatograph fitted with a column (HP-5MS, 30 m $\times$ 0.25 mm i.d.). 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 also used for ^{13}C NMR spectra.

Materials. Commercially available air-dried tips of clove (tyouji) were obtained from Yamada Yakken Co., Ltd. Furylfuramide, 4-nitroquinoline 1-oxide (4NQO), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), and aflatoxin B₁ (Afb₁) were purchased from Wako Pure Chemical Co. S9 (supernatant of 9000g) and coenzyme, NADPH, NADH, and G-6-P were purchased from Oriental Yeast Co. *o*-Eugenol was purchased from Aldrich Chemical Co.

***umu* Test.** The *umu* test for detecting the chemical-induced SOS response was carried out according to the method of Oda

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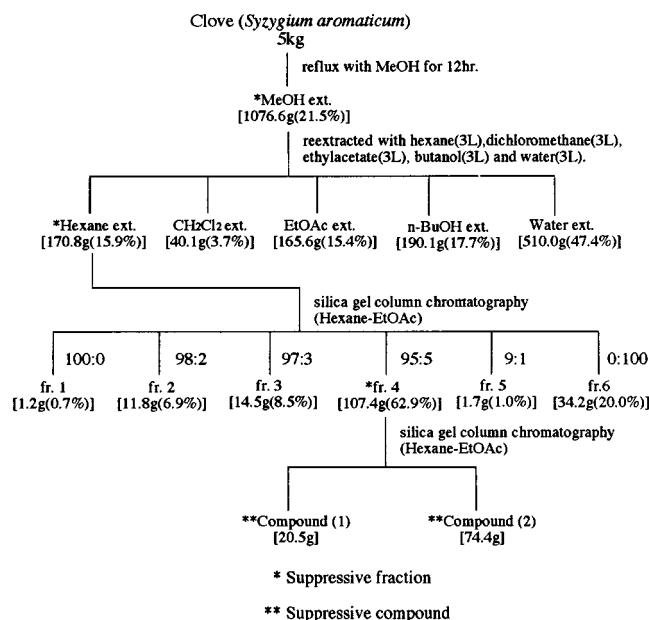


Figure 1. Isolation scheme for suppressive compounds from clove (*S. aromaticum*).

et al. (5) using *Salmonella typhimurium* TA1535/pSK1002, the pSK1002 plasmid of which carries an *umuC'-lacZ* fused gene. The overnight culture of bacterial strain was diluted 50-fold into 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 reached 0.25–0.30 in OD₆₀₀. The bacterial culture was subdivided into 2.1 mL portions in test tubes, and the test compound (50 µL), 0.1 M phosphate buffer (300 µL, pH 7.4), and mutagens, furylfuramide (50 µL, 5 µg/mL in DMSO), 4NQO (50 µL, 20 µg/mL in DMSO), and MNNG (50 µL, 200 µg/mL in DMSO) were added to each tube. In the case of AFB₁ (50 µL, 40 µg/mL in DMSO) and Trp-P-1 (50 µL, 40 µg/mL in DMSO), 300 µL of S9-metabolizing enzyme mixture including the cofactors was added instead of the phosphate buffer. As a positive control an equivalent volume of DMSO was added instead of the test compound, whereas with negative control an equivalent volume of DMSO was added instead of both the test compound and the mutagen. After 2 h of incubation at 37 °C with shaking, the culture was centrifuged (3000 rpm) to collect cells, which were centrifuged in 2.5 mL of PBS. The level of β -galactosidase activity was measured according to a slight modification of Miller's method (Miller, 1972). Fractions (0.25 mL) of the culture were diluted with 2.25 mL of Z buffer, and 0.1% SDS solution (50 µL) and chloroform (10 µL) were added to each fraction. The enzyme reaction was initiated by the addition of 0.25 mL of 2-nitrophenyl β -D-galactopyranoside solution (ONPG; 4 mg/mL in 0.1 M phosphate buffer, pH 7.4) at 28 °C. After 15 min, the reaction was stopped by 0.1 M Na₂CO₃, and the absorbance at OD₄₂₀ and OD₅₅₀ was measured. Using the remainder of the culture, the bacterial density was measured at OD₆₀₀. The units of β -galactosidase activity was calculated according to the method of Miller (1972).³⁴

Preparation of Activated Trp-P-1. Preparation of activated Trp-P-1 was carried out according to the method of Arimoto et al. (16).

Purification and Identification of the Suppressive Compounds. As shown in Figure 1, the dry powder (5 kg) of clove was refluxed with methanol for 12 h to give a methanol extract (1076.6 g). This extract was suspended in water and re-extracted with hexane, dichloromethane, ethyl acetate, butanol, and water, respectively. Each soluble fraction was concentrated under reduced pressure to give hexane (170.8 g), dichloromethane (40.1 g), ethyl acetate (165.6 g), butanol (190.1 g), and water (510.0 g) fractions. To purify the compound responsible for suppression of the SOS-inducing activity, these fractions were evaluated with the *umu* test. The hexane fraction

showed a suppressive effect. The hexane fraction was fractionated to fractions 1–6 by silica gel column chromatography with hexane and ethyl acetate as eluents. Fraction 4 showed a suppression of the furylfuramide-induced SOS response in the *umu* test, and this fraction was repeatedly fractionated by silica gel column chromatography with hexane and ethyl acetate as eluents using the *umu* test as a guide. Finally, suppressive compounds **1** (20.5 g) and **2** (74.4 g) were isolated from clove. These compounds were major components in the hexane fraction. Compounds **1** and **2** were identified as *trans*-isoeugenol and eugenol by GC, GC-MS, IR, and ¹H and ¹³C NMR, respectively.

Compound 1. Compound **1** was a yellow oil: MS, *m/z* 164 (*M*⁺, 100%), 149 (27%), 133 (15%), 131 (20%), 103 (24%), 77 (19%), 66 (11%); IR 3510.8, 2939.7, 2842.9, 1514.9, 1268.6; ¹H NMR (CDCl₃) δ 1.84 (3H, dd, *J* = 2, 6, γ -H₃), 3.85 (3H, s, OCH₃), 5.64 (1H, s, OH), 6.06 (1H, m, *J* = 6, 16, β -H), 6.31 (1H, dd, *J* = 2, 16, α -H), 6.81 (1H, dd, H-3), 6.83 (1H, s, H-5), 6.85 (1H, t, H-6); ¹³C NMR (CDCl₃) δ 146.51 (C-1), 144.70 (C-2), 130.69 (α -C), 130.59 (C-4), 123.31 (C-6), 119.23 (C-3), 114.32 (C-5), 107.87 (β -C), 55.75 (OCH₃), 18.24 (γ -C). Compound **1** was identified as *trans*-isoeugenol [(*E*)-2-methoxy-4-(1-propenyl)phenol] from these spectral data.

Compound 2. Compound **2** was a yellow oil: MS, *m/z* 164 (*M*⁺, 100%), 149 (28%), 137 (20%), 131(27%), 103 (30%), 91 (26%), 77(35%); IR 3509.6, 2939.6, 2851.9, 1514.4, 1267.0; ¹H NMR (CDCl₃) δ 3.23 (2H, d, α -H₂), 3.78 (3H, s, OCH₃), 5.00 (2H, dd, γ -H₂), 5.45 (1H, s, OH), 5.80–6.00 (1H, m, β -H), 6.60 (2H, m, H-3,5), 6.77 (1H, d, H-6); ¹³C NMR (CDCl₃) δ 146.41 (C-1), 143.87 (C-2), 137.79 (β -C), 131.89 (C-4), 121.15 (C-6), 115.47 (γ -C), 114.23 (C-3), 111.08 (C-5), 55.82 (OCH₃), 39.84 (α -C). Compound **2** was identified as eugenol [2-methoxy-4-(2-propenyl)phenol] from these spectral data.

Methyl Esters of Compounds 1 and 2 (1Me and 2Me). Methyl esters of **1** and **2** were obtained by reaction with diazomethane. These structures were identified by GC, GC-MS, IR, and ¹H and ¹³C NMR.

Methyl Ester 1Me. Methyl ester **1Me** was a colorless oil: MS, *m/z* 179 (12.2%), 178 (*M*⁺, 100%), 163 (31.1%), 147 (10.8%), 135 (8.8%), 107 (36.5%), 103 (23.0%), 89 (5.4%), 77 (14.9%); IR 2938.9, 2833.6, 1515.6, 1261.8; ¹H NMR (CDCl₃) δ 1.90 (3H, dd, *J* = 2, 6, γ -H), 3.90 (3H, s, OCH₃), 3.92 (3H, s, OCH₃), 6.14 (1H, m, *J* = 6, 16, β -H), 6.38 (1H, dd, *J* = 2, 16, α -H), 6.81(1H, s, H-3), 6.83 (1H, s, H-5), 6.90 (1H, t, H-6); ¹³C NMR (CDCl₃) δ 148.86 (C-1), 148.06 (C-2), 131.04 (α -C), 130.50 (C-4), 123.65 (C-6), 118.54 (C-3), 111.08 (C-5), 108.36 (β -C), 55.78 (OCH₃), 55.65 (OCH₃), 18.26 (γ -C). Methyl ester **1Me** was identified as *trans*-methyl isoeugenol [(*E*)-1,2-dimethoxy-4-(1-propenyl)-benzene] from these spectral data.

Methyl Ester 2Me. Methyl ester **2Me** was a colorless oil: MS, *m/z* 179 (12.2%), 178 (*M*⁺, 100%), 163 (29.9%), 147 (28.6%), 135 (10.2%), 107 (23.1%), 103 (25.9%), 92 (28.6%), 77 (13.6%); IR 2932.0, 2834.6, 1515.2, 1264.9; ¹H NMR (CDCl₃) δ 3.34 (2H, d, α -H₂), 3.85 (3H, s, OCH₃), 3.87 (3H, s, OCH₃), 5.07 (2H, dd, γ -H₂), 5.97 (1H, m, β -H), 6.73 (2H, m, H-3,5), 6.81 (1H, d, H-6); ¹³C NMR (CDCl₃) δ 148.85 (C-1), 147.33 (C-2), 137.62 (β -C), 132.58 (C-4), 120.34 (C-6), 115.52 (γ -C), 111.83 (C-3), 111.22 (C-5), 55.88 (OCH₃), 55.73 (OCH₃). Methyl ester **2Me** was identified as methyleugenol [1,2-dimethoxy-4-(2-propenyl)-benzene] from these spectral data.

RESULTS

Fractionation of the Extract from Clove and Isolation of Suppressive Compounds 1 and 2. The methanol extract of clove was fractionated to search for suppressive compounds using the *umu* test as a guide (Figure 1). To obtain dose–response data, test samples were evaluated at dose levels of 0.2, 0.1, and 0.04 mg/mL. If test samples showed toxicity at 0.2 mg/mL, test samples were evaluated at dose levels of 0.1, 0.05, and 0.02 mg/mL. As shown in Table 1, the hexane fraction exhibited a suppressive effect of the furylfuramide-induced SOS response in *S. typhimurium* TA1535/

Table 1. Suppression of Furfylfuranide-Induced^a SOS Response by Clove Fractions in *S. typhimurium* TA1535/pSK1002

sample	control ^c	dose response ^b			
		200 μg/mL	100 μg/mL	40 μg/mL	0 μg/mL
MeOH extract ^d	142	341	364	390	407
hexane fraction ^d	142	260	339	374	407
CH ₂ Cl ₂ fraction	142	387	381	373	407
EtOAc fraction	142	303	366	389	407
BuOH fraction	142	379	394	398	407
water fraction	142	366	384	395	407

sample	control ^c	dose response ^b			
		100 μg/mL	50 μg/mL	20 μg/mL	0 μg/mL
fraction 1	261	687	707	716	736
fraction 2	261	693	709	719	736
fraction 3	261	657	674	692	736
fraction 4 ^d	261	592	627	661	736
fraction 5	261	692	712	723	736
fraction 6	261	681	706	718	736

^a Furfylfuranide (5 μg/mL in DMSO) was added at 50 μL. ^b β-Galactosidase activity (units). ^c Control was exposed to DMSO. ^d Suppressive fraction.

pSK1002. After the hexane fraction was fractionated, only the suppressive fraction 4 eluted with 95:5 hexane/ethyl acetate as eluent had a clear-cut dose-response effect in the first fractionation (fractions 1–6). Finally, suppressive compounds **1** (20.5 g) and **2** (74.4 g) were isolated from the suppressive fraction 4. Compounds **1** and **2** were identified as *trans*-isoeugenol and eugenol by GC, GC-MS, IR, and ¹H and ¹³C NMR, respectively.

Inhibition of SOS-Inducing Activity by Compounds 1 and 2. The suppressive effects of compounds **1** and **2** were evaluated in the *umu* test. Compounds **1** and **2** exhibited inhibition on the furfurylamine-induced SOS response (Table 2). Compounds **1** and **2** suppressed 42.3 and 29.9%, respectively, of the SOS-inducing activity on furfurylamine at a concentration of 0.60 μmol/mL, although these compounds showed toxicity at 1.2 μmol/mL (Figure 2). Compounds **1** and **2** were also assayed with other mutagens, 4NQO and MNNG, which do not require a liver-metabolizing enzymes mixture (Table 2). Compounds **1** and **2** suppressed 55.4 and 44.0% of the SOS-inducing activity on 4NQO at a concentration of 0.60 μmol/mL, respectively, and the ID₅₀ (50% inhibitory dose) value of **1** was 0.53 μmol/mL

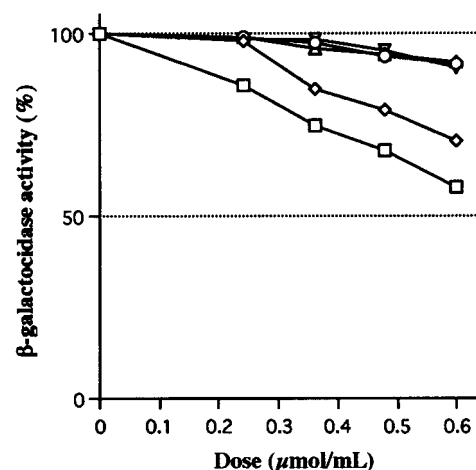


Figure 2. Suppression of furfurylamine-induced SOS response by compounds **1–3**, **1Me**, and **2Me** in *S. typhimurium* TA1535/pSK1002: (□) effect of **1** on furfurylamine; (◇) effect of **2** on furfurylamine; (▽) effect of **3** on furfurylamine; (○) effect of **1Me** on furfurylamine; (Δ) effect of **2Me** on furfurylamine. Furfurylamine (5 μg/mL in DMSO) was added at 50 μL.

(Figure 3). Compounds **1** and **2** showed weak (13.6 and 11.1% 0.60 μmol/mL, respectively) suppressive effects of the SOS-inducing activity on MNNG (Figure 3). The suppressive effects of **1** and **2** on 4NQO are similar to the suppressive effects observed in the case of furfurylamine. These compounds were also assayed with Afb₁ and Trp-P-1, which require liver metabolic activation (Table 3). As shown in Figure 4, compound **1** suppressed 90.4% of the SOS-inducing activity on Afb₁ at a concentration of 0.60 μmol/mL, and the ID₅₀ value was 0.27 μmol/mL. Compound **2** suppressed 61.5% of the SOS-inducing activity on Afb₁ at a concentration of 0.60 μmol/mL, and the ID₅₀ value was 0.48 μmol/mL. Compound **1** suppressed 88.1% of the SOS-inducing activity on Trp-P-1 at a concentration of 0.60 μmol/mL, and the ID₅₀ value was 0.25 μmol/mL. Compound **2** suppressed 60.8% of the SOS-inducing activity on Trp-P-1 at a concentration of 0.60 μmol/mL, and the ID₅₀ value was 0.50 μmol/mL. As shown by the *umu* test, compounds **1** and **2** had greater suppressive effects on the SOS genes against mutagens, furfurylamine, 4NQO, Afb₁, and Trp-P-1 than did MNNG.

Suppressive Effect of Methyl Esters (1Me and 2Me) of Compounds 1 and 2. Methyl esters (**1Me** and

Table 2. Suppressive Effects of Compounds 1–3, 1Me, and 2Me on Furfurylamine,^a 4NQO,^b and MNNG^c Using *S. typhimurium* TA1535/pSK1002

compd	furfurylamine	4NQO	MNNG	control	dose response ^d			
					0.60 μmol/mL	0.48 μmol/mL	0.36 μmol/mL	0.24 μmol/mL
1	1074			282	740	820	873	958
2	1074			282	837	905	953	1032
3	1074			282	995	1034	1057	1060
1Me	1074			282	1007	1022	1053	1062
2Me	1074			282	1015	1024	1040	1064
1		759		194	446	499	548	606
2		759		194	510	566	606	657
3		759		194	701	712	736	740
1Me		759		194	690	697	721	742
2Me		759		194	712	719	727	747
1			697	304	643	657	673	675
2			697	304	653	675	686	688
3			697	304	659	672	680	689
1Me			697	304	435	458	496	554
2Me			697	304	486	503	530	573

^a Furfurylamine (5 μg/mL in DMSO) was added at 50 μL. ^b 4NQO (20 μg/mL in DMSO) was added at 50 μL. ^c MNNG (200 μg/mL in DMSO) was added at 50 μL. ^d β-Galactosidase activity (units).

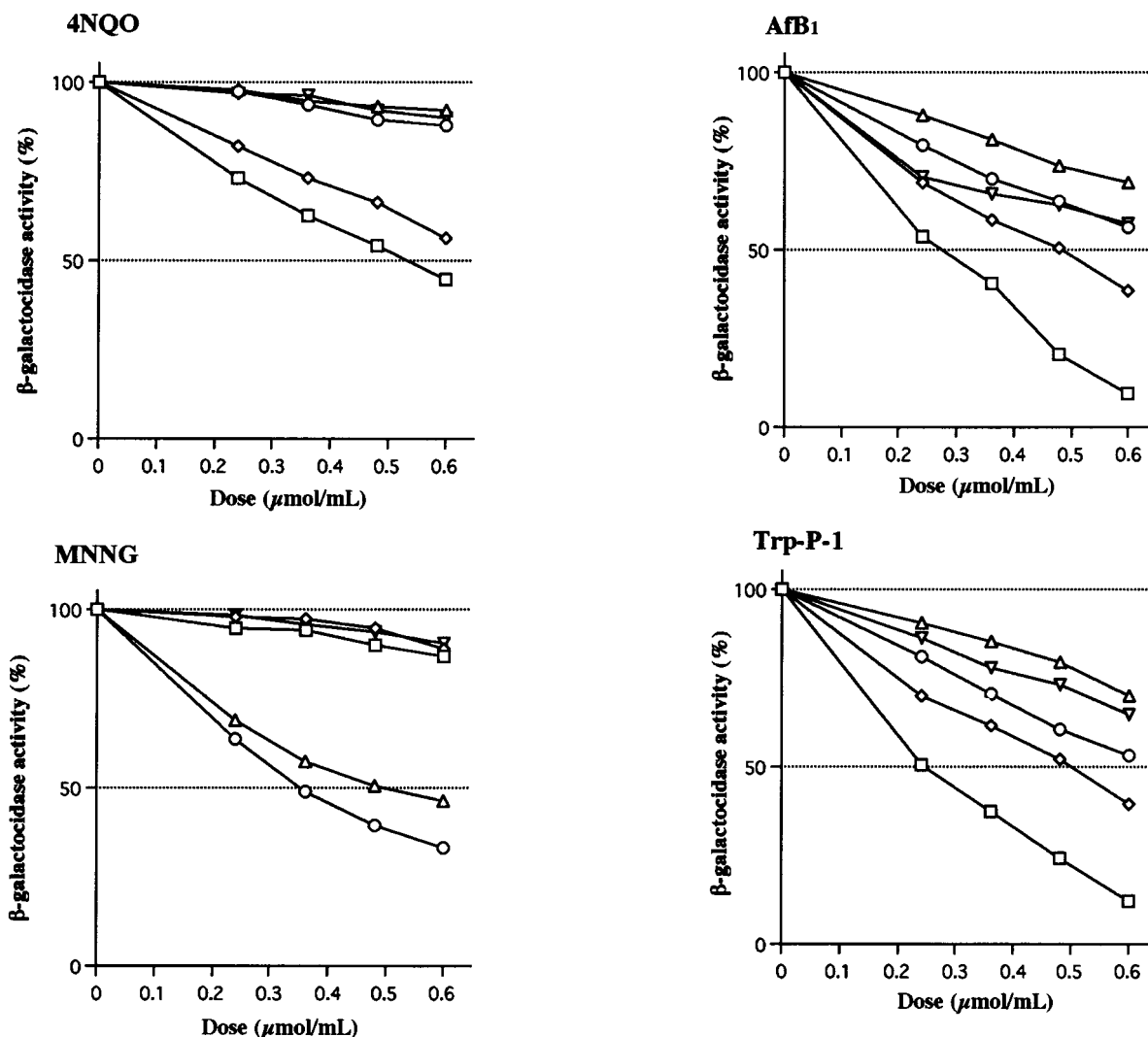


Figure 3. Suppression of 4NQO- and MNNG-induced SOS response by compounds **1–3**, **1Me**, and **2Me** in *S. typhimurium* TA1535/pSK1002: (□) effect of **1**; (◇) effect of **2**; (▽) effect of **3**; (○) effect of **1Me**; (△) effect of **2Me**. 4NQO (20 μg/mL in DMSO) was added at 50 μL. MNNG (200 μg/mL in DMSO) was added at 50 μL.

2Me) of **1** and **2** were examined for their ability to suppress the SOS-inducing activity on furofuramide, 4NQO, and MNNG, which do not require a liver-metabolizing enzymes mixture (Table 2). Methyl esters **1Me** and **2Me** showed weaker suppressive effects on furofuramide and 4NQO than **1** and **2** (Figures 2 and 3). Methyl esters **1Me** and **2Me** showed greater suppressive effects (66.7 and 53.8% at 0.60 μmol/mL, respectively) on MNNG than **1** and **2**, and the ID₅₀ values of **1Me** and **2Me** were 0.35 and 0.49 μmol/mL, respectively (Figure 3). These methyl esters were also assayed with AfB₁ and Trp-P-1, which require liver metabolic activation (Table 3). As shown in Figure 4, methyl esters **1Me** and **2Me** suppressed 44.0 and 31.4%, respectively, of the SOS-inducing activity on AfB₁ at a concentration of 0.60 μmol/mL. These methyl esters suppressed 47.0 and 29.9%, respectively, of the SOS-inducing activity on Trp-P-1 at a concentration of 0.60 μmol/mL. As shown by the *umu* test, methyl esters **1Me** and **2Me** had greater suppressive effects on the SOS genes against MNNG than mutagens, furofuramide, 4NQO, AfB₁ and Trp-P-1.

activated Trp-P-1

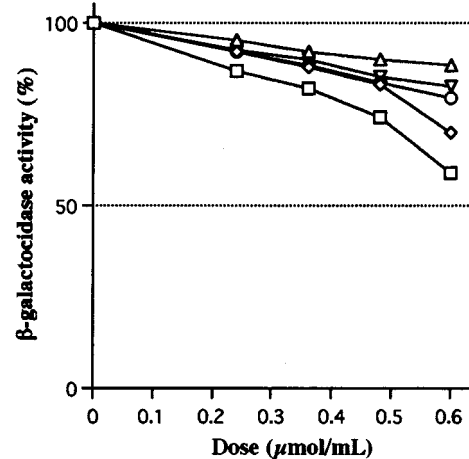


Figure 4. Suppression of AfB₁-, Trp-P-1-, and activated Trp-P-1-induced SOS responses by compounds **1–3**, **1Me**, and **2Me** in *S. typhimurium* TA1535/pSK1002: (□) effect of **1**; (◇) effect of **2**; (▽) effect of **3**; (○) effect of **1Me**; (△) effect of **2Me**. AfB₁ (40 μg/mL in DMSO) was added at 50 μL. Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL. Activated Trp-P-1 was added at 50 μL.

Inhibition of SOS-Inducing Activity by Compound 3. For confirming the structure–activity relationship, o-eugenol [1-methoxy-5-(2-propenyl)phenol] (**3**) was obtained. Compound **3** was also examined for its

Table 3. Suppressive Effects of Compounds 1–3, 1Me, and 2Me on AfB₁,^a Trp-P-1,^b and Activated Trp-P-1^c Using *S. typhimurium* TA1535/pSK1002

compd	AfB ₁	Trp-P-1	activated Trp-P-1	control	dose response ^d			
					0.60 μ mol/mL	0.48 μ mol/mL	0.36 μ mol/mL	0.24 μ mol/mL
1	1292			296	391	501	697	829
2	1292			296	679	798	877	980
3	1292			296	868	920	950	997
1Me	1292			296	854	928	994	1083
2Me	1292			296	979	1028	1100	1167
1		819		387	438	490	548	605
2		819		387	556	611	652	688
3		819		387	666	702	718	758
1Me		819		387	616	648	690	736
2Me		819		387	690	729	754	777
1			558	101	370	439	475	496
2			558	101	420	480	502	520
3			558	101	478	489	493	523
1Me			558	101	464	483	505	519
2Me			558	101	504	510	522	535

^a AfB₁ (40 μ g/mL in DMSO) was added at 50 μ L. ^b Trp-P-1 (40 μ g/mL in DMSO) was added at 50 μ L. ^c Activated Trp-P-1 was added at 50 μ L. ^d β -Galactosidase activity (units).

ability to suppress the SOS-inducing activity on furyl-furamide, 4NQO, and MNNG, which do not require a liver-metabolizing enzymes mixture (Table 2). Compound **3** showed a weaker suppressive effect on furyl-furamide, 4NQO, and MNNG than **2** (Figures 2 and 3). Compound **3** was also assayed with AfB₁ and Trp-P-1, which require liver metabolic activation (Table 3). As shown in Figure 4, compound **3** showed a weaker suppressive effect (42.5% at 0.60 μ mol/mL) on AfB₁ than did **2**. Compound **3** showed a weaker suppressive effect (35.5% at 0.60 μ mol/mL) on Trp-P-1 than did **2**.

Suppressive Effects of 1, 2, 1Me, 2Me, and 3 on Metabolic Activation of Trp-P-1. The suppressive effects of **1**, **2**, **1Me**, **2Me**, and **3** on metabolic activation of Trp-P-1 were determined by using the *umu* test. The value of β -galactosidase activity observed in the absence of these compounds was for activated Trp-P-1. As shown in Table 3 and Figure 4, compounds **1**, **2**, and **3** suppressed 41.0, 30.1, and 17.4%, respectively, of the SOS-inducing activity on activated Trp-P-1 at a concentration of 0.60 μ mol/mL. Methyl esters **1Me** and **2Me** suppressed 20.6 and 11.8%, respectively, of the SOS-inducing activity on activated Trp-P-1 at a concentration of 0.60 μ mol/mL. Suppressive effects of **1**, **2**, **1Me**, **2Me**, and **3** on activated-Trp-P-1 were decreased compared with those of Trp-P-1.

DISCUSSION

The suppressive compounds of SOS-inducing activity in clove were identified as **1** and **2**. These compounds showed suppressive effects on *umu* gene expression of the SOS response in *S. typhimurium* TA1535/pSK1002 against furyl-furamide and 4NQO, which do not require liver-metabolizing enzymes, and AfB₁ and Trp-P-1, which do require liver-metabolizing enzymes. As shown in Tables 2 and 3 and Figures 2–4, compounds **1** and **2** had greater suppressive potencies against furyl-furamide, 4NQO, AfB₁, and Trp-P-1 than did **1Me** and **2Me**. The difference in structure between **1** and **1Me** and between **2** and **2Me** is a hydroxy group. These results indicated that a hydroxy group is an important factor for suppressing the SOS-inducing activity on furyl-furamide, 4NQO, AfB₁, and Trp-P-1. As shown in Tables 2 and 3 and Figures 2–4, compound **2** had more suppressive potency against furyl-furamide, 4NQO, AfB₁, and Trp-P-1 than did **3**. The difference in structure between

2 and **3** is the C-4 position of a hydroxy group. These results indicated that the C-4 position of a hydroxy group is also an important factor for suppressing the SOS-inducing activity on furyl-furamide, 4NQO, AfB₁, and Trp-P-1. As shown in Tables 2 and 3 and Figures 2–4, compound **1** had more suppressive potency against furyl-furamide, 4NQO, AfB₁, and Trp-P-1 than did **2**. The difference in structure between **1** and **2** is the position of an olefinic double bond. These results indicated that the position of an olefinic double bond is an important factor for suppressing the SOS-inducing activity on furyl-furamide, 4NQO, AfB₁, and Trp-P-1.

These compounds were examined for the ability to suppress the metabolic activation of Trp-P-1 by S9. As shown in Figure 4, these compounds suppressed the SOS induction on activated Trp-P-1 more weakly than they did on Trp-P-1. These results suggested the possibility that the inhibition of the SOS-inducing activity on Trp-P-1, which was caused by compounds **1**, **2**, **3**, **1Me**, and **2Me**, was due to the inhibition of metabolic activation by S9.

For mutagenic activation of furyl-furamide (*cis* form), *cis*–*trans* isomerization (17–19) and reduction of the nitro group of 5-nitrofurane (20, 21) are important in the metabolic pathway. The *cis*–*trans* isomerization is based on the formation of nitro anion radicals. *cis*-Furyl-furamide receives a single electron derived from an enzyme system to form the anion radical. Spin density on the olefinic double bond results in free rotation between the olefinic carbons followed by conversion to its thermodynamically more stable *trans* isomer. The nitro group of 5-nitrofurane is activated by the reductive metabolism associated with nitroreductases in bacteria. The main pathway for nitrofurane activation would be via reduction to a hydroxylamine intermediate, which could react with DNA though a nitrenium ion. An alternative reactive intermediate, the ring-opened acrylonitrile derivative, could form through rearrangement of the hydroxylamine intermediate. It has been shown that the acrylonitrile derivative readily forms conjugates with glutathione, mercaptoethanol, and thiol groups of proteins. These conjugates increase the mutation frequency in *S. typhimurium* TA100, suggesting that the acrylonitrile derivative is also capable of interacting with DNA. *trans*-Isoeugenol and eugenol exhibited inhibition of SOS induction on furyl-

furamide. Therefore, *trans*-isoeugenol and eugenol may block these reactive metabolic pathways and/or activation of nitroanion radicals on furylfuramide.

With respect to mutagenic activation of 4NQO, metabolic activation of 4NQO by a nitro reduction enzyme to 4-hydroxyaminoquinoline 1-oxide (4HAQO) (22, 23), which is believed to be a proximate derivative of the potent carcinogen 4NQO, reaction of 4HAQO with DNA to yield three primary adducts (N2-guanine, C8-guanine, and N6-adenine adducts) (24), and access of 4NQO and/or 4HAQO to the target cell of bacteria are necessary. *trans*-Isoeugenol and eugenol exhibited inhibition of SOS induction on 4NQO. Therefore, *trans*-isoeugenol and eugenol may block this reactive metabolic activation of 4NQO, which is reacted with a nitro reduction enzyme in cells of bacteria.

Blocking effects of *trans*-isoeugenol and eugenol may be caused by the possible involvement of their antioxidant and scavenging properties. Isoeugenol had a strong antioxidant effect, and eugenol had a weak antioxidant effect (25). Isoeugenol and eugenol had inhibitory effects on the peroxidation of lecithin induced by reactive oxygen derived from the interaction of ferrous salt and hydrogen peroxide (26). Isoeugenol and eugenol had hydroxyl radical scavenging abilities (27). Therefore, the block by *trans*-isoeugenol and eugenol could arise from their scavenging ability to trap hydroxyl radicals originating from metabolites of furylfuramide and 4NQO and/or activation of nitro anion radicals on furylfuramide with a hydroxy group.

trans-Isoeugenol and eugenol had weak suppressive effects of the SOS-inducing activity on the direct alkylating agent MNNG. On the other hand, *trans*-methylisoeugenol and methyleugenol had great suppressive effects of the SOS-inducing activity on the direct alkylating agent MNNG. It was found that *trans*-methylisoeugenol and methyleugenol were operative to inhibit the SOS-inducing activity on the direct alkylating agent MNNG. As shown in Table 2 and Figure 3, compounds **1Me** and **2Me** had greater suppressive potencies against the direct alkylating agent MNNG than did **1** and **2**. The difference in structure between **1Me** and **1** and between **2Me** and **2** is a methoxy group at the C-4 position. These results indicated that the methoxy group at the C-4 position is an important factor for suppressing the SOS-inducing activity on the direct alkylating agent MNNG. As shown in Table 2 and Figure 3, compound **1Me** had more suppressive potency against the direct alkylating agent MNNG than did **2Me**. The difference in structure between **1Me** and **2Me** is the position of an olefinic double bond. These results also indicated that the position of an olefinic double bond is an important factor for suppressing the SOS-inducing activity on the direct alkylating agent MNNG.

Eugenol inhibited the mutagenicity of aflatoxin B₁ and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *S. typhimurium* tester strain TA100 (28). In this case, eugenol had shown a high suppressive effect on MNNG. On the other hand, in the *umu* test, eugenol showed a low suppressive effect on MNNG. This illustrates the discrepancy between the results of the *umu* test and the Ames test. We think this discrepancy is probably due to the difference of mechanisms of the suppressive effects.

Isoeugenol and eugenol had negative responses of genetic activity in growing yeast cells using strains D7 and XV185-14C without S9 (29). Isoeugenol and eugenol

inhibited the mutagenicity of furylfuramide on the Ames test using *S. typhimurium* TA100 (30). In addition, microsomes or S9 prepared from rats that received eugenol decreased the mutagenic activity of benzo[*a*]pyrene in the Ames test in comparison to microsomes or S9 from untreated rats (31, 32). Eugenol has the antimutagenic effect on the mutagenicity of cyclophosphamide in the rodent bone marrow micronucleus test using male Swiss mice (33). However, inhibition of mutagen-induced SOS response by *trans*-isoeugenol and eugenol has not been reported.

In summary, this research suggests that suppressive compounds on SOS response against mutagens in clove were primarily *trans*-isoeugenol (**1**) and eugenol (**2**). Compounds **1** and **2** showed potent suppressive effects of the SOS-inducing activity by chemical mutagens, and the characteristic activity of the respective compounds was dependent upon the hydroxy group at the C-4 position.

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